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Special issue on Chemical Speciation

Chemical speciation is a science area that seeks to characterize the chemical forms of an element – its species – presents in the environment (water, soil, atmosphere), in living organisms or in materials, in order to understand the transformation between forms which can occur, and to infer from such information the likely consequences for example in terms of risk assessment, reactivity, toxicity or biological activity. As such, there is an increasing interest in speciation analysis in a number of areas such as Environmental Sciences, Occupational Health, Nutrition, Medicine, Biochemistry, among others. Therefore, nowadays, speciation has gained interdisciplinary status and the join venture among different areas of knowledge can lead to a better understanding of extreme complex systems that are still hardly explored and understood.

So, during the last two decades, interest and research in chemical speciation strongly increased and one of the important meetings dedicated to speciation analysis is the International Symposium on Speciation of Elements in Biological, Environmental and Toxicological Sciences. In Brazil, also some research groups worked with chemical speciation, their founding were generally unnoticed due to the lack of a special forum able to give them the convenient highlight.

A workshop on chemical speciation organized in 2006 in the 29th Meeting of the Brazilian Chemical Society was the embryo of the biannual Brazilian Meeting of Chemical Speciation. The "1o Encontro Brasileiro sobre Especiação Quimica - EspeQBrasil-2008" was hold in 2008 in São Pedro (SP), and the second issue of the event - EspeQBrasil-2010" was hold in 2010, also in São Pedro. In each issue, more than 150 participants were presents and many sponsors supported the events, clearly showing the great interest for this so promising area.

Within its 4th issue BrJAC publishes six papers presented at EspeQBrasil-2010 and the "Interview", "Letter" and "Point of view" sections, also bring many information on Chemical Speciation, showing the dynamism of speciation science in Brazil and highlighting its importance in a large field of applications.

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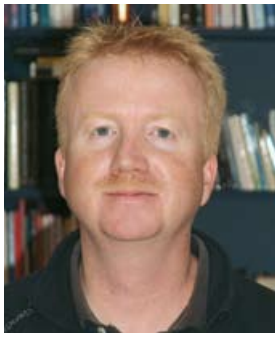
Letter

This section is reserved for you to send comments, suggestions or reviews about the articles or published reports by BrJAC. You may also submit comments on issues related to the Analytical Chemistry in Brazil and abroad. Join us in this project! Be part of that!



Speciation analysis: from academic research to industrial applications

It is with great pleasure that I contribute some reflections on chemical speciation to this new issue of the Brazilian Journal of Analytical Chemistry (BrJAC).

Metals and metalloids play a key role in many fields of our modern industrial societies. Not only as basic material such as steel in many industrial products, but also as trace elements and minerals in industrial processes, in the environment and in living organisms. Thus knowledge on metals is directly related to scientific and technical progress, industrial development and economical growth. Academic research during the last 25 years has shown that not only simple information on the metals, e.g. their total concentrations in a sample, but rather knowledge on chemical forms of metals and metalloids - their species - is an important issue in order to evaluate their reactivity, their transport in the environment or their essentiality and toxicity in organisms. Thus the analytic-chemical research field of speciation analysis was born. Examples illustrating the importance of this research are: mercury as a global pollutant, selenium in nutrition supplements, arsenic in drinking water and rice, metal compounds in catalysts … . In the following, the International Union for Pure and Applied Chemistry (IUPAC) has published guidelines or recommendations for a clear definition of the terms speciation analysis, chemical species, and speciation [1].

The analytical-chemical discipline of speciation analysis is an impressive example how academic research has impact on our society, influences and interacts with industry and leading to new technologies. During the last decade analytical methods for speciation analysis left the academic research laboratories, introduced new analytical instrumentation on the market and found their applications in manifold industrial and societal fields as well as legislation. Examples are: drinking water and food industry (quality of drinking water and food); chemical, semiconductor and petrochemical industry (optimization of processes); pharmacy, medicine, occupational health and hygiene (essentiality and toxicity, metabolic pathways and exposure) … .

With great interest I recognize that BrJAC focus on academic - industrial integration by attracting authors and readers from both areas. This partnership can be a basis to promote technological innovation, higher productivity, development and economical growth in Brazil. Speciation analysis plays an important role in this context; moreover it is a strong research field in Brazil present at many national and international scientific meetings. I wish BrJAC much success for promoting research on chemical speciation in Brazil.

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[1] D.M. Templeton, F. Ariese, R. Cornelis, L.-G. Danielsson, H. Muntau, H.P. van Leeuwen, R. Lobinski, *Pure Appl. Chem*., 72/8 (2000) 1453-1470.

Frontiers of environmental chemistry in Brazil

Marco Tadeu Grassi

Water and air pollution, destination and toxicity of wastes are today political problems that affect the whole world population in different degrees and with different complexities. This is one of the areas of study of professor Marco Tadeu Grassi, from Federal University of Paraná, in southern Brazil. Professor Grassi is a Brazilian chemist dedicated to the field of Environmental Chemistry.

Grassi has created and found infrastructure and financial support for laboratories in the field of Environmental Chemistry in Brazil. He has been researching in the field since the 90s, and has many ongoing research projects dealing with water, oil, instrumentation for chemical analysis, aquatic sediments, and pollutants in public water supply and in rivers and watersheds, plastic containers for water, bioavailability of heavy metals and many other themes. He talked to BrJAC about Environmental Chemistry in Brazil.

How did the Environmental Chemistry begin in Brazil and what is its relationship with the Analytical Chemistry?

In Brazil, Environmental Chemistry has developed from a very close link with the Analytical Chemistry, which was consolidated based on a very strong and traditional school, with a classical vision of its role, and firstly turned

to the development of analytical protocols and elucidation of problems that arise in the laboratory. Much of environmental chemists at that time had a strong connection with the Analytical Chemistry and few had experience in environmental field work and sampling.

In the early 1980's, research in Environmental Chemistry conducted in our country was focused on monitoring of chemical species considered pollutants in the hydrosphere, atmosphere and biosphere. Documents and articles highlight the paucity of research activities dedicated to the study of chemical reactivity and physical behavior of pollutants not only in the three compartments aforementioned, but also in their interfaces[1]. This resulted in a slower development of the Brazilian Environmental Chemistry, compared to other countries, especially the developed ones.

"In Brazil, Environmental Chemistry has developed from a very close link with the Analytical Chemistry"

Despite this, today Environmental Chemistry occupies a prominent position in the national scientific scene, having also reached international prominence. Scientific events, such as the Annual Meetings of the Brazilian Chemical Society (SBQ), the National Meetings of Analytical Chemistry (ENQA), the Brazilian Congress of Environmental Chemistry, subsequently named the International Environmental Chemistry Congress in Brazil, and National Meetings of Environmental Chemistry (ENQAmb), contributed to that. They allowed the discussion about the course of Environmental Chemistry in Brazil.

Other relevant factors were the creation of the Division of Environmental Chemistry of SBQ in 1994, the formation of a significant number of researchers from the groups that had consolidated in the years 80 and 90. These researchers now lead active groups distributed throughout the national territory. Finally, it should also be mentioned the creation of the Environmental Chemistry course, which was included in the curriculum of undergraduate courses in Chemistry in Brazil since the 80's and 90's.

What is the difference between environmental analytical chemistry and analytical chemistry?

They are very distinct areas of knowledge. Briefly we can say that the Environmental Analytical Chemistry is dedicated to the development of protocols and analytical methods aimed at the evaluation of chemical species relevant to humans and the environment. The Environmental Chemistry, in turn, can be defined in many ways, as well as other classic areas of chemistry. Particularly, I like to refer to the definition given on the website of the Division of Environmental Chemistry of SBQ[2], which states: "Environmental Chemistry studies the chemical processes that take place in nature, whether natural or man-made, and that compromise the human health and the planet as a whole." Thus, it is important to say that this area of knowledge is devoted to the elucidation of the mechanisms that define and control the concentration of a particular chemical species in a given environmental compartment, as well as in the interfaces between compartments.

What is the importance of chemical speciation for Environmental Chemistry?

The important role of trace elements in areas such as health and environment, materials science, biogeochemistry, among others, came to

1 *To read more: Andrade, J.B. Química Nova, 15, 1992, 173; Mozeto, A.A. and Jardim, W.F. Química Nova, 25, 2002, 7; Fatibello Filho, O. et al., Química Nova, 25, 2002, 62.*

2 *To see details visit http://ww.sbq.org.br*

be recognized from the development of instrumental analysis, which occurred in the second half of the twentieth century, especially in the area of atomic spectrometry. However, in the environmental and public health contexts it soon became evident that issues such as distribution, mobility and transport, reactivity, bioavailability and consequently the toxicity of chemical species could not be explained solely on the basis of certain chemical inventories, i.e., the total concentration of the species studied. Therefore, it is essential to know the physical and chemical associations in which species are involved. For example, inorganic arsenic is highly toxic, while arsenobetaine, an organic compound, is practically harmless to fish. Another example is chromium. The trivalent chromium is an essential element for humans, while hexavalent chromium is a known carcinogenic agent. Thus, speciation analysis has many implications in environmental chemistry and its interface areas, such as ecotoxicology, biology, soil science, as well as in certain areas of nutrition and medicine.

"Environmental Chemistry occupies a prominent position in the national scientific scene, having also reached international prominence"

How does politics interfere with Environmental Analytical Chemistry?

In Brazil, especially, most studies in the environmental area have a political bias. This is because, with few exceptions, government agencies have been negligent, failing to fulfill their role to monitor and control; governments at the municipal, state and federal levels do not play their role as enforcing the law. If a company develops waste-generating activities, it can be fined or even closed if the effluent does not comply with the law, but what about cities that do not invest in the treatment of domestic sewage, for example? In this case nothing happens in Brazil nowadays. The same can be said about situations involving the presence of compounds that are not provided for by law in a given environmental compartment. The fact that they are not "legislated" makes the situation much worse and shows that there is a lack of action in the environmental and public health fields. Rarely the true culprits are held responsible for it. Fortunately, in some localities the prosecution has sought to intervene in this process, forcing the responsible individuals or companies to fulfill their roles. Ultimately, I believe that the greatest desire of the academic community in the environmental area is to be able to contribute through their research to the improvement of public policies for the environment and extensively the public health.

What are the main developments in the area of "diagnosis" in environmental analytical chemistry in recent years? How did the technology evolve to detect contaminants?

There is no doubt that the environmental analytical chemistry has advanced greatly in recent years. Recently, the American researcher Shane Snyder was asked whether the presence of pharmaceuticals in natural waters was a new problem. In a statement before the U.S. Senate, he was categorical: "contrary to recent reports that characterize pharmaceuticals in water as an entirely new issue, pharmaceuticals were first reported in US waters by the EPA in 1975. The fact that more pharmaceuticals are detected today is not due to greater contamination of our nation's water, but a reflection of the increasingly sensi-

3 To read the whole document visit: http://epw.senate.gov/public/index.cfm?FuseAction=Hearings.Testimony&Hearing_ID=30641a14-802a-23ad-4b51-a10dd439793f&Witness_ID=81d13b7b-c746-4dc7-b97e-c7245922bc8f

tivity analytical technology that allow us to identify and quantify diminishingly minute concentrations of these chemicals in water.[3]" There is no doubt, therefore, that the environmental analytical chemistry, as well as analytical chemistry in general, has achieved enormous progress, both by the rapid development in analytical instrumentation, and because of the advances that have occurred in the area of materials, which has enabled the development and improvement of numerous types of sensors, as well as systems of pre-concentration analysis, for example.

What are the main developments in the area of "treatment", i.e., in the solution for the problem of residuals?

In the area of treatment advances are also unambiguous, making it possible to efficiently treat most of the waste produced by man, whether liquid, solid or gaseous. But today the scientific community has to deal with challenges that mainly involve the development of treatment processes without increasing the pressure on the use of energy and natural resources. In this scenario, the question of water and sewage treatment, for example, is emblematic. Despite all the advances available, about 1 billion people worldwide still lack access to clean water. The availability of good water in quantity and quality is still an indispensable factor for the improvement of life for a good part of the world population. In this field, sanitary engineering has been successful in the treatment of sewage and in the removal of the so-called conventional pollutants. However, the removal of a greater variety of pollutants at different levels of concentration, without increasing energy demand and/or natural resources remains a challenge.

The processes based on biological treatment are still being implemented empirically, grounded on past experience, and this scenario needs to be changed quickly. The traditional microbiology still works, in many cases, considering monocultures, while the microorganisms act in the form of consortia in wastewater treatment systems. This requires a much more detailed assessment on the highly complex microbial community and the advances in molecular biology should contribute to the improvement of treatment processes. In addition, we also have to improve the efficiency of all production processes in general, given that consumption of manufactured goods has grown dramatically over the past 20 years. The production of one kilogram of beef demands, on average, about 15,000 liters of water. This number is frightening and shows us that we urgently need to reflect on our habits of consumption, in all spheres, in order to better manage our water resources.

"The greatest desire of the academic community in the environmental area is to be able to contribute through their research to the improvement of public policies for the environment and extensively the public health"

What are the frontiers of research in environmental analytical chemistry?

As mentioned earlier, the analytical instrumentation has become more and more powerful each year, allowing, for example, the detection of smaller amounts of environmental contaminants. This increase in analytical sensitivity is important in that many of these contaminants are present in public water supplies, air, food, even though we know very little about the long-term effects of their intake at trace or ultra-trace concentrations. On the other hand, one of the main challenges in chemical speciation is the ability to isolate, identify and quantify a given individual species, without tampering with the original compound.

In the environmental area the trend seems to be

to invest heavily in the development of sensors that can be used directly in the field, eliminating the need for collection, preservation and transportation of samples to the laboratory, considered critical steps in the maintenance of the sample's "identity". While such devices are not currently available, efforts have been devoted to the development of semi-automatic equipment based on hyphenated techniques, seeking to include part of the samples pre-treatment stage, in order to offer better reproducibility and also aiming at shortening the time needed for analysis. Another challenge is the development of certified reference materials for speciation analysis, in order to ensure analytical reliability through accuracy and traceability.

How is Brazil positioned in research in this area compared to other developing and developed countries?

Both in the areas of Environmental Analytical Chemistry and Environmental Chemistry, Brazil is very well positioned. We have now consolidated groups developing excellence research in both areas of knowledge, which is the result of the consolidation of chemistry in our country, a consequence of the constant growth and improvement of our graduate programs and the significant improvement in research funding in the last 10 years, particularly at the federal level. However, especially with regard to the Environmental Chemistry, we could be in an even better position. Much of our community continues to develop research of descriptive nature and producing articles that describe only the concentrations of chemical species in a given environmental matrix. Nowadays, taking into account the importance given to impact factors of the scientific journals, the publication of results has to be strongly linked to the development of effectively innovative studies, where the mere characterization of the matrix is no longer relevant. This requires a deep knowledge of chemistry, and a strong academic background.

"Much of our community continues to develop research of descriptive nature and producing articles that describe only the concentrations of chemical species in a given environmental matrix"

What are the possibilities of work today for the newly graduated chemist in the environmental area?

The pressures exerted on our planet by human activities have reached a level never seen before, where abrupt environmental changes clearly can no longer be disregarded. Maintain economic growth and good living conditions for the growing world population without further increasing our demands for energy and raw materials represent a series of challenges for all stakeholders and offer, even in the regional, local level, excellent prospects and opportunities for newly graduated in chemistry in the environmental area. We need urgent answers to a number of issues associated with global warming, acidification of ocean waters, the biogeochemical cycles of nitrogen and phosphorus, global water use, as well as pollution by chemicals, among others. We also need to develop projects based on the principles of green chemistry, i.e., that result in minimization of waste production and decreasing the toxicity. To provide answers and safe paths for humanity, we will certainly need well-formed professionals in chemistry, especially in the environmental area.

Please tell us a little about the history of your career and how (and when) you started to work in the environmental area.

My training took place almost entirely at the Chemistry Institute of Unicamp (Campinas State University). I graduated in Chemistry in July 1986 and soon after that I joined the Masters degree program in the area of Analytical Chem-

istry, working under the guidance of Professor Oswaldo Godinho, a person with a particular characteristic with a strong influence on my training: the search for simple solutions to the problems faced in the laboratory. The Masters degree program represented my first real scientific experiment, for I had met only a short stage-level undergraduate research project in the end of the graduation course, also under Professor Godinho. Living with the teachers and other colleagues in the laboratory was very rich and important to my training, and contributed decisively to the choice of my professional future.

"The pressures exerted on our planet by human activities have reached a level never seen before, where abrupt environmental changes clearly can no longer be disregarded"

Upon completion of the Masters, I decided to go ahead with a Doctoral Degree, working in the field of Environmental Chemistry. I made contact and was accepted to work under the guidance of Professor Wilson de Figueiredo Jardim. In his laboratory I also had excellent opportunities to improve my training. The change of area brought to me the experience of dealing with the real-world problems, with the management of a laboratory, with the development of research projects, something that was not part of our training at the time.

After the PhD, I conducted a post-doctoral training for two years at the University of Delaware, USA, in the Graduate Program in Environmental and Water Resources Engineering, where I also worked as an off campus faculty, having worked under the supervision of Professor Herbert E. Allen. In U.S. I had the opportunity to begin my work in the field of speciation of metals in natural waters, research field to which I still dedicate myself.

Returning to Brazil, I worked for two years as a visiting professor at the Federal University of Maranhão, where, in the company of colleagues and Professor Godinho, I worked on the creation of a research laboratory. There, I have been orienting students at the Masters and Undergraduate research projects.

In 1998 I was admitted to the Department of Chemistry, Federal University of Paraná (UFPR), where I coordinate the Environmental Chemistry Research Group. In UFPR I have been the head of department and vice-coordinator of the graduate program. With support from CNPq, CAPES, Fundação Araucária and also from PETROBRAS, in partnerships in the last 4 years, I have created and gathered infrastructure for a new laboratory. Nowadays, I develop research activities in the area of Trace Analysis and Environmental Chemistry, on the following themes: aquatic and sediment chemistry, speciation of metals and metalloids, behavior and fate of organic and inorganic contaminants in the environment.

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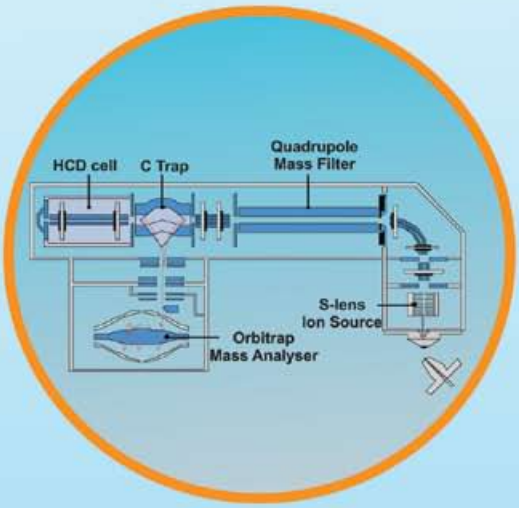

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Concentration of Cu(II) and Cd(II) from natural river water by adsorption on a modified silica surface

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Abstract

A high surface area silica gel (737 ± m^2 g^{-1}) was synthesized modified through a two-step reaction with a 4-amino-2-mercaptopyrimidine ligand and applied to Cu(II) and Cd(II) adsorption from an aqueous medium. The modified material was characterized by FTIR, which showed that attachment of the molecule occurred via thiol groups at 2547 and 2600 cm^{-1}, and by elemental analysis that indicated the presence of 0.0102 mmol of ligand. The data from adsorption experiments were adjusted to a modified Langmuir equation and the maximum adsorption capacity was 6.6 and 3.8 µmol g^{-1} for Cu(II) and Cd(II), respectively. After adjusting several parameters, the material was applied in the preconcentration of natural river water using a continuous flow system before and after sample mineralization, and the results showed a 10-fold enrichment factor. The proposed method was validated through preconcentration and analysis of certified standard reference material (1643e), whose results were in agreement with the values provided by the manufacturer.

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Keywords: metal ions; continuous flow system; surface modification; preconcentration; river water; solid phase extraction;

Introduction

The world's ever growing population and our unsustainable lifestyle are the major causes of environmental contamination. Among the various classes of contaminants, toxic metal species have attracted the attention of numerous scientists around the world because of their distinctive characteristics, such as their persistence in the environment, essentiality and non-essentiality for live organisms, and ability to bioaccumulate in the food chain [1-4]. Heavy metal ions such as cadmium, mercury, lead, nickel and others generally originate from sources such as natural erosion of the earth's crust, mining activities, sewage and industrial effluents that enter the aquatic environment [1,5-8]. Due to the bioaccumulation process, it is important to monitor their concentrations even when metals are present at trace levels.

Materials developed for application in solid phase extraction processes can be used as filters and in concentration procedures [9-14]. The advantages of materials use in concentration are related to the increase in analyte concentration that may reach up to 100-fold. This way, simple equipment such as a flame atomic absorption spectrometer can be used. On the other hand, through concentration the matrix is eliminated ensuring a safe quantification.

Silica-based materials have been used extensively due to the properties of their matrix, such as thermal and mechanical stability and high surface reactivity, which are attributed to the presence of large numbers of silanol groups (4.5 OH/nm^2) [15].

Adsorbent materials are prepared through reaction steps that involve the attachment of organic molecules containing Lewis bases as adsorption sites. This attachment usually occurs via a covalent bond with chlorosilane or aminosilane molecules or through a chlorination reaction with $POCl_3$ [16-21]. The purpose of these modification reactions is to ensure the availability of sites to react with the nucleophilic species existing in the ligand and the major advantage of these modifications is that they improve the adsorption capacity of silica materials toward metal ions.

Several studies have focused on commercial silica [17-19, 22] and its synthesis via a SiO_2 precursor, which can yield materials with desirable characteristics such as large surface area, pores with uniform radii, and ordered structures such as hexagonal mesoporous silica [12,14,23,24].

Actually there is large variety of materials applied to the same purpose, such as silica modified with 2-aminothiazole [12] and oxime molecules [25], cellulose modified with succinic anhydride [26] and natural adsorbents such as coconut coir and agricultural waste material [27,28]. Despite the same application related to metal adsorption/extraction, natural materials presents low cost production and due to this can be used to remove metals from waste water and contaminated systems [29]. Synthetic materials usually present high cost production and are used in concentration systems in order to determine trace concentration of metals.

In this work, a silica gel was synthesized by the sol-gel method and the resulting material was modified in a two-step reaction, resulting in the attachment of 4-amino-2-mercaptopyrimidine to its surface. The modified material (SG-BP) was characterized and applied to determine trace concentrations, through a concentration procedure, of Cu(II) and Cd(II) from a freshwater sample from the Paraná River, Ilha Solteira, SP, Brazil. The accuracy of the proposed method was evaluated by comparison against a standard reference material (SRM-1643-e).

Methodology

Materials and Instrumentation

All the reagents used in the synthesis and modification steps, namely tetraethylorthosilicate (TEOS), 3-chloropropyltrimethoxysilane (CPTS) and dimethylformamide (DMF), were of analytical grade (Aldrich – Steinheim, Germany). The solution of metal ions used in all the experiments was prepared by dissolving their respective nitrate salts (Aldrich-Steinheim, Germany) in deionized water (Millipore, Direct-Q).

A Perkin Elmer AAnalyst 700 atomic absorption spectrometer equipped with electrothermal atomization in a graphite furnace was used in all the metal determinations using 324.8 and 228.8 nm wavelengths as resonance lines for copper and cadmium, respectively. Infrared spectra were recorded on a Nicolet Nexus 670 spectrometer equipped with a Smart Collector, in 200 scans and with 4 cm^{-1} resolution. The surface area was identified using a Micromeritics ASAP-2010 system and the continuous flow experiments were performed with a Gilson Minipuls 3 peristaltic pump.

Preparation of silica gel

Aliquots of 10 mL of TEOS, 20 mL of ethanol and 20 mL of water were transferred into a 250 mL reaction flask and the solution was acidified to pH 1.7 with nitric acid. The solution was stirred for 60 minutes, after which it was transferred to another reaction flask containing 250 mL of nitric acid solution, pH 2. The resulting solution was allowed to rest for 24 hours at 298 K, followed by gradual heating to 323 K to evaporate the solvent until it turned into a gel. This gel was then placed in a drying oven at 333 K and left there until it dried. The resulting silica was stored in a desiccator.

Modification of the silica surface

The reaction steps followed the heterogeneous route, in which the solid matrix is first modified with the organosilane and then attached to the molecule containing Lewis base sites [30,31]. An aliquot of 3 g of the presynthesized silica was activated at 423 K under vacuum for 24 h, followed by immersion in 50 mL of DMF together with 3.37 mL of 3-chloropropyltrimethoxysilane. This suspension was stirred continually for 72 h in a nitrogen atmosphere at 423 K, after which the reaction product (SG-CPTS) was filtered, washed with ethanol and oven-dried at 373 K.

The resulting product (Si-CPTS) was immersed in 50 mL of DMF containing 2.33 g of 4-amino-2-mercaptopyrimidine (MP) ligand, and the mixture was stirred for 72 h in a nitrogen atmosphere at 423 K. The resulting product, called SG-MP, was washed with ethanol and acetone to remove molecules not bonded to the SG-CPTS product, oven-dried at 373 K and stored in a desiccator before use. The two-step reaction involved in the silica modification is depicted in Fig. 1.

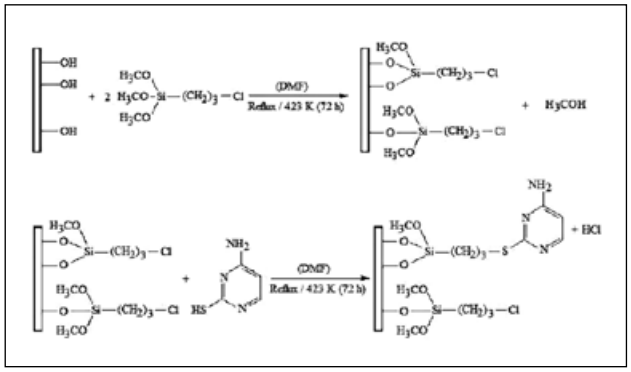

Figure 1. Silica surface modification steps through the attachment of 4-amino-2-mercaptopyrimidine.

Column optimization parameters

The modified material (SG-MP) was characterized in terms of its ability to adsorb Cu(II) and Cd(II), using the continuous flow system illustrated in Fig. 2, and a column packed with 10 mg of SG-MP. In the system, the metal ion solution was stored in the container (1), from where it was pumped through a peristaltic pump (3) to percolate through the SG-MP column (5). After percolation of the metal ion solution, an aliquot of 10 mL of deionized water was percolated to strip the metal species not bound to SG-MP from the column. The commutation switch (4) enabled the eluant (2) to percolate through the column and the eluate was collected in a 2 mL polyethylene flask.

