INVESTIGATION OF ESSENTIAL AND TOXIC HEAVY METAL SPECIES IN BIOLOGICAL SAMPLES BY MEANS OF ONE AND 2-DIMENSIONAL CHROMATOGRAPHY COUPLED TO ICP-MS

Dissertation
zur Erlangung des Grades
"Doktor der Naturwissenschaften"
(Dr. rer. nat.)

am Fachbereich Chemie, Pharmazie und Geowissenschaften
der Johannes Gutenberg Universität
in Mainz

Valeria Loreti
geboren in Camposanpiero (PD), Italien

Mainz 2005

Dekan:

1. Berichterstatter:

2. Berichterstatter:

Tag der mündlichen Prüfung: 26.09.2005

Die vorliegende Arbeit wurde im Zeitraum von Oktober 2002 bis September 2005 am Institut für Anorganische Chemie und Analytische Chemie im Fachbereich Chemie, Pharmazie und Geowissenschaften der Johannes Gutenberg-Universität Mainz unter der Betreuung von PD Dr. J.B. angefertigt.

I would like to thank PD Dr. J.B. for having invited me to continue my university studies with a PhD in Mainz and for his sustain, help and discussion during these three years.

I thank Prof. Dr. K.G.H. for his engagement, the many interesting discussions and for having given me the possibility to meet the analytical community and present my work in national and international conferences.

I would like to thank the Deutsche Forschungsgemeinschaft for financial support of this work, in the framework of the Graduiertenkolleg 826/1 "Spurenanalytik von Elementspezies: Methodenentwicklungen und Anwendungen".

I would like to thank all the members of the Arbeitskreis, for the interesting scientific discussions and above all for the good work climate and the nice moments we spent out of the working time. Among them, my thanks go particularly to Dr. S.B., for all the work we carried on together, and to A.H. and W.B., for their fundamental help (and the heavy work) repairing ELAN.

I would like to thank P.H., for her support in the extremely complicated German bureaucracy.

I thank U.L. for her help and patience the innumerable times I asked her to freeze dry my samples. I would like to thank Prof. Dr. T.H., Dr. J.W. and M.S. for the ESI-MS and the tandem MS measurements; Prof. Dr. W.B., Dr I.S., S.J. and M.S. for their cooperation and help in the other ESI-MS measurements; Prof. Dr. G.H. and Dr. J.M. for their help and availability in the attempt to carry out MALDI-TOF measurements.

I thank Mr O. from the botanic garden for the *S. vulgaris* seeds.

I would like to thank Dr. E.B. and D.T. for the nice and productive collaboration; Dr. G.S. and Dr. E.M. for the constructive discussions, the cooperation and their fundamental contribution to this work with the *P. tricornutum* cultures.

I thank all the friends, near and faraway, who helped me not to feel alone in a new land.

Last but not least, I thank my husband for the force he gave me and for having heard with patience all the complaining on my results.

Che cos'é la chimica analitica, se non travasare
liquidi da un contenitore all'altro?

Prof. F. M., Pisa, 2000

Dedicato ai miei genitori,
a mio marito ed a me stessa.

ABSTRACT

The metabolism of heavy metals, both essential and toxic, is moderated by expression of proteins, or synthesis of molecules, which are able to complex the target ions, in order either to stock them, or to excrete them from the organism. These processes are regulated by a huge number of variables, above all the species in which the heavy metal is present in the organism.

Innovative analytical speciation methods and original approaches are therefore necessary to characterise these species, leading to new information, necessary to understand the whole processes.

Within this work new speciation analysis methods were developed for the investigation of heavy metal species in different matrixes: size exclusion and reversed phase chromatographic separations were coupled to ICP-MS for the study of metallothioneins bound to copper and zinc in bovine liver samples, by which the procedure was optimised and tested. The same procedure was subsequently applied to human thyroid tissue samples, in which trace metal patterns were comparatively investigated, having at disposition both cancer affected and healthy tissues.

On the other side, extracts of the monocellular alga *Phaeodactylum tricornutum* were the substrates for the optimisation of the procedure for investigation of cadmium and other trace metals bound phytochelatins: employing an ion pair chromatography it has been possible to elute and detect via ICP-MS the metal complex. The use of a ^{116}Cd enriched standard solution led to the synthesis of ^{116}Cd spiked phytochelatins, which were fundamental to state the low kinetic stability of the investigated species. Subsequently, the method was employed for the investigation of the same species in more complex samples, like *Silene vulgaris* and oat and wheat shots and roots extracts. The use of ESI-MS as soft ionisation mass spectrometric method was then necessary to characterise unknown species.

List of abbreviations

ac: alternate current
Ala: Alanine
Asn: Asparagine
Asp: Aspartic Acid
CEM: Channeltron Electron Multiplier
Cys: Cysteine
dc: direct current
DTPA: Diethylen Triamine Pentaacetic Acid
ES: Electrospray
ESI-MS: Electrospray Ionisation-Mass Spectrometer
FAAS: Flame Atomic Absorption Spectroscopy
Gln: Glutamine
Glu: Glutamic Acid
Gly: Glycine
GSH: Glutathione (reduced form)
GSSG: Oxidised form of GSH
HPLC: High Performance Liquid Chromatography
ICP-MS: Inductively Coupled Plasma-Mass Spectrometry
ICP-OES: Inductively Coupled Plasma-Optical Emission Spectroscopy
ICP-Q-MS: Inductively Coupled Plasma-Quadrupole-Mass Spectrometer
ICP-SF-MS: Inductively Coupled Plasma-Sector Field-Mass Spectrometer
Ile: Isoleucine
INAA: Instrumental Neutron Activation Analysis
IPC: Ion Pair Chromatography
IS: Ion Spray Potential
LC: Liquid Chromatography
LOD: Limit of Detection
Lys: Lysine
M^{n+}: unspecified metal ion
Met: Methionine
MRI: Magnetic Resonance Imaging
MS: Mass Spectrometer – Mass Spectrometry
MT: Metallothionein(s)
MW: Molecular Weight
NPP: Nuclear Power Plant

PC: Phytochelatin(s)

PCS: Phytochelatin Synthase

PEEK: Polyetherether Ketone

Pro: Proline

RIMS: (high-resolution multi-step) Resonance Ionisation Mass Spectrometry

RF: Radio Frequency

RPC: Reversed Phase Chromatography

RSD: Relative Standard Deviation

SEC: Size Exclusion Chromatography

Ser: Serine

Thr: Tryptophan

INDEX

1. INTRODUCTION

1.1. Motivation

Heavy metals are present in the environment not only in form of minerals, but also as more mobile species, from soluble and ionic compounds to volatile and organic-bound species. Even if small amounts of heavy metals are common in the diet of most organisms (animal and plants) and are actually necessary for good health, large amounts of any of them may have acute or chronic toxicity as consequences.

In human beings, heavy metal toxicity can result in damaged or reduced mental and central nervous functions, lower energy levels, and damages to blood composition, lungs, kidneys, liver, and other vital organs. Long-term exposure may result in slowly progressing physical, muscular, and neurological degenerative processes that mimic Alzheimer's disease, Parkinson's disease, muscular dystrophy, and multiple sclerosis. Among animals and plants, the effects of chronicle or acute toxicity are as well tissue damaging, organism poisoning, various degenerative processes and death[1].

In small quantities, certain heavy metals are nutritionally essential for the healthy life of every organism, and are referred to as the trace elements (e.g., iron, copper, manganese, and zinc). These elements, or some forms of them, are commonly found naturally in foodstuff, in fruits and vegetables, and in commercially available multivitamin products. Moreover, diagnostic medical applications include the use of heavy metal based medicaments; among the others for example dosing with chromium in parenteral nutrition mixtures and the application of stable gadolinium complexes as contrast agents in magnetic resonance imaging practice.

The increasing concentration of mobile heavy metal species in the environment is largely due to anthropogenic causes, first of all industrial pollution. Heavy metals are in fact common in industrial applications such as the manufacture of pesticides, batteries, alloys, electroplated metal parts, textile dyes and steel[1].

Among toxic heavy metals, also the essential ones can reach toxic levels, when their concentration in the organism is too high and they are no longer metabolised. Heavy metals enter plants mainly through the root uptake, animals and the human body through food, water, air, or absorption through the skin.

Table 1: top ten chart of the most poisonous compounds and elements compiled by the Agency for toxic substances and disease registry (ATSDR) in 2003[1]

Rank	Substance name
1	Arsenic
2	**Lead**
3	**Mercury**
4	Vinyl chloride
5	Polychlorinated biphenyls
6	Benzene
7	**Cadmium**
8	Polycyclic aromatic hydrocarbons
9	Benzo(a)pyrene
10	Benzo (b)fluoranthene

As reported in Table 1, three of the ten most toxic substances are heavy metals.

It is not the aim of this work to point out in detail which are the sources of such metals, or the particulars of their effects on plants, animals and humans; it is instead to underline once more that the toxicity of heavy metals depends first of all on their form, their mobility and bioavailability. Every organism reacts producing proteins or peptides able to bind or complex the contaminants, in order to neutralise them, and within this work an attempt has been made to deepen the knowledge of these species, from their chemical stability, to the transformations they undergo within the organisms, with the aim to achieve a better understanding of these processes. The knowledge of the processes and the species' role and behaviour is the basis for a future exploitation of these targets.

Owing to the fundamental role played by the heavy metal species, this work describes several new methods for the investigation of toxic and essential heavy metal species in different matrixes and samples. A new and original approach to the problem, for example in the part of the work that deals with plants, pointed out sides of it which have been up to now not yet studied.

The work is articulated in three main parts, dealing with the speciation analysis of heavy metals bound to proteins and peptides in very different matrixes. Copper and zinc metallothioneins in bovine liver and human thyroids, cadmium phytochelatins in monocellular algae and in higher plants, gadolinium complexes in urine, describe only roughly the wide variety of investigated species of interest. The common denominator between the themes faced was the instrumentation employed and speciation analysis.

In analytical chemistry speciation analysis has gained in the last 50 years more and more importance, leading to a large amount of scientific knowledge on various metal species and a huge instrumental development. The motivation for such a high interest in speciation lies in the information gained on the different roles the same element plays, when present in the system as one or the other species.

Moreover, the analytical challenge in speciation analysis is very high: developing and validating a complete method, starting from an appropriate sample digestion, following with the suitable analytical technique, element and/or species-specific, sensitive and robust, ending finally with precise and accurate quantitative determination of the species, possibly all at the same time, is for an analytical chemist a dream come true.

Speciation analysis is even more than this subtle analytical virtuosity: if we open a book written in a foreign language, we can only see the words it contains, and all we can do is to look at them and maybe to count them. Performing speciation analysis is like learning the grammar of this foreign language: more than only knowing how much of an element is present in a sample, it allows us to see and identify and quantify the species in which the element is present. It allows us to understand the meaning of the single words and of the sentences, establishing relations between one word and the other, giving sense to all the parts of the sentence as a regular, ordinate and sensible structure. The metal species present in a sample have an objective, there are relationships between one and the others, and they rule the effects on the entire system and the equilibrium of an element more than the chemical nature of the element itself.

Speciation analysis is the key to understand the meaning of these species and the connections that bind them one to another. This is the reason why speciation analysis becomes even more challenging, when applied to complex systems: understanding *which* species are there leads, even if in many cases not directly, to understand *why* they are there.

Rationalising a complex system enable us to reproduce it, to employ it for several aims. In the case of plants, for example, to understand which are the rules and the species involved in the processes of metal uptake, mobilisation and storage leads us to the possibility to employ the plants at their maximum capabilities in remediation of contaminated soils.

Interdisciplinary work with the medical, biological and pharmaceutical branches brings the expectations of speciation analysis at their triumph. Understanding what are the species involved in diseases, what is the alteration of metal metabolism in the single organ leads as well to a better and more complete knowledge of the alteration itself.

Speciation analysis is the key to a casket full of gold.

1.2. Summary of results

Heavy metals in mammalian samples

Human samples (i.e. urine, saliva, sweat and hair) were analysed by size exclusion chromatography coupled to ICP-MS, to investigate if a Gd-based medicament underwent transformation or metabolisation during its permanence in the human body. The samples were taken from a patience that received an injection of Gd-DTPA as MRI contrast agent. No other species than Gd-DTPA were detected in the fluid samples. For the hair extraction, several procedures were tested, but none led to a satisfactory extraction without any degradation of Gd-DTPA to Gd^{3+}.

Size exclusion and reversed phase chromatographic separations coupled on-line to the ICP-MS were employed to investigate the metallothioneins (MT) present both in a bovine liver extract, used in principle as a model sample for the method optimisation, and in human thyroids of healthy and cancer affected people. The application of SEC and RPC, respectively, brought us to extrapolate information of various nature about the samples: the presence of MT-molecular weight range, metal complexing, high sulphur-containing species was demonstrated. Although the two dimensional approach did not show significant signals, due to the extreme low concentration, the dramatic dilution, and the short time life of the species of interest, the amount of clues that testify the MT-nature of the detected species is a remarkable result, above all thank to the reliable method developed, able to afford reproducible and stable results.

The analysis of bovine liver extract was aimed to the sample preparation procedure optimisation, as well as the chromatographic separation. The signals detected were attributed to several isoforms of MT, even if no characterisation of the peaks was possible via ESI-MS, due to the low sensitivity of the system. Nevertheless, the system was stable and reliable, optimised for MT analysis, and was therefore employed for the investigation of thyroid samples.

The comparative analysis of healthy and cancer affected thyroid samples, performed on a wide pattern of elements, brought information on the metal distribution differences in healthy thyroids and those removed during the operation of the tumour tissue. Thus, no MT were detected by means of SEC-ICP-MS in the case of cancer affected thyroid samples, while a reproducible signal was detected in the case of healthy tissues. The conspicuous better resolution of RPC-ICP-MS let us achieve more information on the species and metal distribution in thyroid samples: thanks to this more detailed information, a definitive poorness of MT isoforms was stated in the thyroid samples, in

comparison to the liver, depending of course on the difference of the nature and the function of the two organs.

Heavy metals in plants

By means of the high concentrated Cd-PC induced in algae cultures and separated through size exclusion chromatography, an ion pair reversed phase chromatographic separation was optimised, in order to detect the formed Cd-PC complexes on-line via ICP-MS. The method was then applied to plant extracts, and the metal content of the samples investigated for the presence of M^{n+}-PC. The sample preparation procedure for species transformation and/or degradation was also tested employing ^{116}Cd spiked PC.

At the same time, the synthesised ^{116}Cd-PC supplied a new exciting approach for PC research: the possibility to prepare species-specific spiked compounds, for a deeper knowledge of the structure and for a detailed quantification, was investigated. The stability of these species, in order for them to be used to spike natural PC, was the most fundamental characteristic, and therefore the first studied.

For the first time, the high kinetic lability of the complexes was detailed documented, beside the thermodynamic one, and hence the impossibility to use the synthesised ^{116}Cd-PC as a spike: thank to the use of these particular species it was stated that, during the sample preparation and the chromatographic separation itself, the species undergo degradation, leading to loss of the analytes and formation of artefacts.

This approach explained then why almost no PC signal was achieved in the *S. vulgaris* samples, while several low-molecular, PC-derivate complexes like Cd-glutathion arose.

The consequences of the high kinetic instability of PC complexes, stated through these investigations, has the strong analytical consequence that a direct investigation on the metal complexes becomes extremely difficult: from the sample preparation, to the times of analysis, up to the detection of the low concentrated species.

The necessary improvement of the sample preparation procedure was thus carried on by means of an oat and wheat seed mixture, capable to produce a quite high amount of plant material in a relatively short time. The metal uptake and distribution of such a sample were monitored through SEC-ICP-MS, showing a regular uptake of the metals used in the nutritious solutions (Cu, Cd, Mn, Zn) and their regular mobilization, from the roots to the shoots. High amounts of sample were therefore digested and subjected to the two dimensional chromatography (SEC/RPC-ICP-MS) without undergoing the freeze

drying step. This brought to the detection of a PC_5 signal, in which sulphur and several metals correlated.

2. FUNDAMENTALS

2.1. Speciation analysis

In 2000 the unequivocal definition for chemical species and speciation analysis was published[2]:

i. *Chemical species*. Chemical elements: specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure

ii. *Speciation analysis*. Analytical chemistry: analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample

iii. *Speciation of an element; speciation*. Distribution of an element amongst defined chemical species in a system;

bringing order in a field that underwent a lively development in the last decades.

In many modern and challenging fields of interest, such as biological and environmental chemistry, medicine and biochemistry, toxicology or nutrition chemistry, characteristics like reactivity, functionality, mobility, bioavailability and toxicology of an element are extremely dependent on the species in which the element itself is present in the sample.

The oxidation state of the element, the complexes formed or the molecules to which it is bound are only a few examples by which the species could be described. The goal of speciation analysis is to describe, both qualitatively and quantitatively, the elemental distribution among the species present in the sample.

The role of speciation analysis has been achieving more and more importance in the last twenty years, dealing with the most diverse matrixes and focussing first on the branch of organometallic compounds and, in the last years, on biological ligands. DNA fragments, organic acids, proteins and small peptides have a fundamental role in complexing and binding both toxic and essential heavy metals in every cell. Nevertheless, many questions are still open, being the complexity of the samples and the weakness of such bonds the major obstacles in the study of such compounds[3].

2.2. Hyphenated techniques

Among the most suitable techniques for speciation analysis are the hyphenated techniques, based on a first separation step, in which the species are divided one from the other exploiting their characteristics, followed by a detection step, in which an element specific detector is employed. Depending on the analyte's characteristics and

nature, the separation can be performed via gas or liquid chromatography, gel or capillary electrophoresis. The state and form of the sample and the nature of the matrix are only few factors which condition the choice of the separation technique.

On the other hand, the ideal detector should be a versatile, selective and sensitive technique, possibly robust and with a high sample output, with a wide linear dynamic range, low limits of detection and multielemental capabilities. Inductively coupled plasma mass spectrometry (ICP-MS) has become in the last twenty years the dominant speciation detector, being able to fulfil most of these requests. ICP-MS alone brings no information on the species; nevertheless the hyphenation with a chromatographic or electrophoretic separation method is possible and convenient[3].

Nevertheless, the great disadvantage of a destructive detector like ICP-MS is the fact that it brings completely no information on the species' structure. In order to have a complete palette of information on the species of interest, and due to the lack of standards for most of the analytes, a soft ionisation method is in most cases required.

Among the others, electrospray ionisation has proven to be the most interesting technique: typical flow rates for (micro) chromatographic or electrophoretic separations are comparable to the flow rates used for the sample introduction in ESI-MS,becoming therefore suitable techniques for an on line coupling, an ESI-MS offers a good sensitivity and structure and molecular weight information are gained through the spectrum interpretation.

The detection limits achieved by means of this technique and the operative conditions required are regrettably not always satisfactory when applied to species present in low concentration and accompanied by a complex matrix. Alternative techniques can be a pneumatically assisted electrospray source, in which a gas assist nebulization and desolvation of liquids, or a completely different ionisation technique, such as MALDI-TOF (Matrix assisted laser desorption ionisation –time of flight MS)[3].

A schematic summary of the most used hyphenated technique for elemental speciation of biological samples is presented in Figure 1:

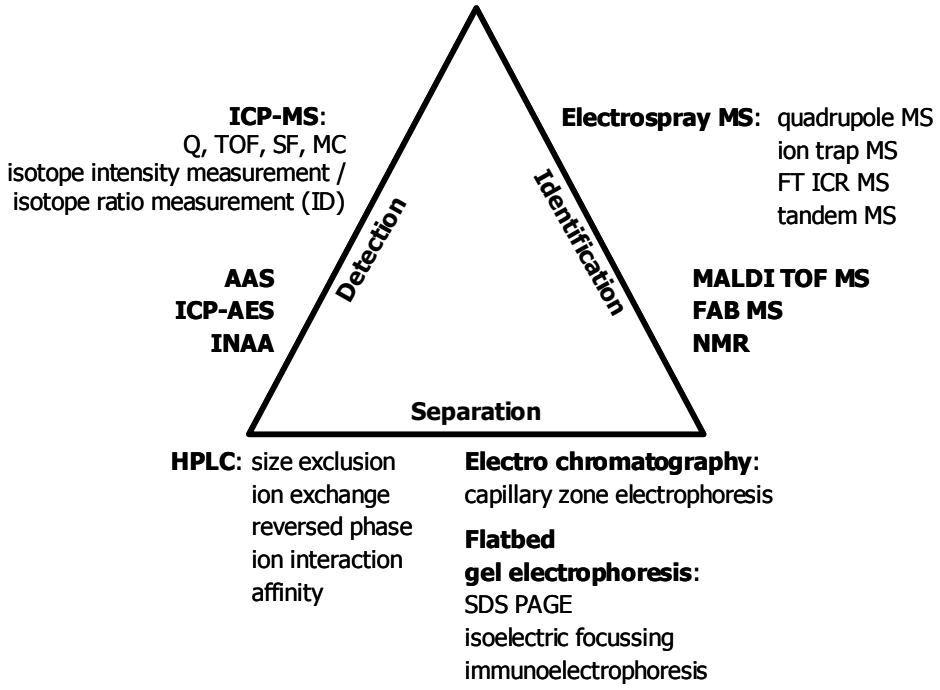

Figure 1: hyphenated techniques available for species-selective analysis of biological material[4,5]

2.3. HPLC

The separation principle on which LC is based is the different interactions of the species injected in the column with the continuously pumped mobile phase and the filling of the column itself, the stationary phase. As a result of their different nature, the species would interact more or less strongly with the two of them, being eluted with different retention times. In the case of HPLC the stationary phase particles have small diameters, of the order of magnitude of 3-5 μm, and being packed they produce a high back-pressure on the mobile phase, as it is pumped through the column.

HPLC is one of the most exploited and widespread separation methods in analytical chemistry and in speciation analysis, due to its capability of separating non-volatile compounds and its broad versatility: both the mobile and the stationary phases may be modified in order to achieve the desired separation; in addition, many different stationary phases are commercially available. Among the mobile phases, the most common are buffer solutions, which allow a strict pH control, mixed with organic solvents like methanol or acetonitrile, or the pure solvents. The solutions have always to be degassed before being employed, to avoid formation of air bubbles in the system.

In addition to the nature of the mobile and the stationary phase and the kind of elution adopted (either isocratic or with a gradient), the most significant parameters that

affect a chromatographic separation, and have to be considered for the system's optimisation, are the pH, the temperature and the flow rate.

Interfacing HPLC with element-specific detectors, like ICP-MS, ICP-OES and FAAS, is quite easy, being the sample introduction flow rate of such detectors comparable to those employed for the elution (0.5 – 2.0 $mL \cdot min^{-1}$). Interfacing the HPLC system to these detectors is easily achieved connecting the end of the chromatographic column with the sample nebulizer using tubing of the inert polymer PEEK.

2.3.1. Size exclusion chromatography (SEC)

The decisive separation parameter in SEC is the size of the molecules, and consequently their molecular weight. Therefore this kind of chromatography is broadly employed in separating macromolecules such as proteins and polymers, whose molecular weight is higher than 2000 Da. In the ideal case there is no chemical interaction between the analyte and the stationary phase, and the separation occurs only depending on the ability of the species to enter its pores. Small molecules spend more time in the column because they can penetrate more deeply the pores; on the contrary, large molecules cannot and are eluted first.

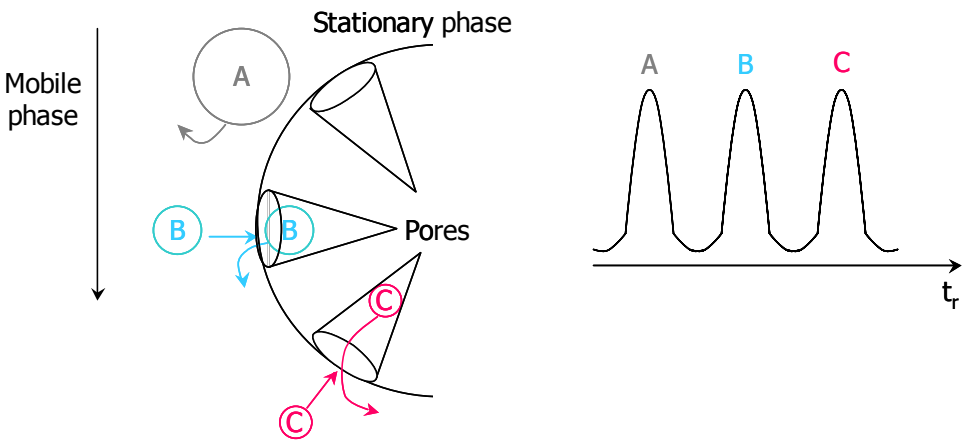

Figure 2: separation mechanism of SEC[6]

Due to the existence of a direct proportionality between the molecular weight of the species and their retention times, a calibration of the SEC column is possible. As standard, molecules similar to the analyte are normally used.

The mobile phase plays a modest role, it is usually selected depending on the solubility characteristics of the species of interest. Due to its simplicity and broad adaptability, SEC is often used as a first step in a two dimensional separation.

2.3.2. Reversed phase chromatography (RPC)

RPC is one of the most widely used chromatographic separation techniques in analytical chemistry. The stationary phase is strongly non polar, characterised by siloxanes derivatised with *n*-butyl, *n*-octyl or *n*-octadecyl (abbreviated as C_4, C_8 and C_{18}) chains, which are perpendicular bound to the inner column surface and parallel one to the other, generating a brush structure. The mobile phases employed in RPC are polar solvents, and the repartition mechanism is therefore based on the polarity difference between the two phases: the first eluted component will be the most polar; increasing the polarity of the mobile phase, the retention times will be increased. Typical mobile phases are water, methanol, acetonitrile, or mixtures of them. The separation mechanism can be considered like a liquid-liquid repartition equilibrium, in which the more polar liquid is the mobile phase, and the less polar one is the hydrocarbon brush, which acts as a non polar fluid[7].

The separation may be optimised in response to the ionic strength, the pH or the polarity of the water-organic solvent constituting the mobile phase. For efficient separations, gradient elutions are often employed, through which the polarity of the mobile phase is increased in time by a gradual higher addiction of methanol or acetonitrile.

2.3.3. Ion pair chromatography (IPC)

IP is able to separate simultaneously mixtures of anions and cations. It is a subgroup of RPC in the fact that the employed stationary phases are the same, while the mobile phase is normally an aqueous solution of an organic solvent like methanol or acetonitrile containing an *ion pairing reagent.* These compounds are ionic species with a cation or anion having a polar head group and a non polar tail, like tetraalkyl or triethylalkyl ammonium salts, or sodium alkyl sulfonates. The ion pairing reagent has a charge opposite to that of the analyte, and combines with it to form an *ion pair*, a neutral species that interacts with the non-polar stationary phase[7].

The separation mechanism is the combination of two effects. The first, that the ion pairing reagent forms a neutral 'ion pair' together with the charged analyte, leading to an increased interaction with the non polar stationary phase; the second, that the

hydrophobic tail of the ion pairing reagent adsorbs to the stationary phase, resulting in a pseudo ion exchange stationary phase.

In Figure 3 is schematically represented the IPC separation mechanism: the analyte, the anion A^-, has no interaction with the mobile phase (Figure 3, part *a*). If the ion pairing reagent tetrabutylammonium hydroxide (TBAH) is added to the mobile phase, the positively charged tetrabutylammonium ion interacts with A^-, forming the neutral ion pair (Figure 3, part *b*). The ion pair can therefore interact with the C_{18} stationary phase and A^- is separated from the other components (Figure 3, part *c*).

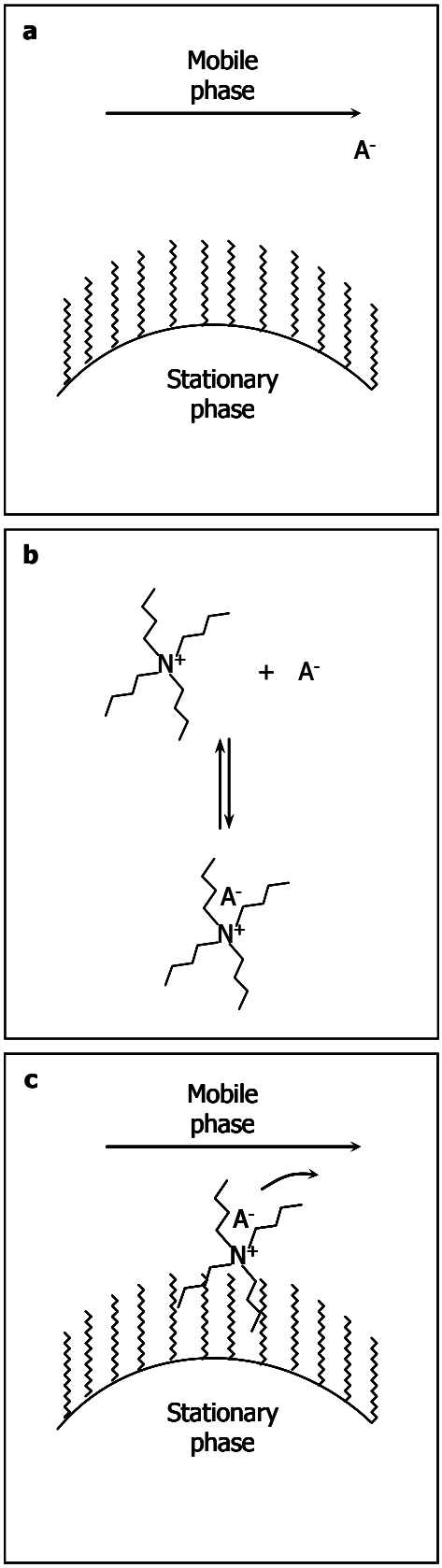

Figure 3: separation mechanism of IPC

2.4. ICP-MS

Coupled to a separation method that is able to characterise the species before they enter the plasma, ICP-MS proves to be one of the most powerful and versatile element-specific detectors employed in (ultra)trace speciation analysis, thanks to its extremely low LODs, the wide linear dynamic range, the high sample throughput and its multielemental capabilities.

ICP has been used for a long time in analytical chemistry as atom source for the optical spectroscopy, and has been coupled to a mass spectrometer for trace elements determination first in 1980[8]. In its 'classical version' ICP-MS is used for the analysis of (aqueous) solutions. These are converted in an aerosol by means of a nebulizer, and only the finest part of it passes through the spray chamber, reaching the plasma (see Figure 4).

The quartz torch in which the plasma is generated and maintained is composed of three concentric tubes; the most external one delivers the *cooling gas* (ca. 15 $L \cdot min^{-1}$), the majority of the Ar alimenting the plasma; through the middle tube flows the *auxiliary plasma gas* (0.7 – 1.3 $L \cdot min^{-1}$), that forms the internal part of the plasma and finally, through the most internal tube, the sample is delivered throughout a third stream of Ar, the nebulizer gas (0.6 – 1.8 $L \cdot min^{-1}$). The plasma is generated at the end of the quartz torch via a Tesla spark and maintained by use of a high-frequency (27.12 or 40.68 MHz) electromagnetic field. The power of 600 – 2000 W is provided through an alternate generator coupled to the load coil.

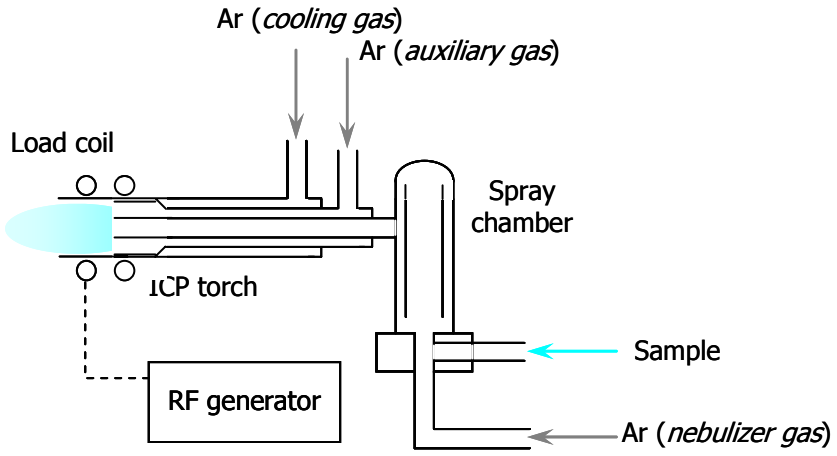

Figure 4: scheme of the sample introduction and ionisation system

Temperatures in the plasma are between 8000 and 10000 K. In this environment, the sample is desolvated, atomised and finally positively ionised[9]. Nevertheless, the largest

part of the sample is lost during its conversion to aerosol, being common cross-flow nebulizer efficiency in the best case 1%[10].

The ions are sampled through the orifices of two water cooled cones (see Figure 5), the *sampler cone* and the *skimmer cone*, constituting the interface between the plasma, at atmospheric pressure, and the low pressure chamber ($10^{-5} \div 10^{-7}$ mm Hg) where the mass analysis takes place. The majority of the gas is subsequently evacuated by means of a mechanical pump, while a thin beam of ions enters the higher vacuum region.

Here the positive ions are collimated by means of the *ion optics*, negatively charged electrodes, in the mass separator (a quadrupole in Figure 5). Depending on the kind of instrument used, it can be a quadrupole, a sector field, a time of flight, an ion trap or a cyclotron.

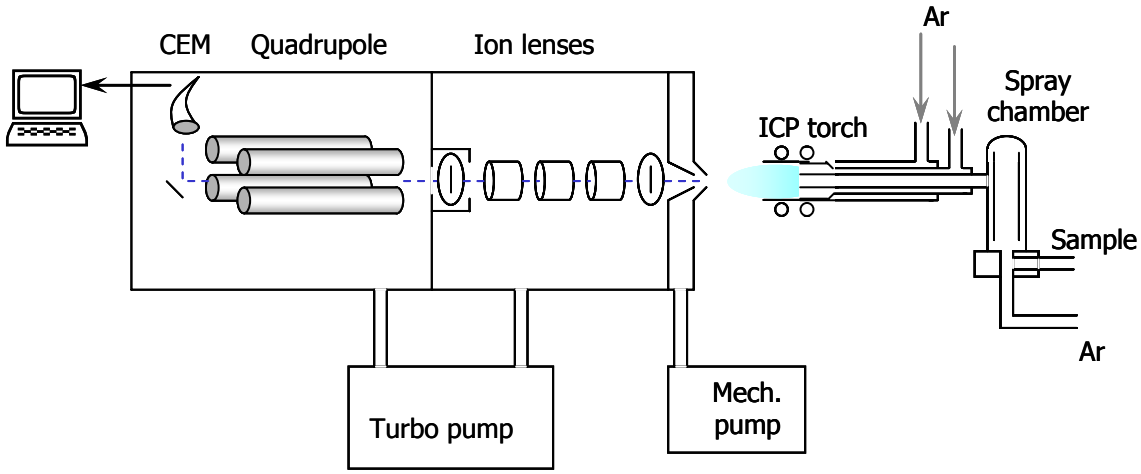

Figure 5: schematic diagram of a quadrupole-based ICP-MS instrument

Finally, the ions have to be detected. This happens commonly with an electron multiplier (CEM), an extremely sensitive detector that allows each individual ion to be counted, while photons' signal is excluded for instance by mounting the detector (or the entire mass analyser) off axis. The ions signals are the converted to electrical ones and elaborated through adequate software.

2.4.1. Quadrupole ICP-MS (Q-ICP-MS)

A quadrupole is composed of four parallel metallic rods, the two opposites electrically bound. To each pair is applied a potential $U(t)$, which results from the composition of a constant potential U_0 and an alternate potential $V(t)$:

eq. 1 $$U(t) = U_0 + V_0 \cos \omega t$$

The regulation of the potentials applied produces in the space between them an electric field that lets through only those ions that have a determined mass to charge ratio. Every other ion has an unstable trajectory and is thus neutralised at the quadrupole rods, not reaching the detector. It works therefore like a band pass mass filter, through which only the restricted m/z window is transmitted.

When the rods have a r_0 distance from the centre, the produced potential Φ can be calculated depending from space (x, y) and time (t):

eq. 2 $$\Phi(x, y, t) = \frac{U(t)}{2r_0^2}(x^2 + y^2)$$

The ion of mass m and charge q follows therefore the trajectory described by eq. 3:

eq. 3 $$\ddot{r} = \frac{q}{mr_0^2}[U_0 + V_0 \cos(\omega t)]r$$

For simplicity the variables a, b and τ are introduced:

eq. 4 $$a = -\frac{8qU}{mr_0^2\omega^2} \qquad b = \frac{4qV}{mr_0^2\omega^2} \qquad \tau = \frac{\omega t}{2}$$

that lead to the differential Mathieu equation:

eq. 5 $$\ddot{r} = [2b\cos(2\tau) - a]r$$

Definite values of a and b lead to solutions (compare Figure 6) of the Mathieu equation which represent stable ion trajectories: if the stability in both x and y dimensions are simultaneously achieved, then the ion reaches the detector[11].

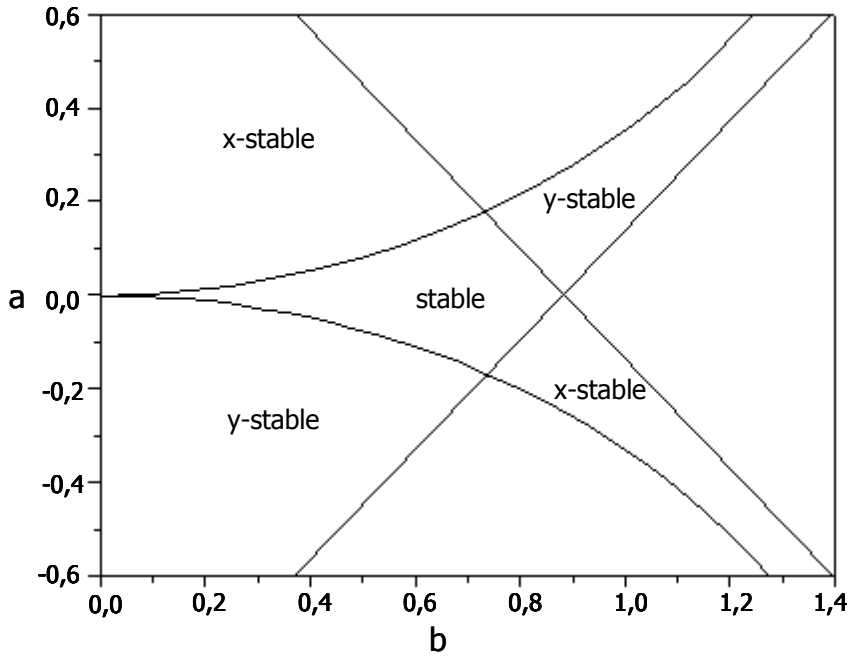

Figure 6: stability diagram of ion trajectories following the Mathieu equation[11]

By changing the applied potentials U and V (and a and b as a consequence) ions with different m/z ratios ($z = q/e_0$) reach the detector: the possibility of changing quite rapidly U and V leads to a quick *scan* of the entire mass spectrum, or a narrow portion of it, or to monitor the intensity of a limited number of pre-selected analyte signals (*peak jumping* or *hopping*).

Interferences

Non spectral interferences are generally caused by the matrix components, leading to enhancement or suppression of the signal. They can be easily reduced by dilution, or compensated by the use of an appropriate internal standard. The most elegant solution is nevertheless represented by the use of the standard addiction or, when possible, of the isotope dilution methods.

Spectral interferences are on the other side more troublesome: they are caused by the overlapping of the selected isotope signal with those of ions showing a difference in mass < 0.5 u. Isobaric interferences take place because ICP ionisation is not element selective, while in addition a large number of molecular ions originated by the combination of matrix elements, the solvent, air and Ar can still survive and get over the mass filter. The problem becomes quite complicated especially in the case of light masses and complex matrixes: examples of spectral interferences are shown in Table 2[3].

Table 2: examples of spectral interferences potentially encountered using Q-ICP-MS for some of the isotopes measured within this work[12]

Isotope	Isotopic abundance (%)	Molecular ions potentially causing spectral interferences
^{32}S	95.02	$^{16}O_2^+$
^{52}Cr	83.79	$^{40}Ar^{12}C^+$, $^{35}Cl^{16}OH^+$
^{53}Cr	9.50	$^{37}Cl^{16}O^+$
^{55}Mn	100	$^{39}K^{16}O^+$, $^{40}Ar^{14}NH^+$
^{58}Ni	68.27	$^{23}Na^{35}Cl^+$
^{60}Ni	26.10	$^{23}Na^{37}Cl^+$
^{63}Cu	69.17	$^{40}Ar^{23}Na^+$, $^{31}P^{16}O_2^+$
^{64}Zn	48.60	$^{32}S_2^+$, $^{40}Ar^{24}Mg^+$, $^{32}S^{16}O_2^+$
^{66}Zn	27.90	$^{40}Ar^{26}Mg^+$

In order to avoid interferences with a Q-ICP-MS, an isotope with low abundance can be selected, although it leads to a decrease in sensitivity.

