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ICP-MS and MALDI-TOF: an integrated approach in the study of metal-protein interactions in biological samples

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ACRONYMS

AS3MT Arsenic 3-methyltranferase

AsB Arsenobetaine

betaME beta-mercaptoethanol

BSA Bovine Serum Albumin

CA Peptide Mass Fingerprint

DMA Dimethylarsinic Acid

DMMT Dimethylmonothioarsenite

DTPAA Anhydride of Diethylenetriamine-Pentaacetic Acid

DTT Dithiothreitol

ESI-MS Electrospray Ionization-Mass Spectrometry

EtHg Ethylmercury

GSH Glutathione

HCCA alpha-Cyano Hydroxy Cinnamic Acid

HPLC-DAD High-performance liquid chromatography-Diode Array Detector

IAA Iodoacetamide

ICP-MS Inductively coupled Plasma Mass Spectrometry

MALDI-TOF Matrix Assisted Laser Desorption/Ionization Time-of-Flight

MeHg Methylmercury

MMA Monomethylarsonic Acid

MMMTA Monomethylmonothioarsenite

MT Metallothioneins

MW Molecular Weight

MWa apparent Molecular Weight

MWCO Molecular Weight Cut-Off

PMSF Phenylmethanesulfonyl Fluoride

PV Parvalbumin

SA Sinapinic Acid

SEC Size Exclusion Chromatography

TEAB Tetraethyl Ammonium Bromide

TFA Peptide Mass Fingerprint

TMAO Trimethyl Arsine Oxide

TRIS Tris(Hydroxymethyl)aminomethane

Ve Elution volume

ICP-MS and MALDI-TOF: an integrated approach in the study of metal-protein interactions in biological samples

Sara Crotti

Chapter 1

1.1 Heavy metals

1.1.1 Arsenic

Elemental arsenic is a metal insoluble in water, while the solubility of its salts is strongly pH dependent. Arsenic can assume several oxidation states (−3, 0, +3 e +5) depending on the oxido/reductive conditions present. For example, in the surface waters, arsenate is the most common form, while the arsenite is more likely to exist under anaerobic conditions (such as ground waters).

In the terrestrial crust As concentrations are relatively low (2ppb) but locally it can increase due to industrial activities (used as semiconductor or wood preservative use) or agricultural uses (as insecticide and herbicide). Usually it is found in association with iron, oxygen and sulphur (as pyrite, chalcopyrite, arsenopyrite).

In developed countries arsenic can be found in contaminated foods (Ruangwises et al. 2012; Li et al. 2011) and drinking waters. An arsenic compound, 4-hydroxy-3-nitrophenylarsonic acid, is used to promote growth, feed efficiency and pigmentation and to control dysentery in pigs and chicken (Sierra Alvarez et al. 2010). The arsenic levels in some countries is preoccupying, e.g in the north and western regions of Bangladesh, there are millions of people who drink water with more than 50ppb of arsenic. Karagas (1998) estimated that up to 12% of available water resources (due tosurface weathering or underground deposits) in the US has As concentration >20ppb. This is twice the recommend value (10ppb expressed as Maximum Contaminant Level, MCL) adopted from the Environmental Protection Agency (EPA) in 2001 and the US Environmental Protection Agency (USEPA) in 2006. In Italy local contaminations have been reported in Lazio and Campania regions by Dall'Aglio (Dall'Aglio 1996). In other countries the levels of

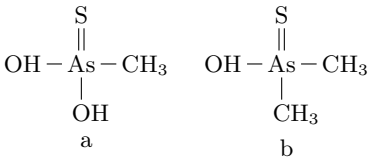
arsenic contamination can reach very hazardous concentrations, as in the Indian subcontinent (in particular in the West Bengal). Here water (and consequently water cultivation such as rice) are the main sources of human exposure (Samal et al. 2011; Rahman 2011). and in these regions the toxicological effects have been reported since 1983.

Even if it is well known in the literature that the arsenic toxicity is species specific, all arsenic values used in legislation refer only to total arsenic, there are no existing regulations that highlight the problem of arsenic species.

Interaction with organisms and detoxification pathways

Arsenic metal has a strong affinity for sulfur and some pentavalent As-S compound have been identified in the biological samples (Hansen et al. 2004). These compounds include generally small molecules bounded to As and no amino acids seem to be involved. *Vice versa* As(III) is more prone to bind thiol groups in peptides and proteins (glutathione (GSH) and metallothioneins, MT) *in vitro* (Kanaki et al. 2008; Schmeisser et al. 2004; Jiang et al. 2003). Amongs the arsenical-proteins species identified *in vivo* up to now, there are phytochelatin complexes in plants (Raab et al. 2004), arsenic-glutathione complexes in rat bile (Gregus et al. 2000) and an arsenic-hemoglobin complex in rat plasma (Naranmandura et al. 2008) are the most significative examples. Recently, the interaction of As(V) compounds with organic biomolecules has been detected. Raab et al showed the ability of As(V) to bind biomolecules after activation by sulfide and they identified the dimethylarsinothioyl-glutathione complex (DMMT(AV)-GSH) in cabbage plants (Raab et al. 2007a), while Suzuki et al. demonstrated the existence of DMMTA(V)-GSH bound in vitro, supporting the data with a theoretical calculation (Suzuki et al. 2008).

The chemical structures of (a) monomethylmonothioarsenite (MMMTA(V)) and (b) dimethylmonothioarsenite (DMMTA(V)), are reported below:



a b

The metabolic pathways of arsenic after the its intake have been extensively studied. Inorganic arsenic is readily absorbed in the organism after oral assumption (more

than 50% of bioavailability), while smaller absorption percentages are obtained after pulmonary and topical exposure. Once As reaches the central compartment (the blood stream), it is captured from the liver where it is subjected to phase I and II metabolic reactions. The first accepted model of metabolic pathway has been proposed by Challenger (Figure 1.1, left panel) and it consists of a sequence of reduction and methylation reactions (Vasken et al. 2004; David et al. 2001). This model has been revised several times, due to its illogical sequence of reactions, and in the 2006 Naranmandura proposed a more logical model (Figure 1.1, right panel). In this model, the arsenic species are bounded to proteins during the methylation process. The methylation is catalyzed by a specific enzyme such as the Arsenic 3 Methyltranferase (AS3MT).

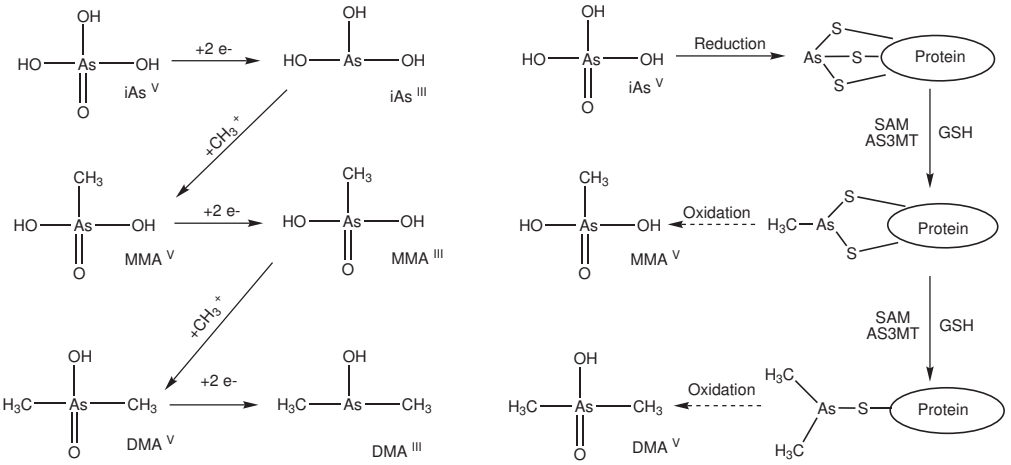


Figure 1.1: Classical model of arsenic phase I metabolic process in mammals proposed by Challenger (Challenger 1945) (left panel) and the revised model proposed by Naranmandura (Naranmandura et al. 2006) (right panel).

The final fate of arsenic metabolites is excretion in urine and, approximately 50% of excreted arsenic is DMA, 25% is MMA and the remainder is inorganic (Buchet et al. 1981) In marine fish, the predominant arsenic form is the arsenobetaine (AsB) ("Arsenic Transformations in Short Marine Food Chains studied by HPLC-ICP MS"). This species is not directly synthesized from arsenate, but is first produced by microbial species (Cullen et al. 1993) and then ingested by marine animals (Edmonds et al. 1988)). Among the other organic arsenicals found in seafood there are MMA, DMA and trimethyl arsine oxide (TMAO), and arsenocholine (AsC) and arsenosugars (Schmeisser et al. 2004).

The chemical form and oxidation state of arsenic is very important with regard to its toxicity. The toxicological impact is divided into two classes: acute and chronic toxicity.

In acute toxicity, high levels of exposure determine a fast absorption into the organism. The primary species of absorbed arsenic (e.g. the inorganic form) and its metabolites are linked to the toxicological effects. The biological mechanisms of acute toxicity have been identified in the disactivation of the citric acid cycle (Krebs cycle) enzymes. As(III) strongly complexes the pyruvate dehydrogenase enzyme drastically lowering the adenosine triphosphate (ATP) synthesis, that is the primary source of energy in the cells. Cell damage and death are direct consequences.

As(V) is a biological competitor of phosphate and it is able to enter in the oxidative phosphorylation chain giving rise to the arsenate ester of ADP, which is highly unstable and undergoes hydrolysis via a non-enzymatic pathway (Dixon 1996). In acute As(V) intoxication, the first damage observed is the blocking of oxidative phosphorylation (the final step in the ATP synthesis process) with consequent cell death.

In the chronic toxicity onset, the methylation pathway plays a key role in damage onset. Because inorganic arsenic is metabolized in organisms, chronic exposure automatically results in chronic exposure to its metabolites (methylated forms). Skin, lung and bladder carcinomas (Chen et al. 2003; Yu et al. 2000) have been detected in people cronically exposed to arsenic, due to chromosomic alteration (clastogenic damage). Actually, there is not a complete knowledge about the damage mechanisms of chronic arsenic exposure and in general, it is well assumed that the acute toxicity is species dependent, chronic toxicity still not as well defined.

The cellular toxicity of arsenic has been positively used as an anticancer treatment. Arsenic trioxide (As_2O_3) is used against acute promyelocytic leukemia (APL) and other hematological malignancies (Shen et al. 1997; Munshi 2001).

1.1.2 Mercury

Metallic mercury is liquid at ambiental temperatures. It is silvery-white coloured and, due to its cohesive forces, it gives rise to spherical droplets. It is considered a poor conductor of heat and a fair conductor of electricity. If used with addition of gold, silver, and tin, it can give rise to more resistant alloys called amalgams.

Mercury is naturally occurring in the Earth's crust. It can be found mainly in the form of sulphides and cinnabar (HgS) is the main source of this metal. Human activities have a strong impact on mercury release into the environment (e.g. with combustion of fossil fuels, waste disposal and industrial activities). A drammatic example of the impact of mercury release into the environment is the Minamata Disaster, in which a prolonged release of methylmercury in the industrial wastewater (from 1932 to 1968) produced over

3,000 victims in the local populace of mercury poisoning.

Even if the local contamination of mercury can be considered as relatively low, some sites that are naturally polluted can be indicated. This is the case of Mediterranean sea, in which about half of world mercury resources are located. In a wide study accomplished in 2005, it has been calculated that the mercury concentration in this sea was about 10^{-9}M. Despite these levels, the impact on marine life is high. This is due to the characteristic *bioaccumulation* process which occurs for mercury. This term defines the increase in the concentration of a substance (toxic or not) that occurs in the food chain. This can be the consequence of several process, such as (i) the inability of environmental processes to break down the substance (also known as persistence), (ii) food chain accumulation and (iii) the low rate degradation/excretion of the substance by the organisms exposed.

The European Food Safety Agency (EFSA) released some recommendations about the fish consumption, evidencing the risk of mercury intake.

Exposure and detoxification pathways

It is well recognized that the main sources of human exposure to mercurials, mainly methylmercury (MeHg), is the consumption of polluted fish. Methylmercury is extremely dangerous since it bioaccumulates in predatory fish, which eat the smaller ones that are exposed to environmental mercury. Bioaccumulation occurs in the lipidic tissues, due to its lipophilic characteristics. Other sources of contaminations for humans are vaccines containing thimerosal (Aschner et al. 2010) that release ethylmercury (EtHg) after administration and inorganic Hg(II) released from dental amalgams (Goering et al. 1992; Mutter 2011). Mercury, differently to arsenic, exists in biological organisms mainly as a limited number of species, all reported below with a charged state at physiological pH.

$$\underset{\text{a}}{Hg^{++}} \qquad \underset{\text{b}}{^{+}Hg-CH_3} \qquad \underset{\text{c}}{^{+}Hg-CH_2CH_3} \qquad \underset{\text{d}}{CH_3\text{-}Hg-CH_3}$$

The Hg_2^{2+} cation, with a strong metal–metal bond, is not known to be important in biological systems and for this reason this species will not be discussed further. The oral absorption of metallic mercury (Hg^0) is low and in case of single-dose administration (acute exposure) there are no toxicological consequences. The inorganic soluble mercuric compounds are absorbed by up to 10% (WHO, 1991) and it was estimated that the bio-accessibility of MeHg is similar (9−17%) (Cabanero et al. 2007). Once absorbed, mercury

enters into the central compartment, and about half of the absorbed metal binds to plasmatic proteins, while the other half enters into red blood cells. Both Hg(II) and MeHg bind to nucleophilic groups of biomolecules, especially with endogenous thiol-containing molecules, such as glutathione (GSH), cysteine, homocysteine, N-acetylcysteine, metallothioneins, and albumin (Bagger et al. 1991). The affinity constants are of the order of 10^{15} to 10^{20}, while its affinity constants for oxygen- or nitrogen-containing ligands (e.g., carbonyl or amino groups) are lower ($<10^{10}$).

Mercury is rapidly distributed into the organism and metabolism occurs as methylation and demethylation reactions until excretion with feces and urine (Figure 1.2).

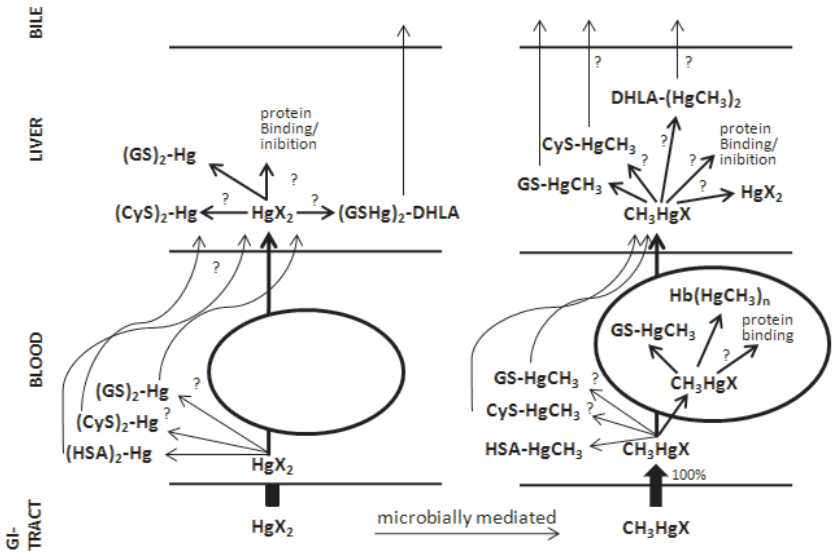


Figure 1.2: Schematic of mercury absorption, metabolism and distribution in mammals. The circle represents erythrocytes (blood cells). Reproduced from (Gailer 2007).

Chronic exposure (even if at low levels) to these compounds may lead to mercury intoxication (Shigeo et al. 2007). The brain is the main target of MeHg and EtHg (Marcelo et al. 2011). and for this reason foetus and children are particularly sensitive to its effects. The damage to the brain cells leads to intellective retardatio, ataxia, hand and feet numbness, damage to hearing and speech disruption. Moreover, mercury is a potent endocrine distruptor and its effects have been observed in humans and animals (Tan et al. 2009). Both species easily penetrate the blood-brain barrier thanks to their aliphatic moiety and, here they undergo to de-methylation processes (Burbacher et al. 2005). The accumulation of organic mercury into the nervous system is the direct cause of its damage,

while (Aschner et al. 2010) the presence of inorganic mercury in the brain seem to have no effect.

To monitor mercury exposure in humans, its levels are analyzed segmentally in hair, and the history of exposure can be obtained (Cernichiari et al. 1995). The hair to blood ratio is approximately 250:1 and usually, up to 80% of the mercury found is in the form of the MeHg-cys complex.

As for arsenic, international legislation concerning food safety is mostly based on total mercury concentrations (expressed as maximum limits). Considering the differences in the toxicity of metal species, changes in the normative implementation with the addition of species-specific limits are strongly recommended from the scientific community.

1.2 Proteins

1.2.1 Structure and function

Proteins are macromolecules responsible for most of the functions of living organisms. They are involved in catalysis, in transport and storage, they give mechanical support to the cells and tissues and are active in the immune response, DNA replication and transmission of signals. From a structural point of view, proteins are a heteropolymer constituted of α-amino acids (AA). In nature substantially 20 different amino acids are encountered. The proteic backbone is build up with a condensation reaction between the amine and the carboxylic groups to form an amide, or so called *peptide bond*, with characteristic planarity, due to the sp^2 structure. In the absence of any other interactions, the sequence of bound amino-acids should give rise to a linear structure. This is not the case of proteins since the residue R, also called side chain, is responsable for proteins three-dimensional structure by the onset of secondary interactions such as: hydrogen bonds, disulfide bridges, electrostatic and hydrophobic interactions. For example, serum proteins in their native environment, tend to adopt a structure in which polar amino acids are exposed, and non-polar amino acids are on the interior.

The three-dimensional structure that a protein adopts in its natural environment, is called *native* structure and four different levels of classification exist (Figure 1.3).

1. The sequence in which amino acids appear defines the *primary structure* of proteins.

2. The local arrangement of some AA sequences to give an alpha helix or beta strand is called *secondary structure*.

3. The complete folding of proteins in its native environment, defines their *tertiary structure*.

4. The assembly of more proteic moyeties determines the *quaternary structure*.

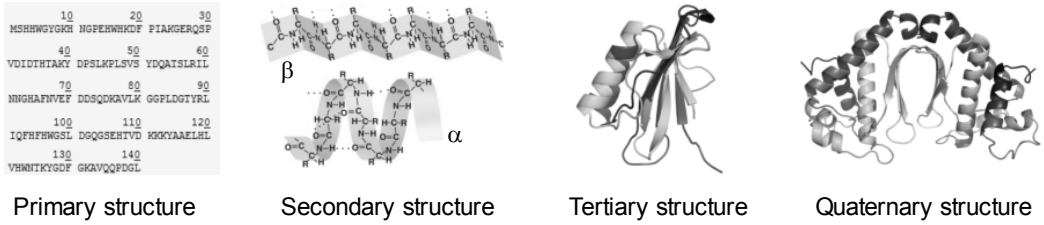


Figure 1.3: Schematic of the four structural classification of proteins.

Beside the structural classification, proteins can be divided into many groups in relation to their function, their localization, or their expression. On the basis of their final shape, all existing proteins can be classified into three families: (i) globular proteins, (ii) transmembrane proteins and (iii) fibrillar proteins.

1.3 Metallomics and metalloproteins

The proteome is defined as the sum of all the proteins in an organism, a tissue, or in a sample. The study of the human proteome started about ten years ago and the availability of new and powerful instrumental approaches led to important results on the knowledge of the physiopathological role of proteins in human body. Recently, the term *metallome* has been introduced (Mounicou et al. 2009) to refer to the study of the entire type of the metal and metalloid species present in a cell or tissue (Figure 1.4), while the metallic component interacting with proteins is called the *metalloproteome* (Mounicou et al. 2008). Metalloproteomics is a branch of proteomics that use all possible approaches to address the expression of metalloproteins/metal-binding proteins and the quantification of their changes in biological systems during physiopathological events.

The importance of metals in the organisms physiology is evident when taking into account that about 1/3 of all well structurally-determined proteins are metal bound. This binding is essential for several protein functions such as gene expression regulation and alteration, signalling processing, metabolic pathways and metal homeostasis, cellular respiration, muscular movement and antioxidant defense. Proteins can contain an element

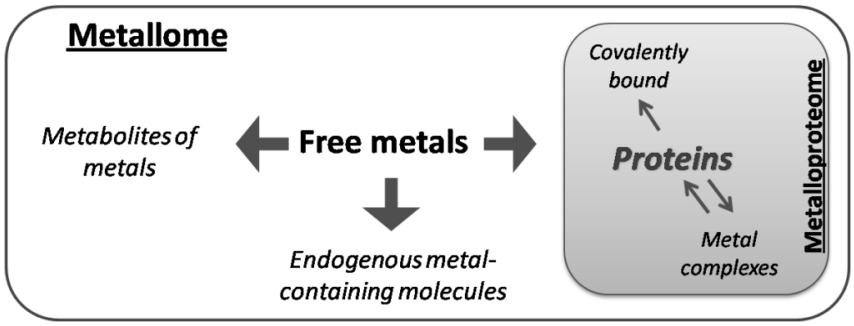


Figure 1.4: The metallome of an organism is represented by all different forms assumed by the metals.

bound in a covalent way or as a coordination complex of a certain thermodynamic stability and kinetic inertness (Figure 1.4). In the last case, the protein-metal interaction is highly dynamic and can be subjected to changes in response to physiological/pathological conditions. The term *metalloproteins* is used to define those that strongly bind metal ions (but not exclusively in a covalent way) and don't undergo dissociation during sample manipulation (e.g. during protein purification), while *metal-protein complexes* is used for those that may lose their metal(Maret 2010).

In the first group are some well characterized domains, such as iron porphyrins, zinc fingers, cysteine-rich residues and EF hands for calcium complexation (Figure 1.5). These structures have been selected during the evolution to specifically bind metals essential for any living system.

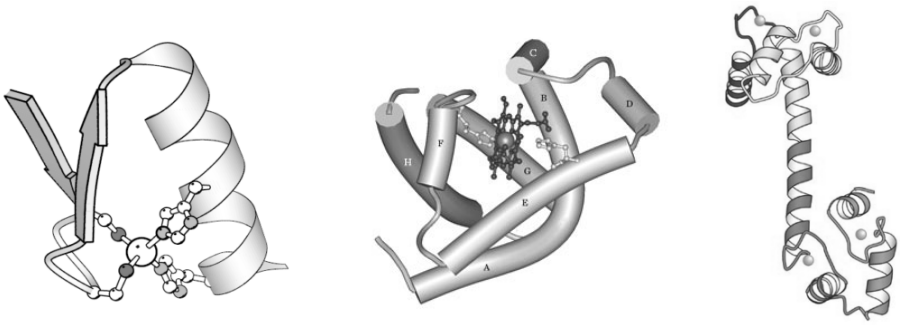


Figure 1.5: Structure of some characteristic domains in protein designed for specific metals coordination: (a) zinc finger, (b) heme-iron porfirins and (c) EF hands.

Metalloproteins can bind several metal atoms contemporary. Metallothioneins (MT)

are a peculiar group of metalloproteins which are characterized by low molecular weight (less that 10kDa) in which up to 20 cysteine residues can be arranged in two domains containing 7 equivalents of metal (e.g. zinc, copper or cadmium). They are naturally occurring in organisms, but their synthesis considerably increases in presence of heavy metals. The presence of a metal domain into a protein sequence may not necessarily give rise to binding with the expected metal. Depending on the environment, the bond with other metals can be observed. For example, if yeast are left to grow in a cobalt(II)-rich medium, the incorporation of cobalt instead of zinc into the zinc fingers of alcohol dehydrogenase (ADH) is observed (Andree et al. 1968).

In the second group falls many proteins involved in pathological state development. Metals interacting with proteins have been indicated as the cause of the onset of some chronic diseases like Alzheimers (AD), Parkinson's or diabetes. In these pathologies, an altered interaction of some transition metals (such as Cu or Zn) with target proteins occurs. For example, transition metals have been found to be responsible for enhancments in the fibrillar assemblies of the amyloids-beta in AD (Yankner et al. 2009), while iron ions are found to enhance the oxidative damage in the case of altered ferritin metabolism (Watt 2011). In diabetes it was observed that Cu and Fe are both implied in the oxidative modifications determining the increase of lipoxidation and glycooxidation products, associated with the pathogenesis of diabetic complications(Baynes et al. 1999).

Chapter 2

2.1 Elemental analysis

Sample preparation procedures traditionally employed in elemental analysis are, in general, quite simple. For the total element quantification, samples are processed to obtain liquid solutions. If the original sample is already in liquid form (e.g. environmental waters) it is generally sufficient to perform sample filtration before the analysis. For solids samples, digestion procedures with strong reactive agents (HNO_3, HCl of H_2O_2) are employed, that lead to the complete solubilization of the matrix. When elemental analysis faces the speciation field, softer conditions are used. Extractive procedures and sample storage are the most important steps in speciation analysis and their evaluation is discussed below:

Storage of collected samples

Before applying any sampling procedure, the physicochemical properties of the metals and the metal species must be considered, to avoid oxido/reductive reactions, precipitation or absorption onto vessels walls. It is usually recognized that by adding acids (such as hydrochloric or nitric) there is a metal preservative effect. This is not the case in speciation analysis, in which strong acids are generally avoided. In the literature, As and Hg speciation in biological fluids or tissues have been reported and different strategies are proposed. Feldmann et al. studied the acidification effect on the arsenical species determination in urine samples. The authors divided some urine samples into two classes, one with added 0.1M HCl and the second one was under unacidified conditions. Both have been stored at different temperatures (-20, 4, and 25°). Unfortunately, the effect of HCl addition on the recoveries of arsenicals has shown the degradation of all arsenical and organoarsenical compounds (Feldmann et al. 1999). A different approach has been followed by Gallagher et al. who developed a storage method for drinking water samples.

They studied the EDTA/acetic acid treatment together with the effect of temperature and oxygen exposure. The best method for arsenical species preservation in samples is based on the use of 0.1M acetic acid and 0.5 ng/μL EDTA. The EDTA preservative action is usually based on the complexation of metals prone to induce oxido/reductive process, such as Fe ions (Gallagher et al. 2001). Even thought it usually used also as protein preservative, no data on its effect on the metal-protein complexes are reported.

Moreover, for certain metal ions in the sample bottle adsorption on the surface or the cation exchange can occurs. For example mercury ions are highly unstable in the presence of any plastic surfaces. For this reason, glass preferred. On the contrary, since arsenic is present in the materials used for glass production, arsenic released from glass must be verified as a possible source of contamination.

For both metals, the storage at low temperatures and in the dark can effectively reduce any alteration.

Samples extraction

Metals and their species are usually present in low concentrations in environmental and biological samples and they are usually classified as micronutrients. Biological samples cannot be analyzed directly since they require at least a filtration step. Plasma samples, even if in liquid form, must be centrifuged to remove cellular components and fibrillar proteins that can cause liquid aggregation (such as fibrinogen). Solid tissues are processed for analytes extraction and usually manual or mechanical (not chemical) digestion are employed to achieve a satisfactory recovery. For the extraction of As, Hg and their species, several procedures have been proposed and a summary is reported in Table 2.1.

A great variety of the extraction procedures are found in the literature, in which both aqueous solutions (in acid or alkaline conditions) and organic solvents have been used. Their use depends on the differences in the nature of the sample and the target species. For example, water (Raml et al. 2006) is used for the extraction of thioarsenical compounds in oyster tissues, giving satisfactory recovery, while methanol is used for arsenolipids extraction (Arroyo et al. 2010). For the mercury extraction, the protocol proposed by Rodrigues et al. is of particular interest, since the addition of thiolic groups to the extraction buffer leads to the prevention of the metals loss during the extraction procedure.

Table 2.1: Some extraction protocols proposed for the speciation analysis of As and Hg.

Extration solutions	Species	Reference
water	Methylarsenicals	Raml et al.,2006
trypsin at 37°C	AsB, DMA, MMA, As(V)	Pardo et al.,2001
MeOH	Arsenolipids	Arroyo et al.,2010
methanol/water	AsB, DMA, MMA, iAs	Sanchez et al.,2006
TMAH at 60 °C	Arsenosugars	Conklin et al.,2006
Acetic acid with MAE	MeHg	Davis et al.,2004
βME , l-cys and HCl	Hg, MeHg	Rodrigues et al.,2010
6M HCl and toluene	MeHg	Vazquez et al.,1997
1M H_2SO_4	Hg, MeHg	Niessen et al.,1999
25% (w/v) (TMAH)	MeHg, EtHg	Gao et al.,2008
6M HCl and 0.25M NaCl	Hg, MeHg	Reyes et al.,2009

Sample analysis

Mercury speciation analysis in biological tissues is usually performed by coupling LC, GC or CE to ICP-MS (Balarama Krishna et al. 2010; Rocha et al. 2001). Usually, Hg speciation with GC as a separative technique, has the disadvantage of requiring a time consuming derivatization step. On the other hand, the advantage of GC CP-MS is the achievement of complete ionization of all the sample introduced into the plasma, while with LC ICP-MS only a little fraction of the eluant reaches the plasma after the nebulization process. LC methods have been developed using C8 or C18 columns, leading to the separation of mercury species as a function of their different lipophilicity (Cairns et al. 2008).

Arsenic speciation in environmental and biological samples has been reported in several works using LC ICP-MS (Byrne et al. 2010; Lin et al. 2008; Grotti et al. 2008). The main difficulty faced in performing identification and quantification of all As compounds is related to the great number of species usually presents in biological samples. This is the direct consequence of the complex metabolic pathway for As which changes for different organisms. The most widely used separation techniques are based on anion and the cation exchange columns. The separation is based on the different pKa values exhibited by the arsenic species. Anion exchanges column methods were developed to separate As(III), As(V), MMA(V), DMA(V), and AsB, while cation exchange has been used to separate AsB, AsC, TMSO and Me_4As (Gong et al. 2002; Xie et al. 2007).

2.2 Proteins analysis

Proteins are extremely complex and fragile and usually undergo fast degradation under non physiological conditions. For this reason, their manipulation requires specific procedures.

Storage of the collected samples

When a biological tissue is sampled, the storing procedure is extremely important: they must be conserved from zero to −130°C, depending on the protein. For example, relatively stables proteins such as those belonging to the heat shock family proteins (e.g. metallothioneins) can be easily stored at zero degrees, while enzymes must be stored at least at −80°C (xx). Such storage is not easy to achieve and samples are kept in special cryocontainers under liquid nitrogen. In some cases, water can be removed from biological tissues (lyophilization) to stop bacterial growth, but with this method of storage the metallic moiety can move with the water.

Samples extraction

Proteins extraction is a complex procedure in which many factors must be taken into account. Firstly, in the tissues sampled most of proteins are not free, but present into the cytosol, in the subcellular organelles or in the intermembrane layer. This means that cellular lysis must be performed before protein recovery. To lyse eucaryotic cells, both physical and mechanical approaches are used.

Table 2.2: Techniques used for cells and tissues disruption.

Lysis Method	Apparatus	Uses
Mechanical	rotating blades	cells and tissues
Liquid Homogenization	potter homogenizer	cells
Sonication	ultrasonic probe	cells and soft tissues
Freezing cycle	freezer or dry ice/ethanol	cells
Manual grinding	mortar and pestle	strong tissue

During the extractive procedure, proteins folding and their oxido/reductive state preservation are factors that can affect the protein function. For correct protein folding, pH, ionic strength and temperature all are important parameters to keep under control. For example, if a large quantity of salt is added to a proteins solution, the ions can disrupt the normal protein-water interactions causing changes in the protein's structure. Under

such conditions a *salting out* process occurs and proteins precipitate in unsoluble pellets. Protein structures can be altered by changes in pH also, since the there is a variation in the charges present in different parts of the protein, and by increases in temperature (e.g. the irreversible unfolding of egg whites proteins during the cooking). In general, during proteins manipulation, the best conditions are: pH 7-8, ion strength 50-100mM and temperature 0-4°C.

2.3 Metal-proteins analysis

To detect trace level concentrations of metalloproteins hyphenated techniques (LC, GC, GE) coupled to MS are largely employed. In these applications the metal species should be kinetically inert (no metals exchange should occur during the separation procedure) and thermodynamically stable (they must be representative of the equilibrium conditions in the original sample). To study the metal-proteins interaction with chromatographic techniques, several parameters must be evaluated:

1. proteins must not be exposed to denaturing conditions during the procedures;
2. metals species must be preserved for the correct determination of the interactions to be studied;
3. both proteins and metals recovery must be evaluated in the extraction procedures;
4. the chromatographic separation must not break the metal-protein interaction.

Metal–biomolecule complexes present in biological samples have been analyzed with reverse phase (RP), size exclusion (SEC) and affinity chromatography (AC). Apart from C18 stationary phases, that require organic solvents as the eluent, both SEC and AC are highly compatible with the use of ICP-MS.

SEC chromatography has been used by several authors working with metalloproteins proteins (Pei et al. 2009; Persson et al. 2009). All eluting peaks are monitored by ICP-MS and the size of biomolecules coupled to a metal have been determined after a column calibration procedure. Proteins identification have been obtained by some authors using only the calculated size (Haraguchi et al. 2008; Munoz et al. 2005; Huang et al. 2004). This method can not be accurate, due to the low resolution of this kind of chromatography. In the same manner, few investigations on the metal species interacting with proteins have been performed. Moreover, the protein interactions and the metal species responsible for the interaction should be identified with more accuracy, as the physiol/pathology of the

organisms is based on their specific interactions. If there is not a deep characterization of both metallic and proteic moiety, the risk to drawing incorrect conclusions is high.

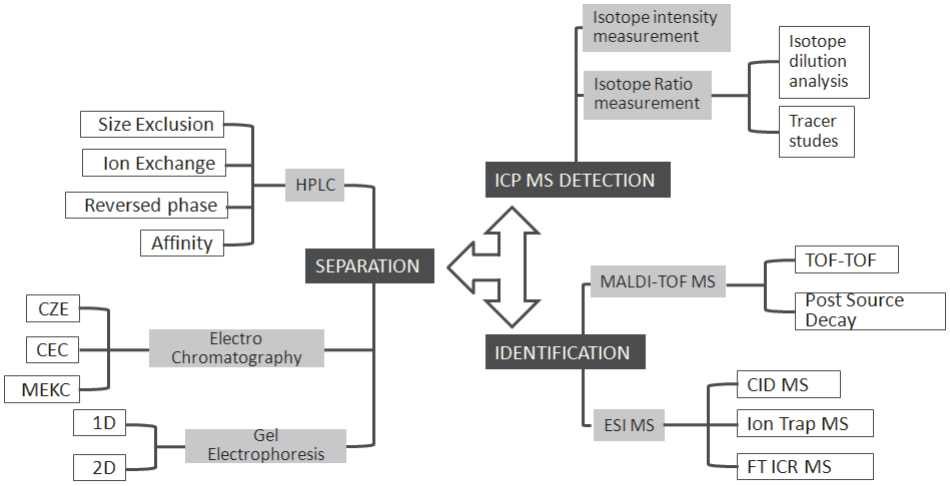


Figure 2.1: The multi-methodological approach to follow in the metal/proteins interaction studies.

This observation suggests that an integration with other techniques should be used and a good strategy to adopt is that summarized in Figure 2.1, reproduced from Szpunar 2005.

2.3.1 Extraction of metal-protein complexes

The specific reactivity of different metals is reflectet in the different stability levels of complexes formed with proteins. As a consequence, the same extraction procedure could not be useful to investigate more than one metal complex. An interesting approach has been tested by Schmidt de Magalhaes et al., that investigates up to 11 different extraction protocols for horse chestnuts (*Aescullus hippocastanum L*). For each procedure, the samples were cut, dried in an oven, and resuspended in deionized water or 1M Tris–HCl (pH 6.8). Among the tested procedures, the most interesting are listed in Table 2.3, with respective their details.

Considering the protein recovery, the protocol f, in which samples have been manually ground in TRIS buffer and immersed into a sonic bath seems to be the most suitable. The same positive results can be found for the iron quantified in the proteic extract (380 pg). Anyway, both for Cr and Mn recoveries give low and for these metals other

Table 2.3: Protein and metals recovery obtained reported by Schmidt et al. 2007. The extraction procedures are: (*a*) water extraction, (*b*) buffer extraction, (*c*) samples manually ground in water, (*d*) samples manually ground in buffer, (*e*) manually ground in water and sonic bath, (*f*) manually ground in buffer and sonic bath.

	Extraction results			
Protocol	Proteins (mg/g)	Cr (pg)	Fe (pg)	Mn (pg)
a	1.81± 0.06	41.8± 1.5	294± 3	22.68± 0.25
b	1.51± 0.06	21± 2	494± 2	25± 2
c	3.04± 0.08	36.0± 1.5	360± 4	135.0± 1.6
d	3.59± 0.2	15.0± 0.5	370± 4	120.0± 1.1
e	3.38± 0.09	<0.054	115± 5	<0.06
f	5.5± 0.1	13.0± 0.5	380± 2	11.0± 0.6

extraction protocols seem to be more effective (prot. *a* and *c* respectively). This clearly demonstrate the complexity of developing a method for the study of more than one metal at the same time.

Buffer additives such as, mercaptoethanol (Sussulini et al. 2007) or dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF) and sodium dodecyl sulfate (SDS) have been reported in the literature. PMSF in particular is used to preserve the proteic extract from the attack of metalloprotease enzymes (Ahmed 2004), that can be activated during the sample manipulation. All these additives should be used carefully, since in some cases, metal-protein purification resulted in the isolation of a protein that does not contain any metal ion since strong chelating (e.g. EDTA), or reducing (DTT) agents have been used (Krezel et al. 2007).

Finally, the possible alteration of samples during analysis must be considered also. This has been evaluated by Wolf et al. They investigated sample variation during repeated analysis in a timespan of 10h and the high variability in the metalloprotein stability has been evidenced. As conclusion of the work, they suggest to analyze the samples immediately after preparation or thawing (Wolf et al. 2007).

2.4 Instrumental techniques

2.4.1 ICP-MS

Plasma-based sources are widely used today and the different kinds of instruments are characterized by the mechanism that is used to induce the plasma generation. A plasma is the fourth state of matter and is constituted of gaseous ions and free electrons. Plasma-

based sources used today are: Inductively coupled Plasma (ICP), Glow discharge Plasma (GDP) and Microwave induced plasma (MIP).

The plasma generation in an ICP source is obtained with an oscillating magnetic field working with megahertz frequencies (27MHz). This field is generated by a metal coil (usually copper) placed around the end of a plasma torch (Figure 2.2). A radio-frequency electric current of 1000-1500 W is applied to the coil for plasma generation. The argon gas is left to flow into the torch during the onset of RF potential, and a spark is applied causing gas ionization. Electrons stripped from Ar are then accelerated by the magnetic field and collide with the neutral gas molecule, generate more ions and more electrons. The mixture of neutral species and ions colliding several time per sec, leads to a stable plasma characterized by different temperature zones (up to 10000°K) that are responsible for sample degradation.

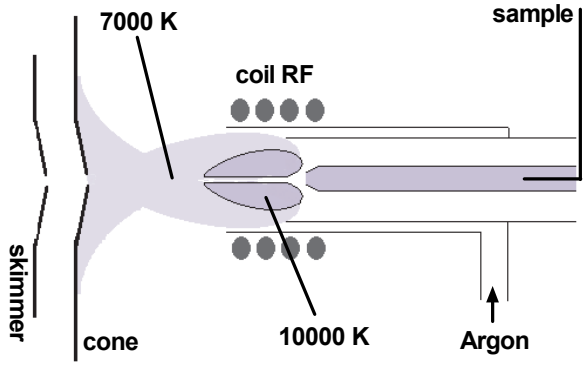


Figure 2.2: Schematic of a plasma torch, in which a gradient of temperatures starting from the injector line are originated. The analytical zone at 7000°K is responsible for analyte ionization.

The schematic representation of a plasma torch reported in Figure 2.2 shows the shape assumed by the plasma between the injection line and the mass spectrometric entrance. In this picture, the region at 10000K and the analytical region of plasma at 7000K have been evidenced. ICP-MS is widely used in several fields, from the environmental analysis (48% of applications) to semiconductor analysis (33%) but, recently, the most interesting applications under development are in clinical, forensic and food analysis (10%). The main characteristics of an ICP source is its capability to ionize practically all the elements present in the periodic table. Using different instrumental setups, a wide variety of information can be obtained: the total elemental content, the speciation of

elements (with hyphenated techniques) and the isotopic ratio of some elements.

The ionization process

In ICP-MS gaseous, solid and liquid samples can be analyzed. In the case of liquid samples, their delivery into the source is obtained with systems generally constituted of three components:

1. Liquid handling systems: there are pumps for operation at low pressures (peristaltic pumps) or in high pressure (HPLC systems). They transport the liquid sample to the nebulizer,
2. Nebulizer: is the component that generates the primary aerosol from the introduced liquid flow. The aerosol generation is pneumatically assisted with argon,
3. Spray chamber: it is placed between the nebulizer and the torch and acts as a dimensional filter, allowing to arrive at the plasma only droplets with low aerodynamic diameter (5/6μm).

The presence of a dimensional filter has as a direct consequence of lowering of the amount of analyte loaded into the plasma source. It was evalued that only a small percentage (from 2 to 5%) of the sample is effectively ionized.

On the other hand, the formation of an homogeneous aerosol has the advantage of give the best ionization percentages, without lowering the plasma performance. Travelling through the different heating zones of the plasma, the aerosol is subjected to several modifications and it is transformed from a liquid into ions, as reported in Figure 2.3. The process is a sequence of sample desolvatation, degradation, atomization and ionization.

