Tutors

Antoni Rúbies Prat

Laboratori de l' Agència de Salut Pública de Barcelona

Mercè Granados Juan

Departament de Química Analítica

Treball Final de Grau

Determination of non-steroidal anti-inflammatory drugs in milk by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

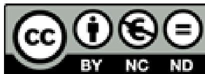
Determinación de antiinflamatorios no esteroideos en leche por cromatografía de líquidos-espectrometría de masa en tándem (LC-MS/MS)

Lili Guo Luo

June 2015

Universitat de Barcelona

Campus d'Excel·lència Internacional B:KC Barcelona Knowledge Campus

Aquesta obra esta subjecta a la llicència de:
Reconeixement–NoComercial-SenseObraDerivada


http://creativecommons.org/licenses/by-nc-nd/3.0/es/

Quiero agradecer a todo el equipo de Agencia de Salud Pública de Barcelona por ofrecerme la oportunidad de realizar este trabajo con ellos y por sus ayudas y soportes. En particular, a Toni Rúbies por guiarme en este trabajo con sus amplios conocimientos, he aprendido muchísimo trabajando con él. También agradezco muchísimo a Mercè Granados por sus ayudas.

Agradezco los ánimos y apoyos que me han dado mi familia y mis amigos, que siempre han estado al mi lado. Especialmente a Dios, gracias por todo.

.

Report

CONTENTS

1. SUMMARY 3
2. RESUMEN 5
3. INTRODUCTION 7
3.1. Non-steroidal anti-inflammatory drugs 7
3.1.1. Use and administration 9
3.1.2. Mechanism of action 10
3.1.3. Adverse effects 10
3.1.4. Legislation 11
3.1.5. Analysis 12
4. OBJECTIVES 15
5. EXPERIMENTAL SECTION 15
5.1. Reagents 15
5.1.1. NSAIDs standards and internal standards 15
5.1.2. Other chemicals 16
5.2. Preparation of standard solutions 16
5.3. Instrumentation 17
5.3.1. Liquid chromatography –tandem Mass Spectrometry (LC-MS/MS) 17
5.3.1.1. Waters Acquity-QqQ Premier 17
5.3.1.2. Agilent Technologies 1290-QqQ 6460 18
5.3.2. Other equipment 20
5.4. Sample preparation 20
5.4.1. Extraction 20
5.4.2. Calibration 20
6. RESULTS AND DISCUSSION 21
6.1. Mass spectrometry detection 21
6.1.1. Preliminary study 21

6.1.2. Optimization of mass spectrometry detection 23
6.1.2.1. Precursor ions 23
6.1.2.2. Product ions 24
6.2. Chromatographic separation optimization 26
6.3. Sample preparation 32
6.3.1. QuEChERS methodology 34
6.3.1.1. QuEChERS methodology optimization 36
7. CONCLUSIONS 43
8. REFERENCES 45
9. ACRONYMS 47

1. SUMMARY

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in human and veterinary medicine because of their main properties: anti-inflammatory, analgesic and antipyretic. The use of these drugs has increased significantly over the world in the last decades. The widespread use of NSAIDs can cause serious side effects such as gastric and intestinal disturbances. The control of the presence of residues of NSAIDs in food of animal origin is mandatory following Europe legislation; for this reason analytical methods for milk and meat analysis have to be set up.

In this work, a method based on the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) approach was developed for the determination of NSAIDs in milk samples by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI) using the Multiple Reaction Monitoring (MRM) mode.

A new sample preparation method was developed, using 5% acetic acid in acetonitrile as extraction system and with a clean-up step by dispersive solid phase extraction (d-SPE) using sorbent C_{18}. Mass spectrometric parameters and chromatographic separation were optimized. Two MRM transitions were monitored for each analyte in positive or negative electrospray mode.

Keywords: Non-steroidal anti-inflammatory drugs,QuEChERS, Milk, Liquid chromatography-tandem mass spectrometry,

2. RESUMEN

Los antiinflamatorios no esteroideos (AINES) son ampliamente utilizados en medicina y veterinaria debido a sus propiedades antiinflamatorias, analgésicas y antipiréticas. El uso de estos medicamentos se ha incrementado significativamente en todo el mundo en las últimas décadas. El uso extendido de los AINES puede causar efectos secundarios, como trastornos intestinales o gástricos. De acuerdo con la legislación europea, es obligatorio el control de la presencia de residuos de AINES en alimentos de origen animal. Por este motivo se debe disponer de métodos para el análisis de AINES en distintos tipos de alimentos.

En este trabajo, se ha desarrollado un método basado en la estrategia QuEChERS para la determinación de AINES en muestras de leche por cromatografía de líquidos-espectrometría de masas en tándem (LC-MS/MS) con ionización por electrospray (ESI). La adquisición de datos se ha hecho en modo Monitorización de Reacciones Múltiples (MRM).

Las muestras se extraen con acetonitrilo con un 5% de ácido acético y se realiza una etapa de clean-up mediante una extracción en fase sólida dispersiva utilizando fase C_{18}. Se han optimizado los parámetros de espectrometría de masas y las condiciones cromatográficas. Se monitorizaron dos transiciones MRM para cada analito, trabajando con ionización en modo positivo y negativo.

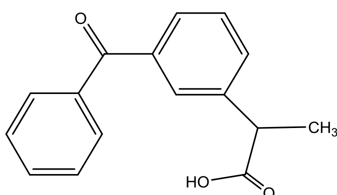
Palabras claves: Antiinflamatorios no esteroideos, QuEChERS, Leche, Cromatografía de líquidos-espectrometría de masa en tándem.

3. INTRODUCTION

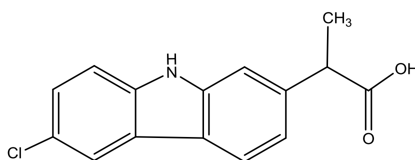
3.1. NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDS)

Non-steroidal anti-inflammatory drugs (NSAIDs) are a heterogeneous group of drugs with quite different chemical structures and with similar effects on human and animal health. They can be classified into several groups depending on their chemical structure [1]:

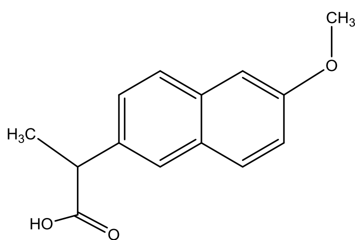
a) Propionic acid derivatives: Ketoprofen (KTP), Carprofen (CPF), Naproxen (NP), and Ibuprofen (IBU)



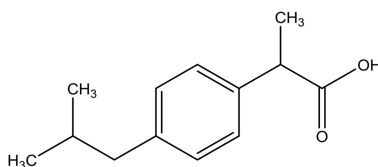
Ketoprofen



Carprofen

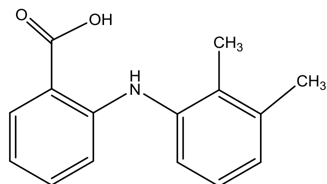


Naproxen

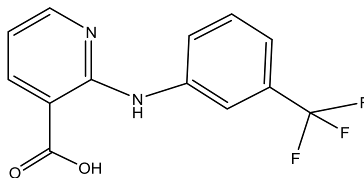


Ibuprofen

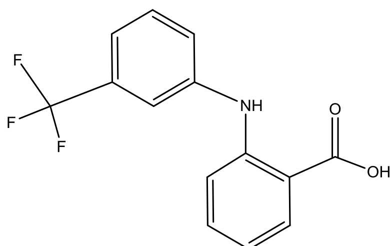
b) Anthranilic acid derivates: Tolfenamic acid (TLF), Mefenamic acid (MF), Meclofenamic acid (MEC), Flufenamic acid (FLUf) and Niflumic acid (NFL)



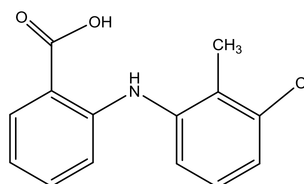
Mefenamic acid



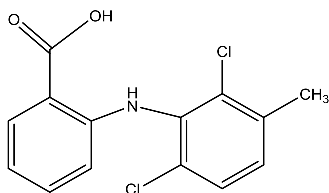
Niflumic acid



Flufenamic acid

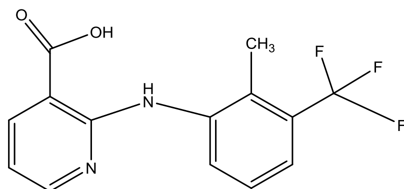


Tolfenamic acid

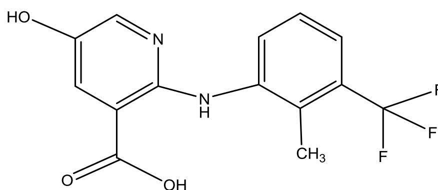


Meclofenamic acid

c) Nicotinic acid derivates: Flunixin (FLU) and 5-hydroxyflunixin (5-FLU)

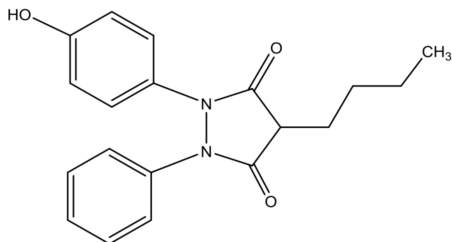


Flunixin

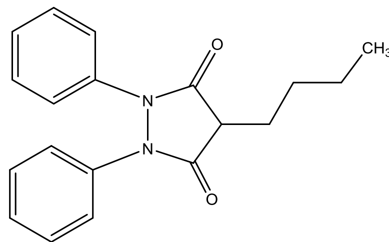


5-hydroxyflunixin

d) Pyrazolones: Phenylbutazone (PBZ) and Oxyphenylbutazone (OPB)