Whenever possible, care should be taken in sample preparation in order to separate the interfering species before the measurement takes place.

If the interfering species is exactly known and produces a signal which does not exceed that of the analyte by a too great amount, a mathematical correction can be used successfully, even if at the cost of rather complex calculations and the use of delicate computer programs.

2.4.2. ICP Sector Field MS (ICP-SF-MS)

Compared to a quadrupole instrument, a sector field offers the advantages of an average higher sensitivity, a lower instrument background and, above all, a sufficient mass resolution to separate many different ions which cannot be distinguished by means of Q-ICP-MS.

The ICP-SF-MS works on the principle of the double focussing, using both an electrostatic field and a magnetic field to separate the ions.

Ions of mass m and charge q flying through a magnetic field B with a speed v are subjected to both the centrifugal and the centripetal forces. When they are equivalent, it is possible to define the ion trajectory's radius r_B as follows:

eq. 6 $$r_B = \frac{m \cdot v}{q \cdot B}$$

In the electrostatic field, on the other side, the potential U would accelerate the ions granting them a kinetic energy $E_k = U \cdot q$. In the ideal case the ions having the same m/q ratio have also the same velocity: substituting it in eq. 6 we obtain that:

eq. 7 $$r_B = \sqrt{\frac{m}{q}\frac{2U}{B^2}}$$

The electrostatic field is subsequently employed due to its capability to separate the ions in dependence on their energy and not on their mass:

eq. 8 $$r_E = \frac{2}{q \cdot E_{el.field}} \cdot E_k$$

As a consequence of the double focussing the ions are separated on the basis of their m/z ratio and the energy dispersion is simultaneously corrected.

The instrument employed for this work, a *Thermo Finnigan Element2,* is built with the inverse Nier-Johnson geometry, as shown in Figure 7: after the ion optics, the ion beam is collimated and enters the magnetic field, while the electrostatic field is the second separator. They pass through an adjustable slit and finally reach the detector.

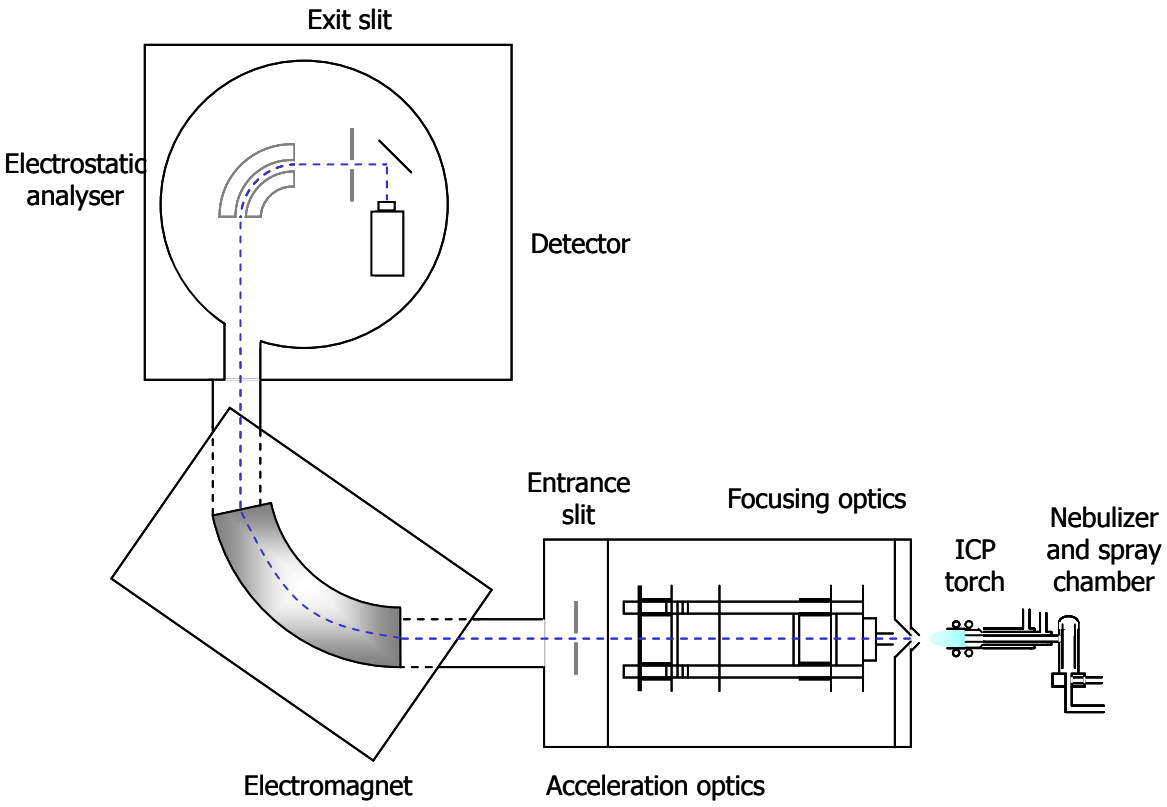

Figure 7: schematic diagram of a sector field-based ICP-MS *Element2* instrument

The first application of an ICP-SF-MS for speciation purposes was that of Rottmann and Heumann[13], for the interference-free determination of Fe after SEC separation.

Interferences

The most satisfactory way to overcome spectral interferences is to use an ICP-SF-MS. With its high resolution, it allows to resolve signals generated by the analyte and those due to the molecular ions that have the same nominal mass, but are actually differing from it for mass unit fractions. The only drawback is that to an increasing mass resolution corresponds a loss in the ion transmission efficiency, leading to a lower sensitivity.

The resolution R is defined as $m/\Delta m$, the m/z value divided by the separable mass difference and is mainly affected by the slit and the ion beam breadth and the kinetic energy spread of the ion beam. It has been shown[14] that for a double focussing instrument R is inversely proportional to the slit width as follows:

eq. 9 $$R \propto ar_e / S$$

where S is the width of the entrance and the exit slit, r_e is the electrostatic analyser radius and a is a proportionality constant approximately 1 - 2. Thus, an instrument of r_e = 10 cm with a slit of 10 μm has a resolution in the order of 10000.

The peak shape is also affected by the dimension of the slits: when the slit is larger than the ion beam, the produced peak has the best precision, being the signal at the top of the peak less affected by small variations in accelerating voltage or magnetic field strenght[3].

2.5. ESI-MS

Even though ICP-MS is unbeatable concerning sensitivity and low detection limits, it brings no information at all on the nature of the species observed in the case of lack of standards to identify the chromatographic peaks. To understand the identity of such molecules a soft ionisation mass spectrometry is needed and in this respect electrospray sources have demonstrated to be the easiest, most widely applicable and exploited in speciation analysis.

ESI-MS has proved to be a suitable ionisation source for metal containing species, and by tandem mass spectrometry a precise determination of molecular weight and structural characterisation of molecule at trace levels are possible.

Being the normally employed sample introduction rates of the same order of magnitude as (micro) HPLC or CE flow rates, the hyphenation of ESI-MS results facile and provides a successful on line analysis.

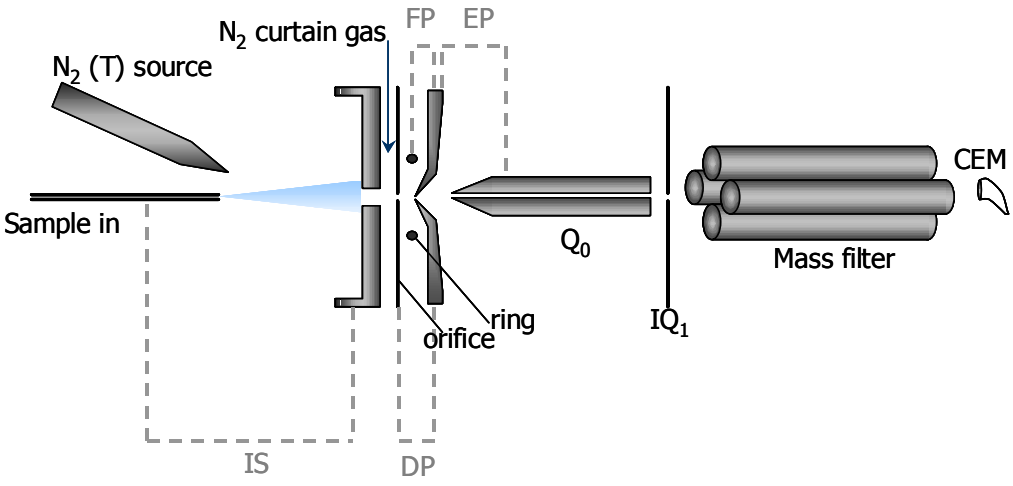

Figure 8: schematic diagram of an electrospray source quadrupole based MS (PE Sciex API 150EX)

The sample solution is delivered through a fused silica capillary held inside a metallic needle, between which and the interface a potential difference (the *ion spray potential*, IS, typically 3-5 kV) is applied. The ideal flow range for the sample, that can be introduced whether by means of a syringe pump or an HPLC system, is between 1 and 100 $\mu L \cdot min^{-1}$. Typical ES solutions are made up of a polar solvent and dissolved electrolytes (10^{-5} - 10^{-3} $mol \cdot L^{-1}$). Applying an adequate IS between the capillary tip and the counter electrode, positive ions would move to the liquid surface and, overcoming the surface tension, form the so called *Taylor-Cone* (see Figure 9). As a consequence the spray would be finally formed.

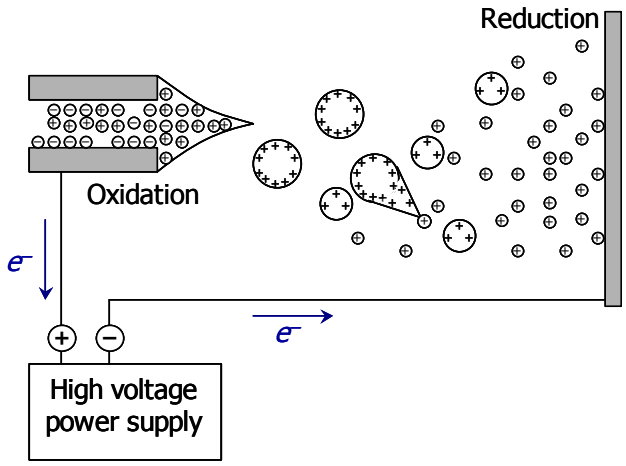

Figure 9: the Taylor cone at the end of the capillary and the formation of the electrospray[15]

A *negative ion mode* can as well be operated, producing and detecting negatives ions. The principles applied are the same, the potentials are inverted. Within this work only the positive ion mode was employed, and is therefore described in detail.

Solvent evaporates and the droplets shrink until the electrostatic repulsion overcomes the surface tension, that results in droplet explosion, the so called Coulomb explosion. The process repeats until gaseous ions are formed. Through the orifice and the following decreasing (in the positive ion mode) potential elements (the *declustering potential* DP 50 - 100 V influences the fragmentation of molecular ions; the *focusing potential* FP 150 – 300 V, and the *entrance potential* EP 10 – 50 V serves focussing the ions inside the skimmer) the ions are sampled through the skimmer cone, then guided in the high vacuum compartment and collimated in the ion optics. The adequate m/z is then selected and finally the ions are detected.

The fission droplets have circa 2% m and 15% of charge of their parents: this process results therefore in multi charged particles that can be easily detected with

regular mass detectors (up to 3000 Da). For this reason ESI-MS is a very adequate technique for small and large protein analysis.

In Figure 8 is shown an ionisation process improved by the use of a hot (T≈200°C) stream of nitrogen, which helps desolvating the droplets: this device leads to the possibility to introduce higher flow rates, up to 200 $\mu L \cdot min^{-1}$, without flow splitting, and is therefore of advantage for HPLC coupling and improving the detection limit.

2.5.1. Ion trap and tandem MS

In the case of an ESI source coupled to another mass analyser, like an ion trap, is not only possible to have better detection limits in comparison to a linear quadrupole, but also to perform tandem MS (MS^n, where n=2-10). This operating mode of an ion trap gives the possibility to isolate a single ion, depending on its m/z ratio, to crash it into daughter ions and to analyse these fragments, leading to a great amount of structural information on the species of interest.

The scheme of an ion trap based ESI-MS is exactly the same as that shown in Figure 8 for a quadrupole based instrument, with the exception that an ion trap mass analyser is present instead of a quadrupole mass filter.

An ion trap mass analyser is the site where ion storage, ion isolation, collision induced dissociation and ion scan out happen. As shown in Figure 10, it is composed of three main electrodes: an entrance *endcap electrode*, the one closest to the ion optics, an *exit endcap electrode*, the one closest to the ion detection system, and the ring electrode, located between the two. The inner surfaces of the electrodes are hyperbolic, in order to form a cavity in which the mass analysis occurs.

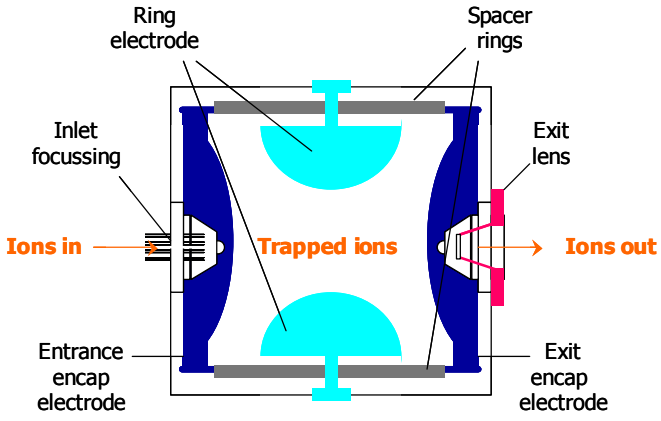

Figure 10: schematic diagram (cross view) of an ion trap mass analyser

Ions produced in the ESI source enter the mass analyser cavity through the entrance endcap electrode; they can be later ejected through either endcap electrodes during the analysis: ions that are ejected through the exit endcap electrode are focused by the conversion dynode accelerating potential through the *exit lens* (at ground potential) towards the ion detection system.

The three electrodes are separated by two quartz *spacer rings,* that position the electrodes at the proper distance and also serve as electrical insulators. Through a nipple on the exit endcap electrode He (damping gas) enters the mass analyser cavity.

An ESI-ion trap mass analyser can be operated either in the positive or in the negative ion mode, depending on the species of interest. In this work only the positive ion mode has been used, therefore this will be described in detail. The negative ion mode, on the other side, is operated exactly in the same way with inverted potentials.

A dc offset voltage is applied to the mass analyser to draw in ions from the ion optics, the magnitude of this voltage is –10 V for positive ion polarity mode. Several other ac voltages are applied to the ring and endcap electrodes to trap, fragment and eject ions according to their mass to charge ratios. One of them is an ac voltage of constant frequency (0.76 MHz) and variable amplitude (0 – 8500 V zero-to-peak) is called the *ring electrode RF voltage*, being an ac potential in the radio frequency (RF) range applied to the ring electrode. It produces a varying three-dimensional quadrupole field within the mass analyser cavity (see Figure 11).

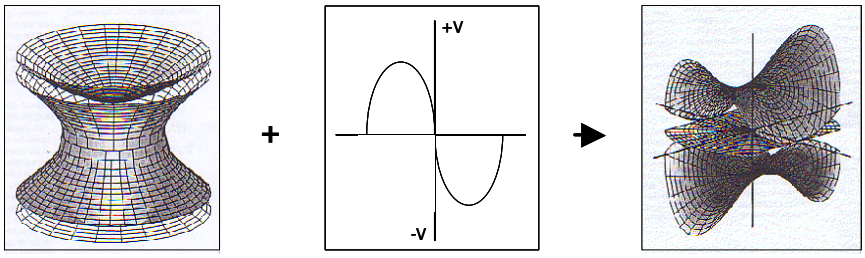

Figure 11: scheme of the time dependent potential generated in the ion trap by the superposition of the applied ac and dc voltages[16]

This time-varying field drives ionic motion in both the axial (toward the endcap) and radial (from the ring electrode toward the centre) directions. Ionic motion must be stable in both directions for an ion to remain trapped, that means, its trajectory must be confined inside the mass analyser. During ion scan out, the system produces a mass dependent instability to eject ions from the mass analyser in the axial direction.

When the amplitude of the ring electrode RF is low, all ions above a minimum m/z ratio are trapped: this RF voltage is referred to as the storage voltage, and the minimum mass-to-charge ratio is usually chosen to be greater than that associated with air, water and solvent ions.

During ion scan out, the ring electrode RF voltage is ramped at a constant rate corresponding to approximately 5500 u·s^{-1}. As this voltage increases, ions of increasing m/z ratio become successively unstable in the axial direction and are ejected from the mass analyser. The voltage at which an ion is ejected is defined as its *resonance voltage*. The ejection of ions of each m/z ratio occurs over a very short time.

Other ac voltages (i.e. the *ion injection waveform voltage,* the *ion isolation waveform voltage,* the *resonance excitation RF voltage* and the *resonance ejection RF voltage*) are applied to the endcap electrodes to stimulate motion of the ions in the axial direction: they are equal in amplitude, but 180° out of phase to one another. When the RF frequency applied to the endcaps equals the resonance frequency of a trapped ion, which depends on its mass, the ion gains kinetic energy up to the point that, when the magnitude of the applied voltage is large enough, the ion is ejected from the mass analyser in the axial direction.

The waveform voltages are a distribution of frequencies between 10 and 380 kHz containing all resonance frequencies except those corresponding to the ions to be trapped, allowing the concentration of target ions and the ejection of all ions except those of a selected m/z ratio or a narrow range of m/z ratio.

The resonance voltages are used to run the ion trap in the MS^n modus: the resonance excitation RF voltage is not strong enough to eject an ion from the mass analyser, however, ion motion in the axial direction is enhanced and the ion gains kinetic energy: after many collisions with the damping gas present in the mass analyser, the ion has gained enough internal energy to cause it to dissociate into daughter ions, that are subsequently mass analysed. During the ion scan out, the resonance ejection RF voltage facilitates the ejection of ions from the mass analyser, improving thus mass resolution.

The He contained in the mass analyser cavity is used as a damping gas and a collision partner. It enters through a nipple on the exit endcap electrode, with a flow rate of 1 mL·min^{-1} regulated by a pressure regulator and a capillary restrictor. The flows into and out of the cavity are matched so that the partial pressure of He in the mass analyser cavity is maintained at approximately 0.1 Pa.

The collisions of the ions entering the ion trap with He slow the ions so that they can be trapped by the RF field in the mass analyser. Furthermore, the presence of He significantly enhances sensitivity and mass spectra resolution: through collisions the

kinetic energy of the target ions is reduced, damping thereby the amplitude of their oscillations and focussing into the centre of the cavity rather then being allowed to spread throughout it. As a collision activator partner, He causes the parent ions to dissociate, as described above[17].

2.5.2. HPLC-ESI-MS coupling

As already observed, the interface of ES is quite suitable for a coupling to HPLC. Nevertheless, not every flow, nor any mobile phase is appropriate for such application. In the literature, the most fitting, and the most described kind of chromatography coupled to ESI-MS is the reversed phase[18,19]. It has been also experimentally proved that the best sensitivity is achieved by means of low flows and small inner column diameters[20].

It must be considered what is the effect of the mobile phase on the ionisation efficiency of the sample, and, in the case of an elution gradient, what are its consequences on the sensitivity and the background signals. The fact that a typical direct ES analysis is carried out in 50 - 50 water – acetonitrile, or that 5 – 10% of methanol or acetonitrile already increases the stability and the efficiency of the nebulization process and the desolvation step, explain the reasons behind this choice. Reversed phase typical mobile phases happen to be exactly what an ES needs to produce a stable spray.

On the other hand, some chemicals often used in RPC are not proper for ESI-MS: for example, buffers widely employed like phosphate or Tris(hydroxymethyl)-aminomethane (Tris) have to be avoided, because already at the trace level they interfere with the ES process. Only volatile buffers like ammonium acetate or ammonium formate can be used; while to adjust the pH of the mobile phase almost only acetic, trifluoroacetic and formic acids are suitable. High concentration of Na^+ or K^+ and detergents cannot be employed: for its nature, the detergent concentrate on the surface of a liquid and in the ionisation process this phenomenon happens in the aerosol droplets, suppressing thus other ions[3].

3. METALLOPROTEINS

Metalloproteins are all those proteins which form complexes with metals that are thermodynamically stable in a given chemical environment and kinetically stable on the time scale of the average analytical procedure[4].

The major group of interest for modern research is composed by metalloenzymes, metal- transport proteins and metal-stress proteins. The first group comprehends those enzymes which are activated and stabilised in the cell by the presence of several trace elements, usually transition metals with small atomic radius (e.g. Cu, Fe, Zn, Mn, Ni…)[4]. To the metal-transport proteins group belong such proteins like albumin or transferrin, cysteine-rich proteins which assure the transport of heavy and essential metals in the organism. Recently intracellular metal trafficking proteins (metallochaperones) have been identified in various organisms, responsible for the delivery of metal ions to specific organelles or target proteins[21,22,23]. The metal-stress proteins are induced in an organism by high concentrations of heavy metals, which provoke the synthesis of proteins able to complex the excess of it.

This work deals with the third group of metalloproteins, both in mammalian and plant samples. In the case of animal and human tissues, metallothioneins are investigated; in the case of plant and algae samples, phytochelatins are the main target.

3.1. Metallothioneins

Metallothioneins (MT) are the best known group of metal-stress proteins. They are non enzymatic, low-molecular mass (6 – 7 kDa) metal binding proteins, whose chain of circa 60 non aromatic amino acids is composed in a large number (up to 30%) of cysteine residuals. The occurrence of Cys-X-Cys tripeptide sequence is typical for these proteins. Further characteristics of MT are the absence of disulfide bonds and their resistance to thermocoagulation and acid precipitation[24,25].

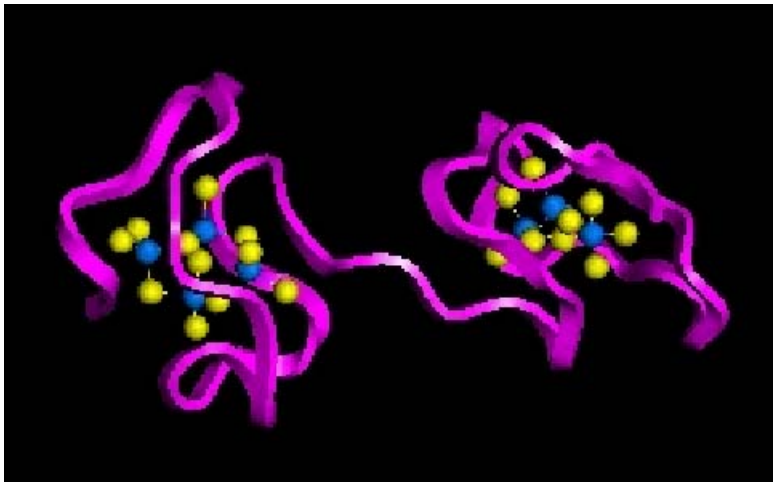

Figure 12: scheme of a metal-binding MT. The rose string is the amino acid chain; the blue spots represent metal ions; the yellow ones represent the –SH groups in cysteine residues.

Since MT were first described in the late 1950s[26], different properties have been investigated, such as structure, physico-chemical and chemical characteristics. From a physiological point of view, clarification of their biological roles in organisms remains a challenge[27]. Furthermore, quantification of MT is a field in which many studies have been carried on, which led to the development of a large number of methods: due to the lack and inadequacy of standards, hyphenated techniques turned out to be the most powerful and information rich method for their study.

3.1.1. Nomenclature

The nomenclature generally adopted for MT is based on the recommendation of the Nomenclature of Multiple Forms of Enzymes, IUPAC-IUB Commission on Biochemical Nomenclature (CBN)[28].

MT are divided into three classes: class I includes all proteinaceous MT with location of Cys closely related to those in the mammalian forms; class II comprehends on the contrary proteinaceous MT with lack of this property; class III subsumes metallopolyisopeptides containing gammaglutamyl-cysteinyl units resembling in their features proteinaceous MT[29]. MT are also classified depending on the organism in which they have been found, i.e. mammalian, mollusc, planta...

The continuously increasing amount of research and results on the topic has shown the necessity to classify MT on a less ambiguous way as the defined classes, i.e. on the basis of their decoded amino acid structure. Therefore MT belong to a *Superfamiliy*, which can be divided into *Families*: the here belonging MT are characterised by similar

primary sequences. Families can be then divided into *Subfamilies* and furthermore into *Subgroups*, where the proteins are distinguished on the basis of their origin and the differences are step by step smaller[30] (see Figure 13).

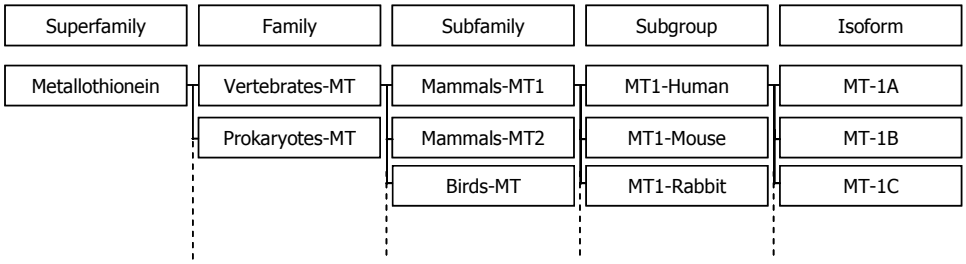

Figure 13: MT classification proposed by Kägi et al[30].

Differences in 2-15 amino acids other than cysteine residues are responsible for the presence of MT i*soforms*, developed during evolution of a species. They are identified through an Arabic number (e.g. MT-1, MT-2...) and are characterised by different isoelectric points and hydrophobicities[24]. Isoforms with minor differences, such as only one amino acid residue, were detected as subgroups and classified as *sub-isoforms*. Whereas sequences of the major isoforms have been decoded[31], the difficulties in the separation and identification of sub-isoforms are responsible for the lack of literature data regarding their identity[32].

3.1.2. Metal binding and structure of MT

Although naturally occurring MT are generally isolated containing Zn(II), Cd(II) and Cu(I), a number of metal ions will bind to the protein in vitro: among the others, Ag(I), Au(I), Bi(III), Co(II), Fe(II), Hg(II), Ni(II), Po and Pt(II)[27]. The metal affinity for the binding sites follows the general order for inorganic thiolates, i.e. Hg(II) » Ag(I) ~ Cu(I) » Cd(II) > Zn(II). Optical, magneto-optical and NMR studies[25, 33] gave the details regarding metal-cluster formation: the metal ions bound to MT are organised in one or two metal-thiolate cluster(s), with –SH groups acting both as a terminal and bridging[27].

In the most studied mammalian MT-1 and MT-2 isoforms the 20 cysteine residues are involved in binding seven divalent metal ions or up to twelve monovalent ions.

Several studies have been carried out for the determination of 3D structures. In the case of mammalian, MT-2 were determined in solution by NMR spectroscopy, investigating the $^{113}Cd_7$-MT-2 form isolated from rabbit, rat and human liver[34,35,36]. An analogue study was performed for recombinant mouse $^{113}Cd_7$-MT-1[37], while native

Zn_2,Cd_5-MT-2 was isolated from cadmium overloaded rat liver, and was successfully used in X-ray structure determination[38].

All the determined structures are similar and reveal a dumbbell-shaped protein with seven metal ions, which are located in two separated metal-thiolate clusters (see Figure 12 and Figure 14). In both clusters the metal ions are tetrahedral co-ordinated by bridging and terminal thiolate ligands.

The seven ions are divided in a three-metal cluster and a four-metal cluster, respectively. The three-metal cluster ($Me_3^{II}S_9$) is located in the N-terminal β-domain (see again Figure 14), in which the residues have been arbitrarily numbered from 1 to 30; while the four-metal cluster ($Me_4^{II}S_{11}$) remains in the C-terminal α-domain, whose residues are numbered from 31 to 61[27].

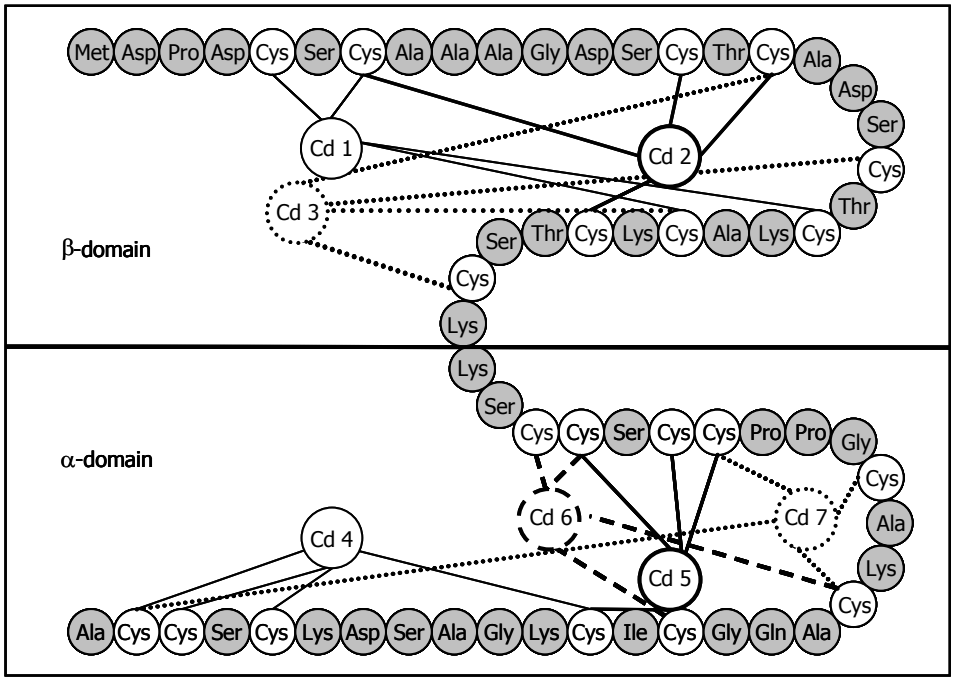

Figure 14: 2-dimensional scheme of a Cd_7-MT-2a, rabbit liver[25]

The other liquid phase NMR structures available are those of crustacean $^{113}Cd_6$-MT[39] and equinodermal $^{113}Cd_7$-MT[40]: both structures reveal a monomeric protein composed of two globular domains, each of them containing a three-metal cluster ($Me_3^{II}S_9$) in crustacean, while a four- and three-metal clusters are present in equinodermal MT. The cluster structure is closely similar to those found in mammalian MT, even if their location along the amino acid chain is inverted, due to differences in the sequence.

The structure of Cu(I)-MT from yeast has been also determined via NMR spectroscopy, showing a single $Cu_7(Cys)_8$ cluster[41,42].

Information on kinetics of MT reactions[43] and the evolution of the various MT species arising from the sequential addition of metal ions, such as Cu(II) and Zn(II), have been obtained through polarographic measurements[27]. Differential pulse polarography (DPP) shows the presence of different signals, that can be assigned to the free metal ions or those associated to MT, forming complexes with one ore more metals at the same time. A similar behaviour has been observed for individual apo-α and apo-β MT domains in the presence of Cd(II) and/or Zn(II)[44,45].

These results have been later confirmed by mass spectrometry, both for purified MT[46,47] and synthesised apo-α and apo-β domains. It provides, moreover, additional information on the stoichiometry presented by the different complexes, including pure and mixed complexes such as $Cd_4\beta$ or $Cd_2Zn_2\alpha$, respectively[48].

3.1.3. Biological role of MT

The main biological roles of MT are to regulate some essential metals (Cu and Zn) homeostasis and to protect the organism and tissues from toxic metal ions (Cd, Hg). It is well known that the exposure of an organism to toxic metals induces MT expression in different tissues. It is also being investigated if different MT isoforms are playing different roles in metals detoxification[49]: in terrestrial gastropod tissues it has been found that the exposure to different factors has as a consequence the induction of two different MT isoforms[50].

Examples of the varied functions of MT in the organism are the cell protection against oxidative damage caused by free radicals, pharmacological agents and mutagens, neuroprotection from ionising radiation, the modulation of cell apoptosis, the MT-3 function in regulation of neuronal outgrowth[27].

Among the others, the toxicity of Cd and the response of MT has been very intensively investigated: here MT are a decisive factor against hepatotoxicity, nephrotoxicity, hematotoxicity, immunotoxicity and bone damage[51,52].

Occurrence

Even if MT have been detected not only in animals, but also in several prokaryotes, eukaryotes microorganisms[53], and genes responsible for MT expression have been found in plants[54], the most explored field for MT is with no doubt that of vertebrates.

In mammals the MT isoforms of the same subfamily have been found in different parts of the organism: MT-1 and MT-2 are spread over all the internal organs[55] (liver, kidneys, spleen) due to their detoxifying aim; on the other side, MT-3 (Growth Inhibitory

factor) is present overall in the brain[56]. MT-3 have been also found in thyroid, in relation to studies on tumour affected tissues[57].

3.1.4. MT speciation analysis

Different methods and techniques have been developed and applied to identification, characterisation and quantification of metallothioneins, allowing the detection of total MT on the basis of several properties of the molecules. Electroanalytical techniques are based on the redox properties of the thiol-metal complexes, but give no information on the initial metal content and may suffer from interferences by other redox systems present in the matrix.

Metal affinity or saturation assays exploit the saturation capability of the molecule with metal ions, by displacing the initially MT-bound metals, generally Cu or Zn, with others of higher S-affinity, like Ag or Hg, for indirect MT determination. The initial supposed stoichiometry could nevertheless lead to false conclusions.

Immunoreactivity with specific antibodies has been successfully used for immunological methods, like RIA (radioimmunoassay) and ELISA (enzyme-linked immunosorbent assay); nevertheless these techniques bring no information on the initial metal content and present difficulties in raising high titres of antibodies.

The colorimetric properties of specific reagents in combination with mercaptanes are the basis for UV-Vis spectrophotometric determinations, even if the relatively poor sensitivity and specificity of the method does not allow proper MT quantification[27,58].

All the method cited, nevertheless, show absolutely no possibility to distinguish between the different MT isoforms or sub-isoforms, leading to a great lack of information. In order to better understand the structure and moreover the specific function of MT in organisms, the analytical procedures applied must include selectivity, not only with regard to MT isoforms and sub-isoforms, but also to the metals, and sensitivity, able to cope with non-induced MT levels in "real-world" samples[58].

Hyphenated techniques offer the solution to the problem, providing a high performance separation through HPLC and CE, information on the molecular structure via ESI or other soft sources –MS, information on the metal content and distribution and challenging LODs via ICP-MS, as schematic illustrated in Figure 1 for biological material in general. Among these, some of the most relevant for MT investigation are listed in Table 3, Table 4 and Table 5:

Table 3: hyphenated techniques for MT speciation based on a soft ionisation MS detector[59]

Sample	Separation	Eluents	Detection
Sheep liver	RPC-C_{18}	up to 30% ACN, 0.1% TFA	FI-ES-MS
Rabbit liver	RPC-C_8	up to 28% ACN, 0.1% TFA	MALDI-MS off line FAB-MS/MS off line
Rabbit liver, horse kidney	RPC-C_8	5mM ammonium acetate pH6, up to 8% ACN	on line μ-bore HPLC-ES-MS
Rat liver MT-1, mouse MT-3 and human MT-3	RPC-SEC	25 mM HCl	ESI-MS off line
Rabbit liver	CZE	40mM ammonium phosphate, 5% 2-propanol pH 2.5	on line ES-MS (ion trap)
Rabbit liver	RP-C18	up to 25% ACN, 0.05% TFA	ES-MS off line, on line HPLC-DAD-ES-MS
Rabbit liver	CZE	10mM acetate, 50% MeOH pH6	on line CZE-ES-MS
Rabbit liver MT	SEC	50 mM phosphate buffer	off line nano spray ES-Q-TOF
Human MT, α and β domains	Desalting, SEC	5mM formate buffer	ES-MS off line

Table 4: hyphenated techniques for MT speciation based on an element specific detector[58]

Sample	Separation	Eluents	Detection
Mussel tissue (digestive gland)	SEC	50mM K2HPO4/KH2PO4 buffer pH 7.5	ICP-AES
Cd-induced rat liver	SEC	10mM $(NH_4)_2CO_3$	AAS
Pig kidney	SEC	120mM Tris-HCl pH7.5	ICP-MS
Osprey blood, mussels	SEC	30mM Tris-HCl pH8.6	AAS, ICP-MS
Cyanobacterium	SEC	50mM Tris-HCl pH7.5, 0.2M $(NH_4)_2SO_4$	ICP-MS
Liver tissue	RP-C_8	20 min lin. grd. 0.5-30% MeOH in 50mM Tris-HCl buffer pH 7.0	ICP-MS
Anacystit nidulans	RP-C_8	ACN/50mM Tris-HCl pH7.5 (9:91), 0.1mM EDTA	ICP-MS
Fish tissue (bream)	RP-C_{18}	15 min lin. grd. 10-30% MeOH in 30mM acetate buffer pH7, isocr. 30% MeOH	ICP-MS
Cd-induced rat liver	Ion exchange	Lin. salt grd. (2:1), limiting buffer 250mM Tris-HCl pH 8.6	AAS
Rabbit and mouse liver tissue	Ion exchange	Lin. grd. 100% 10mM Tris-HCl pH 8.6 to100% 250mM Tris-HCl pH 8.6 in 15 min.	UV, off line AAS

Table 5: hyphenated techniques for MT speciation based on electrophoretic separation[58]

Sample	Capillary	Electrolyte	Voltage & T	Detection (nm)	LOD range
Chicken/sheep liver	Polyimide coated fused silica	100mM borate buffer pH 8.4, 775mM SDS	10 kV; 25°C	200, 214, 254, 280	0.5-1 $mg \cdot mL^{-1}$
Rabbit kidney	Untreated fused silica	20mM $Na_2B_4O_7$,10mM Tris, pH 9	10, 15, 20, 25 kV; 30°C	250	10-50 $\mu g \cdot mL^{-1}$
Chicken liver	Polyamine coated fused silica	0.1M NaH_2PO_4 / Na_2HPO_4, pH 7	20 kV; 30°C	200	5 $\mu g \cdot mL^{-1}$
Purified and semi-purified MT samples from animal species	Uncoated fused silica	20mM Tris-HCl pH 9.1	30 kV; 20°C	200	1 to 10-500 $\mu g \cdot mL^{-1}$

3.2. Phytochelatins

3.2.1. Heavy metals in plants

An increasing heavy metal concentration has been introduced in the environment in the modern times, whose main sources are car traffic, pesticides and fertilizers, industrial and domestic waste and sewage. Several heavy metals have demonstrated to be harmful to plants, affecting their metabolisms and poisoning their enzymes. Several others, even if essential elements, have toxic effects if present in a too high concentration in the environment.

For example, Al, Cd, Ni, Pb, Hg and Cr are directly toxic for plants, while Cu and Fe have an indirect negative effect, being able to induce hydroxyl radicals:

eq. 10

$$M^{n+} + O_2 \longrightarrow M^{(n+1)+} + O_2^{-}\cdot$$

$$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)+} + OH^{-} + OH\cdot$$

$$O_2^{-}\cdot + H_2O_2 \longrightarrow O_2 + OH^{-} + OH\cdot$$

·OH can oxidize biological molecules, produce cellular damage leading to cell death, add or remove H atoms to DNA bases or backbone, are implicated in lipid peroxidation, modify proteins and free amino acids, mainly histidine, arginine, lysine, methionine, cysteine[60].

On the other side, many heavy metals are essential for an equilibrate plant's growth; some examples are given in Table 6. Their uptake and mobilisation are therefore essential mechanisms in the plants' life; however, being such mechanisms not always metal specific, it follows that a not regulated uptake of toxic elements takes place as well.

Table 6: examples of essential heavy metals and their function in the plant

Essential element	Function
Fe(II)/Fe(III) - reversible e^- transfer	photosynthesis, respiration, nitrogen and sulfate reduction, oxygen filtration
Cu(I)/Cu(II) - reversible e^- transfer	cytochrome oxidase in mitochondriae
Mn(VI)/Mn(IV) - reversible e^- transfer	photolysis of water
Mn(II)	co-factor in enzymes involved in cell metabolism
Zn(II)	part of enzymes involved in cell metabolism
Mo(VI)/Mo(IV) - reversible e^- transfer	necessary for nitrate reductase and nitrogenase (oxygen transfer)
Ni(II)	catalyses the transformation of urea into CO_2 and NH_3

Heavy metal uptake and mobilisation

Metals can enter the plant through the roots as chelates (malate, citrate and other small organic acids complexes). Several characteristic proteins can also be synthesised and excreted through the root cells in the exudates: their objective is to complex the target heavy metals and make them mobile.