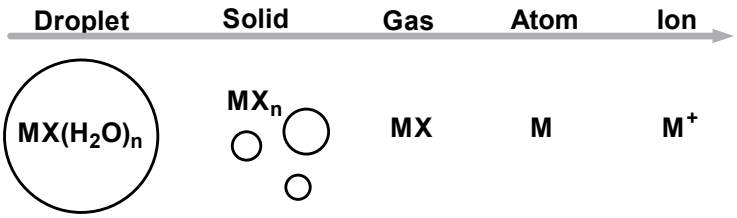


Figure 2.3: Aerosol modification into the plasma torch from the droplet to the ion entering into the mass spectrometer.

The ionization process is carried out in the analytical zone of the plasma, here the plasma temperature reaches 6000–7000 K. Under these conditions all molecules are reduced to ground-state atoms. These atoms are then transformed in ions due to collisions

with argon ions. Argon is extensively used since it is an inert gas with a first ionization potential (15.47eV) that is higher than most other elements (with the exception of He, F and Ne) meaning that Ar ions can ionize these elements. Moreover, its ionization potential is lower than the second ionization potential of most elements, limiting the onset of 2^+ charged ions. It is important to mention that although the ICP source is generally used for positive ions generation, negative ions are also produced. However, the ion lens optics and the final detector of most commercial instruments are not designed to focus and detect them, unlke the past when the first instrument (Elan500) had this option.

Ion detection

Once the ions have been generated, they are separated as a function of the mass to charge ratio (m/z) before reaching the detector. To obtain ion separation, their physical properties are used. In particular, when an ion in motion enters into a magnetic field, its trajectory can be modified. Magnetics fields with the field intensity inversely proportional to the square of the distance are named quadrupolar fields. The majority of commercial ICP instruments are coupled to quadrupolar mass analyzer. A quadrupole was one of the first electro-dynamics mass analyzers introduced. In this mass analyzer ions are separated using a direct current (DC) field and a radio frequency alternating current (AC) applied on opposite pairs of the four rods. If the desired ion enters into the quadrupole, it is driven by the alternating potential onto the rods. The ion selection is obtained by varying the AC/DC ratio on each pair of rods, and for each couple of AC and DC values, just one ion is allowed to pass to the detector, whereas the others will be unstable and, passing through the spaces between the rods, will be ejected from the quadrupole (Figure 2.4, ion m/z =101).

The ions emerging from the quadrupole are then introduced into the detection system, that converts and amplify the ionic current into a electric signal (mass spectrum).

The conventional way for liquid sample introduction in ICP-MS is based on the continuous nebulization of a solution with measurements at the steady-state of signal. In the case of hyphenation techniques such as LC or GG discrete amounts of analytes arrive at the instrument and, as a consequence, the analyte signals have not a steady state but a time-dependent signals (transient signal). In order to describe a chromatographic peak with enough accuracy, a minimum of 10 points are usually requred. To do this, the parameters to take into account are both the number of spectra per time unit (sampling frequency) and the time spent sampling each ion (dwell time). These parameters are correlated and the acquisition frequency is generally adapted to the amplitude of the

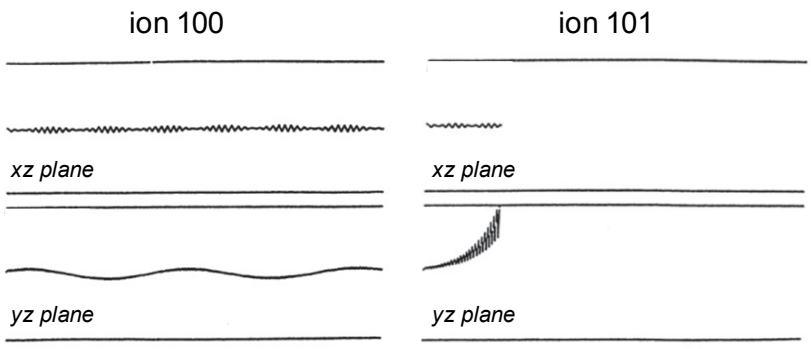


Figure 2.4: Ion motion into a quadrupolar mass filter. The ion at m/z is stable and reach the detector, while the ion m/x 101 is gathered out of the analyzer.

peak to be sampled.

2.4.2 MALDI-TOF

Matrix Assisted Laser Desorption/Ionization (MALDI) coupled to Time-of-Flight (TOF) analyzer and Electrospray ionization (ESI) coupled to Ion Trap mass analyzer are considered the systems of choice for researchers in the proteomics field. The use of ESI-MS/MS lead to fast sequencing of enzymatic digests of complex mixtures of proteins. The advent of nESI source technologies lead to drastic improvement in the sensitivity and has opened the way to high-throughput analysis. On the other hand, MALDI-TOF has the advantage of analyzing both intact proteins (at high molecular weight) and sequencing the enzymatic digests. In addition, the coupling of MALDI with off-line separation techniques lead to competitive results with ESI-MS.

The ionization process

The MALDI laser source produces ions in the gaseous phase from solid samples and compared to ESI, very low amounts of multicharged ions are formed. Laser sources for analysis of biological samples have been tested from 1960, but the first instruments lead to extensive sample degradation, and only small molecules were detectable. Karas and Hillenkamp (Karas et al. 1988) were the first to obtain the spectrum of a biomolecule having MW$>$ 10kDa with the addition of a matrix in a mixture with the sample.

Several ways of mixing the sample with the matrix exist and the dried droplet method was the first developed, but the two-layers and the sandwich method are also employed. In the dried droplet technique, before laser ionisation, peptide/protein samples are mixed

with a saturated matrix solution and the mixture is placed to crystallize as small droplets on a conductive target (usually stainless steal). The matrices most frequently used (Figure 2.5) are sinapinic acid (SA), dihydroxy benzoic acid (DHB), or α-cyano hydroxy cinnamic acid (HCCA).

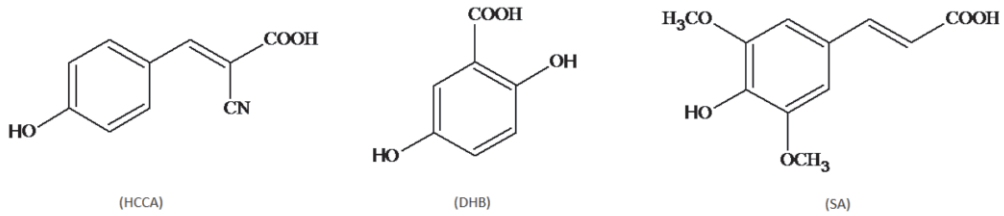


Figure 2.5: Chemical structure of most common matrices used in MALDI sample deposition. SA is used for large peptides and proteins, DHB is used for peptides and nucleic acids and HCCA is used for little molecules and peptides.

In all matrices acidic hydrogens are present (e.g. carboxylic acids) and to obtain good ionization, the sample/matrix ratio must be in the order of 1 to 10000. The sample ionization mechanism is not fully clarified, but it is generally assumed that matrix crystals absorb the energy of the laser shots and transfers it to the sample with proton donor reactions. Since ionization energies needed for the matrix are of the order of 8/9 eV and the laser can give only 3.7 eV of free energy, a more complex mechanisms must be invoked. Actually, two models try to explain the ionization process:

> *Cluster model*: it is assumed that the laser shot produces the ejection of a cluster containing ions pre-formed in the solid sample (Karas et al. 2000).
> *Photoionization model*: based on the pooling of energy captured from the matrix in a few molecules so that the energetical barrier is overcomed Zenobi et al. 1998.

In both models, the resultant process is the chemical ionization of the analytes, that explains the onset of low multicharged states.

MALDI ionization is salt-sensitive, since relatively high buffer or salt concentrations (in the order of millimolar amount) cause loss of ionization and quite poor quality spectra. Samples must be purified before deposition onto the plate. The most commonly used devices are micro column purification (microSPE) made with C18 or C4 stationary phase. The sample is loaded onto the microcolumn and purified in a few steps using acidic deionized water (usually with 0.1% TFA). Once the sample is cleaned, it is eluted from the column with H_2O/CH_3CN solution, mixed with the matrix and deposited.

Ions detection

The Time-of-Flight detector has been for several years the only detector associable to a MALDI source. This is due to the very fast ion production occurring in this source and to the mass range requested for intact proteins ionization. A TOF mass spectrometer is based on accelerating a group of ions into a flight tube under vacuum conditions. The mass (m) of the analytes is determined from the time of arrival. Mass, charge, and kinetic energy (KE) of the ions all play a part in the arrival time at the detector.

Before the introduction of delayed extraction (DE), the TOF resolution was quite low since ions were accelerated out of the ionization source immediately as they were formed, with a broad range of kinetic energies. The delayed extraction allow the ions to cool for some nanoseconds before being accelerated into the flight tube. This generates a discrete amount of ions with a smaller kinetic energy distribution, reduces the temporal spread of ions and increases the resolution. The reflectron has been introduced in TOF systems to increase the path length and to focus the kinetic energy of ions. It is constituted of a sequence of ion mirrors that act as ion repulsor.s Reflectron mode is used for peptides and small molecules and can increase the instrumental resolution up to 10000 (at 1000uma), while the linear mode can be used for medium/high MW proteins. Tandem mass spectra

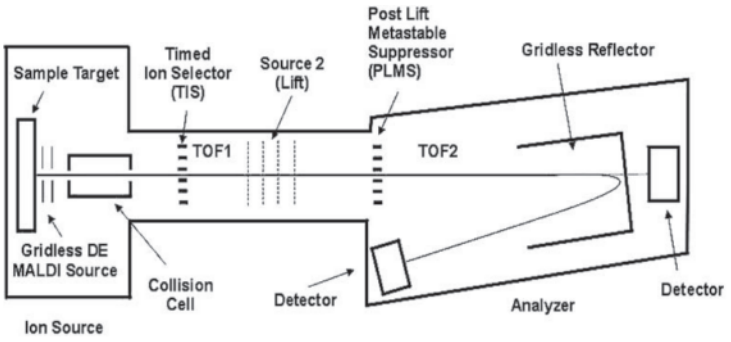


Figure 2.6: Mechanism of MS/MS operated with LIFT technology.

can be obtained with TOF-TOF instruments. In the instrument used for our analysis (Bruker Ultraflex II TOF/TOF) a lift cell is introduced in the ion flight pathway, which is able to select both parent ion and fragments ions. Passing throughout this cell, ions are re-accelerated and then reach the detector at different times (Figure 2.6) producing the MS/MS spectra.

2.4.3 Principles of proteomics

Usually, the proteins sequence (primary structure) can be determined with an enzymatic digestion and subsequent peptide analysis. Two methodological approaches have been developed for protein identification with mass spectrometry: the Peptide Mass Fingerprint (PMS) and peptide tandem-mass sequencing.

PMF

In the first case, the tryptic digest of a protein is analyzed and a list of all m/z is obtained. This list represents the fingerprint of the protein, since the enzymatic cleavage is highly specific and give rise to a sequence of characteristic peptides for each protein. Trypsin, an endoprotease that cut the amino acidic sequence exclusively after the Lys (K) and Arg (R) residues, is largely employed in proteins digestion. The m/z list is compared to those present in databases (e.g. Mascot or ProFound) and the protein identification is associated to a probabilistic score (Berndt et al. 1999; Perkins et al. 1999).

This method is fast and reliable if accurate m/z detection is obtained. Moreover, this method is based on the multiple peptide attribution for unambiguous protein identification.

The main limitations of PMF are in the analysis of complex mixtures of proteins, since low numbers of peptides for each protein can be detected and, moreover, the risk of non significative attributions increases.

Tandem-mass sequencing

In the second approach, the MS/MS spectra of peptide fragments are used for database searching. These spectra are searched against real spectra stored in databases or theoretically obtained fragmentation spectra. Peptide fragmentation with a tandem mass approach follows characteristic pathways and the typical cleavage sites are reported in Figure 2.7. Each cleavage leads to obtain two fragments, the neutral and the charged one, depending on the proton affinities. Only the charged fragment will be detected by the mass spectrometer.

The most common cleavage sites are found at the peptidic bonds which lead us to obtain the b and the y series. Sometime, the onset of side-chain fragmentation or deamidation processes are observed with specific instruments. MALDI source, in particular, can produce more extensive fragmentations with respect to traditional ESI MS instruments (Dupre et al. 2011).

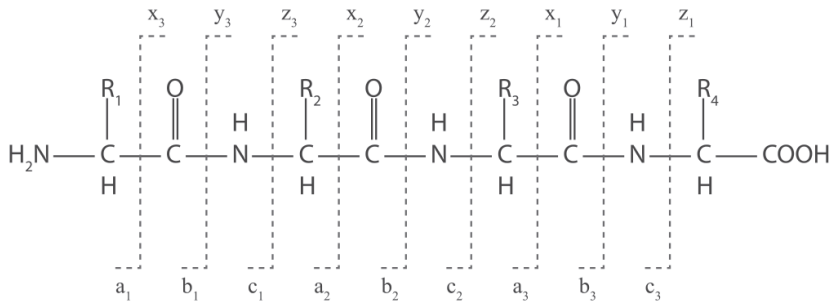


Figure 2.7: Fragmentation typically occurring along the peptide backbone.

The protein sequencing is usually more reliable than PMF since, even in the presence of complex mixtures in the digest, the protein score can be computed with good results. In addition, in the case that no valuable data are obtained, peptide sequences can be deduced via *de novo* sequencing simply by computing the amino acid loss in the fragmentation spectra.

Chapter 3

3.1 Aim

The aim of this work is to study the most suitable conditions for the metal-proteins interaction studies using mass spectrometry-based techniques. ICP-MS has been used due to its advantages in terms of sensitivity and specificity, while MALDI-TOF has been chosen for the proteins analysis, due to its employment in field of proteomics. This study is focussed on the study of metal-proteins interactions either spontaneously occurring, or obtained with labeling techniques. Part of this work has been devoted to study the *in vitro* interactions of proteins-rich foodstuffs with some As and Hg compounds. Both metals are able to interact with proteins, influencing their activity and stability in the biological organisms. The positive results obtained led us to approach the study of *in vivo* interactions, with the analysis of some naturally exposed samples. In a subsequent part, the use of an hetero-atom labeling technique has been investigated. An HPLC ICP-MS method for the identification of intact proteins isolated from painting layers has been developed. The characterization of proteinaceous binders typically used in frescoes and the analysis of some reconstructed pictorial models have been obtained, before to apply the method to unknown paint layers.

Chapter 4

In this chapter, the procedural steps for the development of a method to identify the protein interactions with two heavy metals (As and Hg) will be discussed. The method developed will be used to analyze samples of milk, eggs and fish glue spiked with the metals. The aim is to study the interactions of arsenic and mercury in the presence in these kinds of complex matrix proteins. As the matrices analyzed are liquid, they will be analysed directly, without any extraction procedure. The operative strategy applied consisted firstly of the investigation of the metal (and metal species) binding to biological molecules and then moved to the detection of the proteins. With these aim in mind, chromatographic techniques have been used to separate the proteins in their complex matrix. For the separation of proteins and metal-binding proteins, SEC chromatography has been considered the most apt technique due to its capability to separate large molecules, its simplicity when coupling with ICP-MS and the reduced interaction between the analyte and the relatively inert stationary phase reducing the probability that the metals are stripped out of the complexes. Before sample analysis, all parameters that could affect the reliability of the results have been reduced by optimizing the selectivity, and sensitivity whilst concentrating on preservation of both metallic and proteic moyeties, with particular attention paid to the preservation of any existing metal-protein complex.

4.1 Chromatographic separation of proteins

To achieve a chromatographic separation of proteins and metal-binding proteins with a SEC column, different mobile phases have been tested. Due to the non covalent binding between metals and proteins, all factors that can alter the coordinative bond must be avoided. First of all, since a protein binds metals using different amino-acidic residues present in their structure, to obtain the most stable complex the protein should remain

folded. To keep proteins in their native form, all chromatographic separations have been performed avoiding the use of denaturating agents and, moreover, using buffers and additives that assist protein folding. Finally, due to the coupling with ICP-MS, only compatible buffers have been considered and, among them, we have focused on TRIS and ammonium acetate (NH_4Ac) buffers.

To test the best mobile phase composition for the Biosep S2000 column (250x4.6mm, Phenomenex) the two buffers have been used at different concentrations and with or without the addition of mobile phase modifiers. The modifiers are used to maintain the correct ionic strength in the buffer solution and to avoid electrostatic interactions between column and proteins leading to a lowering of the protein recovery from the column. The parameters evaluated during method development are as follows.

4.1.1 Chromatographic resolution

In a chromatographic separation, the resolution (R) is the distance between two neighboring peaks calculated at the base of the peaks and usually defined by the equation:

$$R = \frac{1}{4} \cdot \sqrt{N} \cdot \frac{a-1}{a} \cdot \frac{k_2}{k_2+1} \tag{4.1}$$

Where α is the separation factor, N is the theoretical plate number and k_2 is the retention factor. It is important to note that, when working with a SEC column, any retention mechanism based on repartition of analytes between the two phases cannot be invoked. This means that k_2 is not alterable to increase the resolution and the alpha coefficient (α) can only be slightly affected by optimizing the pore size and the pore volume of the stationary phase. In our SEC column (Phenomenex Biosep S2000, 4.6x250mm) the particle size is 5µm and pore size is 145Å. The linear range declared by the producer is between 10^3 and 10^5 Dalton. With SEC columns, the resolution can be improved by varying the electrostatic and hydrophobic effects with different mobile phases and by optimizing the flow rate. By lowering the ionic strength, electrostatic interactions between the samples and column surface occur, while at high ionic strengths, hydrophobic effects become dominant. If one of them prevails, the sample components are retained due to the interaction with the packing surface and elute with broadened peaks that affect the chromatographic performances.

Buffer compositions have been studied by varying the ionic strength using different concentrations of ionizable solutes in a range from 10mM to 200mM, by carefully considering the benefit of the increase of salt concentration versus the lowering of ICP-MS performance. In effect, high salt concentrations can cause deposition onto the interfaces

and the inner instrument parts causing loss of sensitivity. To overcome these problems, non volatile salts where tested at the maximum concentration of 25mM, while for volatile salts, concentrations up to 200mM have been tested. The resolution has been evaluated using two globular standard proteins: BSA (bovine serum albumin, MW 66kDa) and CA (carbonic anhydrase, MW 29kDa). Both proteins were diluted in the same buffer used for chromatographic separation at the opportune concentration (see Table 4.1) to obtain equal signals with UV detection. This precaution is necessary to prevent incorrect evaluations of the peak width due to protein load. The proteins elution has been monitored using a Diode Array Detector (HPLC-DAD system, Agilent 1200 Series) operating at λ=280 and 210nm (BW,4nm) with a reference signal at λ 360nm). The λ=280nm is used to detect the absorbance of aromatic groups present in the protein structure, while the λ=210nm is used to detect the absorbance of peptidic bonds. The injection volume was 20μL.

Table 4.1: Concentration of BSA and CA injected into the SEC column for the chromatographic resolution evaluation.

Protein	Concentration (M)
BSA	$1.6 \cdot 10^{-5}$
CA	$3 \cdot 10^{-5}$

TRIS as mobile phase

TRIS (tris(hydroxymethyl)aminomethane) is a commonly used buffer in molecular biology to keep proteins in their native folding (e.g. globular shape). Its buffer range (pH 7-9) coincides with the physiological pH of most living organisms and to obtain the required pH, HCl is used to obtain the TRIS HCl buffer. With TRIS buffer an adequate buffering capacity is reached when working at concentrations at least equal to 25 mM (Durst et al. 1972), lower concentration are not commonly used. Since at a concentration of 25mM, TRIS buffer doesn't provide sufficient ionic strength for protein elution, NaCl is added to the eluent. To obtain the best chromatographic resolution, different salt concentrations and operative flows have been tested. For each condition tested, the chromatographic resolution has been evaluated using the equation:

$$R = \frac{t_2 - t_1}{\frac{w_2 + w_1}{2}} \tag{4.2}$$

Where t_1 and t_2 are the retention time of CA and BSA respectively, while w_1 and w_2 are the peak width at the half height of the eluting protein. In Figure 4.1, the resolution obtained at different NaCl concentrations (0, 10 and 25mM) is plotted against the chromatographic flow tested:

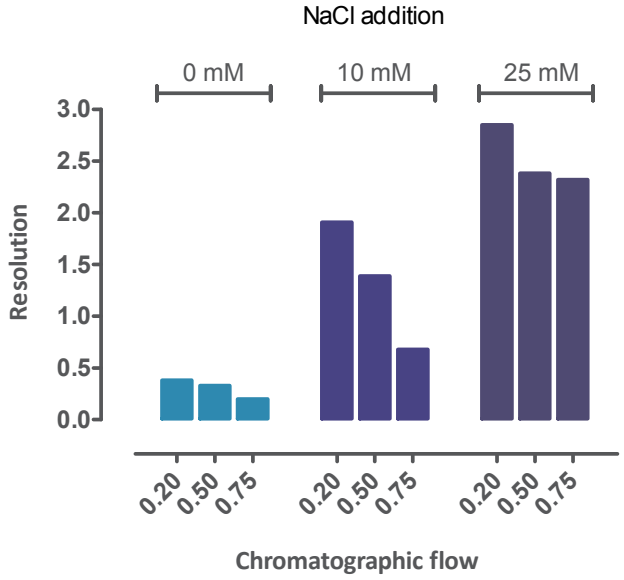


Figure 4.1: Variation in the chromatographic resolution in SEC chromatography with TRIS buffer at 25mM. Different operative flows with NaCl addition have been tested to obtain the best conditions.

With TRIS buffer, the resolution appears dependent both on salt addition (NaCl) and the chromatographic flow. The best resolution (R=2.85) can be achieved when working with 25mM of NaCl at low flow rates (0.2mL/min). Even though in the other conditions the resolution obtained can be considered acceptable (R>1),in this context it is important to work at higher separation performances in order to obtain an accurate size determination. By lowering the flow rate, the separation of standard proteins improves with minimal increases of the peak width. This leads to an improvement in resolution, with a small increase in analysis time. Even though 25mM NaCl resulted better resolution than 10mM, before using this as the definitive solution to the problem, the effect of salt addition to the protein recovery must also be evaluated.

NH_4Ac as mobile phase

Ammonium acetate (NH_4Ac) is an highly volatile buffer not usually employed in molecular biology. Its buffering range is between pH 3.8-5.8 and pH 8.2-10.2. This means that the buffering capacity increases as you move away from pH 7, but if used for protein solubilization, NH_4Ac is adequate for keeping the pH between 6 and 8. This buffer is compatible with ICP-MS instrumentation if present in concentrations up to 0.1/0.2M (similar to those experienced by proteins in biological systems). Due this characteristic, this buffer has been employed to study the effect of ionic strengths > 25mM in our SEC separation (Figure 4.2).

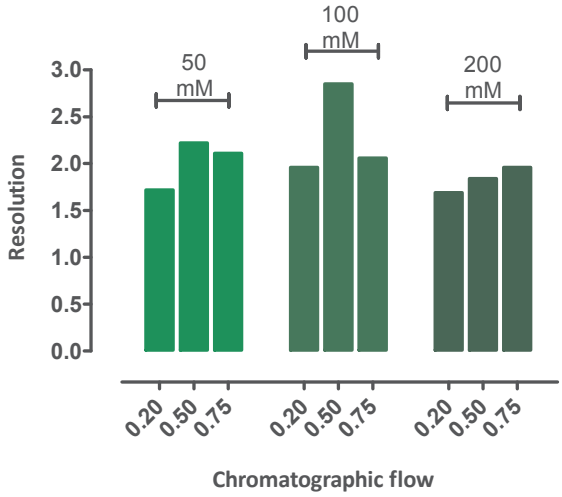


Figure 4.2: Changes in the chromatographic resolution with respect the flow and NH_4Ac mM variation.

The plot of resolution obtained at various buffer concentrations versus the chromatographic flow was used to find the best conditions for NH_4Ac as a mobile phase. As highlighted for TRIS, usually in SEC chromatography the resolution improves with the lowering of mobile phase flow. In the case of ammonium acetate this appears to not be true, since the resolution is worse at low flow rates and the chromatographic peaks shape is highly asymmetric, revealing the presence of an interaction with the stationary phase. The evaluation of protein recovery can demonstrate if these interactions are reversible (high recovery) or not (low recovery). When working with a 100mM buffer concentration, the resolution at a flow rate of 0.5mL/min is comparable to that with TRIS buffer (R 2.85) meaning that the separative performances are equivalent.

4.1.2 Proteins recovery from column

In SEC chromatography non-specific protein adsorption onto the stationary phase can cause a loss of protein recovery, leading to distortion of the peak shape or an enhancement of the retention time, causing underestimation of molecular size. This phenomenon is due to the presence of different functional groups on the protein surface that can interact with the resin surface Arakawa et al. 2010. In case of reversible interactions, if the analyte is not fully recovered it can elute with subsequent injections causing analysis artefacts. If a non-reversible interaction is occurring, there is the possibility of severe loss of analyte during the chromatographic separation. In this case, due to the strong interaction between the column and proteins, the recovery of the analyte is very difficult and so strong eluents or denaturating agents must be employed to clean the column. The problem of reversible interactions can be resolved by using the right mobile phase. In practice, co-solvents can be added to the mobile phase to avoid protein-resin binding and, among them, various salts and amino acids have been successfully used.

Before applying the chromatographic separation developed to real samples, the recovery of proteins injected onto the SEC column was evaluated with the buffers discussed above. This was done by injecting some standard proteins into the instrumentation with and without the column attached to the injection valve, the area obtained after the chromatographic separation was compared with the area obtained after flow injection analysis (FIA) and the recovery % was quantified as:

$$Rec\% = (\frac{A_{SEC}}{A_{FIA}}) * 100 \tag{4.3}$$

Protein recovery has been estimated using the equation 4.2 with repeated 20µL injections (n=3) of BSA and CA with and without the SEC column. The elution of the proteins has been monitored with a DAD at λ=280 and 210 nm.

The NaCl addition that improved the chromatographic resolution seems to have a negative effect on the protein recovery in TRIS buffer (Figure 4.3, left panel). The quantitative recovery desidered (set at 95%) is represented by a dashed line. With NaCl addition the CA recovery is unsatisfactory at all concentrations while for BSA the recovery is under the threshold only with an addition of 25mM of NaCl.

At all the concentrations tested (50, 100 and 200mM), ammonium acetate buffer gave a poor recovery (Figure 4.3, right panel). This clearly indicates the presence of interactions between the proteins and the stationary phase that led to a large protein loss. In addition, the plot shows a clear recovery inversion % between CA and BSA at

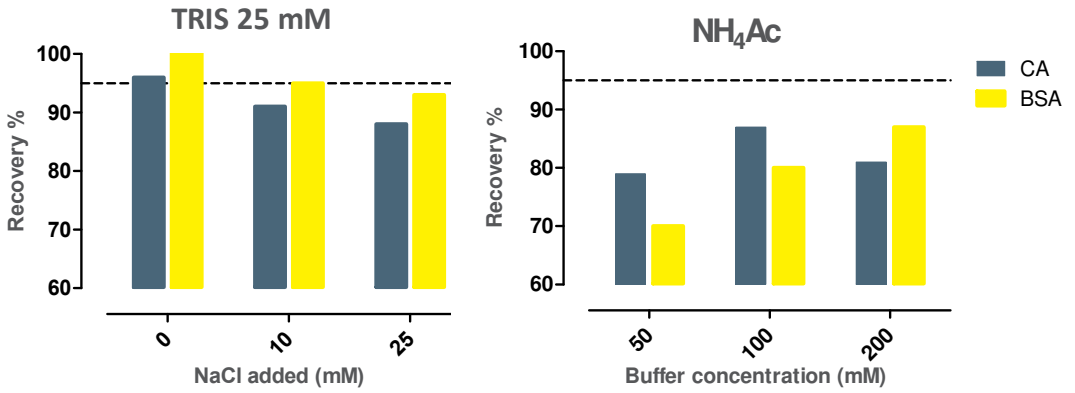


Figure 4.3: Protein recovery from SEC using TRIS and NH_4Ac buffers at different concentrations. The desired threshold (95%) is highlighted with a dashed line.

100 and 200mM buffer concentrations. This suggests that there are selective interactions between the two proteins and the stationary phase in this buffer. For these reasons, this buffer cannot considered appropriate for the aims of this study.

Since TRIS gave good resolution and a better recovery compared to ammonium acetate, we have chosen it for the subsequent SEC chromatography experiments optimize the recovery in the presence of NaCl.

4.1.3 Improvement of protein recovery for TRIS buffer

To improve the protein recovery capacity of the 25mM TRIS buffer mobile phase in the presence of 25mM NaCl, the amino-acid arginine (Arg) has been used as competitor for hydrogen binding. Arginine is a non polarity amino-acid that is used as a refolding agent (Nakakido et al. 2009) in molecular biology due to its ability to stabilize protein folding. This is because when globular proteins are present in the correct folding, their hydrophobic sites are not exposed to the surface, so we can hypothesize that arginine may also prevent hydrophobic binding with the resin surface. Even in this case, due to the final coupling with ICP-MS, a low co-solute concentration (1 millimolar) of arginine has been tested in the 25mM TRIS mobile phase in the presence of both 10 and 25mM of NaCl. The recovery obtained for BSA and CA are reported in Figure 4.4.

As showed in Figure 4.4, when arginine is added to the mobile phase at very low concentrations (1mM), the proteins recovery improves for both BSA and CA. This indicates that the nonspecific protein adsorptions are now occurring much less during the chromatographic separation.

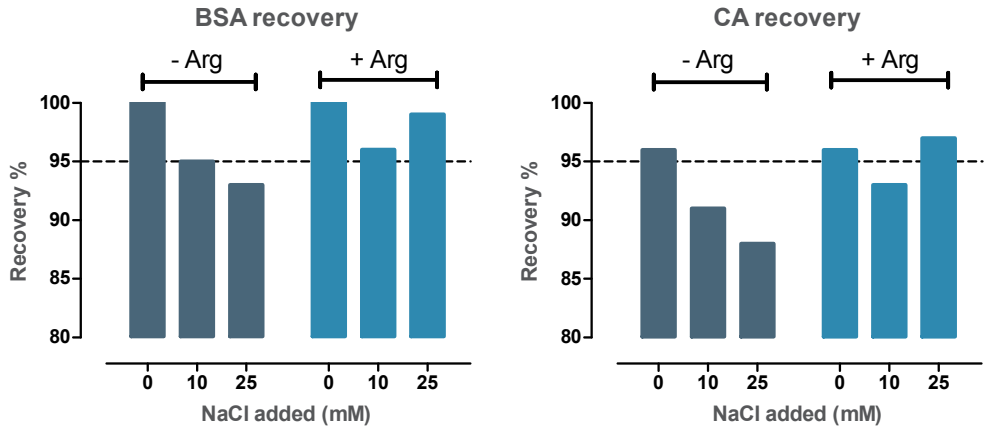


Figure 4.4: Recovery from SEC column with 1mM Arg addition to the 25mM TRIS mobile phase in presence both of 10 and 25mM of NaCl

Considering the performances obtained in terms of resolution and recovery, 25mM TRIS buffer with an addition of 25mM NaCl and 1mM of arginine was finally chosen as the mobile phase. The operational flow rate was set to 0.2mL/min.

4.1.4 SEC column calibration

A SEC column is used to provide quantitative information about the size of eluting molecules. To obtain information about an unknown eluting molecule, the column should firstly be calibrated using a mixture of molecules of different sizes. When working with macromolecules (e.g. proteins or polimes of synthesis) the size may not be not correlated with the molecular weight, since there are several different shapes. For this reason, the SEC separation of proteins should not be correlated to the molecular weight but to the Stokes radius (Rs), as demonstrated by Siegel (Siegel et al. 1966). The Stokes radius (or hydrodynamic radius, RH) represents the radius of a sphere that diffuses at the same rate as the molecule to which it refers. In practice the Stokes radius is usually smaller than the effective radius of a shaped molecule. This means that, with an extended protein like fibrinogen, the resultant Stokes radius will be larger than a globular protein of the same molecular weight. When proteins are considered in solution, the Stokes radius is proportional to the frictional coefficient f and inverse proportional to viscosity η:

$$Rs = \frac{f}{6\pi\eta} \tag{4.4}$$

The Stokes radii for a large number of proteins are well known, and when standard proteins are used for column calibration, they should be chosen considering their Rs in addition of their MW. For simplicity the SEC column vendors refer to the protein MW separation range more often than the Rs range. This can be explained by considering that for proteins identification purposes, it is more important to correctly define the MW more than their Rs and, moreover, Rs can vary if operating in denaturating conditions. An intermediate parameter that can be used is the minimal radius (Rmin) this refers to the minimal radius of a sphere that could contain the mass of protein and it is calculated with the simplified equation (reported from Erickson 2009):

$$Rmin = 0.066 \cdot M^{\frac{1}{2}} \tag{4.5}$$

Where M is expressed in Dalton and the radius is expressed in nm. To give an idea of the existing relationship among the MW, Rs and Rmin of a know protein, in Table 4.2 is listed a selection of standard proteins recommended for SEC calibration.

As evident in Table 4.2 almost all the proteins used are globular (with the exception of fibrinogen) and as a consequence, when an unknown protein is analysed, the molecular weight calculated will be close to the real value only if the protein has a globular shape too. This procedure can introduce an error in MW estimation and for this reason all results should be expressed as 'apparent MW'. To correctly attribute the real MW to an unknown protein, the use of additional techniques are necessary (such as MALDI-TOF). In addition, as discussed in the chromatographic optimization section, the elution of proteins is mobile phase-sensitive and then the calibration should be performed only when all the chromatographic parameters have been defined. From a physical point of view, the separation of analytes injected onto a SEC column depends on the size of the molecules relative to the stationary phase pores.

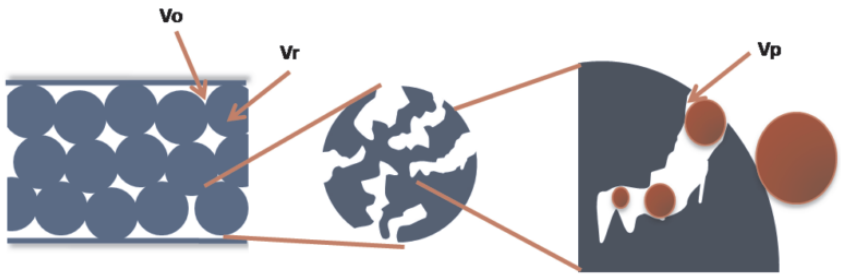


Figure 4.5: Visual representation of internal volumes of a SEC column.

Table 4.2: Proteins recommended for SEC calibration. The MW of proteins refers to the polypeptidic chain without signal-peptides and it is calculated using PAWS®software. No other Post Transitional Modifications (PTM) are considered. The taxonomy of each protein considered can be obtained from the Expasy ID number.

Protein name	MW (kDa)	Rs (nm)	R min(nm)	Expasy ID	NOTE
Ribonuclease A	13.6	1.64	1.59	P61823	
Chymotrypsinogen C	26.1	2.09	1.95	Q7M3E1	
Ovalbumin	42.7	3.05	2.31	P01012	
BSA	66.3	3.55	2.71	P02769	
Aldolase	156.7	4.81	3.56	P00883	homotetramer
Catalase	238.9	5.2	4.10	P00432	homotetramer
Apo-ferritin	409.2	6.1	5.20	P02791(l) Q8MIP0(h)	heterocomplex of 24 subunits
Thyroglobulin	602	8.5	5.59	P01267	
Fibrinogen	380.8	10.7	4.81	P02671 P02675 P02679	heterohexamer

In a typical chromatogram we can observe an initial peak containing the totally excluded molecules and a final peak that contains the totally included solutes. While the totally excluded molecules are bigger than the pore, the totally included molecules are able to penetrate into all the pores present. Since the path for these is much be longer, they are eluted later. In the same manner all intermediate molecules will be separated as a function of their Stokes radius and they represent the linear range of the SEC column. As explained previously, in an ideal SEC separation there will be no retention of molecules, since no interaction between the stationary phase and molecules occurs. In the same way, for protein eluting from a SEC column the measured parameter is not the retention time, but the elution volume (Ve). Before to calculating the correct elution volume for the analytes, all the volumes present in the column must be characterized (Figure 4.5). These volumes are named:

1. Vb= the volume external to the packing material
2. Vo= the volume contained within the porous resin beads
3. Vr= the volume occupied by the resin itself
4. Vt =the sum of Vo and Vp

The values of Vo and Vt can be empirically determined by measuring the elution volume of a large molecule that is totally excluded from the pores and the elution volume of a small solute that has access to all pores of the resin. The elution volume (Ve) of a solute that is partially included in the pores of the resin is related to the void and total volumes of a column by the following equation:

$$k_D = \frac{V_e - V_o}{V_t - V_o} \tag{4.6}$$

Where k_D is the parameter defining the retention factor of molecules in SEC column. In our experiments the Biosep S200 column has been calibrated using some standard proteins chosen amongst those present in Table 4.3, with the addition of Aprotinin and Cobalamin for the calibration of low molecular ranges:

Standard proteins have been injected (20µL) and the elution times obtained have been used to calculate the elution volume (Ve). The elution of the proteins has been monitored by a DAD operating at λ=280nm, while the elution of H_2O was monitored at λ= 210 nm. The TRIS buffer used for the chromatographic separation has an absorbance cut-off at λ=205nm (Abs >0.5 for 10 mM solution) and so water has a negative peak

Table 4.3: Elution volume (Ve) of calibrants injected onto the Biosep S2000 column where 4.2^a represenst the totally included molecule, while 1.6^b represent the totally excluded molecule. The value Ve is calculated by multiplying the retention time by the flow rate. For all proteins, the respective molecular weight (MW) and Stokes radius (Rs) are also reported.

Protein name	Ve (mL)	MW (kDa)	Rs (nm)
H_2O	4.2^a	0.018	0.14
Cobalamin	3.7	1.3	0.65
Aprotinin	2.8	6.5	1.48
Ribonuclease A	2.7	13.7	1.64
Ovalbumin	2.2	44.5	3.05
BSA	2	66.3	3.55
Thyreoglobulin	1.6^b	670	8.5

at its retention time. This behavior is explained by the local dilution of the buffer in the presence of the injected water. The calibration curve reported in Figure 4.6(a) has been obtained by plotting the correlation between k_D and the logarithm of the molecular weight of the eluting molecules. The tabulated data for each molecule are also reported (Figure 4.6(b)).

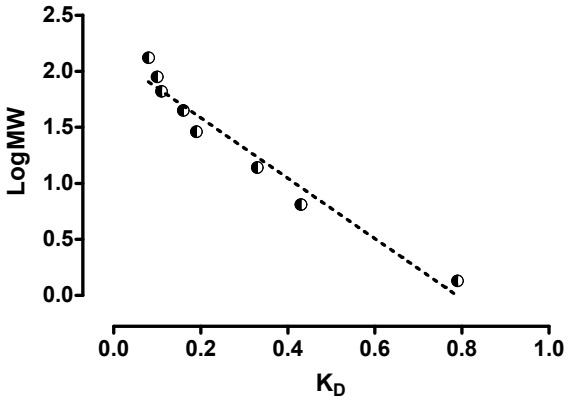
Since the final goal of this study is to identify the interactions that occur between proteins and metals in solution, the chromatographic conditions chosen should be tested to verify that they do not perturb the metal-protein docking. With this aim, the most important parameter to test is the buffer, as the incorrect buffer could interact with the metals or modify the protein folding and hence the disrupt the existing bindings.

4.1.5 Preservation of metal-protein complex

In the formation of metal-proteins complexes, several kind of intermolecular forces can be involved:

1. electrostatic interactions
2. hydrophobic interactions
3. hydrogen bonds
4. Van der Waals forces

To avoid incorrect evaluations of metal and proteins interactions, the buffer chosen should not cause the breakdown or splitting of an complexes that are formed. To evaluate this



Protein	LogMW	K_D
H2O	-1.74	Vt
Cobalamin	0.13	0.81
Aprotinin	0.81	0.46
Ribonuclease A	1.13	0.42
Ovalbumin	1.65	0.22
BSA	1.82	0.15
Thyreoglobulin	2.78	Vo

Figure 4.6: Calibration curve for Biosep S2000, the linear regression is y=-2.64x + 2.21, r^2 0.959. The flow rate was 0.2mL/min.

aspect, some experiments with ESI-MS have been performed. The utility of Electrospay Ionization in studying non covalent complexes has been recognized by several authors and reviewed by Loo in 1997. The complexes observable by ESI are stable in the gas phase but sometimes, this does correlate with their effective stability in solution (Zhang et al. 2004). When the metal-protein complexes are formed in solution, all the intermolecular forces cited above can be present while, for example, no hydrophobic interactions can be observed in the gas phase. Moreover electrostatic interactions can be strengthened in the vacuum, as demonstrated by Feng in 1995. However, if fragile complexes in the gas phase are analyzed, the source parameters (e.g. the desolvation gas or the capillary voltage) can be varied in order to obtain good desolvation of the analytes without destroying the interactions (Potier et al. 2005).

In our experiments, once the best buffer in terms of chromatographic resolution and protein recovery has been selected, the stability of the proteins-metals complexes in the presence of the buffer of choice is evaluated. Data are obtained by direct infusion of a model protein (Lisozyme from egg white, Sigma Aldrich) with and without mercury addition ($HgCl_2$, Romil). To evaluate if the buffer can be destructive of the complex, the same experiment was performed in deionized water and the results were compared.

Complex formation in deionized water

The ESI-MS experiments have been performed using a LCQ DECA instrument (ThermoFisher Scientific, San Josè, CA). Lysozyme was chosen as the protein model for these experiments. The mature form of lysozyme in chicken egg white is composed of 128 amino acids and its molecular weight is approximately 14.31 kDa. The low MW means it is easy to analyse the protein with electrospray ionization thanks to the formation of multicharged ions. Since the ion trap analyzer has a mass range of (50- 2000), analysis of this molecule will not be possible without the presence of multicharged ions.