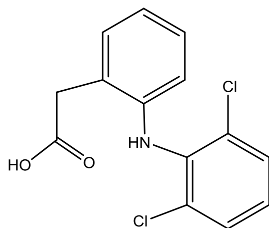


Oxyphenbutazone



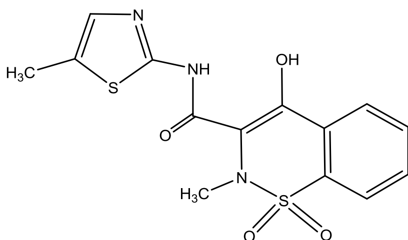
Phenylbutazone

e) Acetic acid derivates: Diclofenac (DC)



Diclofenac

f) The class of oxicams: Meloxicam (MLX)



Meloxicam

3.1.1. Use and administration

NSAIDs are widely used in human and veterinary medicine, because their anti-inflammatory, antipyretic and analgesic properties. The combined analgesic and anti-

inflammatory effects of NSAIDs make them particularly useful for the symptomatic relief of painful and inflammatory processes, including musculoskeletal and joint disorders, such as rheumatoid arthritis, osteoarthritis, and the spondyloarthropathies and in peri-articular and soft-tissue disorders [2].

NSAIDs provide adequate analgesia to relieve inflammation and pain in short-term intermittent therapy or administered as single dose [2].

3.1.2. Mechanism of action

Many of the effects of NSAIDs appear to be due to their inhibition of cyclooxygenases (COX), which are involved in the biosynthesis of prostaglandins [2]. Cyclooxygenase has different isoforms; the most common are cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 has clear physiologic functions. Its activation leads to the production of prostacyclin which when released by the gastric mucosa, is cytoprotective. COX-2 is induced by inflammation and the presence of proinflammatory cytokines and mitogens [4]. The inhibition of COX-1 is associated with adverse gastro-intestinal effects, while inhibition of COX-2 is associated with anti-inflammatory activity. A NSAID which inhibits COX-2 selectively should reduce inflammation and not influence normal physiologic function and thus should cause fewer gastro-intestinal effects [2]. Hence, NSAIDs can be divided into two groups according to their inhibitory selectivity: COX non-selective inhibitors (propionic acid derivates, salicylic acid derivates, pyrazole derivates, nicotinic acid derivates, acetic acid derivates, anthranilic acid derivates and oxicam) and COX-2 selective inhibitors (Celecoxibs, Refecoxibs) [3]. Our present study does not include any of the COX-2 selective inhibitors.

3.1.3 Adverse effects

The most common side effect are gastric and intestinal disturbances. These are usually mild and reversible, but in some patients peptic ulcer and/or severe gastro-intestinal bleeding have been reported. These adverse effects may be associated with inhibition of the cyclooxygenase-1 (COX-1) [2].

Moreover, there are other effects:

- Effects on the central nervous system (CNS)

The most frequent CNS adverse effects are headache and hearing loss, but also nervousness, depression, drowsiness and insomnia.

- Effects on the kidneys

NSAIDs can produce different renal disorders, some of which are due to their inhibition of prostaglandin synthesis.

- Hypersensitivity reactions may occur, including fever, asthma and rashes.
- Haematological adverse effects include anemia, thrombocytopenia, neutropenia, eosinophilia and agranulocytosis.

3.1.4. Legislation

Several NSAIDs are authorized to be administered to food-producing animals. The use of these drugs has increased significantly over the world in the last decades. Residues of these pharmaceuticals in edible products may be a potential risk to consumers. For this reason, the European Union (EU) has established maximum residue limits (MRLs) [5] in food products of animal origin.

Table 1. Maximum residue limits (MRLs) for NSAIDs

	MRLs (µg·Kg^{-1})			
Compound	**Milk**	**Muscle**		
		Bovine	**Porcine**	**Equidae**
5-hydroxyflunixin	40	40		
Flunixin	40	20	20	20
Diclofenac	0.1	5		5
Carprofen		500		500
Tolfenamic acid	50	50		
Meloxicam	15	20		

Furthermore, according to the Community Reference Laboratories (CRLs) guidance paper 12/2007 [6], the recommended concentrations for NSAIDs to be monitored in the laboratory are the following:

Table 2. Recommended concentrations for NSAIDs according to CRLs

Compound	Matrix	Recommended concentration
Phenylbutazone Oxyphenylbutazone	Muscle, Kidney, Liver, Plasma, Milk	5 $\mu g \cdot Kg^{-1}$
Ibuprofen Naproxen Mefenamic acid	Muscle, Kidney, Liver, Plasma, Milk	10 $\mu g \cdot Kg^{-1}$
Diclofenac	Milk	5 $\mu g \cdot Kg^{-1}$

3.1.5. Analysis

It is difficult to analyze NSAIDs from different classes with one single method due to their chemical structure, because they are very different. Table 3 shows some multi-residue methods for the determination of NSAIDs in milk. Sample extraction is performed with acetonitrile [7,8, 11] or methanol [10], and a clean-up step by SPE, using NH_2 [7] or C_8 cartridges [10] can be applied, although sometimes the clean-up step is avoided [8]. These methods allow the detection of NSAIDs according to MRLs, except Diclofenac, for which there only very few are able to quantify this NSAID in milk at the MRL. [5]

Table 3. Sample preparation for determination of NSAIDs

AINES	Sample preparation	Conditions of separation	Instrumental	Reference
Ketoprofen Flunixin Mefenamic acid 5-hyroxyflunixin Oxyphenbutazone Phenylbutazone Diclofenac Naproxen Tolfenamic acid Carpofen Celecoxib Firocoxib Rofecoxib	Extraction: acetonitrile in the presence of ammonium acetate Clean-up: Sep-Pak NH_2 cartridge	Column: phenomenex Luna C_8 (3µm, 2.1mm x 150mm) Phase mobile: MeOH/ACN 8:2 (v /v) (phase A) 0.01 mol/L ammonium formate pH 5.0 (phase B)	Liquid chromatograph (Agilent Techonologies 1200) connected to QTrap 5500 mass spectrometer. MS: ESI+/ESI-	Jedziniak.P et al. 2012 [7]

Ibuprofen Meloxicam Metamizolemetabolites				
Ketoprofen Carprofen Flunixin Mefenamic acid Flufenamic acid 5-hydroxyflunixin Phenylbutazone Oxyphenbutazone Meclofenamic acid Diclofenac Niflumic acid Tolfenamic acid Meloxicam 4-methylaminoantipyrine	Extraction: ACN No Clean-up	Column: Waters symmetry C_{18} (5µm, 2.1 x 150 mm) Phase mobile: Water/ACN (95/5) and 0.1% formic acid (phase A) 0.1% formic acid and ACN (phase B)	Acquity UPLC system connected to MicroMass Quattro Ultima triple quadrupole	E.Daeseleire et al. [8]
Naproxen Carprofen 5-hydroxyflunixin Flunixin Mefenamic acid Flufenamic acid Phenylbutazone Oxyphenbutzaone Meclofenamic acid Niflumic acid Tolfenamic acid Ketorprofen Suxibuzone Flurbiprofen	Extraction: ACN/MeOH + ascorbic acid buffer 0.01M and HCl 1.0M Clean-up: SPE cartridge	Column: Max RP Synergi stainless steel (Phenomenex) Phase mobile: 0.1% acetic acid in water (phase A) 0.1% acetic acid in ACN (phase B)	Agilent 1200 system connected to QTRAP 4000 mass spectrometer	Gallo P et al. [9]
Mefenamic acid Flufenamic acid Phenylbutazone	Extraction: MeOH + HCl 0.24M and ascorbic acid	Column:Acquity UPLC® HSS T3 C_{18} (1.8µm, 50	Waters® LC-MS/MS system	The Laboratory of Public Health Agency in

Oxyphenbutazone Meclofenamic acid Carboxiibuprofen Piroxicam Naproxen Niflumic acid Flurbiprofen Indometacin Suxibuzone	0.02M Clean-up: SPE C_8 cartridge	x 2.1 mm) Phase mobile: formic acid 0.1% pH =4 (phase A) 0.1% formic acid in ACN (phase B)		Valencia [10]
Flunixin Phenylbutazone	Extraction: 1% acetic acid in ACN + sodium acetate $+MgSO_4$ No clean-up	Column phenomenex, Biphenyl, kinetex, 2.6µm, 100mm x 2.1mm Phase mobile: 0.1% formic acid (phase A) 10 mM ammonium acetate in water (phase B)	Waters Acquity UPLC coupled with a Q-TOF Premier	Wang. J et al. 2012 [11]

4. OBJECTIVES

The final aim of this work is to develop a method for the analysis of 15 NSAIDs in milk samples by LC-MS/MS according to the legal requirements.

The objectives are:

- Optimization of mass spectrometry conditions to achieve two suitable multiple reaction monitoring (MRM) transitions for each NSAID.
- Optimization of liquid chromatographic separation
- Set up of a simple multi-residue extraction method of NSAIDs from milk samples.