The most studied plant metal acquisition mechanism is Fe uptake, based on catalysed reduction of external Fe(III) and the transport across the root plasma membrane by Fe(II) transporters. It has also been shown that the iron regulated transporter IRT1 accumulates in *Arabidopsis thaliana* roots not only in response to iron deficiency, but also when stimulated by other transition metals, first of all Cd[60]. Similar carrier system have proved therefore to lack in specificity for the substrate, and transport a broad range of divalent cations into plant root cells, such as Cu^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Pb^{2+} and Cd^{2+} [61].

Once entered in the plant, metal cations are complexed and transported in each part of the plant where they are needed. Particular protein classes are synthesised for this aim, for example the chaperones proteins, that specifically deliver metal ions to organelles and other metal-requiring proteins. Recently, the Cu has been deeply investigated[62]. For long-distance transport also organic acids, especially citrate, are responsible for metal chelation.

Amino acids are also potential metal chelators for Ni and Cd[62]. In the case of Ni, increasing free hystidine enhanced its translocation to the shoots. Nicotinamine has been identified as a shuttle for Fe, Cu, Zn and Mn[63]. Intracellular metal trafficking pathways

are now emerging, pointing out the importance of chaperones also in protein expression, in displacing heavy metals from cytosol in specific organelles and vice versa, and in several enzymatic reactions, where they work as delivering proteins[62].

Compartmentalisation and detoxification

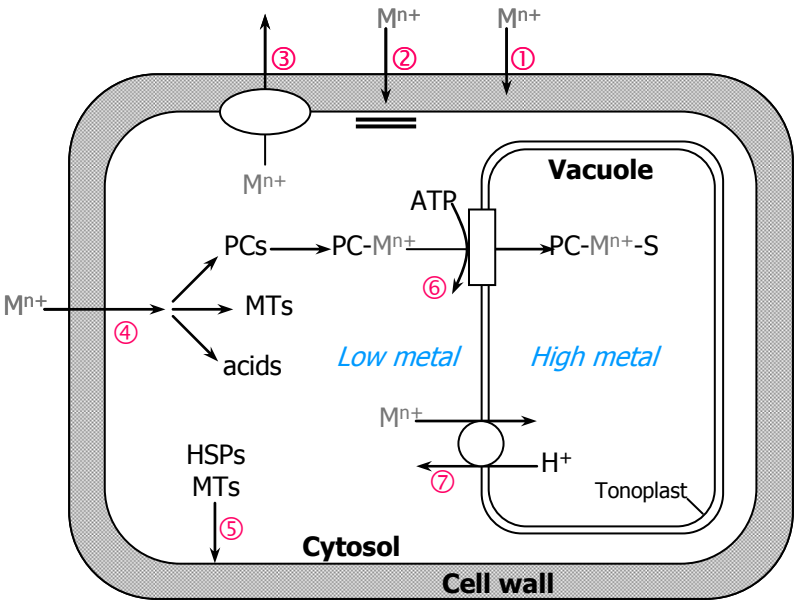

Figure 15: Summary of potential cellular mechanisms available for metal detoxification and tolerance in higher plants. 1. Binding to cell wall and roots exudates. 2. Reduce influx across plasma membrane. 3. Active efflux into apoplaste. 4. Chelation in cytosol by various ligands. 5. Repair and protection of plasma membrane under stress conditions. 6. Transport of PC-Cd complex into the vacuole. 7. Transport and accumulation of metals into the vacuole[64].

Plants have developed various mechanisms that maintain internal concentration of essential metals between deficient and toxic limits and keep non-essential metals below their toxicity thresholds. The principal classes of known metal chelators in plants include phytochelatins, metallothioneins, organic acids and amino acids. Nevertheless the complex with these ligands is not the last step in heavy metal metabolism: metals are transferred to the vacuole and here stored, being no longer harmful for the plant. In his review[67], Zenk describes this process in the case of Cd-PC complexes: once entered the vacuole, due to the lower pH values in this cell compartment, the species undergo a rapid turnover, by which Cd^{2+} is once again set free. The apo-PC are subsequently degraded in order to salvage the valuable reduced sulphur present in the cysteine. The complete cycle is schematic shown in Figure 16.

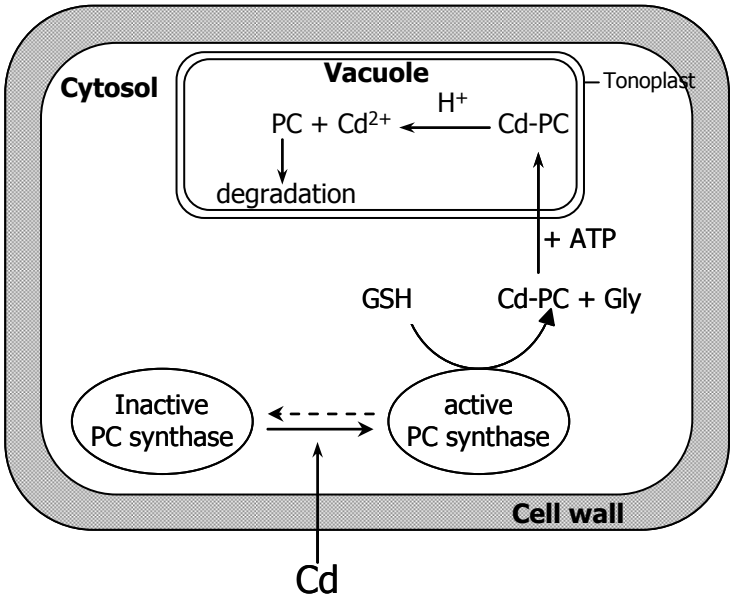

Figure 16: Cd homeostasis in the cell regulated by PC[67]

Phytochelatins are very small (539 Da for PC_2 ≥ MW ≥ 2627 Da for PC_{11}) metal binding peptides of the general structure $(\gamma - Glu - Cys)_n - Gly$ for n=2-11 that have been found virtually in all plants and certain fungi[65,66,67]. Their synthesis takes place in the cells' cytosol in presence of the enzyme γ-glutamylcysteine dipeptidyl transpeptidase, also known as phytochelatin synthase, throughout the following primary reaction[67]:

eq.11 γ-Glu-Cys-Gly + $(\gamma$-Glu-Cys$)_n$-Gly $\xrightarrow{PCS\,/\,M^{n+}}$ $(\gamma$-Glu-Cys$)_{n+1}$-Gly + Gly

It is also known that the reaction eq.11) is regulated by the presence of metal ions: the enzyme PC synthase (shortened with PCS in the reaction equation) is not only activated by the presence of M^{n+}, but also once all M^{n+} have been complexed by the freshly formed PC, the reaction turns to end (see Figure 17) and no further PC are produced.

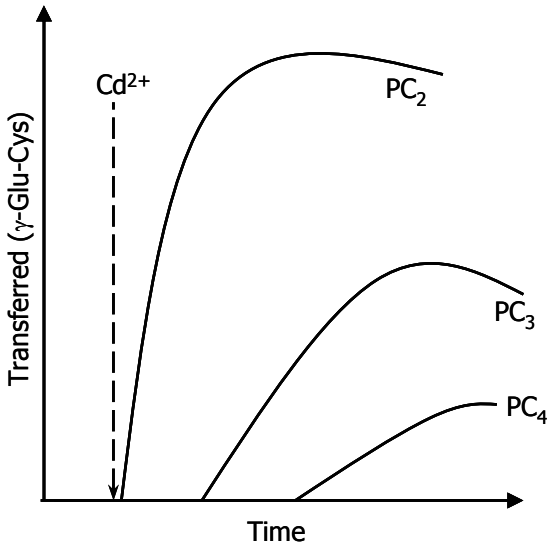

Figure 17: Time course of enzyme catalysed PC synthesis from glutathione before and after (arrow) administration of Cd^{2+} to the incubation mixture[67]

Nonetheless not all the elements behave in the same way. While several elements have no effect on the PC synthesis, others, presented by Zenk in his review[67], are ordered as follows with decreasing efficacy of activation of the PCS: Cd^{2+}> Ag^{+}> Pb^{2+}> Cu^{+}> Hg^{2+}> Zn^{2+}> Sn^{2+}> Au^{3+}> As^{5+}> In^{3+}> Tl^{3+}> Ge^{4+}> Bi^{3+}> Ga^{3+}.

The function of metal ions in the synthesis of PC is of great importance because these peptides play a primary role in M^{n+} homeostasis. Due to their high number of –SH groups, PC are the most effective and important species synthesised by plants to complex and detoxify undesired heavy metal ions.

3.2.2. Phytoremediation

The use of plants in order to remediate contamination of soil with organic or inorganic wastes is called phytoremediation. It has long been known that the life cycle of a plant has profound effects on the chemical, physical and biological processes that occur in its immediate vicinity. Growing, through water and mineral acquisition and finally decaying the plant alters profoundly the surrounding soil. One of the greatest forces driving increased emphasis on research in this area is the potential economy benefit of an agronomy-based technology: on the contrary, conventional remediation engineering techniques are often very expensive and require a lot of work.

Agronomic techniques have to be employed to remediate the contaminated soil for planting; the plants make then the majority of the job: they directly or indirectly adsorb, sequester and/or degrade the contaminant. The nature of the plants, irrigation, fertilization and cropping schemes have to be managed to maximise the remedial effect. On the other side, the technique is still not widely exploited, being the basic plant metabolism subject of research: only a deep knowledge on which are the dominating and the decisive processes happening to the contaminant inside the plants can successfully lead to a clever use of phytoremediation[68].

Remediation of organic and inorganic contaminants

For what concerns organic contaminants, they are generally absorbed by plant roots and in many cases bound into plant tissues in a form that is harmless to the organism. In order to destroy the contaminant the plants can be harvested, dried and incinerated.

Unlike organic pollutants, that can be degraded or mineralised by plants (or their associated microorganisms), inorganic pollutants are immutable at an elemental level: therefore, the remediation must either physically remove the contaminant from the system or convert it into a biologically inert form. Removal could mean a removing of the biomass or, in few cases, the contaminant volatilisation. The cases of Se[69,70] and Hg[71] volatilisation are significant, but not every heavy metal ion can be converted, either through plants metabolism or through a biological approach, into volatile species. Concentrating toxic inorganic pollutants in plants and harvesting them seems to be the most elegant and easy solution to the problem.

The processes involved in phytoextraction are the following (see also Figure 18): the contaminant must be in a biologically accessible form, it must be absorbed by roots and then transported to the shoots: these are the easiest harvestable tissues of the plant.

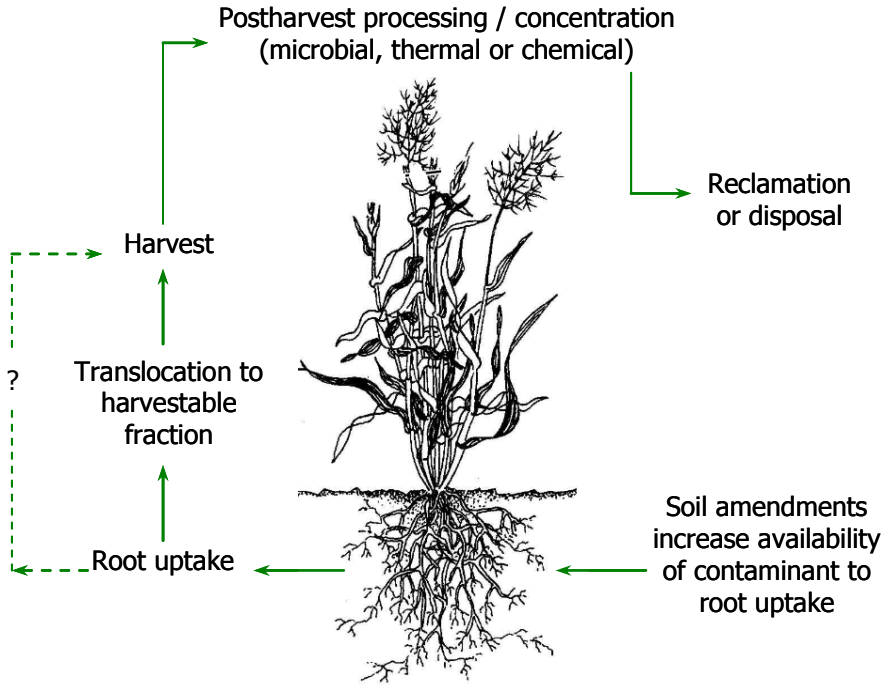

Figure 18: processes involved in phytoexctraction[68]

The actual rate of removal for a contaminant is dependent on the biomass gathered during harvesting, the number of harvests per year and the metal concentration in the harvested portion of the plants.

After harvesting, a biomass-processing step is believed to be practical to recover most metal contaminants. Alternatively, the harvested biomass could be reduced in volume and weight by thermal, microbial, physical or chemical means[68].

In order to optimise the phytoremediation techniques, is necessary an accurate and foregoing study on the agronomics, genetics and breeding potential of these plants. For example, increasing bioavailability and plant uptake of metals by lowering soil pH, adding chelating agents, the use of fertilizers and altering soil ion composition are known agronomic techniques that have to be applied.

On the other hand, genetic modifications of plants would lead to a highly improved performance: all the absorption, sequestration, mobilisation, and detoxification processes have to be clearly understood, with the aim to enhance one or all of them "improving" those aspects that are fundamental for phytoremediation.

3.2.3. PC speciation analysis

The classical approach to PC analysis is a reversed phase HPLC separation followed by post-column derivatisation of the sulfhydryl groups with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) and spectrophotometric detection at 410 nm[72,73,74,75]. Similarly, another post column derivatisation has been carried out with the fluorescent tag monobromobimane, followed by UV detection at 254 nm[76].

However, this way of detection is not specific for PC: any compound containing a sulfhydryl group is able to produce a signal. The identification of the chromatographic peaks need therefore to be based on matching of retention times of the analyte compounds with the corresponding standards, that are not commercially available.

An alternative solution is offered by coupling RPC with a mass spectrometer, in order to identify the peptides specifically and without ambiguity. An elegant solution adopted has been the positive ion fast atom bombardment tandem mass spectrometry (FAB-MS-MS)[77,78], but its low sensitivity let no chance to PC quantification. The obvious evolution has been then the coupling of RPC to electrospray ionisation tandem MS (ESI-MS-MS)[79,80,81], that has proved to be a sensitive species-selective quantification technique.

The investigation of PC-metal complexes has been made via SEC-ICP-MS, leading to information on different heavy metals (Cu, Zn, Cd and Pb) and their importance in the activation of the PC synthase. Moreover, a multielemental study was carried out to analyse their affinity for the synthesised PC under in vivo and in vitro conditions[82,83].

4. EXPERIMENTAL

4.1. Reagents used

All the solutions were prepared with MilliQ purified water (Millipore system, Eschborn, Germany), with conductivity under 0.05 μS. All the reagents and solvents used were of analytical grade or better, in order to avoid contamination.

Special reagents, stock and standard solutions, investigated samples and their preparation are described in the respective following paragraphs. In Table 7 are listed the most common chemicals used throughout the work.

Table 7: chemical reagents and solvents used

Reagent	Content/purity	Producer
Hydrochloric acid	32% p.a.	Merck (Darmstadt, D)
Nitric acid	65% p.a.	Merck (Darmstadt, D)
Hydrogen peroxide	30% Suprapur	Merck (Darmstadt, D)
Methanol	ECD tested	Acros, (Geel, B)
Tris(hydroxymethyl)-aminomethane (Tris)	99+%	Acros (Geel, B)
Tris(hydroxymethyl)-aminomethane-hydrochloride (Tris-HCl)	99+%	Acros (Geel, B)
2-mercaptoethanol	99%	Acros (Geel, B)
Ammonium acetate	99+%, HPLC grade	Fisher Chemicals (Zürich, CH)
Tetrabutylammonium acetate (TBAH)	p.a.	Fluka (Buchs, CH)
Phenylmethanesulfonyl fluoride (PMSF)	>99%	Sigma (München, D)
Ar	5.0	Westfalen AG (Münster, D)
He	5.0	Westfalen AG (Münster, D)

4.2. Instrumentation

4.2.1. Sample preparation

Every sample treated required a centrifugation step, in order to separate efficiently the extract from the solid residues and a Rotanta /AP centrifuge was therefore used (Hettich GmbH & Co.KG, Tuttlingen, Germany).

Another common step for almost every analysed sample was the freeze-drying procedure, carried out at the institute for microbiology of the university of Manz by means of a Lyovac GT2 (Leybold-Heraeus, Köln, Germany).

4.2.2. Chromatography

A three-way Sykam S1000 HPLC delivery system (Fürstenfeldbruck, Germany) and the columns listed in Table 8 were used to perform chromatographic separations. The injections were made via a totally metal-free (PEEK) sample injector (Model 9725i, Rheodyne, Phenomenex, Aschaffenburg, Germany) and a PEEK 50 µL sample loop; connections and other tubing were of PEEK as well.

Table 8: chromatographic columns employed

Chromatography	Column	Properties	Producer
SEC	BioSep-SEC-S3000	300×7.8 mm, 5 µm particle size, 290 Å pores	Phenomenex
SEC (pre column)	BioSep-SEC-S3000	75×7.8 mm, 5 µm particle size, 290 Å pores	Phenomenex
RPC	Nucleosil	150×2.0 mm, 5 µm particle size, C_{18}, 100 Å pores	Phenomenex
IPC	Synergi Polar	150×2.0 mm, 4 µm particle size, ether-linked phenyl with polar end capping	Phenomenex

In the case of RPC and IPC no pre-column was used; a cartridge system (SecurityGuard, 4 mm, C_{18} or Polar-RP phases respectively, Phenomenex) was employed as an alternative, in which contaminants are retained by a disposable cartridge.

Before being pumped in the chromatographic systems, the eluents were degassed by means of a He flow.

In the case of SEC, the correlation between the retention times and the molecular weight was stated via a calibration performed using the compounds listed in Table 9:

Table 9: compounds used for calibration of SEC

Compound	Molecular weight (Da)	Purchaser
Transferrin	79550	Fluka (Buchs, CH)
Haemoglobin	64500	Fluka (Buchs, CH)
CytochromC	12380	Merck (Darmstadt, D)
MT-I (rabbit liver)	6103	Sigma (München, D)
CN-cobalamin	1355	Fluka (Buchs, CH)
$Cu(mercaptoethanol)_2$	220	Acros Organics (Geel, B)

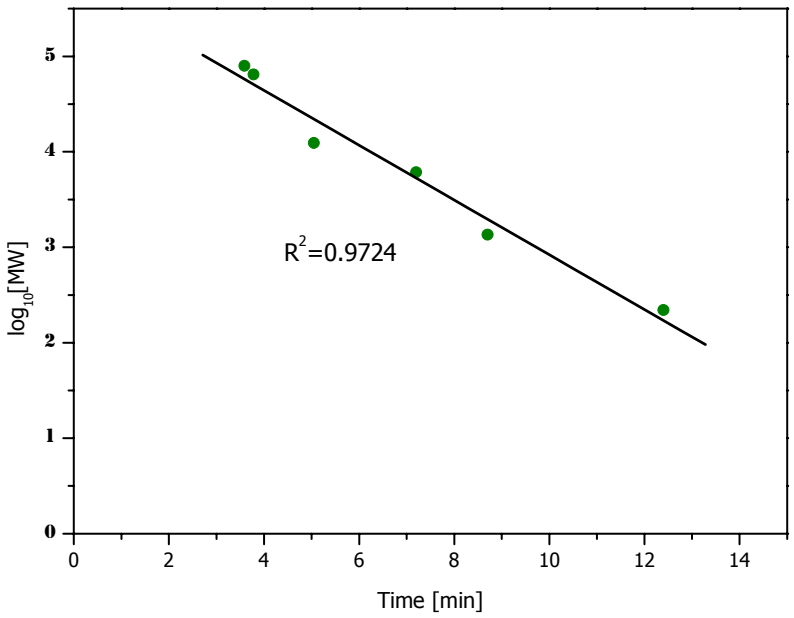

Figure 19: molecular weight calibration for the size exclusion chromatography. The used compounds and their molecular weight are reported in Table 9

4.2.3. ICP-MS

The instruments used throughout the work were the following:

- ELAN 5000 (ICP-Q-MS) (PerkinElmer, Überlingen, Germany) equipped with a cross-flow nebulizer and a Scott spray chamber (both AHF Analysentechnik AG, Tübingen, Germany);
- ELEMENT 2 (ICP-SF-MS) (Finnigan MAT, Bremen, Germany) equipped with a cross-flow nebulizer with a Scott double-pass quartz spray chamber cooled to 3°C. Mass resolutions of 300 (low resolution) and 4000 (medium resolution) were applied.

The instrumental conditions under which the ICP-MS were run are listed in Table 10:

Table 10: operational parameter for the ICP-MS, peak hopping mode

Parameters	ELAN 5000	ELEMENT 2
Nebulizer	Cross-flow	Cross-flow
Spray chamber	Scott type	Scott type, double pass, cooled to 4°C
RF power [W]	1200	1350
Cooling gas flow rate [L·min^{-1}]	15.0	16.0
Auxiliary gas flow rate [L·min^{-1}]	0.95	0.90
Carrier gas flow rate [L·min^{-1}]	1.2	0.95
Dwell time [ms]	100	150

The isotopes monitored and other experimental parameters specific for each section are reported in the respective following paragraphs.

4.2.4. ESI-MS

The instruments used throughout the work were the following:

- Sciex API 150EX (ESI-Q-MS) (Perkin Elmer)
- LCQ (ESI-ion trap-MS) (Finnigan MAT)

The instrumental conditions under which the ESI-MS were run are listed in Table 11 and Table 12:

Table 11: operational parameter for the Sciex API 150EX

Parameter	Value
Ion spray potential	5000 V
Declustering potential	35 V
Focusing potential	200 V
Entrance potential	10 V
Nebulizer gas	8 $L \cdot min^{-1}$
T curtain gas	240 °C
Sample flow rate	40 $\mu l \cdot min^{-1}$

Table 12: operational parameter for the LCQ

Parameter	Value
Ion spray potential	3600 V
Capillary voltage	15 V
Tube lens offset	10 V
Capillary T	200 °C
Sample flow rate	5 $\mu l \cdot min^{-1}$

The experimental conditions for both instruments have been optimised on the basis of a GSH and a GSSG (oxidised glutathione) standard dissolved in ammonium acetate buffer.

5. GD SPECIATION IN HUMAN SAMPLES: GD-DTPA COMPLEX[84]

5.1. Background and motivation

The magnetic resonance imaging (MRI) represents a powerful aid in the medical precocious diagnosis of several pathologies such as rheumatoid arthritis, articulations and soft tissue changing, early stage of tumours. In order for a pathology or any tissue to be better visible in MRI images there must be a decisive difference in signal intensity between it and the adjacent tissue. At the end of the 1980s it was put in evidence that this contrast can be easily enhanced by administration of the so called contrast agents. They can be easily administrated to patients per intravenous injection and enhance the visual contrast between normal and diseased tissues, or indicate organ malfunctions[85,86].

Contrast agents are based on the presence of a paramagnetic element, able to develop a relative large magnetic moment if placed in a magnetic field, which results in a relatively large local magnetic field, able to enhance the relaxation rates of water protons in vicinity. This process has as a consequence the enhancement, in comparison to the adjacent ones, of those tissues where the contrast agent has been accumulated, which can therefore be more easily investigated (see Figure 20).

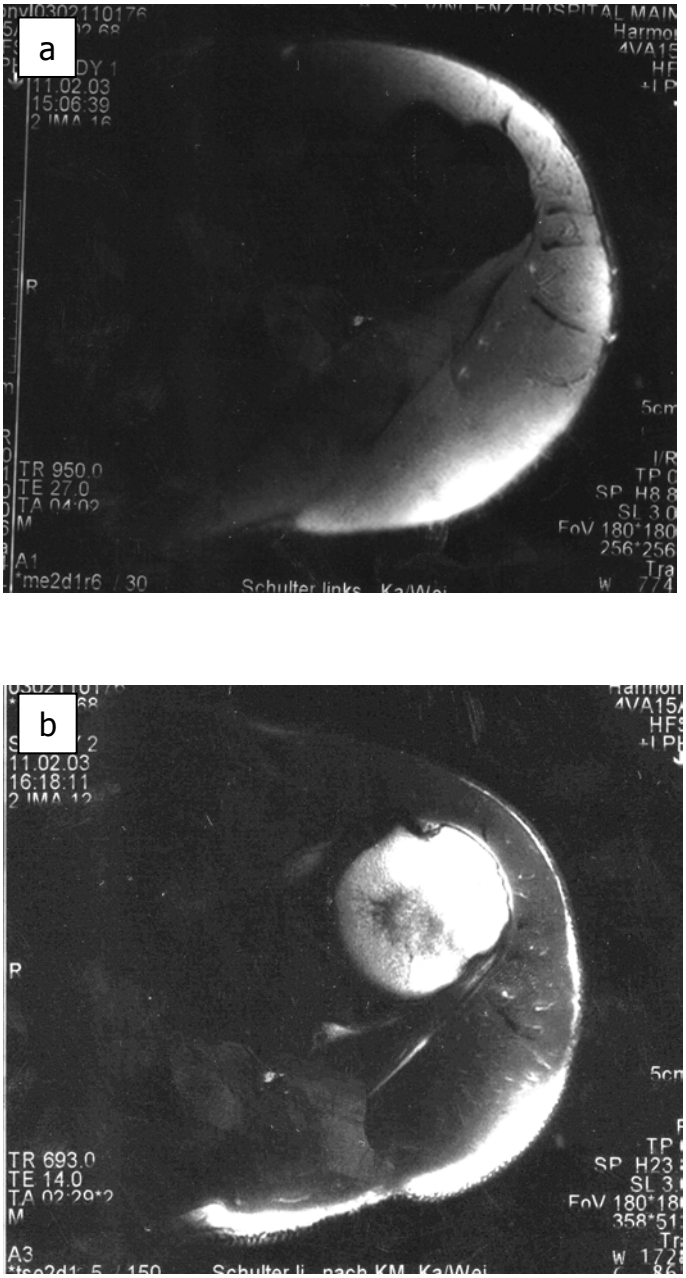

Figure 20: MRI image of a shoulder without (a) and with (b) administration of contrast agent Gd-DTPA

The Gd(III) complex with diethylentriaminepentaacetic acid (Gd-DTPA, shown in Figure 21) is a widely employed contrast agent based on Gd, commercially known as Magnevist® (Schering, Berlin, Germany).

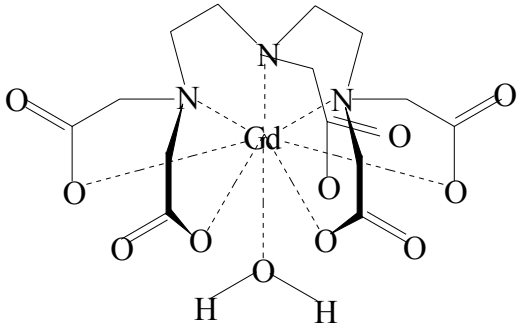

Figure 21: 3-dimensional representation of Gd-DTPA

The seven unpaired Gd(III) 4f-electrons have a magnetic moment larger than that of the surrounding hydrogen atoms in water. Through the polarisation of the O-H bonds, the dipole moment of water would be stronger and the contrast between different tissues is therefore achieved. The need of a strong complexing agent such as DTPA arises from the fact that Gd(III) in the ionic form is highly toxic for the organism: free Gd(III) has proved to provoke acute toxicity, due to its ability to bind to serum proteins and other ligands, such as citrate. It can be later displaced from these proteins and cause ataxia, writhing, laboured respiration, sedation, hypotension, death by cardiovascular collapse and focal necrosis of the liver[87].

On the contrary, the strong complex with DTPA prevent Gd(III) to bind to serum proteins or induce colloidal hydroxides, carbonates or phosphates[84]; furthermore it is extremely water soluble and so suitable for intravenous injection and quick excretion through urine. However, small amounts of Gd-DTPA are probably retained in the organism due to thermodynamic stability of Gd(III) and the kinetics of its release[88].

Gd-DTPA speciation analysis

Gd-based contrast agents have up to now been investigated by means of HPLC coupled to UV-Vis, fluorescence and time-resolved luminescence detectors[84]; HPLC-ESI-MS has been used as molecular specific detector in the investigation of chemical reactions between Gd complexes and oxidative human neutrophil production, induced *in vitro* by phorbol-12-myristate-13-acetate [89], where no evidence of Gd complexes degradation or interaction was observed.

Total Gd determination in biomedical samples has been carried out through ICP-OES, ICP-MS and RIMS (high-resolution multi-step resonance ionisation mass spectrometry)[90,91], while in this work speciation analysis was performed by means of an on-line coupling of SEC and ICP-MS.

Aim of the work

The aim of this work was to investigate the Gd(III) containing species in urine, saliva, sweat and hair samples of a patient that received a Gd-DTPA intravenous injection. The combination of separating Gd-DTPA from other Gd-containing species and the monitoring of several Gd isotopes enabled us to detect conversions and degradation processes of Gd-DTPA and to quantify its excretion process.

5.2. Experimental set up

SEC-ICP-MS was employed to investigate the chosen samples, for which an isocratic elution was performed. The employed mobile phase was a Tris-HCl buffer solution 0.02 $mol \cdot L^{-1}$, pH=7.4, at a flow rate of 1 $mL \cdot min^{-1}$.

Total Gd measurements were performed with a Hewlett-Packard ICP-MS 4500 (Waldbronn, Germany) in combination with a self-aspirating PFA µ-flow nebulizer (AHF Analysentechnik AG, Tübingen, Germany). ICP-MS quantitative analysis of the digested solutions was performed with external calibration.

For microwave digestions the MLS-Ethos plus (MLS, Leutkirch, Germany) was used in order to dissolve urine and hair samples.

The monitored Gd isotopes were m/z =155, 156, 157, 158 and 160 by peak hopping with a dwell time of 100 ms.

5.2.1. Reagents and standard solutions

Standard solution of Gd(III) and Gd-DTPA were prepared daily by dilution of an ICP standard (1 $g \cdot L^{-1}$, CertiPUR, Merk, Darmstadt, Germany) and a stock solution, obtained dissolving the suitable amount of dry salt (DTPA Gd(III) dihydrogen salt hydrate 97%, Aldrich, Taufkirchen, Germay) in 3% (m/m) HNO_3, respectively.

The mobile phase was prepared as described above (paragraph 5.1).

Tetramethylammonium hydroxide (TMAH 25%, p.a., Fluka, Taufkirchen, Germany), 0.1 $mol \cdot L^{-1}$ Tris-HCl (pH 7.4), the enzyme Proteinase K (≥ 90%, Sigma-Aldrich, München, Germany), 30% hydrogen peroxide and 65% nitric acid were tested for the digestion.

5.2.2. Samples and sample preparation

Urine, saliva and sweat samples were collected from a patient after intravenous injection of Gd-DTPA, preserved in a refrigerator (-20°C), and measured at room

temperature diluted in the mobile phase without any previous digestion. 2-mercaptoethanol was added to complex free ions.

Hair samples, unlike the other liquid samples, needed to be digested in order to be analysed. The reagents and digestion procedures tested to destroy the matrix and preserve the existing Gd species are shown in Table 13.

Table 13: digestion procedures tested

Extraction	Digestion reagents	Experimental conditions
1 (total Gd determination)	HNO_3 65%, H_2O_2 30%	MW assisted, 25 min., 200°C
2	Tris-HCl 0.1 mol·L^{-1}	shaking for 5 hours, room T
3	Tris-HCl 0.1 mol·L^{-1}	MW assisted, 6 hours, 60°C
4	MeOH	MW assisted, 6 hours, 60°C
5	TMAH 25%	MW assisted, 2 hours, 60°C
6	Proteinase K 20 mg mL^{-1}, Tris-HCl 0.05 mol·L^{-1} (pH 8)	incubated for 24 hours, 50°C[92]

In order to determine the total Gd content, a complete digestion was performed (extraction 1 in Table 13) both on 0.1 g hair and 1 mL urine.

Both the complete digestion and the MW assisted mild extractions were carried out by means of a MLS-Ethos plus microwave oven (Mikrowellen-Labor-System, Leutkirch, Switzerland).

5.3. Results and discussion

5.3.1. Separation and detection of Gd^{3+} and Gd-DTPA

Our earlier investigations on the separation of the Gd^{3+} and Gd-DTPA were focussed on the reversed-phase chromatography. No separation was possible by means of a RPC, even if different stationary phases have been tested. Therefore, it was decided to apply merely SEC, also due to the easier coupling to ICP-MS instrumentation and the possibility to monitor higher mass fraction containing potentially Gd.

For our experiments a SEC column was used which works in a separation range between about 0.5 and 200 kDa. On the one hand the separation between the compounds of interest is possible, on the other hand the broad range to higher masses enables the detection of eventual present macromolecules which interact with Gd^{3+} or the MRI reagent itself.

For SEC separation Tris-HCl buffer solutions of different concentration and pH were applied as eluents. A concentration of 20 mmol·L^{-1} (pH=7.4) at a flow rate of 1 mL·min^{-1} met the requirement of satisfactory separation of Gd^{3+} and Gd-DTPA standard solutions as demonstrated in Figure 22. The addition of 2-mercaptoethanol (0.01 % *v/v*) to the samples injected is suggested in order to increase the recovery of Gd^{3+}-ions from the SEC column due to complexation.

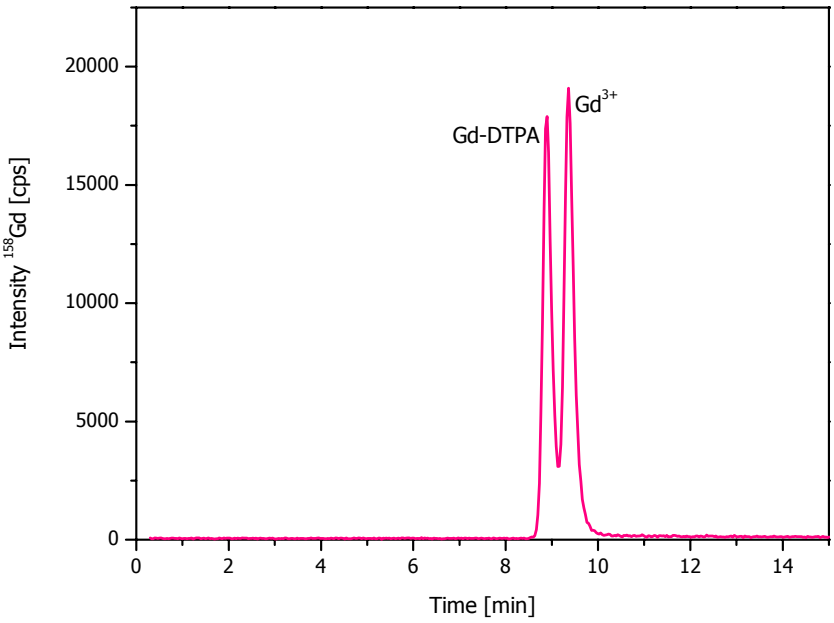

Figure 22: size exclusion chromatogram of a Gd^{3+}/Gd-DTPA standard solution (1000 µg·L^{-1} each as Gd)

The Gd isotopes monitored can suffer from interference from isobaric and/or cluster ions, e.g. $^{156}Gd^{+}$: $^{156}Dy^{+}$, $^{140}Ce^{16}O^{+}$ (see also Table 2), and could not be resolved by quadrupole-MS. However, the use of all naturally occurring isotopes and the continuous monitoring of different Gd isotope ratios by peak area integration enabled selective detection of Gadolinium species in all the samples. It was observed no deviation from the expected ratios.

Analysis of urine samples

It is well known that after intravenous administration, Gd-DTPA quickly diffuses in the extracellular space and is eliminated via the kidney. Thus, urine samples were collected

from a patient whom about 20 mmol Gd-DTPA in total was intravenously administrated as Magnevist®. After collection samples were stored at -20°C; for analysis appropriate aliquots were diluted with 0.1 mol·L^{-1} Tris-HCl (pH=7.4), and after addition of 2-mercaptoethanol (0.01% *v/v*) and filtration (0.45 µm) the solutions were directly injected.

A typical chromatogram of the most abundant Gd isotopes is shown for a diluted urine sample in Figure 23. It indicates that Gd compounds could only be detected at the retention time of Gd-DTPA. Addition of Gd-DTPA standard solution confirmed its presence. No other high-molecular-mass Gd-species could be observed in the analysed urine samples. Furthermore, free Gd^{3+} was below the detection limit for Gd^{3+}: 3.5 µg·L^{-1}.

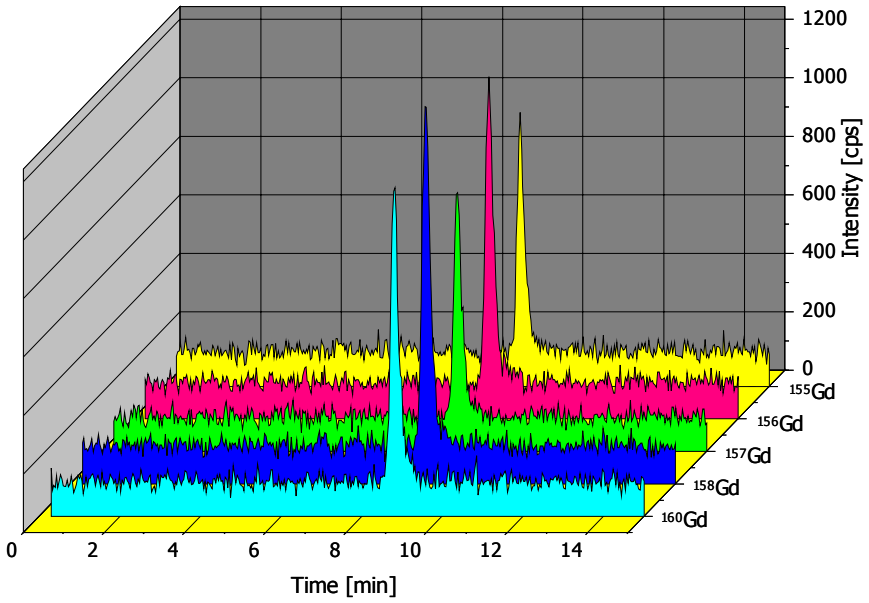

Figure 23: size exclusion chromatogram of a urine sample (200 times diluted) following the most abundant Gd isotopes

Although no compounds other than Gd_DTPA were found in any urine samples, total Gd analysis by ICP-MS was performed in order to evaluate the excretion behaviour of the MRI reagent. For this, microwave assisted digestion was performed to destroy all the organic matrix components as reported in Table 13 (1). External calibration resulted in the values given in Figure 24 after appropriate sample dilution. Each sample was

measured three times; the linear range for Gd^{3+} was 1-1000 $mg \cdot L^{-1}$, the resulting calibration curve had a correlation factor R^2=0.998.

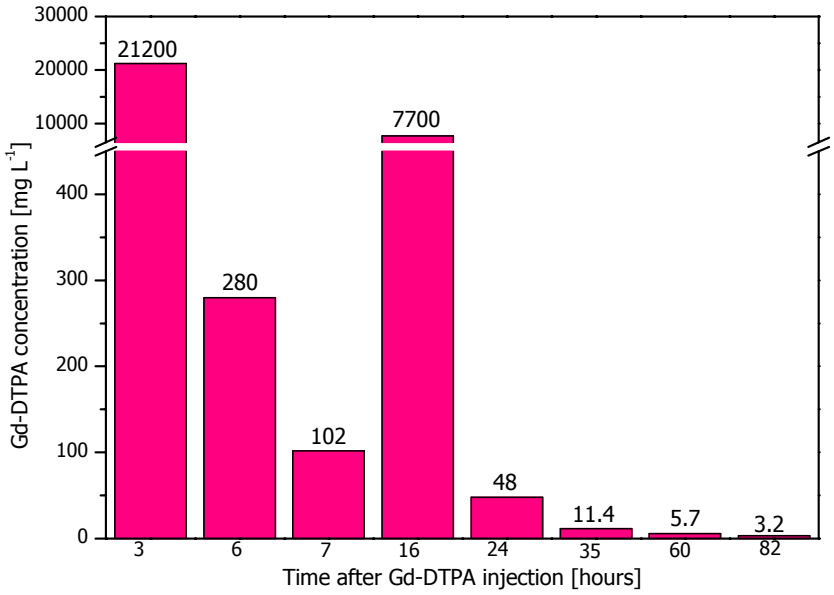

Figure 24: time dependency of Gd-DTPA urine excretion

Figure 24 indicates the time dependence of the excretion in urine. It was calculated that about 70% of the total injected Gd-DTPA was excreted within the first urination, in this case, after 3 hours. Consecutive samples showed an enormous decrease in Gd concentration except for the sample taken after 16 hours. A large increase in Gd concentration could be observed in this sample. Even if this exception has not yet been explained, it is known that more than 90% of Gd-DTPA is excreted within 24 hours from the administration. This result could have been confirmed, if more than one patient's samples could have been analysed.

