Gonçalo Jorge Dias do Vale

FRESHWATER ARSENIC DETOXIFICATION

THROUGH SELENIUM-ENRICHED FOOD SUPPLEMENTS.

A PROTEOMIC APPROACH

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ABSTRACT

Arsenic is a metalloid that occurs naturally in soils and is toxic to living organisms at high concentrations. The arsenic poisoning can occur indirectly (food chain) or directly through drinking water. In humans the chronic exposure to arsenic is linked to cancer, vascular diseases and skin lesions. In countries like Bangladesh the problems related to arsenic poisoning are very dramatic and has become a public health problem.

Selenium is an essential micro-nutrient to humans and it is known for its anti-cancer and anti-oxidant properties. This element is present in nature at small amounts and enters the food chain through the plants that uptake it from the soils. Although there are some references to diseases related with selenium poisoning, they are rarely documented and for this reason the effects of selenium toxicity in humans remains unknown.

Throughout its evolution, many organisms have developed strategies and mechanisms to excrete heavy metals preventing their adverse effects. In the late 90's a study with small mammals showed that an enriched diet in selenium had decreased the arsenic toxic effects on mammals exposed to high concentrations of this metalloid. Afterwards the bio-formation of a metabolite that contained in its composition arsenic and selenium was identified. This metabolite was easily excreted by the organism which suggesting the presence of a biological mechanism for the detoxication of metals in mammals.

The present work studies the possibility to use selenium as an ecological solution to avoid/diminish the toxicity of arsenic in drinking waters, using food supplements as a selenium source. Techniques were developed with the goal of determining the total amount and speciation of selenium in biological samples and food supplements by HPLC and ET-AAS. For solid-liquid selenium extraction it was used an enzymatic digestion accelerated with ultrasonic energy. This methodology, that has reduced the extraction time from hours to minutes, was firstly reported in 2004 in the *Analytical Chemistry* journal and since then it has been extensively used by the scientific community. A bibliographic review has been developed in order to establish the state of the art and to enhance the divulgation of this methodology between the scientific community. To study the antagonistic effects of selenium and arsenic in biological systems, freshwater clams (*C. fluminea*) were exposed during 21 days to different concentrations of these elements. The determination of arsenic and selenium on the clam's soft tissue (digestive gland and remains body) was performed by ET-AAS. For the identification of proteins by peptide mass fingerprint, a new and fast ultrasonication assisted enzymatic digestion with immobilized trypsin (on magnetic particles and glass beads) method was developed

Chapter 1 – In this chapter a general introduction about the main subjects adressed in this thesis is made: The arsenic toxicity problem in drinking waters and their consequences to the human health; the anti-cancer and anti-oxidant properties of selenium and its benefits for the human health; the antagonistic relation between arsenic and selenium; the most common sample pre-treatment and analytical methods for the determination and speciation of selenium and arsenic in biological and environmental samples; and a brief introduction to the applications of the ultrasonic devices.

Chapter 2 – In this chapter a new, fast and reproducible analytical technique for the total determination of Se in biological samples was developed. The selenium extraction was performed using the ultrasonic probe enzymatic digestion method and the Se determination was done by ET-AAS. For the optimization of the ET-AAS conditions, two matrix modifiers (palladium and nickel nitrate) in conjunction with hydrogen peroxide were tested. The best results were obtained with palladium nitrate. The presence of hydrogen peroxide as matrix modifier increase up to 66% the lifetime of the graphite tubes by avoiding the formation of carbonaceous residues inside the tube. It were also tested several types of enzymes for the extraction of selenium from the organic tissues being the best results obtained with Protease XIV.

Chapter 3 – In this chapter a new methodology for selenium speciation in food supplements enriched with selenium is described. An ultrasonic assisted enzyme digestion was performed to extract selenium from the matrix; the different Se-species were separated by HPLC and Se was measured by ET-AAS. It was not noticed any interconvertion of selenium species after ultrasonication. In this work the most common selenium species found in food supplements (Se(IV), Se(VI), SeMet, SeMeSeCys, and $SeCys_2$) were studied. In five of the ten food supplements studied it was found Se(IV) and SeMet. On the other five supplements, the selenium species present did not match the Se standards used in the study. In one of the supplements it was found a selenium specie different from the one granted by the manufacturer. The aforementioned results should alert governments to the need of developing and implementing analytical methods to control the food supplement products. The new method developed could be easily extended to new selenium species and implemented as a routine method for selenium control in food supplements.

Chapter 4 – In this chapter a bibliographic review about the ultrasonic assisted enzymatic digestion was done. Several subjects related to this methodology are outlined and described: type of enzymes and samples used; applications with different ultrasonic devices and variables that can influence the performance of the ultrasonic method.

Chapter 5 – In this chapter is described a new and fast methodology for the identification of proteins by peptide mass fingerprint after ultrasonic assisted enzymatic digestion with immobilized trypsin in glass beads and magnetic particles. The mechanical resistance to ultrasonic energy of several particles was tested being the glass beads and the magnetic particles the ones that presented better performance. The studies have shown a decrease of 39% and 71% in the enzymatic activity of magnetic particles and glass beads, respectively, after ultrasonication with an ultrasonic probe during 2 minutes at a 50% ultrasonic amplitude. For this reason, if ultrasonication is applied, the immobilized trypsin particles could not be reused for another protein digestion. This new ultrasonic assisted method was applied to four different proteins (Bovine serum albumin, Carbonic anhydrase, Ovoalbumin e alfa-lacto albumin) being all identified by peptide mass fingerprint and/or peptide fragment fingerprint.

Chapter 6 - In this chapter freshwater clams (*C. fluminea*) are exposed to selenium and arsenic and the effects are studied. The clams were exposed to different concentrations of selenium and arsenic during 21 days. After determination (by ET-AAS) of these two elements, in the clams soft tissues (digestive gland and remain body), it was found that the *C. fluminea* bio-regulate arsenic and bio-accumulate selenium. The results have showed a higher accumulation of arsenic in the digestive gland and of selenium in the remaining body. It was also reported a detoxification mechanism of arsenic in the presence of selenium.

RESUMO

O arsénio é um metalóide que se encontra naturalmente disponível nos solos sendo, em altas concentrações, tóxico para os seres vivos. A intoxicação com arsénio pode ocorrer de forma indirecta (cadeia alimentar) ou directa através de águas de consumo. No ser humano a exposição crónica a arsénio (arsenólise) encontra-se associada ao cancro, a doenças vasculares e de pele. Em países como o Bangladesh a problemática da intoxicação por arsénio é bastante grave sendo um caso de saúde pública.

O Selénio, por seu lado, é um micro-nutriente essencial aos seres humanos, conhecido pelas suas capacidades anti-cancerígenas e anti-oxidantes. Não são muito documentadas doenças associadas ao excesso deste metal no organismo, pois trata-se de um elemento que se encontra disponível na natureza (solos) em pequenas quantidades. Os seres humanos obtêm o selénio através da alimentação, principalmente pelos vegetais que retiram este elemento dos solos.

Ao longo da sua evolução, muitos organismos desenvolveram mecanismos e estratégias para excretar metais pesados impedindo os seus efeitos nefastos a nível orgânico. No final dos anos 90 um estudo realizado em pequenos mamíferos demonstrou que a adição de suplementos de selénio à alimentação de animais expostos a altas concentrações de arsénio, originava uma diminuição dos efeitos característicos de arsenólise nos mesmos. Posteriormente foi identificada a bio-formação de um metabolito, facilmente excretado pelo organismo, que contém na sua composição arsénio e selénio. Foram assim reveladas novas pistas para uma via metabólica, até então desconhecida, de desintoxicação de metais pesados em mamíferos.

No presente trabalho estuda-se a possibilidade de utilizar selénio como uma solução ecológica para evitar/diminuir a toxicidade do Arsénio em águas de consumo, usando suplementos alimentares como uma fonte de selénio. Foram desenvolvidas técnicas analíticas para a determinação total e especiação de selénio por HPLC e ET-AAS em amostras biológicas e suplementos alimentares enriquecidos neste elemento. Para a extracção de selénio nas amostras aplicou-se um novo método de digestão enzimática acelerado com sonda de ultrasons que permitiu reduzir de horas para minutos o tempo de extracção. Este novo método foi pela primeira vez publicado na revista *Analytical Chemistry* em 2004 tendo sido desde então utilizado por diversos investigadores. Com o objectivo de efectuar uma revisão bibliográfica e aumentar a divulgação pela comunidade científica deste novo método foi efectuado um artigo de revisão sob a forma de tutorial acerca desta nova metodologia. Para o estudo dos efeitos de antagonismo entre o selénio e o arsénio em sistemas biológicos, foram utilizadas amêijoas de água doce da espécie *Corbicula fluminea*. As amêijoas foram colocadas em tanques com diversas concentrações de arsénio e selénio durante 21 dias sendo posteriormente determinado por ET-AAS, nos tecidos moles, o teor destes elementos na glândula digestiva e restantes órgãos do corpo. Por último foi igualmente desenvolvido um novo método de digestão enzimática com a utilização de tripsina imobilizada em esferas de vidro e em partículas magnéticas para identificação de proteínas por "*peptide mass fingerprint*".

Capítulo 1 – Neste capítulo são descritos, numa introdução geral, os principais assuntos abordados neste trabalho: A problemática acerca do excesso de arsénio em águas de consumo, bem como as suas consequências para a saúde pública; os benefícios do Se como agente anti-cancerígeno e anti-oxidante natural; a relação de antagonismo entre estes dois elementos; os métodos analíticos mais comuns para extracção determinação e especiação de selénio e arsénio em amostras biológicas e ambientais; e por último uma breve descrição acerca do funcionamento e aplicações de aparelhos de ultrasons.

Capítulo 2 – Neste capítulo é descrita uma nova, rápida e reprodutível técnica analítica para determinação total de selénio por ET-AAS em amostras biológicas recorrendo a digestões enzimáticas aceleradas com sonda de ultrasons. Para a optimização do método de determinação de Se por ET-AAS, foram testados dois modificadores de matriz (nitrato de níquel e nitrato de paládio) em conjunto com peróxido de hidrogénio. Registou-se como melhor modificador de matriz o conjunto nitrato de paládio e peróxido de hidrogénio. Verificou-se igualmente que a utilização de peróxido de hidrogénio evita a formação de resíduos calcinados de matéria orgânica no interior das *cuvettes*, aumentando o seu tempo de vida útil em cerca de 66%. Para as extracções de Se nas amostras biológicas foram testadas diferentes tipos enzimas tendo os melhores resultados sido obtidos com Protease XIV.

Capítulo 3 – Neste capítulo é descrita uma nova metodologia para a especiação de selénio em suplementos alimentares enriquecidos com o mesmo elemento. Utilizando como método de extracção a digestão enzimática acelerada com sonda de ultrasons, como método de separação, HPLC e como técnica de determinação e quantificação, ET-AAS. As espécies de selénio estudadas foram Se(IV), Se(VI), SeMet, SeMeSeCys, e $SeCys_2$, sendo as mais comummente publicitadas em suplementos alimentares enriquecidos com selénio. Não foi observada interconversão de espécies após utilização da sonda de ultrasons. As espécies Se(IV) e SeMet foram encontradas em cinco dos dez suplementos estudados, sendo que as espécies presentes nos restantes cinco não correspondiam a nenhum dos padrões utilizados neste trabalho. Num dos suplementos alimentares estudados foi detectado uma espécie de selénio diferente da publicitada pelo fornecedor alertando assim para a urgente necessidade de desenvolver e aplicar métodos analíticos de controlo para suplementos alimentares. O método de especiação desenvolvido poderá facilmente ser alargado a novas espécies de selénio podendo igualmente ser utilizado como análise de rotina para o controlo de Se em suplementos alimentares.

Capítulo 4 – Neste capítulo foi desenvolvida uma revisão bibliográfica acerca das aplicações do método de digestão enzimática acelerada por ultrasons, como método analítico para tratamento de amostras, onde são abordados diversos temas, como os diferentes tipos de enzimas, amostras e equipamentos de ultrasons usados bem como as principais variáveis que podem influenciar na performance deste método.

Capítulo 5 – Neste capítulo é descrito um novo método de digestão de proteínas com tripsina imobilizada em esferas de vidro e partículas magnéticas acelerado com sonda de ultrasons para identificação de proteínas por *peptide mass fingerprint*. No desenvolvimento deste trabalho foi testada a resistência mecânica aos ultrasons de diversas partículas, sendo escolhidas as partículas magnéticas e de vidro por serem as mais resistentes. Verificou-se que a actividade enzimática da tripsina imobilizada diminuía cerca de 39% nas partículas magnéticas e 71% nas partículas de vidro após a utilização de sonda de ultrasons tornado assim inviável a sua reutilização. Para verificar a funcionalidade do método foram utilizadas quatro proteínas padrão (Bovine serum albumin, Carbonic anhydrase, Ovoalbumin e alfa-lacto albumin) que após digestão foram identificadas por *peptide mass fingerprint* e *peptide fragment fingerprint*.

Capítulo 6 – Neste capítulo é descrito um estudo acerca dos efeitos de antagonismo do arsénio e selénio em sistemas biológicos. Para tal foi utilizada uma amêijoa de água doce da espécie *C. fluminea* que foi exposta durante 21 dias a diversas concentrações destes metais. Verificou-se a existência de um mecanismo de bio-regulação para o arsénio e de bio-acumulação para o selénio. Nos estudos efectuados sobre os tecidos moles da amêijoa, verificou-se que o arsénio apresenta uma tendência para acumular na glândula digestiva enquanto o selénio acumula preferencialmente nos restantes órgãos. Verificou-se igualmente a presença de um mecanismo de desintoxicação de arsénio na presença de selénio que revelou indícios de ser dependente da forma química em que o arsénio esta presente nos tecidos.

ABBREVIATIONS

AAS – Atomic absoption sepectrometry
AFS - Atomic fluorescente spectrometry
AIDS – Acquired imune deficienty syndrome
AMBIC – Ammonium bicarbonate
As – Arsenic
As(III) –Arsenite
As(V) – Arsenate
AsB – Arsenobetaine
AsC – Arsenocholine
BAEE – Nα-Benzoyl-L-arginine ethyl ester
BSA – Bovine serum albumin
CA – Carbonic anhydrase
CE – Cappilary electrophoresis
CV – Cold vapour
DG – Digestive gland
DMA – Dimethylarsinic
DNA – Deoxyribonucleic acid
ET – Electrothermal
EPS – Enzymatic probe sonication
F-AAS – Flame atomic absorption spectrometry
FI – Flow injection
GC – Gas chromatography
GPx – Glutathione peroxidises
GSH – Glutathione
HFBA – Heptafluorobutanoic acid
Hg – Mercury
HG – Hydride generation
HPLC – High performance liquid chromatography
ICP-MS – Inductively coupled plasma mass spectrometry
ICP-OES – Inductively coupled plasma optical emission spectrometry
LOD – Limit of detection

LOQ – Limit of quantification

MALDI – Matrix assited laser desorption/ionization

MMA – Monomethylarsonic acid

MW – Microwave

OA – Ovoalbumin

PMF – Peptide mass fingerprint

PFF – Peptide fragment fingerprint

PLE – Pressurized liquid extraction

RSD – Relative standard deviation

Se – Selenium

Se (IV) – Selenite

Se (V) – Selenate

SEC – Size exclusion chromatography

SeCm – Selenocystamine

SeCys – Selenocysteine

$SeCys_2$ – Selenocystine

SeEt – Selenoethionine

SeMet – Selenomethionine

SeMeSeCys – Selenomethyl-selenocysteine

SeUr – Selenourea

TCA – Trichloric acetic acid

TFA – Trifluoroacetic acid

TOF – Time of flight

UA – Ultrasonic amplitude

UE – Ultrasonic energy

UP – Ultrasonic probe

US – Ultrasonication

USAED – Ultrasonic assisted enzymatic digestion

UT – Ultrasonic time

UV – Ultraviolet

α-Lact – α-lactoalbumin

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Chapter I

General Introduction

I.1. Arsenic in Freshwater: A worldwide problem.

Arsenic (As) is a natural element which occurs in many environmental compartments, it is found in the atmosphere, soils, rocks, natural waters and organisms. It is situated in the 15th group of the periodic table and is usually described as a metalloid [1]. The metalloids are located between the metals and non-metals lines in the periodic table and exhibit characteristics of both classes, being good semiconductors.

The As mobilization into the environment is often made by two ways: (i) a combination of natural processes such as weathering reactions, biological activities and volcanic emissions; (ii) man activities such as mining, combustion of fossil fuels, arsenical pesticides, herbicides and wood preservers [2-5]. There are several environmental sources contaminated with As however, the one with the major risk to human health are the drinking waters. Arsenic is responsible for the contamination of water supplies in various parts of the world such as India, Nepal, Cambodia, Myanmar, Taiwan, Mongolia, Vietnam, China, Afghanistan, Pakistan, Argentina, Mexico, Chile and United States of America (Figure I.1). In general, the drinking water comes from two sources: surface water (rivers, lakes, water springs) and groundwater. These sources have a variable risk to human health, however is in the groundwater where the highest concentrations of As is found since this metaloid is in the bedrock and is easily dissolved into surroundings. For this reason it is not surprising that inorganic As is normally found at higher concentration in these water sources which has created a major environmental and public health problem in the affected regions. Groundwater contaminated with As, when extensively used for irrigation, can cause detrimental effects since drinking water contamination, food contamination and human health are all interlinked [2, 3, 6, 7].

Arsenic is one of the oldest poisons known to men, it has been used since ancient times (The Greeks and Persians used arsenic as a poison to eliminate political opponents or enemies). Chronic arsenic exposure can cause many skin lesions such as melanosis, leucomelanosis and keratinosis, can also cause vascular diseases, cancer of various organ or tissues (skin, liver, lung and bladder) and other manifestations including neurological effects, obstetric problems and diabetes. The skin seems to be quite susceptible to the effects of this metal. The skin lesions seem to be the most common and initial symptoms to As exposure [8]. However the As toxicity beside the physical harms effects in humans it also have a psychological and social effect. Brinkel et al. [6] in their studies done in Bangladesh have found that chronic exposures to these metalloid can lead to mental retardation, development disabilities such as physical, cognitive, psychological and speech impairments which have serious implications to its victims and families including social instability, discrimination, depression and marriage-related problems.

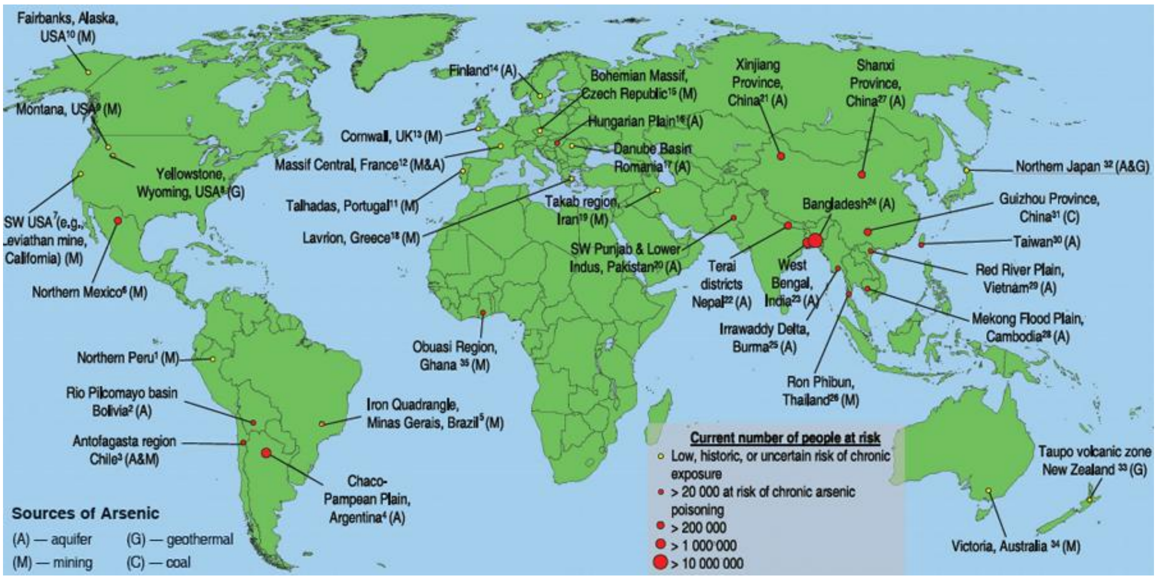


Figure I.1. Worldwide distribution of Arsenic contaminated region. Reproduced with permission [9].

One of the most known cases of arsenic exposure to humans is happening in Bangladesh which has experienced the largest arsenic poisoning known in human history. This country has an abundance of surface and underground water however these sources are highly contaminated with As. The surface water is extremely polluted due to human waste and industrial activities and for this reason the majority of people uses the underground water for consume (about 97% of rural population). Unfortunately, this water source is widely contaminated by natural occurring arsenic to levels higher than 0.05 mg L^{-1}. According to the recommendations of the World Health Organization, the maximum level allowed of arsenic in drinking waters is ≤0.01 mg L^{-1}. Based on estimation, about 35 million and 57 million inhabitants in Bangladesh are at risk of drinking arsenic contaminated water exceeding 0.05 mg L^{-1} and 0.01 mg L^{-1}, respectively (Figure I.2). The groundwater arsenic contamination in Bangladesh continues to be a widespread problem despite the best international efforts of many countries [2, 3, 6-8, 10] to overcome problems. Khan et al. [7] in their review about human arsenic exposure and risk assessment at the landscape level, worn that the As poisoning in South East Asian countries are of international importance, since exposure to As is not a localized phenomena and is not occurring in isolated areas or from point sources, instead is occurring at the landscape level.

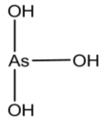
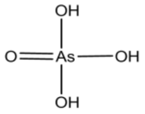
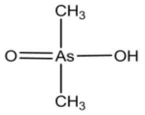
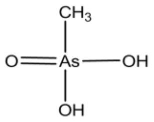
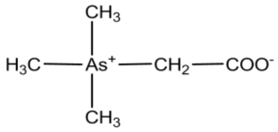
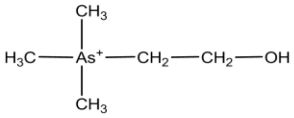
Figure I.2 - A) and B) Manual water pumps system used in Bangladesh to collect underground water. C) Bangladesh women with skin lesions due to Arsenic contamination.

I.1.2. Arsenic in the environment.

Arsenic enters the atmosphere from volcanic emissions, low- temperature volatilisation from soils, wind erosion, marine aerosols and pollution and is returned to the earth's surface by wet and dry deposition [1, 3]. There are many As compounds in the environmental with different levels of toxicity which can be present in the inorganic and organic forms. The most common arsenic species (Table I.1) present in water are arsenite (As(III)), arsenate (As(V)), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), arsenobetaine (AsB), arsenocholine (AsC). In natural waters, As is normally found in the inorganic forms, As(III) and As(V). The organic forms may be produced by biological activities, the inorganic As can be methylated by microorganisms resulting MMA and DMA. In humans the ingestion of As(III) and As(V) led to the formation of DMA and MMA. Thus the inorganic As is transformed in the organic forms both the four species are excreted in the urine. There are more As organic compounds of higher chemical complexity, where As is directly connected with carbon atoms by covalent bonds: AsB, AsC, arsenosugars and arsenic containing lipids. These compounds are very resistant to chemical degradation and have been found in the marine environment. For this reason, the main source of AsB in human diet comes via marine products like shellfish. This arsenic species are relatively non-toxic and does not accumulate in the human body, being excreted by the urine. In a recent work [11] it was found in an isolate lake in California bacteria that substitute As for phosphate to sustain its growth. It was also found that in these microorganism the As was incorporated in macromolecules that usually contain phosphate such as lipids, proteins and nucleic acids. These findings are very important since it is the first time that is reported the substitution of one of the six essential elements to life in a living organism. The incorporation of As in macromolecules, such as DNA, has opened a new chapter on the way that we understand life.

Under natural conditions, arsenic is found in underground waters at high levels. This is a result of the water-rock interactions and the greater tendency in aquifers for the physical and geochemical conditions to be favourable for arsenic mobilization and accumulation [3, 12, 13].

Table I.1. The most common As species present in natural water.

Species name	Species Structure
Arsenite (As(III))	 $\overset{OH}{\underset{OH}{As}}-OH$
Arsenate (As(V))	 $O{=}\overset{OH}{\underset{OH}{As}}-OH$
Dimethyl arsinic acid (DMA)	 $O{=}\overset{CH_3}{\underset{CH_3}{As}}-OH$
Monomethyl arsonic acid (MMA)	 $O{=}\overset{CH_3}{\underset{OH}{As}}-OH$
Arsenobetaine (AsB)	 $H_3C-\overset{CH_3}{\underset{CH_3}{As^+}}-CH_2-COO^-$
Arsenocholine (AsC)	 $H_3C-\overset{CH_3}{\underset{CH_3}{As^+}}-CH_2-CH_2-OH$

At moderate or high redox potentials, As can be present in water as As(V) and at more reducing conditions and lower redox potentials, the As(III) predominates. In lake and river waters As(V) is the dominant species however the concentration and ratio As(V) to As(III) are seasonal and can vary with redox conditions and biological activity [3]. During the summer months and with the increase of the temperature in lake and river waters, there is a boost of microbiological activities, in fact, during this months, the concentration of As(III) may increase due to biological reduction of As(V) [3, 13]. The same characteristics are found in the underground water. Kim et al. [14] in their studies on underground waters in the southeast of Michigan, have found that As(III) was the species at the highest concentration in most of the detected samples. However, the ratio As(III)/As(V) can vary with (i) the redox-active solids,(ii) the microorganisms activity and conversion and (iii) with the diffusion of oxygen from the atmosphere. In arsenic-rich undergrounds water from Bangladesh the As(III)/As(V) ratio can fluctuate between 0.1 and 0.9 [13].

I.1.3. The analytical chemistry of Arsenic.

Arsenic is available on the environment under different inorganic and organic forms. However, the toxicity of As to animal and humans depends on its chemical form and oxidation state. It is known that inorganic forms are more toxic that the organic ones, and that the toxicity of As compounds decrease from As(III) to As(V). The methylated forms of these elements (MMA and DMA) are relatively less toxic than the inorganic forms while AsB and AsC are non-toxic. It is necessary to determine As bioaccumulation, transformation and toxicity to organisms. Arsenic is a trace element in nature and due to the low levels at which many of the As species are present in water, it is necessary to develop analytical methods with the lowest detection limit possible to this element [13, 15].

I.1.3.1. Analytical methods for Arsenic determination and speciation

The term chemical speciation refers to the analytical activities of identifying and/or measuring the quantities of one ore more chemical species of an element in a sample [15]. Historically, the introduction of the chemical speciation analysis of elements has been triggered by a number of environmental accidents. For instance, on of the most well known cases of massive mercury (Hg) poisoning occurred in the village of Minamata, located in Japan. The disaster has become known as ”The Minimata bay disaster” In the 1950s an industrial company in Minimata had released mercury (Hg) wastes into the bay. Inorganic Hg was converted by microorganism into methylmercury. These Hg organic specie was accumulated in the food chain, from shellfish to fish and finally to humans. As a consequence, the local population suffered from Hg intoxication. Incidents like the Minimata Bay revealed the importance of information about the elemental specie in order to understand it biological accessibility and toxicity [16, 17].

The fundamental requirement for elemental speciation is to determine each species without the interference from the others. An ideal speciation method provides the desired information maintaining the original structure of the interest species which includes it, separation and detection. The selection of the proper separation technique depends on the species physico-chemical properties (e.g. volatility, charge, polarity). The most common analytical methodologies used to separate As species are: a) hydride generation; b) capillary electrophoresis;

and c) chromatographic methods. On the other hand, the selection of the analytical method depends on the concentration of the species and the matrix composition of the sample. The most common tecniques used for As determination are: a) inductively coupled plasma mass spectrometry (ICP-MS); b) atomic absorption spectrometery (AAS); and c) atomic fluorescence spectrometery (AFS).

Most of the speciation methods are based on the chromatography coupled to AAS or to AFS [13, 16, 18, 19]. The chromatographic techniques offer excellent possibilities for separation of different As forms, being the most common separation techniques used gas and liquid chromatography where species such as As(III), As(V), DMA, MMA AsB and AsC have been successfully separated with the aforementioned methods [20]. The gas chromatography (GC) techniques require a previous derivatization step to produce volatile As-species which is not always feasible. For this reasons the high performance liquid chromatography (HPLC) is the preferred technique in As speciation, since no previous derivatization is required. Many HPLC-based approaches have been described in the literature for As specation of which the ion-exchange chromatography has become the most popular [21-23]. In this type of chromatography, anion-exchange column is used for As(III), As(V), MMA and DMA separation whereas cation exchange column is used for AsB and AsC species. By using different types of separation columns, As species can be more effectively separated. For instance when an anion-exchange column is used, cationic compounds (AsB, AsC) are co-eluted with As(III), but they are separated when a cation column is used. Therefore some authors have used a combination of chromatographic columns to obtain better results [13, 16, 18, 24-26] in terms of As species separation.

There is a wide range of analytical techniques for As determination and speciation in different matrixes. The most powerful speciation methods derived from hyphened techniques, where previous clean up or separating steps allows a better determination or quantification of elements in the detector. This combination is proven to be a success on lowering the detection and quantification limits allowing the determination of trace elements in environmental samples. Atomic AAS is currently used for total determination and speciation of arsenic due to its high sensivity, selectivity and low detection limit, when hyphened with a separation technique such as chromatography. The AAS is also frequently combined with HG techniques [27, 28] for total As or As speciation. Correia et al. [12] developed a novel procedure for determination of total As in human urine using the flow injection hydride generation atomic absorption spectrometry (FI-HG-AAS) method. It was found that the combination between potassium permanganate ($KMnO_4$), hydrochloric acid (HCl) and high intensity focused ultrasound was a powerful and rapid procedure for degradation of organic matter in human urine. The limit of detection and quantification found was 0.1 µg L^{-1} and 0.4 µg L^{-1}, respectively. Santos et al. [29] have determined the total As concentration in water and in the soft tissues of the freshwater clam *Corbicula fluminea*. In their work this metalloid was determined by electrothermal atomic absorption spectrometry (ET-AAS) with a detection and quantification limit of 3.1 µg L^{-1} and 9.3 µg L^{-1}, respectively. To reduce the background interferences due to the organic matter present in the sample matrix, it was used palladium nitrate as a matrix modifier in conjunction with high ashing temperatures.

Another analytical technique commonly used for As determination is the AFS. This technique has a higher sensivity than AAS, allowing the achievement of lower detection limits. On the other hand, light scattering and background interferences due to sample matrix are the main problems to be overcome when working with this technique. When AFS is used hyphenated with HG the matrix problems are minimised because many interferences are avoided leading to improve detection limits. As some examples, Sarmiento et al. [30] have determined As(III) and As(V) in the surface water of two rivers heavily polluted due to mining exploitations. The As speciation studies were made with an HPLC-HG-AFS system with a detection limit of 0.17 µg L^{-1} to As(III) and 0.46 µg L^{-1} to As(V). Chen et al. [18] have recently published a critical review about HPLC coupled to hydride generation or cold vapour atomic generation atomic fluorescence spectrometry (HPLC-HG/CVAFS) for speciation of various elements, such as arsenic. They have stated that the replacement of AAS by AFS greatly enhances the sensivity of the methods.

Nowadays the ICP-MS system is the most widely used for elemental speciation, because it offers several advantages such as (i) high sensivity, (ii) multi-element capability, (iii) large dynamic range of concentrations and (iv) it can be combined (online or offline) with different separation techniques for speciation analysis, such as liquid chromatography, gas chromatography and capillary electrophoresis. One of the major reasons why ICP-MS technique is having such an acceptance is likely due to their simplicity since sample treatment is easier, no oxidation and pre-reduction steps are required [18]. However, this technique is considered expensive to purchase and maintain, for this reasons it remains hard to be acquired. The hyphened method HPLC-ICP-MS is considered one of the most effective techniques in As speciation. The use of HPLC, has given access to a vast variety of chromatographic columns offering different separation modes [31-34]. However, ion chromatography still represents the most frequent approach to HPLC-ICP-MS in the environmental field studies. As some examples, Duarte et al. [35] have identified and quantified As(III), As(V), DMA and MMA in water from industrial treatment. The As species were separated by HPLC in an anion-exchange column, where parameters like retention times, pH, flow rate and concentration of the mobile phase were optimized. The detection limits (µg L^{-1}) of these system were 0.02, 0.06, 0.04 and 0.10 for As(III), DMA, MMA and As(V), respectively.

In Table I.2 are summarized some of the most used techniques for total determination and speciation of As.

Table I.2. Summary of As determination/speciation methods.

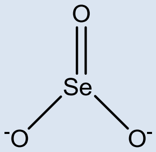
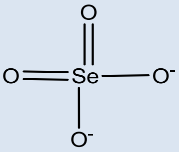
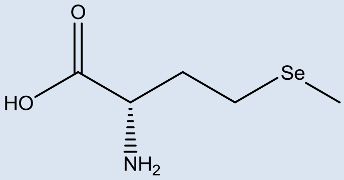
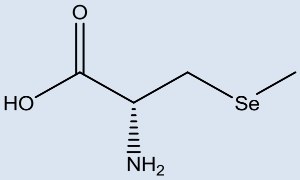
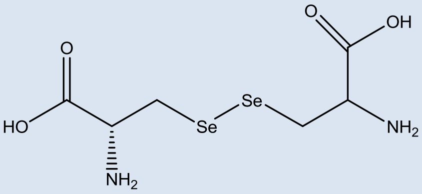
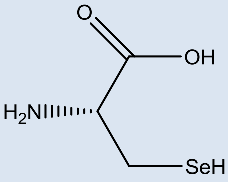
Arsenic species	Analytical Method	Limit of detection (LOD) (µg L^{-1})	Matrix	Reference
Total As	FI-HG-AAS	0.1	Human Urine	[12]
Total As	ET-AAS	3.1	Water and mussel	[29]
As(III); As(V)	HPLC-HG-AFS	0.17, 0.46	River water	[30]
As(III); As(V); DMA and MMA	HPLC-HG-AFS	1.7, 1.5, 1.1 and 1.4	Water and vegetables	[36]
As(III); As(V); DMA and MMA	HPLC-ICP-MS	0.02, 0.06, 0.04 and 0.10	Industrial treated water	[35]
As(III); As(V); DMA; MMA; AsB and AsC	HPLC-ICP-MS	0.2	Hot spring water and fish sample	[33]

I.2. The chemistry and biochemistry of Selenium

Selenium (Se) is an ultra trace element that is environmentally available in small amounts. It is situated in the 16th group of the periodic table and like arsenic it has chemical and physical properties of metals and non-metals. Some authors consider this element a metalloid and others a non-metal, in fact there is no agreement on this matter [1, 37]. The knowledge about Selenium’s importance has changed dramatically over the last century. In the 1930s, this element was found to be responsible for several illnesses and was considered to be a toxic agent in mammals. In 1957 Schwarz and Foltz reported that Se prevent liver necrosis in rats under vitamin E deficit. In the 1960s and 1970s, Se was reported (i) to have anticarcinogenic properties, (ii) to have a role in (a) preventing heart disease, (b) in immune function, (c), viral suppression, (d) in AIDS, (e) in delaying the ageing process and (f) was further confirmed to be a chemopreventive agent in cancer. The aforementioned evidences led to the suggestion that Se was an important nutrient for mammals and for certain bacteria. Nowadays, Se is believed to be an essential element for human health and it has been recognized as antioxidant, being its presence related with the reduction of certain types of cancer and other diseases [38]. This cancer chemoprevention has been associated with inorganic forms of Se, selenoaminoacids and organoselenium compounds [39]. Micke et al. [40] have published a recent review about the role of Se in oncology, where their related a clinical trial about Se supplementation on patients that were subjected to radiotherapy. On this trial, one group of patients had a diet supplemented with Se while the control group remain without this supplementation. The results showed, that after 4 weeks, the supplemented group showed a lower incidence on radiotherapy associated diarrhea. The potential benefits of Se supplementation in tumor patients is undeniable, however further research is needed. As stated before, Se has an ambivalent behaviour ranging from being essential to highly toxic. In fact the toxicity of Se depends on the specie, oxidation sate and concentration. Worldwide, the problems related with Selenosis (Se poisoning) are much lower than the episodes concerning to its deficiency, in fact selenosis are diagnosed rarely and information regarding to its long terms effect is scarce. However, some symptoms and diseases are known to be related with Selenium poisoning. A deficient Se diet in humans is generally related with two endemic diseases: The Keshan disease (endemic cardiomyopathy)[41] and Kashin-Beck disease (deforming arthritis) [38, 42]. Acute exposure to high concentrations of Se results in respiratory effects, dypsnea, bronchitis and pulmonary edema. Chronic exposure to high levels of Se in food and water results in hair loss, discoloration of skin, deformation of nails, weakness and a lack of mental alertness. Selenium exists in food and biological systems in its inorganic (Selenite (Se(IV)) and selenate(Se(VI))) and organic forms (selenoaminoacids, methylated forms and very complex selenoproteins). The most common Se-compounds are decipted in Table I.3.