The lysozyme protein has been dissolved in deionized water and the resulting mass spectrum Figure 4.7 is reported.

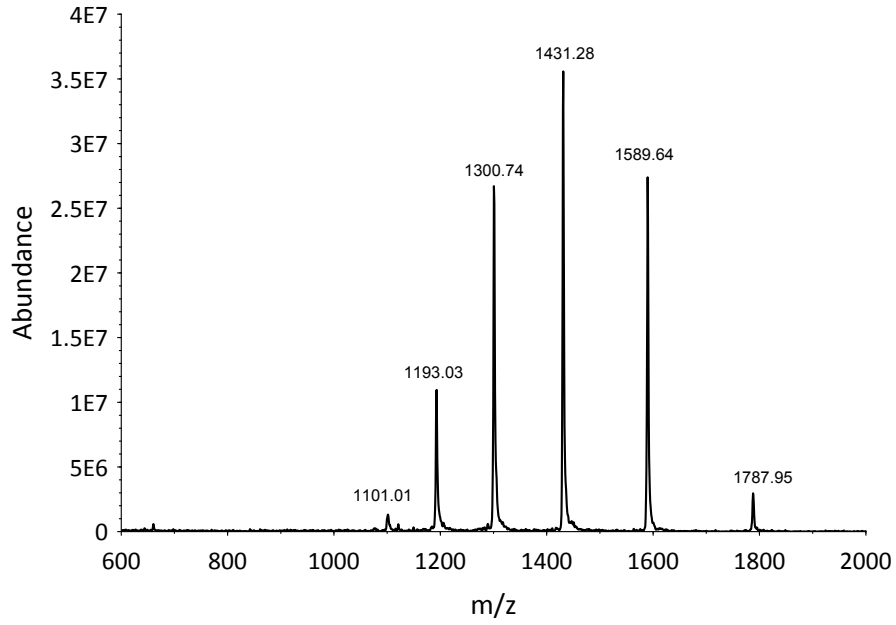


Figure 4.7: ESI-MS spectrum of lysozyme in deionized water. The multicharge distribution is present.

In the protein spectra, each m/z observed is a cluster of differently charged lysozyme molecules. In this kind of analysis with just one m value and multiple z values the spectrum deconvolution is easily obtained using a simple algorithm. The charge state determination for two adjacent peaks, such as a=1589.64 and b=1431.28 is the first step of deconvolution. These peaks correspond to the intact protein (with its original

molecular weight, MW) with z and z+1 charges respectively:

$$a = (MW + z)/z \tag{4.7}$$

and

$$b = (MW + z + 1)/(z + 1) \tag{4.8}$$

the charge state can then be easily calculated using equation 4.9:

$$Z_a = (b - 1)/(a - b) \tag{4.9}$$

For the above selected peaks (a=1589.64 and b=1431.28), the calculated charges are: z(a)=10 and z(b)=9.

The state charge helps us to calculate the MW of the intact protein, which is obtained by (as $z \cdot m$), giving as a result 14.3kDa, which corresponds to the MW of lysozyme.

As the spectrum of uncomplexed lysozyme has been obtained, ionic mercury(II) is added to the solution to verify the mass shift. To verify the Hg-lysozyme binding, the reaction has been performed in the narrow pH range (of 7 to 4), the pH most suitable for the complex formation was found to be pH=5 Figure 4.8. For each multicharged cluster, a minor ion is observable with a difference (ΔM) of several Daltons. In particular, next to the ion 1431.28, the presence of an ion at 1451.54 and (at very low intensity) an ion at 1471.09 m/z is evident. The ΔM observed is 20Da and 40Da respectively. Considering a charge of 10 for these ions, as calculated above, the real mass shifts for the protein are 200 and 400 Daltons. These mass shift correspond to the addition of one or two atoms of mercury (athomic mass=200.59) and confirm the complex formation. Due to the low resolution of ion trap, the isotopic cluster of mercury in the multicharged protein can be not detectable.

Complex formation in TRIS buffer

Having obtained these results, the same reaction was performed in the presence of 25mM TRIS buffer. Since the pKa of TRIS is 8.06, the buffer solution was keept at pH=5 with by the addition of diluted TFA to ensure the same reaction conditions as in Figure 4.8 on the next page. TRIS is a well known non volatile buffer, and to avoid clogging the instrumental parts, it was diluted tenfold immediately before infusion into ESI-MS to concentration of 2.5mM, which is more compatible with the ESI source. The spectrum of lysozyme in TRIS buffer after Hg addition is reported in Figure 4.9 on page 43. The differences with complex formation in deionized water are evident, in this case a

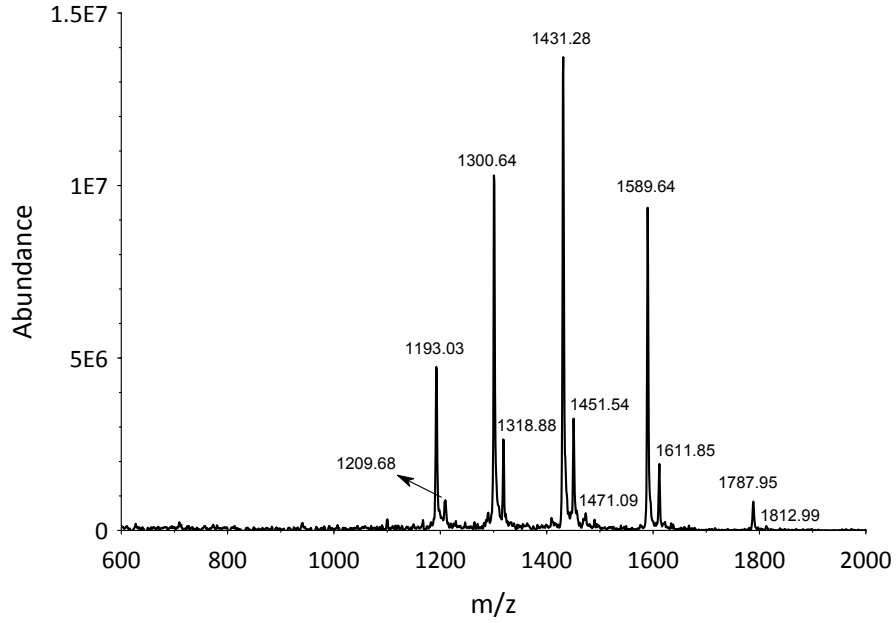


Figure 4.8: ESI-MS spectrum of lysozyme in deionized water in presence of ionic mercury(II).

more intense signal for the protein-metal complex is obtained. In addition, almost all the multicharge clusters show the presence of at least two mercury ions. These differences can be justified by considering that TRIS buffer is able to keep the protein in its native folding. These spectra suggest that the correct folding of lysozyme leads to more interactions with mercury. This is probably due to the closer spatial distribution of the amino-acids that favour complex formation.

With these experiments we can positively confirm that TRIS buffer can be used for these kinds of studies, since it was able to preserve (and in some extent to reinforce) the complexes. The chromatographic method development is concluded here and the final step will be the setting of ICP-MS parameters for metallic moiety detection.

4.2 Metals detection by ICP-MS

The ICP-MS is the instrument of choice for elemental detection thanks to its sensitivity and specificity. Due to the destructive ionization obtained in the plasma source (complete atomization), no molecular information can be deduced. By coupling with HPLC, some molecular information can be obtained due to the use of a chromatographic sep-

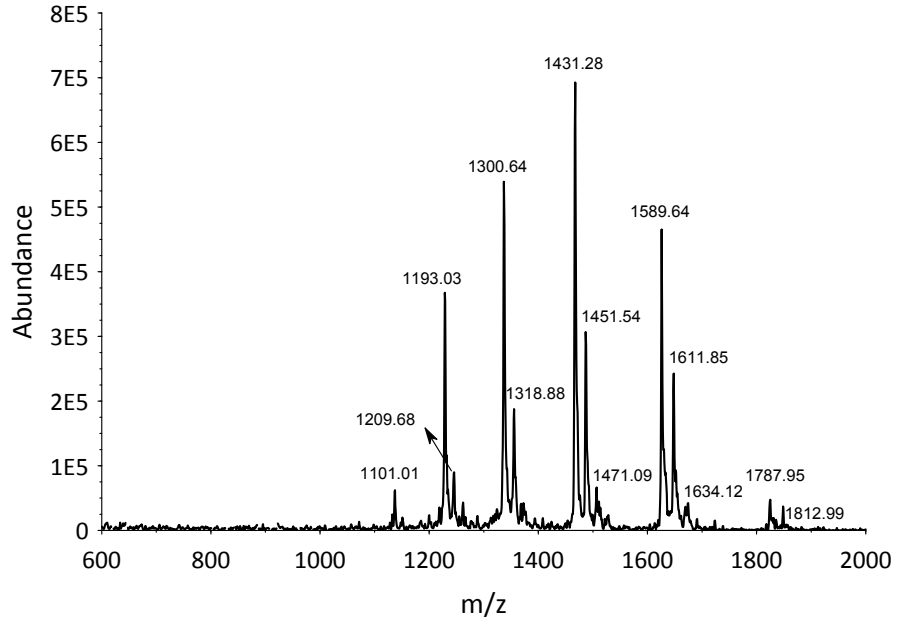


Figure 4.9: ESI-MS spectrum of lysozyme in TRIS buffer in presence of ionic mercury(II). The working pH was 5.

aration. The main application field for this is the so called *elemental speciation*. In our experiments, the metal-protein complex and (in general all metal-binding molecules) eluting from the SEC column will be detected thanks to the coupling with ICP-MS. In this section the instrumental optimization will be briefly discussed.

4.2.1 Instrumental settings

In the analysis of arsenic and mercury with ICP-MS, the presence of spectral interferences must first be verified. In particular, it is well known that arsenic is interfered with by the formation of an in-source adduct between the argon used for plasma generation and the chlorine present in the sample. This polyatomic species has the formula $^{40}Ag^{35}Cl^{+}$ and has the same m/z of the arsenic ion (m/z=75), its presence can reduce quality of the analysis. Under our chromatographic conditions there is a discrete quantity of chlorine originating from the mobile phase composition (NaCl 25mM). When attempting to lower the impact of this spectral interferent on arsenic analysis, the elimination of chlorine must be performed, or the interference has to be removed post source by spectral resolution or by the use of a collision cell.

With the use of a collision cell, it is possible to have a kinetic energy discrimination (KED) between the arsenic ion and the polyatomic $^{40}Ag^{35}Cl^{+}$ ion. This step is performed thanks to the presence of an octopole collision cell, between the plasma source and the mass detector, that can be pressurized with a collision or reaction gas (usually Helium or Hydrogen). Since the polyatomic interferents have a larger cross section than monoatomic analytes, they will collide with the gas atoms more frequently. These collisions lead to a loss of kinetic energy and the instrumental conditions can be adjusted to reduce their passage to the mass analyzer.

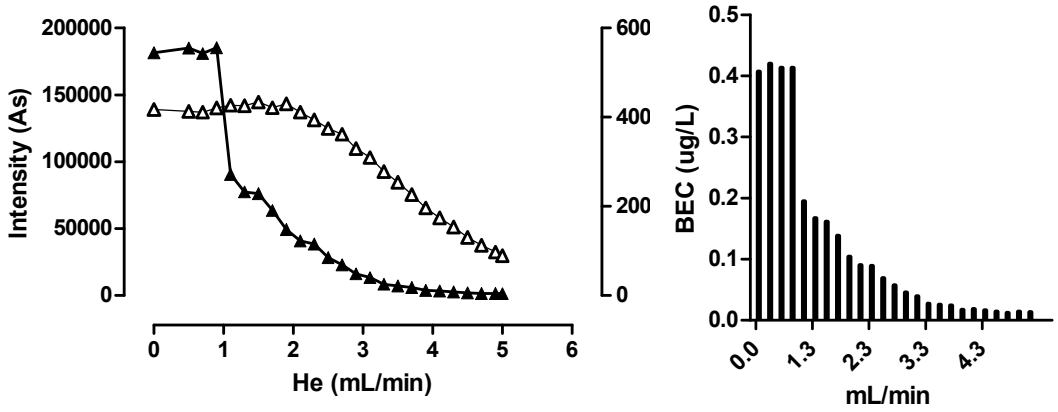


Figure 4.10: (a) Intensity loss of Arsenic (white triangles) and the polyatomic interferent (black triangles) with the increase of Helium flow. (b) BEC values expressed as µg/L.

To positively eliminate the $^{40}Ag^{35}Cl^{+}$ interference, Helium has been chosen as a collision gas. To avoid an excessive loss of As sensitivity, the gas flow has been optimized, whilst a discrimination voltage step of +4 v was set between the octopole ion guide of the collision cell and the quadrupole mass analyser. The optimization results have been expressed as the Background Equivalent Concentration, that defines the intensity of the background as a concentration at the analyte mass monitored, and is indicative of the signal to noise ratio. From a practical point of view, the BEC is calculated as:

$$BEC = \frac{I_B}{I_A - I_B} \cdot C_A \tag{4.10}$$

Where I_B is the intensity of background, I_A is the intensity of the analyte and C_A is the analyte concentration. To perform the BEC analysis, a blank solution which contains just the interferent (such as the 25mM NaCl solution) has been analysed to obtain the I_B value, whilst a solution containing the analyte at 10µg/L has been analysed to obtain

the I_A. As reported in the Figure 4.10, the signal intensity decreases both for analyte and background as the He flow increases. In particular, the chlorine interferent intensity is dramatically reduced even with the addition of a low gas flow (1mL/min). Although the arsenic intensity loss is also evident, the minimal value for the BEC is obtained at a flow of 4mL/min. So, this gas flow has been used during ICP-MS data acquisition.

All of the instrumental parameters for samples analysis are reported below:

Table 4.4: Source and mass analyser parameters used for the analysis of samples.

Parameter	Value
Power RF	1500 W
Sample Depth	7.5mm
Carrier Gas	1.12mL
S/C Temperature	2°C
Helium flow	4mL/min
Monitored Masses	59, 75, 202

As reported in the table, the monitored masses are cobalt (m/z=59), arsenic (m/z=75) and mercury (m/z=202). Cobalt is naturally occurring in the biological samples as cobalamin, or cobalamin binding protein (CBP). For this reason it has been monitored in case of overlapping in the HPLC ICP-MS data.

4.2.2 Metals recovery from the SEC column

The SEC column used for analysis has a silica structure that can be reactive with respect to Hg and As. In particular, ionic mercury can interact with the hydroxylic groups present on the pores surface. This can cause the loss of free mercury, while the mercury interacting with proteins should be preserved. To evaluate the presence of mercury absorption onto the column, some injections of ionic mercury and arsenic have been performed. When ionic mercury is injected onto the SEC column, no can be detected with the ICP-MS. This clearly shows that mercury is strongly retained by this column. This retention is not occurring in the case of arsenic injection, since practically all the metal injected is recovered from the column. These differences can be explained by the positive charge of Hg^2+ that can interact with the hydroxyles present on the silica particles surface.

To confirm the presence of mercury on the column, mercury free βME and l-cysteine have been injected onto the column immediately after these samples. The ICP-MS

chromatograms of eluting mercury and the contemporary elution of βME and l-cysteine (DAD, 254nm) are reported below.

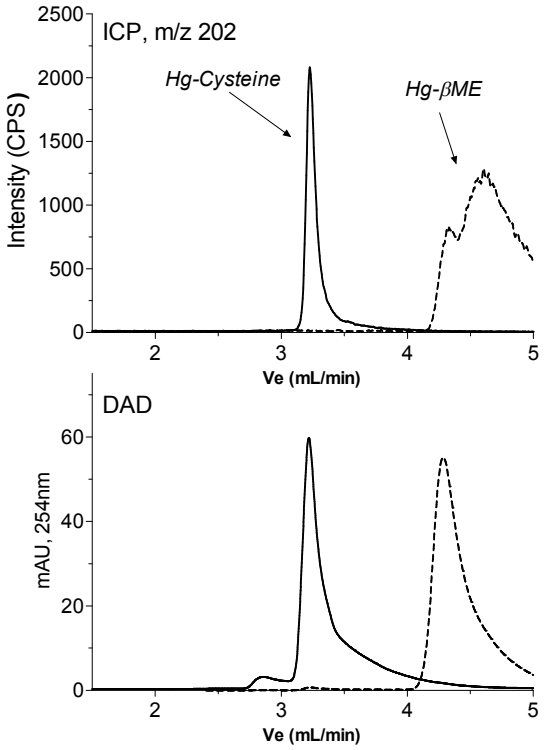


Figure 4.11: Hg elution from SEC column after the injection of clean cysteine and βME. The upper panel is the ICP-MS aquisition, while the lower is DAD detction of thiols at 254nm.

These injections seem to show that Hg was reversibly bound to the column. The difference of Ve of cys-Hg complex and βME complex (about 1.2mL) appears to be very significant with respect to the small difference in the MW between the two complexes (l-cys-Hg MW= 440.89 Da, βME-Hg MW=354.84 Da). This difference can be explained by examining the structure of the two complexes.

These structures have been obtained by assuming a stoichiometry of 2:1 between the thiols and mercury, since the free thiols concentration (10^{-5}M) is in excess with respect the Hg concentration into the column (1μM) calculated on the basis of an injected sample. As shown in Figure 4.12 the complex between mercaptoethanol and mercury has a linear structure that could pass into all the pores present in SEC column. Due to the longer path length, the peak shape is wide and asymmetrical. The Hg-cys peak at Ve=3.25mL

appears to be sharper, even if its Ve reveals an apparent MW not corresponding to the actual one (MWa=3200Da).

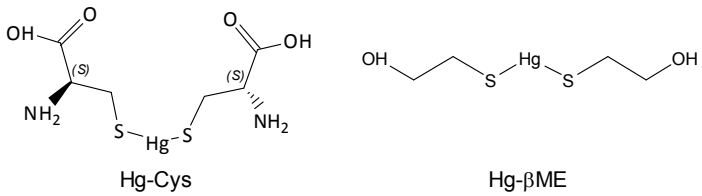


Figure 4.12: Structure of the complexes between ionic Hg(II) and cysteine or βME.

Additional experiments have been performed to verify if the addition of βME or l-cys directly into the mobile phase can be useful to prevent the loss of ionic mercury during SEC chromatography. The quantification of recovery has been obtained by Flow Injection Analysis (FIA) of the samples. The FIA analysis has the advantage of giving sufficient accuracy in the quantification of metals thanks to the use of the collision cell (CRC) that reduces the spectral interferences. The loss of metals due to unspecific absorption of metals in the SEC column can then be calculated by comparing the areas of the eluting peaks in SEC mode with the area obtained by FIA.

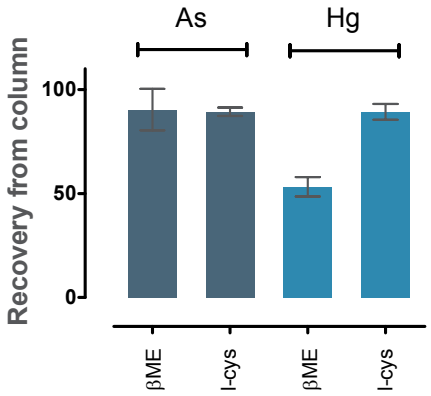


Figure 4.13: Variation of recovery of ionic As and Hg from SEC column with addition of 0.1% βME and 0.1% w/v l-cys.

The advantage of using the thiolic compounds in the mobile phase appears to be evident in the improvement of mercury recovery up to 89% (Figure 4.13). Of the tested thiols, cysteine addition results in the best final recovery, the final mobile phase composition is summarised in Table 4.5 on the following page. It is important to highlight that the cys addition has a positive effect on the proteins moiety thanks to its antioxidative

properties.

Table 4.5: Final composition of the mobile phase for SEC chromatography. Chromatographic flow 0.2mL/min.

TRIS	25mM
NaCl	25mM
Arg	1mM
l-Cys	0.01% w/v

4.3 Application to real samples

To test the above developed method, the analysis of some samples has been performed. All samples chosen are foodstuffs with a known proteic content. Firstly to study the direct interactions with the sample matrix, no extractive procedure have been performed. For this reason, only liquids foods have been considered at this stage and, among them, we have chosen to focus on milk, eggs (white and yolk) and fish glue. These samples have been chosen as they have very different compositions, as shown in Table 4.6.

Table 4.6: Nutritional composition of samples used to study the metal-protein interactions in solution

	Water	Fats	Proteins	Ashes
Egg white	85.7%	0.25%	12.6%	0.59%
Egg yolk	50.9%	31.75%	16.2%	1.09%
Milk	86%	4.4%	3.4%	0.23%
Fish glue	10%	1.3%	88.3%	0.4%

All samples have been analysed by HPLC (UV) ICP-MS before and after the addition of the metal of interest and, in order to verify if this method is able to monitor and quantify specific interactions, the results for the different samples will be compared. The aim is to study mercury and arsenic interactions with proteins directly in their native matrices. Considering that mercury is a positively charged ion (2+) while inorganic arsenic are uncharged (As(III)) or negatively charged (As(V)) at the physiological pH, it is predictable that differences could be found in their reactivity. For this reason, to test the method for the study of metals with a similar behaviour, the metabolites of arsenic (MMA, and DMA) and mercury ($MeHg^+$) have been also been considered. The experiments reported below will allow us to verify if this method could be useful to study

whether metal-protein interactions are species-specific or not. Moreover, a first attempt to identify the proteic moiety of the observed interactions, will also be reported in the next subsections.

4.3.1 In-solution interactions of mercury compounds.

To study the metals-protein interaction under native conditions, $HgCl_2$ and MeHgCl have been added in the molar concentration ratio of 10:1 with respect to the protein in the samples. The metal excess is necessary because of the presence of others molecules that can interact with the metals that are not proteins. The use of a greater metal excess is not useful, since it could lead to the formation of complexes with several atoms per protein, risking an alteration of the protein folding. If the protein folding is altered, precipitation or aggregation could occur, giving unpredictable and difficult to interpret results.

To evaluate the amount of metal needed, the protein concentration in each sample has been quantified by a colorimetric Bradford assay, using different standard proteins to prepare the calibration curve.

Egg white

Egg white (or albumen) is an aqueous solution of proteins, with low number of other components (such as lipids and sugars). The pH of fresh albumen is 7.6-7.9 and its characteristic viscosity is due to the presence of ovomucoid. The major proteins present in the albumen, and their abundance, are listed in Table 4.7. The main protein content in egg white is ovalbumin, and so it should represent the main interaction target for the metals in this study. The reported MWs refer to the protein chain and have been obtained using the online database (http://www.uniprot.org).

Fresh egg white has been sampled from eggs from a domestic farm and has been diluted 1 to 4 with a solution containing 25mM TRIS buffer and 0.1mM of PMSF. The dilution was been performed to preserve the proteinic content (thanks to the presence of PMFS as proteases inhibitor) and to lower the viscosity of sample. This latter is necessary for direct sample injection onto the column. The prepared sample has then been divided into four subsamples, as reported below:

1. Sample for protein quantitation with Bradford assay
2. Unspiked sample as a control
3. Mercury spiked sample

Table 4.7: Major proteins present in the egg white.

Protein	% of dry weigh	MW (Da)	Characteristic
Ovalbumin	54.0	44,500	Phosphoglycoprotein
Conalbumin	12.0	77,700	Metal ions binding
Ovomucoid	11.0	28,000	Trypsin inibitor
Globulin G2	4.0	49,000	
Globulin G3	4.0	49,000	
Ovomucin	3.5	550,000	Sialoprotein
Lysozyme	3.4	14,300	Antibacterical
Avidin	0.05	68,300	Biotine binding

4. Methylmercury spiked sample

Before metals spiking, subsample 1 has been diluted a further 20 fold and subjected to a Bradford assay to obtain the protein content. The assay was performed using an ovalbumin standard for the calibration curve (calibration range 0-1.4 mg/mL). The result given was a protein content in the undiluted albumen of 116mg/mL. This is in good agreement with the medium value calculated from Table 4.6 on page 48 (130mg/mL). Since the results with this colorimetric assay are expressed as weight, the data conversion to the molar concentration have been performed. This was done by taking the weighted average of the MW for the major proteins present in the samples. The concentration obtained was 1.3μM, from this the ten fold excess for metals addition was calculated.

Once mercury and methylmercury had been added to subsamples 3 and 4, the mixture was shaken for several minutes and left to react overnight (o/n) at +4°C in the dark. Immediately before analysis, the solution has been diluted 100 fold with the mobile phase to lower the mercury load into the ICP-MS. The diluted samples have been filtered through 0.45μm cellulose acetate disposable filters, and finally 20μL of the resulting solution have been injected onto the column.

The chromatograms obtained from the spiked samples (Figure 4.14) allowed the near simultaneous detection of the proteins (with UV signal at 280nm) and the metals (ICP-MS detection) after elution. As shown, the Hg^{2+} ion and the $MeHg^{+}$ co-elute with the higher molecular fraction. Both mercury-binding peaks coincide with the most intense peak detected UV, suggesting the presence of the major protein in albumen (Ovalbumin, 54% by dry weight). To confirm this, the elution volume of the observed peaks was used to calculate apparent molecular weight (MWa) of the proteins.

With the Eq. 4.6 on page 37, the value of k_D can be obtained for each peak detected by DAD or MS detector. The MWa is then calculated thanks to the SEC calibration

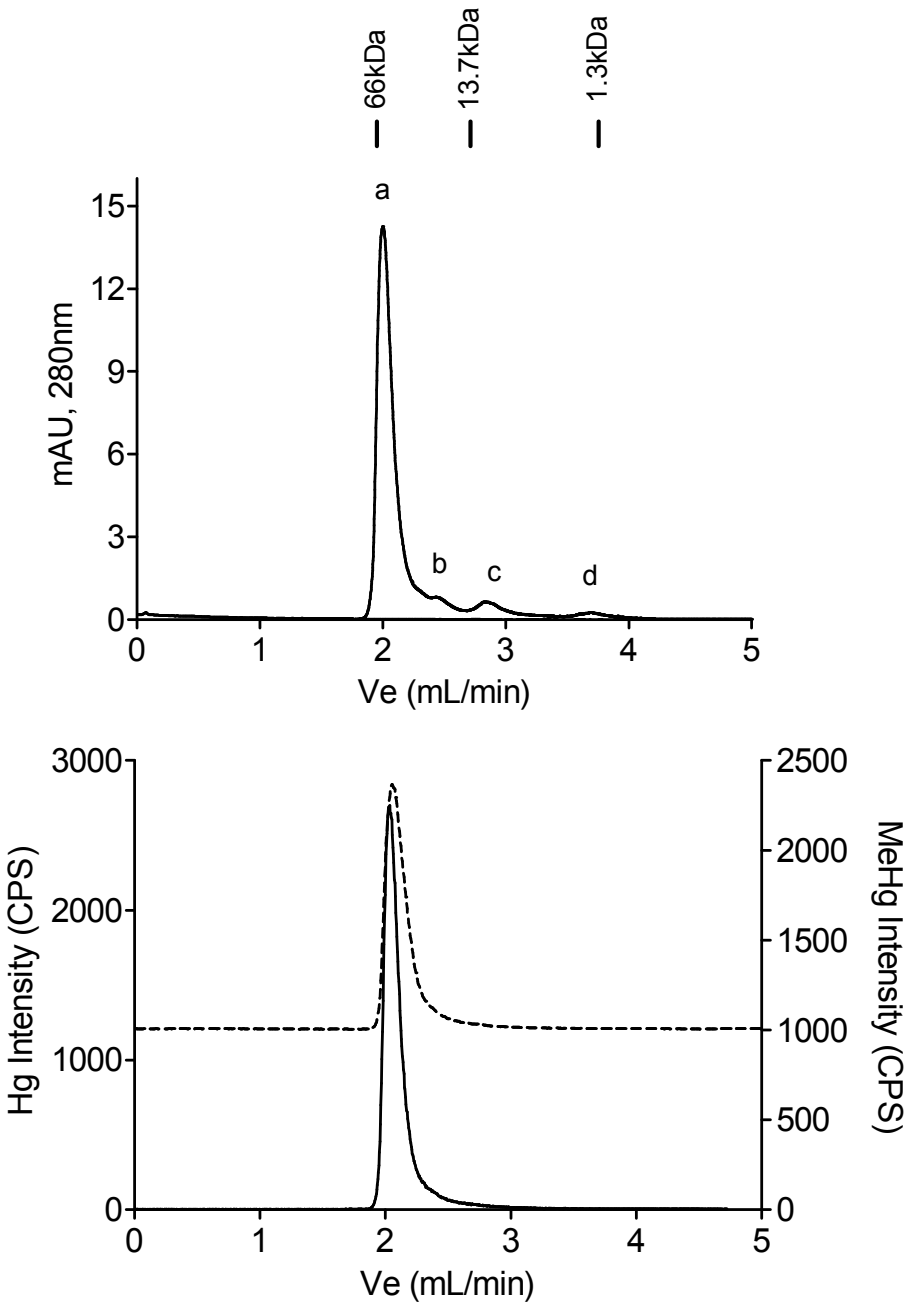


Figure 4.14: DAD (upper) and ICP-MS (lower) analysis of egg white. The Hg elution is reported with a continuous line, while the MeHg is reported with a dashed line.

curve reported in Figure 4.6 on page 39.

The results for both mercury species, are expressed as MWa and are reported in Table 4.8.

Table 4.8: List of eluting peaks from the SEC column. The Ve for elemental species detected by ICP-MS are reported. Peaks without metals are obtained from DAD acquisition only.

	Molecular moiety		Metallic moiety	
Ve	k_D	MWa	Spiked	Label
2.09	0.17	51	Hg, MeHg	a
2.43	0.32	23.1		b
2.85	0.48	8.6		c
3.7	0.80	1.2		d

As shown in Table 4.8, the peak in which Hg^{2+} and $MeHg^{+}$ are co-eluting has an apparent molecular weight of 51kDa, which is close to the theoretical MW of ovalbumin (45kDa). Since no other peaks interacting with Hg/MeHg have been detected, it is possible suppose that ovalbumin is the only reactive protein. The ovalbumin reactivity versus mercury has been showed by Duce et al. 2012 and Kutscher et al. 2008, that used the p-hydroxymercuribenzoic acid as labeling agent.

Egg yolk

The egg yolk is dramatically different from albumen. It is generally described as a fat-in-water solution that contain high levels of lipids (31% of total content) and proteins (16.2%). Due to this lipidic-rich medium (lipids to proteins ratio 2 to 1), the interactions between them result in the formation of particular structures, known as lipoproteins. The High Density Lipoproteins (HDL) and the phosvitins are present in the granular fraction of egg yolk and are responsible for lipids and metals storing. The remaining water-soluble proteins are dispersed in the serum fraction. A list of the major proteinic constituents in the egg yolk are reported in Table 4.9 on the next page.

As for the albumen sample preparation, before mercury spiking, the sample has been diluted with a solution containing 25mM TRIS buffer and 0.1mM of PMSF (dilution factor 1 to 4). The same subsampling procedure has been followed in order to perform a Bradford assay before mercury species addition. Again, ovalbumin has been used for calibration and the protein content in the undiluted yolk has been found to be 150mg/mL. The molar concentration was then calculated as 1.1µM.

Table 4.9: Major proteins present in the egg yolk.

Protein	% on total proteins	MW (Da)	Characteristic
Lipovitellins			
α-lipovitellin	58	195	HDL formation
β-lipovitellin	11	110	HDL formation
Livetins			
α-livetin	4	67	Serum albumin
β-livetin	5	36	Glycoprotein
γ-livetin	2	180	Immunoglobulin
Phosvitins	7	37-45	Phosphoproteins
Lipovitellenins	12	Variable	LDL formation

Even though egg yolk is a more proteic foodstuff compared to egg white (as reported in Table 4.6), the molar protein concentration found is lower than for albumen (1.3μM). This is due to an higher medium MW for egg yolk proteins. After Hg and MeHg addition (in the ratio 10:1), the mixture is shaken for several minutes and left to react o/n at +4°C in the dark. As seen previously, the samples have been diluted (100 fold) and filtered before injection.

For egg yolk analysis, the chromatograms and the table of the peak elution order are reported in Figure 4.15 and Table 4.10 respectively.

Table 4.10: List of egg yolk peaks eluting from the SEC column. The elution volume for metals <1.8 and >4 mL are out of the linearity range.

	Molecular moiety		Metallic moiety	
Ve	k_D	MWa	Spiked	Label
1.70	0.04	>100		a
1.98	0.15	66.1	Hg, MeHg	b
2.39	0.30	25.3		c
2.83	0.47	9		d
3.30	0.76	3		e
4.51	1.13	<0.5		f

The UV chromatogram is slightly different to that obtained from egg white. In particular, the most intense peak (peak b, in the DAD chromatogram) seems to have a MWa of 66kDa. A small peak from a large protein(s) which has a MWa >100kDa has been detected also (peak *a*). In egg yolk, as obtained in the egg white, both Hg and MeHg

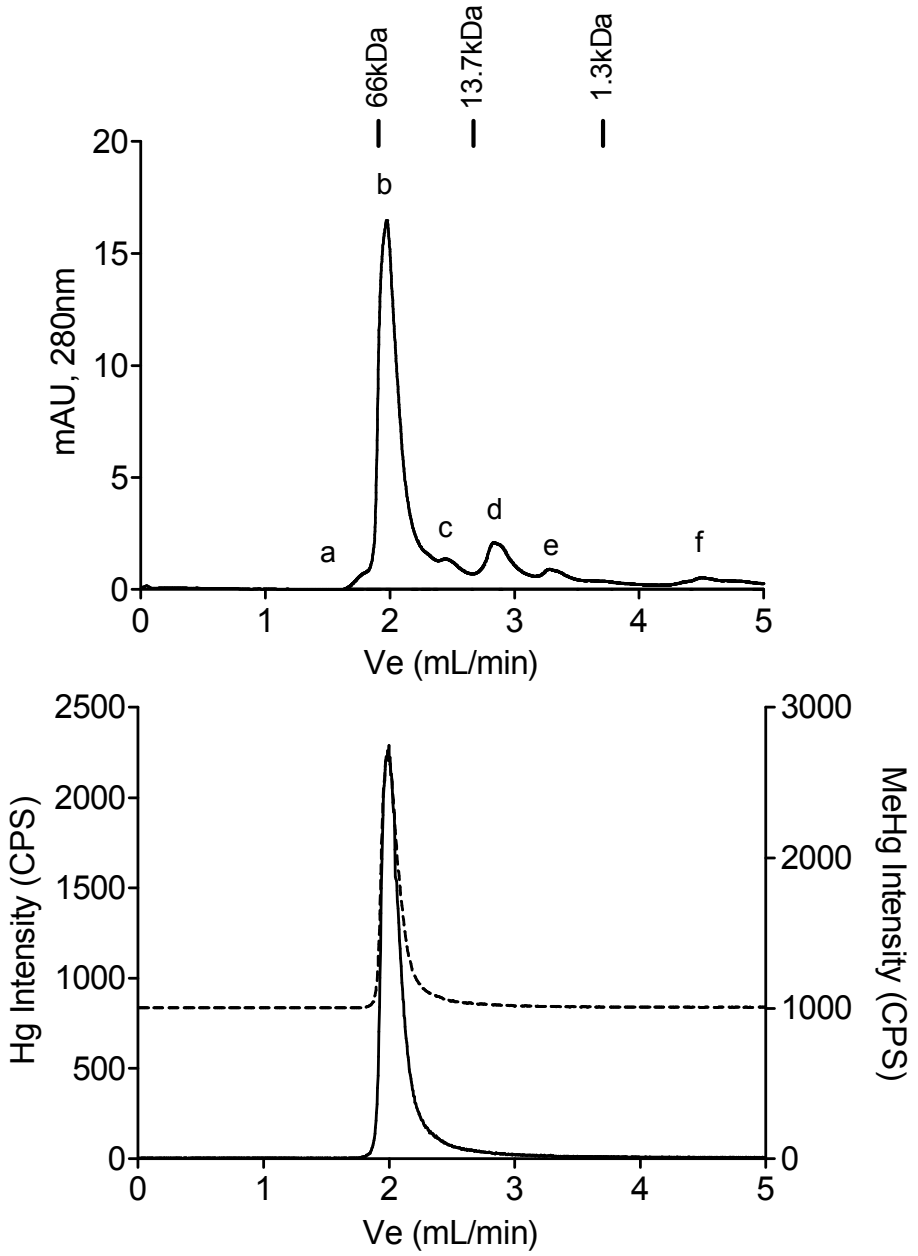


Figure 4.15: DAD (upper) and ICP-MS (lower) analysis of egg yolk. The Hg elution is reported with a continuous line, while the MeHg is reported with a dashed line. The MeHg chromatogram has been shifted upwards for visual clarity.

seem to be reactive only towards the most abundant peak at 2.17mL. By comparing the MWa obtained with the list Table 4.9, a correlation seem possible with serum albumins (e. g. α-livetin). This protein represents only a 4% of proteins present but, as it is dispersed into the serum fraction, it can easily interact with metals in solution. Proteins present into the granules, like lipovitellins of phosvitins, are less accessible for interaction since the metals must be actively transported into the granules. Amongst the serum proteins, livetins and lipovitellenins are the most abundant and their interaction with mercury/methylmercury interactions with them is more than reasonable. Moreover, any data can be found in literature about the mercury- yolk proteins interaction.

Gelatin

Fish glue is a edible gelatin used for thickening liquids. Despite its name, practically all fish glues present on the market are obtained from pig or bovine tissues (skin, bones and cartilage). The latter are used to a minor extent, since the Bovine Spongiform Encephalopathy (BSE) epidemic.

The main characteristics of fish glues are their very high content of proteins (88%). As suggested by the tissues used for gelatin production, the main protein present is collagen. In particular, since gelatins are obtained by thermal denaturing of collagen (e.g. with several thermal cycles from 5°C to 90°C) after pre-treatmen with alkali or acids, the resultant product is a mixture of protein fragments. The fish glue used in these analyses have been purchased at the market. A few grams of gelatin were dissolved in a TRIS/PMSF buffer to obtain a final concentration of 50mg/mL. This concentration was chosen as it is lower than the gelling concentration so the solution remained liquid.

The solution was quantified for the total protein content using the Bradford assay, but the concentration was lower than expected (4mg/mL). This is due to the low content of amino acids that are reactive to the Bradford reagent (arg, lys and hys), leading to an under-estimation of the real protein content. As the sample was obtained by industrial processing, the declared protein content by the manufacturer was used as reference value. After metal addition and incubation o/n, the samples were diluted, filtered and injected as reported previously. The resultant chromatograms are reported in Figure 4.16 on the following page.

The DAD and MS chromatograms are very different from those obtained for egg analysis. In particular, the UV signal reveals the presence of a broad peak from 2 to 3.5mL. This clearly represents the elution of fragments obtained with the denaturation of collagen and it is comparable with results obtained using SDS-PAGE analysis (Zhongkai

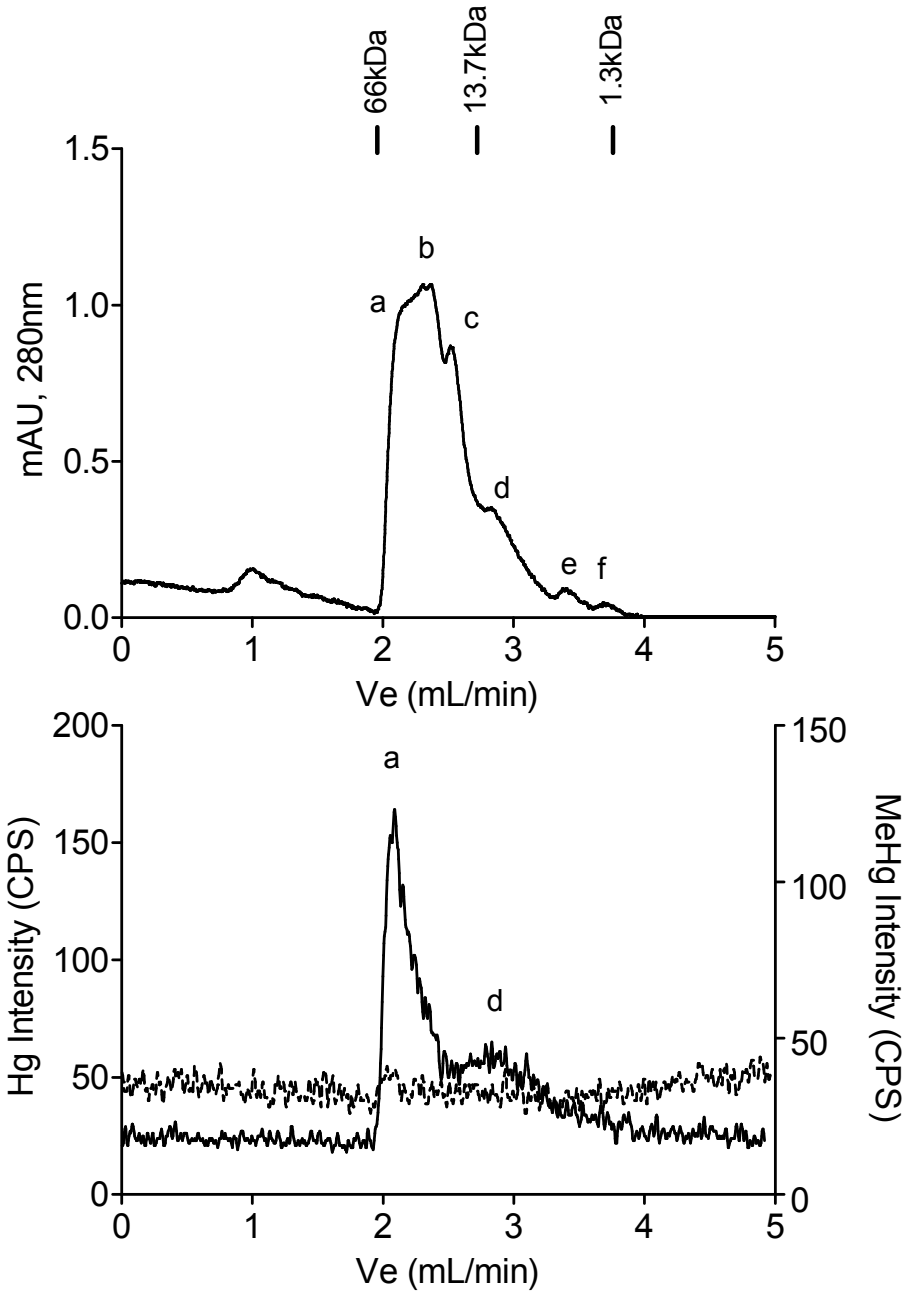


Figure 4.16: DAD (upper) and ICP-MS (lower) analysis of food grade fish glue. The Hg elution is reported with a continuous line, while the MeHg is reported with a dashed line. The MeHg chromatogram has been shifted for visual clarity.

et al. 2011). The calculated MWa are reported in Table 4.11.