Subsequent samples contained insignificant amounts (less than 1% of total Gd-DTPA), so that, as expected, more than 90% of total Gd-DTPA was excreted in the urine within 24 hours under these conditions.

Analysis of other samples

Beside the urine samples, saliva and sweat samples were collected and analysed, but no Gadolinium could be detected. Four weeks after the Gd-DTPA administration hair samples were taken and tested to be analysed for the Gd species.

First, the hair samples were analysed to determine their Gd concentration. In comparison to a hair sample belonging to a person not subjected to the Gd-DTPA administration, it was stated that Gd-compounds were present. The digestion of 100 mg of hair was performed as described in Table 13 for extraction 1.

Representative samples from the patient showed the Gd concentration to be 10±2 $ng \cdot g^{-1}$ whereas no Gd could be found in the untreated person's hair.

To determine which species are present, different extraction approaches in combination with SEC-ICP-MS have been tested for their efficiency (see Table 13). Before the analysis, every solution was adjusted to pH 7.4.

The extraction procedures with Tris-HCl (2, 3), MeOH (4) and the enzymatic digestion (6) were not successful. No Gd-containing compound could be observed in the chromatograms, probably because the dissolution of the sample was incomplete.

A typical chromatogram obtained after sample extraction 5, with TMAH, is shown in Figure 25.

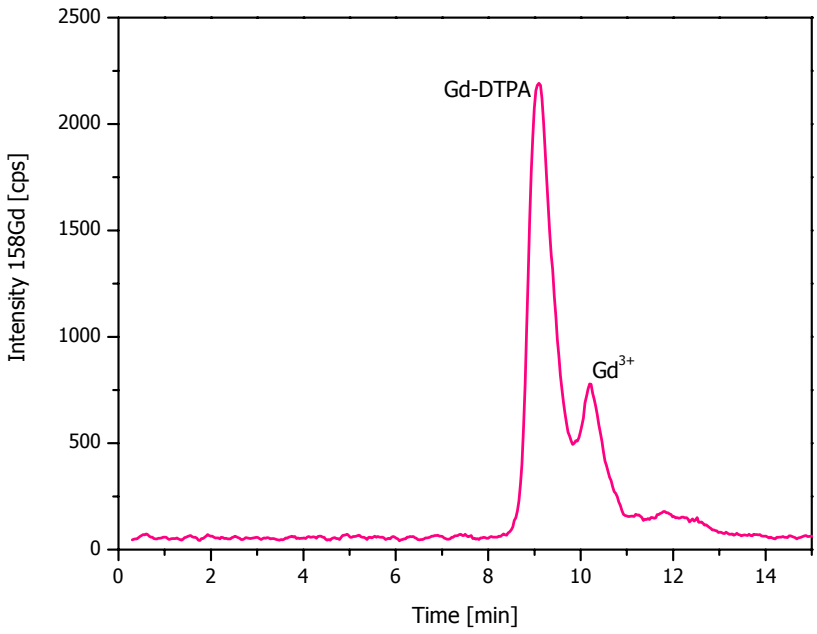

Figure 25: size exclusion chromatogram obtained after TMAH extraction of a hair sample

The peak widths and the retention times were slightly different from those on the other chromatograms probably because of the high matrix concentration. Nevertheless, in contrast to the analysis of the other samples, both species Gd^{3+} and Gd-DTPA could be detected.

Control experiments with standard solution of Gd-DTPA resulted in the same observation: TMAH induces partial degradation of Gd-DTPA to Gd^{3+}. Variation of conditions such as extraction time, temperature or TMAH concentration did not lead to a decrease in the formation of artefact Gd^{3+} but only to incomplete analyte recovery.

Although without an exact scrutiny, it was supposed that Gd-DTPA is transported from blood to hair without any chemical degradation to Gd^{3+}. Whether Gd-DTPA is bound to proteins or other macromolecules could not be verified. For this, a mild extraction method is necessary, which ensures the stability of analyte-protein interactions.

Indicative results have shown that no interaction between Gd-DTPA and macromolecules like transferrin and haemoglobin could be observed. Further investigation should reveal the form in which Gd-DTPA is transported within the body fluids.

5.4. Conclusions

The coupling of SEC and ICP-MS has been proved to be a suitable, element selective and versatile technique for differentiation between Gd^{3+} and Gd-DTPA, often used as MRI reagent. This development enables reliable assessment of the pathways of Gd-DTPA in the body fluids after administration, especially if the analyte is bound to high-molecular-mass biomolecules.

If the kidneys of the patient are healthy, our investigations could show that the injected Gd-DTPA is excreted almost completely (>99%) by urination within one day without any chemical change; only comparably low concentrations of Gd could be measured in hair. In saliva and sweat samples the Gd-DTPA concentration was below the detection limit.

The method can also be readily adapted to analysis of other Gd-containing MRI reagents.

6. HEAVY METAL SPECIATION IN MAMMAL AND HUMAN SAMPLES: METALLOTHIONEINS

6.1. Qualitative investigations on bovine liver and method development

6.1.1. Experimental set up

Samples preparation

The analysed sample was bovine liver, where a high amount of MT is to be expected.

The samples were freshly purchased from a local store, and had to be digested immediately after purchase or the day after. Conservation in a normal refrigerator was not enough to preserve intact the metal binding species, as a dramatic drop in the signals intensity was observed, dependent on the time the organs had been stored. In order to preserve the same sample for more than few analyses, small part of the organs were freeze-dried, sealed under nitrogen in polypropylene centrifuge tubes and conserved at 4°C.

To detect MT and other metal binding species, the samples had to be digested. However, the extraction was carried on under extremely mild conditions, in order to preserve the original species composition.

It is known that the best way to recover MT is a room temperature extraction by means of Tris-HCl buffer: under these conditions, the extraction efficiency and the species stability were found satisfying[93,94,95]. The solution was daily diluted from a stock solution, prepared dissolving a proper amount of Tris and Tris-HCL and adjusting the pH to a value of 7.4. In order to preserve MT and other –SH containing species to be oxidised, degassed water was used. The procedure followed for the extraction is schematically resumed in Figure 26. The addition of 2-mercaptoethanol was necessary to prevent oxidation of –SH groups.

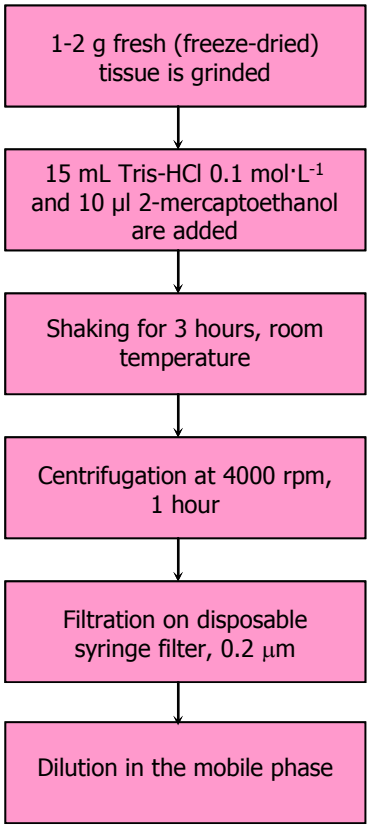

Figure 26: diagram of the applied sample's digestion procedure

Instrumental: chromatography

The chromatographic system described in paragraph 4.2.1. was equipped with the SEC and successively with the RPC column (see Table 8 for details).

For the size exclusion measurements, an isocratic elution was performed. The employed mobile phase was a Tris-HCl buffer solution 0.02 mol·L^{-1}, pH=7.4, at a flow rate of 1 mL·min^{-1}.

For the reversed phase measurements, a gradient elution was optimised, using as eluents the solutions A= Tris-HCl 0.02 mol·L^{-1}, pH=7.4, and B=A 20% / MeOH 80%. The gradient was run at a flow rate of 0.4 mL·min^{-1}, with the following program: 3.5 min A 100%; 20.0 min increasing in B up to 75%; 2.5 min A 25%, B 75%. A cleaning and conditioning procedure (7 min down to A 100%, 10 min A 100%) followed then always the separation gradient, in order to recreate the original column's conditions after every measurement. The complete program is illustrated in Figure 27.

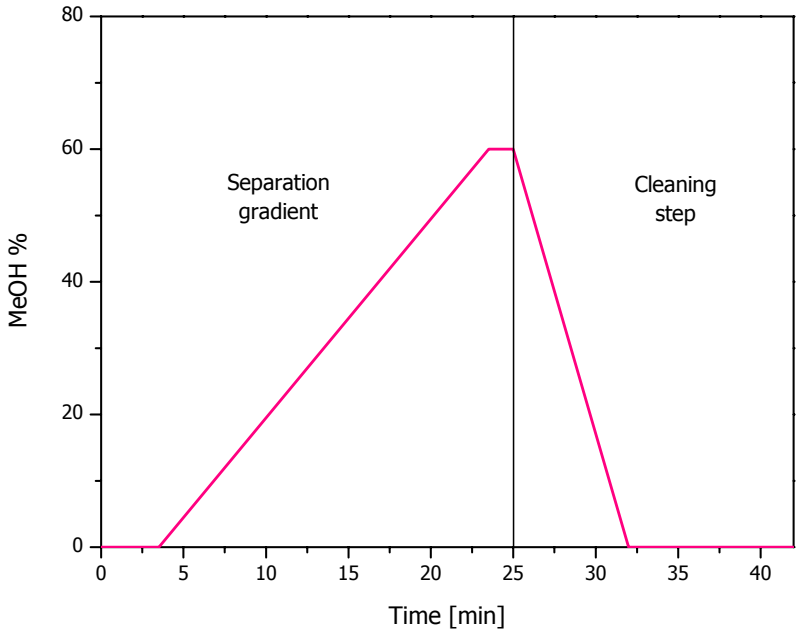

Figure 27: optimised chromatographic conditions for MT separation

Instrumental: ICP-MS

The measurements of bovine liver extracts were run on the ELAN5000 ICP-Q-MS, under the experimental conditions listed in Table 10. Owing to the high methanol amount after the RPC separation, a post column dilution of the eluate was carried out by means of a 10 ng·mL^{-1} Rh and 2% HNO_3 solution supplied via a peristaltic pump, with a flow rate of 0.8 mL·min^{-1}. The methanol amount in the plasma was therefore not higher than 20%.

The isotopes monitored were ^{53}Cr, ^{58}Ni, $^{63, 65}$Cu, $^{64, 66}$Zn, ^{112}Cd and ^{208}Pb. Due to the low resolution of the quadrupole, it was necessary to monitor more than one isotope of the same element, so that the isotope ratios could be checked and possible isobaric interferences (e.g. $^{23}Na^{40}Ar^{+}$ for ^{63}Cu, see also Table 2) revealed. ^{103}Rh was also monitored, in order to compensate the signal intensity changes produced by the increasing methanol concentration.

Other measurements were carried on with the same chromatographic system (and the post column dilution, as well) coupled to the ELEMENT2 ICP-SF-MS, operating at the medium resolution mode ($\Delta m / m = 4000$). In this case, ^{32}S was measured in addiction to the already monitored isotopes.

6.1.2. Results and discussion

SEC results

The main function of liver is to detoxify the organism, therefore MT have to be searched here. To have a very quick, direct and general approach on the heavy metal distribution in the samples, the very first approach made was by means of SEC, which is known as the most powerful tool for a preliminary investigation of unknown samples. Even if the separation is sometimes rough and does not show the necessary resolution, it needs much less time for the optimisation –generally, the mobile phase is chosen only on the basis of its ability to dissolve the sample. The chromatogram's length is fixed and dependent on the stationary phase, i.e. on the chosen column, and information on the molecular weight of the investigated species derives from its calibration, as described in paragraph 4.2.1.

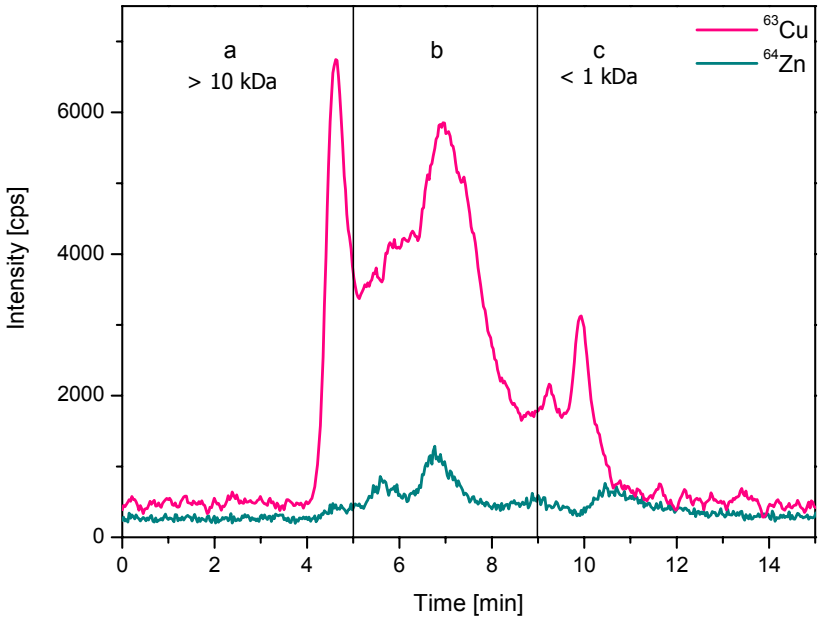

Figure 28: Typical size exclusion chromatogram (SEC-ICP-Q-MS) obtained from a bovine liver extract. **a** retention time range corresponds to high molecular-mass species, MW>10 kDa; **b** retention time range corresponds to 10>MW>1 kDa, and here therefore lie MT (6-7 kDa); **c** retention time range corresponds to low molecular-mass species, MW<1 kDa.

In Figure 28 is shown a typical chromatogram of a fresh digested bovine liver. On the basis of the mass calibration, the time scale was divided in three zones: *a*, for the high molecular weight species (MW>10 kDa); *c*, for the low molecular species (MW<1 kDa); and between the two the *b* range, where belong the MT, being their molecular weight ~ 6-7 kDa.

Cu signal shows three intense peaks. The fraction eluting at 4.6 min belongs to the *a*-zone and can be attributed to transport proteins, high molecular-mass species able to bind heavy metals. The peak at 9.9 min belongs in the low molecular-mass range and represents the "inorganic fraction", i.e. small inorganic complexes of the monitored heavy metals, not bound to proteins or peptides. The peak at 6.8 min (in good correlation to the corresponding Zn peak) can be attributed to MT on the basis that it represent a species able to bind at the same time both Cu and Zn and of a MW comparable to those of MT (peaks in the *b* region).

The exact nature of these peaks can be cleared either through a proper soft ionisation (ESI-MS) mass analysis or by the comparison of retention times to those of a proper standard. An MT (MT-I, rabbit liver, Sigma) standard was commercially available, but its stability and pureness have been often mistrusted, to the point that it is no longer sold.

Therefore, the bovine liver extract were treated at high temperature (75°, 30 min), with the aim to perform a rough but effective pre-column separation: it is known[24,25] that MT are heat resistant, while other proteins are denaturated under these conditions. SEC measurements after this treatment showed the permanence of wide signals in the *b*-zone, while a large part of the *a*-zone signals disappeared and the inorganic fraction (*c*-zone) was increased in intensity. In Figure 29 this phenomenon is shown for ^{63}Cu.

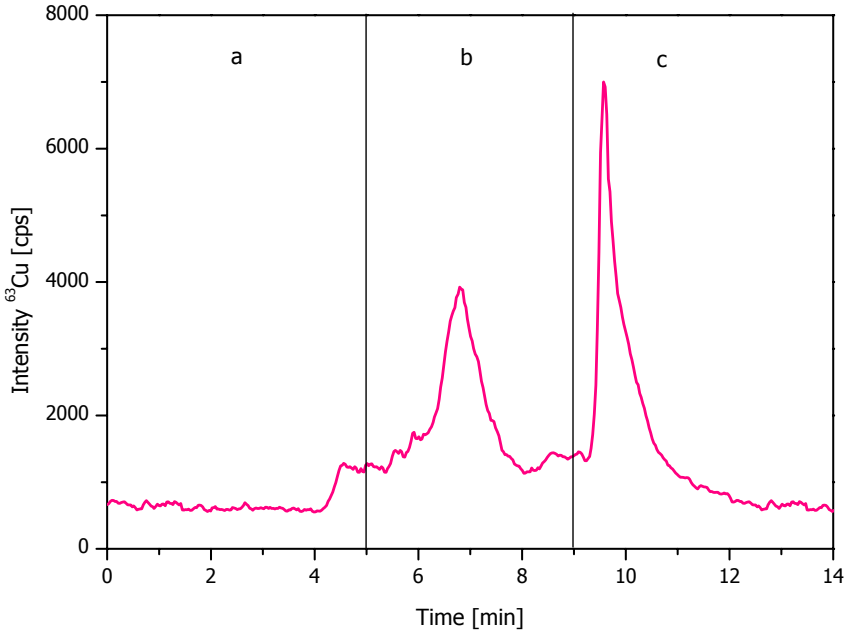

Figure 29: copper trace in a SEC chromatogram of bovine liver extract after heat treatment

The presence of the MT peaks shows that the extraction procedure is able to recover the desired species and that the bound between the metals and the protein is stable under the extraction and chromatographic conditions. Nevertheless, even if the SEC can take apart the MT from a large matrix fraction, its resolution is not sufficient to separate the single isoforms: the wide shape of the peaks and the presence of several shoulders (5.5, also correlating with a Zn signal, 5.8, 6.3 min) are suggesting that more than one species with similar MW are present. For this goal, the reversed phase chromatography was used, as reported in the next paragraph.

RPC results

At the preliminary stage several RP-columns and different buffers (Tris-HCl, acetate, citrate) at different pH (4-8) and concentrations (0.001-0.1 mol·L^{-1}) have been employed. Moreover, different flow rates and MeOH gradients have been checked, and their performance was studied in order to optimise the experimental conditions to achieve a reliable MT isoform separation by means of an RPC. In Figure 30 are shown three attempts of separating the MT isoforms in a bovine liver extract:

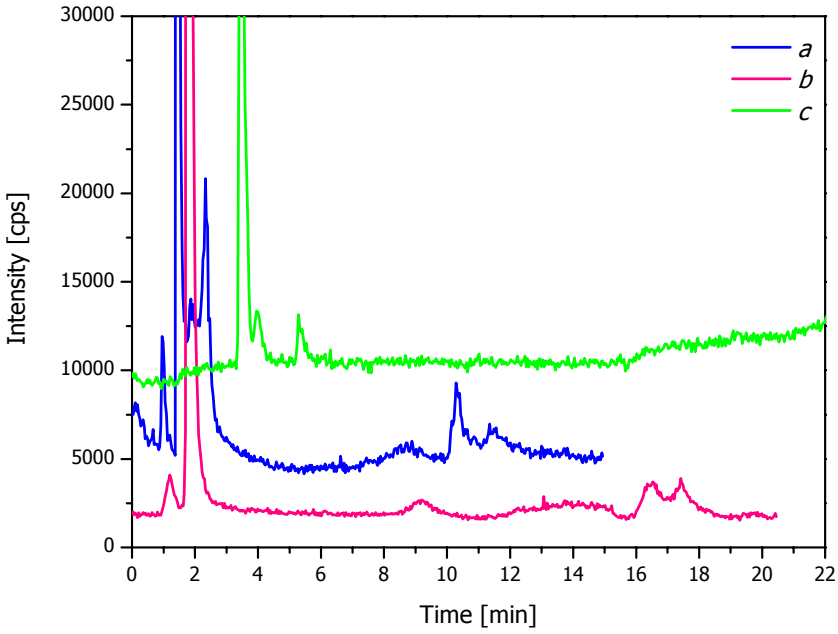

Figure 30: attempt to optimise the chromatographic conditions for the investigation of bovine liver extracts. Conditions *a*: *Jupiter* column; conditions *b*: *Nucleosil* column; conditions *c*: mobile phase at pH=5.5

The blue trace (conditions *a*) represent the attempt to use a different column. In this case the employed column was a Jupiter (150×2.0 mm, 5 µm particle size, C_{18}, 300 Å pores, Phenomenex), but the early co-elution with the inorganic fraction of several species of interest at 2.0 and 2.5 minutes was not avoidable. The pink trace (conditions *b*) indicates a first attempt with the later used Nuclesoil column. In this case the methanol content was different, as well as its increase in time (in 20 minutes, from 0 up to 40% MeOH), and not all the present species are separated, co-eluting in the two wide peaks at 16 and 17.5 minutes. The green trace (conditions *c*) shows an elution with a Tris-HCl buffer which pH was adjusted at a value of 5.5. Under these conditions part of the species is early eluted, part is lost.

Among all the experimental conditions, the optimised and chosen ones (described above, paragraph 6.1.1.) were the best compromise between signal intensity, peaks resolution and time requested. In Figure 31 a typical RPC-ICP-Q-MS chromatogram run under these conditions is shown. The peak eluting at 1.9 min represents the "inorganic fraction", composed by small inorganic complexes of the monitored heavy metals and their 2-mercaptoethanol complex. In the second part of the chromatogram (12-22 min),

where less polar species like proteins are eluted, small peaks were detected. In Figure 32 this region, later called the MT eluting range, has been enlarged.

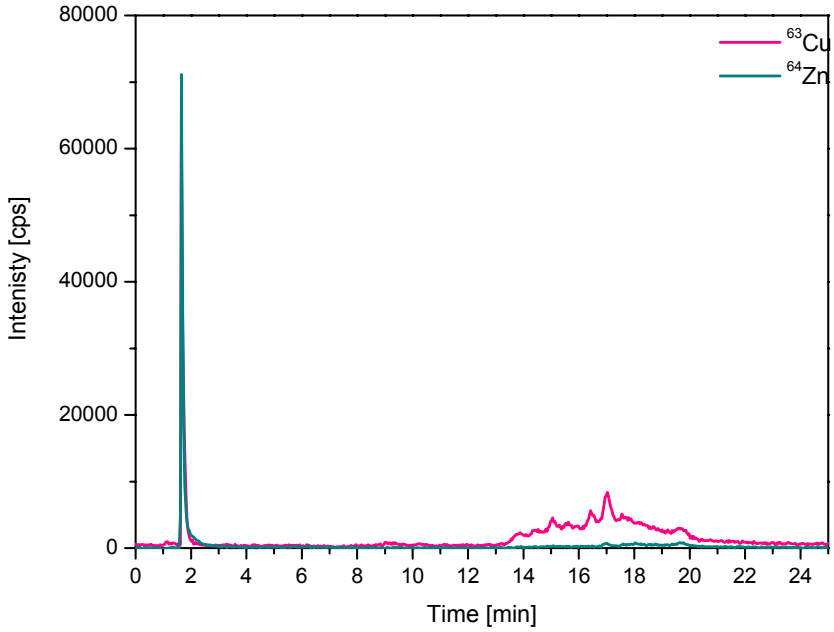

Figure 31: Typical reversed phase chromatogram (RPC-ICP-Q-MS) obtained from a bovine liver extract. The peak at 1.9 min is the "inorganic fraction"; MT isoforms elute between 12 and 22 min.

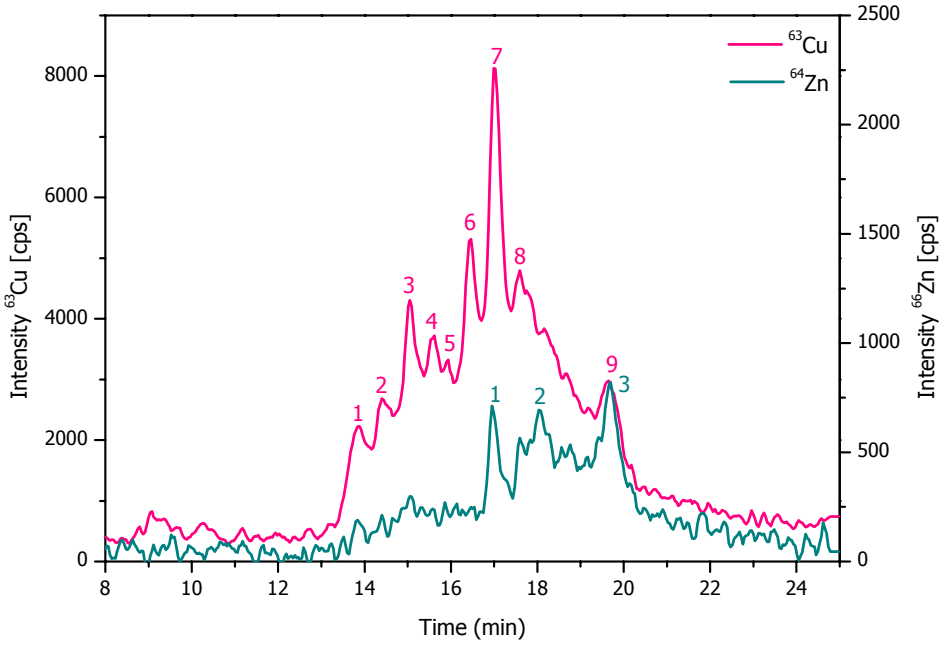

Figure 32: zoom of the previous chromatogram in the MT elution range.

The signals detected and shown in Figure 32 are numerous and in some cases not completely separated, meaning that the samples contain a large variety of species with slightly different polarity. The Zn peaks 1 and 3 correlate exactly with the corresponding Cu signals 7 and 9. This can be explained assuming that the detected signals belong to different isoforms of the same MT binding Cu, Zn or both.

The reliability of the system and the stability of the investigated species were testified by the low RSD of the signals, <1% on the retention times when the same sample was analysed 3 times.

In Figure 33 two aliquots of the same sample were injected in the column before and after the heat treatment: the very good correspondence of the detected peaks is evidence that these metal binding proteins are MT with a reasonable certainty.

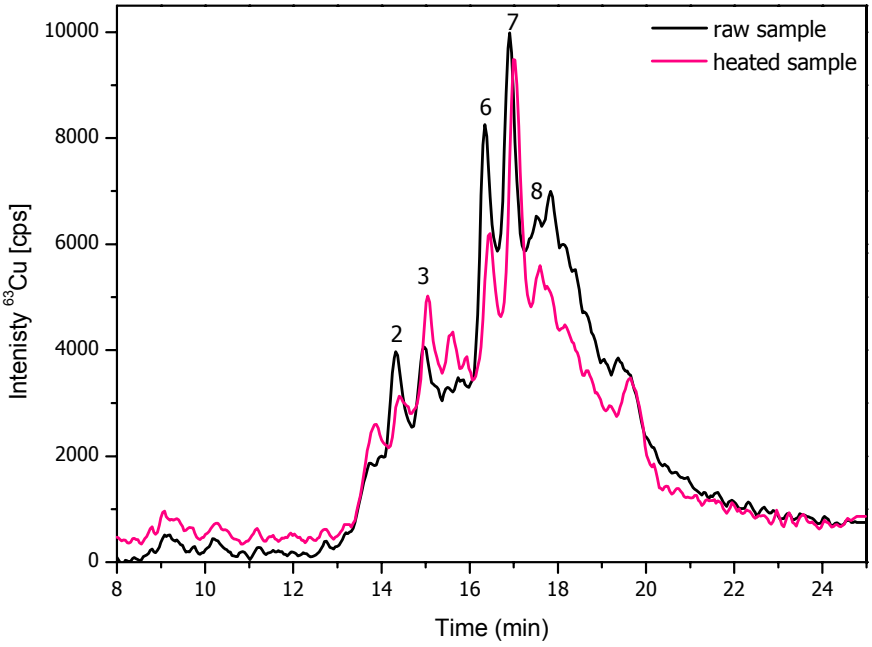

Figure 33: RPC-ICP-Q-MS chromatogram of the same bovine liver sample with (pink) and without (black) heating treatment (75°C, 30 min). The numeration of the signals indicates the peaks matching their correspondence in Figure 32.

In Figure 34 the chromatograms of the extracts of two different bovine liver samples are compared. Considering that they are not standards, it is logical to find differences between the samples, as not all the peaks match. The correspondence between the two samples shown is nevertheless very good, attesting once more the stability and the widespread nature of these species in the analysed samples.

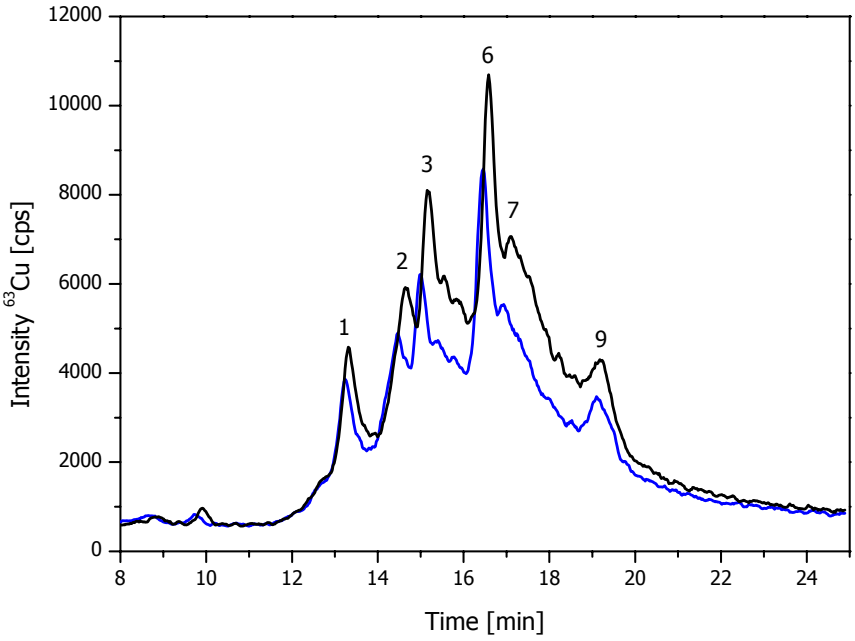

Figure 34: RPC-ICP-Q-MS chromatogram of two different bovine liver samples. The numeration of the signals indicates the peaks matching their correspondence in Figure 32.

It has been observed that the intensity of the inorganic fraction signals tends to increase as the sample gets older, that means that metal binding species are degraded in time, releasing the metal ions. This degradation is dramatic in the case of refrigerated liver pieces or extracts, while it is less strong in the case of freeze-dried samples or extracts. A chromatogram showing this effect is reported in Figure 35:

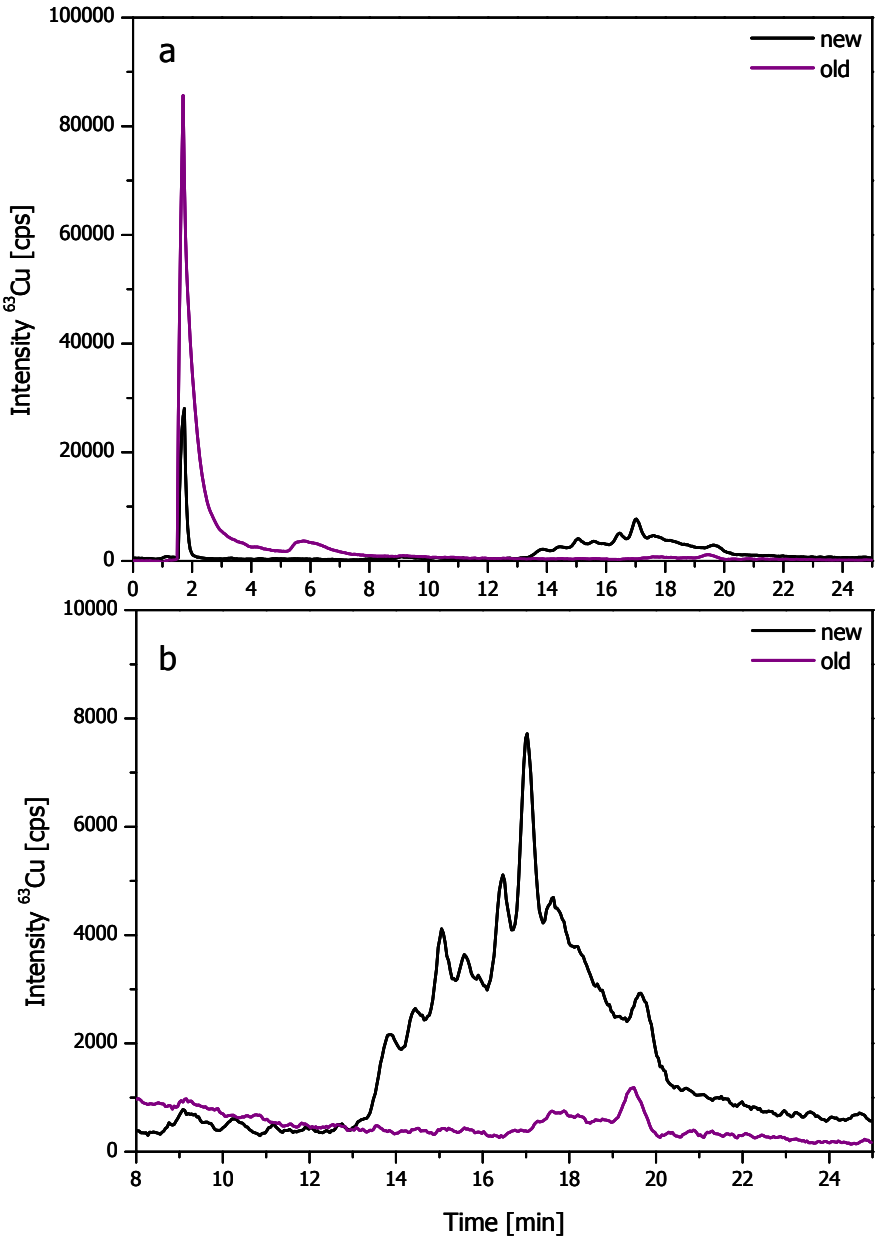

Figure 35: comparison between a new and a two weeks old bovine liver sample: degradation of MT occurs (b) while the inorganic fraction rises (a).

In order to get a more accurate isolation of MT a two-dimensional approach to the problem was employed. By means of the size exclusion separation, the bulk of metal binding proteins with molecular mass of 6-7 kDa was isolated, sampling the eluted

fraction at the right time. The fraction was then injected in the reversed phase column and the signals detected via ICP-MS.

The chromatograms obtained through this approach showed signals of extremely low intensity, not much higher than the background noise and with a bad reproducibility. The results for ^{63}Cu are shown as example in Figure 36:

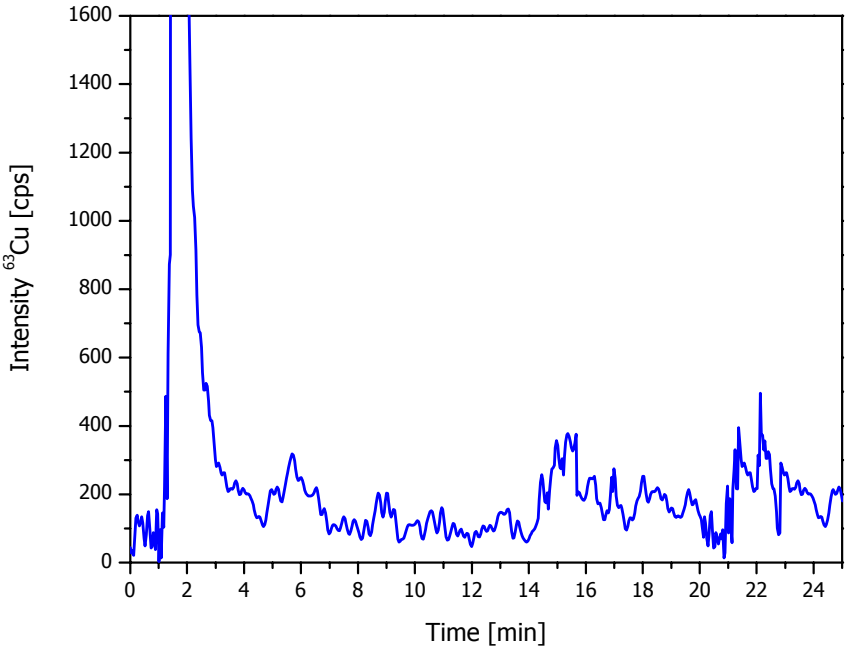

Figure 36: 2-dimensional approach for bovine liver extracts investigation: after the SEC/RPC no ICP-MS signal can be detected. The inorganic fraction peak eluting at circa 2 minutes could arise from degraded species

The fact that the measured signals had such a low intensity, can be explained with the synergic effects of an extreme dilution, due to the application of two chromatographic separations, and a dramatic degree of MT degradation during the long times of analysis required for the two-dimensional chromatography.

The first contribution, dilution, depends on the nature of a chromatographic separation itself: 50 µl of the original sample are injected in the SEC column for the first separation. If this sample were composed at 100% of one species, eluting in a 1 minute-wide peak, the collected fraction volumes would contain the total amount of the injected sample and be of 1 ml.

Only 50 µl of it will be then re-injected in the second dimension (RPC), leading to a 1:20 dilution. Already in this hypothetical case a MT signal would sink in the background

noise, as it can be seen comparing the most intense signal of the previous chromatograms, 10000 cps, that would be reduced to 500 cps (background ~ 500 cps).

The second contribution, the significant degree of degradation of MT isoforms, was already stated for row and digested samples, as commented above and showed in Figure 35. The loss on MT isoforms was increasing in time, and took place in a more relevant way if the samples were conserved in a refrigerator than if they were freeze-dried. Nevertheless, also freeze-dried samples showed a species degradation occurring in time, underlining that the most relevant factor is a kinetic instability: if the analysis takes place within a relative short time (one day), the signals are stable and reproducible, as commented before. If the analysis requires a longer time, as it happens in the case of a two-dimensional chromatography, then the time-degradation assumes a higher weight and the species degrade. The freeze drying step itself requires a relative long time (12-24 hours) and is therefore suspected to give a relevant contribute to species degradation.

This hypothesis is supported also by the inorganic fraction signal eluted at the beginning of the RP chromatogram (compare Figure 36): this fraction should already have been excluded by means of SEC

This explains the difficulty to apply a two-dimensional approach to the analysis of these samples.

Another very relevant characteristic of MT is the elevate presence of sulphur, due to the sulfuryl groups of the very abundant cysteine residues. For this reason experiments with the aim to detect the S signal, together with those of Cu and Zn were carried out.

In order to establish a correlation between the heavy metals and the sulphur in the detected proteins, these measurements have been carried out with the ICP-SF-MS, operating at the medium resolution mode ($\Delta m / m = 4000$). In this way no spectral interferences ($^{16}O^{16}O^{+}$, $^{15}N^{16}OH^{+}$, $^{14}N^{18}O^{+}$) had to be taken in consideration for the analysis of the isotope ^{32}S.

A typical chromatogram measured in this way is reported in Figure 37:

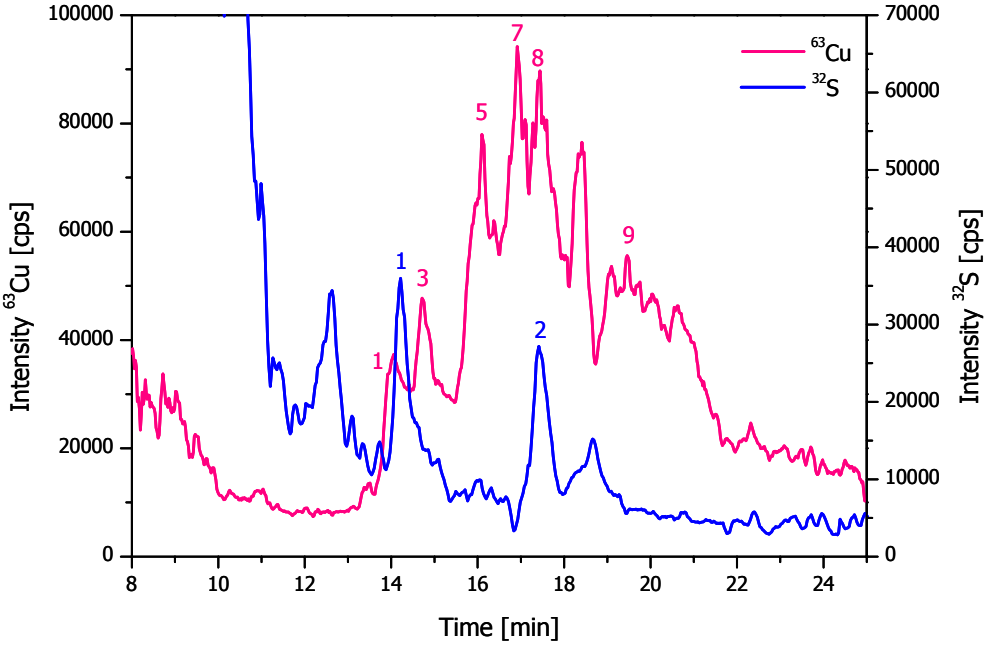

Figure 37: RPC-ICP-SF-MS chromatogram of a bovine liver extract. The numeration of the ^{63}Cu signals indicates the peaks matching their correspondence in Figure 32.

