Novel Specific Receptor-based Techniques for Antibiotic Residue Analysis

Javier Adrián Izquierdo

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J. Adrián Novel Specific Receptor-based Techniques for Antibiotic Residue Analysis

UNIVERSITAT DE BARCELONA
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Nanomedicina

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Memoria para optar al **Grado de Doctor por la Universidad de Barcelona**
presentada por:

Javier Adrián Izquierdo

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Universidad de Barcelona
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^{1}H-NMR	Proton Nuclear Magnetic Resonance
Ab-Ag	Antibodies-Antigen
ACN	Acetonitrile
AE	Active Ester
AESAN	Agencia Española de Seguridad Alimentaria y Nutrición
AIBN	2,2'azobisisobutyronitrile
AMP	Ampicillin
AMRg	Applied Molecular Receptors group
As	Antisera
AUPA	Alliance for the Prudent Use of Antibiotics
BLs	ß-Lactam antibiotics
BM	Biological Monitoring
BSA	Bovine serum albumin
CA	Coating antigen
CAFOs	Confined Animal Feeding Operations
CAP	Chlor(amphenicol)
CC	cyanuric chloride
CCß	Detection capability
CCα	Decision limit
CDC	Centers for Disease Control and Prevention
CDR	Variable region of the immunoglobulin chains
CFZ	Cefazolin
CLIA	Chemiluminescence immunoassay
CLO	Cloxacillin
CONA	Conalbumin
CPX	Ciprofloxacine
CR	Cross-reactivity
CSEM	Swiss Center for Electronics and Microtechnology
CTC	Chlortetracycline
CT-IMB	Chemical Transducers group from the Barcelona Microelectronic Institute
DAP	Diaminopimelate
DC	Doxycycline
DCC	N,N'-dicyclohexyl-carbodiimide
DHPA	Dihydropteroic acid
DHPS	Dihydropteroate synthase
DMF	Dimethylformamide
DMP	dimethyl pimelimidate
DNA	Deoxyribonucleic acid
DOI	Digital Object Identifier
EC	European Commission
EDC	1-(3-dimethylaminopropyl)-2-ethylcarbodiimide
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Safety Authority
EGDMA	Ethylene glycol dimethacrylate
EIA	Enzyme immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay

Abbreviations

ELRA	Enzyme-Linked Receptor Assay
EM	Environmental Monitoring
EMEA	European Medicines Agency
EMIT	Enzyme-Multiplied Immunoassay Technique
ERX	Enrofloxacin
ESAC	European Surveillance of Antimicrobials Consumption
ET	Enzyme tracer
EW	Evanescent wave
Fab	Fragment antigen binding
FAO	Food and Agriculture Organization of the United Nations
Fc	Fragment crystallisable
FDA	Food and Drug Administration
FEDESA	European Federation of Animal Health
FIA	Fluoroimmunoassay
FICT	Fluorescein
FIIA	Flow-injection immunoassay
FN	False negative sample
FP	False positive sample
FQs	Fluoroquinolone antibiotics
Fv	Variable domain
GBEQ-UAH	Biosensors Group from University of Alcalá
GC	Gas Chromatography
HCH	Horseshoe crab hemocyanin
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
HTS	High Throughput Screening
IA	Immunoassay
IAC	Immunoaffinity Chromatography
IC_{50}	50% of the zero dose
IC_{90}	90% of the zero dose
ICFO	The Institute of Photonic Sciences
IFAH	International Federation for Animal Health
IgG	Immunoglobuline G
IRTA	Institut de Recerca i Tecnologia Agroalimentàries
IS	Immunosorbent
IUPAC	International Union of Pure and Applied Chemistry
JETACAR	Joint Expert Advisory Committee on Antibiotic Resistance
Ka	Affinity constant
LC-MS	Liquid Chromatography-Mass Spectrometry
LOD	Limit of Detection
LSPR	Localized surface plasmon resonance
M&NT	Micro and Nano Technologies
MA	Mixed anhydride
MAA	Methacrylic acid
MAb	Monoclonal antibodies
MC	Methacycline

MIA	Magnetic immunoassay
MIPs	Molecular Imprinted Polymers
MISPE	Molecularly Imprinted Solid-Phase Extracion
MNP	Magnetic NanoParticles
MRLs	Maximum Residue Limits
mRNA	Messenger ribonucleic acid
MRPLs	Minimum Required Performance Limits
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	Mass Spectrometry
N	Negative sample
NAG-NAM	N-acetylglucosamine linked b-1,4 to N-acetylmuramic acid
NHS	N-hydroxysuccinimide
NIP	Non imprinted polymer
NMR	Nuclear magnetic resonance
NOX	Norfloxacin
NRC	Nesté Research Centre
OTC	Oxytetracycline
OVA	Ovalbumin
OWLS	Optical waveguide lightmode spectroscopy
P	Positive sample
PAb	Polyclonal antibodies
PABA	p-aminobenzoic acid
PBPs	Penicillin Binding Proteins
PBS	Phosphate buffered saline
PEG	Penicillin G
PFIA	Polarization fluoroimmunoassay
PoC	Point-of-care
PPCPs	Pharmaceutical and Personal Care Products
QDs	Quantum Dots
RIA	Radioimmunoassay
SA1	Sulfonamide hapten containing the whole SAs structure
SA2	Sulfonamide hapten containing the common moiety of the SAs
SAs	Sulfonamide antibiotics
SCP	Sulfachloropyridazine
SD	Standard deviation
SMZ	Sulfamethazine
SPE	Solid-phase extraction
SPR	Surface Plasmon Resonance
SPY	Sulfapyridine
STZ (or STA)	Sulfathiazole
TC	Tetracycline
TCs	Tetracycline antibiotics
TetR	Tetracycline repressor protein
TMB	3,3”,5,5”-tetramethylbenzidine
tRNA	Transfer ribonucleic acid
UB	Universitat de Barcelona
UCC	University College Cork

Abbreviations

UPLC	Ultra performance liquid chromatography
USD	United States dollar
USEPA	U.S. Environmental Protection Agency
USP	United States Pharmacopea
VCSEL	Vertical-cavity surface-emiting laser
VPY	4-Vinylpyridine
WHO	World Health Organization
WIObS	Wavelength Interrogated Optical Biosensor System
WIOS	Wavelength Interrogated Optical System
WTP	WaterTreatment Plants
WWTP	WasteWater Treatment Plants

A SUMMARY

The indiscriminate and/or improper use of veterinary drugs is the cause of potential adverse health effects due to the risk of entering into the food chain and the appearance of residues in food products of animal origin [1, 2]. Moreover, in the case of antibiotics, this fact has been identified as one of the causes for the appearance of antimicrobial resistance mechanisms in bacteria causing human diseases [3, 4], which is the cause of a big concern within the health authorities, distinct governmental agencies and the society in general. There is an increased need to ensure safety and quality of the foodstuff and customers have also started to become more exigent forcing the industry to introduce consumer worries in their daily procedures, in terms of getting more natural, ecological and healthy products with a clear traceable origin of the ingredients.

Nowadays, food control is performed on centralized laboratories that in most cases use very reliable procedures, but involving expensive equipment, specialized personnel and time consuming sample treatment procedures. In order to drastically improve this situation, the European Commission (EC) and the member state agencies are strongly supporting research activities aimed to increase the efficiency of the actual analytical methods. An strategy is to take advantage of the latest bio-micro-nanotechnological advances and of the complementary skills of multidisciplinary research teams to develop more rapid, sensitive and specific methodologies capable of detecting a wide variety of chemical, biological or any other health risk associated to the agrofood industry and along to the whole food chain.

This thesis describes the research performed in respect to the possibility to develop improved alternatives for food residue analysis based on the combination of selective receptors and novel micro/nanotechnologies. Particularly, the final objective of this thesis was addressed to the development of a multiplexed sensor device to detect inappropriate farm practices and or the contamination of food products by antibiotic residues, mainly milk. In this respect, production of selective receptors with a broad recognition of the most important antibiotic families used in the veterinary field has been one of the most important aims of this research work. Thus, we report here the investigation made regarding development of synthetic receptors for sulfonamide antibiotics (SAs), particularly molecular imprinted polymers (MIPs), and their evaluation of a rational approach. Moreover, production of generic (or class-selective) antibodies for SAs and tetracycline antibiotics (TCs) has also been approached through the rational design and synthesis of appropriate haptens. Evaluation of the features of the antibodies produced has been accomplished through the development of microplate-based ELISAs (enzyme-linked immunosorbent assays). The results show that although it has been possible to obtain class-selective antibodies for SAs (up to 11 congeners can be detected), the approach followed for the case of the TCs afforded antibodies with a high selectivity

versus TCs possessing an alkyl/alkene group at position 6 of the C-ring, but lacking the hydroxyl group at this position. The necessary protocols to apply these immunochemical procedures to the analysis of milk and hair samples have been established showing that determination of these antibiotics according to the EC regulations was possible for the case of milk. For the case of hair, no regulations do exist at the moment. However this matrix holds great value regarding their potential use to trace inappropriate treatments through the life of the farm animals. The immunoreagents and immunochemical procedures established have been implemented on an optical sensor device developed by the Centre Suisse for Electronics and Microelectronics Inc. (CSEM, Neuchâtel - Switzerland). This device is based on the evanescent wave principle using a particular technological design based on waveguide grating couplers and it is very sensitive to the changes in the refractive index produced at the surface of the transducer. Moreover, the chip developed by CSEM has 24 sensing pads which allow multiple measurements with the same transducer. As a consequence of this collaboration it has been possible to develop a biosensor device able to detect SA residues in milk samples in compliance with the EC regulations. Further investigation, has led to the development of a multiplexed biosensor device by combining immunoreagents (SAs and fluoroquinolone antibiotics (FQs)) produced at the Applied Molecular Receptors group (AMRg) of the CSIC, with bioreceptors (ß-lactam antibiotics (BLs) and TCs) provided by UNISENSOR S.A. (Liege - Belgium). The results obtained were very good. About 34 antibiotics from four different antibiotic families can be detected in milk samples following all the EC regulations with the device developed. Before, a multianalyte microplate-based ELISA had been developed combining the same bioreagents to evaluate performance and to establish the most appropriate immunochemical procedures. The immunochemical methods developed within this thesis, including immunoassays and immunosensors, have been preliminarily evaluated in collaboration with the Nestlé Research Centre (NRC), at Lausanne in Switzerland, in order to demonstrate performance in real milk samples.

B OBJECTIVES

B.1 Objectives of this thesis

B.1.1 General objective

The final objective of this thesis has been the development of a multiplexed biosensor device for the analysis of antibiotic residues in food sector. A biosensor is the result of merging the knowledge of different scientific areas, and particularly of the integration of a (bio)chemical and a (micro/nano) technological component (see **Figure B.1**).

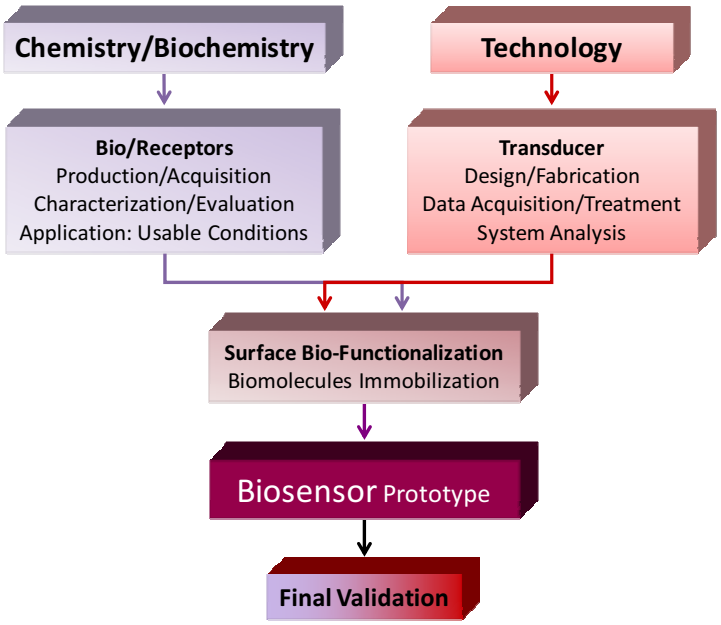


Figure B.1: General scheme of a biosensor development. For the *technology* part it has been crucial the collaboration with other research groups with complementary expertise.

The development of a biosensor requires the integration of biochemistry and technology expertises. Research in biochemistry is addressed to the design, preparation, characterization and evaluation procedures to obtain the specific biological recognition element and the corresponding necessary bioreagents. Further on, these bioreagents can be implemented in sensor devices developed as result of intensive research on microelectronics and other related technological research areas, in which the key element is the design and further fabrication of the transducer which is responsible of providing an indication or a signal processing as result of specific recognition events on its surface. Parallel intense work to produce other biosensor components such as the sample handling, treatment units, electronics or the data acquisition and processing system are

also necessary to reach a robust and reliable device useful in the clinical, food safety, environment or homeland security fields [5].

Research in both biochemistry and technology fields converge towards the development of biofunctional (bio)hybrid materials able to respond selectively to the presence of a target analyte as a single unit. This requires the intimate integration of the biological recognition element on the appropriate designed and developed transducer, making use of suitable well-established surface chemistry procedures. Development of a biosensor is thus a multidisciplinary work for which the expertise of scientists from different fields is required, including chemists, biochemists, physicists, engineers, as well as experts in food sciences, or medicine, depending on the final application field. The research performed in the context of this thesis is at the intersection of all these disciplines and has followed quite faithfully the described scheme which is also summarized as a flowchart in **Figure B.1**, starting from the development of the bioreceptors to their final implementation in an optical transducer integrated on a biosensor device. As a result, a biosensor device able to detect up to 34 antibiotics has been developed being evaluated their performance on milk samples.

Therefore, to accomplish the final objective of this thesis it has been necessary on one side to establish a series of specific chemical and biochemical objectives conducting to the biosensor prototype (see **Figure B.1**) and integrating our achievements on the transducer. For this last activity it has been crucial establishing a tight collaboration between our research group AMR with scientist and institutions from other complementary fields that occurred in the context of 3 different collaborative projects at national and international level.

B.1.2 Specific Objectives

To accomplish the above mentioned general objective, target antibiotic families were selected as a result of a literature search and a small survey conducted among dairy producers, associations and factories at national and international level (see the **Appendix**). The survey aimed to obtain information not only on the most important antibiotics used in this field, but also on the end-user requirements regarding antibiotic residue analysis. In respect of the antibiotics, and their potential residues in food samples, different congeners of SAs, FQs, BLs, and TCs families were identified as the most relevant. On the other hand, the end-users pointed to the necessity of fast and reliable screening analytical methods able to detect a wide number on antibiotic residues in a single run, in order to increase efficiency before the food product arrives to the consumer or to the dairy processing factories (see the **Appendix** for more information).

For the development of a multiplexed biosensor device for the analysis of antibiotic residues in food sector, several key milestones were identified such as the need to obtain or develop of specific receptors, the establishment of reliable analytical methods and procedures using these selective receptors, the demonstration that these receptors are able to detect and quantify the target analytes in real samples, and the possibility of implementation of the selective receptors into a multiplexed analytical sensor platform. According to them, several specific objectives were proposed:

SO1) Preparation and evaluation of specific receptors for antibiotics. With this purpose, it was proposed to investigate two types of specific receptors, from synthetic and natural origin. In respect to the synthetic receptors it was proposed to develop MIPs as an alternative to the most frequently used biological receptors, such as the antibodies. Thus, within this thesis, it has been planned to develop and evaluate MIPs and antibodies for SAs and TCs. Moreover, the necessity to detect a wide range of antibiotics in a single run, pointed out the need to establish as a priority objective, the development of generic selective receptors, or receptors showing a broad recognition profile within the same antibiotic family.

SO2) Evaluation of the performance of the bioreceptors developed to detect and quantify antibiotic residues in food samples. In this respect, milk was proposed as the most important target matrix for these types of residues. Moreover, the analysis of hair extracts has also been considered an interesting sample matrix due to its recognized value to trace veterinary drug treatments performed through the life of the animal.

SO3) Establishment of multiplexed bioanalytical methods based on the combination of several bioreceptors on the sample platform, with the objective to develop a device able to detect a wide range of antibiotics of the most important antibiotic families. With this purpose, bioreceptors with a broad-recognition pattern should be combined into the same bioanalytical assay and its performance to detect a wide range of antibiotics of the four most important antibiotic families (SAs, BLs, FQs and TCs) should be evaluated.

SO4) Implementation of this multiplexed bioanalytical assay on an optical biosensor, by integrating the bioreceptors on the WIOS (wavelength interrogated optical system) transducer, and evaluating its performance in food matrices such as milk.

B.1.3 Context scenario of the thesis

The present research work has been performed in the frame of National and European projects that have supported different aspects of the investigations realized to accomplish the final objective. The following section shows a small summary the projects which have been performed within the present thesis.

B.1.3.1 The ARGOS project

The ARGOS project had as primary objective the development of new technologies to improve the control of FQs and SAs as well as corticosteroids in foodstuffs from animal origin and evaluating the potential of the hair as a matrix useful to track previous treatments of farm animals with these drugs (see **Figure B.2**).

New technologies to immprove the efficiency of the antibiotics and corticosteroids residue controls in foodstuffs from animal origin (ARGOS)

Ministerio de Ciencia y Tecnología (MCYT). Plan Nacional de I+D
Action Line: Programa Nacional de Recursos y Tecnologías Agroalimentarias
Project URL: http://www.iiqab.csic.es/amrg/
COORDINATOR: Prof. Mª Pilar Marco (IQAC-CSIC)

MINISTERIO DE EDUCACION Y CIENCIA

PROJECT DETAILS

Project Acronym: ARGOS
Project Reference: AGL2002-04635-C04-03
Start / End Date: 2002-01-11 / 2005-01-11
Partners: 4
Duration: 36 months
Project Cost: 400.000 €
Contract Type: Integrated Project
Project Status: Completed

Figure B.2: Summary card with primary information about ARGOS project.

The principal aim of the ARGOS project was to provide analytical alternatives to solve some of the limitations of the actual analytical procedures to fulfil with the requirements of the directives 96/23/CE and 2377/90/CE, which care about the frequency or number of substances that have to be monitored and the maximum residue limits (MRLs) established, respectively. The new methodologies to be developed within this project were based on the Antibody-Antigen (Ab-Ag) recognition event to be adapted to the development of several detection techniques, such as ELISA, test-field assays and immunosensors using serigraphiated gold electrodes. The idea was to produce class-selective antibodies against FQs and SAs by preparing immunizing haptens through

rational design of their chemical structure using molecular modelling tools. Moreover, the possibility to develop selective solid-phase extraction procedures based on the preparation of immunosorbents (IS) or molecularly imprinted solid-phase extraction (MISPE) systems for sample treatment was also contemplated. To accomplish these aims, the project consortium was formed by research groups from several disciplines such as the National Reference Laboratory for Foodstuffs (AESAN, Agencia Española de Seguridad Alimentaria y Nutrición), a group specialized on animal nutrition (IRTA, Institut de Recerca i Tecnologia Agroalimentàries) that had access to an experimental farm where controlled real samples from treated and untreated animals were prepared, and one group with known expertise on electrochemical transduction for the analysis of trace elements (GBEQ-UAH, Biosensors Group from University of Alcalá). The AMR group was responsible of the design and preparation of chemical (MIPs) and biochemical (Antibodies) specific receptors, and the development of the immunochemical procedures and of the coordination of the project.

The design, preparation and evaluation of selective receptors for SAs, particularly antibodies and MIPs described in this thesis, were performed in the context of the ARGOS project, in collaboration with Dr. Hector Font and Dr. Raquel Obregón, also from the AMR group. Moreover, preliminary immunochemical determination of SAs residues in hair was realized within the project.

B.1.3.2 The PANOPTES project

The promising results obtained in the ARGOS project encouraged the scientific consortium established previously to continue again in the same direction in the context of a new project. The PANOPTES project had the goal to approach the problems derived from the control of residues of veterinary drugs in food samples with a more global perspective (see **Figure B.3**). The new proposal addressed the development of several novel rapid response immunosensor devices, based on different transducing principles, for the determination of the most important antibiotic families in different points of the food chain. To achieve all these objectives new partners from different research areas joined the initial ARGOS community, CT-IMB (Chemical Transducers Group form the Barcelona Microelectronic Institute) and ICFO (The Institute of Photonic Sciences) providing their expertise in microelectronics and photonic sciences, and the Department of Analytical Chemistry at Universitat de Barcelona (UB) with a recognized expertise in analytical chemistry.

Our research group coordinated this project and, in addition to continue with the investigation on the development of selective receptors (Antibodies and MIPs) for antibiotics (TCs, SAs and FQs), also collaborated with the groups developing technology

(GBEQ-UAH, CT-IMB and ICFO) establishing the necessary chemical procedures to immobilize the biomolecules, of organic nature, on the surface of the transducers, usually of inorganic nature. Moreover, we continued with the production of new immunochemical reagents and MIPs for the detection of chloramphenicol (CAP) and TCs to complement the already developed ones for FQs and SAs families.

Integrated approach to the development of technologies for antibiotic residue control to improve safety from the farm to the table (PANOPTES)

Ministerio de Ciencia y Tecnología (MCYT). Plan Nacional de I+D+I
Action Line: Programa Nacional de Recursos y Tecnologías Agroalimentarias
Project URL: http://www.iiqab.csic.es/amrg/panoptes.htm
COORDINATOR: Dr. F. Sánchez-Baeza (IQAC-CSIC)

PROJECT DETAILS

Project Acronym: PANOPTES
Project Reference: AGL2005-07700-C06-04
Start / End Date: 2006-01-01 / 2009-01-01
Partners: 6
Duration: 36 months
Project Cost: 580.000 €
Contract Type: Integrated Project
Project Status: Completed

Figure B.3: Summary card with primary information about PANOPTES project.

The GBEQ-UAH studied multianalyte configurations based on their experience on the use of amperometric immunosensors constructed as self assembled supramolecular structures for the determination of residues. CT-IMB investigated configurations consisting on arrays of ultra microelectrodes that are characterized for their very short response times. On the other hand, ICFO investigated optical transducer systems based on measuring changes in the dielectric constant of the evanescent field. The suitability of these devices as control tools through the distinct points of the food chain was evaluated according to different features such as portability, response time or detectability. Simultaneously to the development of these immunosensor devices, IRTA prepared controlled real samples by performing appropriate animal treatments with the antibiotics selected. Moreover, UB used all these samples to prepare reference materials, which were necessary for the adequate validation of the new analytical methods developed. Both groups collaborated on the implementation of the corresponding reference analytical methods, preparing extracts compatible (aqueous, hydro alcoholic, etc.) with the new immunochemicals and evaluating with those the selective solid-phase supports (MISPE and IS). The confirmation, at the end of ARGOS project, that remarkable quantities of

antibiotics could be accumulated in pig and calf hair structure of treated animals encouraged the research team to evaluate in this novel matrix the new analytical procedures established. It is worth noting that all these studies were performed under the attentive watch of AESAN, actively involved in ARGOS, and later on in PANOPTES as an external observer, accompanied by other companies and research institutions that were given support with different degrees of implication.

The contribution of this thesis to this project was the design, preparation and evaluation of immunoreagents for TCs. Moreover, a broad selectively indirect ELISA for the SAs residues was developed and evaluated in cattle and pig hair samples collected from treated animals.

B.1.3.3 The GOODFOOD project

This European project aimed at developing a new generation of analytical methods based on micro and nanotechnology solutions for safety and quality assurance along the food chain in the agro food industry (see **Figure B.4**).

Food safety and quality monitoring with microsystems (GOODFOOD)
WP1: Mycrosystems technology solutions for the detection of antibiotic residues

Funded under 6th FWP (Sixth Framework Programme)
Action Line: IST-2002-2.3.1.2 Micro and nano-systems
Project URL: http://www.gooodfood-project.org
COORDINATOR: Prof. Carles Cane (CNM-CSIC)

PROJECT DETAILS

Project Acronym: GOODFOOD
Project Reference: FP6-IST-1-508774-IP
Start / End Date: 2004-01-01 / 2007-09-03
Partners: 29 (WP1: 6)
Duration: 45 months
Project Cost: 17.43 million euro
Contract Type: Integrated Project
Project Status: Completed

Figure B.4: Summary card with primary information about GOODFOOD project.

Current and future concerns related to agro food safety and quality will increasingly require a multidisciplinary and universal approach based on the massive use of simple detection systems able to be used *near to the foodstuff*. As it has been already commented, the technology used nowadays to assess food safety and quality relies on laboratory solutions that are bulky, costly, punctual and time consuming. On the contrary, the GOODFOOD approach intended comply, through the development of innovative

solutions, with the needs of ubiquity, low cost and low power, fast response, simple use and fully interconnection to the decisional bodies. Overall project objectives were firstly to demonstrate to the agro food sector actors (final consumers included) the advantages driven to the complete food chain control by the use of micro and nanotechnology inspired systems, secondly to develop and integrate such systems with improved performances compared to the state of the art, and thirdly to introduce the ambient intelligence paradigm in the agro food chain by developing a vineyard pilot site and by applying the results to the activities of all quality and safety technical solutions of the project.

Thus, about 30 partners from ten different European countries were involved in the GOODFOOD project to evaluate different monitoring scenarios, such as antibiotics, pesticides, mycotoxins and pathogens detection as well as foodstuff emissions and ambient monitoring. A significant part of this thesis was performed in the context of this project within work package 1. Main objective of this work package was developing a portable system for multiple and fast antibiotic residue screening of milk samples at the point of demand like the farm, collection truck or diary. The system was based on an optical biosensor device based on evanescent waves combined with a microfluidics system. UNISENSOR S.A. partner and our group were responsible of providing antibodies and bioreceptors for antibiotics detection. Moreover, both groups worked together in the development of traditional field screening techniques, such as dipsticks or ELISAs. On the other hand, CSEM developed a high sensitivity optical sensor system, named WIOS, using a waveguide sensor chip and a CMOS camera using smart pixel. CEA/LETI, as a major research centre for microelectronics, microtechnologies, optronics and instrumentation in Europe, was the responsible of the microfluidics handling in the system for the sample preparation. The UCC (University College Cork) group assured the electronic development for process management and signal processing of the entire system. Finally, NRC as an end-user provided information on the specifications of the device being also responsible of its final validation.

Based on the knowledge and expertise developed in ARGOS and PANOPTES, the contribution of this thesis to GOODFOOD project was the investigation of the possibility to establish an immunochemical multiplexed procedure for antibiotic residue analysis and its implementation, in collaboration with CSEM, in the multiplexed WIOS device, assessing further on its performance in milk samples.

C STRUCTURE

Attending to the objectives already exposed, this thesis is presented as a compendium of publications. Structurally, it is divided into six chapters, plus an appendix and the final conclusions.

C.1 Structure of this thesis

This chapter shows an overview of how the research work performed and the results obtained are presented in this volume. Basically, **Chapter 1** provides an extensive review of the state of the art on topics such as food safety, particularly for veterinary drug residues, regulations, analytical methods for their control and the new trends regarding development of novel and more efficient analytical approaches based on the use of selective receptors. **Chapters 2** and **3** address the development of broad-selectivity receptors against SAs. **Chapter 2** reports the research work performed to produce and characterize synthetic selective receptors, particularly MIPs, for SAs through a rational approach. **Chapter 3** addresses the same objective but in this case to produce biological receptors, particularly polyclonal antibodies against the same family of antibiotics. The characterization of the antibodies produced has been made by the establishment of ELISAs in different biological samples. **Chapter 4** shows the works carried out to design and prepare polyclonal antibodies against the TCs, including all the difficulties arising while synthesizing of the corresponding haptens and subsequent evaluation of the resultant immunoreagents again by ELISA. **Chapter 5** includes information about fibber-optic biosensor technologies based on integrated optical label-free detection systems. In this sense, we have described how SAs immunoreagents were implemented on the WIOS platform and its subsequent evaluation of the biosensor analyzing milk samples. **Chapter 6** explains the route followed to build a multiplexed WIOS biosensor device to analyze different antibiotics, such as SAs, FQs, BLs and TCs, simultaneously in milk samples, which was required first evaluating performance of the different receptor through the development of a multiplexed microplate-based ELISA. The assays developed were based on ELISA and WIOS analytical techniques. A final **Appendix** includes the results from a survey performed to know the requirements for the optimal detection of antibiotic residues in milk and summarizes the firsts attempts performed during this thesis to develop novel analytical strategies, but complementary to those already described, by labelling the SAs immunoreagents to other units such as magnetic particles, fluorescent dyes or quantum dots (QDs).

The results of this research have been published in several scientific journals of international relevance, as they are indicated in **Figure C.1**.

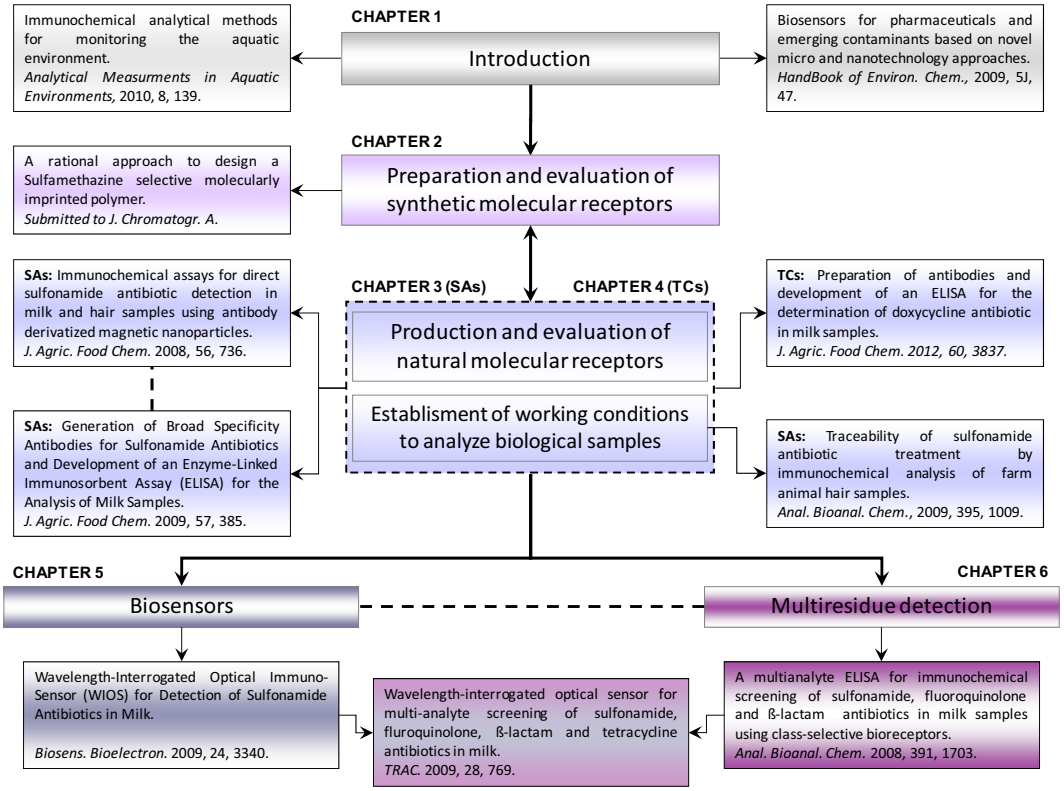


Figure C.1: Flowchart showing the connection between chapters and the corresponding publications.

The following sections intend to provide a general overview of the work described in each chapter and the important results accomplished by inserting the abstracts of the publications.

C.1.1 Chapter 1: Introduction

Two book chapters, related to the state of the art of some of the main topics of this thesis (e.g. immunochemical techniques or biosensors applied to monitor contaminants), have been included in this section to complement the information of the introduction (see **Figure C.2**).

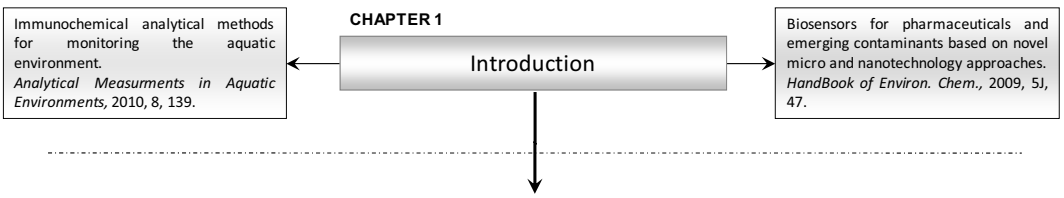


Figure C.2: Scheme of publications related to the introduction section.

C.1.1.1 Immunochemical analytical methods for monitoring the aquatic environment

Javier Adrián, Fátima Fernández, Alejandro Muriano, Raquel Obregon, Javier Ramón-Azcon, Nuria Tort and M.-Pilar Marco *in Analytical Measurements in Aquatic Environments; Namiesnik, J. and Szefer, P., Eds.; CRC Press (Taylor & Francis Group),* 2009; Vol. 5: Water Pollution, Part 5J, pp 139-187. ISBN: 9781843393061.

A review on immunochemical methods for the analysis of pollutants such as industrial contaminants, pesticides, and pharmaceutical and personal care products is presented. A broad range of these pollutants can enter the environment in a great many ways after excretion mainly through industrial, domestic, and hospital wastewater. From there on, the transport, fate, and possible adverse consequences of these pollutants on human health and the ecosystem are frequently unknown or at best not clearly understood. Potential concerns include reproductive impairment, increased incidence of cancer, development of antibiotic-resistant bacteria, or the potentially elevated toxicity of chemical mixtures due to synergistic effects. The aim of regulations and regulatory methods to assess and control the impact of these substances in the aquatic environment is to protect the ecosystem and public health while keeping watch on their contamination levels and potential negative effects. In order to achieve these objectives, more efficient analytical techniques need to be developed. In recent decades, immunochemical techniques have been widely demonstrated to be an interesting alternative to the more conventional analytical methodologies in many areas, but additional work is still necessary to completely adapt them to the analysis of environmental contaminants. On the other hand, considering that the analysis of very complex biological samples with these methods has been successful, the prospects for their application to the analysis of water and soil samples seem highly promising. Technical development should be accompanied by some officially organized efforts to find ways of validating screening immunoassay techniques and recognizing them as practicable routine methods in environmental monitoring laboratories.

C.1.1.2 Biosensors for pharmaceuticals and emerging contaminants based on novel micro and nanotechnology approaches

Javier Adrián, Fátima Fernández, Alejandro Muriano, Raquel Obregon, Javier Ramón-Azcon, Nuria Tort and M.-Pilar Marco *in Biosensors for Environmental Monitoring of Aquatic Systems; Barceló, D. and Hansen, P.-D., Eds.; Springer Berlin: Heidelberg,* 2009; Vol. 5: Water Pollution, Part 5J, pp 47-68. ISBN: 9783540002789.
Digital Object Identifier (DOI): 10.1007/978-3-540-36253-1_3.

The investigation of new sensing principles and technologies for the detection of molecular binding events has created great expectations on numerous major industrial sectors, such as healthcare, food, water and agriculture. Combining many of these advances with the potential of the immunochemical systems has allowed developing novel biosensors that provide interesting advantages against the traditional strategies for analysis, such as the possibility of multianalysis, development of field analytical methods and fabrication of easy end-user devices. Specifically, many efforts have been lately invested to control residues of pharmaceuticals in food and environmental samples, as an indication of the impact of the human activity in the media. Human and veterinary drugs, such as antibiotics, hormones, analgesics, cytostatics or β-blockers, show a high potential risk of negative effects in the

environment and public health. Thus, there is a great need for low-cost and highly efficient tools for quick, reliable, and accurate detection of these contaminating bioactive agents. In particular, the scope of the present chapter is addressed to provide an overview of the potential of novel micro(nano)technology approaches to develop biosensors useful for the analysis of emerging pollutants.

C.1.2 Chapter 2: Synthetic molecular receptors

This section includes a short introduction about the most important and fundamental aspects related the synthesis and recognition mechanisms by imprinted polymers focusing in those ruled by non-covalent interactions. The chapter includes a publication submitted in which the rational approach applied for the preparation and evaluation of a sulfamethazine (SMZ) MIP is reported (see **Figure C.3**).

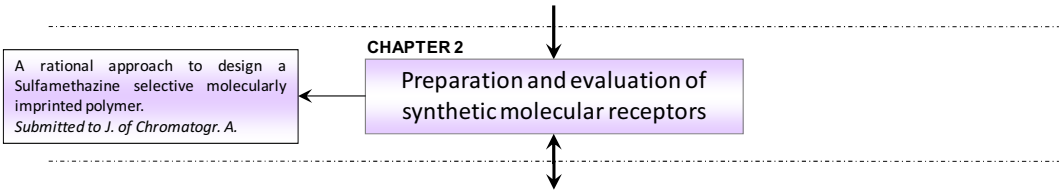


Figure C.3: Scheme of publications related to the synthetic receptors section.

C.1.2.1 A rational approach to design a sulfamethazine selective molecularly imprinted polymer

Javier Adrián, Raquel Obregon, M.-Pilar Marco and F. Sánchez Baeza, *submitted to Journal of Chromatography A*.

A rational development of a MIP for SMZ is described. Theoretical computational and experimental ^{1}H-NMR studies about complex formation between the template and different functional monomers were performed as a previous step to a MIP preparation. A good correlation between both techniques was found offering preliminary information about most convenient reagents and pre-polymer mixture composition to be used in order to obtain a suitable imprinted material. Synthesis on a small scale of a variety of MIPs, using monomers previously selected in the rational design by changing the porogen and mixture proportion, were prepared and evaluated by diverse molecular recognition experiments. This methodology pretends to select, from a large collection of MIPs prepared, the ones with better application properties. It is based on several assays, increasing the complexity, having fewer candidates in each step and with enough efficiency for being introduced in a combinatorial strategy for new MIP development with appropriate affinity and selectivity properties. Finally, affinity and specificity of the MIP not excluded in previous experiments, using SMZ as the template prepared with methacrylic acid and 4-vinylpyridine in acetonitrile, were evaluated by frontal chromatography and batch rebinding experiments.

Keywords: sulfamethazine, antibiotics, molecularly imprinted polymers, rational design.

C.1.3 Chapters 3 and 4: Natural molecular receptors

Main topics of this section have consisted in the explanation of how the production of broad-recognition antibodies for SAs and TCs has been approached, based on a rational hapten design. The preparation and evaluation of the polyclonal antibodies produced is described. The posterior analysis of biological samples shows the features and potential of these immunoreagents. Some of the protocols published (see **Figure C.4**) were really useful for the subsequent development of the bioanalytical techniques developed in this thesis, such as biosensors and multiplexed systems.

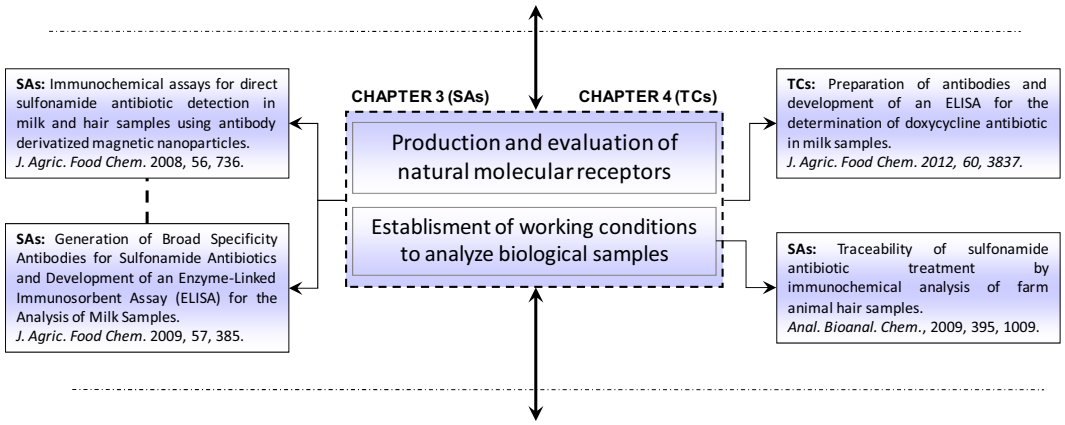


Figure C.4: Scheme of publications related to the natural receptors section.

C.1.3.1 Immunochemical assays for direct sulfonamide antibiotic detection in milk and hair samples using antibody derivatized magnetic nanoparticles

Hector Font, Javier Adrián, Roger Galve, M.-Carmen Estévez, Massimo Castellari, Marta Gratacós-Cubarsí, Francisco Sánchez-Baeza, and M.-Pilar Marco. *Journal Agricultural and Food Chemistry* 2008, 56, 736-743.
DOI: 10.1021/jf072550n.

Two direct ELISAs have been developed for detection of SAs residues in milk samples. One of them is using magnetic nanoparticles for target capture/enrichment, and the second is performed using microtiter plates. Selective polyclonal antibodies, raised against 5-[6-(4-amino-benzenesulfonylamino)-pyridin-3-yl]-2-methyl-pentanoic acid (SA1), used in combination with an enzyme tracer prepared with the same hapten, has allowed us to reach a limit of detection lower than 0.5 μg L^{-1} for both ELISA formats. Sulfapyridine, sulfamethoxypyridazine, sulfathiazole, and sulfachloropyridazine are detected below the MRLs established by the European Union for these antibiotics in milk (100 μg L^{-1}). Matrix effects and accuracy studies performed with full-cream milk and hair extracts indicated a lack of interference from these sample matrices and very good recovery values, especially when using the antibodies coupled to magnetic nanoparticles format. Milk samples and hair extracts

can be measured without any previous treatment. The results demonstrate the high potential of these methods as screening tools for food safety and inspection controls.

Keywords: Sulfonamide antibiotics, sulfapyridine, immunoassay, magnetic particles, milk, hair.

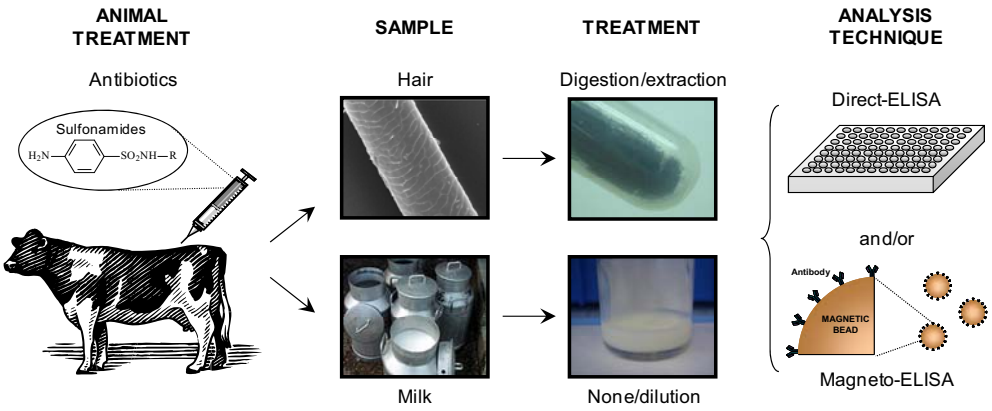


Figure C.5: TOC figure of the manuscript.

C.1.3.2 Generation of broad specificity antibodies for sulfonamide antibiotics and development of enzyme-linked immunosorbent assay for the analysis of milk samples

Javier Adrián, Héctor Font, Jean-Marc Diserens, Francisco Sánchez-Baeza, and M.-Pilar Marco.
Journal Agricultural and Food Chemistry. 2009, 57, 385-394.
DOI: ***10.1021/jf8027655***.

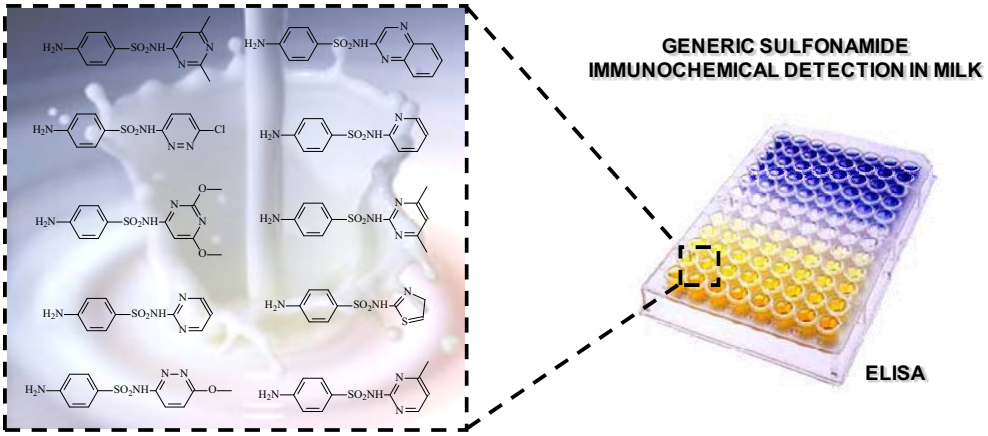


Figure C.6: TOC figure of the manuscript.

Immunoreagents appropriately produced to detect a wide range of SAs have been used to develop a highly sensitive ELISA. The selectivity has been achieved by combining antibodies raised against SA1**,** covalently coupled to horseshoe crab hemocianyn, and *5-[4-(amino)phenylsulfonamide]-5-oxopentanoic acid* (SA2) coupled to ovalbumin, on an indirect ELISA format. The immunizing hapten has been designed to address selectivity against the

common aminobenzenesufonylamino moieties, using theoretical calculations and molecular modelling tools. Hapten SA1 has been synthesized in 4 steps from methyl 5-(4-amino-3-pyridinyl)-2-methyl-4-pentenoate through a Heck reaction, under Jeffery conditions, to avoid introduction of additional epitopes in the linker. The microplate immunoassay developed is able to reach the necessary detectability for the determination of the SAs most frequently used in the veterinary field, in compliance with the 2377/90 EC Regulation. As an example, the IC_{50} and limit of detection values accomplished for sulfapyridine are 2.86 ± 0.24 and 0.13 ± 0.03 μg L^{-1}, respectively. Studies performed with different types of milk samples demonstrate that direct and accurate measurements can be performed in this type of matrix without any previous sample clean-up method.

Keywords: Sulfonamide antibiotics, hapten, molecular modelling, immunoassay, milk, class-selective antibodies.

C.1.3.3 Traceability of sulfonamide antibiotics treatment by immunochemical analysis of farm animal hair samples

Javier Adrian, Marta Gratacós-Cubarsí, Héctor Font, Massimo Castellari, Francisco Sánchez-Baeza, and M.-Pilar Marco. *Analytical and Bioanalytical Chemistry*, 2009, 395, 1009-1016.
DOI: ***10.1007/s00216-009-2878-6***.

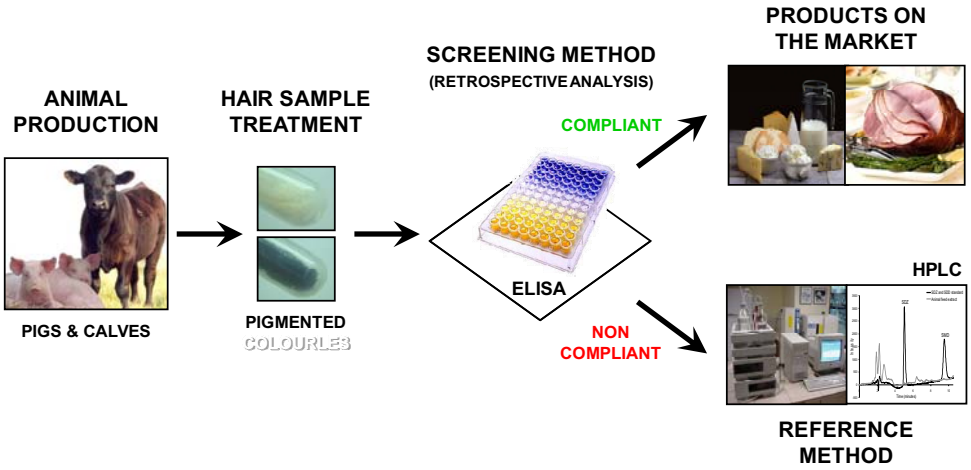


Figure C.7: TOC figure of the manuscript.

The use of hair to trace use of unauthorized substances, therapeutic agents or their misuse is becoming very attractive since residues can be detected for a long time after treatment. For this purpose, an indirect ELISA has been evaluated for its capability to trace SAs treatment by analyzing cattle and pig hair samples. Pigmented and non-pigmented hair samples from control and SMZ treated pigs and calves were collected, extracted under different alkaline conditions and analyzed by ELISA after just diluting the extracts with the assay buffer. Data analysis following the European recommendations for screening methods demonstrates that the ELISA can detect SMZ in hair samples with a limit of detection (90% of the zero dose (IC_{90})) between 30 and 75 ng g^{-1}. Same samples have been analyzed by high performance liquid chromatography after a dual solid-phase extraction. The ELISA results matched very well those obtained by the chromatographic method, demonstrating that the immunochemical

method can be used as a screening tool to trace animal treatments. Between the benefits of this method are the possibility to directly analyze hair extracts with sufficient detectability and its high-throughput capability. Preliminary validation data are reported using an experimental approach inspired on the Commission Decision 2002/657/EC criteria for screening methods.

Keywords: Sulfonamide antibiotics; Sulfamethazine, Hair analysis; ELISA; immunoassay; HPLC-DAD, treatment traceability.

C.1.3.4 Preparation of antibodies and development of an ELISA for the determination of doxycycline antibiotic in milk samples

Javier Adrián, Fátima Fernández, Francisco Sánchez-Baeza, and M.-Pilar Marco. *Journal Agricultural and Food Chemistry* 2012, 60, 3837-3846.
DOI: ***10.1021/jf2053355***.

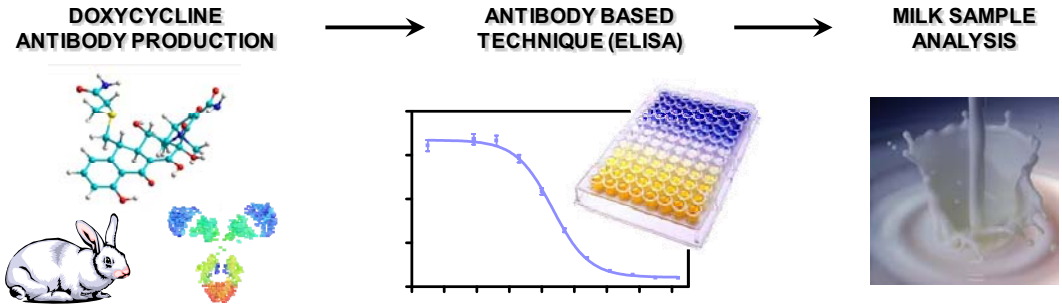


Figure C.8: TOC figure of the manuscript.

This paper reports the development of an immunoassay for the specific analysis of the doxycycline, a congener of the TCs, in milk samples. This is the first time that doxycycline antibodies production is reported, based on a rationally designed and well-characterized immunizing hapten. The chemical structure of the immunizing hapten (*13-[(2-carboxyethyl)thiol]-5-hydroxy-6-α-deoxytetracycline*, TC1) was designed to maximize recognition of the tetracycline characteristic moiety defined as lower periphery of the TCs plus the region of the upper periphery comprised by the hydroxyl group at position C_5 (B ring) and the dimethylamino group in ring A. Polyclonal antibodies raised against TC1 coupled to horseshoe crab hemocianyn were used to develop a homologous indirect competitive ELISA. The microplate ELISA can detect doxycycline in buffer down to 0.1 µg L^{-1}. The ELISA has been proven to tolerate a wide range of ionic strengths and pH values. The assay is very selective for doxycycline with a minor recognition of methacycline (32% of cross reactivity). Experiments performed with whole milk samples demonstrate that samples can be directly analyzed after a simple treatment method reaching detectability values below 5 µg L^{-1}.

Keywords: Doxycycline; immunoassay; ELISA; milk; tetracyclines.

C.1.4 Chapter 5: Development of biosensors

This section initially describes essential information related to optical biosensors followed by the most important fundamental aspects of the WIOS platform, such as the

principle of detection used, biofunctionalization of the sensing layers or the features of the instrument itself. The publication included in this chapter demonstrates, as a proof of concept, the possibility to integrate the previously described immunoreagents for SAs into the WIOS detection system and the subsequent application to the analysis of milk samples (see **Figure C.9**).

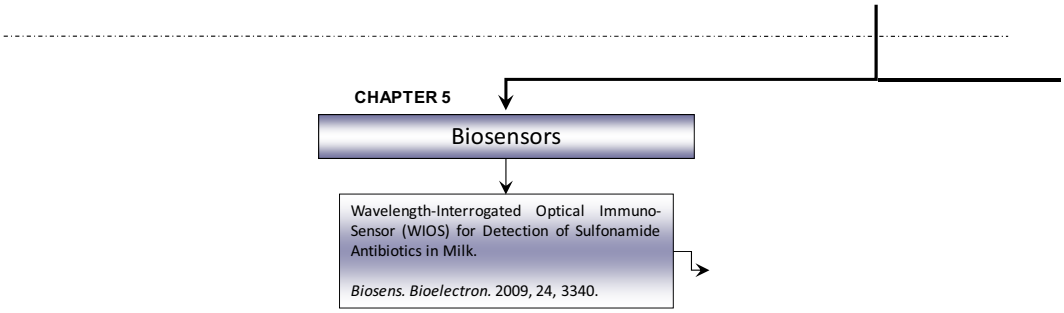


Figure C.9: Scheme of publications related to the biosensor section.

C.1.4.1 Waveguide interrogated optical immunosensor for detection of sulfonamide antibiotics in milk

Javier Adrián, Stéphanie Pasche, Jean-Marc Diserens, Francisco Sánchez-Baeza, Hui Gao, M.-Pilar Marco and Guy Voirin. *Biosensors and Bioelectronics* 2009, 24, 3340-3346.
DOI: 10.1016/j.bios.2009.04.036.

An immunosensor was developed for the detection of SAs in milk. Detection relied on a competitive immunoassay format, using immunoreagents previously developed for the generic detection of SAs and evaluated by enzyme-linked immunosorbent assay. The immunoassay was implemented onto a microsystem platform, the wavelength interrogated optical sensing device, which uses the evanescent field to probe changes at the interface of a waveguiding layer, and thus allows sensitive detection of biomolecule adsorption. The immunoreagents were immobilized onto the surface of the waveguide chip, and a fluidic cell allowed flowing analyte and detection solutions above the surface. Sulfapyridine was used as reference SAs and detected with the immunosensor in buffer and in milk with a limit of detection (IC_{90}) of 0.2 ± 0.1 μg L^{-1} and 0.5 ± 0.1 μg L^{-1}, respectively. The analysis time was below 30 min, including regeneration of the sensing surface, with minimum sample preparation required. The reproducibility of the detection was better than 10%. A blind assay allowed identifying milk samples that were contaminated with different SAs at or above the MRLs established by the EU for these compounds (100 μg L^{-1}). Thus, the developed immunosensor presents great potential as a generic sensing device for the fast and early detection of food contaminants on the field by non-skilled users.

Keywords: Sulfonamide antibiotics; immunoassay; label-free optical biosensor; milk contamination; class-selective antibodies.

C.1.5 Chapter 6: Multianalyte detection approaches

Finally, with the objective to develop a multiplexed platform, on a first instance the performance of a combination of broad-selectivity bioreceptors into the same platform was assessed through the development of a multiplexed ELISA able to detect SAs, FQs and BLs simultaneously in the same microplate. Subsequently, a combination of bioreceptors was also implemented into the WIOS platform (see **Figure C.10**). Those multiplexed detection platforms for antibiotics developed in the framework of the GOODFOOD project (i.e. ELISA, WIOS and dipstick systems) were evaluated in a small inter-lab study performed at the NRC in Lausanne. The results of this preliminary this comparative exercise are also included at the end of this section.

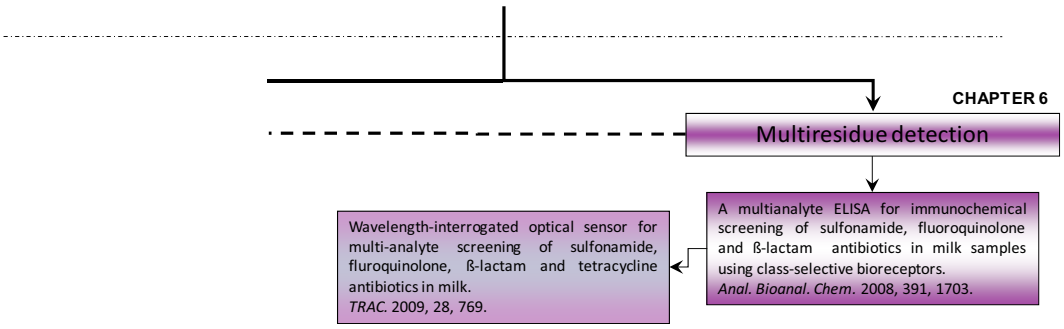


Figure C.10: Scheme of publications related to the multianalyte detection section.

C.1.5.1 A multianalyte ELISA for immunochemical screening of sulfonamide, fluoroquinolone and ß-lactam antibiotics in milk samples using class-selective bioreceptors

Javier Adrián, Daniel G. Pinacho, Benoit Granier, Jean-Marc Diserens, Francisco Sánchez-Baeza, and M.-Pilar Marco. *Analytical and Bioanalytical Chemistry* 2008, 391, 1703-1712.
DOI:* *10.1007/s00216-008-2106-9.

A multianalyte ELISA has been developed for the simultaneous determination of the most frequently used antibiotic families in the veterinary field following the typical planar microarray configuration, where the identity of the target analyte is encoded by its location in the detection platform (Master et al. in Drug Discovery Today 11:1007–1011, 2006). To accomplish this aim, two individual enzyme-linked immunosorbent assays for SAs and FQs and an enzyme-linked receptor assay for BLs have been combined. The strategy uses microplates coated with the corresponding haptenized proteins in specific sections of the microplate. The samples are mixed with a cocktail containing the bioreagents, and distributed in the wells of the microplate. Identification of the antibiotic present in a particular sample is consequently accomplished by detecting a positive response on the corresponding microplate section. Since the bioreceptors used show a wide recognition of the congeners of each antibiotic family, the multianalyte method is able to detect more than 25 different antibiotics from the three most important antibiotic families. The detectability reached in full-fat milk

samples is below the European MRLs. The accuracy and reliability of this multiplexed bioanalytical method have been demonstrated by analyzing blind spiked samples.

Keywords Multianalyte detection, enzyme-linked immunosorbent assay, enzyme-linked receptor assay, antibiotic residues, sulfonamides, fluoroquinolones, ß-Lactams.

C.1.5.2 Wavelength-interrogated optical biosensor for multi-analyte screening of sulfonamide, fluoroquinolone, beta-lactam and tetracycline antibiotics in milk

Javier Adrián, Stéphanie Pasche, Daniel G. Pinacho, Héctor Font, Jean-Marc Diserens, Francisco Sánchez-Baeza, Benoit Granier, Guy Voirin and M.-Pilar Marco. *Trends in Analytical Chemistry* 2009, 28, 769-777.
DOI: 10.1016/j.trac.2009.04.011.

Major research efforts are focusing on the development of multi-analyte-residue analysis and the design of user-friendly analytical devices for continuous or on-site measurements. Advances in microtechnology and nanotechnology make possible novel analytical solutions to meet these needs. With specific bioreceptors and antibodies, immunosensors may become excellent analytical tools. A portable WIOS exploits class-selective bioreceptors for simultaneous screening of the most frequently used antibiotics in the veterinary field (e.g., SAs, FQs, BLs and TCs). The label-free sensor uses the evanescent-wave principle, by which changes in the refractive index close to the modified chip surface are detected by scanning the resonance condition at which a light wave is coupled in the waveguide through a conveniently designed grating. The bioreagents used in this study were developed to detect a wide range of congeners of each selected family of antibiotics below the MRL values established for milk samples. The WIOS made it possible to detect more than 30 different antibiotics and it was successfully applied to analyze different antibiotic residues in milk samples.

Keywords: ß-lactams, Biosensor, Evanescent-wave sensor, Fluoroquinolone, Integrated optical sensor chip, Label-free sensing, Milk, Multi-analyte antibiotic detection, Sulfonamide, Tetracycline.

C.2 Other outputs not included in this thesis

The publications cited below are shown as an indication of the impact of the results of this research in other research projects of the AMR group.

C.2.1 Book Chapters

- **Procedure 34 Electrochemical determination of sulfonamide antibiotics in milk samples using a class-selective antibody.** Emanuela Zacco, Roger Galve, Javier Adrián, Francisco Sánchez-Baeza, M.-Pilar Marco, Salvador Alegret and María Isabel Pividori. *In Electrochemical sensor analysis. Comprehensive Analytical Chemistry series, vol. 49, e237-e241 (2007). Elsevier.*
  DOI: 10.1016/S0166-526X(06)49077-2.
- **Chapter 2.8: Application of bioassays/biosensors for the analysis of pharmaceuticals in environmental samples.** J.-Pablo Salvador, Javier Adrián, Roger Galve, Daniel G. Pinacho, Mark Kreuzer, Francisco Sánchez-Baeza and M.-Pilar Marco. *In Analysis, fate and removal of pharmaceuticals in the water cycle. Comprehensive Analytical Chemistry series, vol. 50, 279-334 (2007). Elsevier.*
  DOI: 10.1016/S0166-526X(07)50009-7.

C.2.2 Journal Publications

- **Electrochemical magneto immunosensing of antibiotic residues in milk.** Emanuela Zacco, Javier Adrián, Roger Galve, M.-Pilar Marco and Maria Isabel Pividori. *Biosensors and Bioelectronics 2007, 22, 2184-2191.*
  DOI: 10.1016/j.bios.2006.10.014.
- **Three-dimensional interdigitated electrode array as a transducer for label-free biosensors.** Andrey Bratov, Javier Ramón-Azcón, Natalia Abramova, Angel Merlos, Javier Adrián, Francisco Sánchez-Baeza, M.-Pilar Marco and Carlos Domínguez. *Biosensors and Bioelectronics 2008, 24, 729-735.*
  DOI: 10.1016/j.bios.2008.06.057.
- **Validation of an Enzyme-Linked Immunosorbent Assay for Detecting Sulfonamides in Feed Resources.** Vanesa Jiménez, Javier Adrián, Jacinto Guiteras, M.-Pilar Marco and Ramon Companyó. *Journal of Agricultural and Food Chemistry 2010, 58 (13), 7526-7531.*
  DOI: 10.1021/jf1011616.
- **Quantum dot-based array for sensitive detection of Escherichia coli.** Nuria Sanvicens, Nuria Pascual, María Teresa Fernández-Argüelles, Javier Adrián, José Manuel Costa- Fernández, Francisco Sánchez-Baeza, Alfredo Sanz-Medel, and M.-Pilar Marco. *Analytical and Bioanalytical Chemistry 2011, 399 (8), 2755-2762.* DOI: 10.1007/s00216-010-4624-5.

C.2.3 Patents

- **Compuestos derivados de doxiciclinas como haptenos, conjugados y anticuerpos de los mismos, y método inmunoquímico para la detección de doxicilina.** M.-Pilar Marco, Javier Adrián, Francisco Sánchez-Baeza (Consejo Superior de Investigaciones Científicas, CSIC). Application number: P201230378 (ES, Spain).

C.3 Bibliography of Chapters A, B and C

[1] Harrison, P. T. C. Links between environment and health: possible future directions. *Sci. Total Environ.* **2000**, *249*, 103-105.

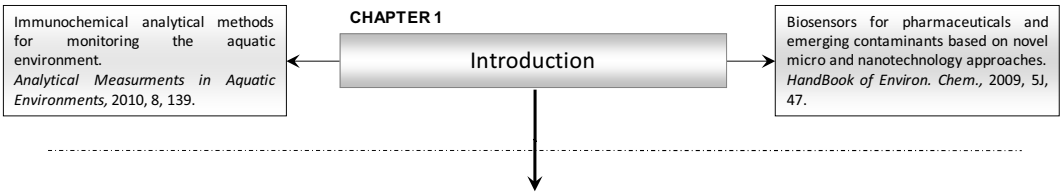
[2] Kemper, N. Veterinary antibiotics in the aquatic and terrestrial environment. *Ecol. Indic.* **2008**, *8*, 1-13.

[3] Catry, B., Laevens, H., Devriese, L. A., Opsomer, G. and Kruif, A. Antimicrobial resistance in livestock. *J. Vet. Pharmacol. Ther.* **2003**, *26*, 81-93.

[4] Aarestrup, F. M. Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. *Basic Clin. Pharmacol. Toxicol.* **2005**, *96*, 271-281.

[5] Velasco-Garcia, M. N. and Mottram, T. Biosensor Technology addressing Agricultural Problems. *Biosystems Eng.* **2003**, *84*, 1-12.

1 STATE OF THE ART



As early as the 1950s, environmental chemists had focused on the study of synthetic or natural chemicals derived from agriculture (e.g. fertilizers, pesticides), the chemical industry (e.g. organohalogenated compounds, heavy metals) as well as factory wastes and secondary by-products (e.g. dioxins, furans) [1]. The focus of attention since the 1970s, of both European Union and United States environmental pollution control programmes, has been devoted to the conventional priority pollutants especially those collectively referred to as *persistent bioaccumulative toxic*, *persistent organic pollutants* and other *bioaccumulative chemicals of concern* [2]. Until the 1990s, non-conventional pollutants were largely ignored mainly because their higher water solubility, relative to other contaminants, complicated their chemical analysis, made them more easily degraded preventing their escape to the atmosphere [3]. A significant portion of these important *unrecognized* or *emerging* pollutants are now widely used in everyday urban activities and includes various pharmaceutical and personal care products (PPCPs).

These PPCPs are a diverse group of chemicals mainly used externally or ingested by humans and domestic animals. They include substances such as human and veterinary drugs, diagnostic agents, nutraceuticals, certain feed and food additives, sunscreen agents, fragrances, cosmetic additives and numerous others, many of which possess profound biochemical activity [4]. So, these compounds have probably been present in water and the environment for as long as humans have been using them. In 2007, Daughton suggested that there might be over 14 million of PPCP substances commercially available worldwide representing a 40 % increase over the prior year [5]. Of these, fewer than a quarter million were regulated by numerous government bodies (less 1.8 % of those found in the market). Thousands of tons of these pharmacologically active substances are used yearly to treat illnesses, to prevent unwanted pregnancy, or to face the stress of modern life. The origins of PPCPs as trace environmental pollutants results largely from their global continual usage by humans and domestic animals as a result of ingestion/excretion as well as the purposeful direct disposal of expired or unwanted PPCPs. Other potential routes to the environment include leaching from municipal landfills, hospital discharges, runoff from confined animal feeding operations (CAFOs), loss from aquaculture, spray-drift from agriculture and oral contraceptives used as soil amendment [6]. The principal pathways of PPCPs into the environment are summarized in **Figure 1.1**.

Most of these substances are entering or are discharged to water and air resources without regulated controls. The prime sources discharge contaminated effluent to both the surface water and foul sewage network with only the latter effluent being likely to receive appropriate treatment. On the other hand, wastewater treatment plants (WWTPs) are often not yet adapted to completely remove them, and therefore these new compounds can be

found to some extent in wastewater effluents as well as in soil and sludge. With advances in technology that improve the ability to detect and quantify these chemicals, we can now begin to control and identify what effects, if any, these chemicals have on human and environmental health. In Europe, regulations and regulatory methods to assess and control the impact of these substances in the entire environment, aim to protect the ecosystem and the public health while keeping a watch of the contamination levels and the potential negative effects.

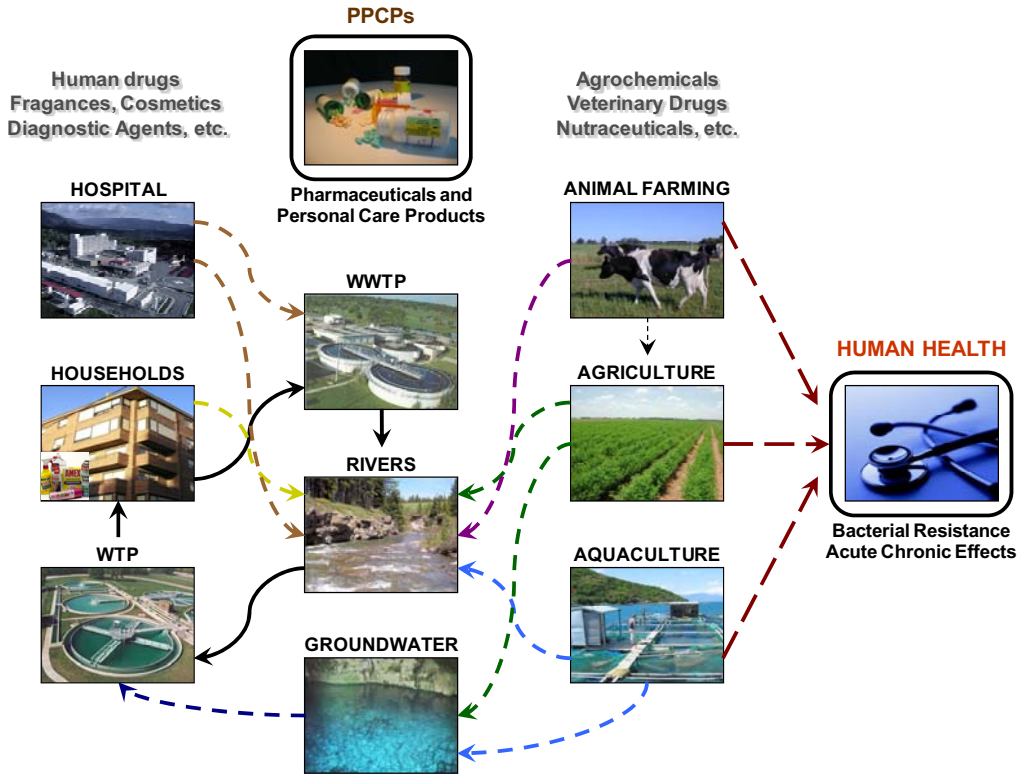


Figure 1.1: Sources and pathways of PPCPs in the environment cycle. (WWTP, WTP: Water treatment plants).

Additional information about PPCP compounds and other industrial contaminants, their regulations and current immunochemical methods to detect them in environmental samples has been included at the end of this section as book chapter publications (see **Section 1.8**).

1.1 Veterinary drug residues in food

In modern intensive farming systems the introduction of several PPCPs, like veterinary pharmaceuticals and pesticides, has been the key element to reach a very high productivity. For example, veterinary drugs are being used, on a large scale and administered as feed additives or via the drinking water, in order to prevent the outbreak of diseases improving health and quality of life of the animals but also increasing the density of population in farms [7]. On the other hand, pharmaceuticals have also been used for non therapeutic treatments, such as prophylaxis or growth promotion (i.e. hormones and certain veterinary drugs, mainly antibiotics). Thus, the excesive improper use of antibiotics has contributed to the remarkable increase of cases of resistance to antibiotic treatments in infections in humans [8]. For this reason, the European Union biggest focus of concern has been centered in the use and control of antibiotics and compounds with hormonal activity, particularly in food animal species, by issuing several Regulations and Directives.

1.1.1 History of antibiotics

Among the diverse groups of pharmaceuticals, antibiotics have played a crucial role for the humanity since their discovery. These kind of antimicrobials are substances that are able to suppress or kill the growth of microorganism, such as bacteria, fungi or protozoa, being an essential part of modern human and veterinary medicine as well as in aquaculture or even in plants for the treatment of infectious diseases [9]. Antibiotic therapy began with the clinical use of SAs against certain bacteria in 1936 and was followed by the development of BLs (1940), TCs (1948), CAP (1949), aminoglycosides (1950), macrolides (1952), glycopeptides (1958), FQs (1980), and others (see **Figure 1.2** for the chemical structures).

Since the middle 1940s, and in parallel with the successful use of antibiotics in human medicine, veterinary use has also provided control of diseases in animal farms. At the annual meeting of the American Chemical Society in Philadelphia in 1950, scientists announced the discovery that continuous sub-therapeutic doses of antibiotics made chickens grow faster [10]. By 1951, the United States Food and Drug Administration (FDA) agency approved the addition of penicillin and TCs to chicken feed as growth promoters, encouraging pharmaceutical companies to mass-produce antibiotics for animal agriculture [11]. This was followed by their frequently use as prophylactics for prevention purposes in many countries, although at present this practice is becoming increasingly controversial and even forbidden in some areas.

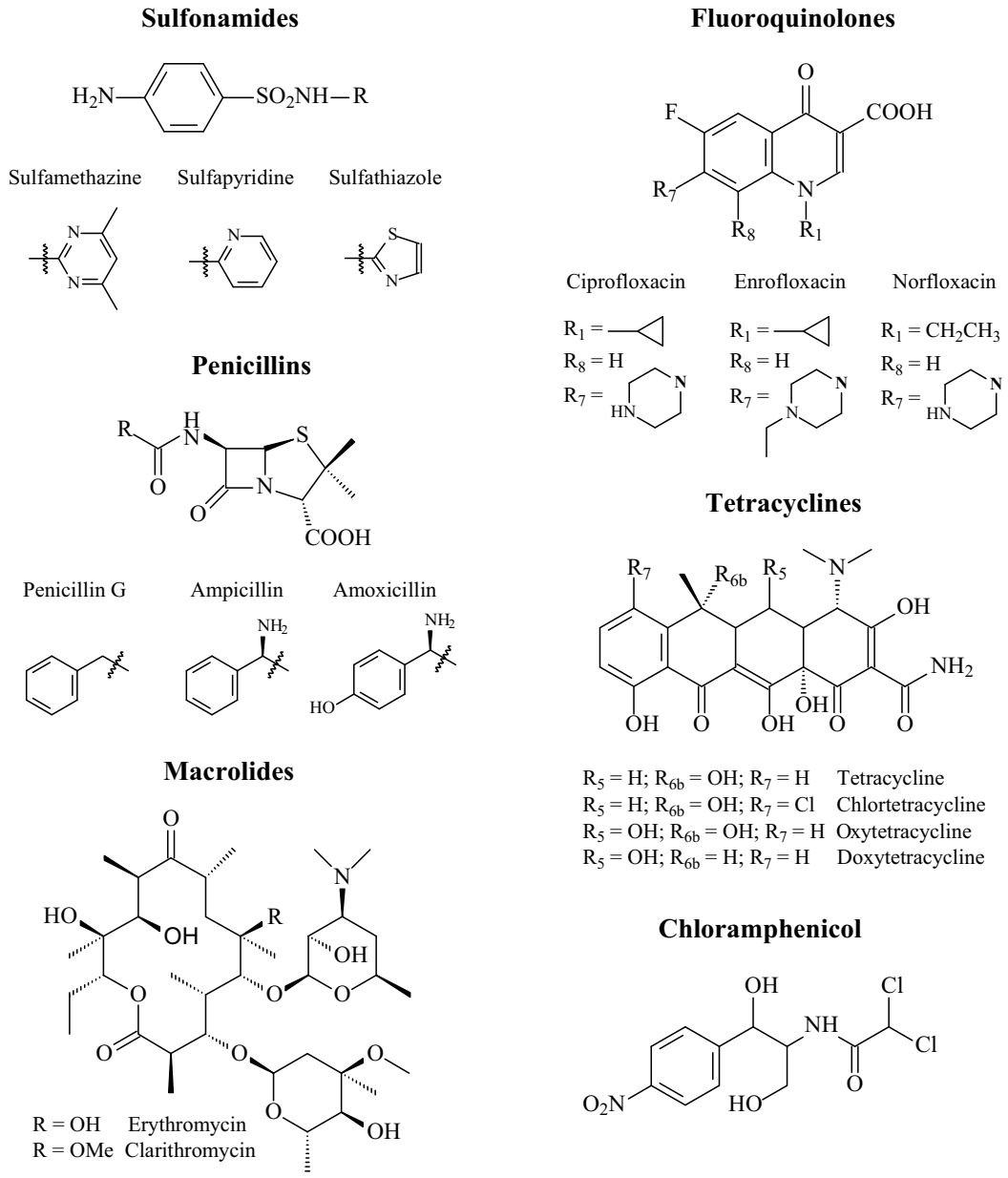


Figure 1.2: Chemical structures of some of the most important antibiotics used nowadays divided into the most representative families including chloramphenicol.

Nobody knows precisely what volume of antibiotics is used worldwide today, but as can be observed in **Figure 1.3**, the international pharmaceutical sales amounted USD (United States dollar) 712 billion in 2007 [12]. During the last decade, about 13.000 tons of antibiotics were consumed each year in Europe and has accelerated rapidly.

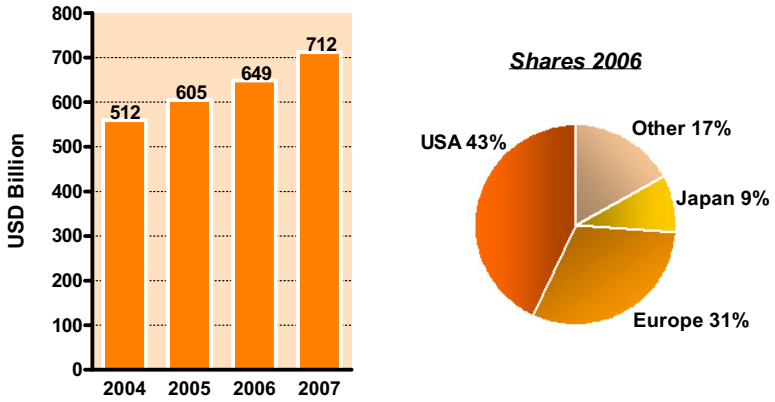


Figure 1.3: The pharmaceutical market worldwide. Sales at manufacturers' prices in the pharmacy market. Source: International Marketing Services Health and German Association of Research-Based Pharmaceutical Companies (Verband Forschender Arzneimittelhersteller e.V).

According to this data, less than a half were due to veterinary purposes (4.000 tons prophylaxis and therapy, and growth promotion about 1.000 tons) being the rest used in medicine. The *European Surveillance of Antimicrobials Consumption* project has recently been able to deliver outpatient data on antibiotic use reporting that all kind of BLs, FQs, macrolides, and aminoglycosides are the most frequently used [13]. On the other hand, among the antimicrobials used in veterinary practice, penicillins, SAs, and TCs (also applied in aquaculture) are the most frequently administered. Unfortunately, although reporting systems have recently been implemented in most of European countries, nothing similar exists for the quantity of veterinary drugs used in food animals in the United States. That's critical because to better understand the human health consequences of the use of antimicrobials, it's very important to evaluate the quantity used in food animals.

Moreover, a number of authors and international health institutions have identified uncontrolled human self-medication and over-the-counter consumption of antibiotics as an extra factor to be considered regarding the improper use of antibiotics [14]. Although community pharmacies in most European countries are not legally allowed to dispense antibiotics without a prescription, this is a common practice at least in some of the European countries. Curiously, data provided by *International Medical Statistics* suggested that total outpatient antibiotic consumption in Spain was among the highest in Europe presenting acute rates of self-antibiotic and over-the-counter consumption in comparison with other European countries [15]. Regarding this, international governmental authorities and well-being care organizations can put a lot pressure on foodstuff producers and farm veterinaries but general health safety begins with the individual actions of each person.

1.1.2 Misuse of antibiotics

The most important negative impact of the general misuse of antibiotics is related to the development of resistance mechanisms on the treatment of infectious diseases. In fact, Alexander Fleming, the discoverer of penicillin, commented in the New York Times in 1945 that "...the microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out which can be passed to other individuals and from them to others until they reach someone who gets a septicemia or a pneumonia which penicillin cannot save" [16]. Antibiotic resistance may be viewed as the ability of microorganisms of a certain species to withstand the presence of a concentration of an antibiotic that is usually sufficient to inhibit or kill bacteria of the same species [17]. Microbial resistance to antibiotics evolves inherently via natural selection acting upon random mutation, but it can also be engineered by applying an evolutionary stress on a population. Once such a gene is originated, bacteria can then transfer the genetic information in a horizontal fashion, between individuals, by plasmid exchange (see **Figure 1.4**).

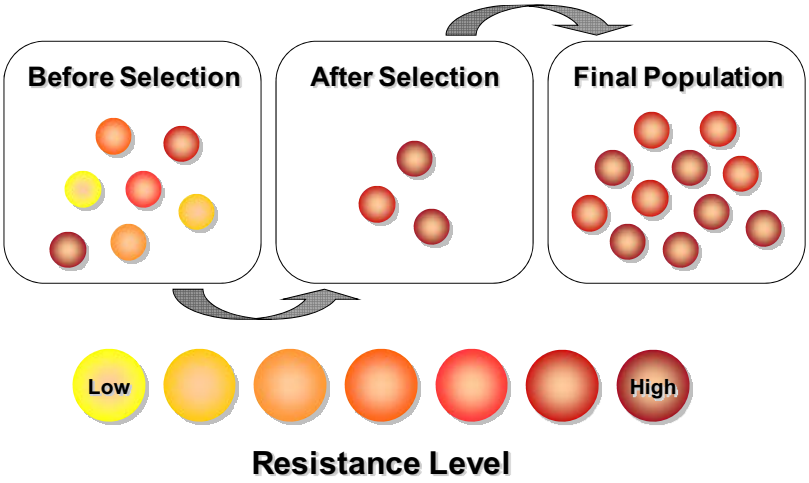


Figure 1.4: Schematic representation of how antibiotic resistance evolves via natural selection. First section represents a population of bacteria before exposure to an antibiotic. The last one shows the distribution of resistance in a new generation of bacteria. *Source: self-made but partially reproduced image from Wikipedia (antibiotic resistance article).*

In this sense, two conditions are needed for antibiotic resistance to develop in bacteria. First, the organism must come into contact with the antibiotic. Then, resistance against the agent must develop, along with a mechanism to transfer the resistance to daughter organisms or directly to other members of the same species [18]. Once arrived at the point, where the bacterial population comes to consist largely or entirely of resistant bacteria, traditional treatments based on presently known antibiotics start to fail. The most notable example of this phenomenon is methicillin-resistant *Staphylococcus aureus*

(MRSA), the first clinical isolate of which was reported as early as 1961, just one year after the launch of methicillin [19]. Since then, MRSA has gradually disseminated, and in the 1970s began causing serious hospital infections worldwide [20]. Regarding the veterinary field, this situation was first documented in 1963, when increased levels of resistance in a particular strain of *Salmonella typhimurium* were observed at several British feedlots [21]. From then on, many examples of antibiotic-resistant microorganism have been emerged. It has been reported that nowadays more than 70% of bacteria are insensitive against at least one antibiotic, causing a serious situation threat for public health [22].

Resistance to antimicrobials is a natural biological phenomenon that can be amplified or accelerated by a wide variety of factors. However, if the use of antibiotics in human medicine is the main source of resistance in the human population, but as antibiotics are extremely active at low doses, direct contact with animals and the consumption of contaminated food of animal origin are recognised to be the main routes of transfer of resistance from animals to humans. Other factors contributing towards resistance include incorrect diagnosis, unnecessary prescriptions, improper use of antibiotics by patients, the impregnation of household items and children's toys with low levels of antibiotics, and the already cited use of antibiotics as livestock food additives for growth promotion [23]. On the other hand, the relative contributions of each of these sources as well as the different routes of transfer remain to be fully understood.

The development of resistance can be minimised provided that a number of measures are observed to prolong the useful life of all antibiotics in both human and veterinary medicine. Antibiotic use should be limited to situations where they are needed and the selection of the right antibiotic should take a number of factors into account. A prudent employ of antibiotics is also an integral part of good veterinary practices because their improper use as prophylactics or disease preventives to avoid, instead of treating, animal infections is also favouring development of antimicrobial resistance. Thus, it is an attitude to maximise therapeutic efficacy and minimise selection of resistant micro-organisms. Principles are a guide for optimal use of antibiotics, but they should not be interpreted so restrictively as to replace professional judgement of practitioners or to compromise animal health or welfare. In all cases, animals should receive prompt and effective treatment as deemed necessary by the prescribing or supervising veterinarian. Furthermore, resistant bacteria may not be confined to those specific scenarios of farms and hospitals, but can be carried thousands of miles away by wind, water, animals, food, or people. And, most importantly, antibiotic resistant organisms that develop in animals, fruits, or vegetables can be passed to humans through the food chain and environment. Nowadays, as it can be observed in **Figure 1.1**, actions like WWTP effluents and CAFOs

represent main important sources of antibiotics to the environment because most percentage of antibiotics are excreted after consumption reaching the terrestrial and aquatic environment at up thousands of tonnes per year [24]. All of these factors have had the effect of changing the balance between antibiotic susceptible and the antibiotic resistant bacteria in our ecosystem, locally and globally. Thus, several studies have been carried out around the world to investigate the occurrence and fate of the antibacterial drugs in sewage treatment plants or surface waters [25]. Antibiotic resistance causes, besides the adverse effects in animals and plants, an important impact on the ecosystem, water, and soil-dwelling organisms [26]. For example, sulfadimethoxine and bacitracin produce loss of weight in roots and leaves in some plants, and oxytetracycline (OTC) and tetracycline (TC) can kill pinto bean plants at a concentration level of 160 mg L^{-1} [27]. CAP can produce pneumonia and SMZ has been evaluated by the Food Agriculture Organization (FAO) of the United Nations expert Committee as a suspected carcinogen.

Macrolide and SAs families are the most prevalent antibiotics found in the environment (μg L^{-1}) due to their relatively high mobility they can enter groundwater and be transported in aquifers and surface waters [28]. On the other hand, FQs, TCs, and penicillins have been detected in fewer cases and usually at low concentrations (ng L^{-1}) [29]. This result is not surprising, since BLs ring of penicillins is chemically unstable to pH, heat or ß-lactamase enzymes [30], and TCs readily precipitate in presence of divalent cations (i.e. Ca^{2+}, Mg^{2+} or Zn^{2+}) being accumulated in sewage sludge or sediments [31]. Regarding FQs conventional wastewater treatment eliminates about 80%-90% of these compounds before they arrive to the rivers indicating a low likelihood for adverse effects to the aquatic habitat of surface waters.

On the other hand, these compounds are also susceptible to photodegradation in aqueous medium, involving oxidation, dealkylation, and cleavage of the piperazine ring [32]. Moreover, during the last decades, antibiotic residues have been detected at significant concentrations levels in many biological samples, such as meat, honey, milk or dairy products. One of the main reasons finding antibiotic residues in these kind of samples has been the dramatic increase of meat and fish production needed for human consumption during the last years which has led some farmers to use none very ethical practices with the aim to improve the productivity of that industry. Continuous administration of sub-therapeutic antibiotic doses to animals produce a growth promoter effect, but also select bacteria resistant strains again which could end-up contaminating animal-derived food.

1.1.3 Social concern and first actions taken

All these situations constitute a potential hazard for the consumer and may cause allergic reactions, interference in the intestinal flora also contributing to the resistant populations

of bacteria in the general population, thereby rendering antibiotic treatment ineffective. Furthermore, both technological problems and important economic losses in diary producers are related with the inhibition of the bacterial fermentation processes involved in the elaboration of cheese and cultured milk products. There is also a big social concern about all this situation that make consumers becoming more exigent in terms of getting more natural and high quality food products. As a result, recent trends in global food production, processing, and distribution are creating an increasing demand for food safety research in order to ensure a safer global food supply (see **Figure 1.5**).

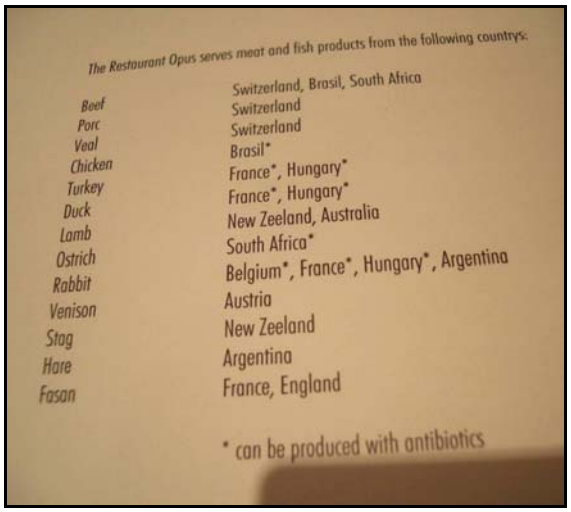


Figure 1.5: Picture of the menu annex from the restaurant Opus (Lucerne - Switzerland) providing information to the costumers of the potential risk of antibiotic contamination of certain meat and fish products depending on the country origin.

Thus, the World Food Safety Organisation supports the European Union, the International Organization for Standardization and the corresponding accreditation schemes initiatives for the implementation of food safety management systems. In this way, *Hazard Analysis and Critical Control Points* is a systematic preventive formula and pharmaceutical safety that addresses physical, chemical, and biological risks as a means of prevention rather than finished product inspection. Achieving a certification for food safety in line with the FAO, the World Health Organization (WHO) and *Codex Alimentarius* supported by local legislation is urgently required [33].

Many international organizations are working closely to address food safety issues along the entire food production chain, which means from manufacture to consumption (see **Figure 1.6**), using new methods of risk analysis benefiting both, public health and economic development. These methods should provide efficient, science based tools for professional consultants, auditors and internal controls to verify and check an effective

food safety management system through a process of assessments and continuous improvements. That means the need to develop new technologies to improve the control of antibiotics in foodstuffs from animal origin nowadays.

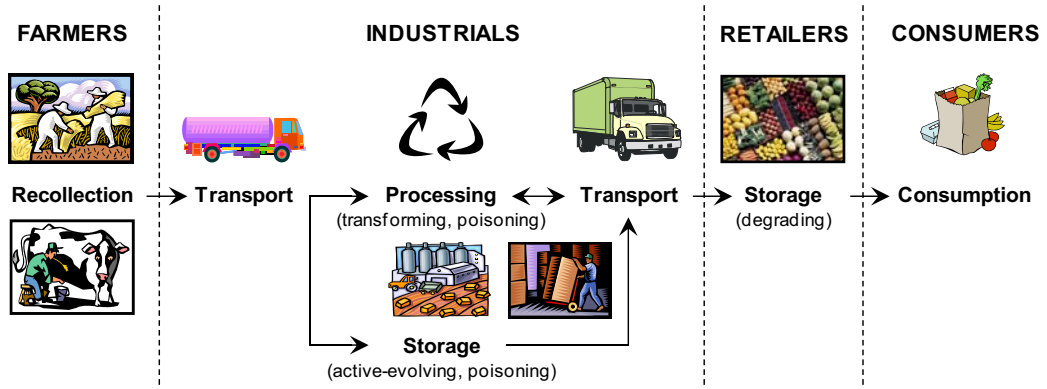


Figure 1.6: Food supply chain in the agro food new marketing industry. *Source: Image inspired from GOODFOOD project memory.*

But on the other hand, food diagnosis and control is performed in most cases with traditional laboratory tools that are time consuming, expensive and used under a sorting basis. In order to drastically improve this situation it is necessary to carry out research activities that take advantage of multidisciplinary skills at national and international level. The development of the so-called *rapid methods* compared to the c*onventional methods* will be the driving force. Rapid methods based on miniaturised kits may benefit from micro and nano technologies and devices [34]. Nowadays, both basic and applied research is being done at national and international level in many countries, with specific plans for agro food research and development activities [35]. In this manner, several major projects have been launched in this area that the European Union supported with research funds totalling €43 million through the Sixth Research Framework Programme. The Seventh Framework Programme is working in the same direction putting big interest on life sciences and biotechnology topics. Moreover, the Spanish National Research Network has also launched many activities which take the *farm-to-fork* approach.

Summarizing, the concern produced within the scientific community, the authorities, and governmental bodies about all these undesirable situations for the public health and the entire environment has prompted the establishment of certain regulations, directives and decisions at different levels [36].

1.2 Governmental regulations

An international approach to the management of antimicrobial resistance is essential for its surveillance. Thus, the WHO, the EC, the Centres for Disease Control and Prevention (CDC), and many other organizations as the ones summarized in **Table 1.1**, have recognized the importance of studying the determinants of resistance as well as the need for control strategies.

Table 1.1: Some examples of active governmental bodies and international organizations who combat the spread antimicrobial resistance problematic.

Antibiotic Resistance Entities	Website
World Health Organization (WHO)	www.who.int
European Commission (EC)	www.ec.europa.eu
European Medicines Agency (EMEA)	www.emea.europa.eu
European Food Safety Authority (EFSA)	www.efsa.europa.eu
International Federation for Animal Health (IFAH)	www.ifahsec.org
Centres for Disease Control and Prevention (CDC)	www.cdc.gov
Food and Drug Administration (FDA)	www.fda.gov
US Environmental Protection Agency (USEPA)	www.epa.gov
Alliance for the Prudent Use of Antibiotics (AUPA)	www.tufs.edu/med/apua

In Europe, antimicrobial resistance of invasive pathogens has been monitored by the European Antimicrobial Resistance Surveillance System. Its counterpart at the United States, the International Network for the Study and Prevention of Emerging Antimicrobial Resistance, was launched by the CDC with a similar goal [37]. The main objectives of both vigilance networks are to serve as an early warning system for emerging resistant pathogens, to facilitate rapid distribution of reliable data and information about emerging multidrug resistant pathogens to hospitals and public health authorities worldwide, and to serve as a model for the development and implementation of infection control interventions to prevent the emergence or transmission of antimicrobial drug resistant pathogens in healthcare facilities. As an example, more than 20 countries and 600 laboratories in Europe provide antibiotic susceptibility data to national and central database [38]. Another important function is to assist microbiologists and infection control personnel in hospitals and countries that lack the expertise needed to conduct microbiologic or epidemiologic studies, as well as, providing the basis for and assess the effectiveness of prevention programs and policy decisions [39]. EFSA, whose principles have been established in EC Regulation 178/2002, is giving also scientific support to food safety concerns like this one [40]. Thus, the European Union has strictly

regulated controls on the use of veterinary drugs, including growth-promoting agents [41], particularly in food-animal species, by issuing several Regulations and Directives (see **Table 1.2**), and, since 1998, has prohibited antibiotics used in human medicine from being added to feed [42].

Table 1.2: List of most important Regulations, Directives and Decisions established by the European Commission to regulate and control the use of veterinary drugs.

European Union Law Documents	Information
Council Regulation 178/2002/EC	General principles and requirements of food safety law
Council Regulation 2377/90/EC (Annexes I, II, III and IV) Update: Commission Regulation 30/2010	Establishment of security residue levels for veterinary medicinal products in foodstuffs
Council Regulation 1831/2003	Regulation on additives for use in animal nutrition
Council Directive 96/22/EC Commission Directive 97/6/EC	Prohibition of growth promoting agents in food-producing animals
Council Directive 96/23/EC	Antibiotic monitoring control requirements
Commission Decision 2002/657/EC	Technical guidelines for residue control
Regulation (EC) 882/2004	Feed and food law, animal health and animal welfare rules
Council Directives 2001/82/EC & 90/167/EC	Conditions governing the preparation, placing on market and use of medicated feeding stuffs

The use of veterinary drugs is regulated through the European Union Council Regulation 2377/90/EC, which describes the procedures for establishing MRLs [43] for veterinary medicinal products in foodstuffs of animal origin (e.g. milk, eggs or meat). Its Annexes present very important additional information [44]:

- Annex I includes substances for which final MRL values have been established.
- Annex II includes substances for which it is not considered necessary for the protection of public health to establish MRL values. These substances are allowed to be used in veterinary medicinal products for food-producing species for the animal species indicated and according to the conditions established.
- Annex III includes substances with provisional MRLs. These are established, for a defined period of time, when not all requirements for the establishment of an MRL have yet been fully addressed.
- Annex IV includes substances for which no MRL could be established because residues of these substances, at whatever limits, in foodstuffs of animal origin constitute a hazard to the health of the consumer. The administration of substances listed in this Annex to food-producing species is prohibited.

This Council Regulation 2377/90/EC was recently updated to the Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin [45]. In this sense, in Europe medicated feeds can only be prepared from authorized pre-mixes complying with the provisions of Council Directive 2001/82/EC [46]. Moreover, the use and marketing of medicated feeds are regulated by the former Directive 90/167/EC [47]. According to the Council Directive 96/22/EC, the European Union has also banned the use of growth-promoting agents, such as ß-agonists or steroid hormones, from use in food-producing animals [48]. In particular, in 1997 avoparcin was banned in all European countries and in 1998 the EC decided to prohibit the use of bacitracin, spiramycin, tylosin and virginiamycin for the same reason (see Commission Directive 97/6/EC [49]). Moreover, it was also approved Council Directive 96/23/EC [48] which regulates the residue control monitoring and sampling, by establishing the procedures for inspection of pharmacologically active compounds.

Considering all these possibilities, for any type of animal or food, there are two main groups of substances that must be monitored (see some examples in **Table 1.3**):

- Group A: Comprises prohibited substances accorded to Council Directive 96/22/EC and Annex IV of updated Council Regulation 2377/90/EC.
- Group B: Comprises all registered veterinary drugs accorded to update Annexes I and III of Council Regulation 2377/90/EC as well as other residues not prohibited.

Technical guidelines and performance criteria (i.e. detection level, selectivity and specificity) for residue control of these substances, in the framework of Directive 96/23/EC, are described in Commission Decision 2002/657/EC [50]. It establishes criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results generated by official laboratories [51]. Moreover, the Decision establishes common criteria for the interpretation of test results and introduces a procedure to progressively establish minimum required performance limits (MRPLs) for analytical methods employed to detect substances for which no permitted limit has been placed [52, 53]. This is in particular important for substances whose use is not authorised or is specifically prohibited in the European Union. In this sense, validation tests shall demonstrate that the corresponding analytical method complies with the criteria applicable for the relevant performance characteristics. Different control purposes require different categories of methods. **Table 1.4** determines which performance characteristic shall be verified for which type of method.

Table 1.3: Residue material groups.

Group A *(Unauthorized substances and anabolics)*	**Group B** *(Veterinary drugs and contaminants)*
Stilbenes and derivatives	***Antibacterial substances***
Antithyroid agents	Other veterinary drugs:
Steroids (hormones and synthetic steroids)	Anthelmintics
ß-agonists	Anticoccidiostats and nitroimidazoles
Annex IV (2377/90/EC) compounds)	Carbamates and pyrethroids
	Carbadox and olaquindox
	Sedatives and others
	Non-steroidal anti-inflammatory drugs
	Environmental contaminants
	Organohalogenated compounds
	Organophophorus compounds
	Chemical elements
	Mycotoxins
	Dyes and others

Table 1.4: Classification of analytical methods by the performance characteristics that have to be determined.

		Detection Capability (CCß)	**Decision limit (CCα)**	**Trueness Recovery**	**Precision**	**Selectivity Specificity**	**Applicability Ruggedness stability**
Qualitative methods	S	+	-	-	-	+	+
	C	+	+	-	-	+	+
Quantitative methods	S	+	-	-	+	+	+
	C	+	+	+	+	+	+

S = screening methods; C = confirmatory methods; + = determination is mandatory

The Directive 96/23/EC also lays down that samples collected for the *National Surveillance Programme* have to be analysed in accredited laboratories. Accordingly, an extensive network of analytical residue laboratories has been created for the purpose of residue inspections. This hierarchical system comprises, at the lowest level, so-called Routine and/or Field Laboratories, next some forty National Reference Laboratories and, at the top, four Community Reference Laboratories located in Germany, France, Italy and The Netherlands, and responsible for a dedicated set of compounds. In addition, the decision takes recent technical developments into account, with multiresidue liquid chromatography-mass spectrometry (LC–MS) approach taking the foremost position.

But although Directive 96/23/EC and Decision 2002/657/EC dictates the frequency, total analyses to be performed and number of substances that have to be monitored, most of the analytical methods available today are insufficient to accomplish these requirements mainly due to problems with biological samples treatment [54].

1.3 Principal antibiotic families to be measured in milk samples

In order to follow current regulations (see **Section 1.2**), about 50 different antibiotics should be tested per analysis. Currently, it is not realistically possible because of the cost of each commercially available test being almost always necessary a different test for each antibiotic family (10 kinds). Even in the same family, different tests are usually needed to detect all congeners. Furthermore, the time needed per most of these analyses is also a limitation for complete testing of antibiotic residues. Besides governmental regulations reported regarding this topic, projects like the ones cited above are necessary to improve the current analytical techniques after being identified, valued and considered the real agro food safety control requirements nowadays. Thus, several market analyses have been carried out previously to advise framework programs and their main project coordinators about the needs that should be attempted in the veterinary drug residue analysis field, such as most important target analytes, level of detection, measurement time or price per assay. Moreover, depending on the type of sample to be analyzed all the scenario presented just before could be completely different being also necessary to select the priority matrices to start working with. In our case, since milk was the main matrix selected to be monitored, Spanish, European and international milk processing units, such as Nestlé, Pascual or Puleva, and reference laboratories throughout Europe were contacted to fill a questionnaire (see **Appendix**) in order to establish specifications for an antibiotic residue monitoring system.

Milk has long been a popular beverage, not only for its flavour, but because of its unique nutritional package [55] containing nine essential nutrients (calcium, potassium, phosphorus, niacin, riboflavin, protein and vitamins A, B_{12}, D). Thus, milk is one of the best sources of calcium also providing high-quality protein, vitamins and other minerals (see **Figure 1.7**).

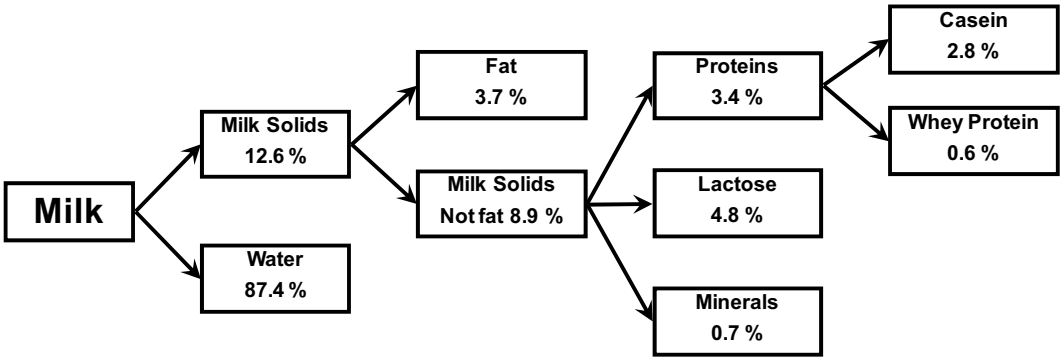


Figure 1.7: Major constituents of milk. Extracted from National Diary Council.

Regarding the analysis of this matrix, parameters such as its chemical and biochemical composition, its physic-chemical nature or its microbial quality could interfere with the screening test used to detect antibiotic residues. According to the literature, matrix effects for the immunochemical techniques in milk are likely produced by the fat and the protein content of the sample. In addition, the target analyte properties can also be crucial for the good performance of the assay in milk. In some cases, when analyzing highly hydrophobic analytes, there are fluctuations in the absorbance values at low analyte values due to the difficulty in spiking raw milk consisting in two-phase fat and aqueous system [56]. On the other hand, microbial interferences are usually originated from a high somatic cell counts or a high plate count, which are very important parameters to determine the quality of milk. Milk products are sold in a number of varieties based on types/degrees of additives, coagulation, farming method, fat content, fermentation, flavouring, homogenization, mammal, packaging, sterilization, water content and others. For all cases, Council Directive 92/46/EEC laid down the health rules for the production and placing on the market of raw milk, heat treated milk and milk based product [57]. Nowadays, European dairy industry employs essentially two, but increasingly three to four, levels of testing for antibiotics (see **Figure 1.8**).

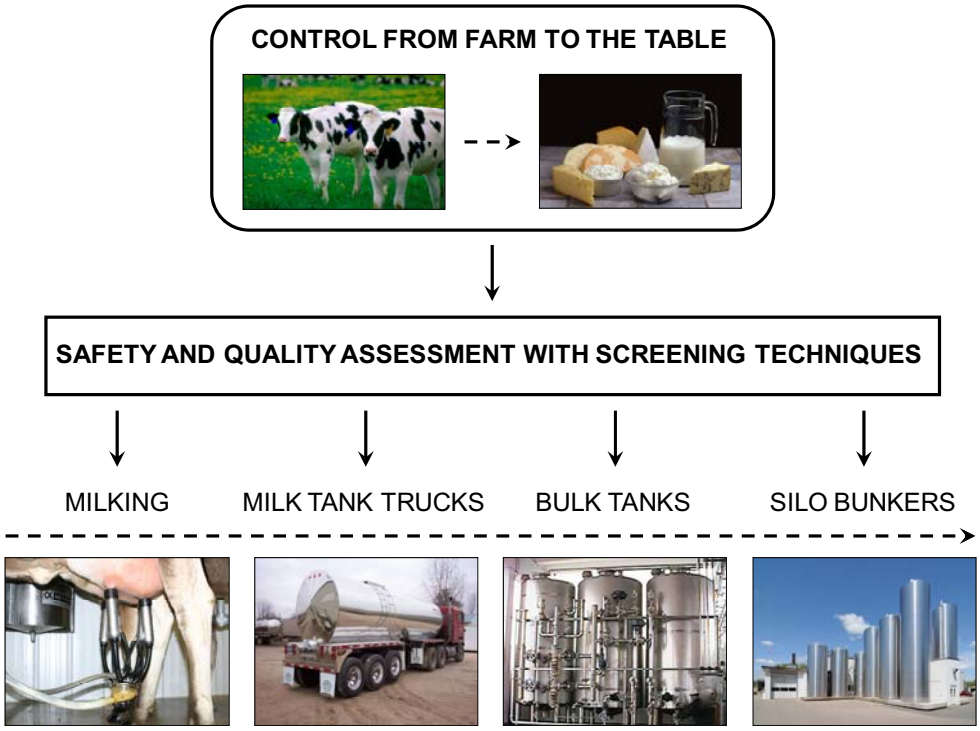


Figure 1.8: Milk chain monitoring and traceability: safety and quality assessment stages. Novel screening contribution should be addressed closeness to the foodstuff, power of analysis and speed by multisensing, multipoint and continuous monitoring, automation or non-specialist intervention.

Firstly, bulk tank samples are tested weekly being the results therefore often retrospective. At that point, there is still no commercial need for a rapid result being the main requirements of a test addressed to low cost, broad recognition and reliability. On a second stage, milk tank trucks are also usually tested on arrival at the dairy where there is a desire to accept or reject the consignment before off-loading. Thus, it is desirable to obtain the test results before the milk is released into production or silo bunkers. In this situation, a rapid result is essential and consequently many dairies prefers to sacrifice a broad spectrum analysis and, to some extent, cost for the benefit of speed. Lately, tests for antibiotics have begun to be applied even at the individual cow level, notably for freshly calved animals that have received dry cow therapy. The ideal tool for this last scenario is a simple broad spectrum, high speed and low cost test, but those currently available do not achieve all three attributes. Thus, at this point the choice is often between speed and broad spectrum. Rapid tests for antibiotic residues in the milk supply have received international approval but sometimes are quite difficult to accomplish.

The diversity of tests now available and the partial fragmentation of the European dairy industry about this issue have created a significant dilemma. Firstly, different tests may be applied by purchaser and supplier, since there is no longer a standardised approach being probable an increased danger that conflicting results could be obtained between two tests undertaken on the same consignment of milk. The supplier-customer relationship often defines which tests are to be used, to ensure that consistent results are obtained by both parties, an aspect that is becoming increasingly complex as the movement of milk across national boundaries expands.

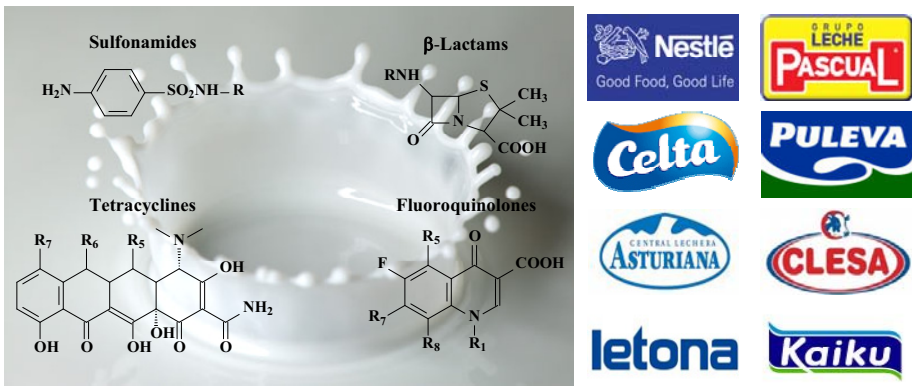


Figure 1.9: General structure of most relevant antibiotic families and some of the milk companies which were contacted to establish the specifications required.

Thus, dairies must decide whether they should screen milk supplies for the widest possible range of substances using a test where the result is obtained retrospectively or whether they should use a rapid test to detect only those antimicrobials most commonly

encountered. Since some antibiotic groups are much more commonly found than others, is seems not to be necessary to screen milk for all antimicrobial groups. For this reason, as it was described above, several well known milk producers, industry representatives and regulatory agencies, from the European market, were contacted in order to establish the necessary specifications of the tools for antibiotic residue monitoring (see the **Appendix**). Besides many other interesting information received regarding the necessary analysis requirements (e.g. easy, broad detection, rapid and low cost), most valuable feedback was regarding the identification of priority antibiotic families to be controlled (see **Figure 1.9**).

Table 1.5: Main antibiotics to be monitors in milk samples.

Family	Main Compounds	MRL
SAs[a]	Sulfamethazine	100 μg kg^{-1}
	Sulfathiazole	100 μg kg^{-1}
	Sulfapyridine	100 μg kg^{-1}
	Sulfachloropyridazine	100 μg kg^{-1}
	Sulfamethoxine	100 μg kg^{-1}
	Sulfamerazine	100 μg kg^{-1}
FQs	Enrofloxacin[b]	100 μg kg^{-1}
	Ciprofloxacin[b]	100 μg kg^{-1}
	Danofloxacin	30 μg kg^{-1}
	Flumequine	50 μg kg^{-1}
	Marbofloxacin	75 μg kg^{-1}
BLs	Ampicillin	4 μg kg^{-1}
	Amoxicillin	4 μg kg^{-1}
	Benzylpenicillin	4 μg kg^{-1}
	Cefazolin	50 μg kg^{-1}
	Cefoperazone	50 μg kg^{-1}
	Ceftiofur	100 μg kg^{-1}
	Cephapirin	60 μg kg^{-1}
	Cloxacillin	30 μg kg^{-1}
	Nafcillin	30 μg kg^{-1}
TCs[c]	Tetracycline	100 μg kg^{-1}
	Oxytetracycline	100 μg kg^{-1}
	Chlortetracycline	100 μg kg^{-1}

[a] *A MRL of 100 μg kg^{-1} should be applied to all compounds of the sulfonamide group. Moreover, the combined residues of all substances in the sulfonamide group should not exceed this value.*
[b] *Marker residue considers sum of enrofloxacin and ciprofloxacin not to exceed 100 μg kg^{-1} in bovine milk samples.*
[c] *MRLs established considering the sum of parent drug and its 4-epimer.*

Thus, main antibiotic families to be controlled were identified to establish cartography of the antibiotic formula used in Europe. SAs, FQs, BLs and TCs were the most relevant antibiotics required to be detected in milk and dairy products. The respective detection limits of the different immunochemical techniques planned should be fixed in function of the MRLs established for each antibiotic family by the European Union (see **Table 1.5**) being a priority for us the analysis of fresh and whole milk type, which seem to be the most complicated (high fat content). On the other hand, the broad illegal use of these antibiotic families within the veterinary field (i.e. prophylaxis and/or enhance growth purposes) will also make necessary their analysis in other biological samples. For this reason, it's crucial to know these primary antibiotic families (i.e. history, structures, mechanism of action, etc.) to better understand which kind of compounds we are working with and their importance in the veterinary field. **Figure 1.10** tries to summarize schematically the antibacterial location of action for the different antibiotic families.

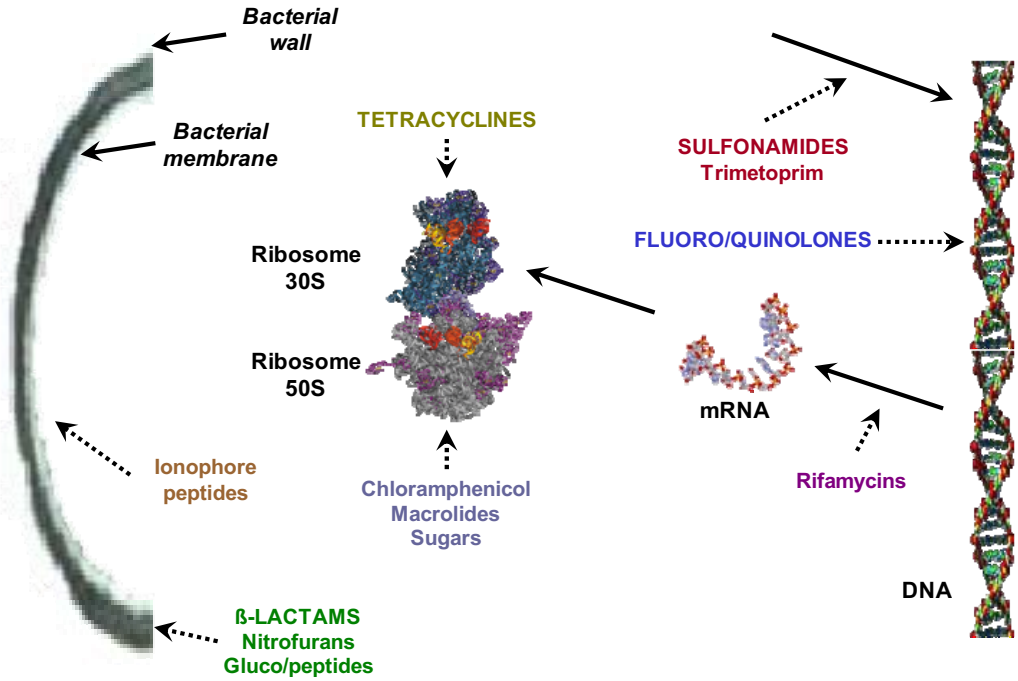


Figure 1.10: Scheme of bacterial structure including wall, membrane, ribosome and nucleic acids together with some antimicrobial examples which act at those levels.

1.3.1 Sulfonamide antibiotics

1.3.1.1 History

SAs are synthetic antimicrobial agents with a wide spectrum encompassing most gram-positive and many gram-negative organisms. These drugs were the first efficient

treatment to be employed systematically for the prevention and cure of bacterial infections in human beings paving the way for the antibiotic revolution in medicine [58]. First studies about these antimicrobial compounds were performed by Gerhard Dogmag in the late 1920s. This German pathologist and bacteriologist turned his attention to azo dyes because some of them attached strongly to protein in fibres or leather, so that they held fast against fading or cleaning. Domagk reasoned that they might also attach themselves to the protein in bacteria, inhibiting if not killing the organisms. Later on, he found the sulfanilamide metabolite from Prontosil dye (see **Figure 1.11**) to be effective on laboratory rats and rabbits, infected with streptococcus bacteria, before treating his own daughter with it saving her the amputation of an arm [59].

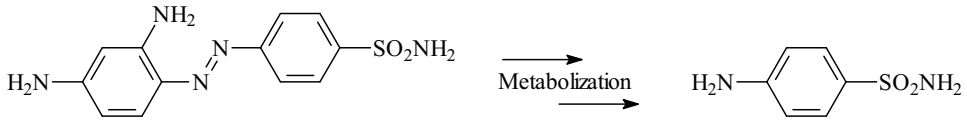


Figure 1.11: Structure of Prontosil and its active metabolite. The discovery and development of this first SAs drug opened a new era in medicine.

Since then, scientist realized a great number of chemical modifications of the sulfanilamide molecule to greatly expand its antibacterial activity and reducing its side effects. Thus, thousands of SAs were studied while dozens finally were used to treat patients. Nowadays, only a small fraction of these are used in humans for urinary tract infections or treatment of meningococcal meningitis, but they are still valuable antibiotics [60]. On the other hand, this antibiotic family is still widely used by the veterinary medicine for prophylactic and therapeutic purposes in farm animals addressed to produce food for human consumption. Furthermore, some farmers use them illegally as growth promoters which result in improved feed efficiency, thereby contributing to lower production costs of meat, eggs and other products. But, the money saved on a short-term could produce human health problems and even more economic losses on a long-term.

1.3.1.2 Structures

The original antibacterial SAs, sometimes called simply sulfa drugs, are synthetic antimicrobial agents that contain the sulfonamide group (-SO_2NH-). Currently used SAs consist of a benzene ring with an aminosulfonyl group on C_1 and amino free group on C_4. The basic structure of the SAs cannot be modified if it is to be an effective competitive mimic for p-aminobenzoic acid. The SAs family group comprises over 20 different drugs (see **Figure 1.12**). Diversity of SAs is based normally on the addition of a heterocyclic aromatic ring (i.e. pyridines, pyridazines, pyrimidines, quinolones, thiazoles, etc.) on N_1

of the aminosulfonyl group with the possibility to have none, one or two substituents such as methyl, methoxy or chloride groups.

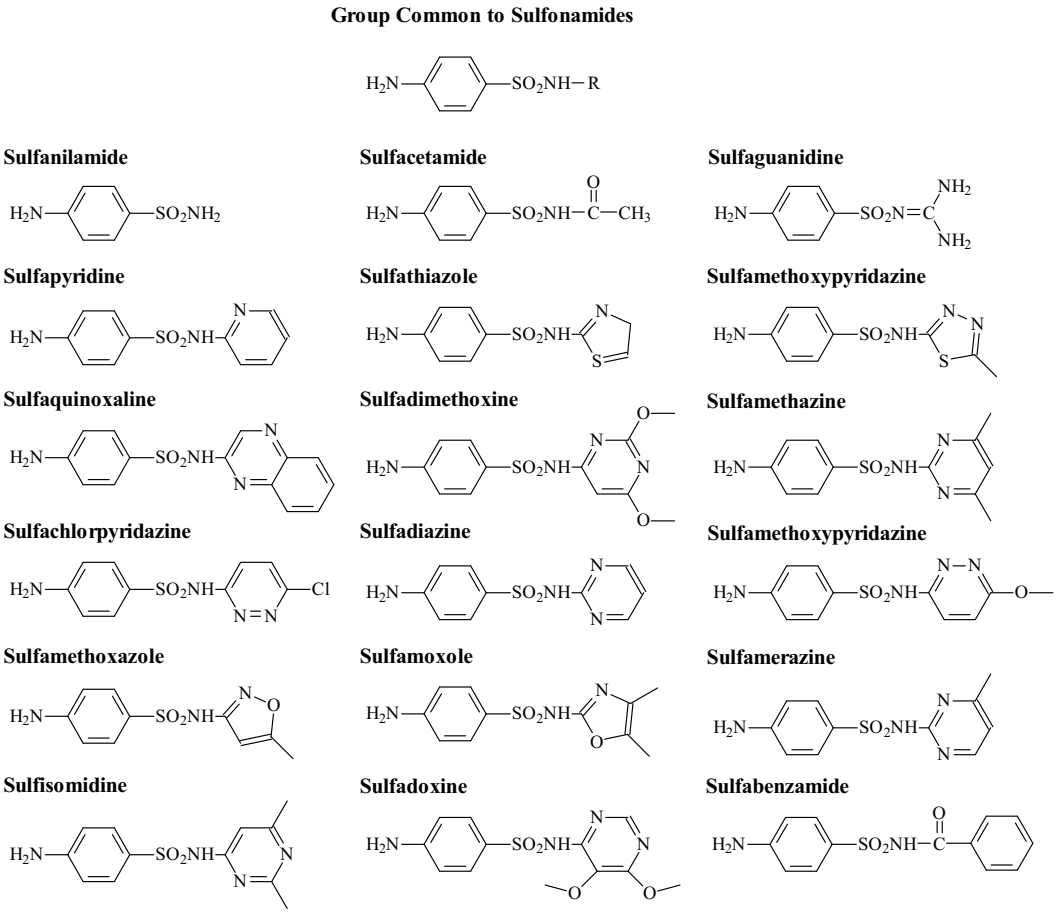


Figure 1.12: Chemical structure of some of the most common SAs.

It has been reported that the sulfonamide group is related to the antibiotic phamarcodynamics being the free amino group responsible of the bacteriostatic properties while pharmacokinetics depends on the different R groups [61].

1.3.1.3 Mechanism of action

SAs exhibit a bacteriostatic rather than bactericidal effect, which means that they inhibit growth and reproduction of bacteria without killing them. This property helps us to define their mechanism of action against bacterial infections (see **Figure 1.13**). The target of SAs is the enzyme dihydropteroate synthase (DHPS), which catalyzes the formation of dihydropteroic acid (DHPA) in bacteria and some eukaryotic cells required for further folic acid (vitamin B_9 or M), ultimately purine and deoxyribonucleic acid (DNA)

synthesis. Humans and animals don't synthesize folic acid but acquire it in their diet, so their DNA synthesis is less affected in that way. This difference is the basis of the selective action of SAs.

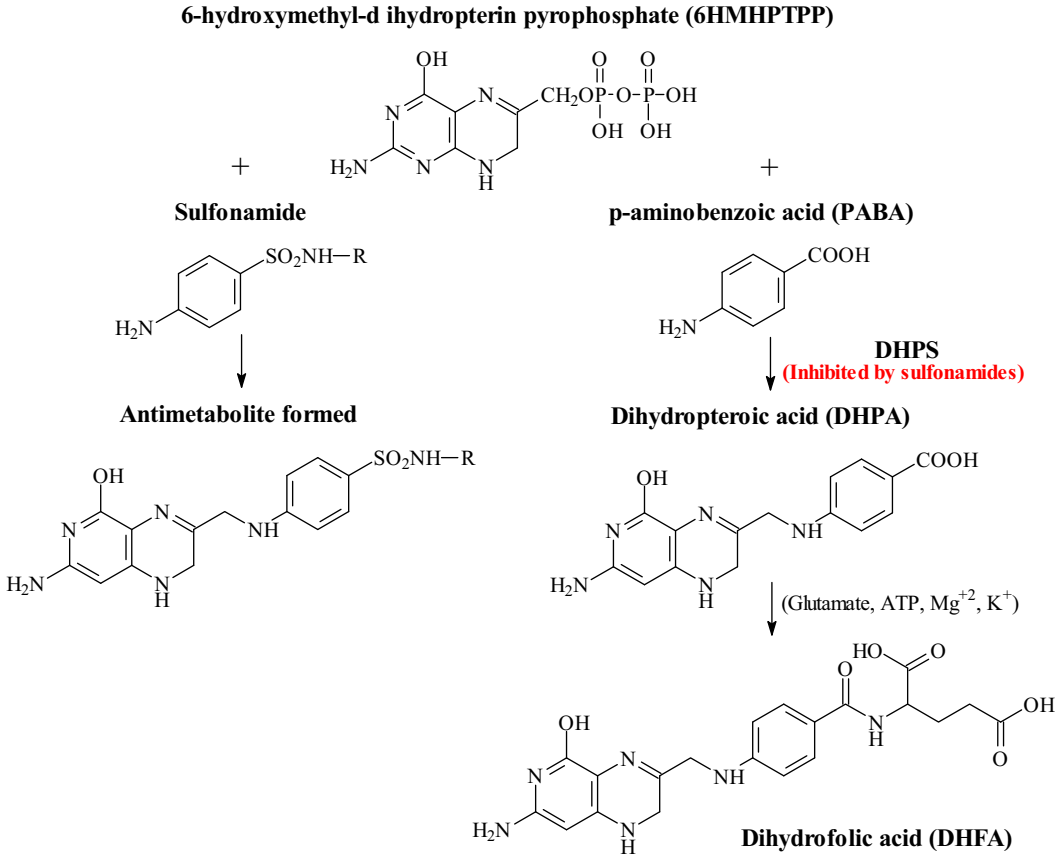


Figure 1.13: Basic scheme of SAs mechanism of action in bacteria.

Thus, SAs are a structural analogous of p-aminobenzoic acid (PABA), the substrate of the DHPS enzyme, and inhibits it competitively [62]. It can also function as an alternative substrate for the production of a SAs-containing pteroate analogous that cannot be used in the subsequent steps of the biosynthetic pathway. The folate cofactor pool in the bacterial cell is consequently depleted, resulting in growth inhibition and cell death. The interaction of several antibiotics against germs can produce synergism, competence, antagonism and the so-called *post antibiotic effect*. Synergism effect is produced when the action of the combination of two or more antibiotics is greater than when they are used individually. Synergism between SAs, which are bacteriostatic agents, and trimethoprim, which is bactericidal agent, has been widely studied. Trimethoprim compound was commonly used in combination with sulfamethoxazole inhibiting an

earlier step in the folate synthesis pathway. If tetrahydrofolic acid, which plays an important role in the synthesis of purine and pyrimidine nucleotides so in this way in DNA repair and synthesis, can't be formed the bacteria tend to die. But most of the antibacterial effects just showed are dose and duration dependent and reversible on drug discontinuation [63].

1.3.1.4 Therapeutic use of sulfonamides

Nowadays, SAs are rarely used in humans being sulfasoxazole and sulfamethoxazole combined with trimethoprim the most commonly prescribed for some urinary, meningitis and eye or ear infections. On the other hand, at least ten SAs are allowed to be legally used in animals (see **Table 1.6**) for the treatment of enteritis, cystitis, mastitis and bacterial pneumonia as well as bird cholera and coccidia [64].

Table 1.6: SAs most applied in veterinary medicine in Europe and some of their brand name products. Data from the United States Pharmacopea (USP).

SAs	Commercial Products	Animals
Sulfathiazole	Sulfamed, Sulfa M-T, Sulfa-2.	bees
Sulfamethazine	Sulfalean, Sulfa-Max III, Triple Sulfa.	bovines, chicken, pigs turkeys
Sulfaquinoxaline	Sulfa Q, Optimed, Sul-Q-Nex.	bovines, chicken, turkeys
Sulfadimethoxine	Albon, Am Tech, Di-Methox, SOM.	chicken, dogs, turkeys
Sulfadiazine	Lantrisul, Neotrizine, Sulfaloid	pigs

These drugs can be also categorized depending on their time of action as short acting, intermediate acting and long acting, although the last ones are not available for use in the United States because of their ability to cause Stevens-Johnson syndrome, which is a is a life-threatening condition affecting the skin in which cell death causes the epidermis to separate from the dermis [65]. The pharmacokinetic profile of SAs ensures that they are quickly eliminated from the organism (40-90%), usually as the parent compound or as bioactive metabolites [66]. Main possible transformations are the N_4 acetylation, deamination, aromatic hydroxylation and N_1-glucuronidation. As with many other pharmaceuticals, SAs are fairly water-soluble, polar compounds which ionize depending on the pH of the matrix (SAs pKa values from 6.36 to 7.99). In addition to hydrophobic partitioning, these compounds can absorb to soils via cation exchange, cation bridging, surface complexes and hydrogen bonding [67].

Hence, SAs will persist in the environment and, because of their relatively high mobility, will enter groundwater and be transported in aquifers and surface waters.

1.3.2 Fluoroquinolone antibiotics

1.3.2.1 History

The origins of the quinolone class lie in the use of chloroquine as an antimalarial agent during World War II. A compound isolated from the commercial preparation of chloroquine proved to have antibacterial activity and was modified to produce the first marketed quinolone in the 1960s, nalidixic acid [68]. This compound was discovered by George Lesher and co-workers being the starting point for the first generation of quinolones (see **Figure 1.14**).

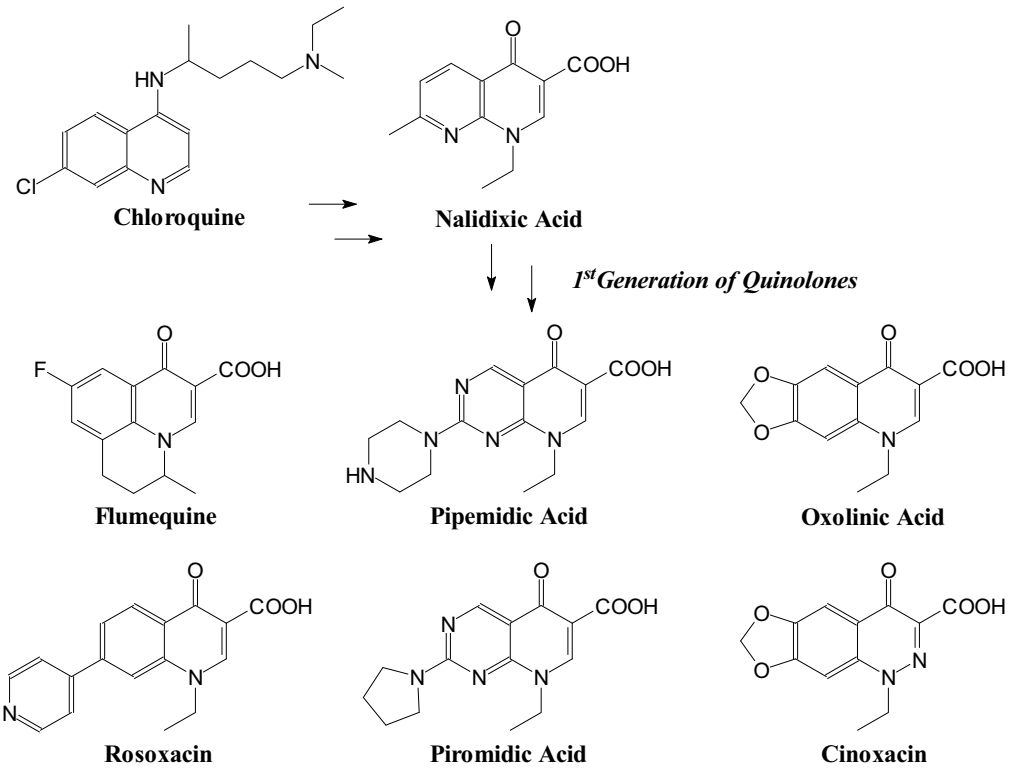


Figure 1.14: Structure of nalidixic acid, discovered accidentally when synthesising chloroquine.

After several attempts to improve the activity of nalidixic acid, the introduction of a piperazinyl side chain at position 7 (to provide pipemidic acid) did improve the activity against gram-negatives organism as well as increasing the ability of the quinolones to penetrate the bacterial cell wall, thus enhancing activity. The first compound with a fluoro group at position 6 was flumequine (patented in 1973) which gave the first indications that activity against gram-positive organisms could be improved in this class [69]. The real breakthrough came in 1978 with the combination of these two features in norfloxacin, a 6-fluorinated compound with a piperazine ring at position 7. Since then,

great discoveries and developments were rapidly superseded by even better compounds and norfloxacin proved to be just the beginning of a highly successful period of research into the modification of the FQs molecule [70]. Thus, new generations of quinolones came along (see **Table 1.7**). Since the development of newer quinolones and their release in the mid 1980s, there has been extensive clinical use of these agents in human medicine. Various quinolones have been approved and used extensively for treatment of a broad range of clinical infections, including those of the genitourinary, gastrointestinal, and respiratory tracts as well as infections of bone, joints, and skin. During the last decades, several FQs have also been available for treatment of animals, poultry and fish in many countries in the world. On the other hand, as it happens with other antibiotic families, their continue misuse in clinical medicine and agricultural feed is promoting resistance of bacteria and is likely to limit the effectiveness of the quinolones in the near future.

Table 1.7: Quinolones are divided into generations based on their antibacterial spectrum. The earlier generation agents are, in general, more narrow than the later ones.

2nd generation	3rd generation	4th generation
Ciprofloxacin	Balofloxacin	Clinafloxacin
Enoxacin	Gatifloxacin	Gemifloxacin
Fleroxacin	Grepafloxacin	Sitafloxacin
Iomefloxacin	Levofloxacin	Trovafloxacin
Nadifloxacin	Moxifloxacin	Prulifloxacin
Norfloxacin	Pazufloxacin	**New**
Ofloxacin	Spartfloxacin	Garenoxacin
Pefloxacin	Temafloxacin	Ecinofloxacin
Rufloxacin	Tosufloxacin	Delafloxacin

This resistance is most commonly associated with genetic alterations in the topoisomerases resulting in decreased drug binding and expulsion of these agents from cell before they reach their intercellular targets [71]. This situation indicates that new FQs will have to be used with prudence if they are to realize their potential in the clinic.

1.3.2.2 Structures

All FQs contain a carboxylic acid side chain at position 3 and the exocyclic oxygen at position 4, both of which have now been found to be essential to activity by binding the DNA gyrase of the bacterial cell. Some attempts have been made to modify this basic structure, together with the hydrogen at position 2, but with any great success. This antibiotic family is also characterised for having a fluorine atom attached to the central ring system, typically at the 6 or 7 position (see **Figure 1.15**).

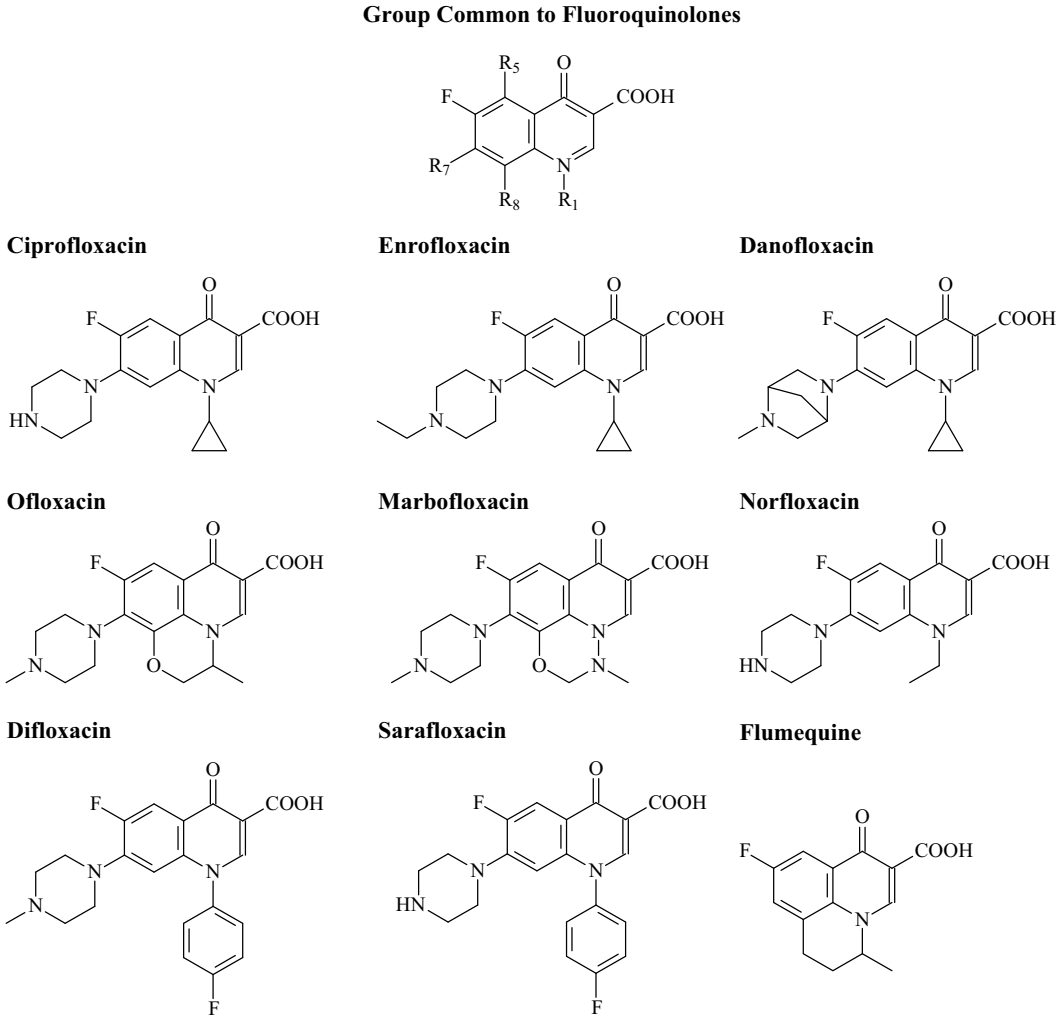


Figure 1.15: Chemical structure of some of the most common FQs.

On the other hand, substituents at the N_1 position vary from agent to agent based normally on the addition of different groups, such as a cyclopropyl, a fluorophenyl, and a ring closing positions 1 and 8. But it's at position 7 where major changes can be seen in potency being well established that heterocyclic nitrogen-containing rings give improved activity that affect the pharmacokinetics [72]. Other modifications at other positions don't improve substantially the activity of these antimicrobial compounds.

1.3.2.3 Mechanism of action

Contrary to SAs, the FQs are bactericidal drugs meaning that they kill bacteria. The targets of this potent group of drugs are the essential bacterial enzymes DNA gyrase and topoisomerases IV both necessary for DNA replication [73]. DNA gyrase is the primary

target of gram negative organisms while the topoisomerase IV is the primary target of gram positive organisms. A general scheme for intracellular quinolone action is sketched in **Figure 1.16**. Gyrase is responsible for introducing negative supercoils into DNA and for relieving torsional stress expected to accumulate ahead of transcription and replication complexes. Topoisomerase IV provides a potent decatenating (unlinking) activity. Both gyrase and topoisomerase IV are essential enzymes, and, therefore, agents that attack them are expected to block bacterial growth [74].

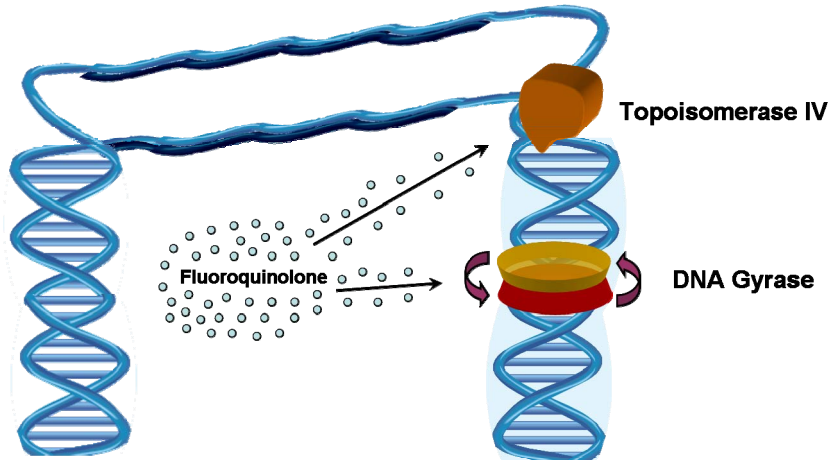


Figure 1.16: Mechanism of action of FQs where antibiotic/enzymes/DNA complexes block DNA movement through replication forks. The formation of these chromosomal complexes correlates with inhibition of intracellular DNA synthesis.

The FQs, however, do not simply eliminate enzyme function since they actively poison cells by trapping these two topoisomerases on DNA as drug/enzyme/DNA complexes in which double-strand DNA breaks are held together by protein. Thus, these ternary complexes interfere with DNA transcription, replication, and repair and promote his cleavage, leading to rapid bacterial cell dead.

1.3.2.4 Therapeutic use of fluoroquinolones

FQs are a synthetic class of antibiotics widely used for both the prevention and treatment of various diseases in animal husbandry and aquaculture, as well as in humans. In this sense, current clinical applications of FQs include the treatment of urinary tract, skin, gastrointestinal, and gynecological infections. Moreover, these antibiotics are also used to treat upper and lower respiratory infections such as sinusitis, pneumonia or bronchitis [75]. Regarding the veterinary use of this antibiotic family (see **Table 1.8)**, FQs are accepted for cystitis, urinary, respiratory (e.g. pneumonia), skin and soft tissue infections. Like aminoglycosides, FQs exhibit concentration dependent bacterial killing. The

pharmacokinetic profile of FQs ensures that they are well absorbed following oral administration, with moderate to excellent bioavility being later excreted as parent compound, as conjugates, or as oxidation, hydroxylation, dealkylation, or decarboxylation products. Thus, the environmental concern of this antibiotic family is not only based on their potential to promote antibiotic resistance, but also on their unfavourable ecotoxicity profile [76].

Table 1.8: FQs most applied in veterinary medicine in Europe and some of their brand name products. Data from the USP.

FQs	Commercial Products	Animals
Enrofloxacin	Baytril	cats, cattle, dogs, horses, pigs, sheep
Marbofloxacin	Marbocyl, Zenequin	cats, dogs, horses
Orbifloxacin	Orbax, Victas	cats, dogs, horses
Danofloxacin	A180, Advocin, Advocid	cattle
Difloxacin	Dicural, Vetequinon	dogs
Ibafloxacin	Ibaflin	goat
Sarafloxacin	Floxasol, Saraflox, Sarafin	poultry

These compounds bind strongly to topsoil, reducing the threat of surface water and groundwater contamination, which suggest that the terrestrial environment is a further relevant exposure pathway [4]. The strong binding of FQs to soil and sediments delays their biodegradation and explains their persistence in the environment. Wastewater treatment eliminates about 79%-87% of these antibiotics before arriving to the rivers indicating a low likelihood for adverse effects to the aquatic habitat of surface waters. On the other hand, these compounds are also susceptible to photodegradation in aqueous medium, involving oxidation, dealkylation, and cleavage of the piperazine ring.

1.3.3 ß-lactam antibiotics

1.3.3.1 History

The discovery and development of the BLs are among the most powerful and successful achievements of modern science and technology. Since Fleming's accidental discovery of the penicillin producing mould, eighty years of steady progress has followed, and today the BLs group of compounds are the most successful example of natural product application and chemotherapy [77]. BLs are considered a broad class of antibiotics that include penicillin derivatives (1940s), cephalosporins (1960s), monobactams (1980s), carbapenems (1980s), and β-lactamase (1990s) inhibitors, that is, any antibiotic agent that contains a BLs nucleus in its molecular structure (see **Figure 1.17**). Each one of these groups has yielded medically useful products and has contributed to the reduction of pain

and suffering of people throughout the world. Continued efforts have resulted in the improvement of these compounds with respect to potency, breadth of spectrum, activity against resistant pathogens, stability and pharmacokinetic properties. BLs have been used extensively both in human and veterinary medicine practices. These compounds are indicated for the prophylaxis and treatment of bacterial infections caused by susceptible organisms. At first, BLs were mainly active only against gram-positive bacteria, yet the recent development of broad-spectrum BLs active against various gram-negative organisms has increased their usefulness.

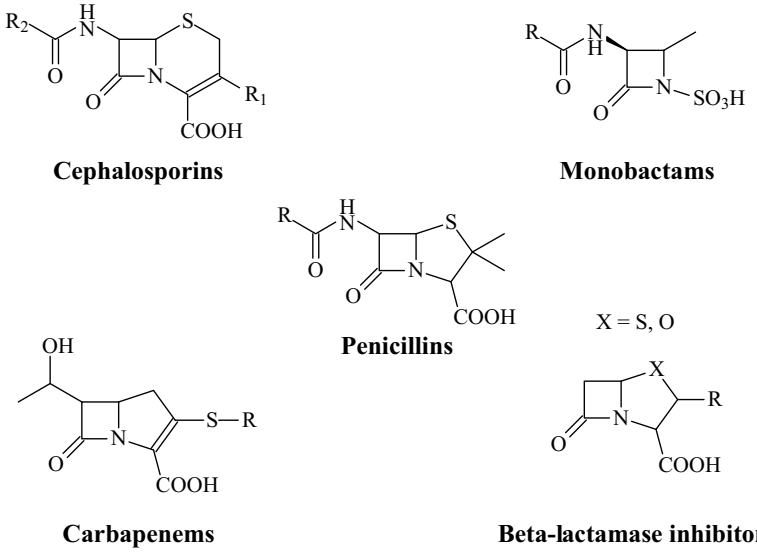


Figure 1.17: Core structures of most common BLs. The R group substituents can be changed to give the molecule different antibacterial properties.

In veterinary medicine, they have been widely as growth promoters as well as chemotherapeutic and prophylactic agents mainly used to treat diseases like mastitis, pneumonia, bacterial diarrhea and bacterial arthritis in food producing animals [78]. But in recent years, bacterial resistance to BLs has also been increasingly observed in bacteria, including those of animal origin. The mechanisms of BLs resistance include inaccessibility of the drugs to their target, target alterations and/or inactivation of the drugs by ß-lactamases [79].

1.3.3.2 Structures

Interestingly, all known classes of BLs share a common structural feature formed by a lactam (i.e. cyclic amide) with heteroatomic ring, consisting of three carbon atoms and one nitrogen atom.

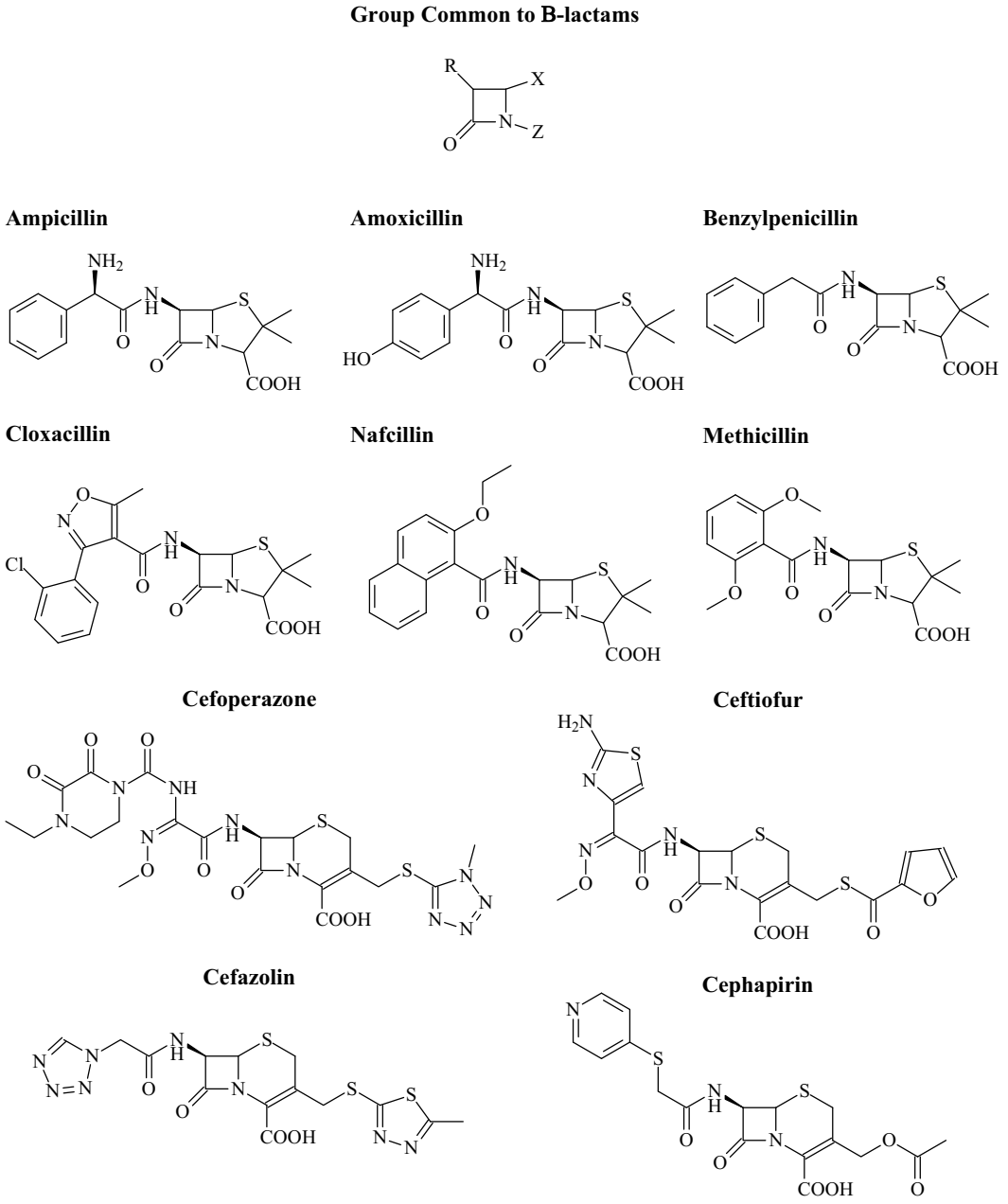


Figure 1.18: Chemical structure of some of the most important BLs.