Table 4.11: Table of eluting peaks of fish glue from SEC column. The elution volume for metals <1.6 is out of the linearity range.

	Molecular moiety		Metallic moiety	
Ve	k_D	MWa	Spiked	Label
1.01	-0.23	>100		
2.09	0.19	51.1	Hg	a
2.45	0.33	22		b
2.56	0.37	17		c
2.88	0.49	8.1	Hg	d
3.51	0.73	1.8		e
3.75	0.83	1.1		f

In the case of ICP-MS analysis, only two very small peaks of mercury can be detected, while methylmercury is not detectable. The reasons for the loss of mercury are not clear, since the same samples injected without an SEC column reveals the presence of the spiked compound. We are not able to explain this phenomenon, but hypothesize that, both Hg^{2+} and $MeHg^{+}$ are interacting with the negatively charged residues of gelatin (Isolectric point 4.8) forming insoluble compounds. Only in the case of Hg^{2+} chromatogram, two small peaks elute, with MWa of 51 and 8kDa. Due to their very low intensity, we can only hypothesize that they are not coming from collagen. Maybe they represent some residual proteins -or fragments of proteins- of the original tissue used for the fish glue preparation. This hypothesis is reinforced when considering the low content of cysteine in the collagen sequence (e.g. in the Bovine collagen α2(I) chain is aound of 0.6%) and the cys oxidation process during the thermal procedures (Perry et al. 1987). This leads to the loss of the most favourable site for the interaction with Hg.

These results suggest the use of ICP-MS in detecting less abundant proteins that are not detected with a DAD, thanks to the presence of a metallic moiety.

Milk

Milk is a complex product containing hundred of substances that are either in solution, in suspension or in emulsion with water. The concentration of proteins in milk can vary from 3.0 to 4.0%. The percentage changes with the breed of animal and in proportion to the amount of fat in the milk.

Milk proteins are divided into two major groups: caseins (representing up to 80%

of whole protein content) and serum proteins (the remaining 20%). The caseins family is composed of different types of caseins (α, β and ϰ) which contains phosphorus and coagulate or precipitate at pH<5. Caseins are not dissolved, but are suspended in milk generating micellular structures. The high phosphate content in the caseins allows them to associate with calcium and for this reason, they are able to interact with several cations. The remaining protein portion, the serum proteins, do not contain phosphorus so are not involved in cheese production. In Table 4.12 below, a summary of the abundances and the MW of the major proteins of cow milk are reported. As for egg proteins, the reported MW are referring to the protein chain and any post transitional modifications have been accounted for.

Table 4.12: Major proteins present in cow milk. The MW is refers to the unprocessed protein. Caseins MW can be greater, as a function of the phosphorylation level.

Protein	% of total protein	MW (Da)	Characteristic
α -Casein s-1	30.6	22,900	Phosphoprotein
α -Casein s-2	8.0	24,300	Phosphoprotein
β-Casein	28.4	23,600	Phosphoprotein
ϰ-Casein	10.1	19,000	Phosphoprotein
α -lactalbumin	3.7	14,100	Ca^{2+} binding
β -lactoglobulin	9.8	18,300	Dimers at neutral pH
BSA	1.2	66,000	
Immunoglobulins	2.1	150,000	IgG major form
Others	6.1		Enzymes

Milk sample has been diluted three fold with TRIS/PMSF buffer. The protein content obtained with a Bradford assay, using β-Casein as a standard for the calibration curve. The concentration was corrected for the weighted MW has been estimated as 2.6μM and a ten-fold excess of Hg/MeHg has been added. After incubation, the samples have been diluted and filtered and the obtained chromatograms are reported in Figure 4.17.

The DAD chromatogram shows the presence of five main peaks, having a MWa from 100kDa to 1kDa. The corresponding ICP chromatogram detecting the mercury ion shows the presence of a marge peak at 2.06mL which corresponds to the labeled peak *b* in the upper chromatogram. In addition to this main peak, two small peaks at 1.8 and 3.06 have been also detected, the first corresponding to the peak *a* in the DAD chromatogram and the latter in correspondence with the shoulder of the peak *e*. The MWa calculated for the above mentioned peaks are shown in table Table 4.13.

The largest peak into the DAD chromatogram (peak *b*) should correspond to the

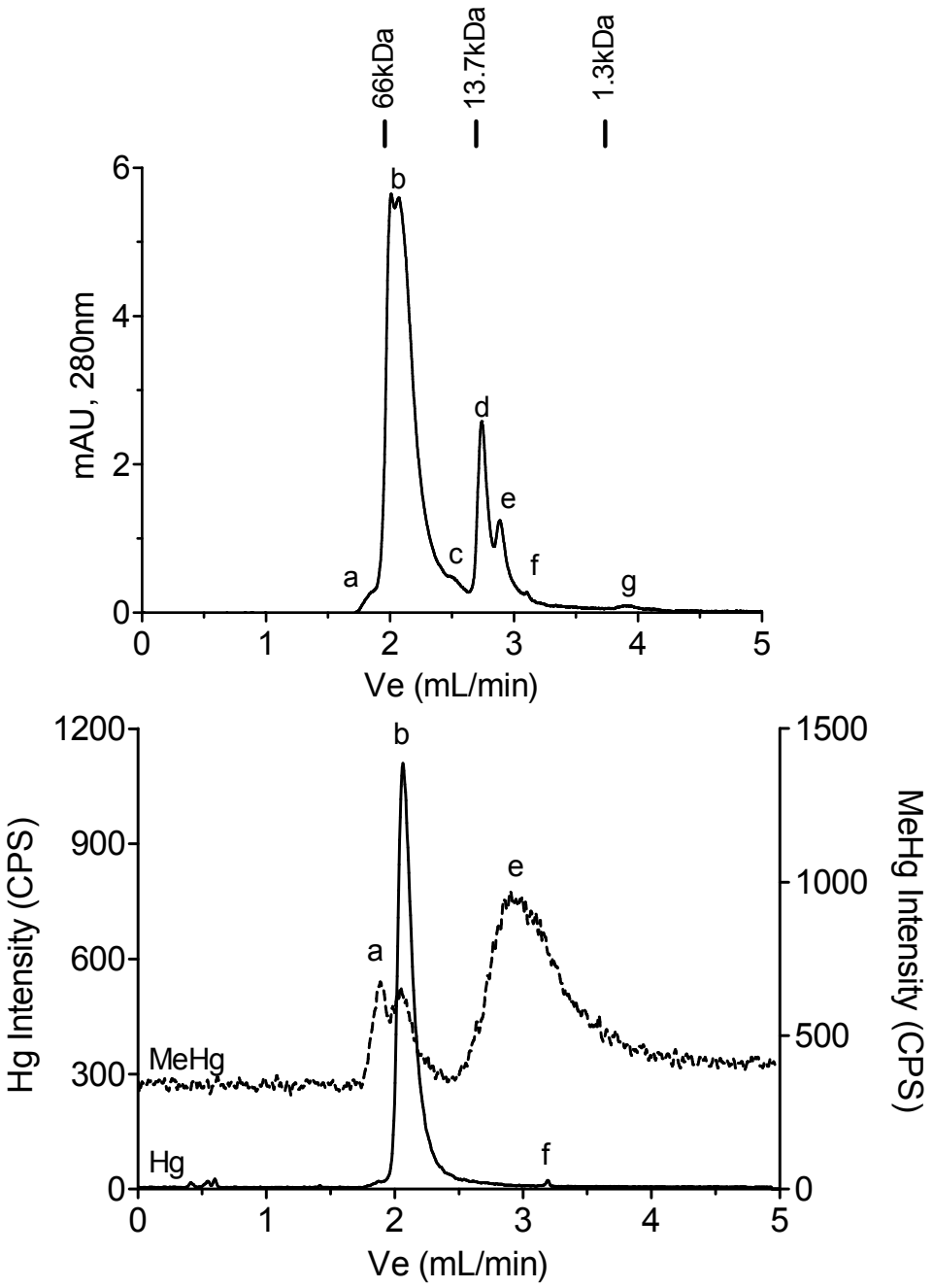


Figure 4.17: DAD (upper) and ICP-MS (lower) analysis of milk. The Hg elution is reported with a continuous line, while the MeHg is reported with a dashed line. The MeHg chromatogram has been shifted for visual clarity.

Table 4.13: Table of eluting peaks of milk spiked with mercury analysis. MWa are expressed as kDa.

	Molecular moiety		Metallic moiety	
Ve	k_D	MWa	Spiked	Label
1.8	0.108	100	Hg, MeHg	a
2.01	0.16	61.6		b
2.06	0.19	55	Hg, MeHg	b
2.48	0.34	20.5		c
2.73	0.43	11.4		d
2.86	0.48	8.4	MeHg	e
3.06	0.56	5.3	Hg	f
3.84	0.86	0.9		g

major milk protein, α -Casein s-1 (30.6%) and β-Casein (28.4%). The MWa attributed (55kDa) is quite different from the real one (approx. 23kDa for both). The phosphorylation process of these proteins is not sufficient to explain this difference. To clarify this, the injection of a standard solution of β-Casein has been performed showing a peak at 2.09, which correspon to 51kDa. This shows that its elution volume is faster than expected, this may be due to the phosphate groups present or to aggregation of caseins. The peak *a* of Hg at 2.06mL has been then identify with milk caseins. Caseins are naturally binding Ca^{2+} and divalent cations Sugiarto et al. 2009. Moreover, milk caseins and serum albumin are able bind mercury and methyimercury. Inorganic mercury is able to form dimers of serum albumin (Sundberg et al. 1999), but in the ICP-MS chromatogram, no peak at 66kDa is present. Probably this is because all Hg ions are interacting with the caseins fraction. The two minors peak of mercury, peak *a* and *f* suggest an interaction with some cys containing protein (MWa 100kDa) and peptide (MWa 5.3kDa). The peak *a* could be attributed to some immunoglobulins, but whitout additional information the protein identification can be not obtained.

4.3.2 In-solution interactions of arsenic compounds.

The experiments with arsenic species have been performed with the same operative procedures shown in the previous subsections. Since the reactive form of arsenic compounds have a valence state of III, the MMA(V) and DMA(V) standards (Cacodylic Acid and Dimethylarsinic acid from Sigma Aldrich) were reduced before use. Briefly, MMA(V) and DMA(V) have been diluted with deionized water and a five-fold excess of cystein was added as a reducing agent (Naranmandura 2008; Bu et al. 2011) . The mixture was

left to react at 70°C for 1,5 hours and the reduction of the standards was verified by ICP-MS.

The chromatographic separation of oxidized and reduced species of arsenic has been performed according to Rabieh et al. (Rabieh et al.,2008), using a C18 column (Figure 4.18). The analytes have been separated using 5% of MeOH, 0.001% of tetrabuty-

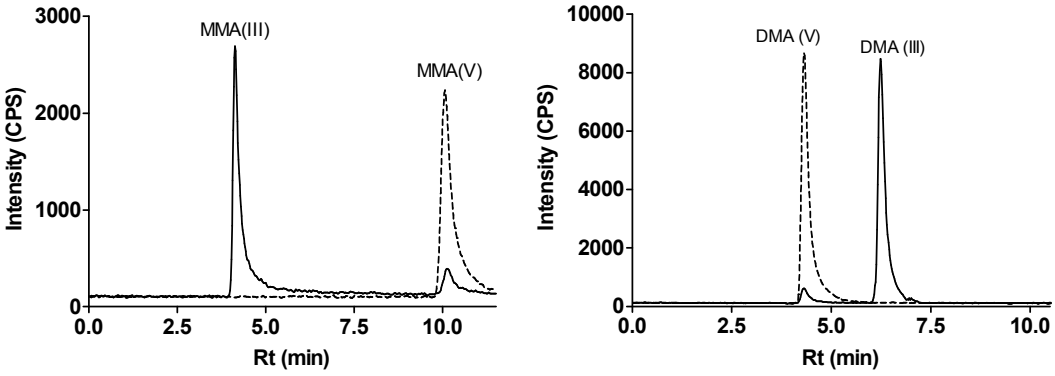


Figure 4.18: Chromatographic separation of oxidized and reduced species of MMA and DMA. The separation has been performed using a SBaq column, 2.1x150mm, Agilent Technologies. Flow 0.3mL/min.

lammonium hydroxide and 2 mM malonic acid. The chromatogram on the left shows the signal of MMA(V) before (dashed line) and after the reduction procedure (continuous line). As reported, the MMA(III) species can be detected since it elutes earlier. In the same manner, in the right figure, the reduction of DMA(V) is shown. The results show the reduction of 91.4% of MMA(V) to MMA(III) and the reduction of 95.1% of DMA(V) to DMA(III).

The reduced forms of the arsenic compounds have been used for spiking reactions as reported in the next sections.

Egg White

Fresh egg white has been treated as described for the mercury addition. The As(III), MMA(III) and DMA(III) have been spiked separately to the diluted sample and, after the reaction o/n, they have been subsequently diluted (50 fold) before filtration and injected into the HPLC (UV) ICP-MS system. The chromatograms obtained are reported in Figure 4.19.

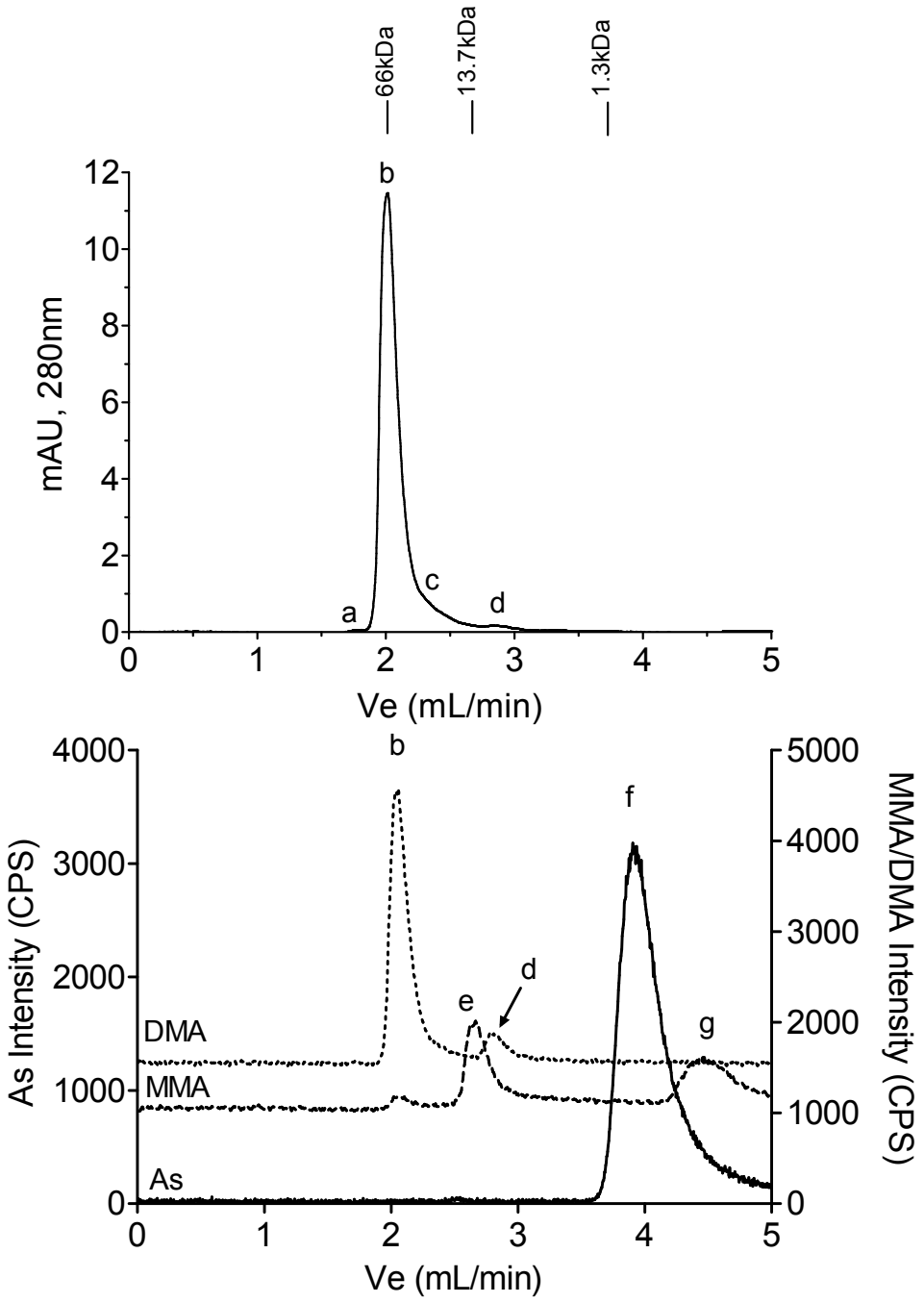


Figure 4.19: Chromatographic separation of egg white spiked with As(III), MMA(III) and DMA(III). The As elution is reported with a continuous line, while the MMA and DMA are reported with a pointed and a dashed line respectively. The MMA and DMA chromatograms have been shifted for visual clarity.

The eluting As(III) peak *f* at Ve=3.96mL reveals that no interactions with proteins (or other matrix components) are occurring (Table 4.14). The MWa calculated for arsenic (0.5kDa) is than that real one (0.1kDa) but, as the Ve of standard As(III) is the same, we can hypothesize a small shift from the SEC linearity for this compound. Arsenic metabolites show different behaviors since both are able to bind to ovalbumin (peak *b*), even if MMA seems to bind to this protein to a minor extent. MMA shows 2 other peaks at 2.66mL (peak *e*) and 4.46mL (peak *g*). The *f* peak correspond to a MWa of 13.5kDa, while the last peak is due to the residual, unbound MMA. It is interesting to note that two very similar compounds (such as As an MMA) have this difference in the Ve. We can attribute this behaviour to the onset of some interactions between the negatively charged MMA with the silica structure of the SEC column that slightly retains the analyte. The peak detected at MWa 13.5kDa has a MW close to lsozyme protein

Table 4.14: Table of eluting peaks during egg white analysis. Peaks without metals are obtained from DAD acquisition only.

Ve	k_D	MWa	Spiked	Label
1.8	0.108	100		a
2.05	0.17	44.1	MMA, DMA	b
2.43	0.32	17.8		c
2.66	0.41	13.5	MMA	e
2.79	0.46	10.4	DMA	d
3.91	0.89	0.5	As	f
4.45	1.1	<0.5	MMA	g

(14.3kDa). The ability of As(III) and its reduced metabolites to bind with lysozyme has been reported by Ramadan et al. in 2009. Even if the author shows *in vitro* interaction with reduced lysozyme, our experiment suggests that similar binding can occur under native conditions. DMA also reacts with other proteins in addition to ovalbumin, as revealed by the presence of a peak *d* with a MWa of 10.4kDa (Table 4.14) but in this case, no correlation could be found with the protein list reported in Table 4.7. For identification of proteins under this peak, additional techniques should be used as we do not have sufficient information to identify it. Finally, the main difference observed with respect to the same experiments performed with mercury is the different behaviours of the arsenic species. While Hg and MeHg have near identical interactions with albumen, arsenic and its metabolites show a selective interaction with matrix proteins.

Egg Yolk

Fresh egg yolk samples have been diluted and the protein content has been quantified as described for mercury analysis in the previous sections. Arsenic compounds have been added to the samples in the molar ratio 10:1 and, after incubation, they have been diluted 50 fold before injection.

The As(III) ion elutes practically at the same Ve (3.8 mL, peak *f*) as egg white, meaning that, in this case, no reaction with matrix protein has occurred. Methylated arsenic compounds show a discrete reactivity against some egg yolk components. MMA addition gives rise to three peaks with different Ve (2.03, 2.66 and 4.44 mL), and their corresponding MWa are reported in Table 4.15.

Table 4.15: Table of eluting peaks of egg yolk analysis. Peaks without metals are obtained from DAD acquisition only.

Ve	k_D	MWa	Spiked	Label
1.77	0.07	85.9		a
2.03	0.17	46.2	MMA, DMA	b
2.29	0.27	24.9		c
2.66	0.41	10.3	MMA	d
2.84	0.48	6.7	DMA	e
3.76	0.83	0.8	As	f
4.44	1.09	<0.5	MMA	g

Both MMA and DMA are co-eluting with the most intense peak in the UV chromatogram, and its identification (46kDa MWa) should correspond to some proteins present in the soluble fraction. Anyway, the egg yolk proteins listed in Table 4.9 show that no correspondence can be found between the 46kDa peak and the livetins. Maybe phosvitins could be indicated as the target since they have a closer MW (MW range 37-45kDa) but, as they are phosphorylated proteins, the negative charge of the phosphate groups should give rise to electrostatic repulsion of arsenic species. In the same manner, the peaks *d* and *e* could not be attributed to any protein listed in the Table 4.9, and the presence of some minor proteins that interact with MMA and DMA could be hypothesized.

Gelatin

Despite the high MW of collagen fibers (300kDa and more), the DAD chromatogram of gelatin (Figure 4.21) shows proteins eluting at lower molecular weights. This, as showed

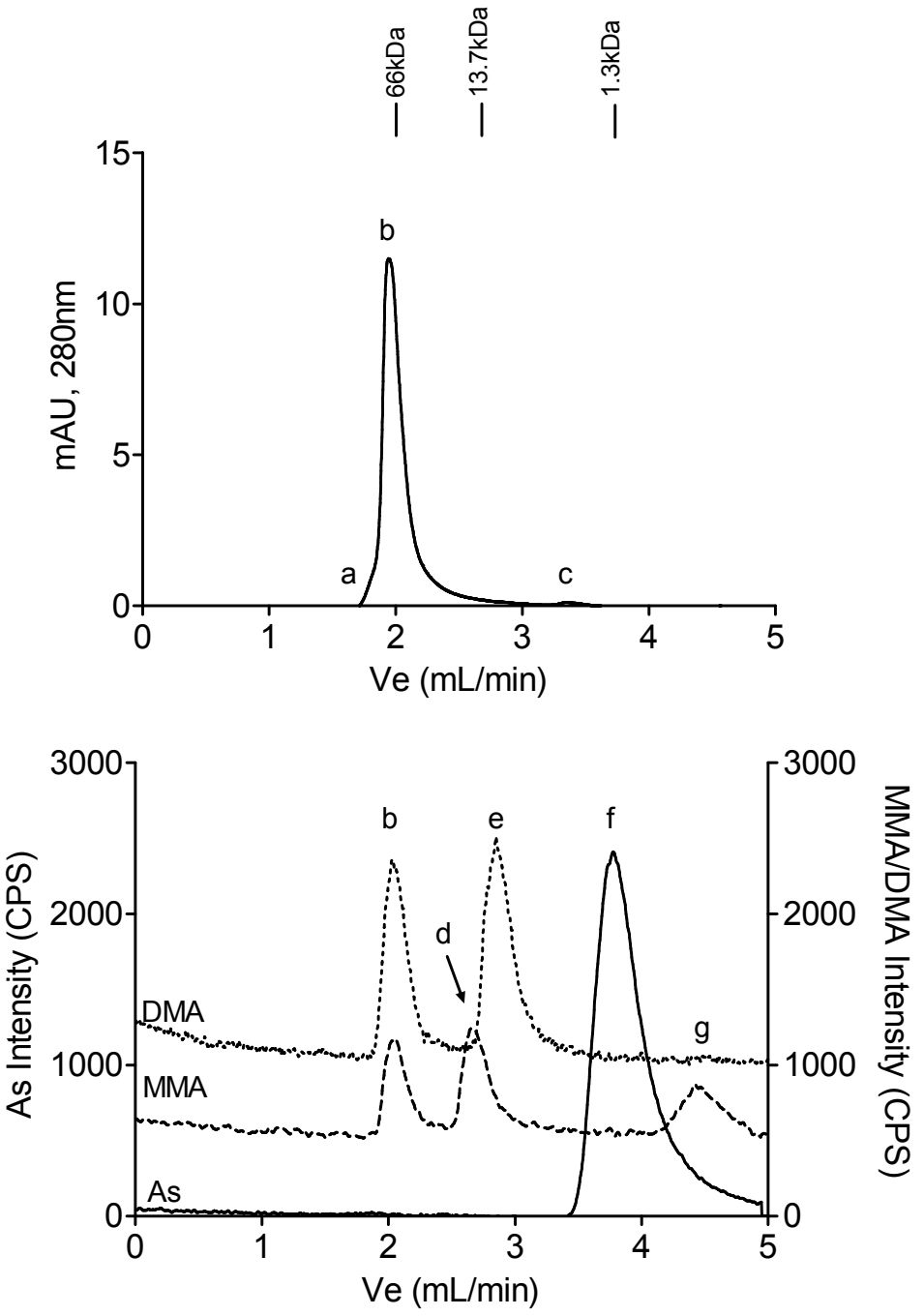


Figure 4.20: Chromatographic separation of egg yolk spiked with As, MMA and DMA. MMA and DMA chromatograms have been shifted for visual clarity.

in mercury-spiked samples, is due to denaturing of the original protein to obtain food gelatins. In the sample spiked with ionic arsenic, a clear peak at Ve=2.56 reveals that some interaction with As(III) has been occurred in this sample. The protein binding arsenic is a small protein with a MWa=22kDa (Table 4.16) and probably, due to the low intensity of this peak, its concentration is quite low. Arsenic(III) binds to cysteines in proteins in a different way with respect to Hg^{2+}, since its uncharged at phisiological pH and moreover, the arsenic bind is obtained via water loss. This reflects in the difference of MW of mercury and arsenic-interacting proteins in the gelatins analysis. The major As(III) fraction, elutes at Ve=3.86, with an apparent MW of 0.8kDa. Suggesting no additional interactions with molecules. In the MMA and DMA analysis, just one peak

Table 4.16: Table of eluting peaks from food grade fish glue spiked with arsenic species analysis.

Ve	k_D	MWa	Spiked	Label
2.09	0.19	51		a
2.45	0.33	22	As	b
2.56	0.37	17	MMA	c
2.88	0.49	8.1	DMA	d
3.51	0.73	1.8		e
3.71	0.81	1.2		f
3.86	0.87	0.8	As	g

can be detected indicating the presence of small proteins (or fragments of proteins) that are able to interact with them. As observed for the mercury analysis, low content of sulphydrile groups is present in the gelatins, and this limit the onset of interactions with trivalent arsenic species. Moreover, since both arsenic metabolites and gelatin are negatively charged at phisiological pH, other kind of proteins should be the target. Even in this case, the identification of proteins under the peaks b, c and d could be very interesting but, with ICP-MS analysis, no other molecular information can be obtained.

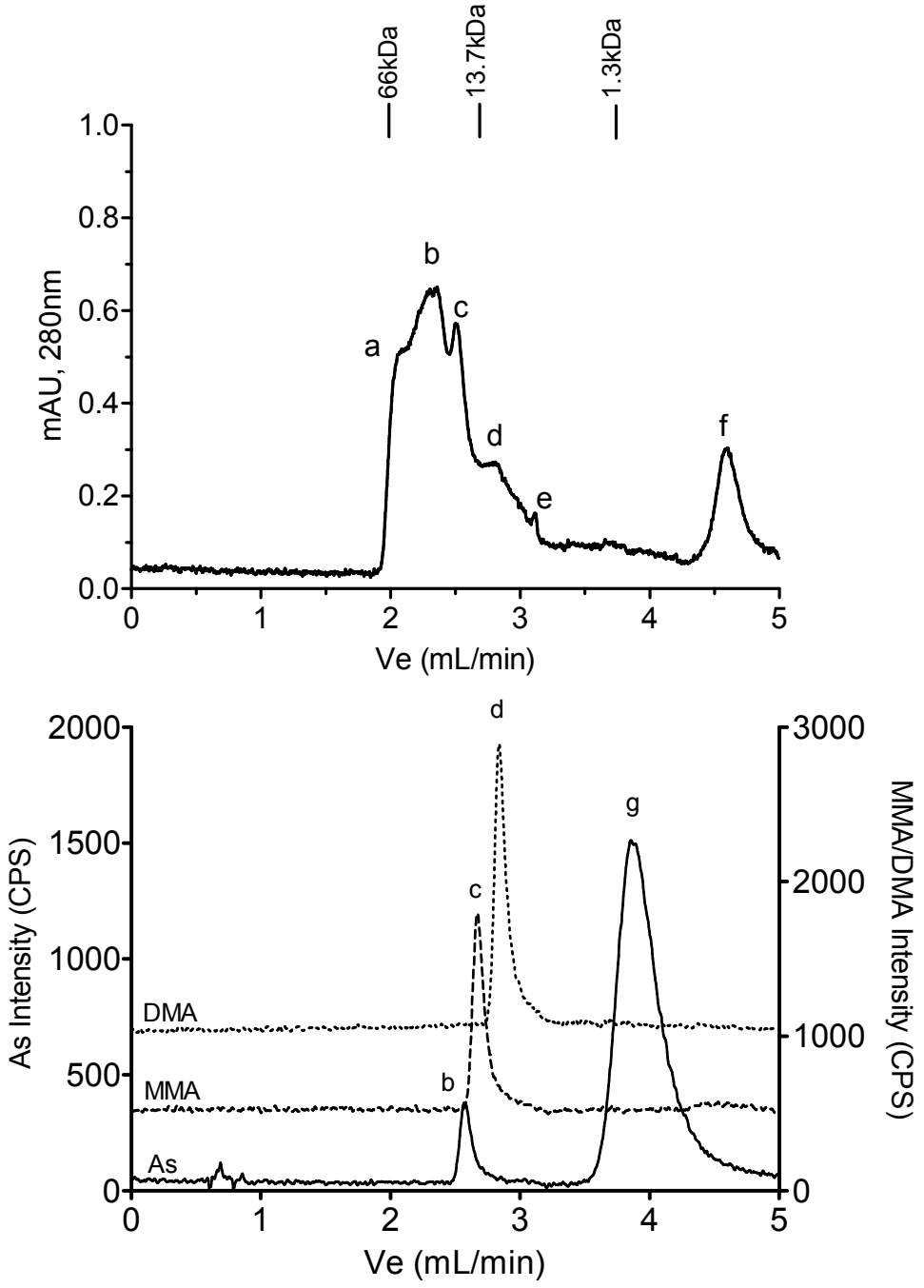


Figure 4.21: Chromatographic separation of edible fish glue spiked with As, MMA and DMA. MMA and DMA chromatograms have been shifted for visual clarity.

Milk

The milk sample has been purchased from the local market and, before arsenic addition, it has been diluted 3 fold with TRIS/PMSF buffer. The protein content has been estimated, and a ten-fold molar excess of arsenic and arsenic species has been added. Before injection, the samples were diluted to lower the metal load into the ICP-MS to avoiding column/instrument contamination. As observed for egg samples analysis, no reactivity can be attributed to the As(III) ion versus any protein.

Table 4.17: Table of eluting peaks from milk analysis.

Ve	k_D	MWa	Spiked	Label
1.82	0.08	96		a
2.13	0.2	46.5	DMA	b
2.45	0.33	22		c
2.67	0.41	13.2	MMA	d
2.83	0.47	9.0	DMA	e
3.96	0.91	0.6	As	f
4.42	1.08	<0.5	MMA	g

The arsenic metabolites are quite unreactives with respect the caseinic moiety of the milk matrix (peak *b*, in the upper panel of Figure 4.22 on the following page). This is explainable considering the negative charge of caseins, due to the high phosphate groups content. As both MMA and DMA have a negative charge (-1) at neutral pH, the electrostatic repulsion can inhibit the onset of the interactions between them. Otherwise, two clear peaks indicating the presence of interactions with non caseinic proteins are observable at Ve 2.67 (peak *d*, with MMA) and Ve 2.83 (peak *e*, with DMA). The apparent MWs calculated for these peaks are 13kDa and 9kDa respectively, suggesting an interaction with two small proteins in the milk serum (whey). In particular, the α-lactalbumine (MW 14.2Da) is know to bind arsenic species *in vitro* Schmidt et al. 2009 and it can be considered as a possible target for MMA. No correspondence was found for peak *e*. Finally, peak *g* is the elution of free MMA, as previously observed.

4.4 Conclusions

The developed method above shows the possibility of studying both the metallic and the molecular moiety of a metal-protein complex. In particular, as both have quite different chemical physical characteristics, each procedural step has been carefully evaluated dur-

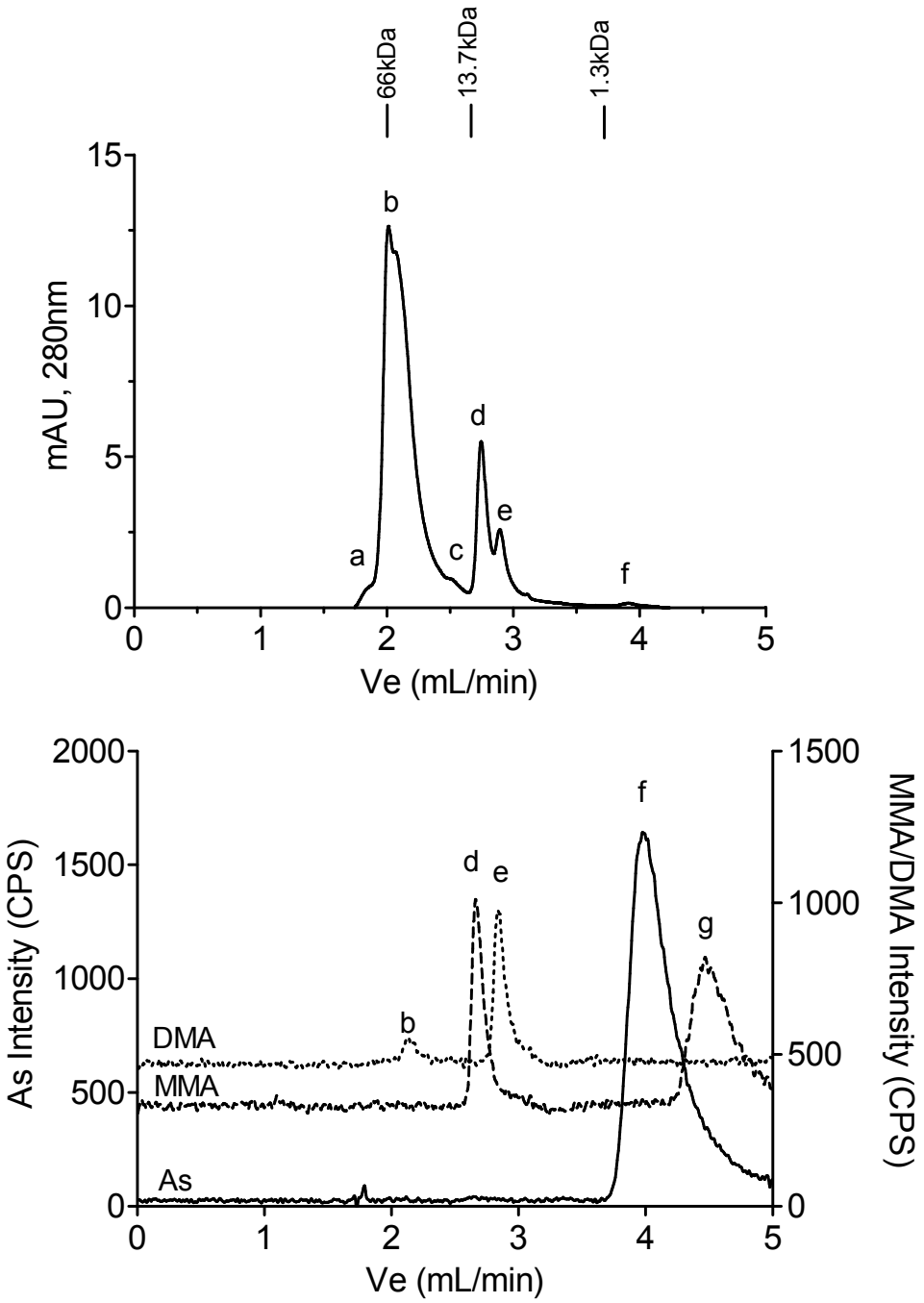


Figure 4.22: Chromatographic separation of milk spiked with As, MMA and DMA. MMA and DMA chromatograms have been shifted for visual clarity.

ing the method development. The analysis of some spiked samples has shown the benefit of the use of HPLC ICP-MS to detect complex formation. In addition, the high ICP-MS sensitivity has lead us to verify the presence of some minor proteins not detected by DAD in the chromatogram.

The comparison of results for arsenic and its trivalent metabolites reveals different behaviours. MMA and DMA showed characteristic interactions with matrix components in all samples analyzed. This has lead us to show that, even a small chemical modification such as the methylation of a metal, give rise to very different reactions in solution that are easily detectable with this method. In a stange manner, pratically no As(III) reactivity versus any proteins has been observed. It must be considered that the presence of complex matrices could alter the onset of interactions. Moreover, the oxidation of As(III) to As(V) by some reactive matrix component can not be excluded. Anyway, some authors funded no As(III) alterations in presence of biological tissues (Currier et al. 2011) or during sample manipulation (Lu et al. 2009) and this suggest deeper investigations of what is occurring in our samples.

For mercury and methylmercury, small differences have been found. As general consideration, their reactivity appears to be similar in pratically all samples analyzed. In the case of milk analysis only, some differences have been found, mainly reconducible to the ability of Hg^{2+} (but not $MeHg^{+}$) to form dimers.

The main limit however, is evident. Even if with the coupling with HPLC some important molecular information can be obtained, this is not sufficient for the identification of the protein moiety. This aspect lead us to consider the use of additional techniques for method improvement, as will be reported in the next chapter.

Chapter 5

The study of non covalent interactions of As/Hg with proteins in their native matrix (Chapter 3) has shown the low of specificity of SEC-based proteins identification. Moreover, even if the use of hyphenated techniques coupled to ICP-MS leads us to obtain some molecular information, more suitable instruments should be considered to complete the results. For method implementation, MALDI-TOF instrumentation has been used in the following experiments.

In order to apply the method to some naturally contaminated samples, the most suitable extraction technique had to be found. To test the best buffer for extraction of metal-protein complexes, two Certified Reference Materials (CRMs) have been used. They have been chosen in order to provide quantitative informations about metals recovery. The certified values reported for the CRMs used are the total content of As/Hg metals and some of their species. Both metals loss and metal species variation during the procedure have been evalued. Metal species preservation is the most important factor in the interaction studies since, as found in the previous chapter, each single specie of a metal can interact differentiallt with a specific protein.

5.1 Extraction buffers and procedure

Certified values and origin of hte CRMs are reported in Table 5.1. Both have been obtained from dorsal tuna fish muscle. The producers declared that the muscle has been sliced, minced and freeze-dried. It was grounded with a mill equipped with ZrO_2 balls and sieved before homogenization to obtain a particle diameter $<125\mu m$.

Since the CRMs used have already been homogenized as a lyophilized powder. No other preparative procedures have been judged necessary. They have been simply extracted with a sonic bath using different solutions. This procedure has lead to the rehydration of micro-particles and to the extraction of soluble components (such as proteins

Table 5.1: Specifications for CRMs used.

	BCR 463	BCR 427
Origin	tuna fish, from Adriatic Sea	tuna fish, from Ionic Sea
Values Certified (μg/g)	$TotalHg$: 2.85 ± 0.16 $MeHg$: 3.04 ± 0.16	AsB: 52 ± 3 μmol/kg $DMA(V)$: 2.0 ± 0.3 $TotalAs$: 4.8 ± 0.3

and metals). Two buffers have been chosen in order to compare the protocols generally used to extract metals (and metals species) with protocols generally used to extract proteins.

Buffer 1

The first extractive solution is a commonly used buffer in molecular biology to extract proteins from tissues. The solution contains 25mM TRIS as a buffer to keep the pH in the optimal range (7-8), NaCl to conserve the sample ionic strength and phenylmethylsulfonyl fluoride (PMSF), as inhibitor of proteases (0.1mM). Proteases are common enzymes present in tissues and in bacteria that are the principal cause of protein degradation. Moreover their inhibition is fundamental in order to obtain a stable protein extract. It is important to note that PMSF is used to stop serine proteases (Gimenez et al. 2000). EDTA is used as an additive for the inhibition of an other important classes of proteases, the *metalloproteases* (MP) (Thompson et al. 2012). In our study, EDTA is not added to the extractive solution since its capability to strongly chelate metals can have negative effects on the preservation of metal-protein interactions.

It is estimated that the PMSF activity persists for no more than 2 hours, due to its rapid degradation in aqueous solutions. For this reason, it was added to the TRIS buffer immediately before sample analysis. Moreover, all samples have been injected as soon as possible after extraction to avoid storage of the extracts.

Buffer 2

The second buffer used has been chosen among those reported in Table 2.1 on page 13. As most of the listed buffers are incompatible with protein stability (e.g. methanol and strong acids), we decided on an aqueous solution of βmercaptoethanol (βME). This composition is of particularly interest in mercury speciation since it acts as a stabilizing

agent. Another important advantage is the antioxidant property of thiols which leads to the preservation of reduced forms both of metals and proteins.