Before determining the metal ion adsorption capacity, the system's operational parameters, such as sample and eluant flow rates, sample and eluant pH, and type of eluant, were optimized by a univariate method. These parameters were established by using 10 mL of 0.05 mg L^{-1} Cu(II) solution and 1 mL of 2.0 mol L^{-1} HNO_3 solution as eluant. After

each elution step, the eluates were analyzed by flame atomic absorption spectrometry (FAAS).

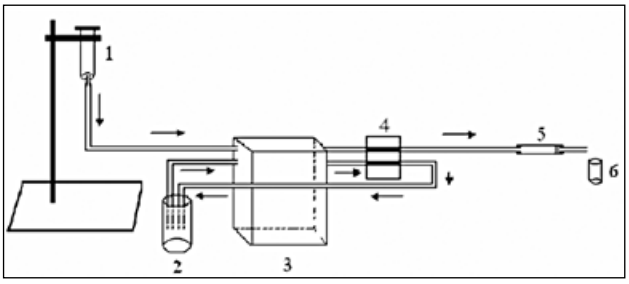

Figure 2. Schematic diagram of the continuous flow system used for the determination of SG-MP adsorption capacity and for the concentration of natural river water: (1) solution/sample container; (2) eluant; (3) peristaltic pump; (4) commutation switch; (5) SG-MP column.

Column adsorption experiments of Cu(II) and Cd(II)

As described in the literature [32], static (solid phase remains fixed such as in column experiments) and dynamic (solid phase and metal species remains under stirring) methods did not show significant differences, so the adsorption experiments were performed using the static (column) method. The percolation and elution flow rate of the metal solutions was fixed at 1.0 mL min^{-1}. To determine the SG-MP adsorption capacity, aliquots of 10 mL of the metal solutions in the range of 0.05 - 1.0 mg L^{-1} were percolated through the column and the eluates (1 mL) were analyzed by FAAS.

The effect of sample volume on adsorption was studied by percolating volumes of 5.0–150 mL of metal ion solution containing the same amount of metal ions, 0.5 µg. After elution, the metal concentration was determined by FAAS.

Preparation of Paraná River water and SRM material

The river water was filtered through a 0.45 µm membrane to eliminate particulate matter, and a filtered aliquot of 50 mL was heated near to dryness (approximately 10 mL) at 80 °C on a hot plate. A digestive solution was prepared by pouring 5 mL of concentrated HNO_3 and 0.5 mL of H_2O_2 into a flask containing the remaining water and keeping the mixture on a hot plate for 30 minutes. After digestion the mixture was filtered, the pH was adjusted to 5 by adding NaOH solution, and it was then transferred to a 50 mL volumetric flask. The results obtained with river water concentration were compared with those obtained directly by GFAAS to estimate the contribution of organic matter that could be associated with the metal species under investigation.

The precision and accuracy of the proposed method were checked by comparing them with a water SRM. Aliquots of 10 mL were percolated through the column and the eluates were analyzed by FAAS.

Results and Discussion

Characterization of modified silica

To confirm the silica surface modification, FTIR spectra of pure silica (SG), silica with 3-chloropropyltrimetoxysilane (SG-CPTS) and silica modified with 4-amino-2-mercaptopyrimidine (SG-MP) were recorded and are depicted in Fig. 3. Pure silica spectra exhibit a broad band in the region of 3500 cm^{-1} that was attributed to OH stretching vibrations from silanol groups as well as that intense band in the region of 3750 cm^{-1}. After silica reaction with 3-chloropropyltrimethoxysilane several changes in the silica spectrum can be observed, such as bands that appears in the region of 2950 and 2850 cm^{-1}, which can be attributed to the symmetrical and asymmetrical vibrations of CH_2 groups from silylating agent, respectively. Through the comparison of the modified silica spectrum (SG-MP) with the other two spectra some observed modifications were attributed to the attachment of the ligand, such as the bands that appear in the region of 1582 and 1541 cm^{-1} and at 3347 cm^{-}, which are attributed, respectively, to N-H and NH_2 vibrations from 4-amino-2-mercaptopyrimidine groups. The presence of the NH_2 band in the SG-MP and the absence of SH absorption bands (2550 – 2600 cm^{-1}) confirm that MP reacted with 3-chloropropyltirmethoxysilane through a chemical bond formed with sulfur atoms (Fig. 1).

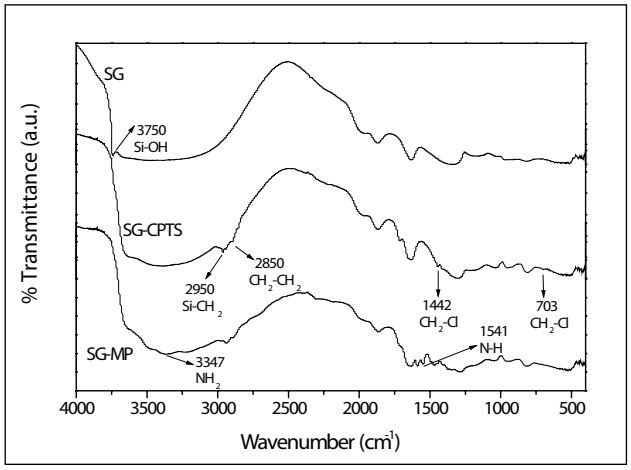

Figure 3. Infrared spectra of pure silica (SG), silica with 3-chloropropyltirmethoxysilane (Si-CPTS) and silica with 4-amino-2-mercaptopyridine (SG-MP).

The attachment of the ligand was also ascertained by measuring the specific surface area of the materials. The decrease in specific surface area from 737 ± m^2 g^{-1} to 399 ± 1 m^2 g^{-1} was ascribed to occlusion of SG pores by bond formation between pendant organic chains, which reduced the adsorption of N_2 molecules used in the S_{BET} measurement process [33].

An important aspect of the information about the immobilization of pendant groups on the silica surface was obtained through elemental analysis, which showed the presence of 0.0102 mmol of 4-amino-2-mercaptopyrimidine attached per gram of silica. The number of ligand molecule was calculated taking into account the SG-BP nitrogen content (in a determined mass of modified material) and the number of nitrogen atoms existing in a molecule of 4-amino-2-mercaptopyrimidine.

Column optimization parameters

The continuous flow system parameters were optimized based on the best Cu(II) ion recovery rate and these conditions were also applied in the concentration of Cd(II). The sample and eluant flow rate were investigated in a range of 1-4 mL min^{-1} and when one parameter was under investigation the other was kept constant at 1.0 mL min^{-1}. The results are shown in Fig. 4.

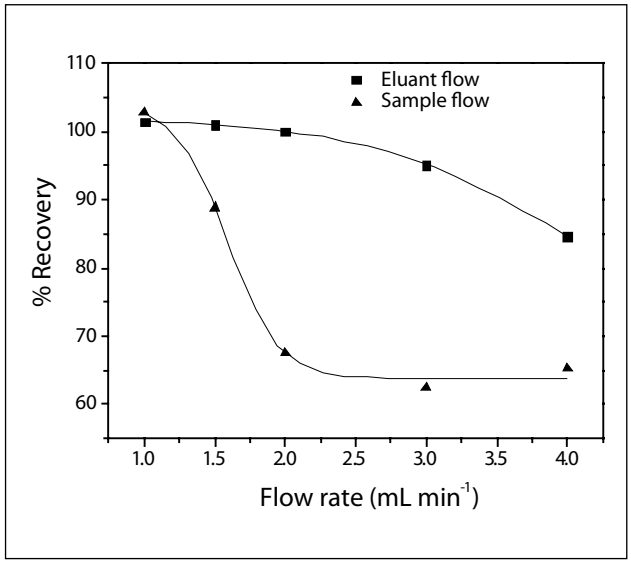

Figure 4. Effect of sample and eluant flow rate on the recovery of metal ions

As Fig. 4 indicates, the best sample flow rate was achieved with 1.0 mL min^{-1}, which corresponds to a recovery rate of about 101%. Higher flow rates led to a decrease in the recovery rate, which was attributed to the shorter contact time between metal species and adsorption sites, preventing the formation of bonds between the electron pair donor atoms in the 4-amino-2-mercaptopyrimidine ligand and the metal ion species. This behavior was also observed in other published works that involved the use of continuous flow systems [12,31].

The effect of the eluant flow rate on the metal ion recovery rate was not significant, since 100% recovery was attained at flow rates of up to 2.0 mL min^{-1}. At higher values, the recovery rate decreased due to the briefer contact between adsorption sites and H^+ species, rendering the protonation ineffective. To render the system easy to use in further experiments, the sample and eluant flow rates were kept constant at 1.0 mL min^{-1}.

The sample's pH was investigated in the range of 1-5, with sample and eluant flow rates kept constant at 1.0 mL min^{-1}. The best recovery rate (about 100%) was achieved at sample pH 5. At pH values lower than 5, the concentration of H^+ species becomes higher and, hence, the number of adsorption sites available to coordinate metal species diminishes, due to the protonation process that may occur in the ligand.

To investigate the best eluant and its concentration, three different types of acid solutions were tested: HNO_3, HCl and $HC_2H_3O_2$ in concentration ranges of 0.5-3.0 mol L^{-1}. The results in Fig. 5 indicate that, at a concentration of 2.0 mol L^{-1}, all acid solutions attained an effective recovery rate of over 96%. However, acetic acid only attained a recovery rate of about 100% at a high concentration of 3.0 mol L^{-1}. Hydrochloric acid showed quantitative recovery starting from 1.5 mol L^{-1} and could be used to elute Cu(II) and Cd(II). Nevertheless, the eluant chosen for further experiments was nitric acid because it showed a high percentage of recovery even at the lowest concentration (1.0 mol L^{-1}), with the advantage of allowing for the simultaneous elution of several elements.

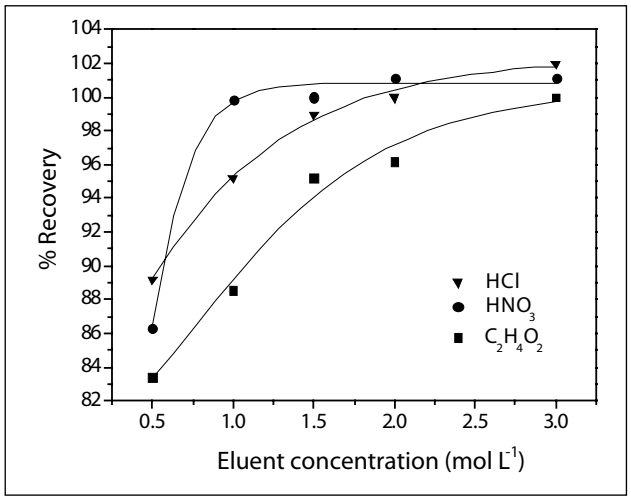

Figure 5. Type of eluant and influence of concentration on the recovery of metal ions

Effect of sample volume

The effect of sample volume on the adsorption of metal species on 10 mg of SG-MP was investigated by passing sample volumes of 5-150 mL containing the same quantity of metal ions (0.5 µg) through the column and measuring the amount of adsorbed metal after elution. The system parameters were the ones described previously. All the sample volumes of both Cu(II) and Cd(II) resulted in a recovery rate of about 100%, indicating that sample volumes of up to 150 mL can be concentrated in the system, provided their metal concentrations are at trace levels [34-36].

Cu(II) and Cd(II) adsorption capacity and enrichment factor

The adsorption capacity was determined using the column system and all the aforementioned parameters. The Si-MP adsorption capacity was calculated according to equation 1 [19]:

$$N_f = \frac{n_e}{m_{SG\text{-}MP}} \quad (1)$$

where N_f is the adsorption capacity, n_e is the mol number of metal species existing in the eluate, and $m_{SG\text{-}MP}$ is the modified silica mass used in the packed column. The experimental data were substituted in the general equation of the modified Langmuir model presented in equation 2 [19]:

$$\frac{C_s}{N_f} = \frac{C_s}{N_s} + \frac{1}{N_s b} \qquad (2)$$

where C_s is the concentration of the solution at equilibrium, N_s is the maximum amount of metal species adsorbed per gram of Si-mod, and b is the equilibrium constant. The N_s values for Cu(II) and Cd(II) calculated from the linearization of adsorption isotherms (Fig. 6) were 6.6 and 3.8 μmol g^{-1}, respectively, for Cu(II) and Cd(II).

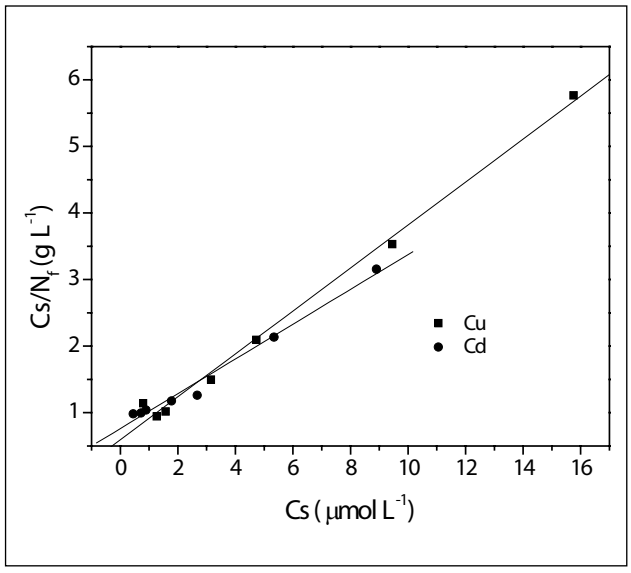

Figure 6. Linearized adsorption isotherms for Cu(II) and Cd(II)

The enrichment factor was calculated by percolating aliquots of 10.0 mL of 0.05 mg L^{-1} of Cu(II) and Cd(II) solutions. After elution, the eluates containing 1.0 mL were subjected to FAAS analysis. The enrichment factor of the metals under investigation was 10.2, which is consistent with the sample volume experiment. The enrichment factor was calculated according to equation 3 [30]:

$$Ef = \frac{Ec}{Sc} \qquad (3)$$

were *Ec* represents the eluant concentration (1.0 mL) and *Sc* represents the solution concentration/sample concentration (before being subjected to the concentration). A comparison of enrichment factor for different type materials are summarized in Table I.

Table I. Comparison of enrichment factor of several materials applied in the extraction of metals from water.

Type material	Mass used (g)	Sample volume (L)	Enrichment factor	Reference
Modified activated carbon	---	0.25	25.0	[37]
Silica with amidoamidoxime	0.04	0.10	20.0	[38]
Activated carbon	---	0.025	30.0	[39]
SG-MP	0.01	0.010	10.0	a

[a] This work

According to the values presented in Table I the enrichment factor of the developed material showed a similar enrichment factor, compared to the other materials. As this parameter is related to the mass and sample volume subjected to concentration, the enrichment factor could be enhanced, although sample volumes larger than 10 mL were not used due to the high percolation time necessary.

Concentration of natural river water

After the digestion procedure, aliquots of 10.0 mL of water were percolated through the column at 1.0 mL min^{-1}. The elution was also performed at 1.0 mL min^{-1} with 1.0 mL of 2.0 mol L^{-1} HNO_3 solution. The procedure was carried out in triplicate for each metal and after recovery, all eluates were analyzed by FAAS. Table II lists the results of the Cu(II) and Cd(II) concentration in natural river water determined directly by electrothermal atomization in the graphite furnace, as well as the concentration results. The results listed in Table II indicate the consistency of the values obtained for the concentration of metal species in water with and without the use of the concentration system. To validate the method, a certified reference material of water was subjected to a concentration, and its results showed a direct relationship with the values provided by the manufacturer, taking into account the preconcentration factor provided by the continuous flow system.

Table II. Metal ion concentrations in river water with and without concentration

Metal	Sample without concentration (μg L-1)*	Concentrated/ mineralized sample(μg L^{-1})	Concentrated/ non mineralized (μg L^{-1})
Copper	8.20 ± 0.67	85.2 ± 4.5**	71.0 ± 4.0**
Cadmium	2.30 ± 0.19	21.0 ± 2.6*	14.1 ± 2.8*
	SRM (μg L^{-1})		SRM PC (μg L^{-1})**
Copper	22.76 ± 0.31		230.0 ± 5.4
Cadmium	6.568 ± 0.073		65.1 ± 3.6

(*) Analyzed by GFAAS, (**) Analyzed by FAAS

Conclusion

The two step reaction of silica with organic molecules resulted in a hybrid material with good separation and concentration capacity. Through the concentration system developed a water sample can be concentrated and metal ions present at trace levels can be determined using flame atomic absorption spectrometry, which is an accessible analytical technique with low cost. The developed concentration system was successfully validated through certified reference material.

Acknowledgments

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Association of Zn, Cd and As with proteins in *Pityrogramma calomelanos* and *Nephrolepis biserrata* submitted to oxidative and hydric stress

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Abstract
This work represents a study of the association of zinc, cadmium and arsenic to proteins in terrestrial plants. Two plants species (*Pityrogramma calomelanos* and *Nephrolepis biserrata)* were collected at a site highly contaminated with to Cd, Zn and As, located in Sepetiba Bay, RJ-Brazil. Plant samples were dried at room temperature and crushed. Then plant proteins were extracted. The fractions were immediately injected into an hyphenated system SEC-UV-ICP-MS, using a Superdex 200 10/300 GL column. The results showed an association of zinc, cadmium and arsenic to three proteins with molar mass ranges of 94.7 ± 0.2 kDa (present in *P. calomelanos*); of 31.6 ± 2.6 kDa and of 20.2 ± 0.6 kDa (in both plant species). Moreover, the results also showed that zinc, cadmium and arsenic are preferentially associated to a protein whose molar mass is close to superoxide dismutase - SOD (32 kDa), normally associated with oxidative stress processes. In both species the arsenic also shows association to a protein whose molar mass is close to that of dehydrins (19 and 24 kDa), which are produced by plants under conditions of high salinity in dry environments.

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Keywords: plants, proteins, metals, SEC-UV-ICP-MS.

Introduction

The determination of chemical species present in low concentrations in living cells is extremely important, since small variations in their concentrations determine the essentiality or toxicity of the species[1,2]. Heavy metals, at high levels, affect plant growth, nutrient uptake and metabolism[2].

Several studies have been made in order to identify plants which can be used for phytoremediation. This technology uses plants that are able to accumulate metals in order to clean up the environment. Even though most of the plants are capable of accumulating metals, only a small number of species is considered hyperaccumulators. According to the literature[3], metal concentrations in plants above 500 mg kg^{-1} indicate hyperaccumulation capability.

Studies involving the relationship of metals and oxidative stress have carried out and the authors concluded that the induction of oxidative stress is the main process underlying metal toxicity in plants[4,5,6,7].

Singh et al. (2006)[5] studied arsenic-induced oxidative stress in two ferns, one hyperaccumulator (*P. vittata*) and another a non-hyperaccumulator specie *(P. ensiformis)*. In this study plants were cultivated in vitro in a nutrient solution containing 133 and 267 mmol As L^{-1}. *P. vittata* accumulated 2.4–2.8 times more arsenic than *P. ensiformis* in the fronds after 1 day exposure, and the difference increased to 10–19 times after 5- and 10-days exposures. Proteins, lipids peroxidation, chlorophyll, total carotenoids, glutathione and ascorbate were determined, and changes in these concentrations explained why *P. vittata* is an As hyperaccumulator and *P. ensiformis* isn't. Concentrations of glutathione and ascorbate, which are known to combat oxidative stress, increased at rates higher for *P. vittata* than *P. ensiformis*, showing that the antioxidant defense system in *P. vittata* is more effective.

According to Bidar et al. (2007)[8] different plant species, *Trifolium repens and Lolium perenne,* can exhibit different behaviors in soil polluted with Cd, Pb and Zn, *L. perenne* being more affected by metal-induced oxidative stress than *T. repens*. In this study two sites were used, one unpolluted environment, distant of about 20 km from the polluted area. The polluted site was situated at 200 m from a closed smelter on former agricultural land. The unpolluted site was defined as control. The plants were cultivated at these sites. The results show that the *T. repens* species accumulates until 1301 ± 55 mg kg^{-1} of Zn, 1222 ± 150 mg kg^{-1} of Pb and 25.86 ± 0.9 mg kg^{-1} of Cd from the polluted site. Moreover, this species shows lowh oxidative damage than *L. perenne*, suggesting that *T. repens* would be more suitable for phytomanagement than *L. perenne*. In this study the superoxide dismutase (SOD) level in plants was also determinated and the authors concluded that SOD has been delineated to be one of the most

important members of enzymatic antioxidant defenses.

It is well known that plants submitted to high metal concentrations can show induced oxidative stress and increased levels of superoxide dismutase in the cell compartments. Oxidative stress is closely related to metal levels and to plants species[8].

Metals such as copper, zinc and manganese are related to the enzymatic oxidative stress defense system. Oxidative stress occurs when there are serious imbalances in some compartments of the cells between the production of reactive oxygen species (ROS) and antioxidant defense. ROS in plants are produced mainly in mitochondria and also in the chloroplast. Because reactive oxygen species can damage proteins, lipids and DNA, anti oxidant enzymes act in defense against oxidative stress[8,9]. The superoxide dismutase is a metalloenzyme crucial in defense against reactive oxygen species, as it acts in the removal of superoxide radical. The metalloenzyme plays an essential role in protecting the cell against harmful effects of oxygen radicals. The form that contains copper and zinc, called copper and zinc dependent superoxide dismutase (CuZnSOD), is very stable and seems to be present in virtually all eukaryotic cells (plants or animals). The CuZnSOD has a molar mass of 32 kDa and consists of two identical protein subunits, with one atom of copper and zinc in each[9,10].

Another factor that interferes in the development of the plant is drought. There are several factors responsible for plant adaptation to dry conditions; however, dehydrins develop an important role in these cases. Dehydrins correspond to a family of hydrophilic proteins, with a wide range of molar masses ranging from 9 to 200 kDa, accumulated in various plant species in environmental conditions of drought, cold and salinity[11]. In cultured *Cynodom dactylum*, a species of grass, Longxing Hu et al. (2010)[11] found six dehydrin species, with molar masses of 74, 40, 31, 24, 19 and 14 kDa.

The presence of these protein-associated metals is closely linked to environmental factors. Thus the aim of work was to characterise As, Zn and Cd species in *P. calomelano*s and *N. biserrata* growing in a highly contaminated area in order to provide some insight into the mechanisms involved in metal tolerance by plants species.

Methodology

2.1 Study area

Sepetiba Bay is a semi-enclosed bay of 519 km^2, situated 60 km from Rio de Janeiro city. It has the second largest area of mangroves in the State of Rio de Janeiro and is where most of the industrial and domestic effluents are drained. The industrial park in Sepetiba Bay contains diversified companies in various industries. Among them metallurgical and chemical industries are of greatest relevance for potential metal contamination[12,13].

Metalworking began production of Cd and Zn in 1974. As raw material, this industry used the calamine ($Zn_2SiO_3(OH)_2$) or willemite (Zn_2SiO_4). The production process was based on the solubilization of the minerals, followed by the precipitation of undesirable metal and, finally, metal electrodeposition[14]. Also according Barcellos[14], this industry used about 2,000 tons $year^{-1}$ of arsenic during the purification step and only 11 t $year^{-1}$ (0.6% of total used) were recovered from the tailings. Moreover, from 2 to 7% of waste was composed of by zinc.

2.2 Sampling and sample preparation

Two plant species (*Pityrogramma calomelanos* and *Nephrolepis biserrata)* were collected at two sampling points: along a drainage channel (DC) and in a waste pile (WP). They were packed separately into paper bags and transported to the laboratory. Immediately, the samples were washed several times with deionised water (Milli-Q, specific resistivity: 18 MΩ cm) and air-dried. Microscopic inspection of plant surfaces did not show any visible deposits of solid matter. A composite sample from each group of four samples was prepared by mechanically grinding all of them, using a stainless steel grinder, and then by careful homogenisation of the powder. These composite samples were stored in clean polyethylene tubes and kept in the freezer (-4 °C) until analysis.

2.3 Total element determinations

Aliquots of 500 mg (n = 4) of each composite plant sample were digested with 6 mL of HNO_3 (twofold sub-boiled) using closed 50-mL graduated polypropylene tubes placed in a heating block at 90 °C for 12 hours. After cooling, 3 mL of H_2O_2 (Merck Suprapur®, 30% v/v) were added and digestion continued for further 3 hours. The sample extract was then completed to a final volume of 10 mL and centrifuged to separate a small insoluble portion. This insoluble fraction was not considered in the further results. The total As, Zn and Cd determination was performed by ICP-MS (ELAN 6000, Perkin Elmer Sciex). The validation of this technique was tested by the use of a standard reference material of *tomato leaves* (SRM 1573a NIST).

2.4 Extraction procedure

Aliquots of 400 mg (n=3) of each composite plant sample were used for protein extraction in three sequential steps, modified from reference[15]. The extraction procedure consisted of 3 steps: Step 1 (E1) - 10 mmol/L Tris-HCl (pH=7.4) for removal of aqueous complexes located in cytoplasm and vacuoles; Step 2 (E2) - 1% solution of SDS in 10 mmol/L Tris-HCl (pH=7.4) for hydrophobic protein removal, and Step 3 (E3) - 10 mmol/L Ammonium acetate solution (pH=4.6) for removal of small organic acids of low molar mass. The extraction procedures were performed with 10 mL of extracting solution under agitation during 1 h, followed by centrifugation for 40 min (12000 rpm) at 5 °C, in the same vessel, in sequence.

In order to investigate extraction efficiency, an aliquot of the extracts obtained were digested with HNO_3 using

the same procedure described in section 2.3. The determination of total element concentrations in these extracts was performed by ICP-MS. The extracts were diluted 100 times before the determination.

2.5 Qualitative characterization of the extracts

For this study a chromatographic system composed of an isocratic pump, LC-200 (Perkin Elmer, NY,USA), a Superdex 200 10/300 GL SEC column (Tricorn, GE-Healthcare Bio-Sciences, Sweden), a UV/VIS detector (Perkin Elmer) for monitoring macromolecules and ICP-MS (ELAN 5000A, Perkin-Elmer-Sciex, NY,USA) detector for monitoring As-75, Cd-114 and Zn-64 were used. The extracts obtained (section 2.3) were immediately injected into this hyphenated system. The column calibration was performed with the following molar mass markers: chymotrypsinogen (21.6 kDa), ovalbumin (45 kDa), bovine albumin (66 kDa) and blue dextran (2000 kDa). The operational parameters are shown in Table I.