The ^{32}S peaks 1 and 2 and the ^{63}Cu peaks 1 and 8 correlate with a good agreement; furthermore, the sulphur signals have the same behaviour as the metal ones, they are good reproducible and stable within one day.

Through this experiment it was demonstrated that at least two of the metal binding species are rich in sulphur, which contributes to corroborate the hypothesis that the detected species are MT isoforms.

6.1.3. Conclusions

A digestion procedure, SEC, RPC and a two dimensional approach coupled to ICP-MS were developed for the investigation of metal binding species in bovine liver extracts.

The detected signals were characterised as follows: they are Cu and/or Zn binding proteins of a molecular weight of circa 6-7 kDa, they have very similar chemical characteristics, they are heat resistant proteins, they appear in different samples showing a reproducible and similar distribution, two of them correlate with the corresponding sulphur signal, showing a high S content of the species.

Even if the monitored elements included Cd among Cu and Zn, no Cd-bound MT was detected, confirming that the animals slaughtered were not Cd contaminated.

The direct coupling of the developed chromatographic separation to an ESI-MS was tried. Even substituting the inadequate Tris-HCl mobile phase with an ESI suitable ammonium acetate based, it has not been possible to detect MT-signals. A reason for it can be the intrinsic detection limit of the instrument, which is higher than that of an ICP-MS, and the low concentration of the investigated species in the bovine liver extract.

Even if the ultimate way to definitively prove the nature of the detected species is a soft ionisation approach like ESI-MS, all the factors deduced by the experiences reported show that the probability that they effectively represent MT isoforms is extremely high.

A quantitative approach on the detected species has not been tested, being the only standard commercially available a different matrix (rabbit liver) and well known for its poor reliability. Besides, an analysis of this standard showed two peaks, not corresponding with the retention times of the MT isoforms contained in the bovine liver.

Another important statement achieved through these experiments concerns the kinetic instability of the MT-metal bounded isoforms. It has been pointed out that the metal binding proteins undergo degradation, both in the entire liver and in the extract, as the sample gets old. The same degradation is therefore to be taken in consideration during the analysis itself: if the experiment needs too much time, a significant fraction of the metal bounded species is lost. This, in addition to the high dilution introduced by means of this procedure, can be one of the reasons why the two-dimensional approach brought us to detect no signal.

The development of an innovative procedure has brought nevertheless new information on the investigated species, and it has revealed to be a reliable and stable method. Both the SEC and the RPC experimental conditions showed their suitability for an ICP-MS coupling, and were therefore used later for the investigation of different samples containing MT, as discussed in the following paragraphs.

6.2. Qualitative investigations on human thyroid samples[96]

6.2.1. Background and motivation

Metalloproteins and metalloenzymes have been acquiring clinical and physiological significance in trace metals speciation research of biological tissues, due to the fact that they are involved in many pathophysiological processes such as metal ion homeostasis and detoxification, protection against oxidative damage, tissue-specific growth inhibition, cell proliferation and apoptosis[49].

Different pathologies can alter the metabolism of trace metals in a biological tissue, resulting in different speciation of trace metals. In particular, MT can be involved in several aspects of the carcinogenic process, cancer development and progression[97]. However, the data about expression of MT isoforms in different tumours are controversial: thus, increased MT expression has been found in subconfluent cultures of human colonic cancer cells[98], liver tumours[99], laryngeal hyperplastic lesions[100] and in peripheral blood samples from children with acute leukemia[101]. On the other hand, underexpression of MT genes in thyroid malignancy was reported[57]. Although metallic species participate in the carcinogenic process, their use as a potential marker of tumour differentiation or cell proliferation, or as a predictor of poor prognosis remains unclear because of a very complicated mechanisms of the action of trace elements on molecular level. Thyroid cancer is the most serious health problem related to the accident at the Chernobyl nuclear power plant (NPP)[102,103], which occurred on April 26, 1986, and affected a population of more than 5 million people[104].

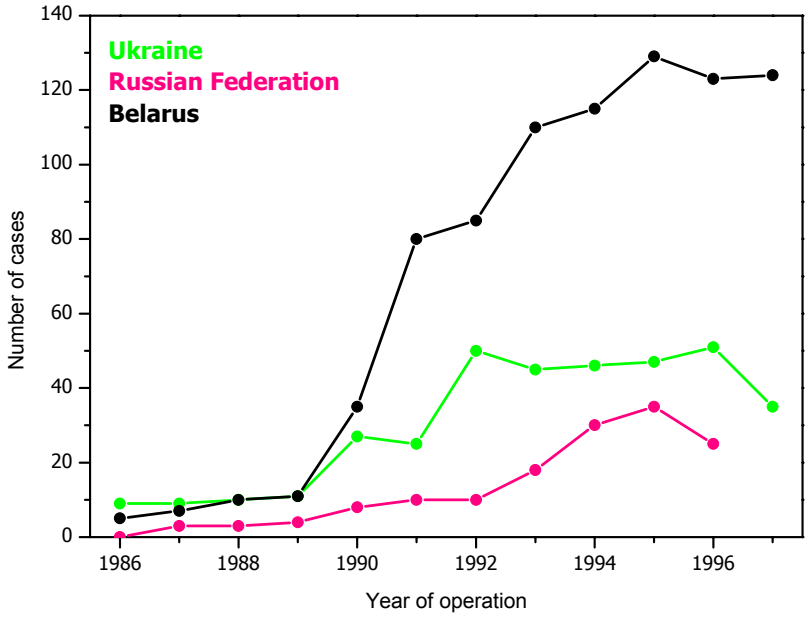

Figure 38: number of the thyroid cancer operated cases after the Chernobyl NPP accident in strong contaminated (Belarus) and less contaminated (Ukraine, Russian Federation) areas[104]

In Figure 38 is schematic shown how rapidly the number of operated thyroid cases increased in the years following the accident, and lets no doubt on the correlation of the occurrence of this pathology and the release in the environment, among long-lived radioactive isotopes, of the radionuclide ^{131}I ($t_{1/2}$ = 8.04 d), which can be accumulated in the thyroid, causing its cancer.

The frequency of this pathology in Belarus raised from about 0.04 cases per 100000 children in 1987 up to 13.5 cases per 100000 radioactively affected children in 2000 (in Gomel region it was higher than 20)[105], exceeding the expected cancer rate by a considerable extent, in particular in the youngest children at the time of exposure. A possible reason for risk underestimation can be caused by differences in metabolism of childhood and adult thyroids.

On the other hand, ecological factors, connected with iodine deficiency and endemic goitre in the southern Belarus could increase cancer risk. Thus, due to a higher absorption of the radioiodine the follicular structures of the goitre thyroids get significantly higher radiation effects than the dose estimated for the whole thyroid, resulting in an increased cell mutation. Cell mutations are usually accompanied by changing chemical composition, including formation of sclerotic sections, calcinosis and formation of psammoma bodies. Previous studies revealed the significant differences in

the elemental composition of healthy and pathological thyroids, where, for example, Zn and Sb were found at much higher concentrations in tumour thyroids[106,107]. Therefore, speciation studies of metals in biological tissues may enlighten mutagenic processes.

Beside the total element analysis in thyroid tissue[106,107], which allowed to evaluate integral changes of chemical composition in cancerous tissue over the period of cancer development, speciation analysis with soft leaching techniques provides more specific information about metal metabolism in cells and/or extracellular fluid excluding non-extractable metal ions (and corresponding metallo-species) accumulated for instance in sclerotic sections. The tumour growth alters both cells and the extracellular environment. Thus, the concentrations of cell growth specific agents in extracellular fluid reflect their intracellular levels; therefore, analysis of both intracellular and extracellular fluids is often of interest for diagnosis of disorders involving altered cell growth. In some cases analysis of extracellular fluids is preferable if the fluid can be obtained easier than the tissue itself.

The aim of the present work was application of SEC-ICP-MS for the study of metal-containing species in digests of cancerous human thyroid tissues in comparison to digests obtained from thyroid tissue of healthy inhabitants of Belarus. Both types of samples were treated in the same way to allow investigating relative differences in the distribution of metal-containing species between the two groups. The suitability of SEC-ICP-MS for the monitoring of changes in trace metal distribution in cancerous tissue is discussed.

6.2.2. Experimental set up

Instrumental

The sector field ICP-MS *Element2* was coupled to a size exclusion and a reversed phase system respectively, under the experimental conditions illustrated in paragraph 4.2. The chromatographic optimised conditions were the same as those employed for the bovine liver analysis, both for the SEC and the RPC, discussed in paragraph 6.1. The monitored isotopes are listed in Table 14:

Table 14: monitored isotopes in thyroid sample analysis

Low mass resolution (R=300)	Medium mass resolution (R=4000)
^{112}Cd, ^{121}Sb, ^{208}Pb	^{52}Cr, ^{58}Ni, ^{63}Cu, ^{64}Zn, ^{127}I

The chosen elements were all essential or toxic trace elements, investigated simultaneously in order to have a pattern of their species in the thyroid tissue. Iodine was also monitored, owing to its relevance in the hormones produced in the gland.

Samples and sample preparation

Healthy human thyroids were obtained from the Bureau of Forensic Medical Expertise from persons died as a result of accidental events from two regions: Minsk, the capital of Belarus, and the Gomel region, which was affected by Chernobyl accident and where goitre is endemic (due to iodine deficiency). The tumour thyroid tissues were obtained from operations on persons in hospitals in Minsk, Belarus. The sampling of all thyroid tissues was performed in 1996–1997. One aliquot of operated thyroid tissue was subject to molecular, cellular and biological studies to prove the cancer diagnosis, and another aliquot of the tissue was analyzed with respect to trace element speciation. The tissue aliquots for trace element analysis (10–100 mg) were freeze-dried after the sampling and sealed in quartz ampoules under nitrogen atmosphere. After that, samples were sterilized in a neutron reactor channel, whereas no significant impairment of proteins in dry tissue samples can be expected under the doses applied. It should be mentioned, that in the case of these thyroid samples the preservation of the original species composition cannot be guaranteed. Nevertheless, both kinds of thyroids (healthy and cancerous) have been treated in exactly the same way. That means after "sampling" important steps like storage, freeze-drying, etc. were performed in a uniform manner. Such uniform sample preparation allowed to perform comparative speciation analysis of healthy and thyroid tissues.

The sample preparation procedure was based on that optimised for the bovine liver extracts, schematically reported in Figure 26, although in this case only 30-60 mg sample were digested in 1 mL Tris-HCl 0.1 mol·L^{-1}.

6.2.3. Results and discussion

SEC results

Before the human thyroid samples were analysed, the digestion and the procedure for chromatographic separation of metal-containing species were optimised by means of the analysis of bovine liver extract, as described above (see paragraph 6.1).

Having no standard to optimise the system, the high amount of samples of bovine liver extract, which in a short time period show reproducible and stable signals, was used as a model substance to optimise the experimental conditions. Even if the matrix can be quite different, the metals investigated were almost the same, as well as the target species, MT. They were therefore considered a suitable mean for the setting of the parameters in the analysis of the thyroid samples.

The bovine liver extracts were good samples to simulate the experimental conditions before subjecting to them the precious samples: sample preparation, chromatographic and ICP-MS, everything was previously tested on the high abundant extracts. In particular, the freeze-drying procedure has been checked for species transformation, but showed no change on the composition of metalloproteins, which agrees with studies by other groups[93,94,108].

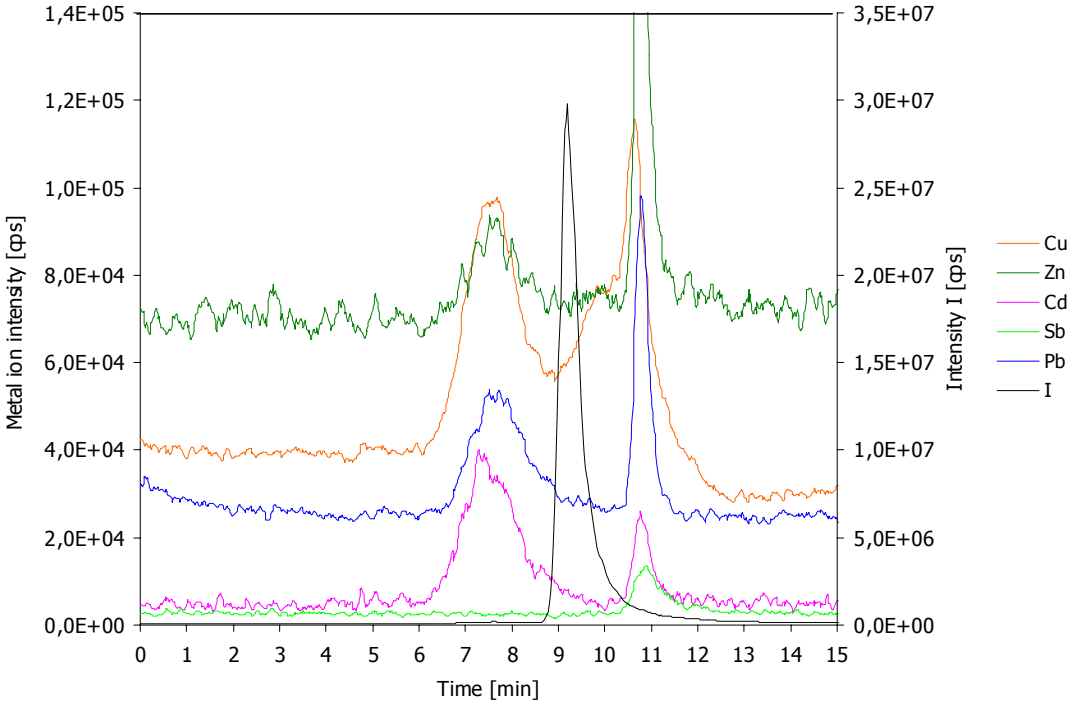

Figure 39: typical SEC-ICP-MS chromatogram obtained from a thyroid sample of an healthy inhabitant of Minsk. Ion intensities were corrected for the total element intensity by the natural isotopic abundance of the measured isotope. In addition, the intensities of the isotopes measured at a mass resolution of 4000 (see Table 14) were multiplied by the factor corresponding to sensitivity difference in low and medium resolution. The Sb intensity scale is extended by a factor of 3, for Cd by a factor of 5[96].

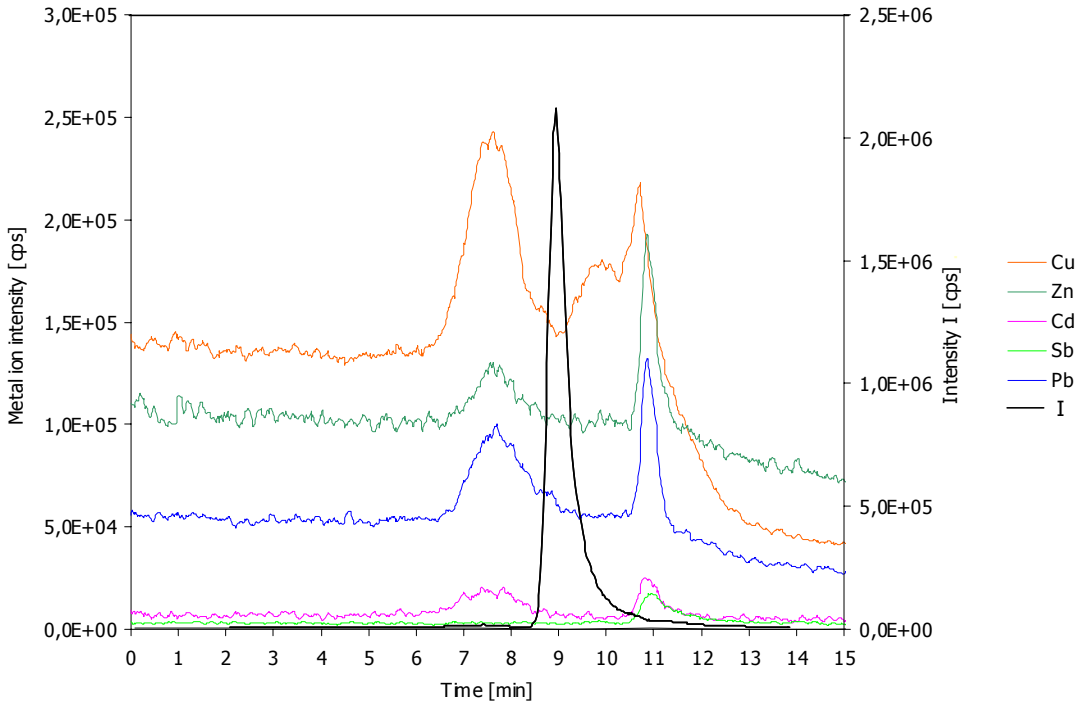

Figure 40: typical SEC-ICP-MS chromatogram obtained from a thyroid sample of a healthy inhabitant of Gomel region (intensity scales and retention time ranges are given as described in Figure 39)

Typical SEC-ICP-MS chromatograms of Cu, Zn, Cd, Sb, Pb and I-binding species in healthy thyroid tissues are reported in Figure 39 and Figure 40. A spread pattern of heavy metals were investigated, being the samples not yet measured with speciation methods. The presence of the monitored metals was determined in other works[106,107], and the aim of the investigations discussed here was to understand how these metals were present in the sample. Once more, speciation analysis faces the problem from a more subtle point of view, bringing to evidence a metal pattern that could not be stated by means of a total-element determination. Thus the necessity to get the more information possible, monitoring also "exotic" elements for a MT study, like Sb.

The high presence of iodine in the thyroid, due to the gland's function, was a reason to monitor this element as well. Furthermore, being iodine only inside the thyroid's produced hormones, its signal was used as an internal standard, producing a sharp and intense peak at very reproducible retention times, and belonging to an approximately known molecular weight species, as discussed later.

The retention time and molecular mass correlation is the same as in the bovine liver-SEC analysis (see Figure 28): between 5 and 9 min compounds of molecular weight of 6-7 kDa, comparable to those of MT, are eluting.

Copper is presented by three peaks; the one eluting with a retention time of about 7.5 min corresponds to MT. The second peak at the retention time of about 9.5 min (which was more pronounced in thyroids of Gomel inhabitants) is close to thyroid specific iodine-binding species (thyroxine with a molecular mass of 776.87 Da and triiodothyronine with 650.98 Da) detected at t=9 min and probably represents Cu-species with a molecular mass of a few hundreds Da. The sharp peak observed at t=11 min is correlated with a well pronounced sulphur peak (not shown here). The latter peak is mainly caused by complexation with mercaptoethanol, which has a good binding capacity for metals.

Zn, Cd and Pb correlate with Cu, whereas Cd/Cu and Pb/Cu ratios are generally lower in the tissue of the Gomel inhabitants (thus, Cd/Cu intensity ratio in MT peaks varied from 0.022 to 0.056 and from 0.045 to 0.11 in tissue samples from Gomel and Minsk, respectively; Pb/Cu intensity ratio in MT peaks varied from 0.25 to 0.37 and from 0.43 to 0.72 in thyroids of Gomel and Minsk inhabitants, respectively).

Thus, an accumulation of heavy metals in MT in thyroids from Minsk inhabitants (an industrial city with about 1,700,000 population) in comparison to Gomel (country region) was observed which may be due to environmental conditions (e.g. industrial contamination of Minsk with these elements[109]).

Iodine concentrations in some thyroids of Gomel inhabitants (endemic goitre region) were lower than those in thyroids of Minsk inhabitants, caused by the inhibition of the thyroid function as shown in a previous work[106]. A chromatogram obtained for a thyroid sample associated with iodine deficiency is presented in Figure 41:

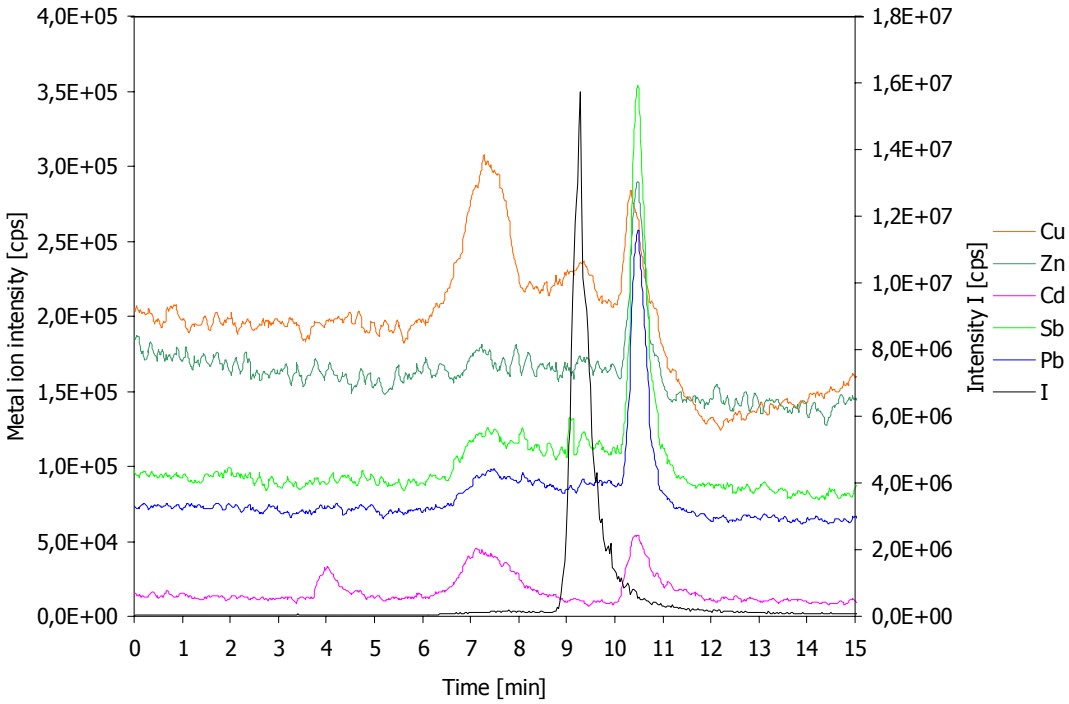

Figure 41: SEC-ICP-MS chromatogram obtained from a goitre thyroid sample of a Gomel inhabitant (intensity scales and retention time ranges are given as described in Figure 39)

In this sample, no zinc peak could be detected at the retention time typical for MT, however, intensities of other metals, in particular Cd and Sb, were more pronounced. It should be noted, that an additional peak of high-molecular Cd-binding species was only detected in the thyroid tissue with iodine deficiency at a retention time of about 4 min. Thus, a pathological state of the organ (in this particular case thyroid goitre) results in different metal metabolism, and consequently, the observed metal species distribution was changed.

It should be noted that zinc speciation analysis by SEC-ICP-MS was affected by a relatively high background baseline, which made Zn-speciation analysis difficult in the retention time range (b) for MT. The high Zn background was probably due to a high concentration of inorganic zinc (total Zn concentration reached 14 mg g^{-1} in dry thyroid tissue[106], which did not completely bind to mercaptoethanol and thus remained in the buffer solution.

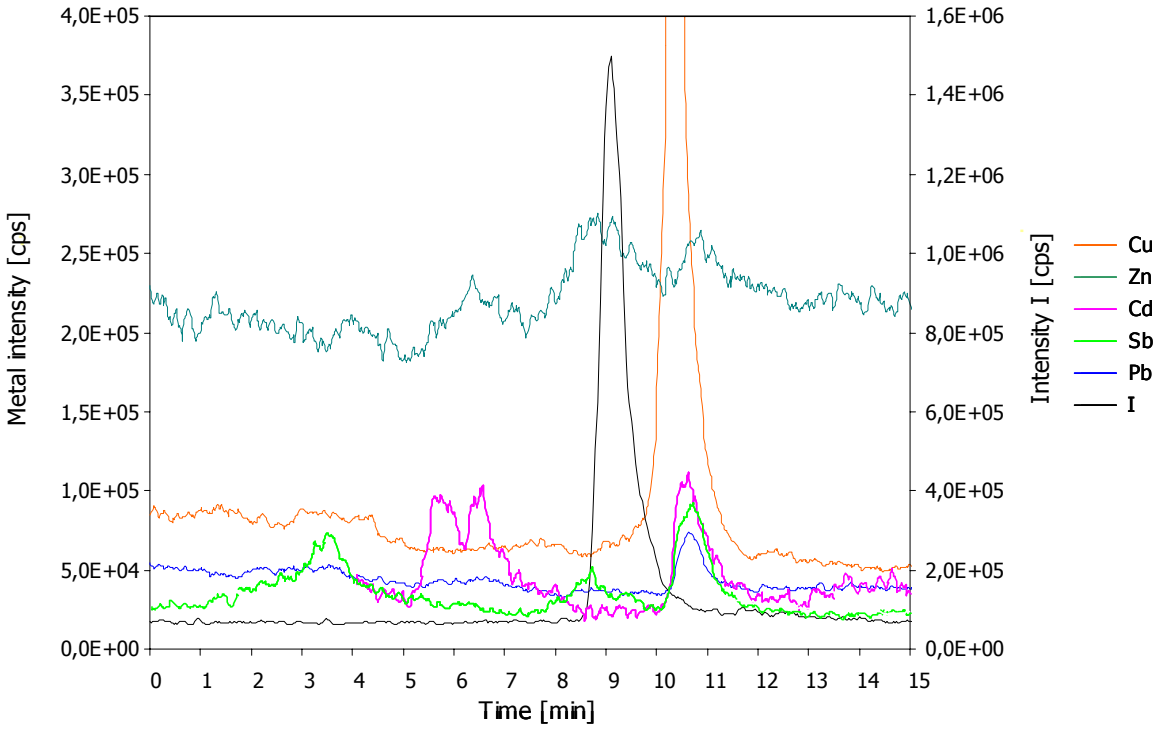

Figure 42: typical SEC-ICP-MS chromatogram obtained from a cancerous thyroid sample (intensity scales and retention time ranges are given as described in Figure 39)

Figure 42 presents the results of the speciation study of trace elements in thyroid tissues of a person with cancer. Measured iodine concentration was lower by more than one order of magnitude in cancer tissue compared to healthy thyroids (see reference 106, Figure 40 and Figure 42), hence, the main function of thyroid, production of iodide containing thyroid hormones T3 and T4, is strongly inhibited in cancer tissue.

The most spectacular result was that, contrary to healthy tissues, no MT were detected in cancer thyroids. However, Cd- and Sb-binding species were observed at the high-molecular protein site, detected at a retention time of 3–4 min. In addition, two Cd peaks were observed at t=5.5 min and t=6.5 min.

Figure 43 presents SEC-ICP-MS chromatograms of species containing Sb, Ni and Cr obtained for another sample of thyroid cancerous tissue. Unlike Cu, Cd and Pb, Cr was bound only to high-molecular species (detected at a retention time of t=3–5 min). In general, metal speciation differed from sample to sample in pathological tissues, and was completely different from metal speciation in healthy thyroids.

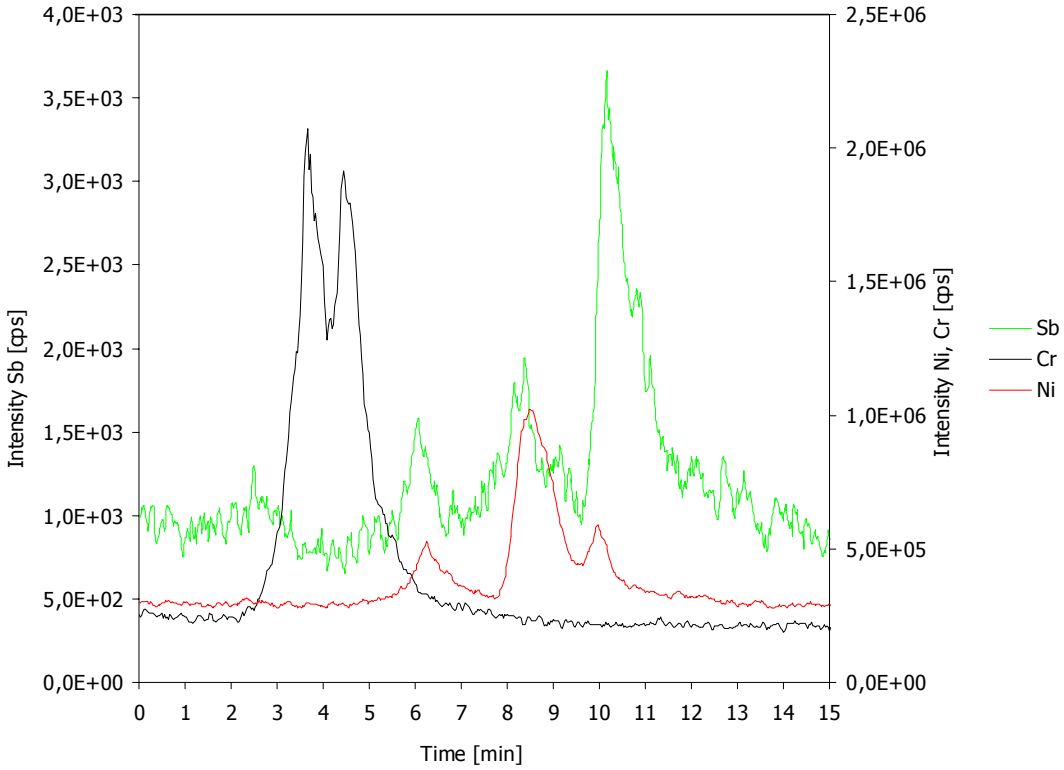

Figure 43: SEC-ICP-MS chromatogram of Ni, Cr and Sb speciation in a cancerous thyroid sample (ion intensities were corrected for the total element intensity by the natural isotopic abundance of the measured isotope. The Ni intensity scale is extended by a factor of 2. Retention time ranges are given as described in Figure 39)

As already obtained for the bovine liver samples, SEC-ICP-MS yielded good reproducibility when analysing repetitiously the same sample (relative standard deviation of retention time for Cu peaks did not exceeded 1%, the RDS of peak area corresponding to MT was 3–5%). Furthermore, a good reproducibility of chromatographic results of Cu, Zn, Cd and Pb-containing species was observed in different thyroid samples (copper chromatograms obtained for healthy and cancerous tissues of three different persons are compared in Figure 44). On the other hand, chromatograms for tumour tissues (Figure 44b) differed remarkably from those obtained for the healthy ones (Figure 44a).

The main difference to the healthy thyroid samples is the *total absence* of the MT fraction at a retention time of about 7 min in SEC-ICP-MS chromatograms. This effect was observed in all available thyroid samples.

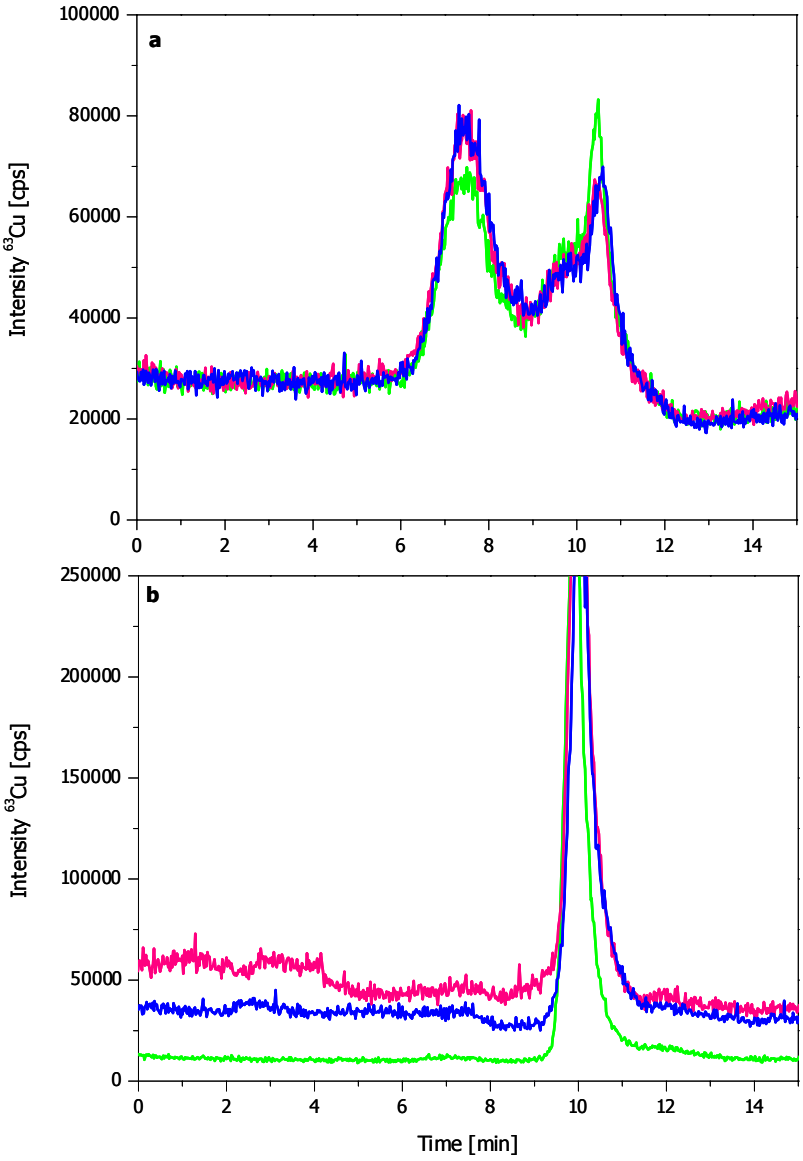

Figure 44: SEC-ICP-MS chromatograms of copper-containing species obtained from (a) thyroid samples from three different healthy Minsk inhabitants; (b) from cancerous thyroid samples of three different persons.

The reproducibility of the signals among different samples is shown for Cu in Figure 44. The identical distribution (no MT in cancer-affected thyroids) was observed for other metals, such as Zn, Cd and Pb, as shown in the following chromatograms (Figure 45):

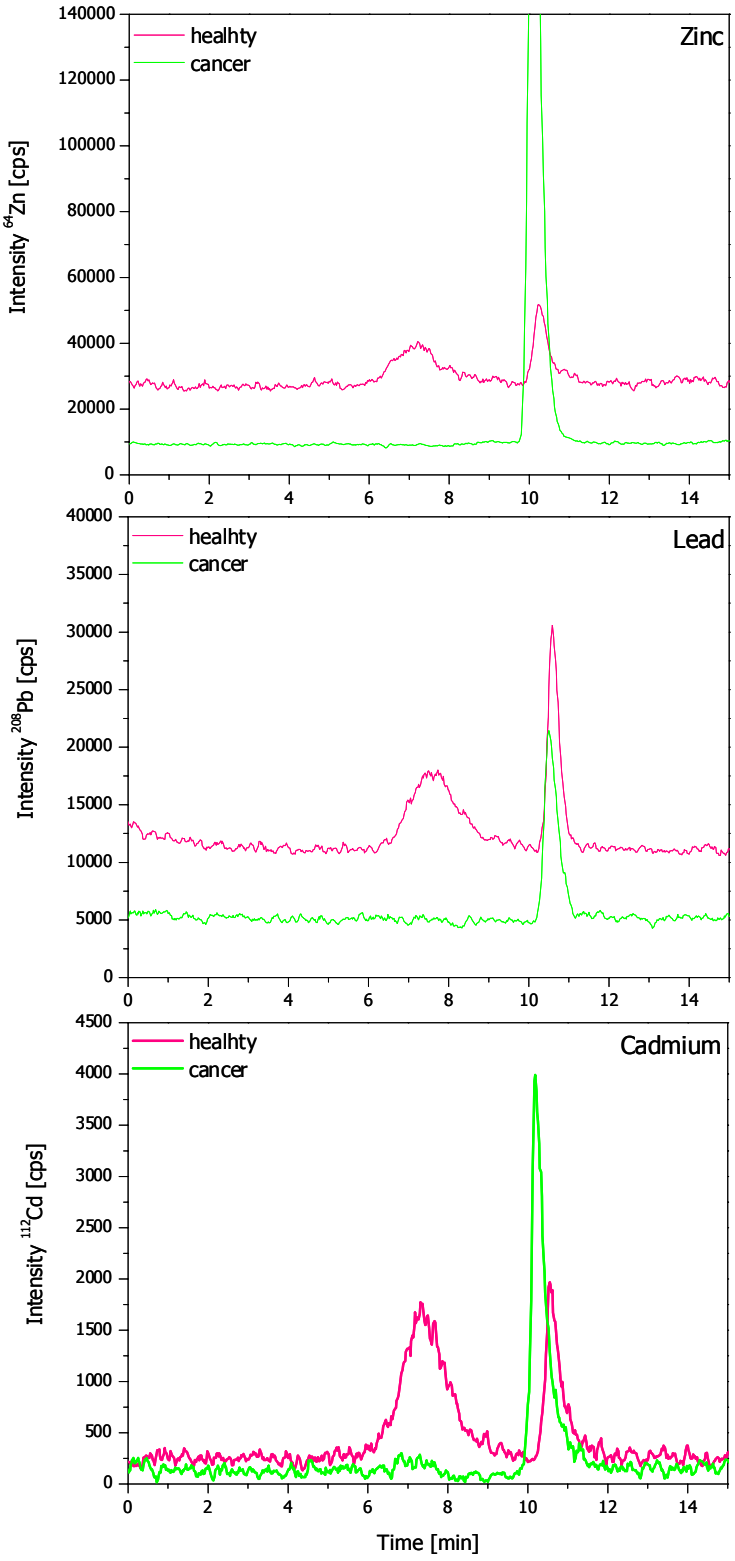

Figure 45: distribution of Zn, Pb and Cd in healthy and cancer affected thyroid samples

RPC results

The healthy thyroid samples analysed by means of RPC-ICP-MS showed the poor variety of MT isoforms, if compared to the abundance of signals registered for the bovine liver extracts (compare Figure 46 and Figure 32).

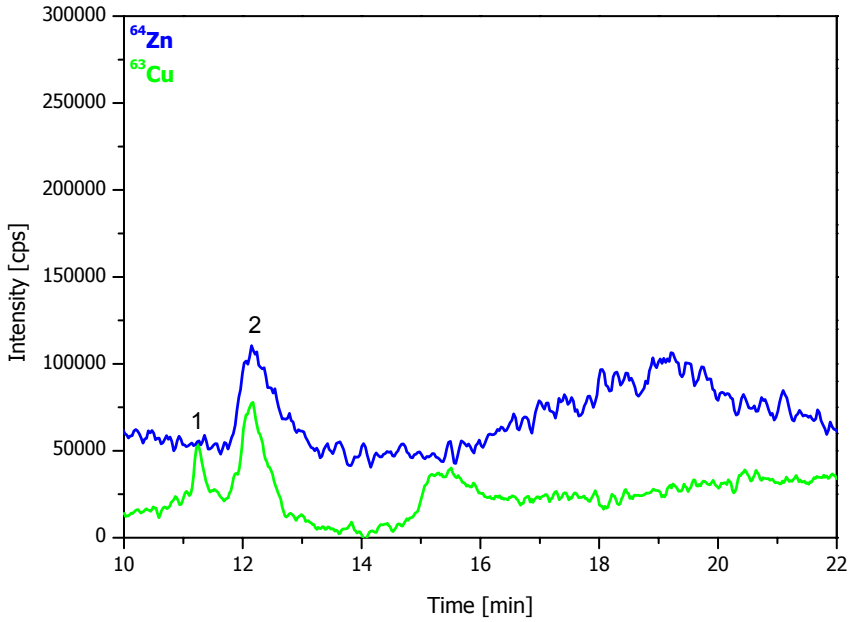

Figure 46: RPC-ICP-MS chromatogram of the Zn and Cu patterns in a healthy thyroid sample (MT elution range)

In the thyroid extract only two MT isoforms bind Cu (peak 1 and 2 in Figure 46) and one of these complexes Zn as well (peak 2), while in a bovine liver extract 9 Cu-MT and 3 Zn-MT isoforms were regularly detected. This result can be explained considering that, unlike liver, the thyroid is not an organ directly involved with metabolism and heavy metals detoxification.