5.1.1 Extraction of metal-protein complexes

To obtain the extracts, both CRMs have been carefully weighed and the tested buffers have been added. The CRM-buffer mix gave rise to a suspensions which has been vigorously shacken and subsequently put in a sonic bath for 20mins. To avoid protein degradation due to heating in the sonic bath, water is kept cold (+4°C) during the extraction with ice addition. Before extract injection, all solutions have been centrifuged at 2000rpm to avoid precipitation of insoluble particles into the SEC column.

Chromatographic separation has been performed using the conditions reported in the previous chapter. The analyte elution has been monitored using both DAD and ICP-MS, for the latter, the elution of As (at m/z 75) and Hg (m/z 202) have been followed using the same instrumental parameters reported in Table 4.4 on page 45. For the apparent molecular weight (MWa) determination the SEC calibration curve reported in Figure 4.6 on page 39 has been used.

Metals and protein recovery has been evaluated for both buffers. In particular, the elution range 1.8<Ve<3 (such as the range of MWa between 100kDa and 5kDa) has been considered as the most indicative in terms of metal-protein binding preservation. It is important to highlight that this parameter has not an absolute value, due to differences in the thermodynamical stability of different metal-protein interactions in a complex sample. Anyway, we can judge it useful to get some information on the preservation of existing interactions.

Arsenic complexes extraction

Arsenic SEC chromatograms relative to the two extractions of BCR 627 (Figure 5.1) appear to be slightly buffer-sensitive. All peaks of arsenic detected with ICP-MS seem to be related to small protein, or proteins fragments and no peaks in the higher molecular ranges (<2.5mL) have been detected. The main peak at Ve 3.73mL has a MWa of 1.1kDa and probably represents the free fraction of arsenic, meaning that no interactions with macromolecules are occurring. The signal of As coupled with molecules eluting under peak *b* (Figure 5.1, right panel) is the only peak with a noticeable difference in the two extracts. TRIS buffer extraction lead to a recovery improvement of 182% with respect the βME buffer. The other peaks extracted (such as peaks *a*, *c* and *d*) are practically superimposable, with just a small improvement for peak *d* with TRIS (2.7%). This could

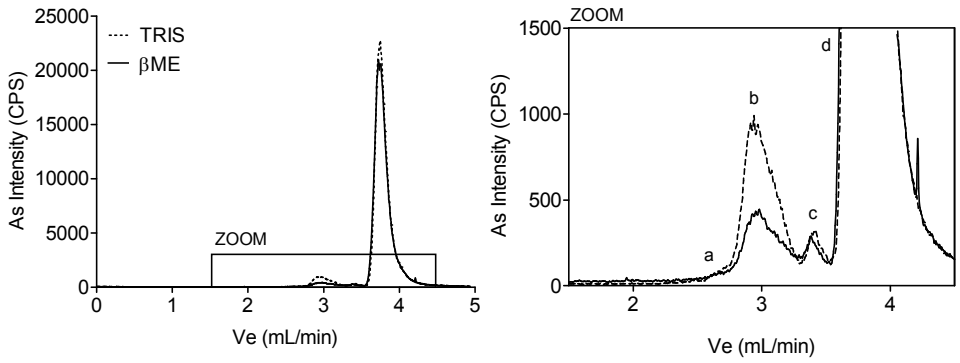


Figure 5.1: ICP-MS chromatogram of BCR 627 extracted with TRIS (dashed line) and βME (continuous line).

indicate both a major stability of arsenic-protein binding in the presence of TRIS buffer or a major capability of this buffer to extract them from tissues. Considering the nature of the CRMs, the effect of buffer addition to the samples seems to be just a resolubilization of lyophilized proteins, more than a real extraction. This leads us to hypothesize that the difference between the two buffers was only in the ability to resolubilize the proteic moiety. the HPLC- DAD chromatograms of the eluted proteins are reported in Figure 5.2 to clarify this point.

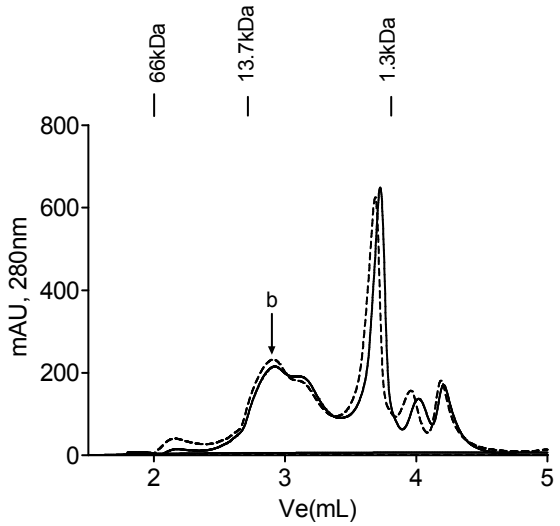


Figure 5.2: DAD chromatogram of BCR 627 extracted with TRIS (dashed line) and βME buffer (continuous line).

As can be observed, the chromatograms are quite identical. Peak *b* detected by ICP-MS has been used as evidence that there are no differences in the proteins recovery between the buffers.

This is clearly shown given that the intensity of the As signal under this peak is larger due to the major stability of the complex in the presence of TRIS buffer with respect to the thiols rich buffer.

Mercury complexes extraction

The evaluation of mercury-protein complex extraction has been performed using BCR 463, certified for total and MeHg content. Differently to what was observed in the arsenic extraction, when mercury is extracted with TRIS or with βME buffer, very different results have been obtained (Figure 5.3). TRIS is clearly shown to be inefficient in extracting the whole mercury fraction from the sample, since practically no signal relative to m/z 202 has been detected. Only a small peak *a* at 2mL shows the presence of some

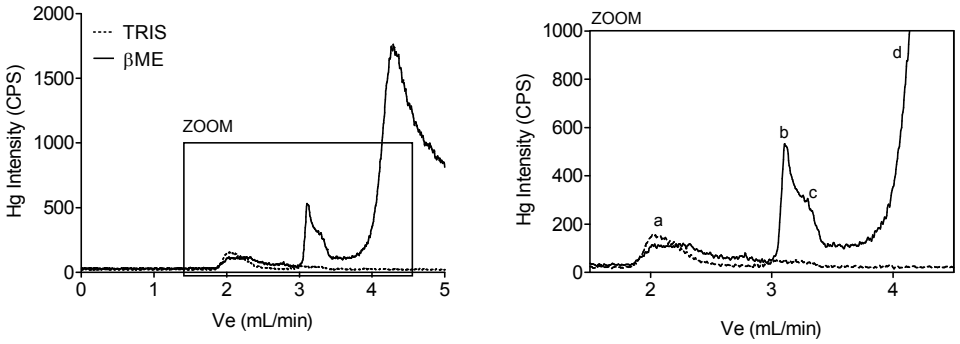


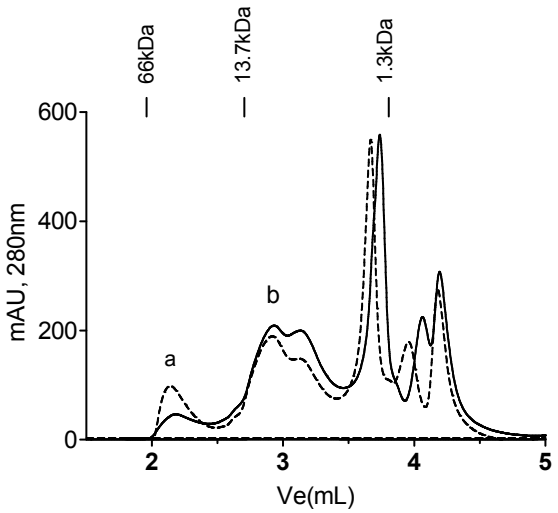
Figure 5.3: ICP-MS chromatogram of Hg extracted from BCR 463 with TRIS and βME/l-cys buffer.

mercury species co-eluting with a macromolecule (MWa 61kDa). For this peak, the recovery obtained with TRIS is just slightly more intense than that obtained with βME (+30%). This means that, in the extraction of metals-proteins complex, the former buffer is still the best one. However, the differences in the region of high Ve (Ve> 3 mL) put in evidence the ability of the thiols-rich buffer to improve the mercury recovery, even in the presence of some molecular interactions (peaks *b* and *c*, of 4.8 and 3.2 kDa respectively).

Protein elution has also been evaluated for this sample, and the relative DAD chromatograms are reported in Figure 5.4. What is observed is very similar to that obtained for arsenic extraction, showing the same origin of the analyzed tissue (i.e. tuna fish

muscle). The presence of a more intense peak *a* in the chromatogram seems to suggest as improved preservation of proteins present in this CRM sample with respect to the previous one.

In the minor MWa ranges (Ve>3.5mL), a peaks shift has been observed, with a major apparent MW in the TRIS buffer extract with respect to βME. The ion current of cobalt, an endogenous metal, is practically superimposable between these samples indicating that no chromatographic alterations occured. Probably this shift is due to the onset of oxidation reactions during sample extraction. In fact, βME is an antioxidant



DAD chromatogram of BCR 463 after TRIS and βME/l-cys extraction.

Figure 5.4: .

that prevents peptide/proteins oxidation. As a consequence, when this buffer is used no condensation reactions are observable since cysteinic residues in the proteins remain in their reduced state. These changes in MW as a consequence of oxidative processes can be easily shown with SEC chromatography (Mirzaei et al. 2008) and, in order to avoid these kinds of alterations, the use of βME buffer can be advantageous.

5.1.2 Quantitative extraction of metals

All extracts obtained with the buffers under investigation have been analysed with ICP-MS to obtain the As and Hg content. A calibration curve prepared in the range of 0-100 ppb was used to calculate the concentrations of As and Hg and the results have been compared with the certified values of each CRM to quantify the recovery%.

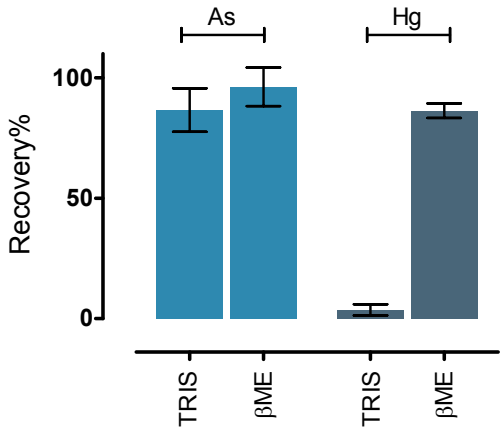


Figure 5.5: Recovery quantification from extracted CRM with TRIS buffer and βME/l-cys buffer.

Data in Figure 5.5 show the relative differences in the recovery of mercury and arsenic during the sample extraction. When a thiolic-rich buffer is used, the antioxidant properties of βME and l-cys have a beneficial effect in the stabilization of mercury and the recovery for the extraction procedure appears to be good (86.3±3%). The same mercury extraction with TRIS buffer gave a loss of practically all the metal content (recovery 3.6±2.3%). This loss can be attributed to the instability of ionic mercury (or methylmercury), which undergoes oxidative processes or absorption onto the vessel surfaces. It is important to highlight that the low recovery is not affecting the metallic moiety bound to the macromolecules, but just the unbounded one.

Arsenic recovery is less affected by the buffer used, since small differences have been observed. The best recovery is obtained with the use of βME buffer (96.3±8%). These data have been used to estimate the global recovery of both metals in the whole analytical procedure, considering the data obtained from the chromatographic method development (Figure 5.6).

Mercury loss during the analytical procedure shows a significative difference between TRIS and βME-based buffers. When TRIS buffer is used, the main mercury loss (96.6%) is due to the extractive procedure. On the other hand, even if βME-based buffer is able to limit the mercury loss during the extraction, additional mercury is lost during the separation procedures. When thiols are used both for the extraction and the chromatographic separation, the global recovery amounts to 75%.

In the arsenic recovery evaluation, the use of a βME buffer leads us to obtain a small improvement but the final values are very similar for both buffers (TRIS 83%, βME 90%).

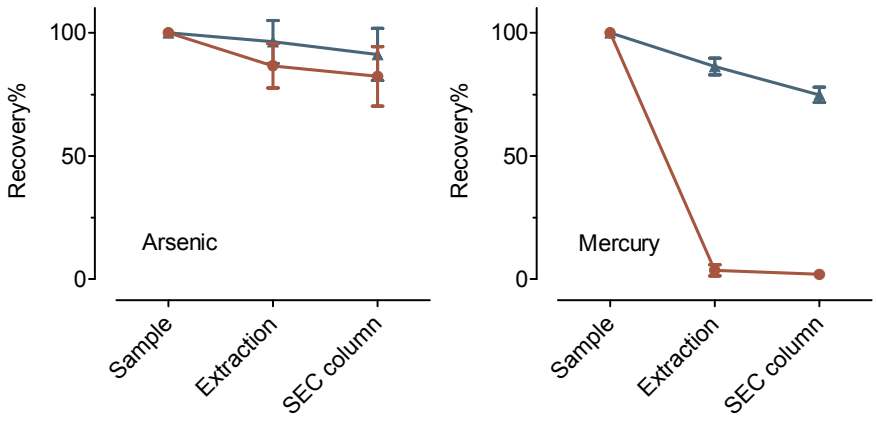


Figure 5.6: Recovery of whole procedure using with TRIS buffer (red line) and βME/l-cys buffer (green line).

With these investigation, some important considerations can be made. Firstly, both buffers provided some advantage regarding the metals or the metal-proteins analysis. A final choice of just only one of them, without considering this is not opportune. For this reason, the use of a TRIS buffer with thiols addition seems to be the best condition for the samples under investigation. Since the mobile phase used for SEC separation contains l-cys, we prefered to add this as a thiols source instead of βME, to work with similar conditions.

With these considerations in mind, all extractions cited below will be performed using a final buffer composition of: 25mM TRIS, 50mM NaCl, 0.1mM of PMSF and 0.1%l-cys.

5.1.3 Metal species preservation

Previous analyses have been all performed considering only the As/ Hg total content . Since in the real samples these metals can be present as different species, their preservation in the CRM extracts has been also evaluated. For As and Hg speciation, the chromatographic separations reported in the literature have been reproduced, with minor modifications.

Arsenic species

Arsenic species separation has been performed using anion exchange chromatography. The method reported in the literature (Ammann 2010), has been modified with pHBA (parahydroxybenzoic acid) addition in order to increase the elution of As(V), that is strongly retained due its charge (2-). Without this additive, column contamination has been observed after a few injections. The method is summarized in Table 5.2.

Table 5.2: Mobile phase composition for arsenic species separation. The mobile phase pH was 9 in all cases.

Phase A	0.02% NH_3
Phase B	0,1M NH_4NO_3 in 1% pHBA
Flow	250µL/min
Injection volume	10µL

Five species of arsenic have been separated. They were: Arsenite (As(III)), arsenate (As(V)), monomethyl arsenic acid (MMA), dimetylarsenic acid (DMA) (all from Sigma Aldrich) and Arsenobetaine (AsB) (BCR 626, from IRMM). The column (PRP X100, 2.1x150mm, Hamilton) in PEEK material has been used. Since the net charge of arsenic compounds is pH dependent, species separation is obtained working at a basic pH. This led us to keep negatively charged most of species. The nitrate anion is responsible for the elution of negatively charged analytes (MMS, DMA and As(V)), while positively charged AsB elutes with the void volume of the column. As(III), that is uncharged at pH=9, is slightly retained and a low concentration of NO_3^- is sufficient for its elution. The separation of standards compound and the %B used for the gradient generation are reported in Figure 5.7 on the following page.

The chromatographic separation reported abive has been used to analyse the BCR 627 extract in order to detect and quantify the arsenic species. BCR 627 (0.5gr) has been extracted with the buffer as reported in 5.1.2 and the surnatant has been injected onto the ion exchange column for arsenic species detection (Figure 5.8).

The species extracted from the CRM are constituted mainly of Arsenobetaine (AsB), with lower amounts of DMA, MMA and As(V). The presence of As(V) and MMA, altrough not certified for this CRM, has been already identified, confirming the validity of extraction procedure of Leufroy et al. 2011. Other small peaks have been detected between the elution of DMA and MMA, indicating the presence of additional arsenic species. Some authors have identified the presence of trimethylarsine oxide (TMAO) or tetramethylarsonium ion (TMAS) (Nakazato et al. 2000; Dufailly et al. 2007) in this

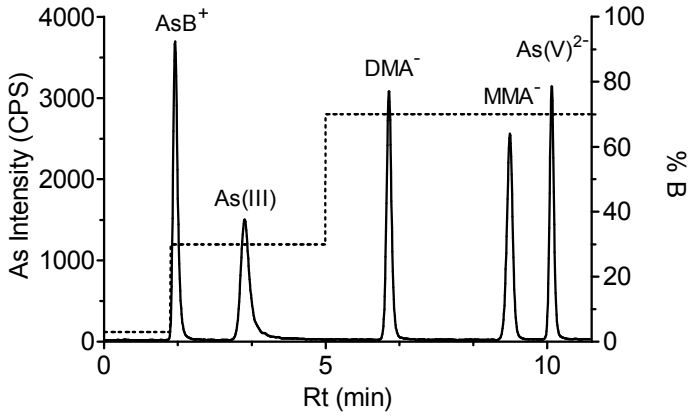


Figure 5.7: Chromatographic separation of five arsenic standards using ion exchange column. The % of mobile phase with respect to the time of analysis is reported on the right axis.

CRM using a different chromatographic approach. Maybe there are the same species responsable for the unknown peaks, but we cannot verify this.

To quantify the extracted species, the standard addition method has been used, and the recovery quantification has been calculated from the certified value reported in Table 5.3 on the next page. Both AsB and DMA gave a satisfactory recovery under these conditions (Figure 5.5). The total arsenic recovery is also positive. For total arsenic quantification, the sum of all eluting peaks in the extract chromatograms has been calculated.

The injections of the same extract into the SEC column leads us to detect several peaks for arsenic (Figure 5.9, SEC panel). The species identified with the ion exchange column are responsible for the As signals observed in the SEC separation, but we have no idea which species were interacting with the biological molecules. For this reason, the CRM extract has been then re-injected onto the SEC column and the fractions containing As have been collected. The peaks collected were *a*, *b* and *c*. These fractions have then injected onto the anion exchange column and arsenic species have been verified (**??**, SAX panels).

This procedure can be considered an off-line 2D chromatography and gives the possibility of verifying the metal species present. A similar approach has been reported in the literature by Li et al., in the analysis of aqueous extract of Antarctic krill. They found the reactive As(III) as the main arsenic species present but, in their samples, this species was not bound to any high molecular weight fraction (Li et al. 2005).

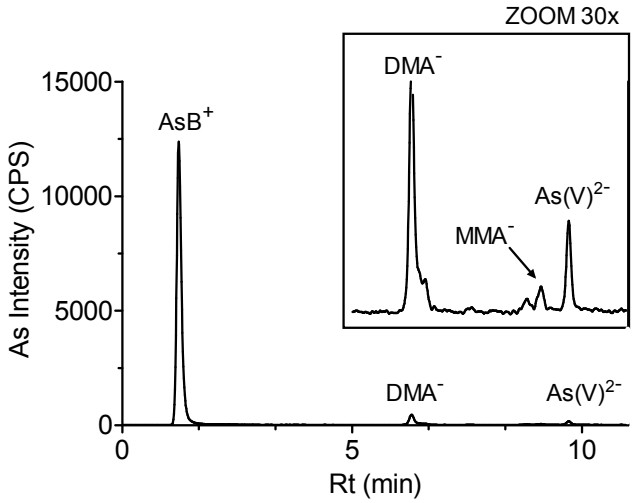


Figure 5.8: Chromatographic separation of arsenic species extracted with TRIS-based buffer using an ion exchange column.

In the CRM fractions analyzed, the arsenic species detected were quite different. Fraction *a* contains just DMA, indicating that this species is interacting with some endogenous molecule around 15kDa MWa, while fraction *b* seems to be more complex, and several species can be detected. In particular, two unknown peaks (Unk1 and Unk2) are co-eluting with DMA, meaning that the unknown species detected in the whole extract are eluting in this fraction. Finally, peak *c* is mainly constituted of unbound arsenobetaine. The presence of "free" arsenobetaine is due to its inability to bind proteins, as reported by Pei K.L (Pei et al. 2009).

Table 5.3: Recovery of arsenic species extracted from the BCR 627.

Total As	$99\% \pm 2.6$
AsB	$93.8\% \pm 5.0$
DMA	$93.4\% \pm 5.4$

Mercury species

For the analysis of mercury species, reverse phase chromatography has been chosen. The chromatographic method has been previously developed in our lab (Cairns et al. 2008) for water analysis and wassubsequently implemented for tissues extract analysis. Species separation has been performed using a SBaq column, 2.1x150mm (Agilent Technologies)

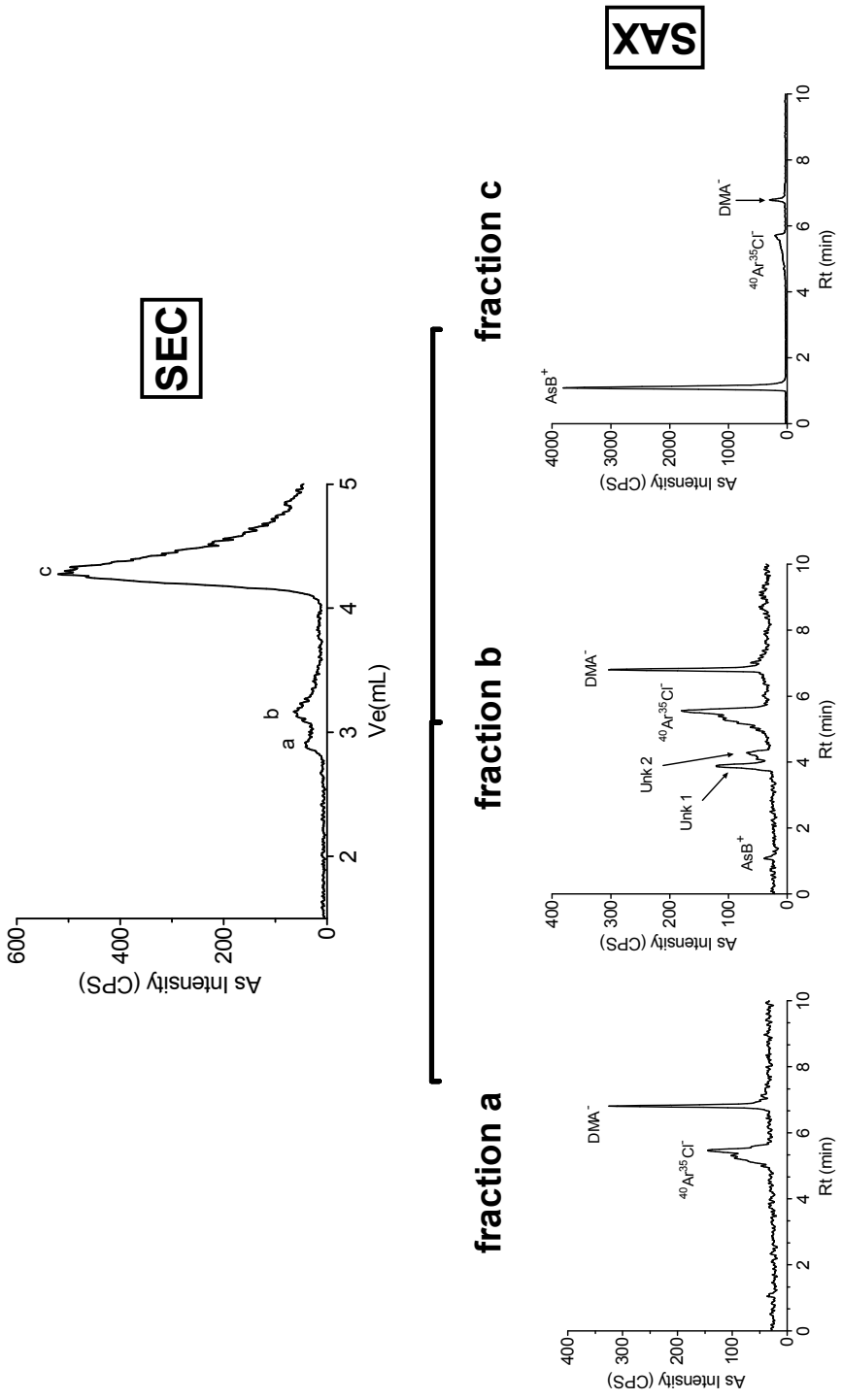


Figure 5.9: Chromatographic separation of arsenic species collected from the SEC column.

under isocratic conditions. This column has been chosen since it is suitable for aqueous mobile phases, as required for this chromatographic separation.

Method parameters and the separation obtained for standard species available are reported in Figure 5.10.

Mobile phase	0.05% βME 0.5% Cys (w/v) 3%MeOH
Flow	300μL/min
Loop	10μL

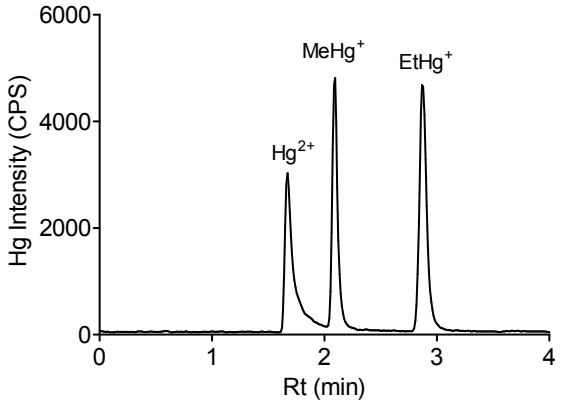


Figure 5.10: Parameters and chromatographic separation of ionic mercury and some organic species with reverse phase column.

BCR 643 has been extracted with TRIS and directly injected onto the C18 column for mercury speciation analysis. Since the presence of dispersed materials can occlude the column pores, sample centrifugation has been performed to pull down debris and insoluble fractions. The obtained chromatogram is reported in Figure 5.11. It shows that, as expected, almost all the mercury present in the sample is in the form of MeHg, while no EtHg has been detected and the Hg^{2+} signal is quite low.

The certified values show that the species recovery, was 92% for total mercury and of 85% for methylmercury. The total Hg recovery is appreciable, but MeHg species recovery seems to be lower than expected.

With additional experiments, we found that a slight conversion of $MeHg^{+}$ to Hg^{2+} (around 2.7%) can occur in the standard solution, if exposed to warmth or light. This MeHg loss increases in the spiked matrices (about 5-10%), probably because mercury demethylation occurs both via chemical and biological causes.

Table 5.4: Recovery of MeHg and total mercury extracted from the BCR 463.

Total Hg	$92\% \pm 6$
MeHg	$84.9\% \pm 2.1$

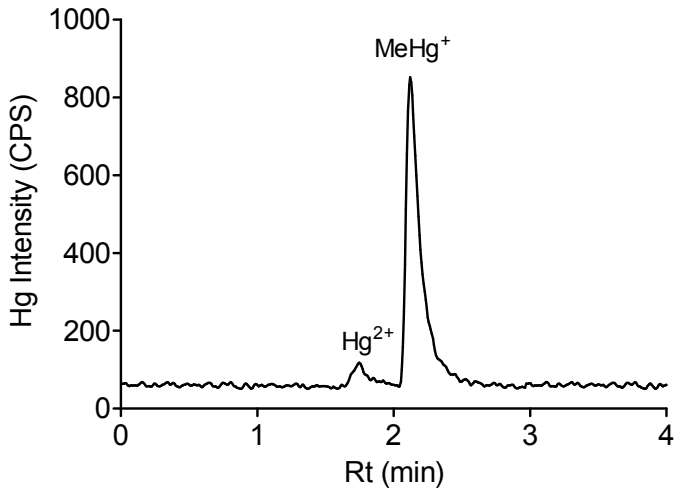


Figure 5.11: Chromatographic separation of mercury species extracted from BCR 463.

Even though this does not affect the total recovery in the speciation studies, it could introduce some erroneous evaluations. In order to keep this interconversion as low as possible, extracts should be immediately analyzed or conserved at +4°C in the dark for short periods.

Fractions obtained from SEC column analysis of the extracts have been injected onto the C18 column and the chromatograms obtained have been reported in Figure 5.12.

MeHg is present under each peak collected from the SEC column. In the previous chapter the chemical interaction established by Hg^{2+} and $MeHg^{+}$ with proteins was found to be practically the same. This suggest that, even if the low levels of inorganic Hg^{2+} led us to hardly detect it, its presence in the same fractions in which $MeHg^{+}$ is eluting can be expected. The small peak of Hg detected in all fractions seem to confirm this hypothesis.

5.2 Analysis of real samples

The real samples analyzed are specimens of fish coming from a polluted lake in the north of Italy (Mergozzo lake, MERGOZZO (VB)). Perch freshwater fish (*Perca fluviatilis*) has been used for this study. This freshwater fish can inhabit estuarine lagoons, lakes and medium-sized streams. Samples analyzed were slices of muscular tissues (n=6) from specimens fished in the period sept-nov 2009 and stored at -20°C until analysis. Due to the levels of mercury (several hundreds of ppb) and the concomitant presence of arsenic

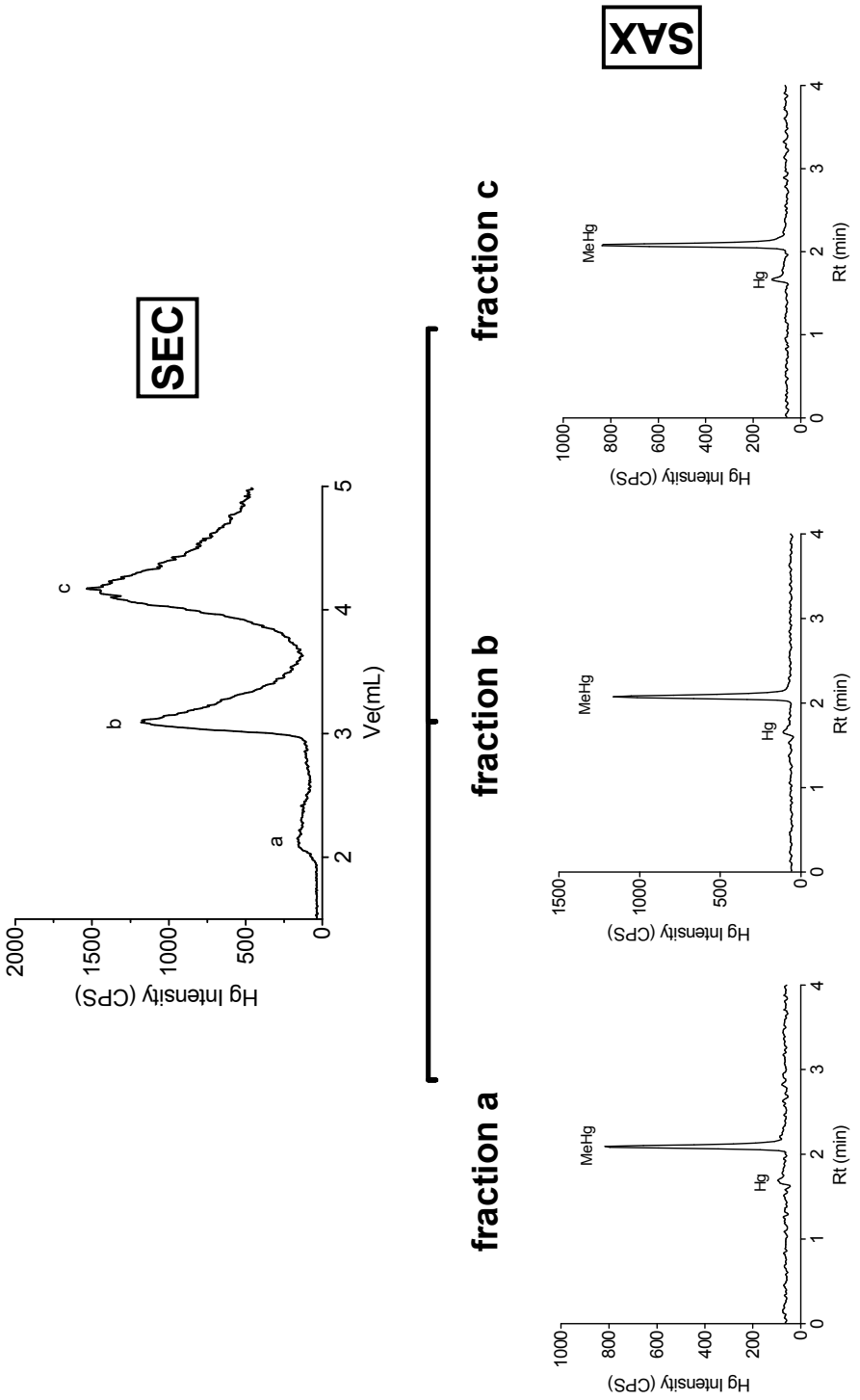


Figure 5.12: Chromatographic separation of mercury species collected from SEC column injection.

in the fish tissues, these samples have been judged helpful to directly investigate the interaction of these metals (and their relative species) with proteins into living systems.

In the literature (De Gieter et al. 2002; Li et al. 2008; Fu et al. 2010; Shiber 2011) several reports of arsenic and mercury speciation in fish samples are present, but very few of them regard the identification of the proteic target in complex tissues sample (Naranmandura et al. 2008; Zhang et al. 2007). Due to the problem of food security, fish represent an important organism for studies of heavy metals interaction. In addition, the high levels of sulphur-containing amino acids (such as methionine and cysteine) with respect to other proteic foodstuffs, make this organism very interesting for the aims of our study.

In our work, we focussed on fish muscle, since it represents the main the edible part. In addition, the high metabolic level (O_2 consumption) and the structural changes which it undergoes during muscular movement, make this tissue very interesting.

From a biological point of view, muscle fibers are made up of different proteins arranged in a specific manner in order to frorm a series of thick and thin filaments. Proteins characterizing this tissue are usually divided into three major groups:

1. *structural proteins*: such as actin, myosin, tropomyosin and actomyosin are involved in the muscular contractions and they represent up to the 75% of all muscle proteins.

2. *sarcoplasmic proteins*: such as myoglobin, parvalbumin and some enzymes are involved in the metabolic process that leads to the muscular contractions. They represent 20% of the muscular proteins.

3. *connective tissue*: is mainly constituted by collagen and its function is structural support of muscular tissue.

Fresh fillet fish analysis

Before metal-protein analysis of contaminated samples, fresh fillet of Perch fish have been purchased at the local market. The sample was homogenized and the MALDI analysis of extracted proteins have been carried out. Few grams of fillet have been immersed in the extraction buffer and processed with a liquid homogenization procedure. To protect the extract from degradation, homogenization is performed at 4°C under a nitrogen flow.

For the intact proteins determination, both the whole extract and the fractions eluting from a SEC column have been analyzed. As the MALDI ionization process is sensitive to the presence of buffers and salts, the sample clean up is a fundamental step to perform before sample deposition onto a MALDI plate. To clean the extract microSPE was used,

equipped with C4 or C18 stationary phases. Samples are loaded onto the fibers and acidic water (0.1% TFA) was used for lowering the salt content. The purified extract has been then eluted with a CH_3CN/H_2O solution and deposited onto the MALDI plate using sinapinic acid. Spectra have been acquired in the linear positive mode (Figure 5.13).

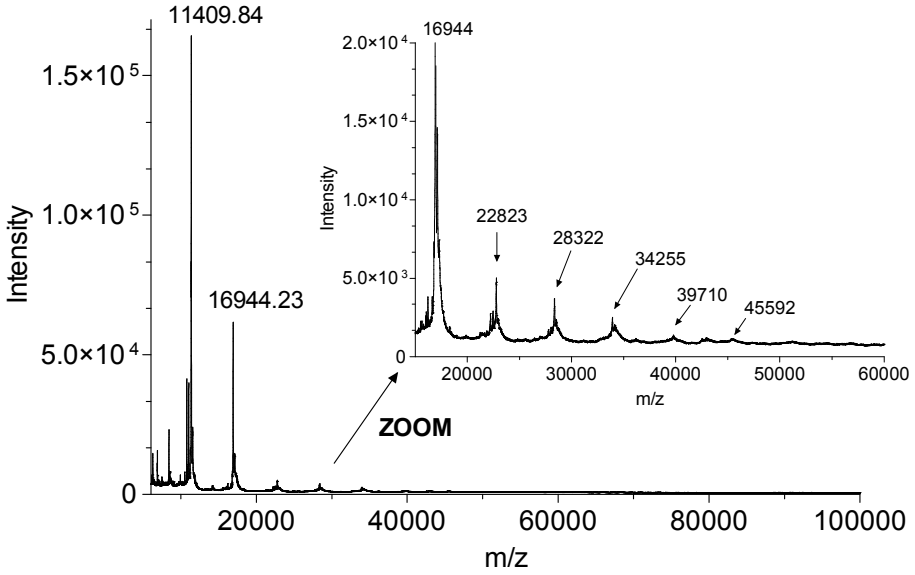


Figure 5.13: Fresh filet extract from a control samples bought at the local market.

This spectrum show that the aqueous extract obtained from muscle tissues with a low ionic strength buffer contains mainly peptides and proteins originating from the sarcoplasmatic reticule, as described for similar protocols (Czok et al. 1960). The major peak at 11.4 kDa correspond to an abundant protein into the muscle tissue: the *parvalbumin*. This protein is present in the sarcoplasmatic reticule and it is responsible for the fast muscular relaxation/contraction due to the presence of two EF domains for calcium complexation. Usually this protein can be found in high concentrations in muscle extract and, as it exist in several isoforms (up to 7) its molecular weight can vary in the range of 11-12.5 kDa Carrera et al. 2010. Parvalbumin (PV) has been detected with MALDI-TOF by some authors as the main peak of muscle extract (Mazzeo et al. 2008).

The second peak detected at m/z 16.9 kDa correspond to myoglobin. This is a monomeric iron-containing protein involved in oxygen transport and its presence is responsible for the red colour of muscular tissues. If in mammalia this protein is very abundant, in fish its concentration is generally lower but, in some very active fish such

as tuna, the levels found are consistent. Other minor peaks can be detected from 20 to 50kDa corresponding to the oligomeric forms of parvalbumin (from 2 to 4 subunits). The oligomerization of parvalbumins is a well know process in proteins of avian origin, and the cause seems to be the two cysteine residues that readily undergo oxidation (Hapak et al. 1994). Fish parvalbumins contain no cysteine (or just one) amino acid, depending on the species. The ability of fish parvalbumins to oligomerize has been demonstrated (Das Dores et al. 2002) in electrophoretic gel runs (SDS-PAGE) of muscle extracts.

If the same sample is analyzed after SEC elution, very interesting differences can be observed. All fractions eluting in the range of 2 and 3 mL, corresponding to an expected molecular size of 70-10kDa, have been collected and the relative MALDI spectra are reported (Figure 5.14).

The MALDI spectra of the first six fractions show quite unexpected results. No peak >of 30kDa has been observed in any fraction (spectra up to 70kDa are reported in the appendix section). By contrast, the peak at 11kDa, (marked with an asterisk) elutes practically in all fractions. This peak still corresponds to PV. The most interesting result is that the MALDI spectrum of whole extract (Figure 5.13) has shown the presence of PV oligomers, but in the SEC fraction there is no trace of them and just the monomer can be found.

What it is found here is that, probably, oligomers are able to dissociate and to restore the monomeric form, after column elution. This could be justified both by the dilution of analytes in the column fraction (e.g. 20µL of injected samples are distributed in 4/5mL of eluent during all the chromatographic run) or with the antioxidant effect of l-cys presents in the mobile phase.

These results suggest that is required a careful approach in the protein identification just on the basis of SEC elution, since the apparent molecular weight (MWa) obtained can be very different from the real one.

5.2.1 Analysis of metals exposed samples

Aliquots of metal-exposed samples have been extracted as previously reported, using cold buffer (+4°C) under N_2. To increase tissue disaggregation, samples have been additionally immersed into a cold sonic bath for 20mins after the liquid homogenization procedure. Before column injection, the extract has been centrifuged at 3000rpm to pull down the debris.