5. EXPERIMENTAL SECTION

5.1. REAGENTS

5.1.1. NSAIDs standards and internal standards

Ketoprofen (KTP)

Carprofen (CPF)

Naproxen (NP)

Ibuprofen (IBU)

Tolfenamic acid (TLF)

Mefenamic acid (MF)

Meclofenamic acid (MEC)

Niflumic acid (NFL)

Flufenmic acid (FLUF)

Flunixin (FLU)

5-hydroxyflunixin (5-FLU)

Phenylbutazone (PBZ)

Oxyphenbutazone (OPB)

Diclofenac (DC)

Meloxicam (MLX)

All were over 98% purity and provide by Sigma Aldrich.

Meloxicam-D_3

Niflumic acid $^{13}C_6$

Flufenamic acid $^{13}C_6$

Phenylbutazone$^{13}C_6$

All were over 98% purity and provide by Sigma Aldrich

5.1.2. Other chemicals

Acetonitrile

Acetic acid

Ammonium acetate

Na_2SO_4

Ascorbic acid

HCl

$MgSO_4$ and

Formic acid

Sorbent C_{18}

All were analytical reagent grade

Water was obtained with a Milli-Q system

5.2. PREPARATION OF STANDARD SOLUTIONS

The individual stock standard solutions were prepared in methanol at concentration of 1000 mg·L^{-1} for each of the 15 NSAID standards. The mixed standard solutions were prepared by dilution of the individual standard solutions at concentration of 20 mg·L^{-1}, working standard solutions (0.4 mg·L^{-1}, 2.0 mg·L^{-1} and 5.0 mg·L^{-1}) were prepared in 0.1% formic acid by dilution of the mixed standard solution of 20 mg·L^{-1}. All standard solutions were stored at -20 ºC and were stable at least for 6 months.

The mixed internal standard solution (IS), including Meloxicam-D_3, Niflumic acid $^{13}C_6$, Flufenamic acid $^{13}C_6$ and Phenylbutazone$^{13}C_6$, was prepared in 0.1% formic acid at concentration 20 mg·L^{-1}. It was stored at -20 ºC and was stable at least for 6 months.

5.3. INSTRUMENTATION

5.3.1. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

5.3.1.1. Waters Acquity-QqQ Premier

The system consisted of Waters Acquity UPLC coupled to Quattro Premier QqQ mass spectrometer and separations were achieved using a Phenomenex Kinetex C_{18} column (1.8µm, 100mm x 2.1mm); the column temperature was kept at 40ºC. Chromatographic separation was carried out using a mobile phase consisting of 0.1% formic acid (phase A) and 0.1% formic acid in ACN (phase B) at a flow rate of 0.4 ml·min^{-1}, with the gradient program shown in Figure 1. The injection volume was 10 µl. The total runtime, including equilibration, was 14 min.

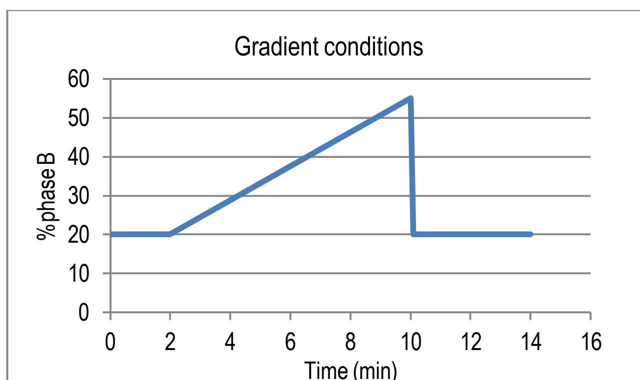


Figure1. Gradient conditions used for LC separation with the Waters system

The MS/MS detection was performed with electrospray ionization in positive mode and using the multiple reactions monitoring (MRM) mode. Two transitions for compound were monitored and one transition for the internal standards. Table 4 shows the MS parameters for each compound.

Table 4. MS/MS parameters for NSAIDs detection with Quattro Premier QqQ mass spectrometer

	Precursor ion (m/z)	**CV (V)**	**Product ion 1 (m/z)**	**CE (V)**	**Product ion 2 (m/z)**	**CE (v)**
Fluninxin	297	35	279	20	265	35
Ketoprofen	255	30	105	25	209	15
Diclofenac	296	20	250	13	278	9
Flufenamic acid	282	23	244	27	264	19
5-hydroxyflunixin	313	35	280	30	295	25
Niflumic acid	283	36	245	28	265	22
Naproxen	231	20	170	25	185	15
Meloxicam	352	29	141	25	115	15
Carprofen	274	15	193	33	228	20
Phenylbutazone	309	30	120	20	211	16
Mefenamic acid	242	23	209	28	242	15
Tolfenamic acid	262	23	209	28	244	15
Oxyphenbutazone	325	25	148	28	204	16
Meclofenamic acid	296	21	243	24	278	12

(a) CV: Cone voltage
(b) CE: Collision energy

5.3.1.2. Agilent Technologies 1290-QqQ 6460

The LC system consisted of an Agilent Technologies 1290 connected to a Triple Quadrupole (QqQ) 6460 with electrospray ionization (ESI). A gradient LC system (Figure 2) using 0.1% formic acid (mobile phase A1) and acetonitrile (mobile phase B1) at a flow rate of 0.4 ml·min^{-1} were used to separate the NSAIDs on a column Phenomenex Kinetex Biphenyl (2.6µm, 100 x 2.1mm). The column temperature was kept at 40ºC and the injection volume was 20 µl. The total runtime, including equilibration, was 14 min.

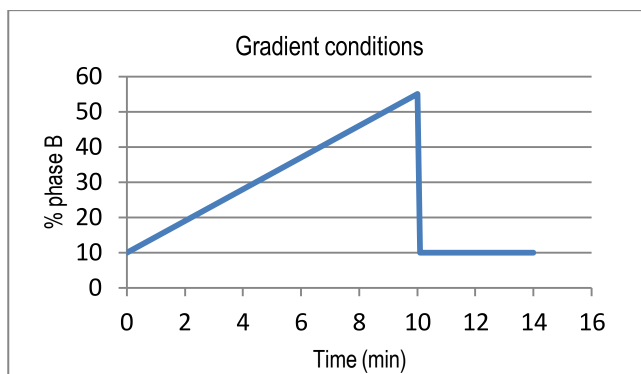


Figure 2. Gradient conditions used for LC separation with the Agilent equipment

The MS/MS detection was performed with electrospray ionization operated in both positive and negative polarities using the multiple reactions monitoring (MRM) mode. Two transitions for compound were monitored and one transition for the internal standards. Table 5 shows the MS/MS parameters for each compound.

Table 5. MS/MS parameters for NSAIDs detection with Agilent QqQ 6460 mass spectrometer

	ESI+/ESI -	**Precursor ion (m/z)**	**Fragmentor energy (V)**	**Product ion 1 (m/z)**	**CE (V)**	**Product ion 2 (m/z)**	**CE (V)**
5-hydroxyflunixin	ESI+	313	110	295	25	280	35
Diclofenac	ESI-	294	90	250	10		
Phenylbutazone	ESI-	307	130	279	15	131	15
Flunixin	ESI-	295	70	251	15	209	35
Flufenamic acid	ESI-	281	130	237	15	216	25
Ketoprofen	ESI+	255	130	105	15	77	50
Meclofenamic acid	ESI+	297	110	279	15	264	40
Mefenamic acid	ESI-	240	70	196	20		
Naproxen	ESI-	229	160	185	50	169	35
Niflumic acid	ESI-	281	130	237	15	177	35
Oxyphenbutazone	ESI-	323	130	295	15	134	15
Carprofen	ESI-	271	130	228	5	226	25
Tolfenamic acid	ESI-	260	110	216	5		

Ibuprofen	ESI-	205	110	161	1		
Meloxicam	ESI+	352	130	141	15	115	25

(a) CE: Collision energy

5.3.2. Other equipment

A multi-tube vortex (VWR, DVX-2500), laboratory centrifuge (Rotanta 460R), vertical agitator (Agitax) and nitrogen evaporator (Turbo Vap) were used for sample preparation.

5.4. SAMPLE PREPARATION

5.4.1. Extraction

5 g of milk were weighed in a centrifuge tube. Next, 10ml of 5% acetic acid in ACN, 1g of ammonium acetate and 5g of Na_2SO_4 were added. The sample was shaken for 5 minutes and it was centrifuged (3000rpm, 10ºC, 10min). Then, the supernatant was transferred to a centrifuge tube containing 150mg of sorbent C_{18} and 1g of $MgSO_4$. 4ml of ascorbic acid 0.02M and HCl 0.24M and 2 g of Na_2SO_4 were added. The mixture was shaken for 4 min and it was centrifuged again under the same conditions. Then, the supernatant was evaporated to 250µl (N_2, 40ºC). Finally, the residue was re-dissolved in 250µl of 0.1 % formic acid filtered through a 0.22µm nylon filter membrane.

5.4.2. Calibration

The quantification of NSAIDs in milk samples was based on the use of surrogate matrix matched standards (SMMS), which consists of blank milk samples spiked with known amount of NSAIDs and submitted to the whole sample treatment procedure. Calibration with SMMS allows overcoming matrix effects on mass spectrometry detection. In this work, three working standards solutions (0.4 mg·L^{-1}, 2 mg·L^{-1} and 5 mg·L^{-1}) were added to spike blank samples at four levels (Table 6) to prepare surrogate matrix method standards.

Table 6. Spiking level (µg·Kg^{-1}) for determination of NSAIDs in milk.