Moreover, the signals are quite low in intensity, if compared to the background noise, than the MT signals of a bovine liver. This can be explained by the fact that a lower quantity of sample was weighed and digested in the case of thyroids, and once more because a lower amount of MT is present in this organ.

In Figure 47 a cancer affected thyroid extract RPC-ICP-MS is reported. Even if the SEC showed clearly that no species were eluting at the MT molecular weight range, with the

use of the RPC alone a signal at 12 min, in the MT-range, was present. The strong difference in concentration of these species is confirmed also in the RPC analysis, by the lower signal intensity of the peaks in Figure 47 if compared to Figure 46, showing once more a damaged metabolism in cancer affected thyroids.

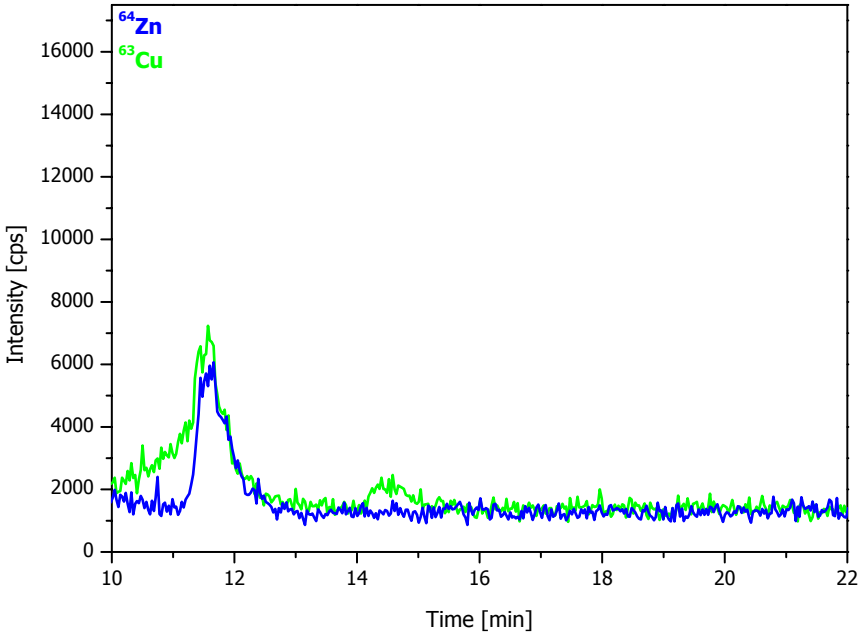

Figure 47: RPC-ICP-MS chromatogram of the Zn and Cu patterns in a cancer affected thyroid sample (MT elution range)

This result, apparently at variance with the SEC results, can be explained comparing the baseline for the cancer affected thyroids in the chromatograms shown in Figure 44 and in Figure 45 with the baseline of Figure 47.

In the case of the SEC chromatogram, the baseline for Zn has an order of magnitude of circa 10000 cps, while in the RPC chromatogram the same signal is assessed at about 1000 cps. For what concerns Cu, the same reasoning is valid: in Figure 44 the baseline varies between 10000 to 50000 cps, while in Figure 47 it is about 1000.

Considering that the amount of MT in the thyroid samples is, as it has been already discussed, very low, a small MT signal could have been swallowed by the background noise, in the case of SEC investigations. The reason for a high background in the SEC analysis can depend on the SEC separation mechanism. Being the separation regulated by a physical effect, inside the column the extremely small non complexed metal ions can

be trapped in the pores, where they remain for a non predictable time, and are then slowly eluted, leading therefore to a poisoning effect.

The mechanism of separation of an RPC makes the column not so dramatical subjected to such effects, having the non complexed heavy metal ions no chance to remain inside the column, differently from what can happen with the pores of the SEC, which produces a lowered background.

This new amount of information leads necessarily to a renewed discussion and revision of the SEC results: are the MT actually absent, in the case of cancer affected tissues, or are they present but hidden under the detection limit? Before answering this question, it must be taken in consideration the high amount of data realized with the SEC-ICP-MS in comparison to the relatively poor pattern from the RPC-ICP-MS analysis; and also the fact that the reproducibility of the SEC results is higher than that of the RPC.

The topic requires clearly more data, with both chromatographic methods, in order to have a comparable amount of cases in RPC and SEC; a different approach is also to foresee in the future: a soft ionisation analysis or a two dimensional separation. By gaining more information about the exact nature of the peaks detected (above all on the RPC signals), it will be certainly possible to explain these discordant results.

As discussed before, different strategies show often parts of the problem which would otherwise remain in the shadows.

To gain more information with the means at disposal, as it was done before for the bovine liver samples, the ^{32}S trace was measured, to determine if there was any correlation between heavy metals and sulphur peaks. The samples have been therefore analysed in the medium resolution mode (R=4000).

The results for a healthy thyroid sample are shown in Figure 48: a very good correlation of Cu-peak 2 and the corresponding S peak is once more a confirmation of the nature of these signals.

Once again, if compared to cancer affected thyroids, healthy thyroid chromatograms show a higher intensity in the MT signals, and a wider variety of species.

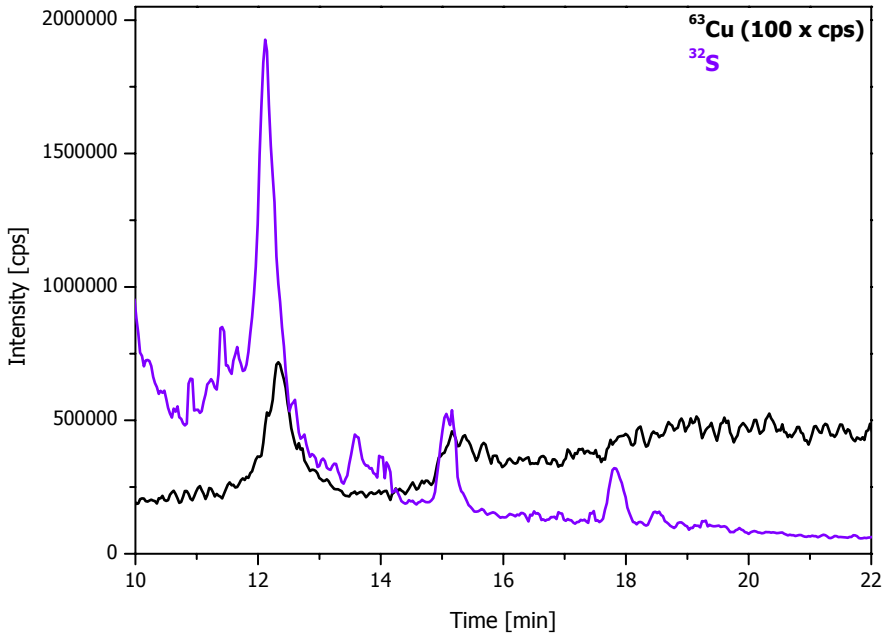

Figure 48: Cu and S pattern in a healthy thyroid sample (MT elution range)

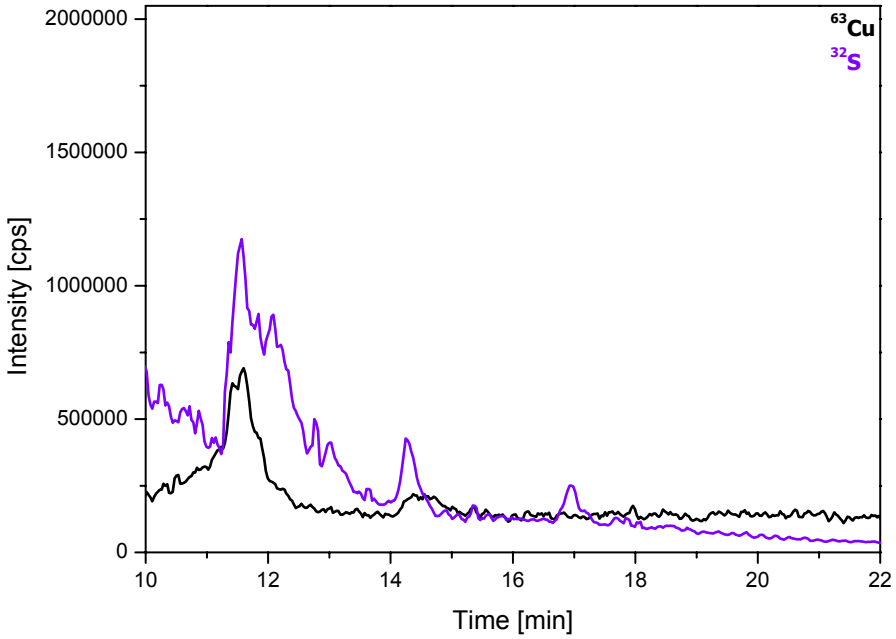

Figure 49: Cu and S pattern in a cancer affected thyroid sample (MT elution range)

A two dimensional approach based on a SEC-RPC separation would finally deliver a significant amount of information on the nature of these species. The procedure followed was the same as for the bovine liver samples: the SEC fraction corresponding to the MT-molecular weight range was collected, freeze dried and re-dissolved in a smaller volume of mobile phase, to afford a pre-concentration of the species. Then this new sample was injected in the RPC column and ICP-MS monitored.

Considering that in general the separation of the species of interest from the matrix without changing their original composition, and the separation of the species themselves are connected with a significant analyte dilution, the detection limit seems to be at a glance the biggest obstacle to overcome.

Actually, in this case even the use of the highly sensitive ICP-MS was not of advantage and a two dimensional chromatography was not successful: no signal at all was detectable in the second dimension, as it is shown in Figure 50:

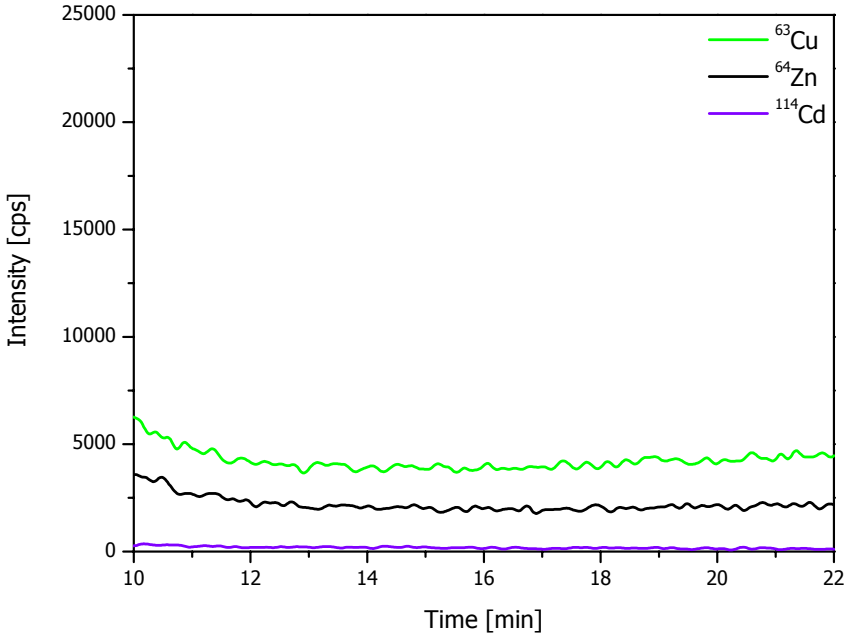

Figure 50: 2-dimensional approach to the thyroid samples: after the SEC/RPC no ICP-MS signal can be detected.

Considering the results on bovine liver samples previously obtained, and the even lower concentration of the MT isoforms in thyroid tissues, the high dilution of the methods can explain the negative results.

Furthermore, the time-stability of the MT-metal complexes inside the samples investigated is still a question mark: the long analysis times required by a 2-D chromatography can lead to species degradation, and thus to the stated lack of signals.

6.2.4. Conclusions

Pathological states of thyroid, e.g. thyroid goitre, seem to affect speciation of trace metals in this organ. Thus, different SEC-ICP-MS chromatograms were observed for several elements (Cd, Sb and Pb) in goitre tissues in comparison to healthy ones.

Even more severe differences in metal speciation were found in tumour thyroids: pronounced MT fractions were found in thyroids of healthy Minsk and Gomel inhabitants, whereas no MT could be detected in cancerous tissues by using SEC-ICP-MS. The observed changes of chemical composition and metal speciation could promote pathology development after it has been initiated by irradiation with radioiodine due to the mutagenic and carcinogenic properties of some elements (Cd, Cr, Ni, Pb, Sb).

In the frame of this work, SEC-ICP-MS showed a good reproducibility of chromatographic results and stability of metal-containing species in the digests over the analysis times required for a monodimensional separation. Even if the identification of metalloproteins was not possible due to a lack of appropriate standards and the incompatibility of the chromatographic conditions with a soft ionisation-MS technique, the detected peaks can be with a high probability MT-isoforms.

The confirmation of this hypothesis comes also from RPC-ICP-MS measurements, which show, in the MT eluting range, quite intense M^{n+} signals correlating with S. In cancer affected tissues a poor variety of species was detected; the MT signals are still present, even if in decidedly lower concentrations.

Unfortunately, through a two-dimensional separation no signal was detectable, as a consequence of one or both the following effects: the species are too strongly diluted and the signals remain under the detection limit; the species are degraded during the long times required for the analysis.

Nevertheless, MT metal binding species have been detected by means of a simple one dimensional chromatography, stating a conspicuous difference between the cancer affected and the healthy samples. To gain more information, to identify definitely the detected species and to explain some contrasting results between RPC and SEC measurements, is required not only a soft ionisation detection, but also a suitable chromatographic separation for this technique, a pre-concentration procedure and a kinetic stability study, to investigate whether the species undergo effectively degradation during the analysis, as supposed, or remain stable.

7. HEAVY METAL SPECIATION IN ALGAE AND PLANTS: PHYTOCHELATINS

7.1. Background and motivation

Investigations on PC synthesis, their function and their structure have been carried out up to now intensively, pointing out that the stability of the Cd-PC_n complex in the cell cytosol is the basis of plants Cd tolerance[110,111,112]. Also for this field's investigations, hyphenated techniques are demonstrated to be a very powerful tool [113 ,83,112, 114]. Nevertheless, the complex matrix of plants extracts, the low stability and the low concentration of PC complexes with metals play a fundamental role in the determination of such species.

With the aim to achieve more information on M^{n+}-PC complexes, as it has been done for bovine liver and the thyroid samples, a new chromatographic method for the separation of the species was developed. In this case a neutral mobile phase assisted by an ion pairing reagent was employed, in order to elute the Cd-PC complexes and detect the Cd-signal via ICP-MS. To optimise this completely new separation method, and being no standard commercially available, a model sample (as it had been for MT the bovine liver) was therefore employed. The extracts of cultures of the monocellular alga *Phaeodactylum tricornutum* incubated with cadmium supplied a stable, characterised and quantified, abundant and simple-matrix sample.

Thank to the cooperation with the Biophysical Institute at the CNR of Pisa (Italy), where the algae were incubated, prepared and purified through a preparative SEC, it was possible to perform a quick and efficient optimisation of the chromatographic separation. The long and deep experience of the Italian group on these samples constituted a great advantage in the sample investigation and in the evaluation of the results.

The complete method was therefore a two-dimensional chromatographic separation, in which the second dimension was represented by the developed ion-pair reversed phase chromatography (IP-RPC), to resolve the different PC in the SEC fractions.

Special care has been taken to investigate the stability of Cd-PC during the sample preparation and for this purpose an isotopically enriched ^{116}Cd-PCs have been synthesised and used to study possible transformations and degradations of the species.

The method was finally applied to more complex plants extract.

7.2. Experimental

7.2.1. Isolation of native Cd-PC complexes from Phaeodactylum tricornutum

Stock cultures of the marine microalga *P. tricornutum Bohlin* (Bacillariophyceae) were grown in axenic conditions, at 21°C, using continuous illumination under fluorescent daylight (100 μmol photons·m^{-2}·s^{-1}). The growth medium was natural seawater added with the f/2 enrichment solution for microalgal culture (f/2 solution contains major nutrients like nitrate and phosphate, essential trace elements and vitamins), at one fifth the normal trace metal concentration and without Cu[115]. Calculated volumes of these stock cultures were used to inoculate 1-liter culture medium. Five days later, at the beginning of the stationary growth phase (cell density $4 \cdot 10^6$ cells·mL^{-1}), Cd was added to obtain a concentration of 10 μmol·L^{-1}.

After 6 hours the cells were harvested by filtration through 1.2 μm membrane filters, rinsed extensively with natural seawater and then re-suspended in 2 mL of 25 mmol·L^{-1} phosphate buffer (pH 7.5). Disruption of the algae was performed by sonication (Sonopuls Ultrasonic Homogenizer, Bandelin) for 3 minutes with a repeating duty cycle of 0.3 s, in an ice bath. The cellular homogenate was centrifuged (35000 g, 60 min, 4°C) and the supernatant was applied to the Hi-Load Superdex 30 preparation grade size exclusion column (60 x 1.6 cm, Pharmacia Biotech) eluted with 25 mmol·L^{-1} phosphate buffer (pH 8) at a flow rate of 1.0 mL·min^{-1}. The SEC system consisted of a Pharmacia Biotech Pump P-500, a Rheodyne model IV-7 injection valve and a photodiode-array detector (SPD-M10A, Shimadzu).

Cd-PC complexes were isolated by heart cutting of the chromatographic peaks and then characterized for their PC and Cd content. The chromatographic fractions were evaporated using a centrifuge (Hetovac) linked to a freeze-dryer (Edwards-Modulyo) and stored at -70°C.

γ-glutamyl peptides were determined by reversed-phase-HPLC after derivatisation with the fluorescent probe mBrB as reported by Scarano and Morelli[111]. Cd was assayed by atomic absorption spectroscopy (Perkin Elmer Model 1100 B equipped with Model HGA 700 graphite furnace).

7.2.2. Silene vulgaris sample preparation and analysis

S. vulgaris seeds were kindly provided by the Botanic Garden of Mainz University. In order to imitate a real plant growth, they have been planted in commercially available soil

purchased in a garden store, and treated with tap water and commercial fertiliser. After their germination, the sprouts have been planted with the same soil in separated vessels, and treated with nutrition solutions containing each Cu^{2+}, Pb^{2+}, Mn^{2+}, Cd^{2+}, Hg^{2+} or Zn^{2+} in the concentration of 1 or 10 $\mu g \cdot g^{-1}$. After 13 weeks the plants produced a sufficient amount of roots, stipes, leaves and flowers that have been separately sampled, washed with water and digested. The weighed sample amount was between 20-30 mg of plant material.

The mild digestion consisted in a previous treatment of the plant material with liquid N_2 with mortar and pestle, followed by the extraction with degassed 0.1 $mol \cdot L^{-1}$ ammonium acetate and 1 $mmol \cdot L^{-1}$ phenylmethanesulfonyl fluoride (PMSF) for 3 hours at room temperature. The samples were finally centrifuged (4000 rpm) and the supernatant filtered (0.2 μm). This solution was adequately diluted in 20 $mmol \cdot L^{-1}$ CH_3CONH_4 and injected into the HPLC system, under the same conditions described for the *P. tricornutum* samples.

7.2.3. Oat and wheat sample preparation and analysis

For further studies on the reliability and the suitability of the sample preparation procedure, a mixture of oat and wheat seeds was treated with a multielemental solution of fertilizer, tap water and 10 $\mu g \cdot ml^{-1}$ Mn^{2+}, Cd^{2+}, Zn^{2+} and Cu^{2+} respectively. This sample was chosen in response to the need of an easy-to-grow, high biomass producing and quickly growing plant, able to produce within less than a week a large amount of both roots and shoots for laboratory analysis.

After 10, 20 and 40 days samples of roots and shoots of 1-2 g in weight were collected and digested with the same procedure described above for the *S. vulgaris* samples.

7.2.4. IPC-ICP-MS detection of Cd-PC

The freeze-dried SEC-fractions were dissolved in 1 mL water, diluted 1:10 in degassed 20 $mmol \cdot L^{-1}$ CH_3COONH_4 and directly injected in the reversed phase column (Synergi Polar, see Table 8 for details). The employed HPLC system was the same as described for the bovine liver samples analysis (see paragraph 6.1.)

The employed mobile phases were A: 20 $mmol \cdot L^{-1}$ CH_3COONH_4 / 0.04% (w/w) TBAH; B: 20% A and 80% MeOH. The optimised gradient was the following: 0-20 min from 12.5 to 75% B; 20-22 min 75% B; the flow rate was 0.4 $mL \cdot min^{-1}$ (see Figure 51).

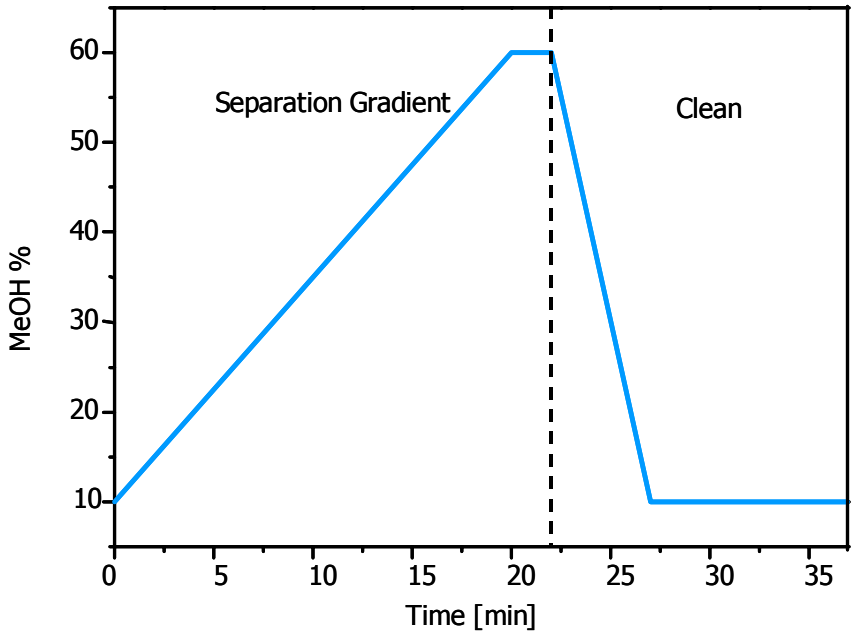

Figure 51: separation gradient optimised for PC speciation with IPC-ICP-MS

Due to the high amount of methanol (up to 60%), again a post-column dilution with a solution containing HNO_3 0.3% (w/w) and 10 $ng \cdot g^{-1}$ Rh was necessary; Rh was used to monitor the sensitivity variations due to the increasing amount of MeOH. The flow rate of this solution was 0.8 $mL \cdot min^{-1}$, provided via a peristaltic pump and a dead-volume-free PEEK t-junction.

The *Element2* sector field ICP-MS was employed for the detection, being run under the operational parameter reported in Table 10. The low resolution mode was chosen, to monitor the following isotopes: ^{55}Mn, ^{60}Ni, ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{64}Zn, ^{112}Cd, ^{114}Cd, ^{116}Cd, ^{202}Hg, ^{208}Pb.

Not all the monitored isotopes are shown in the following chromatograms, but only those significant for the analysis.

7.2.5. ESI-MS characterisation

For some samples ESI-MS measurements were carried out to characterise the nature of the species. The employed instrument was the LCQ (ion trap), under the experimental conditions described in paragraph 4.2.3.

7.3. Results and discussion

7.3.1. Method optimisation

P. tricornutum samples SEC-UV/Vis analysis

Size-exclusion chromatograms of crude extracts of cells of *P. tricornutum* exposed for 6 h in Cd-enriched (10 μmol·L^{-1} Cd) growth medium were characterised by several inducible UV (254 nm) peaks in the middle section of the elution range of the column (chromatogram shown in Figure 52).

Based on previous studies on cellular Cd speciation in this alga[111,115,116,117] these peaks are referable to sulphide-free Cd-PC complexes and are due to the sequential elution according to the molecular weight of the oligopeptide involved in the complex. PC-coated CdS nanocrystallites, Cd-PC complexes in which inorganic sulphide ions are incorporated, also synthesized in this alga in response to Cd[117], were expected to be synthesized at very low extent under actual Cd exposure conditions and to elute earlier than sulphide-free Cd-PC complexes.

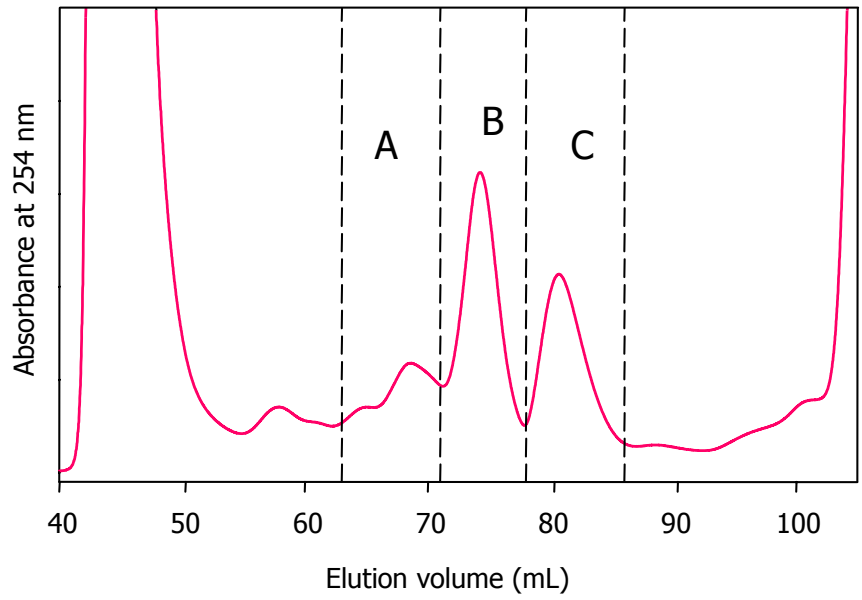

Figure 52: SEC-UV/VIS of a crude extract of *P. tricornutum*

Characterization of γ-glutamyl peptides showed that sulphide-free Cd-PC complexes with n > 4, n = 4 and n = 2-3 were present in the fractions collected under the peak A, B and C, respectively (Table 15).

Table 15: concentration of PC (γ-Glu-Cys units, μmol·L^{-1}) in the size exclusion fractions as reported in Figure 52

Fraction	PC_2	PC_3	PC_4	PC_5	PC_6	PC_7
A	-	-	1.2	5.0	3.8	1.1
B	-	2.2	21.0	0.9	-	-
C	1.0	19.8	0.5	-	-	-

P. tricornutum SEC-fractions IPC-ICP-MS analysis

Due to the lack of standards, the analysis of Cd-PC complexes through a reversed-phase chromatography raises a problem, unlike the SEC, that gives univocal information depending on the molecular weight. Such difficulties can be resolved easily with the characterised algae SEC-fractions, which, as mentioned above, represented a very useful model sample to optimise the experimental conditions for a successful separation and detection of PC via IPC-ICP-MS. In fact, the SEC fractions of *P. tricornutum* presented the following advantages: the order of magnitude of the PC molecular weight, and therefore of the PC identity, was known; the concentration of the PC in the solution was extremely high, if compared to the natural one found in a common plant extract, being not only obtained from a high amount of algae material, but also being the original 7-8 mL fractions freeze-dried and then re-dissolved in 1 mL; they had a simple matrix, having already been purified and separated from other parts of the original matrix by means of the SEC.

Due to these properties, they provided intense signals for the already characterised species. The reproducibility concerning the retention times is better than 1.7 % RSD (n = 5). Typical chromatograms are shown in Figure 53, for which three different fractions of the SEC separation (*a, b* and *c*) were collected and further separated via IPC.

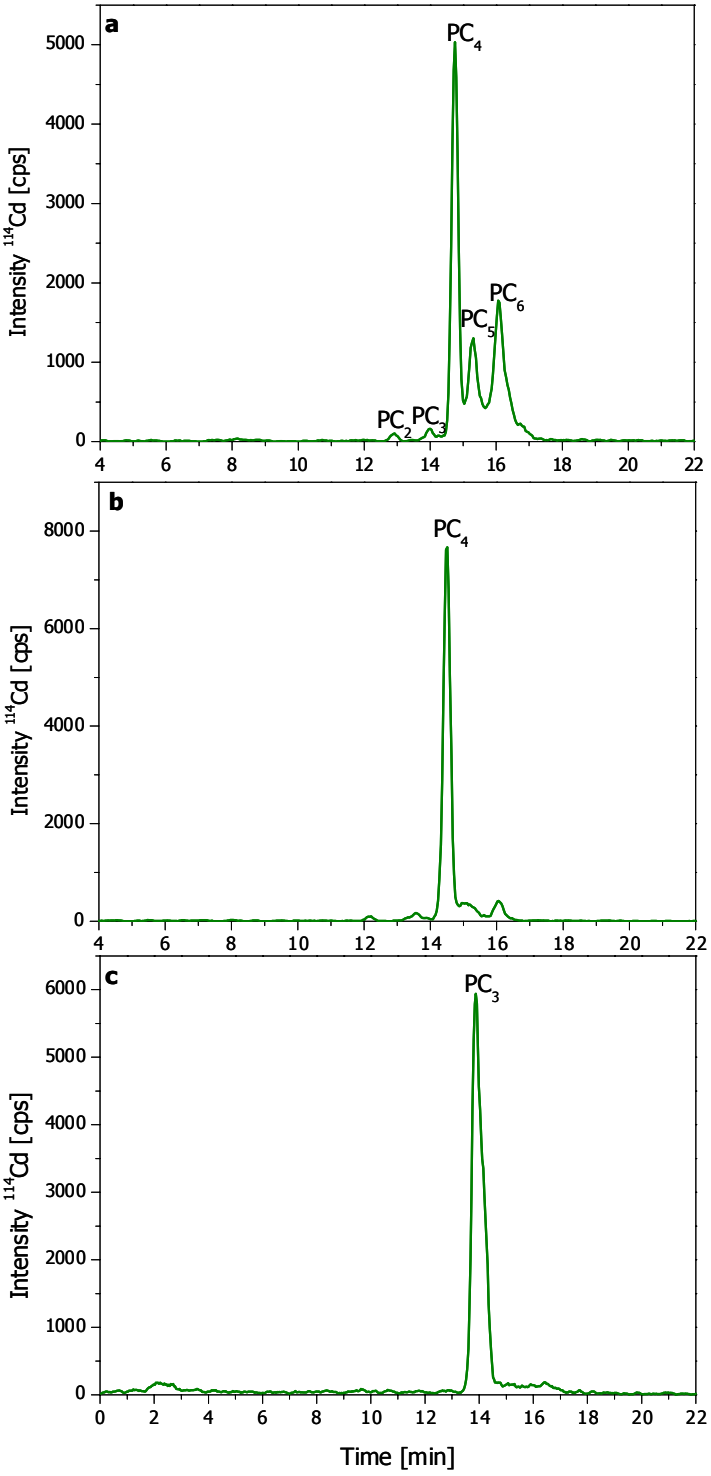

Figure 53: IPC-ICP-MS of fractions A, B and C respectively

While the fractions *B* and *C* contain basically one form of Cd-PC, Cd-PC_4 and Cd-PC_3 respectively, the SEC fraction *A*, a wide signal in the UV chromatogram which partially

overlaps the *B* fraction (see Figure 52), revealed three very intense Cd-PC signals, corresponding to Cd-PC_4, Cd-PC_5 and Cd-PC_6. Two more small peaks appear, matching the retention times of Cd-PC_3 and Cd-PC_2, probably a product of degradation of the longer chains.

The complementary information achieved through the peptide characterisation of the PC after the SEC and the complex detection after the IPC, allowed a comparison of the IPC-ICP-MS chromatographic data with those obtained from the independent determination of the apoproteins (listed in Table 15). The correspondence was excellent for fractions *B* and *C*: as expected, PC_3 and PC_4 were respectively the dominant species which were detected by means of the UV-Vis analysis as well as through ICP-MS, as shown in Figure 53, part *B* and *C*. However, a significant difference for fraction *A* was observed: instead of PC_5 being the dominant species in this fraction, IPC showed Cd-PC_4 as most intensive peak (Figure 53, part *a*). It was supposed that either Cd^{2+} preferably binds to PC_4, or this complex is less prone to degradation or transformation.

On the whole, the peak identification was carried out by means of comparison between the species contained in the SEC fractions; as mentioned above, the reproducibility of the retention times was good and the separation of the Cd-PC_n in the second dimensional chromatography satisfactory: the method was therefore ready for more challenging and complicated samples, like plants extracts.

7.3.2. S. vulgaris analysis

The use of *P. tricornutum* SEC fractions has had as primary goal the optimisation of the separation gradient, in order to elute PC still bound to Cd through IPC and detect the Cd trace through ICP-MS. Subsequently, the method was applied to investigate other samples, the *S. vulgaris* extracts, prepared as described above (paragraph 7.2.2.).

S. vulgaris is a well known and easy-to-grow widespread plant, which is able to resist on contaminated soils (e.g. Cu and Pb) and whose wide adaptability is very well known[83, 118 , 119 , 120]; being a heavy metal tolerant plant, it has been already widely employed for PC studies[118,119].

Heavy metal species pattern

S. vulgaris sprouts have been treated with concentrated heavy metal solutions as reported in paragraph 7.2.2. Once near the end of their life cycle, the plants have been harvested and samples of leaves, stipes, roots and flowers were separately digested and analysed.

The IPC chromatograms obtained from the different plant parts did not show noticeable differences, as it is shown in Figure 54 for Zn. The heavy metal species in the leaves and stipes extracts were in almost all samples lightly more abundant than those in roots and flowers. This can be explained considering the mobilisation of the species inside the mature plants: the largest part of them has left the roots and has been accumulated whether in the leaves' or in the bodies' cells. The low concentration in the flowers was probably owing to the relatively short life of these plants' part at the moment of harvesting.

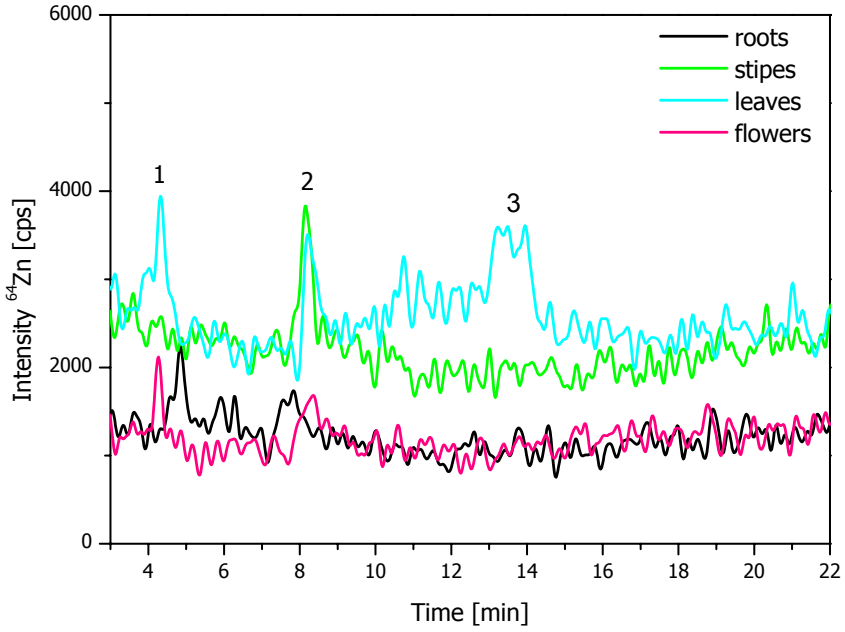

Figure 54: comparison between Zn species distribution in *S. vulgaris* plant parts: roots, stipes, leaves and flowers

Differently to what expected, even if the plants have been treated with solutions containing each a different heavy metal, no appreciable difference was noticed either in the heavy metal enrichment and the species pattern between various plants. The heavy metal uptake was not related to the heavy metal solution used during the growth of the plant.

An example of the similar Cu, Zn and Cd uptake and distribution is given in Figure 55, where a chromatogram of the digested leaves of a plant treated with copper is shown: Zn and Cd present two significant peaks, marked as 1 and 2, while Cu shows an extremely low and wide peak approximately at the same retention time of peak 2, which

cannot be considered an evidence of a copper species, being of the same order of magnitude as the detection limit.

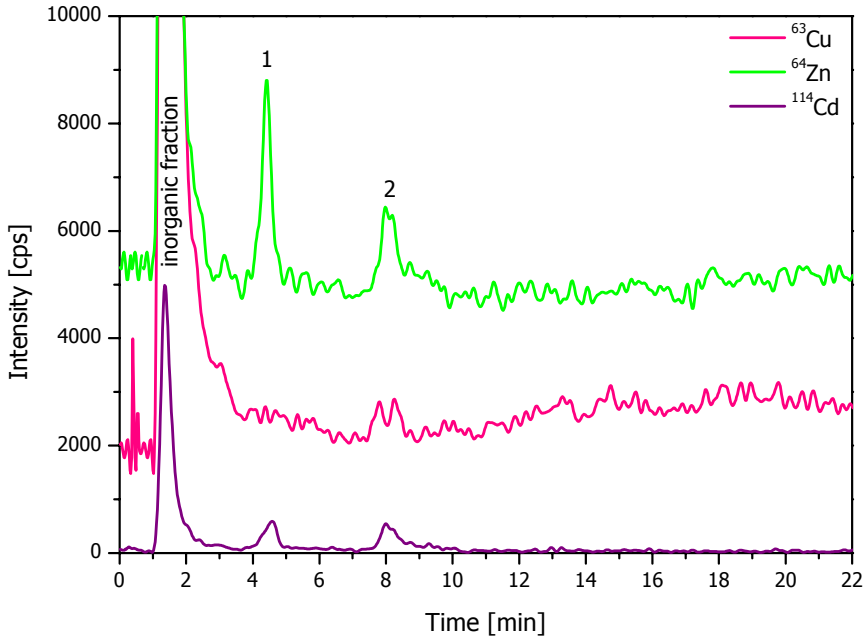

Figure 55: comparison between Cu, Zn and Cd species distribution in *S. vulgaris* leaves; the plant was treated with 10 µg·mL Cu^{2+}

This can be explained considering that in roots exudates the proteins and chelating agents responsible for essential heavy metal uptake are non specific, making them transport into the plant all the heavy metal ions present in soil without distinction.