All extracts have been firstly injected on the SEC column for molecular fraction detection. All metals-containing fractions have been subsequently injected onto the sec-

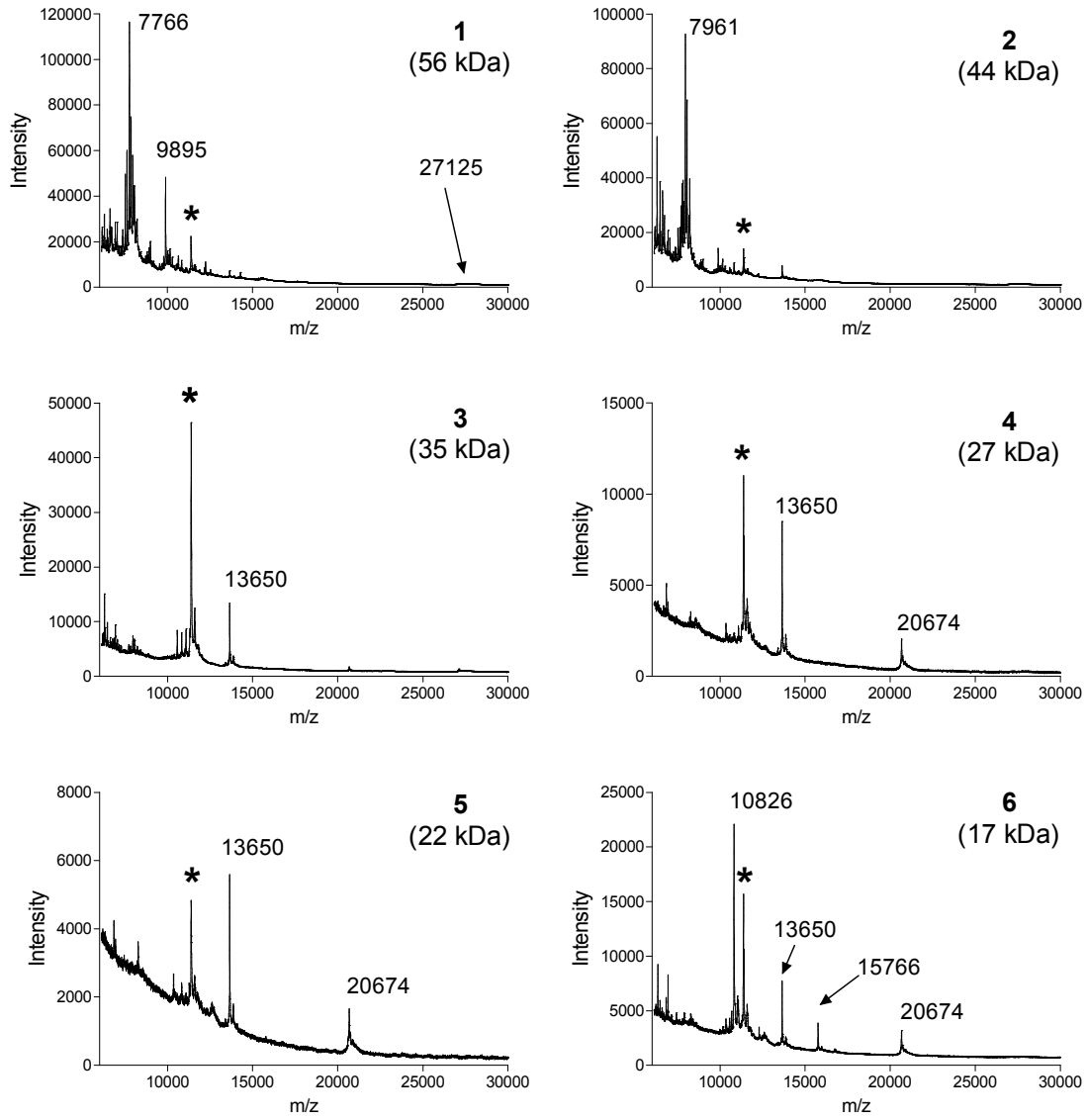


Figure 5.14: Fresh filet analyzed after SEC chromatography. The expected molecular size of eluting protein is reported in the round brackets. No ions have been detected in the m/z>30kDa.

ond column for metal species detection. Finally, MALDI TOF analysis of extracts and fractions have been performed for proteins identification.

5.2.2 HPLC (DAD) ICP-MS

DAD chromatogram of extracted fish samples show a well distributed mass range of eluting molecules, as reported in Figure 5.15. Several peaks have been detected with

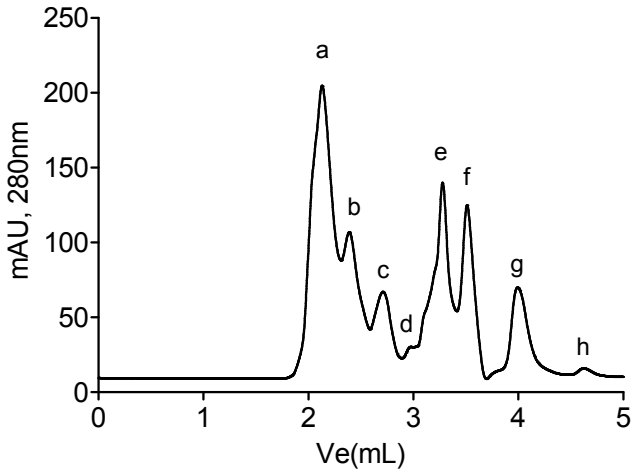


Figure 5.15: DAD detection at 280nm of extract from fish sample living in a polluted environment.

trhee different MW ranges: high range (50-5kDa, peaks from a to d), medium range (5-1kDa, peaks e and f) and low range (less than 1kDa, peaks g and h). The peaks detected with DAD have been compared with the co-eluting metals detected with ICP-MS and a summary is reported in Table 5.5. All peaks detected have a calculated size of less than 50kDa and, which is in agreement with that previously observed by MALDI-TOF analysis.

Arsenic elution from the SEC column is characterized by a complex profile. In all samples analyzed, with the exception of sample 4, al least one peak can be detected. In general, peak a observed in DAD signal (48kDa) has been detected with the As ion current. Other arsenic peaks have been detected at MWa around 12 and 7 kDa (corresponding to peaks c and d in the DAD chromatogram) in at least half of the samples. In this range we expect to find the presence of small proteins such as parvalbumin or enzymes. Finally two arsenic peaks are present at the higher elution volumes (around 4mL). In particular this fraction represents the only peak detectable in sample #6. As

Table 5.5: List of eluting peaks of fish sample extracts. The peak *h* has not been inserted since it is out of the linearity range of SEC.

			Sample #					
Label	Ve	MWa	1	2	3	4	5	6
a	2.11	48.7	As	As, Hg	As, Hg		As, Hg	
b	2.36	27.2					Hg	
c	2.68	12.9		As	As		As	Hg
d	2.92	7.3	As			Hg	Hg	
e	3.26	3.3		Hg				
f	3.49	1.9	Hg , As	As		Hg	As	
g	3.96	0.6		As, Hg	Hg		As, Hg	As

previously discussed in the CRM extracts analysis, these fractions indicate the presence of small complexes of metals (for example with some endogenous molecules) or the unbound (free metals) fraction. Even if these peaks don't give any direct information on the protein-arsenic interaction, their identification could be useful for the determination of species-specific profiles.

As an example, the complete chromatographic separation of two samples (1 and 2) are reported.

In sample 1 (Figure 5.16, upper panel), two very small peaks of arsenic have been detected in the SEC chromatogram. The first peak is in the area of the major MWa (around 48kDa), while the second one seems to have an MWa of about 7kDa. The last peak is more intense and has an apparent MW of less than 2kDa. Under peak *a* the As(V) has been found as major component (Figure 5.16, right panels), even if a small peak of DMA has also been detected. Peak *d* still shows the presence of As(V), while peak *f* is constituted of AsB with a very small peak at 8min, revealing the presence of an unknown arsenic species (Unk 1).

Despite the low signal intensity in the SEC elution (upper panel), in the SAX chromatography the As signals are significantly higher. This is due both to the difference in the mobile phase used and in the difference of diameter of columns used. In the SAX column, the lower i.d. (2.1mm, versus 4.6mm of SEC column) result in smaller dispersion of the analytes and, as a consequence, their concentration increases in the chromatographic band giving a more intense signal. The peak at 7kDa results of particular interest, since it could be due to the presence of a characteristic class of proteins known for its capability in metal binding, such as the metallothioneins (MT). Their expression can be induced by heavy metals exposure, even if there is not a clear relationship (Mieiro et al. 2011).

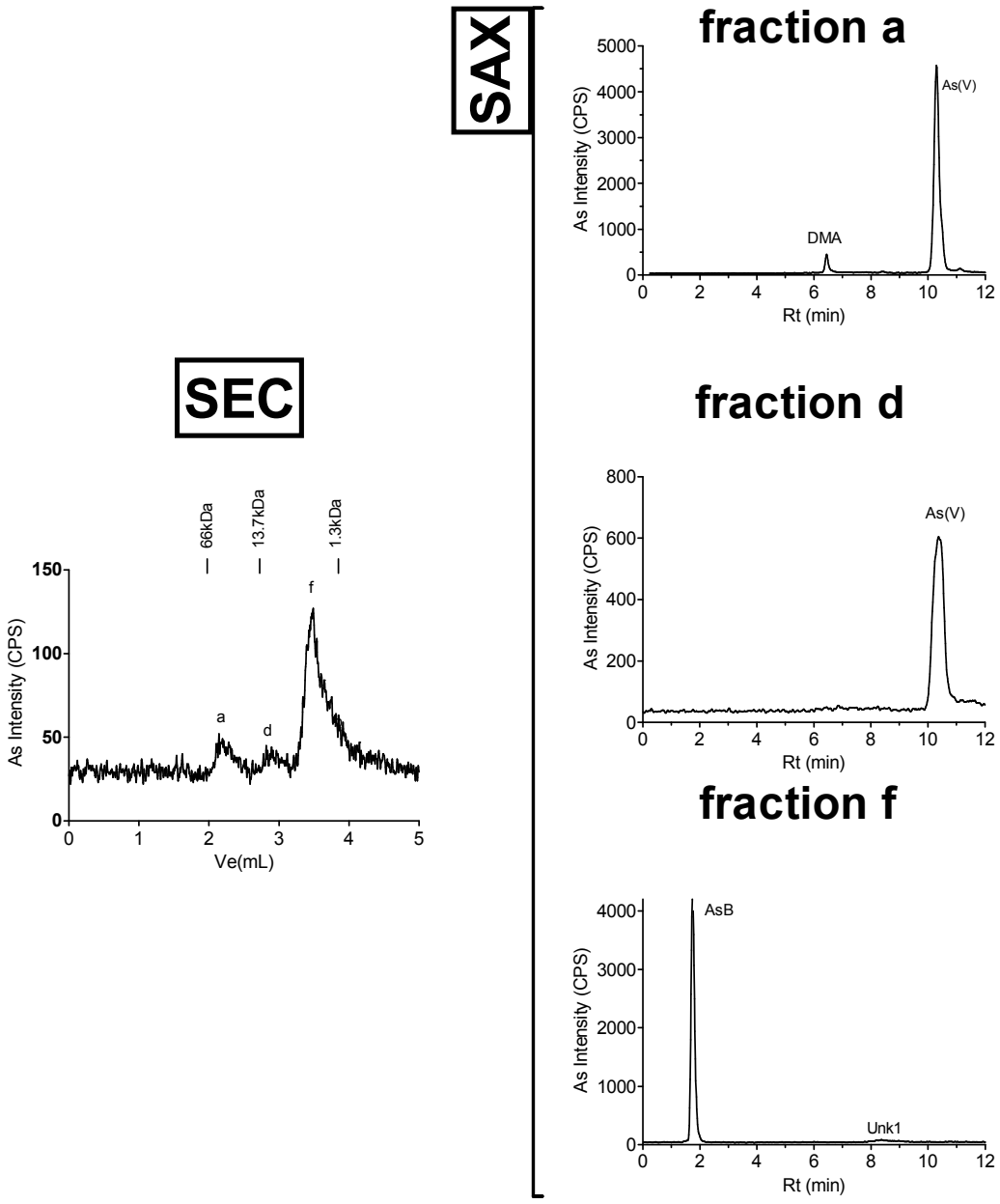


Figure 5.16: Arsenic co-eluting with endogenous molecules (left panel, SEC column) and speciation of arsenic compounds collected from peaks a, d and f (right panels, SAX column).

The attribution of this peak to the presence of MT could only be done effectively after MALDI analysis.

The other chromatograms reported in Figure 5.17 are relative to sample 2. In this sample a different elution profile is present. Several peaks have been detected and, among them, *b* peak was the most intense, while peak *f* detected in the first sample is practically absent. The absence of this peak is renforced by the lack of AsB species (Figure 5.17, fraction *f*). For this sample, As(V) is the main species detected with the SAX column but some other very small peaks relative to AsB (fraction *g*) and some unknown species have also been detected.

At the same manner, all the other samples analyzeddid not have a typical profile, but each sample was characterized by a very different SEC elution profile and arsenic species detection. The presence of As(V) and the Unk1 species seems to be the only similarity in some samples (Table 5.6). As previously described, the Unk peaks could be attributed to thiorsenical compounds, but in the absence of standards we cannot correcty identify them.

Table 5.6: Arsenic species detected in the contaminated freshwater fish samples analyzed.

	Sample #					
MWa	1	2	3	4	5	6
48.7	As(V), DMA	As(V),Unk1, 2 and 3	As(V),Unk1		Unk1	
12.9		As(V),Unk1, Unk2	As(V),Unk1		Unk1	
7.3	As(V)					
1.9	As(V), Unk1	As(V),Unk1			Unk1	
0.6		As(V),Unk1,AsB			As(V)	AsB

Another characteristic was the lack of AsB in almost all samples analyzed. Although AsB is usually considered the main species of arsenic in marine fish, a quite different metabolic behavior has been observed in the freshwater fish. For example, Shiomi et al. (1995) and Slejkovec et al. (2004) both found AsB as the major species in some freshwater fish, while others found only trace amounts of this compound (Zheng et al. 2004). Other studies revealed the major fraction of arsenic found in some fish from the Hayakawa River (Japan) was constituted mainly of inorganic arsenic (Miyashita et al. 2009) and similar results have been reported by other authors (Zheng et al. 2004) during the analysis of samples of *Perca flavescens* (Yellow Perch). These differences can be due, both to a greater spatial/seasonal variability in the arsenic mobilization in freshwater or to specific metabolic pathways in animals. In particular, the enzyme involved in arsenic methylation (AS3MT, arsenic methyl transferase) has been found to be present in different animals

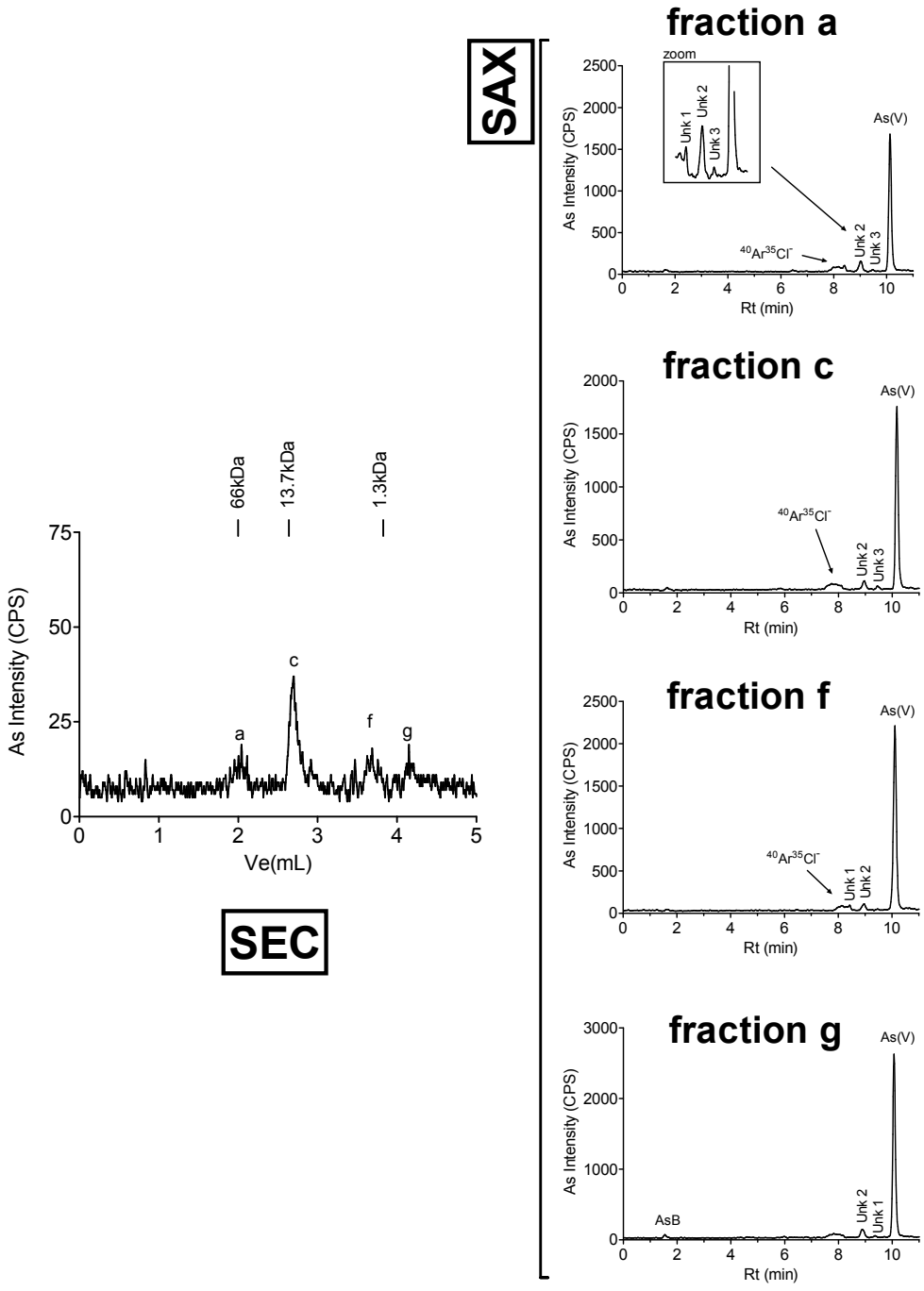


Figure 5.17: Arsenic co-eluting with endogenous molecules (left panel, SEC column) and speciation of arsenic compound collected from peaks *a*, *c*, *f* and *g* (right panels, SAX column).

with a high variability (e.g. marmoset monkeys, chimpanzees and guinea pigs are known to lack this enzyme (Zakharyan et al. 1996; Drobna et al. 2010),while in humans its levels are correlated with the genetical polymorfisms (Hernandez et al. 2008; Agusa et al. 2012). Anyway, in our samples As(V) has been found in several fractions corresponding to the elution of proteins. The presence of As(V) interacting in solution with high a molecular weight fraction is uncommon, since in the literature only trivalent As (and its species) is considered able to interact with proteins. Recently, the interaction of As(V) metabolites (thioarsenicals) with biomolecules has been reported (Raab et al. 2007b) in extracted samples, opening the door to deeper studies in this field. Moreover, As(V) compounds are able to strongly interact with proteins in crystallized form (Retailleau et al. 2003) and in some cases the activation of cysteine residues by the near presence of acidic AA, seem to be involved (Liu et al. 2011). Due to the very low number of samples analyzed, no data of biological relevance can be obtained. Moreover, the presence of arsenite in the proteins extracted should be investigated deeper to exclude the presence of artefacts in the analysis. Maybe the oxidation of As(III) during sample manipulation can be hypothetisized, but the extraction under nitrogen flow and in presence of reductive compounds (l-Cys) in the extraction buffer and in the mobile phase should avoid this.

Mercury elution from the samples injected onto the SEC column has been investigated. Also in this case, a complex profile has been found (Table 5.5), with small similarities among the samples. With difference regarding As elution, all samples have shown the presence of some peaks indicating that the metal is interacting with molecular components. This can be explained by the very strong bound between Hg and -SH groups in biological samples. As these groups can be found not only in proteins, but in other molecules like glutathione, lipoic acid and free amino-acids etc, the dimensional range of Hg targets is very wide (Zalups 2000). This ability is shown in the chromatographic separation of a sample analyzed (Figure 5.18) with a SEC column. Interactions have been found indicating its ability to bind high MW fractions (peak a) and medium MW fractions (peak e),in addition to the free metal (g). Interesting, all these peaks have a quite strange shape with evident tailing of the metal. This could suggest an instability of the complex, with mercury detachment during chromatography and consequent dispersion into the SEC pores. To investigate this aspect, the DAD chromatogram at 254 nm is used to verify the elution profile of -SH groups. In the DAD chromatogram, no tailing has been observed under the peaks e and g, suggesting the presence of mercury

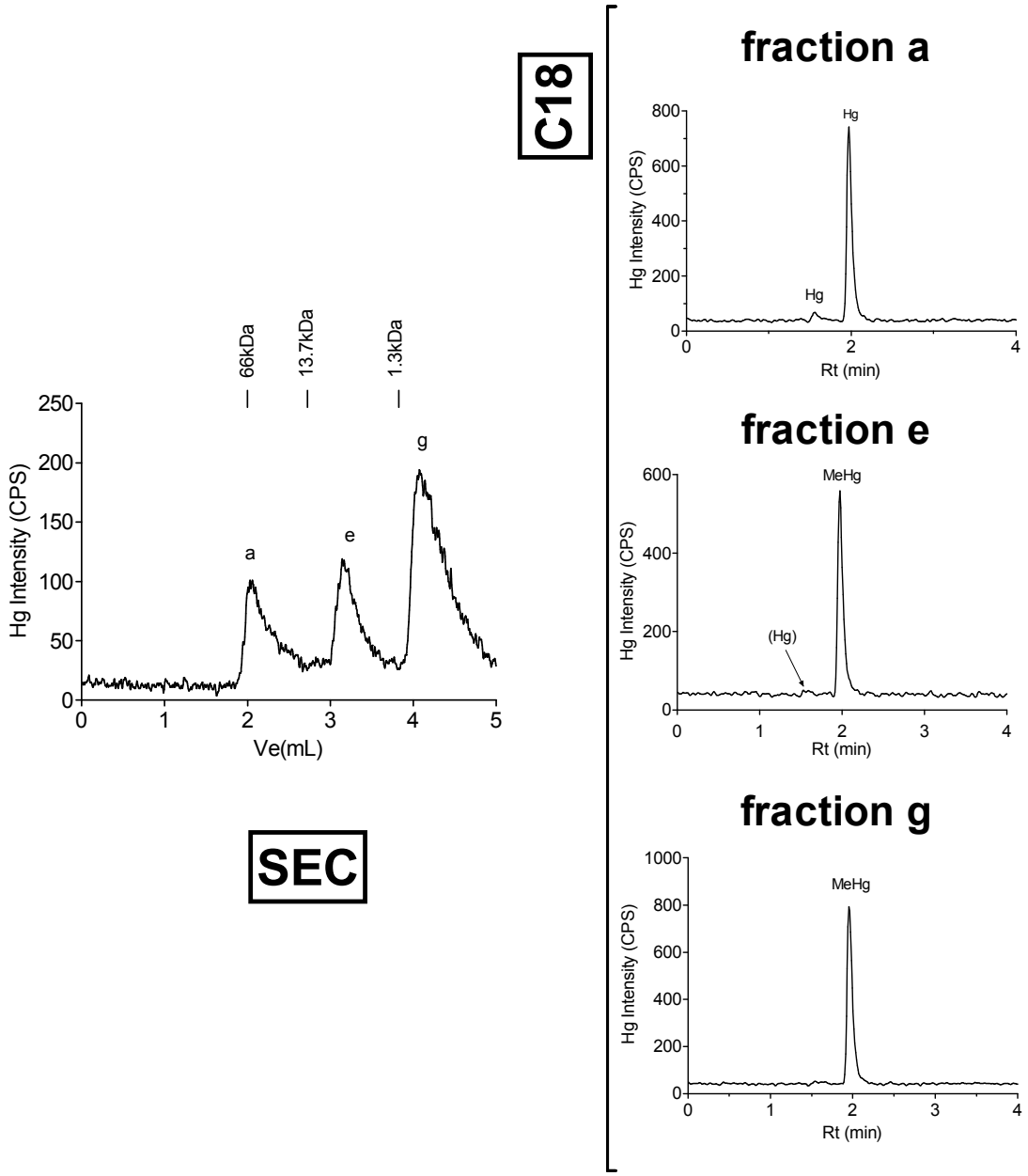


Figure 5.18: Mercury co-eluting with endogenous molecules (left panel, SEC column) and speciation of mercury compounds collected from peaks *a*, *e* and *g* (right panels, C18 column).

release. For peak a no consideration can be made, due to the overlapping of other peaks in the 254nm signal.

Hg speciation of fractions a, e and g has been performed using a C18 column and, for each fraction, results are reported in the right panels of Table 5.5. $MeHg^{+}$, as expected, was the main species detected but a little peak of Hg^{2+} has also been found. A clear Hg^{2+} presence has been observed only in the high molecular weight fraction, indicating a strong bond with the proteins present.

Similar results have been obtained for all samples. Differently to arsenic, in which several species have been detected under the SEC fractions, in the mercury analysis the only species found is MeHg. Generally, the peak of Hg^{2+}, if present is co-eluting only with the major MW fraction (peak a). The main differences have been observed in these samples in the SEC elution profiles (Figure 5.19), since in some cases peaks with the same Ve resulted symmetric (e.g. sample 3) or asymmetric (e.g. sample 1) without any correlation. We can hypothetisize that these differences are due not only to different

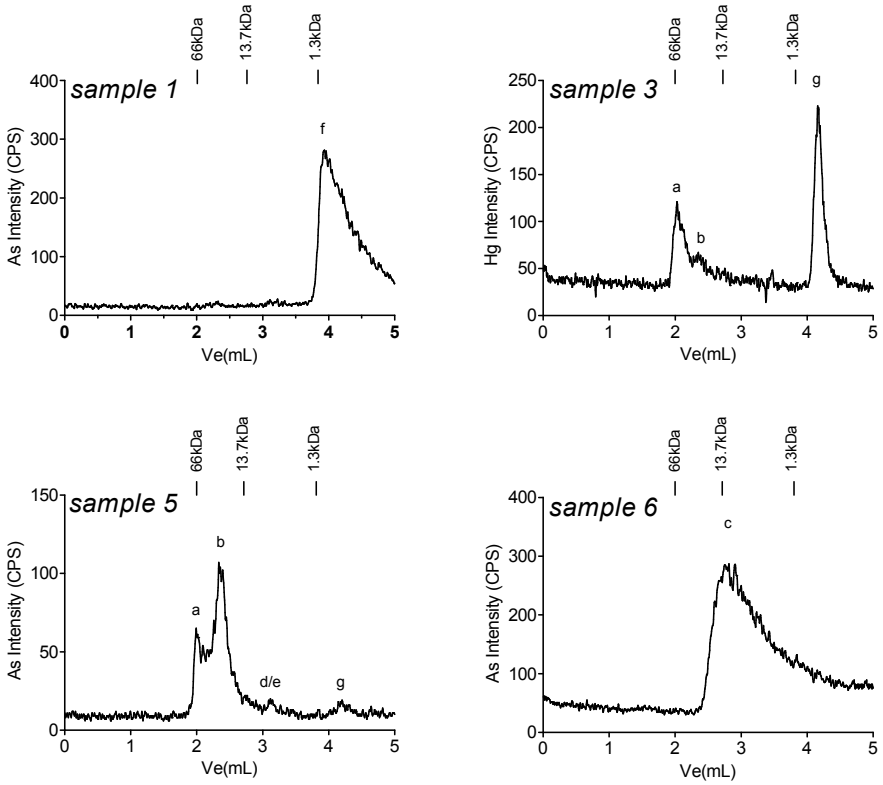


Figure 5.19: Mercury co-eluting with endogenous molecules in the samples analyzed with SEC column.

proteic targets, but also to a different degree of stability of the mercury-complex analyzed (as suggested by the peaks tailoring).

5.2.3 MALDI TOF analysis.

MALDI-TOF and MALDI-TOF/TOF analyses have been carried out for the identification of the molecular moiety eluting with the metals. The extracts have been purified with C18 microSPE and then deposited using sinapinic acid or HCCA as the matrix. Spectra for intact protein analysis have been analyzed in linear positive mode, using two different mass ranges: from 5kDa to 30kDa (Figure 5.20) and from 20kDa to 70kDa Figure 5.21.

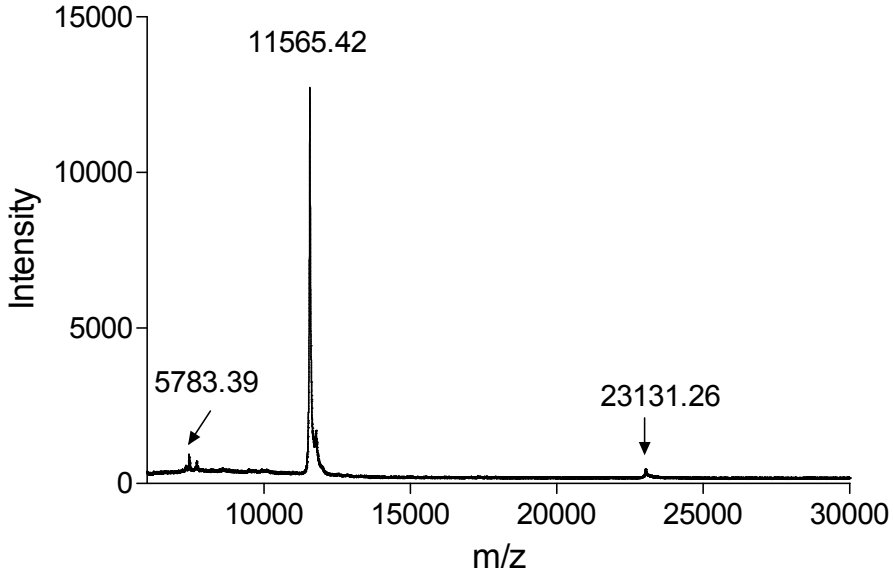


Figure 5.20: Whole extract MALDI-TOF analysis of fish in the mass range 5-30kDa, after purification on zip-tip C18.

In the medium mass range, a big peak at 11565.42 Da is present. This peak corresponds to the parvalbumin (PV) and, its dimer at 23131.26 Da and the doubly charged ion at 5783.39Da has also been detected. The investigation of higher mass ranges shows in this case, the presence of small oligomers of PV, of up to five units (at 57kDa). A third MALDI acquisition has been performed at a very high MW (up to 200kDa) but no protein was detectable in this ranges. This confirm the DAD analysis, in which the first eluting peak has a MWa of about 50kDa. The lack of high MW proteins is due to the

capability of the extraction buffer to extract only the soluble fraction of muscle, not the unsoluble one (collagen).

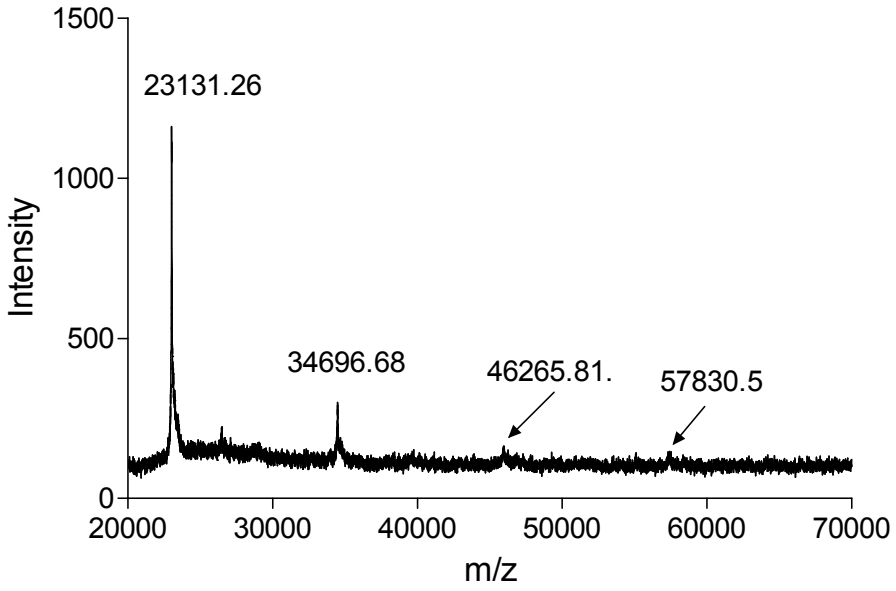


Figure 5.21: Whole extract of fish filet in the mass range 20-70kDa, after purification on zip-tip C4.

Additional considerations can be made comparing the typical DAD profile (Figure 5.15 on page 90) and MALDI-TOF results. In the DAD chromatogram the most intense peak detected (peak *a*) has no correspondence in the MALDI-TOF spectrum (or at least it can be ascribed to the very small ion m/z at 46kDa). The difference in the signal intensity observed, is correlatable to the technique used and not to the absolute protein concentration. In DAD analysis, the signal intensity at 280nm is due to the absorbance of aromatic groups present in the protein. This means that, as the MW of protein increases, it will probably increase the number of aromatic AA per protein molecule. As a consequence, two proteins present at the same concentration will have a proportional signal intensity due to their MW. This is not occurring in MALDI, since in this case the signal intensity is not solely due to the protein concentration but, more properly, it is due to the matrix/analyte ratio.

As evidence for this, the same MALDI spectrum of a whole extract is reported in Figure 5.20 which has been acquired after 10-fold sample dilution (Figure 5.22).

The spectrum reported is very similar to that in Figure 5.20. In contrast to the

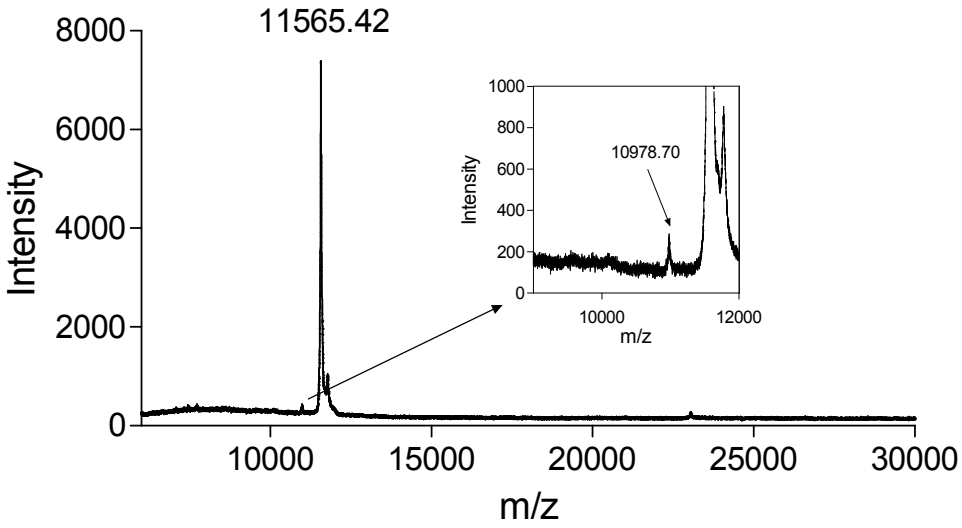


Figure 5.22: Whole extract of fish filet after ten fold dilution with deionized water.

dilution factor, the observed signal intensity at m/z 11kDa is not 10 fold lower, but just half, confirming what was previously described and, moreover, putting in evidence the main limit of MALDI analysis for quantitative purposes.

The diluted spectra show an interesting peak, not detected in the concentrated sample. This peak has m/z of 10.9kDa with a shift of about 580Da with respect to parvalbumin. This difference cannot be explained by calcium loss, since only two ions are usually present in this protein (Hu et al. 1995). More properly this peak should be attributed to undetectable proteins with the former matrix/analyte ratio used.

SEC fractions have been collected and analyzed also in an attempt to verify the mass of co-eluting proteins with metals detected by ICP. Fractions have been collected every 30 sec, corresponding to 0.1mL of elution volume. All the sample have been cleaned-up (microSPE) and deposited using sinapinic acid for the expected proteins with MW>5kDa and using HCCA matrix for those with expected MW<5kDa.

The most significant spectra, corresponding to *a*, *b*, *f* and *g* peaks in the DAD chromatogram have been reported in Figure 5.23 and the identified proteins are reported in Table 5.7.

The first spectrum, relative to peak *a*, indicates that the molecules with lower MW than expected are present. In particular parvalbumin (11.5kDa) and a probable peak

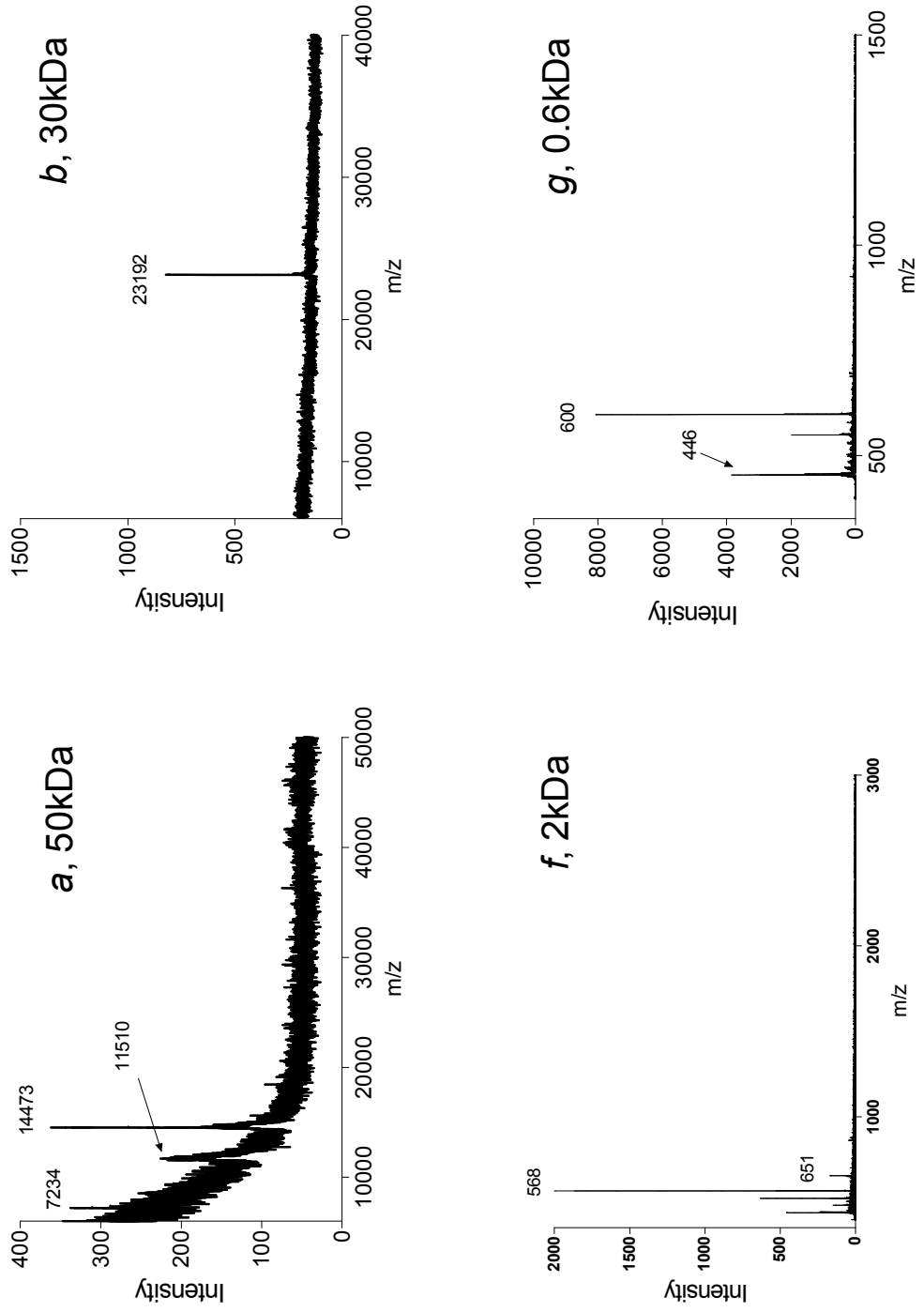


Figure 5.23: Fractions recovered from SEC column of extracted fish. The expected MW in each fraction is reported.

corresponding to myoglobin (14.5kDa) have been observed. The presence of PV in this fraction can easily be explained considering what was found for the fresh sample previously analyzed. This means that peak *a* observed in DAD corresponds to the presence of protein aggregates instead of a real protein having a MW of 48kDa. Both PV and myoglobin are metal binding proteins (they bind Ca^{2+} $Fe^{2+/3+}$ respectively) but at this time, no report of their interaction with As or Hg has been reported in the literature.

Table 5.7: Summary of proteins detected in the SEC fractions of the extracted samples. *Oligomers observed in the whole extract.

Label	DAD (kDa)	MALDI (kDa)	Protein ID
a	48.7	11.5, 16	parvalbumin*, myoglobin*
b	27.2	23	cytochrome c
c	12.9	11.5	parvalbumin
d	7.3	5.8	metallothionein
e	3.3	nd	-
f	1.9	0.5	-
g	0.6	0.6	-

The spectrum of fraction *b* shows that in this case that no presence of PV is detected, but only the presence of a protein with MW at 23kDa is seen. Not having detected any other proteins, this one seem to be the most probable target for the mercury interaction detected in sample #5 (Figure 5.15 on page 90). At this MW, Cytochrome c can be indicated as the most probable protein.

Minor fractions (*f* and *g*) analyzed with the HCCA matrix, show the presence of only very small molecules. In particular, in fraction *f* we expected to find larger molecules (1.9kDa, from SEC evaluation) but, in this case, the real size of the elution molecules is quite different.

5.3 Database searching and proteins identification

Tryptic digestion of the above mentioned samples has been performed. Some aliquots of fish extracts (100µL) have been diluted with 50mM ammonium bicarbonate buffer (pH=8) before trypsin addition (10µL). The mixtures have been left to react over night at 37°C. In order to obtain the best conditions, the tryptic digestions have been performed using three different sample dilutions: 1 to 2, 1 to 10 and 1 to 100.

Before deposition onto the MALDI plate, the digested proteins have been cleaned using C18 microSPE and the purified peptides have been deposited with HCCA matrix.

The tryptic digest spectra are reported in Figure 5.24. In all spectra the most intense fragment obtained has m/z at 1657.9. In the 10-fold and 100-fold diluted samples, the low protein content gave rise to the autilytic trypsin digestion with formation of ions at m/z 2211.1 [peptide: 50-69] and 2284 [peptide: 70-89] marked in the spectra with the asterisks.

Proteins identification has been performed using two different approaches: the peptide mass fingerprint (PMF) and the MS/MS spectra database search.

PMF

For the PMF identification, the peak list of digested peptides has been extracted and the contaminant peaks (such as trypsin auto-digest products or human keratin fragments) have been manually removed. The m/z obtained have been submitted to the MASCOT on line search **http://www.matrixscience.com** Oxidation of methionine and tryptophan amino acids has been added into the search parameters and the taxonomy selected was the Actinoperygii class. For proteins not directly attributed to the Perca fluviatilis but attributed to similar organism with high score, BLAST (Basic Local Alignment Search Tools) **http://blast.ncbi.nlm.nih.gov** has been used. This procedure is possible since in nature proteins with the same functions can contain one or more identical domains (called conserved regions). The comparison of the obtained sequences with those conserved in the BLAST database, increase the possibility of proteins identification.