Selenium enters in the food chain through plants which take it up from soil [41, 43, 44] mainly as selenate, using the same incorporation pathway of sulphate, through enzyme sulphate permease located in the roots cell membranes.

Table I.3. Some of the most relevant Selenium species. Adapted from [38].

Inorganic Selenium Species	**Chemical structure**
Selenite (Se(IV))	
Selenate (Se(VI))	
Organic Selenium Species	**Chemical structure**
Selenomethionine	
Selenomethyl-selenocysteine	
Selenocystine	
Selenocysteine	

Although selenate is accumulated without further transformations, selenite is easily transformed in selenoaminoacids. Some vegetables have the properties to transform inorganic Se into organic species. Broccoli, radish and garlic have the capability to transform the inorganic species into selenomethyl selenocysteine and γ-glutamyl-Se-methyl selenocysteine, these monometylathed forms are known for their anticarcinogenic properties [41]. It is also known that domestic food animals with a poor Se diet develop several metabolic diseases such as reproductive impairment, growth depression and white muscle diseases. These cases occurred in places where the soils have low amounts of Se like in volcanic regions. In these areas with soils poor in Se the agricultural producers have to adopt methods to ensure that their animals obtain an adequate diet intake of this element. The most common measures adopted to overcome this problem have been the introduction of selenium fertilizers in the soils and the use of selenium-enriched food to feed the domestic animals in order to satisfy their Se needs. Irrigation of soils with Se fertilizers for the crop culture occurs in several countries such as Finland, United Kingdom and USA [45]. This biofortification process of food crops is a largely used strategy to increase the amounts of this element in the human diet. In Finland, where this process was implemented in 1984 [45], the amounts of Se have increased in 125 indigenous food items such as wheat bran and fruits [46]. Therefore, associated with the increase of Se in soils, was the increase of this element in meat, fish, daily products and processed food [45]. The irrigation with Se enriched fertilizers is one of the Se introductions in arid and semi-arid regions of the western USA. These agricultural activities have caused high levels of Se released into the local aquatic systems where Se concentration rapidly increase in fish, water fowl and other aquatic organisms bioaccumulating along the food chain. In fact the excess of Se in the environment can be toxic to some species, the complete elimination of some fish communities have been attributed to the excess of Se in aquatic systems [39, 47, 48].

The molecular biology of Se have had a fast development in recent years about 35 selenoproteins have been identified up to know. However, despite of the intensive research in this area many of such proteins still have roles that remain unknown. Selenoproteins are proteins that include selenocysteine (Secyst), this molecule have a similar structure to cysteine, however an atom of Se take the place of sulphur. Most selenoproteins, prevent and repair damage to cellular components, regulate redox state of proteins or have other redox functions. The selenoproteins are divided into two groups, depending on the position of the selenocysteine in the polypeptide. In group one Secyst is located at N-terminus site and in group two is present at the C-terminal sequence. Glutathione peroxidises (GPx) and selenoprotein P and W belong to the first group, thiredoxin reductase and selenoproteins K and I belongs to the second one [42].

The GPx and thiredoxin reductase are very important for the protection of the thyroid from the peroxide hydrogen that is formed for thyroid hormones synthesis [41]. The GPx were the first selenoproteins to be described and characterized, they have an antioxidant function protecting cells against oxidation damage from free radicals and reactive oxygen species. The substitution of Secyst by a normal cysteine at their active center results in an enzymatic activity reduction [49]. In mammal’s four GPx forms were found being the forms GPx-1, 2 and 4 found in the cytosol whilst GPx-3 was found in the plasma where it acts as an extracellular antioxidant [40, 50, 51]. GPx-1 and GPx-2 have similar metabolic functions they can metabolize hydrogen peroxidise, cholesterol and long chain fatty acid peroxidases and cannot metabolize acid hydroperoxides in phospholipids [51]. The GPx are tetrameric proteins with four identical subunits except the GPx-4 form that is monomeric which makes this protein able to bind with a wider range of substrates.

The GPx-4 have an important role on the protection of cellular membranes against oxidative damage by reducing phospholipids hydroperoxides [50].

Selenoprotein P is required for the transport of Se to a number of tissues after its synthesis in the liver [52], this selenoprotein is also required by the brain to avoid neurological dysfunctions and degenerations since the genetic inactivation of Selenoprotein-P leads to a reduction of Se in animal brains which correspond to movement disorder and spontaneous seizures [41]. Though some selenoproteins functions are already known others like Selenoprotein K and I functions remain unknown [42].

Apart from selenoproteins, small-molecular-weight Se compounds like Selenomethyl selenocysteine and γ-glutamyl-Se-methyl selenocysteine are thought to be precursors of the anti-cancer agent methyl selenol [41] that cause cell-cycle arrest and inhibition of tumour cell invasion.

I.2.1. Selenium as an anti-poisoning for mercury and arsenic.

The ingestion of food and water is the major source of essential elements for all living organisms. Due to anthropogenic emissions of toxic metals and metalloid compounds through the global environment, certain populations are exposed to increasing levels of toxic metals and metalloids on their diets. The toxicity of a mixture cannot be accurately predicted since numerous antagonistic and synergetic effects are known to exist between individual compounds. Synergistic toxicity refers to a situation in which the administration of two toxic compounds results in a greater overall toxicity than the sum of the individual response. The antagonistic toxicity refers to a decreased overall toxicity of a mixture as compared to the toxicity of the individual compounds. Therefore many organisms have developed detoxification mechanisms against the effects of the ingestion of toxic metals and metalloids. Some bacteria, like the strain *P. aeruginosa* and *P. fluorescens* are able to detoxify mercury. They have developed a detoxify mechanism for methyl mercury, by the cleavage of C-Hg bond followed by the reduction of the metal to its elemental form (Hg^0) which is less toxic [53-55]. After this reduction the Hg can diffuse through the cell membranes and is excreted from the organism [54].

Different detoxification mechanisms have been studied in other animals such as small mammalians. It was found that the interaction between toxic metals, such as As(III) and Hg(II), and Se, induce to the formation of As-Se and Hg-Se bonds. It was also found that these compounds have an important role on the detoxifications mechanisms by which mammals protect themselves from toxic metals and metalloids. The simultaneous administration of two high toxic compounds in rabbits followed by the analysis of various biological fluids has revealed the structural basis of the antagonistic interactions between the inorganic pollutants As(III), Hg(II) and the essential ultra trace element Se [54]. Both interactions occur in the bloodstream and are driven by L-glutathione, a tripeptide that has several important cell functions such as biosynthesis of macromolecules, transport and drug metabolism [56].

Interactions between arsenite and selenite occur after their exposure to environmentally relevant doses of these elements. The antagonism between Se and As was first reported in the 1938 by Alvin Maxon when drinking water with arsenite have protected rats against the possible liver damage due to a Se enriched diet [57, 58]. Even though Maxon studies didn’t went far from the recommendation to use water with arsenite for Se poisoning prevention, his findings were very important since it was the first time that the mammalian metabolism of Se and As were interlinked.

Later it was found that skin lesions in humans, due to high levels of arsenic, had a significantly decreased after following a Se enriched diet. These data have supported that a Se deficient diet could increase the risk of skin lesions on populations exposed to high amounts of inorganic arsenic [59-62]. It was also found in a study done in China on a population exposed to high concentrations of As due to coal burning, that the inorganic As exposure is associated with oxidative stress, which may be prevented by high Se status via its antioxidative activity and detoxification effect through the formation of an As-Se metabolite in the bloodstream the Seleno-bis(S-glutathionyl) arsenium ion, $[(GS)_2AsSe]^-$ [61]. This metabolite (Figure I.3) is formed from arsenite, selenate and glutathione (GSH).

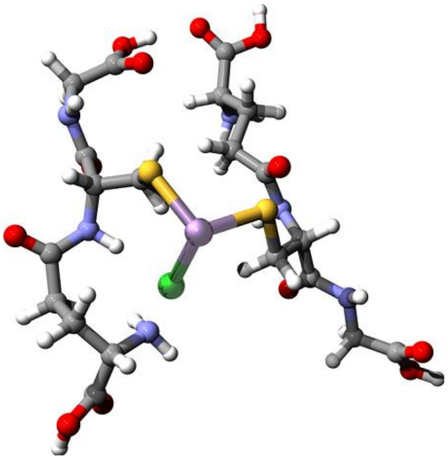


Figure I.3. Structure of $[(GS)_2AsSe]^-$ [57, 63]. Selenium is showed in green, arsenic in mauve, sulphur in yellow, oxygen in red, nitrogen in blue, carbon in grey, and hydrogen in white.

The $[(GS)_2AsSe]^-$ metabolite was firstly found by J.Gailer research team [64]. This metabolite was found on bilie samples of rabbits after intravenous injections of As(III) and Se (IV) [65]. In a subsequent work it was injected on rabbits different concentrations of As(III) and it was found the mobilization of endogenous Se to bilie which established a relationship between exposure to As and Se deficiency in rabbits [66]. The bio-formation of $[(GS)_2AsSe]^-$ in the mammalian bloodstream, followed by its subsequent excretion from the organism via bilie strongly suggests that this represent a natural detoxification metabolic pathway for As(III), though this needs to be confirmed with other animal models [64]. Therefore these studies should be applied to humans too, since these findings are of considerable value. Humans exposed to As could be supplied with Se food supplements or a Se enriched diet in order to remove As from the organism. In fact, since 2007 a human trial is being developed in Bangladesh. This trial aimed to access the oral administration of selenite in humans exposed to high concentrations of Arsenic. If the results turn out to be positive, selenite would represent an antidote for humans in the treatment of the effects of chronic arsenic exposure [64, 67].

In 1967, a $HgCl_2$ induced renal and intestinal necrosis in rats was demonstrated to be completely eliminated by the simultaneous administration of Se(IV) [68, 69]. This experience showed another antagonism between two toxic compounds. In a long-term feeding study, rats were exposed to $HgCl_2$ in drinking water and given Se(IV) on its diet. After seven weeks, it was observed a decreased of Se concentration in the liver and kidneys of the rats[64]. In other work, it was also found that was possible to inactivate mercury in fish with inorganic Se if it was supplied simultaneously with Hg [70]. These and other experiments have suggested that like As some compounds were bio-formed by the reaction of Se and Hg. Yoneda et al. [71] in their studies to clarify this metal biding mechanisms, has found that a complex containing equimolar amount of Se and Hg were formed in the bloodstream by the co-administration of selenite and $HgCl_2$, the $(Hg\text{-}Se)_n$ complex. This complex was found to bind to specific plasma proteins in order to form a highly stable complex. The formation of this stable complex was related to the detoxification mechanism of Se against Hg. In plasma proteins it was found a structurally unknown Hg-Se entity bound as Selenoprotein P. In order to elucidate the structure of these entity a investigation into the reaction of equimolar Hg^{2+} and Se(IV) with increasing amounts of L-glutathione was conducted [68]. The results suggested a core of mercuric selenide (Hg-Se) with the bond of 5 S- glutathionyl (GS) moieties on the surface. These $(Hg\text{-}Se)_{100}(GS)_5$ is formed *in vivo* and it binds to selenoproteins-P.

The As-Se interaction is based on the formation of $(GS)_2AsSe^-$ followed by its diffusion to plasma, liver and bile while the Hg-Se interaction is based on the formation of $(Hg\text{-}Se)_{100}(GS)_5$ in blood plasma which bind to selenoprotein-P. Since Se is an essential element for all higher animals including humans, the exposure to As(III) and Hg(II) will consume a great part of the ingested Se, it is known that these two elements induced Se deficiency on internal organs. Since Se is a known anti-cancer agent the reduction of this element in the organism induce bigger odds for cancer development. In this view As and Hg are not a carcinogens by their own way, instead they deflect Se deficiency on the organisms [57]. So it is easy to understand that the ingestion of As and Hg increase the risk of cancer in humans which represents a conceptually novel mechanism for the chronic toxicity of these elements.

I.2.2. Selenium determination and speciation

The predominant forms of Se found in nature are salts, such as sodium selenate (Se(VI)) and selenite (Se(IV)) and aminoacids such as selenocysteine, (SeCyst). The physiological effects of Se consumption are directly related to its chemical forms, some are preferentially incorporated into selenoprotein and others not, while others are excreted. The need to fulfil the correct Se levels in the human diet has increased the interest into development of functional food products enriched with Se. The functional food term does not have an exact definition, thus it can be defined as fresh or produced food claimed to have a health promoting and or a disease preventing properties beyond the basic nutritional function [72]. Some of these supplements content are inorganic selenium salts, mainly sodium selenate, others are prepared based on selenized enriched yeast *Saccharomyces cerevisae*. These yeasts can accumulate great amounts of inorganic Se and transform it into selenomethionine, (SeMet) [73].

The discovery of the importance of Se to human health has started a pursuit over the Se supplements products and in the response of these demands the commercial houses started to release in large number a great variety of Se enriched supplements. However, due to the lack of legislation about these products there is no monitoring analysis about their content. In 1984, several individuals have ingested a Se supplement where Se concentration value was 200 times higher that the reported on the label [38, 74]. This toxicity episode was an uncommon case but it justifies the extreme importance of the accurate Se content determination in a narrow range of concentration. Nowadays, the number of persons who complement their diets with enriched selenium food supplements continues to grow, for this reason special precautions with the unregulated use and analytical control of selenium supplements should take place to avoid toxicity episodes [38, 75].

For the correct determination and speciation of Se, the stability of Se species in standards and environmental samples is crucial. Sampling and storage affect the reliability of the results since volatilization, adsorption, inter-conversion of species, precipitation or contamination may change Se composition of the sample. When working with Se aqueous solutions is important to take into account the pH, ionic strength and the container material, to avoid Se losses [76]. In the inorganic Se species, light do not have a significant effect however warming can affect these species. Therefore, the organic forms of Se should be stored at temperatures as low as possible (-20ºC) and the inorganic ones at 4˚C. Regarding to organic species SeMet and SeCyst solutions should be dissolved in an acidic solution (e.g 3% HCl). The acid selection is a critical step since it should not interfere with the analytical method used. In general acidification with HCl is the most used and recommended [76]. To avoid the degradation of SeMet solutions, the standard concentration should be higher than 10 µg L^{-1} prepared in a high ionic strength solution and stored in glass or polyethylene containers [77-79]. Despite the considerations about Se species storages it seems to remain a lack of agreement in literature about this matter (Table I.4).

Table I.4. Standard preparation and storage of Se species. Table adapted from [76].

Selenium species	Analytical technique	Standard preparationand storage	Reference
Se(IV), Se(VI), SeMet	LC-HG-AAS	All standards were prepared in Mili-Q water. Stock solutions were stored in a freezer at -20˚C	[80]
Se(IV), Se(VI)	FI-HG-AAS	Se(IV) and Se(VI) were prepared in sulphuric acid pH=0	[81]
SeMet, SeEt, Selenoprotein P	ET-AAS	SeMet was dissolved in 0,14M HNO_3 whereas SeEt was dissolved in water. Selenoprotein P was obtained from human plasma after purification	[82]
Se(IV), Se(VI)	HG-AAS	Se(IV) and Se(VI) were prepared in HCl 10% (v/v) and in water respectively	[83]
Se(IV), Se(VI), SeMet, SeEt, TMSe$^+$	FI-HG-AAS	All standards were prepared in Mili-Q water. Stock solutions were stored in a a freezer at -20˚C	[84]

For the identification and quantification of Se species in food or biota samples it is important to use extraction and separation steps that maintain the integrity of the species involved. The selection of the sample preparation step is dependent of the sample matrix the chemical forms of Se expected and the instrumentation selected for further separation and identification of the different species. The speciation of Se implies three important steps: i) sample pre-treatment, ii) species separation and iii) identification and quantification. Each of these steps will influence the accuracy and quality of the final result and consequently the identification and quantification of the different Se species.

In biological samples Se is mainly present as selenoaminoacids being the most common methodologies used for their extractions the enzymatic and basic hydrolysis [85-88]. The main advantages of enzymatic hydrolysis method are the mild conditions used during the extractions (37˚C, pH 7.0), which prevents interconvertion and degradation of the different Se species. The traditional enzymatic digestion for Se speciation is performed in an incubation bath at 37ºC however, this traditional approach is very time-consuming, at least 24-48 h [38, 89]. Nevertheless, recent trends in sample treatments for Se extraction from biological matrices make use of an ultrasonic probe, USP, to accelerate enzyme digestions whilst preserving the Se species integrity [86, 88, 90-92]. This new method is very easy and fast to perform when comparing with the traditional enzymatic hydrolysis, the extraction of Se species takes place in a range time comprised between 30 s to 2 minutes being obtained the same results that with the traditional procedure that last for after 24/48h of sample treatment with the traditional procedure [85, 86, 90, 93]. The reduced extraction times could be related with the cell disrupting capability of the ultrasonic energy, favouring the contact between the enzymes and the cellular components. These new methodology have been named ultrasonic-assisted enzymatic digestion (USAED) [86, 92].

Total determination of Se with USAED was applied on the extraction of Se from spruce needles, oyster, plankton and mussel tissue with Protease XIV, an enzyme cocktail [85]. The extractions were made in 2 minutes at 50% ultrasonication amplitude using an ultrasonic probe tip of 1 mm. The accuracy of total Se determination was controlled by analysing reference material and comparing the results obtained from USAED with the ones obtained after acidic digestion in a microwave oven. Good Se recoveries were obtained; 111% for oyster tissue, 106% for Plankton and 93% for mussel tissue. Even though Se was found in the spruce needle plant sample after the microwave assisted acid digestion it wasn’t found after the USAED extraction method. This result was not surprising and accented the importance of the enzyme selection, since there is a direct link between the organic sample used and the enzyme chosen for a successfully enzymatic treatment. In another work USAED method was used for total Se extraction in two different Se-enriched plants, the *A. sativum* and *B. juncea* [94]. In addition to the enzymatic hydrolysis it was also tested an acid (HCl) and buffer (Ammonium acetate) extraction of Se with and without the use of an ultrasonic probe. Protease was used for the enzymatic digestion and the ultrasonic extractions were performed with an ultrasonic probe during 3 minutes, the results were compared with the ones obtained after acidic digestion on a microwave oven, in order to calculate Se extraction yield from the different extractions. In general, the results obtained have shown no statistical significant differences between the extracted Se with and without ultrasound, however when applying ultrasounds the extraction time is significantly shorten, from 20 h to 3 minutes. The total Se extraction recoveries were above 75% for acid and buffer extractions and 103% to 127% when using enzymatic hydrolysis. These results have showed that the Se-exposed plants did not accumulate Se in the form of selenoproteins since the enzymatic hydrolysis did not provide extractions significantly higher than the ones obtained with the extraction leaching procedures [94].

Another Se extraction technique used is the pressurized liquid extraction (PLE) [95-97]. The PLE is based on the use of solvents for extractions on solid matrixes at elevated temperatures and pressures. This technique was used on the extraction of selenocompounds (SeCys, SeMet, SeEt, Se(IV) and Se(VI) from spiked yeast [96], where the optimum extraction conditions found for a 11 mL PLE cell were 1600 psi of pressure, 160˚C, solvent mixture of H_2O:MeOH (1:1(v/v)), 10 minutes of static extraction time and 75% of solvent flush.

The next step after the extraction of the Se species from the matrix it is their separation for subsequent identification and quantification. The use of hyphened techniques which allows fast identification and quantification of species in real time is a powerful tool for speciation analysis. The most used hyphened techniques for Se speciation are chromatographic or electrophoretic techniques coupled on or off-line with AAS, AFS or ICP-MS analysers.

The HPLC is the regular method of choice for the separation of selenium species. This is a separation method that offers several advantages over the others such as gas chromatography and electrophoretic methods. It can be directly applied to non-volatile compounds of high and low molecular weight providing great versatility due to the different separation modes than can be used: reversed phase, size exclusion and anion exchange.

In the reversed phase chromatography the separation is made according to the compounds polarity. The reverse phase ion-pairing chromatography is a reverse phase chromatography mode that has been used for Se speciation being trifluoracetic acid (TFA) and heptafluorobutanoic acid (HFBA) the most used ion-paring agents [38]. In this mode an ion-pair is formed between the solute and the counter ion present in the polar phase and it permits a simultaneous separation of anionic, cationic ions and neutral molecules. Zheng et al. [98] have separated Se(IV), Se(VI), SeCyst, SeMet, SeEt, $TMSe^+$, selenourea (SeUr) and selenocystamine (SeCm) by using a mixed ion-pair reagents (Sodium 1-butanosulfotone and tetramethylammonium hydroxide) and a reversed phase column C18. The separation of the 8 species occurred in 18 minutes using an isocratic elution. Do et al. [99] have separated Se(IV), Se(VI), SeCyst, SeCm, SeMet and SeEt using tetrabutylammonium as an ion-pair reagent and a reversed phase C18 column. Tsopelas et al. [100] using a C18 reversed phase column and sodium salt of *n*-octanesulfonic acid as an ion-pair reagent, have separated Se(IV), Se(VI), SeMet, SeCm and dimethyldiselenide in 6 minutes.

The size exclusion chromatography (SEC) is used to separate biomolecules with different molecular weight, in general between 10 and 1000 kDa. The SEC has been applied for Se speciation as a purification step followed by a complementary liquid chromatography method. Therefore, the selenocompounds are firstly fractionated by SEC and the collected fractions are consequently analysed by ion exchange chromatography or reversed phase chromatography. The SEC-HPLC has been used to separate water-soluble Se species present in lyophilized samples of fish, tuna, shellfish, vegetables and yeast that were subsequently digested by enzymatic hydrolysis and analysed by ion exchange chromatography [101]. In another work SEC-HPLC was used for the identification of selenoproteins in *A. schoenoprasum* subsequently the Se aminoacids speciation was performed by reverse phase ion paring chromatography [102].

The ion-exchange chromatography is an efficient tool in the separation of Se species and it is based on their anionic, cationic and zwitterionic forms at the different pH solutions. For instance the selenoaminoacids SeMet and SeCys behave as anions in basic solutions and as cations in acid solutions. At an intermediate pH this two selenoaminoacids behave as zwitterions due to their ammonium and carboxylate groups [103]. Liang et al. [104] have used this chromatography method in the speciation of selenoaminoacids in Se food supplements and urine. Selenomethylselenocysteine (SeMeCys), SeCys, SeMet and Se(IV) were separated with an anionic exchange column using an NaOH solution as eluent and then the different fractions were digested by an on-line ultraviolet (UV) irradiation that destroyed the matrix organic matter. Vale et al. [92] have also used an anionic exchange column for the speciation of Se(IV), Se(VI), SeMet, Seleno-cystine ($SeCys_2$), SeMeCys in Se food supplements. On the other hand, Yathavakilla et al. [105] has used a cationic exchange column in the study of cationic Se compounds in the leaves of *B. juncea* suplementend with Se(IV) where it was found high amounts of methyl-seleno-cysteine.

To improve the separation performance between different Se species, some researchers have used more than one of the aforementioned chromatographic techniques at the same time. Gomez-Ariza et al. [96] have extracted and separate SeCys, SeEt, Se(IV) and Se(VI) from yeast material using a column-switching system that coupled reverse-phase and ion-exchange columns in an on-line system. Petropoulou et al. [106] have separated, selenourea, SeEt, SeMet, Se(VI) and Se(IV) using an anion exchange column and dimethylselenide and dimethyldiselenide with an reversed phase column. Kápolna et al. [107] have used anionic and cationic columns for Se speciation in Se enriched green onions. The cationic exchange column was used on the separation of MeSeCys, SeMet and $SeCys_2$ while the anionic exchange columns was used on the separation of Se(IV) and Se(VI).

Capillary electrophoresis (CE) is another technique generally used for Se speciation. Separations by CE are based on differences in the electrophoretic mobility of target analytes and on electroosmotic transport. It offers several features such as high resolution, low amount of sample and good detection limits. Normally this technique is used hyphened with an ICP-MS analyser [108]. The main disadvantage of this technique is the adaptation of the flow rates required for both techniques, nL min^{-1} for electrophoresis and mL min^{-1} for ICP-MS. An interface system was crucial and many approaches have been applied in the past, however nowadays a commercial interface is available that optimize the electrophoretic and nebulizer flows [109]. The CE-ICP-MS system has been reported for separation of SeMet, Se(IV), Se(VI) and SeCys in nuts, in times as short as 7 minutes [110]. Morales et al. [108] have recently published a review about the Se speciation by CE. They have proposed that the CE hyphened with those detectors could be a suitable alternative to HPLC for the separation of Se species in low concentration levels.

ICP-MS has several advantages for general speciation analysis in comparison with AAS and AFS: multi element detection, high sensivity to a wide linear dynamic range and possibility to automation. The HPLC is generally coupled to an ICP-MS. This hyphened technique has been used for the speciation of Se in different type of samples like vegetables, yeasts and food supplements [39, 87, 98, 102, 105-107, 111]. It has been used for Se speciation on the roots, steam and leafs of Se enriched *A. graveolens,* whereas enzymatic hydrolysis was used for Se extraction, ion pairing reverse phase and cationic exchange chromatography columns were used for Se species separation and ICP-MS for quantification [111]. In other work, $SeCys_2$, SeMet, methylselinic acid (MSA), Se(IV), Se(VI), Se-methyl-selenocysteine (SeMC) and γ-glutamyl-methyl-selenocysteine (γ-glutamyl-SeMC) were separated with an anionic exchange and reversed phase chromatographic column connected in tandem. This HPLC system was coupled to an ICP-MS and the limits of detection found for the different species were 81 ng Kg^{-1} for $SeCys_2$, 80 ng Kg^{-1} for SeMC, 140 ng Kg^{-1} for MSA, 120 ng Kg^{-1} for SeMet, 123 ng Kg^{-1} for Se(IV), 180 ng Kg^{-1} for γ-glutamyl-SeMC and 121 ng Kg^{-1} for Se(VI) [112]. However, as stated before, the ICP-MS apparatus is very expensive and some laboratories cannot afford such expense, the hyphenation of techniques such as chromatography and AAS or AFS is an alternative to HPLC-ICP-MS of great interest.

The direct determination by electrothermal (ET)-AAS of Se can be troublesome, especially when the sample matrix has a high amount of organic matter. For this reason an effective pre-treatment of the sample is a crucial step. In ET-AAS the direct determination of volatile analytes requires the use of efficient chemical modifiers to avoid elemental volatilization and consequently analyte losses, during the different stages of the electrothermal analysis. Different matrix modifiers have been proposed for Se determination with electrothermal atomization such as Pd [82, 85, 92, 113], Ir+Rh, Pd+Mg or Ir+Zr [76, 85, 113]. The ET-AAS has been applied to determine Se in different samples such as selenoproteins in plastic membranes (after protein separation by gel electrophoresis) [82], total Se in seafood and biological samples (after Se extraction with an ultrasonic probe) [77, 85], in vegetables after acidic digestion [114, 115] and Se speciation in food supplements (after Se species separation by HPLC) [92].

The Se determination can also be achieved with HG coupled to AAS or AFS[116]. From all Se species, only Se(IV) forms volatile Se hydride, the H_2Se. For this reason transformation of the different Se compounds into Se(IV) are crucial for a correct quantification. However, Chatterjee et al. [80] have found that not only Se(IV) was HG-active being SeMet HG-active, too. This fact has excluded the previous pre-treatment steps (UV/Microwave/acids) that were necessary to transform SeMet into Se(IV) before the HG. In this work it was developed an HPLC-HG-AAS hyphened technique for the determination of SeMet in human urine with a limit of detection (LOD) of 1.08 ng g^{-1}. The Se compounds were previously separated in an anionic exchange chromatography column and sodium tetrahydroborate ($NaBH_4$) and HCl was used for the generation of the hydride. As aforementioned the HG can be coupled with AFS for Se determination and it has been applied for the Se determination in human urine (LOD and LOQ of 1.4 and 4.7 ng g^{-1})[116] and in plant and peat samples with a LOD of 2.8 ng g^{-1}[117].

I.3. Ultrasonic energy as a tool for the enhancement of chemical reactions.

Ultrasonic energy can greatly enhance chemical reactivity, the very high temperatures and very short times of cavitational collapse make sonochemistry a unique interaction of energy and matter [118-120].

I.3.1. The effects of ultrasonic energy

Ultrasound spans the frequencies of roughly 15 kHz to 1 GHz with typical sound velocities in liquids of 1500 m s^{-1} [121]. The chemical effects of ultrasound do not arise from a direct interaction with molecular species - acoustic wavelengths are much larger (10 to 0.01 cm^{-1}) than molecular dimensions. So no direct coupling of the acoustic field on a molecular level is responsible for sonochemistry. Instead, sonochemistry derives principally from acoustic cavitation. When ultrasonic waves across through a liquid media, the alternating expansive and compressive acoustic waves creates bubbles, these effect is known as cavitation - formation, growth and implosive collapse of bubbles in a liquid. The oscillating bubbles can accumulate ultrasonic energy effectively while growing. Under the right conditions, a bubble can overgrow and implode releasing the concentrated energy stored in a very short time. The compression of the bubbles during cavitation is very quick generating a short-lived localized hot-spot in which temperatures near to 5000ºC and pressures of 1000 atm are reached (Figure I.4). This cavitational implosion is localized and can be considered as micro-reactors.

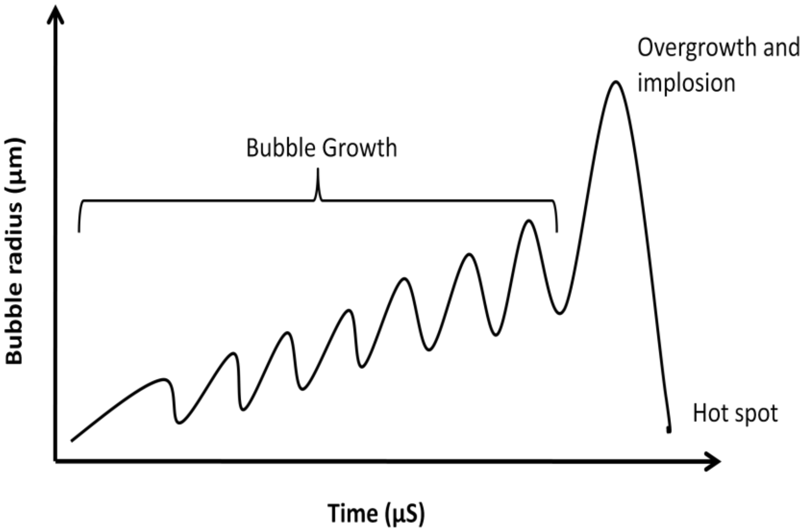


Figure I.4 - The scheme shows the bubble cycle during cavitation phenomena adapted from [122].

Cavity collapse in a homogeneous liquid is very different from cavitation near a liquid-solid interface. In these systems cavitation causes physical phenomena, such as pitting and mechanical erosion, including particles rupture, leading to smaller particles size. There are two mechanisms for the effects of cavitation near surfaces: microjet impact and shockwave damage (Figure I.5). Once cavitation occurs near an extended solid surface, cavity collapse and drives fast-moving stream of liquid with velocities greater than 100 m s^{-1} [123]. These microjets impacts leave behind characteristic microscopic pitting in the solids surface. In the second mechanism the shockwaves created by cavity collapse can create high velocity particle collisions. The impingement of microjets and shockwaves against the solid surface change its morphology, composition and reactivity [120, 124, 125].

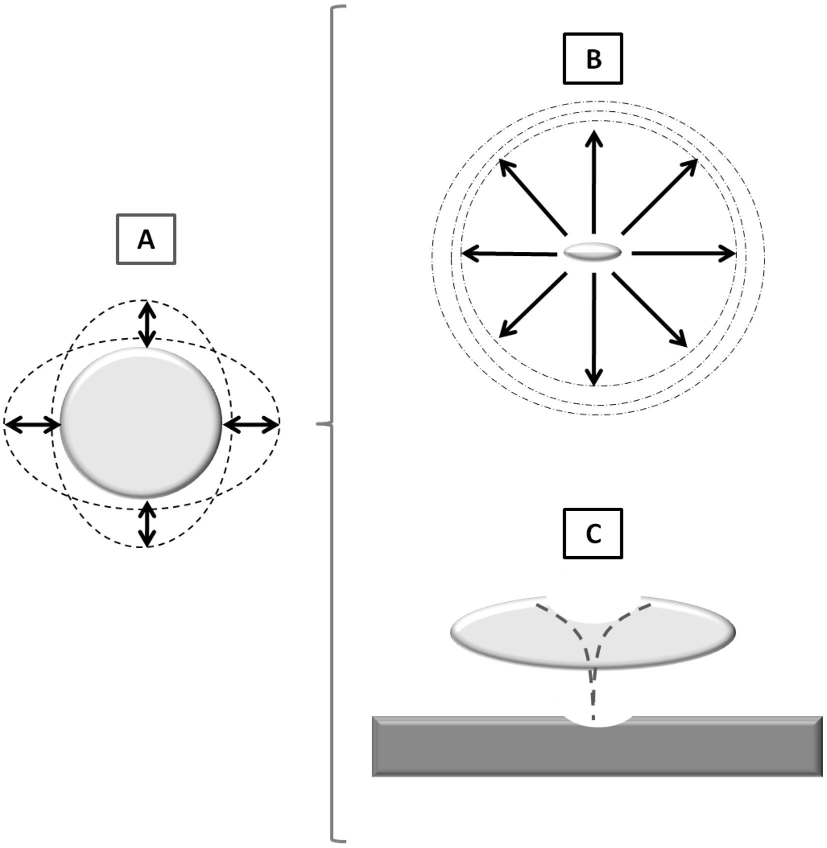


Figure I.5 – Figure adapted from [126]. A) The cavitation bubbles oscillations forms a hot spot with temperature of 5000˚C and pressure of 1000 atm. The bubbles keep oscillating until it overgrow, implode and release the accumulated energy. B) The collapsing of the cavitation bubbles release the energy under the form of shockwaves. C) The collapsing of the cavitation bubbles near a solid surface experience non-uniformities in their surroundings which lead to the formation of microjets that can penetrate into the solids surfaces.

The ultrasonic irradiation of aqueous liquids generates free radicals which can combine to return to their original form. As an example, the primary sonolysis products in water are hydrogen and hydroxyl radicals. These compounds can recombine to produce hydrogen and hydrogen peroxide, but they can also produce water by the combination with oxygen. These strong oxidants and reductants are capable of causing secondary oxidation and reduction reactions which are used for several sonochemical reactions in aqueous solutions [120, 124, 125, 127, 128].

I.3.2. The most common ultrasonic devices for sonochemistry

A variety of ultrasonic devices are commercially available such as: ultrasonic baths, ultrasonic probes and cup horns/sonoreactors (Figure I.6). The ultrasonic baths are the most common sonochemical devices. The normal method for submitting a chemical reaction to ultrasound using a bath is simply to dipping in it the reaction vessel. The sound energy should be intense enough to penetrate the walls of the vessel and cause cavitations in the reaction. The power given by this device is comprised between 1 and 5 W cm^{-2}, very low for the majority of the US applications required in chemical analysis. These devices are useful for liquid-solid reactions and for degassing of solutions like mobile phases used on HPLC. When used for analytical tasks the ultrasonic bath lacks in reproducibility [124, 125, 129]. Despite the limitations of these equipments, the advances on ultrasonic bath technology have brought new remarkable features. The new devices are now equipped with an ultrasonic power controller, different ultrasonication frequencies, different operating modes (which allow a different distribution of ultrasonic waves across the water), a thermostat and a timer [124].

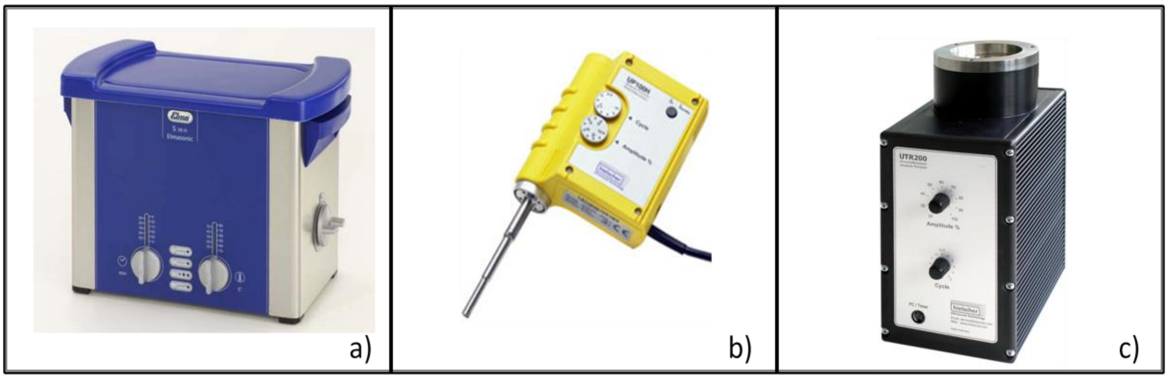


Figure I.6. The most common ultrasonic devices for sonochemistry: a) Ultrasonic bath; b) Ultrasonic Probe/Horn; c) Sonoreactor.

The ultrasonic probes/horns deliver high intensities (50 to 500 W cm^{-2}) of US and are the most efficient method of transmitting ultrasonic energy into a laboratory reaction. The ultrasonic intensity provided by the probe is at least 100 times greater than the one supplied by the bath. The use of this device involves direct immersion of a metal probe (generally a titanium probe) into the reaction mixture. This fact has the advantage that there is no power loss due to transfer through the vessel walls, however, tip erosion may occur leading to contamination by metallic particles. Therefore, new glass probes are being developed sonochemistry applications. These probes will help to avoid the introduction into sample of non-desired elements from the titanium probes [130]. The ultrasonic probe is a good system for the solid-liquid extraction of analytes that can be extracted but can, however, be also degraded. The material used for the probe fabrication should be resistant, have low acoustic loss, resistance to cavitation erosion and be chemical inert. Titanium is the metal that best fulfil all of these needs and generally all the ultrasonic probes are made of this material. The ultrasonic horn is driven by a transducer and the intensity of ultrasonication, (the vibrational amplitude of the tip), can be controlled by changing the power input to the transducer. The amplitude control of the probes allows the ultrasonic vibrations at the probe tip to be set in the desired level, the more higher the amplitude the more intense is the sonication. The sample volume to be treated along with the sample type is crucial to determine the selection of unit and the type of probe (Table I.5). Temperature is a factor that must be controlled because when ultrasonication is applied to a liquid medium a slow but constant increase in the bulk temperature is achieved. However, this problem is easily overcome if the ultrasonication of the sample is done in an ice bath. If longer ultrasonication times are needed the ultrasonic probe can be used in the pulse mode where the power of the probe is switched on and off repeatedly. This procedure avoids the increase of the temperature in the reaction media [124, 129].

Table I.5. Ultrasonic probe diameters and recommended related sample volume.

Probe diameter	**1 mm**	**2 mm**	**3 mm**	**7 mm**	**10 mm**
Sample Volume (mL)	0.1up to 5	2 up to 50	5 up to 100	10 up to 250	20 up to 500

Sonoreactor or cup horns devices provide indirect ultrasonication like the ultrasonic bath, even though they provide 50 times more intense ultrasonic energy. In the cup horn, the titanium probe is held within acrylic cup, filled with water. The samples are placed in sealed tubes into the water and the ultrasonic waves cross the walls of the sample containers. The cavitation produced in the sonoreactor or the cup horn is higher than the one given by the ultrasonic bath but lower than the one provided with the ultrasonic probe.

I.3.3. The enhancement of useful analytical processes through ultrasonication

Nowadays with more legal restrictions about the presence of contaminants in drinking water and food known to be dangerous for public health and environment, many laboratories deals with the need to develop rapid and efficient sample treatment methods that could be used in a different number of matrixes such as biological fluids, food, industrial residues, etc. Most modern analytical devices require homogeneous samples and solutions free or almost free from all the organic matter that could interfere with the analysis. For example, organic matter can interact with both metals, ions and chemical reagents used in analytical procedures for metals determination [89].

I.3.3.1. Solid-liquid extraction of elements and organic compounds

As stated before the ultrasonic probe is very efficient in the solid-liquid extraction of elements also called ultrasonic-assisted acid leaching which has been largely used for this purpose [130-133]. However, reported data about this methodology in the literature, shows contradictory information. The reasons for these facts may be explained on the basis of critical influencing factors such as the ultrasonication devices used and the particle size of the solids. Many papers lack on these experimental details and for this reason sometimes is difficult to find an explanation for controversial data [124, 134].

The particle size of the solids should be as small as possible since there is a direct relation between the area of the solid and the analyte extraction [124, 135]. Sonication time, sonication amplitude, temperature and leaching reagents are important variables for a successful extraction procedure. The time necessary to achieve the total solid-liquid extraction of metals depends on the analyte, in the liquid media and in the ultrasonic device. When using an ultrasonic probe, times shorter than 5 minutes are usually reported [130, 132, 135]. The ultrasonic bath is more matrix dependent, the more inorganic matter is in the matrix, the greater is the time needed to get successful metal extraction with this device. Extraction times spanning from 5 to 180 minutes for the ultrasonic bath are reported in literature [131, 133, 135].

The direct analysis of solids as slurries homogenised with the aid of ultrasonic energy is regularly used for elemental determination in ET-AAS. Accurate results can be obtained using small amounts of solids (5-10 mg) suspended in diluted acid. When the total analyte is extracted from the solid phase, only the supernatant is needed to be analysed. This procedure brings some advantages: 1) the use of the supernatant prevents the build-up of carbonaceous residues in the furnace; 2) sample dilution is simpler, 3) matrix effect are eliminated or diminished if an effective separation from the solids occurs leading to an easier calibration procedure; 4) There is a substantial decrease on the background signal which led to a diminishing on the amounts of matrix modifier used [124, 135].

I.3.3.2. Elemental speciation

The physico-chemical forms in which the elements occur in the environment, may strongly condition their bioavailability and toxicity. Speciation of metals and metalloids in foods and biota is important because of the differences in toxicity and mobility of elemental compounds. For example the As inorganic forms, As(III) and As(V) are highly toxic, while the organic forms (e.g Arsenobetaine , AsB) have varying degrees of toxicity. As a result, total As determination is not an adequate measure for estimating the risk of arsenic toxicity in food [89]. As stated before the analytical techniques for elemental speciation must include an efficiently methodology that permits to liberate the species from the matrix while maintaining their chemical integrity. Extraction must be efficient but it should be made in chemically mild conditions, in order to liberate the analytes of interest from the sample matrix preserving their integrity. Ultrasonic devices have been used for sample preparation in the chemical speciation of elements. Huerga et al. [136] developed a fast method for the speciation of As(III), As(V) , monomethylarsonic (MMA) and dimethylarsinic (DMA) in river sediments using an ultrasonic probe to enhance the extraction. In their work they showed that ultrasonic probe provided the same extractable content as the conventional extraction method without ultrasonic energy. Neither As nor As species interconvertion were observed in the samples subjected to ultrasonic extraction. In other work Sanz et al. [137] have developed an analytical method based on the ultrasonicatic assisted extraction of As in samples of chicken, fish, soil and rice with an ultrasonic probe. An HPLC-ICP-MS hyphened method was used for the speciation and quantification of the As species. The application of the ultrasonic probe diminished the extraction times of As from hours to minutes. No interconvertion of As species were also observed on the samples subjected to ultrasonic energy.

I.3.3.3. Ultrasonication and enzymatic digestion.

The combination of enzymatic digestions and ultrasonication has emerged as a promising tool for total metal/metalloid determination and speciation with environmentally accepted performance [90]. Ultrasonication devices have been used to accelerate the enzymatic kinetics with the purpose to degrade organic matter, in such a way, that elemental organic species are extracted into solution, maintaining their chemical integrity. The enzymes used in analytical chemistry are hydrolytic enzymes. Their catalytic process is based on the introduction of a water molecule at a specific bond of the substrate. The enzymatic hydrolysis presents some advantages over conventional sample treatments methods. One of the most important are the mild conditions of temperature and pH where the hydrolysis occurs and the selectivity of the enzymatic hydrolysis. The mild conditions prevent elemental losses by volatilization and reduce the risk of contamination when using reagents to neutralize excess of acid or alkali while with the enzyme selectivity is possible to distinguish between fractions of elements associated with different components of the matrix. The classical enzymatic hydrolysis is known to have long sample treatment time however, when this process is accelerated with ultrasonic devices the hydrolysis times drops from hours to minutes.

Ultrasonication has been used to improve the digestion performance of enzymes. The parameters affecting the process are cavitation frequency, ultrasound intensity, type of solvent and external temperatures. In the literature has been cited the use of ultrasonic bath, probe and sonoreactor to accelerate enzymatic digestions. Ultrasonic baths have been used to speed up enzymatic hydrolysis of mussel tissue to determine trace elements [138]. The results have showed that with an ultrasonic bath and proteases enzymes the hydrolysis process was finished in 30 minutes, against the conventional hydrolysis in thermostatic devices that took between 12-24 hours. It was also reported that variables such as temperature, pH and ionic strength affect the enzymatic activity and that high ultrasound frequencies decreases it ultrasonic probes have been used to speed up enzymatic hydrolysis. An enzymatic probe sonication method has been developed for total selenium determination and speciation in biological samples [90]. Total Se was extracted from yeast using protease enzyme in 5 seconds while SeMet was extracted in 30 seconds. The extraction was made on water media without pH adjustments, which minimizes the contamination risk of the samples and no interconvertion of Se species was detected. This new sample treatment was successfully applied to Se speciation in yeast using chromatographic separation coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS).

The use of these hyphenated technique have been adopted in the Proteomics field, for protein digestion and subsequent identification by peptide mass fingerprint (PMF). The performance of ultrasonic devices, such as the sonoreactor and ultrasonic probe, has been compared for the acceleration of protein digestions through enzymatic cleavage with trypsin [139]. The results were analysed in a MALDI-TOF- MS and identified by PMF. It was shown that the time needed to digest proteins was shorter with the ultrasonic probe (60 s) than with the sonoreactor (120 s) for the same ultrasonic amplitude. Therefore, cleaner MALDI-TOF spectra were obtained when using the sonoreactor that in addition provides higher sample throughput, since it can ultrasonicate 6 samples at the same time, while the ultrasonic probe can only handle one sample a time. Even though similar results were obtained with the two devices (same number of peptide match and protein sequence coverage), sonoreactor is easier to use, and the sample handling is less time consuming when compared to the ultrasonic probe procedure.

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Chapter II

Enzymatic Probe Sonication as a tool for Solid-Liquid Extraction for Total Selenium Determination by Electrothermal-Atomic Absorption Spectrometry

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II.1. Abstract

A new fast and reproducible approach is described for the application of the enzymatic probe sonication (EPS) methodology [J. L. Capelo, P. Ximénez-Embún, Y. Madrid-Albarrán, C. Cámara, Anal. Chem. 2004, 76, 233-237] for total Selenium determination by electhothermal atomic absorption spectrometry, ET-AAS. $Ni(NO_3)_2$ and $Pd(NO_3)_2$ were studied as matrix modifiers in conjunction with H_2O_2, being the best results obtained with $Pd(NO_3)_2$ plus H_2O_2. The presence of H_2O_2 as matrix modifier increases up to 66% the lifetime of the graphite tubes by avoiding the building-up of carbonaceous residues. BCR-414 plankton; and ERM–CE 278 mussel tissue reference materials were used for proof-of-the-methodology. Different enzymes, protease XIV, substilisin A and trypsin were studied. The use of fresh enzyme was found critical. Good Se recoveries were obtained for oyster tissue, 111%; BCR-414 plankton, 106%; and ERM-CE 278 mussel tissues, 93%, when protease XIV was used. Data regarding microwave digestion *versus* EPS methodology was also presented and discussed.

Keywords: Focused ultrasound, Selenium, Solid-liquid extraction, ET-AAS, Enzymes, EPS

My contribution for this work was the optimization and validation of the EPS and ET-AAS methods and the extraction and quantification of Se in the different biological samples used.

II.2. Introduction

Selenium is known by its antioxidant properties in living organisms throughout enzymes such as glutathione peroxidase and thioredoxin reductase, playing an important role in vital functions, such as thyroid metabolism or selenoprotein synthesis [1]. Moreover, findings reported in literature about Se vital activity links this metal to the following features [1]: (i) immune system, (ii) reproductive function, (iii) protection from heavy metal toxicity and (iv) risk and/or prognosis of certain diseases. The Se functions are directly related to the chemical form in which the metal is present in the organism, i.e., inorganic [*e.g.* Se (VI), Se (IV)] or organic selenium (*e.g.*, seleno-methionine). Therefore, when chemical information is going to be reported for selenium in biological samples it must be included, if possible, not only information about total Se content but also about Se chemical species.

Total selenium has been determined in biological samples after wet acid digestion or acid leaching [2], whereas Se speciation from biological samples has been traditionally done using mass spectrometry and methodologies entailing extraction conditions chemically mild but sufficiently efficient to liberate the metal species from the matrix, preserving chemical forms [2], such as enzymatic or basic hydrolysis, the former being the most widely used [2-4].

The traditional enzymatic hydrolysis treatments for Se speciation used to present the following drawbacks: (i) time-consuming, since the enzymatic reaction requires an overnight incubation at 37 ºC, (ii) the extraction is far from quantitative, highly matrix dependent and is not applicable for total Se determination, and (iii) there is a risk of selenium chemical species inter-conversion [2,4]. Capelo *et al.* [3,4] have recently overcome the former problems by enhancing the enzymatic activity with the aid of an ultrasonic field provided by high intensity focused ultrasound. The methodology has been named Enzymatic Probe Sonication, EPS, and allows total Se extraction from oyster, mussel and selenized yeast, in times as short as 15 s whereas total Se species extraction is done in 30 s [3]. This procedure has been validated by other research groups [5-7]. In addition to speciation, total Se extraction using the EPS methodology presents some advantages over conventional sample pre-treatment procedures based on acid or alkaline digestions, such as (i) mild conditions of temperature and pH, which prevent elemental losses by volatilization and (ii) risk reduction of contamination, since the use of additional reagents to neutralize the excess of acid or alkali is avoided. Furthermore, the selectivity of enzymatic hydrolysis is a powerful tool for distinguishing between fractions of elements associated with different components of the matrix, since enzymes act on certain chemical bonds only. Therefore, EPS can be used within the same sample treatment to measure the total Se content and for Se chemical speciation purposes, thus simplifying sample handling. A detailed description of the EPS procedure is given in reference [4].

To the best of our knowledge the EPS methodology has been applied for total Se determination, since its introduction in 2004 to date, only with ICP-MS [5-7]. Although modern techniques using mass detection can help to a better understanding of the experimental data and to almost confident Se species identification, many analytical laboratories can not support such equipments because of their high price and expensive maintenance. Alternative methods such as electrothermal atomic absorption spectrometry, ET-AAS, can be used for total Se determination at levels of low ng g^{-1}, enough for most of the modern requirements in terms of Se quantification in biological samples, specially enriched Se alimentary supplements, such as yeast or algae [8]. Furthermore, the hyphenation of chromatography with ET-AAS is also a reliable procedure for off-line Se speciation, not extensively used by the analytical community yet [9].

The EPS procedure for total Se determination by ET-AAS presents several drawbacks. Firstly, when EPS is applied in biological samples for Se extraction, a high amount of organic matter is also co-extracted into the liquid media. It is well known that samples with high organic matter content are troublesome in ET-AAS [2,10]. Secondly, different sensitivities were obtained for human blood plasma spiked with different Se-species as follows, Se(IV) = Se(VI) > SeMet > $TMSe^+$ when the Se content was quantified by ET–AAS [11], and finally, Sabe *et al* [12] found that accurate Se determination in 1/5 diluted serum was only possible with the standard addition method, being necessary an analysis time of 45 min per sample. Hence, the application of the EPS methodology in conjunction with ET-AAS, either for total Se determination or Se speciation, deserves dedicated research in order to find conditions that can overcome the aforementioned drawbacks.

The main purpose of the present work is to show the applicability of the EPS methodology for total Se determination using ET-AAS by matching the following items:

(i) Se quantification by calibration with aqueous standards.

(ii) Applicability of the EPS procedure for total Se extraction by applying it to different matrixes, oyster tissue and 3 reference materials: BCR-101 spruce needles; BCR-414 plankton; and ERM-CE-278 mussel tissue.

II.3. Experimental section

II.3.1. Apparatus

A Branson Sonifier 150 ultrasonic cell disruptor-homogeniser (100 W, 22.5 kHz, Branson Ultrasonics Corporation, USA) equipped with a 1 mm titanium microtip was used. Ultrasonic energy irradiation was fixed at any desired level using a power setting at 50%. The Sonifier 150 has a digital LCD display which provides a continuous reading of the watts delivered to the end of the probe (range 5-8 W in this work). Selenium absorbance was measured with a Unicam (Cambridge, UK) atomic absorption spectrometer model SpectrAA-300 *plus* equipped with a graphite furnace, an autosampler and Zeeman background correction. A selenium hollow-cathode lamp from Thermo Electron Corporation (Massachusetts, USA) operated at 15 mA was used as a radiation source. The Se analytical line at 196.0 nm and a slit width of 0.5 nm were used for measurements. Pyrolytic graphite-coated graphite tubes with L´vov platform (Omega Plantaform Extended Lifetime type, Unicam) were used. The electrothermal program is presented in Table II.1. For Microwave Acid Digestions it was used a Samsung MW81W (850W, 2450MHz) microwave apparatus from Samsung Electronics (South Corea) and Parr® (Illinois, USA) microwave pressure digestion bombs. Quantification of the enzyme activity was done using an UV/VIS Spectrophotometer V-530 (JASCO, Japan) with a cell size of 1 cm, and a centrifuge HERMLE Z160 M (Wehingen, Germany).

A vibrational agate ball mill, Restch (Germany) model MM 2000, was used for grinding oyster tissue samples. Sieves made of nylon with mesh-sizes of <150 µm and <200 µm were used.

Tabela II.1. Electrothermal Program.

Program Step	Temperature (ºC)	Time (s)	Ramp (ºC/s)	Gas Flow (L/min)
1	95	40	10	0.2
2	130	10	5	0.2
3	1100	30	90	0.2
4	2300	4	0	Off
5	2600	3	0	0.2

II.3.2 Reagents and standards

Milli-Q ultrapure water was used throughout. All chemicals were of analytical-reagent grade, unless otherwise specified. $Ni(NO_3)_2$ was purchased from BDH; HNO_3 65%, Palladium nitrate matrix modifier and Tris-HCl buffer (pH 7.5) were purchased from Fluka; hydrogen peroxide 30% was purchased from Riedel-de-Haën.

An inorganic Selenium atomic absorption standard solution (Aldrich, 976 mg/ml) was used. All stock standard solutions were stored in a refrigerator at 4ºC and protected from light. Working standard solutions were prepared just before use by appropriate dilution of the stock standard solution.

For enzyme activity determination, casein, trichloroacetic acid 99,5 %, potassium phosphate dibasic trihydrate and sodium acetate were purchased from Merck. L-Tyrosine (N 3754), Folin-Ciocalteu's phenol reagent and calcium acetate were purchased from Sigma. Carbonate sodium was purchased for Riedel-de-Haën.

II.3.3 Sample pre-treatment

Oyster tissue was prepared from oysters purchased in local markets as follows: the samples, once in the laboratory, were homogenized using a mixer and dried for 48 h in an oven at 45°C to constant weight. Then, they were ground in the vibrational agate ball mill for 5 min using a power of 60%. The powdered samples were sieved in order to separate the material into different fractions, the fraction with a size lower than 150 µm was selected for optimization of the process. Powdered samples were stored in capped polypropylene flasks at 4°C.

II.3.4 Certified reference materials

The certified reference materials used in this work, BCR-101 spruce needles, BCR-414 plankton, ERM-CE 278 mussel tissue, were purchased from the Insitute for Reference Materials and Measurements, former BCR, and nowadays ERM, from Europe.

II.3.5. Enzymes

The following enzymes were purchased from Sigma: Protease, Type XIV from Streptomyces griseus (P 5147) (5.8 U/mg solid); Trypsin acetylated, Type V from Bovine Pancreas (T6763) (9300 U/mg solid); and Subtilisin A, type VIII from Bacillus Licheniformis (P5380) (10 U/mg solid).

II.3.6. Sample treatment

Each sample was prepared mixing 10 mg of the biological material with 10 mg of enzyme. One millilitre of ultrapure water or buffered solution (Tris-HCl buffer, pH 7.5) was added and then the mixture was ultrasonicated for 120 seconds. Then the sample was allowed to stand for 10 min. The amount of sample and enzyme as well as the sonication parameters were as follows [3]: 10 mg of sample, 10 mg of enzyme, 120 seconds sonication time, 50% sonication amplitude, 1 mm probe diameter.

II.3.7. Microwave digestion procedure

In order to validate the EPS treatment, a different but common sample treatment procedure, an acid microwave digestion, was done using a domestic microwave oven and Parr® pressurized digestion bombs. Different digestion programs were used and all were variations of a digestion program previously reported by the author [13].

Mussel and oyster were easily digested but plankton and spruce needle samples needed a more elaborated program to be dissolved. Microwave digestion programs were as follows:

(i) Mussel, oyster

70 mg of sample were mixed with 2 mL of HNO_3 conc. plus 25 µL of H_2O_2 conc. The microwave oven was set at 350 W during 2 min.

(ii) Plankton

70 mg of sample were mixed with 2 mL of HNO_3 conc. plus 500 µL of H_2O_2 conc. The microwave program was as follows: (i) 250 W, 5 min; (ii) 250 W, 5 min; (iii) 500 W, 2 min; (iv) 500 W, 2 min. Between each two steps a rest period of 5 minutes was introduced.

(iii) Spruce needles

70 mg of sample were mixed with 2 mL of HNO_3 conc. plus 500 µL of H_2O_2 conc. The microwave program was as follows: (i) 300 W, 3 min; (ii) 300 W, 3 min; (iii) 450 W, 2 min; (iv) 450 W, 2 min. Between each two steps a rest period of 10 minutes was introduced.

After the microwave digestion, a period of at least 3 hours was necessary for the cooling of the samples. Each sample was then diluted up to 5 mL with Milli-Q water.

II.3.8 Enzymatic activity assay

20±2 mg of enzyme was diluted in 100 mL of 10 mM sodium acetate buffer (pH 7.5) with 5 mM calcium acetate (freshly prepared). The solution was diluted in order to obtain a 0.2 U/mL for protease XIV and subtilisin, and 370 U/mL for trypsin.

The estimation of the enzymatic activity was performed according to Sigma protocol (http://www.sigmaaldrich.com/sigma/enzyme%20assay/p5147enz.pdf, last accessed February 2007) as follows: 0.5 mL of 0.65% (w/v) casein and 0.1 mL of previous diluted enzyme solution was incubated at 37 C for 10 min. The reaction was stopped by adding 0.5 mL of 110 mM trichloroacetic acid (TCA). The mixture was then kept at 37 C for 30 min and finally centrifuged at 10 000 rpm for 10 min. 0.4 mL of the supernatant fluid was collected, the volumes of 1 mL of Na_2CO_3 solution plus 0.2 mL of Folin-Ciocalteu reagent added to that fraction and the solution mixed by manual shaking. After 30 min of incubation at 37°C, the solution was centrifuged at 10 000 rpm for 5 min. The colour developed by the solution was then measured at 660 nm. A standard graph was generated using standard tyrosine solutions of 0.022–0.088 µmol. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmol of amino acid equivalent to tyrosine used as standard in 1 min at pH 7.5 and 37ºC. The following equations were used to determine the enzyme units per mg of solid preparation:

$$\left(\mathrm{units/mg\ enzyme}\right) = \frac{\left(\mathrm{units/mL}\right)}{\left(\mathrm{mg\ enzyme/mL\ enzyme}\right)}$$

$$\left(units/mL\right) = \frac{\left(\mu\ mole\ T\right)\left(otal\ volume\right)}{\left(olume\ of\ diluted\ enzyme\right)\left(ncubation\ time\right)\left(olume\ used\ in\ the\ colorimetric\ assay\right)}$$

Where T is Tyrosine, the total volume is equal to 1.1 mL, the volume of diluted enzyme solution is 0.1 mL, the incubation time is 10 min, and the volume used in the colorimetric determination is 0.4 mL.

II.4. Results and discussion.

In ET-AAS, the direct determination of volatile analytes, such as Se in complex matrices, requires the use of efficient chemical modifiers to avoid elemental volatilization and consequently analyte losses during the different stages of the ET process. In order to study matrix modification for Se different chemicals were chosen as follows: (1) $Ni(NO_3)_2$, (2) $Ni(NO_3)_2$+H_2O_2, (3) $Pd(NO_3)_2$, and (4) $Pd(NO_3)_2$+H_2O_2. $Ni(NO_3)_2$ was selected because it is the manufacturer's recommended matrix modifier for Se determinations; $Pd(NO_3)_2$ was used because it is considered an universal matrix modifier [2] and it was successfully used in our laboratory previously [14,15]. In addition, since a high amount of organic matter was expected to be introduced into the graphite tube as consequence of the EPS extraction procedure, H_2O_2 was used as an auxiliary oxidant in order (i) to help to degrade the organic matter and (ii) to avoid building-up of carbonize residues inside the tube. H_2O_2 was successfully used for the aforementioned purposes by Vinas and co-workers [16]. Instead of a time-consuming ash-atomization study, the ashing time, and the ashing and atomization temperatures were initially used as recommended by the manufacturer, and with these conditions, the matrix modifiers were tested in (1) water, (2) buffered water, (3) sample extracts and (4) buffered sample extracts. It is recommended to perform the EPS extractions in a buffered media in order to maintain pH under control, since there is a pH value at which the activity of the enzyme reaches a maximum or a plateau [4]. Sample extracts were used in these experiments as follows: the samples (oyster tissue) were ultrasonicated and allowed to stand for 10 min. Then the supernatant, rich in organic matter, was used for selenium determination. Results, shown in Figures II.1 and II.2, are discussed below.

II.4.1 Standard solutions

As far as Se determination in buffered and non buffered standards concerns, Figure II.1, the highest sensitivities were found with the matrix modification provided by the $Ni(NO_3)_2$ in non-buffered solutions. However, the results showed that the Se absorbance obtained was buffer dependant. For instance, when the amount of $Ni(NO_3)_2$ added was 32 µg, the signal decreases more than 30%, from c. a. 0.130 (in non buffered solution) to 0.090 (in buffered solution). This showed that $Ni(NO_3)_2$, although recommended by the manufacturers, is a non-robust modifier. The addition of H_2O_2 did not change the former conclusion.

Regarding $Pd(NO_3)_2$ modifier, the intensity was not significantly different between buffered or non-buffered standards (n = 3, test *t*, $P = 0.05$). Hence, $Pd(NO_3)_2$ showed a better performance than $Ni(NO_3)_2$, being standard aqueous calibration possible for buffered samples. The addition of H_2O_2 did not change the former results.

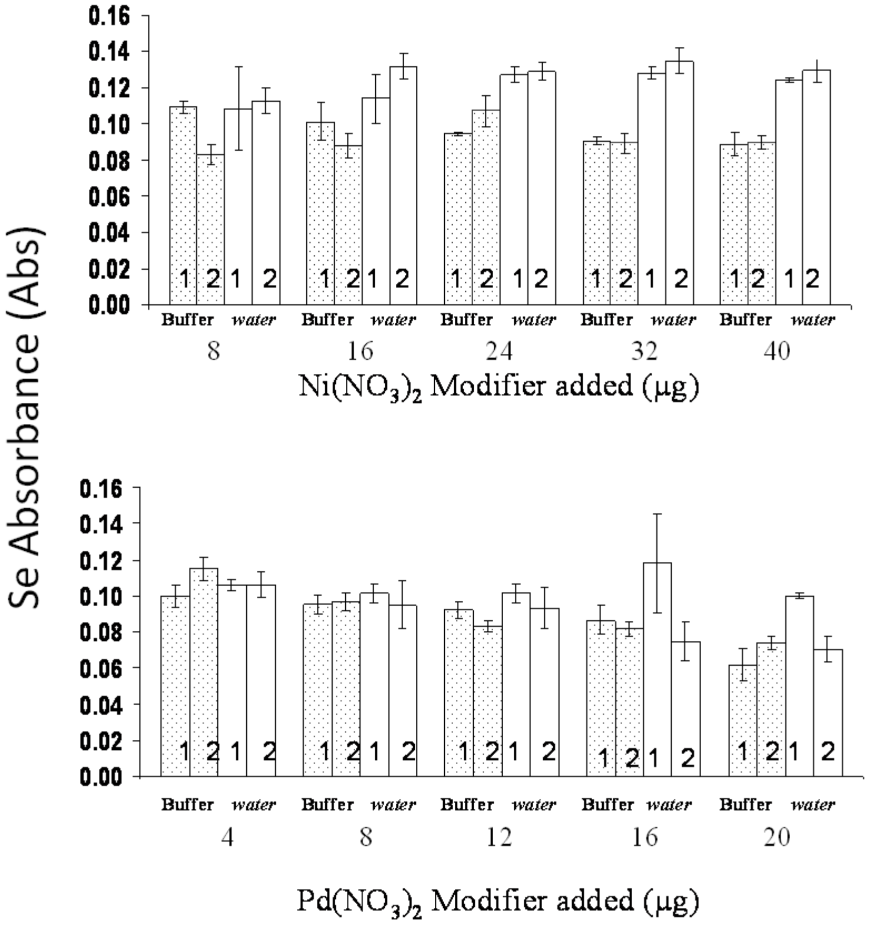

Figure II.1. Matrix modifier efficiency on non-buffered and buffered (pH= 7.5) 25 µg l^{-1} Se standard conc.; 1 = with 5 µL of H_2O_2; 2 = without H_2O_2.

II.4.2. Sample extracts

In Figure II.2 it can be seen that the absorption signal stabilizes and remains constant from 24 up to 40 µg of $Ni(NO_3)_2$, for each set of experimental conditions. In that range, absorbances in non-buffered are slightly higher than in buffered solutions, and in the presence of H_2O_2 absorbances are lower than in its absence. The later finding must be remarked, since this response was not expected on samples rich in organic matter.

As far as $Pd(NO_3)_2$ concerns, for the buffered medium the signal remains constant from 8 up to 20 µg of $Pd(NO_3)_2$, with a little increment when H_2O_2 was added. Regarding non-buffered extracts, when H_2O_2 plus $Pd(NO_3)_2$ was used, an increase was observed for all cases in the signal of *c.a.* 70%. It must be noted that with 16 µg and 20 µg of $Pd(NO_3)_2$, the absorbance obtained was not significantly different (n = 3, test *t*, *P* = 0.05) between the buffered or non buffered extracts in the presence of H_2O_2.

In conclusion, the best matrix modification found in this work was $Pd(NO_3)_2$ plus H_2O_2, and it was used for further experiments [20 µg of $Pd(NO_3)_2$ plus 5 µL of H_2O_2 conc.].

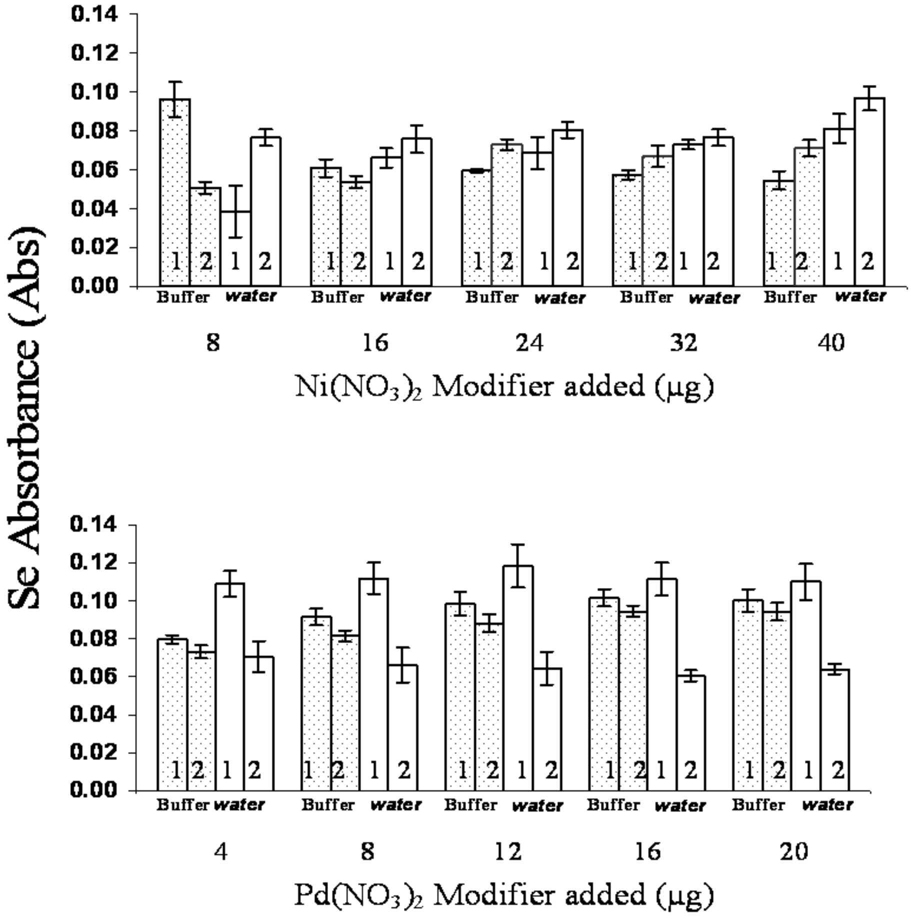

Figure II.2. Matrix modifier efficiency on sample extracts and buffered (pH= 7.5) sample extracts; 1 = with 5 µL of H_2O_2 conc.; 2 = without H_2O_2.

II.4.3. Study of long-term life of the graphite tube

The presence of H_2O_2 creates oxidant conditions inside the graphite tube, which helps to obtain better organic matter degradation and avoids building-up of carbonaceous residues inside the tube. To study the long-term performance of the graphite tube using H_2O_2, the lifetime of two graphite tubes was followed in the presence of oyster extracts using $Pd(NO_3)_2$+H_2O_2 or $Pd(NO_3)_2$, as matrix modifiers (Figure II.3). Se absorbance measurements were done until the difference between the mean of the first 50 values was 20% higher than the mean of the last 50 ones.

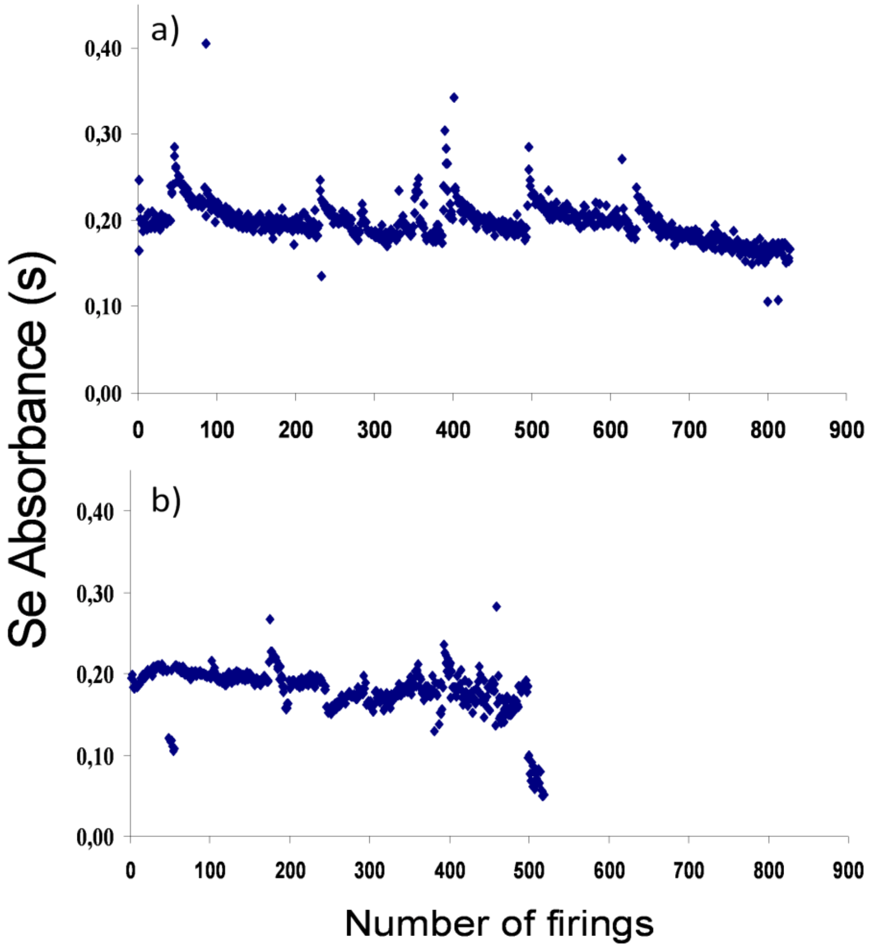

Figure II.3. Study of the long-term life of graphite cuvettes. Buffered (pH 7.5) extracts from oyster tissue were used, with the following matrix modifiers: a) 20 µg of $Pd(NO_3)_2$ plus 5 µL of H_2O_2 conc. b) 20 µg of $Pd(NO_3)_2$.

As can be seen in Figure II.3, when the mixture $Pd(NO_3)_2$+H_2O_2 is used, the signal decreases *c.a.* 20% after 828 firings, from 0.21±0.02 to 0.16±0.01 (n=50), whilst with $Pd(NO_3)_2$ alone the absorbance is lowered *c.a.* 34% after 519 firings, from 0.19±0.02 to 0.13±0.05 (n=50). This means that the presence of H_2O_2 increases at least 66% the tube life. In addition, the relative standard deviation, RSD, of the first 50 firings with $Pd(NO_3)_2$+H_2O_2 was 10%, whilst the RSD for the last 50 firings was 6%, meaning that precision was not degraded after 828 firings. In contrast, when $Pd(NO_3)_2$ was used alone the decrease of precision was remarkable: 11% of RSD for the first 50 firings *vs* 38% for the last 50 ones. Furthermore, the spectrometer has a charge-coupled device, CCD, which allows obtaining high-definition video images of the inside of the graphite tube. This camera makes possible to monitor the different steps of the furnace program [17]. Thus, capillary alignment, sample deposition, sample drying and sample ashing can be easily controlled, avoiding long experimental trials to find the best conditions for the aforementioned steps. For instance, the CCD can be used to detect if all sample has been deposited correctly into the tube or if sample sputtering is happening during the drying step. More interestingly, the long-term performance of the inside of the tube can be followed by monitoring the building-up of organic residues, as showed in Figure II.4, in which it can be seen that after 519 firings the residues accumulated are negligible thanks to the addition of H_2O_2 as matrix modifier. On the contrary, when $Pd(NO_3)_2$ was used alone, the space inside the tube was reduced *c.a.* 50% due to residue accumulation. This effect can be directly linked to the fast deterioration and lack of precision observed for this tube after 519 firings.

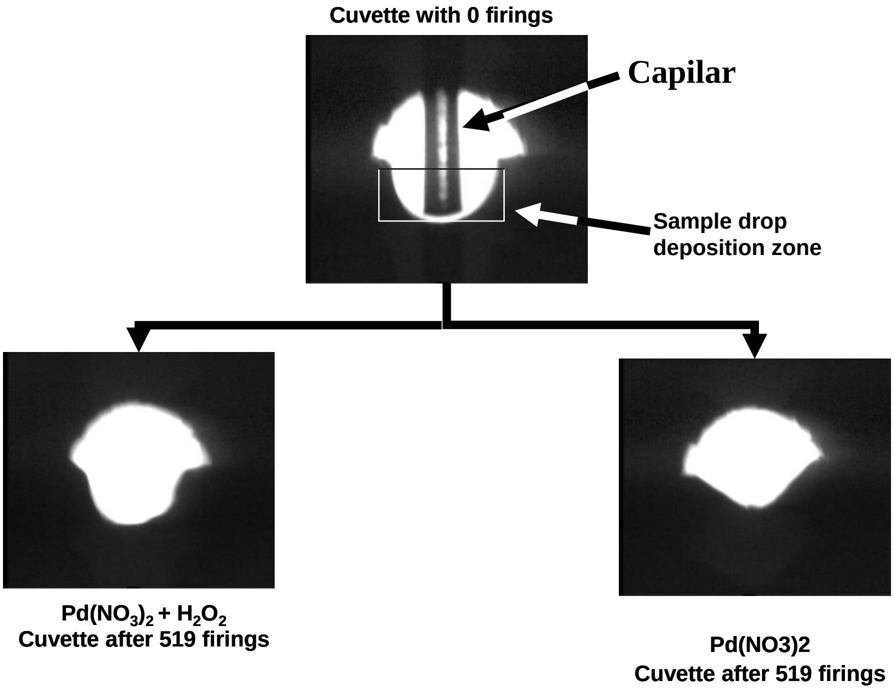

Figure II.4. CCD shots of graphite tube as a function of matrix modification.

II.4.4. Study of Se recovery

Once defined the best matrix modifier to be used, the next step was to check if the conditions were appropriated for the retention of all the selenium present in solution. As stated before, Se sensitivities in ET-AAS could be a function of the Se-species present in the sample [11, 12]. In this set of experiments, oyster extracts were spiked up to 25 µg L^{-1} with Se(IV). Recoveries were calculated as follows:

$$\frac{(\text{Absorbance of spiked samples}) - (\text{Absorbance of samples})}{(\text{Absorbance of } 25\,\mu\text{g/L standard})} \text{x}100$$

As expected, when 24 µg of $Ni(NO_3)_2$ was used as matrix modifier, the Se recoveries achieved were below 42% of the expected values, confirming that $Ni(NO_3)_2$ is not a good choice as matrix modifier. On the contrary, the best results were achieved with $Pd(NO_3)_2$. Recoveries with this matrix modifier comprised from 94% to 111% with or without H_2O_2.

II.4.5. Influence of the type of enzyme

It is well known that enzymes from the same class can have significant differences in terms of activity and extraction efficiency. Consequently, different enzymes of the protease family were studied: Protease XIV, Subtilisin A and Trypsin. The Se recoveries (n=3) from mussel tissue, obtained by the application of the EPS methodology, were: (i) 93±7% for Protease XIV; (ii) 70±9% for subtilisin A and (iii) 28±5% for Trypsin.

As far as enzyme activity concerns, the results (U/mg solid, n = 3) were: Protease XIV, 5.4±0.2; Trypsin, 3.3±0.1; Subtilisin, 8.0±0.9. It must be stressed that enzymes loss activity by ageing, e.g., the activity of Protease XIV after 3 months was 4.4±0.2. In experiments developed in our laboratory, some Se recoveries were unexpectedly low, what was directly linked to the loss in enzymatic activity with protease ageing. Therefore, researchers are encouraged to use fresh enzymes and to control, when possible, the activity of the enzyme. It must be also remarked that subtilisin A was the enzyme used in this work with the highest activity against casein, at the experimental conditions of the enzyme activity assay. However, the extraction efficiency was lower that the one obtained with Protease XIV. This suggests that enzyme specificity plays an important role in EPS, and that enzymes must be carefully chosen.

II.4.6. Influence of the amount of enzyme used

Regarding the amount of enzyme, 10 mg and 20 mg of Protrease XIV were used to extract Se from 10 mg of mussel by EPS. The Selenium recovery felt down from 93% to 77% as the amount of enzyme was increased. It is interesting to note that the background observed in the Se signal was increased *c.a.* 30% (data not shown) as consequence of the higher amount of enzyme used, suggesting that the decreasing in the Se recovery was due to problems with the graphite furnace program (e.g. low ashing time, matrix modification) rather than to a low efficiency in the EPS performance. Therefore, and to avoid this problems, 10 mg of enzyme was used in further experiments.

II.4.7. Influence of ultrasound in protease activity

The influence of the ultrasonic energy in the enzymatic activity (casein hydrolysis) of Protease XIV was studied by applying ultrasound at amplitude of 50% in intervals of 30 to 120 s. interestingly, 30 s of sonication does not affect enzyme activity, however, after 60 s, the activity decreases *c.a.* 20%. A sonication time of 120 s led to the complete inactivation of the enzyme (Table II). Therefore, sonication times larger than 2 minutes are useless.

Tabela II.2 – Influence of ultrasound on enzyme activity.

Sonication time	Enzyme activity (U mg^{-1} solid)	Enzyme activity (%)
0 seconds	4.81 ± 0.03	-
30s	4.8 ± 0.3	99 ± 6
60s	3.8 ± 0.4	78 ± 8
60s (a)	3.5 ± 0.3	73± 5
120s	0.06 ± 0.03	1.2 ± 0.6
120s (a)	0.03 ± 0.02	0.6 ± 0.4

(a) Ultrasonication time was obtained with pulses of 30s followed by a rest period of 30 s

II.4.8. Influence of the temperature.

To study the effect of temperature, the EPS procedure was done under cooling conditions (*e.g.* ice bath) and at room temperature. Interestingly, the recoveries were approximately *c.a.* 20% lower when the procedure was performed under cooling conditions. This result agrees well with the fact that Protease XIV has its highest activity at temperatures in the range of 30-50ºC. On the other hand, similar values were observed in the enzyme activity, when the sonication time of 60 s (or 120 s) was applied in a continuous way, or by pulses of 30 s followed by a rest period of 30 s, in order to decrease the heating produced by sonication (Table II). A continuous sonication time at room temperature was applied in further experiments.

II.4.9. Analytical figures of merit.

Calibration was performed with a series of Se(IV) standards. Sensitivity (m) was the slope value obtained by least-square regression analysis of calibration curves based on peak area measurements. The equation (n = 5) for the calibration curve was as follows:

$$y = (0.0028 \pm 0.00003)\,[Se] + (0.004 \pm 0.002)$$

where *y* is Se absorbance (peak area) and [Se] is the Se concentration in ng g^{-1}. For these conditions, the correlation coefficients of the calibration curves, r^2, was 0.999 within the calibration range (0–100) ng g^{-1}. The limit of detection (LOD), equal to 0.6 ng g^{-1} was defined as 3 s m^{-1}, *s* being the standard deviation corresponding to 10 blank injections and *m* the slope of the calibration graph. The limit of quantification (LOQ), defined as 10 s m^{-1}, was 2 ng g^{-1}. The relative standard deviation (RSD), estimated from aqueous standards (10 replicates) and calculated at concentrations of 20 ng g^{-1} and 50 ng g^{-1}, was 7% and 4%, respectively. The RSDs estimated from 10 replicates for oyster, mussel and plankton were, 6%, 6% and 10%, respectively.

II.4.10.Validation of the methodology.

Although the research described in this work was done using reference materials, additional validation was carried out dissolving the samples in an acid solution using pressure microwave digestion, and then determining the Se content. Results are shown in Table III. As can be seen, recoveries were in agreement with the certified values for mussel and plankton. In addition, this validates the use of Se content in oyster tissue, obtained after microwave digestion, as a reference value. When applying the t-test, it was found that no significant differences (P=0.05) occurred between the certified and EPS concentration values ($t_{exp}<t_{tab}$) for all the materials. For the unknown oyster tissue sample, the Se concentration found by EPS was compared with that obtained by microwave digestion.

In EPS assays using Protease XIV (Table III) total recoveries were found for mussel, plankton and oyster, since Protease XIV was able to degrade the organic matter of those biological samples. No Se was found, as anticipated, for the vegetal sample spruce needles after the enzymatic treatment, thus showing that there is a direct link between the type of the organic sample and the type of enzyme chosen to perform the enzymatic treatment. Thus, it is well known that there are different families of enzymes. In this work, the enzymes used belong to a generic family called proteases that cleavage the peptides bonds of proteins. This type of enzymes can not degrade vegetal samples, such as spruce needles, because they are not specific for hydrolysis the polysaccharides components of vegetables such as starch or glycogen. For the aforementioned reason the EPS extraction with proteases failed for this sample.

Tabela II.3 -Analytical results for Se obtained after Enzymatic Probe Sonication (EPS) and microwave digestion (MW).

Sample	**Certified Content ($\mu g\ g^{-1}$)**	**Found Content EPS[a] ($\mu g\ g^{-1}$)**	**Found Content MW[b] ($\mu g\ g^{-1}$)**	**Recovery (%)**	t_{exp}
ERM-CE 278 Mussel tissue	1.84±0.10	1.71±0.10	1.80±0.07	93	2.25[c]
BCR - 414 Plankton	1.75±0.10	1.86±0.09	1.65±0.09	106	2.11[c]
oyster tissue	no certified	4.94±0.48	4.45±0.11	111	1.76[d]
BCR-101 spruce needles	no certified	n. d.	1.21±0.29	-	-

[a]Average value ($\mu g\ g^{-1}$) of 3 different extractions. [b]Average value ($\mu g\ g^{-1}$) of 3 different microwave digestions. Results are expressed as mean value ± standard deviation. [c] *t* test of significance, t_{tab}= 3.18 for n = 3 (P = 0.05). [d]t_{tab} = 2.78 for n = 4 (P = 0.05). Recovery (%) of Se is determined by comparing the EPS value to the certified content or to the MW content (if the certified value is not available). n. d. = not detected.

II.5. Conclusions

In the present work it was demonstrated that EPS can be successfully used for total Se determination using ET-AAS in samples such as oyster, mussel and plankton, when a correct matrix modifier, e.g.,$Pd(NO_3)+H_2O_2$, is used and the enzyme protease XIV is employed in the extraction process. The use of H_2O_2 helped to obtain a better performance in the Se ET-AAS measurements: precision was improved and a 66% extra-life for the graphite tubes was obtained. Therefore the use of H_2O_2 combined with an appropriate matrix modifier such as $Pd(NO_3)_2$ is highly recommended if high amounts of organic matter are expected to be introduced into the graphite furnace for Se measurements.

The enzymes used with the EPS procedure to extract Se from biological samples must be carefully chosen. Protease XIV is the recommended protease-type enzyme to be used for EPS treatment in samples such as the ones referred in this work. Activity of the enzyme must be regularly checked, since low activities led to low extractability. Once the container is open, fresh protease must be used as soon as possible. EPS must be performed at room temperature, since cooling diminishes enzyme activity. When necessary, the search of another more appropriate enzyme is often a better option rather than the increase of the enzyme amount, which may lead to an increase on the background signal.

II.6. Acknowledgements

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Chapter III

Ultrasonic Assisted Enzymatic Digestion (USAED) coupled with High Performance Liquid Chromatography and Electrothermal Atomic Absorption Spectrometry as a Powerful Tool for Total Selenium and Selenium Species Control in Se-Enriched Food Supplements

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III.1. Abstract

The suitability of hyphenated USAED with HPLC separation and ET-AAS determination as a new rapid methodology for Se control in Se enriched food supplements is demonstrated. Total Se determination and Se speciation are accomplished in a single sample treatment using low sample amounts (ca. 10 mg), and low extracting volume (1 ml). The total Se content in seven of the ten Se enriched supplements studied was in agreement with the values obtained after microwave pressurized acid digestion, MW, (test t, $p = 0.05$). The Se species studied were Se(IV), Se(VI), SeMet, SeMeSeCys, and $SeCys_2$, being some of the most common found in the ten supplements studied. Although SeMet was the Se species expected to be present at the highest concentration in most Se enriched food supplements, we detected it in only three of the ten samples studied. In the other seven samples, two of them had Se(IV) as the main Se species. The other five supplements had Se species that did not match with any of the five standards selected by us. We have also systematically demonstrated that ultrasonication does not alter the following Se-species: Se(IV), Se(VI), SeMet, SeMeSeCys, and $SeCys_2$. The new procedure can be easily adapted to more Se-species and can be routinely used for Se control in Se enriched food supplements. Concerning the supplements studied, our results suggest that stricter control on the Se content in enriched food supplements in terms of Se species will need to become mandatory.

Keywords: Se, Speciation, HPLC, ET-AAS, USAED

My contribution to this work was the optimization and validation of the HPLC coupled with ET-AAS method, the extraction of Se from the samples by USAED and the Se species determination.

III.2. Introduction

Selenium and its related bio-species are regularly studied by the scientific community due to their recognized medicinal properties as powerful antioxidants and as protectors against heavy metal toxicity [Nogueira, Zeni & Rocha, 2004]. In addition, Se has been suggested to play an important role in the immune system, in the reproductive function and has been identified as a therapeutic target for risk and prognosis of certain diseases [Nogueira, Zeni & Rocha, 2004]. Due to these properties, Se is frequently included in food supplements to help maintain a healthy diet. Some examples of such supplements are the following Se-enriched foods: broccoli, yeast and potatoes, and also a plethora of pseudo-medicines claimed to be rich in Se [Cabañero, Madrid & Cámara, 2005]. The majority of the aforementioned products are sold in dedicated health shops without any control regarding the certainty of the Se content.

The Se-species present in Se-enriched supplements can vary, but the most common are generally the following: SeCys, H_3N^+-CH(COO^-)-CH_2-SeH; SeMeSeCys, H_3N^+-CH(COO^-)-CH_2-Se-CH_3; SeMet, H_3N^+-CH(COO^-)-CH_2-CH_2-Se-CH_3 and SeMeSeMet, H_3N^+-CH(COO^-)-CH_2-CH_2-Se-$(CH_3)_2$ [Polatajko, Jakubowski, & Szpunar, 2006; Capelo, Fernandez, Pedras, Santos, Gonzalez, & Vaz, 2006]. The importance of the Se-species present in the food supplement arises from the fact that each one has different benefits for living organisms. For instance, at only 1–3 mg kg^{-1}, Se-methylselenocysteine has been reported to be a better chemopreventive agent than either SeMet or selenite, Se(IV) [Abdulah, Miyazaki, Nakazawa & Koyama, 2005]. Furthermore, the bioavailability of Se depends on its chemical form in food. In addition to the benefits, it must also be taken into account that the range between deficiency, essentiality and toxicity is very narrow [Nogueira, Zeni, & Rocha, 2004].

Total Se can be determined through different analytical techniques including ET-AAS, HG-AAS, ICP-AES; and, HG-AFS [Capelo, Fernandez, Pedras, Santos, Gonzalez, & Vaz, 2006]. Regarding Se speciation, HPLC, associated with ET-AAS; HPLC-UV-(MW)-HG-AAS/AFS; and HPLC-UV-(MW)-HG-ET-AAS are techniques that have also been applied for Se speciation [Capelo, Fernandez, Pedras, Santos, Gonzalez, & Vaz, 2006]. However, due to its speed and low limit of detection, HPLC-ICP-MS, is nowadays the preferred analytical technique used in studies of Se speciation [Capelo, Fernandez, Pedras, Santos, Gonzalez, & Vaz, 2006; Cuderman, Kreft, Germ, Kovacevic, & Stibilj, 2008]. Nevertheless, the high price and running costs of this technology makes it a mandatory technique for studies involving low Se concentrations but only optional for the control in Se enriched supplements, where the concentration of the Se species is high enough to be determined by less expensive techniques [Capelo, Fernandez, Pedras, Santos, Gonzalez, & Vaz, 2006]. In addition, hydride generation is difficult to couple to sample treatments that relay in the use of ultrasonic energy for the solid-liquid extraction of Se (and Se species). When ultrasonic energy is applied to a solid matrix of animal or vegetal origin, organic matter is extracted to the liquid solution. This organic matter hinders the formation of the Se hydride.

As far as Se solid-liquid extraction is concerned, the enzymatic hydrolysis of biological samples accelerated by ultrasound (known as ultrasonic assisted enzymatic digestion, USAED) is currently the most powerful tool used to extract the total content of Se whilst maintaining Se-species integrity. It can be used for total elemental determination and elemental speciation. It is fast (< 2 min per extraction), easy to operate and inexpensive [Capelo, Ximenez-Embun, Madrid-Albarran, & Camara, 2004; Vale, Rial-Otero, Mota, Fonseca, & Capelo, Talanta, 2008]. Up to the present, this method of sample treatment has been applied to a vast number of different matrixes by many different research groups. [Peña-Farfal, Moreda-Piñeiro, Bermejo-Barrera, Bermejo-Barrera, Pinochet-Cancino, & Gregori-Henríquez, 2005; Maduro, Vale, Alves, Galesio, Gomes da Silva, Fernandez, Catarino, Rivas, Mota, & Capelo, 2006; Siwek, Noubar, Bergmann, Niemeyer, & Galunsky, 2006; M. Montes-Bayon, Molet, Gonzalez, & Sanz-Medel, 2006].

In the present work we report on the combination of USAED, for fast Se extraction, together with HPLC, for Se species separation, and ET-AAS, for Se quantification, as a new, fast and inexpensive methodology for monitoring total Se and Se species in Se enriched supplements.

III.3. Experimental

III.3.1. Apparatus

A Branson Sonifier 150 ultrasonic cell disruptor homogeniser (100 W, 22.5 kHz, Branson Ultrasonics Corporation, USA) equipped with a 1 mm titanium microtip was used for ultrasonic applications. The ultrasonic irradiation energy was fixed at a power setting of 50%. The Sonifier 150 has a digital LCD display which provides a continuous read-out of the watts delivered to the end of the probe (range 5–8 W in this work). A Hermle (Wehingen, Germany) model Z160M refrigerated microliter centrifuge was used. Chromatographic separations were done with a Thermo Electron Corporation (Massachusetts, USA) Chromatograph, model Finningan Surveyor equipped with a diode array detector and a 50 µL injection loop. The separation was performed on a 250 x 4.1 mm, 10 µm Hamilton PRP-X100 anion exchange chromatographic column. The Se absorbance was measured with a Unicam (Cambridge, UK) atomic absorption spectrometer model SpectrAA-300 *plus* equipped with a graphite furnace, an autosampler and Zeeman background correction. A Se hollow-cathode lamp from Thermo Electron Corporation (Massachusetts, USA) operated at 15 mA was used as a radiation source. The Se analytical line at 196.0 nm and a slit width of 0.5 nm were used for measurements. Selenium quantification was performed in a peak area mode. Pyrolytic graphite-coated graphite tubes with L'vov platform (Omega Platform Extended Lifetime type, Unicam) were used. The electrothermal program used was (i) step 1-dry 1: 95 132ºC, Time 40 s, ramp 10ºC/s , Gas flow 0.2 L min^{-1}; (ii) step 2-dry 2: 130ºC, Time 10 s, ramp 5 133ºC/s , Gas flow 0.2 L min^{-1}; (iii) step 3-ashing: 1100 ºC, Time 30 s, ramp 90ºC/s , Gas flow 0.2134 L min^{-1}; (iv) step 4-atomization: 2300 ºC, Time 4 s, ramp 0ºC/s , Gas flow off; (v) step 5-Cleaning: 2600ºC, Time 3 s, ramp 0ºC/s , Gas flow 0.2 L min^{-1}. Microwave acid digestions were performed in a Samsung MW81W (850 W, 2450 MHz) microwave apparatus from Samsung Electronics (South Korea) and Parr (IL, USA) microwave pressure digestion bombs. Filtration of sample extracts was performed using a 25 mm diameter and 0.22 µm particle size Cameo syringe Nylon filters purchased from Sigma-Aldrich. The mobile phase was filtered with a 47 mm diameter, 0.45 µm pore size mixed cellulose ester membrane filter (Whatman, Maidstone, U.K.)

III.3.2. Reagents

The enzymatic hydrolysis was performed with a non-specific enzyme, Protease XIV, from Sigma (Sigma-Aldrich, Steinheim, Germany). The ten Selenium enriched food supplements used in this work were purchased in local shops in Lisbon, Portugal. They are listed along with their characteristics in Table III.1. All chemicals used in this work were of analytical-reagent grade, unless otherwise specified. HNO_3 65% w/w and $Pd(NO_3)_2$ matrix modifier were purchased from Fluka (Buchs, Switzerland). A solution of citric acid (3 mM; Panreac, Barcelona, Spain) adjusted to pH 5 with an ammonia solution 25% w/v from Merck (Darmstadt, Germany) was used as the chromatographic mobile phase. An inorganic Selenium atomic absorption standard solution from Aldrich, (976 mg mL^{-1}) was used to prepare Se standards for ET-AAS. All the Selenium standards, sodium selenite (S5261), Se(IV); sodium selenate (S-8295), Se(VI); seleno-L-methionine (S3132), SeMet; Se-(Methyl) selenocysteine (M6680), SeMeSeCys and seleno-DL-cystine (S1650), $SeCys_2$ were purchased from Sigma-Aldrich (Milwaukee, USA). A $SeCys_2$ standard solution was prepared in a HCl solution (3% v/v). The other Se-species standard solutions were prepared in ultra-pure water. Stock solutions of 500 mg L^{-1} were stored in a refrigerator at 4ºC and protected from light. Working standard solutions were prepared immediately before use by appropriate dilution of the stock standard solution.

Table III.1. - Country of origin and source of the supplements studied, as provided by the manufacturers.

S1. Spain. Yeast cultivated in Se enriched medium. 100 µg g^{-1} Se.
S2. United Kingdom. Se provided as complex of aminoacids. Six inorganic compounds are also added.
S3. United Kingdom. Se provided as complex of aminoacids. No further information is given. Twelve inorganic compounds are also present.
S4 USA. Six inorganic substances and twenty three different varieties of fruit and plants. The manufacturers provide no details of the form Se incorporated in this supplement.
S5. United Kingdom Se(VI) and other inorganic compounds. 62.5 µg g^{-1} Se.
S6. USA. Aged garlic extract. Se as L-SeMet.
S7 USA. Spiruline and other 8 plant extracts and 4 inorganic substances.
S8. Belgium. Se added as Se-Met. Ten organic substances and ten inorganic substances.
S9. Belgium. Different inorganic and organic compounds. Se present is claimed to be SeMet.
S10. France. Se enriched yeast. Twenty eight different varieties of fruit and plants. Nine inorganic compounds added.

III.3.3. Sample pre-treatment

Selenium enriched dietary food supplements were prepared as follows: the samples, once in the laboratory, were homogenized and ground with an agate mortar to a fine powder. Powdered samples were stored in capped polypropylene flasks at 4ºC protected from light.

III.3.4. Certified reference material

The certified reference materials used in this work for validation purposes, SELM-1, selenium enriched yeast, was purchased from the National Research Council from Canada, NRC-CNRC. This material is certified in Se as follows: 2059±64 µg g^{-1} total Se; 3389±173 µg g^{-1} SeMet. Reference material BCR-414, plankton, was obtained from the European Institute for Reference Materials and Measurements, Geel, Belgium and was used as a blank matrix due to its low Se content (1.75±0.1 µg g^{-1} total Se).

III.3.5. USAED sample treatment

This sample treatment was optimized in previous work [Vale, Pereira, Mota, Fonseca, & Capelo J. L. 2007]. Briefly, each sample was prepared by mixing 10 mg of the food supplement material with 10 mg of enzyme preparation. One millilitre of ultrapure water was added and the mixture was ultrasonicated for 120 s with a 1 mm diameter probe at 50% ultrasonic amplitude.

Afterwards, the mixture was centrifuged for 3 minutes at 4000 rpm and the supernatant was withdrawn and filtered through a 0.22 µm nylon cut-off filter. 50 µL of this purified extract was introduced directly into the HPLC without further handling. After separation, the eluted fractions were recovered and, after appropriate dilution, the Se was determined using ET-AAS.

III.3.6. Acid Microwave digestion procedure

In order to validate the USAED treatment, a different but common sample treatment procedure for solid sample solubilization, acid microwave digestion, was carried out using a domestic microwave oven and Parr® pressurized digestion bombs. The protocol for this digestion has been previously described. [Vale, Pereira, Mota, Fonseca, & Capelo J. L. 2007]. Briefly, 70 mg of sample were mixed with 2 mL of HNO_3 conc. plus 25 µL of H_2O_2. The microwave oven was set at 350 W for 2 minutes. Afterwards, at least 3 h was necessary to allow the samples to cool. Finally, each sample was then diluted up to 5 mL with Milli-Q water.

III.3.7. Chromatographic conditions

The separation of selenium compounds was carried out in an anion exchange column Hamilton PRP-X100. The flow rate of the mobile phase was 1 mL min^{-1}. The 234 nm wavelength was used to monitor the Se species.

III.4. Results and discussion

III.4.1. Optimization of the chromatographic separation process

In general, the literature describing Se speciation in unknown samples recommends the use of two different chromatographic columns, for instance an anion exchange column and a column combining ion exchange and size exclusion chromatography, to discriminate $SeCys_2$ from SeMetO, since both species elute at the same time when only an anion exchange column is used [Pedrero, Encinar, Madrid, & Camara, 2007]. Furthermore, other authors have also recommended not using only retention times of standards in a single chromatographic method as a unique method to identify the presence of certain Se species [Montes-Bayon, Molet, Gonzalez, Sanz-Medel, 2006]. However, in routine control, when certain Se species can be anticipated or the number of expected species is low, the use of a single chromatographic column is still a valuable option [Siwek, Noubar, Bergmann, Niemeyer, & Galunsky, 2006; Cabañero, Madrid, & Camara, 2005; Cuderman, Kreft, Germ, Kovacevic, & Stibilj, 2008]. For the latter reason we decided to follow the single chromatograph approach in this work.

III.4.1.1 Influence of the composition of the mobile-phase

Se species can be present in solution in the form of cations, anions or zwitterions, hence their chromatographic separation can only be accomplished by choosing optimal operating conditions. Much work addressing Se separations using HPLC describes the use of an anion exchange column in conjunction with citric acid (10 mM + 2% of methanol, at pH 5) as the mobile phase [Stadlober, Sager & Irgolic, 2001; Mazej, Falnoga, Veber & Stibilj, 2006; Pedrero, Encinar, Madrid, Cámara, 2007]. Methanol is claimed to enhance the Se signal when ICP-MS detection is used. Hence, as a first step, we checked the effect of the presence of methanol in both (i) the separation process using HPLC and (ii) the subsequent measurement by ET-AAS. A mixture of the following five selenium compounds Se(VI); Se(IV); SeMeSeCys; $SeCys_2$, and SeMet (50 mg L^{-1} each) was prepared. This mixture was then injected in the HPLC system using the following mobile phases at 1ml/min: (a) 10 mM citric acid (pH ca. 5) with 2% methanol or (b) the same solution without methanol. No effect on the elution times of any of the Se species or on their respective signals in the ET-AAS measurements (data not shown) was observed. Consequently, we selected the mobile phase without methanol.

Precise determination of Se by ET-AAS is not simple. For example, samples with high organic matter content have different sensitivities in ET-AAS as a function of the component Se species [Gammelgaard & Larsen, 1998, Sabe, Rubio, Garcia Beltran, 2001]. In our work, due to the high level of Se, the samples always required dilution prior to Se measurement, thus reducing the organic matter content to levels with no influence on the analysis. Irrespective of the latter, we already had a reliable method for measuring Se using ET-AAS in samples with high organic matter content [Vale, Pereira, Mota, Fonseca, & Capelo, 2007].

Unfortunately, SeMeSeCys and Se(IV) had the same elution time in HPLC making it necessary to optimize the mobile phase to achieve better separation. To this end, we devised a set of experiments in which the different standards were injected individually, to obtain their retention times, at a constant pH of 5 but varying the citric acid concentration in the mobile phase. Afterwards, the same study was repeated, this time injecting all standards at the same time. The results are shown in Figure III.1.

Clearly, as the citric acid concentration increases from 1 mM to 10 mM, the difference in the elution times for four of the five species becomes slightly smaller whilst for Se(VI) the retention time changes 20 min. Apparently this species, whose peak was obtained inverted, is more sensitive to the ionic strength of the solution than the others. The inverted peak (denoted as * in Figure III.1.), is attributed to the presence of HCl in the preparation of $SeCys_2$. To optimize retention time and resolution, we selected the mobile phase with a citric acid concentration of 3 mM at pH 5 since, on one hand, when using the lowest concentration (1 mM) the analysis was too lengthy (30 min *vs* 17 min), to separate all the species, whilst, on the other hand, the highest

10 mM concentration lowered the resolution. The resolution obtained with 5 mM showed no clear advantage over that obtained with the 3 mM concentration (14 min *vs*. 17 min for the total elution time). On the other hand, since low ionic strengths in the mobile phase generally extend the column lifetime, we chose to work with the lower concentration.

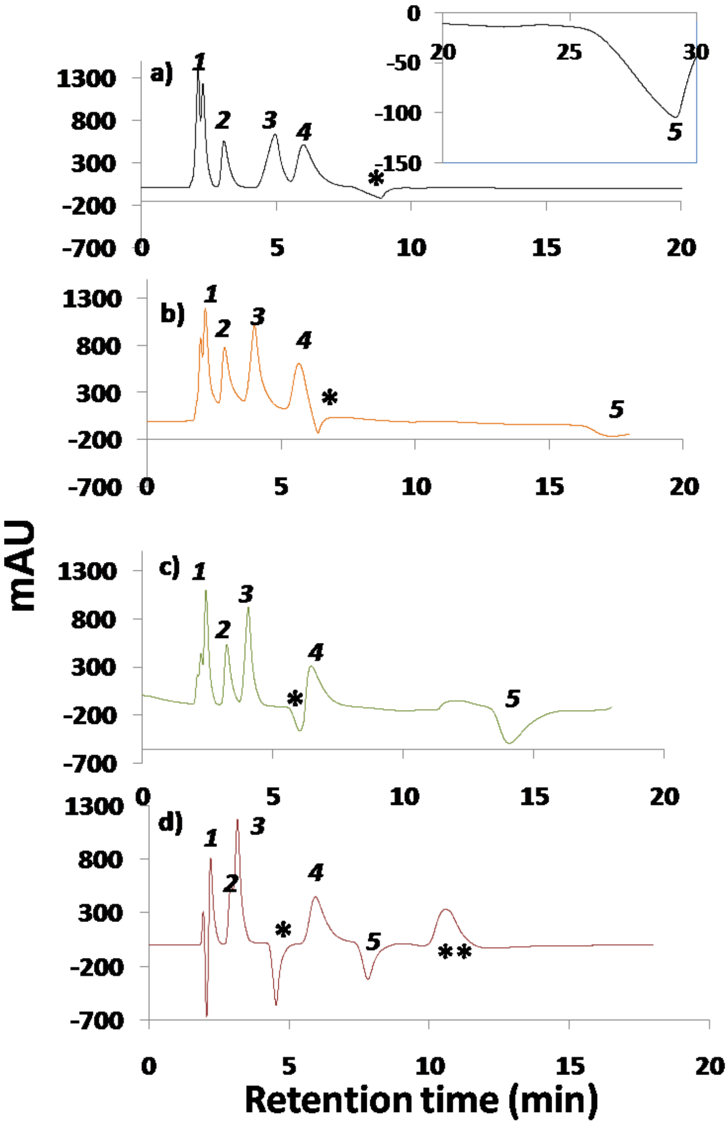

Figure III.1. Chromatograms with different concentrations of citric acid as the mobile phase at pH=5 a) 1 mM; b) 3 mM; c) 5 mM; d) 10 mM. A mixture of selenium standards at 50 mg L^{-1} each, was injected. 1) SeCys, 2) SeMeSeCys, 3) Se(IV), 4) SeMet, 5) Se(VI). * Peak due to HCl used in the preparation of the SeCys$_2$ standard. ** Peak due to citric acid, this peak is only observed at high citric acid concentrations, such as 10 mM

III.4.1.2 Influence of the mobile phase flow rate

Some authors have described improved efficiency in the separation of the Se species at low mobile phase flow rates [Mazej, Falnoga, Veber, Stibilj, 2006]. Therefore, two different flow rates for the mobile phase were assayed, 0.8 mL min^{-1} and 1 mL min^{-1}. The lower flow rate produced a worst resolution and therefore 1 mL/min was chosen as the optimum flow rate for the following experiments. Finally, since the five species studied were separated to our satisfaction, neither the mobile phase flow rates nor the pH were further optimized.

III.4.2 Optimization of Se determination

III.4.2.1. Se recovery determined by ET-AAS after HPLC separation. Influence of the organic matrix

The key to a successful recovery of Se species, once they have been separated by HPLC, is the careful collection of the different fractions obtained in the separation process (Table III.2). Based on the elution times of each standard two sets of experiments were tried. In the first set, the fractions obtained for the standards were tested for Se recovery using the ET-AAS system.

Table III.2 - Effect of organic matter and ultrasonication on the recovery of Se species. 10 mg reference material plankton was used as clean matrix. Se species were spiked (10 µg g^{-1}) and samples were ultrasonicated before Se separation by HPLC and quantification by ET-AAS. Procedure: protease + plankton ultrasonicated with a 1 mm probe at 50% amplitude during 2 min., then centrifuged at 400 rpm and filtered through a 0.22 µm cut off.

Se species	Collecting times (min)	Collected volumes (ml)	Se recoveries from aqueous standards (%)	Se recoveries from spiked plankton samples (%)
$SeCys_2$	1.93-2.60	0.69±0.04	103±10	109.3±5.2
SeMeSeCys	2.60-3.50	0.90±0.04	98.1±8.1	118.1±6.2
Se(IV)	3.50-4.36	0.91±0.05	89.2±7.1	84.1±3.2
SeMet	5.33-7.19	1.87±0.06	93.1±9.1	107.5±9.4
Se(VI)	14.76-16.7	1.47±0.05	97.2±3.1	103.2±5.3

Recovery expressed as ([Se recovered]/[Se spiked])*100. Results are expressed as mean value ± standard deviation (n=3).

The results presented in Table III.2 show recoveries of Se from 89% to 103% depending on the Se specie. In the second set of experiments, the influence of the organic matrix in the separation of Se species was studied. It must be borne in mind that when ultrasound is applied near to a solid, micro-jets of solvent accelerate toward its surface at velocities > 100 m s^{-1}, causing pitting and mechanical erosion, resulting in particle rupture (i.e., disruption) and inducing transfer of a large amount of organic matter into the liquid phase [Wibetoe, Takuwa, Lund & Sawula, 1999]. A, certified reference material BCR-414, Plankton (trace elements; Sigma Aldrich), was used as a representative biological matrix. This sample was chosen as a «blank matrix» because its Se content was too low to interfere with this type of study. Samples of BCR-414 were treated with the Se standards studied in this work at a concentration of 10 µg g^{-1}. The samples were ultrasonicated as described in the sample treatment section and, after centrifugation, the extracts were withdrawn. The results presented in Table 2 showed that recoveries from the spiked extracts were between 93% and 109%, confirming that the presence of organic matter does not change the elution order of the Se species neither their elution time.

Another interesting finding shown in Table III.2 is that ultrasonication does not induce the interconversion of species neither does it alter the elution order or the elution time. This result is very important since it confirms that the brief ultrasonication is too short to induce changes in Se species during the USAED treatment. This conclusion was further confirmed, as reported in the next sections, by fortifying some of the target samples with the standards and studying the effects of ultrasonication on species interconversion.

III.4.2.2. Optimization of total Se and Se species determination

The use of enzymes to extract the Se content under an ultrasonic field is nowadays a well established protocol [Capelo, Ximenez-Embun, Madrid-Albarrán & Camara, 2004; Vale, Rial-Otero, Mota, Fonseca, Capelo, 2008]. Different enzymes or cocktails of enzymes can be used as appropriate to the type of matrix being studied. Thus, if the matrix is of animal origin, proteases are generally the enzymes of choice, since these specifically cleave proteins [Vale, Pereira, Mota, Fonseca & Capelo, 2007]. On the other hand, if the matrix is from vegetal origin, then enzymes that specifically cleavage cellulose are preferred [Sanz, Munoz-Olivas, Dietz, Sanz, & Camara, 2007]. In any case, the most used enzyme for matrixes of animal origin is Protease XIV, a cocktail of at least 10 proteases: five serine-type proteases, two zinc endopeptidases, two zinc leucine aminopeptidases and one zinc carboxypeptidase. In our laboratory we have successfully determined Se by ET-AAS immediately after USAED extraction, using protease XIV in different types of matrixes [Vale, Pereira, Mota, Fonseca & Capelo, 2007]. Although the extracts obtained after USAED treatment can be used without further cleaning for total Se quantification by ET-AAS, the use of HPLC requires some preliminary steps to clean up the extracts before injection, namely centrifugation and subsequent filtering. This cleaning could produce some losses of Se compounds. Therefore, we compared the total Se content of certified material SELM-1, after USAED extraction, following two different clean up processes. In one set of experiments the sample was centrifuged, the extract was withdrawn and used to measure the total selenium content without further cleaning. In another set of experiments the sample was passed through a 0.22 µm nylon filter after the centrifugation step. The recoveries (n=3) were 105±7% and 99±9% respectively, thus ensuring that no losses of Se were taking place. These results validated further the accuracy of USAED extraction for total Se determination.

Next, to proceed with the validation of Se speciation, a set of experiments were performed using the reference material SELM-1, which has a total certified amount of Se of 2059±64 $\mu g.g^{-1}$ of which 66% is certified as SeMet. After USAED extraction, the sample was centrifuged, filtered and injected into the HPLC; the fractions were taken at appropriate times and, after dilution, measured in the ET-AAS system. Total Se recovery (n=3) was 80±9%. Concerning the SeMet, the amount recovered was 92±8% of the certified value. A chromatogram of the extract is shown in Figure III.2 along with a typical ET-AAS signal obtained for the fraction containing SeMet. This result confirms the good performance of the combination USAED-HPLC-ET-AAS, for Se control in enriched food supplements. Furthermore, it also confirms that this sample treatment can be used for total Se determination and/or for Se speciation. Moreover, it also demonstrates that ultrasonication does not destroy the SeMet.

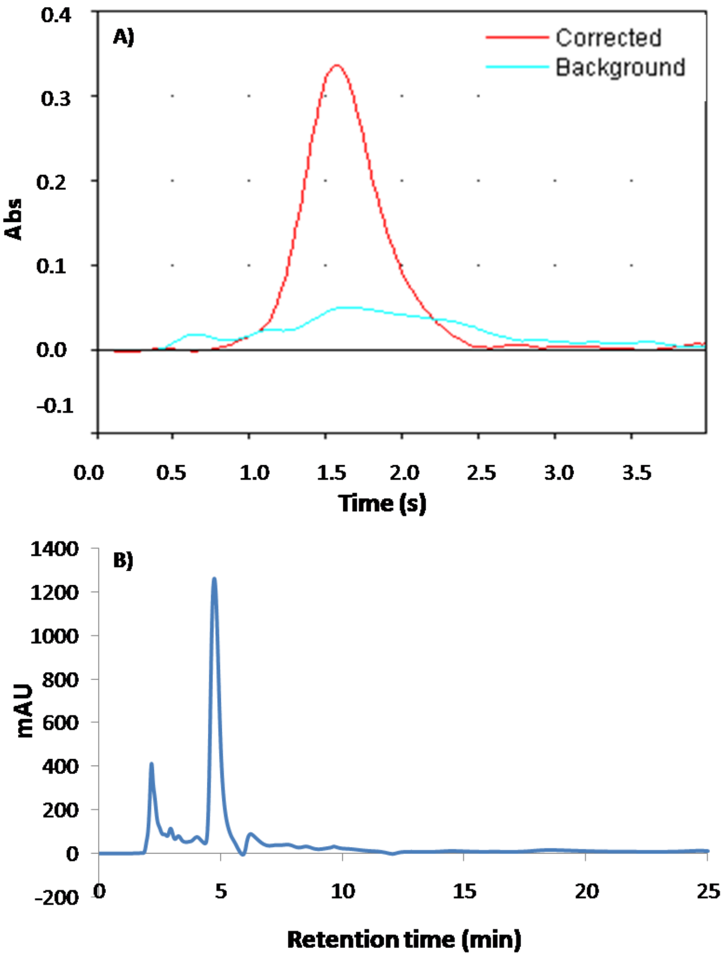

Figure III.2. A) Se ET-AAS signal and B) HPLC-DAD chromatogram for SELM-1 reference material. 10 mg of the sample and 10 mg of Protease XIV were dissolved in 1 mL of ultrapure water. The mixture was ultrasonicated 120 s at 50% ultrasonication amplitude with a 1 mm sonic probe. The sample was centrifuged for three minutes at 4000 rpm and filtered through a 0.22 µm nylon filter. The treated sample was injected in a HPLC equipped with a 50 µL loop and an anionic exchange column. The chromatogram was monitored at 234 nm. ET-AAS conditions: 5 µL of $Pd(NO_3)_2$ (2000 mg kg^{-1}) matrix modifier. 10 µL of sample were introduced into the furnace.

III.4.2.3 - Analytical parameters

The limit of quantification, LOQ, and the limit of detection, LOD, for each Se-species was calculated taking into account their respective LOQ and LOD from the ET-AAS system, the dilutions done for each fraction, the 50 µL loop used in the HPLC and the amount of sample used in the extraction process along with the extracting volume solution. The results were as follows (ng g^{-1}), LOQs: SeCys2, 235;SeMeSeCys, 216; Se(IV), 437; SeMet, 823; Se(VI), 764; LODs: SeCys2, 97; SeMeSeCys, 90; Se(IV), 200; SeMet, 299; Se(VI), 265.

III.4.3. Study cases

To test the applicability of the USAED-HPLC-ET-AAS approach, ten Se enriched dietary food supplements were studied. The characteristics of the supplements given by the manufacturers are shown in Table III.1. According to the manufacturers, eight supplements have organic-Se, S5 has inorganic-Se; information on the form of Se is omitted in sample S4. It must be borne in mind that enzymes help to extract Se-species from matrixes of biological origin. Therefore, it can be anticipated that the extraction will fail in Se supplements prepared from inorganic matrixes.

III.4.4 Total Se content

Table III.3 shows the total Se recovered using the USAED-ET-AAS procedure from the ten Se supplements. Although most manufacturers provide details of the total Se content, we have rejected these values as references since traceability is difficult. Instead, we decided to use pressurized acid microwave digestion to dissolve the supplements as a parallel method of sample treatment to determine the total Se content by ET-AAS. The respective Se concentration thus obtained was considered the value of reference for each supplement. As can be seen in Table III.3, for six of the ten supplements the values obtained for total Se, after sample solubilization through microwave digestion, were in concordance with the ones presented by the manufacturers, within an error below 16%. For samples S1, S3, S5 and S7 although the Se contents were not in agreement with the values declared by the manufacturers, the differences were < 33%.

Regarding total Se extraction using USAED, the values obtained for the majority of the samples were in agreement with those resulting from the microwave digestion procedure. Remarkably, the USAED extraction process completely failed for sample S5, 72±22 µg g^{-1} *versus* 222±28 µg g^{-1}, the latter value found with MW solubilization. Since sample S5 has an inorganic matrix, this result was not a surprise. In conclusion, the USAED methodology was shown to be an appropriate technique for fast and reliable extraction of total Se from enriched food supplements of biological origin, thus confirming the results recently reported on this method of extraction [Vale, Pereira, Mota, Fonseca & Capelo, 2007; Cuderman, Kreft, Germ, Kovacevic, & Stibilj, 2008].

Table III.3. Total Se content determined in 10 Se enriched food supplements using two different methods of sample treatment, a) microwave pressurized acid extraction; b) ultrasonic assisted enzymatic digestion. To compare the two treatments, test *t* was used (t_{table} = 2.920, for 95% confidence level and 2 degree of freedom). If $t_{table} > t_{exp}$ the two values are no significantly different at the confidence level used. Results obtained with a n=3.

Supplements	Bottle labeled Se ($\mu g.g^{-1}$)	Microwave Se ($\mu g.g^{-1}$)	USAED Se ($\mu g.g^{-1}$)	t_{exp}	Expected Se type
S1	103.0	72.5±6.5	75±15	0.27	Organic
S2	585.7	594±28	673±13	4.43	Organic
S3	43.6	30.5±3.5	34.6±1.1	2.25	Organic
S4	14.3	12.5±2.5	15.75±0.35	2.81	Unknown
S5	167.2	222±28	72±22	7.30	Inorganic
S6	38.2	35.5±5.5	40.9±4.3	1.46	Organic
S7	85.2	110.5±8.5	94.0±7.9	2.53	Organic
S8	91.7	89.5±4.5	82.4±5.2	1.19	Organic
S9	109.5	106.5±5.5	97±11	1.44	Organic
S10	22.4	21.65 ± 0.55	24.1±1.5	1.24	Organic

III.4.5. Se speciation

Data on Se speciation is presented in Table III.4. Of the ten supplements studied, Se compounds matching the elution times of our standards were found in five of them. Thus, samples S7, S8 and S9, presented a unique species, SeMet, accounting for the total Se content. Apparently all the Se contained in these particular samples was SeMet. This result agrees with the data provided by the manufacturer. For samples S8 and S9, the major carrier of Se was said to be SeMet whilst S7 was rich in spiruline a free-floating filamentous cyanobacteria that transforms Se into SeMet.

Sample S5 also contained a unique inorganic species, Se(IV) accounting for all the Se extracted, The amount of Se obtained was 69% of the total Se found using USAED extraction but only 23% of the total Se obtained after MW. This means that USAED extraction failed for this inorganic matrix, supporting previous conclusions that USAED is not adequate for Se extraction from inorganic matrices [Vale, Rial-Otero, Mota, Fonseca, & Capelo, Talanta, 2008]. Interestingly, the manufacturer's information about sample S1 ("rich in organic selenium"; "yeast cultivated in Se enriched media") appears fraudulent. We found all the Se content to be Se(IV). For clarification S1 was fortified with the five standards used in this work in a set of new experiments, before USAED extraction. Remarkably, all the species were recovered (recoveries for n=3 comprising

85%-110%, data not shown). Thus, there was no Se species interconversion during ultrasonication and confirming the major species in S1 as Se(IV).

Table III.4 - Results of Se speciation. Concentrations are given in Se (µg g^{-1}, n=3). The total Se, obtained from Se speciation, was compared with the total Se obtained by MW through the t-test (t_{table} = 2.920 for 95% confidence level and 2 degrees of freedom). If $t_{table} > t_{calc}$ the two values are significantly different at the confidence level used.

Se Species	S1	S5	S7	S8	S9
$SeCys_2$	*	*	*	*	*
SeMeSeCys	*	*	*	*	*
Se(IV)	76.0±9.5	50.1±5.1	*	*	*
SeMet	*	*	116±18	77.1±7.2	90.2±6.1
Se(VI)	*	*	*	*	*
Se rec. (%)*	106±13	23.1±2.2	105±16	87.1±8.1	85.2±6.2
t_{exp}	0.56	10.47	0.48	1.88	4.21

* Recovery was calculated as ([Se microwave]/[Se USAED-HPLC-ET-AAS])*100

As for the samples S2, S3, S4, S6 and S10 no Se compounds matching the retention times of our standards were detected. Clearly the Se forms present were different. Samples S2 and S3 are claimed to have the Se associated with a mixture of aminoacids, and no further information was provided. Bearing in mind that we could extract all the Se content in both samples, we speculate that the Se forms present are also different to those in our standards. Regarding S4, this sample is a mixture of inorganic substances with an organic base composed of various plant substrates, such as gin-seng or pepper. This case, another in which no Se forms corresponding to our standards were found, suggests that the organic base may participate in chelation or Se transformation. Such mechanisms may make their identification difficult without a more sophisticated approach, e.g. HPLC-ICP-MS. This hypothesis was further confirmed with two pieces of evidence. Firstly, after sample fortification, all the standards added were recovered in agreement with the expected values, i.e. ultrasonication did not cause species transformation. Secondly, sample 10 was claimed to have yeast as its main Se source. Although we did indeed obtain the total Se content using USAED extraction and ET-AAS, no Se species were found matching any of our five standards, including SeMet. SeMet is the major Se species expected to be found in yeast.

Sample 10 had an extremely complex organic support consisting of thirty varieties of plants and fruits plus ten different inorganic substances. As in previous problematic cases, the sample (S10) was fortified with the pool of standards and submitted to the USAED extraction. The recoveries were again as good as for the other samples (data not shown).

III.5 Conclusions

Joint USAED with HPLC separation and ET-AAS determination of Se is suitable as a new rapid methodology for Se monitoring in Se enriched food supplements. Total Se determination and Se speciation are accomplished in a single sample treatment that does not alter the following Se-species: Se(IV), Se(VI), SeMet, SeMeSeCys, and $SeCys_2$.

Determinations on ten different supplementary materials shows that, although all supplements have total Se contents in agreement with the manufacturers values (within an error of < 30%), this information is almost irrelevant without detailed knowledge of the Se-species present. Although SeMet was expected to be the main Se component in most Se enriched food supplements, it was absent in seven of the ten samples tested. Of these, two of them had Se(IV) as the main Se species. The other five supplements had Se species that did not match any of the five standards employed by us. The manufacturer of supplement S1 confirmed the presence of spirulin, suggesting that the main species should be SeMet. In fact, the Se species found was Se(IV). In summary, the results indicate that more stringent legal controls should be enforced on the manufacturers of Se enriched food supplements to avoid the often unrealistic claims made for their products.

III.6. Ackowledgements

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Chapter IV

Ultrasonic Assisted Enzymatic Digestion (USAED) for Total Elemental Determination

and Elemental Speciation.

A Tutorial

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IV.1. Abstract

Due to its potential as sample treatment for Analytical Chemistry, the ultrasonic assisted enzymatic digestion, USAED, for total elemental determination and elemental speciation is described under the most recent achievements published in literature, focusing in the variables that critically affect the performance of this relatively new sample treatment, such as the type of enzymes or the type of ultrasonic system used for the acceleration of the solid-liquid extraction process. Moreover, analytical chemists are aware of common errors produced in data interpretation concerning USAED. In addition, a guide for the rapid application of this methodology is also provided along with detailed explanations. Finally, future trends regarding USAED are also given and commented.

Keywords: Ultrasonication, Enzymes, Elemental speciation, Elemental determination, Tutorial

My contribution to this work was the bibliographic review, the study of the ultrasonic effects of the different ultrasonic devices on biological tissues and the effect of US and vortex agitation in the recovery of total Se from mussel tissues with Protease XIV.

IV.2. Introduction

The challenges for studies regarding metals and metalloids nowadays are focused on chemical speciation. As an example, Se is supplemented in many diets due to its recognized medical properties [1]. However the same health benefits are not obtained if Se is added in the food as inorganic Se, e.g. Se(VI) or Se(IV), or as organic Se, e.g. Se-methionine.

Chemical speciation is a difficult issue that demands advanced analytical techniques and highly qualified personnel [2]. Therefore, the search for new methods for chemical speciation accomplishing simplicity, robustness and rapidity is an objective of main importance in analytical chemistry.

The use of ultrasounds to speed up enzymatic reactions for chemical speciation was first reported by Capelo et al. in 2004 [3] for Se speciation in yeast. This method is named Ultrasonic Assisted Enzymatic Digestion, USAED, and it meets the requirements of simplicity, robustness and rapidity. Since it was reported for first time the USAED sample treatment has been further applied in different matrixes for chemical speciation of other metals as can be seen in the Table IV.1. In addition, some improvements on the methodology have been also done.

Despite of its simplicity, USAED as sample treatment has been used scarcely since 2004 to date in the field of elemental speciation. In our opinion, the methodology is not known enough among the analytical community. Therefore, the main aim of the present work is to provide a tutorial to teach clearly how USAED must be applied in the analytical laboratory, focusing in to highlight the variables affecting USAED performance, along with its drawbacks and how to overcome them.

IV.3. - Essentials about ultrasonics

When ultrasonic waves cross a liquid solution, different chemical and physical phenomena take place. It is out of the scopes of this review to describe in detail such phenomena. For a complete reading of the effects of ultrasonication for chemical processes, the reading of the books of Professor T. J. Mason, especially the one entitled sonochemistry, are highly recommended [4].

Table IV.1. Literature reporting USAED for total elemental determination and elemental speciation.

	Analytical methodology
(a) [3]. **(b)** Yeast, CRM 710 Oyster tissue and CRM 278 Mussel tissue. **(c)** Protease XIV, 10% w/w **(d)** Total Se and SeMet.	**HPLC-ICP-MS**. Total Se determination and Se speciation. Cationic exchange column Hamilton PRP-X200 (250 x 4.1 mm; 10 µm). Mobile phase: 4 mM pyridine formate solution (pH 2.8) with 3% of methanol at 1 mL min^{-1} in the isocratic mode. Injection volume: 200 µL). USAED procedure: a) for total metal determination: 10 mg of sample + 1 mg protease XIV + 1 mL water + sonication during 5 s at 20 W. The mixture was centrifuged and 20 µL of the supernatant was diluted up to 10 mL with water; b) for metal speciation: 10 mg yeast + 1 mg of protease XIV + 1 mL of water + sonication during 30 s. The mixture was filtered through a 0.45 µm nylon filter. The total Se recoveries found for yeast, oyster and mussel tissue were 105%, 106% and 96%, respectively. In yeast it was found an 87% in SeMet from the total Se.
(a) [14]. **(b)** Spanish rice and Indian basmati. **(c)** 0.33% w/w α-amylase and 1% w/w protease type XIV. **(d)** Total As, As (III), As(V), DMA, MMA	**HPLC-ICP-MS**. Total As determination and As speciation. Anion exchange column Hamilton PRP-X100 (250 x 4.1 mm; 10 µm). Mobile phase: 10 mM $HPO_4^{2-}/H_2PO_4^{-}$ with 2% methanol (pH 6) at 1 mL min^{-1} in the isocratic mode. Injection volume: 100 µL. The analytes were extracted using and UP with a 3 mm titanium microtip. USAED procedure: 1st step: 3 g of sample + 10 mg of α-amylase + 3 mL of water + sonication during 1 min. 2nd step: 30 mg of protease XIV + sonication during 2 min. After sonication, the extracts were centrifuged (4000 rpm, 10 min) and filtered (0.22 µm). For method validation was used the reference material rice SRM 1568a (NIST, USA). The total As recovery was 99.7 ± 0.8%. LODs were 0.05 µg Kg^{-1} for As (III) and 0.2 µg Kg^{-1} for all the other As species.
(a) [15]. **(b)** BCR-414 Plankton, ERM-CE 278 Mussel tissue, oyster tissue. **(c)** Protease XIV, 100% w/w. **(d)** Total Se.	**ET-AAS**. Total Se Determination. The analytes were extracted using and UP with a 1 mm microtip at 50% amplitude. USAED procedure: 10 mg of sample + 10 mg of protease XIV + 1 mL of water or Tris –HCl buffer (pH 7.5) + sonication during 2 min. Matrix modifier $Pd(NO_3)_2$ and H_2O_2. The total Se recoveries found for mussel tissue, plankton and oyster tissue were 93%, 106% and 111% respectively. LOD was 0.6 ng g^{-1} and LOQ was 2 ng g^{-1}.

Table IV.1 (Continued)

	Analytical methodology
(a) [16]. **(b)** Rice and chicken muscle samples. All of them candidate to reference material (European project SEAS-G6RD-CT2001-00473). **(c)** For rice samples: 1% w/w of α-amylase and 10% w/w protease XIV. For chicken samples: 10% w/w of protease XIV. **(d)** Total As, As(III), As(V), AsB, DMA, MMA	**HPLC-ICP-MS**. Total As Determination and As Speciation. The analyte was extracted using and UP with a 3 mm titanium microtip at 30% amplitude. USAED procedure: a) rice sample: 1st step: 0.3 g of rice + 3 mg of α-amylase + 3 mL water + sonication during 1 min; 2nd step: 30 mg ml^{-1} of protease XIV + 2 min sonication time. b) chicken samples: 0.15 g of sample + 15 mg of protease XIV + 3 mL of water + 4 min sonication time. All the extracts were centrifuged (5000 rpm, 10 min) and then filtered (0.22 µm). Anion Chromatography column Hamilton PRP X100 (250 x 4.1 mm; 10 µm). Mobile phase: 10 mM $HPO_4^{2-}/H_2PO_4^{2+}$ with 2% methanol (pH 8.5) at 1.5 mL min^{-1} in the isocratic mode. Injection volume: 100 µL. For method validation the following reference materials were used: rice flour SRM 1568a (NIST, USA). Recoveries: 98-110 % (± 7). LOD: 13-20 ng L^{-1}.
(a) [17]. **(b)** Human Hair. **(c)** Protease XIV and lipase. **(d)** Total As, As(III), As(V), DMA, MMA	**HPLC-ICP-MS**. Total As determination and As speciation. The analyte was extracted using and UP with a 3 mm titanium microtip at 30% of amplitude. USAED procedure: 75 mg of sample + protease and lipase mixture + 3 mL of water + sonication during 10 min. The extracts were centrifuged (5000 rpm, 10 min) and filtered (0.22 µm). Anion Chromatography column Hamilton PRP X100 (250 x 4.1 mm; 10 µm). Mobile phase: 10 mM $HPO_4^{2-}/H_2PO_4^{2+}$ with 2% methanol (pH 8.5) at 1.5 mL min^{-1} in the isocratic mode. Injection mode: 100 µL. For method validation it was used the following reference material: human hair nº 13 (NIES, Japan) and GBW 07601 (GBW, China). Recovery for total Se: 61.6% (± 3.0).

Table IV.1 (Continued)

	Analytical methodology
(a) [18]**. (b)** Rice samples (Chinese rice (Zhenshan 97), Hungarian rice (Risabell) and brown rice sample (Köröstáj-333). **(c)** 0.33% w/w of α-amylase and 10% w/w of protease XIV **(d)** Total As, As(III), As(V), AsC, AsB, DMA, MMA.	**HPLC-ICP-SF-MS**. Total As and As speciation. The analyte was extracted using an UP with a 3 mm microtip. USAED procedure: 1st step: 0.3 g of raw and cooked rice + 10 mg of α-amylase + 3 mL water + sonication during 1 min; 2nd step: 30 mg of protease XIV + 2 min sonication time. The mixture was centrifuged (4000 rpm, 10 min) and then filtered (0.22 µm). Anion Chromatography column Hamilton PRP X100 (250 x 4.1 mm; 10 µm). Mobile phase: $NH_4H_2PO_4$ (pH 5.5 with NH_3) at 1.5 mL min^{-1} in the isocratic mode. Cationic column Suplecosil LC SCX-100 (250 x 4.1 mm; 10 µm). Mobile phase: 25 mM of pyridine (pH 2.7 with formic acid) at 1.5 mL min^{-1} in the isocratic mode. Injection mode: 20 µL. Major As species found were: As(III) (50%); As(V) (25-33%); DMA (8-10%); AsC (6-10%); and AsB (5%) only detected in chinese rice samples. The reference material rice flour sample – NIST SRM 1568a was used for method validation.
(a) [19]. **(b)** Edible seaweed samples **(c)** Trypsin, α-amylase, pepsin 10% w/w enzyme-mass ratio. **(d)** As, Cd, Cu, Cr, Fe, Mn, Ni, Pb, Zn	**ICP-OES**. Multielemental determination. Samples were treated with different types of enzymes: a) with trypsin and α-amylase: 0.2 g of sample + 20 mg of enzyme + 7 mL of PDHP/PHP buffer solution 0.3 M (pH 6 for α-amylase and pH 8 for trypsin) b) with pepsin: 0.2 g of sample + 20 mg of pepsin + 7 mL of 1% (w/v) sodium chloride (pH 1). The mixtures were sonicated at 35 kHz and at 37ºC for 30 min, centrifuged (3000 rpm, 15 min), and the supernatant made up to 10 mL. For method validation it was used the following reference material: focus INAEA-140/TM (INAEA) and sargasso NIES-CRM-09 (NIES).Quantitative recoveries were only reached when using pepsin, while recoveries close to 80% were obtained for the use of α-amylase and trypsin.
(a) [20]. **(b)** plankton, cabbage, oyster tissue, algae. **(c)** Protease XIV, 50% w/w. **(d)** Cd, Pb	**ET-AAS**. Total Cd and Pb determination. The analyte was extracted using and UP with a 3 mm titanium microtip at 50% of amplitude. USAED procedure: 10 mg of sample + 1 mL of water + 5 mg of protease XIV + sonication during 5 min. For method validation it was used the following reference material: BCR-414 plankton, BCR-679 white cabbage, BCR-710 oyster tissue, IAEA-0392, algae. Recoveries between 50 and 100 % for Cd and between 30 and 50 % for Pb.

Table IV.1 (Continued)

	Analytical methodology
(a) [21]**. (b)** Selenium-enriched plants: garlic (*A. Sativum)* and Indian mustard (*B. Juncea*). **(c)** Protease XIV, 25% w/w. **(d)** Total Se, SeMet, SeMetSeCys.	**HPLC-ICP-MS**. Total Se determination and Se speciation. USAED procedure: 0.2 g of sample + 2.5 mL of water + 50 mg of protease XIV + sonication during 3 min. The mixture was centrifuged (2000 rpm, 20 min), filtered (0.45 µm) and then passed through a 3000 Da ultracentrifugation membrane in order to remove the excess of protease. Ion pairing reversed phase column C8 (250 x 2.0 mm; 5 µm). Mobile phase: 0.2% (v/v) of heptafluorobutyric acid, 10% (v/v) MeOH (pH 2.5) at 0.2 mL min^{-1}. Size exclusion column Shodex Asahipack GS-220HQ (300 x 7.6 mm; > 3000 Da). Mobile phase: 10 mM ammonium acetate Buffer (pH 6.5) at 0.6 mL min^{-1}. For method validation it was used an aquatic plant BCR-670 reference material. Recoveries: 103-127 %.
(a) [22]. **(b)** Antartic Krill. **(c)** Pronase E, 5% w/w. **(d)** Total Se and SeMet.	**HPLC-ICP-MS**. Total Se determination and Se speciation. The analytes were extracted using and UP with a 3 mm microtip at 20 W of power, 20 kHz of frequency and 50% of amplitude. USAED procedure: 0.4 g of lyophilised krill + 20 mg of pronase E + 10 mL of 20 mM Tris-HCl (pH 7.5) + sonication during 15 minutes on ice. Reverse-phase column C18 Synergi Hydro (1 x 150 mm; 4 µm). Mobile phase: 0.1% trifluoroacetic acid/methanol (98:2 v/v) at 0.15 mL min^{-1} in the isocratic mode. Injection volume: 5 µL. Recovery of SeMet: 99 %
(a) [23]**. (b)** Chicken muscle, liver, kidney and chicken feed. **(c)** Protease XIV, 20% w/w. **(d)** Total Se and SeMet	**HPLC-ICP-MS**. Total Se determination and Se speciation. The analytes were extracted using and UP with a 3 mm titanium microtip at 20 W power and 10% amplitude. USAED procedure: chicken muscle (0.15 g), chicken liver (0.1 g), chicken kidney (0.05 g) + 20% w/w protease XIV + 3 mL Tris-HCl buffer (pH 7.5) + sonication during 2 min. After sonication the extracts were centrifuged (4000 rpm, 20 min). Cationic column Hamilton PRPX-200 (250 x 4.1 mm; 10 µm). Mobile phase: 4 mM pyridine formiate in water, 3% methanol at 1 mL min^{-1} in the isocratic mode. Injection mode: 100 µL. For method validation it was used as reference material mussel tissue CRM 278. Recoveries: 93-102%. LOD = 2.4 µg Kg^{-1}; LOQ = 8.1 µg Kg^{-1}.

Table IV.1 (Continued)

	Analytical methodology
(a) [29]. **(b)** selenium-enriched lentil plants (*Lens esculenta*). **(c)** Protease XIV. 40% w/w . **(d)** Total Se, Se(IV), Se(VI), SeMetO, SeMetSeCys, SeMet	**HPLC-ICP-MS**. Total Se determination and Se speciation. The analyte was extracted using and UP with a 3 mm titanium microtip at 20 W power and 20 kHz frequency. USAED procedure: 25 mg of sample + 10 mg of protease XIV + 3 mL of water + sonication during 2 minutes. The mixture was centrifuged (7500 g, 30 min) using a 10 kDa cut-off filter. Anion exchange column PRP-X100. Mobile phase: 10 mM citric acid solution in 2% (v/v) methanol (pH 5 adjusted with ammonium hydroxide) at 1 mL min^{-1}. Size exclusion/ion exchange column Shodex Asahipacks Gs-220HQ. Mobile phase: 25 mM ammonium acetate solution (pH 6.7) at 0.6 mL min^{-1}. Biosep-SEC-2000 protein separation column (300 to 1 kDa). Mobile phase: 25 mM Tris-HCl buffer (pH 6.8) at 1 mL min^{-1}. Injection volume: 100 and 200 µL). For method validation it was used as reference material white clover CRM-402.

(a) Reference; (b) matrix; (c) enzyme type and enzyme-sample ratio; (d) metal species

Briefly, the most important properties caused by ultrasonic waves crossing through a liquid medium come from a physical phenomenon called cavitation. Cavitation refers to a process by which numerous micro-gas bubbles are formed, as the ultrasonic wave compress and decompress the liquid media as it passes through it. These bubbles can grow, oscillate, split and implode, causing the following effects:

- Temperatures of ca. 5000ºC;
- Pressures of ca. 1000 atm;
- Formation of highly reactive chemical radicals;
- Mechanical erosion of solids (if present in solution) and particle disruption.

The former properties of ultrasonic energy make it a powerful tool in analytical chemistry, as demonstrated by the increasing number of analytical chemistry applications published during the last decade [5]. Some reviews on analytical uses of ultrasonication can be found in recent literature [5-11], focusing in the following items:

- Solid-liquid extraction of metals, metalloids and organics from solid samples;
- Elemental speciation;
- Shortening of sequential extraction schemes;

- Acceleration of liquid-liquid extraction processes;
- Acceleration of solid-liquid extraction of organics;
- Electroanalytical applications.

It must be pointed out that nowadays there are many types of ultrasonic devices [6]. For instance, ultrasonic probes, ultrasonic baths, sonoreactors, and cup horns, are ultrasonic systems that can be used for sample treatment in analytical chemistry. Nevertheless, a minimum knowledge of their properties is required otherwise unexpected effects can be obtained. As an example, the intensity of sonication, which ultimately dictates the properties of cavitation, is considerable different among the above mentioned systems. Additional examples are given below:

Ultrasonic bath cannot boost enzymatic kinetics in the same way as the ultrasonic probe or sonoreactor does. The capabilities of the aforementioned apparatus to speed the enzymatic reactions follows the same order that their respective intensities of sonication: ultrasonic probe > sonoreactor > ultrasonic bath.

Considering the sample container as 1 mL eppendorf cups, the number of samples that can be treated at once is by large higher for the ultrasonic bath, the number of samples depending on its size: for a 3 L ultrasonic bath, this number is 120. The sonoreactor allows ultrasonication of at least six eppendorf cups at the same time whilst the ultrasonic probe allows only one at a time.

The possibility of destruction of compounds of interest caused by cavitation depends on the sonication device, and it follows the same order than the sonication intensity as described above in (i): the higher the intensity the higher the risk of analyte transformation.

It is necessary to stress that recent advances in technology can help to overcome some of the common drawbacks above addressed for sample treatments using ultrasonication. As an example, ultrasonication with ultrasonic probe can be nowadays done with the so-called multiple probes, allowing ultrasonication of at least 4 samples at the same time. However, those new ultrasonic devices are not very common in the analytical labs yet. A detailed review regarding new ultrasonic devices, their advantages and limitations, and their present and future potential applications for analytical chemistry, has been recently published and is highly recommended [6]. Table IV.2 contains a list of available ultrasonic devices recommended to speed elemental speciation using enzymes along with their main characteristics and suppliers. (Disclaimer: specific company, product, and equipment names are given to provide useful information. Their mention does not imply recommendation or endorsement by the authors).

Table IV.2. Characteristics of the main ultrasonic systems used for sample treatment for analytical chemistry.

Variable	Probe	Bath	Sonoreactor
Sonication time	< 5 min	> 30 min	< 5 min
Thermostat	No	Yes	no
Intensity of sonication for 1.5 mL vial (W)	15	0.01	0.5
Amplitude	Variable	Variable	variable
Solid-Liquid extraction yield	High	low	medium
Destruction of organics	High	Low	medium
Sample throughput	low	High	medium
Accessories	High	Low	medium
On-line applications	Yes	yes	yes
Suppliers	(1) www.bandelin.com; (2) www.hielscher.com; (3) www.bransonultrasonics.com; (4) www.equilabcanada.com. (5) www.misonix.com, (6) www.elmaultrasonic.com		

IV.4. Ultrasound and enzymes for elemental speciation

One of the most remarkable advances in the uses of ultrasonic energy for sample treatment for analytical chemistry was reported in 2004, when Capelo et al. demonstrated that by joining enzymes and ultrasonication provided by an ultrasonic probe, total selenium could be extracted from biological matrixes in times as short as 30 s, maintaining the Se-bounded species integrity. Previous time-consuming protocols for Se speciation used to need times as long as 12 h. The methodology proposed was fast and of easy implementation, saving time and money [3].

Although enzymes were previously reported in literature as a tool for chemical speciation, never before the enzymatic reaction kinetics had been accelerated by means of an ultrasonic probe [8] for this objective. Formerly works done with enzymes for chemical speciation purposes used ultrasonic baths or heating baths at 37 ºC for improving enzymatic reactions. Sample treatment times were comprised between 5 to 12 h [8].

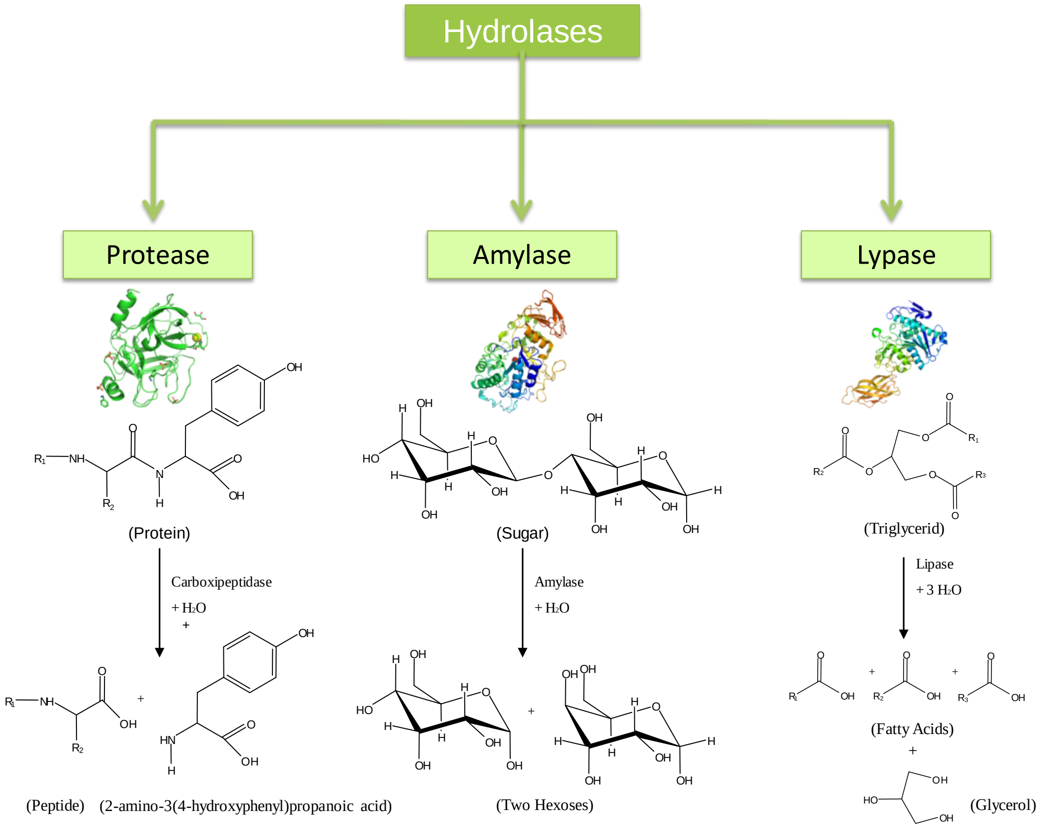

Figure IV.1 - Typical reaction of hydrolases.

The enzymes used for chemical speciation belongs to the family of hydrolases [8]. Hydrolases act at specific bonds of the substrate, breaking molecules through catalyzing the introduction of water. Figure IV.1 shows schematically how hydrolases work. In the following sections detailed information is given to explain how to take advantage of USAED for elemental speciation purposes.

IV.5. Does ultrasonication enhance enzymatic activity?

Studies regarding enhancement of enzymatic kinetics by ultrasonication have been reported in dedicated literature to this task. Results put forward a dependence of the enzymatic enhancement with the intensity of sonication rather than with frequency of sonication [12]. Apparently, cavitation promotes the increment in reaction rates rather than a change in the reaction constants. As an example, Talukder et al reported an increment in the maximum reaction rate (V_{max}) and unalteration for the Michaelis constant (K_m) when the hydrolytic activity of lipase was enhanced by ultrasonication [13]. It has been also stated that ultrasonication in short times enhances enzymatic activity whilst long times lead to the inactivation of the enzymes [13].

Concerning studies focused on elemental speciation, most works done to date highlight the efficiency of the extraction process when enzymes and ultrasonication are applied together in short times, generally lower than 4 min, as can be seen in Table IV.1. At this point some recommendations must be given to avoid erroneous data interpretation. Thus, when studying the efficiency of USAED versus ultrasonication time, it is critical to stop the enzymatic activity after the ultrasonication process. It must be borne in mind that, although the ultrasonication had been stopped, the enzyme can still be active. This problem can be understood better by looking at data provided in Table IV.3. As can be seen, different sample treatments were done using protease XIV over mussel tissue for the extraction of total Se. Measurements of Se were done by electrothermal atomic absorption spectrometry (ET-AAS).

The extraction experiments were done with and without lowering the pH with formic acid to stop the enzymatic activity after ultrasonic treatment. As can be seen, when the USAED was done by joining the enzymes and the ultrasound in one single step, experiment number 1, the extraction efficiency was ca. 18% higher than when the enzymatic activity was stopped immediately after the ultrasonic treatment ended. It must be taking into account that the Se measurements were done at least 30 minutes after the ultrasonic treatment was finished. This is, the enzyme had 30 min more of activity, degrading further the sample and thus, increasing the ratio of metal extracted. This finding is critical to obtain accurate results. Therefore, some studies reported in literature, regarding enzymatic elemental extractions accelerated by ultrasound versus time of sonication, must be considered as inconclusive, since the enzymatic activity was not stopped after the ultrasonic process [14].

Table IV.3. Effect of ultrasound and vortex agitation in the recovery of total Se from mussel tissue with protease XIV.

Experiment number; (lasting time until the first measurement was done by ET-AAS, min)	**Without stopping the enzyme activity (X ± SD, n=3)***	**Stopping the enzyme activity (X ± SD, n=3)***
1 (120)	100.6 ± 2.7	81.9 ± 5.0
2 (60)	48.9 ± 6.0	39.2 ± 2.6
3 (25)	17.3 ± 2.7	25.4 ± 2.0
4 (110)	25.3 ± 8.0	25.3 ± 8.5
5 (35)	34.6 ± 1.5	44.2 ± 11.1

Measurements of Se were done by ET-AAS with and without stopping enzyme activity with formic acid 5% v/v. (1) Mussel tissue and protease XIV in water. Sonication time 2 minutes. (2)

Mussel tissue and protease XIV in water. Vortex agitation during 2 minutes. (3) Mussel tissue sonication during 2 minutes in water. Then protease XIV was added and mixed in a vortex during 2 minutes. (4) Vortex agitation of the mussel tissue in water during 2 minutes. Then protease XIV was added and mixed in vortex during 2 minutes. (5) Vortex mixing of mussel tissue and protease XIV during 4 minutes.

* % of Se recovery

Table IV.3 also shows that when the mussel tissue was ultrasonicated during 2 min before the addition of protease XIV, the Se extracted was only ca. 20-25 % (experiment 3). In addition, the results obtained with vortex mixing (experiment 4) were similar to the ones get it when the enzymes and ultrasonication were applied to the solution separately, showing that to obtain boosting of enzymatic activities for elemental extraction in minutes, ultrasonication and enzymes must be jointed.

Other remarkable conclusion is that, although some studies focusing on enzyme inactivation caused by ultrasonication have reported inactivation of protease XIV after 2 min of treatment [15], this inactivation seems to be linked to the substrate being studied rather to the enzyme itself. Thus, ultrasonic inactivation of enzyme protease XIV towards casein substrate was obtained in two minutes [15]. However, for mussel tissue, after two minutes sonication time, protease XIV was still active, extracting ca. 18% more of Se than when the enzyme was inactivated with formic acid after the 2 min ultrasonic treatment, as stated above (experiment 1 in Table 3).

IV.6. Factor affecting USAED efficiency

IV.6.1. Correlation between samples and enzymes

The first key to attain a good result using the methodology we are describing deals with the correct enzyme choice. The family of hydrolytic enzymes comprises the following: lipases, amylases and proteases. Theoretically, each type of enzyme hydrolyses a different kind of substrate. Lipases hydrolyze fats; amylases hydrolyze starch and glycogen, and proteases proteins and peptides. Therefore, it can be understood that there is no enzyme for universal use. However, it must be stressed that even for the same family of enzymes, some differences in the extraction efficiency can be expected. Thus, three proteases were studied in conjunction with ultrasonication with probe for the extraction of Se from mussel tissue. Under the same conditions of sample treatment, the Se extractions were as follows: (i) 93 ± 7% for protease XIV; (ii) 70 ± 9% for subtilisin A and (iii) 28 ± 5% for trypsin [15].

Some authors have reported the use of enzyme cocktails to improve the extraction of elements from plants or animal tissues. Thus, Sanz et al. have described As speciation in rice and hair using (α-amylase + protease type XIV) and (protease XIV + lipase) respectively [16, 17]. Later, Mihuez et al have validated the As speciation in rice using the same enzyme cocktail reported by Sanz et al. [18].

Regarding total metal extraction, Cd, As, Cu, Cr, Fe, Mn, Ni, Pb, and Zn were determined in Atlantic seaweed Dulse by Inductively Coupled Plasma with Optical Emission Spectroscopy (ICP-OES) with the extraction done in a medium containing trypsine, α-amylase and pepsine [19]. Table IV.1 shows a detailed description of the works done to date concerning USAED and metal extraction/speciation.

IV.6.2. pH and temperature

The activity of most enzymes is pH and temperature dependent; therefore these parameters must be always taking into account. Regarding pH, information available to date in literature suggests that enzymatic kinetics, for those enzymes having their optimum pH activities around 7, can be boosted with ultrasonication even if the extracting solution is not buffered. This finding, which was first reported in the pioneer work and related ones of Capelo et al, [3, 15, 20] and later by further authors [14, 16, 17, 18, 21, 29], is very important since facilitates chromatographic separations and it helps to avoid contaminations caused by buffer solutions. For some matrixes, however, buffering the medium has been shown critical to attain total elemental recovery, as such it was the case reported by Cabanero et al., regarding Se speciation in chicken samples [23]. For the latter matrix, the Se extraction was 28% higher when the extraction was done in buffered than in non-buffered media with protease XIV at pH 7.5. Therefore, in order to access the extraction efficiencies, to compare trials done in buffered and non-buffered media are recommended for new matrixes.

Some enzymes require low pH to work properly, as an example; pepsine was used at pH 1 to extract As, Cd, Cu, Cr, Fe, Mn, Ni, Pb, and Zn from Atlantic Seaweed Dulse [19]. Concerning temperature, equilibrium between the optimum one for an efficient enzyme activity and the optimum for a good cavitation to occur must be obtained. Although heating in excess should be avoided to obtain good ultrasonic performance, the best temperature for some enzymes is around 37ºC or even higher. Some experiences has shown that doing USAED under cooling conditions lead to lower recoveries than if doing it at room temperature. Thus, to study the effect of temperature on Se recovery from mussel tissue, the USAED procedure was done under cooling conditions (e.g. ice bath) and at room temperature [15]. Interestingly, the recoveries were approximately ca. 20% lower when the procedure was performed under cooling conditions. As a general role a continuous sonication time at room temperature for short times, 2-3 min, is recommended for USAED treatment.

IV.6.3. Ratio substrate/enzyme

The substrate to enzyme ratio is one of the most important parameters to be optimized. This ratio needs to be high enough to guarantee total sample degradation maintaining the compromise of not to be expensive. It must be borne in mind that enzymes are expensive reagents. Data regarding substrate/enzyme ratios is not consistent through the published literature. However, it seems that to obtain accurate results low ratios must be avoided. Thus, Cabanero et al. [23] have shown that doubling the amount of enzyme used, the Se recoveries from chicken muscle, liver and kidney were increased from 86% to 97%; 84% to 93% and 81% to 95%, respectively. Table IV.1 provides a rapid guide to the different ratios substrate/enzyme as a function of the matrix reported in literature to date. It is strongly recommended to the analyst to do a study of metal extraction as a function of the amount of enzyme used when the USAED is applied for first time in a new matrix with two main aims: (i) to establish the amount of enzyme that is necessary for an accurate total extraction, and (ii) to avoid the use of an excess of enzymes. Due to that, besides of saving money, as the amount of enzyme increases for a constant amount of substrate, the amount of organic matter co-extracted to the solution as a result of the ultrasonication process. It is important to remind that high intensity focused ultrasound solubilizates organic matter from the matrixes studied. High organic matter content in solution can negatively affect the signal of numerous analytical techniques. For example, when the amount of enzyme protease XIV was double, from 10 mg to 20 mg for a constant amount of 10 mg of mussel, the Se extracted was a 20% lower. Rather that a lack in extractability caused by the increment in the amount of enzyme used, the problem was related with the system of measurement employed, Electrothermal Atomization. This technique is highly sensible to the amounts of organic matter present in the sample introduced in the graphite tube [15].

IV.6.4. Cleaning procedures

As it was explained in the previous section, high in-solution organic matter contents caused by ultrasonic-based sample treatments on samples such as biological tissues, is a drawback for analytical techniques such as Cold Vapour Atomic Absorption Spectrometry, CV-AAS; Hydride Generation Atomic Absorption Spectrometry, HG-AAS; Electrothermal Atomic Absorption Spectrometry, ET-AAS; or High Performance Liquid Chromatography Inductively Coupled Plasma Mass Spectrometry, HPLC-ICP-MS. To avoid this problem, three main strategies can be performed:

- Sedimentation;
- Centrifugation;
- Filtration.

Sample sedimentation occurs as soon as the ultrasonication ends. When the element is associated to the low fraction mass, the sample can stand for hours, even 24 h, to be analyzed, without problems related with the settling down of the element under study. However, this works only for some elements. In addition, for some analytical techniques, the amount of organic matter remaining in solution stills a problem. In fact, this approach is only recommended for ET-AAS, which can be used for samples with high organic matter content. ICP-Optical Emision Spectrometry, ICP-OES; and Flame-AAS, F-AAS; are analytical techniques which also allow low amounts of organic matter in solution without compromising the analytical measurement [19].

Centrifugation is a rapid approach that can expand USAED to some analytical techniques by lowering the amount of organic matter present into solution [19], however, it has been demonstrated that for some elements, such as Cd, the concentration obtained after USAED is dependent of the speed of centrifugation [20].

The methodology most used for cleaning purposes after USAED treatment is filtration after centrifugation at low velocity, normally 5000 rpm. This is the cleaning procedure that we recommend. It must be stressed that when speciation is the main objective, HPLC will be used, so far, the sample must have the lower level of non desired compounds possible [3, 18]. The latter task is only achieved with centrifugation plus filtration. The cut-off of the filters commonly used is 0.22 µm although some authors have used 0.45 µm filters after membrane ultracentrifugation [21]. Filters are made of nylon.

Since USAED is a relatively novel technique, it is recommended to obtain always information regarding the total content of the element(s) under study in the target sample by some classic analytical sample treatment such as sample dissolution by microwave pressurized acid digestion. This is because, after all, the cleaning procedure can retain the element(s) in certain degree. Therefore, the sum of the different elemental species must be always compared with the total elemental content in order to asses the efficiency of the USAED treatment.

IV.6.5. Enzyme ageing

Enzyme ageing is a parameter in USAED recently highlighted in literature [15]. It was demonstrated that the extraction efficiency of protease XIV for the extraction of Se from mussel tissue felt down a 20% after 3 months [15]. It is recommended to purchase the enzymes in amounts as low as possible to avoid storage, and once the enzyme container is opened, the enzyme must be used as soon as possible. The use of enzymes stored for long time lead to inconsistent results [15].

IV.6.6. Type of ultrasonication device

Although it is not well understood the phenomenon by which ultrasonication accelerates the enzymatic kinetics, some authors have pointed out that there is an increased contact area between phases caused by cavitation allowing a reduction of mass-transfer limitations in the enzyme-substrate system. In addition, the reduction of the sample size caused by high intensity focused ultrasound (i.e. particle disruption), allows more substrate area to be in contact with the enzyme per second of sample treatment [7, 22]. Furthermore, the enhancement reaction rates caused by ultrasonication have been attributed to the increase in collisions between enzyme and substrate [24].

As recently pointed out [6], the different sonication devices available in the market nowadays lead to different results when are used for USAED applications. This phenomenon can be linked with the difference in the intensities of ultrasonication, which leads to different ultrasonic cavitation efficiencies. Ultrasonic baths can not boost the enzymatic kinetics in short times (2-4 minutes), so far are not recommended for USAED. Sonoreactor and ultrasonic probes are the devices of choice for a correct USAED application [6].

It was previously explained that different ultrasonic devices can cause different effects in the enzyme/substrate kinetics. To illustrate this problem Figure IV.2 shows microscopy photos of mussel sample, 20 mg, plus protease XIV, 10 mg, after 2 min of sample treatment with (a) vortex agitation, (b) ultrasonic bath, (c) sonoreactor, (d) ultrasonic probe and (e) with ultrasonic probe only (no enzyme added). The ratio protein/enzyme selected was 2 in order to show the sonication effects more clearly. As it can be seen, the sample size is reduced accordingly with the intensity of the sonicator device as follows: ultrasonic probe > sonoreactor > ultrasonic bath. In addition, it is clearly shown the synergic effect of the combination between enzymes and ultrasound: when no enzymes are used, Figure IV.2 e), the reduction of the sample size produced by the ultrasonic probe is considerably lower than when enzymes and ultrasounds are jointed, Figure IV.2 d).

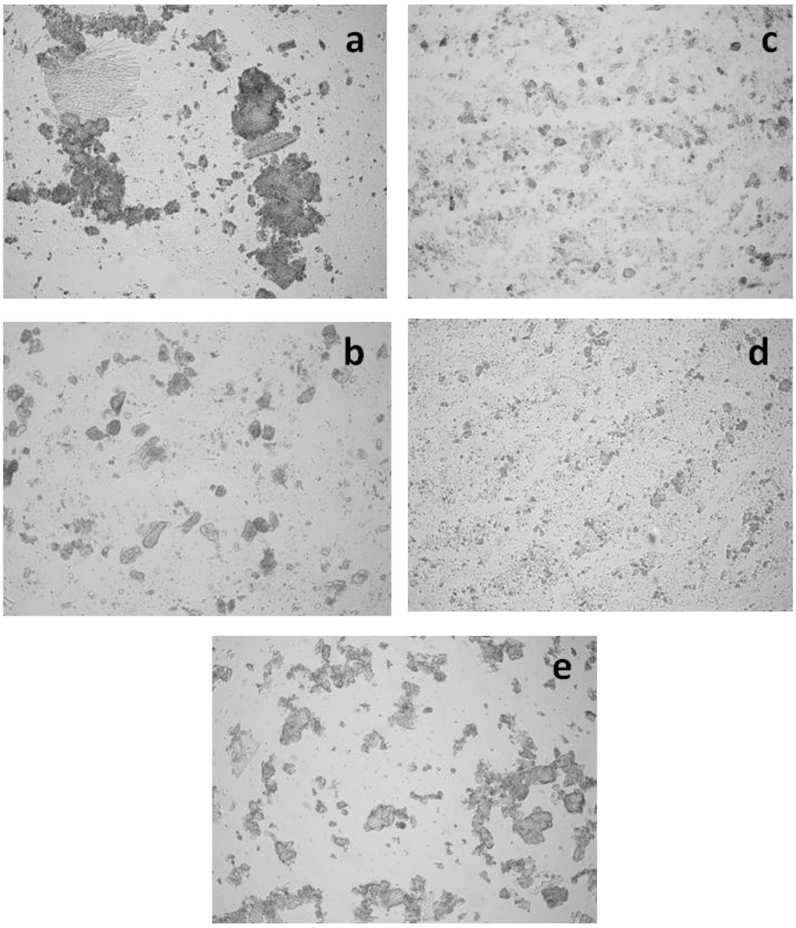

Figure IV.2.-Microscopy photo of 20 mg of mussel tissue plus 10 mg of enzyme after sample treatment with: (a) vortex mixing, (b) ultrasonic bath, 35 kHz, 100% sonication amplitude, 2 min sonication time; (c) sonoreactor, 35 kHz, 100% sonication amplitude, 2 min sonication time; (d) ultrasonic probe, 20 kHz, 50% sonication time, 2 min sonication time; (e) ultrasonic probe, 20 kHz, 50% sonication time, 2 min sonication time, no enzyme was added.

IV.6.7. Ultrasonication time

As a general role (Table IV.1) times lower than 4 min. are enough to obtain total elemental extraction. However, Sanz et al reported to require a sonication time of 10 min to extract As from human hair [17]. In addition, Siwek et al. needed sonication times of 15 min to extract Se from Antarctic krill with Pronase E [22]. Although Penal-Farfal et al reported a time of ultrasonication of 30 min [19], this can be considered normal as the ultrasonic device used was an ultrasonic bath, which usually takes longer times than the ultrasonic probe.

IV.6.8. Ultrasonication amplitude

As a trend (Table IV.2), for a common ultrasonic probe of 50 or 100 W of power, amplitudes comprised between 25-50% are enough to attain reliable results, whilst for ultrasonic probes of high power, such as 200 W, 250 W or even 600 W, amplitudes of 10-20% are recommended. For the latter case, higher amplitudes should not be used, since they are not necessary to boost the enzymatic kinetics and the sonication tip is keep away of damage caused by the excessive mechanical stress promoted by high ultrasonication amplitudes. In addition, excess sample heating is avoided when low ultrasonic amplitudes are required to boost enzymatic reactions.

IV.6.9. Type of container

USAED must be performed, as all solid-liquid extraction procedures using ultrasonication, in conical-bottom shapes vessels [6]. While any type of vessel can be used to hold the sample, the shape of the vessel is often determined primarily by the volume to be processed. For small volumes the smallest diameter vessel that allows the probe to be inserted without risk of touching the sides of the vessel must be chosen. The minimum diameter raises the height of the liquid sample, thus the probe can be inserted more deeply into the process sample. In addition, the called dead zones, where no cavitation is actually done, are diminished. Furthermore, lowering the probe into the solution avoids aerosoling and foaming since both generally occur when the probe tip is not immersed deep enough into the solution. Aerosoling and foaming have the effect of "de-coupling" the probe from the process sample.

IV.7. Joining ultrasonication and enzymes for elemental speciation

Two different ways of joint enzymes and ultrasound have been described to date in literature for elemental speciation. On one hand, one enzyme or one cocktail of enzymes are mixed with the substrate and then the mixture is ultrasonicated in one single step for a time comprised between 2 or 4 min. On the other hand, for some type of samples a multi-step enzymatic digestion is the only way to obtain accurate results. Let us see how this works. For a botanical sample, the enzyme amylase is firstly used to break the wall of the cells, made mainly of starch and glycogen. Through this first step the protein content of the sample is released to the liquid media, then the speciation is completed is a second step, in which a different type of enzymes, a protease, is used to digest the proteins releasing by this way the elemental species associated to the proteins [18].

IV.8. Elemental determination

IV.8.1. Total elemental determination

It must be stressed that USAED is a methodology that extracts a high amount of organic matter from biological samples due to the properties of ultrasonication, as it was described in section 5, therefore this methodology can not be used with the majority of the analytical techniques of measurement without a previous cleaning procedure that diminish the organic content of the extract. Nevetheless, the cleaning step may lead to diminish the metal content in the extract.

USAED has been used to date for the total elemental determination of the following elements: As, Se, Cd, Cu, Cr, Fe, Mn, Ni, Pb, and Zn, as shown in Table IV.1. The matrixes in which the USAED methodology has been successfully applied for metal extraction include material from botanical and animal origin, including both marine and terrestrial. Nevertheless, more data need to be reported by more research groups for further validation of the USAED methodology. Furthermore, the methodology needs to be expanded to more metals and more matrixes.

Regarding instrumental techniques used to determine total metal content, ET-AAS and ICP-OES or ICP-MS are the ones reported by now in conjunction with USAED. As far as ET-AAS concerns, it is well known that the higher the organic matter content introduced into the graphite tube, the higher the possibilities to loose an element during the pyrolisis and or atomization stages. Additionally, chemical interferences in the atomization step can take place as a result of the incomplete degradation of organic matter during the pyrolisis step. The faster and easier way to avoid high organic matter contents after USAED, is by using centrifugation, always that the metal or metals under study do not settle down during centrifugation. This is the recommended cleaning procedure when working with ET-AAS and USAED. If centrifugation must be avoided, such as the case for Pb in oyster tissue [20], then it is recommended the use of robust matrix modifiers, such as palladium nitrate, in conjunction with hydrogen peroxide. The latter reagent helps to obtain better organic matter degradation inside the graphite tube. In addition, the life time of the tube is enlarged twice [15].

Concerning ICP-OES, to the best of our knowledge, this technique for elemental determination has been reported in a sample treatment in which enzymatic degradation of mussel tissue was done with an ultrasonic bath. The elements were measured by ICP-OES (axial configuration). A lack in accuracy for some elements was reported when using scandium as internal standard, probably due the organic content of the extracts, since the cleaning procedure was centrifugation at low speed, 3000 rpm. The problem was overcome through using the standard addition technique [6, 19, 25].

As far as ICP-MS concerns, this technique is the most widely used, since allows rapid total elemental determination and, in conjunction with HPLC, fast elemental speciation. Since this technique is very influenced by the chemical and physical conditions of the sample, the cleaning procedures is more elaborated than for the above mentioned techniques for metal determination. Generally, centrifugation during 10 min at 4000-5000 rpm followed of filtration through 0.22 µm nylon filters [17, 18].

Finally it must be remarked that USAED should not be used only for total elemental determination. For this purpose this technique is economically non competitive with the ultrasonic assisted acid solid-liquid extraction methodology, which already has proven to be a powerful tool [26]. As it has been pointed out previously, USAED is a useful sample treatment for speciation purposes, but in addition, the total content can be also determined, helping in this way to close the element mass balance and avoiding extensive sample treatments, one for speciation and another one for total content [15].

IV.8.2. Elemental speciation

The main challenge for any sample treatment aiming elemental speciation is to extract all the metal content maintaining the chemical forms in which the analyte is present in the sample. To the latter requirements it must be added, when possible, the characteristics of analytical minimalism, as defined by Halls [27]:

(1) low cost;

(2) low sample requirements;

(3) low reagent consumption; and

(4) low waste production.

All the items highlighted above are meet by USAED as described by Bermejo et al [8]. Saving time and improving sample handling are two additional advantages of the sample treatment we are dealing with.

USAED has been used to date for the elemental speciation of As and Se. It is mandatory to spread this methodology to other metals of environmental and healthy concern such as Cr, Zn, Sn or Li. Furthermore, some controversial results has been reported in literature dealing with elemental speciation and ultrasonication, and further data reported by the research community is needed urgently to get unambiguously interpretation of the robustness of USAED.

It must be remembered that to prove the suitability of the USAED method for speciation studies, it is necessary to ensure species integrity during the whole extraction/separation/detection process. Some authors have stressed that matching retention times of standards in a single chromatographic method should not be used as conclusive evidence of the presence of a certain species in the sample. Therefore, samples should be run through two different chromatographic columns with different retention mechanism [21].

The chemical forms of As that have been reported after USAED sample treatment in rice [14, 16, 18] and human hair [17] are As(III), As(V), arsenochlorine (AsC), arsenobetaine (AsB), dimethyl-arsonic acid (DMA), monomethyl arsonic acid (MMA), and As(III), As(V), DMA, MMA respectively. It must be emphasized that no As-species inter-conversion was claimed to occur by the authors. However, it has been reported DMA and MMA degradation in human urine [28], after joint ultrasonication with a solution containing $KMnO_4$/HCl. Thus, it is expected in the near future dedicated studies focused on As-species stability under USAED conditions.

Regarding Se speciation, most data available in literature concerning USAED are dedicated to this metal, which has been studied in yeast [3], garlic [21], antartic krill [22], chicken [23], and lentil [29]. Selenomethionine (SeMet) was found in yeast; seleno methyl selenocysteine (SeMetSeCys) and SeMet were found in garlic samples; SeMet in chicken and antartic krill; and Se (IV), Se (VI), selenomethionine Se-oxide (SeMetO), SeMetSeCys, and SeMet were found in lentil. It has been stressed the stability of selenium species under the effects of an ultrasonic field provided by an ultrasonic probe, so neither species degradation nor species inter-conversion was described for this metal. However, Pedrero et al. using USAED, have pointed out that Se speciation corresponding to stem and roots from plants grown in presence of selenite and selenate Se(VI), and done by anion exchange chromatography, was not able to discriminate selenocystine ($SeCys_2$) from SeMetO, both co-eluting at a retention time of 2.1 min. It was mandatory the use of a second chromatography column combining ion-exchange and size-exclusion mechanisms for unambiguous species identification [29].

IV.9. A guide for USAED application

In Figure IV.3 it is shown a comprehensive scheme for USAED application.

The sample mass used should be comprised between 10 and 50 mg. Sample mass depends mainly on the limit of detection of the analytical technique, on the target analyte concentration in the sample and on sample homogeneity. Sample size should be as low as possible in order to increase the total area in contact with the enzyme. A comparison of the amount of metal extracted for buffered and no buffered aqueous solutions should be done always. If buffered solutions are required to obtain accurate results, the type of buffer used must be chosen taking into account the subsequent speciation process thought HPLC. The first attempt for total elemental extraction with the USAED methodology must be always compared with a classic method for total elemental extraction.

When the use of enzymes for elemental extraction gives low recoveries, some changes can be done to the sample treatment to try to increase the levels of metals extracted. Thus, the time of sonication or the amount of enzyme can be increased. Always plot a curve of % of metal extracted *versus* amount of enzyme added for a fixed amount of sample. Other approaches include to choose another type of enzyme or to try an enzyme cocktail. If the metal extracted is still low, the analytical criteria of the chemist must be used to decide if the amount of metal extracted is high enough to justify further research regarding chemical speciation.

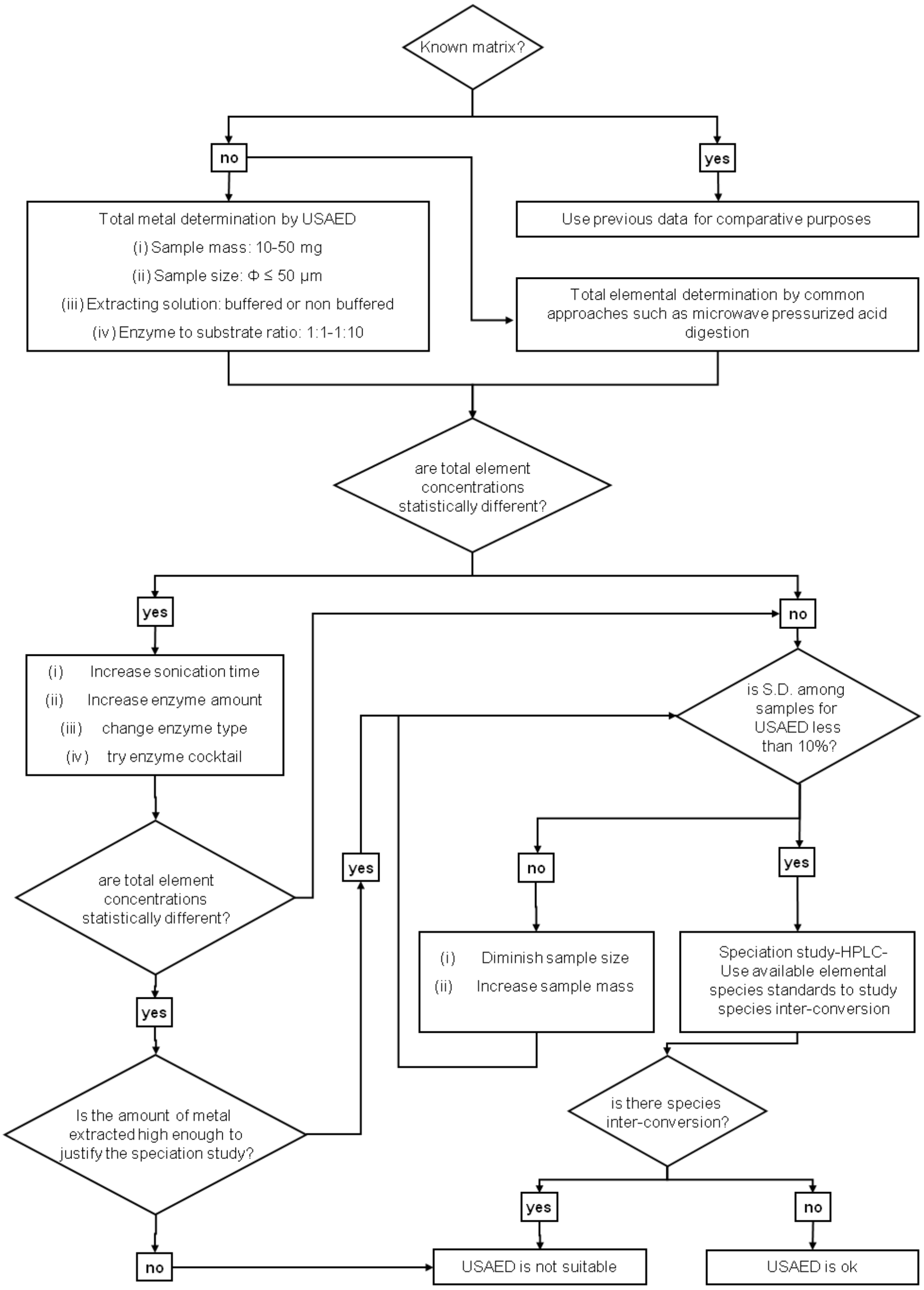

Figure IV.3. Comprehensive guide for a rapid USAED application. See explanation on the text.

Once it has been proved that the extraction is accurate or more than 80% of the total expected metal, with a reasonable standard deviation, lower than 10%, the next step is to ensure that there is no species inter-conversion due to the sample treatment. To do this, the samples must be spiked with standards of the elemental-species, such as DMA or MMA for As, and subjected to the sample treatment. Recoveries will indicate whether there are transformations or not.

Once demonstrated that (i) most than 80% of the element is extracted and that (ii) there are no species inter-conversion, then the methodology can be applied for elemental speciation.

IV.10. Future trends

Regarding future applications, the spreading of the USAED methodology to other metals is expected. Thus, metals of great environmental concern, such as Cr or Hg, or metals with remarkable effects in health care, such as Li or Zn, will be investigated to access the applicability of the methodology we are dealing with to their chemical speciation. Furthermore, USAED online applications for speciation purposes entailing analytical techniques others that HPLC-ICP-MS are expected. So far, HPLC-FI-CV-AAS or HPLC-FI-HG-AAS will also take advantage of the possibilities of joint enzymes and ultrasound for chemical speciation. Vapor generation or hydride generation in conjunction with flow injection designs are approaches economically viable for laboratories that can not support ICP-MS, which is an expensive analytical technique. It must be remarked that schemes entailing flow injection systems and atomic fluorescence spectrometry can provided detection limits low enough to be a competitive approach when comparing it with ICP-MS.

Another approach predicted is the linking between USAED and imprinting polymers. On this item a pioneer work reporting limited success regarding speciation of organotin compounds with imprinted polymers has been reported by Gallegos-Gallegos et al. [30]. However, this research area deserves more dedication from the analytical community to elucidate for which elements and for which conditions the linking between imprinted polymers and USAED is a valuable methodology.

IV.11. Conclusions

Ultrasonic assisted enzymatic digestion has proven to be a powerful methodology for chemical speciation. However, the methodology described in the present manuscript deserved more dedication from the analytical community in order to clarify its field of use; this is, for which metals and for which matrixes this method can be used.

The variables affecting the performance of USAED has been described and critically discussed. In addition, a scheme providing a practical guide for the application of USAED has been given in Figure IV.3.

Finally, future applications of USAED, including online systems and different analytical techniques of measurement than the ones used to date with the methodology we are dealing with has been suggested and commented.

IV.12. Acknowledgments

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Chapter V

An Assessment of the Ultrasonic Probe-Based Enhancement of Protein Cleavage with Immobilized Trypsin: Does it Work?

Submitted for publication on Proteomics:
G. Vale, H. M. Santos, L. Fonseca, M. Miró, V. Cerda, J. L. Capelo

V.1. Abstract

The use of ultrasonic probe in conjunction with immobilized trypsin has been explored in this work for potential enhancement of protein digestion. Several solid supports commonly used to immobilize trypsin were subjected to different ultrasonication amplitudes (UA) and times (UT), in order to investigate their mechanical resistance to ultrasonic energy (UE), when provided by ultrasonic probe. Glass beads and magnetic particles were found to remain intact in most conditions. It was found that immobilized trypsin cannot, however, be reused after ultrasonication (US) since the enzymatic activity was greatly diminished after sonication for 2 min at 50% UA. For comparative purposes, vortex shaking was also explored for protein cleavage.

Four standard proteins - bovine serum albumin (BSA), alfa-lacto albumin (α-lact), carbonic anhydrase (CA) and ovoalbumin (OA) – were successfully identified using peptide mass fingerprint (PMF) or peptide fragment fingerprint (PFF). In addition, depleted human plasma was digested. The plasma profile was similar to that obtained by classical protein cleavage (overnight, 12h), and the vortex shaking method whenever preceded by US-assisted protein reduction and alkylation.

I contributed to this chapter with all the experimental dara reported.

V.2. Introduction

Nowadays, protein identification in modern proteomics workflows relay mostly in mass spectrometry-based approaches [1]. In such workflows, as a general trend, proteins in complex samples are first separated by gel-based electrophoresis or 1-D or 2-D column chromatographic methods [2]. Proteins are then submitted in bottom-up or shotgun proteome assays to four main steps, namely, denaturation, reduction, alkylation and digestion with the aim to obtain a set of peptides which is interrogated against databases in order to identify the protein(s) [1, 2]. A classic proteomic workflow based on the aforementioned steps might last for 12 to 24 hours or even more, depending on the type of protein(s). To overcome this bottleneck in sample processing, the proteomics community has dedicated many resources and efforts to develop new procedures allowing simplicity of handling, high sample throughput and expeditious assays. This has been witnessed by the publication of a vast number of fast procedures that use different tools to make proteomics workflows matching the above mentioned demands. Heating [3, 4], microspin columns [5], ultrasonic energy [6, 7], high pressure [8], infrared energy [9], microwave energy [10, 11], alternating electric fields [12], and on-target sample treatment [13, 14] embrace the majority of such tools.

Digestion of proteins is by far the most important step in protein identification, which is accomplished on a regular basis with the enzyme trypsin. Digestion can be conducted either in homogeneous phases, where both protein(s) and enzyme are in solution, or in heterogeneous phases, where the protein(s) are in solution and the enzyme is immobilized onto a solid support [1, 2]. Digestion in heterogeneous phases has a number of advantages over homogeneous phases. Enzyme immobilization makes autolysis almost negligible and therefore the presence of peptides belonging to the enzyme is overcome. Hence, sensitivity is increased and interpretation of sample spectra is streamlined. Because trypsin autolysis is greatly minimised the amount of immobilized trypsin for the assay becomes less critical whereby trypsin can be used at our will. In addition, immobilized trypsin can be reused for several times. Different approaches have been described in the literature to exploit immobilized trypsin in proteomics workflows including open tubular enzyme reactors, synthetic polymeric monoliths and reactors in lab-on-a-chip platforms [15]. Trypsin immobilized onto magnetic particles combined with magnetic separation has been presented as a fast and efficient approach in the identification of a huge number of proteins from complex samples [16, 17]. Other types of solid supports in the form of beads have been also reported in the literature with the same goal of making sample digestion more amenable to proteomics workflows [18, 19]. In an attempt to speed-up protein(s) digestion with immobilized-based trypsin, external energy sources involving infrared energy[20], microwave energy [19], ultrasonic energy[18] and heating [21] have been implemented in the analytical procedure.

In the present work, we report on the effects of ultrasonic fields provided by ultrasonic probes in the integrity of the bead-based materials commonly used to immobilize trypsin; in the activity of the immobilized trypsin; in the number of times that the immobilized trypsin can be reused and in the effectiveness in the acceleration of the digestion process. The assessment was done using bovine serum albumin (BSA), alfa-lacto albumin (α-lacto), carbonic anhydrase (CA) and ovoalbumin (OA) as model proteins to optimize the conditions of this methodology. Finally, human plasma was selected to evaluate the reliability of the proposed approach in a complex proteome.

V.3. Experimental

V.3.1. Apparatus

Protein digestions were made in capped microtubes of 500 µL from Eppendorf (Hamburg, Germany). A vacuum concentrator centrifuge UNIVAPO 100H with a refrigerated aspirator vacuum pump model Unijet II from UniEquip (Martinsried, Germany) was used for sample drying and pre-concentration. Centrifuges and vortex models Sky Linea and Spectrafuge Mini from Biogen Cientifica (Madrid, Spain) were used for sample treatment. An ultrasonic probe UP 100H from Dr Hielscher (Teltow, Germany) was used to accelerate sample treatment. A Leica-ATC (Wetzal, Germany) optic microscope model was used to observe the physical effects of the ultrasonic energy in several particles. For the trypsin activity assay a V-530 UV/Vis spectrophotometer from Jasco (Maryland, USA) was utilized and equipped with a cell of 1 cm optical path.

V.3.2. Standards and reagents

Milli-Q water was used throughout. All the chemicals used were analytical-reagent grade, unless otherwise specified. Bovine serum albumin (BSA), carbonic anhydrase from, bovine erythrocytes (CA), alfa-lacto albumin (α-lact) and human plasma were purchased from Sigma (Steinheim, Germany). Albumin from hen egg white (Ovoalbumina, OA) was purchased from Fluka (Buchs, Switzerland). Aminohexyl speharose 4B, silica (SiO_2) and non-porous glass beads (particle size ≤ 106 µm) were purchased from Sigma (Steinheim, Germany). All material was used without further purification. Immobilized trypsin enzymes in magnetic particles were purchased from Clontech (Mountain View. USA). α-Cyano-4-hydroxycinnamic acid (α-CHCA) *puriss.* for MALDI-MS from Fluka (Buchs, Switzerland) was used as MALDI matrix. Mass standard kit for the 4700 proteomic analyzer from Applied Biosystems (Foster City, USA) was used as mass calibration standard for MALDI-TOF- (TOF) – MS. For protein digestion, acetonitrile (ACN), iodoacetamide (IAA), DL-dithiothreitol (DTT) and proteomic grade trypsin purchased from Sigma were used. Ammonium Bicarbonate (AMBIC) and trifluoroacetic acid (TFA) was purchased from Fluka and Riedel-de-Haën (Seelze, Germany), respectively. The following reagents were used for protein immobilization in glass particles: 65% (w/w) nitric acid from Fluka (Buchs, Switzerland), aminopropyltriethoxysilane, glutaraldehyde and trypsin from bovine pancreas from Sigma (Steinheim, Germany). For the trypsin activity assay, Nα-Benzoyl-L-Arginine Ethyl Ester (BAEE) and sodium phosphate purchased from Sigma (Steinheim, Germany) were used.

V.3.3. Sample preparation

Four standard proteins, BSA, α-Lacto, CA and OA, were prepared in 1 mL of a 12.5 mM AMBIC (pH 6.8) solution with 50% of ACN to a concentration of 1mg/ml. A 20 µL aliquot was then denaturated for 1 min with an ultrasonic probe, reduced and alkylated with 2 µL of 110 mM DDT solution and 2 µL of a 600 mM IAA solution, respectively. The reduction and alkylation processes were assisted with an ultrasonic probe (equipped with a 1 mm titanium tip) for 1 min at 50% UA. After sample pre-treatment the protein was diluted with 76 µL of a 12.5 mM AMBIC solution to a final concentration of 0.2 µg µL^{-1}.

From the commercial human plasma sample a 40 µL aliquot was taken and mixed with 200 µL of acetone. The mixture was kept at -20°C for 3 hours and spinned down for 15 min at 12500 rpm. The pellet was resuspended in 70 µL of an AMBIC 12.5 mM solution with 30% of ACN. The sample was then reduced and alkylated as described above.

V.3.4. Trypsin immobilization

The spherical glass particles (200 mg, 80-100 µm), used as a support to immobilize trypsin, were washed with a 5% nitric acid solution for 8h at 80-100ºC. The particles were then collected and introduced in 5 mL of a 8% (v/v) 3-aminopropyltriethoxysilane solution (pH 3-4) for 8h at 80-85ºC. The aminopropyl-functionalized glass particles were washed with water several times until obtaining a neutral pH. The particles were heated overnight at 115ºC and resuspended in 4 mL of a 2.5% (v/v) glutaraldehyde solution under gentle stirring for 1h at room temperature. The particle washed consecutively with water and with a 20 mM AMBIC solution (pH 7). The functionalized glass particles were introduced in a vessel with 5 mL of a trypsin solution (20 mg mL, in 20 mM AMBIC (pH 7)) and incubated for 24h at 4ºC. The particles were collected and stored at 4ºC in a 20 mM AMBIC buffer (pH 7) solution.

V.3.5. Protein cleavage

After protein reduction and alkylation a 10 µL aliquot was taken and mixed with 10 µL of immobilized trypsin. In the overnight procedure, the mixture was placed for 8h in a thermostatized bath at 37°C. In the ultrasonic accelerated method, the mixture was ultrasonicated for 2 min with an ultrasonic probe (50% UA) equipped with a 1 mm titanium tip. In the stirred procedure, the mixture was placed in a vortex for 2 min. After protein cleavage, the immobilized trypsin was separated from the peptide solution, with a magnet, that was subsequently dried.

V.3.6. Trypsin activity

To determine the enzymatic activity of the immobilized trypsin particles, we exploited the Sigma quality control test procedure for enzymatic assay of trypsin (EC 3.4.21.4). To this end, a 0.25 mM BAEE solution was prepared in 50 mL of a 67 mM phosphate buffer (pH 7.6). Thus, 2.8 mL of BAEE solution and 40 µL of immobilized trypsin suspension (5%) were introduced in a quartz cell. The solution was mixed in end-over-end mode and the increase of absorbance at 253 nm was monitored for 5 min (15-20 seconds of interval frequency between measures). The cell was hand-stirred in the course of the assay to maintain the immobilized trypsin particles in suspension

V.3.7. MALDI-MS/MS analysis

For MALDI analysis, the samples were mixed with a matrix solution of α-CHCA (10 mg mL^{-1}) prepared in 50% ACN with 0.1% TFA. Then, 5 µL of the matrix solution was mixed with 5 µL of sample and stirred in a vortex. 0.5 µL of each sample was spotted on the wells of the MALDI plate and dried. To obtain the mass spectra for protein identification by peptide mass fingerprint, PMF, or by peptide fragment fingerprint, PFF, an MALDI mass spectrometer 4700 from Applied Biosystems (Foster City, USA) was used. The measurements were performed in the reflector positive ion mode and the MALDI mass spectra were acquired as recommended by the manufacturer. The proteins were identified with the Swiss-Prot database. The complete experimental procedure is schematized in Figure V.1.

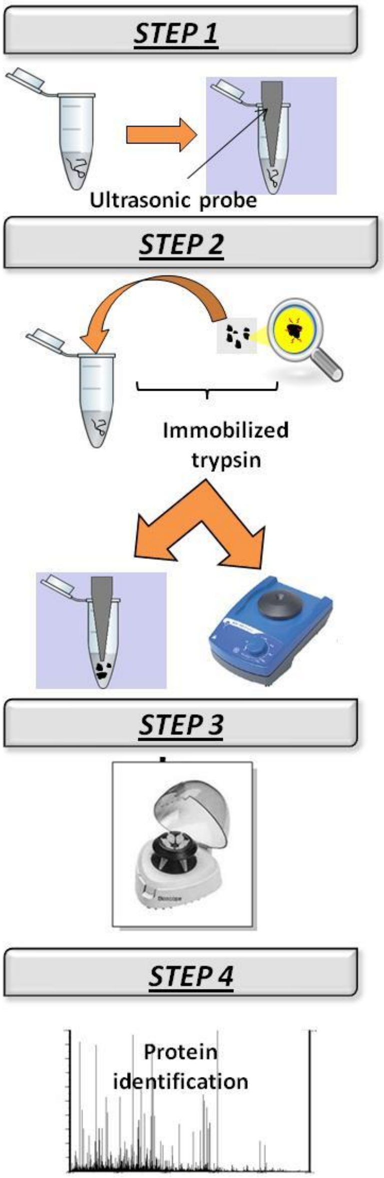

Figure V.1. Ultrasonic probe-based experimental scheme for protein(s) identification with immobilized trypsin. Step 1: Sample preparation: UT and UA for the steps of protein denaturation, protein reduction and protein alkylation: 1 min and 50% respectively (for each step). Step 2: Protein(s) cleavage: Mixing the bead-based immobilized trypsin in the sample solution; protein digestion was enhanced with the aid of an ultrasonic probe (ultrasonication time and amplitude: 2 min and 50% respectively); or with the aid of a vortex shaker. Step 3: Bead separation by centrifugation. Step 4: Sample mixing with matrix and spotting onto the target plate. Protein identification through peptide mass fingerprint of through peptide fragment fingerprint.

V.4 Results and discussion

V.4.1. Influence of ultrasonic amplitude on particle integrity

One of the characteristics of ultrasonic energy is that it might disrupt solids [22]. Therefore, one of our concerns was to test the integrity of the solid supports to be used in this work when submitted to an ultrasonic field provided by ultrasonic probes.

Table V.1. Different particles (200 mg) were prepared in 1 mL of MiliQ water and were ultrasonicated with an ultrasonic probe at different ultrasonication amplitude and times

Ultrasonication Amplitude (%)	**Silica particles Ultrasonication time (seconds)**			**Glass particles Ultrasonication time (seconds)**			**Magnetic particles Ultrasonication Time (seconds)**			**Sepharose particles Ultrasonication Time(seconds)**		
	60	120	240	60	120	240	60	120	240	60	120	240
20	+	+	+	-	-	-	-	-	+	-	+	+
50	+	+	+	-	-	-	-	-	+	-	+	+
80	+	+	+	-	-	-	+	+	+	-	+	+

(-) The particles remained intact; (+) the particles were disrupted

For a given frequency of ultrasound, the effect of solid disruption is directly proportional to the ultrasonic amplitude, UA, because as the UA is increased so the energy delivered to the bulk media is also increased [22]. For that reason we devised a set of experiments in which the solid supports under assessment were submitted to the effects of ultrasonic energy, UE, under different conditions of UA and ultrasonication time, UT. The UA was varied within the range of 20-80% whilst the UT was varied between 0 s and 240 s for each UA. The results of this set of experiments are shown in Table V.1 and Figure V.2. As seen in Figure V.2, silica particles are very easily disrupted due to the effects of ultrasound. It was found that this material was degraded in all the experimental conditions tested. Thus, the lower amplitude and time tested were enough to disrupt the silica particles. According to these findings, the use of this material was not further attempted. As far as the integrity of the glass particles concerns, they remained intact for all the conditions tested, even for the longest amplitude and time assessed 80% and 240 s, respectively. Therefore this material was selected for subsequent experiments. Concerning the magnetic particles, their structure remained intact for low (20%) to medium (50%) amplitudes (scale 0-100%) and for low (60 s) and medium (120 s) times of ultrasonication. For that reason this material was also selected for further studies. Finally, as to sepharose particles, disruption can be avoided if times below 60s are used. However, sepharose particles tend to form aggregates (see Figure V.2) which makes the handling of this material difficult. Based on the aforementioned results we selected the glass and magnetic particles for further experiments.

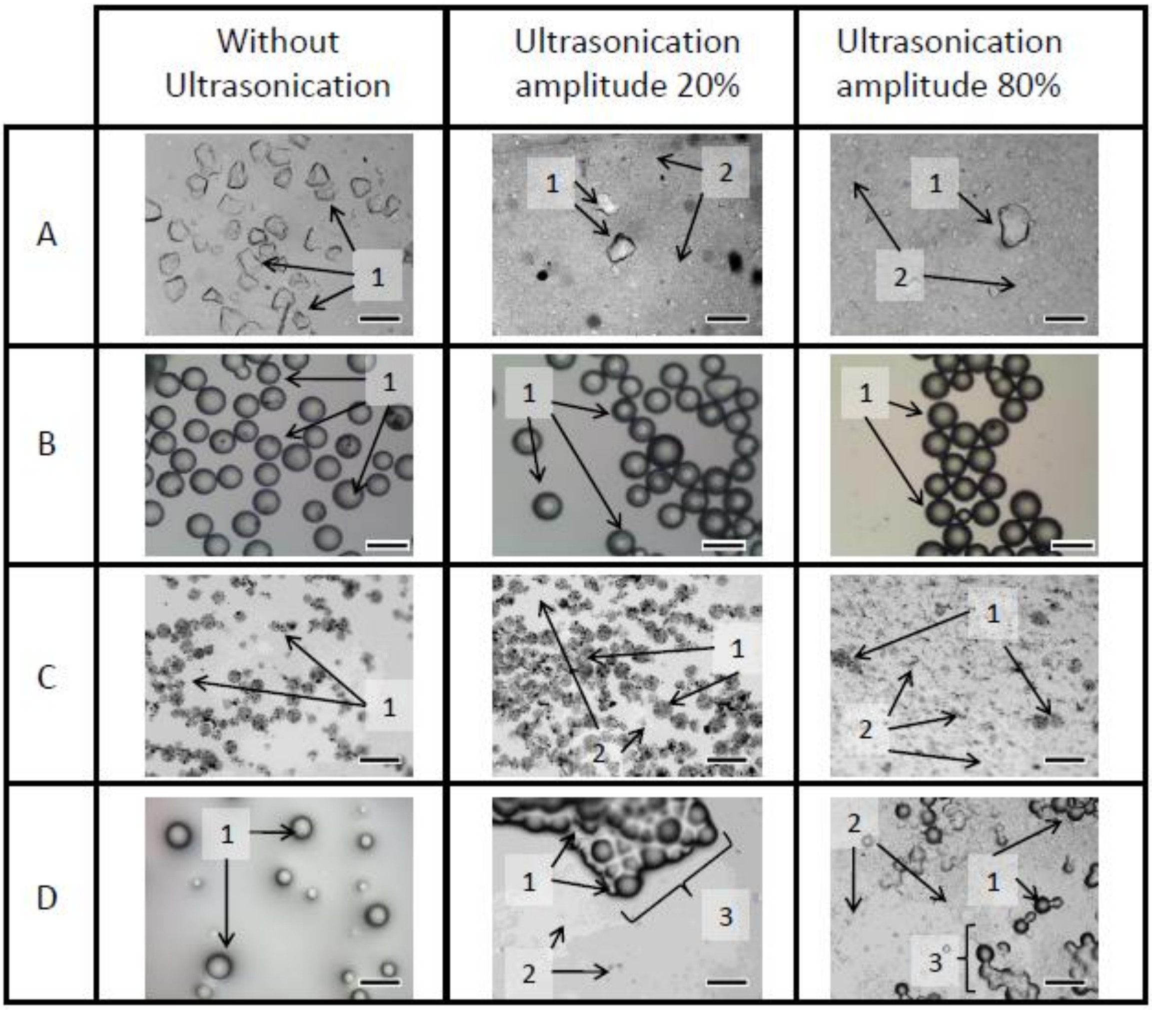

Figure V.2. Different particles (200 mg) were prepared in 1ml of MiliQ water and ultrasonicated during 2 minutes with an ultrasonic probe at different ultrasonic amplitudes. The microscopic pictures were taken with a magnification of 100 times and the scale corresponds to 200 µm. A-Silica particles; B–Glass particles; C–Magnetic particles; D–Sepharose particles; 1-Intact particles; 2-Disrupted particles; 3-Agregated particles.

V.4.2. Effect of ultrasonic energy on the activity of the immobilized trypsin

The set of experiments described in the previous section provided useful information regarding the stability of the particles studied when submitted to the effects of an ultrasonic field. However, the information retrieved was not enough to get knowledge about the effects of UE on the activity of the immobilized enzymes. Previous experiments done in our laboratory have demonstrated that, depending on the substrate, UE inactivates solubilized trypsin in times as short as two minutes [23]. Therefore, we devised a set of experiments to measure the activity of the immobilized trypsin after different exposure times from 0 to 120 s to ultrasonic energy. The activity was measured against BAEE as explained in the experimental section. The UA selected, 50%, was the amplitude regularly used in our laboratory to enhance the in-solution trypsin cleavage of proteins when probe sonication is used [6, 7]. Results are shown in Table 2. Experimental results revealed that the activity of the immobilized trypsin decreases as the exposure time to ultrasound increases. As far as the magnetic particles concerns, the activity was diminished by 39%, from 610 to 373 relative units. Concerning glass beads the activity varied by 71%, from 233 down to 68 relative units. We concluded from this set of experiments that although the glass beads were more resistant to ultrasonic energy than the magnetic beads, the enzymatic activity was less affected in the latter.

Table V.2. Enzymatic activity of immobilized trypsin after exposure to UE. The activity was measured against BAEE (see experimental section for further details). The represented data correspond to a mean (±SD) of three independent technical replicates.

	Enzymatic activity (BAEE U/mL of enzyme)	
	Mag-trypsin®	**glass beads**
Referenced values	> 150	None
Without ultrasonication	610±55	233±19
Ultrasonication 20 seconds	421±42	168±14
Ultrasonication 60 seconds	422±29	68±26
Ultrasonication 120 seconds	373±52	without activity

V.4.3. Enhanced ultrasonic protein cleavage with tryspsin immobilization in glass particles

V.4.3.1. Influence of the temperature of trypsin immobilization

Two main procedures are currently described in literature for the immobilization of trypsin in glass particles: immobilization at room temperature or at 4ºC [24-26]. To test the influence of the method used to immobilize trypsin, a set of experiments was devised in which immobilized trypsin prepared at room temperature or at 4ºC was used to digest BSA overnight (12h) or for 120 s with UE. Results shown in Table V.3 (conditions A-B, and C-D) reveal that the cleavage efficiency was identical regardless of of the temperature in immobilization, and that the ultrasonic method promotes a faster cleavage, the same results being obtained with ultrasonication by 60 s than overnight (compare A-B with C-D). This result is in agreement with data published elsewhere [18].

V.4.3.2. Influence of the ultrasonic treatment in particle reutilization

In a different set of experiments the possibility of re-using immobilized trypsin was investigated. In this regard, it was found that the number of peptides obtained when trypsin bearing particles were used twice decreased by 60% in the second assay, and no appreciable cleavage was observed in the third assay data not shown). This result is in agreement with that reported in section V.4.2., where it was shown that after 120 s of ultrasonication the activity of the immobilized trypsin diminished by 71%. Therefore, the particles to digest proteins were used once for further experiments.

Table V.3. Comparison of different conditions for the use of glass-bead immobilized trypsin to speed BSA digestion under the effects of an ultrasonic field. The values correspond to a mean (±SD) of three independent technical replicates.

Conditions	**Score**	**Coverage**	**Number of peptides matched**
A	109±23	37±9	21±5
B	99±13	34 ±5	21±4
C	113±35	34±8	22±6
D	153±7	28±6	16±5
E	130±4	27±4	16±3
F	153±37	28±6	16±5
G	115±6	37±1	23±1
H	113±35	34±8	22±6
J	1814	34±5	22±5

A: Overnight digestion with trypsin immobilized at room temperature; **B:** Overnight digestion with trypsin immobilized at 4 ºC; **C:** Ultrasonic digestion with trypsin immobilized at room temperature. 2 min UT.; **D:** Ultrasonic digestion with trypsin immobilized at 4 ºC. 2 min UT.; **E:** for reduction, alkylation and digestion, UT: 1 min, UA: 20%; **F:** for reduction, alkylation and digestion, UT 1 min, UA: 50%.; **G:** for reduction, alkylation and digestion, UT 2 min, UA: 20%; **H:** for reduction, alkylation and digestion, UT 2 min, UA: 50%; **J:** for reduction and alkylation, UT 2 min, UA: 50%. For digestion: 2 min. stirring;

V.4.3.3. Influence of the ultrasonic treatment on the reduction, alkylation and digestion steps

Concerning reduction and alkylation of proteins, contradictory information can be found in the literature dealing with its application in proteomics workflows using ultrasonication. It has been, for example, claimed that those steps can be circumvented [18] whilst the opposite finding has also been reported [27, 28]. In our case, when US-based reduction and alkylation were not done, BSA was not identified by PMF. Therefore in subsequent experiments proteins were always alkylated and reduced for 1 min at 50% of UA.

As far as the digestion step concerns, differences were not found between the use of an UA of 20% or 50% in terms of BSA peptides matched or protein coverage, as shown in Table V.3 (G and H) . Regarding UT, it was found that 120 s provide better results that 60s. Therefore for further experiments, an UT of 120s and an UA of 50% were used.

V.4.3.4. Influence of the amount of immobilized trypsin used

To find out the influence of the amount of trypsin in protein identification, different volumes of immobilized trypsin were used to digest BSA. To 10 µL of BSA samples previously reduced and alkylated with UE, volumes of immobilized trypsin of 5 µL, 10 µL and 20 µL were added. The best results in terms of protein coverage and number of peptides identified was obtained with 10 µL, which was chosen for the remainder of the work.

V.4.3.5. Application to other proteins

The next step was to use the best conditions found for BSA in the identification of other proteins, namely, α-lact, CA, and OA. Results depicted in Figure V.3 demonstrate that although the three proteins were digested; only OA was identified through PMF. However, as will be showed below, the number and intensity of the peptides obtained was sufficient to identify all the proteins through PFF.

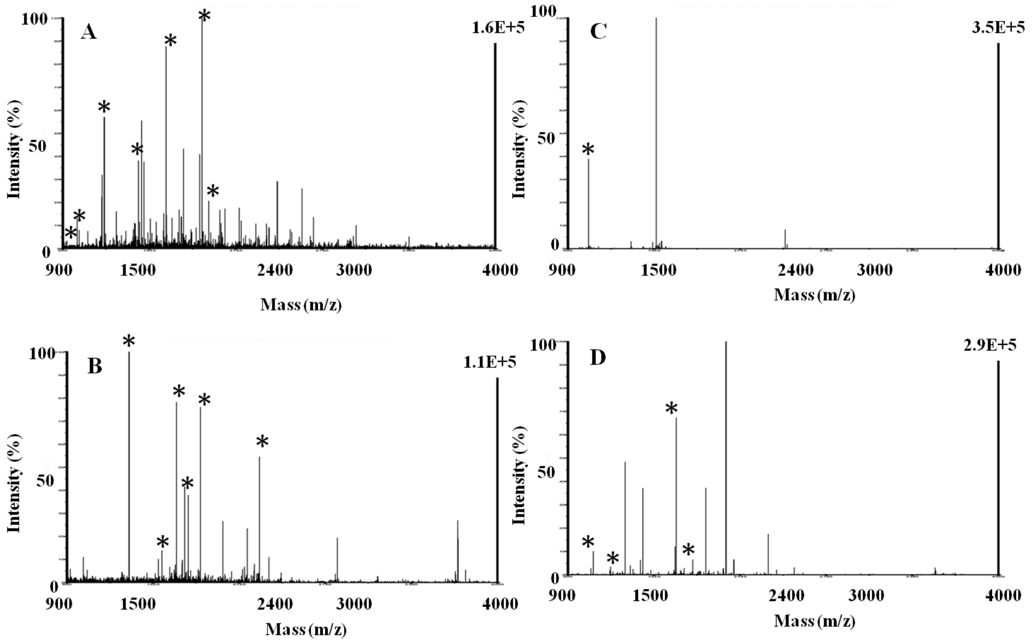

Figure V.3. - Protein cleavage with immobilized trypsin on glass-beads accelerated with ultrasonic probe (2 minutes at 50% UA). A – Bovine serum albumin (BSA); B – Albumin from egg (OA); C –Carbonic anhydrase, CA; D – Alfa-lacto albumin, α-Lacto. BSA and OA were identified by PMF and CA and α-Lacto were identified by PFF.

V.4.3.6. Ultrasonic energy versus shaking with a vortex

UE can produce different effects of which the most important is cavitation. The explanation of ultrasonic cavitation is out of the scope of this manuscript and the reader is referred to the works of Mason et al on this item [22]. A lesser known effect of UE is its capability to promote mixing of phases by agitation. Therefore it is important to test always that is not agitation but cavitation the cause of the observed effects. Consequently, an additional set of experiments was done, in which ultrasonication was replaced by vortex shaking. Unexpectedly, the results obtained were similar to those achieved with ultrasonication or with overnight digestion (see Figure V.4B and C). This finding suggests that the enhancement promoted by UE in the digestion of proteins using immobilized enzymes might be due to the mass transfer promoted by agitation of the bulk solution rather than the cavitation effects of ultrasound. In other words, although UE can promote the digestion a simpler system such as a vortex shaker can produce a similar enhancement.

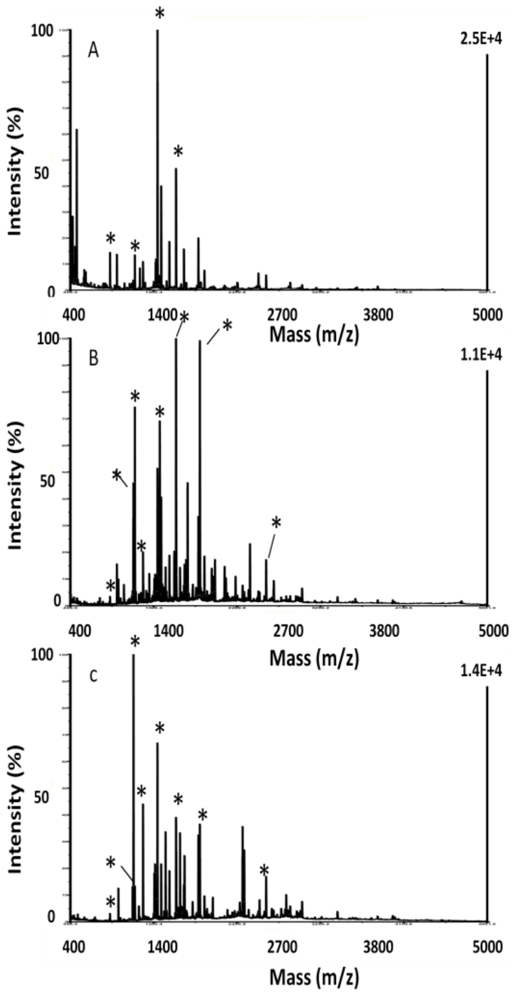

Figure V.4 BSA mass spectra obtained after digestion with an ultrasonic probe (50% ultrasonication amplitude) or shaking in a vortex. A –1 min UT ; B –2 min UT; C – 2 min. Shaking on a vortex. Peaks identified with an asterisk correspond to the same m/z charge.

V.4.4. Enhanced protein cleavage with trypsin immobilized magnetic particles

Based on the results obtained with the glass beads, a set of experiments was devised to investigate the best amount of magnetic particles to be used. As above for the glass particles, 2 µL, 5 µL, and 10 µL, were added to 10 µL of CA, or BSA or α-lact. The conditions for ultrasonication used were the best obtained in the assessment of the glass particles. Thus reduction, alkylation were conducted for 1 min at 50% UA and digestion for 2 min at 50% UA. For comparative purposes the cleavage was allowed to take place overnight in a shaker at 37˚C.

Both, ultrasonic and overnight methods provided similar results concerning protein identification, i.e. protein score and number of peptides. Identification through PMF was successfully obtained for BSA and α-lacto for a 10 µL volume of magnetic particles, whilst for CA identification trough PMF was not attained, although digestion was clearly achieved as visualized in the MALDI spectra (data not shown). As the results were similar to the ones obtained with glass beads no further optimization was done.

V.4.5. Protein identification through peptide fragment fingerprint

It was verified throughout this work that protein cleavage was always achieved, although for some proteins, such as CA, digestion yields were too low and consequently the number of peptides obtained was not sufficient to acquire spectra with enough quality to ensure protein identification through PMF (see Figure V.3). However, in all analysis some peptides were obtained with an intensity and resolution adequate to explore their fragmentation and identification using PFF. As showed in Table V.4, the proteins were identified with a minimum of two peptides per protein. It must be stressed that in those cases were protein identification through PMF failed due to a poor digestion, PFF was always successfully applied.

Table V.4 MS/MS data for α-lacto and CA after enzymatic digestion (immobilized trypsin in magnetic particles) accelerated with an ultrasonic probe (UT 2min, UA 50%).

Acession Number	Protein name	MW/pI	Peptide matched	Sequence coverage (%)	Sequence	Score
P00711	α-lactoalbumin from bovine	16692/4.93	4	28±2	K.VGINYWLAHK.A K.ILDKVGINYWLAHKA.A K.ALCSEKLDQWLCEK.L K.CEVFRELK.D	61±16
P00921	Carbonic anhydrase 2 from bovine	29096/6.41	2	6	K.HNGPEHWHK.D K.DFPIANGER.Q	72±3

V.4.6. Application to a complex proteome: human plasma profiling

With the aim to ensure that the ultrasonic enhanced digestion does not have indeed differences with the overnight digestion, we used human plasma to find out potential differences between both methods. In addition, we also used shaking in a vortex, SV, instead of UE, for comparative purposes. We selected magnetic particles instead of glass beads in this set of experiments because of their potentialities in simplification of sample treatment in proteomics with the aid of a magnet for particle retrieval. The results in Figure V.5, demonstrate that the serum profile obtained after digestion was the same either for the treatment with UE or SV. The profile in the overnight digestion was also similar, but it seemed to be obtained with higher digestion efficiency, as the intensity and number of peptides with masses higher than 2000 seems to be less intense than those of UE or SV.

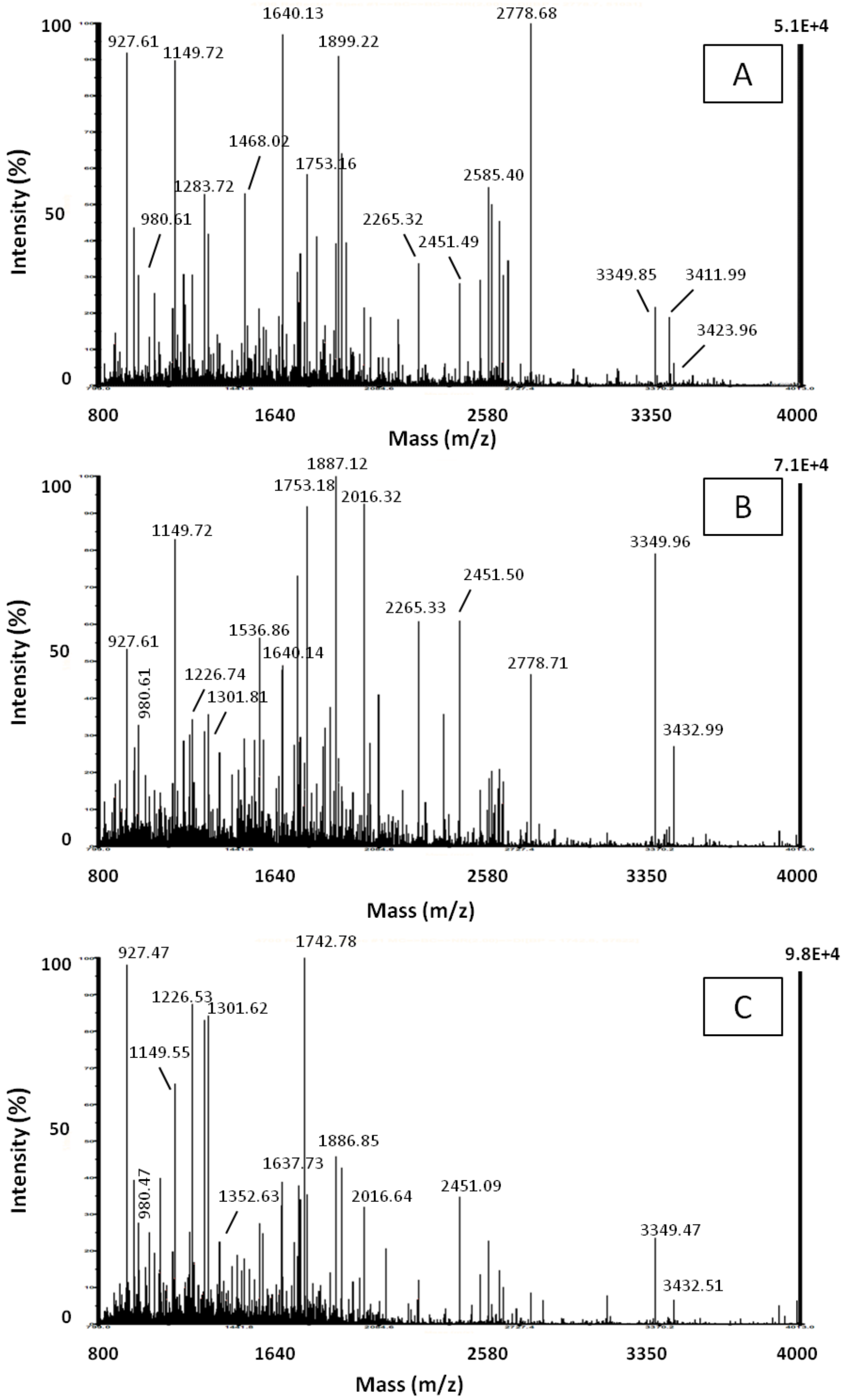

Figure V.5. - Human plasma mass spectra obtained after different treatments. A) Plasma digestion with ultrasonic probe; B) Protein digestion with shaking in a vortex; C) Protein digestion with overnight method. Peaks identified with an asterisk correspond to the same m/z charge.

V.5. Conclusions

Ultrasonication with an ultrasonic probe must be applied with extremely care over immobilized trypsin, as the solid support can be disrupted. Glass beads and magnetic beads were found appropriate to immobilize trypsin for subsequent ultrasonic treatment. Immobilized trypsin cannot be however reused after ultrasonication, as the activity of the enzyme decreases in each US treatment. The results presented in this work clearly demonstrate that ultrasonic energy delivered by an ultrasonic probe can be used to speed proteomics workflow entailing reduction, alkylation and digestion with immobilized trypsin from overnight (12h) to merely 6 minutes. On the overall, when using ultrasonication in the aforementioned steps for 120s with UA of 50%, protein cleavage using immobilized trypsin was consistently obtained. BSA, OA, CA and α-lact were identified by resorting to PFF. Experimental results also showed that the digestion efficiency in human plasma was similar between overnight, ultrasonic and shaking protocols, yet reduction and alkylation were significantly accelerated with the aid of UE.

V.6. Acknowledgements

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Chapter VI

Influence of selenium in arsenic accumulation by the freshwater clam *Corbicula fluminea*

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VI.1. Abstract

Arsenic (As) is one of the most common toxic elements found in the environment, both aquatic and terrestrial, from geological and anthropic sources. Selenium (Se) is an element well known by its anti-cancer and antioxidant properties. The aim of the present work was to study the hypothesis of using selenium as a mediator to avoid damage caused by high levels of As in freshwater bivalves (*Corbicula fluminea*). The organisms were exposed to different concentrations of As (1000 μg L^{-1}), Se (100, 500 and 1000 μg L^{-1}) and a mixture of these elements. The concentration of As and Se in the soft tissues were determined by ET-AAS (Electrothermal Atomic Absorption Spectrometry) after acidic digestion. The results show that the organisms are able to regulate As and accumulate significant levels of Se. The histochemical and histological analysis revealed the presence of As granules in the soft tissues complementing and supporting our findings.

I contributed to this chapter with all the experimental dara reported.

VI.2. Introduction

Arsenic (As) is a natural element that occurs in many environmental compartments (atmosphere, soils, rocks, natural waters, organisms) and is one of the oldest poisons known by man [1, 2]. Chronic exposure can cause many skin lesions such as melanosis, leucomelanosis and keratinosis, can also cause vascular diseases, cancer on various organs or tissues (skin, liver, lung and bladder) and other manifestations including neurological effects, obstetric problems and diabetes [3]. From the several environmental sources of As, contaminated drinking water is the one that presents more risks to human health, particularly when it is from groundwater supplies since it has higher concentrations of As due to bedrock dissolution into surrounding water [1, 4, 5]. One of the best known cases of As contamination in waters occurs in Bangladesh, where millions of people suffer from As exposure diseases. Due to the high levels of pollutants in the surface water, the Bangladesh population have to use the groundwater for consumption. However, due to the geological nature of the bedrock, this groundwater have high amounts of As, that surpass 50 times the maximum advised limit in drinking waters (10µg As/L) by the World Health Organization [6]. With respect to selenium (Se), an essential element for human health, it has been recognized as antioxidant and is generally associated to the diminishing of certain types of cancer and other diseases [7-9]. This element occurs in soils and enters in the food chain essentially through the plants. Selenium has an ambivalent behaviour ranging from being essential to highly toxic, depending on the species, oxidation sate and concentration. Problems related with selenosis (Se poisoning) are much lower than the episodes concerning to its deficiency. However, selenosis is rarely diagnosed and information regarding to its long terms effects is limited. Nonetheless, some symptoms and diseases are known to be related with Selenium poisoning such as respiratory disturbances (e.g. dyspnea, bronchitis and pulmonary edema) [10]. High amounts of Se are usually released in the environment by coal-fired power plants and are known to be responsible for adverse reproductive effects and local extinction of birds and fishes [11]. Many organisms have developed detoxification mechanisms against the effects of the ingestion of toxic elements hindering the ingested elements of reaching their target organs. For instance, some studies [12, 13] have shown that the interaction between arsenic and selenium in mammals induce the bioformation of Seleno-bis(S-gluthionyl) arsenium ion $(GS)_2AsSe^-$, which has an important role on the natural detoxification mechanisms for these elements. The formation of $(GS)_2AsSe^-$ in the mammalian bloodstream followed by its excretion from the organism via billie, reinforce the concept that this could be a natural detoxification metabolic pathway for arsenic [13, 14]. However this mechanisms needs to be more studied in other organisms such as aquatic species which are exposed to arsenic through the water, like the filter-feeders (mollusks) which are known for their capabilities to bioacummulate metals and trace metals from the environment. In fact bivalve mollusks have been used as bioindicators of aquatic pollution providing information about the impact and bioavailability of chemicals in natural systems [15-17]. However, most of the ecotoxicological studies using bivalves have been carried out with marine species, being cadmium, zinc and cooper, the most studied metals [18-20], while the freshwater species have been often forgotten. If we think that clean fresh/drinking water is essential for the health of all animals (including humans) then we easily understand that the study of freshwater environments should be a priority especially in what concerns to toxic elements such as arsenic.

The *Corbicula fluminea* is a freshwater filter-feeding bivalve native from the Southeast of Asia. It lives in the three top layers of the sediment and makes constant exchanges with the media water through a siphon that control the inhalant and exhalant water flow[15]. This freshwater mollusk as spread worldwide colonizing the rivers, channels and lakes of the European and American continents. The first report of this bivalve in Europe dates back to 1980 in France (river Dordogne) [15]. However, nowadays this bivalve is widespread through other European countries such as Portugal (Tagus and Minho rivers), Germany, Netherlands and Spain. This species present a strong invasive dynamics, high adaptation capabilities and high resistant against pollutants. These characteristics make the *C. Fluminea* a great threat to native freshwater species and a model to ecotoxicological studies in freshwaters, being a biological indicator of contaminants. This bivalve has been subjected only to a few ecotoxicological assays concerning As and Se contaminations [15-17]. To our knowledge this is the first study reporting a hypothetical mechanism of As detoxification by interaction with Se in this species. Therefore, the main objective of the present work, is to increase the knowledge and understanding about the As and Se *in vivo* interaction using *C. fluminea* as a biological model, to assess the bioaccumulation dynamics of theses elements and to discuss the possibility to use Se to avoid damage caused by high amounts of As in drinking waters. Furthermore, histochemical and histological analysis complements and supports the assessment of As and Se presence in *C. fluminea*.

VI.2. Experimental

Experimental design

The freshwater mussels, *C. Fluminea*, were hand-collected on Tagus river (Portugal, 39˚04’02.18’’N; 8˚45’26.50’’W) near Escaroupim village. On arrival at the laboratory, five bivalves were randomly selected, identified as T0 organism and stored at -20°C. The remain organisms were maintained inside a system of tanks equipped with water filters and air pumps. After one month of acclimatization time in tap water (without chlorine), a homogeneous group of 80 clams were selected (shell size = 3±1cm, body and shell weight 7±3g) and distributed randomly into 2L polyvinyl tanks aerated with an air pump system. The mussels were exposed to different concentrations of Arsenic (1000 μg L^{-1}), Selenium (100, 500 and 1000 μg L^{-1}) and a mixture of this two elements (As 1000 μg L^{-1} + Se 100 μg L^{-1}; As 1000 μg L^{-1} + Se 500 μg L^{-1}.;

As 1000 μg L^{-1} + Se 1000 μg L^{-1}), as schematized in figure VI.1, during 21 days. Sodium selenite, Se(IV) and arsenite, As(III) purchased from Aldrich (Milwaukee, USA) were used to prepare the water solutions for aforementioned assays All the tanks were prepared in duplicate and the controls were filled with tap water,. The bivalves were daily fed with commercial pellets and the tanks were subjected to continuous aeration and a 12h light photoperiod. The temperature (18°C), pH (6-7) and ammonia levels (< 0.6 mg mL^{-1}) were daily monitored. Ammonia was determined using an ammonia test Kit from Nutrafin (Montereal, Canada).

Water samples

Water samples from the different tanks were collected weekly to capped microtubes of 0.5 ml from Eppendorf (Hamburg, Germany), acidified at 10 μL mL^{-1} with nitric acid 65% w/w purchased from Fluka (Buchs, Switzerland) and stored at 4˚C. The water samples were used for monitoring As and Se in water during the assay.

Soft tissues digestion

After 21 days of exposure, *C. fluminea* soft tissue were excised from the shell and dissected into two parts: digestive gland (DG) and the remain body (B). Sub-samples of soft tissues were taken for histological and histochemical evaluation and the remain dried in an oven (72h at 70˚C). After dried, the digestive gland and the remain body were digested with nitric acid (5ml) in closed Teflon cells during 24h at room temperature, followed by heating at 90˚C during 5h and an additional hour after adding 1mL of hydroxide peroxide 30% (Riedel-de-Haën (Seelze, Germany)). The digested samples were diluted with 10mL of ultrapure water.

Selenium and arsenic determination by ET-AAS

The total determination of As and Se concentration in water and dry tissues were measured with an Unicam (Cambridge, UK) atomic absorption spectrometer model SpectrAA-300 *plus* equipped with a graphite furnace, an autosampler and Zeeman background correction. A Se and As hollow-cathode lamp from Thermo Electron Corporation (Massachusetts, USA) operated at 15 mA was used as radiation source. Pyrolytic graphite-coated graphite tubes with L'vov platform (Omega Platform Extended Lifetime type, Unicam) were used. An inorganic Se and As atomic absorption standard solution purchased from Aldrich (Milwaukee, USA), were used to prepare standard for ET-AAS. All stock standard solutions were stored in a refrigerator at 4˚C and protected from light. Working standard solutions were prepared just before use by appropriate dilution of the stock standard solution. Palladium nitrate matrix modifier for AAS was purchased from Fluka (Buchs, Switzerland). Se and As quantification were performed in peak area mode. The water and the digested tissues were directly measured in the ET-AAS system after proper dilution with ultrapure water. The limit of detection (LOD) and limit of quantification (LOQ) [21] was 6.6 μg L^{-1} and 11.8 μg L^{-1} for Se and 3.3 μg L^{-1} and 8.4 μg L^{-1} for As, respectively.

Certified reference material

For validation purposes it was used DOLT-3, dog fish liver certified reference material for trace elements, from the National Research Council from Canada, NRC-CNRC. This material is certified in a large number of elements, such as As (10.2±0.5 mg Kg^{-1}) and Se (7.1±0.5 mg Kg^{-1}). The certified material was digested in capped Teflon tubes as described previously and the As and Se amounts determined by ET-AAS. The results obtained (n=3) were (10.1±0.3 mg Kg^{-1}) for As and (7.8±0.4 mg Kg^{-1}) for Se witch showed a good agreement with the certified values.

Histology and histochemistry analysis

The detection of As granules in soft tissues was carried out using the Castel’s method [22]. Briefly, samples were fixed during 5 days in a solution of 10% formalin containing 2.5% of copper sulfate. After fixation, the samples were treated according with the usual histological procedures[23]. After immersion in paraffin, sections of 5-7μm were cut by a microtome (Leica-ATC; Wetzal, Germany) and stained with hematoxyline and eosin purchased from Riedel-de-Haën (Seelze, Germany). The histological and histochemical observations were performed in an optical microscope (Leica-ATC, Wetzal, Germany).

Statistical analysis

The nonparametric Mann-Whitney U test was used to determine differences between the treated samples and the control samples. Statistical analyses were performed with a significance level of 5%, using the software Statistica 5.0 (statsoft, 1995, Tulsa, OK, USA).

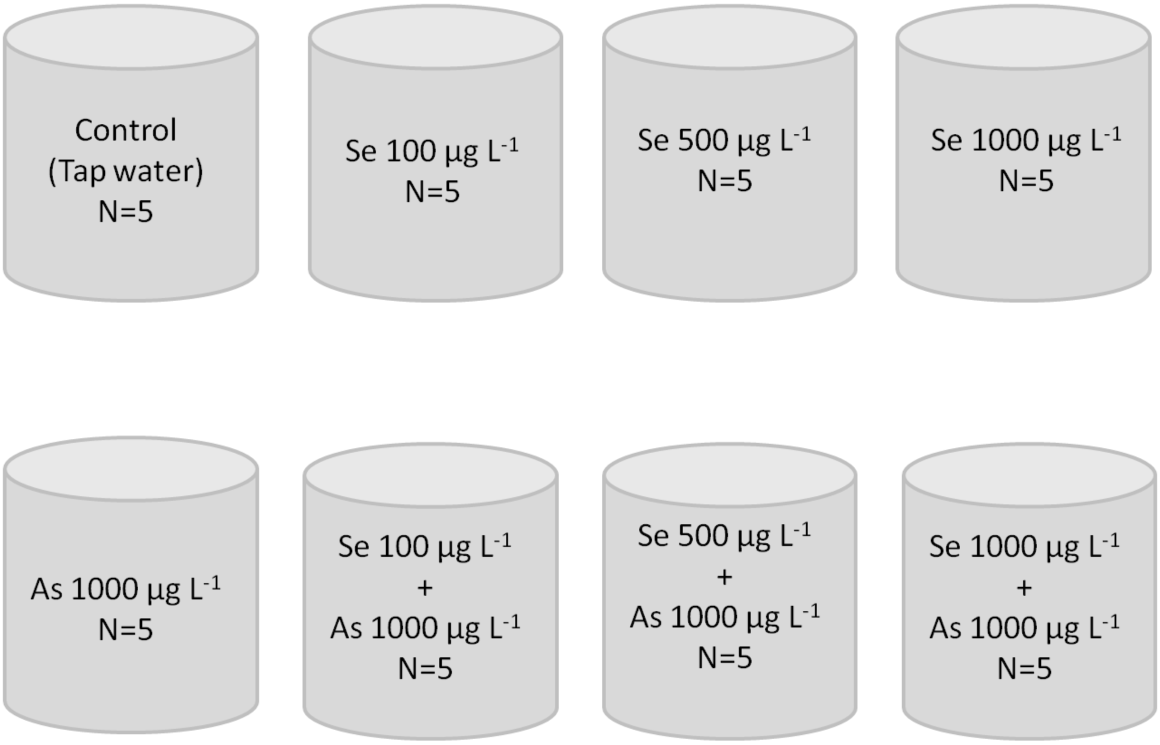


Figure VI.1. Experimental design

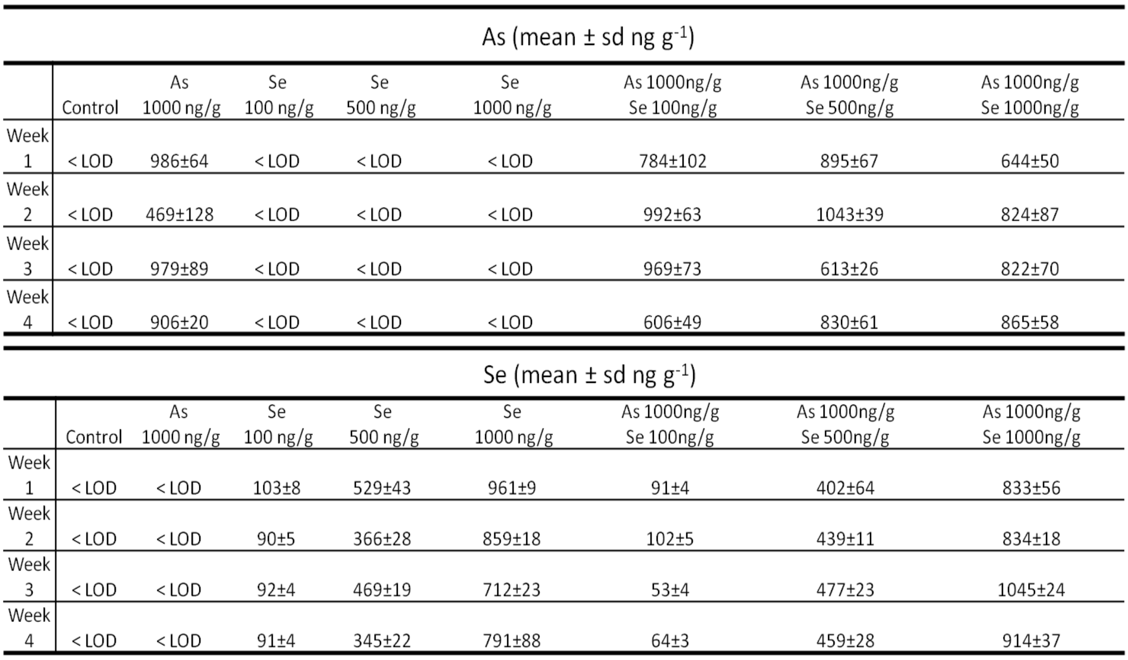
VI.3. Results and discussion

VI.3.1. Arsenic and selenium analysis

Water

Water samples were collected every week to monitorize the levels of the target elements available in water. Samples were analyzed by ET-AAS and the results obtained are summarized in table VI.I. With respect to control tanks, As and Se were not detected (< LOD). The results from the tanks with different concentrations and/or mixtures of the both elements are in agreement with the nominal concentrations. Moreover, in general the variability found in Se and As concentrations in the water throughout the experiment was not conclusive or statistically significant. However, weekly variability found in the concentrations of the elements could be related with adsorption to the internal walls of the tanks, uptake by microorganisms or some lost by evaporation [24].

Table VI.1. Concentration of As and Se (Mean ± SD ng g^{-1}) measured in water samples during 4 weeks. LOD – limit of detection; LOQ – limit of quantification.



As (mean ± sd ng g^{-1})

	Control	As 1000 ng/g	Se 100 ng/g	Se 500 ng/g	Se 1000 ng/g	As 1000ng/g Se 100ng/g	As 1000ng/g Se 500ng/g	As 1000ng/g Se 1000ng/g
Week 1	< LOD	986±64	< LOD	< LOD	< LOD	784±102	895±67	644±50
Week 2	< LOD	469±128	< LOD	< LOD	< LOD	992±63	1043±39	824±87
Week 3	< LOD	979±89	< LOD	< LOD	< LOD	969±73	613±26	822±70
Week 4	< LOD	906±20	< LOD	< LOD	< LOD	606±49	830±61	865±58

Se (mean ± sd ng g^{-1})

	Control	As 1000 ng/g	Se 100 ng/g	Se 500 ng/g	Se 1000 ng/g	As 1000ng/g Se 100ng/g	As 1000ng/g Se 500ng/g	As 1000ng/g Se 1000ng/g
Week 1	< LOD	< LOD	103±8	529±43	961±9	91±4	402±64	833±56
Week 2	< LOD	< LOD	90±5	366±28	859±18	102±5	439±11	834±18
Week 3	< LOD	< LOD	92±4	469±19	712±23	53±4	477±23	1045±24
Week 4	< LOD	< LOD	91±4	345±22	791±88	64±3	459±28	914±37

Tissues

After 21 days of exposure to the different concentrations of As and Se, the mortality rate of the *C. fluminea* was less than 7% for all conditions assayed. These results are in agreement with dedicated literature since freshwater clams are known to be very resistant to a large diversity of contaminants [15, 25, 26]. The DG of *C. fluminea* was excised from the remaining parts of the whole body B and, after sample dissolution, extracts of both parts were analyzed by ET-AAS to assess the As and Se concentrations accumulated in these portions. Regarding the As measurements in the controls (figure VI.2), the results show that although this element was not detected in the water samples (table VI.1) it is present in control organisms soft tissues (DG= 10.91±0.43 ng mg^{-1}; B= 8.21±0.33 ng mg^{-1}). A possible reason is that the freshwater clams had already some As when they were collected in the Tagus river which is supported by the concentrations found in T0 organisms (DG=7.8±0.7 ng $mg^{-1;}$ B=10.1±1.6 ng mg^{-1}). The concentrations of As found in control organisms are in agreement with data reported in literature [15; 27]. Although the organisms were acclimated in clean water over one month, in our laboratory, they still had As in their constitution which suggests that this organisms seems to be able to regulate this element [27]. We must also notice that the Tagus river is known to be highy polluted due to industrial explorations which discharg through the water several contaminants such as arsenic, mercury, cooper, zinc, cadmium, nickel which are found in the river sediments [28], and that therefore could it be the source of this element found in the clams collected.

The amount of As found in the control samples is similar to the one found in the tissues of the clams that were exposed to 1000 µg L^{-1} of As, which indicates that introducing higher amounts of this element in the water column did not increased the accumulation of these element in the soft tissues of *C. fluminea*. The statistical analysis show that there are no significant differences ($p>0.05$) between the amount of As found in the control soft tissues (B) and in the soft tissues (B) from the clams exposed to As 1000 ug L^{-1}. Thus, data suggests that there is a bioregulation mechanism for As in this freshwater clam which permits that the As concentration in the tissues remains constant independently of higher amounts of As being added to the water. Similar findings have been reported by Sebesvari et al [29], in their works where they report that As concentration is maintained constant in *C. fluminea* soft tissues with the organism continuously uptaking and excreting As being these element actively regulated.

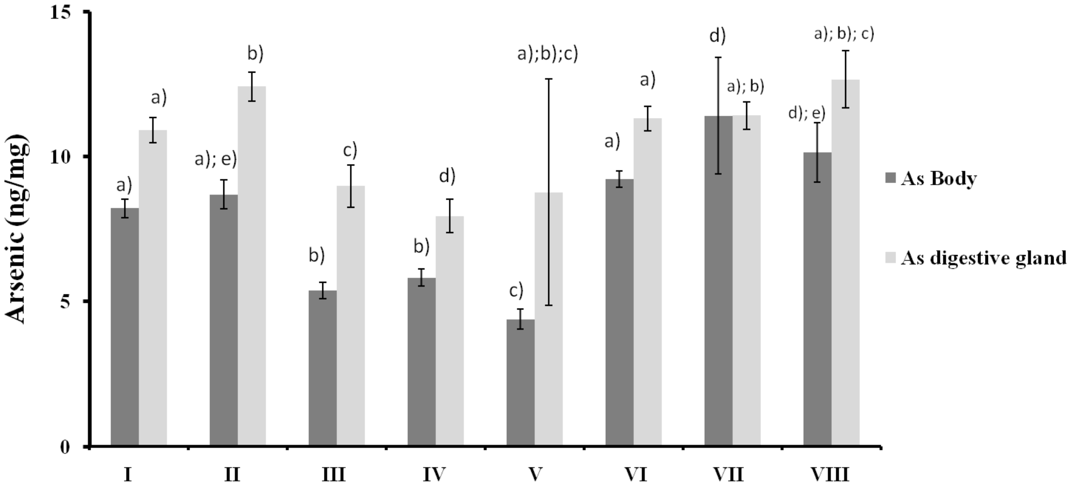


Figure VI.2. Concentration of arsenic found in the freshwater clam tissues body (B) and digestive gland (DG) after 21 days of exposure (median±SE). In the comparation between B-B and DG-DG the data shown is significantly different if letters are different for $P<0.05$. I – Control tank; II- As 1000 ng g^{-1}; III – Se 100 ng g^{-1}; IV – Se 500 ng g^{-1}, V – Se 1000ng g^{-1}, VI – As 1000 ng g^{-1}+Se 100 ng g^{-1}, VII - As 1000 ng g^{-1} + Se 500 ng g^{-1}; VIII – As 1000 ng g^{-1} + Se 1000 ng g^{-1}

The bioaccumulation of As in the clam tissue is higher in the DG than it the remain parts of the whole body. This supports for the existence of a bioregulation mechanism, since it is known that the DG has metabolic and excretion functions [16, 24] .The analysis of Se (figure VI.3) shows that this element was not detectable in the control samples being only detectable in the clams that were exposed to this element. The Se concentrations found in tissues increased according to tested concentrations in the water. Data shows that Se accumulates in the organism´s soft tissues with higher levels in the B than in DG. Fournier et al [17] reported similar findings, where after exposing *C. fluminea* to different species of Se, selenomethionine, a organic species, was found to be the most bioaccumulated form followed by selenate and selenite, both inorganic species.

With respect to the tested binary mixtures, the results shown in figure 3, suggests that the presence of As did not have any significant effect on the bioaccumulation of Se. There were no significant differences ($p>0.05$) between the amounts of Se found on the clam tissues exposed singly to this element and those exposed to the binary mixture. In fact the results obtained for As and Se in the clams that were exposed to the mixture and those exposed singly to each element are not statistically different ($p>0.05$) which may suggest that there is no antagonistic effect between these elements. Nonetheless, the results from the organisms exposed singly to Se show that the basal concentrations of As (similar to controls) decreased significantly ($p<0.05$) to levels below the controls as a consequence, these results may suggest that the presence of Se had some effect in diminishing As concentrations in the clam´s soft tissues. A possible explanation may rely on the species of As present within tissues, where the mechanism of detoxication can be triggered depending on the chemical form present. In previous studies [30-32] a similar antagonist was reported in rabbits after their exposure to inorganic forms of As and Se however to the best of our knowledge no similar studies were performed in bivalves. Given that we added to the water column an inorganic As species it is possible that Se does not interact with the inorganic forms of As but is capable to interact with organic species. Since we do not know the form of As basally present in the clams, it is essential to develop further speciation studies to clarify if the species of As has some influence on the Se ability to interact with As.

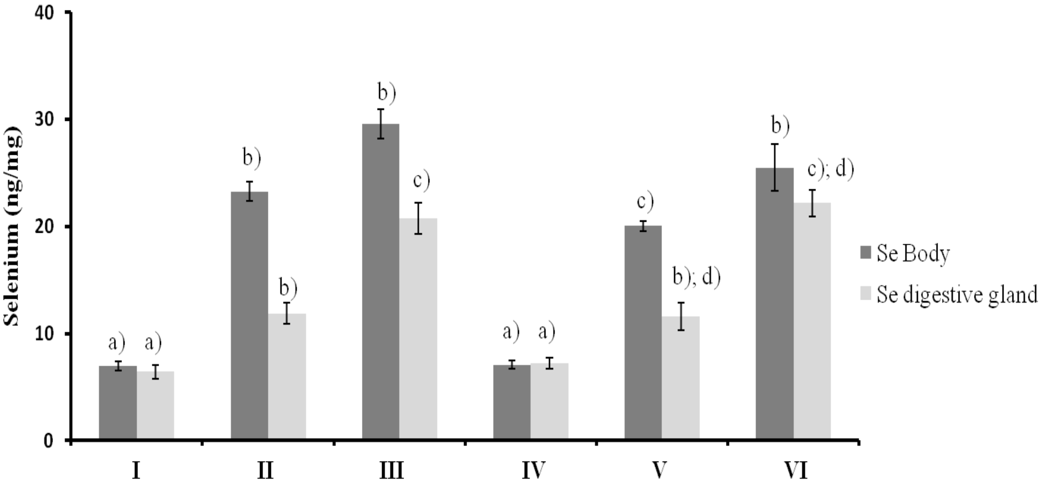


Figure VI.3 Concentration of selenium found in the freshwater clam tissues after 21 days of exposure (median±SE). In the comparation between B-B and DG-DG the data shown is significantly different if letters are different for $P<0.05$. I – Se 100 ng g^{-1}; II- Se 500 ng g^{-1}; III – Se 1000 ng g^{-1}; IV –As 1000 ng g^{-1} + Se 100 ng g^{-1}; V – As 1000 ng g^{-1} + Se 500 ng g^{-1}; VI – As 1000 ng g^{-1} + Se 1000 ng g^{-1}

Histology and histochemistry

The results from histochemical evaluation (figure VI.4) show that As granules are present in all the freshwater clam soft-tissues (figure VI.1). However, when comparing with the other tissues, the concentration of granules is higher in the digestive gland cells, which is in agreement with data already reported [24].

It was not possible to establish a correlation between the As and Se granules and the different concentration used not even between the presence and absence of selenium in the tissues. The histological and histochemical results add complementary information on the elements distribution in the organisms and the tissue damage caused by the studied elements such as the degeneration of the DG cells [24].

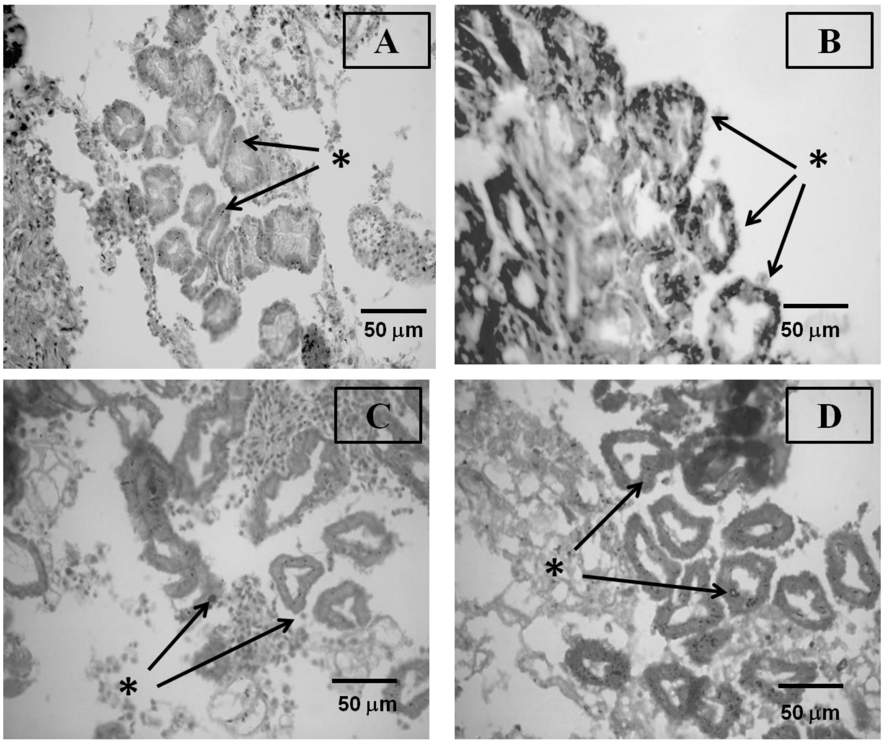


Figure VI.4. Microscopic images of *C. fluminea* digestive gland (400X magnify) after arsenic granules staining with castel's method and counter staining with nuclear fast red.
A –Control; B – As 1000 ng g^{-1}; C –As 1000 ng g^{-1}+Se 500 ng g^{-1}; D- As 1000 ng g^{-1}+Se 1000 ng g^{-1}. * Arsenic granules

VI.4. Conclusions

The present study focused on the effects of two elements, As and Se, in the freshwater clam *C. fluminea*. Selenium is bio-accumulated in the clam tissues, showing higher concentrations in some parts of the body (e.g. muscle, gills) than in the digestive gland. In contrast, As seems to be efficiently regulated by the clams and accumulates in higher concentrations in the digestive gland than in the remains parts of the whole body, which is in agreement with its metabolic and excretion function. Histochemical and histological studies complemented the information revealing higher concentration of arsenic granules in the digestive gland supporting the results.

Regarding the binary mixtures of the elements, the results suggest that no interaction exists with inorganic species for triggering an eventual mechanism of detoxification. However, the added Se seems to have an effect on the basal As concentration, diminishing As levels which may suggests the existence of a possible As detoxification mechanism dependent on chemical forms of the elements. Thus, there is a future need to perform speciation analysis in order to understand which Se and As species are involved and how they interact to clarify the existence or not of a detoxification mechanism.

VI.5. Acknowledgements

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CONCLUSIONS AND FUTURE PROSPECTS

In this work it was developed and optimized an ET-AAS-based technique for the determination of Se in biological samples. It was found the conjunction of $Pd(NO_3)_2$+H_2O_2.as the best matrix modifier, since of the modifiers tested, the best results were obtained with $Pd(NO_3)_2$ and it was demonstrated that the use of H_2O_2 increased over 66% the lifetime of the graphite tubes, avoiding the build-up of carbonaceous residues Therefore the use of H_2O_2 combined with $Pd(NO_3)_2$ is highly recommended if high amounts of organic matter are expected to be introduced into the graphite furnace for Se measurements. The developed ET-AAS technique was used in all the Se determinations performed in this work (chapter II, III and VI).

The USAED technique was firstly reported in 2004 and it was developed in this work for the Se extraction in biological samples (plankton, yeast, mussel and oyster tissue) and food supplements. It was demonstrated that this method could be successfully applied for total determination and speciation of Se (chapter II and III) using HPLC and Electrothermal Atomization. The enzymes used with the USAED procedure to extract Se from biological samples must be carefully chosen, because they must be robust enough to extract Se from the different matrixes but preserving at the same time the integrity of the species. Of all enzymes tested (Protease XIV, substilisin A and trypsin) protease XIV, an enzyme cocktail, was the one performing best. Therefore protease XIV is the recommended protease-type enzyme to be used for USAED treatment in samples such as the ones referred in this work. In addition, the developed methodology was also proved to be effective in speciation studies, since it was possible to extract Se from Se enriched food supplements, maintaining the integrity of the different species without their interconversion as it was reported in the USAED -HPLC- ET-AAS method (chapter 3). With this technique Se(IV), Se(VI), SeMet, SeMeSeCys, and $SeCys_2$ were successfully separated and quantified. The survey of Se content in enriched food supplements showed that total Se content was in agreement with the manufacturers values (within an error of 30%), being. SeMet and Se (IV) the most common species found. One of the analyzed enriched supplements had erroneous information about the Se species present. It was referred in the label the presence of SeMet when in fact only Se(IV) was found. An increment on legal controls should be enforced on the manufacturers of Se enriched food supplements to avoid the often unrealistic claims made for their products.

After developing analytical techniques to extract, detect and speciate Se from biological tissues a method to identify possible expressed proteins, due to selenium or arsenic exposure in living organisms, was still needed. In order to fulfill that gap a new, fast and clean approach was studied for the identification of proteins by PMF after ultrasonic assisted enzymatic digestion with immobilized trypsin in glass beads and magnetic particles (chapter V). It was verified that the utilization of ultrasonic energy lowered the activity of the immobilized trypsin by a 71% and 39% in the glass and magnetic particles, respectively. For this reason the reuse of the immobilized trypsin after ultrasonication is not advised. With the methodology developed the proteins BSA, OA, CA and α-lact were all identified by PMF and PFF. On the other hand, it is suggested a simpler method for the enzymatic digestion with immobilized trypsin, namely shacking in a vortex, which allows the same speed eficiency as the UP does, even for more complex samples such as the human plasma.

The methodologies described in chapters II, III and V, where developed in order to establish analytical tools to study the effects of exposure to Se and As in biological systems and consequently trying to answer the question of wether is possible to use selenium as an ecological solution to diminish or avoid the effects of arsenicosis due to the exposure to high concentrations of arsenic in drinking waters. A first approach to answer this question was developed in chapter 6, where it was found some evidences that suggested a detoxification mechanism for As in the freshwater clam *C. Fluminea*. The results have shown different reactions on the exposure to As and Se, since Se is bio-accumulated being more concentrated in the muscle, gonads and gills, while As levels are bio-regulated being more accumulated in the digestive gland, that has an excretion function. The histological analysis had shown arsenic and selenium granules distributed along the mussel soft tissue, confirming the presence of this elements.

In the present work the foundations for a successfully study of the As detoxification mechanism have been established. The preliminary results obtained with *C. fluminea* reveled to be promising. In fact, the studies about this interesting relation between As and Se are far for being concluded. Regarding future applications, speciation studies should be performed in order to study the As and Se species involved in the bio-accumulation, bio-regulation and detoxification mechanism on *C. fluminea*. The USAED-HPLC-ET-AAS method, should be applied for Se species determinations and could be optimized and extend for As speciation analysis. Another interesting study, in the freshwater clams, could include the study of proteins that are over-expressed due to the exposure to metals; and to identify the peptides to which the metals are binding. The proteins could be fractionated and separated by chromatographic and gel electrophoresis methods and then identified by peptide mass fingerprint after ultrasonic assisted enzymatic digestion with immobilized trypsin in magnetic particles. These studies could reveal the identification of new biomarkers for metal toxicity and the proteins involved in the As detoxification mechanism.