The peaks 1, 2 and 3 were present with reproducible retention times in almost all the plants for Cu, Zn, Cd and Mn, while no Pb or Hg species have ever been detected. Examples for Cd, Zn and Mn species distribution are given in Figure 56, Figure 57 and Figure 58:

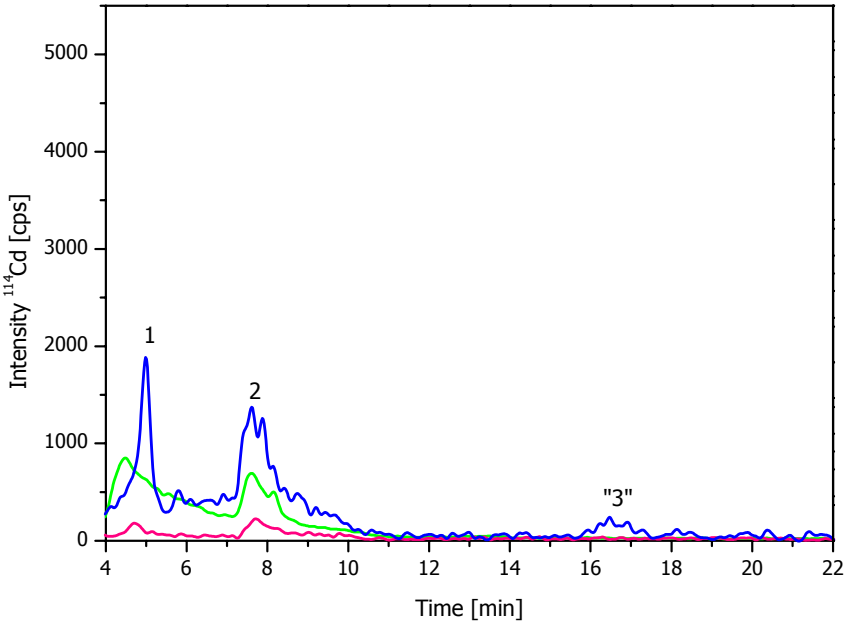

Figure 56: IPC-ICP-MS Cd-bounded species pattern in *S. vulgaris* extracts (leaves) obtained from three different plants

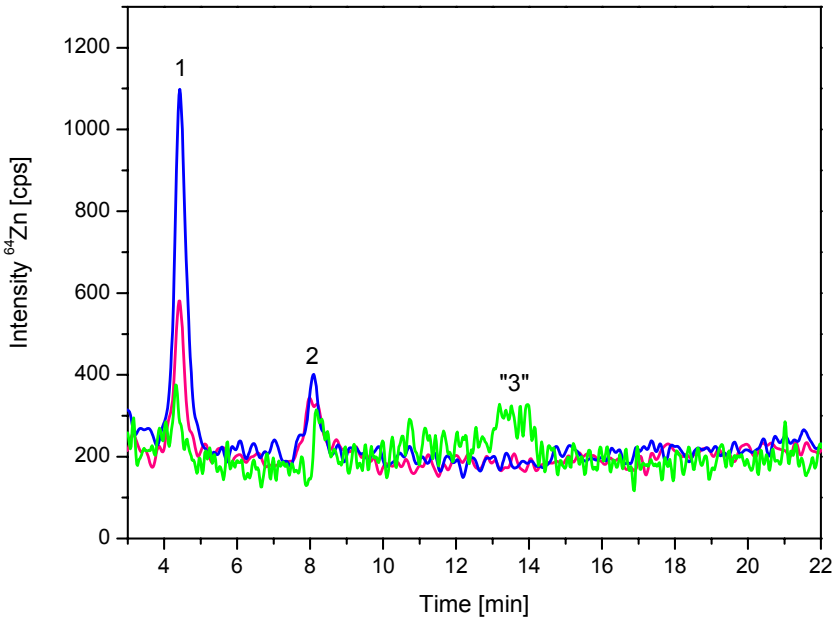

Figure 57: IPC-ICP-MS Zn-bounded species pattern in *S. vulgaris* extracts (leaves) obtained from three different plants

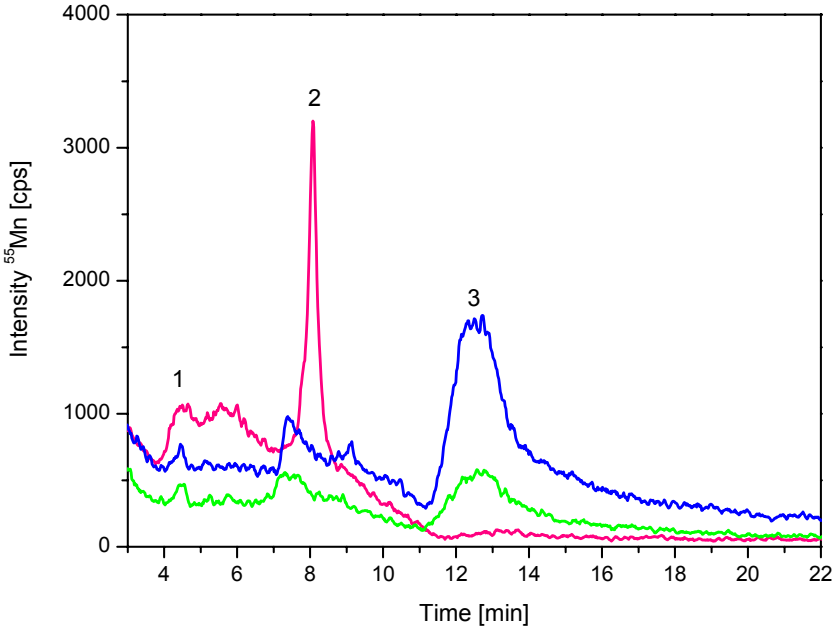

Figure 58: IPC-ICP-MS Mn-bounded species pattern in *S. vulgaris* extracts (leaves) obtained from three different plants

In every chromatogram the first and intense signal (often excluded from the figure) eluting at 1.4 minutes is the inorganic fraction of the metal, composed, as it was for the bovine liver and thyroid samples, by small matrix and buffer complexes of M^{n+}.

As clearly shown in the reported chromatograms (see Figure 54-Figure 58) no significant PC signal was detected in the expected retention time range (12-17 minutes), while the dominant species showed to bind almost all the metals investigated and eluted at about 4 and 8 minutes.

The very small signals eluting at about 13 minutes (peak 3 in Figure 54, in Figure 57 and in Figure 58) and at 16.5 minutes in Figure 56 could be M^{n+}-PC complexes, but in all the cases, except for Mn (Figure 58), the extremely low intensity let arise appreciable doubts on their nature and determinability. In the case of Cd and Zn the "peak 3" lies almost in the detection limit range. On the other hand, the Mn signal is very broad and it can be reasonably supposed that more than one species are co-eluting at this time. Up to now, no *in-vivo* Mn-PC have been detected.

To characterise the peaks, more information on them has to be gained through a two dimensional approach, or an ESI-MS analysis.

An IPC-ESI-MS direct coupling was not possible, due to the mobile phase characteristics: the presence of TBAH, a detergent, as the ion pairing reagent, makes an ESI-MS analysis of the eluate impossible. Nevertheless, peak 2 was identified through an indirect ESI-MS approach, as discussed in the following section (paragraph 7.3.3.).

For the two-dimensional chromatography, an attempt to separate the *S. vulgaris* extracts first with a SEC run and then by means of the developed IPC-ICP-MS was made. The SEC applied conditions were the same as for the bovine liver samples (see paragraph 5.1.1.), employing ammonium acetate buffer instead of Tris-HCl. The fractions were collected 5-10 times, freeze-dried, re-dissolved in 1 mL mobile phase and injected for the second separation.

The multiple collection and the freeze-drying steps were taken in consideration for a pre-concentration of the analytes. Unfortunately, no M^{n+}-PC signal was detected after the second separation, as shown in Figure 59: the causes can be the same as discussed for the bovine liver and thyroid samples; a too strong dilution and the species instability.

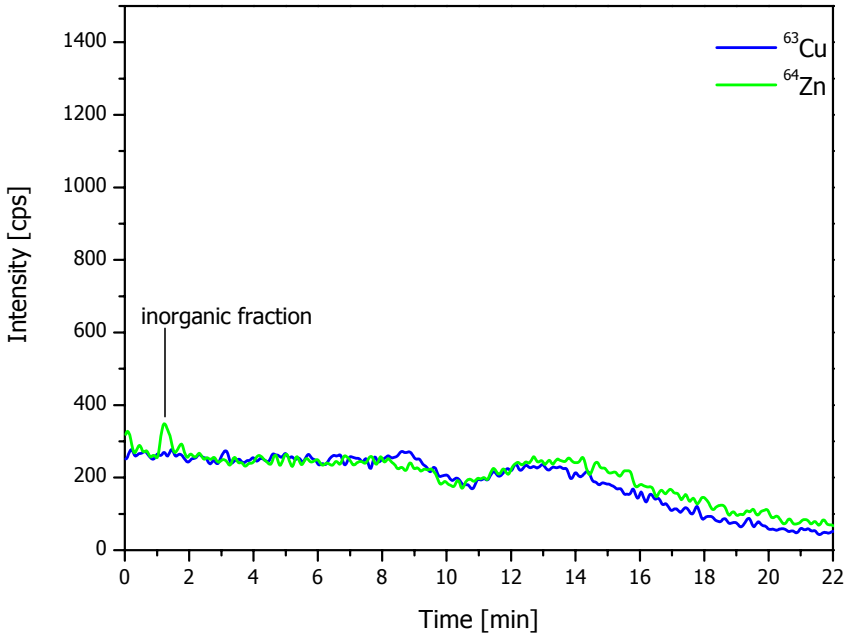

Figure 59: 2-dimensional approach for S. vulgaris extracts investigation: after the SEC/IPC no ICP-MS signal can be detected. The small inorganic fraction Zn peak at 1.4 minutes could arise from degraded species

The complex stability was therefore taken in consideration as the main cause not only for the absence of signals in the chromatograms of the 2-dimensional approach, but even

for no Cd or M^{n+}-PC signal in the *S. vulgaris* extracts. The stability of the complex during the sample preparation and the IPC-ICP-MS analysis was deeply studied and is discussed in the following paragraph 7.3.4.

7.3.3. Peak characterisation via ESI-MS

The presence of a detergent (TBAH) in high concentration in the eluate, allows elution of the M^{n+}-PC complexes but no coupling of the developed chromatographic separation to an ESI-MS. For this reason it was not possible to identify directly the eluted peaks with an on-line LC-MS analysis, or even sampling the fractions and injecting them in the ESI-MS. Therefore, an alternative path for peak characterisation was performed, taking the Cd species (chromatogram in Figure 56) as reference.

The eluted species were low-molecular, quite hydrophilic Cd complexes. They were either synthesised in the plant, or originated from the degradation of PC complexes. On the basis of these considerations, they were supposed to be glutathione (GSH) or cysteine complexes, as they are both known to be involved in metal tolerance[121] and as a part of PC synthesis[116,122] and/or an alternative detoxification way.

Thus, a solution containing Cd and GSH was prepared, injected and eluted under the same IPC conditions employed for the samples. The obtained chromatograms shown in Figure 60:

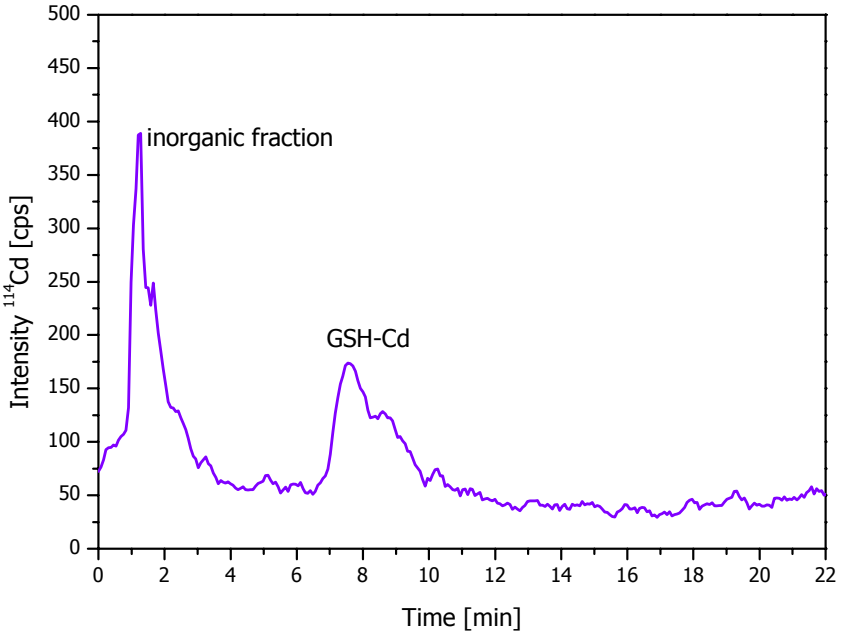

Figure 60: IPC-ICP-MS of an aqueous GSH/Cd solution

The peak eluting at 1.3 minutes is the inorganic fraction, while the second peak, eluting at 7.5 minutes, was attributed to a GSH-Cd complex. To prove this hypothesis, the same solution was directly analysed by means of ESI-MS. The spectrum is shown in Figure 61:

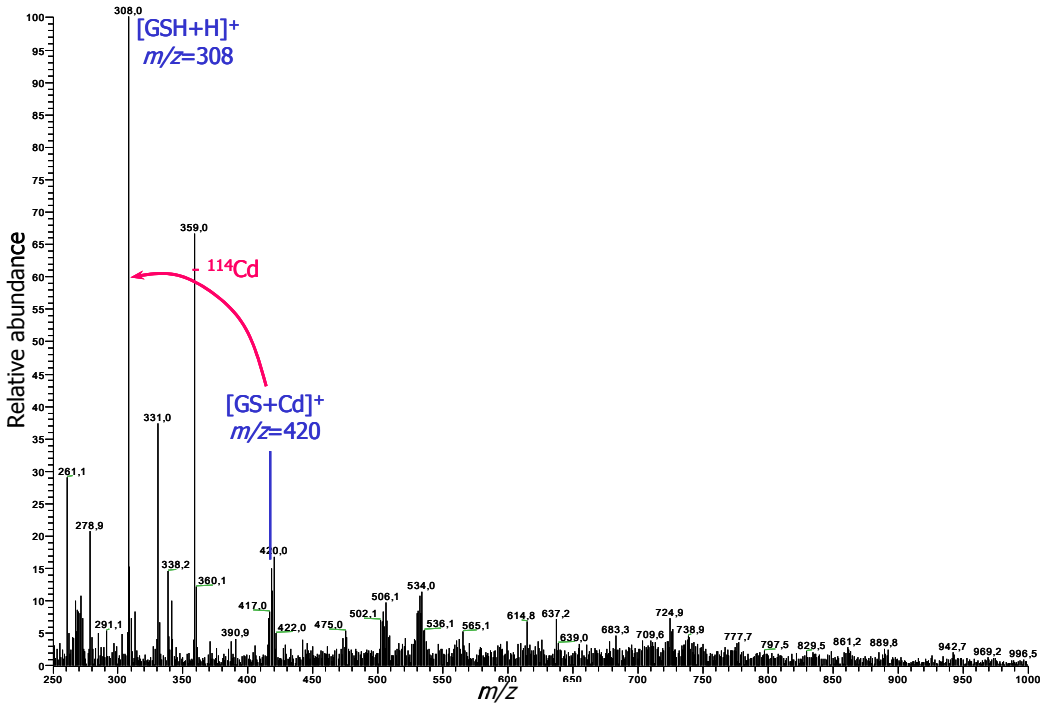

Figure 61: ESI-MS spectrum of a GSH/Cd solution

The most intense peak with m/z =308 arises from the ion $[GSH+H]^+$, while the peak with m/z =420 is due to the combination of the deprotonated GSH and a Cd atom, $[GS+Cd]^+$, where 114 is the nominal mass for Cd. The presence of Cd in this ion can be verified comparing the Cd-pattern to that of the detected signal. The good agreement between the two is shown in Figure 62 and is the confirmation on its nature.

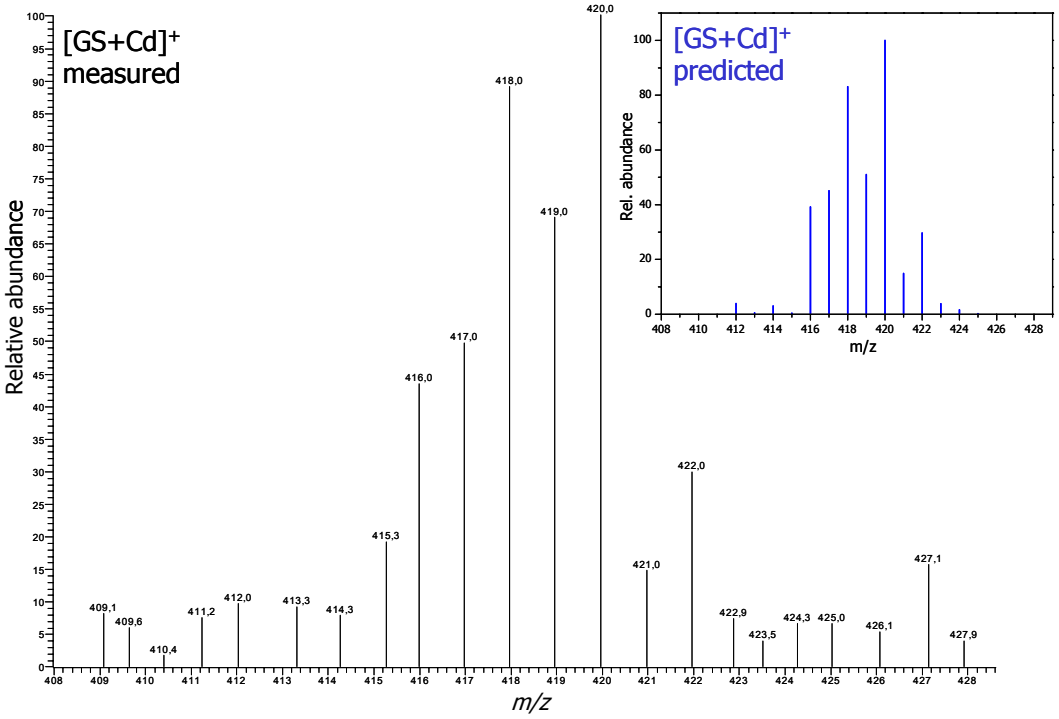

Figure 62: isotope pattern of the $[GS+Cd]^+$ and comparison with the calculated isotope pattern (small window)

To prove that the detected species contains one Cd atom, the experiment was repeated using a ^{116}Cd enriched standard. The results are shown in the chromatograms reported in Figure 63 and Figure 64:

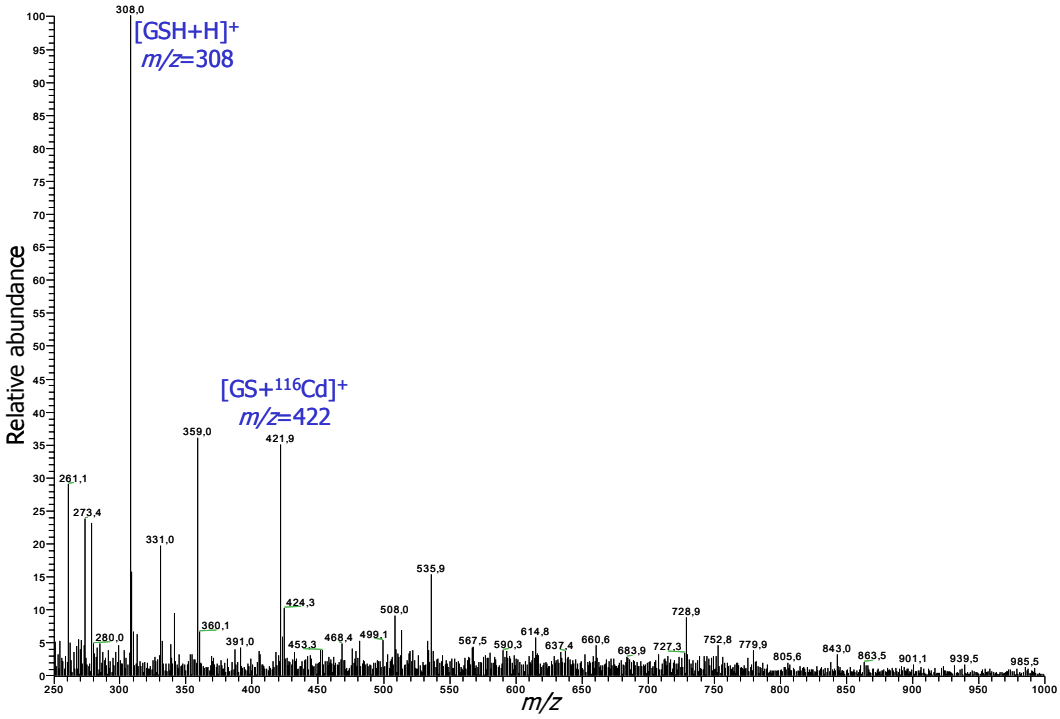

Figure 63: ESI-MS spectrum of the aqueous solution of GSH/^{116}Cd

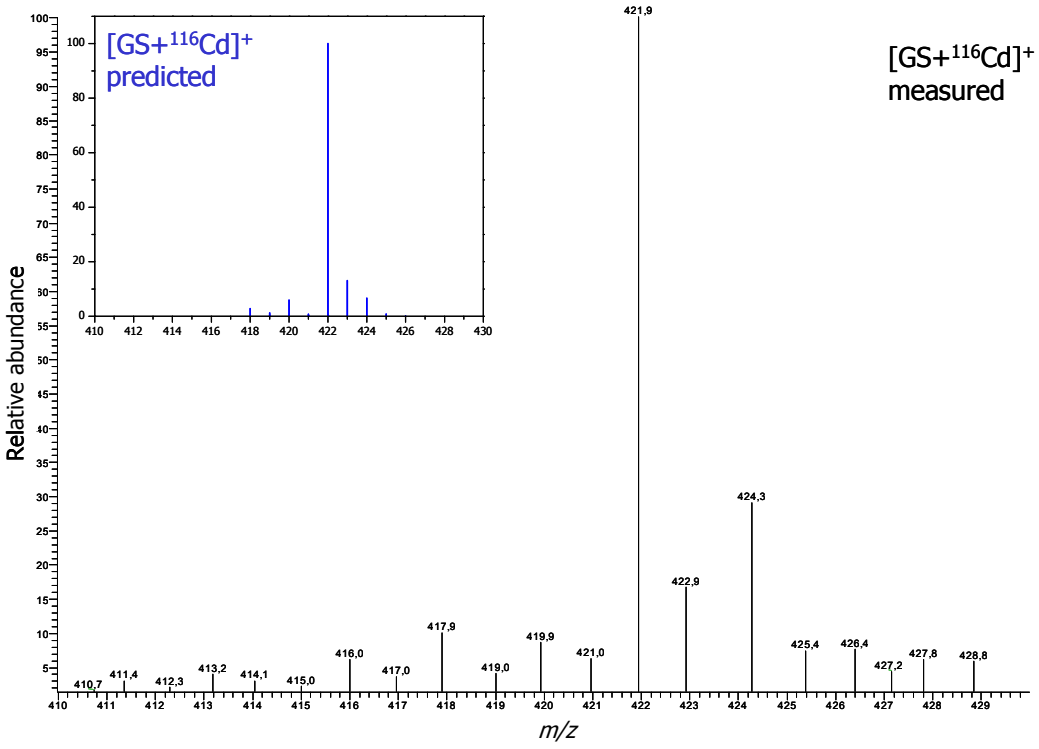

Figure 64: isotope pattern of the [GS+^{116}Cd]$^{+}$ and comparison with the calculated isotope pattern (small window)

The information on how many metal ions are bound to the investigated species derives not only from the molecular weight, but also from the fingerprint of the peak itself, which results modified and reproduces that of the metal ion, if a 1:1 complex is formed; which will be differently modified of more metal ions are present. In the case of Cd, the high number of isotopes, which occur in a discrete percent, is of course an advantage. The ^{116}Cd enriched solution, which contains 89.6% of ^{116}Cd, 5.3% of ^{114}Cd, 2.5% of ^{112}Cd and less than 1% for the other isotopes, looks more like a shifted GSH peak: this case is similar to monoisotopic metal ions, which do not produce the characteristic fingerprint modelled on the composition of the organic and metal spectra.

The detected $[GSH+H]^+$ (m/z=308) signal was subsequently isolated in the ion trap and fragmented, performing MS^2 and MS^3 measurements, which are shown in Figure 65.

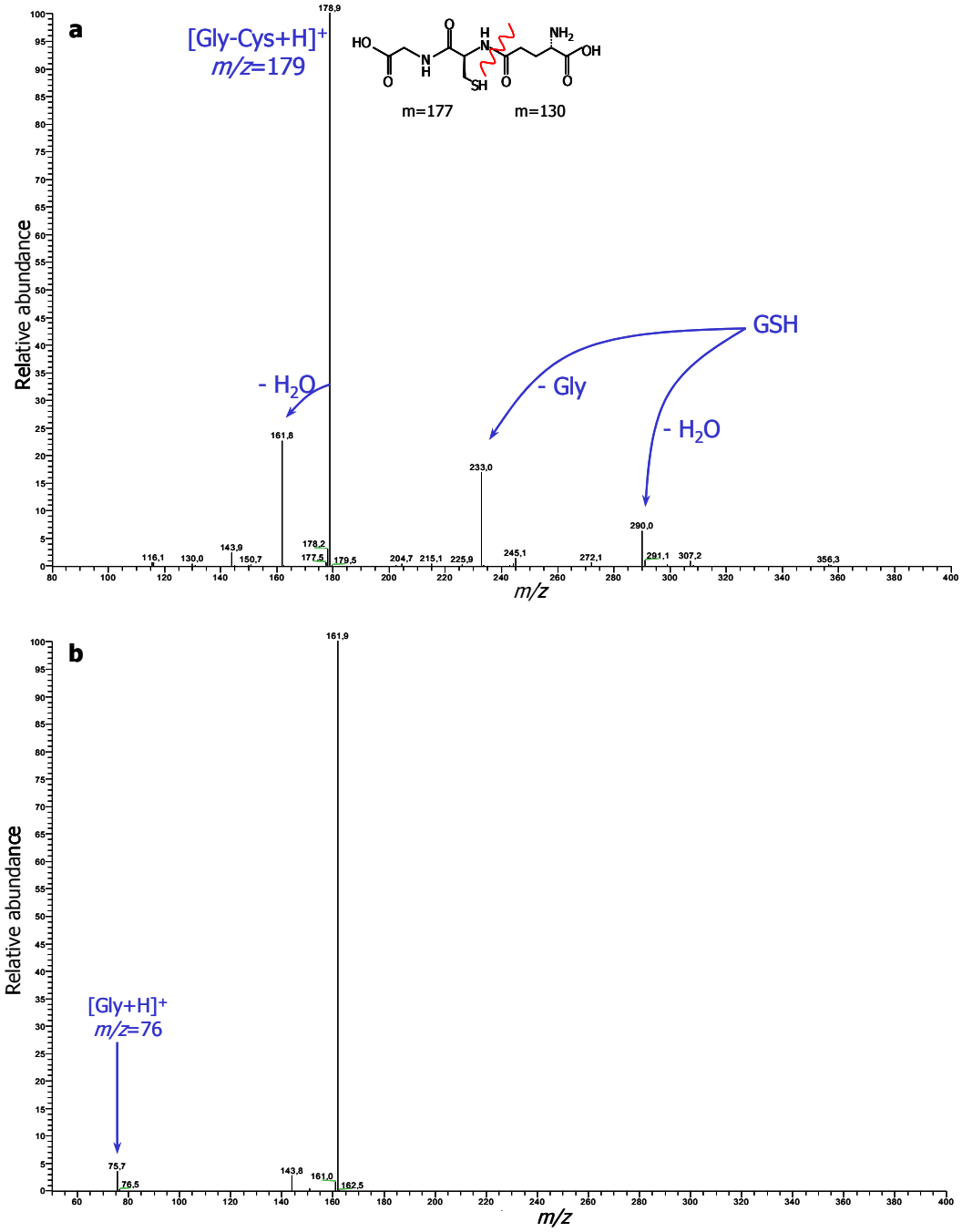

Figure 65: ESI-MS^2 (*a*) and ESI-MS^3 (*b*) of the GSH peak, m/z=308 in Figure 61.

In Figure 65, part *a*, the signal with *m/z*=290 represents the loss of a water molecule from GSH, while *m/z*=233 is the GSH fragmentation and loss of glycine (Gly). The dominant signal is the fragmentation of GSH as shown in the figure, through which it loses a glutamic acid (Glu) molecule. The signal with *m/z*=162 represent the loss of a water molecule from this species. The part *b* represent the isolation of the peak with

m/z=179 and its fragmentation. In this case, the small signal of protonated glycine arises at *m/z*=76.

The MS-MS analysis of the $[GS+Cd]^+$ (m/z=420) and $[GS+^{116}Cd]^+$ (m/z=422) signals was also performed, and the obtained spectra show, as confirmation of the nature of the species, analogous peaks differing from a $\Delta m = {}^{114}Cd - {}^{116}Cd = 2$, as it is reported in Figure 66 and Figure 67:

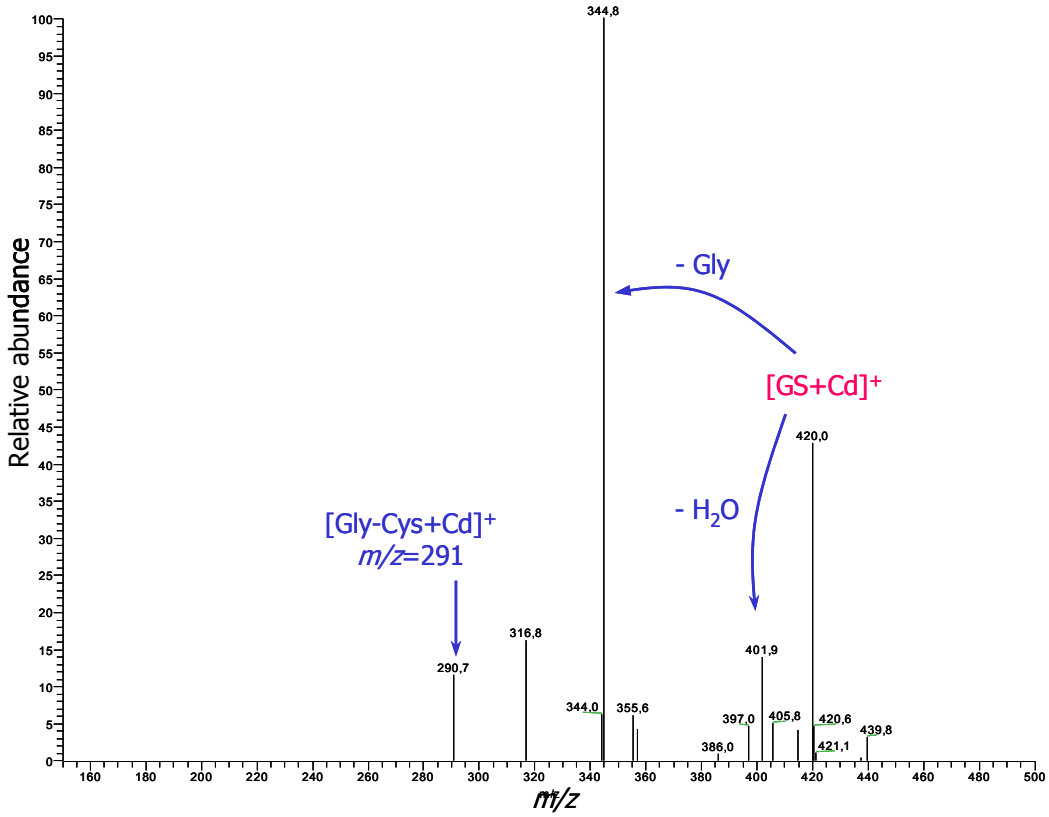

Figure 66: ESI-MS^2 of the GSH and Cd complex, peak m/z=420 of Figure 61

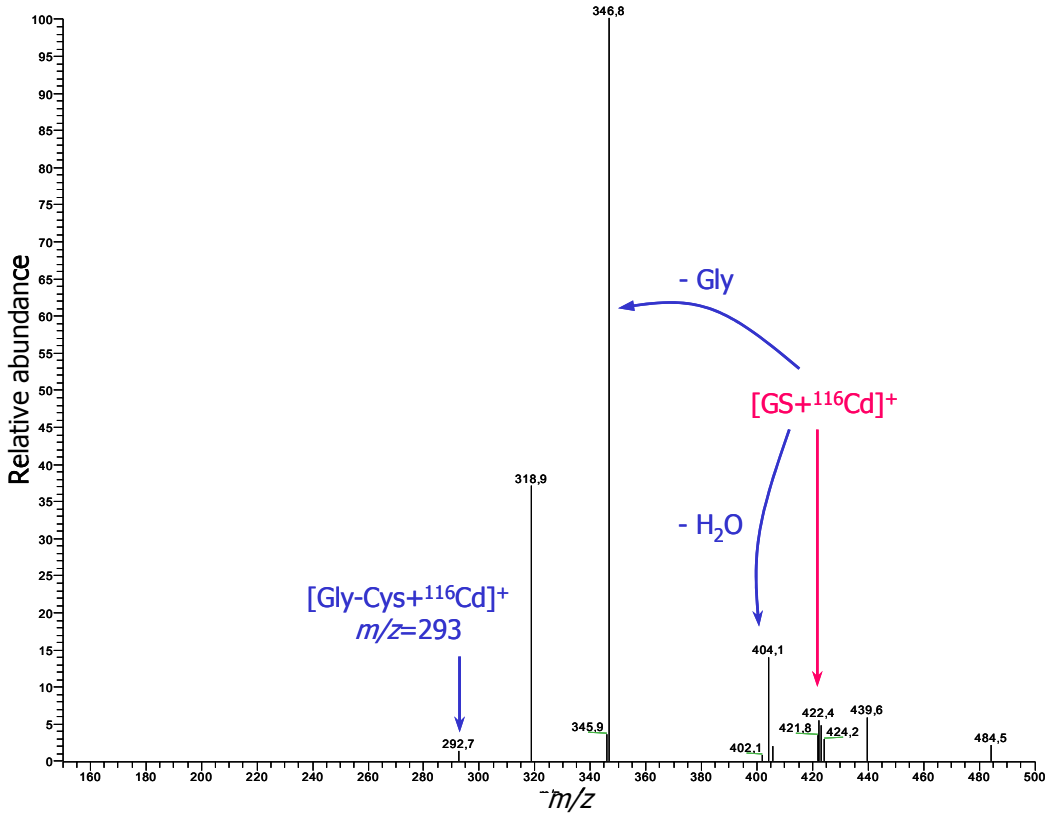

Figure 67: ESI-MS^2 of the GSH and ^{116}Cd complex, peak m/z=422 of Figure 63

Comparing the two figures, the above mentioned peaks can be interpreted as follows: the mother peak, with *m/z*=420 for natural Cd (422 for ^{116}Cd), loses a water molecule producing the peak at *m/z*=402 (404), while the dominant species at *m/z*=345 (347) is due to the fragmentation of the mother ion and its loss of glycine. The small signal at m/z=291 (293) can be attributed to the protonated fragment Gly-Cys complexed with Cd.

The ESI-MS analysis led to the identification of the peak eluting at 7.5 minutes as a Cd-glutathion complex. By means of retention time matching, it was therefore possible to identify this as one of the three species constituting the *S. vulgaris* extract, forming complexes not only with Cd, but also with Cu, Zn and Mn, as it can be seen by the presence of this peak in the chromatograms shown in Figure 54 to Figure 58.

7.3.4. Method stability

None of the chromatograms obtained from the *S. Vulgaris* extracts showed significant presence of M^{n+}-PC, nor Cd-PC, while the GSH complexes of several of the metals investigated gave in many samples reproducible and stable signals. This fact can be explained with the single or combined effect of one of the following hypothesis: Cd-PC (M^{n+}-PC) have not been synthesised by *S. vulgaris* and Cd (M^{n+}) has been complexed with some other species, e.g. glutathione; on the other hand, Cd-PC (M^{n+}-PC) have been synthesised, but a significant percentage of the labile compounds did not survive the extraction and/or the chromatographic separation.

Having been M^{n+}-PC complexes separated and detected for the first time within this work, their stability in time and above all to the experimental conditions remains an unknown characteristic.

Going back to the analysis of Cd-PC in *P. tricornutum* fractions, the problem of the complex degradation never appeared; it has nevertheless to be taken in consideration that no quantitative recovery of the measured Cd-PC could be carried out, being the Cd amount in the samples not known. Nevertheless, it was possible to measure the Cd-recovery after the chromatographic run, measuring the Cd concentration in the total eluate after the separation, and the Cd concentration in the original sample diluted under the same conditions. It was thus stated that during the IPC 20% of the Cd was lost. Moreover, within one day of measurements, the Cd-PC in the *P. tricornutum* fractions underwent degradation, as new unknown peak arose and the PC peaks became smaller.

These effects had no dramatic influence on the carried out measurements, on the basis that the aim of these investigations were the optimisation of the experimental conditions, and the concentration of such samples was high enough to allow the detection of sharp peaks, even if a huge amount of the species disappeared.

The concentration of the Cd-PC in these samples was extremely high, if compared to the concentrations expected in *S. vulgaris* extracts. This has as a consequence that in the case of a *P. tricornutum* samples a loss of the species leads only to a signal decreased in intensity, while in the *S. vulgaris* samples it can let the signal disappear at all: if the total amount of the M^{n+}-PC contained in the *S. vulgaris* sample gives a 800 cps signal, and its 20% is lost due to degradation in the chromatographic column, and another unknown amount is lost due to the low kinetic stability of the complex, maybe during the sample preparation, the signal would decrease, and in the worst case sink under the detection limit.

To find out if these were the reasons for no M^{n+}-PC signal in the *S. vulgaris* extract, an experiment to study the kinetic stability of the species during extraction and analysis was carried out.

^{116}Cd spiked phytochelatins

The biosynthesis of PC in *P. tricornutum* cultures was repeated using an isotopically enriched $^{116}Cd^{2+}$ characterised standard. The synthesis was performed under the same conditions described in paragraph 7.2.1., as well as the SEC and IPC separation. The three Cd isotopes 112, 114 and 116 were then detected by means of ICP-MS. A typical chromatogram of fraction C (containing almost only Cd-PC_3) is shown in Figure 68:

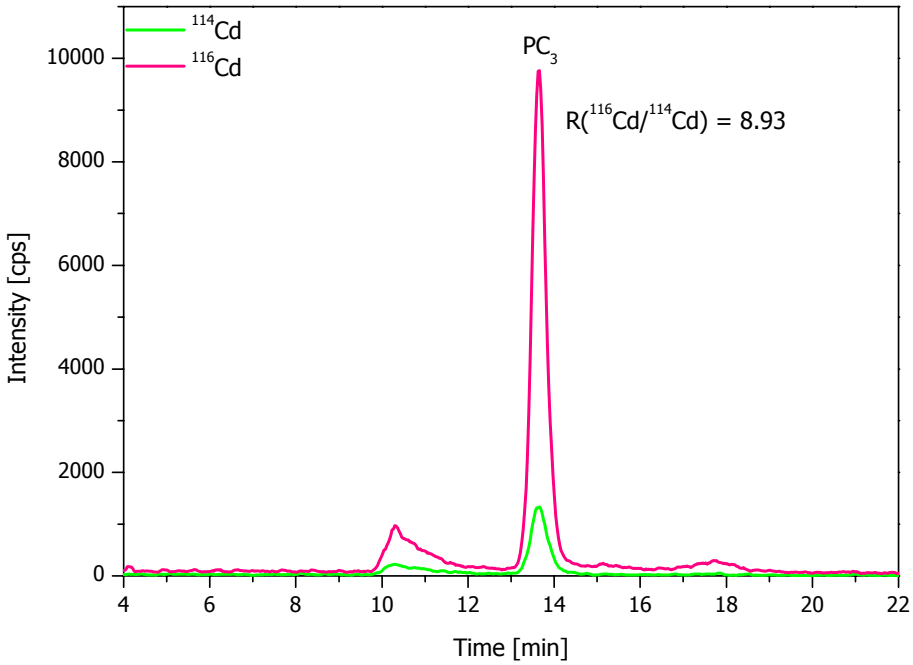

Figure 68: IPC-ICP-MS of fraction C ^{116}Cd-spiked

As expected, besides an unknown compound eluting at 10.2 min, probably an artefact or a PC-degradation product, PC_3 is the dominant species originated from the enriched $^{116}Cd^{2+}$.

The measured isotope ratios were, in the case of the ^{116}Cd enriched solution R(^{116}Cd/^{114}Cd) = 8.93; in the case of the natural Cd abundance, R(^{116}Cd/^{114}Cd) = 0.26.

In order to prove the stability of the species during the sample preparation and separation, this compound was added to a *S. vulgaris* leaves sample, which has been grown up with the addition of Cd^{2+}. The extraction was carried out under the usual conditions, and the IPC-ICP-MS analysis gave the chromatogram shown in Figure 69:

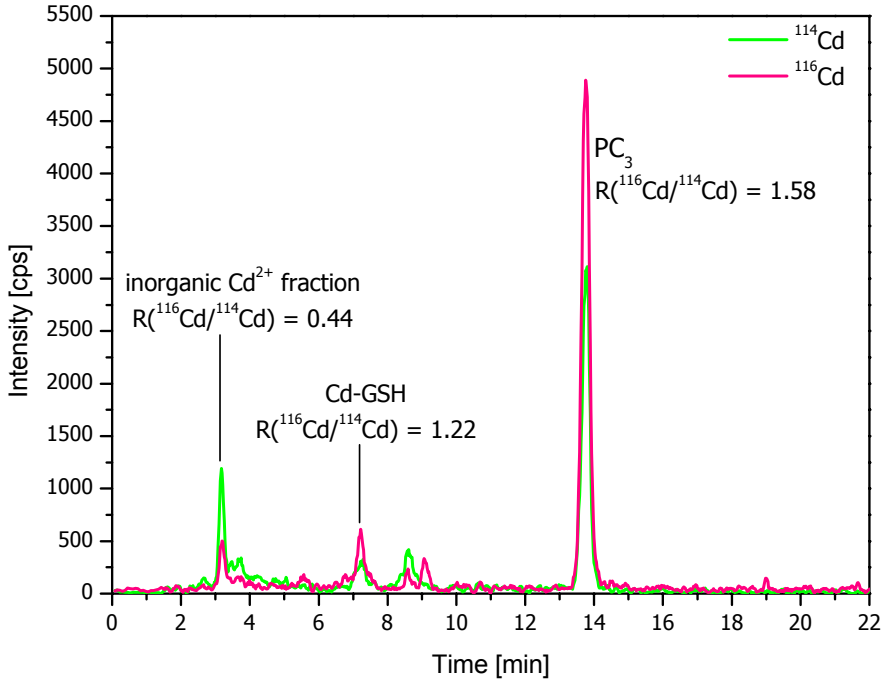

Figure 69: IPC-ICP-MS of *S.vulgaris* (leaves) with the addition of ^{116}Cd enriched PC_3

Cd-PC_3 produces the most intensive signal in the chromatogram, confirming that at least a part of the analyte could be recovered. Furthermore, peaks for "inorganic" Cd^{2+} and Cd-GSH could be detected. For these peaks, the isotope ratios R(^{116}Cd/^{114}Cd) were determined by peak integration (the standard deviations for the given values were between 5 and 10 % (n=3)).