The results obtained are listed in Table 5.8, in which the theoretical MW of the detected protein has also been reported.

Table 5.8: PMF analysis of tryptic digest from Perca fluviatilis samples. The database searching has been performed using MASCOT.

protein name	MW (kDa)	seq. coverage	peptides
α-globin	15.6	20%	2
ATP synthase f	8.3	50%	2
Ca binding protein 39	39.7	22%	6
Cytochrome c	23	23%	3
glyc-3-phospate dehydrogenase	28	19%	3
myoglobin	15.5	38%	4
myosin l	19.8	60%	3
NADH dehydrogenase	12.7	22%	4
parvalbumin	11.4	25%	1
40s ribosomal	21.9	23%	4
10kDa heat shook protein	10.9	44%	4

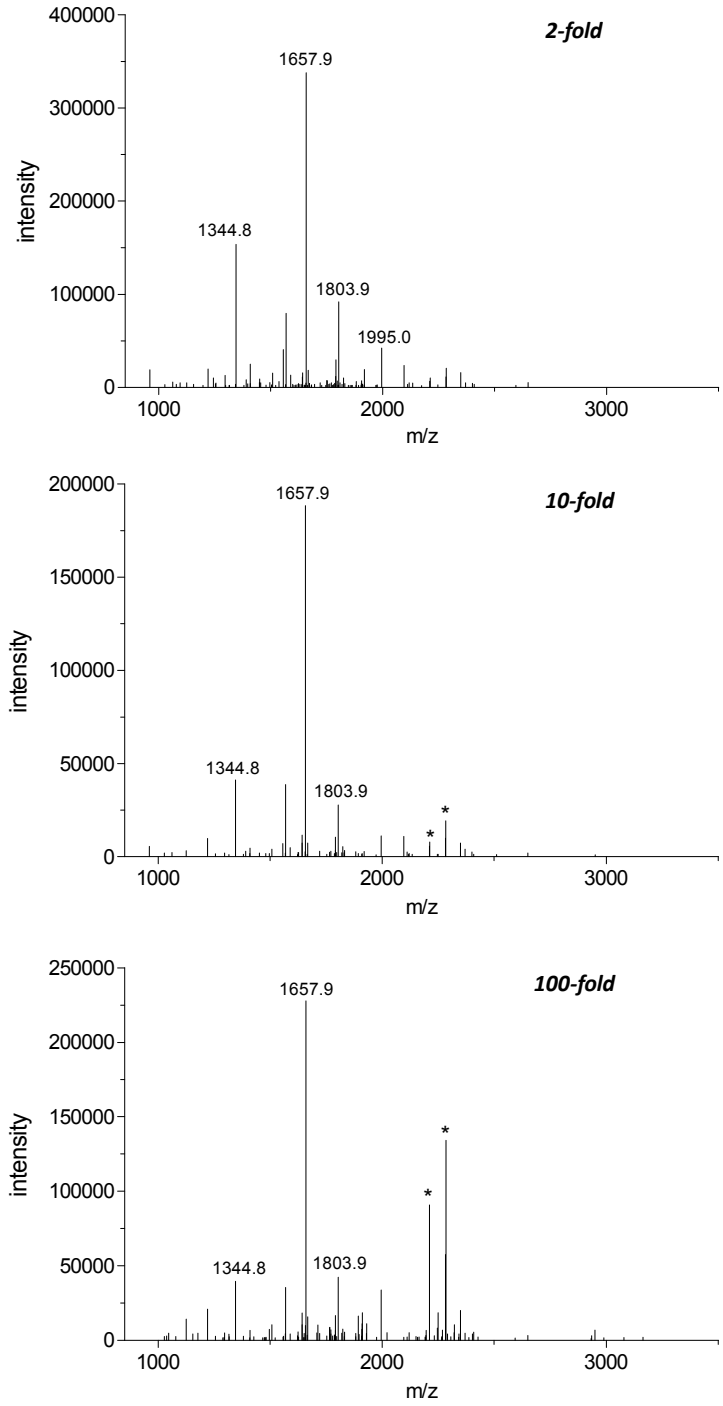


Figure 5.24: Tryptic digest of fish sample extracts using three different sample dilutions. The peaks indicated with the asterisk are originating from the autolysis of trypsin.

Among the identified proteins we found the parvalbumin (responsible for the major peak in the MALDI spectrum) and myoglobin detected in fraction *a*. Other proteins responsible for metabolic processes (such as cytochrome c, ATP synthase or NADH dehydrogenase) have also been identified. The number of peptides necessary to identify a protein with a good score is variable and depends on the mass accuracy data. In some protein (such as parvalbumin or α-globin) the number of identified peptides is low. This is due to the small proteins size that give rise to a very low number of peptides during the tryptic digestion. For this reason, peptide sequencing can be useful to confirm, or improve the obtained results.

MS/MS spectra

The most intense peaks in the digest spectra, have been subjected to sequencing using TOF-TOF fragmentation. The spectra of the most intense fragments (m/z 1647.9, m/z 1344.8 and m/z 1803.9) are reported in Figure 5.25. The precursor ion is still evident in all cases and the major fragmentation pathway observed gave rise to a number of small fragments. This behaviour is very common in TOF-TOF fragmentations.

When peptide fragments are submitted to Mascot for protein identification, an ions score matching is reported. This result is based on the calculated probability (P) that the matching between the acquired data and databases sequence is not a random event. The final reported score is calculated as -10Log(P). When more than one peptide is attributed to the same protein, the probabilistic score decrease, giving more valuable results.

Sometimes, if the quality of the acquired MS/MS spectrum is poor, the match could have a relatively high score but not exceeding the significative threshold. Moreover it appears to be well separated from the distribution of random scores. This relatively high threshold is reported as "homology threshold", while the higher threshold is reported as "identity threshold". A visible result is also reported, in which the protein found falls in the significative score region. This is what has occurred, for example in the searching of ions obtained in our tryptic digest (Figure 5.26).

In our analysis, even if the instrumental accuracy give reliables results, the proteins database available for Perch fish is very small, limiting the possibility of positive matchings. Anyway, the MS/MS search has led us to identify the main proteins observed in the MALDI spectrum, such as myoglobin eluting in fraction *a*. In addition new proteins not found with PMF procedure have been attributed, such as the MHC of class II, representing the major allergenic protein in the fish and metallothionein (MT). If the MHC protein has been not detected in the MALDI spectra of whole extracts, the MT

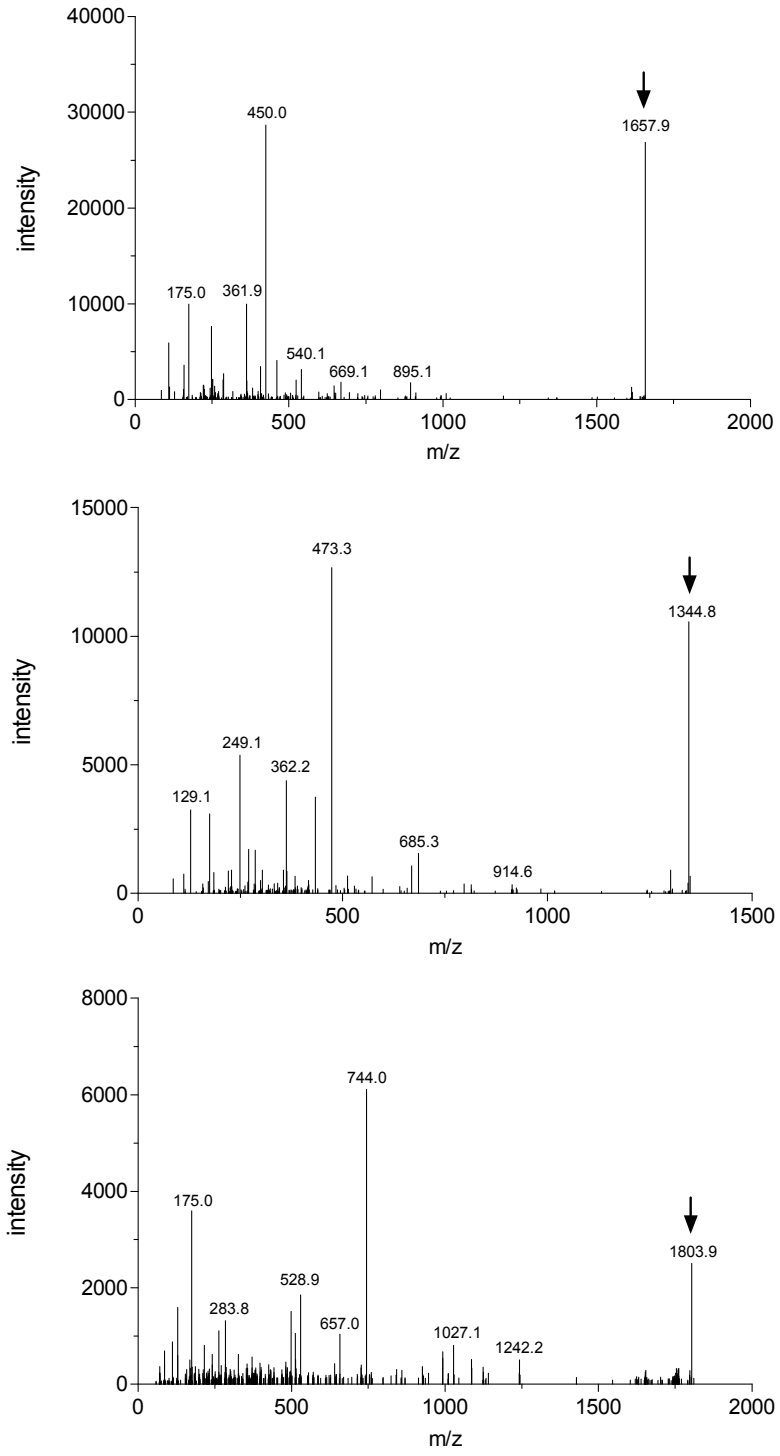


Figure 5.25: TOF-TOF spectra of peptides at m/z=1647, 1344.8 and 1803.9 Da.

protein has been identified as the small protein at 5.8kDa present in Figure 5.20. The most intense peak at m/z 1657.9 has been attributed to the creatine kinase enzyme (creatine phosphokinase (CPK)). This enzyme catalyses the conversion of creatine to create phosphocreatine (PCr) with adenosine triphosphate (ATP) consumption. This enzyme is present in tissues and cells that consume ATP rapidly, and is found especially in the muscle tissues.

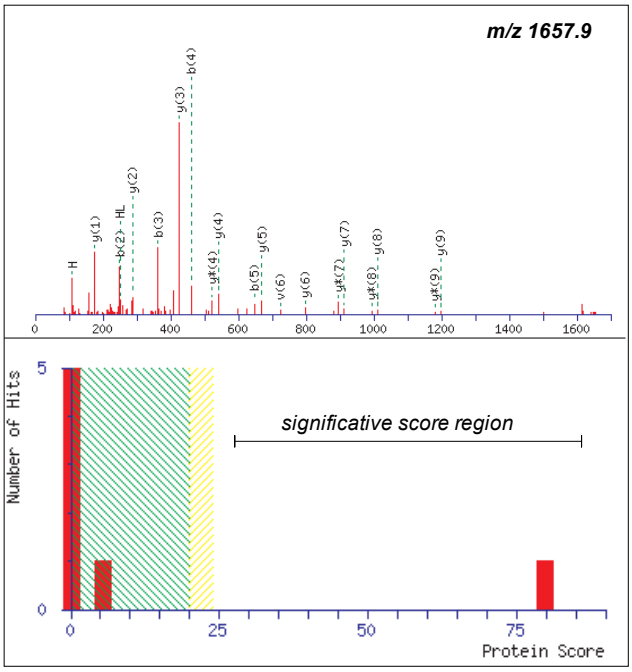


Figure 5.26: MASCOT fragments attribition and visive report of the significative score obtained for m/z 1657.9. The significative peptide has been identified as Creatine kinase enzyme.

5.4 Conclusions

In the HPLC (DAD) ICP-MS analysis several hypothetical interactions between metals and endogenous molecules in the fish samples have been found. In fact, for practically all samples, more than one metal peak has been detected with ICP-MS during elution. As just the soluble protein fraction can be recovered with the extraction performed, the metal-proteins complex that we expect to found, are only those relative to this fraction. An in depth investigation using organic mass spectrometry techniques, shows that the real interactions are less than expected.

The proteins found in the MALDI-TOF analysis have been reported by comparing the observed MWa (DAD) with the real one (MALDI). In the higher fractions, some proteins have been detected, in the lower mass fractions no peptide has been found, in contrast with DAD detection. For these fractions only very small molecules have been found (less than 0.6kDa) showing a behaviour out of the linear range of the SEC column. In addition to the proteins detected in the fractions, other minor proteins have been identified with MALDI TOF-TOF analysis of the tryptic digest. Comparing the ICP and MALDI data (Table 5.9), some important considerations can be made.

Table 5.9: Summary of proteins and metals detected in the extracted samples. The label is referring to the peaks detected in the DAD chromatograms.

Label	As	Hg	Protein ID
a	As(V), DMA, Unks	Hg, MeHg	parvalbumin, myoglobin
b		MeHg	cytochrome c
c	As(V), Unks	MeHg	parvalbumin
d	As(V)	MeHg	metallothionein
e		MeHg	-
f	As(V), Unks	MeHg	-
g	As(V),AsB, Unks	MeHg	-

As and Hg species eluting under peak *a*, can be found interacting with parvalbumin and/or myoglobin. Similar interactions have been found even in fraction *c*, in which just PV is present, suggesting this last is the most probable target. The second peak at 23kDa contains only mercury and the only protein found in this fraction was the cytochrome c. Finally, metallothioneins at MW 5.8 kDa are responsible for the As and Hg peaks detected by ICP analysis, as they are well recognized heavy metal binding proteins. The other proteins detected by MALDI could represent a probable target for As and Hg but, without information about their Ve, no specific considerations can be made.

In conclusion, with this work the best approach to study interaction between the metals and proteins in a biological organism has been evaluated. The whole analytical procedure has been developed in order to obtain the best operating conditions for the analytes identification. The obtained results suggest that the method above developed leads us to obtain valuable results in the metal-protein interaction analysis. In particular, the use of MALDI has led us to verify the real nature of the protein and moreover, has allowed us to recognize sample alteration (such as aggregation of parvalbumin) that could affect

the results evaluation. Moreover, the use of organic mass spectrometry techiques led us to hypothtisize some potential targets for arsenic and mercury, on the basis of their co-elution. In order to directly probe these interactions, several biochemical (e.g. the Immobilized Metal Affinity Chromatography), biophysical (e.g. the X-ray diffraction of protein crystals) or theoretical (e.g. the bioinformatics tools) methods can be adopted. Nevertheless for the identification of the interaction in such a complex matrices like the biological tissues, mass spectrometry still represents the most powerful technique. For this reason, additional efforts will be devoted in the future to directly probe the metal-proteins interaction in these samples.

The differences existing in the metal metabolisms and in the interactions with endogenous molecules in some fish samples has been positively recognized. In addition, the different behaviors of the same metals in different specimens of the same organism has been detected (e.g. due to biological variability). These data are promising, but to give them more weight, a more consistent number of specimens should be analyzed. Moreover, the As (V) detected in all samples suggest the need for deeper investigations, to verify if it is present in the original samples or if it came from sample manipulation. At this stage two possible causes can be hypothetisized: it can derive from As(III) detached from thiolic groups in the presence of free l-cys in the mobile phase, that can reduce proteins, or it derive from microbical degradation during the -20°C storage of samples before analysis. These hypoteses are now under investigation before judgement on whether the method is ready for its application to other biological samples.

Chapter 6

6.1 Elemental labeling

The use of ICP-MS for the detection/quantification of biomolecules based on the detection of heteroatoms (either naturally presente or chemically added as a label) is a developing field (Szpunar 2005). In fact, LC ICP-MS applications have been recently developed to quantify proteins by chemical labeling using rare earth elements such as lanthanides (Esteban−Fernandez et al. 2011; Careri et al. 2007). Most of labeling agents used come from the radio-pharmacology field (radioactive labeling) and were initially employed to derivatize antibodies for immunohistochemical assays (Feng−Bo et al. 2003). In the proteins sequence several natural element tags and reactive sites are present (Figure 6.1.

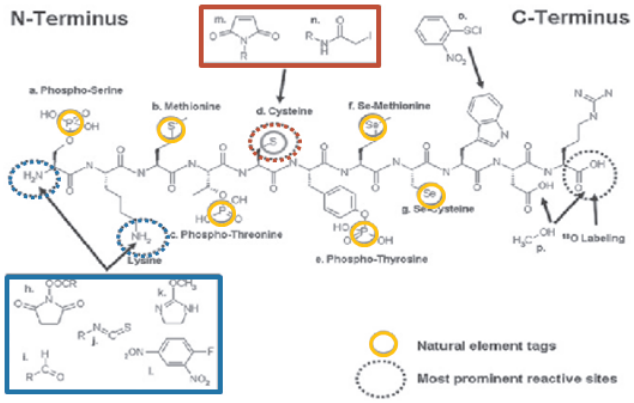


Figure 6.1: Natural element tags and reactive sites present in the proteins sequence, reported from Prange et al. 2008.

Cysteine and lysineamino acids have been used as targets (Wang et al. 2010). Re-

garding the cysteine labeling, the *element-coded affinity tag* (ECAT), based on use of the macrocycle 1,4,7,10-tetraazacyclododecane-N,N',N",N"'-tetraacetic acid (DOTA) has been used (Ahrends et al. 2007; Whetstone et al. 2004). The primary amine (lysine) labeling has been obtained using the *metal element-chelated tag* (MECT), based on the bicyclic anhydride of diethylenetriamine-N,N,N',N",N"'-pentaacetic acid (DTPA) has been developed (Patel et al. 2008). The advantages of DTPA reactive are its low cost and the possibility to use more than one lanthanide for the protein labeling (Liu et al. 2006).

The protocol for the protein-labeling procedure with DTPA reactive is reported in Figure 6.2. Because of the labeling procedure leads to directly detect (and quantify)

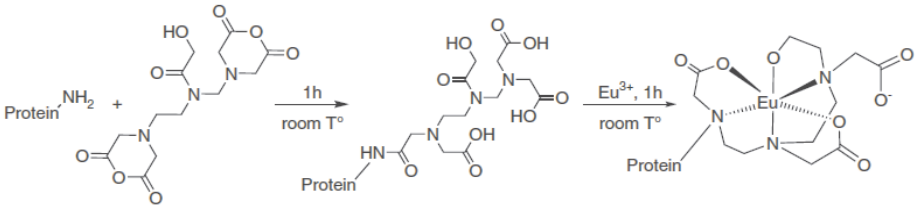


Figure 6.2: Outline of the protein derivatization procedure showing: the attack of DTPA to the lysine residue and the europium chelation.

virtually all proteins by ICP-MS, we decided to investigate on its use for the analysis of proteins used as binders in art painting.

Painting binders are used in artworks to bind and apply pigments onto the pictorial support. In the past centuries artists prepared their binders starting from simple materials such as eggs, milk and some animal tissues (skin, bone, tendons). Proteinaceous materials were used as reported below: egg wes just diluted and applied as egg yolk, egg white or whole egg. The animal-based glues were obtained by boiling the tissues for long periods, after alkalis or acids addition, while milk was processed to obtain caseins.

The analytical procedure was firstly developed working with some standard proteins. The effectiveness of the derivatization process and the chromatographic separation of labeled proteins were optimized. The method was then applied for the analysis of binders and pictorial models before analyzing some unknown samples.

6.1.1 Labeling of standard proteins and binders

The DTPA attack and the europium chelation have been monitored separately. The DTPA attack causes a mass shift of 375 Da (and multiples) leading the formation of a DTPA-protein complex. Due to the presence of this mass shift, MALDI-TOF was

used to monitor the reaction. BSA, a 66kDa protein that contains 59 lysine residues, has been used as model protein. This protein was reduced (DTT, 1 h at 37°C) and alkylated (IAA, 1 h at 37°C) to obtain a good exposure to the reactive sites. In order to obtain the sample derivatization, DTPA was dissolved in anhydrous DMSO and added to the protein solution in a final stoichiometry of 5 : 1 (DTPA to lysine). To assure the lysine reactivity, the working pH was kept at 8. Before MALDI-TOF analysis BSA was purified through a microSPE-C18 and then mixed with sinapinic acid (0.1%TFA) for the deposition onto the plate. DTPA caused a MW shift of among 4000Da (Figure 6.3), corresponding to the addition of at least 10 molecules. Because of the labeled protein has a larger shape than the unlabeled one, it was supposed that the DTPA attack was not homogeneous, and more lysine residues seemed to be involved (Ogawa et al. 2007).

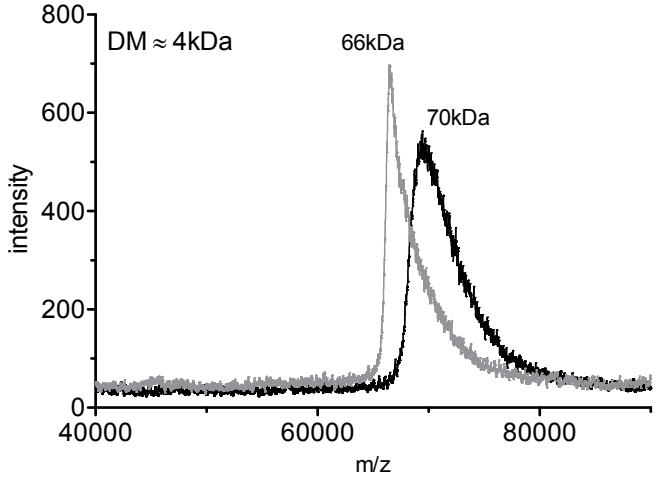


Figure 6.3: MALDI-TOF spectra of BSA (grey line) showing the mass shift caused by DTPA attack (black line).

After the BSA analysis, the DTPA attack was evaluated in the commercial binders labeling. Animals' glues (from sturgeon, rabbit, and skin and bone tissues of mammalians) and ammonium caseinate were purchased from Kremer Pigmente (Aichstetten, Germany), while fresh eggs were obtained from a local market.

All binders have been prepared before labeling as reported below:

Animal glues: suspended in water at 50°C,

Egg yolk: recovered by piercing the vitelline membrane,

Egg white: beaten until stiff, then the bottom liquid was recovered,

Casein: dissolved in 4% NH3 (w/w).

The derivatization procedure has been performed as previously described and the results obtained for lysozyme (in the egg white) and for casein are reported (Figure 6.4).

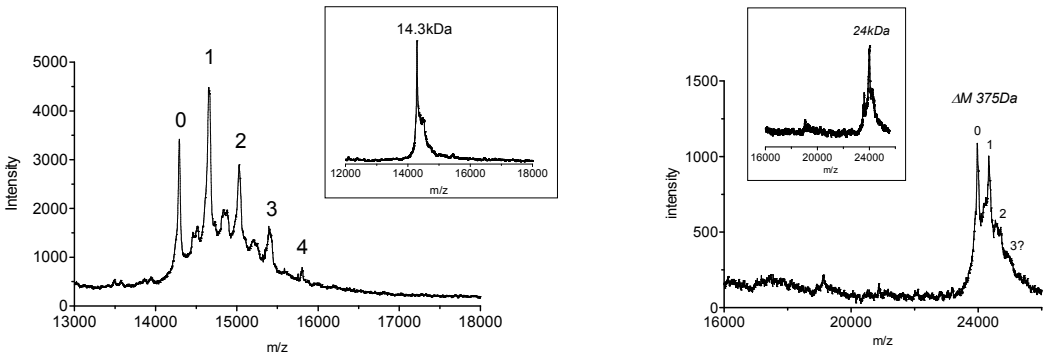


Figure 6.4: MALDI-TOF spectra of lysozyme (left panel) present in the egg white and casein (right panel) showing the number of DTPA molecules added. The mass shift for each DTPA added was 375Da. The unlabeled proteins are reported in the inserts.

DThe low MW of thess proteins, with respect to BSA, let us to observe, each DTPA addition. Both proteins contain several lysine residues, thus multiple DTPA molecules were present. In particular the lysozyme showed four Lys labeled, while for casein they were at least two, but a third attack was suspected. With respect to the other binders, the animal glues analysis by MALDI-TOF gave no results, in fact any peak was detected. This was probably due to the low ionization efficacy of glues obtained with MALDI. For this reason any consideration on the labeling performances on these samples can be made. The analysis of proteinaceous binders after DTPA attack has shown that, due the different accessibility to the sites in the folded protein, not all amines were derivatized. As an example, the attack of 4 lysine residues (on the 6 present) means for lysozime a labeling efficiency around 60%, while for casein only 2 residues on the 11 present have been derivatized, meaning a labeling efficiency around <29%. For quantitative purposes the derivatization of all residues should be obtained, in order to reach the maximal sensitivity. This is not necessary in this kind of analysis, since it is not possible (and moreover, meaningless) to quantify the proteinaceous binder present in a paint. For this reason, even if the reaction was not complete, it was enough for our purposes.

To complete the labeling procedure ionic europium was added using a lanthanide/

DTPA ratio of 5 : 1. The europium solution was prepared by dissolving Eu_2O_3 in 37% HCl and diluting the obtained suspension with deionized water. Since the formation of Eu-DTPA complex could not be detected with MALDI, this step was monitored by HPLC-ICP-MS.

Chromatographic separation of labeled-proteins

In order to obtain the chromatographic separation of a complex mixture of proteins several techniques can be used. SEC chromatography, which has been used and described in the Chapter 3 for similar samples, has not enough resolution for this kind of analysis. Moreover, the onset of proteins aggregation during the labeling procedures can alter the results. For these reasons this kind of chromatography has been excluded. The use of cation exchange chromatography has also been excluded, since we found that this column strongly retained europium and gave complex instability.

RP chromatography has been finally chosen and the proteins were separated using a linear gradient from 5% to 60% of CH_3CN + 0.025% TFA in 18mins.

Instrumental optimization

The coupling of reverse phase chromatography with ICP-MS demands attention, due to the reductive properties of the ICP. The organic solvent introduced into the ICP source is reduced into elemental carbon, which can deposit onto the the instrumental parts (sampler cone, skimmer or ion lenses).

Since the proteins elution requires a large amount of CH_3CN (up to 70%), we needed to reduce the amount of solvent arriving to the torch. With the Symmetry 300, 2.1x150mm column (Waters Corp.), the flow used was 250µl/min and, under these conditions, the CH_3CN arriving to the source is still high. Usually the best wayto solve this proble is the use of capillary or nano-columns for which very low flow is required (few µl/min or less), but in order to operate in these conditions specific HPLC systems or post column flow split are required. Some authors reported the use of membrane desolvation (Bluemlein et al. 2009) or cold spray chambers (Rivas et al. 1996) to additionally decrease the amount of solvent arriving to the plasma. For this reason, we decided to set the spray chamber to -5°C during the analysis (Table 6.1).

A constant flow of oxygen was added to overcome the carbon formation within the plasma. In the ICP-MS 7500es (Agilent Technologies) used the oxygen has been added as optional gas, using a modified connector placed between the spray chamber and the ICP torch. The amount of O_2 added should be proportional to the organic solvent load

Table 6.1: Source and mass analyzer parameters used for the analysis of samples.

Parameter	Value
Power RF	1500 W
Sample Depth	8.3mm
S/C Temperature	-5°C
Monitored Masses	151, 153 and 167
Torch ID	1mm
Sampler cone and Skimmer	Platinum

in order to convert the CH_3CN in the gasseous CO/CO_2. In the presence of O_2 the plasma changes from reductive to oxidative conditions. This means that, if too much oxygen is added, the excess can be reactive versus the metal (usually nikel) constituting the instrumental parts (sampler cone & skimmer). The amount of oxygen to add must be carefully done, especially when chromatographic gradients are performed. To do this, the instrumental acquisition method was set in the multitune modality and different levels of O_2 were used during the CH_3CN gradient (Table 6.2). Before the analysis the instrument was tuned for each O_2 level with 10ppb of europium solution, to obtain the best sensitivity.

Table 6.2: Parameters used in presence of different load of organic solvent and chromatographic gradient (continuous line) with O_2 addition (dashed line).

%CH_3CN	Instrumental parameters	
20%	Carrier Gas	0.95
	Optional gas	15%
	Torch -V	-0.3mm
40%	Carrier Gas	0.0.87
	Optional gas	25%
	Torch -V	-0.4mm
60%	Carrier Gas	0.69
	Optional gas	36%
	Torch -V	-0.5mm

With these intrumental parameters no signal variations was observed even in the presence of 60% of CH_3CN.

Europium recovery

The silanol activity can influence the metal recovery from the C18 column. This phenomenon is due to the cation exchange properties of silica (Smith et al. 1984), which is based on coulombic interactions:

$$-SiO - C^+ + X^+ = -SiO - X^+ + C^+ \tag{6.1}$$

where X^+ represents the cation under investigation and C^+ represents H^+ (or other mobile phase cations). In the presence of aqueous electrolyte solutions (such as the starting conditions of our gradient) this equilibrium appears to be a simple ion exchange. Usually the end-capped columns are less prone to interact with metals since the silanols are de-activated. The analytical column used (a Symmetry 300, Waters corp.) is fully end-capped and gave an Eu recovery of 106% for the injections in presence of 5%CH_3CN. The pre-column used gave unfortunately some metal retention (Figure 6.5). Probably this is due to a different silica used for the pre-column packing. For this reason we decided to operate without using the pre-column, in order to limit the europium loss, even if this can drastically lower the column life.

The Eu recovery from column was investigated under the high CH_3CN percentages present in the chromatographic gradient (60%). In this case the recovery obtained was lower (74%) than the one obtained with the 5% of CH_3CN, suggesting that the organic solvent was affecting the europium elution. It is a well known phenomenon occuring in column without endcapping (Sugrue et al. 2005), but this should be not occurring in the fullt endcapped columns. Nevertheless, in our chromatographic separation when the 60% of CH_3CN is reached, there was not free europium eluting form the column, but only the Eu-DTPA-protein complex, which was not affected at all.

6.2 Proteinaceous binders analysis

Some standard proteins and the proteinaceous binders have been labeled with the above described procedure. The m/z 151 ion (corresponding to europium) was monitored in order to characterize all samples by their RP ICP-MS chromatogram.

Standard proteins (BSA, β-casein and ovalbumin from egg white, all purchased from Sigma Aldrich) have been diluted in 10mM tetraethyl ammonium bromide (TEAB) buffer to obtain 10^{-5} M solutions. After the labeling procedure, proteins were injected into the RP column as soon as possible in order to avoid sample changes. When a labeled sample is injected the excess of europium elutes in the void volume of the column, while a second

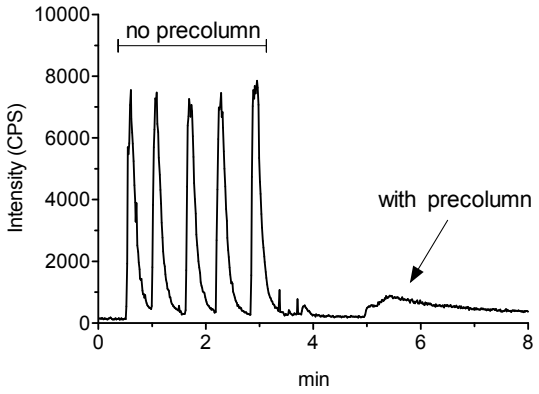


Figure 6.5: Injection of 20ppb europium solution in presence of pre-column. The mobile phase was 5%CH_3CN with 0.025% TFA. Flow 0.25ml/min.

large peak, corresponding to the Eu-DTPA complex, is usually detected. In the panel (a) of Figure 6.6 the chromatogram of labeled β-casein is reported. This protein gave a single peak at 13.3 min which was not overlapped to the excess of Eu-DTPA complex. At the same manner, BSA analysis has shown only one sharp peak at 15.4 min (reported in the panel (c)).

On the contrary, in the analysis of standard egg ovalbumin (panel (b)), several peaks were detected, suggesting the presence of more than one protein. Additional MALDI-TOF investigations revealed the presence of others egg white proteins in the purchased standard and, moreover, the same results have been found by other authors (Tokarski et al. 2006).

The amount of Eu-DTPA-protein complex formed in the standard protein labeling was evaluated. The europium quantified in the protein peak, is used to calculate the concentration of the labeled protein. Consequently, its comparison with the initially employed protein concentration (10^{-5}M) enabled to obtain the labeling efficiency, which was practically complete.

The analysis of standard proteins suggested that it is possible to discriminate different labeled proteins in function of their retention times. This was judged promising for the subsequent investigation of real binders used in art painting.

Proteinaceous binders have been prepared as reported in 6.1.1. Ammonium caseinate and animal glues were diluted in 10mM TEAB in order to obtain 10^{-5} M solutions, while egg yolk and egg white were diluted 4-fold in 10mM TEAB and purified with centrifugal filters (3000MWCO) before labeling. This step was performed in order to purify and

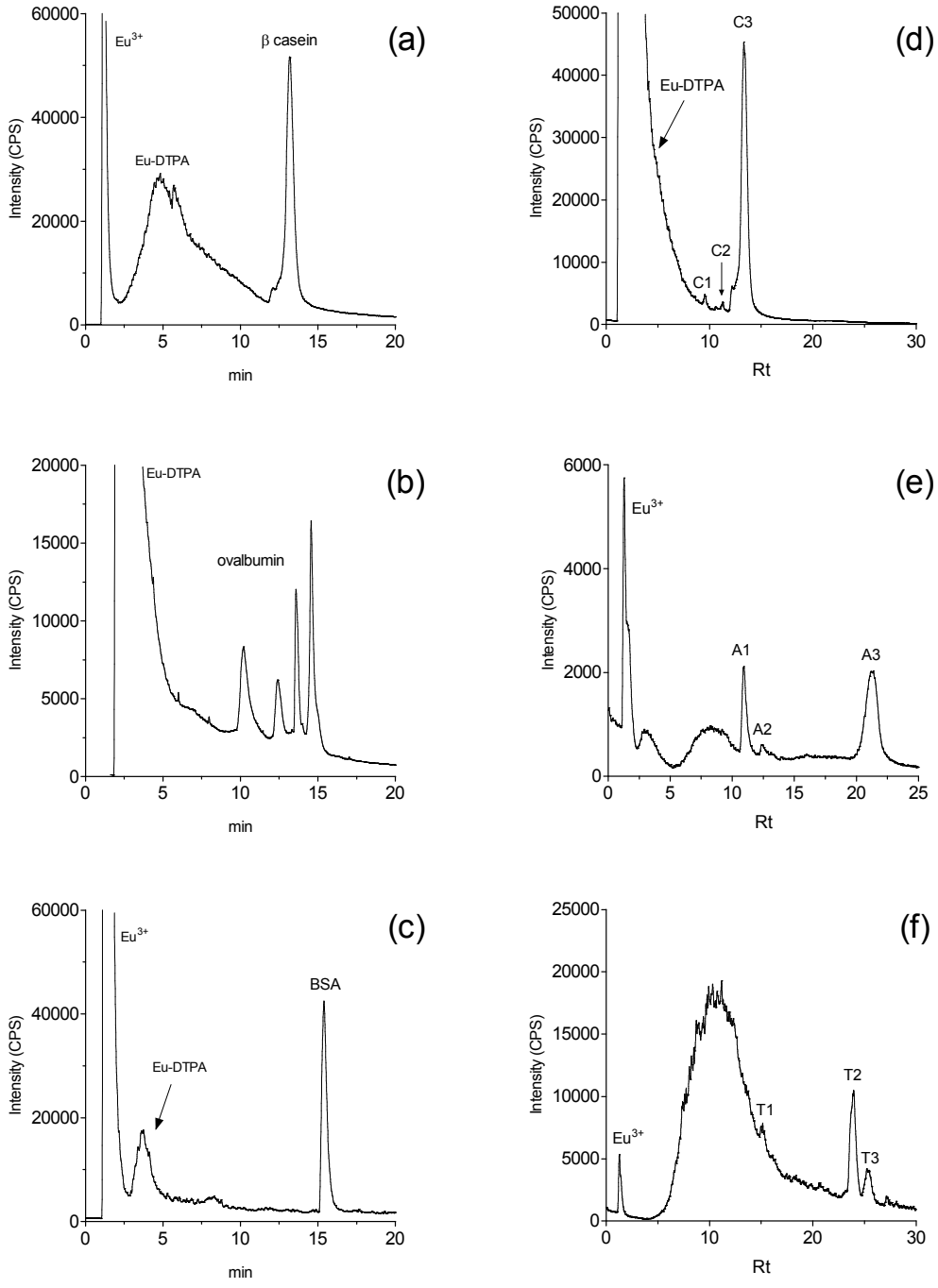


Figure 6.6: RP-ICP-MS chromatogram obtained from the analysis of: (a) β-casein, (b) ovalbumin and (c) BSA standard proteins, and (d) casein, (e) egg white, and (f) egg yolk binders.

remove the numerous reactive amines present in the fresh binder that could react with DTPA. Casein (panel (*d*)) gave an intense peak (peak C3) at 13.3 min corresponding to β-casein isoform. Other peaks (C1 and C2), corresponding to different casein isoforms have also been detected (α-casein and ϰ-casein).

Egg yolk and egg white gave quite different chromatograms (Figure 6.6, panel (*e*) and (*f*)). Egg yolk was characterized by a broad peak at 10min. This probably corresponding to the interference of some matrix component, which reacted with the DTPA excess. Since the yolk samples were previously purified with 3000MWCO filters, it is possible that small proteins were responsible for this peak. Nevertheless, two clear peaks characterizing this binder have been found (peak T2 and T3) that eluted later in the chromatogram.

The egg white analysis showed two characteristic peaks (A1 and A3) which were also found in the ovalbumin standard analysis. These results positively confirm that all the above mentioned binders can be identified using their characteristic retention time.

Unfortunately, this was not the case for the animal glues (Figure 6.7). All samples of

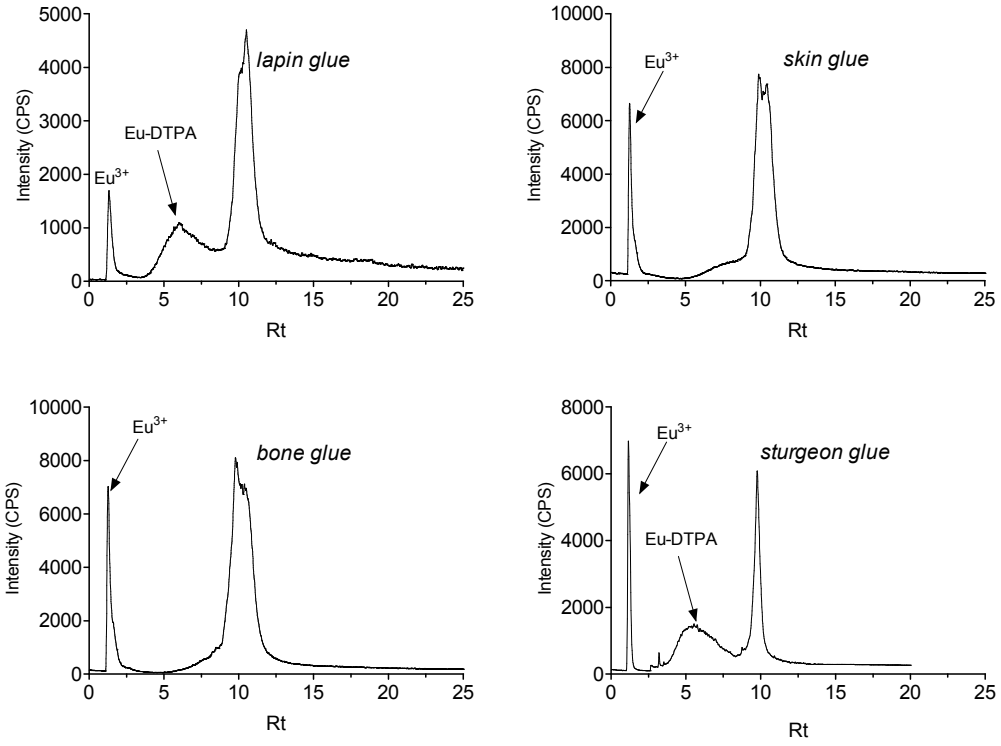


Figure 6.7: RP-ICP-MS chromatogram obtained from the analysis of: lapin glue, skin glue, bone glue and sturgeon glue.

glues analyzed gave a similar chromatogram profile. Lapin (panel (*a*)), skin (panel (*b*)) and bone glue (panel (*c*)) chromatograms showed only the broad peak at 10 min. The sturgeon glue gave also one peak at the same Rt, but more sharp. Even if several gradients have been tested the results otained were similar. The impossibility of distinguishing glues is explained by the fact that all of them are obtained by thermal degradation of collagen, one of the most conserved proteins in animal bodies.

Moreover, in the RP chromatography the analytes are retained according to their lipophilicity. Since in the collagen half of the AA sequence is constituted by glycine and proline (or hydroxyproline), there is a low variation in the chemical-physical characteristic of collagens coming from different sources. The only difference detected in these samples was in the characteristic peak shape of sturgeon glue (fish collagen is usually less cross-linked) which differs from the peak shape of glues extracted from terrestrial animals. However, this diference could be not significative in the analysis of unknown samples.

All these results suggest that different classes (milk, egg and glues) of proteinaceous binders were recognized with this method. Unfortunately it was not possible to differentiate binders belonging to the same family, such as the animals glues. Anyway, these results were comparable to those obtained with the traditional spectroscopic techniques used for binders analysis (Domenech-Carbo 2008).

6.2.1 Pictorial models

A painting is usually made of different layers (preparative layers and paint layers) and the proteinaceous binder represents a minimal portion of the paint (<10% w/w). Moreover paints are exposed to environmental conditions that can alter the binders, causing protein oxidation and/or degradation(Leo et al. 2011; Romero−Pastor et al. 2012).