	Concentration (µg·Kg^{-1})	Blank milk (g)	IS (µl)	Standard solution (µl)
ME1	2.5	5	100	31.3 (0.4 mg·L^{-1})

ME2	5	5	100	12.5 (2 mg·L^{-1})
ME3	10	5	100	25 (2 mg·L^{-1})
ME4	25	5	100	25 (5 mg·L^{-1})

6. RESULTS AND DISCUSSION

6.1. MASS SPECTROMETRY DETECTION

According to UE decision 657 [12], for a confirmatory method based on LC-MS/MS, the acquisition of two transitions is required. Therefore, two transitions for compound were monitored, the most sensitive transition was selected for quantification and the other was used for confirmation. Only one transition was monitored for the internal standards.

6.1.1. Preliminary study

In the beginning of this work, some preliminary experiments were performed with the Waters Acquity-Quattro Premier QqQ LC-MS equipment with the electrospray ionization in positive mode.

First of all, cone voltage for the precursor ion has to be optimized. Afterwards, product ions should be selected and the corresponding collision energy optimized.

The optimization consisted of the direct infusion of the individual standard solution of NSAID at 2 mg·L^{-1} with the following conditions:

- Mobile phase: 0.1% formic acid (phase A) and 0.1% formic acid in ACN(phase B)
- Mass spectrometry: MS Scan mode was used to optimize precursor ion and cone voltage, MS daughter Scan mode was used to optimize product ions and collision energies.

As an example, in the following figures the optimized precursor ion and product ions for Ketoprofen are shown:

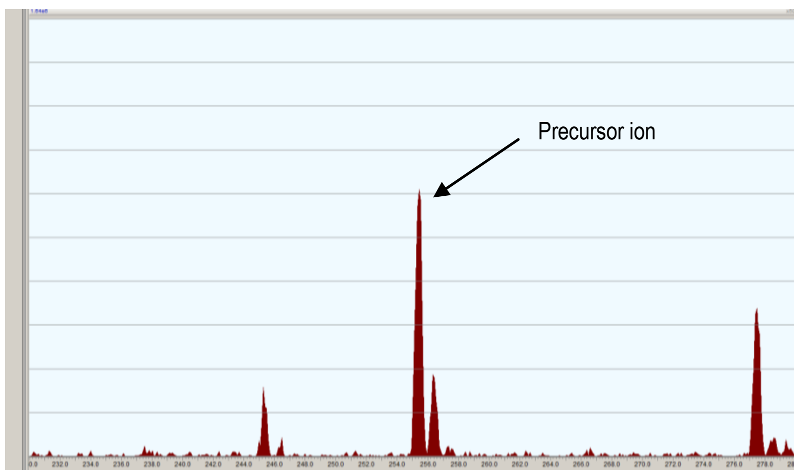


Figure 3. Precursor ion of Ketoprofen (m/z= 255)

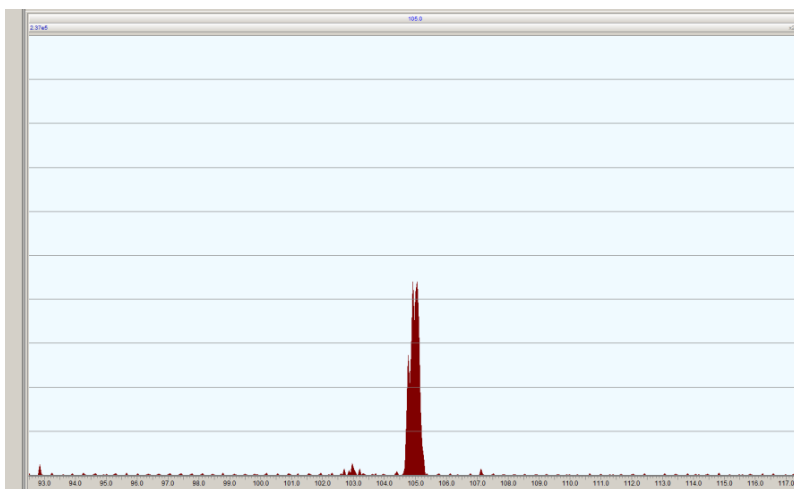


Figure 4. Product ion 1 of Ketoprofen (m/z= 105)

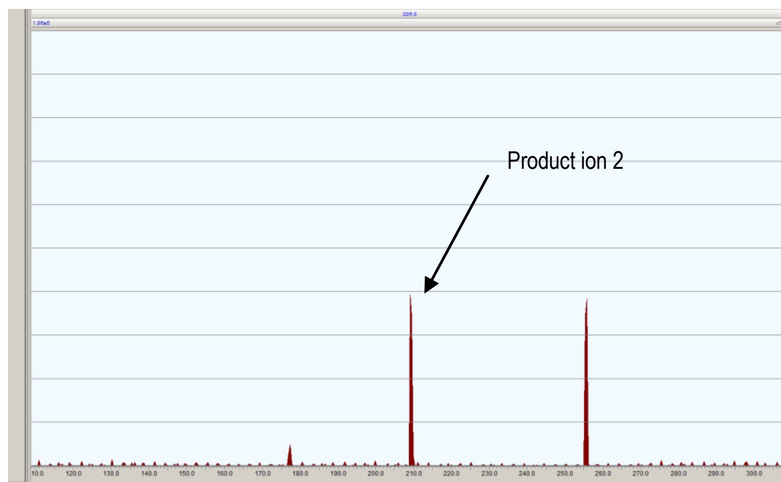


Figure 5. Product ion 2 of Ketoprofen (m/z =209)

Some NSAIDs were not detected in positive ion mode. Since with the Waters instrument it was not possible to detect compounds in negative polarity, further experiments were performed with the Agilent Technology 1290 infinity-QqQ 6460 Instrument.

6.1.2. Optimization of mass spectrometry detection

In order to achieve the optimum MS/MS conditions for NSAIDs detection, fragmentor energy and collision energies for each individual compound were optimized using the Agilent Technologies 1290 -QqQ 6460.

The optimization was performed using electrospray ionization operated in both positive and negative polarities. It is consisted of the injection of the NSAIDs mixture standard solution at 0.4 $mg\cdot L^{-1}$ in the LC- MS/MS system with the following conditions:

- Column: Phenomenex Kinetex C_{18}, (1.8µm, 100mm x 2.1mm)
- Mobile phase: water (phase A) and ACN (phase B)
- Mass spectrometry: precursor ion Scan or product ion Scan modes with positive and negative ionization.

6.1.2.1. Precursor ions

The optimization of precursor ion was performed using precursor ion SCAN mode with different fragmentor energies (70V, 90V, 110V, 130V) for each NSAIDs. Intensities of different

fragmentor energies were compared, and the voltage that provided the highest response was selected.

As an example, figure 6 shows a mass spectra obtained in the optimization process of the fragmentor energy for the precursor ion of Meloxicam (m/z=352), and the highest response was obtained with 130V.

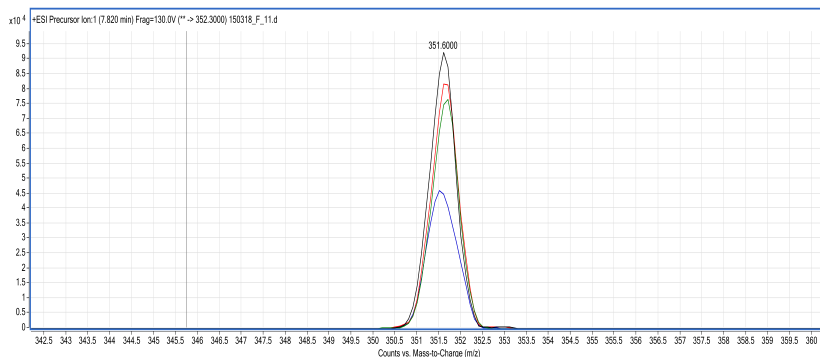


Figure 6. Mass spectra of the optimization process of the fragmentor energy for the precursor ion of Meloxicam (m/z =352) (70V-90V-110V-130V)

6.1.2.2. Product ions

After optimization of the fragmentor energy, the mass spectrometer was operated in product ion SCAN mode to optimize the detection of product ions with different collision energies (5V, 15V, 25V, 35V, 40V, 45V, 50V). Then intensities obtained for the product ions at different collision energies were compared, and the collision energy that provided the highest response was selected. Two transitions were monitored for each compound, the most sensitive transition was selected for quantification and the other was used for confirmation. Only one transition was monitored for the internal standards.