As it can be observed in **Figure 1.17**, the ring is very strained while the corresponding bond between the carbonyl and the nitrogen is very labile and hence makes the molecule reactive. Since they were first discovered, thousands of BLs derivatives have been designed around the simple 2-azetidinone ring (see **Figure 1.18**). Thus, many potent antibacterial agents have been developed and millions of lives have been saved as a

consequence of fundamental and applied chemistry on and peripheral to the BLs ring. On the other hand, it's very difficult to establish structure/activity relationships in this antibiotic family from the fact that the targets in various bacteria exhibit widely different sensitivities. Moreover, some bacteria produce ß-lactamases enzymes capable of destroying the antibiotics. But the rates of the reactions with the ß-lactamases and the target enzymes are not necessarily related.

1.3.3.3 Mechanism of action

BLs show bactericidal effect, and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls (i.e. inactivating the peptidoglycan transpeptidase enzyme). This peptidoglycan layer is important for cell wall structural integrity, especially in gram positive organisms. Thus, if cell walls are improperly made cell walls allow water to flow into the cell causing it to burst. Gram-positive bacteria possess a cell wall is composed of a cellulose like structural sugar polymer covalently bound to short peptide units in layers.

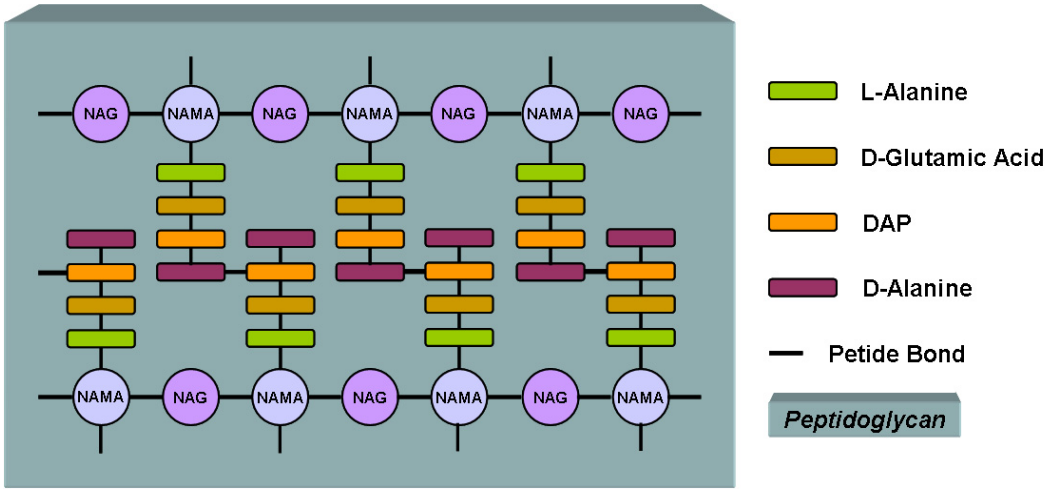


Figure 1.19: Representation of a cell wall. Gram-positive is composed by 50 to 100 molecules thick while it's only 1 or 2 molecules thick in gram-negative microorganisms. *Source: self-made but partially reproduced image from WikiChem (penicillin article).*

The polysaccharide portion of the peptidoglycan structure is made of repeating units of N-acetylglucosamine linked b-1,4 to N-acetylmuramic acid (NAG-NAM). The peptide varies, but begins with L-alanine and ends with D-alanine. In the middle is a dibasic amino acid, diaminopimelate (DAP) which provides a linkage to the D-alanine residue on an adjacent peptide. The bacterial cell wall synthesis is completed when a cross link between two peptide chains attached to polysaccharide backbones is formed (see **Figure 1.19**). The cross linking is catalyzed by the enzyme transpeptidase. First, the terminal

alanine from each peptide is hydrolyzed and secondly one alanine is joined to lysine through an amide bond (see **Figure 1.20**).

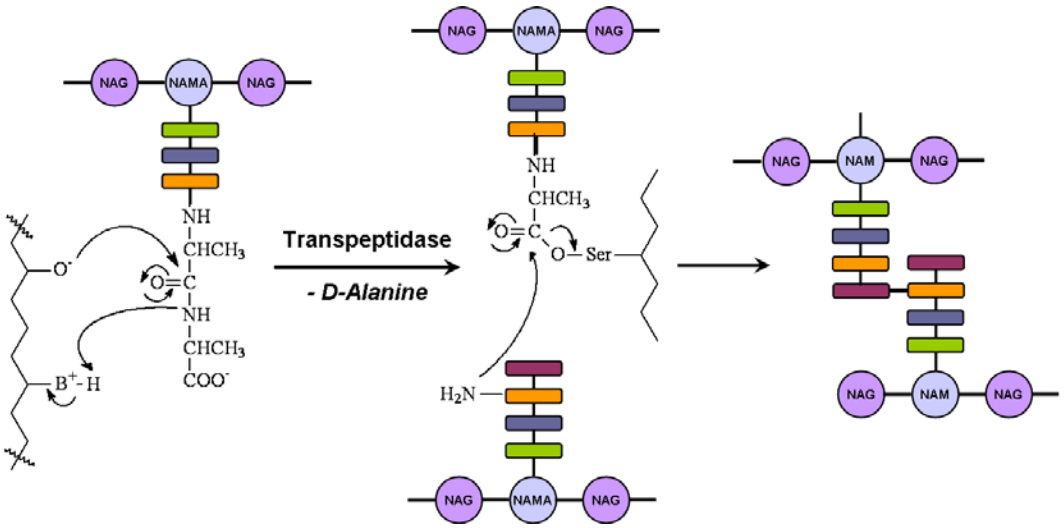


Figure 1.20: Formation of peptidoglycan cell wall.

Most of BLs are analogues of D-alanyl-D-alanine, which is the terminal amino acid residue on the precursor NAM-NAG peptide subunits of the nascent peptidoglycan layer. This structural similarity facilitates their binding to the active site of penicillin binding proteins (PBPs).

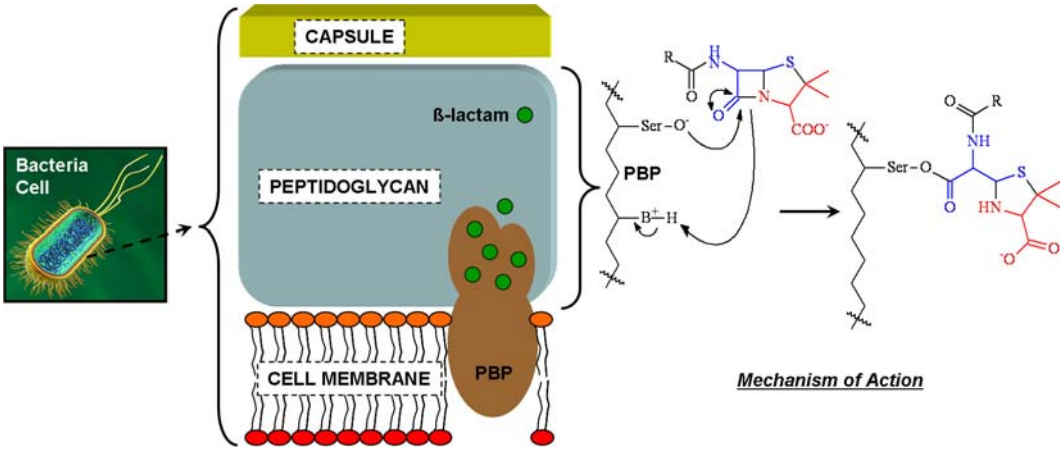


Figure 1.21: Simple representation of bacterial cell wall structure of gram-positive organisms together with the reaction involved in the inhibition synthesis of the peptidoglycan layer.

These PBPs have been shown to catalyse a number of reactions involved in the process of synthesising cross-linked peptidoglycan from lipid intermediates and mediating the removal of D-alanine from the precursor of peptidoglycan. In contrast, the BLs nucleus of

the molecule irreversibly binds to (acylates) the Ser403 residue of the PBP active site (see **Figure 1.21**). This irreversible inhibition of the PBPs prevents the final crosslinking (transpeptidation) of the nascent peptidoglycan layer, disrupting cell wall synthesis. Since mammal cells do not have the same type of cell walls, penicillin specifically inhibits only bacterial cell wall synthesis. Under normal circumstances peptidoglycan precursors signal a reorganisation of the bacterial cell wall and, as a consequence, trigger the activation of autolytic cell wall hydrolases. Inhibition of cross-linkage by BLs causes a build-up of peptidoglycan precursors, which triggers the digestion of existing peptidoglycan by autolytic hydrolases without the production of new peptidoglycan. Thus, the action of BLs on sensitive bacteria can be regarded as a two stage process. In the first stage, the antibiotics bind to the PBPs which are involved in central roles in the cell cycle-related, morphogenetic synthesis of cell wall peptidoglycan. Inactivation of these PBPs by bound antibiotic has immediate, biochemically definable effects on their function. The second stage encompasses the physiological effects on the sensitive cell initiated by this primary receptor ligand interaction [80].

1.3.3.4 Therapeutic use of ß-lactams

After several decades of clinical use, BLs family continues to be an important and frequently used antibiotic. Although increasing resistance to the drug has appeared in several bacterial species in the years since its introduction, many important pathogens remain exquisitely susceptible to these compounds [81]. Furthermore, this group is one of the most important families of antibiotic used in veterinary medicine in the treatment of septicaemia, urinary and pulmonary infections.

Table 1.9: BLs most applied in veterinary medicine in Europe and some of their brand name products. Data from the USP.

BLs	Commercial Products	Animals
Ampicillin	Plyflex	calves, cattle, dogs, horses
Cefadroxil	Cefa-Drops	cats, dogs,
Cephalexin	Apo-Cephalex, Biocef, Cefanox	cats, dogs
Amoxicillin	Amoxi-Drop, Amoxil Tablets	cats, cows, dogs
Penicillin G	Agri-cillin, Aquacillin, Combipen 48, Depocillin, Derapen	cattle, dogs, horses, pigs, sheep, turkeys
Ceftiofur	Excede, Excenel, Naxcel	cattle, chicks, dogs, goats, horses, pigs, sheep
Cephapirin	Cefa-Dri, Cefa-Lak, ToDay	cattle
Hetacillin	Hetacin-K Intramammary Infusion	cows
Cefpodoxime	Simplicef	dogs
Cefazolin	Ancef, Cefacidal, Cefamezin	dogs

In **Table 1.9** is summarized the list of most used BLs in veterinary medicine as well as their commercial brand names. The bewildering variety of BLs currently in clinical or veterinary use or under evaluation attests to the fact that the design of these selective inhibitors has still not been perfected. This continuing effort is necessary because of the intrinsic variation in bacterial susceptibility and because of the humbling capacity of bacteria to render the latest modified BLs ineffective by acquiring resistance. The first mode of BLs resistance is due to enzymatic hydrolysis of the BLs ring. The genes encoding these enzymes may be inherently present on the bacterial chromosome or may be acquired via plasmid transfer, and β-lactamase gene expression may be induced by exposure to BLs. The second mode of BLs resistance is due to possession of altered PBPs. BLs cannot bind as effectively to these altered PBPs, and, as a result, the BLs are less effective at disrupting cell wall synthesis [82].

1.3.4 Tetracycline antibiotics

1.3.4.1 History

Discover of penicillin at the late 1920s initiated a rush throughout the 1930s and ‘40s by scientists to discover other antibiotics and what it was commonly called *wonder drugs*, financed by large pharmaceutical companies. Specifically in 1947, Benjamin Minge Duggar, a botanist emeritus from the University of Wisconsin, was hired by the Lederle Laboratories Division of American Cyanamid, to bio prospect for soil microorganisms producing new antibiotic substances. Out of the many samples he obtained, one from a timothy hay field near Columbia produced an unusual yellow coloured microorganism from the genus *Streptomyce aureofacienss* [31]. He reported that this strain was able to produce in high yield a chemical of unknown identity and structure, which was named *aureomycin*, capable to be effective against many bacteria, rickettsias and viral pathogens. Nowadays, this compound is commonly known as chlortetracycline. The discovery of *terramycin*, currently called oxytetracycline, produced by *Streptomyces rimosus*, followed shortly thereafter in 1950 by Finlay and co-workers [83]. Subsequently in 1953, Conover was the first chemist who took aereomycin and chemically modified it by catalytic hydrogenation to produce the more stable and usable antibiotic *tetracyn deschloroaureomycin*, known as tetracycline [84], the generic structure for which the TCs family of molecules is named (see **Figure 1.22**). Then, research on the TCs family in this era of basic discovery, the 1950s and 1960s, primarily focused on the isolation and chemical characterization of novel compounds within the TCs from microorganism sources or chemical synthesis. This global research effort was fuelled by the hopes of increasing the potency of these antibacterial agents being the first major class of therapeutics to earn the distinction of broad-spectrum antibiotic due to their higher

effectiveness against both gram-negative and gram-negative pathogens. The early success of this antibiotic family contributed to their widespread production and use in both human and animal medicine in the decades following their discovery. As a result, this subsequently led to emergence some cases of resistance mechanisms and decreased effectiveness of TCs as frontline antibiotics. Currently, there are 40 known TCs resistance genes [85].

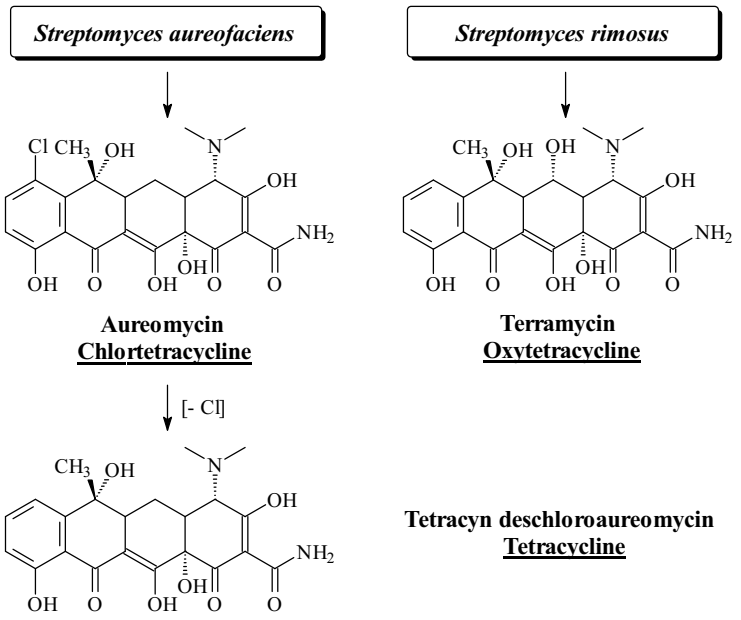


Figure 1.22: Industrial producers Streptomyces aureofaciens and Streptomyces rimosus and the primary TCs they biosynthesize.

On the other hand, development of new generation of effective compounds like tigecycline, which has been recently approved by FDA, demonstrates that the TCs scaffold remains a valuable starting point for further drug discovery. Nowadays, maybe other antibiotic families have considerably more citations but primarily within the bacteriological discipline and none showing the wide cross-utility as have the TCs. Besides the bacteriology field, their technological fronts are emerging in dental and enzymatic pharmacology, genetics and transgenics, cellular physiology of eukaryotic cells, neurology, oncology, reproductive biology and virology [86].

1.3.4.2 Structures

TCs comprise four annelated six-membered rings denoted as A, B, C and D, by the IUPAC (International Union of Pure and Applied Chemistry) convention, where the nucleus is perhydrogenated and only the D-ring retains aromatization.

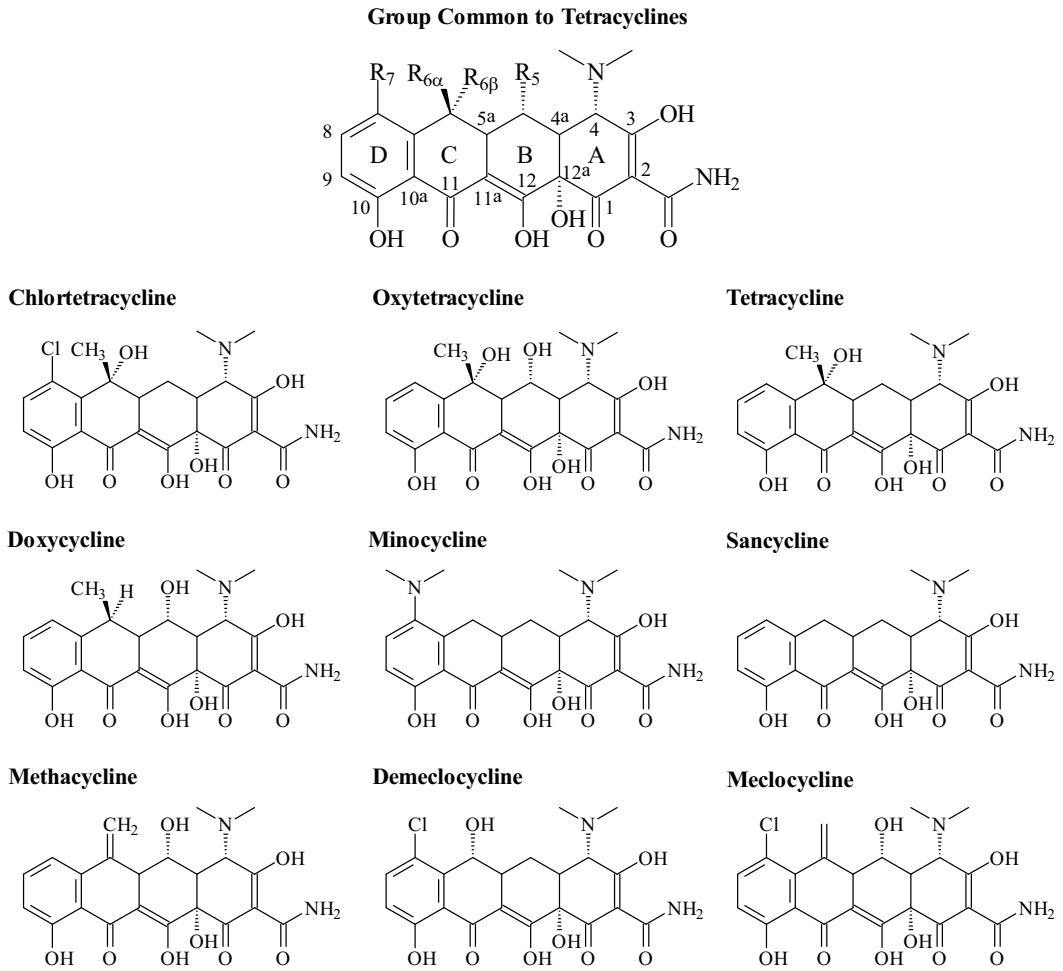


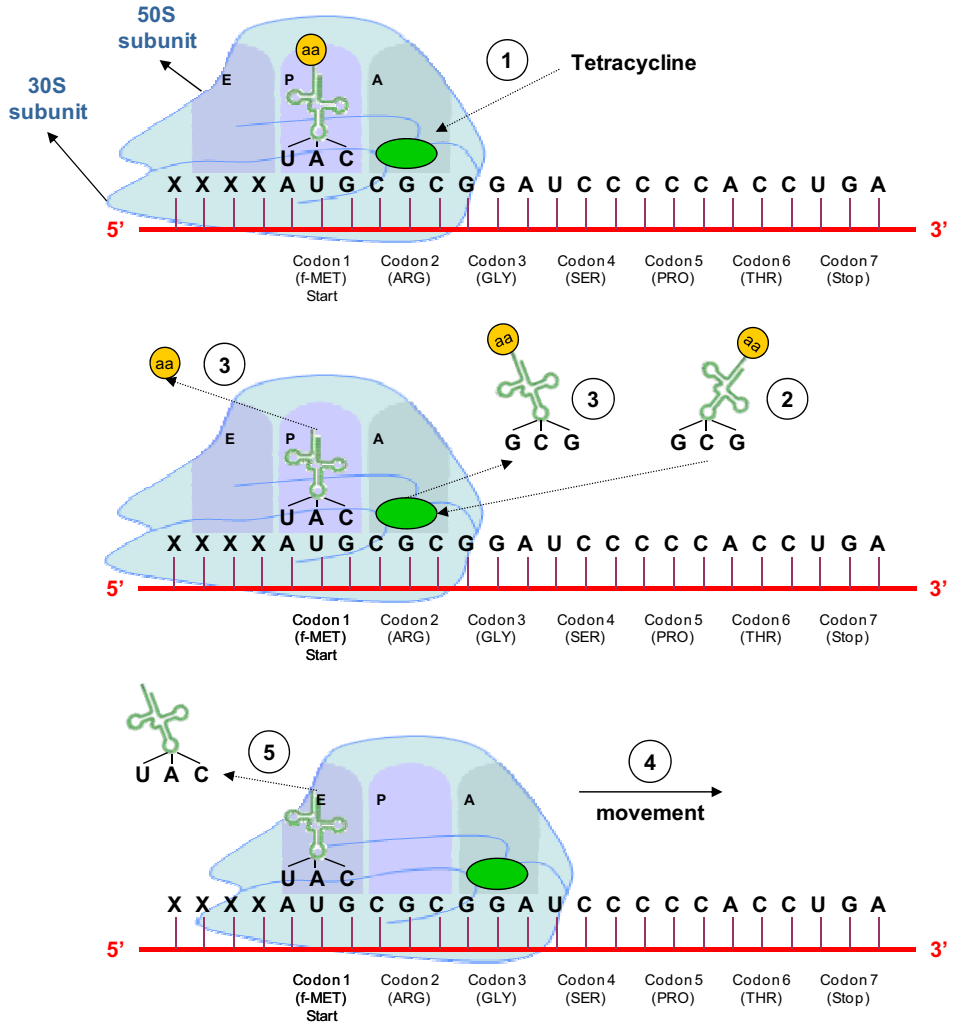
Figure 1.23: Chemical structure of some of the most common TCs.

These compounds are numbered starting at C1 on the A-ring moving counter clockwise, where all skeletal and exocyclic carbon atoms receive a number designation. The presence of a heavily oxidized lower periphery, that includes a C11, C12, and C11a keto-enol configuration, has an impact on the physicochemical and three dimensional features of these compounds being the major region responsible for the pharmacological properties of the TCs family [87]. Several well-known TCs, including both naturally produced and semi-synthetically derived, are shown in **Figure 1.23**. On the other hand, modifications of molecular functional groups within the upper peripheral region of TCs seem to alter their bioactivity drastically [88]. Thus, the linear arrangement of the ABCD rings is crucial for their properties, and as a general rule, chemical modification along the lower peripheral region decreases bioactivity, whereas the upper peripheral region can be usually chemically modified to produce other bioactive semi-synthetic TCs.

1.3.4.3 Mechanism of action

TCs, as well as SAs compounds presented before, exhibit a bacteriostatic rather than bactericidal effect, which means that they inhibit growth and reproduction of bacteria without killing them. **Figure 1.24** shows the TCs mechanism of action in bacteria.

TCs bind primarily but reversibly to the small 30S ribosomal subunits of bacterial where they inhibit protein synthesis by interfering and even blocking the binding of aminoacylated transfer ribonucleic acid (tRNA) to the A (aminoacyl) site [86]. One of the central roles of that subunit is to discriminate cognate from no cognate tRNAs by monitoring base pairing between the codon of messenger RNA (mRNA) and the anticodon on tRNA in its A site in a process called decoding. Then, more precisely TCs are inhibitors of this codon-anticodon interaction.



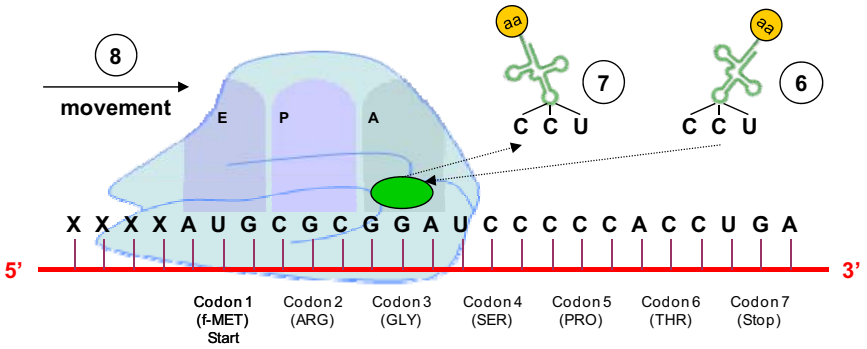


Figure 1.24: Mechanism of action of TCs: they block the bacterial translation by binding to the 30S ribosomal unit. Numeration indicates the order in which all the process is done. *Source: self-made but partially reproduced image from Pharmacology Corner (Animation: Protein synthesis inhibitors: tetracyclines mechanism of action animation. Classification of agents).*

This antibiotic family can also inhibit protein synthesis in the host, but are less likely to reach the concentration required because eukaryotic cells do not have a TCs uptake mechanism. Other antibiotics, such as pactamycin and hygromycin B, can also interact with the 30S ribosomal subunit but with E (exit) and P (peptidyl) binding sites inhibiting the growth of bacteria [87].

1.3.4.4 Therapeutic use of tetracyclines

TCs are used in human medicine for the treatment of infections related to the respiratory tract, sinuses, middle ear, urinary tract, intestines, and also gonorrhoea, especially in patients allergic to BLs and macrolides. More recently, biologic nonantimicrobial actions affecting inflammation, proteolysis, angiogenesis, apoptosis, metal chelation, ionophoresis, and bone metabolism have been also researched [88]. Their most common current use is in the treatment of moderately severe acne and rosacea. DC is also used as a prophylactic treatment for infection by *Bacillus anthracis* (anthrax) and is effective against *Yersinia pestis*, the infectious agent of bubonic plague. Additionally, can be used for malaria treatment and prophylaxis, as well as treating elephantiasis and reducing the duration and severity of cholera. Moreover, TCs remain the treatment of choice for infections caused by *chlamydia* (trachoma, psittacosis, salpingitis, urethritis and *Lymphogranuloma venereum* infection), *Rickettsia* (typhus, Rocky Mountain spotted fever), brucellosis, and spirochetal infections (borreliosis, syphilis, and Lyme disease). However, their use for some of these indications is less popular than it once was due to widespread resistance development in the causative organisms. On the other hand, TCs are even more widely used in veterinary medicine mainly for the treatment of gastrointestinal, respiratory and skin bacterial infections, infectious diseases of locomotive organs and of genito-urinary tract as well as systemic infections and sepsis

[92]. In this case, TCs preparations registered in the EU contain TC, DC, chlortetracycline (CTC) and OTC compounds [93] to be applied in an extensive list of target animal species (see **Table 1.10**).

Table 1.10: TCs most applied in veterinary medicine in Europe and some of their brand name products. Data from the Joint Expert Advisory Committee on Antibiotic Resistance and from the USP convention.

TCs	Commercial Products	Animals
Chlortetracycline	Aureomycin, Chlor Max, Chlorosol	beef cattle, cats, dogs, pigs, poultry, sheep
Oxytetracycline	Agrymycin, AmTech Maxim, Biomycin	beef cattle, bees, cats, dogs, fish, goat, horse, pigs, poultry, rabbit, sheep
Tetracycline	AmTech Tetracycline, Duramycin	beef cattle, poultry, rabbit
Doxycyclines	Vibramycin, Vibra-Tabs	cats, dogs

On the other hand, this antibiotic family compounds are contraindicated in several cases, such as oral administration to ruminants for reasons of the destruction of their microflora and the attenuation of the digestive processes [94] or in pregnant and young animals, because TCs form chelates with calcium on the surface of teeth and bones, which results in the discoloration of teeth and in a retarded development of the skeleton [95]. In contrast to the US, the application of TCs as growth promoters is not allowed in the EU (Council Directive 70/524/EEC [96]), and since 1975, no tetracycline has been used for this purpose. Consumption of TCs in veterinary medicine is relatively high as compared with other classes of antibiotics ranking the first positions in consumption statistics in the EU according to European Federation of Animal Health (FEDESA). Thus, in **Table 1.10** is summarized the list of most used TCs in veterinary medicine as well as their commercial brand names.

As with most types of antibiotics, only small portions of the TCs administered are actually metabolized or absorbed in the body, and most of the drug is eliminated in faeces and urine in unchanged form [97]. Normally, TCs are not found at high levels in the environment: because of their chelation properties, they readily precipitate in the presence of divalent cations (e.g. Ca^{2+}, Mg^{2+} or Zn^{2+}) and are accumulated in sewage sludge or sediments. On the other hand, TCs residues have also been detected in many surface water resources that receive discharges from municipal wastewater treatment plants and agricultural runoff [29]. Besides the demonstrated persistence of these antibiotics in agricultural soils that have received manure containing antibiotics, the biodegradation of these compounds to even more toxic substances must activate new strategies to improve their control and the efficiency of their removal in wastewater plants.

1.4 Environmental and human biological monitoring

Environmental toxicology deals with complex interactions between thousands of chemicals and multitude of potential adverse effects. Thereby, environmental monitoring (EM) provides physical, chemical and biological information about the use, fate, degradation, and concentration of these contaminants in scenarios like air, soil, plant and foods to which the population is exposed [98]. All these knowledge obtained can be only used as an estimation of the external body exposure to xenobiotic and anthropogenic compounds. The assessment of the absorbed or internal accumulation dose of these substances by using EM approaches is frequently under suspect basically due to the extrinsic effect combination of other environmental (i.e. temperature, humidity and wind) and human factors (i.e. smoking, work and alimentary habits, etc.) [99]. Nowadays, biological monitoring (BM) procedures can closely estimate and confirm internal individual exposure to external chemicals from the environment by measuring parent compounds, metabolites or protein adducts in biological samples regardless of the route [100]. EM and BM should be totally complementary for early detection of exposure, risk, and prevention of future health effects (see **Figure 1.25**). Thus, BM has been promoted as a valuable method for exposure assessment since the 1950s, but gaining increasing higher reputation in occupational and environmental health in order to determine individual chemical vulnerabilities during the last decade [101]. Biomarkers are defined as indicators of actual or possible changes of systemic organ, tissue, cellular and functional integrity, which can be used singly or in batteries to monitor health and exposure to compounds in populations and individuals [102]. These signs are used by the biomonitoring techniques in body fluids [103], tissues [104] or exhaled breath [105]. There are two major types of biological markers: biomarkers of exposure, which are used in risk prediction, and biomarkers of disease, which are used in screening and diagnosis and monitoring of disease progression. Those used in risk prediction, in screening, and as diagnostic tests are well established, and they offer distinct and obvious advantages. Some of the desired characteristics of an ideal biomarker of exposure are: sensitivity and specificity in relation to the xenobiotic, stability in both vivo and in vitro even for any length of time storage, availability by non-invasive sampling, relevance to the dose of exposure, presence in concentrations detectable by current analytical methods at a reasonable cost in a reasonable time [106]. Most exposure biomarkers are well suited for the identification of the exposed individuals and groups, however this requires analytical, metabolic and source specificity. Analytical specificity means that the analytical method is capable of measuring exclusively the chemical parent or metabolite that it purports to be evaluated in a complex matrix. We are talking about metabolic specificity when the

marker measured is the product of interest derived exclusively from the parent chemical of interest.

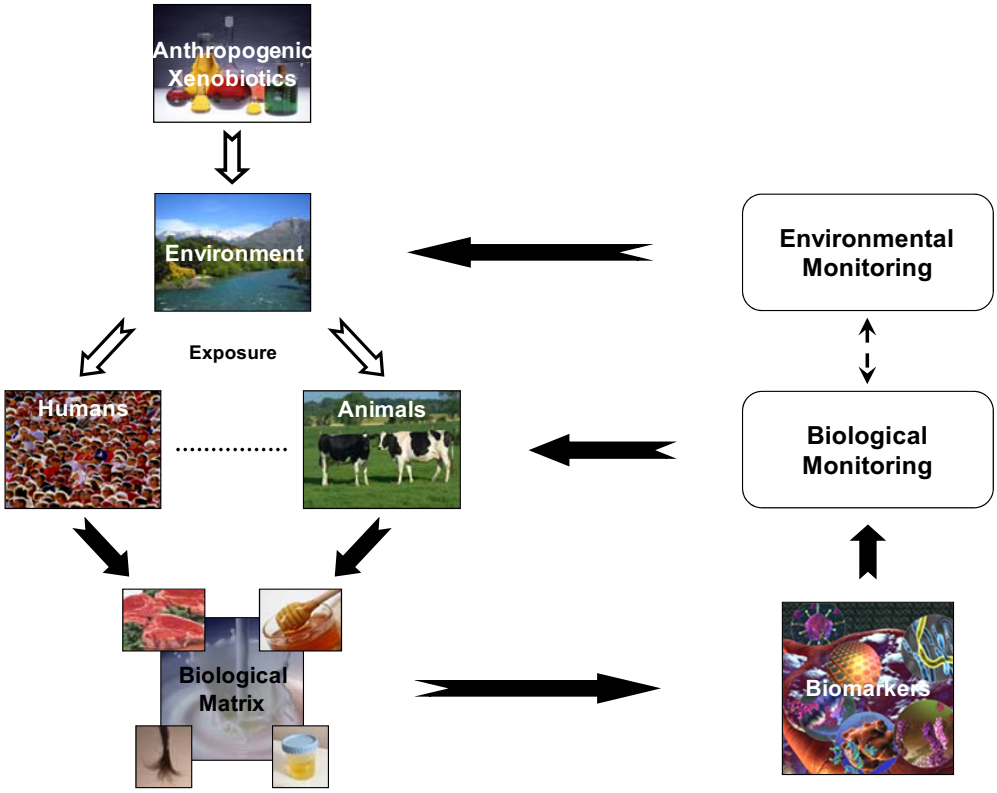


Figure 1.25: EM and BM to assess health risk evaluation and future adverse effects.

Finally, the source specificity concept regards the extent to which the source of exposure can be attributed directly to the workplace, lifestyle factors, diet, ecological exposures or others [107]. In order to select the best biological medium or sample for monitoring the chemicals of interest, scientists have to consider aspects such as excretion patterns of the selected analyte, the complexity for obtaining or treat the sample, the sampling time and cost, compatibility with the analysis technique, and the availability of data relating excretion to exposure [108].

The control of veterinary drugs, as well as other type of contaminants, is commonly performed not only on tissue samples obtained after slaughter but also on biological fluids taken from living animals. The collection of these samples is sometimes invasive besides requiring a carefully storage and dispatch for not having undesired cross contamination. Traditionally, urine has been the preferred matrix for the analysis of Group A compounds (see **Section 1.2**), because is the route of excretion for many xenobiotics, it is relatively easy to collect, and the measured concentration of the parent chemical or metabolite is

proportional to the adsorbed dose for the majority of xenobiotics [109]. Additionally, urine samples can be taken before slaughter having the advantage that, when *non-compliant* (positive) results are obtained, the animals can be destroyed to prevent them reaching the market. For Group B compounds, it is observed that, next to bovine meat, liver, kidney and milk samples, there is increasing interest in monitoring drug residues in samples of fish, egg and honey and also animal feed. In recent years, the analysis of hair and retina has gained popularity because residues are detectable for a long time after treatment. Thus, the increasing number of potentially harmful pollutants in the environment, as well as in biological samples, for fast and cost-effective analytical techniques to be used in extensive monitoring programs.

1.4.1 Analytical techniques

Analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food producing samples have been extensively reviewed [42, 110]. Traditional procedures used to identify and quantify these substances have been mainly based on dipsticks or microbiological tests, immunoanalytical and chromatographic techniques. Depending on the type of analysis it is recommended use one technique or another (see **Figure 1.26**).

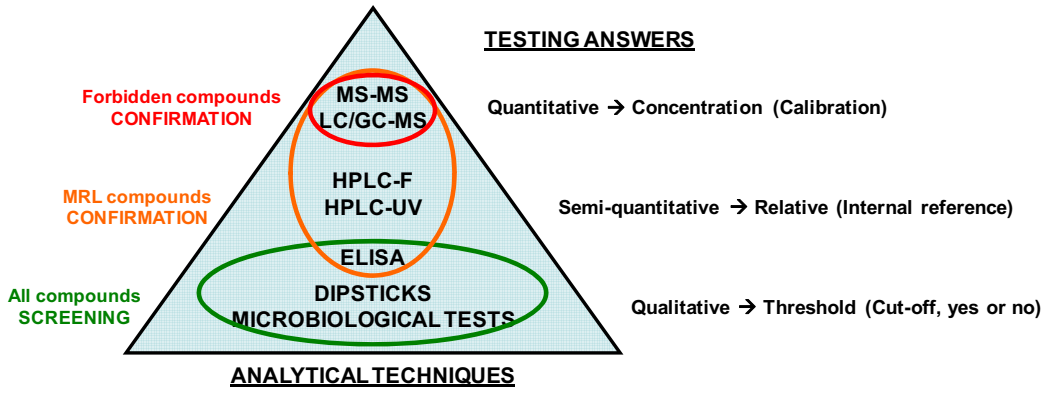


Figure 1.26: Outline of the analytical techniques most used for the detection of antibiotics starting from screening assays (i.e. qualitative answer) up to final confirmation analysis (i.e. quantitative answer).

For example, quantitative confirmation methods (e.g. chromatographic techniques coupled to MS detectors) must be automated, process monitoring and labour-free but their speed is sometimes a limitation needing also a little training before use them. Besides, semi-quantitative confirmatory methods (e.g. chromatographic techniques not coupled to MS detectors) are required to be ready for multianalysis in real time, on-line and cost-efficient. Nowadays, what is essential is to improve the screening qualitative techniques

(e.g. ELISA, dipsticks or microbiological tests) to be very easy to use, rapid, accurate, reliable, precise, obtaining clear negatives and being able to detect several contaminants simultaneously.

Microbiological tests are non-expensive, easy to perform, broad spectrum assays, adaptable for large screening and do not require specialized equipment or toxic solvents. On the other hand, main disadvantages are being not a very specific technique (i.e. can't tell what compound is concern), having the possibility to obtain high percentage of false positives and negatives due to the influence of the matrix and limited detection levels to many antibiotics. Thus, the use of confirmatory techniques is always necessary in this case. The instrumental analyses combine the high resolution of the chromatographic methods (e.g. gas chromatography (GC) and high performance liquid chromatography (HPLC) with the excellent detectability of sophisticated detectors such as those based on mass spectrometry (MS). These techniques are extremely specific with accurate mass capability which allows the reconstruction of highly selective precise mass chromatograms of target residues being very useful for confirmation analysis and identification of *unknowns*. On the other hand, they require extensive sample preparation, skilled laboratory personnel, thereby limiting their use as fast as efficient high-throughput screening methods. Additionally, all these factors lead to an increase of the final cost of these methodologies and the analysis time. Alternatively, immunochemical techniques are simple, fast, very specific and sensitive being excellent tools to be exploited in monitoring programs where a great number of samples need to be analyzed in a short period of time [4]. One of their drawbacks is often the fact that specific and nonspecific signals are not so easy to distinguish leading to overestimation results. Contrariwise, false negatives are very seldom seen in these techniques. Thus, as effective screening techniques, these methods are complementary to the standard analytical chromatographic ones.

Today requirements, both in terms of time and costs, of most traditional analytical methods (i.e. chromatographic techniques) often constitute an important impediment for their application on regular basis. In this context, biosensor portable devices and real time monitors, as well as field assays like test kits, dipsticks, and indicators appear as suitable complementary analytical tools.

1.5 Analytical techniques based on molecular receptors

Molecular recognition plays a very important role in biological systems, like for example enzyme-substrate, DNA-protein, and Ab-Ag interactions, being essential for the existence of life [108]. All these bindings are often referred to as the *lock and key* model where both the receptor and the substrate possess specific interactions and complementary geometric shapes that fit exactly into one another (see **Figure 1.27**). All these specific interactions refers to noncovalent bonding, such as hydrogen bonding, metal coordination, hydrophobic forces, *van der Waals* forces, π-π interactions, and/or electrostatic effects [109]. This selective molecular recognition phenomenon has been also widely used to detect and quantify veterinary drugs, at trace levels in environment and biological samples. Most of these analytical systems are based on the use of antibodies, as the recognition element, giving rise to the known immunochemical techniques employed in both EM and BM.

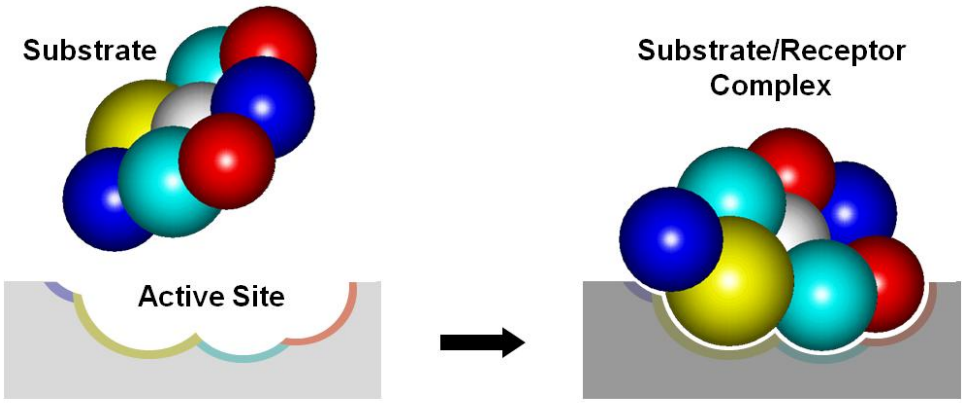


Figure 1.27: Schematic representation of Fischer's *lock and key* model. The substrate binds specifically to its receptor, forming a complex, to produce some kind of response to the system.

The elegance of molecular recognition in nature has been spurring many scientists to mimic it. This issue has led to the establishment of the new field of biomimetic chemistry, in which imitations of natural binding entities are being studied. As the structures and mechanisms of biochemical systems become known, scientists are attempting to transfer this knowledge to new synthetic strategies to reduce the degree of complexity of some biological systems and their limitations mainly related to their low stability in organic solvents [110]. One of the greatest advantages of the artificial receptors over naturally occurring ones is freedom of molecular design where the stability, flexibility, and other properties are freely modulated according to need. Several factors are necessary for obtaining an artificial receptor with accurate molecular recognition capacities. Firstly, functional residues of the target analyte and receptor must be complementary to each

other, then conformational freedom of both components should be minimized and finally chemical circumstances should appropriately regulated. In many cases, various functional residues are covalently attached to cyclic host molecules, such as crown ethers [114] and cyclodextrins [115]. Although each of the interactions involved in previous examples is rather weak, remarkably high selectivity and binding strengths are accomplished when all of them work cooperatively. During the last few years MIPs have appeared to be one of the most used synthetic receptors mainly due to their easy, fast and economic preparation. MIPs are polymers possessing specific cavities designed for a specific target molecule. By a mechanism of molecular recognition, the MIPs are used as selective tools for the development of various analytical techniques such as liquid chromatography, capillary electrochromatography, SPE, binding assays and biosensors [113]. Nowadays, the requirements both in terms of time and costs of most traditional analytical methods, such as the chromatographic ones, often constitute an important impediment for their application on regular basis. Thus, the methodologies to be developed for this purpose are based on molecular recognition systems for the chemical of interest. Traditionally, most of these interactions already applied on analytical procedures take profit of the affinity and selectivity characteristics of antibodies generated against the desired compound by vertebrate immune systems.

Antibody production for a target analyte is very well established, but with some limitations including the high cost and time requirements as well as host animal variability due to the intrinsic genotypic elements of each species or individual [116]. Although having a great potential, this last factor may have been contributing largely to the slow or poor implementation of the immunochemical methods in the reference laboratories. The polymeric nature of molecularly imprinted polymers results in some advantages over natural antibodies. For instance, the physical and chemical resistance of imprinted polymers leads to the possibility of sterilising the polymers, the high durability ensures a high stability of the recognition properties, and the production cost is considerably lower. Another obvious advantage is the obviation of the need for host animals in the antibody production. Moreover, sensors based on a specific biological/artificial recognition element in combination with a transducer for signal processing also appear as suitable alternative or complementary analytical tools expecting to play a significant analytical role in medicine, agriculture, food safety, homeland security, environmental and industrial monitoring [117].

1.5.1 Synthetic receptors: Molecular Imprinted Polymers

Nowadays MIPs could be considered as the most promising approach towards artificial Antibodies. These materials have been prepared against a wide number of compounds

showing pretty good molecular affinity and selectivity properties but still far away from the antibody case. On the contrary, their chemical and thermal stability, easy preparation and low cost make them attractive for numerous applications. There are two main molecular imprinting approaches to prepare MIPS depending on the nature of adducts between functional monomers and template, which can be even combined. Typical examples of these two kinds of methods are presented in **Figure 1.28**. Both have advantages and disadvantages and thus the choice of the best method strongly depends on various factors (see **Table 1.11**).

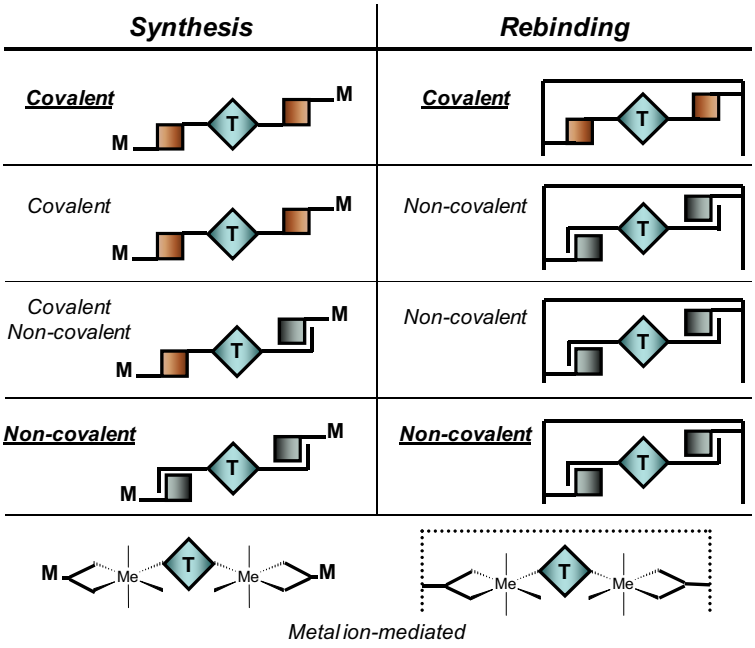


Figure 1.28: Molecular imprinting synthesis and subsequent rebinding approaches. (T = template, M = monomer, Me = metal).

Wulff and his co-workers reported the first covalent imprinting in 1977 [118]. In this approach, functional polymerizable monomer and template are bound to each other by labile covalent linkage prior to the polymerization. The template molecule is fixed in spatial arrangement within the polymer matrix by means of this chemical bond. After the polymerization process, the labile bond is then cleaved to remove the template molecule, leaving binding pockets that are claimed to be more uniform in placement of a single complementary functional group than those obtained by the non-covalent approach. The covalent approach is more difficult to apply since it requires multiple heteroatom functionality to be available in the template molecule. In the most common approach, firstly described by Mosbach and co-workers [119], the synthesis of MIPs involves first the complexation in solution of a template molecule with functional monomers, through

non-covalent bonds, followed by polymerisation of these monomers around the template with the help of a cross-linker in the presence of an initiator.

Table 1.11: Advantages and disadvantages of covalent and non-covalent imprinting.

Molecular imprinting procedure	Covalent	Non-covalent
Synthesis of monomer-template conjugate	Necessary	Unnecessary
Polymerization conditions	Rather free	Restricted
Removal of template after polymerization	Difficult	Easy
Guest-binding and guest-release	Slow	Fast
Structure of guest-binding site	Clearer	Less clearer

Therefore, the choice of the chemical reagents making up the MIP must be judicious in order to create highly specific cavities designed for the template molecule. Polymerization is then affected usually in bulk, forming a highly cross-linked polymer around the template molecule. Then, template molecules are removed by extensive washing steps to disrupt the interactions between the template and the monomers leaving binding *pockets* that have size and functional memory of the template molecule [120]. By virtue of its simplicity and generality, the non-covalent approach is by far the most widely used for molecular imprinting [121].

1.5.1.1 Theoretical and experimental methods to prepare MIPs

The main concerns regarding experimental procedures of molecular non-covalent imprinting are the reagents involved, reaction conditions and the factors to lead high imprinting efficiency. Moreover, the precise evaluation of the imprinting is also an important aspect to have in account. To prepare a MIP all necessary chemicals, besides a template, are the functional monomers, crosslinking agents, porogens for the polymerization, and solvents to remove the templates from the final polymer. The reaction should occur under the conditions where all the components remain intact. The radical polymerization is the most commonly used because of its versatile applicability and experimental easiness. The key issue in the synthesis of MIPs is the optimisation of the main factors that affect the material structure and its molecular recognition properties. Lately, some groups combine previous information obtained from computational chemistry models calculations and ^{1}H-NMR experimental data [122] to then apply it on experimental design and multivariate analysis methods [123] to evaluate most promising theoretical configurations. Finally, the guest-binding activity of the imprinted polymer obtained is measured mainly by chromatographic experiments with the correspondent activity for a non-imprinted polymer (NIP), prepared in absence of template, the magnitude of the molecular imprinting effect is evaluated.

1.5.1.2 Applications of molecularly imprinted polymers

Many compounds, such as antibiotics, pesticides hormones, ß-receptors or even sugars, peptides, proteins and whole cells, have been used as template molecules in molecular imprinting. In fact, over 1500 references related to the use of MIPs in a large range of application areas have been recently collected [124] being used as separation media in most cases. So, these materials have been applied in several analytical techniques, such as liquid chromatography separation [125], capillary electrochromatography [126], capillary electrophoresis [127], and MISPE procedures [128]. Versatility of imprinting methodologies has made possible the development of new applications beyond the *traditional* uses of MIPs. Special note in preparative organic chemistry are the applications of these materials in synthesis, where the imprinted polymers can act as heterogeneous scavengers or clean-up aids, as passive or reactive supports, as *protecting groups* and perhaps most importantly as catalysts [129]. Moreover, the characteristics of MIPs make possible their use to develop highly stable sensors which can be used in harsh environments. These materials can be even the only alternative if there are no natural recognition elements available. Applications of MIPs to sensor technology can be categorized according to the transducers which convert the signals of polymer recognition into physical ones, such as electrochemical, optical, and piezoelectric [130-132]. The methods most widely used are based on optical detection, including fluorimetry, spectrometry, luminescence or even surface plasmon resonance (SPR). Substantial works on electrochemical sensors (i.e. conductimetry, potenciometry, and amperometry detection) utilizing MIPs have also been developed. Considerable effort has been taken to substitute antibodies with robust and inexpensive synthetic receptors, but it should be considered only as complementary analytical resources to overcome some of their limitations but not to entirely replace them.

1.5.2 Natural receptors: Antibody

Nowadays, the worldwide market for therapeutic and diagnostic immunology involves more than 100.000 research antibodies commercially available [133]. Their specificity enables them to react with their respective antigenic determinants and not with others being very attractive to be applied in numerous applications.

1.5.2.1 Structure and properties

Antibodies are gamma globular proteins that are found in blood or other fluids of vertebrates, and are used by the immune system to identify and neutralize foreign agents, such as bacteria's and viruses. Mammalian antibody structure varies depending on their

isotype (IgA, IgD, IgE, IgG and IgM) being the immunoglobulin G subclass the most abundant in serum and consequently the most used in immunochemical applications [134]. The immunoglobulin G (IgG) are nano molecules (15 nm x 12 nm x 5 nm; Mw ~ 150 kDa) built of two heavy (H; Mw ~ 50 kDa) and two light (L; Mw ~ 25 kDa) identical polypeptide chains interconnected by disulfide bonds (see **Figure 1.29**).

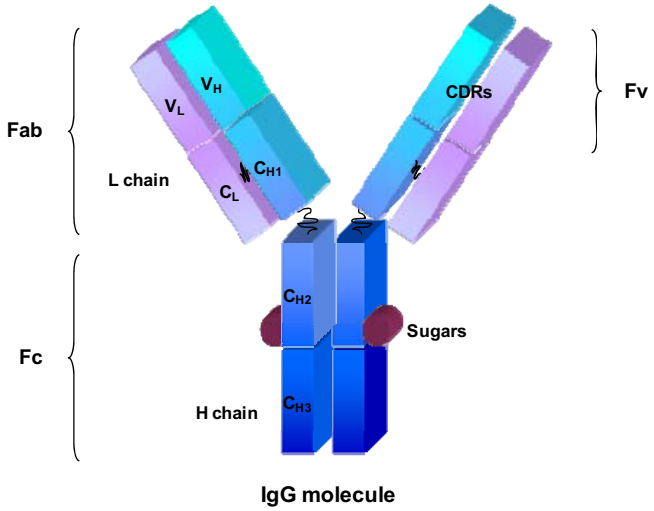


Figure 1.29: Schematic antibody structure. Constant domains: C_L; C_{H1}, C_{H2} and C_{H3}. Variable domains: V_L and V_H. Antigen binding site: CDR (hypervariable regions).

The Fc (fragment crystallisable) region, composed of two heavy chains, have the ability to bind to various cell receptors to regulate the immune response. On the contrary, the fragment antigen binding (Fab) region is composed of one constant and one variable domain from each heavy (C_{H1}, V_H) and light chain (C_L, V_L). The most important regions of the antibody are the ones which recognize the antigen epitope, being shaped at the amino terminal end of the antibody monomer by the variable domains (Fv) from the heavy and light chains [135]. The immunochemical techniques are based on the interaction between Ab-Ag which involve non-covalent binding of the antigenic determinant (epitope) to the variable region (CDR) of the immunoglobulin chains. To describe the strength of the Ab-Ag interaction at the equilibrium, the affinity constant (Ka) can be defined as it is showed below in **Equation 1.1**.

$$Ab + Ag \longleftrightarrow AbAg \qquad Ka = \frac{[AbAg]}{[Ab]\cdot[Ag]}$$

Equation 1.1: [AbAg] is the antibody-antigen complexes, [Ab] the unbound antibody, and [Ag] is the free antigen not bound.

In this case, Ka values range from 10^{-7} to 10^{-11} M [136]. The high affinity showed makes possible detection of compounds at very low concentrations, using also little sample

volume amounts (from μL to mL). Thus, based on these interactions several analytical tools have been developed for both analysis or samples clean-up.

1.5.2.2 Antibody production

Proteins, peptides or small chemical compounds can be used to generate antibodies being a great number of them commercially available. On the other hand, many research groups decide to raise their own antibodies against new target compounds or to improve the ones found in the market. Nowadays, antibodies can be produced by three different techniques (see **Table 1.12**), yielding polyclonal (PAb), monoclonal (MAb), and recombinant antibodies following, in all cases, very well established procedures.

Table 1.12: Comparison between polyclonal, monoclonal and recombinant antibodies[a].

Type / Properties	Polyclonal	Monoclonal	Recombinant
Production	Limited	Unlimited	Unlimited (No immunization)
Affinity	Highly uniform	Highly/poorly uniform	Highly/poorly uniform[b]
Cross-reactivity	Variable, depending on immunization hapten	Variable, depending on each individual	Variable, depending on each individual[b]
Class	Typical range	Defined isotype	Variable, depending on molecular design
Ag requirement	High purity for a specific serum	Immunization: im/pure Screening: pure	Immunization: im/pure Screening: pure
Cost	Low	High	High

[a]*Extracted from the Encyclopedia of Analytical Chemistry (A. Dankwart).*
[b]*Property that can be modified.*

PAb are produced in vivo in response to multiple injections of the antigen along with an enhancer of the immune response. The procedure can be performed in a wide range of mammal animals (e.g. rabbits, goats or sheep) to obtain a serum with several different populations of IgGs exhibiting high affinity binding properties to different epitopes (antigenic determinants) on the immunogen. Main drawback of PAb is the limitation to obtain them in reproducible quantities from different host animals. MAb are typically made by fusing myeloma cells (cancerous murine plasma cells) with the spleen cells from a mouse that has been immunized with the desired antigen. In this manner, MAb contain a unique defined IgG molecule produced by a single cell clone providing single epitope specificity and potentially limitless amounts of identical antibody. However, generally MAb have lower affinities to small molecules than PAb and are more expensive and difficult to produce [137]. Although their main use has been conducted mainly for therapeutic application, recombinant antibodies can provide another source of high quality detection reagents to identify and detect contaminants in environmental samples.

These are similar to MAb but consist of only the antigen binding domains and are produced from immune tissue or hybridoma cell lines in vitro through the use of recombinant DNA technology. Thus, genetic engineering provides to the biorecognition molecules an elegant way for the alteration of their existing properties and the supplementation with additional functions. Moreover, the ability to produce a large number of recombinant antibodies in bacterial cells and to select for antibodies that bind to unique or non-dominant epitopes demonstrates the power of a recombinant approach to antibody development. Expression and purification of recombinant antibodies by bacterial fermentation is normally less expensive, easier to perform, and less time consuming than production of either PAb or MAb [138].

1.5.2.3 Antibody-based analytical methods

Antibodies are the key component in immunochemical techniques, whether used in bioanalytical determinations or bioseparation procedures of a target compound from a sample. Biosensors, based on Ab-Ag interaction phenomenon, are also of great interest because of their potential for future automation and easier field application of these immunodetection strategies [139]. Immunochemical methods have been widely used because they are rapid, sensitive, simple, low in cost, with high throughput and easy to use being more practical that conventional analytical procedures. On the other hand, the progress of development of new immunochemical techniques is in some way also limited by the availability of antibodies with the desired affinities and specificities for given applications. Efforts are still to be made for developing new antibodies for both common and new emerging contaminants and pollutants. As any other detection technique, antibody-based analytical methods have advantages and disadvantages, but overall impression is that they constitute an excellent tool to be exploited in monitoring programs.

1.5.2.3.1 Immunoassays

Nowadays, the immunoassay (IA) is probably the most frequently used immunochemical methodology. Since the first IA reported in 1961, where Yalow and co-workers developed an assay to detect human insulin [140], this bioanalytical technique has gradually spread to agriculture, forensic, medicine, pharmaceutical veterinary and other areas including environmental and food analyses. In all these areas, where large a number of samples are required to be analyzed, high sample throughput features of IAs makes them particularly suited to large-scale monitoring efforts. The general strategy of IA to quantify small molecules involves a competition step between a fixed amount of labelled Ag and the free analyte for a limited amount of antibodies, in contrast with determination

of large substances which is not always necessary. At the end of the reaction the amount of analyte can be indirectly determined by measuring the labelled antigen. The label may consist of radioisotopes (RIA, radioimmunoassay [141]), an enzyme (EIA, enzyme immunoassay [142]), fluorophores (FIA, fluoroimmunoassay [143]), chemiluminescent reagents (CLIA, chemiluminescence immunoassay [144]) or magnetic markers (MIA, magnetic immunoassay [145]). Most common IAs require the use of a solid support, such as microtiter plates, tubes, microspheres or magnetic particles, to immobilize one of the immunoreagents before the analysis process [146]. This configuration is known as heterogeneous format. On the contrary, when the assay is performed directly in solution we are talking about the homogeneous format. In this case, experiments are faster, simpler, and can be easily adapted to the available automated analyzers often used in clinical chemistry. However, they are often less sensitive and are more exposed to matrix interferences than the heterogeneous ones. Although RIA technique is extremely sensitive, it has the big disadvantage of handling and producing radioactive residues, they tend to be replaced by more by more environmentally friendly and less hazardous label substances. Nowadays, enzymes like horseradish peroxidase (HRP), alkaline phosphatase, and glucose oxidase are most common labels used in IAs. The use of these labels makes possible the signal amplification in presence of a substrate and subsequent increase of the assay detectability. ELISA is the most common and frequently used EIA as analytical method being also very popular for the evaluation or characterization of antibodies. The most usual configurations (direct and indirect formats) for the analysis of small molecules will be showed in posterior chapters of this thesis. On the contrary, FIAs use fluorescent labels instead of enzymes, such as fluorescein, rhodamine, cyanines or earth chelates (Eu^{3+}, Tb^{3+}). They were firstly developed mainly because of their theoretical superior sensitivity in comparison with the spectrophotometric methods but final results achieved in many cases have been not so good as expected due to the presence of many interferences in real samples that drive to a quenching effect of the signal [147]. The enzyme-multiplied immunoassay technique (EMIT) and polarization fluoroimmunoassay (PFIA) are examples of IAs working under homogeneous conditions, so less commonly used but being both methods successfully applied in the analysis of environmental pollutants [148]. In recent years, flow-injection systems coupled to immunoassays (FIIA) offer rapid analysis of a great number of samples, providing rapid results, good sensitivity, and allowing continuous monitoring working in both homogeneous and heterogeneous conditions [149]. This first integrated approach for automated IAs was the starting point of many innovative research areas to developed new devices for the analysis of chemical contaminants.

1.5.2.3.2 Immunosensors

In recent years many efforts have been made to develop biochemical techniques, integrating specific recognition elements and electronic components to obtain small devices with the ability to carry out direct, selective, and continuous measurements. Thus, a biosensor is a self-contained integrated device, consisting of a biological component (enzymes, protein receptors, antibodies, nucleic acids, whole cells or even biomimetic receptors) in direct contact with a physical transducer, which converts the biological recognition event into a useable output signal (see **Figure 1.30**).

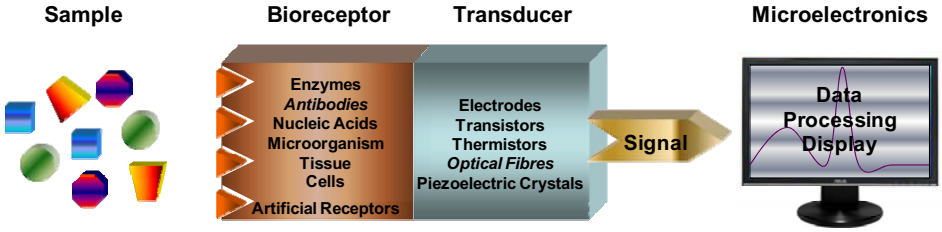


Figure 1.30: Essential components of a biosensor formed by the biorecognition element surface, a transducer and the electronic device involved in the data processing and display.

In this sense, immunosensors are affinity-based sensors designed to detect the direct binding of an antibody or an antigen to form an immunocomplex at the transducer surface. These devices are usually classified into various basic groups according to the method of signal transduction (see **Table 1.13**): electrochemical [150], optical [151], piezoelectric [152], and thermometric [153].

Table 1.13: Transducers and principle of detection in biosensors.

Transducer	Type	Principle/Change	Detection	Ref.[a]
Electro chemical	Amperometric	Oxidation state	Indirect	[154]
	Conductimetric	Conductivity	In/Direct	[155]
	Impedimetric	Conductivity	In/Direct	[156]
	Potentiometric	Surface potential	In/Direct	[157]
Optical	Fibber Optic	Optic properties	Indirect	[158]
	Photon correlation spectroscopy	Scattered light	Direct	[159]
	Surface plasmon resonance	Refractive index	Direct	[160]
	Total internal reflection	Evanescent wave	In/Direct	[161]
Piezo electric	Bulk acoustic	Frequency	Direct	[162]
	Cantilever	Surface stress	Direct	[163]
	Surface Acoustic Wave	Frequency	Direct	[162]
Thermometric	Calorimetric	Temperature	Indirect	[164]

[a]*Examples of each type of transducer has been included.*

Extensive information on the transducer principles, methods, and examples of all these types of immunosensor can be found in recent reviews [165-168] as well as in a chapter book prepared included at the end of this chapter (see **Section 1.9**). Analysis of small organic molecules by the immunosensors are usually performed under competitive assay configurations (indirect measurements) and/or using labels, such as enzymes, fluorescent chemicals or electrochemically active substances. Detectability and selectivity parameters are mainly determined by the immunoreagents used, not by the assay configuration, so immunosensors after being optimized should be capable to achieve at least the same detection limits as the ones obtained in other detection platforms. Successful biosensors must be versatile to support interchangeable biorecognition elements, and in addition miniaturization must be feasible to allow automation for parallel sensing with ease of operation at a competitive cost. Biosensors have been mainly reported for clinical purposes in medicine [169], but slowly their application has been extended to agriculture, food safety, homeland security, environmental and industrial monitoring [117]. However, the final commercialization of biosensor technology has significantly lagged behind the research output as reflected by a plethora of publications and patenting activities.

1.5.2.4 Bioseparation techniques

Methods that use immunoaffinity chromatography (IAC) for sample preparation or detection are becoming increasingly popular as tools in the analysis of biological and non-biological compounds. There are many different procedures that can be used for the antibody immobilization to both low and high performance supports. Main advantage over SPE formats is that IAC provides a highly selective extraction based on specific molecular recognition [170]. Thus, antibody cross-reactivity allows targeting the parent compound and structurally related analytes. Moreover, pre-concentration of analytes from large sample volumes resulting in low detection limits is also possible. On the other hand, the high price of some of these immunoreagents increases the cost of the IAC respect other techniques. The most common applications include the use of IAC in the direct detection of analytes, the extraction of samples prior to on/off-line detection by other methods, their use in chromatographic-based immunoassays, and the development of post column reactors based on IAC for the detection of analytes as they elute from other types of chromatographic columns [171]. This separation technique has been successfully implemented in the environmental and bioclinical fields.

1.5.3 Multi-residue detection approaches

In the last years, the progress of development of new efficient analytical methods has grown considerably to protect public health by controlling environmental pollutants. To

go further, today main research efforts are focused in creating new multiresidue analytical procedures able to analyze in a single run an important number of chemicals. Most of them combine the high resolution of the chromatographic methods (GC, HPLC, ultra performance liquid chromatography (UPLC)) with powerful detectors such as those based on mass spectrometry (QqQ). As a curiosity, several of these multiresidue chromatographic methods have been validated to determine more than one hundred pesticides after a fast extraction method and a short instrumental analysis [172]. Regarding molecular receptors, the current trend is for development of imprinted techniques, natural and artificial receptors or aptamers that enable detection of several targets within a single test avoiding tedious sample treatments. Considering these approaches, there is no doubt that classical antibodies will play an important role even in the future since they exhibit a high potential for sensitivity and desired broad selectivity. The simultaneous analysis of many samples is possible by incorporating the immunoreagents on formats using 96- or 384-well microplates or immunoaffinity columns for developing platforms with high throughput capabilities [173]. On the other hand, an uncontrolled high cross-reactivity pattern of an antibody could increase the tendency of these techniques toward false positive results also commonly produced by matrix effects when measuring real samples.

1.6 Immunochemical techniques for antibiotic substances

A great number of immunochemical methods have been developed for many veterinary drugs. Some examples for the immunodetection of the most important antibiotics in environmental samples have been lately reviewed in our research group [4, 168] and an update is included in the published book chapter at the end of this chapter. In here, recent examples for the immunodetection of these compounds in biological food samples are presented in **Table 1.14**.

Table 1.14: Immunochemical techniques for veterinary drug substances in milk and other biological samples.

Compound/s	Immunochemical technique	Matrix	Ref.
Chloramphenicol	ELIFA[a], Dipstick EIA	Milk	[174]
	ELISA	Milk, eggs, muscle, honey	[175]
	SPR	Milk, prawn, muscle, honey	[176]
	ELISA,GC-MS^2/LC-MS^2	Shrimp	[177]
FQs	ELISA	Milk	[178]
	ELISA	Chicken liver	[179]
	IAC	Chicken muscle and liver	[180]
	SPR	Egg, fish, poultry meat	[181]
	Impedance sensor	Buffer	[182]
Macrolides	ELISA	Milk, kidney	[183]
	ELISA	Swine tissue	[184]
	ELISA	Bovine meat	[185]
Penicillins	SPR	Milk	[186]
	Time-resolved FIA	Milk	[187]
	Amperometric sensor	Milk	[188]
	Impedimetric sensor	Milk	[189]
SAs	Flow cytometric IA	Milk	[190]
	Magneto-ELISA	Milk	[154]
	ELISA	Honey	[191]
	IAC	Muscle	[192]
TCs	ELISA	Milk	[193]
	Dips-tick	Milk, serum, meat	[194]
	ELISA	Honey	[195]
	ELISA	Honey	[196]

[a]*Enzyme-linked immunofiltration.*

Moreover, there are several commercially available antibody-based analytical methods for several of these substances. See a few examples in **Table 1.15**.

Table 1.15: Some representative commercial assay kits based on immunodetection for the most important veterinary drugs in milk samples.

Analyte	IA Kit Test	Supplier	Contact
Chloramphenicol	MaxSignal ELISA	BIOO Scientific Co.	www.biooscientific.com
	CAP EIA	Euro-Diagnostica AB.	www.eurodiagnostica.com
	L'screen CAP	Tecna Srl.	www.tecnalab.it
FQs	MaxSignal ELISA	BIOO Scientific Co.	www.biooscientific.com
	Charm Rosa Test	Charm Sciences Inc.	www.charm.com
	FQs ELISA Test	Randox Laboratories	www.randox.com
Macrolides	MaxSignal ELISA	BIOO Scientific Co.	www.biooscientific.com
	Multi-AGP EIA	Euro-Diagnostica AB.	www.eurodiagnostica.com
	L'screen Tylosin	Tecna Srl.	www.tecnalab.it
Penicillins	Delvo-X-PRESS	DMS Food Specialties	www.dsm.com
	Parallux	IDEXX Laboratories	www.idexx.com
	SNAP ß-Lactam	IDEXX Laboratories	www.idexx.com
SAs	MaxSignal ELISA	BIOO Scientific Co.	www.biooscientific.com
	SNAP SMZ	IDEXX Laboratories	www.idexx.com
	Sulfa Combo	Int. Diagnostic Systems	www.ids-kits.com
TCs	MaxSignal ELISA	BIOO Scientific Co.	www.biooscientific.com
	Delvotest	DSM Food Specialties	www.dsm.com
	SNAP Tc	IDEXX Laboratories	www.idexx.com

Many other veterinary drug residues Test Kits, applied in other biological samples, can be found in the next web link: http://www.aoac.org/testkits/testedmethods.html#antibiotics.

This brief overview shows that immunochemical techniques have been widely proven to be an interesting alternative to other analytical methodologies to asses risk and protect public health from the adverse effects of many pollutants. However, additional work is necessary in order to expand the number of families of compounds that can be detected using these methods, and especially to adapt them to the analysis of complex samples. From now on, big efforts are focused to develop multi-analyte immunochemical systems, wherein more than one compound or group of compounds could be detected simultaneously, and the design of new analytical user-friendly devices for continuous or *on site* measurements. Technical development should be accompanied by some officially organized efforts to find ways to validate and recognize the screening immunoassay techniques as practicable routine methods in control laboratories.

Thus, the main goal of the present thesis consisted of complementing the already existing developed immunochemical techniques, starting from the antibody design, preparation, and fully evaluation in biological samples to its final implementation in other devices such as dipsticks or biosensors. All this work has been complemented by developing a rational strategy to prepare MIPs using the SAs as a model. But before, in order to complete this introduction two book chapters have been included in this section containing, 1) supplementary information about most relevant contaminants nowadays, their regulations, and examples of new immunochemical methods to detect them in environmental samples, and 2) information about transducer principles, methods, and examples of biosensors based on novel micro and nanotechnology approaches.

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1.8 Book Chapter 1: Immunochemical Analytical Methods for Monitoring the Aquatic Environment

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A review on immunochemical methods for the analysis of pollutants such as industrial contaminants, pesticides, and pharmaceutical and personal care products is presented. A broad range of these pollutants can enter the environment in a great many ways after excretion mainly through industrial, domestic, and hospital wastewater. From there on, the transport, fate, and possible adverse consequences of these pollutants on human health and the ecosystem are frequently unknown or at best not clearly understood. Potential concerns include reproductive impairment, increased incidence of cancer, development of antibiotic-resistant bacteria, or the potentially elevated toxicity of chemical mixtures due to synergistic effects. The aim of regulations and regulatory methods to assess and control the impact of these substances in the aquatic environment is to protect the ecosystem and public health while keeping watch on their contamination levels and potential negative effects. In order to achieve these objectives, more efficient analytical techniques need to be developed. In recent decades, immunochemical techniques have been widely demonstrated to be an interesting alternative to the more conventional analytical methodologies in many areas, but additional work is still necessary to completely adapt them to the analysis of environmental contaminants. On the other hand, considering that the analysis of very complex biological samples with these methods has been successful, the prospects for their application to the analysis of water and soil samples seem highly promising. Technical development should be accompanied by some officially organized efforts to find ways of validating screening immunoassay techniques and recognizing them as practicable routine methods in environmental monitoring laboratories.

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8 Immunochemical Analytical Methods for Monitoring the Aquatic Environment

Javier Adrian, Fátima Fernández, Alejandro Muriano, Raquel Obregón, Javier Ramón, Nuria Tort, and M.-Pilar Marco

CONTENTS

8.1 INTRODUCTION

In recent decades, efforts to better understand the occurrence, fate, and environmental effects of anthropogenic chemicals have focused largely on industrial compounds and agricultural pesticides. This emphasis has been in response to the increasing production of these chemicals, including the so-called emerging contaminants, such as pharmaceutical active ingredients and personal care products, their concentrated use, the potential risk of persistence, and their sometimes unknown acute and chronic toxic effects (see Table 8.1 for the most important groups of emerging pollutants).[1]

Pollutants can enter the environment in a great many ways.[2] Some compounds, such as pesticides, are deliberately released during agricultural applications; others, like industrial by-products, get into our water and air resources during regulated and unregulated industrial discharges. On the other hand, pharmaceuticals and biogenic hormones are normally discharged into the environment through wastewater treatment plants (WWTPs), which are often not designed to remove them from the effluent. From there on, the transport, fate, and possible adverse consequences of these pollutants on human health and the ecosystem are frequently unknown or at best not clearly understood.[3] Potential concerns include reproductive impairment,[4–6] increased incidence of cancer,[7] development of antibiotic-resistant bacteria,[8] or the potentially elevated toxicity of chemical mixtures due to synergistic effects;[9] these concerns demand extensive investigation of all possible situations.

The aim of regulations and regulatory methods to assess and control the impact of these substances in the aquatic environment is to protect the ecosystem and public health while keeping watch on their contamination levels and potential negative effects. In addition to the REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) law (EC 1907/2006) regarding chemicals and their safe use, which came into force on June 1, 2007, there are specific regulations for protecting health and ensuring the good quality of all water resources, such as the Drinking Water Directive (DWD, the Council Directive 98/83/EC), the Bathing Water Directive (2006/7/EC), or the Urban Waste Water Directives (91/271/EEC and 98/15/EC). Moreover, the intention of the Water Framework

TABLE 8.1
Some of the Most Important Contaminants Needed to be Monitored

Industrial Contamintants	Pesticides	Pharmaceuticals
Surfactants and metabolites	*Insecticides*	*Antibiotics*
Nonanionic	Organochlorines	Sulfonamides
Anionic	Organophosphorus	Fluoroquinolones
Cationic		Amphenicols
		Tetracyclines
		ß-Lactams
		Macrolides
Organohalogenated compounds	*Herbicides/plant growth regulators*	*Other drugs*
Polychlorinated	Triazines	Diclofenac
Chlorophenols	Phenylurea compounds	Indomethacin
Dioxins	Chloroacetanilides	Nitrofurantion
Polybrominated biphenyl ethers	Sulfonylureas	Paclitaxel
Bromophenols		Spectinomycin
Heavy metals and metalloids		*Steroid hormones*
As, Cd, Cr, Cu, Pb, Hg, Ag, Se, Zn, etc.		Estrogens
		Androgens
		Gestagens
		Corticosteroids
Industrial additives and others		
Phthalate esters		
Bisphenol A		

Directive (WFD, 2000/60/EC) is to provide an overall framework for a cleaner and safer aquatic ecosystem, particularly with regard to surface freshwater and groundwater bodies (i.e., lakes, streams, rivers, estuaries, coastal waters, etc.). Thus, in line with this directive, the Marine Strategy Directive (2008/56/EC) aims to achieve a good environmental status of the EU's seawaters by 2021. Similarly, a River Basin Management Plan (RBMP) is being developed for implementation in each river basin district; each such plan must include knowledge of the particular pressures and impacts of human activities on the river basin in question, as well as protection programs, controls, and remediation measures. The first RBPM is scheduled to be published at the end of 2009; in parallel with it, the new Groundwater Directive (2006/118/EC) establishes a regime that sets underground water quality standards (QSs) and introduces measures to prevent or limit inputs of pollutants into groundwater.

The aim of all these directives is to maintain water quality in all its aspects, including chemical pollution, on which this chapter will focus. Community policy regarding dangerous or hazardous substances in European waters was introduced almost three decades ago by Council Directive 76/464/EEC. This Directive is now integrated in the WFD (codified as 2006/11/EC). The main strategy against the pollution of surface waters involves the identification of substances and development of control measures. More than 100,000 existing chemicals have been identified in the European Inventory of Existing Commercial Chemical Substances (EINECS). Data collection on the potential adverse effects of these chemicals has led to 141 of them being classified as priority substances. The latest list of priority substances presenting a significant risk to or via the aquatic environment was published in Council Decision No 2455/2001/EC. The list identifies a further 33 substances or groups of substances that have been shown to be of major concern. Moreover, in July 2006, the EC adopted proposals for a new Directive to protect surface water from pollution by setting up Environmental Quality Standards (EQS, concentrations of pollutants which should not be exceeded) and measures to monitor pollution (COM(2006)397 final). Although for surface waters the WFD aims to ensure at least a minimum chemical quality, particularly in relation to very toxic substances, the case regarding groundwater is somewhat different: the assumption is that groundwater should not be polluted at all. Therefore, the WFD strategy includes a prohibition on direct discharges to groundwater and, to cover indirect discharges, requires groundwater bodies to be monitored so that any change in chemical composition can be detected.

In order to achieve these objectives, more efficient analytical techniques need to be developed. This is so that the public health can be protected by ensuring that levels of pollutants remain below EQS concentrations and by providing, on a continuous basis, information on the fate of existing chemicals. A variety of multiresidue analytical procedures capable of analyzing an important number of chemicals[10–14] in a single run have been reported in recent years. Most of them combine the high resolution of chromatographic methods (gas chromatography (GC), HPLC, and UPLC) with the excellent detectability of sophisticated detectors like those based on mass spectrometry. In spite of this, these procedures lack the requisite sample throughput for a really effective assessment of the health of aquatic environments. The main limitations are due to the sequential nature of these analytical methods as well as the need to clean up and preconcentrate samples prior to analysis. In this context, immunochemical methods, which are based on the affinity of an antibody for an antigen (Figure 8.1), should be looked at as an interesting alternative for a number of reasons.

Firstly, measurements are performed by default in aqueous media, which makes these methods excellent tools for the analysis of aqueous samples. Secondly, owing to high specificity and detectability of certain substances or groups of substances, it is often possible to analyze them directly in the same matrix without having to purify, extract, and preconcentrate the sample. It is also possible to develop a variety of immunochemical configurations targeted to particular analytical requirements. Thus, a great number of immunochemical methods for the on-site analysis of environmental pollutants have been reported or are commercially available.[15–19] On the other hand, simultaneous analysis of many samples is possible by incorporating the immunoreagents on formats using 96- or 384-well microplates and developing formats with high sample-throughput capacities.[20–22] Selective sample treatments based on immunosorbents yield very clean aqueous extracts; such techniques can then be applied in tandem with some other type of immunochemical or chromatographic analysis.[23–26]

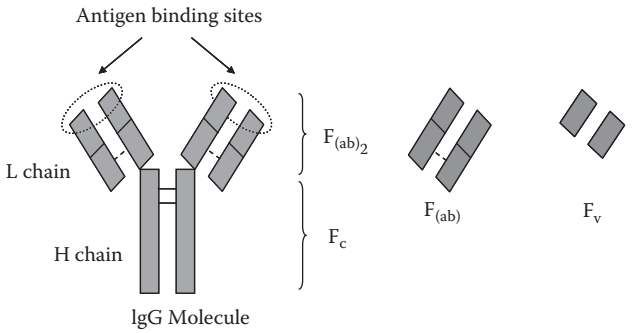

FIGURE 8.1 Basic H_2L_2 structure of the G immunoglobulins (IgG). It is formed by two pairs of polypeptide chains interlinked by disulfide bonds. The F_c fragment is the constant region and is involved in immune regulation, whereas the $F_{(ab)}$ (antibody binding fraction) fragment is the region that contains the variable fraction (F_v) with specific binding sites that allow interaction with Ag.

Recent years have witnessed interesting advances in the incorporation of selective immunoreagents into electronic devices, which emit optical or electrical signals in response to the presence of an analyte. Immunosensors have made it possible to develop integrated devices capable of handling data and of taking automated remedial action when the analyte is present.[3,27–33] Since the reader will find extensive information in recent reviews on the fundamentals[34–38] of these immunochemical analytical methods and examples of their application in environmental analysis,[29,30,33,39–44] we will now concentrate our attention on the applicability of these methods to the determination of some of the most important pollutants in aquatic environments—specifically, marine and freshwater ecosystems and their biota. The chapter does not pretend to be an exhaustive review of what has been reported in the literature; rather, its aim is to provide the reader with representative examples of immunochemical analytical technologies that have either been applied to the aquatic environment or exhibit great potential in this respect. Examples of the several groups of chemicals have been selected according to their toxicological risk, relevance, and regular use or production.

8.2 IMMUNOCHEMICAL DETERMINATION OF INDUSTRIAL CONTAMINANTS

Worldwide industrial activity generates vast amounts of chemical residues such as metals, polycyclic aromatic hydrocarbons (PAHs), polyhalogenated biphenylethers, and surfactants. One of the main concerns regarding the contamination caused by such activity is the enormous amount of unknown substances generated as by-products during the manufacture of other chemicals. The highly toxic dioxins, for example, are released into the environment largely as the unwanted by-products of industrial processes. In addition, there is the risk of major accidents, like the Seveso accident in 1976, with dramatic consequences for the population. Immunochemical methods for following up and monitoring the emissions of some of these pollutants into the aquatic environment have been reported.

8.2.1 Polycyclic Aromatic Hydrocarbons

PAHs are chemical compounds consisting of fused aromatic rings without any heteroatoms or substituents. They are formed mainly from the incomplete combustion of organic carbon-containing fuels such as wood, coal, diesel fuel, fat, or tobacco. PAHs can be found airborne in the gaseous phase or adsorbed to particles,[45] in aqueous matrices like groundwater, wastewater, drinking water, or river water,[46] and even adsorbed to solids in sediments or soils.[47] The U.S. Environmental Protection Agency (EPA) includes 16 PAHs in the list of priority pollutants in wastewater to be

TABLE 8.2
Immunochemical Techniques Developed for the Detection of PAHs

Target Analyte	Technique	Matrix	Sensitivity		Reference
			LOD	IC_{50}	
		PAHs			
Benzo(a)pyrene[a]	ELISA	Water and sediments	0.7 μg L^{-1}[b]	20.5 μg L^{-1}[b]	50
	ELISA	Tap, lake, and river water	24 μg L^{-1}[b]	65 μg L^{-1}[b]	51
	ELISA[c] (SDI, Newark, USA)	Soil		3.2 μg L^{-1}[b]	54
	ELISA[c] (SDI, Alton, UK)	Sediment	5.5 μg kg^{-1}	255 μg kg^{-1}	55
PAHs	ELISA[c] (Ohmicron, PA, USA)	River water		0.1–37 μg L^{-1}[b]	57
	Immunosorbent-LC	Surface water	0.002 μg L^{-1}		48
	Immunoaffinity and GC-MS	Corals	25 μg kg^{-1}		62
Phenanthrene[a]	Amperometric immunosensor	River water	5.0 μg L^{-1}	18.0 μg L^{-1}	58
		Tap water	6.3 μg L^{-1}	26.0 μg L^{-1}	
	Amperometric immunosensor	Sea, river, and tap water	1.4 μg L^{-1}[b]	29.3 μg L^{-1}[b]	59

[a] Indicator of total PAHs.
[b] LODs and IC_{50} calculated in buffer solution.
[c] Commercial kit.

monitored[48] because of their carcinogenic, mutagenic, and toxic properties.[7] The European Council Directive 98/83/EC concerning the quality of water intended for human consumption (WIHC) established a limit of 0.01 μg L^{-1} for benzo(a)pyrene, the lowest limit set for any individual chemical parameter in this directive.

As can be seen in Table 8.2, several immunochemical techniques, principally enzyme-linked immunosorbent assays (ELISAs), have been developed and applied to environmental samples such as water, soil, and sediments with very good limits of detection (LOD).[49–51]

EPA has included commercially available ELISA kits in its list of official methods (Method 4035)[52] capable of detecting PAH concentrations >1 mg kg^{-1} in soil samples.[53–56] The usual ELISA configurations for the analysis of small molecules are shown in Figure 8.2.

Most of the antibodies described were produced against one particular PAH congener, but because of their structural similarities they are in fact able to detect several PAHs. For this reason, different assays employ distinct target analytes as indicators of total PAH content, for example, phenanthrene,[53,56] benzo(a)pyrene,[50,51,54,55] or pyrene.[49] When real samples are immunoassayed, PAH levels have often been overestimated due to cross-reactivity with other congeners in the same sample.[50,54,57] In contrast, the underestimated PAH levels reported following the analysis of sediments[55] or contaminated soil samples[56] can be explained by the low extraction efficiency of the methods recommended in most commercial kits, where samples are merely shaken manually in the presence of an organic solvent. Nevertheless, the possibility must also be entertained that the sample contains PAHs with a low response factor to the particular antibody employed in the kit.[49,53] Amperometric immunosensors employing electrodes directly printed with an immobilized competitor of the target analyte have also been investigated to detect phenanthrene in environmental samples. Following this approach, Fahnrich et al.[58] achieved detection limits of 5 μg L^{-1} in river water and 6 μg L^{-1} in tap water samples. Moore et al.[59] applied the same transducing principle to

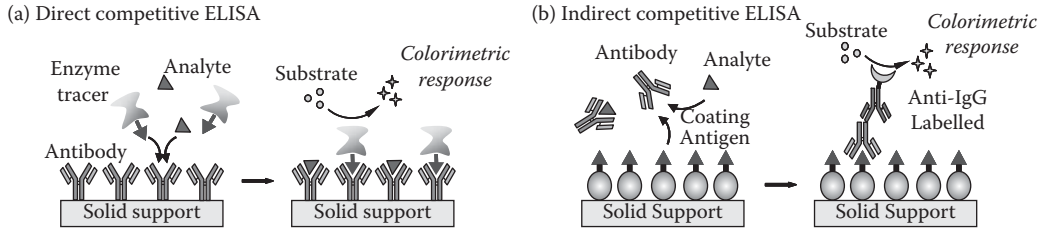

FIGURE 8.2 Scheme of the ELISA formats most frequently used for the analysis of low-molecular-weight analytes. Direct competitive ELISA: the Ab is coated on the surface and a competition is established between the analyte and the enzyme tracer. After the washing step, a substrate is added to produce a chromogen product that is easily quantified. Indirect competitive ELISA: a coating antigen is immobilized on the solid support while the specific IgG and the analyte are in solution during the competition step. After the removal of unbound reagents, a secondary IgG labeled with the enzyme (IgG-enzyme), which specifically recognizes the Ab, is added. Finally, after another washing step, the amount bound is also quantified by the addition of the substrate solution.

analyze phenanthrene in sea, river, and tap water samples, obtaining an LOD of 1.4 μg L^{-1}. These biosensors have great potential in field assays, where simple, quick detection systems are required: unlike the microplate ELISA methods, the measurements do not involve many operational steps. Biosensors based on surface plasmon resonance (SPR) have also been reported, although they have not often been used to analyze PAHs in real environmental samples. Figure 8.3 illustrates both the SPR principle and the schematic representations of a typical sensogram obtained with this technique. Gobi et al.[60] used this methodology to analyze benzo(a)pyrene in buffer with promising results (LOD = 0.1 μg L^{-1}). Liu et al.[61] report the use of an immunosensor based on piezoelectric signal transduction for the analysis of benzo(a)pyrene, pyrene, and naphthalene compounds; when measuring buffered samples, this device can achieve an LOD in the nM range.

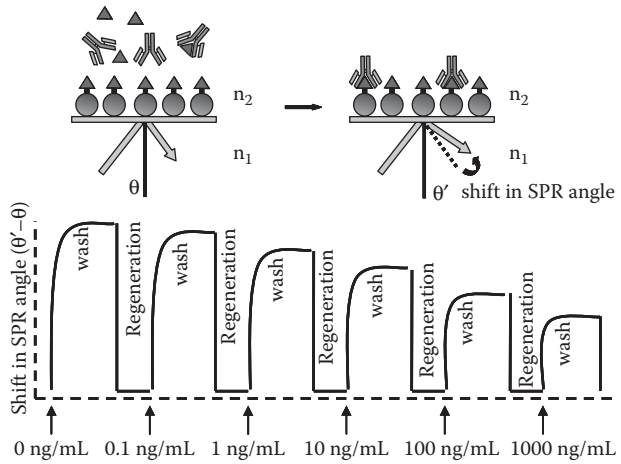

FIGURE 8.3 Sensogram generated by SPR principle for the constant concentration of antigen and antibody, and the varying concentration of analyte. Surface plasmons are excited by the light energy at a critical angle (θ) causing an oscillation and the generation of an evanescent wave. Under this condition a decrease in the reflected light intensity is observed. The angle θ depends on the dielectric medium close to the metal surface and is therefore strongly affected by molecules directly adsorbed on the metal surface. This principle allows the direct detection of the interaction between the analyte and the antibody.

As an alternative, immunoaffinity procedures have been developed to selectively extract PAHs from environmental samples. Thomas and Li[62] demonstrated the greater efficiency of immunoaffinity methods in comparison with conventional extraction procedures. Bouzige et al.[48] prepared an immunosorbent for use in an on-line analytical procedure, followed by liquid chromatography coupled to fluorescence detection, to monitor surface water samples. The sensitivity of the fluorescence detection in combination with the selectivity of the immunoextraction (IS-HPLC-UV) enabled PAH compounds to be detected at levels between 2 and 10 ng L^{-1}.

8.2.2 Surfactants

Surfactants are very common water pollutants, mainly because of their extensive use in detergent formulations that are directly discharged into the environment via wastewater.[63] They are usually organic compounds described as being amphiphilic, that is, containing both hydrophobic and hydrophilic groups. The most commonly accepted and scientifically sound classification of a surfactant is based on the charge present in the hydrophilic portion of the molecule after its dissociation in aqueous solution. Anionic surfactants are based mainly on sulfate, sulfonate, or carboxylate compounds: examples include linear alkylbenzene sulfonates (LAS), fatty alcohol sulfates (FAS), alkyl sulfonates (AS), alkyl ether sulfates (AES), and sodium dodecyl sulfate (SDS).[64] Commercial LAS, which represent more than 40% of all surfactants, consist of a mixture of at least 20 compounds, including isomers.[65] Residues of LAS, as well as their metabolites, the sulfophenyl carboxylates (SPCs), have been found in marine sediments[66] and in surface waters from the low µg L^{-1} up to the 500 µg L^{-1} range.[67–69] On the one hand, the toxicity of these compounds is low, but on the other, they may assist the permeation of other pollutants into aquatic biota.[70] Nonionic surfactants, such as alkylphenol ethoxylates (APEs) and alkyl ethoxylates (AEs), do not ionize in aqueous solution; they are extensively used in detergent formulations or as stabilizers in plastics. The main components of APEs are isomers of nonylphenol ethoxylate (NPE) and to a lesser extent, compounds related to octylphenol ethoxylate (OPE). Alkylphenols (APs), APE metabolites, and their halogenated derivatives are much more persistent in the aquatic environment and thus give reason for concern. Nonylphenol (NP) and octylphenol (OP) are listed as surface water priority substances; they are endocrine disruptors with powerful estrogenic effects.[71,72] Accordingly, the OSPAR commission (from the Oslo and Paris conventions),[73] whose brief it is to protect the marine environment of the North-East Atlantic, decided to phase out the use of APEs by the year 2000.[74] Nevertheless, monitoring of APEs will still be necessary in the coming years. Finally, the class of cationic surfactants includes nitrogen compounds such as fatty amine and quaternary ammonium salts (QAC), in which the hydrophobic groups are attached to positively charged nitrogen. These surfactants, which also have a biocide effect, are in general more expensive than the anionic ones and therefore less often used.[64] Their poor solubility and tendency to adsorb to solids or to form complexes with anionic substances reduce the risk to the aquatic environment. The application of immunoassays to the analysis of surfactants started in 1982 with the determination of Triton X, one of the best known APEs, with ELISA achieving detection limits in the µg L^{-1} range.[75] Since then, numerous immunochemical techniques have been developed, mainly to detect anionic and nonionic surfactants in the environment (Table 8.3).

With regard to anionic surfactants, Ramón-Azcón et al.[76,77] reported an immunoassay for LAS determination in wastewater samples (LOD = 2 µg L^{-1}). The assay also identified, with a high level of cross-reactivity, the long SPC chain that is formed after LAS degradation. Analyses can be performed directly in wastewater merely by diluting the samples 10-fold to eliminate matrix interferences. Similarly, Estevez and co-workers recently reported an immunoassay for short alkyl chain SPCs, which are the final degradation products of LAS.[78] Because these products are highly polar, it is quite difficult to analyze SPCs if an extraction/preconcentration step is necessary. In contrast, immunochemical methods allow the direct determination of these chemical markers in aqueous samples. Thus, Zhang et al.[79] developed a sequential injection analysis (SIA) combined with a

TABLE 8.3
Immunochemical Techniques Developed for the Detection of Surfactants

Target Analyte	Technique	Matrix	Sensitivity		Reference
			LOD	IC_{50}	
		Surfactants			
AEs	ELISA (tube assay)	Tap and river water	2 μg L^{-1}[a]	12 μg L^{-1}[a]	89
	ELISA (plate assay)	Tap and river water	20 μg L^{-1}	71 μg L^{-1}	89
APEs	ELISA	River water	16 μg L^{-1}[a]	79 μg L^{-1}[a]	63
	ELISA	Polluted and tap water	10 μg L^{-1}[a]	246 μg L^{-1}[a]	72
	Sequential injection CL assay	River water	10 μg L^{-1}	30 μg L^{-1}[a]	86
APs	ELISA	Surface river water	μg L^{-1} range		87
	SPR	Marine products, shellfish	10 μg kg^{-1}		90
LASs	ELISA	Wastewater	1.8 μg L^{-1}[a]	28,1 μg L^{-1}[a]	76
	IS fluorescence detection assay	Waste- and groundwater	7 μg L^{-1}		81
	FPIA	Waste- and groundwater	30 μg L^{-1}		394

[a] LODs and IC_{50} calculated in buffer solution.

chemiluminescence detector and neodymium magnet to perform magneto-immunoassay experiments for the analysis of LAS; they achieved an LOD of 25 μg L^{-1}. Moreover, Sáchez-Martínez et al.[80] developed a very sensitive fluorescence polarization immunoassay (FPIA) for analyzing LAS in wastewater and groundwater samples. Previously, the same authors had developed an immunoaffinity chromatography procedure followed by fluorescence detection to analyze LAS in tap water, groundwater, and wastewater samples.[81] Recoveries were between 86% and 111% with dodecylbenzenesulfonate (LDS) as analyte (LOD = 7 μg L^{-1}).

The immunochemical determination of APEs and AP has been undertaken by several research groups[63,72,82,83] (for further details, see the review by Estevez-Alberola and Marco[84]), although the detectability achieved is not good enough to analyze NP at the EQS value established for all surface waters (0.33 μg L^{-1}).[85] An LOD of 10 μg L^{-1} has recently been reported for a chemiluminescent immunoassay of APE.[86] Previously, a highly sensitive and reproducible ELISA method had been described for NP, but the LOD obtained (2.3 μg L^{-1}) was again still insufficient.[82] This is why it is often necessary to include a preconcentration step prior to immunochemical analysis. The precision and accuracy of most of these ELISAs have therefore been evaluated by measuring these surfactants in spiked river samples, following a solid-phase extraction (SPE) procedure; recoveries have been good: 85–118%.[63,87] The validation studies carried out by several groups have also shown good correlation with chromatographic methods,[72,76,88] although some samples analyzed were clearly overestimated. The accuracy of the APE immunoassay mentioned above[86] was also successfully evaluated by measuring spiked river samples. Usually, a wide cross-reactivity pattern is observed, in which APEs with a distinct number of ethoxylate units are detected in addition to AP and certain carboxylate metabolites. A highly selective ELISA for AEs has also been developed and evaluated by measuring different spiked water matrices, such as distilled, tap, and river water samples, with recoveries reported to be in the 75–134% range.[89] Furthermore, an SPR Biacore sensor was applied by Samsonova et al.[90] to detect NPs in different shellfish matrices like mussels, oysters, cockles, and scallops.

The detection limits obtained for all the aquatic biota samples were around 10 μg kg^{-1},[90] which is good enough considering the QS estimated for aquatic biota [10 mg NP kg^{-1} food (wet weight)].[91] Several amperometric biosensors have been described, but none which might be suitable for aquatic

environment monitoring. Rose et al.,[92] for example, analyzed APE and AP in buffer using a capillary immunoassay with subsequent amperometric detection, and Evtugyn et al.[93] developed another amperometric immunosensor for the analysis of NP with an LOD of 10 μg L^{-1}.

Several ELISA methods for determining cationic surfactants have been reported although we have not found any examples of their application to real environmental samples. The detectability obtainable by these methods is very good: an LOD of 0.04 μg L^{-1} has been reported for benzyldomethyldodecylammonium chloride (BDD12AC), a component of benzalkonium chloride (BAK).[94]

8.2.3 Organohalogenated Compounds

The toxicity, bioaccumulative potential, and ecological impact of organohalogenated substances such as polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs), polychlorinated dibenzo-*para*-dioxins (PCDDs), or polybrominated diphenylethers (PBDEs) have been extensively reviewed.[95–98] All are referred to as persistent organic pollutants (POPs), that is, chemical substances that remain in the environment, bioaccumulate through the food chain, and pose a risk to human health and the environment. The international community is calling for action to reduce and then eliminate the production or formation of these substances and to monitor their emission. In this case, the detectability obtainable by analytical methods should be very low, since the limits established for these residues are in the ng per liter range.

PCBs, which have been commonly used as lubricants, immersion oils, or fire retardants, are normally formed by the chlorination of biphenyl in the presence of an $FeCl_3$ catalyst. PCB production was banned in the 1970s because of the high toxicity of most PCB congeners and mixtures. The number and the location of chlorine atoms in a specific PCB congener determine its physicochemical properties, environmental pathways, and toxicity. Isomers with a higher chlorine content bind preferentially to organic matter present in the solid phase; consequently, they are not easily degraded and are also poorly leached from sediments by water.[99] On the other hand, some PCBs are more mobile, hence their tendency to volatilize, and therefore to circulate, in gaseous form, through different environmental compartments.[100] PCBs are classified as a probable human carcinogen by EPA, which has established a maximum contaminant level goal of zero and a maximum contaminant level and practical quantification limit of 0.5 μg L^{-1} in drinking water.[101] In the USA, regulatory limits for soil remediation vary according to state and site, but in general are 5 or 10 μg g^{-1} for industrial restricted access areas and 1 or 2 μg g^{-1} for residential access areas.[102] For this reason, EPA has established method 4020, a procedure for screening soils and nonaqueous waste liquids, to determine when total PCBs are present at concentrations above 5, 10, or 50 mg kg^{-1}.[103] Table 8.4 shows other results regarding the analysis of organohalogenated compounds in environmental matrices. A commercially available ELISA test (PCB RaPID Assay®) has also been used to analyze these compounds; an LOD of 0.6 μg g^{-1} was obtained in mussel tissues.[104] Lawruk et al. applied the same kit but with super paramagnetic particles as the solid support to analyze soil and water samples; they obtained detection limits of 0.2 μg L^{-1} and 500 μg kg^{-1}, respectively.[105] Another commercial ELISA kit (EnBioTec Laboratories) was evaluated for PCB 118 determination in retail fish samples (LOD = 0.05 μg L^{-1}).[106]

Now let us take a brief look at other approaches. Zhao et al.[107] developed an optical immunosensor consisting of a quartz crystal fiber coated with partially purified polyclonal antibodies to detect PCBs in soil and water samples. The optical signal was generated by the fluorescence produced when the 2,4,5-trichlorophenoxybutyrate fluorescein conjugate binds to the previously coated antibody.[107] Electrochemical immunosensing strategies using carbon-based screen-printed electrodes as transducers in a direct competitive immunoassay have also been applied in the analysis of PCBs in marine sediment extracts[108,109] (LOD = <1 μg L^{-1}). Pribyl et al.[110] developed a piezoelectric quartz crystal (PQC) immunosensor for the *in situ* determination of different PCB congeners in soil toluene extracts without any additional purification step. Also, a high-performance immunochromatographic (HPIAC) procedure has been successfully used as a cleanup method to isolate PCBs from water samples.[111]

TABLE 8.4
Immunochemical Techniques Developed for the Detection of Organohalogenated Compounds

Target Analyte	Technique	Matrix (Pretreatment)	Sensitivity: LOD	Sensitivity: IC_{50}	Reference
		Organohalogenated Compounds			
PCBs	PQC immunosensor	Soil samples		6 μg L^{-1}	110
118	ELISA (EnBioTec Labs)	Fish muscle tissue		0.05 μg L^{-1}[a]	106
Aroclor® 1248	Immunomagnetic	Marine sediment extracts	24 μg L^{-1}	0.4 μg L^{-1}	108,109
Aroclor® 1242	amperometric		8 μg L^{-1}	0.5 μg L^{-1}	
Aroclor® 1016	immunosensor		94 μg L^{-1}	0.8 μg L^{-1}	
Aroclor® 1254	ELISA[a] (PCB Assay®)	Mussel tissue		600 μg kg^{-1}	104
	MAG particle IA	Water Soil		0.2 μg L^{-1}	105
	(PCB RaPID Assay® kit)			500 μg L^{-1}	
Aroclor® 1242	Fiber optic immunosensor	Soil, river, and bay water		10 μg L^{-1}	107
PCDDs (TMDD)	ELISA	Soil	0.123 μg kg^{-1}	0.028 μg kg^{-1}	121

[a] Commercial kit.

Other immunochemical techniques, not applied to environmental samples as yet, could be interesting for the future analysis of these residues.[112,113]

Immunochemical methods have also been reported for PCDDs and PCDFs. These substances are unintentionally formed during combustion processes and in the synthesis of chlorine gas and other chemicals used in the bleaching procedures of the pulp or paper industry.[114,115] PCDDs have 75 positional congeners with different grades of toxicity, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) being the most toxic. The extremely low water solubility of these compounds,[116] which is approximately 1000-fold lower than that of PCBs and PAHs, has significant downstream effects on the development of immunoassays because they are applied in aqueous media. EPA has established an official immunoassay method (4025) based on a commercially available ELISA kit that uses polyclonal antibodies for the analysis of these compounds in soil samples at 0.5 μg kg^{-1} levels.[117] Harrison and Carlson developed both a tube test and a microplate assay using one of Stanker's monoclonal antibodies[118,119] and obtained detection limits of 167 and 50 pg L^{-1} for TCDDs in soil samples.[120] The use of accelerated solvent extraction (ASE) followed by ELISA for the rapid screening of dioxin-contaminated soils has been reported recently.[121] An immunoaffinity chromatography method for the purification of polychlorinated dibenzo-*p*-dioxins and furans from biological samples was explored with the aim of simplifying the cleanup procedure and thereby reducing the time and cost of analysis.[122] A study of the effect of organic solvents on the development of an ELISA has also been reported; an IC_{50} of 0.24 μg L^{-1} for TCDD was obtained.[123] Additionally, a piezoelectric immunosensor system was developed for the rapid detection of PCDDs primarily in buffer. In this case, the antibodies deposited in the quartz crystal resonator were able to quantitatively detect concentrations between 0.01 and 1.30 μg L^{-1}.[124]

Finally, PBDEs—mainly three commercial mixtures known as Penta-BDE, Octa-BDE, and Deca-BDE—are still widely used as flame retardants in products such as polymers, resins, electronic devices, building materials, textiles, and the polyurethane foam padding used in furniture and carpets. The intensive production and use of these compounds has made them ubiquitous in the environment and in biota.[125,126] EPA is working with industry, governments, and environmental and public health groups to research and better understand the potential health risks posed by these substances.[127] The European Commission is also aware of these risks to the environment and public health and has established EQS in the low ppt level. Thus, for Penta-BDE, the annual average (AA) EQS is 0.0005 μg L^{-1} for inland surface waters and 0.0002 μg L^{-1} for other surface waters. There

have been a few attempts to develop immunochemical methods for polybrominated flame retardant compounds. An ELISA kit for the analysis of PBDE is commercially available from Abraxis LLC. Based on the use of magnetic particles, this kit is addressed to the 47th and 99th congeners that compose the Penta-BDE formulation; LOD for the 47th congener is <25 ng L^{-1}. Shelver et al.[128] have reported an ELISA with an IC_{50} value of 28 μg L^{-1} for BDE-47 in buffer.

8.2.4 Heavy Metals and Metalloids

Heavy metals are also considered dangerous and persistent environmental contaminants. At least 20 are known to be toxic in some way and fully half of these, including As, Cd, Cr, Cu, Pb, Hg, Ni, Ag, Se, or Zn, are released into the environment in sufficient quantities to constitute a risk to human health. Metals bind easily to soils or sediments, and in this form they are relatively nontoxic; but changes in the weather or medium pH in combination with other environmental factors can mobilize them, thereby increasing their availability and effective toxicity. For this reason, sites contaminated with heavy metals must be monitored regularly. By way of example, mercury exceeds the 1 mg kg^{-1} action level, established by the U.S. Food and Drug Administration (FDA), in many marine and freshwater fish samples. For mercuric chloride used in medicine, the minimum risk level for Hg exposure is 0.007 mg kg^{-1} per day.[129] EPA has therefore certified an immunoassay (Method 4500) that provides a screening procedure for the determination of mercury in soils at concentrations up to 0.5 mg kg^{-1}. On the other hand, the Lead and Copper Rule (LCR), introduced by the EPA in 1991, established an action level of 0.015 mg L^{-1} for lead and 1.3 mg L^{-1} for copper based on the 90 percentile level of tap water samples.[130] Another method (4510), again proposed by EPA, also determines lead in water and soil by means of an immunoassay.[131] Nowadays, many of the heavy metals mentioned above are also regulated by the European Union; in water for human consumption, the permitted levels of chromium and mercury are 50 and 1 μg L^{-1}, respectively.[132] Moreover, cadmium and its compounds are among the 33 priority substances with an EQS of 0.08 μg L^{-1} in water listed in the proposal for a Directive [COM(2006)398 final] presented by the European Commission.

Basically, there are two ways of producing antibodies against heavy metals. Firstly, when the immunogen consists of a heavy metal bound to a chelator like EDTA, the antibodies raised do not recognize the metal itself but identify the entire structure.[133–135] On the other hand, if the antibodies are produced directly against the heavy metal attached to a suitable immunogen,[136,137] the free metal can be recognized instead of the cage-like chelate structure. Wylie et al.[137] developed highly specific antibodies for mercury using a glutathione complex, which is the basis of the only commercially available metal ion immunoassay (BiMelyze® Mercury Immunoassay). Alternatively, Lerner et al.[138] reported the isolation of recombinant antibody fragments that preferentially recognize certain metals complexed to iminodiacetic acid. Some examples of immunochemical techniques applied to detect heavy metals in environmental samples are given in Table 8.5. ELISAs for detecting cadmium(II),

TABLE 8.5
Immunochemical Techniques Developed for the Detection of Heavy Metals

Target Analyte	Technique	Matrix	Sensitivity		Reference
			LOD	IC_{50}	
		Heavy Metals			
Cd(II)	ELISA	Environmnetal water samples	7 μg L^{-1}	—	134
Hg(II)	ELISA	Water (EPA samples)	0.5 μg L^{-1}	—	137
	ELISA[a] (BiMelyze®)	Scallop tissue extract	100 μg kg^{-1}	—	395
Pb(II)	FPIA	Soil	20 ng kg^{-1}	—	140

[a] Commercial kit.

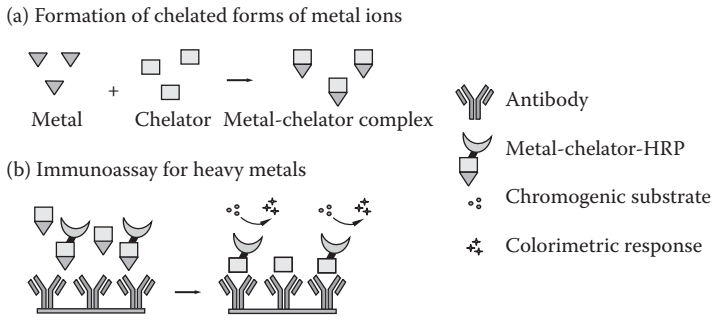

FIGURE 8.4 Scheme of the ELISA format most commonly used for the analysis of heavy metals, where antibodies recognize chelated forms of metal ions.

nickel(II), lead(II), and mercury(II) in water samples have also been reported.[134,139] The most common ELISA format used for the analysis of these compounds can be seen in Figure 8.4.

Similarly, an FPIA used polyclonal antibodies raised against the lead(II)-EDTA chelate to detect the metal in soils, solid waste leachates, airborne dust, and drinking water samples.[140] Moreover, the Kin ExA™ 3000 automated immunoassay instrument was adapted to analyze Cd(II), Co(II), Pb(II), and U(VI) metals in groundwater samples.[141] On the other hand, monoclonal antibodies, raised against the Cd-EDTA complex, have been used to develop an immunochromatography (IC) procedure for the quick testing of Cd in food (LOD = 0.3 $\mu g\ kg^{-1}$).[135] The development and validation of a one-step immunoassay for the determination of Cd(II) in human serum with an LOD of 0.24 $\mu g\ L^{-1}$ has also been described,[133] as has the optimization and validation of an immunoassay that measures soluble indium at 0.005 $\mu g\ L^{-1}$ in buffer.[142] An alternative way of detecting the presence of heavy metals is to use molecular biomarkers such as the diagnostic and prognostic tools used in marine pollution monitoring. Metallothioneins (MTs) are synthesized by toxic metals such as Cd, Hg, and Cu by chelation through cysteine residues and act as biomarkers of metal exposure in both vertebrates and invertebrates. These biomarkers are used with a range of molecular approaches to evaluate the exposure of various sentinel marine organisms, for example, mussels, clams, oysters, snails, and fishes, to metal contaminants.

The demonstration that MTs from a wide variety of fish species are recognized by an antiserum raised against one piscine MT has enabled the development of immunotechniques based on ELISA[143] and radioimmunoassay (RIA) procedures[144] for the quantification of these compounds. A competitive solid-phase assay based on dissociation-enhanced lanthanide fluoroimmuno-detection (DELFIA) of anti-MT monoclonal antibody bound to a solid phase has been reported.[145] An electrochemical determination of MTs by square wave cathodic stripping voltammetry has also been developed and optimized.[146]

8.2.5 Other Industrial Pollutants: Bisphenol A

Among the emerging pollutants of industrial origin, Bisphenol A [2,2 bis(4-hydroxydiphenyl)propane] (BPA) has special relevance since it was one of the first chemicals discovered to mimic estrogens as endocrine disrupters.[147] This compound was first reported by Dianin et al.[148] in 1891. BPA is produced in large quantities worldwide, mainly for the preparation of polycarbonates, epoxy resins, and unsaturated polyester-styrene resins.[149] The final products are used in many ways, such as coatings on cans, powder paints, additives in thermal paper, in dental composite fillings, and even as antioxidants in plasticizers or polymerization inhibitors in PVC. To a minor extent, BPA is also used as precursor for flame retardants such as tetrabromobisphenol A or tetrabromobisphenol-S-bis(2,3-dibromopropyl) ether.[150] This substance can enter the environment via the effluent from the factories

TABLE 8.6
Immunochemical Techniques Developed for the Detection of Bisphenol A

			Sensitivity		
Target Analyte	**Technique**	**Matrix**	**LOD (μg L^{-1})**	**IC_{50} (μg L^{-1})**	**Reference**
Bisphenol A	TIRF immunosensor	Water	0.005[a]	—	163
	SPR immunosensor	MilliQ water	0.014	0.86	162
		Groundwater	0.168	5.12	
		River water	0.292	8.38	

[a] LODs and IC_{50} calculated in buffer solution.

producing it because it is not completely removed during wastewater treatment.[151,152] Several studies have demonstrated that BPA released to ground or surface water may be strongly adsorbed to soil or sediments.[153–155] Several ELISA applications have been developed to determine BPA in environmental and industrial waste samples:[56,157] Zhao et al.[158] obtained an LOD of 0.1 μg L^{-1} in real water samples. Recently, immunosensors have appeared on the market to complement conventional immunoassays for the analysis of this compound (Table 8.6). Optical SPR immunosensors have been reported to analyze BPA in buffer,[159–161] while a fully automated sensor called River ANAlyzer (RIANA), based on a combination of fluorescent labels and the evanescent wave principle, achieved an LOD of 14 ng L^{-1} in natural water samples.[162,163] Moreover, an impedimetric immunosensor, based on label-free direct detection of BPA with a quartz crystal microbalance, has been reported to obtain a detection limit of *ca*. 0.3 μg L^{-1} in human serum.[164]

Park et al. demonstrated the effectiveness of piezoelectric immunosensors as a valuable alternative screening method for BAP environmental monitoring, achieving an LOD of 0.1 μg L^{-1}, although so far, only in buffer.[165] Further immunoaffinity chromatographic methods have been developed with the aim of improving the analytical procedure of BPA in biological fluid samples[166] and in canned food.[167] The same approach could be used to shorten cleanup steps, as well as the cost and time of analysis of other environmental samples.

8.3 IMMUNOCHEMICAL METHODS FOR PESTICIDES

Pesticides are chemical substances used for preventing or limiting the damage caused by pests. Thus, unlike other groups of chemicals, pesticides are intentionally released into the environment. Moreover, there is a high risk of these chemicals turning up in the food chain: foodstuffs may become contaminated during agricultural production, processing, packaging, and storage. Owing to the sheer volume of their usage, coupled with their universal distribution, environmental persistence, and toxicological properties, pesticides are considered a major public health hazard. Agricultural pesticides may lead to contamination of surface and groundwaters by drift, runoff, drainage, and leaching.[168] Surface water contamination may have ecotoxicological effects on aquatic flora and fauna as well as on human health.[169,170] In the aquatic ecosystem, there is a continuous interchange of these compounds between the land, sediment, sediment–water interface, interstitial waters, aquatic organisms, and air–water interface. The distribution of pesticides between water and biotic materials can affect their dynamics in the ecosystem. Thus, their mobility, possible transformation, and biomagnification constitute a real threat to human health, wildlife, and the entire environment. This situation is reflected by the number of analyses on the influence of pesticides on particular aquatic ecosystems,[171–179] and also by the presence of these biocides on most governmental priority lists of compounds that should be monitored. Intensive research has been carried out during the last 20 years aiming to develop analytical immunochemical technologies with improved

capabilities regarding detectability and sample-throughput capabilities for pesticide analysis in environmental matrices.[41,180–185] In the following, we will take a brief look at some of the most important of these methodologies and their application to the aquatic environment; the reader will find more detailed information in recent reviews.[25,41,43]

8.3.1 Insecticides

Used in agriculture to combat insect pests since the 1940s, insecticides include several chemical families that constitute a serious environmental risk. Thus, organochlorine (OC) or organophosphorus (OP) insecticides, the first generation of pesticides, are known to be highly persistent in the environment, unlike the pyrethroids that break down quickly in direct sunlight, usually just a few days after application. Nowadays, the use of OC has been banned in most developing countries because of concerns about their environmental impact and human health effects; nevertheless, their residues are still present in many environmental and biological compartments. The most important immunochemical techniques developed to analyze these compounds are listed in Table 8.7.

In a recent study, we reported on how the general population is still exposed to these substances, as evidenced by the excretion of chlorophenols and bromophenols in urine.[186] The study used antibodies developed for trichlorophenol,[187–189] an insecticide used as a wood and textile preservative, to extract these analytes from urine and to analyze them by combining immunosorbent cartridges[190] and an ELISA on a 96 setup, in such a way that 96 samples could be immunoextracted and analyzed in parallel. The results obtained from the immunochemical analyses were validated by gas chromatography-mass spectrometry (GC-MS), showing excellent correlation.[186] One of the best known OC pesticides is dichloro-diphenyl-trichloroethane (DDT), and many research groups have attempted to develop antibodies to detect this compound.[191,192] Beasley et al.,[193] in 1998, were the first to apply a DDT ELISA to environmental samples (LOD = 0.3 $\mu g\ L^{-1}$). Later, Amitarani et al.[194] reported on another ELISA where DDT was detected in river water samples at levels close to 1 $\mu g\ L^{-1}$. An FPIA for the detection of DDT and its isomers in drinking water was developed by Eremin et al.,[195] who achieved LODs of 12 $\mu g\ L^{-1}$ and 30 $ng\ L^{-1}$, respectively. Several immunochemical analytical methods have also been developed for chlorinated cyclodienes (CCDs), such as endosulfan, heptachlor, chlordane aldrin, endrin, and dieldrin, since the pioneering work of Langone and Van Vunakis,[196] who designed a RIA for dieldrin and aldrin in 1975. Manclus et al.[197] produced monoclonal antibodies against endosulfan (α/β), which recognized almost all structurally related cyclodiene insecticides with good detectability. On the other hand, Stanker et al.[198] adapted a commercially available ELISA kit for the analysis of endosulfan in environmental water samples with very good results. Lee and Kennedy[199] produced polyclonal antibodies to develop an ELISA to detect endosulfan in runoff water and soil extracts with an LOD of 0.2 $\mu g\ L^{-1}$. A direct ELISA was also developed for screening aldrin, dieldrin, and endrin compounds in tap and Nile river water samples: LODs of 5 and 10 $\mu g\ L^{-1}$ were obtained for aldrin and dieldrin, respectively.[200] Finally, a fiber optic immunosensor was developed, which can detect most of the cyclodiene congeners at ppb levels in soil extracts and environmental water samples, using rabbit polyclonal antibodies raised against the chlorendic caproic acid hapten.[201]

Several qualitative and quantitative immunochemical methods and their application to the analysis of environmental samples have been described for OP insecticides, a family that includes widely used pesticides such as azinphos-ethyl/methyl, dichlorvos, fenitrothion or fenthion, malathion, mevinphos, and parathion. Mercader and Montoya[202] produced monoclonal antibodies against azinphos-methyl and developed an ELISA that was used for the analysis of water samples from different sources, reaching detectability levels near 0.05 $\mu g\ L^{-1}$. Watanabe et al.[203] reported the production of polyclonal antibodies and ELISA procedures to analyze fenitrothion in river, tap, and mineral water (LOD = 0.3 $\mu g\ L^{-1}$). Banks et al.[204] produced polyclonal antibodies against dichlorvos, an organophosphate insecticide used for stored grain, which also cross-reacts with fenitrothion. Nishi et al.[205] reported the first immunoassay for malathion. Residues of this insecticide have

TABLE 8.7
Immunochemical Techniques Developed for the Detection of Insecticides

Target Analyte	Technique	Matrix	Sensitivity		Reference
			LOD	IC_{50}	
		Insecticides			
Aldrin	ELISA	Tap and river water	5 μg L^{-1}		200
	Optical immunosensor	Soil and water samples		5 μg L^{-1}	201
Azinphos-ethyl/ methyl	ELISA	Well, tap, channel, cistern, and drinking water	0.05 μg L^{-1}	0.33 μg L^{-1}	202
Carbaryl	ELISA	Cucumber and strawberry		0.13 μg L^{-1}	396
	SPR	Ground, river, and tap water	0.86 μg L^{-1}	3.97 μg L^{-1}	397
Carbofuran	Magneto-ELISA	Water and soil	5 μg L^{-1}	0.056 μg L^{-1}	213
	Planar array evanescent immunosensor	Ground and river water	0.1 μg L^{-1}		222
	SPR immunosensor	Drinking water	0.03 μg L^{-1}	1.06 μg L^{-1}	223
Deltamethrin	ELISA	River sample	1.1 μg L^{-1}	17.5 μg L^{-1}	398
Endosulfan	ELISA[a]	Environmental water		2 μg $well^{-1}$	198
	ELISA	Soil and runoff water	0.2 μg L^{-1}		399
Esfenvalerate	ELISA	Tap and river water		30 μg L^{-1}	400
	Fluorescence immunoassay	River water	0.04 μg L^{-1}	0.8 μg L^{-1}	233
Fenitrothion	ELISA	Fruit extracts	40 ng $well^{-1}$	297 ng $well^{-1}$	401
	ELISA	River and tap water	0.3 μg L^{-1}	6 μg L^{-1}	402
	ELISA	Rice extracts	3 μg L^{-1}	14 μg L^{-1}	403
Fenthion	Dipstick immunoassay	Food samples	0.5 μg L^{-1}	15 μg L^{-1}	404
Flucythrinate	ELISA	River water and soil	10, 0.2 mg L^{-1}		405
Malathion	Sol-gel immunosorbent	Surface water	0.50 μg L^{-1}	0.10 μg L^{-1}	406
Mevinphos	ELISA	Buffer	52 ng $well^{-1}$	3700 μg L^{-1}	407
Parathion	ELISA	Buffer		600 μg L^{-1}	407
Parathion-methyl	Commercial kit	Water		0.3 μg L^{-1}	408
Triazophos	ELISA	Buffer	0.11 μg L^{-1}	5.51 μg L^{-1}	409
	ELISA	Buffer	0.10 μg L^{-1}	0.65 μg L^{-1}	410

[a] Commercial kit.

been detected in ground and surface water at levels up to 6.1 μg L^{-1}.[206,207] Brun's group[208] recently developed an assay with an LOD of *ca*. 0.1 μg L^{-1} that was successfully applied to the analysis of river and groundwater samples. In contrast, monoclonal antibodies have been produced to develop an ELISA for the analysis of parathion and parathion-methyl compounds in water and milk samples at levels around 1 μg L^{-1}.[209] Similarly, a commercially available ELISA kit (EnviroGard Parathion Plate Kit) has been validated for application in water samples: LODs of 0.03 and 0.05 μg L^{-1} were obtained for parathion and parathion-methyl, respectively.

Since their commercial introduction in the early 1960s, *N*-methylcarbamate pesticides (carbaryl, carbofuran (CF), methiocarb, etc.) have been used worldwide as substitutes for OCs because of their excellent efficiency as insecticides and nematicides, their relatively low mammalian toxicities in

many cases, and their low bioaccumulation potentials. In recent years, several ELISAs have become commercially available or have been developed to determine these pesticides in water samples,[210–215] as well as in fruits and vegetables.[214,216–219] For example, the performance of two ELISA formats (microtiter plates and magnetic particles) were compared with the EPA method 531.1 (liquid chromatography-postcolumn derivatization-fluorescence detection, LC-PCR-FD) for the determination of carbaryl in groundwater samples of the Campo de Nijar aquifer (Almeria, Spain).[212] A close correspondence was found for the results obtained when spiked and well water samples were split for analysis by ELISA and by LC-PCR-FD, but the absence of a matrix effect and the high throughput capability of the ELISA formats pointed to the superiority of these immunochemical methods for screening purposes. The presence of CF during a year in lake, well, and irrigation ditch water in an agricultural area south of Milan has been evaluated using a fluorescent immunoassay with a time-resolved revelation system. Results show that CF peaked at around 87 ng mL^{-1} in September and October.[220] Another interesting approach is the homogenous immunoassay developed for CF by the same group. In this case, the determination used liposomes and a mastoparan (Mast)-hapten conjugate as cytolitic agent. Dipicolinic acid (DPA) was used as fluorescent chelating agent. Liposome lysis was proportional to the standard concentrations in a dynamic range between 10 pg and 10 ng. The assay was applied to the analysis of tap water and environmental water samples taken from the same agricultural area, with recoveries of between 90% and 105%.[221] Automated methods and immunosensors have also been reported.[215,220,222–227] Mauriz et al.[226] described the application of a commercial optical sensor system based on SPR detection to the direct analysis of carbaryl in different environmental water samples without any sample pretreatment. Detection limits obtained for ground, river, and tap water were 1.3, 1.2, and 0.9 μg L^{-1}, respectively, whereas the IC_{50} values obtained were in the 4.0–4.6 μg L^{-1} range.

Immunochemical analytical methods have also been developed for pyrethroid insecticides. Lee et al.[228,229] developed an immunoassay for analyzing pyrethroids of the second group that was applied to detect deltamethrin and bifenthrin in water and soil samples as well as deltamethrin in wheat grain. Watanabe et al.[230] and Mak's group[231] developed a class-specific immunoassay for the first and second group of pyrethroids respectively, obtaining very good detection limits. A competitive ELISA has recently been developed for the detection of the pyrethroid insecticide cyhalothrin, giving an LOD of 4.7 μg L^{-1}; it was evaluated using fortified tap water, well water, and wastewater samples with recoveries between 80% and 114%.[232] Another interesting example is the fluorescence-quenching competitive immunoassay in microdroplets reported for the sensitive detection of the pyrethroid insecticide esfenvalerate using laser-induced fluorescence from a rhodamine hapten conjugate. The competitive immunoreaction was performed in microdroplets generated by a vibrating orifice aerosol generator system with a 10 μm diameter orifice. The fluorescence emitted from the droplets was detected by a 1/8 inch imaging spectrograph with a 512 × 512 thermoelectrically cooled, charged-coupled device. A very small mass of analyte could be detected with this method; thus, monitoring of a picoliter droplet sample enabled detection down to ~0.1 nM. Matrix interferences were negligible when this technique was applied to the analysis of river water samples.[233] Sasaki et al.[234] developed a novel SPR biosensor chip by using a plasma-polymerized ethylene diamine film over a gold layer sputtered onto glass. Antietofenprox antibody was immobilized on the glass surface using glutaraldehyde, and the response of the SPR biosensor was compared to that of a commercial chip. The result was not so different from that obtained with the commercial chip, but the fact that the plasma polymerized membrane is optically homogeneous might have helped to produce a higher response.

8.3.2 Herbicides and Plant Growth Regulators

Herbicides are used to get rid of unwanted plants like weeds, brush, unproductive trees, and other vegetation that may deprive crops and other "useful" plants of nutrients. Numerous immunological techniques for the analysis of triazines, such as atrazine, propazine, simazine, ametryn, and cyanazine, have been developed recently (Table 8.8).[185,235–237] Owing to their environmental persistence and their

TABLE 8.8
Immunochemical Techniques Developed for the Detection of Herbicides

Target Analyte	Technique	Matrix	Sensitivity		Reference
			LOD	IC_{50}	
		Herbicides			
2,4-D	Optical immunosensor	River and lake water	0.1 μg L^{-1}		248
	Dipstick immunoassay	Pond water	0.5 μg L^{-1}	6 μg L^{-1}	249
2,4,5-T	ELISA	Soil and water samples		11.6 μg L^{-1}	246
Acetochlor	Polarization fluoroimmunoassay	Drinking water	9 μg L^{-1}		271
Atrazine	ELISA	Soil, ground, and water	1 ng L^{-1}	20 ng L^{-1}	238
	ELISA	River, lake, and tap water		5 μg L^{-1}	411
	ELISA	Creek and drinking water	0.2 μg L^{-1}		412
	ELISA[a]	River, estuarine, and sea water	0.05 μg L^{-1}	0.3 μg L^{-1}	239
	FIIA	Estuarine and sea water	75 ng L^{-1}	470 ng L^{-1}	413
	ELISA		60 ng L^{-1}	9 ng L^{-1}	
	Amperometric immunosensor	Drinking water	6 ng L^{-1}	0.17 μg L^{-1}	240
	SPR immunosensor	Well, river, and tap water	26 ng L^{-1}	0.18 μg L^{-1}	243
Isoproturon	Optical immunosensor	River and estuarine water	0.14 μg L^{-1}	1.65 μg L^{-1}	255
Metsulfuron-methyl	ELISA	Drinking water	40 ng L^{-1}	1.4 μg L^{-1}	274
Propanil	RIANA	Drinking water	0.6 ng L^{-1}	52 μg L^{-1}	163
Simazine	ELISA	Ground and tap water	50 ng L^{-1}	0.1 μg L^{-1}	414
	ELISA	Lake, rain, and mineral water	0.01 μg L^{-1}	0.07 μg L^{-1}	415
	ELISA	Ground and well water		2.03 μg L^{-1}	416
	ELISA	Tap water		0.75 μg L^{-1}	417
		Putah creek water			
		Bay water			
	Magneto-ELISA	Distilled water	0.349 μg L^{-1}	1.76 μg L^{-1}	418
		Groundwater	0.402 μg L^{-1}	2.09 μg L^{-1}	
		Estuarine water	0.416 μg L^{-1}	2.10 μg L^{-1}	
	FIIA	Drinking water	0.02 μg L^{-1}		419
	Optical immunosensor	Drinking water	0.026 μg L^{-1}		420
	Sol-gel immunosorbent	Surface water	0.25 μg L^{-1}	0.05 μg L^{-1}	406
	m-ISLMA_1	Surface water	1×10^{-5} μg L^{-1}	0.25 μg L^{-1}	421
	m-ISLMA_2		2×10^{-2} μg L^{-1}	1×10^{-4} μg L^{-1}	
	m-ELISA		1×10^{-1} μg L^{-1}	13.8 μg L^{-1}	
	μ-IA	Mineral water	0.2 ng L^{-1}		244
	μ-ISLMA		0.1 ng L^{-1}		
Trifluralin	ELISA	Surface water	0.85 μg L^{-1}	5.78 μg L^{-1}	422
	ELISA (OWLS)	Surface water	0.8 μg L^{-1}	2.87 μg L^{-1}	278
Linuron	RIANA	River and MilliQ water	0.01 μg L^{-1}	1.03 μg L^{-1}	255

water solubility (33 mg L^{-1}), triazines are distributed mainly in groundwaters and surface waters. For this reason, a large number of reported immunoassays have focused on the analysis of natural water samples.

Wittmann and Hock[238] measured atrazine in drinking and groundwater samples, reaching detection limits close to 1 ng L^{-1} without using any preconcentration or cleanup step. The presence of atrazine has also been analyzed in estuarine and seawater samples using other immunological techniques like magneto ELISA and FIIA with very low detection limits—close to 50 ng L^{-1}.[239] Several electrochemical immunosensors have been described for atrazine detection in food and aquatic matrices, like the one presented by Zacco et al.,[240] who developed an amperometric immunosensor based on modified magnetic particles with antibodies that are captured by a graphite-epoxy magneto composite, also used as the transducer for the electrical immunosensing; the LOD for drinking water samples was 6 ng L^{-1}. Figure 8.5 shows a schematic representation of the biosensor.

Several authors[185,237,241] developed an impedimetric immunosensor based on interdigitated electrodes without the use of any label; this system achieved detection limits of 0.04 and 0.19 μg L^{-1} in buffer and wine samples, respectively. Following up the same idea, but exploring a conductometric transduction system, Valera et al.[242] produced an immunosensor using antibodies labeled with gold nanoparticles; the LOD in buffer was 0.1 μg L^{-1}. Both systems should be easily adaptable for the analysis of environmental samples. Recently, Farré et al.[243] developed another immunosensor based on the SPR principle to analyze atrazine in well, river, and drinking water; detection limits were *ca.* 26 ng L^{-1} in all cases. A micro-immune-supported liquid membrane assay (μ-ISLMA) based on chemiluminescent detection has been developed to detect simazine in a single miniaturized cartridge system.[244] This chapter also discusses the influence of using different SAMs and different kinds of antibodies (polyclonal, affinity purified polyclonal, and monoclonal) on extraction parameters and assay sensitivity. LODs obtained for mineral water samples were at the ng L^{-1} level. Tschmelak et al.[163,245] applied the RIANA biosensor to detect propanil, a selective postemergent herbicide, in water samples without any pretreatment (LOD = 0.6 ng L^{-1}).

Chlorophenoxy acid herbicides are also widely used to control broadleaf weeds and grass plants. Several immunoassays have been reported for 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T).[246,247] Several immunosensors have been described using a transducing principle similar to the RIANA system already described in this chapter. Thus, Meusel et al.[248] reported the use of monoclonal antibodies in a sensor chip to analyze river and lake water samples, obtaining detection limits of 0.1 μg L^{-1}. Moreover, monoclonal antibodies, produced by Cuong et al.,[249] were used in a dipstick immunoassay format to analyze pond water samples. When applied to the 2,4-D compound, this semiquantitative method yielded for an IC_{50} of 6 μg L^{-1} and an LOD of 0.5 μg L^{-1}.

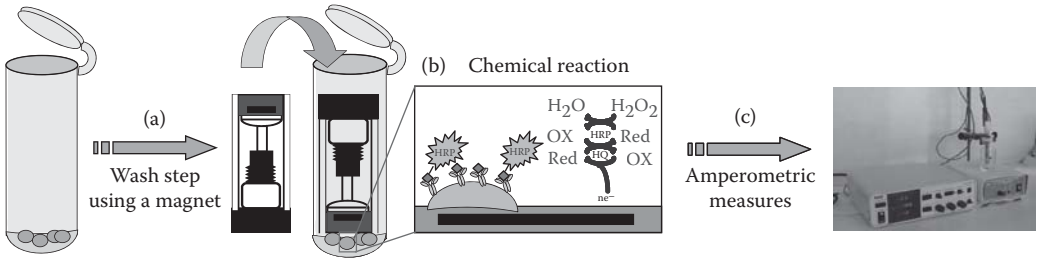

FIGURE 8.5 Schematic representation of an electrochemical magneto immunosensing strategy for the detection of low-molecular-weight compounds. After the immunoreaction, the antibody-modified magnetic beads are captured by the m-GEC electrode. Chemical reactions occurring at the m-GEC surface polarized at −0.150 V (versus Ag/AgCl) upon the addition of H_2O_2 in the presence of mediator (hydroquinone) are recorded. (From Zacco, E. et al. 2006. *Anal. Chem.* 78: 1780–1788.)

Immunochemical methods have also been reported for the analysis of phenylurea herbicides in different matrices, including food and environmental samples.[250–254] Thus, recombinant antibodies have been applied to the analysis of the phenylurea herbicide diuron with very good detectability (IC_{50} = 2 and 12 μg L^{-1} in the indirect and direct ELISA formats, respectively).[251] Similarly, isoproturon has been analyzed in soil extracts using an ELISA.[254] An ultrasensitive time-resolved fluorescence immunoassay (TR-FIA) for diuron in water samples has been recently reported. This assay was performed using the diuron-specific polyclonal antibody raised in sheep; rabbit antisheep IgG was used as fluorescent marker, conjugated with a chelating molecule complexed with Eu^{3+}. Even though the sensitivity of the lanthanide chelate was up to 10 times better than in other techniques, this level was 20 ng L^{-1} below the European Community limits. Water samples collected monthly from an agricultural area showed that peak diuron concentrations were 65 pg mL^{-1} in ditch water samples in June and 180 pg mL^{-1} in lake water samples in September.[220] On the subject of immunosensors, it is worth mentioning the work by Mallat et al.,[255] who again applied the RIANA system to monitor isoproturon, diuron, and linuron in Ebro delta waters (Tarragona, Spain) (LOD = 0.01 μg L^{-1}). A flow-through fluoroimmunosensor has also been developed for isoproturon in well water with a detectability in the μg L^{-1} range.[256] The use of antibodies in SPE methods against phenylurea herbicides has been investigated by immobilizing the antibodies on different solid supports[257–259] or encapsulating them in sol-gel matrices.[260] These immunosorbents have been applied as both a cleanup and a preconcentration step of these herbicides from ground,[252] drinking,[259] and surface[258,259] water samples, using on- and off-line procedures. Thus, with the immunosorbent conveniently packed in a C18 column coupled to a liquid chromatography (LC) system, about 10 phenylureas were monitored from the Seine River.[259] The class-selectivity profile demonstrated by these immunosorbents makes them useful for multiresidue analysis procedures of this particular family of herbicides.

Several immunoanalytical techniques have been developed for the analysis of chloroacetanilides, another important family of herbicides, such as alachlor,[261,262] metolachlor,[263–265] and their metabolites[266] in various matrices. Immunoassays for detecting butachlor and acetochlor have received less attention than the above-mentioned analogs, although some immunochemical developments have also been reported.[267–269] An electrochemical immunosensor[270] and a fluororimmunoassay[271] have also been described for acetochlor. Interesting are the interlaboratory collaborative field experiments[264] carried out to compare solid-phase extraction-gas chromatography (SPE-GC), solid-phase microextraction-gas chromatography (SPME-GC), and ELISA tests for the analysis of metolachlor. Runoff water samples were collected during the first rain event following herbicide application and analyzed using different methods. Larger metolachlor concentrations were found in surface runoff (1.4–54.9 μg L^{-1}) than in tile drainage (0.01–8.5 μg L^{-1}). The results demonstrated that although ELISA overestimated the concentration of this chloroacetanilide herbicide, correlation with the chromatographic methods was very good. An amperometric immunosensor for acetochlor detection[270] based on screen-printed electrodes has been reported, although the detectability achieved was not sufficient for the direct analysis of drinking water. The LOD described were around 25 and 60 μg L^{-1} for drinking and surface water, respectively.

Several ELISAs have been developed for the analysis of sulfonylurea herbicides like chlorsulfuron,[272] triasulfuron,[273] and metsulfuron methyl.[274] Schlaeppi et al.,[273] for example, developed an immunoassay using monoclonal antibodies for the analysis of fortified soil samples. The sensitivity of the assay, after an optimized extraction procedure, was 0.1 pg kg^{-1}. Eremin et al.[195] developed a FPIA for chlorsulfuron detection in MilliQ water samples; the LOD obtained in 50 μL of sample was 10 μg L^{-1}. Another example of a fluoroimmunoassay was the one developed by Wang et al.[275] consisting of a time-resolved fluoroimmunoassay (TR-FIA) method for bensulfuron-methyl based on fluorescence resonance energy transfer (FRET) from a Tb^{3+} fluorescent chelate to an organic dye, Cy3 or Cy3.5; this method achieved a detection limit of 2.1 μg L^{-1}. The same author[276] developed a new immunoassay method by using graphite furnace atomic absorption spectrometry with an EDTA-Cd^{2+} chelate as the label; bensulfuron-methyl was analyzed using this technique (LOD = 0.95 μg L^{-1}). Dzantiev et al.[277] developed an electrochemical immunosensor for analyzing chlorsulfuron herbicide

in just 15 min. The working range for the quantitative detection of chlorsulfuron was from 0.01 to 1 μg L^{-1}. Finally, Szekacs et al.[278] developed a highly sensitive immunosensor using optical waveguide lightmode spectroscopy (OWLS) to detect trifluralin, a selective pre-emergence herbicide, with an IC_{50} of 1.0 μg L^{-1}. The principle is based on the precise measurement of the resonance angle of polarized laser light, diffracted by grating and coupled onto a thin waveguide.

8.4 IMMUNOCHEMICAL DETERMINATIONS OF PHARMACEUTICAL AND PERSONAL CARE PRODUCTS

The term "pharmaceutical and personal care products (PPCPs)" refers to any product used for personal health or cosmetic reasons or used in agriculture to enhance the growth or health of livestock;[279] it comprises a diverse collection of thousands of chemical substances.[40] The overall pharmaceutical production in Europe, Japan, and the United States amounted to USD373 billion in 2005.[280] PPCPs have probably been present in the environment and water for as long as humans have been using them. While an important number of these substances enter the environment directly from industry, treated and untreated domestic sewage containing excreted PPCPs and their metabolites following human use is a major source of these compounds in the environment.[39] Nowadays, sewage systems and municipal WWTPs are still not equipped for the complete removal of PPCPs or other unregulated contaminants.[281] In addition to the framework provided by the Water Directive mentioned in the introduction, Directive 2001/82/EC regulates the requirements for the ecotoxicity testing of pharmaceuticals. With the advances in technologies that have improved the ability to detect, control, and quantify these chemicals, we can now begin to identify what effects, if any, these chemicals have on human and environmental health.

8.4.1 Antibiotics

Antibiotics are chemical substances extremely active at low doses that kill or slow the growth of bacteria. Since their discovery, antimicrobials have been an essential part of modern human and veterinary medicine as well as in aquaculture or even in plants for the treatment of infectious diseases produced by bacteria. In the last decade, the general misuse of antibiotics as growth promoters or for prophylactic purposes[282] has become a decisive factor favoring the increase of bacterial resistance. This risk situation may spread from animals to humans through the food chain[8] but may also have a crucial impact on the ecosystem itself by producing adverse effects in animals and plants. At present, WWTP effluents and confined animal feeding operations (AFOs) represent the prime sources of antibiotics entering the environment:[281,283] the greatest percentage of antibiotics are excreted after consumption, and thousands of tonnes of them reach the terrestrial and aquatic environment every year.[1] Governmental agencies have therefore set limitations on the levels of residues in accordance with available toxicological data by laying down specific regulations or proposals that are to complement the restrictions already in place with respect to animal foods destined for human consumption. Antibiotics are classified into several families, for example, penicillins, fluoroquinolones (FQs), sulfonamides (SAs), tetracyclines (TCs), macrolides, and chloramphenicols (CAPs). In general, all these compounds are quite resistant to biodegradation since they were designed to demonstrate a certain metabolic stability during their pharmacological action; they are likely to remain in the environment in unchanged form or as persistently active metabolites.[284] In this context, different techniques based on wholly divergent principles have been developed to deal with the problems relating to antibiotic residues.[286,287] Most of them, like growth inhibition tests, take advantage of their antibacterial activity. Others, such as chromatographic methods, are highly specific and sensitive but require extensive sample preparation, sophisticated and therefore expensive equipment, and skilled laboratory staff. On the other hand, immunochemical techniques can be excellent tools for assessing antibiotic contamination in different environmental matrices as a result of their excellent detectability, specificity, and throughput capacities.[44,287] Table 8.9 summarizes some of these techniques reported for the detection of several families of antibiotics.

TABLE 8.9
Immunochemical Techniques Developed for the Detection of Antibiotics

Target Analyte	Technique	Matrix	Sensitivity		Reference
			LOD (μg L^{-1})	IC_{50} (μg L^{-1})	
		Antibiotics			
Sulfonamides	ELISA	Fish muscle	<100[a]		303
	Charm II RIA test	Drinking water sources	0.05		310
	RIANA	Water samples	0.01	100	311
Sulfamethazine	RIA	Lagoon and river samples	5.00		309
	SPIE with MALDI-TOF MS	Drinking water	0.10		313
		Soil	1.00		
		Manure	1.00		
Sulfamethizole	AWACSS	River water samples	0.02		312
Fluoroquinolones	ELISA	Shrimp tissues	~4.00		323
	ELISA	Fish and shrimp sample	0.70[b]	<10[c]	322
Ciprofloxacin	ELISA	Milk, chicken, and pork		0.32[a]	324
Enrofloxacin	RIA	Lagoon and river samples	5.00		309
Tetracyclines	Charm II RIA test	Hog lagoon	1.00	1–20[d]	348
		Surface water			
		Groundwater			
	Charm II RIA test	Drinking water sources	0.05		310
Tetracycline	ELISA[e] (IDS Corporation)	Surface and groundwater	0.20		345
	ELISA[e] (Ridascreen)		0.10		
Tetracycline	ELISA[e] (R-Biopharm GmH)	Manure samples from hog lagoons and cattle feedlots	0.38[a]	1.02[a]	346
Anhydrotetracycline			0.25[a]	5.40[a]	
Chlortetracycline			0.01[a]	0.21[a]	
Anhydrochlor-TC			0.01[a]	6.92[a]	
Oxytetracycline			0.05[a]	0.97[a]	
Chloramphenicol	ELISA[e] (5091CAP1p, EDiagnostica)	Shrimp tissue	0.10	0.22[c]	334
			0.13[b]		
	SPR Biosensor (Biacore Q)	Prawn samples	1.00[a]	0.07[d]	336
			0.04[b]		
	Membrane-based CL sensor	Shrimp samples	3.23		339
Chloramphenicol	TR-FIA	Shrimp samples	0.05		423
CAP succinate			0.10		
Thiamphenicol	SPR Biosensor	Shrimp tissue	0.5[b]	0.13[a]	337
Florefenicol	(Biacore Q, sensor chip		0.2[b]	0.47[a]	
Florefenicol amine	CM5)		250[b]	887[a]	
Chloramphenicol			0.1[b]	1.26[a]	
Chloramphenicol	SPR Biosensor	Prawn	0.04[b]	0.07[c]	338
CAP glucuronide	(Biacore Q and Qflex® Kit)			76% CR	
Pencillin G	RIA	Lagoon and river samples	1.00		309
Pencillin G	Fluoro immunoassay	Wastewater	2.40	30.0	354
Amoxicillin		Sewage water	~5.00	58.0	
Tylosin	ELISA (IDS Corporation)	Surface and groundwater	0.20		345
	ELISA (Ridascreen)		0.10		
Erythromycin	RIA	Lagoon and river samples	10.0		309

[a] LODs and IC_{50} calculated in buffer solution.
[b] General decision limit.
[c] Detection capacity.
[d] Linear range.
[e] Commercial kit.

A significant number of immunochemical methods for antibiotic residue analysis with narrow or broad specificity, or even possessing multianalyte capabilities, have been described, and some of them are commercially available.[39,40,287,288] We recently reported a microplate-based ELISA method that can detect 25 antibiotics from the ß-lactam (BL), SA, and FQ families.[22] Moreover, the assay based on a dipstick platform is becoming the new simple, rapid and easy-to-use sensing device for on-site measurements. The biological recognition elements normally used in this user-friendly technology are the receptors;[289–292] applications using antibodies have appeared in recent years[254] but have so far been implemented only in the analysis of food samples, not yet in environmental samples.