Before to applying the above developed method to real paints layers, the effect of proteins modification and, moreover, the effect of pigments present in the paint layer must be verified.

With this aim some pictorial models were prepared and the binders under investigation were applied onto three kinds of support models:

- *glass slide*,
- *wooden support* with the *intonachino* layer prepared following the method described by Chiavari et al. Chromatographia,
- *wooden support* covered by a layer of gypsum and glue.

The glass slide represents the simplest model and it was used to test the extraction procedure for binders recovery, while the other supports have been prepared in order to test the feasibility of whole procedure in the analysis of complex paint layers. The *intonachino* layer was obtained with a mixture of lime and thin sand (1 to 1.5 w/w) and water was added until the mixture reached the proper consistency. This model was prepared in order to simulates the structure of a fresco painting. The gypsum ground was obtained by mixing $CaSO_4 \cdot 2H_2O$ with a tepid rabbit glue solution (1 to 1 w/w) and was prepared in order to simulates a panel painting.

All binders were then applied onto the supports with or without pigment (*ochre yellow*, Fe_2O_3 and *zinc white*, ZnO). The paints were prepared by mixing pigments with the binder in a ratio of 3 to 1 w/w, in order to evaluate their interference in the procedure. The pictorial models were naturally aged by keeping them exposed to light and humidity for 2/3 months before being sampled.

Pictorial models extraction and derivatization

The proteins extraction has been evaluated using Bradford assay and both acidic and basic conditions were tested. The binders applied onto the glass slides were excised, and about 1mg of sample was weighed for analysis. The calibration curves have been prepared in the same buffer used for the extraction. Standard BSA was used for egg yolk and casein quantification, while ovalbumin standard was used for the egg white quantification. Animals glues gave very low signal intensity with the Bradford assay, due to the scarce presence of reactive amino acids. Nevertheless their recovery % was obtained using a calibration curve prepared with fresh glue solutions. In Table 6.3, the recovery obtained using two different extraction buffers are reported.

Table 6.3: Recovery of binders from the glass slides using two different extraction protocols.

Binders	1% TFA	2% NH_3
Casein	0%	76%
Rabbit glue	13%	83%
Skin glue	88%	102%
Bone glue	5%	90%
Sturgeon glue	25%	110%
Egg yolk	19%	86%
Egg white	6%	93%

The extraction with TFA gave very low recovery for almost all binders. Moreover,

on the contrast of what reported by some authors (Tokarski et al. 2006), casein was not re-solubilized under acidic conditions. This is due to the high content of phosphate groups, which led proteins precipitation after acid addition (e.g. what occurs in the cheese production). On the other side ammonia solution gave good recovery for all binders analyzed, so this buffer has been used for the subsequent experiments.

The more complex pictorial models (binders and paint onto the supports) have been extracted. After ammonia addition an ultrasound-assisted extraction (15 min) was performed. The supernatant was recovered and washed with 3000MWCO centrifugal filters using 10mM TEAB buffer in order to eliminate pigments. The obtained solutions were finally diluted to a final volume of 50µl before the labeling procedure. The paint layers (binders in presence of pigments) extracted from the glass slides have been easily recovered and labeled. Moreover, the presence of pigments (Fe_2O_3 and ZnO) gave no interference in the labeling procedure and instrumental detection. In general the signal intensity was very low, because of the small amount of proteins in the samples (around 1mg of layer of which 3/4 were pigments). Nevertheless, the characteristic peaks of binders have been detected, as it can be seen in Figure 6.8 for the case of egg white in presence of ochre yellow (panel (a)).

The analysis of extracts from the *Intonachino* and gypsum ground preparations revealed that most of the binders were correctly recognized, even if the quality of chromatograms was scarce (Figure 6.8). The panels from (b) to (f) were obtained from the analysis of:

- (b): egg white from *Intonachino*,
- (c): casein from *Intonachino* with ZnO pigment,
- (d): casein from gypsum&glue with Fe_2O_3 pigment,
- (e): bone glue from *Intonachino* with Fe_2O_3 pigment,
- (f): lapin glue from gypsum&glue with ZnO pigment.

Unfortunately the egg-based binders were scarcely recovered (due to the adhesive properties) and the characteristic peaks were not detectable (Figure 6.8, panel (b)) even in absence of pigments. Moreover, the sampling of the intonachino and the gypsum ground preparation was not an easy process, since the sampling of the preparative layers can occur. This is clear for the chromatogram of sample (d) in which the characteristic peak of glue was detected in conjunction with the casein. The results obtained for all binders and supports have been summarized in Table 6.4.

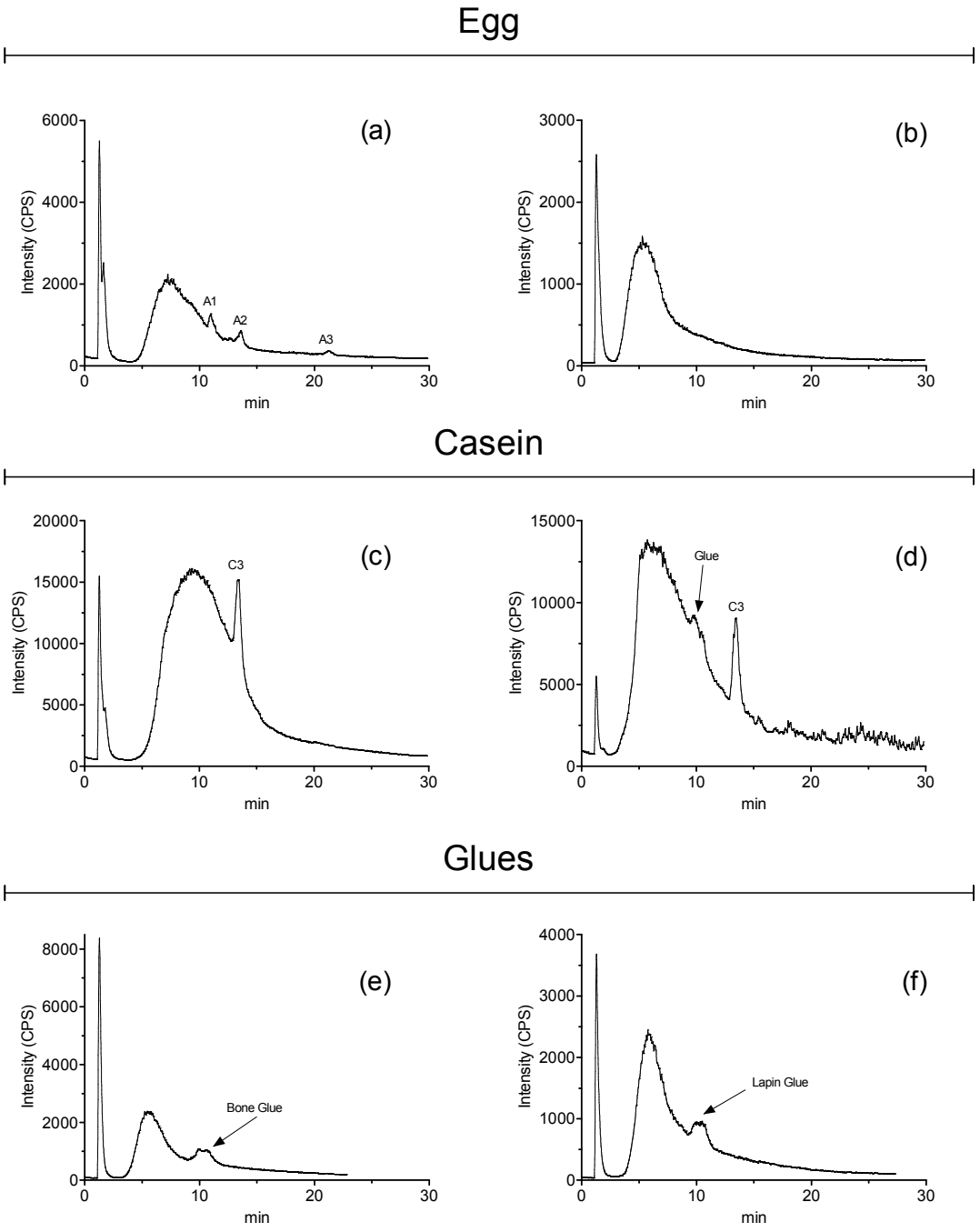


Figure 6.8: RP-ICP-MS chromatogram of binders recovered from different supports. Samples description is reported in the text.

Table 6.4: Binders recovery from supports.

	Glass slide		*Intonachino*		Gypsum	
	Alone	Pigments	Alone	Pigments	Alone	Pigments
Casein	+	+	+	+	+	+
Glues	+	+	+	+	+	+/-
Egg	+	+	-	-	-	-

With respect to the extraction of binders from glass slides, no quantitative evaluation were obtained for extractions of binders from the supports. This is due both to the presence of residual material after the extraction (pigments and preparative layers) that is not easily quantifiable, and to the slight interference of pigments with the Bradford assay. On the other hand the quantitative analysis is not applicable to paintings, as demonstrated by Zangrando et al. 2010 so this aspect was not further investigated.

Analysis of unknown samples

A first attempt to evaluate the effectiveness of this methodology has been done analyszing some unknown paint layer samples. The samples, obtained from the Scientific Research Department of The Metropolitan Museum of Art, NY, were two paint layers with no artistic value. They were characterized by enzyme-linked immunosorbent assay (ELISA) and the presence of proteinaceous binders was revealed. The first sample (called sample 1) was a white colored fragment of around 1.7 mg, whereas the second (sample 2) was a light blue colored fragment of 0.6 mg. The extraction and the labeling procedures were performed as described previously and the chromatograms obtained are reported in Figure 6.9.

For both samples, a peak with an Rt of 10 min has been found revealing that animal glue was used. Unfortunately, due to their similar chromatographic behavior, no information about the specific source of animal glue could be obtained. In the sample 1 the glue signal was good, while in the sample 2 the signal intensity was scarce, due to the very low amount of this sample, but still clearly identifiable.

6.3 Conclusions:

The application of HPLC-ICP-MS for the identification of proteinaceous binders (glue, casein, egg yolk and egg white) traditionally used in art painting has been developed. The method was tested on fresh prepared binders and some pictorial models. The analysis

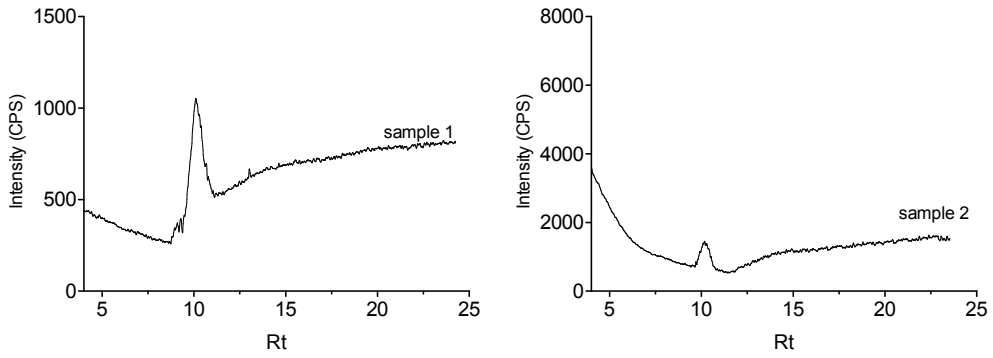


Figure 6.9: RP-ICP-MS chromatogram obtained from the analysis of two unknown paint layers. The peak at 10 min elution time is typical of the animal glues.

show that it was possible to correctly identify the binders present in pictorial layers after extraction and labeling procedures.

The recovery of all binders from the supports must be improved (e.g. in the case of egg-based binders) and other extraction procedures are now under investigation. Despite this, binders identification was obtained using very low sample amounts (<1mg of sample), making the technique micro invasive and very useful for this kind of analysis. In addition, we found that this method appears to be comparable with other mass spectrometric techniques used which have are currently used (such as: GC-MS, LC-MS and MALDI-TOF) with the advantage of being a faster procedure. Moreover in all these techniques, binders identification occurs after hydrolysis or trypsinic digestion and this method represent the first attempt in this field to do the analysis of intact proteins.

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Elemental labeling for the identification of proteinaceous-binding media in art works by ICP-MS†

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In the history of art, artists have used many different organic compounds to dissolve pigments and apply them onto a support to obtain a paint layer. Proteins were used with success from the Middle Ages up to the Renaissance, and the traditional protein sources were animal parts (skins, tendons and bones) or milk and eggs. Moreover, some of these materials are commonly used as adhesive. In this paper, the first application of the metallomic analytical technique to the identification of proteins in artworks is reported. Samples were derivatized with DTPA/Eu and the derivatization procedure was evaluated by matrix-assisted laser desorption/ionization time-of-flight before high performance liquid chromatography inductively coupled plasma MS analysis. This study has been carried out on laboratory models prepared in-house for method development, resulting in the correct identification of the different classes of proteinaceous binders typically used. In addition, some unknown paint layer samples have been analyzed demonstrating that the method is applicable to very small sample amounts (0.6 mg), which are compatible with the amount normally available for this kind of analysis. The results obtained demonstrate the effectiveness of the method, suggesting the potential future use as novel diagnostic tool in the scientific study of artworks.

Keywords: elemental tagging; proteins; painting; ICP-MS; MALDI-TOF

INTRODUCTION

Painting binders are used in artworks to bind pigments so they can be applied in a coherent film onto a pictorial support (such as canvas or plaster). Artists generally prepared the proteinaceous binder starting from very simple and easily available materials such as eggs, milk and animal tissues (skin, bone, tendons). Egg was used as a binder mainly as egg yolk or whole egg, although it is reported in treaties that when egg white was used as paint binder, it was mixed with fig tree latex to obtain a less fragile film.[1] Animal-based glues were obtained by boiling animal's skin, tendons or bones, for long periods followed by steps to obtain a more purified collagen product. Milk was also minimally processed to obtain a product that could be used as glue and binder.

The chemical characterization and identification of proteins present in the pictorial layers are extremely important for understanding the painting technique used by the artist. This is useful both in authentication studies and when carrying out diagnostic analyses prior to restoration. The correct identification of an artwork and the techniques used is important so correct and compatible restoration procedures can be applied. It must be taken into consideration that a painting is made of different layers (including a preparative layer and one or more paint layers). Furthermore, biological and chemical degradation of the original material has occurred because of the environment, natural aging and possible past conservation treatments that were incompatible with the original artwork.[2] Taking all these factors into account, the complexity of protein analysis in artworks is evident. In addition, binder generally represents a minimal portion of the paint (less than 10% w/w) and the presence of pigments can interfere with the analytical technique decreasing the amount of protein recovered.[3]

Many different analytical methods can be used for the identification of proteins, but no single technique is immune to the challenges listed previously. Techniques such as Fourier transform infrared spectroscopy, Near-infrared spectroscopy and Raman spectroscopy allow analysts to generically distinguish among oils, proteins and other species of binders, but cannot differentiate between binders of same family,[4,5] for example oil but not linseed or safflower oil, and protein but not animal skin glue or casein binder. Chromatographic techniques coupled with mass spectrometric detection [i.e. GC, high performance liquid chromatogarphy (HPLC), pyrolysis (Py)-GC][6–8] and more recently matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)[9,10] have been successfully applied to binder identification. However, the identification can only occur after acidic hydrolysis followed by deconvolution of the amino acid profile to identify the proteins present or trypsinic digestion followed by peptide mass fingerprint. Thus for these techniques, the sample preparation time is long and, mainly for chromatographic techniques, the results can be biased

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by pigment interferences or by structural modification of the proteins during hydrolysis and derivatization reactions.[11–13]

The use of inductively coupled plasma (ICP) MS for the quantification of organic biomolecules based on the detection of heteroatoms is a developing field. These heteroatoms are either naturally presented (i.e. S, P and Se) or chemically added as a label.[14–17] In fact, recently mass spectrometric techniques such LC-ESI-MS/MS and in particular LC-ICP-MS applications have been developed to quantify peptides/proteins by chemical labeling using rare earth elements such as lanthanides.[18,19] This technique was born in the radio pharmacology field as radioactive labeling and was initially employed to derivatize antibodies for immunohistochemical assays.[20–23] Different strategies have been studied. One of the first methods employed was a Cys-specific labeling technique called element-coded affinity tag based on tags containing different element-coded metal chelates that incorporate rare earth elements into the macrocycle 1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid (DOTA).[24,25] More recently, a new method using a metal element-chelated tag (MECT) has been developed.[26–28] In the MECT approach, to introduce the lanthanides the bicyclic anhydride of diethylenetriamine-N,N,N′,N″,N‴ pentaacetic acid (DTPA) is used. With respect to DOTA, this is a less expensive labeling agent that reacts with primary amines (amino terminus and internal Lys) present in the proteins. Once the DTPA–protein reaction is performed, the complex with rare earth metal of interest is obtained by adding the metal to the solution.[29]

The coupling of labeling procedures with surface analysis techniques, such as laser ablation ICP-MS, could be an attractive method for analysis of the painting layers. However, we must consider that laser ablation is a micro-destructive technique and even if the laser spot size can reach few microns, the surrounding surface can be burnt causing visible damage to the artworks. In addition, to detect and recognize the class of proteins without a separation step (e.g. chromatographic separation), other solutions must be hypothesized (e.g. the use of labeled antibodies against proteinaceous binders) but surely the derivatization *in situ* of dry proteins present in pictorial layers is the major limitation. For these reasons, we judge that use of laser ablation in this field not helpful.

In this paper, the first use of the metallomic analytical technique of heteroatom labeling of proteins in the field of artistic protein recognition is presented. Lanthanide was selected as chemical labeling because naturally occurring heteroatoms such as sulfur are often present in pigments (as sulfides or sulfates) and this can cause interference problems. Furthermore, phosphorus is present in a very low number of pigments but some proteinaceous binders used by the artists are not phosphorylated (e.g. glues).

The analytical procedure was first tested by using protein standards to evaluate the effectiveness of the derivatization process and to optimize the chromatographic separation. Then, the characterization of proteins binders was performed by analyzing commercially available and freshly prepared binders. Finally, before applying the method to unknown paint samples, the effectiveness of the protein extraction procedure and the optimization of sample preparation were evaluated using reconstructed pictorial models.

EXPERIMENTAL

Instrumentation

The ICP-MS instrumentation used was an Agilent 7500is (Agilent Technologies, Tokyo, Japan) fitted with a standard quartz spray chamber and a PolyPro-ST concentric nebulizer (Elemental Scientific Inc. Omaha, NE, USA). Chromatographic separations were performed on a Symmetry300 C18 2.1 × 100 column (Waters, Dublin, Ireland) with an Agilent 1100 series HPLC pump (Agilent, Waldbronn, Germany) fitted with a manual injection valve (9125i, Rheodyne, CA, USA) with a 10μL (PEEK) sample loop. The proteins were separated using a linear chromatographic solvent gradient starting from 5 to 70% CH_3CN with 0.05% TFA. Chromatographic parameters and instrumental condition are reported in Table 1. MALDI/MS measurements were carried out on a MALDI-TOF Ultraflex II (Bruker Daltonics, Bremen, Germany) operating in the linear positive ion mode. Ions were formed using a pulsed ultraviolet laser beam (nitrogen laser $\lambda = 337$ nm).

Standards and reagents

Albumin from bovine serum (BSA), ovalbumin from egg white, β-casein from bovine milk, europium (III) oxide, dimethylsuphoxide (DMSO), sinapinic acid and micro SPE Supel-tips C18 were purchased from Sigma Aldrich (Milan, Italy). Dithiothreitol (DTT), tetraethylammonium bromide (TEAB), ammonium carbonate $(NH_4)_2CO_3$, dianhydride of diethylenetriaminepentaacetic acid (DTPA) and trifluoracetic acid (TFA) were purchased from Fluka (Milan, Italy). Iodoacetamide (IAA) was purchased from Carlo Erba Reagenti (Rodano, Italy). Centrifugal filters Amicon Ultra 3000 MWCO were purchased from Millipore (Molshiem, France). Sturgeon, rabbit, skin and bone glues and casein were purchased from Kremer Pigmente (Aichstetten, Germany). Ochre yellow pigment was purchased from Bresciani (Milan, Italy), and zinc white pigment was purchased from Dolci (Verona, Italy). Fresh chicken eggs were purchased from a local market. The ultrapure water was produced by a Purelab Ultra system (Elga, High Wycombe, UK), and Ultrapurity Solvent grade acetonitrile was purchased from Romil Ltd (Cambridge, UK).

Sample preparation

Bovine serum albumin and ovalbumin standard proteins were dissolved in a 10 mM TEAB solution to obtain a final concentration of 1×10^{-5} M. The β-casein standard and casein extract were

Table 1. Instrumental conditions and chromatographic parameters

ICP-MS instrumental parameters	
RF power	1500 W
Sample depth	8 mm
Carrier gas	0.87 L/min
Optional gas (O_2)	35%
S/C temperature	−5 °C
Monitoring masses	151, 167
HPLC gradient program	
Time	**% B**
0	5
3	5
10	45
16	45
25	70
29	70
29.1	5
Flow	0.25 mL/min

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dissolved in 4% w/w ammonia solution, both solutions were finally diluted in 10 mM TEAB to obtain the working solutions (1×10^{-5} M). Animal glues were dissolved in water at 50 °C and then diluted in TEAB to avoid any repolymerization processes.

Proteins were reduced (DTT, 1 h at 37 °C) and alkylated (IAA, 1 h at 37 °C) to keep them unfolded and to obtain a good exposure to the reactive sites. Sample derivatization was performed by adding the cyclic anhydride of DTPA dissolved in anhydrous DMSO in order to obtain a final stoichiometry of derivatizing agent to number of primary amines (e.g. Lys) present for each protein of 5 : 1. The mixture obtained was adjusted to pH ~8 by adding $(NH_4)_2CO_3$ and was left to react at room temperature for 1 h. After the derivatization reaction was complete, europium was prepared by dissolving europium oxide (Eu_2O_3) in 37% HCl and then diluting in deionized water. The europium solution was added to the derivatized sample to obtain the chelated complex (1 h at room temperature) before ICP-MS analysis. The final lanthanide/DTPA ratio used was 5 : 1. A schematic of the protein-labeling procedure is reported in Fig. 1 for clarity.

Samples prepared for MALDI-TOF analysis were purified through a microSPE tip before deposition with sinapinic acid in order to remove all salts. Samples for HPLC-ICP-MS analysis were injected without desalting as it is. Because a drop in the signal was observed after 3 h of chelating reaction, all samples obtained were analyzed as soon as possible after the derivatization process.

Pictorial models reconstruction

Pictorial models were constructed by working with commercially available and freshly prepared binders on the basis of recipes in the literature.[9,30,31] In Table 2, the proteinaceous binder preparation procedures are reported. Each binder was applied with a paintbrush onto three kinds of pictorial support models: (1) a glass slide, (2) a wooden support with a layer simulating the traditional composition of *intonachino* (plaster) used for *fresco* paintings and (3) a layer of ground gypsum ($CaSO_4$ $2H_2O$) and rabbit glue simulating a panel painting. The *intonachino* layer was prepared following the method described in Chiavari *et. al*[32] using lime and thin sand in a ratio of 1 : 1.5 w/w with addition of water until the mixture reached the proper consistency, and the ground gypsum was obtained by mixing the $CaSO_4$ $2H_2O$ with tepid rabbit glue in a ratio of 1 : 1 w/w. The binders were then applied onto the supports with or without pigment. The paints were prepared by mixing pigments with each binder in a ratio of 3 : 1 w/w, to simulate possible interference effects in the analytical procedure. Among the several pigments available, our choice was focused on ochre yellow (composed of hydrated iron oxide containing small quantities of alumina and silica) and zinc white because they are very common pigments. These models were naturally aged by keeping them exposed to light in a laboratory at ambient temperature and humidity for 2–3 months before being sampled to simulate a museum atmosphere. The schematic structure of the pictorial models preparation is reported in Fig. 2.

Table 2. Preparation procedures of different proteinaceous binders used in this study

Binder	Procedure
Animal glues	2 g of granular glue was suspended in 25 mL of water until swelling, then heating in a water bath at 50 °C to obtain complete solubilisation.
Egg yolk	Yolk was recovered by piercing the vitelline membrane without contaminating it with egg white.
Egg white	Egg white was beaten until stiff, then left to rest overnight. The day after, the superficial foam was removed to recover the bottom liquid.
Whole egg	Egg yolk and egg white were separately prepared as previously reported and then mixed together in a 1 : 1 v/v ratio.
Casein	20 g of casein was dissolved in 4% NH_3 (w/w).

For the analysis, the pictorial model painting layers were excised with a scalpel to obtain about 1 mg of sample and weighed. Protein extraction was performed by adding (2×100 μL) of a 1% ammonia solution to the sample followed by ultrasound-assisted extraction (15 min), and the supernatant was recovered and was washed with a TEAB buffer onto 3000 Da MWCO centrifugal filters to eliminate pigments. The recovered protein solutions from centrifugal filters were diluted to a final volume of 50 μL. The derivatization procedure was performed as reported previously, and 10 μL of the sample was injected onto the C18 analytical column.

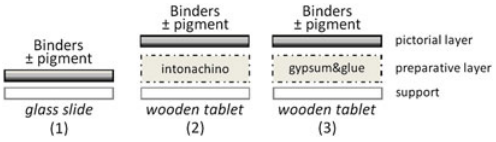

Figure 2. Schematic of the pictorial models prepared in the laboratory using different kinds of supports; the stratification of the preparative and pictorial layers was made to simulate the typical complexity of a painting.

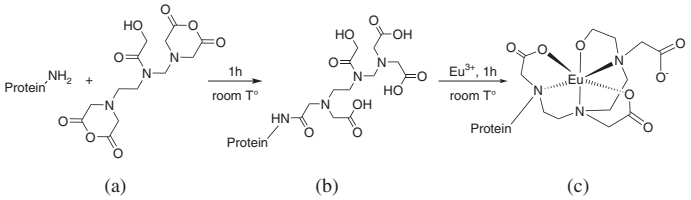

Figure 1. Schematic of protein derivatization procedure showing (a) the $-NH_2$ residue and the cyclic anhydride of DTPA, (b) the resultant DTPA-protein complex and (c) the europium chelation.

Pictorial layer samples

To evaluate the effectiveness of the methodology, we analyzed two unknown paint layer samples from the Scientific Research Department of The Metropolitan Museum of Art, NY. The paints had no artistic value and were previously characterized by enzyme-linked immunosorbent assay (ELISA) before sending them to us. The first (called sample 1) was a white fragment weighing 1.7 mg, whereas the second (sample 2) was a light blue fragment weighing 0.6 mg. The samples were extracted and subjected to the purification and derivatization process as aforementioned.

RESULTS

Method development

Because the derivatization method is a sequential procedure, it is essential to correctly evaluate each step to determine the overall procedural effectiveness. For this, MALDI-TOF measurements were performed to monitor the DTPA–protein reaction, whereas the chelation reaction with europium was monitored directly by RP-HPLC-ICP-MS. The first reaction step was verified by observing the mass shift of 375 Da (and multiples) of protein standards (BSA, β-casein and ovalbumin) and freshly prepared binders before and after the derivatization with DTPA. For example, Fig. 3 shows the MALDI-TOF spectra of egg lysozime protein present in the egg white binder before (a) and after (b) derivatization. The (b) spectra demonstrate that multiple DTPA molecules are present. Because of the accessibility to the different site of the proteins, not all the primary amines present were derivatized. Because quantitative analysis is not applicable to artistic paintings, as demonstrated in a previous work in our laboratory,[8] we can judge it as fit for the purpose. The reason for this is that the painting is a highly inhomogeneous surface. Artists prepared paints each day, following recipes based on measures such as spoonfuls or by eye until a certain consistency is reached. This implies that in different sections of an artwork there could be one or more paint layer superimposed on each other and paints may have been prepared on different days, meaning that a quantitative analysis of proteins in paintings could require the sampling of almost the entire artwork.

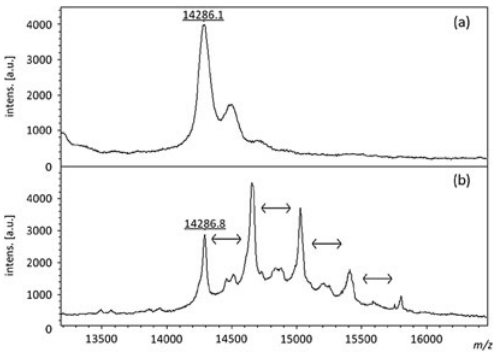

Figure 3. Matrix-assisted laser desorption/ionization time-of-flight spectra of lysozime before (a) and after (b) the derivatization process. Each single addition of DTPA ($\Delta M = 375$ Da) is evident.

After the addition of the europium solution, the amount of Eu-DTPA-protein complex formed was evaluated by ICP-MS. The europium quantified in the protein peak can be correlated with the concentration of the labeled protein in the sample. Consequently, its comparison with the initially employed protein concentration enabled the determination of labeling efficiency, which was practically complete. The correlation of labeling efficiency and europium concentration is possible because ICP is an element-specific ion source and the ionization efficiency is compound independent. As a consequence, free europium and protein-europium complexes respond in the same manner during the ionization process and this is considered the great advantage when using ICP-MS for quantification of metals and organometallic compounds.

Chromatographic separation of standard proteins, commercial and fresh-prepared binders was performed to obtain the characteristic retention times (Rt) of all the proteinaceous binders. The m/z 151 ion corresponding to europium was monitored. A typical chromatogram has a peak that corresponds with free Eu that elutes in the void column as it has no interaction with the C18 stationary phase. Even though it can be assumed that the complex at low pH releases some Eu, in our system the free Eu present is mainly due to the excess of europium used to ensure that all the reactive sites in the proteins are occupied. In Fig. 4, the chromatograms obtained from animal glues (collagen-based) are reported. Sample (a) was glue obtained from a mixture of different rabbit parts, samples (b) and (c) were from rabbit skin and bone, respectively, and sample (d) from sturgeon glue. Even though several gradients were tested, glues (a), (b) and (c) all gave a similar chromatogram profile with a broad peak centered at 10 min, and only in the chromatogram of sturgeon glue there is a unique sharp peak. The impossibility of distinguishing glues (a), (b) and (c) can be explained by the fact that the animal glues are obtained by thermal denaturing of collagen, one of the most conserved proteins in animal bodies. When collagen degradation is performed in an unspecific manner (such as not using collagenase enzymes), what is obtained is a mixture of tropocollagen strands that have a molecular weight of about 150 kDa.

Casein powder (Fig. 5(a)) was analyzed without any purification step. It gave an intense peak at 13.3 min corresponding to β-casein and other smaller peaks corresponding to other kinds of caseins (α-casein and κ-casein), which were subsequently verified by MALDI-TOF analysis (data not shown).

Finally, freshly prepared egg yolk and egg white were diluted 1 : 4 in buffer (10 mM TEAB) and purified with centrifugal filters before the derivatization process. This step was fundamental in purifying the sample to remove the numerous reactive amines present in the biological medium (amino acids or peptides) that could react with the derivatizing agent. The egg white chromatogram obtained is reported in Fig. 5(b). As a comparison in Fig. 5 (c), the ovalbumin standard chromatogram is reported in which more than one protein can be seen. The presence of other egg proteins in the standard was subsequently verified by MALDI-TOF, and this was in line with previous observations.[9]

Application to painting layers

Pictorial model samples

Because the proteinaceous binders considered in this study can be correctly identified by the method, pictorial model samples were analyzed to evaluate the recovery procedure. The extraction

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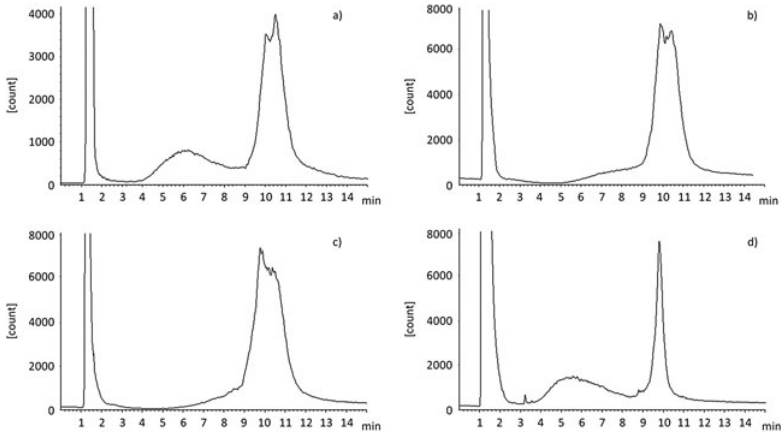

Figure 4. High performance liquid chromatography inductively coupled plasma MS chromatogram of animal glues commercially available: (a) lapin glue, (b) skin glue, (c) bone glue and (d) sturgeon glue. The ion monitored was m/z 151 corresponding to europium.

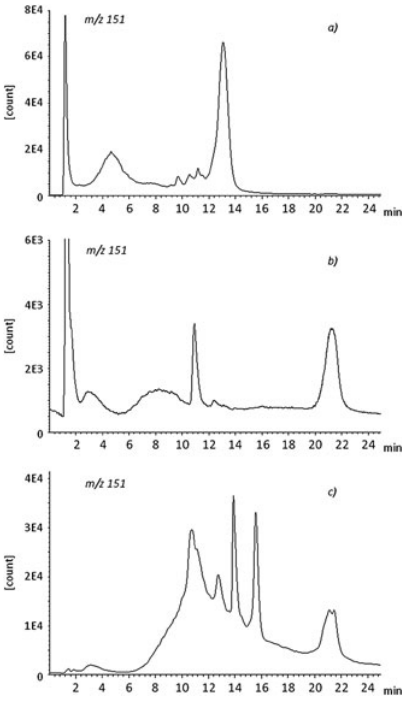

Figure 5. High performance liquid chromatography inductively coupled plasma MS chromatogram of (a) a caseinic extract commercially available, (b) fresh prepared egg white and (c) ovalbumin standard, showing the presence of other egg proteins. The ion monitored was m/z 151 corresponding to europium.

procedure was evaluated both in acidic (with TFA addition) and basic conditions (with NH_3 addition), and finally, ammonia was chosen because casein is highly soluble as ammonium caseinate. Analysis of the proteins recovered from pants on the glass slide reveals that all proteins were easily resolubilized and recovered. Even in presence of pigments (Fe_2O_3 and ZnO), protein extraction and derivatization occurred without interference. This is promising but other pigments should be evaluated in the future.

Whereas sampling of the glass slide is an easy process, sampling of the *intonachino* and the ground gypsum/glue preparation required more attention in order to avoid including a portion of the preparation layer as well. The proteins extracted from the supports were almost all recovered and easily recognized. Also in this case, no interference was found in the presence of pigments during the purification and the derivatization procedures. Only egg was scarcely recovered from this kind of supports, probably due to its highly adhesive properties.

Unknown layer samples

To evaluate the applicability of the method, we analyzed two unknown samples of pictorial layers obtained from the Scientific Research Department of The Metropolitan Museum of Art, NY. The samples were previously characterized with ELISA assay and revealed the presence of proteinaceous binders. Samples appeared as a single pigmented layer and no preparative layer has been observed. Samples extraction was performed with an ammonia solution and the supernatant was washed in centrifugal filters as detailed previously. The chromatograms obtained after derivatization are reported in Fig. 6(b) (sample 1) and (c) (sample 2). Analysis of sample 1 showed a significant peak with an Rt of 10 min, revealing that animal glue was used as the binder. As discussed previously, no information about the specific source of animal glue can be obtained, because they are so similar among them. Analysis of sample 2 also shows that animal glue was used (Fig. 6(c)) even if the signal intensity was very low, and this probably is due to the scarce amount of sample (0.6 mg).

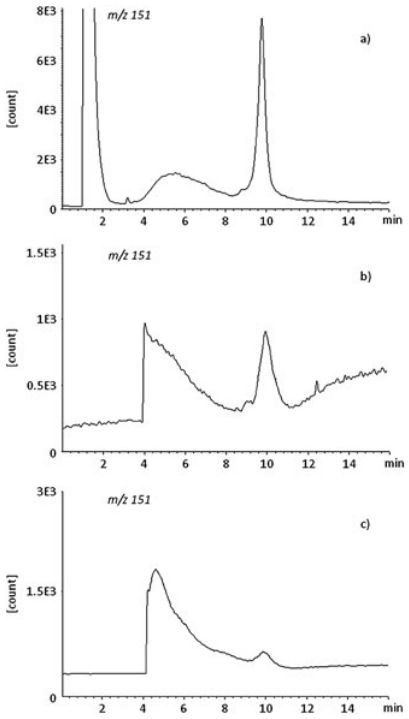

Figure 6. Analysis of pictorial layer samples compared with (a) animal glue standard, (b) sample 1 and (c) sample 2. For samples 1 and 2 to avoid contamination of the instrument with Eu, from t = 0–4 min of chromatographic run, the eluent flowed to waste.

It should be noted that, to avoid contamination of the instrument with Eu, for the first 4 min of the chromatographic run, the eluent flowed to waste.

CONCLUSIONS

The identification of proteinaceous paint binders is one of the most challenging tasks in the analysis of works of art. This work presents for the first time the application of HPLC-ICP-MS for the identification of all classes of binder (glue, casein, egg yolk and egg white) traditionally used. The method was tested on pictorial models prepared using the typical procedures. These analyses show that almost all binders could be successfully recovered and that the pigments do not interfere with the analytical procedure. By HPLC-ICP-MS, the binders present in pictorial layers were correctly identified by using sample amounts that are typically available for this kind of analysis (<1 mg of sample), making the technique micro invasive and so acceptable to museum curators. These results show that this method appears to be comparable with other analytical techniques used [e.g. HPLC/(Py) GC-MS] with the advantage of being a faster procedure. In addition, the analysis of intact proteins offers the advantage of less susceptibility to single amino-acid modifications occurring during aging (e.g. oxidation) and simplifies the identification of mixtures of proteins present in the samples without the use of statistical approaches (such as chemometry). Even though we found that the recovery of egg was not an easy process and the extraction procedures need to be further investigated to improve this step, we can affirm that analysis of proteins in artworks can be considered a novel and interesting application field for ICP-MS-based metallomic techniques.

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ICP-MS and MALDI-TOF: an integrated approach in the study of metal-protein interactions in biological samples.

The activity and stability of most of proteins in a biological organism are subject to metal ion binding. Heavy metals such as arsenic and mercury are able to interact with proteins and, due to their impact on human health and environmental pollution, they represent a priority investigation field. Moreover, the contribution of these metals to the pathological processes, requires an in-depth understanding of the protein targets. Studying the nature of these interactions require the most appropriate analytical method. In order to verify the most suitable operational conditions, the *in vitro* interactions in proteins-rich foodstuffs (such as: milk, eggs and food grade gelatin) with some As and Hg compounds have been evaluated. Then, some naturally exposed samples have been analyzed in order to study any *in vivo* interactions. Samples have been separated using SEC chromatography and metals eluted have been detected by ICP-MS, while proteins have been identified by MALDI-TOF. The results showed that arsenical compounds had strictly species-specific interactions, while for mercury compounds fewer differences have been found. Since the use of ICP-MS for the analysis of biomolecules containing heteroatoms can give us several advantages in terms of sensitivity and specificity, a part of this PhD thesis has been devoted to protein labeling techniques. The study has been focussed on the development of an HPLC-ICP-MS hetero-atom tagging method for the identification of intact proteins isolated from painting layers. The proteins labeling has been performed using the *metal element-chelated tag* (MECT) procedure. The analytical procedure has been firstly tested on proteins standard. Then, the characterization of proteinaceous binders typically used in paints from past centuries has been performed. The proteins extraction and purification procedures have been evaluated using reconstructed pictorial models before testing the effectiveness of the method on some unknown samples.

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ICP-MS e MALDI-TOF: utilizzo di un approccio integrato per lo studio delle interazioni metallo-proteina in campioni di natura biologica.

Arsenico e mercurio metalli noti perla loro capacità di interagire con le proteine e, a causa dell' impatto che hanno sulla salute e sull'inquinamento ambientale, essi rappresentano un campo d'indagine prioritario. Data la natura dell'interazione metallo-proteina, è stata valutata la metodica più adatta per lo studio di questo tipo di interazioni. Le condizioni operative sono state inizialmente sviluppate *in vitro* utilizzando alimenti ricchi di proteine quali: latte, uova e colla di pesce in presenza di alcune specie di As e Hg. Successivamente, alcuni campioni naturalmente contaminati sono stati analizzati con lo scopo di individuare *in vivo* le interazioni che questi metalli hanno instaurato con la componente proteica. Gli analiti sono stati separati con colonna SEC. L'eluizione dei metalli è stata rilevata tramite ICP-MS, mentre le proteine sono state identificate con strumentazione MALDI-TOF. I risultati ottenuti mostrano che la reattività chimica dell'arsenico è strettamente specie-specifica, mentre per le specie mercurio minori differenze sono state trovate.

Poiché l'uso della strumentazione ICP-MS per lo studio delle biomolecole contenenti eteroatomi ha offerto innegabili vantaggi in termini di sensibilità e specificità, nella seconda parte della tesi di dottorato è stato valutato l'utilizzo delle tecniche di marcatura delle proteine con metalli. Lo studio si è focalizzato nello sviluppo di una metodica HPLC-ICP-MS per l'identificazione delle proteine estratte da strati pittorici. La derivatizzazione delle proteine è stata effettuata utilizzando la tecnica del *metal element-chelated tag* (MECT). La procedura analitica è stata sviluppata utilizzando inizialmente delle proteine standard. Successivamente è stata eseguita la caratterizzazione delle proteine presenti nei leganti pittorici tipicamente utilizzati negli affreschi e sono state valutate le procedure di estrazione più appropiate utilizzando dei modelli pittorici ricostruiti in laboratorio. Infine, per testare l'efficacia del metodo sviluppato, alcuni campioni incogniti di strati pittorici sono stati analizzati.

Firma: