UNIVERSITAT DE BARCELONA

Determination of mercury and antimony in environmental and food matrices: development of analytical methodology and migration studies

Sergio Carneado Moreno

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Programa de doctorat

Química Analítica del Medi Ambient i la Pol·lució

Tesi Doctoral

DETERMINATION OF MERCURY AND ANTIMONY IN ENVIRONMENTAL AND FOOD MATRICES: DEVELOPMENT OF ANALYTICAL METHODOLOGY AND MIGRATION STUDIES

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FAN CONSTAR

que el present treball d'investigació que porta per títol:

Determination of mercury and antimony in environmental and food matrices: development of analytical methodology and migration studies

ha estat realitzat sota la seva direcció per Sergio Carneado Moreno per optar al Títol de Doctor per la Universitat de Barcelona.

Barcelona, desembre del 2016

Dr. José Fermín López Sánchez   Dra. Angels Sahuquillo Estrugo

A mis seres queridos

Between ingenuity and the analytic ability there exists a difference far greater, indeed, than that between the fancy and the imagination, but of a character very strictly analogous. It will be found, in fact, that the ingenious are always fanciful, and the truly imaginative never otherwise than analytic.

The murders in the rue morgue, Selected tales

Edgar Allan Poe

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ACRONYMS

AAS: Espectroscòpia d'Absorció Atòmica / Atomic Absorption Spectroscopy

ACN: Acetonitril / Acetonitrile

AED: Detector d'Emissió Atòmica / Atomic Emission Detector

AFS: Espectroscòpia de Fluorescència Atòmica / Atomic Fluorescence Spectroscopy

APDC: Pirrolidinditiocarbamat d'amoni / Ammonium pyrrolidinedithiocarbamate

ASV: Voltametria de Redissolució Anòdica / Anodic Stripping Voltammetry

CBT: 1,3-bis (2-cianobenzè) triazè / 1,3-bis (2-cyanobenzene) triazene

CE: Electroforesi Capil·lar / Capilar Electroforesis

C_{LD}: Concentració en el Límit de Detecció / Concentration in the Limit of Detection.

C_{LQ}: Concentració en el Límit de Quantificació / Concentration in the Limit of Quantification.

CONTAM: Comissió Tècnica sobre els Contaminants a la Cadena Alimentària / Scientific Panel on Contaminants in the Food Chain.

CRM: Material de Referència Certificat / Certified Reference Materials

CSV: Voltametria de Redissolució Catòdica / Cathodic Stripping Voltammetry

CV-AAS: Generació de Vapor Fred amb Espectroscòpia d'Absorció Atòmica / Cold Vapour Atomic Absorption Spectroscopy

CV-AFS: Generació de Vapor Fred amb Espectroscòpia de Fluorescència Atòmica / Cold Vapour Atomic Fluorescence Spectroscopy

DART: Anàlisi Directe en Temps Real / Direct Analysis on Real Time.

DDTC: Dietilditiocarbamat / Diethyldithiocarbamate

DESI: Desorció per Electrosprai / Desorption Electrospray

DESI-HRMS: Desorció per Electrosprai acoblada a Espectrometria de Masses d'Alta Resolució / Desorption Electrospray coupled to High Resolution Mass Spectrometry.

DMSb: Dimetilantimoni / Dimethylantimony

DPP: Polarografia Diferencial d'Impulsos / Diferential Pulse Polarographic

EC: Comissió Europea / European Comission

EC: Conductivitat elèctrica / Electric conductivity

EDC: Detector de Captura d'Electrons / Electron Capture Detector

EDTA: àcid etilendiamonotetraacètic / Etilenediaminetetraacetic acid

EFSA: Autoritat Europea de Seguretat Alimentària / European Food Safety Authority

ESI-MS: Espectrometria de Masses amb Ionització per Electrosprai / Electrospray Ionisation – Mass Spectrometry

ESI-MS: Espectrometria de Masses amb Ionització per Electrosprai / Mass spectrometry with electrospray ionization.

ETAAS: Espectroscòpia de Absorción Atómica Electrotérmica o amb Forn de Grafit / Electrothermal or Grafite Furnace Atomic Absorption Spectroscopy

$EtHg^+$: Monoetilmercuri o etilmercuri / Monoethylmercury or ehtylmercury

EtOH: Etanol / Ethanol

EU: Unió Europea / European Union

FAO: Organització de les Nacions Unides per a l'Agricultura i l'Alimentació / Food and Agriculture Organization of the United Nations

GC: Cromatografia de Gasos / Gas Chromatography

HDPE: Polietilè d'alta densitat / High-density polyethylene

HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol

HG-AAS: Generació d'Hidrurs amb Espectroscòpia d'Absorció Atòmica / Hydride Generation Atomic Absorption Spectroscopy

HG-AFS: Generació d'Hidrurs amb Espectroscòpia de Fluorescència Atòmica / Hydride Generation Atomic Fluorescence Spectroscopy

HRMS: Espectrometria de Masses d'Alta Resolució / High Resolution Mass Spectrometry

IARC: Agència Internacional per a la Investigació del Càncer / International Agency for Research on Cancer

ICP-AES: Plasma Acoblat Inductivament amb Espectroscòpia d'Emissió Atòmica / Inductive Coupled Plasma with Atomic Emision Spectroscopy

ICP-MS: Plasma Acoblat Inductivament amb Espectrometria de Masses / Inductive Coupled Plasma with Mass Spectrometry

ICP-OES: Plasma Acoblat Inductivament amb Espectroscòpia d’Emissió Òptica / Inductive Coupled Plasma with Optic Emision Spectroscopy

id: diàmetre intern / internal diameter.

ID: Dilució Isotòpica / Isotopic Dilution

iHg: Mercuri inorgànic / Inorganic mercury

ISE: Elèctrode d’ions sel·lectiu / Ion Selective Electrode

IUPAC: Unió Internacional de Química Pura i Aplicada / International Union of Pure and Applied Chemistry.

JECFA: Comitè Mixt FAO/WHO d'Experts en Additius Alimentaris / Joint FAO/WHO Expert Committee on Food Additives

LC: Cromatografia de Líquids / Liquid Chromatography

LC-ESI-MS: Cromatografia de Líquidds acoblada a Espectrometria de Masses amb Ionització per Electrosprai / Liquid Chromatography coupled to Mass spectrometer with Electrospray Ionisation.

LC-HG-AFS: Cromatografia de Líquids acoblada a Generació d’Hidrurs amb Espectroscòpia de Fluorescència Atòmica / Liquid chromatography coupled to hydride generation – atomic fluorescence spectroscopy.

LC-ICP-MS: Cromatografia de Líquids acoblada a Plasma Acoblat Inductivament amb Espectrometria de Masses / Liquid Chromatography coupled to Inductive Coupled Plasma with Mass Spectrometry

LC-MS/MS: Cromatografia de Líquids acoblada a Espectrometria de Masses en Tàndem / Liquid Chromatography coupled to Tandem Mass Spectrometry

LC-UV-CV-AFS: Cromatografia de Líquids acoblada a Irradiació Ultraviolada i Vapor Fred amb Espectroscòpia de Fluorescència Atòmica / Liquid chromatography coupled to Ultraviolet irradiation and cold vapour – atomic fluorescence spectroscopy

LD: Límit de Detecció / Limit of Detection.

LDPE: Polietilè de baixa densitat / Low-density polyethylene

LLE: Extracció Líquid-Líquid / Liquid-Liquid Extraction

LLME: Microextracció Líquid-Líquid / Liquid-Liquid Microextraction

LOL: Límit de linealitat / Limit of Linearity

LPME: Microextracció en Fase Líquida / Liquid Phase Microextraction

LQ: Límit de Quantificació / Limit of Quantification.

m/z: Relació massa càrrega / mass to charge ratio

MAPA: Ministeri d’Agricultura, Ramaderia i Abastiment / Ministry of Agriculture, Livestock and Food Supply

MBI: 2-mercaptobenzimidazol / 2-mercaptobenzimidazole

MCL: Nivell de Concentració Màxim / Maximum Concentration Level

MeHg$^+$: Monometilmercuri o metilmercuri / Monomethylmercury or methylmercury

MeOH: Metanol / Methanol

MPD: Emissió de Plasma induït per Microones / Microwave-induced Plasma Emission

MPMP: 2-[(2-mercaptofenilimino)metil] fenol / 2-[(2-mercaptophenylimino)methyl] phenol

MS: Espectrometria de Masses / Mass Spectrometry

MSb: Metilantimoni / Methylantimony

NAA: Anàlisi d’Activació de Neutrons / Neutron Activation Analysis

od: diàmetre extern / outside diameter.

ODS: Octadecilsilà / Octadecylsilane

OML: límit global de migració / overall migration limit

PC: Component Principal / Principal Component

PC: Policarbonat / Polycarbonate

PCA: Anàlisi de Components Principals / Principal Component Analysis

PE: Polietilè / Polyethylene

PEEK: Poliéter éter cetona / Polyether ether ketone

PET: polietilentereftalat / polyethylene terephthalate

PF: Factor de Preconcentració / Preconcentration factor

$PhHg^+$: Monofenilmercuri o fenilmercuri / Monophenylmercury or phenylmercury

PP: Polipropilè / Polypropilene

PRP: Fase Inversa Polimèrica / Polymeric Reversed Phase

PS: Poliestirè / Polystyrene

PSTH-cpg: [1,5-bis(2-piridil)-3-sulfofenil metilè] tiocarbonohidrazida immobilitzada en vidre de porus controlat amb aminopropil / [1,5-bis(2-pyridyl)-3-sulfophenyl methylene] thiocarbonohydrazide immobilised on aminopropyl-controlled pore glass

PTFE: Politetrafluoroetilè / Polytetrafluoroethylene

PVC: Clorur de polivinil / Polyvinyl chloride

QM: quantitat máxima permesa / maximum permitted quantity

R: Recuperació / Recovery.

R^2: Coeficient de determinació / Determination coefficient

RP: Fase inversa / Reversed Phase

rpm: revolucions per minut / revolutions per minute

rps: revolucions per segon / revolutions per second

RSD: Desviació Estàndard Relativa / Relative standard deviation.

RSD_r: Repetitivitat / Repeatability

RSD_R: Reproductibilitat / Reproducibility.

S: Pendent / Slope.

S_B: Senyal del Blanc / Blank Signal.

SIMS: Espectrometria de Masses de Ions Secundaris / Secondary Ion Mass Spectrometry

S_{LD}: Senyal en el Límit de Detecció / Signal of the Limit of Detection.

S_{LQ}: Senyal en el Límit de Quantificació / Signal of the Limit of Quantification.

SML: límit de migració específica / specific migration limit

SPE: Extracció en Fase Sòlida / Solid Phase Extraction

SPME: Microextracció en Fase Sòlida / Sòlid Phase Microextraction

SSI: Ionització amb Esprai Sònic / Sonic spray ionisation.

TIC: Cromatograma Total d'Ions / Total Ion Chromatogram

TMAOH: Hidròxid de tetrametilamoni / Tetramethylammonium hydroxide

$TMSb(OH)_2$: Hidròxid de trimetilantimoni / Trimethylantimony hydroxide

TMSb: Trimetilantimoni / Trimethylantimony

$TMSbBr_2$: bromur de trimetilantimoni / Trimethylantimony bromide

$TMSbCl_2$: clorur de trimetilantimoni / Trimethylantimony chloride

TMSbO: Òxid de trimetilantimoni / Trimethylantimony oxide

TPPP: 5,10,15,20-tetra-(4-fenoxifenil)porfirin / 5,10,15,20-tetra-(4-phenoxyphenyl)porphyrin

TWI: Ingesta Setmanal Tolerable / Tolerable weekly intake

USEPA: Agència de Protecció Mediambiental del Estats Units / United States Environmental Protection Agency

UV: Absorció molecular Ultraviolada-Visible / Ultraviolet-Visible Spectroscopy

V-EASI-MS: Venturi easy ambient sonic-spray ionization mass spectrometry.

WHO: Organització Mundial de la Salut / World Health Organisation

XAFS: Absorció de Raigs X d'Estructura Fina / X-ray Absorption Fine Structure

XANES: Absorció de Raigs X "Near-Edge Structure" / X-ray Absorption Near-Edge Structure

XRF: Fluorescència de Raigs X / X-ray Fluorescence

σ_B: Desviació Estàndard del Senyal del Blanc / Standard Deviation of the Blank Signal.

ABSTRACT

Food Safety is an issue of vital importance in the world. It must be sustained so as to avoid pollution episodes in food, which subsequently could cause potential risks to human, animal and environmental health. Due to the high importance to know the presence, behaviour and effects of the contaminants, the main approach of this thesis consist of the study of two particular food contamination cases: primary contamination of the aquatic media and the corresponding seafood with mercury, and direct contamination of beverages due to polyethylene terephthalate containers (PET) with antimony.

To carry out these studies, the work was structured in three objectives: development of analytical methodology for the determination of mercury and antimony, the application of the developed methodology in real samples and the assessment of the potential migration of antimony in PET bottled beverages.

In the first part of the thesis, the development of methods for the determination of total mercury and antimony was carried out by atomic fluorescence spectroscopy (AFS), with hydride generation in the case of antimony (HG) and with cold vapour generation in the case of mercury (CV); and by inductively coupled plasma with mass spectrometry (ICP-MS). For speciation, the liquid chromatography (LC) was coupled to these techniques. Regarding antimony determination, a method for the separation of the inorganic species (Sb(V) and Sb(III)) was developed using the coupled systems LC-HG-AFS and LC-ICP-MS, whereas for mercury, a method for the separation of two species (methylmercury, $MeHg^+$, and inorganic mercury, Hg(II)) or three (ethylmercury, $EtHg^+$, together with the other two species) was assessed by LC-UV-CV-AFS. These methods, together with the corresponding techniques for the determination of total content, were validated by calculating several quality parameters, including limits of detection, quantification and linearity.

Additionally, since the studied matrices present low concentrations of mercury and antimony, online preconcentration methods for speciation were developed using the AFS detection systems aforementioned. The developments were performed systematically studying several variables such as the precolumn length, complexing agent type and concentration, pH and breakthrough volume, among others. In this way, the optimum conditions of analysis were established with a minimal sample pretreatment, which consists of the addition of 2-mercaptoethanol as complexing agent, and a low sample volume (20 mL for antimony and 4 mL for mercury preconcentration). These conditions provide good recoveries, preconcentration factors and reproducibility. The methods have been applied to tap water and PET bottled water samples, obtaining preconcentration factors approximately of 10 and 70 for Sb(V) and Sb(III), species, respectively, and 30 for the three mercury species.

In the second part of the thesis, the aforementioned methods were applied for the determination of antimony in PET bottled waters, juices and spirits, as well as the same PET bottles, and for the determination of mercury in seafood. For each matrix, different approximations were used for the determination of antimony and mercury, such as minor changes in the analytical methodology. In addition, different strategies were used for the interpretation of the results obtained, such as the comparison with the literature, the application of chemometric tools and the use of other techniques, such as the mass spectrometry, in order to elucidate the structure of the compounds.

Regarding antimony in beverages, the total concentrations found in the aforementioned matrices did not exceed the maximum limit permitted by the European Union in waters (5 µg L^{-1}). Speciation analyses, with the help of mass spectrometry, showed that the predominant form in waters corresponds to non-complexed Sb(V), for juices is a complex of Sb(III) with citrate and for spirits is and organic complex of Sb(V). In the case of plastic, although elevated concentrations of antimony in PET bottles were found (200 – 300 mg kg^{-1}), the extractable content is much lower (10 – 25 mg kg^{-1}), and both Sb(V) and Sb(III) were found, but the structure in the PET surface is still unknown. Regarding the determination of mercury in seafood, concentrations higher than the limit (1 mg kg^{-1}) were found in four predator fish, being $MeHg^+$ species the predominant form.

In the third part of the thesis, antimony migration studies in waters, juices and spirits were carried out in order to understand the mechanisms of migration from PET to the beverage, and which variables potentially affect the antimony release. These studies were performed at different times and temperatures. The results obtained pointed out that high temperatures (60ºC) enhance the migration. Water samples exceeded the maximum limit after 30 days of storage and spirits after a week, whereas juices did not exceed the limit. The differences among matrices and tendencies observed throughout time showed that, apart from the temperature, migration could depend on other variables, such as matrix or PET bottle type. To study this, a crossed migration experiment at elevated temperature was performed with waters and juices, in which the matrices were stored in bottles different from the original ones. This experiment demonstrated that antimony migration is also dependent on the PET characteristics and beverage matrix.

As a result of the research carried out in this thesis, three articles have been published in peer-reviewed scientific journals, which are attached in the annex of the thesis. Additionally, four more articles are in preparation, whose abstract is also attached in the annex.

The results obtained in this thesis, especially the information on Sb and Hg species present in food matrices and migration experiments; contribute to improve the knowledge of the behaviour of these elements for future legislation development and for the proposal of threshold limits for food safety.

RESUM

La seguretat alimentària és un tema de vital importància en el món sencer. Aquesta s'ha de preservar per tal d'evitar episodis de contaminació en els aliments, els quals, a la vegada, poden provocar riscos a la salut humana, animal i mediambiental. Donada la importància de conèixer l'existència, el comportament i els efectes que els contaminats poden tenir, la present tesi té com a principal enfoc l'estudi de dos casos particulars de contaminació dels aliments: la contaminació primària del medi aquàtic i dels peixos que hi viuen amb mercuri, i la contaminació directa de begudes deguda als envasos de polietilentereftalat (PET) amb antimoni.

Per tal de realitzar aquests estudis, el treball s'ha estructurat en tres grans objectius: desenvolupament de metodologia analítica per a la determinació de mercuri i antimoni, aplicació de la metodologia desenvolupada a mostres reals i la realització d'estudis per avaluar la potencial migració d'antimoni en diferents begudes embotellades en PET.

En la primera part de la tesi, s'ha dut a terme el desenvolupament de mètodes per la determinació de mercuri i antimoni mitjançant espectroscòpia de fluorescència atòmica (AFS), amb prèvia generació d'hidrurs pel cas de l'antimoni (HG) i amb prèvia generació de vapor fred pel cas del mercuri (CV), i plasma acoblat inductivament amb espectrometria de masses (ICP-MS) per conèixer el contingut total. Per l'especiació, s'ha acoblat la cromatografia de líquids (LC) a aquestes tècniques. Pel que fa a l'antimoni, s'ha creat un mètode per a la separació de les espècies inorgàniques (Sb(V) i Sb(III)) mitjançant els acoblaments LC-HG-AFS i LC-ICP-MS, mentre que pel mercuri s'ha avaluat un mètode per a la separació de dues espècies de mercuri (metilmercuri, $MeHg^+$ i mercuri inorgànic, Hg(II)) o de tres (etilmercuri, $EtHg^+$ junt amb les altres dues) mitjançant LC-UV-CV-AFS. Aquests mètodes, junt amb les corresponents tècniques per la determinació del contingut total, s'han validat mitjançant el càlcul de diversos paràmetres de qualitat, com els límits de detecció, quantificació i linealitat.

Addicionalment, donat que les matrius a estudiar presenten concentracions baixes de mercuri i antimoni, s'han desenvolupat mètodes de preconcentració online per a la seva especiació utilitzant els sistemes de AFS abans esmentat com a detecció. Aquests s'han realitzat de manera sistemàtica estudiant diferents variables, com ara la longitud de precolumna, el tipus i concentració d'agent complexant, el pH, i el volum de ruptura, entre d'altres. D'aquesta manera, s'ha aconseguit establir unes condicions òptimes d'anàlisi amb un mínim pretractament de mostra, que consisteix en l'adició de 2-mercaptoetanol com a agent complexant i un volum baix de mostra (20 mL pel cas de l'antimoni i 4 mL pel cas del mercuri). Aquestes condicions proporcionen bones recuperacions, factors de preconcentració i reproductibilitat. Els mètodes s'han aplicat a mostres d'aigua d'aixeta i envasada i s'han obtingut uns factors de preconcentració aproximadament de 10 i 70 per a les espècies d'antimoni Sb(V) i Sb(III), respectivament, i de 30 per a les tres espècies de mercuri.

A la segona part de la tesi, els mètodes esmentats s'han aplicat per determinar antimoni en aigües, sucs i licors envasats en PET, així com en el propi plàstic, i mercuri en peixos. Per cada matriu, s'han utilitzat diferents aproximacions per la determinació d'antimoni i mercuri, com ara la modificació lleugera d'algun punt dels mètodes d'anàlisi. També s'han fet servir diferents estratègies per a la interpretació dels resultats obtinguts, com la comparació amb les dades bibliogràfiques, l'aplicació d'eines quimiomètriques i la utilització d'altres tècniques, com l'espectrometria de masses, per tal d'elucidar l'estructura dels compostos.

En quant a l'antimoni en begudes, les concentracions totals trobades en les matrius esmentades no superen el límit màxim permès per la Unió Europea (5 µg L^{-1}) en aigües. L'especiació, amb l'ajut de l'espectrometria de masses, va posar de manifest que la forma predominant en aigua correspon a Sb(V) sense complexar, pels sucs és un complex de Sb(III) amb citrat i pels licors un complex orgànic de Sb(V). En el cas del plàstic, tot i que s'han trobat concentracions elevades d'antimoni total en les ampolles de PET (200 – 300 mg kg^{-1}), la quantitat extraïble és molt inferior, (10 – 25 µg kg^{-1}) i es pot trobar en qualsevol de les dues espècies inorgàniques, desconeixent la seva estructura en la superfície. Pel que fa a la determinació de mercuri en peixos, es van trobar concentracions superiors a les permeses (1 mg kg^{-1}) en quatre mostres de peixos depredadors, sent el l'espècie de $MeHg^+$ la forma predominant.

A la tercera part de la tesi, s'han realitzat estudis de migració d'antimoni en aigües, sucs i licors per tal de conèixer els seus mecanismes del PET cap a la beguda i quines són les variables que més potencien el seu alliberament. Aquests s'han dut a terme a diferents temps i temperatures. Els resultats van demostrar per una banda que les temperatures altes (60ºC) afavoreixen la migració. Les aigües van superar els límits permesos al cap de 30 dies d'emmagatzematge i els licors al cap d'una setmana, mentre que els sucs no el van superar. Les diferències entre matrius i tendències observades al llarg del temps van demostrar que, a més de la temperatura, la migració pot dependre depèn d'altres variables, com ara la matriu o el tipus de PET de l'ampolla. Per estudiar aquest fet, es va realitzar un experiment de migració creuada a elevada temperatura amb aigües i sucs, en el qual es van envasar les matrius en ampolles diferents de les originals. Aquest experiment va demostrar que la migració d'antimoni també depèn de les característiques del PET així com de la matriu de la beguda.

Com a resultat de la recerca duta a terme en la present tesi, s'han publicat tres articles en revistes científiques, els quals es troben a l'annex de la tesi. Addicionalment, quatre articles més es troben en preparació, el resum dels quals també es troben a l'annex.

Els resultat obtinguts en aquesta tesi, especialment la informació relativa a les espècies de Sb i Hg presents en matrius alimentàries i els experiments de migració, contribueixen a millorar el coneixement del comportament d'aquests elements per al desenvolupament de legislació futura i per proposar nivells màxims per garantir la seguretat alimentària.

PREFACE

La química dels elements traça ha estat un tema de gran interès des de fa molts anys. Els elements generalment són persistents al medi ambient i el principal tema d'interès és l'estudi de la seva presència i transport als diferents compartiments mediambientals, i la seva possible biodisponibilitat i toxicitat per la biota.

Durant els últims anys, una eina indispensable per a l'estudi d'aquests temes ha estat l'especiació. El concepte d'especiació elemental, interpretat com a reconeixement de l'existència de diverses formes d'un metall, data de la dècada dels anys cinquanta, quan un grup d'investigadors geoquímics va separar les fraccions del que en aquell temps s'anomenava "metall dissolt" i "metall particulat" amb una simple filtració a través d'una membrana de 0,45 mm [Rodriguez-Baena, 2001].

Uns anys més tard, al 1980 Florence i Batley van definir l'especiació com la determinació de les concentracions de les diferents formes o espècies químiques en les que es troba un determinat element en una mostra. Concretament, el terme "espècie química" fa referència a compostos químics definits mentre que el terme "forma" es fa servir de manera més flexible [Florence et al., 1980].

L'any 1988, Buffle va introduir el terme "grup de compostos homòlegs", que es defineixen com a compostos amb funcions o comportaments mediambientals i biològics similars [Buffle et al., 1988]. Tres anys més tard, Ure y Davidson van diferenciar tres tipus d'especiació: la química, l'operacional i la funcional. L'especiació operacional va ser definida com la caracterització de grups formats per compostos diferents entre sí, però que reaccionen de forma similar a una operació metodològica determinada. En quant al terme d'especiació funcional, la definició diu que aquests grups de diferents compostos provoquen una resposta biològica idèntica en un organisme [Ure et al., 1991].

L'any 2000 la "International Union of Pure and Applied Chemistry" (IUPAC) va limitar el concepte d'especiació a "especiació química", i va substituir les definicions d'especiació operacional i funcional pel terme "fraccionament". Aquest es va definir com el procés de classificació d'un analit o d'un grup d'analits en una mostra en base a les seves propietats físiques o químiques [Templeton et al., 2000]. Cinc anys més tard, la IUPAC va publicar les definicions avui dia acceptades en quant al camp de l'especiació:

- **Espècie química**: Forma específica d'un element definida per la seva composició isotòpica, estat electrònic o d'oxidació i/o estructura molecular.
- **Anàlisi d'especiació**: activitat analítica implícita en la identificació i/o quantificació d'una o més espècies químiques individuals en una mostra.
- **Especiació d'un element**: Distribució d'un element entre les diferents espècies químiques definides en una mostra o matriu en particular.

Addicionalment, durant aquest mateix any, van Leeuwen va introduir un nou concepte: el d'especiació dinàmica. Aquest té en compte la labilitat de les espècies químiques en relació amb els aspectes funcionals, com per exemple, la biodisponibilitat de les espècies [Van Leeuwen et al., 2005].

A dia d'avui, en el camp de l'anàlisi d'especiació s'han descrit estudis on les nanopartícules es consideren com una espècie química [Laborda, 2014].

A la present tesi, s'ha estudiat concretament l'especiació d'antimoni i mercuri en matrius ambientals, alimentàries i biològiques amb l'objectiu d'identificar i quantificar les espècies d'aquests elements en les matrius estudiades.

La present tesi doctoral s'ha estructurat en 7 capítols. El capítol 1 correspon a la Introducció de la tesi, la qual resumeix informació general relativa a la seguretat alimentària i els tipus de contaminació en aliments, arribant així a dos casos particulars: la contaminació amb mercuri de compartiments mediambientals afectats i la contaminació amb antimoni deguda a l'envasament d'aliments. També s'ha resumit informació més específica relativa al mercuri i a l'antimoni. El capítol finalitza presentant els principals objectius de la tesi.

Seguidament, en el capítol 2 es resumeixen els materials i mètodes utilitzats a la tesi doctoral. Els resultats obtinguts en aquesta tesi junt amb la seva discussió s'han presentat en els capítols 3, 4 i 5. Al capítol 3 es descriu el desenvolupament de metodologia analítica per a la determinació d'espècies d'antimoni i mercuri. El capítol 4 tracta de la determinació de mercuri i antimoni en begudes i aliments, aplicant els mètodes descrits i desenvolupats al capítol previ. Al capítol 5 es resumeixen altres aplicacions corresponents a estudis de migració d'antimoni.

Finalment, les conclusions de la tesi es recullen al capítol 6. Al final es troben les referències bibliogràfiques consultades per la redacció de la tesi i un apartat d'annexos, en el qual es recullen els articles publicats, i els que estan enviats a una revista o en procés de redacció.

Cal esmentar que aquesta Tesi opta a la Menció Internacional de Doctoral per haver realitzat una estada de 4 mesos a la Facultat de Química de la Universitat de Creta, a Heraklion (Πανεπιστήμιο Κρήτησ, Ηράκλειο) sota la tutela del Professor Spiros A. Pergantis. La temàtica de l'estada efectuada s'emmarca perfectament dins el projecte de la present tesi, ja que amplia els estudis realitzats a la Universitat de Barcelona. Els apartats de la tesi corresponents amb el treball efectuat durant l'estada són la secció 4.3 del capítol 4 i la secció 5.3 del capítol 5.

Chapter I

INTRODUCTION

1.1. SEGURETAT ALIMENTÀRIA

La seguretat dels aliments, juntament amb la nutrició, és un tema de vital importància en el món sencer. Els ciutadans d'avui en dia volen conèixer cada vegada més els productes que mengen i beuen, no solament per estar segurs de la seva composició i propietats, sinó també per sentir-se segurs de qualsevol risc per la salut [Pla de seguretat alimentària de Catalunya, 2015]. Segons la Organització de les Nacions Unides per a l'Agricultura i l'Alimentació (FAO), la seguretat alimentària s'assoleix quan totes les persones tenen, en tot moment, accés físic, social i econòmic als aliments suficients, innocus i nutritius que satisfacin les seves necessitats energètiques diàries i les preferències alimentàries per a portar una vida activa i sana. Així doncs, la seguretat alimentària compren els quatre següents aspectes importants, els quals s'han de donar simultàniament per tal de garantir-la:

1) La disponibilitat física dels aliments, ja que és funció del nivell de la seva producció, dels nivells d'existències i del comerç net.
2) L'accés econòmic i físic als aliments, el qual depèn del poder adquisitiu i les infraestructures disponibles i que, a més, no ha d'estar restringit per causes socials.
3) La utilització dels aliments, que es basa en la combinació de la ingesta i el bon ús biològic d'aquest, amb el que s'obté la condició nutricional dels individus.
4) L'estabilitat en el temps dels tres aspectes anteriors.

A partir de la seguretat alimentària, és útil definir i classificar dues categories d'inseguretat alimentària: la crònica i la transitòria. La primera és el resultat de llargs períodes de pobresa, de manera que les persones no tenen capacitat de satisfer les seves necessitats alimentàries durant un llarg període de temps. Es pot superar mitjançant mesures correctives a llarg termini. La segona és resultat d'un impacte a curt termini en la disponibilitat i l'accés als aliments, de manera que es dóna una caiguda sobtada de la capacitat de produir o accedir a una quantitat d'aliments suficient per mantenir un bon estat nutricional. Aquesta inseguretat és més difícil de superar degut al seu caràcter imprevisible, de manera que es recomana una bona capacitat d'alerta [FAO, 2011].

Les principals causes que poden provocar inseguretat alimentària són la manca d'aigua o la seva dificultat en accedir-hi, la degradació de sòls, la contaminació atmosfèrica, el canvi climàtic, l'explosió demogràfica, les epidèmies i pràctiques governamentals inapropiades. Així doncs una manca de la seguretat alimentària comporta episodis de contaminació en els aliments, els quals, a la vegada, poden provocar certs riscos en la salut humana, animal i mediambiental [FAO, 2011].

La contaminació es sol classificar partint de dos punts de vista diferents. Un d'ells és el punt de vista del tipus de medi al que afecta, on generalment es distingeixen com a principals tipus l'aire, l'aigua i el terra. L'altre punt de vista és l'efecte en el medi d'un contaminant en particular. En aquest darrer cas es solen agrupar els contaminants segons el tipus de contaminació que produeixen, com per exemple, la contaminació química, radioactiva, genètica, electromagnètica, acústica, lumínica, tèrmica, visual o difusa [SBS, 2016]. Des d'un enfoc químic, segons la IUPAC, la contaminació es defineix com la presència de substàncies en un determinat medi, ja sigui com a resultat de l'activitat humana o processos naturals, en una concentració suficient, durant un temps suficient i sota unes circumstàncies tals que hi hagi una interferència en la comoditat, la salut o el benestar de les persones o el medi ambient [IUPAC, 1997].

Aleshores, el concepte de contaminació en un aliment s'entendria com tota la matèria que s'incorpora al aliment sense ser pròpia d'ell i amb la capacitat de produir alguna malaltia o disfunció al consumidor. Aquestes matèries bàsicament, poden ser de tipus biològic (bactèries, paràsits o virus), químic (qualsevol substància química) o físic (partícules físiques provinents de utensilis o altres objectes) [ANMAT, 2011].

La contaminació dels aliments es pot produir de diverses maneres, però bàsicament es distingeixen tres tipus de contaminació:

1) Contaminació primària o d'origen: és aquella en la qual els contaminats esdevenen en el moment mateix de la producció de l'aliment.
2) Contaminació directa: és la més comú i prové generalment per manipulació i/o contacte amb contaminants.
3) La contaminació creuada: és el pas de qualsevol contaminant des d'un aliment o matèria primera prèviament contaminats a un altre aliment que no ho està mitjançant el contacte indirecte a través de superfícies o entitats netes [ANMAT, 2011].

D'aquesta manera, de forma general es pot dir que els contaminants provenen d'un sistema que els transfereixi, ja sigui un medi que ja es troba contaminat o un producte físic.

1.2. CONTAMINACIÓ MEDIAMBIENTAL: EL CAS DEL MERCURI

La contaminació, tal i com s'ha comentat, s'estudia des de dos punts de vista diferents: segons el medi i segons els efectes. En quant al medi al que afecta, es distingeixen principalment l'aire, l'aigua i la terra o sòl. D'aquesta manera, la contaminació es pot classificar com a:

- Contaminació atmosfèrica: és la introducció de partícules, molècules biològiques o altres materials nocius a l'atmosfera terrestre, els quals causen malalties o danys als éssers humans i altres formes de vida com animals i vegetals.
- Contaminació dels sòls: és la degradació física o química d'aquest medi, la qual comporta la pèrdua del sòl o un canvi significatiu en el seu ecosistema. Aquesta es produeix generalment per la presència d'agents químics xenobiòtics depositats per l'ésser humà o altres alteracions del sòl a arrel de l'activitat industrial, els agents químics emprats en l'agricultura o un mal tractament de residus.

- Contaminació aquàtica: és la modificació de les característiques d'unes aigües donades (com ara aigua potable, llacs, rius o oceans) de manera que es provoqui una alteració significativa d'aquestes, en especial si això comporta un canvi a l’ecosistema que provoqui un perill per a la salut humana o altres formes de vida [Bradford, 2015; CEF, 2016].

Concretament, la contaminació en el medi aquàtic és un problema important a nivell global, ja que l’aigua cobreix un 70% de la superfície terrestre i és un recurs important per a l’ésser humà i el medi ambient. Aquesta requereix una avaluació i revisió contínua de les polítiques de recursos hídrics a tots els nivells [Directive 2008/105/EC]. Tot i que la contaminació de l’aigua pot no danyar la salut de forma immediata, podria ser perjudicial a llarg termini. Algunes fonts afirmen que la contaminació de l’aigua és la causa principal de morts i malalties arreu del món [West, 2015]. D’aquesta manera, la contaminació aquàtica es pot considerar com un cas de contaminació primària o d’origen, donat que l’aigua, així com els ecosistemes que en puguin derivar d’ella, poden esdevenir o produir aliments per a l’ésser humà i altres organismes vius.

La contaminació del medi aquàtic esdevé quan no es practica un tractament adequat de les aigües per tal d’eliminar uns certs contaminants introduïts prèviament en aquest medi. La introducció d’aquests pol·luents es produeix de forma directa o indirecta i prové generalment de fonts antropogèniques, com ara els abocaments de residus (rebuigs industrials, vessaments o fuites accidentals), tot i que també pot provenir de fonts naturals com ara la pol·lució tèrmica deguda al escalfament global, la qual provoca canvis de temperatures en les aigües que poden afectar el medi, deposicions atmosfèriques o processos d’eutrofització. Els pol·luents principals corresponen a substàncies orgàniques i inorgàniques. Existeix un gran nombre de contaminants, entre els quals els més rellevants són els hidrocarburs del petroli, els pesticides i els metalls pesants.

Els metalls pesants provenen principalment dels processos industrials. Aquests són altament tòxics i els més perillosos són aquells que són persistents, ja que es poden acumular no només en l’aigua de medis com llacs o rius, sinó que també ho fan en els sediments o les formes de vida que habiten el medi aquàtic. Entre els metalls pesants, el mercuri és un element força comú, i, a més, és altament conegut per la seva toxicitat, persistència i acumulació en diversos medis [The Guide Networks, 2016].

1.2.1. Propietats del mercuri

El mercuri és un element químic de nombre atòmic 80 que es troba situat en el 6è període i el grup 12è de la taula periòdica del elements.

Aquest element és un metall pesant de color blanc platejat. En comparació amb els altres metalls, és un mal conductor de la calor, però si que és bon conductor elèctric [Hammond, 2005]. El mercuri té un punt de fusió de -38,829ºC i un punt d'ebullició de 356,619ºC [RSC, 2016], que són valors excepcionalment baixos per a un metall. A més, el punt d'ebullició és el més baix de qualsevol metall. Això és degut a què el mercuri té una única configuració electrònica on els electrons omplen tots els orbitals disponibles des de *1s* fins a *6d*. A causa d'aquesta configuració, l'eliminació d'un electró es troba fortament resistida, de manera que el mercuri es comporta d'una manera similar als gasos nobles, els quals formen enllaços febles i que per tant es fonen a baixes temperatures.

El mercuri no reacciona amb la majoria dels àcids, encara que si que es dissol en àcids oxidants com ara l'àcid sulfúric concentrat i l'àcid nítric o aigua règia per donar sulfats, nitrats o clorurs. El mercuri reacciona amb el sulfur d'hidrogen de l'atmosfera, al igual que la plata, i també reacciona amb els flocs de sofre sòlid, els quals es fan servir en els kits de vessament de mercuri per a la seva absorció.

La Taula 1.1 mostra les principals propietats fisicoquímiques del mercuri.

Taula 1.1. Propietats físiques i químiques del mercuri elemental [RSC, 2016].

PROPIETAT	DADA
Nombre atòmic	80
Massa atòmica (u)	200,592
Configuració electrònica	[Xe] $4f^{14}5d^{10}6s^{2}$
Punt de fusió (ºC)	-38,829
Punt d'ebullició (ºC)	356,619
Densitat ($g·mL^{-1}$)	13,5336
Entalpia de vaporització ($kJ·mol^{-1}$)	59,11
Entalpia de fusió ($kJ·mol^{-1}$)	2,29
Pressió de vapor a 77ºC (Pa)	10
Electronegativitat (escala de Pauling)	2,00
Calor específic ($J·K^{-1}·kg^{-1}$)	140
Conductivitat tèrmica ($W·K^{-1}·m^{-1}$)	8,34
Radi atòmic (pm)	151
Radi covalent (pm)	132 ± 5
Radi de Van der Waals (pm)	155
Energies de ionització ($kJ·mol^{-1}$)	
1a	1007,1
2a	1810
3a	3300

El mercuri disposa de diferents isòtops naturals i estables i de radioisòtops sintètics, que es troben resumits a la Taula 1.2. Hi ha 7 isòtops naturals i estables de mercuri, sent el ^{202}Hg el més abundant d'ells (29.86%). Existeixen 4 radioisòtops sintètics. El ^{194}Hg i el ^{203}Hg presenten el temps de vida més llarg mentre que la resta presenten temps de vida inferiors a un dia.

Taula 1.2. Isòtops de mercuri [WebElements, 2016].

ISÒTOP	ABUNDÀNCIA NATURAL (%)	TEMPS DE VIDA MITJANA
^{194}Hg	Sintètic	520 anys
^{195}Hg	Sintètic	9,5 hores
^{196}Hg	0,15	Estable
^{197}Hg	Sintètic	2.672 dies hores
^{198}Hg	9,97	Estable
^{199}Hg	16,87	Estable
^{200}Hg	23,10	Estable
^{201}Hg	13,18	Estable
^{202}Hg	29,86	Estable
^{203}Hg	sintètic	46,61 dies
^{204}Hg	6,87	Estable

El mercuri pot prendre tres estats d'oxidació principals: 0, +I i +II. Pel que fa als compostos de mercuri (I) (a diferència dels elements veïns cadmi i zinc), la majoria d'ells són diamagnètics i prenen la forma del catió dimèric Hg_2^{2+}, els quals són estables i estan formats amb enllaços metall-metall. Els seus derivats més estables corresponen a compostos de clorur i nitrat. El tractament d'aquests compostos de Hg(I) amb lligands forts per tal de complexar-los, com per exemple el sulfur o el cianur, indueix a la seva desproporció en mercuri (II), Hg(II), i mercuri elemental [Henderson, 2000].

El mercuri (II) és l'estat d'oxidació més comú i també és el principal que es troba a la naturalesa. Els halurs de mercuri són dels compostos més coneguts. Adopten una geometria de coordinació lineal, similar al que tendeix a passar amb els compostos de plata (I) (Ag^+). El més conegut és el clorur de mercuri (II), $HgCl_2$, un sòlid blanc que sublima amb facilitat i forma complexos de coordinació tetraèdrica. A més, donat que el mercuri és un metall tou, forma derivats molt estables amb els altres elements del grup dels calcògens. El més rellevant és el sulfur de mercuri (II), HgS, que existeix a la naturalesa en el mineral de cinabri [Rogalski, 2000].

Els estats d'oxidació del mercuri superiors a +II són molt estranys de trobar. Un dels exemples més rellevants és el fluorur de mercuri (IV), HgF_4, que va ser sintetitzat [Wang et al., 2007].

Per altra banda, l'existència dels compostos orgànics de mercuri és important des del punt de vista toxicològic tot i no tenir gaire valor industrial. Aquests compostos s'anomenen organomercúrics, ja que són organometàl·lics degut a l'enllaç mercuri – carboni. Aquest enllaç és estable a l'aire i la humitat, però és sensible a la llum. Els compostos organomercúrics són sempre divalents i de geometria lineal. A diferència dels compostos organometàl·lics de zinc i de cadmi, aquests no reaccionen amb l'aigua. La seva fórmula general és HgR_2, els quals solen ser volàtils, o HgRX, els quals solen ser sòlids, on "R" és un aril o un alquil i "X" és un halur o un acetat. El més rellevant és el monometilmercuri, (CH_3Hg^+ o $MeHg^+$) també conegut com a metilmercuri. Els compostos de metilmercuri són molt perillosos. Es troben sovint en aigües contaminades i es generen mitjançant processos de biometilació [National Research Council, 2000]. Altres compostos rellevants són el etilmercuri, $C_2H_5Hg^+$, el fenilmercuri, $C_6H_5Hg^+$, el dimetilmercuri, $(CH_3)_2Hg$, el dietilmercuri, $(C_2H_5)_2Hg$ i la merbromina, el tiomersal i el nitromersal. A la Figura 1.1 es mostra l'estructura del compostos organometàl·lics de mercuri esmentats.

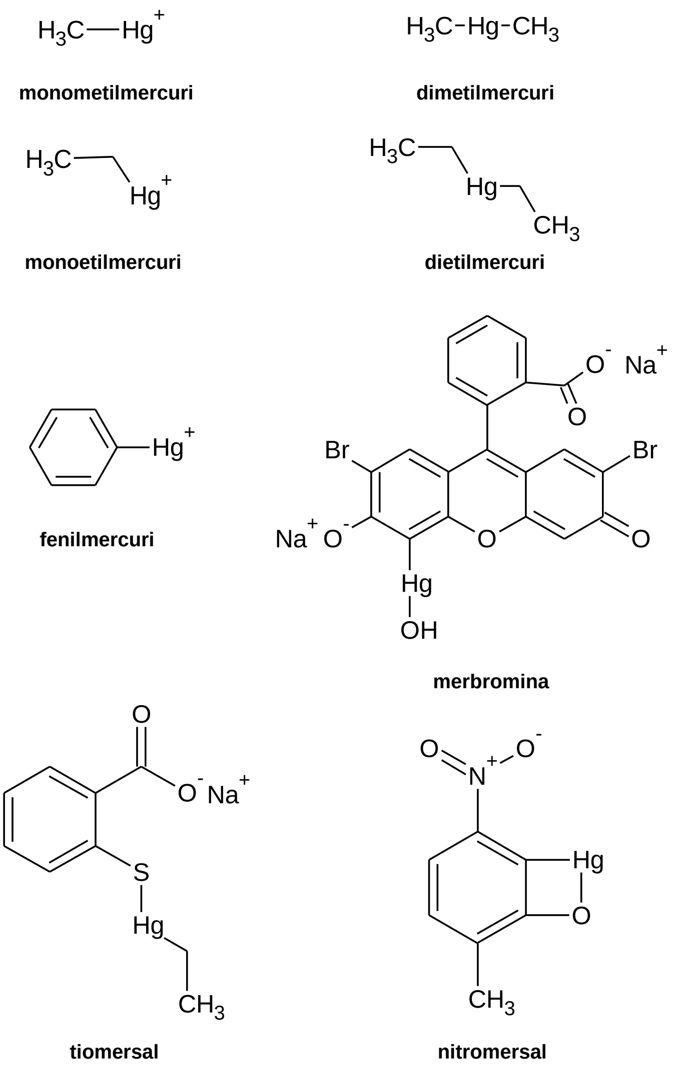


Figura 1.1. Principals compostos organometàl·lics de mercuri.

1.2.2. Toxicitat del mercuri

El mercuri pot entrar a l’organisme de diferents maneres. Les seves vies d'ingrés són la dèrmica, la respiratòria, la intravenosa i la digestiva. Els seus compostos químics, especialment els compostos organomercúrics, són absorbits fàcilment per contacte directe amb la pell, o fins i tot en alguns casos, com el dimetilmercuri, amb pell insuficientment protegida. A més de les dades de concentració i temps, altres factors a tenir en compte en l'exposició per avaluar la toxicitat són l’edat, el gènere, l’estat fisiològic, la massa corporal, l’estat de nutrició i la preexistència de malalties [UBA, 2008].

El mercuri és un agent tòxic altament reactiu. Els seus efectes inicials són difícils d'identificar i encara queda conèixer molta informació sobre els seus mecanismes de toxicitat. Danya el sistema nerviós central, el sistema endocrí, els ronyons, altres òrgans i afecta a la boca, les genives i les dents. També s’han trobat símptomes de decoloració de la pell, inflamació i descamació. La ingesta durant llargs períodes de temps o una forta exposició al vapor de mercuri pot causar dany al cervell i finalment la mort. El mercuri i els seus compostos són particularment tòxics pels fetus i els nadons [Roberts et al., 2013]. Existeixen també evidències de que l'enverinament per mercuri pot donar lloc al síndrome de Young: homes amb bronquièctasis i recompte baix d'espermatozoides [Hendry et al., 1993]. També s’ha associat a efectes com infarts de miocardi, fibromiàlgia, síndrome de fatiga crònica, demència, mal de Parkinson, Alzheimer i esclerosi múltiple [UBA, 2008].

Tot i així, la toxicitat del mercuri depèn de la seva forma química i, per tant, els símptomes i signes varien segons es tracti d’exposició a mercuri elemental, als compostos inorgànics de mercuri, o als compostos orgànics de mercuri (en particular els compostos d’alquilmercuri). Cada espècie química dóna lloc a efectes característics sobre la salut.

El mercuri metàl·lic líquid no s’absorbeix bé ni per ingestió ni per contacte amb la pell. És perillós pel seu potencial d'alliberament de vapors de mercuri. Els estudis realitzats en animals indiquen que menys del 0,01% del mercuri ingerit és absorbit pel tracte gastrointestinal. En els éssers humans, aproximadament el 80% del vapor de mercuri inhalat s'absorbeix a través de les vies respiratòries i entra en el sistema circulatori des d'on es distribueix per tot el cos. L'exposició crònica per inhalació, fins i tot a baixes concentracions, ha demostrat que causa efectes com ara tremolors, deteriorament de les habilitats cognitives i trastorns de la son [Ngim et al., 1992; Linag et al., 1993]. La inhalació aguda d'altes concentracions causa una àmplia varietat de trastorns de les habilitats cognitives, sensorials i motores. Es pronuncien excessivament els símptomes abans descrits, i a més s’han determinat casos de labilitat emocional, pèrdua de memòria, canvis neuromusculars, dolor de cap i polineuropatia.

El mercuri inorgànic es produeix en forma de sals. Aquestes afecten principalment al tracte gastrointestinal i els ronyons, i poden causar danys renals greus. No obstant això, aquestes sals causen poc dany neurològic llevat que es pateixi una exposició contínua o alta ja que no poden travessar la barrera hematoencefàlica amb facilitat [Langford et al., 1999]. Les sals de mercuri (II) són generalment més tòxiques que les de mercuri (I) perquè la seva solubilitat en aigua és més gran, de manera que s'absorbeixen més fàcilment en el tracte gastrointestinal.

Els compostos orgànics de mercuri tendeixen a ser molt més tòxics que no pas l'element en si i les formes inorgàniques. Aquests són capaços d'induir danys en el sistema nerviós central ja que migren al cervell i també al fetge [Aschner et al., 1990]. Tot i que existeixen diversos compostos organomercúrics, tal i com s'ha comentat a l'apartat 1.2.1, el monometilmercuri, el dimetilmercuri i el dietilmercuri es consideren les úniques espècies significativament neurotòxiques, essent el més perillós el dimetilmercuri. També es sap que aquests compostos inhibeixen el transport d'aminoàcids, fet que pot conduir a efectes de ecotoxicitat [Brookes, 1988].

El principal compost organometàl·lic de mercuri que es pot trobar és el metilmercuri, tal com s'ha descrit a l'apartat 1.2.1. Existeix un llarg període de latència entre l'exposició al metilmercuri i l'aparició dels símptomes en casos d'enverinament als adults, des d'unes setmanes fins a uns cinc mesos. No hi ha explicació per a aquest període de latència. El primer símptoma que apareix és en general la parestèsia, una sensació de formigueig, picor o adormiment en la pell. Aquest és seguit ràpidament pels efectes més greus, que de vegades acaben en coma i la mort. El dany sembla ser determinat més per la durada de l'exposició que no pas per concentracions elevades de mercuri.

1.2.2.1. Casos rellevants sobre la toxicitat del mercuri

A partir d'activitats especialment relacionades amb l'agricultura i la mineria, s'ha donat lloc a episodis de contaminació del medi ambient o d'aliments per al consum humà. Alguns d'aquests antecedents van donar lloc a epidèmies tòxiques considerades veritables catàstrofes per la seva magnitud, impacte o permanència en el temps. A continuació, es descriuen alguns d'aquests exemples en els quals ha intervingut el mercuri:

- Japó, badia de Minimata: entre els anys 1932 i 1968, una companyia química va abocar aproximadament unes 27 tones de compostos de mercuri a la badia de Minimata. El mercuri present en els sediments de la badia va esdevenir disponible per les persones a través dels peixos contaminats, causant un síndrome neurològic greu i permanent. Els símptomes inclouen alteracions greus de l'equilibri, de la sensibilitat en les mans i els peus, el deteriorament del sentit de la vista i la oïda, debilitat general i, en casos extrems, paràlisi i la mort. El 1956, l'any en què es va detectar la malaltia, van morir 46 persones. A la dècada dels 60, s'havien identificat més de 100 víctimes mortals, sumades als més de 400 casos amb problemes neurològics greus. Hi va haver mares sense cap símptoma preocupant durant l'embaràs que van tenir fills greument malalts. L'any 1968, el govern japonès va anunciar oficialment que la causa de la malaltia era la ingestió de peix i marisc contaminats amb mercuri pels abocaments de l'empresa petroquímica. Fins l'any 2001 es van diagnosticar al Japó a prop de 3000 casos de la malaltia de Minimata, dels quals prop de 2300 havien viscut a la zona contaminada. [Harada, 1995].

- Iraq: entre l'any 1971 i el 1972 van emmalaltir més de 5000 persones degut a un enverinament provinent de llavors de blat i ordi importades des de Mèxic i els EUA. L'inici de la tragèdia es va originar en la falta de grans per proveir la població com a conseqüència d'un prolongat període de sequera. Els grans importats havien estat tractats amb metilmercuri i l'advertència a cada sac sobre la prohibició d'utilitzar aquests grans com a aliments estava retolada en anglès i en castellà. Els grans van ser distribuïts entre els agricultors i els van utilitzar per preparar aliments; sense saber el significat dels rètols. Tres mesos després d'haver-se iniciat la distribució, els casos d'enverinament i de morts es van multiplicar al país. La crisi es va estendre al comprovar que la contaminació s'havia estès al bestiar, de manera que el consum de la seva carn contaminada va esdevenir una nova i imprevista via de propagació. [Octopus, 1983].
- Perú, Yanacocha: la mina de Yanacocha és la més gran i més productiva mina d'or a l'Amèrica Llatina. En els primers dies de juny de l'any 2000, un camió vinculat a l'empresa encarregada d'explotar el jaciment miner va abocar a Choropampa, ciutat situada als voltants de la mina, uns 150 quilograms de mercuri al llarg de diversos quilòmetres. Els pobladors, pensant que el mercuri contenia or, el van recollir i fins i tot alguns d'ells el van portar a casa seva. 72 hores després, van aparèixer els primers casos d'intoxicació en els pobladors. A finals de juliol, s'havien identificat aproximadament 67 llars amb dades de contaminació ambiental per mercuri [UBA, 2008].
- La conca de l'Amazones: la Conca de l'Amazones és una de les més afectades per processos d'extracció artesanal d'or. Milers de miners independents que realitzen treball no regulat fan servir un procés rudimentari que consisteix a barrejar el mercuri en el seu estat elemental amb els sediments i el sòl del riu, per tal de separar les partícules d'or que hi ha a l'aigua. Aquesta mineria a petita escala o artesanal, concentrada majoritàriament en els països de l'Hemisferi Sud, afecta les vides de 80 a 100 milions de persones. Segons estudis científics duts a terme conjuntament per universitats del Brasil i del Canadà, el mercuri es trobava present en el sòl, els sediments fluvials, els peixos i les persones que vivien a les rodalies del riu. Els resultats d'un seguiment realitzat en aquestes persones van demostrar una reducció en la destresa manual i en certes funcions visuals. Aquestes disfuncions estaven associades amb un increment dels nivells de mercuri, focalitzat principalment en els cabells, tot i que també es va comprovar que aquestes sorgien a nivells d'exposició inferiors [UBA, 2008].

1.2.3. Presència de mercuri en el medi ambient

A la biosfera, la presència i les emissions de mercuri poden provenir de fonts naturals, fonts antropogèniques o mixtes abans d'entrar en un cicle i arribar al medi aquàtic i a altres medis.

Les principals fonts naturals de mercuri són les emissions volcàniques i l'erosió de roques, les quals impliquen una transferència de massa de mercuri als compartiments del medi mitjançant processos naturals. Les principals fonts antropogèniques provenen d'activitats relacionades amb l'extracció de recursos naturals, com la recuperació de metalls preciosos o el treball en jaciments petrolífers i carbonífers, i la incineració de residus o la disposició d'aquests, com piles, làmpades, termòmetres, etc. També s'inclouen les emissions i els efluents provinents d'indústries, com per exemple, la combustió de derivats del petroli i del carbó, i de serveis, com els residus líquids no tractats de les pràctiques o accidents en unitats mèdiques i odontològiques.

Les fonts mixtes estan constituïdes per aquelles emissions que impliquen una transferència de massa de mercuri a l'atmosfera mitjançant processos biològics i geològics mobilitzant el mercuri que anteriorment havia estat dipositat a la superfície terrestre per activitats humanes o naturals. Els processos més importants que alliberen mercuri d'origen mixt al medi ambient són la combustió de biomassa i l'emissió de mercuri dels sòls i l'oceà [MFE, 2009].

Des d'aquestes fonts, el mercuri contamina sòls, aire, aigües i la cadena tròfica. La Figura 1.2 representa el cicle del mercuri al medi ambient.

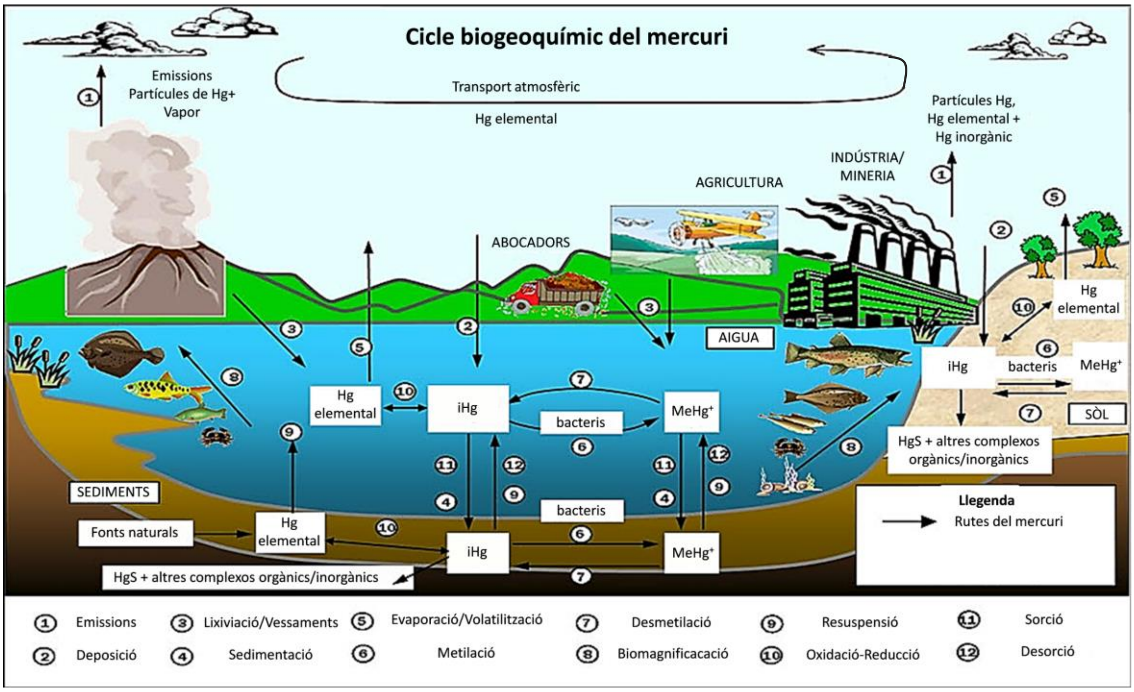


Figura 1.2. Cicle del mercuri, adaptat de la referència [MFE, 2009]. iHg: mercuri inorgànic; $MeHg^+$: metilmercuri.

Aquest cicle es pot resumir de la següent manera: el vapor de mercuri alliberat per les fonts és estable a l’atmosfera, i per tant es distribueix de manera global, fins a convertir-se en formes hidrosolubles que permetin el seu retorn a la terra amb l’aigua de la pluja. El mercuri també pot entrar a l’aigua a través de processos de lixiviació i abocaments provinents de l’agricultura i dels abocadors. En aquesta etapa, el metall pot reduir-se novament i tornar com a vapor a l’atmosfera, o pot ésser metilat.

Aquesta metilació és una reacció química que té lloc en els sistemes biològics mitjançant la qual s’aconsegueix l’addició de un o més grups metil (CH_3), donant lloc a compostos de metilmercuri. Són els microorganismes naturals dels sòls, sediments i aigua dolça i salada els que duen a terme aquesta reacció de metilació, en la qual hi intervenen diverses poblacions microbianes. Aquests compostos orgànics de mercuri formats en aquests processos de metilació tenen com a principal característica que poden ingressar a la cadena alimentària a partir de la seva assimilació pel plàncton, el qual els torna disponibles per a la resta dels organismes del sistema, de manera que es converteix en un autèntic reservori de mercuri [Craig et al., 2003]. Aleshores, a la cadena tròfica, els compostos orgànics de mercuri troben la possibilitat de bioacumular-se, bioconcentrar-se i biomagnificar-se, de manera que passen a les parts més altes de la cadena, com els peixos, i consegüentment als humans mitjançant el consum d’aquest peix contaminat amb mercuri.

D’aquesta manera la bioacumulació es defineix com la capacitat d’un organisme per acumular als seus teixits algun compost químic. La bioconcentració és la capacitat d’alguns organismes de concentrar nivells creixents d’una substància tòxica sense que aquesta provoqui danys evidents. Aquesta característica és típica de molts organismes aquàtics. A causa d’això, les substàncies tòxiques queden disponibles per a la resta de la cadena tròfica, en el curs de la qual el procés de bioconcentració continua. La biomagnificació és l’augment continu de la concentració d’un compost en cada nivell de la cadena alimentària, fins a assolir una concentració potencialment letal per a algun organisme pertanyent a la mateixa [UBA, 2008].

1.2.4. Presència de mercuri en aigües i peixos

Tal i com s’ha comentat anteriorment, el mercuri alliberat al medi ambient entra en un cicle entre l’aire, la terra i l’aigua. Aquesta última és un dels medis més importants per estudiar l’efecte del mercuri en el medi ambient donat el gran nombre de processos i transformacions que hi esdevenen. L’aigua no només presenta una forta rellevància a la naturalesa, sinó que també la té a la salut humana donat que és essencial en la nostra vida quotidiana.

La Directiva Europea de l'Aigua [Directive 2008/105/EC] busca establir un marc per a la protecció de les aigües subterrànies i superficials i inclou el mercuri i els seus compostos en una llista de substàncies prioritàries i perilloses. Per tant, el mercuri és un dels elements considerats en l'avaluació de la qualitat de l'aigua. Generalment, les concertacions de mercuri en aigua són molt baixes [Ibáñez-Palomino et al., 2012b]. A més, els nivells de metilmercuri tendeixen a ser molt inferiors als de mercuri (II) a causa de la descomposició dels compostos orgànics per la llum ultraviolada solar i la dificultat de les reaccions de metilació en fase aquosa. La concentració de Hg mitjana reportada a l'aigua és de 2 ng L^{-1} [Martínez-Trinidad et al., 2013]. El metilmercuri correspon al 1 % d'aquest valor i la resta a mercuri inorgànic en forma de "mercuri (II)". En l'aigua d'alta mar, la concentració de mercuri es troba normalment en el rang de 1 a 20 ng L^{-1}, mentre que en les aigües costaneres la concentració pot arribar fins a 100 ng L^{-1} a causa de les descàrregues antropogèniques [Jia et al., 2012]. No obstant això, la Directiva Europea relativa a l'aigua de beguda només considera actualment la concentració de mercuri total, i estableix un valor màxim de 1 µg L^{-1} [Directive 98/83/EC].

Dins del medi aquàtic, els organismes que hi viuen poden captar el mercuri i transformar-lo en espècies organometàl·liques mitjançant processos de biometilació, tal i com s'ha comentat a l'apartat anterior. Aquest procés consisteix en la producció de $MeHg^+$ o d'altres compostos organomercúrics per les bactèries presents a l'aigua com a mecanisme de defensa que tenen davant aquest metall. Aquesta metilació bacteriana, que converteix el Hg metàl·lic en un compost orgànic, liposoluble i capaç de travessar barreres biològiques amb facilitat, és el mecanisme a través del qual el Hg queda disponible per a ingressar a la cadena alimentària partir dels organismes aquàtics.

Els peixos acumulen mercuri però no semblen patir enverinament. El $MeHg^+$ s'acumula principalment a les vísceres dels peixos, tot i que també ho fa en els teixits musculars i el teixit adipós [Cocoros et al., 1973]. En conseqüència, el mercuri es bioacumula a cada nivell de la cadena tròfica, des de les algues i el zooplàncton, els peixos de mida petita, i d'aquests als seus depredadors més grans. Com més gran és l'edat del peix, la concentració de mercuri acumulada és més elevada, de manera que qualsevol animal (inclòs l'ésser humà) que el consumeixi ingeriria la màxima quantitat de mercuri acumulada en el peix. Aquest procés justifica que els peixos depredadors, com per exemple, el peix espasa (*Xiphias gladius*) o el caçó (*Galeorhinus Galeus*) mostrin concentracions de mercuri per gram de pes majors que el que es podria esperar per simple contacte amb el mercuri ambiental. Com a regla empírica es pot assumir que la concentració de mercuri augmenta deu vegades en cada baula de la cadena tròfica. Per exemple, la concentració de mercuri en l'arengada (*Clupea harengus*) és aproximadament 0,1 mg kg^{-1}, mentre que al caçó és aproximadament de 1,0 mg kg^{-1} [EPA, 1997]. S'han trobat fins i tot concentracions superiors a 10 mg kg^{-1} de $MeHg^+$ en alguns treballs [Von Burg et al., 1991]. Consegüentment, el consum de peixos o organismes depredadors comporta un risc d'ingesta d'elevades concentracions de mercuri. Els consum de carn de balena i dofins és una pràctica habitual al Japó, de manera que és una font d'alts nivells d'enverinament per mercuri. En conclusió, el consum de peix, marisc o altres tipus d'organismes aquàtics no només esdevé una altra font d'exposició al mercuri, sinó que n'és la principal degut als possibles alts nivells de Hg presents en els seus teixits i a la presència de les espècies més tòxiques, especialment el metilmercuri.

El Codex Stan 193-1995, estipulat per la FAO i la WHO (Organització Mundial de la Salut), recomana uns nivells màxims de metilmercuri en peixos no depredadors i depredadors de 0,5 i 1 mg kg^{-1}, respectivament. El Codex indica el nivell màxim de substàncies tòxiques permeses en el comerç internacional d'aliments [CODEX STAN, 2009]. El Comitè Mixt FAO/WHO d'Experts en Additius Alimentaris (JECFA) va proposar una ingesta setmanal tolerable (TWI) provisional pel $MeHg^+$ de 1,6 µg kg^{-1} en el pes corporal. No obstant això, la Comissió Europea va demanar a l'Autoritat Europea de Seguretat Alimentària (EFSA) de revisar el valor tolerable de $MeHg^+$. Per tant, la EFSA va publicar al 2012 un dictamen científic sobre el risc de l'exposició humana al mercuri i el metilmercuri. Aleshores, la Comissió Tècnica de la EFSA sobre els Contaminants a la Cadena Alimentària (CONTAM) va establir una TWI pel $MeHg^+$ de 1,3 µg kg^{-1} en el pes corporal, expressada com a mercuri, segons uns nous estudis epidemiològics realitzats en els nens [EFSA, 2012]. Pel que fa a la Unió Europea, encara que el Reglament de la Comissió 1881/2006/EC no estipula límits per a la concentració $MeHg^+$, aquesta dóna uns límits pel Hg total de 0,5 mg kg^{-1} o de 1 mg kg^{-1} per a diversos tipus de peixos i marisc, depenent dels seu tipus i classe [Regulation 1881/2006/EC].

D'aquesta manera, donat que diferents concentracions de mercuri i de les seves espècies es poden trobar en peixos depenent de l'espècie de peix, del medi on hi habitin i del seu grau de contaminació, existeix la necessitat de determinar de manera acurada la concentració de mercuri i de les seves espècies present en aquestes matrius per tal de garantir així el possible consum d'aquests productes.

1.3. CONTAMINACIÓ DEGUT A L'ENVASAMENT: EL CAS DE L'ANTIMONI

1.3.1 Els envasos

L'envasament dels aliments és una estratègia fonamental per a conservar la qualitat dels aliments, reduir al mínim el seu deteriorament químic i físic i limitar l'ús d'additius. Qualsevol tipus d'envàs, ja sigui una llauna, un recipient de vidre o un envàs de cartró, contribueix a protegir els aliments de la contaminació per microorganismes, insectes i altres agents contaminants. Així mateix, l'envàs preserva la forma i la textura de l'aliment que conté, evita que perdi sabor o aroma, allarga el temps d'emmagatzematge i regula el contingut d'aigua o humitat de l'aliment. En alguns casos, el material seleccionat per a l'envàs pot afectar la qualitat nutricional del producte. Per exemple, els envasos opacs com els cartrons en els que s'envasen els productes lactis eviten la pèrdua de riboflavina, una vitamina fotosensible, per exposició del producte a la llum solar. L'envàs també permet oferir informació al consumidor sobre les característiques del producte, el seu contingut nutricional i la seva composició.

Un aspecte important de l'envasament en quant a la seguretat alimentària és la identificació dels productes que puguin haver-se manipulat de manera inadequada o fet malbé involuntàriament durant el seu procés de producció o transport. Alguns fabricants utilitzen un tipus d’envàs que permet detectar si aquest ha estat danyat o obert, com el tancament al buit o taps o obertures especials. D’aquesta manera, per exemple, els aliments continguts en llaunes bonyegudes o embolcalls trencats no han de consumir-se ja que poden estar contaminats per microorganismes o agents perjudicials [EUFIC, 2016].

Durant els últims anys, les investigacions sobre la forma més segura i eficaç d’envasar aliments per tal de millorar la qualitat del producte i del procés d’envasament en general han progressat de manera considerable. En particular, s’han realitzat nombrosos estudis sobre els materials més adequats per a contenir els diversos aliments, centrant-se en la interacció entre l’aliment, l’envàs i l’impacte mediambiental dels diferents materials. D’aquesta manera, l’envasament experimenta constantment canvis degut a la introducció de nous materials, així com en la tecnologia i el seu processament.

Els principals materials per fabricar envasos i les seves propietats es resumeixen a continuació. Cal remarcar que en la majoria d’envasos o procediments d’envasament es sol fer servir una combinació d’aquests materials per tal de aprofitar al màxim les propietats desitjades de cada material [Coles et al., 2003]:

- Vidre: aquest material és rígid, inert respecte els aliments, impermeable a gasos i vapors externs. Pot ésser fàcilment reutilitzat ja que és trencadís i fràgil i es fa servir àmpliament com a envàs de transport.
- Alumini: aquest material és també rígid i té una bona resistència a la tracció. Presenta una excel·lent barrera a la llum, líquids i el traspàs del propi aliment. Té diverses aplicacions en quant a l’envàs, ja que no només és fa servir com a llaunes per aliments i begudes, sinó que també per contenir altres tipus de productes com els aerosols. Malauradament, aquests materials poden reaccionar amb el producte contingut i causar la dissolució del metall
- Paper i cartró: són materials rígids de baixa densitat que presenten una barrera pobre a la llum, líquids, gasos i vapors a menys que estiguin recoberts, laminats o embolicats. No obstant, són resistents al greix i manejables, ja que s’arruguen, es pleguen i s’enganxen amb facilitat. No són materials que es trenquin amb facilitat.
- Plàstics: Els plàstics són típicament polímers orgànics sintètics o semi-sintètics d'alta massa molecular amb unes propietats interessants que els hi donen un ús molt ampli. Les matèries primeres més importants utilitzades en la fabricació de plàstics són el petroli i el gas natural, els quals es sotmeten a un procés de clivellament en el qual es produeixen monòmers d'hidrocarburs. Aquests s’enllacen en cadenes polimèriques i de la combinació final d'aquests monòmers en resulta una àmplia gamma de propietats i característiques. Els plàstics són en general químicament inerts i emmotllables. Tenen unes propietats de resistència i tenacitat que els permeten ser utilitzats en un gran interval de temperatures.

A causa d'aquestes propietats, els plàstics són àmpliament utilitzats com a materials d'envasament, especialment en el sector de l'alimentació. A Europa, gairebé el 40% de tots els plàstics s'utilitza en el sector de l'embalatge i al voltant del 50% dels aliments d'Europa és envasat en plàstic [Coles et al., 2003; Barnes et al., 2007]. Per a l'envasament d'aliments, es poden trobar recipients de plàstic rígids (ampolles, cubs...), films flexibles de plàstic i bosses per protegir els aliments del deteriorament i components d'altres tipus de recipients, com ara taps, entre d'altres [Coles et al., 2003].

Existeixen diferents tipus de plàstic que es fan servir en l'envasament d'aliments. Els més rellevants són els següents: polietilè (PE) de baixa i alta densitat (LDPE; HDPE), polipropilè (PP), polietilentereftalat (PET), poliestirè (PS), policarbonat (PC) i clorur de polivinil (PVC). Al mercat Europeu dels envasos, el PE és el plàstic més comercialitzat, amb un valor del 56% en pes en el mercat. El segueixen el PP, PET, PS i PVC, que corresponen a la major part del tant per cent restant. El PET és el plàstic que presenta cada cop més aplicacions per a l'envasament d'aliments com a resultat del seu ús popular en la substitució del vidre, en especial per ampolles de beguda [Coles et al., 2003].

1.3.2. Síntesi i característiques del PET

El PET és un tipus de plàstic derivat del petroli. Pertany al grup dels materials sintètics anomenats polièsters. Aquest plàstic va ser sintetitzat per primera vegada l'any 1941 pels científics britànics Whinfield i Dickson. Aquest polímer presenta una aparença transparent i cristal·lina, encara que pot admetre alguns colorants. És lleuger, impermeable i no és gens fràgil. Aquests científics el van patentar com a polímer per a la fabricació de fibres ja que en aquell temps el país es trobava en guerra i es necessitava trobar substituts del cotó.

El PET es fabrica fonamentalment per policondensació en estat fos. Es realitza una esterificació directa de l'àcid tereftàlic o del tereftalat de dimetil amb diols, especialment amb etilenglicol. La reacció es realitza entre 200 i 260ºC, i s'accelera amb catalitzadors com el triacetat d'antimoni o el triòxid d'antimoni (Sb_2O_3) [Reimschuessel, 1980; Duh, 2002].

La síntesi s'inicia quan un dels grups alcohol del diol ataca al grup carbonil de l'àcid tereftàlic, de manera que es desprèn una molècula d'aigua. L'altre grup alcohol restant que pertany al diol original ataca a un altre grup carbonil d'una altra molècula, fent així que el polímer creixi. El polímer fos és viscós i surt posteriorment del reactor en forma de cinta, es refreda i es granula.

Per a augmentar la longitud de la cadena, el granulat es continua condensant, mitjançant una condensació en fase sòlida, dins del seu interval de rebliment, entre 210 i 240ºC. Aquests polièsters lineals, segons la seva composició i condicions de transformació són termoplàstics amorfs o semi cristal·lins, amb un grau de cristal·lització del 30-40 %. Cal destacar també que, si s'obté una longitud de cadena alta, s'obtindrà un plàstic amb una viscositat intrínseca elevada. D'aquesta manera es pot fabricar PET amb més o menys grau de polimerització (PET dur o "hard PET" i PET suau o "soft PET", respectivament) [POLIMAT].

Els usos del PET s'han anat ampliant al llarg del temps. A partir de l'any 1946, degut a la seva elevada resistència al plegat i la seva baixa absorció de humitat, es va fer servir industrialment com a fibra, i el seu ús tèxtil ha continuat fins el present. El PET no és un material tòxic, a més es va demostrar que és inert al seu contingut, estable a la intempèrie i que té una alta resistència química a greixos i olis presents en aliments, solucions diluïdes d'àcids minerals, àlcalis, sals, surfactants, hidrocarburs alifàtics i alcohols. D'aquesta manera, al 1952 es va començar a utilitzar en forma de film per a envasar aliments. El 1976, degut a la seva duresa i resistència al desgast, va aparèixer la seva principal aplicació en envasos rígids per begudes poc sensibles a l'oxigen, com l'aigua mineral i els refrescos carbonatats. Des de l'any 2000 també es fa servir per envasaments de cervesa. També s'ha demostrat que el PET no es deforma a temperatures moderades tot i que no presenta bona resistència a dissolvents halogenats, aromàtics i cetones de baix pes molecular. Per aquesta raó també es fa servir per a fabricar recipients destinats al cuinat i a l'escalfament d'aliments. Un gran avantatge d'aquest polímer és la capacitat de reciclar-lo. A més, també existeix una certa tendència a reutilitzar-lo [Andra et al., 2011; Tukur et al., 2012]. Per aquesta raó, els envasos de PET destinats als usos abans esmentats es fan servir per emmagatzemar multitud d'aliments. En molts llocs és freqüent preservar begudes alcohòliques o menjars preparats en envasos de PET [Χανιώτικα νέα, 2015]

1.3.3. Fabricació d'envasos de PET

A partir del PET es fabriquen envasos destinats a preservar aliments, aigua i begudes. Per fabricar aquests recipients de plàstic es fa servir el mètode d'extrusió – bufat o injecció – bufat [Ashby, 1988; Biros et al., 2002; Duh, 2002]. És una variant dels models d'extrusió i injecció simples. El plàstic va entrant en un motlle per una obertura estreta i es comprimeix. A mesura que aquest es va omplint de PET, s'injecta aire a pressió, de manera que el plàstic es posiciona cap a les parets del motlle. Així, s'acaba obtenint una peça buida amb la forma del motlle. Amb aquest mètode, si es sincronitza bé l'obertura i tancament del motlle amb la velocitat de sortida del material, es poden fabricar de manera contínua i automatitzada objectes com ampolles, flascons, i tot tipus de recipients buits impossibles de formar-se per un altre procediment. De manera similar, es poden fabricar bosses fent que el plàstic que surt de la màquina injectora o d'extrusió sigui de parets molt fines. A la figura 1.3 es detalla l'esquema de la fabricació d'una ampolla pel mètode extrusió – bufat [Calvo, 2016].

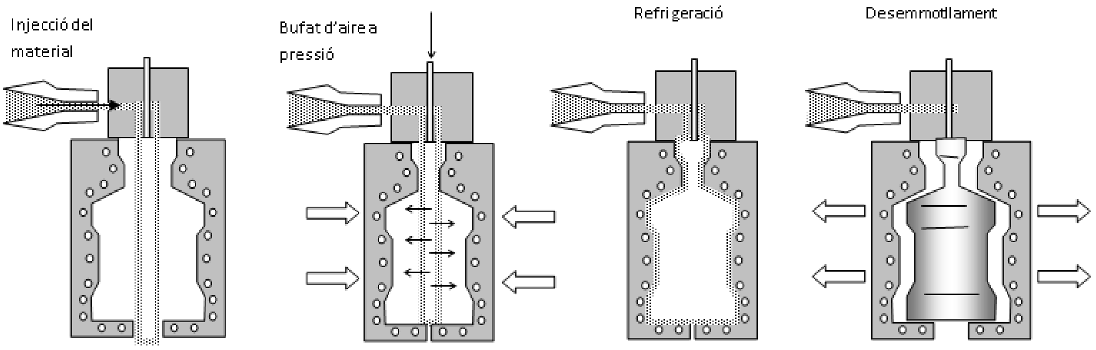


Figura 1.3. Esquema de la fabricació d'una ampolla de PET pel mètode extrusió – bufat.

El PET i altres materials plàstics es solen acolorir durant el procés de fabricació mitjançant un procediment de concentrat de color, també anomenat "masterbatch". Consisteix en una mescla concentrada de pigments o additius dispersats en forma de partícules molt fines incorporades en un suport plàstic igual o compatible a la resina que es vol acolorir. A partir del "masterbatch", es fabrica el plàstic corresponent i s'aconsegueixen coloracions de gran uniformitat. També es pot aplicar un masterbatch d'additius, la finalitat del qual és obtenir unes característiques concretes, com per exemple l'absorció o estabilització de la llum ultraviolada per incrementar la vida útil, la facilitat de lliscament i evitar el trencament o la formació i acumulació de càrregues estàtiques. Aquest tipus de coloració es fa servir especialment en tots aquells processos que comportin un modelat previ del material a acolorir, com l'extrusió – bufat. No existeix un masterbatch universal, però si que n'hi ha algun concret per certs polímers tècnics.

Els avantatges que presenta l'ús de masterbatch com a mètode per acolorir el plàstic són una gran uniformitat, bona estabilitat i resistència de color a la temperatura, a l'envelliment, a la llum i a la intempèrie. Tampoc afecta a les propietats mecàniques de les poliolefines, encara que estigui un bon temps d'exposició a la intempèrie. També s'assegura una total resistència a la migració del colorant al contingut del plàstic i una reducció en l'absorció de la humitat. El concentrat de color també permet una neteja ràpida a l'àrea de producció del colorant, fàcil manipulació i pesada, dosificació automàtica i eliminació de grumolls durant la seva aplicació.

Per una altra banda, el masterbatch presenta com a desavantatges un elevat preu a més d'un augment del temps de fabricació. No existeix un únic concentrat universal i alguns fabricants poden fer servir resines base de segona qualitat o reprocessada. En quant a la seva manipulació, la dosificació del concentrat de color ha de ser superior a 1 %. Addicionalment, presenta una alta variació de tonalitats a més d'una difícil correcció i alteració d'aquesta [Recubrimientos plásticos, 2016; Proquimac color, 2016]. Un clar exemple d'aquests recipients fabricats i acolorits tal i com s'ha explicat són les ampolles d'aigua.

Els pigments que fan servir per a la fabricació de concentrats de color per acolorir ampolles de PET estan formats bàsicament per compostos orgànics, en especial per colorants azoics.

També n’hi ha alguns que contenen sals, com per exemple el blau ultramarí, que està format per sulfurs [IQAP, 2016].

Com s’ha comentat anteriorment, l’envasament dels aliments té com a funció principal la protecció dels aliments de la contaminació. No obstant això, s’ha de tenir en compte que el propi material d'envàs ha de ser prou inert per evitar la contaminació de l’aliment al llarg de tot el període de contacte, des del procés d’envasament fins al moment del consum. Per aquesta raó és necessari un estudi detallat dels mecanismes de migració de possibles compostos del plàstic a l’aliment. En el cas del PET, es poden trobar quantitats significatives d’antimoni en el plàstic, les quals provenen del seu ús com a catalitzador en forma de triòxid d’antimoni o triacetat d’antimoni durant la producció del polímer, tal i com s’ha comentat abans, sent el primer el principal utilitzat. Per tant, a causa de l’ús del PET com a material d'envasament en la indústria alimentària, l’antimoni esdevé amb freqüència en contacte amb el menjar i beguda. En conseqüència, en els productes pel consum humà s’ha observat la presència d’antimoni.

Diferents treballs han determinat la concentració total d’antimoni en envasos de PET, la majoria dels quals tenen com a ús l’envasament d’aigua mineral. S’han trobat valors entre 100 i 400 mg kg^{-1}. [Shotyk et al., 2006; Shotyk et al., 2007; Takahashi et al., 2008; Westerhoff et al., 2008; Keresztes et al., 2009; Welle et al., 2011; Hureiki et al., 2012; Tukur et al., 2012; Rungchang et al., 2013, Brandao et al., 2014]. Això s’explica perquè, un cop acabada la producció del PET, una quantitat significativa del semimetall queda a la superfície del producte. Aquest residu es pot eliminar mitjançant el rentatge. Tot i així el Sb també roman en el propi material i pot migrar cap a l’aliment o la beguda que contingui el propi plàstic.

1.3.4. Propietats de l’antimoni

L’antimoni és un element químic de nombre atòmic 51 que es troba situat en el cinquè període i el grup 15è de la taula periòdica dels elements. Aquest element es considera un semimetall i presenta quatre formes al·lotròpiques. La seva forma estable, corresponent a antimoni metàl·lic elemental (antimoni romboèdric o β-antimoni), és un sòlid cristal·lí gris platejat brillant que s’evapora a altes temperatures i presenta una conductivitat elèctrica i tèrmica baixa. Tot i ser resistent als àcids, pot ésser atacat per àcids oxidants i halògens. S’assembla als metalls en quant a les característiques físiques, però químicament es comporta com un no-metall. La seva duresa no és gaire elevada, per la qual cosa aquesta forma d’antimoni és massa tova per a la fabricació d’objectes que vulguin presentar una duresa considerable. Les altres formes al·lotròpiques de l’element són no metàl·liques i inestables. L’antimoni groc o α-antimoni és amorf i passa ràpidament a temperatures superiors a -90ºC a antimoni negre, el qual s’oxida espontàniament a l’aire i es converteix en la forma metàl·lica estable. La quarta forma al·lotròpica correspon a antimoni explosiu i es pot obtenir a partir del clorur [Cotton et al., 1980].

L'antimoni té dos isòtops estables a la naturalesa: el ^{121}Sb amb una abundància relativa de 57,36% i el ^{123}Sb amb un 42,64%. També existeixen altres isòtops d'antimoni en l'atmosfera generats de forma sintètica a causa d'explosions de bombes nuclears o accidents en reactors nuclears, com per exemple el ^{125}Sb, el qual té una vida mitjana curta (2,73 anys) [Boyle et al., 1984].

La taula 1.3 mostra les principals propietats fisicoquímiques de l'antimoni.

Taula 1.3. Propietats físiques i químiques de l'antimoni elemental.

PROPIETAT	DADA
Nombre atòmic	51
Massa atòmica (u)	121,760
Configuració electrònica	[Kr] $4d^{10}$ $5s^2$ $5p^3$
Punt de fusió (ºC)	630,5
Punt d'ebullició (ºC)	1635
Densitat ($g\cdot mL^{-1}$)	6,69
Entalpia de vaporització ($kJ\cdot mol^{-1}$)	77,14
Entalpia de fusió ($kJ\cdot mol^{-1}$)	19,87
Pressió de vapor a 886ºC (kPa)	0,133
Electronegativitat (escala de Pauling)	2,05
Calor específic ($J\cdot K^{-1}\cdot kg^{-1}$)	230
Conductivitat tèrmica ($W\cdot K^{-1}\cdot m^{-1}$)	24,3
Radi atòmic (pm)	133
Radi covalent (pm)	138
Radi de Van der Waals (pm)	220
Energies de ionització ($kJ\cdot mol^{-1}$)	
1a	834
2a	1595
3a	2440
4a	4260
5a	5400
6a	10400

L'antimoni pot mostrar els següents estats d'oxidació: -III, 0, +III i +V. Tot i així, aquest element sol perdre tres o cinc electrons, de manera que Sb(III) i Sb(V) són les formes predominants i presents en materials ambientals, geoquímics i biològics, juntament amb algunes formes organometàl·liques [Cotton et al., 1980].

Les espècies inorgàniques es troben principalment en el medi aquàtic, terrestre i atmosferic. En quant el medi aquàtic, dins l'interval de pH de la majoria de les aigües naturals, l'antimoni es troba en solució en sistemes oxigenats de la següent forma: $[Sb(OH)_3]$, $[Sb(OH)_6]^-$ i $[Sb(OH)_5]$

[Meima et al., 1998; Filella et al., 2002b; Shotyk et al., 2005; Nakamaru et al., 2006]. Les formes pentavalents són les predominants, però la forma trivalent també s'ha detectat en aigües marines, dolces, subterrànies i de pluja. Aquest fet és contradictori als principis de la termodinàmica, ja que les espècies trivalents són termodinàmicament inestables en aquest medi, de manera que les formes de Sb(V) haurien de trobar-se a concentracions de diversos ordres de magnitud per sobre a les de Sb(III). Aquest fet és degut a l'estabilització de les espècies de Sb(III) per alguns compostos existents en els sistemes oxigenats. Un possible candidat és la matèria orgànica, ja que hi ha diversos compostos orgànics, com l'àcid tartàric, que eviten la seva oxidació. Per altra banda, en sistemes no oxigenats, l'espècie predominant és la de Sb(III), tot i que hi ha evidències de l'existència de la forma de Sb(V) degut a una possible oxidació [Filella et al., 2002a].

També es pot trobar la forma sulfurosa: $[H_2Sb_2S_4]$, $[Sb_2S_4]^{2-}$. Al medi terrestre el Sb es presenta com a òxid o com a sulfur, sent els més predominants el Sb_2O_3, Sb_2O_5, Sb_2S_3. A l'atmosfera l'espècie predominant és l'hidrur d'antimoni (III), SbH_3.

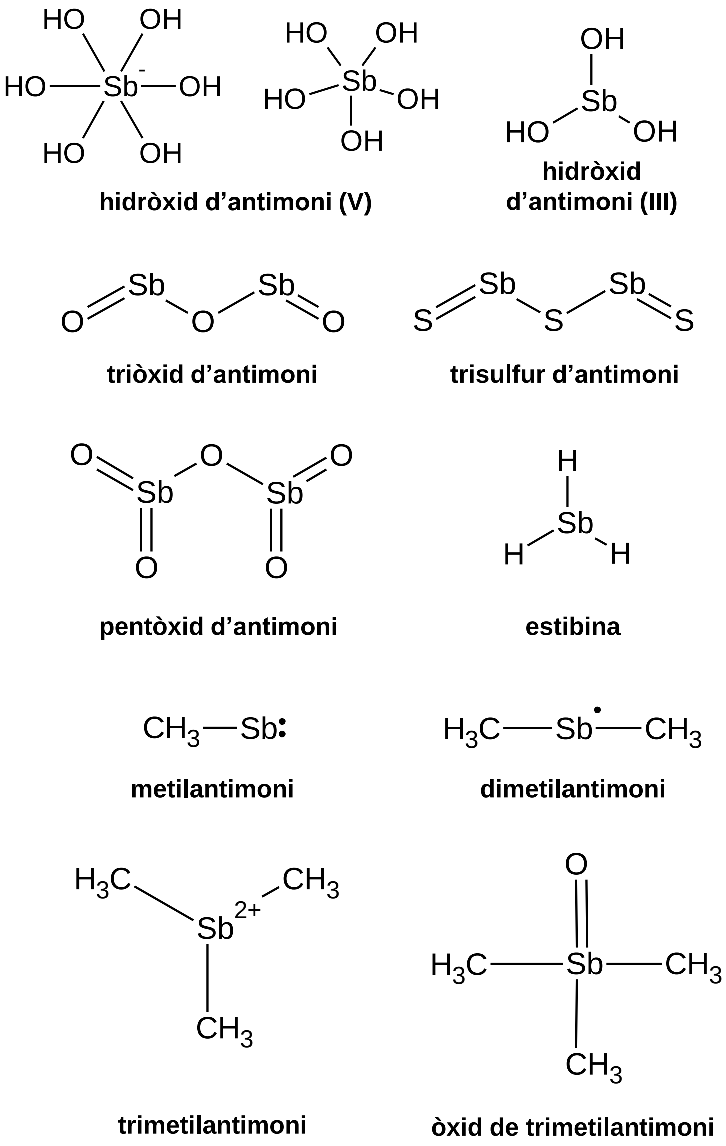
Les espècies organometàl·liques estan presents principalment al medi aquàtic, als sòls, als sediments i a la biota. Les més rellevants són: metilantimoni (MSb), dimetilantimoni (DMSb), òxid de trimetilantimoni (TMSbO), hidròxid de trimetilantimoni ($TMSb(OH)_2$), bromur de trimetilantimoni ($TMSbBr_2$) i clorur de trimetilantimoni ($TMSbCl_2$). A la Figura 1.4 es mostra l'estructura del compostos més rellevants d'antimoni.

L'antimoni i els seus compostos es consideren pol·luents d'interès prioritari per l'Agència de Protecció Mediambiental del Estats Units (USEPA) i la Unió Europea (UE) ja que són agents contaminants i tòxics pel medi ambient i la salut humana [Filella et al., 2002a].

Tot i no tenir cap funció biològica coneguda, l'antimoni és tòxic i les seves propietats toxicològiques depenen del seu grau d'oxidació, la seva solubilitat i la unió amb altres lligands. L'antimoni elemental és més tòxic que no pas les seves sals. Els compostos de Sb(III) són més tòxics (aproximadament unes 10 vegades) que els de Sb(V), i aquests a la vegada són més tòxics que els compostos organometàl·lics d'antimoni [Zheng et al., 2000, Filella et al., 2002a]. Alguns estudis han demostrat que el Sb(III), a diferència del Sb(V), presenta afinitat pels glòbuls vermells i pels grups tiol de les cèl·lules [Ulrich, 1998].

La quantitat d'antimoni que s'ingereix entra a la sang després d'unes hores, i la que es troba present als pulmons hi arriba després de diversos dies o setmanes. Des de la sang, l'antimoni va cap al fetge, els pulmons, els intestins i la melsa i s'excreta després de diverses setmanes.

Aquest element es troba present a nivells inferiors a 1 $\mu g\ g^{-1}$ en els teixits de l'ésser humà. Els pulmons, els ganglis limfàtics i el cabell solen contenir les majors quantitats [Filella et al., 2002a].



hidròxid d'antimoni (V)

hidròxid d'antimoni (III)

triòxid d'antimoni

trisulfur d'antimoni

pentòxid d'antimoni

estibina

metilantimoni

dimetilantimoni

trimetilantimoni

òxid de trimetilantimoni

Figura 1.4. Principals compostos d'antimoni.

El contacte de la pell amb l'antimoni a llarg termini pot provocar dermatitis. L'exposició a 9 mg de Sb per metre cúbic d'aire durant un temps pot causar irritació als ulls, la pell i els pulmons. Respirar uns 2 mg m^{-3} de Sb durant llarg temps pot causar problemes pulmonars (com la pneumoconiosis), cardíacs (irregularitat en el ritme), dolors estomacals (fins i tot úlceres), diarrea i vòmits. Estudis demostren que la ingesta de més de 19 mg kg^{-1} de Sb causa vòmits. No es sap quins altres efectes causa l'antimoni sobre la salut de les persones. S'està estudiant si és cancerigen, provoca defectes congènits o afecta a la reproducció. En alguns estudis s'ha observat l'aparició de càncer de pulmó en rates que van respirar altes concentracions d'antimoni. L'Agència Internacional per a la Investigació del Càncer (IARC) classifica el Sb_2O_3 i el $SbCl_3$ com a substàncies sospitoses de ser carcinògenes pels humans. Les persones que han consumit molta quantitat de Sb com a medicina han experimentat efectes negatius en la seva salut quan aquest va ésser injectat a la sang o als músculs [ASTDR, 1992].

1.3.5. Nivells d'antimoni en aigües

Tot i que les concentracions d'antimoni en PET són significativament elevades, la concentració de Sb en els corresponents aliments envasats és molt inferior. A la bibliografia, la gran majoria de treballs sobre presència d'antimoni en aliments envasats en PET es centra en aigües. Les concentracions d'antimoni en aigües embotellades són majoritàriament inferiors a 1 µg L^{-1}, tot i que en alguns casos es poden trobar valors una mica més elevats. En tots aquests estudis es conclou que la presència de Sb en aigua embotellada és deguda a la seva migració des del PET, ja que s'ha demostrat que les aigües provinents de fonts, pous o envasades en altres tipus de plàstic, com LDPE, HDPE, PS o PC, no contenen Sb [Shotyk et al., 2006; Shotyk et al., 2007; Ristić et al., 2011; Andra et al., 2012] Tot i així, existeixen algunes excepcions: hi ha un estudi que ha trobat concentracions de Sb de fins a 127 µg L^{-1} en aigua d'aixeta i en afluents provinents d'una regió específica, la qual es troba altament contaminada d'antimoni [Ghassemzadeh et al., 2006].

El consum d'aigua embotellada és un fet comú en la societat d'avui dia. A partir de l'any 2002, el sector industrial de l'aigua embotellada ha experimentat un creixement anual del 7,9%. A l'any 2008, el consum global d'aigua embotellada va arribar a la xifra de 200 bilions de litres. Degut al creixent consum d'aigua embotellada i a la toxicitat de l'antimoni, s'han establert unes recomanacions sobre la ingesta diària d'antimoni. L'Organització Mundial de la Salut va suggerir en un article de l'any 2003 una ingesta mitjana de 6 µg per quilogram de pes corporal al dia de Sb en aigua de beguda. Per altra banda, donat que aquest valor es considera bastant alt, el Departament Federal de la Salut de Canadà ("Health Canada") va estimar al 2008 una ingesta diària de 2,8 µg, la qual s'ha calculat a partir del valor mitjà de Sb en aigües i d'un consum mitjà diari d'aigua de 1,5 L.

Per una altra part, diverses institucions han establert uns nivells màxims permesos d'antimoni en aigua de beguda:

- Unió Europea: 5 µg L^{-1} [EU, 2003].
- USEPA, i "Health Canada": 6 µg L^{-1} [EPA, 2009; Health Canada, 1997].
- Japó: 1,5 µg L^{-1} [MHLW, 2005].
- Organització Mundial de la Salut: 20 µg L^{-1} [WHO, 2003a].

1.3.6. Assajos de migració en PET: legislació

1.3.6.1. Simulants alimentaris

D'acord amb el Reglament 1935/2004 de la Comissió Europea [Regulation 1935/2004/EC], els materials i objectes destinats a entrar en contacte amb els aliments, com el plàstic, han de ser fabricats seguint una sèrie de requisits per tal d'assegurar la protecció de la salut dels consumidors. Hi ha requisits específics per a cada tipus de material que han de garantir que no es transfereixin els seus components als aliments, i que tampoc causin canvis ni en la seva composició, ni en les seves característiques organolèptiques. Pel cas del plàstic, aquests requisits es troben establerts en el Reglament 10/2011 [Regulation 10/2011]. Per tant, el plàstics han de complir amb la Directiva 2002/72/CE, la qual inclou límits globals de migració (OML) i els límits de migració específica (SML) en monòmers i additius, junt amb la seva quantitat màxima permesa en el material acabat (QM). En begudes envasades en PET, el límit de migració específica (SML) pel Sb_2O_3 d'acord amb la present Directiva és de 0,04 mg kg^{-1} com a Sb total. En cap cas hi ha referències en quant a possible legislació sobre especiació de Sb.