As a result all ratios were different from the natural one, which indicates a strong Cd^{2+} exchange within the different complexes: the inorganic fraction and the GSH complex showed a Cd composition enriched in ^{116}Cd, due to ^{116}Cd-PC degradation during the sample preparation or the analysis. This change could also be observed for the PC_3 complex itself (R(^{116}Cd/^{114}Cd)=1.58): in this case, the enrichment was on ^{114}Cd, due to Cd-exchange between the complex and the other species synthesised by *S. vulgaris*.

The observed Cd exchange confirms the hypothesis of a low kinetic stability of the Cd-PC complex. Within this work was nonetheless possible to establish if this instability leads to degradation of the peptides into more stable species such as GSH, or if GSH was already the most abundant Cd-complexing species synthesised in the *S. vulgaris* leaves, and an equilibrium between Cd-GSH and Cd-PC_3 was then established, as the two species came in contact. No ICP-MS measurement could prove that the peptide itself undergoes

degradation, but the measured isotopic ratios prove that this happens to the metal-complexes species.

The low kinetic stability of the Cd-PC complexes towards other competing chelators was for the first time put in evidence: the rapid Cd exchange between the *P. tricornutum* PC, the *S. vulgaris* PC and/or the GSH are proved by the altered isotopic ratios.

No kinetic constant for the complex was measured within this work, but the attention was shifted to the possibility itself to detect M^{n+}-PC species in complex samples: even under mild extraction and elution conditions, the characterisation of a plant extract sample, maintaining its original species composition, was once more revealed to be a very delicate and difficult goal. Moreover, when the species are present at low concentrations, a significant amount of analyte is lost owing to exchange equilibriums.

The kinetic instability of the Cd-PC has also the consequence that the synthesised ^{116}Cd enriched species cannot be used as species-specific indicators. One of the most important characteristics of these substances should be in fact an extremely low tendency to exchange metal ion. Nevertheless, the lack of this characteristic in the carried out experiment represented the basis to cast a new light on an up to now disregarded characteristic of Cd-PC.

In the light of the proved instability of the species, the fact that almost no M^{n+}-PC signal in the *S. vulgaris* extract was detected can be explained with a species degradation within the entire procedure. A part of the species was lost during the chromatographic separation, but a higher amount was lost during the sample preparation. Being their concentration in the *S. vulgaris* samples already low, the complete signal was in the end lost. This spurred the development of a slightly different sample preparation procedure and analysis strategy for the plant samples, which was tested analysing roots and shoots extracts of oat and wheat samples. The results of this new experiment are discussed in the following paragraph.

7.3.5. Oat and wheat samples analysis

All the harvested S. vulgaris was prepared and analysed following the procedures reported in 7.2.2., and was therefore no longer available for further experiments which required row material. To carry on a new experiment in a relatively short time, another sample was chosen. A mixture of oat and wheat seeds required one week only to produce a high amount of roots and shoots: this new sample had then the advantage to save much time in the growing phase, and to deliver a higher amount of plant material

once harvested. This second characteristic has not to be underestimated, being the species of interest present only in low concentrations: the higher the digested material weight, the higher the PC amount.

The digestion procedure was exactly the same as the one employed for the *S. vulgaris*, with the only difference that the oat and wheat extracts were directly measured via SEC/IPC-ICP-MS, while the *S. vulgaris* extracts were freeze-dried twice: once after the plant digestion, once after the SEC separation.

The freeze-drying step was avoided because the stated low kinetic stability of the species could lead to a dramatic loss during the long time requested for this procedure. Being anyway this step among the others a pre-concentration step, it was necessary to perform the analysis with a high amount of plant material.

The metal enriched solution employed contained only Mn, Zn, Cu and Cd and the digested plant amount was 1-2 g of shoots or roots, instead of 20-30 mg of *S. vulgaris* plant material. The two-dimensional chromatography was directly performed without the freeze drying treatment: the extracts were prepared and injected in the SEC; The fractions corresponding to 500-2000 kDa were immediately once collected, and an RPC-ICP-MS analysis was directly run.

In Figure 70 are shown the results of the two dimensional chromatography:

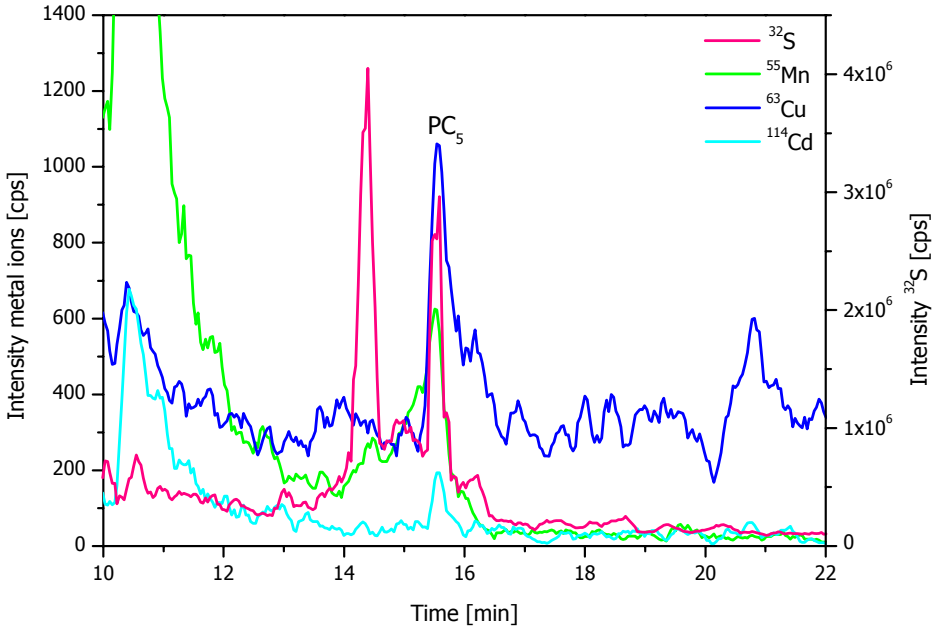

Figure 70: SEC/IPC-ICP-MS of an oat and wheat shoots sample performed immediately after the extraction and without any freeze drying in between

A multi-metal peak (Cd, Cu and Mn) correlating with sulphur is eluted at 15.5 min, which makes it a PC_5 by the method of retention times matching. Another S signal is present at 14 min, the retention time of PC_4. Nonetheless, it co-elutes with no metal, and, on the basis of the analysis carried out, it is not possible to make any hypothesis on its nature. The concentration of the species is low, but nevertheless the signals are clear, sharp and reproducible peaks.

In the roots extracts no PC peak was detected.

Even if the kinetic stability of M^{n+}-PC was demonstrated to be very low, through this experiment a M^{n+}-PC_5 was detected: the tested experimental conditions proved therefore to be suitable for this research.

Through this analysis it was verified that even if the species are unstable and occur in a low concentration in the samples without any pre-concentration step like the freeze-drying it is possible to detect them.

Without any freeze-drying step, the samples have to undergo SEC directly after the digestion, as well as the SEC fraction have to be collected and immediately RPC analysed, making in the end the entire procedure time consuming: no more than few (2-3) samples per day can be measured; moreover, no pre-concentration of the samples or the fraction is possible. In the future, further improvements of this method will surely concern this aspect.

7.4. Conclusions

Size-exclusion and ion-pairing chromatography as two dimensional separation were successfully applied to the analysis of Cd-PC synthesised in *Phaeodactylum tricornutum* cell cultures. In the presence of Cd^{2+} these algae produce Cd-PC (PC_2-PC_6) in very high concentrations.

The species were first separated through SEC, and the fractions have been subsequently used for the optimisation of the second dimensional chromatography, through which a better separation of the Cd-PC was possible. Due to the neutral pH of the mobile phase, it has been possible to detect the Cd-PC complex by means of ICP-MS, while up to now only the apoproteins or covalently bound PC have been characterised. This information was complementary to that gained through SEC-UV-VIS. By the comparison of the two, the Cd-PC complexes were identified in the IPC-ICP-MS chromatogram.

The IPC-ICP-MS was subsequently applied to detect M^{n+}-PC in *Silene vulgaris* samples, which had been digested under mild conditions. The observation that no M^{n+}-PC were present in the extracts led to new experiments, with the aim to test the stability of the PC-metal complexes to the sample preparation and analysis conditions.

A 20% Cd loss during the second chromatographic separation was determined by comparing the Cd content of a SEC fraction before and after IPC. In order to scrutinise both the extraction and separation steps, *S. vulgaris* leaves samples were spiked with isotopically enriched ^{116}Cd-PC, obtained incubating *P. tricornutum* cells with a ^{116}Cd enriched solution and performing the normal SEC separation. As a result, an intensive Cd exchange between the PC and other complexing species, like GSH, was detected. The very high kinetic instability of the Cd-PC complexes was therefore responsible for species degradation in the analysed *S. vulgaris* samples: the low amount of such species in the raw sample, in comparison to that of Cd-PC observed in the *P. tricornutum* fractions, led to complete degradation of the species during the preparation and the analysis.

Within this work, the kinetic instability of Cd(M^{n+})-PC was investigated and pointed out for the first time. This characteristic makes the ^{116}Cd-PC a not suitable species specific indicator for the analysis of such species, but was at the basis of the results obtained. Moreover, the employ of the ^{116}Cd enriched species in the ESI-MS investigations allowed a rapid and simple identification of the Cd- containing species: through the comparison of the spectra obtained in the both cases, of the natural and the spiked complexes, peaks related to Cd were immediately detected due to the 2 mass unit differences.

The analytical procedure for plant extracts investigation was then slightly changed, excluding the freeze-drying steps: the plant material was digested, SEC analysed, the fraction collected once end immediately IPC-ICP-MS analysed. This modified procedure did not foresee any pre-concentration step, and was therefore applied to a large amount of plant material. The chosen samples were oat and wheat shoots and roots.

A PC_5 was detected in the extract, characterised by sulphur co-eluting with Cd, Cu and Mn, by retention time matching. For the first time a Mn-PC *in vivo* produced was detected.

Through the new information gained, a new light was cast upon the processes that regulate heavy metal sequestration and detoxification in plant cells.

8. CONCLUSIONS

This work was articulated in three big sections, centred on the investigation of three different species' classes in human, mammal and vegetal samples. The species of interest were, respectively, the Gd-DTPA complex, metallothioneins and phytochelatins.

Efficient and reliable chromatographic separations for metallothioneins in mammalian organs were elaborated: the resolution of the different MT isoforms was very good, the retention times were good reproducible, even for different samples. The nature of the peak as MT isoforms was stated through indirect validation, e.g. the heath resistance of the species was proved, as well as their molecular weight and their high sulphur content. These entire characteristic corroborate the hypothesis of the peaks representing MT isoforms, but cannot demonstrate it. A definitive confirmation of the nature of the detected peaks, which can be achieved only through a soft ionisation mass spectrometric analysis, was actually not possible. In this case, the huge sensitivity difference in ESI-MS and ICP-MS instrument leads to a dramatic loss in information for those species that occur in extremely low concentration, and even if still detectable by means of ICP-MS, produce indeed no signal by ESI-MS analysis.

The same problem was later faced, in the investigation of phytochelatins in plants and monocellular algae extracts: an innovative and satisfactory separation of the PC in the algae samples, which contained Cd-PC in large concentrations and were therefore used as model samples for the analytical procedure's optimisation, was achieved.

The very labile species Cd-PC complex was detected, delivering complementary and innovative information on these species in comparison to the apoprotein detection. In this case, the chromatographic conditions were absolutely not compatible with the ESI-MS, being the SEC separated species in presence of phosphate buffer, and the IPC separated species in presence of the ion pairing reagent, both an insurmountable obstacle for a fruitful ESI-MS analysis. Therefore, once more the species could be indirectly identified, thanks to the independent determination of the derivatised apoproteins by means of UV-VIS detection. This lack of information can be fulfilled only looking forward for new and different chromatographic separation methods, which leave the complex intact *and* are ESI-MS compatible.

Deepened work was carried out on the investigation of the Cd-PC (and M^{n+}-PC) complex stability during the sample preparation procedure and the chromatographic separation of the species. The use of ^{116}Cd enriched PC samples, synthesised by the same monocellular alga, was fundamental to verify the high kinetic instability of the Cd-

PC complexes. Nevertheless, the same characteristic undermines the possibility to use this compound as a species specific indicator.

The analysis of *S. vulgaris* samples led to no detection of the M^{n+}-PC complexes, due to this stated kinetic instability, pointing out once more how fundamental is a correct validation of the entire analytical procedure for speciation analysis. The production of artefacts or species transformation and degradation is at the basis of huge interpretation errors.

Nevertheless, appreciable efforts have been made in improving the sample preparation for the PC analysis skipping the freeze dry step, which enabled the detection of at least one PC complex, bound to Mn, Cu and Cd. Much of new information on the processes regulating the sequestration and detoxification on heavy metals in plant cells can be gained following this approach, even if it is only at the early state, and in need of further developments: nevertheless, for the first time the research has been focussed on the integrity and the stability of the complex itself, and not only on the apoprotein.

Through these studies another piece of information has been added to the knowledge of the complex processes regulating toxic and essential heavy metals metabolism, in plants, in liver and in organs not directly involved in detoxification, like the thyroid. Highly toxic heavy metal can result in advantageous and practical applications if they occur in the organism as a stable species, like the Gd-DTPA complex.

The development of new analytical approaches opens windows to look at the problem from new points of view, illuminating step by step all those unknown particulars of the system, and contributing therefore to its complete knowledge.

9. BIBLIOGRAPHY

[1] ATSDR (Agency for toxic substances and disease registry), http://www.atsdr.cdc.gov/

[2] Templeton D.M., Ariese F., Cornelis R., Danielsson L.G., Muntau H., Van Leeuwen H.P., Lobinski R., "Guidelines for terms related to chemical speciation and fractionation of elements. Definitions, structural aspects, and methodological approaches (IUPAC recommendations 2000)" *Pure Appl. Chem.*, **72**: 1453–1470, (2000)

[3] Cornelis R., Caruso J., Crews H., Heumann K.G. (ed), Handbook of Elemental Speciation, John Wiley & Sons, Chicester, (2003)

[4] Szpunar J.: "Advances in analytical methodology for bioinorganic speciation analysis: metallomics, metalloproteomics and heteroatom-tagged proteomics and metabolomics" *Analyst*, **130**: 442-483, (2005)

[5] Szpunar J.: "Bio-inorganic speciation analysis by hyphenated techniques" *Analyst*, **125**: 963-988, (2000)

[6] Macherey – Nagel, HPLC Flüssigkeitschromatographie, Düren, (1996)

[7] Skoog D.A., Leary J.J., Principle of Instrumental Analysis IV ed., Saunders College Publishing, New York, (1992)

[8] Houk R.S., Fassel V.A., Flesh G.D., Scev H.J., Gray A.L., Taylor C.E.: "Inductively coupled argon plasma for mass spectrometric determination of trace elements", *Anal. Chem.*, **52**: 2283-2289, (1980)

[9] Date A.R., Gray A.L.: Applications of inductively coupled plasma mass spectrometry, Blackie, Glasgow, 17-36, (1989)

[10] Jarvis K.E., Gray A.L., Houk R.S., "Instrumentation for inductively coupled plasma mass spectrometry" in Handbook of inductively coupled plasma mass spectrometry, 10-57, Blackie, London, (1992)

[11] Marx G., *Doktorarbeit*, Mainz, (2000)

[12] Finnigan MAT, *ICP-MS Interferenz-Tabelle, 2. Version*, (1995)

[13] Rottmann L., Heumann K.G.: "Determination of heavy metal interactions with dissolved organic materials in natural aquatic systems by coupling a high-performance liquid chromatography system with an inductively coupled plasma mass spectrometer" *Anal. Chem.*, **66**: 3709-3715, (1994)

[14] Roboz J., Introduction to Mass Spectrometry; Instrumentation and Techniques, Wiley-Interscience, New York, (1968)

[15] http://www.unige.ch/cyberdocuments/unine/theses2001/PerretC/images/image048.jpg

[16] McLuckey S.A., Van Berkel G.J., Goeringer D.E., Glish G.L.: "Ion Trap Mass Spectrometry of Externally Generated Ions" *Anal. Chem.*, **66**: 689A-696A, (1994)

[17] Finnigan, *LCQ™ MS detector Hardware Manual*, *Revision C*, (1997)

[18] Wachs T., Conboy J.C., Garcia F., Henion J.D.: "Liquid chromatography-mass spectrometry and related techniques via atmospheric pressure ionization", *J. Chromatogr. Sci*, **29**: 357-366, (1991)

[19] Niessen W.M.A., Tinke A.P.: "Liquid chromatography-mass spectrometry: general principles and instrumentation" *J. Chromatogr. A*, **703**: 37-57 (1995)

[20] Whitehouse C.M., Dreyer R.N., Yamashita M., Fenn J.B.: "Electrospray interface for liquid chromatographs and mass spectrometers" *Anal. Chem.*, **57**: 675-679, (1985)

[21] Urvoas A., Amekraz B., Moulin C., Le Clainche L., Stöcklin R., Moutiez M.: "Analysis of the metal-binding selectivity of the metallochaperone CopZ from *Enterococcus hirae* by electrospray ionization mass spectrometry" *Rapid Commun. Mass Spectrom.*, **17**: 1889-1896, (2003)

[22] Rosenzweig A.C.: "Metallochaperones: bind and deliver" *Chem. Biol.*, **9**: 673-677, (2002)

[23] O'Halloran T.V., Culotta V.C.: "Metallochaperones, an intracellular shuttle service for metal ions" *J. Biol. Chem.*, **275**: 25057-25060, (2000)

[24] Stillman M.J., Shaw C.F., Suzuki K.T., Metallothioneins Synthesis, Structure and Properties of Metallothioneins, Phytochelatins and Metalthiolate Complexes, VCH, New York, (1992)

[25] Stillman M.J.: "Metallothioneins" *Coord. Chem. Rev.* **144**: 461-511, (1995)

[26] Margoshes M., Vallee B.: "A cadmium protein from equine kidney cortex" *J. Am. Chem. Soc.*, **79**: 4813-4814, (1957)

[27] Dabrio M., Rodríguez A.R., Bordin G., Bebianno M.J., De Ley M., Šestáková I., Vašák M., Nordberg M.: "Study of complexing properties of the α and β metallothioneins domains with cadmium and/or zinc using electrospray ionisation mass spectrometry" *Anal. Chim. Acta,* **435**: 319–330, (2001)

[28] IUPAC-IUB Commission on Biochemical Nomenclature (CBN): "Nomenclature of multiple forms of enzymes. Recommendations (1976)" *J. Biol. Chem*, **252**: 5939-5941, (1977)

[29] Kojima Y., Binz P.A., Kägi J.H.R., Nomenclature of metallothionein: proposal for a revision, in Metallothionein IV, Birkhäuser Verlag, Basel, (1999)

[30] Kojima Y., Binz P.A., Kägi J.H.R., Metallothionein: Molecular Evolution and Classification, in Metallothionein IV, Birkhäuser Verlag, Basel, (1999)

[31] Kägi J.H.R. in Metallothionein III: "Biological Roles and Medical Implications", Suzuki K.T., Imura N., Kimura M., Birkhäuser Verlag, Basel, (1993)

[32] Chassaigne H., Lobinski R.: "Polymorphism and identification of metallothionein isoforms by reversed-phase HPLC with on-line ion-spray mass spectrometric detection" *Anal. Chem.*, **70**: 2536-2543, (1998)

[33] Vašák M., Kägi J.H.R., "Metallothioneins" in Encyclopedia of Inorganic Chemistry, Wiley, New York, (1994)

[34] Arseniev A., Schultze P., Wörgötter E., Braun W., Wagner G., Vašák M., Kägi J.H.R., Wüthrich K.: "Three-dimensional structure of rabbit liver [Cd_7]metallothionein-2a in aqueous solution determined by nuclear magnetic resonance" *J. Mol. Biol.*, **201**: 637-657, (1988)

[35] Schultze P., Wörgötter E., Braun W., Wagner G., Vašák M., Kägi J.H.R., Wüthrich K.: "Conformation of [Cd_7]-metallothionein-2 from rat liver in aqueous solution determined by nuclear magnetic resonance spectroscopy" *J. Mol. Biol.*, **203**: 251-268, (1988)

[36] Messerle B.A., Schaffer A., Vašák M., Kägi J.H.R., Wüthrich K.: "Three-dimensional structure of human [$^{113}Cd_7$]metallothionein-2 in solution determined by nuclear magnetic resonance spectroscopy" *J. Mol. Biol.*, **214**: 765-779 (1990)

[37] Zangger K., Oz G., Otvos J.D., Armitage I.M.: "Three-dimensional solution structure of mouse [Cd_7]-metallothionein-1 by homonuclear and heteronuclear NMR spectroscopy" *Protein Sci.*, **8**: 2630-2638, (1999)

[38] Robbins A.H., McRee D.E., Williamson M., Collett S.A., Xuong N.H., Furey W.F., Wang B.C., Stout C.D.: "Refined crystal structure of Cd, Zn metallothionein at 2.0 Å resolution" *J. Mol. Biol.*, **221**: 1269-1293, (1991)

[39] Narula S.S., Brower M., Hua Y., Armitage I.A.: "Three-dimensional solution structure of *Callinectes sapidus* metallothionein-1 determined by homonuclear and heteronuclear magnetic resonance spectroscopy" *Biochem.*, **34**: 620-631, (1995)

[40] Riek R., Prêcheur B., Wang Y., MacKay E.A., Wider G., Güntert P., Liu A., Kägi J.H.R., Wüthrich K.: "NMR Structure of the Sea Urchin (*Strongylocentrotus purpuratus*) Metallothionein MTA" *J. Mol. Biol.*, **291**: 417-428, (1999)

[41] Peterson C.W., Narula S.S., Armitage I.M.: "3D solution structure of copper and silver-substituted yeast metallothioneins" *FEBS Lett.*, **379**: 85-93, (1996)

[42] Bertini I., Hartmann H.J., Klein T., Gaohua L., Luchinat C., Weser U.: "High resolution solution structure of the protein part of Cu7 metallothionein" *Eur. J. Biochem.*, **267**: 1008-1018, (2000)

[43] Stillman M.J., Thomas D., Trevithick C., Guo W., Siu M.: "Circular dichroism, kinetic and mass spectrometric studies of copper(I) and mercury(II) binding to metallothionein" *J. Inorg. Biochem.*, **79**: 11-19, (2000)

[44] Dabrio M., Rodríguez A.R.: "Complexing properties of the β metallothionein domain with cadmium and/or zinc, studied by differential pulse polarography" *Analusis*, **28**: 370-381, (2000)

[45] Dabrio M., Rodríguez A.R.: "Study of complexing properties of the α-metallothionein domain with cadmium and/or zinc, using differential pulse polarography" *Anal. Chim. Acta*, **424**: 77-90, (2000)

[46] Van Vyncht G., Bordin G., Rodríguez A.R.: "Rabbit liver metallothionein subisoform characterisation using liquid chromatography hyphenated to diode array detection and electrospray ionisation mass spectrometry", *Chromatographia*, **52**: 745-752, (2000)

[47] Chassaigne H., Vacchina V., Lobinski R.: "Elemental speciation analysis in biochemistry by electrospray mass spectrometry " *Trends Anal. Chem.*, **19**: 300-313, (2000)

[48] Dabrio M., Van Vyncht G., Bordin G., Rodríguez A.R.: "Study of complexing properties of the α and β metallothionein domains with cadmium and/or zinc using electrospray ionisation mass spectrometry" *Anal. Chim. Acta*, **435**: 319-330, (2001)

[49] Nordberg M.: "Metallothioneins: historical review and state of knowledge" *Talanta*, **46**: 243-254, (1998)

[50] Bordin G. (Ed.): "Metallothionein: a remarkable molecule for multidisciplinary approach" *Cell. Mol. Biol.*, **46** (2), (2000)

[51] Klaasen C.D., Choudhuri S.: "Use of genetically altered animal models in understanding the role of metallothionein in cadmium toxicity" *Pure Appl. Chem.*, **72**: 1023-1026, (2000)

[52] Nordberg G.F., Jin T., Leffler P., Svensson M., Zhou T., Nordberg M.: "Metallothioneins and diseases with special reference to cadmium poisoning" *Analusis*, **28**: 396-401, (2000)

[53] Kojima Y., "Definitions and Nomenclature of Metallothionein" in Methods in Enzymology 205, Metallobiochemistry Part B: Metallothionein and related Molecules, Academic Press Inc., (1991)

[54] Cobbett C.S., Goldsbrough P.B.: "Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis" *Ann. Rev. Plant Physiol. and Plant Mol. Biol.*, **53**: 159-182, (2002)

[55] Vašák M., Hasler D.W.: "Metallothioneins: new functional and structural insights" *Curr. Opin. in Chem. Biol.*, **4**: 177-183, (2000)

[56] Uchida Y., Takio K., Titani K., Ihara Y., Tomonaga M.: "Metallothioneins: new functional and structural insights" *Neuron*, **7**: 337-347, (1991)

[57] Huang Y., Prasad M., Lemon W.J., Hampel H., Wright F.A., Kornacker K., LiVolsi V., Frankel W., Kloos R.T., Eng C., Pellegata N.S., de la Chapelle A.: "Gene expression in papillary thyroid carcinoma reveals highly consistent profiles" *Proc. Natl. Acad. Sci. USA*, **98**: 15044-15049, (2001)

[58] Lobinski R., Chassaigne H., Szpunar J.: "Analysis for metallothioneins using coupled techniques" *Talanta*, **46**: 271-289, (1998)

[59] McSheehy S., Mester Z.: "The speciation of natural tissues by electrospray mass-spectrometry. II: bioinduced ligands and environmental contaminants" *Trends Anal. Chem.*, **22**: 311-326, (2003)

[60] Briat J.F., Lebrun M.: "Plant responses to metal toxicity" *CR Acad. Sci. Paris.,* **322**: 43-54, (1999)

[61] Clarkson D.T., Lüttge U.: "Divalent cations, transport and compartmentation" *Prog. Bot.*, **51**: 93-112, (1989)

[62] Clemens S.: "Molecular mechanisms of plant metal tolerance and homeostasis" *Planta*, **212**: 475-486, (2001)

[63] Von Wirén N., Klair S., Bansal S., Briat J.F., Khodr H., Shioiri T., Leigh R.A., Hider R.C.: "Nicotianamine chelates both Fe^{III} and Fe^{II}. Implications for metal transport in plants" *Plant Physiol.*, **119**: 1107-1114, (1999)

[64] Hall J.L.: "Cellular mechanisms for heavy metal detoxification and tolerance" *J. of Exp. Bot.*, **53**: 1-11, (2002)

[65] Grill E., Winnacker E.L., Zenk M.H.: „Phytochelatins" *Meth. Enzymol.*, **205**: 333-341, (1991)

[66] Rauser W.E.: "Phytochelatins" *Annu. Rev. Biochem.*, **59**: 61-86, (1990)

[67] Zenk H.M.: "Heavy metal detoxification in higher plants - a review" *Gene*, **179**: 21-30, (1996)

[68] Cunningham S.D., Ow D.W.: "Promises and prospects of phytoremediation" *Plant Physiol.*, **110**: 715719, (1996)

[69] Banuelos G.S., Cardon G., Mackey B., Ben-Asher J., Wu L., Beuselinck P., Akohoue S., Zambrzuski S.: "Boron and selenium removal in boron laden soils by four sprinkler irrigated plant species" *J. Environ. Qual.*, **22**: 786-792, (1993)

[70] Zayed A.M., Terry N.: "Selenium volatilization in roots and shoots: effect of shoot removal and sulfate level" *J. Plant Physiol.*, **143**: 8-14, (1994)

[71] Rugh C.L., Wilde H.D., Stack N.M., Thompson D.M., Summers A.O., Meagher R.B.: "Mercuric ion reduction and resistance in transgenic *Arabidopsis thaliana* plants expressing a modified bacterial merA gene" *Proc. Natl. Acad. Sci. USA,* **93**: 3182-3187, (1996)

[72] Grill E., Thumann J., Winnacker E.L., Zenk M.H.: "Induction of heavy-metal binding phytochelatins by inoculation of cell cultures in standard media" *Plant Cell Rep.*, **7**: 375-378, (1988)

[73] Harmens H., Den Hartog P.R., Bookum W.M.T., Verkleij J.A.C.: "Increased zinc tolerance in *Silene vulgaris* (Moench) garcke is not due to increased production of phytochelatins." *Plant Physiol.*, **103**: 1305-1309, (1993)

[74] Tukendorf A., Rauser W.E.: "Changes in glutathione and phytochelatins in roots of maize seedlings exposed to cadmium" *Plant Sci.*, **70**: 155-166, (1990)

[75] Rai U.N., Tripathi R.D., Gupta M., Chandra P.: "Induction of phytochelatins under cadmium stress in water lettuce (*Pristia stratiotes L*)" *J. Environ. Sci. Health*, **A30**: 2007-2026, (1995)

[76] Morelli E., Scarano G.: "Synthesis and stability of phytochelatins induced by cadmium and lead in the marine diatom *Phaeodactylum tricornutum*" *Mar. Environ. Res.*, **52**: 383-395, (2001)

[77] Klapheck S., Fliegner W., Zimmer I.: "Hydroxymethyl-phytochelatins [(gamma-glutamylcysteine)n-serine] are metal-induced peptides of the Poaceae" *Plant Physiol.*, **104**: 1325-1332, (1994)

[78] Isobe M., Uyakul D., Liu K., Goto T.: "FAB-MS/MS spectrometry in determining the primary structure of γ-glutamyl-containing peptides" *Agric. Biol. Chem.*, **54**: 1651-1660, (1990)

[79] Hofstadler S.A., Bakhtiar R., Smith R.D.: "Electrospray ionization mass spectroscopy: part I. Instrumentation and spectral interpretation" *J. Chem. Educ.*, **73**: A82-A91, (1996)

[80] Yates J.R., III, McCormack A.L., Link A.J., Schieltz D., Eng J., Hays L: "Tutorial review. Future prospects for the analysis of complex biological systems using micro-column liquid chromatography–electrospray tandem mass spectrometry", *Analyst*, **121**: 65R, (1996)

[81] Vacchina V., Chassaigne H., Oven M., Zenk M.H., Lobinski R.: " Characterisation and determination of phytochelatins in plant extracts by electrospray tandem mass spectrometry" *Analyst*, **124**: 1425-1430, (1999)

[82] Leopold I., Günther D.: "Investigation of the binding properties of heavy-metal-peptide complexes in plant cell cultures using HPLC-ICP-MS" *Fresenius' J. Anal. Chem.*, **359**: 364-370, (1997)

[83] Leopold I., Günther D., Neumann D.: „Application of high performance liquid chromatography - inductively coupled plasma mass spectrometry to the investigation of phytochelatin complexes and their role in heavy metal detoxification in plants" *Analusis* **26** (6): M28-M31, (1998)

[84] Loreti V., Bettmer J.: " Determination of the MRI contrast agent Gd-DTPA by SEC–ICP–MS" *Anal. Bioanal. Chem.*, **379**: 1050-1054, (2004)

[85] Runge V.M., Gelblum D.Y., Pacetti M.L., Carolan F., Heard G., *Radiol.*: "Gd-HP-DO3A in clinical MR imaging of the brain" **177**: 393, (1990)

[86] Seiler H., Sigel A., Sigel H. (eds), "Handbook on metals in clinical and analytical chemistry", Dekker M., New York, (1994)

[87] Rocklage S.M., Worah D., Kim S.: "Metal ion release from paramagnetic chelates: what is tolerable?" *Magn. Reson. Med.*, **22**: 216-221 (1991)

[88] Cacheris W.P., Quay S.C., Rocklage S.M.: "The relationship between thermodynamics and the toxicity of gadolinium complexes" *Magn. Reson. Imaging*, **8**: 467-481, (1990)

[89] Behra-Miellet J., Briand G., Kouach M., Gressier B., Cazin M., Cazin J.C.: "On-line HPLC-electrospray ionization mass spectrometry: a pharmacological tool for identifying and studying the stability of Gd^{3+}" *Biomed. Chromatogr.*, **12**: 21-26, (1998)

[90] Skelly Frame E.M., Uzgiris E.E.: "Gadolinium determination in tissue samples by inductively coupled plasma mass spectrometry and inductively coupled plasma atomic emission spectrometry in evaluation of the action of magnetic resonance imaging contrast agents" *Analyst*, **123**: 675-680, (1998)

[91] Blaum K., Geppert C., Schreiber W.G., Hengstler J.G., Müller P., Nörtershäuser W., Wendt K., Bushaw B.A.: "Trace determination of gadolinium in biomedical samples by diode laser-based multi-step resonance ionization mass spectrometry" *Anal. Bioanal. Chem.*, **372**: 759-765, (2002)

[92] Wrobel K., Kannamkumarath S.S., Wrobel K., Caruso J.A.: "Hydrolysis of proteins with methanesulfonic acid for improved HPLC-ICP-MS determination of seleno-methionine in yeast and nuts" *Anal. Bioanal. Chem.,* **375**: 133-138, (2003)

[93] Nischwitz V., Michalke B., Kettrup A.: "Optimisation of extraction procedures for metallothionein-isoforms and superoxide dismutase from liver samples using spiking experiments" *Analyst*, **128**: 109, (2003)

[94] Nischwitz V., Michalke B., Kettrup A.: "Identification and quantification of metallothionein isoforms and superoxide dismutase in spiked liver extracts using HPLC-ESI-MS offline coupling and HPLC-ICP-MS online coupling" *Anal. Bioanal. Chem.*, **375**: 145-156, (2003)

[95] Rivero Martino F.A., Fernàndez-Sànchez M.L., Sanz-Medel A.: "Multi-elemental fractionation in milk whey by size exclusion chromatography coupled on line to ICP-MS" *J. Anal. At. Spectrom.*, **17**: 1271-1277, (2002)

[96] Boulyga S.F., Loreti V., Bettmer J., Heumann K.G.: "Application of SEC-ICP-MS for comparative analyses of metal-containing species in cancerous and healthy human thyroid samples" *Anal. Bioanal. Chem.*, **380**: 198-203, (2004)

[97] Miyazaki I., Asanuma M., Higashi Y., Sogawa C.A., Tanaka K., Ogawa N.: "Age-related changes in expression of metallothionein-III in rat brain" *Neurosci. Res.*, **43**: 323-333, (2002)

[98] Nagel W.W., Vallee B.L.: "Cell cycle regulation of metallothionein in human colonic cancer cells" *Proc. Natl. Acad. Sci. USA*, **92**: 579-583, (1995)

[99] Deng D.X., Chakrabarti S., Waalkes M.P., Cherian M.G.: "Metallothionein and apoptosis in primary human hepatocellular carcinoma and metastatic adenocarcinoma" *Histopathology,* **32**: 340-347, (1998)

[100] Hellquist H.B.: "Apoptosis in epithelial hyperplastic laryngeal lesions" *Acta Otolaryngol.*, **527**: 25-29, (1997)

[101] Tsangaris G.T., Vamvoukakis J., Politis I., Kattamis A.C., Tzortzatou-Stathopoulou F.: "Metallothionein expression prevents apoptosis. II: evaluation of the role of metallothionein expression on the chemotherapy-induced apoptosis during the treatment of acute leukaemia" *Anticancer Res.*, **20**: 4407-4411, (2000)

[102] Karaoglou A., Desmet G., Kelly G.N., Menzel H.G. (eds) "The Radiological Consequences of the Chernobyl Accident. Part IIIB: thyroid cancer in children living near Chernobyl" EUR 16544, Luxembourg, 669–834, (1996)

[103] Williams E.D., Tornko N.D. (eds) "Molecular, cellular, biological characterisation of childhood thyroid cancer", EUR 16538, Luxembourg, (1996)

[104] UNSCEAR*,* 'Exposures and effects of the Chernobyl accident, in sources and effects of ionising radiation" United Nation Publication, E.00.IX.4, 451-565, (2000)

[105] Malko M.V., *Report KUR-KR-79*, Kyoto University, Japan, (2002)

[106] Boulyga S.F., Petri H., Zhuk I.V., Kanash N.V., Malenchenko A.F.: "Neutron-activation analysis of trace elements in thyroids" *J. Radioanal. Nucl. Chem.*, **242**: 335-340, (1999)

[107] Boulyga S.F., Becker J.S., Malenchenko A.F., Dietze H.J.: "Application of ICP-MS for multielement analysis in small sample amounts of pathological thyroid tissue" *Microchim. Acta*, **134**: 215-222, (2000)

[108] Mota J.P.V., Arias A.R.L., de la Campa M.R.F., Alonso J.I.G., Sanz-Medel A.: "Development of a stable isotope approach for the inductively coupled plasma–mass spectrometry determination of oxidized metallothionein in biological materials" *Anal. Biochem.*, **282**: 194-199, (2000)

[109] Malenchenko A.F. (ed), "Ecological problems of pediatrics in a large industrial city, Asveta", Minsk, (1999)

[110] Yen T.Y., Villa J.A., DeWitt J.G.: "Analysis of phytochelatin-cadmium complexes from plant tissue culture using nano-electrospray ionization tandem mass spectrometry and capillary liquid chromatography/electrospray ionization tandem mass spectrometry" *J. Mass Spectrom.,* **34**: 930-941, (1999)

[111] Scarano G., Morelli E.: "Characterization of cadmium- and lead- phytochelatin complexes formed in a marine microalga in response to metal exposure" *BioMetals*, **15**: 145-151, (2002)

[112] Vacchina V., Polec K., Szpunar J.: "Speciation of cadmium in plant tissues by size-exclusion chromatography with ICP-MS detection" *J. Anal. At. Spectrom.,* **14**: 1557-1566, (1999)

[113] Günther K., Waldner H.: "Speciation of zinc and cadmium in ordinary vegetable foodstuffs" *Anal. Chim. Acta*, **259**: 165-173, (1992)

[114] Wei Z., Wong J.W., Chen D.: "Speciation of heavy metal binding non-protein thiols in Agropyron elongatum by size-exclusion HPLC–ICP-MS" *Microchem. J.*, **74**: 207-213, (2003)

[115] Guillard R.R.L. in Smith W.L., Chanley M.H. (Ed) "Culture of marine invertebrates animals", Plenum Press, New York (1975)

[116] Morelli E., Cruz B.H., Somovigo S., Scarano G.: "Speciation of cadmium-γ-glutamyl peptides complexes in cells of the marine microalga *Phaeodactylum tricornutum*" *Plant Sci.*, **163**: 807-813, (2002)

[117] Morelli E., Scarano G.: "Properties of phytochelatin-coated CdS nanocrystallites formed in a marine phytoplanktonic alga (*Phaeodactylum tricornutum*, Bohlin) in response to Cd" *Plant Sci.*, **165**: 803-810, (2003)

[118] Bringezu K., Lichtenberger O., Leopold I., Neumann D.: "Heavy metal tolerance of *Silene vulgaris*" *J. Plant. Physiol.*, **154**: 536-546, (1999)

[119] Leopold I., Günther D., Schmidt J., Neumann D.: "Phytochelatins and heavy metal tolerance" *Phytochem.,* **50**: 1323-1328, (1999)

[120] Wierzbicka M., Szarek-Lukaszewska G., Grodzinska K.: "Highly toxic thallium in plants from the vicinity of Olkusz (Poland)" *Ecotox. Environ. Safety.* **59**: 84-88, (2004)

[121] Freeman J.L., Persans M.W., Nieman K., Albrecht C., Peer W., Pickering I.J., Salt D.E.: "Increased glutathione biosynthesis plays a role in nickel tolerance in *Thlaspi* nickel hyperaccumulators" *Plant Cell*, **16**: 2176–2191, (2004)

[122] Gupta D.K., Tohoyama H., Joho M., Inouhe M.: "Possible roles of phytochelatins and glutathione metabolism in cadmium tolerance in chickpea roots" *J. Plant Res.,* **115**: 429–437, (2002)