Figures 7 and 8 show mass spectra of the optimization process of the collision energy for product ions of Meloxicam (m/z=141 and m/z = 115) and the highest response was obtained with 15V and 25V

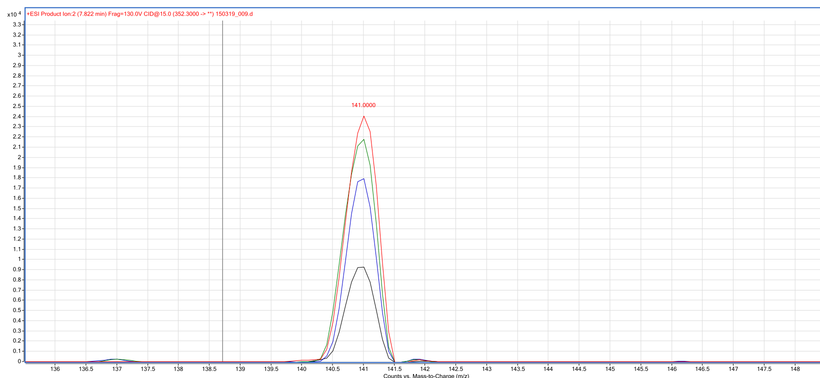


Figure 7. Mass spectra of the optimization process of the collision energy for the product ion 1 of Meloxicam (m/z = 141) (5V-15V-25V-35V)

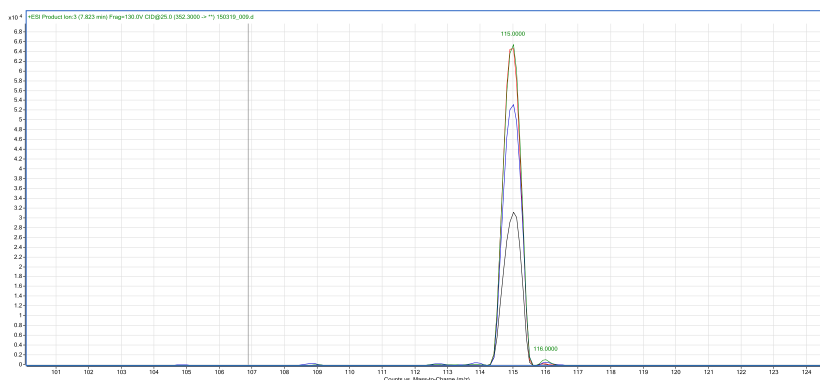


Figure 8. Mass spectra of the optimization process of the collision energy for the product ion 2 of Meloxicam (m/z =115) (5V-15V-25V-35V)

Most NSAIDs were ionized in the negative ion mode with acceptable sensitivity and low baseline noise, but for some compounds (Ibuprofen, Tolfenamic acid, Diclofenac and Mefenamic acid) only one product ion was obtained. Positive ionization was finally selected for 5-hydroxyflunixin, Ketoprofen, Meclofenamic acid and Meloxicam.

The optimized MS conditions for NSAIDs detection are summarized in Table 7.

Table 7. MS/MS parameters for NSAIDs detection

	ESI+/ESI-	Precursor ion (m/z)	Fragmentor energy (V)	Product ion 1 (m/z)	CE (V)	Product ion 2 (m/z)	CE (V)
5-hydroxyflunixin	ESI+	313	110	295	25	280	35
Diclofenac	ESI-	294	90	250	10		
Phenylbutazone	ESI-	307	130	279	15	131	15
Flunixin	ESI-	295	70	251	15	209	35
Flufenamic acid	ESI-	281	130	237	15	216	25
Ketoprofen	ESI+	255	130	105	15	77	50
Meclofenamic acid	ESI+	297	110	279	15	264	40
Mefenamic acid	ESI-	240	70	196	20		
Naproxen	ESI-	229	160	185	50	169	35
Niflumic acid	ESI-	281	130	237	15	177	35
Oxyphenbutazone	ESI-	323	130	295	15	134	15
Carprofen	ESI-	271	130	228	5	226	25
Tolfenamic acid	ESI-	260	110	216	5		
Ibuprofen	ESI-	205	110	161	1		
Meloxicam	ESI+	352	130	141	15	115	25

(a) CE: Collision energy

6.2. CHROMATOGRAPHIC SEPARATION OPTIMIZATION

The selection of column and gradient conditions affects peak shape, retention time and sensitivity. In this work, different gradient conditions and different columns were tested.

First experiments were performed with a Phenomenex Kinetex C_{18} column (1.8µm, 100mm x 2.1mm) and different gradient conditions were compared to achieve the optimum conditions for chromatography separations. The optimization consisted of the injection in the LC- MS/MS system of the standard mixture solution at 0.4 $mg \cdot L^{-1}$ and mobile phases were based on water (phase A) and ACN (phase B).

Table 8. Gradient conditions 1

Time (min)	%A	%B

0.00	80	20
2.00	80	20
10.00	45	55
10.10	80	20
14.00	80	20

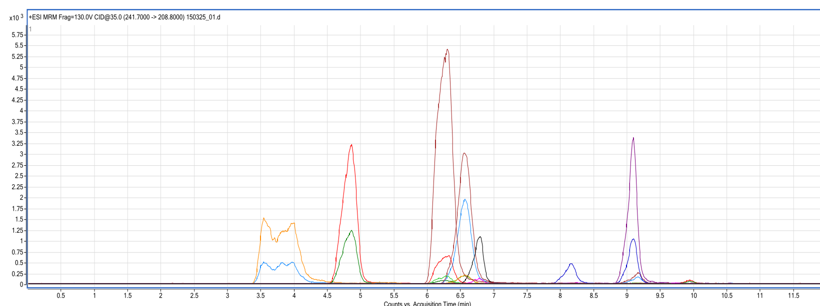


Figure 9. Chromatogram (MRM) of NSAIDs in standard solution (0.4 mg·L^{-1}) with gradient conditions 1.

Table 9. Gradient conditions 2.

Time (min)	%A	%B
0.00	80	20
2.00	80	20
12.00	45	55
12.10	80	20
20.00	80	20

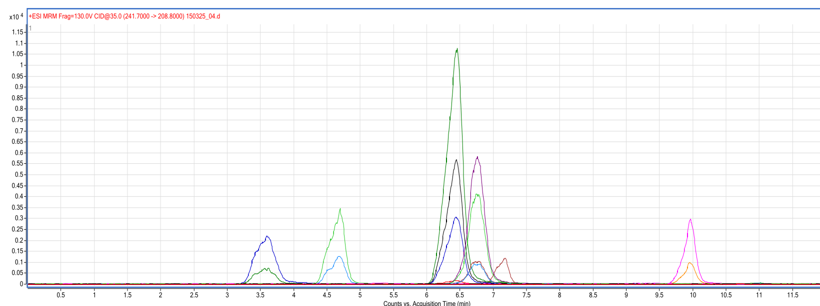


Figure 10. Chromatogram (MRM) of NSAIDs in standard solution (0.4 mg·L^{-1}) with gradient conditions 2.

With the both gradient 1 and 2, broad peaks were obtained and this reduced the sensitivity due to reduced peak height (Figure 9 and 10). Different gradient time did not improve chromatographic separation.

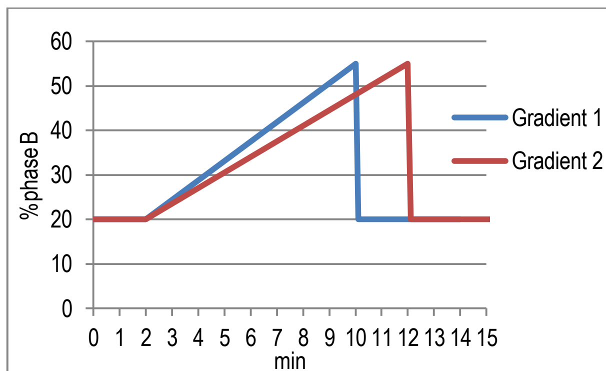


Figure11. Gradient conditions

Then, the initial percentage of organic solvent (phase B) was reduced (gradient 3), elution times were delayed. Because NSAIDs are soluble in the organic solvent, a reduction of the percentage of organic solvent improved the retention of compounds in the stationary phase, on the contrary, when the percentage of organic solvent increases, the elution of NSAIDs is speed up. Moreover, elution times were delayed due to the initial isocratic conditions.

Table 10. Gradient conditions 3

Time (min)	%A	%B
0.00	90	10
2.00	90	10
10.00	45	55
10.10	90	10
14.00	90	10

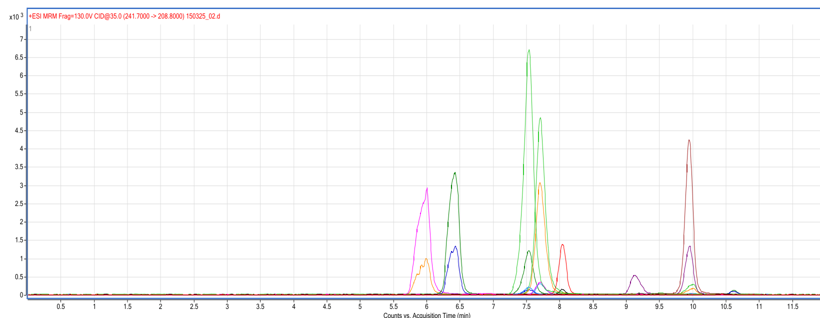


Figure 12. Chromatogram (MRM) of NSAIDs in standard solution (0.4 mg·L^{-1}) with gradient conditions 3.

Elution conditions without the initial isocratic zone were tested (Gradient 4) and retention times decreased.

Table 11. Gradient conditions 4

Time (min)	%A	%B
0.00	90	10
10.00	45	55
10.10	90	10
14.00	90	10

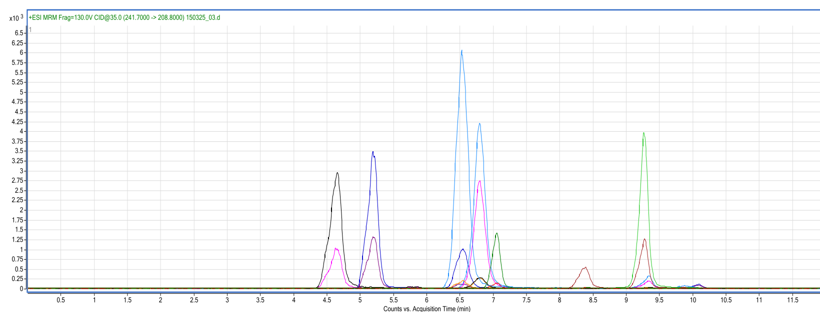


Figure 13. Chromatogram (MRM) of NSAIDs in working standard solution (0.4 mg·L^{-1}) with gradient conditions 4.