Els paràmetres OML i SML per a un compost en un aliment específic que es troba en contacte amb un determinat plàstic s'estableixen mitjançant uns assajos de migració. Aquests assajos es realitzen amb uns determinats simulants alimentaris, resumits a la Directiva 85/572/EEC [Directive 85/572/EEC] els quals estan relacionats amb el tipus d'aliment. Tanmateix, les condicions de l’assaig estan relacionades amb les condicions reals d’ús de l’aliment envasat. Aquestes s'estableixen a la Directiva de la UE 97/48/CE [Barnes et al., 2007; Directive 97/48/EC]. A mode d’exemple, en el cas dels aliments aquosos amb pH ≤ 4,5 el simulant alimentari corresponent és l'àcid acètic al 3%. Les condicions de contacte del plàstic amb el simulant (temperatura i temps) varien d'acord amb les condicions reals de conservació del aliment assajat. L’assaig s’ha de realitzar a la temperatura i temps més alts en els quals l’aliment hi pugui estar sotmès durant tot el seu període d’emmagatzematge.

A la bibliografia s’han trobat diversos treballs en els quals s’ha avaluat l’extracció d’antimoni en plàstics utilitzant els assajos descrits a la Directiva 82/711/CE amb diferents finalitats. [Thompson et al., 1997; Takahashi et al., 2008; Welle et al., 2011; Rungchang et al., 2013; Haldimann et al., 2013; Sánchez-Martínez et al., 2013; De Jesus et al., 2016]. En el primer cas, Thompson et al., 1997, van avaluar dues estratègies per comparar la migració de diversos metalls, entre ells el Sb, en mostres de PET i de LDPE: els assajos estipulats a la Directiva 82/711/CE i un nou mètode d’Anàlisi d’Activació de Neutrons (NAA) amb el qual s’irradiaven les mostres abans de la seva exposició al simulant alimentari corresponent. No es van observar diferències entre les dues estratègies.

Takahashi et al., 2008, Sánchez-Martínez et al., 2013 i De Jesus et al., 2016 van aplicar aquests assajos en recipients de PET per tal d’avaluar els SML. En el primer cas, es van trobar alguns valors molt proper al límit, mentre que els dos darrers autors van trobar valors més baixos i llunyans a aquest.

Per altra banda, Welle et al., 2011, Rungchang et al., 2013 i Haldimann et al., 2013 proposen diferents models teòrics de migració per tal de predir la concentració de Sb en begudes o menjars precuinats envasats en PET en funció del temps i la temperatura d’emmagatzematge. Les variables incloses en els corresponents models són la forma de l’envàs (també considerada com a relació superfície / volum), la concentració de Sb en el PET, el coeficient de difusió i l'energia d'activació. Aquests dos últims paràmetres es van establir experimentalment mitjançant els assajos de migració i els simulants alimentaris esmentats. Les concentracions obtingudes a partir del model matemàtic es corresponen als valors experimentals de migració trobats per altres autors. Aquests últims estudis demostren que la migració d'antimoni del PET als aliments o begudes es troba afectada per diverses variables, les quals són un objecte d'estudi interesant.

1.3.6.2. Migració en aliments i variables que afecten a la migració

Els estudis de migració d'antimoni del PET cap a les begudes i aliments donen una informació molt útil considerant el risc potencial associat amb la seva aparició i existència sota les diferents condicions d'emmagatzematge. La gran majoria d'aquests estudis s'han realitzat en mostres d'aigua de consum. A la bibliografia, s'ha trobat que hi ha diversos factors fisicoquímics que poden tenir una influència en la migració de Sb total, com per exemple el temps d'emmagatzematge, la temperatura, la irradiació amb llum natural o artificial, el color del PET, la superfície de contacte i les característiques de la matriu (pH, contingut de CO_2 i contingut de sals).

La variable més estudiada és el temps d'emmagatzematge (des de 3 mesos fins a un any i mig), principalment quan les mostres es troben a temperatura ambient i també a baixa temperatura (2ºC). Tots els estudis trobats a la bibliografia van determinar que la concentració de Sb en l'aigua mineral augmentava amb el temps, incrementant els valors entre un 25% o un 140%. Tot i així, els nivells màxims permesos no es sobrepassen a la majoria de casos [Shotyk et al., 2006; Shotyk et al., 2007; Westerhoff et al., 2008; Keresztes et al., 2009; Perić-Grujić et al., 2010; Reimann et al., 2010; Ristić et al., 2011; Andra et al., 2012; Hureiki et al., 2012; Reimann et al., 2012; Tukur et al., 2012; Aghaee et al., 2014; Brandao et al., 2014].

L'altre paràmetre que ha estat més estudiat és la temperatura i els efectes descrits són variables. S'han realitzat estudis en mostres d'aigua emmagatzemades a temperatures des de 30ºC fins a 80ºC, durant un temps des de unes hores fins a un any i mig. Per una banda, alguns treballs han determinat que es produeix un lleuger augment en la concentració de Sb a temperatures inferiors o iguals a 65ºC [Westerhoff et al., 2008; Keresztes et al., 2009; Hureiki et al 2012; Tukur et al., 2012; Aghaee et al., 2014], mentre que a temperatures de 80ºC, s'ha observat una migració ràpida del Sb després d'un temps de 5 a 24 hores d'emmagatzematge [Westerhoff et al., 2008; Tukur et al., 2012; Aghaee et al., 2014]. Per altra banda, altres autors van demostrar que la migració ràpida de Sb es produeix a partir dels 60ºC de temperatura a partir de 7 dies d'emmagatzematge [Reimann et al,. 2012, Bach et al., 2013].

La resta de variables ha estat estudiada amb menys profunditat. Pel que fa al volum de l'envàs, en tots els casos en què es va estudiar aquesta variable es va determinar que es produeix una major migració en volums d'envàs petits, ja que la superfície de contacte entre l'aigua i el recipient és més elevada [Keresztes et al., 2009; Hureiki et al., 2012]. També s'ha estudiat en alguns casos l'efecte que té la reutilització d'un envàs en la migració de Sb. En els pocs casos estudiats s'han trobat evidències contradictòries: concentracions de Sb més elevades [Andra et al 2011] o cada cop més inferiors [Tukur et al., 2012] com més es reutilitza l'envàs.

Pel que fa a la resta de variables (l'efecte de la irradiació solar, el color del PET, el pH de l'aigua, el contingut de CO_2, i el contingut de sals), els resultats obtinguts pels autors no demostren evidències clares o, fins i tot, s'han trobat conclusions contradictòries.

La majoria dels autors van concloure que la irradiació de les mostres amb llum solar (tant natural com artificial) causa un petit però significant increment en la migració de Sb [Keresztes et al., 2009; Tukur et al., 2012; Westerhoff et al., 2008; Aghaee et al., 2014; Perić-Grujić et al., 2010; Andra et al., 2011]. No obstant, Hureiki et al. 2012 no van trobar que la migració estigués afavorida amb la irradiació.

En el cas del color de l'ampolla, el pH o contingut de CO_2 i el contingut de sals, una discussió més exhaustiva sobre els resultats obtinguts pels diferents autors es troba detallada en el capítol 4.1 d'aquesta tesi.

Els estudis de migració d'antimoni en aigües envasades en PET que s'han trobat a la bibliografia es troben resumits a la taula 1.4. Aquesta s'ha centrat principalment en destacar les variables més rellevants: la temperatura i el temps. També descriu els tipus i nombre de mostres analitzats, les concentracions de Sb trobades i alguna altra informació important sobre altres variables que influeixen en la migració d'antimoni.

Pel que fa a la presència d'antimoni en altres tipus de begudes envasades en PET, les quals presenten una matriu més complexa degut a la presència de sals i / o àcids orgànics, hi ha molta menys informació descrita a la bibliografia. S'han trobat estudis sobre la determinació del contingut total d'antimoni en refrescos, vi negre i sucs de fruita. Per una part, les concentracions de Sb trobades en vins, refrescos i sucs de fruita no superen els límits màxims permesos en aigües de beguda per la Unió Europea [Tukur et al., 2012; de Jesus et al., 2016; Shakerian et al., 2014], tot i que els valors en refrescos són superiors als trobats en aigües [Tukur et al., 2012]. Per altra banda, s'han trobat concentracions de Sb superiors a aquest límit en alguns sucs de fruita [Hansen et al., 2010]. En aquest últim estudi es comenta que les concentracions de Sb poden dependre del contingut de carbohidrats del suc i del temps que ha passat des que el suc es troba caducat.

Fins el moment, tots els estudis descrits tracten sobre el contingut d'antimoni total. S'ha trobat un estudi sobre la identificació i quantificació d'espècies d'antimoni en 2 aigües minerals envasades en PET. Aquest treball utilitza com a estratègia la retenció selectiva de Sb(III) en sorbents amb superfície polimèrica, amb posterior desorció i anàlisi per ETAAS. La determinació de Sb(V) es realitza mitjançant la diferència entre Sb total i Sb(III). Els resultats van mostrar la presència de totes dues espècies, amb concentracions al voltant de 0.1 µg L^{-1} per la suma d'espècies [Shakerian et al., 2014]

Pel que fa a altres matrius, s'han trobat alguns estudis en begudes i en altres tipus d'aliments líquids. S'ha determinat la presència de diferents complexos de coordinació de Sb(III) i Sb(V) en sucs de fruita, iogurts i vinagre envasats en PET mitjançant cromatografia de líquids acoblada a plasma acoblat inductivament amb espectrometria de masses (LC-ICP-MS) i cromatografia de líquids acoblada a espectrometria de masses en tàndem (LC-MS/MS). El tipus d'espècies trobades són compostos de Sb(V) sense complexar en forma de $Sb(OH)_6^-$ o complexos d'elevada estabilitat amb diferent nombres de coordinació entre el Sb i citrat, adenosina, lactat [Hansen et al., 2006a; Hansen et al., 2007] i acetat [Sánchez-Martínez et al., 2013].

En quant als estudis de migració en begudes envasades en PET, hi ha un únic treball a la bibliografia que estudia l'efecte de la temperatura (50ºC) durant 14 dies en la migració d'espècies d'antimoni en dos sucs amb i sense gas [Hansen et al., 2006b]. Es va observar un augment més significatiu en la concentració de Sb a les mostres amb gas, atribuït a l'espècie de Sb(III).

Degut als pocs estudis relacionats amb l'especiació d'antimoni en aigües i altres begudes envasats en PET, una major investigació en aquest camp seria un tema d'elevat interès.

Taula 1.4. Resultats de la migració d'antimoni en aigües envasades en PET.

Temperatura	Temps	Matriu	Nombre de mostres	Concentració ($\mu g\ L^{-1}$)	Altres variables	Referències
Temperatura ambient	2 mesos	Aigua comercial de Sudàfrica	Amb gas: N = 11 Sense gas: N = 39	Inicial: 0,012 – 0,358 Final: 0,169 – 0,715	Lleuger efecte de pH	Brandao et al., 2014
25, 31 i 40ºC	8 setmanes	Aigua comercial d'Iran	N = 5	1 – 4,5	-	Aghaee et al., 2014
60 i 80ºC	6 setmanes	Aigua comercial d'Iran	N = 5	60ºC: 3 – 9 80ºC: 9 – 22	-	Aghaee et al., 2014
20, 40, 50 i 60ºC	10 dies	Aigua amb i sense gas Aigua ultrapura envasada en PET	N = 2	20ºC: fins a 1 40ºC: fins a 2 50ºC: fins a 2,5 60ºC: fins a 4 (sense gas) i fins a 8 (amb gas)	Més migració a pH àcid	Bach et al., 2013
23ºC	60 dies	Aigua comercial amb gas, sense gas i sense gas enriquida dels EEUU	N = 23	Sense gas: 0,001 – 0,634 Amb gas: 0,0055 – 0,617 Sense gas enriquida: 0,077 – 1,191	Més migració a pH àcid	Andra et al., 2012
Temperatura ambient	11 mesos	Aigua comercial de Nigèria	N = 11	> de 5	-	Tukur et al., 2012
Temperatura ambient	19 mesos	Aigua comercial del Regne Unit	Amb gas: N = 5 Sense gas: N = 5	Fins a 2,92	No efecte de pH ni de color	Tukur et al., 2012
40, 60 i 80ºC	48 hores	Aigua comercial del Regne Unit	Amb gas: N = 4 Sense gas: N = 2	40ºC: fins a 1 60ºC: fins a 4 80ºC: fins a 23	No efecte de pH	Tukur et al., 2012
22 i 45ºC	544 dies	Aigua comercial del Líban	N = 8	22ºC: 0,2 – 5,1 45ºC: 0,2 – 5,5	Més migració en volums petits. No efecte de la llum del sol. No efecte de pH	Hureiki et al., 2012
2, 22, 45, 60 i 80ºC	1 setmana	Aigua ultrapura envasada en PET	N = 3	2ºC: 0,00276 22ºC: 0,0117 45ºC: 1,24 60ºC: 2,75 80ºC: 10,9	-	Reimann et al., 2012
-	-	Aigua mineral	Sèrbia: N = 9 Resta d'Europa: N = 7	0,12-1,81	-	Ristic et al., 2011

Temperatura	Temps	Matriu	Nombre de mostres	Concentració (μg L^{-1})	Altres variables	Referències
20, 30 i 40ºC	-	Aigua ultrapura envasada en PET	N = 20	Fins a 0.355	Efecte radiació UV Efecte reutilització ampolla	Andra et al., 2011
25ºC	24 hores	Aigua ultrapura envasada en PET	N = 7	0,675-1,652	-	Cheng et al., 2010
25ºC	7 dies	Aigua ultrapura a pH 4 envasada en PET	N = 7	0,049-3,794	Més migració a pH àcid	Cheng et al., 2010
2ºC	150 dies	Aigua ultrapura a pH 3,5 i 6,5 envasada en PET	N = 74	Fins a 0,25	No efecte de pH Efecte color	Reimann et al., 2010
22ºC	3 mesos	Aigua comercial	N = 9	Rang: 0,095 – 0,521 Inicial: : 0,195 ± 0,116 Final: 0,226 ± 0,160	No efecte de pH Efecte color	Westerhoff et al., 2010
40, 60 i 80ºC	7 dies	Aigua comercial	N = 1	40ºC: fins a 0,5 60ºC: fins a 2 80ºC: fins a 14,4	-	Westerhoff et al., 2010
Temperatura ambient	65 dies	Aigua comercial	Sèrbia: N = 9 Resta d'Europa: N = 7	0,15-1,8	-	Perić-Grujić et al., 2010
22ºC	Poc menys d'un any	Aigua mineral amb i sense gas	N = 20	Sense gas: 0,26 ± 0,16 Amb gas: 0,40 ± 0,22	Més migració a pH àcid i a volums petits	Keresztes et al., 2009
40, 50 i 60ºC	24 hores	Aigua mineral sense gas	N = 5	40 – 50ºC: fins a 0,5 60ºC: fins a 2	-	Keresztes et al., 2009
Temperatura ambient	6 mesos	Aigua subterrània comercial de Canadà, Europa i Japó	N = 132	Fins a 0,6	-	Shotyk et al., 2007
-	-	Aigua de font (F) Aigua de beguda (B) Aigua amb gas (G)	F: N = 43 B: N = 6 G: N = 3	F: 0,5 – 5 (3,37 ± 1,95) B: 5 G: 1 – 5 (3,67 ± 2,31)	-	Guler 2007
Temperatura ambient	4 mesos	Aigua subterrània comercial	Canadà: N = 12 Europa: N = 48	0,112-0,375 Canadà 0,006-0,725 Europa	-	Shotyk et al., 2006

1.4. ANÀLISI DE MERCURI I ANTIMONI: PROBLEMÀTICA I SITUACIÓ ACTUAL

1.4.1. Tècniques instrumentals de mesura: determinació del contingut total

Existeix un ampli ventall de tècniques analítiques per a la determinació d'antimoni i mercuri total en mostres aquoses o líquides, així com extractes de mostres sòlides. Els sistemes de detecció més utilitzats per el seu anàlisi són les tècniques d'espectroscòpia atòmica, entre les quals es troben l'espectroscòpia d'absorció atòmica (AAS), l'espectroscòpia de fluorescència atòmica (AFS), i el plasma acoblat inductivament, amb espectroscòpia d'emissió atòmica o òptica (ICP-AES – ICP-OES) o espectrometria de masses (ICP-MS).

Els sistemes de detecció per AAS i per AFS són dels més emprats per a la determinació d'antimoni i mercuri en diversos tipus de mostres tot i que presenten limitacions en quant a sensibilitat. No obstant, la incorporació d'una etapa de generació d'hidrurs, pel cas de l'antimoni, o de vapor fred, pel cas del mercuri, anterior a la detecció proporciona resultats excel·lents en termes de sensibilitat i selectivitat per aquests analits, en especial pel cas de la fluorescència. D'aquesta manera, els sistemes acoblats de HG-AFS i CV-AFS presenten uns límits de detecció comparables als obtinguts per les tècniques més sensibles, com l'ICP-MS. Per altra part, s'han trobat diversos treballs que fan servir el forn de grafit (ETAAS) per a la determinació d'aquests elements en aigües, plantes, vapors i mostres biològiques [Koch et al., 1997; Bermejo-Barrera et al., 1999; Shiowatana et al., 1999; Saber-Tehrani et al., 2007; Hagarova et al., 2012]. Aquesta tècnica ofereix una sensibilitat més baixa que no pas HG-AFS o CV-AFS. Addicionalment, uns pocs autors s'han dedicat a la determinació d'antimoni acoblant la generació al sistema ETAAS per a la determinació d'antimoni en mostres d'aigua [Moreda-Piñeiro et al., 2001a; Moreda-Piñeiro et al., 2001b; Freschi et al., 2005], entre altres matrius, obtenint resultats comparables i del mateix ordre als obtinguts per HG-AFS.

Els sistemes detecció de ICP-AES i ICP-MS són les eines més emprades per a l'anàlisi multielemental. Proporcionen una resposta ràpida, robustesa i elevada selectivitat. Pel que fa a la tècnica de ICP-AES, la sensibilitat que proporciona no és l'adequada per la determinació d'alguns analits, com l'arsènic, el plom, l'antimoni, el seleni i el mercuri ja que els límits de detecció són elevats i no permeten una determinació acurada en la majoria de mostres. Per altra banda, l'ICP-MS avui dia representa la millor tècnica per a la determinació de metalls i semimetalls a nivells ultratraça en un ampli ventall de mostres. Alguns treballs han aconseguit arribar a límits de detecció de l'ordre de ng L^{-1} [Krachler et al., 2004]. L'acoblament del sistema HG o CV a l'ICP-MS és una aproximació que a priori hauria d'incrementar la sensibilitat de detecció. No obstant, l'eficiència d'aquest pas de derivatització sovint és problemàtic en matrius complexes [Zhang et al., 1996] tot i que alguns autors han aconseguit bons resultats [Santosa et al., 1997; Liu et al., 2013; Balarama-Krishna et al., 2012a; Kenduzler et al., 2012; Pyhtilae et al., 2012].

Tots aquests fets donen lloc a l’aplicació d’aquestes tècniques per a la determinació d’antimoni i mercuri en una gran varietat de mostres ambientals, alimentàries i biològiques. S’han reportat treballs recents amb resultats satisfactoris en la determinació d’antimoni per HG-AFS en mostres com aigües, sòls, plantes i bolets [De Gregori et al., 2001; Ferreira et al., 2009; Wang et al., 2010a; Liu et al., 2012], i per ICP-MS en aquests mateix tipus de mostres i també en matrius biològiques com sang, cabell i orina [Garbos et al., 2000; Miekeley et al., 2002; Tamasi et al., 2008; Telford et al., 2008; Rahman et al., 2012]. També s’han reportat treballs recents en la determinació de mercuri per CV-AFS en mostres com aigües, peixos, teixits humans i combustibles [Carvalho et al., 2008; Aranda et al., 2009; Torres et al., 2014; Straka et al., 2016] i per ICP-MS en aquest tipus de mostres i també en matrius com l’arròs, plantes i altres teixits biològics [Balarama-Krishna et al., 2012b; Ebrahim et al., 2012; Rahman et al., 2012; Drennan-Harris et al., 2013; Ostertag et al., 2013; Jallad, 2015; Herrmann et al., 2016].

Pel cas del mercuri, la introducció d’Analitzadors de Mercuri, basats en una detecció per AAS, ha estat realitzada durant els últims anys. Aquests instruments són espectròmetres d’absorció atòmica dissenyats específicament per a una ràpida, segura i precisa determinació de traces de mercuri en diverses matrius sòlides o líquides. Es basen en la combustió de la matriu sense pretractament o preconcentració de mostra, seguida de la formació d’un amalgama de mercuri amb or que atrapa selectivament el mercuri, rebutjant així altres productes de la combustió que hi podrien interferir. Posteriorment, l’amalgama s’escalfa i el mercuri s’allibera ràpidament cap el sistema de detecció. D’aquesta manera, es proveeix un temps d’anàlisi inferior a 5 minuts i un baix cost, inferior als convencionals CV-AFS o ICP. Aquest sistema s’ha aplicat recentment en una gran varietat de matrius sòlides, especialment peixos, bolets, sòls i altres matrius biològiques [Khoshnamvand et al., 2013; Sysalova et al., 2013; Sedlackova et al., 2014; Sipkova et al., 2014; Borosova et al., 2014; Knezovic et al., 2016].

Existeixen altres estratègies per a la determinació d’antimoni i mercuri, basades en tècniques electroanalítiques: Voltametria de Redissolució Anòdica (ASV) i Catòdica (CSV) i Polarografia Diferencial d’Impulsos (DPP). Els avantatges que presenten aquestes tècniques són la seva elevada selectivitat i sensibilitat, oferint límits de detecció baixos.

1.4.2. Tècniques acoblades: determinació d’espècies

L’ús de les tècniques acoblades basades en la combinació d’un mètode de separació amb un sistema de detecció adequat representa una de les eines més fiables i emprades per a l’especiació analítica de les diferents formes d’un element. Pel que fa a les estratègies emprades en l’especiació d’un determinat element, com és el cas de l’antimoni i el mercuri, existeix molta informació a la literatura degut al creixent interès científic en l’estudi de les diverses formes analítiques existents o espècies d’un element concret.

El desenvolupament de metodologia analítica per a l'especiació d'antimoni s'ha dut a terme en una extensió menor comparat amb el cas del mercuri o el d'altres elements com l'arsènic o el seleni. Pel cas de l'antimoni, s'ha trobat a la literatura alguns mètodes basats en la determinació de Sb(III) i l'estimació de la concentració de Sb(V) com la diferència entre l'antimoni total i el contingut de Sb(III), i viceversa [Garbos et al., 2000; Shakerian et al., 2014]. No obstant, aquests mètodes no són adequats per a l'especiació en matrius complexes. Pel que fa al mercuri, existeixen diverses estratègies. Algunes d'elles es basen en la separació de mercuri inorgànic i organometàl·lic, sense discriminar entre espècies [Ramalhosa et al., 2008], mentre que d'altres sí que permeten la determinació individual de cada espècie.

D'aquesta manera, el desenvolupament de tècniques acoblades per identificar i/o quantificar les espècies de mercuri i antimoni amb una sensibilitat adequada és un tema d'elevat interès per tal de determinar la seva distribució en una matriu determinada. A més, l'especiació desenvolupa un paper important en els estudis de la toxicitat i exposició d'un determinat element en organismes vius aportant informació de gran rellevància. L'espècie química també influeix en el seu propi transport dins l'organisme cap els teixits en els quals té efectes tòxics, així com la seva acumulació i modificació.

L'estratègia més utilitzada i més fiable per a l'especiació analítica d'antimoni i mercuri entre les diferents emprades consisteix en l'acoblament en línia de les tècniques cromatogràfiques amb detectors d'espectroscòpia atòmica, entre els quals es troben els sistemes esmentats a l'apartat anterior (AAS, AFS, ICP) o l'espectrometria de masses (MS). També existeixen tècniques de separació no cromatogràfiques, com l'electroforesi capil·lar (CE), emprades en menor extensió en aquest camp.

1.4.2.1. Tècniques cromatogràfiques per l'especiació

Pel que fa les tècniques cromatogràfiques, s'ha trobat que la cromatografia de líquids (LC) és la més emprada per a l'especiació d'antimoni en front de la cromatografia de gasos (GC), mentre que pel mercuri, les dues tècniques han estat àmpliament utilitzades, tot i que recentment, molts treballs opten per l'ús de LC en front de GC ja que no es requereix una etapa de derivatització.

Els mètodes d'especiació basats en l'ús de la GC s'apliquen en espècies que són volàtils o que poden formar compostos volàtils i comporten alguns o tots els passos següents: derivatització en espècies volàtils, separació cromatogràfica, piròlisi per atomitzar i detecció [Ramalhosa et al., 2008].

Per a la determinació d'espècies no volàtils, aquestes s'acostumen a reduir prèviament amb borohidrur de sodi ($NaBH_4$) per tal de formar, en el cas de les espècies d'antimoni i les organometàl·liques de mercuri, l'hidrur volàtil corresponent, i en el cas del mercuri inorgànic, el vapor de mercuri elemental [Kock et al., 1998; Craig et al., 1999; Hirner et al., 1998; Tseng et al., 1998; Puk et al., 1994a; Ritsema et al., 1994]. Aquest mètode té com a avantatge la generació d'hidrogen, el qual té una funció de purga dels derivats formats en entrar cap a la columna [Tseng et al., 1998; Puk et al., 1994b] i com a inconvenient la possible formació de radicals en presència d'oxigen o d'àcid nítric, els quals dificultarien la posterior detecció [Reuther et al., 1999]. Pel que fa a les espècies orgàniques d'antimoni, com les esmentades a l'apartat 1.3.4, aquestes són volàtils i poden ésser fàcilment separades per GC sense derivatització prèvia. En el cas del Hg, un altra variant molt utilitzada en diversos treballs és l'etilació, en la qual les espècies de mercuri es derivatitzen en compostos dialquilats emprant tetraetilborat de sodi ($NaBEt_4$) [Wasik et al., 1998]. Existeixen també agents derivatitzants com altres borats o reactius de Grignard [Ramalhosa et al., 2008].

Pel que fa a les columnes emprades, s'ha trobat l'ús tant de columnes de rebliment com capil·lars, i fins i tot multicapil·lars, les quals resulten de la unió de diverses columnes capil·lars en paral·lel i permetre així una injecció de volums més alts [Pereiro et al., 1998]. Les fases estacionàries més utilitzades són les formades per silicones o polimetilsiloxans. Després de la separació, és necessària una etapa de piròlisi per tal de formar antimoni o mercuri elemental i així ésser conseqüentment atomitzat, excitat i detectat. Si al sistema de detecció és fa servir un plasma, aquest ja fa la funció de piròlisi, de manera que aquesta etapa no seria necessària. [Prange et al., 1995; Reuther et al., 1999]. La cromatografia de gasos acoblada a detectors d'espectroscòpia atòmica i espectrometria de masses ha estat aplicada per la determinació de mercuri en una àmplia varietat de mostres alimentàries, ambientals i biològiques, mentre que pel cas de l'antimoni, ha estat bàsicament aplicada a cultius de microorganismes [Jenkins et al., 1998; Andrewes et al., 1999a; Andrewes et al., 1999b], fongs [Koch et al., 1998], plantes [Craig et al., 1999], i aigües de diferents orígens [Hirner et al., 1998] per a l'estudi sobre la producció biològica de compostos volàtils d'aquest element.

El fet d'haver d'incloure una etapa de derivatització a elevada temperatura dels compostos d'antimoni o de mercuri quan la separació es realitza amb GC ha portat a desenvolupar mètodes de separació mitjançant la cromatografia de líquids. La LC permet un tractament més senzill de la mostra que en el cas de la GC, treballa a temperatura ambient i les pèrdues d'analit així com el temps d'anàlisi són menors. A més, la LC ofereix una gran versatilitat, ja que existeix una àmplia gamma tant de fases estacionàries com mòbils que es poden variar per tal de separar una gran varietat d'espècies, volàtils i no volàtils. Els mètodes d'especiació mitjançant LC consten d'alguns o de tots els passos següents: preconcentració dels analits, separació cromatogràfica, oxidació o reducció post-columna i detecció [Ramalhosa et al., 2008].

En diverses ocasions, l'anàlisi de determinades mostres requereix la incorporació d'una etapa de preconcentració prèvia a la separació cromatogràfica per tal d'assolir nivells de concentració quantificables. A la literatura, la preconcentració s'ha aplicat en mostres ambientals, essencialment en aigües, tal i com es descriurà de manera més extensa a l'apartat 1.4.3.

Pel que fa a la separació cromatogràfica, s'han trobat estratègies diferents a la literatura. Pel cas del mercuri, principalment s'han trobat treballs en fase inversa, en les que la fase estacionària és una columna amb octadecilsilà (C18) i la fase mòbil consisteix en una mescla d'un solvent orgànic polar i una solució amortidora o de sals que controlin la naturalesa dels analits. Els solvents orgànics més utilitzats són el metanol i l'acetonitril, mentre que per les solucions amortidores són les mescles àcid acètic / acetat [Ramalhosa et al., 2008]. Tanmateix, s'afegeixen agents complexants a la fase mòbil per tal de formar complexos estables amb les espècies de mercuri, i així proporcionar una sensibilitat i selectivitat adequada [Wilken et al., 1991]. Els complexants més utilitzats són el 2-mercaptoetanol, la cisteïna i els ditiocarbamats [Ramalhosa et al., 2008]. També es fa servir en alguns casos la cromatografia iònica per a l'especiació de mercuri. S'ha demostrat que l'ús de sals d'amoni quaternari com a formador de parells iònics és un mètode efectiu per a la separació tant de mercuri inorgànic com de compostos organomercúrics [Yin et al., 1998].

En el cas de l'especiació d'antimoni, la LC s'ha fet servir principalment per a la separació de les formes inorgàniques, Sb(V) i Sb(III). Aquesta es realitza majoritàriament en una columna de bescanvi aniònic fort. Entre els mètodes trobats a la bibliografia, la fase mòbil representa la principal diferència en quant a la separació. En general, es podria dir que la elució del Sb(V) es pot aconseguir fàcilment amb diferents tipus de fases mòbils, ja que presenta poca interacció amb aquest sistema cromatogràfic. No obstant, el principal problema es troba en la separació del Sb(III), ja que s'obté un pic molt ample amb la majoria d'eluents. Per això, una alternativa freqüent consisteix en fer servir fases mòbils complexants, com l'àcid etilendiamintetraacètic (EDTA), o el tartrat d'amoni. En aquestes condicions, el Sb(III) forma un complex amb una bona interacció amb el sistema cromatogràfic, el que permet millorar la forma dels pics i separar-lo del Sb(V) en un temps de retenció més curt [Miravet et al., 2010]. Per altra banda, la separació simultània de les espècies inorgàniques i organometàl·liques d'antimoni presenta dificultats degut al diferent comportament que aquestes presenten en solució. L'ús de fases mòbils alcalines, com l'hidròxid de potassi (KOH) o de tetrametilamoni (TMAOH), amb una columna de bescanvi aniònic permet una bona separació del Sb(V) i les espècies metilades però no del Sb(III), ja que es queda altament retingut [Lintschinger et al., 1998]. Addicionalment, s'han obtingut resultats poc satisfactoris en estudis realitzats amb altres tipus de columnes cromatogràfiques com ara de bescanvi aniònic feble i de bescanvi catiònic, on els compostos metilats d'antimoni no són retinguts [Nash et al., 2006].

Després de la separació, es requereix que l'antimoni o el mercuri arribi al detector en forma elemental. Per tal d'assolir aquest objectiu, es fan servir etapes d'oxidació o reducció. Aquestes consisteixen en la incorporació de sistemes que facin reaccionar les espècies a una forma capaç de ser atomitzada al sistema de detecció. Les estratègies emprades més sovint són l'ús de la generació d'hidrurs o de vapor fred, esmentades a l'apartat anterior. Pel cas del mercuri, de vegades és necessari primer una oxidació prèvia a la reducció per generar el vapor de mercuri, especialment pel cas de les espècies organometàl·liques. Si al sistema de detecció es fa servir un plasma, aquest ja és suficient per tal de produir l'atomització de les espècies, de manera que aquesta etapa no seria necessària en principi.

La cromatografia de líquids acoblada a detectors d'espectroscòpia atòmica i espectrometria de masses ha estat aplicada per la determinació de mercuri i antimoni en mostres alimentàries, ambientals i biològiques, com ara aigües [Kozak et al., 2008], plantes [Mueller et al., 2009], sòls i sediments [Telford et al., 2008; Ge et al., 2013; Leng et al., 2013; Yang et al., 2015], aliments [Pilz et al., 2011; Koplik et al., 2014], i en aplicacions biològiques [Mantrangolo et al., 2013; Wang et al., 2013; Truempler et al., 2014].

Per altra banda, la detecció per MS ha estat utilitzada per a obtenir informació estructural sobre espècies organometàl·liques, en especial per a compostos d'antimoni desconeguts presents en mostres biològiques i aliments [Hansen et al., 2008]. Tot i que els sistemes d'espectrometria de masses amb ionització per electrosprai com a font de ionització (ESI-MS) d'avui dia proporcionen una elevada sensibilitat, aquesta no és suficientment elevada per a la detecció de les espècies presents en matrius biològiques, de manera que en els exemples esmentats s'ha treballat amb mostres fortificades. D'aquesta manera, és necessària l'aplicació d'un pretractament i preconcentració de mostra adequats, tals que no canviïn la integritat de les espècies, per tal d'arribar als límits de detecció de la tècnica.

1.4.3. Determinació de mercuri i antimoni a nivells traça: preconcentració

El medi aquàtic, tal i com s'ha comentat anteriorment, pot presentar diferents contaminants entre els qual es troben els metalls pesants. La presència d'aquests metalls es sol trobar a uns nivells de concentració traça o ultratraça, els quals poden ser significatius des del punt de vista toxicològic pels éssers vius

Les concentracions de mercuri en aigua no són elevades. Tal i com s'ha comentat a l'apartat 1.2.4 la mitjana de Hg total en aigua és de 2 ng L^{-1}. La major part del mercuri correspon a mercuri (II), ja que les formes inorgàniques prevalen en aquest medi en front de les organometàl·liques.

Pel que fa a les concentracions d'antimoni trobades tant en aigua natural com en aigua de beguda, aquestes són generalment molt baixes (inferiors a 1 µg L^{-1}), tal i com s'ha comentat a l'apartat 1.3.5. A més, en quant a l'especiació, s'espera que els nivells de les formes pentavalents siguin molt més elevats que no pas els de les formes trivalents, ja que aquesta última espècie és inestable en aquest medi, tal i com s'ha comentat a l'apartat 1.3.4.

Tots aquests nivells de concentracions de Hg i de Sb no són suficientment elevats per arribar a ésser detectats directament per tècniques atòmiques com la generació d'hidrurs o de vapor fred acoblats a l'espectroscòpia d'absorció / de fluorescència atòmica (HG-AAS; HG-AFS; CV-AAS; CV-AFS). Per tant, es requereixen mètodes d'elevada sensibilitat per tal d'arribar a determinar aquestes concentracions baixes d'antimoni i mercuri, el que fa necessari el desenvolupament de sistemes de preconcentració.

A la bibliografia, s'han trobat diversos treballs sobre mètodes d'extracció i preconcentració d'espècies de mercuri i d'antimoni a nivells traça. Les principals estratègies per a la preconcentració d'aquestes espècies són l'extracció líquid-líquid (LLE) [Bozkurt et al., 2011, Serafimovska et al., 2011a; Serafimovska et al., 2011b] i l'extracció en fase sòlida (SPE). Comparant les dues estratègies, el SPE és una extracció més respectuosa amb el medi ambient ja que no comporta l'ús de solvents orgànics com a agents extractants. A més, el seu potencial per a la complexació dels analits en diversos tipus de matrius la fa adequada per a la seva aplicació en línia [Jia et al., 2012]. En quant a l'extracció en fase sòlida com a etapa de preconcentració, la fase estacionaria més utilitzada ha estat la C18 pel cas del mercuri, mentre que per l'antimoni, s'han fet servir principalment diferents tipus de fases modificades.

La retenció de les espècies de mercuri s'ha realitzat tant de forma directa com amb l'ús de diferents agents complexants per a la seva derivatització, la majoria dels quals contenen àtoms de sofre a la seva estructura. Aquests corresponen a compostos de 2-mercaptoalcohols, ditiocarbamats, ditizones, triazens, i fins i tot bactèries. Pel que fa a la retenció d'espècies d'antimoni, la principal estratègia utilitzada consisteix en la retenció de l'espècie Sb(III) i la retenció de l'antimoni total mitjançant una reducció prèvia de la resta d'espècies a Sb(III). El contingut de Sb(V) s'estima per diferència del Sb total i Sb(III). Per una banda, s'ha realitzat la retenció en presència de pirrolidinditiocarbamat d'amoni (APDC). Per altra banda, aquesta s'ha fet de forma directa en fases modificades, ja sigui en suspensió o en un suport sòlid. Aquestes modificacions s'han fet principalment en presència de sofre.

Pel que fa a l'etapa d'elució, s'ha utilitzat una àmplia varietat de solucions per tal de desorbir les espècies de la fase estacionària. Pel que fa al mercuri, aquestes corresponen principalment a dissolucions àcides, dissolucions de tiourees, fases mòbils amb una quantitat determinada de modificador orgànic, solucions aquoses de diferents agents que contenen sofre, i fins i tot mescles d'aquestes darreres amb solvents orgànics. En el cas de l'antimoni, s'han utilitzat majoritàriament dissolucions àcides diluïdes, en presència de modificadors orgànics en alguns casos, tot i que també s'han fet servir dissolucions bàsiques.

Alguns treballs, després de l'elució descriuen l'aplicació d'una etapa de separació de les espècies de mercuri, com per exemple, la cromatografia de gasos o de líquids. En el cas de l'antimoni no s'ha aplicat la cromatografia en cap cas. En altres casos s'ha aplicat una retenció o elució selectiva de les espècies de mercuri o antimoni fent servir diferents agents complexants o eluents per cada espècie en concret.

En quant a la quantificació final de les espècies de mercuri o d'antimoni, s'han emprat diversos tipus de detectors, ja sigui després de la preconcentració en línia (online) o fora de línia (offline). Els sistemes de detecció més rellevants són l'absorció molecular Ultraviolada-Visible, l'ICP-MS, HG-AAS, HG-AFS, CV-AAS i CV-AFS. Entre l'absorció atòmica (AAS) i la fluorescència atòmica (AFS), es troba que el segon sistema de detecció és més sensible que no pas el primer perquè el senyal de fluorescència presenta un soroll de fons més baix, més selectivitat i linealitat en un ampli interval de concentracions. Entre tots els mètodes de detecció esmentats, l'ICP-MS és el més sensible. No obstant, un sistema de preconcentració en línia acoblat a la detecció CV-AFS o HG-AFS pot no solament proveir una sensibilitat equivalent, sinó que també comporta una senzilla posada a punt del mètode i un cost inferior.

Les taules 1.5 i 1.6 mostren els treballs sobre preconcentració d'espècies de mercuri i d'antimoni en aigües trobats a la bibliografia, respectivament. Aquestes resumeixen les espècies de mercuri i antimoni analitzades, el tipus d'aigua, l'agent complexant emprat, el volum de mostra, el tipus de preconcentració, les condicions de retenció i elució, la tècnica instrumental i els límits de detecció obtinguts. Existeixen nombrosos treballs no només sobre aigua, sinó que també s'han trobat aplicacions a altres matrius, especialment pel cas de l'antimoni. Per tant, en aquest cas s'ha decidit centrar la recerca en els treballs publicats a partir de l'any 2009, aplicats principalment a aigües de diferent naturalesa.

Taula 1.5. Metodologies per la preconcentració d'espècies de mercuri en aigua.

Espècies de mercuri	Matriu	Agent complexant	Volum mostra (mL)	Preconcentració	Retenció/Elució	Mètode instrumental	LD (ng L^{-1})	Recuperació (%)	Referència
MeHg$^+$ Hg(II)	Aigua d'aixeta Aigua de riu Aigua d'estuari	-	10	Offline	Retenció mitjançant fibra buida-LPME	LC-ICP-MS	110 230	71-99	Moreno et al., 2013
MeHg$^+$ Hg(II)	Aigua fortificada	-	50	Offline	Retenció mitjançant SPE i elució amb HCl 0,1 mol L^{-1} pel MeHg$^+$ i HCl 3 mol L^{-1} + tiourea 0,2 mol L^{-1} pel Hg(II)	CV-AAS	0,56 0,44	-	Türker et al., 2013
MeHg$^+$ EtHg$^+$ Hg(II)	Aigua de mar	-	30	Online	Retenció en microcolumna de bescanvi catiònic i elució amb MeOH: L-cisteïna 10 mmol L^{-1} a pH 8 (4:96)	LC-ICP-MS	0,016 0,008 0,042	87-102	Jia et al., 2012
Hg(II)	Aigua d'aixeta Aigua de riu Aigua mineral	5,10,15,20-tetra-(4-fenoxifenil)porfirin (TPPP)	20	Offline	Extracció líquid-líquid	Fluorescència	80	98	Bozkurt et al., 2012
MeHg$^+$ Hg(II)	Aigua embotellada Aigua d'aixeta	Dietilditiocarbamat (DDTC)	5,3	Online	Retenció en reactor nuat i elució amb DDTC, irradiació UV i argó	CV-AFS	4 4	90-110	Gao et al., 2011
MeHg$^+$ Hg(II)	Aigua d'aixeta Aigua de llac Aigua de mar Aigua subterrània	-	0,1	Online	Retenció en microcolumna de polianilina i elució amb HCl 0,5 % pel MeHg$^+$ i HCl 2 % + tiourea 0,02 % pel Hg(II)	CV-ICP-MS	0,0324 0,0252	96-102	Balarama-Krishna et al., 2010
MeHg$^+$ EtHg$^+$ Hg(II)	Aigua d'aixeta Aigua de riu Aigua de mar Aigua residual de rentats de carbó	Ditizona	100	Offline	Retenció en microcolumna C18 modificada amb ditizona i elució amb $Na_2S_2O_3$	LC-ICP-MS	3 3 3	71,7-93,7	Yin et al., 2010

Espècies de mercuri	**Matriu**	**Agent complexant**	**Volum mostra (mL)**	**Preconcentració**	**Retenció/Elució**	**Mètode instrumental**	**LD (ng L^{-1})**	**Recuperació (%)**	**Referència**
MeHg$^+$ Hg(II)	Aigua d'aixeta Aigua de deu Aigua de mar	2-[(2-mercaptofenilimino)metil] fenol (MPMP)	250-1500	Offline	Retenció en disc C18 modificat amb MPMP i elució amb EtOH	CV-AAS	3,8 3,8	97-99	Ashkenani et al., 2009
MeHg$^+$ Hg(II)	Aigua d'aixeta Aigua de mar Aigua de riu Aigua de deu	Bactèries	250	Offline	Retenció en microcolumna modificada amb bactèries (*Streptococcus pyogenes, Staphylococcus aureus*) i elució amb HCl 0,1 mol L^{-1} pel MeHg$^+$ i 2 mol L^{-1} pel Hg(II)	CV-AAS	1,5 2,1	96-97	Tuzen et al., 2009
MeHg$^+$ Hg(II)	Aigua de llac	2-mercaptoetanol	20	Online	Retenció en microcolumna C18 modificada amb 2-mercaptoetanol i elució amb H_2O amb L-cisteïna al 0,5 % i 2-mercaptoetanol al 0,05 %	LC-ICP-MS	0,03 0,12	84-108	Cairns et al., 2008
MeHg$^+$ PhHg$^+$ Hg(II)	Aigua de mar Aigua de riu Aigua sintètica	1,3-bis (2-cianobenzè) triazè (CBT)	250	Offline	Retenció en disc C18 amb 10% de PTFE modificat amb CBT i elució amb ACN	LC-UV	1,0 0,8 1,3	90-105	Hashempur et al., 2008
MeHg$^+$ EtHg$^+$ PhHg$^+$ Hg(II)	Aigua de riu	2-mercaptofenol	100-500	Offline	Retenció en microcolumna C18 modificada amb 2-mercaptophenol i elució amb MeOH	LC-CV-AAS	4,3 1,4 0,8 0,8	> 90	Margetínová et al., 2008
MeHg$^+$ Hg(II)	Aigua fortificada	APDC 4,1 mM	90	Online	Retenció en suspensió de C18 i elució amb HCl 1 mol L^{-1} pel MeHg$^+$ i HCl 5 mol L^{-1} amb Triton 0,02 % pel Hg(II)	CV-AAS	0,96 0,25	> 95	Río Segade et al., 2007
MeHg$^+$	Aigua superficial d'estany Aigua de cores de sediments	Tiol	40	Offline	Retenció en microcolumna amb resina de tiol funcionalitzada i elució amb tiourea àcida al 2 %	LC-CV-AFS	0,007	89,2-100,4	Vermillion et al., 2007

Espècies de mercuri	Matriu	Agent complexant	Volum mostra (mL)	Preconcentració	Retenció/Elució	Mètode instrumental	LD (ng L^{-1})	Recuperació (%)	Referència
MeHg$^+$ Hg(II)	Aigua biològica Aigua natural	Dietil ditiofosfat 0,1 % pel MeHg$^+$ Ditizona 0,002 % pel Hg(II)	9	Online	Retenció en reactor nuat i elució amb HCl 15 %	CV-AFS	2,0 3,6	94-110	Wu et al., 2006
Hg(II)	Aigua natural	Ditizona	1,1	Online	Retenció en microcolumna amb cotó i elució amb aigua a 60ºC	UV	14	97,8-104,9	Garrido et al., 2004
MeHg$^+$	Aigua d'estany Aigua de pluja	-	50-150	Online	Retenció en un tub de quars i elució mitjançant desorció tèrmica	GC-CV-AFS	0,01	90-100	Tseng et al., 2004
MeHg$^+$ Hg(II)	Aigua de riu	2-mercaptobenzimidazol (MBI)	1000	Online	Retenció en microcolumna Vyon-P amb gel sílice amb MBI i elució amb HCl 2 mol L^{-1} pel MeHg$^+$ i KCN 0,05 mol L^{-1} pel Hg(II)	UV-CV-AFS	0,05 0,07	90-102	Bagheri et al., 2001
MeHg$^+$ Hg(II)	Aigua de riu	Dietil Ditiocarbamat (DDTC)	50	Offline	Retenció en C18 modificat amb DDTC de sodi i elució amb tiourea àcida al 5%	LC-ICP-MS	5,6 5,2	80-96	Martinez-Blanco et al., 2000
MeHg$^+$ Hg(II)	Aigua salobre	APDC 2 mmol L^{-1}	50	Online	Retenció en microcolumna C18 i elució amb MeOH:APDC 1,5 mmol L^{-1} (50:50)	LC-CV-AAS	0,034 0,068	-	Qvanström et al., 2000
MeHg$^+$ PhHg$^+$ Hg(II)	Aigua de mar	Ditiziona	50-500	Offline	Retenció en microcolumna C18 modificada amb ditizona i elució amb MeOH	LC-UV	140 160 140	98-100	Sánchez et al., 2000
MeHg$^+$ EtHg$^+$ PhHg$^+$ Hg(II)	Orina humana	APDC 2 mmol L^{-1}	58,5	Online	Retenció en microcolumna C18 i elució amb MeOH:ACN:APDC 1,5 mmol L^{-1} (38:30:32)	LC-CV-AAS	9 6 10 5	92-106	Yin et al., 1998

Espècies de mercuri	Matriu	Agent complexant	Volum mostra (mL)	Preconcentració	Retenció/Elució	Mètode instrumental	LD (ng L^{-1})	Recuperació (%)	Referència
$MeHg^+$ $EtHg^+$ Hg(II)	Aigua de mar Aigua de pantà Aigua dolça	Ditiocarbamat	200	Offline	Retenció en una microcolumna amb resina de ditiocarbamat i elució amb tiourea àcida al 10%	GC-MPD	0,05 0,05 0,15	89,5-95,4	Etemborg et al., 1993

Taula 1.6. Metodologies per la preconcentració d'espècies d'antimoni en aigua.

Espècies d'antimoni	**Matriu**	**Agent Complexant**	**Volum mostra (mL)**	**Preconcentració**	**Retenció/Elució**	**Mètode Instrumental**	**LD (ng L^{-1})**	**PF**	**Referència**
Total Sb(III)	Aigua de llac Aigua de riu	-	100	Offline	Retenció en una suspensió de TiO_2 a pH 6 i elució amb HCl 2%	HG-AFS	13 10	20	Wang et al., 2014
Total Sb(III)	Aigua d'aixeta Aigua mineral Aigua de deu	-	50	Offline	Retenció en microcolumna de tetraetilenpentamina enllaçada a sílice a pH 6 i elució amb 5 mL HNO_3 2M	ETAAS	20	50	Mendil et al., 2013
iSb V and III	Aigua sintètica Aigua mineral Aigua de rierol CRM aigua de llac	-	50-90	Offline	Retenció en una suspensió de TiO_2 a pH 2 i elució amb aigua doblement desionitzada	ETAAS	100	10-30	Hagarová et al., 2012
Total Sb(III)	Aigua embotellada Aigua d'aixeta Aigua de riu Aigua de deu Aigua de mar	APDC	15 50	Offline	Retenció en nanotubs de carboni amb HNO_3 0,01M i elució amb NH_3 1 M	ETAAS	50	75-250	López-García et al., 2011
Sb(V) Sb(III)	Aigua de mar CRM aigua de riu CRM aigua de llac	-	2,2	Online	Retenció en Amberlita per Sb(V) i en PSTH-cpg per Sb(III) a pH 8,4 i elució amb tiourea 0,04% amb HNO_3 5%	HG-ICP-MS	21 13	3,9 5,5	Calvo-Fornieles et al., 2011
Total Sb(III)	Aigua fortificada Aigua de riu	-	25	Offline	Retenció en microcolumna en una Fibra de cotó amb tiols amb HCl 0,5M i elució amb 5 mL HCl 6 M	ICP-MS	-	-	Asaoka et al., 2011

Espècies d'antimoni	**Matriu**	**Agent Complexant**	**Volum mostra (mL)**	**Preconcentració**	**Retenció/Elució**	**Mètode Instrumental**	**LD (ng L^{-1})**	**PF**	**Referència**
Sb(III)	Aigua sintètica Aigua de beguda Aigua de riu Aigua mineral Aigua de mar	-	50	Offline	Retenció en microcolumna de sílice modificada a pH 7 i elució amb 50 mL de HCl 0.1M en medi acetona i etanol 1:1	ICP-MS	25	50	Urbánková et al., 2011
Total Sb(III)	Aigua de riu Aigua de llac Aigua de mar Aigua d'aixeta	APDC	100	Offline	Microextracció Líquid-líquid amb xilé. pH 0-1,2 pel total i pH 5-8 pel Sb(III)	ETAAS	2	400	Serafimovska et al., 2011a
Total Sb(III)	Aigua de riu	APDC	100	Offline	Microextracció Líquid-líquid amb xilé. pH 0-1,2 pel total i pH 5-8 pel Sb(III)	ETAAS	2	400	Serafimovska et al., 2011b
Sb(III)	Aigua de riu Aigua de font Aigua d'aixeta Aigua de beguda	-	10	Offline	Retenció en un polímer sulfonat a pH 6 i elució amb metanol i HNO_3 1 M	ETAAS	-	-	Kapakoglou et al., 2010
Total Sb(III)	Aigua sintètica Aigua de font	-	10	Offline	Microextracció *headspace single-drop* en una gota de HNO_3 1,5% amb $Pd(NO_3)_2$ mitjançant la formació de estibina amb NaBH4 1% en medi HCl 2M	ETAAS	25	176	Pena-Pereira et al., 2009

OBJECTIVES

S’ha detectat un creixent interès en quant a la determinació de mercuri i antimoni en matrius ambientals i alimentàries contaminades degut als efectes potencialment tòxics que aquests elements poden produir en els humans. Segons les publicacions a la bibliografia existent, s’han dut a terme diversos treballs per a la seva correcta determinació, tot i que és necessària una investigació més profunda per tal de entendre millor aquest tema.

D’acord amb l’estat del tema en qüestió, el principal objectiu de la present tesi és la determinació de mercuri i antimoni en diverses matrius ambientals i alimentàries per tal de mesurar i tenir informació rellevant de la presència i comportament d’aquests contaminants en els diferents escenaris, com ara episodis de contaminació i situacions de risc potencial per la salut humana. En tots els casos, es determina la concentració total i les principals espècies presents en aquestes matrius.

L'objectiu general es pot dividir en objectius específics, els quals es detallen a continuació:

- Establiment i validació de mètodes per a la determinació d'antimoni i mercuri (total i contingut de les espècies) i posterior aplicació en mostres reals.
- Desenvolupament de mètodes de preconcentració en línia per a la determinació de les espècies d'antimoni i mercuri.
- Estudi de la potencial migració d'antimoni en begudes embotellades en PET.

Chapter II

MATERIALS AND METHODS

2.1. STANDARDS

This section describes the procedures for the preparation of the stock standard solutions of antimony and mercury used in the present work. Working standard solutions were prepared daily from these stock standard solutions by appropriate dilution in the corresponding media and were used for the preparation of calibration curves, method development assays and sample spiking tests.

2.1.1. Antimony standards

(i) Certified Sb standard solution: prepared from metallic antimony (99.999% "purum", *Inorganic Ventures*), dissolved and stabilised in high purity acids (5 % (v/v) nitric acid, HNO_3, and 0.1% (v/v) hydrofluoric acid, HF). The solution contains 1000 ± 4 mg L^{-1} of Sb.

(ii) Sb(III) standard solution: 0.2743 g of potassium antimony (III) oxide tartrate hemihydrate ($C_4H_4KO_7Sb{\cdot}0{,}5H_2O$, 99,0 – 103 %, *Fluka, Neu-Ulm, Germany*) were dissolved in 100 mL of double deionised water at room temperature. The resulting solution contains approximately 1000 mg L^{-1} of Sb(III) in the form of $[Sb(OH)_3]$.

(iii) Sb(V) standard solution: 0.2157 g of potassium hexahydroxoantimonate (V) ($K[Sb(OH)_6]$, 99,0 %, *Riedel de-Haën, Seelze, Germany*) were dissolved in 100 mL of double deionised water at 40ºC. The resulting solution contains approximately 1000 mg L^{-1} of Sb(V) in the form of $[Sb(OH)_6]^-$.

These stock standard solutions were stored in polyethylene bottles at 4°C. Sb(III) and Sb(V) solutions were standardised against the certified standard solution of 1000 ± 4 mg L^{-1} of Sb.

2.1.2. Mercury standards

(i) Certified Hg standard solution: prepared from metallic mercury (99.9997% "purum", *Inorganic Ventures*), dissolved and stabilised in high purity acid (5% (v/v) HNO_3). The solution contains 1000 ± 6 mg L^{-1} of Hg.

(ii) Inorganic mercury (Hg(II)) standard solution: 0.1353 g of mercury chloride ($HgCl_2$, 99%, *Merck, Darmstadt, Germany*) were dissolved in 100 mL of 1% (v/v) HNO_3 (*Merck, EMSURE ISO, máx 0.005 ppm Hg*). The resulting solution contains approximately 1000 mg L^{-1} of inorganic mercury in the form of Hg(II).

(iii) Methylmercury ($MeHg^+$) standard solution: 0.1252 g of methylmercury chloride (CH_3HgCl, 98%, *Carlo Erba, Milan, Italy*) were dissolved in 100 mL of 3% methanol. The resulting solution contains approximately 1000 mg L^{-1} of methylmercury in the form of $MeHg^+$.

(iv) Ethylmercury ($EtHg^+$) standard solution: 0.1322 g of ethylmercury chloride (C_2H_5HgCl, 98%, *Carlo Erba, Milan, Italy*) were dissolved in 100 mL of 20% acetonitrile. The resulting solution contains approximately 1000 mg L^{-1} of ethylmercury in the form of $EtHg^+$.

These stock standard solutions were stored in dark glass bottles at 4°C. Hg(II), $MeHg^+$ and $EtHg^+$ solutions were standardised against the certified standard solution of 1000 ± 6 mg L^{-1} of Hg.

2.2. CERTIFIED REFERENCE MATERIALS

The Certified Reference Materials (CRM) described in this section were used for method development assays and quality control purposes.

2.2.1. CRMs for antimony

(i) TM-23.4: Lake Ontario water from *National Research Council Canada (Canada)*, with a certified value of 3.26 ± 0.318 µg L^{-1} for total Sb. According to the certificate, the lake water was preserved with 0.2 % of HNO_3 to stabilise the trace amounts of the antimony together with the rest of the certified elements. The material should be stored refrigerated and in the dark.

(ii) ERM-EC681k: Low density polyethylene from *Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre (Belgium)*, with a certified value of 99 ± 6 mg kg^{-1} for total Sb. According to the certificate, the granulate low-density polyethylene (LDPE) was spiked with various pigments to obtain the desired element contents. The material should be stored below 18°C in the dark.

2.2.2. CRMs for mercury

(i) ERM-CA-713: Effluent wastewater from the *Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre (Belgium)*, with a certified value of 1.84 ± 0.11 µg L^{-1} for total Hg. According to the certificate, the wastewater was acidified with HNO_3 to about pH 2 to stabilise the trace amounts of the mercury together with the rest of the certified elements. The material should be stored at 18°C ± 5ºC in the dark.

(ii) DOLT-4: Dogfish liver from the *National Research Council Canada (Canada)*, with a certified value of 2.58 ± 0.22 mg kg^{-1} for total Hg and 1.33 ± 0.12 mg kg^{-1} for $MeHg^+$. The material should be stored in a cool and dark location.

(iii) TORT-2: Lobster hepatopancreas from the *National Research Council Canada (Canada)*, with a certified value of 0.27 ± 0.06 mg kg^{-1} for total Hg and 0.152 ± 0.013 mg kg^{-1} for $MeHg^+$. The material should be stored in a cool and dark location.

(iv) TORT-3: Lobster hepatopancreas from the *National Research Council Canada (Canada)*, with a certified value of 0.292 ± 0.022 mg kg^{-1} for total Hg and 0.137 ± 0.012 mg kg^{-1} for $MeHg^+$. The material should be stored in a cool and dark location.

(v) BCR-463: Tuna fish from the *Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre (Belgium)*, with a certified value of 2.85 ± 0.16 mg kg^{-1} for total Hg and 3.04 ± 0.16 mg kg^{-1} for $MeHg^+$. The material should be stored at 4ºC and in the dark.

2.3. INSTRUMENTATION

This section describes the different instrumentation used in the present thesis. They were mainly used for the analysis of Sb and Hg and also for the analysis of several anions and cations content.

2.3.1. Total analysis

2.3.1.1. Cold Vapour Atomic Fluorescence Spectroscopy (CV-AFS) for Hg determination

Total Hg content was determined using cold vapour atomic fluorescence spectroscopy (CV-AFS) using a *PS Analytical model 10.004.* The reagents for cold vapour generation, 4% HCl (HCl, *Panreac, hyperpure*) and 1.5% tin(II) chloride ($SnCl_2$, *Panreac, p.a.*), were continuously pumped at 8 mL min^{-1} and 4 mL min^{-1}, respectively. The reagents were mixed in a reaction coil together with the sample, pumped at 8 mL min^{-1}. The corresponding mercury vapour is formed by the following reaction:

$$Hg^{2+} + Sn^{2+} \rightarrow Hg^0 + Sn^{4+}$$

The generated mercury vapour was driven by an argon flow (300 mL min^{-1}) to the AFS detector through the gas-liquid separator. Before detection, the argon stream was passed through a Perma pure drying membrane with N_2 as drying gas (2.5 L min^{-1}), which prevents droplets being transmitted into the transfer line. Detection was carried out in a P.S Analytical Merlin Atomic Florescence Spectrometer equipped with a low pressure Hg lamp. For the instrument control and data acquisition *Avalon 2.15* software was used.

For the analysis of the samples, a pre-oxidation step is needed to transform the Hg content in the samples to Hg(II), so the mercury vapour could be then generated. For carrying out the pre-oxidation, a 10 mL aliquot of sub-sample was mixed with 0.5 mL of a 0.55 % potassium bromate/bromide solution ($KBrO_3$/KBr, *Panreac, p.a.*) and was diluted up to 20 mL in 4% HCl medium. Samples were quantified by means of an external calibration curve from Hg(II) standards. Analyses in triplicate were performed for each sample. For quality control purposes, the standards of the calibration curve were run before and after each sample series.

2.3.1.2. Hydride Generation Atomic Fluorescence Spectroscopy (HG-AFS) for Sb determination

Total Sb content was determined using hydride generation atomic fluorescence spectroscopy (HG-AFS) using a *Millennium PS Analytical (Kent-UK), model 10.055.* The reagents for hydride generation, 2 mol L^{-1} hydrochloric acid (HCl, *Panreac, hyperpure*) and 0.7% (w/v) sodium borohydride ($NaBH_4$, *Sigma Aldrich, for the determination of hydride formers*) with a 0.4% (w/v) sodium hydroxide (NaOH, *Merck, p.a.*) were continuously pumped at 8 mL min^{-1} and 4 mL min^{-1}, respectively. The reagents were mixed in a reaction coil for the generation of the flame. The sample was also pumped at 8 mL min^{-1} and mixed in the reaction coil with the reductant. The corresponding hydride is formed by the following reactions:

$$NaBH_4 + 3H_2O + HCl \rightarrow H_3BO_3 + NaCl + 8H^*$$

$$8H^* + Sb^{3+} \rightarrow SbH_3 + \frac{5}{2} H_2$$

Where "H*" is the forming hydrogen [Morand et al., 2002]. The generated stibine was driven by an argon flow (300 mL min^{-1}) to the AFS detector through the gas-liquid separator. Before detection, the argon stream was passed through a Perma pure drying membrane with N_2 as drying gas (2.5 L min^{-1}), which prevents droplets being transmitted into the transfer line. Detection was carried out in the atomic fluorescence spectrometer with a diffusion flame and a Sb-boosted discharge lamp. For the instrument control and data acquisition Millenium software was used.

For the analysis of the samples, a pre-reduction step is needed to reduce the Sb content in the samples to Sb(III), so the stibine could be then generated. For carrying out the pre-reduction, a 10 mL aliquot of sub-sample was mixed with 0.5 mL of a 50 % potassium iodide (*KI, Panreac, p.a.*) and 10 % ascorbic acid (*Panreac, p.a.*) solution and was diluted up to 20 or 25 mL in HCl 2 mol L^{-1} medium. Samples were quantified by means of an external calibration curve from Sb(III) standards. Analyses in triplicate were performed for each sample. For quality control purposes, the standards of the calibration curve were run before and after each sample series.

Figure 2.1 shows a schematic diagram of the whole system, either hydride generation or cold vapour.

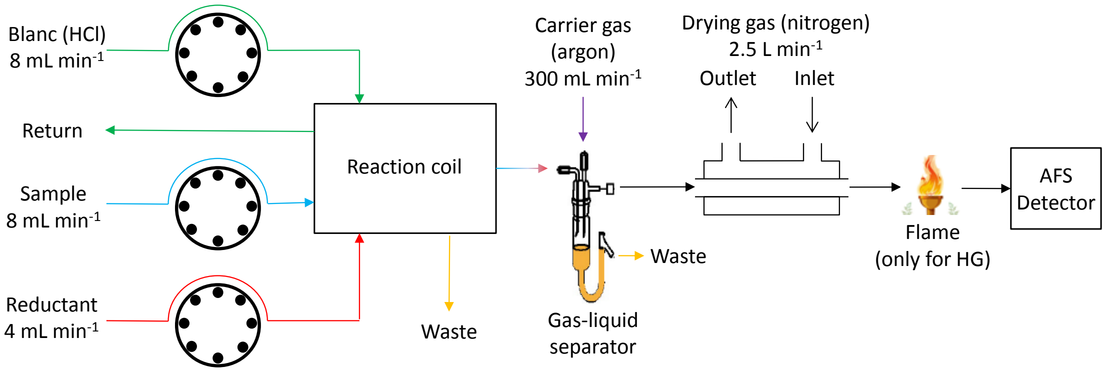


Figure 2.1. Schematic diagram of the HG-AFS/CV-AFS system.

2.3.1.3. Inductive Coupled Plasma Mass Spectrometry (ICP-MS)

ICP-MS is a commonly used technique for multielemental determination which presents high precision and sensitivity. In the present work, an *Agilent 7500ce series Octopole Reaction System* was used for total Sb and Hg determination in low concentration level matrices. ICP-MS presents the following parts:

- Nebulizer: is the component responsible for the sample introduction into the system. An aerosol is formed in the nebuliser which allows the incorporation of the liquid sample into a gas stream. The nebulizer used is Burgener Ari Mist HP model, which allows working in both low and high flow rates for total and speciation determination, respectively.
- Spray chamber: the main function is the separation and removal of the big drops (more than 10 µm) formed during the nebulisation. They are directed to the walls of the chamber and are finally removed with a drainage system, so a 99% of sample as solution is discarded.
- Torch and induction coil: the torch is formed with three concentric quartz tubes through which the argon current passes. The torch is surrounded by the induction coil, a spiral which generates an electromagnetic field. Ionization is produced in this part.
- Interface: is responsible for the transfer of the gas from the plasma, which is representative of the original sample. Interface has two cones which inject plasma and ions to the mass spectrometer zone through an increasing vacuum.
- Ionic lens: transport the ions to the mass spectrometer zone.
- Collision cell: consists of an octopole where polyatomic interferences are removed.
- Quadrupole: the function of this part consists of separating ions according to their mass-to-charge ratio (m/z). Only some ions with a specific m/z present a stable trajectory and arrive to the detector.
- Detector: is the responsible for recording the corresponding signal of the ion. The detector used in this ICP-MS is *Channeltron electron multiplier*.

The following operating parameters, summarised in Table 2.1, were applied for the analysis of either antimony or mercury.

Table 2.1. ICP-MS Operating Parameters.

General	
Radio frequency power	1550 W
Radio frequency Matching	1.66 V
Peristaltic pump speed	0.1 rps
Stabilization delay	30 s
Sampler and skimmer cones	Nickel
Nebulizer	BURGENER Ari Mist HP
Number of replicates	3
Spray chamber (type and temperature)	Scott-type and 15^{o}C
Gas flows	
Carrier gas flow, Ar	0.75 L min^{-1}
Make up gas flow, Ar	0.33 L min^{-1}
Torch alignment	
Sampling depth	7.5 mm
Ion lenses	
Extract 1	0 V
Extract 2	-130 V
Omega Bias-ce	-18 V
Omega Lens-ce	1.2 V
Cell entrance	-26 V
QP Focus	2 V
Cell exit	-36 V
Quadrupole and octopole parameters	
QP/OctP bias difference	3 V
Mass-to-charge ratio	
Sb and Hg	m/z 121 (^{121}Sb); 202(^{202}Hg)
Quality control standard	m/z 9 (Be), m/z 103 (Rh) and m/z 205 (Tl)

Non-gas mode was applied for mercury determination whereas helium-gas mode was applied for antimony. 20 µg L^{-1} of a rhodium, beryllium and thallium standard solution in 1% HNO_3 media was used to correct some possible measure deviations during the analysis. For the instrument control and data acquisition *ChemStation* software was used. Figure 2.2 shows the different parts of the ICP-MS instrument.

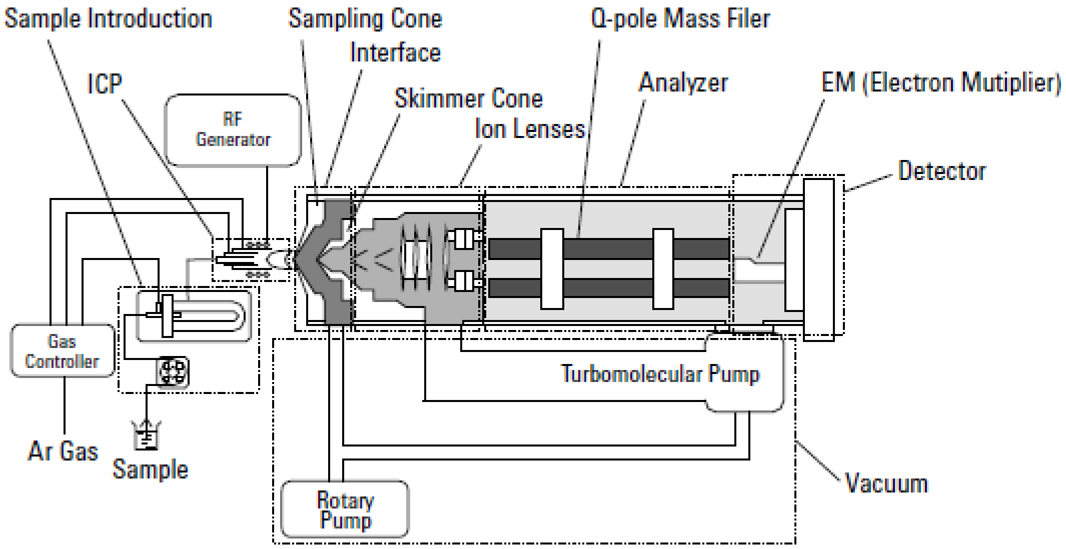


Figure 2.2. Schematic representation of the ICP-MS instrument.

During the four-month stay in the research group from the *Laboratory of Speciation Analysis of Elements* in the *Environmental Chemical Processes Laboratory* from the *University of Crete (Πανεπιστήμιο Κρήτης)*, a *Perkin Elmer NexION 300X Series* was used for Sb determination applying the same measurement conditions described above. For the instrument control and data acquisition *Syngistix* software was used.

For the analysis of samples an aliquot of sub-sample was appropriately diluted up in HNO_3 1% (*Panreac, hyperpure*). Samples were introduced to the ICP-MS system at the conditions aforementioned. Samples were quantified by means of an external calibration curve from certified Sb or Hg standards. Analyses in triplicate were performed for each sample. For quality control purposes, the standards of the calibration curve were run before and after each sample series.

2.3.2. Speciation analysis

2.3.2.1. Mercury speciation using LC-UV-CV-AFS

Hg species content was determined using liquid chromatography coupled to Ultraviolet irradiation and cold vapour – atomic fluorescence spectroscopy (LC-UV-CV-AFS). The liquid chromatography system consists of a quaternary pump model *Agilent 1100 Series*, equipped with a manual stainless steel sampler injector (Rheodyne Model 7725i) with a 100 µL loop. The separation of mercury species was performed in an analytical RP-C18 column (ODS Hypersyl 250 mm x 4.6 mm id, 5 µm particle size, Thermo Hypersil-Keystone). The mobile phase used was a mixture of methanol (MeOH, *Panreac*, LC grade) and a buffer solution of ammonium pyrrolidine dithiocarbamate (APDC, *Fluka, p.a.*) 0.0015 mol L^{-1} and ammonium acetate (NH_4CH_3COO, *Merck, p.a.*) 0.01 mol L^{-1} adjusted to pH 5.5, at flow 1.5 mL min^{-1}. The ratio of the mobile phase components was 80% and 20%, respectively, for the separation of $MeHg^+$ and Hg(II) with a chromatographic time of 10 minutes, and 75% and 25%, respectively, for the separation of $MeHg^+$, $EtHg^+$, and Hg(II) with a chromatographic time of 12 minutes.

After separation, a photo-oxidation step was performed in a 12 meter length x 0.5 mm id polytetrafluoroethylene (PTFE) tube coiled around a UV lamp with a power irradiation of 150 W (Heraeus TQ 150). This step allows the fragmentation of the separated organomercuric species, which is necessary for the subsequent cold vapour formation. The outlet of the tube was coupled to the CV-AFS system described in section 2.3.1.1. For the instrument control and data acquisition *Pendragon* software was used.

Sub-samples were directly injected to the system at the conditions aforementioned. The samples were quantified by means of external calibration curves from $MeHg^+$, $EtHg^+$ and Hg(II) standards. Mercury species were identified by comparison of retention times with the standards and confirmed by spiking. All the samples were analysed in triplicate. For quality control purposes, the standards of the calibration curves were run before and after each sample series.

2.3.2.2. Antimony speciation using LC-HG-AFS

Sb species content was determined using liquid chromatography coupled to hydride generation – atomic fluorescence spectroscopy. The liquid chromatography system consists of a quaternary pump model *Perkin Elmer 200 Series*, equipped with a manual stainless steel sampler injector (Rheodyne Model 7125i) with a 100 µL loop. The separation of antimony species was performed in analytical anion exchange columns (Hamilton PRP-X100, 4.1 mm id, 10 µm particle size). The mobile phase used was a buffer solution of etilenediaminetetraacetic acid (EDTA, *Panreac, p.a.*) 10 mmol L^{-1} with a 0.5% of methanol. After separation, the outlet of the column was coupled to the HG-AFS system described in section 2.3.1.2. For the instrument control and data acquisition *SAMS* software was used. This technique was used for the carrying out of preliminary experiments for the method development for Sb(V) and Sb(III) determination.

Figure 2.3 shows a schematic diagram of the whole system, either LC-UV-CV-AFS or LC-HG-AFS.

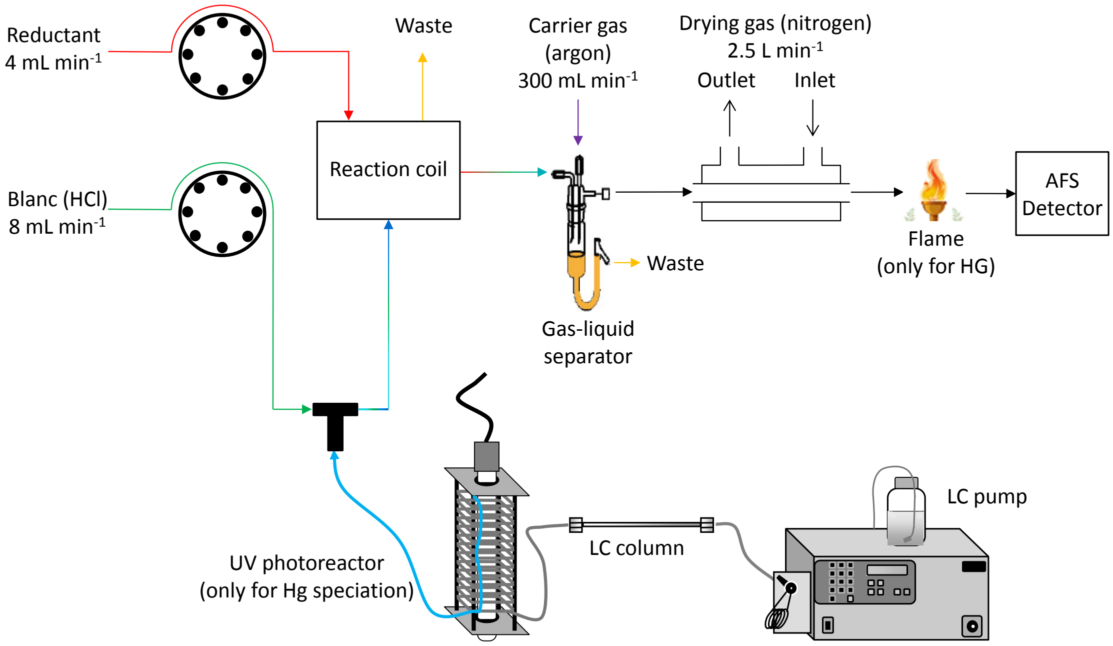


Figure 2.3. Schematic diagram of the LC-HG-AFS/LC-UV-CV-AFS system.

2.3.2.3. Preconcentration system

An online preconcentration system was coupled prior to both LC-UV-CV-AFS and LC-HG-AFS for the determination of trace mercury and antimony species in waters, respectively. The original sample loop was replaced with a short precolumn with the same characteristics as the separation column (RP-C18 ODS Hypersyl for Hg and anion exchange Hamilton PRP-X100 for Sb) which was connected by an isocratic LC pump (*Agilent 1260 Series*) and a six channel valve (Rheodyne model 7000 6-port). This system alternates the sample flow and the mobile phase passing through the precolumn, which allows the loading of different sample volumes to the precolumn so as to preconcentrate Hg or Sb species. When the valve is in the loading position, the sample passes through the precolumn and species are adsorbed onto the stationary phase. In the injection position, the mobile phase passes through the precolumn and elutes the retained species to the LC-AFS system for determination. Figure 2.4 shows a schematic diagram of the online preconcentration system coupled to either LC-UV-CV-AFS or LC-HG-AFS, respectively.

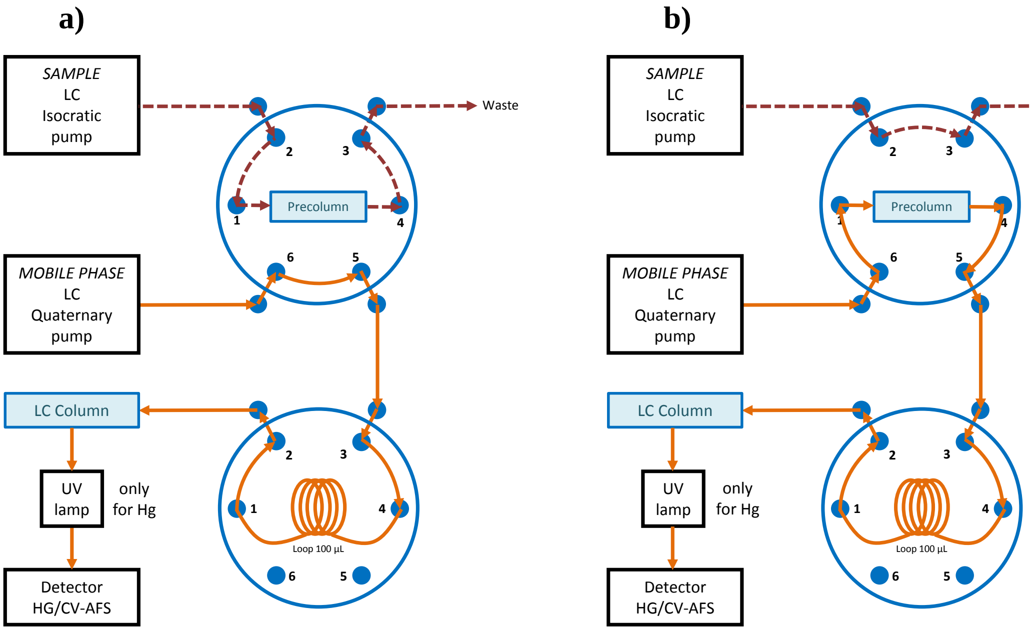


Figure 2.4: Schematic representation of the online preconcentration system: (a) load of the sample on the precolumn, and (b) elution of the sample to the separation column.

2.3.2.4. Antimony speciation using LC-ICP-MS

Sb species content was determined using liquid chromatography hyphenated to inductive coupled plasma – mass spectrometry. The liquid chromatography system consists of a quaternary pump model *Agilent 1200 Series*, equipped with an auto-sampler with an injection volume between 0.01 and 2000 µL. An injection volume of 100 µL was used. The separation of antimony species was performed in analytical anion exchange columns (Hamilton PRP-X100 4.1 mm id, 10 µm particle size). The mobile phases used were a buffer solution of etilenediaminetetraacetic acid (EDTA, *Panreac*) 10 mmol L^{-1} adjusted to pH 4.0 with a 0.5% of methanol, at flow 1.5 mL min^{-1} and a buffer solution of ammonium formate 8 mmol L^{-1} ($HCOONH_4$, *Fluka*) with a 2% of methanol, at flow 1.5 mL min^{-1}. After separation, the outlet of the column was coupled by a PEEK tube to the nebuliser of the ICP-MS instrument described in section 2.3.1.3. For the instrument control and data acquisition *ChemStation* software was used.

During the stay in the research group from the *Laboratory of Speciation Analysis of Elements* in the *Environmental Chemical Processes Laboratory* from the *University of Crete (Πανεπιστήμιο Κρήτης)*, a quaternary pump model *Perkin Elmer Flexar* equipped with an autosampler with an injection volume between 0.01 and 2000 µL was coupled to the *NexION 300X* ICP-MS. The measurement conditions employed were the same as mentioned before. For the instrument control and data acquisition *Chromera* software was used.

Sub-samples were directly injected to the system at the conditions aforementioned. The samples were quantified by means of external calibration curves from Sb(III) and Sb(V) standards. Antimony species in the extracts were identified by comparison of retention times with the standards and confirmed by spiking. All the samples were analysed in triplicate. For quality control purposes, the standards of the calibration curves were run before and after each sample series.

2.3.3. Antimony structural elucidation

2.3.3.1. Low Resolution ESI-MS and LC-ESI-MS

A low resolution mass spectrometer was used for the characterization of the structure of antimony complexes during the research stay in the University of Crete. A *Thermo Scientific LCQ Advantage* ion trap mass spectrometer with electrospray (ESI-MS) source was employed with the following modified configuration developed by the research group: a Meinhard pneumatic nebulizer which has been used extensively for aerosol formation in atomic spectrometry (ICP-MS and ICP-AES), was placed next to the sampling orifice of the ion trap mass spectrometer instead of the ESI source of the instrument. This configuration allows the performance of sonic spray ionisation (SSI) mass spectrometry (MS), which is based on the formation of gas-phase ions without applying an electric field to the sample capillary, as required in electrospray ionization [Hirabayashi et al., 1994]. The configuration of the present work is specifically a version of SSI which is referred to as Venturi easy ambient sonic-spray ionization mass spectrometry (V-EASI-MS). V-EASI has the potential to become a competitive ionization technique to ESI for the purpose of molecular mass spectrometry of inorganic compounds as coordination and redox active compounds [Antonakis et al., 2013].

The following optimised conditions were used for the V-EASI-MS: a 15 – 20 cm long polyimide coated fused-silica capillary (100 μm id. × 200 μm od) was inserted into the pneumatic nebulizer's sample uptake channel till it is aligned with the glass nebulizer spray tip. The fused silica capillary is secured in place by tightening a PEEK sleeve around it in the back of the nebulizer. The tip of nebuliser was placed approximately 1 – 3 mm from the mass spectrometer orifice. Figure 2.5 shows the nebuliser (a) and the coupling to the MS (b). The temperature of the heated ion transfer capillary was settled on 300ºC, the source induced dissociation (SID) voltage applied was 5V, the collision energy was 20% and the nitrogen gas pressure used was between 40-60 psi [Antonakis et al., 2013]. Samples were introduced by direct infusion through the capillary as depicted in Figure 2.5b.

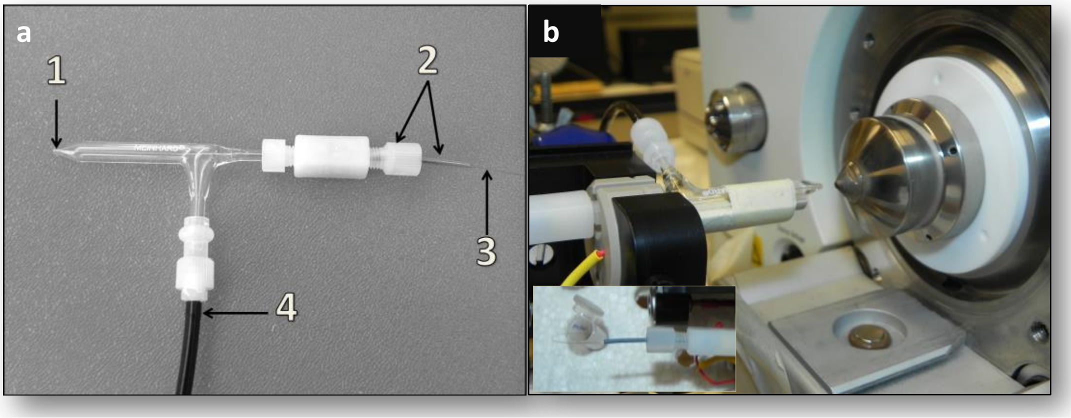


Figure 2.5: (a) Modified Meinhard pneumatic glass nebulizer used for V-EASI-MS: (1) fused silica capillary located at glass nebulizer tip; (2) nut and PEEK sleeve used to secure fused silica capillary in place; (3) fused silica capillary directed to sample; (4) 40-60 psi N_2 gas line. (b) Meinhard V-EASI source coupled to mass spectrometer [Antonakis et al., 2013].

For antimony speciation, a liquid chromatography system was coupled to the V-EASI-MS by a PEEK sleeve for the separation of Sb species. A quaternary pump model *Shimadzu LC-20AD* equipped with a manual stainless steel sampler injector (Rheodyne Model 7125i) with a 2 µL loop. The separation of antimony species was performed in analytical RP-C18 columns Shim-pack XR-ODS. The mobile phase used was a mixture of methanol and 0.1% formic acid (HCOOH, *Fluka, p.a.*) at flow 0.1 mL min^{-1}. For the instrument control and data acquisition *Xcalibur* software was used. Sub-samples were directly injected to the system at the conditions aforementioned.

2.3.3.2. High resolution ESI-MS

High resolution mass spectrometry (HRMS) was also used for the characterization of the structure of antimony complexes. In the present work, two different mass spectrometers were used for this purpose:

Thermo Fisher Scientific Q-Exactive Orbitrap equipped with a thermally assisted electrospray ionization source. The instrument was operated in negative mode, applying the following parameters: ESI voltage was -2.5 kV; capillary and vaporizer temperatures were 320ºC; sheath gas, auxiliary gas and sweep gas flow rate were 40, 10 and 2 arbitrary units, respectively, and the tube lens was held at 50 V.

Thermo Fisher Scientific LTQ Orbitrap Velos equipped with a thermally assisted electrospray ionization source. The instrument was operated in negative node applying the same parameters described before. For targeted tandem MS experiments, nitrogen was used as collision gas (1.5 mTorr) and the normalised collision energy (NCE) ranged from 20 to 50%, depending on the compound.

Figure 2.6 depicts an schematic diagram of the mass spectrometers, highlighting the most relevant parts of the system. The mass spectrometers were operated in profile mode (scan range, m/z 100-1000) with a resolving power of 70000 FWHM (full width half maximum) (at m/z 200) and an automatic gain control setting of 3 x 10^6 with a maximum injection time of 200 ms. For the instrument control and data acquisition, *Thermo Xcalibur Qual Browser* software was used.

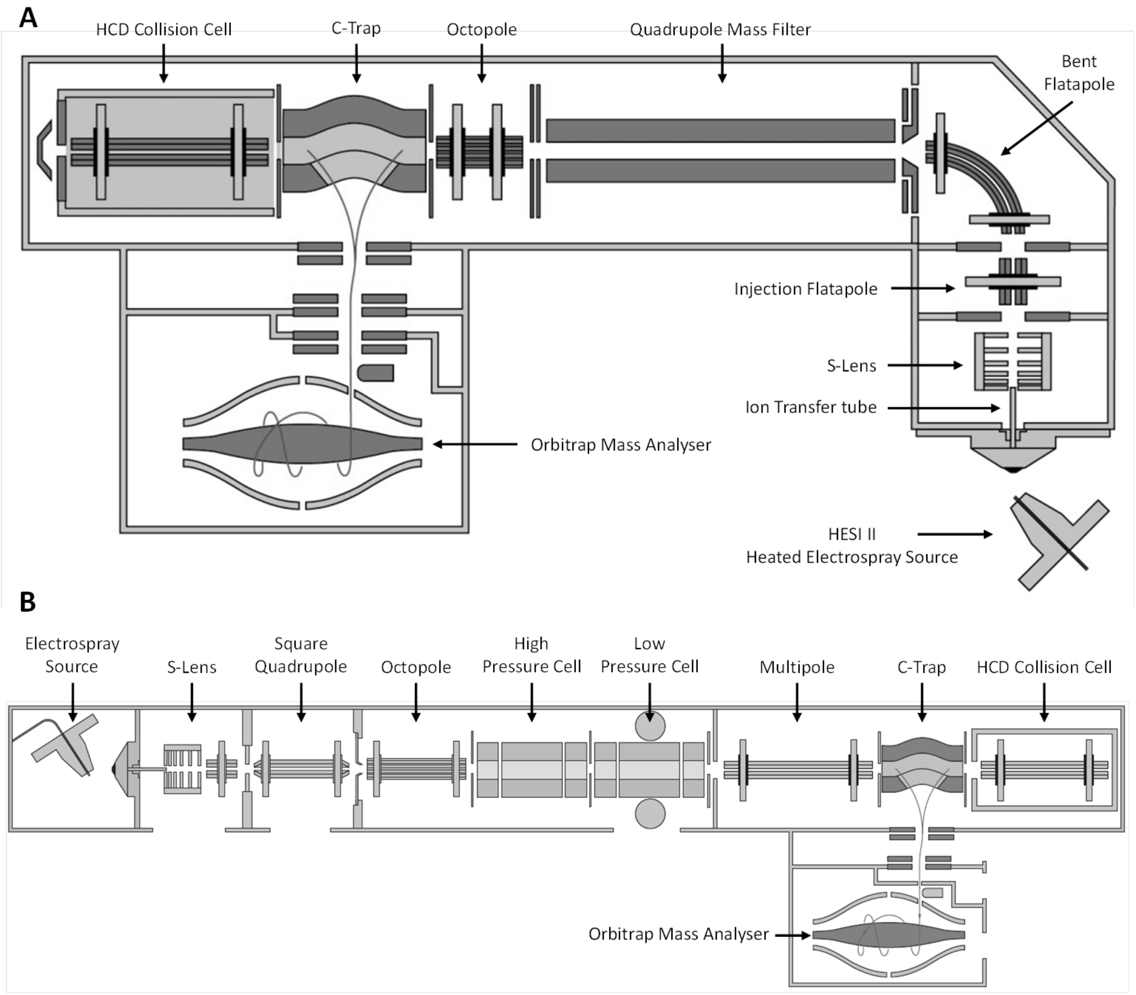


Figure 2.6. Schematic for the Q-Exactive Orbitrap (A) and LTQ Orbitrap Velos (B).

2.3.4. Ambient ionisation for antimony determination: DESI-HRMS

As a result from a collaboration with the research group *Chromatography, Capillary Electrophoresis and Mass Spectrometry* from the *University of Barcelona*, a desorption electrospray ionization (DESI) source (*Prosolia Inc.*) equipped with a 1D moving stage and coupled to the Q-Exactive Orbitrap (*Thermo Fisher Scientific*) described in the previous section was used for the determination of antimony compounds on the surface of PET bottles. The principle of operation of DESI is carried out by directing high velocity charged droplets produced from a pneumatically-assisted electrospray onto a surface to be analysed at atmospheric conditions. Ions of chemical species present on the surface are produced through the interaction of the charged droplets and the sample. The resulting mass spectra are similar to ESI mass spectra [Prosolia, 2015]. Figure 2.7 depicts a schematic diagram of the DESI.

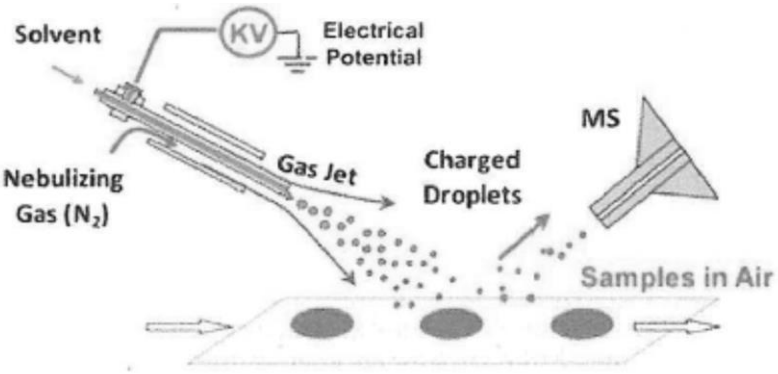


Figure 2.7. Schematic diagram of the DESI principle of operation [Wiley et al., 2014].

The DESI solvent was infused by a syringe pump at 2.5 µL min^{-1} and N_2 gas was used as nebulizer gas at a pressure of 9 bar. DESI solvent was directed onto the sample surface at a nebulization capillary angle of 55° and a distance of approximately 9.2 mm between the mass spectrometer inlet and the spray tip. The electrospray voltage was ±4.8 kV (positive/negative). The transfer capillary temperature was set at 250 °C. Samples were analysed either directly directing the emitter onto the PET surface or with a previous dissolution and deposition onto microscope glass slides of 7.1 mm^2 polytetrafluoroethylene (PTFE, Teflon) printed spots. In this second case, the acquisition is carried out via a scan along the spots printed on the glass slide, during which the instrument is registering sample and blank positions consecutively. Thus, the mass spectra form samples would correspond to those obtained when the emitter is just focused on the zone where the sub-sample droplet was placed, whereas blank mass spectra would correspond to those obtained when the emitter is focusing outside the droplets.

The QExactive mass spectrometer was operated in positive and negative ion mode within a scan range of 100–1000 m/z. *Omni Spray ion source v2.0* software was used to control the DESI source, while data acquisition and data processing were performed with *Xcalibur* software described in the previous section.

2.3.5. Complementary matrix characterisation techniques

The concentration of the major anions and cations present in the water samples used for the preconcentration studies (section 3.3) and for the analysis of Sb (section 4.1) was analysed using the following techniques.

Fluoride content was determined by ion-selective electrode (ISE) using an *Orion Dualstar* equipment. Samples were diluted 1:1 with a Total Ionic Strength Adjustment Buffer (TISAB) and quantified by means of an external calibration curve.

Chloride, nitrate and sulphate were determined by anion exchange liquid chromatography (*Waters*, model 512) coupled to a conductivity detector (*Waters*, model 432) (LC-CD). Anions were separated using a borate/gluconate buffer at a flow of 2 mL min^{-1} and a temperature of 35ºC and quantified by means of external calibration curves.

Calcium, magnesium, sodium and potassium were determined by ICP-OES (*Perkin Elmer*, model *Optima 3200 RL*). Samples were diluted 1:1 with nitric acid 2% and quantified by means of external calibration curves.

2.4. Sample pretreatments

2.4.1. Analysis of liquid samples

2.4.1.1. Drinking water, *raki* and *tsipouro*

Determination of Sb and Hg in drinking water samples and determination of Sb in spirit samples was carried out with a previous filtration of the samples. Water, *raki* and *tsipouro* were filtered through 0.45 µm prior to analysis. Analyses of Sb and Hg content in waters were carried out using the techniques and procedures described in section 2.3.1 and 2.3.2 whereas analyses of Sb content in spirits were carried out during the research stay in the University of Crete, using the techniques and procedures of sections 2.3.1.3, 2.3.2.4 and 2.3.3.

2.4.1.2. Juices

Determination of total Sb and species in juice samples was carried out with a previous centrifugation and subsequent filtration of the samples, as further discussed in section 4.2.1. All the juice samples were centrifuged at 3500 rpm during 10 minutes. The supernatant was filtered through 0.45 µm prior to analysis. Analyses of total Sb were performed by ICP-MS with the conditions and procedure described in section 2.3.1.3 whereas analyses of Sb species were performed by LC-ICP-MS by direct injection of the sub-sample in the system operating at the conditions mentioned in section 2.3.2.4.

2.4.2. Analysis of plastics

2.4.2.1. Microwave digestion

Determination of total Sb content in PET bottles and the LDPE certified reference material was carried out with a closed microwave digestion (*Milestone Ethos Touch Control*) with temperature controller and a constant power of 1000 W. The program used was optimised by *Milestone* and it is suitable for the digestion of plastics. The following procedure was performed: PET bottles were previously cut into square pieces of 1cm^2. 0.1500 g of sample were weighed in the digestion vessels and were subjected to a pre-digestion with 3.0 mL of concentrated sulphuric acid (H_2SO_4, *Merck, Suprapur*) for 5 minutes at 300 W. Afterwards, the sample was digested with 8.0 mL of concentrated nitric acid (HNO_3, *Panreac, hyperpure*) using the temperature program depicted in Figure 2.8.

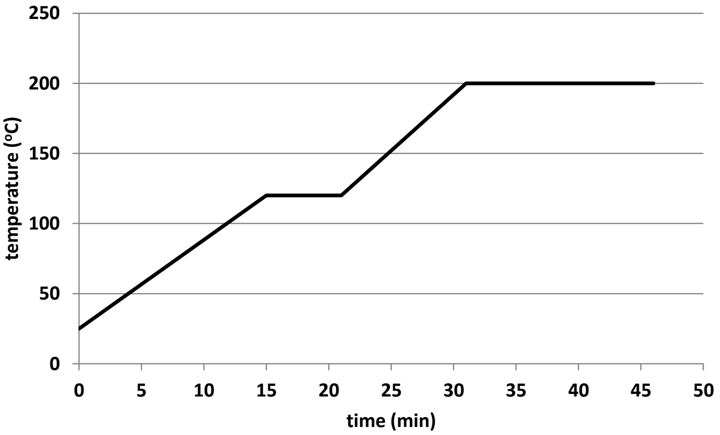


Figure 2.8. Program used for the digestion of plastic in the analysis of total Sb.

Once the digestion was finished, samples were cooled at room temperature, filtered through a filter paper (*Whatman 40*) into 50-mL glass flask and diluted with double deionised water. Analyses of total Sb content were performed by HG-AFS following the procedure described in section 2.3.1.2.

2.4.2.2. End over end extraction

Determination of leachable Sb content in PET bottles was carried out by “end over end” extraction. The following procedure was performed: PET bottles were previously cut into square pieces of 1cm^2. 1.500 g of sample were shaken with 10 mL of HCl 10 mmol L^{-1} as the extractant solution in Teflon tubes for 16 hours at 30 rpm at room temperature [Miravet et al., 2006]. Once the extraction was finished, total Sb was determined either by HG-AFS and ICP-MS using the procedures described in section 2.3.1.2 and 2.3.1.3. Analyses of Sb species were performed by

LC-ICP-MS by direct injection of the sub-sample in the system operating at the conditions mentioned in section 2.3.2.4.

2.4.2.3. Migration assays

Determination of leachable Sb content in PET bottles was carried out according to the Directive 97/48/EC using an appropriate food simulant stipulated by the Directive 85/72/EEC. PET bottles were previously cut into square pieces of 1 cm^2. 1.500 g of sample were put in contact with 10 mL of 3% acetic acid as food simulant in glass containers for 10 days at 40ºC [Directive 97/48/EC; Directive 85/72/EEC]. Total Sb was determined by ICP-MS using the procedure described in section 2.3.1.3. Analyses of Sb species were performed by LC-ICP-MS by direct injection of the sub-sample in the system operating at the conditions mentioned in section 2.3.2.4. The calibration curve for the quantification was also performed in 3% acetic acid media so as to suppress the matrix effect caused by the acetic acid.

2.4.3. Analysis of seafood samples

2.4.3.1. Microwave digestion

Determination of total Hg content in seafood samples and CRMs was carried out with a closed microwave digestion (*Milestone Ethos Touch Control*) with temperature controller and a constant power of 1000 W. The program used was previously optimised by the research group. The following procedure was performed: 0.1 – 1 g of sample were weighed in the digestion vessels, after which 8 mL of concentrated nitric acid (HNO_3, *Panreac, hyperpure*) and 2 mL of hydrogen peroxide (H_2O_2, *Merck, Suprapur*) were added. The microwave digestion procedure applied is depicted in Figure 2.9.

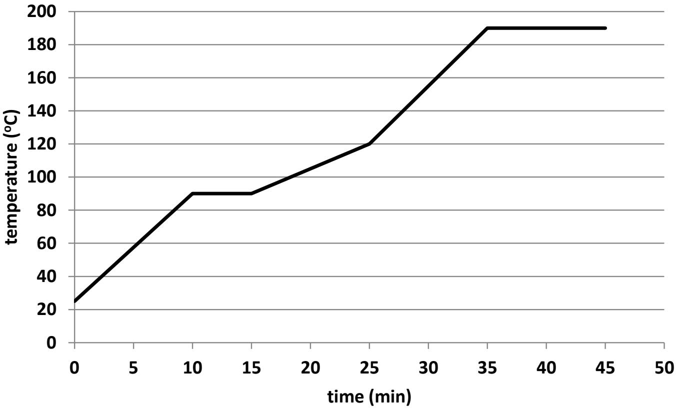


Figure 2.9. Program used for the digestion of seafood in the analysis of total Hg.

Once the digestion was finished, samples were cooled at room temperature, filtered through a filter paper (*Whatman 40*) into 20-mL HDPE vials and diluted with double deionised water. Analyses of total Hg content were performed by ICP-MS following the procedure described in section 2.3.1.3.

2.4.3.2. Microwave extraction

Determination of Hg species content in seafood samples and CRMs was carried out by microwave assisted extraction. The procedures used were previously tested by the research group: 0.1 – 1 g of sample and 10 mL of either hydrochloric acid or nitric acid 4 mol L^{-1} were weighed in the digestion vessels. The extraction was performed as follows: 2 minutes from room temperature to 100ºC and 10 minutes maintained at 100ºC. After the extraction and cooling to room temperature, samples were filtered through a filter paper (*Whatman 40*) into 20-mL HDPE vials and diluted with double deionised water. Analyses of Hg species were performed by LC-UV-CV-AFS by direct injection of sub-samples to the system operating at the conditions mentioned in section 2.3.2.1.

2.4.3.3. Moisture determination

The moisture of seafood samples was determined in triplicate by drying 0.5 g aliquots in an oven (*JP Selecta*) at 102 ± 3°C until constant weight.

Chapter III

DEVELOPMENT OF ANALYTICAL METHODS

The hyphenated techniques are widely used to get information on analytical speciation. They consist of a separation technique coupled with different atomic spectroscopy detectors, such as AFS, AAS, ICP-MS and ETAAS [Szpunar et al., 1999]. Among them, AFS provides a good selectivity and sensitivity which is suitable for the determination of some elements, such as Sb, Hg, As and Se in environmental samples. Moreover, this detection technique provides suitable analytical performance; it is user-friendly and requires lower investment and maintenance costs than ICP-MS, so it is a good approach in daily routine laboratory analyses. On the other hand, ICP-MS provides high robustness, low limits of quantification and a wide linear range. Besides, matrix effects are less important as they are easily reduced in the plasma, and mass spectrometry provides suitable selectivity. Therefore, in this thesis, the analytical development of methods for antimony speciation using LC-HG-AFS and LC-ICP-MS has been carried out.

In the first part of this section, a study for the separation of inorganic Sb species has been performed. These species are the main forms present in the aquatic environment. Besides, methods for the separation of mercury species have been assessed.

As mention in the introduction, Sb and Hg concentrations in environmental and food matrices are generally low. AFS techniques are sensible enough to detect and quantify the total concentration levels of Sb or Hg in this kind of matrices. However, when they are coupled to the liquid chromatography, there is a dilution factor coming from the column, which increases the limits of detection. Consequently, the detection technique becomes not sensible enough for the detection of Sb or Hg species at the concentration levels present in waters. Therefore, in order to detect these low concentrations, a preconcentration step is required. Thus, in the second part of this chapter, a systematic study for the development of an online preconcentration followed by LC-UV-CV-AFS and LC-HG-AFS determination for the mercury and antimony speciation, respectively, was carried out. These methods were used for the analysis of water samples.

3.1. METHOD DEVELOPMENT FOR THE DETERMINATION OF INORGANIC ANTIMONY SPECIES

3.1.1. General background

The determination of total antimony is one of the main issues which has been performed in the research group the last years using methodologies based on ICP-MS or HG-AFS techniques, which have been previously detailed in Chapter 2. These methodologies were applied for the analysis of environmental, biological and food samples aiming the total antimony determination at the ng L^{-1} range.

Besides, antimony speciation has also been focused in the group since the last years. One of the most relevant studies was the development of a method for the determination of three antimony species (the inorganic species, Sb(V) and Sb(III) and the organometallic species trimethylantimony, TMSb) by LC-ICP-MS [Nataren-Hernandez et al., 2011]. The chromatographic conditions used were an anion exchange column (Hamilton, 250 x 4.1 mm id) as stationary phase and EDTA 10 mmol L^{-1} with 0.5% of methanol at pH 4.0 with a 1.5 mL min^{-1} flow rate as mobile phase. The conditions of the ICP-MS system were those mentioned in section 2.3.1.3. It was observed that Sb(V) is a non-retained species because it was detected at the dead time of the column, whereas the other species presented some affinity with the stationary phase. The retention times for Sb(V), TMSb and Sb(III) were 2, 3 and 7.5 minutes, respectively, with a total analysis time of 10 minutes approximately. Figure 3.1 shows a chromatogram of a standard of the three species with a concentration of 2, 7 and 7 µg L^{-1}, respectively.

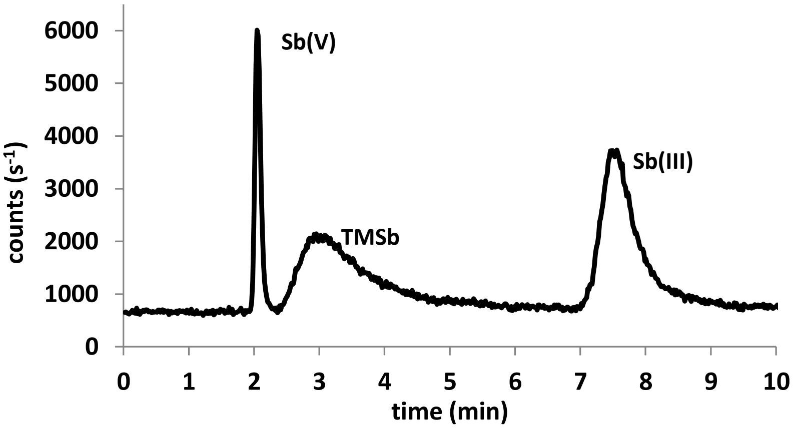


Figure 3.1. Chromatogram of a Sb(V), TMSb and Sb(III) standard obtained by LC-ICP-MS.

It was observed that TMSb showed a different elution profile with respect to the other species as it presents a non-Gaussian distribution with a long tail. It could be due to a possible interaction not only with the stationary phase, but also with the support.

As TMSb species is rarely present in most of environmental samples [Nataren-Hernandez et al., 2011], in this thesis it was decided to modify the existing analytical method in the research group for the determination of the inorganic species.

3.1.2. Optimisation of the method

First of all, preliminary studies were carried out, which consisted of establishing the optimum working conditions for the separation of Sb(V) and Sb(III) in a shorter analysis time and good resolution. The first test was performed with the same column and mobile phase used for the separation of Sb(V), TMSb and Sb(III), mentioned before. Initially, pH of the mobile phase was the variable optimised, ranging values from 3 to 8. Detection was carried out by HG-AFS using the conditions mentioned in section 2.3.2.2. Figure 3.2 shows the chromatograms obtained in this test.

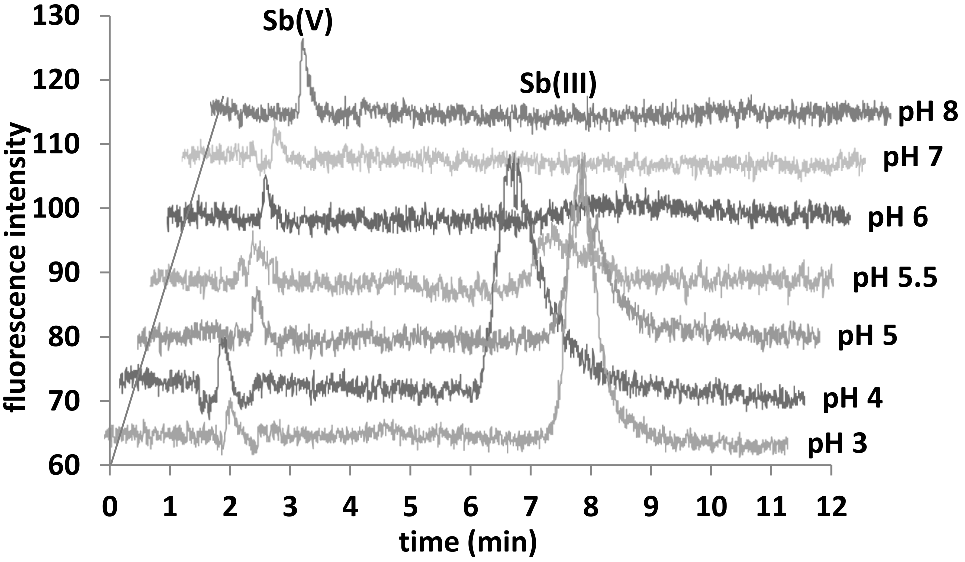


Figure 3.2. Sb(V) and Sb(III) separation by LC at different mobile phase pH values and detection by HG-AFS. Flow rate: 1.5 mL min^{-1}. Sb(V) and Sb(III) concentration: 20 µg L^{-1}.

It was observed that Sb(V) presented the same retention time in all cases, 2 minutes approximately, whereas Sb(III) presented always a stronger retention. This fact is due to the form in which species are present in the working conditions. For the interpretation, Sb(V) and

Sb(III) equilibrium pH diagrams were represented in the presence of EDTA (Figure 3.3) using the “Chemical Equilibrium” software *Hydra* and *Medusa*, which contains a database with logK data at 25°C [Chemical equilibrium diagrams, 2013].

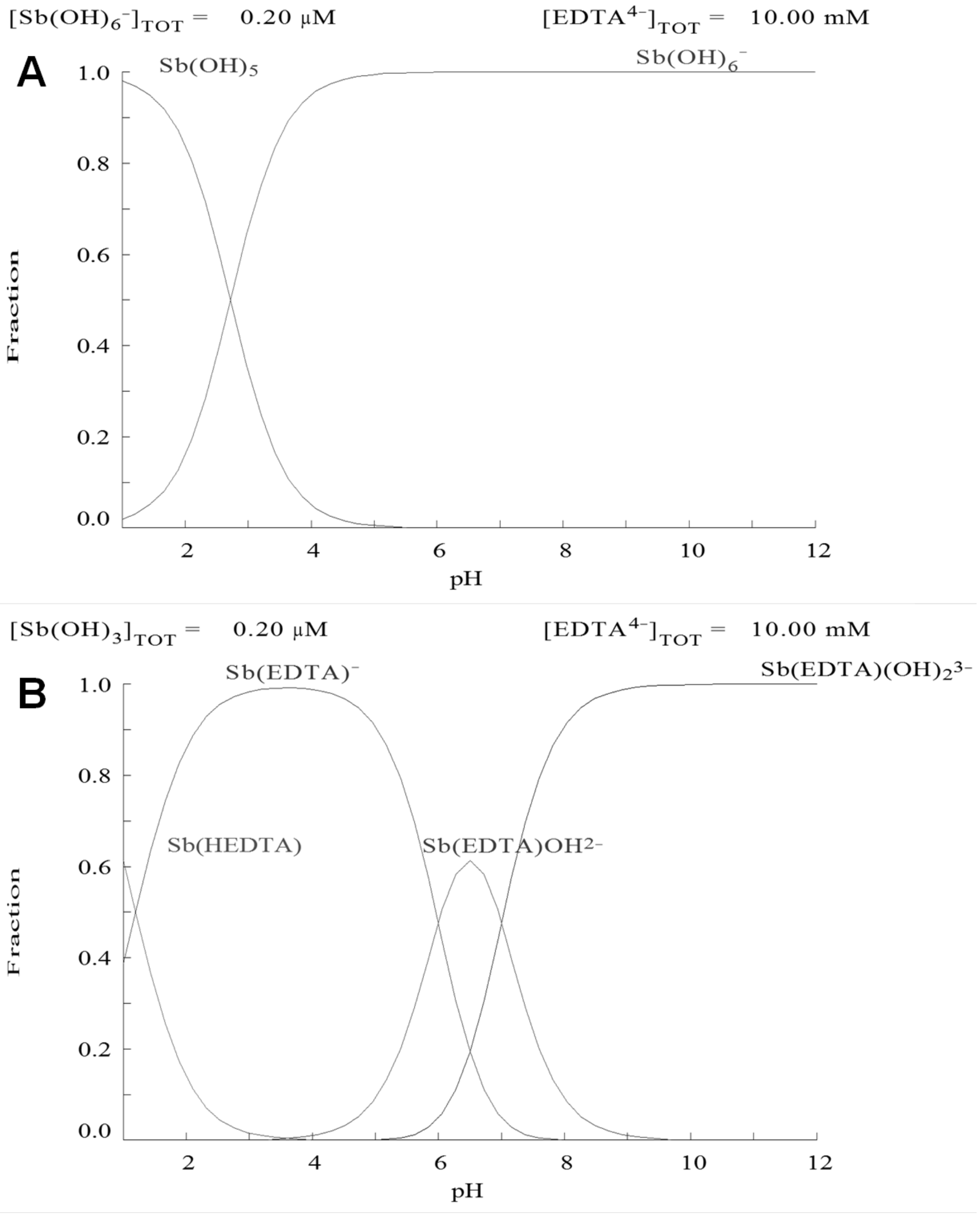


Figure 3.3. Equilibrium pH diagrams of Sb(V) – EDTA (A) and Sb(III) – EDTA systems (B).

Sb(V) species is not complexed with EDTA and it is in the same form in most of the pH range studied. However, Sb(III) is complexed with EDTA when injecting into the column and a soluble negatively charged complex is formed [Krachler et al., 2001]. This complex is not only negative charged but also higher in mass than the Sb(V) species. Therefore, Sb(III) species have more affinity for the anionic exchange stationary phase in the presence of EDTA, which explains the higher retention of Sb(III) by comparison with Sb(V). The antimony complexes formed in the presence of EDTA were verified experimentally by High Resolution Mass Spectrometry (HRMS), by analysing standards of 1 mg L^{-1} of Sb(V) and Sb(III) with the presence of the stoichiometric concentration of EDTA: for pentavalent species, non-complexed Sb(V) ($[Sb(OH)_6]^-$) and free EDTA ($C_{10}H_{15}N_2O_8^-$) were detected with mass-to-charge (m/z) values of 222.92035 and 291.08308, respectively, whereas for trivalent species, the complex Sb(EDTA) ($C_{10}H_{12}N_2O_8Sb^-$) was detected with a m/z value of 408.96353. The structure of this complex is depicted in Figure 3.4.

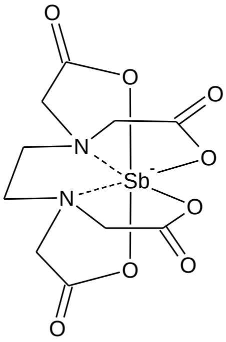


Figure 3.4. Structure of the complex formed between Sb(III) and EDTA. ($C_{10}H_{12}N_2O_8Sb^-$; m/z 408.96353).

It was also observed that Sb(III) retention time does not follow a clear trend with pH. The shorter retention time was observed at pH 4. Moreover, from pH 4 the intensity of the corresponding signal to Sb(III) decreased: at pH 5, the peak intensity is slightly lower, at pH 5.5 the peak is even lower and loses its Gaussian profile, and from pH 6, the species could not be detected. This fact can be also explained with the pH diagrams. At pH 4, the only species present in solution is the negative charged Sb(III)-EDTA complex aforementioned. However, from this pH, the presence of two-negative and three-negative charged complexes is significant. This species may not be eluted from the column as they may keep strongly retained on the stationary phase. The strong retention of Sb(III) in anion exchange phases under basic conditions was also observed by other authors [Miravet el al., 2004].

Another aspect which can be observed in the chromatogram profiles obtained at pH 3 and 4 from Figure 3.2 is that although standards have the same concentration, Sb(III) peak intensity is much higher than the Sb(V) one, which is due to a higher yield in the hydride formation with Sb(III) species [Chen et al., 2010a].

Thus, a good separation of the species was achieved at pH range 3 – 5.5, with a 9 – 10 minutes analysis time. However, this analysis time could be shortened as time difference between Sb(V) and Sb(III) is considerable (about 5 minutes). For this reason, a second test was performed assessing a shorter anion exchange column (Hamilton, 125 x 4.1 mm id) with the same mobile phase adjusted at pH 4.0, in which the shortest time and highest sensitivity was previously observed. At these conditions, flow rate was changed from 1.5 to 3.5 mL min^{-1}. As mentioned before, Sb(V) is less sensitive than Sb(III), therefore, Sb(III) standard used for this test is 4 times less concentrated than Sb(V) standard. Figure 3.5 shows the chromatograms obtained in this test.

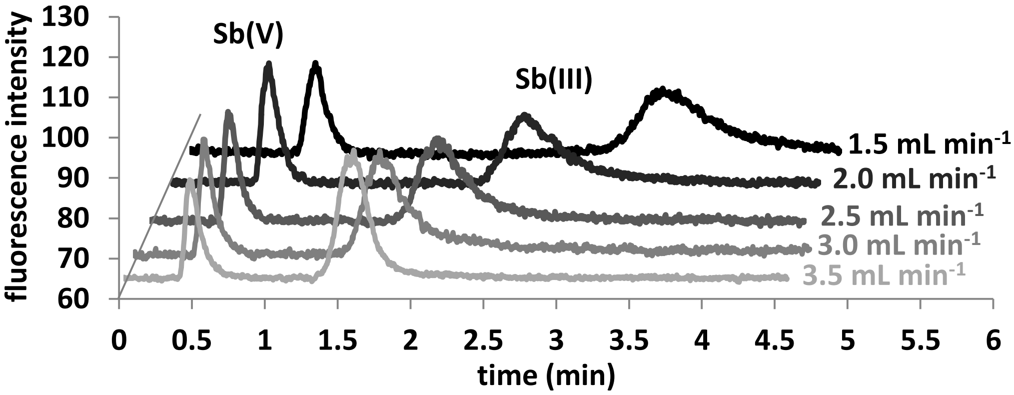


Figure 3.5. Sb(V) and Sb(III) separation by LC with a Hamilton PRP-X100 column (125 x 4.1 mm) at different flow rates and detection by HG-AFS. Mobile phase pH: 4.0. Sb(V) concentration: 20 µg L^{-1}. Sb(III) concentration: 5 µg L^{-1}.

As expected, analysis time is almost twice shorter (about 5.5 minutes) than those obtained before when working at the lowest flow rate, 1.5 mL min^{-1}. The analysis time was improved when a higher flow rate is used. A 2.5-minute analysis time was achieved using the highest flow rate (3.5 mL min^{-1}), which corresponds to the maximum pressure (200 bar) that is recommendable to work with this column.

Once developed the chromatographic method for Sb(V) and Sb(III) separation using HG-AFS as detection, the adjustment of the optimum chromatographic conditions to the ICP-MS detection was undertaken. This instrument provides more robustness and sensitivity than the AFS, therefore it is more suitable for the analysis of samples with low concentration levels. In order to adapt ICP-MS to the LC, some conditions need to be modified because of the ICP features, such as the nebuliser design or the plasma stability. In the first case, a flood of the nebulised has to be prevented; thus, a high mobile phase flow rate cannot be used. A flow rate of 1.5 mL min^{-1}, which is the maximum allowed for the nebuliser, was selected. In the second case, organic solvents cause instability in the plasma, so, low ratios of them are allowed to be used. For this reason, from the beginning of the experiment when assessing the retention of the species by LC-HG-AFS, inorganic antimony speciation was always carried out with the presence of only a 0.5 % of methanol as organic modifier assuring in that way the plasma stability in the torch when analysing by LC-ICP-MS. Hence, the Sb species eluted in chromatogram obtained using these conditions presented the same retention times to those depicted in Figure 3.5 using a flow of 1.5 mL min^{-1}: Sb(V) presents a retention time of about 1 minute and whereas the Sb(III) one is a little bit less than 4 minutes, with a total analysis time of 5.5 minutes. Figure 3.6 shows a chromatogram of a standard of 0.25 µg L^{-1} of Sb(V) and 1.0 µg L^{-1} Sb(III) obtained by LC-ICP-MS using the conditions described in Chapter 2.

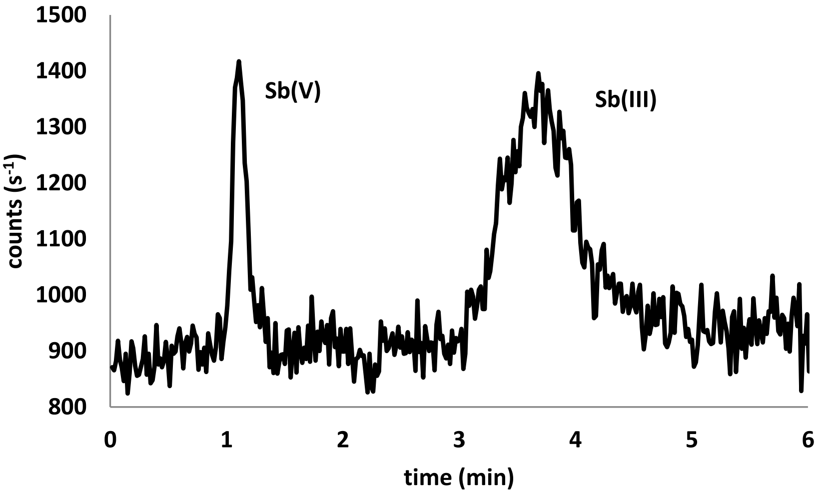


Figure 3.6. Sb(V) and Sb(III) separation by LC-ICP-MS. Column: Hamilton PRP-X100 (125 x 4.1 mm). Mobile phase: EDTA 10 mmol L^{-1} at pH 4.0 with a 0.5% of methanol at 1.5 mL min^{-1} flow rate. Sb(V) concentration: 0.25 µg L^{-1}. Sb(III) concentration: 1 µg L^{-1}.

Thus, the optimum conditions established for the determination of inorganic antimony species are summarised in Table 3.1.

Table 3.1. Experimental conditions of the chromatographic separation for the determination of antimony inorganic species.

	LC-HG-AFS	LC-ICP-MS
Column	Hamilton PRP-X100 (125 × 4.1 mm)	
Mobile phase	EDTA 10 mmol L^{-1} at pH 4.0 with a 0.5 % of methanol	
Flow rate	3.5 mL min^{-1}	1.5 mL min^{-1}
Retention time	Sb(V): 0.6 min Sb(III): 1.5 min	Sb(V): 1.1 min Sb(III): 3.8 min
Analysis time	2.5 min	5.5 min

3.1.3. Quality parameters

The quality parameters both for the total Sb determination techniques and the speciation methods optimised were established. These parameters were the limit of linearity (LOL), the repeatability (RSD$_r$), the reproducibility (RSD$_R$), and the limits of detection (LD) and quantification (LQ).

3.1.3.1. Limit of linearity

The calculation of the limit of linearity was performed with the analysis of the corresponding standards, both for total and speciation, at concentrations between 5 and 1000 µg L^{-1}. In this case, only the HG-AFS technique was used for the determination of the LOL as it is well known the wider linear ranges provided by ICP-MS [Wilbur, 2002].

The results obtained for the limit of linearity showed that the fluorescence intensity corresponding to peak area either for total Sb and inorganic species followed a linear behaviour up to 1000 µg L^{-1}, which is the whole concentration range studied. As an example, Figure 3.7 depicts the calibration curves for Sb(V) and Sb(III) standards analysed by LC-HG-AFS. The differences in sensitivity previously observed between Sb(V) and Sb(III) were corroborated by the slope values obtained. It can be observed that R^2 determination coefficient is higher than 0.999 for both species.

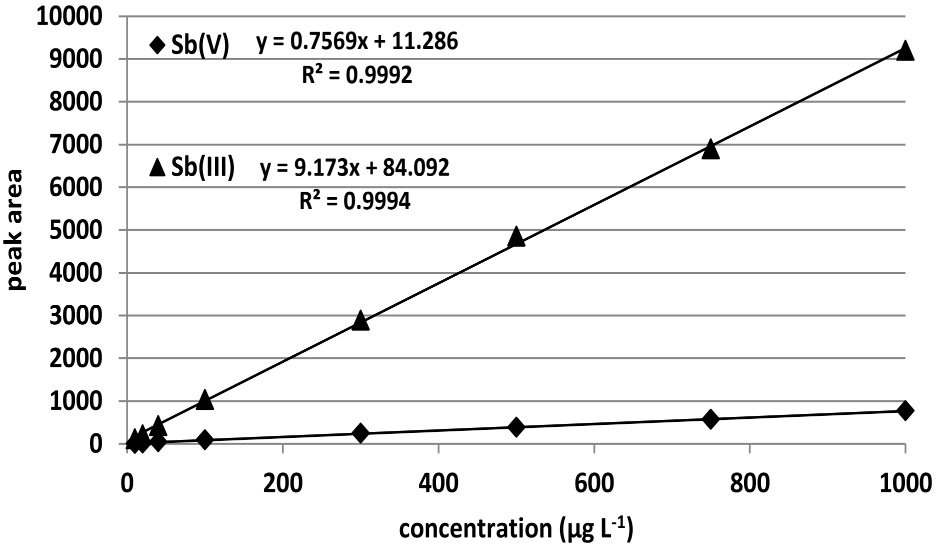


Figure 3.7. Sb(V) and Sb(III) calibration curves obtained by LC-HG-AFS.

From data obtained, RSD_r and the RSD_R were assessed. The calculation of these parameters was performed using duplicate values obtained from two standards used for the calibration curve in three different sessions. The antimony concentrations were selected according to those described in the literature for the samples studied in the present thesis: 0.5 and 5 µg L^{-1} for total determination and antimony speciation by LC-ICP-MS (both species); 10 and 100 µg L^{-1} for Sb(V) and 3 and 30 for Sb(III), in the case of antimony speciation by LC-HG-AFS. The values obtained for the RSD_r and RSD_R are summarised in Table 3.2. Slightly high RSD_R values were observed in the HG-AFS techniques, which demonstrate that ICP-MS provides a signal with higher stability. This fact is not surprising as the fluorescence signal depends on a lot of variables, such as the aging of the lamp, the flame status and the concentration and purity of hydride generation reagents, which can lead to significant variations in the signal throughout days.

Table 3.2. Repeatability and reproducibility values in terms of Relative Standard Deviation (RSD, %) obtained from 2 concentration levels of the calibration curves with both total and speciation techniques.

Quality parameter		**ICP-MS**	**LC-ICP-MS**		**HG-AFS**	**LC-HG-AFS**	
		Sb total	**Sb(V)**	**Sb(III)**	**Sb total**	**Sb(V)**	**Sb(III)**
RSD_r	low level	8.0	5.5	8.1	6.2	5.3	8.1
	high level	5.3	6.4	4.0	4.8	3.1	7.8
RSD_R	low level	6.0	13.1	11.7	27.2	21.2	14.7
	high level	4.3	10.7	9.4	24.1	13.4	16.5

3.1.3.2. Limits of detection and quantification

For total determination the signal of the limit of detection and quantification (S_{LD}; S_{LQ}, respectively) needs to be established. In the used techniques, these parameters are defined as the signal obtained in a blank (S_B) plus three or ten times, respectively, its standard deviation (σ_B):

$S_{LD} = S_B + 3 \cdot \sigma_B$

$S_{LQ} = S_B + 10 \cdot \sigma_B$

Secondly, the concentration in the limit of detection and quantification (C_{LD}, C_{LQ}, respectively) is calculated as the signal in the limit divided by the slope of the calibration curve (S) constructed in the lower part of the working range corresponding to the total antimony standards:

$C_{LD} = S_{LD}/S$

$C_{LQ} = S_{LQ}/S$

For speciation, the standard deviation of the background of the different chromatogram profiles from standards used for the establishment of a calibration curve in the lower part of the working range was established. Then, concentrations in the limit of detection and quantification are defined as three or ten times this standard deviation, respectively, divided by the slope of the calibration curves mentioned (S), in terms of peak height, for each species:

$C_{LD} = 3 \cdot \sigma_{BG}/S$

$C_{LQ} = 10 \cdot \sigma_{BG}/S$

The values of the concentrations in the limit of detection and quantification in the instrumental techniques mentioned used in this thesis for antimony determination were summarised in Table 3.3. The values were assessed at least in four independent working sessions with fresh standard and blank solutions, showing the stability of both measuring techniques.

Table 3.3. Instrumental limits of detection and quantification for total Sb and speciation.

Quality parameter	**ICP-MS**	**LC-ICP-MS**		**HG-AFS**	**LC-HG-AFS**	
	Sb total	**Sb(V)**	**Sb(III)**	**Sb total**	**Sb(V)**	**Sb(III)**
LD (µg L^{-1})	0.01	0.01	0.03	0.11	2.04	0.55
LQ (µg L^{-1})	0.02	0.03	0.09	0.14	6.8	1.82

The values obtained by ICP-MS are lower than those obtained by HG-AFS, which evidences that the first technique is more sensible than the second one. This difference is not quite significant for total determination, but for speciation. It can be observed that the values obtained by LC-HG-AFS are quite higher than the corresponding ones obtained by LC-ICP-MS, especially for Sb(V) species. This could be related to a low yield in the hydride formation with Sb(V) species, as previously mentioned.

3.2. ANALYTICAL PERFORMANCE OF METHODOLOGY FOR MERCURY DETERMINATION

3.2.1 State of the art

The determination of total mercury is also other of the research topics which has been studied in the research group using methodologies based on ICP-MS or CV-AFS techniques, previously described. These methodologies were applied for the analysis of environmental, biological and food samples for total mercury determination in the ng L^{-1} range.

Besides, mercury speciation is an interesting topic which has been also studied. The most recent studies dealt with the development of a method for the determination of methylmercury and mercury (II) by LC-UV-CV-AFS [Ibáñez-Palomino et al., 2012a]. The reversed phase chromatographic conditions used were an ODS Hypersyl column (250 mm x 4.6 mm id) with a C18 as stationary phase and a mixture of a buffer solution (APDC 0.0015 mol L^{-1} and NH_4CH_3COO 0.01 mol L^{-1} at pH 5.5) and methanol 20:80 with a 1.5 flow rate mL min^{-1} as mobile phase. The conditions of the CV-AFS system were those mentioned in section 2.3.1.1. It was observed the good separation of $MeHg^+$ and Hg(II) with retention times of 4.5 and 7.5 minutes, respectively with a total chromatographic time of 10 minutes. Figure 3.8A shows a chromatogram of a standard of the two species with concentrations of 10 µg L^{-1}. From this conditions, the development of a method for the determination of three mercury species, $MeHg^+$, $EtHg^+$ and Hg(II) was carried out. Although the presence of ethylmercury is much less frequent in environmental and biological samples, it could be found in these matrices if they are sampled from a zone which has undergone immediate contamination which come from recent polluted zones. When analysing the three species, simultaneously, $EtHg^+$ eluted just after

$MeHg^+$ without enough peak resolution, as can be seen in Figure 3.8B. Thus, for the achievement of well-resolved chromatographic peaks, the mobile phase ratio was modified to a 75% of methanol and 25% of the buffer solution mentioned. The retention times for $MeHg^+$, $EtHg^+$ and Hg(II) were 5, 6.5, and 9, respectively with a total chromatographic time of 12 minutes [Ibáñez-Palomino, 2012]. Figure 3.8C shows a chromatogram of a standard of the three species with concentrations of 10 µg L^{-1}.

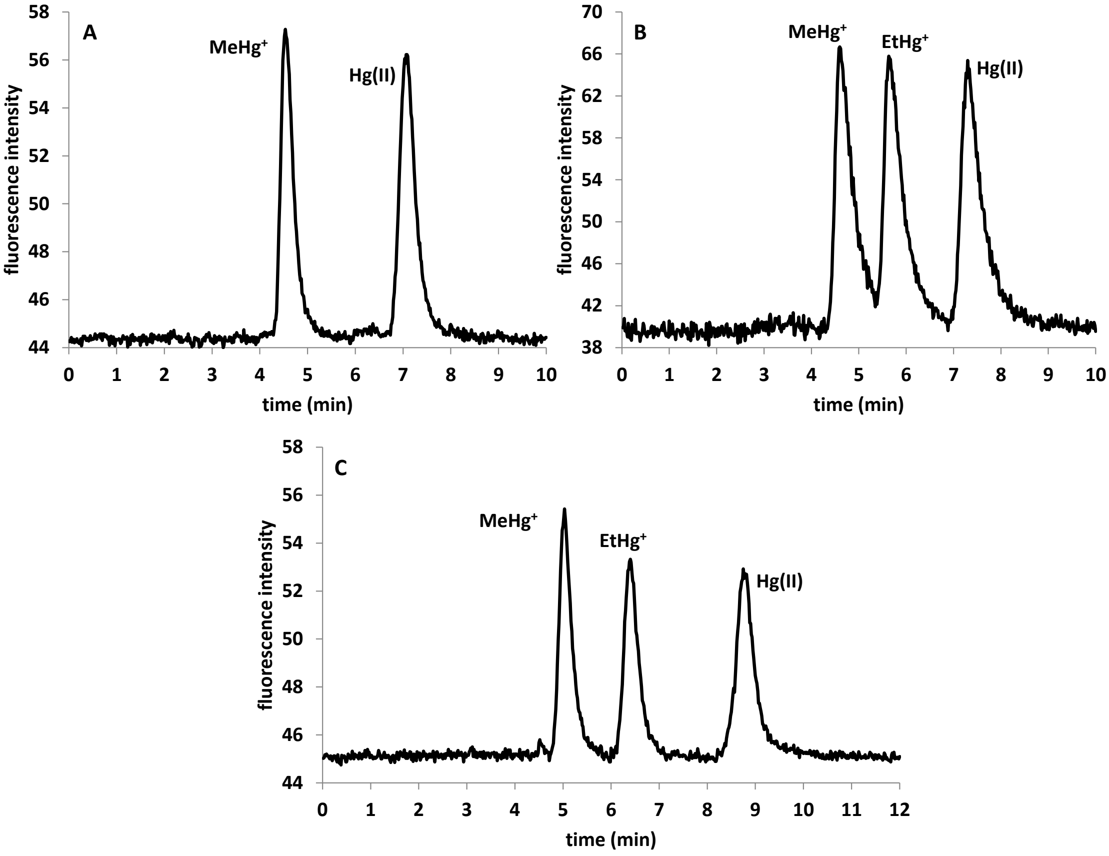


Figure 3.8. Chromatograms of mercury species using a mobile phase ratio of 80% methanol and 20% of buffer solution (A, B) and a mobile phase ratio of 75% methanol and 25% of buffer solution (C) obtained by LC-UV-CV-AFS.

These methods are suitable for the determination of mercury species in a wide range of polluted samples. Thus, the quality parameters were reassessed for these methods.

3.2.2. Quality parameters

The quality parameters both for the total Hg determination techniques and the speciation methods were established. These parameters were the limit of linearity (LOL), the repeatability (RSD_r), the reproducibility (RSD_R), and the limits of detection (LD) and quantification (LQ).

3.2.2.1. Limit of linearity

The calculation of the limit of linearity was performed with the analysis of the corresponding standards, both for total and species, at concentrations between 5 and 1000 µg L^{-1}. Once again, only the fluorescence technique was used for the determination of the LOL.

The results obtained for the limit of linearity showed that the fluorescence intensity both for total Hg and species followed a linear tendency up to 750 µg L^{-1}. In this case, it was observed a significant decrease in the linearity from this concentration. As an example, Figure 3.9 depicts the calibration curves for $MeHg^+$, $EtHg^+$ and Hg(II) standards analysed by LC-UV-CV-AFS. R^2 coefficients are 0.99998, 0.999995 and 0.99995, respectively, when performing the calibration curves up to 750 µg L^{-1}, whereas these values become lower than 0.99 when performing the curves up to 1000 µg L^{-1}.

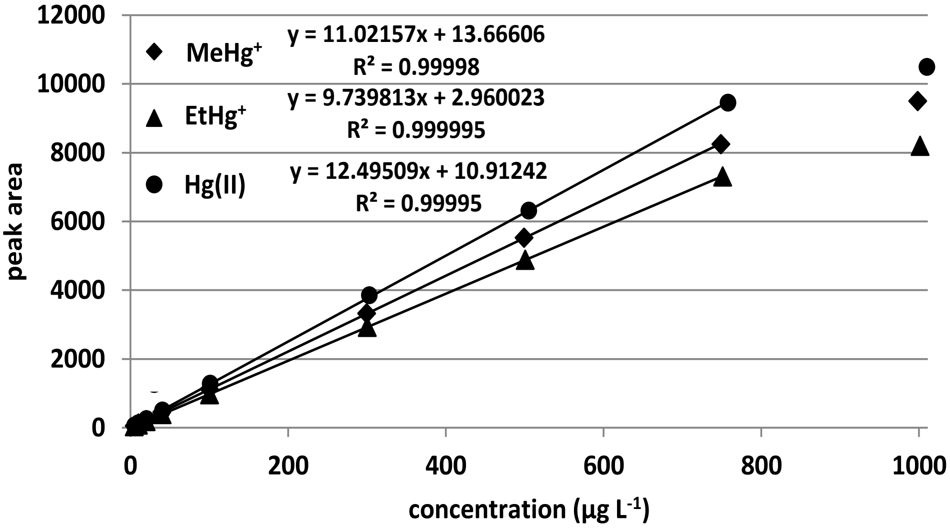


Figure 3.9. $MeHg^+$, $EtHg^+$ and Hg(II) standards calibration curves obtained by LC-UV-CV-AFS.

From data obtained, RSD_r and the RSD_R were assessed. The calculation of these parameters was performed using duplicate values obtained from two standards used for the calibration curve in three different sessions. The concentrations were selected according to those described in the literature for the samples studied in the present thesis: 0.5 and 5 µg L^{-1} for total determination and 10 and 100 µg L^{-1} for mercury speciation. The values obtained for the RSD_r and RSD_R are summarised in Table 3.4. Once again, quite high RSD_R values were observed in the CV-AFS techniques, since slight variations in the conditions cause significant changes in the fluorescence signal.

Table 3.4. Repeatability and reproducibility values in terms of RSD obtained from 2 concentration levels of the calibration curves with both total and speciation techniques.

Quality parameter		**ICP-MS**	**CV-AFS**	**LC-UV-CV-AFS (2 species)**		**LC-UV-CV-AFS (3 species)**		
		Hg total	**Hg total**	**$MeHg^+$**	**Hg(II)**	**$MeHg^+$**	**$EtHg^+$**	**Hg(II)**
RSD_r	low level	6.3	3.2	10.0	9.2	15.5	16.0	11.7
	high level	3.6	2.4	5.0	2.4	10.3	10.4	16.1
RSD_R	low level	12.9	27.0	7.7	7.0	11.3	11.4	9.3
	high level	10.2	13.7	5.7	4.1	7.6	9.8	16.0

3.2.2.2. Limits of detection and quantification

The calculations of the limits of detection and quantification were performed using the same equations described in section 3.1.3.2. The values of the concentrations in the limit of detection and quantification in the instrumental techniques mentioned used in this thesis for mercury determination are summarised in Table 3.5. The values reported correspond to the mean obtained from five different working sessions.

Table 3.5. Instrumental limits of detection and quantification for total Hg and speciation.

Quality parameter	**ICP-MS**	**CV-AFS**	**LC-UV-CV-AFS (2 species)**		**LC-UV-CV-AFS (3 species)**		
	Hg total	**Hg total**	**$MeHg^+$**	**Hg(II)**	**$MeHg^+$**	**$EtHg^+$**	**Hg(II)**
LD (µg L^{-1})	0.02	0.1	0.53	0.57	0.38	0.42	0.44
LQ (µg L^{-1})	0.05	0.23	1.8	1.9	1.3	1.4	1.5

Once again, the values obtained by ICP-MS are lower than those obtained by CV-AFS; and the values obtained by speciation are slightly higher than the corresponding ones obtained by total analysis.

3.3. DEVELOPMENT OF ONLINE PRECONCENTRATION METHODS

Regarding all the results obtained in the previous section, it can be considered on the one hand that the proposed antimony speciation LC-ICP-MS method is suitable for the analysis of antimony species in a wide range of environmental samples, such as waters. On the other hand, the limits obtained by LC-AFS techniques may not be high enough for the determination of Sb and Hg in samples with low-concentration levels. Thus, a preconcentration step should be coupled in order to improve them and this section deals with the development of the online methods for Sb determination by LC-HG-AFS and Hg determination by LC-UV-CV-AFS after short-column preconcentration.

For the performance of the preconcentration methods, the previously antimony and mercury chromatographic methods described were adapted, as described in section 2.3.2.3. The online preconcentration system developed in this thesis was based in the use of a chromatographic precolumn controlled by a six-port injection valve.

During the development of the online preconcentration step, several variables were studied to obtain the optimum species recoveries (R), preconcentration factors (PF), the breakthrough volume and good reproducibility. PFs were calculated as the ratio between the concentration obtained after preconcentration and the initial concentration. Recovery values were calculated as the ratio between the experimental concentration obtained and the theoretical. The breakthrough volume is the sample volume loaded from which the signal does not increase and thus, the linearity range between the load volume and the signal intensity is lost. Once established the most suitable conditions of the preconcentration methods on the basis of these indicators, they were applied to determine antimony and mercury species in drinking water samples of different matrix composition.

The drinking water samples for the validation of the preconcentration methods consisted of three samples with different origin and mineral content: tap water, weak mineralised and strong mineralised bottled waters. Prior to Sb and Hg preconcentration, samples were filtered through a filter with a pore size of 0.22 µm and pH and conductivity values for each sample after filtration were measured together with the main anion and cation contents determined by anionic exchange chromatography and ICP-OES, respectively, as described in section 2.3.5. Theses parameters are summarised in Table 3.6.

Moreover, the preconcentration methods were evaluated for quality control purposes using two CRMs. The CRMs of wastewater ERM-CA713 and lake water TM-23.4 were used for the mercury and antimony preconcentration with certified values of 1.84 ± 0.11 µg Hg L^{-1} and 3.26 ± 0.318 µg Sb L^{-1}, respectively.

Table 3.6. Characteristics of the water samples tested.

	Weak mineralised water	Strong mineralised water	Tap water
pH	6.8	7.8	8.1
Conductivity (μS cm^{-1})	66	767	547
Cl^- (mg L^{-1})	1.8	7.1	34.1
F^- (mg L^{-1})	0.06	0.16	0.10
NO_3^- (mg L^{-1})	1.7	0.56	5.6
SO_4^{2-} (mg L^{-1})	5.5	120	43.5
Ca^{2+} (mg L^{-1})	3.2	94	52.9
Mg^{2+} (mg L^{-1})	3.5	43	9.0
Na^+ (mg L^{-1})	1.6	7.7	20.7
K^+ (mg L^{-1})	1.4	2.5	3.3

3.3.1. Antimony preconcentration method

In a previous study in the research group, an attempt to select suitable complexing agents for both Sb(V) and Sb(III) species and retention and elution conditions was carried out. Following this study, in this thesis, some initial tests were performed in order to check that the proposed preconcentration system works, using the following conditions: a short preconcentration column Hamilton PRP-X100 (50 x 4.1 mm), 2-mercaptoethanol at a concentration of 220 µmol L^{-1} as complexing agent for the antimony species and a sample load volume of 2 mL. The concentration of the complexing agent was pre-established as the corresponding to an excess for the total complexation of the antimony species. Using these parameters, these initial tests were carried out using several replicates of working standard solutions containing 10 µg L^{-1} of Sb(V) and Sb(III), that is a concentration slightly higher than the limits of quantification of the LC-HG-AFS technique for the species.

The results indicated that the preconcentration system increased the signal for the working standards. However, at the established separation conditions, the chromatographic peaks obtained presented a sharp and irregular profile as it is depicted in Figure 3.10.

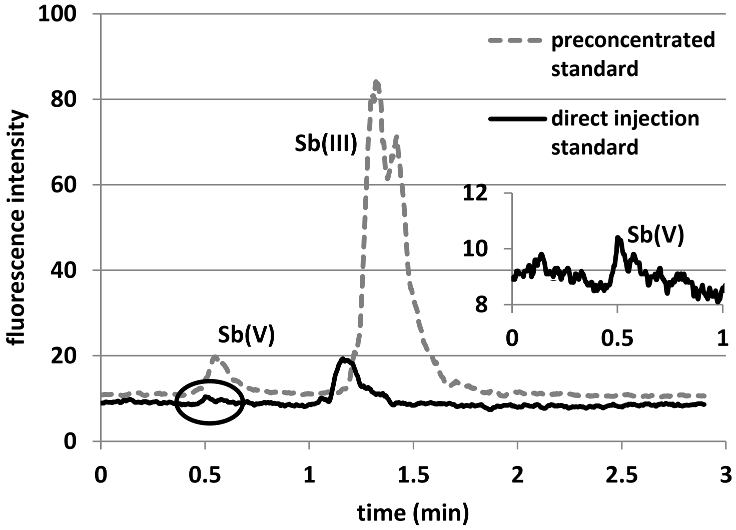


Figure 3.10. Chromatogram profiles obtained from a 10 µg L^{-1} of Sb(V) and Sb(III) standard together with a direct injection (insert for Sb(V)): mobile phase EDTA 10 mmol L^{-1} at pH 4 with 0.5% of methanol.

Thus, systematic studies of several variables were undertaken so as to assess the load volume, preconcentration factors (PF) and recoveries. The variables studied were the concentration and flow rate of the mobile phase, the precolumn length and type, the concentration of the complexing agent and the pH of the working standard solutions.

3.3.1.1. Assessment of the preconcentration step

Mobile phase concentration and flow rate

To decrease the peak irregularity, the concentration of EDTA was increased, since the more concentrated the mobile phase is, the higher the elution strength. Thus, using the conditions described, the retention and the elution of antimony species were assessed using mobile phase concentrations of 30 and 40 mmol L^{-1} and varying the flow rate from 1 to 3.5 mL min^{-1}.

Figures 3.11 and 3.12 depict the results obtained in this test. Each picture represents the preconcentrated 10 µg L^{-1} standard together with the direct injection of the same standard.

It was observed that the chromatographic peaks when using EDTA 40 mmol L^{-1} were more irregular than those obtained when using EDTA 30 mmol L^{-1}, even double peaks for Sb(III) were produced. Regarding the flow rate, even if analysis time diminished with increasing values, the pressure of the system became too high (> 200 bar) when working at a flow of 2.5 mL min^{-1} or greater than it. Thus, a 2 mL min^{-1} flow rate and a 30 mmol^{-1} EDTA concentration were selected as working parameters. The PFs obtained for Sb(V) and Sb(III) were 6.0 ± 0.5 and 9.1 ± 0.4, respectively, which demonstrated that the system provides better results for Sb(III) than for Sb(V).

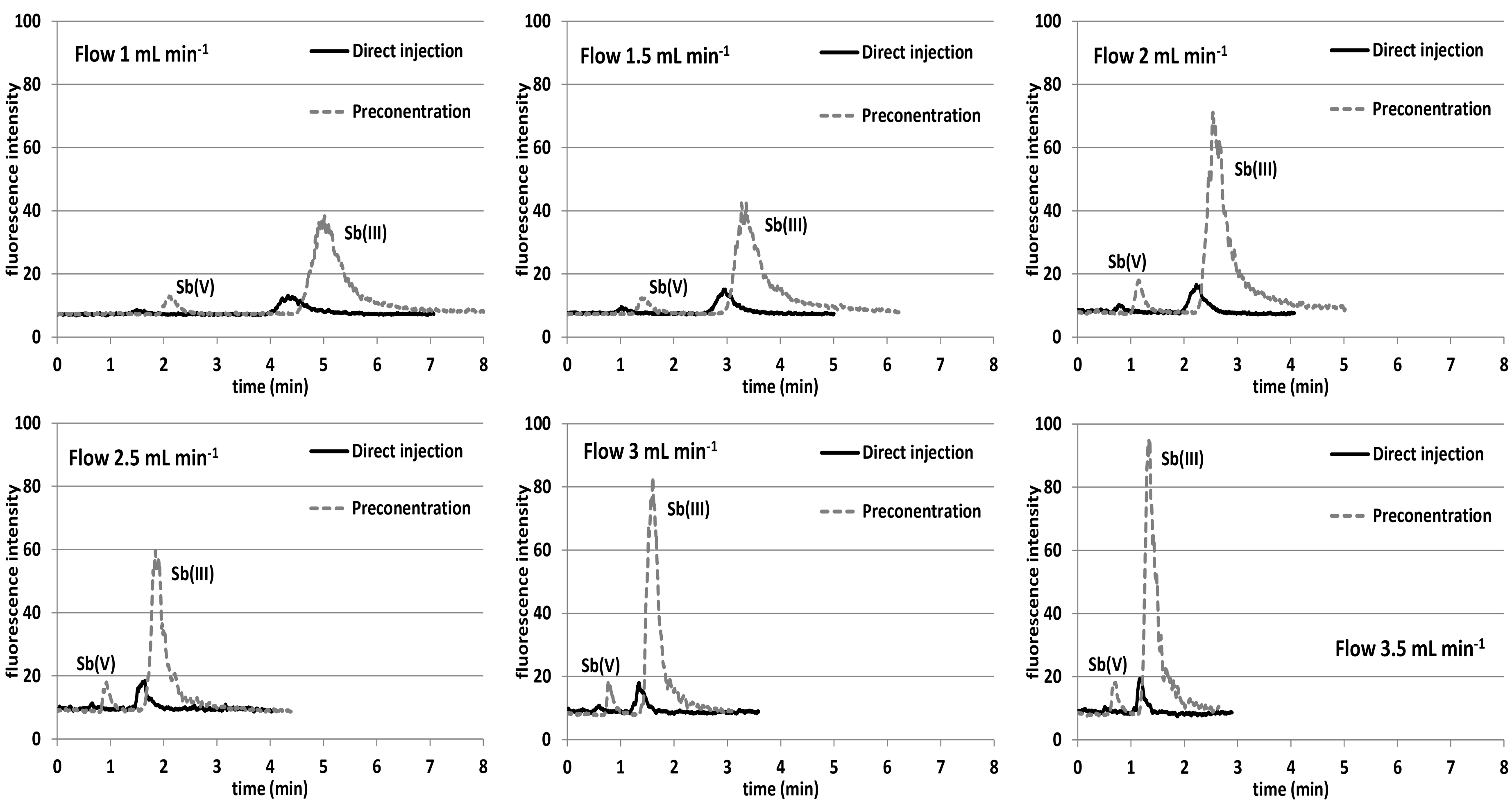


Figure 3.11. Sb(V) and Sb(III) chromatograms of a 10 µg L^{-1} preconcentrated standard obtained using as mobile phase EDTA 30 mmol L^{-1} at pH 4 with 0.5% of methanol based on flow rate, together with a direct injection.

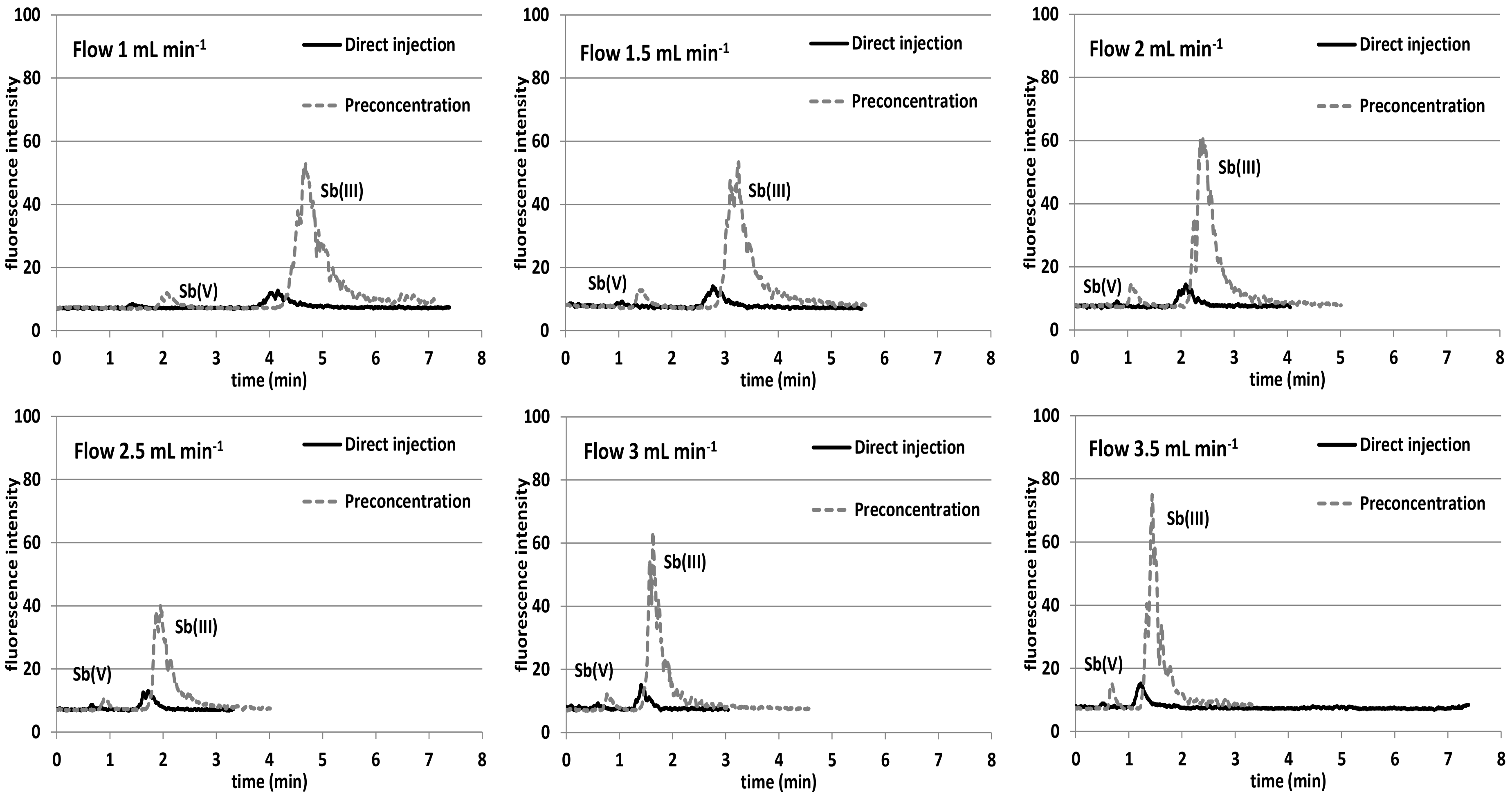


Figure 3.12. Sb(V) and Sb(III) chromatograms of a 10 µg L^{-1} preconcentrated standard obtained using as mobile phase EDTA 40 mmol L^{-1} at pH 4 with 0.5% of methanol based on flow rate, together with a direct injection.

Precolumn length

The aim of this test is the selection of a column length which provides a good retention capability of the species and the optimum preconcentration. Two columns of different lengths were selected: 2 and 5 cm. Working standard solutions of Sb(V) and Sb(III) at concentrations of 10 µg L^{-1} of both species were initially prepared in 2-mercaptoethanol 220 µmol L^{-1}. Increasing volumes of these solutions were tested until the breakthrough point.

The antimony species concentrations obtained in the preconcentration step versus the load volume in the working standard solutions are represented in Figure 3.13 for both column lengths. As can be observed, the 2 cm-long precolumn does not preconcentrate Sb(V) as the signal does not increase with the load volume. Sb(III) species is preconcentrated and has a breakthrough volume of 7 mL. However, in 5 cm-long precolumn, both species were preconcentrated and the breakthrough volumes were increased up to 9 mL for Sb(V) and 11 mL for Sb(III). It can be observed that the breakthrough volumes were higher for Sb(III) than for Sb(V), due to the higher affinity of this species for the anion exchange stationary phase.

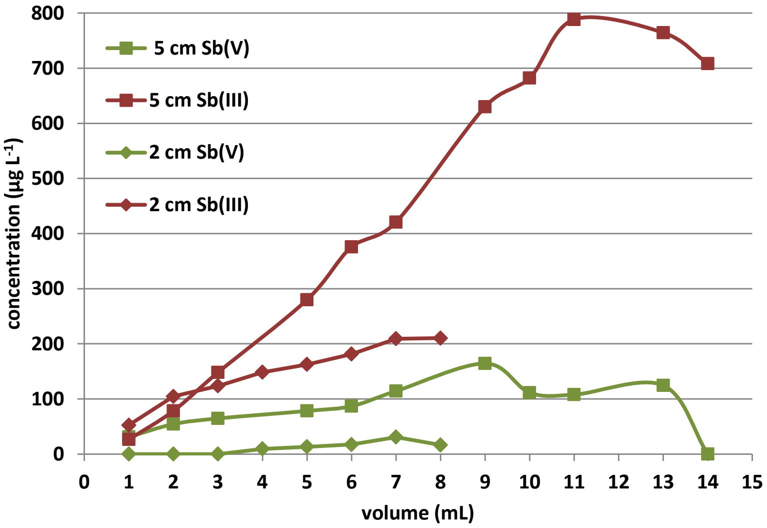


Figure 3.13. Inorganic antimony species concentrations obtained versus volume preconcentrated on working solutions of 10 µg L^{-1} in the assessment of 2 and 5 cm precolumns.

Preconcentration factors and recoveries at the breakthrough volume including the standard deviation for triplicate experiments are summarised in Table 3.7 for both species in the two precolumns and Figure 3.14 depicts the chromatographic profiles at the corresponding volumes together with the direct injection of the 10 µg L^{-1} standard. Higher PFs were obtained when 5 cm-long precolumn was used, due to the higher retention capability in relation to the 2 cm-long precolumn. Regarding percentage recoveries, values lower than 20% were obtained for pentavalent species whereas a significant recovery (72%) was achieved for Sb(III) when using the 5 cm-long precolumn. As it can be seen in the Figure 3.14, the chromatographic behaviour for the two species was different, as Sb(III) species signal is highly increased after preconcentration but the Sb(V) one was not high enough compared to the signal of the direct injection standard. Thus, it can be concluded that a 5 cm precolumn is the most suitable for the preconcentration method and further assays are proposed for achieving a better preconcentration of the pentavalent species.

Table 3.7. Sb(V) and Sb(III) breakthrough volumes, recoveries and preconcentration factors determined in the column length test.

Precolumn length	Species	Breakthrough volume	Concentration (µg L^{-1})	PF	Recovery (%)	RSD (%)
2 cm	Sb(V)	7	30.8 ± 8.5	3.1 ± 0.9	4.4 ± 1.2	27.5
	Sb(III)	7	209.2 ± 4.6	20.9 ± 0.5	29.9 ± 0.7	2.2
5 cm	Sb(V)	9	164.3 ± 9.4	16.4 ± 0.9	18.3 ± 1.0	5.7
	Sb(III)	11	788.6 ± 26.0	78.9 ± 2.6	71.7 ± 2.4	3.3

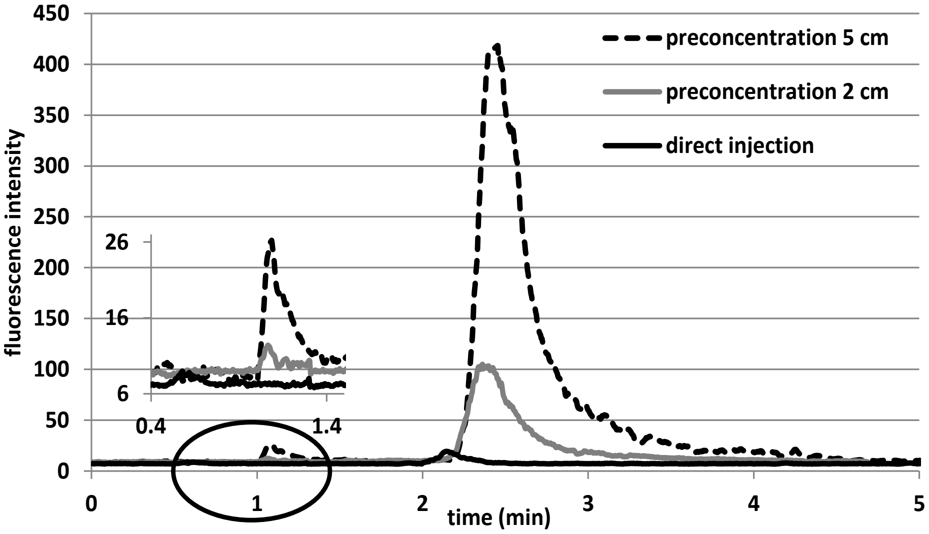


Figure 3.14. Chromatograms obtained from a 10 µg L^{-1} standard at the breakthrough volume in each precolumn, together with a direct injection of this standard. The insert shows an enlargement of the chromatograms.

Complexing agent concentration

The previous experiments have been performed using working standard solutions with a concentration of 10 µg L^{-1} for both antimony species and the concentrations obtained for the trivalent species after preconcentration were much more sensitive than those obtained for the pentavalent one. Taking into account that the limit of quantification for Sb(III) is lower than the Sb(V) one, (1.82 and 6.80 µg L^{-1}, respectively), it was decided to decrease the Sb(III) concentration of the working standard solution at 3 µg L^{-1} whereas the Sb(V) concentration was maintained at 10 µg L^{-1} hereinafter.

In this test, concentrations of 2-mercaptoethanol from 220 µmol L^{-1}, which is the concentration used in the previous tests, to 0.22 µmol L^{-1}, which is the corresponding stoichiometric value for complexing both Sb(III) and Sb(V) at the concentrations used in the working standard solution, were assessed. Increasing sample volumes of these standard solutions were preconcentrated using the 5 cm-long precolumn until reaching the breakthrough volume. Figure 3.15 depicts the Sb concentration obtained at each complexing concentration versus the load volume. As can be seen, the lower 2-mercaptoethanol concentration is, the higher sample volumes could be loaded in the precolumn before achieving the breakthrough point. Consequently, higher concentrations and preconcentration factors were obtained. Especially, the retention capability of Sb(V) species is improved when the complexing agent concentration is decreased and even comparable to the Sb(III) one, obtaining the same breakthrough volume for both species.

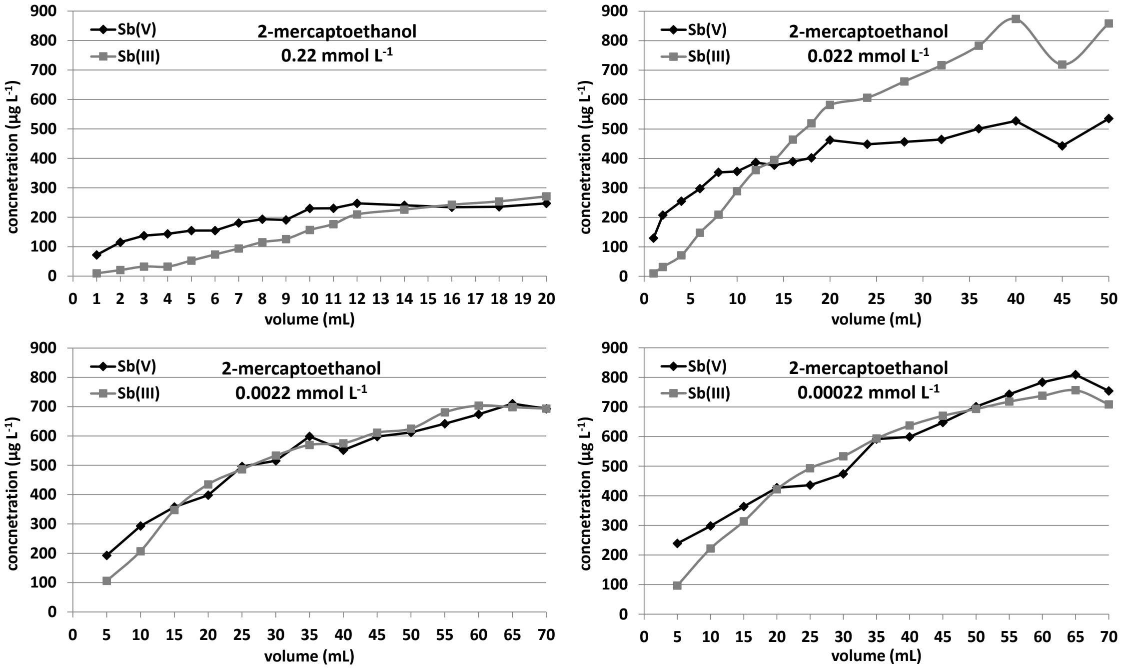


Figure 3.15. Inorganic antimony species concentrations obtained versus volume preconcentrated on working solutions of 10 µg L^{-1} for Sb(V) and 3 for Sb(III) in the assessment of 2-mercaptoethanol concentration. Precolumn length: 5 cm.

Table 3.8 summarises the breakthrough volumes obtained at each 2-mercaptoethanol concentration tested together with the concentrations and the PFs obtained. The optimum values for both Sb(V) and Sb(III) were obtained with the lowest 2-mercaptoethanol concentration. Thus, 0.22 µmol L^{-1} was selected as the working concentration.

Table 3.8. Sb(V) and Sb(III) breakthrough volumes (V), concentrations (C), PFs and recoveries determined in the complexing concentration test.

		Sb(V)				Sb(III)			
Complexing agent (μmol L^{-1})	**V (mL)**	**C (μg L^{-1})**	**PF**	**R (%)**	**RSD (%)**	**C (μg L^{-1})**	**PF**	**R (%)**	**RSD (%)**
220	12	241 ± 6	24 ± 1	20 ± 2	2.4	241 ± 21	80 ± 7	54 ± 2	8.9
22	40	502 ± 36	50 ± 4	24 ± 2	7.3	808 ± 62	270 ± 21	96 ± 1	7.7
2.2	65	692 ± 15	69 ± 2	60 ± 1	2.1	694 ± 9	231 ± 3	88 ± 2	1.2
0.22	65	782 ± 23	78 ± 2	65 ± 1	2.9	734 ± 20	245 ± 7	94 ± 3	2.7

Sample pH and precolumn type

After the establishment of the 2-mercaptoethanol concentration at 0.22 µmol L^{-1}, the pH of the working solution to be preconcentrated was assessed. Different pHs were tested: acidic (pH 3), alkaline (pH 12) and pH 5, this last corresponding to the double deionised water available in the laboratory without any adjustment. Since the variation of the pH changes the nature of the Sb(V) and Sb(III) species, as it has been reported [Tella et al., 2012; Filella et al., 2005], the assay was carried out both with and without the presence of 2-mercaptoethanol 0.22 µmol L^{-1} in the working standard solution. Furthermore, for this same reason, two different types of 5 cm-long precolumn were tested: the anion exchange Hamilton PRP-X100 and a RP-C18 ODS Hypersyl.

Figure 3.16 depicts the Sb concentration obtained versus the load volume at each pH tested using the C18 precolumn. It was observed that the concentration of pentavalent species does not increase with the load volume, at any pH and with or without 2-mercaptoethanol in the solution. The maximum PF and recovery obtained was 12.2 and 27%, respectively. Therefore, it can be concluded that Sb(V) was not retained in this kind of column at any of the conditions assayed. Regarding the trivalent species, it was observed a good retention when using the complexing agent at pH 3 or 5, as the concentration is greatly increased with the load volume. PFs obtained were 183 and 85, respectively and recoveries were 53% and 34%. However, similar behaviour as Sb(V) was observed when working at pH 12, with a slight increase of the concentration, showing no good retention.

Figure 3.17 depicts the Sb concentration obtained versus the load volume at each pH tested using the anion exchange precolumn. Using this type of column, the retention for the two species is considerably improved, except for the case of pH 12. At pH 3 and pH 5 with the presence of the complexing agent, Sb(III) presents the optimum retention capability, whereas the greatest retention of Sb(V) is observed mainly at pH 5 with the presence of 2-mercatoethanol 0.22 µmol L^{-1}, since it followed a retention profile similar to Sb(III). The PFs obtained in this case are 75 and 242 for Sb(V) and Sb(III), respectively, and the recoveries obtained are 63% and 90%, respectively.

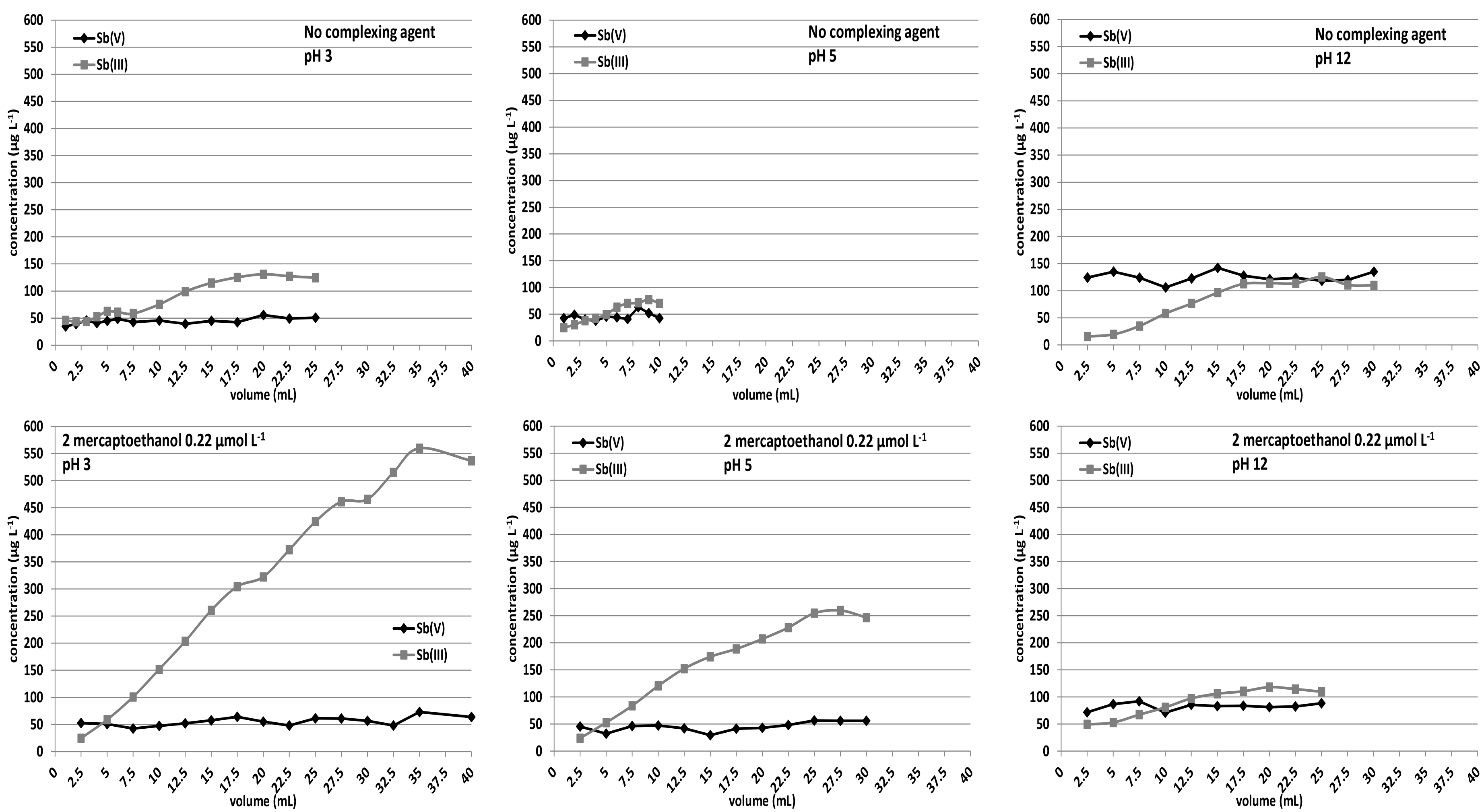


Figure 3.16. Inorganic antimony species concentrations obtained versus volume preconcentrated on working solutions of 10 µg L^{-1} for Sb(V) and 3 for Sb(III) in the assessment of the pH and precolumn type. Precolumn used: **C18**.

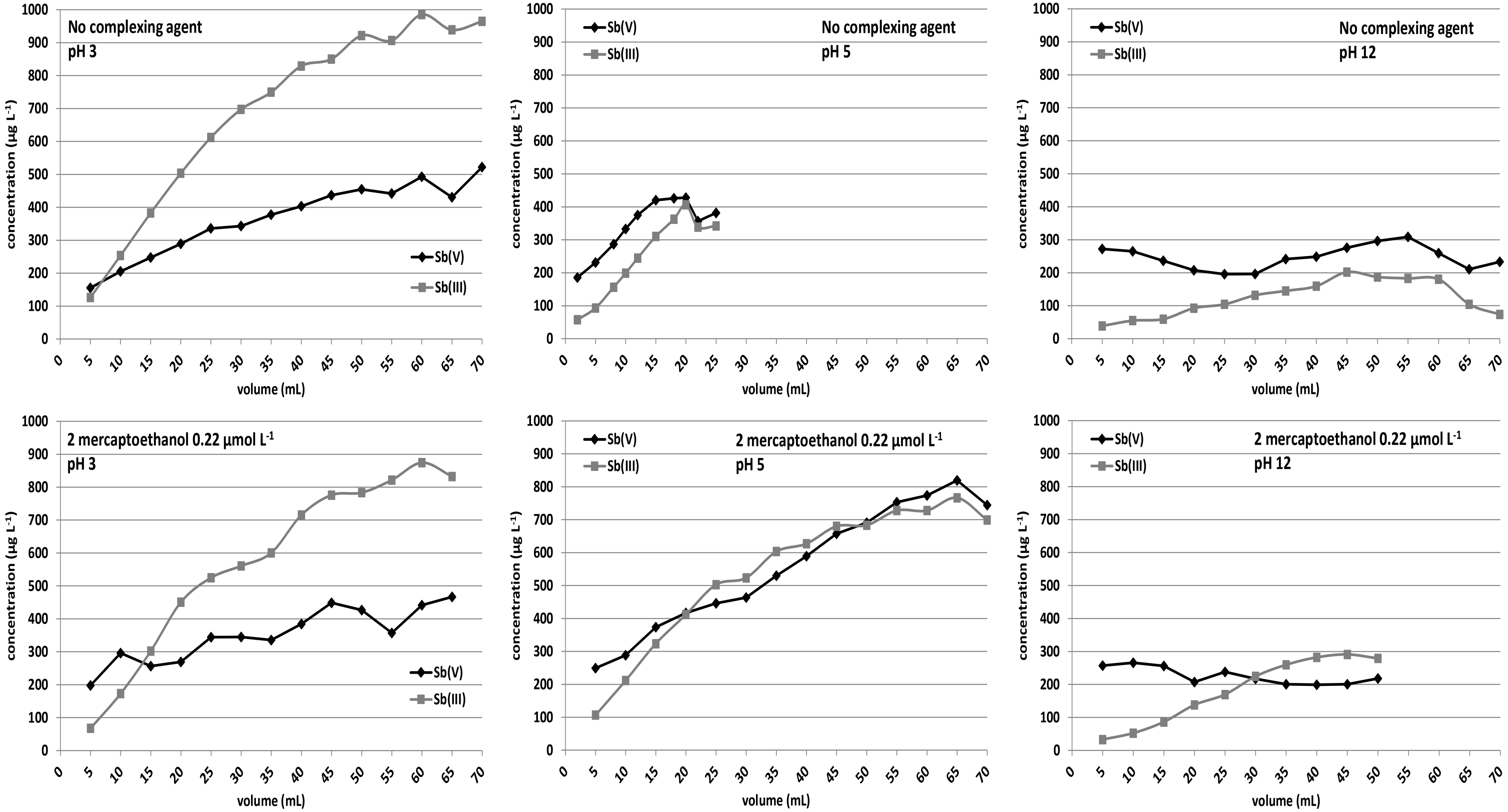


Figure 3.17. Inorganic antimony species concentrations obtained versus volume preconcentrated on working solutions of 10 µg L^{-1} for Sb(V) and 3 for Sb(III) in the assessment of the pH and precolumn type. Precolumn used: **anion exchange**.

The differences observed in the retention behaviour of the inorganic antimony can be explained from a difference in the retention capability of the species according to the pH. Figure 3.18 depicts the distribution of inorganic species of Sb(III) and Sb(V) in aqueous solution.

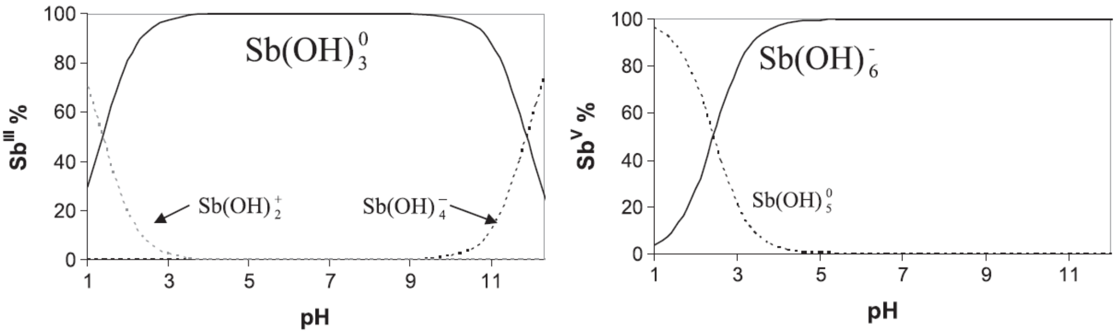


Figure 3.18. Distribution of inorganic species of Sb(III) and Sb(V) in aqueous solution as a function of pH at 25 °C [Tella et al., 2012].

In absence of complexing agent, no significant retention was observed in C18 stationary phase at any pH as the antimony species present free hydroxyl groups, which are polar and not well retained in this kind of phase. When 2-mercatoethanol is added, Sb(V) forms a 1:1 complex with the complexing agent whereas Sb(III) forms a 1:2 complex [Filella et al., 2005]. The 1:1 complex should be a polar complex which still presents free hydroxyl groups and consequently a poor retention for C18. However, the 1:2 complex contains less free hydroxyl ligands, and consequently is much less polar than the first one, which could explain the higher retention capability achieved at pH 3 and 5 for Sb(III).

Taking into account the anion exchange precolumn, some protonation and deprotonation equilibriums are produced both in absence and presence of 2-mercaptoethanol, which could be attributed to the different retention behaviours observed. These equilibriums involve not only to the Sb species, but also the complexing agent, which leads to the modification of the charge of the formed complexes. Regarding basic pH, the complex formed might present several negative charges, which allows a strong retention but prevents the elution from the precolumn.

Table 3.9 summarises the breakthrough volumes reached in each test together with the corresponding concentrations obtained and the preconcentration factors. RSDs obtained were ≤ 11% in all cases.

Table 3.9. Sb(V) and Sb(III) breakthrough volumes, concentrations, preconcentration factors determined in the pH and precolumn type test.

			Sb(V)				**Sb(III)**			
			V (mL)	**C (µg L^{-1})**	**PF**	**RSD (%)**	**V (mL)**	**C (µg L^{-1})**	**PF**	**RSD (%)**
C18	**Without 2-mercaptoethanol**	**pH3**	-	49 ± 5	4.9 ± 0.5	9.6	20	127 ± 3	42.4 ± 0.8	1.9
		pH5	-	43 ± 3	4.3 ± 0.3	7.8	8	73 ± 3	24 ± 1	4.3
		pH12	-	122 ± 3	12.2 ± 0.3	2.6	17.5	113.5 ± 0.5	37.8 ± 0.2	0.4
	With 2-mercaptoethanol	**pH3**	-	54 ± 6	5.4 ± 0.6	11.4	35	548 ± 12	183 ± 4	2.1
		pH5	-	56.2 ± 0.2	5.6 ± 0.1	0.3	27.5	254 ± 5	85 ± 2	2.1
		pH12	-	84 ± 2	8.4 ± 0.2	2.7	20	113 ± 3	38 ± 1	3.0
Anion exchange	**Without 2-mercaptoethanol**	**pH3**	60	472 ± 37	47 ± 4	7.9	60	963 ± 19	321 ± 6	2.0
		pH5	15	389 ± 30	39 ± 3	7.6	20	363 ± 31	121 ± 10	8.6
		pH12	-	293 ± 13	29 ± 1	4.6	45	188 ± 8	63 ± 3	4.5
	With 2-mercaptoethanol	**pH3**	45	439 ± 9	43.9 ± 0.9	2.1	60	843 ± 23	281 ± 8	2.7
		pH5	65	782 ± 23	78 ± 2	2.9	65	734 ± 20	245 ± 7	2.7
		pH12	-	226 ± 25	23 ± 3	11	45	284 ± 5	95 ± 2	1.8

The obtained results showed that the optimum conditions for the retention of Sb(V) and Sb(III) correspond to the use of the anion exchange precolumn, and a pH 5 for the working solution with the presence of the 2-mercapthoethanol 0.22 µmol L^{-1} as complexing agent, providing a breakthrough volume of 65 mL.

Figure 3.19 depicts a chromatogram working at the optimum conditions (with complexing agent at pH 5) with respect to three chromatograms obtained at other non-optimum conditions: anion exchange without complexing agent at pH 5 and C18 with and without complexing agent at pH 5. It can be observed that the peak signals obtained using the optimum conditions provide higher fluorescence intensity than those obtained in the rest of the chromatograms.

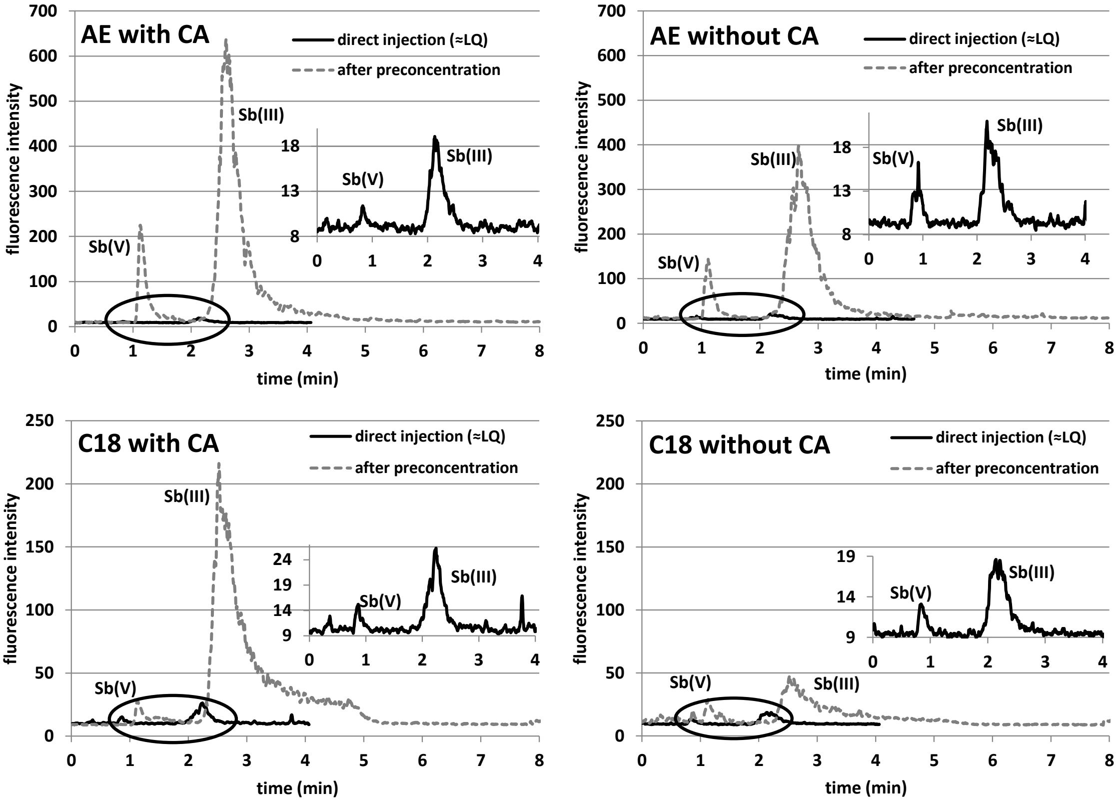


Figure 3.19. Chromatograms obtained from a preconcentrated standard with a concentration of 10 µg L^{-1} for Sb(V) and 3 µg L^{-1} for Sb(III) adjusted at pH 5, together with the direct injection (AE: anion exchange; CA: complexing agent).

Table 1.6 from the Introduction (Chapter 1, pages 42 – 43) compiles the published preconcentration Sb methods in the last five years. It can be observed that all the works published, except one, used offline systems without the simultaneous retention of the inorganic Sb species on the stationary phase. The strategies proposed consist of the direct retention of Sb(III) on the one hand, and the retention of total Sb using a reduction step of all antimony forms to Sb(III) on the other hand. Sb(V) concentration is then estimated as the difference of total Sb and Sb(III). In the present work, the simultaneous retention of Sb(V) and Sb(III) is proposed using an online preconcentration step for the retention of the species and the liquid chromatography for their elution, which should provide more reliable speciation results than those obtained by the other works using the estimations mentioned.

3.3.1.2. Application in water samples

Once the most appropriate conditions for online preconcentration had been selected, the water samples aforementioned in this section (see Table 3.6) were tested. Antimony species content was determined in all samples by LC-ICP-MS (n=3) as the quantification was not possible when coupling to HG-AFS. The only species present was Sb(V) in all drinking water matrices. The concentrations obtained were: 0.212 ± 0.011 µg L^{-1} in tap water; 0.335 ± 0.008 µg L^{-1} in weak mineralisation water; and 0.911 ± 0.007 µg L^{-1} in strong mineralisation water. Taking into account these results, it was decided to spike these water samples at a concentration of 10 µg L^{-1} of Sb(V) and 3 µg L^{-1} of Sb(III) in order to assess the new preconcentration efficiency with real samples. Increasing volumes up to the breakthrough volume were used, together with the direct injection of the corresponding spiked sample and the PFs were calculated.

The results obtained after the online preconcentration method showed great differences between the inorganic species. For Sb(V) species, the preconcentration of 1 mL of any water sample matrix presented PFs around 10. Peak signals were not improved with higher sample volumes, which means that the preconcentration efficiency is not increased. Regarding Sb(III) species, its presence in spiked tap water was not observed due to the easy oxidation even just after the spiking process in this kind of matrix. For bottled waters the preconcentration efficiency of Sb(III) is increased until a volume of 20 mL, obtaining PFs around 70. Regarding percentage recoveries, the values obtained were values were 14% for Sb(V) and 54% for Sb(III). As an example, Figure 3.20 depicts the inorganic antimony species concentrations obtained versus volume preconcentrated on the strong mineralised water spiked sample (A) and the chromatograms obtained with a 20 mL sample volume together with the direct injection of the sample (B).

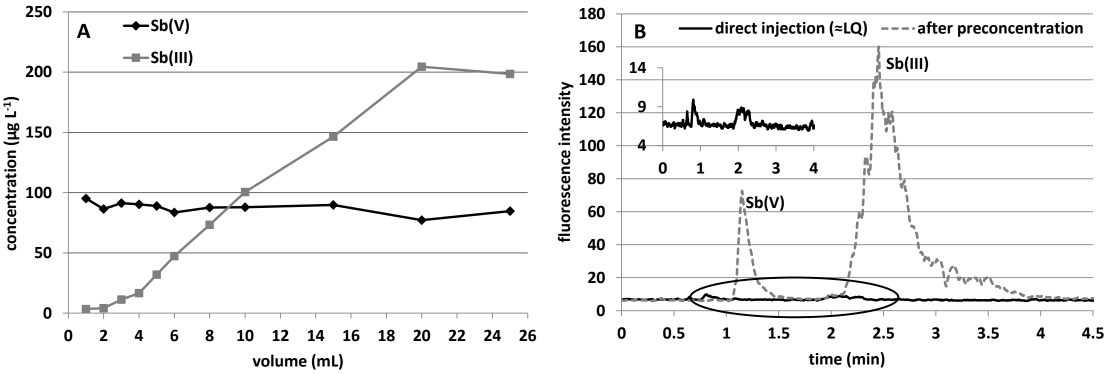


Figure 3.20. (A) Inorganic Sb species concentrations obtained versus volume preconcentrated on strong mineralised spiked water samples (10 µg L^{-1} Sb(V) and 3 µg L^{-1} Sb(III)). (B) Chromatogram obtained from the sample using a 20 mL as loading volume, together with a direct injection of this sample. The insert shows an enlargement of the direct injection chromatogram.

The achievement of smaller breakthrough volumes and poor recoveries may be due to the matrix characteristics, which decrease the precolumn retention capacity. However, it should not be disregarded that the system still provides a significant preconcentration of both species in spite of the efficiency loss.

As Sb(V) PFs obtained did not present variations with the volume loaded, a volume of 20 mL for both species could be established as the breakthrough volume. Table 3.10 summarises the preconcentration factor and recovery mean values together with the standard deviation for a 20 mL preconcentration volume performed in duplicate.

Table 3.10. Preconcentration parameters obtained for each species in water samples for a 20 mL preconcentration volume (n=2).

Species	Sample (water)	PF	Recovery (%)	RSD (%)
Sb(V)	Tap	9.1 ± 0.3	13.2 ± 0.5	4
	Weak mineralised	8.8 ± 0.2	13.9 ± 0.4	3
	Strong mineralised	8.5 ± 0.5	14.2 ± 0.8	6
	Overall Average	8.8 ± 0.4	13.9 ± 0.7	5
Sb(III)	Tap	<LD	-	-
	Weak mineralised	66 ± 4	55 ± 3	6
	Strong mineralised	67 ± 3	53 ± 2	4
	Overall Average	67 ± 3	54 ± 2	5

As the PFs and recoveries obtained were similar for each matrix and the standard deviation values were suitable, it was decided to set these conditions as the optimum ones for the determination of Sb(V) and Sb(III) by LC-HG-AFS following online preconcentration. The parameters established are summarised in Table 3.11.

Table 3.11. Final selected conditions for online preconcentration of Sb(V) and Sb(III) by LC-HG-AFS.

Optimum conditions	
Mobile phase	EDTA 30 mmol L^{-1} (with 0,5% of MeOH) at 2 mL min^{-1}
Complexing agent	2-mercaptoethanol 0.22 µmol L^{-1}
Sample pH	5
Precolumn	Anion exchange, 5 cm
Preconcentration volume	20 mL

3.3.1.3. Method assessment

The preconcentration method proposed was assessed with the analysis of a CRM and a non-spiked bottled water sample. Firstly, the most suitable CRM found was a lake water with certified values for the total content of 26 elements including antimony (TM 23.4: 3.26 ± 0.318 µg Sb L^{-1}). Total Sb content was analysed in the CRM by HG-AFS, which provided an antimony concentration of 3.01 ± 0.03 µg L^{-1} (n=3). No significant difference was found between the certified and experimental total content (t-test at 95% confidence level). Secondly, taking into account that the system allows the preconcentration of approximately ten times the Sb(V) species, a bottle water with a concentration of Sb(V) around 1 µg L^{-1} was selected so as to obtain a Sb(V) preconcentrated signal around 10 µg L^{-1}, which is slightly above the limit of quantification of the technique (see Table 3.3). Thus, among the bottled waters analysed in the previous section, the selected sample was the strong mineralisation water.

Antimony species in the CRM and the strong mineralisation bottled water sample were analysed using direct injection both by LC-ICP-MS and LC-HG-AFS, and after the online preconcentration step by LC-HG-AFS, using the previously established optimised conditions. Increasing volumes were preconcentrated until reaching a volume of 20 mL and the PFs obtained for the water samples were applied for the calculation of Sb(V) and Sb(III) concentration. The analyses were performed in triplicate. Table 3.12 summarises the results obtained. Regarding the direct analysis, both inorganic antimony species were present in the CRM, obtaining quantifiable results in the case of ICP-MS detection. In the preconcentration method, the species were well-quantified, as the concentrations obtained were not significantly different than those obtained by direct analysis (t-test, 95% confidence level). Figure 3.21 shows some of the chromatograms obtained as an example. Moreover, there was no significant difference between the total certified content and the sum of species for both detection techniques.

Table 3.12. Sb(V) and Sb(III) concentrations obtained in TM 23.4 (certified value: 3.26 ± 0.318 µg Sb L^{-1}) and PET-bottled strong mineralisation water by direct injection and online preconcentration.

Sample	**Species**	**Direct injection LC-ICP-MS**		**Direct injection LC-HG-AFS**	**Online preconcentration LC-HG-AFS**	
		C (µg L^{-1})	**RSD (%)**	**C (µg L^{-1})**	**C (µg L^{-1})**	**RSD (%)**
TM 23.4	Sb(V)	2.02 ± 0.05	2.5	< LQ	1.98 ± 0.15	7.6
	Sb(III)	0.93 ± 0.03	3.2	< LQ	0.85 ± 0.03	3.5
	Sum of species	2.95 ± 0.08	2.7	-	2.84 ± 0.18	6.3
Strong mineralised	Sb(V)	0.911 ± 0.007	0.8	<LD	0.87 ± 0.08	9.6
	Sb(III)	<LD	-	<LD	<LD	-

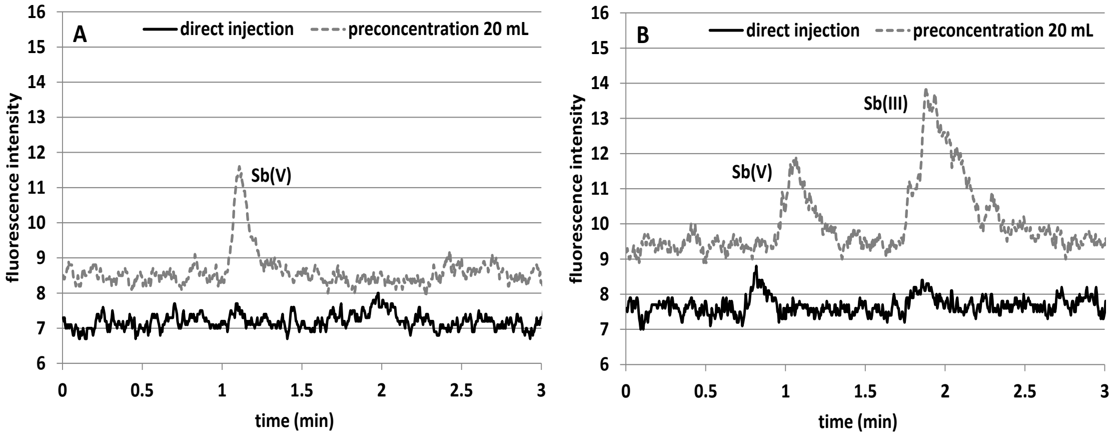


Figure 3.21: Chromatograms obtained volume from the strong mineralisation bottled water (A) and the CRM lake water (B) after the preconcentration of a 20 mL, together with the direct injection of the sample.

These results indicate that the online proposed method allows the preconcentration and reliable quantification of both inorganic Sb species in drinking waters. Although the analysis of the CRM and the non-spiked bottled waters proved the reliability for the analysis of real water samples, further studies about these work could be developed in the research group so as to improve the preconcentration efficiency, especially for Sb(V) species.

Limits of detection and quantification for the online preconcentration method were calculated using the PFs summarised in Table 3.10. The calculated LDs were 232 ng L^{-1} for Sb(V) and 8 ng L^{-1} for Sb(III), whereas the LQs were 773 ng L^{-1} and 27 ng L^{-1}, respectively. The limits were also assessed experimentally by injecting Sb standards from 25 ng L^{-1} to 1000 ng L^{-1} for Sb(V) and from 1 ng L^{-1} to 40 ng L^{-1} for Sb(III). Some of the obtained chromatograms are depicted in Figure 3.22 as an example. It was observed that Sb(V) was detected between 250 and 375 ng L^{-1}, whereas for Sb(III) it was detected between 30 and 40 ng L^{-1}. These results pointed out that the calculated limits for Sb(V) are quite accurate from the experimental point of view, whereas for Sb(III), the experimental limits were slightly higher (one order of magnitude) than the calculated ones.

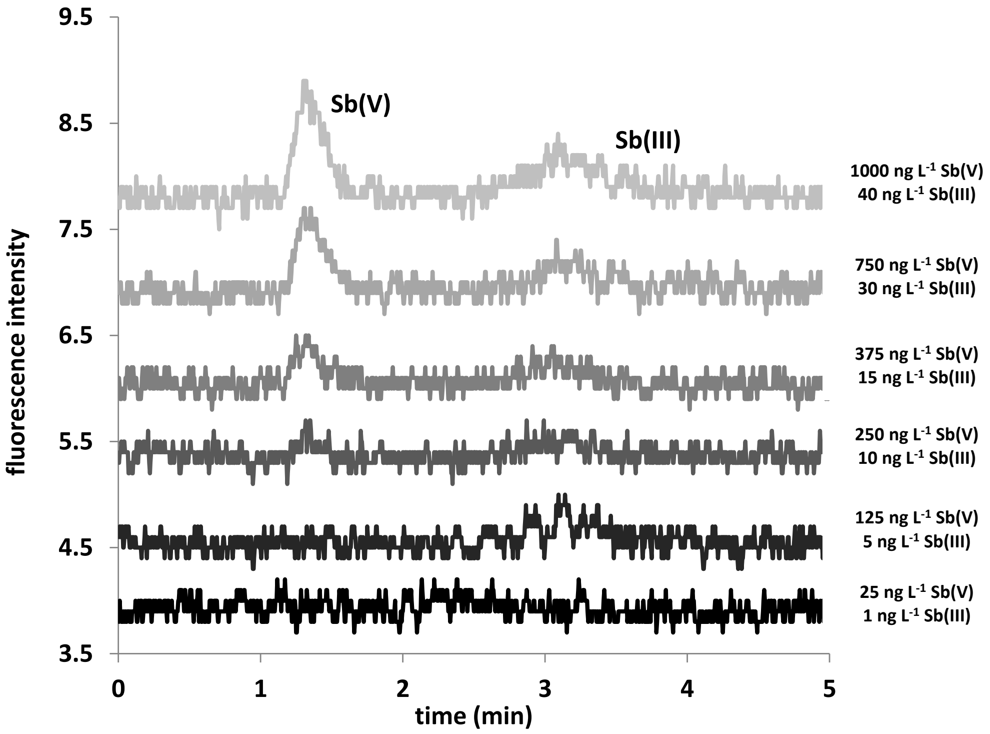


Figure 3.22. Chromatograms obtained from antimony standard solutions (Sb(V): 25 – 1000 ng L^{-1}; Sb(III): 1 – 40 ng L^{-1}) using the preconcentration method.

These values are lower than those obtained by the direct injection method, at µg L^{-1} level, and are in agreement with the PFs obtained for each species. Moreover, the absolutes LDs were calculated considering a volume of 20 mL, obtaining values of 4 – 5 and 0.04 – 0.1 ng for Sb(V) and Sb(III), respectively. Nevertheless, it should be taken into account that the preconcentration of samples with a low complexity matrix would decrease the limits of detection and quantification in the online preconcentration method as a higher load volume could be used.

The detection limits obtained in this thesis are compared with those previously reported in the literature using similar methodology (see Table 1.6 from the Introduction). The limits of detection for Sb(III) species are comparable and of the same order of magnitude than those reported by the works summarised, independently of the detection system used. Besides, all the preconcentration methods reported, with the exception of one work, perform an indirect determination of Sb(V), as it is estimated as the difference between the total Sb and Sb(III) species. This approach may not be an accurate form of determination as this difference might be related to other kind of Sb species. It should be also noted that the system proposed in this work (HG-AFS) provided as much sensitivity as the other works who used more sensitive detection systems (ICP-MS, ETAAS), which require higher investment and maintenance costs.

3.3.2. Mercury preconcentration method

The first assays were performed with a column RP-C18 ODS Hypersyl (10 x 4.6 mm), in order to ensure that the proposed preconcentration system could also work with the separation method previously optimised by LC-UV-CV-AFS. Thus, several increasing volumes, from 1 mL till reaching the breakthrough point, of working standard solutions containing 5 µg L^{-1} of $MeHg^+$ and Hg(II), that is a concentration slightly higher than the established limits of quantification, were preconcentrated.

The results of this initial test showed that the operational approach was suitable as the signal of the standard solution was increased. However, a lack of reproducibility was observed as strong memory effects were observed and peak signals were sometimes not eluted or eluted at any retention time. Thus, systematic studies of several variables, such as the use of a complexing agent, type and concentration, precolumn conditioning and length, and sample flow were undertaken to assess the preconcentration step.

3.3.2.1. Assessment of the preconcentration step

Complexing agent type and concentration

As the initial preconcentration test working with standards showed a lack of reproducibility of the signal of the species in the elution step when no complexing agent was added to the working standard solutions, the use of an agent which could complex mercury species was studied. The use of complexing agents have been evaluated for the retention of mercury species in many works, as mentioned in the introduction. Regarding the works reported in Table 1.5 (see page 38), it was observed that APDC and 2-mercaptoethanol, were two of the most used complexing agents for the retention of mercury. The concentrations of the complexing agents tested were 2 mmol L^{-1} and 14 mmol L^{-1}, respectively [Qvarnström et al., 2000; Margetínová et al., 2008]. Thus, APDC and 2-mercaptoethanol were selected and the preconcentration of mercury standards with the presence of the complexing agents was tested. Working standard solutions of 5 µg L^{-1} of $MeHg^+$ and Hg(II), and also 0.5 and 0.05 µg L^{-1}, which are concentrations lower than the limit of detection, were prepared with the corresponding complexing agent, and increasing volumes were preconcentrated in different working sessions with the same working conditions than those used in the initial test.

As an example, the chromatograms obtained for a 5 µg L^{-1} preconcentrated standard in presence of APDC and 2-mercaptoethanol are depicted in Figure 3.23.

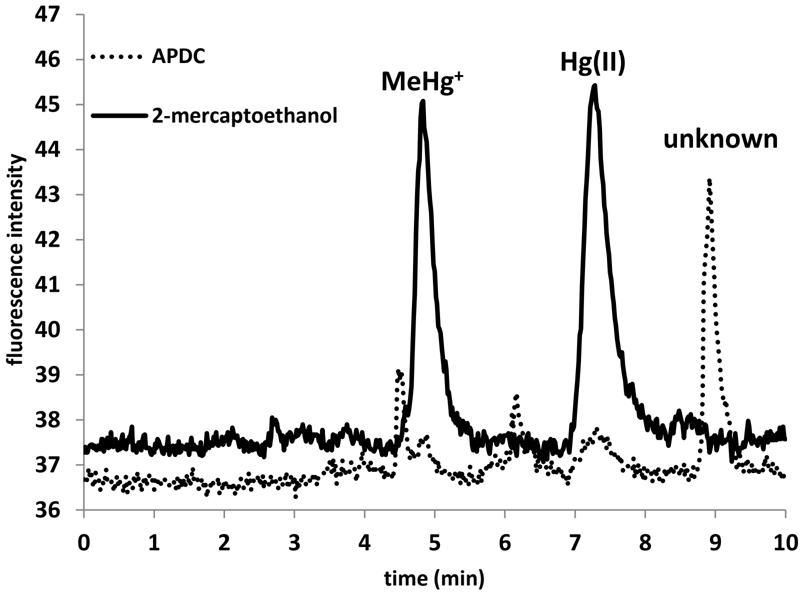


Figure 3.23. Chromatogram profiles of a 5 µg L^{-1} preconcentrated standard using APDC and 2-mercaptoethanol as complexing agent.

It can be observed that when working with APDC, the presence of low peak signals at retention times different from those attributed to the mercury species in the separation method were observed with a lack of reproducibility. Even if a significant retention can be achieved with APDC, an incomplete elution of the species as well as memory effects were still observed.

When using 2-mercaptoethanol as complexing agent, both species were eluted at the expected retention times, with a good signal overcoming the previous observed problems. Thus, further studies were performed to establish the optimum working concentration of 2-mercaptoethanol.

Five concentrations ranging from 140 to 0.07 mmol L^{-1}, which is the lowest possible concentration of 2-mercaptoethanol for the formation of the mercury complexes, were tested. Increasing sample volumes of the mercury standard solutions with the corresponding amount of 2-mercaptoethanol were preconcentrated in three working sessions using the same conditions previously mentioned till reaching the breakthrough volume.

As it can be seen in Figure 3.24, for a 5 µg L^{-1} standard solution, when the highest concentration of 2-mercaptoethanol (140 mmol L^{-1}) was used, different signals that did not correspond neither to MeHg^{+} nor to Hg(II) were observed. These additional signals could be due to the formation of undesired complexes of Hg(CH_3OH):mercaptoethanol at elevated concentrations of the reagent in the working solution [Balarama-Krishna et al., 2007]. Concentrations of the complexing agent from 0.07 to 14 mmol L^{-1} did not show any additional signals, apart from mercury species.

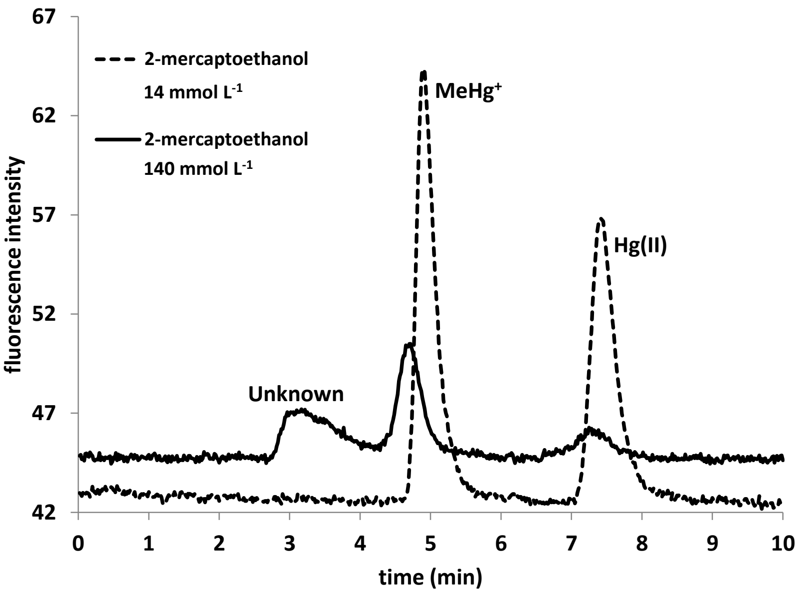


Figure 3.24. Chromatogram profiles obtained after the preconcentration of a 5 µg L^{-1} $MeHg^+$ and Hg(II) standard with 140 and 14 mmol L^{-1} of 2-mercaptoethanol (volume loaded: 6 mL).

In the test with the four concentration values of 2-mercaptoethanol up to 14 mmol L^{-1}, different breakthrough volumes were achieved, which are shown in Figure 3.25. As it can be seen, at the 0.07 mmol L^{-1} concentration, higher sample volumes could be loaded in the precolumn before achieving the breakthrough point, especially for Hg(II), consequently leading to higher preconcentration factors. Thus, 0.07 mmol L^{-1} was selected as the optimum working concentration.

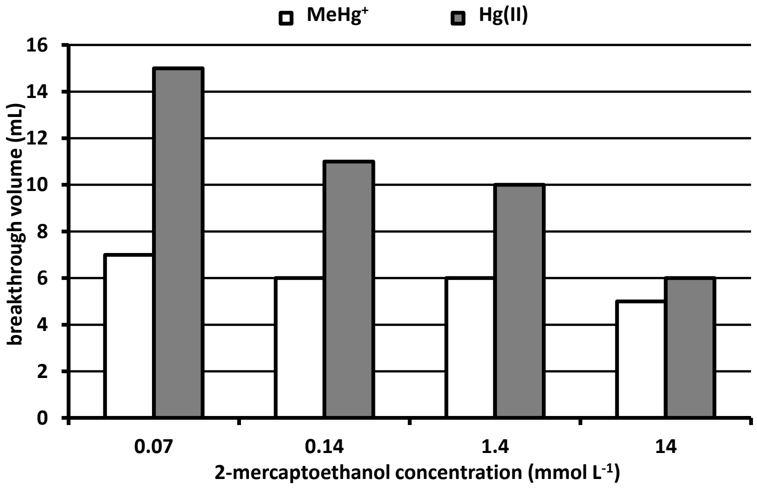


Figure 3.25. Breakthrough volume obtained versus complexing agent concentration in a 5 µg L^{-1} $MeHg^+$ and Hg(II) standard. Precolumn length: 1 cm.

Precolumn conditioning

The effect of conditioning the precolumn before the sample loading was also studied in order to assess the potential of mercury species sensitivity. The same complexing agent used in the working standard solutions (2-mercaptoethanol 0.07 mmol L^{-1}) was assessed for conditioning the precolumn and the results were compared to those obtained without using this step. The conditioning step consisted of loading a specific volume of the complexing agent solution on the precolumn before loading the same volume of the working standard solution. Increasing volumes were preconcentrated before achieving the breakthrough point.

The use of the conditioning step with 2-mercaptoethanol caused a decrease of more than 50% in the intensity peaks. Figure 3.26 depicts the chromatograms after the preconcentration of 5 µg L^{-1} $MeHg^+$ and Hg(II) standards, in which the intensity differences can be observed.

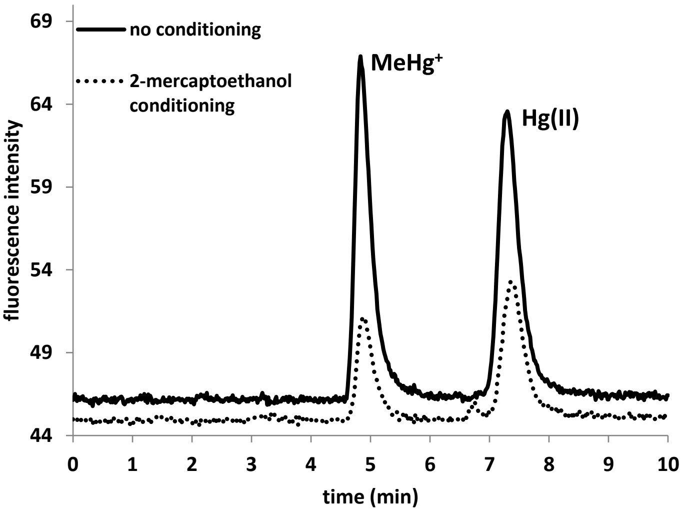


Figure 3.26. Chromatogram profiles of a 5 µg L^{-1} preconcentrated standard after conditioning the precolumn with 2-mercaptoethanol 0.07 mmol L^{-1} and in absence of this step.

This fact could be due to the effect of the 2-mercaptoethanol in the precolumn. The complexing agent could be retained in the precolumn and occupy the stationary phase sites where the mercury species should have been retained. Therefore, there is less stationary phase available for the retention of $MeHg^+$ and Hg(II) when the 2-mercaptoethanol is used to condition the precolumn. Consequently, the breakthrough point was achieved faster and the retention capacity of the precolumn decreased. Thus, in further experiments, the precolumn was not conditioned with the complexing agent and only mobile phase was used for the stabilization of both LC separation column and preconcentration column.

Sample flow

The sample loading at different flows was also assessed to study the possible impact of this variable on mercury species signals. Two different sample volumes, 2 and 5 mL, were preconcentrated at five different flows, from 1 to 5 mL min^{-1} using a 1 cm-long precolumn. The peak signals obtained at 1 and 2 mL min^{-1} flow were of the same order of magnitude, but from 3 mL min^{-1} flow, the signal of both species decreased 25% or even 50% when the highest flow was tested. A possible explanation could be that when the flow rate was increased, the residence time was not enough to achieve equilibrium between the mobile and stationary phases. Thus, 2 mL min^{-1} was selected for further assays.

Precolumn length

The effect of precolumn length was studied to assess the retention capability of mercury species in the stationary phase. Three columns of different lengths were selected: 1, 2 and 5 cm. Two working standard solutions of $MeHg^+$ and Hg(II) at concentrations of 0.5 and 5 µg L^{-1} of both species were initially prepared in 2-mercaptoethanol 0.07 mmol L^{-1}. Increasing volumes of these solutions were tested up to a total volume of 20 mL, which corresponds to a volume higher than the breakthrough point in all cases. As an example, Figure 3.27 represents the mercury species concentrations obtained in the preconcentration of a given volume in working solutions of 5 µg L^{-1}. As can be observed, the 1 cm-long precolumn breakthrough volume for both species was lower than 8 mL. However, in 2 and 5 cm-long precolumns, these volumes increased up to 14-18 mL. In all cases, the breakthrough volumes were higher for Hg(II) than for $MeHg^+$, due to the higher affinity of this species for the C18 stationary phase. The higher affinity of Hg(II) for the C18 could be due to the kind of the formed complex. Hg(II) forms 1:2 complexes with 2-mercaptoethanol and APDC whereas $MeHg^+$ forms 1:1 complexes. The 1:2 complex presents more retention in C18 than 1:1 complex because the first one should be less polar, and consequently has more affinity in the phase.

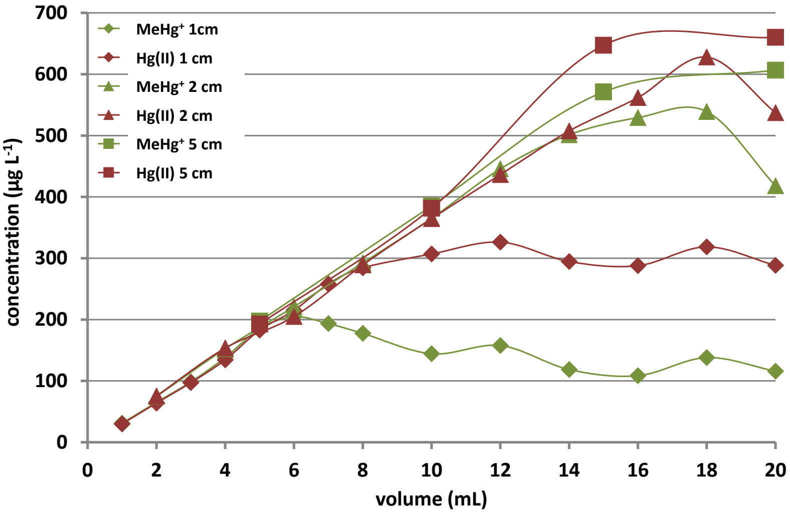


Figure 3.27. Hg species concentrations versus volume preconcentrated on working solutions of 5 µg L^{-1}.

Preconcentration factors and recoveries at the breakthrough volume including the standard deviation are plotted in Figure 3.28A for both species in each precolumn. Higher preconcentration factors were obtained when 2 and 5 cm precolumns were used, due to the fact that their retention capability is higher than that of the 1 cm precolumn. Regarding percentage recoveries, similar values were obtained among the three precolumns and they ranged from 60 to 80%.

Even if preconcentration factors provided by 2 and 5 cm precolumns were suitable, the observed chromatographic behaviour of both systems was different, as shown in Figure 3.28B. Direct injection of 5 µg L^{-1} standard has also been included for comparison purposes. As can be seen, mercury (II) did not present Gaussian behaviour when 15 mL were preconcentrated in a 5 cm precolumn. This effect could be because the precolumn is long enough for the mercury (II) separation process to start before the analytical column is reached. Thus, it can be concluded that a 2 cm column is most suitable for the preconcentration method.

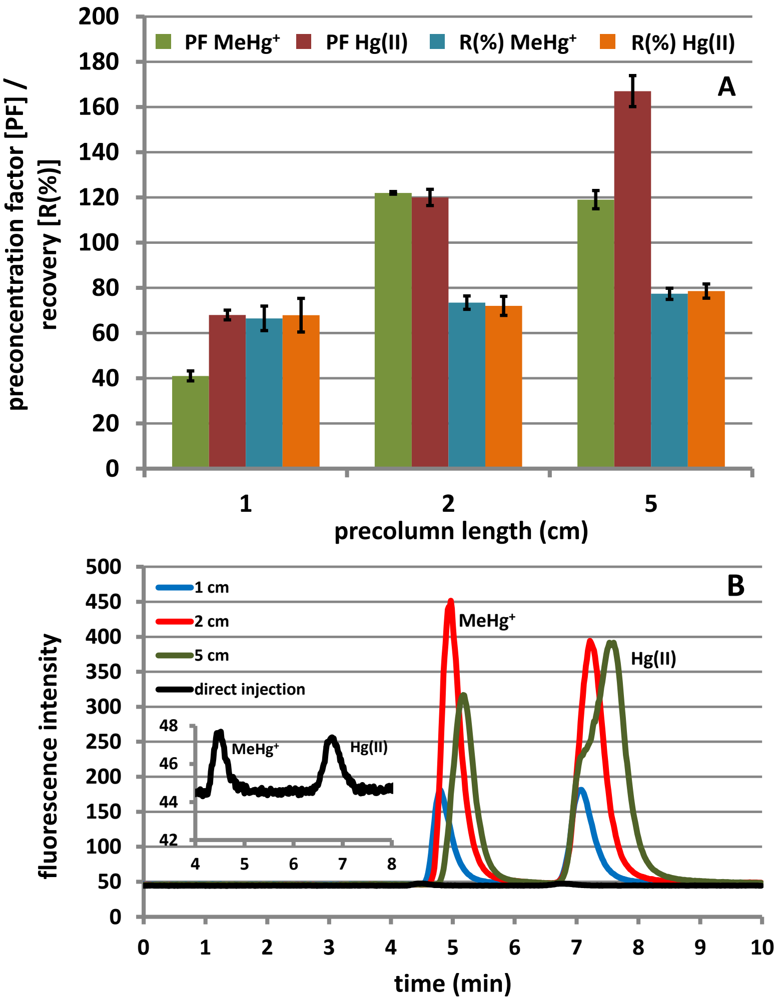


Figure 3.28. MeHg$^+$ and Hg(II) recoveries, preconcentration factors (A) and chromatograms obtained (B) from a 5 µg L^{-1} standard at the breakthrough volume in each precolumn, together with a direct injection of this standard, which is enlarged in the insert graphic.

3.3.2.2. Application in water samples

Once the most appropriate conditions for online preconcentration have been established with working standard solutions, the water samples previously described were tested (see Table 3.6). To ensure if the samples could have or not trace amounts of mercury, total mercury content was determined in all matrices by ICP-MS, and the Hg content was under the limit of detection (0.02 µg L^{-1}). Thus, samples were spiked at three concentration levels with the appropriate amount of complexing agent: low-level (0.5 µg L^{-1} of both species), medium-level (0.5 µg L^{-1} of $MeHg^+$ and 5 µg L^{-1} of Hg(II)) and high-level (5 µg L^{-1} of both species). The samples were then preconcentrated until the breakthrough volume was achieved for each matrix.

Due to a matrix effect, both breakthrough volumes and preconcentration factors were lower in water samples (about 7 mL and about 50, respectively) than in double deionised water (16 mL and about 120, respectively). This effect may be due to a possible competition of other substances present in water samples in addition to mercury with the precolumn stationary phase, which can lead to a decrease in its retention capacity. However, recovery values of both species were of same order of magnitude as those previously described in the previous section and ranged from 67 to 86%, regardless of the type of water, which may indicate that this parameter is independent from matrix composition. Higher PF and recoveries for Hg(II) were also observed. As an example, Figure 3.29 depicts the concentrations obtained after preconcentration of a given volume in the water samples spiked at high-level.

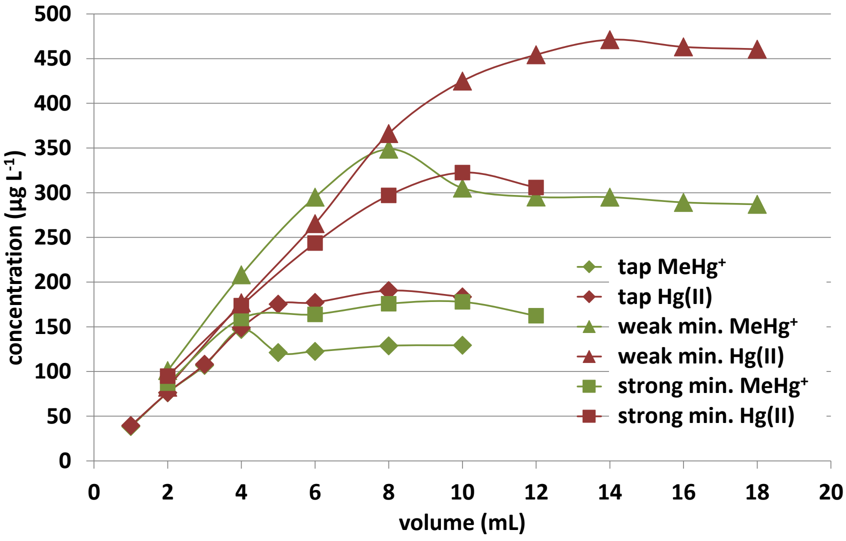


Figure 3.29. Mercury species concentrations obtained versus volume preconcentrated on high-level spiked water samples (5 µg L^{-1}).

From the data obtained, it was considered that the most suitable breakthrough volume for routine analysis should be the obtained for the most complex matrix (tap water) and for the least retained species ($MeHg^+$), which are the worst retention conditions: 4 mL. This volume allowed us to work under reproducible conditions with good recoveries, regardless of the type of sample and the concentration levels.

Table 3.13 shows mean values and standard deviation for the preconcentration factor and recovery using a 4 mL preconcentration volume performed in triplicate in different working sessions. The overall average represents the mean of all assays performed (n = 12) regardless the water matrix. The PF values were 30 ± 1 and 32 ± 3 for $MeHg^+$ and Hg(II), respectively. The recovery values were 72% for $MeHg^+$ and 81% for Hg(II) and the RSD means were below 15%. As it can be seen, methylmercury recoveries are always lower than those obtained for the Hg(II). The possible justification to this behaviour is that $MeHg^+$-mercaptoethanol complexes present less affinity for C18 than the Hg(II) ones, as mentioned in the previous sub-section.

Table 3.13. Preconcentration parameters obtained for each species in water samples for a 4 mL preconcentration volume (n=3).

Species	Sample (water)	PF	Recovery (%)	RSD (%)
$MeHg^+$	Double deionised	29 ± 2	73 ± 5	7
	Weak mineralised	30 ± 2	74 ± 6	8
	Strong mineralised	30 ± 4	67 ± 9	13
	Tap	27 ± 4	74 ± 2	2
	Overall Average	30 ± 1	72 ± 3	4
Hg(II)	Double deionised	32 ± 2	80 ± 4	5
	Weak mineralised	34 ± 2	86 ± 4	5
	Strong mineralised	34 ± 2	84 ± 5	6
	Tap	35 ± 4	87 ± 9	10
	Overall Average	32 ± 2	81 ± 5	6

Considering that the waters which were analysed had different matrices, the standard deviations obtained were suitable and the similarity between the PF and recovery values demonstrates the robustness of the established conditions for the online preconcentration system.

Thus, the optimum conditions for the determination of $MeHg^+$ and Hg(II) by LC-UV-CV-AFS following online preconcentration are summarised in Table 3.14. A chromatogram obtained of the preconcentrated strong mineralised water spiked at high-level is depicted in Figure 3.30 together with its direct injection. The great difference in the signal intensity between the direct injection and the preconcentration shows the high efficiency of the developed method.

Table 3.14. Final selected conditions for online preconcentration of $MeHg^+$ and Hg(II) by LC-UV-CV-AFS.

Optimum conditions	
Precolumn conditioning	Mobile phase
Complexing agent	2-mercaptoethanol 0.07 mmol L^{-1}
Sample flow	2 mL min^{-1}
Precolumn length	2 cm
Preconcentration volume	4 mL

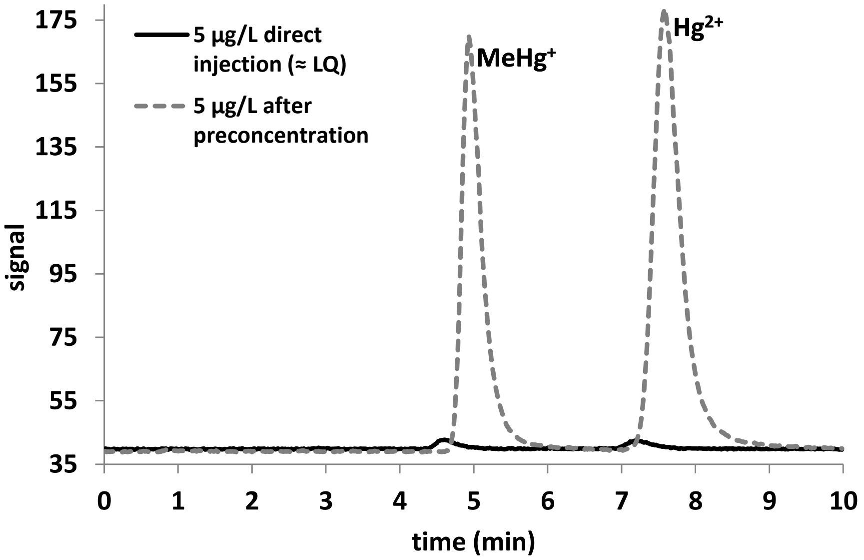


Figure 3.30. chromatogram obtained from a 5 µg L^{-1} spiked strong mineralised water sample using the optimum conditions, together with a direct injection of this sample.

3.3.2.3. Method assessment

Accuracy

The accuracy of the method was assessed by the analysis of a certified reference material (CRM). To our knowledge there are no CRMs for Hg(II) and $MeHg^+$ species in natural waters. Most of the CRMs available for total mercury consist of spiked water samples [Ibáñez-Palomino et al., 2012b]. To evaluate the preconcentration method, the most suitable CRMs would be waters with a total mercury level close to the limit of quantification of the analytical technique without the preconcentration step. It was only found wastewater with certified values for the total content of 10 elements including mercury (ERM-CA713, 1.84 ± 0.11 µg Hg L^{-1}). Total Hg content was analysed in the CRM by CV-AFS, which provided a mercury concentration of 1.81 ± 0.03 µg L^{-1} (n=3). No significant difference was found between the certified and experimental total content (*t*-test at 95% confidence level).

Mercury species in the CRM were analysed by direct injection and after the online preconcentration step, using the previously established optimised conditions. A total of 4 mL of wastewater were preconcentrated and the PF obtained in the previous section, summarised in Table 3.13, were applied. The analyses were performed in triplicate.

Table 3.15 summarises the results obtained by the two speciation methods. Regarding the direct injection method, the concentration of methylmercury was below the limit of detection, whereas the concentration of mercury (II) in the CRM was close to the limit of quantification.

Table 3.15. Methylmercury and inorganic mercury concentration obtained in ERM-CA713 (certified value: 1.84 ± 0.11 µg Hg L^{-1}) by direct injection and online preconcentration.

Species	**Direct injection**		**Online preconcentration**	
	C(µg L^{-1})	**RSD (%)**	**C(µg L^{-1})**	**RSD (%)**
MeHg^{+}	< LD	-	0.28 ± 0.01	2.9
Hg(II)	1.71 ± 0.02	1.2	1.72 ± 0.06	3.6
Sum of species	1.71 ± 0.02	1.2	2.00 ± 0.06	3.0

In the preconcentration method, both species were well-quantified. Regarding the sum of species, a t-test (95% confidence level) with respect to the certified value showed no significant difference. The results show that the preconcentration method can quantify all mercury species, and the sum of them can be used to determine the total mercury content in water.

Limits of detection and quantification

Limits of detection and quantification for the online preconcentration method were both calculated using the PFs summarised in Table 3.13 and assessed experimentally by injecting standard solutions from 1 to 500 ng L^{-1}. The calculated LDs and LQs for both species were 18 ng L^{-1} and 60 ng L^{-1}, respectively. Regarding the experimental limits, several chromatograms of the standards are depicted in Figure 3.31. Hg(II) was detected between 2.5 and 5 ng L^{-1} whereas MeHg^{+} was detected between 10 and 25 ng L^{-1}. Limits of quantification were about 50 ng L^{-1}. The experimental limits obtained are of the same order of magnitude than the calculated ones, except the LD of Hg(II), which is lower. This fact could be due to the stronger affinity of the inorganic species with respect to the methylated one, as discussed in section 3.3.2.1.

Limit of detection and quantification concentrations at ng L^{-1} were considerably lower than those obtained by the direct injection method at µg L^{-1}. Moreover, the absolutes LDs were calculated considering a volume of 4 mL, obtaining values of 40 - 100 and 10 - 20 pg for MeHg^{+} and Hg(II), respectively.

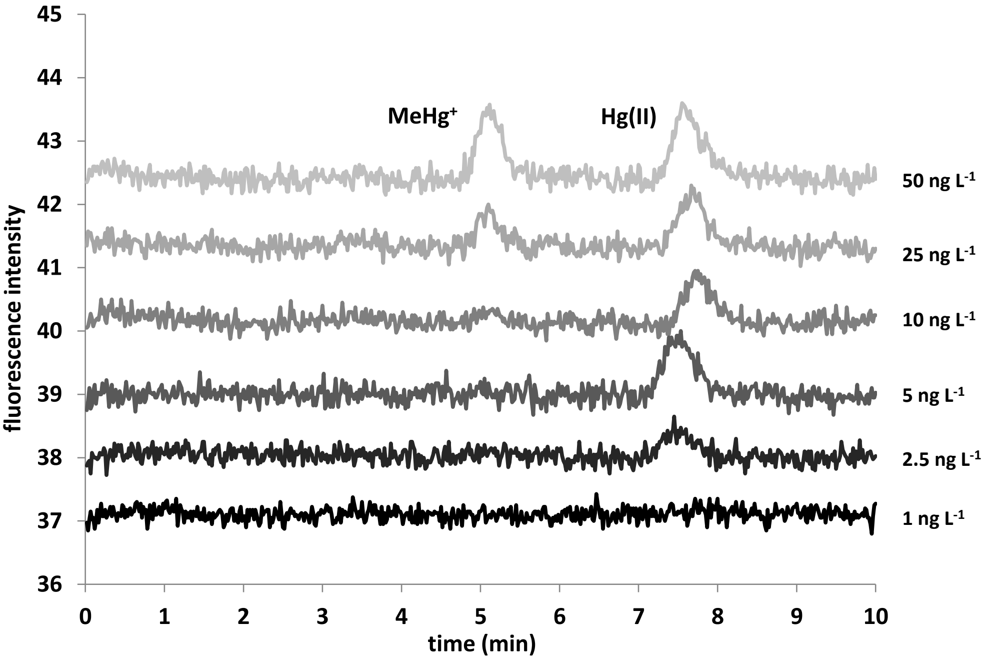


Figure 3.31. Chromatograms obtained from mercury standard solutions (1 – 500 ng L^{-1}) using the preconcentration method.

However, the preconcentration of samples with a low complexity matrix would decrease the limits of detection and quantification in the online preconcentration method as a higher load volume could be used.

The detection limits and recoveries obtained in this thesis are compared with those previously reported in the literature using similar methodology (see Table 1.5 from the Introduction). The recoveries obtained are comparable, and the detection limits are of the same order of magnitude when the detected absolute mass of mercury is considered. As expected, the detection limits obtained with ICP-MS are lower than those obtained with AAS or AFS. Nevertheless, CV-AFS provides suitable analytical performance, is user-friendly and requires lower investment and maintenance costs than ICP-MS, so it is a good approach in daily routine laboratory analysis.

The results obtained in sections 3.3.2.1 – 3.3.2.3 were published in the journal *Analytical Methods* as a research article, entitled *Mercury(II) and methylmercury determination in water by liquid chromatography hyphenated to cold vapour atomic fluorescence spectrometry after online short-column preconcentration*, which is attached in the Annex 1.

3.3.2.4. Preconcentration studies with three mercury species

The preconcentration studies performed have been focused on the speciation of two mercury species: methylmercury and mercury (II). However, some works deal with other organometallic mercury species, such as ethylmercury. In the works summarised in Table 1.5 from the Introduction where $EtHg^+$ is also studied, this species is spiked in the samples, as it can rarely appear in this kind of samples [Etemborg et al., 1993; Yin et al., 1998; Margetínová et al., 2008; Yin et al., 2010; Jia et al., 2012]. However, as mentioned before, the presence of $EtHg^+$ can be found in some biological samples from zones which have suffered immediate contamination [Ibáñez-Palomino, C., 2012].

Thus, the developed preconcentration method has been modified and adapted in order to perform the simultaneous determination of three mercury species in case the presence of $EtHg^+$ could appear in any sample.

For the performance of the preconcentration method, a previously developed and validated LC-UV-CV-AFS method for the separation of the three mercury species was adapted as described in section 2.3.2.3. The experimental conditions of the hyphenated technique were optimised by the research group and are described in Ibáñez-Palomino, C., 2012, which were summarised in section 2.3.2.1.

The same water samples analysed in the previous section were used except from the tap water. This sample was discarded because previous studies in the research group showed that $EtHg^+$ species is not stable in this medium [Ibáñez-Palomino, C., 2012]. Thus, weak and strong mineralised water samples were spiked at two levels, with the appropriate amount of complexing agent: high (5 µg L^{-1} of the three species) and low (0.5 µg L^{-1} of the three species). The samples, together with the corresponding standard solutions prepared with double deionised water of the same concentrations, were then preconcentrated using the optimum conditions, summarised in Table 3.14, until the breakthrough volume was achieved for each matrix.

Breakthrough volumes, preconcentration factors and recovery values are comparable to those obtained in the study with two mercury species. As an example, Figure 3.32 depicts the concentrations obtained of a given volume in the water samples spiked at high-level together with the results obtained in the standard. It can be observed that $EtHg^+$ presents a retention capability comparable or even higher that the corresponding to the mercury (II), as it reaches a higher breakthrough volume and consequently higher PFs. It can be also considered that the most suitable breakthrough volume is 4 mL, obtained for $MeHg^+$, the least retained species, in the strong mineralised water, as described in the previous section.

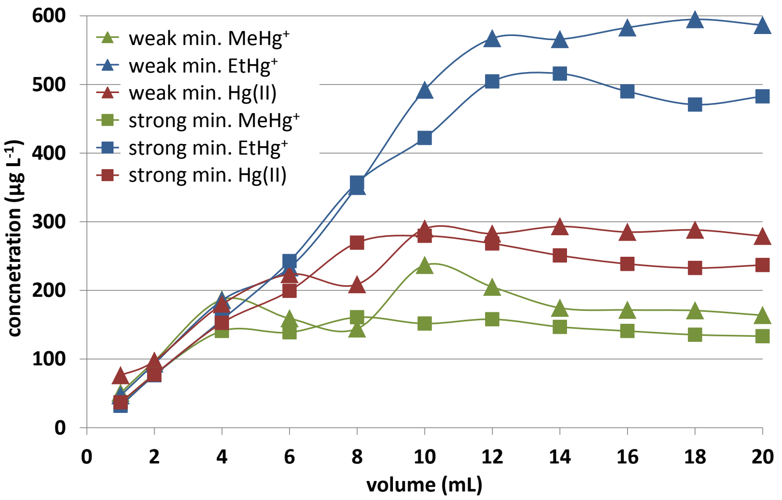


Figure 3.32. Mercury species concentrations obtained versus volume preconcentrated on high-level spiked water samples (5 µg L^{-1}) together with a standard solution.

Table 3.16 summarises the preconcentration factor, recovery, mean values and standard deviation for a 4 mL preconcentration volume. The overall average represents the mean of all water samples. The PF values were 28 ± 3, 32 ± 2 and 32 ± 3 for $MeHg^+$, $EtHg^+$ and Hg(II), respectively. The recovery values were 69% for $MeHg^+$, 75% for $EtHg^+$ and 81% for Hg(II) and the RSD means were below 15%.

Table 3.16. Preconcentration parameters obtained for each species in water samples for a 4 mL preconcentration volume (n=3).

Species	Sample (water)	PF	Recovery (%)	RSD (%)
$MeHg^+$	Double deionised	29 ± 3	73 ± 8	10
	Weak mineralised	27 ± 3	67 ± 8	11
	Strong mineralised	27 ± 1	68 ± 3	5
	Overall Average	28 ± 3	69 ± 7	10
$EtHg^+$	Double deionised	30 ± 3	74 ± 8	11
	Weak mineralised	29 ± 1	71 ± 2	3
	Strong mineralised	32 ± 2	80 ± 4	5
	Overall Average	30 ± 3	75 ± 7	9
Hg(II)	Double deionised	34 ± 1	86 ± 3	4
	Weak mineralised	32 ± 2	79 ± 6	8
	Strong mineralised	31 ± 2	76 ± 6	8
	Overall Average	32 ± 3	81 ± 7	8

A chromatogram obtained of the preconcentrated weak mineralised water spiked at 5 µg L^{-1} is depicted in Figure 3.33 together with its direct injection. It can be also observed that the difference in the signal intensity between the direct injection and the preconcentration implies an efficient developed method.

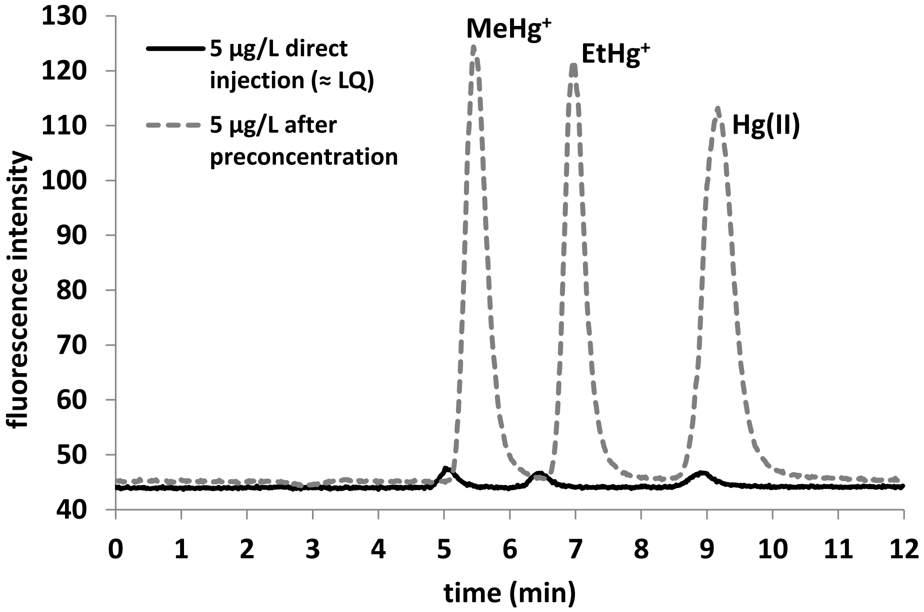


Figure 3.33. Chromatogram obtained from a 5 µg L^{-1} spiked weak mineralised water sample using the optimum conditions, together with a direct injection of this sample.

The behaviour of the species is similar to the obtained when the preconcentration was performed with two species, which explains that the values obtained are of the same order of magnitude as those obtained before, allowing the quantification of a third species with a good chromatographic resolution.

The calculated limits of detection using the new PFs obtained were 14 ng L^{-1} and the limits of quantification were 45 ng L^{-1} for $MeHg^+$ and Hg(II), and 47 for $EtHg^+$. Once again, the results demonstrate that this system works as efficient as the proposed method for the preconcentration of 2 species.

3.3.3. Remarks

The development of online preconcentration methods for the determination of trace Sb and Hg amounts was carried out in order to improve the limits of detection and quantification of the LC-AFS techniques. From the results obtained, several differences were observed between the two methods.

Table 3.17 summarises the optimum working conditions of the online preconcentration methods for the determination of Sb and Hg species in drinking waters together with the recoveries, PFs, sample volumes and quality parameters obtained.

Regarding the achievement of the highest possible retention of the species, from the data obtained, it can be observed the high applicability of 2-mercaptoethanol as complexing agent, since it provided the highest retention, good elution and suitable reproducibility for both elements. In the case of antimony, the pH of the sample with the complexing agent played an important role, as acidic and alkaline pHs decreased the retention capability of the species, especially for Sb(V). By contrast, for mercury preconcentration, the pH of the sample did not affect significantly in the retention of the species, as good results were observed without the pH adjusting in the working solutions and samples. With respect to the column length, for Sb, the best retention capability was achieved using a 5 cm precolumn, whereas in the case of Hg, a 2 cm length precolumn was suitable enough for the obtention of good results, as, in this case, the use of a 5 cm precolumn led to a loss in peak efficiency for Hg(II) species.

In the analysis of water samples, it can be observed that it is possible to load a sample volume five times higher for Sb with respect to Hg. This is mainly due to Sb(III) species retention capability, which has always showed a high affinity for the anion exchange phase. However, the behaviour of Sb species in anion exchange, and Hg species in C18, cannot be compared as the retention mechanisms are different. Regarding the results, the highest preconcentration factor, which led to the lowest LD, was obtained for Sb(III): it was possible to preconcentrate almost 70 times. The lowest PF was attributed to Sb(V), which led to the highest LD: it was only possible to preconcentrate almost 10 times. In the case of Hg, PFs around 30 were obtained, which demonstrates for this case similar retention behaviour for both species. However, the highest recoveries were obtained for both Hg species in spite of providing lower PFs than Sb(III). Taking into account the LQs obtained, water samples with Sb(V), Sb(III), $MeHg^+$ and Hg(II) concentrations as low as 0.8, 0.1, 0.05 and 0.05 µg L^{-1}, respectively, can be analysed.

Table 3.17. Working conditions of the online preconcentration methods for Sb and Hg species determination.

Variable	**Sb**	**Hg**
Separation column	Hamilton PRP-X100 (125 x 4.1 mm)	ODS Hypersil column (250 mm x 4.6 mm)
Mobile phase	EDTA 30 mmol L^{-1} at pH 4 (99.5%) Methanol (0.5%)	APDC 0.0015 mol L^{-1} and NH_4CH_3COO 0.01 mol L^{-1} at pH 5.5 (20%) Methanol (80%)
Flow	2 mL min^{-1}	1.5 mL min^{-1}
Precolumn	Hamilton PRP-X100 (50 x 4.1 mm)	ODS Hypersyl column (20 mm x 4.6 mm)
Conditioning	No (only mobile phase)	No (only mobile phase)
Complexing agent	2-mercaptoethanol 0.22 µmol L^{-1}	2-mercaptoethanol 0.07 mmol L^{-1}
Sample pH	5	No adjusting
Sample Volume	20 mL	4 mL
PF	Sb(V): 8.8 ± 0.8 Sb(III): 67 ± 3	$MeHg^+$: 30 ± 1 Hg(II): 32 ± 2
Recovery	Sb(V): 13.9 ± 0.7 Sb(III): 54 ± 2	$MeHg^+$: 72 ± 3 Hg(II): 81 ± 5
LD	Sb(V): 250 ng L^{-1} Sb(III): 30 ng L^{-1}	$MeHg^+$: 10 ng L^{-1} Hg(II): 2.5 ng L^{-1}
LQ	Sb(V): 800 ng L^{-1} Sb(III): 100 ng L^{-1}	$MeHg^+$: 50 ng L^{-1} Hg(II): 50 ng L^{-1}

In addition, the mercury preconcentration method was reassessed for the simultaneous analysis of a third species: $EtHg^+$. The results showed that it is possible to analyse $EtHg^+$ together with the rest of mercury species obtaining PFs, recoveries and limits of the same order of magnitude than those summarised in Table 3.17.

Thus, it can be concluded that the online preconcentration methods can be applied for the determination of antimony and mercury species in a wide range of water samples.

Chapter IV

OCCURRENCE OF ANTIMONY IN BEVERAGES AND MERCURY IN SEAFOOD

On the one hand, the interest for the determination of antimony and its species is mainly found in PET-bottled food samples due to the migration of the element from the containers. The use of PET for food packaging has been growing faster than any other plastic and two of the main foodstuffs that are commonly stored in PET containers are water and juices. Moreover, in some countries there is an increasing trend of the reuse of PET containers for the storage of several foodstuffs, especially bulk goods. A clear example consists of the storage of alcoholic beverages in PET bottles. Although alcoholic beverages tend to be stored in glass containers, in some countries, such as Greece or Turkey, it is typical to store local homemade spirits, such as *raki* or *tsipouro*, in PET bottles in order to ease their transport.

Drinking waters have basically different salts content depending on the mineralisation degree, whereas juices and spirits present a more complex matrix due to the presence of different organic acids, among other organic substances. Thus, once antimony has migrated to the sample, the element can be found as Sb(V) or Sb(III) complexes, depending on the matrix of the foodstuff.

On the other hand, the interest for the determination of mercury and its species is mainly focused on environmental samples due to their persistence in the environment. Soils, waters and aquatic sediments are considered the main deposits of mercury in the environment. The mercury present in the soil water environment is found mainly as inorganic forms and can be combined with the organic matter.

As mentioned in the introduction, the mercury content in water media presents a great bioaccumulation and biomethylation in the aquatic biota, mainly in animals. Consequently, seafood becomes the main responsible for the highest source of mercury and the element arrives to the human body with its consumption. Thus, in this section the implementation of the developed analytical methodology to these matrices is described: Sb is determined in waters, juices, spirits and PET bottles whereas Hg is determined in seafood.

4.1. ANALYSIS OF ANTIMONY IN PET BOTTLED WATERS

After the development of the chromatographic method for the separation of inorganic antimony species, real water samples were analysed using this method. A total 38 samples of plastic bottled water were purchased randomly in eleven different countries. The plastic of the bottles was the same in all cases: polyethylene terephthalate (PET). Seventeen water samples were purchased from local markets in Spain whereas the rest of the samples were purchased in other countries. Total and antimony speciation was carried out when purchasing and after one year of storage of the samples. Moreover, some physicochemical parameters were also determined when purchasing the samples in order to establish a possible correlation between antimony concentration and the physicochemical variables studied.

4.1.1. Samples characterisation

The characterisation of every PET-bottled water sample is summarised in Table 4.1. Samples were ordered by the geographical origin: national waters from Spain, international waters from Europe, and from America. A second classification of the samples is based on the bottle colour. Waters were stored in colourless or blue bottles, except one which is stored in green. Blue bottles were classified in three groups of colour: light blue, blue and dark blue. Figure 4.1 depicts some bottled water samples of each colour group.

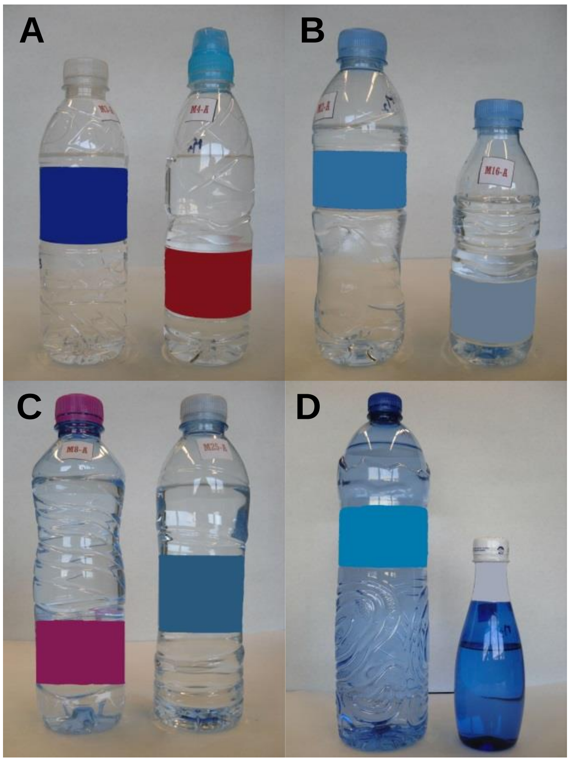


Figure 4.1. Water samples stored in coloured bottles: (A) colourless, (B) light blue, (C) blue and (D) dark blue.

Several physicochemical parameters of the PET-bottled samples were experimentally determined. The pH and the EC were measured in all the samples. The main anions (F^-, Cl^-, SO_4^{2-}, NO_3^-) and cations (Ca^{2+}, Mg^{2+}, K^+, Na^+) content were determined by anionic exchange chromatography and ICP-OES, respectively. For HCO_3^-, concentration was not measured as it was labelled in every sample except three, in which the concentration was estimated using a charge balance (marked with asterisk in Table 4.1). Mineralisation degree in waters is divided into various categories, according to what is stated in the Directive 80/777/EEC [Directive 80/777/EEC]:

Very weak mineralised water: the dry residue is lower than 50 mg L^{-1}.

Weak mineralised water: the dry residue is between 50 and 500 mg L^{-1}. These values are recommended for the prevention of kidney stones, easing the movement of the stones and their expulsion.

Moderate mineralised water: the dry residue is between 500 and 1500 mg L^{-1}.

Rich mineralised water: the dry residue is higher than 1500 mg L^{-1}.

As dry residue in water samples is not labelled in 40% of the samples, it was decided to establish the mineralisation category for each sample taking as reference value the sum of anions and cations. It was checked that this value is of the same order of magnitude than the dry residue value for the samples in which this parameter is labelled.

Table 4.1. Physicochemical parameters of the 38 bottled water samples (Values of anions and cations in mg L^{-1}; values of conductivity in µS cm^{-1}).

Sample	Country	Bottle colour	pH	HCO_3^-	F^-	Cl^-	SO_4^{2-}	NO_3^-	Ca^{2+}	Mg^{2+}	K^+	Na^+	Sum	Mineralisation	EC
SCb	Spain	dark blue	7.82	285	0.13 ± 0.01	8.40 ± 0.11	22.7 ± 0.5	8.5 ± 0.1	34.3 ± 0.1	27.4 ± 0.1	1.39 ± 0.07	4.53 ± 0.03	392	Weak	333 ± 4
FV1	Spain	light blue	7.75	149	0.37 ± 0.01	10.94 ± 0.09	12.4 ± 0.4	4.0 ± 0.2	33.5 ± 0.4	8.6 ± 0.4	1.44 ± 0.09	11.66 ± 0.04	232	Weak	238 ± 3
Aq	Spain	colourless	7.79	120	0.90 ± 0.01	8.07 ± 0.04	8.2 ± 0.6	6.9 ± 0.4	30.2 ± 0.2	6.9 ± 0.3	1.66 ± 0.01	9.71 ± 0.01	193	Weak	206 ± 2
Vd	Spain	colourless	7.83	113	0.76 ± 0.02	8.1 ± 0.3	7.8 ± 0.2	6.0 ± 0.4	29.8 ± 0.1	7.0 ± 0.3	1.64 ± 0.01	9.85 ± 0.01	184	Weak	207 ± 2
Ek	Spain	blue	7.87	297.2	0.17 ± 0.01	33.0 ± 0.4	43.8 ± 0.5	12.1 ± 0.5	47.5 ± 0.3	23.4 ± 0.1	1.51 ± 0.02	16.96 ± 0.02	476	Weak	454 ± 7
Cb	Spain	dark blue	6.92	119	0.97 ± 0.01	9.00 ± 0.13	10.9 ± 0.3	9.00 ± 0.03	12.6 ± 0.1	6.02 ± 0.06	3.23 ± 0.01	46.4 ± 0.1	217	Weak	288 ± 5
Fc	Spain	light blue	7.15	285	1.62 ± 0.01	35.28 ± 0.11	6.0 ± 0.2	1.8 ± 0.1	1.1 ± 0.1	1.45 ± 0.01	3.70 ± 0.04	112.0 ± 0.1	448	Weak	447 ± 5
Ab	Spain	light blue	7.46	150	0.11 ± 0.01	15.43 ± 0.06	6.7 ± 0.3	16.4 ± 0.1	39.2 ± 0.2	7.6 ± 0.3	2.14 ± 0.06	11.06 ± 0.01	249	Weak	255 ± 4
SCz	Spain	light blue	7.48	408.7	0.09 ± 0.01	4.21 ± 0.09	14.4 ± 0.3	6.8 ± 0.2	37.5 ± 0.1	38.4 ± 0.2	0.78 ± 0.01	0.87 ± 0.01	512	Moderate	384 ± 2
FV2	Spain	light blue	7.7	309	0.14 ± 0.01	15.36 ± 0.06	26.2 ± 0.1	14.0 ± 0.2	22.9 ± 0.2	24.0 ± 0.1	1.34 ± 0.01	5.68 ± 0.04	419	Weak	439 ± 4
AR	Spain	blue	7.8	146	0.15 ± 0.01	7.38 ± 0.02	41.0 ± 0.5	7.5 ± 0.1	45.5 ± 0.1	8.1 ± 0.3	1.11 ± 0.03	9.96 ± 0.02	267	Weak	276 ± 1
I	Spain	blue	7.98	156.4	0.15 ± 0.01	37.61 ± 0.07	954.1 ± 0.6	< LQ	163.1 ± 0.3	29.4 ± 0.2	2.12 ± 0.01	13.56 ± 0.01	1356	Moderate	904 ± 14
Ea	Spain	light blue	6.47	14*	0.04 ± 0.01	11.80 ± 0.05	< LQ	28.7 ± 0.5	3.4 ± 0.1	1.35 ± 0.01	0.39 ± 0.01	1.74 ± 0.01	47	Very weak	32.2 ± 0.1
SA	Spain	light blue	7.83	114	0.14 ± 0.01	98.0 ± 0.4	17.0 ± 0.2	55.4 ± 0.4	13.4 ± 0.1	11.2 ± 0.1	4.29 ± 0.01	3.60 ± 0.01	316	Weak	201 ± 4
Ln	Spain	dark blue	7.72	306.9	0.20 ± 0.01	11.3 ± 0.2	27.5 ± 0.4	2.6 ± 0.2	70.0 ± 0.2	39.1 ± 0.3	2.81 ± 0.04	15.97 ± 0.01	476	Weak	691 ± 5
Ve	Spain	dark blue	7.89	197	0.04 ± 0.01	29.9 ± 0.2	< LQ	4.5 ± 0.5	67.2 ± 0.2	1.14 ± 0.08	0.65 ± 0.02	0.69 ± 0.01	301	Weak	284 ± 8
Lj	Spain	colourless	6.83	107	0.17 ± 0.03	7.3 ± 0.1	< LQ	< LQ	23.4 ± 0.2	9.6 ± 0.3	1.24 ± 0.04	7.47 ± 0.01	156	Weak	183 ± 1
Vt1	Belgium	blue	7.66	399	0.21 ± 0.01	6.81 ± 0.03	397 ± 1	7.03 ± 0.03	128.0 ± 0.5	44.38 ± 0.06	2.53 ± 0.02	5.00 ± 0.07	990	Moderate	800 ± 10
Ch1	Belgium	blue	7.45	305	0.58 ± 0.01	36.33 ± 0.12	43.0 ± 0.1	1.9 ± 0.2	43.3 ± 0.1	18.5 ± 0.1	3.15 ± 0.02	26.9 ± 0.2	479	Weak	465 ± 6
Ch2	Belgium	blue	5.46	305	0.45 ± 0.01	3.96 ± 0.01	< LQ	< LQ	58.8 ± 0.2	18.3 ± 0.2	2.96 ± 0.04	26.9 ± 0.5	416	Weak	508 ± 4
Cx	France	blue	7.56	375	0.19 ± 0.04	6.16 ± 0.01	1393.7 ± 0.9	< LQ	386 ± 1	78.8 ± 0.3	3.64 ± 0.02	9.09 ± 0.09	2253	Rich	1729 ± 5
Ev	France	light blue	7.58	360	0.06 ± 0.01	8.29 ± 0.02	10.8 ± 0.8	0.64 ± 0.02	27.4 ± 0.2	26.3 ± 0.2	1.41 ± 0.01	6.73 ± 0.04	442	Weak	373 ± 7
Vt2	France	colourless	7.41	399	0.16 ± 0.01	7.1 ± 0.1	360.5 ± 0.1	0.56 ± 0.01	134.8 ± 0.1	43.15 ± 0.12	2.50 ± 0.01	5.09 ± 0.04	953	Moderate	767 ± 10
ViO	Germany	blue	8.04	150	0.13 ± 0.01	11.9 ± 0.1	3.6 ± 0.5	< LQ	43.5 ± 0.1	6.95 ± 0.06	2.13 ± 0.01	10.81 ± 0.03	229	Weak	260 ± 4
Av	Greece	colourless	8.18	225	0.36 ± 0.04	38.85 ± 0.04	< LQ	1.0 ± 0.1	33.7 ± 0.3	8.0 ± 0.1	2.26 ± 0.03	12.21 ± 0.01	321	Weak	259 ± 4
Ly	Greece	colourless	8.04	200.5	0.05 ± 0.01	7.31 ± 0.01	< LQ	5.8 ± 0.1	43.2 ± 0.6	10.5 ± 0.1	1.08 ± 0.01	13.50 ± 0.01	282	Weak	311 ± 4
KS	Ireland	dark blue	7.49	228	0.04 ± 0.01	46.7 ± 0.3	5.1 ± 0.3	10.4 ± 0.7	70.4 ± 0.9	9.79 ± 0.06	2.67 ± 0.02	19.12 ± 0.07	392	Weak	442 ± 8
SnB	Italy	colourless	7.53	311	0.04 ± 0.01	2.72 ± 0.01	< LQ	11.71 ± 0.01	32.6 ± 0.5	30.14 ± 0.06	1.32 ± 0.01	6.5 ± 0.2	396	Weak	347 ± 9
Bc	Italy	blue	7.83	324	0.09 ± 0.01	1.15 ± 0.04	21.8 ± 0.3	3.22 ± 0.04	40.1 ± 0.2	28.60 ± 0.06	0.41 ± 0.01	0.75 ± 0.01	420	Weak	345 ± 7
AP	Italy	colourless	7.87	106	0.06 ± 0.01	8.5 ± 0.2	23.5 ± 0.5	3.5 ± 0.2	30.3 ± 0.1	7.1 ± 0.1	1.27 ± 0.01	6.88 ± 0.01	187	Weak	193 ± 2
SnE	Italy	blue	7.71	354	0.11 ± 0.01	2.01 ± 0.11	3.7 ± 0.3	1.14 ± 0.06	36.1 ± 0.2	26.45 ± 0.06	2.22 ± 0.02	15.35 ± 0.01	441	Weak	411 ± 5
SnP	Italy	green	5.36	239	0.46 ± 0.02	6.42 ± 0.02	< LQ	< LQ	161 ± 2	50.13 ± 0.06	2.82 ± 0.01	12.08 ± 0.01	472	Weak	1004 ± 5
SdE	Portugal	light blue	6.46	16.5	0.09 ± 0.01	65.12 ± 0.12	54.1 ± 0.5	4.4 ± 0.2	0.96 ± 0.01	0.30 ± 0.01	0.78 ± 0.07	4.0 ± 0.7	146	Weak	25.5 ± 0.1
CN	Switzerland	blue	7.66	306	1.43 ± 0.01	3.49 ± 0.08	51.4 ± 0.9	0.45 ± 0.02	79.1 ± 0.9	41.23 ± 0.06	2.57 ± 0.01	44.3 ± 0.1	530	Moderate	673 ± 9
Bf	Mexico	colourless	7.65	<10*	0.23 ± 0.01	10.3 ± 0.2	4.4 ± 0.1	< LQ	10.8 ± 0.2	13.99 ± 0.06	4.16 ± 0.01	20.71 ± 0.05	65	Weak	228 ± 2
AA	Mexico	colourless	7.14	<10*	0.05 ± 0.01	35.9 ± 0.1	< LQ	18.9 ± 0.2	0.73 ± 0.01	1.0 ± 0.1	1.50 ± 0.03	1.06 ± 0.01	59	Weak	53.8 ± 0.8
AV1	Panama	blue	7.75	10	0.08 ± 0.01	1.5 ± 0.1	< LQ	0.45 ± 0.03	1.11 ± 0.01	4.4 ± 0.1	2.34 ± 0.01	3.84 ± 0.05	24	Very weak	65 ± 1
AV2	Panama	blue	7.51	56.2	0.09 ± 0.01	2.13 ± 0.15	< LQ	0.15 ± 0.02	1.20 ± 0.01	4.5 ± 0.1	2.37 ± 0.01	3.99 ± 0.02	71	Weak	67 ± 1

*Estimated by charge balance

The pH of the samples ranges between 5.36 and 8.18. Although pH usually has no direct impact on consumers, it is one of the most important operational water quality parameters. The pH values in mineral water will vary in different supplies according to the composition and the source of the water, but it is usually in the range 4.5 – 9.5 [Real Decreto 1798/2010]. No health-based guideline value has been proposed for pH.

Fluoride concentration in samples ranged between 0.04 and 1.62 mg L^{-1}. Traces of fluorides are present in many waters, with higher concentrations often associated with groundwater. Fluor in water is always present as fluoride ions, either from natural sources or from artificial fluoridation. The amounts added to drinking-water are such that final concentrations are usually between 0.5 and 1 mg L^{-1}. Although the guideline value of fluoride concentration in water is 5 mg L^{-1}, waters with concentrations higher than 1 mg L^{-1} are considered as fluoridated waters, and if the content is higher than 1.5 mg L^{-1}, it has to be indicated in the label. [Real Decreto 1798/2010; WHO, 2006]. Concentrations above the guideline value lead to an increasing risk of dental fluorosis, and much higher concentrations lead to skeletal fluorosis.

Chloride was present in samples between 1.15 and 98.0 mg L^{-1}. High concentrations of chloride give a salty taste to water and beverages. Chloride-containing waters are those which present values higher than 200 mg L^{-1} [Real Decreto 1798/2010]. Concentrations above 250 mg L^{-1} are increasingly likely to be detected by taste, but some consumers may become accustomed to low levels of chloride-induced taste. No health-based guideline value is proposed for chloride in drinking-water [Real Decreto 1798/2010; WHO, 2003b].

Sulphate concentration in samples was below the limit of detection in 29% of the samples and between 3.6 and 1394 mg L^{-1} in the rest of them. Sulphated waters are those which present values higher than 200 mg L^{-1} [Real Decreto 1798/2010]. The presence of sulphate in water can cause noticeable taste, as some authors demonstrated that the taste of water could depend on the chemical composition of the salt content by the performance of chemometric analysis on sensory studies [Platikanov et al., 2013]. Very high sulphate levels might cause a laxative effect in unaccustomed consumers. It is generally considered that taste impairment is minimal at levels below 250 mg L^{-1}. Five samples contained sulphate concentration above this value. No health-based guideline value has been derived for sulphate [Real Decreto 1798/2010; WHO, 2004].

Nitrate concentration in samples was below the limit of detection in 18% of the samples and between 0.15 and 55.4 mg L^{-1} in the rest of them. The guideline value for nitrate is 50 mg L^{-1} as nitrate ion or 11 mg L^{-1} if reported as nitrate-nitrogen. Only one sample presented a concentration higher than the limit. This value has been considered for the protection against methaemoglobinaemia in bottle-fed infants at short-term exposure. This disease is a block of oxygen transport consequence of the reaction of nitrite, which is produced by the previous reduction of nitrate, with haemoglobin in the red blood cells to form methaemoglobin, which binds oxygen tightly and does not release it [Real Decreto 1798/2010; WHO, 2011].

Calcium and magnesium concentration in samples ranged between 0.73 - 386 mg L^{-1} and 0.30 – 78.8 mg L^{-1}, respectively. The presence of calcium and magnesium in water is related to the hardness, which is the traditional measure of the capacity of water to react with soap. Calcium-containing and magnesium-containing waters are those which present values higher than 150 and 50 mg L^{-1}, respectively [Real Decreto 1798/2010].The taste limit for calcium ion is in the range of 100–300 mg L^{-1}, depending on the associated anion and the taste limit for magnesium has not been evaluated although is probably lower than that for calcium. In some instances, consumers tolerate water hardness in excess of 500 mg L^{-1}. No health-based guideline value is proposed for hardness in water [WHO, 2009a].

Potassium concentration in water samples was between 0.39 and 4.29 mg L^{-1}. Currently, there is no evidence that potassium levels in waters are likely to pose any risk for the health of consumers. Therefore, it is not considered necessary to establish a health-based guideline value for potassium in water. Although potassium may cause some health effects in susceptible individuals, potassium intake from drinking water is well below the level at which adverse health effects may occur [WHO, 2009b].

Sodium concentration in samples ranges between 0.69 and 112 mg L^{-1}. The taste limit concentration of sodium in water depends on the associated anion and the temperature of the solution. At room temperature, waters with concentrations higher than 200 mg L^{-1} are considered sodic waters [Real Decreto 1798/2010] and may give rise to unacceptable taste. No health-based guideline value has been derived as the contribution from drinking water to daily intake is small [WHO, 2003c].

Electric conductivity in samples ranges between 25.5 and 1729 µS cm^{-1}. As expected, it was observed that rich mineralisation samples are those which present the highest values of conductivity whereas the samples with a “very weak” mineralisation degree are those which present the lowest values. A value of 2500 µS cm^{-1} is established as a threshold value [Real Decreto 1798/2010].

4.1.2. Determination of Sb

Total antimony and speciation were analysed by triplicate analysis when purchasing the samples. The quantification technique for total analysis was HG-AFS. In the case of speciation it was taken into account that some drinking waters could present antimony concentration levels lower that the limit of detection calculated for the hyphenated technique LC-HG-AFS. Regarding this fact, the determination of Sb(V) and Sb(III) was carried out by LC-ICP-MS. The analysis of total Sb and speciation was carried out following the procedures described in sections 2.3.1.2 and 2.3.2.4 (Chapter 2).

The concentrations of total Sb in the 38 samples are summarized in Figure 4.2. Total Sb concentration ranges between 0.07 and 1.05 µg L^{-1}, with a mean concentration of 0.49 µg L^{-1}. It should be taken into account that 5 samples (Ln, Ly, SnB, AP and SnP) presented concentrations lower than the LQ of the HG-AFS technique. Thus, they were analysed by ICP-MS, and the Sb depicted values correspond to the concentration determined by this technique. RSD values obtained were below 10 % in all cases. The values of Sb obtained are comparable and of the same order of magnitude of those obtained by other works which carried out the analysis of total Sb in PET bottled waters. [Shotyk et al., 2006; Güler, 2007; Shotyk et al., 2007; Westerhoff et al., 2008; Keresztes et al., 2009; Perić-Grujić et al., 2010; Ristić et al., 2011; Andra et al., 2012; Tukur et al., 2012; Brandao et al., 2014].

Regarding speciation analysis, the only species observed was Sb(V) in all samples. Sb(V) concentration ranged between 0.14 and 1.12 µg L^{-1}, with a mean concentration of 0.49 µg L^{-1}. RSD values obtained were below 10 % in all cases. Sb(III), the most toxic species was not detected in any sample. A dependent Student's t-test for paired samples, at the 95% confidence level, demonstrated that there was no significant difference between total Sb content and Sb(V). This fact evidences that the total Sb present in waters is all in the form of Sb(V). Although some studies have dealt with antimony speciation in spiked or polluted waters [De Gregori et al., 2005; Ghassemzadeh et al., 2006], only one study dealt with antimony speciation in two mineral water samples [Shakerian et al., 2014], as mentioned in the Introduction. Comparing with the results obtained by Shakerian et al., 2014, some differences were observed: as they found the presence of both Sb(V) (estimated as difference between total Sb and trivalent forms) and Sb(III) analysed by ETAAS after a SPE using an antimony ion imprinted polymer, only the pentavalent species was found in the present work.

The effect of storage time in antimony concentration was assessed by reanalysing both total Sb and speciation after one year of storage in the laboratory at room temperature. The concentrations obtained are also depicted in Figure 4.3. After 1 year of storage at room temperature, total Sb concentration in the samples ranged between 0.17 and 2.12 µg L^{-1} with a mean concentration of 1.04 µg L^{-1}. It can be observed higher antimony concentration in waters after one year of storage, with an increase of a 0.05 – 1.26 µg L^{-1} with a mean increased concentration of 0.54 µg L^{-1} with respect to the mean initial concentration. For speciation analysis, Sb(V) was the only species observed. Concentrations ranged between 0.14 and 2.08 µg L^{-1}, with no significant difference between the total concentrations (95% confidence level). RSD values obtained were below 10 % in all cases.

All the values obtained are below the health limit value for Sb in the European Union directive on mineral water (5 µg L^{-1}) and accomplish the standards of the World Health Organisation (WHO) for drinking water [Real Decreto 1798/2010]. However, some of the authors aforementioned reported Sb concentration slightly higher than the obtained in the present work, up to 5 µg L^{-1}. This difference may be attributed to the fact that the presence of antimony in PET bottled waters is due to the several factors which could affect to the migration of the semimetal present in the polymer to the water (PET bottle characteristics, type of water, storage time, among others), as mentioned in the Introduction. Thus, a systematic migration study will be undertaken in this thesis (see Chapter 5).

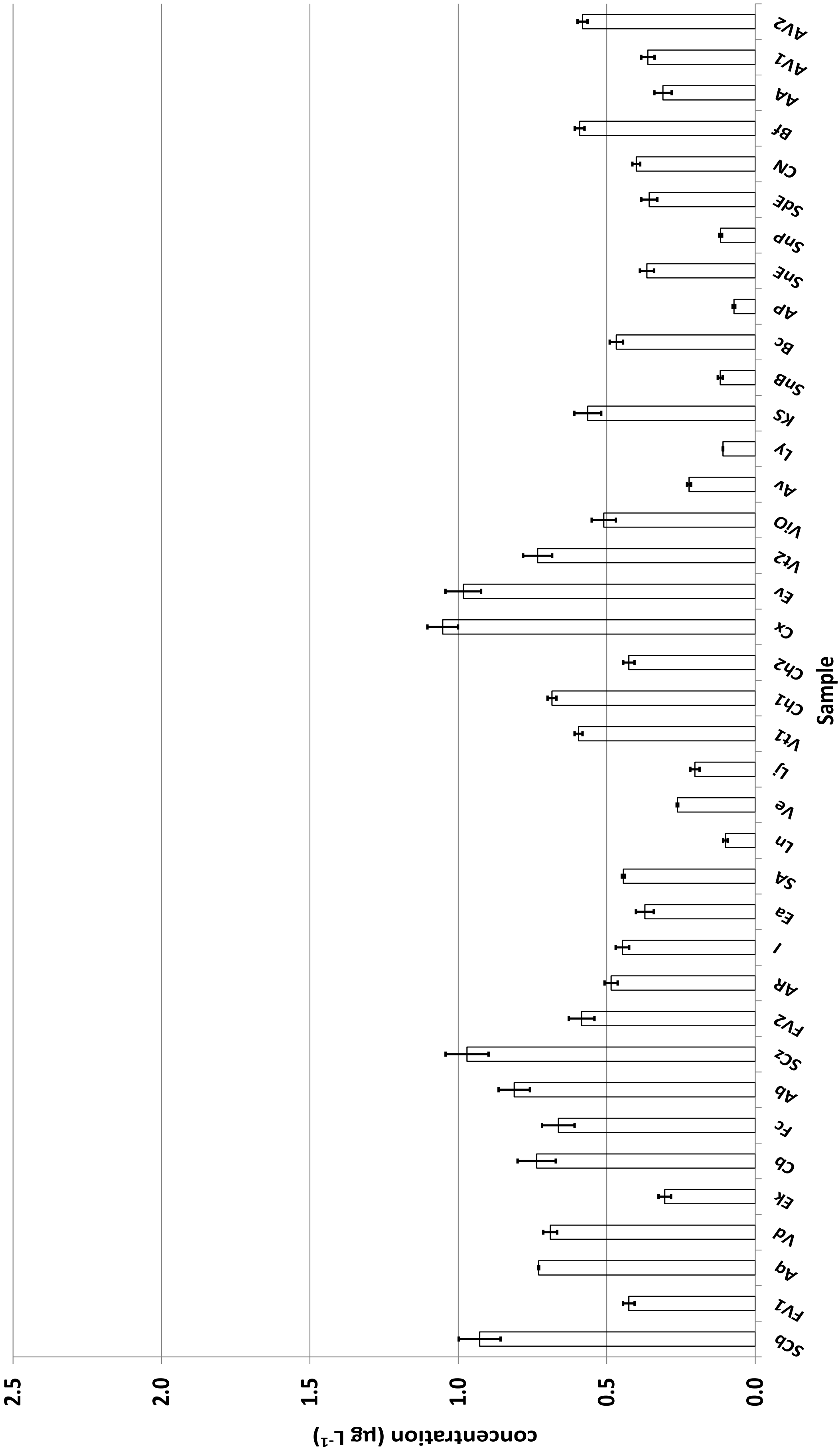


Figure 4.2. Total Sb concentration in the 38 PET bottled water samples after purchasing.

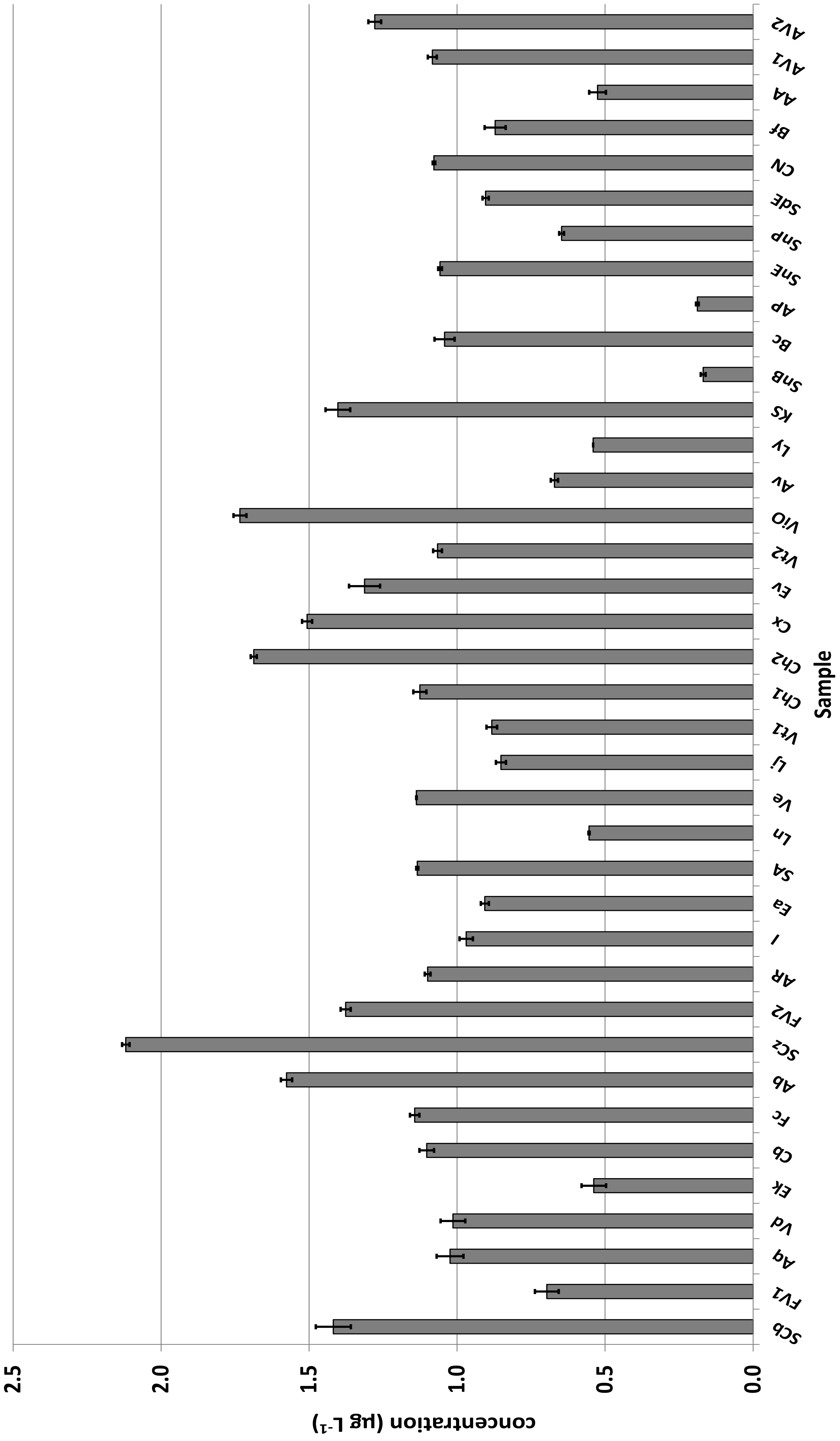


Figure 4.3. Total Sb concentration in the 38 PET bottled water samples after 1 year of storage.

4.1.3. Data analysis

In this section, the results obtained from the PET bottled water samples were evaluated by means of pattern recognition methods. They can provide a powerful tool for analysing a large amount of data. Consequently, the data treatment increase the quality of results, eases interpretation and bases the conclusions rigorously. These methods can be applied to test the physicochemical parameters and antimony concentration data and determine if samples can be related or grouped into distinct populations that may be significant from a statistical point of view.

In this study, two tools were used: correlation analysis and Principal Component Analysis (PCA). Correlation analysis was performed by the calculation of Pearson *r* coefficients to describe the degree of relation between two water physicochemical parameters. Correlation coefficient is a dimensionless number that ranges between -1.0 and +1.0. A value of +1.0 is given when the two variables react in exactly the same way as their values change, whereas -1.0 represents a perfect inverse relationship between the two variables [Ramis-Ramos et al., 2001]. A correlation coefficient of zero suggests that the two variables are independent of each other. PCA reduces a large number of variables (in this case the physicochemical parameters) to a fewer number defined as principal components (PCs). More concisely, PCA linearly combines two or more correlated variables into one variable. This approach has been used to extract related variables. The defined new variables can be then displayed in scores and loading diagrams, which represent the individual samples and the physicochemical parameters, respectively, as points in a lower dimensional space. PCA is useful for data reduction and to assess the continuity or overlap of clusters or similarities in the data [Guler et al., 2007]. The *Solo* commercial software package was used for the PCA analysis. Therefore, these tools could help in understanding better if antimony concentration released from PET bottles to water has relationship with a physicochemical parameter described before.

4.1.3.1. Correlation analysis

Pearson correlation coefficients were calculated for all possible pairs of variables with a pre-established significance level of 5%, and are summarised in Table 4.2. For data treatment, the values assigned to the degree of colour was zero for colourless bottles, 1 for light blue and green, 2 for blue and 3 for dark blue. Evident strong correlations (>0.70) between pairs of anions and cations together with the EC and mineralisation were observed, marked in bold on the table. However, no strong correlations were observed between antimony concentration and any variable. The highest Pearson correlation coefficient values obtained for initial concentration and final concentration of Sb were 0.36, which are related to mineralisation and colour, respectively. This fact means that a possible weak relation exists between antimony concentration and these two variables.

Table 4.2. Pearson correlation coefficients between pairs of the variables studied in the 38 bottled water samples.

R	pH	HCO_3^-	F^-	Cl^-	SO_4^{2-}	NO_3^-	Ca^{2+}	Mg^{2+}	K^+	Na^+	EC	Min	colour
pH	1												
HCO_3^-	0.08	1											
F^-	-0.14	0.09	1										
Cl^-	0.05	-0.24	-0.04	1									
SO_4^{2-}	0.11	0.26	-0.08	-0.01	1								
NO_3^-	0.03	-0.24	-0.18	0.59	-0.17	1							
Ca^{2+}	-0.05	0.45	-0.04	-0.14	**0.88**	-0.25	1						
Mg^{2+}	-0.05	**0.73**	-0.03	-0.30	0.63	-0.23	**0.80**	1					
K^+	-0.11	0.07	0.40	0.24	0.25	0.05	0.28	0.24	1				
Na^+	-0.15	0.14	**0.82**	0.07	-0.07	-0.19	-0.08	-0.10	0.49	1			
EC	-0.09	0.63	0.12	-0.16	**0.79**	-0.28	**0.94**	**0.89**	0.40	0.12	1		
Min	0.09	0.58	0.00	-0.05	**0.93**	-0.20	**0.92**	**0.81**	0.29	0.03	**0.92**	1	
colour	-0.06	0.25	0.01	-0.01	0.13	-0.11	0.24	0.22	0.16	0.11	0.30	0.22	1
iSbT	0.11	0.29	0.16	-0.11	0.32	-0.03	0.23	0.25	0.20	0.10	0.23	0.36	0.13
fSbT	-0.07	0.24	0.04	-0.05	0.13	-0.03	0.12	0.11	0.13	0.05	0.13	0.19	0.36

Min: mineralisation
iSbT: initial Sb total
fSbT: final Sb total (1 year later)

4.1.3.2. PCA analysis

PCA technique was used to reduce the number of dimensions present in the data matrix (reducing 14 variables to 3 PCs in this study), to select the most discriminating parameters, and to investigate the overall variation of data.

The first principal component (PC1) contains 30.57% of the total variance, the second component (PC2) represents 17.90% of the total variance, the third component (PC3) a 12.12% and the forth (PC4) a 9.55%. PC1 is concerned mainly with Ca^{2+}, Mg^{2+}, SO_4^{2-}, HCO_3^-, mineralisation and EC, followed by colour and initial and final concentration of Sb. Therefore, as expected, electric conductivity and the degree of mineralisation are mainly given by Ca^{2+}, Mg^{2+}, SO_4^{2-}. PC2 is basically concerned with antimony concentration and PC3 is related to Na^+, F^-, and K^+. PC4 presents a lower value of explained variance and is related to Cl^-, NO_3^- and repeated variables, as colour and K^+.

PC1 may be considered as an index of water hardness, whereas PC3 appears to represent the water saltiness. PC2 can be considered as antimony release. Thus, the three principal components PC1, PC2 and PC3 were chosen for building the model, which explain the 60.59% of the total variance, and PC4 was not included as it does not represent any new and significant information. Figures 4.4 and 4.5 show the result of the PCA analysis of the 38 bottled water samples, in which the first one depicts the scores and loadings biplot graphic of PC1 vs PC2, whereas the second one represents PC1 vs PC3.

As Figure 4.4 shows, in general terms, waters with high mineral content are grouped on the positive side of the PC1 whereas waters with a very low mineral content are grouped on the opposite side. Samples with the highest mineral content are close to the variables related to the mineralisation degree, as expected. It can also be observed in Figure 4.4, as expected, that samples with the highest concentration of antimony (ViO, Cb, Fc, Ch2, SCb, Ev and SCz,) are grouped close to Sb concentration variables. However, sample Cx, which presents the highest concentration of Sb, is not located close to Sb variables because it presents a very high mineral content. Therefore, this last variable prevails over the antimony concentration, and consequently, sample Cx is far located along PC1 in the plot. Besides, antimony concentration variables, (initial, final and the difference) are grouped among them but are not grouped with any other variables. This fact demonstrates that Sb concentration and release is independent from physicochemical variables, therefore, it could be related to other factors that are not contemplated in this set of data.

By contrast, when plotting PC1 vs PC3 in Figure 4.5, antimony concentration gets close to the variables Ca^{2+}, Mg^{2+}, SO_4^{2-}, HCO_3^-, mineralisation, EC, and colour. Moreover, samples with the highest degree of mineralisation group close to Sb variables. However, this fact does not mean that Sb concentration could be strongly related to the mineralisation degree and bottle colour, since antimony variables are mainly explained by PC2 (not included in this biplot). In any case, it should be taken into account that PC1 is partially related with Sb concentration and colour, therefore, a partial relation between Sb concentration and mineralisation degree and/or bottle colour in the range studied (3 grades of colour) could be possible. This fact gives some support to the obtained results, as more than half of the samples stored in clear bottles contained lower amounts of Sb than those stored in coloured bottles. In addition, the representation of PC2 vs PC3 does not give new information, as Sb variables get again grouped among them but not with other variables. For this reason, this biplot is not shown.

When comparing the results obtained with the literature, contradictory conclusions were found regarding the relation between antimony presence and the variables mineral content, pH and bottle colour. Table 4.3 summarises the conclusions obtained by the works who evaluated these relations together with those obtained in this work.

Firstly, regarding the relation between antimony and the mineral content, Westerhoff et al., 2008 concluded that higher salt content tended to result in higher antimony concentrations, and Hureiki et al., 2012 concluded that antimony leaching increased with the calcium concentration. Secondly, regarding the bottle colour, Tukur et al., 2011 reported no dependence of the Sb migration with the PET colour bottle whereas Reimann et al., 2010 reported colour dependence in migration. Taking into account these contradictory conclusions and the non-consistent evidences resulted in the present work on almost 40 samples; further studies should be carried out.

No correlation was observed between Sb concentration and the natural water pH in the range studied (5.36 - 8.18). These facts were also observed by Tukur et al., 2011 and Hureiki et al., 2012, whereas Kerestzes et al., 2009, Andra et al., 2012 and Brandao et al., 2014 reported that antimony migration from PET in water is favoured at lower pHs. In addition, some works studied the pH effect by adjusting experimentally. Westerhoff et al., 2008 and Reimann et al., 2010 did not find evidences about pH effect while Cheng et al., 2010 found significant variation. It has to be taken into account that neither sparkling nor acidified waters were analysed in this study. Therefore, regarding this fact and the contradictory evidences reported in the literature, no consistent conclusions can be deduced about the Sb migration at low pH waters.

With all the results obtained, it can be concluded that antimony migration is an important fact to be taken into account in PET-bottled waters as Sb concentration increases throughout time. As weak correlation with the mineralisation degree and bottle colour was observed, these variables cannot explain Sb migration, since they should not be the only responsible of this process. Sb migration could also depend on the sample, as it is not possible to know the conditions in which PET-bottled waters were undergone before being supplied in the stores. In addition, it can be remarked that mineral waters are little contaminated with antimony independently of the type of sample. As it has been shown, there are several variables which could play a role in Sb migration process, and their potential cross effects make difficult to draw definitive conclusions on the driving force of Sb migration.

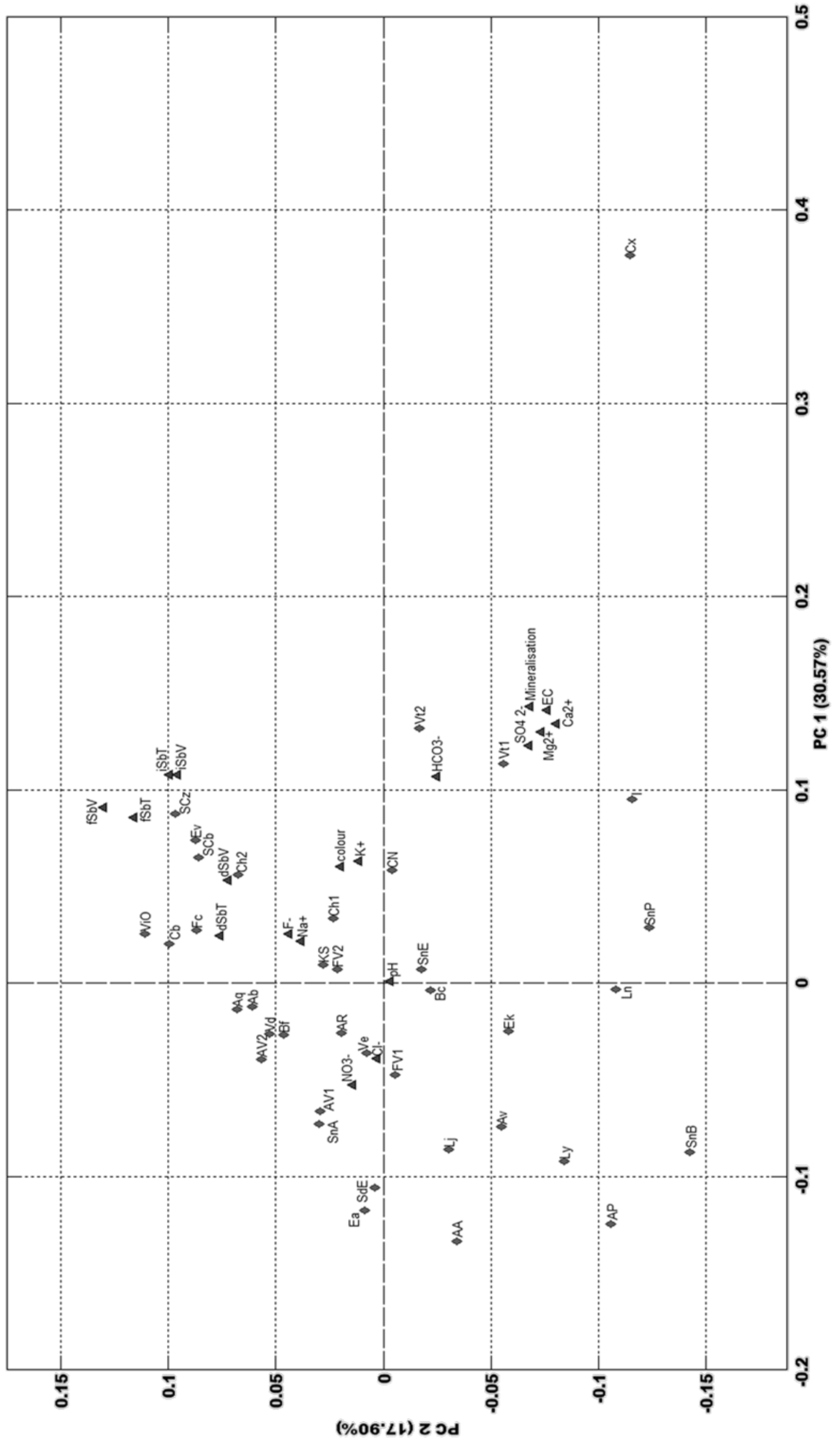


Figure 4.4. PCA analysis of the 38 water samples. Biplot graph PC1 vs PC2 (rhombus: water samples; triangle: characterisation parameters).

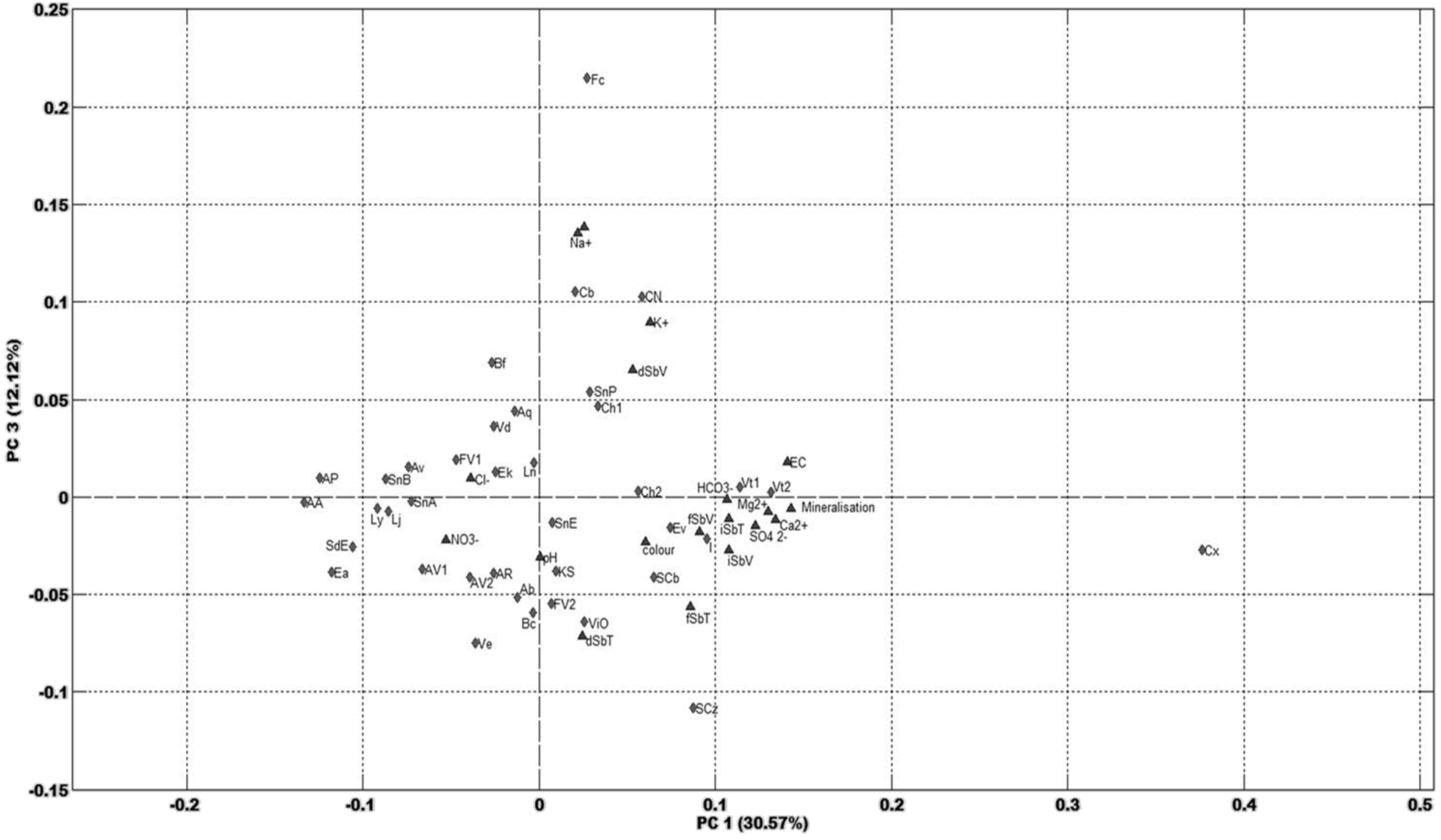


Figure 4.5. PCA analysis of the 38 water samples. Biplot graph PC1 vs PC3 (rhombus: water samples; triangle: characterisation parameters).

Table 4.3. Conclusions obtained in the literature about variables affecting on the antimony presence in PET-bottled water.

Author	Nº samples	Mineralisation	Colour range	pH	Correlation Sb – mineral content	Correlation Sb - colour	Correlation Sb – acid pH
Keresztes et al., 2009	20	-	-	10 samples still 10 samples sparkling	-	-	YES
Cheng et al., 2010	14	-	-	7 samples neutral 7 samples pH 3.5*	-	-	YES
Reimann et al., 2010	74	-	Colourless, brown, green and blue	15 samples pH 6.5 59 samples pH 3.5*	-	YES (blue)	NO
Westerhoff et al., 2010	9	2 samples high mineralised	Colourless, blue	1 sample at pH 6.3, 7.3 and 8.3*	YES	YES (colourless)	NO
Andra et al., 2012	14	-	-	3 samples still 11 samples sparkling	-	-	YES
Tukur et al., 2012	20	-	5 samples colourless, 5 samples green	5 samples still 5 samples sparkling	-	NO	NO
Hureiki et al., 2012	8	Ca^{2+}: 21-65.5 mg L^{-1} Mg^{2+}: 5-20 mg L^{-1} HCO_3^-: 80-293 mg L^{-1}	-	From 7 to 8	YES	-	NO
Brandao et al., 2014	50	-	-	39 samples still 11 samples sparkling	-	-	YES
This work	38	6 samples high mineralised	10 samples colourless, 28 samples blue	From 5.36 to 8.18	YES (weak)	YES (weak)	NO

*pH adjusted

4.2. ANALYSIS OF ANTIMONY IN JUICES

4.2.1. Determination of total Sb

As mentioned in the Introduction, little information has been published on the determination of antimony in juices: total Sb concentrations in red fruit juices and Sb species in lemon and juices were determined obtaining values of the same order of magnitude that those obtained in water samples [Hansen et al., 2010, Hansen et al., 2006a]. Therefore, in order to find the best sample preparation procedure and the best instrumental detection technique for the analysis of total Sb in the juice samples, several PET-bottled juices were tested using different analytical approaches. Seven commercial juices of different flavours of a same Spanish brand, which correspond to the full range of flavours from it, were purchased in a local shop: apple, grape, pear, pineapple, peach, orange and tomato.

Some authors recommend a digestion procedure as a pretreatment for the analysis of total Sb in juices [Dehelean et al., 2013; Shakerian et al., 2014]. However, it has been reported that there is no difference among a digestion procedure (wet digestion with HNO_3 – H_2O_2), extraction (sonication with aqua regia) and a simpler pretreatment (direct analysis after centrifugation, or direct analysis after dilution with HNO_3 with subsequent centrifugation or filtration) in the analysis of Sb in spiked fruit juices (apple, grape, grapefruit, orange, pineapple, pear and plum) [Welna et al., 2014]. Thus, the following analytical approaches were assessed for the analysis of juices: filtration followed by HG-AFS determination and microwave digestion followed by HG-AFS.

For the first procedure, the juices were filtered using ashless filter paper. Apple and grape juice were easily filtered, whereas pineapple and pear juice took longer. However, it was not possible to filter peach, orange and tomato juice, since the pulp blocked the passage of the liquid. Therefore, prior to filtration, both a sieve and a PTFE press machine were used to remove part of the pulp. Nevertheless, the filtration also took longer, as with pineapple and pear juice. Once filtered, the samples were analysed by HG-AFS using the procedure described in section 2.3.1.2 (Chapter 2). During sample measurement, white foam was observed to appear in the gas-liquid separator, mainly for pineapple and orange juices. This foam prevented antimony hydride formation, which led to the flickering or even disappearance of the flame. The results obtained for the remaining juice samples using this procedure ranged between 0.156 – 0.778 $\mu g\ L^{-1}$ and presented RSDs up to 22.5%. In view of all these facts, it can be concluded that this method is not reliable for the analysis of juices.

Microwave digestion with two different mixtures of concentrated acids (HNO_3 – H_2O_2 and HCl – H_2O_2 5:1) was tested as sample preparation. The juice samples were digested for 15 minutes from room temperature to 170ºC and maintained at 170ºC for 10 minutes. The extracts were made up to a 20 mL volume, resulting in a final dilution factor of 10. When analysing the samples, foam in the gas-liquid separator was not observed in any case. However, the signals obtained using this procedure were around the instrumental limit of quantification of the technique, or even lower, leading to high RSD values (> 20%). Therefore, this method is not suitable for the analysis of juices as the dilution factor involved in the microwave digestion led to Sb concentration in the extracts too low for measurement by HG-AFS.

Since it has been proved that HG-AFS technique is not suitable for the analysis of juices; ICP-MS detection was assayed. Taking into account the reported results by Welna et al., 2014, summarised in the previous page, sample digestion was rejected and sample dilution was again chosen. In this case, as the previous filtration method was rather slow, it was decided to centrifuge the samples prior to the filtration of the supernatant. Then 2 mL of the filtrated supernatant were taken and diluted 1:1 in HNO_3 1%, prior to ICP-MS analysis.

The results, summarised in Table 4.4, were low in all cases but quantifiable; concentrations of Sb were of the same order of magnitude for all the samples (0.166-0.937 µg L^{-1}) and good RSD values were obtained (≤ 5%). The maximum level of Sb established by the European Union in drinking water (5.0 µg L^{-1}) was not exceeded in any case.

Table 4.4. Total antimony concentration in juices determined by ICP-MS using as pretreatment a sample centrifugation (n = 3).

Juice	**Concentration (µg L^{-1})**	**RSD (%)**
Apple	0.343 ± 0.002	0.7
Grape	0.937 ± 0.018	1.9
Pear	0.522 ± 0.008	1.6
Pineapple	0.338 ± 0.002	0.5
Peach	0.273 ± 0.009	3.2
Orange	0.364 ± 0.005	1.3
Tomato	0.166 ± 0.007	4.2

In view of all the results obtained using the three procedures mentioned, it can be concluded that the most suitable and reproducible procedure consists of the centrifugation and filtration of the samples followed by ICP-MS determination. These results are comparable with those reported in the literature, with Sb concentrations ranging from 0.28 to 1.05 µg L^{-1} in orange and lemon juice contained in PET bottles [Hansen et al., 2006b] and 0.02 to 1.20 µg L^{-1} in apple, plum and sour cherry juice [Dehelean et al., 2013]. Sb concentrations reported in 25 PET-bottled red fruit juices are lower than 0.5 µg L^{-1} in 10 of samples analysed; whereas 7 of them were above the maximum level established by EU for drinking water (5 µg L^{-1}) [Hansen et al., 2010].

4.2.2. Identification and quantification of Sb species

To analyse the antimony speciation in juices, the LC-ICP-MS method for the separation and determination of inorganic Sb species in water samples developed in section 3.1 needs to be reassessed for the Sb species present in juices. As it has been reported by Hansen et al., 2006b and Hansen et al., 2007 that different antimony species from those present in water can be present in juice because of the formation of complexes with the organic acids as citrate, the separation process in a reasonable time analysis was assessed.

To start this study, the same conditions of the method previously described in section 3.1 were applied. Firstly, for the identification of the species, the analyses of individual Sb(V) and Sb(III) standard solutions of 0.25 µg L^{-1} diluted in the mobile phase (EDTA 10 mmol L^{-1}) and citric acid 40 mmol L^{-1} (simulating the concentration in juice matrix [Penniston et al., 2008]) were carried out. The results of analysing the standards, depicted in Figure 4.6, enabled us to see that Sb species in the mobile phase medium present different retention times from those prepared in citrate medium. This effect is due to the formation of different complexes between antimony and EDTA or citrate, which present different structures and consequently retention capacity. When using EDTA standards, retention times were approximately 1 minute for Sb(V), which corresponds to non-complexed Sb(V); and slightly less than 4 minutes for Sb(III), which corresponds to an Sb(III)-EDTA complex, as explained in section 3.1 for the method development for water analysis. Meanwhile, when using citrate standards, the elution order is exchanged: retention times were slightly more than 4 minutes for Sb(III) and approximately 8 minutes for Sb(V). The pentavalent species corresponds to an antimony-citrate complex [Hansen et al 2006b]. However, it is not clear if the Sb(III) peak observed in citrate media standards corresponds to an antimony-citrate or antimony EDTA, as the retention time is similar.

The analysis of the 7 different flavoured PET-bottled commercial juices used in the previous section for the total Sb determination was carried out after centrifugation and filtration, as described in the previous section. Samples were analysed with the conditions described in section 2.3.2.4 (Chapter 2). As an example, the upper part of the Figure 4.6 depicts the chromatograms obtained for the pineapple, peach and apple samples, together with the chromatograms of the standards previously mentioned. In the results, chromatographic peaks with the same retention times as Sb(III) standards were observed and small amounts of non-complexed Sb(V) were observed at 1 minute.

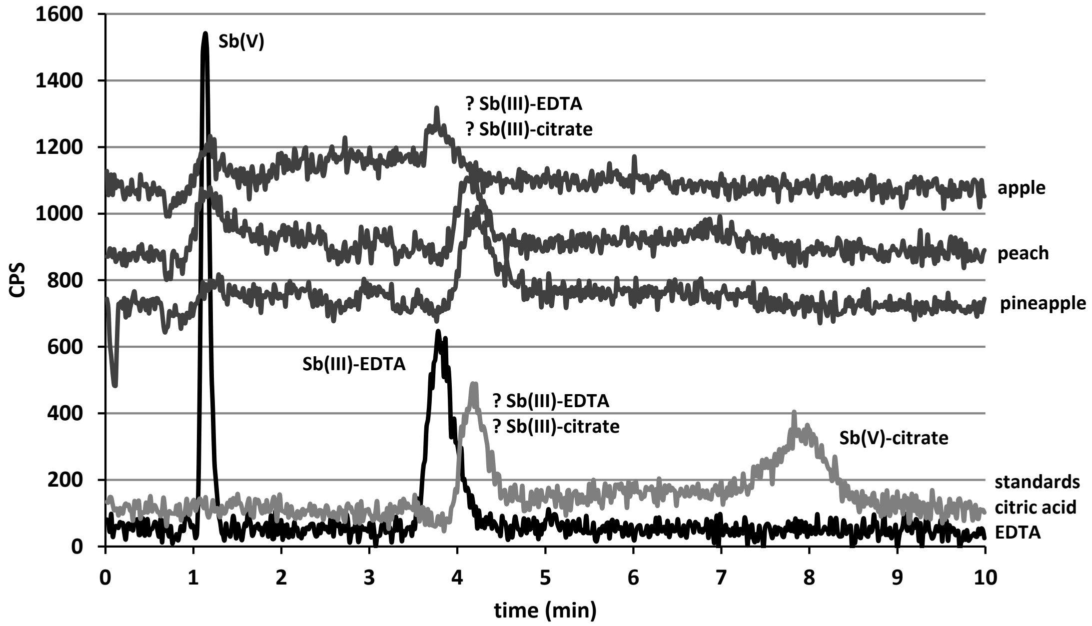


Figure 4.6. Chromatograms obtained from apple, pineapple and peach juice samples together with 0.25 µg L^{-1} antimony standards in mobile phase or citrate medium.

To ensure that the identity of the species observed in the juices corresponds to the complexes mentioned, spiking tests were performed. Samples were spiked with individual Sb(V) and Sb(III) standards diluted in both EDTA and citric acid. As representative results, spiked pineapple and peach juice samples are depicted in Figure 4.7. When adding the Sb(V)-citrate, the 8-minute peak corresponding to this standard appeared. Besides, when adding the standard of Sb(V) in EDTA media, a small peak corresponding to non-complexed Sb(V) at a retention time of 1 minute could be observed together with the peak of Sb(V)-citrate (approximately 70% of added Sb). This fact demonstrated that Sb(V) is complexed with citrate present in the juice.

Furthermore, it can be observed that the intensity of the main peak present in the samples increased either when Sb(III)-citrate or Sb(III)-EDTA was added. This behaviour only demonstrates that this peak corresponds to Sb(III) species, but it could not be still discerned if the species observed in the chromatogram is a Sb(III)-citrate or Sb(III)-EDTA. However, it is suspected that this peak corresponds to an Sb(III)-EDTA complex as it has been found in the literature that, although Sb(III)-citrate complexes are well known [Hansen et al 2006a], Sb(III) complexes convert to Sb(III)-EDTA complexes on the column when EDTA is part of the mobile phase [Hansen et al 2006b].

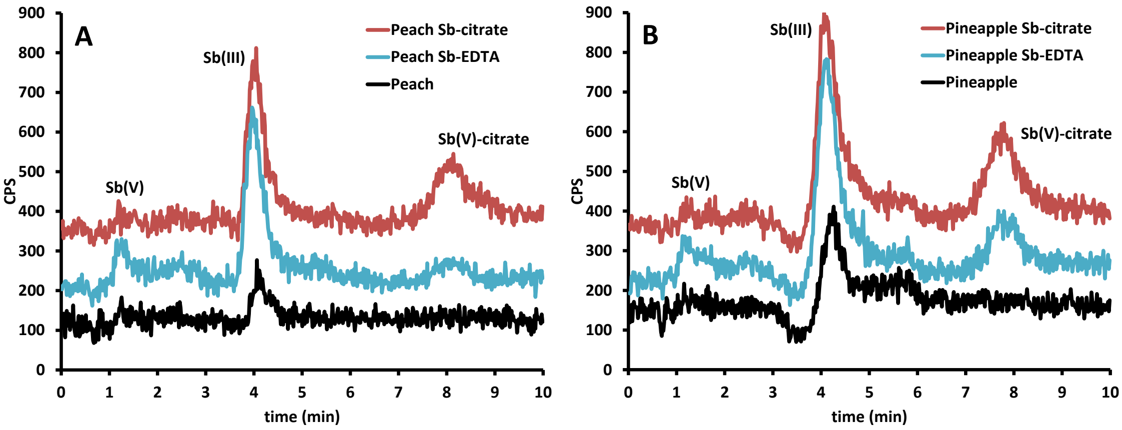


Figure 4.7. Spiking test performed with Sb standards prepared in EDTA and citrate medium in peach juice (A) and pineapple juice (B).

Therefore, to check if the Sb(III) species observed, both in the standards prepared with citric acid and the juice samples, corresponds to Sb(III)-EDTA or to Sb(III)-citrate complexes, two different approaches were carried out. Firstly, decreasing injection volumes of 1 µg L^{-1} Sb(III) standards prepared in EDTA and citrate media and 1 µg L^{-1} Sb(III) spiked pineapple and peach samples were injected: 100, 50, 25, 10 and 5 µL. In the chromatogram profiles, it was observed on the one hand that the retention time of EDTA media standard was the same at each volume injected. On the other hand, regarding the citrate media standard and the samples, as lower volumes were injected, the retention time of the Sb(III) peak get closer to the corresponding one to Sb(III)-EDTA standard. From an injection volume of 10 µL, the retention time was the same as the Sb(III)-EDTA standard. As an example, Figure 4.8 depicts the chromatographic profiles of the standards prepared with citric acid and EDTA and the spiked samples, injecting volumes of 100 and 10 µL. Thus, it can be concluded that Sb(III) species eluted with an EDTA mobile phase from the standard prepared with citric acid and also from juice samples convert to Sb(III)-EDTA complexes, which, at higher working volumes and due to matrix effect, eluted at higher retention times.

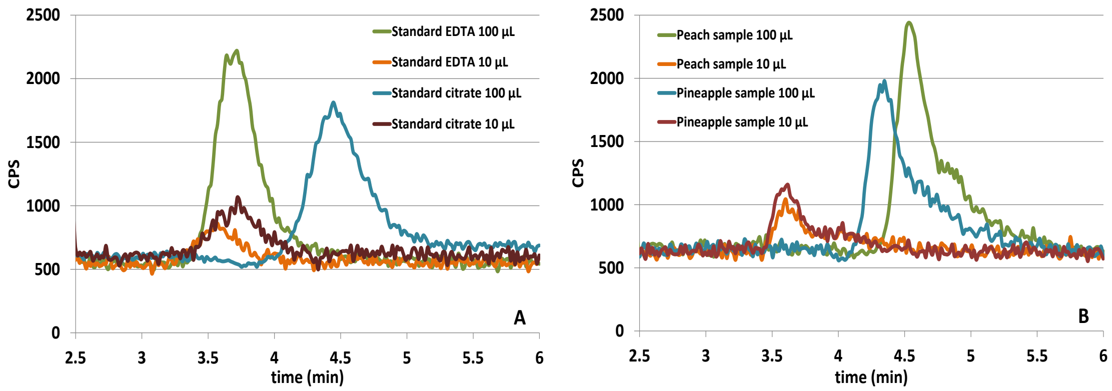


Figure 4.8. Chromatographic profiles obtained from a 100 and 10 µL injections of 1 µg L^{-1} standards of Sb(III) prepared in EDTA and citric acid media (A) and 1 µg L^{-1} spiked peach and pineapple samples (B).

However, the conversion of Sb(III)-citrate species to Sb(III)-EDTA complexes was confirmed using High Resolution Mass Spectrometry (HRMS), by analysing standards of Sb(III) with the presence of the stoichiometric concentration of EDTA and 10 times the stoichiometric concentration of citric acid for the correct formation of the complex [Hansen et al., 2006a]. The complex Sb-citrate ($[Sb(citH_2)_2]^-$; $C_{12}H_{12}O_{14}Sb^-$), with an expected m/z value of 500.9260, was not detected whereas the complex $[Sb(EDTA)]^-$ ($C_{10}H_{12}N_2O_8Sb^-$) was detected with a m/z value of 408.9634 with an error lower than 5 ppm. These results confirmed that trivalent forms of antimony are preferably complexed with EDTA instead citrate in the presence of both substances in the conditions where separation is performed.

Therefore, the same separation and measuring conditions proposed for the analysis of Sb in waters by LC-ICP-MS can be also applied to the analysis of Sb species in juices, with a 10-minute analysis time. For quantification, calibration curves from Sb(V) standards prepared both in the mobile phase and citrate media are necessary, as the presence of non-complexed Sb(V) and Sb(V)-citrate can be only quantified using the EDTA-media curve and citrate media curve, respectively. In the case of the trivalent species, they can be quantified with both Sb(III) calibration curves, as the slopes obtained from the curves prepared with Sb(III)-EDTA and Sb(III)-citrate standards are not significantly different.

Limits of detection and quantification were assessed for Sb(III) and Sb(V)-citrate with the same procedure described in section 3.1.3.2. The values obtained were: LD Sb(III): 0.03 µg L^{-1}; LQ Sb(III): 0.09 µg L^{-1}; LD Sb(V)-citrate: 0.06 µg L^{-1}; LQ Sb(V)-citrate: 0.20 µg L^{-1}.

Thus, with the established conditions, the seven juice matrices were quantified. The concentrations of the non-complexed Sb(V) were near the limit of detection in all samples and the concentrations of Sb(III), summarised in Table 4.5, ranged between 0.12 and 0.42 µg L^{-1}, with a mean concentration of 0.29 µg L^{-1}. A dependent Student's t-test for paired samples, at the 95% confidence level, demonstrated that total Sb content was significantly higher than Sb(III) species content. This can be attributed to the small amounts of Sb species found in the measuring extracts, which were close to the limit of quantification where the widespread of expected results is high. Column recoveries obtained were ≥ 70%, which are acceptable for speciation in complex matrices except for the grape sample (44%).

Table 4.5. Sb(III) species concentration in juices determined by LC-ICP-MS using as pretreatment a sample centrifugation (n = 3).

Juice	Sb(III) concentration (µg L^{-1})	RSD (%)	Column recovery (%)
Apple	0.254 ± 0.016	6.3	74
Grape	0.412 ± 0.007	1.7	44
Pear	0.416 ± 0.003	0.7	80
Pineapple	0.288 ± 0.018	6.3	85
Peach	0.192 ± 0.018	9.4	70
Orange	0.324 ± 0.008	2.5	89
Tomato	0.120 ± 0.012	10	72

4.2.3. MS characterisation

After the identification and quantification of the antimony species observed in the juice samples, further experiments were performed so as to study the formation of different antimony complexes in juice matrices by HRMS. To perform this study, peach and pineapple juices were selected among the matrices aforementioned.

Peach and pineapple juices present matrices with a high content of organic acids. The main acids present in these matrices are citric and malic acids, at concentrations around 40 mmol L^{-1} [Penniston et al., 2008]. As mentioned in the beginning of this section, the presence of Sb-citrate complexes was found in PET-bottled juices by Hansen et al., 2006b. Thus, it is thought that the antimony present in PET-bottled juices may not only be complexed with citric acid, but also with malic acid or other organic acids present in the matrix.

Taking into account this information, first of all, standards of Sb(V) and Sb(III) were prepared in three different media (double deionised water, citric acid and malic acid) so as to identify the most probable Sb complexes formed in juice matrix. Standards of 1 mg L^{-1} for each species were prepared with the corresponding stoichiometric concentration of citric or malic acid, except for the Sb(III) – acid citric standard, in which the concentration of the acid was ten times higher than the corresponding to the Sb(III) for the correct formation of the complex, as mentioned above [Hansen et al., 2006a]. Standards were analysed by HRMS using the conditions described in section 2.3.3.2 (Chapter 2).

Secondly, peach and pineapple samples were analysed by HRMS. Samples were directly injected and spiked with 1 mg L^{-1} of Sb(V) and Sb(III). Prior to the spiking step, the juice matrix was diluted up to the stoichiometric concentration of citric and malic acids (1 mg L^{-1}) except for the Sb(III) spiked samples, in which the matrix was diluted up to a concentration of 10 mg L^{-1}.

Table 4.6 summarises the major molecular ions obtained in the standards and the samples.

Table 4.6. Assignment for prominent ions observed for inorganic Sb standards in double deionised water, citric acid and malic acid media and direct and spiked peach and pineapple juices.

Standards		**molecular ion (m/z)**	**interpretation**	**z**	**assignment**
H_2O	Sb(V)	222.9207	H_6O_6Sb	1	$[Sb(OH)_6]^-$
	Sb(III)	149.0091	$C_4H_5O_6$	1	tartaric acid-H
		266.8893	$C_4H_2O_6Sb$	1	$[Sb(tar)]^-$
citric	blank	191.0189	$C_6H_7O_7$	1	citric acid-H
	Sb(V)	**360.9171**	$\mathbf{C_6H_8O_{10}Sb}$	1	$\mathbf{[Sb(OH)_3(citH)]^-}$
	Sb(III)	**500.9257**	$\mathbf{C_{12}H_{12}O_{14}Sb_2}$	1	$\mathbf{[Sb(citH_2)_2]^-}$
malic	blank	133.0141	$C_4H_5O_5$	1	malic acid-H
	Sb(V)	320.9212	$C_4H_8O_9Sb$	1	$[Sb(OH)_4(mal)]^-$
	Sb(III)	No Sb(III)-malic complex			
Juice samples		**molecular ion (m/z)**	**interpretation**	**z**	**assignment**
Peach	direct	133.0138	$C_4H_5O_5$	1	malic acid-H
		191.0523	$C_6H_7O_7$	1	citric acid-H
	Spiked Sb(V)	222.9200	$[Sb(OH)_6]^-$	1	$[Sb(OH)_6]^-$
		360.9151	$\mathbf{C_6H_8O_{10}Sb}$	1	$\mathbf{[Sb(OH)_3(citH)]^-}$
	Spiked Sb(III)	266.8898	$C_4H_2O_6Sb$	1	$[Sb(tar)]^-$
		500.9255	$\mathbf{C_{12}H_{12}O_{14}Sb_2}$	1	$\mathbf{[Sb(citH_2)_2]^-}$
Pineapple	direct	133.0139	$C_4H_5O_5$	1	malic acid-H
		191.0191	$C_6H_7O_7$	1	citric acid-H
	Spiked Sb(V)	222.9214	$[Sb(OH)_6]^-$	1	$[Sb(OH)_6]^-$
		360.9167	$\mathbf{C_6H_8O_{10}Sb}$	1	$\mathbf{[Sb(OH)_3(citH)]^-}$
	Spiked Sb(III)	266.8892	$C_4H_2O_6Sb$	1	$[Sb(tar)]^-$
		500.9249	$\mathbf{C_{12}H_{12}O_{14}Sb_2}$	1	$\mathbf{[Sb(citH_2)_2]^-}$

The Sb compounds determined in the standards prepared with double deionised water present the same structure than the corresponding to the solid standard: hexahydroxoantimonate (V) ($[Sb(OH)_6]^-$) for Sb(V) and antimony (III) oxide tartrate ($[Sb(tar)]^-$) for Sb(III). Both compounds are depicted in Figure 4.9. With the presence of citric acid and malic acid, Sb(V) is complexed 1:1 with both compounds as $[Sb(OH)_3(citH)]^-$ and $[Sb(OH)_4(malH)]^-$, respectively. However, Sb(III) is only complexed 1:2 with the citric acid as $[Sb(citH_2)_2]^-$, as mentioned before. Small amounts of non-complexed Sb(V), non-complexed Sb(III), citric and malic acid were observed in all respective cases. Figure 4.10 represents a possible structure of the mentioned complexes.

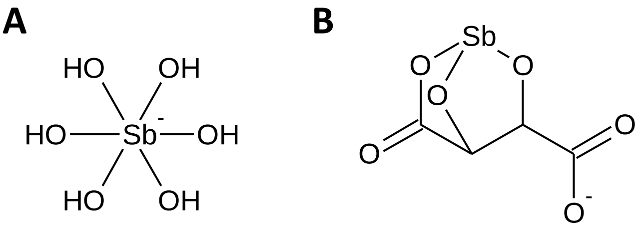


Figure 4.9. Sb(V) (A) and Sb(III) (B) standards structures

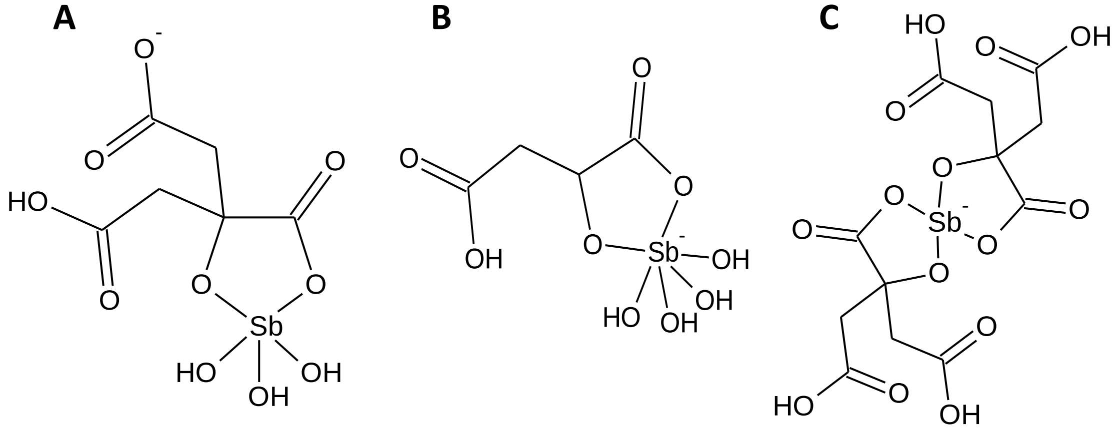


Figure 4.10. 1:1 Sb(V)-citrate (A), 1:1 Sb(V)-malate (B) and 1:2 Sb(III)-citrate (C) structures.

Regarding the results obtained in juice samples, the mass spectra obtained for the direct injected samples showed high amounts of citric acid and malic acid without the presence of any Sb complex, as expected. This is related to the sensibility of the instrument, which is not high enough to detect the Sb trace levels present. Thus, to identify the Sb complexes formed in juice matrices, the spiked samples were analysed. The Sb(V) spiked juices showed the presence of the 1:1 Sb(V)-citrate compound ($[Sb(OH)_3(citH)]^-$), whereas Sb(III) spiked juices showed the 1:2 Sb(III)-citrate compound ($[Sb(citH_2)_2]^-$). The presence of Sb(V)-malic compounds was not observed in any case. Small amounts of non-complexed Sb(V) and non-complexed Sb(III) were also observed in the respective cases. Thus, with the data obtained, it can be determined that the unique antimony compound present in PET-bottled juices is the 1:2 Sb(III)-citrate compound.

With all the results obtained, it can be concluded that antimony compounds which migrated from PET to the juice samples are complexed with the acid citric present in the matrix forming the molecule of $[Sb(citH_2)_2]^-$, depicted in Figure 4.10C. When juice samples are analysed by LC-ICP-MS using EDTA as mobile phase, the Sb(III) species are then complexed with the EDTA molecules, forming the $[Sb(EDTA)]^-$ molecules aforementioned.

4.3. DETERMINATION OF ANTIMONY IN *RAKI* AND *TSIPOURO*

This section deals with the work carried out during the research stay in the Laboratory of Speciation Analysis Elements of the Division "Environmental Chemical Processes Laboratory (ECPL)"" from the Department of Chemistry of the University of Crete. The studies performed were focused on the determination of the presence of antimony and its species in PET-bottled homemade spirits, specifically *raki* and *tsipouro*.

Raki (ρακί) is a traditional double-distilled anise-flavoured Turkish spirit with a 'protected designation of origin'. It is made from double distillation of ethyl alcohol produced from the fermentation of grapes or raisins in the presence of star anise. There is a variant of *raki*, produced in Greece, which is prepared without anise [Gueven, 2013]. *Tsipouro* (τσιπουρο) is a Greek traditional alcoholic drink produced by distillation of the residual from wine-making, mostly the marc. *Tsipouro* is similar to other Mediterranean countries alcoholic beverages, such as Spanish *orujo*, the Italian *grappa*, the Turkish *ouzo* or Greek *raki* in terms of organoleptic properties, chemical composition and manufacturing techniques. In general, it can be considered that *tsipouro* is a homemade version of *ouzo*, which is produced on an industrial scale [Anli et al., 2010]. The final products of *raki* and *tsipouro* ready-to-consume contain approximately a 40% of ethanol. Their production could be either industrial or homemade, and they are typically sold in bulk on grocery stores. As mentioned in the Introduction, homemade spirits tend to be stored in PET containers to ease their transport, as it can be seen in Figure 4.11.

Figure 4.11. *Raki* for sale in a grocery store from the centre of Heraklion, Crete.

This section deals with the study of the presence of antimony in PET bottled *raki* and *tsipouro*.

4.3.1. Analysis of total Sb

Little information has been published on the determination of metals in alcoholic beverages and there is no information about the determination of antimony in PET bottled spirits. Since these type of samples present a complex matrix due to the significant alcohol content, the analysis of total Sb in *raki* and *tsipouro* samples was assessed in order to find the best sample preparation procedure and the instrumental conditions.

A total of 12 spirit samples from local stores and suppliers were selected for the determination of total Sb: 9 samples of *raki* and 3 samples of *tsipouro*. Among these, 2 samples of both *raki* and *tsipouro* were stored in glass bottles for comparison purposes in terms of antimony concentration. Figure 4.12 depicts some of the samples mentioned.

Figure 4.12. *Raki* and *tsipouro* samples stored in PET and glass bottles.

Total Sb content was analysed by ICP-MS using the conditions described in section 2.3.1.3 (Chapter 2). In this case, both external calibration and standard addition were used for quantification. All samples were analysed using 2 different sample pretreatments: direct analysis and analysis after a previous evaporation of the alcoholic part of the samples with appropiate dilution, in order to reduce the ethanol content in the samples in case the torch gets unstable. The evaporation process was performed in a water bath at a temperature of 50ºC for 1 hour. Before injection, direct samples and evaporated samples were diluted four and two times, respectively, in the corresponding media.

First of all, no difference was observed between the two calibrations methods. Sb content in glass bottled samples was lower than the limit of detection, whereas PET-bottled samples showed higher concentrations. The Sb concentrations obtained in the PET bottled samples are plotted in Figure 4.13. Significant differences were observed between the pretreatments: the concentrations obtained with the direct analysis were between 0.4 – 4 µg L^{-1}, whereas the values obtained using the evaporation process were lower: 0.2 – 3 µg L^{-1}. RSD values were ≤

10 % in all cases. This difference could be due to a possible loss of the Sb content during the alcohol evaporation process. Therefore, for the correct analysis of Sb in these spirits, samples must be analysed directly, with only appropriate dilution. It was observed that more than the half of the PET-samples analysed showed concentrations higher than 1 µg L^{-1}. These values are higher than those obtained in water and juice samples (sections 4.1.2, and 4.2.1). This difference could be partially related to the matrix because of its high ethanol content. Indeed, ethanol is used as a food simulant for alcoholic foodstuffs to perform the migration test stipulated in the EU Directive 97/48/CE. Spirits present a high content of fusel alcohols, organic volatile molecules and sugar, which could ease the migration of Sb from the PET matrix. Besides, the historical background of the samples and the PET bottles should could also be potentially related to the migration, as it is not known how long samples were stored in the PET bottles neither the bottle aging or characteristics. Moreover, 3 PET-bottled *raki* samples presented Sb concentrations close to 4 µg L^{-1}, which are quite close to the limit established by the EU in drinking waters (5 µg L^{-1}).

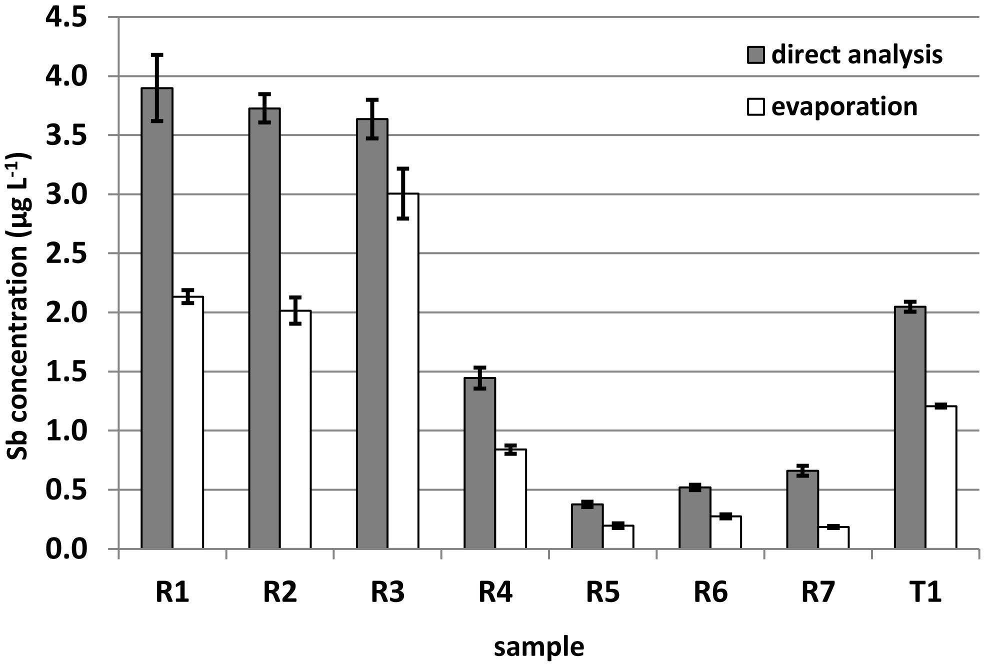


Figure 4.13. Total Sb concentration in PET-bottled *raki* and *tsipouro* samples. R1 – R7: PET-bottled *raki*; T1: PET bottled *tsipouro* (n=3).

4.3.2. Analysis of Sb species

After the analysis of total Sb in *raki* and *tsipouro*, the determination of Sb species in the PET-bottled samples was carried out by LC-ICP-MS by direct injection after appropiate dilution, using the instrumental conditions summarised in section 2.3.2.4 (Chapter 2). As no information was found about the possible antimony species present in spirits, two different separation conditions were assessed for the separation of Sb species. The first one was the procedure developed in section 3.1: Hamilton PRP-X100 (125 x 4.1 mm) as stationary phase and EDTA 10 mmol L^{-1} at pH 4.0 with a 0.5 % of methanol as mobile phase. The second separation conditions selected consist of the use of the same column as stationary phase and ammonium formate 10 mmol L^{-1} with 2 % of methanol as mobile phase. This mobile phase was previously used in the Greek research group for the separation of several Sb(V) complexes in spiked yogurt samples [Hansen et al 2007]. The analyses were performed at a flow of 1 mL min^{-1} to avoid the nebuliser flood. Standards of Sb(V) and Sb(III) were also analysed using both separation methods in order to identify the species.

The results obtained showed chromatograms with a maximum of 3 peaks for both methods. As an example, Figure 4.14 depicts the chromatograms of the *raki* sample R2 with the two separation conditions. With EDTA as mobile phase, the first and the third peaks have the same retention time than Sb(V) and Sb(III) standards, respectively. Thus, they correspond to Sb(V) and Sb(III) species. The trivalent species is only present in *raki* samples R1 – R3, which have presented the highest total Sb content. The second peak, which is the main species in all samples and has a retention time slightly higher than the Sb(V) one, corresponds to an unknown species. The peaks obtained with the second method correspond all to species of Sb(V), as Sb(III) cannot be eluted using these separation conditions [Hansen et al 2007]. The second peak, which is the predominant in all samples, has the same retention time than the Sb(V) standard, therefore, it corresponds to non-complexed Sb(V) species. The other peaks may well correspond to unknown complexes of Sb(V).

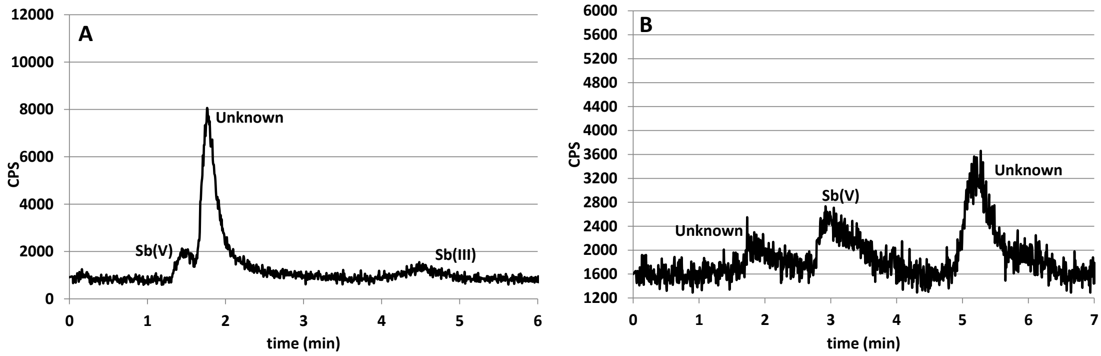


Figure 4.14. Chromatograms obtained from *raki* R2 by LC-ICP-MS using as mobile phase: A) EDTA 10 mmol L^{-1} pH 4 with 0.5% of methanol, and B) Ammonium formate 8 mmol L^{-1} with 2% of methanol.

To verify the identity of the species present in samples, spike tests were performed with Sb(V) and Sb(III) standards. Samples were spiked with a concentration of 2 µg L^{-1} for both species separately. On the one hand, it was verified that the third peak observed when using EDTA as mobile phase corresponds to Sb(III) species, as the intensity increased when spiking with the trivalent species. On the other hand, spikes with Sb(V) were performed using the two separation procedures. The results obtained are depicted in Figure 4.15. When samples were analysed just after spiking, the intensity of the non-complexed Sb(V) only was increased, as can be seen with respect to the non-spiked sample. However, when analysing 30 minutes later after spiking, the signal of Sb(V) decreased whereas the intensity of the second peak, in the case of EDTA mobile phase, and the third peak, in the case of ammonium formate one, increased. This fact demonstrates that these species correspond to Sb(V) complexes. Thus, further studies dealing with the mass spectrometry to elucidate the structure of the complex are described in the following section.

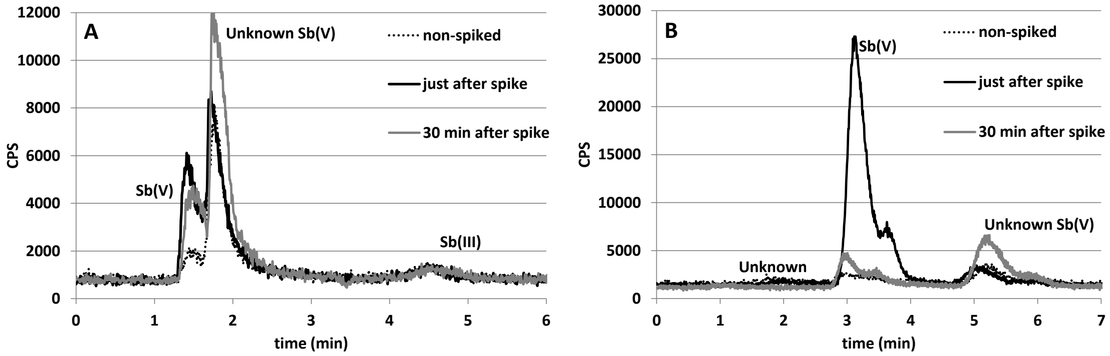


Figure 4.15. Spike tests with 2 µg L^{-1} of Sb(V) using A) Mobile phase EDTA 10 mmol L^{-1} pH 4 with 0.5% of methanol, and B) Mobile phase ammonium formate 8 mmol L^{-1} with 2% of methanol. Continuous line: analysis just after spiking. Dotted line: analysis after 30 minutes of spiking.

Moreover, it should be taken into account that the loss and gain of the area of Sb(V) and the unknown Sb(V) species, respectively, after 30 minutes of spiking, is different between the two methods. With EDTA mobile phase, although the signal gain for the unknown species is slightly higher than the signal loss for Sb(V) species, the % of gain and loss, respectively, in terms of area and concentration is similar. However, with the ammonium formate method, the signal loss for non-complexed Sb(V) is much more significant than the signal gain for the unknown species, which demonstrates that there is a loss of the sensitivity for the species with the second separation method.

From the results obtained, it is better to use the EDTA mobile phase rather than the ammonium formate one for the determination of antimony species in these samples by LC-ICP-MS due to the presence of Sb(III). All the same, the use of ammonium format is still a good tool, as it is capable of eluting the unknown Sb(V) species, which is the main one in every samples, specially coupled to other kind of detection methods, such as ESI-MS, which cannot accept non-volatile salts or buffers like the EDTA.

Once selected the separation procedure, antimony species in samples were quantified by means of external calibration curves of Sb(V) and Sb(III), which were prepared in 40% of ethanol to simulate sample matrix. As the unknown species has a retention time very similar to the Sb(V) one, it was quantified with the Sb(V) calibration curve. The concentrations obtained are depicted in Figure 4.16 together with the sum of species. RSD values were ≤ 10 in all cases.

Column recoveries calculated with respect to total Sb contents ranged from 70 – 110% except for sample R1, which presented a slightly lower value (62%). This fact could be due to the presence of other minor Sb species that could not be quantified or even eluted from the column. However, for those three samples (R5 – R7) with total Sb content around 0.5 µg L^{-1} or even lower, high recovery values were obtained, which evidenced analytical problems due to the sample dilution factor needed for analysis.

Even though the unknown species, which is the predominant in all samples, was quantified with a different standard, the recoveries obtained for five of the samples analysed were acceptable. This fact evidences that this approximation can be a good tool as it provided acceptable results.

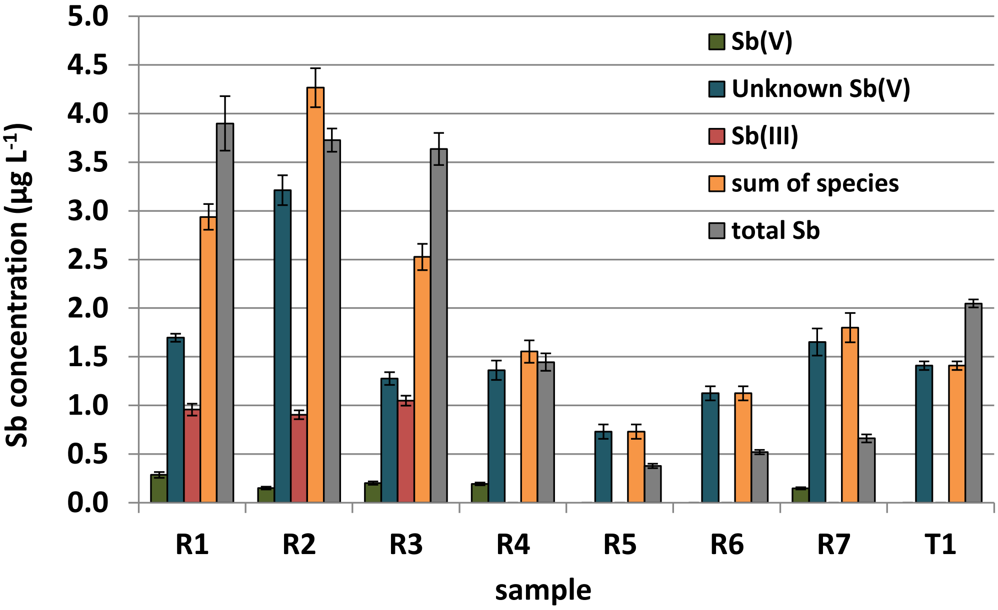


Figure 4.16. Sb species concentrations in *raki* and *tsipouro* samples.

4.3.3. Elucidation of the unknown Sb(V) species

4.3.3.1. Determination of Sb species and optimisation

The analysis of Sb species by LC-ICP-MS showed that *raki* samples had an unknown complex of Sb(V) as the major species. In order to determine the structure of this complex, several studies by ESI-MS were performed. As EDTA is not compatible with ESI due to its low volatility, a mobile phase with ammonium formate was used for the liquid chromatography. Specifically, the following conditions, previously optimised by the research group, were used: a mixture of 0.1% formic acid and methanol 80:20 as mobile phase, a 3-cm length C18 column as stationary phase and an injection volume of 2 µL, as detailed in section 2.3.3.1 (Chapter 2).

First of all, the two raki samples with the highest concentration of Sb, R1 and R2, were selected and analysed by ESI-MS with direct infusion. The analysis conditions are described in section

2.3.3.1 (Chapter 2). The samples were analysed both directly and spiked at four different concentration levels of Sb(V): 10 µg L^{-1}, 100 µg L^{-1}, 1 mg L^{-1} and 10 mg L^{-1}. The spectra showed plenty of signals and a high level background in all cases. Therefore, it was decided to analyse the samples by LC-ESI-MS with the conditions described in section 2.3.3.1 (Chapter 2). The results showed a peak signal with the same retention time as the dead volume (0.8 min). Figure 4.17 shows the Total Ion Chromatogram (TIC) of the non-spiked raki R1 and the spiked ones at 10 mg L^{-1} level together with the corresponding spectra average of the peak signal. For spiked samples from 1 mg L^{-1}, this signal corresponded to antimony complexes with mass-to-charge ratio (m/z) of 367-369, 457-459, 475-477. In the non-spiked sample and the spiked ones at a concentration level lower than 1 mg L^{-1}, any signal with the isotopic distribution of the antimony was observed.

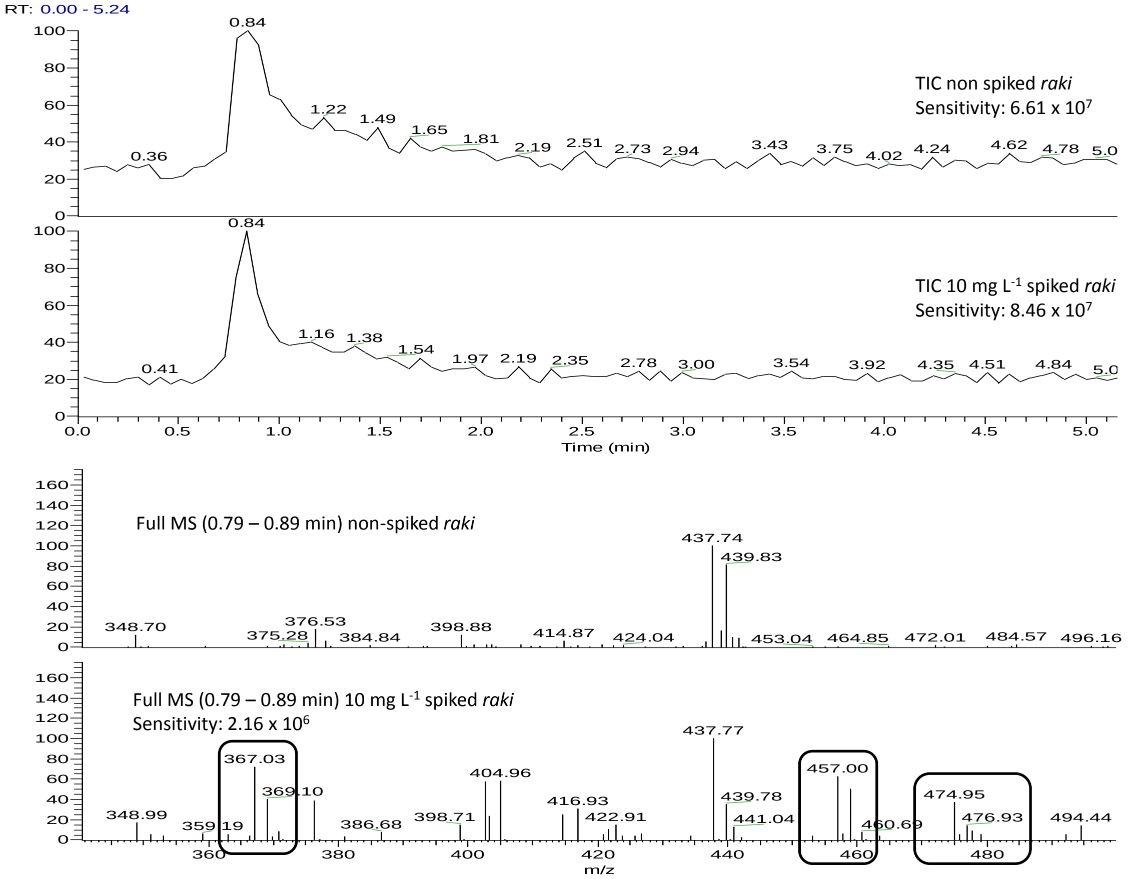


Figure 4.17. TIC of non-spiked and 10 mg Sb(V) L^{-1} spiked *raki* R1 together with the average spectra of the 0.84-minute peak signal.

In order to improve the retention of the antimony complex, two chromatographic parameters were assessed and modified: the composition of the mobile phase and the column length. First of all, *raki* sample R1 spiked with 10 mg L^{-1} of Sb(V) was analysed using different ratios of formic acid - methanol. The ratio of formic acid 0.1% was increased from the fixed value used in the previous test (80%) to 100%. The TICs obtained in this test are depicted in Figure 4.18. It can be observed that as the percentage of methanol decreases, the retention of the Sb peak is slightly higher. However, for organic solvent content lower than 10%, the Sb peak present deformation. Thus, the optimum mobile phase ratio was 90% of formic acid and 10% of methanol. However, the retention and sensitivity of the organic Sb complex was not enough, therefore, a longer column was assessed.

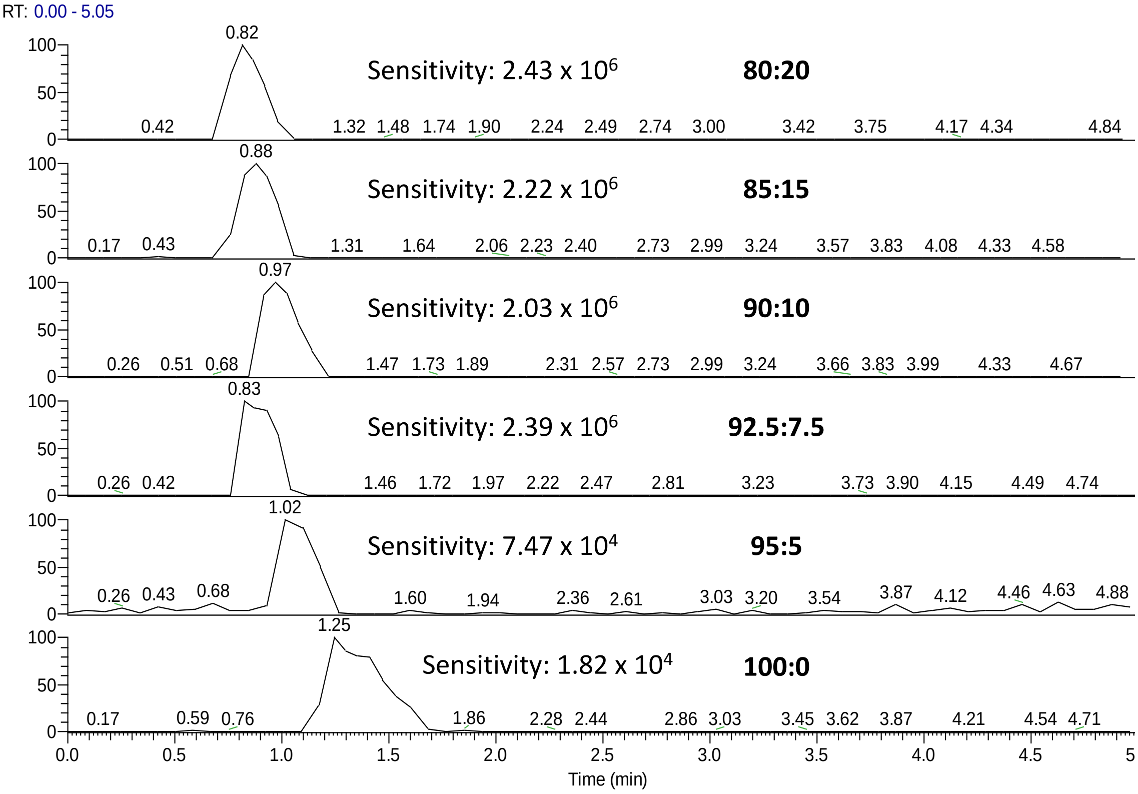


Figure 4.18. TICs obtained from 10 mg Sb(V) L^{-1} spiked R1 sample using ratios of formic acid 0.1% and methanol as mobile phase from 80:20 to 100:0.

A 10-cm length column was secondly assessed and the results obtained were compared to the obtained with the 3-cm length column. The spiked R1 was analysed and the mobile phase with the optimum ratio (90% of formic acid and 10% of methanol) was used. The TICs obtained are depicted in Figure 4.19. As expected, it can be observed that the retention time for the organic Sb peak is increased with the 10-cm length column.

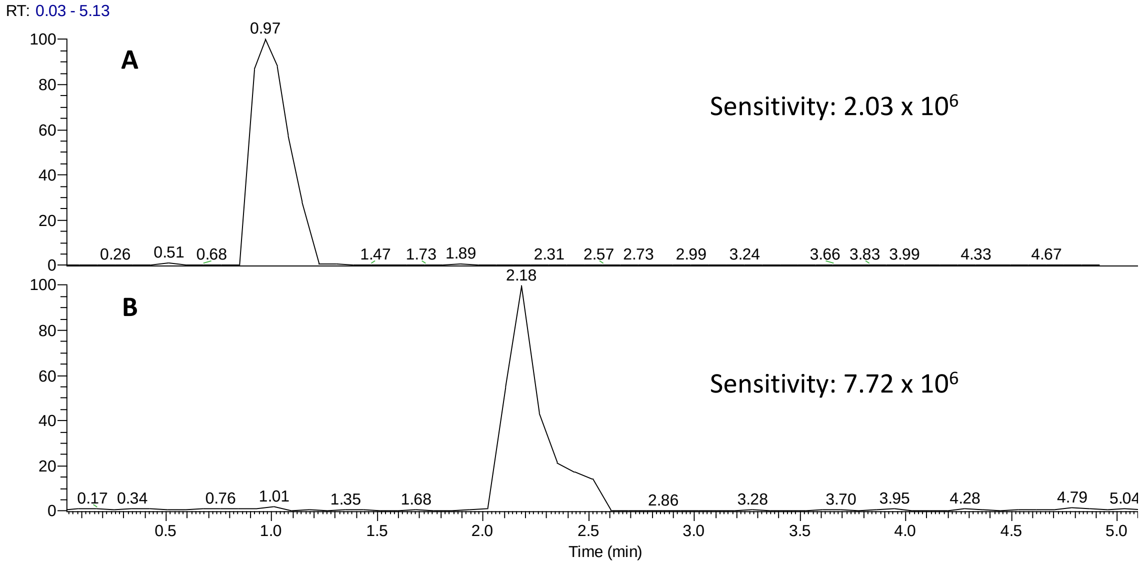


Figure 4.19. TICs obtained from 10 mg Sb(V) L^{-1} spiked R1 sample using as mobile phase formic acid 0.1% and methanol 90:10 and a column length of (A): 3-cm; (B) 10-cm.

Thus, the optimum conditions for the analysis of Sb by LC-ESI-MS consist of a separation with a mobile phase of formic acid 0.1% and methanol 90:10, a column length of 10 cm and an injection volume of 2 µL.

4.3.3.2. Structure characterisation

After the assessment of the optimum conditions for the determination of the organic Sb complex in *raki* spiked with Sb(V), the confirmation and identification of the corresponding Sb species was carried out with the study of the fragmentation of the complex by using the tandem mass spectrometry. First of all, the ion with a m/z 367 was selectively monitored using the mode “secondary ion mass spectrometry” (SIMS). By using this approach it was possible to obtain the Sb-containing mass spectra. The obtained fragments with the Sb isotopic distribution were selected and fragmented in a subsequent tandem mass spectrometry. The number of tandem performed to obtain the last fragment with the isotopic distribution of Sb (m/z 153) was 5. The mass spectrum of the fragment corresponds to an atom of antimony bounded to 2 oxygens. Figure 4.20 summarises the spectra obtained by tandem mass spectrometry.

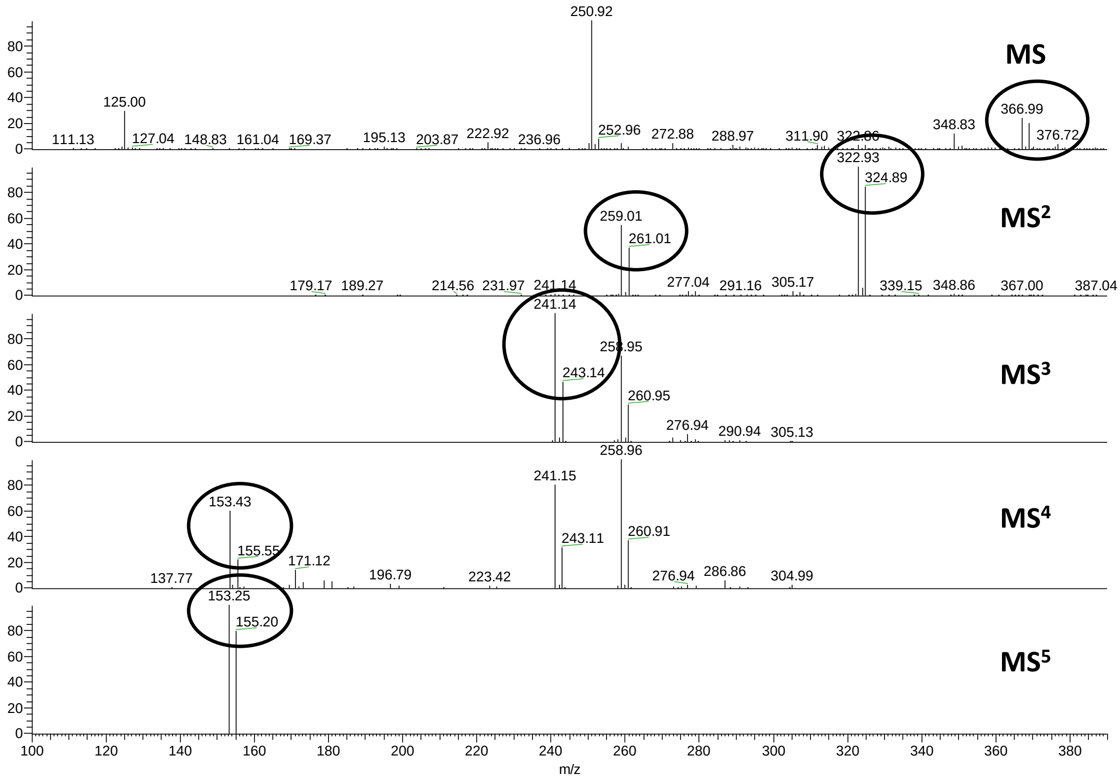


Figure 4.20. MS, MS^2 on m/z 367, MS^3 on m/z 259, MS^4 on m/z 241 and MS^5 on m/z 153 spectra of the organic Sb complex in *raki* R1 spiked sample with 10 mg L^{-1} of Sb(V). Collision energy for MS and MS^2: 20%. Collision energy for MS^3 and MS^4: 25%.

In MS^2, it can be observed that the complex with m/z 367-369 yielded the ions at m/z 323-325 and 259-261 with the isotopic distribution typical of the Sb. In MS^3, the fragment with m/z 323-325 yielded the ion at m/z 259-261 and the fragment with m/z 259-261 yielded the ion at m/z 241-243. In MS^4, the fragment with m/z 241-243 yielded the ion at m/z 153-155. It was not possible to yield any ion from this last Sb fragment in MS^5 at any collision energy. In the analysis by tandem mass spectrometry, it has been observed a mass loss of 108 in MS^2, from ion 367-369 to ion 259-261; and a mass loss of 88 in MS^4 from ion 241-243 to ion 153-155.

Sb(V) complexes may present a coordination number of 5 or 6. There is a study which characterised the products of the reaction of inorganic Sb(V) with citric acid by the use of various chromatographic methods [Hansen et al 2006a] and another study regarding the presence of Sb-citrate complexes in PET bottled juices by LC-ES-MS [Hansen et al 2006b]. In both works, the m/z of the Sb(V)-citrate complex is 361-363 and its structure is elucidated as $[Sb(citH)(OH)_3]^-$, whose structure is depicted in Figure 4.21A. This proposed complex could be five or six coordinated. Thus, the organic Sb(V) complex present in *raki*, assuming a similar structure, could also be five or six-coordinated. In this case, the most probable structure of the complex corresponds to the structure depicted in Figure 4.21B.

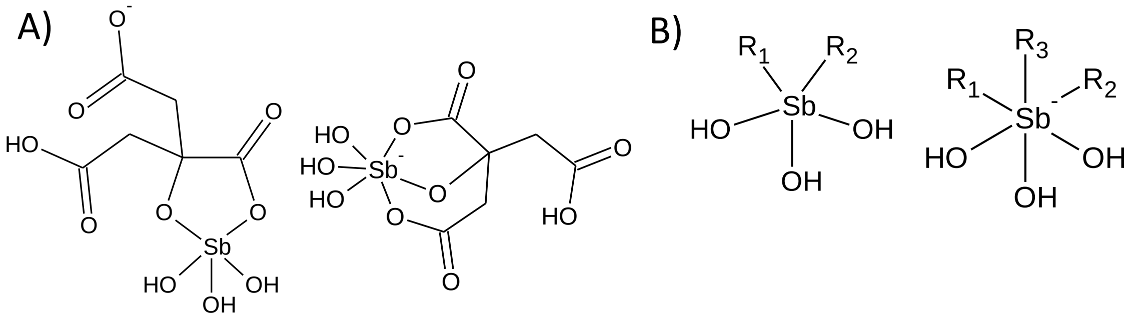


Figure 4.21. (A) Sb(V)-citrate complex and (B) Proposed structure of the Sb(V) complex present in *raki*.

After the fragmentation of the Sb complex, the mass losses were studied and compared to the possible compounds present in the *raki* matrix. As neither possible molecule among those found in the literature matched with the mass losses observed and it is really difficult to propose a structure without any molecular formula, it was decided to perform the tandem mass spectrometry by HRMS using a LTQ-ESI-Orbitrap to determine the exact mass of the compound and the fragments obtained (see section 2.3.3.2, Chapter 2). In this way, the elemental composition of the obtained ions can be estimated and subsequently determine the composition of the mass losses. This approach was not only applied for the fragmentation of the “m/z” 367, but also to the other antimony masses observed: 457 and 475.

The sample R1 was analysed by direct infusion. As this operation mode provides less sensitivity than the V-EASI-MS, used in the previous section, it was decided to spike the sample R1 at a concentration of 100 µg L^{-1}. Figure 4.22 depicts the full mass spectra obtained from the mentioned sample. It can be observed the same masses obtained before with low resolution (366.90710; 456.88419; 474.89456).

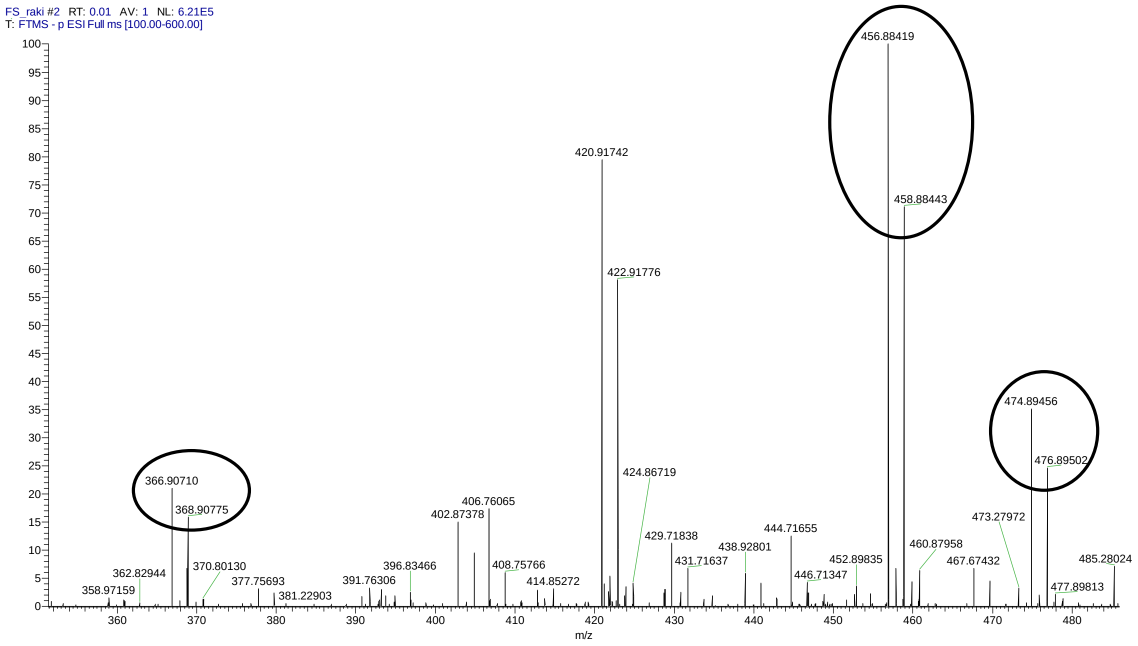


Figure 4.22. Enlargement of the full high resolution mass spectrum of *raki* sample R1, spiked with Sb(V).

After this, tandem mass spectrometry was carried out on the obtained masses to also obtain the exact “m/z” of all the fragments. For the m/z 366.90710, the same fragmentation route than the observed by low resolution was obtained. The m/z 456.88419 fragmented to the 366.90710 and to another one with the antimony isotopic distribution profile: 348.89706. The m/z 474.89456 fragmented to the 456.88419. Figure 4.23 depicts the most relevant tandem mass spectra obtained in this experiment.

These results demonstrate that the ions 366.90710 and 456.88419 were yielded from the precursor ion at m/z 474.89456 in spite of appearing in the full MS spectrum. According to this, the elemental composition of the ion 474.89456 and the fragments obtained was determined using the appropriate software (*Thermo Xcalibur Qual Browser*). Firstly, the program provided more than 10 possible molecular formulas for the target mases, with an error lower than 5 ppm. However, after analysing the obtained fragments in the different spectra, only two of the proposals were feasible: $C_7H_{14}O_{12}S_2Sb$ and $C_8H_{18}O_6S_4Sb$, with an error of 2.0 and 4.7 ppm, respectively. It is thought that the antimony complex present in *raki* corresponds to the first formula, as it present a lower error and less number of sulphurs. The isotopic cluster was simulated so as to verify the proposed formulas by the software, obtaining good agreement. Figure 4.24 depicts the real spectrum together with the simulated isotopic cluster.

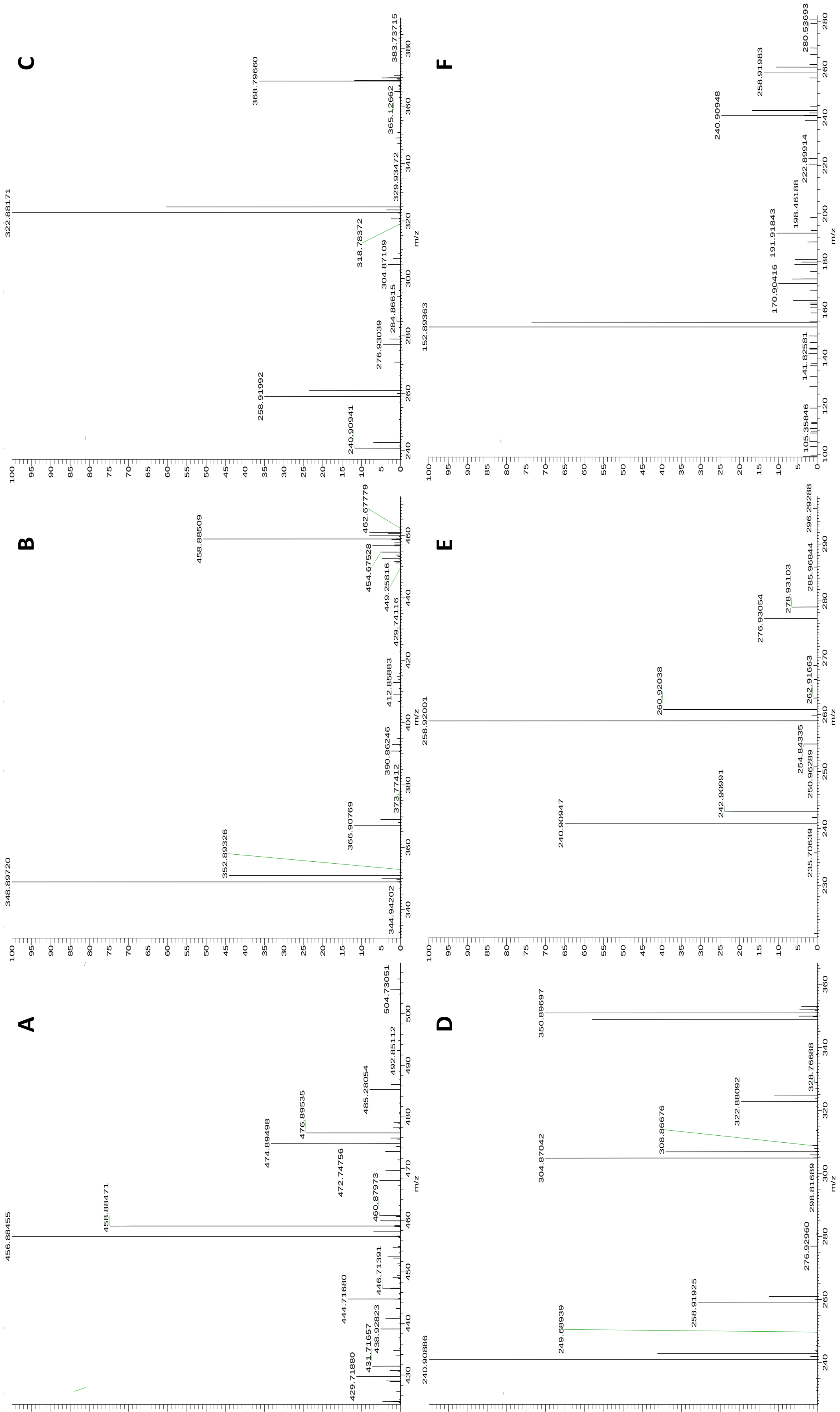


Figure 4.23. Tandem mass spectra of the target m/z. A: MS^2 on m/z 475; B: MS^3 on m/z 457; C: MS^4 on m/z 367; D: MS^4 on m/z 349; E: MS^5 on m/z 259; F: MS^5 on m/z 241.

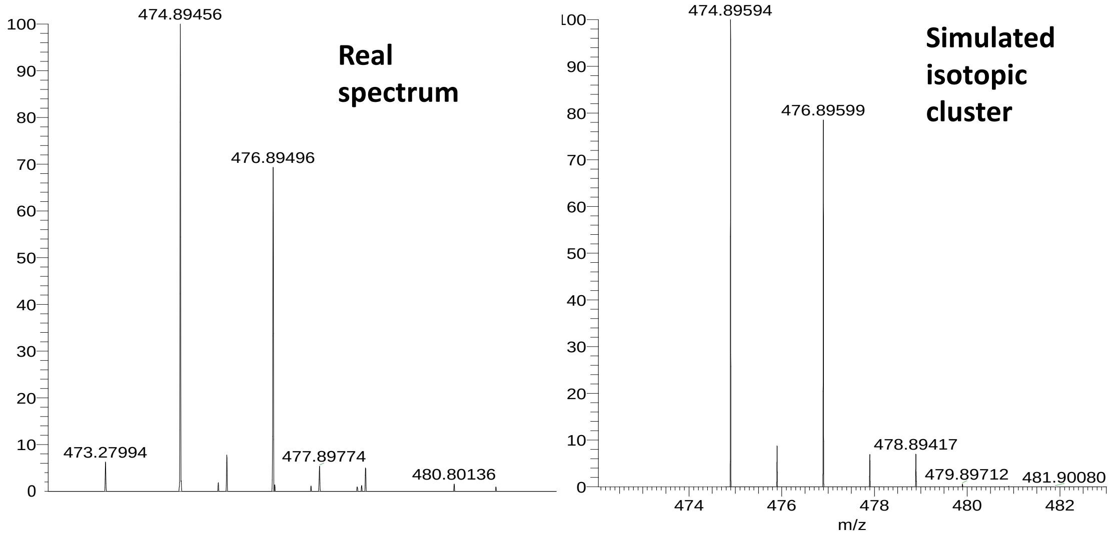


Figure 4.24. Enlargement of full MS of *raki* sample spiked with Sb(V) and simulated isotopic cluster.

After the obtention of the molecular formula, the assignment of the ions obtained in the full MS and the tandem MS was performed. Table 4.7 summarises the m/z obtained with the typical isotopic distribution of the antimony and the molecular formulas obtained, together with the corresponding product ions and losses.

It was observed that the most significant mass losses were 108 and 88 (see Figure 4.23). Checking the results obtained, these losses correspond to the following formulas: $C_2H_4O_3S$ and $C_3H_4O_3$. As described in the literature, sulphites are commonly used as an additive for several foodstuffs and beverages, such as grapes, wines or beer [García-Ruiz et al., 2013; Ramos et al., 2016]. Thus, sulphites may be added during the production of the *raki* and may have reacted with the ethanol present, forming the corresponding compound. A possible structure of this product is depicted in Figure 4.25A. The second molecular formula may correspond to pyruvic acid, a product from the alcoholic fermentation. In conclusion, according to these results, the unknown antimony compound present in *raki* with a mass of 475 may be the complex depicted in Figure 4.25B. Thus, according to this structure and the mass losses observed, a proposed fragmentation route, from the first ion (m/z 474.89456) to the last (m/z 152.89632) is depicted in Figure 4.26.

Table 4.7. Assignment of prominent ions observed for the Sb(V) unknown complex in 10 mg L^{-1} spiked *raki*

MS tandem	Molecular ion (m/z)	Interpretation	Product ion (m/z)	Assignment
2	474.89456-476.89502	$C_7H_{14}O_{12}S_2Sb$ $[Sb(C_2H_4O_3S)_2(C_3H_3O_3)(OH)_3]^-$	456.88419-458.88443	$[M - 18]^-$ $[M - H_2O]^-$
3	456.88419-458.88443	$C_7H_{12}O_{11}S_2Sb$ $[Sb(C_2H_4O_3S)(C_2H_3O_3S)(C_3H_3O_3)(OH)_2]^-$	366.90710-368.90755	$[M - 90]^-$ $[M – (C_2H_2O_2S)]^-$
			348.89706-350.89719	$[M - 108]^-$ $[M – (C_2H_4O_3S)]^-$
4	366.90710-368.90755	$C_5H_{10}O_9SSb$ $[Sb(C_2H_4O_3S)(C_3H_3O_3)(OH)_3]^-$	322.88165-324.88183	$[M - 44]^-$ $[M – (C_2H_4O)]^-$
			258.91977-260.92005	$[M - 108]^-$ $[M – (C_2H_4O_3S)]^-$
4	348.89706-350.89719	$C_5H_8O_8SSb$ $[Sb(C_2H_3O_3S)(C_3H_3O_3)(OH)_2]^-$	304.87091-306.87125	$[M - 44]^-$ $[M – (C_2H_4O)]^-$
			240.90912-242.90948	$[M - 108]^-$ $[M – (C_2H_4O_3S)]^-$
5	322.88165-324.88183	$C_3H_6O_8SSb$ $[Sb(O_2S)(C_3H_3O_3)(OH)_3]^-$	258.91977-260.92005	$[M - 64]^-$ $[M – (O_2S)]^-$
	258.91977-260.92005	$C_3H_6O_6Sb$ $[Sb(C_3H_3O_3)(OH)_3]^-$	240.90912-242.90948	$[M - 18]^-$ $[M - H_2O]^-$
	304.87091-306.87125	$C_3H_4O_7SSb$ $[Sb(O_2S)(C_3H_3O_3)(OH)(O)]^-$	240.90912-242.90948	$[M - 64]^-$ $[M – (O_2S)]^-$
	240.90912-242.90948	$C_3H_4O_5Sb$ $[Sb(C_3H_3O_3)(OH)(O)]^-$	152.89632-154.89395	$[M - 88]^-$ $[M – (C_3H_4O_3)]^-$
6	152.89632-154.89395	O_2Sb $[Sb(O)_2]^-$	-	-

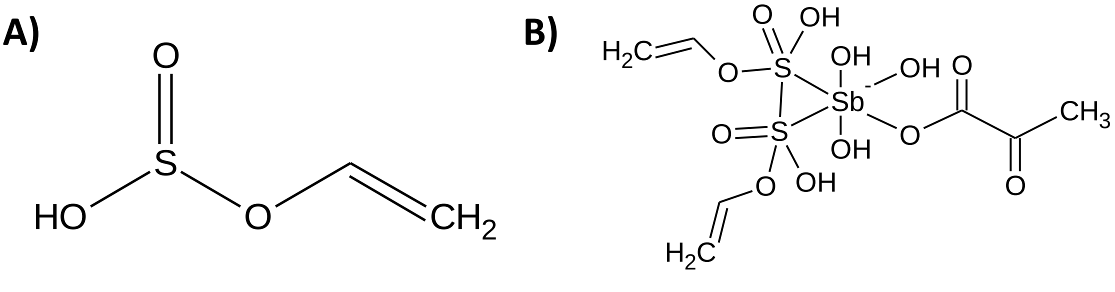


Figure 4.25. A: Possible reaction product formed from sulphites and ethanol. B: Proposed organometallic compound of Sb(V) present in *raki*, in which the reaction product bonds to the antimony with the sulphur atom.

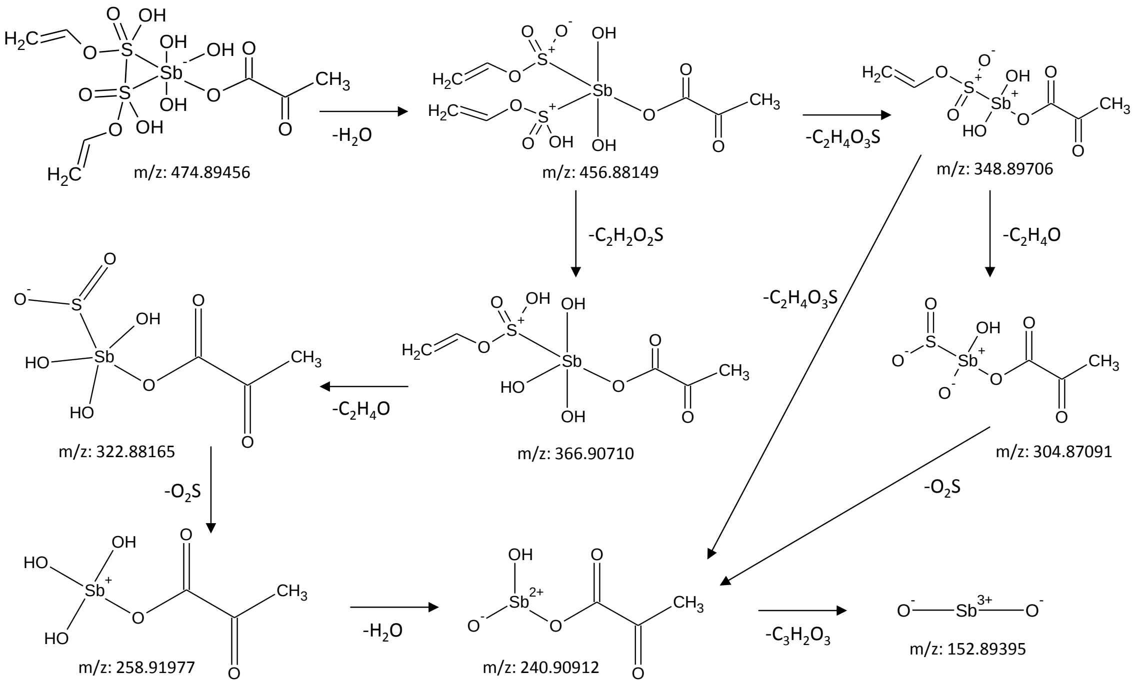


Figure 4.26. Proposed fragmentation route observed for the organometallic compound of Sb(V) present in *raki*.

4.4. DETERMINATION OF ANTIMONY IN PLASTIC

4.4.1. Digestion and extraction techniques

As mentioned in the Introduction, the presence of this element in the polymer is attributed to its use as a catalyst during PET manufacture. It can be added as glycolate or antimony trioxide. When it is added as Sb_2O_3, there is a reported mechanism in which it is readily converted to glycolate [Duh, 2002]. At the end of the manufacturing process, Sb remains bound in the polymer chain as antimony glycolate complexes [Duh, 2002; Takahashi et al., 2008; Welle et al., 2011]. Nevertheless, it is possible that some Sb_2O_3 remains unconverted [Sánchez-Martínez, et al., 2013]. However, although several works reported total Sb concentrations in PET relatively high, between 100 and 400 mg·kg^{-1}, the levels of Sb present in water and juice in contact with PET are much lower (around 1 µg L^{-1}, as seen in sections 4.1 and 4.2), and there is little information about the migration potential and mechanisms of different Sb chemical forms [Welle et al., 2011]. Thus, in this study, on the one hand, a digestion for the total determination of Sb in PET was carried out, whereas on the other hand, a comparison of the information provided by the leaching test described in the Directive 97/48/EC with another extraction procedure was assessed for the determination of extractable Sb.

First of all, for the determination of total Sb content in PET bottles a microwave digestion procedure proposed by *Milestone* was selected and the analysis of total antimony was carried out by HG-AFS using the procedure described in section 2.4.2.1 (Chapter 2). Six PET bottles used for the storage of mineral water and two PET bottles used for the storage of juice were analysed. The samples were purchased randomly from different Spanish local stores. PET bottles used for the storage of water samples are named according to the nomenclature used in section 4.1. PET bottles used for the storage of juice correspond to pineapple (J.Pnp) and peach flavour (J.Pch). Mineral water and juice was removed from the bottles and they were cut into square pieces of 1cm^2 before the digestion procedure and subsequent analysis.

For quality control purposes, total Sb was determined in the polyethylene CRM (ERM-EC681k; certified value: 99 ± 6 mg kg^{-1}). To our knowledge, there are no CRMs for antimony in PET and thus, polyethylene was considered the most similar material to PET available. The total Sb concentration value obtained was 101.4 ± 2 mg kg^{-1} with a RSD of 2%. No significance difference was found between certified and experimental total content (dependent t-test for paired samples at 95% confidence level). The results obtained for the tested samples are shown in Table 4.8.

Table 4.8. Total antimony concentrations in PET bottles (n = 3) after microwave digestion. Results are expressed as mean Sb value ± Standard Deviation, and the relative standard deviations are expressed in % in parentheses.

PET-type	Sb total (mg kg^{-1})	PET-type	Sb total (mg kg^{-1})
PET-Vd	239 ± 20 (8.5%)	PET-Ek	299 ± 5 (1.7%)
PET-FV	249 ± 3 (1.0%)	PET-Ea	294 ± 38 (13.0%)
PET-SCb	225 ± 24 (10.4%)	PET-J.Pnp	227 ± 8 (3.4%)
PET-AR	210 ± 9 (4.1%)	PET-J.Pch	215 ± 15 (6.8%)

As it can be seen, PET bottles contain between 210 and 299 mg kg^{-1} of total Sb. The Sb contents measured were comparable with those reported by the authors cited before (100–400 mg kg^{-1}).

Secondly, for the determination of extractable Sb from PET, the migration assays summarised in the Directive EU 97/48/EC were performed using the appropriate food simulant, time and temperature conditions. Specifically, according to the Directive, PET square pieces were put in contact with 3% acetic acid at 40ºC during 10 days [Directive 97/48/EC]. However, the Directive 97/48/CE does not specify the procedure to perform the migration assays, such as the way of contact with the extractant, shaking type and mass / volume ratio, which clearly have an influence on the obtained results. In this study, the procedure followed is described in section 2.4.2.3 (Chapter 2).

Additionally, another method was also assessed. The extraction of semimetals species in environmental and food matrices has been studied for the last years by the research group. Previous studies developed an extraction method for antimony speciation in fly ashes with aqueous solutions of hydrochloric acid at different concentrations by “end over end” stirring for 16 hours at 30 rpm in Teflon tubes at room temperature. The results obtained concluded that the highest extraction efficiencies were achieved with the 10 mmol L^{-1} HCl solution [Miravet et al., 2006]. Thus, the extraction of PET was also performed using this procedure, as described in section 2.4.2.2 (Chapter 2).

Both determinations of leachable Sb were performed on 2 of the PET bottles used for the storage of mineral water and the 2 PET bottles used for the storage of juice. Analysis of total Sb and species were determined by ICP-MS and LC-ICP-MS, respectively, and the results are summarised in Table 4.9. The speciation analysis was carried out using the conditions described in section 3.1.

Table 4.9. Antimony concentrations in PET bottles (n = 3). Results are expressed as mean Sb value ± Standard Deviation, and the relative standard deviations are expressed in % in parentheses.

PET type	Procedure	Extractable content		
		Sb total (µg kg^{-1})	Sb(V) (µg kg^{-1})	Sb(III) (µg kg^{-1})
PET-FV	MA*	18.0 ± 1.1 (6.1%)	18.3 ± 0.1 (0.5%)	< LD
	EA**	19.8 ± 1.2 (6.1%)	16.3 ± 1.7 (10.4%)	< LD
PET-SCb	MA	14.5 ± 0.7 (4.8%)	14.2 ± 1.0 (7.0%)	< LD
	EA	9.2 ± 0.9 (9.3%)	8.9 ± 1.0 (11.7%)	< LD
PET-J.Pnp	MA	303 ± 24 (8.0%)	286 ± 23 (7.9%)	< LD
	EA	394 ± 24 (6.1%)	360 ± 23 (6.3%)	< LD
PET-J.Pch	MA	68 ± 4 (5.2%)	82 ± 4 (5.3%)	< LD
	EA	259 ± 29 (11.3%)	238 ± 35 (14.8%)	< LD

*MA: Migration Assay according to the Directive EU 97/48/EC (Extracting agent: CH_3COOH 3%; 10 days).
**EA: End over end extraction (Extracting agent: HCl 10 mmol L^{-1}; 16 h).

From the values obtained, slightly lower antimony concentrations were obtained in most of the cases in the migration assays, although in comparison with those obtained in the HCl-extraction; the values were of the same order of magnitude, except for PET-J.Pch sample. This fact means that the 16-hour end over end extraction, which is an easy-to-use and user-friendly experiment, provides the same information than the migration assays performed at 40ºC for 10 days. In conclusion, the agitation procedure was selected as less time is required.

The extraction procedure using HCl was subsequently applied to the eight PET samples and the CRM aforementioned. The analysis of total leachable Sb and species were determined in all cases by HG-AFS and LC-ICP-MS, respectively. It should be taken into account that the speciation technique used is not LC-HG-AFS because it is not sensitive enough to detect or quantify antimony species because the concentrations in the extracts are below the limit of detection of the hyphenated technique. The LD and LQ values obtained for the extraction procedure were the following, expressed on PET sample:

LD Sb total HCl extraction = 2.29 µg kg^{-1}; LQ Sb total HCl extraction = 2.92 µg kg^{-1}; LD Sb (V) = 0.5 µg kg^{-1}; LQ Sb (V) = 1.6 µg kg^{-1}; LD Sb (III) = 2.5 µg kg^{-1}; LQ Sb (III) = 8.3 µg kg^{-1}.

The concentrations of extractable antimony (total and speciation) in the eight samples are shown in Table 4.10.

Table 4.10. Extractable antimony concentrations in PET bottles (n = 3) after end-over-end extraction. Results are expressed as mean Sb value ± Standard Deviation, and the relative standard deviations are expressed in % in parentheses.

Samples	Sb total (µg kg^{-1})	Sb(V) (µg kg^{-1})	Sb(III) (µg kg^{-1})
PET-Vd	21.1 ± 0.2 (1.1%)	19.5 ± 1.4 (7.4%)	< LD
PET-FV	19.3 ± 0.3 (1.4%)	17.6 ± 0.6 (3.6%)	< LD
PET-SCb	8.0 ± 0.4 (4.8%)	6.4 ± 0.5 (7.5%)	< LD
PET-AR	24.5 ± 2.8 (11.6%)	20.8 ± 1.3 (6.0%)	< LD
PET-Ek	10.2 ± 1.4 (13.8%)	9.4 ± 0.9 (9.3%)	< LD
PET-Ea	21.2 ± 0.2 (1.1%)	15.3 ± 0.8 (5.4%)	< LD
PET-J.Pnp	393.8 ± 24.1 (6.1%)	360.2 ± 22.6 (6.3%)	< LD
PET-J.Pch	258.7 ± 29.3 (11.3%)	237.9 ± 35.1 (14.8%)	< LD
ERM-EC681k	40.2 ± 1.3 (3.3%)	14.1 ± 0.3 (2.3%)	26.1 ± 0.9 (3.6%)

The total HCl-extractable Sb content, ranged from 8.0 to 24.5 µg kg^{-1} in PET bottles used for the storage of mineral water, whereas for those used for the storage of juices, concentrations ranged from 259 to 394 µg kg^{-1}. These differences may be attributed to the different types of PET used when manufacturing the bottles: as the PET bottles used for the storage of mineral water were rigid and monolayer, those used for the juices were less rigid and multilayer. Moreover, the shapes for each bottle, and consequently the surface / volume ratio, were different. This different design may be the cause of the more potential antimony leaching in the extraction of the PET plastic used for juice storage, as some Sb amounts may remain between layers, which could be easily removable.

It should be noted that Sb concentration in PET-water type bottles are below the SML stipulated by the European Commission in the Regulation 10/2011 when using food simulants (40 µg kg^{-1}), whereas values for PET used for juice storage exceeded this limit. Although this fact would mean that this plastic should not be used for food packaging, it should be noted that Sb concentrations in juices (see section 4.2) are far below the guidelines stipulated by the EU for drinking waters. These variations in Sb concentrations may be attributed to differences between the plastics used for each layer of the bottle: the inner layers which are in contact with foodstuffs may leach less Sb amounts whereas the outer layer seemed to present a different composition, which may be the main part that causes the Sb leaching.

Additionally, the CRM provided concentration values of 40.2 µg kg^{-1}. These values corresponded to 0.0035–0.034% of total Sb in PET, and 0.0396 % in polyethylene sample showing that only a small amount of the antimony present in PET bottles leached into the extractant solution, as indicated by other authors [Welle et al., 2011].

The speciation analysis of HCl extracts for PET samples revealed that Sb(V) was the only species present for every sample. This is consistent with the results of Takahashi et al., 2008, who concluded that, although antimony is added as a trivalent species during PET manufacture, it can be partially oxidized (up to 50%) to Sb(V). Total Sb leachable are of the same order of magnitude than the Sb(V) ones, which demonstrates the recovery of the species during the measure step. When performing HCl extraction on the polyethylene CRM, 65% of total Sb was present as Sb(III). This different behaviour can be attributed to the procedure described for the preparation of CRM batches, which is different from the manufacture of PET. In this case, Sb_2O_3 was added to the polyethylene powder once manufactured, together with other pigments which contained the rest of the certified elements. Thus, speciation behaviour of CRM cannot be compared with the PET samples analysed.

As can be seen from the results in Tables 4.8 and 4.10, total Sb content in PET was not directly correlated with the extractable content: sample with the lowest total Sb content (AR) showed the highest Sb leachability for the case of PET bottles used for water storage. Comparison of total Sb extractable content with the speciation analysis (i.e. total Sb versus Sb (V)) via a dependent Student's t-test for paired samples showed statistically significant differences at the 95% confidence level. This can be attributed to the small amounts of Sb species found in the measuring extracts, which were close to the limit of quantification where the widespread of expected results can be higher.

With the results obtained, it is not possible to know if the species observed in the extract are those present on the PET or if they correspond to an oxidation from Sb(III) forms to Sb(V) ones or to another reaction process which may occur during the extraction. Therefore, in order to assess the possible oxidation of Sb(III) during extraction procedure, leaching experiments with addition of 1 mL EDTA 10 mmol L^{-1} in the extractant solution (HCl 0.01 mol L^{-1}) were performed on one of the PET samples (FV) and the CRM, as this is a good complexing agent for Sb(III) species [De Gregori et al., 2005]. In these extracting conditions, 39% of total extracted Sb was present as Sb(III) in FV, whereas for the CRM, the proportion of Sb(III) increased up to a 95%. This fact evidenced that the potential Sb(III) form extracted from the PET can be easily oxidised to Sb(V). However, it cannot be guaranteed that the oxidation only occurs during the extraction procedure, as it could also be yielded during the PET production. Therefore, it would be interesting to investigate the structure or the form of antimony compound present in the raw PET material.

4.4.2. Surface techniques: Desorption Electrospray Ionisation (DESI)

4.4.2.1. State of the art

As mentioned in the previous sections, the results obtained cannot determinate which is the oxidation state and the structure of the antimony compounds present in the PET bottles because oxidation processes may occur during or after the extraction of antimony. Consequently, the use of an analytical method which does not transform the antimony compounds present in the PET structure would help in this aim.

Scarce information related to non-destructive techniques which can be used for the Sb determination in PET has been found. A useful tool for determining the chemical and structural arrangement of the various elemental components of a material with detection limits in the μg kg^{-1} range is the X-ray analysis. In this field, X-ray fluorescence (XRF) provides 2-D maps of elemental distribution while X-ray absorption near-edge structure (XANES) spectroscopy can be used to determine the oxidation state of individual elements [Martin et al., 2010]. Martin et al 2010 determined the Sb distribution in 10 x 10 μm clusters in a PET bottle sample using XRF and determined the oxidation state using XANES, concluding that the predominant Sb oxidation state present in PET is +3 and that a slight oxidation to +5 may have occurred. Takahashi et al., 2008 arrived to the same conclusion regarding the Sb oxidation state in PET, as explained in the previous section.

However, X-ray analysis does not allow the elucidation of the structure of the antimony present in PET. The use of a desorption electrospray ionisation (DESI) source combined with mass spectrometry could be explored. Takats et al., 2004, introduced this new ionisation technique in 2004. DESI is an excellent technique for performing in situ surface analysis in real-time, under atmospheric pressure, at room temperature, with low sample-consumption and without or minimal sample pretreatment [Chen et al., 2010b]. DESI coupled to mass spectrometry (DESI-MS) has been used in a wide variety of applications, including analyses of intact tissues [Laskin et al., 2012, Gerbig et al., 2012], foods [Nielen et al., 2011], drugs [Thunig et al., 2012], pharmaceuticals and personal care products [Fabrizi et al., 2012; Campbell et al., 2012], and forensic applications [Morelato et al., 2012]. Recently and extensive review on fundamentals and applications of this technique has been published [Wiley et al. 2015]. However, studies that employ these techniques for elemental speciation are yet scarce. Lin et al., 2010 demonstrated that DESI-MS can be employed to detect arsenic species in plant leaves and vegetables and more recently de Abreu et al., 2013 applied to the speciation of arsenic in fern leaves. For antimony speciation there is not any work published and the performance of the technique has been explored in this work.

4.4.2.2. Preliminary studies on Sb determination in PET by DESI-HRMS

For this study, two PET bottles were selected among those analysed in the previous section: PET bottles from SCb sample (mineral waters), and from pineapple sample (juice). The PET bottle SCb is blue coloured whereas the pineapple one is colourless. PET bottles were cut into pieces of 1 cm^2. First of all, analyses by DESI-HRMS were performed focusing the source directly to the inner part of the plastic, which is the zone in contact with the beverage. A mixture of methanol and water 80:20 was used as solvent and the parameters described in section 2.3.4 were applied, acquiring both in positive and negative mode. In the results obtained, no difference was observed between the spectrum profile obtained from the plastic and blank (focusing the source on the PTFE spots from the surface of the slides described in section 2.3.4). A mixture of acetonitrile and water 80:20 was also tested without any changes in the results. This fact means that no molecules from the PET are desorbed in the conditions applied. As PET is a rigid plastic and DESI is not an aggressive ambient technique, the desorption step may be difficult to be produced.

In view of these facts, in order to ease the desorption, the following attempts were assessed to deform the plastic without destroying its structure: high temperature, a droplet of sulphuric acid (both concentrated and 1:1 diluted), and a droplet of acetone. The high temperature was applied with a heat gun and the droplets of the tested solutions were placed with a glass capillary. When applying the high temperature, the plastic turned darkened and when applying the acetone, no differences on the plastic surface were eye-observed. On the other hand, when applying the sulphuric acid droplets, the PET surface was attacked turning white and rough. No differences were observed between the spectra obtained from the blank and from the sample regardless of the treatment, which could mean that these sample treatments did not ease the antimony desorption from the plastic surface. Taking into account these facts, it was opted to look for an organic solvent which could dissolve totally or partially the PET.

A bibliographic research in the literature was carried out to find an organic solvent for the PET dissolution. The following organic solvents were found to be able to dissolve this plastic: 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), a mixture of dichloromethane with small amounts of 1,4-dioxane, o-cresol and dimethyl sulfoxide. With this information, it was decided to test these solvents on both types of plastic together with other common organic solvents which are summarised in Table 4.11. The effects observed are included in the last column.

Table 4.11. Physical changes observed in PET samples after contact with organic solvents.

Organic solvent	LogP*	Physical changes	Observations
Dimethyl sulfoxide	-1.412	NO	-
Methanol	-0.690	NO	-
Acetonitrile	-0.334	NO	-
Acetone	-0.042	NO	-
Tetrahydrofuran	0.473	YES	Partial colour loss for SCb
Ethyl acetate	0.785	NO	-
Diethyl ether	1.041	NO	-
Dichloromethane	1.405	YES	Colour loss for SCb
Dichloromethane + 1,4-dioxane 80:20	1.405 -0.275	YES	Colour loss for SCb
HFIP	1.567	YES	Total dissolving of PET
Nitrobenzene	1.921	NO	-
Chloroform	1.935	YES	PET deformation and colour loss for SCb
o-Cresol	1.962	NO	-
Anisole	2.170	YES	Partial colour loss for SCb
Toluene	2.720	NO	-
Chlorobenzene	2.843	YES	Partial colour loss for SCb
Carbon tetrachloride	2.919	NO	-
Cyclohexane	3.163	NO	-
Hexane	3.764	NO	-
Isooctane	4.373	NO	-

*Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2016 ACD/Labs)

The most significant effects observed where the following: the use of anisole, chlorobenzene and tetrahydrofuran led to a partial loss of the blue colour in the SCb sample after 24 h of contact. The use of dichloromethane and the mixture of dichloromethane and 1,4-dioxane 80:20 led to the total loss of the colour after 24 hours of contact and with the use of chloroform, PET colour was lost after 4 hours and it was also slightly twisted. The HFIP solvent was the only one which caused the total dissolution of PET, in approximately 10 minutes. With the rest of the organic solvents, no physical differences were observed on the PET samples. Thus, according to these results, it was decided to dissolve the PET in the HFIP.

For DESI-MS analyses, taking into account that HFIP is corrosive and could react with some parts of the whole instrument, specifically with the PEEK connections, it was decided to evaporate the solvent and reconstitute with chloroform ($CHCl_3$), as it was the organic solvent which provided the second best results in the previous test. For this procedure, 0.1g of PET were dissolved in 200 µL of HFIP. Afterwards, the solvent was evaporated with nitrogen and the sample was reconstituted with 100 µL of $CHCl_3$. Sub-samples were deposited on the PTFE printed spots from the glass slides and analyses were performed as described in section 2.3.4.

Different spectra profiles between blank and sample were obtained both in positive and negative mode. Some masses which were not present in the blank one were detected with a good sensitivity in the sample spectra. As an example, Figures 4.27 and 4.28 depicts the profiles obtained from the TIC during the emitter acquisition scan, where sample and blank positions were measured consecutively, together with the mass spectra obtained for the SCb sample (dissolved in HFIP, evaporated and reconstituted with $CHCl_3$) both in positive and negative mode, respectively. For the ions observed in the sample spectra with an intensity higher than 10^3, a mass filter was applied so as to check if their presence in the spectrum corresponds to the sample or they are part from the background signal. With this filter, the profiles from the ions which are not present in the sample are similar to the TIC, as they are continuous, whereas those corresponding to ions present in the sample are different. To be more concise, these profiles represent the distribution of the spots, as the relative abundance increases when the emitter passes onto the zone where the sample was deposited. As an example, mass filters for two ions present in the sample and for other two ions corresponding to the blank are depicted in the left part of figures 4.27 and 4.28 (positive and negative mode, respectively). It can be eye observed the discontinuous profile for the ions yielded from the PET sub-sample (791.15820 and 599.11557 for positive mode; 951.57359 and 265.14756 for negative mode) and the continuous ones for the ions which were not from the sample (1199.77748 and 791.15820 for positive mode; 726.90363 and 822.88445 for negative mode).

In addition, for comparison purposes, a 1 cm^2 PET sub-sample with a droplet of HFIP was also analysed by DESI-HRMS. A mixture of methanol and water 80:20 was used as solvent, acquiring both in positive and negative mode. Besides, the use of formic acid in positive mode and ammonia in negative mode on the solvent was tested and led to the increase of the sensitivity of some of the ions obtained. Mass filters were applied in all cases and the target m/z obtained for the samples in both modes is summarised in Table 4.12. It should be noted that less sensitivity was obtained in the analysis of the PET with the HFIP droplet in negative mode. Even some target m/z observed in the dissolved samples were not detected when analysing the direct plastic. This fact demonstrates the difficulty of the desorption from the direct plastic, even if a droplet of a potential solvent is used.

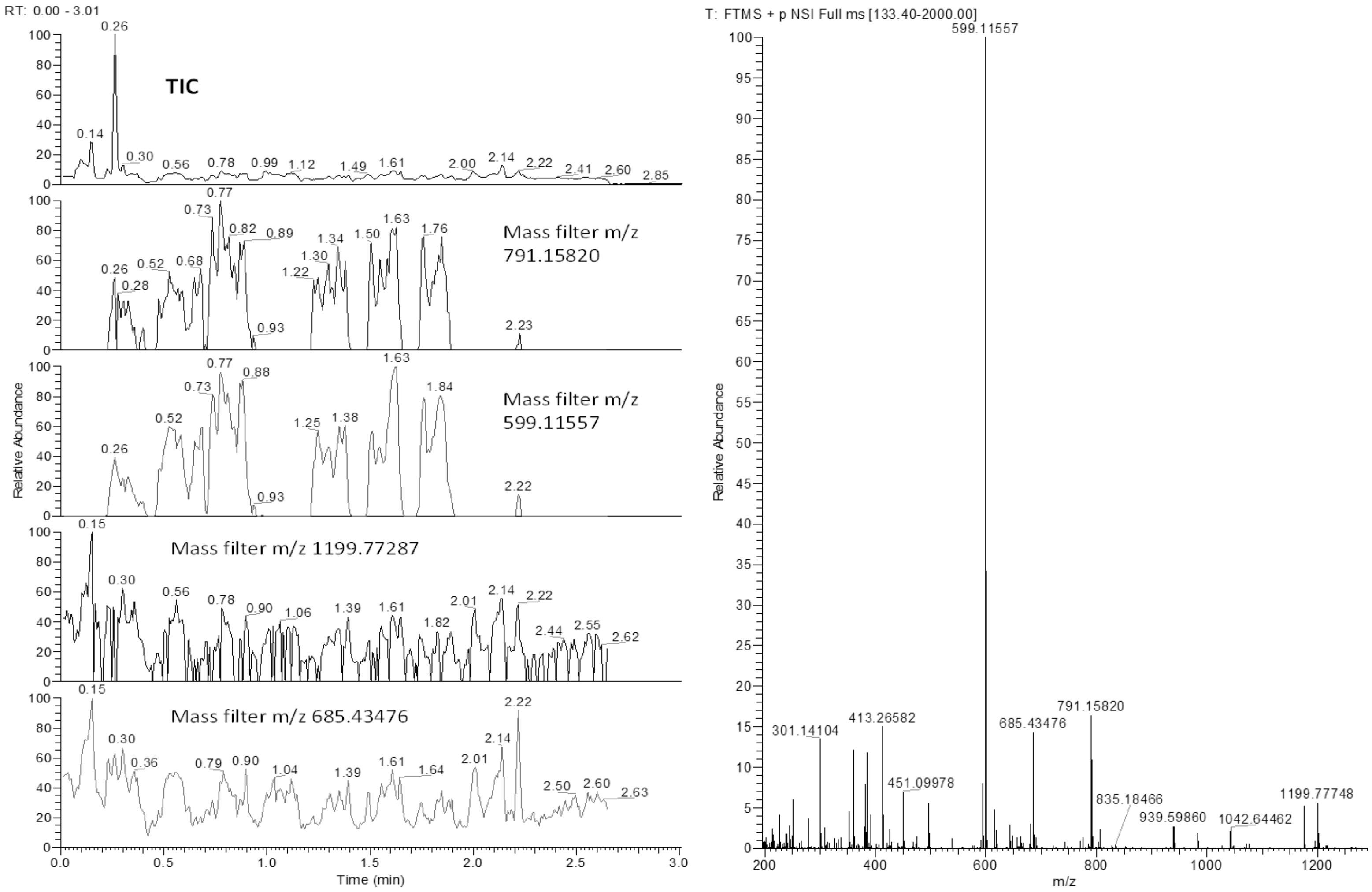


Figure 4.27. TIC profile and mass filters obtained from the analysis of SCb sample dissolved in HFIP, evaporated and reconstituted with $CHCl_3$, together with the mass spectrum obtained from the sample in **positive** mode.

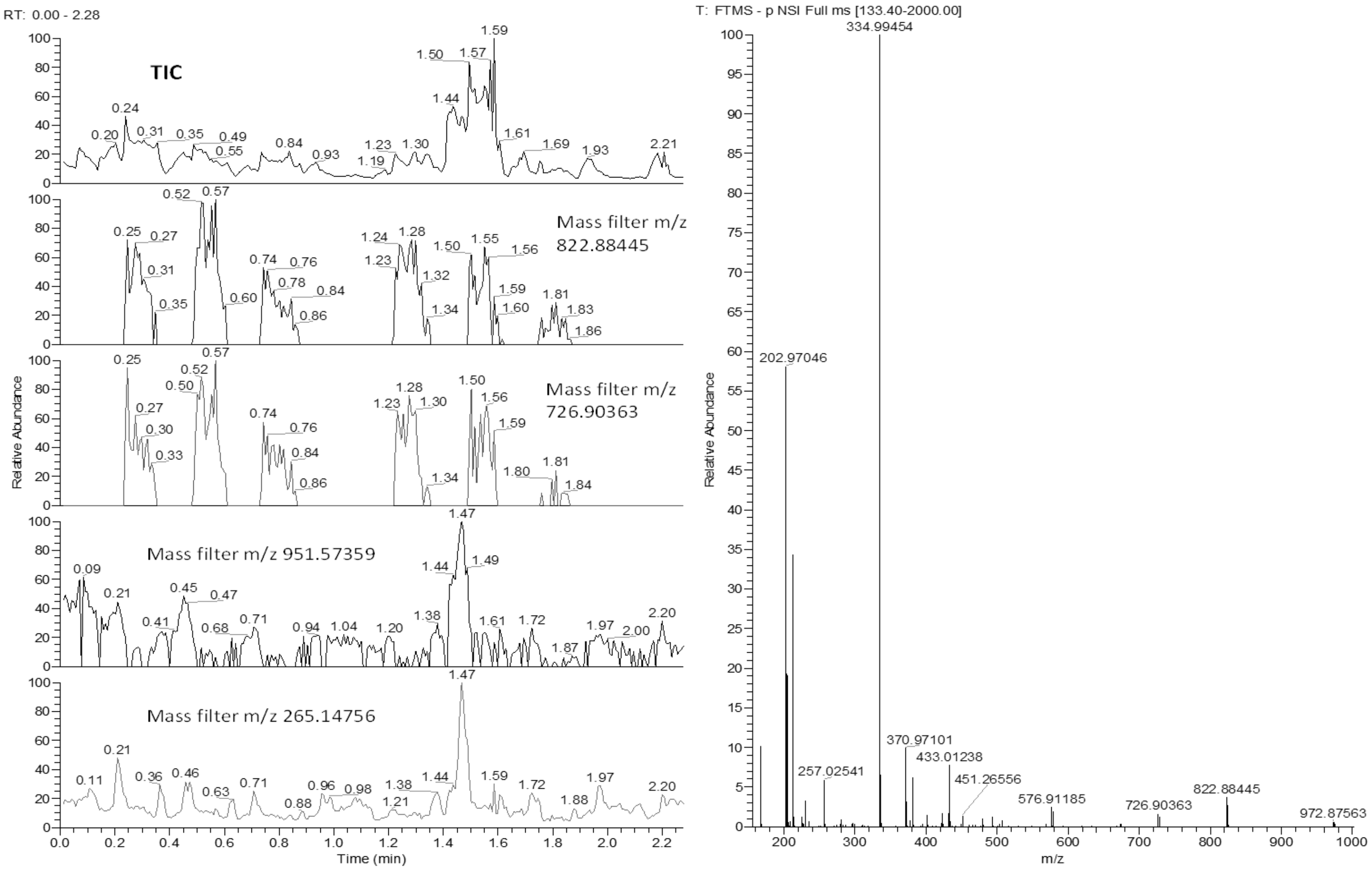


Figure 4.28. TIC profile and mass filters obtained from the analysis of SCb sample dissolved in HFIP, evaporated and reconstituted with $CHCl_3$, together with the mass spectrum obtained from the sample in **negative** mode.

Table 4.12. Target m/z obtained from PET samples in both positive and negative mode.

Positive mode				
m/z	Spot: dissolved plastic		Bare plastic: PET + HFIP droplet	
	MeOH:H_2O 80:20	MeOH:H_2O 80:20 + HCOOH	MeOH:H_2O 80:20	MeOH:H_2O 80:20 + HCOOH
1175.24061	5.14 x 10^3	4.12 x 10^3	2.94 x 10^4	6.67 x 10^3
983.19691	1.66 x 10^3	1.99 x 10^3	8.70 x 10^3	2.10 x 10^3
791.15631	9.21 x 10^3	1.08 x 10^4	3.97 x 10^4	1.09 x 10^4
667.10334	1.09 x 10^3	1.11 x 10^4	1.54 x 10^4	1.03 x 10^4
599.11495	5.77 x 10^4	6.56 x 10^4	4.19 x 10^5	8.14 x 10^4
497.22074	4.64 x 10^5	3.85 x 10^3	6.54 x 10^3	5.26 x 10^3
227.15730	4.25 x 10^5	3.34 x 10^3	5.26 x 10^5	7.43 x 10^4
Negative mode				
m/z	Spot: dissolved plastic		Bare plastic: PET + HFIP droplet	
	MeOH:H_2O 80:20	MeOH:H_2O 80:20 + NH_3	MeOH:H_2O 80:20	MeOH:H_2O 80:20 + NH_3
972.87519	1.59 x 10^3	-	-	-
822.88263	1.41 x 10^4	6.78 x 10^3	-	-
726.90342	3.73 x 10^3	6.00 x 10^3	-	1.09 x 10^4
672.89290	1.84 x 10^3	2.80 x 10^3	-	-
576.91238	4.21 x 10^3	1.64 x 10^4	-	4.35 x 10^4
507.20157	3.69 x 10^3	-	-	-
493.18459	5.64 x 10^3	-	-	-
479.16813	3.83 x 10^3	-	-	-
461.17863	1.53 x 10^3	-	-	-
433.01170	1.84 x 10^4	5.90 x 10^4	-	1.15 x 10^6
334.99411	1.60 x 10^5	-	-	7.39 x 10^6
296.93565	3.54 x 10^3	7.53 x 10^3	-	2.41 x 10^4
202.97051	4.73 x 10^5	-	-	3.83 x 10^5

However, the masses obtained in this experiment did not correspond to a compound with antimony, as the typical isotopic cluster profile of this element was not observed. Therefore, the masses observed may be related to the PET polymer or to other compound that may form part of the bottle.

Consequently, further research should be performed so as to desorb and detect the antimony present in the PET. One approach which could be applied for the determination and characterisation of antimony in PET is the Direct Analysis on Real Time (DART). It is an ionisation technique which uses a heated and metastable gas, mainly helium, to desorb ions from a surface under ambient conditions. This technique has demonstrated success in the determination of hundreds of chemicals in many different surfaces, such as asphalt, human skin, currency, airline boarding passes, business cards, fruits, vegetables, spices, beverages, body fluids, horticultural leaves, cocktail glasses, and clothing [Cody et al., 2005].

4.5. REMARKS

The developed and optimised methods described in the previous section were applied for the analysis of total content and speciation of antimony in PET-bottled beverages, comprising mineral waters, juices, spirits and the PET container; and mercury in seafood, comprising fish, crustaceans and bivalves. Table 4.13 summarises the Sb concentration ranges obtained for the matrices described.

Table 4.13. Total Sb and species concentrations obtained in the studied samples. All values are given in µg L^{-1}, except the total content in plastic, which is given in mg kg^{-1} (marked with asterisk).

<table>
<tr><th>Sample</th><th colspan="2">Total content</th><th>Non-complexed Sb(V)</th><th>Unknown Sb(V)</th><th>Sb(III)</th><th>Recovery (%)</th></tr>
<tr><td>Mineral water</td><td colspan="2">0.07 - 1.05</td><td>0.14 - 1.12</td><td>< LD</td><td>< LD</td><td>90 – 110</td></tr>
<tr><td>Juice</td><td colspan="2">0.17 - 0.94</td><td>< LQ</td><td>< LD</td><td>0.12 - 0.42</td><td>44 – 89</td></tr>
<tr><td><i>Raki Tsipouro</i></td><td colspan="2">0.38 - 3.90</td><td>< LD - 0.19</td><td>0.73 - 3.21</td><td>< LD - 1.05</td><td>62 – 110</td></tr>
<tr><th rowspan="2">Sample</th><th rowspan="2">Total content*</th><th colspan="4">Extractable content</th><th rowspan="2">Recovery (%)</th></tr>
<tr><th>Total Sb</th><th colspan="2">non-complexed Sb(V)</th><th>Sb(III)</th></tr>
<tr><td>Plastic</td><td>210 - 299</td><td>water: 8.0 - 24.5
juice: 258.7 - 393.8</td><td colspan="2">water: 6.4 - 20.8
juice: 237.9 - 360.2</td><td>< LD</td><td>72 – 93</td></tr>
</table>

From the results obtained, it can be observed that total Sb concentration in mineral waters and juices are similar (up to 1 µg kg^{-1}), whereas the obtained for spirits is slightly higher (up to 4 µg kg^{-1}). This difference could be partially related to the matrix because of its high ethanol content. Indeed, ethanol is used as a food simulant for alcoholic foodstuffs to perform the migration test stipulated in the EU Directive 97/48/EC. Spirits present a high content of fusel alcohols, volatile molecules and sugar, which could ease the migration of Sb from the PET matrix. Besides, the historical background of the samples and the PET bottles should could also be potentially related to the migration, as it is not known how long samples were stored in the PET bottles neither the bottle aging or characteristics, before analyses.

When comparing the results obtained between beverages and plastic, total Sb content in plastic is much higher (in the order of mg kg^{-1}) than Sb content in beverages, and also than Sb extractable content (in the order of µg kg^{-1}). These values show that only a small part of the antimony present in plastic bottles is extracted or migrated. As mentioned in section 4.4.1, Sb might be bounded to the polymer, which is not easy to break. This could be the reason why only small amounts are extracted or migrated. In addition, it could be also observed that Sb extractable content from plastic is approximately one or two orders of magnitude higher than the Sb concentrations obtained in the beverages. It is thought that the extraction experiment might simulate the whole Sb possibly removable from PET bottles to the beverage exposed to a very long time. As mentioned in the introduction, some authors found evidences that elevated temperatures accelerate antimony migration. Thus, further studies about antimony migration with time and temperature in the matrices studied were developed.

Regarding the speciation results, different species were observed depending on the matrix. Non-complexed Sb(V) was the only species observed in mineral water and HCl PET extracts, whereas Sb(III) species was the predominant in juices. These differences may be due to either the form how Sb is extracted (as pentavalent or trivalent) or oxidation processes occurred during or after the extraction or migration. In the case of spirits, the main species was an unknown complex of Sb(V) and small amounts of non-complexed Sb(V) and Sb(III) were observed in some cases. In this way, simultaneous oxidation of the trivalent species and formation of the unknown complex of Sb(V) with some of the components from the spirit matrix may have happened during or after migration.

Although antimony is added in the trivalent form in the manufacture of PET, Takahashi et al., 2008 reported that it can be partially oxidised to Sb(V). According to this, antimony in PET should be mainly as Sb(III) rather than Sb(V), and these are the forms that must be observed in the extracts or the studied matrices if no more processes occurred. However, Sb(III) can be easily oxidised in oxygenated media. Thus, in mineral water and HCl extracts, an oxidation process from Sb(III) to Sb(V) may have occurred whereas in juice matrices, Sb(III) was prevented from oxidising because of the organic acids present in the matrix, which easily stabilised Sb(III). These facts were proved by adding EDTA to the solution used for the extraction of Sb in plastics before the extraction on one of the samples. In this case, 39% of total extracted Sb was present as Sb(III).

Regarding the recoveries obtained for every matrix, the best recoveries were obtained for mineral water samples, without significant difference between the sum of species and the total value. For the other matrixes, slightly lower recoveries were obtained in some cases, which may be due to either the quantification of signals close to the limit of quantification or matrix effects which could affect to the signal sensibility.

Comparing the antimony concentrations with the maximum level established by the European Union for drinking waters (5 µg L^{-1}), any beverage presented values above this limit. However, it should be noted that 3 *raki* sampled presented concentrations slightly lower than 4 µg L^{-1}, which are closer to the limit.

4.6. DETERMINATION OF MERCURY IN SEAFOOD

There is an interest for the determination of mercury species in biological samples due to the bioaccumulation and the biomethylation of Hg by the biota, which leads to the presence of considerable amounts of organic mercury, specifically methylmercury in their tissues. As mentioned in the Introduction, seafood can be considered as one of the main dietary source of mercury due to these processes, which makes them targets of great interest.

In the framework of a collaboration with the “Instituto de Química” from the “Universidade Federal do Rio Grande do Sul”, came up the possibility of applying the speciation techniques developed in the research group in seafood. Thus, the goal of this study was to determine total Hg and Hg species in 24 seafood samples comprising fish, crustaceans and bivalves. The study focused on the extraction, identification and accurate quantification of $MeHg^+$, the most toxic form, which was selectively separated and determined by LC-UV-CV-AFS, using the analytical methodology previously established for the analysis of water samples, described in section 2.3.2.1. Sample preparation was optimized to be as simple as possible.

4.6.1. Seafood samples

Five fresh fish muscle samples were provided by the Laboratory of Trace Metals and Contaminants (LANAGRO/RS) of the Ministry of Agriculture, Livestock and Supply (MAPA/Brazil). These samples were initially washed with Milli-Q water, cut and then lyophilized for a period of 5 hours. They were then ground in a vibratory mill and sieved through 85 µm polyester mesh to improve the homogeneity. Eleven fish samples and a clam sample were supplied by the Public Health Agency Laboratory, Barcelona (ASPB, Barcelona, Spain). Three crustacean samples and four bivalve samples were purchased from local supermarkets in Barcelona, Spain, during 2013. All samples were washed with Milli-Q water, cut and homogenized using a blender. After homogenization, samples were stored in the refrigerator at 4–10°C until analysis.

The moisture of samples was determined using the procedure described in section 2.4.3.3 (Chapter 2). The average value was 5% for lyophilized samples. The value for fish fresh samples was around 30% whereas for crustaceans and bivalves ranged from 68% to 89%.

4.6.2. Determination of total mercury

4.6.2.1. Quality parameters

First of all, the trueness of the method for total Hg determination was assessed. The following CRMs were used to verify this parameter and guaranteed the quality control of acid digestion in the sample pretreatment: DOLT-4 (Dogfish; total Hg certified value: 2.58 ± 0.22 mg kg^{-1}), TORT-2 (Lobster Hepatopancreas; total Hg certified value: 0.27 ± 0.06 mg kg^{-1}) and BCR-463 (Tuna fish; total Hg certified value: 2.85 ± 0.16 mg kg^{-1}).

The analysis of total Hg in the CRMs aforementioned was performed by ICP-MS with a previous digestion procedure described in section 2.4.3.1 (Chapter 2). The instrument detection and quantification limits were calculated and expressed on sample by multiplying by the extraction dilution factor. The results obtained were 1 µg Hg kg^{-1} for LD and 3 µg Hg kg^{-1} for LQ. The values of total Hg concentration obtained were 2.68 ± 0.11 mg kg^{-1} for DOLT-4, 0.30 ± 0.02 mg kg^{-1} for TORT-2; 2.86 ± 0.15 mg kg^{-1} for BCR-463. According to the Student's t-test, no significant difference at a 95% confidence level was found between the experimental and certified values. Repeatability was checked by analysis of CRMs (different replicates) 6 times throughout the day. The RSD obtained were: 7% for TORT-2, 4% for DOLT-4 and 5 % for BCR-463.

4.6.2.2. Analysis of seafood samples

Total Hg concentration was determined in the 24 seafood samples aforementioned. They were classified as fish (n=16), crustaceans (n=3) and bivalves (n=5). The values found for total Hg in the seafood samples are depicted in Figure 4.29. Total Hg concentration ranged from 0.007–2.33 mg kg^{-1}, with the crustaceans and bivalves showing less total Hg than fish samples. Three of the shellfish samples showed concentrations lower than the LQ, marked with asterisk on the graphic. Comparison of total Hg concentration means showed that crustaceans and bivalves had 0.07 mg kg^{-1} in dry mass and 0.012 mg kg^{-1} in wet mass, while fish had a mean of 0.71 mg kg^{-1} in dry mass and 0.59 mg kg^{-1} in wet mass. These results are consistent with the literature [Krystek et al., 2006; Batista et al., 2011; Clemens et al., 2011]. According to Krystek et al., 2006, significant differences in Hg levels are found in different seafood species analysed. Fish at high trophic levels in the food chain, like large predatory fish, accumulate more Hg and contain significantly higher concentration levels. Two predatory Brazilian fish samples (red porgy-1 and red porgy-2) and two predatory Spanish fish samples (tuna-3 and swordfish-1) showed the highest levels of total Hg: 1.63 ± 0.04 mg kg^{-1} (red porgy-1), 1.15 ± 0.01 mg kg^{-1} (red porgy-2), 2.33 ± 0.03 mg kg^{-1} (tuna-3) and 1.04 ± 0.03 mg kg^{-1} (swordfish-1).

The Brazilian government, through MAPA [PNCRC, 2009; Damin et al., 2013], and the European Union, through the Regulation 1881/2006 established a reference value of 0.5 mg kg^{-1} for total Hg in fish farming and 1 mg kg^{-1} for predator fish. Two of the five Brazilian samples (red porgy-1 and red porgy-2) and two of the Spanish samples (tuna-3 and swordfish-1), which correspond to predatory fish, presented values higher than the recommended ones by the institutions. These data demonstrate the need to carry out speciation in these samples to discern the more toxic species.

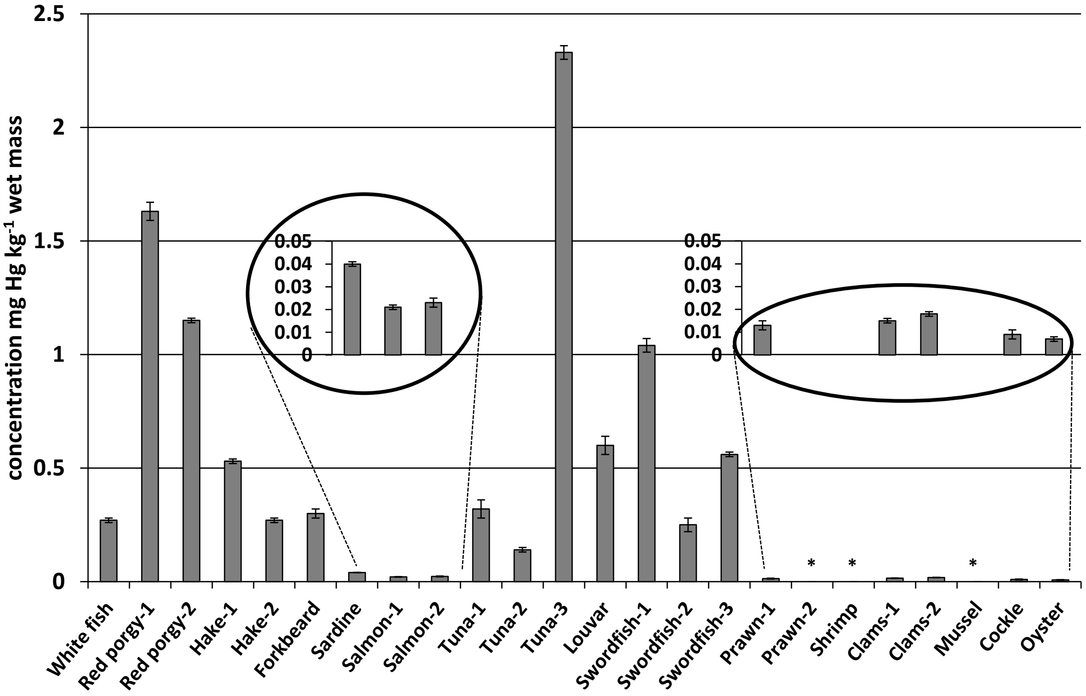


Figure 4.29. Total mercury in seafood samples, concentrations are expressed as mg Hg kg^{-1} wet mass (* < LQ).

4.6.3. Determination of mercury species

4.6.3.1. State of the art

The determination of mercury species in seafood has been carried out during the last years by several strategies. As there is a wide number of works about this topic, a bibliographic research was performed from the works published from 2009 onwards. They mainly focused on the extraction of the mercury species, specifically $MeHg^+$ species, followed by chromatographic separation and detection. Table 4.14 summarises the works published by other authors. It can be observed that the mercury extraction and determination is focused mainly on fish samples, as only 3 works dealt with shellfish samples [Batista et al., 2011; Clémens et al., 2011; Zhang et al., 2012]. Stirring, ultrasonication, water bath, wet digestion and microwave assisted extraction were performed for Hg extraction. These procedures consist of alkaline or acidic extractions. The alkaline extractions were performed using potassium hydroxide or tetramethyl ammonium hydroxide together with an organic solvent or a salt, in some cases, whereas acidic extractions were mainly performed using hydrochloric acid, also with salts or organic solvents in some cases, although some works reported extractions with nitric or sulphuric acid.

Table 4.14 also summarised methylmercury concentrations in the samples analysed together with the percentage with respect to the total mercury concentration. It can be observed that, in spite of being reported a wide range of proportions, the percentage of $MeHg^+$ tends to be high.

Table 4.14. $MeHg^+$ concentrations in seafood samples reported since 2009.

Mercury species	Sample	Extraction procedure	Extracting agent	Technique	$MeHg^+$ (mg kg^{-1})	$MeHg^+$ (%)	Reference
$MeHg^+$	Fish	Stirring manually with a glass rod	Toluene	GC-EDC	<0.5	49-99	Sedláčková et al., 2014
$MeHg^+$ Hg(II)	Fish	Ultrasonication	2-mercaptoethanol, L-cysteine and HCl	ID-LC-ICP-MS	0.04- 1.92	83-98	Montero-Alvarez et al., 2014
$MeHg^+$ Hg(II)	Fish	Microwave	L-cysteine	LC-CV-ICP-MS	0.01- 1.00	50-100	Schmidt et al., 2013
$MeHg^+$ Hg(II)	Fish	Water bath	HNO_3	GC-CV-AFS	0.5-1.5	-	Barst et al., 2013
$MeHg^+$ $EtHg^+$ $PhHg^+$ Hg(II)	Fish	Ultrasonication	HCl + Sodium 3-mercapto-1-propanesulfonate	LC-ICP-MS	0.0032-0.75	> 86	Chen et al., 2013a
$MeHg^+$ $EtHg^+$ $PhHg^+$ Hg(II)	Fish	Ultrasonication	HCl + L-cysteine	LC-ICP-MS	0.17-0.75	> 86	Chen et al., 2013b
$MeHg^+$ $EtHg^+$ Hg(II)	Fish	Microwave	HCl	CE-ICP-MS	1.2-3.2	96	Zhao et al., 2012
$MeHg^+$ Hg(II)	Shellfish	Ultrasonication	HCl	CV-AFS	0.022-0.034	100	Zhang et al., 2012
$MeHg^+$	Fish	Stirring	Toluene	GC-ECD	0.05-0.8	46-100	Kenšová et al., 2012
$MeHg^+$ Hg(II)	Fish	Microwave	TMAH	GC-AFS	0.001-1.16	60-88	Nevado et al., 2011
$MeHg^+$	Fish	Alkaline digestion	KOH–MeOH	GC-CV-AFS	0.007-0.12	37-81	Liang et al., 2011

Mercury species	Sample	Extraction procedure	Extracting agent	Technique	$MeHg^+$ ($mg\ kg^{-1}$)	$MeHg^+$ (%)	Reference
$MeHg^+$ $EtHg^+$ $PhHg^+$ Hg(II)	Fish	Ultrasonication	HCl	LLME-CE-UV	0.004-0.027	100	Li et al., 2011
$MeHg^+$ Hg(II)	Fish	a) Solid-liquid extraction b) Microwave c) Extraction at room temperature	TMAOH	ID-GC-ICP-MS	0.002-0.58	84-97	Clémens et al., 2011
$MeHg^+$ Hg(II)	Shellfish				0.001-0.033	28-98	
$MeHg^+$	Fish	Water bath	KOH	HS-SPME-GC-AFS	0.76	74	Carrasco et al., 2011
$MeHg^+$ $EtHg^+$ Hg(II)	Shellfish	Ultrasonication	HCl + L-cysteine + 2-mercaptoethanol	LC-ICP-MS	0.004-0.037	88-100	Batista et al., 2011
$MeHg^+$ $EtHg^+$ Hg(II)	Fish				0.03-0.16	92-100	
$MeHg^+$	Fish	Alkaline digestion	KOH–MeOH	GC-AED	0.006-0.5	14-100	Kuballa et al., 2011
$MeHg^+$	Fish	Volatilization	H_2SO_4 + KBr + toluene + cysteine	GC-ECD	0.002-0.1	40-110	Miklavčič et al. 2011
$MeHg^+$ $EtHg^+$ Hg(II)	Fish	Stirring	Alkaline extraction	LC-CV-AFS	0.1-0.6	84-89	Wang et al., 2010b
$MeHg^+$	Fish	Stirring	Alkaline extraction	LC-UV-AFS	0.1-0.4	35-76	Fu et al., 2010
$MeHg^+$	Fish	Shaking and centrifugation	H_2SO_4 + KBr + toluene + L-cysteine	GC-ECD	0.29-0.69	70-82	Hajeb et al., 2010
$MeHg^+$	Fish	Alkaline digestion	KOH–MeOH	CV-AFS	0.02-0.09	7.4-93	Qiu et al., 2009
$MeHg^+$ Hg(II)	Fish	Ultrasonication	$HClO_4$ + L-cysteine + toluene + MeOH	LC-ICP-MS	0.05-0.3	80	Santoyo et al., 2009

4.6.3.2. Selection of extractant agent

For speciation analysis, first of all, the study of a quantitative species extraction method system for seafood matrices, suitable for the LC-UV-CV-AFS determination is needed. According to the extraction methods summarised before (Table 4.14), it can be seen that in 50% of the cases acidic digestions are used. Furthermore, there is no knowledge about standardised extraction methods in seafood matrices.

It was found in the literature a standardised extraction method for the determination of mercury species in soils and sediments, the EPA 3200, which consists of the use of HNO_3 4 mol L^{-1} as extractant agent [EPA, 2005]. However, a previous work in the research group demonstrated that the presence of nitric acid in the sediments extracts caused “quenching” effects on the mercury signal provided by the AFS system [Guevara-Riba et al., 2006]. In this way, to perform the extraction of Hg species in seafood, the EPA 3200 method was tested using both nitric acid and another acid substitute, HCl 4 mol L^{-1}. The present method is based on a microwave-assisted extraction, whose procedure is described in section 2.4.3.2 (Chapter 2). For this study, the CRM DOLT-4 (Dogfish) was used, in which total Hg and $MeHg^+$ content are certified.

The chromatograms obtained from hydrochloric and nitric acid DOLT-4 extracts are shown in Figure 4.30. The recoveries obtained when comparing the sum of the species with the total Hg certified value were 97% for both extractions. These values demonstrate that the fluorescence signal in the separation media during speciation analysis is not affected by quenching, as did not happen for total analysis.

However, it can be seen that the ratios of $MeHg^+$ and Hg(II) concentrations are different in each extraction procedure. Recoveries obtained for $MeHg^+$ with respect to the certified value were 95% and 86%, using HCl and HNO_3, respectively. Hg(II) content is not certified, but it can be estimated as the difference between the total Hg content and the $MeHg^+$, as no more species were observed. Taking this value as reference, the recoveries obtained for Hg(II) were 95% and 108%, using HCl and HNO_3, respectively. This latter slightly higher recovery could be attributed to the oxidising action of HNO_3: as Hg(II) recovery with the HNO_3 extraction method is slightly higher than the difference between the total Hg and $MeHg^+$ certified values, there is evidence that this difference could be caused by $MeHg^+$ conversion to Hg(II). Therefore, HCl 4 mol L^{-1} was selected as the extractant agent.

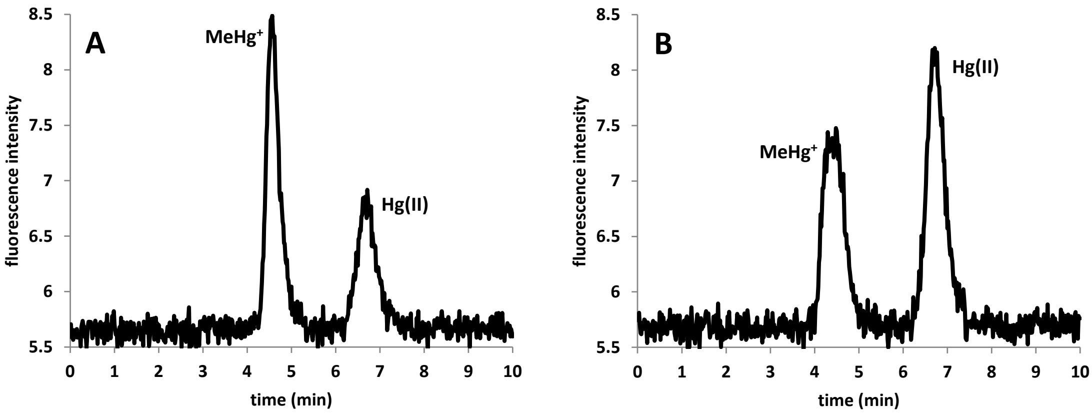


Figure 4.30. Chromatograms obtained for DOLT-4 using microwave assisted extraction with (A) HCl 4 mol L^{-1} and (B) HNO_3 4 mol L^{-1}.

4.6.3.3. Quality parameters

The analysis of Hg species in TORT-2, DOLT-4 and BCR-463 was performed by LC-UV-CV-AFS with the previous extraction procedure optimised. In this case, the accuracy was verified for $MeHg^+$ species as its certified value is given for all the CRMs assessed. $MeHg^+$ and Hg(II) were the species presents in the materials. Limits of detection and limits of quantification for mercury species in samples were estimated. The instrument limits were converted to sample limits by multiplying by the extraction dilution factor. The LDs were 0.3 and 0.4 µg Hg kg^{-1} for $MeHg^+$ and Hg(II), respectively. The LQs were 1 and 1.2 µg Hg kg^{-1} for $MeHg^+$ and Hg(II), respectively.

The values for Hg species concentration are represented in Figure 4.31 together with the results obtained in total analysis and the certified values. The experimental concentrations did not differ significantly at a 95% confidence level from certified values. The recovery for each CRM was calculated by comparing the sum of $MeHg^+$ and iHg concentration, obtained by the proposed speciation method (LC-UV-HG-AFS) and total Hg concentration (ICP-MS). The total Hg concentration was taken as 100% in the calculation of recovery values. The recoveries analysed for CRMs were 80% for TORT-2, 92% for DOLT-4 and 102% for BCR-463. It can be observed that the recovery value for TORT-2 is slightly lower than those obtained for the other two CRMs. This face could be due to the small signals of Sb species found in the measuring extracts, which were close to the limit of quantification where the widespread of expected results can be higher.

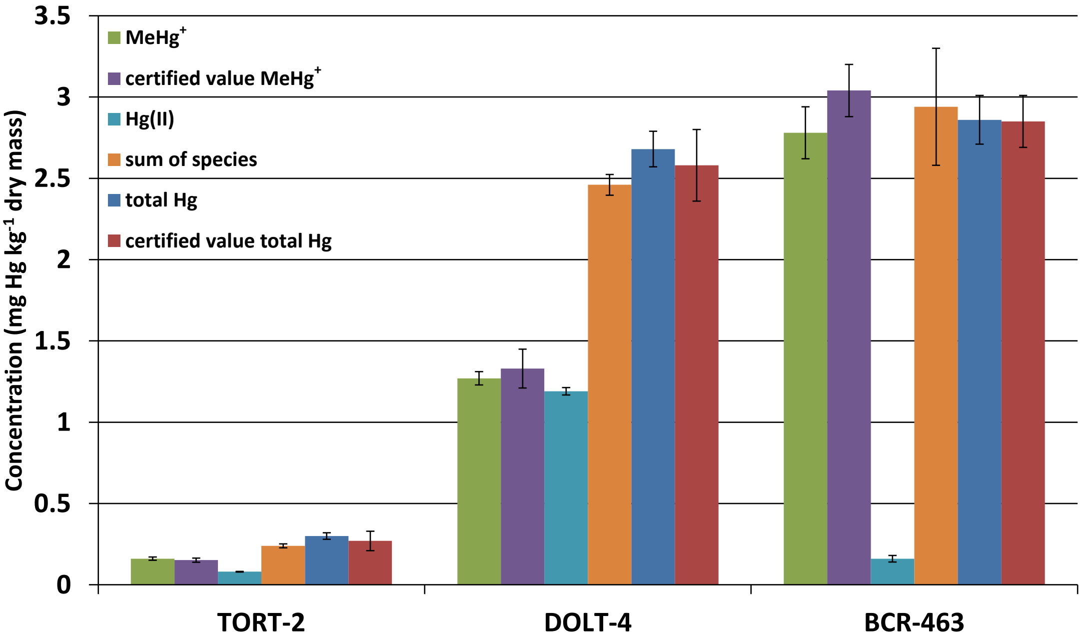


Figure 4.31. Total mercury and mercury species in CRMs; concentrations are expressed as mg Hg kg^{-1}.

In order to assess the extraction recovery, spiked test were performed on four representative seafood matrices (2 fish, 1 crustacean and 1 bivalve) together with a CRM. Standards of $MeHg^+$ were spiked in solid samples of each type of matrix and a CRM: tuna-2, forkbeard, prawn-1, cockle and BCR-463. After addition of standards, the solid samples were homogenized manually and the extraction procedure was carried out 30 minutes after spiking. As an example, Figure 4.32 shows the chromatograms of tuna-2, forkbeard, prawn-1 and cockle. The recoveries found for tuna-2, forkbeard, prawn-1, cockle and BCR-463 were 93 ± 3, 85 ± 5, 93 ± 2, 87 ± 4 and 97 ± 2 (mean % ± standard deviation, n=3), respectively. These recovery values for $MeHg^+$ evidence the good performance of the analytical procedure.

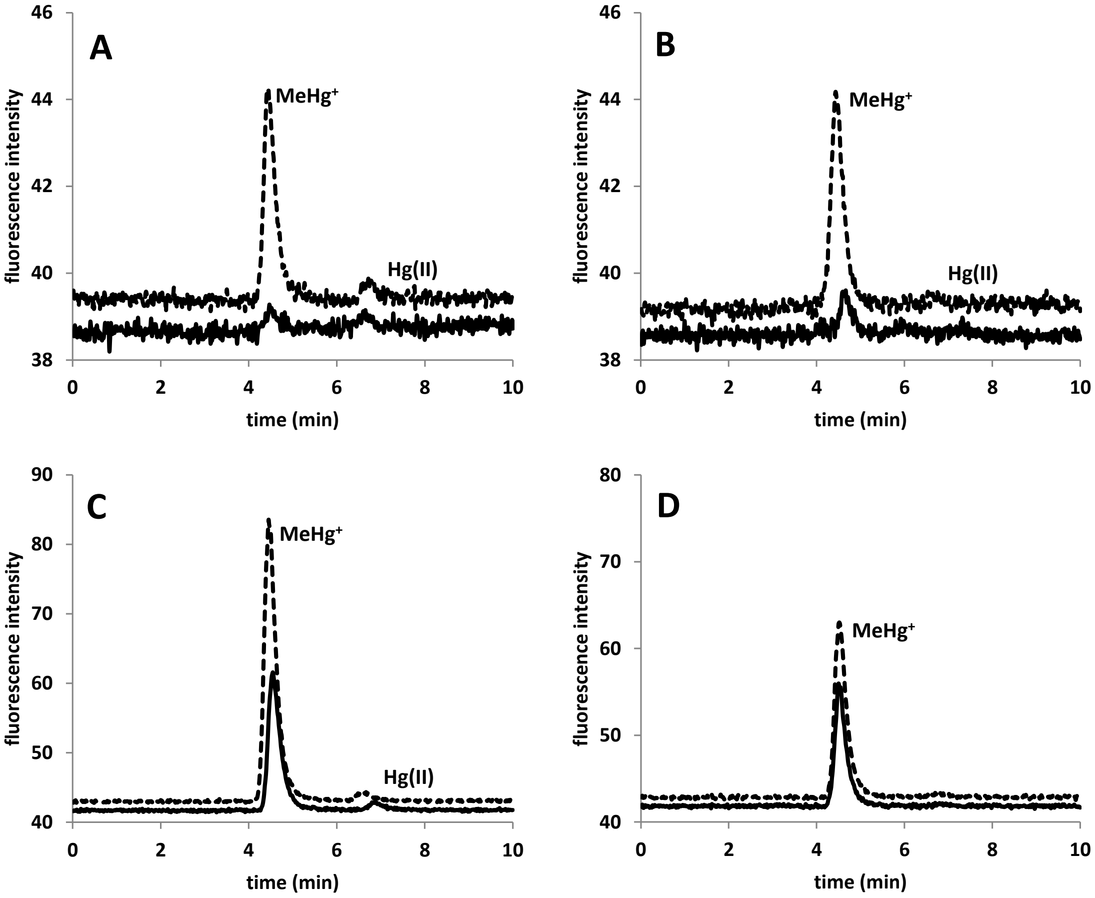


Figure 4.32. Chromatograms of (A) cockle extract (B) prawn-1 extract (C) forkbeard extract (D) tuna-1 extract (continuous line: non-spiked sample; dotted line: sample spiked with $MeHg^+$) by LC-UV-CV-AFS.

4.6.3.4. Analysis of seafood samples

Mercury species concentration in the seafood samples together with the sum are summarised in Table 4.15. For all samples, the sum of $MeHg^+$ and Hg(II) concentration (obtained by the proposed speciation method, LC-UV-CV-AFS) was compared with total Hg concentration (obtained by ICP-MS), and percentage recoveries were calculated, taking as 100% the total Hg. The values are summarised in the last column of Table 4.15.

Table 4.15: Mercury speciation analysis of selected seafood samples; concentrations are expressed as mg Hg kg^{-1} wet mass (mean ± SD, n = 3).

Sample	MeHg$^+$	Hg(II)	Sum of Hg species	Recovery (%)
White fish	0.30 ± 0.02	<LQ	0.30 ± 0.02	114
Red porgy-1	1.67 ± 0.04	0.061 ± 0.009	1.73 ± 0.05	105
Red porgy-2	1.13 ± 0.06	0.035 ± 0.001	1.17 ± 0.06	101
Hake -1	0.62 ± 0.02	0.019 ± 0.002	0.64 ± 0.02	117
Hake -2	0.31 ± 0.04	<LQ	0.31 ± 0.04	114
Forkbeard	0.32 ± 0.01	0.010 ± 0.003	0.33 ± 0.01	109
Sardine	0.040 ± 0.002	<LQ	0.040 ± 0.002	100
Salmon-1	0.022 ± 0.001	<LD	0.022 ± 0.001	103
Salmon-2	0.025 ± 0.003	<LD	0.025 ± 0.003	120
Tuna-1	0.30 ± 0.05	0.011 ± 0.003	0.31 ± 0.05	95
Tuna-2	0.136 ± 0.008	<LD	0.136 ± 0.008	97
Tuna-3	2.23 ± 0.04	0.085 ± 0.004	2.31 ± 0.04	99
Louvar	0.64 ± 0.03	0.011 ± 0.001	0.65 ± 0.03	108
Swordfish-1	1.04 ± 0.04	0.02 ± 0.002	1.06 ± 0.05	102
Swordfish-2	0.26 ± 0.03	<LD	0.26 ± 0.03	102
Swordfish-3	0.58 ± 0.04	<LQ	0.58 ± 0.04	103
Prawn-1	0.011 ± 0.003	<LD	0.011 ± 0.003	88
Prawn-2	<LD	<LD	-	-
Shrimp	<LD	<LD	-	-
Clams-1	<LD	0.016 ± 0.004	0.016 ± 0.004	108
Clams-2	0.013 ± 0.001	0.008 ± 0.001	0.021 ± 0.002	110
Mussel	<LD	<LD	-	-
Cockle	0.003 ± 0.001	0.006 ± 0.002	0.009 ± 0.003	110
Oyster	<LD	0.007 ± 0.001	0.007 ± 0.001	100

LD MeHg$^+$: 0.3 µg kg^{-1}; LD Hg(II): 0.4 µg kg^{-1}.
LQ MeHg$^+$: 1 µg kg^{-1}; LQ Hg(II): 1.2 µg kg^{-1}.

The mean values of percentage and concentration of MeHg$^+$ in fish and shellfish samples were calculated. For fish, a mean percentage of 98%, a mean concentration of 0.71 mg MeHg$^+$ kg^{-1} in dry mass and a mean concentration of 0.60 mg MeHg$^+$ kg^{-1} in wet mass were found; and for shellfish, a mean percentage of 24%, a mean concentration of 0.027 mg MeHg$^+$ kg^{-1} in dry mass and a mean concentration of 0.009 mg MeHg$^+$ kg^{-1} in wet mass. Inside the fish sample group, the highest concentrations of MeHg$^+$ in wet mass were found for red porgy-1 and red porgy-2 (mean value: 1.4 mg kg^{-1}), tuna-3 (2.23 mg kg^{-1}) and swordfish-1 (1.04 mg kg^{-1}). In shellfish, the highest levels of MeHg$^+$ were found for clams-2 (0.013 mg kg^{-1}). The concentrations of all samples were within the maximum levels established in the Codex Stan 193-1995 for $MeHg^+$ [CODEX STAN, 2009], except for red porgy-1 and -2 (Brazilian fish samples), tuna-3 and swordfish-1 (Spanish fish samples), which showed concentrations higher than 1 mg kg^{-1}.

In some samples, Hg(II) was also identified. Values of Hg(II) concentration ranged from 0.010 to 0.085 mg Hg(II) kg^{-1} in wet mass in fish samples, with a mean value of percentage of 1.6%; and from 0.006 to 0.016 mg Hg(II) kg^{-1} in wet mass in shellfish samples, with a mean percentage of 76%. However, Hg(II) was quantified only in four of the eight shellfish samples. These data underline the need to introduce maximum levels of $MeHg^+$ in seafood in Brazilian and European legislation should be considered in further Directives.

All samples analysed showed recovery values between 88 and 120%, which are comparable and of the same order of magnitude with those found in the literature [Clémens et al., 2011; Kenšová et al., 2012; Chen et al., 2013a]. As could be observed from the table, High recoveries were observed for salmon, hake and whitefish samples (fatty samples), with values of 120%, 117% and 114%, respectively. These values are in good agreement with those observed by Clémens et al., 2011, who obtained recoveries between 90 and 110% for matrices with low-fat content and values around 120% for fatty matrices. Thus, close correlation between total and sum of species is achieved, regardless of sample matrix composition.

Regarding the distribution of the species, the percentage of $MeHg^+$ and Hg(II) with respect to the sum of the species was depicted in Figure 4.33.

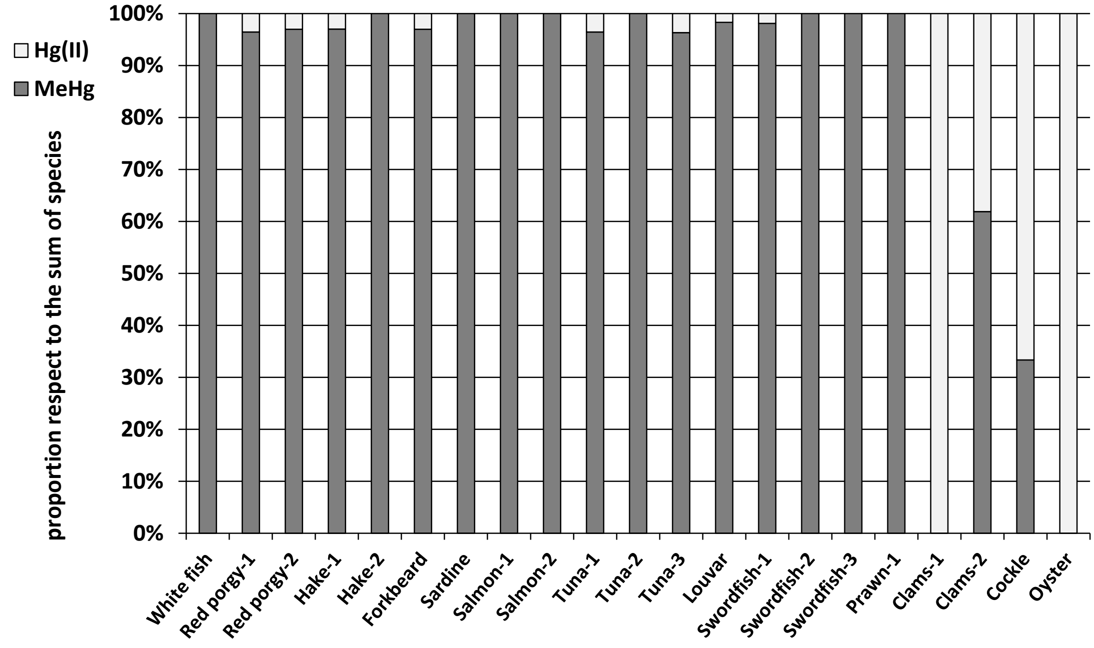


Figure 4.33. Mercury speciation analysis in the selected seafood samples.

The presence of $MeHg^+$ was detected in 19 analysed samples. As it can be seen, whereas for fish and crustaceans $MeHg^+$ was the predominant form of mercury, for the quantified bivalves, the presence of Hg(II) was much more significant, whose contents ranged from 40 to 100% of total mercury

According to data obtained in this work, and as described in the literature, the concentrations of $MeHg^+$ are higher in fish than shellfish being predatory fish those samples showing the highest values. Higher $MeHg^+$ content in fish samples could be related to the fat content. Methylmercury is a fat-soluble substance and therefore can be accumulated in the fatty tissues more easily than inorganic mercury. Bluefish samples, such as salmon and tuna with high fat content, present high levels of $MeHg^+$. Whitefish and shellfish, with lower fat content, present lower $MeHg^+$ concentration and in the case of some shellfish samples the predominant mercury species is Hg(II).

The concentrations of $MeHg^+$ in seafood found in the literature since 2009, summarised in Table 4.14, vary widely, as the chemical behaviour of Hg in the marine environment and the number of predatory fish analysed explain the differences between the mean values of $MeHg^+$ found in several studies. The conditions of the water environment, the age of each species and the time of exposure to Hg contaminants are also factors that affect the results [Fitzgerald et al., 2007].

The concentrations of $MeHg^+$ ranged from 0.001 to 3.2 mg kg^{-1} with percentages with respect to the total value between 30 and 100% for seafood samples, with few exceptions. However, bivalves, molluscs and crustaceans have lower $MeHg^+$ concentration than fish. Zhang et al., 2012 found 100% of $MeHg^+$ concentrations between 0.022 and 0.034 mg kg^{-1} for mussel and clam samples. Clémens et al., 2011 found concentrations of 0.001 and 0.033 mg kg^{-1} for mussel, oyster, scallop and shrimp, with ratios between 28 and 98%; and Batista et al., 2011, of 0.003 and 0.037 mg Hg kg^{-1} for mussel, octopus, shrimp and squid samples, with ration between 88 and 100%.

When comparing the concentrations found in this study for $MeHg^+$ in fish with the literature (Table 4.14), the values were similar [Clémens et al., 2011; Chen, et al., 2013a; Chen et al., 2013b; Montero-Alvarez et al., 2014] or higher [Kenšová et al., 2012; Carrasco et al., 2011; Kuballa, et al., 2011; Liang et al., 2011; Miklavcic et al., 2011; Nevado et al., 2011; Fu et al., 2010; Hajeb et al., 2010; Wang et al., 2010b; Qiu, et al., 2009; Santoyo et al., 2009]. The shellfish analysed had similar levels of $MeHg^+$ to those found by Clémens et al., 2011. The results obtained are in agreement with those reported by Kuballa et al., 2011 and Clémens et al., 2011, showing a great variability in $MeHg^+$ concentration in different fish and shellfish species, respectively. These differences reaffirm the need to monitor $MeHg^+$ concentrations in seafood species more frequently and in different areas, in order to contribute to the protection of human health.

The results obtained in sections 4.6.1 – 4.6.5 were published in the journal *Food Control* as a research article, entitled *Method development for the simultaneous determination of methylmercury and inorganic mercury in seafood*, which is attached in the Annex 2.

4.6.4. Preconcentration studies in seafood samples

The developed methods for the analysis of mercury species after short-column preconcentration described in section 3.3.2 were validated for the determination in drinking water. These methods are suitable for the quantification of mercury species in a wide range of environmental waters. On account of the low Hg species concentrations present in some of the seafood samples analysed in the previous section, it was decided to test the preconcentration system in these complex samples.

Firstly, three samples among the 24 analysed previously and a CRM were selected according to the following criteria: one sample with total Hg concentration below the LD (shrimp), one sample with concentrations of both species close to the limit of quantification (cockle), and one sample with a concentration of Hg(II) close to the limit of quantification (tuna-1). The CRM selected was TORT-3, as it also presents low levels of $MeHg^+$ and Hg(II). The corresponding extracts after microwave extraction were reanalysed by LC-UV-CV-AFS using both direct injection and the preconcentration step using the optimum conditions, summarised in Table 3.14 from section 3.3.2.2, until the breakthrough volume was achieved for each matrix.

The results obtained in the online preconcentration showed an increase of the peak signal for both species when loading the minimum sample volume of 1 mL. Increasing the sample load volume from 1 to 5 mL, did not only presented no significant changes on the maximum signal obtained, but also it did not follow a lineal behaviour. This fact means that the column is saturated with 1 mL of sample volume. This effect may be due to the seafood matrix, which decrease the precolumn retention capacity. It can be eye-observed that the extracts present dark brown or dark orange colour, which demonstrates that seafood could present high matrix effects.

Figure 4.34 depicts the chromatograms obtained in all cases with a 1 mL load volume together with the direct injection of the sample.

As it can be seen, for the shrimp sample which was not quantified after direct injection, the preconcentration step would allow the determination of the species. The system would also permit to quantify those matrices with Hg species concentration levels below the LQ, even though in samples with the presence of considerable amounts of one of the species, as happen with the $MeHg^+$ concentration in tuna sample, a double peak could be observed.

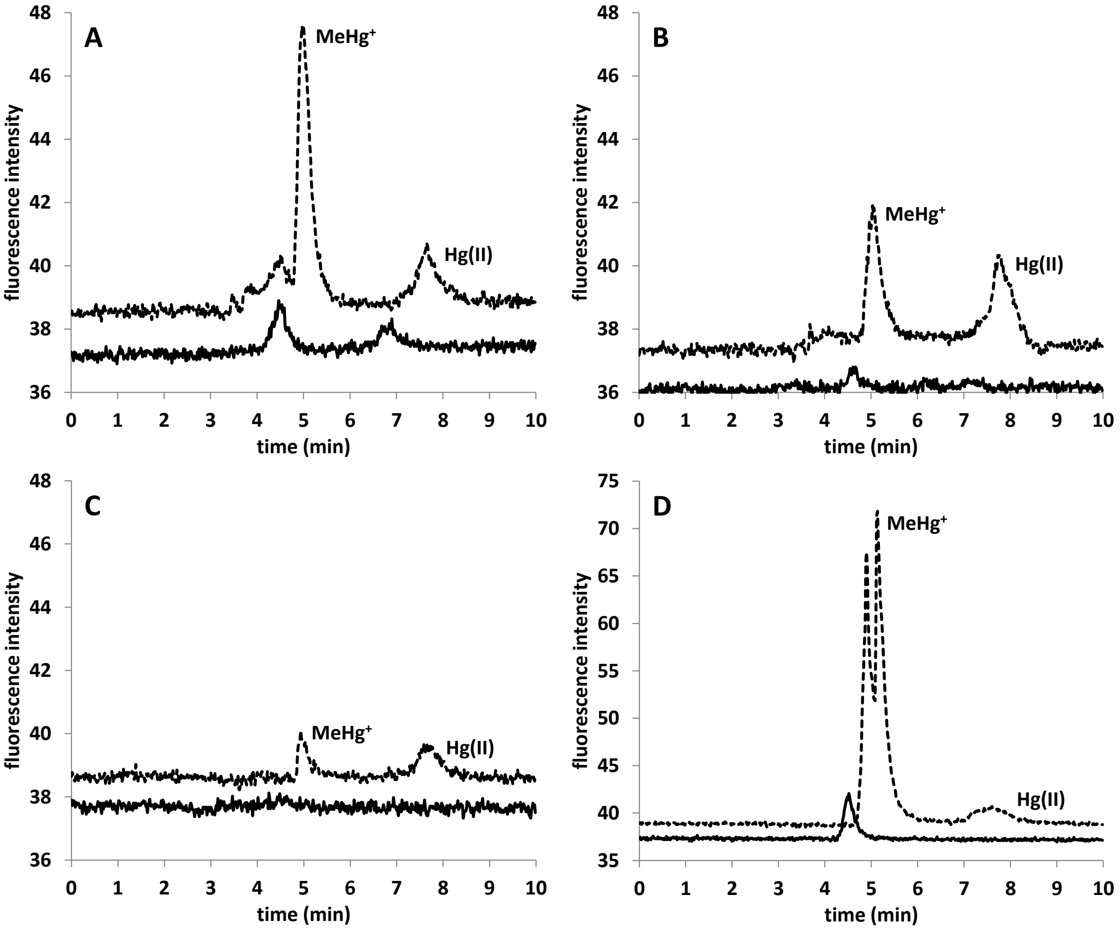


Figure 4.34. Chromatogram obtained using a 1 mL as loading volume, together with a direct injection of this sample from (A) TORT-3, (B) prawn-1, (C) shrimp and (D) tuna-1 (continuous line: direct sample injection; dotted line: preconcentrated sample)

These preliminary results indicate that the system allows the preconcentration of mercury species with similar efficiency than that observed in samples with a low complex matrix, as mineral water, even if when analysing more complex matrixes, as seafood extracts, the efficiency is lost due to a high matrix effect, which prevents a good retention of the species. In all cases, the reproducibility of the preconcentration was not suitable enough for a reliable quantification.

However, it is possible to quantify the presence of trace amounts of mercury species with the proposed preconcentration system, which cannot be detected by direct injection.

All the same, further studies could be developed in the research group so as to improve the preconcentration efficiency in seafood, developing, for example, clean-up strategies. Nevertheless, it will suppose a significant cost and an analytical effort for the determination of trace concentrations of mercury species lower than those stipulated in the legislation, which could be questionable.

Chapter V

ANTIMONY MIGRATION STUDIES

As mentioned in the introduction, antimony content in PET bottled waters or other beverages depend on several variables such as storage time, temperature among others. Although evidences were found about a rapid release of the Sb from PET to bottled waters in a few days from 60ºC of storage, exceeding in some cases the limit established by the EPA and EU for Sb in drinking waters, the observed effects among the published papers are widely variable, which is hindering clear consistent conclusions. In this chapter, antimony migration studies in PET bottled beverages are carried out. Samples studied were mineral drinking water, juice and *raki*. Different variables which can have an effect on Sb migration are assessed.

5.1. MIGRATION IN WATERS

In this section, the influence of various physicochemical factors, including storage time, temperature and PET colour on Sb migration in bottled waters, was investigated. A systematic study was undertaken to assess the effect of storage time around 7 months, and storage temperatures from 4ºC to 60ºC on bottled drinking waters. Total Sb and speciation analysis at some specific times and temperatures was carried out on water samples bottled in clear and blue PET containers.

Three commercial brands of PET-bottled water were purchased. Regarding the previous results obtained in section 4.1, in which more than half of the samples stored in clear bottles contained lower concentration of Sb than those stored in coloured bottles, the selection of brands was based on the colour of the PET bottle: clear (CL), light blue (LB) and dark blue (DB). Each brand was noncarbonated, weak mineralised and the water volume was 0.5 L.

5.1.1. Temperature stress test

The potential effect of temperature on Sb release into water during storage was first studied in a stress short term conditions on the three commercial bottled water brands selected to assess the antimony migration potential with temperature in these matrices. An elevated extreme temperature was selected so as to simulate the worst-case conditions reported. Although some authors consider migration studies at high temperatures (60ºC) to be unnecessary for water samples [Sánchez-Martínez et al., 2013], others have indicated that they are necessary given that the temperatures inside cars, garages and closed storage areas can arrive to 60ºC or even exceed 65ºC in some cases during the summer in some regions [Westerhoff et al., 2008]. Thus, a temperature of 60ºC was selected.

Thus, total and speciation Sb in the drinking waters were analysed by HG-AFS and LC-ICP-MS, respectively, at the beginning of the test and after 15 days of storage at 60ºC. After that, samples were then stored for another 15 days at 4ºC and the Sb contents were measured again so as to evaluate whether the Sb concentration after the migration at elevated temperature is constant, increases because Sb keeps on migrating, or decreases as it is re-sorbed on the plastic bottle. The analyses were performed in triplicate in all cases.

The results are shown in Figure 5.1, where the total Sb and speciation analyses are plotted for each brand, together with the standard deviation. In all cases, the relative standard deviation ranged from 0.07% to 9.90%. Accordingly, antimony was detected in all samples before the storage (day 0). Total Sb ranged from 0.3 to 0.7 µg kg^{-1}. After storage at 60ºC, a significant increase in total Sb concentration was observed (2.6 – 4.2 µg L^{-1}). Subsequent storage at 4ºC gave similar values to those obtained after storage at 60ºC. In the case of speciation studies, Sb(V) was initially the only species present. After 15 days storage at 60ºC, the Sb(V) concentration increased, and Sb(III) appeared in all samples at detectable but not quantifiable levels. From the reduced number of units analysed, it can be stated that waters stored in coloured bottles contained similar Sb concentrations, but they were slightly higher than those stored in clear bottles. A dependent Student's t-test for paired samples, at the 95% confidence level, demonstrated that there was no significant difference between total Sb content and Sb(V). Furthermore, using the same statistical t-test it was shown that Sb concentrations after storage at 4ºC did not differ significantly from those previously obtained after storage at 60ºC. These results showed that, as expected, the exposure of water to a high temperature leads to significant Sb migration from the bottles to the water. It was also observed that antimony is not leached at low temperatures as the Sb concentration remained stable at the lower storage temperature. During these 30 days at these temperatures, there was no change in the Sb species present in water. The Maximum Concentration Level (MCL) established by the European Union (5.0 µg L^{-1}) was not exceeded in any case. Thus, based on the results obtained in the short-term stress test, a more systematic study was undertaken.

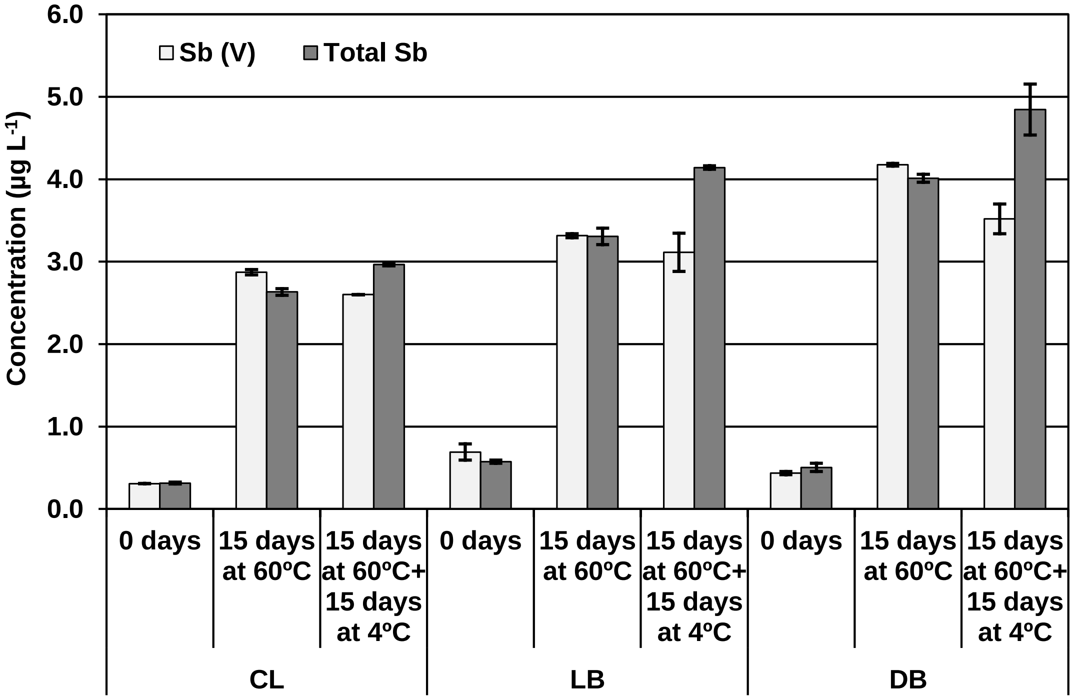


Figure 5.1. Total Sb and Sb (V) concentration (µg L^{-1}) in bottled mineral water samples from three different-coloured PET brands. Times and temperature are mentioned for each brand. Legend for bottle colours: CL: clear; LB: light blue; DB: dark blue.

5.1.2. Migration study

The influence of storage time and temperature on the Sb concentration in water in long-term migration studies was studied on the three commercial bottled water brands mentioned above. Figure 5.2 depicts a schematic design of this study. The temperatures studied have been increased and all series of samples were analysed in 12 different periods of time: three bottles of each brand were stored at 4, 20, 40 and 60ºC and speciation and total antimony were analysed by triplicate analysis until the bottles ran out of water (220 days).

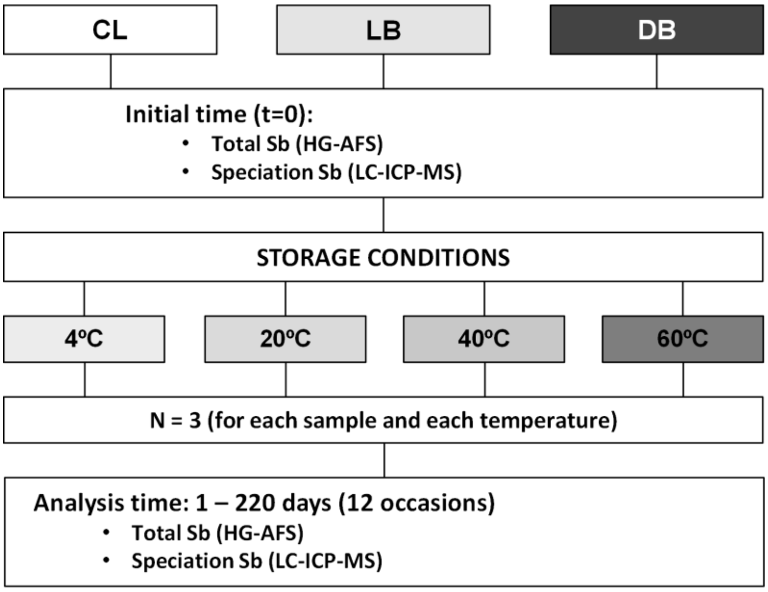


Figure 5.2. Experimental design for the antimony migration study from PET to water with temperature and time.

At the beginning of the test (day 0), all samples contained small concentrations of antimony and Sb(V) was the only species present.

The concentrations of total Sb and Sb(V) species for samples stored at 4 and 20ºC are depicted in Figure 5.3. Samples contained total Sb concentrations below 1 µg L^{-1} throughout the experiment. Thus, the maximum level established by the European Union (5.0 µg L^{-1}) was not exceeded. The only species present was Sb(V). A dependent Student's t-test for paired samples at the 95% confidence level for the 12 different times assayed showed no significant differences in Sb concentration in water over time in all samples.

The concentrations of total Sb and the results of the speciation analysis from the long-term migration experiments at 40 and 60ºC are summarized in Figure 5.4. Figure 5.4A shows the total Sb concentration in each brand stored from day 0 to day 220 and Figure 5.4B shows the Sb(V) concentration under the same conditions.

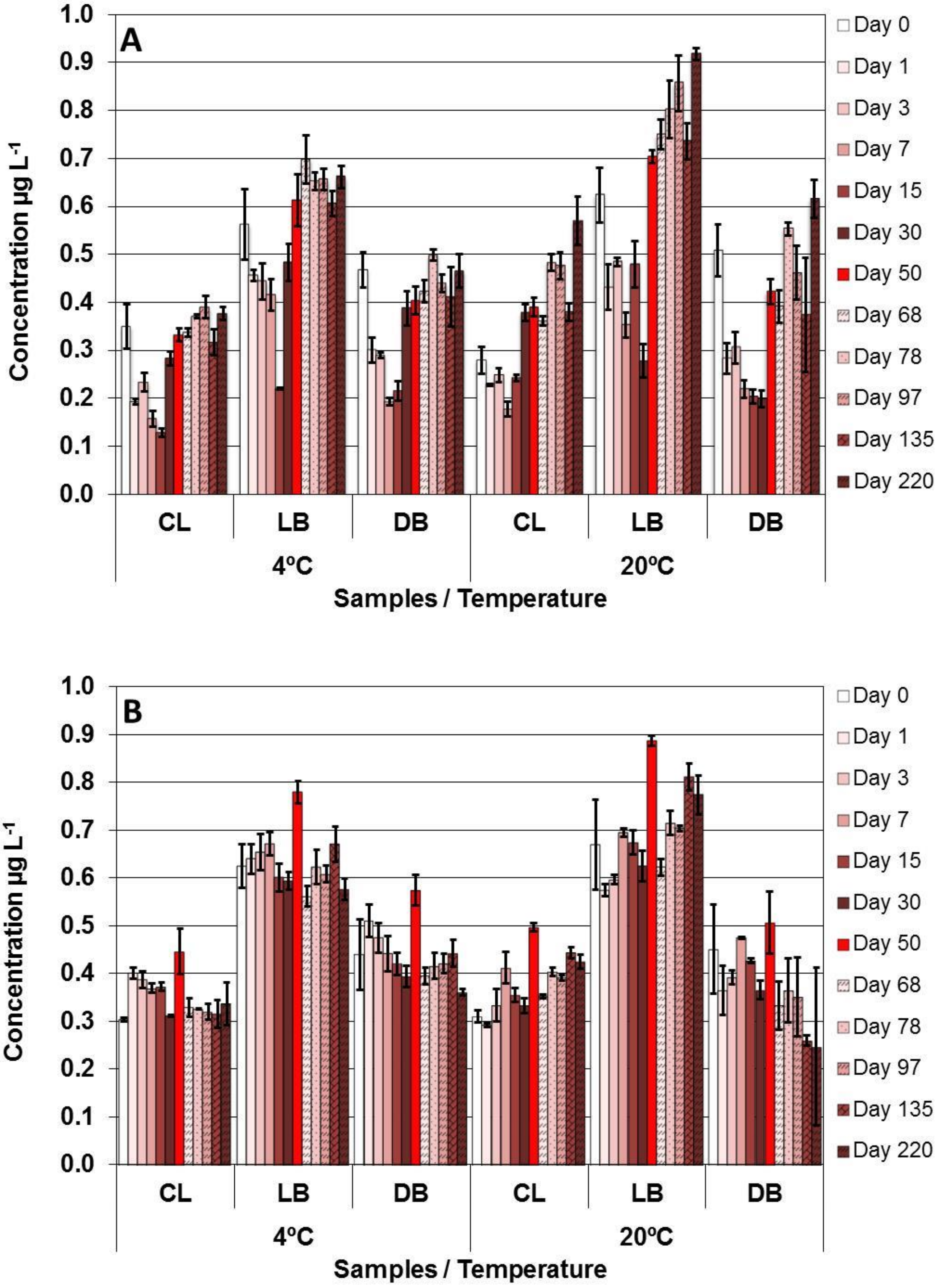
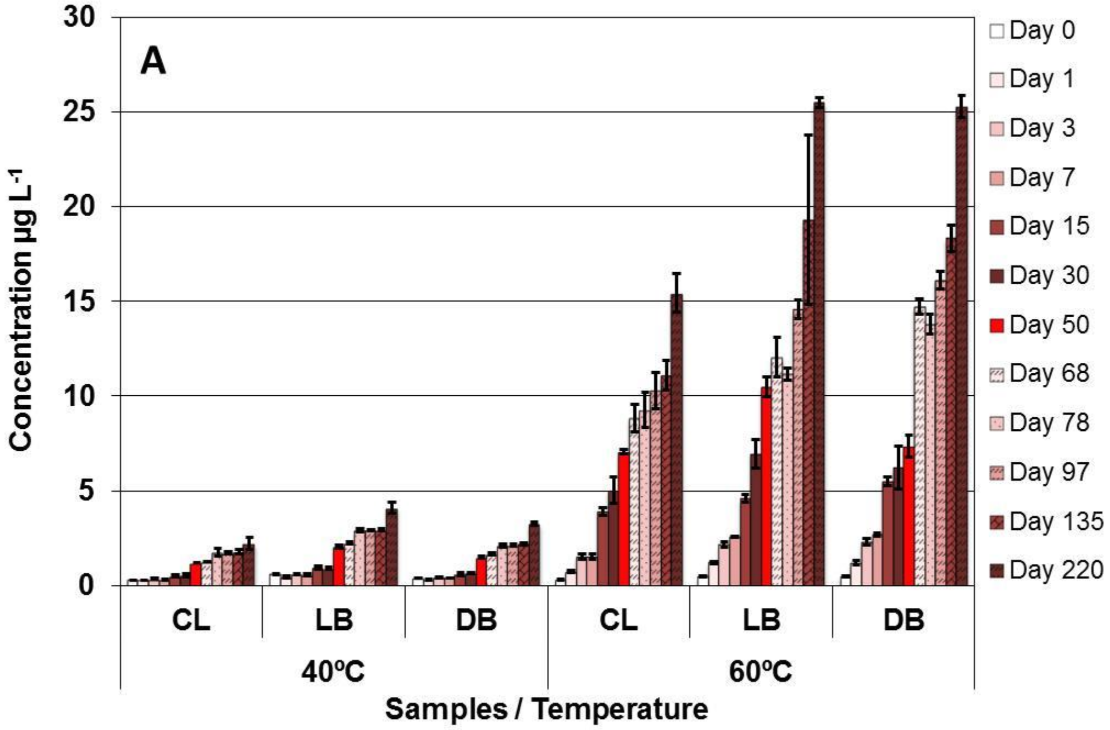


Figure 5.3. Evolution of total Sb (A) and Sb(V) (B) in bottled drinking water stored at 4 and 20ºC for 220 days. Legend for bottle colours: CL: clear; LB: light blue; DB: dark blue



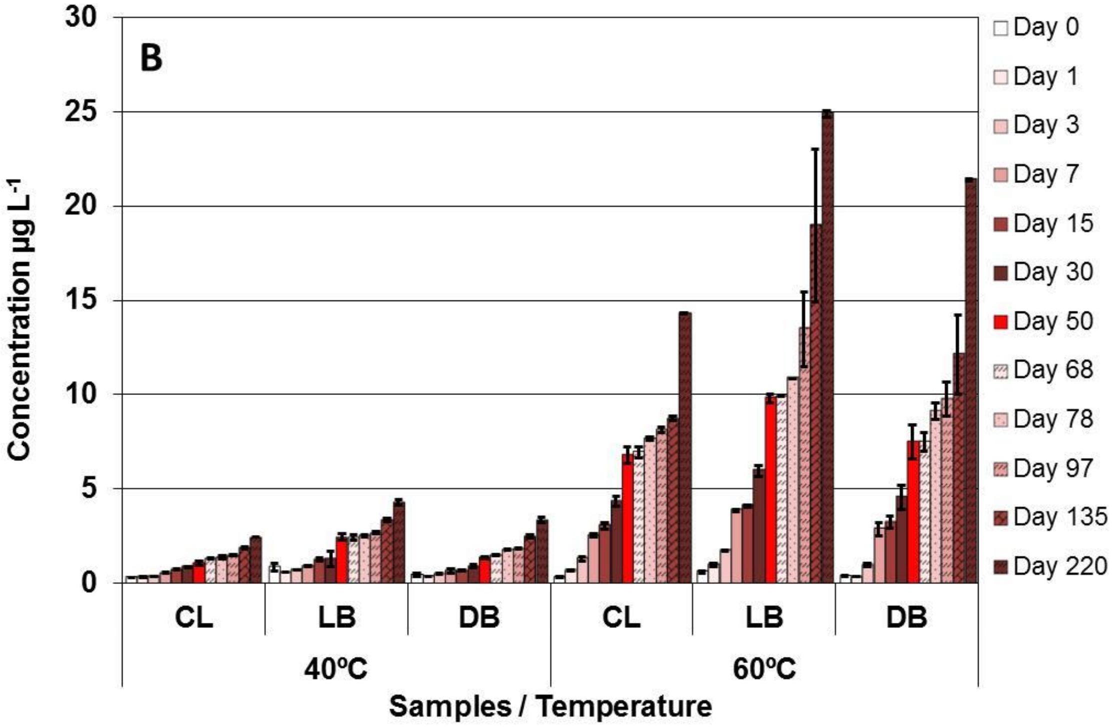


Figure 5.4. Evolution of total Sb (A) and Sb(V) (B) in bottled drinking water stored at 40 and 60ºC for 220 days. Legend for bottle colours: CL: clear; LB: light blue; DB: dark blue.

Samples stored at 40ºC showed a significant increase in antimony concentration after 15 days of storage. From 50 days, this increase became quite pronounced and Sb(III) was also detected in light blue and dark blue bottled samples at concentrations below the limit of quantification. However, Sb(III) was not detected from day 97 onwards. The regulated level was not exceeded in any case.

Samples stored at 60ºC showed a significant increase in total antimony concentration from the first day of storage. The dark blue samples began to exceed the limit for Sb according to the European Union (5 µg L^{-1}) after 15 days, and after 30 days, all samples exceeded the limit. This is consistent with the results obtained by Westerhoff et al., 2008; Keresztes et al., 2009 and Tukur et al., 2012 regarding the increase in total Sb concentration in water samples stored at 60ºC for a few hours to 7 days. As far as the PET colour is concerned, the results obtained in this study seem to indicate that Sb release was slightly lower in non-coloured bottles than in coloured ones, regardless of the colour intensity. This is consistent with Shotyk et al., 2007 and Tukur et al., 2012, who reported that there was no correlation between PET colour and Sb migration potential.

Speciation analysis of the samples at 60ºC showed that Sb(III) was detected from the third to the fiftieth day. After day 50, the concentration of this species was slightly above the limit of quantification in clear and light blue samples but clearly higher in dark blue samples. As an example, chromatograms obtained from the light blue water sample stored at 60ºC are shown in Figure 5.5. The insert shows the Sb(III) peaks obtained at day 0 and 7, in which the species could not be quantified.

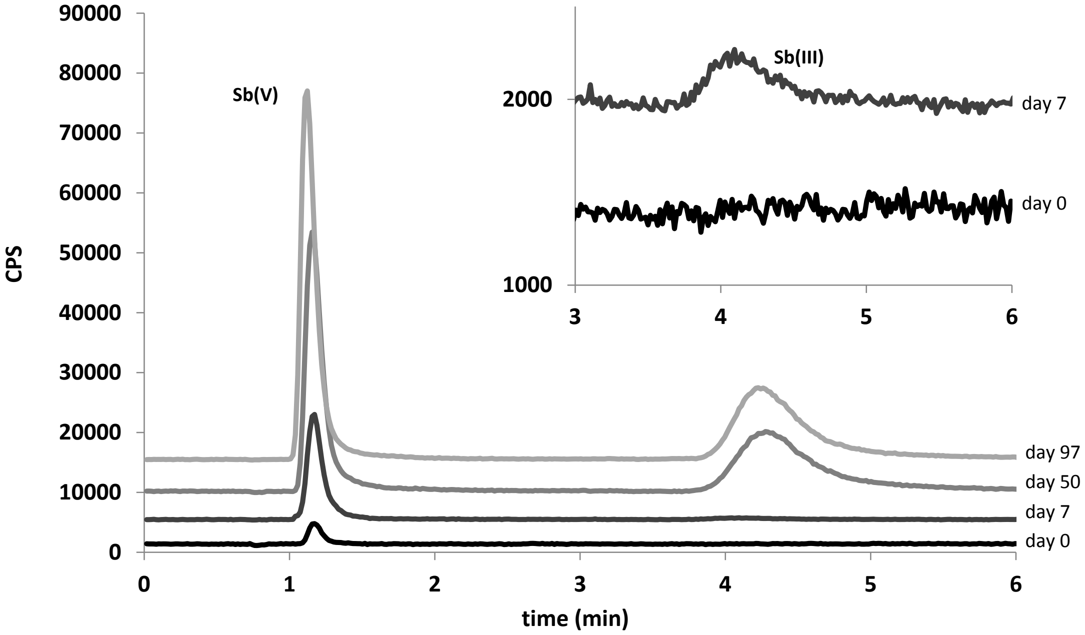


Figure 5.5. Chromatograms obtained from LB sample stored at 60ºC by LC-ICP-MS at days 0, 7, 50 and 97. The insert shows the chromatograms obtained at days 0 and 7, indicating the presence of Sb(III).

The Sb(III) concentration in samples stored for longer than 50 days is depicted in Figure 5.6. As shown, the amounts of trivalent antimony species were similar from day 68 to day 78 and, from day 78 to 220, it decreased slightly or even disappeared in the case of LB samples. As an internal quality control check, a mass balance comparing the sum of species and total antimony was carried out. A dependent Student's t-test for paired samples (95% confidence level) proved that there was no significant difference between these values.

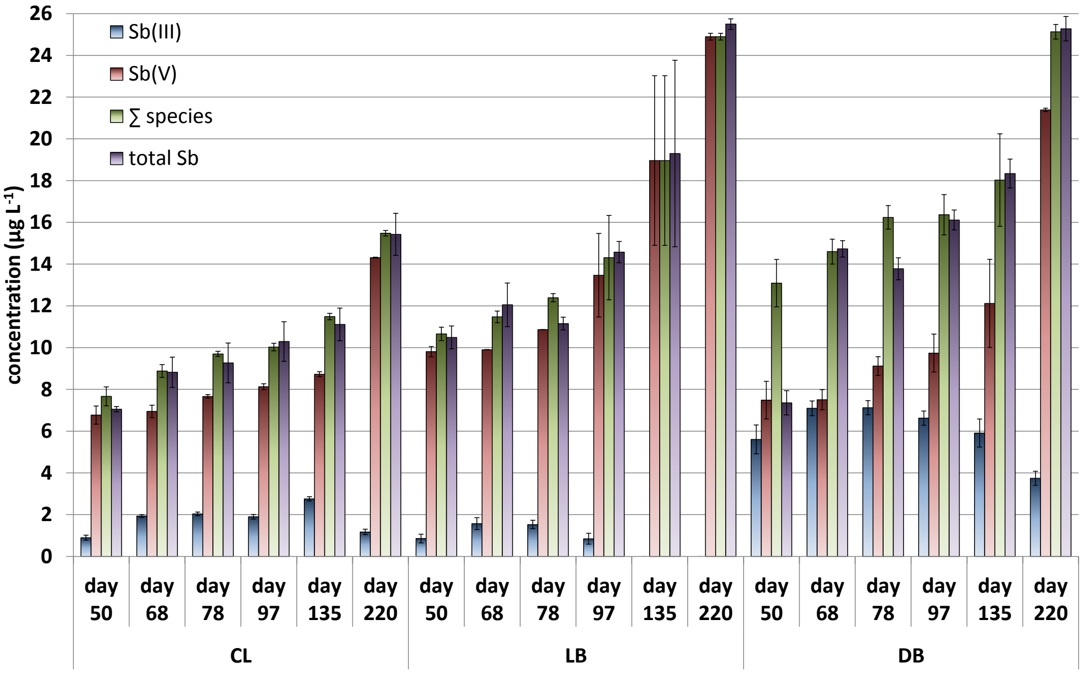


Figure 5.6. Concentration of Sb(III) species in bottled water samples stored at 60ºC from 50 days of storage, together with concentrations of Sb(V), sum of species and total Sb.

The disappearance of the trivalent species with time, when stored at both 40 and 60ºC, could be due to oxidation to the pentavalent form, as it has been previously discussed in section 4.4. It has been shown that Sb(III) is easily oxidized to Sb(V) within a short time at low concentrations [Filella, et al., 2002b; Miravet, et al., 2004].

The results clearly show that at storage temperatures of 40 and 60ºC, Sb migration from PET to drinking water accelerates over time. This effect can be attributed to the degradation of PET with temperature. Degradation by thermo-oxidative and/or thermomechanical processes at temperatures higher than 250ºC is well known [Romao et al., 2009] but has also been described at 40ºC. According to X-ray absorption fine structure (XAFS) measurements, the leaching behaviour of Sb into drinking water after 45 days at 40ºC is mainly due to the degradation of PET [Takahashi et al., 2008]. Thus, antimony could be more easily removable if PET is partially degraded. Moreover, this degradation was also observed visually, as the sample bottles stored at 60ºC presented physical deformation.

However, additional variables apart from temperature can also affect the migration behaviour. Total Sb concentration in PET, bottle volume and wall thickness, activation energy and diffusion coefficients of Sb were used to propose a mathematical model for calculating migration with temperature and time by Welle et al., 2011. On account of these facts, the results obtained in the present work were represented in the same way. Figure 5.7 depicts the tendency of antimony concentrations in water versus days of storage for every sample and temperature.

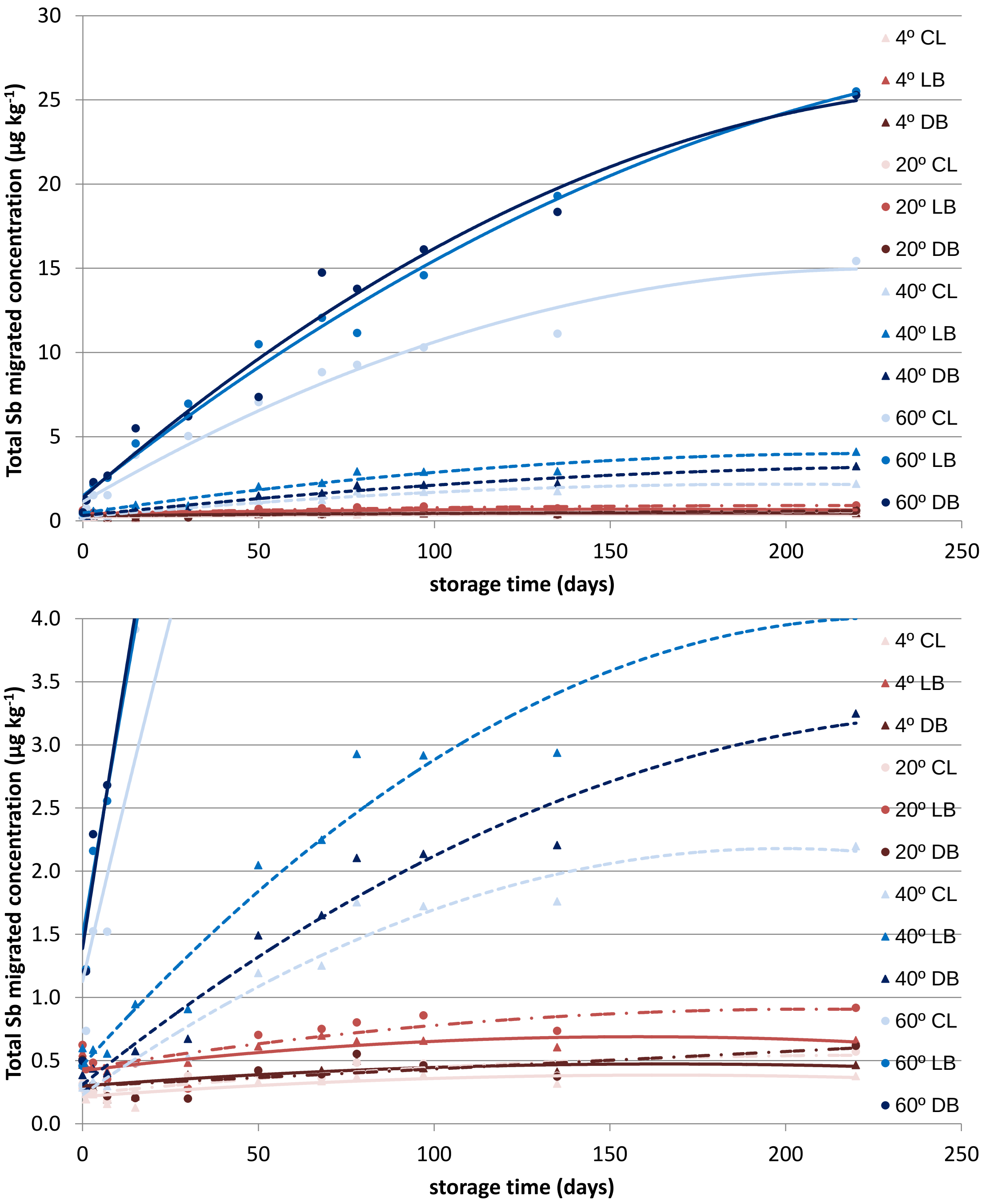


Figure 5.7. Sb migrated concentration versus storage time for every brand at the temperatures studied. The lower part shows an enlargement of the curves.

Even if experimental values obtained in this work at 20ºC and 40ºC are slightly lower than those proposed by this model, the tendency up to 220 days is comparable in all cases to those obtained by Welle et al., 2011. Although no data at 4ºC and 60ºC are available in their work, it can be observed that the tendency at 4ºC is comparable to the obtained at 20ºC, and that at 60ºC, higher concentrations are reached in less time following the same behaviour that the observed at the rest of the temperatures. In addition, Fan et al., 2014 constructed kinetic curves for antimony release using the following equation:

$C = C_{max} (1 – e^{-bt})$

where "C" is the released antimony concentration at storage time "t" (days), "C_{max}" is the maximum concentration of released antimony (µg L^{-1}), and "b" is the kinetic constant (day^{-1}). Values of "C_{max}" and "b" can be estimated by fitting the data obtained in the equation [Fan et al., 2014]. Therefore, the releasing rate (µg L^{-1}/day) at a particular time can be derived from the previous equation through differentiation as the following expression:

$C_{max} \cdot b \cdot e^{-bt}$

Thus, maximum concentration released, kinetic constants, releasing rates and the corresponding determination coefficients were calculated for the three mineral water samples stored during 220 days at the four temperatures, which are summarised in Table 5.1.

Table 5.1. Maximum concentrations and kinetic constant of antimony in PET bottled drinking waters used for the migration study

Sample	C_{max} (µg L^{-1})	b (day^{-1})	Releasing rate (µg L^{-1} day^{-1})	R^2
CL 4ºC	0.392	0.0147	2.27×10^{-4}	0.3840
LB 4ºC	0.695	0.0155	3.56×10^{-4}	0.3898
DB 4ºC	0.513	0.0149	2.88×10^{-4}	-0.2222
CL 20ºC	0.555	0.0115	5.08×10^{-4}	0.7458
LB 20ºC	1.017	0.0086	1.32×10^{-3}	0.5762
DB 20ºC	0.985	0.0105	1.03×10^{-3}	0.3006
CL 40ºC	4.268	0.0078	5.99×10^{-3}	0.8220
LB 40ºC	4.649	0.0086	6.03×10^{-3}	0.9531
DB 40ºC	4.024	0.0067	6.17×10^{-3}	0.9626
CL 60ºC	16.664	0.0096	1.94×10^{-3}	0.9827
LB 60ºC	37.889	0.0049	6.32×10^{-2}	0.9873
DB 60ºC	32.717	0.0064	5.12×10^{-2}	0.9713

Firstly, regarding R^2 values, it can be observed that the constructed kinetic curves match better with the experimental results at high temperatures, as it could be eye-observed from the representations in Figure 5.7. Regarding the C_{max} and releasing rate calculated values, they get higher with temperatures, whereas kinetic constants diminished. These results are in good agreement with the experimental results obtained and confirm, as expected, that migration depends on the temperature.

Regarding the kinetic constants, this parameter is inversely proportional to the time which means that small values are related to faster kinetics. This is consistent with the results obtained, as high temperatures led to rapid kinetics (small "b" values). Regarding releasing rate values, higher antimony concentrations per day migrate from PET to mineral water with temperature, which again is consistent and expected with the experimental facts.

Maximum antimony concentration values calculated for samples stored at 4 and 20ºC are of the same order of magnitude than those obtained experimentally. This fact is due to the reaching of the maximum allowable migrated concentration, and consequently the easy reaching of the equilibrium at low or room temperatures at the studied time, in spite of a low migration speed. However, at 40 and 60ºC, the maximum antimony concentration calculated values are higher than those obtained experimentally. In this case, the maximum allowable migrated concentration was not already reached, as at high temperatures the migration process is enhanced and consequently the equilibrium takes a considerable storage time to be reached.

It should be noted that these facts are consistent with those observed in the stress test performed in the previous section, as Sb migration was enhanced at high temperature, whereas the subsequent storage at cool temperature led to the reaching of the equilibrium, as Sb concentration was not increased.

In conclusion, temperature is the variable which affects potentially to antimony migration for samples stored in a same type of PET container.

Part of the results obtained in section 4.4.1 and 5.1 were published in the journal *Food Chemistry* as a research article, entitled *Migration of antimony from polyethylene terephthalate used in mineral water bottles*, which is attached in the Annex 3.

5.2. MIGRATION IN JUICES

In this section, the influence of storage time and temperature on Sb migration in peach and pineapple bottled juices, was investigated. A systematic study was undertaken to assess the effect of storage time up to 60 days and storage temperatures from 4ºC to 60ºC on the bottled juices. Both total Sb and speciation analysis was carried out using the procedures optimised in section 4.2.1 and 4.2.2.

Two different flavoured PET-bottled juices from a commercial brand were purchased for the migration study. Pineapple and peach juice were selected amongst the samples analysed in section 4.2 as two of the bottled fruit juices most commonly consumed in Spain [Asozumos, 2014]. The effect of different storage temperatures (4ºC, 20ºC, 40ºC and 60ºC) on the migration of total Sb and Sb speciation was performed on the samples. Three bottles of each flavour were stored at the corresponding temperatures and samples were analysed by triplicate analysis on 9 occasions until 60 days, when juice matrix was totally deteriorated. Figure 5.8 depicts a schematic design of this study.

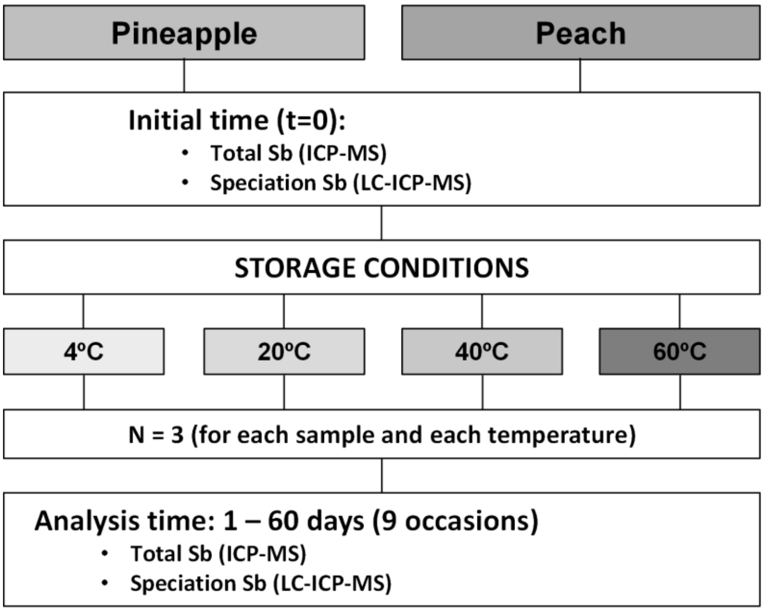


Figure 5.8. Experimental design for the antimony migration study from PET to juice with temperature and time.

5.2.1. Total Sb

At the beginning of the test (day 0) all the samples were analysed and significant amounts of total Sb were present in all of them (0.36 ± 0.01 for peach juice and 0.50 ± 0.02 µg Sb L^{-1} for pineapple juice).

For the samples stored at 4ºC and 20ºC, no variations in Sb concentration were observed over time up to 30 days; and in no case did they exceed the maximum level established by the European Union for drinking water (5.0 µg L^{-1}). Concentrations are depicted in Figure 5.9A. A two factor F-test at the 95% confidence level for the different times assayed showed no significant differences in total Sb concentrations both with respect to time and temperature.

Results of the experiments performed on samples stored at 40ºC and 60ºC are represented in Figure 5.9B, which summarises Sb concentrations in juices over the two months. Samples stored at 40ºC showed a slight increase in Sb concentration over time, reaching a concentration around 0.55 ± 0.01 µg L^{-1} for peach samples and 0.92 ± 0.04 µg L^{-1} for pineapple samples at 30 days of storage. The highest Sb migration was observed in the samples stored at 60ºC, which reached a concentration of 0.95 ± 0.04 µg L^{-1} in peach and 2.71 ± 0.09 µg L^{-1} in pineapple juice, after 21 days of storage.

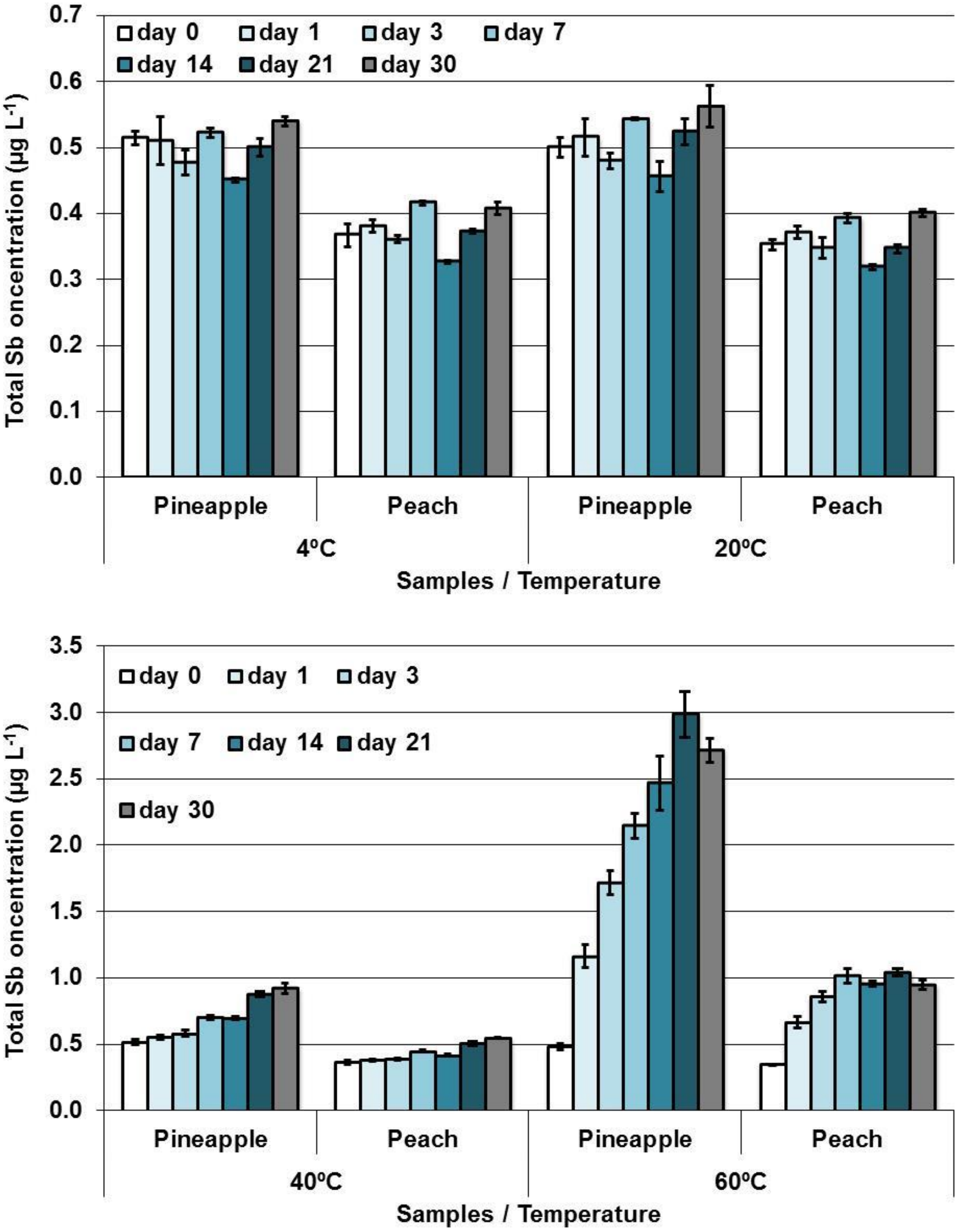


Figure 5.9. Total antimony concentration (µg L^{-1}) in peach and pineapple juice samples stored at 4ºC, 20ºC, 40ºC and 60ºC.

Independently of the expiry date, manufacturers recommend storing the juice at 4ºC and consuming it within a week of opening the bottle, as samples get damaged with time and high temperatures. Therefore, there is concern that long time and high temperature storage could damage the juice. Indeed, such possible effects were observed visually: the juices clearly deteriorated over the length of the experiment. After a month of storage of the bottles, the juices stored at 20ºC appeared spoiled. Meanwhile, after just 7 days of storage, the juices stored at 40ºC and 60ºC had darkened; and from day 30 to the end of the experiment, these juices were almost black and had a foul smell. Moreover, from day 30 to the end of the experiment the Sb concentrations in the juices stored at 40ºC and 60ºC decreased slightly. As many processes are involved in juice decomposition, it is difficult to establish a possible correlation with antimony migration or the causes of further Sb concentration decrease. However, as the juices were already decomposed in the second month of the experiment, there was no use analysing and reporting data from these samples.

The only information regarding the effect of temperature on Sb migration in the literature presents no clear conclusion, as only in some of the juice samples stored at 50ºC for 14 days did the Sb levels increase [Hansen et al 2006b]. In the present work, similar behaviour was always found at a fixed temperature.

The results show again that temperature is the main factor that accelerates Sb migration into a fruit juice, which speeds up the degradation of the PET bottle, as happened in the migration study of water samples. The degradation was again observed visually in samples stored at 60ºC, as the juice bottles presented physical deformation. However, the Sb migration also seemed to be dependent on the matrix, since it is more pronounced in pineapple than in peach juice; as can be observed in Figure 5.9.

The total antimony concentration was represented versus the storage time, as performed by Welle et al., 2011 and Fan et al., 2014 (Figure 5.10). Although juice samples were stored less time than water samples and consequently, fewer data was obtained, it can be concluded that an analogous tendency to that obtained from the water samples was observed. It should be taken into account that these representations reach only 30 days of storage whereas those reported by Welle et al., 2011 arrive till 900 days. Therefore, the current data would represent the beginning of the curves from Welle et al., 2011. The curves obtained from samples stored at 4ºC and 20ºC are less accentuated than those obtained at 40 and 60ºC, as a longer time is needed to reach high concentrations, therefore, they could be considered linear.

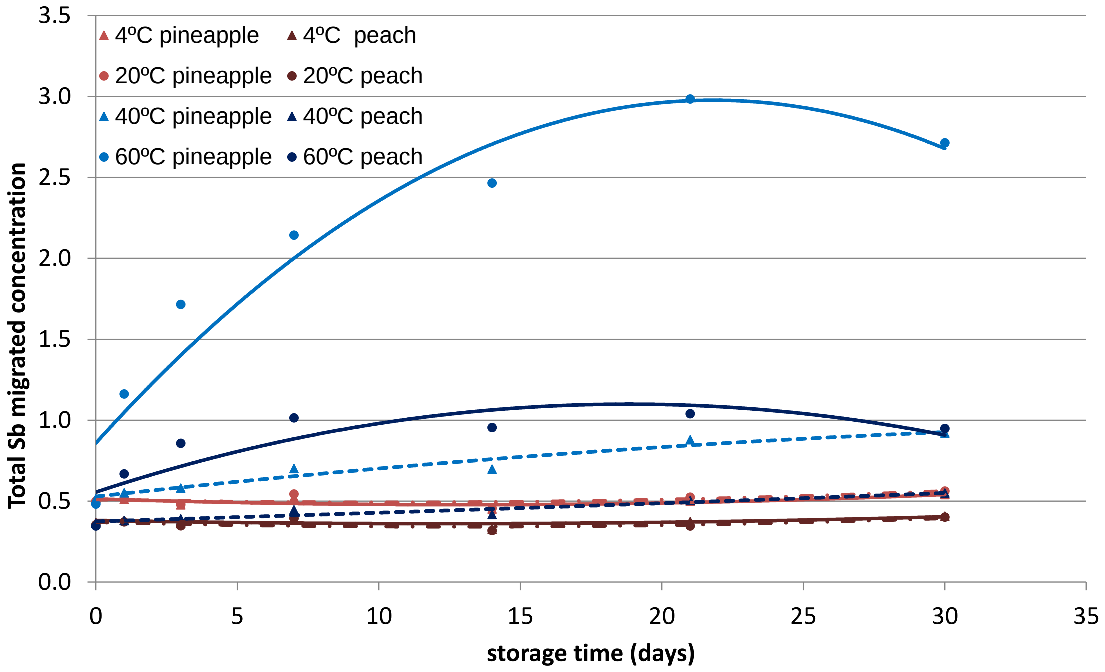


Figure 5.10. Sb migrated concentration versus storage time for peach and pineapple at the temperatures studied.

In addition, kinetic curves for antimony release were constructed according to the exponential equation used in section 5.1.2, proposed by Fan et al., 2014. "C_{max}", "b" constant and releasing rates were calculated with the data obtained in this experiment (Table 5.2).

Table 5.2. Maximum concentrations and kinetic constant of antimony in PET bottled juices used for the migration study

Sample	C_{max} (µg L^{-1})	b (day^{-1})	Releasing rate (µg L^{-1} day^{-1})	R^2
Pineapple 4ºC	0.496	0.438	4.27×10^{-7}	-0.0252
Peach 4ºC	0.304	0.617	1.72×10^{-9}	-0.4315
Pineapple 20ºC	0.501	0.216	1.66×10^{-4}	0.0908
Peach 20ºC	0.355	0.772	2.40×10^{-11}	0.0417
Pineapple 40ºC	1.184	0.031	1.45×10^{-2}	0.9270
Peach 40ºC	0.987	0.620	5.11×10^{-9}	0.8611
Pineapple 60ºC	2.766	0.011	2.19×10^{-2}	0.9500
Peach 60ºC	1.045	0.357	8.33×10^{-6}	0.9670

Once again, kinetic curves at high temperatures matched better with the experimental values as R^2 values obtained were higher. In this case, considerable fewer differences in C_{max} and kinetic constants were obtained throughout temperature, which evidences that temperature is not the unique variable that affects migration in this specific case.

What is more, C_{max} were of the same order of magnitude than the experimental maximum concentrations obtained. This fact demonstrates that all samples have reached the maximum allowable migrated concentration. Sb concentration in samples from day 30 onwards, if they had been possible to be analysed, valued should have been equal or of the same order of magnitude, as the results obtained in the kinetic curve showed that the maximum Sb concentration was reached. Regarding the kinetic constants, values are of the same order in all cases, which means that the migration process is similar at all temperatures. In the case of releasing rate values, considerable different values between types of juice and among temperature were obtained, which evidences the differences between matrices and temperatures experimentally observed. It should be noted that this migration behaviour is different from that observed in mineral water samples. This fact may be due to the differences between matrices and between the PET containers in each case.

All these facts pointed out that the antimony migration behaviour is not only ruled by matrix composition or temperature, but also by the PET container characteristics. This is the possible explanation for the obtained results in mineral waters and juices, which present different PET containers. Further studies are presented in section 5.4.

5.2.2. Sb speciation

The analysis of Sb species in the samples showed that all the juices presented Sb(III) as the major species at any temperature during the whole experiment. The concentration levels followed the same tendency as described for total Sb analysis. Small amounts of non-complexed Sb(V) were also observed near the limit of detection, together with some unidentifiable signals of very low intensity. Storage at 4ºC and 20ºC did not result in a significant evolution of Sb concentrations; while storage at 40ºC and 60ºC presented a slight increase in Sb(III) concentration up to day 30, and a decrease from this point till the end of the experiment due to the reasons mentioned above.

Hansen et al., 2006b obtained similar results, as they found that the major species present in lemon and orange juices was Sb(III). According to those migration results at 50ºC, the antimony increase was mainly due to Sb(III), which is consistent with the results obtained in this study.

Sb(III) average concentrations in the samples stored at 4ºC and 20ºC were 0.13 ± 0.02 and 0.27 ± 0.04 µg Sb L^{-1} for peach and pineapple respectively, which are depicted in Figure 5.11 together with the concentrations in the samples stored at 40ºC and 60ºC. It can be observed that the concentrations of Sb(III) are below of those obtained in the total Sb determination: the peach samples reached Sb concentrations of 0.17 ± 0.01 and 0.18 ± 0.02 µg Sb L^{-1} at 40ºC and 60ºC, respectively; whereas for the pineapple samples, these values are 0.53 ± 0.04 and 1.02 ± 0.09 µg Sb L^{-1}, respectively. RSD values were ≤ 10% in all cases.

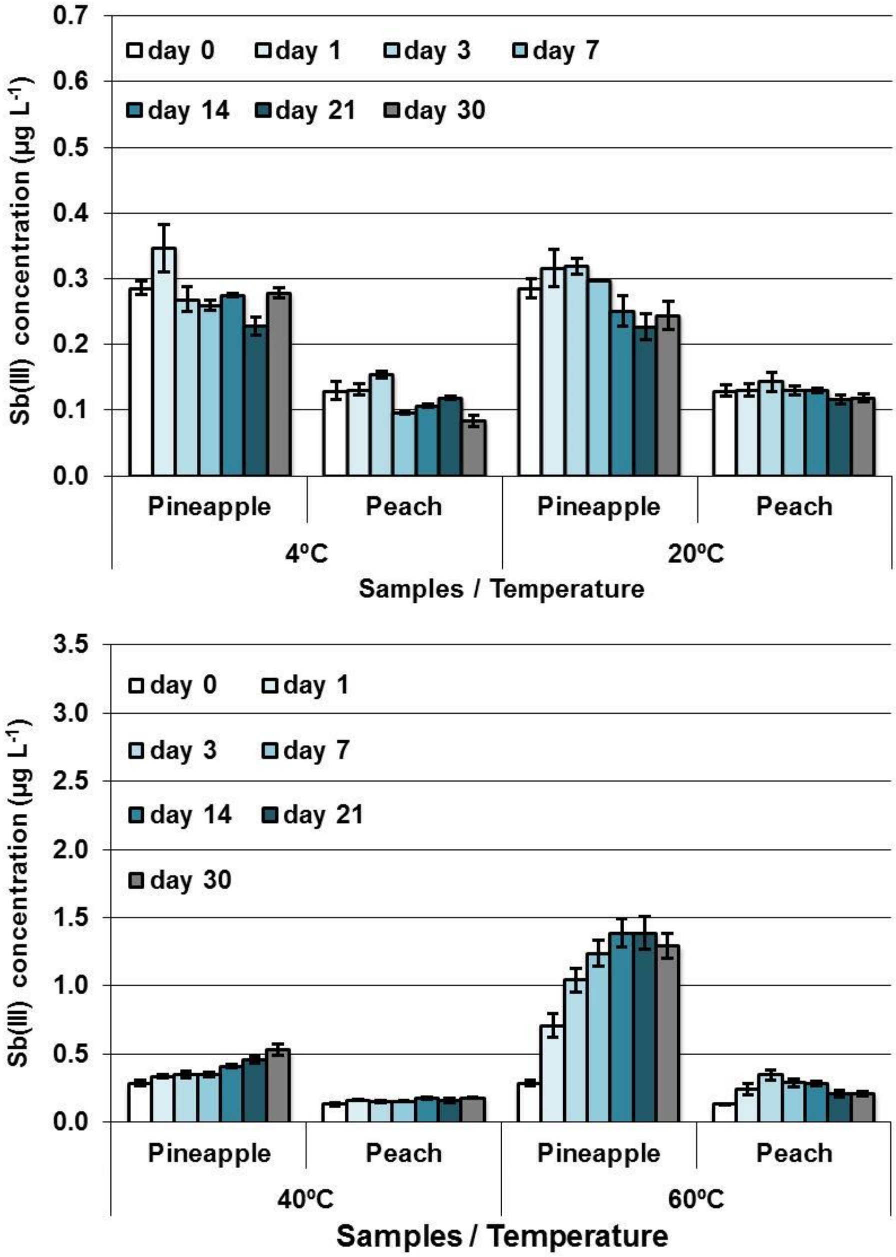


Figure 5.11. Sb(III) concentration (µg L^{-1}) in peach and pineapple juice samples stored at the studied temperatures.

The values obtained throughout the experiment for Sb(III), which is the unique species present at concentrations higher than the LQ, correspond approximately to 40%-65% of the total antimony concentration for pineapple juice and to 20%-45% of the total antimony concentration for peach samples. The lack of mass balance could be attributed to the minors signals which correspond not only to non-complexed Sb(V), but also to unidentified antimony species. Figure 5.12 shows the chromatograms obtained from peach and pineapple juice stored at 60°C at different times. Apart from the increase of the main peak, Sb(III), with time, it can be eye-observed that the baseline from the samples is not plain compared to that obtained from the blank. These slight gain in the background signal may be attributed to different Sb(V) complexes similar to those identified by Hansen et al., 2007 in spiked yogurts and juices, which cannot be separated correctly with the method conditions (strong anion exchange liquid chromatography). Besides, specifically for peach juices, the Sb(III) signal is close to the limit of quantification, where the widespread of expected results can be higher.

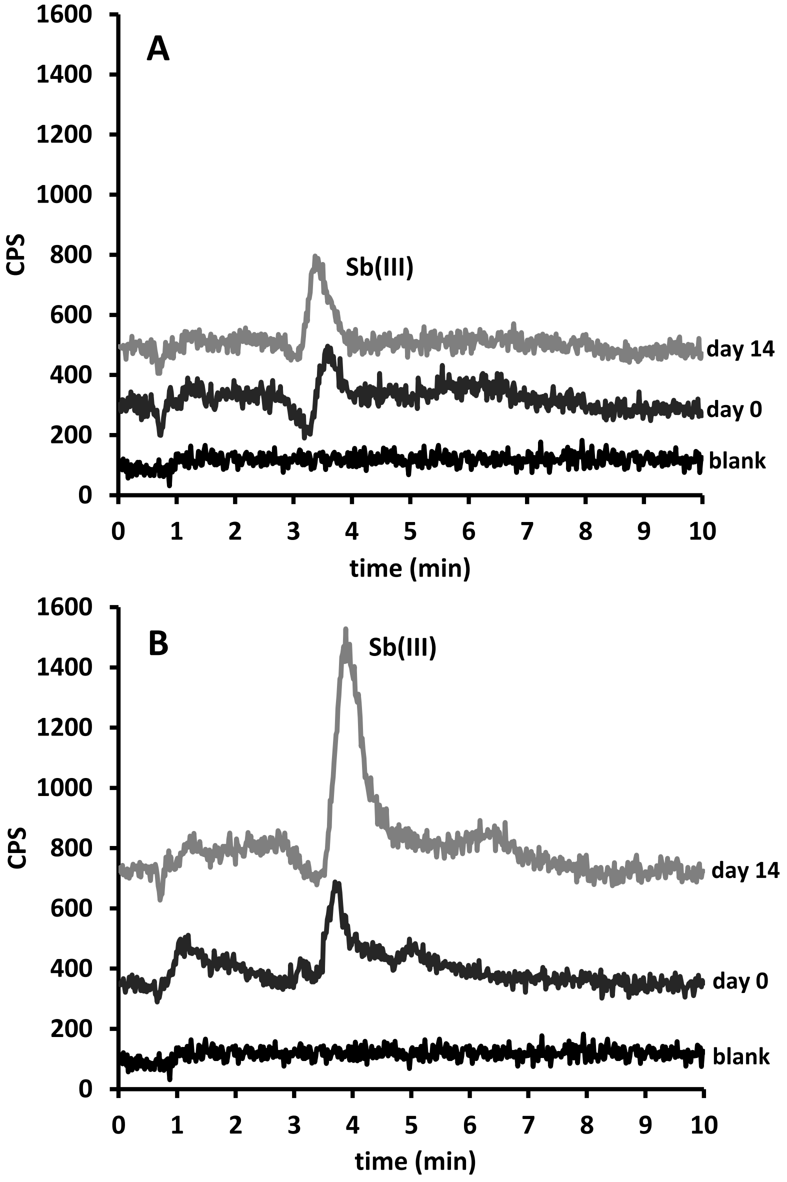


Figure 5.12. Chromatograms obtained by HPLC-ICP-MS from peach juice (A) and pineapple juice (B) stored at 60ºC at different days.

5.3. MIGRATION IN *RAKI*

This section deals with the second part of the work carried out during the research stay in the Laboratory of Speciation Analysis Elements of the Division “Environmental Chemical Processes Laboratory (ECPL)”” from the Department of Chemistry of the University of Crete. The studies performed were focused on the antimony migration in PET-bottled *raki*.

In this section, the influence of storage time and temperature on Sb migration in *raki*, was investigated. Due to sample availability, six PET bottled *raki* samples were selected among the samples analysed in section 4.3: R1, R2, R3, R4, R6 and R7. The effect of storage time up to 2 weeks and a storage temperature of 60ºC was investigated. *Raki* samples were stored in bottles used for mineral water storage of the same brand and type of PET. Both total Sb and speciation analysis was carried out using the same procedures from sections 4.3.1 and 4.3.2. The Sb content was determined at the beginning of the experiment, after 7 and 15 days of storage.

5.3.1. Total Sb

Total antimony concentrations throughout the experiment are plotted in Figure 5.13. *Raki* samples at the beginning of the test (day 0) showed significant concentrations of total Sb (0.5 – 3.9 µg L^{-1}).

After a week of storage at 60ºC, *raki* samples showed a considerable increase in Sb concentration, exceeding the maximum level established by the EU in drinking waters in all cases. Sample R4 showed the highest concentration, reaching a value of 28 µg L^{-1}. After 2 weeks of storage, Sb concentrations keep increasing sharply in samples R1 and R4 reaching values of 36 and 51 µg L^{-1}, respectively, whereas in the rest of the samples this increase was not very pronounced, or even the values obtained were of the same order of magnitude that those obtained after 1 week.

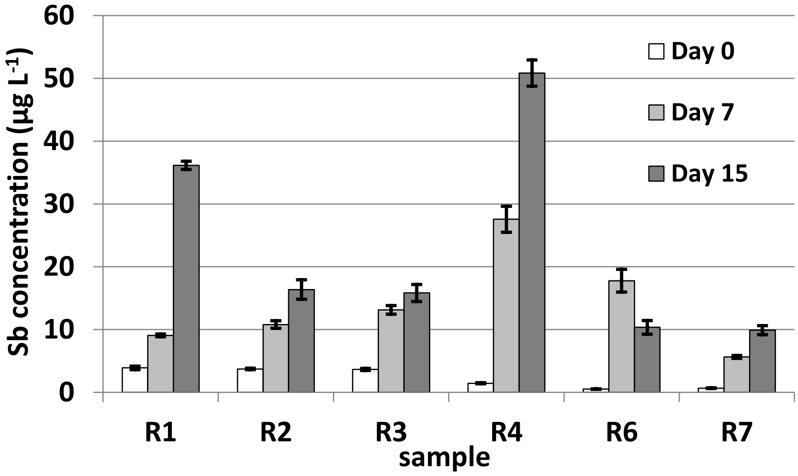


Figure 5.13. Evolution of total Sb concentration in PET bottled *raki* stored at 60ºC for 15 days.

5.3.2. Sb speciation

The analysis of Sb species in *raki* samples during the whole experiment is depicted in Figure 5.14. Concentrations of the organic complex of Sb(V) were calculated by means of the external curve calibration of Sb(V) standards. At the beginning of the experiment, the predominant species was the organic Sb(V) complex in all the *raki* samples. However, after a week of storage, the concentration of this species decreased slightly or even disappeared in the case of R2 sample. Sb(V) concentration increased slightly in all samples (0.2 – 2.1 µg L^{-1}) whereas the Sb(III) one increased sharply (2.9 – 11.6 µg L^{-1}). After 2 weeks of storage, the presence of the organic complex of Sb(V) kept decreasing and disappeared in samples R1, R3 and R4. Sb(V) concentration, with respect to the values obtained after 1 week of storage, increased slightly (0.8 – 26 µg L^{-1}) whereas Sb(III) concentration had no specific behaviour: values increased or decreased depending on the sample. Sample R4 showed the highest concentration, reaching a value of 22 µg L^{-1}.

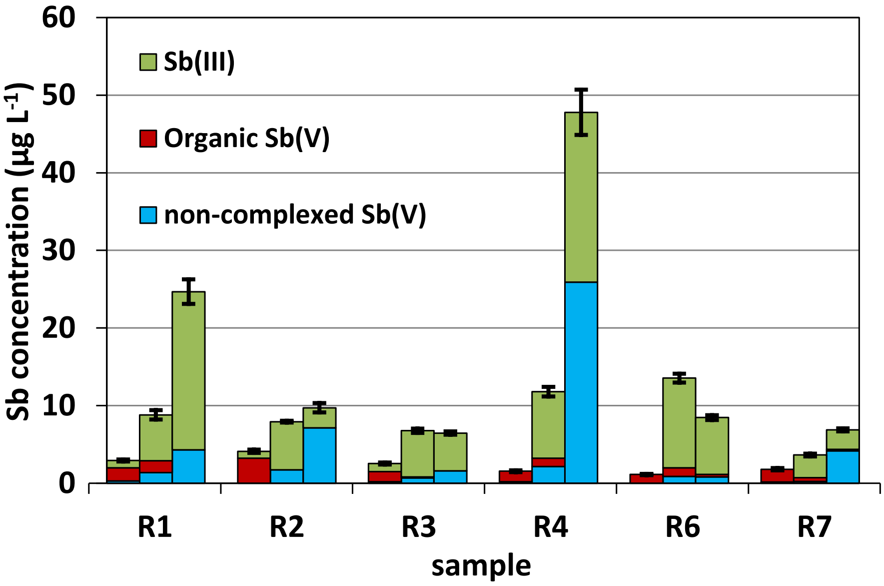


Figure 5.14. Evolution of Sb species concentration in PET bottled *raki* stored at 60ºC for 15 days. For the groups of 3 bars grouped in each X-axis label, each column corresponds to the species concentrations measured at day 0, 7 and 15, from left to right.

This fact could be due to a simultaneous release of antimony from PET and a conversion of the Sb species into others. *Raki* samples present high amounts of allyl alcohols and other organic substances which can stabilise or even complex the antimony released from PET, as demonstrated in section 4.3. However, the exposure at high temperature may decompose this complexes to the inorganic pentavalent species, and simultaneously, Sb(III) may be released and part of it converted into the pentavalent ones. As an example, chromatograms obtained from the *raki* sample R1 are shown in Figure 5.15.

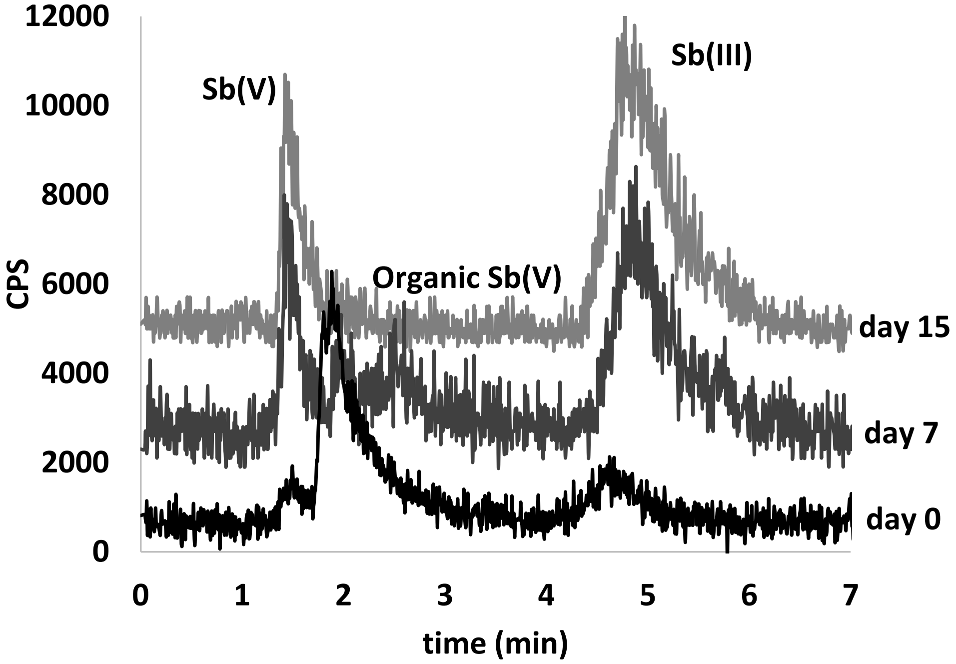


Figure 5.15. Chromatograms obtained from R1 by LC-ICP-MS at days 0, 7, and 15.

Table 5.3 summarises the total Sb concentration and the sum of the species obtained together with the recovery. As observed in section 4.4.2, good recoveries, from 70 to 110%, were observed in some cases, specifically in the 61%, whereas in the 28% the values were slightly lower (down to 40%). Besides, for samples R6 and R7 at day 0 of storage, extremely high recovery values were obtained again, as they present low total concentrations due to the need of a sample dilution to decrease the EtOH content for correct analyses, which led to the quantification around the LQ. These facts demonstrate again that some other minor species could be present in the *raki* samples but not detected, or even not eluted from the column. These high Sb concentrations obtained in the migration experiment exceed the maximum limit of the total antimony in water (5 µg L^{-1}). Moreover, Sb(III) species, the most toxic one, is the predominant in some of the samples. Thus, it is recommendable not to expose spirit beverages at elevated temperatures if they are stored in PET, as their matrix characteristics may easily enhance the antimony migration from PET.

Table 5.3. Total Sb concentration and sum of the species (µg L^{-1}) together with the column recovery in PET bottled *raki* samples stored at 60ºC for 15 days.

Time	Sample	Total Sb	Sum of species	Column recovery (%)
Day 0	R1	3.91 ± 0.28	2.94 ± 0.13	75.3
	R2	3.73 ± 0.12	4.116 ± 0.200	110.4
	R3	3.64 ± 0.16	2.526 ± 0.134	69.5
	R4	1.45 ± 0.09	1.553 ± 0.114	107.5
	R6	0.52 ± 0.02	1.124 ± 0.073	216.5
	R7	0.66 ± 0.04	1.799 ± 0.151	272.4
Day 7	R1	9.07 ± 0.21	8.81 ± 0.62	97.2
	R2	10.79 ± 0.60	7.92 ± 0.12	73.4
	R3	13.13 ± 0.68	6.77 ± 0.28	51.5
	R4	27.57 ± 2.07	11.79 ± 0.62	42.8
	R6	17.77 ± 1.81	13.54 ± 0.57	76.2
	R7	5.63 ± 0.23	3.63 ± 0.17	55.4
Day 15	R1	36.16 ± 0.65	24.68 ± 1.58	68.3
	R2	13.37 ± 1.56	9.72 ± 0.61	59.4
	R3	15.83 ± 1.35	6.46 ± 0.21	40.8
	R4	50.85 ± 2.09	47.79 ± 2.93	94.0
	R6	10.36 ± 1.09	8.46 ± 0.31	81.7
	R7	9.92 ± 0.71	6.87 ± 0.20	69.3

5.4. REMARKS

The migration of antimony from PET bottles to mineral water, fruit juices and *raki* was performed at different times of storage and different temperatures.

Table 5.4 summarises the mean total Sb concentrations obtained in the migration experiments for the matrices described.

Table 5.4. Mean antimony concentrations (µg L^{-1}) obtained for the three matrices studied (mineral water, fruit juice and *raki*) at each storage temperatures throughout days.

Sample	Temperature	**Day 0**	**Day 1**	**Day 3**	**Day 7**	**Day 15**	**Day 21**	**Day 30**
Mineral water	4ºC	0.46	0.32	0.32	0.26	0.19	-	0.38
	20ºC	0.47	0.31	0.35	0.25	0.31	-	0.28
	40ºC	0.42	0.35	0.45	0.42	0.67	-	0.70
	60ºC	0.42	1.05	1.99	2.25	4.66	-	6.06
Fruit juice	4ºC	0.44	0.45	0.42	0.47	0.39	0.44	0.47
	20ºC	0.43	0.44	0.41	0.47	0.39	0.44	0.48
	40ºC	0.44	0.47	0.49	0.57	0.56	0.69	0.74
	60ºC	0.41	0.91	1.29	1.58	1.71	2.01	1.83
Raki	60ºC	2.31	-	-	14.0	23.2	-	-
Sample	Temperature	**Day 50**	**Day 68**	**Day 78**	**Day 97**	**Day 135**	**Day 220**	**-**
Mineral water	4ºC	0.45	0.49	0.51	0.50	0.44	0.50	-
	20ºC	0.51	0.50	0.61	0.60	0.50	0.70	-
	40ºC	1.58	1.72	2.26	2.26	2.30	3.18	-
	60ºC	8.30	11.87	11.40	13.66	16.25	22.07	-

From the results obtained, it can be observed that both mineral water and juices stored at 4ºC and 20ºC presented antimony concentrations of the same order of magnitude during the whole experiment. The mean values calculated are the following: 0.40 µg L^{-1} for water at 4ºC, 0.45 µg L^{-1} for water at 20ºC and 0.44 µg L^{-1} for juices stored both at 4ºC and 20ºC.

Regarding the storage at high temperatures, 40 and 60ºC, it can be observed that both water and juice presented the same trend at 40ºC of storage, as both reached a mean concentration around 0.7 µg L^{-1} at 30 days of storage. However, at 60ºC of storage, mineral water reached higher Sb concentrations throughout time than juice samples. It can be observed that at 30 days of storage, juices presented a mean Sb concentration of 1.83 µg L^{-1}, whereas mineral water presented a mean value of 6.06 µg L^{-1}.

Regarding the migration studies performed on *raki* at 60ºC, it can be observed that the migration of antimony is much higher than the observed in water and juice matrices. A mean concentration of 23 µg L^{-1} was reached in these matrices. This fact could be attributed to several facts, as discussed in section 4.3.1: historical background of the PET-bottle and the samples, or the matrix characteristics, which enhances the potential migration of the antimony from the container.

Regarding speciation results, it was observed in general terms that the main species in mineral water samples throughout the migration experiment was non-complexed Sb(V), whereas in fruit juices was Sb(III). However, for *raki* samples and in some specific cases for mineral waters, the arising of other species was observed. The presence of Sb(III) was detected in water samples stored at 40 and 60ºC after 50 and 3 days of storage, respectively, whereas in *raki* samples at 60ºC, a great variability was observed: the presence of the organic Sb(V) complex decreased throughout days until reaching levels lower than the LD in some cases as Sb(III) concentrations increased together with the presence of non-complexed Sb(V) in some cases.

To summarise, it should be noted that the predominant species in the migration experiment in juice and spirits is Sb(III), the most toxic, whereas in mineral waters is Sb(V). In the case of mineral waters, an oxidation of the trivalent forms to the pentavalent ones process may have occurred after migration. In the case of juices and *raki*, Sb(III) may have been stabilised by some compounds of the matrix, which prevented the oxidation to Sb(V). Moreover, in the case of *raki*, the organic complex of Sb(V) may have dissociated to the non-complexed Sb(V) while simultaneous release of Sb(III) and partially oxidation into the non-complexed Sb(V) may occur because of the elevated temperature.

The maximum level established by the European Union for total antimony in drinking waters (5 µg L^{-1}) was almost reached in mineral waters stored at 60ºC after 15 days of storage, and after this time, the level was exceeded, whereas for juice samples, this level was not exceeded in any case. Regarding *raki* samples, the level was exceeded after one week of storage, obtaining concentrations approximately twice higher than the stipulated value. These facts point out that PET-bottled beverages should not be kept in places where elevated temperature could be reached, since antimony release from PET to the beverage is favoured. Moreover, juice and *raki* matrices tend to stabilise the trivalent forms of antimony, which means that it is preferable not to store these beverages in PET at high temperatures.

Thus, with all the results obtained, it can be concluded that high temperatures are not only the main variable which increases antimony migration from PET bottles. Matrix characteristics have an important role, as significant differences were observed in the Sb concentration throughout days at 60ºC among the matrices studies in this thesis. In addition, the type of PET is equal or even more important than matrix, as the experimental results obtained in mineral waters and fruit juices were consistent with the predicted model of Welle et al., 2011, which contemplate several PET bottle characteristics. In order to get a better understanding of the Sb migration process considering the potential variables altogether, a new experimental approach was undertaken.

5.4.1. Crossed migration experiments

In order to study in which extent Sb migration is ruled by matrix and PET characteristics, a crossed migration experiment was carried out using the same brands of mineral drinking water (CL, LB and DB) and fruit juice (pineapple and peach) previously studied. For this experiment, five 0.33 L containers of each brand were used (four brands: PET-CL, PET-LB, PET-DB and PET-Juice). The content of each container was discarded and bottles were cleaned three times with double deionised water and dried at room temperature. Subsequently, each set of containers of a brand (for instance, PET-CL) were filled with all studied matrices (CL, LB, DB, peach and pineapple), that is, each matrix was put inside all types of PET containers. For filling, a 1.5L container of the studied samples was used. The content of the 1.5L containers was initially characterised in terms of total and speciation Sb content by ICP-MS and LC-ICP-MS, respectively.

After filling, they were stored at 60ºC for 30 days. Total Sb and speciation were analysed by ICP-MS and LC-ICP-MS, respectively after 7, 15 and 30 days of storage. As reference, another set of bottles, prepared as previously described, were kept at 20ºC and total Sb content was measured after 30 days of storage.

The concentrations obtained at the beginning of the experiment, before the storage of the samples in the respective small PET bottles (day 0) are summarised in Table 5.4. Total Sb concentrations in waters were slightly lower than those obtained in juices. Regarding speciation, the results obtained were the same than those obtained in the migration experiments described in previous sections 5.1 and 5.2: non-complexed Sb(V) was the only species present in waters whereas in juices, Sb(III) was the predominant species and non-complexed Sb(V) was present under the LQ.

Table 5.4. Antimony concentrations obtained in water samples (CL, LB and DB), and juice samples (pineapple and peach flavour) at the beginning of the experiment (day 0). Results are expressed as mean Sb value ± Standard Deviation, and the relative standard deviations are expressed in % in parentheses.

Sample	**Total Sb (μg L^{-1})**	**Sb(V) (μg L^{-1})**	**Sb (III) (μg L^{-1})**
CL (water)	0.47 ± 0.03 (6.9%)	0.46 ± 0.02 (5.0%)	< LD
LB (water)	0.61 ± 0.04 (6.2%)	0.63 ± 0.06 (8.9%)	< LD
DB (water)	0.49 ± 0.05 (9.8%)	0.50 ± 0.05 (9.2%)	< LD
Peach (juice)	0.99 ± 0.11 (10.7%)	< LQ	0.74 ± 0.04 (5.7%)
Pineapple (juice)	1.45 ± 0.15 (10.1%)	< LQ	1.09 ± 0.03 (3.0%)

Regarding the results obtained after 30 days of storage at 20ºC, summarised in Figure 5.15 together with the Sb concentrations obtained at day 0, they were in agreement with the previous migration studies, regardless PET type container: total Sb concentration did not increased throughout time. This fact evidences that no Sb migration was observed at room temperature independently of matrix sample and container type. Antimony concentrations in water samples ranged approximately from 0.4 to 0.7, from 0.9 to 1.2 in peach samples and from 1.3 to 1.6 in pineapple samples.

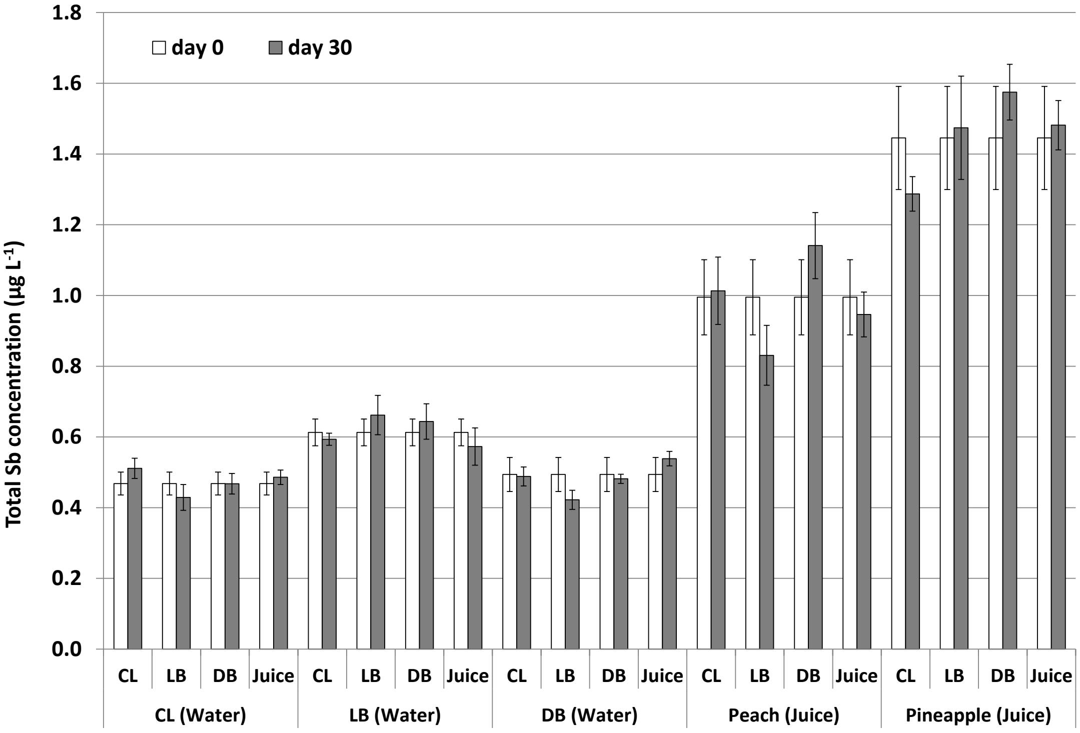


Figure 5.15. Total Sb concentration obtained in the cross migration experiment at 20ºC. In X-axis, the outer labels correspond to the beverage (water or juice) and the inner labels correspond to the PET-bottle.

The total Sb concentrations obtained at 60ºC with time are summarised in Figure 5.16. Firstly, as expected, antimony concentrations showed a significant increase throughout days. Secondly, it can be observed that samples stored in bottles used for keeping mineral water presented a more pronounced migration than those stored in bottles used for keeping juice (the last group of columns for each matrix), as they reach higher Sb concentrations throughout days. After 30 days of storage, mineral water samples stored in clear and dark blue PET bottles reach concentrations between 8 and 10 µg L^{-1}, when stored in light blue PET bottles the concentration reached is approximately 4 µg L^{-1}, and when stored in juice PET-type bottles it is smaller (around 2 µg L^{-1}).

Peach juice samples stored in clear PET bottles reach a concentration around 5 µg L^{-1}, when stored in light and dark blue PET bottles around 3 µg L^{-1}, and when stored in juice PET-type bottles lower than 2 µg L^{-1}. Regarding pineapple juice, Sb concentrations when storing in clear and dark blue PET bottles are approximately 15 µg L^{-1}, when storing in light blue PET bottles around 7 µg L^{-1}, whereas when the sample is in the juice PET-type bottle the concentration is smaller (around 5 µg L^{-1}).

According to these results, it can be concluded on the one hand that antimony release depends on the PET type where the sample is stored, as it has been demonstrated samples stored in clear and dark blue bottles suffer more migration potential, followed by the light blue ones and then by the juice bottles. On the other hand, it can also be concluded that focusing on each PET container, the antimony migration is also dependent on the sample matrix, as antimony concentrations after 30 days of storage were higher in pineapple juice samples, followed by the mineral waters, and finally by the peach juice.

Regarding the speciation results, antimony species concentrations obtained in the cross migration experiment are summarised in Figure 5.17. For water samples, as mentioned before, non-complexed Sb(V) was the only species present at the beginning of the experiment. After storage time non-complexed Sb(V) concentration keep increasing and Sb(III) species appeared in waters stored in clear, light blue and dark blue PET bottles, those which are used for keeping mineral water. Sb(III) species was not observed in waters stored in juice PET-type bottles. Column recoveries of the sum of species calculated with respect to the total Sb concentration were generally between 90 and 100% except in some cases, in which the values are between 80 and 90%.

For juice samples, non-complexed Sb(V), under the limit of quantification, and Sb(III) were the species present at the beginning of the experiment. After the storage at 60ºC, Sb(III) concentrations increased and continued being the main species present in the samples. Non-complexed Sb(V) concentrations increased and reached values slightly higher than the limit of quantification. Besides, in pineapple juice samples stored in clear, light blue and dark blue PET bottles, Sb(V)-citrate species appeared, reaching values between 1 and 1.5 µg L^{-1} at the end of the experiment. Column recoveries calculated for juice samples, ranged between 70 and 85%, which are acceptable for speciation in complex matrices.

Crossed-migration experiments pointed out that antimony migration behaviour can only be explained taking into account both matrix composition (content) and PET bottle characteristics (container). The variety of the species observed in the cross migration experiment could be attributed to both the form in which antimony is present in the PET, to which species are extracted and also to the oxidation or stabilisation of the species by the sample matrix. As all these processes may occur simultaneously, it could not be discerned whether antimony is present in PET as pentavalent, trivalent or both oxidation states.

The crossed-migration strategy used to study antimony release proved to be a useful approach to assess the possible role of the matrix and the PET type simultaneously. Both, the proposed methodology and the obtained results, could be considered when updating the present legislation with migration assays using food simulants, as it only considers total concentrations, and there is a lack of information about the performance of the migration assays, such as the sample/extractant ratio recommended to run the experiment.

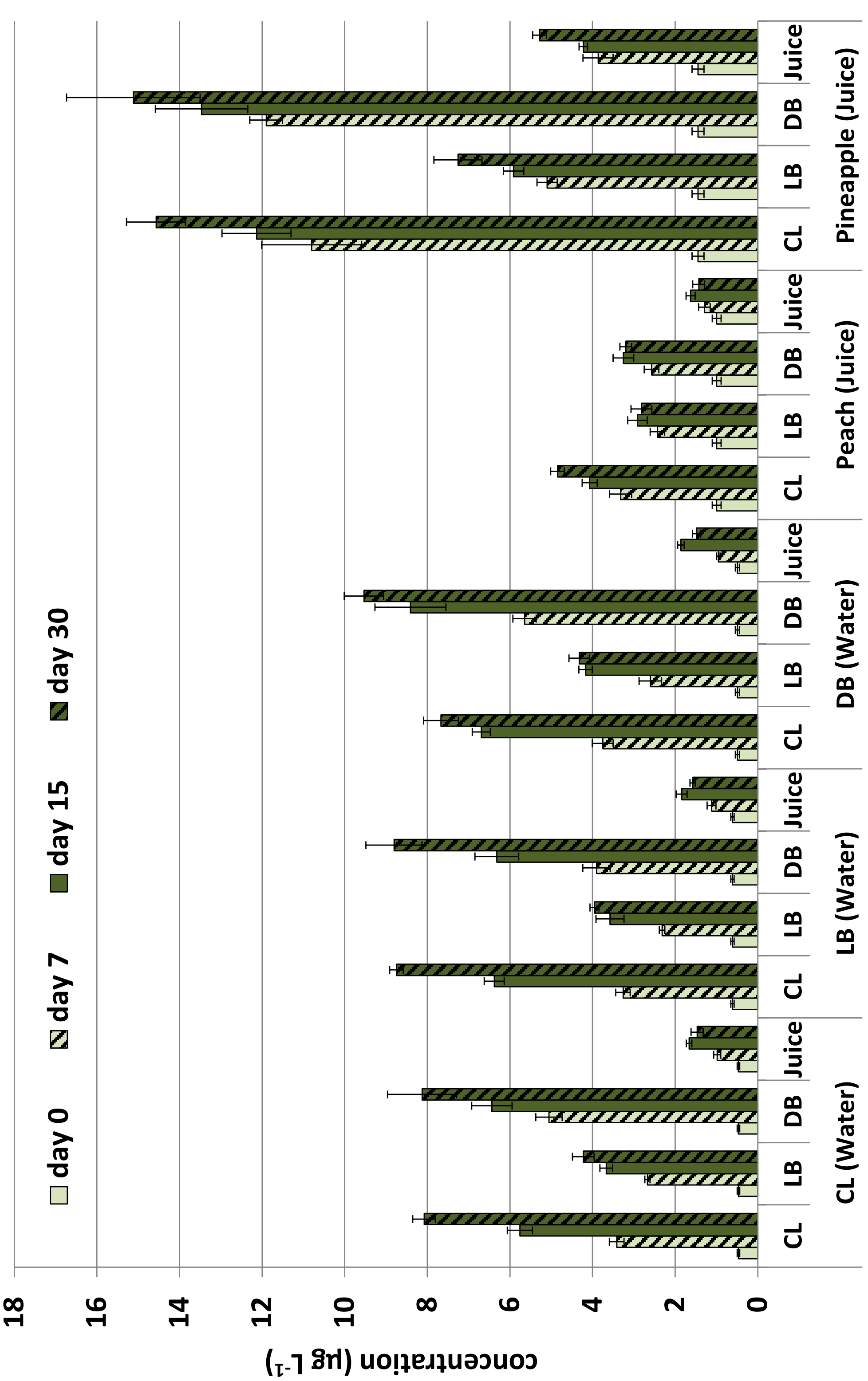


Figure 5.16. Total Sb concentrations in drinking mineral waters and fruit juices samples used for the cross migration experiment, stored at 60ºC for one month. In X-axis, the outer labels correspond to the beverage (water or juice) and the inner labels correspond to the PET-bottle.

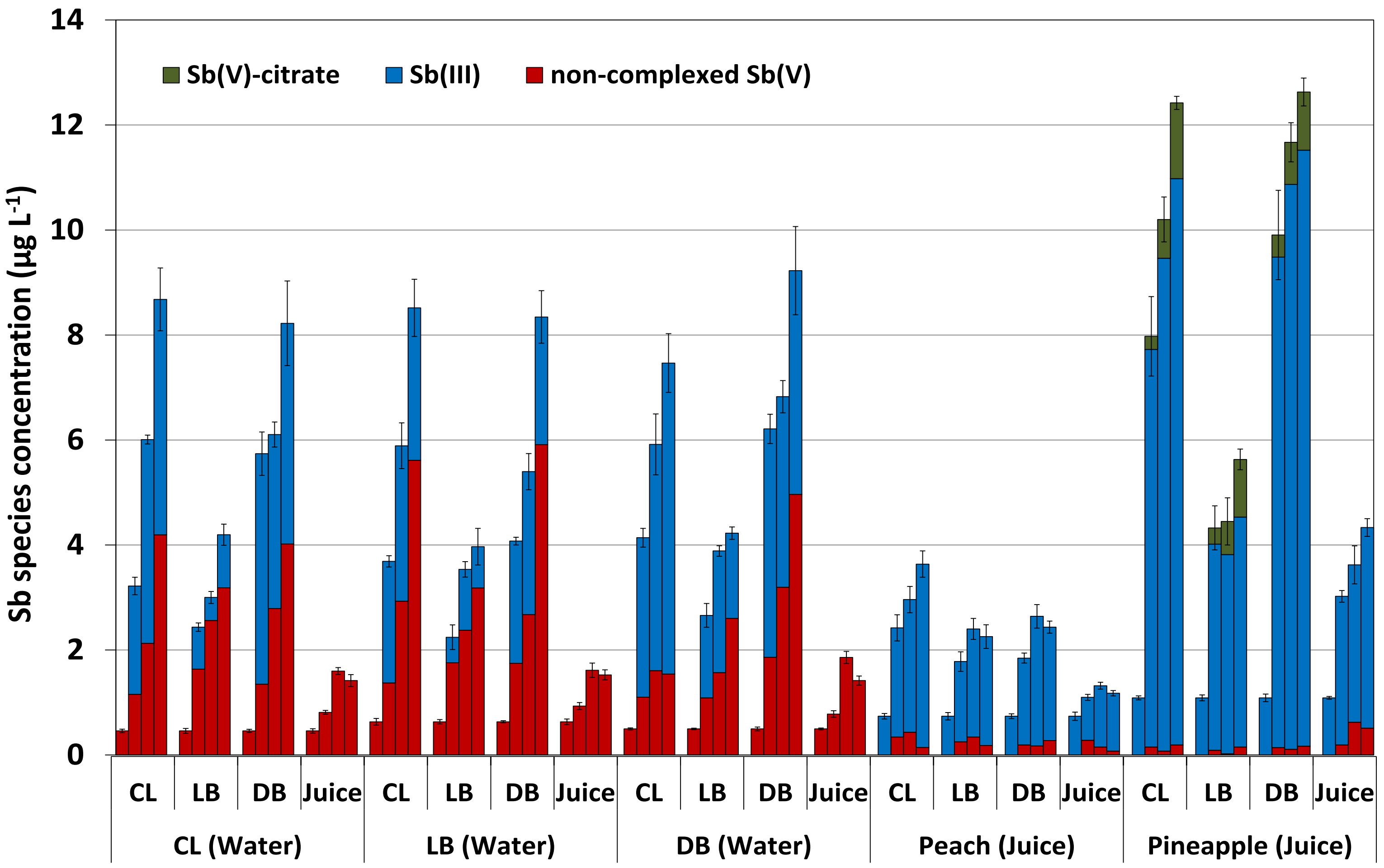


Figure 5.17. Sb species concentrations in drinking mineral waters and fruit juices samples used for the cross migration experiment, stored at 60ºC for one month. In X-axis, the outer labels correspond to the beverage (water or juice) and the inner labels correspond to the PET-bottle. For the groups of 4 bars grouped in each inner label, each column corresponds to the species concentrations measured at day 0, 7, 15 and 30, from left to right.

Chapter VI

CONCLUSIONS

The following conclusions can be drawn from the results obtained and discoursed in the present dissertation:

Regarding the analytical methodology:

1. A chromatographic method for the separation and determination of inorganic antimony species, Sb(V) and Sb(III), has been developed by LC-HG-AFS and LC-ICP-MS with a short analysis time, 2.5 and 5.5, respectively, which allows the analysis of a large number of samples in a short period of time.
2. An online preconcentration method for Sb(V) and Sb(III) determination, the most relevant antimony species present in the environment was developed, using a 5-cm Hamilton PRP-X100 (anion exchange) precolumn in the preconcentration step. These precolumns are commercially available and widely used in routine laboratory analysis. The method requires a sample volume of 20 mL and a simple sample pretreatment (addition of 2-mercaptoethanol 0.22 µmol L^{-1}). The online preconcentration LC-HG-AFS system provides recoveries of 14 ± 1% and 54 ± 2% for Sb(V) and Sb(III), respectively, These recoveries were obtained regardless of the matrix composition.
3. An online preconcentration method for $MeHg^+$, Hg(II), and $EtHg^+$ determination has been developed using a 2-cm ODS Hypersil (C18, reverse phase) precolumn in the preconcentration step. The method provides good recoveries (>70%) regardless of the matrix composition. Low sample volume is required and sample treatment consist only of the addition of 2-mercaptoethanol.
4. The limits of detection and quantification established for the preconcentration methods are suitable for the analysis using environmental samples at the ng L^{-1} level. The Hg-preconcentration method is widely applicable, highly precise and accurate, and could be useful for $MeHg^+$, $EtHg^+$ and Hg(II) determination, in response to any future legislation on mercury species whereas the Sb-preconcentration method needs the improvement of the preconcentration efficiency, especially for Sb(V) species.

Regarding the occurrence of antimony and mercury in beverages and foodstuffs:

1. Sb in waters:
    a. Reproducible and reliable determination of antimony in PET bottled waters has been assessed by HG-AFS in around 40 samples of different origin, obtaining significant amount of Sb due to the migration from the bottle. After one year of storage at room temperature, Sb content showed a significant increase. However, the maximum level established by European legislation was not exceeded in any case.
    b. Speciation analysis showed that Sb(V) was present in all the samples, with no significant differences with the total content, whereas Sb(III) was not detected in any case.

   c. The use of correlation analysis and PCA revealed that a weak correlation was established between Sb concentration and the variables mineralisation degree and colour whereas no correlation was found between Sb concentration and the pH range studied.

2. Sb in juices:
   a. The determination of total antimony in juices was carried out by ICP-MS with a simple sample pretreatment, centrifugation and subsequent filtration of the samples, providing reproducible and reliable results.
   b. The identification and determination of antimony species was also successfully performed, being the trivalent forms the predominant in the samples. This form is present in samples as a 1:2 Sb(III)-citrate complex. Citrate is displaced by the EDTA from the mobile phase yielding a 1:1 Sb(III)-EDTA complex during the analysis by LC-ICP-MS, and it is a matter of concern, as it is the most toxic form of antimony.
   c. The study of the formation of antimony complexes in pineapple and peach samples showed the formation of 1:1 Sb(V)-citrate and 1:2 Sb(III)-citrate complexes when spiking with non-complexed Sb(V) and Sb(III), respectively.

3. Sb in *raki*:
   a. Reliable and reproducible determination of antimony in spirit samples has been assessed by ICP-MS with a direct injection of samples. Concentrations up to 4 µg L^{-1} were found, which is close to the maximum limit established by the EU in drinking waters.
   b. The predominant antimony species is the pentavalent one in the form of an organic Sb(V) complex.
   c. The elucidation of the structure of the organic Sb(V) complex was established using HRMS. The proposed structure resulted in a six-coordination complex with m/z 474.89456, taking as ligands one molecule of pyruvic acid and two molecules of a reaction product between sulphites and ethanol from the spirit matrix.

4. Sb in PET bottles:
   a. Concentrations of total Sb in PET material used for water and juice bottling were between 200 and 300 mg kg^{-1}, regardless of the PET type.
   b. An “end over end” extraction process overnight yielded comparable results to those obtained when performing the migration assays described in the legislation, which take a longer time. Only small quantities of antimony compared with the total content were extracted.
   c. Sb(V) was the only species extracted. This fact was could be related to a partial oxidation of Sb(III) into Sb(V).

   d. The comparison between the total Sb concentrations in PET with those obtained in waters, juices and spirits, points out that only small amounts of Sb migrate into the foodstuff.
   e. The determination of the structure of the Sb compounds present in the PET surface was attempted by DESI-HRMS. The total dissolution of PET in HFIP provided good mass spectra, showing several ions from the PET matrix, but not from the Sb compounds.

5. <u>Hg in seafood</u>:
   a. The presence of mercury in different seafood samples was found. The present study determined total Hg, $MeHg^+$ and Hg(II) species in fish, crustaceans and bivalves. As expected, $MeHg^+$ was the predominant species in all fish samples.
   b. Figures of merit (LD, LQ, reproducibility and trueness) of the proposed LC-UV-CV-AFS method were satisfactory for the determination of $MeHg^+$ and Hg(II) in fish and shellfish.
   c. All $MeHg^+$ concentrations are below the maximum levels set by Regulation (EC) No. 1881/2006 except for two Brazilian and two Spanish fish samples.
   d. The present results have increased the availability of reliable results on $MeHg^+$ in seafood and could be used in further Directives on $MeHg^+$ in food commodities. Thus, the present method could be a valuable tool for food control laboratories that assess $MeHg^+$ in seafood samples.

Regarding the antimony migration studies:

1. Antimony migration at low or room temperature in mineral waters and fruit juices is insignificant and independent from the container in which they are stored, as Sb concentrations did not increase throughout time.
2. Significant amounts of Sb migrated into the water when bottles were stored at 60ºC and remained in the water matrix once they had been released.
3. Migration of antimony from the plastic into the mineral water and juice gives rise to concentrations under the maximum allowed by the EU at storage temperatures below 60ºC, whereas at 60ºC Sb migration is more rapid and the limit is exceeded in 15 days in water samples.
4. The migration data is in good agreement with the mathematical models proposed in the literature, concerning mainly the PET container characteristics.
5. Sample matrix also affects migration, as antimony release in pineapple juice is greater than in peach juice.
6. Sb(V) was the predominant species in mineral waters whereas Sb(III) was the main species in juices. Although the most toxic species, Sb(III), was also present in mineral waters at 40

and 60ºC from the fiftieth and the third day, respectively, its presence decreased onwards due to the oxidation of Sb(III) to Sb (V).

7. Regarding migration studies in *raki*, higher concentrations than those obtained in water and juice were obtained after two weeks of storage at 60ºC, far exceeding the limit stipulated for waters in just one week. Speciation analysis showed the dissociation of the unknown complex of Sb(V) into the non-complexed Sb(V) and simultaneous release of Sb(III) and partial oxidation of the trivalent species into the pentavalent ones.
8. The cross migration experiment demonstrated that the type of PET has the same importance, or even more, on antimony migration than matrix characteristics, as the same samples stored in different kinds of bottles not only showed different migration potential, but also different species were observed. Thus, for a correct performance of migration experiments, the studied variables which potentially affect Sb migration (temperature, matrix and PET type) should be assessed altogether.
9. It is highly recommendable not to expose PET-bottled beverages at elevated temperatures, as a rapid release of antimony has been proved, exceeding the maximum limit established for waters in some cases. The results obtained in this thesis could be used in the proposal of further Directives on Sb in other beverages.

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ANNEXES

ANNEX 1

Article: *Mercury(II) and methylmercury determination in water by liquid chromatography hyphenated to cold vapour atomic fluorescence spectrometry after online short-column preconcentration*

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Mercury(II) and methylmercury determination in water by liquid chromatography hyphenated to cold vapour atomic fluorescence spectrometry after online short-column preconcentration

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This paper reports a method developed for the simultaneous determination of methylmercury ($MeHg^+$) and mercury(II) (Hg^{2+}) species in water by liquid chromatography coupled to online UV irradiation and cold vapour atomic fluorescence spectrometry (LC-UV-CV-AFS) after online short-column preconcentration. This work focused on systematic studies of several variables to establish the maximum species recoveries, preconcentration factors and good reproducibility. The optimum results obtained were the following: 0.07 mmol L^{-1} 2-mercaptoethanol as a complexing agent, precolumn conditioning with the mobile phase: a mixture of 80% of methanol (MeOH) and 20% of the following buffer: 0.0015 mol L^{-1} ammonium pyrrolidine dithiocarbamate (APDC) and 0.01 mol L^{-1} ammonium acetate (NH_4CH_3COO) at pH 5.5, 2 cm precolumn length and 2 mL min^{-1} sample flow. This method was applied to three water samples with different mineralisation contents. Various tests, based on spikes, were performed on each sample. A breakthrough volume of 4 mL was found. The recovery values of 72 ± 3% and 81 ± 5% for $MeHg^+$ and Hg^{2+}, respectively, were obtained regardless of the matrix composition, and the PF values were 30 and 32 for $MeHg^+$ and Hg^{2+}, respectively. The accuracy of the preconcentration method was verified by analysing a certified reference material. The detection limits (LDs) obtained were 15 ng L^{-1} for $MeHg^+$ and 2 ng L^{-1} for Hg^{2+}. The quantification limits (LQs) were 50 ng L^{-1} for both species. The established analytical online preconcentration method is suitable for the quantification of mercury species in a wide range of environmental waters.

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Introduction

The determination of mercury species in environmental samples is of global concern, because of their natural persistence in the environment and the distinct mechanisms whereby they change their chemical form,[1] which affects their distribution and toxicity. The most relevant species in the environment are elemental mercury (Hg^0), mercury(II) (Hg^{2+}), monomethylmercury (CH_3Hg^+, $MeHg^+$), dimethylmercury (DMeHg) and monoethylmercury ($EtHg^+$). Organic mercury compounds tend to be much more toxic than mercury(II), and mercury(II) is more toxic than the elemental form. $MeHg^+$ is the form in which mercury accumulates and biomagnifies in the aquatic food chain due to its high liposolubility.[2] It is absorbed, transported through biological membranes and accumulated in nerve cells. Due to the decrease in the production and use of organomercurials, methylmercury ($MeHg^+$) is by far the most common organomercury compound in the environment.[3]

Mercury is released into the environment from both natural sources and as a result of human activities. Once it has entered the environment, mercury cycles occur between air, land and water. In these cycles, mercury species may be converted.[1] A relevant transformation process in aquatic environments is the conversion of mercury(II) into monomethylmercury by microorganisms and microalgae.[4] Therefore, water is one of the most relevant studied environmental compartments. It not only has a great impact on the environment, but also on human health because safe water is essential to human activity.

The European Water Directive,[5] which seeks to establish a framework for the protection of groundwater and surface water, includes mercury and its compounds in a list of priority and hazardous substances. Therefore, it is one of the elements to be considered in the evaluation of the status of physico-chemical water quality. However, at present, the European Drinking Water Directive considers only total mercury concentration, and establishes a parametric value of 1 μg L^{-1}.[6]

Mercury concentrations in water are expected to be very low.[7] Besides, methylmercury levels tend to be much lower than those of mercury(II), due to the decomposition of organic compounds by solar UV light and the difficulty of methylation reactions in the aqueous phase. The mean reported for the Hg concentration in water is 2 ng L^{-1}.[8] The $MeHg^+$ concentration corresponds to 1% of

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this value, and the rest is mercury(II). The concentration of mercury is normally in the range of 1–20 ng L^{-1} in open-ocean water, while up to 100 ng L^{-1} is usually found in coastal water, owing to anthropogenic discharges.[9] In the literature, analytical methods using CV-AFS or CV-AAS detectors without a preconcentration step have limits of quantification higher than the Hg concentrations in water.[3,10–13] Therefore, because of the extremely low concentrations of mercury in this type of sample, highly sensitive, simple and rapid methods are required. Consequently, preconcentration systems need to be developed.

Several extraction and preconcentration methods have been reported for the enrichment of mercury species applied mainly in environmental waters. The main approaches for the preconcentration of trace elements from water are liquid–liquid extraction (LLE)[14] and solid phase extraction (SPE). Comparatively, SPE is more environmentally friendly, as it is free of toxic organic extraction reagents. Most importantly, its stronger tolerance to complex matrices endows it with better capability of online applications.[9] In solid phase extraction as a preconcentration step, C18 cartridges have been the most widely used stationary phase, both directly and after derivatisation[15–24] with a wide range of complexing agents, most of which contain sulphur, such as 2-mercaptoalcohols, dithiocarbamates, dithizones, triazenes, and even bacteria.[15–32] A wide variety of eluting agents have also been used to desorb mercury species from the stationary phase, such as acidic solutions, thiourea solutions, mobile phases with organic-modifiers, aqueous solutions with a reagent containing sulphur, and even a mixture of these kinds of solutions with an organic solvent, among others.[9,14–35]

After elution, a separation procedure has sometimes been applied. In some cases, gas-chromatography or liquid chromatography was performed to separate mercury species.[9,15–18,20–22,24,25,29,33,36] In others, selective retention or elution of mercury species was carried out using different complexing agents or eluting agents for each species.[19,26,28,30,31,34,35] A wide variety of detectors have been used, either for offline preconcentration or online flow injection preconcentration. Ultraviolet detection (UV), ICP-MS and atomic absorption or fluorescence spectrometry with cold vapour generation (CV-AAS and CV-AFS) are the most relevant systems of detection reported.[9,15–24,26–36] ICP-MS is the most sensitive of these detection methods. However, an online preconcentration system coupled to CV-AFS could provide a similar analytical performance by using a simpler set-up and with a lower investment.

As the reported methods for the mercury preconcentration are mainly applied to natural waters, such as sea, river, spring, lake, rain and underground waters, among others, there is a lack of studies applied to drinking water. A few studies are applied to tap water. Thus, the aim of this paper is to develop an online method for mercury(II) and methylmercury determination by high-performance liquid chromatography hyphenated to cold vapour atomic fluorescence spectrometry after short-column preconcentration. The established method was applied to determine mercury species in drinking water samples of different matrix compositions, including a certified reference material of wastewater.

Experimental

Instrumentation

The LC system consisted of a quaternary pump and degasser (Agilent Technologies 1100, Waldbronn, Germany), equipped with a manual stainless steel sampler injector (Rheodyne Model 7725i) and a 100 μL sample loop.

The separation of mercury species (Hg^{2+} and $MeHg^+$) was achieved in an analytical RP-C18 column (ODS Hypersyl 250 mm × 4.6 mm id, 5 μm, Thermo Hypersil-Keystone).

After separation, a photo-oxidation step was performed in a 12 meter length × 0.5 mm id PTFE tube coiled around a UV lamp with a power irradiation of 150 W (Heraeus TQ 150).

The reduction step was achieved in a cold vapour generator (CV) 10004 (P.S. Analytical, Orpington, UK), in which the effluent was mixed with the reducing agent. The metallic mercury vapour that was obtained reached the gas–liquid separator, from which it was dragged into the detector by an argon stream (300 mL min^{-1}) and dried in a PermaPure membrane with nitrogen (2.5 L min^{-1}). Measurements were carried out using a Merlin Mercury Atomic Fluorescence Detector model 10023 (P.S. Analytical).

Reagents and standards

Only analytical grade reagents were used. The standards and reagents in this study were prepared with doubly deionised water (Elix & Rios 5–15 MΩ cm^{-1}, total organic carbon < 30 μg L^{-1}) obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

A mercury(II) stock standard solution of 1000 mg L^{-1} was prepared by dissolving appropriate amounts of mercury chloride and $HgCl_2$ (Merck, Darmstadt, Germany) in 1% (v/v) HNO_3, from nitric acid 69% (Panreac, Hiperpur). A methylmercury stock standard solution of 1000 mg L^{-1} was prepared by dissolving appropriate amounts of CH_3HgCl (Carlo Erba, Milan, Italy) in 3% methanol (Panreac, p.a.). All stock standard solutions were stored at 4 °C. The working standard solutions were prepared daily from the stock standard solutions by appropriate dilution.

For the cold vapour generation, $SnCl_2$ solution was prepared daily from tin chloride 2-hydrate (Panreac, p.a.) to 1.5% concentration, in 4% of HCl, from hydrochloric acid 35% (Panreac, Hiperpur).

The mobile phase was prepared daily by dissolving appropriate amounts of ammonium pyrrolidine dithiocarbamate, APDC (Fluka, p.a.), ammonium acetate, and NH_4CH_3COO (Merck, p.a.) in water. The pH was adjusted to 5.5 with diluted acetic acid (Panreac, p.a.) and then filtered on a 0.45 μm filter paper (Millipore type HA). The final mobile phase composition was a mixture of 80% of MeOH LC gradient grade (Panreac, p.a.) and the prepared buffer: 0.0015 mol L^{-1} APDC and 0.01 mol L^{-1} NH_4CH_3COO.

For the preconcentration step, appropriate amounts of 2-mercaptoethanol and APDC (Fluka, p.a.) were used as a complexing agent for mercury species in working solutions and water samples.

Certified reference material (CRM) of wastewater effluent acidified with HNO_3 to about pH 2 to stabilise the trace amounts (ERM-CA713) was used for quality control. It was obtained from the Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre, Geel, Belgium.

Samples

Three samples, tap water and weak and strong mineralised bottled waters, were filtered through a filter with a pore size of 0.22 µm. The origin, pH and conductivity values for each sample after filtration are shown in Table 1, together with some anion and cation content determined by anionic exchange chromatography and ICP-OES, respectively. The final solutions of 0.5 µg L^{-1} and 5 µg L^{-1} for the two mercury species with the appropriate amount of complexing agent were prepared by making up the volume with the corresponding water matrix: double deionised water, weak and strong mineralised bottled water or tap water, prior to the preconcentration step.

Table 1 Characteristics of the water samples tested

	Weak mineralised water	Strong mineralised water	Tap water
pH	6.8	7.8	8.1
Conductivity (µS cm^{-1})	66	767	547
Cl^- (mg L^{-1})	1.8	7.1	34.1
F^- (mg L^{-1})	0.06	0.16	0.10
NO_3^- (mg L^{-1})	1.7	0.56	5.6
SO_4^{2-} (mg L^{-1})	5.5	120	43.5
Ca^{2+} (mg L^{-1})	3.2	94	52.9
Mg^{2+} (mg L^{-1})	3.5	43	9.0
Na^+ (mg L^{-1})	1.6	7.7	20.7
K^+ (mg L^{-1})	1.4	2.5	3.3

Preconcentration system

A previously developed and validated LC-UV-CV-AFS method for the separation of mercury species was adapted. The experimental conditions of the hyphenated technique are described in study of Ibáñez-Palomino *et al.*[3]

In order to couple the online preconcentration system prior to LC-UV-CV-AFS, the original sample loop was replaced with a short RP C18 precolumn with the same characteristics as the separation column: ODS Hypersyl 10, 20 or 50 mm × 4.6 mm id, 5 µm, Thermo Hypersil-Keystone, which was connected by an isocratic LC pump (Agilent Technologies 1260, Waldbronn, Germany) and a six channel valve (Rheodyne model 7000 6-port). This system alternates the sample flow and the mobile phase passing through the precolumn, which allows the loading of different sample volumes to the precolumn, so as to preconcentrate mercury species. When the valve is in the loading position, the sample passes through the precolumn and mercury species are adsorbed onto the stationary phase. In the injection position, the mobile phase passes through the precolumn and elutes the retained mercury species to the LC-UV-CV-AFS system for determination.

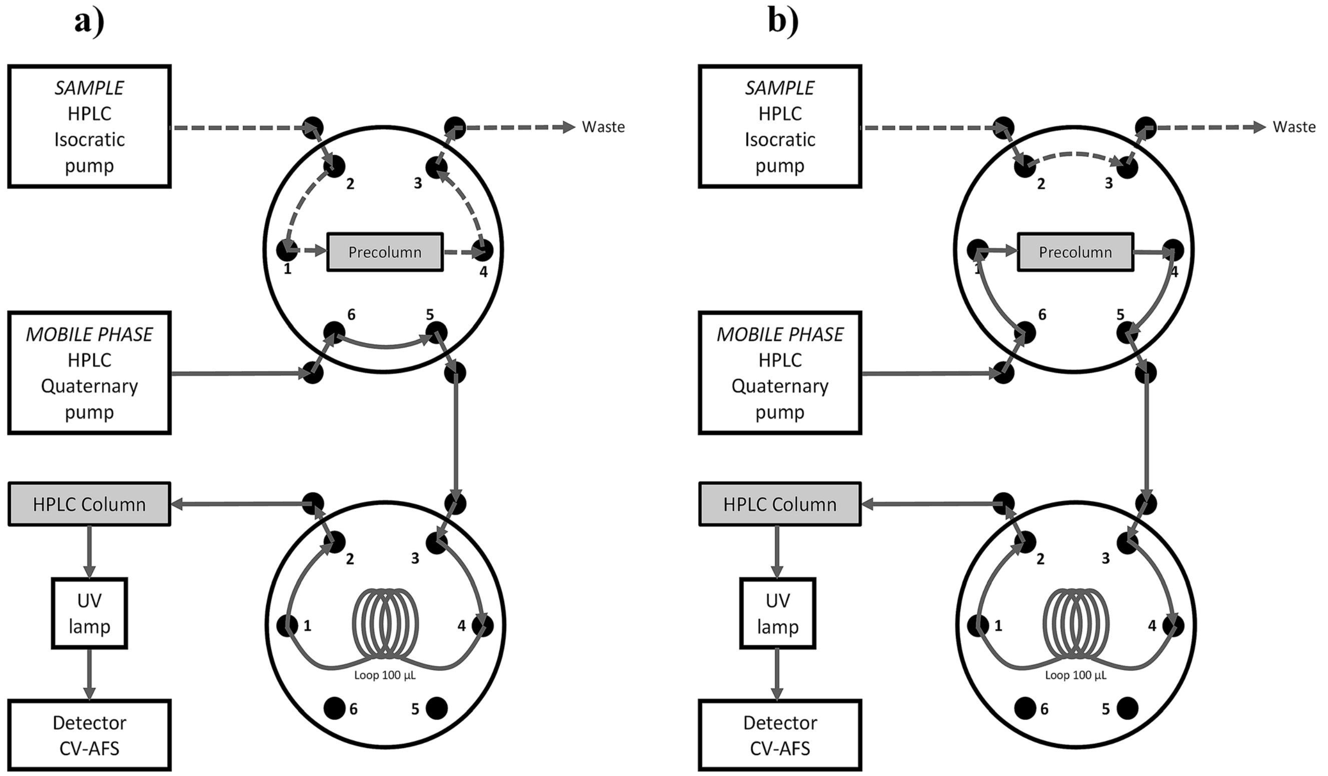

Fig. 1 Schematic representation of the online preconcentration system hyphenated to LC-UV-CV-AFS: (a) load of the sample on the precolumn, and (b) elution of the sample to the separation column.

Fig. 1 shows a schematic diagram of the online preconcentration system coupled to LC-UV-CV-AFS for the determination of trace mercury species in water samples.

The samples were quantified by means of an external calibration curve from methylmercury and mercury(II) standards from 2.5 μg L^{-1} to 750 μg L^{-1}. They were prepared by appropriate dilution in MeOH : APDC 80 : 20 and they were injected into the LC-UV-CV-AFS system using the 100 μL loop represented in Fig. 1.

Results and discussion

To set the working standard concentration for the preconcentration studies, detection and quantification limits of the previously established LC-UV-CV-AFS method[3] were assessed again under the current instrumental conditions. The detection limits (calculated as 3 SD_{BLANK}/slope; $n = 23$) were 0.53 and 0.57 μg L^{-1} for $MeHg^+$ and Hg^{2+}, respectively. The quantification limits (calculated as 10 SD_{BLANK}/slope; $n = 23$) were 1.80 and 1.90 μg L^{-1} for $MeHg^+$ and Hg^{2+}, respectively. The values were of the same order of magnitude of those previously reported. The linearity range was observed to be lineal up to 750 μg L^{-1}.[3]

Different tests using several replicates of working standard solution containing mercury species at a concentration of 5 μg L^{-1}, which is slightly higher than the limit of quantification, were performed to establish the preconcentration method. Even if the preconcentration system increased the signal for the working standards, a lack of reproducibility and strong memory effects were observed. Thus, systematic studies of several variables were undertaken to assess the load volume, preconcentration factors (PFs) and recoveries. PFs were calculated as the ratio between the concentration obtained after preconcentration and the initial concentration. Recovery values were calculated as the ratio between the experimental concentration obtained and the theoretical concentration.

Assessment of the preconcentration step

Initial preconcentration tests working with standards showed a lack of reproducibility of the signal or even no detection of the species in the elution step when no complexing agent was added to the working standard solutions. Thus, the use of a complexing agent which is able to retain mercury species was studied. Two complexing agents, APDC and 2-mercaptoethanol, commonly used in the literature,[17,22] were tested at concentrations 2 mmol L^{-1} and 14 mmol L^{-1}, respectively. Working standard solutions of 0.05, 0.5 and 5 μg L^{-1} of $MeHg^+$ and Hg^{2+} were prepared with the complexing agent, and were preconcentrated in different working sessions.

When working with APDC, the presence of low peak signals at retentions times different from those attributed to the mercury species in the separation method has been observed with a lack of reproducibility. Even if a significant retention can be achieved with APDC, an incomplete elution of the species as well as memory effects were observed. When using 2-mercaptoethanol as a complexing agent, both species were eluted at the expected retention times, with a good signal and overcoming the previous observed problems. Thus, further studies were performed using 2-mercaptoethanol as the complexing agent to establish its concentration.

The concentrations of 2-mercaptoethanol from 0.07 to 140 mmol L^{-1} were tested. Different sample volumes of these standard solutions were preconcentrated in three working sessions using a 1 cm-long precolumn. When the highest concentration of 2-mercaptoethanol (140 mmol L^{-1}) was used, different signals that did not correspond to either $MeHg^+$ or Hg^{2+} were observed. These additional signals could be due to the formation of undesired complexes of $Hg(CH_3OH)$: mercaptoethanol.[37] The concentrations of the complexing agent from 0.07 to 14 mmol L^{-1} did not show any additional signals, apart from mercury species. Fig. 2 shows the breakthrough volume obtained. As can be seen, at the lower 2-mercaptoethanol concentration, higher sample volumes could be loaded in the precolumn before achieving the breakthrough point. Consequently, higher preconcentration factors were obtained. Thus, 0.07 mmol L^{-1} was selected as the working concentration. Conditioning the precolumn with 0.07 mmol L^{-1} 2-mercaptoethanol caused a decrease in peak intensity, because the retention of the free thiol groups in the C18 precolumn decreased the amount of stationary phase available for the retention of $MeHg^+$ and Hg^{2+} complexes. Thus, in further experiments, the precolumn was only conditioned with the mobile phase.

The sample loading at different flows was also assessed to study the possible impact of this variable on mercury species signals. Two different sample volumes, 2 and 5 mL, were preconcentrated at five different flows, from 1 to 5 mL min^{-1} using a 1 cm-long precolumn. The peak signals obtained at 1 and 2 mL min^{-1} flow were of the same order of magnitude, but from 3 mL min^{-1} flow, the signal of both species decreased gradually. When the flow rate was increased, the contact time was not enough to achieve equilibrium between the mobile and stationary phases. Thus, 2 mL min^{-1} was selected for further assays.

The effect of precolumn length was studied to assess the retention capability of mercury species in the stationary phase. Three columns of different lengths were selected: 1, 2 and 5 cm.

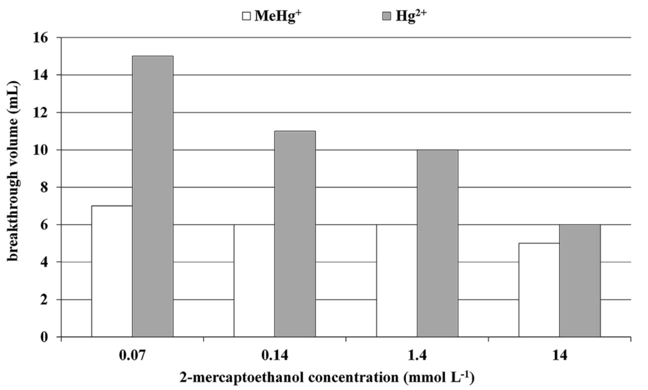

Fig. 2 Breakthrough volume obtained *versus* complexing agent concentration in a 5 μg L^{-1} $MeHg^+$ and Hg^{2+} standard. Precolumn length: 1 cm.

Two working standard solutions of $MeHg^+$ and Hg^{2+} at concentrations of 0.5 and 5 μg L^{-1} of both species were initially prepared in 0.07 mmol L^{-1} 2-mercaptoethanol. Increasing volumes of these solutions were tested until the breakthrough point. As an example, Fig. 3 represents the mercury species concentrations obtained in the preconcentration of a given volume in working solutions of 5 μg L^{-1}. As can be observed, the 1 cm-long precolumn breakthrough volume for both species was lower than 8 mL. However, in 2 and 5 cm-long precolumns, these volumes increased up to 14–18 mL. In all cases, the breakthrough volumes were higher for Hg^{2+} than for $MeHg^+$, due to the higher affinity of this species for the C18 stationary phase.

Preconcentration factors and recoveries at the breakthrough volume including the standard deviation are plotted in Fig. 4A for both species in each precolumn. Higher preconcentration factors were obtained when 2 and 5 cm precolumns were used, due to the fact that their retention capability is higher than that of the 1 cm precolumn. Regarding percent recoveries, similar values were obtained among the three precolumns and they ranged from 60 to 80%.

Even if preconcentration factors provided by 2 and 5 cm precolumns were suitable, the observed chromatographic behaviour of both systems was different, as shown in Fig. 4B. The direct injection of the 5 μg L^{-1} standard has also been included for comparison purposes. As can be seen, mercury(II) did not present Gaussian behaviour when the breakthrough volume was preconcentrated in a 5 cm precolumn. This effect could be because the precolumn is long enough for the mercury(II) separation process to start before the analytical column is reached. Thus, it can be concluded that a 2 cm column is most suitable for the preconcentration method.

Application in water samples

Once the most appropriate conditions for online preconcentration had been selected, the water samples were tested. Three water samples of increasing complexity (weak mineralisation, strong mineralisation and tap water) were characterised following the procedure described in the "Samples" section. To ensure if the samples could have or not trace amounts of mercury, the total mercury content was determined in all matrices by ICP-MS, and the Hg content was under the detection limit (0.05 μg L^{-1}). Samples were spiked at three levels: low-level (0.5 μg L^{-1} of both species), medium-level (0.5 μg L^{-1} of $MeHg^+$ and 5 μg L^{-1} of Hg^{2+}) and high-level (5 μg L^{-1} of both species). The samples were then preconcentrated until the breakthrough volume was achieved for each matrix.

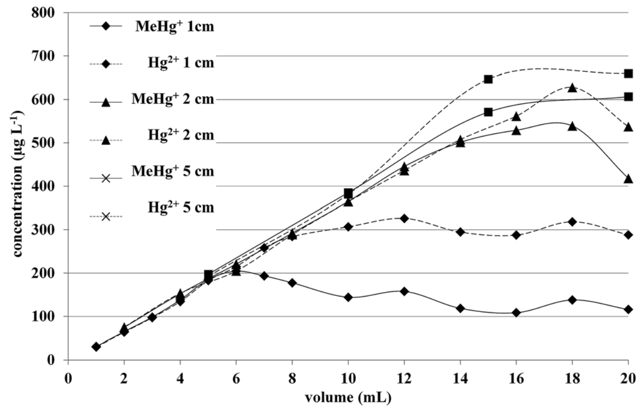

Fig. 3 Mercury species concentrations obtained *versus* volume preconcentrated on working solutions of 5 μg L^{-1}.

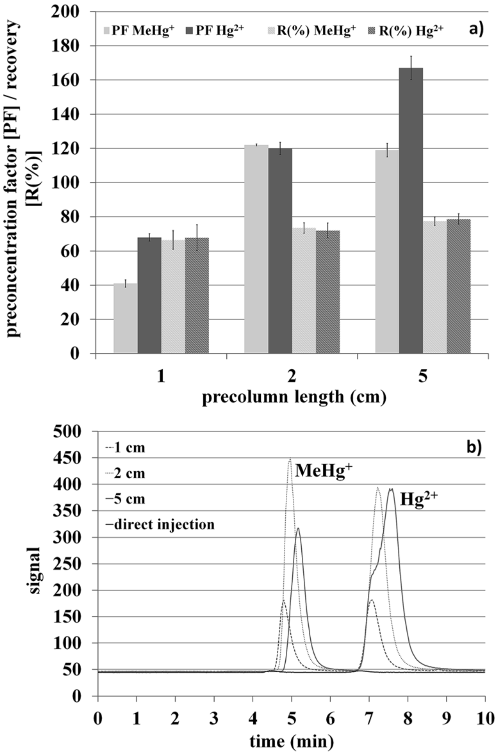

Fig. 4 $MeHg^+$ and Hg^{2+} recoveries, preconcentration factors (A) and chromatograms obtained (B) from a 5 μg L^{-1} standard at the breakthrough volume in each precolumn, together with a direct injection of this standard.

Due to a matrix effect, both breakthrough volumes and preconcentration factors were lower in water samples (≈7 mL and ≈50, respectively) than in double deionised water (16 mL and ≈120, respectively). This effect may be due to a possible competition of other substances present in water samples in addition to mercury with the precolumn stationary phase, which can lead to a decrease in its retention capacity. However, recovery values of both species were of the same order of magnitude as those previously described in the "Precolumn length" section and ranged from 67 to 86%, regardless of the type of water, which may indicate that this parameter is independent of the matrix composition. Higher PF and recoveries for Hg^{2+} were also observed.

From the data obtained, it was considered that the most suitable breakthrough volume for routine analysis is that obtained for the most complex matrix (tap water) and for the least retained species ($MeHg^+$), which are the worst retention conditions: 4 mL. This volume allows us to work under reproducible conditions with good recoveries, regardless of the type of sample and concentration levels.

Table 2 shows the preconcentration factor, recovery, mean values and standard deviation for a 4 mL preconcentration volume. The overall average represents the mean of each replicate. The PF values were 30 ± 1 and 32 ± 3 for $MeHg^+$ and Hg^{2+}, respectively. The recovery values were 72% $MeHg^+$ and 81% for Hg^{2+} and the RSD mean values were below 15%. As it can be seen, methylmercury recoveries are always lower than those obtained for Hg^{2+}. The possible justification for this behaviour is that $MeHg^+$–mercaptoethanol complexes present less affinity for C18 than the Hg^{2+} ones. The higher affinity of Hg^{2+} for the C18 could be due to the stoichiometry of the formed complex. Hg^{2+} forms 1 : 2 complexes with 2-mercaptoethanol and APDC whereas $MeHg^+$ forms 1 : 1 complexes. The 1 : 2 complex presents more retention in C18 than the 1 : 1 complex because it has more sulphur atoms in the structure, which are mainly responsible for the retention process in C18.

Considering that the waters that were analysed had different matrices, the standard deviations obtained were suitable and the similarity between the PF and recovery values demonstrates the robustness of the established conditions for the online preconcentration system.

Thus, Table 3 summarises the optimum conditions for the determination of $MeHg^+$ and Hg^{2+} by LC-UV-CV-AFS following online preconcentration.

Analytical figures of merit

Accuracy. The accuracy of the method was assessed by the analysis of a certified reference material (CRM). To our knowledge there are no CRMs for Hg^{2+} and $MeHg^+$ species in natural waters. Most of the CRMs available for total mercury consist of spiked water samples.[7] To evaluate the preconcentration method, the most suitable CRMs would be water with a total mercury level close to the limit of quantification of the analytical technique without the preconcentration step. It was found only wastewater with certified values for the total content of 10 elements including mercury (ERM-CA713, 1.84 ± 0.11 μg Hg L^{-1}). The total Hg content was analysed in the CRM by CV-AFS, which provided a mercury concentration of 1.81 ± 0.03 μg L^{-1} ($n = 3$). No significant difference was found between the certified and experimental total content (*t*-test at 95% confidence level).

Mercury species in the CRM were analysed by direct injection and after the online preconcentration step, using the previously established optimised conditions. A total of 4 mL of wastewater were preconcentrated and the PFs obtained in section "Application in water samples" (see Table 2) were applied. The analyses were performed in triplicate.

Table 4 summarises the results obtained by the two speciation methods. Regarding the direct injection method, the concentration of methylmercury was below the limit of detection, whereas the concentration of mercury(II) in the CRM was close to the limit of quantification.

In the preconcentration method, both species were well quantified. Regarding the sum of species, a *t*-test (95% confidence level) was performed with respect to the certified value. No significant difference was found. The results show that the preconcentration method can quantify all mercury species, and their sum can be used to determine the total mercury content in water.

Limits of detection and quantification. Limits of detection and quantification for the online preconcentration method were assessed experimentally by injecting standard solutions from 1 to 500 ng L^{-1}. Hg^{2+} was detected at about 2 ng L^{-1} whereas $MeHg^+$ was detected at about 15 ng L^{-1}. Experimental limits of quantification were 50 ng L^{-1}.

Limits of detection and quantification concentrations were considerably lower than those obtained by the direct injection method: values were in the order of μg L^{-1}, compared to values in the order of tens of ng L^{-1}, using a non-expensive technique.

However, the preconcentration of samples with a low complexity matrix would decrease the limits of detection and quantification in the online preconcentration method, by using a higher load volume.

Table 2 Preconcentration parameters obtained for each species in water samples for a 4 mL preconcentration volume

Species	Sample (water)	PF[a]	Recovery[b] (%)	RSD[b] (%)
$MeHg^+$	Double deionised	29 ± 2	73 ± 5	2
	Weak mineralised	30 ± 2	74 ± 6	8
	Strong mineralised	30 ± 1	67 ± 9	13
	Tap	27 ± 4	74 ± 2	2
Overall average		30 ± 1	72 ± 3	4
Hg^{2+}	Double deionised	32 ± 2	80 ± 4	5
	Weak mineralised	34 ± 1	86 ± 4	5
	Strong mineralised	34 ± 2	84 ± 5	6
	Tap	35 ± 5	87 ± 9	10
Overall average		32 ± 3	81 ± 5	6

[a] Preconcentration factor. [b] $n = 3$.

Table 3 Final selected conditions for online preconcentration of $MeHg^+$ and Hg^{2+} by LC-UV-CV-AFS

Optimum conditions	
Precolumn conditioning	Mobile phase
Complexing agent	0.07 mmol L^{-1} 2-mercaptoethanol
Sample flow	2 mL min^{-1}
Precolumn length	2 cm
Preconcentration volume	4 mL

Table 4 Methylmercury and mercury(II) concentrations obtained in ERM-CA713 (certified value: 1.84 ± 0.11 μg Hg L^{-1}) by direct injection and online preconcentration

	Direct injection		Online preconcentration	
Species	*C* (μg L^{-1})	RSD (%)	*C* (μg L^{-1})	RSD (%)
$MeHg^+$	<LD	—	0.28 ± 0.01	2.9
Hg^{2+}	1.71 ± 0.02	1.2	1.72 ± 0.06	3.6
Sum of species	1.71 ± 0.02	1.2	2.00 ± 0.06	3.0

Table 5 Online preconcentration of mercury species in water samples

Mercury species	Matrix	Complexing agent	Retention/elution	Instrumental method	Absolute LD (pg)	Recoveries (%)	Reference
$MeHg^+$ Hg^{2+}	Bottled water Tap water	2-Mercaptoethanol 0.07 mM	Retention in a C18 microcolumn and elution with MeOH : ADPC 1.5 mM pH 5.5 (80 : 20)	LC-UV-CV-AFS	60 8	69–86	Present method
$MeHg^+$ $EtHg^+$ $PhHg^+$ Hg^{2+}	Human urine	APDC 2 mM	Retention in a C18 microcolumn and elution with MeOH : ACN : APDC 1.5 mM (38 : 30 : 32)	LC-CV-AAS	526.5 351 585 292.5	92–106	15
$MeHg^+$ Hg^{2+}	Brackish water	APDC 2 mM	Retention in a C18 microcolumn and elution with MeOH : APDC 1.5 mM (50 : 50)	LC-CV-AAS	1.7 3.4	—	17
$MeHg^+$ Hg^{2+}	Seawater from lagoon	2-Mercaptoethanol	Retention in a microcolumn C18 modified with 2-mercaptoethanol and elution with H_2O with 0.5% L-cysteine and 0.05% 2-mercaptoethanol	LC-ICP-MS	0.6 2.4	84–108	20
$MeHg^+$ $EtHg^+$ Hg^{2+}	Sea water	—	Retention in a cation exchange microcolumn and elution with MeOH : L-cysteine 10 mM pH 8 (4 : 96)	LC-ICP-MS	0.48 0.24 1.26	87–102	9

Table 5 compares the detection limits and recoveries obtained in this paper with those previously reported in the literature using a similar methodology. The recoveries obtained are comparable, and the detection limits are of the same order of magnitude when the total amount of mercury detected is considered. As expected, the detection limits obtained by ICP-MS are lower than those obtained by AAS or AFS. Nevertheless, CV-AFS provides suitable analytical performance, which is user-friendly and requires lower investment and maintenance costs than ICP-MS, so it is a good approach in daily routine laboratory analysis.

Conclusions

An online preconcentration method for $MeHg^+$ and Hg^{2+} determination, the most relevant mercury species present in the environment, was developed using a 2 cm ODS Hypersil (C18; reverse phase) precolumn in the preconcentration step. These precolumns are commercially available and widely used in routine laboratory analysis. The method requires a low volume (4 mL) and a simple sample pre-treatment (addition of 2-mercaptoehtanol 0.07 mmol L^{-1}). The online preconcentration-LC-UV-CV-AFS system provides recoveries of 72 ± 3% and 81 ± 5% for $MeHg^+$ and Hg^{2+}, respectively, which were obtained regardless of the matrix composition.

The sum of the species in the proposed method matched with the total mercury content. The limits of detection and quantification established are suitable for analytical performance using environmental samples. Thus, the method is widely applicable, highly precise and accurate, and could be useful for $MeHg^+$ and Hg^{2+} determination, in response to any future legislation on mercury species.

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ANNEX 2

Article: *Method development for the simultaneous determination of methylmercury and inorganic mercury in seafood*

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Method development for the simultaneous determination of methylmercury and inorganic mercury in seafood

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ABSTRACT

This paper reports the method development for the simultaneous determination of methylmercury ($MeHg^+$) and inorganic mercury (iHg) species in seafood samples. The study focused on the extraction and quantification of $MeHg^+$ (the most toxic species) by liquid chromatography coupled to on-line UV irradiation and cold vapour atomic fluorescence spectroscopy (LC-UV-CV-AFS), using HCl 4 mol L^{-1} as the extractant agent. Accuracy of the method has been verified by analysing three certified reference materials and different spiked samples. The values found for total Hg and $MeHg^+$ for the CRMs did not differ significantly from certified values at a 95% confidence level, and recoveries between 85% and 97% for $MeHg^+$, based on spikes, were achieved. The detection limits (LODs) obtained were 0.001 mg Hg kg^{-1} for total mercury, 0.0003 mg Hg kg^{-1} for $MeHg^+$ and 0.0004 mg Hg kg^{-1} for iHg. The quantification limits (LOQs) established were 0.003 mg Hg kg^{-1} for total mercury, 0.0010 mg Hg kg^{-1} for $MeHg^+$ and 0.0012 mg Hg kg^{-1} for iHg. Precision for each mercury species was established, being $\leq$ 12% in terms of RSD in all cases.

Finally, the developed method was applied to 24 seafood samples from different origins and total mercury contents. The concentrations for Total Hg, $MeHg^+$ and iHg ranged from 0.07 to 2.33, 0.003–2.23 and 0.006–0.085 mg Hg kg^{-1}, respectively. The established analytical method allows to obtain results for mercury speciation in less than 1 one hour including both, sample pretreatment and measuring step.

1. Introduction

Within the elements that are toxic for humans and the environment, mercury is a well-known pollutant due to the high toxicity of its species (Ibáñez-Palomino, López-Sánchez, & Sahuquillo, 2012a; Leopold, Foulkes, & Worsfold, 2010). All Hg forms are toxic, with the organic species being in most cases more dangerous than the inorganic ones (Gochfeld, 2003; Leopold et al., 2010). However, it is very important to identify which chemical form is more or less toxic, so as to evaluate its impact on environment and human health (Ibáñez-Palomino et al., 2012a). The akyl compounds of Hg are more toxic than the inorganic ones, particularly methylmercury ($MeHg^+$), the most toxic species (Ibáñez-Palomino et al., 2012a; Leopold et al., 2010). $MeHg^+$ bioaccumulates in the food chain, with its concentration higher in some fish species than in the water environment (Leopold et al., 2010).

Bioaccumulation occurs in most cases of human exposure (Gochfeld, 2003). Seafood is responsible for the highest source of Hg, especially $MeHg^+$ (Baer et al., 2011; Capar, Mindak, & Cheng, 2007). Concentrations higher than 10 mg kg^{-1} of $MeHg^+$ are found in the muscle of some fish species (Von Burg & Greenwood, 1991). The consumption of fish located at the top of the food chain is not recommended for pregnant women, due to the potential risk of $MeHg^+$ contamination (Baer et al., 2011; EFSA, 2004). As a consequence of $MeHg^+$ exposure, neurological problems in adults and blindness and mental retardation in infants were reported in the victims of Minamata disease (Gochfeld, 2003). Other countries, such as Iraq, Guatemala and Brazil, also had serious problems with exposure to organic mercury (Amin-Zaki et al., 1974; Dolbec, Mergler, Sousa Passos, Sousa de Morais, & Lebel, 2000; Gochfeld, 2003; Storelli, Busco, & Marcotrigiano, 2005).

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Health Organization), stipulates the maximum levels of methylmercury in fish and predatory fish (0.5 and 1 mg kg^{-1}, respectively) (CODEX STAN 193-1995, 2009). The Codex indicates the maximum level for toxicants permitted in food trade internationally (CODEX STAN 193-1995, 2009). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) proposed a provisional tolerable weekly intake for $MeHg^+$ of 1.6 μg kg^{-1} in body weight. However, the European Commission asked the European Safety Authority (EFSA) to review the tolerable value of $MeHg^+$ (EFSA, 2012). Thus, the EFSA published in 2012 a scientific opinion on the risk of human exposure to mercury and methylmercury (EFSA, 2012). According to new epidemiological studies in children, the EFSA Panel on Contaminants in the Food Chain (CONTAM) established a tolerable weekly intake (TWI) for $MeHg^+$ of 1.3 μg kg^{-1} in body weight, expressed as mercury (EFSA, 2012).

Although the Commission Regulation (EC) N° 1881/2006 does not provide limits for $MeHg^+$ concentration, total Hg limits of 0.5 mg kg^{-1} and 1 mg kg^{-1} are given for distinct seafood (according to seafood type) (Commission Regulation (EC) No 1881/2006). The Brazilian Normative Instruction N° 14 (May 2009) regulates the maximum level of total Hg in fish farming and predator fish. The established limits are 1 mg kg^{-1} for predator fish and 0.5 mg kg^{-1} for fish farming (Damin, Santo, Hennigen, & Vargas, 2013; PNCRC, 2009).

The toxicity of metals and their bioavailability depend on the chemical form of the metals. Thus, an accurate analytical method for Hg speciation is required to assess the real toxicity of samples (Harrington, 2000). Mercury speciation is generally performed by chromatographic separation techniques coupled with different detectors (Zhang, Yang, Dong, & Xue, 2012). The chromatographic separation techniques include: gas chromatography (GC) (Barst et al., 2013; Kenšová, Kružíková, & Svobodová, 2012; Nevado et al., 2011), liquid chromatography (HPLC) (Batista, Rodrigues, De Souza, Oliveira Souza, & Barbosa Jr., 2011; Chen, Han, Cheng, Liu, et al., 2013; Chen, Han, Cheng, Wang, et al., 2013) and ionic chromatography (IC) (Shade & Hudson, 2005). The most commonly used detectors are: inductively coupled plasma mass spectrometry (ICP-MS) (Batista et al., 2011; Clémens, Monperrus, Donard, Amouroux, & Guérin, 2011), atomic absorption spectroscopy (AAS) (Naozuka & Nomura, 2011; Sarıca & Türker, 2012), atomic fluorescence spectrometry (AFS) (Nevado et al., 2011; Zhang et al., 2012), electron capture detector (ECD) (Kehrig et al., 2009; Kenšová et al., 2012), microwave induced plasma-atomic emission spectrometry (MIP-AES) (Sanz, De Diego, Raposo, & Madariaga, 2003), atomic emision detection (AED) (Kuballa, Leonhardt, Schoeberl, & Lachenmeier, 2011) and isotope dilution mass spectrometry (IDMS) (Demuth & Heumann, 2001).

The goal of the study was to determine total Hg and Hg species in seafood samples comprising fish, crustaceans and bivalves. The study focused on the extraction, identification and accurate quantification of $MeHg^+$, the most toxic form, which was selectively separated and determined by liquid chromatography coupled to on-line UV irradiation and cold vapour atomic fluorescence spectroscopy (LC-UV-CV-AFS). Sample preparation was optimized to be as simple as possible, but still provide adequate sensitivity and specificity for the routine analyses of seafood.

2. Materials and methods

2.1. Instruments

Total Hg was measured by an Agilent 7500ce ICP-MS (Agilent, Germany) with a BURGENER Ari Mist HP type nebulizer. For Hg speciation, an HPLC system with a quaternary pump and degasser (Agilent Technologies 1100, Waldbronn, Germany) equipped with a manual stainless steel sampler injector (Rheodyne 7725i) and a 100 μL sample loop was used. Mercury species (iHg and $MeHg^+$) were separated in an analytical RP-C_{18} column (ODS Hypersyl 250 mm × 4.6 mm id, 5 μm, Thermo Hypersil-Keystone). After separation, a photo-oxidation step was performed in a 12 meter-long × 0.5 mm id PTFE tube coiled around a UV lamp with 150 W of power irradiation (Heraeus TQ 150). The reduction step was achieved in a cold vapour generator (CV) 10004 (P.S. Analytical, Orpington, UK), in which the effluent is mixed with the reducing agent. The metallic mercury vapour obtained reaches the gas–liquid separator, from which it is dragged into the detector by an argon stream and dried in a PermaPure membrane with nitrogen. A Merlin Mercury Atomic Fluorescence Detector, model 10023 (P.S. Analytical), was used for measurements. A microwave (Milestone Ethos Touch Control) was used for digesting and extracting the samples. The fish samples supplied by MAPA (Brazil) were lyophilized in a ModulyonD Freeze Dryer lyophilizer (Thermo Electron Corporation, USA) and milled in an A 11 Basic micro-mill (IKA – Werke, Germany).

2.2. Reagents and standards

Only analytical grade reagents were used in this study. The standards and reagents were prepared with doubly deionized water (Elix&Rios 5–15 MΩ cm^{-1}, Total Organic Carbon <30 μg L^{-1}) obtained from the Milli-Q water purification system (Millipore, Bedford, MA, USA). An inorganic mercury stock standard solution of 1000 mg L^{-1} was prepared by dissolving appropriate amounts of mercury chloride, $HgCl_2$ (Merck, Darmstadt, Germany), in 1% (v/v) HNO_3, from 69% nitric acid (Panreac, Hiperpur). A methylmercury stock standard solution of 1000 mg L^{-1} was prepared by dissolving appropriate amounts of CH_3HgCl (Carlo Erba, Milan, Italy) in 3% Methanol (Panreac, p.a.). All stock standard solutions were stored at 4 °C. The working standard solutions were prepared daily from the stock standard solutions by appropriate dilution. For cold vapour generation, $SnCl_2$ solution was prepared daily from tin chloride 2-hydrate (Panreac, p.a.) to 1.5% concentration, in 4% of HCl, from 35% hydrochloric acid (Panreac, Hiperpur). Mobile phase was prepared daily by dissolving appropriate amounts of pyrrolidinedithiocarbamate, APDC, (Fluka, p.a.) and ammonium acetate, NH_4CH_3COO, (Merck, p.a.) in water. pH was adjusted with diluted acetic acid (Panreac, p.a.) and then filtered in a 0.45 μm filter (HA-type Millipore). The final mobile phase composition was 20% of the APDC and NH_4CH_3COO solution and 80% of methanol HPLC-gradient grade (Panreac, p.a.). For microwave digestion samples, 31% H_2O_2 (Merck, Selectipur) and 69% HNO_3 (Panreac, Hiperpur) were used. For microwave extraction, 4 M HCl was prepared from 35% hydrochloric acid (Panreac, Hiperpur).

2.3. Reference materials and samples

The following certified reference materials (CRM) were used for quality control: DOLT-4 (Dogfish), TORT-2 (Lobster Hepatopancreas) (both from the National Research Council, Canada) and BCR-463 (Tuna fish) (Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre, Geel, Belgium). DOLT-4 was also used to assess the selection of extractant agent.

Five fresh fish muscle samples were provided by the Laboratory of Trace Metals and Contaminants (LANAGRO/RS) of the Ministry of Agriculture, Livestock and Supply (MAPA/Brazil). These samples were initially washed with Milli-Q water, cut and then lyophilized for a period of 5 hours. They were then ground in a vibratory mill and sieved through 85 μm polyester mesh to improve the particle size distribution.

Eleven fish samples and a clam sample were supplied by the Public Health Agency Laboratory, Barcelona (ASPB, Barcelona, Spain). Three crustacean samples and four bivalve samples were purchased from local supermarkets in Barcelona, Spain, during 2013. All samples were washed with Milli-Q water, cut and homogenized using a blender (non-contaminating kitchen mixer; Multiquick 5 Hand Processor, Braun, Barcelona, Spain). After homogenization, samples were stored in the freezer at −18 °C until analysis.

2.4. Procedures

2.4.1. Moisture determination

The moisture of samples was determined in triplicate by drying 0.5 g aliquots in an oven at 102 ± 3 °C until constant weight. Moisture ranged from 5% (lyophilized samples) to 88% (fresh samples); all results were expressed as dry mass.

2.4.2. Total mercury analysis

The total mercury content in seafood and CRM samples was determined by ICP-MS following microwave digestion. Initially, 0.1–1 g of samples were weighed in digestion vessels, after which 8 mL of concentrated nitric acid and 2 mL of hydrogen peroxide were added. The microwave digestion procedure was as follows: 10 min from room temperature to 90 °C, maintained for 5 min at 90 °C, 10 min from 90 °C to 120 °C, 10 min from 120 °C to 190 °C and 10 min maintained at 190 °C. After cooling to room temperature, the digested samples were diluted in water up to 20 mL.

Total Hg was measured in the digested samples by ICP-MS. Helium gas was used in the collision cell to avoid interference in the ICP-MS measurements. A solution of ^{9}Be, ^{103}Rh and ^{205}Tl was used as the internal standard. The samples were quantified by means of an external calibration curve from inorganic mercury standards. Analyses in triplicate were performed for each sample. For quality control purposes, the standards of the calibration curve were run before and after each sample series. The corresponding digestion blanks (one for each sample digestion series) were also measured. Quality control standard solutions at two concentrations were measured at the end of the sequence to ensure stable instrument sensitivity. To assess the accuracy of the ICP-MS method, three CRMs (DOLT-4, TORT-2 and BCR-463) were analysed.

2.4.3. Mercury speciation analysis

The mercury speciation content in seafood and CRM samples was determined by LC-UV-CV-AFS following microwave extraction. The samples and CRMs were weighed in digestion vessels (0.1–1 g) and 10 mL of hydrochloric acid 4 mol L^{-1} were added to perform a microwave-assisted extraction (MAE). The microwave extraction procedure was as follows: 2 min from room temperature to 100 °C and 10 min maintained at 100 °C. After cooling to room temperature, the extraction samples were filtered through paper filters (Whatman 40). Mercury species were measured in the extracts by LC-UV-CV-AFS. The performance characteristics of the hyphenated system are those described by Ibañez-Palomino et al. (2012). Mercury species in extracts were identified by comparison of retention times with standards. External calibration curves quantified $MeHg^{+}$ and iHg, according to the corresponding standards. All samples were analysed in triplicate. Extraction blanks were also analysed by LC-UV-CV-AFS in each work session. In each speciation run, two quality control standard solutions were measured at the end of the sequence to ensure stable instrument sensitivity. To assess the accuracy of the LC-UV-CV-AFS method, three CRMs (DOLT-4, TORT-2 and BCR-463) were analysed.

3. Results and discussion

3.1. Selection of extractant agent

This assay focused on the study of a quantitative species extraction method system for seafood matrices, suitable for the subsequent determination technique. Extraction methods performed by several authors during the last five years are summarised in Table 1. Both acidic and basic extraction methods are described. However, there is no knowledge about standardised extraction methods in seafood matrices. Therefore, to perform the extraction of Hg species in seafood, a preliminary test selecting two different extractant agents was run, to assess the main Hg species extracted. However, there is knowledge about a standardised extraction method for sediments: EPA 3200 (EPA, 2005). This method uses HNO_3 4 mol L^{-1} as extractant agent. It has also been taken into account that, in almost half the studies summarised in Table 1, hydrochloric acid was used as the extractant agent. Therefore, the extractants tested were HNO_3 4 mol L^{-1}, such as EPA 3200 employs, and HCl 4 mol L^{-1}, as an adaptation of this method. The present method is based on a microwave-assisted extraction, whose procedure is described in Section 2.4.3. For this study, the CRM DOLT-4 (Dogfish) was used. In DOLT-4, $MeHg^{+}$ and iHg were the species present in the extracts. Fig. 1 shows chromatograms obtained from hydrochloric and nitric acid DOLT-4 extracts, in which Hg species are highlighted. Recoveries obtained for $MeHg^{+}$ were 95% and 86%, using HCl and HNO_3, respectively. On measuring iHg, the concentration obtained when using HCl was 46% of the total certified content; whereas, when using HNO_3, the figure was 85%. This increase could be attributed to the oxidising action of HNO_3. As $MeHg^{+}$ recovery with the HNO_3 extraction method is 9% less than recovery with HCl extraction, there is evidence that this difference could be caused by $MeHg^{+}$ conversion to iHg. Therefore, HCl 4 mol L^{-1} was selected as the extractant agent.

3.2. Quality parameters

3.2.1. Analysis of the total Hg

Three CRMs (TORT-2, DOLT-4 and BCR-463) were analysed to verify the accuracy of the proposed method. Concomitant analyses of TORT-2, DOLT-4 and BCR-463 verified the accuracy of the determination of total Hg (Table 2). The analysis of one CRM for each sample group was used in total Hg measurements. The use of CRMs guaranteed the quality control of acid digestion (sample pretreatment). The values for total Hg concentration, together with the corresponding certified value, are given in Table 2. According to the Student's *t*-test, no significant difference at a 95% confidence level was found in the data shown in Table 2. Repeatability was checked by analysis of CRMs (different replicates) 6 times throughout the day (Table 2). The RSD (%) values were: 7% for TORT-2 and 4% for DOLT-4. The instrument detection (LOD) and quantification limits (LOQ) were calculated as three times the standard deviation (3σ) and ten times the standard deviation signal (10σ) of ten digestion blanks, respectively (Llorente-Mirandes, Calderón, Centrich, Rubio, & López-Sánchez, 2014). The results obtained were 0.001 mg Hg kg^{-1} for LOD and 0.003 mg Hg kg^{-1} for LOQ.

3.2.2. Analysis of Hg species

The accuracy of the method proposed for $MeHg^{+}$ speciation was verified by analysis of BCR-463 (Tuna fish), DOLT-4 (Dogfish liver) and TORT-2 (Lobster hepatopancreas) CRMs. The values for each CRM are given in Table 2 and did not differ significantly at a 95% confidence level from certified values. The amount of iHg was also analysed in the BCR-463, DOLT-4 and TORT-2 CRMs. The recovery for each CRM was calculated by comparing the sum of $MeHg^{+}$ and

Table 1
MeHg$^+$ concentrations in seafood samples found in literature since 2009.

Type of seafood	Samples	Extraction procedure	Extracting agent	Technique	MeHg$^+$ (mg kg^{-1})	MeHg$^+$ %	Reference
Fish	Chub muscle	Stirring manually with a glass rod	Toluene	GC-EDC	<0.5	–	Sedláčková, Kružíková, & Svobodová, 2014
Fish	Tuna Emperor fish Red grouper Bass Aquarium fish Snook black Grunt Bream Dogtooth herring Mackerel Nurse shark	Ultrasonication	2-mercaptoethanol, L-cysteine and HCl	ID-LC–ICP-MS	0.04–1.92	83–98	Montero-Alvarez et al., 2014
Fish	Bearded brotula Tuna Pirarucu Salmon Whitemouth croaker Mullet	Microwave	L-cysteine	LC-CV-ICP-MS	0.01–1.00	–	Schmidt, Bizzi, Duarte, Dressler, & Flores, 2013
Fish	Arctic char Spotted gar Largemouth bass Bowfin Catfish	Water bath	HNO_3	GC-CVA-FS	0.5–1.5	–	Barst et al., 2013
Fish	Pomfret Hairtail Croaker Japanese seabass	Ultrasonication	HCl + L-cysteine	LC-ICP-MS	0.17–0.75	More than 86%	Chen, Han, Cheng, Wang, et al., 2013
Fish	Pomfret Hairtail Croaker Japanese seabass Silver carp Black carp Goldfish Northern snakehead	Ultrasonication	HCl + Sodium 3-mercapto-1-propanesulfonate	LC-ICP-MS	0.0032–0.75	More than 86%	Chen, Han, Cheng, Liu, et al., 2013
Fish	Tapertail Anchovy	Microwave	HCl	CE-ICP-MS	1.2–3.2	–	Zhao et al., 2012
Shellfish	Mussel Razor clam Baby clam	Ultrasonication	HCl	EVG-AFS	0.022–0.034	–	Zhang et al., 2012
Fish	Chub Pike Bream Roach Asp Carp Eel Perch Tench Trout Grayling	Stirring	Toluene	GC-ECD	0.05–0.8	46–100	Kenšová et al., 2012
Fish	Nase Carp Catfish	Microwave	TMAH	GC-AFS	0.001–1.16	60–88	Nevado et al., 2011
Fish	Red snapper Orange-spotted grouper Snubnose pompano	Wet digestion	KOH–methanol	GC-CV-AFS	0.007–0.12	37–81	Liang et al., 2011
Fish	Sea fish from local markets (Wuhan, China)	Ultrasonication	HCl	LLME-CE-UV	0.004–0.027	–	Li, Zhang, & Hu, 2011
Fish	Saithe Salmon Smoked salmon Tuna Canned tuna	a) Solid–liquid extraction b) Microwave c) Extraction at room temperature	TMAH	ID-GC-ICP-MS	0.002–0.58	84–97	Clémens et al., 2011
Shellfish	Mussel Oyster Scallop Shrimp				0.001–0.033	28–98	
Fish	Catfish Carp	Water bath	KOH	HS-SPME-GC-AFS	0.76	74	Carrasco et al., 2011

Table 1 (*continued*)

Type of seafood	Samples	Extraction procedure	Extracting agent	Technique	$MeHg^+$ (mg kg^{-1})	$MeHg^+$ %	Reference
Shellfish	Mussels Octopus Shrimps Squids	Ultrasonication	HCl + L-cysteine + 2-mercaptoethanol	LC-ICP-MS	0.003–0.037	–	Batista et al., 2011
Fish	Tuna				0.03–0.16		
Fish	German market	alkaline digestion	Methanolic potassium hydroxide solution	GC-AED	0.006–0.5	14–100	Kuballa et al., 2011
Fish	Canned fish	According to reference	According to reference	GC-ECD	0.002–0.1	40–110	Miklavčič et al. 2011
Fish	Fish from Qinghai and Tibet plateau	Shaking	Alkaline extraction	LC-CV-AFS	0.1–0.6	84–89	Wang et al., 2010
Fish	Common carp Crucian carp Catfish Java tilapia Chinese soft shell turtle	Shaking	Alkaline extraction	LC-UV-AFS	0.1–0.4	35–76	Fu et al., 2010
Fish	Tuna and mackerel	Shaking and centrifugation	H_2SO_4 + KBr + toluene + cysteine	GC-ECD	0.29–0.69	70–82	Hajeb et al., 2010
Fish	Grass carp	Over digestion	KOH–methanol	CV-AFS	0.02–0.09	7.4–93	Qiu et al., 2009
Fish	King mackerel Red snapper	Ultrasonication	Perchloric acid + L-cysteine + toluene + methanol	LC-ICP-MS	0.05–0.3	80	Santoyo et al., 2009

iHg concentration, obtained by the proposed speciation method (LC-UV-CV-AFS) and total Hg concentration (ICP-MS). The total Hg concentration was taken as 100% in the calculation of recovery values. The recoveries analysed for CRMs showed a range between 80 and 102% (Table 2).

Additionally, standards of $MeHg^+$ were spiked in solid samples of tuna-2, forkbeard, prawn-1, cockle and BCR-463. After addition of standards, the solid samples were homogenized. The extraction procedure was carried out only 30 minutes after the spiking procedure. The recoveries found for tuna-2, forkbeard, prawn-1, cockle and BCR-463 were 93 ± 3, 85 ± 5, 93 ± 2, 87 ± 4 and 97 ± 2 (mean % ± standard deviation, $n = 3$), respectively. These recovery values were calculated according to the literature (Santoyo, Figueroa, Wrobel, & Wrobel, 2009) and show good recovery of $MeHg^+$. As an example, Fig. 2 shows the chromatograms of tuna-2, forkbeard, prawn-1 and cockle. The tuna-2 was fortified with 0.20 mg Hg kg^{-1} of $MeHg^+$; the forkbeard, with 0.35 mg Hg kg^{-1} of $MeHg^+$; and the prawn-1 and cockle, with 0.10 mg Hg kg^{-1} of $MeHg^+$. As can be seen, $MeHg^+$ was recovered successfully from the four samples.

Limits of detection (LOD) and limits of quantification (LOQ) for mercury species were estimated. To calculate LOD and LOQ, the standard deviation of the base line and the chromatographic peak base of each analyte (SD_{BLANK}), multiplied by 3 or 10 (LOD and LOQ, respectively), were interpolated in the slope of the height calibration curve (Ibáñez-Palomino, López-Sánchez, & Sahuquillo, 2012b), which is expressed as: LOD = 3 SD_{BLANK}/slope; LOQ = 10 SD_{BLANK}/slope. The instrument limits were converted to sample limits by multiplying by the extraction dilution factor. The LODs were 0.0003 and 0.0004 mg Hg kg^{-1} for $MeHg^+$ and iHg, respectively. The LOQs were 0.0010 and 0.0012 mg Hg kg^{-1} for $MeHg^+$ and iHg, respectively.

3.3. *Total Hg in samples*

Total Hg concentration was determined in 24 seafood samples: 5 Brazilian fish samples and 19 Spanish seafood samples. The samples were classified as fish ($n = 16$), crustaceans ($n = 3$) and bivalves ($n = 5$); the values found for total Hg in seafood samples are given in Table 3. Total Hg concentration ranged from 0.07 to 2.33 mg kg^{-1}, with the crustaceans and bivalves showing less total Hg than fish samples. Comparison of total Hg concentration means showed that crustaceans and bivalves had 0.07 mg kg^{-1} dry mass (dm) and

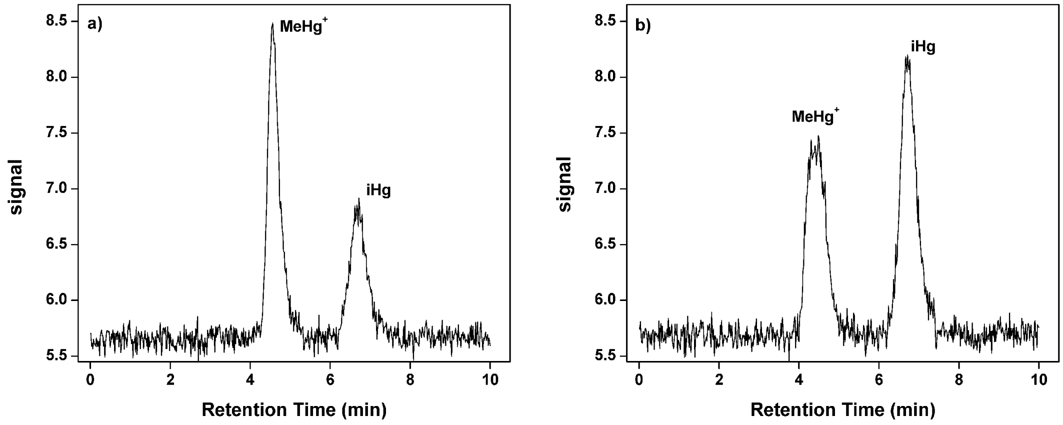

Fig. 1. Chromatograms obtained for DOLT-4 using microwave assisted extraction with (a) HCl 4 mol L^{-1} and (b) HNO_3 4 mol L^{-1}.

Table 2
Total mercury and mercury species in certified reference materials; concentrations are expressed as mg Hg kg^{-1} dry mass (mean ± SD, $n = 3$).

Sample		Total Hg	MeHg^{+}	iHg	Sum of Hg species	Recovery %
TORT-2	measured value	0.30 ± 0.02	0.161 ± 0.010	0.081 ± 0.002	0.242 ± 0.012	80
	certified value	0.27 ± 0.06	0.152 ± 0.013			
DOLT-4	measured value	2.68 ± 0.11	1.27 ± 0.04	1.19 ± 0.02	2.46 ± 0.06	92
	certified value	2.58 ± 0.22	1.33 ± 0.12			
BCR-463	measured value	2.86 ± 0.15	2.78 ± 0.16	0.16 ± 0.20	2.94 ± 0.36	102
	certified value	2.85 ± 0.16	3.04 ± 0.16			

0.12 mg kg^{-1} wet mass (wm), while fish had a mean of 0.71 mg kg^{-1} dm and 0.59 mg kg^{-1} wm. These results are consistent with the literature (Batista et al., 2011; Clémens et al., 2011; Krystek & Ritsema, 2006). According to Krystek and Ritsema (2006), significant differences in Hg levels are found in different seafood species analysed. Fish at high trophic levels in the food chain, like large predatory fish, accumulate more Hg and contain significantly higher concentration levels. Two predatory Brazilian fish samples (red porgy-1 and red porgy-2) and two predatory Spanish fish samples (tuna-3 and swordfish-1) showed the highest levels of total Hg: 1.63 ± 0.04 mg kg^{-1} (red porgy-1), 1.15 ± 0.01 mg kg^{-1} (red porgy-2), 2.33 ± 0.03 mg kg^{-1}(tuna-3) and 1.04 ± 0.03 mg kg^{-1} (swordfish-1).

The Brazilian government, through its Ministry of Agriculture, Livestock and Food Supply (MAPA), instituted a reference value of 0.5 mg kg^{-1} for total Hg in fish farming and 1 mg kg^{-1} for predator fish (Damin et al., 2013; PNCRC, 2009). Two of the five Brazilian samples (red porgy-1 and red porgy-2) were above the values recommended by the Brazilian government (Table 3). All Spanish samples had concentrations of total Hg below the maximum levels set by EC Regulation No 1881/2006 (Commission Regulation (EC) No 1881/2006), except for tuna-3 and swordfish-1 samples (2.33 ± 0.03 mg kg^{-1} and 1.04 ± 0.03 mg kg^{-1}, respectively). These data demonstrate the need to carry out speciation in seafood samples to discern the more toxic species.

3.4. Hg species in seafood samples

The concentrations of MeHg + found in the literature since 2009 are given in Table 1. These concentrations vary widely, depending on the extraction and detection method. According to Table 1, the concentrations of MeHg^{+} ranged from 0.001 to 3.2 mg kg^{-1} for seafood samples. However, bivalves, mollusks and crustaceans have lower MeHg^{+} concentration than fish. Zhang et al. (2012) found concentrations between 0.022 and 0.034 mg Hg kg^{-1} (in the form of MeHg^{+}) for mussel and clam samples. Clémens et al. (2011) found concentrations of 0.001 and 0.033 mg Hg kg^{-1} (in the form of MeHg^{+}) for mussel, oyster, scallop and shrimp; and Batista et al. (2011), of 0.003 and 0.037 mg Hg kg^{-1} (in the form of MeHg^{+}) for mussel, octopus, shrimp and squid samples.

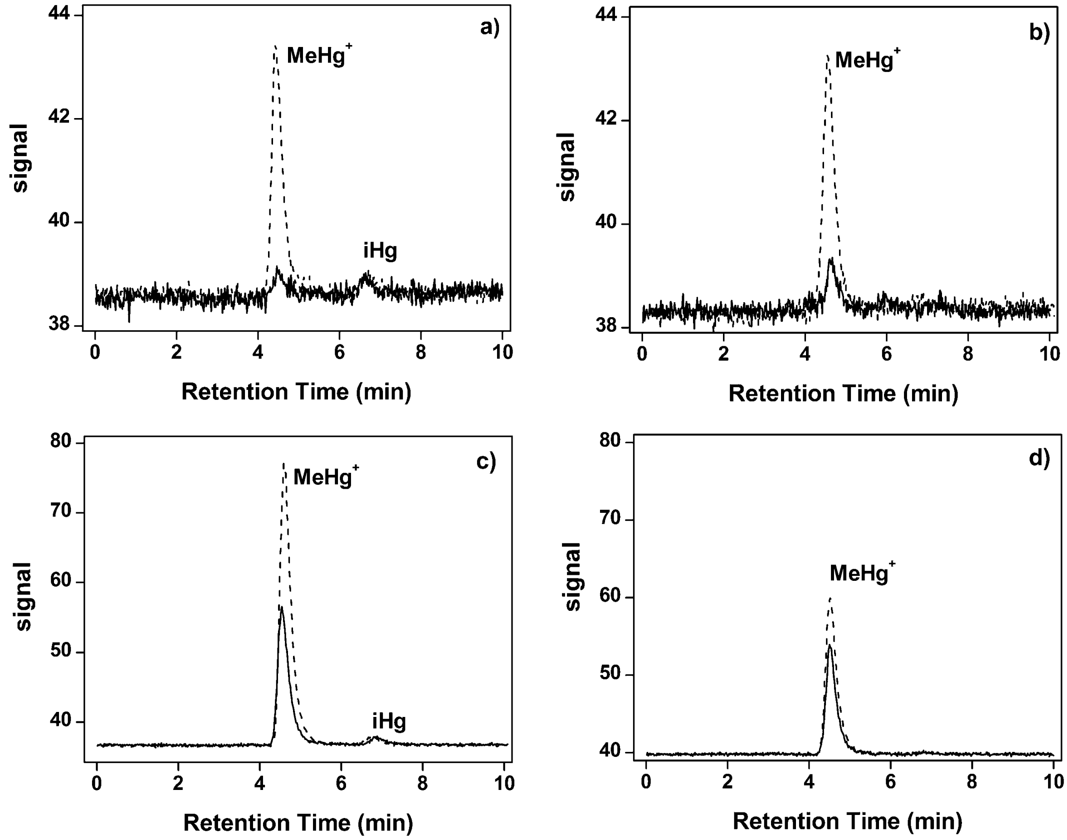

Fig. 2. Chromatograms of a) cokle extract b) prawn-1 extract c) forkbeard extract d) tuna-2 extract (continuous line: non-spiked sample and dotted line: sample spiked with MeHg^{+}) by LC-UV-CV-AFS.

Table 3
Total mercury in seafood samples, concentrations are expressed as mg Hg kg^{-1} wet mass (mean ± SD, $n = 3$).

Samples	Species	Trade name	Origin	Total Hg
Fish	*Urophycis cirrata*	White fish	Brazil	0.27 ± 0.01
	Pagrus pagrus	Red porgy-1	Brazil	1.63 ± 0.04
	Pagrus pagrus	Red porgy-2	Brazil	1.15 ± 0.01
	Merluccius hubbsi	Hake-1	Brazil	0.53 ± 0.01
	Merluccius gayi	Hake-2	Brazil	0.27 ± 0.01
	Phycis blennoides	Forkbeard	Spain	0.30 ± 0.02
	Sardina pilchardus	Sardine	Spain	0.040 ± 0.001
	Salmo sp.	Salmon-1	Spain	0.021 ± 0.001
	Salmo sp.	Salmon-2	Spain	0.023 ± 0.002
	Thunnus sp.	Tuna-1	Spain	0.32 ± 0.04
	Thunnus sp.	Tuna-2	Spain	0.14 ± 0.01
	Thunnus sp.	Tuna-3	Spain	2.33 ± 0.03
	Luvarus imperialis	Louvar	Spain	0.60 ± 0.04
	Xiphias gladius	Swordfish-1	Spain	1.04 ± 0.03
	Xiphias gladius	Swordfish-2	Spain	0.25 ± 0.03
	Xiphias gladius	Swordfish-3	Spain	0.56 ± 0.01
Crustaceans	*Aristeus antennatus*	Prawn-1	Spain	0.013 ± 0.002
	Aristaeopsis edwardsiana	Prawn-2	Spain	<LOQ
	Crangon crangon	Shrimp	Spain	<LOQ
Bivalves	*Tapes pullastra*	Clams-1	Spain	0.015 ± 0.001
	Tapes Decussatus	Clams-2	Spain	0.018 ± 0.001
	Mytilus edulis	Mussel	Spain	<LOQ
	Cerastoderma edule	Cockle	Spain	0.009 ± 0.002
	Ostrea sp.	Oyster	Spain	0.007 ± 0.001

In this study, the Hg species were analysed from a selection of 24 seafood samples, including crustaceans, bivalves and fish. The results are given in Table 4. For all samples, the sum of MeHg$^+$ and iHg concentration (obtained by the proposed speciation method, using LC-UV-CV-AFS) was compared with total Hg concentration (obtained by ICP-MS). The total Hg concentration was taken as 100% in the calculation of recovery values. All samples analysed showed recovery values between 88 and 120% (Table 4), which are corroborated by the literature (Chen, Han, Cheng, Liu et al., 2013; Clémens et al., 2011; Kenšová et al., 2012). Clémens et al. (2011) observed recoveries between 90 and 110% for matrices with low-fat content. High recovery values were observed for salmon, hake and whitefish samples (fatty samples), with values of 120%, 117% and 114%, respectively (Clémens et al., 2011). Thus, close correlation between total and sum of species is achieved, regardless of sample matrix composition.

The presence of MeHg$^+$ was detected in 19 analysed samples. MeHg$^+$ was the predominant form of mercury in all fish samples and one shellfish sample (prawn-1). The clam-2 and cockle samples had only 13% and 36% of MeHg$^+$, respectively. The mean values of percentage and concentration of MeHg$^+$ in fish and shellfish samples were calculated. For fish, a mean percentage of 98%, a mean concentration of 0.71 mg MeHg$^+$ kg^{-1} in dry mass (dm) and a mean concentration of 0.60 mg MeHg$^+$ kg^{-1} in wet mass (wm) were found; and for shellfish, a mean percentage of 49%, a mean concentration of 0.027 mg MeHg$^+$ kg^{-1} in dm and a mean concentration of 0.009 mg MeHg$^+$ kg^{-1} in wm. Inside the fish sample group, the highest concentrations of MeHg$^+$ in wm were found for red porgy-1 and red porgy-2 (mean value 1.4 mg kg^{-1}), tuna-3 (2.23 mg kg^{-1}) and swordfish-1 (1.04 mg kg^{-1}). In shellfish, the highest levels of MeHg$^+$ were found for prawn-1 (0.011 mg kg^{-1}). The concentrations of all samples were within the maximum levels set by (EC) No. 1881/2006 for MeHg$^+$ (Commission Regulation (EC) No 1881/2006), except for red porgy-1 and -2 (Brazilian fish samples), tuna-3 and swordfish-1 (Spanish fish samples), which showed concentrations higher than 1 mg kg^{-1}. In some samples, iHg was also identified. Table 4 shows that values of iHg concentration ranged from 0.010 to 0.085 mg iHg kg^{-1} in wm in fish samples; and from 0.006 to 0.016 mg iHg kg^{-1} in wm in shellfish samples. However, iHg was quantified only in four of the eight shellfish samples. These data underline the importance of speciation in seafood samples. Speciation makes it possible to establish which the most harmful form to humans is and, therefore, whether the seafood is suitable or not for consumption. Likewise, the need to introduce maximum levels of MeHg$^+$ in seafood in Brazilian and European legislation should be considered in further Directives.

According to data obtained in this work, and as described in the literature, the concentrations of MeHg$^+$ are higher in fish than shellfish being predatory fish those samples showing the highest values.

Table 4
Mercury speciation analysis of selected seafood samples; concentrations are expressed as mg Hg kg^{-1} wet mass (mean ± SD, $n = 3$).

Sample	MeHg$^+$	%MeHg$^+$	iHg	Sum of Hg species	Recovery (%)
White fish	0.30 ± 0.02	100	<LOQ	0.30 ± 0.02	114
Red porgy-1	1.67 ± 0.04	96	0.061 ± 0.009	1.73 ± 0.05	105
Red porgy-2	1.13 ± 0.06	97	0.035 ± 0.001	1.17 ± 0.06	101
Hake-1	0.62 ± 0.02	97	0.019 ± 0.002	0.64 ± 0.02	117
Hake-2	0.31 ± 0.04	100	<LOQ	0.31 ± 0.04	114
Forkbeard	0.32 ± 0.01	98	0.010 ± 0.003	0.33 ± 0.01	109
Sardine	0.040 ± 0.002	100	<LOQ	0.040 ± 0.002	100
Salmon-1	0.022 ± 0.001	100	<LOD	0.022 ± 0.001	103
Salmon-2	0.025 ± 0.003	100	<LOD	0.025 ± 0.003	120
Tuna-1	0.30 ± 0.05	98	0.011 ± 0.003	0.31 ± 0.05	95
Tuna-2	0.136 ± 0.008	100	<LOD	0.136 ± 0.008	97
Tuna-3	2.23 ± 0.04	96	0.085 ± 0.004	2.31 ± 0.04	99
Louvar	0.64 ± 0.03	99	0.011 ± 0.001	0.65 ± 0.03	108
Swordfish-1	1.04 ± 0.04	98	0.02 ± 0.002	1.06 ± 0.05	102
Swordfish-2	0.26 ± 0.03	100	<LOD	0.26 ± 0.03	102
Swordfish-3	0.58 ± 0.04	100	<LOQ	0.58 ± 0.04	103
Prawn-1	0.011 ± 0.003	100	<LOD	0.011 ± 0.003	88
Prawn-2	<LOD	–	<LOD	–	–
Shrimp	<LOD	–	<LOD	–	–
Clams-1	<LOD	–	0.016 ± 0.004	0.016 ± 0.004	108
Clams-2	0.013 ± 0.001	13	0.008 ± 0.001	0.021 ± 0.002	110
Mussel	<LOD	–	<LOD	–	–
Cockle	0.003 ± 0.001	36	0.006 ± 0.002	0.009 ± 0.003	110
Oyster	<LOD	–	0.007 ± 0.001	0.007 ± 0.001	100

Higher $MeHg^+$ content in fish samples could be related to the fat content. Methylmercury is a fat-soluble substance and therefore can be accumulated in the fatty tissues more easily than inorganic mercury. Bluefish samples, such as salmon and tuna with high fat content, present high levels of $MeHg^+$. Whitefish and shellfish, with lower fat content, present lower MeHg + concentration and in the case of some shellfish samples the predominant mercury species is iHg.

When comparing the concentrations found in this study for $MeHg^+$ in fish (Table 4) with the literature (Table 1), the values were similar (Chen, Han, Cheng, Liu, et al., 2013; Chen, Han, Cheng, Wang, et al., 2013; Clémens et al., 2011; Montero-Alvarez, Fernández de la Campa, & Sanz-Medel, 2014) or higher (Carrasco et al., 2011; Fu, Wang, Zhou, & Jiang, 2010; Hajeb, Jinap, & Ahmad, 2010; Kenšová et al., 2012; Kuballa, Moellers, Schoeberl, & Lachenmeier, 2011; Liang et al., 2011; Miklavčič et al., 2011; Nevado et al., 2011; Qiu, Feng, Wang, Fu, & Shang, 2009; Santoyo et al., 2009; Wang et al., 2010). The shellfish analysed had similar levels of $MeHg^+$ to those found by Clémens et al. (2011). According to Fitzgerald, Lamborg, and Hammerschmidt (2007), the behaviour of Hg chemistry in the marine environment and the number of predatory fish analysed explain the differences between the mean values of $MeHg^+$ found in several studies. The conditions of the water environment, the age of each species and the time of exposure to Hg contaminants are also factors that affect the results (Fitzgerald et al., 2007). The results obtained are in agreement with those reported by Kuballa et al. (2011), showing a great variability in $MeHg^+$ concentration in different fish species. These differences reaffirm the need to monitor $MeHg^+$ concentrations in seafood species more frequently and in different areas, in order to avoid human contamination.

4. Conclusion

This study determined total Hg, $MeHg^+$ and iHg species in different seafood samples, including fish, crustaceans and bivalves. Figures of merit (LOD, LOQ, reproducibility and trueness) of the proposed LC-UV-CV-AFS procedure were satisfactory for the determination of $MeHg^+$ and iHg in fish and shellfish. $MeHg^+$ was the predominant species in all fish samples. The highest levels of $MeHg^+$ in fish were found in two Brazilian fish samples and two Spanish fish samples. All concentrations are below the maximum levels set by Regulation (EC) No. 1881/2006 for $MeHg^+$ except for these four fish samples, which showed concentrations higher than 1 mg kg^{-1}. Despite the lack of Brazilian legislation regulating the maximum levels of $MeHg^+$ in seafood, the present results have increased the availability of reliable results on $MeHg^+$ in seafood and could be used in further Directives on $MeHg^+$ in food commodities. Thus, the present method could be a valuable tool for food control laboratories that assess $MeHg^+$ in seafood samples.

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ANNEX 3

Article: *Migration of antimony from polyethylene terephthalate used in mineral water bottles*

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Migration of antimony from polyethylene terephthalate used in mineral water bottles

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ABSTRACT

The influence of storage time and temperature on Sb migration from PET bottles into mineral water was studied in short-term tests lasting up to 15 days and long-term studies lasting up to 220 days. Samples purchased were stored in three different coloured bottles: clear (CL), light blue (LB) and dark blue (DB). Sb migration was assayed by HG-AFS for total determination and HPLC-ICP-MS for speciation analysis. Migration studies showed that waters stored at 4 and 20 °C were not subject to Sb migration. At 40 °C there was a significant increase in Sb concentration, although the maximum limit established by the European Union (5.0 μg L^{-1}) was not exceeded, whereas at 60 °C samples were subject to considerable Sb migration after 30 days of storage. In this case, the maximum limit established by the European Union was exceeded and both Sb (V) and Sb (III) were detected.

1. Introduction

In recent decades, bottled water consumption has increased considerably, raising concerns over water quality and the packaging material (Biros, Bridgewater, Villeges-Estrada, Tanski, & Parkin, 2002).

Polyethylene terephthalate (PET) is a thermoplastic material that is manufactured for a wide variety of applications, especially food and beverage packaging, including drinking water bottles (Ashby, 1988; Biros et al., 2002; Duh, 2002). The first industrial step in the synthesis of PET is a prepolymerization reaction that generates low-weight oligomers and bis(hydroxyethyl)terephthalate (BHET) as an intermediate compound. A second polycondensation reaction use various catalysts. Of these, antimony trioxide (Sb_2O_3) is widely used because it is efficient, presents a minimal tendency to produce side effects and does not create colour in the polymer (Aharoni, 1998; Duh, 2002). The use of this catalyst can lead to residual Sb contents ranging from 200 to 300 mg kg^{-1} (Keresztes et al., 2009; Shotyk & Krachler, 2007; Shotyk, Krachler, & Chen, 2006; Tukur, Sharp, Stern, Tizaoui, & Benkreira, 2012; Welle & Franz, 2011; Westerhoff, Prapaipong, Shock, & Hillaireau, 2008).

Antimony is found as organic and inorganic compounds in two oxidation states. Its other properties, such as solubility and adsorption behaviour, are similar to those of arsenic (Filella, Belzile, & Chen, 2002a; Gebel, 1997). Organic antimony compounds are the less toxic forms and between the inorganic forms, Sb (III) is 10-fold more toxic than Sb (V) (Poon et al., 1998; Rivas, López-García, & Hernández-Córdoba, 2009; Seiler, Sigel, & Sigel, 1988). Therefore, Sb is considered a priority pollutant by the United States Environmental Protection Agency (USEPA) (USEPA, 2009) and the European Union (EU). It has been included in European regulations concerning the quality of water intended for human consumption, among others (Council of the European Union, 1998).

European regulations on plastic materials and articles intended to come into contact with food (European Parliament and the Council of European Union, 2004) have established specific requirements concerning the substances authorised to be present in these materials, such as monomers, additives, polymer production aids and macromolecules. For these authorised substances, specific migration limits (SML) have been established on the basis of migration tests performed on the plastic material. These tests are based on the risk assessment of the single substance able to migrate, simulating the worst case of the foreseeable conditions, in order to ensure the safety of the final material. Specific migration tests should be carried out directly in food or using different food simulants according to the food type (acetic acid, ethanol, vegetable oil and poly(2,6-diphenyl-p-phenylene oxide)), and different storage times (up to 10 days) and temperatures (5–175 °C) depending on the foreseen

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contact time. In PET-packaged food, the SML for Sb_2O_3 is 40 μg kg^{-1} as total Sb (European Commission, 2011).

Studies of total Sb content in drinking waters conducted in recent years have found that although the total residual Sb content is relatively high, the Sb concentration in water samples is well below the Maximum Concentration Levels (MCL) for drinking waters (5 or 6 μg L^{-1}) established by USEPA, Canada and the EU, among others (Shotyk et al., 2006; Shotyk & Krachler, 2007; Cheng, Shi, Adams, & Ma, 2010; Westerhoff et al., 2008). Such studies concluded that the presence of Sb in bottled water was due to its migration from PET, as fresh water obtained directly from a spring, a well or stored in other types of plastic, such as polypropylene (PP) or low density polyethylene (LDPE), did not contain high Sb levels (Shotyk et al., 2006; Shotyk & Krachler, 2007). Although the influence of PET colour was also studied by several authors, its potential effect remains unclear. On the one hand, it was reported that there was no correlation between PET colour and the Sb migration potential in more than 40 and 100 water samples analysed (Shotyk et al., 2006; Shotyk & Krachler, 2007; Tukur et al., 2012). On the other hand, Sb migration was found to be higher in water stored in dark coloured bottles than that stored in clear bottles (Reimann, Birke, & Filzmoser, 2010), although the opposite effect has also been described when comparing colourless and blue bottles (Westerhoff et al., 2008).

Physicochemical factors that can affect Sb migration from the container to the water have also been studied. Storage time (from 3 months up to 3 years) has been the most studied variable, mainly for samples kept at room temperature. All studies showed that the Sb concentration in mineral water increased over time by 25–90%. However, the maximum concentration limit was not exceeded in any case (Keresztes et al., 2009; Shotyk & Krachler, 2007; Tukur et al., 2012; Westerhoff et al., 2008).

Other short-term stability studies have examined water samples stored from a few hours to 7 days at temperatures ranging from −20 to 80 °C. Whereas a slight increase in Sb content was observed in samples stored at 60 °C, rapid release of Sb into the matrix was observed after 5–24 h of storage at 80 °C. The irradiation of samples with sunlight (either natural or simulated) also caused a small but significant increase in Sb leaching (Keresztes et al., 2009; Tukur et al., 2012; Westerhoff et al., 2008).

Other factors, such as the presence of carbon dioxide in bottled waters was studied by Bach et al., 2013. Sb concentration in carbonated water was twice as high as non-carbonated water, suggesting that Sb release was accelerated by carbon dioxide.

Other studies have dealt with the total Sb determination in bottled fruit juices and other soft drinks. In these matrices, the reported Sb concentration values were either over or under the maximum level established for drinking waters (Hansen et al., 2010; Tukur et al., 2012).

Few studies have dealt with the identification, quantification and fate of Sb species migrating from PET into beverages. However, the presence of different coordination complexes of Sb (V) has been confirmed, in spiked non-complexed Sb (V) samples of fruit juices stored in PET, using HPLC-ICPMS and HPLC-ES-MS(/MS). The complexes described were of high stability and with different coordination numbers between Sb (V) and citrate, adenosine and lactate. Whereas inorganic Sb (III) or Sb (V)-citrate were the main species present in citrus juices, only non-complexed inorganic Sb (V) was observed in drinking waters, all of them stored in PET (Hansen & Pergantis, 2007). The effect of temperature (50 °C) on the leaching of these species was described for two carbonated and two non-carbonated juices stored for up to 14 days. The leached Sb concentration only increased in carbonated samples and was mainly due to an increase in Sb (III) (Hansen & Pergantis, 2006). Such speciation studies have not been reported for drinking water samples.

In this paper, the influence of various physicochemical factors, including storage time, temperature and PET colour on Sb migration in bottled waters, was investigated. A systematic study was undertaken to assess the effect of storage time longer than 6 months, and storage temperatures from 4 to 60 °C on bottled drinking waters. Total Sb and speciation analysis was carried out on water samples bottled in clear and blue PET containers.

2. Materials and methods

2.1. Instrumentation

Microwave digestion of the PET bottles was performed using a Milestone Ethos Touch control instrument, with a temperature controller and a power of 1000 W. For this procedure, twelve Teflon pressure vessels were used simultaneously.

Total Sb content was determined using hydride generation-atomic fluorescence spectrometry (HG-AFS). Hydride generation was performed using a Millennium PS Analytical (Kent-UK), model 10.055. The reagents for stibine generation, HCl 2 mol L^{-1} and $NaBH_4$ 0.7 (w/v) were continuously pumped at 8.0 and 4 mL min^{-1}, respectively. Samples were introduced at 8.0 mL min^{-1}. After reaction in a coil the generated stibine was driven by an argon flow (300 mL min^{-1}) to the AFS detector through the type 'ME' gas–liquid separator. Before detection, the argon stream was passed through a Perma pure drying membrane (Perma Pure Products, Farmingdale, NJ, USA) which prevents droplets being transmitted into the transfer line. N_2 was used as drying gas at a flow rate of 2.5 L min^{-1}. Detection was carried out in a P.S Analytical Excalibur Atomic Florescence Spectrometer equipped with a diffusion flame and a Sb-boosted hollow cathode lamp (Super Lamp Photron Teknokroma).

A quaternary pump auto-sampler (Agilent 1200) with an injection volume of between 0.01 and 2000 μL, and an anion exchange column with ammonium quaternary salt and methyl groups as substituents, were used for the separation of Sb species. The outlet of the column was coupled with inductive coupled plasma-mass spectrometry (ICP-MS) (Agilent 7500 ce), with a reaction cell and Burgener Ari Mist HP nebulizer. The ion intensity at m/z 121 (^{121}Sb) was monitored using time-resolved analysis software.

The experimental conditions of HPLC-ICP-MS are the following: PRP-X100 column (125 × 4.1 mm Hamilton, 10 μm particle size, USA) working at room temperature using 10 mM ethylenediaminetetraacetic acid (EDTA) at pH 4.0 with 0.5% methanol as mobile phase; injection volume of 40 μL; flow rate of 1.5 mL min^{-1}.

2.2. Reagents and standards

The standards and reagents used in this study were prepared with deionized water (Elix&Rios 5–15 M Ω cm^{-1}, Total Organic Carbon < 30 μg L^{-1}) obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

A 1000 mg L^{-1} stock standard solution of Sb (III) was prepared by dissolving appropriate amounts of potassium antimony (III) oxide tartrate hemihydrate (Fluka, Neu-Ulm, Germany) in water. The solution was diluted to 100 mL.

The 1000 mg L^{-1} stock standard solution of Sb (V) was prepared by dissolving potassium hexahydroxoantimonate (Riedel de-Haën, Seelze, Germany) in water. The solution was diluted to 100 mL.

Both standard solutions were standardised against a certified standard solution of 1000 ± 4 mg L^{-1} of Sb, prepared from metallic antimony 99.999% "purum", dissolved and stabilised in high purity acids (nitric acid (HNO_3) 5% and hydrofluoric acid (HF) 0.1%). All stock standard solutions were stored in polyethylene bottles at 4 °C. The working standard solutions were prepared daily by dilution in mobile phase for speciation analysis and in acidic medium for total analysis.

For hydride generation in total Sb determination by HG-AFS, sodium borohydride solution was prepared daily from $NaBH_4$ (Aldrich, >97% purity) to a 0.7% concentration containing 0.4% sodium hydroxide prepared from NaOH (Merck, $\geqslant$99% purity). The HCl solution at 2 M was prepared from concentrated HCl (Panreac, hyperpure reagent grade 35%).

Aqueous solutions of the pre-reducing agent in total Sb determination by HG-AFS were prepared daily from both ascorbic acid 99% (Panreac) and potassium iodide, KI, 99.5% (Panreac), at concentrations of 10% and 50%, respectively.

For PET extraction based on end-over-end agitation, a solution of HCl 10 mmol L^{-1} was prepared from concentrated HCl (Panreac, hyperpure reagent grade 35%).

For microwave digestion of PET samples, concentrated HNO_3 (Panreac, hyperpure reagent grade 69%) and sulphuric acid, H_2SO_4, 96% (Merck) were used.

The mobile phase was prepared daily by dissolving EDTA (Panreac) in water. The pH was adjusted with diluted ammonia (Panreac) and then passed through a 0.45 μm filter (Millipore type HA).

2.3. Selection of samples

Three commercial brands of PET-bottled water were purchased in a local market. A previous study screened 38 non-carbonated samples with different degrees of mineralisation and stored in different coloured bottles. The results of this study are summarised in Table S1 as Supplementary material. No relationship was found between Sb concentration and mineral content. However, more than half of the samples stored in clear bottles contained lower amounts of Sb than those stored in coloured bottles. On this basis, the selection of brands was based on the colour of the PET bottle: clear (CL), light blue (LB) and dark blue (DB). Each brand was non-carbonated and weak mineralised. Analysis of total Sb and speciation in PET was performed in triplicate for each brand.

Polyethylene certified reference material (CRM) ERM-EC681k from Institute for Reference Materials and Measurements in Geel, Belgium was used for quality control purposes. To our knowledge there are no CRMs for antimony in PET and thus, polyethylene was considered the most similar material to PET available. Antimony certified value for ERM-EC681k is 99 ± 6 mg Sb kg^{-1}.

2.4. Migration experiments

The effect of temperature stress on the Sb concentration in water was also carried out in the three commercial bottled water brands. Total and speciation Sb in water were analysed at the beginning of the test and after 15 days at 60 °C. Samples were then stored for another 15 days at 4 °C and the Sb contents were measured again. The analyses were performed in triplicate in all cases.

The same samples were used to study the influence of storage time and temperature on the Sb concentration in water in long-term migration studies.

Three bottles of each brand were stored at 4, 20, 40 and 60 °C. Speciation and total antimony were analysed by triplicate analysis on 12 occasions until the bottles ran out of water (220 days). The quantification techniques were HG-AFS for total analysis and HPLC-ICP-MS for speciation.

2.5. Determination of antimony in water samples

2.5.1. Total Sb by HG-AFS

A 10 mL aliquote of bottled drinking water was pre-reduced with the ascorbic acid and KI solution in order to achieve the maximum yield of stibine in the hydride generation step and was diluted up to 25 mL in HCl medium. Samples were introduced to the system at conditions mentioned in Section 2.1. The samples were quantified by means of an external calibration curve from Sb (III) standards. Analyses in triplicate were performed for each sample. For quality control purposes, the standards of the calibration curve were run before and after each sample series. Quality control standard solutions at two concentrations were measured at the end of the sequence to ensure stable instrument sensitivity.

2.5.2. Speciation Sb analysis by HPLC-ICP-MS

Samples were analysed in triplicate without pre-treatment. A 40-μL aliquot of sample was directly injected into the HPLC system operating at the conditions mentioned in Section 2.1. A solution of rhodium was used as the internal standard. The samples were quantified by means of an external calibration curve from Sb (III) and Sb (V) standards. Antimony species in extracts were identified by comparison of retention times with standards. External calibration curves quantified Sb (V) and Sb (III), according to the corresponding standards. All samples were analysed in triplicate. For quality control purposes, the standards of the calibration curve were run before and after each sample series In each speciation run, two quality control standard solutions were measured at the end of the sequence to ensure stable instrument sensitivity. Data acquisition was carried out using ICP-MS Chemstation software, and the peak areas were calculated using the ICP-MS Chromatographic Data Analysis.

2.6. Determination of antimony in PET

2.6.1. Total Sb

PET bottles were cut into pieces measuring 1.0 × 1.0 cm^2, and 0.1500 g of sample was subjected to microwave-assisted closed pre-digestion with 3.0 mL H_2SO_4 at 300 W for 5 min. Afterwards the sample was digested with 8.0 mL HNO_3 at 200 °C for 15 min; the samples were filtered through a filter paper (Whatman 40) into a 50-mL flask glass, diluted with de-ionised water and the antimony content of a 10-mL aliquote was analysed as described in Section 2.5.1. The corresponding digestion blanks (one for each sample digestion series) were also measured. To assess the accuracy of the method, a CRM (ERM-EC681K) was analysed following the same procedure.

2.6.2. Extraction species

A 1.5-g sample was shaken with 10 mL HCl 10 mM as the extractant solution in Teflon tubes for 16 h at 30 rpm (Miravet, López-Sánchez, & Rubio, 2006a). The antimony species in the extracts were determined by HPLC-ICP-MS and total antimony by HG-AFS as described in Section 2.5. Extraction blanks were also analysed in each work session. To assess the accuracy of the method, a CRM (ERM-EC681K) was analysed following the same procedure.

3. Results and discussion

3.1. Antimony in PET material

The concentrations of antimony in the three samples are shown in Table 1. The total Sb content obtained by microwave extraction is included in the first column. Values of Sb species obtained after an HCl extraction together with the total leachable Sb in the extracts are reported in columns 2–4.

Total Sb content was also analysed in polyethylene CRM (ERM-EC681k) providing a concentration value of 101.4 ± 0.2 mg kg^{-1} ($n = 3$) and a RDS of 0.2%. No significance difference was found

Table 1
Total antimony concentration and speciation in PET.

Samples	Sb total[a] (mg kg^{-1})	Sb total[b] (μg kg^{-1})	Sb (V)[b] (μg kg^{-1})	Sb (III)[b] (μg kg^{-1})
CL	203 ± 1.4 (0.7%)	11.1 ± 1.4 (12.4%)	8.6 ± 1.4 (16.4%)	<LD
LB	191 ± 2.7 (1.4%)	18.2 ± 2.1 (11.4%)	16.5 ± 2.7 (16.4%)	<LD
DB	268 ± 2.0 (2.0%)	10.0 ± 1.5 (14.7%)	6.6 ± 0.4 (6.8%)	<LD

Samples were clear (CL), light blue (LB) and dark blue (DB). The relative standard deviations are expressed in% in parentheses. LD Sb total HCl extraction = 2.29 μg kg^{-1}; LQ Sb total HCl extraction = 2.92 μg kg^{-1}; LD Sb (V) = 0.5 μg kg^{-1}; LQ Sb (V) = 1.6 μg kg^{-1}; LD Sb (III) = 2.5 μg kg^{-1}; LQ Sb (III) = 8.3 μg kg^{-1}.

[a] Concentrations obtained by microwave digestion. Results are expressed as mean Sb value ± Standard Deviation in mg kg^{-1} ($n = 3$).

[b] Concentrations obtained by HCl extraction. Results are expressed as mean Sb value ± Standard Deviation in μg kg^{-1} ($n = 3$).

between certified and experimental total content (dependent *t*-test for paired samples at 95% confidence level).

As it can be seen in the first column of Table 1, PET bottles contain between 191 and 268 mg kg^{-1} of total Sb, the dark blue sample containing slightly higher levels than the other two samples. The Sb contents measured were comparable with those reported by other authors (154–311 mg kg^{-1}) (Keresztes et al., 2009; Tukur et al., 2012; Welle & Franz, 2011; Westerhoff et al., 2008; Takahashi, Sakuma, Itai, Zheng, & Mitsunobu, 2008).

The presence of this element in the polymer can be attributed to its use as a catalyst during PET manufacture, as mentioned in the introduction. It can be added as glycolate or antimony trioxide. When it is added as Sb_2O_3, it is readily converted to glycolate (Duh, 2002). At the end of the manufacturing process, Sb remains bound in the polymer chain as antimony glycolate complexes (Duh, 2002; Welle & Franz, 2011; Takahashi, Sakuma, Itai, Zheng, & Mitsunobu, 2008). Nevertheless, it is possible that some Sb_2O_3 remains unconverted (Sánchez-Martínez, Pérez-Corona, Cámara, & Madrid, 2013). However, although total Sb in PET is relatively high (up to 300 mg kg^{-1}), the levels of Sb present in beverages in contact with PET are much lower, and there is little information about the migration potential and mechanisms of different Sb chemical forms (Welle & Franz, 2011).

Therefore, a preliminary test was performed to assess the total Sb and main Sb species extracted from PET. HCl 10 mM was used as the extractant, which after being previously identified as the optimum extractant in other environmental samples (Miravet et al., 2006a). The total HCl-extractable Sb content (second column in Table 1) ranged from 10.0 to 18.2 μg kg^{-1}. Additionally, same experiment was performed on polyethylene CRM providing concentration values of 40.2 μg kg^{-1}. These values corresponded to 0.0037–0.0095% of total Sb in PET, and 0.0396% in polyethylene sample showing that only a small amount of the antimony present in PET bottles leached into the extractant solution, as indicated by other authors (Welle & Franz, 2011).

The speciation analysis of HCl extracts for PET samples (third and fourth columns in Table 1) revealed that Sb (V) was the only species present. This is consistent with the results of Takahashi et al. (2008), who concluded that, although antimony is added as a trivalent species during PET manufacture, it can be partially oxidized (up to 50%) to Sb (V). When performing HCl extraction on the polyethylene CRM, 64.9% of total Sb was present as Sb (III). This different behaviour can be attributed to the procedure described for the preparation of CRM batches, where Sb_2O_3 pigment was added to the polyethylene powder once manufactured. Thus, speciation behaviour of CRM cannot be compared with the PET samples analysed.

As can be seen from the results in Table 1, total Sb content in PET was not directly correlated with the extractable content: light blue samples with the lowest total Sb content showed the highest Sb leachability. Comparison of total Sb extractable content with the speciation analysis (i.e. total Sb versus Sb (V)) via a dependent Student's *t*-test for paired samples showed statistically significant differences at the 95% confidence level, especially with regard to dark blue bottles. This can be attributed to the small amounts of Sb species found in the measuring extracts, which were close to the limit of quantification where the widespread of expected results can be higher. Thus there was a significant difference between total extractable contents and species in dark blue bottles.

In order to assess the possible oxidation of Sb (III) during extraction procedure, leaching experiments with addition of 1 mL EDTA 10 mM in the extractant solution (HCl 0.01 mol L^{-1}) were performed on LB PET sample. In these extracting conditions, 39% of total extracted Sb was present as Sb (III). This fact evidenced that the potential Sb (III) form present and extracted from the PET can be easily oxidised to Sb (V).

3.2. Temperature stress test

The potential effect of temperature on Sb release into water during storage was first studied in the short term. The experimental conditions for this study are described in Section 2.4. The results are shown in Fig. 1, where the total Sb and speciation analyses are plotted for each brand, together with the standard deviation. Accordingly, antimony was detected in all samples before the storage (day 0). Total Sb ranged from 0.3 to 0.7 μg L^{-1}. After storage at 60 °C, a significant increase in total Sb concentration was observed (2.6–4.2 μg L^{-1}). Subsequent storage at 4 °C gave similar values to those obtained after storage at 60 °C.

In the case of speciation studies, Sb (V) was initially the only species present. After 15 days storage at 60 °C, the Sb (V) concentration increased, and Sb (III) appeared in all samples at detectable but not quantifiable levels.

From the reduced number of units analysed, it can be stated that waters stored in coloured bottles contained similar Sb

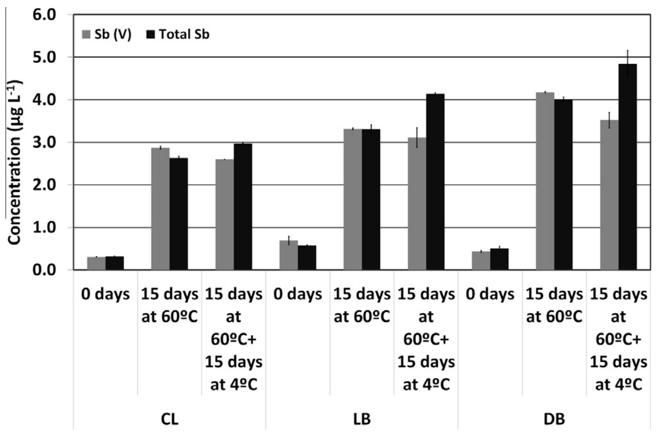

Fig. 1. Total Sb and Sb (V) concentration (μg L^{-1}) in bottled mineral water samples from three different-coloured PET brands. Sb (III) was not detected at day 0 and was not quantified thereafter. Times and temperature are mentioned for each brand. Legend for bottle colours: CL: clear; LB: light blue; DB: dark blue. LD Sb total = 0.11 μg L^{-1}; LQ Sb total = 0.14 μg L^{-1}; LD Sb (V) = 0.05 μg L^{-1}; LQ Sb (V) = 0.16 μg L^{-1}; LD Sb (III) = 0.25 μg L^{-1}; LQ Sb (III) = 0.83 μg L^{-1}.

concentrations, but they were slightly higher than those stored in clear bottles. The relative standard deviation ranged from 0.07% to 9.90%. A dependent Student's *t*-test for paired samples, at the 95% confidence level, demonstrated that there was no significant difference between total Sb content and Sb (V). Furthermore, using the same statistical *t*-test it was shown that Sb concentrations after storage at 4 °C did not differ significantly from those previously obtained after storage at 60 °C.

These results showed that, as expected, the exposure of water to a high temperature leads to significant Sb migration from the bottles to the water. It was also observed that antimony is not leached at low temperatures as the Sb concentration remained stable at the lower storage temperature. During these 30 days, there was no change in the Sb species present in water, indicating that they maintained their integrity throughout the experiment. The Maximum Concentration Level (MCL) established by the European Union (5.0 μg L^{-1}) was not exceeded in any case.

Although some consider migration studies at high temperatures (60 °C) to be unnecessary for water samples (Sánchez-Martínez et al., 2013), others have indicated that they are necessary given that the temperatures inside cars, garages and closed storage areas can exceed 65 °C during the summer in some regions (Westerhoff et al., 2008). Thus, based on the results obtained in the short-term stress test, a more systematic study was undertaken.

3.3. Migration study

The effect of different storage temperatures (4, 20, 40 and 60 °C) and times (up to 220 days) on the Sb concentration was assessed. At the beginning of the test, all samples were analysed and Sb (V) was the only species present.

For samples stored at 4 and 20 °C, a dependent Student's *t*-test for paired samples at the 95% confidence level for the 11 different times assayed showed no significant differences in Sb concentration in water over time. All samples contained total Sb concentrations below 1 μg L^{-1}. Thus, the maximum level established by the European Union (5.0 μg L^{-1}) was not exceeded. The only species present was Sb (V).

The concentrations of total Sb and the results of the speciation analysis from the long-term migration experiments at 40 and 60 °C are summarized in Figs. 2 and 3. The first figure shows the total Sb concentration in each brand stored from day 0 to day 220. The second figure shows the Sb (V) concentration under the same conditions.

Samples stored at 40 °C showed a significant increase in antimony concentration after 15 days of storage. From 50 days, this increase became quite pronounced and Sb (III) was also detected in light blue and dark blue bottled samples at concentrations below the limit of quantification. However, Sb (III) was not detected from day 97 onwards. The regulated level was not exceeded in any case.

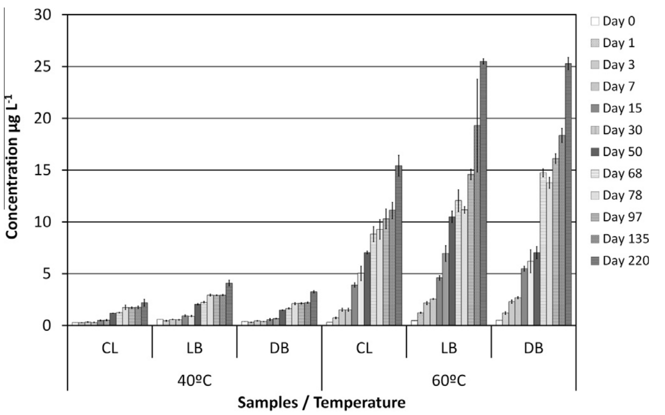

Fig. 2. Evolution of total Sb in bottled drinking water stored at 40 and 60 °C for 220 days. Legend for bottle colours: CL: clear; LB: light blue; DB: dark blue.

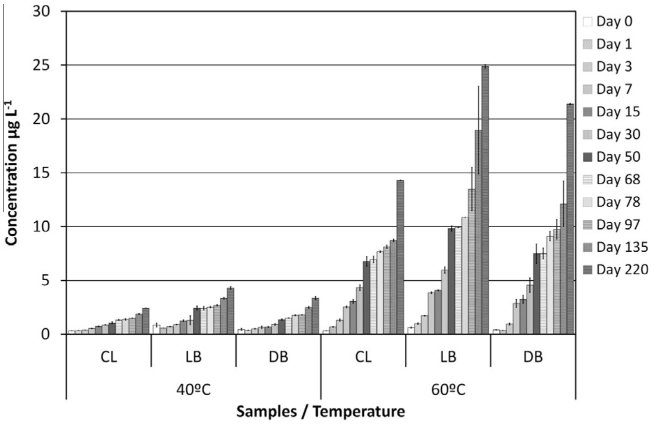

Fig. 3. Evolution of Sb (V) in bottled drinking water stored at 40 and 60 °C for 220 days. Legend for bottle colours: CL: clear; LB: light blue; DB: dark blue.

Samples stored at 60 °C showed a significant increase in total antimony concentration from the first day of storage. The dark blue samples began to exceed the limit for Sb according to the European Union (5 μg L^{-1}) after 15 days, and after 30 days, all samples exceeded the limit. This is consistent with the results obtained by Keresztes et al. (2009), Tukur et al. (2012) and Westerhoff et al. (2008) regarding the increase in total Sb concentration in water samples stored at 60 °C for a few hours to 7 days. As far as the PET colour is concerned, the results obtained in this study seem to indicate that Sb release was slightly lower in non-coloured bottles than in coloured ones, regardless of the colour intensity. This is consistent with Shotyk and Krachler (2007) and Tukur et al. (2012), who reported that there was no correlation between PET colour and Sb migration potential.

Speciation analysis of the samples at 60 °C showed that Sb (III) was detected from the third to the fiftieth day. After day 50, the concentration of this species was slightly above the limit of quantification in clear and light blue samples but clearly higher in dark blue samples. Chromatograms obtained from a water sample stored at 60 °C are shown in Fig. 4. The insert shows the Sb (III) peaks obtained at day 0 and 7, in which the species could not be quantified. The Sb (III) concentration in samples stored for longer than 50 days is summarized in Table 2. As shown, the amounts

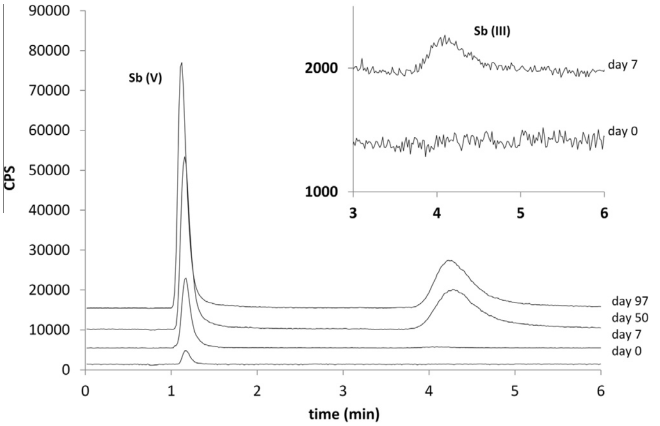

Fig. 4. Chromatograms obtained from samples stored at 60 °C by HPLC-ICP-MS at days 0, 7, 50 and 97. The insert shows the chromatograms obtained at days 0 and 7, indicating the presence of Sb (III).

Table 2
Leaching of antimony (III) from PET into water samples stored at 60 °C for 50–220 days.

Sample	Day 50	Day 68	Day 78	Day 97	Day 135	Day 220
CL	0.90 ± 0.12	1.93 ± 0.07	2.04 ± 0.09	1.90 ± 0.11	2.76 ± 0.10	1.17 ± 0.13
LB	0.86 ± 0.21	1.99 ± 0.28	1.53 ± 0.20	0.85 ± 0.26	<LD	<LD
DB	5.61 ± 0.69	7.10 ± 0.35	7.12 ± 0.34	6.63 ± 0.34	5.91 ± 0.67	3.74 ± 0.34

The concentration is in μg L^{-1}. Samples were stored in clear (CL), light blue (LB) and dark blue (DB) bottles.

of trivalent antimony species were similar from day 68 to day 78 and, from day 78 to 220, it decreased slightly or even disappeared (LB samples). As an internal quality control check, a mass balance comparing the sum of species and total antimony was carried out. A dependent Student's *t*-test for paired samples (95% confidence level) proved that there was no significant difference between these values.

The disappearance of the trivalent species with time, when stored at both 40 and 60 °C, could be due to oxidation to the pentavalent form, as it has been previously discussed in Section 3.1. It has been shown that Sb (III) is easily oxidized to Sb (V) within a short time at low concentrations (Filella, Belzile, & Chen, 2002b; Miravet, Bonilla, López-Sánchez, & Rubio, 2006b; Miravet, López-Sánchez, & Rubio, 2004).

The results clearly show that at storage temperatures of 40 and 60 °C, Sb migration from PET to drinking water accelerates over time. This effect can be attributed to the degradation of PET with temperature. Degradation by thermo-oxidative and/or thermo-mechanical processes at temperatures higher than 250 °C is well known (Romao et al., 2009) but has also been described at 40 °C. According to X-ray absorption fine structure (XAFS) measurements, the leaching behaviour of Sb into drinking water after 45 days at 40 °C is mainly due to the degradation of PET (Takahashi et al., 2008).

However, additional variables can also affect the migration behaviour. Total Sb concentration in PET, bottle volume and wall thickness, activation energy and diffusion coefficients of Sb were used in a mathematical model for calculating migration with temperature and time by Welle and Franz (2011). Even if experimental values obtained in this work at room temperature and 40 °C are slightly lower than those proposed by this model, the tendency up to 220 days is comparable. No data at 60 °C are available in Welle's study (Welle & Franz, 2011).

Migration behaviour is also dependent on the type of water (Bach et al., 2013). As all samples were non-carbonated waters, this parameter was not studied in this paper and future investigations will be focused on the effect of the type of sample in migration behaviour.

4. Conclusions

Although the concentration of Sb in PET material used for water bottling was between 191 and 268 mg kg^{-1}, only small quantities of antimony were released from the plastic into the water.

Significant amounts of Sb migrated into the water when bottles were stored at 60 °C and remained in the water matrix once they had been released.

Migration of antimony from the plastic into the mineral water gives rise to concentrations under the maximum allowed by the EU at storage temperatures below 60 °C, whereas at 60 °C Sb migration is more rapid and the limit is exceeded in 15 days.

After migration experiments, Sb (V) was the predominant species in the measured extracts. Although the most toxic species, Sb (III), was also present at 40 and 60 °C from the fiftieth and the third day, respectively, its presence decreased onwards due to the oxidation of Sb (III) to Sb (V).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.06.041.

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ANNEX 4

Abstract of the articles sent to a journal or in preparation

Antimony(V) and antimony(III) determination in water by liquid chromatography hyphenated to hydride generation atomic fluorescence spectroscopy after online short-column preconcentration

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ABSTRACT

This paper reports a method developed for the simultaneous determination of antimony(V) , Sb(V), and antimony(III), Sb(III) in water by liquid chromatography coupled to hydride generation atomic fluorescence spectroscopy (LC-HG-AFS) after online short-column preconcentration. This work focused on systematic studies of several variables to establish the maximum species recoveries, preconcentration factors and good reproducibility. The optimum results obtained were the following: 0.22 µmol L^{-1} 2-mercaptoethanol as a complexing agent, precolumn conditioning with the mobile phase: 30 mmol L^{-1} etilenediaminetetraacetic acid (EDTA) at pH 4 with 0.5% of methanol (MeOH), 5 cm precolumn length and a sample pH of 5. This method was applied to three water samples with different mineralisation contents. Various tests, based on spikes, were performed on each sample. A breakthrough volume of 20 mL was found. The recovery values of 13.9 ± 0.7% and 54 ± 2% for Sb(V) and Sb(III), respectively, were obtained regardless of the matrix composition, and the PF values were 9 and 67 for Sb(V) and Sb(III), respectively. The accuracy of the preconcentration method was verified by analysing a certified reference material. The detection limits (LDs) obtained were 250 for Sb(V) and 30 for Sb(III). The established analytical online preconcentration method is suitable for the quantification of antimony species in a wide range of water samples with Sb concentrations about 1 g L^{-1}, which is a common value found in most of PET bottled waters.

Keywords: antimony speciation, waters, LC-HG-AFS, preconcentration.

Assessment of the physicochemical parameters affecting the presence and release of antimony in drinking mineral waters bottled in PET containers

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ABSTRACT

Antimony concentrations were determined in 38 PET bottled mineral water samples from eleven different countries. Total Sb was performed by HG-AFS and speciation analysis by LC-ICP-MS. Antimony concentrations in the mineral water samples were up to 1 g L^{-1}. These concentrations increased a till a hundred percent after one year of storage at room temperature, but they did not exceed. The EU guideline values for drinking water. Sb(V) was the only species present in the water samples with no significantly different values with respect to the total Sb. In addition, the concentration of the major ion chemistry variables in waters (calcium, magnesium, sodium, potassium, hydrogencarbonate, chloride, sulphate, nitrate, and fluoride) were determined together with the pH. These parameters, together with the mineralisation degree and the colour of the PET bottle, were evaluated using a correlation analysis and a principal component analysis (PCA) to establish relationships with the antimony concentration. These approaches showed a weak positive correlation between Sb concentration and the variables mineralisation degree and colour.

Keywords: antimony speciation, water, polyethylene terephthalate bottle, inductively coupled plasma-mass spectrometry.

Antimony in PET-bottled beverages: the migration puzzle

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ABSTRACT

The use of polyethylene terephthalate (PET) for food packaging is growing faster than that of any other plastic. Antimony trioxide (Sb_2O_3) is commonly used as catalyst in its manufacture, leading to the presence of significant amounts of this metalloid in the plastic. The objective of this study consists of proposing an experimental strategy to investigate the factors affecting the migration of antimony from PET containers to beverages. The kinetic factors (time and temperature) were studied by storing some units of peach and pineapple juices at 4ºC, 20ºC, 40ºC and 60ºC up to 30 days. Antimony analyses were performed by ICP-MS for total determination and LC-ICP-MS for speciation in seven occasions during the migration study period. A significant increase on Sb concentration was observed in units stored at 60ºC without exceeding the maximum limit established in drinking waters by the European Union. Sb(III) was the predominant species present in the juices.

The different Sb migration behaviour observed on juices with respect to results reported for mineral waters, pointed out the need of a second approach where each matrix (three low mineralised drinking waters and two juice samples) were kept in different PET containers in order to understand the role of the PET type and matrix. For this purpose, samples were kept at 20 and 60ºC during one month. This study demonstrated that the type of bottle also has a great effect on antimony migration: the three PET bottles used for water storage released more antimony than the two bottles used for the storage of juice. At 60 ºC, the EU limit for drinking waters was exceeded in all pineapple juice samples regardless of the container and for all water matrices stored in two of the PET-water type bottles.

Keywords: antimony migration; antimony speciation; juice; water; polyethylene terephthalate bottle; crossed-migration experiments.

Identification of a new Sb(V) complex in PET-bottled *raki* and *tsipouro*: assessment of antimony concentrations (total and species) and migration studies.

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ABSTRACT

The determination of total antimony and the Sb(V) and Sb(III) species was assessed in twelve spirit samples stored in polyethylene terephthalate bottles, comprising Greek *raki* and *tsipouro*. Reliable and reproducible results were obtained with direct analysis by ICP-MS providing results between 0.4 – 4 µg L^{-1} for total Sb contents. The speciation analysis by LC-ICP-MS was assessed and showed the presence of both inorganic species together with an unknown organic complex of Sb(V), which is the predominant in all samples. The structure of this complex was determined by LC-ES-MS, with a previous optimisation of the analysis parameters, and by high-resolution tandem mass spectrometry, resulting in a six coordinated complex with the formula: $[Sb(C_2H_4O_3S)_2(C_3H_3O_3)(OH)_3]^-$, in which the ligands are organic substances present in the *raki* matrix.

Besides, the influence of high temperature storage and time exposure up to two weeks on Sb migration from PET bottles into six *raki* samples was studied. Total and species content was determined by ICP-MS and LC-ICP-MS, respectively. The concentrations obtained were much higher than the limit established by the European Union in drinking waters after a week of storage at 60ºC and the inorganic Sb(V) and Sb(III) became the predominant species with respect to the organic Sb(V) complex.

Keywords: antimony speciation, raki, polyethylene terephthalate bottle, inductively coupled plasma-mass spectrometry, liquid chromatography-mass spectrometry.