8.4.1.1 Sulfonamides

SAs are an important group of broad-spectrum synthetic bacteriostatic antibiotics, whose chemical structure contains a 4-aminobenzensulfonamide functional group with different heterocycles attached to the N1-position of the SA bridge. This antibiotic family is widely used in animal husbandry in most European countries.[294] The pharmacokinetic profile of SAs ensures that they are quickly eliminated from the organism (40–90%), usually as the parent compound or as bioactive metabolites.[295] As with many other pharmaceuticals, SAs are fairly water-soluble, polar compounds that ionize depending on the pH of the matrix. In addition to hydrophobic partitioning, these compounds can absorb to soils via cation exchange, cation bridging, surface complexes, and hydrogen bonding.[296] Hence, SAs will persist in the environment and, because of their relatively high mobility, will enter groundwater and be transported to aquifers and surface waters;[284] relevant methodologies for monitoring environmental samples are therefore necessary. Besides the large number of chromatographic methods reported,[294,297–300] several immunochemical techniques have been developed for the analysis of water samples. Several ELISAs with broad[301–304] or narrower selectivity profiles[305–308] within the SA family have been applied to the analysis of these residues in various food matrices in compliance with legislation. Campagnolo et al.[309] studied the presence of different antimicrobials in wastewater samples from pig and poultry farms using a commercial radioimmunoassay (Charm II RIA). Prior to the analysis, samples were simply filtered through a 0.45 µm glass fiber filter; an LOD of 5 µg L^{-1} was obtained for sulfamethazine. In order to achieve lower detection limits, Yang and Carlson[310] coupled SPE cartridges to the same RIA test as a preconcentration technique. This method was optimized to detect SA and TC (see below) compounds in water samples from rivers and the influent/effluent of a WWTP. The detection limit for sulfamethazine was 0.05 µg L^{-1} using the SPE/RIA method; quantification of sulfamethoxazole, sulfadimethoxazone, and sulfathiazole was also possible. Initially developed for biochemical studies, RIA has the disadvantage of handling and producing radioactive residues, so their use should be avoided whenever possible. On the other hand, the already-mentioned RIANA immunosensor was used to detect SAs in drinking, ground, and surface water samples.[311] With this biosensor and a mixture of antibodies, it was possible to achieve detection limits <10 ng L^{-1}, limits of quantification (LOQ) <100 ng L^{-1}, and IC_{50} values between 0.5 and 5 µg L^{-1} for five SAs without sample pretreatment. The automated water analyser computer supported system (AWACSS) instrument represents a development of the RIANA sensor in that the multianalyte analysis capability has been expanded, theoretically permitting simultaneous measurements of up to 30 analytes from the groups of modern pesticides, endocrine disrupting compounds, and pharmaceuticals. With this system, Tschmelak et al.[184,312] achieved an LOD of <0.02 µg L^{-1} for sulfamethoxazole in river water samples. On the other hand, Grant et al.[313] described a method for detecting residues of sulfamethazine and its major metabolite N_4-acetylsulfamethazine in water, aqueous suspensions of soil, and composted manure samples, using solid-phase immunoextraction (SPIE) coupled with MALDI-TOF MS. The LODs for both compounds in all kinds of samples were <1 µg L^{-1}. No further immunochemical methods for the direct detection of SAs in environmental samples were found; nonetheless, the application to environmental water samples of those currently applied in complex biological matrices[305,314–316] is predicted to be straightforward. Several novel immunosensing strategies for detecting SAs have been developed by our group. Zacco et al.[317] immobilized class-specific anti-SA antibodies to magnetic

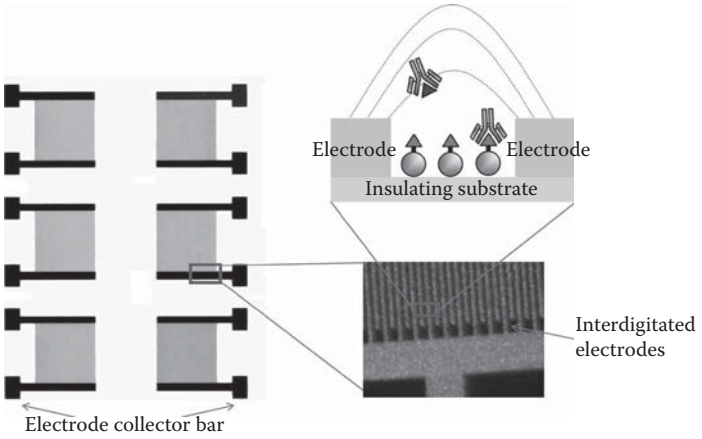

FIGURE 8.6 Schematic representation of a new transducer for biosensor application based on a three-dimensional IDEA. Binding of molecules to the chemically modified and biofunctionalized transducer surface induces important conductivity changes between the electrodes, which can be monitored. (From Ramón-Azcón, J. et al. 2008. *Biosens. Bioelectron.* 23: 1367–1373.)

particles to be captured, after the immunochemical reaction, by a magneto sensor made of graphite-epoxy composite (m-GEC) that is also used as the transducer for the electrochemical detection. The LOD obtained for sulfapyridine in milk was 1.4 μg L^{-1}. Another example, using the same immunoreagents, is presented by Ramón-Azcón et al.:[318] a new transducer for biosensor applications based on a three-dimensional interdigitated electrode array (IDEA) with electrode digits, separated by an insulating barrier. The binding of molecules to the chemically modified surface of the transducer induces important changes in conductivity between the electrodes; impedance measurements with this immunosensor detected sulfapyridine with an IC_{50} of 5.6 μg L^{-1} in buffer. As can be seen in Figure 8.6, using this strategy, it was possible to place the immunoreaction where most of the electric field is, instead of using just a small percentage.

Finally, class-selective immunoreagents for SA detection were implemented in a waveguide interrogated optical system (WIOS). The label-free sensor, developed by the Swiss Center for Electronics and Microtechnology (CSEM), is based on the evanescent wave principle, where changes in the refractive index of the modified chip surface are detected by scanning the resonance condition at which a light wave is coupled in the waveguide through a conveniently designed grating.[319] Monitoring of the resonance wavelength allows real-time monitoring of the binding of nonlabeled molecules to the waveguide grating surface, previously modified with the immunoreagents by means of a photopolymerizable dextran layer. The LOD obtained with this methodology for sulfapyridine in milk was 0.5 μg L^{-1}.

8.4.1.2 Fluoroquinolones

FQs are a synthetic class of antibiotics widely used for both prevention and treatment of various diseases in animal husbandry and aquaculture, as well as in humans. The environmental concern regarding FQs is evinced not only by their potential to promote antibiotic resistance, but also by their unfavorable ecotoxicity profile.[320] FQs are excreted as parent compounds, as conjugates, or as oxidation, hydroxylation, dealkylation, or decarboxylation products. FQs bind strongly to topsoils, thereby reducing the threat of surface water and groundwater contamination; this implies, however, that the terrestrial environment is a further relevant exposure pathway.[40] The strong binding of FQs to soils and sediments delays their biodegradation and explains their persistence in the environment. Wastewater treatment eliminates 79–87% of FQs before their arrival in rivers; adverse effects on the

aquatic habitats of surface waters are thus rather unlikely. On the other hand, these compounds are also susceptible to photodegradation in water: this involves the oxidation, dealkylation, and cleavage of the piperazine ring.[321] Fluoroquinolone residues in marine products are an important analytical target because they are regarded as good indicators of environmental quality. In this context, Huet et al.[322] reported the development of an ELISA for the detection of 15 fluoroquinolones in fish and shrimps, as well as in other samples (kidney, eggs, and muscle). The pretreatment required for the analysis of the marine products involved sample centrifugation and solvent extraction prior to 10-fold dilution. The assay was characterized in accordance with the recommendations of the European Commission (Commission Decision 2002/657/EC) by calculating the general decision limit (CCα) and detection capacity (CCß). CCα was calculated to be 0.70 μg L^{-1}, whereas CCß for most of these compounds was <10 μg L^{-1}, except in the case of sarafloxacin, oxolinic acid, flumequine, and cinoxacin, the detection capacities of which did not exceed 4, 25, 100, and 200 μg L^{-1}, respectively. An ELISA using monoclonal antibodies with a broad specificity for fluoroquinolone antibiotics (12 FQ congeners) was described by Wang et al.[323] for chicken, honey, egg, and shrimp samples: IC_{50} in buffer varied from 2.1 μg L^{-1} (norfloxacin) to 4.4 μg L^{-1} (lomefloxacin). Shrimp samples were fortified at different levels (50, 100, and 200 μg L^{-1}), separately with fluoroquinolones such as enrofloxacin, ciprofloxacin, norfloxacin, ofloxacin, flumequine, and danofloxacin; recoveries were between 63% and 90%. The last example found of an immunoassay applied to the analysis of fluoroquinolones in environmental samples is the already-mentioned immunosensor developed by Campagnolo et al.;[309] these authors achieved an LOD for enrofloxacin of 5.0 μg L^{-1}. Other immunoassays performed to detect FQs in complex biological samples[322,324–326] should a priori be easily adaptable for monitoring these substances in environmental water samples. The polyclonal antibodies developed and evaluated by Pinacho et al.[327] have been implemented in different immunochemical techniques to analyze a wide range of fluoroquinolone congeners. The same authors developed an ELISA capable of analyzing milk samples after a very simple dilution step, obtaining detection limits for most important fluoroquinolones of <0.4 μg L^{-1}.[328] Other uses of these immunoreagents have focused on electrochemical devices.[329,330] One example is an amperometric immunosensor that follows the same format as the one described in the sulfonamide section;[317] with this instrument LODs of 5.3 ng L^{-1} for ciprofloxacin in whole milk were obtained.[331] Impedance spectroscopy combined with immunosensor technology has been used to detect ciprofloxacin at 10 ng L^{-1} levels in buffer.[332] In this approach, the sensor electrode was based on the immobilization of the antibodies by chemical binding onto a poly(pyrrole-NHS) film electrogenerated on a solid gold substrate. The final immunoreaction triggers a signal via impedance spectroscopy measurements. Again, the application of these new analytical approaches to environmental samples should be straightforward.

8.4.1.3 Amphenicols

CAP, a bacteriostatic antimicrobial originally derived from the bacterium *Streptomyces Venezuelae*, was the first antibiotic to be manufactured synthetically on a large scale. Although CAP is effective against a wide variety of microorganisms, its use has been banned in the EU since 1994 because of certain toxicological side effect problems such as aplastic anemia, brown marrow suppression, or the so-called gray baby syndrome.[39] For this reason, a zero tolerance was established for the presence of these residues in any kind of animal products. On the other hand, this antibiotic has been widely used in the last 10 years by many low-income Asian countries for aquaculture disease treatment, because of its exceedingly low price. Although the use of this antibiotic in animal production has recently been prohibited in these countries too, CAP residues have been detected in marine products intended for the EU market.[333] According to several European Commission Decisions (2001/699/EC, 2001/705/EC, 2002/249/EC, 2002/250/EC, and 2002/251/EC), certain fishery and aquaculture products imported for human consumption must be subjected to a test in order to ensure the absence of CAP residues.[334] Thus, the main efforts have focused on the study of CAP residues in marine food products to control the problems mentioned above. Impens et al.[334] described the use

of a commercial ELISA kit (5091CAP1p) to detect CAP in shrimp tissue after organic/aqueous extraction, obtaining an LOD of 0.1 μg L^{-1}. The method was revalidated according to Commission Decision 2002/657/EC,[335] which has been more commonly used for chromatographic techniques; CCα and CCß values of 0.13 and 0.22 μg L^{-1}, respectively, were obtained. On the other hand, a commercial SPR immunosensor (Biacore Q) has been used by several authors to detect CAP residues in different kinds of matrices.[336–338] For example, Ferguson et al.[338] reported the implementation of a commercial detection kit (Qflex) in the cited biosensor to accurately determine CAP residues in milk, poultry muscle, honey, and prawn. CCα and CCß values for prawn samples, after a tedious pretreatment, were 0.04 and 0.07 μg L^{-1}, respectively, while the glucuronide form of CAP cross-reacted 76% in this matrix. Using the same biosensor, Ashwin et al.[336] obtained similar parameters for CAP detection in prawn samples but with a simpler sample pretreatment procedure. Furthermore, the immunoreagents developed by Dumont et al.[337] were implemented in the same SPR sensor as described above for the simultaneous residue detection of several fenicol antibiotic congeners in shrimps from a single sample extract. The IC_{50} values obtained for thiamphenicol (TAP), florefenicol (FF), and CAP were 0.13, 0.47, and 1.26 μg L^{-1}, respectively. CCß values were also estimated for each compound in this study (TAP: 0.13, FF: 0.47, and CAP: 1.26 μg L^{-1}). In a different context, Park and Kim described the development of a membrane-based chemiluminescent immunosensor for the analysis of very low levels of CAP residues in different samples of animal food for human consumption, such as pork, beef, chicken, milk, and shrimps.[339] The shrimp samples were simply filtered through Whatman paper to avoid undesirable matrix effects (LOD = *ca.* 3 μg L^{-1}). Alternatively, a large number of immunoassay screening methods for CAP detection in foods (e.g., milk, eggs, and meat) and other related complex matrices have been reported in the literature.[339–341] Despite the use of immunochemical methods to analyze residues in marine biota, we have not found examples of their application to analyze CAP residues in environmental samples, although these methodologies should be readily adaptable to the analysis of these types of matrices.

8.4.1.4 Tetracyclines

TCs are an important group of broad-spectrum antibiotics used against *Gram*-negative and *Gram*-positive microorganisms in modern human and veterinary medicine practice for both prevention and treatment of diseases, as well as additives in animal foodstuffs to promote growth in concentrated animal feeding operations (CAFOs). As with most types of antibiotics, only small portions of the tetracyclines administered are actually metabolized or absorbed in the body, and most of the drug is eliminated in feces and urine in unchanged form.[342] Normally, tetracyclines are not found at high levels in the environment: because of their chelation properties, they readily precipitate in the presence of divalent cations (i.e., Ca^{2+}, Mg^{2+}, or Zn^{2+}) and are accumulated in sewage sludge or sediments.[343] On the other hand, tetracycline residues have also been detected in many surface water resources that receive discharges from municipal WWTPs and agricultural runoff.[2,344] Besides the demonstrated persistence of TCs in agricultural soils that have received manure containing antibiotics, the biodegradation of these compounds to even more toxic substances must activate new strategies to improve their control and the efficiency of their removal in wastewater plants. To this end, a commercially available ELISA kit (RIDASCREEN®), commonly used for detecting tetracycline residues in meat and milk samples, was easily adapted for the ultratrace analysis of surface and groundwaters.[345] The assay was found to be highly sensitive to tetracycline and chlortetracycline with detection limits of 0.1 μg L^{-1} in lake waters, runoff samples, and soil saturation extracts. Furthermore, Aga et al.[346] evaluated another commercial ELISA kit (R-Biopharm GmbH) for investigating the occurrence and fate of tetracyclines in the environment. In this case, the potential use of class generic antibodies led to the multiple recognition of several TCs such as tetracycline, chlortetracycline, and oxytetracycline, and also their epimers and corresponding dehydration by-products with IC_{50} values from 0.2 to 6.9 μg L^{-1}. Subsequently, the same immunochemical detection kit was evaluated by measuring the presence of tetracyclines in samples from different manured

soil surface layers (0–5 cm).[347] Only trace amounts (<1 μg L^{-1}) of oxytetracycline were recorded in these samples and none was detected in water samples from field lysimeters; tetracyclines thus have a low mobility in soil, as suggested before.[342] The Charm II RIA method was applied to environmental samples but with the focus on tetracycline detection.[348] This Charm II RIA, previously developed as a screening tool for detecting tetracycline residues in serum, urine, milk, and tissues, was adapted for the analysis of water samples by Meyer et al.[348] who achieved an LOD of 1 μg L^{-1} and a semiquantitative analytical range of 1–20 μg L^{-1}. In this study, liquid waste samples were obtained from several hog lagoons, and the surface and groundwater samples were from areas given over to intensive poultry production; the analytical results were well correlated with those acquired by means of liquid chromatography-mass spectrometry (LC-MS) techniques. The same RIA technique, again applied by Campagnolo et al.[309] to different aqueous environmental samples, was able to detect chlortetracycline at sensitivity levels of 1 μg L^{-1}. Yang and Carlson[310] used SPE as a preconcentration technique in conjunction with Charm II RIA to obtain lower detection limits for tetracycline measurements in water matrices. In this case, detection limits of 0.05 μg L^{-1} for tetracycline, oxytetracycline, and chlortetracycline were obtained in the analysis of different wastewater samples. Other immunochemical methods developed to analyze manifold tetracycline residues, mainly in honey, milk, and animal tissues intended for human consumption,[349] could be adapted to analyze water samples.

8.4.1.5 ß-Lactams

The BL group is one of the most important families of antibiotics used in veterinary medicine for the treatment of septicemia, urinary infections, and pulmonary infections. The presence of penicillin residues in food of animal origin, such as milk or meat, can have the same drawbacks as other antibiotics: unfavorable microbiological effects in the dairy industry, possible hypersensitivity reactions in consumers, and antibiotic resistance.[350] On the other hand, their persistence in environmental samples should be very low, mainly because of the chemically unstable BL ring, which is highly sensitive to pH, heat, and ß-lactamase enzymes.[351] Some authors therefore point out the absence of this kind of antibiotic residue in water samples, but aim to detect their degradation products in order to evaluate possible future environmental risks. Several immunochemical techniques, based on different detection principles, have thus been developed to detect BL compounds in food samples of animal origin.[309,352–354] Many of these technologies are applied to the analysis of milk samples, because this antibiotic family is the most frequently used for the treatment of mastitis in dairy cows. Gaudin et al.[352] applied the Biacore SPR sensor, described previously for chloramphenicol detection, to detect ampicillin in milk samples using commercial monoclonal antibodies. Samples were pretreated to facilitate the opening of the BL ring; final detection limits of 5.9 and 12.5 μg L^{-1} for ampicillin in buffer and in milk, respectively, were obtained. This immunoassay revealed high cross-reaction values for other BL antibiotics such as penicillin G and M. The same biosensor was also used by Gustavsson et al.[353] to assay the activity of a carboxypeptidase and antibodies against the enzymatic product generated in milk samples. Detection limits for penicillin G were *ca.* 1 μg L^{-1}, and seven BL compounds were detected below their MRLs. It can be assumed that, as in the case of the antibiotic analyses mentioned earlier, application of immunoassays originally developed for other biological samples to environmental water samples should produce even fewer matrix effects. Benito-Pena et al.[354] prepared polyclonal antibodies to develop an automated flow-through fluoroimmunosensor for the analysis of penicillin antibiotics in wastewater samples from influent and effluent sewage water; LOD and IC_{50} values for penicillin G and amoxicillin in buffer were 2.4, 5.0, and 30, 58 μg L^{-1}, respectively. This immunosensor was applied to the analysis of both compounds in wastewater samples passed through 0.45 μm glass fiber filters; the technique was validated by chromatography. Moreover, as in the case of SAs, fluoroquinolone, tetracycline, and chloramphenicol compounds, Campagnolo et al.[309] measured BLs in water samples taken from the vicinity of a farm; they obtained a detection limit of 2 μg L^{-1} for pencillin G.

8.4.1.6 Macrolides

Macrolide antibiotics, such as tylosin, roxithromycin, and erythromycin, are an important group of pharmaceuticals used in human and veterinary medical practice. Their activity stems from the presence of a large macrocyclic lactone ring containing 14, 15, or 16 atoms, with deoxy sugars, usually cladinose and desosamine, linked via glycosidic bonds. After application, a certain fraction of these macrolides is metabolized to inactive compounds, but a significant amount is excreted as active metabolites.[355] Most macrolide structures enter the environment via animal manure, which limits their mobility and bioactivity. In any case, control of these residuals in the environment is necessary so as to avoid future negative impacts on public health. Kumar et al.[345] reported on the detection of tylosin, used extensively in pig production for both growth promotion and therapeutic purposes, using two commercial ELISAs for surface and groundwater samples. Samples were diluted twice in buffer prior to their analysis; an LOD of 0.2 μg L^{-1} was obtained. Several antibiotic growth promoters, including tylosin, were analyzed in ground feed samples using a multianalyte ELISA after a cleanup step on OASIS cartridges by Situ et al.[356] Polyclonal antibodies were developed for this purpose. With this method, LOD and CCß values for five banned substances in animal feeds were respectively 0.28 and 0.30 mg kg^{-1} for bacitracin, 1.02 and 1.50 mg kg^{-1} for olaquindox, 0.21 and 0.60 mg kg^{-1} for spiramycin/tylosin, and 0.09 and 0.20 mg kg^{-1} for virginiamycin. Campagnolo et al.[309] measured erythromycin macrolides along with five other antibiotics, achieving RIA detection limits of around 10 μg L^{-1}.

8.4.1.7 Other Drugs

This section deals with immunochemical methods developed to determine drugs that do not belong to any of the most common antibiotic family groups described above, but because of their importance and general use require to be considered, too. Table 8.10 shows a few examples of ELISAs for the analysis of these compounds in environmental samples.

A highly sensitive and specific ELISA for the determination, in different types of water samples, of diclofenac, a commonly used nonsteroidal anti-inflammatory drug (NSAID), has been developed by Deng et al.[357] This analyte belongs to the most frequently detected, pharmaceutically active compounds in the water cycle. The immunoassay was able to measure tap water samples directly—respective LOD and IC_{50} values were 6 and 60 ng L^{-1}. On the other hand, surface water samples required fivefold dilution and the wastewater samples 10-fold dilution in buffer to be analyzed correctly; the LODs were then 20 and 60 ng L^{-1}, respectively. Recently, the development and validation of a highly sensitive and specific ELISA for the detection of pharmaceutical indomethacin in

TABLE 8.10
Immunochemical Techniques Developed for the Detection of Other Drugs

			Sensitivity		
Target Analyte	**Technique**	**Matrix**	**LOD (μg L^{-1})**	**IC_{50} (μg L^{-1})**	**Reference**
		Other Drugs			
Diclofenac	ELISA	Tap water	6×10^{-3}	60×10^{-3}	357
		Surface water	19×10^{-3}		
		Wastewater	60×10^{-3}		
Indomethacin (acemetacin 92% CR)	ELISA	Tap water	0.01	<0.25	358
		Driking water	0.01	<0.25	
		Surface water	0.01	<0.25	
		Wastewater	0.10	<2.50	
Nitrofurantoin	ELISA	Animal fed water	0.20	3.20	359

water samples from different sites in the Chengdu area was presented by Huo et al.[358] This commonly used compound is also included in the NSAID group. Although indomethacin is considered stable in the environment, its long-term presence in aquatic systems may increase chronic toxicity and more insidious effects, like endocrine disruption, growth inhibition, and cytotoxicity, in aquatic animals. The study measured tap water and drinking water samples directly (LOD = 0.01 μg L^{-1}); the same LOD was obtained for surface water samples after these had been filtered through a 0.45 μm nylon cartridge. Wastewater samples required a 10-fold dilution step prior to analysis with the immunoassay (LOD = 0.1 μg L^{-1}). In all cases, around 90% of acemetacin cross-reacted. Liu et al.[359] prepared polyclonal antibodies for the immunochemical detection of nitrofurantoin residues in water samples. Nitrofurans are a group of synthetic broad-spectrum antibiotics frequently employed in animal production to treat and prevent gastrointestinal infections caused by *Escherichia coli* and *Salmonella*. They are also used as growth promoters in pig, poultry, and fish production. Using the relevant ELISA, LOD and IC_{50} values of 0.20 and 3.20 μg L^{-1}, respectively, were obtained in drinking water fed to animals. A fluorescence-based continuous-flow immunosensor for the sensitive, precise, accurate, and fast determination of paclitaxel was developed by Sheikh and Mulchandani.[360] A natural product, this compound is known to be one of the most active anticancer agents approved by FDA for application in clinical oncology practice. The assay is based on the displacement and detection downstream of rhodamine-labeled paclitaxel by a flow-through spectrofluorometer, as a result of the competition with paclitaxel introduced as a pulse into the stream of carrier buffer flowing through the system. The detection limit found in buffer and human plasma samples was around 4 μg L^{-1}. Finally, a fluorescence immunoassay to detect spectinomycin, which is used as an oral treatment to control bacterial enteritis in pigs and to prevent and control losses due to chronic respiratory disease in chickens, was developed by Medina et al.[361] The antibodies and secondary immunoreagents implemented in the assay enabled an LOD of *ca.* 5 μg L^{-1} in buffer to be obtained.

8.4.2 Steroid Hormones

Steroid hormones are a group of biologically active compounds controlling human body functions related mainly to the endocrine and immune systems. Synthesized from cholesterol, they have a cyclopenta-*o*-perhydrophenanthrene ring in common.[40] Mammalian steroid hormones, which are secreted by the adrenal cortex, testicles, ovary, and placenta, can be classified into different groups, such as estrogens, gestagens, androgens, and glucocorticoids, depending on the intracellular receptor to which they bind in order to become active.[6] Apart from the endogenous hormones, many synthetic steroids have been produced for their high bioactivity. Thus, the consumption of natural and synthetic steroids in human medicine and animal farming has increased steadily in recent decades. On the other hand, humans and animals excrete hormone steroids from their bodies, which readily enter the aquatic environment through sewage discharge and animal waste disposal, mainly via effluents from WWTPs.[4,6] Once in waterways, they may adsorb to solid particles, like bed sediments or soils, where steroids may persist for long periods.[363] The increasing number of steroid hormones in the environment may interfere with the normal functioning of endocrine systems, thus affecting reproduction and development in wildlife.[363] Apart from the standard chromatographic techniques, many examples can be found in the literature of the immunochemical determination of steroid residues, mainly in biological samples, but also in environmental matrices.[364,365] Table 8.11 shows some examples of the immunological methods described for these compounds.

8.4.2.1 Estrogens

Estradiol is one of the main female sexual hormones; it is also the structural backbone for the engineering of some synthetic estrogens, such as ethynyl estradiol or mestranol, used in human hormone treatments. Both natural and synthetic estrogens are classified as endocrine disrupting chemicals (EDCs).[6,362] Many of these substances and their metabolites end up in the environment where

TABLE 8.11
Immunochemical Techniques Developed for the Detection of Steroid Hormones

Target Analyte	Technique	Matrix	Sensitivity		Reference
			LOD	IC_{50}	
		Steroid Hormones			
Estradiol	ELISA[a] (Abraxis, USA)	Urban wastewater River and groundwater	0.05 μg L^{-1}		370
	SPEoptical sensor	Seawater (sewage plants) Seawater	1.5 μg L^{-1} 0.16 μg L^{-1}		372
	SPE-CLEIA	Tap and wastewater	1.5 μg L^{-1}		369
	TIRF	Wastewater	0.16 μg L^{-1}	1.84 μg L^{-1}	371
	ETIA	Wastewater	0.85 μg L^{-1}	1.2 μg L^{-1}	371
Estriol	ELISA[a] (Abraxis, USA)	Urban wastewater River and groundwater	0.05 μg L^{-1}		370
Estrone	ELISA[a] (Abraxis, USA)	Urban wastewater River and groundwater	0.05 μg L^{-1}		370
	TIRF	River water Groundwater	0.08 μg L^{-1} 0.08 μg L^{-1}	0.53 μg L^{-1} 0.56 μg L^{-1}	424
	TIRF	Wastewater	0.01 μg L^{-1}	0.51 μg L^{-1}	371
	ETIA	Wastewater	0.50 μg L^{-1}	0.81 μg L^{-1}	371
Ethynylestradiol	ELISA[a] (Abraxis, USA)	Urban wastewater River and groundwater	0.05 μg L^{-1}		370
	TIRF	Wastewater	0.07 μg L^{-1}	1.07 μg L^{-1}	371
	ETIA	Wastewater	0.01 μg L^{-1}	2.70 μg L^{-1}	371
Noresthindrone	EIA	River and potable water	10 ng L^{-1}		386
Progesterone	RIA	River and potable water	5 ng L^{-1}		386
	CLEIA	River and potable water	15 pg per tube		385
	TIRF	MilliQ water	0.96 ng L^{-1}		364
Testosterone	RIANA	Drinking water River water	0.2 ng L^{-1} 0.2 ng L^{-1}		375

[a] Commercial kit.
Notes: SPE, solid-phase extraction; SFE, supercritical fluid extraction; and GHD, glucose dehydrogenase.

they can have adverse effects on wildlife organisms even at very low concentrations.[366–368] Thus, environmental monitoring programs on estrogens call for analytical techniques capable of achieving very low detection limits. With regard to immunochemical methods, there are several commercial tests on the market, addressed mainly to food residue analysis. Nevertheless, application to the analysis of aquatic ecosystems should be easy to implement. Zhao et al.[369] developed a chemiluminescence enzyme immunoassay (CLEIA) for the determination of 17β-estradiol in wastewater samples; the working linear range obtained was from 2.5 to 1600 ng L^{-1}, with a detection limit of 1.5 ng L^{-1}. Recoveries of spiked tap water and wastewater samples at 0, 2.5, 10, and 50 ng L^{-1} were in the 80–110% range. Results were compared with the commercially available radioimmunoassay kit (MARCA): a good correlation ($R^2 = 0.997$) was obtained. Farré et al.[76] evaluated four different commercially available ELISAs for the rapid screening of estrogens in different water matrices, including natural and spiked samples from urban wastewater, river water, and groundwater from the vicinity of Barcelona. ELISA kits[370] were configured to measure 40 samples per plate with sufficient sensitivity, high cross-reactivity with other congeners, and reproducibility. All the samples extracted

by SPE yielded recoveries from 79% to 86%; assay validation was carried out by comparison with HPLC-MS/MS using a triple quadrupole (QqQ) instrument. Coille et al.[371] described the use of two fluorescence immunochemical methods to detect different estrogenic compounds in synthetic wastewater. In the first one, the immunosensor is based on the TIRF principle (LOD for estradiol = 0.16 μg L^{-1} and for estrone and ethynylestradiol = 0.07 μg L^{-1}). The other method is an energy transfer immunoassay (ETIA), in which the specific antibody is labeled to a donor fluorescent dye, while the antigen is coupled to an acceptor dye via a BSA molecule. The fluorescence is quenched as a consequence of the biorecognition reaction. In this case, the respective detection limits obtained were 0.5, 0.85, and 0.01 μg L^{-1} for estrone, estradiol, and ethylestradiol. Recovery rates for both techniques when measuring spiked wastewater samples were between 70% and 112%. Recently, Zhang et al.[372] have developed a sensitive and simple immunoassay based on the SPR technique for monitoring 17β-estradiol. Previous to their analysis, seawater samples were hydrolyzed with HCl/methanol solution and preconcentrated using C18 SPE; recovery values were *ca*. 92%. Subsequent studies showed that the precision and repeatability of the SPR assay were good; cross-reactivity with other estrogens was very low.

8.4.2.2 Androgens

The main applications of immunochemical techniques for androgens are in the doping control of athletes, forensic chemistry, farm animals for human consumption, and food analysis.[373,374] However, there have been a few applications in the environmental field for natural or synthetic androgens. For example, Barel-Cohen et al.[4] monitored natural steroids in sewage and fishpond effluents, finding levels of testosterone between 2.1 and 7.8 ng L^{-1} at different collecting points along a river. A TIRF immunosensor has been developed for reliable sub-ng L^{-1} detection of testosterone in aquatic environmental matrices without sample pretreatment. Thus, direct analysis of spiked lab water, drinking water, and river water samples gave an LOD of 0.2 ng L^{-1} with recovery rates between 70% and 120%.[375] This sensor system was therefore shown to be a suitable warning tool in environmental analysis, in addition to the standard analytical methods. On the other hand, several ELISA procedures have been developed for the analysis of testosterone and related compounds in biological samples.[365,376] In the case of performance-enhancing anabolic steroids, such as stanozolol, nandrolone, and the recently designed steroid known as tetrahydrogestrinone, immunochemical assays have also been performed on equine and human urine samples during doping controls.[377–379] Additionally, powerful techniques combining multiresidue immunoaffinity chromatography with GC-MS or ELISAs are available for the simultaneous identification and semiquantification of various androgens in samples of urine and feces.[39,378,379] Figure 8.7 shows a scheme of a multi-IAC procedure.

Immunosensors have made a great contribution in the field of androgenic steroid detection, giving detection limits comparable to those obtained with standard ELISA procedures. Several electrochemical immunosensors have been developed for detecting testosterone, methyltestosterone,

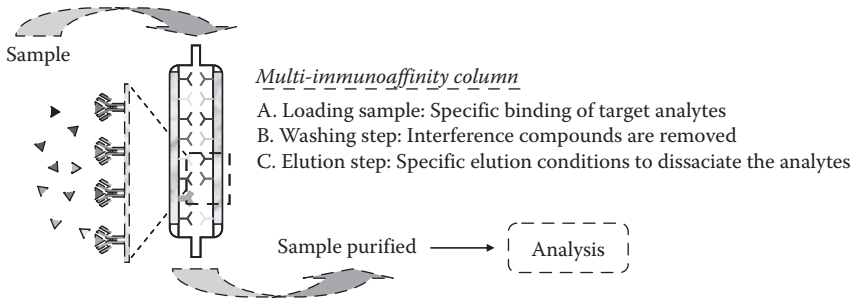

FIGURE 8.7 Schematic sequential step procedure for chromatographic determination after multiimmunoaffinity column purification.

19-nortestosterone, boldenone, and methylboldenone in spiked bovine urine using screen-printed electrodes.[380–382] Once again, the techniques already used to analyze these compounds in complex biological matrices can be adapted for the analysis of environmental samples.

8.4.2.3 Gestagens

Gestagens are hormones that produce similar effects to those of endogenous progesterone, which is excreted from the ovary to act as a balancer in the menstrual cycle, pregnancy, and embryogenesis. As with estrogens, most of the immunochemical techniques described in the literature have been developed to analyze these compounds in biological matrices.[383–385] On the other hand, Aherne et al. described a RIA for progesterone and norethindrone detection in water samples, obtaining LODs of 6 and 17 ng L^{-1}, respectively.[386] This group also reported that norethindrone underwent 28% biodegradation in an activated sludge system in 6 h and was completely degraded in one day, which is the time required to purify river water for drinking purposes. Furthermore, Käppel et al.[364] developed an immunosensor based on TIRF detection to analyze progesterone in spiked MilliQ water. The assay was optimized to obtain results in 5 min with an LOD of 0.96 ng L^{-1}. All these results show fairly well the usefulness of immunochemical techniques for the determination of micro- and nanogram per liter quantities of gestagens in aqueous samples.[387]

8.4.2.4 Corticosteroids

Endogenous corticosteroids are produced by the adrenal glands in response to stressors such as exercise, illness, and starvation.[5] Synthetic cortisone derivatives were synthesized in the late 1940s for therapeutic purposes. Lately, these products have found their way into the world of sports because of their anti-inflammatory properties, but they are now on the list of substances banned by the International Olympic Committee (IOC). Moreover, corticosteroids like dexamethasone are used not only in veterinary practice for the treatment of respiratory and gastrointestinal disorders, but also as illegal growth promoters in animal feedstuffs. To control this undesirable situation, efficient screening procedures, based mainly on ELISA methods, have been described for the analysis of most important corticosteroids in biological matrices,[388–390] but they have not yet been applied to environmental samples. Pujos[390] reported the analysis of 18 human corticosteroids, both endogenous and synthetic, in spiked urine samples. The samples required a pretreatment based on simple 1/50 dilution. ELISA is a suitable technique for the systematic detection of corticosteroids by the food and agriculture industries in many different sample matrices;[391–393] their implementation in the analysis of aquatic environmental samples seems appropriate.

8.5 GENERAL SUMMARY

In recent decades, immunochemical techniques have been widely demonstrated to be an interesting alternative to the more conventional analytical methodologies in many areas, but additional work is still necessary to completely adapt them to the analysis of environmental contaminants. On the other hand, considering that the analysis of very complex biological samples with these methods has been successful, the prospects for their application to the analysis of water and soil samples seem highly promising. In this relatively new situation, where data on the occurrence, risk assessment, and environmental toxicity of most of these emerging pollutants are not available, collaboration and interchange of expertise between analytical and immuno chemists are needed to achieve this objective. The benefits accruing from these methods (i.e., high sensitivity, selectivity, cost-effectiveness, high sample processing capabilities vis-à-vis target analytes) are nowadays available for assessing risk and protecting public health from the adverse effects of these types of pollutants. From now on, research efforts should focus on the development of multianalyte immunochemical systems, in which more than one compound or group of compounds can be detected simultaneously, and on the design of new analytical user-friendly devices (i.e., immunosensors) for continuous or on-site measurements. Technical development should be accompanied by some officially organized efforts

to find ways of validating screening immunoassay techniques and recognizing them as practicable routine methods in environmental monitoring laboratories. For the time being, directives are issued, regulations enacted, and conferences held to ensure water quality, protect water resources, and ensure the good health of the entire environment.

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1.9 Book Chapter 2: Recent Advances on Biosensors for Pharmaceuticals and Emerging Contaminants Based on Novel Micro and Nanotechnology Approaches

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The investigation of new sensing principles and technologies for the detection of molecular binding events has created great expectations on numerous major industrial sectors, such as healthcare, food, water and agriculture. Combining many of these advances with the potential of the immunochemical systems has allowed developing novel biosensors that provide interesting advantages against the traditional strategies for analysis, such as the possibility of multianalysis, development of field analytical methods and fabrication of easy end-user devices. Specifically, many efforts have been lately invested to control residues of pharmaceuticals in food and environmental samples, as an indication of the impact of the human activity in the media. Human and veterinary drugs, such as antibiotics, hormones, analgesics, cytostatics or β-blockers, show a high potential risk of negative effects in the environment and public health. Thus, there is a great need for low-cost and highly efficient tools for quick, reliable, and accurate detection of these contaminating bioactive agents. In particular, the scope of the present chapter is addressed to provide an overview of the potential of novel micro(nano)technology approaches to develop biosensors useful for the analysis of emerging pollutants.

Keywords Amperometric biosensor, Antibody, Antibiotics, Biosensor, Evanescent wave, Hormones, Impedimetric biosensor, Microcantilevers, Nanobiotechnology, Pesticides, Piezoelectric crystal, Plasmon resonance

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Biosensors for Pharmaceuticals and Emerging Contaminants Based on Novel Micro and Nanotechnology Approaches

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Abstract The investigation of new sensing principles and technologies for the detection of molecular binding events has created great expectations on numerous major industrial sectors, such as healthcare, food, water and agriculture. Combining many of these advances with the potential of the immunochemical systems has allowed developing novel biosensors that provide interesting advantages against the traditional strategies for analysis, such as the possibility of multianalysis, development of field analytical methods and fabrication of easy end-user devices. Specifically, many efforts have been lately invested to control residues of pharmaceuticals in food and environmental samples, as an indication of the impact of the human activity in the media. Human and veterinary drugs, such as antibiotics, hormones, analgesics, cytostatics or β-blockers, show a high potential risk of negative effects in the environment and public health. Thus, there is a great need for low-cost and highly efficient tools for quick, reliable, and accurate detection of these contaminating bioactive agents. In particular, the scope of the present chapter is addressed to provide an overview of the potential of novel micro(nano)technology approaches to develop biosensors useful for the analysis of emerging pollutants.

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Contents

1 Introduction

Biomolecular analytical devices have evolved rapidly over the last years. Novel molecular diagnostic approaches to refine and extend the limits of detection have emerged as a consequence of the combination of micro(nano)technological and biotechnological advances. Biosensors are among the potential applications of new materials and devices and of novel nanobiotechnological approaches. Thus, subtle changes in properties such as the dielectric field or the refractive index produced after biomolecular recognition events can be detected if they are taking place at the surface of appropriately developed transducers. New knowledge of the unique properties of nanostructured materials has opened up the possibility to investigate the influence that biorecognition phenomena have on the new optical and/or electrical properties of these systems, possibly leading to the development of more sensitive and flexible biosensing systems.

Among biosensors, immunosensors using antibodies as biorecognition elements have fascinating features such as the possibility to respond selectively to biological or bioactive substances and the capability to respond in a physiological manner. The unique properties of certain nanomaterials combined with the excellent features of the antibodies allow envisaging novel exquisitely sensitive chemical and biological sensors. Thus, antibodies are natural molecules with inherent capabilities to specifically react with their counter antigen. Antibodies can be produced, in principle, against all kinds of substances and their features can be tailored according to the requirements of each application. Their homogeneity regarding chemical structure and properties allow standardization of several procedures. Moreover, antibodies show greater improved stability, when compared to other biomolecules.

A very important aspect in biosensor development is the construction of biofunctional surfaces. Sensor solid surfaces are in general solid inorganic matter and per se not suitable for immobilizing biomolecules. Hence, further modification is required to adapt them for the immobilization of biomolecules. In addition, functional sensor surfaces place several demands such as biocompatibility, homogeneity, stability, specificity, and functionality. Thus, a challenge in biosensor development

is to construct adequate surfaces as well as to design molecules suitable for site-directed immobilization. Surface architecture depends on the nature of the transducer and on the features of the biomolecule, as well as the type of measurements needed [1–4]. The surface has to be activated appropriately for further tethering of the proteins with a particular immobilization method. Subsequent layers can be generated in place, and textured following specific demands.

Environmental monitoring of chemical and biological hazard is a potential application of biosensors, and therefore several developments can be found in the literature trying to provide better analytical tools to assess any risk derived from contamination. This interest in developing more efficient bioanalytical devices responds to the increasing production of these chemicals, including so-called emerging contaminants such as pharmaceutical active ingredients and personal care products (PPCPs) [5] and the potential toxicological risks derived from them. Pharmaceuticals and biogenic hormones are normally discharged to the environment through wastewater treatment plants (WWTP), which are often not designed to remove them from the effluent. From there on, transport, fate, or possible adverse consequences of these pollutants on human health and on the ecosystem, are frequently unknown or not clearly understood [6]. Potential concerns include reproductive impairment [7–9], increased incidence of cancer [10], development of antibiotic-resistant bacteria [11], or the potential increased toxicity of chemical mixtures due to synergistic effects [12].

In Europe, regulations and regulatory methods to assess and control the impact of these substances in the aquatic environment, aim to protect the ecosystem and public health while monitoring contamination levels and any potential negative effects. In addition to the REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) law (EC 1907/2006), regarding chemicals and their safe use, which entered into force on 1st June 2007, there are specific regulations addressed to protect health and ensure quality of all water resources such as the Drinking Water Directive (DWD, Council Directive 98/83/EC), the Bathing Water Directive (2006/7/EC), and the Urban Waste Water Directives (91/271/EEC and 98/15/EC). Moreover, the Water Framework Directive (WFD, 2000/60/EC) intends to provide an overall framework for a cleaner and safer water ecosystem, particularly regarding surface freshwater and ground water bodies (i.e., lakes, streams, rivers, estuaries, coastal waters etc.). Thus, consistent with this directive, the Marine Strategy Framework Directive (2008/56/EC) aims to achieve good environmental status of the EU's marine waters by 2021. Similarly, a River Basin Management Plan (RBMP) is being developed to be implemented for each river basin district which must include knowledge of the particular pressures and impacts of human activity, protection programs, controls, and remediation measures. The first RBPM is scheduled to be published at the end of 2009 and according to it the new Groundwater Directive (2006/118/EC) establishes a regime which sets underground water quality standards and introduces measures to prevent or limit input of pollutants into the groundwater.

On the other hand, pharmaceutical residues can also contaminate food through animal treatment with veterinary drugs. During their lifetime animals may have to be treated with medicines for prevention or cure of diseases. In food-producing

animals such as cattle, pigs, poultry, and fish this may lead to residues of the substances used for the treatment in the food products derived from these animals (e.g., meat, milk, eggs). The residues should, however, not be harmful to the consumer, for which reason EC legislation requires that the toxicity of potential residues is evaluated before use of a medicinal substance in food-producing animals is authorized. If considered necessary, maximum residue limits (MRLs) are established and in some cases the use of the relevant substance is prohibited. Thus, Directive 96/23/EC establishes the frequencies and level of sampling and the groups of substances to be controlled for each food commodity. Moreover, Commission Decision 2002/657/EC (and the following amending directives) establishes criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results generated by official laboratories. Additionally, the Decision introduces a procedure to progressively establish minimum required performance limits (MRPL) for analytical methods employed to detect substances for which no permitted limit (maximum limit) has been established, such as hormones.

Pharmaceuticals are by definition very active substances and therefore there is an increased demand for new and more efficient analytical methods in order to protect ecosystems and public health. Herein we aim to illustrate this with examples of recent developments of new biosensor devices reported to detect certain emerging pollutants, antibiotics, and androgenic anabolic steroids. The physical principles and micro/nanotechnological approaches behind the examples presented herein should be contemplated as universal and with general applicability to the analysis of other chemical or biological hazards as soon as a bioreceptor is available to specifically detect these substances.

2 Electrochemical Biosensors

Because of its simplicity electrochemical transduction has given rise to a great variety of low-cost devices based on different formats and configurations (for recent reviews see [13–16, 17]). From all possible electrochemical transduction systems those based on recording the amperometric signal generated after an enzymatic reaction have seen the greatest development [16]. However, new approaches have been described aimed at directly detecting biomolecular recognition reactions without using enzyme labels, such as the impedimetric devices, or incorporating nanoparticles to generate or increase the electrochemical signal. As an example, gold nanoparticles that provide a suitable microenvironment for biomolecule immobilization whilst retaining biological activity, and to facilitate electron transfer between the immobilized proteins and electrode surfaces, have led to an intensive use of this nanomaterial for the construction of electrochemical biosensors with enhanced analytical performance with respect to other biosensor designs [13, 15]. Similarly, the particular structure of carbon nanotubes and their unique properties make them a very attractive material for the design of electrochemical biosensors. Among the potential advantages are the promotion of different electron transfer reactions, especially those related to

biomolecules and the increase of the active surfaces which has led to different strategies for constructing carbon nanotube-based electrochemical sensors [14].

2.1 Amperometric Biosensors

Amperometric biosensors are based on the measurement of the current generated by oxidation/reduction of redox species at the electrode surface, which is maintained at an appropriate electric potential. The current observed has a linear relationship with the concentration of the electroactive species. The electrode is usually constructed of platinum, gold, or carbon. Adjacent to the electrode, entrapped by a membrane or directly immobilized, the bioreceptor is placed. Label enzymes used in electrochemical immunoassays are usually oxidoreductases such as horseradish peroxidase (HRP), or hydrolytic enzymes, such as alkaline phosphatase (AP), which yield electroactive species as a product of the enzymatic reaction (see Fig. 2). Other enzymes used as sensing elements for environmental monitoring are tyrosinase, laccase, aldehyde dehydrogenase, etc. Sometimes the substrate or the product of the enzymatic reaction can be monitored amperometrically, without the need for a mediator. These electrodes are called *unmediated amperometric enzyme biosensors*. However, a number of factors must be taken into account when assessing the suitability of an enzyme substrate to be used in an electrochemical detection system: the electrochemistry of the substrate, the electrochemistry of the product of the enzymatic reaction, the medium in which the measurements will be performed, and the electrochemistry of endogenous materials in the test sample. A problem often encountered with unmediated sensors is that other species present in the samples being analyzed are also electroactive at the potential applied. For example ascorbic acid and uric acid, present in many biological samples, are oxidized at an anodic potential of +0.35 V. AP combined with *p*-aminophenyl phosphate (PAPP) as a substrate has been shown to be a good alternative when measuring with such a system. Although *p*-aminophenyl phosphate has an irreversible wave in cyclic voltammetry at around 0.45 V vs. Ag/AgCl, its hydrolysis product *p*-aminophenol (PAP) shows a reversible electrochemistry with a half wave potential of –0.065 V vs. Ag/AgCl. Consequently, measurements on biological matrices can take place at lower potentials avoiding interference of endogenous compounds. Choosing an alternative electron transfer acceptor can also circumvent these problems. Usually the *mediator* is a species of low molecular weight (MW) that shuttles electrons between the redox center of the enzyme and the working electrode. These sensors are called *mediated amperometric enzyme biosensors*. A mediator should react rapidly with the enzyme, exhibit reversible heterogeneous kinetics, possess a low overpotential for regeneration, and be stable at a certain range of pH, temperature, redox state, and dioxygen. Some mediators frequently used are I^-, $[Fe(CN)_6]^{-4}$, *o*-phenylenediamine, diaminobenzidine, hydroquinone, and 5-aminosalicylic acid.

A recent example has been reported to detect xenoestrogens using an amperometric immunosensor based on a quinoprotein glucose dehydrogenase (GDH)-modified

thick-film sensor. The immunosensor is used to detect the trazer used in a capillary immunoassay (CIA) [18]. With this configuration, detection of the alkylphenols and their ethoxylates in μg L^{-1} range is possible using a β-galactosidase tracer and anti alkylphenol ethoxylate antibodies. The assay is performed off-line in small disposable PVC capillaries coated with immobilized antibodies, which allows the combination of the assay with a small portable device potentially useful for on-site environmental monitoring. The bioelectrocatalytic properties of this biosensor offer an additional amplification and allow a very sensitive quantification of the 4-aminophenol, generated by the β-galactosidase. However, in the last few years, the advances and possibilities presented by easily fabricated screen-printed electrodes (SPEs) has given rise to a significant number of examples demonstrating the potential and the flexibility of this technology to develop low-cost biosensor devices for a variety of targets including microorganisms, pesticides, and also pharmaceuticals. As an example, disposable carbon SPE immunosensors have been described for the detection of anabolic androgenic steroids (AAS) using horseradish peroxidase as the enzyme label and tetramethylbenzidine/hydrogen peroxide (TMB/H_2O_2) as the substrate for chronoamperometric detection at +100 mV [19–21]. The immunosensors were fabricated by immobilizing hapten-bovine serum albumin conjugates on the surface of the SPEs and followed by the competition between the free analyte and coating conjugate with corresponding antibodies. The signal is recorded after an anti IgG-HRP conjugate determining the degree of competition. By using different antibodies with different affinities and cross-reactivity patterns the immunosensor detects different AAS with limits of detection (LOD) below 200 pg mL^{-1}. Thus, LODs of 30.9 ± 4.3 pg mL^{-1} for boldenone and 120.2 ± 8.2 pg mL^{-1} for methylboldenone, respectively, have been reported. Testosterone can be detected in buffer with a LOD of 26 and 1.8 pg mL^{-1} in a complex matrix such as bovine urine. Similarly, nortestosterone can be detected in the same matrix with a LOD of 15 pg mL^{-1}.

The possibility to analyze progesterone using amperometric immunosensors has been recently reported using a colloidal gold-graphite-Teflon-tyrosinase composite biosensor as the amperometric transducer [22]. A sequential competitive configuration between the analyte and progesterone labeled with alkaline phosphatase was selected using in this case phenyl phosphate as the AP-substrate and the enzyme reaction product, phenol, was oxidized by tyrosinase to o-quinone, which was subsequently reduced at −0.1 V at the biocomposite electrode. A LOD of 0.43 ng mL^{-1} was accomplished while the time needed to reach the steady-state current from the addition of phenyl phosphate was 30–40 s. A lifetime of 14 days with no need to apply any regeneration procedure was reported. The immunosensor was applied to the detection of progesterone in milk spiked after just sample dilution.

Recently, several papers have been published combining magnetic particles with electrochemical amperometric detection. Magnetic beads are known to be a powerful and versatile tool in a variety of analytical and biotechnology applications. The use of magnetic beads greatly improves the performance of the immunological reaction, minimizing the matrix effect due to improved washing and separation steps. Additionally, the magnetic beads can be easy magnetically manipulated by using permanent magnets or electromagnets. Therefore, the analysis of samples performed

on magnetic beads can be easily achieved without any pre-enrichment, purification, or pretreatment steps, which are normally necessary for standard methods. In this context, an electrochemical immunosensing strategy based on magnetic sensors is presented as a sensitive, simple, cheap, and user-friendly analytical alternative to classical methods for the detection of residues. The strategy combines the advantages taken from immunological assays, magnetic bead separation, and electrochemical transduction and is based on the immunochemical competitive immunoreactions between the contaminant and the enzyme tracer with the specific antibody immobilized on magnetic beads followed by its separation from the biological matrix by the use of a magnetic field. Thus, after the immunological reaction, the magnetic beads can be easily captured by a novel electrochemical and magnetic transducer, based on a magneto graphite-epoxy composite [12, 13] (m-GEC), by merely dipping the magneto electrode in the solution where the beads are suspended (Fig. 1). This strategy has been used for the detection of sulfonamide antibiotics in milk samples [23]. The antibodies raised to provide an appropriate recognition pattern of this antibiotic family [24] are immobilized on the surface of magnetic beads through covalent attachment using tosyl-activated commercially available magnetic beads. Other immobilization strategies tested before also gave suitable results, but this strategy was demonstrated to be the one providing the best performance [25]. The immunological reaction for the detection of sulfonamide antibiotics is based on a direct competitive assay using a peroxidase (HRP) tracer as the enzymatic label. Excellent results were accomplished detecting sulfamethazine directly in a diluted raw full cream sample with detectability values far below the MRLs established by the EC in those types of samples (IC50 of 9.8 μg L^{-1} and LOD of 0.36 μg L^{-1}). The same results were obtained with UHT (ultra high temperature) milk, such as full cream (about 3.25% fat), semi-skimmed (about 1.5–1.8% fat), and

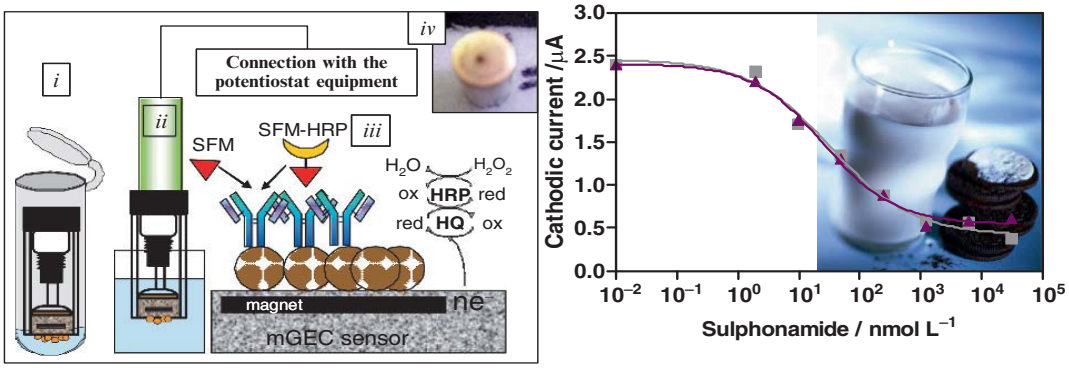

Fig. 1 *Left:* Schematic representation of the electrochemical magnetoimmunosensing strategy for the detection of sulfonamides. After the immunoreaction, the antibody-modified magnetic beads were captured for the m-GEC electrode (i). Chemical reactions occurring at the m-GEC surface polarized at –0.150 V (vs. Ag/AgCl) upon the addition of H_2O_2 in the presence of mediator (HQ) (ii and iii). Appearance of the m-GEC electrode covered with the magnetic beads (iv). *Right:* Calibration curves for sulfapyridine run in PBS and in 1:5 whole milk. Parts of this figure are reprinted from Zacco et al. [23] with permission from Elsevier

skimmed (0.1% fat). This strategy has been proven to be useful for the analysis of other residues in complex matrices such as atrazine in orange juice [25] or fluoroquinolone antibiotics in milk (unpublished results).

The same strategy has also been applied to the detection of polychlorinated biphenyls (PCBs) though in this case using SPEs [26, 27]. The sensor was based on a direct competitive immunoassay scheme in which antibody-coated magnetic beads were used on SPEs to detect the product of the enzymatic reaction of alkaline phosphatase used as the tracer. α-Naphthyl phosphate was used as the substrate and the α-naphthol produced during the enzymatic reaction was detected using differential pulse voltametry (DPV). Aroclor 1248 PCB mixture could be measured with a detection limit of 0.4 ng mL^{-1}. The performance of the sensor was demonstrated analyzing PCBs on marine sediment extracts and spiked soil samples.

2.2 Impedimetric Biosensors

Different sensor approaches have been accomplished to avoid the use of labels when the target analyte is a biomacromolecule, bacteria, or virus, but only on a few occasions has the performance of these transduction principles been demonstrated to detect small organic molecules at low concentration levels. In this context, *electrochemical impedance spectroscopy* (EIS) is being rapidly developed because of the possibility to record directly information on biorecognition events occurring at the electrode surfaces and inducing capacitance and resistance changes [28, 29]. Thus, when a biological receptor binds to its counterpart, there is a change in the impedance of the system that enables direct measurement of an electrical signal, allowing the development of label-free biosensing devices. A major drawback of early sensors was that impedance changes due to biorecognition are very small. However, new electrode configurations have significantly increased the detectability of these devices. Thus, impedimetric label-free devices have been described to detect DNA hybridization [30] or biomolecules such as interferon-gamma [31], but direct detection of contaminants and residues in complex matrices is still a challenge. Label-free detection of bisphenol A (BPA) based on the impedance measurement has also been achieved with an impedimetric immunosensor [87]. The immunosensor was fabricated by the covalent immobilization of a polyclonal antibody on carboxylic acid group-functionalized nano-particle comprised conducting polymer and bisphenol A hapte, (4,4-bis(4-hydroxyphenyl) valeric acid, BHPVA) BSA conjugate. The immunosensor showed specific recognition of BPA with a LOD of 0.3 ± 0.07 ng mL^{-1}. The proposed immunosensor was applied to the analysis of BPA in human serum samples.

In this context, interdigitated microelectrodes (IDμE) have recently drawn great attention since their sensitivity is higher than conventional electrodes [32–35]. Using thin Au/Cr (~200 nm thickness) IDμE's (3.85 μm thick and with an electrode gap of 6.8 μm) on a Pyrex 7740 glass substrate, our group has recently reported detection of atrazine without the use of any label with a limit of detection of 0.04 μg L^{-1}[36]. The sensor has been evaluated to assess its potential to analyze pesticide residues in a complex sample matrix, such as red wine. An atrazine hapten-BSA

conjugate was covalently immobilized within the μ-electrodes on the glass substrate. An appropriate functionalization of the transducer surfaces was crucial in this case. A key problem is the nonspecific binding of molecules present in the sample matrix to the surface of the sensor. This concerns any surface from a lower to higher extent, but particularly, gold is very suitable to capture nonspecifically organic molecules and components from the media. For this reason, a two-step activation procedure based on the distinct properties of the gold (electrodes) and the silicon oxide surfaces (between the electrodes) was used. The whole activation procedure was monitored by impedance spectroscopy using IDμE as is shown in Fig. 2 In a first step, modification of the gold surfaces was accomplished making use of thiol chemistry using N-acetylcysteamine to cover the gold electrodes and to protect the sensor from undesired nonspecific absorptions (Step I). Following this, immobilization of the glass material between the electrodes was activated making use of silane chemistry, using (3-glycidoxypropyl)trimethoxysilane (GOPS) (Step II). The epoxy group provided the necessary reactivity for further attachment of the immunoreagents through a nucleophylic attack of the amino groups of the lysine

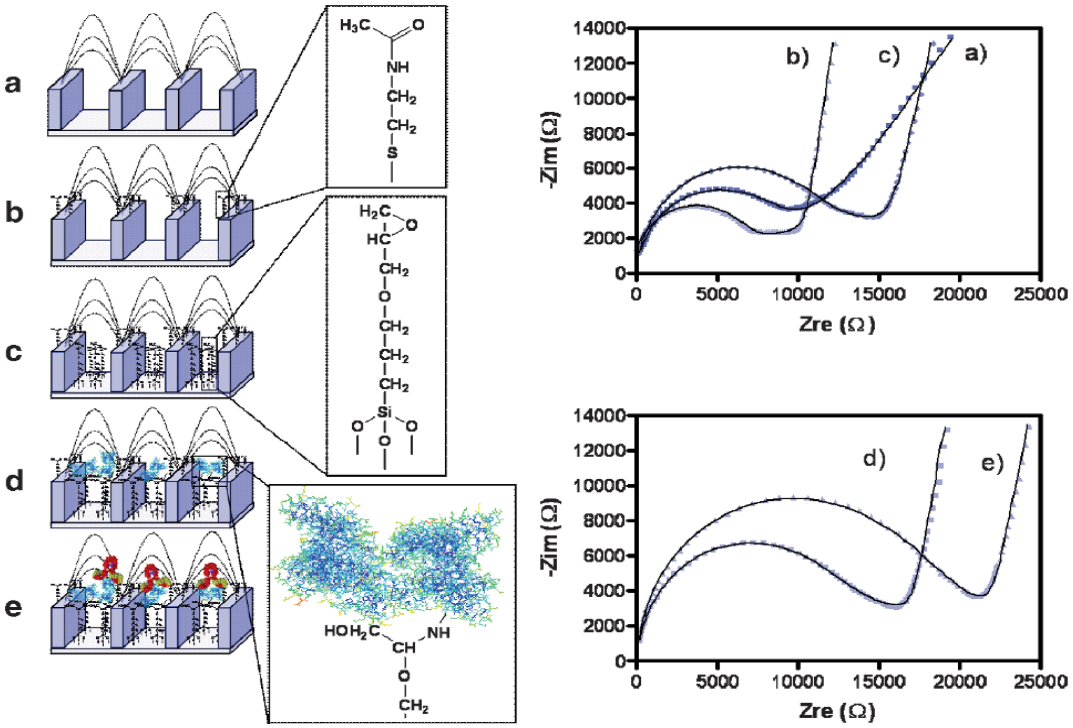

Fig. 2 *Left:* Scheme showing steps used to prepare the immunosensor surfaces and antibody binding. *Right:* Nyquist plots of impedance spectra corresponding to: (**a**) IDμE, (**b**) Step I: N-acetylcysteamine, gold protection, (**c**) Step II: functionalization of Pyrex substrate with (3-glycidoxypropyl)trimethoxysilane, (**d**) Step III: coating antigen 2d-BSA, covalent immobilization (1 μg mL^{-1}), and (**e**) Step IV: Antibody Ab11, incubation step (1 μg mL^{-1}). *Symbols* represent the experimental data. *Solid curves* represent the computer fitting data with the parameters calculated by the commercially available software Zplot/Zview (Scibner Associates). Parts of this figure are reprinted from [36] with permission from Elsevier

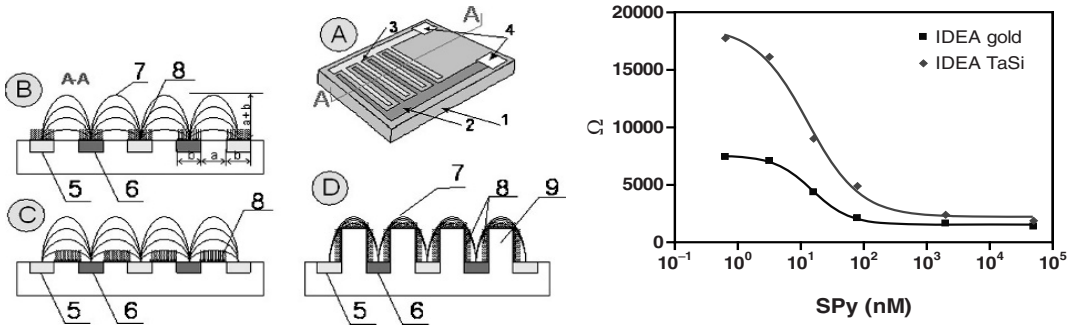

Fig. 3 *Left:* Schemes of the planar and barrier electrodes: (A) Planar IDEA device; (B), (C) cross-section A-A of the planar IDEA device; (D) IDEA device with insulating barriers between electrode digits. The numbers represent: 1-Insulating substrate; 2, 3-electrode collector bars; 4-contact pads; 5, 6-electrode "digits"; 7-electric field lines; 8-immobilized biomolecules; 9-insulating barrier. *Right:* Calibration curves obtained for sulfapyridine using either the planar electrodes or the electrodes with barriers. Parts of this figure are reprinted from Bratov et al. [37] with permission from Elsevier

residues of the antigen. As can be observed, the covalent immobilization of the atrazine antigen 2d-BSA (Step III) produced a significant change in the Nyquist plot. Finally, exposure of the immunosensor to a solution of the specific antibody (Step IV) produced a shift in the impedance spectra that is dependent on the concentration of atrazine present in the sample.

Also based on IDμE an improved immunosensor for sulfonamide antibiotic residue analysis has been reported [37, 38]. This new transducer used a three-dimensional interdigitated electrode in which the different electrode digits are separated by insulating barriers. Using this strategy it was possible to use most of the electric field instead of using just a small percentage (Fig. 3, left). Moreover, there is a significant increase of the available area to immobilize the immunoreagents. On the other hand, the electordes in this case are made of TaSi instead of gold, diminishing the nonspecific adsorptions on the surface. The immunosensor allows direct detection of the antibiotic sulfapyridine (SPY) and shows an IC_{50} value of 5.3 μg L^{-1} in buffer. As can be observed in Fig. 3 (right), the three-dimensional sensor shows considerable improvement compared with a standard planar InterDigitated Electrode Array (IDEA) design.

3 Optical Biosensors

Optical biosensors show great promise for residue analysis. Although first developments took advantage of the flexibility and low cost of the optical fibers measuring the absorption or emission of light of one of the components of the bioreaction [39], later developments were mainly based on micro/nanotechnology and on the possibility

to fabricate complex micro/nanostructures to guide light which has opened up the possibility to investigate novel optical phenomena and the optical properties of materials at the nanometric level (for reviews on new optical biosensing systems see [40–44]). Some of these developments have found application in the detection of emerging pollutants and also pharmaceuticals (i.e., [45–47]) and herein we will describe some of these examples.

3.1 Biosensors Based on the Evanescent Wave Optical Principle

An evanescent wave (EW) is produced in the external media (refractive index, n_2) of a waveguide (n_1) by the electromagnetic field associated to the light guided by total internal refection (TIR). The electromagnetic field does not abruptly switch to zero at the interface between the two media ($n_1 > n_2$), but decays exponentially with distance from the interface. The penetration depth of the evanescent field is defined as the distance where its strength is reduced to 1/e of its value at the interface and generally has a value around a hundred nanometers. The penetration depth is dependent on the incidence angle at the interface and is proportional to the wavelength of the excitation light. When molecules with an absorption spectrum including the excitation wavelength are located in the evanescent field, they absorb energy leading to attenuation (attenuated total reflection, ATN) in the reflected light of the waveguide. One of the advantages of the biosensors based on this principle is that possible interferences from the bulk media are avoided since only directly absorbed substances interfere with the electromagnetic field.

With the aim to improve detectability, many reported immunosensors combine this principle with the use of labeled molecules that are able to re-emit the absorbed evanescent photons at a longer wavelength as fluorescence. Part of this emission is coupled back to the waveguide and in this way is transmitted to the receptor. This phenomenon is known as total internal reflection fluorescence (TIRF). As an example a fully automated TIRF-based biosensor study for progesterone in bovine milk has recently been reported measuring progesterone levels in daily milk samples for 25 days, covering the whole estrus cycle [48]. The immunochemical assay was designed as a binding-inhibition test with a progesterone derivative covalently immobilized on the sensor surface and a monoclonal antiprogesterone antibody as the biological recognition element. Progesterone levels can be measured in about 5 min with a LOD of 0.04 ng mL^{-1}. The biosensor can be used in-line in the milking parlor and thus could be an important tool for reproductive management of dairy cattle detecting heat and predicting pregnancy, which are critical parameters in milk production. Similarly, the River Analyzer (RIANA) is also a highly sensitive fully automated biosensor able to detect multiple organic targets rapidly and simultaneously [49–51]. Thus, the system has been used to analyze a variety of analytes in environmental samples with very good limits of detection. Hormones, pesticides, antibiotics, and endocrine-disrupting chemicals are among the organic pollutants that have been detected with this biosensor system [47, 49, 52, 53].

More recently, other EW immunosensor approaches such as *Grating Couplers* (i.e., [54–56]) or *Mach-Zehnder Interferometers* (MZI) [57–60] have been investigated in order to make possible direct measurements of small analytes without the use of fluorescent labels. Thus, planar optical waveguides with an input grating coupler, or input/output gratings, have been reported with the aim to be used as label-free biodetection devices. Nanoimprint lithography is used to integrate the grating patterns with thin-film waveguides [55]. An optical waveguide lightmode spectroscopy (OWLS)-based biosensor is a recently developed device in the field of integrated optics, and exploits the science of light guided in structures smaller than the wavelength of the light. The system OWLS has been commercialized by MicroVacuum Ltd (Budapest, Hungary). The immobilization of a bioligand to an OWLS-based biosensor occurs by the silanization of the waveguide layer and the coupling of it to the silanized sensor surface. The structure of the chip consists of an input grating and a waveguide layer. The light of a He-Ne laser (λ = 632.8 nm) is diffracted by the grating at the interface and starts propagating via TIR inside the waveguide. At a well-defined incident angle (α), the phase shift during one internal reflection equals zero (constructive interference) and a guided mode is excited, which generates an evanescent field penetrating into the covering medium. A change in refractive index at the surface as a consequence of a biorecognition reaction is monitored on-line by precise measurement of incoupling angle as a function of time with the operating software of the immunosensor system. This principle has been used to analyze sulfamethazine, by immobilizing the antibody on the surface of the chip and reaches a LOD of 10^{-8} M.

A similar system has been developed by the Centre Suisse d'Electronique et de Microtechnique (CSEM, Neuchâtel, Switzerland) [61–63]. The wavelength interrogated optical immunosensor (WIOS) also measures changes in the refractive index of the evanescent wave. In this case, the chip has an input and an output grating (Fig. 4). Light from a VCSEL laser emitting at around 763 nm is incident on a first grating and when coupling takes place, the waveguide mode is excited and propagates into the waveguide layer until the second grating where the light couples out

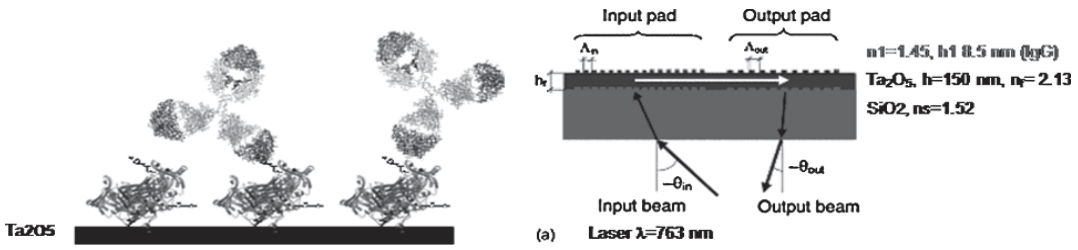

Fig. 4 Scheme of the WIOS system. The chip is a customized platform that contains 24 sensing sites. Each site is waveguide grating. Light from a VCSEL laser emitting at around 763 nm is incident on a first grating. The waveguide mode is excited and propagates into the waveguiding layer. The second grating couples out the guided light (at a different angle), which is collected with large plastic optical fibers. Electronics controls the laser wavelength modulation and amplifies the detected signals. A computer does the data acquisition and processing

of the waveguide (at a different angle) and is collected with large plastic optical fibers. Since the resonant coupling occurring at the first grating is also dependent on the wavelength (λr) of the incident light, an optical configuration has been envisaged that allows working at a fixed incidence angle (θ) while the wavelength at which coupling takes place is monitored through the output grating. This allows recording of effective index variations of the waveguide by continuously monitoring this parameter. On the basis of this principle, an immunosensor to detect sulfonamide antibiotic residues in milk samples has been developed. Immunoreagents appropriately produced to detect a wide range of sulfonamide antibiotic congeners [64] have been incorporated on the surface of the chip making use of a photopolymerizable dextran layer. The WIOS immunosensor is able to reach the necessary detectability to determine the most frequently used sulfonamide antibiotics used in the veterinary field, in compliance with the EC regulations. Thus, sulfapyridine can be detected in milk with a LOD value of 0.50 ± 0.12 and 0.24 ± 0.06 $\mu g\ L^{-1}$ and buffer samples, respectively. Other sulfonamides tested showed LODs below the MRLs values [65].

On a *Mach-Zehnder interferometer* the propagating light is split into two arms, one with the appropriate sensing layer and the other acting as a reference. The evanescent field of the measuring arm collects information regarding the bioreaction via the change produced in the refractive index. Consequently, the velocity of the wave in this arm varies. At the end recombination of the waves from both arms allows observation of a constructive or destructive interference, which is related to the extent of the bioreaction that has occurred on the sensing arm. Although there is intensive research in this field to our knowledge no applications to the development of biosensors for pharmaceuticals or emerging pollutants have been reported [57–60].

3.2 Plasmon Resonance Biosensors

The plasmon resonance is an evanescent electromagnetic field generated at the surface of a metal conductor (usually Ag or Au) when excited by the impact of light of an appropriate wavelength at a particular angle (θ_p). Surface plasmons are generated by electrons at the metal surfaces that behave differently from those in the bulk of the metal. These electrons are excited by the incident light, producing an oscillation (resonance) at different frequency from that in the bulk of the metal film. The absorption of light energy by the surface plasmons during resonance is observed as a sharp minimum in light reflectance when the varying angle of incidence reaches the critical value. The critical angle depends on the wavelength and polarization state of the incident light, but also on the dielectric properties of the medium adjacent to the metal surface and therefore is affected by analytes binding to that surface. This principle has been extensively investigated for monitoring of biological interactions in different types of configuration. We will provide herein some examples of late developments regarding detection of pharmaceuticals using this principle, however for additional information the reader is directed to some recent reviews (i.e., [39, 43, 66]).

Since distinct surface plasmon resonance (SPR) prototypes (Biacore, IASys, etc.) have appeared in the market, a significant number of applications of this principle have been reported during the last year. For example, in a recent paper surface plasmon resonance has been used to analyze 17β-estradiol (E2) in sewage in the coastal marine environment [67]. Using the commercial Biacore system, the small molecule E2 is immobilized on a CM5 sensor chip for an indirect competitive immunoassay. The LOD achieved was 0.445 μg L^{-1} although to analyze environmental samples, the SPR was couplet to a solid-phase extraction system [67]. Similarly, several examples of the use of SPR commercialized by Sensia to analyze environmental organic pollutants (DDT, atrazine, chlorpyrifos, and carbaryl) in natural water samples have been reported [46, 68–70]. BPA has also been analyzed using a SPR biosensor immobilizing a BPA-ovalbumin (BPA-OVA) conjugate on the Au thin film of the SPR sensor chip by just physical adsorption [71]. The lowest detection limit for BPA by SPR was around 1 ppb, almost the same as the LOD reached by ELISA (enzyme-linked immunosorbent assay) using the same immunoreagents.

An interesting example also reports the development of a novel surface plasmon resonance-based biosensor assay for the bioeffect-related screening of chemicals with thyroid-disrupting activity [72]. For this purpose two thyroid transport proteins (TPs), thyroxine-binding globulin (TBG) and recombinant transthyretin (rTTR), were applied in an inhibition assay format in a Biacore 3000 using CM5 biosensor chips coated with l-thyroxine (T4), the main hormone of the thyroid system. Known thyroid disruptors and structurally related compounds such as halogenated phenols, halogenated bisphenols, bisphenol A, 3,5-dichlorobiphenyl, and its hydroxylated metabolite 4-hydroxy-3,5-dichlorobiphenyl (4-OH PCB 14) were tested in this system to their relative potency (RP) with T4. The TBG-based assay was highly specific for T4, and the rTTR-based assay was sensitive toward several compounds, the highest sensitivity (RP = 4.4) being obtained with 4-OH PCB 14, followed by tetrabromobisphenol A (RP = 1.5) and tetrachlorobisphenol A (RP = 0.75).

Regarding antibiotic residues, a rapid and sensitive screening qualitative method using a surface plasmon resonance biosensor has been reported that detects all fenicol antibiotic residues in shrimps from a single sample extract [88]. This method requires ethyl acetate extraction followed by a single wash with isooctane/chloroform. Each sample extract is injected over the surfaces of two biosensor chip flow cells, one surface having the capability to detect florefenicol amine (FF amine), florefenicol (FF), and thiamphenicol (TAP) and the second surface for chloramphenicol (CAP) detection. The estimated detection capabilities (CCβ) were 0.1, 0.2, 250, and 0.5 ppb for CAP, FF, FF amine, and TAP, respectively. This quick, simple test allowed the detection of CAP residues in shrimps at the minimum required performance limit of 0.1 μg kg^{-1} for this compound and of FF, FF amine, and TAP below their maximum residue limits. Previously, detection of chloramphenicol and the mammalian metabolite chloramphenicol glucuronide (CAP-Glu) with the Biacore Q biosensor has also been reported. Honey, porcine kidney, and prawn extracts were analyzed after solvent extraction and solid-phase clean-up. Similarly, the determination of β-lactams using

Biacore has been reported, by immobilizing either an enzyme receptor [73] or a specific antibody [74]. In the first case, the specific recognition is based on the enzymatic activity of a carboxypeptidase converting a 3-peptide into a 2-peptide, a reaction that is inhibited in the presence of β-lactams. Antibodies were used to measure either the amount of formed enzymatic product or the amount of remaining enzymatic substrate. Different β-lactams were detected at or below European maximum residue limits. Thus, the LOD for penicillin G was 1.2 and 1.5 g kg^{-1}, depending on whether the 2- or 3-peptide was detected, respectively. The Biacore biosensor was also employed to detect β-lactams using a commercial antibody against ampicillin which had much higher affinity for the open β-lactam ring than for the closed ring. Two different pretreatments were tested prior to the biosensor assay to open the ring in order to increase the assay sensitivity. The LODs achieved for ampicillin in milk were 33 and 12.5 μg L^{-1} after enzymatic and chemical pretreatment, respectively. Conversely, penicillins G and M could be detected at or below their respective MRLs (4 and 30 μg L^{-1} in milk, respectively) and ampicillin and amoxicillin could be detected only at about the MRLs after chemical pretreatment.

A step forward in the use of plasmon resonant (PR) properties of materials is the use of particles for biological detection. Thus, colloidal gold or silver particles, typically 10–120 nm in diameter, are plasmon resonant. The particle geometric confinement of the surface plasmon gives rise to a spectral resonance in the light scattering that does not occur in thin films such as those used in the Biacore. The light scattered by nanometer-sized colloidal metal particles is dominated by the collective oscillation of the conduction electrons induced by the incident electric field (light), as mentioned above for the plasmon resonance of flat surfaces. However, in this case, the frequency band (specific color) of the scattered light is a function of the size, shape, and material properties of the particle. Thus, silver or gold nanoparticles, with diameters of 30–120 nm, efficiently scatter light in the visible spectrum. Variations in the refractive index around the nanoparticle surface also affect the resonance peak (frequency band of the scattered light), for which reason biomolecular interactions can be detected using nanoparticles (Fig. 5). This physical phenomenon is known as localized surface plasmon resonance (LSPR). An example of the application of this physical transducer principle to the analysis of an anabolic androgenic steroid such as stanozolol, has been recently reported [75, 76]. The chip could be fabricated in house by first activating the glass substrate with a silanizing agent possessing either a NH_2 or a SH group to bind the gold nanoparticles on the top of a flat SiO_2 (glass) surface without the observation of clusters. Following this, a stanozolol hapten-BSA conjugate was used to coat the gold nanoparticles and the detection of the hormone was made under competitive conditions by just mixing the sample with the antibody and adding a drop on top of the LSPR chip. The extinction spectra of the gold colloids were measured by conventional dark-field spectroscopy, illuminating the sample with a halogen lamp (100 W). The light scattered by the particles was analyzed with a microspectrometer and a CCD camera. Using this simple set-up stanozolol could be measured to a level below the minimum required performance level.

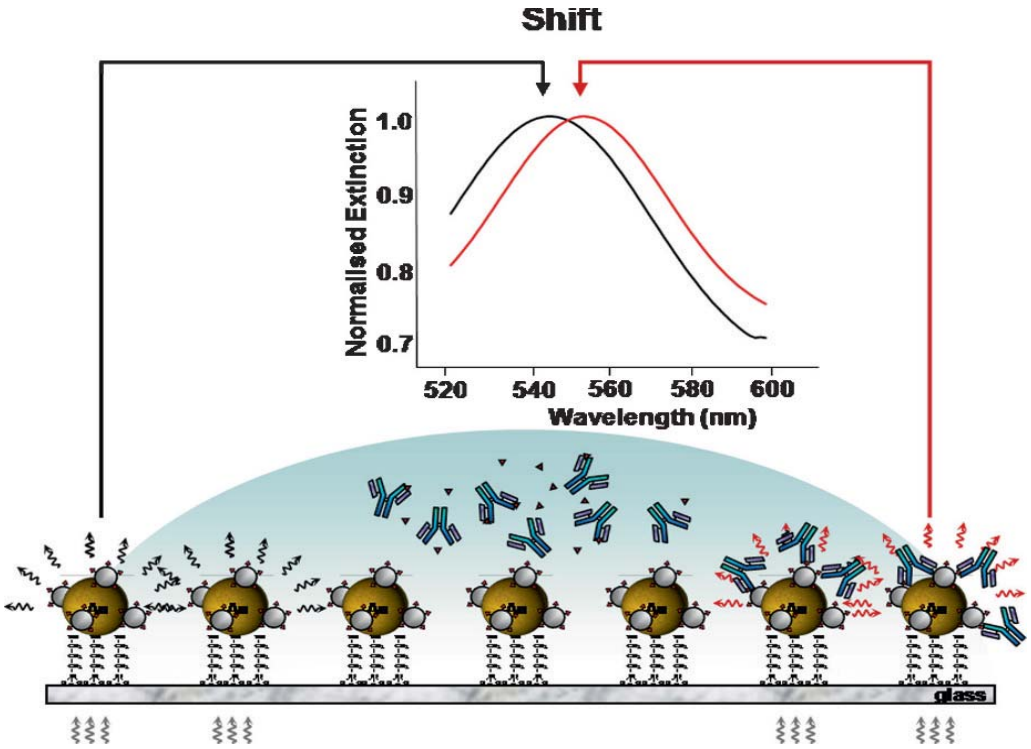

Fig. 5 Schematic of the chip using Au nanoparticles on top of a glass substrate used to detect stanozolol, and androgenic steroid used illegally to increase meat production or athletic efficiency in sport competitions. Specific binding of the antibody to stanozolol hapen-BSA coated on top of the nanoparticles, produces a shift in the resonance peak of the nanoparticles that has been used to detect this anabolic androgenic steroid. Reproduced from [75]

3.3 Nanomechanical Biosensors

Finally, biosensors based on microcantilevers (MCLs) have become a promising tool for directly detecting biomolecular interactions. Microcantilevers translate molecular recognition of biomolecules into nanomechanical motion that is commonly coupled to an optical or piezo-resistive read-out detector system. Biosensors based on cantilevers are a good example of how nanotechnology and biotechnology can go together. Microcantilever sensors rely on their deflection to indicate sensing. Thus, molecular adsorption onto the sensing element shifts the resonance frequency and changes its surface forces (surface stress). Surface stress due to conformation change of proteins and other polymers has been a recent focus of MCL research. Since conformational changes in proteins can be produced through binding of analytes at specific receptor sites, MCLs that respond to conformational change-induced surface stress are promising as transducers of chemical information and are ideal for developing microcantilever-based biosensors. On the other hand, high-throughput platforms using arrays of cantilevers have been developed for simultaneous measurement and read-out of hundreds of samples. Although many interesting applications in the environmental and clinical diagnostic field have been reported

(for recent reviews see [42, 77–80]), examples of their use to detect pharmaceuticals are still scarce.

4 Piezoelectric Immunosensors

Piezoelectrics are materials that may be brought into resonance by the application of an external alternating electric field. The frequency of the resulting oscillation is determined by the mass of the crystal. By coating a piezoelectric with an appropriate biomolecule such as an antibody, these kinds of immunosensors can, in principle, directly detect the binding of the corresponding analyte (for reviews see [81, 82]). Piezoelectric immunosensors may adopt two modes: (1) *Bulk acoustic (BA) devices* where adsorption of the analytes occurs on the coated surface of a piezoelectric crystal connected to an oscillator circuit. Resonance occurs on the entire mass of the crystal. If for example an antibody-coated crystal is placed in an atmosphere containing the selected analyte the immunoreaction will produce an increase in the mass of the crystal. The resonant frequency will therefore decrease according to the Sauerbrey equation: $\Delta f = -2.3 \times 10^6 f^2(\Delta m/\mathrm{A})$, where f is the oscillation frequency in Hz, Δm is the adsorbed mass in g, and A is the sensing area in cm^2. (2) *Surface acoustic wave (SAW) devices* where an acoustic wave moves just at the surface of the crystal. Mass loading on the acoustic path between two sets of electrodes will alter the phase wave velocity and cause a shift in the frequency. This technology can, in principle, detect binding events produced at the surface of the piezoelectric material.

Several examples can be found in the literature regarding the use of this type of sensor for environmental, food safety, and clinical applications. Liu et al. reported the detection of various polycyclic aromatic hydrocarbons (PAHs) by immobilizing the antigen (benzo[a]pyrene-BSA conjugate, BaP-BSA) on gold-coated quartz crystals, with a basic resonant frequency of 10 MHz, through thioctic acid [83]. The monoclonal antibody 10c10 (mAb10c10) allowed one to determine benzo[a]pyrene (BaP) in the flow injection system by using a competitive pattern, down to the nanomolar level. Similarly, a piezoelectric immunosensor system was developed for the rapid detection of polychlorinated dibenzo-p-dioxins (PCDDs) using a direct enzyme immunoassay format [84]. The purpose of using the enzyme was just to increase the mass of the binding molecule. The mouse monoclonal antibody against 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) was deposited on a 10-MHz AT-cut quartz crystal resonator modified with a self-assembly monolayer of dithiobis-N-succinimidyl propionate. Competition took place between the analyte and a conjugate of a dioxin-like competitor coupled to the enzyme horseradish peroxidase. Measurement of the frequency responses allowed detection of 2,3,7,8-TCDD in the concentration range of 0.01–1.3 ng mL^{-1}. In another example BPA was detected down to 10 ng mL^{-1} using antibodies coupled to nanoparticles, approximately 200 nm in diameter, with the aim to increase the mass change on the surface of the immunosensor and thereby increase the frequency shift detected [85]. PCBs have also been detected using a piezoelectric immunosensor, by immobilizing a polyclonal antibody on a gold electrode via protein A. Detection in the range of 1–50 ppm was achieved using either

enzyme or polystyrene particles to increase the response of the sensor [86]. As a representative example of the use of this principle to analyze pharmaceutical residues, Park et al. reported the direct detection of chloramphenicol by covalently immobilizing specific antibodies onto the gold electrode surface of piezoelectric crystals using a self-assembled monolayer, obtaining a significant shift of the resonance frequency in about 10 min [85]. However, the detectability achieved was much worse than in those formats where a competitive configuration was used.

5 Conclusions

Future advances in biosensor development will need scientists from different disciplines combining their research efforts. First, improvement of transducer technology is necessary in order to allow direct detection on environmental samples and to confer the necessary flexibility to develop field analytical methods. Frequent new micro/nanotechnological advances are opening up new and exciting possibilities that should be investigated in order to find novel ways to convert biomolecular recognition events into electrical or optical signals. Second, increasing the number of analytes that can be measured using the biosensor technology is necessary. Especially interesting are those compounds that are difficult to measure using currently accepted methods. Antibody-based biosensors continue to show great potential due to the excellent features of this type of biomolecule (stability, chemical homogeneity etc.) and the capability to produce them against almost any type of analyte, which may broaden the number of compounds that can be analyzed. This versatility of the immune system combined with the progress that recombinant DNA technology is making in this field may increase the potential of these bioreceptors. A third trend should be developing integrated units able to perform multianalyte measurements. And finally the extensive research that is taking place in this area will not give the expected fruit without performing rigorous validation studies with environmental samples. All these facts will contribute to improving investment by private companies to finally deliver reliable devices into the market.

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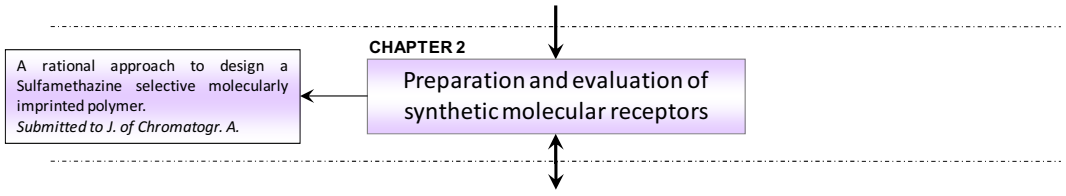
2 ARTIFICIAL MOLECULAR RECEPTORS



The molecular imprinting method is quite simple, cheap and easy to perform in a tailor-made fashion. As it will be described later (see **Section 2.1.1.1**), all we need are functional monomers, templates, solvents and crosslinking agents. Polymerization is followed by the removal of the template obtaining well-defined supramolecular structures and satisfactorily showing both high selectivity and high binding activity towards the target guest compound. On the other hand, rational approaches for the preparation of MIPs based in combined data from computational chemistry models and ^{1}H-NMR experimental data can help us to select the most suitable reagents and their concentrations. Subsequent application of this information on experimental design and multivariate analysis methods make closer the possibility to obtain a final material with good imprinting efficiency.

The analytical challenge associated with these synthetic recognition materials to define their real potential is obvious from the large number of European sponsored projects on this subject. In this manner, an important effort has been focused in developing alternative methodologies for sample purification and preconcentration. After all these considerations, our research group was encouraged to start a new investigation line in 2000 with the idea to establish a general optimized procedure to obtain molecular imprinted materials based on a previous rational design. Considering all these points, during this thesis we have pretended to develop a general methodology, going from the theoretical calculations to the experimental studies, in order to select, from a large collection of MIP combinations for a specific template, the ones with better application properties. This proposal has been proved with MIPs for SAs using SMZ, which is one of the most antibiotic residues found in milk samples, as the template.

2.1 Preamble

When this work started, the number of original papers published related to MIPs had increased considerably in comparison with previous years [1]. In particular, there were few MIPs reported for SAs based on trial an error experiments always on a very similar way of preparation independently which is the target analyte [2-5], but none of them following an optimized procedure to obtain molecular imprinted materials based on a previous rational design. Thus, we have pretended not only to obtain an imprinted material with a certain affinity for its template, but also rationalize the basis about how to design, prepare and evaluate MIPs with high affinity being useful for different application fields.

In this sense, first attempts were made in our group by Dr. Mª Carmen Estevez who prepared a MIP for irgarol, which is a pesticide acting as a highly specific and effective inhibitor of photosynthesis. That investigation helped the group to establish general conditions for MIPs rational design, preparation and evaluation. Subsequently, Dr. Juan Pablo Salvador was able to develop a MIP for testosterone proving that the methodology previously defined for irgarol was robust enough to be successfully applied independently of the template used [6]. The present thesis continues this research line by developing new molecular imprinted materials for antibiotics, focusing in SAs, and trying to improve the entire process.

2.1.1 Non-covalent MIPs for sulfonamides

From all the SAs congeners, SMZ was selected as the template to prepare molecular imprinted polymer for being the most commonly drug used in veterinary medicine to treat mastitis, respiratory tract infections and gastrointestinal diseases. From the imprinting strategies available it has become evident that the use of non-covalent interactions between the print molecule and the functional monomers is the more versatile. The apparent weakness of these interaction types, when considered individually, may be overcome by allowing a multitude of interaction points simultaneously. Together with the fast association and dissociation kinetics of these bond types, so that in a short time many possible combinations can be checked before the correct partners associate, this protocol has proven advantageous. Furthermore, the use of non-covalent interactions in the imprinting step closely resembles the recognition pattern observed in nature [7].

2.1.1.1 Molecular imprinting reagents

In a non-covalent approach all the chemicals we need are functional monomers, templates, crosslinking agents, initiators, porogens for the polymerization and solvents to

remove the templates from the polymers. All components are essential in obtaining a final polymer with appropriate properties to become a specific receptor. The template induces a certain organization of monomers around it, through the complementary interaction of the functional groups of one and other. To maintain the molecular aggregates between template and monomers a crosslinker is required. This is the major component of the mixture being responsible for the structure of the polymer giving strength and rigidity among other properties. Thus, we get any specific locations previously created will not disappear once remove the template and retain their spatial arrangement (see **Figure 2.1**).

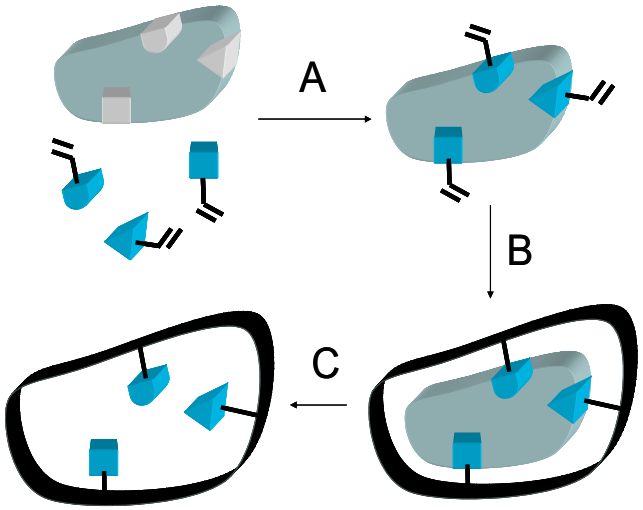


Figure 2.1: Schematic illustration of molecular imprinting. A: Interaction process between template and monomers; B: Polymerization of this monomer-template conjugates (or adduct) in presence of the crosslinking agent; C: Removal of the template from the polymer.

Main components evaluated to prepare sulfonamide MIPs were:

A) Template. Target of interest needs to have functional groups susceptible to establish the higher amount of interactions with the higher number of monomer units in order to create acute specificity cavities [8]. In our case, the SMZ compound is characterized by an aniline group and the sulfonamide bond, besides the two nitrogen atoms present in the heterocyclic ring, being all possible interaction points. Moreover, the methyl substituents can define a cavity in the polymer big enough to be accessible for the heterocycles of most commonly used SAs.

B) Monomers. For non-covalent imprinting, vinyl monomers bearing appropriate functional groups have been designed and synthesized [9] being most of them commercially available (see **Figure 2.2**).

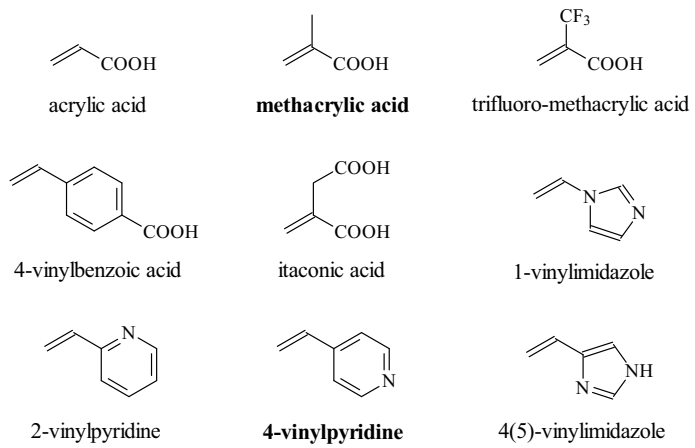


Figure 2.2: Typical monomers used to prepare molecular imprinted polymers.

SMZ template provide both lightly acid (-SO_2NH-) and basic (-NH_2) functional groups which means that acid and basic monomers should be considered to obtain interactions between these components. Carboxylic acid present in methacrylic acid (MAA) can establish bond hydrogen interactions (hydrogen donor) and work as a hydrogen transfer (Bronsted acid/basic interaction) while 4-vinylpyridine (VPY), as a good hydrogen acceptor, can be a complementary system in acid-basic relations besides been able to form π-π interactions between complementary aromatic systems.

C) Crosslinkers. The crosslinking agent is the majority component of the reaction mixture being the responsible of polymer structure configuration and contributing hardly in its physical and mechanical properties [10].

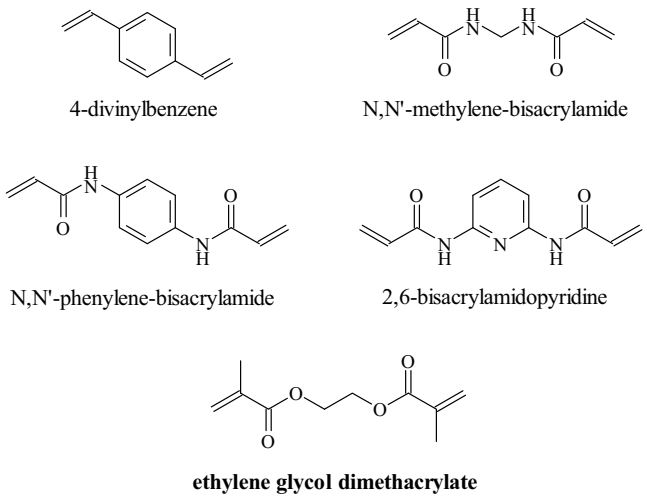


Figure 2.3: Typical crosslinking agents used to prepare molecular imprinted polymers.

For efficient imprinting, the reactivity of this compound should be similar to that of the functional monomers. By choosing an appropriate crosslinker (see **Figure 2.3**), random co-polymerization occurs successfully, and the functional residues derived from the monomers are uniformly distributed in the three-dimensional polymer network. For molecular imprinting in organic solvents, ethylene glycol dimethacrylate (EGDMA) are often used with good results.

D) Porogen. The porogen has the important role in solubilising the template, blending the polymerisation mixture, disperse the heat generated during the polymerization reaction [11] but also creating internal cavities inside MIP structure where will be placed or distributed all possible receptor points formed (see **Figure 2.4**).

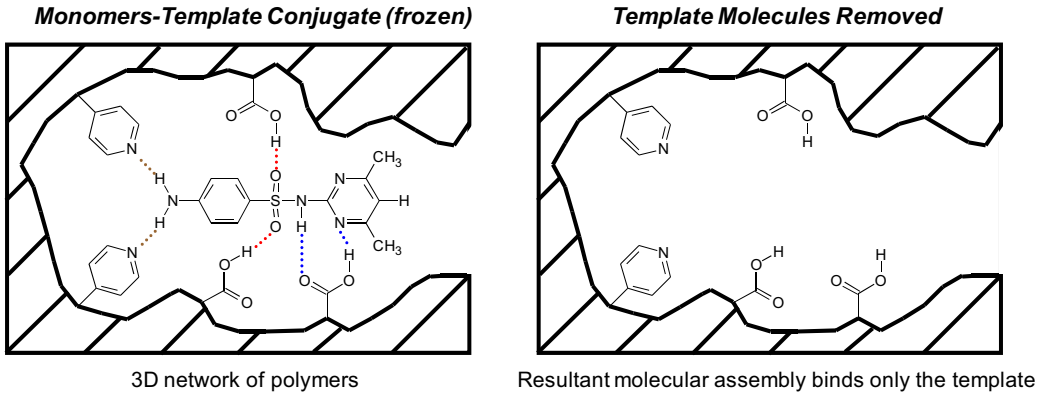


Figure 2.4: Example of non-covalent imprinting.

Potential porogens like acetonitrile (ACN) dimethylformamide (DMF), dioxane, MeOH, EtOH and H_2O + 2-propanol were tested to evaluate their influence in final imprinted polymer properties. Others, like CH_2Cl_2, CH_3Cl, toluene, AcOEt and Cl_3CF were finally discarded because they could not dissolve completely the template in the mixture proportions assessed. Moreover, solvent volume in MIP preparation has to be enough to keep all the components homogeneous in solution before polymerisation and in a high concentration. Thus, final polymer porous structure depends on solvent used and its proportion against amount of polymer prepared.

E) Initiation. Polymerization can be initiated by using thermal or UV decomposition of radical initiators (see **Figure 2.5**). The initiation radicals formed by the decomposition, attack the monomer, producing the propagating radicals. These reactions are very simple and economical, however it's important to remove molecular oxygen from polymerization mixtures (i.e. degassing with nitrogen or argon, as well as freeze-and-thaw cycles under reduced pressure), since it traps the radical and retards or even stops the polymerization procedure [11]. AIBN is the radical initiator most used to activate

polymerization process due to its moderate stability, half life time properties and reasonable thermal activation conditions (65 ºC).

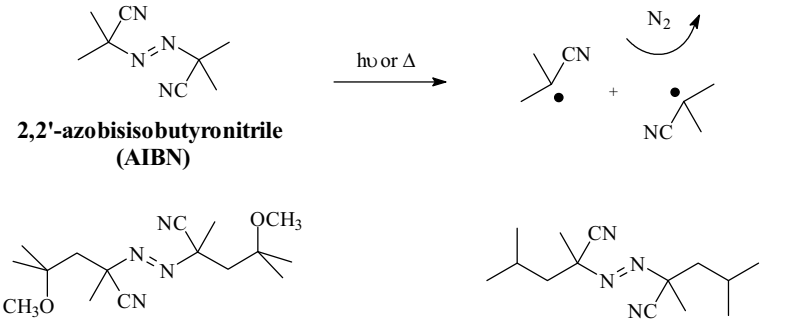


Figure 2.5: Typical free radical initiators used in molecular imprinting. AIBN most characteristic radical reaction is a thermal decomposition, eliminating a molecule of nitrogen gas (N_2) to form two 2-cyanoprop-2-yl radicals.

Under our experience, it was enough to use 1 % of molar initiator radicals respect double bonds present in the mixture. Free radical reactions are little affected by the presence of acids or bases or by changes in the polarity of solvents. This allows molecular imprinting to be carried out under a variety of different conditions.

Thus, MAA and VPY monomers, EGDMA cross-linker and AIBN initiator were selected as the most suitable components to prepare SMZ MIPs by means of non-covalent interactions and temperature induced polymerisation in different solvents.

The present chapter describes the work performed in relation to the **Specific Objective 1** of this thesis (i.e. preparation and evaluation of specific receptors such as MIPs) in respect to SAs. Taking into account that the analytical challenges associated with these synthetic recognition materials is obvious for being used as alternative methodologies for sample purification and preconcentration, the main objective here was a) to develop a methodology for the rational design of MIPs, b) to establish a methodology to select, from a large collection of MIPs prepared, the ones with better application properties, and c) to obtain a MIP able to interact selectively with SMZ.

2.2 A rational approach to design a SMZ selective MIP

Submitted to J. Chromatogr A

A rational approach to design a Sulfamethazine selective molecularly imprinted polymer

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ABSTRACT

A rational development of a molecular imprinted polymer (MIP) for sulfamethazine (SMZ) is described. Theoretical computational and experimental ^{1}H-NMR studies about complex formation between the template and different functional monomers were performed as a previous step to a MIP preparation. A good correlation between both techniques was found offering preliminary information about most convenient reagents and pre-polymer mixture composition to be used in order to obtain a suitable imprinted material. Synthesis on a small scale of a variety of MIPs, using monomers previously selected in the rational design by changing the porogen and mixture proportion, were prepared and evaluated by diverse molecular recognition experiments. This methodology pretends to select, from a large collection of MIPs prepared, the ones with better application properties. It is based on several assays, increasing the complexity, having fewer candidates in each step and with enough efficiency for being introduced in a combinatorial strategy for new MIP development with appropriate affinity and selectivity properties. Finally, affinity and specificity of the MIP not excluded in previous experiments, using SMZ as the template prepared with methacrylic acid (MAA) and 4-vinylpyridine (VPy) in acetonitrile (ACN), were evaluated by frontal chromatography and batch rebinding experiments.

1. Introduction

Antibiotics are chemical substances extremely active at low doses that kill or slow the growth of bacteria. Sulfonamides have been identified between the four antibiotic families most used in human and veterinary medicine treatments for prophylactic and therapeutic purposes. In the last decade, the irresponsible use of antibiotics to prevent diseases derived from the intensive farming is favouring the growth of bacterial resistance [1-5]. This phenomenon is becoming at dangerous levels because residues of these antimicrobial substances could also enter to the human food chain causing drug allergies and favouring the raising of bacterial resistant lines directly related with humans that can no longer be treated in humans with presently known drugs [6-10].

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Thus, the presence of sulfonamides or their derived metabolites in meat, milk or dairy products is considered potentially harmful for the consumer [11,12], so an important concern exists regarding the effects of these residues in the public health and in the economy of these food processing industries.

At present, the European Union has not only established maximum residue limits (MRLs) allowed for these types of compounds in different food and feed samples (Council Regulation 2377/90/CE [13]) but also procedures for inspection dictating the frequency and number of substances that have to be monitored (Council Directive 96/23/CE [14]). On the other hand, analytical procedures available today do not reach these latter requirements, so new techniques need to be developed to solve this deficiency. The main bottleneck in chromatographic methods, such as HPLC, is sample preparation and purification, especially with those from biological or environmental origin. Extraction procedures based on selective stationary phases can be very interesting to isolate specific compounds. In the recent years,

molecular imprinting has become a newly developed methodology which provides molecular assemblies of desired structures and properties [15,16].

Molecular imprinted polymers can be described as artificial recognition systems (receptors), trying to mimic nature, and normally based on non-covalent forces [17-19]. A MIP is produced by polymerisation of a solution containing functional monomers, a cross-linker and a template, which is the target compound desired to be recognized in the future. Before polymerisation process, the functional monomers interact with the template by e.g. hydrogen bonding, electrostatic interaction, and coordination-bond formation, depending of the case in hand. Subsequently, the monomers are cross-linked *in situ* to freeze the possible molecular complexes formed When template is removed from the polymer, the final material contains specific cavities which can selectively bind compounds very similar in structure, with regard to shape and functionality, to the template used [18,20]. The final affinity and specificity of the polymer obtained towards the target molecule are dependent on the choice of cross-linker and functional monomers, the solvent that is used as a porogen and proportions of all components used in the MIP preparation [21].

Working within this frame of knowledge the researcher is still faced with many options before deciding on which chemicals to choose for preparing a MIP with good affinity and specificity properties towards the template. Any MIP preparation usually requires a lengthy process of experimental trial and error before finding the optimal recipe. To circumvent as much as possible this situation, computational methods and proton NMR model studies can be used to obtain preliminary information about possible complex formation between the template and potential functional monomers [22,23]. This may provide useful information for a more directed choice of MIP ingredients, which in turn should lead to better and faster results. Thus, the work described in this paper shows a rational development of a MIP, following a non-covalent approach, using SMZ compound as the template.

2. Experimental

2.1. General Methods and Instruments

^{1}H NMR spectra were obtained with a Varian Inova-500 (Varian Inc., Palo Alto, CA) spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). Data obtained was analyzed with GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA). HPLC analyses were performed with a Merck Hitachi pump L-7100, a diode array detector L-7455, an auto sampler L-7200, and an interface D7000 (Merck, Darmstadt, Germany). The chromatograms were processed with HSM software (Merck, Darmstadt, Germany). The detection wavelength was set at 265 nm. A C18 Purosphere column (15 cm x 4.6 mm, 5 µm particle size) (Merck, Darmstadt, Germany) was used in all samples analysis. The oven employed for the polymerization was from Digitronic (JPSelecta, Abrera, Spain). After the polymerisation, mill process was performed with a Mixer Mill MM200 (Retsch GmbH, Haan, Germany). The sieving used was supplied by SieveShaker As200 basic (Retsch GmbH, Haan, Germany).Antibiotics are chemical substances extremely active at low doses that kill or slow the growth.

2.2. Material and chemicals

Glass vials of 20 mL (Height: 45 mm; diameter: 27 mm), with silicone septa, from Perkin Elmer, were used as the polymerization reactors. Glass vials of 3 mL (Height: 30 mm; diameter: 16 mm) from Trallero & Schlee were used for batch rebinding experiments. Omnifit columns (6.6 mm x 100 mm) were supplied by Omnifit (Cambridge, England) while stainless steel ones (4 mm x 50 mm) came from Teknokroma (Barcelona, Spain). SMZ and sulfapyridine (SPY) were provided by Riedel-de Haën. MAA, VPy and ethylene glycol dimethacrylate (EGDMA) were purchased from Aldrich (Milwaukee, US). Monomers and the cross-linker were distilled before being used to separate the radical inhibitor present. The radical initiator, 2,2'-azobisisobutyronitrile (AIBN) supplied by Fluka (Neu-Ulm, Switzerland) was recrystallized from diethyl ether prior to use. Chemical compounds used for "chromatographic retention studies" were obtained from Sigma Chemical Co (St. Louis, MO). Solvents (MeOH, ACN) were obtained at HPLC grade from Merck (Darmstadt, Germany).

2.3. Molecular modelling and theoretical calculations

Computational methodology to study interactions between potential monomers and SMZ, for obtaining theoretical geometries and electronic distributions of possible clusters formed, were carried out using the Hyperchem 6.03 molecular modelling software package (Hypercube Inc, Gainesville, FL). The semi empirical quantum model (PM3), which is a good model for hydrogen bonding, was used without considering solvents effects, to calculate the molecular geometries and electronic properties. Molecular cluster geometries were determined by a usage directed method instead a random search. Different starting geometries of separated monomers to the template were evaluated to locate possible molecular interaction points between them, calculating in all cases the enthalpy of reaction for each cluster formation. Final combination of binary complexes previously originated was studied to obtain new complexes with more components being more stable than the initial cluster.

2.4. NMR Interaction Study

Titration experiments recording NMR spectra of monomer-template mixtures, by systematic increments of monomer concentration, were investigated to determine optimal MIP composition. Template concentration used was 10 mM while concentration range of monomers ranged between 100 mM to 1.25 mM in either ACN, MeOH and acetone deuterated solvents. Acquisition of ^{1}H chemical shifts are in ppm, relative to 0.05 % TMS (v/v), at 25 ºC.

2.5. Sulfamethazine Analysis by HPLC

Calibration graph for the determination of SMZ was constructed over a concentration range of 0.5 µg mL^{-1} to 100 µg mL^{-1} using SPY as an internal standard (10 µg mL^{-1}). Linear correlation coefficient was 0.999 with a very high reproducibility in area relationship.

2.6. MIPs Preparation

SMZ imprinted polymers were prepared with MAA and VPy as functional monomers and EGDMA as cross-linker using different porogen solvents (ACN, dimethylformamide (DMF), dioxane, EtOH and water/2-propanol). In each case, the total amount of polymerization was 1g while solvent volume tested was 1, 2 and 4 mL. The molar ratios of template, functional monomers (MAA/VPy), cross-linker and initiator were 1:3:3:24:0.27, respectively. Correspondent non-imprinted polymers were parallel and simultaneously prepared. Polymerization mixture was purged by several cycles of argon and vacuum to eliminate the oxygen present in the reaction media. To activate the polymerization process vials were introduced to an oven heated to 60 ºC overnight. Once MIPs were obtained possible residual porogen was removed by evaporation to dryness.

2.7. MIPs Evaluation

Static desorption of template, In this experiment, we added 20 mL of ACN to the glass vials, where MIPs have been previously prepared and dried, and were sonicated for 1 hour. Then, we left them overnight to reach equilibrium being the supernatants then analyzed to evaluate indirectly the amount of template retained in the MIP. This process was repeated two more times with the same vials. For non-imprinted polymers (NIPs), the ACN used was spiked with the same amount of SMZ used in MIP preparation to obtain preliminary and qualitative information about non-selective retention of the template by the NIP.

Dynamic desorption of template (column wash experiment). Omnifit columns, packed with polymer particles (250 mg) of a new batch of MIPs milled, sieved but not washed, were coupled to the HPLC pump at a flow of 0.5 mL min^{-1} of ACN. Eluate fractions (5 mL) were collected and analyzed by HPLC to study dynamic desorption of the template. NIPs were not assessed in that type of experiments.

Chromatography selective retention. Homogeneous polymer particles (20-40 µm) were again packed into Omnifit columns, coupled to the HPLC pump, to remove the template. Washes consisted of 50 mL ACN and 50 mL of a 5 % acetic acid in MeOH (2 cycles) finishing with 50 mL MeOH and 50 mL ACN (1 cycle). Finally, MIP and NIP columns were coupled to the HPLC system with an UV detector (265 nm) to be used as stationary phases using ACN as solvent at a 0.3 mL min^{-1} flow rate. Chromatographic retention of SMZ, related and other non-related compounds were evaluated in that experiment. Relative capacity factor (Ki) was determined in accordance with standard chromatographic procedures [24]. The void volume was calculated using acetone as a non-retained marker.

2.8. MIP Characterization

Physical. Available surface and pore size of some polymers was determined by nitrogen absorption porosimetry using a ASAP 2000 (Micromeritics Inc.) instrument using BET and BJH models for the analysis of the nitrogen absorption isotherms.

Chemical. Final polymer chemical characterization was performed by two different methodologies evaluating and comparing all binding isotherms obtained. In the first approach, MIP/NIP columns were coupled to the HPLC system with an UV detector (310 nm) to evaluate their affinity and capacity properties by frontal chromatography [25,26] using ACN at a 0.3 mL min^{-1} flow. Manual injector was modified introducing a big injection loop of 25 mL. Different concentration solutions of SMZ (1 mM, 0.5 mM, 0.25 mM, 0.125 mM, 0.0625 mM) were loaded to the cited loop and injected to the chromatographic system. With that experiment configuration, sigmoid curves from zero absorbance to a saturation value of receptor points were obtained. The void volume was calculated using cyclohexanone (2 mM) as a non-retained marker. In the second approach, 50 mg of processed MIP/NIP selected, were introduced in 3 mL glass vials together with 1 mL of different SMZ concentrations (range from 30 to 500 µM) in ACN to perform a batch rebinding [27,28]. Glass vial contents, stored at room temperature for 24 hours, were transferred into centrifuge tubes to separate the solid particles. Free SMZ, present in supernatant solutions, was determined by a HPLC.

2.9. Study of Reproducibility

Reproducibility of MIP preparation was studied, in terms of extraction data, from two different points of view: reproducibility between different MIP batches and reproducibility in the same MIP. A new batch of ten MIPs, using 2 mL of ACN as a porogen in their preparation, were grounded, sieved and subsequently evaluated in parallel in this experiment. Glass vials, containing 1 gram of each MIP together with 20 mL ACN, were sonicated for 1 hour at room temperature. Supernatant was analyzed by HPLC to quantify free SMZ. Similar methodology was used in the second approach, where 50 mg of polymer particles from the same batch were analyzed as described above for different MIP batches reproducibility assays.

3. Results and discussion

3.1. Molecular Modelling and Theoretical Calculation

Computer assisted molecular modelling tools have been used in the MIPs field to obtain information on the most stable cluster structure, its relative composition and distribution of all components in the space [29]. Target analyte chosen was SMZ characterized by an aniline group (lightly basic) a sulphonamide bond (lightly acid) and two nitrogen atoms present in the heterocycle, being all of them potential interaction points. MAA and VPy would be selected as the most suitable monomers able to interact with the template using simple chemical intuition. Carboxylic acid present in MAA can establish bond hydrogen interactions (hydrogen donor) and work as a hydrogen transfer (Bronsted acid/basic interaction) while VPy, as a good hydrogen acceptor, can be a complementary system in acid-basic relations besides been able to form π-π interactions between complementary aromatic systems.

In this work we calculated the formation enthalpies of numerous complexes (ΔH_{sm}) as a result of interaction between the selected monomers and the template functional groups. The enthalpies of formation of SMZ (ΔH_s) and monomers (ΔH_m) were also calculated at their minimum energy geometry which corresponds to the most populated molecular conformation. The

enthalpies of reaction for all situations were calculated as follows: $\Delta\Delta H_r = \Delta H_{sm}-(\Delta H_s+\Delta H_m)$. Finally, two clusters were selected, based in energetic terms, from all possible molecular interactions calculated where polymer components (SMZ:MAA:VPy) molar ratios were 1:2:3 and 1:3:2. Results corresponding to the most favoured cluster and its space distribution are showed in Fig. 1.

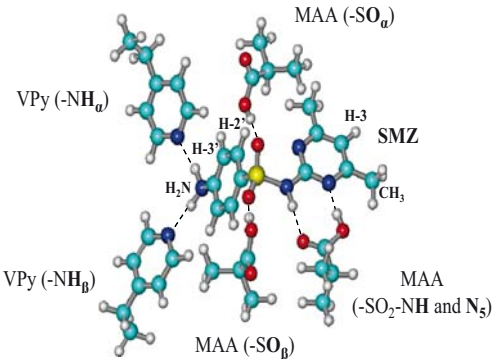

Fig. 1. Model of the most stable cluster of SMZ-MAA/VPy, calculated using PM3 semiempirical method (carbon, oxygen, sulfur, nitrogen and hydrogen are presented by turquoise, red, yellow, blue and grey spheres respectively).

Surface of the minimal box enclosing that cluster was also calculated by the theoretical model to make estimation about cross-linker amount necessary to surround all the structure. Most suitable molar proportion found between SMZ and EGDMA was around 1 to 20-25 approximately.

The first calculation results were surprising because they were contrary to the simple chemical intuition, because the pyridine monomer interacted with the aniline hydrogens and the monomer acid with the sulfonamide region. Then, we decided to modify the calculation models and geometry optimization strategies but obtaining in all cases similar results as before. Thus, we accepted those results as valid instead those based in chemical intuition. It is important to keep in mind that strictly calculations using explicit solvent molecules would be necessary to accurate calculations of the template:monomers affinity That estimation is complex to perform because high calculation effort is required. By that reason, we performed the calculations considering that the molecules interact in vacuum, which means no other molecules took part in the process. On the other hand, we considered that modelling calculations carried out could contribute with valuable information about polymer components to be used in the final MIP preparation and a good approximation to the potential molecular clusters, but they could not replace experimental data. Thus, computational results were complemented with those from an experimental technique, such as the nuclear magnetic resonance (NMR) titration experiments.

3.2. NMR Interaction Study

The nuclear magnetic resonance spectroscopy is usually used to characterize organic compounds but can also be a good system to evaluate and study molecular associations. Advantage of NMR, with respect to other techniques, is providing experimental data about template-monomer affinity in a specific media being very useful to decide, not only, which monomers can be more appropriate for MIP preparation, but also the porogen where cluster/s formed can be more stabilized. The method depends mainly in cluster half time life with respect to the observation in NMR timescale. If final cluster association/dissociation kinetics is fast enough, chemical shift changes of the signals can be observed, obtaining molecular affinity information [21,30]. In the case of template-monomers associations, molecular clusters formation has a short life which means that their signal could not be directly observed. However, it induces a change in the average of chemical shifts which depend on the actual concentration of the active species related by the affinity constant and their initial concentrations in the reagent mixture. On the other hand, the chemical shift changes could be related with the interaction point between the species in relation with the relative amount of the interacting species, because in general hydrogens close to the interaction point are more affected than the distant ones.

Results showed chemical shifts displacements due little modification of proton surroundings in the cluster. If graph representation of these displacement values (Δδ) obtained against template-monomer ratio used, in each case, can be adjusted to a Langmuir binding model, and then molecular association parameters could be calculated. First qualitative evaluation of the results obtained, when deuterated ACN was used, showed significant differences of some SMZ ^{1}H chemical shifts depending of the monomer used (see Fig. 2).

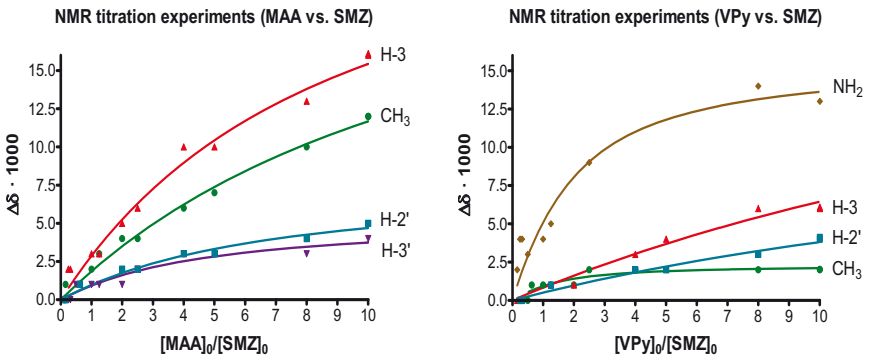

Fig. 2. Chemical shift variation (Δδ) of SMZ proton signals according to different monomer concentrations in NMR titration experiments.

Hydrogen signals corresponding to the heterocycle (-CH_3 and H-3) were more affected when MAA monomer was introduced showing that interaction between this compound with SMZ is close to that cycle area. When VPy monomer was studied, the SMZ free amino group (-NH_2) proton chemical shifts suffered a significant displacement compared to other template signals. It means that this particular monomer interacts preferably with the aniline structure, affecting indirectly to the global electronic system of the molecule, what produced little changes in proton signals corresponding to the heterocycle (-CH_3 and H-3). These results obtained matched very well with those of the molecular modelling calculation. Binding model fitting, of protons more affected in the experiment, were used to calculate association constants for each template:monomer relation to design final polymerisation mixture ($Ka_{SMZ/VPy}$ = $2.8 \cdot 10^3$ M^{-1} & $Ka_{SMZ/MAA}$ = $5.9 \cdot 10^2$ M^{-1}). Theoretically, optimal excess of monomers can be found in the binding isotherms where the slope is close to 1 just because from this point on an increase of monomer is translated in more non-selective binding points and not into more cluster formation. From all calculations used in sulfamethazine MIP rational design it was necessary at least 3 equivalents of MAA and VPy monomers. The experimental NMR approach performed validates the theoretical work previously developed indicating that the joint use of the two methodologies can be an interesting tool in future rational design of MIPs.

Several examples of SMZ MIPs have been also published in the literature [32-37]. Although some of the preparation procedures described are similar in some way (e.g. the use of MAA and VPy monomers together or separated or ACN solvent used as porogen), the authors have followed a trial and error approach. The best MIP compositions found were similar to those derived from our rational approach, thus, the methodology proposed here could be a powerful tool in the design of molecularly imprinted polymers making easier the selection of the MIP mixture in order to be successful for obtaining an imprinting polymer with good selective retention properties faster. The improvement of our model is based on the simultaneous consideration of simplified computational methods and NMR experimental binding assays. The two approaches are complementary and help to better understand the mechanism under the template – monomers cluster formation and stability at least in a semi-quantitative way. It consider not only the template – monomer but also the solvent what is an improvement in respect other models.

3.3. ACN MIPs Preparation

Regarding to theoretical calculation and NMR experiments, polymerization was carried out with the ratio 1:3:3 (SMZ/MAA/VPy) for molecular interaction using 24 equivalents of the cross-linker selected (EGDMA) to obtain a three-dimensional net. The cross-linker agent is the majority component of the reaction mixture being the responsible of polymer structure configuration and contributing hardly in its physical and mechanical properties. EGDMA has been used in numerous works published with good results [31]. The porogen (ACN) has the important role of dissolving the template, blending the polymerisation mixture but also creating internal cavities inside MIP structure where will be placed or distributed all possible receptor points formed. The AIBN was the radical initiator used to activate polymerization process due to its stability, half time life properties and reasonable thermal activation conditions (65 ºC). Under our experience, it was enough to use 1 % of molar initiator radicals respect double bonds present in the mixture. Common amount of polymer obtained in previous works published (~ 5 grams) was reduced to 1 gram expecting few variations in MIP properties. That scale change was proposed in order to prepare large MIP batches to easily explore, and with low cost, numerous polymer mixture combinations, which improves possibilities to locate a MIP with good properties independently of the rational design methodology performed. Normally, after polymerization process, polymers were milled, sieved to have a particle size between 20-40 µm, packed in chromatographic columns and hardly washed for further experiments. Additionally, preparation and NIPs processed was the same that for the MIPs to be compared.

3.4. Determination of Porogen Proportion in MIP Prepolymer Mixture

Besides using ACN solvent for MIPs preparation, previously studied in NMR experiments, several potential porogens like DMF, dioxane, MeOH, EtOH and H_2O + 2-propanol were also tested to evaluate their influence in final imprinted polymer properties. Others, like CH_2Cl_2, CH_3Cl, toluene, AcOEt and Cl_3CF were finally discarded because they could not dissolve completely the template in the mixture proportions assessed. In MIP research area, it's usual to work with organic solvents where hydrogen bond interactions are most favoured. Nowadays, there is a big interest in working with aqueous media in favour to perform green chemistry and to work with hydrophobic type of interactions [38] so aqueous media (H_2O + 2-propanol) were also tested as a porogen.

Solvent volume in MIP preparation has to be enough to keep all the components homogeneous in solution before polymerisation and in a high concentration. Final polymer porous structure depends on solvent used and its proportion against amount of polymer prepared (1 gram). Different porogen volumes (1 mL, 2 mL and 4 mL) were used to prepare a batch of polymers to evaluate final material characteristics and properties obtained in each case. Depending on proportion used in MIP preparation, the void volume and the total volume in the final MIP changes, which was determined in all cases. MIP prepared with 1 mL g^{-1} (2 mL of final mix volume) yielded 0.75 mL of polymer, the one prepared with 2 mL g^{-1} (3 mL of final mix volume) yielded 1.95 mL of polymer while the last prepared with 4 mL g^{-1} (5 mL of final mix volume) yielded 4.65 mL of polymer.

Battery of MIPs/NIPs using solvents and proportions cited above were prepared obtaining very similar external appearance in all cases except the ones prepared with water + 2-propanol. After being processed, polymers prepared with 1 and 2 mL g^{-1} proportion presented a more rigid structure in comparison to 4 mL g^{-1} ones which seemed more spongy and weaker. Then, several polymers were selected to characterize their internal structure by nitrogen absorption porosimetry observing that both solvent nature and proportion used during MIP preparation influence clearly in the material internal structure. Polymers prepared with more polar solvents or with less proportion of porogen, yielded the internal channels structures with less

diameter porous and porosimetry (i.e. meso porous volume). Polymers prepared with ACN showed intermediate porous properties (1 mL/2 mL ACN MIPs = surface: 197/271 $m^2 g^{-1}$, porous diameter: 7.2/8.7 nm, mesoporous surface: 111/97 $m^2 g^{-1}$, microporous volume: 0.014/0.032 mL g^{-1}, mesoporous volume: 0.258/0.361 mL g^{-1} and mesoporous diameter: 9.3/15.0 nm, respectively) while the ones prepared with DMF or dioxane seemed to be the most porous material. These experiments confirmed what we suspected before regarding the connectivity between the use of higher amounts of porogen when preparing the MIPs and the increase of porous size, so the surface interaction contacts. An explanation of these results could be link to the relative volatility of the solvent used and about the differences in dipole moment between solvent and cross-linker used.

3.5. MIP Evaluation

The combined results from chemical computation models and NMR affinity experiments could be very profitable tools to design new MIPs but it is also necessary to optimize the processes used to evaluate them. The set of methods described in this work could help to check easily the prepared MIP batches and their reproducibility but also would help finding new MIPs when the rational approach can't be used (e.g. very high molecular weight compounds). Then, a combinatorial approach could be the best way to overcome this situation but it requires a good methodology to evaluate the properties and the performance of the potential MIPs. The interaction between target analyte and the MIP is a complex process with at least two different association mechanisms, the molecular selective one (i.e. the desired interaction) and non-specific (i.e. simple adsorption or phase partition). Moreover, the solvent component plays an important role because MIP and analyte are solvated. Thus, during MIP evaluation the parameters to be estimated have to be related to the analyte specifically retained by the MIP in respect to the one engaged by the NIP (i.e. the reference material) being also very important an accurate selection of the solvent or media used in these affinity assays. Based on these considerations, we propose to use a cascade of three different assays with increasing complexity to evaluate a set of potential MIPs.

The first assay intended is a simple desorption test performed just after the polymer preparation to evaluate the ratio desorbed template in both MIP and NIP materials. Adequate MIP must show a lower template desorption than NIP because the extra selective receptor cavities formed during the imprinting. A problem in this kind of experiment is the amount of template covalently linked to the polymer structure or trapped in the polymer network which is not available to desorption. A second question arises from the low selective receptors concentration in respect to the total amount of template or the non-specific adsorption. The accuracy and reproducibility of classical HPLC could not be enough to reveal the minute changes in the liberated template. This problem could be avoided if two or more desorption steps are performed. Finally, the combined data of the template desorption assay (see Fig. 3) allowed us to remove MIPs prepared in EtOH and H_2O/2-propanol.

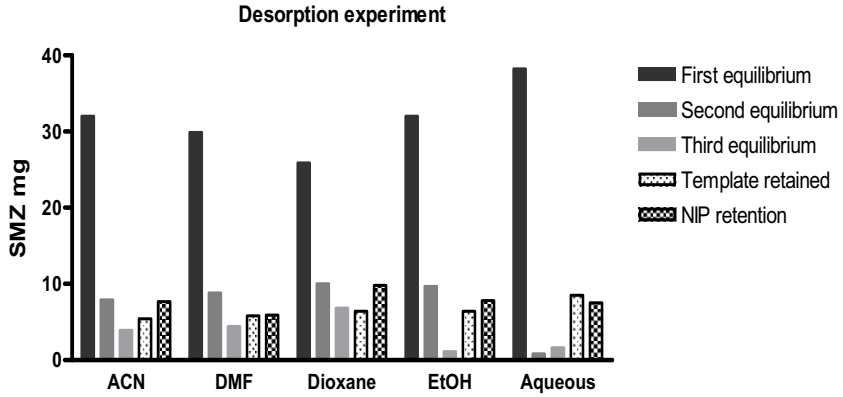

Fig. 3. Study of SMZ desorption over ACN for MIPs freshly prepared (2 mL g-1 proportion). Horizontal axe represents solvent used in the polymerization. Equilibrium bars indicate template liberated in the process. Template retained bar indicates template fixed in the polymer while the NIP retention bar shows non selective SMZ retention by the non imprinted material. Total template used in the polymerization was 49 mg.

The next suggested assay is mainly a washing trial in which the liberation of the template is monitorized in relation to the amount of solvent passed through a bed of MIP particles. The release of template is depending on its linking to the stationary phase and thus, it could be expected that a slower release must correspond to a MIP with better affinity to the template. That could be true if the accessibility of the bounded template is the same for all the MIPs. In this sense the porosity of the MIP and the average size of the channels could modify the amount of the solvent in contact with the bounded template. At this stage, (see Fig. 4) MIPs prepared in DMF and dioxane showed a poor template retention and only the MIP in ACN showed a slow decreasing profile indicating that it would be a good MIP candidate. Additionally, also the discarded MIPs in the previous step were checked and they also show a very fast template releasing in concordance with the previous result. Last evaluation was performed using a chromatography retention test in which the material is tested as a selective stationary phase referenced to the corresponding NIP. The non molecular specific retention (i.e. the standard phase partition mechanism) will be more or less equal for the MIP and

the NIP whereas selective retention must be only showed by MIPs. Then, the ratio of the Ki parameters between MIP and NIP is a good indicator for selective retention properties.

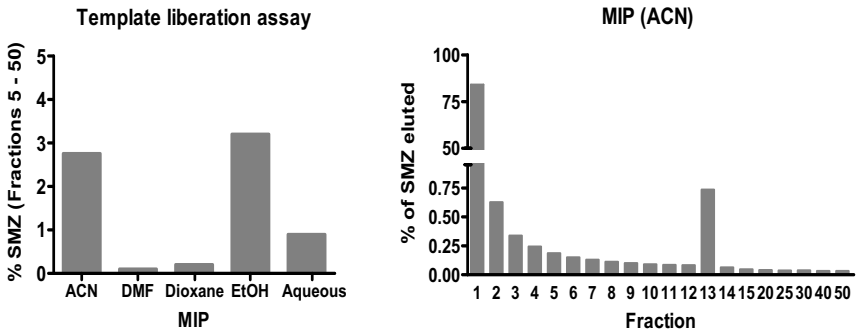

Fig. 4. Results regarding wash experiments using ACN as the mobile phase. Left graph shows the percentage of SMZ retained in the polymers after the first five fractions (25 mL) collected. Representation of SMZ elution behaviour for the polymer prepared with ACN is showed on the right graph.

Thus, if template and other structurally non-related compounds (see Fig. 5) are sequentially injected we could find easily the polymer with the higher selective retention. MIP prepared with ACN as a porogen showed better selective retention for the SMZ under conditions assessed while the rest of polymers did not show enough selectivity. Moreover, SPY also showed certain retention in the ACN MIP due to its chemical and structural similarity to the template, so other sulfonamide compounds were subsequently tested again using this experiment using only this particular polymer. Results regarding chromatographic behaviour (Ki) of these new compounds analyzed are described elsewhere [39].

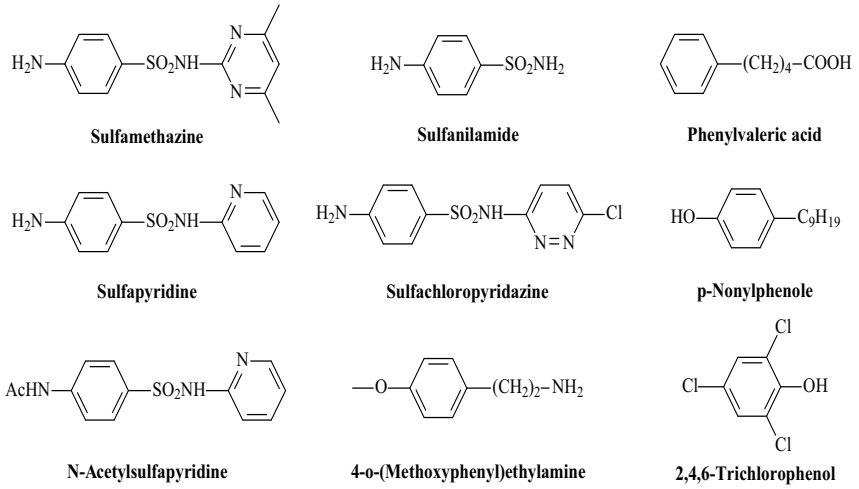

Fig. 5. Compound structures used in the first retention chromatographic experiment.

From the above results, we could conclude that the evaluation of a large set of MIPs could be done efficiently using a sequence of three methods of increasing complexity. All of them yield similar results when they were performed over the whole set of MIPs that we prepared (i.e. they differ only in the porogen nature). The selection strategy tries to remove the worst materials from the assay set instead to select the best ones. Then, the remaining materials go to the next evaluating level which would be run with a fewer number of polymers. Finally, the solvent used in the evaluation assay plays a very important role. Some authors claims that the best solvent is the same used in the imprinting process. This is a biased point of view because the true that complex set of interactions between the template and polymer components would be more or less the same in each case but does not imply that those could enhance the molecular selective recognition being very difficult to compare the polymer performance when the porogen nature is systematically changed in the polymerization mixture. In fact, one of the trickiest points in the development of MIPs applications is the selection of solvents or media used in each step of the process. In our case, we select ACN because we have the NMR studies data that confirm this solvent as the media in which the template monomer interactions were unequivocal.

3.6. Frontal and batch rebinding experiments

The most characteristic properties of a heterophase chemical receptor are their affinity for its substrate and concentration of

these receptors in that material. For MIPs prepared with ACN, frontal chromatography methodology was selected to determine their adsorption and kinetic parameters from simply breakthrough experiments. This technique has been applied previously for studying specific interactions such as enzyme-cofactor, enzyme-inhibitor and antibody-antigen as well as MIPs. For MIPs, the study should be understood as a molecular association between the analyte and the receptors structured in the polymer cavities, which are equivalent to the specific receptors previously cited. Strictly, Langmuir binding model is not the most appropriate in that case because MIPs have a receptor distribution of different affinities, just like polyclonal antibodies [40]. On the other hand, that model can be used as a first approach to provide interesting information about an average of all existing interactions between substrate and polymer (specific and non specific) from a dynamic point of view. Experiment was focused in evaluating amount of analyte retained for the polymers by comparing analyte amount flowed through the packed MIP or NIP with the total analyte eluted when the phase is saturated (see Fig. 6). Only the imprinted polymer showed different saturation chromatograms depending on the SMZ concentration used in the experiment.

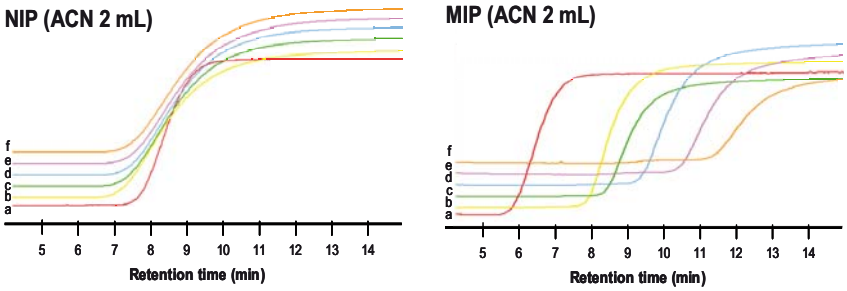

Fig. 6. Saturation chromatograms obtained for polymers (MIP: right graph; NIP: left graph) prepared with ACN (2 mL g-1 proportion). a: cyclohexanone (2 mM), b: SMZ 1 mM, c: SMZ 0,5 mM, d: SMZ 0,25 mM, e: SMZ 0,125 mM, f: 0,0625 mM.

Batch rebinding experiments were also performed to complement these results from a static equilibrium point of view. Constant weights of imprinted polymer prepared with ACN, previously grounded and sieved, together with solutions containing different concentrations of the template where studied until reach equilibrium conditions. Then, substrate in solution was measured to evaluate, in each case, analyte amount linked to the polymer obtaining binding isotherms. This experiment follow the same principles explained in desorption assays section where template desorption from polymers freshly prepared was assessed.

Then, binding curves were calculated from data obtained in frontal chromatography and rebinding experiments indicating association molecular constants and amount of receptors present in the polymer (see Fig. 7).

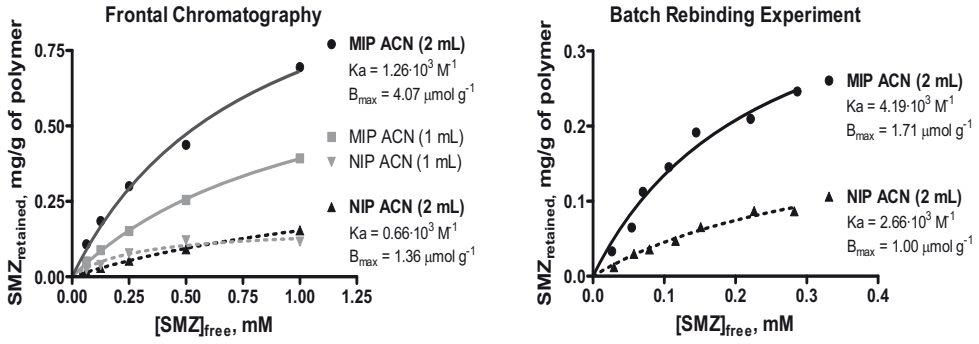

Fig. 7. Binding isotherms obtained from polymers prepared with ACN (2 mL g-1 proportions for both experiments and 1 mL g-1 only for the frontal chromatography approach).

Although parameters calculated are the same in both experiments, the affinity values are not exactly the same when static and dynamic methodologies are compared because in batch rebinding experiments, analyte adsorption to the binding sites is not forced by a constant solvent to flow through micro porous polymer channels like happens in the frontal chromatography. Specific template binding sites present in MIPs provide higher affinity properties which are reflected in curvature changes of the material binding isotherms [41]. In all cases studied, MIPs showed more SMZ fixed compared to the respective NIPs. Association constants are promising (10^3 M^{-1} order) which means that imprinted polymer has good affinity and retention properties for the SMZ.

3.7. MIPs Preparation Reproducibility

Molecular imprinted polymers preparation is known as none very reproducible technique because many variables take part in the process. Thus, experiments focused to check reproducibility were performed to study template desorption behaviour, between different MIPs prepared with the same analyte and between different fractions of the same MIP. As can be observed in the MIPs evaluation section, the desorption assay is easy to perform and its results are robust enough to be used as a control method in MIP preparation. Problems from non-accessible template could be considered similar in the portions of the same batch and also between different batches. If there is some doubts about that a sequential desorption assay could be performed in order to check the amount of non-available template.

A batch of ten polymers was prepared, milled, sieved under 63 µm particle sizes and analyzed for template liberation. All MIPs presented similar behaviour in the amount of analyte not retained under the experiment conditions, which corresponds to an average of 39.9 ± 2.1 mg (81 %). The associated error to the assay was around 5.3 %. In a second run, ten milled and sieved fractions (50 mg), fewer than 63 µm particle sizes, from the same MIP batch were evaluated. Fractions showed similar behaviour where an average of 1.99 ± 0.11 mg (81 %) of template was not retained by the MIP. Associated error to this new assay was around 5.5 %. Experiments showed no significant differences between MIPs, in terms of template liberation (error preparation below 5.5 % in both cases), when high purity reagents are used, residual oxygen is removed and if the polymerisation temperature is regulated during polymers preparation, ensuring batch reproducibility.

4. Conclusion

Combination of computational approaches and NMR experiments has been used to perform rational design of a SMZ molecular imprinted polymer. From these predictive methods, porogen and potential monomers ideally suited for the polymer formation have been selected, interaction molecular points localized and optimal template:monomers ratio calculated. Molecular imprinted polymer preparations based on rational design have superior performance in comparison with typical MIP preparation through a lengthy process of experimental trial and error before finding the most suitable recipe. Subsequently, a combinatorial strategy based on the development of simple sequential experimental assays, but increasing their complexity, was planned to exclude, from a big batch of polymers prepared but using different porogens after the rational design, those MIPs with worse recognition properties having fewer candidates in each step without having to perform an accurate characterization of all candidates. The resulting MIP was able to interact selectively with SMZ showing very good affinity and chromatography properties. These material characteristics allow us to follow up on future MIP application/development in chemical sensors field and selective phase extraction for sample treatment. This polymer has been prepared by means of non-covalent interactions approach by temperature induced polymerisation in ACN, using AIBN as the initiator, MAA and VPy as monomers and EGDMA as the cross-linker. MIP preparation has been optimized by checking all the parameters that can affect the physic-chemical characteristics of the resultant material. Good polymer reproducibility has been achieved having a better control of the polymerisation and the imprinting process. Once obtained a good imprinted material, further work was addressed to develop a molecular imprinted solid phase extraction (MISPE) procedure, which is nowadays one of the most advanced MIP application area for the selective extraction of a target analyte [42,43], for detection of sulfonamides in milk samples [39].

Acknowledgements

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2.3 Results and discussion

In this chapter we have presented the rational design, preparation and evaluation of a MIP for SMZ to establish a general and robust procedure to obtain these kinds of artificial receptors independently of the template used. **Figure 2.6** shows the procedure followed by our research group to obtain MIPs with good recognition properties.

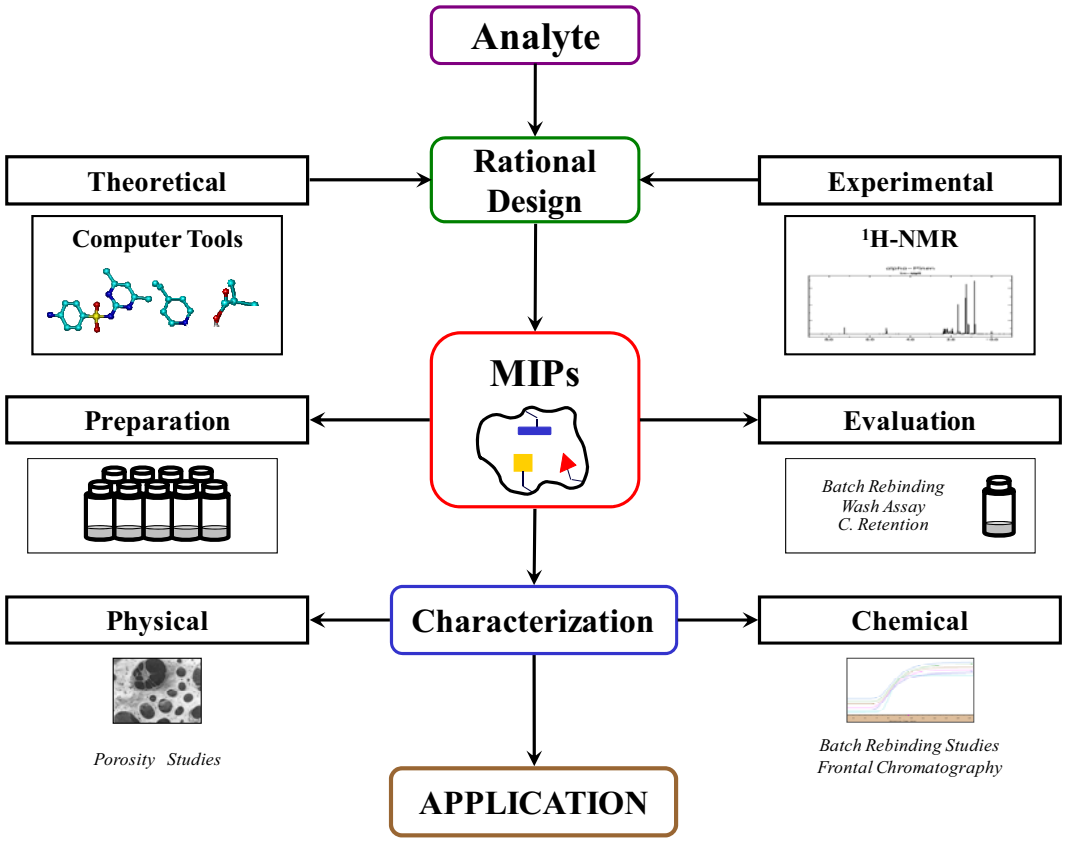

Figure 2.6: Flowchart of all process presented by our group to obtain a molecular imprinted material with good properties for the desired target analyte.

2.3.1 Molecular modelling and theoretical calculations

Computer assisted molecular modelling tools have been used to obtain information on the molecular geometries and the nature of the monomer-template interaction. Before all the theoretical calculations it was necessary to find the most stable conformations for all mixture components to then analyze possible approximation of the monomers to the template (see **Figure 2.7**).

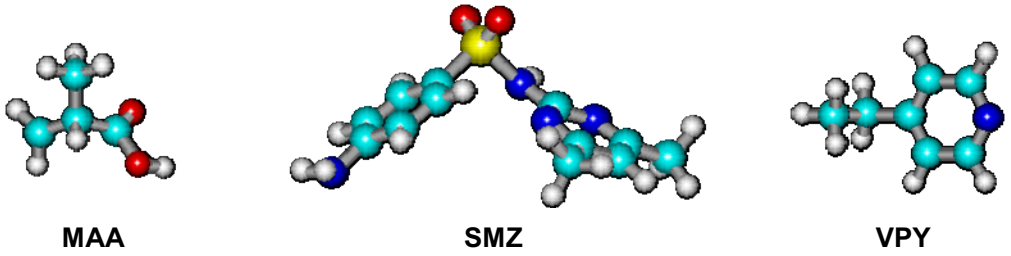

Figure 2.7: MAA, SMZ and VPY chemical structures. PM3 computational models of their most stable conformations. Colours: (C = turquoise blue, N = blue, O = red, S = yellow, H = white).

Formation enthalpies of numerous molecular complexes were calculated as a result of interaction between selected monomers and functional groups of the template from different space approximations.

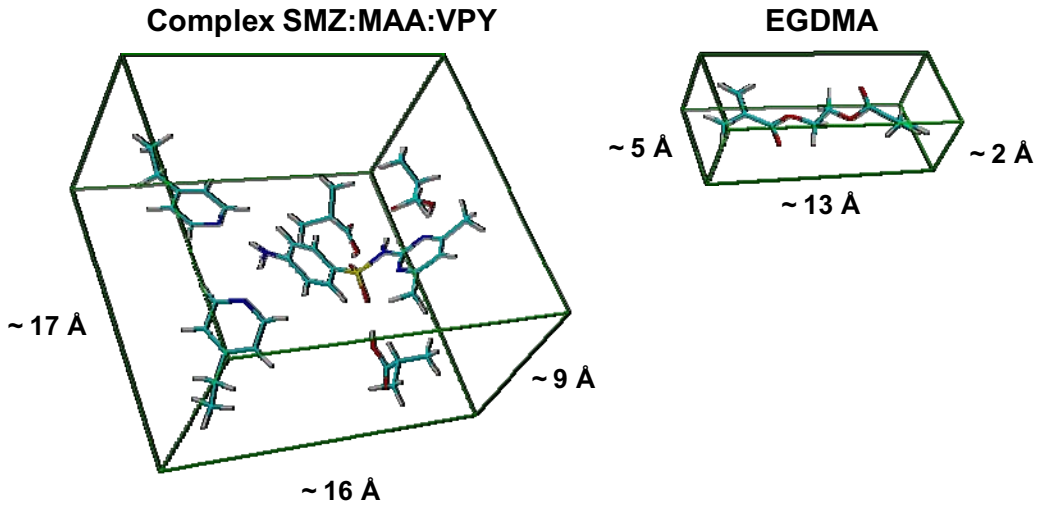

Figure 2.8: Comparison between minimal box surface dimensions of SMZ:MAA:VPY complex and EGDMA compound.

Finally, a cluster was mainly favoured (see **Fig. 1** in **Section 2.2**), in energetic terms, from all possible molecular interactions calculated where polymer components SMZ:MAA:VPY ratios were 1:3:2. **Figure 2.8** shows the most favoured cluster, its space distribution and the surface of the minimal box enclosing it, which was also calculated by the theoretical model to make estimation about crosslinking agent amount necessary to surround all the structure (20-25 to 1). We should consider all the theoretical calculations performed as a quite non-realistic situation since organic molecules usually interact on a media containing solvent molecules that readily favour or hinder these interactions mentioned. On the other hand, modelling experiments carried out can contribute with

valuable preliminary information about polymer components to be used in the final MIP preparation [12].

2.3.2 NMR interaction studies

Experimental studies were performed to validate all the theoretical work previously developed indicating if it could be or not an interesting tool in future rational design of MIPs and to obtain additional data referred to interactions between template and monomers in the presence of a solvent. Nuclear magnetic resonance (NMR) titration experiments were used to provide us experimental data about template-monomer affinity in a specific media being very useful to decide the most promising mixture components [13]. Moreover, strict data was obtained from NMR experiments to fix template:monomers relative affinity and to know the exact monomer excess required to displace the equilibrium to the cluster formation without creating a big number of non selective points. That was determined by observing the systematic change in the chemical shifts (Δδ) of these signals close to the binding points. Qualitative evaluation of the results obtained showed significant differences of some SMZ ^{1}H chemical shifts depending of the monomer used (see **Figure 2.9**).

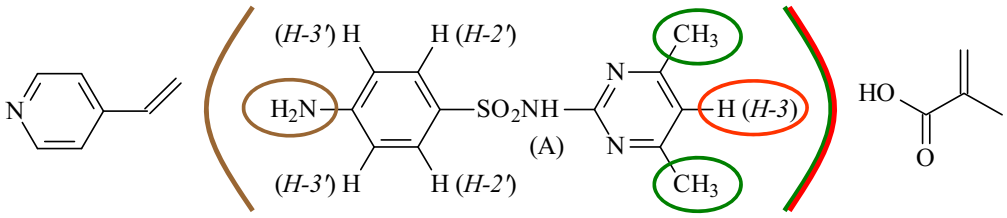

Figure 2.9: Qualitative scheme of interactions between monomers and template obtained with NMR experiments. **Fig. 2** in **Section 2.2** describes these results in more detail.

Proton signals corresponding to the heterocycle (-CH_3 and H_3) were more affected when MAA was introduced while proton chemical shifts of the SMZ free amino group (-NH_2) suffered a significant displacement when VPY was studied. Contrary to our initial thoughts, the acid monomer (MAA) interacts preferably with the SMZ heterocycle instead of the aniline, which also has an unforeseen manner of interaction with the basic monomer (VPY). These results obtained matched very well with those of the molecular modelling calculation and therefore validating the entire procedure.

2.3.3 MIPs preparation

After computational and NMR studies, both SMZ imprinted polymers and the corresponding NIPs were prepared with the functional monomers and crosslinking agent selected but using different porogen solvents (see **Table 2.1**).

Table 2.1: Amount of reagents required to prepare the polymers planned. ACN, DMF, EtOH and water/2-propanol were the porogens tested (24 MIPs in total). Molar ratio of template and initiator was 1:0.27.

Polymer	SMZ	MAA	VPY	EGDMA	Porogen vol.
MIP IA	0.175 mmol	0.524 mmol	0.524 mmol	4.19 mmol	1 mL
NIP IA'	-----	0.524 mmol	0.524 mmol	4.19 mmol	1 mL
MIP IIA	0.175 mmol	0.524 mmol	0.524 mmol	4.19 mmol	2 mL
NIP IIA'	-----	0.524 mmol	0.524 mmol	4.19 mmol	2 mL
MIP IIIA	0.175 mmol	0.524 mmol	0.524 mmol	4.19 mmol	4 mL
NIP IIIA'	-----	0.524 mmol	0.524 mmol	4.19 mmol	4 mL

Glass vials of 20 mL with silicone septa were used to prepare all the polymers. In all cases, before activating the polymerization (sand bath heated to 60 ºC overnight) the mixtures were purged to eliminate the oxygen present in the reaction media. Polymer reproducibility was also tested by template liberation experiments (error preparation obtained below 5.5 %). Depending on the following experiment requirements, bulk polymers were milled and sieved between 20-40 μm and packed into columns (see **Figure 2.10**).

Figure 2.10: Molecular imprinted polymer after being milled and sieved. On the right a picture obtained with an optical microscope of the homogeneous particle material.

2.3.4 MIPs evaluation

Once prepared such a big batch of different polymers we pretended to develop a methodology to exclude those with worse molecular recognition properties without having to perform an accurate characterization of all candidates. That methodology was based on several sequential assays with increasing complexity and having fewer candidates in each step with enough efficiency for being introduced as a combinatorial strategy for new MIP development (see **Figure 2.11**). First experiments were based on batch rebinding tests to analyze template retention of imprinted polymers freshly prepared without being processed (see **Fig. 3** in **Section 2.2**). Second evaluation experiments were thought to be performed with that polymers not discarded from the first ones. With that in

mind, we pretended to evaluate template liberation behaviour of the imprinted polymers prepared after being milled, sieved and column packed, under elution of different organic solvents (see **Fig. 4** in **Section 2.2**). Finally, polymers packed again into columns were evaluated as chromatography stationary phases to calculate their respective molecular selective parameters against different compounds structurally related, and non-related, to the template (see **Fig. 5** in **Section 2.2**). Under all these experiment conditions, only MIP prepared with ACN (2 mL) showed interesting properties with a good imprinting factor and better molecular selectivity.

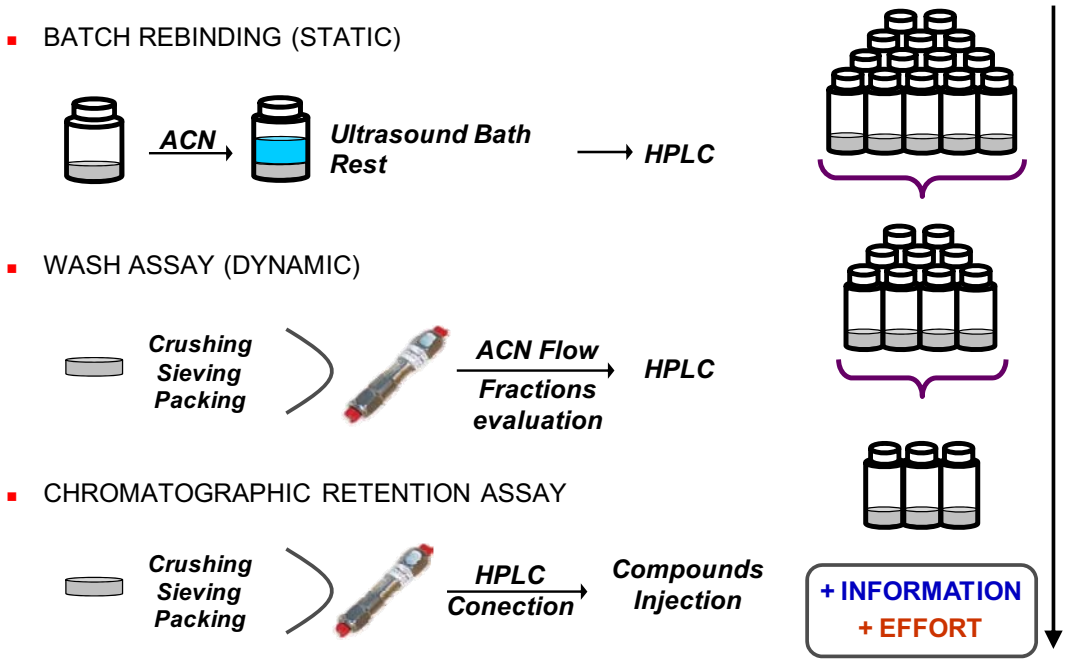

Figure 2.11: Serial molecular recognition experiments planned. This methodology pretends to exclude, from a large collection of MIPs prepared, the ones with worse application properties.

Set of results obtained in the previous steps of selection is coherent and consistent which allows designing an interesting system to select a MIP from a numerous batch polymer preparation.

2.3.5 MIPs characterization

The next step was the characterization of imprinting efficiency, whether or not our imprinted polymers adequately and accurately remember the template. Porosity studies (physical characterization) and frontal chromatography tests (chemical characterization) were performed to better define the final properties of polymers obtained.

2.3.5.1 Physical

As it was explained in **Section 2.1.1.1**, the solvent is the mixture reaction component responsible for the porosity of MIPs. During the polymerization, the solvent molecules are incorporated into the polymer but being eliminated later in the subsequent treatment. In this process, spaces that were originally occupied by solvent molecules remain as pores in the polymer. We could say that MIPs are rigid foam with large empty cavities in its interior volume formed by a network of canals whose size depends mainly on the nature of solvent used. Their distributions are random and tour the interior of the polymer. The sites of interaction associated with mesoporous (> 20 Å) is expected to be more easily accessible (area most likely to have high affinity receptors and / or selectivity) than those located in the micropores (<20 Å) where diffusion is low.

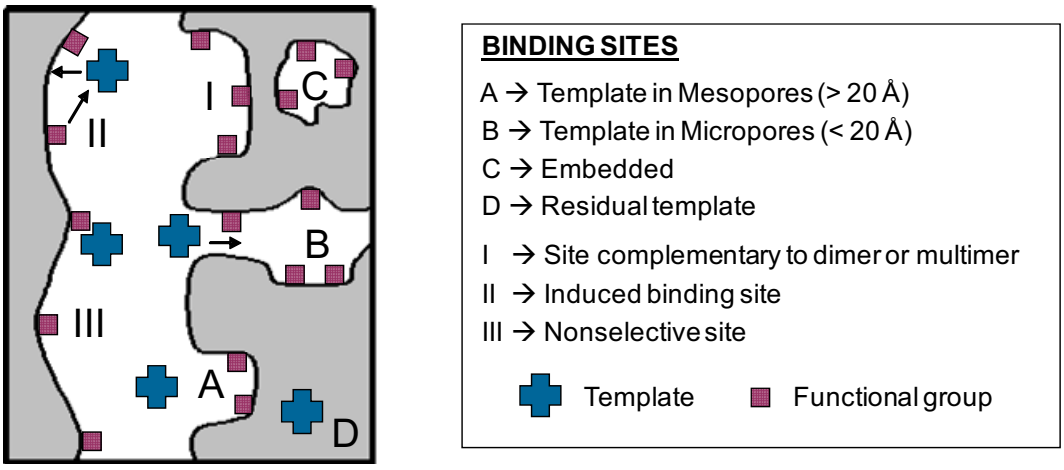

Figure 2.12: Different types of binding sites in polymers containing micro-, meso- and macro-pores. Retention mechanisms can be explained by presence of selective receptors (A, I, II), molecular exclusion (B) or non selective partition between phases (III). Picture inspired from [11].

The porosity analysis was performed to most of polymers firstly prepared just to have more detailed information about differences between MIP internal structure parameters respect the solvent and their amount used to prepare them. Thus, available surface and pore size of some polymers was determined by nitrogen absorption porosimetry. The imprinted polymer with higher presence of macro and mesopores (see **Figure 2.12**), which are the areas where the binding sites are mainly created during the polymerization, was the one prepared with ACN (2 mL). That results maybe explains why we selected that polymer as the one with better properties against SMZ. Thus, the sites associated with mesopores are expected to be easily accessible compared to sites located in the smaller micropores.

2.3.5.2 Chemical

On the other hand, chemical characterisation was only performed to the polymer not discarded in the selection procedure methodology explained, which supposed to be the one with best affinity and selectivity properties (ACN, 2 mL). The procedure consisted in coupling the MIP/NIP columns to the HPLC system with an UV detector (310 nm) to evaluate their affinity and capacity properties [14] using acetonitrile at a 0.3 mL/min flow. Manual injector was modified introducing a big injection curl of 25 mL to then load and inject different concentration solutions of SMZ (1 mM, 0.5 mM, 0.25 mM, 0.125 mM, 0.0625 mM) to the chromatographic system. With that experiment configuration, sigmoid curves from zero absorbance to a saturation value of receptor points were obtained. Results provided interesting information about an average of all existing interactions between substrate and polymer (specific and non specific) from a dynamic point of view (see **Fig. 6** in **Section 2.2**). MIPs showed more SMZ fixed compared to the respective NIPs. Association constant is promising (10^3 M^{-1} order) which means that imprinted polymer has good affinity and retention properties for the SMZ (see **Fig. 7** in **Section 2.2**).

We can summarize that theoretical binding models joined to NMR experiments could be a powerful tool in the design of molecularly imprinted polymers. The theoretical calculations could be employed for localize the points of interaction between the template and different functional groups as potential monomers and to determine the size of the molecular cluster. The NMR experiments let us to know if potential monomers interact in a specific way or not in solution. Also, quantitative data on the monomer:template binding could be obtained that help us to select the monomer:template ratio and to check if the theoretical model is enough correct or not. Using the combined data from these predictive methods we could prepare with a very good reproducibility a polymer which showed a good molecular selectivity (in comparison with the corresponding NIP) for the imprint molecule or structurally closely related compounds. Moreover, different types of serial molecular recognition experiments, based on several assays increasing the complexity having fewer candidates in each step and with enough efficiency, were validated for being introduced as a combinatorial strategy.

2.3.6 Main contributions

The main contributions of this chapter to the existing knowledge at the time of starting this research are:

- First time that it has been performed a rational design of a SMZ MIP by combining computational approaches and NMR experiments.

- The development of a MIP that can interact selectively with SMZ showing very good physic-chemical and chromatography properties.
- The establishment of a combinatorial strategy based on the development of simple sequential experimental assays capable to exclude, from a big batch of polymers prepared after the rational design, those MIPs with worse recognition properties.
- The improvement of the control and reproducibility for the polymerisation and the imprinting process has been achieved by checking all the parameters that can affect the physic-chemical characteristics of the resultant material.

2.3.7 Further considerations

Promising results obtained were the starting point for Raquel Obregón to perform her PhD studies within this field. Since then, she was able to develop a separation application, using this polymer, based on a MISPE procedure to treat milk and hair extract samples contaminated with sulfonamides for their subsequent analysis by chromatographic techniques. Nowadays, investigations in our group regarding this field are concentrated in the preparation of new MIPs for other antibiotics, such as FQs and TCs, as well as developing monolithic columns with imprinting materials.

2.4 Bibliography of Chapter 2

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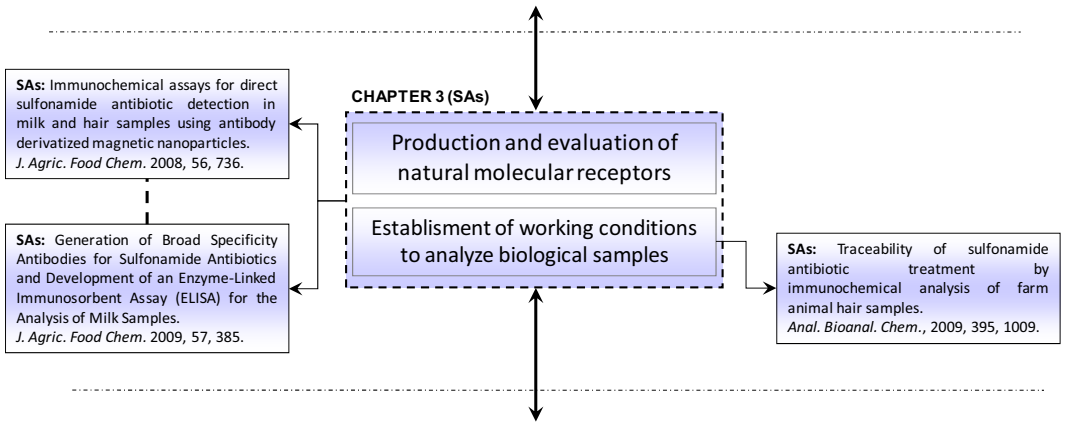
3 BIOLOGICAL RECEPTORS FOR SAs



The most crucial step in the development of an immunochemical technique for low molecular weight environmental pollutants is the hapten design. IA detectability, specificity and selectivity are mainly determined by the antibody and the chemical structure of the competitor hapten used [1, 2] used in competitive direct or indirect assay formats respectively. Many examples in the literature prove that an appropriate design of the immunizing hapten determines the features of the resulting antibodies [3, 4]. Small organic compounds (MW < 2000 Da) are not able to raise an immune response in a host animal, so it's necessary to modify the analyte to an immunogenic molecule, which consists of a hapten covalently attached to a much bigger protein (see **Figure 3.1**).

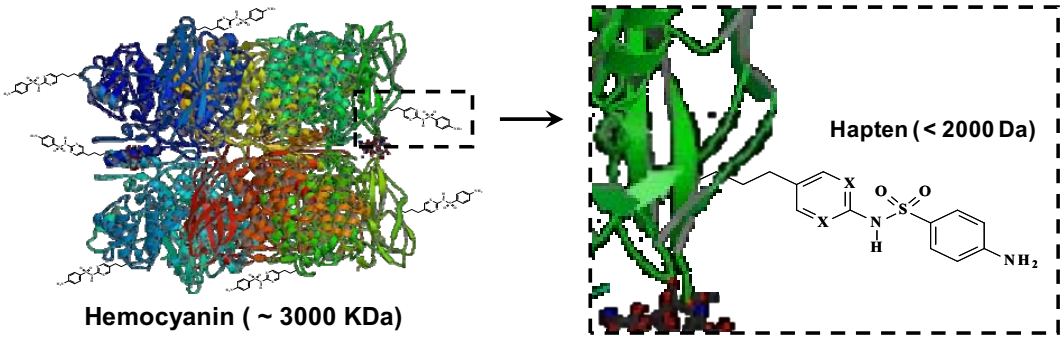


Figure 3.1: Example of an immunogen which consist of a hapten coupled to a hemocianyn carrier protein. It can be observed molecular weight differences between both compounds.

A hapten is a molecule analogous to the target analyte and properly functionalized to allow covalent coupling to a carrier protein compound. An ideal hapten is one that preserves most of the steric and electronic characteristics of the analyte and mimics the target analyte in chemical structure as closely as possible. The attachment point to the carrier should be placed far from the important sites of the target analyte and separated by a spacer arm to avoid hindrance by the carrier. A 3-6 atom spacer length has often been considered as optimum size [5, 6], but several IAs have been described when antibodies had been raised using haptens with shorter spacer arms [7, 8]. Ideally, a spacer arm should be a chain of methylen groups terminated by a chemical functional group (-COOH, -OH, -SH, $-NH_2$) through which the carrier protein will be attached. The synthetic effort needed to produce antibodies against an organic molecule should be carefully evaluated in relation to the chances to obtain a good antibody being the chemical synthesis of the hapten often the most time-consuming step. Nowadays, it is possible to make computer assisted theoretical and molecular modelling studies in order to predict the suitability of a particular chemical structure as a hapten to raise antibodies against the target analyte/s [9-11]. This facilitates the logical comparison of the 3D structure and the electrostatic properties of the designed hapten with those of the target

analyte, increasing considerably the changes to be successful in the entire antibody obtention process. During this thesis we have pretended to use this valuable modelling approach in the choice of ideal SAs and TCs hapten chemical structures (see **Chapter 3** and **Chapter 4**, respectively) before their synthesis and the subsequent development of the immunochemical assays in buffer and different biological samples.

The basic structure of SAs consists of a benzene ring with an aminosulfonyl group on C_1 and a free amino group on C_4. In this sense, although SAs are not complex molecular entities, in comparison with other antibiotics such as TCs or macrolides, they have particular physic-chemical properties that should be considered when approaching the synthesis of a representative immunizing hapten. Thus, SAs are fairly water-soluble, polar compounds which ionize within a pH range, and therefore the actual state will vary depending on the pH of the matrix (see **Figure 3.2**).

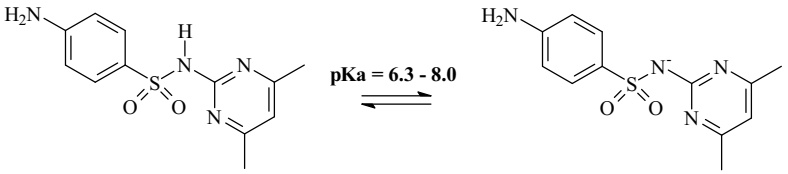


Figure 3.2: Example of SMZ pKa values that range from 6,3 to 8,0.

SAs are conformationally stable with regard to geometry meaning that differences between all congeners of these chemical structures are not so great. On the other hand, depending of the medium, SAs can suffer transformations such as the N_4-acetylation, deamination, aromatic hydroxylation and N_1-glucuronidation (see **Figure 3.3**).

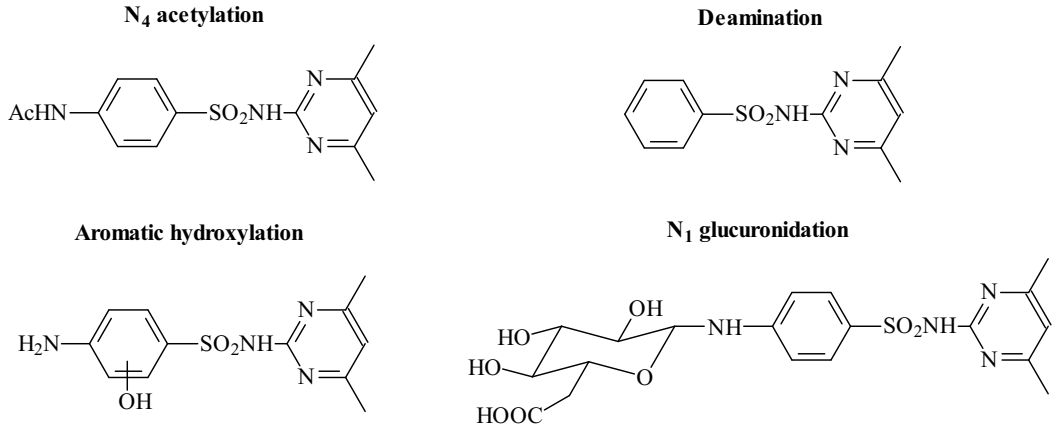


Figure 3.3: Most important degradation reactions of SAs.

All these aspects were considered at the time of designing an immunizing hapten. Particularly, in this case the objective was to produce antibodies with a broad recognition of the most important congeners of the SAs family. Therefore, the chemical structure designed to raise antibodies had to preserve most of the common features of the components of this antibiotic family. On the other hand the immunochemical analytical method established had to reach a detectability below the MRL values established in milk by the EC (see **Table 3.1** and **Section 1.3**) for this group of antibiotics (MRL = 100 μg L^{-1}, for each single antibiotic and for the total of SAs present on a sample).

Table 3.1: Main SAs to be monitored in milk samples.

Family	Main SAs Compounds	MRL
SAs[a]	Sulfamethazine	100 μg kg^{-1}
	Sulfathiazole	100 μg kg^{-1}
	Sulfapyridine	100 μg kg^{-1}
	Sulfachloropyridazine	100 μg kg^{-1}
	Sulfamethoxine	100 μg kg^{-1}
	Sulfamerazine	100 μg kg^{-1}

[a]*An MRL of 100 μg kg^{-1} should be applied to all compounds of the sulfonamide group. Moreover, the combined residues of all substances in the sulfonamide group should not exceed this value.*

3.1 Preamble

The objective of the research work described in this chapter has been the establishment of an immunochemical assay able to detect a wide range of SAs in sample matrices of interest in the food sector. By the time this work started (2003), there was few antibodies reported for SAs [12-15], and most of them addressed to the determination of a single analyte, in most of the cases SMZ [16-18] or sulfathiazole (STZ) [19, 20]. There was only one group that had reported the production of generic antibodies for SAs using DNA recombinant techniques [21, 22]. However, according to certain information received (personal communication) performance and stability of these antibody fragments was not completely satisfactory.

The objective of producing generic antibodies for SAs was initiated in our group by Dr. Héctor Font in the context of his thesis. He succeeded on designing and synthesizing an immunizing hapten, and several competitor haptens, as well as the corresponding protein conjugates. The present thesis continues this project starting from the moment when antibodies were being produced, although their activity and recognition capabilities had not been tested. In order to facilitate comprehension of this chapter, **Sections 3.1.1** to **3.1.4** describe part of the work developed by Dr. Hector Font, which has been to some extent reproduced within this thesis. **Sections 3.1.5** and **3.1.6** provide general information related to basic aspects of competitive immunochemical assays. **Sections 3.2, 3.3 and 3.4** contains the research articles that have been published in respect of this work and **Section 3.5** provides a summary of the most significant results obtained.

3.1.1 Design of immunizing haptens to raise antibodies against sulfonamides

In respect to the immunochemical detection of SAs, the main goal was to establish a broad-selectivity immunochemical assay. For this purpose, immunizing haptens were rationally-designed to maximize recognition of the common structural moieties present in most of the congeners of this antibiotic family. These immunizing haptens were synthesized and coupled to proteins in order to use these bioconjugates as immunogens for raising antibodies. Moreover, design and synthesis of the corresponding secondary immunoreagents was also required for setting-up the necessary immunochemical conditions.

Based on chemical criteria, generic recognition of the SAs family required to maximize recognition of the common aniline moiety. Therefore, a chemical group for covalent attachment to the carrier protein had to be placed at the opposite site of the molecule (see **Figure 3.4**).

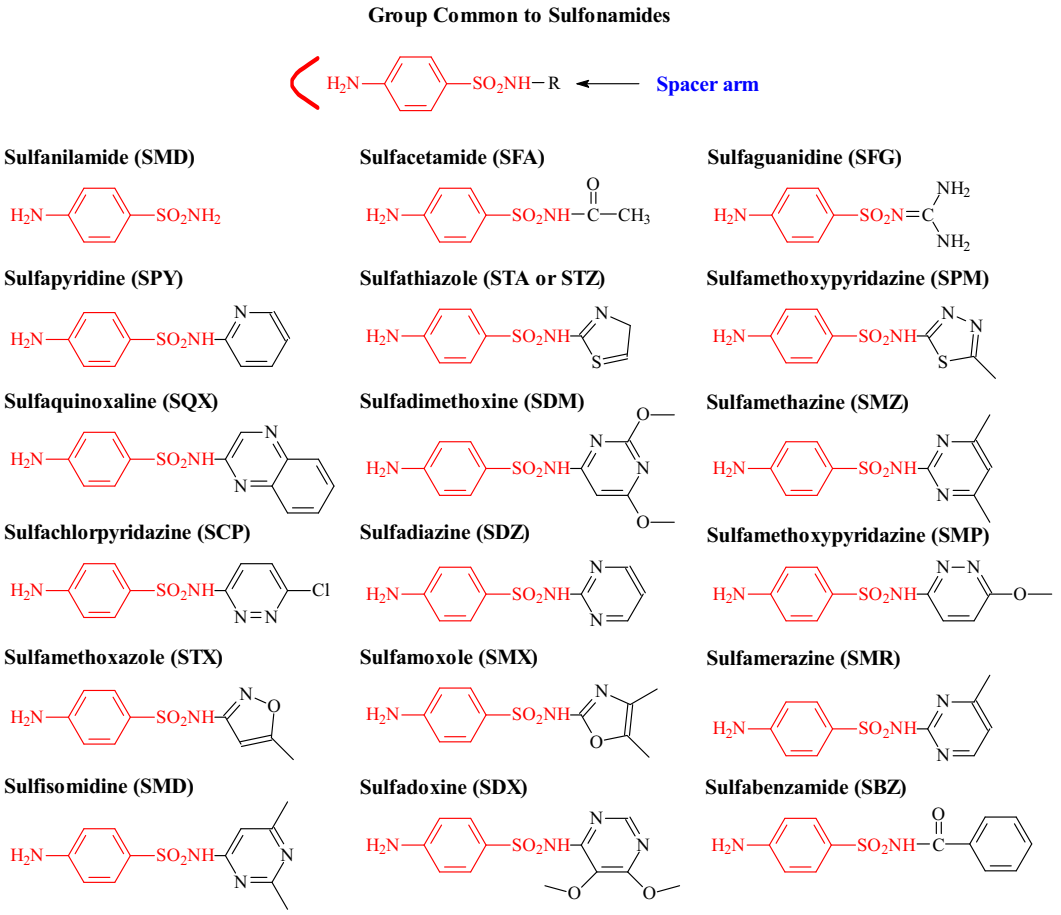


Figure 3.4: Chemical structure of most important sulfonamide congeners. Area marked in red represents the basic chemical structure common for all sulfonamides.

Moreover, it was considered relevant that most of the sulfonamide chemical structures had at least one electron-withdrawing atom close to the carbon situated in the alpha position to the sulfonamide bond. With this criteria it was defined the fundamental chemical structure moiety that had to be present in the immunizing hapten. Thus, we considered only one possible position in the chemical structure of SAs where a spacer arm had to be introduced which led on proposing two different immunizing haptens. Immunizing haptens planned (SA1 and SA2) possessed the mentioned area, however while hapten SA1 contained the whole structure (including the already commented electron-withdrawing atom), hapten SA2 had just the common moiety of the sulfonamide antibiotics (see **Figure 3.5**). The strategy of using just a fragment of the chemical structure was already been used before [12, 23], although not always had provided the desired results regarding antibody selectivity.

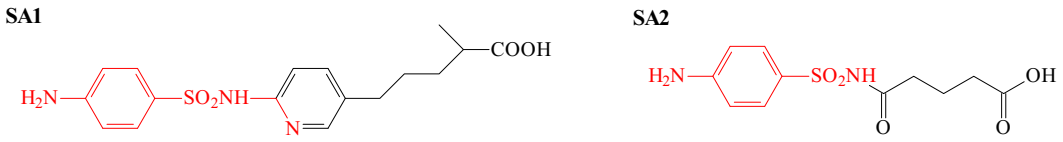


Figure 3.5: Structure of the possible immunizing haptens planned for the production of antibodies against SAs.

For this reason and with the aim to obtain additional information regarding the possible geometric deformations produced by the introduction of a space arm into the target molecule, we made use of the theoretical and molecular modelling studies. Based on semi empirical models PM3 we evaluated the possible geometries and electronic distributions for the two haptens in order to compare them to the ones of most important SAs. Moreover, pKa values for all compounds were also calculated to predict a distinct behaviour of the haptens at the physiological or the usual pH assay (pH 7.5). Results obtained showed that both SA1 and SA2 molecules could be considered excellent immunizing haptens for the production of promising SAs antibodies.

3.1.2 Haptens synthesis

Both haptens were prepared following the procedures established previously by Dr. Héctor Font [24]. Hapten SA1 was prepared in four steps from *methyl 5-(4-amino-3-pyridinyl)-2-methyl-4-pentenoate* through a Heck reaction under Jeffery conditions [2, 25]. The reaction consisted of a sp2-sp2 coupling between an iodoaromatic compound, in this case the *2-amino-5-iodopyridine*, and an alkene group, the *methyl 2-methyl-4-pentenoate*, using palladium acetate as catalyst, tetrabutylammonium chloride and potassium formate. The mixture obtained was hydrogenated and subsequently purified by HPLC to obtain the necessary desired synthon**.** Subsequently the sulfonamide bond was formed by reacting the amino group with *N-acetylsulfanilyl chloride* with the amino group of the heterocycle. The simultaneous hydrolysis of the acetyl group and the methyl ester, under basic conditions, allowed obtaining SA1 hapten, with a moderate yield (26%) due to the partial hydrolysis of the sulfonamide bridge. The key step was the Heck reaction since the conditions had to be carefully controlled to obtain the *methyl 5-(4-amino-3-pyridinyl)-2-methyl-4-pentenoate* on a reproducible manner. The global yield for the preparation of this hapten was 7%. On the other hand, the synthesis of SA2 was much more simple since it was obtained by reacting *N-4-(aminosulfonyl)phenyl)acetamide* with *methyl 4-(chloroformyl)butyrate*, followed by the hydrolysis of the ester and the acetyl group. As before the hydrolysis conditions had to be carefully controlled to avoid breaking the sulfonamide bridge. The global yield for the preparation of this hapten was 25%. The preparation of haptens SA1 and SA2 is accurately described in **Section 3.3**.

3.1.3 Antibody production

Haptens SA1 and SA2 were coupled to HCH (horseshoe crab hemocyanin) following the active ester method (AE) and the HCH conjugates were used to raise antibodies in white New-Zealand rabbits. The obtained antisera (As) were named As154, As155, As156 and As167 against hapten SA1 while those obtained against hapten SA2 were named As157, As158 and As159. With the aim to prove the results of the theoretical studies we decided to evaluate both groups of antiserum for their ability to bind SAs on a competitive ELISA.

3.1.4 Competitor haptens

The immunoassays for the analysis of macromolecular compounds, such as hormones, bacteria and proteins are performed under non-competitive conditions [26, 27]. On the other hand, the strategy used for the analysis of small organic molecules, such as antibiotics, needs the use of competitive assays based on the competition between the free analyte and the labelled antigen for a limiting number of antibody binding sites. What we call enzyme tracer (ET) is the labelled antigen used in the direct competitive format while the coating antigen (CA) is the one used for the indirect (see **Figure 3.9** and **Figure 3.10** respectively). Requirements regarding similarities between the competitors and the target molecule are not so rigorous, contrary to what we have explained for the immunizing haptens. Although this is not always true, it has been often reported that chemical structure of the immunizing hapten is the main determining factor for the immunoassay specificity, while the chemical structure of the competitor defines the final detectability. Thus, introducing a certain degree of heterology in the chemical structure of the competitor hapten in respect to that of the immunizing hapten could increase the assay detectability [9, 10]. Consequently, by increasing the hapten heterology, the equilibrium constant defining the formation of the immunocomplex competitor-antibody would be lower than the one directing the reaction between the analyte and the antibody (see **Figure 3.6**), but that principle is not always obeyed [10]. Therefore, homologous assays using the same chemical structure of the immunizing and competitor hapten have been reported, whose detectability has not been improved by increasing hapten heterology [9, 28]. On the other hand, based on the same principle, it has been reported that increasing hapten heterology may decrease specificity, due to the improved conditions for other structurally related analytes to compete for the antibody biding sites in respect to the competitor hapten [11, 29]. Depending on the desired immunochemical assay requirements, appropriate heterologous immunochemical systems are usually accomplished by the screening of several haptens coupled to enzymes or proteins.

Although there have been some attempts to rationalize the effect of the competitor hapten chemical structure, nowadays is still difficult to predict which one will provide the desired immunochemical assay features [30, 31].

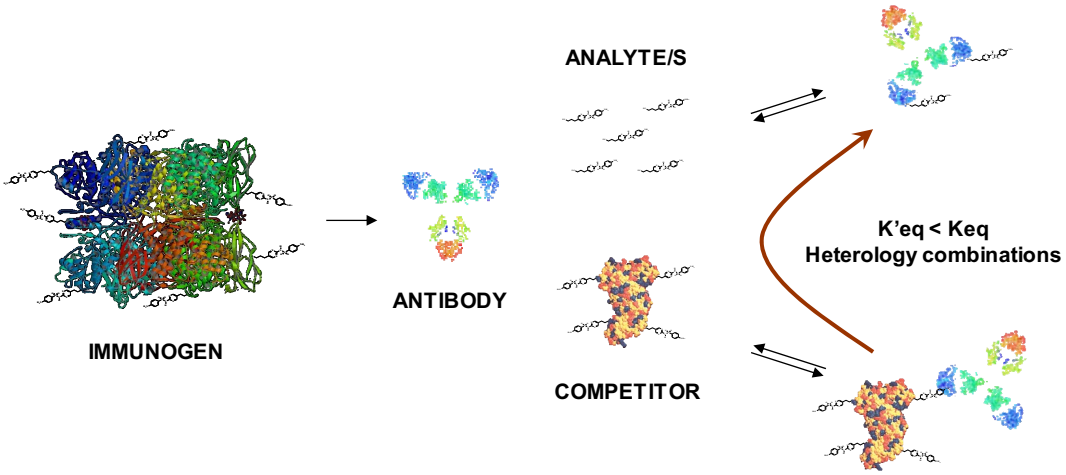


Figure 3.6: Hapten heterology principle.

Thus, we prepared eight new haptens (SA3-SA10), having different degree of heterology by changing the chemical structure and/or the position of the spacer arm, to be used as competitors (for information on the chemical structures and preparation of these haptens see **Section 3.3** and particularly **Section 3.3.1**). Depending of the competitor, the coupling to the correspondent enzymes or proteins through their free available lysine groups was performed by AE or the mixed anhydride (MA) methods (see **Figure 3.7** and **Figure 3.8**).

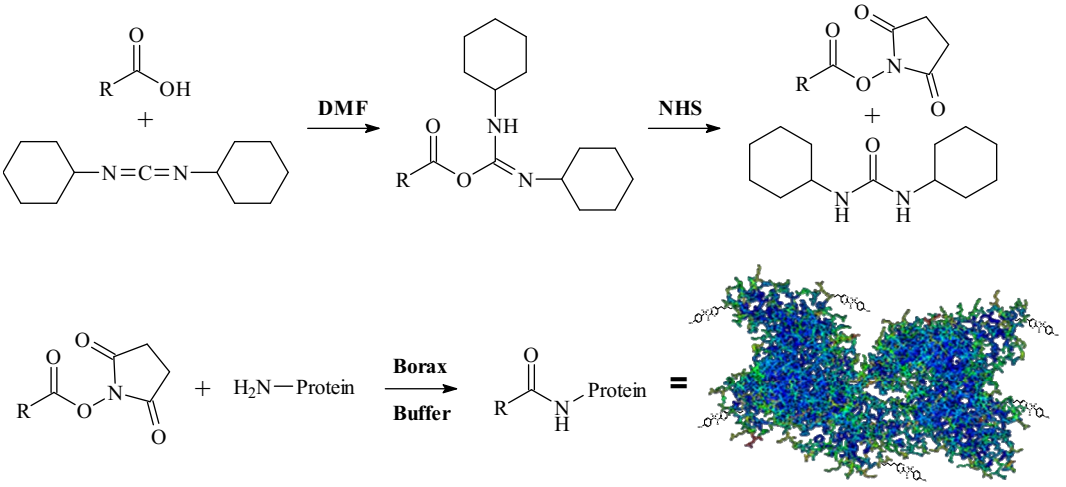


Figure 3.7: General scheme for the preparation of immunogens and most ET and CA following the AE method.

Normally it is advisable to use different conjugation methods for the immunizing hapten and competitor in order to avoid possible interferences in the assay due to secondary products that may be formed during the immunogen and other conjugates preparation. Since the immunizing haptens were covalently prepared by the AE method, we intended to use an alternative methodology like the MA.

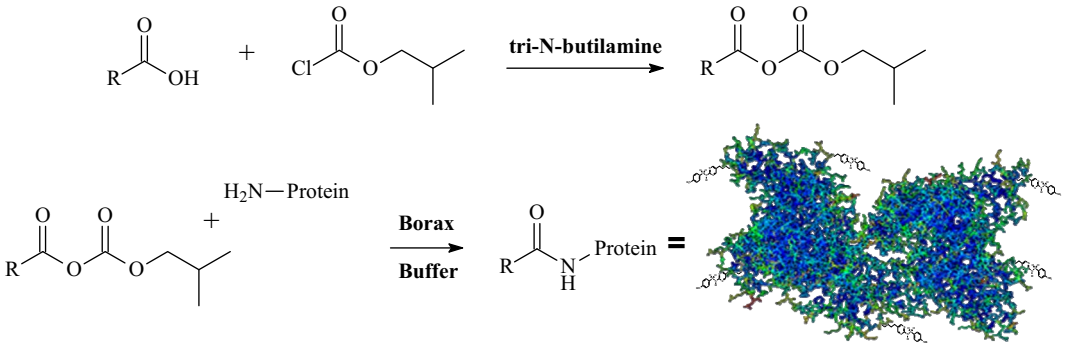


Figure 3.8: General scheme for the preparation of some CA following the mixed anhydride.

However, spectrometric MALDI-TOF analysis showed that conjugation did not take place for some haptens when using this procedure. Bioconjugation studies performed using 2-(4-methoxyphenyl)ethyl amine as model of the lysine groups of the carrier protein, demonstrated that the mixed anhydride formed reacted intra or intermolecularly with the aromatic amine group of the haptens preventing their subsequent coupling to proteins. In this manner, only SA3, SA4, SA5 and SA6 haptens, which have the correspondent space arm introduced at this amino group position (Y-HN-Ar-Z), could be coupled to the carrier proteins by the MA method.

3.1.5 Development of competitive ELISAs

We developed direct and indirect competitive ELISA formats (see **Figure 3.9** and **Figure 3.10** respectively) by testing the As produced for the analysis of SAs. Each ELISA format has a priori advantages and disadvantages, that can be found summarized in **Table 3.2**.

In the direct competitive ELISA format the equilibrium is established between the antibodies immobilized to the plate, the analyte and the ET that are in solution (see **Figure 3.9**). The 3,3¨,5,5¨-tetramethylbenzidine (TMB) was the substrate used for the enzymatic reaction. The absorbance was measured at a wavelength of 450 nm. In this case, haptens were conjugated to HRP, which is an enzyme with two free lysine groups accessible for bioconjugation [32].

Table 3.2: Comparison of Direct and Indirect ELISA detection methods.

Direct Detection (antibody coated microplate format)	
Advantages	Quick methodology since only one step is used
	Cross-reactivity of 2nd antibody is eliminated
Disadvantages	Possible partial loss of antibody activity when it is coated onto solid supports
	Limited stability of the hapten ETs
	Immunoreactivity of the 1st antibody may be reduced as a results of labelling
	Labelling of every 1st antibody can be time-consuming and expensive. Previous purification on the case of PAbs is often required
	Less flexibility in choice of 1st antibody label from one experiment to another
	Lower signal amplification
Indirect Detection (antigen coated microplate format)	
Advantages	Wider variety of labelled 2nd antibody are available commercially
	Versatile, since many 1st antibody can be made in one species and the same labelled 2nd antibody can be used for detection
	Immunoreactivity of the 1st antibody is not affected by labelling
	Sensitivity is increased because each 1st antibody contains several epitopes that can be bound by the labelled 2nd antibody, allowing for signal amplification
	Different visualization markers can be used with the same 1st antibody. Wider antibody recognition in front of different hapten competitors immobilized on the plate is often observed, increasing the possibilities to establish distinct heterologous combinations.
Disadvantages	Cross-reactivity may occur with 2nd antibody, resulting in nonspecific signal
	An extra incubation step is required in the procedure

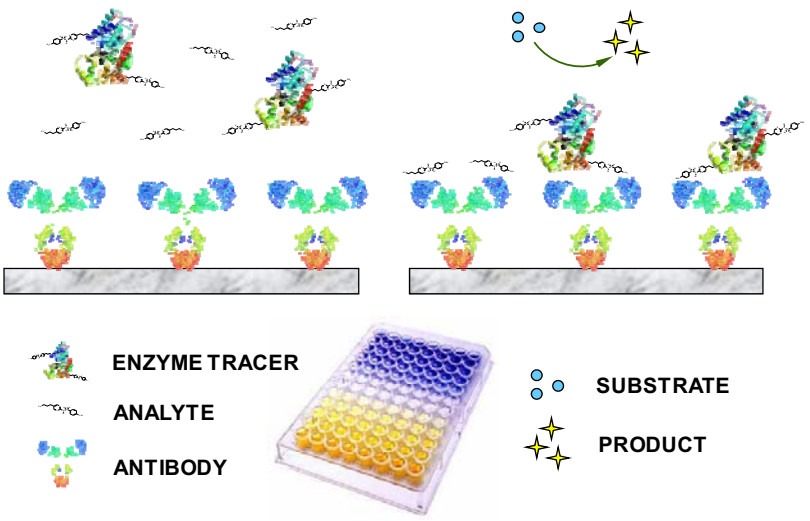


Figure 3.9: Scheme of direct competitive ELISA which is one of the most commonly employed for detection of low molecular weight analytes.

Contrary to the direct ELISA, in the indirect format the equilibrium is established between the CAs immobilized in the plate, the analyte and the antibodies that are in solution.

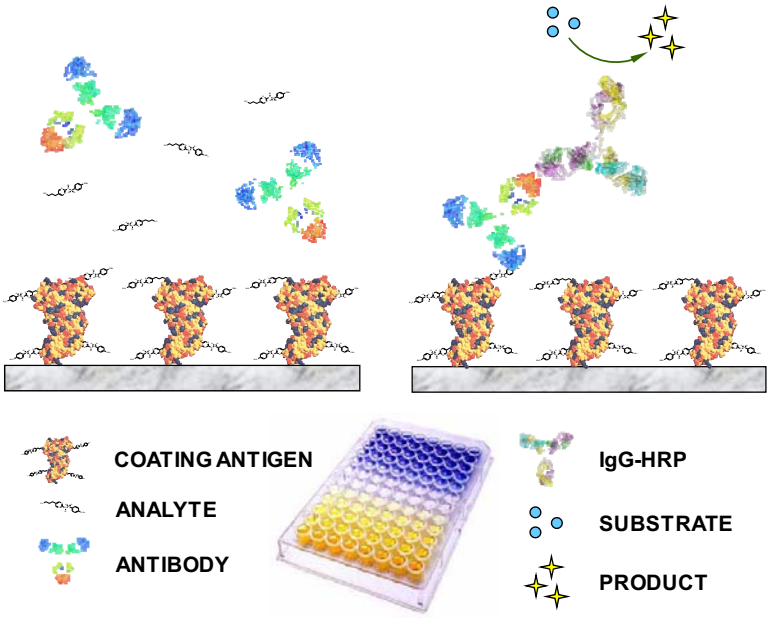


Figure 3.10: Scheme of indirect competitive ELISA which is one of the most commonly employed for detection of low molecular weight analytes.

In this case, the concentration of the analyte present in the samples is measured indirectly by the quantification of the bound specific antibody by using a secondary antibody labelled with an enzyme (anti-IgG-HRP) (see **Figure 3.10**). Again all haptens were conjugated covalently to the free lysine groups of the selected carrier proteins (BSA: bovine serum albumin, OVA: ovalbumin and CONA: conalbumin), and the hapten densities of the BSA conjugates were determined by MALDI-TOF-MS technique.

Finally, we ended with 40 different bioconjugates, between the haptenized proteins (30 CAs) and haptenized enzymes (10 ET), which meant that 70 combinations for the direct format (10 ET x 7 As, see **Section 3.2**) and 210 combinations for the indirect configuration (30 CA x 7 As, see **Section 3.3**) had to be evaluate to find out the most appropriate competitor/antibody combinations.

3.1.6 Calibration curve fitting in ligand binding assays

Final objective in both ELISA formats was to build up calibration curves in order to measure the analyte in real samples, but in this case the objective was also to know the features and behaviour of our immunoreagents before being implemented on a

multiplexed biosensor device. Calibration curves for ligand binding assays [33], such as a competitive ELISA, are generally characterized by a nonlinear relationship between the mean response and the analyte concentration (see **Table 3.3**).

Table 3.3: Differences between calibration curves from chromatographic and immunoassay techniques.

Chromatography	Immunoassay
Direct response	Indirect response
Direct concentration-response relationship	Direct or inverse concentration-response relationship
High precision	Lower precision due to higher inter-assay imprecision
Extended assay range	Limited assay range (frequent need for dilution)
Generally linear calibration curve	Nonlinear calibration curve

Typically, the response exhibits a sigmoidal relationship with concentration. In our case, the standard curves were fitted to a four-parameter equation according to the following formula: $y = (A-B/[1-(x/C^D])+B$ where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve. **Figure 3.11** shows a representative calibration curve with the most important parameters including those of the fitting equation plus those usually employed to define the limit of detection (LOD) or the lineal range.

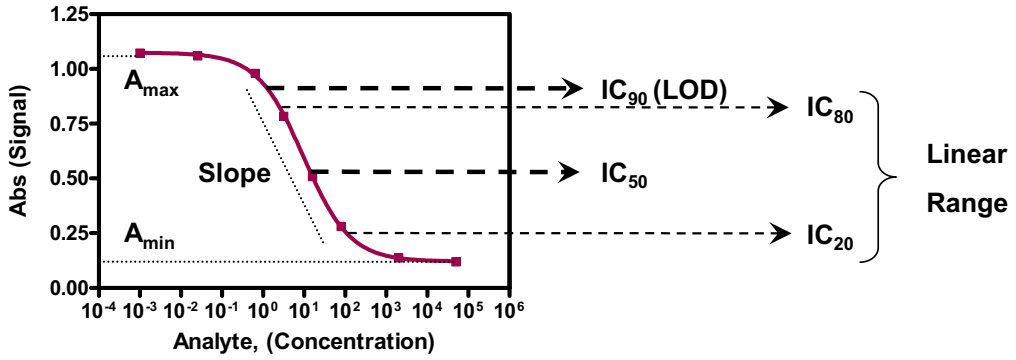


Figure 3.11: Typical calibration curve of a competitive ELISA and the corresponding most important parameters.

The present chapter describes the work performed in relation to the **Specific Objective 1** (i.e. preparation and evaluation of specific receptors) and **Specific Objective 2** (i.e. evaluation of performance of the bioreceptors in real matrices) of this thesis in respect to SAs. Taking into account that the ELISA technique is considered an essential method to characterize the features of antibodies, the main objective here was a) to asses that the antibodies produced were able to recognize a significant number of SAs congeners (see **Section 3.3)**, and b) to develop and establish reliable immunochemical protocols to analyze these antibiotics in milk (see **Sections 3.2** and **3.3**) and hair samples (see **Section 3.4**).

3.2 SAs direct ELISA format (Milk, hair, magnetic particles)

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Immunochemical Assays for Direct Sulfonamide Antibiotic Detection In Milk and Hair Samples Using Antibody Derivatized Magnetic Nanoparticles

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Two direct enzyme-linked immunosorbent assays (ELISAs) have been developed for detection of sulfonamide antibiotic residues in milk samples. One of them is using magnetic nanoparticles (MNP) for target capture/enrichment (Ab-MNP-ELISA), and the second is performed using microtiter plates. Selective polyclonal antibodies, raised against 5-[6-(4-amino-benzenesulfonylamino)-pyridin-3-yl]-2-methyl-pentanoic acid (SA1), used in combination with an enzyme tracer prepared with the same hapten, has allowed us to reach a limit of detection (LOD) lower than 0.5 μg L^{-1} for both ELISA formats. Sulfapyridine, sulfamethoxypyridazine, sulfathiazole, and sulfachloropyridazine are detected below the maximum residue limits established by the European Union for these antibiotics in milk (100 μg L^{-1}). Matrix effects and accuracy studies performed with full-cream milk and hair extracts indicated a lack of interference from these sample matrices and very good recovery values, especially when using the Ab-MNP format. Milk samples and hair extracts can be measured without any previous treatment. The results demonstrate the high potential of these methods as screening tools for food safety and inspection controls.

KEYWORDS: Sulfonamide antibiotics; sulfapyridine; immunoassay; magnetic particles; milk; hair

INTRODUCTION

Following their 20th century triumph in human medicine, antimicrobials have also been increasingly used for the treatment of bacterial diseases in animals, fish, and plants. In addition, they became an important element for intense animal husbandry because of their observed growth-enhancing effect, when added in subtherapeutic doses to animal feed. However, the widespread use of antimicrobials outside human medicine is the cause for the alarming emergence in humans of bacteria that have acquired resistance to antimicrobials. This situation is causing a serious threat to the public health, as more and more infections can no longer be treated with the presently known antidotes (*1, 2*). However, although the amount of antimicrobials used in food animals is not known precisely, it is estimated that about half of the total amount of antimicrobials produced globally is used in food animals. The increase in meat production in many developing countries is mainly due to intensified farming, which is often coupled with increased antimicrobial usage for both disease therapy and growth promotion. Some of the newly emerging resistant bacteria in animals are transmitted to humans, mainly via meat and other food of animal origin or through direct contact with farm animals. The best-known examples are the foodborne pathogenic bacteria *Salmonella* and *Campylobacter* and the commensal (harmless in healthy persons and animals) bacteria *Enterococcus*.

Sulfonamides are an important group of antimicrobials, whose chemical structure contains a 4-aminobenzensulfonamide functionality with different heterocycles attached to the N1-position of the sulfonamide bridge (see **Figure 1**). During the last decades, sulfonamide residues have been detected at significant concentrations levels in many biological samples, such cow's milk (*3–5*). To safeguard the public, maximum residues limits (MRL) of sulfonamides have been established in different matrices. The European Union, Canada, and the USA have stipulated MRLs of 100 μg Kg^{1-} for total sulfonamides in edible tissues (*6, 7*). On the other hand, antibiotic residues in milk samples is the cause of important economical losses in the dairy products industry because of the inhibition caused in the fermentation processes (*8*).

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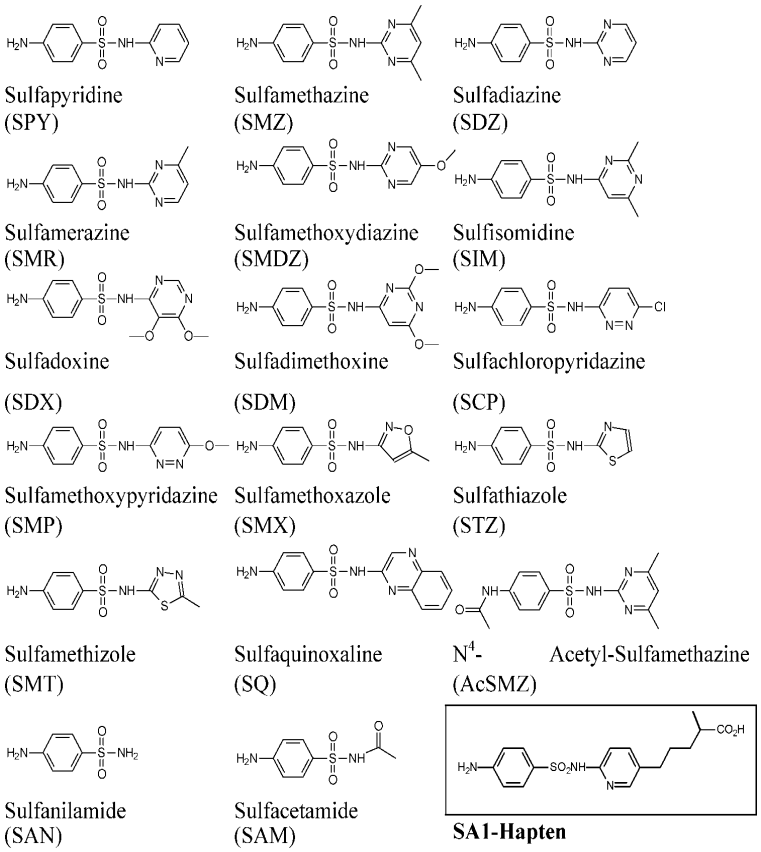

Figure 1. Chemical structures and abbreviations of some of the most common sulfonamides. The selected immunizing hapten (SA1) is also shown.

Table 1. Features of the Three Immunoassays Developed against Sulfapyridine[a]

	As154/SA1-HRP	As167/SA1-HRP	Ab167-MNP/SA1-HRP
A_{max}	1.04 ± 0.08	0.71 ± 0.23	0.86 ± 0.35
A_{min}	0.04 ± 0.01	0.05 ± 0.03	0.07 ± 0.01
IC_{50}, $\mu g\ L^{-1}$	11.21 ± 2.88	5.35 ± 2.40	8.37 ± 2.10
dynamic range, $\mu g\ L^{-1}$	1.6 ± 0.5 to 72.8 ± 23.7	0.7 ± 0.3 to 36.8 ± 16.5	1.2 ± 0.3 to 57.3 ± 21.1
LOD, $\mu g\ L^{-1}$	0.44 ± 0.13	0.22 ± 0.11	0.38 ± 0.12
slope	−0.75 ± 0.05	−0.69 ± 0.07	−0.76 ± 0.14
R^2	0.972	0.985	0.974
N	6	4	6

[a] Data correspond to the average and standard deviation of the parameters extracted from the logistic equation used to fit the standard curves. Assays were performed using three-well replicates on different days.

Usually, sulfonamide antibiotic residues are analyzed in samples such as milk, liver, meat, eggs, and feedstuff. However, recently, other animal target samples such as hair are being seriously considered to be taken and used to control misuse of contaminants in animals due to their lower metabolic and degradation activity as compared with conventional biological samples. This point could be very attractive for the official control organizations because hair analysis could increase the time window in retrospective detection of veterinary drug residues. Over the last 20 years, hair testing has gained increasing attention and recognition for the retrospective investigation of chronic drug abuse as well as intentional or unintentional poisoning (for reviews see refs *9–13*). Originally used to evaluate human exposure to heavy metals, hair analysis is used for criminal court proceedings (*9, 14*), clinical purposes (*15*), and doping control (*16–19*). Whereas urine analysis allows the detection of residues during a period ranging from several hours to 2–3 weeks, hair testing permits long-term detection, sometimes up to several months. Deposition of anabolic hormones (*16, 19*) and β-agonists (*20*) was confirmed in bovine hairs. The accumulation of different antibiotics, such as fluoroquinolones and sulfonamides, particularly in racehorse hair (*15, 21*), has also been reported. Recently, some of us have described that deposition of fluoroquinolones and sulfonamides antibiotics also occurs in cattle and pig hair after a veterinary administration of these drugs (*22, 23*).

Traditionally, most of the tests used to detect antibiotic residues have taken advantage of their antibacterial activity. Thus, growth inhibition tests have been used in different animal matrices; however, they are time-consuming and no conclusion may be drawn about the identity of the antibiotic or its concentration (*24*). Other methods for the analysis of sulfona-

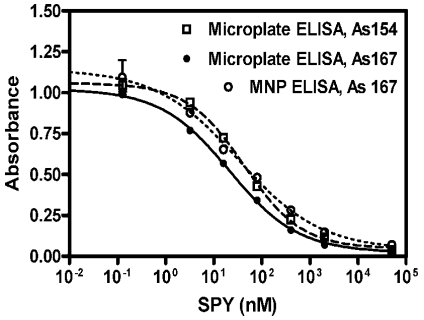

Figure 2. Standard curves of the microplate (As154/SA1-HRP and As167/SA1-HRP) and Ab-MNP-ELISAs (Ab167/SA1-HRP) using SPY as standard analyte. The curves were prepared using three-well replicate. The features of the assays are summarized in **Table 1**.

mides, such as HPLC (*25–27*) and GC (*28*), are highly specific techniques but require extensive sample preparation, sophisticated equipment, and skilled laboratory personnel, thereby limiting their use as fast and efficient high-throughput screening (HTS) methods. As an alternative, immunochemical techniques can be excellent HTS tools for residue analysis in different matrices because of their high detectability and specificity. Attending to it, the main objective of this work was the establishment of a rapid and reliable immunochemical method for the detection of sulfonamides in complex biological samples while avoiding sample preparation methods.

Several monoclonal (*29–32*), polyclonal (*33–35*), and recombinant (*36–38*) antibodies against sulfonamide antibiotics with different recognition patterns versus the different congeners have been reported. However, in very few occasions, direct performance of these antibodies in complex biological samples has been demonstrated. Thus, for tissue immunochemical analysis, several procedures involving different extraction/cleanup methods have been reported (i.e., refs *39 and 40*.). Direct analysis of honey samples by enzyme-linked immunosorbent assays (ELISAs) has also been attempted, but high dilution factors (100 times) had to be applied to avoid matrix interferences. Alternatively, solid-phase extraction methods have been introduced prior the immunochemical analysis. Regarding milk, homogenization and protein removal, by precipitation with trichloroacetic acid or acetone followed by neutralization or dilution, have been some of the sample preparation methods reported to analyze this sample by immunoassay (*36, 41*). Antibody features strongly determine the extent of these undesired effects. On the other hand, it has been reported that the use of magnetic beads may assist in minimizing matrix interferences, increasing efficiency of washing and separation steps, besides improving performance due to an increase in the surface area and the faster assay kinetics, because the immunoreaction takes place in a suspension. In this paper, we describe antibody production by using suitable immunizing hapten combined with the use magnetic nanoparticles with the objective to achieve direct immunochemical detection of sulfonamide antibiotics in milk and hair samples.

MATERIALS AND METHODS

Chemicals and Supplies. All the sulfonamides used in this work were from Riedel-de Haën (Seelze, Germany). The synthesis of the hapten SA1 (5-[6-(4-amino-benzenesulfonylamino)-pyridin-3-yl]-2-methyl-pentanoic acid), will be described elsewhere. Horseshoe crab hemocyanin (HCH), bovine serum albumin (BSA), horseradish peroxidase (HRP), and other biochemical reagents were from Sigma Chemical Co. (St. Louis, Missouri). Magnetic nanoparticles (MNP) modified with carboxyl groups (MP−COOH) with 196 nm of diameter and active chemical functionality of 0.155 mmol g^{-1} were purchased from Estapor (Product No. 00–39, Merck). The filters were Millex-GV 0.22 μm filters (Millipore Corp., Bedford, Massachusetts). Polystyrene microtiter plates used in the ELISA were purchased from Nunc (Maxisorp, Roskilde, Denmark). In the Ab-MNP-ELISA, round-bottom well low-binding microtiter plates were used (Product 900010, U-form individually wrapped, Daslab).

Buffers and Solutions. Unless otherwise indicated, phosphate-buffered saline (PBS) is 0.01 M phosphate buffer and 0.8% saline solution, pH 7.5. PBST is PBS with 0.05% Tween 20. Borate buffer is 0.2 M boric acid/sodium borate, pH 8.7. Coating buffer is 0.05 M carbonate/bicarbonate buffer, pH 9.6. Citrate buffer is a 0.04 M solution of sodium citrate, pH 5.5. The substrate solution contains 0.01% TMB (3,3′,5,5′-tetramethylbenzidine) and 0.004% H_2O_2 in citrate buffer.

Instrumentation. ^{1}H and ^{13}C NMR spectra were obtained with a Varian Inova 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C) (Varian Inc. Palo Alto, California). Thin-layer chromatography (TLC) was performed on 0.25 mm, precoated silica gel F_{254} on aluminum sheets (Merck, Darmstadt, Germany). HPLC analysis was performed with a Merck Hitachi pump L-7100, a diode array detector L-7455, an autosampler L-7200, and an interface D7000 (Merck, Darmstadt, Germany). The matrix-assisted laser desorption ionization-time-of-flight mass spectrometer (MALDI-TOF-MS) used for analyzing the protein conjugates was a Perspective BioSpectrometry Workstation provided with the Voyager-DE-RP software. Washing steps were performed on a SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). Absorbances were read on a SpectramaxPlus (Molecular Devices, Sunnyvale, California). For the Ab-MNP-ELISA, Dynal MPC-S (Product No. 120.20, Dynal Biotech ASA, Norway) or 96-well plate magnetic separation racks (CD1001, Cortex Biochem, CA, USA) were used. The oven employed for the hair alkaline digestion was from Digitronic (JPSelecta, Abrera, Spain).

Immunoreagents. The *haptenized proteins* (SA1-HCH, SA1-BSA, and SA1-HRP) were prepared by covalently coupling the hapten SA1 to the proteins using the active ester method. Briefly, SA1 (10 μmol), N-hydroxysuccinimide (NHS) (25 μmol), and 1,3 dicyclohexylcarbodiimide (DCC) (50 μmol) were left to react in 200 μL of dry DMF at room temperature (RT) for 3 h. The suspension was centrifuged for 10 min at 10 000 rpm, and the supernatant was slowly added to a solution of the protein (10 mg of protein or 2 mg of HRP in 1.8 mL of borate buffer. The mixture was left to react for 3 h at RT. The conjugates were purified using a Sephadex G-25 desalting column and were stored at 4 °C. Stock solutions of the conjugates (1 mg mL^{-1} in PBS) were stored at −20 °C and working aliquots at 4 °C. The hapten density of the conjugates was assessed by MALDI-TOF-MS by comparing the molecular weight of the bioconjugates with those intact proteins. *Antibodies* As155–157 and As167 were raised against SA1-HCH using the active ester method. Four female New Zealand white rabbits, weighting 1–2 Kg, were immunized following the immunizing protocol already described (*42*). The evolution of the antibody titer was assessed by measuring the binding of serial dilutions of the antisera to a microtiter plate coated with SA1-BSA. After an acceptable antibody titer was observed, the animals were exsanguinated and the blood was collected on vacutainer tubes provided with a serum separation gel. Antisera were obtained by centrifugation and stored at −40 °C in the presence of 0.02% NaN_3. *Antibody derivatized magnetic nanoparticles* (Ab-MNP) were prepared by coupling a purified fraction of immunoglobulins, prepared by ammonium sulfate precipitation (*43*) from As167, to magnetic beads. A solution (2.5 mL) of 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (EDC, 45 mg, 0.23 mmol) and NHS (27 mg, 0.23 mmol) in PBS (3 mL, pH 6) was mixed with a suspension of MNP (250 μL, 50 mg mL^{-1}), previously washed twice with PBS (pH 6), and the mixture was shaken for 15 min at RT. The magnetic beads were then washed with HCl (2 mM, 1 mL) and resuspended in PBS (20 mM pH 7.5, 1.5 mL). Then, a PBS solution of the antibody (5 mg mL^{-1}, 71 μL) was added to the suspension of activated MNP, and the reaction was allowed to proceed for two hours at RT with gentle mechanical shaking to minimize the sedimentation of beads. The particles were washed again three times with PBS (20 mM, pH 7.5)

Table 2. Selectivity of As154/SA1-HRP and As167/SA1-HRP Direct ELISA[a]

	microplate ELISA				Ab-MNP ELISA	
	As154/ SA1-HRP		As167/SA1-HRP		Ab167/SA1-HRP	
sulfonamide	IC_{50}[b]	% CR	IC_{50}[b]	% CR	IC_{50}[b]	% CR
sulfapyridine (SPY)	11.7	**100**	4.6	**100**	6.3	**100**
sulfaquinoxaline (SQX)	933.7	2	547.5	1	≫MRL	<0.1
sulfachloropyridazine (SCP)	94.6	**14**	88.8	**6**	79.7	**8**
sulfamethoxazole (SMX)	1298.3	1	204.7	2	≫MRL	<0.1
sulfisomidine (SID)	972.5	1	>13916	<0.01	≫MRL	<0.1
sulfathiazole (STZ)	36.4	**33**	97.7	**5**	7.9	**80**
sulfadiazine (SDZ)	779.6	2	344.7	1	≫MRL	<0.1
sulfadimethoxine (SDM)	1752	1	646.8	1	390.9	2
sulfamerazine (SMR)	649.9	2	616.4	1	133.2	5
sulfadoxine (SDX)	>15516	<0.1	976.6	1	≫MRL	<0.1
sulfamethoxypyridazine (SMP)	26.8	**49**	8.5	**61**	15.7	**40**
sulfamethazine (SMZ)	>13916	<0.1	1623.2	0.3	≫MRL	<0.1
N^4-acetyl-sulfamethazine	>16005	<0.1	1132.8	1	≫MRL	<0.1
sulfanilamide (SIA)	>8600	2	401.2	1	≫MRL	<0.1

[a] The percentage of recognition has been expressed as cross reactivity (CR%) according to the expression [IC_{50}(SPY)/IC_{50}(crossreactant)] × 100. Chemical structures of the different sulfonamides are shown in **Figure 1**. [b] Concentrations are expressed in $\mu g\ L^{-1}$.

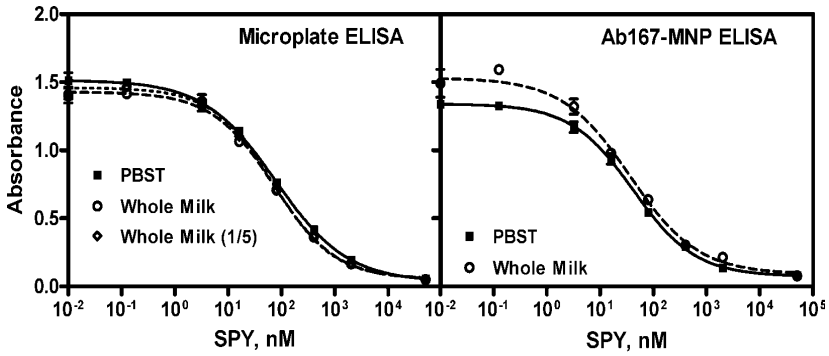

Figure 3. Matrix effect produced by full-cream fresh milk on the As167/SA1-HRP ELISA (left panel) and Ab-MNP-ELISA (right panel). Milk was directly used without including any previous pretreatment and was measured undiluted or after 5× dilution with the assay buffer. Curves were constructed in milk using SPY as standard. The points are the average of three-well replicates.

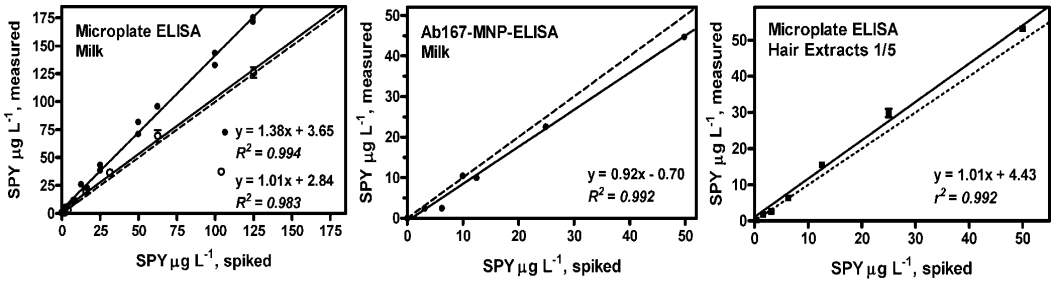

Figure 4. Accuracy studies performed using blind spiked samples prepared in full-cream fresh milk and used directly (solid symbols) in the ELISAs or after 5× dilution with the assay buffer (open symbols) with the As167/SA1-HRP microtiter-plate (left panel) and Ab-MNP (center panel) ELISAs. Accuracy of As167/SA1-HRP microtiter-plate ELISA measuring SPY in hair extracts is also shown (right panel). Blind spiked samples were prepared with the pigmented calf hair extracts. Before ELISA measurements, the extract was buffered and diluted 5× with the assay buffer. In all cases, graphics show the correlation between the spiked concentrations and the values measured. The data shown correspond to the average of at least two well replicates. The dotted line corresponds to a perfect correlation (slope = 1).

and finally resuspended in the same buffer containing 100 mM Glycine, 0.05% (w/v) BSA, and 0.02% NaN_3 to reach a 50 mg mL^{-1} stock solution of Ab167-MNP. The efficiency of the coupling was evaluated using the Bradford test (*44*), analyzing the protein concentration in the supernatant before and after the coupling reaction, using pure IgG to construct the standard curve.

Checkerboard Titration Experiments. The avidity of the antibodies for the enzyme tracer SA1-HRP was determined using two-dimensional (2D) checkerboard titration experiments by measuring the binding of serial dilutions of the enzyme tracer SA1-HRP (1−0.015 $\mu g\ mL^{-1}$ and zero in PBST) to microtiter plates coated with 12 different dilutions of the antisera (1/1000–1/1024000 in coating buffer). After 30 min of incubation at RT, the plates were washed four times with PBST, and the substrate solution (100 μL/well) was added and incubated for 30 min more before stopping the reaction with H_2SO_4 4N (50 μL/well) and reading the absorbances at 450 nm. For the Ab-MNP, a solution of Ab167-MNP (2 mg mL^{-1} in PBST) was prepared, sonicated, and washed three times with PBST. Solutions (50 μL/well) of Ab167-MNP

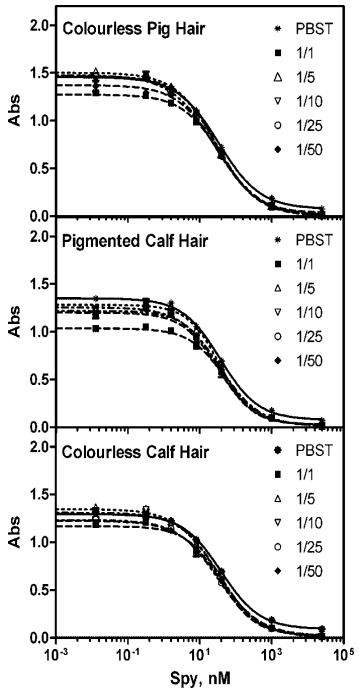

Figure 5. Matrix effect produced by extracts prepared using different types of hair (pigmented or colorless) from calf or pig in the microtiter plate ELISA. Samples were obtained from untreated controlled animals. Aqueous extracts were prepared with 0.1 M NaOH or 0.2 M NH_4OH. Before ELISA measurements, the extracts were buffered and diluted with the assay buffer several times. Curves were constructed using SPY as standard. The points are the average of three-well replicates.

(2–0.031 mg mL^{-1} in PBST) were added to wells of a 96 round-bottom well microtiter plate containing different concentrations of SA1-HRP (1–0.015 μg mL^{-1} in PBST, 50 μL/well) and incubated for 30 min at RT and under slight shaking. The Ab167-MNP were separated and washed three times with PBST (100 μL/well) using a magnetic separation rack. Then, the substrate was added, and the absorbances were read as described above. In both formats, optimal concentrations were chosen to produce absorbances around 0.7–1 units of absorbance under nonsaturating conditions.

Direct ELISA. *Microtiter Plate-ELISA.* The microtiter plates were coated with the antisera appropriately diluted in coating buffer (100 μL/well) overnight at 4 °C and covered with adhesive plate sealers. The day after, the plates were washed (four times with PBST 300 μL/well), and the sulfapyridine standard solutions (0.125 nM −50 μM and zero in PBST, 50 μL/well) followed by SA1-HRP appropriately diluted in PBST (50 μL/well) were added; the plates were then incubated for 30 min at RT. The plates were washed, and the substrate solution was added (100 μL/well). The plates were incubated for 30 min protected from light at RT before the enzymatic reaction was stopped by adding H_2SO_4 4N (50 μL/well). The absorbances were read at 450 nm. The standard curve was fitted to a four-parameter logistic equation according to the formula $y = (A - B/[1 - (X/C)D] + B$, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve. To improve the immunoassay features, using the best antiserum/enzyme tracer combination, a set of experimental parameters (detergent concentration, ionic strength, length of the competition time, and preincubation effect) were sequentially studied as previously described (*45*).

Ab-MNP-ELISA. The competitive experiments were performed in a similar way as described above. The Ab167-MNP suspension (0.25 mg mL^{-1}, 50 μL/well) was first added to the microtiter plate, followed by the standard solutions of SPY (from 50 μM to 0.125 nM and zero, 25 μL/well) and the SA1-HRP solution (0.025 $\mu m L^{-1}$, 25 μL/well), and the mixture was incubated for 30 min at RT and under gentle shaking. After washing, the assay proceeded as described before.

Selective Studies. Stock solutions of thirteen sulfonamides (10 mM in DMSO) were prepared and stored at 4 °C. Standard curves (0.125 nM to 50 μM and zero) were prepared in PBST by serial dilution. Each IC_{50} was determined in the competitive experiments following the optimized protocol described above. The cross-reactivity (CR) values were calculated according to the equation [IC_{50}(SPY)/IC_{50}(cross-reactant) × 100].

Samples. *Milk samples* were obtained from a normal grocery store and were used directly for the analysis or after dilution with PBS.

Hair samples, finely cut, were obtained in the Centre de Tecnologia de la Carn (IRTA, Girona, Spain) from different untreated controlled farm animals. Thus, different types of animal hair (colorless pig, colorless calf, and pigmented calf hair, 50 mg) were taken and extracted with 0.1 M NaOH (4 mL, 1 h at 60 °C for pig hair) or 0.2 M NH_4OH (4 mL, 24 h at 60 °C, for calf hair). Subsequently, extracts were centrifuged and filtrated through a paper filter as described (*23*).

ELISA Matrix Effect Studies. *Full-cream fresh milk* was used, either directly of after dilution with PBS, to prepare SPY standard curves and to analyze them in both immunoassay formats as described previously. The sigmoidal curves obtained were compared to that prepared in the assay buffer to evaluate the extent of the interferences caused by the matrix.

Hair extracts prepared as described above were buffered by adding 10% (v/v) of 100 mM PBS and adjusting the pH to 7.5 with few drops of 5N HCl, if necessary. Then, the extracts were diluted with PBS and used to prepare SPY standard curves that were measured in the microplate ELISA as previously described. The sigmoidal curves obtained with hair extracts were compared to the buffer standard assay to evaluate the extent of the interferences caused by the matrix.

Accuracy Studies. *Milk.* Different blind spiked samples were prepared in full-cream fresh milk and measured directly using the microtiter plate ELISA (undiluted or diluted 5× in PBS) and the Ab-MNP-ELISA (undiluted). Analyses were done in triplicate. The correlation was evaluated by establishing a linear regression between the spiked and the measured values.

Hair. Blind spiked samples were prepared using extracts from pigmented calf finely cut hair. The samples were buffered with 10% (v/v) 100 mM PBS, diluted 5× with the same buffer and measured using the microtiter plate ELISA as described before. Analysis were performed in duplicate. Accuracy was evaluated by establishing a linear regression between the spiked and the measured values.

RESULTS AND DISCUSSION

Microplate and Ab-MNP ELISAs. Immunoreagents were prepared by covalently coupling hapten SA1 to HCH, BSA, and HRP with good yields (∼14 mols of hapten per mol of BSA according to MALDI-TOF-MS). SA1-HCH was used to raise antibodies (As154, As155, As156, and As167) in white New Zealand rabbits. All of these immunoreagents were used to establish competitive immunochemical protocols to detect sulfonamide antibiotics in milk samples, using the standard microtiter plate as solid phase or MNP. Ab-MNP were prepared using the IgG fraction obtained by ammonium sulfate precipitation.

In the microtiter plate ELISA format, As154 and As167 provided the assays with the best detectability using SPY as analyte, with IC_{50} values lower than 10 $\mu g L^{-1}$. Factors such as preincubating the analyte with the coated antibody or varying the length of the competitive step did not affect the assay, although incubation times shorter than 20 min led to a decrease in the maximum signal without improving the IC_{50} value. No significant effect on the immunoassay detectability was observed when varying the percentage of Tween 20 in the assay buffer. The effect on the ionic strength on the assay performance was evaluated in media with conductivity values ranging from 0 to

70 mS/cm (0− 50 mM PBS). The assay tolerated variations in the ionic strength of the media quite well, although the total absence of salts produced a drastic decrease of the maximum signal. According to these studies, a direct ELISA protocol was established consisting of coating the microtiter plates with the antibodies overnight at 4 °C, followed by a 30 min competition step between the enzyme tracer and the analyte for the antibody. The assay buffer was 10 mM PBS (15 mS cm^{-1}) with 0.05% of Tween 20.

The features of the As154/SA1-HRP and As167/SA1-HRP immunoassays summarized in **Table 1** are the average of four assays performed on different days. The LOD (0.49 and 0.22 $\mu g\ L^{-1}$, respectively) and IC_{50} values (8.25 and 5.35 $\mu g\ L^{-1}$, respectively) were considered sufficiently good to analyze milk samples, considering that the MRL established by the EC (Regulation 2377/90) in this matrix is 100 $\mu g\ Kg^{1-}$.

Antibody As167 was selected to further investigate performance of the assay using magnetic nanoparticles because, in combination with SA1-HRP, it had provided the ELISA with the best detectability. Partially purified antibodies (ammonium sulfate IgG fraction, Ab167) were covalently coupled through their amino groups to the magnetic particles (196 nm of diameter) functionalized with carboxylic groups (MP−COOH) following standard carbodiimide reaction conditions. The efficiency of the coupling was evaluated using the Bradford test, comparing the IgG concentration in the supernatant after the coupling procedure (20 $\mu g\ mL^{-1}$) with the initial value (98 $\mu g\ mL^{-1}$), and indicated that conjugation had taken place. Checkerboard titration experiments were used to select the appropriate concentrations of Ab167-MNP and SA1-HRP to be employed in the competitive experiments. All of the incubation steps were performed under a gentle shaking to avoid the deposition of the magnetic beads on the bottom of the wells and to facilitate the interaction with the other species. The experiments were performed using the same buffer media as that in the microplate ELISA format. As it can be observed in **Table 1**, the immunoassay detectability was slightly lower than that accomplished by the same immunoreagents in the microtiter plate format. However, with a LOD of 0.38 $\mu g\ L^{-1}$ and considering the excellent values of the rest immunoassay parameters (high maximum signal, low background and good slope, see **Table 1**), the Ab-MNP-ELISA was considered suitable to assess its performance in real matrices. **Figure 2** shows the calibration curves of the three immunoassays established.

Selective Studies. A set of 13 common sulfonamides were assessed using the two antibody/enzyme tracer combinations. As it can be observed in **Table 2**, SPY, sulfamethoxypyridazine (SMP), sulfathiazol (STZ), and sulfachlorpyridazine (SCP) were the antibiotics better recognized in both assays. This recognition pattern demonstrates the importance of the substitution group in the N1 position. Other sulfonamides containing a pyrimidine group, such as sulfadiazine, sulfamerazine, sulfamethazine, sulfadimethoxine, sulfadoxine, and sulfaisomidine were not recognized, probably because of the presence of two electroattractive groups close to the carbon linked to the sulfonamide bond, which affects the charges of nitrogen and the aromatic carbon. In spite of the chemical similarities between SMP and SCP (see **Figure 1** for chemical structures), the first one was recognized much better in both assays. The chlorine atom, at the para position in respect to N1, instead of the methoxy group, drastically reduced the recognition by both antibodies (from 49 to 14% and from 61 to 6% for As154 and As167, respectively). STZ was better recognized by As154 than by As167. Thus, in general, As 167 was more selective for SPY and SMP than As 154. It should be remarked that it has already been reported that homologous antibody/competitor combinations (same hapten as immunogen and as competitor) tend to rend more specific immunoassays (*46–48*).

Analysis of Milk Samples. The applicability of the developed immunoassays to measure milk samples was initially evaluated by determining the potential interference of this matrix on the parameters of both microplate and Ab-MNP- ELISAs. For this purpose, fresh full-cream milk was used to prepare standard curves with SPY. As can be observed in **Figure 3** the curve made in pure milk mimicked very well the standard curve in buffer, achieving almost a perfect superposition. Only a slight reduction of the maximum signal was observed in the Ab-MNP-ELISA, although the IC_{50} was not affected. The same results were observed when analyzing different brands of fresh milk samples. These results suggested the possibility of directly analyzing full-cream milk with both ELISA formats without any kind of sample pretreatment. The absence of significant interferences from the milk samples seemed independent from the format used (microplate or Ab-MNP), and it may be related to the affinity and selectivity of the antibodies used. To demonstrate performance of the ELISAs in milk, blind spiked samples were prepared and measured with both formats. **Figure 4** shows the correlation observed between the measured and the spiked values. The results demonstrate that the accuracy of the Ab-MNP-ELISA is better (center panel), whereas a slight overestimation was observed when the samples were measured undiluted with microtiter-plate ELISA (left panel, solid symbols). The greater accuracy of the Ab-MNP-ELISA could be related to the matrix effect caused by milk. Because detectability of these assays is high enough, same samples were measured after a 1/5 dilution with the assay, and as it can be observed in **Figure 4** (left panel, open symbols), this treatment completely reduced the undesired effect observed before with the microplate-based ELISA.

Analysis of Hair Samples. Similarly, colorless hair samples obtained from pig and pigmented and colorless hair samples from calf were finely cut and extracted under alkaline conditions. The extracts were buffered to adjust the pH and then diluted several times in PBS. SPY standard curves were prepared with these extracts and measured in the microplate ELISA to assess the parallelism with the standard curve prepared in buffer. **Figure 5** shows that just a 5× dilution of the buffered hair extracts was sufficient to avoid undesired matrix effects caused from the matrix, independent of the type of hair. Following this consideration, accuracy was evaluated by measuring blind spiked samples of the hair extracts. A very good correlation between the measured and the real values was obtained as shown in **Figure 4** (right panel). Results obtained demonstrate the suitability of the immunoassay for determination of SPY in hair samples.

In conclusion, antibodies have been raised against a hapten possessing a linker in the pyridine group of the sulfapyridine. The antibodies have been used to establish two ELISAs, one using microtiter plates as solid-phase, whereas the other uses magnetic nanoparticles. Both formats allow detection of two important sulfonamide antibiotics (SPY and SMP) with LODs values below 0.5 $\mu g\ L^{-1}$, which is much more below the MRLs set by the EC for residues of these antibiotics in food samples. Milk samples can directly be measured without any sample treatment with both formats, although the use of magnetic beads has demonstrated more accurate results. This lack of accuracy was solved in the microplate-based ELISA by just diluting the

sample 5× with the assay buffer. Similarly, sulfonamide residues can be easily analyzed in hair extracts with very good detectability. The results shown in this paper demonstrates that direct analysis of complex matrices is possible with the antibodies developed in this work. Both ELISA formats perform well, although the results obtained with the Ab-MNP-ELISA open the door to development of a variety of other immunochemical techniques (i.e.immunosensors) involving extraction steps with MNP, or microlfuidic components in which the Ab-MNP can be driven with a magnet to the detector, or just immunosensors based on magnetic principles.

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3.3 SAs indirect ELISA format (Milk)

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Generation of Broad Specificity Antibodies for Sulfonamide Antibiotics and Development of an Enzyme-Linked Immunosorbent Assay (ELISA) for the Analysis of Milk Samples

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Immunoreagents appropriately produced to detect a wide range of sulfonamide antibiotic congeners have been used to develop a highly sensitive enzyme-linked immunosorbent assay (ELISA). The selectivity has been achieved by combining antibodies raised against 5-[6-(4-aminobenzenesulfonylamino)pyridin-3-yl]-2-methylpentanoic acid (SA1), covalently coupled to horseshoe crab hemocyanin (HCH), and 5-[4-(amino)phenylsulfonamide]-5-oxopentanoic acid (SA2), coupled to ovalbumin (OVA), on an indirect ELISA format. The immunizing hapten has been designed to address selectivity against the common aminobenzenesulfonylamino moieties, using theoretical calculations and molecular modeling tools. Hapten SA1 has been synthesized in four steps from methyl 5-(4-amino-3-pyridinyl)-2-methyl-4-pentenoate through a Heck reaction, under Jeffery conditions, to avoid introduction of additional epitopes in the linker. The microplate immunoassay developed is able to reach the necessary detectability for the determination of the sulfonamide antibiotics most frequently used in the veterinary field, in compliance with the EC Regulation 2377/90. As an example, the IC_{50} and LOD values accomplished for sulfapyridine are 2.86 ± 0.24 and 0.13 ± 0.03 $\mu g\ L^{-1}$, respectively. Studies performed with different types of milk samples demonstrate that direct and accurate measurements can be performed in this type of matrix without any previous sample cleanup method.

KEYWORDS: Sulfonamide antibiotics; hapten; molecular modeling; immunoassay; milk; class-selective antibodies

INTRODUCTION

Antibiotics are chemical substances extremely active at low doses that kill or slow the growth of bacteria. In the past decade, the irresponsible use of antibiotics in human medicine added to their inappropriate use in the veterinary field to prevent diseases and to improve productivity has favored the growth of bacterial resistance (*1*). Antibiotic-resistant bacteria may arrive in humans through the food chain or by contact with animals, causing diseases that can no longer be treated with presently known antibiotics (*2*). Moreover, antibiotic resistance causes an important impact on the ecosystem, water, and soil-dwelling organisms, producing adverse effects in the ecosystem. Actually, actions such as wastewater treatment plant effluents and confined animal feeding operations represent important sources of antibiotics to the environment (*3*). According to some environmental studies reported, as much as 90% of the sulfonamides are excreted after consumption, contaminating the subsoil, ground, and superficial water resources (*4*).

Governmental agencies have set limitations on the levels of residues attending to toxicological data. In Europe, EC Regulation 2377/90 sets maximum residue limits (MRL) for the majority of antibiotics in different sample tissues, and Directive 96/23/CE establishes procedures for inspection, dictating the frequency and number of substances that have to be monitored. However, the analytical procedures today available cannot respond to the requirements of these regulations because of their low throughput capabilities. Most of the traditional tests based on bacteria growth inhibition are slow and do not have the necessary detectability to comply with the legislation (*5*). Chromatographic techniques such as HPLC-UV (*6*) and/or HPLC-MS detector (*7*) are in contrast highly specific and can reach an excellent detectability. However, they usually require extensive sample preparation, sophisticated equipment, and skilled laboratory personnel. With this panorama, the need to develop alternative analytical technologies becomes clear, and

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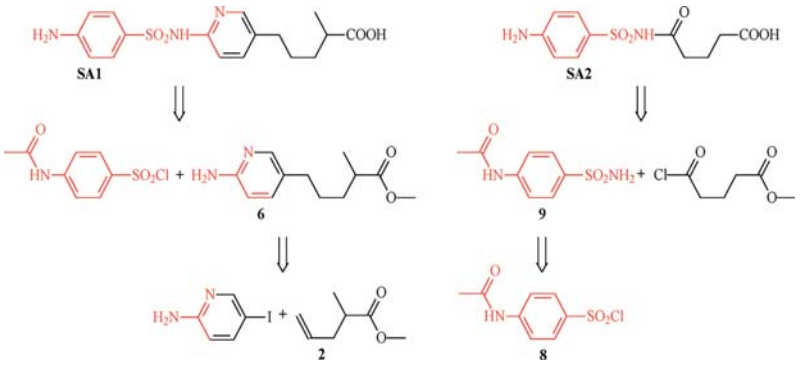


Figure 1. Structure and retrosynthetic analysis of sulfonamide **SA1** and **SA2** haptens.

for this reason, the EC and other governmental agencies are strongly supporting research in this area.

The milk and dairy products industries are between the sectors most affected by the presence of antibiotic residues, not only because of the adverse effects caused to the public health and ecosystems but also because of the economical losses derived by the effect of these biocides in the fermentation processes. Sulfonamides are among the four antibiotic families most frequently detected in these products. This antibiotic family is a wide group of synthetic antimicrobial agents that contain the sulfonamide group. A generic receptor able to interact with most of these congeners would allow development of new efficient and reliable biosensors or friendly user detection platforms. However, despite the efforts made by many research groups to produce class-selective antibodies (*8*−*13*), immunoassays recognizing a wide list of compounds of the same family with sufficient sensitivity to comply with the regulations have only seldom been reported (i.e., ref *14*). Similarly, most of the marketed immunoassay kits for sulfonamides detect only a few sulfonamide specimens below the MRLs (i.e., SulfaMRL by Charm Sci.; sulfamethazine sulfadimethoxine, sulfadiazine, and sulfathiazole; SNAP by IDEXX Laboratories is mainly addressed to sulfamethazine, etc.).

On the basis of our previous experience with molecular modeling tools to assist in hapten design and immunoassay development (*15, 16*), this paper reports the development of an immunochemical analytical method with a broad specificity for this antibiotic family. The approach consisted of raising polyclonal antibodies against an appropriately designed hapten and their use on an indirect competitive ELISA under heterologous conditions (different haptens as immunogen and as competitor). Moreover, we demonstrate the excellent performance of this ELISA to analyze these antibiotics in milk samples after just simple dilution treatment.

EXPERIMENTAL PROCEDURES

Chemistry. *General Methods and Instruments.* Thin-layer chromatography (TLC) was performed on 0.25 mm precoated silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany). Unless otherwise indicated, purification of the reaction mixtures was accomplished by "flash" chromatography using silica gel as the stationary phase. ^{1}H and ^{13}C NMR spectra were obtained with a Varian Inova-500 (Varian Inc., Palo Alto, CA) spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). Liquid chromatography−electrospray ionization/mass spectrometry (LC-ESI/MS) was performed in a Waters (Milford, MA) model composed of an Acquity UPLC system directly interfaced to a Micromass LCT Premier XE MS system equipped with an ESI LockSpray source for monitoring positive ions. Data were processed with MassLynx (v 4.1) software (Milford, MA).

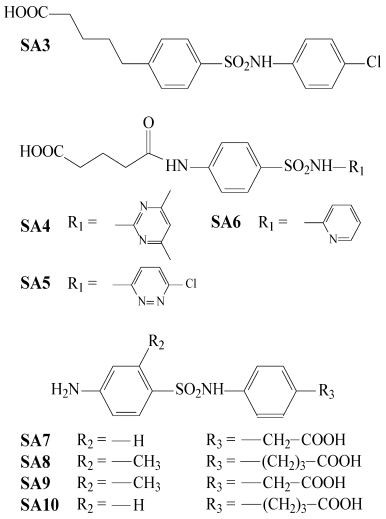


Figure 2. Chemical structures of the competitor haptens.

Molecular Modeling and Theoretical Calculations. Computational methodology was carried out by running the Hyperchem 6.03 software package (Hypercube Inc., Gainsville, FL) to compare physical−chemical features of the haptens with 12 of the most common sulfonamides. Minimum energy conformation, geometry, and molecular charge distribution were calculated using a semiempirical quantum model (PM3).

Preparation of Sulfonamide Haptens. Immunizing and competitor haptens **SA1** and **SA2** were synthesized following the retrosynthetic scheme shown in **Figure 1**. Experimental details on the synthetic and purification procedures together with their spectroscopic and spectrometric characterization can be found below. Preparation of haptens **SA3**−**SA10** (see **Figure 2**) also tested as competitors is described in the Supporting Information (see Figure A).

*Synthesis of the Immunizing Hapten SA1. Methyl 2-methyl-4-pentenoate (***2***).* Dimethyl sulfate (5 mL, 53 mmol) was added to a freshly prepared solution of 2-methyl-4-pentenoic acid **1** (5 mL, 44 mmol) in 44 N NaOH (40 mL), and the mixture was vigorously stirred at room temperature. After 4 h, a new equivalent of dimethyl sulfate was added (5 mL, 44 mmol), and the mixture stirred for 8 h more. The pH of the reaction was constantly controlled and adjusted to pH 8 with 1 N NaOH. When reaction was complete, the organic layer was separated by liquid−liquid extraction and washed with 1 N NaOH to afford a pale yellow liquid corresponding to the pure ester **2** (3.9 g, 66%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.13 (d, J = 7 Hz, 3H, CH_3), 2.15 (m, 1H, CH_2), 2.38 (m, 1H, CH_2), 2.50 (m, 1H, CH), 3.64 (s, 3H, $COOCH_3$), 4.99 (d, J = 11 Hz, 1H, H_{cis}), 5.02 (d, J = 18 Hz, 1H, H_{trans}), 5.71 (ddt, J = 18 Hz, 11 Hz, 7 Hz, 1H, =CH−); ^{13}C NMR

(125 MHz, $CDCl_3$) δ 16.4 ($-\mathbf{C}H_3$), 37.7 ($-\mathbf{C}HCOO$), 39.1 ($-\mathbf{C}H_2$), 51.5 ($-O\mathbf{C}H_3$), 116.8 ($\mathbf{C}H_2=$), 135.4 ($=\mathbf{C}H-$), 176.5 ($-\mathbf{C}OO^-$).

*Methyl 5-(4-Amino-3-pyridinyl)-2-methyl-4-pentenoate (**3**).* A solution of tetrabutylammonium chloride (160 mg, 0.57 mmol) and $Pd(AcO)_2$ (13 mg, 0.06 mmol) in anhydrous dimethylformamide (anh DMF, 3 mL) was slowly added to a solution of 2-amino-5-iodopyridine (500 mg, 2.27 mmol) and potassium formate (153 mg, 1.82 mmol) in the same solvent (3 mL). Subsequently, a solution of the ester **2** (1.3 g, 11.4 mmol) in anh DMF (2 mL) was added to the mixture, and the reaction was kept at 65 °C under Ar atmosphere for 6 h. Additional equivalents of potassium formate (50 mg, 0.59 mmol) and the ester **2** (300 mg, 2.63 mmol) were added, and the progress of the reaction was monitored by TLC using ethyl acetate (AcOEt) as mobile phase. After the reaction was finished, the crude material was cooled and the solvent evaporated. The oil obtained was redissolved in MeOH (25 mL) and filtered through Celite to remove all of the palladium formed, and the solvent was evaporated. The residue obtained was dissolved again with AcOEt (50 mL) and washed with saturated $NaHCO_3$ (3 × 25 mL). The organic layer was separated, dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. The red oil obtained was purified by silica gel column chromatography using a 1:1 to 1:3 polarity gradient of hexane/AcOEt as mobile phase to obtain a yellow oil (250 mg) of a 4:1 mixture of the methyl 5-(4-amino-3-pyridinyl)-2-methyl-4-*trans*-pentenoate **3** (186 mg, 36% yield) and methyl 4-(4-amino-3-pyridinyl)-2-methyl-4-methylene-butanoate **4** (62 mg, 12% yield). **3**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.19 (d, J = 7 Hz, 3H, CH_3), 2.31 (m, 1H, CH_2), 2.53 (m, 1H, CH_2), 2.58 (m, 1H, CH), 3.67 (s, 3H, $COOCH_3$), 5.95 (dt, J = 16 Hz, J = 7 Hz, 1H, $=CH-$), 6.28 (d, J = 16 Hz, 1H, $-CH=$), 6.45 (d, J = 8.5 Hz, 1H, Ar_{meta}), 7.49 (dd, J = 8.5 Hz, J = 2.5 Hz, 1H, Ar_{ortho}), 7.99 (d, J = 2.5 Hz, 1H, Ar_{ortho}). **4**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.12 (d, J = 7 Hz, 3H, CH_3), 2.45 (m, 1H, CH_2), 2.90 (m, 1H, CH_2), 2.56 (m, 1H, CH), 3.63 (s, 3H, $COOCH_3$), 4.99 (d, J = 1 Hz, 1H, $CH_2=$), 5.20 (d, J = 1 Hz, 1H, $CH_2=$), 6.50 (d, J = 8.5 Hz, 1H, Ar_{meta}), 7.48 (dd, J = 8.5 Hz, J = 2 Hz, 1H, Ar_{ortho}), 8.12 (d, J = 2 Hz, 1H, Ar_{ortho}).

*Methyl 5-(4-Amino-3-pyridinyl)-2-methylpentanoate (**6**).* The mixture of esters **3** and **4** (250 mg) in MeOH (12 mL) was reduced under H_2 at atmospheric pressure in the presence of Pd/C (23 mg, 10% Pd, 0.018 mmol of Pd) for 12 h at room temperature. The reaction was passed through Celite, and the solvent was evaporated until dryness. The yellow oil obtained was purified by column chromatography using a 1:1 to 1:3 polarity gradient of hexane/AcOEt as mobile phase to isolate 180 mg of a yellow oil containing a 5:1 mixture of the desired ester **6** and methyl 4-(4-amino-3-pyridinyl)-2,4-dimethylbutanoate. Further purification of the mixture by preparative HPLC using a C_{18} column (25 × 1 cm, 5 μm, Kromasil 100) as stationary phase and ACN/H_2O (Et_3N/CH_3COOH, 0.2 M, pH 7.6) 30:70 as mobile phase allowed us to isolate the pure ester **6** (81 mg, 40% yield): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.13 (d, J = 7 Hz, 3H, CH_3), 1.42 (m, 1H, CH_2), 1.53 (m, 2H, CH_2), 1.65 (m, 1H, CH_2), 2.44 (m, 1H, CH), 2.45 (t, J = 8 Hz, 2H, CH_2), 3.65 (s, 3H, $COOCH_3$), 6.44 (d, J = 8.5 Hz, 1H, Ar_{meta}), 7.24 (dd, J = 8.5 Hz, J = 2.5 Hz, 1H, Ar_{ortho}), 7.85 (d, J = 2.5 Hz, 1H, Ar_{ortho}); ^{13}C NMR (125 MHz, $CDCl_3$) δ 17.4 ($\mathbf{C}H_3CH$), 29.3 ($\mathbf{C}H_2CH_2COO$), 32.2 ($\mathbf{C}H_2CH_2Pyr$), 33.9 ($\mathbf{C}H_2Pyr$), 39.6 ($\mathbf{C}HCOO$), 51.5 ($COO\mathbf{C}H_3$), 108.7 (C_3), 127.9 (C_5), 138.2 (C_4), 147 (C_6), 156.9 (C_2), 176.9 ($\mathbf{C}OOCH_2$).

*Methyl 5-[6-(4-Acetylaminobenzenesulfonylamino)pyridin-3-yl]-2-methylpentanoate (**7**).* Ester **6** (250 mg, 1.13 mmol) in anhydrous dioxane (2 mL) was added to a solution of *N*-acetyl-*p*-aminobenzenechlorosulfonic acid (290 mg, 1.24 mmol) in the same solvent (8 mL). Subsequently, triethylamine (175 μL, 1.24 mmol) was added slowly, and the mixture was kept under argon atmosphere for 15 h at room temperature. When the reaction was finished, the solvent was evaporated, and the oil obtained was redissolved with AcOEt (10 mL) and washed with saturated $NaHCO_3$ (3 × 5 mL). The organic layer was dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure to obtain yellow oil. Purification of the product was performed using a silica gel column, with 1:4 hexane/AcOEt as mobile phase to obtain the desired sulfonamide **7** (239 mg, 35% yield): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.13 (d, J = 7 Hz, 3H, CH_3), 1.41 (m, 1H, CH_2), 1.53 (m, 2H, CH_2), 1.65 (m, 1H, CH_2), 2.20 (s, 3H, CH_3CO), 2.43 (m, 1H, CH), 2.49 (t, J = 8 Hz, 2H, CH_2), 3.64 (s, 3H, $COOCH_3$), 7.52 (s, 1H), 7.57 (d, J = 9 Hz, 2H), 7.68 (d, J = 9 Hz, 2H), 8.13 (s, 1H), 8.28 (s, 1H).

*5-[6-(4-Aminobenzenesulfonylamino)pyridin-3-yl]-2-methylpentanoic acid (**SA1**).* A solution of the sulfonamide **7** (165 mg, 0.39 mmol) in 1 N NaOH (5 mL, 5 mmol) was kept under reflux for 4 h under argon atmosphere. The crude was cooled, acidified with concentrated HCl to pH 3, and extracted with AcOEt (3 × 5 mL). The organic layer was dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure to obtain the desired hapten **SA1** (105 mg, 73% yield) as a pale yellow oil: ^{1}H NMR (500 MHz, CD_3OD) δ 1.13 (d, J = 7 Hz, 3H, CH_3), 1.40 (m, 1H, CH_2), 1.58 (m, 2H, CH_2), 1.62 (m, 1H, CH_2), 2.41 (m, 1H, CH), 2.52 (t, J = 7.5 Hz, 2H, CH_2), 6.61 (d, J = 8.5 Hz, 2H), 7.12 (d, J = 8.5 Hz, 1H), 7.53 (dd, J = 8.5 Hz, J = 2.5 Hz, 1H), 7.56 (d, J = 8.5 Hz, 2H), 7.88 (d, J = 2.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 17.6 ($CH(\mathbf{C}H_3)$), 29.7 ($\mathbf{C}H_2CH_2COOH$), 32.6 ($\mathbf{C}H_2CH_2Pyr$), 34.2 ($\mathbf{C}H_2COOH$), 40.4 ($\mathbf{C}H_2Pyr$), 114.2 (C_3), 114.7 ($C_{meta\ SO_2NH_2}$), 127.9 ($C_{ortho\ SO_2NH_2}$), 130.1 (C_5), 132.6 ($C_{SO_2NH_2}$), 140.9 (C_4), 145.5 (C_6), 152.2 (C_2), 154.1(C_{NH_2}), 180.5 (COOH). HRMS (+EI) calcd for $C_{17}H_{22}N_3O_4S$ (M+): 364.1331; found, 364.1319.

*Synthesis of the Competitor Hapten SA2. N-(4-(Aminosulfonyl)phenyl)acetamide (**9**).* A 30% aqueous solution of NH_3 (5.6 mL, 4.3 mmol) was added slowly to a freshly prepared solution of *N*-acetyl-*p*-aminobenzenechlorosulfonic acid **8** (2 g, 8.6 mmol) in anhydrous acetonitrile (10 mL), and the mixture was kept for 2 h until the complete disappearance of the starting material by TLC analysis (1:1 AcOEt/CH_2Cl_2). The solvent was removed under reduced pressure, and the residue redissolved in water (20 mL) and extracted with AcOEt (3 × 10 mL). The organic layer was dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness to obtain the desired compound **9** (1.5 g, 76% yield) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$, 14:1) δ 2.17 (s, 3H, CH_3-), 7.71 (dd, J = 9.2 Hz, J = 2.1 Hz, 2H, Ar_{meta}), 7.84 (dd, J = 9.2 Hz, J = 2.1 Hz, 2H, Ar_{ortho}); ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$, 14:1) δ 24.6 (CH_3), 119.5 ($C_{meta\ SO_2Cl}$), 127.51 ($C_{ortho\ SO_2Cl}$), 139.6 ($C_{SO_2NH_2}$), 143.6 (C_{NHCOCH_3}), 171.9 (CO).

*Methyl 5-(4-(Acetylamino)phenylsulfonamide-5-oxopentatanoate (**10**).* Methyl 4-(chloroformyl) butyrate (0.65 mL, 4.69 mmol) was added slowly to a freshly prepared solution of **9** (1 g, 4.69 mmol) in pyridine (2 mL), and the mixture was kept under argon atmosphere for 2 h. Then, the reaction was stopped by adding concentrated HCl in ice (30 mL). The aqueous layer was extracted with AcOEt (3 × 15 mL), and the organic layer was dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure to obtain a pale yellow solid. Isolation of compound **10** was performed by crystallization with 9:1 2-propanol/water (0.85 g, 50% yield): ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$, 14:1) δ 1.79 (m, J = 7.2 Hz, 2H, $-CH_2-$), 2.17 (s, 3H, $-CH_3$), 2.25 (t, J = 7.2 Hz, 2H, CH_2CO), 2.27 (t, J = 7.2 Hz, 2H, $-CH_2Ph$), 3.65 (s, 3H, $COOCH_3$), 7.75 (dd, J = 9.2 Hz, J =2.1, Hz 2H, 2H, Ar_{meta}), 7.95 (dd, J = 9.2 Hz, J = 2.1 Hz, 2H, 2H, Ar_{ortho}); ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$, 14:1) δ 19.2 (CH_3), 23.6 ($\mathbf{C}H_2$), 32.4 ($NHCO\mathbf{C}H_2$), 34.5 ($\mathbf{C}H_2COOH$), 51.4 ($-OCH_3$), 119.1 ($C_{meta\ SO_2NH_2}$), 127.1 ($C_{ortho\ SO_2NH}$), 132.8 (C_{SO_2NH}), 143.4 (C_{NHAc}), 170.19 ($\mathbf{C}ONH$), 171.1 ($\mathbf{C}OOH$).

*Preparation of 5-[4-(Amino)phenylsulfonamide]-5-oxopentanoic Acid (**SA2**).* A solution of compound **10** (130 mg, 3.9 mmol) in 1 N NaOH (8 mL) was heated at 75 °C for 6 h until the total disappearance of the starting material was observed by TLC (9:1:1 EtAc/CH_2Cl_2/MeOH; 1% AcOH). The crude mixture was acidified with 1 N HCl to pH 2 and extracted with AcOEt (3 × 10 mL). The organic layer was finally washed with water, dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. Isolation of **SA2** was performed using a silica gel column and 1:2 AcOEt/CH_2Cl_2 (1% acetic acid) as mobile phase to obtain **SA2** (71 mg, 65% yield): ^{1}H NMR (500 MHz, CD_3OD) δ 1.79 (m, J = 7.2 Hz, 2H, $-CH_2-$), 2.25 (t, J = 7.2 Hz, 2H, CH_2COOH), 2.27 (t, J = 7.2 Hz, 2H, $-CH_2Ph$), 6.67 (dd, J = 7.2, J = 2.1 Hz, 2H, $2H_{Ar\ meta}$), 7.66 (dd, J = 7.2, J = 2.1 Hz, 2H, 2H, Ar_{ortho}); ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$, 9:1) δ 20.74 ($\mathbf{C}H_2$), 32.4 ($NHCO\mathbf{C}H_2$), 34.5 ($\mathbf{C}H_2COOH$), 113.8 ($C_{meta\ SO_2NH_2}$), 125.8 (Cortho SO2NH2), 131.6 (CSO2NH2), 154.9 (CNH2), 172.9 (CO). HRMS (+EI) calcd for C11H15N2O5S (M+): 287.0702; found, 287.0693.

Table 1. Hapten Densities of the BSA Conjugates Calculated by MALDI-TOF-MS

immunoreagent	δ-hapten[a]	% conjugation[b]
SA1−BSA	22.2	63−74
SA2−BSA	18.0	51−60
SA3−BSA	13.5	39−45
SA4−BSA	10.9	31−36
SA5−BSA	6.5	19−22
SA6−BSA	8.4	24−28
SA7−BSA	7.5	21−25
SA8−BSA	9.7	28−32
SA9−BSA	18.0	51−60
SA10−BSA	5.7	16−19

[a] Moles of hapten per mole of protein. [b] The conjugation is calculated on the basis of the assumption that BSA has 30−35 free lysine groups.

Immunochemistry. *General Methods and Instruments.* The matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS) used for analyzing the protein conjugates was a Perspective BioSpectrometry Workstation provided with the software Voyager-DE-RP (version 4.03) developed by Perspective Biosystems Inc. (Framingham, MA) and Grams/386 (for Microsoft Windows, version 3.04, level III) developed by Galactic Industries Corp. (Salem, NH). The pH and conductivity of all buffers and solutions were measured with a pH-meter pH 540 GLP and a conductimeter LF 340, respectively (WTW, Weilheim, Germany). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). Washing steps were performed on an SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). A Heidolph Titramax 1000 vibrating platform shaker (Brinkmann Instruments, Westbury, NY) was used to shake the microplates at 900 rpm. Absorbances were read on a SpectramaxPlus (Molecular Devices, Sunnyvale, CA). The competitive curves were analyzed with a four-parameter logistic equation using the software SoftmaxPro v4.7 (Molecular Devices) and GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). The chemical reagents used in the synthesis were obtained from Aldrich Chemical Co. (Milwaukee, WI). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The preparation of the immunoreagents used is described below. Sulfonamides used for cross-reactivity studies were kindly supplied by Riedel-de Haën.

Buffers. Phosphate-buffered saline (PBS) is 0.01 M phosphate buffer on an 0.8% saline solution (137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl), and the pH is 7.5. PBST is PBS with 0.05% Tween 20. For milk experiments, 2× PBST is 20 mM PBS with 0.10% of Tween 20. Borate buffer is 0.25 M boric acid−sodium borate, pH 8.7. Coating buffer is 0.05 M carbonate−bicarbonate buffer, pH 9.6. Citrate buffer is a 0.04 M solution of sodium citrate, pH 5.5. The substrate solution contains 0.01% 3,3′,5,5′-tetramethylbenzidine (TMB) and 0.004% H_2O_2 in citrate buffer.

Immunoreagents. Protein conjugates were prepared following described procedures (*17*) by activating the haptens (10 μmol) with *N*-hydroxysuccinimide (NHS, 12.5 μmol) and dicyclohexylcarbodiimide (DCC, 25 μmol) in anhydrous DMF (200 μL) and reacting with the protein (HCH, BSA, CONA, or OVA, 10 mg) in 0.2 M borate buffer (1.8 mL). The protein conjugates were purified by dialysis against 0.5 mM PBS (4 × 5 L) and Milli-Q water (1 × 5 L) and stored freeze-dried at −40 °C. Unless otherwise indicated, working aliquots were stored at 4 °C in PBS at 1 mg mL^{-1}. Hapten densities of the bioconjugates were estimated by measuring the molecular weight of the native proteins to that of the conjugates by MALDI-TOF-MS. Thus, MALDI spectra were obtained by mixing 2 μL of the freshly prepared matrix (*trans*-3,5-dimethoxy-4-hydroxycinnamic acid, 10 mg mL^{-1} in CH_3CN/H_2O 70:30, 0.1% TFA) with 2 μL of a solution of the conjugates or proteins in CH_3CN/H_2O 70:30, 0.1% TFA (10 mg mL^{-1}). The hapten density (δ-hapten) was calculated according to the following equation: {MW(conjugate) − MW(protein)}/MW(hapten)}. The coupling efficiency evaluated by MALDI-TOF-MS of the corresponding haptenized BSA immunoreagents is shown in **Table 1**.

Table 2. Immunoassay Features of the Best Competitive ELISA[a]

immunogen	assay	A_{max}	A_{min}	IC_{50}[b]	slope	R^2
SA1−HCH	As154/**SA2**−BSA	1.02	0.16	2.51	−0.84	0.99
	As154/**SA2**−OVA	0.86	0.25	1.80	−1.03	0.98
	As155/**SA2**−BSA	1.03	0.02	2.95	−0.66	0.99
	As155/**SA2**−OVA	0.92	0.07	1.41	−0.82	0.98
	As156/**SA2**−BSA	1.00	0.12	11.40	−0.77	0.98
	As167/**SA8**−CONA	0.67	0.01	3.57	−0.61	0.99
	As167/**SA9**−BSA	0.58	0.01	2.90	−0.54	0.99
	As167/**SA2**−OVA	0.91	0.09	4.06	−0.74	0.99

[a] Only some assays showing reasonable parameters and IC_{50} values below 100 μg L^{-1} are shown. [b] IC_{50} values are expressed in μg L^{-1}.

Polyclonal Antisera. As154−156 and As167 were obtained by immunizing female white New Zealand rabbits weighting 1−2 kg with **SA1**−HCH following a protocol already described (*17*).

As157−159 were produced in the same way but using **SA2**−HCH as immunogen. The evolution of the antibody titer was assessed using a noncompetitive indirect ELISA, by measuring the binding of serial dilutions of each antiserum to microtiter plates coated with **SA1**−BSA or **SA2**−BSA. After an acceptable antibody titer was observed, the animals were exsanguinated, and the blood was collected on vacutainer tubes provided with a serum separation gel. Antiserum were obtained by centrifugation and stored at −80 °C in the presence of 0.02% NaN_3.

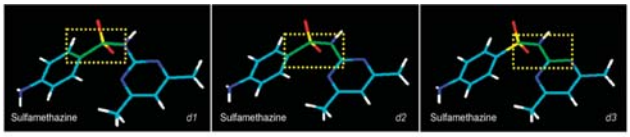
This research has the approval of the Ethical Committee of the CSIC, and all of the experiments have been performed following Animal Research Advisory Committee (ARAC) guidelines.

*Indirect As155/**SA2**−OVA ELISA. General Protocol.* The detectability and specificity of the antibodies raised were assessed through competitive ELISA experiments following a general protocol. Previously, the concentrations of the immunoreagents were selected by two-dimensional checkerboard titration experiments, where the avidity of the antisera for the better recognized coating antigens (CAs) was assessed (*15*). The features of best combinations able to provide competitive assays within the studied concentration interval are included in **Table 2**. Finally, microtiter plates were coated with **SA2**−OVA (0.625 μg mL^{-1} in coating buffer, 100 μL/well) overnight at 4 °C and covered with adhesive plate sealers. The next day, plates were washed four times with PBST (300 μL/well), and solutions of the sulfapyridine (SPY) standards (from 50000 to 0.0256 nM) or cross-reactants (same concentration range) in PBST (50 μL/well) and the antiserum As155 (1/4000 diluted in PBST, 50 μL/well) were added and incubated for 30 min at room temperature, under shaking. The plates were washed as before, and a solution of anti-IgG−HRP (1/6000 in PBST) was added to the wells (100 μL/well) and incubated for 30 min more at room temperature. The plates were washed again, and the substrate solution was added (100 μL/well). Color development was stopped after 30 min at room temperature with 4 N H_2SO_4 (50 μL/well), and the absorbances were read at 450 nm. The standard curves were fitted to a four-parameter equation according to the following formula: $y = (A - B/[1 - (x/C)^D]) + B$, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve. Unless otherwise indicated, the data presented correspond to the average of at least two well replicates.

Specificity Studies. Stock solutions of different sulfonamides were prepared in DMSO at a concentration of 10 mM and kept at 4 °C. Standard curves were prepared in PBST and run in the ELISA following the protocol described before. The cross-reactivity (CR) values were calculated according to the equation {IC_{50} [nM] (SPY)/IC_{50} [nM] (cross-reactant)} × 100. Accuracy Studies.

This parameter was assessed by preparing different blind spiked samples in PBST buffer and measuring them in duplicate in the ELISA.

Immunochemical Analysis of Milk. Samples. Skimmed, semi-skimmed, and whole milk samples free of antibiotics were supplied by the Agencia Española para la Seguridad Alimentaria (AESA; Spanish Agency for Food Security, Madrid, Spain). Contaminated blind samples were prepared at the Nestlé Research Center (Lausanne, Switzerland) from fresh milk samples.



Structures	*Dihedral Angle*			*Charge Distribution*		*pKa*
	d1	*d2*	*d3*	*SO_2NHC*	*SO_2NHC*	
Sulfamethazine	94.0	-76.1	-161.3	-0.394	0.078	7.03
Sulfadiazine	97.2	-74.8	-162.4	-0.392	0.071	6.84
Sulfaquinoxaline	98.1	-72.7	17.2	-0.413	0.095	7.61
Sulfadoxine	-100.3	-69.8	13.2	-0.396	0.109	7.05
Sulfamerazine	-97.1	-75.4	-161.5	-0.393	0.079	6.93
Sulfamethoxydiazine	98.8	-77.8	158.4	-0.393	0.035	7.21
Sulfapyridine	81.5	-94.5	148.7	-0.410	0.044	7.99
Sulfathiazole	93.9	-73.0	18.8	0.376	0.110	7.83
Sulfamethiazole	95.2	-67.8	16.2	-0.367	-0.135	7.05
SA1 Hapten	*80.8*	*-95.4*	*-147.4*	*-0.409*	*0.039*	*8.20*
SA2 Hapten	*88.8*	*-99.5*	*-166.8*	*-0.530*	*0.322*	*5.62*

Figure 3. Results from the theoretical studies performed to assess the suitability of the immunizing hapten to produce class-selective antibodies. Comparison of dihedral sulfonamide angle, charge distribution, and calculated pK_a values for the most important commercial sulfonamides and the haptens proposed. Sulfamethazine dihedral angles are shown as an example.

Matrix Effect Studies. SPY standard curves were prepared in milk samples diluted with Milli-Q water in different proportions and used to study the parallelism with respect to the curves prepared in buffer.

Sample Measurements. Milk samples were analyzed in duplicate with the As155/**SA2**−OVA ELISA using SPY calibration curves and following the general protocol described above. Alternatively, milk samples were also measured using SPY calibration curves prepared in whole milk free of antibiotics diluted 5 times with Milli-Q. In this case, samples and standards were added to the coated plates (50 μL/well) followed by the antibody solution (1/2000 in 2× PBST, 50 μL/well). Subsequently, the plates were processed as described before. Concentration results were expressed as immunoreactivity equivalents of SPY (SPY IR equiv).

RESULTS AND DISCUSSION

Sulfonamide class-selective antibodies and immunoreagents have been developed with the objective of detecting a significant number of sulfonamide antibiotic congeners used in the veterinary field. The chemical structure of the immunizing hapten has been designed on the basis of chemical criteria and information extracted from theoretical models and calculations (*11, 18, 19*) to assess how the proposed immunizing hapten chemical structure mimics that of most of the sulfonamide congeners. On the basis of chemical criteria, generic recognition of the sulfonamide antibiotic family is required to maximize recognition of the common aniline moiety. Therefore, a chemical group for covalent attachment to the carrier had to be placed at the opposite site of the molecule. Moreover, it was considered to be relevant that most of the sulfonamide chemical structures show with the presence of one electron-withdrawing atom close to the carbon situated in the α-position to the sulfonamide bond. With this criteria was defined the fundamental chemical structure moiety that had to be present in the immunizing hapten (see area marked in red in **Figure 1**). Both haptens **SA1** and **SA2** possessed the mentioned area; however, whereas hapten **SA1** contained the whole structure, hapten **SA2** had just the common moiety of the sulfonamide antibiotics. The strategy of using just a fragment of the chemical structure has been used before (*11, 20, 21*), although it has not always provided the desired results with regard to antibody selectivity. For this reason and with the aim of obtaining additional information, we made use of theoretical and molecular modeling studies. **Figure 3** shows dihedral sulfonamide angles in the sulfonamide bridge of the haptens and the most important sulfonamide antibiotics, where it can be observed that with regard to geometry the differences between all of these chemical structures are not so great. Moreover, in the figure can also be found molecule charge distribution in the area close to the sulfonamide bridge, where it can be observed that the main difference is at the carbon α to the sulfonamide group of hapten **SA2**. Finally, the pK_a values calculated also allowed us to predict a distinct behavior of this hapten at the physiological or the usual assay pH of 7.5.

Hapten **SA1** was prepared in four steps from methyl 5-(4-amino-3-pyridinyl)-2-methyl-4-pentenoate **6** through a Heck reaction under Jeffery conditions (*22, 23*). The reaction consists of an sp2−sp2 coupling between an iodoaromatic compound, in this case the 2-amino-5-iodopyridine, and an alkene group, the methyl 2-methy l-4-pentenoate, using palladium acetate as catalyst, tetrabutylammonium chloride, and potassium formate. The mixture obtained was hydrogenated and subsequently purified by HPLC to obtain the necessary synthon **6**. Subsequently, the sulfonamide was formed by reacting the amino group with *N*-acetylsulfanilyl chloride. The simultaneous hydrolysis of the acetyl group and the methyl ester, under basic conditions, allows us to obtain **SA1** (see **Figure 1**). On the other hand, the synthesis of **SA2** was accomplished by reacting *N*-4-(aminosulfonyl)phenyl)acetamide **9** with methyl 4-(chloroformyl)butyrate, followed by the hydrolysis of the ester and the acetyl group (see **Figure 1**).

Both haptens **SA1** and **SA2** were covalently coupled through their carboxylic groups to the lysine amino acid residues of the HCH and used to raise antibodies (As154−156 and As167 from **SA1**−HCH; As157−159 from **SA2**−HCH). Similarly, both haptens were conjugated to BSA, CONA, and OVA to use them as coating antigens to develop the competitive ELISA. As has been reported (*18, 24−28*), the chemical structure of the competitor hapten plays a crucial role in the sensitivity and specificity of the competitive assays. The screening of all possible antiserum/coating antigen (As/CA) combinations was performed using SPY as analyte and the general ELISA protocol described under Experimental Procedures. In all cases, the antisera raised against **SA1**−HCH provided the best assays. In contrast, no usable assays (IC_{50} > 100 μg L^{-1}) were obtained with the As raised against **SA2**−HCH, demonstrating that the presence of the heterocycle, and the electron-withdrawing group next to the carbon atom in α to the sulfonamide group, played an important role in antibody recognition. On the other hand,

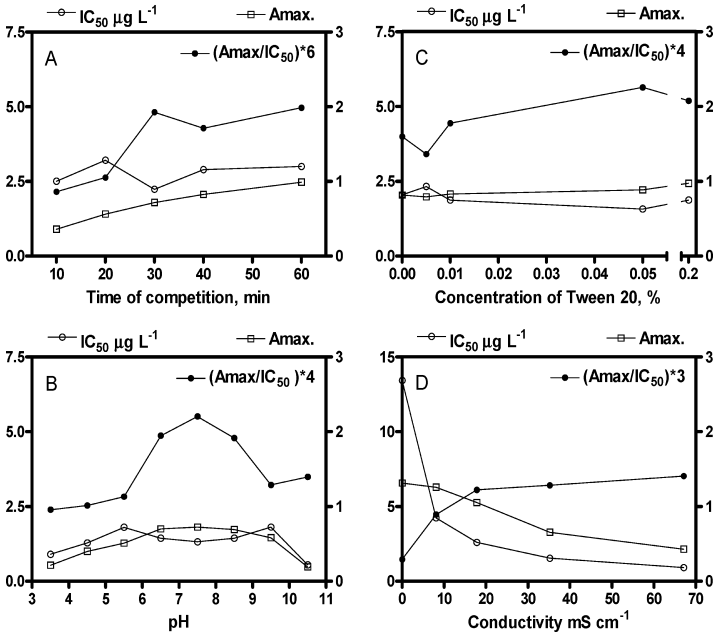


Figure 4. Effect of the length of the competition step, Tween 20, pH, and conductivity on the As155/**SA2**−OVA immunoassay features for the sulfapyridine (SPY) analysis.

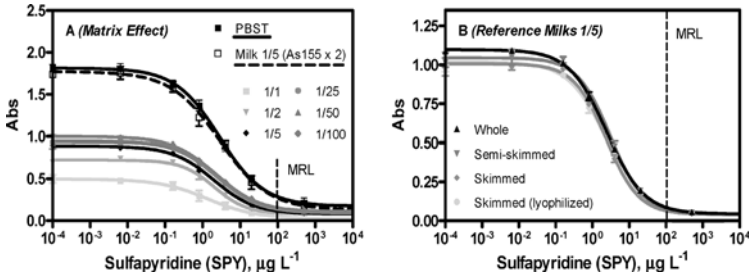


Figure 5. (**A**) Calibration curve of the As155/**SA2**−OVA immunoassay in PBST buffer. The data shown are the averages of six assays performed on different days. The parameters of the assay are shown in **Table 3**. Matrix effect was caused by milk in the As155/**SA2**−OVA ELISA. Whole milk and PBST diluted whole milk were used. Parallelism was found between the standard curve prepared in buffer and the one prepared in 1/5 PBST diluted milk using a double concentration of the antibody in the competition step. (**B**) Comparison of the immunoassay response when different types of milk samples were analyzed after being diluted 5 times with PBST.

as predicted by the theoretical studies, the charge of the carbon α to the sulfonamide group and the pK_a of hapten **SA2** may also have also been some of the reasons for the results obtained.

Table 2 shows the features of the best competitive indirect ELISAs obtained. As can be observed, the best antibody/coating antigen combinations are heterologous. The assays obtained under homologous conditions provided IC_{50} values far from the required detection limits. Although several combinations rendered usable assays, As155/**SA2**−OVA was finally selected for further studies because of the excellent features and reproducibility observed on repetitive experiments. Evaluation of the effect of different physicochemical parameters (time, pH, ionic strength, etc.) showed that no improvement in the detectability was obtained after an overnight preincubation of the antisera with the analyte before the competitive step, by varying the length of the competitive step or by changing the concentration of surfactant Tween 20 in the buffer, with respect to the standard conditions used while screening the different antisera/coating antigen combinations (see **Figure 4A**,**C**). Concerning pH, the assay tolerated quite well pH values between 6 and 9. Outside this pH range the assay is almost inhibited (see **Figure 4B**). With regard to ionic strength, in the absence of salts the detectability is very bad (high IC_{50} value), whereas a marked increase of the detectability was observed with the increase of the ionic strength. However, a slight decrease of the maximum absorbance is also produced, which makes the assay not usable after a certain ionic strength value (see **Figure 4D**).

Figure 5A shows a standard calibration curve corresponding to the average of six assays performed on different days using two-well replicates, and immunoassay features are shown in **Table 3**. The IC_{50} and limit of detection (LOD; 90% of the zero dose) values accomplished were 2.86 ± 0.24 and 0.13 ± 0.03 μg L^{-1}, respectively, with a working range between 0.42 ± 0.06 and 19.2 ± 4.35 μg L^{-1} (20−80% of the assay response at zero doses).

Specificity studies were performed by preparing the calibration curves with several structurally related compounds and measuring them with the assay. As can be observed, most of the sulfonamides tested were highly recognized in this assay, with LOD values far below the MRLs of the EC (see **Table 4**). Only

Table 3. Features of the SPY ELISAs in Buffer and Milk Samples after Evaluation[a]

parameter	PBST curve	milk curve
$signal_{min}$	0.17 ± 0.05	0.04 ± 0.01
$signal_{max}$	1.81 ± 0.05	1.03 ± 0.04
slope	0.74 ± 0.07	0.93 ± 0.07
R^2	0.991 ± 0.005	0.997 ± 0.002

sample measured	buffer	milk (1/5)[b]	milk (1/5)[b]
IC_{50}, $\mu g\ L^{-1}$	2.86 ± 0.24	14.3 ± 1.20	13.0 ± 1.45
working range, $\mu g\ L^{-1}$	0.42 ± 0.06 to 19.2 ± 4.35	2.10 ± 0.30 to 96.0 ± 21.75	2.75 ± 0.70 to 59.5 ± 7.00
LOD, $\mu g\ L^{-1}$	0.13 ± 0.03	0.65 ± 0.15	1.10 ± 0.45

[a] Values obtained correspond to the average and standard deviation of each parameter of at least six assays performed on different days. [b] Real assay parameters of both methods for the analysis of milk samples (5 times diluted).

Table 4. Cross-Reactivity of Related Sulfonamide Compounds in the As155/**SA2**−OVA ELISA

	ELISA		
compound	IC_{50} ($\mu g\ L^{-1}$)	LOD ($\mu g\ L^{-1}$)	% CR[a]
sulfapyridine	2.25	0.15	100
sulfaquinoxaline	79.15	1.17	4
sulfachloropyridazine	61.81	1.34	5
sulfisomidine	2.10	0.23	140
sulfathiazole	1.30	0.13	202
sulfadiazin	14.05	0.38	15
sulfadimethoxine	41.22	0.99	8
sulfamerazine	3.73	0.43	79
sulfamethoxypyridazine	4.95	0.33	61
sulfamethazine	1.78	0.15	119
sulfamethoxazole	>MRL	>MRL	<1
sulfadoxine	>MRL	>MRL	<1
N^4-acetylsulfamethazine	>MRL	>MRL	<1
sulfanilamide	>MRL	>MRL	<1

[a] Cross-reactivity is expressed as a percent of the IC_{50} (nM) of the sulfapyridine divided by the IC_{50} (nM) of the other compounds tested.

a few congeners such as sulfamethoxazole or sulfadoxin were not sufficiently recognized, probably due to the methoxy groups present in these chemicals. Similarly, the N^4-acetylsulfamethazine was not recognized, which demonstrated the relevance of the free amino group of the *p*-aminobenzenesulfonamide moiety. The heterologous indirect ELISA format increased significantly the number of sulfonamide congeners detected in comparison with the direct homologous ELISA reported before (*29*), where only SPY, sulfamethoxypyridazine (SMP), sulfathiazole (STZ), and sulfachloropyridazine (SPC) antibiotics were recognized. It should be noted that it has already been reported that homologous antibody/competitor combinations tend to render more specific immunoassays (*28, 30*).

Due to the interest of controlling the presence of antibiotic residues in milk samples, studies were performed to assess potential nonspecific matrix effects. With this purpose, standard curves were prepared in milk diluted several times with Milli-Q water and run in the ELISA (see **Figure 5**). Surprisingly, under these conditions, where the pH and conductivity of the milk samples were close to those of the buffer, the assay response did not reach that of the assay run in buffer and remained inhibited independent of the dilution applied (see **Figure 5A**). This matrix effect observed was overcome with small modifications of the analytical protocol, such as the concentration of the immunoreagents or using a reference milk free of antibiotics to build the calibration curves. Thus, almost identical curves were obtained by just increasing the concentration of the antibody and diluting the milk 5 times with Milli-Q water, as can be observed in **Figure 5A**. Thus, by increasing the antibody concentration added to the sample we accomplish the same maximum absorbance as that of the assay run in buffer. Therefore, by using this strategy the concentration of sulfonamide antibiotics in milk samples can be quantified using the calibration curve run in buffer. With these conditions, the LOD achieved in milk was $0.65 \pm 0.15\ \mu g\ L^{-1}$. The same procedure was applied to different kinds of milk samples (whole, semi-skimmed, and skimmed), certified by AESA, to assess if the strategy was suitable to analyze any type of milk. As can be observed in **Figure 5B**, reproducible calibration curves with excellent features could be obtained. Moreover, the immunochemical response was very similar independent of the type of milk used, which pointed to the possibility of using a representative milk as a reference material to build standard calibration curve for subsequent milk measurements. The almost negligible matrix effect caused by the milk samples has been attributed to the excellent features of the antibodies produced, being also crucial the fact of shaking the microtiter plates during the competition step, which minimized the formation of fat/protein layers in the bottom of the microtiter wells, improving assay reproducibility and accuracy (*31*−*33*). Thus, although several monoclonal (*9, 34, 35*), polyclonal (*36*−*38*), and recombinant antibodies (*10, 14*) against sulfonamide antibiotics with different recognition patterns versus the different congeners have been reported, on only a very few occasions has direct performance of these antibodies in complex biological samples been demonstrated. Thus, for tissue immunochemical analysis, several procedures involving different extraction/cleanup methods have been reported (i.e., refs *39* and *40*). Direct analysis of honey samples by ELISA has also been attempted, but high dilution factors (100 times) had to be applied to avoid matrix interferences (*37*). Alternatively, solid-phase extraction methods have been introduced prior to the immunochemical analysis. With regard to milk, homogenization and protein removal by precipitation with trichloroacetic acid or acetone followed by neutralization or dilution have been as some of the sample preparation methods reported to analyze this sample by immunoassay (*13, 14*).

The *accuracy* of the assay was first evaluated in buffer by measuring several blind samples prepared in PBST. The results shown in **Figure 6** (left) correspond to the correlation found between the measured and the spiked concentration values. As can be observed, results obtained matched very well the spiked values. A slope near 1 was obtained (0.96) with a coefficient of correlation of $R^2 = 0.997$. Similarly, the accuracy of the assay when measuring whole milk blind samples was excellent. The correlation studies provided slopes close to 1 independent of whether the PBST or the whole milk calibration curves were used as reference (slope values were 1.07 and 0.95, respectively). Moreover, the coefficients of correlation were very good (PBST, 0.996; milk, 0.993), indicating the good accuracy of both methods to analyze milk samples.

Finally, for a preliminary evaluation of the performance of the ELISA as screening method, a set of milk samples spiked with distinct sulfonamides at different concentration levels was prepared at the Nestlé Research Centre (Lausanne, Switzerland) and measured with ELISA using SPY as the reference analyte. According to the immunoassay response the samples were classified as positive ($\geq 100\ \mu g\ L^{-1}$) or negative ($< 100\ \mu g\ L^{-1}$). Data were collected as SPY immunoreactivity equivalents (SPY IR equiv) because this sulfonamide was used as reference in the standard curve. Subsequent data treatment consisted of the

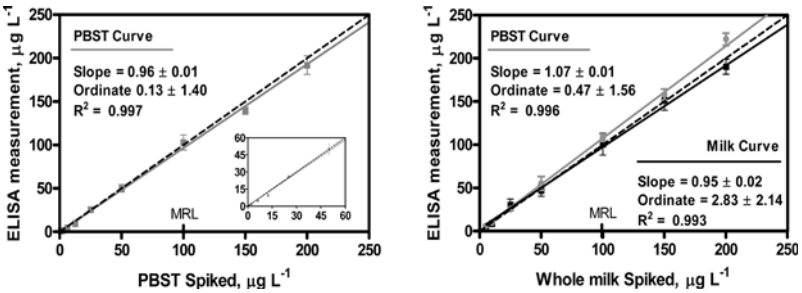


Figure 6. Results from the accuracy studies. The graphs show the correlation between the spiked and measured concentration values using the ELISA format to analyze buffer (left) and milk samples (right). Milk samples were measured both with the buffer and with reference milk calibration curves following the protocols described. The dotted line corresponds to a perfect correlation (slope = 1). The data correspond to the average of at least two replicates.

Table 5. Preliminary Evaluation of the ELISA as Screening Method

		calibration curve measurement[a]			
milk sample[b]		PBST (μg L^{-1})		milk (μg L^{-1})	
GF1 (SMZ, 10 ppb)	N	21 ± 2	N	11 ± 1	N
GF2 (STA, 10 ppb)	N	19 ± 1	N	12 ± 1	N
GF3 (SCP, 10 ppb)	N	75 ± 1	N	55 ± 9	N
GF4 (SMZ, 25 ppb)	N	35 ± 3	N	22 ± 2	N
GF5 (STA, 25 ppb)	N	44 ± 5	N	29 ± 3	N
GF6 (SCP, 25 ppb)	N	120 ± 9	FP	71 ± 6	N
GF7 (blank)	N		N		N
GF8 (SMZ, 50 ppb)	N	70 ± 4	N	69 ± 5	N
GF9 (STA, 50 ppb)	N	76 ± 6	N	56 ± 4	N
GF10 (SCP, 50 ppb)	N	137 ± 23	FP	106 ± 19	FP
GF11 (SMZ, 100 ppb)	P	127 ± 14	P	120 ± 11	P
GF12 (STA, 100 ppb)	P	146 ± 16	P	109 ± 15	P
GF13 (SCP, 100 ppb)	P	153 ± 32	P	165 ± 23	P

[a] Results are expressed as follows: first as equivalents of the sulfonamide spiked in the sample using sulfapyridine as the reference analyte to build the calibration curves (left) and as positive (P), negative (N), false positive (FP), and false negative (FN) according to MRLs established for sulfonamides by the EU (right). Antibiotics spiked in milk samples were sulfamethazine (SMZ), sulfathiazole (STA), and sulfachloropyridazine (SCP). [b] Blind samples were prepared at the Nestlé Research Center (Lausanne, Switzerland).

transformation of the equivalents of SPY detected in the milk samples to the respective sulfonamides using the assay cross-reactivity values shown in **Table 4**. The results obtained are summarized in **Table 5**. It is important to note the lack of false negatives for both protocols. The only false positives obtained corresponded to samples contaminated with sulfachloropyridazine (SPC); however, results indicated the presence of sulfonamides, although in reality they were below the MRLs. Confirmatory methods should in this case provide the final result to say if the levels of antibiotics in these samples are acceptable for consumption.

The analysis of the data obtained in this preliminary evaluation study of the use of the ELISA as a generic sulfonamide screening method evidence that the concentration of sulfonamides that cross-react far from the analyte selected to be used as the reference (SPY), like the already cited SPC, are more likely to be over- or underestimated, increasing the risk of false-positive or false-negative results. This situation could be overdriven by including on each microplate control samples of sulfonamides with different cross-reactivity patterns. This strategy, already suggested and demonstrated in a previous publication (*41*), would considerably increase the efficiency of the assay as screening method. Moreover, when the calibration curve prepared in milk is used as reference, the accuracy seems to be slightly better than if PBST is used as reference, which can be due to the particular properties of each type of milk samples. However, the approach of quantifying with a PBST curve provides acceptable results for use of this method with screening purposes.

In conclusion, high-quality class-selective antibodies have been produced using hapten **SA1** coupled to HCH as immunogen. The use of heterologous conditions using hapten **SA2** as competitor may have also played an important role in the recognition pattern obtained. The excellent features of these immunoreagents have allowed the setting up of a robust immunoassay that improves considerably, in terms of detectability and number of sulfonamides recognized, the direct ELISA format previously reported. The immunoassay performs very well in milk samples without any prior treatment other than dilution of the sample. Evaluation studies show that the immunoassay is able to detect 10 sulfonamides directly in milk samples at the MRL values. No false negatives have been observed, whereas false positives indicate the presence of sulfonamides, and these samples should always be followed by the use of confirmatory methods. However, further research has to be made for a complete validation in compliance with Commission Decision 2002/657/EC, which is still not completely clear for the evaluation of screening methods. Considering the successful analysis of sulfonamides in a complex biological sample, such as milk, there is great promise regarding the potential application of this immunoassay on new matrices such as water and soil samples, where antibiotics are considered to be emerging pollutants. Moreover, the immunoreagents produced in this work have been subsequently incorporated with success onto novel transducers with the objective of developing new biosensor devices for on-site measurements (*42, 43*). On the other hand, investigations were also addressed to build a multiplexed device able to screen simultaneously for the presence of other relevant antibiotics that may contaminate milk or other dairy products.

Supporting Information Available: Preparation of haptens **SA3**−**SA10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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3.3.1 SAs indirect ELISA format - Supporting Information

EXPERIMENTAL SECTION

Competitor haptens

Figure A shows the chemical structure of all the haptens synthesized. Detailed explanation of SA1 and SA2 hapten synthesis is included in the experimental section of the manuscript. On the other hand, schemes showing the entire procedure to obtain both haptens (SA1 and SA2) are summarized in Figures B and C, respectively. The competitors SA3, SA4, SA5 and SA6 were functionalized by the amino group exposing the no common part of the sulfonamide antibiotic family. Synthesis of SA4-SA6 compounds was started from commercial sulfonamides, such as sulfapyridine, sulfamethazine and sulfachloropyridazine. Moreover, trying to find more heterogeneity, compound SA3 was prepared without the common aniline present in all sulfonamide congeners. On the other hand, the proposed haptens SA7, SA8, SA9 and SA10 have the free amino group exposed with different large of the space arm introduced in the other side of the compound structure.

SA3 (See Figure D)

methyl 5-phenylpentanoate (11). A solution of 5-phenylpentanoic acid (5.0 g, 28.2 mmol) in MeOH (30 mL) was kept at 0 ºC before adding slowly thionyl chloride (10.2 mL, 141.2 mmol). Solution was kept for 1 hour at room temperature. The solvent was evaporated to obtain the desired product as yellow oil (5.3 g, 98 % yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.69 (m, 4H, -CH_2CH_2-), 2.34 (t, 2H, -CH_2COO-), 2.63 (t, 2H, -CH_2Ph-), 3.67 (s, 3H, $COOCH_3$), 7.28-7.16 (ac, 5H, Ar). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 24.6 (-**C**H_2CH_2COOH), 30.9 (-**C**H_2CH_2Ph), 33.9 (-**C**H_2COOH), 35.5 (-CH_2Ph), 51.5 (-OCH_3), 125.7 ($C_{para\ CH2}$), 128.3 ($2CH_{meta\ CH2}$), 128.4 ($2CH_{orto\ CH2}$), 142.1 ($C_{ipso\ CH2}$), 174.1 (-COO).

methyl 5-[4-(chlorosulfonyl)phenyl]pentanoate (12). A round bottom flask with chlorosulfonic acid (5 mL, 76.5 mmol), under Ar atmosphere and connected to a security trap line containing 1N NaOH, was introduced in an ice bath prior to start the reaction. Compound **11** (4.8 g, 25.5 mmol) was slowly added during 30 min. Chlorosulfonic acid (3.4 mL, 51.0 mmol) was added again. After 2 hours the crude was drop wise to a water-ice solution and extracted with CH_2Cl_2 (30 mL). The organic layer was washed with a saturated solution of $NaHCO_3$ (3 x 15 mL) and dried with anh. MgSO4, filtered and evaporated to dryness under reduced pressure. Isolation of compound **12** was performed by crystallization with hexane (5.3 g, 72 % yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.69 (m, 4H, -CH_2CH_2), 2.36 (t, 2H, -CH_2COOH), 2.76 (t, 2H, -CH_2Ph-), 3.68 (s, 3H, -$COOCH_3$), 7.42 (d, J = 8.4 Hz, $2H_{meta\ SO2Cl}$), 7.95 (d, J = 8.4 Hz, $2H_{orto\ SO2Cl}$). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 24.3 (**C**H_2CH_2COOH), 30.8 (-**C**H_2CH_2Ph), 33.6 (-**C**H_2COOH), 35.6 (CH_2Ph), 51.5 (-OCH_3), 127.1 ($2C_{orto\ SO2}$), 129.5 ($2C_{meta\ SO2}$), 141.6 (C_{SO2}), 150.7 ($C_{para\ SO2}$), 173.7 (-COO).

5-(4-{[(4-chlorophenyl)amino]sulfonyl}phenyl)pentanoate (13). A solution of compound **12** (100 mg, 3.44 mmol), 4-chloroaniline (0.44 mg, 3.44 mmol) and Et_3N (50 µL, 3.44 mmol) in anh. dioxane was kept for 3 hours at 70 ºC under argon atmosphere. The solvent was evaporated and the residue was redissolved in basic water (10 mL) and washed with AcOEt (3 x 5 mL). The aqueous layer was acidified to pH 3 prior to be extracted with AcOEt (3 x 10 mL). The organic layer was washed again with a saturated solution of NaCl (3 x 5 mL), dried with anh. $MgSO_4$, filtered and evaporated to dryness under reduced pressure obtaining the desired product **13** (114 mg, 87 % yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.63 (m, 4H), 2.32 (t, 2H, CH_2COOH), 2.66 (t, 2H, CH_2Ph), 3.66 (s, 3H, -OCH_3), 7.01 (d, J = 9 Hz, 2H, $H_{orto\ NH}$), 7.20 (d, J = 9 Hz, 2H, $H_{meta\ NH}$), 7.24 (d, J = 8.4 Hz, 2H, $H_{meta\ SO2}$), 7.65 (d, J = 8.4 Hz, 2H, $H_{orto\ SO2NH2}$).

5-(4-{[(4-chlorophenyl)amino]sulfonyl}phenyl)pentanoic acid (SA3). A solution of compound **13** (50 mg, 0.13 mmol) in 1N NaOH (2.5 mL) and THF (2 mL) was kept for 2 h until observing the total disappearance of the starting material by TLC (1:1, CH_2Cl_2:AcOEt). The THF was evaporated and the crude mixture was acidified with 1N HCl to pH 3 and extracted with AcOEt (3 x 10 mL). The organic layer was finally washed with water (3 x 5 mL), dried with anh. $MgSO_4$, filtered and evaporated to dryness under reduced pressure obtaining the **SA3** (40 mg, 90 % yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.63 (m, 4H), 2.32 (t, 2H), 2.66 (t, 2H), 7.01 (d, J = 9 Hz, 2H, $H_{orto\ NH}$), 7.20 (d, J = 9 Hz, 2H, $H_{meta\ NH}$), 7.24 (d, J = 8.4 Hz, 2H, $H_{meta\ SO2}$), 7.65 (d, J = 8.4 Hz, 2H, $H_{orto\ SO2}$).

SA4 (See Figure E)

5-[(4-{[(4,6-dimethylpyrimidin-2-yl)amino]sulfonnyl}phenyl)amino]-5-oxopentanoic acid (SA4). A solution of commercial sulfamethazine (400 mg, 1.42 mmol) and glutaric anhydride (162 mg, 1.42 mmol) in anh. DMF (6 mL) was kept at 100 ºC for 2 hours. The solvent was evaporated, the residue redissolved in basic water (pH 9, 30 mL) and washed several times with AcOEt (3 x 10 mL). The aqueous layer was acidified with 1N HCl to pH 3 and extracted with AcOEt (3 x 20 mL). The organic layer washed with a saturated solution of NaCl (3 x 5 mL), dried with anh. $MgSO_4$, filtered and evaporated to dryness to obtain the desired product **SA4** (530 mg, 95 % yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.79 (m, 2H), 2.25 (s, 6H, $2CH_3$), 2.27 (t, 2H, -CH_2CONH-), 2.38 (t, 2H, -CH_2COOH), 6.75 (s, 1H, $H_{para\ NH}$), 7.72 (d, J = 8.4 Hz, 2H, $H_{meta\ SO}$), 7.91 (d, J = 8.4 Hz, 2H, $H_{orto\ SO2}$).

^{13}C NMR (125 MHz, $CDCl_3$) δ: 20.2 (pyr-**C**H_3), 23.1 (**C**H_2COOH), 29.6 (**C**$H_2$$CH_2$Ph), 32.9 (**C**$H_2$COOH), 35.8 ($CH_2$Ph), 113.6 ($C_{para\ NH}$) 118.6 ($C_{orto\ SO2}$), 129.5 ($C_{meta\ SO2}$) ,134.9 ($C_{SO2}$), 142.9 ($C_{para\ SO2}$), 156.6 ($C_{NH}$), 163.7 ($C_{meta\ NH}$), 168.2 (-CONH-) , 172.9 (COOH).

SA5 (See Figure E)

5-[(4-{[(6-chloropyridazin-3-yl)amino]sulfonyl}phenyl)amino]-5-oxopentanoic acid (SA5). A solution of commercial sulfachloropyridazine (500 mg, 1.76 mmol) and glutaric anhydride (200 mg, 1.76 mmol) in anh. DMF (5 mL) was kept at 100 ºC for 1 hour until observing the total disappearance of the starting material by TLC (14:1, CH_2Cl_2:MeOH). The solvent was evaporated, the residue redissolved in basic water (pH 9, 30 mL) and washed with AcOEt (3 x 10 mL). The aqueous layer was acidified with 1N HCl to pH 3 and extracted with AcOEt (3 x 20 mL). The organic layer washed with a saturated solution of NaCl AcOEt (3 x 5 mL), dried with anh. $MgSO_4$, filtered and evaporated to dryness to obtain the desired product **SA5** (511 mg, 73 % yield). ^{1}H NMR (500 MHz, $CDCl_3$, MeOD) δ: 1.98 (m, 2H), 2.40 (t, 2H,-CH_2CONH), 2.48 (t, 2H,-CH_2COOH), 7.60-7.70 (m, 2H, $H_{orto/meta\ NH}$) ,7.77 (d, J = 8.4 Hz, 2H, $H_{orto\ SO}$), 7.92 (d, J = 8.4 Hz, 2H, $H_{meta\ SO2}$).

SA6 (See Figure E)

Preparation of 5-oxo-5-(4-[(pyridin-2-ylamino)sulfonyl]phenyl}amino)pentanoic acid (SA6). A solution of commercial sulfapyridine (100 mg, 0.44 mmol) and glutaric anhydride (76 mg, 0.66 mmol) in anh. DMF (2 mL) was kept at 100 ºC for 1 hour until observing the total disappearance of the starting material by TLC (14:1, CH_2Cl_2:MeOH). The solvent was evaporated, the residue redissolved in basic water (pH 9, 10 mL) and washed several times with AcOEt (3 x 5 mL). The aqueous layer was acidified with 1N HCl to pH 3 and extracted with AcOEt (3 x 15 mL). The organic layer washed with a saturated solution of NaCl (3 x 5 mL), dried with anh. $MgSO_4$, filtered and evaporated to dryness to obtain the desired product **SA6** (40 mg, 25 % yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.905 (d, J = 6.5 Hz, 1H, H_{6het}), 7.84 (d, J = 8.8 Hz, 2H, H_{orto}), 7.68 (d, J = 8.8 Hz, 2H, H_{meta}), 7.63 (dd, J = 6.5 Hz, 1H, H_{4het}), 7.23 (d, J = 6.5 Hz, 1H, H_{3het},), 6.82 (dd, J = 6.5 Hz, 1H, H_{5het}), 2.42 (d, J = 7.33 Hz, 2H, -CH_2-COOH,), 2.36 (d, J = 7.33 Hz, 2H, -CH_2CONH_2), 1.97 (m, 2H, -C<u>H</u>$_2$ CH_2 CH_2-).

SA7 (See Figure F)

methyl (4-aminophenyl)acetate (14). Sulphuric acid (1 mL) was added to a solution of 2-(4-aminophenyl)acetic acid (300 mg, 2.64 mmol) in MeOH (15 mL) and kept for 12 hours at room temperature. The solvent was removed under reduced pressure and the residue was redissolved with AcOEt (25 mL), washed with 1N NaOH (3 x 10 mL). The organic layer was dried with anh. MgSO4, filtered and evaporated to dryness under reduced pressure to obtain the desired compound **14** (252 mg, 77 % yield).^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.53 (s, 2H, CH_2), 3.69 (s, 3H, OCH_3), 6.68 (d, J = 8.4 Hz, 2H, $H_{orto\ NH2}$), 7.10 (s, J = 8.4 Hz, 2H, $H_{meta\ NH2}$). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 40.6 (CH_2), 52.2. (OCH_3), 115.5 ($C_{orto\ NH2}$), 124.0 ($C_{ipso\ alkyl}$), 130.0 ($C_{meta\ NH2}$), 145.6 ($C_{ipso\ NH2}$), 172.9 (C=O).

methyl methyl [4-({[4-(acetylamino)phenyl]sulfonyl}amino)phenyl]acetate (15). A solution of compound **14** (250 mg, 1.50 mmol), N-acetyl-p-aminobenzenechlorosulfonic acid (354 mg, 1.50 mmol) and anh. Et_3N (211 µL, 1.50 mmol) in anh. dioxane (1.5 mL) was heated at 100 ºC under argon atmosphere. After 2 hours, reaction was left at room temperature overnight. The solvent was evaporated and the residue was redissolved in acid water (pH = 3, 20 mL) before being extracted with AcOEt (3 x 15 mL). The organic layer was washed with a saturated solution of NaCl (3 x 5 mL), dried with anh. $MgSO_4$, filtered and evaporated to dryness under reduced pressure obtaining the product as yellow solid (320 mg, 60 % yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.01 (s, 3H, CH_3CO), 3.50 (s, 2H, CH_2COOH), 3.64 (s, 3H, $COOCH_3$), 7.01 (d, J = 8.5 Hz, 2H, $H_{orto\ NH}$), 7.07 (d, J = 8.5 Hz, 2H, $H_{meta\ NH}$), 7.61 (d, J = 9.0 Hz, 2H, $H_{orto\ SO2}$), 7.65 (d, J = 9.0 Hz, 2H, $H_{orto\ SO2}$). ^{13}C NMR (125 MHz, $CDCl_3$) δ : 40.6 (**C**H_2), 116.5 ($C_{meta\ SO2NH2}$), 114.1 ($C_{orto\ NHSO2}$), 122.2 ($C_{ipso\ alkyl}$), 120.8 ($C'_{meta\ SO2NH2}$), 130.2 ($C_{meta\ NHSO2}$), 141.0 ($C_{ipso\ NHCO}$), 137.4 ($C_{ipso\ NHSO2}$), 154.2 ($C_{ipso\ SO2NH2}$), 168.0 (CONH), 176.3 (**C**OO).

(4-{[(4-aminophenyl)sulfonyl]amino}phenyl)acetic acid (SA7). A solution of compound **15** (300 mg, 0.83 mmol) in 5N NaOH (13 mL) was kept under reflux for 4 hours. The crude was acidified with 5N HCl to pH 3 and extracted with AcOEt (3x 10 mL). The organic layer was washed with a saturated solution of NaCl (3x 5 mL) and evaporated to dryness to obtain **SA7** (239 mg, 95 % yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.01 (s, 3H, CH_3CO) 3.5 (s, 2H, CH_2COOH), 3.64 (s, 3H, $COOCH_3$), 7.01 (d, J = 8.5 Hz, 2H, $H_{orto\ NH}$), 7.07 (d, J = 8.5 Hz, 2H, $H_{meta\ NH}$), 7.61 (d, J = 9.0 Hz, 2H, $H_{orto\ SO2}$), 7.65 (d, J = 9.0 Hz, 2H, $H_{orto\ SO2}$). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 40.6 (CH_2), 107.0 ($C_{orto\ SO2NH2}$), 114.1 ($C'_{orto\ NHSO2}$), 122.2 ($C_{ipso\ alkyl}$), 125.8 ($C_{meta\ SO2NH2}$), 130.2 ($C'_{meta\ NHSO2}$), 130.7 ($C_{ipso\ NH2}$), 137.4 ($C_{ipso\ NHSO2}$), 154.2 ($C_{ipso\ SO2NH2}$), 176.3 (COO).

SA8 (See Figure G)

4-(acetylamino)-2-methylbenzenesulfonyl chloride (16). A round bottom flask with chlorosulfonic acid (700 µL, 10.0 mmol), under argon atmosphere and connected to a security trap line containing 1N NaOH, was introduced in an ice bath prior to start the reaction. The compound 3'-methylacetanilide (500 mg, 3.3 mmol) was slowly added during the next 30 min. After 2 hours the crude was drop wise to a water ice solution observing the appearance of a white precipitate and the formation of gaseous HCl. The solid was extracted with AcOEt (3 x 20 mL) and washed with abundant water. Organic layer was evaporated obtaining the desired product (581 mg, 70 % yield). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 2.19 (s, 3H, CH_3CONH), 2.75 (s, 3H, CH_3Ph), 7.61 (dd, J = 9.3 Hz, J = 1.7 Hz, 1H, $H_{orto\ AcNH}$,), 7.72 (d, J = 1.7 Hz, 1H, $H_{orto\ CH3}$), 7.98 (d, J = 9.3Hz, 1H, $H_{orto\ SO2Cl}$).

4-[4-({[4-(acetylamino)-2-methylphenyl]sulfonyl}amino)phenyl]butanoic acid (17). A solution of compound **16** (454 mg, 1.83 mmol), 4-(4-aminophenyl)butyric acid (329 mg, 1.83 mmol) and anh. Et_3N (255 µL, 1.83 mmol) in anh. dioxane (3.5 mL) was heated at 100 ºC under argon atmosphere. After 3 hours, reaction was left at room temperature overnight. The solvent was evaporated and the residue was redissolved in acid water (pH = 3) before being extracted with AcOEt (3 x 20 mL). The organic layer was dried with anh. $MgSO_4$, filtered and evaporated to dryness under reduced pressure obtaining the desired compound (150 mg, 20 % yield). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 1.85 (m, 2H, -CH_2-), 2.12 (s, 3H, CH_3CO-), 2.23 (t, J = 7.4 Hz, 2H, -CH_2COOH), 2.54 (t, J = 7.4 Hz, 2H, -CH_2Ph), 2.56 (s, 3H, CH_3Ph), 6.96 (d, J = 8.3 Hz, 2H, $H_{orto\ NH}$), 7.02 (d, J = 8.3 Hz, 2H, $H_{meta\ NH}$), 7.47 (dd, J = 8.5 Hz, 1H, $H_{orto\ AcNH}$), 7.52 (s, 1H, $H_{orto\ CH3}$), 7.81 (d, J = 8.5 Hz, 1H, $H_{orto\ SO2NH}$).

4-(4-{[[4-amino-2-methylphenyl)sulfonyl]amino}phenyl)butanoic acid (SA8). A solution of compound **17** (150 mg, 0.38 mmol) in 5N NaOH (5 mL) and THF (2 mL) was kept under reflux for 2 hours. The crude was acidified with 5N HCl to pH 4 and extracted with AcOEt (3x 10 mL). The organic layer evaporated to dryness to obtain **SA8** (60 mg, 45 % yield). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 1.81 (m, 2H, -CH_2-), 2.23 (t, J = 7.4 Hz, 2H, -CH_2COOH), 2.50 (s, 3H, CH_3Ph), 2.56 (t, J = 7.4 Hz, 2H, -CH_2Ph), 6.64 (dd, J = 8.5 Hz, 1H, $H_{orto\ AcNH}$), 6.68 (s, 1H, $H_{orto\ CH3}$) 6.96 (d, J = 8.3 Hz, 2H, $H_{orto\ NH}$), 7.02 (d, J = 8.3 Hz, 2H, $H_{meta\ NH}$), 7.81 (d, J = 8.5 Hz, 1H, $H_{orto\ SO2Cl}$).

SA9 (See Figure H)

[4-({[4-(acetylamino)-2-methylphenyl]sulfonyl}amino)phenyl]acetic acid (18). A solution of compound **16** (250 mg, 1.07 mmol), 2-(4-aminophenyl)acetic acid (162 mg, 1.07 mmol) and anh. Et_3N (150 µL, 1.07 mmol) in anh. dioxane (2 mL) was heated at 100 ºC under argon atmosphere. After 3 hours, reaction was left at room temperature overnight. The solvent was evaporated and the residue was redissolved in acid water (pH = 3, 20 mL) before being extracted with AcOEt (3 x 10 mL). The organic layer was dried with anh. $MgSO_4$, filtered and evaporated to dryness under reduced pressure obtaining the desired compound (310 mg, 78 % yield). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 2.14 (s, 3H, CH_3CO-), 2.57 (s, 3H, CH_3Ph), 3.5 (s, 2H, HOOCCH_2Ph), 6.97 (d, J = 8.3 Hz, 2H, $H_{orto\ NH}$), 7.02 (d, J = 8.3 Hz, 2H, $H_{meta\ NH}$), 7.36 (d, J = 8.5 Hz, 1H, $H_{orto\ AcNH}$), 7.51 (s, 1H, $H_{orto\ CH3}$), 7.84 (d, J = 8.5 Hz, 1H, $H_{orto\ SO2Cl}$).

(4-{[(4-amino-2-methylphenyl)sulfonyl]amino}phenyl)acetic acid (SA9). A solution of compound **18** in 5N NaOH (2 mL) and THF (2 mL) was kept under reflux conditions for two hours observing the total disappearance of the starting material by TLC (AcOEt). The crude was acidified with 5N HCl to pH 4 and extracted with AcOEt (3x 10 mL). The organic layer evaporated to dryness to obtain **SA9** (21 mg, 70 % yield). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 2.49 (s, 3H, CH_3Ph), 3.5 (s, 2H, HOOCCH_2Ph), 6.45 (d, J = 8.5 Hz, 1H, $H_{orto\ AcNH}$), 6.46 (s, 1H, $H_{orto\ CH3}$), 6.97 (d, J = 8.3 Hz, 2H, $H_{orto\ NH}$), 7.02 (d, J = 8.3 Hz, 2H, $H_{meta\ NH}$), 7.84 (d, J = 8.5 Hz, 1H, $H_{orto\ SO2Cl}$).

SA10 (See Figure I)

4-[4-({[4-(acetylamino)phenyl]sulfonyl}amino)phenyl]butanoic acid (19). A solution of N-acetyl-p-aminobenzenechlorosulfonic acid (130 mg, 0.59 mmol), 4-(4-aminophenyl)butyric acid (100 mg, 0.59 mmol) and anh. Et_3N (73 µL, 0.59 mmol) in anh. dioxane (1 mL) was heated at 100 ºC under argon atmosphere. After 1 hour, reaction was left at room temperature overnight. The solvent was evaporated and the residue was redissolved in acid water (pH = 3, 20 mL) before being extracted with AcOEt (3 x 10 mL). The organic layer was dried with anh. $MgSO_4$, filtered and evaporated to dryness under reduced pressure obtaining a solid (151 mg, 70 % yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.85 (m, 2H, -CH_2-), 2.27 (t, J = 7.4 Hz, 2H, -CH_2COOH), 2.58 (t, J = 7.4 Hz, 2H, -CH_2Ph,) 7.07 (d, J = 8.55 Hz, 2H, $H_{orto\ NH}$), 7.11 (d, J = 8.55 Hz, 2H, $H_{meta\ NH}$), 7.61 (d, J = 9.03 Hz, 2H, $H_{orto\text{-}SO2}$), 7.65 (d, J = 9.03Hz, 2H, $H_{orto\ SO2}$).

4-(4-{[(4-aminophenyl)sulfonyl]amino}phenyl)butanoic acid (SA10).

A solution of compound **19** (130 mg, 0.35 mmol) in 5N NaOH (8 mL) was kept under reflux for 4 hours. The crude was acidified with 5N HCl to pH 4 and extracted with AcOEt (3x 10 mL). The organic layer evaporated to dryness to obtain **SA10** (130 mg, 82 % yield). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 1.85 (m, 2H, -CH_2-), 2.27 (t, J = 7.4 Hz, 2H, -CH_2COOH), 2.58 (t, J = 7.4 Hz, 2H, -CH_2Ph), 7.01 (d, J = 8.79 Hz, 2H, $H_{meta\ SO2}$), 7.07 (d, J = 8.55 Hz, 2H, $H_{orto\ NH}$), 7.11 (d, J = 8.55 Hz, 2H, $H_{meta\ NH}$), 7.69 (d, J = 8.79 Hz, 2H, $H_{orto\ SO2}$). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 175.0 (-COOH), 138.3 ($C_{ipso\ NHSO2}$), 129.2 ($C_{meta\ NH}$), 129.1 ($C_{orto\ SO2}$), 121.9 (C), 120.7 ($C_{orto\ NH}$), 108.5 ($C_{meta\ SO2}$) 34.4 (CH_2Ph), 30.8 (**C**H_2COOH), 26.4 (-CH_2-).

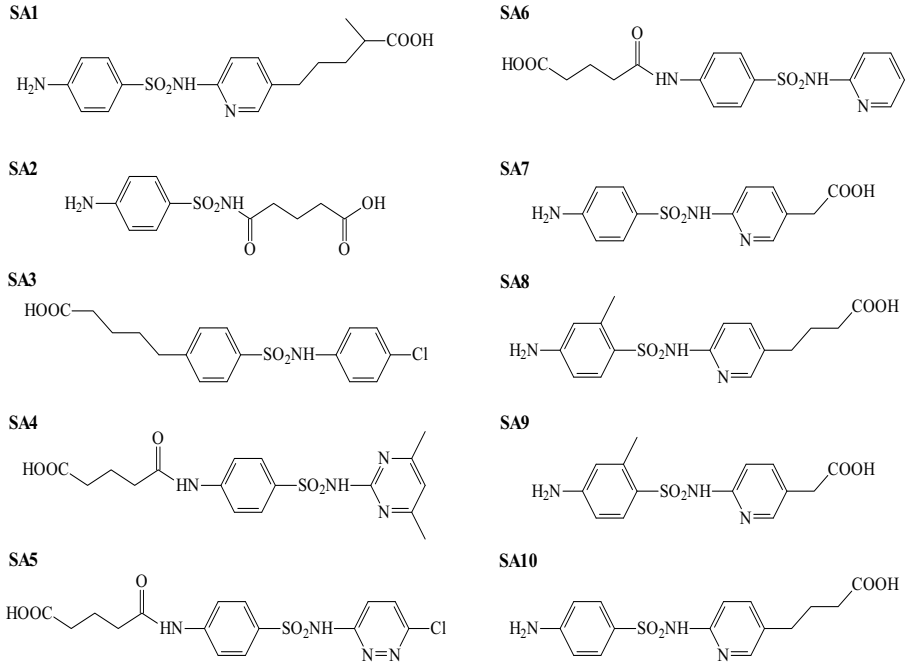

Figure A: Chemical structure of haptens synthesized.

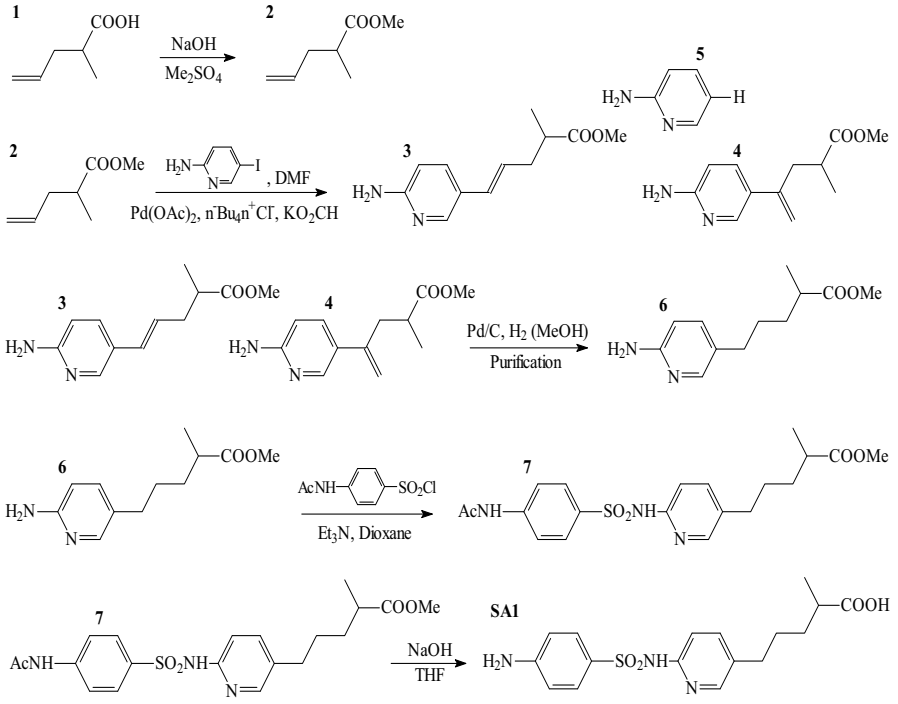

Figure B: Scheme showing the synthesis of hapten SA1.

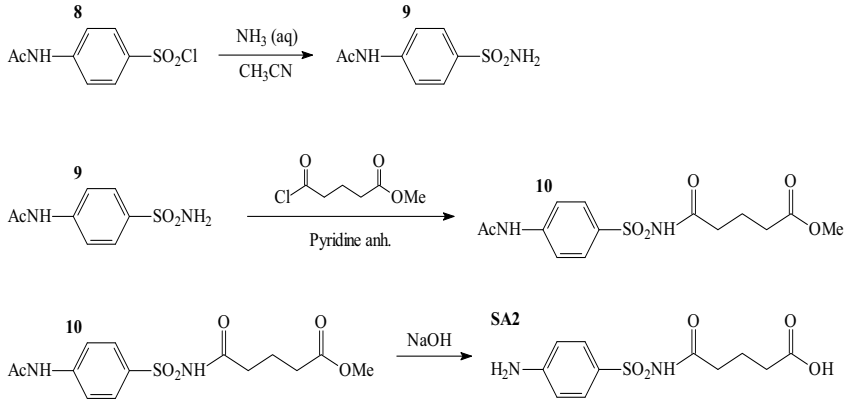

Figure C: Scheme showing the synthesis of hapten SA2.

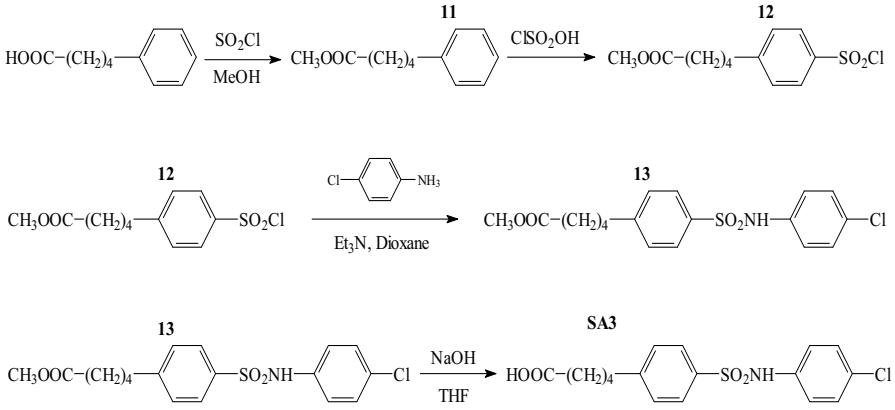

Figure D: Scheme showing the synthesis of hapten SA3.

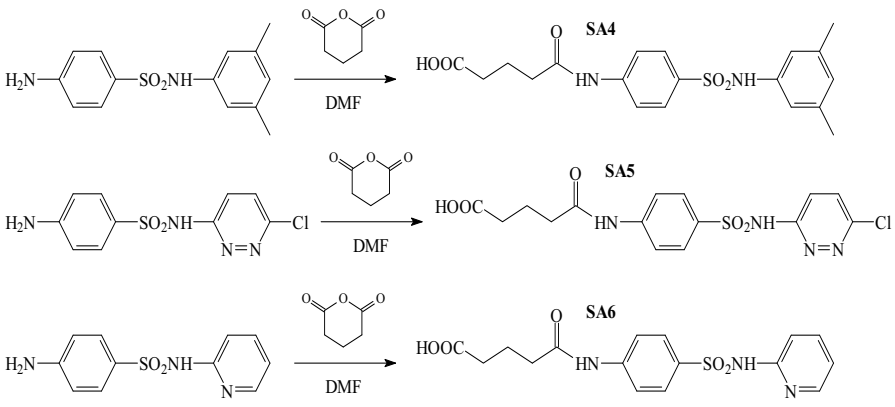

Figure E: Scheme showing the synthesis of haptens SA4, SA5 and SA6.

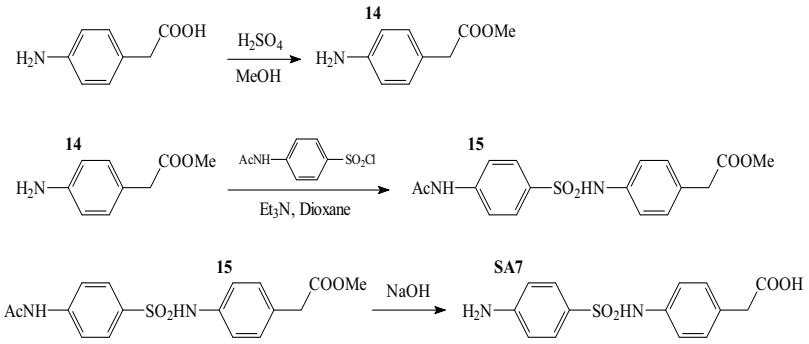

Figure F: Scheme showing the synthesis of hapten SA7.

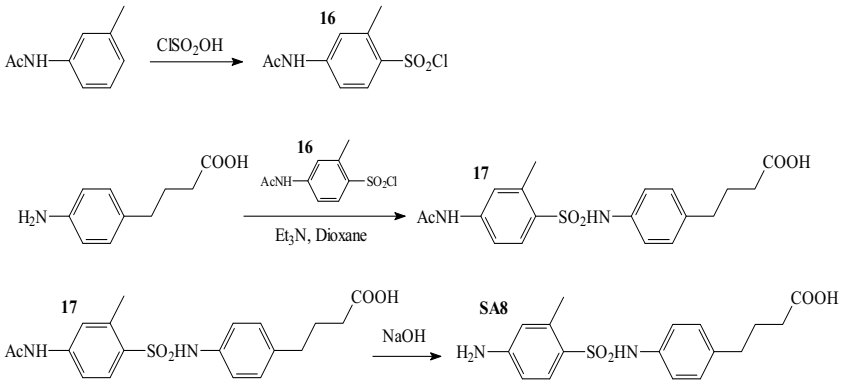

Figure G: Scheme showing the synthesis of hapten SA8.

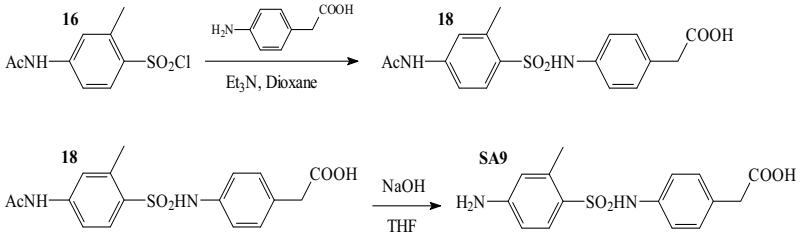

Figure H: Scheme showing the synthesis of hapten SA9.

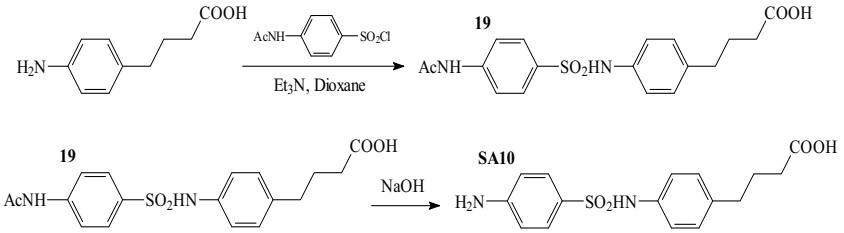

Figure I: Scheme showing the synthesis of hapten SA10.

3.4 SAs indirect ELISA format (Hair)

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Traceability of sulfonamide antibiotic treatment by immunochemical analysis of farm animal hair samples

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Abstract The use of hair to trace use of unauthorized substances, therapeutic agents, or their misuse is becoming very attractive since residues can be detected for a long time after treatment. For this purpose, an indirect enzyme-linked immunosorbent assay (ELISA) has been evaluated for its capability to trace sulfonamide antibiotic treatment by analyzing cattle and pig hair samples. Pigmented and non-pigmented hair samples from control and sulfamethazine (SMZ)-treated pigs and calves were collected, extracted under different alkaline conditions, and analyzed by ELISA after just diluting the extracts with the assay buffer. Data analysis following the European recommendations for screening methods demonstrates that the ELISA can detect SMZ in hair samples with a limit of detection (90% of the zero dose (IC_{90})) between 30 and 75 ng g^{-1}. The same samples have been analyzed by HPLC after a dual solid-phase extraction. The ELISA results matched very well those obtained by the chromatographic method, demonstrating that the immunochemical method can be used as a screening tool to trace animal treatments. Between the benefits of this method are the possibility to directly analyze hair extracts with sufficient detectability and its high-throughput capability. Preliminary validation data are reported using an experimental approach inspired on the Commission Decision 2002/657/EC criteria for screening methods.

Keywords Sulfonamide antibiotics · Sulfamethazine · Hair analysis · ELISA · Immunoassay · HPLC-DAD · Treatment traceability

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Introduction

Nowadays, development of new technologies to improve the control of antibiotics in foodstuffs from animal origin is required. The increase of meat production for human consumption has led, in some cases, to nonethical practices with the aim to improve the productivity of that industry and to prevent diseases derived from farm stacking practices. The abusive use of antimicrobials has led to the appearance of alarmingly growing antimicrobial resistance cases of commonly used antibiotics to fight against human diseases. Extremely active at low doses, these antibiotics can arrive to humans through foodstuffs of animal origin and continue having a negative impact on the health status of the population [1, 2].

The European Food Safety Authority, whose principles have been established in the EC 178/2002 regulation, has been coordinating and giving scientific support to food safety concerns. In this manner, the European Commission has established procedures for inspections dictating the frequency and number of substances that have to be monitored (Directive 96/23/CE). However, the analytical procedures today available do not have sufficient sample throughput capabilities to analyze the huge number of samples that is required. Thus, it is necessary to provide the reference laboratories with fast and high-throughput screening techniques with sufficient detectability to sense the presence of residues over the maximum residue levels

(MRL) established by the UE for foodstuffs according to toxicological data (Directive 2377/90/CE). Alternatively, farms can be inspected to detect inappropriate practices before the animal arrives to the slaughterhouse. Thus, veterinary drug residues and other contaminants are controlled not only in tissue samples obtained after the animal has been killed (i.e., kidney, muscle, liver, etc.) but also in urine, plasma, and milk samples taken from living animals. However, the collection of these samples is not always simple besides requiring careful storage and dispatching. Moreover, the presence of residues does not provide information on the inappropriate use of these substances on earlier animal growing phases.

Hair analysis is being seriously proposed to obtain retrospective information of veterinary drugs treatments since residues may be found for a long time after the treatment due to their lower metabolic activity [3, 4]. Moreover, sampling is much less invasive and easier to handle and to store in comparison with other biological samples [5, 6]. Hence, several studies support the use of hair for identification and quantification of therapeutic and illicit drugs used in humans [7, 8]. Deposition of sulfonamides in hair samples was firstly described by Dunnet and co-workers, who developed and validated a chromatographic method for the detection of different sulfonamides and trimethoprim in equine tail hair [3]. Moreover, a recent study demonstrated that sulfamethazine is also deposited in the hair of pigs and cattle at nanograms per gram levels. In this context, using a direct enzyme-linked immunosorbent assay (ELISA) for sulfonamides [9], we have recently demonstrated its applicability to detect sulfonamide residues in hair samples using spiked extracts [9]. The potential of immunoassays has also been demonstrated, tracing the use of drugs of abuse (amphetamines, benzodiazepines, cocaine, or opium alkaloids) by analyzing hair samples [10–18]. In this paper, we present the results of a study addressed to demonstrate that immunochemical analysis of hair is an excellent strategy to trace sulfonamide antibiotic treatment. Using antibodies with a broad selectivity spectrum for the sulfonamide antibiotic family [9, 19], sulfamethazine has been detected in the hair of treated animals. The method has been evaluated by comparing with the results obtained by HPLC using the same samples. Moreover, since no MRLs are established for hair, a preliminary proposal validation study has been performed following the recommendations of the Commission Decision 2002/657/EC criteria established for qualitative screening methods of substances for which no permitted limit has been established [20].

Experimental section

General methods and instruments The pH and the conductivity of all buffers and solutions were measured with a pH 540 GLP pH meter and a LF 340 conductimeter, respectively (WTW, Weilheim, Germany). The oven employed for the hair alkaline digestion was from Digitronic (JPSelecta, Abrera, Spain). Hair extracts were filtrated through 0.22- or 0.45-μm nylon syringe filters [21] (GE Osmonics Labstore, Minnetonka, MN, USA). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). Washing steps were performed on a SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). A Heidolph Titramax 1000 vibrating platform shaker (Brinkmann Instruments, Westbury, NY, USA) was used to shake the microplates at 900 rpm. Absorbances were read on a SpectramaxPlus (Molecular Devices, Sunnyvale, CA, USA). The competitive curves were analyzed with a four-parameter logistic equation using the software SoftmaxPro v4.7 (Molecular Devices) and GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Unless otherwise indicated, ELISA data presented correspond to the average of at least two well replicates. The HPLC analysis was carried out on an 1100 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump and a diode array detector. Chromatographic separation was performed on a Luna RP-C18 (150 mm×2.1 mm i.d.) column (Phenomenex, Torrance, CA, USA). All samples extracts were injected in triplicate. Solid-phase extraction (SPE) RP polymeric cartridges (HLB-Oasis, 6 cm^3/200 mg) were obtained from Waters (Milford, MA, USA), while strong cationic exchange cartridges (SCX II Accubond, 6 cm^3/500 mg) were obtained from Agilent Technologies S.L. (Madrid, Spain).

Chemicals and immunoreagents Immunochemicals were obtained from Sigma Chemical (St. Louis, MO, USA). Preparation of the immunoreagents required for the ELISA development, like the polyclonal antiserum (As155) and the coating antigen (SA2-OVA), is described elsewhere [19]. Stock solution of sulfamethazine, kindly supplied by Riedel-de Haën, was prepared in DMSO at a concentration of 10 mM and kept at 4 °C.

Buffers Phosphate-buffered saline (PBS) is 0.01 M phosphate buffer on a 0.8% saline solution (137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl), and the pH is 7.5. PBST is PBS with 0.05% tween 20. For hair experiments, PBS2T is PBS with 0.10% of tween 20. Borate buffer is 0.25 M boric acid-sodium borate, pH 8.7. Coating buffer is 0.05 M carbonate-bicarbonate buffer, pH 9.6. Citrate buffer is a 0.04 M solution of sodium citrate, pH 5.5. The substrate solution contains 0.01% 3,3′,5,5′-tetramethylbenzidine and 0.004% H_2O_2 in citrate buffer.

Samples Hair samples from four Friesian calves (colorless and pigmented) and six Landrace pigs (colorless), un/treated with

sulfamethazine, were collected in the Centre de Tecnologia de la Carn (IRTA; Institute for Food and Agricultural Research and Technology) at different periods of a controlled pharmacological treatment. Hair samples were rinsed with abundant milliQ water, dried, and cut finely before being analyzed. Additional information on the procedure used to collect and process the sample can be found in a recent publication [4]. For the evaluation studies, hair filaments were split into three portions (3×50 mg), extracted, and analyzed separately.

Sample treatment Extracts—Hair samples (50 mg) were extracted with 0.1 M NaOH (4 mL, 1 h at 60 °C) and 0.2 M NH_4OH (4 mL, overnight at 60 °C) in 10 mL amber glass tubes. ELISA analysis—Extracts were filtered through 0.22 μm nylon filters and analyzed after adjusting the pH and buffering the sample. HPLC analysis—Extracts were loaded onto a SPE Oasis-HLB cartridge, previously activated, subsequently washed with 2 mL of 5% aqueous methanol, and then dried under vacuum during 2 min. SMZ was eluted with 4 mL of acetic acid/acetonitrile (5:95, *v*/*v*) and loaded directly onto an activated Accubond SCX cartridge. The SCX cartridge was washed with 5 mL methanol/acetonitrile (1:1, *v*/*v*), then SMZ was eluted with 5 mL of ammonium hydroxide/methanol (5:95, *v*/*v*) solution. Eluates containing SMZ were evaporated under nitrogen at 25 °C, reconstituted with 400 μL of ammonium acetate 0.01 M pH 4.5/acetonitrile (88:12, *v*/*v*) and filtered through a 0.45 μm nylon filters prior to the chromatographic analysis.

ELISA: general protocol Microtiter plates were coated with SA2-OVA (0.625 μg mL^{-1} in coating buffer, 100 μL/well) overnight at 4 °C and covered with adhesive plate sealers. The next day, plates were washed four times with PBST (300 μL/well), and the sulfamethazine standards (from 13,900 to 0.0007 μg L^{-1} in PBST) and/or the samples were added to the microplate (50 μL/well) followed by the antiserum As155 (1/8,000 diluted in PBST, 50 μL/well) and incubated for 30 min at room temperature while shaking. The plates were washed as before, and a solution of anti IgG-HRP (1/6,000 in PBST) was added to the wells (100 μL/well) and incubated for 30 min more at room temperature. The plates were washed again, and the substrate solution was added (100 μL/well). Color development was stopped after 30 min at room temperature with 4 N H_2SO_4 (50 μL/well), and the absorbances were read at 450 nm. The standard curves were fitted to a four-parameter equation according to the following formula: $y = (A - B/\left[1 - (x/C)^D\right] + B$, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve. Hair samples were analyzed in triplicates.

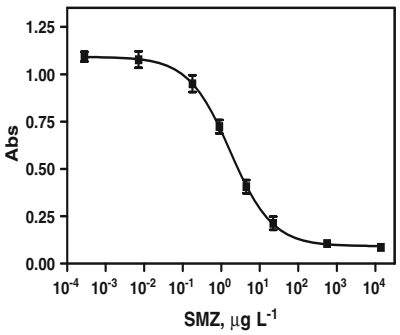

Fig. 1 Calibration curve of the As155/SA2-OVA immunoassay. The data shown in the graph are the averages and standard deviations of five assays performed in different days. Each datum is the result of the analysis of the same sample on two well replicates on every assay. The parameters of the assay are shown in Table 1

Table 1 Features of the sulfamethazine ELISA assay in buffer and hair samples[a]

Parameter	Sample Measured		
	Buffer[b]	Hair (1/5)[c,d]	Hair (1/10)[c,d]
IC_{50}	1.75±0.02	0.70±0.01	1.40±0.02
Working range	0.28±0.03	0.11±0.01	0.22±0.02
	10.2±1.83	4.1 ± 0.7	to 8.2±1.4
LOD	0.09±0.02	36.0±8.0	72.0±16.0

[a] Values obtained correspond to the average and standard deviation of each parameter of five assays performed in different days. Other immunoassay parameters (A*max*, A*min*, Slope and R^2 are similar in all assays. As an example, in PBST, the *Amax* is 1.09±0.02, the A*min* is 0.09±0.01, the slope is –0.80±0.05 and R^2 is 0.996±0.002

[b] For the buffer the values are expressed in μg L^{-1}

[c] For hair samples values are expressed in ng mg^{-1}

[d] Assay paremeters corresponding to the concentration in the matrix.

ELISA: accuracy studies This parameter was assessed by preparing eight, different, blind, spiked samples in PBST buffer and measuring them by duplicate in the ELISA.

ELISA: matrix effect studies Hair extracts prepared as explained above were buffered by adding 10% (*v*/*v*) of 100 mM PBS and adjusting the pH to 7.5 with few drops of 5 N HCl, if necessary. Then, the extracts were diluted with PBS (1/1, 1/5, 1/10, 1/25, and 1/50) and used to prepare SMZ standard curves that were run in the ELISA, preparing the As155 solution in PBS2T, with the objective to evaluate the parallelism with the buffer standard assay estimating the extent of the interferences caused by the matrix.

HPLC: procedure Sulfamethazine HPLC analyses were carried out using a binary gradient elution at a flow rate

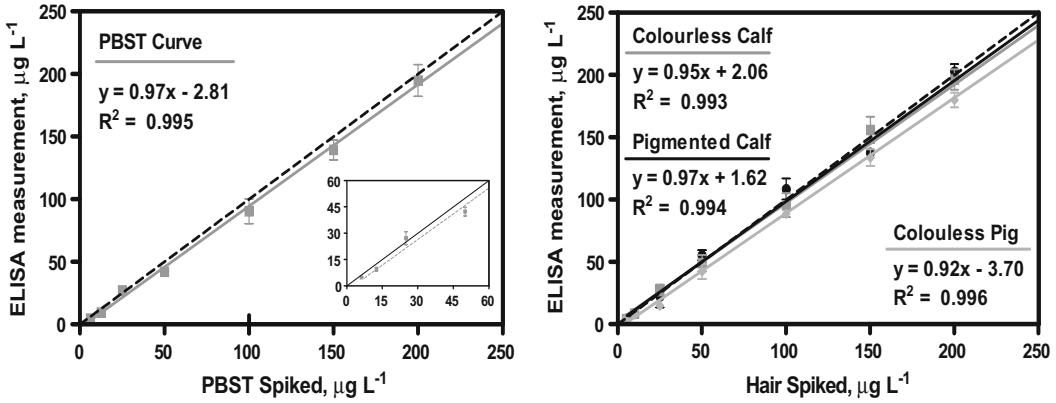

Fig. 2 Results from the accuracy studies. The graph shows the correlation between the spiked and the measured concentration values using the As155/SA2-OVA ELISA to analyze SMZ in buffer (*left*) and hair extract samples diluted ten times (*right*). The *inset* shows the results at lower concentration. Each spiked sample was tested using three well replicates on every assay. The *dotted lines* correspond to a perfect correlation (slope=1)

of 0.35 mL min^{-1}, varying the composition of the mobile phase linearly from 88% A (ammonium acetate 0.01 M solution, acidified to pH 4.5 with acetic acid glacial) and 12% B (acetonitrile) to 82% A and 18% B at 12 min. An external standard calibration curve was created by injecting known amounts of the sulfonamide. Under the chromatographic conditions established, the SMZ peak showed an average t_R of 9.3 min (RSD 2%, n=12). The identification of SMZ peak in hair extracts was made comparing both retention time and spectra with those of the SMZ pure standard solutions. Peak purity was also evaluated with the Chemstation software utility. SMZ was quantified at a wavelength of 270 nM (16 nm bandwidth) with a reference signal of 450 nm (80 nm bandwidth). An external standard calibration curve was built by injecting known amounts of SMZ (0.1–60 ng mg^{-1}). More details of this procedure are already described by Gratacós-Curbasi and co-workers [4]. Analysis of hair extracts were performed per triplicate.

Decision limit (CCα) and detection capability (CCβ) Different hair samples (20 blanks) collected from different control cows, known to be free of sulfonamides, were repetitively analyzed on three different days by ELISA as described above to calculate the average and standard deviation (SD) of the signal afforded and further on determining the CCα and CCß parameters. CCα was calculated as the concentration that provides a signal equivalent to subtracting 2.33 times the standard deviation to the average signal given by the blank samples (α=1%) prior to the hair curve interpolation. CCß was determined, in the same way, using the equation CC = CCa(signal) – 1.64 × SD, being SD the standard deviation obtained for above blanks.

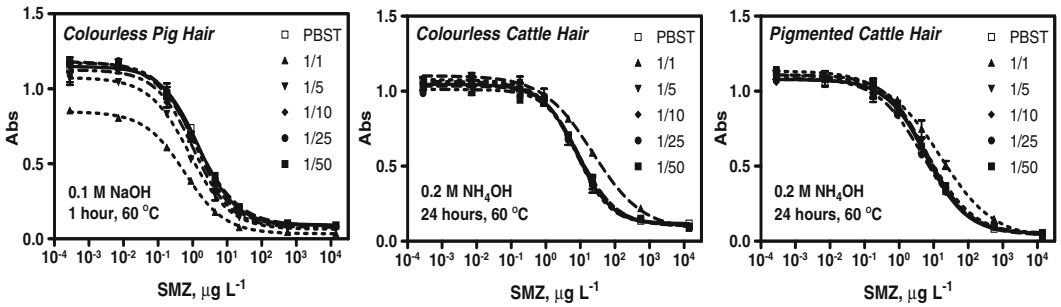

Fig. 3 Matrix effect caused by extracts prepared using different types of hair (colorless and/or pigmented) from calf or pig in the As155/SA2-OVA ELISA. Samples were obtained from untreated controlled animals. Aqueous extracts were prepared with 0.1 M NaOH or 0.2 M NH_4OH. Before ELISA measurements, the extracts were buffered and diluted several times to build the calibration curves using SMZ as standard. The points are the average of two well replicates

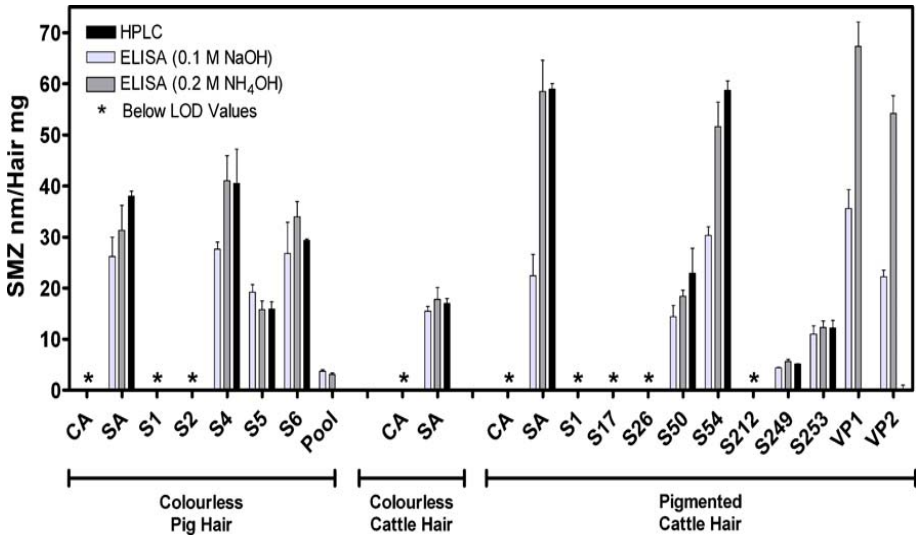

Fig. 4 Results from the analysis of real hair samples. Samples were extracted with both NaOH and NH_4OH treatments. Total content of sulfamethazine determined by ELISA is compared with HPLC-DAD analysis. For each analysis, three hair samples were extracted and subsequently analyzed per triplicate. Samples named as Pool (colorless pig hair) and VP1 and VP2 (pigmented cattle hair) were not analyzed by HPLC

Results and discussion

Accumulation of sulfonamides in the structure of pig and calf hair, after a therapeutic administration, was firstly demonstrated by Gratacós-Curbasí and co-workers [4] using HPLC-DAD after SPE purification. Recently, using sulfapyridine spiked hair extracts, we have demonstrated that ELISA may be suitable to detect sulfonamide residues in hair samples [9]. A direct ELISA able to detect four different sulfonamides was used. Here, we present further work performed in this direction using an indirect immunoassay (As155/SA2OVA) able to detect up to 11 different sulfonamide antibiotic congeners [19] and measuring hair samples collected from SMZ-treated animals at therapeutic doses. Using this ELISA, SMZ can be detected in buffer with a limit of detection (LOD) of 0.09±0.02 μg L^{-1} (90% response of the zero dose) and with a working range between 0.28±0.03 and 10.2±1.8 μg L^{-1} (20% to 80% of the assay response at zero doses, see Table 1). Figure 1 shows a SMZ standard curve corresponding to data recorded from five assays performed in buffer in different days. Additionally, the accuracy of the assay was evaluated by measuring repetitively eight blind samples prepared in buffer during different days. The results shown in Fig. 2 (left graph) correspond to the correlation found between the measured and the spiked concentration values. As it can be observed, results obtained match very well the spiked values as it is demonstrated by the coefficient of correlation (R^2=0.995) and a slope value near 1 (0.97), which indicates excellent recovery values in all cases.

Hair extracts were prepared by alkaline digestion, at temperatures no higher than 60 °C and at different contact times. As reported [3, 4], the higher extraction efficiency (>80%) was obtained with 0.2 M NH_4OH (60 °C, overnight) although 0.1 N NaOH (60 °C, 1 h) also provided good recoveries (75–80%). In contrast, poor extraction efficacy was accomplished when using acidic or alcoholic solutions (<40%). Nonspecific interferences on the immunoassay were evaluated using hair extracts from nontreated animals. Prior the immunochemical analysis, the extracts were filtered and buffered by adding 10% (*v*/*v*) of 100 mM PBS (few drops of 5 N HCl were added when necessary to adjust the pH to 7.5). Further dilutions of the hair extracts were performed in 10 mM PBS. SMZ standard curves were prepared with these extracts in order to evaluate the parallelism with the standard curve prepared in buffer. Results are shown in Fig. 3 where it can be observed that, undesirable matrix effects of the 0.1 M NaOH, extracts could be removed by diluting ten times with the assay buffer independently from the type of hair. SMZ residues could be detected on these extracts with a LOD of 72.0±16.0 ng g^{-1} (see Table 1). NH_4OH extracts were cleaner and allowed performing measurements after just five times dilution of the extract reaching LOD values of 36.0± 8.0 ng g^{-1} (Fig. 3, centered and right graphs), almost in the same range as the HPLC-DAD method [4]. Therefore, these preliminary experiments indicated that the detectability achieved was sufficient to analyze hair sample without the need to preconcentrate the sample. Moreover, the extraction procedure was easy to perform, and the matrix interferences could be removed by just a simple dilution with the assay

Table 2 Results from the ELISA analysis of SMZ in the different type of hairs compared to the HPLC analysis

Hair samples[a]	Calibration curve measurement				
		0.1M NaOH treatment		0.2M NH_4OH treatment	
	HPLC (*A*)	ELISA	*B*/*A*	ELISA (*C*)	*C*/*A*
Colorless pig					
Sample CP_CA	nd[b]	nd[b]	–	nd[b]	–
Sample CP_SA	37.5±4.3	26.2±3.8	69%	31.3±4.9	82%
Sample CP_S1	nd[b]	nd[b]	–	nd[b]	–
Sample CP_S2	nd[b]	nd[b]	–	nd[b]	–
Sample CP_S4	40.5±6.7	27.6±1.4	68%	41.0±0.3	101%
Sample CP_S5	15.9±1.4	19.2±1.5	120%	15.8±1.7	99%
Sample CP_S6	29.3±0.3	26.8±6.1	91%	34.0±2.9	116%
Sample CP_Pool	–[c]	3.7±0.3	–	3.1±0.3	–
Pigmented cattle					
Sample PC_CA	nd[b]	nd[b]	–	nd[b]	–
Sample PC_SA	58.7±1.9	22.4±4.2	69%	58.5±6.1	98%
Sample PC_S1	nd[b]	nd[b]	–	nd[b]	–
Sample PC_S17	nd[b]	nd[b]	–	nd[b]	–
Sample PC_S26	nd[b]	nd[b]	–	nd[b]	–
Sample PC_S50	22.9±4.9	14.4±2.2	63%	18.4±1.2	80%
Sample PC_S54	58.7±1.9	30.3±1.7	52%	51.6±4.8	88%
Sample PC_S212	nd[b]	nd[b]	–	nd[b]	–
Sample PC_S249	5.1±0.1	4.4±0.1	86%	5.6±0.5	109%
Sample PC_S253	12.2±1.5	11.0±1.6	90%	12.3±1.3	101%
Sample PC_VP1	–[c]	35.6±3.7	–	67.3±4.8	–
Sample PC_VP2	–[c]	22.2±1.3	–	54.2±3.5	–
Colorless cattle					
Sample CC_CA	nd[b]	nd[b]	–	nd[b]	–
Sample PC_SA	16.7±0.5	15.5±0.9	91%	17.8±2.3	105%

[a] Results are expressed as SMZ nanograms per hair milligram

[b] SMZ not detected (concentration below the LOD for the technique applied)

[c] Sample not analyzed by HPLC

buffer, while for HPLC-DAD, the alkaline extract had to be purified through a dual SPE.

Accuracy was assessed by measuring blind spiked hair extracts. As it can be observed in Fig. 2 (right graph), a very good correlation was observed between the measured and the real concentration values (right graph) providing slopes close to 1 (colorless pig hair, 0.92; colorless cattle hair, 0.95; pigmented cattle hair, 0.97) and very good coefficients of correlation (colorless pig hair, 0.996; colorless cattle hair, 0.993; pigmented cattle hair, 0.994). The results obtained demonstrate the suitability of the immunoassay to determine of SMZ in different types of hair samples.

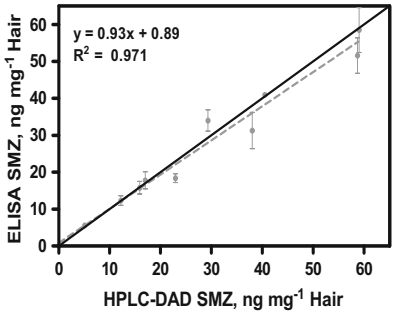

Fig. 5 Correlation between SMZ determined by ELISA and HPLC-DAD for the hair samples analyzed (n=22) after NH_4OH treatment. The *black line* indicates and ideal correlation (x=y) between both methods

Finally, with the aim to further evaluate the potential of the immunochemical method, alkaline extracts were prepared from hair samples (n=22) from different treated and not treated animals, obtained at different collection times. Samples were split in two fractions to be analyzed by ELISA and HPLC-DAD. The ELISA measurements were performed on serially PBS diluted samples (to ensure measurements within the dynamic range of the immunoassay, 0.28–10.2 μg L^{-1}). The ELISA analyses revealed that the hair of treated animals contained high levels of SMZ with concentration values ranging between 5 and 60 ng mg^{-1}. These high levels demanded for applying dilution factors much higher than those necessary to eliminate nonspecific matrix effect (1/5 or 1/10). Moreover, as it can be observed in Fig. 4 and Table 2, the results obtained by

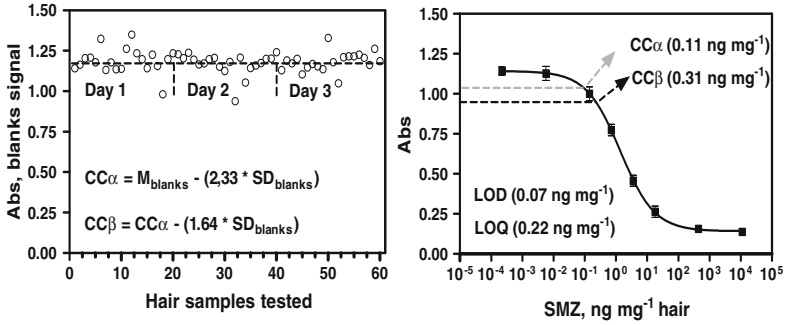

Fig. 6 Determinations of decision limit (CCα) and detection capability (CCß) parameters of the SMZ ELISA in hair (*left*). CCα was calculated from the hair cattle extracts (n=20, 3 days) by subtracting 2.33 times the corresponding standard deviation to the average signal when measuring the blank samples (α=1%) before estimating the concentration in the calibration curve. CCß was determined using the equation CC = CCa(signal) − 1.64 × SD_{blanks}, in terms of response values before assay curve interpolation. A typical matrix matched calibration curve for SMZ antibiotic in hair cattle samples (*right*). CCα and CCß values obtained are included as sulfonamide nanograms per gram of hair and compared with the corresponding LOD and LOQ of the assay

ELISA matched very well with those obtained by HPLC-DAD, particularly for the NH_4OH extracts. The concentration of SMZ in hair samples extracted with NaOH was clearly underestimated by the immunoassay at levels over 15 ng mg^{-1} (see Fig. 4 and Table 2), probably due to lower extraction efficiency or partial destruction/degradation of the analyte under these conditions. Moreover, more complex HPLC chromatograms and greater ELISA interferences were observed when NaOH was used for extraction.

A linear regression analysis was also performed with all hair samples analyzed by ELISA and HPLC-DAD. As can be observed in Fig. 5, an excellent correlation between both techniques was observed (y=0.93x + 0.89, R^2=0.971). Moreover, samples labeled as not contaminated were confirmed to be free of sulfonamides by both methods.

Finally, we intended to characterize the assay following criteria of the Commission Decision 2002/657/EC for qualitative screening methods, even if in this case no regulation exists regarding sulfonamide residue levels in hair. The criteria was just followed as an alternative to provide objective data of the reliability of the immunochemical method presented here as a tool to detect SMZ residues in hair. From the not very clear different approaches, suggested by the European Commission [20, 22, 23] for screening methods of substances for which no permitted limit have been established yet, we have used here a close alternative. Thus, blank hair samples (n=20) have been analyzed for 3 days by ELISA to obtain the background values for the noncontaminated samples (see Fig. 6, left graph). A CCα value of 0.11 ng mg^{-1} was obtained by subtracting 2.33 times the SD of the blank signals to the mean signal to noise level and subsequently interpolated this value in the standard hair calibration curve. This value gives the level at which using this method, the sample can be considered positive, although it may exist a low risk of false positives and a certain risk of false negative, which is not desirable for a screening method. The detection capability, which reduces the probability of false negatives, is more useful. In this case, this parameter was calculated from the equation CC = CCa(signal) − 1.64 × SD_{blanks}, in ELISA response terms as before, obtaining a value of 0.31 ng mg^{-1} in the reference curve. The CCα and the CCß values obtained (see Fig. 6, right graph) were slightly higher than the LOD (0.07 ng mg^{-1}) and limit of quantitation (LOQ; 0.22 ng mg^{-1}) values calculated as the concentrations producing 90% and 80% of the maximum response, but considering that the SMZ levels found in hair, are sufficient to analyze this residues with sufficient reliability. Further work needs to be addressed to confirm that using the CCβ value, the probabilities for false negatives is below 5% as it is proposed in the regulation.

Conclusions

An immunochemical analytical method to analyze SMZ in different types of hair samples has been presented. The whole analytical procedure shows high-throughput screening capabilities since the extraction procedure is fast and simple and completely compatible with the ELISA method. The immunoassay takes about 90 min and can process many samples simultaneously. Thus, in the actual microplate setup used in this study, about 16 samples could be analyzed in one plate using two well replicates and two different dilution factors for each extract (four measurements for one sample). As155/SA2-OVA immunoreagent combination has shown as an excellent detectability for SMZ (LOD=0.09±0.02 μg L^{-1} in buffer). The nonspecific interferences caused by the hair extracts can be removed by just buffering and diluting the samples, obtaining a LOD in the range of 36–72 ng g^{-1}, depending on the extraction procedure used and applied (1/5 for NH_4OH or 1/10 for NaOH). Moreover, the broad specificity of the ELISA used

for this study allows detecting up to 11 sulfonamide antibiotics, as previously reported. The excellent correlation between ELISA and HPLC techniques demonstrates that the present immunoassay is a reliable and accurate tool for SMZ quantification in hair extracts obtained from NH_4OH digestions. These results prove the suitability of the ELISA method presented to trace sulfonamide antibiotic treatment in live animals without using invasive methods by analyzing hair samples, which are very easy to obtain. Thus, as routine qualitative screening of SMZ in hair, the proposed shows a CCα and CCß below 0.11 and 0.31 ng mg^{-1} for hair samples, respectively. Finally, considering the successful analysis of sulfonamides in complex biological samples, such as milk [19] or hair, there is a great promise regarding the potential application of this immunoassay on new matrices like feed, water, and soil samples where lately antibiotics have been considered as emerging pollutants.

Both the ELISA and the chromatographic method used in this study have shown to be useful methods to detect sulfonamide residues in hair samples after animal treatment. For the retrospective detection of inappropriate administration of these antibiotics, further studies should be addressed to understand the kinetics of deposition in hair and the relation with the antibiotic treatment, regarding time and therapeutic protocol used. The society of hair testing has given some recommendations about the examination of contaminants in human hair, even though there are still questions to be solved to set up a consensus opinion and interpretation criteria. Transferring that into animals is not easy because nowadays knowledge on veterinary drug deposition is poor for the great number of active compounds and livestock species besides the effect of multiple endo- and exogenous factors that influence their accumulation in hair. Nowadays, there are few evidences in the literature of accumulation of substances such as antibiotics or anabolic steroids in livestock hair [21, 24–27]. Many studies in that aspect should be performed in order to clarify more this field but meanwhile, development of different methodologies to analyze hair could be interesting to help, facilitate, and understand better most aspects of this novel matrix.

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3.5 Results and discussion

This chapter reports the research performed in relation to the establishment of immunochemical protocols for the analysis of SAs residues in biological samples. **Figure 3.12** shows a flow-chart indicating common steps involved on the development of an immunochemical assay for small organic molecules. As mentioned before, the contribution of this thesis starts at the time when antibodies against two different immunogens, SA1 and SA2 had been produced, but its activity and features had not been evaluated.

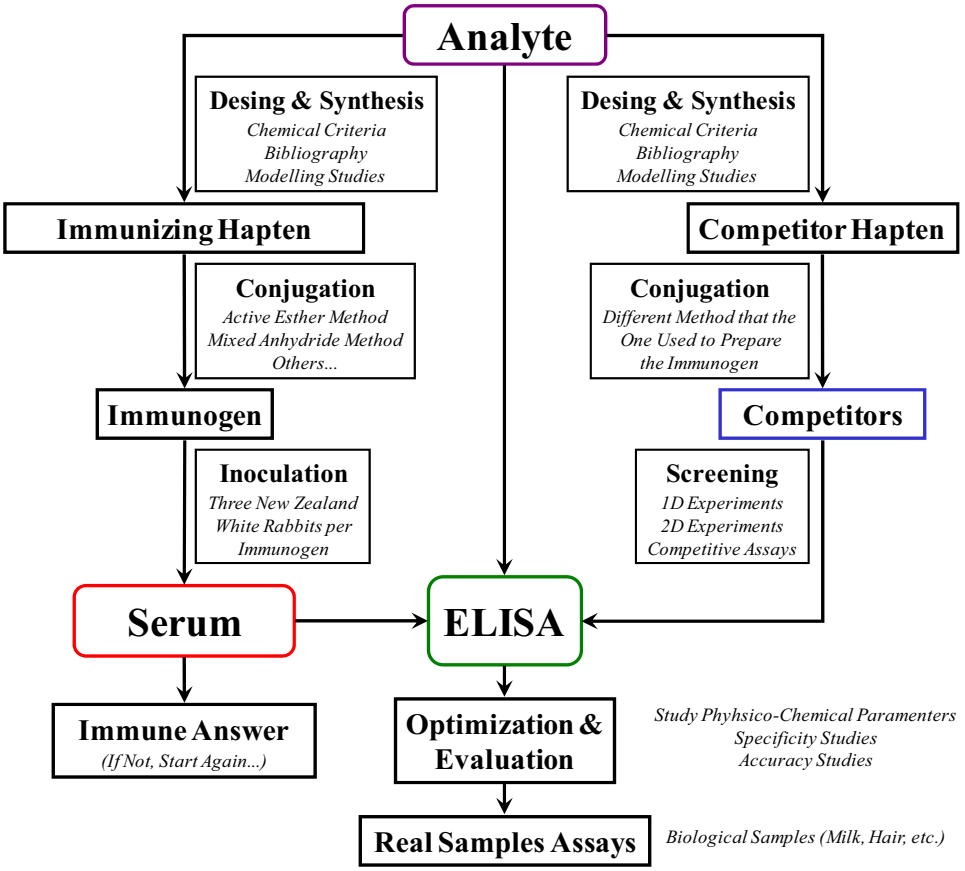

Figure 3.12: The scheme shows the entire strategy followed in our research group to develop an immunoassay based on PAb against a specific analyte or family of compounds. It consists on preparing from one side all the reagents necessary to obtain the antibodies (left side) and on the other hand the secondary immunoreagents (right side) required to establish an ELISA procedure.

3.5.1 Screening, Evaluation and Optimization

The recognition for the competitors (haptenized enzymes and proteins), ETs (direct format) and CAs (indirect format), by the As was firstly evaluated through 1D (one dimension) experiments using non-competitive ELISAs. In those experiments the recognition of the competitors is tested by measuring the binding of several dilutions of the different antisera (indirect format) or the distinct ETs (direct format) to CA or As microplates coated at a constant concentration, respectively. As a result of these experiments the As/CA (indirect format) and As/ET (direct format) providing the highest signal are selected. Further on, the avidity of the As for the best recognized competitors was subsequently evaluated by two-dimensional checkerboard titration experiments (2D). From these experiments, we selected the appropriate concentrations to perform further competitive assays, in both formats (see **Section 3.1.5**), using a representative sulfonamide antibiotic such as sulfapyridine (SPY).

No competitive assays were obtained when using the antisera generated against SA2-HCH, in contrast all combinations chosen for the case of the antisera against SA1-HCH gave competitive assays. The selection of the SA2 hapten for antibody production was made in order to maximize recognition of the common epitopes of the SAs, however, these results indicate that in this particular case, the use of just one fragment of the molecule provided antibodies with insufficient affinity for the target analytes. On the other hand, these results support the criteria used to design the immunizing hapten SA1, for which it was considered crucial to keep at least, one electron-withdrawing atom close to the carbon situated in the alpha position of the sulfonamide bond (see **Figure 3.5**).

For the direct ELISA the best detectability (low IC_{50}) values were afforded by the homologous antibody/antigen combinations (same hapten as immunogen and as competitor). On the contrary, the best competitive assays for the indirect ELISA format were afforded by the heterologous combinations (different hapten as immunogen and as competitor) playing this parameter an important role in the sensitivity pattern obtained (see **Table 3.4**).

The selections of the best antibody/competitor combinations was made based on the parameters of the fitting equation, selecting those antibody/competitor combinations providing a high A_{max}, a high A_{max}/A_{min} ratio, a low A_{min}, a low IC_{50} value, a slope between 0.7 and 1.2, and the $R^2>0.90$. Moreover, other parameters such as reproducibility of the assays between days were also considered. Thus, several combinations were found suitable for the analysis of SAs, however, after repetitive experiments, one antibody/competitor combination was selected for each format in order to pursuit with the

characterization and evaluation of these immunoreagents on subsequent experiments (direct ELISA: As154/SA1-HRP, indirect ELISA: As155/SA2-OVA).

Table 3.4: Immunoassay features of some competitive direct and indirect ELISAs for SAs.

Immunogen	**As** [a]	**ET (direct)**	**Amax**	**Amin**	**IC_{50}** [b]	**Slope**	**R^2**
SA1-HCH	**154**	**SA1-HRP**	**0,814**	**0,064**	**19,78**	**-0,801**	**0,981**
	154	SA7-HRP	0,755	0,297	28,88	-0,747	0,855
	156	SA1-HRP	0,513	0,007	25,73	0,539	0,993
	156	SA7-HRP	0,831	0,160	43,58	-0,665	0,994
Immunogen	**As** [a]	**CA (indirect)**	**Amax**	**Amin**	**IC_{50}** [b]	**Slope**	**R^2**
SA1-HCH	154	SA5-OVA	0,858	0,038	13,97	-0,861	0,989
	154	SA8-BSA	0,736	0,169	9,855	-0,875	0,961
	155	SA2-BSA	1,031	0,017	5,948	-0,660	0,997
	155	**SA2-OVA**	**0,896**	**0,045**	**3,919**	**-0,749**	**0,994**
	167	SA5-BSA	0,665	0,006	13,10	-0,643	0,995
	167	SA7-OVA	0,546	0,008	21,15	-0,763	0,994

[a] *No competition was observed for As raised against SA2-HCH.*
[b] *IC_{50} values are expressed in µg L^{-1}. Boxes represented in bold show the immunoassay features of the best combinations used for further optimization.*

Then, a set of experimental parameters (e.g. incubation time, detergent concentration ionic strength, pH, among others) was studied sequentially in both ELISA formats in order to improve the immunoassays detectability and to study their performance under several conditions. Factors determining the best immunoassays performance for the selected antibody/competitor combinations in both formats and their parameters are summarized in **Table 3.5**.

Table 3.5: Optimum conditions and features of the SPY immunoassays for As155/SA2-OVA and As154/SA1-HRP combinations. [a] Concentrations are expressed in µg L^{-1}.

Condition	**Values**	**Parameter**	**As155 SA2-OVA**	**As154 SA1-HRP**
Preincubation time	0 min	A_{max}	1.07 ± 0.07	1.04 ± 0.08
Competition time	30 min	A_{min}	0.04 ± 0.02	0.04 ± 0.01
pH	7.5	IC_{50}[a]	2.35 ± 0.72	11.21 ± 2.88
Ionic strength	10 mS/cm (10 mM PBS)	Dynamic range[a]	0.4 ± 0.1 to 16.9 ± 3.5	1.6 ± 0.5 to 72.8 ± 23.7
Tween 20	0.05%	Slope	-0.73 ± 0.10	-0.75 ± 0.05
		LOD[a]	0.15 ± 0.08	0.44 ± 0.13
		R^2	0.997	0.972

The data presented correspond at least to the average of 6 calibration curves run in different days.

Moreover, these studies provided information of the response of the immunochemical assay under different physic-chemical conditions, which it is very useful for further application of this assay to real samples matrices. Thus, it is worth mentioning that the direct ELISA tolerated very well variations in the ionic strength of the media within the range of 0 to 70 mS cm^{-1}, although a total absence of salts produced a drastic decrease of the signal. In contrast, for the indirect assay the increase in the ionic strength produced an improvement of the detectability, but also a decrease of the A_{max}. In the absence of salts, the indirect assay showed a high A_{max}, but the analyte failed to compete under these conditions. The corresponding standard curves are presented in **Figure 3.13**. It can be observed that the heterologous indirect ELISA has a significantly greater detectability than the SPY detectability achieved by the homologous direct format. As mentioned before, this can be explained based on the heterology principle shown above in **Figure 3.6**. The same behaviour has been found in other competitive assays [30, 34].

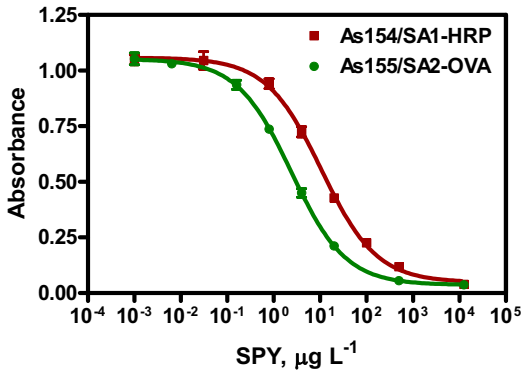

Figure 3.13: Standard calibration curves for the SPY immunoassays to compare direct (As154/SA1-HRP) and indirect (As155/SA2-OVA) ELISA formats.

In both ELISA formats the detectability achieved (LOD 0.15 and 0.4 µg L^{-1}, respectively for the indirect and direct formats) is far below the requirements of the EC in respect to the MRL allowed in milk (100 µg L^{-1}, see **Table 3.1**) which pointed to the fact that the immunoassays developed could be useful as screening tools for antibiotic residue analysis. However, this fact had to be demonstrated also running the assays on milk samples (see **Section 3.5.4.1**).

3.5.2 Specificity Studies

Specificity of both assays was assessed by preparing standard curves with a battery of 14 SAs congeners. As it can be observed in **Table 3.6** the heterologous indirect ELISA format increased notably the number of sulfonamide congeners detected in comparison

with the direct homologous ELISA. Thus, this last format was found to be quite specific for SPY, only sulfamethoxypyridazine, sulfathiazole and sulfachlorpyridazine did cross-react in this assay. This pattern recognition demonstrates the importance of the substitution group in the N_1 position. The highest selectivity of immunoassays with homologous combinations has been reported before [11, 29], and it can be explained based on the difficulties of the other congeners to efficiently compete with the competitor for the binding sites of the antibodies. In contrast, on heterologous format, the decrease in the affinity of the antibody for the competitor favours that other structurally related analytes can compete.

Table 3.6: Interference caused by structurally related sulfonamide antibiotics, expressed by their IC_{50} and the percentage of cross-reactivity.

Sulfonamide antibiotic	As155 SA2-OVA		As154 SA1-HRP	
	IC_{50} (µg L^{-1})	% CR	IC_{50} (µg L^{-1})	% CR
Sulfapyridine	**2.25**	**100**	**11.7**	**100**
Sulfaquinoxaline	**79.15**	**4**	933.7	2
Sulfachloropyridazine	**61.81**	**5**	**94.6**	**14**
Sulfamethoxazole	> MRL	< 1	1298.3	1
Sulfisomidine	**2.10**	**140**	972.5	1
Sulfathiazole	**1.30**	**202**	**36.4**	**33**
Sulfadiazine	**14.05**	**15**	779.6	2
Sulfadimethoxine	**41.22**	**8**	1752	1
Sulfamerazine	**3.73**	**79**	649.9	2
Sulfadoxine	> MRL	< 1	> 15000	< 0.1
Sulfamethoxypyridazine	**4.95**	**61**	**26.8**	**49**
Sulfamethazine	**1.78**	**119**	> 13000	< 0.1
N4-acetyl-sulfamethazine	> MRL	< 1	> 16000	< 0.1
Sulfanilamide	> MRL	< 1	> 8000	2

[a]The percentage of recognition has been expressed as cross reactivity (% CR) according to the expression [IC_{50}(SPY)/IC_{50}(cross reactant)] × 100 as nanomolar (nM) concentration. Chemical structures of most of these sulfonamides are shown in ***Figure 3.4****.*

Other sulfonamides containing a pyrimidine group were not recognized by direct ELISA format, probably because of the presence of two electron attractive groups close to the carbon linked to the sulfonamide bond, which affects the charges of nitrogen and the aromatic carbon. On the other hand, hapten SA2 used to prepare the corresponding CAs played an important role in the pattern recognition obtained within the indirect ELISA format.

The excellent features of these immunoreagents have allowed setting up a robust immunoassay which improves considerably, in terms of detectability and number of

sulfonamides recognized, the direct ELISA format. During the evaluation of all SAs immunoreagents, we observed only a slightly variability in terms of detectability and specificity between the antisera from different animals (e.g. As154, As155, As156 and As167) raised against same immunizing hapten (SA1-HCH). The recognition pattern (data not shown) is the same on each format (i.e. direct and indirect) independently from the As used (i.e. As154, As155, As156 and As167 versus SA1-HRP and As154, As155, As156 and As167 versus SA2-BSA, SA2-CONA and SA2-OVA).

Thus, in respect to the assay specificity the results obtained demonstrated that the goal of producing broad-recognition immunoreagents had been achieved. This is the first time that an ELISA with a wide SAs recognition capability has been established. Previously, there was only one precedent in which several SAs could be detected based on the use of recombinant antibodies [21, 22].

3.5.3 Accuracy studies for direct & indirect SAs ELISA formats

The accuracy of As154/SA1-HRP and As155/SA2-OVA assays was evaluated by measuring, repetitively 10 blind samples prepared in buffer during different days. The results shown in **Figure 3.14** correspond to the correlation found between the measured and the spiked concentration values. As it can be observed, results obtained matched very well the spiked values.

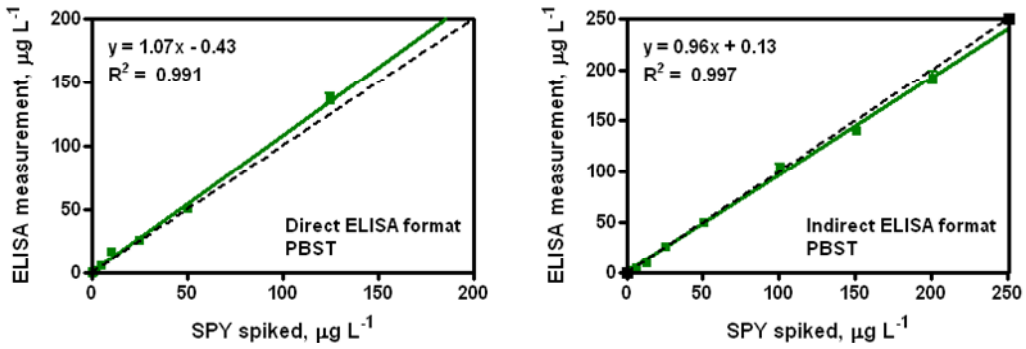

Figure 3.14: Correlation between the spiked and the measured concentration values. The dotted line corresponds to a perfect correlation (slope = 1).

3.5.4 Evaluation of the effect of biological samples on the immunochemical determination of sulfonamides

In the selection of any biological medium for monitoring previous considerations, such as the excretion patterns of the selected analytes, the complexity for obtaining the sample, the sampling time, and the availability of data relating excretion to exposure, should be

taken on account. In this work, milk and hair samples were selected as target matrices to evaluate the performance of the ELISAs developed to analyze SAs residues [35]. The appearance of SAs residues in milk samples is regulated by the Directive 96/23/EC (see **Section 1.2** in **Chapter 1**), establishing that the concentration should always be lower than 100 μg L^{-1}. However, for the case of hair there are not regulations established since it is not a food product. The value of this matrix for veterinary drug residue analysis is mainly for traceability purposes.

3.5.4.1 Milk

The metabolism of sulfonamide veterinary treatment given by intravenous injection was firstly examined in cows by Nielsen and co-workers [36]. They found that sulfonamides were present mainly unchanged in blood samples collected 2h after administration. Moreover, the N_4-acetyl sulfonamide derivative was majority observed in urine samples apart from considerable quantities of the correspondent non-modified drug. On the other hand, sulfonamides were excreted in the milk mainly as unchanged drugs and partly as conjugated metabolites whereas only few small amounts of N_4-acetyl derivates were found.

With the aim to evaluate the effect matrix, skimmed, semi-skimmed and whole milk samples, certified free of antibiotics by AESAN where evaluated in both ELISA formats. The pH range found for the different brand milks assessed was between 6.4 and 6.8 while conductivity values (~ 6 mS cm^{-1}) were lower than that of the assay buffer. As mentioned, both assays were quite robust within the pH range of the milk samples. In respect of the conductivity, the indirect assay showed lower detectability when ionic strength decreased (see **Figure 4** in **Section 3.3**). However, any of these effects could be circumvented by buffering the samples or by dilution with the assay buffer. Due to the high detectability of the ELISA developed, it was very likely that the samples would need to be diluted before the immunochemical analysis. However, the extent of the potential nonspecific interferences caused by the matrix had to be evaluated first.

Left graph in **Figure 3.15** shows several standard curves prepared in milk diluted in PBS using the ELISA direct format. As it can be observed that the curve made in pure milk mimicked very well the standard curve in buffer, achieving almost a perfect superposition. Same experiments were performed using the indirect ELISA format but always the signal was half inhibited, in comparison with that run in buffer, even if high milk dilution factors were applied (see **Figure 5**, graph A in **Section 3.3**). Therefore, in this case the direct ELISA seemed to be more robust in respect to the matrix effects than the indirect ELISA. This is at the opposite of the general belief, since usually direct formats have been claimed to be more sensitive to the matrix interferences due to the fact

that the enzyme tracer is in contact with the sample matrix, which does not occur in the indirect format.

Due to the broad selectivity profile of the indirect assay and the greater detectability, we were very interested on establishing and immunochemical analytical protocol to analyze SAs residues in milk samples using this ELISA. Several strategies could have been applied to circumvent the matrix effect caused by the milk on the indirect format. Since the interferences could not be removed by dilution, an option would have been to develop a clean-up procedure compatible with the immunochemical protocol. In fact, other authors that have reported immunochemical assays for SAs do perform fat-removal, protein precipitation or C_{18} clean-up steps before milk analysis [37]. However, introducing a clean-up step would have been decrease the high-throughput capabilities of the ELISA while increasing the analysis time. Alternatively, an immunochemical protocol in which the calibration curve would have always been prepared in milk diluted 5 or 10 times could have been established, since the ELISA readily works in this media. However, this would have required ensuring a permanent access to certified SAs negative milk samples. Thus, we proposed to establish a procedure in which the milk could mimic the behaviour of the buffer. This was accomplished by using milk diluted five times with milliQ water and duplicating the concentration of As respect the reference curve prepared in buffer. This strategy was proven to be good and reproducible in all types of samples analyzed. Thus, this procedure was applied to different kind of milk samples (whole, semi-skimmed and skimmed) to assess if the strategy was suitable to analyze any type of milk sample. As it can be observed in **Figure 3.15** right graph, the response was very similar in all cases.

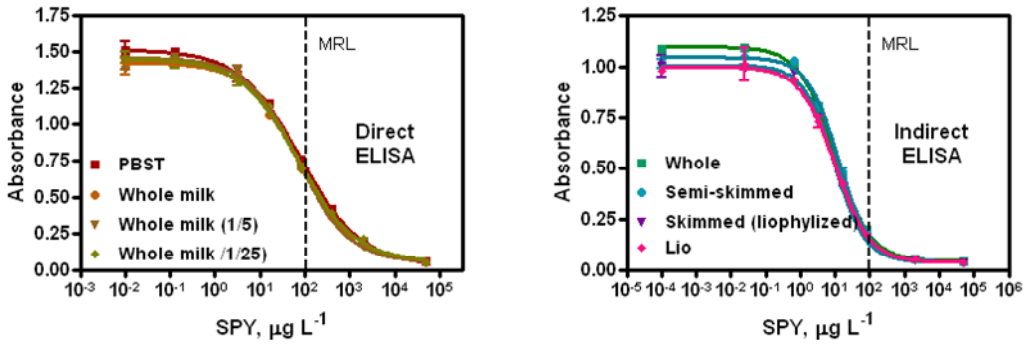

Figure 3.15: Results from the studies of the matrix effect produced by milk on the direct (left graph) and indirect (right graph) ELISAs. Previous to the analysis the samples were buffered by adding 10% (v/v) of 100 mM PBS, diluted and measured with the ELISA. For the indirect ELISA the graph shows how different types of milk samples do perform on an identical manner after 5 times dilution with PBS.

In order test accuracy and to probe performance of the ELISA protocols established, blind milk samples spiked with SPY at several concentration levels were measured by both ELISAs. **Figure 3.16** shows that both assays are characterized by an excellent accuracy. The slope is in both cases very close to 1 and the coefficient of regression is very good. On the other hand, the results show that the strategy selected to avoid the nonspecific interferences produced in the indirect ELISA is suitable for these kinds of measurements.

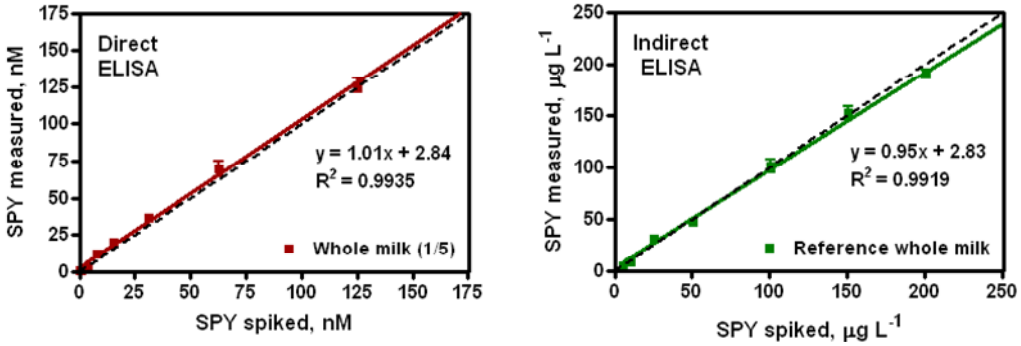

Figure 3.16: Results from the accuracy studies. The graphs show the correlation between the spiked and the measured concentration values using the ELISA formats. The dotted line correspond to a perfect correlation (slope = 1). Accuracy evaluated by several spiked samples and subsequently conditioned as already described before (milk diluted five times in 2x PBS) being then analyzed by both ELISA formats (direct: PBST curve; indirect: milk reference curve).

Finally, as a preliminary evaluation of the performance of the indirect ELISA format as screening method, a set of blind milk samples spiked with distinct SAs at different concentration levels were prepared at the Nestlé Research Centre and measured using SPY as the reference analyte. As it can be observed in **Section 3.3** (**Table 5**) no false negatives were observed while false positives (sample GF10; sulfachloropyridazine, 50 ppb) indicated the presence of SAs although below the MRL values. Nevertheless, a positive value obtained with a screening analytical method should always be confronted by a confirmatory method. The almost negligible matrix effect caused by the milk samples in both ELISA formats has been attributed to the excellent features of the antibodies and immunoreagents produced being also crucial the fact of shaking the microtiter plates during the competition step which minimized the formation of fat/protein layers in the bottom of the microtiter wells, improving assay reproducibility and accuracy [38, 39].

Summarizing, although several antibodies against SAs with different recognition patterns *versus* the different congeners have been reported [13, 21, 22, 40-42], on only a very few occasions has direct performance of these antibodies in complex biological samples has been demonstrated. With regard to milk, homogenization and protein removal by

precipitation with trichloroacetic acid or acetone followed by neutralization have been some of the sample preparation methods reported to analyze this sample by immunoassay [22, 37]. On the contrary, the immunoassay presented here performs very well in milk samples without any prior treatment other than dilution of the sample, being possible to detect these residues far below of the MRL values established by the EC.

3.5.4.2 Hair

Hair is a protein filament that grows through the epidermis from follicles deep within the dermis found exclusively in mammals. The hair shaft consists of three different layers of cells, an inner medulla, a protective external cuticle and a central cortex. Hairs contain cross-linked and orientated fibrous proteins, basically α-keratins rich in cysteine (85-93%), melanins (0.3-1.5%), water (3-5%), lipids (1-9%) and mineral compounds (0.25-0.95%) [43]. Although hair looks like a primitive structure, it is actually a very complex part of human and animal body and its biology is still unclear at many points [44]. Nowadays, hair is being recognized as the third fundamental biological specimen for drug testing besides urine and blood. Although more than 450 papers on hair analysis for drugs have been published since 1954 [45], most of them have appeared only in this decade [46]. In the 1990s, it was reported the use of hair analysis for the detection of fraudulent uses of anabolics and ß-agonists in animals [47] being considered an interesting tissue in veterinary control, because active compounds can be retained for a longer time than in urine or blood, due to the low metabolic activity of this tissue [48]. Since then, several studies have investigated whether or not hair analysis could be considered a useful matrix to control veterinary drug residues in food producing animals (see **Figure 3.17**). However, aspects like hair sampling, pre-treatment, extraction and purification are very important before the analysis of this kind of matrix.

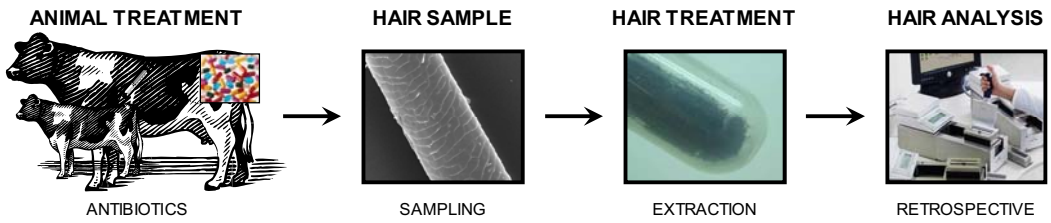

Figure 3.17: Hair matrix cycle from the animal treatment to their analysis.

Governmental authorities have not established MRLs for these compounds in hair samples yet, basically because there is no complete knowledge on how veterinary drug deposition occurs. Therefore, nowadays is still not possible establishing a reliable correlation between the levels in found in hair and the potential levels in the food samples

derived from a particular animal. On the other hand, hair analysis has been proposed lately as a possible technique to increase the time window in retrospective detection of veterinary drug residues to discover an illicit use of sulfonamides during the first growing phases of the animals. The target samples commonly taken for the official controls at the time of slaughter (meat, kidney, fat, liver and others) and taken from live animals (milk, urine, faeces and others) can only reveal a recent administration of sulfonamides due to their limited half-life in these biological samples [49]. Deposition of SAs in hair samples was firstly described by Dunnet and co-workers, who developed and validated a chromatographic method for the detection of different sulfonamides and trimethoprim in equine tail hair [50]. Subsequently, Gratacós and co-workers from IRTA Institute reported for the first time that remarkable quantities of sulfonamides and fluoroquinolones can be accumulated in pig and calf hair after their administration to the animals [51, 52]. Moreover, a recent study performed by the same group demonstrated the accumulation of SMZ in other different animal hair samples at ppm levels [53]. Thus, different types of animal hair samples (colourless pig, colourless calf, and pigmented calf hair), collected from control animals grew at the experimental farm of IRTA, were extracted under different conditions (e.g. alkaline solutions, time, temperature), and the effect of the extract on both ELISA formats was evaluated to study the extent of the interferences caused by this kind of hair extracts (see **Figure 3.18**).

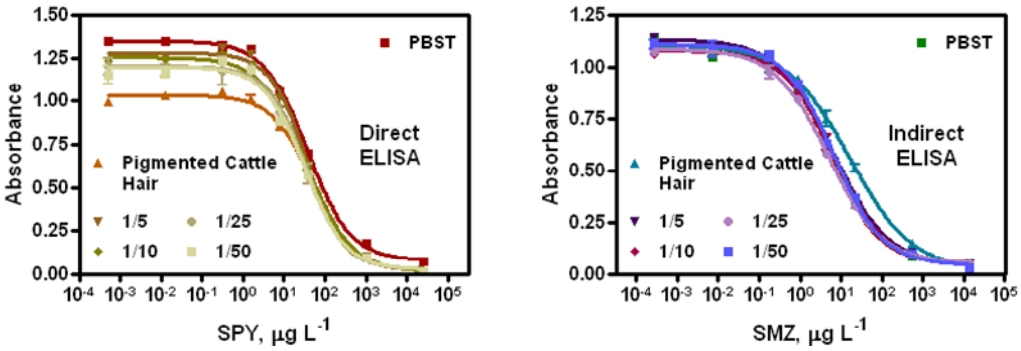

Figure 3.18: Matrix effects on the SPY direct ELISA and SMZ indirect ELISA caused by pigmented cattle hair extracts.

For the indirect ELISA format the evaluation of the matrix effect was performed using SMZ as standard, since this sulfonamide was one of the most frequently employed in the veterinary field and IRTA had treated animals with SMZ in their experimental farm to assess deposition on the hair. The same experiments could not be done with the direct ELISA since this antibiotic is not recognized with this format (see **Table 3.6**).

The experiments were done using hair samples from treated and untreated animals, particularly pig and cattle. For the last cases, we also investigated the effect of extracts

prepared from pigmented and colourless hair. The results obtained demonstrated that the interference of the matrix, in both ELISAs was almost negligible independently from the type of hair analyzed. **Figure 3.18** shows the calibration curves build with pigmented hair extracts where it can be observed that, particularly on the indirect ELISA format, the extracts could be analyzed almost undiluted.

Accuracy of the ELISAs for the determination of sulfonamides in hair extracts was evaluated by measuring SPY and SMZ spiked extracts, for the direct and indirect formats, respectively (see **Figure 3.19**). Correlation between the spiked and the measured concentration values was very good in both ELISA formats. The slope of the regression lines was close to 1 (direct: 1.01; indirect: 0.97) and the coefficients of correlation were also very good (R^2 = 0.993 and 0.994) that is an indication of the assay accuracy. To complete the indirect ELISA evaluation of hair samples, alkaline extracts were prepared from of different un/treated animals, obtained at different collection times. Same samples were also evaluated by HPLC-DAD in collaboration with IRTA. Results obtained by the immunochemical technique for SMZ matched very well with those obtained by the reference chromatographic (HPLC-DAD) methodology. An excellent correlation between both techniques was observed (y = 0.93x + 0.89, R^2 = 0.971, see **Figure 5** or **Table 2** in **Section 3.4**).

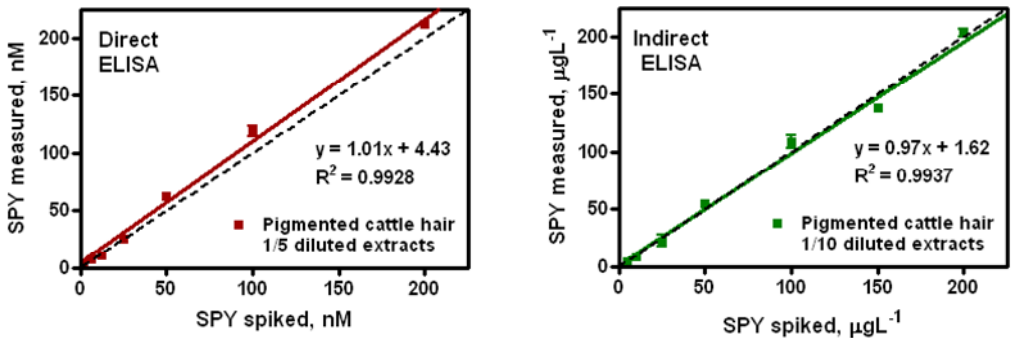

Figure 3.19: Correlation between the spiked and the measured concentration values. The dotted line corresponds to a perfect correlation (slope = 1).

The excellent results obtained regarding analysis of SAs in complex biological samples, such as milk or hair, show great promise regarding the potential application of these immunoassays on new matrices like feed, water and soil samples where lately antibiotics have been considered emerging pollutants.

3.5.5 Preliminary Proposal for validation of ELISA immunochemical techniques (Commission Decision 2002/657/EC)

A preliminary general proposal of a validation study, using cattle hair samples as a model, was performed for the ELISA methodologies according to the Commission Decision 2002/657/EC criteria (see **Section 1.2**) established for qualitative screening methods of substances for which no permitted limits have been established [54]. Contrary to other analytical techniques, the response obtained with the ELISA is not lineal observing a decrease in the signal directly proportional to the amount of analyte present in the sample making difficult, in this case, the procedure election for the best estimation of CCα and CCß parameters. CCα means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant. On the other hand, CCβ means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. In our case, where we are analyzing substances for which no permitted limit has been established (i.e. antibiotics in hair samples), the CCβ is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1 – β [54]. Thus, following the main points of the Decision we proposed a method to determine these parameters for the ELISA technique. As it has been showed in **Section 3.4**, results obtained satisfied 2002/657/EC Commission Decision criteria for the performance and general validation of qualitative screening analytical methods.

3.5.6 Main contributions in respect to the immunochemical analysis of SAs

The main contributions of this chapter to the existing knowledge at the time of starting this research are:

- This is the first time that the preparation of class-selective antibodies for SAs through a rational design of the immunizing hapten has been described.
- An immunochemical technique has been developed able to detect up to 11 SAs in milk samples without any kind of sample preparation and in accordance with the current EC legislation.
- It has been demonstrated for the first time the usefulness of an immunochemical method to trace treatments of SAs, through the analysis of hair extracts without performing any previous clean-up procedure.

While doing this research several papers have been published reporting the production of generic antibodies for SAs [37, 55-64] but in all cases the immunoassays did not improved the work here presented in terms of increasing the number of recognized compounds of the SAs family with sufficient detectability to comply with the current regulations for biological samples from edible animals [65].

3.5.7 Further considerations

A part from milk and hair samples, some other matrices have been monitored with the antibiotic immunoreagents developed within this thesis. For example, an interesting collaboration with Prof. Ramón Compaño (Department of Analytical Chemistry of the UB) at the end of this thesis, made possible the implementation of the ELISA procedures developed to determine SAs in animal feed materials [66]. Medicated feeds are prepared in the same production lines as regular feeds leading to a high risk of cross-contamination. Full removal of these antibiotic residues before switching back to a normal production is sometimes complicated being necessary the control of antibiotic residues. Moreover, other matrices like honey have also been investigated by other members of the AMR group, within the context of a project addressed to develop rapid test for chemical contaminants (CONffIDENCE, EC-FP7) [67]. Nowadays, honey is becoming an attractive matrix to be monitored because several groups have reported that antibiotic residues have been also found in this matrix, mainly the ones imported from Asian countries.

Moreover, the immunoreagents produced in this work are being incorporated onto novel transducers with the objective of developing new biosensor devices for on-site measurements for the analysis of not only milk and hair samples but also other relevant biological or environmental matrices. In this sense, at the time of presenting this thesis already an amperometric magneto immunosensor device [68], a 3D interdigitated electrode array as a transducer for label-free biosensors [69] and a portable multichannel SPR immunosensor [70] have been reported using the immunoreagents and immuno chemical procedures developed (see **Section C.2** in **Chapter C**). Moreover, several companies have shown interest for using these reagents and implementing them on their systems (confidential information).

3.6 Bibliography of Chapter 3

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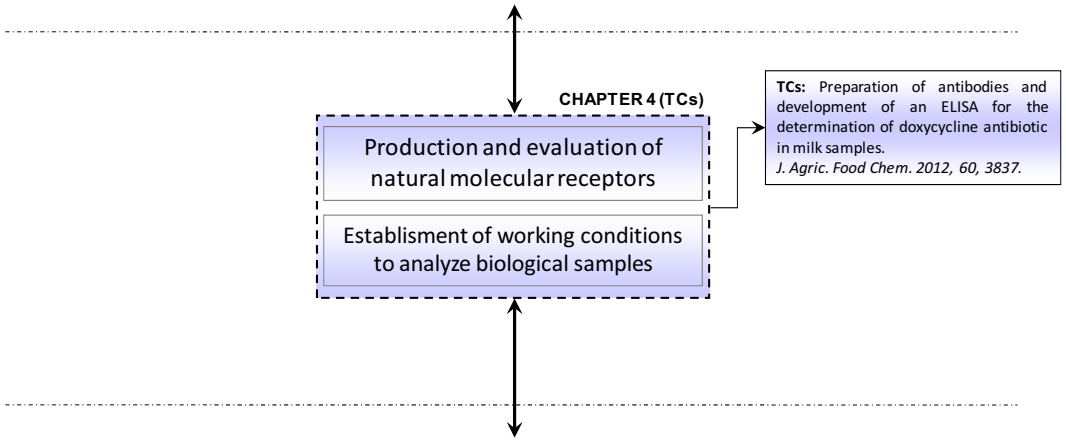
4 BIOLOGICAL RECEPTORS FOR TCs



As complex molecular entities, TCs have different physic-chemical properties and pharmacological aspects which makes difficult to work with, particularly when attempting to synthesize, purify and characterize them. Firstly, most derivatives contain three functional groups which can be subject to protonation-deprotonation equilibrium being the final conformation of the drug dependent on the protonation state (see **Figure 4.1**).

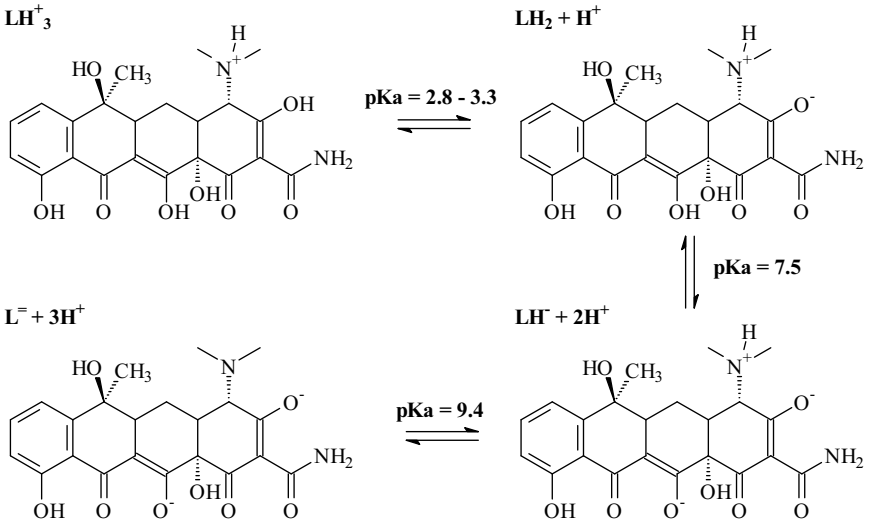


Figure 4.1: Deprotonation scheme (Triprotic system) of TCs.

Between pH 4 and 7, the zwitterionic species $LH^{2\pm}$ are the dominants. Around pH 9 about 80% of the molecules are present as anionic species LH^- [1].

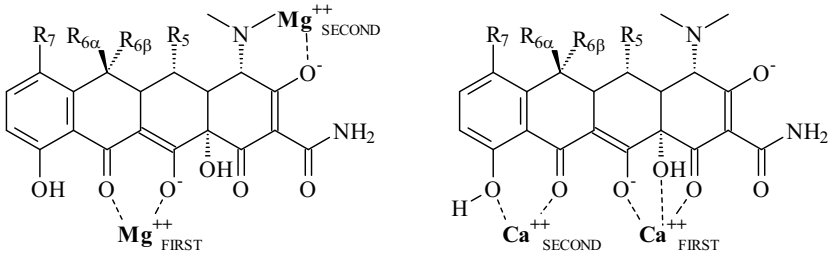


Figure 4.2: TC complexation sites for Mg^{2+} and Ca^{2+} proposed by Schneider [2].

TCs are conformationally labile, so they can adopt various geometries exhibiting heats of formation which differ only by a few kJ mol^{-1}. Thus, in fully protonated form conditions they adopt a twisted conformation whereas in the fully deprotonated form they adopt an extended conformation. Secondly, it is well established that TCs bind to many proteins preferentially, if not exclusively, as complexes with divalent metal ions like Mg^{2+} or Ca^{2+}

(see **Figure 4.2**). Thus, the relative content of the different complexes varies with pH and ionic strength of the solution, the nature and concentration of the metal ions and, to a certain degree, also with the history of the solution. And last, but not least important, is the easy thermal and photochemical degradation of these antibiotics (see **Figure 4.3**).

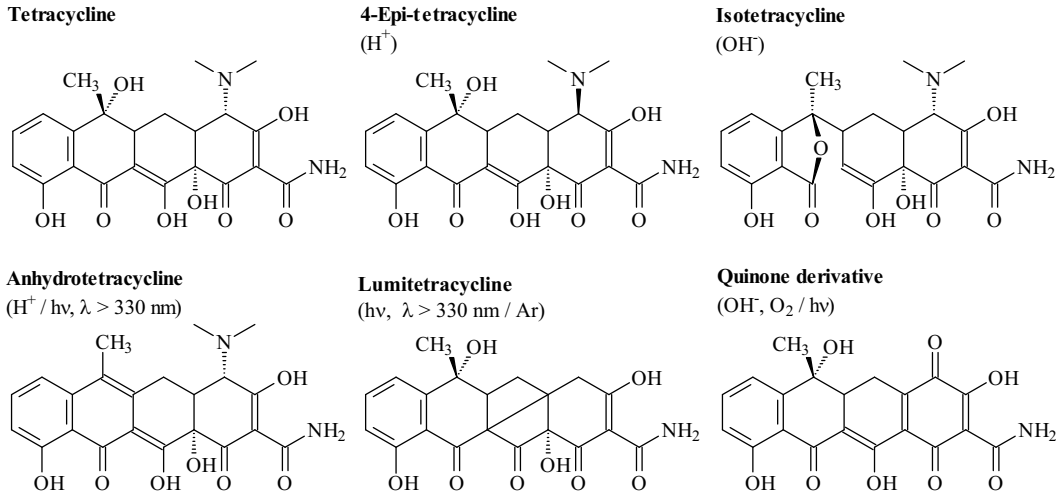


Figure 4.3: Most important degradation reactions of TCs.

Summarizing, it is practically impossible to find conditions under which only one specie is present nearly exclusively. As a consequence, it is also extremely difficult to determine experimentally the thermodynamic and spectroscopic properties of each specie defined by state of protonation or metal ion chelation, conformation of the four annelated rings, or rotation and hydrogen bonding of functional groups. From the early days of tetracycline research onward, it was suggested that it is the flexibility of these molecules that makes them such a successful and efficient drugs. On the other hand, this flexibility makes also very difficult to establish a structure-function relationship, as long as its precise structure has not been determined at the site of action. Summarizing, all these previous considerations forecasted us that working with this compounds, in terms of designing and synthesizing representative haptens for subsequent antibody production, was a scientific challenge.

4.1 Preamble

As mentioned before, when we started both, the ARGOS and the PANOPTES projects there were several examples regarding the use of immunoassays for detecting CAP [3, 4] and some individual SAs [5-7] mainly based on commercial kits. However, very few papers reported production and use of antibodies capable to detect FQs [8] and TCs [9, 10]. Since then, our group has tried to design, prepare and evaluate antibodies to broadening the class-selectivity and improving the features of immunochemical assays for antibiotic residue analysis. However, production of antibodies against TCs poses many challenges due to their physic-chemical features, mentioned before and to their rapid and complex degradation in aqueous solutions. Within this thesis, we decided to take the challenge of overcoming all these adverse considerations in order to generate class-selective antibodies for TCs and to establish the corresponding class-selective immunochemical procedures. The aim of the following work was focused as a challenge to overcome all these adverse considerations for developing an ELISA for TCs. While doing this research several papers have been published reporting the production of antibodies for TCs [11-14] although not always provided immunoassays with the desired features regarding detectability and/or selectivity for the analysis of biological samples from edible animals. On the other hand, several commercial ELISA kits, capable to detect residues of TCs in different agro food samples, have become available. Thus, the Biopharm test for TC and rolitetracycline in milk, honey and meat or the IDEXX test addressed to screen TC, OTC and CTC in milk. This fact demonstrates the interest that this family of antibiotics raises also in the food safety field. Therefore, in this chapter we report the research performed addressed to the production of broad specificity antibodies for TCs with the aim to establish an immunochemical procedure to analyze these residues in milk samples. As with the SAs, our goal was to reach a detectability below the MRLs established for TCs in milk (see **Table 4.1**).

Table 4.1: Main TCs to be monitors in milk samples.

Family	Main TCs Compounds	MRL
TCs[a]	Tetracycline	100 μg kg^{-1}
	Oxytetracycline	100 μg kg^{-1}
	Chlortetracycline	100 μg kg^{-1}

[a] *MRLs established considering the sum of parent drug and its 4-epimer.*

The present chapter describes the work performed in relation to the **Specific Objective 1** (i.e. preparation and evaluation of specific receptors) and **Specific Objective 2** (i.e. evaluation of performance of the bioreceptors in real matrices) of this thesis in respect to TC. Taking into account that the ELISA technique is considered an essential method to characterize the features of antibodies, the main objective here was a) to understand better the behaviour and chemistry of TCs, b) to asses that the antibodies produced were able to recognize a significant number of TCs congeners and, c) to develop and establish a reliable immunochemical protocol to analyze these antibiotics in milk samples.

4.2 TCs indirect ELISA format (Milk)

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Preparation of Antibodies and Development of an Enzyme-Linked Immunosorbent Assay (ELISA) for the Determination of Doxycycline Antibiotic in Milk Samples

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ABSTRACT: This paper reports the development of an immunoassay for the specific analysis of doxycycline (DC), a congener of the tetracycline antibiotic family (TCs), in milk samples. This is the first time that DC antibody production is reported, based on a rationally designed and well-characterized immunizing hapten. The chemical structure of the immunizing hapten (13-[(2-carboxyethyl)thiol]-5-hydroxy-6-α-deoxytetracycline, TC1) was designed to maximize recognition of the tetracycline characteristic moiety defined as lower periphery of the TCs plus the region of the upper periphery composed by the hydroxyl group at position C_5 (B ring) and the dimethylamino group in ring A. Polyclonal antibodies raised against TC1 coupled to horseshoe crab hemocianyn (HCH) were used to develop a homologous indirect competitive enzyme-linked immunosorbent assay (ELISA). The microplate ELISA can detect DC in buffer down to 0.1 μg L^{-1}. The ELISA has been proven to tolerate a wide range of ionic strengths and pH values. The assay is very selective for DC with a minor recognition of methacycline (32% of cross-reactivity). Experiments performed with whole milk samples demonstrate that samples can be directly analyzed after a simple treatment method, reaching detectability values below 5 μg L^{-1}.

KEYWORDS: *doxycycline, immunoassay, ELISA, milk, tetracyclines*

INTRODUCTION

Tetracycline antibiotics (TCs) are nowadays extremely popular in human and veterinary medicine for the treatment of gastrointestinal, respiratory, and urinary, among others, infections.[1] This antibiotic family has a broad range of activity against a variety of Gram(+) and Gram(−) bacteria by oral administration via water and feed.[2] Only a small proportion of the TC dose administered is actually metabolized or absorbed in the body, a significant part being eliminated in feces and urine unaltered,[3] which has an important impact on the ecosystem, water, and soil-dwelling organisms. TCs are not usually found at high levels in the environment due to their chelating properties; they readily precipitate in the presence of divalent cations (e.g., Ca^{2+}, Mg^{2+}, or Zn^{2+}) accumulating in sewage, sludge, or sediments. On the other hand, TC residues have also been detected in many surface water resources that receive discharges from municipal wastewater treatment plants and agricultural runoff.[4] Besides the demonstrated persistence of these antibiotics in agricultural soils that have received manure containing antibiotics, the biodegradation may lead to even more toxic substances. Therefore, new strategies for improving tetracycline residue control and the efficiency of their removal in wastewater plants are necessary. Additionally, their widespread use together with the irresponsible and inappropriate administration of these antibiotics (and others) in animal husbandry, either for the prevention and treatment of diseases or as feed additives to promote growth,[5] favors the increase of bacterial resistance,[6] causing an important risk for human health due to diseases that can no longer be treated with the presently known antibiotics.[7] Moreover, consumption of food contaminated with antibiotic residues may cause allergic reactions and interferences with the intestinal flora while contributing to the appearance of resistant populations of bacteria. Furthermore, antibiotic residues cause important technological problems and economic losses in the diary industry related to the inhibition of the bacterial fermentation processes involved in the elaboration of cheese and cultured milk products.

There is also a social concern about all of these situations that is making consumers more exigent in terms of getting more natural and high-quality food products. As a result, recent trends in global food production, processing, and distribution are creating an increasing demand for food safety research to ensure a safer global food supply. An international approach to the management of antimicrobial resistance is essential for its surveillance, ensuring the safety of food for consumers. In Europe, the use of veterinary drugs is regulated through European Union (EU) Council Regulation 2377/90/EC and its annexes,[8] which describe the procedures for establishing maximum residue limits (MRLs)[9] for veterinary medicinal products in foodstuffs of animal origin (e.g., milk, eggs, or meat). In particular, Europe has established MRLs for tetracycline (TC), chlortetracycline (CTC), and oxytetracycline (OTC) compounds at 100 μg L^{-1} in milk samples. On the contrary, nowadays no MRL value has been settled for doxycycline (DC) or methacycline (MC) in these kinds of samples because they should not be used in farm animals for

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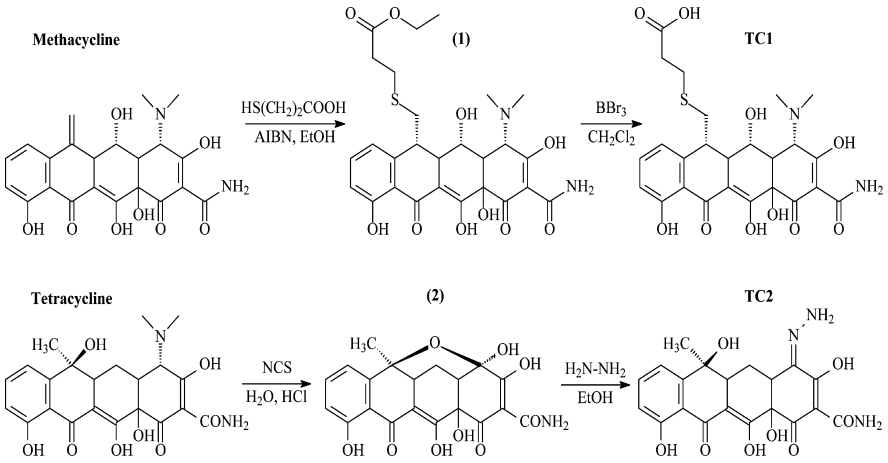

Figure 1. Synthetic pathways used to prepare TC1 and TC2 haptens.

milk production.[9] Thus, according to the Veterinary Medicine Evaluation Unit of the European Agency for the Evaluation of Medicinal Products, DC is indicated in cattle, pigs, poultry, turkeys, dogs, and cats for the treatment of infections, but not for use on lactating cattle and layers.[10] On the other hand, the U.S. Food and Drug Administration has restricted the use of DC for just pet dogs, and no use is permitted in pregnant, nursing, or growing animals.

Despite this, there exists a risk for illegal or inappropriate use of DC, which claims for the availability of analytical procedures to determine these residues in several tissues. Chromatographic techniques such as HPLC-UV[11] and/or HPLC-MS[12] provide high specificity and an excellent detectability but are not suitable for rapid residue analysis or screening of many samples. They usually require extensive sample preparation, sophisticated equipment, and skilled laboratory personnel. Alternatively, receptor-based assays such as dipsticks (i.e., lateral flow chromatography devices) are easy to use, being nowadays the most traditional on-site screening tests for TCs.[13,14] Current commercially available TC dipsticks are based on the use of DNA-regulatory proteins as receptors and show a quite broad recognition character for tetracycline congeners, including doxycycline. On the other hand, immunochemical analytical methods can also provide the necessary selectivity and detectability while offering the possibility to develop a wide variety of reliable, low-cost, and easy-to-use analytical configurations.[15] A few immunoassays for tetracyclines have been reported during recent years used to analyze preferentially honey[16,17] and milk[18,19] but also liver and muscle samples.[20] In particular, some of these immunoassays have proven to be suitable for the determination of single tetracycline congeners in milk samples. Thus, this paper presents the work performed addressed to produce polyclonal antibodies and the necessary immunoreagents to establish an immunochemical method for the specific analysis of DC residues. This is the first time that DC antibody production based on a rationally designed and well-characterized immunizing hapten is reported. Performance of the developed microplate-based ELISA has been demonstrated with milk samples.

MATERIALS AND METHODS

Chemistry. *General Methods and Instruments.* Thin-layer chromatography (TLC) was performed on 0.25 mm, precoated silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany) predeveloped with saturated aqueous ethylenediaminetetraacetic acid solution (Na_2EDTA) followed by activation for 2 h at 130 °C. Unless otherwise indicated, purification of the reaction mixtures was accomplished by "flash" chromatography using silica gel as the stationary phase, being previously impregnated with saturated aqueous Na_2EDTA solution by dipping and further activated for 2 h at 130 °C. ^1H and ^{13}C NMR spectra were obtained with a Varian Inova-500 (Varian Inc., Palo Alto, CA) spectrometer (500 MHz ^1H and 125 MHz for ^{13}C). HPLC analyses were performed with a Merck Hitachi pump L-7100, a diode array detector L-7455, an autosampler L-7200, and an interface D7000 (Merck). The chromatograms were processed with HSM software (Merck). The column used was a C18 Purosphere column 15 cm × 4.6 mm, 5 μm, particle size (Merck), and the analyses were performed in isocratic mode using 0.01 M oxalic acid/acetonitrile (ACN)/MeOH 70:20:10 as mobile phase at a flow rate of 1.0 mL min^{-1}. The detection wavelengths to monitor the reactions were set at 250 and 365 nm. Liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS) was performed in a Waters (Milford, MA, USA) model composed by an Acquity UPLC system directly interfaced to a Micromass LCT Premier XE MS system equipped with an ESI LockSpray source for monitoring positive ions. Data were processed with MassLynx (V 4.1) software (Waters).

Molecular Modeling and Theoretical Calculations. Computational methodology was carried out by running Hyperchem 6.03 software package (Hypercube Inc., Gainsville, FL, USA) to compare physical−chemical features of the haptens with TC and DC. Minimum energy conformation, geometry, and molecular charge distribution were calculated using the semiempirical quantum model (PM3). Geometry was calculated for each acid−base form that could coexist at neutral (or close) pH. Conformational search was performed using a Monte Carlo method exploring all of the dihedral angles for the ring system and for the main substituents. Deprotonation enthalpies were calculated as a measure of acidity for comparison between haptens and analytes. The calculation results were also compared with the available bibliography data on the structures of tetracyclines and the immunoassay results.

Preparation of Tetracycline Haptens. Immunizing and competitor haptens TC1 and TC2 were synthesized following the scheme shown in Figure 1. Experimental details on the synthetic procedures and purification processes can be found below. Spectroscopic and spectrometric characterizations of the haptens are also provided.

Synthesis of Hapten TC1. (a) Ethyl 13-[(thio)propanoate]-5-hydroxy-6-α-deoxytetracycline (1). 3-Mercaptopropionic acid

(2.4 mL, 27.59 mmol) and azobis(isobutyronitrile) (AIBN, 25 mg) were added to a suspension of MC hydrochloride (0.5 g, 1.04 mmol) in EtOH (10 mL) placed in a round-bottom flask. The reaction mixture was kept on reflux with stirring for 12 h under N_2, until the complete disappearance of the starting material by TLC (9:1 CH_2Cl_2/MeOH as mobile phase) and HPLC-UV analysis and subsequently suspended in cold Et_2O (100 mL). The precipitate obtained was centrifuged to facilitate removal of 3-mercaptopropionic acid, and the solid collected was dissolved in water (3 mL) with the pH adjusted to 4.5. The aqueous solution was washed with $CHCl_3$ (5 mL) to remove the remaining mercaptan and finally extracted with CH_2Cl_2 (50 mL × 3). The organic layer was evaporated to dryness under vacuum to obtain a brownish-green solid (184.4 mg, 30.5%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.17 (3H, t, $-CH_2\mathbf{CH_3}$), 2.31 (2H, d, C_6-CH_2-S-), 2.43 (6H, s, $-N(CH_3)_2$), 2.55 (2H, m, $-CH_2-COOEt$), 2.69 (2H, m, $-S-\mathbf{CH_2}-CH_2-$), 3.04 (1H, m, $C_{4a}H$), 3.08–3.51 (3H, m (under water signal), C_5H, $C_{5a}H$, C_6H), 3.75 (1H, m, C_4H), 4.05 (2H, q, $-\mathbf{CH_2}CH_3$), 6.80 (1H, d, C_9H), 6.97 (1H, d, C_7H), 7.48 (1H, t, C_8H).

(b) 13-[(2-Carboxyethyl)thio]-5-hydroxy-6-α-deoxytetracycline (TC1). A solution of substrate **1** (308 mg, 0.54 mmol) in CH_2Cl_2 (15 mL) was cooled to −10 °C and then added dropwise to a stirred solution of BBr_3 (1 M in hexane, 3 mL, 3 mmol) followed by CH_2Cl_2 (15 mL). The reaction mixture was kept at −10 °C for 1 h under N_2 atmosphere and then at 25 °C until the complete disappearance of the starting material by TLC analysis (9:1 CH_2Cl_2/MeOH as mobile phase). The reaction was terminated by careful dropwise addition of water (30 mL), the organic phase was separated and washed with H_2O (3 × 20 mL), and the combined aqueous layers were evaporated to dryness. The residue was taken up in H_2O and chromatographed on Macro Prep, High Q Supgrat (ion-exchange resin) using a 5% acetic acid solution as mobile phase. Concentration in vacuum of the positive fractions led to the product as a brownish-yellow solid (214.1 mg, 73.0%): ^{1}H NMR (500 MHz, CD_3OD) δ 2.20 (2H, d, C_6-CH_2-S-), 2.51 (6H, s, $-N(CH_3)_2$), 2.63 (2H, m, $-CH_2-COOEt$), 2.69 (2H, m, $-S-\mathbf{CH_2}-CH_2-$), 3.13 (1H, m, $C_{4a}H$), 3.20–3.48 (3H, m (under water signal), C_5H, $C_{5a}H$, C_6H), 3.99 (1H, m, C_4H), 6.66 (1H, d, C_9H), 6.84 (1H, d, C_7H), 7.47 (1H, t, C_8H); HRMS (+EI) calcd for $C_{25}H_{29}N_2O_{10}S$ (M+) 549.1543, found 549.1523.

*Synthesis of Hapten TC2. (a) 4-Oxo-4-dedimethylaminotetracycline-4,6-hemiketal (**2**).* Powdered *N*-chlorosuccinimide (3.7 g, 26.2 mmol) was added to a solution of tetracycline hydrochloride (5 g, 10,4 mmol) in water (500 mL) containing concentrated HCl (1 mL) under vigorous stirring and at room temperature. The crude product that began to precipitate after a few minutes was recovered by filtration after 30 min of reaction and washed with water. Partial purification of the crude air-dried product (3.1 g) was achieved by adding water (75 mL) and Et_2O (200 mL) to the solid. The organic layer was washed with water (5 × 20 mL) and evaporated to dryness on a rotary vacuum evaporator. The residue was treated with water, and the product recovered by filtration was air-dried to obtain **2** partially purified (2.8 g, 65% yield) suitable for further transformations: ^{1}H NMR (500 MHz, DMSO-$d3_6$) δ 1.65 (s, 3H, CH_3), 2.29–2.34 (m, 1H, C_5H_β), 2.38–2.44 (m, 1H, C_5H_α), 2.80 (dd, 1H, C_4H), 2.92 (m, 1H, $C_{5a}H$), 6.96 (1H, d, C_9H), 7.19 (1H, d, C_7H), 7.66 (1H, t, C_8H).

(b) 4-Hydrazono-4-dedimethylaminotetracycline (TC2). A solution of **2** (2.7 g, 6.5 mmol) in 95% ethanol (19 mL) was added dropwise to a solution of hydrazine hydrate (0.4 mL, 12.6 mmol) in the same solvent (19 mL), and a precipitate began to appear immediately. The mixture was stirred for 1 h more and then left to stand overnight. The day after, the solid product was recovered by low-speed centrifugation and the crude was purified by a Na_2EDTA-impregnated silica gel column (after activation) with 9:1 CH_2Cl_2/MeOH as mobile phase to obtain the desired product (397.4 mg, 14% yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.46 (s, 3H, $-CH_3$), 1.48–1.57 (m, 1H, C_5H_β), 1.84–1.89 (m, 1H, C_5H_α), 2.96 (dd, 1H, $C_{4a}H$), 3.21–3.24 (m, 1H, $C_{5a}H$), 6.91 (1H, d, C_9H), 7.09 (1H, d, C_7H), 7.53 (1H, t, C_8H); HRMS (+EI) calcd for $C_{20}H_{20}N_3O_8$ (M+) 430.1250, found 430.1239.

Immunochemistry. *General Methods and Instruments.* The matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS) used for analyzing the protein conjugates was a Perspective BioSpectrometry Workstation provided with Voyager-DE-RP software (version 4.03) developed by Perspective Biosystems Inc. (Framingham, MA, USA) and Grams/386 (for Microsoft Windows, version 3.04, level III) developed by Galactic Industries Corp. (Salem, NH, USA). The pH and the conductivity of all buffers and solutions were measured with a pH-meter pH 540 GLP and a conductimeter LF 340, respectively (WTW, Weilheim, Germany). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). Washing steps were performed on a SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). A Heidolph Titramax 1000 vibrating platform shaker (Brinkmann Instruments, Westbury, NY, USA) was used to shake the microplates at 900 rpm. Absorbances were read on a SpectramaxPlus (Molecular Devices, Sunnyvale, CA, USA). The competitive curves were analyzed with a four-parameter logistic equation using the software SoftmaxPro v4.7 (Molecular Devices) and GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). The chemical reagents used in the synthesis were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The preparation of the immunoreagents used is described below. Tetracyclines used for cross-reactivity studies were kindly supplied by Unisensor S.A. (Liege, Belgium).

Buffers. Unless otherwise indicated, phosphate-buffered saline (PBS) is 0.025 M phosphate buffer in a 2% saline solution (343 mmol L^{-1} NaCl, 6.8 mmol L^{-1} KCl) containing calcium divalent cation (1 mmol L^{-1} $CaCl_2$), and the pH is 5.5. PBST is PBS with 0.001% Tween 20. Borate buffer is 0.25 M boric acid–sodium borate, pH 8.7. Coating buffer is 0.05 M carbonate–bicarbonate buffer, pH 9.6. Citrate buffer is a 0.04 M solution of sodium citrate, pH 5.5. The substrate solution contains 0.01% 3,3′,5,5′-tetramethylbenzidine (TMB) and 0.004% H_2O_2 in citrate buffer. The McIlvaine buffer contains citric acid (0.1 M) and Na_2HPO_4 (0.2 M), pH 3.

Immunoreagents. (a) Immunogens. (1) TC1–HCH. Hapten TC1 was coupled to HCH following the mixed anhydride (MA) method as previously described.[21] Briefly, the carboxylic acid of TC1 (10 μmol) was activated with isobutyl chloroformate (14 μmol) in the presence of tributhylamine (12 μmol) in anhydrous dimethylformamide (DMF, 200 μL) and added to a solution of the protein (10 mg) in borate buffer (1.8 mL). The mixture was stirred for 3 h at room temperature overnight at 4 °C.

(2) TC2–HCH. Hapten TC2 (10 μmol in 100 μL of PBS) was added to a solution of HCH (10 mg in 0.9 mL of PBS) followed by a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 50 μmol in 100 μL of PBS), and the mixture was reacted for 3 h at room temperature.

(b) Bioconjugates. (1) TC1–BSA, TC1–CONA, TC1–OVA, and TC1–HRP were prepared following the active ester (AE) procedure[22] by activating the hapten (10 μmol) with *N*-hydroxysuccinimide (NHS, 12.5 μmol) and dicyclohexylcarbodiimide (DCC, 25 μmol) in anhydrous DMF (200 μL) and adding the solution to horseradish peroxidase (HRP, 2 mg), bovine serum albumin (BSA, 10 mg), conalbumin (CONA, 10 mg), or ovalbumin (OVA, 10 mg) proteins in borate buffer (1.8 mL).

TC2–BSA, TC2–CONA, TC2–OVA, and TC2–HRP were prepared following different strategies. *Strategy A:* TC2 (10 μmol), in anhydrous DMF (100 μL) was coupled to HRP (2 mg) or to the proteins (10 mg each) in borate buffer (1.8 mL) by adding a freshly prepared solution of dimethyl pimelimidate dihydrochloride (DPM, 125 μL 10 μmol in borate buffer).[23] *Strategy B:* The proteins (BSA, CONA, OVA, 10 mg, each) or the enzyme (HRP, 2 mg) was coupled

to cyanuric chloride (CC, 10 μmol) in coating buffer as previously described[24,25] and in a second step a solution of TC2 (10 μmol) in anhydrous DMF (100 μL) was added to CC previously derivatized proteins (3 mg each) in coating buffer (675 μL).

All of the protein conjugates were purified by dialysis against 0.5 mM PBS (4 × 5 L) and Milli-Q water (1 × 5 L) and stored freeze-dried at −40 °C. Unless otherwise indicated, working aliquots were stored at 4 °C in 0.01 M PBS at 1 mg mL^{-1}. Hapten densities of the bioconjugates were estimated by measuring the molecular weight of the native proteins relative to that of the conjugates by MALDI-TOF-MS. Thus, MALDI spectra were obtained by mixing 2 μL of the freshly prepared matrix (*trans*-3,5-dimethoxy-4-hydroxycinnamic acid, 10 mg mL^{-1} in CH_3CN/H_2O 70:30, 0.1% TFA) with 2 μL of a solution of the conjugates or proteins in CH_3CN/H_2O 70:30, 0.1% TFA (10 mg mL^{-1}). The hapten density (δ hapten) was calculated according to the following equation: {MW(conjugate) − MW(protein)}/MW-(hapten)}. Coupling efficiency evaluated by MALDI-TOF-MS of the corresponding haptenized BSA immunoreagents is shown in Table 1.

Table 1. Hapten Densities of the BSA Conjugates[a]

immunoreagent	δ-hapten[b]	% conjugation[c]
TC1−BSA (MA)	3.3	9−11
TC2−BSA (EDC)	6.0	17−20
TC1−BSA (EA)	10.8	31−36
TC2−DMP−BSA	2.1	6−7
TC2−CC−BSA	1.0	2−3

[a]Analyses were performed by MALDI-TOF-MS. [b]Moles of hapten per mole of protein. [c]The conjugation is calculated on the basis of the assumption that the BSA has 30−35 free lysine groups.

Polyclonal Antisera. As180−As182 and As183−As185 were obtained by immunizing female white New Zealand rabbits weighing 1−2 kg with TC1−HCH and TC2−HCH, respectively, following a protocol already described.[22] The evolution of the antibody titer was assessed on a noncompetitive indirect ELISA, by measuring the binding of serial dilutions of each antiserum to microtiter plates coated with TC1−BSA or TC2−BSA. After an acceptable antibody titer was observed, the animals were exsanguinated, and the blood was collected on vacutainer tubes provided with a serum separation gel. Antisera were obtained by centrifugation and stored at −80 °C in the presence of 0.02% NaN_3.

Competitive Indirect TC1−OVA/As181 ELISA. Microtiter plates were coated with TC1−OVA (0.625 μg mL^{-1} in coating buffer, 100 μL/well) overnight at 4 °C and covered with adhesive plate sealers. The next day, the plates were washed four times with PBST (300 μL/well), and milk samples or standard (DC or other cross-reactants, from 50000 to 0.005 nM in PBST) were added to the wells (50 μL/well) followed by the antiserum As181 (1/500 diluted in PBST, 50 μL/well) and incubated for 30 min at room temperature, under shaking. The plates were washed as before, and a solution of anti-IgG−HRP (1/6000 in 10 mM PBST) was added to the wells (100 μL/well) and incubated for 30 min more at room temperature. The plates were washed again, and the substrate solution was added (100 μL/well). Color development was stopped after 30 min at room temperature with 4 N H_2SO_4 (50 μL/well), and the absorbances were read at 450 nm. The standard curves were fitted to a four-parameter equation according to the following formula: $y = (A - B/[1 - (x/C)^D] + B$, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve. Unless otherwise indicated, data presented correspond to the average of at least two well replicates.

(a) Specificity Studies. DC, TC, MC, and OTC analytes used as standards were prepared from 10 mM stocks in dimethyl sulfoxide (DMSO) and CTC in 50 mM aqueous HCl and kept at 4 °C. Standard curves were prepared in PBST and run in the ELISA following the protocol described before. The cross-reactivity (CR) values were calculated according to the equation {IC_{50} [nM] (DC)/IC_{50} [nM] (cross-reactant)} × 100.

(b) Accuracy Studies. This parameter was assessed by preparing eight different blind spiked samples in PBST buffer and measuring them in duplicate in the ELISA.

Milk Experiments. (a) Samples. Skimmed milk samples free of antibiotics were supplied by the Agencia Española para la Seguridad Alimentaria y Nutricion (AESAN; Spanish Agency for Food Security).

(b) Sample Treatments. (i) Saturated ammonium sulfate (0.45 mL) or (ii) McIlvaine buffer (0.30 mL) solutions were added dropwise to whole milk samples (1 mL). The mixtures were shaken (0.5 min at room temperature), centrifuged (5000*g*, 10 min), and filtered (0.45 μm pore size) to separate the milk whey from the precipitate.

(c) Matrix Effect Studies. Nonspecific interferences produced by the milk have been assessed by preparing standard curves in milk whey at several dilution factors and running them in the ELISA to compare the parallelism with the standard curve prepared in buffer.

(d) Recovery Studies. The recovery of the analyte after sample treatment was assessed by spiking blank milk samples in triplicates at four concentration levels (25, 50, 100, and 200 μg kg^{-1}). After precipitation, the samples were analyzed using as reference standard curves prepared in milk whey.

(e) Accuracy Studies. This parameter was assessed by preparing again diverse blind spiked samples at different concentration levels and measuring them in the ELISA. In all cases data were corrected with the corresponding recovery factor.

■ RESULTS AND DISCUSSION

Immunoreagents for TCs have been developed with the objective to detect specifically doxycycline residues in food products. DC-specific polyclonal antibodies have been raised against two immunizing haptens. The chemical structures of the immunizing haptens were designed following chemical criteria with the aim to maximize exposure of the most important epitopes in the lower periphery of the tetracycline structure, an area with a great proportion of keto−enolic and hydroxyl groups and a primary amide. Moreover, attending to the chemical structure of DC, the presence of one hydroxyl group situated in the C_5 position (ring B) and that of the dimethylamino groups were considered to be relevant. Thus, hapten TC1 was designed to maximize recognition of these groups by introducing a spacer arm at the C_6 position in ring C. On the other hand, hapten TC2, although lacking the hydroxyl group at the C_5 position, allowed the preservation of the methyl group at C6, which could also be an important epitope for antibody recognition, by introducing the spacer arm through the amino group of ring A (see Figure 1 for chemical structures).

Hapten TC1 was synthesized following the procedures previously described by Blackwood et al.[26−29] to slightly modify 6-methylenetetracyclines. Reaction consisted on an anti-Markovnikov free radical addition of the 3-mercaptopropionic acid to the MC C_6 exocyclic double bond, using a catalytic amount of AIBN as radical initiator in ethanol. Contrary to the results reported by Nelson and co-workers,[30] the ethyl ester ($MC-CH_2-S-(CH_2)_2-COOEt$) instead of the carboxylic derivative was obtained in moderate yield. The ester was purified by flash column chromatography (silica gel pretreated with Na_2EDTA to avoid excessive retention of the product on the solid phase due to the formation of chelates with divalent cations) and hydrolyzed under strong basic conditions to obtain the desired TC1 hapten. On the other hand, synthesis of TC2 was accomplished by reacting TC with *N*-chlorosuccinimide, followed by the addition of the hydrazine.[29] The final mixture was also purified by EDTA-treated silica gel flash

column chromatography. Although the final yield of this reaction is very low, we suspect that a significant amount is lost during the purification procedure probably because of the lack of stability of the intermediate or final product.

TC1 was covalently coupled through its carboxylic groups to the lysine amino acid residues of the HCH using the mixed anhydride method, and the TC1−HCH bioconjugate was used to raise three antisera, As180, As181, and As182. On the contrary, TC2 was covalently coupled to the glutaric and aspartic residues of the same carrier protein but using 1-(3-dimethylaminopropyl)-3-ethylcabodiimide (EDC) in buffer solution, and the TC2−HCH bioconjugate was used to raise As183, As184, and As185. Similarly, both haptens were conjugated to HRP, BSA, CONA, and OVA proteins to use them as coating antigens or enzyme tracers to further develop the competitive ELISAs (see details under Immunochemistry/Immunoreagents). The screening of all possible antiserum/enzyme tracer (As/ET) and antiserum/coating antigen (As/CA) combinations was performed using DC as analyte. Previously, the concentrations of the immunoreagents were selected by two-dimensional checkerboard titration experiments in which the avidity of the antisera for the better-recognized coating antigens (CAs) was assessed.[15] Subsequently, the different competitor/antibody combinations were evaluated under competitive configuration to assess their capability to detect DC and to select the best coating antigen/antibody combination. In all cases, the antisera raised against TC1−HCH provided the best assays. In contrast, no usable assays (IC_{50} > 100 μg L^{-1}) were obtained with the As raised against TC2−HCH, indicating that the dimethylamino group played an important role in antibody recognition. Moreover, the indirect ELISA format was found to be more robust and reproducible.

Because of the simplicity we first tried to develop a direct competitive ELISA format; however, we did not succeed in establishing a good competitive immunochemical assay. On the contrary, Table 2 shows the features of the best competitive indirect ELISAs obtained, showing that DC could be detected under homologous conditions. Although several combinations rendered usable assays, As181/TC1−OVA was selected for further studies because of the excellent features and reproducibility observed on repetitive experiments. The TC1−OVA bioconjugate had a hapten density of 11 mol per mole of protein according to MALDI-TOF-MS analysis. The As181/TC1−OVA ELISA was further investigated with the aim to improve performance and to characterize their behavior in media with different physicochemical parameters (pH, ionic strength, etc.). No significant effects on the immunoassay detectability were observed after an overnight preincubation of the antisera with the analyte before the competitive step or by varying the length of the competitive step from 10 min to 1 h. In contrast, the concentration of Tween 20 in the assay buffer readily influenced the detectability of the assay. Thus, the best detectability was accomplished when the percentage of this detergent was reduced (see Figure 2A). With regard to the pH, the assay tolerated quite well pH values between 4 and 9, having the best performance at pH 5.5, which corresponds to the maximum concentration of the zwitterionic form according to the pK_a values reported for the TCs.[1] Above pH 9, the signal of the assay is almost inhibited, showing unexpectedly high IC_{50} values (see Figure 2B). With regard to ionic strength, in the absence of salts the signal of the detectability of the assay was significantly affected. On the other hand, when the ionic strength increased, an improvement of the detectability was observed. A conductivity of around 32 mS/cm was considered to be the most appropriate for this assay (see Figure 2C). Finally, the addition of Ca^{2+} seems to stabilize the TC analytes in terms of structure, owing to their tendency to form chelates, and improves the reproducibility of the assay (see Figure 2D). On the contrary, the addition of Mg^{2+} or Mg^{2+}/Ca^{2+} did not affect so much the assay and therefore did not improve the results obtained previously. The attempts to reduce the A_{min} and to improve signal-to-noise ratio with different blocking agents, such as BSA, casein, or milk powder, after coating the microplates with the antigen were unsuccessful.

According to the above studies, on further experiments a PBS, 0.025 M phosphate, 2% saline solution (343 mmol L^{-1} NaCl, 6.8 mmol L^{-1} KCl) containing calcium divalent cation (1 mmol L^{-1} $CaCl_2$) and 0.001% Tween with a pH of 5.5 was used. Figure 3 shows a standard calibration curve corresponding to the average of four assays performed on different days using two-well replicates using these conditions in the competitive step. The immunoassay features are shown in Table 3. As can be observed, the *detectability* accomplished is very good. The assay shows an IC_{50} of 1.26 ± 0.05 μg L^{-1} and a limit of detection (LOD (90% of the zero dose) of 0.10 ± 0.03 μg L^{-1}). The working range was defined to be between 0.25 ± 0.06 and 6.70 ± 0.59 μg L^{-1} (20−80% of the assay response at zero doses). Moreover, *specificity* studies performed measuring other tetracycline antibiotics (DC, TC, MC, OTC, CTC) showed that only DC and MC were significantly recognized within the concentrations evaluated (see Table 4). The recognition pattern demonstrates the importance of the hydroxyl group in the C_5 position at the B ring. Other TCs not containing this functional group, such as TC, CTC, or OTC, were not recognized.

This result is difficult to explain, although it is well-known that bioreceptors could be very sensitive to subtle modifications on particular functional groups (the so-called methyl group effect). Examination of the recognition profile of immunoassays for tetracyclines described in the literature[16−18,20,31,32] distinguishes two main groups: on the one hand, the immunoassays developed with antibodies raised against immunizing haptens keeping the 6-hydroxy group; on the other hand, those using antibodies generated against immunizing haptens lacking this group. The presence/absence of this group completely determined the recognition profile on each group of assays, recognizing for each of them only those congeners keeping or lacking this group, respectively. This observation deserves a more in-depth study to try to justify it. Computational chemistry could be a good tool for that because it could yield

Table 2. Immunoassay Features of the Best Competitive ELISA (TC1−HCH Immunogen)[a]

assay	A_{max}	A_{min}	IC_{50}[b]	slope	R^2
As182/TC1−BSA	0.533	0.158	4.22	−0.728	0.986
As181/TC1−BSA	0.619	0.189	2.00	−0.852	0.956
As181/TC1−OVA	0.808	0.129	5.27	−1.012	0.995
As182/TC1−CONA	0.701	0.234	3.82	−0.734	0.962
As180/TC1−OVA	1.061	0.243	5.13	−0.932	0.994
As182/TC1−OVA	1.214	0.340	8.26	−0.802	0.947
As181/TC1−CONA	1.251	0.556	2.42	−0.756	0.986

[a]Only some assays showing reasonable parameters and IC_{50} values are shown. [b]IC_{50} values are expressed in μg L^{-1}.

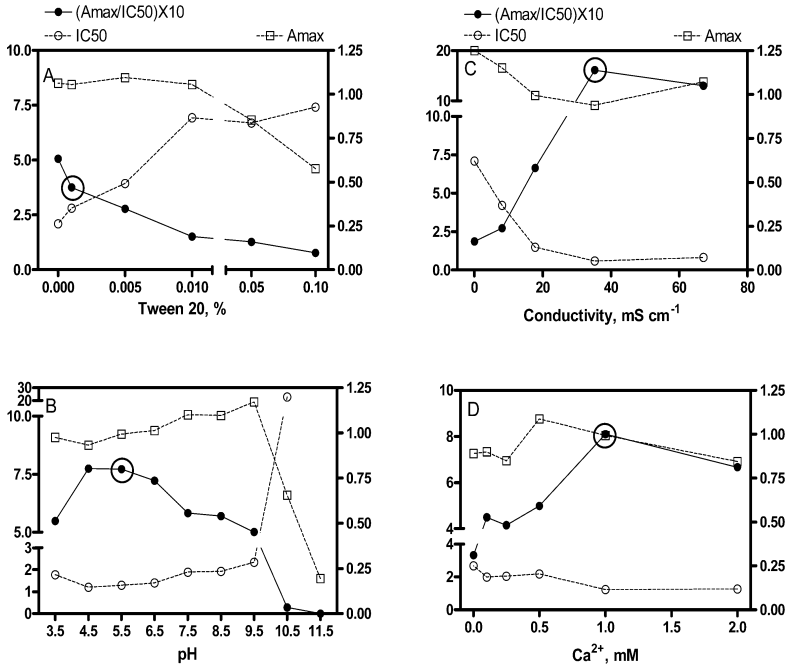

Figure 2. Effect of different physical–chemical parameters on the As181/TC1–OVA immunoassay: (A) concentration of Tween 20 in the assay buffer; (B) effect of pH; (C) effect of ionic strength; (D) effect of concentration of divalent cations (Ca^{2+}). The studies were performed by varying the composition of the buffer used in the competitive step. To evaluate the effect of the ionic strength, the concentration of NaCl was varied while the concentration of phosphate salts remained constant. To evaluate the effect of the concentration of divalent Ca^{2+}, Cl_2Ca was dissolved in PBS at different concentration values. All experiments were performed building calibration curves under different conditions. At least two-well replicates were employed for each standard concentration. Left axes indicate the value of the IC_{50} and the ratio $(A_{max}/IC_{50}) \times 10$. Right axes indicate the maximum absorbance. The IC_{50} values are expressed in nM.

information not only about the geometry of the haptens but also their electronic distribution, polarization, and, in some degree, comparative values for acidity. From the computational chemistry results it is evident that the conformation of the four-ring tetracycline system is highly conserved for tetracycline, doxycycline, and the immunizing hapten (it is taken as the reference structure), whereas the hydrazine derivative showed a large difference for rings A and B (see Figure 4 and Table 5). This fact is independent of the tetracycline protonation state. If we compare only the atoms in the ring system, the geometry differences between tetracycline, doxycycline, and the immunizing hapten are nonsignificant. If we add the common heavy atoms, the difference increases slightly but not enough to consider that the structures are different. In fact, the most important change is the 6-hydroxy group, which is perpendicular to the C/D ring system. If we compare the charge distribution between the four compounds another time, the hydrazine is clearly different for any protonation state. The other three compounds are very similar, with the obvious exception of the C-6 for tetracycline. Finally, if we compare the deprotonation enthalpies (an estimation of the acidity), the values are only appreciably different for the hydrazine compound. In this sense at pH 7 the populations of the different ionization states must be very different for this compound in relation to the other three.

The above data also explain the fact that TC2 did not provide usable assays. Thus, the hydrazine hapten shows large differences in geometry, electronic and charge distribution, and acidity. TC2 keeps the structure and properties of the target analytes with only one exception, the 6-hydroxy group, which is projected perpendicular to the ring system and highly exposed for interactions. This group is not trivial and could interact apparently at very large distance with respect to other parts in the molecule (in fact, the distance is not so large) as it is demonstrated in the oxidation reaction with NCS, in which this oxygen atom bridges with the C-4 (see Figure 1). Also, in the alkali-catalyzed TC isomerization this oxygen reacts with the carbonyl group at C-11, making a lactonization rearrangement that yields isotetracycline. This hypothesis of the highly interacting 6-hydroxy group is also coherent with the empirical observation from the published tetracycline immunoassays.

Finally, the assay *accuracy* was assessed by measuring several blind samples prepared in buffer. The results shown in Figure 5 correspond to the correlation found between the measured and the spiked concentration values. As can be observed, results obtained matched very well the spiked values. A slope near 1 was obtained (0.94) with a coefficient of correlation of R^2 = 0.989.

Due to the interest in controlling the presence of antibiotic residues in milk samples, studies were performed to assess potential nonspecific matrix effects. Results revealed that, at this point, milk samples could not be directly analyzed with the developed assay. Thus, the application of dilution factors of up to 500 times was needed to eliminate the nonspecific response observed (see Figure 3). For this reason, simple sample treatment methods were evaluated with the aim to implement this assay for high-throughput screening of milk samples. As can be observed in Figure 3, a simple precipitation procedure with

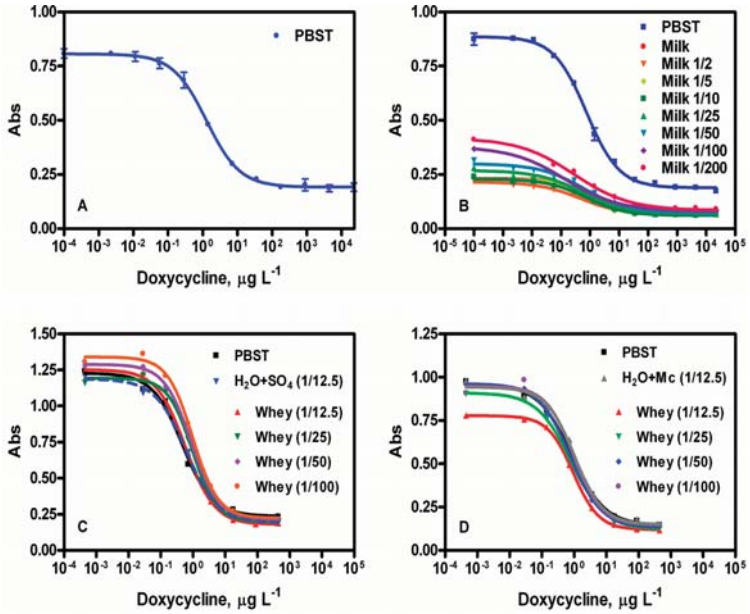

Figure 3. Graphs showing the calibration curve of the As181/TC1−OVA established ELISA for doxycycline under different conditions: (A) assay buffer; (B) untreated whole milk samples diluted to different proportions in the assay buffer; (C) whey milk prepared by treating whole milk with ammonium sulfate and further diluted with the assay buffer; (D) whey milk prepared by treating whole milk with the McIlvaine buffer and further diluted with the assay buffer. The data shown in graph A correspond to the averages of four assays performed on different days (see parameters in Table 3). The data shown in graphs B−D are the average and standard deviation of at least two-well replicates.

Table 3. Features of the Doxycycline ELISA

condition	value	As181/TC1−OVA assay parameter	value[a]
preincubation time	0 min	$signal_{min}$	0.19 ± 0.01
competition time	30 min	$signal_{max}$	0.81 ± 0.01
pH	5.5	slope	−0.90 ± 0.11
ionic strength	35.2 mS/cm (25 mM PBS)	R^2	0.998 ± 0.002
Tween 20	0.001%	IC_{50}, $\mu g\ L^{-1}$	1.26 ± 0.05
Ca^{2+} ($CaCl_2$)	1 mM	working range, $\mu g\ L^{-1}$	from 0.25 ± 0.06 to 6.70 ± 0.59
		LOD, $\mu g\ L^{-1}$	0.10 ± 0.03

[a]Values obtained correspond to the average and standard deviation of six calibration curves performed in four different days. Calibration curves were measured using three-well replicates.

Table 4. Cross-Reactivity of Related Compounds in the As181/TC1−OVA ELISA

	ELISA		
compound	IC_{50} ($\mu g\ L^{-1}$)	LOD ($\mu g\ L^{-1}$)	% CR[a]
doxycycline	1.26	0.10	100%
methacycline	3.89	0.28	32%
oxytetracycline	816.3	31.1	<1%
chlortetracycline	443.7	26.7	<1%
tetracycline	3605.0	127.8	<1%

[a]Cross-reactivity is expressed as a percent of the IC_{50} (nM) of the DC divided by the IC_{50} (nM) of the other compounds tested.

saturated ammonium sulfate or the McIlvaine buffer, followed by the corresponding centrifugation and filtration steps, was sufficient to minimize these undesired interferences. In both cases, the matrix effect, with respect to the PBS buffer, is almost negligible when milk whey is diluted 25 times. Moreover, as can be observed in the figure, milk could be directly measured with a lower dilution factor, because the effect observed after protein precipitation is very low. Thus, preparation of the standards in milk whey allowed the samples to be measured undiluted. Although both protein purification methods were found to perform very well, the use of the McIlvaine buffer was found to be slightly more reproducible. The DC recovery mean after the treatment was 42 ± 9%, being taken into account in subsequent LOD and accuracy studies. Moreover, shaking the microtiter plates during the competition step was found to be crucial because it minimized the formation of whey layers in the bottom of the microtiter wells, improving assay reproducibility and accuracy. Under these conditions, diluting the milk 25 times, the LOD achieved was close to 5 $\mu g\ L^{-1}$, which is still quite below the limits established for most of the tetracyclines allowed to be used on cattle producing milk for human consumption. Thus, preliminary experiments were performed by spiking milk samples at several concentrations around the MRL values of the rest of the tetracyclines, treated as described above and measured with the ELISA. Table 6 shows that the results obtained match very well the spiked concentration values.

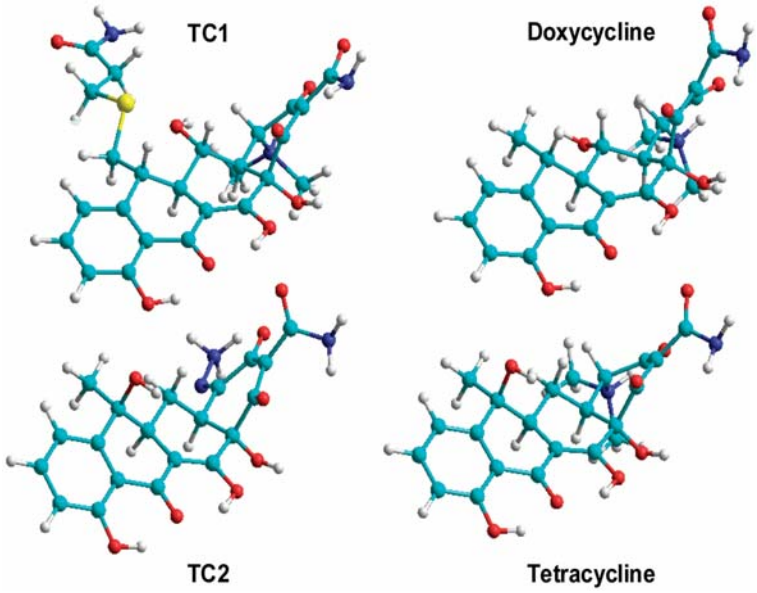

Figure 4. Calculated lower energy structures for the four compounds studied at the zwitterionic state.

Table 5. Geometry Fitting Error[a], Formation Enthalpy, and Deprotonation Enthalpy for TC1, TC, DC, and TC2

compound[b]	rms-rings (Å)	rms-common (Å)	ΔH_f (kJ/mol)	$\Delta\Delta H_{dp}$ (kJ/mol)
TC1+H			−545.5	
TC1+H−H			−1064.2	−518.7
TC1−H			−1195.4	−131.2
TC+H	0.153	0.218	−531.6	
TC+H−H	0.176	0.177	−1059.6	−528
TC−H	0.201	0.193	−1190.4	−130.8
DC+H	0.155	0.172	−523.0	
DC+H−H	0.184	0.178	−1056.7	−533.7
DC−H	0.169	0.156	−1190.7	−134.0
TC2+H	0.519	0.964	−334.6	
TC2+H−H	0.648	1.002	−922.4	−587.8
TC2−H	0.609	0.891	−1247.2	−324.8

[a]Expressed as rms for selected atoms referenced to the geometry of the corresponding hapten taken as reference. [b]+H or −H indicates the acid and base form with respect to the neutral tetracycline structures (+H is the form at high acidic pH, +H−H is the zwitterionic form, and −H is the form at moderate alkaline pH).

In summary, the immunochemical method reported here is a promising tool that could be used as an analytical screening method for monitoring the presence of DC residues in food samples. The antibodies raised, using TC1 immunizing hapten, have proven to be very specific for DC within the tetracycline antibiotic family. By combining these antibodies with a suitable competitor such as TC1−OVA, it has been possible to establish a competitive ELISA with excellent detectability. DC can be detected down to 0.1 μg L^{-1} in the assay buffer. Knowledge of the physicochemical properties of the TC antibiotic family has been critical to establishing a reproducible and robust method. Although the extent of nonspecific interferences caused by the milk samples is very high, a simple sample treatment method such as precipitating proteins using McIlvaine buffer has been sufficient to circumvent this drawback, yielding a reproducible

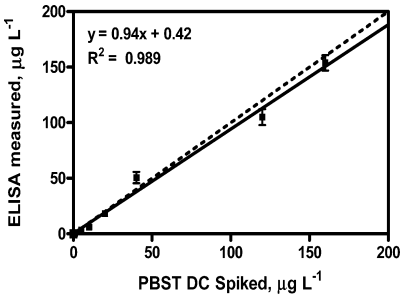

Figure 5. Results from the accuracy study performed in PBST. The graph shows the correlation between the spiked and measured concentration values using the DC ELISA. The dotted line corresponds to a perfect correlation (slope = 1). The data correspond to the average of at least three replicates.

Table 6. Accuracy of the DC Microplate-Based ELISA[a]

		measured	
spiked	*N*	PBST	whey (1/25)
200	3	220.4 ± 38.4	206.7 ± 29.3
100	3	118.7 ± 13.4	129.5 ± 32.3
50	3	81.43 ± 7.9	71.4 ± 5.7
25	3	31.15 ± 9.0	21.5 ± 7.0

[a]PBST and milk whey samples were spiked at distinct concentrations and measured with the DC ELISA. Spiked and measured DC concentration values are expressed in μg L^{-1}. Both types of samples were quantified using the calibration curve prepared in PBST buffer.

and accurate immunochemical analytical method. A LOD of 5 μg L^{-1} for DC in milk was achieved using the established immunochemical method and considering the recovery rate of the sample treatment step. Relative to the MRL values established for other TCs in milk, the detectability achieved by this ELISA is very good. Further research has to be performed for a complete validation in compliance with

Commission Decision 2002/657/EC. In view of the excellent performance demonstrated by this assay to analyze DC in a complex biological sample, such as milk, there is great promise regarding the potential application of this immunoassay on new matrices such as water and soil samples in which antibiotics are considered to be emerging pollutants.

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Notes

The authors declare no competing financial interest.

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4.3 Results and discussion

4.3.1 Design of immunizing haptens to raise antibodies against tetracyclines

As mentioned before in **Section 3.1.1**, the most crucial step in the development of an immunochemical technique for detecting low molecular weight molecules is the hapten design since the chemical structure selected strongly modulates the affinity and selectivity of the resulting antibodies. As described for SAs, in order to produce generic antibodies, selectivity has to be addressed against common parts of the molecules.

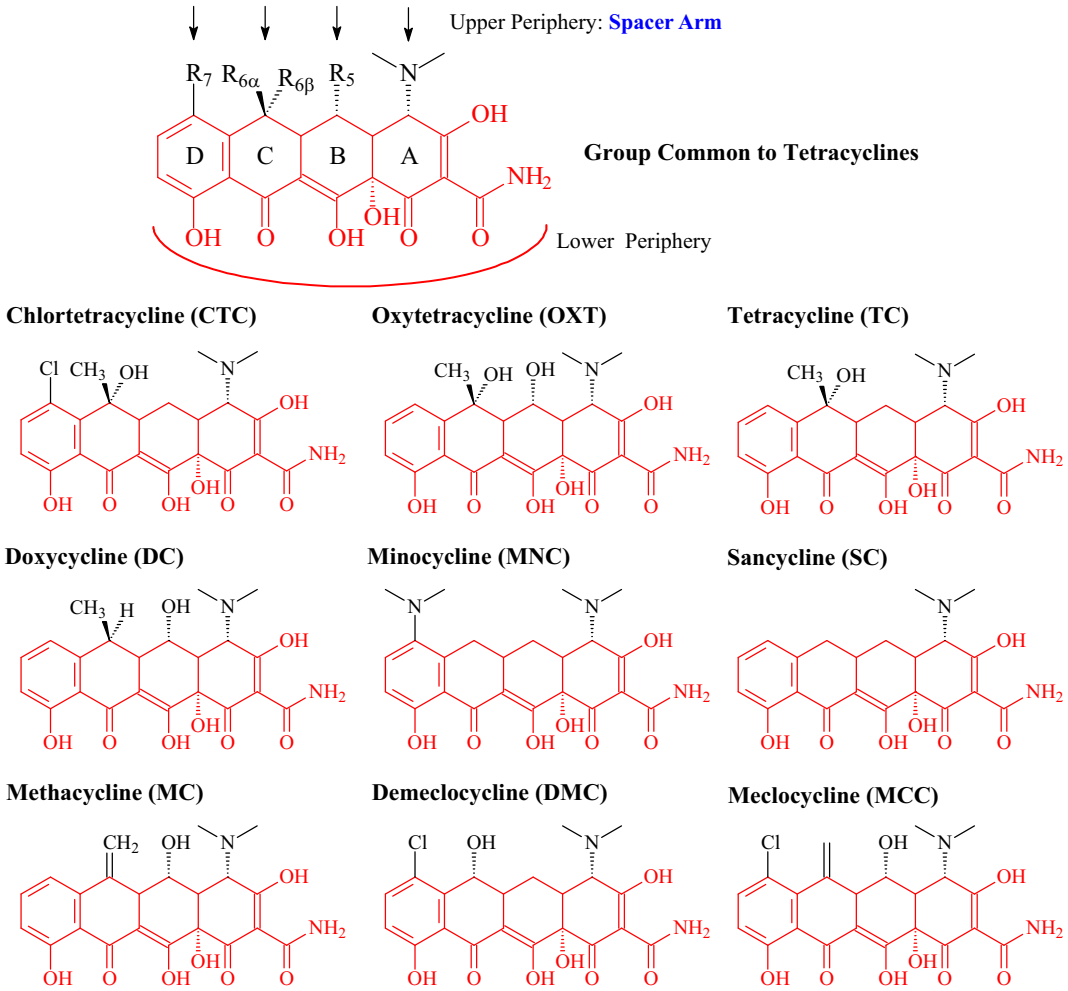

Figure 4.4: Chemical structure of most important TC congeners. Area marked in red represents the basic chemical structure common for most TCs.

In this case, the so-called the ***lower periphery*** of the TCs, characterized for being heavily oxidized, is common to all the congeners (see **Figure 4.4**). Thus since recognition is maximized on the opposite site to the location of the spacer arm, the hapten had to contain a linker with a chemical group for covalent attachment to the carrier protein on the so-called ***upper periphery*** (see **Figure 4.4**). With this criteria the fundamental chemical structure moiety that had to be present in the immunizing hapten was defined. Thus, several positions where a spacer arm could be introduced in the chemical structure of the TCs were considered, leading to propose two different potential immunizing haptens (TC1 and TC2). Both haptens maximized the mentioned common area, however while hapten TC1 was modified through the C ring, hapten TC2 had the spacer arm introduced in the A ring (see **Figure 4.5**).

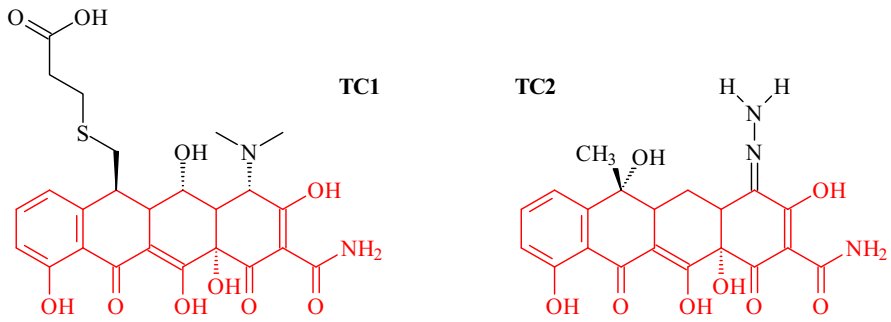

Figure 4.5: Structure of the possible immunizing haptens planned for the production of antibodies against tetracyclines.

The introduction of the linker in ring C for hapten TC1 was selected because several congeners already had an alkyl group at this position. Moreover, the greatest variability for TCs was observed on the upper periphery of rings C and D, while variations on rings A and B was lower.

4.3.2 Haptens synthesis

Hapten TC1 was synthesized using the procedure previously described by Blackwood et al. to modify 6-methylenetetracyclines [15-17] with slight modifications. The key step consisted on an anti-Markovnikov free-radical addition of the 3-mercaptopropionic acid to the methacycline (MC) C-6 exocyclic double bond, using catalytic amounts of AIBN as radical initiator in ethanol (see **Figure 2.5** in **Section 2.1.1.1** and **Figure 4.6**). Contrary to the results reported by Nelson and co-workers [18] the mixture obtained did not contain the desired carboxylic acid, but the ethyl ester (MC-CH_2-S-$(CH_2)_2$-COOEt) which was spontaneously formed in the reaction media with a yield of 31% (see **Figure 1** in **Section 4.2**).

Thus, the ethyl ester was purified by flash column chromatography packed with silicagel intensely pre-treated with ethylenediaminetetraacetic acid (EDTA). Precoating of the silica gel with EDTA has been advised in order to avoid complexation with divalent cations [19] present in the stationary or the mobile phase. Subsequent, hydrolysis of the purified ester under strong basic conditions allowed obtaining TC1 compound. This hapten is obtained with R configuration on C_6 instead of the racemic mixture because of the chiral groups located next to this position which guide the addition of 3-mercaptopropionc acid perfectly oriented. In parallel we tried to optimize this reaction by different strategies changing the reaction conditions (e.g. time, temperature, type of inert atmosphere), reagents (e.g. butyl 3-mercaptopropionate, neutral forms (≠ pH), cleavage reagents) or solvents (e.g. MeOH, ButOH) but being not successful.

On the other hand, the synthesis of TC2 was much simpler. The hapten was obtained by reacting TC with N-chlorosuccinimide, followed by the addition of the hydrazine [20]. Again, the final mixture was purified by flash column chromatography previously impregnated with EDTA in order to block all the cations present in the silica avoiding undesired retention of TCs in the stationary phase (see **Figure 4.6**).

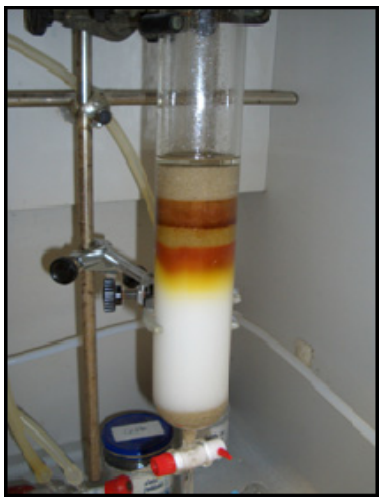

Figure 4.6: Picture of a typical flash column purification used where the product was applied into the system by impregnating the silica, previously treated with EDTA, with the tetracycline.

Both TC1 and TC2 were obtained in low yields due to the low stability of their intermediate and final products, which experimented unexpected transformations during the reaction and/or the purification steps (see **Figure 4.3**). Thus, it has been described by Blackwood et al. that under the action of mercaptans, 6-methylenetetracyclines undergoes into side reactions such as the initial rearrangement of the double bond into the ring to form 5a, 6-anydro compounds [21] affecting the formation of TC1 compound (see **Figure**

4.3). It has been proven that the amount of side reaction increases with (1) decreasing acid stability of the methylene compound, (2) increasing acidity of the mercaptan, and (3) decreasing reactivity of the mercaptan. On the other hand, under the action of N-chlorosuccinimide in aqueous media, TC undergoes deamination with the formation of a 4-oxo derivative forming a 4, 6-semiketal, also known as tetracycloxide [22]. Under our experience, this intermediate compound had to be rapidly used to synthesize TC2 in order to avoid undesired side reactions and degradation.

4.3.3 Antibody production

Hapten TC1 (R-COOH) was coupled through its carboxylic group to the lysine residues of the HCH protein following the standard MA method, while hapten TC2 (R-NH_2) was directly coupled to the glutaric and aspartic residues of the same carrier protein but using 1-(3-dimethylaminopropyl)-2-ethylcarbodiimide (EDC) in buffer solution. Both hemocyanin conjugates were used to raise antibodies in white New-Zealand rabbits. The obtained As were named As180, As181, and As182 for those obtained against hapten TC1 while those obtained against hapten TC2 were named As183, As184 and As185.

4.3.4 Competitors

As it was already described in **Chapter 3**, the immunochemical analysis of small organic compounds, such as the TCs in this case, requires the use of competitive formats. Then, the structural heterology concept between the immunizing and competitor haptens was considered again in order to have more chances of success to achieve required detectability and selectivity profiles. Contrary to the SAs case, the extremely high difficulties that we found regarding the introduction of other spacer arms to TCs have limited the number of competitor haptens synthesized. Therefore, it was proposed to use TC1 and TC2 as competitors by preparing the corresponding CAs and ETs. Again, different coupling strategies, to link the haptens to the correspondent enzymes and proteins, were defined to avoid possible interferences later in the immunoassay due to secondary products that may be formed during the conjugates preparation.

Since the TC1 immunizing hapten was covalently prepared by the MA method, we used alternative methodology like the AE, to attach the hapten to HRP, BSA, CONA and OVA proteins. On the other hand, new TC2 conjugates were prepared in the same way as the correspondent immunogen by using EDC. Alternatively, linkers such as dimethyl pimelimidate (DMP) and cyanuric chloride (CC), which have been previously used with success by other groups for similar purposes [23, 24], were also tested here to couple TC2 hapten with the carrier proteins (see **Figure 4.7**) at different molar ratios.

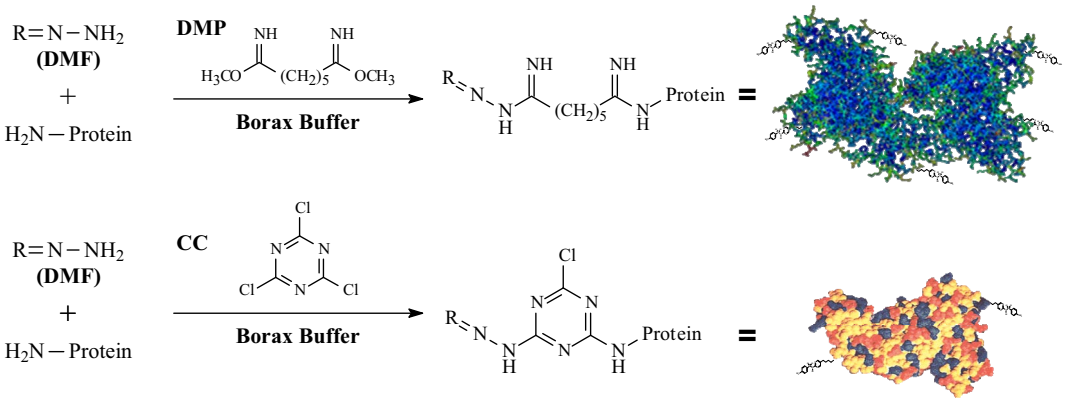

Figure 4.7: Additional strategies followed to couple TC2 hapten with the carrier proteins, in addition to that used to prepare the immunogen based on the use of EDC.

Table 4.2 summarizes the bioconjugates prepared for the development of a competitive ELISA for TCs.

Table 4.2: Bioconjugates prepared for both direct and indirect ELISA formats, including hapten densities (δ) of the BSA conjugates measured by MALDI-TOF-MS.

Bioconjugates for the Direct ELISA[a]	
TC2-DMP-HRP	
TC1-HRP-I (EA)	
TC1-HRP-II (AE)[b]	
TC2-HRP (EDC)	
TC2-CC-HRP	
Bioconjugates for Indirect ELISA[c]	
TC1-BSA (MA) / 3.3 hapten δ (9-11%)	TC2-OVA (EDC)
TC1-BSA-I (AE) / 10.8 hapten δ (31-36%)	TC2-CONA (EDC)
TC1-BSA-II (AE) / 11.6 hapten δ (33-39%)	TC2-DMP-BSA / 2.1 hapten δ (6-7%)
TC1-OVA-I (AE)	TC2-DMP-OVA
TC1-OVA-II (AE)	TC2-DMP-CONA
TC1-CONA-I (AE)	TC2-CC-BSA / 0.9 hapten δ (2-3%)
TC1-CONA-II (AE)	TC2-CC-OVA
TC2-BSA (EDC) / 6.0 hapten δ (17-20%)	TC2-CC-CONA

Conjugation method used is indicated in brackets: DMP, dimethylpimelidate; AE: active ester active; MA, mixed anhydride; EDC, ethyl diisopropyl carbodiimide; CC, cyanuric chloride. [a]*unless otherwise indicated the bioconjugates ratio was 10 µmol of hapten per 2 mg of HRP;* [b]*the bioconjugates ratio was 20 µmol of hapten per 2 mg of HRP;* [c]*for the indirect format, the type I bioconjugates ratio was 10 µmol of hapten per 10 mg of protein while the type II bioconjugates ratio was 20 µmol of hapten per 10 mg of protein. Hapten density (δ): Mols of hapten per mol of protein. The % conjugation is calculated based on the assumption that the BSA protein has between 35 and 30 free lysine groups.*

The hapten densities of the CAs were assessed by measuring the BSA conjugates by MALDI-TOF-MS technique. and comparing the molecular weight with that of the control BSA. Since all BSA, CONA and OVA bioconjugates had been prepared simultaneously, measuring just the BSA conjugates was considered at this step sufficient to probe of the extent of the bioconjugation reaction. The HRP bioconjugates were not measured by MALDI-TOF-MS since it has been described that just 1-2 residues are available for conjugation, which could produce variations on the molecular weight of the protein below the resolution of the equipment used at that moment.

Finally, we had 30 combinations (5 ET x 6 As) to evaluate with the direct ELISA format and 96 combinations (16 CA x 6 As) to evaluate with the indirect ELISA format (see **Section 4.2**). Some of the bioconjugates presented low solubilities being necessary the filtration to obtain working aliquots with a concentration of just1 $\mu g\ mL^{-1}$ (concentration estimated using the Bradford assay [25]). The reason for this low solubility has not been found since the hapten density recorded on the MALDI-TOF-MS measurements cannot completely justify this behaviour.

4.3.5 Screening, Evaluation and Optimization

The recognition for the ETs (direct format) and CAs (indirect format) by the As were firstly evaluated by non-competitive ELISAs using 1D experiments. Further on, the avidity of As for the better recognized competitors was evaluated by 2D checkerboard titration experiments. From these experiments, the most appropriate concentrations of the immunoreagents to perform competitive assays, in both formats (see **Section 3.1.5**), were selected. From these experiments about 15 homologous As/competitor combinations were chosen for each ELISA format. Unfortunately, in this case no heterologous combinations were afforded and therefore reducing, a priori, the chances to decrease specificity of the subsequent assays. Since the objective of this work was to produce antibodies with a wide selectivity, the screening for competitive assays was in this case performed using DC, OTC, CTC, TC and MC as analytes. Because of the simplicity we first tried to develop a direct competitive ELISA format, however we did not succeed on establishing a competitive immunochemical assay.

Unluckily, as it can be observed in **Figure 4.8**, using the heterologous As181/TC2-CC-HRP combination as an example, none of the TCs tested were recognized in the direct format (left graph) with acceptable detectability (see **Section 4.3.6**). Same experiments performed varying several physicochemical experimental conditions such as pH, conductivity, the concentration of the detergent (tween 20) or the preincubation time effect did not improve the results.

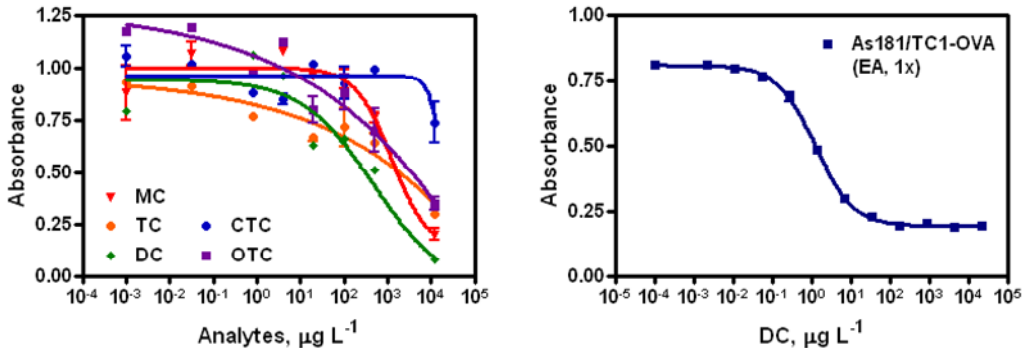

Figure 4.8: Calibration curves obtained when analyzing different TCs on the direct ELISA (left graph) and on the indirect ELISA (right graph). As an example, the graphs show the results of the As181/TC2-CC-HRP and the As181/TC1-OVA-I (AE) combinations.

In respect to the indirect format, the screening was in this case performed initially with DC. Only As181 and As182, raised against hapten TC1, were able to provide competitive assays within the antibiotic concentrations evaluated (from 50000 to 0.0256 nM). The best competitive assays were afforded by homogeneous combinations. Finally, As181/TC1-OVA-I (AE) was selected for further evaluation (see **Table 2** in **Section 4.2**). Using the selected combination, under standard conditions (10 mM PBST, 0.05% Tween, pH 7.5) DC was detected with an IC_{50} of 5,70 µg L^{-1}. A set of experimental parameters (incubation time, detergent concentration ionic strength or pH) were studied sequentially in order to improve the immunoassay detectability and to study the immunoassay performance under several different conditions. The conditions determining the best immunoassay performance for the As181/TC1-OVA-I (AE) combination are summarized in see **Table 3** in **Section 4.2** and the corresponding standard curve is presented in **Figure 4.8**.

As a results of these studies it was possible to increase the detectability around four times up to 1,26 of IC_{50} as well as the reproducibility. Thus, the recognition of DC seemed to be favoured at pH 5.5, when the zwitterionic specie is dominant (see **Figure 4.1**) and thus being unified the conformation of the DC present in the buffer solution assessed. The addition of Ca^{2+} to the buffer seemed to stabilize the tetracycline improving the reproducibility of the assay. The same effect had been observed before for the FQ assay developed in our research group [26]. The other two factors, % of detergent and ionic strength, also affected positively in the detection and the reproducibility of the assay. On the other hand, the attempts to reduce the A_{min} and to improve signal-to-noise ratio by with different blocking agents, such as BSA, casein or milk powder, after coating the microplates with the antigen, were unsuccessful.

4.3.6 Specificity Study

Immunoassay specificity was evaluated by testing, in addition to DC, the same battery of TCs as in the direct ELISA format MC, OXT, CTC and TC. Standard curves for all the selected compounds were build in buffer ranging from 0.005 to 50000 nM and run in the ELISA. As it can be observed in see **Table 4** in **Section 4.2** only DC and MC were recognized with sufficient detectability. MC cross-reacted 32% in respect to DC.

Thus, unfortunately we did not reach the objective to develop a broad-selectivity immunochemical method for TCs. Although the chemical structure of the selected immunizing haptens was chosen to maximize exposure of the common moieties of this family of antibiotics, the antibodies obtained did only recognize those with an alkyl group at position C-6, even if in MC is a methylene and in DC is a methyl. Moreover, both tetracyclines have an hydroxyl group at position 5. Surprisingly, OTC possessing also a methyl group in this position and the hydrozyl at position C-5, was almost not recognized under these conditions, even if the only significant difference was the hydroxyl position at C-6. In fact CTC and TC, with the same functional groups and estereochemical configuration at this position were also not recognized. CTC has additionally a chlorine atom at C-7 and TC lacks the OH group at C-5. The structural differences between the TCs did not justify the lack recognition of this family, since the variations above mentioned occur precisely at the upper periphery that was apparently less exposed to antibody recognition considering the immunizing hapten TC1. At the light to the results obtained we performed some molecular modelling studies to find out an explanation (see **Table 5** and **Figure 4** in **Section 4.2**).

The corresponding results exposed in the publication (**Section 4.2**, pages 279-280) let us to conclude that the hydrazine hapten (TC2) is not a good competitor hapten for TCs due to the large differences in geometry, electronic and charge distribution and acidity. On the other hand, the TC1 proposed immunizing hapten keep the structure and properties of the target analytes with only one exception, the 6-hydroxy group which is projected perpendicular to the ring system and highly exposed for interactions. This hypothesis of the highly interacting 6-hydroxy group is also coherent with the empirical observation from the published tetracyclines immunoassays.

4.3.7 Accuracy studies for indirect TC ELISA format

The accuracy of the assay As181/TC1-OVA-I (AE) was evaluated by measuring 8 blind samples prepared in buffer in different days. The results shown in **Figure 5** in **Section 4.2** correspond to the correlation found between the measured and the spiked concentration

values. Results obtained matched very well the spiked values. A slope near 1 was obtained with a coefficient of correlation of $R^2 = 0.989$.

4.3.8 Evaluation of the effect of milk samples on the immunochemical determination of TCs

There are important differences in the pharmacokinetic properties of the TCs in animals being absorbed from the gastrointestinal tract at the range from 30% to 100%. Unabsorbed TCs can potentially disrupt the gut flora and can therefore give rise to infections. This absorption is reduced by the presence of food and this is an important factor to consider in livestock medication where the drug is normally administered in feed. Moreover, the presence of divalent or trivalent metal ions also significantly reduces absorption as a result of the formation of complexes making easier their excretion in the faeces. On the other hand, this antibiotic family is generally well distributed in tissues and body fluids, but plasma protein binding varies between the different agents. TCs readily cross the placenta and high concentrations are found in the milk of lactating females [27]. The routes and rates of excretion of the different tetracyclines vary considerably, although all undergo renal excretion by filtration to some degree and all concentrate in the liver and appear in the bile. Half-lives of TCs range from 7 to 17 hours [28]. TCs normally administered to cattle comprise CTC, TC and OTC (see **Section 1.3.4.5**), although more recently a increased use DC has been reported. There are few immunochemical techniques to analyze these compounds in milk [13, 29] but on the other hand it has raised the interest to analyze other TCs not supposed to be administered (e.g. DC, MC) to fight against their fraudulent use for non therapeutic purposes. Thus, the indirect ELISA was evaluated as screening tool to detect DC residues in milk samples. For this purpose standard curves were prepared using skimmed milk samples previously diluted several times (1/2, 1/5, 1/10, 1/25, 1/50, 1/100 and 1/200) with 2x PBS (PBS buffer two times concentrated) and measured with the developed DC ELISA (see **Figure 3 B** in **Section 4.2**) shows the calibration curves obtained when running the ELISA in milk samples.

Although under pH (~ 6.6 – 6.8) and the conductivity (~ 4.0 – 5.5 mS cm^{-1}) values of the milk samples were closed to those of the buffer, the signal was completely inhibited contrary to what happened with the SAs ELISA (see **Chapter 3.5.4.1**). Thus, the milk affected significantly this assay. A potential reason for this fact could be the high protein content of the milk, even if this feature did not affect the SAs ELISA and other assays developed in the lab. Thus, with the aim to evaluate this effect and to eliminate the observed interferences we decided to remove proteins by precipitation. Two different procedures were tested: procedure A used saturated ammonium sulfate (0.45 mL of $(NH_4)_2SO_4$ per mL of milk) followed by centrifugation (500G for 10 min) and filtration of

the supernatant (0.45µm pore size); procedure B consisted on using the Mc Ilvaine Buffer (0.30 mL of Mc Ilvaine buffer, pH 3, per mL of milk) also followed by centrifugation and filtration as in procedure A.

In each case, the milk whey obtained, previously diluted several times with water, was used to prepare standard curves that were run in the ELISA. Both procedures succeeded on diminishing significantly the matrix effect which was completely avoided when milk whey is diluted 25 times (see **Figure 3 C** and **D** in **Section 4.2**). However, for practical purposes the milk could be measured almost undiluted, since the matrix effect is almost negligible. The reproducibility in the assay was found to be better using milk whey prepared with Mc Ilvaine Buffer (±9%), but unfortunately only a 42% of recovery was obtained. The calcium of the casein micelles [30, 31] may, through complexation with TCs, have a major role in the co-precipitation of the analyte with the proteins, decreasing the recovery. Thus, some authors have reported the use of EDTA to capture the calcium with the aim to avoid this effect [29, 32-34]. Probably this could have been a way to improve recovery. However, we did not invest more time to investigate this aspect because the detectability of our assay did not require improving this step. Thus, considering that no MRL value has been settle for DC in this matrix since it should not be used in farm animals for milk production, the LOD achieved was close to 5 µg L^{-1}, that is still quite below the limits established for most of the TCs which use is allowed on cattle addressed to produce milk for human consumption [35]. The good sensibility of the ELISA developed makes possible the specific control of the potential illegal or inappropriate use of DC. In this sense, preliminary experiments were performed spiking milk samples at several concentrations around the MRL values of the rest of TCs, treated as described above and measured with the ELISA. **Table 6** in **Section 4.2** shows that the results obtained match very well the spiked concentration values.

4.3.9 Main contributions

The main contributions of this chapter to the existing knowledge at the time of starting this research are:

- Specific antibodies for DC, with a lesser recognition degree of MC, have been developed for the first time.
- An immunochemical analytical protocol has been established for the analysis of DC residues in milk samples and in compliance with the EC regulations in respect to the required detectability.

While doing this research several papers have been published reporting the production of antibodies for TCs (see **Section 4.1**) but few have reported the application of these assays

to the analysis of milk samples [13, 29]. In both cases, the immunoassays developed were able to detect only the TC compound (i.e. not showing cross-reactivity with other structurally similar compounds) below the corresponding MRL. Any other immunochemical assay capable of detecting DC in milk samples has been reported. On the other hand, to our knowledge, no reports have been found describing the production of bread selectivity antibodies or immunochemical assays for the TCs family, which was the objective of the present work.

4.3.10 Further considerations

Much effort has been invested in our group with the aim to produce generic antibodies and establishing immunochemical protocols for the broad recognition of the TCs family. Up-to know these efforts have not become successful although other members in our research group do keep working. Nevertheless, the results obtained have called the attention of several research groups and companies (confidential information) since there aren't any immunoassay available in the market capable to detect specifically DC in biological samples. The illegal use of this antibiotic is emerging lately being therefore justified the interest of developing new screening techniques to detect it. The good results obtained in milk samples open the possibility to explore other biological samples such as honey where TCs residues have been found.

4.4 Bibliography of Chapter 4

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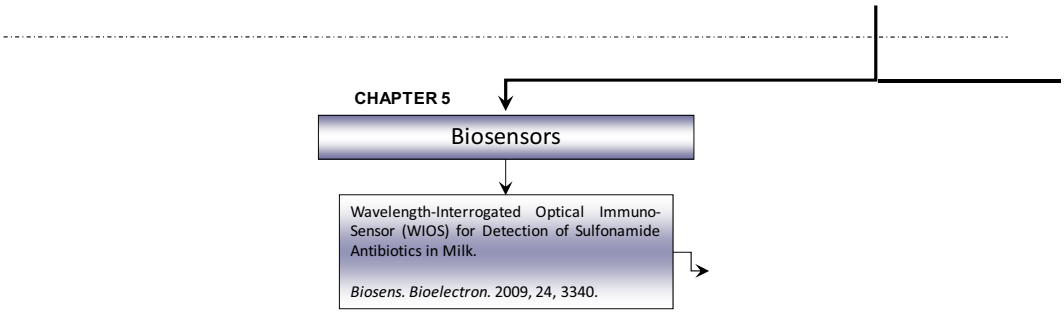
5 IMMUNOSENSOR DEVELOPMENT



Technology used nowadays to assess food safety and quality relies mainly on solutions that involve bulky and expensive equipment and long and complex laboratory procedures. As in the clinical field, in the food sector there is a demand for developing a new generation of bioanalytical tools and devices based on simple detection systems able to be used "on site", which means bringing the laboratory to the foodstuff in the different points of the food chain, providing means to control de quality and safety of the food in all the steps, from the farm to the table [1, 2]. The possibility to combine the potential of immunochemical methods, developed and described in previous chapters, with the current advances in micro/nanoelectronics allows developing rapid methods based on miniaturised kits, such as biosensors, that provide interesting advantages as alternative analytical methods to those more conventional.

As mentioned in **Chapter 1** (see **Section 1.5.2.3.2**), biosensors are electrical, optical, chemical or mechanical devices which are modified with biological entities to provide them with the desired selectivity. Examples of biological recognition molecules include enzymes, receptors, antibodies, and oligonucleotides [3]. Immunosensors are a particular type of biosensors in which the biological molecule is an immunoreagent. The ideal biosensor not only has to respond to low concentrations of analytes, but also must have the ability to discriminate among species according to the recognition molecules that are immobilized on its transducer surface. Nowadays, biosensors have wide applications, including biomarker detection for medical diagnostics, and pathogen and toxin detection in food and water. From the multiple options (see review in **Section 1.8**), optical biosensors show great promise as devices for bioanalysis or diagnostic [4, 5]. Although first developments took advantage of the flexibility and low cost of the optical fibbers measuring the absorption or emission of light of one of the components of the bioreaction [6], late developments are mainly based on micro/nanotechnology and on the possibility to fabricate complex micro/nanostructures to guide light which have opened up the possibility to investigate novel optical phenomena and the optical properties of materials at the nanometric level [7-10].

Nowadays, the most widespread fibber-optic label-free evanescent detection systems (see **Section 5.1.1**) are based on the SPR phenomenon [11] and on the evanescent wave (EW) optical principle [6]. Within this thesis we have focussed on the implementation of the immunochemical procedures established previously on a particular type of EW sensor know as WIOS, developed by Cottier et al. [12, 13] at CSEM.

5.1 Preamble

Food safety and quality assurance is a relative new business area, partly because the market is not well developed, and also partly, since there is a lack of cost effective solutions [14]. Thus, high quality investigation and development activities are the basis for future marketable developments. In the last years, the AMR group has been also working in this direction by doing basic research focused on developing novel strategies to detect different substances in collaboration with top national or international research groups with complementary expertises and similar interest regarding development of new strategies for chemical analysis in the food and in the clinical field. In this sense, the following chapter describes part of the work done during a stay of three months in the Biosensing group, lead by Dr. Guy Voirin, in the Nanotechnology and Life Sciences division of CSEM, in order to develop a biosensor for the detection of SAs in milk samples, using their WIOS platform.

By the time this work started, there wasn't described a biosensing device addressed to the determination of generic detection of SAs and even less in biological samples such as milk. On the contrary, similar studies such as detection of SMZ with the optical waveguide lightmode spectroscopy (OWLS) system was limited to one sulfonamide antibiotic but in buffer samples [15].

5.1.1 Label-free evanescent biosensors

At present, most screening techniques that are used in agro food analysis are performed by systems that require some type of fluorescent, radioactive or light absorbing labels to report the binding of a ligand to its receptor. Despite their high sensitivity and many other advantages, this labelling step sometimes imposes extra time and cost demands, and can also interfere in some cases with the molecular interaction by occluding a binding site, which leads to false negatives [16]. That's one of the reasons why the trend in these kinds of detection techniques has recently turned towards label-free systems [13]. For example, in SPR systems biochemical interactions at the sensor surface are monitored in real time with no labelling requirements by observing the changes in the optical reflectivity, both versus wavelength at fixed angle or versus angle at fixed wavelength, of guided waves at a thin flat (2D) metal film (typically gold and silver). Therefore, by functionalizating metal surfaces with specific bioreceptors, SPR spectroscopy has become a common tool in biochemistry and bioanalytical chemistry especially for determining the on- and off-rates and equilibrium binding constants or for investigating the effects of various cofactors or inhibitors on these binding constants. Since distinct SPR prototypes

(Biacore™, IASys™, IBIS™, etc) have appear in the market, a significant number of the application of this principle have been reported during the last years, and particularly also for the analysis of pharmaceuticals [17, 18]. A variation of this transduction system is the so-called localized surface plasmon resonance (LSPR) based on the light in-coupling phenomenon into the resonance oscillation of charge density on noble metallic nano-particles (3D) and nano structures [19]. The wavelength of the LSPR phenomena is characteristic of the type of material and is sensitive to the size, distribution and shape of the nano-structures, as well as to the dielectric environment. Thus, nano-structures can be generally fabricated on a variety of substrates allowing for easy integration with other biosensor components [20].

Other label-free detection technologies found in the market include those biosensors based on the EW optical principle. An EW is produced in the external media of a waveguide by the electromagnetic field associated to the light guided by total internal refection (for more details see review in **Section 1.8**).

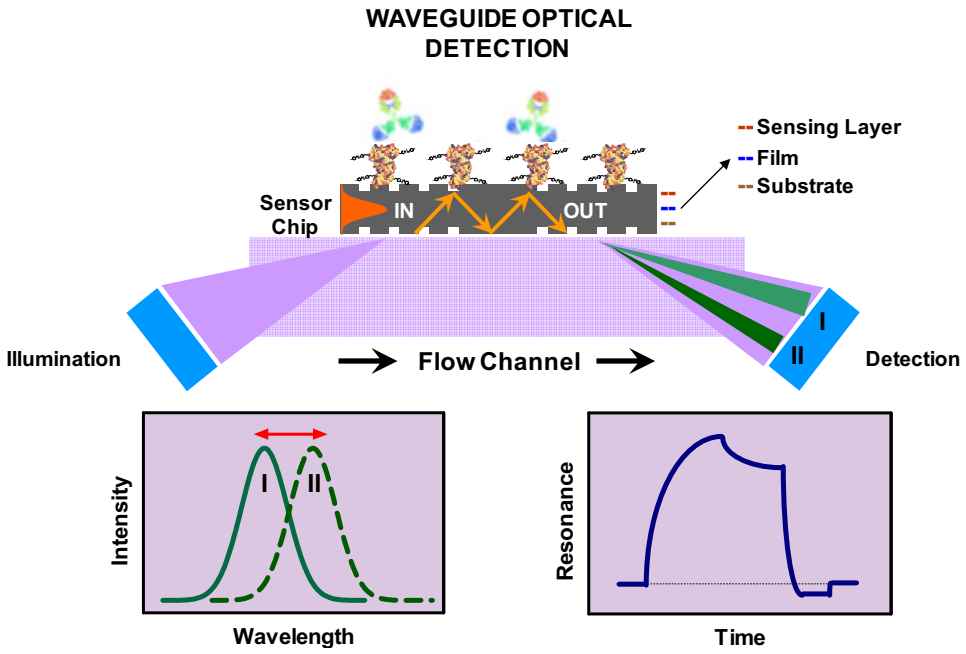


Figure 5.1: Scheme of a biosensor which detects changes in the refractive index in the evanescent wave of a straight waveguide. Light from a laser is incident (angle fixed) on a first grating where the waveguide mode is excited. The second grating couples out the guided light which is collected by the optical detector. This change in resonant wavelength for which coupling occurs can be also monitored non-invasively in real time as a plot of resonance signal (proportional to mass change) versus time.

When molecules with an absorption spectrum including the excitation wavelength are located in the evanescent field, they absorb energy leading to attenuation in the reflected

light of the waveguide. Contrary to the River Analyser RIANA biosensor [21-23], recent EW immunosensor approaches, such as, Mach-Zehnder Interferometers [24-26] or Grating Couplers (i.e. [27-29]) have been investigated in order to make possible direct measurement of small analytes without the use of fluorescent labels. On the other side, planar optical waveguides with an input grating coupler, or input/output gratings (see **Figure 5.1**), have also been reported with the aim to be used as label-free biodetection devices. The OWLS-based biosensor [30] and the WIOS [31] are just few examples following this principle. Some authors have reported that label-free biosensors based on planar dielectric waveguides have several advantages over SPR devices, by means of having higher degrees of freedom for sensing design [12, 32]. For example, SPR is not optimal for studying large molecules such as living cells because their size considerably exceeds the penetration dept of visible-range surface plasmon waves (around 200 nm). In the case of planar dielectric waveguides, this property can be modulated through an adequate design, enhancing the application fields to the detection of biomolecules in the micrometer range [33]. Another disadvantage for the SPR sensors is found when combining the label-free with luminescent detection because the metal film required by this technology can produce a quenching effect (energy transfers to the metal). Furthermore, biosensors based on waveguide gratings have the possibility to use arrays built with a high number of sensing regions on the same chip being able to detect simultaneously several target analytes (this was not possible in SPR devices at the time that the work related to the WIOS was started in 2005).

Thus, label-free sensors based on planar waveguides appear as promising candidates for future detection systems to be applied in fields such as pharmaceutical industry, medical diagnostics, food quality safety or environmental control. As it will be showed in the following sections they can provide not only yes-or-no answers, but quantification of the concentration of the target analyte plus the possibility to obtain detailed information about affinity, specificity and rate of binding through time-resolved monitoring of the binding processes. Moreover, these devices can provide quantitative data regarding concentration of the counter-part, the analyte, if calibrated appropriately with standards of know concentration values.

5.1.1.1 Waveguide grating sensors

There is a silent revolution going on with other optical components where freely propagating beams are replaced by guided waves, and refractive elements such as classical lenses are being replaced by diffractive components such as diffractive lenses and gratings. Obviously, these new components, which are currently gaining more and more importance in telecommunication as well as in sensor technology, allow for much

higher degrees of integration and miniaturisation. Thus, the grating coupler has been implemented in a variety of configurations: input, output, and reflective modes [32] as well as various resonance approaches [34]. As an example, **Figure 5.2** shows the operating principle of the input grating coupler where a collimated laser beam is incident at an angle onto a waveguide deposited on a transparent substrate. When the coupling conditions are satisfied, the light beam is coupled into the waveguide, propagates, and is detected at the edge. When the grating is exposed to the analytes, adsorption causes changes in the refractive index at the grating surface and results in a shift in the laser beam coupling conditions, detected either as angular shift or wavelength shift [35].

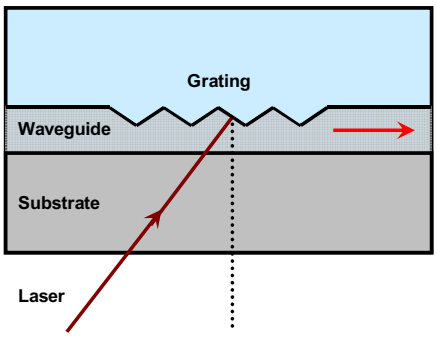


Figure 5.2: Scheme of the input grating coupler principle.

Then, by incorporating a grating in a planar optical waveguide a device can be created, with which the spectrum of guided lightmodes can be measured, that means when the surface of the waveguide is exposed to different solutions, the peaks in the spectrum shift (see **Figure 5.1**) due to molecular interactions with the surface (see more details related to the corresponding coupling light equation in **Section 5.1.2.1**).

5.1.2 WIOS

A novel optical detection platform called WIOS based on a waveguide grating coupling with wavelength modulation was reported by CSEM. With an input-output configuration this platform had been proved to be very sensitive to changes in the refraction index of the adjacent media [13, 31]. During the last years CSEM had been working on integrating this transducer on a portable sensor device with the necessary microfluidics, optical and electrical components as well as of a data acquisition system. Thanks to the collaboration established between AMRg and CSEM in the context of the GOODFOOD project (see **Section B.1.3.3**), it was possible to investigate the possibility to use the WIOS as a biosensor (WIObS) for antibiotic residue detection, based on the changes produced in the refractive index as a consequence of a selective biomolecular interaction.

5.1.2.1 Sensing principle

WIOS detects the refractive index changes in the evanescent wave of a straight waveguide grating using a wavelength sweep. It is composed by a high refractive index waveguide (Si_3N_4, TiO_2, Ta_2O_5) combined with a fine grating period (300 to 700 nm). In this case, the grating is used as a light coupler and/or as a waveguide effective refractive index transducer being possible both the label-free or fluorescence detection. **Figure 5.3** shows schematically the detection WIOS principle. Firstly, light from a VCSEL (vertical-cavity surface-emitting laser) source emitting at around 763 nm is incident on a first grating. Then, the waveguide mode is excited and propagates into the waveguiding layer. The second grating couples out the guided light (at a different angle), which is collected with large plastic optical fibbers. Electronics controls the laser wavelength modulation and amplifies the detected signals while a computer does the data acquisition and processing [31].

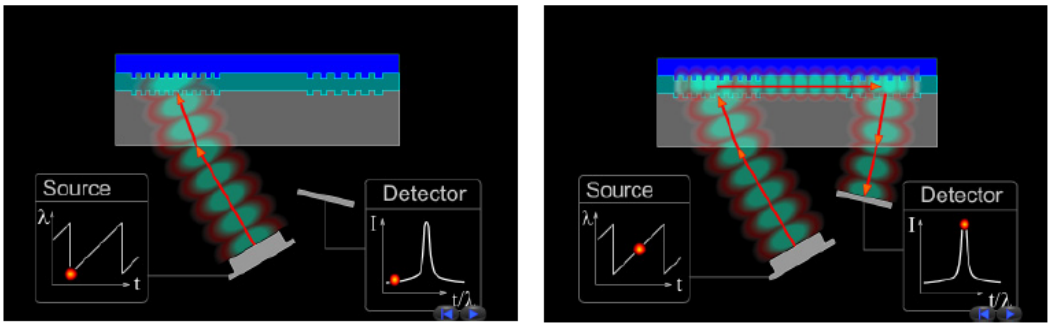


Figure 5.3: Scheme of WIOS detection principle. The sensor chip is a waveguide structured with two gratings. A wavelength tuneable laser is used as source while a photodiode as detector. If the wavelength does not match the grating resonance, no light enters the waveguide (left graph). Upon resonance, light is coupled into the waveguide and can be detected (right graph). Rapid wavelength scanning reveals the complete resonance peak. Finally, analyte solution properties applied to the chip surface are observed by measuring peak position

Thus, the resonant coupling occurring at the first grating is governed by the following grating equation:

$$\lambda_r(t) = \Lambda[n_e(t) - \sin(\theta)]$$

with λ_r as the resonance wavelength for which coupling occurs; n_e as the effective refractive index of the waveguide, θ as the incident angle and Λ as the period of the grating. In this case, for a given optical configuration (θ, fixed), monitoring λ_r will give access to effective index variations of the waveguide, and consequently give access to bulk refractive index variations of the liquid in the fluidic cell or/and to the thickness and refractive index of a sensing layer deposited on the waveguide.

5.1.2.2 Instrument

The developed system consists of a small benchtop device (22 x 10 x 10 cm^3) with the optical system and electronics assembled (see **Figure 5.4**). Data acquisition is made with a personal computer via a data acquisition card. The optical system is composed of a VCSEL laser with a tuneable wavelength range of 2 nm, an actuated rotating mirror for adjusting the coupling angle and a fibber ribbon for collecting the light coming out of the different transducers (pads).

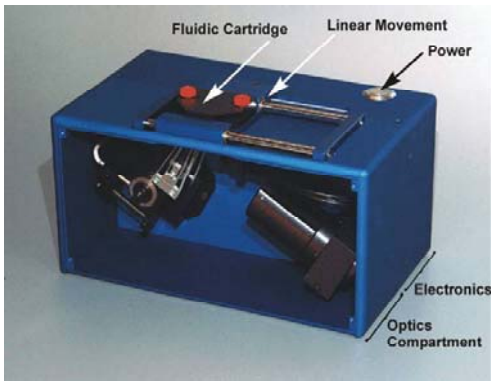


Figure 5.4: CSEM's WIOS research instrument.

Electronics box consists of the temperature regulation for the laser diode and a current oscillator to create the wavelength modulation. The chip holder and sample container is positioned on top of the system. Static and fluidic cells can be adapted on the system.

5.1.2.3 Sensing layer

Waveguide chips used to perform all the experiments were supplied by Oerlikon (Balzers - Lichtenstein.), and consisted in 17.5 x 17.5 x 0.7 mm^3 glass slides with several pads at the surface (grating periodicity of each pad, 360 nm), coated with a 150-300 nm layer of tantalum oxide (see **Figure 5.5**).

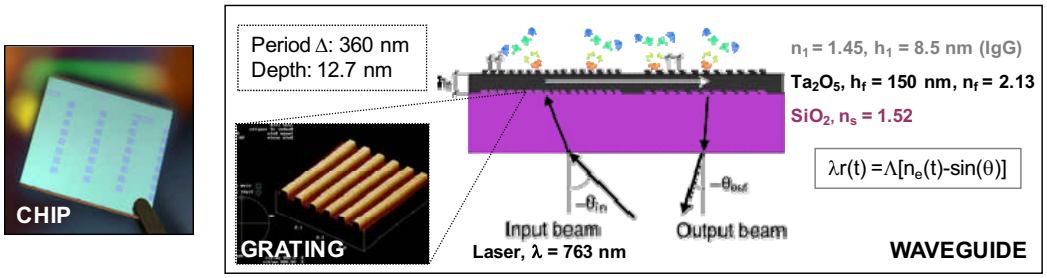


Figure 5.5: Picture of the chip. On the right there is a scheme of the design of the waveguide and grating applied to the chip configuration and subsequent fabrication.

Each chip comprised an array of 24 independent sensing zones (8 pads distributed in 3 different columns). These chips are introduced in a solid plastic support (up and down covers) together with a silicone tape mask, to separate physically different sensing areas (see **Figure 5.6**). Then, this solid support, including all pieces assembled, is fixed magnetically to the top of the integrated WIOS system. Measurements were made using stop-flow mode for all assays.

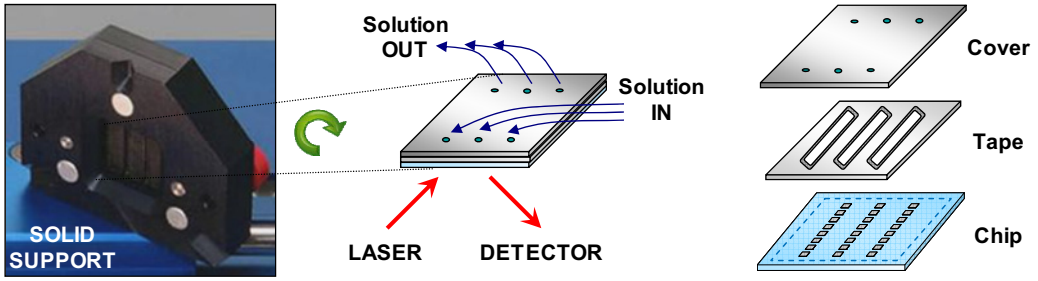


Figure 5.6: Picture of the solid support used during the experiments to fix the chip. Furthermore, a schematic representation of the other components required to assemble successfully the chip to the WIOS instrument.

The chips used in this work allowed either physisorption or covalent immobilization of the recognition biomolecules to the sensing surfaces. Binding events of the immobilised receptor were detected as a change in the refractive index at the surface of the waveguide producing thus a variation on the λ_r according to the equation in **Section 5.1.2.1**. Molecules with molecular weights from many thousands to a few hundred Daltons could in principle be measured [13, 31], although for our purposes we decided to use competitive configurations due to the small size of the antibiotics.

The present chapter describes the work performed in relation to the first steps taken to achieve the **Specific Objective 4** (i.e. integration of bioreceptors on the WIOS transducer and its performance in milk samples) of this thesis in respect to SAs. Considering the precedents mentioned above, in the present chapter we describe the work performed addressed to demonstrate the possibility of using the WIOS system as a biosensor device (WIObS). According to the general objective of this thesis, the WIOS system was envisaged as an attractive platform due to the fact that the chip contained 24 sensing pads which, in case this platform would show the necessary features, could open the possibility to use this platform as a system for simultaneous determination of multiple antibiotics. Therefore the main objectives addressed have been a) to implement the SAs immunoreagents in the WIOS detection platform and to prove suitability as biosensor

device (WIObS), and particularly to quantitatively analyze small organic molecules such as the SA antibiotics, b) to establish the optimal conditions to perform reliable and repetitive measurements of SAs with the same chip and, c) to assess performance of the SAs WIObS analyzing milk samples without or minimum sample treatment and in compliance with the EC regulations (see **Section 5.2)**.

5.2 WIObS for detection of SAs in milk

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Waveguide interrogated optical *immuno*sensor (WIOS) for detection of sulfonamide antibiotics in milk

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ABSTRACT

An immunosensor was developed for the detection of sulfonamide antibiotics in milk. Detection relied on a competitive immunoassay format, using immunoreagents previously developed for the generic detection of sulfonamide antibiotics and evaluated by enzyme-linked immunosorbent assay. The immunoassay was implemented onto a microsystem platform, the wavelength interrogated optical sensing device, which uses the evanescent field to probe changes at the interface of a waveguiding layer, and thus allows sensitive detection of biomolecule adsorption. The immunoreagents were immobilized onto the surface of the waveguide chip, and a fluidic cell allowed flowing analyte and detection solutions above the surface. Sulfapyridine was used as reference sulfonamide and detected with the immunosensor in buffer and in milk with a limit of detection (IC_{90}) of $0.2 \pm 0.1\ \mu g\,L^{-1}$ and $0.5 \pm 0.1\ \mu g\,L^{-1}$, respectively. The analysis time was below 30 min, including regeneration of the sensing surface, with minimum sample preparation required. The reproducibility of the detection was better than 10%. A blind assay allowed identifying milk samples that were contaminated with different sulfonamide antibiotics at or above the maximum residue limits established by the European Union for these compounds ($100\ \mu g\,L^{-1}$). Thus, the developed immunosensor presents great potential as a generic sensing device for the fast and early detection of food contaminants on the field by non-skilled users.

1. Introduction

Early detection of contaminants in food requires fast, cost-effective and reliable analytical methods that should ideally be accessible to non-skilled users. Sulfonamides consist in a family of broad-spectrum synthetic bacteriostatic antibiotics that are widely used in veterinary medicine as feed additives in most European countries (Raich-Montiu et al., 2007). Consequently residues of sulfonamides can be present in food products of animal origin, particularly in cow milk and other dairy products. A maximum residue limit (MRL) for sulfonamides has been established by the authorities to protect the consumer's health, e.g. against bacterial resistance (Kelly et al., 2004; Wegener, 2003). The European Commission (EC Regulation 2377/90) has thus set up the MRL for sulfonamides to 100 ppb ($\mu g\,L^{-1}$) (EC (18 August 1990)). As a consequence, suitable analytical methods have been developed for the detection of sulfonamide antibiotics in milk. High-performance liquid chromatography (HPLC) (Furusawa, 2003; Stoev and Michailova, 2000), gas chromatography–mass spectrometry (GC–MS) (Reeves, 1999) and enzyme-linked immunosorbent assay (ELISA) (Cliquet et al., 2003) are sensitive and specific techniques commonly used for the detection of sulfonamide, although they are laborious, expensive and require trained personnel. More recently, immunochromatographic assays relying on lateral flow on dipstick formats have led to fast and simple detection of sulfonamides (Wang et al., 2007). Alternatively, the Copan Milk Test (CMT), a microbial inhibition assay, allows sensitive detection of a wide range of antibiotics, including sulfonamides (Le Breton et al., 2007).

The investigation of new sensing principles for the detection of molecular binding events has created great expectations in the clinical and food safety areas. The combination of the advances in micro/nanoelectronics with the potential of the immunochemical methods has allowed developing biosensors that provide interesting advantages as alternative analytical methods (Adanyi et al.,

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[1] Contribution of these authors has been 50%.

2006; Baeumner, 2003). Traditionally, immunoanalytical methods have relied on the use of labels covalently linked to the molecules, allowing detection of molecular recognition event with a high detectability. Such methods however requiring labelling, leading to extra costs and complications. Thus, the development of label-free immunosensing devices may improve speed and analytical efficiency. The most widespread label-free detection systems found in the market are based on the surface-plasmon resonance (SPR) phenomena (Pattnaik and Srivastav, 2006) of a gold film (e.g. Farre et al., 2007) or of gold nanoparticles (LSPR, localized surface-plasmon resonance, e.g. Kreuzer et al., 2006). Another way is to use the evanescent wave of a dielectric waveguide, compared to SPR it offers the possibility to measure the interaction at the waveguide surface with several waveguide modes of different polarization (Nellen and Lukosz, 1991) and therefore the possibility to determine several parameter simultaneously (refractive index and thickness of the layer at the interface). Another advantage with waveguide is the freedom with the optical structure design that allows adjusting the evanescent wave to the thickness of the layer to monitor (Horvath et al., 2005; Kunz and Cottier, 2006). Other optical detection methods relying on evanescent wave sensing with grating couplers are comparable to SPR. The analysis of sulfonamides using the evanescent wave has been recently described by Kim and co-workers. An optical waveguide lightmode spectroscopy (OWLS)-based biosensor has been developed to detect sulfamethazine, by immobilizing an antibody on the surface of the chip, reaching a limit of detection (LOD) of 3 $\mu g\,L^{-1}$ in buffer, with an analysis time around 10 min (Kim et al., 2008). Alternatively, non-optical methods such as quartz crystal microbalance (QCM) (Ricci et al., 2007) and impedance spectroscopy (Garifallou et al., 2007; Ramón-Azcón et al., 2008) are interesting technologies for label-free detection (Gauglitz, 2005).

Many optical methods use the evanescent field to probe changes in the refractive index at the transducer–liquid interface, using different optical configurations (e.g. total internal reflection in waveguides or surface plasmons). In this sensing approach, the part of the electromagnetic field associated to the propagating guided light penetrates into the sensing area allowing the detection of biomolecular interactions at the surface of the waveguide. Adsorption of biomolecules at the surfaces changes the refractive index at the interface, affecting the propagation of the light. An elegant way to monitor these optical changes is to combine a single mode waveguide and a grating coupler. In this case the refractive index change will affect directly the coupling condition into or from the waveguide (Cottier et al., 2006; Tiefenthaler and Lukosz, 1989). Different configurations have been used, from monitoring the input coupling angle, output grating angle, coupling position when using a chirped grating and resonance wavelength interrogation (Kunz and Kempen, 1994; Lukosz et al., 1991). In the wavelength interrogation approach, the resonance is scanned by observing the resonance wavelength at a fixed angle of incidence, therefore the mechanical scanning adjustment during the measurements is not necessary. This type of sensors has been shown to allow monitoring biorecognition events occurring at the surface (Cottier et al., 2003; Gauglitz, 2005; Kim et al., 2007). Recently this wavelength interrogation approach was extended to the measurement of several modes combining therefore all the advantages of the dielectric waveguide (Nielsen et al., 2006).

CSEM has developed a label-free detection technology (WIOS), based on a waveguide grating coupling with wavelength modulation (Wiki and Kunz, 2000). The wavelength at which the resonant coupling occurs depends on the refractive index and thickness of the layer formed during the binding of the target on the waveguide grating. By monitoring the resonance wavelength at which light coupling into the waveguide occurs, extremely small optical changes at the sample waveguide interface can be observed with high sensitivity (Cottier et al., 2003). An immunoassay has been previously developed for the generic detection of sulfonamide antibiotics, relying on class-selective antibodies and immunoreagents specifically developed for the sulfonamide family, and tested previously with an ELISA (Adrian et al., 2009). The immunoreagents are selective to the 4-aminobenzensulfonamide functionality, common for all sulfonamides. Differences among sulfonamides lay in the heterocycles attached to the N1-position of the sulfonamide bridge.

Here we present the use of the recently developed wavelength interrogated optical sensing system, composed by a waveguide and a grating, for the analysis of sulfonamide antibiotic residues. Details of the immunoassay implementation on the system together with experimental results for the measurement of different antibiotics from the sulfonamide family in buffer and milk samples are reported.

2. Experimental section

2.1. Materials

Chemicals were obtained from Sigma–Aldrich (Buchs, Switzerland). The immunoreagents used in this study (As155 and SA2-OVA) were prepared as described previously (Adrian et al., 2009; Font et al., 2008). Polyclonal As155 was obtained from rabbits immunized with *5-[6-(4-amino-benzenesulfonylamino)-pyridin-3-yl]-2-methyl-pentanoic acid* (SA1) covalently coupled to horseshoe crab hemocyanin (HCH). As155 was purified, by ammonium sulfate precipitation followed by affinity chromatography using a HiTrap Protein A HP column, before being used in the WIOS system (Baines and Thorpe, 1992). SA2-OVA consists in *5-[4-(amino)phenylsulfonamide]-5-oxopentanoic acid* coupled to ovalbumin (OVA) by the active ester method. Sulfapyridine (SPY) was supplied by Riedel-de-Haën (Sigma–Aldrich, Buchs, Switzerland). Stock solutions containing each sulfonamide at a concentration of 10 mM were prepared in dimethyl sulfoxide. Anti rabbit IgG (α-rIgG) was purchased at Jackson ImmunoResearch (Suffolk, England). All experiments were performed using phosphate-buffered saline (PBS, 10 mM phosphate buffer, 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl), pH 7.4. Commercial, pasteurized milk (Cremo, Switzerland) was used for the test experiments with milk, whereas lyophilized uncontaminated and contaminated fresh milk samples were supplied by Nestlé (Nestlé Research Center, Switzerland).

2.2. WIOS instrument

The wavelength interrogated optical sensing (WIOS) technique uses the evanescent field of light propagating in a single mode waveguide to probe changes in the refractive index at the surface of the waveguide, upon adsorption of biomolecules from solution on the surface (Cottier et al., 2003). The resonance condition of a waveguide grating is scanned by wavelength modulation of a vertical cavity surface emitting laser (VCSEL) emitting at 763 nm and with a tunable range of 2 nm allowing a refractive index change measurement of 14×10^{-3} (Cottier et al., 2003). For a given optical configuration (fixed incidence angle), the collimated light of the VCSEL is incident on a first grating. At a specific wavelength (resonance wavelength), the waveguide mode is excited and propagates into the waveguide layer (Fig. 1). The second grating couples out the guided light, which is collected with large plastic optical fibers and guided onto photodiodes. The peak intensity during a wavelength scanning is used to determine the resonance wavelength, which is translated into "WIOS signal" (the 2 nm scanning range corresponding to 330 "WIOS units"). Its monitoring gives access to effective index variations of the waveguide mode, and consequently allows real-time monitoring of the binding of non-labelled

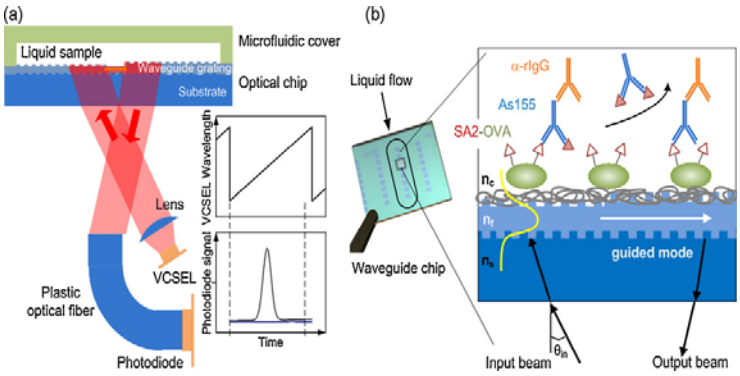

Fig. 1. Working principle of the WIOS instrument: (a) optical scheme showing the incoming tunable light from the VCSEL, the detection photodiode, and the structure of the waveguide chip with the gratings; a flow cell is mounted on top of the waveguide and allows flowing the liquid sample; (b) waveguide chip with the flow path of the liquid over the eight measurement pads, and sensing chip surface for the competitive immunoassay, with immobilized immunoreagents; the input and output gratings on the waveguide chip allow coupling of the light in the waveguide.

molecules on the waveguide grating surface. Combining the optical system with a fluidic cell, consisting on three 7.2 µL channels, *in situ* measurements can be performed, and allow following kinetics of biomolecule adsorption on the surface, with a sensitivity smaller than 1 ng cm^{-2} (<1/100th of a protein monolayer for large proteins (MW > 20 kDa)). The WIOS instrument used for the measurements was fabricated at CSEM (Neuchâtel, Switzerland), and the data were analyzed with CSEM proprietary software DataView 0.86. The WIOS technology is now commercialized by Dynetix AG (Landquart GR, Switzerland) (BR-8 instrument).

2.3. Surface functionalization

Waveguide chips were supplied by Oerlikon (Balzers, Lichtenstein), and consisted in 17.5 × 17.5 × 0.7 mm^3 glass slides with a grating at the surface (periodicity 360 nm), coated with a 150–300 nm layer of tantalum pentoxide (Kunz and Cottier, 2006). The surface of the chips was functionalized with a polymeric layer, OptoDex® (Arrayon Biotechnology SA, Neuchâtel, Switzerland), a photopolymerizable dextran layer that allows covalent binding of biomolecules after exposure to UV light (Barie et al., 1998; Caelen et al., 2002). In addition, the OptoDex® layer prevents non-specific adsorption from molecules in solution. The dry thickness of the OptoDex® was estimated to ~20 nm with XPS and ellipsometry (unpublished data). As the waveguide chips contain eight distinct measurement zones, different biomolecules can be spotted on each separate measurement area, using a Nanoplotter™ NP 2.0/E (GeSiM, Dresden, Germany) (Angeloni et al., 2005). The SA2-OVA coating antigen was covalently bound to the surface, by spotting 50 nL of a 100 µg mL^{-1} SA2-OVA solution in 1/100 PBS (1% (v/v) dilution of PBS in water), and exposing the surface to UV light.

2.4. WIOS measurements

As the WIOS response signal is proportional to the mass coverage of the biomolecule on the sensor surface (Cottier et al., 2003; Kunz et al., 1994), direct detection of small biomolecules (MW < 1 kDa) is difficult (less mass coverage for a monolayer). Therefore a competitive assay format was considered for the detection, with the hapten conjugate (SA2-OVA) immobilized on the chip surface and the antibiotic together with the antibody (As155) in solution, monitoring the immunoassay *in situ* with the WIOS instrument. The chip was mounted in the flow cell and placed on the WIOS instrument. Measurements were made using stop-flow mode for all assays. The cell was equilibrated with PBS buffer (80 µL) for 5 min. Solutions (80 µL) containing a mixture of sulfonamide standard (SPY 0.01– 10,000 µg L^{-1}) and As155 (50 µg mL^{-1}) in PBS were injected and incubated on the cell for 5 min. The chip surface was rinsed with PBS (80 µL) for 5 min and a solution of α-rIgG (50 µg mL^{-1} in PBS, 80 µL) was flown and incubated for another 5 min. After rinsing with PBS the signal variation before and after injection of α-rIgG was recorded. The active surface was regenerated after 5 min exposure to 200 mM NaOH (80 µL) followed by PBS (80 µL) before starting a new measurements cycle. The total analysis time, including the regeneration step, was about 30 min. The variation in the WIOS signal related to adsorption of α-rIgG was recorded, and the response signal observed with SPY standards at various concentrations allowed establishing a calibration curve, where the measured values were adjusted to a four-parameter equation according to Eq. (1):

$$y = \left(\frac{A - B}{[1 - (x/C)^D]} \right) + B \quad (1)$$

where A is the maximal WIOS signal, B the minimum WIOS signal, C the concentration producing 50% of the maximal WIOS signal and D is the slope at the inflection point of the sigmoid curve. The fit was done using the software GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). Quantification of the samples was performed using this calibration curve after recording a reference measurement at zero concentration of the analyte. This allowed for variations between chips in the absolute signal.

2.5. Evaluation of the WIOS system for the analysis of milk samples

In a first stage, matrix effects in milk were evaluated by comparing the maximal signal response obtained from samples prepared in buffer with the ones obtained with milk following the same analysis procedure. Commercial milk was used for the tests, following the same procedure as described above, but increasing the incubation time for the competition step to 10 min and changing the PBS rinsing step to two injections of 2.5 min each (total 80 µL).

2.5.1. Accuracy studies

Spiked milk samples were prepared by diluting SPY in commercial milk, and measured by the WIOS system. The resulting response signal was normalized to the response obtained by the reference free-antibiotic milk sample. Comparison with the calibration plot allowed quantifying the antibiotic concentration in these samples and assessing the assay sensitivity.

2.5.2. Blind samples measurements

Final evaluation of the WIOS was performed using blind samples of fresh milk spiked with different concentrations of various sulfonamide antibiotics supplied by the Nestlé Research Center. Due to the excellent detectability achieved by the immunosensor, sulfonamide references, initially prepared in milk at 100 $\mu g\,L^{-1}$, as well as the milk blind samples, were diluted 20 times (sulfonamide at 5 $\mu g\,L^{-1}$) with PBS to ensure measurements within the dynamic range of the immunosensor. Detection of various sulfonamide antibiotics allowed assessing the assay specificity.

3. Results and discussion

An immunosensor for the detection of sulfonamide antibiotics was developed using an indirect competitive format, combining immunoreagents developed for sulfonamide antibiotic detection (Adrian et al., 2009; Font et al., 2008) and the WIOS system based on optical detection of biomolecule adsorption at the surface (Fig. 1). The immunoreagents were developed with the objective to detect a significant number of sulfonamide congeners used as antibiotics in the veterinary field. Class-selective antibodies against sulfonamides and immunoreagents were prepared by designing an immunizing hapten based on chemical criteria and on the information extracted from theoretical models and calculations performed to assess suitability of the proposed immunizing hapten chemical structure to mimic most of the sulfonamide congeners (Ballesteros et al., 1998; Galve et al., 2000; Spinks et al., 1999). Thus, the antibodies were widely evaluated with ELISA techniques in various media, such as milk (Adrian et al., 2008; Font et al., 2008) and hair samples (Adrian et al., in press, accepted manuscript; Font et al., 2008). In the WIOS instrument, adsorption of biomolecules onto the surface results in a shift in the resonance wavelength at which light coupling into the waveguide occurs. Monitoring of the resonance wavelength allows real-time monitoring of the binding of non-labelled molecules on the waveguide grating surface.

3.1. Immunoassay

The immunoreagents were implemented in the WIOS detection system by covalently immobilizing the SA2-OVA hapten conjugate onto the surface of waveguide chips, using the Optodex® surface chemistry. Quantitative detection of sulfonamide antibiotics was performed after exposure of the surface to a mixture of antibody and analyte for 5 min, followed by exposure to α-rIgG, which amplified the response of the immunosensor, thus increasing the sensitivity and improving the reliability of the assay. A complete WIOS competitive measurement in real-time is shown in Fig. 2. The presence of antibiotic in solution results in a decrease of the maximum signal observed after exposure to α-rIgG in the absence of antibiotic. Optimum concentrations for As155 and SA2-OVA were selected through two-dimensional checkerboard experiments by measuring the binding of several antibody solutions (1–1000 $\mu g\,mL^{-1}$ in PBS) to the surface chips functionalized with four different concentrations of SA2-OVA (5–100 $\mu g\,mL^{-1}$, 50 nL spotted on 1 mm^2) (data not shown). Due to variations from chip to chip, the signal response was always related to the maximum reference response signal observed in the absence of antibiotics. The absolute value for the absolute signal varied between 6 and 18 WIOS units [a.u.] for all measurements. As mentioned before, the WIOS system uses the evanescent field of light to probe changes in the refractive index at the surface of a waveguide (Cottier et al., 2003) by scanning the resonance condition of the waveguide grating through the wavelength modulation of a laser diode. Monitoring of the resonance wavelength allows real-time monitoring of the binding of non-labelled molecules on the waveguide grating surface. As it can be observed in the sensogram shown in Fig. 2, binding of the As155 to the SA2-OVA functionalized surface chip was evidenced by a shift in the wavelength at which resonance condition occur. The use of α-rIgG (optimized to 50 $\mu g\,mL^{-1}$ in PBS) amplified the response of the immunosensor allowing to increase sensitivity and therefore to improve reliability.

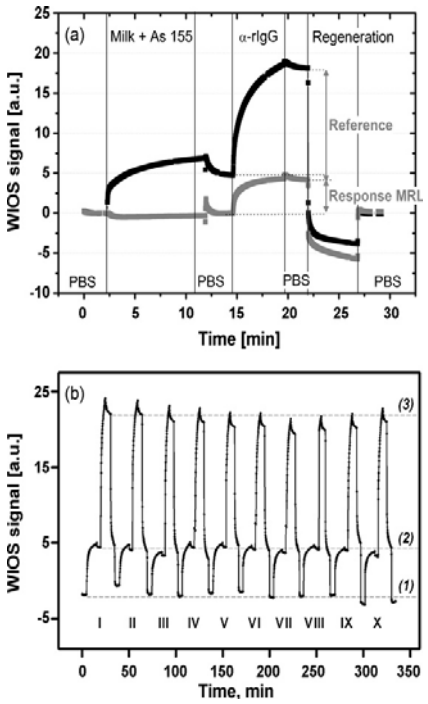

Fig. 2. (a) Typical WIOS adsorption curve showing the competition between the immobilized hapten (SA2-OVA) and the antibiotic (SPY) in solution with the specific antibody (As155). Adsorption of the secondary antibody (α-rIgG) provides an amplified response signal, which is inversely proportional to the concentration of antibiotic in solution. Exposure to 200 mM NaOH in the end of the assay allows regeneration of the active surface. The upper curve shows the sensogram recorded when an antibiotic free sample was injected. The lower curve corresponds to the sensogram recorded when a milk sample spiked with SPY at the MRL concentration was injected. The absolute values may vary from chip to chip. (b) WIOS response for ten consecutive measurements (I to X) after regeneration of the chip surface with 200 mM NaOH: baseline level (*1*), after sample injection (*2*), and response after α-rIgG injection (*3*).

3.2. Surface regeneration

Regeneration is crucial as it allows using the same chip surface several times, not only decreasing the assay cost but reducing the need for calibration after each measurement. Regeneration of the active sensor surface aimed at removing the antibodies interacting with the antigen SA2-OVA printed on the chip while preserving the functionality of the surface layer. Regeneration of a single chip surface was studied by performing several consecutive measurement cycles and testing different agents. Efficient regeneration was achieved with 5 min exposure to 200 mM NaOH, showing no significant variation in the immunosensor response after regeneration (≤10% variation in the subsequent cycles).[2] No apparent degradation of the surface was observed after at least ten measurement

[2] Greater differences (up to 30% variations) were observed between different chips, most likely related to the chip surface chemistry and to the biomolecule spotting method. These deviations could be corrected by performing reference measurements on each chip area.

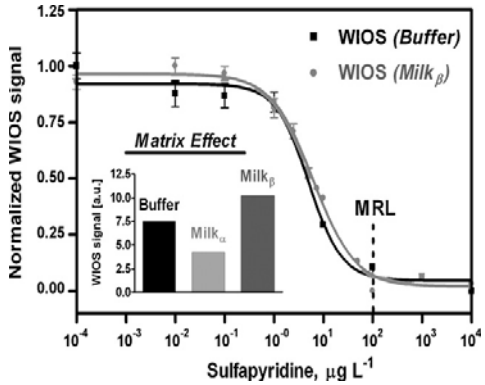

Fig. 3. Normalized calibration curves for the detection of SPY in buffer and in milk performed with the WIOS instrument. The inset graph shows the decrease of the maximal signal observed in the absence of antibiotic in milk compared to PBS, for the same conditions (milk$_\alpha$). The standard curve in milk was obtained after optimization of the analysis procedure, increasing the competitive step to 10 min (milk$_\beta$). The IC_{50} values in PBS (5.1 µg L^{-1}) and in milk (6.3 µg L^{-1}) are below the MRL of 100 ppb (100 µg L^{-1}) for sulfonamide antibiotics.

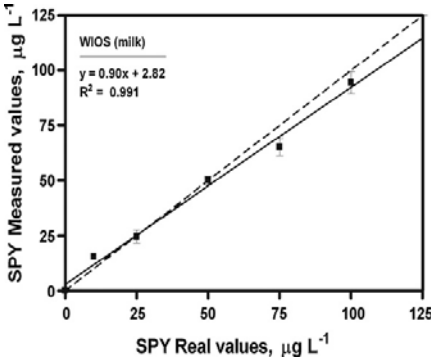

Fig. 4. Results from the accuracy studies for the detection of SPY in milk, showing the correlation between the spiked concentration (*x*-axis) and the concentration measured with the WIOS system (*y*-axis). The dotted line corresponds to a perfect correlation (slope = 1).

cycles using the treatment selected (WIOS signal due to binding of α-rIgG = 16.7 ± 0.9 [a.u.], *n* = 10), confirming full activity of the surface-immobilized SA2-OVA hapten conjugate (Fig. 2b). Complete removal of adsorbed As155 was verified with the absence of interaction of the regenerated surface with α-rIgG (WIOS signal variation = 0.7 ± 0.1 [a.u.], *n* = 10).

3.3. WIOS calibration for sulfonamide detection

A calibration curve was performed to relate the sensor response to the antibiotic concentration. In a first stage, a series of measurements was performed in PBS containing various concentrations of SPY (representative sulfonamide antibiotic selected as reference). The signal related to binding of α-rIgG was plotted as function of the SPY concentration, and fitted to Eq. (1). The response signals were normalized to the maximum signal, obtained in the absence of antibiotics (Fig. 3). The IC_{50} and LOD values in buffer were respectively 5.1 µg L^{-1} ± 0.6 and 0.2 µg L^{-1} ± 0.1 (Table 1). Each measurement constitutes an average of at least six measurement zones on the same chip. Although variations were observed in the absolute signal, the inhibition features were reproducible. The response of the immunosensor was very similar to that of the ELISA using the same bioreagents (Adrian et al., 2009). Specificity of the assay with other sulfonamide antibiotics was shown with ELISA, allowing detection of 10 structural related compounds below the MRLs. Detection of sulfamethazine (SMZ), sulfathiazole (STA) and sulfachloropyridazine (SCP) is demonstrated in a following Section with WIOS, showing comparable results to the ELISA. Generic detection of sulfonamides with a biosensing device had not yet been shown. Similar studies such as detection of sulfamethazine with optical waveguide lightmode spectroscopy (OWLS) (LOD 3 µg L^{-1} in buffer) was limited to one sulfonamide antibiotic (Kim et al., 2008).

Table 1
Features of the SPY WIOS assay in buffer and milk samples after evaluation.[a]

Parameter	WIOS	
	Buffer	Milk
$Signal_{min}$[b]	0.05 ± 0.03	0.02 ± 0.02
$Signal_{max}$[b]	0.92 ± 0.03	0.96 ± 0.02
Slope	−1.16 ± 0.35	−1.04 ± 0.03
IC_{50} (µg L^{-1})	5.1 ± 0.6	6.3 ± 0.6
Dynamic range (µg L^{-1})	0.7–21.0	1.4–26.4
LOD (IC_{90}) (µg L^{-1})	0.2 ± 0.1	0.5 ± 0.1
R^2	0.961 ± 0.008	0.980 ± 0.004

[a] Values obtained correspond to the average and standard deviation of 6-pad replicates from the same chip.
[b] Normalized immunosensor signals.

3.4. Analysis of milk samples

In order to assess the detection sensitivity in milk, a calibration curve in milk had to be established, taking into account possible matrix effects. A significant decrease in the response signal was observed in milk compared to the assays in PBS (Fig. 3, inset graph; milk$_\alpha$), which could be corrected by increasing the contact time for the competitive step to 10 min (Fig. 3, inset graph; milk$_\beta$). This decrease was attributed to matrix effects from the milk, which contains various species, possibly interfering with the competitive reaction. Therefore, a longer incubation time was required for the competitive step. A new calibration curve was established by diluting SPY in antibiotic-free milk, showing that milk samples could be quantified without any pre-treatment (Fig. 3). The LOD obtained for SPY in milk was 0.5 ± 0.1 µg L^{-1}, and the IC_{50} 6.3 ± 0.6 µg L^{-1} (Table 1), which are comparable to the measurements in buffer, and allow detection below the MRLs established by the European Union for sulfonamide in milk.

3.5. Accuracy studies

As the IC_{50} values obtained from the calibration curves are far below the MRLs for sulfonamide antibiotics (100 µg L^{-1}), milk samples were diluted 20 times in PBS buffer in order to allow detection at the MRLs. SPY spiked milk samples were measured with the immunoassay in the WIOS instrument as reported in the experimental section. The results are reported in Fig. 4, showing a good correlation between spiked measured values (correlation coefficient R^2 = 0.991) indicating the good accuracy of the method to analyze milk samples.

3.6. Evaluation of the WIOS system with blind samples

Finally, a preliminary evaluation of the WIOS system for the detection of sulfonamide antibiotic residues was performed with a set of blind milk samples spiked with different sulfonamides, at the Nestlé Research Centre (Lausanne, Switzerland), and measured using the immunosensor and SPY as reference. The signal given for each sample was compared to that provided by a sulfonamide

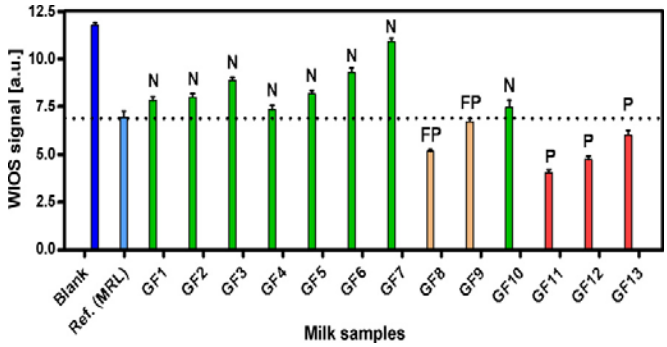

Fig. 5. Evaluation of the WIOS immunosensor as a tool to analyze fresh milk by measuring blind spiked samples prepared at the Nestlé Research Center (Lausanne, Switzerland). Results are expressed as follows: positive (P), negative (N), false positive (FP) and false negative (FN) according to MRLs established for each compound by the EU. Sulfonamide antibiotics spiked in these milk samples were sulfamethazine (SMZ; GF1 = 10 $\mu g\,L^{-1}$, GF4 = 25 $\mu g\,L^{-1}$, GF8 = 50 $\mu g\,L^{-1}$, GF11 = 100 $\mu g\,L^{-1}$), sulfathiazole (STA; GF2 = 10 $\mu g\,L^{-1}$, GF5 = 25 $\mu g\,L^{-1}$, GF9 = 50 $\mu g\,L^{-1}$, GF12 = 100 $\mu g\,L^{-1}$), and sulfachloropyridazine (SCP; GF3 = 10 $\mu g\,L^{-1}$, GF6 = 25 $\mu g\,L^{-1}$, GF10 = 50 $\mu g\,L^{-1}$, GF13 = 100 $\mu g\,L^{-1}$). Sample GF7 was not contaminated with sulfonamide antibiotics (blank). All samples were measured using the same chip surface, with regeneration of the surface.

control at the MRL concentration. After running the assay, the signal given by a particular milk sample was compared with that of the MRL control. Response signals below the control value indicate potential contamination by the sulfonamide antibiotics and *vice versa*. Results shown in Fig. 5 indicate that the WIOS system was able to detect contamination of milk samples with sulfonamides over the MRL in less than 30 min. Detection of four different sulfonamide antibiotics demonstrates the generic specificity of the assay for many common sulfonamides. Whereas there were no false negatives, false positives indicated the presence of the antibiotic, although below the MRLs. The use of standard methods for confirmation would be only necessary for the potential positive samples allowing the discrimination of these false positive results while dramatically reducing the number of analyses.

3.7. *Immunosensor performance*

In summary, an immunosensor for sulfonamide detection has been developed. The features of the antibiotic analysis performed in buffer and milk are not significantly affected as can be observed in Table 1. The assay reproducibility showed less than 10% variation between measurements. Greater variations were observed between different chips (<30%) for the absolute signal values, thus requiring reference measurements for each new chip. Besides, normalization of the signal response to the signal obtained in the absence of antibiotic allows compensating these variations. Reproducibility between the assays could be optimized both by controlling the surface chemistry and improving the fluid homogeneity. Fluidics developments would improve the fluid handling and contribute to the development of a "field" device. Additionally, in order to further ameliorate the assay efficiency, main efforts should be focused in reducing the time analysis from 30 min to 10 min. Nevertheless, compared to current methods used for the detection of contaminated milk, the WIOS system presents great advantages. The WIOS system for sulfonamide antibiotic detection developed here profits from the enhanced freedom design of planar dielectric waveguides, with no mechanical movement during the measurement. The possibility to adapt the penetration depth of the electromagnetic field associated to the guided waves through an appropriate design of the waveguide grating is one of the main advantages of this type of sensor. Moreover, waveguide gratings allow building sensor arrays with a high number of sensing regions on the same chip, thus offering perspectives for multiplexed detection (8 simultaneous measurements), resulting in a cheap instrument adapted for multisensing. The small dimension of the system allows point-of-care analysis with low skill personnel, when combined with a micro fluidic circuit. Consequently, the WIOS immunosensor for antibiotics detection stands as a transition between expensive, accurate laboratory techniques (e.g. chromatography, ELISA), and easy-to-use, non-quantitative field methods (e.g. dipstick).

4. Conclusions

An immunosensor was developed as a tool for the detection of antibiotic residues in the food chain. The immunosensor combines generic immunoreagents developed for the detection of the sulfonamide antibiotic family with a WIOS label-free optical biosensor. Using a competitive format, traces of SPY were detected in buffer and in milk, with a LOD of 0.2 ± 0.1 and 0.5 ± 0.1 $\mu g\,L^{-1}$, respectively (IC_{50} of 5.1 ± 0.6 in buffer and 6.3 ± 0.6 $\mu g\,L^{-1}$ in milk) with a good reproducibility ($\leq$10%). The developed system allowed discriminating between milk contaminated with sulfonamide antibiotics at or above the MRL (100 $\mu g\,L^{-1}$) in less than 30 min, including regeneration of the sensing surface. The WIOS system has shown to be able to perform accurate measurements of sulfonamide antibiotics in milk samples. No sample treatment was necessary to obtain reliable data regarding the potential presence of this family of antibiotics. The WIOS system has shown comparable detection sensitivity and specificity as previously reported ELISA tests, but is more suitable for automated on-site measurements (i.e. at the dairy farm). Research is currently ongoing to improve the design of the fluidic chamber, and reduce the handling of solutions for future use by non-skilled personnel. Additional improvements such as data processing, economical considerations and compliance with the Commission Decision 2002/657/EC are needed for the system to be exploitable in the field and accepted. As waveguide gratings allow building sensor arrays with a high number of sensing regions on the same chip. Thus, further investigations will be addressed to build a multiplexed device able to screen simultaneously the presence of other relevant antibiotics such as fluoroquinolones, β-lactams and tetracyclines in milk and other dairy products.

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5.3 Results and discussion

In this chapter we present the development of a WIObS for SAs in milk samples using the immunoreagents described in **Chapter 3**.

5.3.1 Preliminary work for WIObS experiments

5.3.1.1 Chip functionalization

First attempts for the biofunctionalization of the WIOS chips were performed by immobilizing the biomolecules on the surface by physical adsorption (see **Figure 5.7**) and evaluating the WIOS response on competitive format. For this purpose different SAs As/CAs combinations performing well in the ELISA (see **Chapter 3**), were assessed in the WIOS system by performing experiments in buffer and immobilizing the distinct CAs by physisorption.

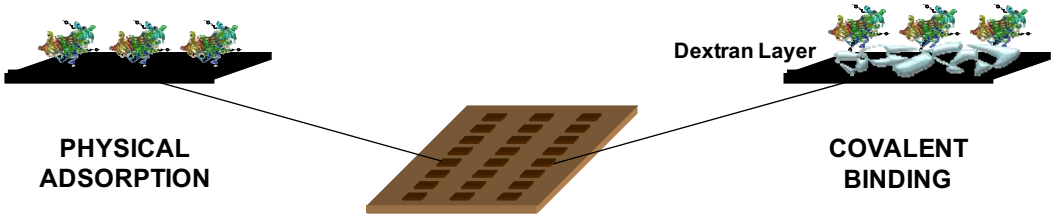

Figure 5.7: Types of chip surface modification.

Based on the results obtained, the As155/SA2-OVA combination was selected for further studies using covalent immobilization. Moreover, this combination had also been evaluated in the microplate-based ELISA which allowed future comparison between both methodologies. Thus, the SA2-OVA antigen was covalently immobilized on the WIOS transducer through a photoactivable dextran polymer (Optodex®), developed by Dr. Hui Gao from Arrayon Biotechnology S.A. (Neuchâtel - Switzerland). Due to the high hydrophilic character of this polymer the nonspecific adsorptions to the sensing area of the chip surface were expected to be minimised [36, 37], while increasing the stability of the sensing layer, opening the possibility to use the same chip in many consecutive analytical cycles. The specific Optodex® technology consisted in polysaccharide dextran matrices combined with secondary chemical functional groups (see **Figure 5.8**). A great number of diverse functional platforms can be generated with the Optodex® matrices, which are commercially available, since they can carry different chemical groups (e.g. carboxyl, amino, thiol, biotin, and lactose or cyanine fluorescent platforms) and proteins [38]. As it can be observed in **Figure 5.8**, the Optodex® used in this work was a dextran

polymer modified with diazirine functional groups, which rely on the generation of carbene groups under the action of light.

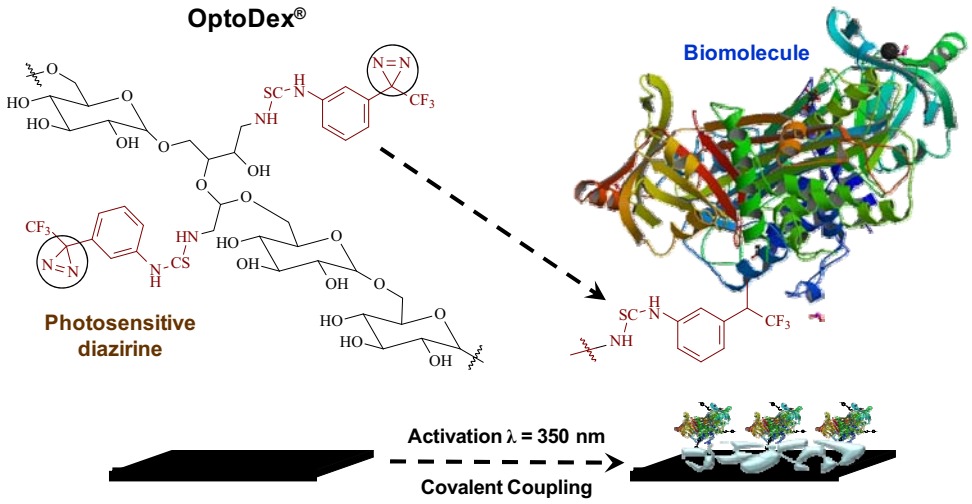

Figure 5.8: Scheme of Optodex® surface coating procedure for covalent immobilization of biomolecules to the chip sensing zone.

Figure 5.9 shows the chemical reactions involved in the procedure. In this way, one side of the polymer is immobilized on the waveguide zones of the chip surface while the other attaches the biomolecules by covalent binding. The advantage of this strategy is the possibility to immobilize the biomolecules on specific sites of the transducer by the action of light, allowing the creation of multiplexed platforms.

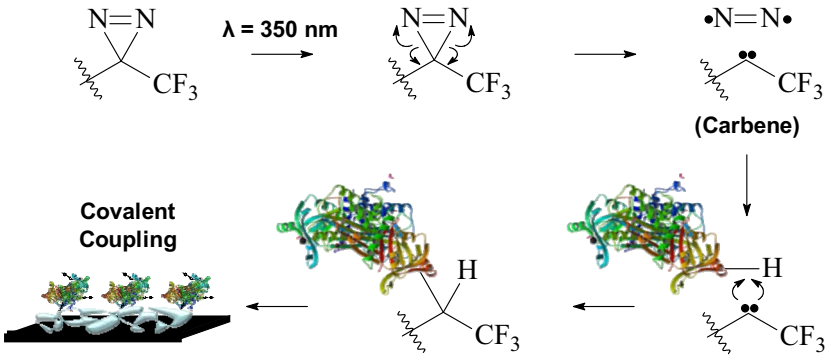

Figure 5.9: Reaction mechanism proposed for carbene formation for subsequent covalent couplings.

5.3.1.2 Biosensor development

Optimum concentrations, for the As155, SA2-OVA and anti-IgG assay components to achieve enough signal variation, were selected through associated 2D checkerboard

experiments by measuring the binding of several antibody solutions to the surface of the chips previously spotted with different concentrations of SA2-OVA in combination with diverse anti-IgG concentrations (see **Figure 5.10**).

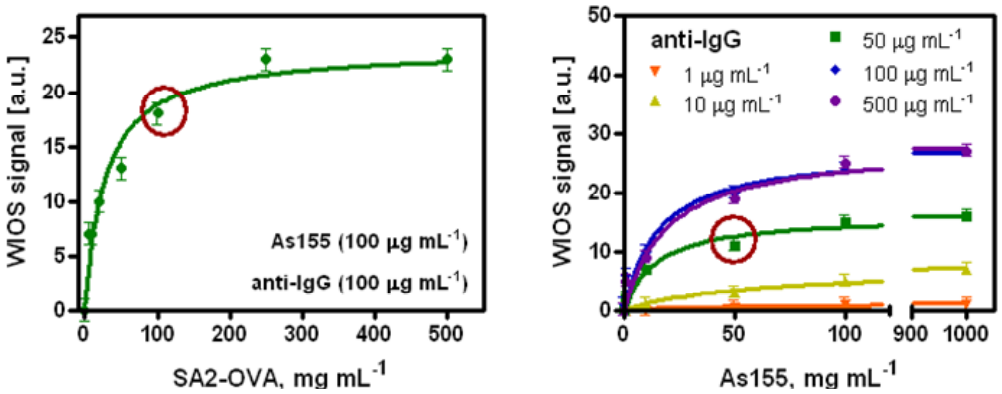

Figure 5.10: Representative 2D experiments performed with WIOS using sulfonamide immunoreagents. Red circle shows the assay conditions chosen.

Optimal concentrations of immunoreagents chosen by using this procedure were subsequently used to build calibration curves for the analysis of SAs in buffer and milk samples. A critical point for all biosensors is trying to regenerate the sensor surface. The method has to be strong enough to remove the antibodies interacting with the antigen printed on the chip but preserving the functionalized surface layer.

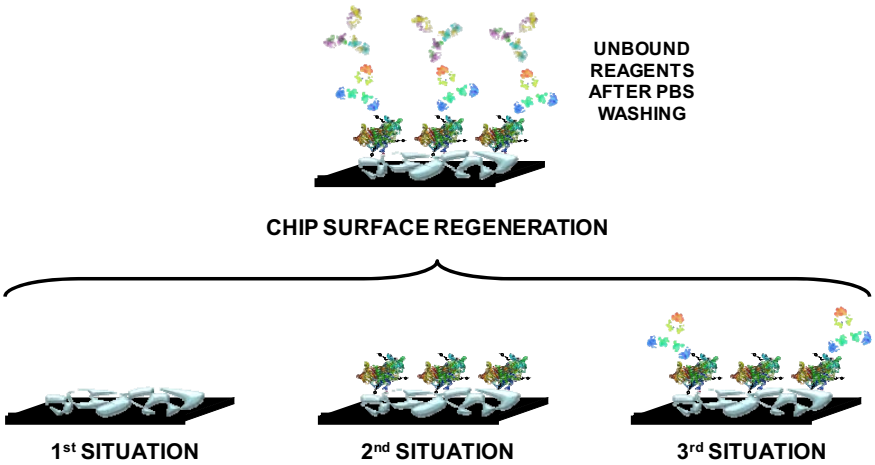

Figure 5.11: Representation of most common possibilities regarding sensing area states after chip regeneration treatment. First situation shows the destruction of the antigen recognition layer (treatment too strong). In the second situation is shown the removal of primary and secondary antibodies keeping invariable the antigen linked to the surface (good regeneration process). Last example explains complete removal of secondary antibody but partial elimination of the primary (treatment too soft).

That simple idea or concept is sometimes limited by the high affinity of the bioreceptors (see **Figure 5.11**). Regeneration of a single chip surface was studied by performing several consecutive measurement cycles and testing different agents (see **Section 5.2**). Finally, 200 mM NaOH was chosen, since no significant variation was observed in the immunosensor responses.

5.3.2 Performance of the WIObS in buffer

A series of consecutive measurements were performed in PBS containing in the competition step As155 and different concentrations of sulfapyridine (0.01 µg L^{-1} – 10000 µg L^{-1}), selected as a representative SAs as reference. Binding of the antibody produced a change in the refractive index on top of the waveguide, which produced ended a change on the wavelength at which the light is coupled on the waveguide. The extent of the wavelength deviation is proportional to the variation of the refractive index as a result of the antibody binding. **Figure 5.12** shows a representative example of the typical WIOS adsorption curve.

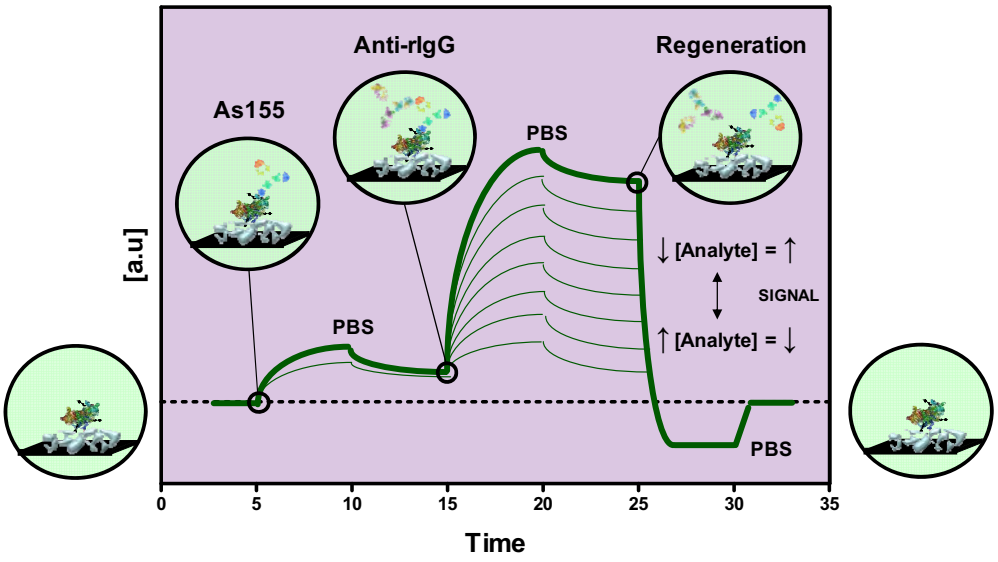

Figure 5.12: A typical binding cycle observed with an optical biosensor. In this case, the SA2-OVA protein is covalently immobilized on the sensor surface. The entire binding cycle is normally repeated several times at varying concentrations of analyte to generate a robust data set for global fitting to an appropriate binding algorithm. Finally, a pulse of a regeneration solution to disrupt binding and regenerate the chip surface. After each step, solutions are replaced again with PBS.

First the sample is mixed with the antibody and subsequently injected into the system. The initial part of the sensogram shows the response resulting from the binding of the primary antibody (As155) to the transducer during competition between the antigen

immobilized covalently and the analyte in solution for the specific antibody (5 min). As it can be observed the shift in the signal produced is very small, therefore it was needed to add a secondary antibody in order to improve the signal. After a washing step (5 min), adsorption of the secondary antibody provides an amplified response signal (5 min), which is inversely proportional to the concentration of antibiotic in solution. The total analysis time including all washing steps and the regeneration process (5 min), before starting a new measurements cycle, was about 30 min. The variation in the WIOS signal related to adsorption of anti-IgG is recorded to be plotted as function of the SPY concentration and fitted to the corresponding $y = (A-B/[1-(x/C^D])+B$ equation showed in **Section 3.1.6** (see **Figure 5.13**).

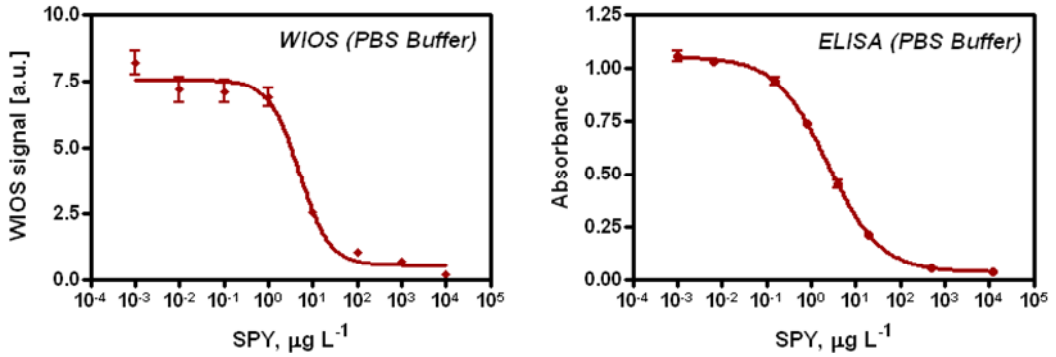

Figure 5.13: Calibration curves for the detection of SPY using As155/SA2-OVA combination in buffer comparing WIOS instrument with the ELISA format. The signal of each standard corresponds to the average and standard deviation of 6-pad replicates from the same chip (consecutive injections of the analyte after regeneration of the surface).

As it can be observed, the response of the immunosensor was very similar to that of the ELISA using the same bioreagents (see **Table 5.1** and **Section 5.2**).

Table 5.1: Comparison of optimum features of the SPY assay, using WIOS and ELISA formats, with As155/SA2-OVA combination, in buffer. [a]Concentrations are expressed in µg L^{-1}.

WIOS Calibration Curve in buffer		**ELISA Calibration Curve in buffer**	
Parameter	**As155/SA2-OVA**	**Parameter**	**As155/SA2-OVA**
A_{max} (WIOS a.u.)	8.2 ± 0.9	A_{max} (Abs)	1.07 ± 0.07
A_{min} (WIOS a.u.)	0.2 ± 0.1	A_{min} (Abs)	0.04 ± 0.02
IC_{50}[a]	5.1 ± 0.6	IC_{50}[a]	2.35 ± 0.72
Dynamic range[a]	0.7 ± 0.1 to 21.0 ± 1.2	Dynamic range[a]	0.4 ± 0.1 to 16.9 ± 3.5
Slope	-1.16 ± 0.35	Slope	-0.73 ± 0.10
LOD[a]	0.2 ± 0.1	LOD[a]	0.15 ± 0.08
R^2	0.986	R^2	0.997

Specificity of the As155/SA2-OVA combination with other SAs was already studied by ELISA (see **Section 3.3**), demonstrating that detection of 10 structural related compounds below the MRLs was possible. On the other hand, further experiments performed later on in milk samples with distinct SAs showed that the recognition pattern was the same as with the ELISA (see **Section 5.2**), demonstrating that the selectivity is mainly determined by the immunoreagents, not by the assay or device configuration.

5.3.3 Performance of the WIObS in milk samples

A slight decrease in the response signal was observed when the assay was run in milk compared to the assay in buffer, which was corrected by increasing the contact time of the competitive step and changing the PBS washing step by rinsing the sensor twice during 2.5 minutes (see **Table 5.2**), instead of just one for 5 min.

Table 5.2: Optimum conditions of the WIOS assay (As155/SA2-OVA) in milk.

Assay Step	Solution Injected (Concentration)	Incubation Time
Equilibration (80 µL)	PBS (10 mM)	5 minutes
Competition in Milk (80 µL)	As155 (50 µg mL^{-1}) SPY samples; ≠ concentration	10 minutes
First Washing (80 µL)	PBS (10 mM)	2.5 minutes
Second Washing (80 µL)	PBS (10 mM)	2.5 minutes
Signal Amplification (80 µL)	Anti-IgG (50 µg mL^{-1})	5 minutes
Washing (80 µL)	PBS (10 mM)	5 minutes
Regeneration (80 µL)	NaOH (200 mM)	5 minutes
Begin the cycle again	**For each point → calibration curve**	**35 minutes**

The calibration curve of SPY prepared using non-diluted whole milk samples showed that measurements could be made without any pre-treatment (see **Figure 5.14**).

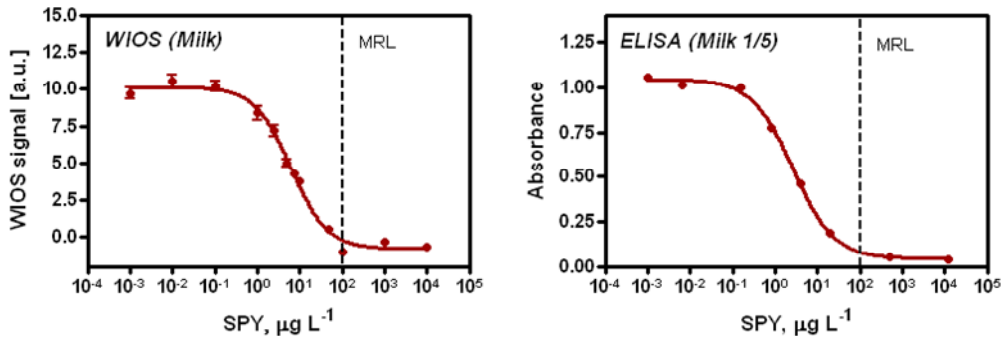

Figure 5.14: Calibration curves for the detection of SPY using As155/SA2-OVA combination in milk comparing WIOS instrument with the ELISA format. The signal of each standard corresponds to the average and standard deviation of 6-pad replicates from the same chip (consecutive injections of the analyte after regeneration of the surface).

Biosensor analytical parameters in milk samples are summarized in **Table 5.3**. SPY could be detected with an IC_{50} of 6.3 μg L^{-1} and a LOD of 0.5 μg L^{-1} which are concentration values quite below the MRLs established by the EU for this antibiotic family in this matrix (MRL for SAs is 100 μg L^{-1}). Milk samples could be measured undiluted by the WIOS system which allowed obtaining better detectability values than with the ELISA format (see **Table 5.3**) since a 1/5 dilution factor had to be applied in this second case.

Table 5.3: Comparison of optimum features of the SPY assay, using WIOS and ELISA formats, with As155/SA2-OVA combination, in milk. [a]Concentrations are expressed in μg L^{-1}.

WIOS Calibration Curve in milk		**ELISA Calibration Curve in milk (1/5)**	
Parameter	**As155/SA2-OVA**	**Parameter**	**As155/SA2-OVA**
A_{max} (WIOS a.u.)	9.2 ± 0.2	A_{max} (Abs)	1.03 ± 0.04
A_{min} (WIOS a.u.)	-0.7 ± 0.2	A_{min} (Abs)	0.04 ± 0.01
IC_{50}[a]	6.3 ± 0.6	IC_{50}[a]	13.0 ± 1.5
Dynamic range[a]	1.4 ± 0.2 to 26.4 ± 0.9	Dynamic range[a]	2.8 ± 0.7 to 59.5 ± 7.0
Slope	-1.04 ± 0.03	Slope	-0.93 ± 0.07
LOD[a]	0.5 ± 0.1	LOD[a]	1.1 ± 0.5
R^2	0.991	R^2	0.997

A preliminary evaluation of the WIObS system was performed by analyzing a set of blind milk samples (see **Figure 5** in **Section 5.2**), prepared at the NRC that had been spiked with different SAs at different contamination levels. The samples were measured and the signal given compared to that provided by SPY used as reference at the MRL concentration (100 μg L^{-1}). Thus, response signals below the control value indicate potential contamination by SAs and *vice versa*. As it is shown in the paper, it is important to notice the lack of false negatives. On the other hand, false positives indicated the presence of the antibiotic, although below the MRLs. At this point, it is important to notice that as generally happens with the immunochemical and other bioanalytical methods, the different cross-reactivity (CR) values of the SAs prevents from providing quantitative values, and the results have to be expressed as SPY IR equiv. (SPY immune reactivity equivalents). Due to the fact the other SAs showed were less recognized than SPY, there exist the risk to miss samples contaminated by these other antibiotic congeners. Therefore, for further studies it was proposed to use other SAs with lower CR as reference in order to ensure avoiding *false negatives*.

By doing this is very much possible that samples contaminated with small amounts of high CR (e.g. SPY) would provide a positive response, even if their concentration was below the MRL, and therefore the sample was compliance with the EC regulations.

However, the biosensor developed here is addressed to perform screening and therefore positive samples will be further analyzed by chromatographic confirmatory methods allow discriminating these false positive results. On the other hand, this selective biosensor method would readily reduce the number of samples to be analyzed by these confirmatory methods, while improving the efficiency of the control due to its higher through-put capabilities. After all these experiments, we can conclude that the good features of the sulfonamide immunoreagents prepared, in combination with the WIOS instrument, has allowed to develop a biosensor device able to perform reliable automatic measurements detecting the presence of antibiotic residues in milk samples without any previous sample clean-up method in less than 30 minutes.

5.3.4 Main contributions

The main contributions of this chapter to the existing knowledge at the time of performing this research are:

- It has been demonstrated for the first time that the WIOS platform can be used as an effective biosensor (WIObS).
- It has been developed for the first time an optical biosensor able to detect up to 11 SAs.
- Probably, it may also be the first biosensor able to analyze directly SAs in milk in accordance with the current EC regulations.

At the time when the current thesis was written, we had only found an example in the literature regarding a biosensor development to detect sulfonamides which was based on a similar detection principle as the one used by the WIOS instrument. The OWLS system was able to achieve a LOD of 3 $\mu g\ L^{-1}$ for SMZ in buffer samples but being not tested in real matrices yet [15].

5.3.5 Further considerations

Generic detection of SAs with a biosensing device has not been reported yet. In our case, the WIObS system has shown comparable detection sensitivity and specificity as previously reported ELISA tests in buffer and milk samples (see **Section 3.3**), but seems to be promising and more suitable for automated on-site measurements (i.e. at the dairy farm or milk tank trucks). In this sense, research at CSEM is currently ongoing to improve the design of the fluidic chamber, and reduce the handling of solutions for future use by non-skilled personnel. From here on, further investigations were addressed to build multiplexed analytical tools and devices able to screen simultaneously the presence

of other relevant antibiotics such as FQs, BLs or TCs in milk. On the other hand, as it was posted in **Section 3.5.7**, the SAs immunoreagents produced along this thesis have been incorporated onto other novel transducers (a part from the WIObS) with the objective of developing new biosensor devices for on-site measurements for the analysis of relevant biological or environmental matrices [39-41].

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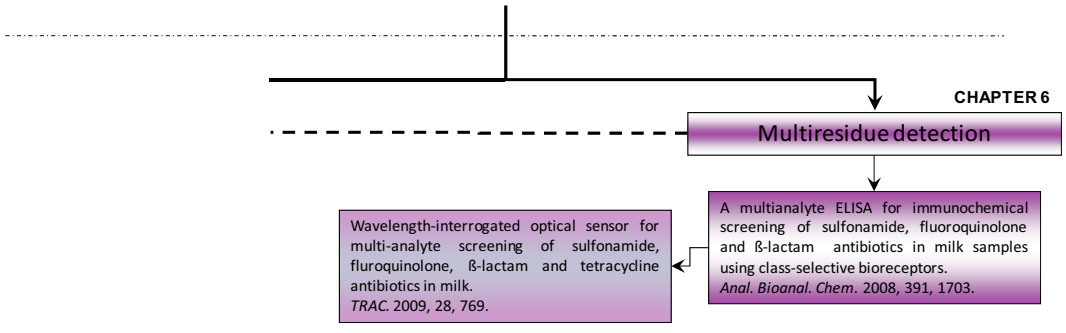
6 MULTIRESIDUE DETECTION



Biological receptor based techniques are founded on the premise that they specifically bind to their target antigens. This interaction allows development of quantitative and qualitative assays for the desired target compounds. Particularly, on antibody based methods, the orthogonal features of hapten–antibody/receptor combinations seem to be ideal for creating array structures with different immunoreagents for different analytes [1, 2]. This kind of parallelization reaches its physical and economical limits with the increasing number of analytes that could be measured at the same time. In this sense, besides of the SAs and TCs immunoassays described in previous chapters, our research group has also developed other specific and/or class-selective antibodies against different groups of contaminants, such as FQs [3], synthetic steroids [4], industrial residues [5] or pesticides [6]. Thus, combining generic bioreceptors for simultaneous detection of a wide range of antibiotics from the most relevant families, below the MRLs, on a single array configuration, could provide an excellent tool for the screening of these veterinary drug residues, increasing the efficiency of the analysis of the food control laboratories.

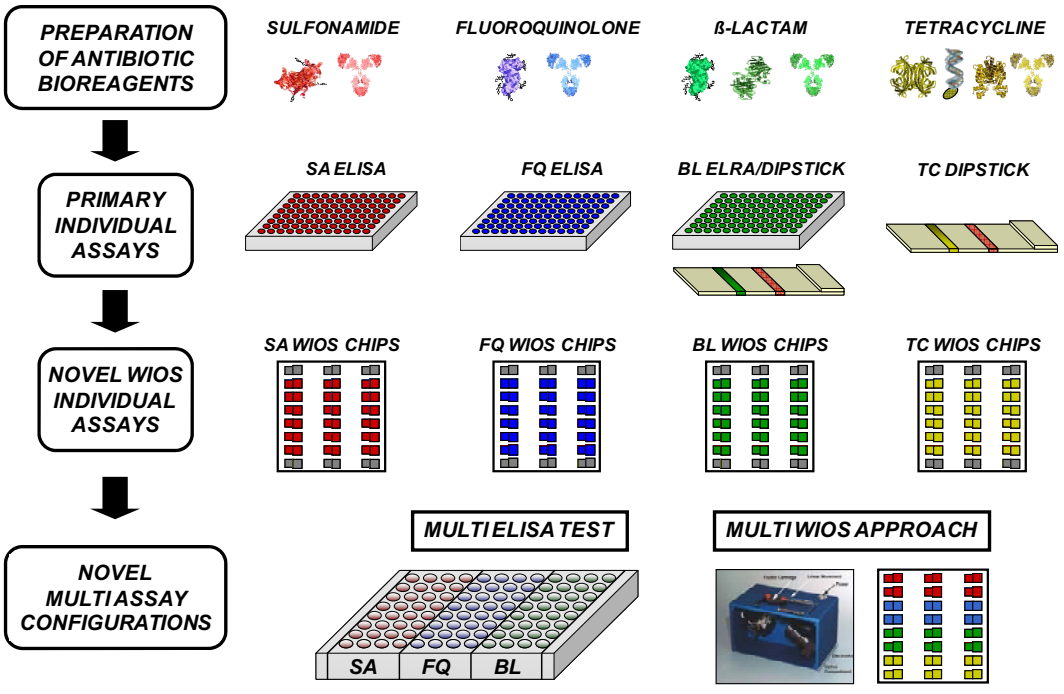


Figure 6.1: Working flow conducting to the development of distinct multiplexed bioanalytical approaches starting from the development of the corresponding specific bioreceptors, their individual evaluation on the different platforms and their combination on a single device.

In this sense, this chapter describes the research performed in this thesis to achieve the final objective of this thesis, which has been the development of multiplexed immunoanalytical approaches (see **Figure 6.1**) with the aim of providing fast, robust,

easy to handle, high throughput screening tools and devices for the analysis of antibiotic residues in food samples. The work has started with the establishment first of a multianalyte ELISA, in which different antibiotic receptors have been combined, and it has followed with the implementation of this strategy on the WIOS device, described in the previous chapter.

6.1 Preamble

Antibody and receptor based assays are techniques often used to detect single analytes but often simultaneous detection of different congeners of same chemical family is required. The use of bioreceptors with a generic recognition character may improve the screening efficiency of bioanalytical methods developed. Thus, combining class-selective antibodies against the most important antibiotic families on a same assay would allow increasing the efficiency of the actual antibiotic residue screening methods and developing fast and efficient multiplexed analytical tools and devices. In this respect, one of the main challenges is to demonstrate that the bioreceptors will show the same features used individually or mixed on a kind of "immunoreagent cocktail". Consequently, there exists a risk for synergic effects of cooperative responses. Thus, testing the orthogonal compatibility of different antigen–antibody/receptor combinations of bioreceptors against the most important antibiotic families has been one of the primary specific objectives to further on implementing these assays on a multiplexed optical sensor, such as the WIOS platform.

6.1.1 Most relevant antibiotic families

According to the information found in the literature, our discussion with our co-workers, experts in the field (Dr. Jean-Marc Diserens from the NRC at Switzerland) and the survey performed at the beginning of this thesis (see the **Appendix**), SAs, FQs, BLs and TCs are the main antibiotic families used to treat caws and therefore, more likely to be found in milk and dairy products in Europe. Hence, the immunoreagents developed for SAs (see **Section 3.1.5)**, and FQs (developed by Daniel G. Pinacho of the AMR group), both with a broad recognition spectra [3] and bioreceptors for BLs and TCs, both provided by UNISENSOR S.A. [7-11], were selected with the objective to develop a multiplexed biosensor for antibiotic residue analysis. All assays (SAs, FQs, BLs, and TCs) showed a quite broad recognition character for their respective family congeners.

Unfortunately, we did not succeeded on the preparation of antibodies for TCs with a broad selectivity spectrum, and UNISENSOR is proprietary of a technology based on the use of a regulatory protein with capability to detect a significant number of this antibiotic family. Similarly, for the case of the BLs, for which UNISENSOR has developed one of the most popular test-strip methods based on a bioreceptor. **Figure 6.2** shows SAs and FQs immunoreagents developed in our research group while **Figure 6.3** includes the BLs and TCs bioreagents supplied by UNISENSOR.

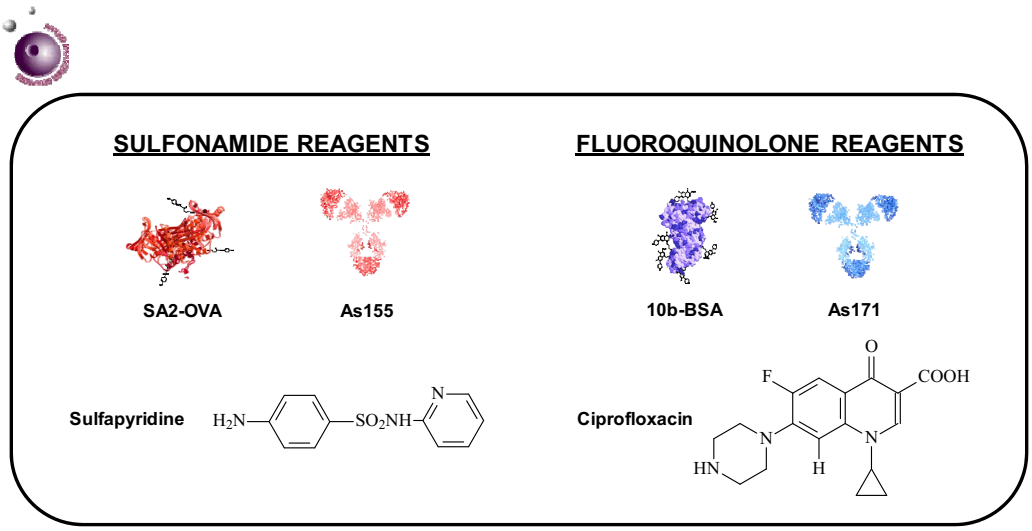


Figure 6.2: Schematic representation of SAs and FQs immunoreagents (following the same format) developed by AMRg and used for the antibiotic multidetection strategy planned.

Immunochemical detection of SAs and FQs is based on competitive indirect immunoassay configurations where the analytes had to compete with the CAs (SA2-OVA or 10b-BSA, 10b is 7-(2-aminoethylamino)-6-fluoro-4-oxo-1-propyl-1,4-dihydroquinoline-3-carboxylic acid) for the corresponding antibodies (As155 or As171).

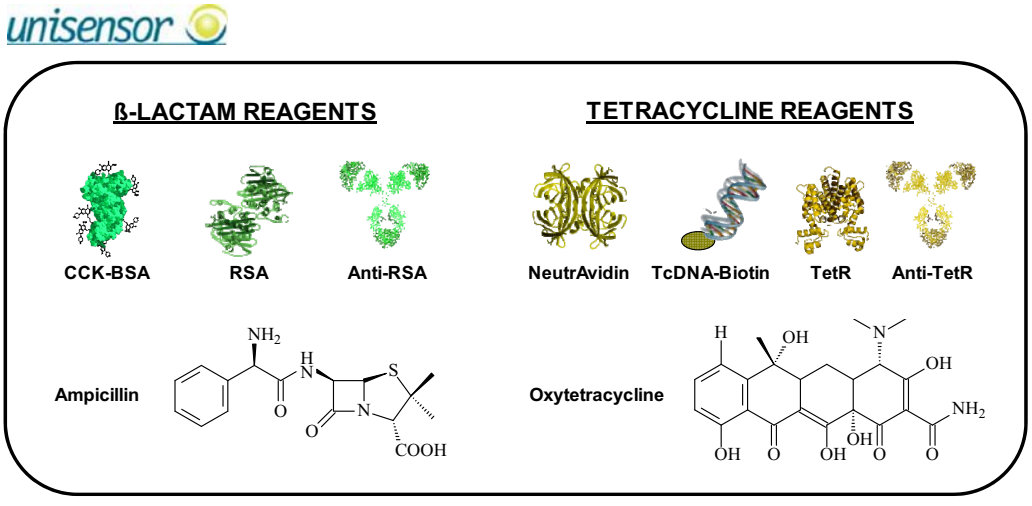


Figure 6.3: Schematic representation of BLs and TCs bioreagents supplied by UNISENSOR. (confidential information) within the frame of collaboration inside GOODFOOD project.

In this sense, the bioreceptor used to detect BLs [12] also works following on a similar manner, using a hapten-protein bioconjugate (CCK-BSA) as competitor [10] to interact with the BLs bioreceptor (RSA), but in this case, the assay has an step more since the

signal is obtained as a result of the recognition of this bioreceptor by an specific antibody (IgG, anti RSA) and further on by a secondary antibody labelled with HRP (antiIgG-HRP). For TCs detection, a recombinant tetracycline repressor protein (TetR) is also used as a binding receptor on a particular assay configuration, in which the microplates are coated with NeutrAvidin followed by a DNA-biotin conjugate (TcDNA-Biotin). The detection of TCs is in this case based on the ability of TetR to bind the defined DNA sequence of this conjugate used as competitor in the assay [9, 13]. As before, the receptor assay requires a specific antibody (IgG, anti TetR) and the antiIgG-HRP to record the signal.

6.1.2 Multisensing strategies

Nowadays (bio)chemical analyses call for methods able to simultaneously measure different molecular species present on a sample. Additionally, new transducer principles for biosensor development have derived from the new properties of the materials at the nanolevel. Multiplexation, miniaturization and detectability are goals pursued by many researchers. Improvement in the analytical efficiency and the consequent reduction of the necessary time and cost of the analysis are some of the advantages of multiplexing. Advances in nanobiotechnology have provided the possibility to develop multiplexing bioassays mainly using two strategies: a) achieving spatial multiplexing (planar microarrays) or b) using multiple quantitation tags (non-planar microarrays). This has been possible due to the knowledge acquired about the physic-chemical properties of the materials at the nanometer scale and also due to the development of sophisticated equipment to know what it takes place at the nanolevel.

A widely used approach for spatial multiplexing is the use of planar microarray, in which the identity of the target analyte is encoded by its location. This is the most well known technology and relevant growing in areas such as proteomics (large-scale study of proteins, particularly their structures and functions), genomics (study of the genome of an organism, investigation of single genes, their functions and roles) and pharmacogenomics (study of expression in individual genes relevant to disease susceptibility as well as drug response at cellular, tissue, individual or population levels) has been possible thanks to the availability of protein and DNA microarrays (more information can be found in recent reviews and books dealing with this topic [14-18]). Planar microarrays have found application in the pharmaceutical, biotechnology, agrochemical and diagnostics fields, in addition to academic and other non-profit research institutes. Analytical microarrays are typically used to profile complex mixtures of proteins in order to measure binding affinities, specificities, and protein expression levels of the proteins in the mixture, but only in few occasions has been reported their use to analyze small organic molecules of

interest in the food safety field. On planar microarrays, a library of receptors (antibodies, antigens, aptamers, etc.) is arrayed on a glass microscope slide and then probed with a solution containing the mixture of substances to analyze. Detection in planar microarrays can be accomplished either using labels or non-labelled procedures. In the first case microarrays have been combined with the use of organic fluorophores [19-21], being among others the cyanines like Cy3 and Cy5 the most commonly used ones, or in general with fluorescence detection, gaining importance the use of fluorescent nanoparticles in order to increase the signal amplification. Chemiluminescent probes [22] or colorimetric substrates [21, 23] are also often used. On the other hand, non-labelled microarrays can also be developed based on already well-known (MS, SPR, grating couplers, piezoelectric substrates, atomic force microscopy, electrochemical impedance spectroscopy or ellipsometry) or new biosensing principles under investigation or even derived from the particular properties of nanostructured materials [24-28]. Such is the case of the WIOS platform used in this thesis.

The typical planar array sometimes suffers from non-uniformity and slow diffusion of targets to the binding surface [29]. Moreover, several authors have demonstrated that binding reactions in solution results in a gain of sensitivity and improves accuracy and reproducibility of the bioanalytical technologies [30]. Thus, an approach to overcome this problem consists on the use of encoded microparticle arrays to perform the biorecognition reactions in a "solution-like" environment. Unlike positional or spatial encoding, in which it is the exact location on the array surface that allows the identification of the analyzed molecule, in non-planar arrays, each microcarrier has to be encoded to assess the identity of each probe. The microcarriers with different probes attached to their surface can be mixed in the same vial that contains the target analyte. The unique code on each microcarrier allows either the ligand or the compound attached to the carrier surface to be unequivocally identified. Various strategies developed for microcarrier encoding have been reviewed [31], between them the use of organic fluorophores with distinct emission wavelengths, but also nanoparticles which optical properties can be tuned by varying their nature, material or size. Thus, as an example colloidal semiconductor QDs have been the first nanomaterials used as labels [32-34]. Traditionally made of II-Vi or III-V elements, may be synthesized in different colours by changing the Bohr excitation radius (2-50 nm size) to tune then the wavelength of the emitted light.

Within this thesis, we have focused on the development of a multiplexed platform for multiple antibiotic simultaneous detection based on a typical planar array for sensors. The WIOS transducer principle has been used for this purpose making use of a 24 array chip. To accomplish this aim, orthogonality of the selected bioreceptors and performance,

when used as cocktail for simultaneous antibiotic determination, has been assessed first through the development of a multianalyte microplate-based ELISA (see **Figure 6.4**).

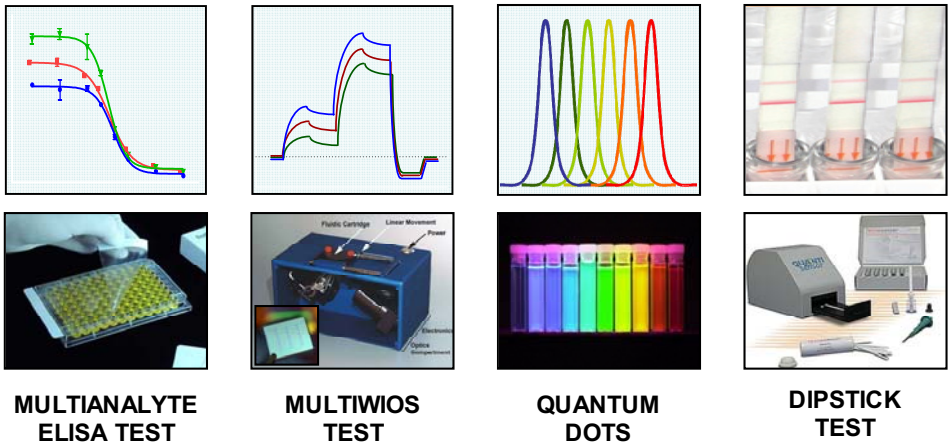


Figure 6.4: Schematic picture showing the multianalyte techniques considered within this thesis.

Further on, these bioreceptors and the conditions established have been implemented in the WIOS platform. Alternatively, some preliminary studies have been performed in order to develop non-planar microarray approaches. The results of these studies can be found in the **Appendix**.

6.1.2.1 Multianalyte ELISA

ELISA methods have been traditionally addressed to single analyte detection (see **Sections 1.6** and **3.1.5**). On the other hand, orthogonal hapten-antibody combinations seem to be ideal to create array structures with different antibodies for different analytes, such as in this case the antibodies/bioreceptors against different antibiotic families. In order to assess orthogonality of the bioreagents, a multianalyte ELISA was developed combining initially three antigen/bioreceptor combinations for on a further step incrementing the number of antibiotic families. The assay was based on a strategy that consisted on coating a microplate with the three different coating antigens of the antibiotic families selected but distributed in separated rows or columns. The sample is mixed with a cocktail of the bioreagents and distributed in the three different ones of the plates. Thus, identification of the antibiotic present in a particular sample is consequently accomplished by detecting a positive response on any of the microplate sections (**Fig. 2** in **Section 6.2)**. The multianalyte microplate-based ELISA developed on a first instance uses two indirect ELISAs (SAs and FQs) and an ELRA (enzyme-linked receptor assay) for BLs. The results of this study show that it is possibly to combine these bioreceptors to develop a multiplexed analytical method. The multiplexed ELISA is able to accurately

quantify simultaneously the presence of SAs, FQs and BLs residues in milk samples in a single microplate. These results are described in the paper of **Section 6.2.** Additionally, the investigation reported established the bases to combine and implement the multiplexed assay on the WIOS transducer.

6.1.2.2 Multi WIOS

Nanobiotechnology involves the processing, fabrication, and packaging of organic, biomaterial devices or assemblies in which the dimension of at least one functional component lies between atomic lengths and the wavelength of visible light [35]. The WIOS approach, described in the previous chapter, is not an exception since it combines the last advances in the micro/nanoelectronics with the excellent features of specific bioreceptors. The excellent performance of the WIOS system for the analysis of SAs residues in milk samples has been demonstrated in the previous chapter. In that case SAs haptenized protein was immobilized on the chip surface (see **Section 5.2**). However, these waveguide gratings allow building sensor arrays with 24 independent sensing regions on the same chip allowing the fabrication of multiplexed devices able to screen simultaneously the presence of different targets. Thus, based on the results obtained by combining the different immunoreagents and receptors on the multiplexed ELISA, we approached their implementation on the WIOS platform with the objective to develop a multiplexed biosensor device. The investigation performed in this respect and the results obtained are described in **Section 6.3**.

The present chapter describes the work performed in relation to the first steps taken to achieve the **Specific Objective 4** (i.e. integration of bioreceptors on the WIOS transducer and its performance in milk samples) of this thesis in respect to SAs, FQs, BLs and TCs. Considering the precedents mentioned above, the main objective here was a) to test and prove the orthogonal features of the bioreceptors developed for the detection of SAs, FQs, BLs and TCs, b) to develop a multianalyte ELISA test to detect simultaneously SAs, FQs and BLs families in milk samples, c) to implement the bioreceptors for SAs, FQs, BLs and TCs in the WIOS platform to further analyse milk samples, and d) to perform a preliminary evaluation and comparative study of the multiplexed ELISA, the multiWIObS and the dipstick (UNISENSOR) approaches by measuring unknown milk samples.

6.2 Multianalyte ELISA (SAs, FQs and BLs in milk)

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A multianalyte ELISA for immunochemical screening of sulfonamide, fluoroquinolone and ß-lactam antibiotics in milk samples using class-selective bioreceptors

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Abstract A multianalyte ELISA has been developed for the simultaneous determination of the most frequently used antibiotic families in the veterinary field following the typical planar microarray configuration, where the identity of the target analyte is encoded by its location in the detection platform (Master et al. in *Drug Discovery Today* 11:1007–1011, 2006). To accomplish this aim, two individual enzyme-linked immunosorbent assays for sulfonamide and fluoroquinolone antibiotics and an enzyme-linked receptor assay for ß-lactam antibiotics have been combined. The strategy uses microplates coated with the corresponding haptenized proteins in specific sections of the microplate. The samples are mixed with a cocktail containing the bioreagents, and distributed in the wells of the microplate. Identification of the antibiotic present in a particular sample is consequently accomplished by detecting a positive response on the corresponding microplate section. Since the bioreceptors used show a wide recognition of the congeners of each antibiotic family, the multianalyte method is able to detect more than 25 different antibiotics from the three most important antibiotic families. The detectability reached in full-fat milk samples is below the European maximum residue limits. The accuracy and reliability of this multiplexed bioanalytical method have been demonstrated by analyzing blind spiked samples.

Keywords Multianalyte detection · enzyme-linked immunosorbent assay · enzyme-linked receptor assay · antibiotic residues · sulfonamides · fluoroquinolones · ß-Lactams

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Introduction

Nowadays, antibiotics are frequently used in veterinary practice not only for treatment of bacterial infections but also for prophylactic and prevention purposes to improve the productivity of foodstuffs. In the last decade, this irresponsible misuse of antibiotics as a preventive measure has been a decisive factor favoring the growth of bacterial resistance [1–3]. This risk situation may spread from animals to humans through the food chain (meat, milk, etc.), leading to an increase in the failure of antibiotics to treat usual human infections. This phenomenon is a major public health issue and a reason for great concern within health authorities. It has also been reported that antibiotics excreted by farm animals after consumption, besides their

metabolites or degradation products generated, are reaching the terrestrial and aquatic environment at up to a 1,000 t per year [4, 5]. Moreover, antibiotic residues are known to be among the most frequently detected contaminants in milk and diary products and cause important problems in this industrial sector at both economical and technological levels.

To prevent the negative impact of the antibiotic residues on human health and on the entire ecosystem, procedures for the establishment of maximum residue limits (MRLs) of veterinary drugs in foodstuffs of animal origin are regulated by European Council (EC) Regulation no. 2377/90. Although the European Union has also dictated the frequency and number of analyses to be performed (Directive 96/23/CE), the analytical methods available today are insufficient to accomplish these requirements. Thus, unspecific and time-consuming microbiological tests based on cylinder plate methods are frequently used for screening [6–8]. Chromatographic techniques, such as high-performance liquid chromatography (HPLC)–UV spectroscopy [9, 10] and/or HPLC–mass spectrometry (MS) [11, 12], normally used to confirm the results of samples suspected of being contaminated, show high specific and excellent detectability; however, their inherent sequential nature and the need to be preceded by sample preparation procedures limit their efficiency to ensure food safety.

Immunochemical techniques can offer important advantages as screening methods owing to their simplicity, high-throughput capabilities and low cost [13–18]; they are already being used by some food safety reference laboratories. Enzyme-linked immunosorbent assay (ELISA) and enzyme-linked receptor assay (ELRA) tests are examples of the methods most commonly used to detect antibiotic contaminants in milk samples although they are limited sometimes by their high specificity. Hence, immunoassay techniques are often used to detect a single analyte. On the other hand, orthogonal hapten–antibody/receptor combinations seem to be ideal for creating array structures with different immunoreagents for different analytes [19, 20]. This kind of parallelization reaches its physical and economical limits with the increasing number of analytes that could be measured at the same time.

The combination of generic immunoassays for simultaneous detection of a wide range of sulfonamides, fluoroquinolones and ß-lactams below the MRLs on a single array could be an excellent tool for antibiotic residue screening. Here we report the development and evaluation of a multianalyte ELISA combining immunoreagents specifically developed to detect a wide range of congeners of the three most important antibiotic families found in the veterinary field. The multianalyte ELISA has been evaluated by testing blind full-fat milk samples contaminated (or not) with different antibiotics.

Experimental

General methods and instruments

The pH and the conductivity of all buffers and solutions were measured with a pH 540 GLP pH meter and an LF 340 conductimeter, respectively (WTW, Weilheim, Germany). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). Washing steps were performed using an SLY96 PW microplate washer (SLT Labinstruments, Salzburg, Austria). Absorbances were read at 450 nm with a SpectramaxPlus (Molecular Devices, Sunnyvale, CA, USA). A Heidolph Titramax 1000 vibrating platform shaker (Brinkmann Instruments, Westbury, NY, USA) was used to shake the microplates at 900 rpm. The competitive curves were analyzed with a four-parameter logistic equation using the programs SoftmaxPro version 4.7 (Molecular Devices) and GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA). The standard curves were fitted to a four-parameter equation according to the following formula: $Y = [(A - B)/1 - (x/C)^D] + B$, where A is the maximum absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximum absorbance and D is the slope at the inflection point of the sigmoid curve. Unless otherwise indicated, data presented correspond to the average of at least two well replicates.

Milk samples

Bovine full-fat milk samples testing free of antibiotics (B-milk) were provided by the Agencia Española para la Seguridad Alimentaria (AESA; Spanish Agency for Food Security). Contaminated blind samples were prepared at the Nestlé Research Center from bovine fresh full-fat milk.

Buffers and solutions

Phosphate-buffered saline (PBS) was 0.01 M phosphate buffer in a 0.8% saline solution (137 mM NaCl, 2.7 mM KCl), and the pH was 7.5. PBST was PBS with 0.05% Tween 20. Borate buffer was 0.25 M boric acid–sodium borate, pH 8.7. The coating buffer was 0.05 M carbonate–bicarbonate buffer, pH 9.6. Citrate buffer was a 0.04 M solution of sodium citrate, pH 5.5. The substrate solution contained 0.01% 3,3′,5,5′-tetramethylbenzidine and 0.004% H_2O_2 in citrate buffer. D-Milk was B-milk diluted 5 times with Milli-Q water.

Chemicals and biochemicals

Immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The preparation of the sulfonamide

and fluoroquinolone haptenized protein conjugates (SA2-OVA and 10b-BSA, respectively) and antiserums (As155 and As171, respectively) is described elsewhere [21]. The β-lactam haptenized protein (CCK-BSA), the bioreceptor (RSA) and the antibody (anti-RSA) were kindly provided by Unisensor. Sulfonamides and fluoroquinolones used as standards or for cross-reactivity (CR) studies were supplied by Riedel-de Haën and Aldrich Chemical Co. (Milwaukee, WI, USA), respectively, while the β-lactams were obtained from Unisensor. The analytes used as standards were prepared from 10 mM stocks in dimethyl sulfoxide (DMSO) (sulfapyridine, SPY; ampicillin, AMP) and in 50 mM aqueous NaOH (ciprofloxacin, CPX). For the multianalyte ELISA, a 100 μM stock was prepared in PBST containing the three analytes (cocktail An) and was used to prepare the standard curve. Similarly, the bioreagents (As155, diluted 4,000 times; As171, diluted 32,000 times; RSA, diluted 8,000 times; and anti-RSA, diluted 2,000; all in PBST) were used in combination (cocktail BR). For the semiquantitative multianalyte ELISA, the following controls prepared in PBST, at the MRLs established in milk, were used: sulfachloropyridazine (SCP, 100 μg L^{-1}), sulfamethazine (SMZ, 100 μg L^{-1}), enrofloxacin (ERX, 100 μg L^{-1}), difloxacin, prohibited (DFX, 10 μg L^{-1}) and AMP (4 μg L^{-1}).

Individual sulfonamide indirect ELISA (SA2-OVA/As155)

Microtiter plates were coated with SA2-OVA (0.625 μg mL^{-1} in coating buffer, 100 μL per well) overnight at 4 °C and covered with adhesive plate sealers. The following day, the plates were washed with PBST (four times, 300 μL per well). SPY standards (0.0256–50,000 nM in PBST) or milk samples were added to the microtiter plates (50 μL per well), followed by the antiserum (As155, 1:4,000 in PBST; 50 μL per well) and incubated for 30 min at room temperature, under shaking. The plates were washed again as before, and a solution of anti immunoglobulin G (IgG)–horseradish peroxidase (HRP) (1:6,000 in PBST) was added (100 μL per well) and incubated for 30 min more at room temperature. After a new washing step, the substrate solution was added (100 μL per well) and the color development stopped after 30 min at room temperature with 4 N H_2SO_4 (50 μL per well). The absorbances were read at 450 nm.

Individual fluoroquinolone indirect ELISA (10b-BSA/As171)

Microtiter plates were coated with 10b-BSA (0.25 μg mL^{-1} in coating buffer, 100 μL per well) overnight at 4 °C and covered with adhesive plate sealers. The following day, the plates were washed with PBST (four times, 300 μL per well) and the CPX standards (0.0256–50,000 nM in PBST) or the milk samples were added to the microtiter plates (50 μL per well), followed by the antiserum (As 171, 1:32,000 in PBST; 50 μL per well). After 30 min of incubation at room temperature under shaking, the plates were processed as described earlier.

Individual ß-lactam) indirect ELRA (CCK-BSA/RSA/anti-RSA)

Microtiter plates were coated with CCK-BSA (0.5 ng mL^{-1} in coating buffer, 100 μL per well) overnight at 4 °C and covered with adhesive plate sealers. The following day, the plates were washed with PBST (four times, 300 μL per well). The AMP standards (0.0256–50,000 nM in PBST) or the milk samples were added to the microtiter plates (50 μL per well), followed by a mixture of the β-lactam receptor (RSA, 1:8,000) and the anti-RSA (1:2,000) solution in PBST (50 μL per well). The mixture was incubated for 30 min at room temperature under shaking. The plates were processed as described earlier.

Multianalyte ELISA

Three distinct sections (sulfonamides, fluoroquinolones and β-lactam) were defined in the microtiter plates and coated with the three different antibiotic antigens (sulfonamides, SA2-OVA, 1.0 μg mL^{-1}; fluoroquinolones, 10b-BSA, 0.5 μg mL^{-1}; β-lactam, CCK-BSA, 0.03 μg mL^{-1} in coating buffer; 100 μL per well in all cases) overnight at 4 °C and covered with adhesive plate sealers. The next day, cocktail An (0.0256–50,000 nM in D-milk) was added to the microplates (50 μL per well), followed by cocktail BR (50 μL per well). After 30 min of incubation at room temperature the plates were processed as described earlier.

Semiquantitative multianalyte ELISA

The ELISA was performed as described earlier according to the following set-up. Prior to starting the test, the controls were diluted in D-milk (SCP, 1:10; SMZ, 1:100; ERX, 1:500; DFX, 1:10; AMP 1:5). Similarly, B-milk and the unknown milk samples were diluted 5 times (Mx-5), 10 times (Mx-10), 100 times (Mx-100) and 500 times (Mx-500) in D-milk. The ELISA was run by adding the controls (sulfonamides SCP and SMZ; fluoroquinolones ERX and DFX; β-lactam AMP) and the samples (sulfonamides Mx-5 and Mx-100; fluoroquinolones Mx-500 and Mx-10; β-lactam Mx-5) in duplicate (50 μL per well) to their corresponding section of the microplate as indicated, followed by the cocktail BR (50 μL per well). After 30 min of incubation at room temperature the plates were processed as described earlier.

Matrix effect studies

B-milk samples were supplied by AESA and used to assess interferences caused by the sample matrix in the multianalyte ELISA test. Milk samples were serially diluted in 2× PBS and used to prepare standard curves to compare the parallelism with the curves obtained in PBST.

Specificity studies

Stock solutions of different sulfonamides (10 mM in DMSO), fluoroquinolones (10 mM in 50 mM NaOH) and ß-lactams (10 mM in DMSO) were prepared and stored at 4 °C. Standard calibration curves for each one were prepared by serial dilutions (50,000–0 nM) in noncontaminated reference fresh milk diluted 1:5 in Milli-Q water and measured with the multianalyte ELISA test (same procedure as described earlier). The CR values were calculated according to the equation [IC_{50}(SPY, CPX or AMP)/ IC_{50}(antibiotic family cross-reactant)×100].

Accuracy studies

Blind samples prepared in PBST and in B-milk were measured using the multianalyte ELISA protocol described earlier. Analyses were performed in duplicate. Accuracy was evaluated by establishing a linear regression between the spiked and the measured values.

Results and discussion

Individual assays

Two ELISAs for sulfonamide (As155/SA2-OVA) and fluoroquinolone (As171/10b-BSA) antibiotics and an ELRA for β-lactams (CCK-BSA, RSA receptor and anti-RSA) were employed for the development of a multianalyte method. All three assays were developed and evaluated previously and showed their capabilities to recognize a wide variety of congeners of each antibiotic family in compliance with the EC regulations [21] (D.G. Pinacho, F. Sanchez-Baeza and M.-P. Marco, unpublished results). About 11 sulfonamides were detected in full-fat milk samples below 100 μg L^{-1} (MRLs for sulfonamides in milk). Fluoroquinolone and ß-lactam antibiotics were also detected although different safety levels have been established in these families for each individual congener (i.e., CPX 100 μg kg^{-1}, marbofloxacin 75 μg kg^{-1}, danofloxacin prohibited, AMP 4 μg L^{-1}, ceftiofur 100 μg L^{-1}). All three assays were performed on indirect formats (plates were coated with a haptenized protein and detection took place through a secondary anti-rabbit IgG labeled with HRP), although in the ELRA of the β-lactams, based on the use of a protein receptor (RSA), there is an additional step in which this bioreceptor is recognized by a specific antibody (anti-RSA). Thus, our first goal was to equalize the protocols, reducing the number of steps of the ELRA. Mixing the RSA and anti-RSA in the competitive step allowed us to obtain an assay with features similar to those of the original ELRA for β-lactams (Fig. 1). Attempts to accomplish this aim by mixing the anti-RSA and the anti IgG-HRP in the last step produced a considerable decrease in the signal. The features of the individual assays, once the protocols had been equalized in the same buffer system, are shown in Table 1.

Antibiotic multianalyte ELISA

For the multianalyte ELISA the microplates were divided in three different sections, each of them used to give a response to each family of antibiotics, and the bioreceptors (sulfonamides, As155; fluoroquinolones, As171; and β-lactams, RSA and anti-RSA) were mixed, at the appropriate dilution, with a solution named "cocktail BR." As a standard, an equimolar mixture named "cocktail An" of three representative analytes (SPY, CPX and AMP) of each antibiotic family (sulfonamides, fluoroquinolones and AMP) was used.

Evaluation of the response of the individual assays in a multianalyte format

The first objective was to assess if an orthogonal system could be established for each independent antigen–antibody/receptor combination. This meant that each antigen immobilized separately on the microplate had only to be

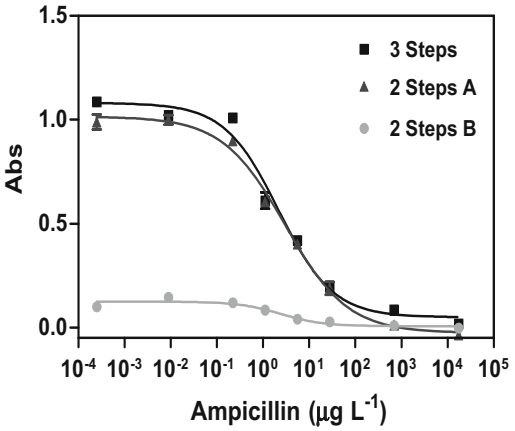

Fig. 1 Standard calibration curves of ß-lactam individual enzyme-linked receptor assay comparing the protocol performed in two or three steps: *A*) mixing RSA and anti-RSA in the same step; *B* mixing anti-RSA and anti immunoglobulin G–horseradish peroxidase in the same step

Table 1 Features of the individual immunoassays compared with those obtained with the multianalyte enzyme-linked immunosorbent assay (ELISA) test

Parameter	Assay values					
Assay, analyte	Sulfonamide, sulfapyridine		Fluoroquinolone, ciprofloxacin		ß-Lactam, ampicillin	
Coating antigen (μg mL^{-1})	SA2-OVA (0.63)		10b-BSA (0.25)		BSA-CCK (5×10^{-4})	
Receptor, dilution	–		–		RSA, 1:32000	
Antibody, dilution	As155, 1:8,000		As171, 1:64,000		Anti-RSA, 1:4,000	
Assay configuration	Individual	Mixture	Individual	Mixture	Individual	Mixture
A_{max}	1.80	1.79	2.11	2.16	1.50	1.49
A_{min}	0.12	0.19	0.17	0.19	0.04	0.12
IC_{50} (μg L^{-1})	2.80	2.93	2.36	2.54	3.70	3.91
Slope	-0.62	-0.75	-0.97	-0.94	-0.73	-0.90
R^2	0.99	0.99	0.99	0.99	0.99	0.99

Assays run individually under the same physicochemical conditions

recognized by its counter partner of antibody/receptor (Fig. 2). If more than one kind of antibody bound to an immobilized antigen, the so-called shared reactivity would lead to distorted signals depending on the differences between the recognition elements (antibody/receptor) for a particular antigen. Thus, testing the absence of possible shared reactivity was critical. For this purpose, the multianalyte ELISA was run with the objective to compare the response of the different bioreceptors of cocktail BR in the distinct microplate sections, in comparison with the individual response of each bioreceptor and binary mixtures of them. The results of these studies demonstrated that a specific response is obtained independently of whether the bioreceptors are used individually or as mixtures, indicating the absence of a shared-reactivity effect (Table 2). Calibration curves were prepared and used for microplates using the individual and the multianalyte protocols. As can be observed in Fig. 3 the calibration curves obtained for each antibiotic family were identical irrespective of whether they were obtained for the individual assay or for the multianalyte ELISA using cocktail BR and cocktail An. Similarly, the features of the assays are not significantly affected as can be observed in Table 1.

Evaluation of the specificity of the response of the multianalyte ELISA

Although the individual assays had shown no recognition for antibiotic congeners of other families, specificity of the multianalyte ELISA was evaluated to probe if the use of cocktail BR had no effect on the recognition profile. The results demonstrated that the multianalyte ELISA responded selectively on each corresponding section for samples containing single antibiotic congeners (i.e., SPY, CPX or AMP) or combinations of them. The specificity was maintained on each section even if high concentrations (up to 50 μM) of the other antibiotics were present in the same sample (Table S1). Thus, when calibration curves of a particular antibiotic were obtained by mixing it with increasing concentrations of antibiotics of the other two families no significant variations were observed in the calibration curves obtained (Fig. S1).

Accuracy of the multianalyte ELISA

A collection of blind PBS samples, spiked individually or with mixtures of representatives of the three antibiotic

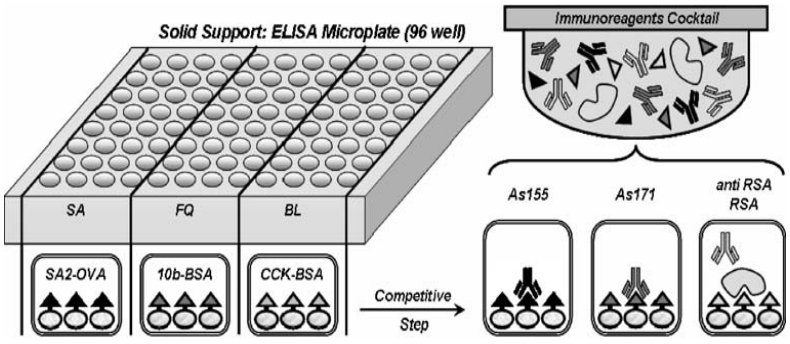

Fig. 2 The multianalyte enzyme-linked immunosorbent assay (*ELISA*). Different sections of the microtiter plate are coated with coating antigens of the three antibiotic families. The immunoreagents/bioreceptors and the analytes are then distributed through the whole plate as a mixture of reagents

Table 2 Response of the bioreceptors used individually or as mixture in the distinct sections of the microplate

Microplate ELISA sections			Bioreceptors
SA	βL	FQ	
■			As155 *(SAs)*
		■	As171 *(FQ)*
	■		RSA/anti RSA *(βL)*
■		■	As155 + As171 *(SA and FQ)*
■	■		As155 + RSA/anti RSA *(SA and BL)*
	■	■	RSA/anti RSA + As171 *(BL and FQ)*
■	■	■	As155 + RSA/anti RSA + As171 *(SA, BL and FQ)*
			PBST (no reagents)

Each section of the microplate was coated with a distinct coating antigen of the three antibiotic families. The color scale indicates absorbance:

Absorbance 0.0 0.2 0.6 0.8 1.0

SA sulfonamide, *FQ* fluoroquinolone, *BL* ß-lactam, *PBST* phosphate-buffered saline with 0.05% Tween 20

families (SPY, CPX and AMP), were analyzed with the multianalyte ELISA. As can be observed in Table 3 the multianalyte ELISA recognized in all cases the type of analyte/s present in the samples. Moreover, the antibiotic concentration could be quantified with good accuracy, except for some samples spiked with CPX and/or AMP, whose concentration was underestimated. As consequence two false negatives were obtained, which compromised the usefulness of the method as a screening method. As can be seen in Fig. 4 (left graph), although the coefficient of correlation was very good in all cases (R^2>0.96), slope values below 0.8 were in agreement with the underestimation observed. A potential explanation of this behavior was found in the buffer systems used in the multianalyte ELISA, which were slightly modified with respect to the original individual assays in order to equalize the conditions. Previous studies performed while developing the

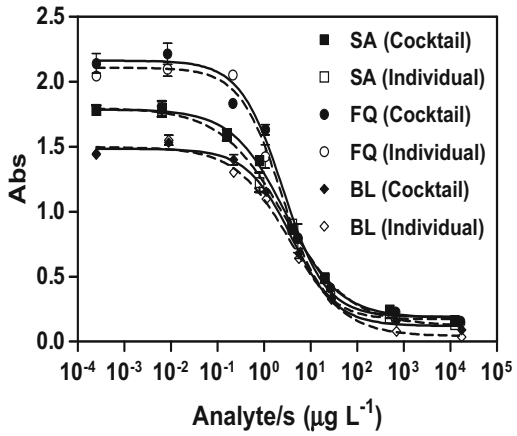

Fig. 3 The standard curves obtained with the individual immunoassays for each antibiotic family and with the multianalyte ELISA where immunoreagents and receptors are mixed as a cocktail. *SA* sulfonamide, *FQ* fluoroquinolone, *BL* ß-lactam

Table 3 Study of spiked samples in PBST with the multianalyte ELISA test

Spiked sample	Antibiotic detected	Measured (µg L^{-1})	Spiked (µg L^{-1})	Results
Sulfapyridine	Sulfonamide	192	100	P
Sulfapyridine	Sulfonamide	64	50	N
Ampicillin	ß-Lactam	36	37	P
Ampicillin	ß-Lactam	301	371	P
Ciprofloxacin	Fluoroquinolone	60	146	FN
Ciprofloxacin	Fluoroquinolone	22	36	N
Sulfapyridine	Sulfonamide	15	25	N
Ciprofloxacin	Fluoroquinolone	125	367	P
Sulfapyridine	Sulfonamide	166	250	P
Ampicillin	ß-Lactam	28	37	P
Ciprofloxacin	Fluoroquinolone	85	146	FN
Ampicillin	ß-Lactam	94	148	P
Sulfapyridine	Sulfonamide	19	25	N
Ciprofloxacin	Fluoroquinolone	211	367	P
Ampicillin	ß-Lactam	58	74	P
Blank	None	0	0	N
Ampicillin	ß-Lactam	3	14	FN
Ampicillin	ß-Lactam	7.6	7.4	P
Sulfapyridine	Sulfonamide	212	250	P
Ciprofloxacin	Fluoroquinolone	24	36	N
Ampicillin	ß-Lactam	31	37	N
Sulfapyridine	Sulfonamide	24	25	N
Ciprofloxacin	Fluoroquinolone	184	367	P
Ampicillin	ß-Lactam	5	7	FP
Blank	None	0	0	N

Results are expressed as positive (*P*), negative (*N*), false positive (*FP*) and false negative (*FN*) according to maximum residue limits (MRLs) established for each compound in milk (sulfapyridine 100 µg L^{-1}, ciprofloxcacin 100 µg L^{-1}, ampicillin 4 µg L^{-1}) by the European Union.

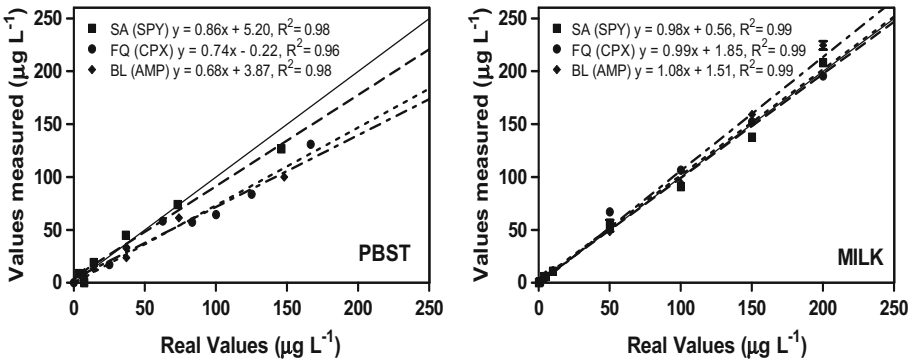

Fig. 4 Correlation between the spiked and measured antibiotic concentration values. Blind spiked samples were prepared using a single antibiotic or mixtures of antibiotics from the three families. *Left*: Samples were prepared by spiking phosphate-buffered saline with 0.05% Tween 20 (*PBST*) with different concentrations of sulfapyridine (SPY), FQ and ampicillin (AMP) and measured with the ELISA. *Right*: Full-fat milk samples were spiked with different concentrations of SPY, FQ and AMP and measured with the multianalyte ELISA. The *continuous line* corresponds to perfect correlation (slope 1)

fluoroquinolone assay showed that a superior performance was obtained if a certain concentration of divalent cations (i.e., Ca^{2+} or Mg^{2+}) [22] (D.G. Pinacho, F. Sanchez-Baeza and M.-P. Marco, unpublished results) was present owing to the tendency of these antibiotics to form chelates. Since the multianalyte ELISA protocol was used for the analysis of milk samples, where a significant concentration of Ca^{2+} is present, attempts to solve the underestimation were not addressed at this point. On the other hand, false positives do not represent a problem, since positive findings from the screening should always be followed by the use of confirmatory methods. On the other hand, these results usually indicate the presence of the antibiotic, although at a concentration below the MRL.

Performance of the multianalyte ELISA in milk samples

According to the literature [23] matrix effects in milk are likely produced by the fat and the protein content of the sample; however, the analyte properties can also be crucial for good performance of the assay in milk. It was found that shaking the microtiter plates [24, 25] minimized the formation of fat/protein layers in the bottom of the microtiter wells, improving assay reproducibility. To assess potential nonspecific interferences, B-milk was diluted several times in PBS and used to prepare standard curves. As can be observed in Table 4 (see also Fig. 5), the features of the standard calibration curves prepared for D-milk are almost identical to those obtained for the buffer.

Table 4 Features of the different immunoassays in 1:5 diluted bovine full-fat milk samples free of antibiotics

Parameter	Assay values		
	Multianalyte ELISA test (cocktail of immunoreagents)		
Assay	Sulfonamide	Fluoroquinolone	ß-Lactam
Analyte	Sulfapyridine	Ciprofloxacin	Ampicillin
Coating antigen (µg mL^{-1})	SA2-OVA, (1.00)	10b-BSA, (0.50)	BSA-CCK, (0.03)
Receptor, dilution	–	–	RSA, 1:8,000
Antibody, dilution	As155, 1:4,000	As171, 1:32,000	Anti-RSA, 1:4,000
A_{max}	1.24	1.01	1.28
A_{min}	0.12	0.02	0.01
IC_{50} (µg L^{-1})	0.81	0.77	2.97
Slope	-0.93	-0.90	-0.98
R^2	0.99	0.99	0.99

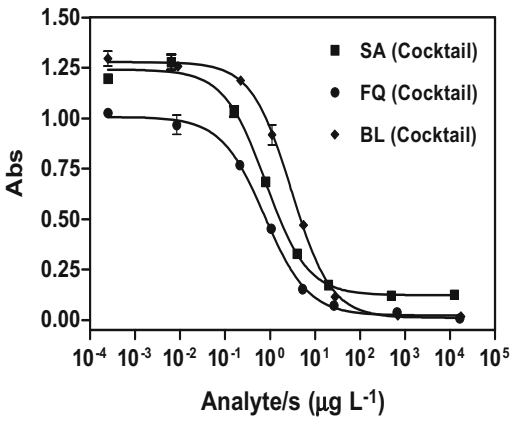

Fig. 5 Standard curves for the different immunoassays performed (multianalyte ELISA test) using the cocktail of immunoreagents in bovine full-fat milk samples free of antibiotics diluted 5 times with Milli-Q water and shaking the plate during the competition step

Specificity of the multianalyte ELISA in milk

Standard curves were prepared for D-milk with 25 antibiotic congeners and used in the multianalyte ELISA to assess the specificity of the method. Table 5 shows the results of these studies, expressing the recognition of each of those compounds by their IC_{50} and their percentage of CR in respect of the IC_{50} of SPY, for sulfonamides, CPX, for fluoroquinolones, and AMP for the ß-lactams. Each antibiotic was only recognized on the corresponding microplate ELISA section. Moreover, as expected regarding the recognition profile of the individual ELISAs, most of the related compounds tested were highly recognized in the multianalyte ELISA, with IC_{50} values under the MRLs established by the European Union. The results demonstrate the generic character of the multianalyte ELISA since a significant number of antibiotics from the three most important families can be detected in milk.

Table 5 Interference presented by different related compound of each antibiotic family in the multianalyte assay

Analyte	Multianalyte ELISA test (cocktail of immunoreagents)					
	Sulfonamide coating (SA2-OVA)		Fluoroquinolone coating (10b-BSA)		ß-Lactam coating (CCK-BSA)	
	IC_{50} (μg L^{-1})	CR (%)	IC_{50} (μg L^{-1})	CR (%)	IC_{50} (μg L^{-1})	CR (%)
Sulfapyridine	0.61	100	> MRL	<<1	> MRL	<<1
Sulfaquinoxaline	23.22	3	> MRL	<<1	> MRL	<<1
Sulfachloropyridazine	31.32	2	> MRL	<<1	> MRL	<<1
Sulfamethoxazole	> MRL	<1	> MRL	<<1	> MRL	<<1
Sulfisomidine	3.77	18	> MRL	<<1	> MRL	<<1
Sulfathiazole	0.45	138	> MRL	<<1	> MRL	<<1
Sulfadiazin	12.61	5	> MRL	<<1	> MRL	<<1
Sulfadiazin	12.61	5	> MRL	<<1	> MRL	<<1
Sulfadimethoxine	41.88	2	> MRL	<<1	> MRL	<<1
Sulfamerazine	1.56	41	> MRL	<<1	> MRL	<<1
Sulfadoxine	> MRL	<1	> MRL	<<1	> MRL	<<1
Sulfamethoxypyridazine	1.40	49	> MRL	<<1	> MRL	<<1
Sulfamethazine	1.17	58	> MRL	<<1	> MRL	<<1
N^4-Acetyl-Sulfamethazine	> MRL	<1	> MRL	<<1	> MRL	<<1
Trimetroprim	> MRL	<1	> MRL	<<1	> MRL	<<1
Sulfanilamide	> MRL	<1	> MRL	<<1	> MRL	<<1
Ciprofloxacin	> MRL	<<1	0.27	100	> MRL	<<1
Enrofloxacin	> MRL	<<1	0.36	72	> MRL	<<1
Enrofloxacin	> MRL	<<1	0.36	72	> MRL	<<1
Danofloxacin	> MRL	<<1	2.45	11	> MRL	<<1
Difloxacin	> MRL	<<1	0.30	89	> MRL	<<1
Marbofloxacin	> MRL	<<1	1.48	18	> MRL	<<1
Flumequine	> MRL	<<1	1.08	18	> MRL	<<1
Oxolinic Acid	> MRL	<<1	8.03	3	> MRL	<<1
Norfloxacin	> MRL	<<1	0.67	39	> MRL	<<1
Sarafloxacin	> MRL	<<1	0.49	64	> MRL	<<1
Ofloxacin	> MRL	<<1	1.29	23	> MRL	<<1
Ampicillin	> MRL	<<1	> MRL	<<1	2.97	100
Amoxicillin[a]	> MRL	<<1	> MRL	<<1	3.34	93
Benzylpenicillin	> MRL	<<1	> MRL	<<1	1.86	153
Cefazolin[a]	> MRL	<<1	> MRL	<<1	16.71	23
Cefoperazone[a]	> MRL	<<1	> MRL	<<1	1.86	296
Ceftiofur[a]	> MRL	<<1	> MRL	<<1	9.28	48
Cephapirin[a]	> MRL	<<1	> MRL	<<1	4.45	81
Cloxacillin	> MRL	<<1	> MRL	<<1	4.45	83
Nafcillin[a]	> MRL	<<1	> MRL	<<1	33.41	11

[a] Cross-reactivity (*CR*) provided by UNISENSOR

Accuracy of the multianalyte ELISA in milk

A battery of blind spiked milk samples was measured according to the protocol described in "Experimental." As can be observed in Fig. 4 (right graph), excellent correlation between the spiked and measured values was obtained in this case. Moreover, as can be observed, the slope values are now close to 1, indicating that the composition of the buffer was one of the reasons for the underestimation observed when samples were prepared in buffer. The presence of Ca^{2+} ions in milk has now favored the formation of the fluoroquinolone complexes that are better recognized by the antibody. The multianalyte ELISA reported here is able to discriminate the type of antibiotic present in the sample and to provide accurate results in respect of the concentration in milk. With a coefficient of correlation of $R^2 \sim 0.99$ and all slopes close to 1, we can conclude that the multianalyte ELISA reported here can be an excellent tool for screening residues of antibiotics in milk samples.

Semiquantitative multianalyte ELISA

Finally, for screening purposes the multianalyte protocol was modified with a double objective: (1) to increase the efficiency of a single ELISA microplate by reducing the number of wells used for the calibration curves and (2) to ensure that also antibiotics with different CR profiles or MRL values would be detected. For this purpose, representative compounds with different CR values for each antibiotic family were selected and used as controls at their respective MRL concentrations. Thus, instead of using standard curves of cocktail An for each section of the microplate, the multianalyte ELISA now used just a discrete number of positive controls. Particularly, SCP (2% CR; 100 μg L^{-1} MRL) and SMZ (58% CR; 100 μg L^{-1} MRL) were used for sulfonamide antibiotics, ERX (79% CR; 100 μg L^{-1} MRL) and DFX (85% CR, prohibited) were employed as controls for fluoroquinolone antibiotics and AMP (AMP, 100% CR; 4 μg L^{-1} MRL) was used for β-lactam residues. A negative control (B-milk) was also included. Prior the assay, all controls were appropriately diluted in D-milk to place them within the working range of the assay as described in "Experimental." Similarly, the unknown milk samples were diluted with D-milk in the corresponding proportions for each microplate section (see "Experimental"). After running the assay, the absorbance given by a particular milk sample was compared with that of the control. Absorbances below those of the controls indicated potential contamination by the antimicrobial of that particular microplate section.

Following this procedure, a study was carried out using spiked blind milk samples prepared at the Nestlé Research Center (Lausanne, Switzerland). The results obtained are shown in Table 6, where it can be observed that using this semiquantitative format, the multianalyte ELISA was able to find milk samples contaminated with antibiotics over the MRL values and also identify the type of antibiotic present. This format increases considerably the efficiency of the microplate to detect contaminated samples in compliance with European legislation. From 19 milk samples analyzed, only one false negative was obtained. Further validation studies will need to be performed to confirm if this ratio remains below the 5% of false negatives allowed by Commission Decision 2002/657/EC.

Table 6 Results obtained with the multianalyte ELISA test when measuring spiked full-fat milk samples prepared by Nestlé

Milk sample	Detection technique		
	ELISA (HTS)		
	Sulfonamide	Fluoroquinolone	ß-Lactam
GF1 (SMZ, 10 ppb)	N	N	N
GF2 (STA, 10 ppb)	N	N	N
GF3 (SCP, 10 ppb)	N	N	N
GF4 (SMZ, 25 ppb)	N	N	N
GF5 (STA, 25 ppb)	N	N	N
GF6 (SCP, 25 ppb)	N	N	N
GF7 (blank)	N	N	N
GF8 (SMZ, 50 ppb)	FP	N	N
GF9 (STA, 50 ppb)	FP	N	N
GF10 (SCP, 50 ppb)	N	N	N
GF11 (SMZ, 100 ppb)	P	N	N
GF12 (STA, 100 ppb)	P	N	N
GF13 (SCP, 100 ppb)	FN	N	N
GF20 (blank)	N	N	N
GF21 (CPX, 100 ppb)	N	P	N
GF22 (SMZ, 100 ppb) (PENG, 4 ppb) (NOX, 100 ppb)	P	P	P
GF23 (SPY, 100 ppb)	P	N	N
GF24 (CFZ, 50 ppb) (ERX, 100 ppb)	N	P	P
GF25 (AMP, 4 ppb)	N	N	P

Results are expressed as positive (*P*), negative (*N*), false positive (*FP*) and false negative (*FN*) according to MRLs established for each compound by the European Union. Antibiotics spiked in full-fat milk samples were sulfamethazine (*SMZ*), sulfathiazole (*STA*), sulfachloropyridazine (*SCP*) and sulfapyridine (*SPY*) for sulfonamides, ciprofloxacin (*CPX*), norfloxacin (*NOX*) and enrofloxacin (*ERX*) for fluoroquinolones and penicillin G (*PENG*), ampicillin (*AMP*) and cefazolin (*CFZ*) for ß-lactams
HTS high-throughput screening

Conclusions

A multianalyte ELISA for screening antibiotic residues in full-fat milk samples has been developed following the

typical planar microarray configuration where the identity of the target analyte is encoded by its location in the detection platform. The bioreceptors used as a mixture (cocktail BR) are mixed with the sample (or standard) and added to the microplate. The assay can be run in about 2 h and provides a specific response on a different microplate section depending on the antibiotic family. The specificity of the response remains even when the sample contains mixtures of the other antibiotics and is independent of their concentration. The multianalyte ELISA performs very well in milk samples without any prior sample treatment other than dilution of the sample. Two protocols have been established, one for screening purposes to find contaminated samples and another one to quantify the concentration of the antibiotic in the sample. Final evaluation of the multianalyte ELISA shows that the assay is able to give a positive response in the presence of antibiotics in milk samples, indicating additionally the type of antibiotic family and the level of contamination. CR studies demonstrate that more than 25 different antibiotics can be detected in compliance with the regulations of the EC. Further work will address miniaturization of the present immunochemical multiplexed protocol on a microarray system. Moreover, these results will be the base for developing a multianalyte immunosensor system.

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6.2.1 Multianalyte ELISA - Supporting Information

Electronic Supplementary Material

A Multianalyte ELISA for Immunochemical Screening of Sulfonamide, Fluoroquinolone and ß-Lactam Antibiotics in Milk Samples Using Class-Selective Bioreceptors

Javier Adrian, Daniel G. Pinacho, Francisco Sánchez-Baeza, M.-Pilar Marco,
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Figure S1: Standard calibration curves obtained when testing different concentrations of analytes not involved in the specific wells assay. (i.e. Interferences of AMP and CPX in the sulfonamide multianalyte assay using the immunoreagents cocktail).

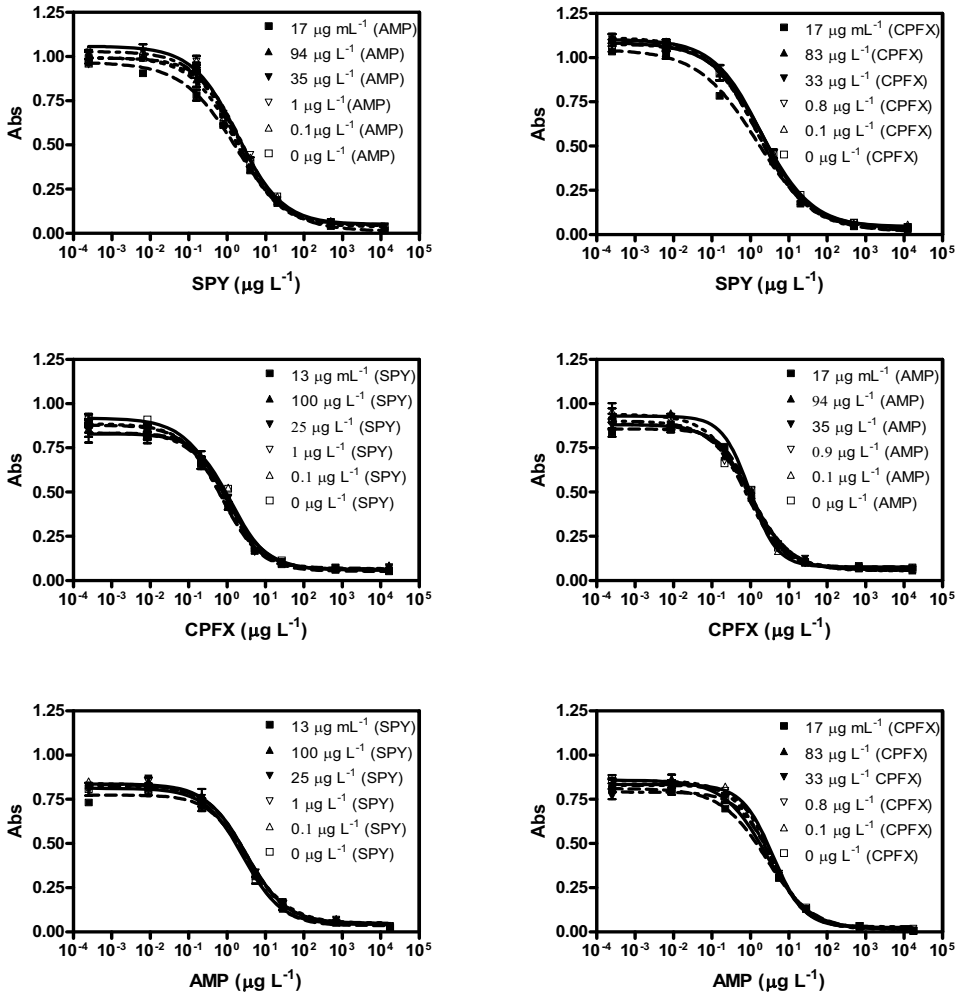
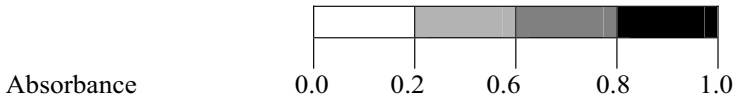


Table S1: Response observed on the distinct sections of the microplate by the antibiotics[a]

Microplate ELISA sections			**Immunoreagents**	**Analytes**
SA	**βL**	**FQ**		
■	■	■	*Abs/R cocktail*	*None (Abs max)*
	■	■	*Abs/R cocktail*	*SPY*
■	■		*Abs/R cocktail*	*CPX*
■		■	*Abs/R cocktail*	*AMP*
			Abs/R cocktail	*SPY + CPX + AMP*
			PBST (no reagents)	

[a] PBS samples containing SPY, CPX and AMP (50 μM) individually or as mixture were measured with the multianalyte ELISA to assess the specificity of the response.



6.3 Multianalyte WIObS (SAs, FQs, BLs and TCs in milk)

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Wavelength-interrogated optical biosensor for multi-analyte screening of sulfonamide, fluoroquinolone, β-lactam and tetracycline antibiotics in milk

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Major research efforts are focusing on the development of multi-analyte-residue analysis and the design of user-friendly analytical devices for continuous or on-site measurements. Advances in microtechnology and nanotechnology make possible novel analytical solutions to meet these needs. With specific bioreceptors and antibodies, immunosensors may become excellent analytical tools.

A portable wavelength-interrogated optical system (WIOS) exploits class-selective bioreceptors for simultaneous screening of the most frequently used antibiotics in the veterinary field (e.g., sulfonamides, fluoroquinolones, β-lactams and tetracyclines). The label-free sensor uses the evanescent-wave principle, by which changes in the refractive index close to the modified chip surface are detected by scanning the resonance condition at which a light wave is coupled in the waveguide through a conveniently designed grating. The bioreagents used in this study were developed to detect a wide range of congeners of each selected family of antibiotics below the maximum residue limit (MRL) values established for milk samples.

The WIOS made it possible to detect more than 30 different antibiotics and it was successfully applied to analyze different antibiotic residues in milk samples.

Keywords: β-lactam; Biosensor; Evanescent-wave sensor; Fluoroquinolone; Integrated optical sensor chip; Label-free sensing; Milk; Multi-analyte antibiotic detection; Sulfonamide; Tetracycline

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1. Introduction

The investigation of new sensing principles for the detection of molecular binding events has created great expectations in the clinical and food-safety areas. The combination of advances in the microelectronics and nanoelectronics with the potential of the immunochemical methods hassled to the development of immunosensors that provide interesting advantages as alternative analytical methods [1]. Traditionally, immunoanalytical methods have relied on labels that covalently bind to one of the molecules to display the molecular recognition event

with high detectability. However, methods requiring labeling impose extra cost due to the need to develop labeled reagents that perform appropriately in the assay (i.e. due to their hydrophobic nature some fluorescent compounds produce significant background signal). Research therefore developed label-free immunosensor devices to improve sensitivity, speed and analytical efficiency. The most widespread label-free detection systems found in the market are based on surface-plasmon resonance (SPR) [2] of a gold film [3] or localized SPR (LSPR) of gold nanoparticles [4]. Quartz-crystal microbalance [5] and impedance spectroscopy [6,7] are among other label-free detection systems. A recent review provides more information and examples [8].

It is well known that the evanescent field created by the light traveling through a waveguide can be used to probe changes in the refractive index to record binding events in real time without labels. Evanescent waves can be found in different optical configurations (e.g., total internal reflection, or surface-plasmon or waveguide mode). The advantage of this sensing approach is that part of the propagating guided light penetrates into the sensing area (where target and analyte interact) and is affected by optical changes occurring only in this region. Recently, integrated optical (IO) sensors have been developed in which light is guided in structures smaller than the wavelength of the light. In this wavelength-interrogation approach, the resonance is scanned by observing the resonance wavelength at a fixed angle of incidence. Different research groups [8–10] investigating this sensing mode have demonstrated that biorecognition events occurring at the surface can be detected using this principle. As well as flexibility, specificity, detectability and the possibility of automation, multiplexing is also a goal pursued by many researchers. There have been few reports on multi-analyte biosensor devices, but nanoscience and nanotechnology offer opportunities for direct development of analytical tools compared with microarray formats. Apart from optically-encoded microarrays, for which interesting recent examples can be found in the literature [11–13], placing different bioreceptors at different locations on a substrate produces site-encoded multiplexed devices [14,15].

To meet these demands, we present a wavelength-interrogated optical multiplexed biosensor, comprising an array of waveguide gratings, in which different bioreceptors have been immobilized with the aim of detecting a wide range of antibiotic residues in food samples. Antibiotics are chemical substances extremely active at low doses that kill or slow the growth of bacteria. Since their discovery, antimicrobials have been an essential part of human and veterinary medicine, and have being used in aquaculture or even in plants for the treatment of infectious diseases produced by bacteria. The most important impact of the misuse of antibiotics [16] relates to the development of mechanisms of bacterial resistance that cause a serious threat to public health [17], significant technological and economical problems in dairy industry [18] and have a strong negative impact on the entire ecosystem [19]. In Europe, European Community (EC) Regulation 2377/90 sets maximum residue limits (MRLs) for the majority of antibiotics in different sample tissues to control this undesirable situation.

Previously, one of our groups (CSEM) [9] reported a label-free detection technology [wavelength-interrogated optical system (WIOS)], which is based on a waveguide grating and wavelength modulation. More recently, we demonstrated the potential of this technology to analyze antibiotic sulfonamide (SA) residues in milk samples [20] by immobilizing immunoreagents specifically produced to detect a wide range of SA congeners [21]. In parallel, we demonstrated the possibility of simultaneously detecting more than 25 antibiotics from three different families in milk samples in compliance with the EC regulation, by combining bioreceptors for antibiotic SAs, fluoroquinolones (FQs) and β-lactams (BLs), using a typical planar microarray configuration. Now, we have implemented these reagents, together with new bioreagents for detecting antibiotic tetracyclines (TCs) on the WIOS platform to prove that is possible to use it for screening efficiently in food-safety laboratories.

2. Experimental

2.1. Materials

Unless otherwise indicated, chemicals and biochemicals were supplied by Sigma-Aldrich (Buchs, Switzerland). The analytes used as standards were prepared from 10 mM stocks in dimethyl sulfoxide (DMSO) [sulfapyridine (SPY); ampicillin (AMP); oxytetracycline (OTC)] and in 50 mM aqueous NaOH [ciprofloxacin (CPFX)]. We describe elsewhere [21,22] the preparation and the evaluation of immunoreagents used in this study for SA and FQ detection – hapten-protein conjugates, (SA2-OVA and 10b-BSA, respectively) and polyclonal antibodies (As155 and As171, respectively). Bioreagents for the detection of βL (CCK-BSA, hapten-protein conjugate; RSA, bioreceptor; anti RSA, polyclonal antibody from rabbit) and TC (TcDNA-Biotin; oligonucleotide; TetR, bioreceptor; anti TetR, monoclonal antibody from mouse) were obtained from Unisensor [23]. Anti-rabbit IgG (α-rIgG) and anti-mouse IgG (α-mIgG) were supplied by Jackson ImmunoResearch (Suffolk, England).

2.2. Buffers and milk samples

Phosphate-buffered saline (PBS) was 0.01 M phosphate buffer on a 0.8% saline solution (137 mmol/L NaCl, 2.7 mmol/L KCl), and the pH was 7.5. Commercial fresh milk (Cremo, Switzerland) was obtained from a grocery store. Blank and blind spiked fresh milk samples were prepared at the Nestlé Research Center (Lausanne, Switzerland) and supplied freeze-dried.

2.3. General methods and procedures

Calibration curves were fitted with a four-parameter logistic equation:

$$y = (A - B/[1 - (x/C)^{D}]) + B$$

where A is the maximal WIOS signal, B is the minimum WIOS signal, C is the concentration producing 50% of the maximal WIOS signal and D is the slope at the inflection point of the sigmoid curve using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). Unless otherwise indicated, the results given are the average and standard deviation of at least two measurements.

2.4. Instrumentation and the WIOS principle

The WIOS instrument (see Fig. 1) uses the evanescent wave of light to probe changes in the refractive index at the interface of a waveguide surface upon adsorption of biomolecules [9]. The resonance condition of a waveguide grating is scanned by wavelength modulation of a vertical cavity-surface-emitting laser (VCSEL) emitting at 763 nm and with a tunable range of 2 nm. A first grating couples the light into the waveguide layer. The waveguide mode is excited at the resonance wavelength, and propagates into the waveguide layer. The second grating couples out the guided light, which reaches photodiodes. Effective refractive-index variations of the waveguide are determined by the resonance wavelength and relate to the binding of non–labeled molecules on the waveguide grating surface [9,20]. The optical system is combined with a fluidic cell, comprising three 7.2-µL channels, allowing in situ measurements of biomolecule adsorption on the surface in real time, with detectability values smaller than 1 ng/cm^2. The WIOS instrument used for the measurements was fabricated at CSEM (Neuchâtel, Switzerland) with all components assembled in a $22 \times 10 \times 10$ cm^3 bench-top device. A data-acquisition card (National Instruments, PCI-6024E) allowed direct data analysis on a separate computer.

2.5. Biofunctionalization of the chips

Waveguide chips were supplied by Oerlikon (Balzers, Lichtenstein), and comprised $17.5 \times 17.5 \times 0.7$ mm^3 glass slides with a grating at the surface (periodicity 360 nm), coated with a 150–300 nm layer of tantalum oxide. Each chip comprised an array of 24 independent sensing zones (8 pads distributed in 3 different columns)

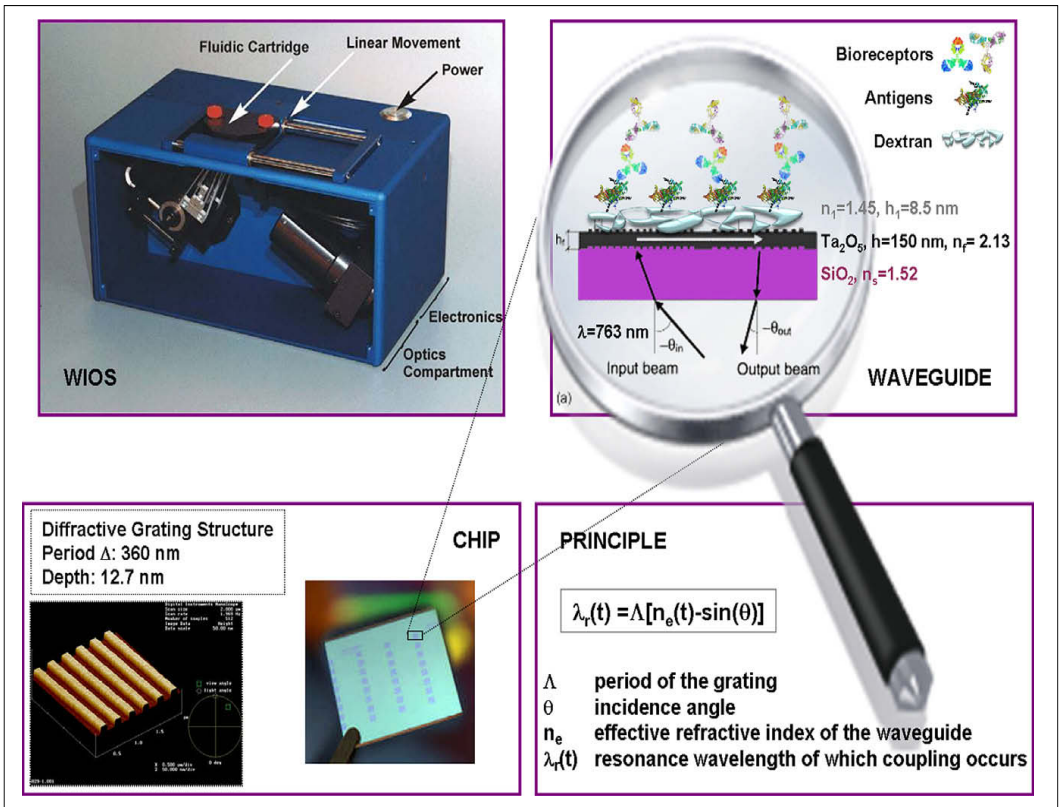


Figure 1. The wavelength-interrogated optical system (WIOS). The chip is a customized platform that contains 24 sensing sites. Each site is waveguide grating. Light from a VCSEL laser emitting at around 763 nm is incident on the first grating. The waveguide mode is excited and propagates into the waveguiding layer. The second grating sends out the guided light (at a different angle), which is collected by large plastic optical fibers. Electronics controls the laser-wavelength modulation and amplifies the signals detected. A computer acquires and processes the data.

[24]. The surface of the waveguide chips was functionalized with a photopolymerizable dextran layer (Optodex®, Arrayon Biotechnology SA, Neuchâtel, Switzerland) [25,26], which allows covalent binding of biomolecules and at the same time prevents non-specific adsorption of biomolecules. Coating antigens, SA2-OVA (100 µg/mL), 10b-BSA (200 µg/mL), CCK-BSA (25 µg/mL) and NeutrAvidin (200 µg/mL) for detection of SA, FQ, BL and TC, respectively, were covalently immobilized on the surface on the selected waveguide zones, by spotting 50 nL of printing solution on each pad with a Nanoplotter NP 2.0/E (GeSiM, Dresden, Germany) and exposing the surface to ultraviolet (UV) light. Printing solutions of antigens were prepared in 1% PBS.

2.6. General procedure for the WIOS

Before starting each measurement cycle, the cell was equilibrated with PBS (80 µL) for 5 min. For TC measurements, TcDNA-Biotin (0.1 µM in PBS, 80 µL) had to be injected and incubated in the cell for 10 min and then washed with PBS (80 µL) for 5 min. Then, milk solutions (80 µL) containing a mixture of the standards (SPY, 0.001–10,000 µg/L; CPFX 0.001–50,000 µg/L; AMP 0.001–100,000 µg/L; OTC 0.001–50,000 µg/L) or sample and specific bioreceptors and immunoreagents [SAs, As155 (50 µg/mL); FQs, As171 (50 µg/mL); βLs, RSA receptor 0.3 µM and anti-RSA antibody (50 µg/mL); TCs, TetR receptor (1.0 µM) plus anti-TetR antibody (10 µg/mL)] were injected into the cell. After incubating the mixture for 10 min, the chip surface was rinsed with PBS (80 µL) twice for 2.5 min and α-rIgG (for SAs, FQs, βLs) or α-mIgG (for TCs) solutions (50 µg/mL in PBS, 80 µL) were introduced and incubated for 5 min more. The chip was rinsed again and the signal variation resulting from the binding of α-rIgG was recorded. The surface was regenerated with 5-min exposure to 200 mM NaOH (80 µL) followed by PBS (80 µL) before starting a new measurement cycle. Regeneration was complete in all pads except for those addressed to βLs, since binding of the receptor to the antigen coating is irreversible. Measurements could be performed for each antibiotic family individually or simultaneously (see Fig. 2). When performing simultaneous measurements, the standards and the bioreagents were injected as cocktails using the same concentrations as for individual measurements. The total analysis time for analyzing an individual antibiotic family, including the regeneration step, was 30 min, except for TCs (40 min), due to immobilization of the antigen coating through neutravidin-biotin binding. For same reason, total analysis time of multi-analyte measurements was 40 min.

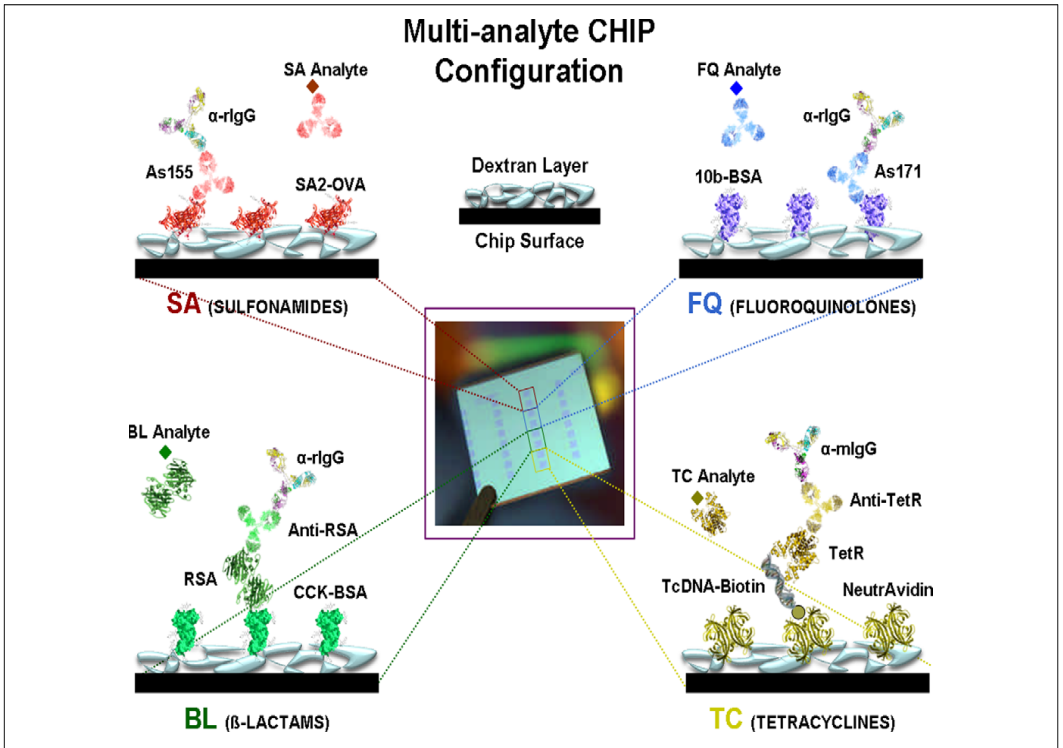


Figure 2. Assay configurations employed for the detection of sulfonamide, fluoroquinolone, β-lactam and tetracycline (SA, FQ, BL and TC). Sensor-chip areas are first coated with the correspondent antigens by using a photopolymerizable dextran layer. The pictures show the bioreagents involved in the immune/biochemical reactions taking place on each sensor pad.

2.7. Tests with milk samples

In order to evaluate the WIOS instrument for detection of antibiotics, tests were performed at the Nestlé Research Center (Lausanne, Switzerland). Milk samples were diluted five times in PBS buffer and mixed with the detection-reagent cocktail. The response signal relating to the binding of the secondary antibody (α-rIgG or α-mIgG) was compared to the response signals obtained in the absence of antibiotics and with the antibiotic standards (SPY, CPFX, OTC) at 100 µg/L according to MRLs established for each compound in milk except for AMP (MRL = 4 µg/L). As regeneration of the BL surface was not possible, the test was followed by direct exposure to a reference (non-contaminated milk) and the response signal of the sample was normalized to this reference signal.

3. Results and discussion

We developed a bench-top biosensor for fast screening of antibiotic residues in milk at the point of demand (farm, collection truck or dairy) or in the laboratory (see Fig. 1). The system uses an optical transducer based on evanescent wave combined with class-selective bioreceptors designed to recognize a wide variety of antibiotics. We have already shown this biosensor system to be efficient for the detection of SA antibiotics in milk samples in compliance with EC regulations [20]. By using generic antibodies produced after careful hapten design, it was possible to detect up to 11 SA congeners [21,27]. The success of this study led to the development of a biosensor for the simultaneous detection of the most frequently used antibiotics in the veterinary field. For this purpose, we produced bioreagents to recognize the most important antibiotic families (SA [21], FQ [22], BL [28] and TC [29]) on the optical transducing system. Previously, we had demonstrated that these bioreceptors could be used in combination to detect more than 25 different antibiotics simultaneously on a multi-analyte microplate-based enzyme-linked immunosorbent assay (ELISA) [30].

Haptenized proteins for SAs (SA2-OVA), FQs (10b-BSA), βLs (CCK-BSA) and TCs (TcDNA-biotin) were immobilized on the surface of a chip with an array of 24 independent sensing zones (8 pads distributed in 3 different columns). Site-directed biofunctionalization was

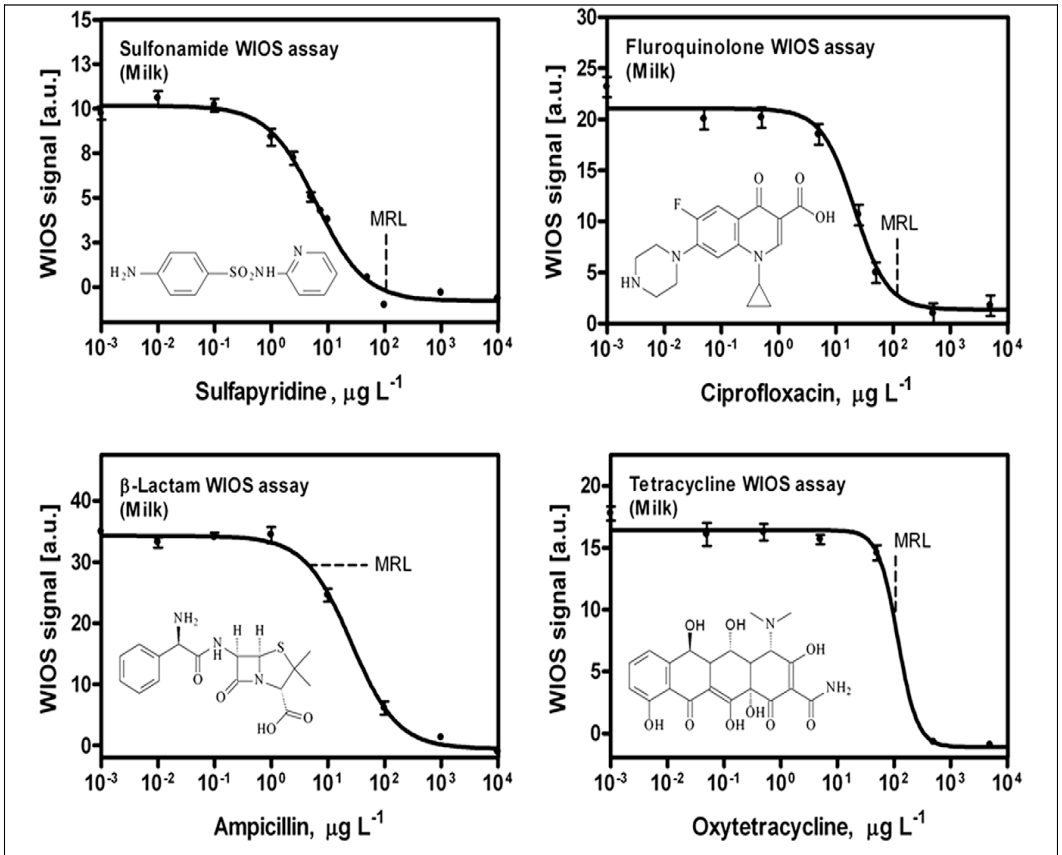


Figure 3. Standard curves recorded for each family of antibiotics in milk. The chemical structures correspond to the representative congeners used to build the standard curves for each family of antibiotics. Table 1 summarizes the parameters of each curve.

accomplished with a nanospotter via a photoactivable dextran polymer (Optodex®). All bioconjugates were directly immobilized, except for TCs, for which biofunctionalization was accomplished through neutravidin followed by binding of TcDNA-biotin conjugate (see Fig. 2). The chip was placed on the flow chamber of the device in which all the measurements were made.

All bioreceptor and immunoreagent systems worked under competitive configurations on indirect formats. Each analyte had to compete with the antigen coating for the corresponding antibody-bioreceptor binding sites. Only TC detection was based on a slightly different principle, since, in this case, the TC receptor (TetR) could bind a defined DNA sequence in the absence of TC [31]. In most cases, there was significant enhancement using a secondary antibody (anti-rabbit IgG for SAs, FQs and βLs and anti-mouse for TCs).

Regeneration of waveguide-sensing surfaces was studied for each antibiotic family by testing several agents (acid, basic, hydro-alcoholic and surfactant media). The aim was to find out conditions for regeneration that would not affect activity of the immobilized reagents. Regeneration would decrease both time and cost per assay, while avoiding the need for calibration on each measurement. Flowing a 200 mM NaOH solution for 5 min efficiently regenerated all the surfaces without damaging the biofunctionalty of the immobilized bioreagents. Only the βL-sensing sites could not be regenerated due to the covalent binding of the receptor (RSA) to the haptenized protein (CCK-BSA), which makes formation of this complex irreversible. Regeneration efficiency was verified for the other pads by considering the variations in the biosensor response after several consecutive measurement cycles. Sensing sites for SA and FQ showed variations <10% after regeneration (n = 10 cycles), whereas the TC assay showed a slight decrease in the final signal in subsequent measurements due to a partial destruction of the NeutrAvidin dextran layer. Although we had found that a 100 mM NaOH solution was sufficient for this system, after considering the multi-analyte purpose of this system, we selected 200 mM NaOH for regeneration.

Calibration curves were obtained for each individual bioreceptor system after optimizing the assay conditions with respect to the maximum signal observed in the absence of antibiotic and to the inhibition of the signal at the MRL value [MRLs for bovine milk according to the EC Regulation EC2377/90: SPY 100 μg/kg; CPFX (value corresponds to the sum of enrofloxacin and CPFX according to EC Regulation EC2377/90), 100 μg/kg; AMP, 4 μg/kg; OTC, 100 μg/kg]. The conditions of use of the biosensor system were initially evaluated in buffer and subsequently using blank milk samples. Fig. 3 shows the calibration curves recorded on each sensor pad when measuring milk samples, and Table 1 shows the features of the WIOS system for each antibiotic family. As can be observed, the IC_{50} values and the limits of detection (LODs) accomplished were low enough for antibiotic-residue analysis. Data presented is the average of measurements made on six different grating pads. Although these values are slightly higher than the ones reported with the same reagents measured with ELISA or dipsticks [21,22,29,30], they are still below MRL values established for the EC. Moreover, as previously reported [30,32], the wide spectrum of selectivities showed by the bioreceptors used in this study allows us to envisage the possibility of detecting the presence of more than 30 different antibiotics with the multi-analyte WIOS approach (see Table 2).

Compatibility and absence of shared recognition between all reagents was tested by recording the signal on each pad when each set of bioreceptors was individually tested on each functionalized zone of the sensor chip. The results shown in Fig. 4 demonstrate that the signal recorded in the absence of antibiotic is specific on each

Table 1. Features of the individual wavelength-interrogated optical system (WIOS) calibration assays[a] in whole milk

Parameter / Assay	Assay values			
	Sulfonamide	**Fluoroquinolone**	**β-lactam**	**Tetracycline**
Analyte	Sulfapyridine	Ciprofloxacin	Ampicillin	Oxytetracycline
Coating antigen	SA2-OVA 100 μg/mL	10b-BSA 200 μg/mL	CCK-BSA 25 μg/mL	NeutrAvidin 200 μg/mL
Oligonucleotide	- - - - -	- - - - -	- - - - -	TcDNA-Biotin 0.1 μM
Receptor	- - - - -	- - - - -	RSA 0.3 μM	TetR 1.0 μM
Antibody	As155 50 μg/mL	As171 50 μg/mL	anti RSA 50 μg/mL	anti TetR 10 μg/mL
α-rIgG, α-mIgG	50 μg/mL	50 μg/mL	50 μg/mL	50 μg/mL
$Signal_{min}$	0.7 ± 0.2	0.7 ± 0.1	0.5 ± 0.1	1.1 ± 0.1
$Signal_{max}$	9.2 ± 0.2	21.1 ± 0.3	34.1 ± 1.5	16.4 ± 1.4
Slope	−1.04 ± 0.03	−1.14 ± 0.01	−1.06 ± 0.27	−2.40 ± 0.02
IC_{50}, μg/L	6.3 ± 0.6	19.7 ± 4.8	26.2 ± 2.6	114.8 ± 2.7
Dynamic range, μg/L	1.4 ± 0.2 to 26.4 ± 0.9	4.4 ± 0.5 to 70.3 ± 13.7	7.1 ± 3.1 to 100.0 ± 11.3	56.8 ± 7.9 to 193.3 ± 13.4
LOD (IC_{90}), μg/L	0.5 ± 0.1	1.3 ± 0.2	3.1 ± 0.2	34.2 ± 5.4
R^2	0.991 ± 0.004	0.991 ± 0.003	0.992 ± 0.008	0.993 ± 0.005

[a]Values obtained correspond to the average and standard deviation of at least 6 pad replicates from the same chip.

Table 2. Interference presented by different related compound of each antibiotic family on the multi-analyte wavelength-interrogated optical system (WIOS) assay in milk

Analyte	SA coating (SA2-OVA)		FQ coating (10b-BSA)		BL coating (CCK-BSA)		TC coating (NeutrAvidin)	
	IC_{50}[c]	LOD[c]	IC_{50}[c]	LOD[c]	IC_{50}[c]	LOD[c]	IC_{50}[c]	LOD[c]
Sulfapyridine	*6.3*	*0.5*	-----	*> MRL*	-----	*> MRL*	-----	*> MRL*
Sulfaquinoxaline	77.1	3.9	-----	> MRL	-----	> MRL	-----	> MRL
Sulfachloropyridazine	73.3	4.7	-----	> MRL	-----	> MRL	-----	> MRL
Sulfisomidine	6.6	0.8	-----	> MRL	-----	> MRL	-----	> MRL
Sulfathiazole	1.7	0.5	-----	> MRL	-----	> MRL	-----	> MRL
Sulfadiazin	15.0	1.5	-----	> MRL	-----	> MRL	-----	> MRL
Sulfadimethoxine	69.8	3.2	-----	> MRL	-----	> MRL	-----	> MRL
Sulfamerazine	5.8	0.4	-----	> MRL	-----	> MRL	-----	> MRL
Sulfamethoxypyridazine	5.1	0.3	-----	> MRL	-----	> MRL	-----	> MRL
Sulfamethazine	3.3	0,1	-----	> MRL	-----	> MRL	-----	> MRL
Trimetroprim	-----	> MRL	-----	> MRL	-----	> MRL	-----	> MRL
Ciprofloxacin	-----	*> MRL*	*20.0*	*1.3*	-----	*> MRL*	-----	*> MRL*
Enrofloxacin	-----	> MRL	30.1	2.0	-----	> MRL	-----	> MRL
Danofloxacin	-----	> MRL	196.1	12.7	-----	> MRL	-----	> MRL
Difloxacin	-----	> MRL	27.1	1.8	-----	> MRL	-----	> MRL
Marbofloxacin	-----	> MRL	121.5	7.9	-----	> MRL	-----	> MRL
Flumequine	-----	> MRL	87.6	5.7	-----	> MRL	-----	> MRL
Oxolinic acid	-----	> MRL	525.6	34.2	-----	> MRL	-----	> MRL
Norfloxacin	-----	> MRL	49.4	3.2	-----	> MRL	-----	> MRL
Sarafloxacin	-----	> MRL	36.3	2.4	-----	> MRL	-----	> MRL
Ofloxacin	-----	> MRL	94.8	6.2	-----	> MRL	-----	> MRL
Ampicillin	-----	*> MRL*	-----	*> MRL*	*34.1*	*3.1*	-----	*> MRL*
Amoxicillin[b]	-----	> MRL	-----	> MRL	38,3	3.5	-----	> MRL
Benzylpenicillin	-----	> MRL	-----	> MRL	21,3	1.9	-----	> MRL
Cefazolin[b]	-----	> MRL	-----	> MRL	192,9	17.5	-----	> MRL
Cefoperazone[b]	-----	> MRL	-----	> MRL	21,3	1.9	-----	> MRL
Ceftiofur[b]	-----	> MRL	-----	> MRL	106,4	9.7	-----	> MRL
Cephapirin[b]	-----	> MRL	-----	> MRL	52,5	4.8	-----	> MRL
Cloxacillin	-----	> MRL	-----	> MRL	50,1	4.6	-----	> MRL
Nafcillin[b]	-----	> MRL	-----	> MRL	374,4	34.0	-----	> MRL
Oxytetracycline	-----	*> MRL*	-----	*> MRL*	-----	*> MRL*	*114.8*	*34.2*
Chlortetracycline[b]	-----	> MRL	-----	> MRL	-----	> MRL	119.4	35.6
Doxycycline[b]	-----	> MRL	-----	> MRL	-----	> MRL	55.4	16.5
Methacycline[b]	-----	> MRL	-----	> MRL	-----	> MRL	73.5	21.9
Tetracycline[b]	-----	> MRL	-----	> MRL	-----	> MRL	147.8	44.0

Cross-reactivity values were extracted from ELISA experiments performed with the same bioreagents [30], except [b], for which the cross-reactivity was provided by Unisensor. [c] Results are expressed in µg/L.

sensor pad. Moreover, the maximum signal recorded on each individual pad is comparable to that obtained when the bioreceptors are used as a cocktail on the multi-analyte system. However, combination of several analytes included in Table 2 resulted in a greater signal decrease in the corresponding sensing sites for antibiotic families (data not shown). These results are in agreement with the previously reported data using these bioreceptors on the multi-analyte microplate-based ELISA [30].

3.1. Multi-analyte-assay evaluation and tests

Simultaneous detection of four families of antibiotics was performed in milk using the WIOS instrument in combination with the bioreagents tested independently. The assay comprised mixing the contaminated milk with the cocktail of bioreagents containing the specific antibodies and bioreceptors and letting them react with the surface-immobilized reagents (competitive step). The response signal was recorded after amplification with a secondary reagent (α-rIgG or α-mIgG), and, in some cases, the surface was regenerated with 200 mM NaOH (not possible for BL detection). By normalizing the signal, the reproducibility of the assays was better than 10%.

The final evaluation of the WIOS as a bench-top screening method consisted of analysis of blind milk samples prepared at the Nestlé Research Centre (Lausanne, Switzerland) using the multi-chips proposed. Prior to the

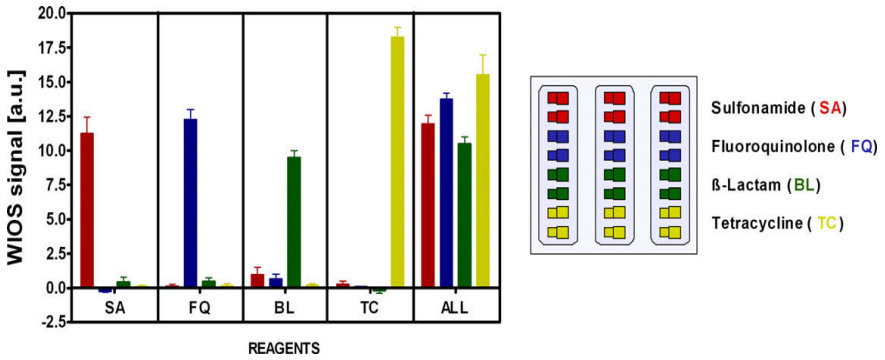
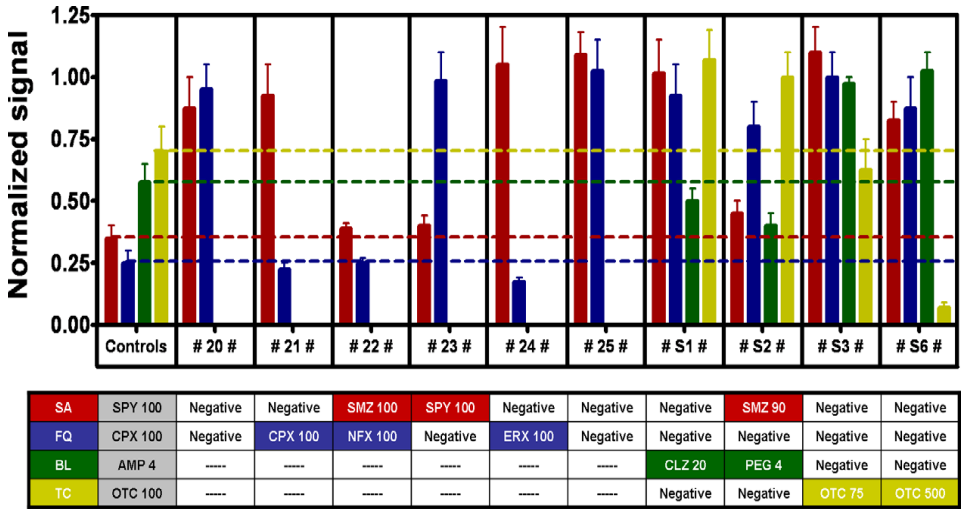


Figure 4. Results from the shared recognition experiments show that the response of each sensor pad is specific for each bioreagent system. Bars show the WIOS response of the bioreagents, used individually or as mixtures in chips coated with different antigens. The color indicates the biosensor signal obtained for each sensing section, while the horizontal axes give information about the reagents used each time.



SA	SPY 100	Negative	Negative	SMZ 100	SPY 100	Negative	Negative	Negative	SMZ 90	Negative	Negative
FQ	CPX 100	Negative	CPX 100	NFX 100	Negative	ERX 100	Negative	Negative	Negative	Negative	Negative
BL	AMP 4	-----	-----	-----	-----	-----	-----	CLZ 20	PEG 4	Negative	Negative
TC	OTC 100	-----	-----	-----	-----	-----	-----	Negative	Negative	OTC 75	OTC 500

Figure 5. Preliminary evaluation of the multi-analyte WIOS-biosensor configuration as a screening method. For each sample, results are expressed in column bars by representing the sensor response for each sensing zone. The signal obtained is compared with the corresponding reference controls. Signals below the control values indicate potential antimicrobial contamination and *vice versa*. Blind samples were prepared at the Nestlé Research Center (Lausanne, Switzerland). Antibiotics used to prepare the samples and their concentrations(µ/L) are indicated in the bottom square (SMZ, Sulfamethazine; SPY, Sulfapyridine; CPX, Ciprofloxacin; NFX, Norfloxacin; ERX, Enrofloxacin; CLZ, Cefalozin; PEG, Benzylpenicillin; OTC, Oxytetracycline).

assay, a blank sample and positive controls for each antibiotic family were analyzed with the WIOS to calibrate the system. Then, after running the assay, the response signal given by a particular milk sample was compared with that of the controls. Signals below those of the controls indicated potential contamination by the antimicrobial family and vice versa. Using this approach, 10 fresh milk samples were analyzed with the WIOS system. Fig. 5 shows the results, which indicate that, in one assay, the WIOS system could discriminate milk samples contaminated with antibiotics over the MRL values and could identify the type of the antibiotic family present.

4. Conclusions

We developed a label-free biosensor as a screening method for antibiotic-residue detection in the food-safety field. The WIOS system combined with generic bioreagents against antibiotics SA, FQ, BL and TC can detect 34 antimicrobials in milk samples in compliance with EC regulations. The results shown indicate that the WIOS system is a promising candidate for fast screening of antibiotics in milk. No sample treatment is necessary to obtain reliable data regarding the potential presence of these families of antibiotics.

At present, a whole analysis of a sample with the WIOS takes 30 min for the individual and SA/FQ combined chips, including regeneration of the sensing surface. By contrast, the entire cycle for the multi-analyte SA/FQ/BL/TC chip takes 40 min as it is not yet possible to regenerate the BL assay. However, we believe that the entire assay time in both configurations could possibly be reduced by a factor of 2.

In addition, further optimization of the assay should improve the reproducibility between different chips and homogenize the LODs of all antibiotics.

The WIOS system, which used bioreagents previously implemented in other formats or analytical techniques, seems to be suitable for automated on-site determinations (i.e. on the farm before loading milk into the truck). However, further improvements are needed to develop a field instrument (e.g., fluid handling, chip fabrication and functionalization costs, and data processing). We are currently focusing on the design of a fluidic cartridge, including temperature control and optimization of the bioreagent conditions, to accomplish common regeneration of the sensor zones for all the antibiotic families.

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6.4 Results and discussion

This chapter reports the research performed in relation to the establishment of multiplexed analytical platforms to detect simultaneously different antibiotic families in milk samples.

6.4.1 Multianalyte ELISA Test.

6.4.1.1 Standardize individual assays

On a first instance the objective was to accomplish individual assay protocols working under the same conditions (i.e. buffer, number of incubation steps and periods, etc.) and reaching the necessary detectability according to the EC regulations. Main efforts were addressed to adapt the ELRA assay, since as mentioned above, included an additional step due to the use of the receptor (RSA) and a specific antibody (anti-RSA). After investigating several strategies, we found that combining the RSA and anti-RSA in a single step allowed obtaining an assay with a very good detectability and the same number of steps as the SAs and FQs immunoassays (see **Fig. 1** in **Section 6.1.2.1**). Adjusting time, buffer composition and reagents concentrations of all three individual assays was also addressed in order to have compatible settings and conditions on the final multianalyte configuration.

6.4.1.2 Evaluation of shared reactivity

The next important task was driven to assess the orthogonality of the bioreagents selected in order to ensure that each antigen immobilized on the plate was only recognized by its antibody/receptor counter partner. If more than one recognition bioelement would bind to an immobilized haptenized protein, it would result on interferences on the recorded signal that would affect the accuracy of the assay (see **Figure 6.5**).

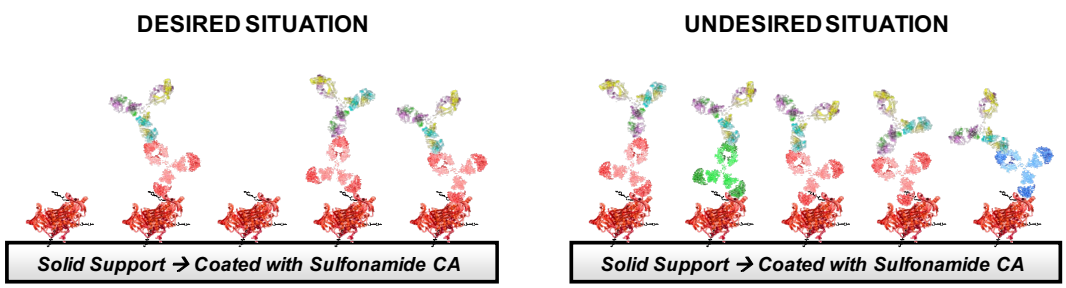

Figure 6.5: Concept of shared reactivity.

Thus, the first objective was to study the avidity and selectivity of the different bioreceptors for their respective immobilized coating antigens when they are added mixed as a cocktail on the microwell plates. Initially, qualitative experiments were planned to compare the immunoassay signals obtained from the individual assays (i.e. SAs, FQs or BLs reagents) to those where the reagents from different antibiotic families were combined in pairs (i.e. SAs/FQs, SAs/BLs or FQs/BLs) and on a second step all mixed as a cocktail (i.e. SAs/FQs/BLs), but in absence of the target analytes. As a result of these experiments, it was observed that in all cases, the antibiotic bioreagents had the same avidity for their correspondent coating antigens independently of whether they were used individually or combined. No shared-reactivity was observed due to the use of a mixture of bioreceptors (see **Table 2** in **Section 6.2**). Next objective was addressed to test the absence of cross-recognition of the different antibiotic analytes when using the bioreceptor cocktail. As expected, the results obtained showed that each antibiotic inhibited specifically the binding of the corresponding bioreceptor to its coating antigen, indicating that the bioreceptors cocktail maintained the same specificity pattern. Same results were obtained when mixtures of antibiotics were mixed with the cocktail and added to the coated microplate (see **Table S1** in **Section 6.2.1**). Thus, after all these preliminary experiments, we were able to postulate the possibility to build orthogonal systems using antibodies, receptors and the corresponding secondary reagents in buffer.

6.4.1.3 Individual assays vs. Multianalyte experiments

Next set of experiments were addressed to determine if the response of the multianalyte ELISA was the same as the individual ELISA analyzing samples containing mixtures of antibiotics. For this purpose, calibration curves were prepared in buffer using standards containing the individual analytes or equimolecular mixtures of antibiotics, and measured on the multiplexed ELISA. The features of the calibration curves obtained in the multianalyte ELISA were very similar to those obtained measured with the individual assays (see **Fig. 3** in **Section 6.2**). Final experiments were planned to asses if the response of the multianalyte ELISA was the same for each antibiotic family independently of the concentration ratio of the other analytes present in the sample. The study was planned to evaluate if high concentrations of antibiotics (over their respective MRLs), could interfere the quantification of each individual antibiotic. With this purpose, calibration curves were prepared for each antibiotic in the presence of increasing concentrations of one of the antibiotics of the other two antibiotic families. As it can be observed in **Figure S1** (**Section 6.2.1**), in all cases the standard curves were identical independently of the concentration of the non-related analyte which ensured the viability of the multianalyte ELISA approach.

6.4.1.4 Performance of the multianalyte ELISA test in milk samples

Bovine full-fat milk tested free of antibiotics, provided again by AESAN, was used to evaluate the multianalyte ELISA test. To assess potential nonspecific interferences in the multianalyte approach, milk was diluted several times with milliQ water and used to prepare the corresponding standard curves. **Fig. 5** in **Section 6.2** shows the calibration curves obtained using milk samples diluted 5-times with milliQ water, SPY, ciprofloxacin (CPX) and ampicillin (AMP) were selected as representatives of the three antibiotic families. In this case, features of the optimized standard calibration curves prepared for the diluted milk were almost identical to those obtained in buffer. The accuracy and reliability of this multiplexed bioanalytical method was also demonstrated by analyzing blind samples prepared in PBS and milk (see **Fig. 4** in **Section 6.2**). Samples were spiked individually or with mixtures of representative congeners of the three antibiotic families (SPY, CPX and AMP). The results demonstrate that the multianalyte ELISA was able to unequivocally identify the type (antibiotic family) of analyte/s present in the samples and to quantify them accurately.

Results obtained show the generic character of the assay since a considerable number of antibiotics from the three antibiotic families were detected in compliance with current regulations of the EC. Finally, simultaneous detection of the antibiotics from the three families was verified by analysing blind milk samples within a workshop meeting performed at NRC confirming the potential of the multiplexed ELISA developed to be used as screening tool on antibiotic residue analysis (see **Table 6** in **Section 6.2** and **Section 6.5.2**).

6.4.2 WIOS

The multiplexed biosensor platform was developed in collaboration with CSEM by using the same optical transducer as for the SAs (see **Chapter 5**), but implementing this time additional bioreceptors produced for other antibiotic families by AMRg and UNISENSOR, based on the experience and knowledge acquired while developing the multianalyte ELISA.

6.4.2.1 Individual antibiotic detection using WIOS

The performance of the bioreagents for the different antibiotic families selected (FQs, BLs and TCs) were initially tested individually in the WIOS platform, following a similar strategy as for the SAs (see **Chapter 5**). Thus, several waveguide chips were functionalized with the already described OptoDex® photopolymerizable dextran layer using the same coating equipment (see **Section 5.3.1.1**). For each assay, optimum

concentrations of the reagents were previously selected through 2D checkerboard experiments by measuring the binding of several bioreceptor solutions to the surface of the chips functionalized covalently with different concentrations of the antigen (see **Figure 6.6**).

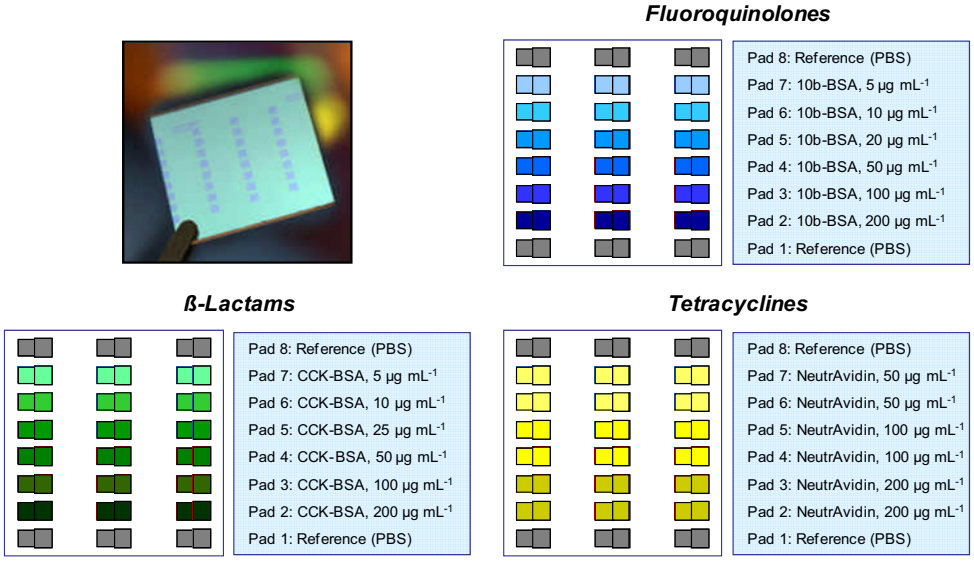

Figure 6.6: Picture of an example sensor array together with schematic configurations of chips coated with different antigen concentrations.

Finally, coating antigens 10b-BSA (200 µg mL^{-1}), CCK-BSA (25 µg mL^{-1}) and NeutrAvidin (200 µg mL^{-1}) were covalently immobilized individually on the surface of different waveguide chips for FQs, BLs and TCs antibiotic detection, respectively. The FQs WIObS was based exactly on the same immunochemical configuration than the SAs biosensor. Regeneration of the surface was also successfully accomplished with 5 min exposure to 200 mM NaOH followed by PBS before starting a new measurement cycle. Regeneration efficiency was verified by considering the variations in the biosensor response after several consecutive measurement cycles. The chips showed a variation of the signal below 10% on the different measurement cycles (n = 10). Total analysis time including the regeneration step was about 35 minutes. As occurred with the multianalyte ELISA, the development of the BLs WIObS required again mixing the RSA and anti-RSA compounds in the competitive step (see **Section 6.4.1.1**) in order to have the same steps as the other assays in the final multianalyte configuration. In this case, the analyte competes with the receptor instead of the antibody as it happens with the SAs and FQs analysis. Moreover, none of the surface regeneration treatments tested (e.g. acid, basic, hydro-alcoholic and surfactant media) succeeded to regenerate the BLs chips due to the covalent interaction produced between the CCK-BSA and the RSA [10, 12]. Thus,

calibration of the biosensor had to be achieved by using several chips instead of regenerating the sensing chip areas. Contrary to the SAs, FQs and BLs biosensor chips, where all bioconjugates were directly immobilized to the sensing chip zones, biofunctionalization of TCs chip was accomplished through NeutrAvidin followed by binding of TcDNA-Biotin conjugate. Thus, detection of TCs was based on the ability of the TC receptors (TetR) to bind the defined DNA sequence in the absence of the analyte [13]. Here, regeneration of the surface was successfully accomplished with 5 min exposure to 100 mM NaOH followed by PBS before starting a new measurement cycle. Again chips showed less than 10% signal variation between the different measurement cycles (n = 10). As it happened with the SAs WIOS assay (see **Section 5.2**), a significant enhancement was observed in FQs, BLs and TCs cases by using a secondary antibody (anti-rabbit IgG for FQs and BLs and anti-mouse IgG for TCs). Under these conditions the detectability accomplished was slightly lower than with the ELISA, but in all cases allowed measuring these antibiotics below the MRL values established by the EC (see **Figure 3** and **Table 1** both included in **Section 6.3**, and also **Table 1.5** in **Section 1.3**).

6.4.2.2 WIOS specificity studies

The wide spectrum of selectivities showed by the bioreceptors used in this study allows envisaging the possibility to detect the presence of more than 30 different antibiotics with the multianalyte WIOS approach. Although, not all of them were evaluated at this preliminary stage of the biosensor development those that were tested showed the same relative responses as in the ELISA demonstrating again that selectivity parameters are mainly determined by the bioreagents used and not by the detection principle (see **Table 2** in **Section 6.3**).

6.4.2.3 Multicoating Experiments

Future advances on biosensor development are mainly addressed to improve the transducer technology to build integrated units able to perform multianalyte analysis. In this sense, the WIOS system allows fast and accurate multi-channel measurements by combining non-mechanical scanning approach with multisensing photochips. Each chip comprises an array of 24 independent sensing zones (8 pads distributed in 3 different columns) for the simultaneous immobilization of different (bio)components (**Figure 2** in **Section 6.3**). Thus, our first goal was addressed to adapt the individual WIOS system, described previously in **Section 6.4.2.1**, to the multiplexed WIOS configuration in order to have a system able to screen simultaneously the 4 antibiotic families in milk. This goal was addressed based on the excellent results obtained on the previous studies performed combining SAs, FQs and BLs bioreagents in the multiELISA (see **Section 6.2**).

6.4.2.3.1 SA and FQ multi WIOS chips

First studies were focussed on combining the SAs and FQs bioreagents since in both cases the determination of these antibiotics is based on immunochemical indirect configurations. The chips were functionalized by spotting the corresponding antigens (SA2-OVA and 10b-BSA) on different sensing areas using the external pads as references (see **Figure 6.7**).

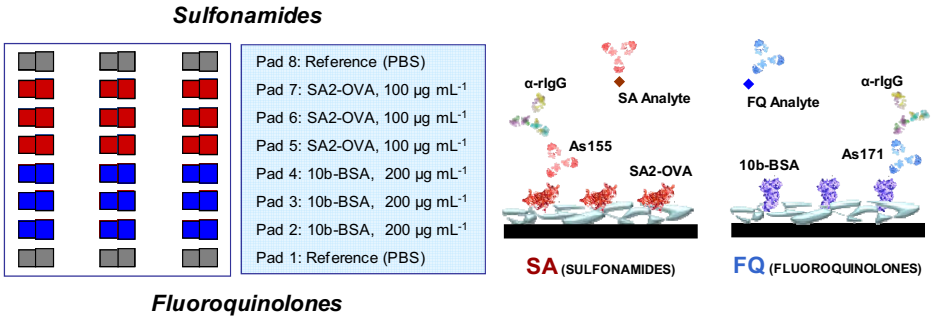

Figure 6.7: Scheme of a chip functionalized with the optimized concentrations of the corresponding sulfonamide and fluoroquinolone antigens. Reference pads were only treated with PBS.

Compatibility and absence of shared recognition between all reagents was tested by recording the signal on each pad when each set of bioreceptors was individually tested on each functionalized zone of the sensor chip. Results shown in **Figure 6.8** demonstrate that the signal recorded in the absence of antibiotic is specific on each sensor pad.

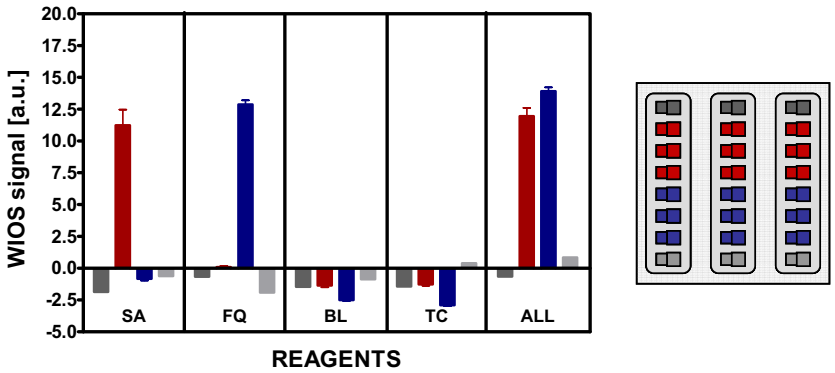

Figure 6.8: Shared recognition experiments show that the response of each SAs (red) and FQs (blue) sensor pads is specific for each bioreagent system. The colour indicates the biosensor signal obtained for each sensing section while the horizontal axes give information about the reagents used each time. Each section indicates the response recorded on each group of sensor pads when a particular bioreagent solution (SAs and FQs antibodies or BLs and TCs bioreceptors) flowed through the cell were the sensor chip was placed. The section named "Cocktail" shows the response obtained when a mixture of bioreagents was used.

Moreover, the maximum signal recorded on each individual pad is comparable to that obtained when the bioreceptors are used as cocktail on the multianalyte system. Best experimental conditions to analyze both SAs and FQs in milk samples are summarized in **Table 6.1**.

Table 6.1: Measurement conditions used on the SA and FQ WIOS system to analyze milk samples[a].

Assay Step	Solution Injected (Concentration)	Incubation Time
Equilibration (80 µL)	PBS (10 mM)	5 minutes
Competition in Milk (80 µL)	As155 (50 µg mL^{-1})/As171 (50 µg mL^{-1}) SPY/CPX samples; ≠ concentration	10 minutes
First Washing (80 µL)	PBS (10 mM)	2.5 minutes
Second Washing (80 µL)	PBS (10 mM)	2.5 minutes
Signal Amplification (80 µL)	α-rIgG (50 µg mL^{-1})	5 minutes
Washing (80 µL)	PBS (10 mM)	5 minutes
Regeneration (80 µL)	NaOH (200 mM)	5 minutes
Begin the cycle again	**For each sample measurement**	**35 minutes**

[a]*The sensor pads were spotted with SA2-OVA (SAs) and 10b-BSA (FQs) as it is shown in the scheme of* ***Figure 6.2****. The milk samples were then mixed with a solution containing a mixture the corresponding antibodies, As155 (SAs) and As171 (FQs) at the appropriate concentrations. Then the measurement cycle proceeded as described in the table.*

The whole analysis time was 30 min including regeneration of the sensing surface, and the samples could be tested for the presence of both SAs and FQs residues. Reproducibility of the measurements using these combined chips was very similar to that observed with the corresponding individual WIOS experiments (coefficients of variation below 10%). With these results, on a second step we faced the investigation of the WIOS system combining these immunoreagents with the protein bioreceptors addressed to BLs and TCs detection.

6.4.2.3.2 SAs, FQs, BLs and TCs multi WIOS chips

The different pads of the chip were biofunctionalized as it is shown in **Figure 6.9** with the different competitor bioconjugates with a spotter and using the photoactive Optodex polymer (**Figure 2** in **Section 6.3**). The milk samples were mixed with cocktail of immunoreagents and bioreceptors and measured following the sequence described in **Table 6.2**. No cross-reactivity or shared-recognition was observed from the WIOS response recorded, which confirms the specificity of the reagents (see **Fig. 3** in **Section 6.2**), which pointed to the possibility to perform simultaneous specific measurements of the four antibiotic families on a single run and using a single chip.

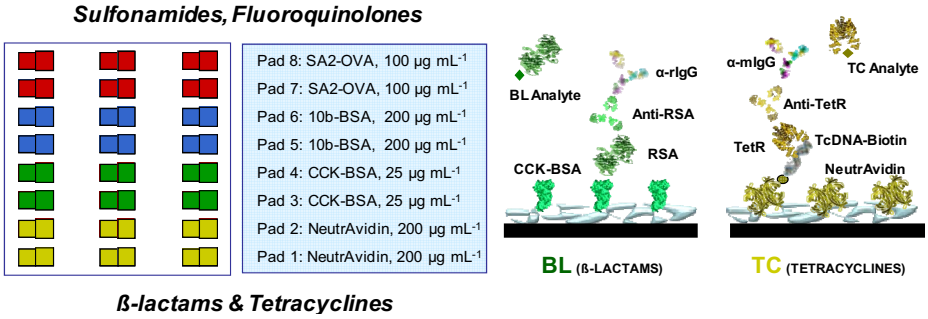

Figure 6.9: Scheme of a chip functionalized with the optimized concentrations of the corresponding SAs, FQs and BLs competitor bioconjugates. For the case of the TCs, the corresponding pads were biofunctionalized with Neutravidin.

Based on the previous studies performed with these bioreceptors and due to the generic character of most of them, we envisaged that up to 34 antimicrobials could be screening at the same time in milk samples in compliance with EC regulations (see **Table 2** on **Section 6.3**).

Table 6.2: Sequence of steps of each measurement cycle on the multiplexed WIObS system[a].

Assay Step	Solution Injected (Concentration)	Incubation Time
Equilibration (80 µL)	PBS (10 mM)	5 minutes
Pre-competition Step	TCDNA-Biotin (10^{-5} M)	10 minutes
Competition in Milk (80 µL)	As155 (50 µg mL^{-1})/As171 (50 µg mL^{-1}) RSA (0.3 µM), Anti-RSA (50 µg mL^{-1}) TetR (1.0 µM), Anti-TetR (10 µg mL^{-1}) Milk samples; ≠ concentration	10 minutes
First Washing (80 µL)	PBS (10 mM)	2.5 minutes
Second Washing (80 µL)	PBS (10 mM)	2.5 minutes
Signal Amplification (80 µL)	α-rIgG (50 µg mL^{-1})	5 minutes
Washing (80 µL)	PBS (10 mM)	5 minutes
Regeneration (80 µL)	NaOH (200 mM)	5 minutes
Begin the cycle again	**For each sample measurement**	**45 minutes**

[a]*The sensor pads were spotted with SA2-OVA (SAs), 10b-BSA (FQs), CCK-BSA (BLs) and Neutravidin (TCs) as it is shown in the scheme of **Figure 6.9**. The chip was pre-conditioned to bind the TC-DNA-biotin competitor on the sensor pads biofunctionalized with Neutravidin. The milk samples were then mixed with a solution containing a mixture the corresponding antibodies, As155 (SAs) and As171 (FQs), bioreceptors, RSA (BLs) and TetR (TCs), and the respective antibodies against these bioreceptors, anti-RSA and anti-TetR, at the appropriate concentrations. Then the measurement cycle proceeded as described in the table.*

Finally, performance of the multiplexed WIOS system was verified by analysing blind milk samples within a workshop meeting performed at the NRC. It is possible to simultaneously/selectively detect the presence of residues of distinct antibiotic families on a milk sample using the WIObS (see **Figure 5** on **Section 6.3** and **Section 6.5.3**).

6.5 Critical evaluation of the analytical methodologies developed: GOODFOOD workshop at NRC

The multianalyte analytical methods described in this chapter (i.e. ELISA, WIOS) and dipstick for antibiotic residue analysis, developed by UNISENSOR [36] were experimentally contrasted on a technical workshop performed at the NRC at the end of the GOODFOOD project. The idea was to execute some kind of preliminary validation for the three techniques by measuring a significant number of milk samples, contaminated or not with different antimicrobials, with the three methods at the same time, with the objective to perform a critical analysis of the performance of all the methods developed. Thus, the three methods were evaluated in respect to parameters such as detectability, sensitivity, reproducibility, simplicity or easy-to-use features, or analysis time. Thus, the group at the NRC prepared blind samples and each research group performed the analysis with their own detection platforms (AMRg: multiplexed ELISA; CSEM/AMRg: multi-WIO*b*S and UNISENSOR; dipstick). This last common activity culminated the tight and successful collaboration accomplished between the distinct WP1 partners of the GOODFOOD European project (see **Figure 6.10**).

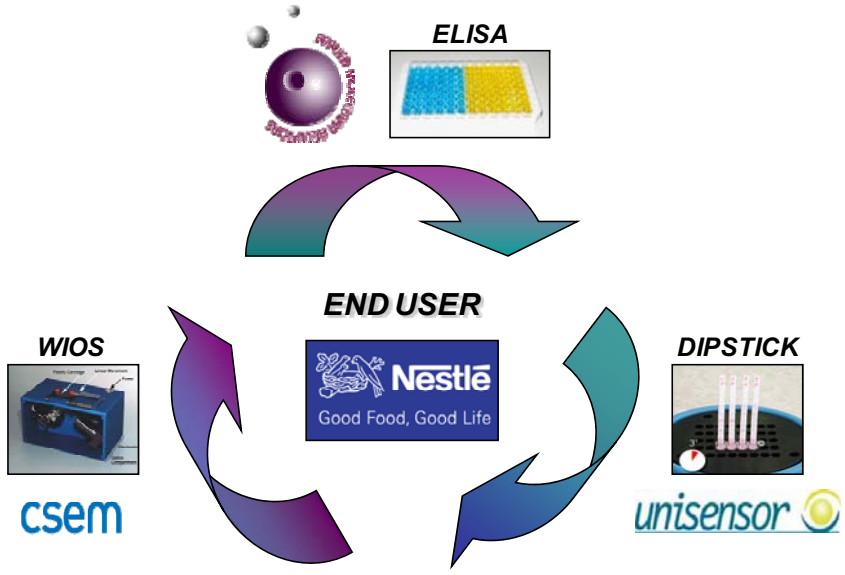

Figure 6.10: GOODFOOD consortium formed to accomplish all the objectives within the project.

6.5.1 End-User Partner (NRC)

The group from the Nestlé Quality and Safety Assurance Department, coordinated by Dr. Jean-Marc Diserens, was the keystone of the antibiotic working group within the GOODFOOD project. Thus, the group provided not only its great scientific and

technological know-how but also its expertise and relevance in the agro food sector. During the three years-period supported the group with very valuable information about composition and physical properties of milk as well as guiding on establishing the required specifications for the antibiotic residue fast screening systems (see the **Appendix**). Moreover, the NRC promoted training, dissemination and demonstration activities along the project. One of their most important actions consisted in organizing this particular technical workshop to evaluate the different methods developed during the project as well as preparing the correspondent reference milk samples for the analysis. The experimental meeting was performed in the analytical laboratory facilities of the NRC (see **Figure 6.11**). The NRC prepared a set of blind milk samples that contained the most common antibiotics found in real milk samples (e.g. SMZ, STA, SPY and sulfachloropyridazine (SCP) for SAs; CPX, norfloxacin (NOX) and enrofloxacin (ERX) for FQs; AMP, penicillin G (PEG), cloxacillin (CLO) and cefazolin (CFZ) for BLs; and OTC for TCs, among others). These antibiotics were spiked in fresh full-fat milk at different levels and sometimes even combined.

Figure 6.11: On the left the NRC (Lausanne - Switzerland). On the right the milk samples prepared to be analyzed.

The experiments performed for each technique, during the validation workshop, are briefly described in the following sections.

6.5.2 Multianalyte ELISA Test (AMRg)

The microplate-based ELISA methods established within this thesis have been used to obtain information about the characteristics of the antibodies before using them in other platforms, but at the same time this analytical method itself constitutes a an efficient screening tool. For screening purposes, the multianalyte ELISA protocol was slightly modified to both increase the efficiency of a single microplate (i.e. reducing number of wells used for calibration curves) and to ensure that antibiotics with different cross-

reactivity profiles or MRL values would be detected. For this purpose, representative compounds for each antibiotic family were selected and used as positive controls at their respective MRL concentrations (see **Figure 6.12**).

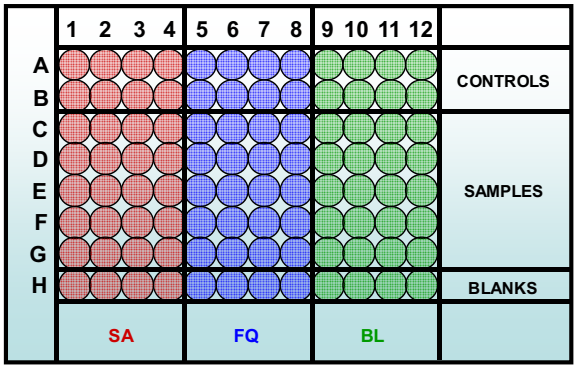

Figure 6.12: Scheme of the microplate-based multiplexed ELISA for qualitative screening of antibiotic residues in milk samples. The figure shows the distribution of the distinct controls used, the blanks and the unknown samples. Positive controls used were SCP (2% CR; 100 μg L^{-1} MRL), SMZ (58% CR; 100 μg L^{-1} MRL), ERX (79% CR; 100 μg L^{-1} MRL), danofloxacin (85% CR, prohibited) and AMP (100% CR; 4 μg L^{-1} MRL). Negative controls were also included.

This format increases considerably the efficiency of the microplate to detect contaminated samples in compliance with European legislation.

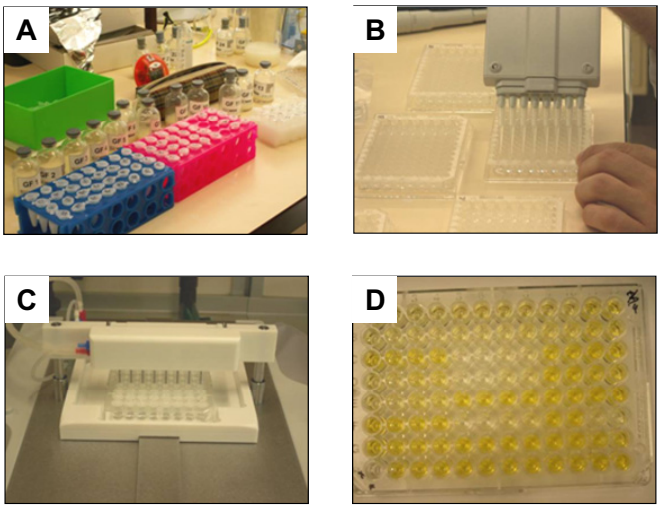

Figure 6.13: Multianalyte ELISA procedure. Prior the assay, all controls and unknown milk samples were appropriately diluted in to place them within the working range of the assay (A). Bioreagents and milk samples are placed in their corresponding microplate section (B). Washing step to remove the unbound reagents (C). Example of the final colorimetric response obtained (D).

Thus, with this microplate set-up, about 60 milk samples could be simultaneously screened per operator/day (10 samples per microplate) for their potential contamination

by up to 25 antibiotics from three of the most important families, on a semi-quantitative manner. On the other hand, the use of fully-automated ELISA workstations would increase considerably the number of samples analyzed on a row. A scheme of the main steps is summarized in **Figure 6.13**. After running the assay, those milk samples providing an absorbance below that of the controls is classified as suspicious of a potential contamination by the antimicrobial of that particular microplate section. The results obtained using this method are shown in **Table 6.3**, where it can be observed that using this semi-quantitative format, the multianalyte ELISA was able to find milk samples contaminated with antibiotics over the MRL values and also identify the type of antibiotic present.

6.5.3 MultiWIOS Test (CSEM-CSIC)

CSEM main contribution in the GOODFOOD project was focused in developing a wavelength-interrogated optical multiplexed sensor, comprising an array of waveguide gratings. The implementation of this platform with the bioreceptor and the bioanalytical procedures developed in collaboration with AMRg and UNISENSOR ending on the multiplexed biosensor device (WIObS) described above developed to detect a wide range of antibiotic residues in milk samples.

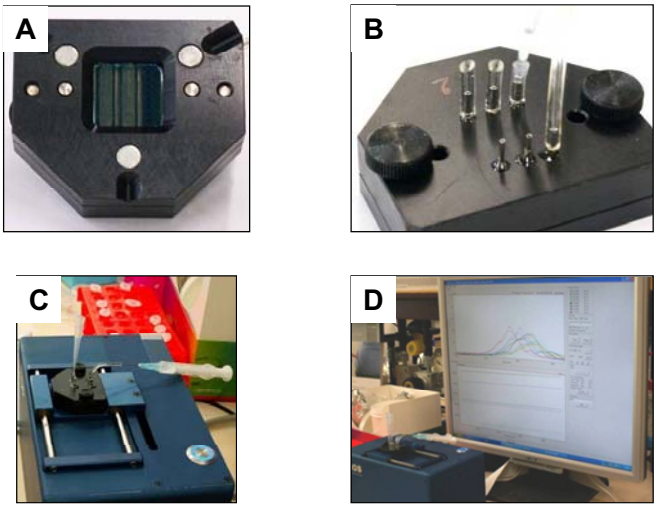

Figure 6.14: Multianalyte WIOS components. Down side of the chip cartridge holder (A). Up side of the chip cartridge holder (B). Chip cartridge coupled to the WIOS during a measurement cycle while a simple is injected to the system (C). Example of the final biosensor response (D).

Both multianalyte chips (i.e. SAs/FQs and SAs/FQs/BLs/TCs) were evaluated during this workshop at Nestlé (see **Figure 6.7** and **Figure 6.9**). The WIObS was used to analyze the blind milk samples prepared by the NRC. No sample treatment was necessary to obtain

reliable data regarding the potential presence of antibiotics in milk. The scheme of the main steps for WIOS measurements is summarized in **Figure 6.14**. It is possible to analyse 12 samples per day using the current assay protocol. Similar to the multianalyte ELISA test, most representative compounds of each antibiotic family were used as controls at the corresponding MRL values. Then, after running the assay, the response signal given by a particular milk sample was compared with that of the controls. Signals below those of the controls indicated potential contamination by the antimicrobial family and vice versa. 40 samples can be analysed per day per analyst. The results obtained during the workshop are shown in **Table 6.3**, where it can be observed that multianalyte WIOS approach was also able to find milk samples contaminated over the MRL values identifying the type of antibiotic family.

6.5.4 Multidipstick Test (UNISENSOR-AMRg)

UNISENSOR used an immunochromatographic lateral-flow device specially developed by this company for the simultaneous detection of SAs, BLs and TCs families (see **Figure 6.15**). The bioreceptors used were the same as those employed to develop the multiplexed ELISA and WIObS systems. Undesired shared-reactivities or cross-recognition reactions were not observed either in this format. In this case, final measurements consisted in mixing milk samples with the lyophilised bioreagents in microtiter wells during 10 minutes at 37ºC. After this incubation, dipsticks were put in those wells and after 10 minutes the reading was made (see **Figure 6.16**). If the antibiotic was present in milk, the recognition molecules (i.e. receptors or antibodies) labelled with gold nanoparticles will not be able anymore to bind the antigen deposited on the dipstick and therefore preventing the appearance of a signal at the capture line. On the contrary, with blank samples the labelled molecules are bound to the corresponding antigen capture lines (see **Figure 6.15**).

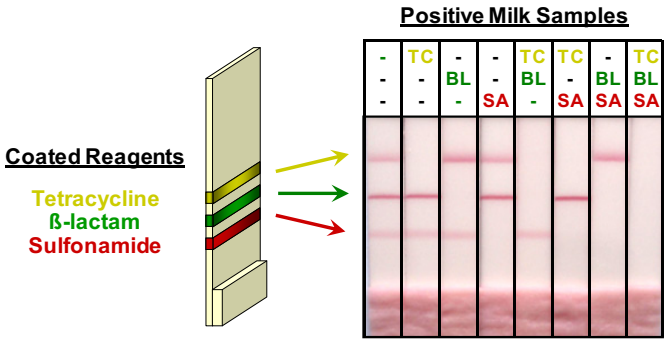

Figure 6.15: On the left, a scheme of the multidipstick test where three lines of antibiotic haptens (one for each family) have been deposited. On the right, an example of the multidipstick response when analysing different milk samples contaminated with all possible antibiotic combinations.

Once performed the entire experiment, dipsticks can be read either visually or with a special optical reader that analyse the optical density of each line. According to UNISENSOR 40 samples can be analysed per day per analyst. The results obtained with dipsticks during the workshop are shown again in **Table 6.3**.

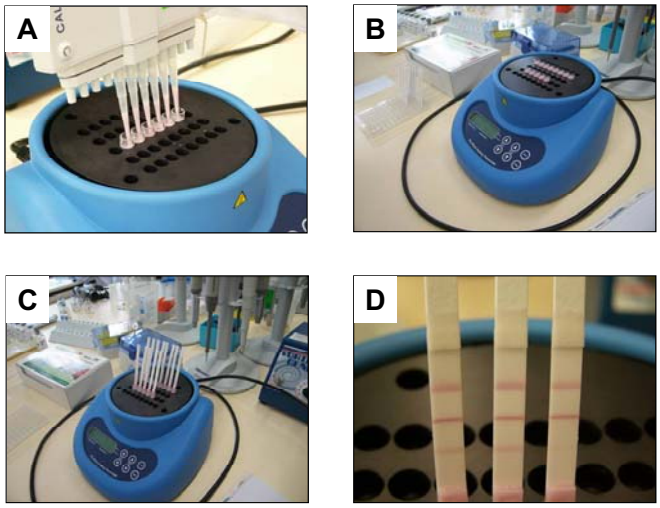

Figure 6.16: Multianalyte Dipstick procedure. Prior the assay, all unknown milk samples were placed in the wells using a multichannel pipette (A). Incubation of milk samples together with the bioreagents (B). Dipsticks are placed in the corresponding wells for the lateral flow diffusion of the sample (C). Example of the final colorimetric response obtained (D).

6.5.5 Results from the comparative study performed at the NRC

When the workshop was finished, results obtained in each platform were exchanged and discussed between the partners of the GOODFOOD antibiotic group. A summary of the critical comparison regarding performance of the 3 methods is shown in **Table 6.4**. Results obtained are expressed as positive (P), negative (N), false positive (FP) and false negative (FN) according to MRLs established for each compound by the European Union (see **Table 6.3**). Although not all samples were analyzed by the three techniques due to the duration of the workshop, the number of common samples analyzed and the fact that the three techniques were run simultaneously, allowed establishing some conclusions in respect to the capabilities, strong and weak points of each technique. From the 25 milk samples analyzed with the different detection techniques, only one FN was obtained with the multiplexed ELISA. According to the Commission Decision 2002/657/EC the number of FNs should remain below 5%, which was the case in this comparative study, although further evaluation is required. Moreover, seven FPs were also recorded, four with the dipstick method, two with the multiplexed ELISA and just one with the multiplexed WIObS.

Table 6.3: Results obtained with all three multianalyte analytical methods developed during the GOODFOOD project when measuring spiked full-fat milk samples prepared by Nestlé.

	MultiELISA Test				MultiWIOS Test				MultiDipstick Test				Spiked Milk Samples (μg L^{-1})			
Sample	**SA**	**FQ**	**BL**	**TC**	**SA**	**FQ**	**BL**	**TC**	**SA**	**FQ**	**BL**	**TC**	**SA**	**FQ**	**BL**	**TC**
GF1	N	N	N	---	N	N	---	---	FP	---	N	N	SMZ 10	---	---	---
GF2	N	N	N	---	N	N	---	---	---	---	---	---	STA 10	---	---	---
GF3	N	N	N	---	N	N	---	---	---	---	---	---	SCP 10	---	---	---
GF4	N	N	N	---	N	N	---	---	FP	---	N	N	SMZ 25	---	---	---
GF5	N	N	N	---	N	N	---	---	---	---	---	---	STA 25	---	---	---
GF6	N	N	N	---	N	N	---	---	---	---	---	---	SCP 25	---	---	---
GF7	N	N	N	---	N	N	---	---	---	---	---	---	---	---	---	---
GF8	FP	N	N	---	N	N	---	---	FP	---	N	N	SMZ 50	---	---	---
GF9	FP	N	N	---	N	N	---	---	---	---	---	---	STA 50	---	---	---
GF10	N	N	N	---	N	N	---	---	---	---	---	---	SCP 50	---	---	---
GF11	P	N	N	---	N	N	---	---	P	---	N	N	SMZ 100	---	---	---
GF12	P	N	N	---	N	N	---	---	P	---	N	N	STA 100	---	---	---
GF13	FN	N	N	---	N	N	---	---	P	---	N	N	SCP 100	---	---	---
GF20	N	N	N	---	N	N	---	---	N	---	N	N	---	---	---	---
GF21	N	P	N	---	N	P	---	---	N	---	N	N	---	CPX 100	---	---
GF22	P	P	P	---	P	P	---	---	P	---	P	N	SMZ 100	NFX 100	PEG 4	---
GF23	P	N	N	---	P	N	---	---	P	---	N	N	SPY 100	---	---	---
GF24	N	P	P	---	N	P	---	---	N	---	P	N	---	ERX 100	CFZ 50	---
GF25	N	N	P	---	N	N	---	---	N	---	P	N	---	---	AMP 4	---
GFS1	---	---	---	---	N	N	P	---	N	---	P	N	---	---	CLO 20	---
GFS2	---	---	---	---	P	N	P	N	P	---	P	N	SMZ 90	---	PEG 4	---
GFS3	---	---	---	---	N	N	N	FP	N	---	N	FP	---	---	---	OTC 75
GFS4	---	---	---	---	---	---	---	---	N	---	N	N	---	---	---	---
GFS5	---	---	---	---	---	---	---	---	N	---	N	N	---	---	---	---
GFS6	---	---	---	---	N	N	N	P	N	---	N	P	---	---	---	OTC 500

As screening methods, the presence of FPs does not constitute a big issue, unless the number is extremely high, since the priority is detecting samples that may be contaminated at levels over the MRL values. On the other hand, in most cases the FPs were samples readily contaminated by the antibiotic, although below the MRLs.

Summarizing, the **multianalyte ELISA assay** was adapted to measure a large number of samples in parallel being the only method able to analyze all the blind samples prepared during the duration of the workshop. Once optimized the multianalyte ELISA protocol established for screening purposes, the entire analysis for all samples was run in about 2 hours by providing a specific response on a different microplate sections depending on the antibiotic family. The assay performs very well in milk samples without any prior sample treatment other than dilution of the sample. Moreover, specificity studies demonstrate that more than 25 different antibiotics can be detected in compliance with the regulations of the EC. Apart from the specific bioreagents (i.e. antigens, antibodies and receptors), and the typical ELISA tools no special equipment is required.

The **multianalyte WIObS assay** was not fast but was the only method able to measure simultaneously the four antibiotics families and to perform several measurements sequentially on the same sensing surface. The entire cycle for the multianalyte WIObS approach takes 40 min for the analysis of one milk sample as it is not yet possible to regenerate the BLs assay. On the other hand, we believe that sooner the entire experimental time could possibly be reduced by a factor of 2. In addition, further optimization of the assay should improve the reproducibility between different chips and homogenize the LODs of all antibiotics. Technical and performance characteristics showed by the WIOS system seem to be a type of biosensor suitable for automated on-site determinations (i.e. on the farm before loading milk into the truck). However, few modifications are needed to develop a more robust instrument for on-site use (e.g., fluid handling, chip fabrication and functionalization costs, and data processing). At present, a spin-off company of CSEM (Dynetix AG, www.dynetix.ch) is trying to improve the set-up and performance of this sensor to make it suitable for PoC (point-of-care) and *on site* analysis for a wide variety of applications. The estimated cost of this prototype device is not defined yet but to this price it has to be added the cost of the bioreceptors and the biofunctionalization of the chip.

The **multianalyte dipstick** technique allows the simultaneous detection of three antibiotic families, within the MRLs established by the EU, in just 20 minutes. Numerous measurements can be made in parallel by using different dipsticks together with several microtiter wells for the incubation step. At the moment, the multianalyte approach of this technique could probably arrive to the market with the most competitive price since no special equipment is necessary being everything included within the kit. However, the

assay must be optimized for the analysis of SAs to adapt the concentration of the immunoreagents to the MRLs since its detectability is too high at the moment and this is the reason because the number of FP is the highest.

A summary of the critical comparison of the three techniques in respect to different parameters can be found in **Table 6.4**. One of the main conclusions was that the dipstick platform is nowadays the most suitable for field screening. Moreover, the dipstick was able to perform rapid, inexpensive and reliable screening of antibiotic residues in milk samples. At the moment, no other detection platform can execute *in situ* simultaneous analysis in as many samples as dipsticks are used, without sample pre-treatments and in less than 20 minutes. Secondly, non-experienced laboratory technicians are necessary to perform the analysis, which means dipsticks could be used daily by farmers to control their antibiotic treated animals or even by the same drivers of milk tanks when this is collected.

Table 6.4: Qualitative comparison of the different approaches developed to analyze milk samples within their current configuration format.

PARAMETERs	ELISA	WIOS	DIPSTICK
End-user skills required	Medium-High	High	Low
Matrix effect	Low	Low	Low
Limits of detection in milk	Low	Low	Low
False negative rate	Low	Low	Low
False positive rate	Medium	Medium	High
Semi-quantitative results	Medium-High	Medium	Medium
Portability of the assay	Medium	Low	High
Estimated price per assay	Low	High	Low
Speed/HTS (Samples/day/operator)	High (60)	Low (12)	High (40)

On the other hand, the ELISA technique is an excellent tool for evaluating the quality of the bioreagents, establishing the assay conditions to reach the maximum detectability, assess potential matrix interferences and all the necessary studies before the implantation on other individual or multianalyte analytical detection platforms. However, additionally, this technique still offers great high-through-put screening capabilities within laboratory facilities, and to a lesser degree as a field-test in comparison to dipsticks, since several assays can be performed in parallel by using different microtiter plates. Although ELISAs can be performed by non-skilled operators, an entire analysis of, for example, biological samples is much easier to handle when using dipsticks. Finally, though not in the format used in this workshop, this ELISA can provide semi-quantitative data which offers

superior capabilities if compared with the visual detection used by dipsticks which in questionable samples can lead to erroneous readings.

Finally, the WIOS approach appears as the farthest screening method from being placed on the market, considering the complexity and cost of the technology. On the other hand, from the three developed techniques, this biosensor seems to be the most suitable approach for automated on-site determinations (i.e. on the farm before loading milk into the truck). Research is currently ongoing to develop a field instrument such as improving the design of the fluidic chamber, data processing, or reducing the handling of solutions for future use by non-skilled personnel. In this sense, tackling new scientific challenges, such as combining bioreagents with exceptional features together with the latest in biosensor technology, is the key point to improve the current available analytical techniques.

The price per assay for the three techniques evaluated (i.e. ELISA, WIOS, dipstick), considering the time factor as part of the total cost, will be always lower using the multiplexing formats than the individual ones. Moreover, the possibility to process in parallel several microplates, chips and dipsticks reduces the operator time. The main advantage of multiplexing is the capability to analyse different antibiotic families using the same protocol, and not using as many protocols as number of antibiotic families studied, simplifying a lot the work.

6.5.6 Main contributions

The main contributions of this chapter to the existing knowledge at the time of starting this research are:

- It has been described for the first time the successful combination of SAs, FQs, BLs and TCs class-selective bioreceptors to screen simultaneously the potential presence of distinct antibiotic family residues on a same sample. In this respect the data shown here through the multiplexed ELISA and WIObS systems demonstrates the orthogonal features of the bioreceptors used.
- A multianalyte ELISA able to detect more than 25 different antibiotic congeners from SAs, FQs and BLs families in milk samples has been developed and described for the first time. The analysis of milk samples can be performed without any kind of sample preparation other than dilution and the detectability reached is in accordance with the current EC legislation in respect to the possibility to detect antibiotic residues over the MRLs. Two immunochemical protocols have been established for the case of the microplate-based ELISA. The first one has the aim to be used for screening purposes to just detect the presence

of antibiotic residues in the unknown milk samples, over the MRLs. The second one is addressed to quantify the concentration of the antibiotic in the sample, before submitting the samples to confirmatory chromatographic methods. In spite of the quantitative character of the second approach, the multiplexed immunochemical method developed keeps high throughput screening capabilities.

- A multiplexed WIObS label-free biosensor for antibiotic screening able to detect up to 35 antibiotic congeners from four different antibiotic families (SAs, FQs, BLs and TCs) has been developed and reported for the first time. The biosensor can perform measurements in milk samples, without any prior treatment other than dilution and the detectability reached is in compliance with EC regulations. The biosensor chip can be regenerated and used multiple times by flowing a solution of 200 mM NaOH (5 min). Sensing sites for SAs and FQs showed variations <10% after regeneration (n = 10 cycles). Only the BLs sensor pads could not be regenerated due to the irreversible character of the RSA-BL binding reaction.

While doing these studies some of the immunoreagents produced in our research group (SAs, FQs and CAP (i.e. detection of phenicols)) were implemented successfully by Fátima Fernández on a novel portable six channel SPR biosensor based on the plasmon of gold diffraction grating surface for simultaneous multianalyte antibiotic detection in milk samples [37]. This approach doesn't need the use of a secondary antibody to induce a final signal enhancement and thus slightly reducing the time per assay in comparison with the WIObS. On the contrary, each WIObS chip currently comprises an array of 24 independent sensing zones (8 pads distributed in 3 different columns), while the corresponding SPR chips include just 6 independent sensing zones, and therefore being able to analyse more samples and/or replicates per assay. Concerning the regeneration procedures there were obtained similar results with both systems.

6.6 Bibliography of Chapter 6

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7 APPENDIX

In this appendix it is reported the complementary research performed during this thesis. Although part of the work here described has not directly contributed to the development of the WIObS (see **Chapters 5** and **6**), the studies realized really provided know-how and useful information that were crucial to make decisions in respect to its final configuration. Thus, in this chapter there are reported the results of a questionnaire elaborated with the aim to define the specifications of the device, and the investigation addressed to prepare novel bioconjugates based on magnetic beads and different types of fluorescent probes (see **Figure 7.1**).

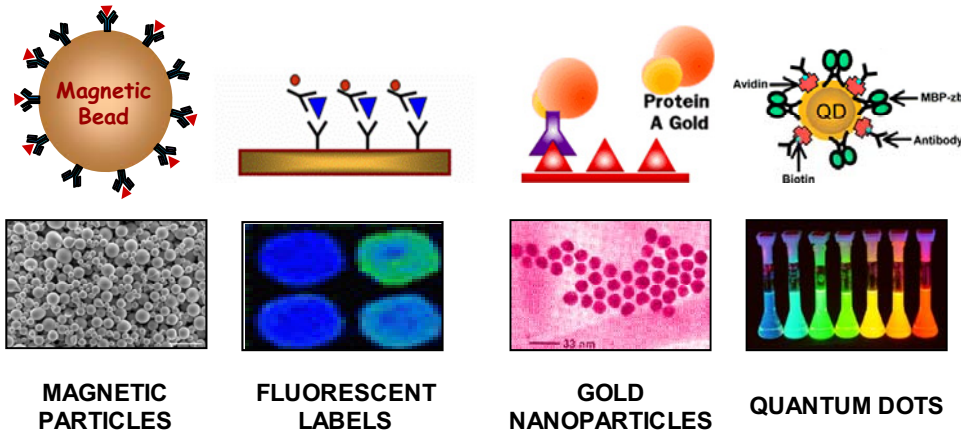

Figure 7.1: Examples of possible antibody chemical modifications useful to develop new immunochemical techniques.

7.1 Preamble

By the time this work started, there were no clear the specifications that the biosensor device had to accomplish to respond to the real needs of the dairy field. For this reason the elaboration of a questionnaire addressed to milk producers and food safety agencies was considered a priority. The results of a European survey, covering aspects such as the type of drugs used to treat mastitis, selection of the food chain points where the analysis should be performed and the time at disposal to test the milk, were used to define the specifications of the technology solutions that have been developed in this thesis (see **Section B.1.2**).

On the other hand, it must be noticed that in the original device scheme proposed by CSEM within the GOODFOOD project, the analytes were captured from the sample matrix making use of magnetic beads and the detection was based on fluorescent labelling (see **Figure 7.3**). In EW sensing, the waveguide mode is perturbed by the binding of the target analyte or any other molecules to the transducer surface. The transducing mechanism will transform this perturbation into a useful electrical signal. EW sensing can be accomplished by different mechanisms:

- **Absorption:** After a given propagation length, the spectrum of the light is analysed which will give information on the absorption properties of the volume seen by the evanescent wave. This method can be use with a large light spectra and the signature of the spectra will give information on the type of absorbed molecules or at one wavelength specific to the molecule to be detected.
- **Waveguide resonance analysis:** This consists in the measurement of the effective refractive index of the waveguide mode which is dependent on the biochemical surface reaction. The coupling using a grating coupler can be monitored either by monitoring the coupling angle, the resonance wavelength or the resonance periodicity. This is the approach finally used in the WIObS for antibiotic detection reported in **Chapters 5** and **6**.
- **Interferometry:** The binding of molecules affects the propagation constant of the waveguide. The optical beam is split into two arms, one being in contact with the sample and the other being protected; the two beams are then superposed and interfere. The interference pattern is analysed in order to extract the phase change due to difference of propagation constant between the two arms.
- **Fluorescence:** In the special case of waveguide, the evanescent wave can be used to excite the fluorescent molecules, to collect the fluorescent light or both. The

general scheme is to excite the waveguide mode by means of a grating coupler. The waveguide mode will excite the fluorescent labels located in the evanescent part of the mode, the excited fluorescent molecules will behave like source for waveguide modes at the fluorescence wavelength, a second grating outcouples the fluorescent light on the detector (**Figure 7.2**, left). Another alternative is to excite the fluorescent labels directly and collect the fluorescent light with the waveguide mode (**Figure 7.2**, right).

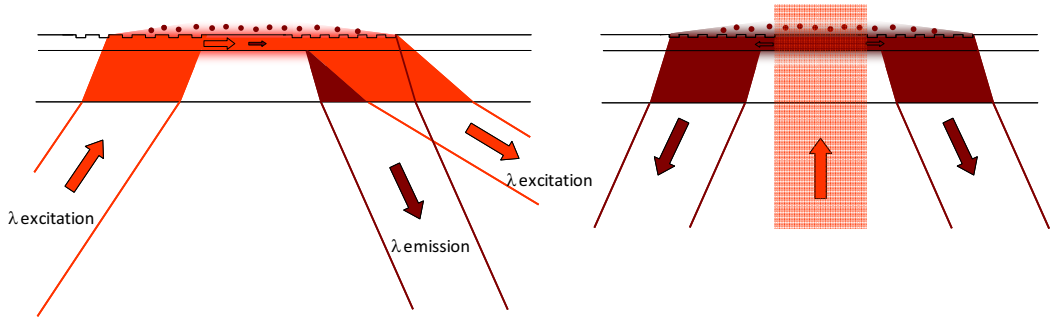

Figure 7.2: Schemes of fluorescent reading on waveguide evanescent sensor. Left: Waveguide excitation and waveguide collection of the fluorescence. The incident bean is coupled into the waveguide through the first grating. The fluorescent molecules excited by the evanescent wave emit light that is coupled back into the waveguide and it is outcoupled at a different angle. Right: Free space excitation and waveguide collection. The incident beam excites the fluorescent molecules. The light emitted in the evanescent waveguide is collected and it is outcoupled through the gratings.

Thus, on the biosensor device initially envisaged, sample handling relied on a microfluidic device that collected and concentrated the antibiotics by means of a magnet. A smart CMOS/CCD camera, initially developed for "3D-vision" [1] was proposed to be used for optical detection of the different measurement pads. This type of camera allowed detecting the phase difference between the emitted to the reflected light signals, which allows recording fluorescence emission with an efficient filtering of the parasitic noise. In a fluorescence-based sensor, waveguide grating sensing pads are imaged on the camera through a filter at the fluorescence wavelength. The modulation of the fluorescence light detected by the pixels is proportional to the concentration of fluorescent labelled molecules present near the surface and is used to determine this concentration.

Considering the design of the first prototype proposed the contribution of our group was addressed to establish immunochemical assays involving the use of magnetic beads and fluorescent labels. For the first case, an ELISA using magnetic beads (magneto-ELISA) was established initially for atrazine as a model analyte, in the context of a research project performed in our group by Dr. Emanuela Zacco. Her research was focused to

develop a novel electrochemical immunosensing strategy, based on magnetic beads and a magneto sensor made of graphite–epoxy composite (m-GEC) electrode. Atrazine magneto-ELISA [2] showed excellent features (IC_{50} of 0.11 mg L^{-1} and LOD of 0.06 mg L^{-1}), encouraged us to extend this approach to use the same approach for the antibiotic residues (see **Section 3.2**). For the case of the fluorescent labelling, we investigated the use of organic fluorophores and QDs but being limited by the excitation wavelength of the device developed by CSEM. Thus, we had to use dyes absorbing and emitting at high wavelengths such as Cy5 or Alexa 750. The advantage of the QDs is their high quantum efficiency and that the emission of the fluorescence can be modulated by the size of the nanoparticle.

Considering thus the first scenario where the present thesis started, in the following sections we will describe the work and research performed in the initial steps that not only contributed significantly to the progress of our research, but also established the bases for the final sensor set-up or on the decision making process in order to select the most appropriate WIOS configuration.

7.2 Survey on the specifications of new techniques for antibiotic residue analysis in milk samples.

The specifications of the analytical techniques for antibiotic residue analysis should respond to the user needs. Therefore in collaboration with the rest the partners of the antibiotic group of the GOODFOOD project a questionnaire was elaborated and distributed on different European countries (see questionnaire below). The aim of the survey was to obtain a view of the user requests and of what is important today concerning the antibiotic residue detection by contacting with reference laboratory groups and also some important dairy players throughout the world - 19 countries - (confidential information). The questions addressed aspects such as the relevance of the different drug families, the place where the control should be made, what should be the length of the analysis, the desired detection limits and the most frequently used antibiotic for intramammary treatments.

SIXTH FRAMEWORK PROGRAMME
PRIORITY 2
INFORMATION SOCIETY TECHNOLOGIES

GoodFood
Food Safety and Quality Monitoring with Microsystems

Deliverable D2- WP1D1:
"Sensor Specification and Waveguide Design"

Partner Responsible of the deliverable:
Other Partners: NESTEC, CSIC_AMR, CEA_LETI, CEA_SPI, NMRC, APIBIO

GOODFOOD project

WP1-Antibiotics: Questionnaire

GOODFOOD - WP1

Microsystems technology solutions for the detection of antibiotic residues in milk

Questionnaire on the required performance of a microsystem for the detection of antibiotic residues in milk

Remarks: with each question, several answers can be given, extra lines can be filled, etc.

Antibiotic families to be searched

Drug families to be searched	Yes	No	Priority level 1 to 8, 1 = high priority
Aminoglycosides			
Amphenicols			
Beta-lactams/ Cephalosporins			
Macrolides			
Nitrofuran metabolites			
Quinolones			
Sulfonamides			
Tetracyclines			

Place where to analyse the milk

Several answers can be given, the systems will be adapted or improved to be used at the most convenient place.

Where to control the milk	
Farm	
Collecting point	
Lorry	
Dairy/factory	

Time at diposal

The time necessary or at disposal to analyse the milk is usually short. It is important to define the longest time an analysis could take.

Time at disposal to test the milk	
5 – 10 min	
10 – 20 min	
60 min	
180 min	

Detection limits and MRL's

The detection limits should be close or below the MRL.
Which antibiotics should be used to validate the systems. Of course it will not be possible to determine the detection limits of all antibiotics of the legislation in a first step. Please fill the table with the most important drugs.
The table with the MRL is from Dr. Walter Heeschen former chairman of the International Dairy Federation Joint Action Team Antimicrobial & Other Veterinary Medicinal Residues.

Maximum residue limits of antimicrobial drugs in milk
Antibiotics for which a detection limit should be determined in priority.
(µg/kg)

Substance (-group)	MRL EU	Yes	Substance (-group)	MRL EU	Yes
ß-Lactams			**Macrolides**		
Penicillin	4		Erythromycin	40	
Penethamat	4		Pirlimycin	150	
Ampicillin	4		Spiramycin	200	
Amoxicillin	4		Tilmicosin	50	
Nafcillin	30		Tylosin	50	
Cloxacillin	30		**Aminoglycosides**		
Dicloxacillin	30		Gentamicin	100	
Oxacillin	30		Neomycin	500	
Cefacetril	125		Spectinomycin	200	
Cefalexin	100		DH/Streptomycin	200	
Cefalonium	10		**Quinolones**		
Cefoperazon	50		Enrofloxacin	100	
Ceftiofur	100		Danofloxacin	30	
Cefquinome	20		Marbofloxacin	75	
Cephapirin	60		**Various**		
Cephazolin	50		Bacitracin	150	
Tetracyclines			Baquiloprim	30	
Chlortetracycline	100		Clavulanic acid	200	
Doxycycline	0		Colistin	50	
Oxytetracycline	100		Lincomycin	150	
Tetracycline	100		Novobiocin	50	
Sulfonamides	100		Rifaximin	60	

	Thiamphenicol	50
	Trimethoprim	50

Antibiotics used in various intramammary injectors

The drugs used in 180 intramammary injectors available in France, Italy, UK, USA and Switzerland are listed in the following table. Choose which antibiotics should be detected with a screening method.

Drugs used for mastitis treatment Need to develop a screening method		
Antibiotic family	**Active drug**	**Yes**
Aminoglycosides	Aminosidin	
	Dihydrostreptomycin	
	Gentamicin	
	Kanamycin	
	Neomycin	
	Streptomycin	
Bacitracins	Bacitracin	
Beta – lactams	Amoxycillin	
	Ampicillin	
	Benzylpenicillin	
	Cloxacillin	
	Dicloxacillin	
	Hetacillin	
	Nafcilin	
	Oxacillin	
	Penethamate hydroiod	
Cephalosporins	Cefacetril	
	Cefalexin	
	Cefalonium	
	Ceffazol	
	Cefoperazon	
	Céfuroxime	
	Cephapirin	
Chloramphenicols	Chloramphenicol	
Diaminopyrimidines	Trimetoprim	
Lincosamides	Lincomycin	
	Pirlimycin	
Macrolides	Erytromycin	
	Oleandomycin	
	Spiramycin	
Novobiocins	Novobiocin	
Polymixins	Colistin	
	Polymixin	
Rifomycins	Rifampicin	
	Rifamycin	
	Rifaxymin	
Sulfonamides	Sulfadimidine	
Tetracyclines	Oxytetracycline	
	Tetracycline	

The analysis of the received questionnaires offered the following summarized results. BLs, TCs, SAs, and CAP seem to be the priority antibiotics to be detected by a new technique (see **Table 1**). Moreover, apparently, macrolides and aminoglycosides are usually prescribed in cocktails containing one of the previously mentioned antibiotics. Although FQs should not be used on lactating cows there exists a concern due to the widespread use in animal husbandry.

Table 7.1: Antibiotic detection priorities.

Time	Number of answers with priorities 1 to 3
BLs / Cephalosporins	31
TCs	23
SAs	20
Aminoglycosides	18
Macrolides	13
Amphenicols (including **CAP**)	12
FQs	3
Nitrofuran metabolites	2

35 answers (1: high priority – 8: low priority)

As it can be observed in **Table 7.2**, answers concerning the time at disposal to perform the test, most of the responders agreed that a 10 minutes test is ideal as it is not a problem to have a lorry waiting this time before unloading. On the other hand, the 3 hours test are would only be used for tankers of a factory before using the stored milk, it could never be used at lorry level at the entrance of a factory/dairy.

Table 7.2: Time at disposal.

Time	Number of answers with priorities 1 to 3	Developed equivalent Technique
5 – 10 minutes	26	Dipstick
11 – 20 minutes	12	Dipstick
60 minutes	0	ELISA, WIObS
180 minutes	22	ELISA, WIObS

36 answers (1: high priority – 8: low priority)

Finally, regarding place where the analysis should be performed (see **Table 7.3**), all the responders pointed about the need of techniques that could be performed at the reception of the dairy factory. However, a need for methods that could be performed at the farm seems also very clear. In respect to the type of methods, used on each scenario, dipstick appears as a suitable technology to be performed at the farm, by the lorry driver, or at the milk reception dairy factory. However, other automated options such as the WIObS

system seems to be more addressed to the analytical laboratories of the food safety agencies or of the factories.

Table 7.3: Where to control the milk.

Where	Number of answers with priorities 1 to 3	Developed equivalent Technique
Farm	15	Dipstick
Collecting point	12	Dipstick, ELISA
Lorry	11	Dipstick,
Milk reception of diary/factory	28	Dipstick, ELISA
Diary/factory (bulk milk)	11	WIObS

35 answers (1: high priority – 8: low priority)

In all cases there was a clear demand to accomplish detection limits of as close as possible to the corresponding antibiotic MRLs.

According to these results, and together with the GOODFOOD antibiotic group, it was proposed to address the development of different analytical methodologies (i.e. ELISA, WIObS, and dipstick) able to provide alternative analytical techniques in the distinct scenarios identified. In this thesis we have presented the work performed regarding antibiotic residue detection using microplate based ELISAs and the WIObS system. Additionally, we also supported the work addressed to develop dipstick methods, although this work was mainly performed by UNISENSOR using some of our immunoreagents (data not shown due to confidential issues).

7.3 Development of a SAs immunoassay using antibody-biofunctionalized magnetic particles (magneto-ELISA)

New solid supports like magnetic particles have been used in combination with immunoassay with the aim to improve sample purification and immunoreaction kinetics, while offering the possibility of being implemented in new detection devices in combination with microfluidics for on-line measurements. As mentioned above, the initial strategy proposed for antibiotic residue analysis in the GOODFOOD project involved the use antibody-modified magnetic particles to capture the antibiotics from the matrix, using microfluidics, in combination with an optical transducer based on specific fluorescence detection (see **Figure 7.3**).

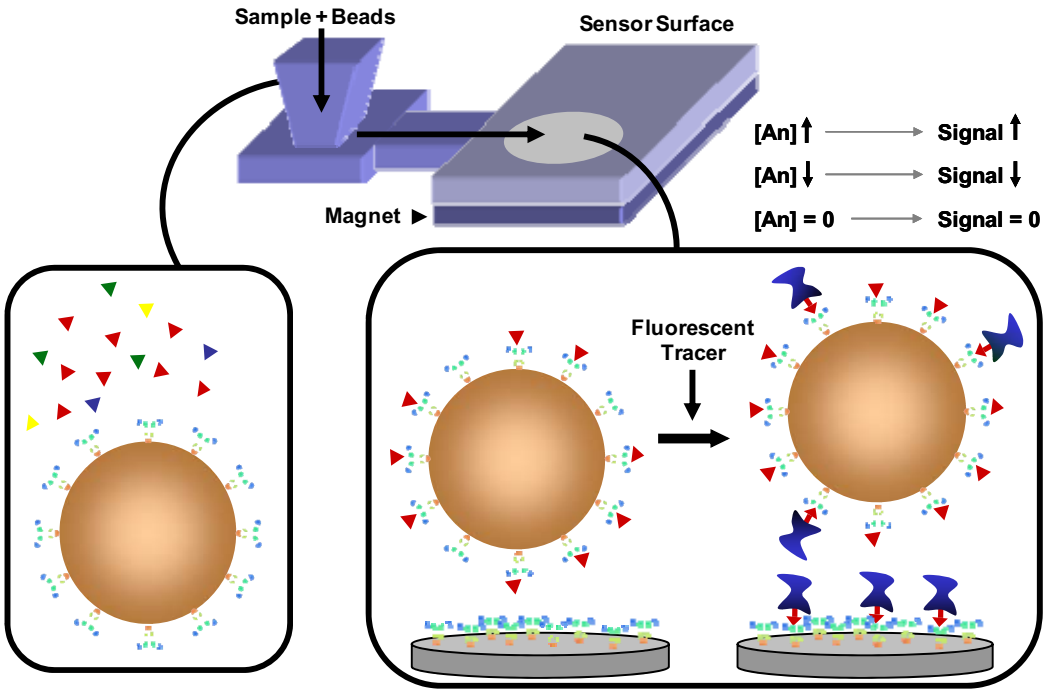

Figure 7.3: Sensor configuration proposed by CSEM within the GOODFOOD project.

Therefore, initial research work was addressed to evaluate performance of the sulfonamide antibodies on magnetic particles. Thus, antibodies were covalently attached to magnetic particles (antibodies-MNP) (see **Figure 7.4**) and evaluated on an ELISA (magneto-ELISA), in this case on a direct format, according to the sensor configuration proposed by CSEM (see above), in which the final signal would be fluorescent. However, on a first instance, the magnetic beads were evaluated using a HRP-SAs-hapten bioconjugate.

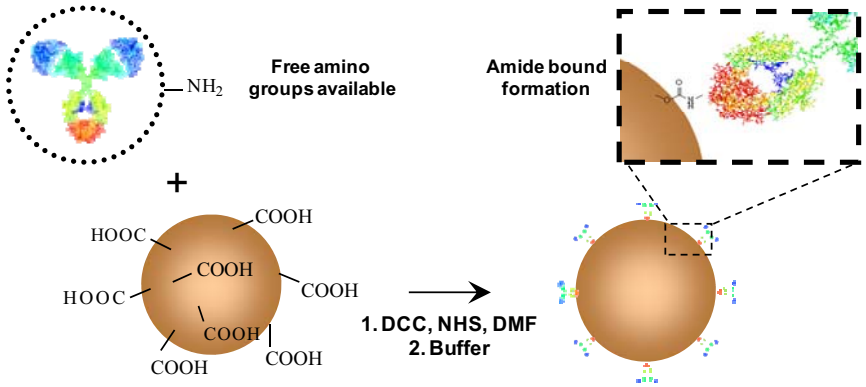

Figure 7.4: Scheme used to prepare antibody-biofunctionalized magnetic particles. The commercial particles were selected with carboxylic acid groups and coupled to the amino groups of the antibodies using the carbodiimide method using N,N'-dicyclohexyl-carbodiimide (DCC) and N-hydroxysuccinimide (NHS).

Purified fractions of immunoglobulins from As167, achieved by ammonium sulfate precipitation, were coupled covalently to the correspondent magnetic particles (~ 200 nm of diameter) following the standard carbodiimide reaction conditions (between free amino groups of antibodies and MNP functionalized with carboxylic groups). The appropriate concentrations of Ab167-MNP and SA1-HRP for the competitive immunoassay in milk samples were selected again by 2D experiments. All incubation steps were performed in solution under gentle shaking to avoid the deposition of the magnetic beads on the bottom of the wells and to facilitate the interaction with the other species.

The experiments demonstrated that the magneto-ELSA was able to reach the detectability required by the EC legislation for the analysis of milk samples. The IC_{50} value for SPY (6.3 µg L^{-1}) was below the MRL (see **Table 2** and **Figure 3** (right) in **Section 3.2**)) and the accuracy was very good (see **Figure 4** (centre) in **Section 3.2**), left and right graphs, respectively). Sensitivity was slightly lower than that accomplished by the microplate ELISA (IC_{50} = 4.6 µg L^{-1}) using the same immunoreagents, however the magneto-ELISA was more robust to the matrix effect at the time of analyzing undiluted whole milk samples. In the microplate ELISA milk samples had to be diluted 5 times in order to accomplish a good accuracy (IC_{50} = 23.0 µg L^{-1}). More information about the magneto-ELISA can be found in the paper published around this topic (see **Chapter 3.2**). These results pointed to the possibility to develop the immunosensor configuration proposed, in addition to opening the door to develop of a variety of other immunochemical techniques involving easier extraction steps. Thus, next step consisted on establishing a similar assay but using a fluorescent label, instead of an enzyme, as it was required for the sensor.

7.4 Development of a SAs fluoroimmunoassay (SAs-FIA)

Due to the type CMOS camera of the sensor prototype being developed by CSEM, the excitation/emission wavelengths of the fluorophor used had to be around 500/650 nm, for which reason Cy5 was selected. However, due to the high cost of this dye, on a first instance we proposed as proof of concept to develop the assay using a less expensive fluorophor such as fluorescein. Thus, initially we assessed performance of the indirect SAs ELISA developed (see **Chapter 3**) using a commercial secondary antibody labelled with fluorescein (FICT), instead of HRP. Previous characterization of this compound was performed using opaque microtiter plates with the Gemini XPS spectrofluorometer (see **Figure 7.5**). With those experiments we were able to choose the optimal excitation and emission wavelengths.

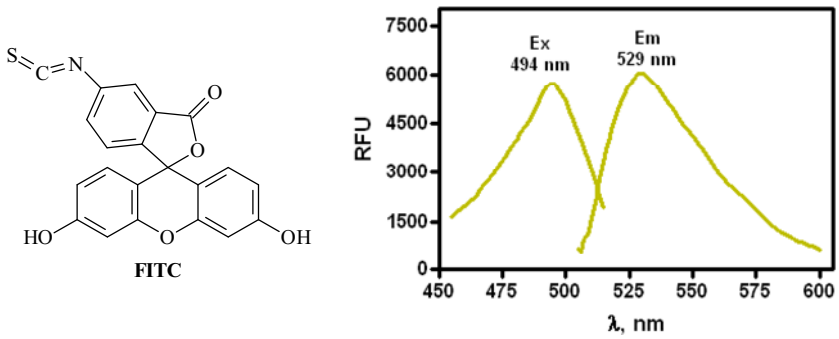

Figure 7.5: Structure of fluorescein compound and its excitation and emission spectra in buffer.

As before, the optimum concentrations were selected on 2D experiments and further on the competitive ELISA was established and evaluated building the corresponding calibration curves (see **Figure 7.6**).

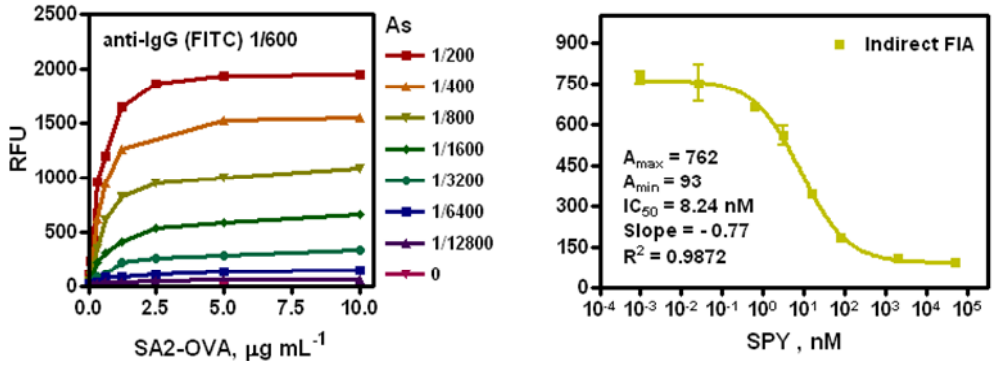

Figure 7.6: 2D experiments and competitive assay obtained using secondary antibody-FITC.

As can be observed, detectability achieved by this fluoroimmunoassay was very similar to that of the indirect ELISA format (see **Chapter 3.3**), although the concentrations of

immunoreagents required were much higher. With these precedents, we proceeded with the development of the fluorescence immunoassay using the cyanine dye. A carboxy-derivatized Cy5 was acquired from commercial sources and characterized to know the excitation/emission spectra in media with different pH values, in order to know its behaviour. Thus, it is known that these dyes show a good aqueous solubility and tolerance to DMSO, exhibit low non-specific binding and are more photostable than fluorescein, but they are pH insensitive between pH 3–10 (see **Figure 7.7**).

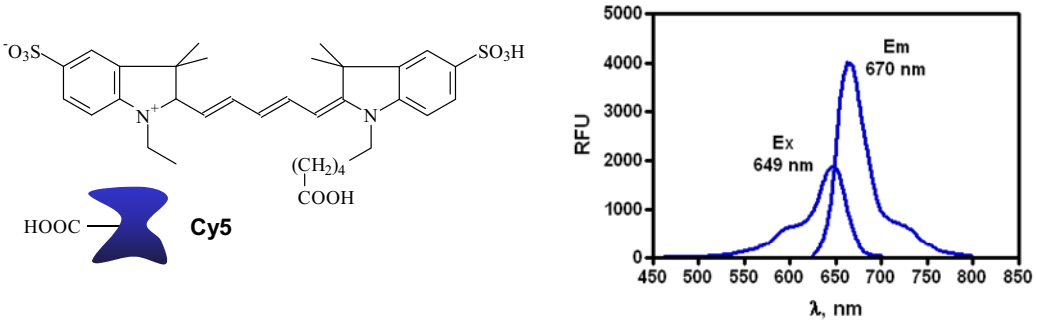

Figure 7.7: Structure of Cy5 compound and its excitation and emission spectra in buffer.

Subsequently, we addressed the preparation of both, hapten- (SA2) and antibody-Cy5 (As155) bioconjugates in order to assess performance of both direct and indirect immunoassay configurations for SAs (see **Figure 7.8**).

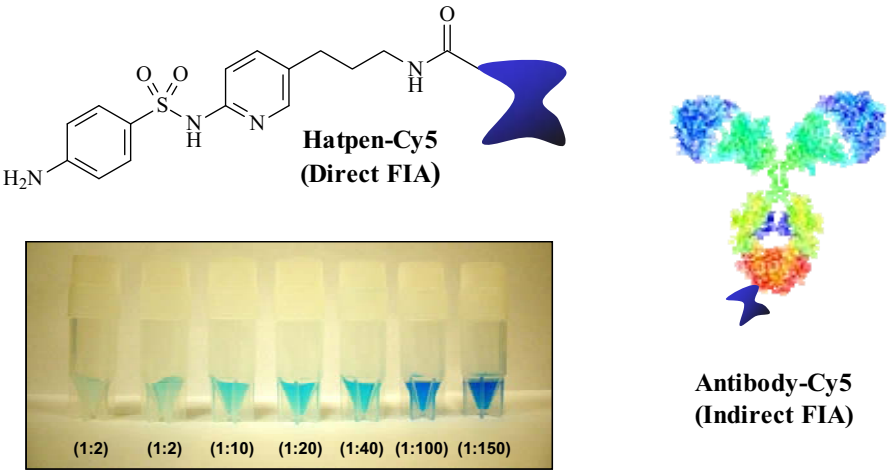

Figure 7.8: Fluorescent Cy5 labelled reagents prepared at different dye ration.

First the sulfonamide competitor was conjugated with the fluorescent label (SA2-Cy5) and being purified the final compound by preparative HPLC and characterized by NMR (see **Figure 7.9** and **Figure 7.10**).

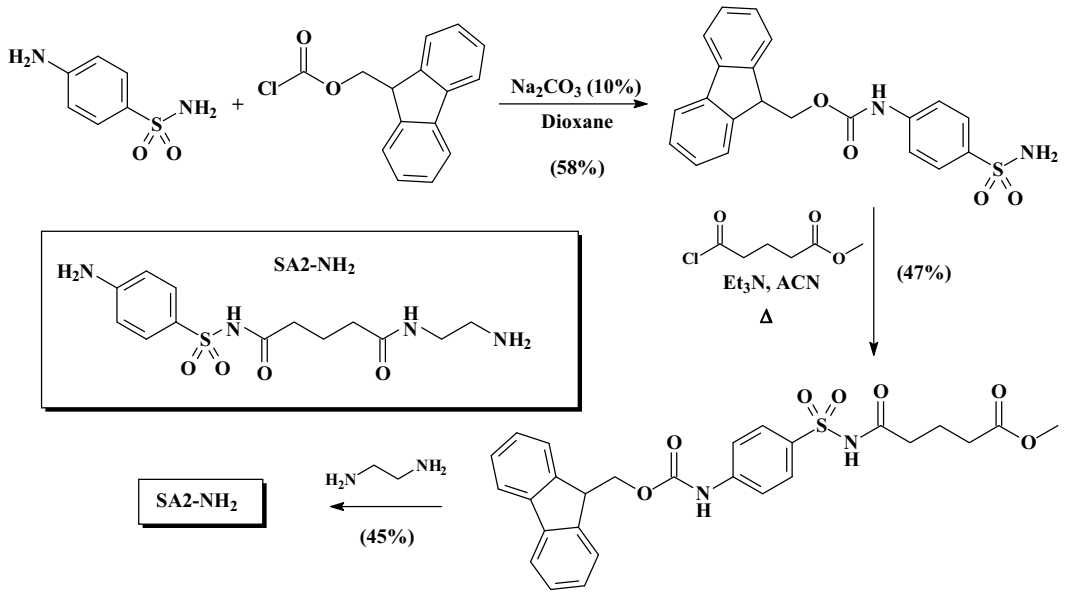

Figure 7.9: Ssynthesis of SA2-NH_2 compound (precursor of fluorescent SAs competitor).

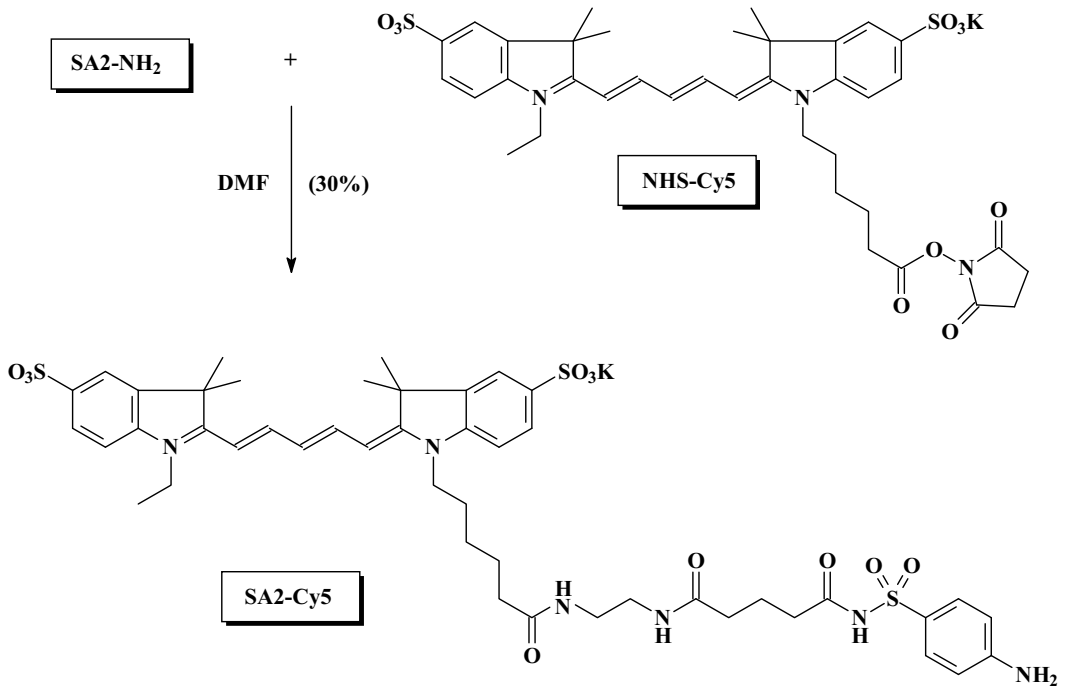

Figure 7.10: Scheme showing the synthesis of fluorescent labelled SAs hapten (SA2-Cy5).

Second, purified fractions of As155 and pre-immune serum were conjugated with different ratios of NHS-Cy5 dye (see **Figure 7.8** and **Figure 7.11**) being purified the final conjugates by dialysis and characterized by UV and fluorescent experiments.

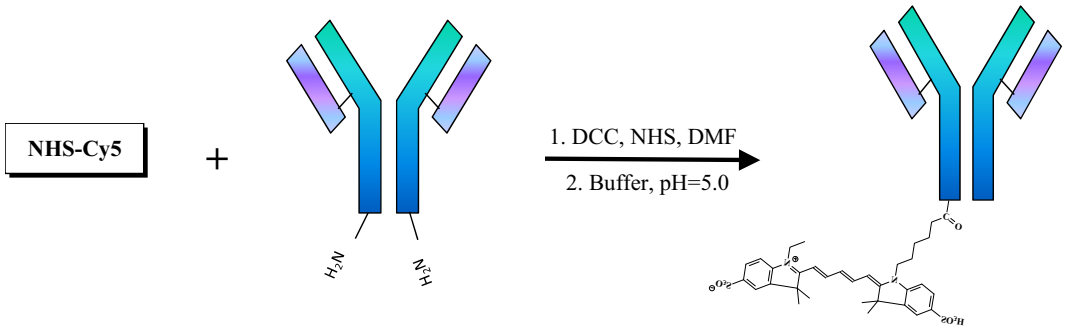

Figure 7.11: Scheme showing the preparation of fluorescent labelled SAs antibody (As155-Cy5).

Although the spectral data of both conjugates (SA2-Cy5 and As155-Cy5) had the same profile that the isolated dye (see **Figure 7.7**), indicating that bioconjugation had taken place, we were not able to establish a competitive immunoassay in any FIA format. Our hypothesis was that the lower quantum yield of Cy5 (0.28 in PBS) in comparison to fluorescein (0.88 in PBS), combined with a potential lack of sensitivity of our fluorescent reader, could have been the reason of this undesirable result. In this respect, it should be noticed that subsequent experiments performed by Núria Tort, from our research group, using microarray glass slides and a microarray scanner (ScanArray Gx PLUS, Perkin Elmer, USA) showed that these kind of fluorescent reagents performed well in that configuration (data not shown), which is in support of our initial hypothesis.

7.4.1 Quantum Dots

Alternatively, we evaluated the possibility to use QDs as fluorescent labels. QDs are a novel class of inorganic fluorophores which are gaining widespread recognition as a result of their exceptional photophysical properties [3]. These compounds are nanometer-scale semiconductor crystals composed of groups II–VI (e.g. CdSe, CdTe, CdS, and ZnSe) or III–V (e.g. InP and InAs) elements, and are defined as three dimensional particles with physical dimensions smaller than the exciton Bohr radius [4]. When these particles are photo-excited, electron-hole pairs are generated and upon their recombination fluorescence light is emitted. Due to their small size quantum effects play an important role, which finally results in size dependent wavelengths of fluorescence (see **Figure 7.12**). The smaller the particles, the more blue-shifted their fluorescence [5]. In this way all colours in the visible and infrared spectra can be obtained by synthesizing nanoparticles of different sizes. Furthermore, QDs have a continuous absorption spectrum for wavelengths shorter than the wavelength of fluorescent emission. Their emission spectra are quite narrow and symmetric, and do not show any red-tail. In this manner, many different colours can be excited with just one wavelength of excitation and can be spectrally well resolved [6]. Between other advantages it has been claimed their greater

photostability (which determines the excitation intensity to be used and the number of possible measurement cycles) and their high quantum yields, in respect to the organic fluorophores. For this reason, within the last years, QDs are increasingly employed, on fluorescence assays [7], fluorescence imaging [8-10] and as biosensors [11].

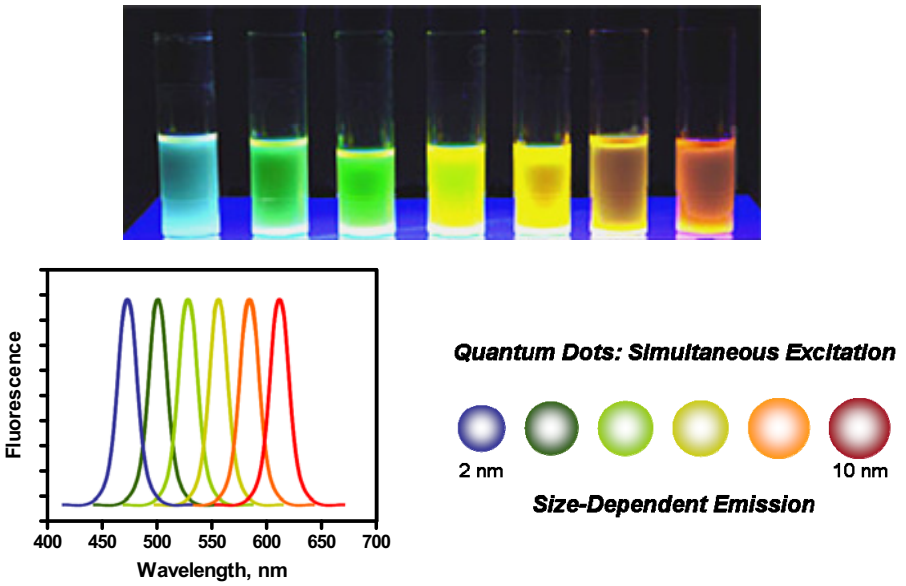

Figure 7.12: Fluorescence spectra of quantum dots of different sizes.

Moreover, the possibility to tune their optical properties QDs offer the possibility to implement them on multiplexed bioanalytical techniques [12].

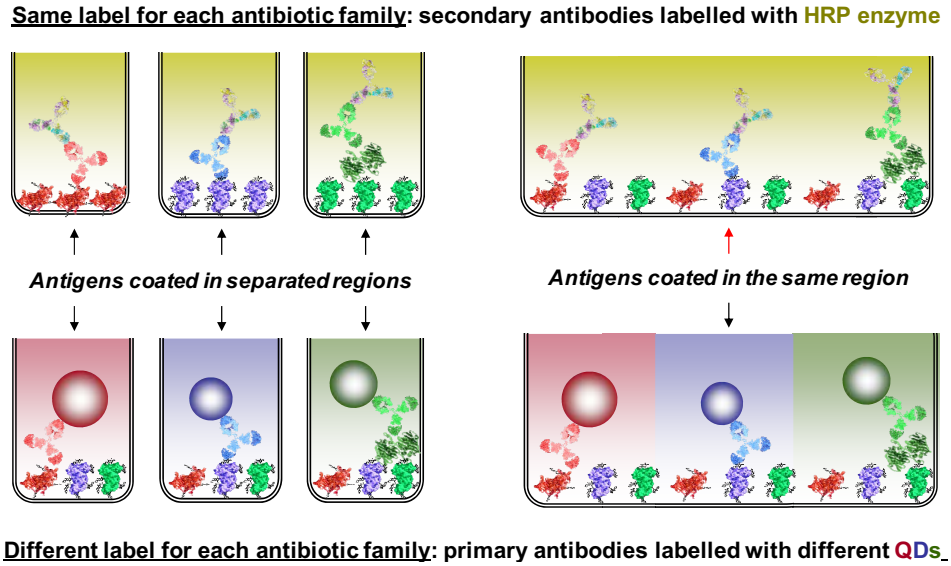

Figure 7.13: Scheme of different detection strategies for antibiotic multianalysis. If the antigens are in the same ELISA microtiter plate regions it's not possible to use the same label.

Thus, the multianalyte ELISA and WIObS techniques were based on planar microarray configurations where the identity of the target analyte is encoded by its location in the detection platform. QDs could allow developing multianalyte detection schemes using encoded antibodies or bioreceptors (non-planar microarray) which may have enhanced features due to the possibility to simplify the detection platform (see **Figure 7.13**).

7.4.1.1 Quantum dots introduction, principle and applications

At the light of the potential advantages of using QDs as labels, we envisaged an scenario in which if we could succeed on preparing such kind of probes, those could be used for the development of the initially proposed optical biosensor, but also to develop a non-planar or encoded multiplexed analytical platform for antibiotic detection linking different QDs to distinct primary antibiotic family antibodies (see **Figure 7.14**).

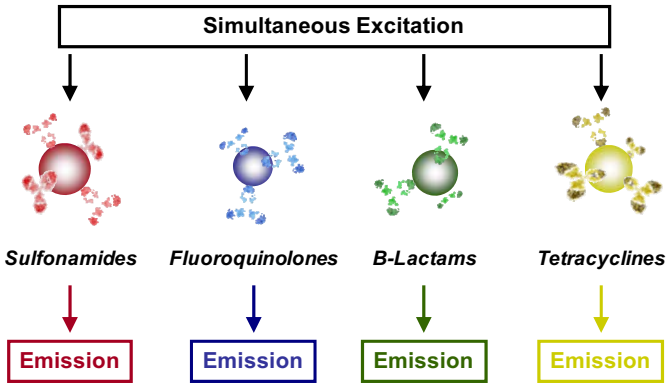

Figure 7.14: Multisensing strategy thought to be implemented by binding covalently antibodies for each antibiotic family to QDs of different sizes.

This research work could be approached thanks to the collaboration established with the group of the Professors A. Sanz-Mendel and J.M. Costa-Fernández of the Department of Physical an Analytical Chemistry from the University of Oviedo.

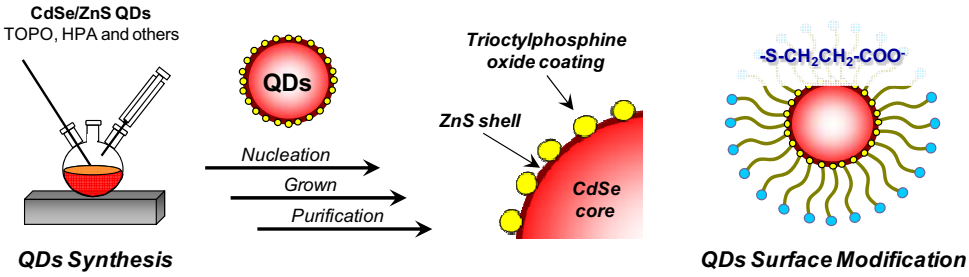

Figure 7.15: Scheme of QDs synthesis to obtain CdSe/ZnS nanoparticles capped with carboxylic functional groups as mercaptoacetic acid (Image courtesy from the Oviedo's group).

At that moment, they were conducting a very interesting research focussed on the preparation and modification of QDs to make them water soluble and use them on different analytical applications [13-15] (see **Figure 7.15**). Thus, they provided us with water soluble QDs made of CdSe with a shell of ZnS. Our challenge would be to be able to prepare biofunctional QD probes useful for our purposes. Thus, we decided to start with an indirect immunoassay format, as with the multianalyte ELISA developed for multianalyte antibiotic analysis, which meant that the QDs had to be conjugated to the primary antibody. As we did with the organic fluorophor (see **Figure 7.7**), the QDs were characterized in solution using opaque microtiter plates with a Gemini XPS spectrofluorometer to obtain the emission spectra and to chose the optimal excitation and emission landa to perform further experiments (see **Figure 7.16**).

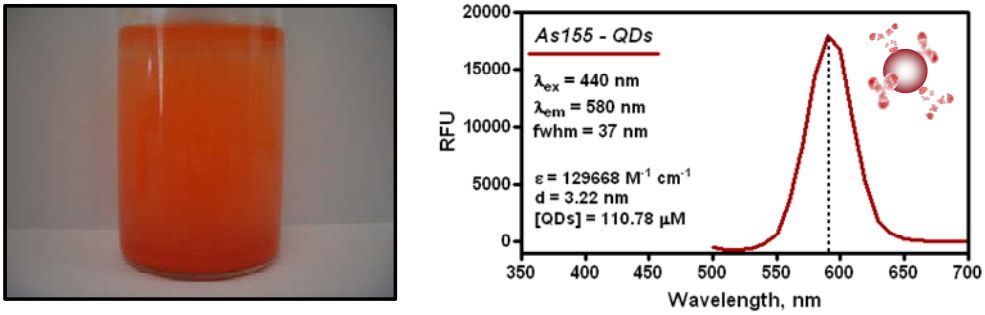

Figure 7.16: Picture of QDs supplied by the group from the Oviedo University as well as their most important parameters evaluated before their bioconjugation.

The QDs provided by the group of the University of Oviedo had carboxyl functional groups on their surfaces, for which reason coupling to the amino groups of As155 (ammonium sulphate purified fraction) was performed via EDC chemistry (see materials and methods in **Section 4.2**). For this purpose, the appropriate volume of the antibody solution (specific and pre-immune) was mixed with several QDs solutions at different concentrations, together with an excess of the carbodiimide, and the mixture was kept under magnetic stirring conditions at room temperature for four hours (see **Figure 7.17**). The study of different antibody:QDs ratios had the aim to accomplish a bioconjugated providing sufficient fluorescence signal while avoiding conformational changes of the antibodies molecules that could negatively affect their recognition capabilities. Moreover, bioconjugates were prepared with both, specific and non-specific (pre-immune serum) antibodies in order to have negative controls and to assess potential non-specific signals. Purification of the conjugates, was initially performed by multiple and consecutive centrifugation and resuspension steps in PBS in order to exclude the free antibody fractions, and/or using size exclusion chromatography (Superdex 200 phases) although in

any case it was possible to not separate completely the QDs-As155 and from the non-conjugated QD fraction.

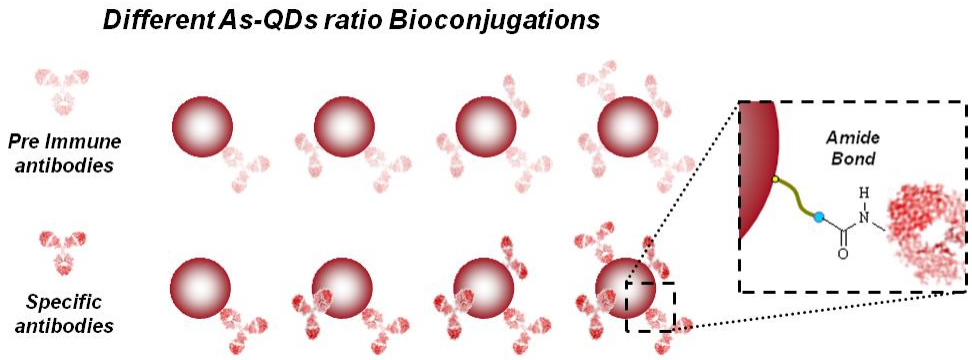

Figure 7.17: Scheme of the strategy planned to prepare different QDs – antibody conjugate ratios. Preimmune antibodies were also coupled to QDs to further evaluate non-specific interactions in the FIA experiments.

The separation of both fractions has been described to be accomplished by HPLC using high-resolution size-exclusion columns [15], but since we did not have access to these columns and the previously mentioned methods had been described by other authors as the usual way to prepare these bioconjugates, we decided to proceed even if the purity QD probes was not complete. In fact, the presence of a small fraction of unlabelled QDs should not interfere in the FIA proposed. On the other hand, the antibody:QDs ratio employed should have ensured an almost complete biofunctionalization of the QDs since the antibody was used in excess. The optical properties of the resultant bioconjugates were compared to those of the intact QDs observing no significant variations of the optical properties. Moreover, qualitative and quantitative experiments performed to evaluate coupling of the antibody employing the Bradford test (see **Section 4.3.4**) analyzing the supernatants indicated that coupling to the nanoparticles had occurred. Unfortunately, when these bioconjugates were employed to develop the FIAs, the results were negative since the signal was always really low. As before, we doubted on the sensitivity of the fluorescence microplate-reader, although it also should be noticed the discordance in the quantum yield values reported for these nanoparticles. Using standard methods [16], fluorescence quantum yields for CdSe are in the range between 0.65 and 0.85 for CdSe [17, 18] or 0.1−0.8 for PbSe [19, 20] Apparently, these values are extremely dependent of parameters such as the size, shell thickness, ligands, but also on the methods and instruments used to characterize them. In our case, the QDs used had a core of CdSe, and a shell of ZnS, and at that moment it was not available any other information in respect to the quantum yield or the absorption coefficient. Thus, it could have been that, as with Cy5, the sensitivity of our reader could have been insufficient.

7.4.1.2 Recapitulation

At this point of the research, it was considered necessary to propose a new technological approach in order to reach the objective planned in the GOODFOOD project. It should also be noticed that when the Cy5-labelled immunoreagents were sent to CSEM to evaluate performance in their sensor set-up provided of an appropriate laser and a very sensitive CMOs camera, the results were also not satisfactory. Then the proposed system was a label-less optical biosensor based on the physical phenomenon already explained in **Chapters 5** and **6**. To simplify the set-up, the use of magnetic nanoparticles was in principle rejected. In this respect, the results further on obtained in all formats and techniques evaluated (see **Chapters 6.2** and **6.3**) demonstrated that there was no need to treat or to extract selectively the antibiotics from milk samples, since the interferences caused by the matrix were negligible thanks to the great features of the immunoreagents developed. As it has been described before the WIObS resulted an excellent device for automated antibiotic screening although further technological improvements were necessary at the time the work performed in this thesis finalized.

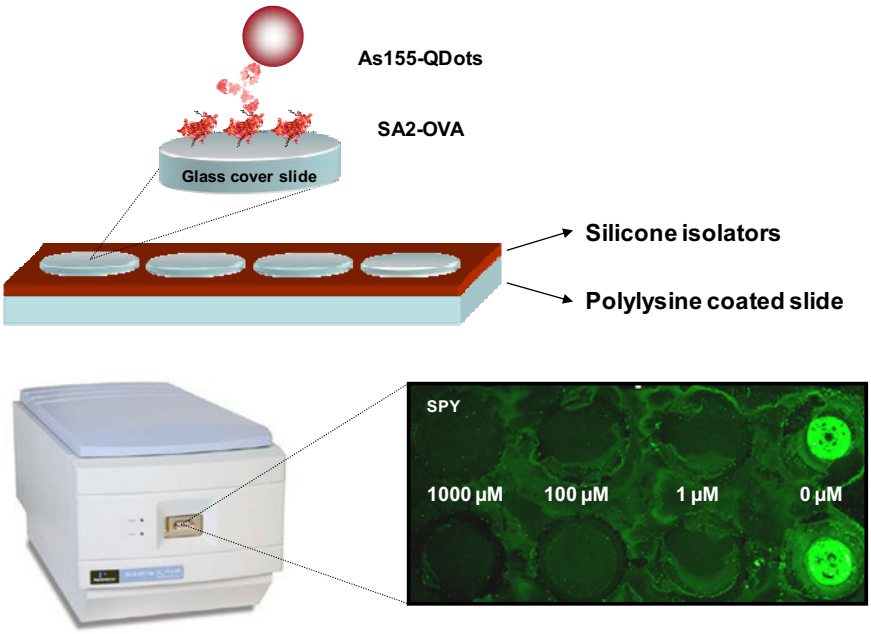

Figure 7.18: Representation of the new solid support used to perform the QDs experiments, which consisted on polylysine coated slides separated by silicone membranes. Picture of the scanner equipment used to run the experiments (down on the left). Results obtained show a decrease in the fluorescence intensity of the QDs in presence of SPY (down on the right).

In respect to the QDs, subsequent studies performed by Dr. Núria Sanvicens using microarray glass slides spotted with the antigen (SA2-OVA) and As155-QDs

bioconjugates demonstrated, as with the case of Cy5, that the use of a more potent laser (excitation wavelength at 535 nm) of an appropriate reader (ScanArray Gx PlusMircroarray Perkin Elmer) could in part solve the problems encountered. Thus, initial experiments performed on polylysine coated glass-lides (Thermo Scientific) covered with silicon gaskets to define the bioassay zones, rendered very promising results (see **Figure 7.18**). However, a lack of reproducibility associated to a low stability of the antibody-conjugates, prompted her to test commercially available QDs. Although similar difficulties were encountered with these types of nanoparticles, it was possible to demonstrate that the use of QDs can significantly enhance the analytical performance of fluorescent-based bioanalytical methods. Thus, by using these commercially available QDs, the appropriate fluorescent reading equipment, and the antibody bioconjugation procedure developed in this thesis, Dr. Nuria Sanvicens was able to enhance significantly the detectability of an assay developed for *Escherichia coli,* a microorganism widely distributed with some pathogen strains (see **Figure 7.19** and **Chapter C.2.2**).

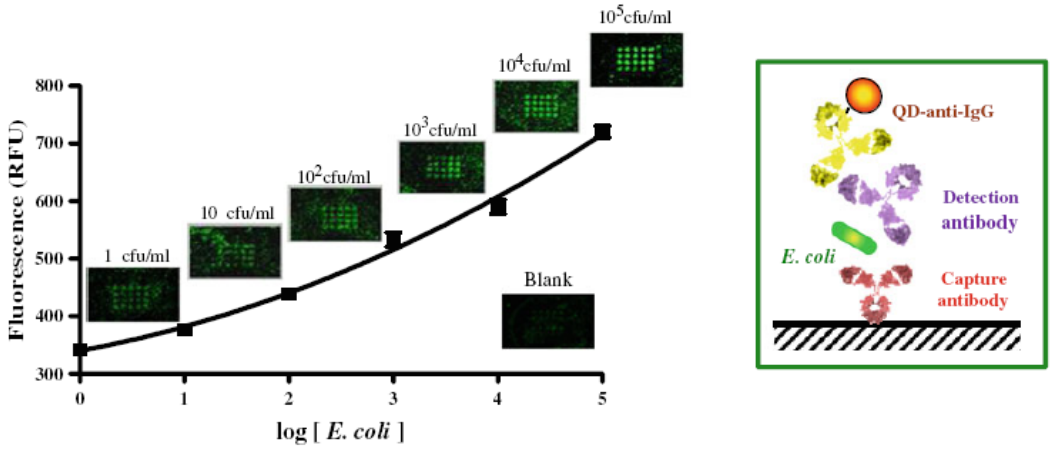

Figure 7.19: Dose-response curve for E. coli O157:H7 in a sandwich array-based assay. The standard curve was fitted to a quadratic polynomial equation as indicated by Herman et al [21] and de LOD calculated using the statistical approach reported by Long and Winefordner [22]. Results correspond to the average and standard deviation of four assays run on 4 different days in duplicate (n=12).

The bacteria could be detected down to 2CFU mL^{-1} in buffer samples using this technology, when the same immunoreagents used in the microplate-ELISA did reach detectability around 10^3 CFU mL^{-1} [7].

7.4.2 Main contributions

The main contribution of the work described in this chapter has been the input to the final design of the multiplexed immunosensor reported in **Chapter 6**. However, additionally

there has been acquired sufficient knowledge in other areas, which have been crucial for subsequent developments and achievements of our research group:

- The support given to Nestlé partner by preparing and distributing a questionnaire in order to know the main end user requirements for the optimal detection of antibiotic residues in milk samples.
- The establishment of a reproducible procedure to covalently attach the anti-SAs class-specific antibodies to carboxyl-modified magnetic beads, and the development of a magneto-ELISA with similar features than the standard ELISA, and much more robust to the matrix interferences caused by the milk samples.
- The establishment of bioconjugation procedures to label the SAs immunoreagents of fluorescein and Cy5.
- The establishment of a bioconjugation procedure to prepare QDs probes. The procedure was developed using SAs antibodies, although due the corresponding bioanalytical procedure could not be established. However, using the procedure developed within this thesis, it has been possible to develop a fluorescent quantum dot-based antibody microarray to detect *Escherichia coli* [7]. Although there are still many questions to be solved in respect to the reproducibility and stability of the QDs and their bioconjugates, these results are promising and point to the possibility to develop multiplexed bioanalytical techniques with enhanced features in respect to the detectability that it may be possible to achieve.

7.5 Bibliography of Appendix

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8 CONCLUSIONS

8.1 Conclusions of Chapter 2: MIPs for SAs

- It has been developed a new methodology, by combining computational approaches and NMR experiments, for the rational design of the polymerization mixture to obtain a MIP able to interact selectively with SMZ, by means of non-covalent interactions approach, and showing very good affinity and chromatography properties.

- A combinatorial strategy based on the development of simple sequential experimental assays, but increasing their complexity, was established to exclude, from a big batch of polymers prepared but using different porogens after the rational design, those MIPs with worse recognition properties and having fewer candidates in each step without having to perform an accurate characterization of all candidates.

- The MIP preparations based on rational design have superior performance in comparison with typical MIP preparation through a lengthy process of experimental trial and error before finding the most suitable recipe.

8.2 Conclusions of Chapter 3: Natural receptors for SAs

- Although most of antibiotics, such as SAs, FQs, BLs or TCs, are compounds that are regulated or prohibited in the agro food field, the survey data collected and analyzed demonstrate the continued use of fraudulently, hence it is interesting to continue research on new methodologies and techniques for detecting these compounds.

- Using a hapten that preserve the whole sulfonamide structure (hapten SA1) produced antibodies with better detectability and wide range of recognition for this antibiotic family than those obtained when the immunization hapten contained only the common fragment of the SAs (hapten SA2). Thus, with these antibodies it has been possible to develop IAs with detection limits below the European regulations regarding the detection of residues of this antibiotic family in agro food products. The results indicate that keeping in the immunizing hapten, the basic structure defined by the aniline, the sulfonamide bridge and a heterocycle with, at least, one nitrogen at

position β in respect to the sulfonamide bridge, has been crucial to accomplish the broad recognition range of the antibodies produced.

- From the results of this thesis, it is not possible to provide support to any of the distinct hypothesis related to the convenience of using homologous or heterologous antibody/competitor combinations on the competitive immunochemical analytical methods, to reach the highest detectability. Thus, the detectability reached by the direct SAs ELISA has found to be higher when using homologous combinations of antibody/enzyme tracer. On the other hand, in the indirect format the detectability is greater when using heterologous combinations of antibody/coating antigen.

- In contrast, our results support the rule stated previously by this and other research groups, in respect to the effect of the assay heterology on the specificity of the assay. Thus, it has been demonstrated that using heterologous combinations it is possible to reach a broader recognition profile of the different SAs congeners. This is in accordance with data reported literature pointing to lower affinity of the antibody versus the competitor as the main reason to favour recognition of other related compounds that can interact with the antibody.

- The excellent features of the antibodies generated against SA1 hapten, and their high recognition capabilities against the SAs have been identified as the most important reason of the robustness of the ELISA methods developed in front of complex samples. Thus, the SAs ELISA formats developed can effectively detect traces of SAs residues in milk and hair extracts, without any sample treatment other than dilution, and in spite of the complexity of the composition of these samples. These results point to the possibility of using these immunochemical methods to the analysis of SAs residues in other matrices.

- The antibodies generated against SAs have displayed better affinity properties than the corresponding MIPs and therefore being more suitable for the further development of a biosensor. On the contrary, MIPs have presented physical and chemical stability under organic solvents and thus fitting better as selective materials for stationary phases or for solid-phase extraction applications.

8.3 Conclusions of Chapter 4: Natural receptors for TCs

- Although the structures of the tetracycline haptens for immunization were designed with the aim of obtaining with class-selective antibodies, the results show that to achieve this goal would be crucial to take into account other considerations than just the position of the spacer arm on the upper periphery.

- It has been achieved an immune response with the two immunizing haptens prepared against TCs but only those raised against TC1 provided the possibility to develop competitive ELISAs. An ELISA has been developed able to detect DC and MC compounds with sufficient detectability, although the avidity of the antibodies raised for the different competitors prepared was not so high as for the case of the SAs antibodies.

- The ELISA developed can be applied to the analysis of milk samples, in compliance with the European regulations established for these compounds. However, in this case it has been necessary to develop a sample treatment consisting of precipitating milk proteins followed by a dilution step. This fact is in accordance with our hypothesis in respect to the greater tolerance to the matrix when the antibodies show higher avidity for the analyte and/or the competitors (see above).

8.4 Conclusions of Chapter 5: Immunosensor development

- It is possible to detect low traces of antibiotic residues by combining the WIOS transducer with specific bioreceptors. The WIOS has proved to be sensitive enough to detect variations in the refractive index produced within the evanescent field generated by light travelling in the waveguide, due the binding of the antibody to the antigen biofunctionalized sensor chip. However, it has been necessary using a secondary antibody in order to enhance the signal and to obtain more accurate response.

- The features of the immunoreagents have a strong influence on the performance of the sensor. Thus, in the present study the SAs WIObS system developed shows comparable features, in terms of detectability and specificity, if compared to the ELISA previously developed.

- The SAs WIObS system is sufficiently robust to selectively detect binding interactions in the surface of the waveguide, even in the presence of complex samples such as the case of milk. Thus, SAs residues could be detected with the biosensor without any sample treatment. The developed system allows discriminating between milk contaminated samples with SAs at or above their MRLs, in less than 30 min, including regeneration of the sensing surface.

8.5 Conclusions of Chapter 6: Multiresidue detection

- Bioreceptors can be successfully combined and used as cocktail on multiplexed bioanalytical methods, due to their high specificity and avidity for their counter partner. Thus, within this thesis, SAs, FQs and BLs class-selective bioreceptors have been successfully combined for a multiresidue detection on an orthogonal ELISA system, where the identity of the target analyte is encoded by its location in the detection platform. Similarly, on the multiplexed WIObS system, with the addition of four bioreceptor for TCs. We believe that the excellent features of the bioreceptors, together with the structural differences of these four antibiotic families, have been determinant to be able to establish these multiplexed bioanalytical technologies. Specific responses are obtained independently of whether the bioreceptors are used individually or as mixtures, indicating the absence of a shared-reactivity effect. The sensitivity and specificity of the response remains even when the sample contains mixtures of the other antibiotics and is independent of their concentration.

- The use of bioreceptors individually or in combination does not affect the matrix tolerance, as soon as the avidity of all these bioreceptors for their targets is sufficiently high, as it has been in this case. Thus, both the multianalyte ELISA and

the multiplexed WIObS perform very well in milk samples without any prior sample treatment other than dilution.

- Both multiplexed systems have demonstrated to be useful as screening tools in antibiotic residue analysis, being able to detect simultaneously a high number of antibiotic congeners on a single run. Thus, for the case of the microplate-based ELISA, two protocols have been established, one for screening purposes to detect samples with residue levels over the MRL values, and another one to quantify the concentration of the antibiotic in the sample. In both cases, more than 25 different antibiotics (SAs, FQs and BLs families) can be detected in compliance with the regulations of the EC. On the other hand, the multiplexed WIObS system has the potential to detect 34 antimicrobials in milk samples in compliance with EC. No sample treatment other than dilution is necessary to obtain reliable data regarding the potential presence of these families of antibiotics in this matrix.

- Detection of the four antibiotic families using the WIOS platform showed the same relative responses as by ELISA or dipstick individual formats demonstrating that the selectivity is mainly determined by the bioreagents, not by the assay or device configuration.

- All the bioanalytical methods have both advantages and drawbacks. Thus, within this thesis, three bioanalytical methods (ELISA, WIOS and dipstick) using the same bioreagents but basing their response on different principles, have been compared. All of them are able to straightforwardly detect simultaneously antibiotics from different antibiotic families in milk samples. Dipsticks are currently the best screening method for in-situ measurements since they are simply to use, rapid and easy to read the results. Although the ELISA technique can be used as a screening technique, nowadays we consider it is the ideal methodology to evaluate the quality of the bioreagents prepared prior being implemented in other analytical detection platforms. WIOS platform need further improvements, such as fluid handling, costs, data processing, and optimization of bioreagent conditions, to be used as a field instrument.

8.6 Conclusions of Chapter 7: Appendix

- It is possible to prepare biofunctional magnetic beads with ability to detect SAs. Thus, anti-sulfonamide class-specific antibodies have been successfully covalently coupled to carboxyl-modified magnetic beads, achieving an excellent antibody coupling efficiency. These magnetic beads have been used to develop a magneto-ELISA showing similar features to that of the standard microplate-based ELISA. This results opens the door to the implementation of these bioreagents on a variety of biosensor configurations more suitable for complex samples analysis inside microfluidic systems. Thus, the use of biofunctional magnetic particles allows performing extraction steps and moving the bioreagents within different microfluidic chambers, by driving the antibody-MNP with a magnet.

- It is possible to prepare fluorescent probes by covalently linking fluorescent organic dyes or nanoparticles to antibodies. Thus, Cy5 and QDs have been successfully coupled, via covalent bonds, to both SAs hapten and/or antibodies, as demonstrated by the excitation/emission spectra recorded. Unfortunately it has been not possible within the context of this thesis to develop to use these reagents for establishing immunochemical analytical based on fluorescent principles.

9 RESUMEN

9.1 Introducción

En los años 50, científicos y expertos en medio ambiente estaban centrados en el estudio de sustancias químicas sintéticas o naturales derivadas de la agricultura (fertilizantes, pesticidas), la industria química (compuestos organohalogenados, metales pesados), así como residuos de fábricas y subproductos secundarios (dioxinas, furanos) [1]. El foco de atención en los 70 para los programas de control de contaminación del medio ambiente, tanto de la Comisión Europea (EC) como de los Estados Unidos, estaba dedicado a los contaminantes prioritarios convencionales, especialmente los referidos colectivamente como tóxicos bioacumulativos persistentes, contaminantes orgánicos persistentes y otras sustancias químicas bioacumulativas preocupantes [2]. Hasta la década de los 90, otros contaminantes no convencionales fueron ignorados en gran medida debido a su mayor solubilidad en agua, en relación con otros contaminantes, complicado así su análisis químico, siendo más fácilmente degradados y previniendo su escape a la atmósfera [3]. Una parte significativa de estos importantes contaminantes ambientales no reconocidos o emergentes son ahora ampliamente utilizados en actividades urbanas diarias e incluye varios productos llamados de atención farmacéutica y personal (PPCP). La introducción de varios PPCPs, como por ejemplo productos farmacéuticos veterinarios y plaguicidas, ha sido el elemento clave para alcanzar una productividad muy alta en los modernos sistemas de agricultura intensiva. Por otro lado, el uso indiscriminado y/o inadecuado de estos productos es la causa de posibles efectos adversos para la salud debido al riesgo que existe en que entren en la cadena alimentaria a través de la aparición de residuos en productos alimenticios de origen animal [4, 5].

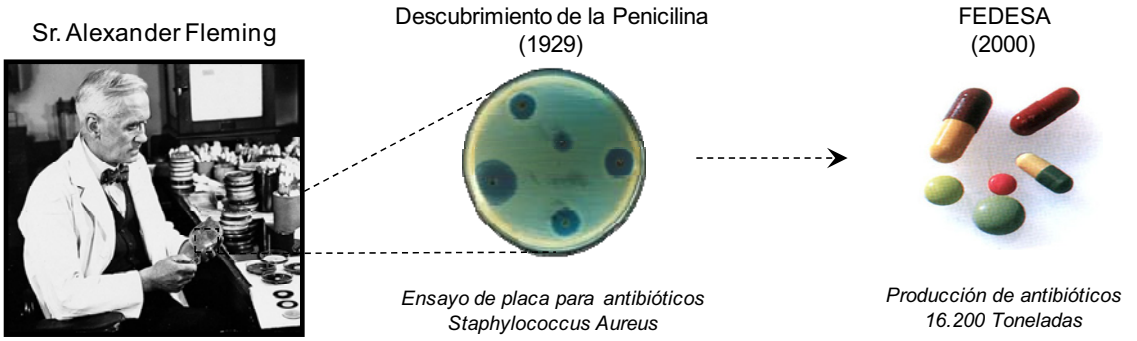


Figura 9.1: Evolución de los antibióticos.

En el caso concreto de los antibióticos, este hecho ha sido identificado como una de las causas de la aparición de mecanismos de resistencia para estos compuestos en bacterias que causan enfermedades humanas [6-8], lo cual es motivo de gran preocupación por parte de las autoridades responsables de proteger la salud de los ciudadanos, de distintos organismos

gubernamentales y de la sociedad en general. Por otra parte, muchos de los problemas tecnológicos e importantes pérdidas económicas en los productores lácteos están relacionados con la inhibición de los procesos de fermentación bacteriana que intervienen en la elaboración de productos lácteos, queso y derivados. En este sentido, hay una creciente necesidad de garantizar la seguridad y calidad de los productos, además los clientes también han empezado a ser más exigentes obligando a la industria a considerar las preocupaciones de los consumidores en sus procedimientos diarios, en términos de conseguir productos más naturales, ecológicos y saludables con una clara trazabilidad del origen de los diferentes ingredientes utilizados. Por esta razón, el foco principal de preocupación de la EC se ha centrado en el uso y control de antibióticos y compuestos con actividad hormonal, especialmente en especies animales para ser consumidas, mediante la presentación de varios reglamentos y directivas (véase **Tabla 9.1**).

Tabla 9.1: Lista de las regulaciones, directivas y decisiones más importantes establecidas por la Comisión Europea para regular y controlar el uso de medicamentos veterinarios.

Documentos legales de la UE	Información
Regulación 178/2002/EC	Principios generales y requisitos de la ley de seguridad alimentaria
Regulación 2377/90/EC (Anexos I, II, III y IV)	Establecimiento de niveles de residuos de seguridad para los medicamentos veterinarios en los alimentos
Regulación 1831/2003	Reglamento sobre los aditivos en la alimentación animal
Directiva 96/22/EC Directiva 97/6/EC	Prohibición de promotores del crecimiento en animales productores de alimentos
Directiva 96/23/EC	Requisitos de supervisión del control de los antibióticos
Decisión 2002/657/EC	Directrices técnicas para el control de residuos

En Europa se han regulado estrictamente los controles del uso de medicamentos veterinarios, incluidos los agentes de la promoción del crecimiento [9], sobre todo en las especies animales destinadas al consumo humano, mediante la emisión de varios reglamentos y directivas, incluso prohibiendo desde 1998 que los antibióticos utilizados en medicina humana sean incluidos en piensos para animales [10]. El uso de medicamentos veterinarios se regula a través del Reglamento 2377/90/EC, que describe los procedimientos para el establecimiento del límite máximo de residuos (MRL) [11] de los medicamentos veterinarios en los alimentos de origen animal (por ejemplo, leche, huevos o carne). Pero si bien la Directiva 96/23/CE [12] y la Decisión 2002/657/CE [13] dictan la frecuencia, el número de análisis a realizar y las sustancias que tienen que ser controlados, la mayoría de métodos de análisis disponibles en la actualidad no permiten cumplir con estos requisitos, principalmente debido a la baja capacidad de procesado de muestras, lo cual suele estar relacionado con problemas derivados de la necesidad de efectuar un tratamiento de muestras biológicas, previo a su análisis [14].

Con el fin de seguir la normativa vigente, alrededor de 50 antibióticos diferentes deberían ser detectados en diferentes productos alimentarios. En la actualidad, esta no es una posibilidad

realista debido al coste de los análisis siendo casi siempre inevitable el uso de diferentes procedimientos y metodologías analíticas para cada familia de antibióticos (10 clases). Incluso en la misma familia, en ocasiones no es posible detectar todos los congéneres empleando un único procedimiento. Además, el tiempo para la mayor parte de estos análisis es también una limitación para un estudio completo de residuos de antibióticos en un gran número de muestras. Por eso se han llevado a cabo análisis de mercado para establecer prioridades en el campo de análisis de residuos de medicamentos veterinarios, como por ejemplo para poder seleccionar los analitos más relevantes o indicadores de contaminación, los niveles de detección, el tiempo de medición o el precio por ensayo óptimo dependiendo del tipo de muestra a analizar. En nuestro caso, ya que la leche fue la matriz principal seleccionada para ser estudiada, contactamos con productores lácteos (inter)nacionales, como Nestlé, Pascual o Puleva, y varios laboratorios de referencia Europeos con el fin de establecer las especificaciones para un sistema de vigilancia de residuos de antibióticos (véase la sección **Appendix**). Según los resultados obtenidos en las encuestas realizadas, sulfonamidas (SAs), fluoroquinolonas (FQs), ß-lactamas (BLs) y tetraciclinas (TCs) fueron las familias de antibióticos prioritarias para ser detectadas en leche y productos lácteos. Por otro lado, el posible uso ilegal de estas familias de antibióticos en el sector veterinario (es decir, profilaxis y/o mejora de los efectos del crecimiento) también hacen necesario su análisis en otras muestras biológicas como por ejemplo el pelo (**Figura 9.2**).

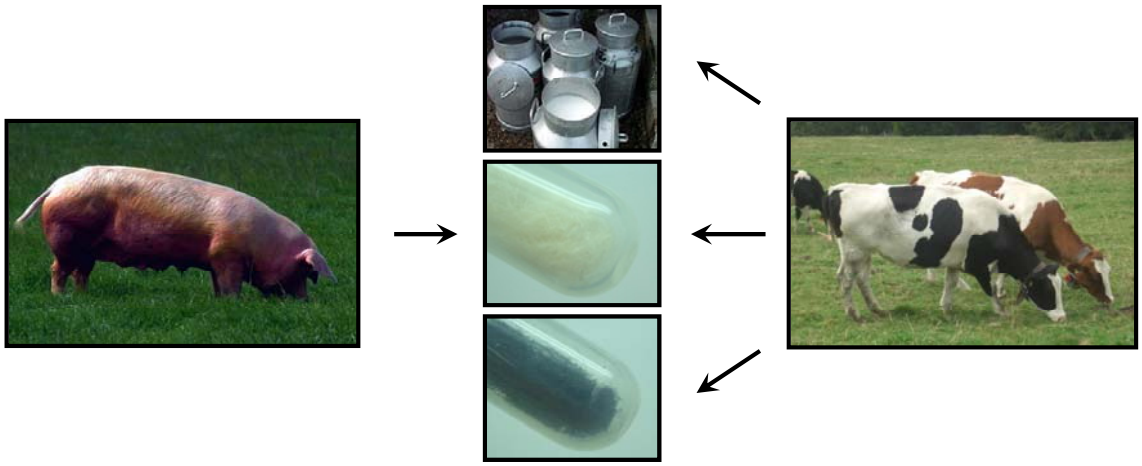


Figura 9.2: Muestras biológicas analizadas con los diferentes ELISAs desarrollados (Leche: SAs y TCs; Pelo: SAs).

Por otro lado, el control de estos antibióticos en alimentos se realiza normalmente en laboratorios centralizados, que en la mayoría de casos usan procedimientos muy fiables, pero que requieren equipos costosos, personal especializado y procedimientos de tratamiento de muestra muy largos. Con el fin de mejorar drásticamente esta situación, la EC apoya firmemente actividades de investigación destinadas a mejorar la eficiencia de los métodos de análisis

actuales. Una estrategia consiste en aprovechar los últimos avances bio-micro-nanotecnológicos y las capacidades complementarias que puedan aportar diferentes grupos de investigación multidisciplinarios para desarrollar metodologías más rápidas, sensibles y específicas capaces de detectar una amplia variedad de substancias químicas, biológicas o cualquier otro riesgo para la salud asociados a la industria agroalimentaria a lo largo de toda la cadena alimentaria (animales individuales, tanques de leche, camiones cisterna, etc.). Hoy en día los requisitos, tanto en términos de tiempo y costes, de los métodos analíticos más tradicionales (es decir, técnicas cromatográficas) a menudo constituyen un obstáculo importante para su aplicación de manera regular. En este contexto, dispositivos biosensores portátiles y equipos de monitorización en tiempo real, así como los ensayos de campo como kits de ensayo, tiras reactivas, e indicadores aparecen como herramientas adecuadas de análisis complementarias a las ya existentes.

Esta tesis describe la investigación realizada, en el marco de proyectos nacionales del Ministerio de Ciencia e Innovación (ARGOS y PANOPTES) y europeos del sexto programa marco (GOODFOOD) que han apoyado diferentes aspectos de las investigaciones realizadas, con respecto a la posibilidad de desarrollar alternativas más adecuadas para el análisis de residuos de antibióticos en alimentos basadas en la combinación receptores selectivos y novedosas micro/nano-tecnologías.

9.2 Objetivos

El objetivo final de esta tesis ha sido el desarrollo de un dispositivo biosensor multiplexado para el análisis de residuos de antibióticos en el sector de la alimentación. Teniendo en cuenta las consideraciones presentadas anteriormente, sobre problemática, legislación, muestras contaminadas de interés y requisitos de análisis, se propusieron varios objetivos específicos (SO):

SO1) Preparación y evaluación de receptores específicos, tales como los polímeros de huella molecular (MIPs) y los anticuerpos para la detección de SAs y TCs.

SO2) Evaluación del funcionamiento de bioreceptores en matrices de interés para el sector de la alimentación, tales como la leche y el pelo, mediante el establecimiento de los consiguientes protocolos inmunoquímicos de análisis como los de tipo ELISA (Ensayo por inmunoabsorción ligado a enzimas).

SO3) Establecimiento de métodos bioanalíticos multiplexados basados en la combinación de varios bioreceptores para llegar a la detección de una amplia gama de antibióticos (SAs, BLs, FQs y TCs) y la correspondiente evaluación de su funcionamiento (ELISA multianalito).

SO4) Implementación de este ensayo bioanalítico multiplexado en un biosensor óptico, mediante la integración de la bioreceptores en el transductor del sistema WIOS (wavelenght interrogated optical system), y la evaluación de su funcionamiento en matrices alimentarias reales, tales como la leche.

9.3 Resultados

9.3.1 Preparación de MIPs para sulfametazina

Durante los últimos años los MIPs han surgido como uno de los receptores sintéticos más utilizados hasta el punto de haber sido considerados como el enfoque más prometedor hacia el desarrollo de anticuerpos artificiales. Los MIPs son polímeros que poseen cavidades específicas diseñadas para una molécula objetivo determinada. Estos materiales se han preparado para un gran número de compuestos mostrando una afinidad molecular bastante buena y propiedades de selectividad, pero aún lejos de las capacidades de los anticuerpos. Por el contrario, su estabilidad química y térmica, su fácil y bajo coste de preparación los hace atractivos para numerosas aplicaciones, tales como la cromatografía líquida, electrocromatografía capilar, extracción en fase sólida (SPE) y biosensores [15]. El método de impresión molecular es simple, barato y fácil de realizar ya que en principio todo lo que necesitamos son monómeros funcionales, disolventes y agentes de reticulación además del analito o de los compuestos de interés. La polimerización es seguida por la eliminación del analito obteniendo estructuras moleculares bien definidas con una alta selectividad y afinidad hacia el complejo de evaluación objetivo. Por otro lado, enfoques racionales para la preparación de MIPs basados en datos combinados de modelos químicos computacionales y de datos experimentales mediante ^{1}H-NMR pueden ayudar a seleccionar los reactivos más adecuados y sus concentraciones (ver **Figura 9.3**).

Figura 9.3: Métodos y herramientas utilizadas para el diseño previo de MIPs.

La posterior aplicación de esta información en el diseño experimental y métodos de análisis multivariantes aumenta la posibilidad de obtener un material final con una buena eficacia de impresión molecular.

9.3.1.1 Diseño racional

Dentro del campo de investigación de MIPs se han utilizado herramientas de modelización asistidas por ordenador para obtener información sobre la estructura de los clústeres más

estables, su composición relativa y la distribución de todos los componentes en el espacio [16]. En este caso, el analito elegido fue la sulfametazina (SMZ) que se caracteriza por un grupo anilina (ligeramente básico), un enlace sulfonamida (ligeramente ácido) y dos átomos de nitrógeno presentes en el heterociclo, siendo todos ellos puntos de interacción potencial. El ácido metacrílico (MAA) y la 4-vinilpiridina (VPY) fueron seleccionados como los monómeros más adecuados capaces de interactuar con el analito. El ácido carboxílico presente en el MAA puede establecer puentes de hidrógeno (donante de hidrógeno) y actuar como dador de hidrógeno (ácido de Bronsted/interacción básica), mientras que la VPY, como buen aceptor de hidrógeno, puede ser un sistema complementario en las relaciones básicas de los ácidos, además de poder formar interacciones π-π entre los sistemas aromáticos complementarios. Finalmente, de todas las posibles interacciones moleculares calculadas, dos clústeres fueron seleccionados, en base a términos energéticos, siendo 1:2:3 y 1:3:2 la relación de los componentes de los polímeros (SMZ:MAA:VPY). La superficie del espacio mínimo que encierra ese grupo también se calculó a nivel teórico para hacer estimaciones sobre la cantidad de agente reticulado necesario para rodear todo el clúster. Encontramos que la proporción molar más adecuada entre SMZ y etilenglicol dimetacrilato (EGDMA) está alrededor de 1 a 20-25 aproximadamente (ver **Figure 2.9** y **Fig. 1** en **Section 2.2** del **Chapter 2**).

9.3.1.2 Estudios de interacción mediante NMR

La espectroscopia de resonancia magnética nuclear (NMR) generalmente se utiliza para caracterizar compuestos orgánicos, pero también puede ser una buena herramienta para evaluar y estudiar asociaciones moleculares. Ventajas de la NMR, con respecto a otras técnicas, es que pueden proporcionar datos experimentales acerca de la afinidad analito-monómero en un medio específico siendo muy útil para decidir, no sólo que monómeros pueden ser más adecuados para la preparación del MIP, sino también el porógeno en el que el/los clúster/es pueden ser más estables. Los resultados mostraron cambios en desplazamientos químicos debidos a pequeñas alteraciones en los alrededores de los protones del clúster. La primera evaluación cualitativa de los resultados obtenidos, cuando se utilizó acetonitrilo (ACN) deuterado, mostró diferencias significativas de algunos cambios en los desplazamientos químicos de los protones de la SMZ dependiendo del monómero utilizado. Las señales de los protones correspondientes al heterociclo (-CH_3 y -H) fueron las más afectadas en presencia del monómero MAA mostrando que la interacción entre este compuesto con SMZ se produce mayoritariamente en esta zona de ciclo. Cuando se estudió el monómero de la VPy, los desplazamientos químicos de los protones más próximos al grupo amino (-NH_2) de la SMZ fueron los que sufrieron una significativa variación en comparación con otras señales del analito. Esto significaba que este monómero interacciona preferentemente con la estructura de la anilina, afectando indirectamente al sistema electrónico global de la molécula, lo que produce pequeños cambios en las señales de protón

correspondiente al heterociclo (ver **Figure 2.10**). Estos resultados concuerdan de manera satisfactoria con los de los cálculos de modelización molecular. De todos los cálculos realizados en el diseño racional de MIPs para SMZ era necesario utilizar por lo menos 3 equivalentes de monómeros MAA y VPY. El enfoque experimental de la NMR valida el trabajo teórico desarrollado previamente lo que indica que la utilización conjunta de ambos métodos puede ser una herramienta interesante en el futuro diseño racional de MIPs.

9.3.1.3 Preparación de MIPs con ACN

A partir de los cálculos teóricos y experimentos de NMR, la polimerización se llevó a cabo con la relación de 1:3:3 (SMZ/MAA/VPY) para la interacción molecular usando 24 equivalentes de EGDMA para obtener una red tridimensional. El porógeno (ACN) tiene el importante papel de disolver el analito, armonizar la mezcla de polimerización, además de crear cavidades internas dentro de la estructura del MIP, donde se colocarán/distribuirán los posibles receptores formados. El azobisisobutironitrilo (AIBN), debido a su estabilidad, sus propiedades de tiempo de vida medio y sus razonables condiciones de activación térmica (65 ºC), fue utilizado (1%) como iniciador radicalario y así poder activar el proceso de polimerización. Decidimos reducir a 1 gramo la cantidad de polímero obtenido respecto a trabajos previos publicados (~ 5 gramos) esperando observar pocas variaciones en las propiedades del MIP. Este cambio de escala se propuso con el fin de preparar diferentes lotes de MIPs para explorar más fácilmente, y con menor coste, numerosas combinaciones de mezcla de polímeros, lo que mejora las posibilidades de localizar un MIP con buenas propiedades independientemente de la metodología de diseño racional. Normalmente, después de un proceso de polimerización, los polímeros fueron triturados, tamizados para tener un tamaño de partícula entre 20 a 40 micras, empaquetados en las columnas de cromatografía y lavados a fondo para posteriores experimentos. Además, en todos los casos se prepararon y procesaron polímeros por partida doble pero uno sin el analito (NIPs) para ser comparados con los correspondientes MIPs. Durante la preparación de polímeros, si se utilizan reactivos de alta pureza, se elimina el oxígeno residual y se regula la temperatura de polimerización, se asegura la reproducibilidad del lote (preparación de error por debajo de 5,5%).

9.3.1.4 Determinación de la proporción de porógeno en la mezcla prepolímero del MIP

Además de utilizar ACN y MeOH para la preparación de MIPs, previamente estudiado en los experimentos de NMR, otros potenciales porógenos como DMF, dioxano, EtOH y H_2O/2-propanol, CH_2Cl_2, CH_3Cl, tolueno, AcOEt y Cl_3CF también fueron probados para evaluar su influencia en las propiedades del polímero final obtenido. Por otro lado, diferentes volúmenes de porógeno (1 mL, 2 mL y 4 mL) fueron utilizados para preparar un gran lote de MIPs/NIPs

para evaluar las características finales de materiales y propiedades obtenidas en cada caso (ver **Table 2.1** en el **Chapter 2**). Los polímeros preparados con 1 y 2 mL g^{-1} presentaron una estructura más rígida en comparación con los de 4 mL g^{-1} que parecían más esponjosos y débiles.

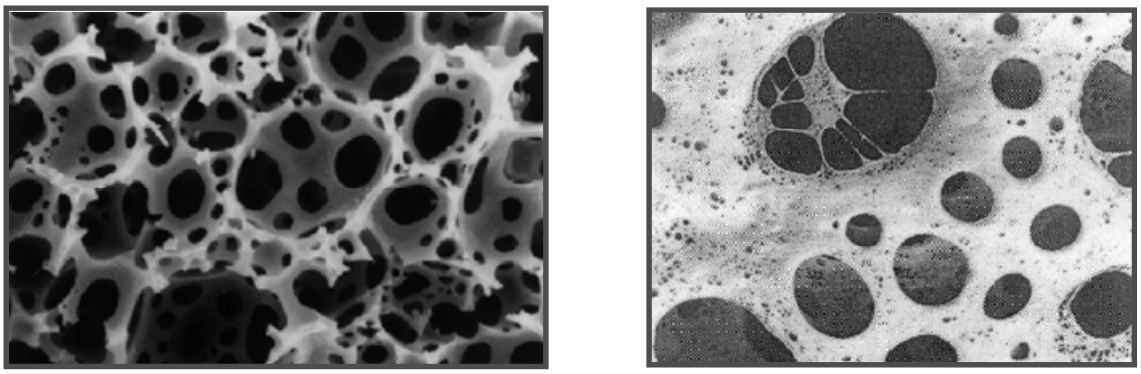


Figura 9.4: Podríamos definir que los MIPs son como una especie de espuma rígida con grandes cavidades vacías en su volumen interior formado por una red de canales cuyo tamaño depende principalmente de la naturaleza del disolvente utilizado. Su distribución es aleatoria y recorre el interior del polímero. Los sitios de interacción asociadas a mesoporosos (> 20 Å) se esperan que sean más fácilmente accesible (la zona más probable que haya receptores de alta afinidad y/o selectividad) que los ubicados en los microporos (< 20 Å), donde la difusión es baja.

Varios polímeros fueron seleccionados para caracterizar su estructura interna por porosimetría de absorción de nitrógeno observando que tanto la naturaleza como disolvente y proporción utilizada en la preparación de MIPs influencia claramente en la estructura del material interno (ver **Figura 9.4**).

9.3.1.5 Evaluación de MIPs

La interacción entre analito y MIP es un proceso complejo con al menos dos mecanismos de asociación diferentes, el selectivo (es decir, la interacción deseada) y el no específico (es decir, adsorción simple o partición de fase). Además, el disolvente juega un papel importante porque MIP y analito son solvatados. Así, durante la evaluación de MIPs los parámetros a estimar tienen que estar relacionados con el analito específicamente retenido por el MIP respecto al atrapado por el NIP (es decir, el material de referencia) siendo también muy importante una cuidadosa selección de los disolventes o el medio utilizado en ensayos de afinidad. Con base a estas consideraciones, se propuso utilizar una cascada de tres ensayos diferentes con una complejidad cada vez mayor para evaluar un conjunto de MIPs potenciales (ver **Figure 2.12** en el **Chapter 2**). El primer ensayo es una simple prueba de desorción realizada justo después de la preparación de polímeros para evaluar la relación desorbida del analito, tanto en MIPs como en NIPs (estudio contrario evaluando la relación adsorbida del analito). El siguiente ensayo

sugerido consiste principalmente un proceso de lavado en el que se monitoriza la liberación del analito en relación con la cantidad de disolvente que pasa a través de un lecho de partículas MIP empaquetadas en una columna. La liberación del analito está en función de su interacción con la fase estacionaria y por lo tanto, se podría esperar que una liberación más lenta deba corresponder a un MIP con una mejor afinidad por el analito. La última evaluación se realizó mediante una prueba de retención cromatográfica en la que se prueba el material como una fase estacionaria selectiva en comparación con el correspondiente NIP. La retención molecular no específica (es decir, el mecanismo de la fase de partición estándar) será más o menos igual para el MIP y el NIP, mientras que la retención selectiva debería ser mostrada únicamente por el MIP. Esta estrategia de selección trata de eliminar los peores materiales de cada test en lugar de seleccionar los mejores. Entonces, el resto de materiales irían al siguiente nivel de evaluación que podría realizarse con un menor número de polímeros. En nuestro caso, el MIP preparado con ACN fue el que obtuvo mejores resultados en las todas las condiciones ensayadas.

9.3.1.6 Caracterización química del MIP seleccionado

La propiedad más característica de un receptor químico es la afinidad que presenta por su substrato y en el caso de receptores en heterofase la concentración de receptores en ese material. Una técnica poderosa para el estudio de interacciones entre solutos y fases estacionarias y para la investigación y caracterización de los parámetros de estas interacciones es el análisis por cromatografía frontal [17]. Este método permite determinaciones de propiedades de adsorción y cinéticos a partir de simples experimentos de determinación de volumen de ruptura o "breaktrough" habiéndose probado la validez de esta técnica en un gran número de estudios previos (ver **Figura 9.5**).

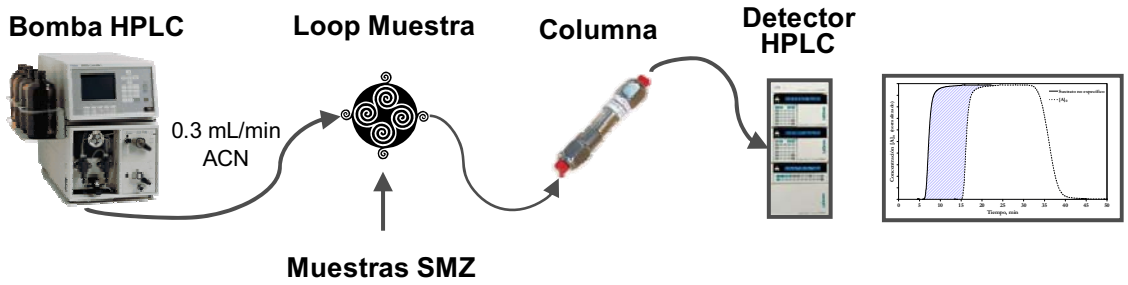


Figura 9.5: Esquema de realización de cromatografía frontal.

Para los MIPs, el estudio se debe entender como una asociación molecular entre el analito y las unidades de monómeros estructuradas situadas en las cavidades y que equivalen a un receptor específico similar a los de los casos anteriormente comentados. El empleo de la cromatografía permite evaluar fácilmente la cantidad de analito fijado a la fase estacionaria. De este modo, el

MIP preparado con ACN fue evaluado mediante cromatografía frontal obteniendo constantes de asociación muy prometedoras (del orden de 10^3 M^{-1}) lo que significa que el polímero impreso tiene buena afinidad y propiedades de retención respecto a SMZ (ver **Fig. 7** en **Section 2.2** del **Chapter 2**). Los prometedores resultados obtenidos fueron el punto de partida de Raquel Obregón para realizar sus estudios de doctorado en este campo. Desde entonces, ella ha sido capaz de desarrollar una aplicación de separación basada en un procedimiento MISPE (fase de extracción sólida molecularmente impresa) para el tratamiento de extractos de leche y pelo contaminados con SAs para su posterior análisis por técnicas cromatográficas.

9.3.2 Producción y evaluación receptores moleculares naturales y posterior desarrollo de técnicas inmunoquímicas

Uno de los pasos cruciales en el desarrollo de una técnica inmunoquímica es el diseño del hapteno empleado como inmunógeno, ya que la estructura química del mismo influye directamente en la especificidad y selectividad de los anticuerpos resultantes y en consecuencia en las características del ensayo final [18]. Compuesto orgánicos de bajo peso molecular (<2000 Da) no son capaces de provocar directamente respuesta inmune en un animal huésped, por lo que es necesario modificar el analito en una molécula inmunogénica, que consiste en un hapteno unido covalentemente a una proteína mucho más grande. En este sentido, uno de los objetivos de esta tesis ha sido la preparación y evaluación de anticuerpos contra SAs y TCs.

9.3.2.1 Sulfonamidas

Las SAs son agentes antimicrobianos sintéticos de amplio espectro que abarcan la mayoría de organismos gram-positivos y gran número de gram-negativos. Estos fármacos fueron el primer tratamiento eficaz que fue empleado de forma sistemática para la prevención y cura de infecciones bacterianas en los seres humanos allanando el camino para la revolución de los antibióticos en la medicina actual. Las SAs originales, a veces llamadas coloquialmente sulfas, son agentes antimicrobianos sintéticos que contienen el grupo sulfonamida (-SO_2NH). Las SAs utilizadas en la actualidad consisten en un anillo de benceno con un grupo aminosulfonilo en el grupo C1 y un grupo amino libre en C4 (ver **Figura 9.6**).

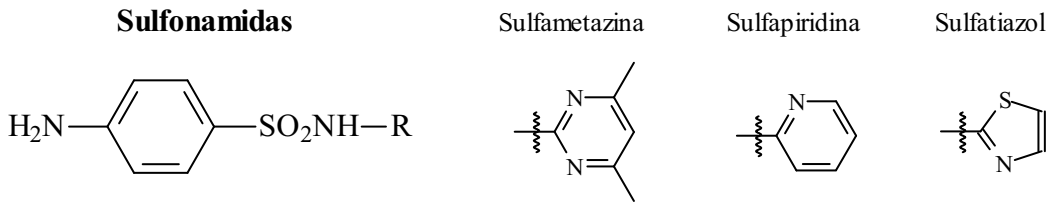


Figura 9.6: Estructura general de las SAs. Se añaden los ejemplos más representativos.

Hoy en día las SAs rara vez se utilizan en medicina humana siendo el sulfisoxazole y el sulfametoxazol combinado con trimetoprim una combinación muy común para el tratamiento de meningitis meningocócica, e infecciones del tracto urinario, oculares o auditivas. Por otra parte, al menos diez SAs están permitidas actualmente en la medicina veterinaria para el tratamiento de la enteritis, cistitis, mastitis o neumonía bacteriana, así como el cólera aviar y coccidios [19].

9.3.2.1.1 Diseño de haptenos de inmunización para generar anticuerpos contra sulfonamidas

El objetivo principal era por lo tanto producir anticuerpos de familia para SAs y los inmunoreactivos secundarios correspondientes con el objetivo final de detectar un número significativo de congéneres de SAs que se utilizan en el sector veterinario (ver **Figura 9.7**). Esta investigación fue iniciada en nuestro grupo por el Dr. Héctor Font. En el contexto de su tesis doctoral, se realizaron estudios de modelización y química teórica para diseñar y posteriormente sintetizar un hapteno de inmunización. La presente tesis ha contribuido a esta investigación evaluando los anticuerpos resultantes de esta inmunización y desarrollando las técnicas inmunoquímicas de análisis que a continuación se describen en más detalle.

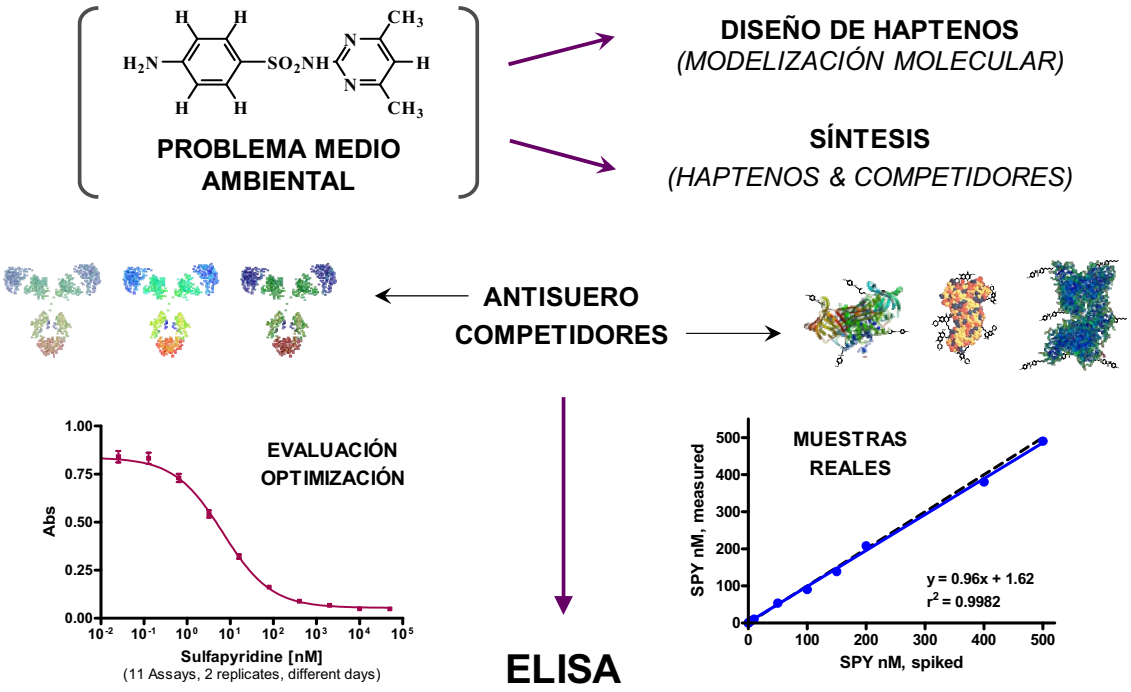


Figura 9.7: Esquema representativo de los diferentes procesos y etapas necesarios para desarrollar un ELISA para un determinado compuesto o familia de compuestos.

Sobre la base de criterios químicos, el reconocimiento genérico de la familia de antibióticos de SAs requiere maximizar el reconocimiento de la fracción común de anilina. Por lo tanto, un

grupo funcional para la unión covalente a la proteína transportadora tendría que ser colocado en el lado opuesto de la molécula. Los haptenos de inmunización propuestos (SA1 y SA2) poseían el área mencionada, sin embargo mientras el hapteno SA1 conservaba toda la estructura, el hapteno SA2 únicamente contenía la fracción común de las SAs (ver **Figura 9.8** y **Figura 9.9**).

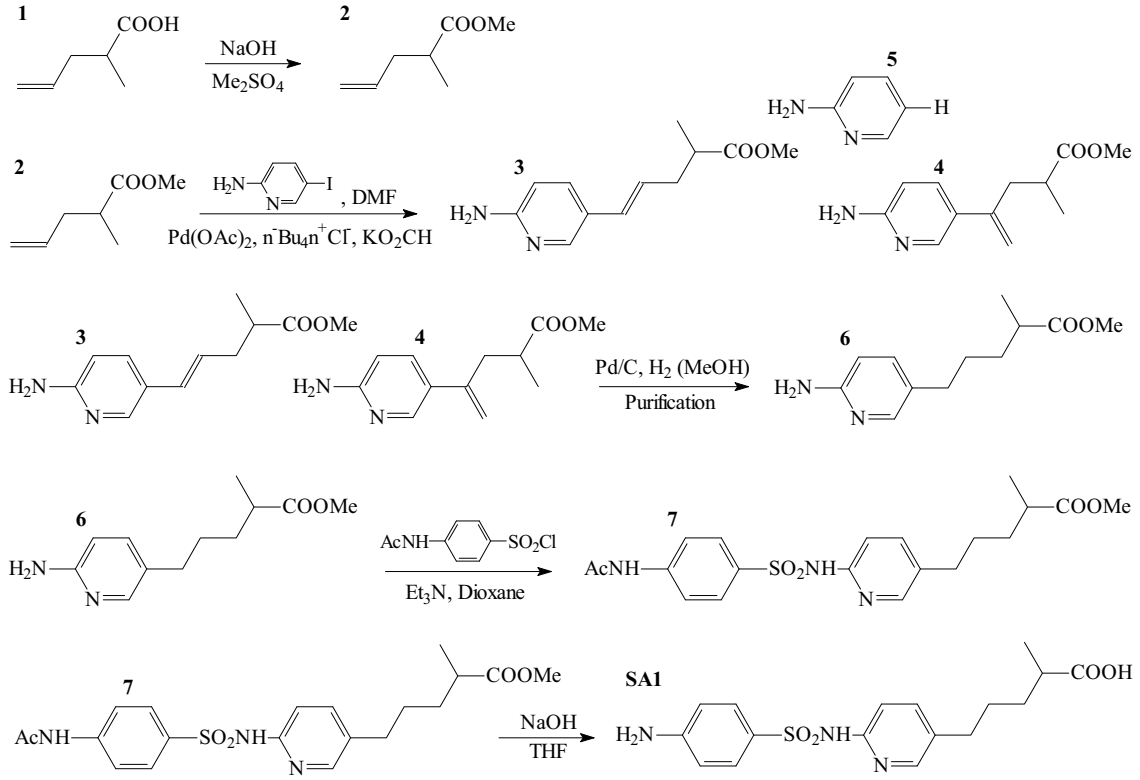


Figura 9.8: Esquema mostrando la síntesis del hapteno SA1.

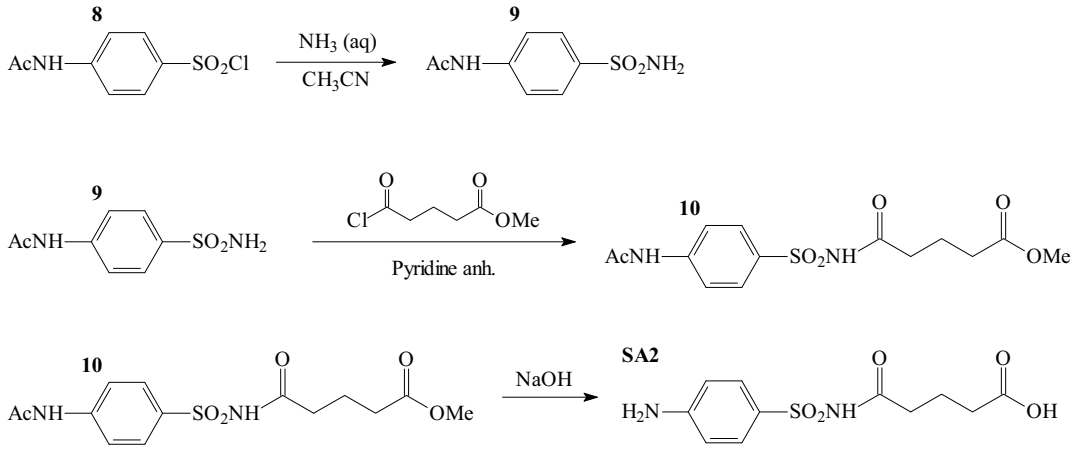


Figura 9.9: Esquema mostrando la síntesis del hapteno SA2.

Con el objetivo de obtener información adicional sobre las posibles deformaciones geométricas producidas por la introducción de un brazo espaciador en la molécula objetivo, se usaron estudios de modelización teórica y molecular. Sobre la base de modelos semiempíricos PM3 se evaluaron las posibles geometrías y distribución electrónica de los dos haptenos con el fin de compararlos con las SAs más importantes. Los resultados obtenidos mostraron que ambas moléculas SA1 y SA2 podían considerarse excelentes haptenos de inmunización para la producción de prometedores anticuerpos de familia para SAs.

Pero para poder analizar moléculas orgánicas de bajo peso molecular (como los antibióticos) mediante técnicas inmunoquímicas es necesario el uso de ensayos basados en la competencia entre el analito libre y un antígeno por un limitado número zonas de enlace del anticuerpo. Con frecuencia se ha publicado que la estructura química de los haptenos de inmunización es el principal factor determinante de la especificidad inmunológica, mientras que la estructura química de los competidores define la detectabilidad final. Por lo tanto, la introducción de un cierto grado de heterología en la estructura química de los competidores podría aumentar la detectabilidad del ensayo [20, 21]. El sistema heterólogo óptimo se consigue normalmente mediante el cribado de varios haptenos, enlazados enzimas o proteínas, porque, *a priori*, es casi imposible predecir qué estructura química daría el mejor competidor. Por lo tanto, se prepararon ocho haptenos nuevos (SA3-SA10), con diferente grado de heterología cambiando la estructura química y/o la posición del brazo espaciador, para ser utilizados como competidores (ver **Figure A** en **Section 3.3.1** del **Chapter 3**).

Los haptenos SA1 y SA2 fueron acoplados a la proteína HCH (hemocianina de cangrejo de herradura) siguiendo el método de éster activo y los conjugados obtenidos fueron utilizados para generar anticuerpos en conejos blancos de la variedad New Zeland. Los antisueros obtenidos fueron nombrados As154, As155, As156 y As167 contra el hapteno SA1, mientras que los obtenidos contra SA2 hapteno fueron nombrados As157, As158 y As159. Con el objetivo de demostrar los resultados de los estudios teóricos, decidimos evaluar la capacidad los dos grupos de antisuero a unirse a SAs mediante ELISAs competitivos. Por otro lado, los 10 haptenos se conjugaron covalentemente a los grupos de lisina libre de las proteínas seleccionadas (HRP: peroxidasa de rábano, BSA: albúmina de suero bovino, OVA: la ovoalbúmina y la CONA: conalbúmina). En resumen, se prepararon 40 bioconjugados diferentes, 30 proteínas haptenizadas (CAs: antígenos de tapizado) y 10 enzimas haptenizadas (ETs: trazadores enzimáticos), lo que significa que se pudieron estudiar 70 combinaciones (10 ETs x 7 antisueros) en formato de ELISA directo y 210 (30 CAs x 7 antisueros) en formato de ELISA indirecto.

9.3.2.1.2 Evaluación de los inmunoreactivos y desarrollo de un ELISA en formato competitivo para la determinación de sulfonamidas

El reconocimiento de los ETs (formato directo) y de los CAs (formato indirecto) por cada antisuero se evaluó en primer lugar en sus correspondientes configuraciones de ELISA no competitivo a través de experimentos 1D (una dimensión). Las combinaciones que mostraron mejor avidez de los antisueros por los antígenos marcados fueron posteriormente evaluadas mediante experimentos no competitivos bidimensionales (2D). De este modo, se seleccionaron las concentraciones apropiadas para llevar a cabo los ensayos competitivos, en ambos formatos, usando la sulfapiridina (SPY) como SAs modelo. Aunque todas las combinaciones elegidas a partir de los anteriores experimentos del ELISA directo ofrecieron ensayos competitivos, los mejores fueron conseguidos por las combinaciones homólogas (mismo hapteno como inmunógeno y como competidor). Por el contrario, los mejores ensayos competitivos para el formato indirecto del ELISA fueron obtenidos por combinaciones heterólogas (diferente hapteno como inmunógeno y como competidor) jugando este parámetro un papel importante en el patrón de detectabilidad obtenido. Finalmente, una combinación de ELISA para cada formato fue seleccionada para experimentos posteriores (ELISA directo: As154/SA1-HRP, ELISA indirecto: As155/SA2-OVA). Los factores que determinan el mejor rendimiento de los inmunoensayos para las combinaciones seleccionadas en ambos formatos y sus parámetros se resumen en la **Tabla 9.2**.

Tabla 9.2: Condiciones óptimas y características de los inmunoensayos de SPY para las combinaciones As155/SA2-OVA y As154/SA1-HRP.

Condiciones	**Valores**	**Parámetros**	**As155 SA2-OVA**	**As154 SA1-HRP**
Tiempo de preincubación	0 min	A_{max}	1.07 ± 0.07	1.04 ± 0.08
Tiempo de competencia	30 min	A_{min}	0.04 ± 0.02	0.04 ± 0.01
pH	7.5	IC_{50}[a]	2.35 ± 0.72	11.21 ± 2.88
Fuerza iónica	10 mS/cm (10 mM PBS)	Rango dinámico[a]	0.4 ± 0.1 to 16.9 ± 3.5	1.6 ± 0.5 to 72.8 ± 23.7
Tween 20	0.05%	Pendiente	-0.73 ± 0.10	-0.75 ± 0.05
		LOD[a]	0.15 ± 0.08	0.44 ± 0.13
		R^2	0.997	0.972

Los datos presentados corresponden al menos a la media de las seis curvas de calibración realizada en diferentes días. [a]Las concentraciones se expresan en μg L^{-1}.

Podemos observar que el formato de ELISA indirecto heterólogo alcanza una detectabilidad significativamente superior a la conseguida por la el formato directo en condiciones homólogas. La especificidad de ambos ensayos se evaluó posteriormente mediante la realización de curvas de calibrado usando una batería de 14 congéneres de SAs. El formato de ELISA indirecto presento un patrón de detectabilidad notablemente más amplio, al permitir detectar mayor

número de SAs (SPY, sulfaquinoxalina, sulfacloropiridazina, sulfisomidina, sulfatiazol, sulfadiazina, sulfadimetoxina, sulfamerazina). El formato directo mostró ser en cambio más específico, detectando un número inferior de sulfonamidas (SPY, sulfametoxipiridazina, sulfatiazol y sulfacloropiridazina). Este patrón de reconocimiento demuestra la importancia del grupo de sustitución en la posición N1. Posteriormente, la precisión de los ensayos de As154/SA1-HRP y As155/SA2-OVA se evaluó mediante la medición repetida de 10 muestras ciegas preparadas en tampón durante diferentes días. Los resultados respecto a la correlación encontrada entre la medida y los valores de concentración dopada fueron muy buenos (ELISA directo, $y = 1.07x – 0.43$ ($R^2 = 0.991$); ELISA indirecto, $y = 0.96x + 0.13$ ($R^2 = 0.997$).

9.3.2.1.3 Implementación del ELISA de SAs al análisis de muestras biológicas de interés en el sector alimentario

En este trabajo, la leche y muestras de pelo fueron seleccionadas como matrices objetivo para analizar SAs. En el primer caso, la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) nos suministró muestras de leche desnatada, semidesnatada y entera todas libres de antibióticos para ser evaluadas por los diferentes ELISAs desarrollados. En ambos casos se prepararon varias curvas de calibrado estándar de la leche diluida en tampón PBS. En el formato directo la curva de calibración en leche imitaba muy bien la curva estándar en el tampón consiguiendo una superposición casi perfecta. Los mismos experimentos se realizaron con el formato de ELISA indirecto pero la señal se inhibía a la mitad en la mayoría de los casos en comparación con la preparada en tampón. Tratando de eliminar las interferencias observadas, y después de diferentes pruebas, decidimos diluir la leche cinco veces con agua milliQ y duplicar la concentración de antisuero respecto a la curva preparada en tampón. Se obtuvieron buenos resultados después de este tratamiento sencillo. Como alternativa, decidimos también usar la leche entera (no contaminadas con antibióticos) como material de referencia (diluida cinco veces, parámetros de sensibilidad x5) para preparar curvas de calibrado estándar proporcionando también muy buenos resultados. El procedimiento se aplicó a diferentes tipos de muestras de leche (entera, semidesnatada y desnatada) para evaluar si la estrategia era adecuada para analizar cualquier tipo de muestra de leche obteniendo respuestas similares en todos los casos.

En la **Figura 9.10** se muestran las curvas de calibración obtenidas en ambos ensayos con diferentes tipos de leche empleando los protocolos inmunoquímicos establecidos. Puede observarse que los parámetros del ELISA no parecen verse afectados por esta matriz. En estas condiciones, la detectabilidad alcanzada para la SPY se encuentra muy por debajo de los niveles permitidos en leche de acuerdo con las MRLs establecidas por la EC (100 $\mu g\ L^{-1}$ para el total de residuos de SAs presentes en una muestra).

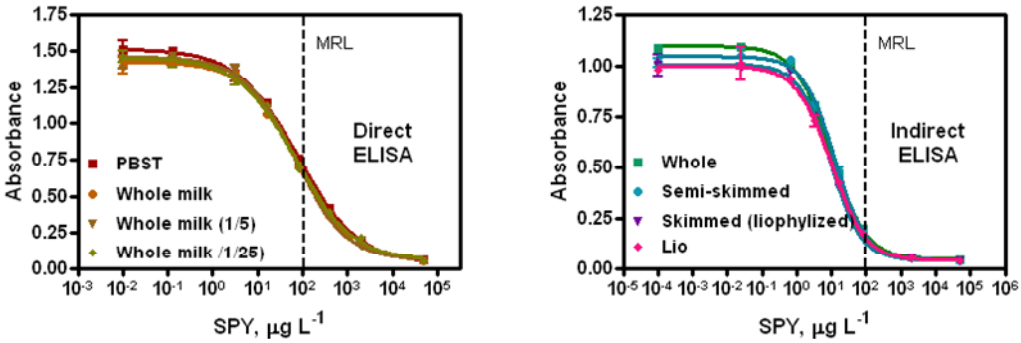


Figura 9.10: Resultados de los estudios del efecto de la matriz producida por la leche en los ELISAs directo en indirecto. Las muestras fueron tamponadas añadiendo 10% (v/v) de PBS 100 mM, diluida y utilizada en el inmunoensayo.

Para probar la fiabilidad de los métodos de ELISA desarrollados en leche, ésta una vez diluida 5 veces en tampón se dopó con SPY a varios niveles de concentración. Los resultados obtenidos en ambos ensayos se caracterizan por una excelente precisión con pendientes cercanas a 1 y coeficientes de regresión muy buenos (Directo, y = 1.01x – 2.84 (R^2 = 0.994); Indirecto, y = 0.95x + 2.83 (R^2 = 0.992)).

Por otro lado, diferentes tipos de muestras de pelo de animales (cerdo incoloro, ternero incoloro y ternero pigmentado), recogidas de animales control en la granja experimental del IRTA, fueron extraídas bajo diferentes condiciones (por ejemplo, tipo de soluciones alcalinas, tiempo, temperatura), para ser posteriormente evaluadas en ambos formatos ELISA. Los resultados obtenidos demostraron que las interferencias de la matriz en ambos ensayos eran prácticamente inapreciables, sobretodo en el formato indirecto, en el que los extractos podían analizarse casi sin ser diluidos (ver **Figura 9.11**).

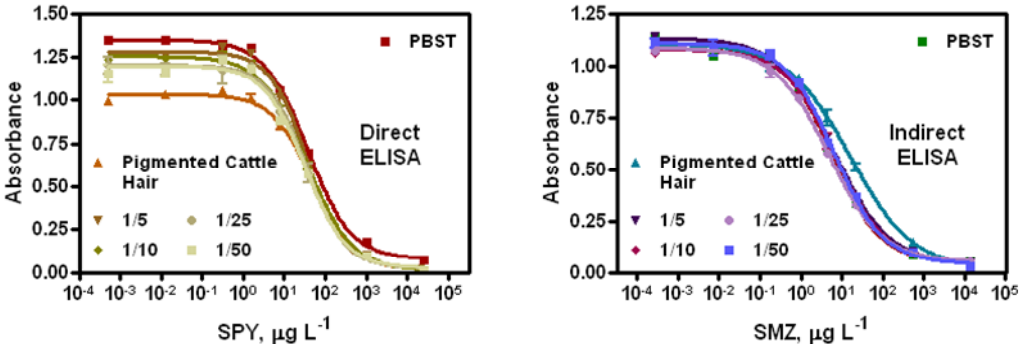


Figura 9.11: Estudios de efecto matriz de extractos de pelo bovino pigmentado en los formatos directo (SPY) e indirecto (SMZ) del ELISA de SAs.

La precisión de los ensayos para la determinación de SAs en extractos de pelo fue evaluada mediante la medición de extractos dopados con SPY y SMZ, para los formatos directos e

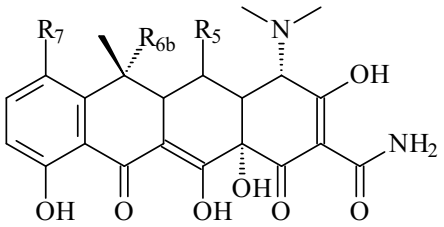
indirectos, respectivamente (Directo, y = 1.01x + 4.43 (R^2 = 0.993); Indirecto, y = 0.97x + 1.62 (R^2 = 0.994)). Las muestras analizadas con el formato indirecto también fueron evaluadas por cromatografía líquida (HPLC-DAD), en colaboración con el IRTA, obteniendo una excelente correlación entre ambas técnicas (y = 0.93x + 0.89, R2 = 0,971, ver **Figure 5** o **Table 2** en **Section 3.4**). Para concluir este trabajo, realizamos también una propuesta preliminar de un estudio de validación, utilizando muestras de ganado de pelo como un modelo, para las metodologías de tipo ELISA de acuerdo con los criterios de la Decisión 2002/657/CE (ver **Section 3.5.5**).

A parte de las muestras de leche y el pelo, otras matrices como piensos, miel y suero humano han sido posteriormente estudiadas por otros miembros del grupo de investigación empleando los inmunoreactivos y protocolos inmunoquímicos de SAs desarrollados en esta tesis doctoral. Además estos reactivos se han incorporado con éxito a novedosos transductores con el objetivo de desarrollar nuevos dispositivos biosensores para mediciones *in* situ (ver **Section C.2**). Por otra parte, varias empresas han mostrado interés para el uso de estos reactivos y su aplicación en sus propios sistemas de detección (información confidencial).

9.3.2.2 Tetraciclinas

Estos antibacterianos fueron considerados la primera clase de agentes terapéuticos de amplio espectro debido a su elevada efectividad contra patógenos gram-negativos y gram-negativos. El éxito inicial de esta familia de antibióticos ha contribuido a su amplia producción y utilización en la medicina humana y animal en las décadas posteriores a su descubrimiento. Las TCs están compuestas por cuatro anillos de seis miembros denominados A, B, C y D, por la IUPAC (Unión Internacional de Química Pura y Aplicada), donde el núcleo es perhidrogenado y sólo el anillo D conserva aromatización (ver **Figura 9.12**).

Tetraciclinas



R_5 = H; R_{6b} = OH; R_7 = H Tetraciclina
R_5 = H; R_{6b} = OH; R_7 = Cl Clorotetraciclina
R_5 = OH; R_{6b} = OH; R_7 = H Oxitetraciclina
R_5 = OH; R_{6b} = H; R_7 = H Doxitetraciclina

Figura 9.12: Estructura general de las TCs. Se incluye también los ejemplos más representativos.

La presencia de una periferia inferior muy oxidada, que incluye una configuración cetoenólica en C11, C12, y C11, tiene un impacto sobre las propiedades fisicoquímicas y características tridimensionalidades de estos compuestos siendo la principal zona responsable de las

propiedades farmacológicas de esta familia [22]. Por otra parte, las modificaciones moleculares de los grupos funcionales dentro de la región periférica superior parecen alterar drásticamente la bioactividad de estos compuestos [23]. De esta manera, la disposición lineal de los anillos ABCD es crucial para sus propiedades, y como regla general, la modificación química a lo largo de la región inferior periférica disminuye la bioactividad, mientras que la región periférica superior puede ser por lo general modificada químicamente para preparar otras TCs semi sintéticas. Desde el descubrimiento de las TCs y en adelante, se ha sugerido en varias ocasiones que la flexibilidad (propiedades físico-químicas, aspectos farmacológicos, etc.) de estas moléculas es la que hace que sean medicamentos tan exitosos y eficientes. Por otra parte, esta flexibilidad hace también muy difícil establecer una relación estructura-función, siempre y cuando su estructura exacta no ha sido determinada en el sitio de acción. En resumen, todas estas consideraciones anteriores nos pronosticaron que trabajar con estos compuestos, en términos de modificar su estructura para sintetizar haptenos y la producción de anticuerpos posteriores, sería un gran desafío científico.

9.3.2.2.1 Diseño de haptenos de inmunización para generar anticuerpos contra tetraciclinas

Como se ha comentado anteriormente (ver **Sección 9.3.2.1.1**), el paso más crucial en el desarrollo de una técnica inmunoquímica para la detección de moléculas de bajo peso molecular es el diseño del hapteno ya que la estructura química seleccionada modula fuertemente la afinidad y selectividad de los anticuerpos que se obtengan. En el caso de las TCs, la zona denominada periferia inferior es común a todos sus congéneres. Así, empleando criterios químicos se deduce que el hapteno de inmunización debería maximizar el reconocimiento de esta zona, para conseguir anticuerpos con un amplio reconocimiento de la familia de TCs. Para ello, la funcionalización de la molécula con la proteína transportadora debe realizarse por la parte de la periferia superior. Consideramos varias posiciones donde podría ser introducido el brazo espaciador en las TCs, dando lugar a dos potenciales haptenos de inmunización (TC1 y TC2). Ambos haptenos poseían el área mencionada, sin embargo mientras hapteno TC1 fue modificado a través del anillo C, el hapteno TC2 tenía el brazo espaciador introducido en el anillo A (ver **Figure 4.5** en **Section 4.3.1**).

El hapteno TC1 se sintetizó utilizando el procedimiento descrito previamente por Blackwood y col. para modificar 6 metilen tetraciclinas [24-26] pero con ligeras modificaciones. El paso clave consistió en una adición anti-Markovnikov de radicales libres del ácido 3-mercaptopropiónico a la metaciclina (MC), en el doble enlace exocíclico del carbono 6, usando cantidades catalíticas de AIBN como iniciador de radicales en etanol (véase la **Figura 9.13**). Contrariamente a los resultados reportados por Nelson y col. [27], el crudo obtenido no contiene

el ácido carboxílico deseado, pero sí el éster etílico (MC-CH2-S-(CH2) 2-COOEt) que se forma espontáneamente en el medio de reacción, con un rendimiento del 31%.

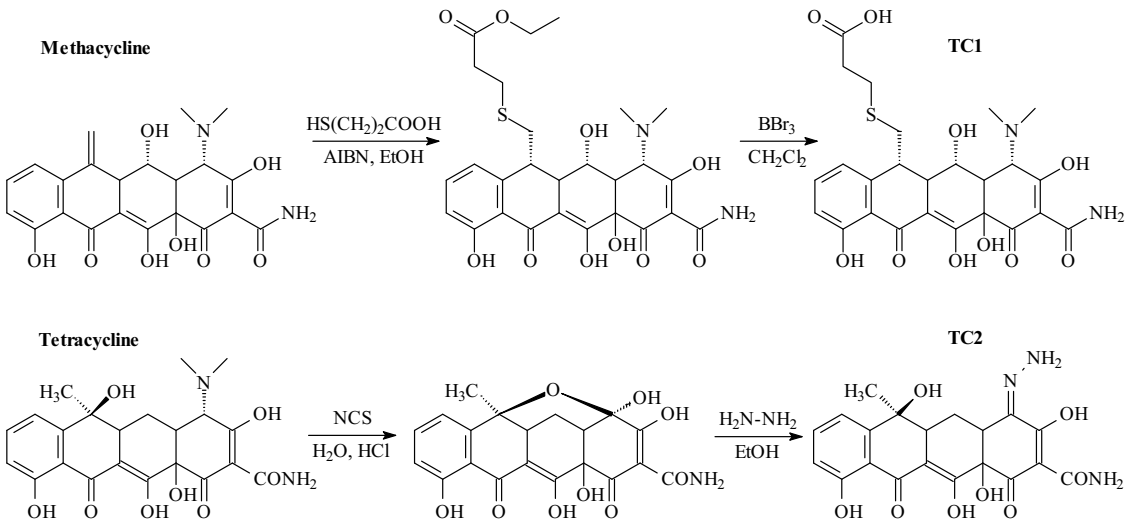


Figura 9.13: Esquema mostrando la síntesis de los haptenos TC1 y TC2.

Así, el éster etílico se purificó por cromatografía en columna de gel de sílice impregnada de ácido etilendiaminotetraacético (EDTA) con la finalidad de evitar la tetraciclina forme complejos con cationes divalentes presentes en la fase estacionaria o en la fase móvil. La hidrólisis del éster purificado, en condiciones fuertemente básicas, permite la obtención del hapteno TC1. Por otro lado, la síntesis de TC2 fue menos complicada. El hapteno se obtuvo por reacción de tetraciclina con N-clorosuccinimida, seguida por la adición de la hidrazina [28]. Una vez más, la mezcla final se purificó por en columna de gel de sílice impregnada de EDTA con el fin de bloquear todos los cationes presentes en la sílice y evitar la retención de las TCs en la fase estacionaria. Al igual que en el caso de las SAs, los haptenos TC1 y TC2 fueron sintetizados y posteriormente utilizados para preparar bioconjugados con la proteína HCH y así generar anticuerpos en conejos blancos de la variedad New Zeland.

9.3.2.2.2 Evaluación de los inmunoreactivos y desarrollo de un ELISA en formato competitivo para la determinación de TCs

Los antisueros obtenidos contra el hapteno TC1 fueron nombrados As180, As181 y As182, mientras que los obtenidos contra TC2 hapteno fueron nombrados As183, As184 y As185. Estos mismos haptenos se conjugaron covalentemente a las proteínas seleccionadas (HRP, BSA, OVA y CONA) mediante diferentes métodos de conjugación (ver **Section 4.3.4**). Con esta batería de bioconjugados se estudiaron 30 combinaciones (5 ETs x 6 antisueros) en formato de ELISA directo y 96 (16 CAs x 6 antisueros) en formato de ELISA indirecto.

Como en el caso de las SAs los diferentes formatos fueron evaluados inicialmente mediante experimentos 1D y 2D no competitivos. De este modo, se seleccionaron las concentraciones apropiadas para llevar a cabo los ensayos competitivos, en ambos formatos, usando doxiciclina (DC), oxitetraciclina (OTC), clorotetraciclina (CTC), tetraciclina (TC) y MC como analitos, puesto que nuestro objetivo era el de desarrollar un ensayo inmunoquímico con especificidad de clase. Desafortunadamente, como se puede observar en la **Figura 9.14**, en el formato directo, ninguna de las combinaciones estudiadas proporcionó ensayos con una detectabilidad aceptable para nuestros propósitos. En cambio, en formato indirecto, la mayoría de combinaciones homólogas ofrecieron ensayos competitivos. Después de un estudio exhaustivo de este ensayo se consiguió establecer unas condiciones en las que el ELISA se comportaba de forma reproducible y con suficiente detectabilidad para nuestros propósitos.

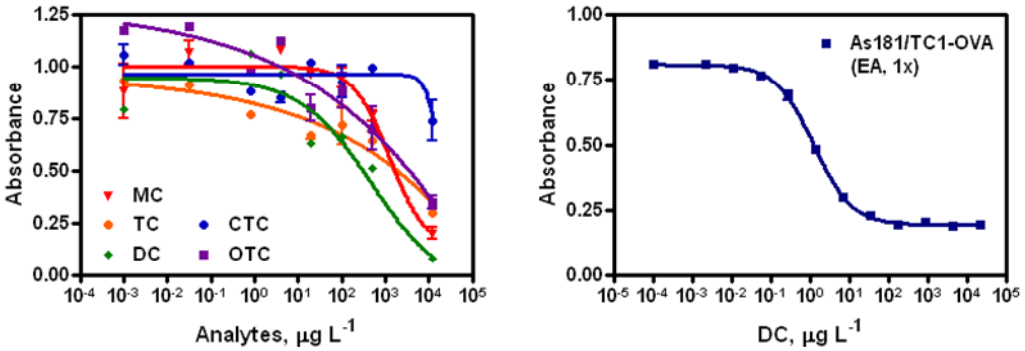


Figura 9.14: Izquierda: Ejemplo de resultados obtenidos para el formato directo del ELISA. Derecha: Ejemplo de una curva de calibración estándar para la detección de DC usando el formato indirecto (As181/TC1-OVA).

Los parámetros extraídos de la curva de calibración, así como las condiciones del ensayo se resumen en la **Tabla 9.3.**

Tabla 9.3: Condiciones óptimas y características de los inmunoensayos de DC para las combinación seleccionada.

Condiciones	Valores	Parámetros	As181 TC1-OVA
Tiempo de preincubación	0 min	A_{max}	0.19 ± 0.01
Tiempo de competencia	30 min	A_{min}	0.81 ± 0.01
pH	5.5	IC_{50}[a]	1.26 ± 0.05
Fuerza iónica	35.2 mS/cm (25 mM PBS)	Rango de trabajo[a]	De 0.25 ± 0.06 a 6.70 ± 0.59
Tween 20	0.001%	Pendiente	-0.90 ± 0.11
Ca^{2+} ($CaCl_2$)	1 mM	LOD[a]	0.10 ± 0.03
		R^2	0.998 ± 0.002

Los datos presentados corresponden al menos a la media de las cuatro curvas de calibración realizada en diferentes días. [a]Las concentraciones se expresan en µg L^{-1}.

Como puede observarse el ensayo alcanza una detectabilidad que para DC, permite su detección a una concentración de 1.26 ± 0.05 μg L^{-1} en el punto medio del ensayo, con un límite de detección de 0.10 ± 0.03 μg L^{-1}. Desafortunadamente los estudios de especificidad realizados con las mismas TCs anteriormente utilizadas para el cribado de las combinaciones, revelaron que en el ensayo indirecto tan sólo la DC y en menor grado la MC (32% de reactividad cruzada) eran reconocidas. Por lo tanto, no hemos podido alcanzar el objetivo de desarrollar un método inmunoquímico de clase para todas las TCs. Aunque la estructura química de los haptenos de inmunización seleccionados pretendía maximizar la exposición de los restos comunes de esta familia de antibióticos, los anticuerpos obtenidos reconocen sólo aquellos con un grupo alquilo en la posición C-6, aunque en la MC haya un metileno y en la DC haya un grupo metilo. A la luz de los resultados obtenidos se realizaron estudios de modelización molecular para descubrir una explicación (ver **Table 5** y **Figure 4** en **Section 4.2**). El hapteno de inmunización TC1 mantiene la estructura y propiedades de los analitos diana con sólo una excepción, el grupo 6-hidroxil que se proyecta perpendicularmente al sistema del anillo pudiendo afectar altamente a las características de reconocimiento de los correspondientes anticuerpos. Aún así, en este estadio resultaba interesante tener un ELISA con una detectabilidad adecuada para la determinación de DC. Por otro lado, los estudios de precisión realizados mediante la medición repetida de 8 muestras ciegas preparadas en tampón durante diferentes días, mostraron una buena correlación entre los valores medidos y los valores reales ($y = 0.94x + 0.42$ ($R^2 = 0.989$).

9.3.2.2.3 Implementación del ELISA de DC al análisis de muestras de leche

Se prepararon curvas de calibrado del ELISA en leche diluida en tampón PBS (leche, 1/2, 1/5, 1/10, 1/25, 1/50, 1/100 y 1/200). Aunque en estas condiciones el pH y la conductividad de las muestras de leche eran muy similares a las preparadas en tampón, la señal del ensayo en presencia de leche quedaba casi completamente inhibida, contrariamente al comportamiento observado con el ELISA de SAs, que mostró ser mucho más robusto al efecto de esta matriz. Este efecto podría estar relacionado con la grasa, el elevado contenido proteico de la leche, con su estructura micelar o bien con el alto contenido en calcio, entre otros posibles factores. Así pues iniciamos un estudio dirigido en primer lugar a comprobar el efecto del contenido proteico. Con este objetivo se procedió a separar las proteínas por precipitación usando dos procedimientos diferentes, tales como (i) el tratamiento con sulfato de amonio saturado (0.45 mL de $(NH_4)_2SO_4$ por mL de leche, centrifugación (500G durante 10 minutos) seguido de filtración (tamaño de poro 0.45μm)) y (ii) el tratamiento con tampón Mc Ilvaine (0.30 mL a pH 3 por mL de leche, centrifugación (5000G durante 10 minutos), seguido también de filtración (tamaño de poro 0.45μm)). El suero de leche obtenido se diluyó en varias proporciones con tampón PBS y se utilizó para evaluar el efecto de esta nueva matriz preparando curvas de calibración y comparando la respuesta con la curva preparada en tampón. Tal como puede

observarse en la **Figura 9.15,** ambos tratamientos consiguen eliminar una parte significante de las interferencias después de una dilución 1/12.5. Aún así, y puesto que el ELISA tenía suficiente detectabilidad, se decidió realizar las medidas en suero de leche diluido 25 veces, en cuyas condiciones no se observa ningún tipo de interferencia no específica.

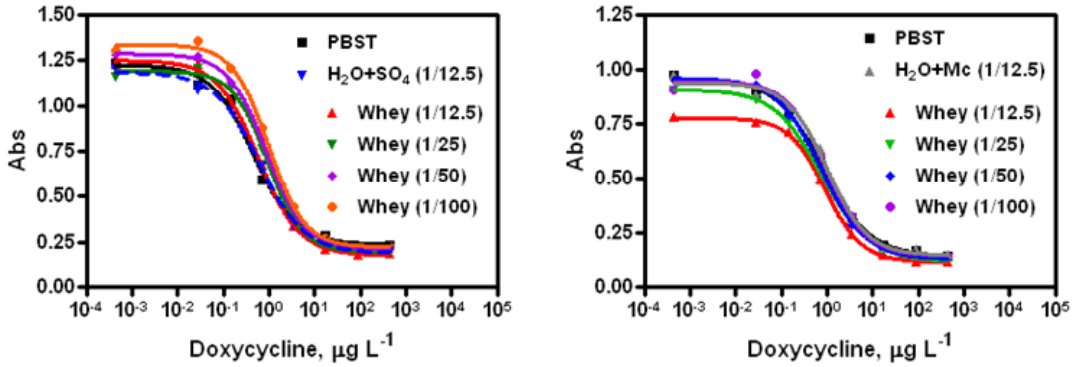


Figura 9.15: Efecto matriz de la leche en el ensayo de DC una vez precipitadas las proteínas. Izquierda: Resultados obtenidos cuando se utiliza el tratamiento con sulfato de amonio saturado. Derecha: Resultados cuando se utiliza el tampón Mc Ilvaine.

Desafortunadamente la recuperación de la DC, después del tratamiento con el tampón Mc Ilvaine, no era cuantitativa sino que se encontraban alrededor del 42%. A pesar de ello, la buena reproducibilidad del procedimiento (± 9%) y la detectabilidad del ELISA apuntaban a la posibilidad de emplear este procedimiento inmunoquímico de análisis para la determinación de DC en muestras de leche. Así pues, incluso en estas condiciones el límite de detección (LOD) alcanzado era cercano a 5 µg L^{-1} que se encuentra aún muy por debajo de los MRLs establecidos para la mayoría TCs permitidas para tratar el ganado vacuno dirigido a producir leche para el consumo humano (DC actualmente está prohibida en animales de granja destinados a producir leche para el consumo humano). Para comprobar la fiabilidad del procedimiento desarrollado se midieron muestras de leche dopadas con DC a varios niveles obteniendo una muy buena correlación de resultados (ver **Table 6** en **Section 4.2**). Actualmente, pese a su dificultad, en el grupo de investigación se sigue trabajando para sintetizar nuevos haptenos de inmunización que consigan la producción de anticuerpos más genéricos para la determinación de un amplio espectro de TCs. Por otra parte, los resultados obtenidos han llamado la atención de varios grupos de investigación y pequeñas empresas (información confidencial), ya que no hay inmunoensayo disponibles en el mercado capaz de detectar específicamente DC en muestras biológicas.

9.3.3 Desarrollo de un inmunosensor (WIObS)

Los biosensores son dispositivos de tipo eléctrico, óptico, químico o mecánico, modificados con entidades biológicas que les proporcionen la selectividad deseada. Las moléculas de

reconocimiento biológico pueden ser enzimas, receptores, anticuerpos y oligonucleótidos [29]. Los llamados inmunosensores son un tipo particular de biosensores en el que la molécula biológica de reconocimiento es un inmunoreactivo. El biosensor ideal no sólo tiene que responder a bajas concentraciones de analitos, sino que también debe tener la capacidad de discriminar entre especies de acuerdo a las moléculas de reconocimiento que se inmovilizan en la superficie del transductor. Hoy en día, los biosensores tienen amplias aplicaciones, incluyendo la detección de biomarcadores para el diagnóstico médico o la detección de patógenos y toxinas en alimentos y agua. De las múltiples opciones, los biosensores ópticos son dispositivos muy prometedores para el bioanálisis o el diagnóstico [30, 31]. Actualmente su desarrollo se basa principalmente en micro/nanotecnologías y en la posibilidad de fabricar micro y nanoestructuras complejas para guiar de luz, lo que han abierto la posibilidad de investigar nuevos fenómenos ópticos y propiedades de los materiales a nivel nanométrico [32, 33]. Durante los últimos años el CSEM (Centre Suisse for Electronics and Microelectronics Inc., Neuchâtel - Switzerland) ha estado trabajando en una novedosa plataforma de detección óptica llamada WIOS, basada en el principio de acoplamiento de guía de ondas con modulación de longitud de onda. También han trabajado en la integración de este transductor en un dispositivo portátil (22 x 10 x 10 cm^3) que incluye los componentes ópticos, eléctricos y de microfluídica, así como de un sistema de adquisición de datos. Gracias a la colaboración establecida entre el AMRg y CSEM en el contexto del proyecto GOODFOOD, fue posible realizar una estancia dentro de mis estudios de doctorado en sus laboratorios e investigar la posibilidad de utilizar el WIOS como un biosensor (WIObS) para la detección de residuos de antibióticos.

9.3.3.1 Sensor, principio de detección, chips del WIOS

El sistema WIOS es capaz de detectar cambios en el índice de refracción de la onda evanescente en una guía de onda recta en la que se acopla la luz mediante un barrido de longitudes de onda.

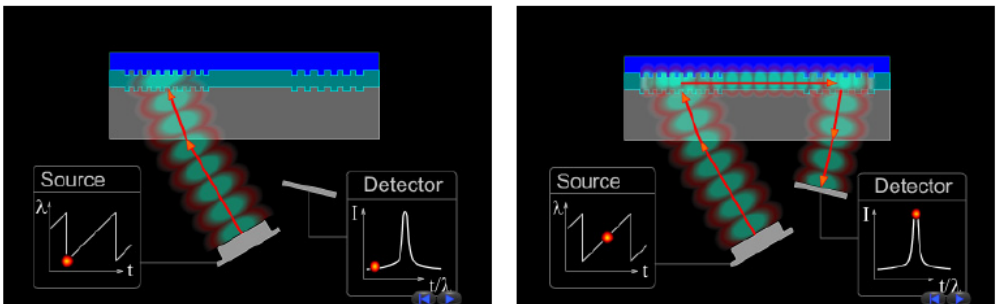


Figura 9.16: Esquema del principio de detección WIOS.

Se compone de una guía de onda con alto índice de refracción (Si_3N_4, TiO_2, Ta_2O_5) en la que se integran dos redes de refracción (entrada y salida) de período comprendido entre los 300 y 700

nm. El láser de la fuente que incide sobre la primera rejilla, se acopla y propaga a través de la guía de onda (ángulo diferente), y vuelve a desacoplarse a través de la segunda rejilla, donde la luz es recogida por el detector (ver **Figura 9.16**). El acoplamiento inicial de la luz tiene lugar bajo condiciones concretas dependientes de la longitud de onda del láser, el ángulo de incidencia, el periodo de la red de difracción y el índice de refracción efectivo de la guía de ondas, tal como aparece definido en la siguiente ecuación:

$$\lambda_r(t) = \Lambda\left[n_e(t) - \sin(\theta)\right]$$

siendo λ_r la longitud de onda de resonancia a la que el acoplamiento se produce, n_e el índice de refracción efectivo de la guía de onda, θ el ángulo de incidencia y Λ o el período de la red de difracción.

En esta configuración de sensor, el ángulo de incidencia se mantiene fijo y se escanea un rango de longitudes de onda de forma continua con el objetivo de monitorizar la resonancia en la guía de ondas. A través de la red de difracción de salida de la luz, la longitud de onda a la cual se ha producido el acoplamiento es determinada mediante un espectrómetro. La monitorización de λ_r permitirá observar las variaciones del índice de refracción efectivo de la guía de onda, producidas en este caso como consecuencia de reacciones biomoleculares de reconocimiento específico. La electrónica controla la modulación de la longitud de onda del láser y amplifica las señales detectadas, mientras que un equipo hace la adquisición y procesamiento de datos [34].

Los chips de guía de onda utilizados para realizar todos los experimentos fueron facilitados por Oerlikon (Balzers -. Lichtenstein), y consistieron en láminas de vidrio (17,5 x 17,5 x 0,7 mm^3) con varias zonas sensoras en la superficie (periodicidad de rejilla de cada zona sensora, 360 nm), cubiertas con una capa de óxido de tantalio de 150-300 nm. Cada chip está compuesto por un conjunto de 24 zonas independientes de detección (8 zonas sensoras distribuidas en 3 columnas distintas). Los chips utilizados en este trabajo permitían una biofuncionalización covalente o por fisisorción (véase **Section 5.1.2**).

9.3.3.2 Desarrollo de un inmunosensor óptico para la detección de SAs

El biosensor se desarrolló en base a los estudios previos realizados por ELISA, en los que se puso en evidencia que la configuración de ensayo inmunoquímico indirecto era la más apropiada para conseguir la máxima detectabilidad de un amplio grupo de congéneres de SAs. Varias combinaciones de anticuerpos/CAs fueron inicialmente estudiadas en el WIOS mediante la realización de sencillos experimentos en tampón e inmovilizando las biomoléculas (CAs) por adsorción física. Con base en los resultados obtenidos, la combinación As155/SA2-OVA fue seleccionada para estudios posteriores por inmovilización covalente a través de un polímero de dextrano foto activable Optodex ® (véase **Figure 5.5** en **Section 5.1.2.2**). Entonces, al igual que en el caso de los ELISA, se seleccionaron las concentraciones óptimas, de los diferentes

componentes del ensayo a través de experimentos 2D. Posteriormente, se estudió la regeneración de los chips mediante la realización de varios ciclos consecutivos de medición probando diferentes agentes (ácidos, básicos, alcalinos, etc.). Finalmente, escogimos NaOH 200 mM, ya que no se observó una variación significativa en las respuestas del inmunosensor en los diferentes ciclos consecutivos de regeneración y medida. El procedimiento desarrollado consistía en mezclar la muestra con el anticuerpo e inyectarla en la celda de flujo del biosensor, en el que se encontraba el chip convenientemente biofuncionalizado de manera covalente con SA2-OVA. Las variaciones en el índice de refracción producidas como consecuencia de la unión del anticuerpo primario (As155) al transductor biofuncionalizado, podían observarse en el sensograma. Debido a que la señal de esta interacción primaría tenía muy poca amplitud, se decidió añadir una etapa adicional, en la que se inyecta una solución del anticuerpo secundario (antiIgG), lo que permite obtener una respuesta amplificada que, como en todos los ensayos competitivos, es inversamente proporcional a la concentración de antibiótico en solución. El tiempo de análisis total, incluyendo todas las etapas de lavado y el proceso de regeneración, antes de comenzar un ciclo de nuevas mediciones, fue de alrededor de 30 min (véase **Figure 5.12** en **Section 5.3.2**). En estas condiciones el biosensor respondía de una manera reproducible a la presencia de SAs en la muestra. En la **Tabla 9.4**, se encuentran los parámetros de la calibración del biosensor, en comparación con los del ELISA.

Tabla 9.4: Parámetros obtenidos como resultado de calibrar el biosensor con SPY en tampón mostrados en comparación con los parámetros de calibración del ELISA, utilizando la misma combinación As155/SA2-OVA.

Curva de calibrado WIOS en tampón		**Curva de calibrado ELISA en tampón**	
Parámetros	**As155/SA2-OVA**	**Parámetros**	**As155/SA2-OVA**
A_{max} (WIOS a.u.)	8.2 ± 0.9	A_{max} (Abs)	1.07 ± 0.07
A_{min} (WIOS a.u.)	0.2 ± 0.1	A_{min} (Abs)	0.04 ± 0.02
IC_{50}[a]	5.1 ± 0.6	IC_{50}[a]	2.35 ± 0.72
Rango de trabajo[a]	0.7 ± 0.1 to 21.0 ± 1.2	Rango de trabajo[a]	0.4 ± 0.1 to 16.9 ± 3.5
Pendiente	-1.16 ± 0.35	Pendiente	-0.73 ± 0.10
LOD[a]	0.2 ± 0.1	LOD[a]	0.15 ± 0.08
R^2	0.986	R^2	0.997

[a]*Las concentraciones se expresan en* $\mu g\ L^{-1}$.

Al realizar los experimentos de calibración en leche se observó una ligera disminución de la señal de respuesta. Este efecto se corrigió aumentando el tiempo de la etapa de la competencia y modificando la etapa de lavado, lo que permitió realizar medidas directamente en leche con el WIObS, sin necesidad de diluir la muestra y por consiguiente obteniendo valores ligeramente mejores de detectabilidad que con el formato de ELISA (ver **Tabla 9.5**), ya que en este último

caso se tenía que aplicar un factor de corrección (x5) por el pretratamiento realizado en la muestra (dilución 1/5).

Tabla 9.5: Parámetros obtenidos como resultado de calibrar el biosensor con SPY en leche mostrados en comparación con los parámetros de calibración del ELISA, utilizando la misma combinación As155/SA2-OVA.

Curva de calibrado WIOS en leche		Curva de calibrado ELISA en leche (1/5)	
Parámetros	**As155/SA2-OVA**	**Parámetros**	**As155/SA2-OVA**
A_{max} (WIOS a.u.)	9.2 ± 0.2	A_{max} (Abs)	1.03 ± 0.04
A_{min} (WIOS a.u.)	-0.7 ± 0.2	A_{min} (Abs)	0.04 ± 0.01
IC_{50}[a]	6.3 ± 0.6	IC_{50}[a]	13.0 ± 1.5
Rango de trabajo[a]	1.4 ± 0.2 to 26.4 ± 0.9	Rango de trabajo[a]	2.8 ± 0.7 to 59.5 ± 7.0
Pendiente	-1.04 ± 0.03	Pendiente	-0.93 ± 0.07
LOD[a]	0.5 ± 0.1	LOD[a]	1.1 ± 0.5
R^2	0.991	R^2	0.997

[a]*Las concentraciones se expresan en μg* L^{-1}.

Finalmente, se realizó una evaluación preliminar del biosensor WIObS mediante el análisis de un conjunto de muestras ciegas de leche, preparadas en NRC (Nestlé Research Centre, Lausanne - Switzerland), dopadas con diferentes SAs a diferentes niveles. Las muestras fueron medidas y la señal obtenida fue comparada con la proporcionada por la SPY que fue usada como referencia a la concentración de MRLs (100 μg L^{-1} para el total de residuos de SAs presentes en una muestra). De esta manera, las señales de respuesta por debajo del valor del control indicaban la posible contaminación de SAs y viceversa. De las 13 muestras analizadas no obtuvimos ningún falso negativo. Por otra parte, los falsos positivos indicaban la presencia del antibiótico, aunque por debajo de los MRL (véase **Figure 5** en **Section 5.2**). Como pasa en muchas técnicas bioanalíticas los diferentes valores reactividad cruzada dificulta la posibilidad valores cuantitativos, siendo necesario en muchas ocasiones expresar los resultados como equivalentes del compuesto control seleccionado. En este sentido es necesario utilizar analitos como referencia/control que tengan reactividades cruzadas bajas a fin de garantizar evitar falsos negativos. El dispositivo WIObS para SAs desarrollado en esta tesis doctoral ha mostrado unos parámetros de detectabilidad y especificidad comparables a los del ELISA presentados anteriormente, tanto en muestras preparadas en tampón como en leche. Por otro lado, parece ser el método más prometedor y conveniente para medidas automatizadas en lugares específicos (es decir, en granjas lecheras o camiones cisterna de leche).

9.3.4 Desarrollo de técnicas para detección multianalítica

La combinación de haptenos-anticuerpos/receptores con características ortogonales parece ser la solución ideal para la creación de plataformas de detección con diferentes inmunoreactivos para

diferentes analitos [35, 36]. Este tipo de paralelización optimizaría los límites físicos y económicos de dichos dispositivos con el creciente número de analitos que se podrían medir al mismo tiempo. Actualmente se están realizando grandes esfuerzos en el desarrollo de sistemas analíticos multianalito para la detección de múltiples residuos y en el diseño de nuevas técnicas de detección fáciles de usar. Para lograr estos objetivos, hemos combinado diferentes bioreceptores para la detección simultánea de los antibióticos usados con más frecuencia en el ámbito veterinario, en diferentes formatos analíticos. De acuerdo a la encuesta realizada a principios de esta tesis (ver **Appendix**), las SAs, FQs, BLs y TCs se identificaron como las principales familias de antibióticos utilizadas para el tratamiento de vacuno y por lo tanto, con más probabilidades de ocasionar la aparición de residuos en leche y productos lácteos en Europa. Los reactivos para la detección de SAs y FQs fueron desarrollados en nuestro laboratorio, mientras que los bioreactivos de BLs y TCs usados fueron suministrados por UNISENSOR S.A. (Liege – Belgium). Dentro de esta tesis, nos hemos centrado en el desarrollo de una plataforma multiplexada basada en el WIOS gracias a la posibilidad de trabajar con chips que contienen 24 zonas sensoras. Para lograr este objetivo, previamente se ha evaluado, mediante el desarrollo de un ELISA multianalito en microplacas, la ortogonalidad de los bioreceptores seleccionados (SAs, FQs, BLs) y su funcionalidad cuando se utilizan conjuntamente para la determinación simultánea de diferentes familias de antibióticos. Una vez establecidas las condiciones óptimas estos bioreceptores fueron implementados con éxito en la plataforma WIOS.

9.3.4.1 ELISA multianalito

En este caso la estrategia ha consistido en un ELISA de formato indirecto pero usando tres CAs diferentes en la etapa de tapizado, correspondientes a las familias de antibióticos seleccionadas (SAs, FQs y BLs). Los CAs de cada familia de antibióticos fueron distribuidos en filas o columnas separadas. Luego, la muestra se mezcla con un cóctel de los bioreactivos siendo distribuido en las tres diferentes zonas de la placa anteriormente tapizadas. Por lo tanto, la identificación de un determinado antibiótico presente en una muestra se consigue mediante la detección de una respuesta positiva del ensayo en alguna o varias de las zonas de las microplacas correspondientes a los reactivos tapizados anteriormente (ver **Figura 9.17**). El multianalito ELISA desarrollado se basó en la puesta a punto de manera simultánea de dos ensayos ELISA de formato indirecto (SAs y FQs) y un ELRA (ensayo por receptor ligado a enzimas) para BLs. El primer paso consistió en que las condiciones de trabajo de los diferentes ensayos individuales fueran las mismas para los tres casos (es decir, tipo de tampón usado, el número de etapas de los ensayos, tiempos de reacción, etc.). Posteriormente era importante evaluar si se podía realmente establecer un sistema ortogonal una vez combinados los reactivos de las familias de antibióticos seleccionados.

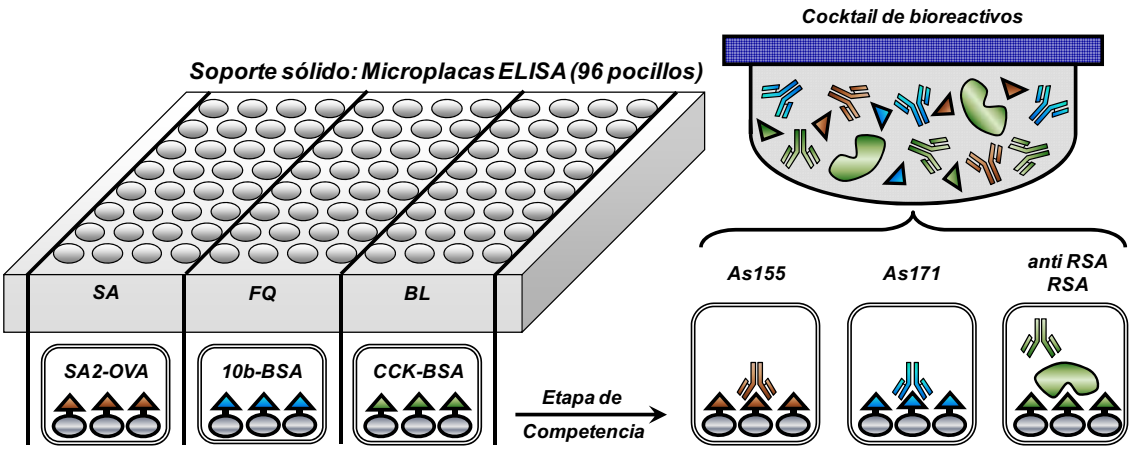


Figura 9.17: Esquema del funcionamiento del ELISA multianalito.

Esto significa que cada CA inmovilizado en la placa sólo debe ser reconocido por el correspondiente anticuerpo/receptor para el que ha sido desarrollado. Los resultados obtenidos mostraron que, usando el cóctel de bioreceptores, la respuesta del ensayo a la presencia de estos antibióticos era muy específica para cada familia, tanto si la muestra contenía tan solo un antibiótico, como si contenía una mezcla de ellos. Finalmente, se estudió si la respuesta del ELISA multianalito (usando el cóctel de bioreactivos) era similar que la de los correspondientes ELISAs individuales. Se prepararon curvas de calibrado en tampón usando por un lado muestras individuales de los analitos y por otro mezclas equimoleculares de los mismos analitos.

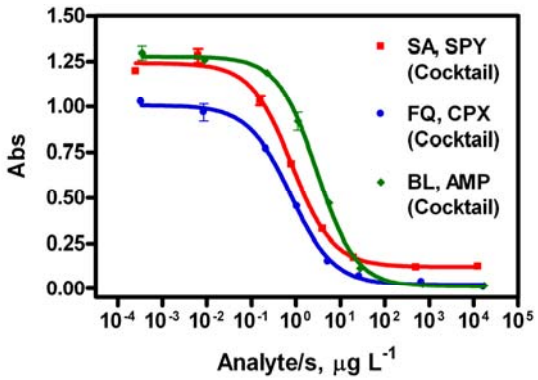


Figura 9.18: Las curvas de calibrado para el ELISA multianalito usando el cóctel de bioreactivos en leche diluida 5 veces, agitando la placa durante la etapa de la competencia.

Las características de las curvas de calibración del ELISA multianalito obtenidas fueron muy similares a las obtenidas en los correspondientes ensayos individuales. Después, para evaluar el efecto matriz de la leche en este ensayo, se prepararon varias curvas estándar de calibrado ELISA con leche diluida en tampón PBS. La **Figura 9.18** muestra los resultados obtenidos con muestras de leche diluida cinco veces con agua milliQ para construir curvas de calibrado con

SPY, ciprofloxacina (CPX) y ampicilina (AMP) como modelos de las tres familias de antibióticos. Cabe destacar que los resultados obtenidos fueron casi idénticos a los correspondientes en tampón, lo cual apuntaba a la posibilidad de realizar medidas fiables en leche, sin ningún tratamiento adicional de la muestra. La precisión y fiabilidad de este método bioanalítico multiplexado también se ha demostrado mediante el análisis de muestras ciegas preparadas en tampón y en leche. Las muestras fueron dopadas de forma individual o con mezclas de los congéneres seleccionados de las tres familias de antibióticos (SPY, CPX y AMP). El multianalito ELISA fue capaz de reconocer en todos los casos el tipo de analito/s presentes en las muestras (véase **Fig. 4** in **Section 6.2**).

9.3.4.2 WIOS multianalito

Antes de intentar trabajar en el biosensor multianalito, debíamos desarrollar de manera satisfactoria ensayos WIObS individuales para cada familia de antibióticos (FQs, BLs, TCs).

Tabla 9.6: Características de las curvas de calibrado (individuales) en el WIOS en leche [a].

Parámetros	**Valores del ensayo**			
Ensayo	**SAs**	**FQs**	**BLs**	**TCs**
Analito	SPY	CPX	AMP	OTC
Antígeno tapizado	SA2-OVA 100 µg mL^{-1}	10b-BSA 200 µg mL^{-1}	CCK-BSA 25 µg mL^{-1}	NeutrAvidin 200 µg mL^{-1}
Oligonucleotido	-----	-----	-----	TcDNA-Biotin 0.1 µM
Receptor	-----	-----	RSA 0.3 µM	TetR 1.0 µM
Anticuerpo	As155 50 µg mL^{-1}	As171 50 µg mL^{-1}	anti RSA 50 µg mL^{-1}	anti TetR 10 µg mL^{-1}
α-rIgG **α-mIgG**	50 µg mL^{-1}	50 µg mL^{-1}	50 µg mL^{-1}	50 µg mL^{-1}
Señal$_{min}$	0.7 ± 0.2	0.7 ± 0.1	0.5 ± 0.1	1.1 ± 0.1
Señal$_{max}$	9.2 ± 0.2	21.1 ± 0.3	34.1 ± 1.5	16.4 ± 1.4
Pendiente	- 1.04 ± 0.03	- 1.14 ± 0.01	- 1.06 ± 0.27	- 2.40 ± 0.02
IC_{50}, µg L^{-1}	6.3 ± 0.6	19.7 ± 4.8	26.2 ± 2.6	114.8 ± 2.7
Rango de trabajo, µg L^{-1}	1.4 ± 0.2 a 26.4 ± 0.9	4.4 ± 0.5 a 70.3 ± 13.7	7.1 ± 3.1 a 100.0 ± 11.3	56.8 ± 7.9 a 193.3 ± 13.4
LOD, µg L^{-1}	0.5 ± 0.1	1.3 ± 0.2	3.1 ± 0.2	34.2 ± 5.4
R^2	0.991 ± 0.004	0.991 ± 0.003	0.992 ± 0.008	0.993 ± 0.005

[a] *Los valores obtenidos corresponden a seis réplicas del mismo chip.*

De este modo, seguimos una estrategia similar a la que se utilizó anteriormente para el WIObS de SAs en este formato, lo que significaba trabajar en configuraciones competitivas y también en formato indirecto, e inmovilizando (mediante polímeros de dextrano) las proteínas

haptenizadas en el los sensores del chip. Para cada ensayo se seleccionaron las concentraciones óptimas de los reactivos a través de experimentos 2D midiendo la unión de varias soluciones de bioreceptores a la superficie de los chips funcionalizados covalentemente con diferentes concentraciones de antígeno. Las concentraciones seleccionadas para cada antígeno fueron 10b-BSA (200 µg mL^{-1}), CCK-BSA (25 µg mL^{-1}) y neutravidina (200 µg mL^{-1}) para la detección de FQs, BLs y TCs, respectivamente. La **Tabla 9.6** muestra los resultados obtenidos en el WIOS para cada caso una vez optimizado todo el proceso para cada ensayo individual.

En algunos casos, los valores de IC_{50} y el LOD conseguidos en muestras de leche fueron ligeramente superiores a los obtenidos cuando se utiliza el mismo reactivo en sus formatos y configuraciones originales (ELISA, ELRA o tiras de ensayo), pero estaban todavía por debajo de los valores MRL establecidos por la EC. Por otro lado, el amplio espectro de selectividad mostrada por los bioreceptores utilizados en este estudio permite prever la posibilidad de detectar la presencia de más de 30 antibióticos diferentes con el WIObS multianalito. Posteriormente se trabajó en el sensor WIObS con la idea de adaptar estos ensayos individuales a un formato multiplexado y poder detectar simultáneamente las cuatro familias de antibióticos en leche. Para conseguir este objetivo, diferentes chips del WIOS fueron funcionalizados tal y como se indica en la **Figura 9.19**.

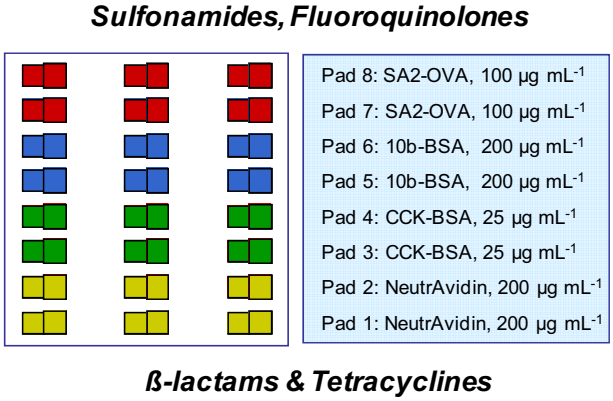


Figura 9.19: Esquema de un chip funcionalizado con las concentraciones de los antígenos de SAs, FQs, BLs y TCs ya optimizadas.

Las condiciones experimentales que figuran en la **Tabla 9.7** se utilizaron para todos los experimentos, diluyendo los diferentes reactivos en la leche, en presencia o ausencia de antibióticos. En ningún caso se observaron reactividades cruzadas inesperadas, lo que confirma la especificidad de los reactivos con los correspondientes antígenos inmovilizados en las superficies sensoras.

Tabla 9.7: Condiciones optimas del WIOS multianalito en leche.

Etapa	Solución Inyectada(Concentración)	Tiempo
Equilibrio (80 µL)	PBS (10 mM)	5 minutos
Etapa previa a la competencia	TCDNA-Biotina (10^{-5} M)	10 minutos
Etapa de competencia en leche (80 µL)	As155 (50 µg mL^{-1})/As171 (50 µg mL^{-1}) RSA (0.3 µM), Anti-RSA (50 µg mL^{-1}) TetR (1.0 µM), Anti-TetR (10 µg mL^{-1}) Muestras de leche; ≠ concentración	10 minutos
Primer lavado (80 µL)	PBS (10 mM)	2.5 minutos
Segundo lavado (80 µL)	PBS (10 mM)	2.5 minutos
Anticuerpo 2º (80 µL)	α-rIgG (50 µg mL^{-1})	5 minutos
Lavado (80 µL)	PBS (10 mM)	5 minutos
Regeneración (80 µL)	NaOH (200 mM)	5 minutos
Iniciar el ciclo de nuevo	**Para cada muestra**	**45 minutos**

De este modo, el sistema WIOS combinado con bioreactivos genéricos contra antibióticos de tipo SAs, FQs, BLs y TCs ha permitido el desarrollo de un biosensor (WIObS) capaz de detectar simultáneamente 34 antimicrobianos en muestras de leche de acuerdo con la normativa EC (véase **Figure 5** en **Section 6.3** y **Section 6.5.3**).

9.3.5 Workshop realizado en NRC (ensayos multianalitos)

Los diferentes métodos analíticos multianalito desarrollados en el contexto del proyecto GOODFOOD (es decir, ELISA, WIObS y las tiras reactivas (ver **Figura 9.20**) fueron evaluados experimentalmente dentro de un taller técnico realizado en el NRC.

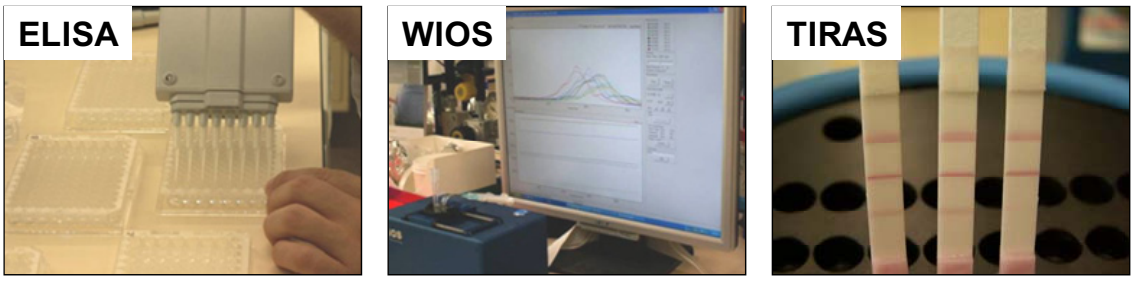


Figura 9.20: Técnicas multianalito desarrolladas en el proyecto GOODFOOD.

La idea era el poder realizar algún tipo de validación preliminar de las tres técnicas mediante el análisis de un número significativo de muestras de leche, contaminadas o no con diferentes antibióticos, y así comparar los resultados obtenidos (sensibilidad, reproducibilidad, facilidad de uso, o el tiempo de análisis) por cada método. En este sentido, cada grupo de investigación adaptó la técnica que había desarrollado para llevar a cabo las diferentes mediciones planteadas

por Nestlé con el fin de evaluar las ventajas e inconvenientes de cada plataforma de detección. El desarrollo de las jornadas fue muy interesante tanto por la interacción entre los grupos como por los resultados obtenidos. De las 25 muestras de leche analizadas con las tres técnicas de detección, sólo se obtuvo un falso negativo por el ELISA multianalito. Por otro lado, se obtuvieron siete falsos positivos entre todos los formatos, pero la prioridad se centró en la búsqueda de muestras contaminadas en vez de intentar minimizar el número de falsos positivos. Aunque esta situación es menos problemática, es necesario seguir trabajando para reducir este porcentaje de falsos positivos. Los diferentes resultados obtenidos por cada plataforma de detección fueron discutidos entre los grupos (ver **Tabla 9.8**).

Tabla 9.8: Comparación cualitativa de las diferentes técnicas desarrolladas para analizar muestras de leche.

PARÁMETROS	ELISA	WIOS	TIRAS ENSAYO
Dificultad de uso	Media-Alta	Alta	Baja
Efecto matriz	Bajo	Bajo	Bajo
Límite de detección	Bajo	Baja	Baja
Relación de falsos negativos	Baja	Baja	Baja
Relación de falsos positivos	Media	Media	Alta
Resultados semi-cuantitativos	Medio-Alto	Medio	Medio
Portabilidad del ensayo	Media	Baja	Alta
Precio estimado por ensayo	Bajo	Alto	Bajo
Velocidad (muestras/día/operador)	Alta (60)	Baja (12)	Alta (40)

Por el momento, ninguna plataforma de detección puede ejecutar el análisis simultáneo de contaminantes *in situ*, en tantas muestras como tiras de ensayo se puedan usar, sin pretratamiento de muestras y en menos de 20 minutos. Por lo tanto, muchos productores de leche y fábricas, como Nestlé, están alentando a los grupos de investigación e invirtiendo en proyectos como el de GOODFOOD para el desarrollo de tiras reactivas para la detección simultánea de diferentes familias de antibióticos. Por otra parte, consideramos que la técnica de ELISA es el método ideal para evaluar la calidad de bioreactivos, en términos de sensibilidad y especificidad, en diferentes muestras reales antes de ser implementados en otras plataformas de detección analítica ya sea individual o multianalito. Además, esta técnica sigue ofreciendo grandes capacidades de cribado de gran número de muestras cuando se usa en un laboratorio, y en menor medida como ensayos de campo en comparación con tiras reactivas, ya que varios análisis se pueden realizar en paralelo mediante el uso de diferentes microplacas. A pesar que un ELISA puede ser realizado por operadores no calificados, un análisis completo de, por ejemplo, muestras biológicas sigue siendo por el momento más fácil de realizar utilizando las tiras reactivas. Por otro lado, el ELISA puede proporcionar medidas cuantitativas y semi-

cuantitativas en contra de la detección de tipo visual ofrecida por tiras reactivas que en muestras complicadas puede dar lugar a lecturas erróneas. Por último, el WIObS es actualmente el método de cribado de muestras aquí presentado más alejado de llegar al mercado, debido a la complejidad de la técnica que todavía ha de mejorarse para poder competir en simplicidad con el ELISA o las tiras reactivas. Por otra parte, de las tres técnicas desarrolladas, este biosensor parece ser el más adecuado para determinaciones automatizada. Actualmente se está trabajando en la elaboración de un instrumento WIOS para ensayos de campo, mejorando el diseño de la cámara de fluidos, el procesamiento de datos, e incluso reduciendo el manejo de soluciones de reactivos para su uso futuro por personal no cualificado. En este sentido, abordar nuevos retos científicos, como la combinación de bioreactivos con características de detección excepcionales, junto con lo último en tecnología de biosensores, es el punto clave para mejorar las actuales técnicas analíticas disponibles. De este modo, dentro del proyecto GOOFOOD se ha trabajado muy duro en esta plataforma WIOS hasta conseguir estos excelentes resultados que eran impensables antes del inicio del proyecto.

Por otra parte, además de trabajar con técnicas inmunoquímicas más convencionales descritas anteriormente, en esta tesis se han intentado otro tipo de experimentos con los inmunoreactivos desarrollados con el fin de explorar metodologías alternativas para analizar antibióticos. Las interesantes características de estos bioreceptores junto con sus capacidades de acoplamiento permite la posibilidad de ser conjugados a otras unidades tales como partículas magnéticas, puntos cuánticos (QDs) o marcadores fluorescentes, por lo que se pueden llegar a utilizar en múltiples plataformas de detección (véase **Appendix**).

9.4 Conclusiones

9.4.1 MIPs para SA

- Se ha desarrollado una nueva metodología, mediante la combinación de métodos computacionales y experimentos de NMR, para el diseño racional de la mezcla de polimerización para obtener un MIP capaz de interactuar selectivamente con SMZ, por medio de interacciones no-covalentes, y mostrando muy buena afinidad y propiedades cromatográficas.
- Se ha establecido una estrategia combinatoria basada en el desarrollo de simples ensayos experimentales secuenciales, pero incrementando su complejidad, para excluir, de un lote grande de polímeros preparados con diferentes porógenos después del correspondiente diseño racional, aquellos MIPs con peores propiedades de reconocimiento y por tanto teniendo menos candidatos en cada paso sin tener que realizar una caracterización precisa de todos los candidatos.
- La preparación de MIPs basados en el diseño racional tienen un rendimiento superior en comparación con la preparación típica de MIPs a través de un largo proceso experimental de prueba y error antes de encontrar el protocolo/condiciones más adecuado.

9.4.2 Anticuerpos para SAs

- Aunque la mayoría de antibióticos, como las SAs, FQs, BLs o TCs, son compuestos que están regulados o prohibidos en el ámbito agroalimentario, los datos de las encuestas recogidos y analizados demuestran su uso continuado de manera fraudulenta, por lo tanto, es interesante continuar investigando en nuevas metodologías y técnicas para la detección de estos compuestos.
- El uso de un hapteno que conserva toda la estructura de sulfonamida (hapteno SA1) ha producido anticuerpos con una mejor detectabilidad y un rango amplio de reconocimiento para esta familia de antibióticos que los obtenidos cuando el hapteno de inmunización sólo contenía el fragmento común de las SAs (hapteno SA2). Por lo tanto, con estos anticuerpos ha sido posible el desarrollo de inmunoensayos con límites de detección por debajo de los

límites determinados por la normativa europea en relación con la detección de residuos de esta familia de antibióticos en los productos agroalimentarios. Los resultados indican que manteniendo en el hapteno de inmunización, la estructura básica definida por la anilina, el puente de sulfonamida y un heterociclo con, al menos, uno nitrógeno en la posición de β con respecto al puente de sulfonamida, ha sido fundamental para lograr el amplio rango de reconocimiento de los anticuerpos producidos.

- De los resultados de esta tesis, no es posible proporcionar sustento a cualquiera de las distintas hipótesis en relación con la conveniencia de utilizar combinaciones homólogas o heterólogas de anticuerpo/competidor en los métodos analíticos inmunoquímicos competitivos, para llegar a la mayor detectabilidad. Por lo tanto, la detectabilidad alcanzada por el ELISA directo para SAs ha sido mejor cuando se utilizan combinaciones anticuerpo/trazador enzimático homólogas. Por otro lado, en el formato indirecto la detectabilidad es mejor cuando se utilizan combinaciones de anticuerpo/antígeno de tapizado heterólogas.
- Por contra, nuestros resultados apoyan la norma establecida previamente por este y otros grupos de investigación, en cuanto a los efectos de la heterología en el ensayo sobre la especificidad del ensayo. Por lo tanto, se ha demostrado que usando combinaciones heterólogas, es posible llegar a un perfil de mayor reconocimiento de los diferentes congéneres de SAs. Esto concuerda con los datos reportados en la literatura que apunta a la baja afinidad de los anticuerpos frente al competidor como la razón principal para favorecer el reconocimiento de otros compuestos relacionados que pueden interactuar con el anticuerpo.
- Las excelentes características de los anticuerpos generados contra el hapteno SA1, y sus elevadas capacidades de reconocimiento contra SAs han sido identificadas como la razón más importante de la robustez de los métodos de ELISA desarrollados en muestras complejas. Por lo tanto, los formatos de ELISA desarrollado para SAs pueden detectar de manera efectiva restos de residuos de SAs en leche y extractos de pelo, sin ningún tipo de tratamiento de la muestra que no sea la dilución, y a pesar de la complejidad de la composición de estas muestras. Estos resultados apuntan a la posibilidad de utilizar estos métodos inmunoquímicos para el análisis de residuos SAs en otras matrices.

- Los anticuerpos generados contra SAs han mostrado tener mejores propiedades de afinidad que los correspondientes MIPs preparados para la misma familia de antibióticos y por lo tanto siendo los receptores más adecuados para el desarrollo de un biosensor. Por el contrario, los MIPs presentan una mejor estabilidad física y química en disolventes orgánicos y, por tanto se pueden ajustar mejor como materiales selectivos para fases estacionarias o para aplicaciones de extracción en fase sólida.

9.4.3 Anticuerpos para TCs

- Aunque las estructuras de los haptenos de TCs para el posterior proceso de inmunización fueron diseñados con el objetivo de obtener anticuerpos de familia, los resultados muestran que para lograr este objetivo sería fundamental tener en cuenta otras consideraciones además de la posición del brazo espaciador en la periferia superior de este tipo de compuestos.
- Se ha logrado una respuesta inmune con los dos haptenos de inmunización preparados contra TCs, pero sólo aquellos obtenidos contra el TC1 proporcionaron la posibilidad de desarrollar ELISAs competitivos. Se ha desarrollado un ensayo ELISA capaz de detectar DC y MC con detectabilidad suficiente, aunque la avidez de los anticuerpos de los diferentes competidores preparados no fue tan elevada como en el caso de los anticuerpos de SAs.
- El ensayo ELISA desarrollado puede ser aplicado para el análisis de muestras de leche, de conformidad con las normas europeas establecidas para estos compuestos. Sin embargo, en este caso ha sido necesario desarrollar un tratamiento de muestra que consta de precipitar las proteínas de la leche seguido de una etapa de dilución. Este hecho, está de acuerdo con nuestra hipótesis con respecto a la mayor tolerancia a la matriz de estudio cuando los anticuerpos muestran una mayor avidez por el analito y/o competidores (véase más arriba).

9.4.4 Biosensor WIOS para SAs

- Es posible la detección de trazas de residuos de antibióticos mediante la combinación del transductor WIOS con bioreceptores específicos. El WIOS ha demostrado ser lo suficientemente sensible como para detectar variaciones en el índice de refracción

producidas en el campo evanescente generado por la luz que viaja en la guía de onda, debido a la unión de anticuerpos al chip sensor biofuncionalizado con antígenos. Sin embargo, ha sido necesario utilizar un anticuerpo secundario con el fin de mejorar la señal y obtener una respuesta más precisa.

- Las características de los inmunoreactivos tienen una fuerte influencia en el rendimiento del sensor. Por lo tanto, en el presente estudio el sistema WIObS desarrollado para SAs muestra características similares, en términos de detección y especificidad, si lo comparamos con el ELISA desarrollado previamente.
- El sistema WIObS para SAs es lo suficientemente robusto para detectar selectivamente las interacciones de unión en la superficie de la guía de onda, incluso en presencia de muestras complejas, como el caso de la leche. De este modo, residuos de SAs pueden ser detectados con el biosensor sin ningún tipo de tratamiento de la muestra. El sistema desarrollado permite discriminar entre muestras de leche contaminadas con SAs en o por encima de sus MRLs, en menos de 30 minutos, incluyendo la regeneración de la superficie de detección.

9.4.5 Detección multianalito

- Diferentes bioreceptores pueden ser combinados con éxito y ser utilizados en forma de cóctel en métodos bioanalíticos multiplexados, debido a su alta especificidad y avidez por su pareja opuesta. De este modo, dentro de esta tesis, bioreceptores de familia para SAs, FQs y BLs se han combinado con éxito para la detección múltiple de residuos en un sistema ELISA ortogonal, donde la identidad del analito objetivo se codifica por su ubicación en la plataforma de detección. Se han obtenido resultados similares en el sistema WIObS multiplexado, con la adición de un cuarto de bioreceptor para TCs. Consideramos que las excelentes características de los bioreceptores junto con las diferencias estructurales de estas cuatro familias de antibióticos, han sido determinantes para poder establecer estas tecnologías de bioanálisis multiplexadas. Respuestas específicas se obtienen independientemente de si los bioreceptores son utilizados individualmente o en mezclas, lo que indica la ausencia de un efecto reactividad compartido. La sensibilidad y la especificidad de la respuesta se mantiene incluso cuando la muestra contiene mezclas de otros antibióticos y es independiente de su concentración

- El uso de bioreceptores individualmente o en combinación no afecta a la tolerancia de la matriz, tan pronto como la avidez de todos estos bioreceptores por sus objetivos es suficientemente alta, como lo ha sido en este caso. Así, tanto el ELISA multianalito como el WIObS multiplexado funcionan muy bien en muestras de leche sin ningún tratamiento previo de la muestra que no sea el de dilución.

- Ambos sistemas multiplexados han demostrado ser útiles como herramientas de cribado en el análisis de residuos de antibióticos, siendo capaz de detectar simultáneamente un elevado número de congéneres de antibióticos en un único análisis. Por lo tanto, para el ELISA en microplacas, se han establecido dos protocolos, uno con fines de cribado para detectar muestras con niveles de residuos en los valores de residuos máximos permitidos, y otro para cuantificar la concentración del antibiótico en la muestra. En ambos casos, más de 25 antibióticos de diferentes familias (SAs, FQs y BLs) pueden ser detectados cumpliendo la normativa de la EC. Por otro lado, el sistema WIObS multiplexado tiene el potencial de detectar 34 antimicrobianos en muestras de leche de acuerdo con la EC. No es necesario realizar un tratamiento de muestra que no sea el de dilución para obtener datos fiables sobre la posible presencia de estas familias de antibióticos en esta matriz.

- La detección de las cuatro familias de antibióticos utilizando la plataforma WIOS mostró las mismas respuestas relativas que las obtenidas por los correspondientes formatos individuales de ELISA o tiras de ensayo demostrando que la selectividad está determinada principalmente por los bioreactivos, no por el tipo de ensayo o la configuración del dispositivo.

- Todos los métodos bioanalíticos tienen ventajas y desventajas. Así, dentro de esta tesis, se han comparado los tres métodos bioanalíticos (ELISA, WIOS y varilla), utilizando los mismos bioreactivos pero basando su respuesta en principios diferentes. Todos ellos son capaces de detectar simultánea y directamente antibióticos de diferentes familias de antibióticos en muestras de leche. Las tiras reactivas son actualmente el mejor método de detección para mediciones in situ, ya que su uso es simple, rápido y fácil de leer los resultados. Aunque la técnica de ELISA se puede utilizar como técnica de cribado, hoy en día consideramos que es la metodología ideal para evaluar la calidad de los bioreactivos preparados antes de ser implementados en otras plataformas de detección analíticas. La

plataforma WIOS necesita de nuevas mejoras, como la manipulación de fluidos, los costes, el procesamiento de datos y la optimización de las condiciones de los bioreactivos, para ser utilizado como un instrumento de campo.

9.4.6 Otros experimentos (Appendix)

- Es posible preparar partículas magnéticas (MNP) biofuncionalizadas con capacidad para detectar SAs. Por lo tanto, se ha realizado con éxito la unión covalente de anticuerpos de familia para sulfonamidas a partículas magnéticas modificadas previamente con grupos carboxilo, logrando una eficacia de acoplamiento excelente. Estas partículas magnéticas se han utilizado para desarrollar un magneto-ELISA con características de detección similares a las del ELISA estándar realizado en microplacas. Estos resultados abren la puerta a la aplicación de estos bioreactivos en una variedad de configuraciones para biosensores más adecuadas para el análisis de muestras dentro de sistemas microfluídicos. Por lo tanto, el uso de partículas magnéticas biofuncionalizadas permite realizar los pasos de extracción y mover los bioreactivos dentro de las diferentes cámaras microfluídicas, mediante la conducción de anticuerpos-MNP con imanes.
- Es posible preparar sondas fluorescentes mediante la unión covalente de compuestos orgánicos fluorescentes o nanopartículas con anticuerpos. Por lo tanto, Cy5 y QDs se han unido con éxito, a través de enlaces covalentes, a haptenos y anticuerpos de SAs, como demuestran los espectros de excitación/emisión obtenidos. Por desgracia, en el contexto de esta tesis no ha sido posible desarrollar el uso de estos reactivos para el establecimiento de análisis inmunoquímicos basados en principios fluorescentes.

9.5 Referencias

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