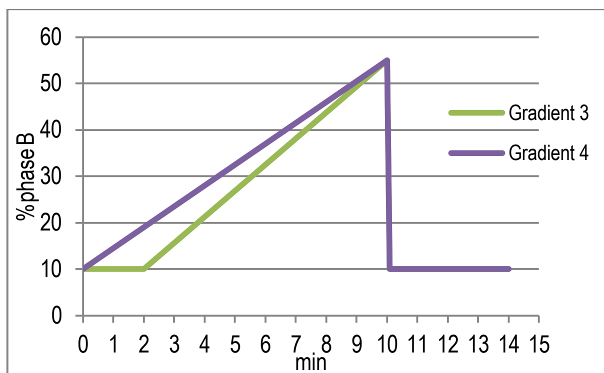


Figure 14. Gradient conditions

In all the assayed conditions, the chromatographic peaks obtained with the Kinetx C18 column were quite broad, and some experiments were performed with a Phenomenex biphenyl column (2.6µm, 100mm x 2.1mm) and peaks were narrower than those obtained previously with the kinetex C18 column (Figure 15)

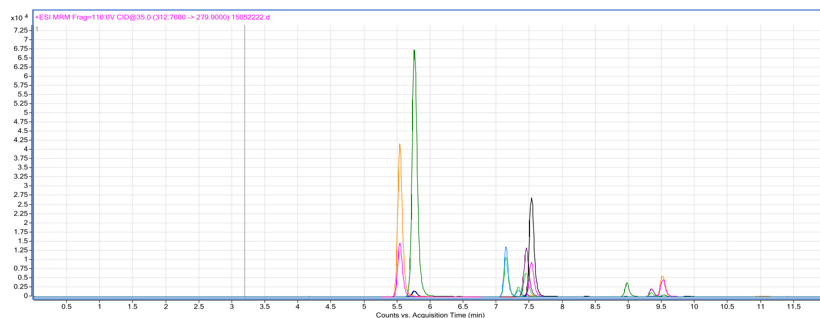


Figure 15. Chromatogram (MRM) of NSAIDs in standard solution (0.4 mg·L^{-1}) using Phenomenex Biphenyl column

With Phenomenex biphenyl column, when compared to the kinetex C_{18} column, there is a change of selectivity; the analytes interact with the stationary phase due to formation of π-π bonds between aromatic groups of NSAIDs and stationary phase. The elution order of the analytes changed, because of different column selectivity.

The narrower peaks in biphenyl column, leads to an improved sensitivity, which is advantageous for the detection of very low concentration levels of NSAIDs.

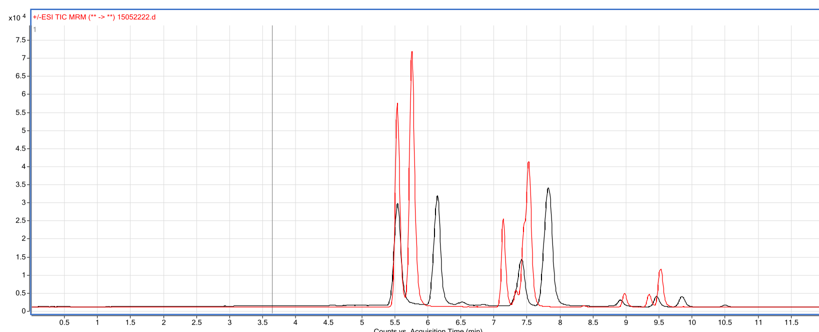


Figure16. Comparison of MRM chromatograms from Phenomenex Kinetex C18 column (black) and Phenomenex Biphenyl column (red)

When extracts from milk samples containing NSAIDs were injected into the Biphenyl column and gradient 4, based on water and ACN was applied, splitted peaks were obtained for some analytes, as can be observed in figure 17, probably due to the dissociation equilibrium. For this reason, water containing 0.1% formic acid and ACN were selected as mobile phases. In this acid conditions, the analytes had only one single form (protonated), and the peak shape improved (Figure 18). Moreover, formic acid improves the ionization of NSAIDs and baseline noise was reduced.

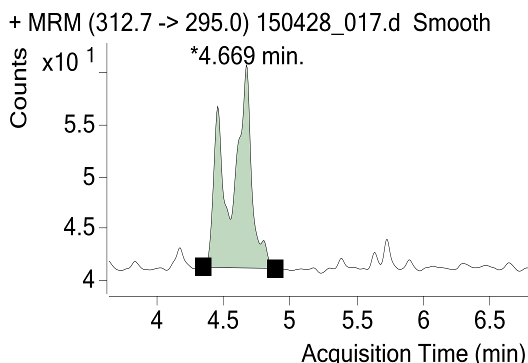


Figure 17. Ion chromatogram (transition used for quantification) of 5-hydoxyflunixin using water/ACN as mobile phase

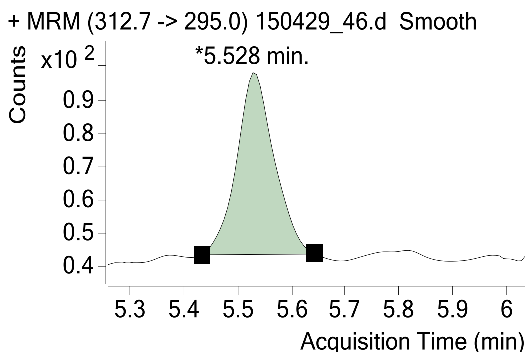


Figure 18. Ion chromatogram (transition used for quantification) of 5-hydoxyflunixin using 0.1% formic acid /ACN as mobile phase

Figure 19 shows a chromatogram of a mixture of NSAIDs (0.4 mg·L^{-1}) obtained in the selected conditions: biphenyl column, water containing 0.1% formic acid and acetonitrile as mobile phases and gradient 4.

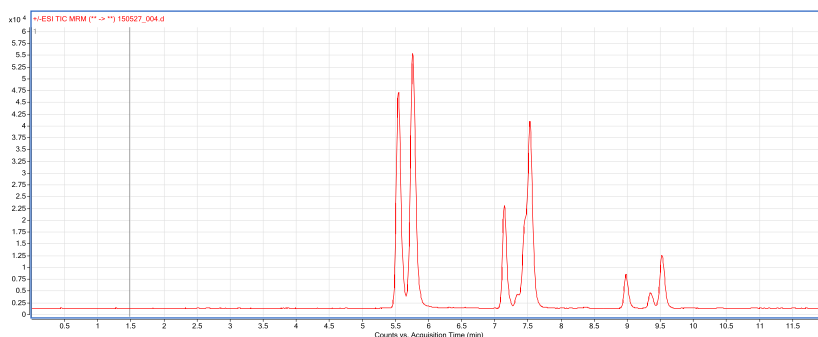


Figure 19. Chromatogram of NSAIDs in standard solution 0.4 mg·L^{-1} using 0.1% formic acid and ACN as mobile phase and Phenomenex biphenyl column

6.3. SAMPLE PREPARATION

Several methods for the analysis of NSAIDs in milk samples have been published (Table 3), and two of them were tested in this work. Briefly, the procedures assayed in this work were:

1) Extraction with ACN and clean-up by SPE using NH_2 cartridge

This procedure was published by Jedziniak.P et al, [7]:

1. 10 g of milk were weighed in a polypropylene centrifuge tube.
2. The sample was mixed (vortex) and left for 10min.
3. 10 ml ACN and 2g ammonium acetate were added and centrifuged (3000g, 10min, 10ºC)
4. The supernatant was cleaned-up on a Sep-Pak NH_2 cartridge:
   4.1. 2 g sodium sulfate were added into the cartridge (an additional layer)
   4.2. The cartridge was preconditioned with 5 ml ACN
   4.3. Analytes were eluted with 6ml of 5% formic acid in ACN.
5. The extract was evaporated to 250 µl (N_2, 40ºC)
6. The residue was re-dissolved with 250 µl water.
7. The sample was transferred into a vial with filter.

Blank samples for calibration were spiked in the concentration range between 1-10 $\mu g \cdot Kg^{-1}$.

2) Extraction with methanol and clean-up by SPE using C_8 cartridge

The method was developed by the Laboratory of Public Health Agency in Valencia [10]:

1. 2.5 g of milk were weighed in a polypropylene centrifuge tube.
2. 6 ml MeOH were added, the sample was mixed for 10min, then centrifuged (11000rpm, 5min, 10ºC).
3. The supernatant was evaporated to 2ml (N_2, 50ºC).
4. 4ml solution of HCl 0.24M and ascorbic acid 0.02M were added to the sample.
5. The extract was cleaned on C_8 cartridge :
   5.1. The cartridge was preconditioned with 4 ml MeOH and 4ml solution of HCl 0.24M and ascorbic acid 0.02M
   5.2. The extract loaded into the cartridge.
   5.3. The cartridge was washed with 4ml MeOH/water (30:70)
   5.4. The cartridge was dried under vacuum for 15min
   5.5. Analytes were eluted with 4ml ethyl acetate.
6. The eluate was evaporated to dryness (N_2, 50ºC)
7. The extract was re-dissolved with 250µl mixture of 0.1% formic acid (pH=4) and ACN (50/50).

Blank samples for calibration were spiked in the concentration range between 1-10 μg·Kg^{-1}.

The figure 20 shows responses obtained with both sample preparation methods. As it can be observed, the procedure based on extraction with ACN and clean-up by SPE using NH_2 cartridges gave higher responses.

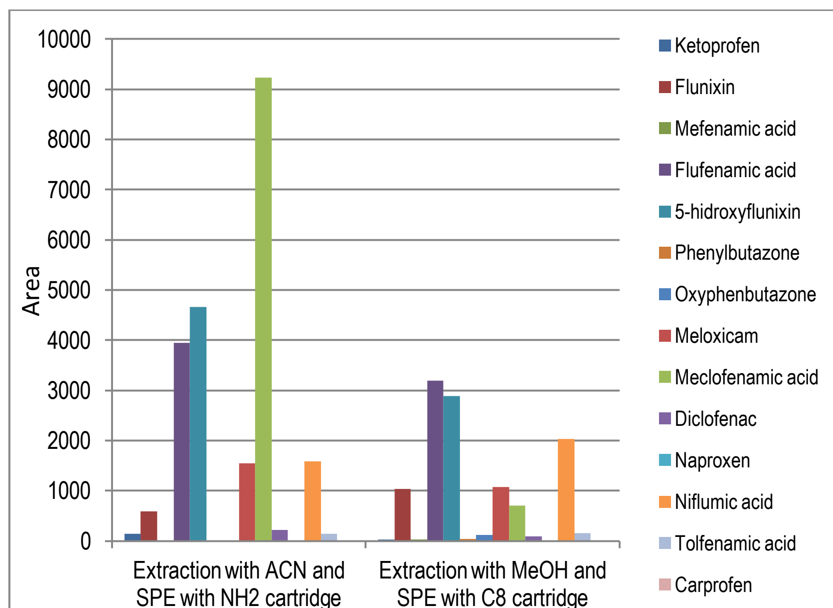


Figure 20. Areas of NSAIDs in milk fortified at 1 μg·Kg^{-1} analyzed with different solid-phase extraction cartridges.

Then, a procedure based on extraction with MeOH and clean-up by SPE using C_{18} was tested. Compared to the procedure using C_8 cartridges, similar responses were obtained. On the basis of these results, it was concluded that, among the assayed methods, the best results were achieved with the method based on acetonitrile and amine cartridges.

6.3.1. QuEChERS methodology

Because of its simplicity and convenience, the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) approach was investigated.

No references were found in the literature about the application of QuEChERS strategies to analysis of NSAIDs in milk. The procedure initially applied was the following:

1. 5 g of milk were weighed in a centrifuge tube. Next, 10 ml of 5% acetic acid in ACN, 1g of NaCl and 5g of Na_2SO_4 were added.
2. The tube was shaken for 5 minutes (vortex). Then it was centrifuged (4000 rpm, 10ºC, 10min).
3. The supernatant was transferred into a centrifuge tube containing 150mg of sorbent C_{18} and 1g $MgSO_4$ (Agilent QuEChERS kits), the tube was shaken for 4 min (vortex).
4. The mixture was centrifuged again under the same conditions.
5. The supernatant was evaporated to 250µl (N_2, 40ºC).
6. The residue was re-dissolved in 250µl of 0.1% formic acid, filtered through a 0.22µm nylon filter membrane.

Responses of QuEChERS and NH_2-SPE methods were compared (Figure 21). Meclofenamic acid and 5-hydroxyflunixin gave higher response with NH_2-SPE method, but other NSAIDs gave higher responses with the QuEChERS method. Moreover, the QuEChERS method is easier and more effective method. So the QuEChERS approach was further investigated for the sample extraction.

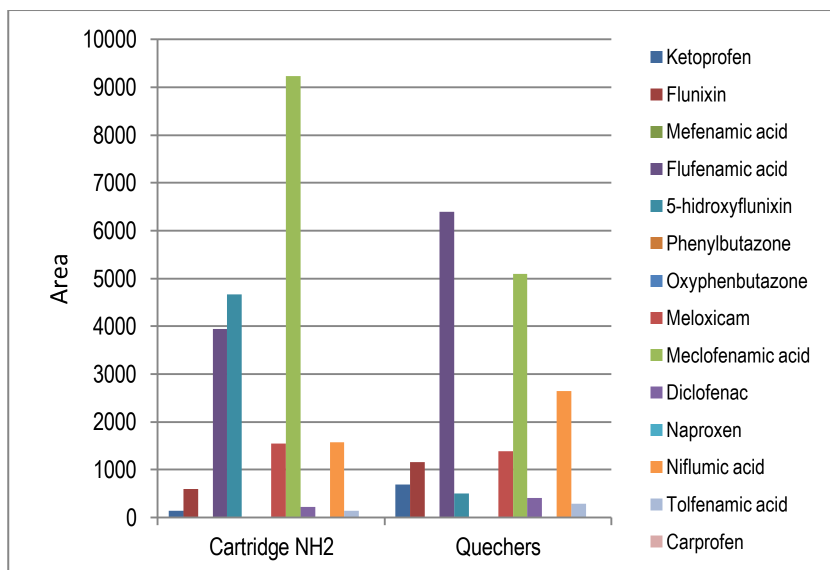


Figure 21. Area of NSAIDs in milk fortified at 1 µg·Kg^{-1} analyzed with different methods

6.3.1.1. QuEChERS methodology optimization

The QuEChERS method was optimized in this work. Initially, the clean-up was performed using commercial sorbent C_{18} and salts supplied in tubes (Agilent QuEChERS kits), but during agitation, samples were not homogenized properly because centrifuge tubes were small (1.5 cm diameter), For this reason, "in house" preparation with large centrifuge tube (2.5 cm diameter) was tested in the clean-up step, and NSAIDs responses improved.

Figure 22. Agilent QuEChERS kits (left) and laboratory centrifuge tube (right)

Then, acetonitrile extraction with no clean-up step was compared with the QuEChERS method with the clean-up procedure using sorbent C_{18} (Figure 23). It can be observed that clean-up is a critical step in the sample preparation. The procedure without clean-up detected fewer analytes and provided lower response.

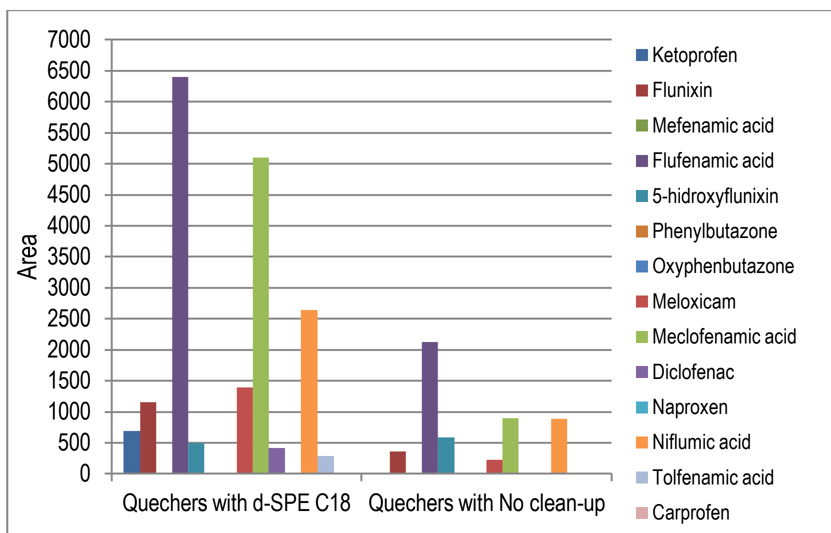


Figure 23. Area of NSAIDs in milk fortified at 1µg·Kg^{-1}

In a further step the primary secondary amines (PSA) sorbent was used in the dispersive solid-phase extraction (d-SPE) step, but in general results were worse than those obtained with C_{18} sorbent (Figure 24), except for Meclofenamic acid and Meloxicam.

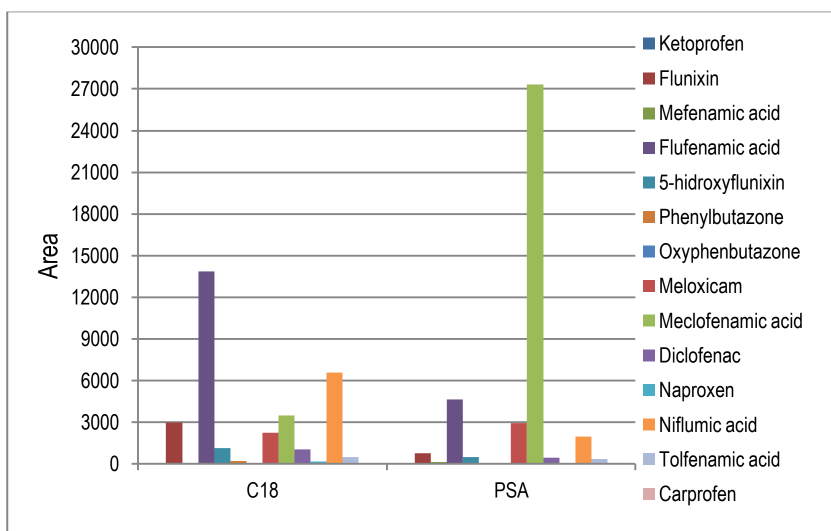


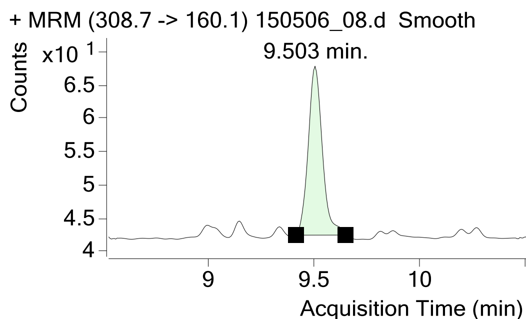
Figure 24. Area of NSAIDs in milk fortified at 2.5 µg·Kg^{-1} analyzed with Quechers method using different sorbent.

In all the assayed conditions the peak area for Phenylbutazone and Oxyphenbutazone was very low. According to some authors [1,9,10], the addition of ascorbic acid in the clean-up step avoids oxidation of Phenylbutazone and it was investigated. Extraction procedure was the following:

1. 5g of the milk were weighed in a centrifuge tube. Next, 10 ml of 5% acetic acid in ACN, 1g of NaCl and 5g of Na_2SO_4 were added.
2. The tube was shaken for 5 minutes (vortex). Then was centrifuged (3000rpm, 10ºC, 10min).
3. The supernatant was transferred to a centrifuge tube containing 150mg of sorbent C_{18} and 1g of $MgSO_4$.
4. 4ml of ascorbic acid 0.02M and HCl 0.24M were added.
5. The mixture was shaken for 4 min (vortex) and it was centrifuged again under the same conditions.
6. The supernatant was evaporated to 250 µl (N_2, 40ºC).
7. The residue was re-dissolved in 250µl of 0.1% formic acid, filtered through a 0.22µm nylon filter membrane.

The addition of ascorbic acid improved the signal of Phenylbutazone and Oxyphenbutazone (Figure 25), but the sensitivity for Diclofenac, Tolfenamic acid and Mefenamic acid decreased, but were still detected in the low µg·Kg^{-1} range. We decided to use ascorbic acid because Phenylbutzone and Oxyphenbutazone are important analytes to determine after the scandal of the horse meat in UK.

a)



b)

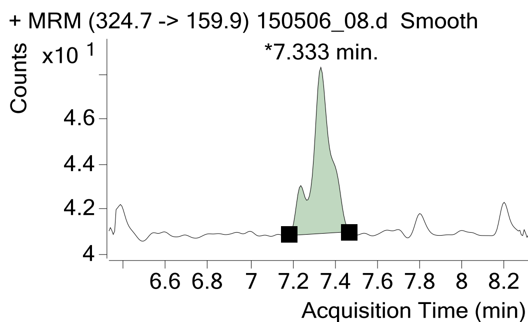
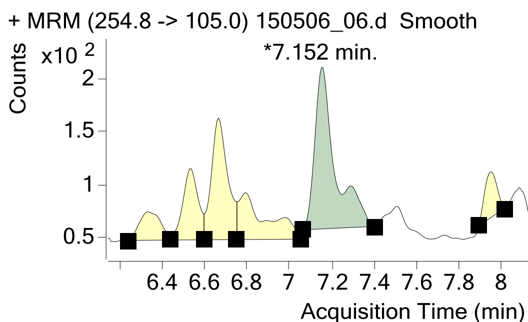


Figure 25. Ion chromatograms (transitions used for quantification) of Phenylbutazone (a) and Oxyphenbutazone (b)

According to Jedziniak.P et al [7], ammonium acetate is more suitable than NaCl in the extraction step, because NaCl can promote formation of sodium adducts and reduce sensitivity in MS detection (Figure 26.a). Therefore we tested the addition of ammonium acetate and as a result the response of NSAIDs and baseline noise improved (Figure 26.b).

a)



b)

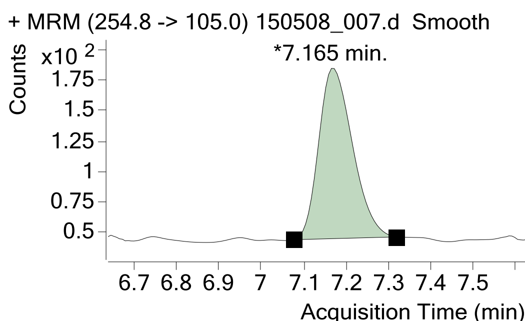


Figure 26. Ion chromatograms (transitions for quantification) of Ketoprofen using NaCl (a) and ammonium acetate (b)

Evaporation was a long step (2h approximate). The addition of anhydrous Na_2SO_4 to the organic extract allowed to reduce the evaporation time (about 1h) while similar responses were obtained.

Finally, the QuEChERS method using sorbent C_{18} was selected for the sample preparation. In the procedure, the sample is extracted with acetonitrile under the acidic conditions for protein precipitation, and ammonium acetate is added before ensured separation of organic and water phase in the extraction. Then, Na_2SO_4 is added to the organic phase to remove water. The addition of ascorbic acid before d-SPE step avoids to the oxidation of Phenylbutazone and Oxyphenbutazone.

Summarizing, the optimized QuEChERS method procedure was the following:

1. 5g of milk were weighed in a centrifuge tube. Next, 10 ml of 5% acetic acid in ACN, 1g of ammonium acetate and 5g of Na_2SO_4 were added.
2. The tube was shaken for 5 minutes (vortex). Then, it was centrifuged (3000rpm, 10ºC, 10min).
3. The supernatant was transferred to a centrifuge tube containing 150mg of sorbent C_{18} and 1g of $MgSO_4$.
4. 4ml of ascorbic acid 0.02M and HCl 0.24M and 2g of Na_2SO_4 were added.
5. The mixture was shaken for 4 minutes, then it was centrifuged again under the same conditions.
6. The supernatant was evaporated to 250µl (N_2, 40ºC).

7. The residue was re-dissolved in 250µl of 0.1% formic acid, filtered through a 0.22µm nylon filter membrane.

7. CONCLUSIONS

1. Mass spectrometry with ESI in positive and negative modes allows the sensitive detection of NSAIDs, in the low µg kg^{-1} range.
2. Phenomenex biphenyl column (2.6µm, 100mm x 2.1mm) is suitable for the separation of NSAIDs, using 0.1% formic and ACN as mobile phase.
3. The QuEChERS methodology is a suitable approach for the extraction of NSAIDs from milk samples, but optimization is required. The QuEChERS and LC-MS/MS method allows the confirmatory analysis of 5-hydroxyflunixin, Flunixin, Phenylbutazone, Flufenamic acid, Ketoprofen, Meclofenamic acid, Niflumic acid, Oxyphenbutazone, Carprofen and Meloxicam according to the legal limits.

8. REFERENCES

1. Dubreil-Chéneau.E, Pirotais.Y, Bessiral.M, Roudaut.B, Verdon.E. *J.Chromatogr.A*. 2011, 1218, 6292-6301.
2. Martindale.W, E.F.Reynolds.J.*Martindale the extra pharmacopoeia*,31st edition, 1996
3. Peng.T, Zhu.A, Zhou.Y, Hu.T, Yue.Z, Chen.D, Wang.G, Kang.J, Fan.C, Chen.Y, Jiang.H, *J. Chromatogr.B*. 2013, 933, 15-23
4. Vane.J.R. *British Journal of Rheumatology*. 1996, 35(suppl.1), 1-3
5. Commission Regulation EU No.37/2010.
6. CRL Guidance paper, 12/2007
7. Jedziniak.P, Szprengier-Juszkiewicz.T, Pietruk.K, Sledzinska.E, Zumdzki.J. *Anal. Bioanal.Chem.* 2012, 403, 2955-2963
8. Daeseleire.E, Van Pamel. E. *Development and validation of a multi-residue LC-MS/MS analysis of Non-steroidal anti-inflammatory drugs in milk and meat*. 2012, P9
9. Gallo.P, Marco.S, Vicenzo.D, Grazia.G, Mauro.E, Serpe.F, Serpe.L. *A sensitive multi-residue method for determination of NSAIDs in milk by LC/ESI-QTRAP-MS/MS.* 2012, P166
10. The laboratory of public health agency in Valencia. *Método de determinación de antinflamatorios no esteroideos (AINEs) por UPLC-MS/MS*. 2014, edición 4.
11. Wang.J, Leung.D. *Drug Test. Analysis* 2012, 4(suppl.1), 103-111
12. Commission Decision 2002/657/EC

9. ACRONYMS

NSAIDs: Non-steroidal anti-inflammatory drugs

QuEChERS: Quick, Easy, Cheap, Effective, Rugged and Safe

MRM: Multiple reaction monitoring

ESI: Electrspray ionization

d-SPE: Dispersive solid-phase extraction

KTP: ketoprofen

CPF: Carprofen

NP: Naproxen

IBU: Ibuprofen

TLF: Tolfenamic acid

MF: Mefenamic acid

MEC: Meclofenamic acid

NFL: Niflumic acid

FLUF: Flufenamic acid

FLU: Flunixin

5-FLU: 5-hydroxyflunixin

PBZ: Phenylbutazone

OPB: Oxyphenbutazone

DC: Diclofenac

MLX: Meloxicam

COX-1: Cyclooxygenase-1

COX-2: Cyclooxygenase-2

CNS: Central nervous system

MRLs: Maximum residue limits

CRLs: Community reference laboratories

ACN: acetonitrile

MeOH: methanol

IS: Internal standard solution

CV: cone voltage

CE: collision energy

PSA: primary secondary amines

SPE: solid-phase extraction