Table I. The Superdex 200 10/300 GL operational parameters

Separation range	100 – 600 kDa
Mobile phase	30 mmol L^{-1} TRIS, 10 mmol L^{-1} NaCl; pH 7.4
Injection volume	100 µL
Flow rate	0.8 mL/min
Total exclusion limit (determine by blue dextran)	10.3 min (2000 kDa)

Results and discussion

To evaluate the methodology, including acidic digestion of the samples and Zn, Cd and As determination by ICP-MS, a standard reference material (SRM 1573a NIST, USA) was analysed. Appropriate recoveries were obtained, ranging from 92.2 to 118% with similar repeatability to that reported by NIST, thus validating the employed methodology (Table II).

Table II. Average concentration (mg g^{-1}), standard deviation (n=4) and analytical recovery (%) of the elements in standard reference material analyzed by ICP-MS.

	Element	Zn	Cd	As
SRM 1573a (tomato leaves)	This study	28.5 ± 0.5	1.80 ± 0.03	0.128 ±0.021
	Certified value	30.9 ± 0.7	1.52 ± 0.04	0.112± 0.004
	Recovery	92.2	118	114

The results obtained for total Zn, Cd and As concentrations in plants species, as well as after protein extraction, are shown in Table III. In general it can be seen that *P. calomelanos* presents higher Zn, Cd and As contents compared to *N. biserrata*. P. *calomelanos* belongs to the same order (Pteridales) as *Pteris vitatta*, which is recognised world-wide as a species that hyperaccumulates As[16].

When compared to the total Zn, Cd and As concentrations in the plant species (Table III) it can be seen that approximately 60% of As, 10% of Zn and 6% of Cd in *P. calomelanos* specie is extractable, regardless of their concentrations. In general, *N. biserrata* showed higher metals extraction efficiency (60% of As, 19% for Zn and 15% for Cd). It also shows that the main portion of total metals is extracted with 10 mmol/L Tris-HCl (pH=7.4, E1) from both plant species. The results on As extraction efficiency are in agreement with data from the literature[17, 18].

Table III. Total average concentrations, standard deviations (n = 4) and total concentrations in each extract (mg g^{-1}) of the elements zinc, cadmium and arsenic in four plant samples.

Sample		Pityrogramma calomelanos		Nephrolepis biserrata	
		DC	WP	DC	WP
Zn	Total	2128 ± 66	6294 ± 363	565 ± 33	3845 ± 198
	Extract 1	174	328	69.6	578
	Extract 2	51,6	168	18.6	146
	Extract 3	37.1	75.0	11.1	63.0
Cd	Total	128 ± 3.9	50.8 ± 2.1	23.7 ± 0.9	58.0 ± 2.9
	Extract 1	3.26	1.29	2.36	5.26
	Extract 2	2.16	1.66	0.819	2.17
	Extract 3	1.68	0.704	0.538	1.13
As	Total	2271 ± 47	510 ± 32	26.4 ± 5.8	36.7 ± 2.7
	Extract 1	854	202	7.60	23.7
	Extract 2	278	78.9	1.95	13.2
	Extract 3	266	35.8	1.22	5.35

Note. (DC): drainage channel and (WP): waste pile.

Even though the extraction efficiencies of Zn and Cd were low, the extracts of the *P. calomelanos* species were submitted to chromatographic analysis on the Superdex 200 column, in order to characterize those proteins that were associated to the metals. The retention times obtained for the peaks were used to identify the molar mass range of the proteins. The results are shown in Table IV.

Table IV. Average retention times, standard deviations (n=6) and molar mass ranges of proteins associated with metals.

Pityrogramma calomelanos			Nephrolepis biserrata		
Peak	Retention time (min)	molar mass range (kDa)	Peak	Retention time (min)	molar mass range (kDa)
1	10.2 ± 0.02	94.5- 94.9	1	10.5 ± 0.3	91.1 – 95.4
2	23.9 ± 1.0	28.9 – 34.2	2	21.8 ± 0.06	37.5 – 37.1
3	29.4 ± 0.4	19.6 – 20.8	3	25.1 ± 1.0	26.3 – 31.1

It can be seen that the qualitative composition of *P. calomelanos* in the drainage channel (Figure 1) presents two predominant protein peaks (2 and 3) in the UV-VIS chromatograms, whose retention times coincide with the ones

also shown by As in the chromatograms obtained by ICP-MS. These peaks are also present in extracts E2 and E3 (not shown). Moreover, these extracts (E2 and E3) present another peak (peak 1) that is not associated to any element of this sample. Peak 1, whose molar mass range is of 94.7 ± 0.2 kDa (Table IV), presents an elution volume close to the exclusion limit which make it difficult to characterize. Peak 2, whose molar mass range is 31.6 ± 2.6 kDa (Table IV), characteristic of superoxide dismutase - SOD (32 kDa), is mostly associated to As. Peak 3 has a molar mass range 20.2 ± 0.6 kDa (Table IV), that resembles the molar masses of dehydrins (19 and 24 kDa according to literature)[20].

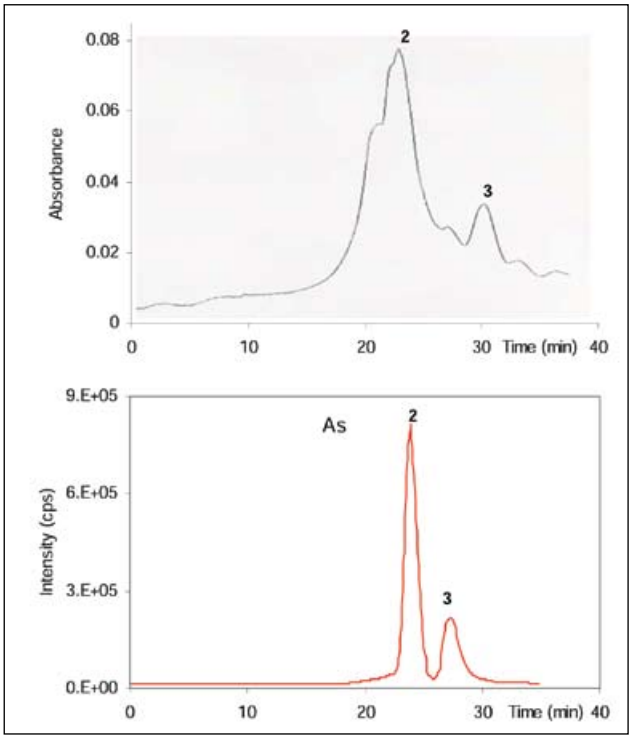

Figure 1. Chromatographic analysis of sample extract 1 (E1) *P. calomelanos*, drainage channel. Upper Figure: UV-VIS detector, monitoring macromolecules; lower figure: ICP-MS detector monitoring the isotope indicated.

The qualitative composition of *P. calomelanos* in the waste pile (Figures 2, 3 and 4; E1, E2 and E3, respectively) for proteins is similar to that obtained for the same species in different locations, showing three protein peaks (1, 2 and 3) in the UV-VIS chromatogram, at the same retention times as those of peaks obtained for the drainage channel. Again, arsenic is associated to peak 2 and 3 in extracts E1, E2 and E3. However, Zn also showed association with peaks 1 and 2 (Figures 2, 3 and 4). The Zn presence associated to proteins in this sample site can be understood due to the higher concentration of total Zn (Table III), as the waste pile has high contents of Zn. Moreover, it can be seen in Figure 3 that peak 1 is associated to the presence of Cd. It is important to note that the specie *P. calomelanos* demonstrated quite different behavior between the two

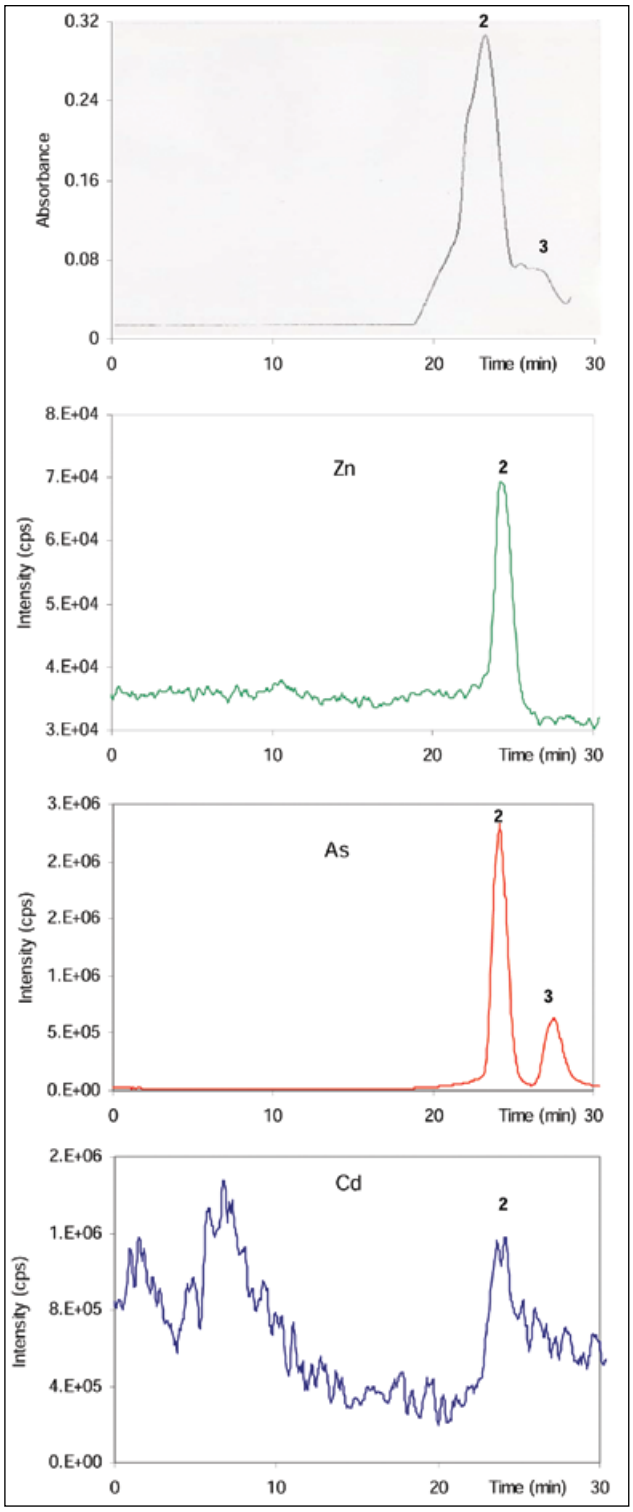

Figure 2. Chromatographic analysis of sample extract 1 (E1) *P. calomelanos*, waste pile. Upper figures: UV-VIS detector, monitoring macromolecules; lower figures: ICP-MS detector monitoring the isotopes indicated.

sampling sites with respect to the association of these metals with proteins. Zn and Cd association with protein was higher in the drier environment (waste pile), which probably promotes greater oxidative stress in plant cells.

It is also important to observe the significant association of Zn (Figure 2) with peak 2, with the mass range of SOD, the protein naturally associated with Zn and related with oxidative stress.

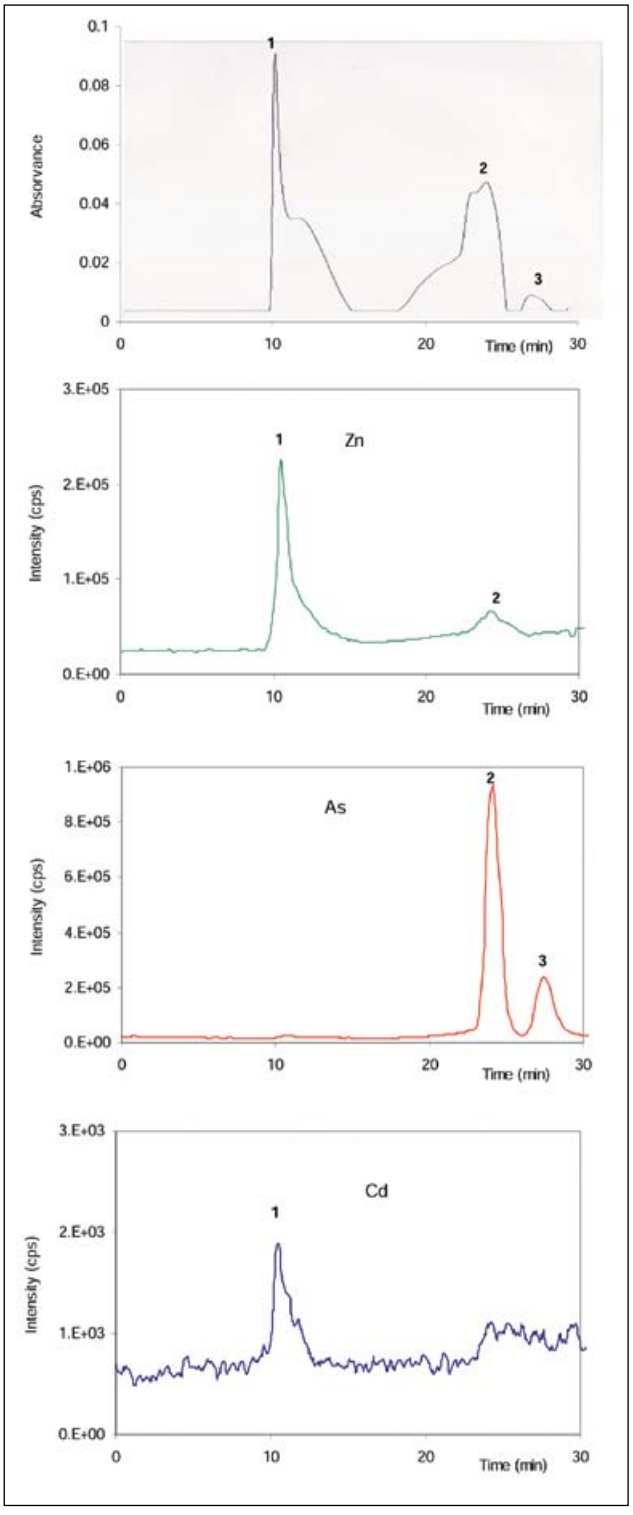

Figure 3. Chromatographic analysis of sample extract 2 (E2) *P. calomelanos*, waste pile. Upper figures: UV-VIS detector, monitoring macromolecules; lower figures: ICP-MS detector monitoring the isotopes indicated.

Even considering its lower concentration Cd shows (Figure 3) an association with peak 1 whose molar mass range is 94.7 ± 0.2 kDa, too close to the column exclusion limit to be identified. Zn also shows an association to this protein.

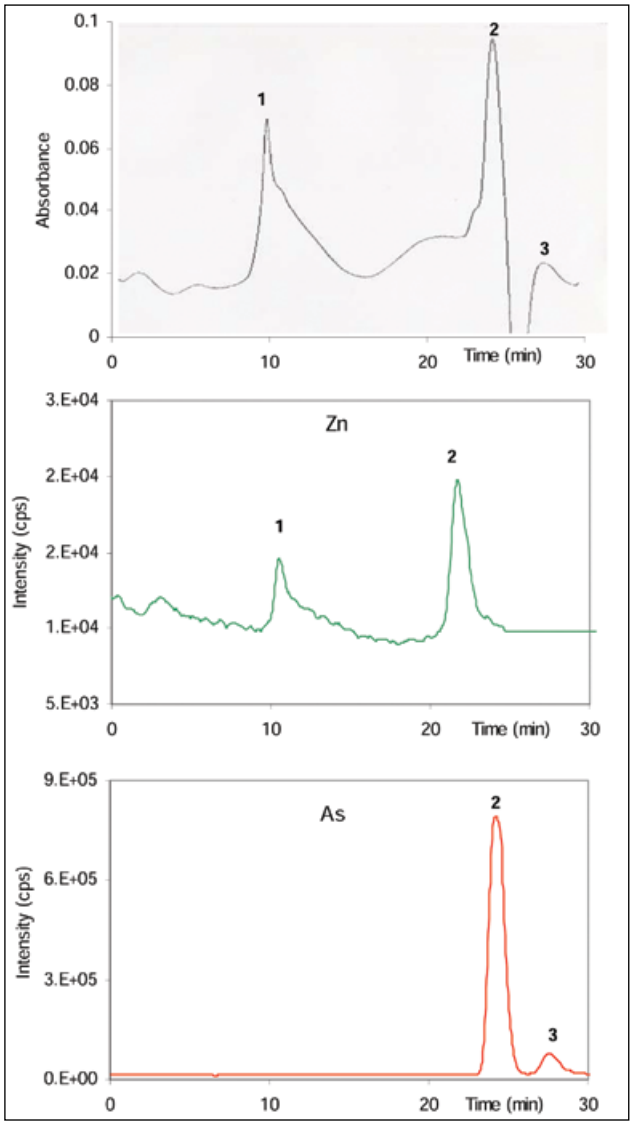

Figure 4. Chromatographic analysis of sample extract 3 (E3) *P. calomelanos*, waste pile. Upper figure: UV-VIS detector, monitoring macromolecules; lower figures: ICP-MS detector monitoring the isotopes indicated

The *N. biserrata* species in the drainage channel showed low metal concentrations that were not associated to proteins. Regarding the *N. biserrata* species in the waste pile it is interesting to point out that although the Cd concentration was too low, this element clearly showed association with peak 2 (Figure 5), whereas *P. calomelanos* showed larger Cd association to peak 1, which indicates different responses from different plant species for the same element, which has also been observed in previous studies[8].

It can be seen that As and Zn are mostly associated to peak2, whose molar mass range is 31.6 ± 2.6 kDa, character-

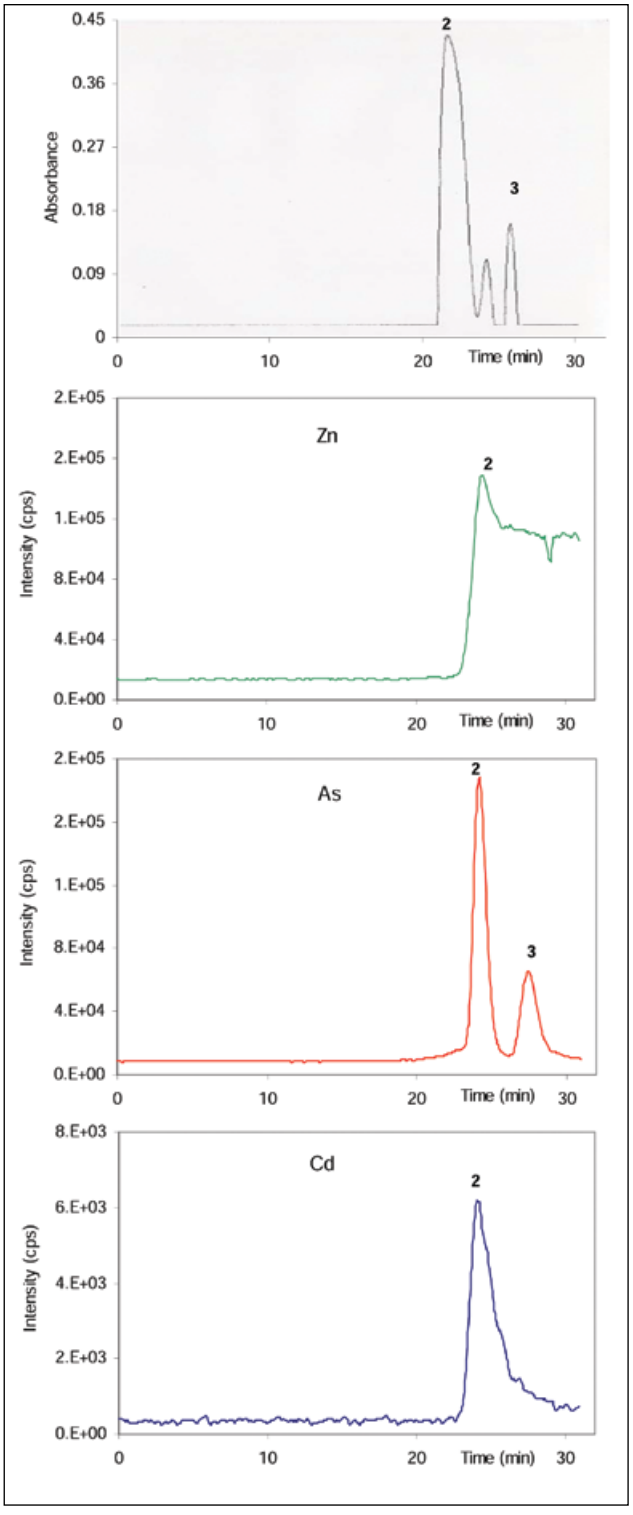

Figure 5. Chromatographic analyses of sample extract 1 (E1) *N. biserrata*, waste pile. Upper figure: UV-VIS detector, monitoring macromolecules; lower figure: ICP-MS detector monitoring the isotopes indicated.

istic of superoxide dismutase - SOD (32 kDa), an antioxidative enzyme found in many plants[19,9].

This shows that, besides the production of phytochelatins, substances which are widely known in detoxification mechanism in plants, there still may be other defense mechanisms involving proteins, which depend on the environmental condition of the plants.

Conclusions

The results showed that total Zn, As and Cd levels in plants and in each extract depend on the plant species and that the protein associations are more depend on plant location than on species. It also showed that plants have different tolerance mechanisms to cope with the presence of metals.

Although the use of complementary techniques for protein identification was not possible, the preliminary results of this research clearly shows that these plant species developed defense mechanisms to adapt and to survive in a highly contaminated, dry and saline environment, since they did not present phytotoxicity symptoms. These mechanisms seem to be related to oxidative and hydric stress.

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Potentiality of the use of montmorillonite in diffusive gradients in thin film (DGT) devices for determination of labile species of Cu, Cr, Cd, Mn, Ni, Pb, and Zn in natural waters

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Abstract
Diffusive gradients in thin films (DGT) were used for characterization of the lability of metallic species in synthetic natural waters. We investigated the possibility of using an alternative low cost binding phase based on montmorillonite (MT) in DGT devices to determine labile fractions of Cu^{2+}, Cr^{3+}, Cd^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} in natural waters. Evaluation of the applicability of montmorillonite for use in DGT devices was performed for metallic species under similar conditions to those found in freshwaters. The percentage of sorption and desorption obtained ranged from 78 ± 5% to 100 ± 2% for the elements studied, indicating that MT exhibited excellent sorption and desorption properties for all elements. DGT devices with montmorillonite were evaluated by recovery tests, with results ranging from 91 ± 6% to 103 ± 6%, being considered satisfactory. The lability of elements in model systems was comparatively evaluated using conventional DGT and MT-DGT devices. Both devices remained between 24 and 72 h in synthetic freshwater samples. The results obtained with both conventional and modified DGT devices showed no statistically significant differences, with 95% confidence. The labile fraction of elements decreased with time, indicating that kinetics is a relevant factor to be considered in studies of speciation. It can be concluded that the MT-DGT devices can be used satisfactorily in determination of labile species of various elements in natural waters.

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1. Introduction

Many trace metals are micronutrients to living organisms, but can become toxic at higher concentrations; others are highly toxic to various life forms [1]. Regardless of their role, the bioavailability and toxicity of elements is related to their chemical form, i.e., their speciation [2,3].

Trace metals can be present in aquatic systems and distributed between different physicochemical forms or species when dissolved, ranging from free hydrated ion to inorganic and organic complexes, as well as in the particulate form [1,4]. Among these forms, labile species – free metal ion and reversible complexes – have been observed to be the bioavailable ones [5]. Therefore, there is a direct relationship between the speciation of elements and their biovailability, dymanics, mobility and toxicity [6]. As a result, speciation studies can provide information about the potential ecotoxicological impact of metals on an environment.

A variety of methods have been used and proposed for the speciation analysis of metallic species in aquatic systems, such as electrochemical, chromatographic and, chemical equilibrium models, among others [3]. However, dynamic metal speciation analysis can be required because natural waters are recognized as mutable and equilibrium conditions are often not achieved [7].

The technique of diffusive gradients in thin films (DGT) has been used for dynamic metal speciation analysis in aquatic systems, taking into account kinetic features and the interconversion of different species [7,8]. Since its invention in the mid-1990s by Davison and Zhang, DGT is an approach for *in situ* determinations of labile metal species in natural waters [9]. DGT rely on diffusion of elements through a layer and accumulation on a binding agent (ion-exchange resin). In general, the advantages of DGT include *in situ* deployment, dynamic speciation capabilities, concentration, detectivity, time-integrated signal and minimization of sample contamination during sampling and handling.

Traditional DGT devices comprise a filter membrane (0.45 μm), a polyacrylamide hydrogel as diffusion layer and a Chelex-100 resin impregnated hydrogel as a binding agent. During the deployment of the DGT devices in aquatic environments, the filter membrane isolates the hydrogel surface from solid particles in the water. Labile metals diffuse across the gel layer and are continuously

accumulated, providing an *in situ* concentration of elements on the resin. The accumulated species are quantified in the laboratory and used to calculate the labile concentrations using Fick's law of diffusion [10,11].

DGT devices with other binding agents have been developed to measure various analytes [12,13]. For example, Fan *et al.* [13] used sodium polyacrylate as binding phase in DGT devices for the measurement of Cu and Cd in waters. In several cases, other materials have been impregnated in the polyacrylamide hydrogel. Chang *et al.* [14] used AG50W-X8 cation exchange resin for the determination of Cs and Sr. Zhang *et al.* [15] studied Fe-oxide for sampling phosphates. Ammonium molybdophosphate was used in DGT devices in order to measure radiocesium [16]. In addition, Menegário *et al.* [17] described the use of *Saccharomyces cerevisiae* immobilized in agarose gel as a new binding agent for DGT.

Clay minerals such as montmorillonite are widely used for various technological developments and numerous studies have demonstrated the efficiency of these materials in the sorption of metal species [18,19]. Thus, clay minerals may be appropriate in DGT sensors because these sorbents have low cost, wide availability, little use in analytical applications as well as high ion exchange capacity.

An alternative binding phase of low cost based on montmorillonite (MT) was investigated and applied as a sorbent in DGT devices (MT-DGT) in this work. Evaluation of the applicability of montmorillonite in DGT devices was performed for Cu^{2+}, Cr^{3+}, Cd^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} under conditions similar to those found in freshwater. The new MT-DGT devices were developed and evaluated by recovery tests and application in synthetic river water.

2. Experimental

2.1. Reagents and apparatus

All reagents were of analytical grade or better and all solutions were prepared with ultra-pure water (Milli-Q, 18 MΩ cm). The standard solutions of Cu^{2+}, Cr^{3+}, Cd^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, and Y^{3+} were prepared by dilution of standard stock 1000 mg L^{-1} (AccuStandard) solutions. DGT polyacrylamide hydrogel diffusion layer, Chelex-100 impregnated hydrogel as binding phase, and nylon DGT holders were purchased from DGT Research Ltd [20]. Cellulose nitrate membranes, 0.45 µm (Sartorius), were used in the DGT devices. K-10 montmorillonite (MT) was supplied by Aldrich.

The determination of all metals was carried out by inductively coupled plasma optical emission spectroscopy (ICP OES) with a Thermo Scientific iCAP 6500. The instrumental conditions were: RF power 1150 W; argon nebulization flow rate 0.2 L min^{-1}; argon main flow 12 L min^{-1}; argon auxiliary flow 0.5 L min^{-1}; axial mode for torch configuration. Measurements of Cu^{2+}, Cr^{3+}, Cd^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} were carried out at 324.754; 283.563; 241.439; 257.610; 231.604; 220.353; and 213.856 nm, respectively. Analytical curves were prepared with 0.10 mg L^{-1} Y as internal standard and its signal was measured during all the experiments at 324.228 nm. The analytical curves were from 0.001 to 1.0 mg L^{-1} for Cu^{2+}, Cr^{3+}, Cd^{2+}, and Mn^{2+}. For Ni^{2+} and Pb^{2+}, the range was from 0.002 to 1.0 mg L^{-1} and for Zn^{2+} it was from 0.005 to 1.0 mg L^{-1}.

The limit of quantification (LOQ) determination followed the recommendations of INMETRO [21]. According to this reference the LOQ is considered as the lowest concentration of an element than can be determined with an acceptable level of accuracy. In this work, LOQ for all elements was determined as the lowest concentration that was able to present an analytical signal greater than the one for the analytical control. The LOQ for Cu^{2+}, Cr^{3+}, Cd^{2+}, and Mn^{2+} was 1.0 µg L^{-1}. The LOQ for Ni^{2+} and Pb^{2+} was 2.0 µg L^{-1}. Finally, the LOQ for Zn^{2+} was 5.0 µg L^{-1}.

2.2. Montmorillonite and membrane treatment

Montmorillonite was treated with 1.0 mol L^{-1} HNO_3 at room temperature to remove inorganic impurities. After the acid treatment, the MT was washed with Milli-Q water and then saturated with 1.0 mol L^{-1} $NaNO_3$ in order to obtain Na^+ homoionic MT. Solids were sieved using a 330 mesh plastic sieve. 1.0 mol L^{-1} HNO_3 was passed under vacuum through 0.45 µm pore size cellulose nitrate membranes to remove inorganic impurities. The membranes were subsequently rinsed thoroughly with Milli-Q water.

2.3. Evaluation of the applicability of MT as sorbent in DGT devices

Sorption and desorption tests were conducted to assess the applicability of montmorillonite as sorbent in DGT devices. Cu^{2+}, Cr^{3+}, Cd^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} were used under similar conditions to those found in fresh waters.

The sorption studies were performed using a batch method and without stirring in which 2.0 g L^{-1} of MT were equilibrated with standard solutions of metals, providing total concentrations of elements between 10.0 and 20.0 µg L^{-1} in 20 ± 5 ºC [22]. The pH and ionic strength of the suspensions were adjusted at 7.0 ± 0.5 and 0.01 mol L^{-1} $NaNO_3$, respectively. After 24 h, the suspensions were centrifuged and the concentrations of metals remaining in solution were determined by ICP OES.

Immediately after the sorption tests, the solid phase obtained from the centrifugation was equilibrated with 1.0 mol L^{-1} HNO_3 for 24 h. The suspensions were centrifuged and the liquid phase was analyzed by ICP OES.

2.4. Preparation and performance evaluation of MT-DGT devices

A disc (2.5 cm diameter and 0.05 cm thickness) was prepared with 0.2 g of montmorillonite, previously dried at 80ºC, using a membrane filter as support in order to assemble DGT sensors. The disc was placed over the base of the conventional holder and it was protected by another layer of membrane filter. Carefully, a poly-

acrylamide hydrogel diffusion layer of 0.08 cm thickness and a cellulose nitrate membrane were arranged on the top of the sorbent layer, respectively. Next, the cap was placed to seal the components of the device. DGT units were stored in clean plastic bags containing drops of 0.01 mol L^{-1} $NaNO_3$ solution and these bags were stored in the refrigerator (4 ºC).

MT-DGT devices were deployed in well-stirred aqueous solutions containing 1.0 mmol L^{-1} and 10.0 mmol L^{-1} of $NaNO_3$, 15.0 and 20.0 µg L^{-1} of each of Cu^{2+}, Cr^{3+}, Cd^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} at pH 7.0 to evaluate the performance of MT-DGT devices. Triplicate DGT sensors were exposed to the test solution for 12 h and 24 h at room temperature (20 ± 5 ºC). After exposure, 1.0 mol L^{-1} HNO_3 was used to desorb the accumulated species for 24 h. The suspensions were centrifuged and the liquid phase was separated and analyzed by ICP OES. The masses of metals (*M*) in MT were obtained using equation 1 [10].

$$M = C_e V_{acid} / f_e \qquad (1)$$

where C_e is the concentration of eluted metals, V_{acid} is the volume of HNO_3 added for desorption and f_e is the elution factor. The concentration of labile species measured by DGT (C_{DGT}) was calculated using equation 2 [10].

$$C_{DGT} = M \Delta g / D A t \qquad (2)$$

where Δg is the thickness of the diffusive phase, D is the diffusion coefficient of the species in the hydrogel, t is the deployment time and A is the exposure area (3.14 cm^2). The values of Δg and D used in this study were those recommended by DGT Research Ltd [20].

2.5. Determination of labile species in the model system employing conventional DGT and MT-DGT devices.

Both types of devices were deployed in 10 L well-stirred synthetic freshwater at pH 7.0 with known composition of metallic species, cations, inorganic and organic ligands. Enrichment with metals to simulate a river sample was performed after 24 h of sample preparation. The composition of the synthetic sample is shown in Table I [22].

Triplicate DGT and MT-DGT units were exposed to the model solution for 24 h and 72 h after sample preparation at room temperature. After exposure, the metals were desorbed with 1.0 mol L^{-1} HNO_3 for 24 h. The suspensions from the desorption experiments were centrifuged and the liquid phase was analyzed by ICP OES. For any DGT device, the mass accumulated is related only to the labile species, so it is the sum of the contributions from free metal, both labile inorganic metal complexes and labile small organic metal complexes. The concentration of labile species measured by DGT (C_{DGT}) was calculated using equations 1 and 2, assuming that the diffusion coefficient (D) of a simple labile metal (free and labile inorganic species) also applies to the labile organic species [2].

Table I. Composition of the synthetic freshwater.

Element	Concentration (mg L^{-1})	
	Initial	After 24 h
Cu^{2+}	0.010	0.015
Cr^{3+}	0.012	0.020
Cd^{2+}	0.010	0.020
Mn^{2+}	0.005	0.015
Ni^{2+}	0.010	0.015
Pb^{2+}	0.015	0.035
Zn^{2+}	0.020	0.040
Ca^{2+}	26.0	26.0
Na^{+}	6.2	6.2
K^{+}	11.0	11.0
Mg^{2+}	7.4	7.4
CO_3^{2-}	56.0	56.0
NO_3^{-}	2.1	2.1
SO_4^{2-}	13.0	13.0
Cl^{-}	8.7	8.7
EDTA	0.25	0.25
Humic Acid (HA)*	2.0	2.0
Fulvic Acid (FA)*	0.15	0.15

* HA: Aldrich, FA: IHSS standard

3. Results and discussion

3.1. Applicability of MT as sorbent phase in DGT devices

The knowledge of sorption and desorption characteristics of metals on/from montmorillonite was important to determine the capacity and applicability of this sorbent in DGT sensors. Indeed, the term "sorption" encompasses adsorption, retention, absorption, surface precipitation and fixation processes. Figure 1 shows the average percentage of sorption and desorption of Cu^{2+}, Cr^{3+}, Cd^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} on/from MT.

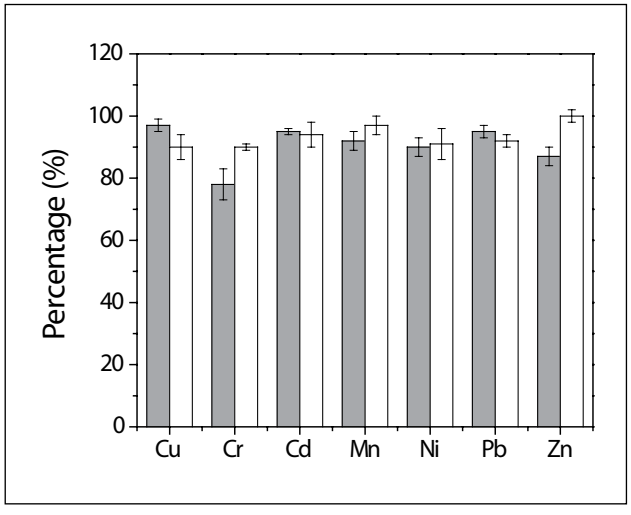

Figure 1. Sorption (gray) and desorption (white) of metals on/from MT. Bars represent the standard deviation. Sorption was evaluated with multi-element solutions containing 10.0 and 20.0 mg L^{-1} of Cu^{2+}, Cr^{3+}, Cd^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}, pH 7.0 and 0.01 mol L^{-1} ionic strength. 1.0 mol L^{-1} HNO_3 was used as extractor in the desorption test.

Figure 1 indicates that the sorption of metal species on montmorillonite was effective, ranging from 78 ± 5% (Cr) to 97 ± 2% (Cu), suggesting an almost complete retention of most species on MT. Thus, montmorillonite has a high efficiency to remove metallic species from the solution under conditions normally found in natural waters, especially regarding the presence of a wide range of elements and concentrations near μg L^{-1}. Numerous studies have demonstrated the efficiency of clay minerals for the sorption of metallic species. However, such studies describe the sorption results for single or few species usually at mg L^{-1} concentration [19,23].

Figure 1 also presents the desorption percentage of previously sorbed elements from MT that was performed with an acidic solution as extractant. The desorption percentage ranged from 90 ± 1% (Cr) to 100 ± 2% (Zn), indicating that a single extraction with 0.1 mol L^{-1} was sufficient for the recovery of all metallic species with high efficiency. Zhang and Davison [10] showed that the elution efficiency was 80% for Chelex 100. As a result, the elution factor (f_e) was considered to be 0.8 for this binding phase in conventional DGT devices. Despite the great number of studies related to the metal retention process onto clay minerals, it is recognized that studies involving the desorption behavior from these sorbents are quite limited [24].

From Figure 1 it is possible to observe that montmorillonite exhibited satisfactory sorption and desorption properties for the seven elements studied. Moreover MT can be regenerated and reused as sorbent. Thus, montmorillonite can be strongly supported as an alternative sorbent phase in DGT devices.

3.2. Performance evaluation of MT-DGT devices

The MT-DGT performance was assessed with recovery tests. MT-DGT measurements of labile metal concentration (C_{DGT}) were performed with aqueous solutions containing the aforementioned metallic species and $NaNO_3$ at pH 7.0, as shown in Figure 2.

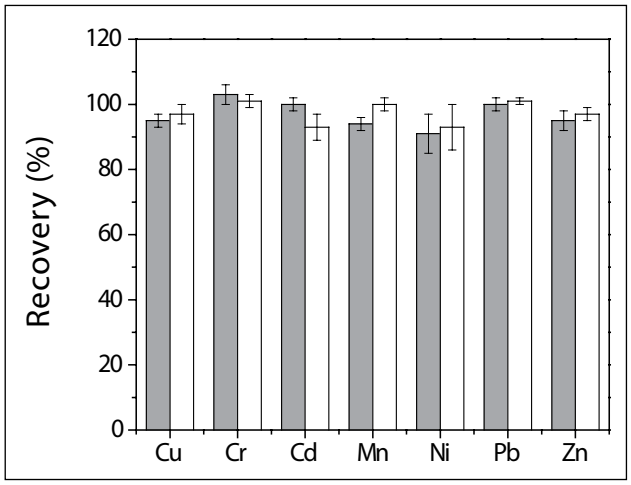

Figure 2. Recovery tests performed in the validation of MT-DGT devices. Gray bars: 5.0 mg L^{-1} of each element, pH 7.0, 1.0 mmol L^{-1} $NaNO_3$ and 24 h of contact time. White bars: 10.0 mg L^{-1} of each element, pH 7.0, 10.0 mmol L^{-1} $NaNO_3$ and 12 h of contact time. The bars represent the standard deviation.

Figure 2 shows the results for tests carried out under different experimental conditions for the evaluation of MT-DGT devices. Recoveries ranged from 91 ± 6% to 103 ± 6% for all species and can be considered adequate. According to the literature recoveries greater than 90 ± 10% are desirable when conventional DGT devices (Chelex 100 as binding phase) are used [20].

These results demonstrate that MT-DGT devices are suitable for determination of labile metallic species in aqueous media.

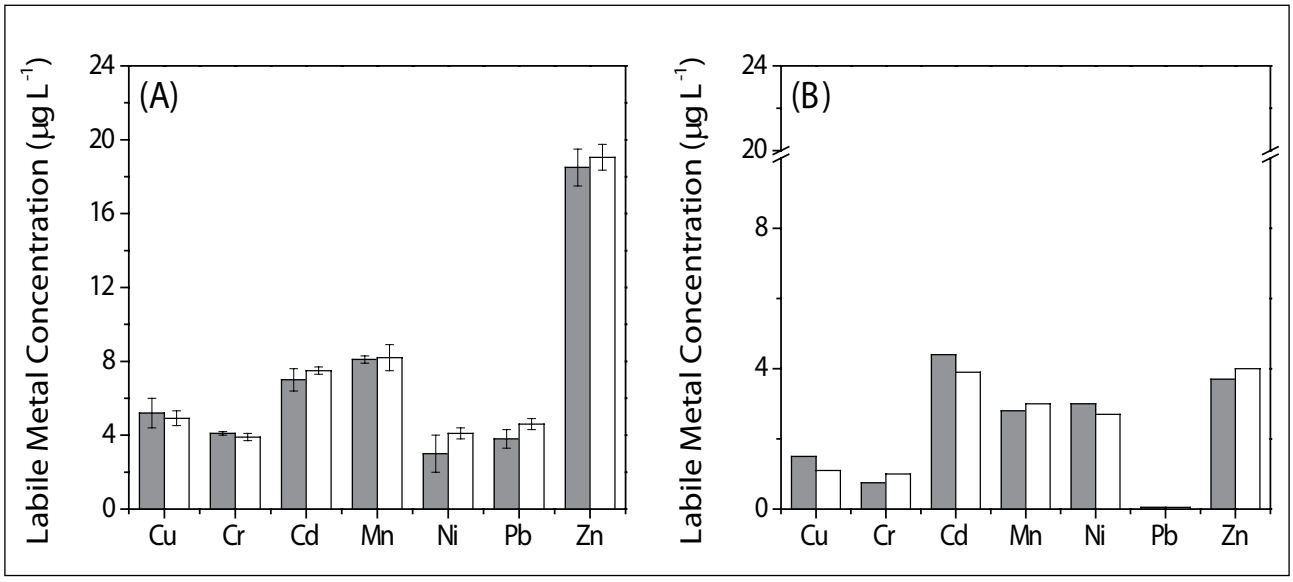

Figure 3. Labile metal concentration in a model system using DGT (gray) and MT-DGT (white) devices after 24 h (A) and 72 h (B). The bars represent the standard deviation.

3.3. Determination of lability of trace elements with conventional DGT and MT-DGT devices.

Lability of Cu^{2+}, Cr^{3+}, Cd^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} in synthetic freshwater was comparatively evaluated using conventional DGT and MT-DGT. Both devices were immersed in the sample, and removed after 24 and 72 hours to assess the association kinetics between metal ions and ligands in the sample.

Figure 3 shows the average labile concentration for metallic species in the synthetic freshwater obtained with conventional DGT and MT-DGT devices. For 24 h device deployment (Figure 3A), the concentrations of metals measured using DGT and MT-DGT ranged from 3 ± 1 to 19 ± 1 μg L^{-1} and 3.9 ± 0.2 to 19.1 ± 0.7 μg L^{-1}, respectively. For 72 h device deployment, the concentrations of metals measured using DGT and MT-DGT ranged from 0.05 ± 0.005 to 4.4 ± 0.6 μg L^{-1} and 0.05 ± 0.005 to 4.0 ± 0.1 μg L^{-1}, respectively. Furthermore, it can be observed in Figures 3A and 3B that the repeatability of measurements with both DGT devices can be considered adequate. For 24 h, the relative standard deviation (RSD) ranged from 2% (Cr) to 33% (Ni) and 1% (Cu) to 9% (Mn) for DGT and MT-DGT, respectively. For 72 h, the RSD values for DGT and MT-DG ranged from 1% (Pb) to 33% (Cu) and 1% (Pb) to 10% (Mn), respectively. Statistical analyses (F Test) were used to detect differences between the results obtained with conventional and modified DGT devices and, in all cases, no significant differences (95% confidence level) were detected as far as the labile concentrations are concerned.

A comparison of metal concentrations determined by both DGT devices (Figure 3) with the values reported in Table I suggests that conventional and MT-DGT devices only measure labile species in the sample (MLabile), i.e., free ions and reversible metallic complexes. The operational definition of lability in DGT is characterized by the thickness of the diffusive gel layer. Free metal ions (M) and metal complexes diffuse through the hydrogel and are concentrated in the resin or montmorillonite, in our case. Metal complexes may also contribute to the metal accumulated in DGT when they are able to dissociate within the diffusive gel layer, and this is the operationally defined labile fraction. DGT measures both labile inorganic and organic species when the non-restrictive hydrogel is used in the devices. Metal complexes that are not able to dissociate within the gel layer are non-labile [9,10]. Thus, the presence of organic ligands (EDTA, humic and fulvic acids) in the sample undoubtedly influences the speciation of the elements. In addition, Figure 3 (A and B) shows that there was a decrease in the lability of metal species with time. The speciation of the elements determined by both DGT devices in the sample model at different times can be evaluated in Figure 4.

Figures 4A and 4B show the distribution of metallic species between complexed and labile fractions in the model sample. It is observed that the labile fraction of elements (MLabile) decreased with time, indicating that a more significant association between metallic species and organic ligands occurred at longer times of the experiments (72 h). For example, the labile fraction of cadmium decreased from 73% to 21% of the total concentration after 24 h and 72 h, respectively.

For the elements shown in Figure 4A, the highest percentages of labile species were found after 24 h of contact. Therefore, these metals were initially found weakly complexed or not yet fully complexed by organic ligands. On the other hand, after 72 h the main fraction was the metal-ligand complex. For the elements in Figure 4B, the labile fraction was lower during the experiment. For example, the labile fraction of lead represented 28% and 0.1% of total concentration after 24 h and 72 h, respectively. As a consequence, these results suggest that there are differ-

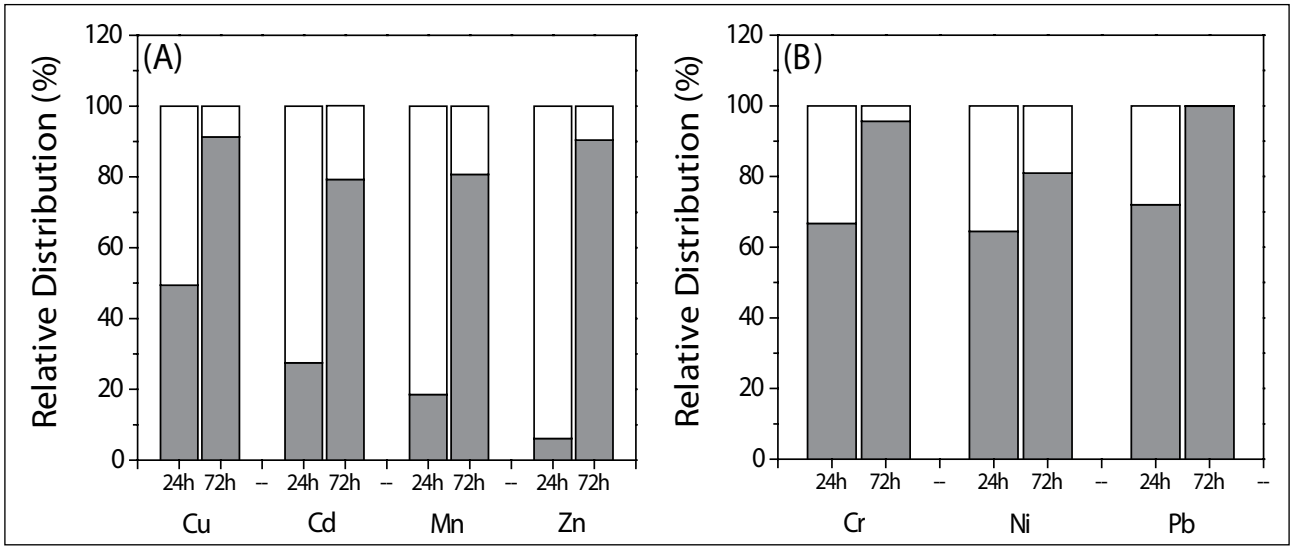

Figure 4. Relative distribution between the labile (MLabile) forms (white) and metal-ligand (ML) complex (black) in a synthetic freshwater sample for (A) Cu^{2+}, Cd^{2+}, Mn^{2+}, Zn^{2+} and (B) Cr^{3+}, Ni^{2+}, Pb^{2+}.

ences in the kinetics of association between the elements and organic ligands in the sample. Cr, Ni and Pb showed fast association kinetics with organic ligands, while Cu, Cd, Mn and Zn showed slower kinetic associations.

Despite the time of the experiment, it was noted that the more labile species in the model system were the elements Zn^{2+}, Mn^{2+} and Cd^{2+}, indicating that the organic ligands showed a lower affinity for these elements. Cr^{3+} and Pb^{2+} showed stronger interactions with organic ligands. These results are consistent with several other studies [25,26]. Humic substances present in the aquatic environment play a major role in the behavior of metallic species due to complexation, which can minimize the bioavailability and toxicity of these elements [1,4,5,26].

In general, it was observed that the lability of elements in the model system depends on the affinity between metals and ligands and, mainly, the kinetics of the complexation process. Thus, kinetics is a relevant factor to be considered in studies of speciation. However, evaluations related to the kinetic nature can only be performed with dynamic techniques of speciation, such as diffusive gradients in thin films.

4. Conclusions

This work has demonstrated that montmorillonite can be used as an alternative and low cost sorbent phase in DGT devices. These sorbents exhibited excellent sorption and desorption properties for seven elements. MT-DGT can accumulate and measure labile metallic species with a suitable recovery under similar conditions to those found in freshwaters. Speciation of several elements in synthetic freshwater was compared using DGT devices with Chelex and montmorillonite and the results demonstrate that data obtained with both devices are comparable. Moreover, our results also have shown that kinetics is a relevant factor to be considered in speciation studies.

Finally, it can be concluded that DGT devices with montmorillonite as a new sorbent phase is an effective and inexpensive alternative for speciation analysis of trace elements in natural waters.

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Chromium speciation in waters using silica nanoparticles organofunctionalized with APTES and graphite furnace atomic absorption spectrometry

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Abstract
A method for the Cr(VI) speciation in waters using silica nanoparticles organofunctionalized with (3-aminopropyl)triethoxysilane (APTES) and graphite furnace atomic absorption spectrometry is proposed. The best conditions for analysis were using 150 mg of silica nanoparticles organofunctionalized with 2.0% (v v^{-1}) of APTES and pH~8. For the heating program the pyrolysis and atomization temperatures were fixed at 1400 °C and 2400 °C, respectively, 10 µg Mg were used as chemical modifier. The detection limits (n=10, 3σ) were 0.06 µg L^{-1} for total Cr and Cr(VI) determinations. The reliability of the proposed method was checked by addition and recovery of Cr(VI) and the results showed recoveries between 91 and 105%.

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Keywords: chromium, speciation analysis, water, APTES, graphite furnace

1. Introduction

Chromium co-exists mainly in two oxidation states, Cr(III) that is essential for the metabolism of glucose, lipids and proteins and Cr(VI), which is highly toxic due to its allergic, carcinogenic, mutagenic and teratogenic effects on humans. Thus, speciation analysis becomes a powerful tool for separation, identification and determination of chromium species present in a sample to recognize the real toxicity [1-3].

Both chromium species can enter into the environment as a result of effluent discharge from steel industries, electroplating, tanning industries, oxidative dyeing, chemical industries, and cooling water towers. Drinking water supply systems can be contaminated by corrosion inhibitors used in water pipes and containers or by contamination of the underground water from sanitary landfill leaching [4].

The level of Cr(VI) concentration has been monitored by many countries, such as, Brazil, Germany, USA, EU, Japan, among others. In the USA, the maximum concentration of total Cr in drinking water is 2.5 µg L^{-1}, considering that up to 7% of this total may be Cr(VI) [1]. The regulation of water quality standards in Brazil is done by the Conselho Nacional do Meio Ambiente (CONAMA, resolution 397/2008). The maximum permissible concentration of total Cr in fresh water is 0.05 mg L^{-1} and the discharged of Cr in waste water is 0.1 mg L^{-1} for Cr(VI) and 1.0 mg L^{-1} for Cr(III).

Speciation analysis of Cr(VI) has been performed by using different analytical methods, such as co-precipitation, ion exchange, liquid–liquid extraction, solid–liquid extraction, cloud point extraction and other techniques. Table I summarizes a short revision, based on the last 10 years (2000-2010) of the main methods used for Cr(III) and Cr(VI) speciation analysis in waters [5-59]. With solid-phase extraction, the use of silica modified with a sililant agent is more common. Silica organofunctionalized with (3-aminopropyl) triethoxysilane (APTES) has been successfully proposed for separation and concentration of chromium species [40, 43 and 56]. APTES incorporates the primary amino group, which can give stable complexes with transition metals or can adsorb metals on the organofunctionalized silica. Considering the performance of this reagent for organofunctionalization and selective complexation of metal species, this work proposes a method for the Cr(VI) speciation in waters using silica nanoparticles organofunctionalized with APTES followed graphite furnace atomic absorption spectrometry.

2. Experimental

2.1. Instrumentation

All measurements were performed with a graphite furnace atomic absorption spectrometer, model SIMAA-6000®, equipped with a longitudinal Zeeman-effect background correction system, Echelle optical arrangement, solid-state detector and standard THGA® graphite tubes with pyrolytically coated integrated platform (Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA). An AS-72 autosampler (Perkin Elmer) was used for transferring reference solutions from polypropylene cups to the graphite tube. The spectrometer was operated using a hollow cathode lamp for chromium (λ: 357.0 nm, lamp current: 25 mA, and bandpass: 0.7 nm). Argon 99.98% (v v^{-1}) (Air Liquide Brasil S/A,

Table I. Revision (2000-2010) of the main methods used for Cr(III) and Cr(VI) speciation in waters.

Method	Water Sample	Separated specie	Method characteristics	Detection	LOD Cr(III) ($\mu g\ L^{-1}$)	LOD Cr(VI) ($\mu g\ L^{-1}$)	Ref.
CE	Tap and river	Cr(III)	Monomeric and polymeric Cr(III) species	ICP-MS	1-10	-	5
Cloud point extraction	Tap and river	Cr(VI)	1,5-diphenylcarbazide and SDS Triton X-114 as an extracting agent. It was obtained a preconcentration factor of 92.	GF AAS	-	0.001	6
	Tap, lake and underground	Cr(VI)	Aliquat-336, Triton X-114, preconcentration factor of 10	ICP-DRC-MS	0.025	0.01	7
	Tap and river	Cr(III)	Bis-[2-Hydroxy-1-naphthaldehyde] thiourea in Triton X-100	F AAS	0.18	-	8
	River	Cr(III)	PAN, Triton X-114 with preconcentration factor of 48	F AAS	0.7	-	9
	Tap and lake	Cr(III)	PMBP with preconcentration factor of 42	GF AAS	0.021	-	10
Spectrophotometric	Tab and top	Cr(III)	α-benzoin oxime and Triton X-100	UV-Vis	0.8	-	11
	River	Cr(VI)	1,5-diphenylcarbazide and flow electrolysis cell	UV-Vis	-	0.014	20
Coprecipitation	Tap, hot mineral spring and sea	Cr(III)	EPHBAT with preconcentration factor of 50	F AAS	1	-	13
	Natural	Cr(III)	3-phenyl-4-o-hydroxybenzyl-idenamino-4,5-dihydro-1,2,4-triazole-5-one	F AAS	0.5	-	14
	Tap	Cr(III)	Dysprosium hydroxide with preconcentration factor of 100	F AAS	0.65	0.78	15
	Tap and mineral	Cr(III)	Ni^{2+}/2-Nitroso-1-naphthol-4-sulfonic acid with preconcentration factor of 50	F AAS	1.33	-	16
Eletrochemical	Tap and sewage	Cr(VI)	Nanostructured gold modified glassy carbon electrode	Au_{nano}/GCE	-	0.01	17
	Sea	Cr(VI)	1,5-diphenylcarbazide	Voltammetry	-	-	18
	River, lake and waste	Cr(III) Cr(VI)	Determination of Cr(III) active and non-active	CCSV-DTPA and GF AAS	-	-	19
	Canal	Cr(VI)	Graphite screen printed macroelectrodes	graphite screen	-	19	20
	Tap and river	Cr(III)	EDTA and DTPA	ASV	-	1.98	21
Liquid-liquid Extration	Waste	Cr(III) Cr(VI)	Triton X-114 and cetylpyridinium bromide	ICP OES	0.02	0.05	22
	Tap and lake	Cr(III)	Nanometre-scale zirconium phosphate coated capillary tube	GF AAS	0.042	-	23
	Tap and river	Cr(VI)	APDC with Pt-195 as an internal standard. I was determined Cr-52 and Cr-53	ICP-MS	0.11	-	24
	Waste	Cr(VI)	Tetrabutylammonium bromide and $(NH_4)_2SO_4$ was used for Cr(VI) extraction	UV-Vis	-	60	25
	Waste	Cr(VI)	Tribenzylamine	UV-Vis	-	80	26
	Waste	Cr(III)	di 2-ethylhexyl phosphoric acid		-	-	27
	Lake and tap	Cr(VI)	Ammonium pyrrolidinedithiocarbamate	GF AAS	-	0.07	28
	Waste	Cr(VI)	TBAI and MIBK		-	10	29
	Waste	Cr(VI)	TBAB	UV-Vis	-	-	30
	Tap , river and sea	Cr(VI)	Ethanol and carbon tetrachloride with preconcentration factors up to 275 and 262 for Cr(VI) and total Cr, respectively	F AAS	-	0.07	31
	River, tap, sea and rain	Cr(VI)	Ethyl acetate	GF AAS	-	0.02	32
Fluorescence	Natural	Cr(VI)	Glutathione capped CdTe quantum dots as fluorescent probes	Fluorescence	-	8	33

Method	Sample	Separated specie	Method characteristics	Detection	LOD Cr(III) ($\mu g\ L^{-1}$)	LOD Cr(VI) ($\mu g\ L^{-1}$)	Ref.
Solid-phase extraction	Natural	Cr(III)	Granular calcite as adsorbent	GF AAS	1.5	0.8	34
	Natural	Cr(III)	DGT and DET in thin-films combined in a single probe	GF AAS	0.0082	0.3	35
	Natural	Cr(III) Cr(VI)	Iminodiacetate extraction disk placed, 1,5-diphenylcarbonohydrazide	MF AAS	-	-	36
	Tap and river	Cr(III)	Chelex-100 by slurry	GF AAS	-	-	37
	Drinking	Cr(VI)	Aliquat 336-AC	X-ray fluorescence	-	-	38
	Drinking	Cr(VI)	TiO_2 using flow injection	GF AAS	0.006	0.01	39
	Deionized, osmosis, mineral, effluent and river	Cr(VI)	Hybrid mesoporous silica	FI–SPE–GF AAS	-	0.0012	40
	Underground	Cr(III)	Alumina	F AAS	-	-	41
	Tap , lake , spring and waste	Cr(III)	Poly N-(4-bromophenyl)-2-methacrylamide-co-2-acrylamido-2-methyl-1-propanesulfonic acid-co-divinylbenzene	F AAS	1.58	-	42
	Drinking	Cr(III) Cr(VI)	magnetic immobilization of aminefunctionalized magnetite (Fe3O4) microspheres, preconcentration factors of 96 for Cr(III) and 47 for Cr(VI)	ICP-MS	0.0015	0.0021	43
	Drinking, tap and waste	Cr(III)	Alumina and 4-aminoantipyrene	F AAS	-	-	44
	Waste water	Cr(III)	ENVI-18 DSK	F AAS	0.02	-	45
	Spring, well and river	Cr(VI)	XAD-7 resin impregnated with brilliant green	UV-Vis	-	-	46
	Mineral and Salinas	Cr(VI)	Diaion HP-2MG resin	GF AAS	-	-	47
	Lake and tap	Cr(III)	Crosslinked chitosan-bound FeC nanoparticles with preconcentration factor of 100	F AAS	0.0524	-	48
	Ground	Cr(III)	Carbon Nanofiber-Packed Microcolumn	ICP-MS	0.015	0.033	49
	Tap and lake	Cr(III)	Nanometer titanium dioxide micro-column with preconcentration factor of 50	ICP OES	0.32	-	50
	River and tap	Cr(III)	Silica gel chemically modified with niobium(V) oxide	F AAS	0.34	-	51
	Sea	Cr(VI)	Diethyldithiocarbamate using micro-column packed with C18 bonded silica gel	F AAS	-	0.02	52
	Solution	Cr(VI)	Modified activated carbons	GF AAS	-	-	53
	Sample	Cr(III)	Microcolumn packed with tetrahydroxyflavanol-modified TiO2 nanoparticles	ICP OES	-	-	54
	Tap and snow	Cr(VI)	1-chlorovinyl-3-methylimidazolium chloride (PVC-NmimCl)	GF AAS	-	0.003	55
	Drink and synthetic river	Cr(VI)	Chromabond NH2 column	F AAS	-	11.7	56
	River	Cr(III)	Saccharomyces cerevisiae immobilized on sepiolite	F AAS	94	-	57
Spectro fluorimetry	Tap, drinking and spring	Cr(VI)	I^{3-} and rhodamine 6G in Tween-80	Spectrofluorometry	-	0.37	58
	Tap and drinking	Cr(VI)	Quercetin	Spectrofluorometry	-	0.47	59

São Paulo, Brazil) was used as protective and purge gas. The analytical signals were based on the integrated peak area (AA – BG).

2.2 Reagents and material

All glassware, polypropylene flasks (Falcon® tubes) and autosampler cups were cleaned with detergent solution, soaked in 10% (v v^{-1}) HNO_3 for 24 h, rinsed with high-purity deionized water, dried and stored in a closed polypropylene container. All solution and sample manipulations were conducted in a class 100 laminar flow bench (Veco, Campinas, Brazil).

The solutions were prepared with analytical-reagent grade materials unless otherwise specified. High-purity deionized water with a final resistivity of 18.2 MΩ cm was provided by a Milli-Q® water purification system (Millipore, Bedford, MA, USA). 65% nitric acid (w w^{-1}) and 37% hydrochloric acid (w w^{-1}) (Merck, Darmstadt, Germany) were used for silica cleaning. Hydrochloric acid and sodium hydroxide (Merck, Darmstadt, Germany) were used for pH adjustment. The analytical reference solutions were prepared by successive dilution of 1000 mg L^{-1} of Cr(III) ($CrCl_3$ prepared in 4.2% v v^{-1} HCl) and Cr(VI) ($K_2Cr_2O_7$ prepared in H_2O), both from Titrisol® standard solutions (Merck).

HDK® T40 nanoparticles of silica (Wacker, Germany) with surface area around 400 m^2 g^{-1}, and primary particle size calculated from BET of ~7 nm were used. The 98% (v v^{-1}) (3-aminopropyl)triethoxysilane (APTES) (Sigma-Aldrich) was used for silica organofunctionalization. This APTES solution was dissolved in high-purity water before use.

2.3 Sample collection and treatment

The river water samples were filtered in a vacuum filtration system of borosilicate glass, model XX15 047 00 (Millipore, USA) using a 0.45 μm nylon membrane filter (Millipore, USA) and stored in a freezer at -4 °C until analysis. Samples from Saltão and Sorocaba rivers, Capivara stream, Saltão waterfall, mineral water from São José dos Campos, and tap and drinking fountain water from Sao Paulo were analyzed.

2.4. Optimization of Cr(III) and Cr(VI) separation

In the first step, 50 g of silica was washed with 200 mL of an acid mixture (0.2 mol L^{-1} HCl + 0.044 mol L^{-1} HNO_3), under agitation (350 rpm) for 24 h. Afterward, the silica was washed twice with high-purity deionized water and centrifuged at 3000 rpm for 30 min. The supernatant was separated and silica was dried at 70 °C for 12 h.

Three portions (~ 1 g) of the washed silica were placed in Falcon® tubes with 0.2, 2.0 and 10% (v v^{-1}) APTES for organofunctionalization. The suspensions were agitated at 350 rpm for 24 h. After this, the supernatant was separated and silica was washed twice with high-purity deionized water, dried at 70 °C for 12 h and kept in a dry container.

The amount of APTES supported over the silica surface was determined by thermal gravimetric analysis (TG), differential thermal analysis (DTG) and elemental analysis for C and N contents.

The silica organofunctionalized with 0.2, 2.0 and 10% (v v^{-1}) APTES were separated in small portions (20-160 mg) to investigate the influence of mass on Cr(III) complexation. To each portion was added 2.5 mL of 20 μg L^{-1} Cr(III). The suspensions were manually shaken, centrifugated at 3000 rpm for 1 min, and the supernatand was analyzed by GF AAS. For the silica organofunctionalized with 2.0% (v v^{-1}) APTES, the same experiment was carried out with 20 μg L^{-1} Cr(VI).

The influence of pH (2, 4, 6 and 8) on chromium species adsorption was investigated using 150 mg of the silica organofunctionalized with 2.0% (v v^{-1}) APTES in the presence of 20 μg L^{-1} of Cr(III) and 20 μg L^{-1} of Cr(VI).

2.5. Analyical method

The GF AAS heating program optimization was achieved using 20 μg L^{-1} of Cr(VI) in deionized water. 10 μL reference solutions were used to obtain the pyrolysis and atomization temperatures in the presence of 10 μg of Mg.

For total Cr and Cr(VI) determination, 10 μL of the analytical calibration solutions (0.5-20 μg L^{-1} Cr(VI) prepared in deionized water) were co-injected into the graphite tube with 10 μg of Mg as chemical modifier. The speciation analysis was carried out by Cr(III) separation after complexation on the organofunctionalized silica and Cr(VI) determination in the supernatant by GF AAS.

For chromium speciation analysis in the samples, 150 mg of silica organofunctionalized with 2.0% v v^{-1} APTES were added to 2.5 mL of water sample. The suspension (pH = 8) was manually shaken, centrifuged at 3000 rpm for 1 min and the supernatant was analyzed for Cr(VI) determination by GF AAS.

3. Results and discussion

3.1. Characterization of APTES-silica

The TG/DTG curves of pure silica nanoparticles (Fig. 1A) showed a mass loss of about 20.1% at temperatures ranging from 25 to 100 °C due to the loss of physically adsorbed surface water. A slow and gradual mass loss (2.2%) between 200 and 900 °C can be attributed to the elimination of water adsorbed with moderate force and also the dehydration of silanol groups that are converted to siloxanes (600 to 900 °C). The TG/DTG curves of APTES-silica (Fig. 1B) showed the same behavior in temperatures ranging from 25 to 100 °C. However, the lower amount of water eliminated (4.2%), suggests modification of the silica surface. The more pronounced mass loss above 200 °C confirms the interaction of the APTES group with the silica surface. The results indicated that at least 2.5% (w w^{-1}) of APTES is presented on the silica surface.

Elemental analysis giving C=1.94% m/m and N=0.42% m/m for the APTES-silica samples confirm the incorporation of the APTES group onto the nanosilica particles.

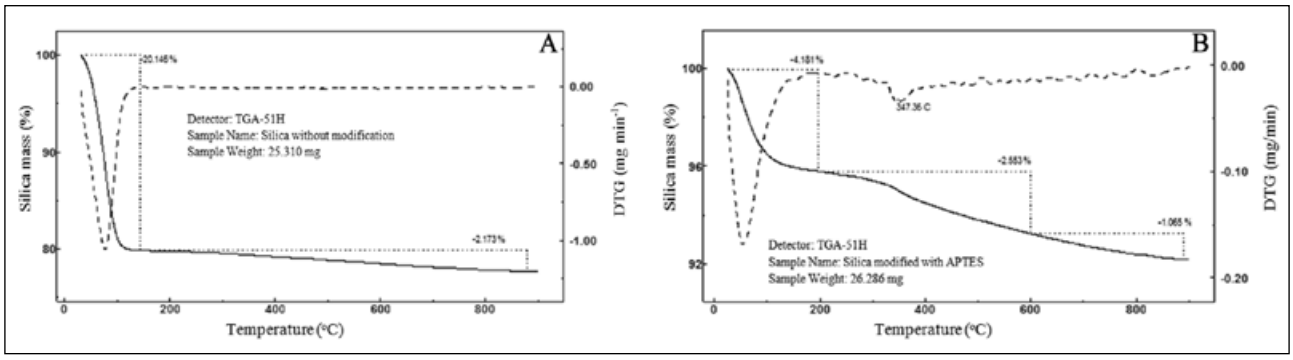

FIGURE 1. TG/DTG CURVES OBTAINED AT 10 °C/MIN AND DYNAMIC AIR ATMOSPHERE: A = SILICA WITHOUT MODIFICATION AND B = SILICA MODIFIED WITH 2% (V V^{-1}) OF APTES.

3.2. THE INFLUENCE OF APTES CONCENTRATION ON CR SPECIATION

The performance of silica prepared from the dispersion of 0.2%, 2.0% and 10% (v v^{-1}) APTES and the effects of different masses of APTES-silica on the complexation of 20 μg L^{-1} of Cr(III) were investigated. In this study the pH was fixed at 8, using NaOH solution. It was observed that silica organofunctionalized with 0.2% and 2.0% (v v^{-1}) APTES presented the best results. In these conditions, the percentage of Cr(III) complexation increases with the mass of organofunctionalized silica (20 to 160 mg). However, for silica prepared with the dispersion of 2.0% (v v^{-1}), the percentage of complexation was above 80% for masses higher than 120 mg. The silica organofunctionalized with 10% (v v^{-1}) presented low capacity for Cr(III) complexation (~60%) for all masses. In this case, hydrolysis of silica during the organofunctionalization step can be related to the lower Cr(III) complexation percentage.

The influence the mass of organofunctionalized silica on Cr(VI) complexation was also investigated. Complexation below to 8% was observed for masses below 100 mg. On the other hand, Cr(VI) complexation was undetectable for masses higher than 100 mg. Considering the best Cr(III) complexation, a mass of 150 mg of silica organofunctionalized with 2.0% (v v^{-1}) APTES was selected to investigate the pH effect.

3.3. PH EFFECT

The pH effect on Cr(III) and Cr(VI) complexation by organofuncionalized silica are shown in Fig. 2. It was observed that the complexation of Cr(VI) decreased and Cr(III) increased with increasing of pH solution. These species are dependent on pH and can exist in different forms in aqueous solutions: $Cr_2O_7^{2-}$, $HCrO_4^-$, CrO_4^{2-} and $HCr_2O_7^-$ for Cr(VI) and Cr^{3+}, $CrOH_2^+$, $Cr(OH)_3^0$, $Cr(OH)_2^+$ and $Cr(OH)_4^-$ for Cr(III). At pH=2, the protonation of the $-CH_2NH_2$ group occurs to form $-CH_2NH_3^+$ [60,61], allowing the electrostatic attraction of Cr(VI) and repulsion of Cr(III). In this condition Cr(VI) complexation was around 60% (Fig. 2). The increase in solution pH decreases the protonation of the amino group and, consequently, the electrostatic attraction of Cr(III) and repulsion of Cr(VI) can occur. PH=8 was chosen for the speciation analysis because in this condition the absorption of Cr(III) on the organofuctionalized silica was ~ 90% while Cr(VI) was undetectable by GF AAS.

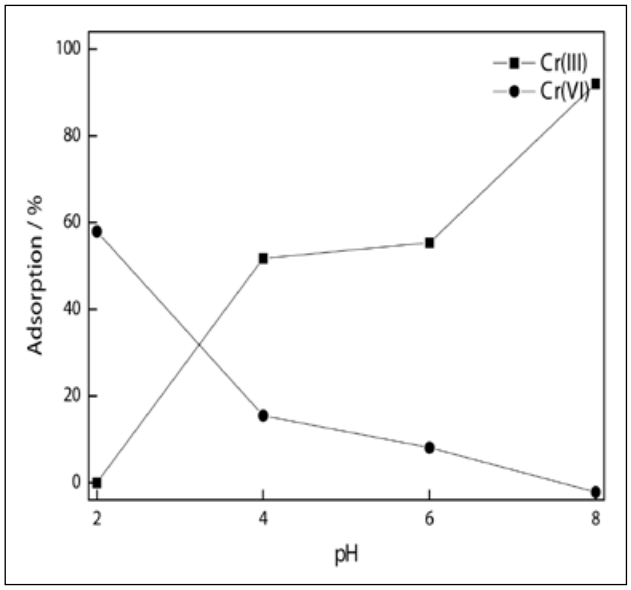

FIGURE 2. INFLUENCE OF THE PH ON CR(III) AND CR(VI) COMPLEXATION ON SILICA ORGANOFUNCIONALIZED WITH 2% (V V^{-1}) APTES.

3.4. FIGURES OF MERITS

The heating program is depicted in Table II. The best pyrolysis and atomization temperatures were 1400 °C and 2400 °C, respectively, using 10 μg of Mg as chemical modifier.

The characteristic parameters of the analytical calibration curve were: slope = 0.0068 sL μg^{-1}, correlation coefficient = 0.9985, and detection limits = 0.06 μg L^{-1} for total Cr and Cr(VI). The LOD for total Cr determination was calculated considering the variability of 10 consecutive measurements of deionized water (blank), according to 3 s_{blk}/b (s_{blk} = standard deviation of the blank and b = calibration curve slope). To estimate the LOD of Cr(VI), 2.5 mL of deionized water was manually shaken with 150 mg of silica organofunctionalized with 2.0% v v^{-1} APTES, cen-

trifuged at 3000 rpm for 1 min and the supernatant was measured (n=10) by GF AAS.

Method reproducibility, calculated by the relative standard deviation (RSD) of the slope on different days was 3% (n=3).

Table II. Heating program for the determination of Cr by SIMAA®-6000

Step	T (°C)	Ramp (s)	Hold (s)	Ar (mL/min)	Read
Drying 1	110	10	25	250	No
Drying 2	130	5	15	250	No
Pyrolysis	1400	10	20	250	No
Atomization	2400	0	5	0	Yes
Cleaning	2600	1	3	250	No

3.5. Analysis of samples

Eight water samples (Saltão river from Itapira-SP, Sorocaba river from Laranjal-SP, Capivara stream from Fernandópolis-SP, Saltão waterfall from Itapira-SP, mineral from São José dos Campos-SP and tap and drinking fountain waters from our laboratory) were analyzed by the proposed speciation method. The results are presented in Table III. All samples presented total Cr below the maximum permissible concentration adopted by CONAMA 397/08. After addition (2 μg L^{-1}) the recovery of Cr(VI) ranged between 91-105% for all samples.

Table III. Concentrations of total Cr and Cr(VI) speciation in water samples.

Water Sample	Total Cr (μg L^{-1})	Cr(VI) (μg L^{-1})	Recovery* (%)
Saltão river (Itapira city)	1.14 ± 0.02	1.12±0.01	92
Sorocaba river (Ibiuna city)	0.71 ± 0.02	<LOD	96
Sorocaba river (Laranjal city)	0.68 ± 0.01	<LOD	101
Capivara stream	0.90 ± 0.01	0.89±0.1	94
Saltão waterfall	0.94 ± 0.02	<LOD	105
Mineral Water (São José dos Campos city)	0.40 ± 0.05	<LOD	100
Tap Water (São Paulo city)	0.09 ± 0.01	<LOD	91
Drinking Fountain Water (São Paulo city)	0.09 ± 0.02	<LOD	104

* Addition of Cr(VI)= 2 μg L^{-1}; LOD = 0.06 μg L^{-1}

Conclusion

Nanoparticles of silica organofunctionalized with 2.0% v v^{-1} APTES were efficient for speciation analysis of Cr(VI) in waters. The organofunctionalization procedure is simple and easy for implementation and does not need to use organic solvents and heat. This method presented good selectivity, precision and reproducibility for routine Cr(VI) speciation analysis.

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The feasibility of coupling graphite furnace with solid phase microextraction aiming at speciating organotin compounds

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Abstract

The goal of this work was to perform the coupling between solid phase microextraction using a SPME fiber device and graphite furnace atomic absorption spectrometry for extracting organotin compounds volatilized during the drying step of the graphite furnace temperature program. In this way, evaluations of volatilization temperatures, ranging from 60 to 120°C, and fiber exposure times (190, 390, 590, 790 and 990 s) were carried out for optimizing the separation of DBT and TBT from sediment matrices. In order to identify the species extracted using the graphite furnace, GC-MS was used. Additionally, a separation/retention model was proposed regarding the DBT and TBT species on the SPME fiber inserted into the graphite furnace

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Keywords: Organotin Species, Speciation, Graphite Furnace, SPME, Extraction

1. Introduction

The literature reports a variety of applications of graphite furnace atomic absorption spectrometry, denoting GF AAS as a consolidated and mature technique.[1] Besides other advantages, the accurate control of both time and temperature in the drying, pyrolysis and atomization stages confers a kind of in situ thermal pre-treatment, so that it can be applied not only to aqueous, but also to slurries and solid samples, as well as to either refractory or volatile analytes.[2] In this way, accurate results are currently obtained, even for samples with complex matrices.

As there are some species that present different degrees of volatility (*i.e.* metallic, organometallic and metallo-biomolecules), and graphite furnaces provide the desirable control of time and temperature, it is easy to conclude that graphite furnaces could be better explored, since the results are expected to be acquired after the last heating step (atomization) only.

In chemical speciation involving inorganic and organometallic species, the vapor pressure can play an important role as an element of selectivity.[3] Then, significant differences between the vapor pressure of each species, allied to the fine control of the temperature of the GF AAS technique, can contribute to chemical speciation based on sequential volatilization. As some organometallic species are thermally labile,[4] the drying step of the GF AAS could be used for separating these species, while the determination of the remaining inorganic species could be determined in the atomization step. Then, the furnace could be employed for separating the labile from the refractory species while performing the temperature program.

Solid phase microextraction is another consolidated technique as well,[5] and some applications can be found for organometallic species in the literature.[6] The insertion of a SPME fiber into the graphite furnace during the drying stage seems to be an alternative in terms of analytical strategy for chemical speciation.

In this way, the present work demonstrates the feasibility of coupling SPME and graphite furnace (GF), focusing on organotin speciation analysis with in situ derivatization. The sorption of dibutil (DBT) and tributiltin (TBT) volatilized during the drying stage of the graphite furnace temperature program is demonstrated as well as their further identification by GC-MS.

2. Experimental

2.1. Instruments and apparatus

For DBT and TBT determinations, a gas chromatograph equipped with a mass spectrometer (GC-MS, Shimadzu QP5000, Kyoto, Japan) based on a quadrupole analyzer and a photomultiplier detector was used. The equipment was fitted with a HP-5MS (Supelco, Bellefonte, USA) capillary column (30 m x 0.25 mm i.d., 0.25-μm film thickness), a split/splitless injector operated in the splitless mode and a SPME glass liner.

The coupling between the SPME fiber device and the graphite furnace (Figure 1) was made using a Perkin-Elmer

AAnalyst 600 Graphite Furnace Atomic Absorption Spectrometer (GF AAS, Perkin-Elmer, Shelton, USA) with an end-capped transversely heated graphite tube atomizer (THGA) with integrated coated pyrolytic graphite platforms, and an AS-800 autosampler.

2.2. Reagents and standard solutions

Analytical grade chemicals such as sodium acetate, glacial acetic acid and methanol (Merck, Darmstadt, Germany) were used. The distilled/deionized water (> 18 MΩ cm) was obtained from a Milli-Q Water Purification System (Millipore, Bedford, USA); 96 % di-*n*-butyltin dichloride (DBTCl) and 96 % tri-*n*-butyltin chloride (TBTCl) were supplied by Aldrich, and their stock solutions were prepared by dissolving appropriate amounts in methanol and used for not longer than three months. The derivatization reagent (sodium tetraethylborate, $NaBEt_4$, Alfa Aesar, Ward Hill, USA) was prepared daily in deionized water, and used up to 6 h after preparation, and the temperature was kept at 5 ± 1 °C during its manipulation and chromatographic runs. Safety and environmental considerations regarding the handling of toxic compounds (tin species and $NaBEt_4$) were considered.

2.3. Extraction of the DBT and TBT from sediments

The sediment samples were collected from the Atibaia river (São Paulo, Brazil) and were kindly provided by P. C. Favaro [7]. The sediment samples were dried at 60°C, ground and sieved to obtain particle size < 63 μm. Spiked sediments samples were prepared according to Muñoz *et al.*[8] with modifications. A 3 mL-volume of 25 mg L^{-1} of each species (DBTCl and TBTCl) dissolved in methanol were added to 1.0 g of sediment samples, and the mixture was stirred for 2 h. Then, the solvent was evaporated at room temperature.

The extraction of the species from the sediments was performed according to Nemanič *et al.*[9] Glacial acetic acid (10 mL) was added to 1.0 g of sample, and the formed slurry was then sonicated using a Branson 2510 model (Markham, Canada) ultrasound bath (frequency, 40 kHz) during 30 min at 50 ± 4 °C. The supernatant was dried under nitrogen flow, and the remaining pellet dissolved into 250 μL of 0.2 mol L^{-1} CH_3COONa / CH_3COOH buffer (pH 5.0). The derivatization reagent was dissolved in deionized water.

2.4. SPME-GF coupling

The commercial fiber PDMS-65μm / DVB (polydimethylsiloxane/divinylbenzene) obtained from Supelco (Bellefonte, USA) was used for evaluating the volatilized species from the graphite furnace.

The SPME device was coupled to the fume extraction of the GF (Figure 1). The fume extractor moves downward, when the temperature program starts, and this mechanism provides the insertion of the needle of the fiber into the furnace, and the fiber is exposed manually to the released vapors. For species capture, *ca.* 1/3 of the fiber length (1.0 cm total length) was inserted into the graphite furnace during the temperature program. The distance between the fiber and the graphite furnace platform was *ca.* 2 mm. The initial temperature program used is shown in Table I, and the atomization temperature was not considered since this stage was not used.

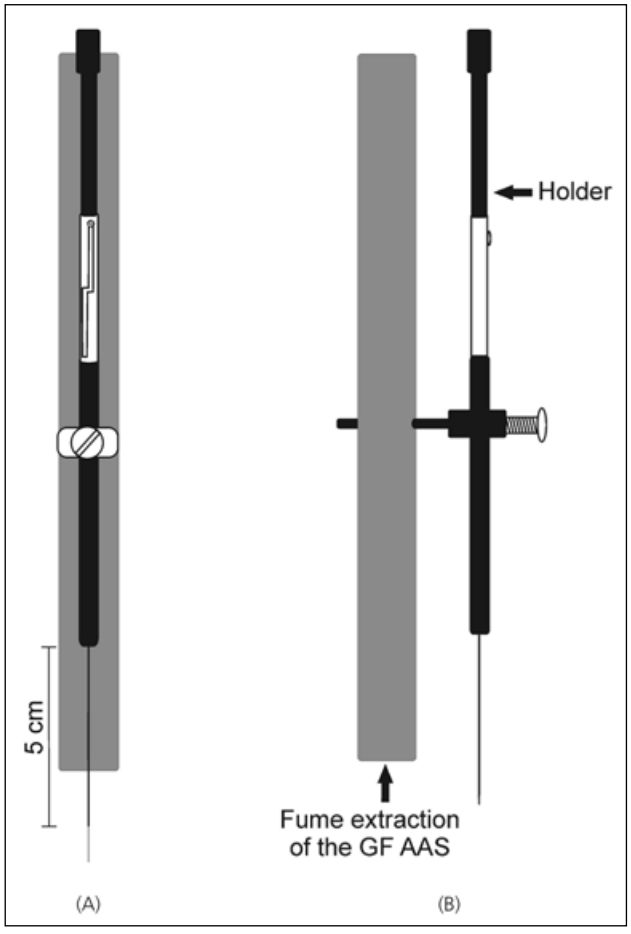

Figure 1. (A) Frontal view of the holder coupled to fume extraction. (B) Lateral view of the coupling.

Table I. Graphite furnace temperature program applied to SN species extraction by SPME

Step	Temperature (°C)	Time (s)	Argon flow-rate (mL min^{-1})
1	60	40	0
2	60-90	950	0
3	300	45	250
4	1000	30	250
5	2200	5	0
6	2450	4	250

Volumes of 49 μL of the sample plus 50 μL of 0.2 % (m/v) $NaBEt_4$ were sequentially aspirated and delivered into the atomizer. The ethylated species was then formed inside the graphite furnace, and the temperature program was

immediately applied. The fiber was exposed during the first and second stages, for a total of 990 s (Table I). The argon flow was stopped during fiber exposure.

After the extraction, the SPME fiber was introduced into the GC-MS for separation and identification of the species. The temperature of the chromatographic oven, 80 °C, was held during 1 min, followed by a 5 °C min^{-1} ramp to 200 °C (1 min hold), and a new 40 °C min^{-1} ramp to 280 °C (1 min hold). The last heating step was used to guarantee the cleaning of the chromatographic column. The GC injection port and GC-MS interface were maintained at 270 and 290 °C, respectively. Helium (99.999 %, origen!) was used as the carrier gas at a constant flow rate of 1 mL min^{-1}. Desorption time for SPME extraction was 15 min, and the time for solvent delay was set to 4 min. The mass spectrometer was operated in the total ion chromatogram mode (*m/z* 40-500) with electron ionization at 70 eV. GC-MS data treatment was carried out using an AMDIS-NIST software (Automated Mass Spectral Deconvolution and Identification System v. 2.61 and Mass Spectral Search Program v. 1.6 d, Washington, DC, USA, 1999), and Class 5000 (1993) from Shimadzu (Kyoto, Japan).

3. Results and discussion

3.1. Ethylation parameters

The $NaBEt_4$ concentration was evaluated in the range of 0.1 – 2.0% (m/v), and no statically significant variation was observed in the range studied. The same result was observed for studies reported in the literature, in which $NaBEt_4$ concentrations were used in the range of 0.002 – 0.1% (m/v), considering the dilution factors involved in the extraction procedures. Then, in the present work, the $NaBEt_4$ concentration was fixed at 0.2% (m/v). The pH range studied was from 4.0 – 5.5. The better analytical signals for the species were obtained at pH = 5.0, close to the results from the literature.

3.2. SPME-GF coupling: evaluation of volatilization temperature and fiber exposure time

The SPME-GF coupling was applied to the in situ ethylated DBT (dibutiltin) and TBT (tributiltin), after their extraction from sediment matrices, as described in 2.3. The drying stage after graphite furnace temperature program and the fiber exposure time were studied for attaining good separation and retention. After the sorption of the species onto the SPME fiber, they were separated by gas chromatography and the peak areas were compared.

Figure 2A shows the variation of the extracted amounts, expressed as peak area of the species obtained by GC-MS, as a function of the furnace temperature. According to this figure, only analytical signals for DBT and TBT were detected. Higher peak areas were observed for temperatures ranging from 90 to 100 °C, with 990 s of exposure time of the fiber. For temperatures lower than 80 °C no analytical signal was detected, and for temperatures higher than 100 °C lower peak areas were obtained. This behavior of the organotin compounds in relation to temperature can be explained by taking into account the solvent of the sample solution obtained after sample preparation (item 2.3). In the 90-100 °C temperature range, solvents (water and acetic acid, solvents used for sample preparation) are evaporated and eliminated from the furnace. Then, the vapor of these solvents could help in carrying the organotin species to the fiber both by convection and diffusion. At lower temperatures this process is less intense, and at higher ones the contact between the released vapors and the fiber decreases. Thus, the optimum temperature range was from 90 to 100°C.

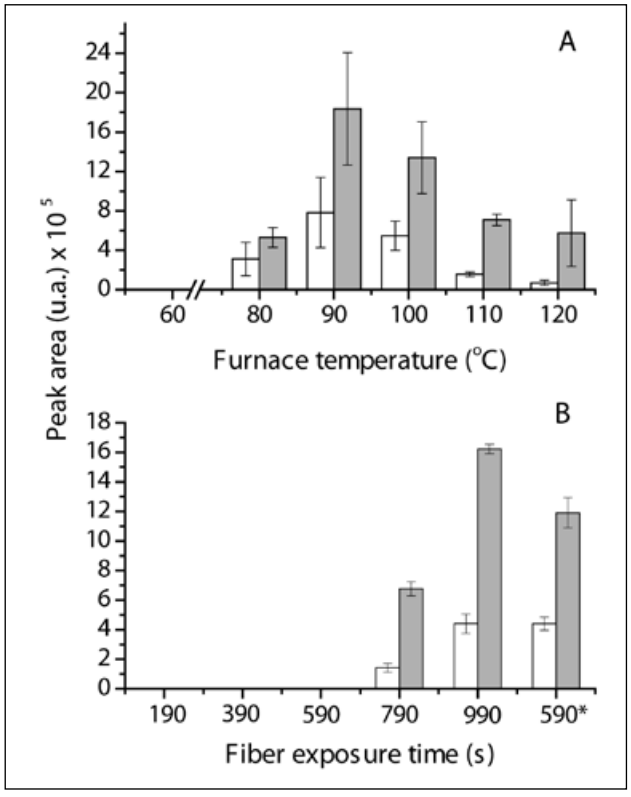

Figure 2. Evaluation of the graphite furnace temperature program. Figura A, using 990 s of fiber exposure time, and Figure B, using 90 °C as the temperature, for ethylated organotin extractions using the SPME-GF coupling. (□) DBT, (■) TBT. In Figure B: asteristic means that the experiment was evaluated in the absence of the solvent while others were carried out in the presence of solvent.

Figure 2B shows the results of the fiber exposure time inside the furnace. Six experiments were performed, where the fiber exposure time was varied from 190 to 990 s (steps 1 and 5 in Figure 2B), and another experiment was carried out exposing the fiber after solvent evaporation (step 6, Figure 2B) during 590 s. Solvent evaporation could be controlled by a mirror positioned close to the furnace of the GF AAS. In this experiment the fiber was exposed only after no liquid portion of the solvent could be noted. According to Figure 2B, no analytical signal was observed for shorter exposure

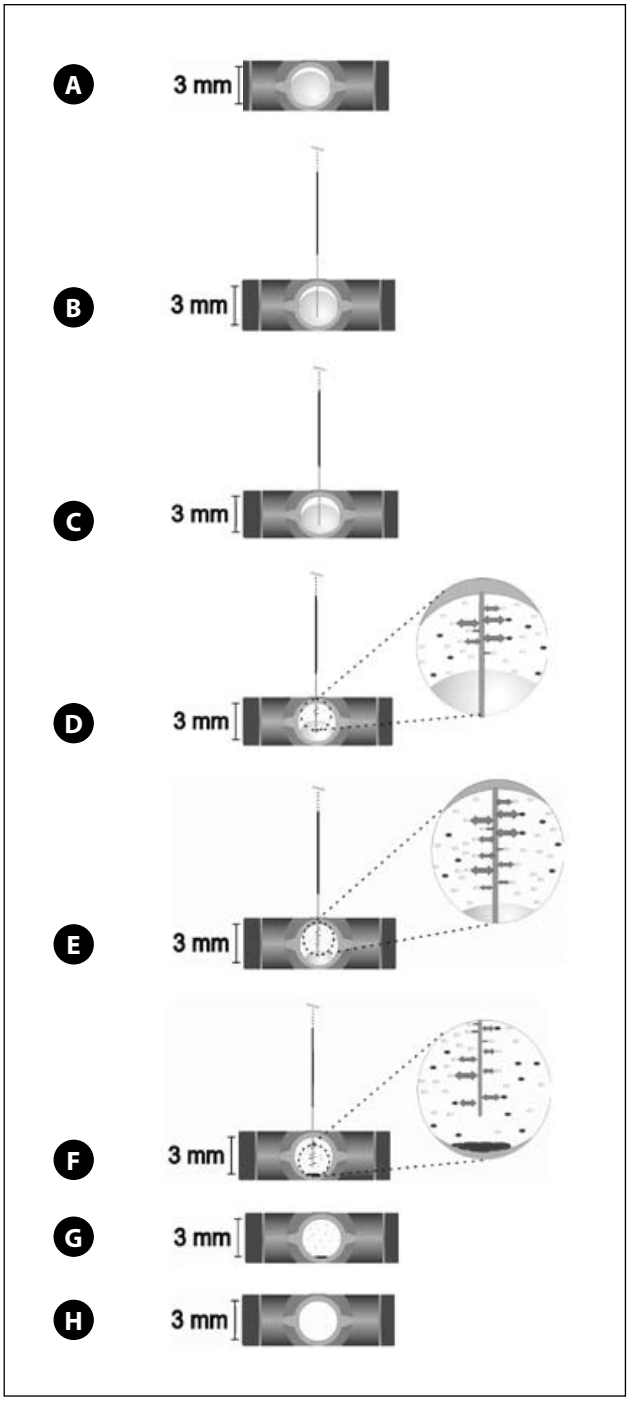

Figure 3. Schematic diagram of the extraction process. Black and grey circles represent the analyte and the solvent vapor molecules, respectively.

times (from 190 to 590 s), and the largest peak area was obtained for TBT at the longest exposure time studied (990 s). However, good TBT recoveries were also obtained when the fiber was exposed only after the solvent evaporation (step 6, Figure 2B). For DBT, good results were noted for 990 s and 590 s after solvent evaporation. This result supports the influence of the solvent (liquid or vapor) on the absorption of species onto the fiber, since similar exposure times (590 s) were applied in the presence and in the absence of the solvent (see steps 3 and 6, Figure 2B, respectively).

For DBT, the efficiency of the separation was similar in the absence or in the presence of the solvent. This fact can indicate a later volatilization of DBT than of the solvent itself, and the vapor pressure of the solvent was not a decisive factor for DBT extraction. However, as noted for the TBT behavior, a larger exposure time (990 s) can also contribute to the release of this species, considering the hypothesis that while the solvent (liquid or vapor) is eliminated, the analyte molecules are transported together, and the analyte is absorbed onto the fiber.

3.3. Separation/retention model

Taking into account the already commented hypothesis, a separation model was proposed for DBT and TBT retention onto the SPME fiber during their exposure in the graphite furnace.

Two parameters can be pointed out as affecting the signal of organotin species on the SPME-GF AAS coupling. The peak areas are maximized with longer exposure to the graphite furnace. Fiber exposure times shorter than 590 s (in the presence of the solvent) were not enough to separate ethylated DBT and TBT, as already mentioned. Additionally, according to the literature,[10] extractions under dynamic headspace conditions are useful only for analytes with high partition coefficients - *K* (ratio between concentrations of the analytes on vapor and liquid phase), or in the presence of trapping systems; mass transfer is inadequate and the accuracy is lost for low partition coefficients.

Another factor involved in the extraction is the purging of the species when the partial vapor pressure of the solvent was increased. The systematically larger measurable peak areas observed for the ethylated TBT relative to ethylated DBT can be explained due to the presence of the solvent vapor that sweeps the analyte, escaping from the furnace in the 90-100 °C temperature range. Direct extraction is other process to be pointed out: in the beginning of the exposure time, the fiber remains in contact with the liquid sample at the furnace platform. For ethylated DBT, which presents higher vapor pressure (0.0170 torr at 25 °C) than TBT, the presence or absence of the solvent does not contribute to the extraction, and major factors governing the retention are the equilibration time between the analyte molecules and the fiber surface, as well as mass transfer, to which the temperature is the unique contributing parameter. In the conventional SPME procedure with headspace extraction, an efficient mass transfer is attained by shaking the solution.[11]

Taking into account these observations, an extraction model for the SPME-GF coupling was proposed, which can be visualized in Figure 3. It shows an illustrative frame-to-frame schematic diagram of the retention process. Figure 3A shows the graphite furnace after the introduction of the sample aliquot (49 µL) plus the derivatizing agent (50 µL). These volumes correspond to *ca.* 100 % of the furnace inter-

nal volume. In Figure 3B, the fiber, which is in contact with the solution, was manually introduced, and a direct extraction is carried out by this contact. A decrease in the volume is attained in few seconds (Figure 3C), and the solvent vapor keeps in contact with the external fiber fraction, close to the entrance of the furnace. When the partial vapor pressure of the solvent increases (Figure 3D and 3E) the species can be transported. This process contributes positively to the extraction of species presenting low vapor pressures, such as TBT (0.0022 torr at 25 °C). After the evaporation of the solvent, the analytes, the matrix and some solvent vapor are supposed to remain in the furnace (Figure 3F). It is supposed that DBT species were extracted at this moment, since no difference was noted in the analytical signal for this species in presence or in the absence of the solvent (as liquid). Figure 3G shows the decomposition of the matrix at 300 °C, and after the fiber removal. In Figure 3H, the furnace is heated at 2450 °C, and no residue is supposed to be found.

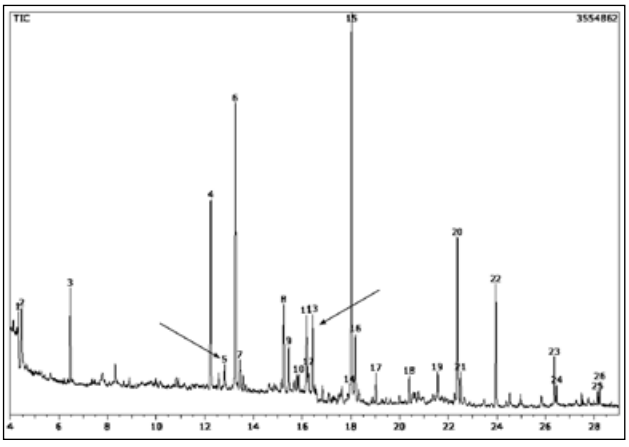

Figure 4. Total ion chromatogram obtained after the extraction of the species by SPME-GF AAS. Peak 5: dibutyldiethyltin-DBT; Peak 13: tributylethyltin-TBT.

Figure 4 shows the GC-MS chromatogram obtained after the extraction of the species using the SPME-GF coupling. TBT was identified with similarities of 87-93 % when considering reference mass spectra, and DBT was identified by its retention time due to its similarity with the TBT mass spectrum.

4. Conclusion

The SPME-GF coupling offers an alternative to separate semivolatile species which can be lost during the drying step of the graphite furnace temperature. This coupling was successfully applied for DBT and TBT, and opens new possibilities in terms of chemical speciation of organometallic species (exemplified by organotin species in this work) using low temperature (90 °C) and an appropriate fiber exposure time. Although monobutiltin (MBT) species were also tested in this work, they were not extracted due to the low equilibration time and/or its higher vapor pressure, as compared to those of the other species studied. Screening non-chromatographic GF AAS methods for partial speciation analysis can be devised, furnishing the concentrations of the volatile and non-volatile species, as well as the total concentration by the use of an adequate modifier.

Some limitations are also pointed out, such as the low mass transfer of the species between the platform and the headspace, and the long equilibration time, *ca.* 18 min is the maximum available time provided by the graphite furnace program. We are presently improving these conditions.

Besides the chemical speciation analysis, the SPME-GF coupling can be an important tool in studies focusing on graphite furnace atomization routes, as well as the identification of organometallic/semimetallic species easily volatilized and lost in the GF AAS steps. Finally, this coupling can also contribute to improving the total determination of elements that present volatile species, which could be lost during drying and pyrolysis steps, when considering the GF AAS technique.

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Metallothioneins and metallothionein-like proteins as biomarkers of environmental contamination: techniques for extraction, separation and quantification - a review

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Abstract
Even after fifteen years of studies of metallothioneins (MTs) and metallothionein-like proteins (MTLPs) as biomarkers of trace metal exposure, the methods for extraction, separation and detection of these proteins are still without an appropriate standardization, which has generated questions about the effectiveness of these methods. This review describes available methods of extraction, separation and detection for these proteins that have been applied with marine invertebrates used in biomonitoring of trace metal contamination in the environment. Extraction procedures using heat treatments seem more appropriate due to lower loss of MTs and MTLPs during the pre-treatment step when compared with solvent precipitation. As for MTs and MTLPs separation, electrophoretic and chromatographic methods have proven to be convenient techniques as these allow determination and speciation of trace metals associated with these proteins and also allow their identification by mass spectrometry. Nevertheless, after a characterization of these proteins in a selected biological matrix, indirect methods for MT and MTLP determination could be applied because these are relatively simple and less expensive, ideal for routine analysis as is demanded for biomonitoring studies.

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1. Introduction

Contamination results from the direct or indirect introduction of substances or energy that has deleterious effects on living resources and on human health. In recent years, some authors have recognized the paradoxical role of water as simultaneously a vital resource and a vehicle for pollutant elimination. This paradox can be considered part of the present environmental crisis that arises from the conflict between nature and technology [1].

Currently, levels of contaminants in marine environments have increased as a consequence of anthropogenic activities. The decline of water and sediment quality can involve a decrease in natural resources, and it is of environmental concern since a high variation in several abiotic factors imposes severe restrictions on organisms living in these areas. Contamination of ecosystems and exposure to toxic metals are major concerns all over the world. Metals and metalloids are significant pollutants of the atmosphere, aquatic systems and terrestrial environments. Depending on the source, they can enter the environment via a variety of inorganic species, which can be bioconverted by the biota [2]. Some of these metals are ubiquitously encountered in the environment due to their release in substantial amounts as a consequence of geological activities and/or anthropogenic impacts [3]. Metal toxicity occurs if the cellular concentration reaches a critical level when either essential or non-essential metals can bind non-specifically with targeted molecules (e.g., sulfhydryl groups of proteins) or substitute for (other) essential metals in metalloenzymes and metalloproteins. An important defense for organisms to respond or adapt to metal toxicity is to store these metals after sequestration and detoxification. Intracellular metals are often sequestered in two different ways, by cytosolic proteins and by formation of "insoluble" deposits, although these mechanisms may be interconnected [4].

Thus, there is an increasing need to develop methods for the identification, estimation, comparative assessment and management of the risks caused by chemical pollut-

ant discharges to the environment and natural resources. As recognized by international organizations and environmental agencies, risk assessment cannot only be based on the chemical analysis of environmental samples because this approach does not provide any indication of the deleterious effects of contaminants on the biota. Therefore, measuring the biological effects of pollutants to determine quality of the environment has become very important [1,5].

In a classic paper by Magnum and Towle (1977), which has been cited by Monserrat *et al.*, the term "enantiostasis" was introduced for the first time; it was defined as a type of regulation that occurs when the effect of a change in one chemical and/or physical property experienced by an animal is counteracted by an opposite change in another variable(s), which preserves the stability of a particular physiological system [1].

Recently, the use of biological markers or biomarkers at the molecular or cellular level has been proposed as sensitive "early warning" tools used to measure biological effects and to assess environmental quality [5].

2. Biomarkers in marine invertebrates: metallothioneins and metallothionein-like proteins

there are two main pathways used to study trace metal contamination in aquatic environments. The first one refers to understanding the physical and chemical processes that act on the bioavailability of metals. For example, studies have been conducted on the interaction of trace metals between the solid phase (sediment) and the aqueous phase (interstitial water), where predictive results about the bioavailability of trace metals in the aquatic environment have been determined [6]. The second pathway is attributed to studies of the interaction between trace metals in abiotic compartments and biota. In general, these studies have focused on the determination of element concentrations accumulated in tissues of aquatic organisms [7,8] and their effects through ecotoxicological assays. Trace metals may accumulate in aquatic organisms through different mechanisms: directly from water via the uptake of suspended particles or by the consumption of lower trophic level organisms [9].

The choice of biological matrix for biomonitoring studies is fundamentally important because it can be possible to draw inferences about the current state of contamination of an aquatic environment. Organisms classified as "sentinels" are the most promising candidates that have been used in biomonitoring studies because of features such as their ability to accumulate high concentrations of trace metals without killing the organism; these organisms are sessile and have a wide geographical distribution [7].

Although this kind of study is a powerful tool to assess the bioavailability of metals in aquatic organisms, the responses provided by organisms of different levels of biological organization can be considered to reverse a moderated or advanced state of contamination in an aquatic environment. This occurs because the studies using higher levels of biological organization, such as organisms and populations, provide information on changes that occur in aquatic environments only when a community has been impacted [10]. However, there are lower levels of biological organization that have been considered promising for an early warning indicator of environmental contamination [11].

In both environmental contamination and biomonitoring studies, lower levels of biological organizations are being considered for immediate responses as biomarkers. Several definitions have been given for the term biomarker; this term is generally used in a broad sense to include almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological [12].

A biomarker can be defined as the measurement of body fluids, cells, or tissues that indicate the presence of contaminants in biochemical or cellular terms. This definition has been broadened to include behavioral parameters now recognized to be extremely important in establishing ecological inferences with observed biochemical and/or physiological responses [1]. The selected biomarkers should indicate that the organism has been exposed to pollutants (exposure biomarkers) and/or the magnitude of the organisms' response to the pollutant (effect biomarkers or stress biomarkers) [5]. Measurements at the biochemical or physiological level more quickly and specifically detect the presence of several toxic compounds, which allow an earlier identification of change before deleterious effects reach higher organization levels [1]. Thus, a biomarker can also be defined as a change in the biological response, which ranges from the molecular to cellular and physiological responses to behavioral changes and can be related to exposure or toxic effects of environmental chemicals [13, 14].

One of the most important features of molecular/cellular biomarkers is that they have the potential to anticipate changes at higher levels of biological organization, i.e., population, community or ecosystem. Thus, these "early warning" biomarkers can be used in a predictive way that allows the initiation of bioremediation strategies before irreversible environmental damage occurs. Therefore, biomarkers can also be defined as short-term indicators of long-term biological effects [5,15,16].

Usually, biomarkers are classified as specific or non-specific. The use of toxicant-specific biomarkers, such as metallothioneins (MTs), has been widely employed to indicate the presence of metals. In the animal kingdom and especially in the marine environment, MTs are widely regarded as biochemical environmental indicators of metal contamination [17]. The determination of MTs and other biomarkers has been successfully employed in field studies aimed to characterize impacted areas, where com-

plex mixtures of pollutants are usually found [1,15,16].

In addition, biological monitoring involves the evaluation of the physiological status of "sentinel" organisms (bioindicators) living in the monitored environment by determining the values of selected biological parameters, which are known to vary in response to the toxic effects of pollutants. These biological parameters are often reported as stress indices or biomarkers [1,15,16,17,18].

According to some researchers [10,12,14], biomarkers can be subdivided into three classes. Exposure biomarkers, such as MTs, MTLPs, cytochrome P450 and some antioxidant enzymes, cover the detection and measurement of exogenous substances, their metabolites or the products of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism [9,19,20,21,22]. Effect biomarkers include measurable biochemical, physiological or other alterations within tissues or body fluids of an organism that can be recognized as associated with an established or possible health impairment or disease (e.g., histopathological alterations, DNA-damage and bulky DNA-adducts) [23,24,25]. Susceptibility biomarkers indicate the inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance, including genetic factors and changes in receptors, which alter the susceptibility of an organism to that exposure, including gene toxicity and susceptibility to oxidative stress by oxyradical scavenging [22,26].

Research disciplines that play an important role in risk assessment and the development of specific biomarkers are toxicogenomics, genomics, proteomics, metallomics, metabolomics and bioinformatics [27]. Moreover, these disciplines have been responsible for an increase in interaction between analytical chemistry and applied ecology fields, which make it possible to more specifically understand the interaction between xenobiotics and aquatic biota. Therefore, it is essential to validate biomarkers in biological matrices before they are applied in field studies. Once validated, biomarkers can provide direct measurements of actual effects of chemicals on living organisms in the field, thereby overcoming large areas of uncertainty that are implicit in normal risk assessment [28, 29].

3. Metallothioneins and metallothionein-like proteins

Metallothioneins (MTs) are non-enzymatic proteins with a low molar mass, high cysteine content, no aromatic amino acids and are heat stable. The high cysteine content that is present in MTs provides these proteins with a high affinity for different divalent metals because of the presence of reactive sulfhydryl (-SH) in their amino acid structure [30]. Many fish and aquatic invertebrate species possess proteins that are better referred to as metallothionein-like proteins (MTLPs) when they have not been purified and are characterized at sufficiently high levels [31]. They play an important role in known biological functions, such as Zn and Cu homeostasis and the detoxification of non-essential metals like Cd, Hg and Pb [18]. Table I. presents the main characteristics and functions of MTs.

Table I. Some characteristics and functions of metallothioneins (adapted from Ref. 32).

Characteristics	Functions
Molar mass 6000-7000 Da, 61 amino acids	Transport of metals
20 Cysteines (30%), N-acetylmethionine, and C-alanine amino acids, no aromatics, no histidine	Detoxification of metals
Tertiary structure: metal clusters	Protection from metal toxicity
Metal content: Cd, Zn, Cu, Hg; 5–10% (w/w)	Free radical scavenger
UV Absorption: 250 nm (Cd), 225 nm (Zn), 275 nm (Cu), 300 nm (Hg)	Storage of metals
Induced synthesis by Cd and Zn	Metabolism of essential metals
Heat stability	Immune response
Cytoplasmic localization	Genotoxicity
Presence of Isoforms	Carcinogenicity

In many marine invertebrate species, such as annelids, mollusks, and crustaceans, the induction of MTs or MTLPs synthesis by non-essential metals (e.g., Ag, Cd, Cu, Hg) has been demonstrated, which suggests the potential use of these protein concentrations in organisms as specific biomarkers of metal exposure [16]. The mechanism of MT-induction by non-essential metals has been studied [33], and models have been created to explain the specificity of these proteins that are induced by metals like cadmium, copper and mercury.

Even though MTs or MTLPs have been studied in marine invertebrates for almost two decades, many questions about the validation of these proteins as specific biomarkers still exist. Since their discovery by Margoshes and Vallee in 1957 [34], it has been difficult to validate these proteins because of different features, functions, and expressions in several biological matrices as well as different procedures of extraction and quantification that have been applied [16]. There are a variety of factors in the study of proteins that can be considered difficult to standardize for the methodology of the proposed application. In the literature, an intercalibration exercise conducted in field-work using marine invertebrates as biological matrices is rarely found [18]. However, some extraction and determination procedures applied to MTs or MTLPs are commonly used in marine invertebrates for their application as specific biomarkers for trace metal contamination in aquatic environments.

4. Metallothionein and metallothionein-like protein extraction procedures

Protein extraction is one of the most critical steps needed for the study of these biomolecules in organisms where dif-

ferent procedures can be applied to obtain better recovery. The type of extraction in biological matrices will primarily depend on the complexity of the matrices and specific characteristics of the target proteins.

Different marine invertebrates have been used for in vivo assays including mollusks, crustaceans and sponges [35,36,37]. Most of the proteomic studies on marine invertebrates have focused on tissues where metals tend to bioaccumulate. Although the extraction of proteins from animal tissues is easier than from plant tissues, sample preparation procedures are quite similar in both cases, and they involve centrifugation (to remove cell debris), subcellular fractionation, sonication, mechanical homogenization and protein precipitation using solvents [3]. However, due to the complexity of the biological matrices, it is essential for the protein extraction procedure to effectively remove compounds that are not of interest and to obtain a purified analyte for the subsequent separation method.

MTs or MTLPs have specific characteristics that allow the use of procedures during the extraction steps that favor the pre-separation of these proteins. The two features that have been used as target factors to obtain proteins from marine invertebrates are their low molar masses and thermostabilities. Figure 1 shows the main analytical steps that must be fulfilled during extraction of MTs.

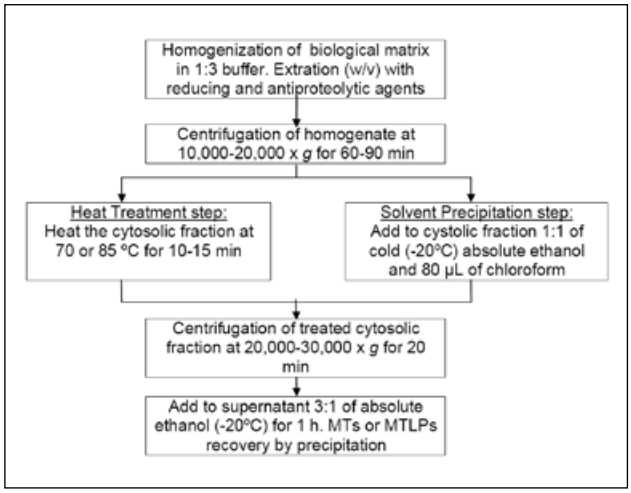

Figure 1. Schematic representation of MTs or MTLPs extraction procedures comparing the heat treatment and the solvent precipitation steps (adapted from Ref. 40 and 41).

Solvent precipitation (ethanol/chloroform) and heat treatment are the methods that have been used in most published studies where these proteins are used as specific biomarkers for trace metals in aquatic environments [16]. The extraction procedure using solvent precipitation is a well-known method that has been used for protein extraction in biological matrices from animal origin; the solvents that are primarily used consist of trichloroacetic acid, acetone, methanol and ethanol [39]. The solubility of proteins in aqueous buffers depends on the distribution of hydrophilic and hydrophobic amino acid residues. Proteins that have highly hydrophobic amino acid contents on the surface have low solubility in aqueous solvents, while charged and polar surface residues interact with ionic groups in the solvent and increase solubility [39]. Ethanol causes protein precipitation primarily because it significantly lowers the dielectric constant of the aqueous solution; thus, ionic compounds are more soluble in solvents with high dielectric constants [40]. Through its polar group, ethanol interacts with the polar group of the protein in competition with water. In addition, the hydrophobic groups may disrupt the intramolecular hydrophobic interaction. Finally, a large volume of ethanol reduces the effective concentration of water, leaving only a small amount for protein hydration. Upon dehydration by ethanol, protein molecules attract each other to a sufficient degree by van der Waals forces and, thus, they become insoluble in the ethanol-water mixtures [40]. The solvent precipitation method, which utilizes ethanol/chloroform, is commonly used to extract MTs or MTLPs in marine invertebrates and was described by Viarengo at al. (1997) [41] based on the study published by Kimura at al. (1979) [40]. Using ethanol instead of other organic solvents such as methanol becomes relevant for studies that require a laboratory routine, such as biomonitoring studies; this is because exposure to organic solvents can cause general health problems. Chloroform allows the elimination of low molar mass soluble thiols, which can react with specific reagents and interfere with the quantification of metallothionein and with the partial purification and concentration of MTs, which are often present at very low levels in the tissues of uncontaminated matrices [41].

The thermal stability of the MTs and MTLPs allows pre-purification with a heat treatment during extraction. Heating increases the kinetic energy and disrupts hydrogen bonds and non-polar hydrophobic interactions, which causes denaturation of the protein. This denaturation is responsible for the decrease in solubility and communal aggregation of proteins that have no relevance in the determination of the MTs or MTLPs. The thermal step in the extraction protocol of MTs or MTLPs consists of heating the sample at a specific temperature for a pre-set time. A study published by Erk et al. (2002) [42] used a heat treatment (at 70 and 85°C) as a pre-purification step for the determination of MTLPs in the digestive glands of mussels. This study has been widely cited as a reference in studies involving MT or MTLPs. A comparison between these two procedures has been performed by some authors [42,43,44], where heat treatment was considered more appropriate than solvent precipitation as a pre-purification step. According to Erk et al. (2002) [42], both heat (at 70 and 85 °C) and solvent treatments efficiently remove high molar mass proteins. However, the MT20 isoform is significantly reduced by heat treatment and drastically reduced by

solvent precipitation. Because MT20 is considered the "target", the MT isoform is used to determine the effects of metal exposure, and authors recommend the use of a heat treatment. In accordance with Geret et al. (1998) [44], this difference in the recovery of MTs between the two methods can be attributed to the coprecipitation of MTs with hydrophobic compounds during the first addition of solvent. Alternatively, the MTs could have partitioned into the supernatant of the second wash with ethanol, together with the highly hydrophilic compounds. Although the results obtained by these authors have been achieved by differential pulse polarography (DPP), other techniques for MTLPs determination have shown that there is a better recovery using heat treatment compared to the solvent precipitation procedure. Figure 2 shows some results obtained for the extraction of MTs using the solvent precipitation and heat treatment methods from marine sponge species [45].

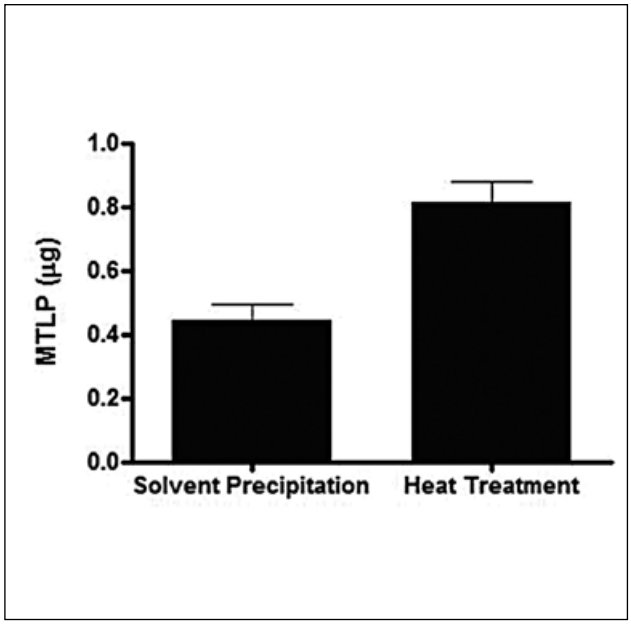

Figure 2. MTLPs obtained by SDS-PAGE, comparing between solvent precipitation and heat treatment from marine sponge *Haliclona sp*. previously exposed to cadmium. Error bars = standard deviation [45].

Thompson and Sutherland (1992) [43] showed that these two extraction procedures might have different applications where heat denaturation is the preferred treatment for the quantitative recovery of metal-binding proteins, while the solvent precipitation method is useful for the purification of MTs or MTLPs due to its ability to remove proteins from biological extracts. Our research group has obtained the best results by using the heat treatment extraction for MTs, as can be seen in Figure 3 for marine sponge *Haliclona* sp. previously exposed to cadmium [45]. The highest removal capacity of protein extract was observed by Erk et al. [42] and Geret et al. [44] using differential pulse polarography.

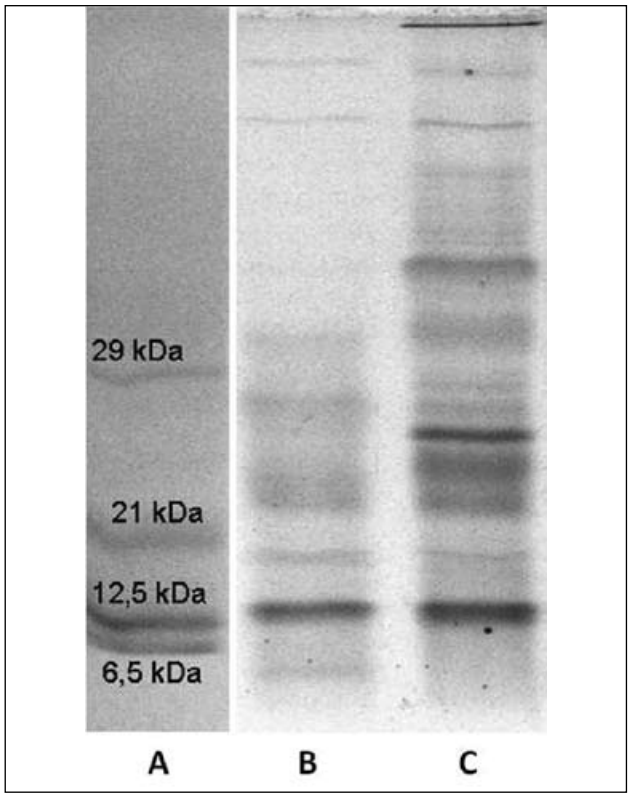

Figure 3. SDS-PAGE protein profile stained by Comassie Blue showing molar mass markers (A), the solvent precipitation profile (B), and heat treatment profile (C). The biological matrix used was marine sponge *Haliclona sp*. previously exposed to cadmium [45].

The differences between these two extraction procedures (solvent and heat treatment) need to be better studied using separation techniques that allow the simultaneous detection of various proteins from the same extract, for example, by using powerful separation techniques such as gel electrophoresis. Gel electrophoresis in the two-dimensional mode enables identification of proteins using isoelectric point in the first dimension and molar mass for the second dimension, so that the relative intensities of the spots of the proteins in a biological extract can be observed. Furthermore, the target proteins can be quantified, identified and further analyzed by mass spectrometry. This type of characterization allows a better comparison between the heat treatment and solvent precipitation procedures in a biological matrix to be used in environmental studies.

5. MTs/MTLPs and associated metals: separation, detection and quantification applied to environmental studies

Considerable attention has been focused on the involvement of these proteins in various biochemical pathways and their use as markers of stress or disease. To fulfill the requirements of multidisciplinary studies, a wide range of bioanalytical techniques are needed. Isolation, separation, detection and/or quantification of MTs and MTLPs are not easy tasks because they have low molar masses and unique primary structures. However, these are the commonly used methods

for detecting proteins that lack specificity and sensitivity. The most frequently used methods for the detection of MTs are indirect and based on quantifying the metal ions that are found in their structures or the high content of sulfhydryl groups [46].

Classical techniques used for the determination of MTs/MTLPs include metal-saturation and immunological assays, i.e., the radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), respectively, and polarography [32,47]. However, these techniques show a lack of selectivity to different MT isoforms and may suffer from interferences as well as be unable to provide information on metal compositions [48]. Selectivity of isoform separation can be achieved using high performance separation techniques, and various modes of high performance liquid chromatography (HPLC) or electrophoresis have become standard. The relatively poor detectivity of quantification based on UV detection or dye staining does not generally allow the non-induced concentrations of MTs to be determined. More importantly, the lack of reliable pure calibration standards does not allow for unambiguous identification of MT-isoforms in HPLC and CZE, so that further studies are needed for the accuracy of molar mass assessment by SEC [32,47].

In recent years, a universally accepted approach to speciation analysis has been offered using hyphenated (e.g., coupled and hybrid) techniques, which are undergoing rapid and continuous developments. They are based on a combination of separation techniques, such as gas chromatography (GC), high performance liquid chromatography (HPLC), or capillary zone electrophoresis (CZE), with a sensitive and species-selective detector (e.g., atomic absorption spectrometry (AAS), inductively coupled plasma optical emission spectrometry (ICP OES) or mass spectrometry (ICP-MS, ESI-MS and MALDI-MS)). This analytical approach is becoming a fundamental tool for the functional characterization of trace elements in biological systems. As calibration standards are unavailable for most trace element species in living organisms, the recent explosion of soft ionization techniques, such as MALDI-MS and ESI-MS, is necessary to complete the characterization of the species detected by HPLC-ICP-MS and opens new exciting possibilities for the detailed characterization of MTs [32,49,50].

Table II shows selected papers found in the literature dealing with extraction, separation and detection procedures mainly applied for MTs and MPLTs in environmental issues.

Table II. Some examples of extraction, separation and detection of MTs and/or MTLPs and associated metals applied to environmental studies.

Organism	Extraction	MTs or MTLPs Separation	MTs or MTLPs Detection	Reference
Tissues after Zn induction *(Daphnia magna)*	Heating	No separation (total MTs)	Modified silver radioisotope saturation assay (^{110m}Ag-glycine)	[51]
Fish hepatopancreas (*Mullus barbatus* and *Merluccius merluccius*)	Buffer (sucrose,tris-HCl, $MgCl_2$, PMSF)	SDS-PAGE	Chemiluminescence	[52]
Oyster gills and digestive glands (*Crassostrea angulata*)	Heating	No separation (total MTs)	Differential pulse polarography	[53]
Green mussels (*Perna viridis)* after Ag, Cd and Cu induction	Heating	No separation (total MTs)	Silver saturation assay	[54]
Nematodes (*Caenorhabditis elegans)*	-	-	Electrospray ionization mass spectrometry	[55]
Sea bream tissues (*Sparus aurata)*	Solvent treatment (ethanol/ chloroform)	No separation (total MTs)	Spectrophotometry	[56]
Gills of mussels (*Mytilus galloprovincialis)*	Heating	No separation (total MTs)	Differential pulse voltammetry	[57]
Clam tissues (*Megapitaria squalida*) after Cd induction	Heating	RP high performance liquid chromatography		[58]
Digestive glands and gills of molluscs (*Anodonta cygnea)*	Heating	No separation (total MTs)	Differential pulse polarography	[59]
Nematodes (*Caenorhabditis elegans)* (*in vitro* and *in vivo*)	Solvent treatment (ethanol/ chloroform)	-	Mass spectrometry (TOF and ion trap)	[60]
Chub fish livers (*Leuciscus cephalus* L.)	Heating	No separation (total MTs)	Electrochemistry	[61]
Vent mussel gills and digestive glands (*Bathymodiolus azoricus*)	Heating	No separation (total MTs)	Differential pulse polarography	[62]
Digestive glands *(Perna viridis)*	Heating	No separation (total MTs)	Differential pulse polarography	[63]
Polychaetes (*Perinereis aibuhitensis)*	Buffer (tris)	No separation (total MTs)	Ag saturation method	[64]

Organism	Extraction	MTs or MTLPs Separation	MTs or MTLPs Detection	Reference
Marine bivalves digestive glands and gills (*Mytilus edulis* and *Mya arenaria*)	Heating	No separation (total MTs)	Differential pulse polarography	[65]
Red mullets (*Mullus barbatus*)	Heating	No separation (total MTs)	Differential pulse polarography	[66]
Digestive glands and gills mussels (*Mytilus galloprovincialis)*	Solvent treatment (ethanol/ chloroform)	No separation (total MTs)	Spectrophotometry	[67, 68]
Blue crab hepatopancreas and gills (*Callinectes sapidus*)	Solvent treatment (ethanol/ chloroform)	No separation (total MTs)	Spectrophotometry	[69]
Gills, digestive glands and tissues of clam (*Ruditapes decussates)*	Heating	Size-exclusion chromatography	Differential pulse polarography	[70]
Gills and digestive glands of clams (*Ruditapes decussates)*	Heating	No separation (total MTs)	Differential pulse polarography	[71]
Shore crabs hepatopancreas and gills (*Carcinus maenas*)	Heating	No separation (total MTs)	Differential pulse polarography / Anodic stripping voltammetry	[72, 73]
Rabbit liver MT, MT-1 and MT-2 and horse kidneys	-	Capillary electrophoresis	Spectrophotometry	[74]
MT-1, MT-2 and MT from rabbit liver standard	-	Liquid chromatography	Electrospray ionization mass spectrometry / UV spectrophotometry	[75, 76]
Mussel tissues *(Mytilus galloprovincialis*)	Heating	No separation (total MTs)	Differential pulse polarography	[77]
MT-1 and MT-2 rabbit liver standard	-	No separation (total MTs)	Cathodic stripping voltammetry / Inductively coupled plasma optical emission spectrometry (sulfur content)	[78]
Sponges (*Spongia officinalis*)	Heating	Size-exclusion chromatography	Differential pulse polarography	[79]
Rabbit liver (MT-1 and MT-2), horse kidney and MT standards	-	Capillary zone electrophoresis	-	[80]
Earthworms (*Eisenia andrei)*	Heating	RP High performance liquid chromatography	UV spectrophotometry	[81]
Rabbit liver MT-1 and MT-2 standards	Heating	RP High performance liquid chromatography	UV spectrophotometry / Electrospray ionization mass spectrometry	[82,83]
Nematodes (*Caenorhabditis elegans)*	Solvent treatment (ethanol/ chloroform)	Gel permeation and ion-exchange chromatography	UV detection	[84]
Springtails (*Orchesella cincta)*	Solvent treatment (acetone)	Gel filtration and reversed-phase chromatography	UV detection	[85]
Red mullet livers (Mullus barbatus)	Solvent treatment (ethanol/ chloroform)	No separation (total MTs)	Spectrophotometry	[86]
Rabbit liver MT-2 standard (previously separated from MT-1 by SEC)	-	RP High performance liquid chromatography	Electrospray ionization (quadrupole) mass spectrometry	[87]
Mussel tissues (*Mytilus galloprovincialis*)	Pressurized liquid extraction	Anion exchange high performance liquid chromatography	UV detection / Inductively coupled plasma optical emission spectrometry	[88]
Rabbit liver MT-2 isoform standard (and its sub-isoforms) / pig kidneys exposed to CdS nanoparticles	-	RP High performance liquid chromatography	Electrospray ionization (tandem) mass spectrometry / Inductively coupled plasma mass spectrometry	[89]
Crab hepatopancreas tissues	Buffer (phosphate)	-	Surface enhanced laser desorption ionization / Time of flight mass spectrometry	[90]

Organism	Extraction	MTs or MTLPs Separation	MTs or MTLPs Detection	Reference
Rabbit liver MT-1 isoform standard / earthworms	Heating	RP High performance liquid chromatography	UV detection / SDS-PAGE	[91]
Digestive glands (*Mytilus galloprovincialis)*	Solvent precipitation	Size exclusion and anion exchange chromatography	UV detection / Atomic absorption spectrometry	[92]
Rabbit liver MT-1 isoform standard	-	RP-HPLC	Electrospray ionization mass spectrometry / Inductively coupled plasma mass spectrometry	[93]
Tissues (*Mytilus galloprovincialis)*	Solvent precipitation	SE and RP chromatography	UV detection / Graphite furnace atomic absorption spectrometry	[94]
Rabbit liver MT standard	-	Capillary electrophoresis	Electrospray ionization mass spectrometry / Matrix assisted laser desorption ionization / Time of flight mass spectrometry	[95]
Purified rabbit liver MT-1 isoform	-	Capillary electrophoresis / High performance liquid chromatography	Inductively coupled plasma mass spectrometry / Electrospray ionization mass spectrometry	[96]
Mussels hepatopancreas	-	SE chromatography	High resolution Inductively coupled plasma mass spectrometry	[97]
Fish tissues	-	SE chromatography	Isotope dilution / Inductively coupled plasma (time of flight) mass spectrometry	[98]
Fish livers	Heating	Capillary electrophoresis	Inductively coupled plasma (quadrupole) mass spectrometry	[99]
Fish livers *(Cyprinus carpio)*	-	RP High performance liquid chromatography	Inductively coupled plasma (time of flight) mass spectrometry / Electrospray ionization mass spectrometry	[100]

5.1. Electrochemical methods

The determination of MTs/MTLPs by electrochemical methods is based on the electroactivity of -SH groups, which tend to be oxidized or to catalyze the evolution of hydrogen from a supporting electrolyte. To prevent interferences and lower limits of detection, an adsorptive transfer stripping technique (AdTS) is often coupled with electrochemical methods. The main improvement made by the AdTS is based on removing the electrode from solution after accumulating a target molecule on its surface, rinsing the electrode and transferring it to a pure supporting electrolyte, where no interferences are present [48]. To detect MTs, linear sweep, cyclic, differential pulse and square-wave voltammetries have been used, even differential pulse voltammetry with the "Brdička reaction". In this one, sulfhydryl-containing peptides (proteins) in the presence of an ammonia-buffered cobalt (III) solution give rise to several electrochemical processes (including catalytic hydrogen evolution - Cat) with characteristic electrochemical signals that can be observed at mercury electrodes. This is the most commonly used electrochemical method for detection of MTs in various types of samples [46,101,102]. Olafson and Sim [103] have documented that the polarographic response by the modified Brdička procedure is independent of the metal concentration or the metal type bound to the MTs and that low molar mass compounds containing thiol groups like glutathione, mercaptoethanol and cysteine have a negligible polarographic response compared to the same amount of MTs. A modified Brdička procedure is convenient for determining the amount of MT after its isolation from various tissues of environmentally exposed and unexposed organisms [101,103].

5.2. Saturation assays

Metal saturation assays are based on the competitive displacement and the subsequent determination of the initially MT-bound metal (usually Cd and Zn) by a metal with higher affinity to MTs (usually Hg and Ag), which depends on the stoichiometry of each metal and have lack of selectivity with regard to the individual MT-isoforms [47]. This affinity decreases in order: Hg (II) > Ag (I) ~ Cu (I) > Cd (II) > Zn (II). When using silver, higher chloride levels cause precipitation. Saturation assays are the first methods used for the quantification with enough LODs; however, there are several limitations in using this method primarily when the copper content is high [46].

5.3. Immunochemical methods

This group of methods for the detection of MTs includes the use of antibodies. It comprises techniques based on the immunological detection of MTs in whole tissues and particularly enzyme-linked immunosorbent assay (ELISA) with enzymatically labeled antibodies, radioimmunoassay (RIA) using isotopically labeled antibodies and western blotting [32,46]. The main obstacles in using ELISA and other immunological methods are the need to avoid the degradation of the target molecule, cross reactivity of polyclonal antibodies, possible interference from higher metal contents, inability to quantify individual MT-isoforms and to provide information about the original metal composition. These methods are more suitable for qualitative detection of MTs than their quantification [46].

5.4. Separation methods

The separation techniques are usually the key components in the speciation of metalloproteins, although sensitive element-specific detectors provide the necessary information about the different forms of a particular element in a sample when coupled with the separation devices [49]. Among the separation techniques used for MTs analysis, several modes of electrophoresis and chromatography can be used [46].

Separation techniques can be divided into those applied for the isolation and purification of MTs from biological matrices (described before) and those involving the subsequent separation of different MT-isoforms within the purified sample. Capillary electrophoresis (CE) and chromatographic methods, such as ion exclusion chromatography (IEC), fast protein liquid chromatography (FPLC), or high performance liquid chromatography (HPLC), are employed in combination with different detection systems (UV, fluorometric) and/or other techniques either off-line or on-line (hyphenated systems) for the determination of MTs/MTLPs [46].

5.4.1. Electrophoretic separation methods

5.4.1.1 Gel electrophoresis

Since the paper published by Laemmli (104), SDS-PAGE has been much used for protein separation. For blue native (BN) gels, protein complexes are solubilized directly from the organelle and mixed with a dye (e.g., Coomassie dyes) that binds to the isolated complexes before PAGE separation. BN-PAGE allows proteins to be efficiently resolved under native conditions and permits the direct quantitative assessment of differential changes in a given proteome [3]. Native or denaturating protocols can be utilized for MT detection. Due to the low molar mass of MTs and their facile reoxidation during an electrophoretic run, it is necessary to use gels with acrylamide concentrations between 15 and 17.5% or gradient gel electrophoresis [46].

Among the separation methods, SDS-PAGE [104] using derivatized samples with a fluorescent marker (monobromobimane - mBBr) has been considered very efficient for MTs/MTLPs determinations in marine invertebrates [38,105], according to O'Kefee (1994) [106]. Its advantages include its rapidity and the high affinity of mBBr for sulphydryl groups, which are abundant in MTs, and it is cheaper, compared to HPLC or CZE. As an example, Fan et al. [107] showed a gel electrophoresis method using tricine-based gel for sensitive determination of MTs, based on mBBr, able to analyze proteins between 3.5 and 26 kDa in only one hour and with more sensitivity when compared with Coomassie blue G-250 staining.

Classical two-dimensional gel electrophoresis (2-DE) is the most frequently used technique in metal-toxicity related proteomic studies. It is a powerful separation technique, which allows the simultaneous resolution of thousands of proteins. The high-resolution capability of 2-DE arises because the first and second dimensions are based on two independent protein characteristics. In the first dimension, the proteins are separated according to their charges, i.e., using isoelectric focusing (IEF), while in the second dimension, the proteins are separated orthogonally by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) based on their molar masses [3].

Following gel electrophoresis, the next steps are protein staining and image analysis to quantify each protein and to allow a qualitative comparison of samples by densitometry. Different staining reagents have been used, such as Coomassie brilliant blue (CBB) and silver-staining visualization. After scanning, selected spots can undergo an in-gel tryptic digestion and the proteins can be identified by MS analysis of the resulting peptides [3,46]. 2-DE in combination with MS plays an important role in proteomics and in metal toxicity-related studies. However, this classical approach has several limitations, and not every protein is amenable to separation. Low-abundant proteins, small and very large proteins, alkaline, hydrophobic and especially membrane proteins are underrepresented in 2-DE. One of the main problems associated with 2-DE is reproducibility due to inherent gel-to-gel variability. To circumvent this problem, difference gel electrophoresis (DIGE) was developed. In DIGE, different samples are labeled with different fluorescent dyes, thus allowing a comparison of two or three protein samples on a single gel. Images of the 2D gels are acquired using as many as three different excitation/emission filters, and the ratio of the differently colored fluorescent signals is used to find protein differences among the samples. The acquisition of fluorescent images of a DIGE gel allows the relative quantitation and a selection of gel spots containing differentially expressed proteins [3,108,109,110]. DIGE has been employed to assess proteome changes in a wide variety of samples, including animal tissues and fluids, and also for sub-cellular compartments. However, to date no paper was found concerning DIGE and MTs.

5.4.1.2. Capillary electrophoresis (CE)

CE is a hot topic in proteomics primarily for its excellent resolution, rapidity, low sample volume demands and ability to differently separate charged and neutral molecules during a run [46]. CE has been applied as an alternative to chromatographic techniques for the quantification of individual MT-isoforms [111]. The basic parameters that affect the resolution of CZE include surface characteristics (e.g., untreated or coated capillaries), and mobile phase composition (i.e., the buffer, its pH, concentration, and the presence of zwitterionic reagents, inorganic salts, metal ions, ion-pairing agents and organic solvents). The choice of capillary and running conditions depends on the complexity of the MT mixture to be resolved. CZE in uncoated silica capillaries provides a rapid analysis of non-heterogeneous MT-isomers in neutral or alkaline buffers. Most heterogeneous isomers and those in complex matrices can be separated using high ionic strength buffers (e.g., 0.5 mol L^{-1}). Uncoated capillaries offer a high-speed analysis and durability, but their resolution needs more work [47].

An alternative technique for the qualitative evaluation of purified MT samples involves the addition of an ionic surfactant to the electrolyte. This technique is known as micellar electrokinetic capillary chromatography (MECC). The principle of analyte separation in MECC involves their partitioning between the hydrophilic medium of the aqueous electrolyte and the hydrophobic interior of the negatively charged SDS micelles. Hence, the separation is made on the basis of differences in hydrophobicity as well as charge [112]. Neutral capillaries are very well suited for protein separations and are very useful for the qualitative evaluation of MT isoforms. By changing the polarity appropriately, the separation of multiple MT isoforms can be obtained at low and neutral pH values, and a wide range of different electrolyte buffers is compatible with this type of capillary. Both phosphate and mixed zwitterionic buffers have been used to obtain good separations of MTs from a range of different species [113].

When using UV detection with coated or uncoated capillaries, careful thought should be given to the buffer used in the electrolyte. Characteristically, MTs have no UV absorbance at 280 nm due to the absence of aromatic amino acids, but some organic buffers, e.g., tricine, will strongly absorb below 230 nm, which will limit the use of lower wavelengths for greater detection sensitivity. While this may not be important for qualitative work, it is a distinct disadvantage for quantification. Sodium phosphate and borate do not significantly absorb UV light and are very suitable for quantification studies [113].

The basic drawback of electrophoretic techniques is poor detectivity resulting from the very small (a few nanoliters) samples that can be injected onto a capillary. This problem can be overcome by on-line pre-concentration prior to the capillary inlet [47]. The great advantage of CE in detection and quantification of MTs is its ability to distinguish MT-isoforms in crude samples.

Several papers can be found in the literature dealing with CE and MT separation. Virtanen et al. [74] separated MT-isoforms using CZE with an uncoated capillary and investigated several parameters that control the separation. Best conditions were obtained by using tris-borate buffer and 11 kV power. For horse kidney and rabbit liver MTs they found two and three isoforms. The authors conclude that this method is very useful but, unfortunately, they did not characterize the peaks found and so that there is less confidence in these findings. Virtanen and Bordin [80] also developed a simple CZE method for MT-isoforms using tris-tricine buffer. After optimizing experimental parameters, they were able to separate rabbit MT-isoforms better than the traditional method that uses tris-borate buffer. Andon et al. [95] have separated several MT-isoforms from rabbit liver, showing three sub-isoforms within MT-1 and MT-2, which were confirmed using MS (on-line). This paper shows the great ability of CE in separation of species coming from different organisms.

Chassaigne et al. [96] also showed MT-sub-isoform detection using CZE coupled to ICP-MS (using a customized interface) with excellent resolution. This method allows different metal complexes of the same isoform be separated by CZE. Álvarez-Llamas et al. [99] proposed a large volume stacking methodology to by pass low CE detectivity. The detection limits were increased by 9 to 13 times, allowing metal speciation in MT-isoforms present in eel liver of exposed and non-exposed eel group organisms.

5.4.2. Chromatographic methods

5.4.2.1. Size-exclusion / Gel permeation chromatography

Size-exclusion chromatography (SEC) is based on the molecular sieve effect and enables species to be separated according to their size and, to a lesser extent, their shape. The average time a substance spends in the pores is determined by its size, which for a given shape, can usually be related directly to its molar mass. The resolution of SEC is insufficient for the discrimination of small heterogeneities in amino acids; thus, this technique serves in the separation of the MT fraction from samples but cannot distinguish among the different isoforms [47]. A separation by SEC should be independent of the analyte charge, but in practice, the stationary phase surface displays charged properties; thus, a mixed mode separation is observed. This makes the choice of packing material critical. The two categories of packing used include silica and organic polymers. At nanogram metal levels, significant silanophilic effects can occur, including metal losses in the presence of low ionic strength eluents on silica-based SEC supports. The average pore size of the packing used varies from 100 to 1000 Å [47]. The optimum eluent should ensure minimum competition between buffer and cytosolic ligands and between these ligands and

the gel. The separation by SEC with water as mobile phase prevents structural changes, denaturation of proteins and destruction of protein–metal complexes. In practice, various aqueous mobile phases of fairly high ionic strength have been used to avoid interactions with the packing material. In general, dilute buffers may cause the adsorption of low molar mass proteins by the column packing. When silica-based packing cannot be avoided, the addition of a non-complexing salt (e.g., NaCl) to the mobile phase was shown to suppress the residual silanol activity of the column packing. Under these conditions no significant exchange of Cd occurred but occasional (for Hg) or appreciable losses (for Zn) can be observed for other metals [47]. The wide variety of buffers reported in the literature makes it relatively easy to choose a suitable one for the detection technique. Up to 30 mol L^{-1} tris-HCl was found to be tolerated in ICP-MS, whereas 20 mol L^{-1} formate or acetate buffer in 10 % methanol was found to be acceptable for ESI-MS.

The analysis time is a function of the column size and the eluent flow-rate. Even though columns up to 120 cm lengths were used, the standard 30 mm x 7.6 mm column is a good choice. At a flow rate of 1 mL min^{-1}, the separation requires approximately 20 min to go to completion. The choice of small-bore columns with size-exclusion packing on the market is still limited; such columns enable the rapid characterization of various metal-containing molar mass fractions in unknown cytosols by direct injection nebulization ICP-MS or ESI-MS.

As examples of papers concerning MT separations by SEC, some can be highlighted. The work developed by Berthet et al. [79] in which MTs were separated from sponge (*Spongia officinalis*) tissue using Sepharose and Sephadex columns found proteins with 6.7 and 15 kDa. The metals associated with these MT fractions were determined and the authors found a relationship between metals (Cd, Zn and Hg) and MTLPs contents. You et al. [84] also used SEC for pre-purification of MTs from worms (*Caenorhabditis elegans*) exposed to Cd to express the two MT-isoforms (CeMT-I and CeMT-II). These isoforms were further separated by ion-exchange chromatography. In the same way, Hensbergen et al. [85] studying MTs in insects (*Orchesella cincta*), used a Superose column and further separation by RPC. Cd was also determined by GF AAS and the authors showed a good relationship between MT and Cd concentrations. Other papers dealing with SEC can be found in Table II. As can be seen in the above mentioned literature, SEC is normally used for pre-separation (purification) of MT fractions due to its poorer resolution. It is mandatory to employ a better separation technique, such as RPC or IEC, for further MT-isoform separations.

5.4.2.2. Reverse-phase chromatography (RPC)

The separation of MT-isoforms is most frequently performed using reverse-phase chromatography between a non-polar stationary phase (usually a covalently bound C8 or C18 linear hydrocarbon) and a relatively polar mobile phase. Reverse-phase HPLC seems to be superior to SEC and ion-exchange because the packing material for RPC is primarily free of ligands for metals. Because the hydrophobicity of a polypeptide primarily dictates its retention in RPC, the gradual elution of individual MT-isoforms of a mixture is achieved by decreasing the polarity of the mobile phase by the addition of methanol or acetonitrile [47].

Wide-bore (4–5 mm) 15–25 cm long columns are the most frequently used. Narrow-bore and microbore columns are expected to gain significance because of their higher sensitivity and resolution. The presence of a buffer is necessary. Tris–HCl is the most widely used in the separation of metallated isoforms. Apo-MT-isoforms have been separated using trifluoroacetic acid (TFA) or acetic acid media. The resolution of isoforms is affected by pH. Thus far, UV detection has been used in most cases. This is because the high content of organic modifier hinders the compatibility between RP-HPLC and ICP-MS. A set of Pt-cones and the addition of oxygen to the nebulizer gas are highly recommended. Loss of detectivity is unavoidable. However, RP separation conditions are close to ideal for ESI-MS detection, which is gaining popularity [47].

Besides some papers discussed in the previous section, other papers can be found in the specialized literature dealing with MTs and RPC. Shen et al. [87], working with Zn-rich rabbit MT, separated a MT-2a isoform using a Zorbax 300 SB C18 column. After separation it was analyzed by positive ionization mode (which maintains the metal integrity) ESI-MS, showing a protein with 6568 Da and a structure as Zn_7-MT-2a. Mounicou et al. [89] used RPC (Vydac C 8 microcolumn) for the isolation of pig kidney MT-isoforms and its subsequent characterization by ICP-MS and ESI-MS (orbitrap system). This approach allows simultaneous access to inorganic and organic information that is of concern for biological cytosol studies. Infante et al. [100] also used RPC for isolation of two MT-isoforms from Cd-exposed carp tissue. These authors also used a coupled configuration with ICP-MS and ESI-MS, and simultaneously determined the metals (Cd, Cu, Zn and Pb) associated to each MT-isoform.

5.4.2.3. Anion exchange chromatography

MT-1 and MT-2 fractions, which have a negative charge in aqueous solutions, can be separated using anion exchange chromatography. Frequently, MTs, before being applied to anion exchange, are first pre-separated using SEC [47]. A weak-anion exchanger with diethylaminoethyl functional groups is the only type of support used. Aqueous buffers are used with a linear concentration gradient. The high potential separation of the MT-1 and MT-2 isoforms by anion exchange is not fully exploited with coupled systems because the common end-concentration of

0.25 mol L^{-1} buffer is not tolerated well by ICP-MS and suppresses electrospray ionization [47].

Among the published papers discussed in section 5.4.2.1, in which anion-exchange chromatography was used to separate MT-isoforms, a very good work developed by Santiago-Rivas et al. [88] describes this field. In this work MT-1 and MT-2 isoforms from mussel tissue (*Mytilus galloprovincialis*) were separated using a Protein Pak DEAE-SPW column. As an improvement in this work, a pressurized liquid extraction for cytosolic MT extraction was used and the concentrations of Cu-MT-1, Mn-MT-1, Sr-MT-1 and Zn-MT-1 could be determined by ICP OES.

5.5. Coupling separation and detection techniques

Development and application of "hybrid" techniques to obtain speciation information in environmental samples were soon extended to the speciation of trace elements in biological material of the most varied living organisms. However, the complexity of chemical speciation in biological samples is greater than in environmental sources. For a given element, many unknown compounds (e.g., peaks of unidentified species containing that element) were detected that result from its metabolism in the living organism under study [49,112].

Chemical species of a trace element are often unstable and concentrations found in different matrices of interest are often on the ng g^{-1} range, whereas inorganic forms can simultaneously be present at 1000-fold higher levels. Thus, sensitive and selective analytical atomic spectrometric techniques have been used as detectors for speciation and are generally coupled to some gas or liquid chromatographic step for the time-resolved introduction of analytes into the atomic detector [49].

Two types of separation-detector couplings are usually considered in metalloprotein species-selective analyses: (a) off-line detection of metal(oid)-proteins or other charged macromolecules bound to some metal or metalloid, which are separated by gradient gel electrophoresis or isoelectric focusing for flame atomic absorption spectrometric (FAAS) determination. Other discrete detection techniques can also be preferred when very high detectivity is needed, using an LC connected to electrothermal AAS (ET AAS) or when LC or CE devices are connected to ICP-MS (off-line), especially when a graphite furnace is used for ET sample introduction at the plasma device inlet; and (b) on-line couplings of separation column techniques, such as LC or CE, with some very sensitive elemental detector, especially ICP OES or ICP-MS, in which the hyphenation is completed via a nebulizer [49].

The choice of a hyphenated system primarily depends on the research objective. The separation component of the coupling system is favored when the characterization of the maximum number of iso- or sub-isoforms is of interest, whereas the detector component becomes crucial when high detectivity is required [49].

The presence of a metal bound to MT is a prerequisite when using an "inorganic" atomic spectrometric detector, such as atomic absorption, ICP atomic emission or ICP-MS spectrometers. The linear range and multi-element capability of these detectors make them a rapid alternative to metal-saturation assays. An electrospray mass spectrometer is unmatched when characterizing MT isoforms in terms of molar mass. A collision-induced dissociation (CID) cell and another mass spectrometer in series are necessary to provide structural information in terms of the amino acid sequence. An important problem often encountered is the interface between chromatography and spectrometry. LC mobile phases typically consist of some combination of organic solvents, salts from buffer solutions and/or ion-pair reagents, and the separation conditions may not be compatible in terms of flow-rate and mobile phase composition with those acceptable/required by the detector. This criterion is often much more difficult to fulfill compared to the UV detector, where the optical transparency of the mobile phase above 200 nm is the only condition. High concentrations of organic solvents in reversed-phase HPLC negatively affect plasma but enhance the ESI process. Conversely, the relatively concentrated buffers used in ion-exchange chromatography suppress the signal in electrospray and can also clog nebulizers. The nanoliter sample volume and low microliter per minute flow-rate makes the dead volume and the detectivity critical in AAS and plasma techniques, whereas they are not far from the optimum nebulization techniques in ESI-MS. Because of these considerations, the choice and optimization of the best hyphenated system for a given task is difficult and requires some experience [47].

5.5.1. Atomic Absorption Spectrometry (AAS)

The metals that preferentially bind to MTs (e.g., Cd, Zn, Cu) are among the elements that provide the most intense response in AAS. Off-line analysis of MT-bound metals after intense HPLC by fraction collection and GF AAS analysis has been a common approach. Being less sensitive than GF AAS, F AAS offers the possibility of an on-line approach. This technique is compatible with both flow-rates and mobile phase composition, which typically includes organic solvents, commonly used in HPLC. Taking the widespread availability of this technique into account, it is no wonder that HPLC-AAS was the first hyphenated technique to be used in the determination of MT isoforms [47]. The primary interest in HPLC-AAS lies in its simplicity, wide availability and compatibility with the mobile phases used in HPLC. AAS is not truly a multi-element technique; nevertheless, up to four elements can be measured simultaneously with the latest instruments, which is sufficient for practical applications. Despite the fact that detection limits obtained with advanced interfaces almost match those of ICP-MS, the primary field of application of HPLC-AAS is the characterization of MT-isoforms after metal saturation protocols [47].

5.5.2. Inductively coupled plasma optical emission spectrometry (ICP OES)

Compared with F AAS, ICP OES offers better detection limits, at the 1 ng mL^{-1} level, but lower solvent compatibility. High organic solvent (> 10%) contents may not be easily tolerated. The unmatched advantage of ICP OES is the possibility of monitoring sulfur together with metals. ICP OES instruments with an axial view seem to offer lower detection limits for transition metals. In addition, its multielement capability is a great advantage for the quantitation of several metals simultaneously.

5.5.3. Inductively coupled plasma mass spectrometry (ICP-MS)

Currently, ICP-MS is the most powerful elemental detector used to "screen" the presence of an element in known or unknown large biomolecules. ICP-MS is a specific detector for metals (and metalloids) at very low levels. It can conduct selective, real-time monitoring of a given element associated with a compound eluted from a chromatographic column or electrophoretic capillary. Its synergic use with a previous high-resolution separation of the protein (or its complexes) offers a revolutionary analytical tool [112,114,115]. In ICP-MS, the signal measured is an elemental ion; the species-selectivity is a function of the arrival time of the analyte to the ionization source. Consequently, the identification requires prior knowledge about the possible species and the availability of a standard to calibrate the column in terms of retention time. For a large majority of compounds present in biological materials, these conditions are not fulfilled, and a need arises for a technique able to inherently generate a signal that would be specific to an element and to a species or its fragment [115].

Despite its potential, some care should be taken in case of coupling with a separation technique. The eluent from the LC instrument may be rich in organic constituents and these can cause further problems when connected to an ICP-MS. Organic solvents can adversely affect the stability of the plasma, deposit soot on the faces of the sampler and on the skimmer of a MS instrument, which would result in increased noise and varying responses. Organic solvents can also produce high-reflection power that can result in plasma extinction. The nebulizer can also become blocked if the solvents contain high concentrations of salt. The problem of organic solvents entering the plasma can be overcome by reducing the quantity of solvent reaching the plasma by employing micro LC separations or by increasing the amount of O_2 plasma gases, although this significantly reduces the lifetime of the skimmers and of the sampler [116].

When coupling with CZE, the interface must provide an electrical connection and a stable electrical current for reproducible electrophoretic separations. The major problem is the small sample volume (typically 10–100 nL), which requires a very sensitive detector to cope with the low naturally occurring analyte levels, and the low flow-rates (1 - 5 nL min^{-1}) limit the choice of nebulizer. In addition, the interface should prevent the occurrence of laminar flow inside the CE capillary caused by the nebulizer; this is potentially a very serious problem because it could disturb the high resolving power of the capillary electrophoresis. In terms of the practical applications of the characterization of MT isoforms, the high resolution of CZE has long been off-set by the lack of suitable element/species selective detectors. Recent instrumental developments regarding the coupled techniques of CZE-ICP-MS and CZE-ESI-MS are likely to result in numerous contributions to this field [47,48].

5.5.4. Electrospray-ionization mass spectrometry (ESI-MS)

For protein analysis by MS, two main ionization techniques are used: electrospray ionization (ESI) and matrix-assisted laser desorption-ionization (MALDI). These techniques are characterized by a soft ionization that lowers the probability of protein destruction.

ESI is based on electro-nebulization (i.e., a mechanism that involves the evaporation of ions) of a sample performed at atmospheric pressure. The technique was shown to produce gas-phase ions of highly labile and non-volatile compounds, such as peptides, proteins, oligonucleotides and oligosaccharides, without any fragmentation. Metal complexes of Cd, Ag and Cu were transferred into the gaseous phase and detected by the mass spectrometer [115,117]. Soft ionization using ESI can preserve the formation of the metal-protein complexes; thus, coupling ESI with MS can help in the identification of protein isoforms and the stoichiometry of metal complexes [46,51]. Depending on how the generated ions are further handled, different types of information on the identity of the molecule of interest can be obtained. In the simplest case (i.e., in the molecular ion or single MS mode), one or more protons can be attached to the analyte molecule leading to the formation of a single-charged or multicharged ion. This allows an accurate measurement of the molar mass of the analyte species of interest. At a sufficiently high ionization energy, elemental ions of metals or metalloids present in the sample can be obtained. Finally, the molecular ion, which can be isolated at the first mass filter, can be fragmented by collisions with molecules of a neutral gas in a process referred to as collision-induced dissociation (CID). The resulting fragments identified by molar masses in a second mass analyzer (tandem MS or MS/MS mode) allow information on the structure of the molecule to be obtained [115].

In ESI-MS/MS, the triple quadrupole mass spectrometer is a very popular instrument, in which the second quadrupole is used as a collision cell. The analytes are fragmented in the collision cell by a collision gas, and the fragments are analyzed by the third quadrupole to obtain further structural information. Triple quadrupole mass spectrometers have

mass resolutions up to 3000, but even the analysis of MTs with molecular masses of 6 - 7 kDa (and other large proteins) is possible because the MTs acquire charges of +4 and +5 in the ESI and this expands the mass range after the deconvolution of the mass spectra. If the third quadrupole is replaced by a time-of-flight mass spectrometer (ESI-MS/Q/TOF), a mass resolution of up to 10000 can be achieved. This provides the mass accuracy required for definitive mass assignment and structural elucidation [48].

Despite its enormous potential, this technique is just starting to become popular for the characterization of MT-isoforms and their metal complexes. It was used for the determination of the major rabbit MT isoform II. ESI mass spectra of purified rabbit MT-2 revealed the presence of two isoforms in the sample that differed by 30 mass units, and the characteristic spectra for the apoproteins and metal-saturated proteins in acidic and alkaline pH gave information about the number and the identity of metals bound to the protein [47].

Because the metal binding capacity of MTs is strongly pH-dependent, a step-wise or complete demetallation can be achieved by lowering the pH to below 2. Both the disappearance of the different isoforms of the MTs (with the formation of the corresponding apo-MTs) and the higher degree of protonation lead to a significant increase in sensitivity for the detection of the protein at low pH. If the sequence of the ligand, such as the apo-MTs, is already known and exists in a protein database, the determination of its molar mass is already sufficient for its identification. In the case of a previously unknown protein, a CID with a tandem MS instrument can be used for the elucidation of its amino acid sequence [4].

5.5.5. Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) analyzer

The MALDI time-of-flight analyzer (MALDI-TOF) is a soft ionization technique suitable for protein analysis and is more tolerant than ESI to higher sample mass and to salt contents, buffers and other substances [46]. By means of a MALDI-TOF mass spectrometer, 2-DE has been coupled with Edman sequencing or peptide-mass fingerprinting (PMF). Although this approach has been extensively used, it is important to note that PMF measures the masses of the peptides derived from the trypsinized parent-protein spot. Thus, if several proteins happen to be in the same gel area, the spectra could become very complicated, which would make it difficult to identify the protein of interest. To overcome this potential problem, a few authors have chosen to use tandem MS (MS^2) - MALDI-TOF/TOF or LC-MS^2, which allows the sequencing of the peptides and providing more confident protein identification [3].

5.5.6. Surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) analyzer

Surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) analysis has also been used to evaluate protein changes induced by exposure to arsenic and lead. A variation of MALDI-TOF, SELDI-TOF uses a modified target to achieve biochemical affinity. Thus, the SELDI surface acts as a separation step, which allows the analysis of a subset of proteins and reduces sample complexity. SELDI-TOF has been widely used to identify potential disease biomarkers and its potential makes it interesting for identifying candidate biomarkers for toxicity, especially when working with body fluids [3].

6. Conclusion

There is an increasing need to develop techniques, methods and procedures for the identification, estimation, comparative assessment and management of the risks caused by chemical pollutant discharges to the environment and natural resources. The measurement of the biological effects of pollutants upon the biota has become of major importance for the assessment of the quality of the environment. Thus, the use of biomarkers as a sensitive "early warning" tool to measure biological effects in environmental quality seems to be of concern.

Nowadays, MTs and MTLPs have being considered as specific biomarkers for trace metal exposure in the marine environment. However, the extraction, separation and detection methods for these proteins lack appropriate standardization, which has generated questions about the effectiveness of these methods. Studies performed for comparison between the extraction procedures pointed out the use of the heat treatment method. This trend is due to a relative loss of MTs and MTLPs reported when compared with the solvent precipitation method. However, more studies about the comparison between these two procedures need to be made, using other separation techniques that allow the identification of MTs and MTLPs.

Electrophoretic and chromatographic methods have a potential to be attractive methods to perform good separation and to allow the characterization of MTs in a biological matrix before it is applied for biomonitoring studies. Coupling a separation with a detection technique is the best way to perform the speciation analysis of MTs and associated metals and metalloids. Thus, the hyphenation HPLC-ICP-MS and ESI-MS or MALDI-MS are the best tools to study the presence of MTs and trace metals in biological bioindicators to assure effective environmental monitoring of marine environments.

Also, after the initial characterization of MTs and MTLPs in a bioindicador organism, the use of indirect methods to detection of these proteins, such as by derivatization with monobromobimane associated with separation by SDS-PAGE, may be applied to identify such proteins. This method seems to be sensitive for detection of molar mass and cysteine-rich proteins and it is a relatively rapid and cheaper method, ideal for routine studies such as biomonitoring.

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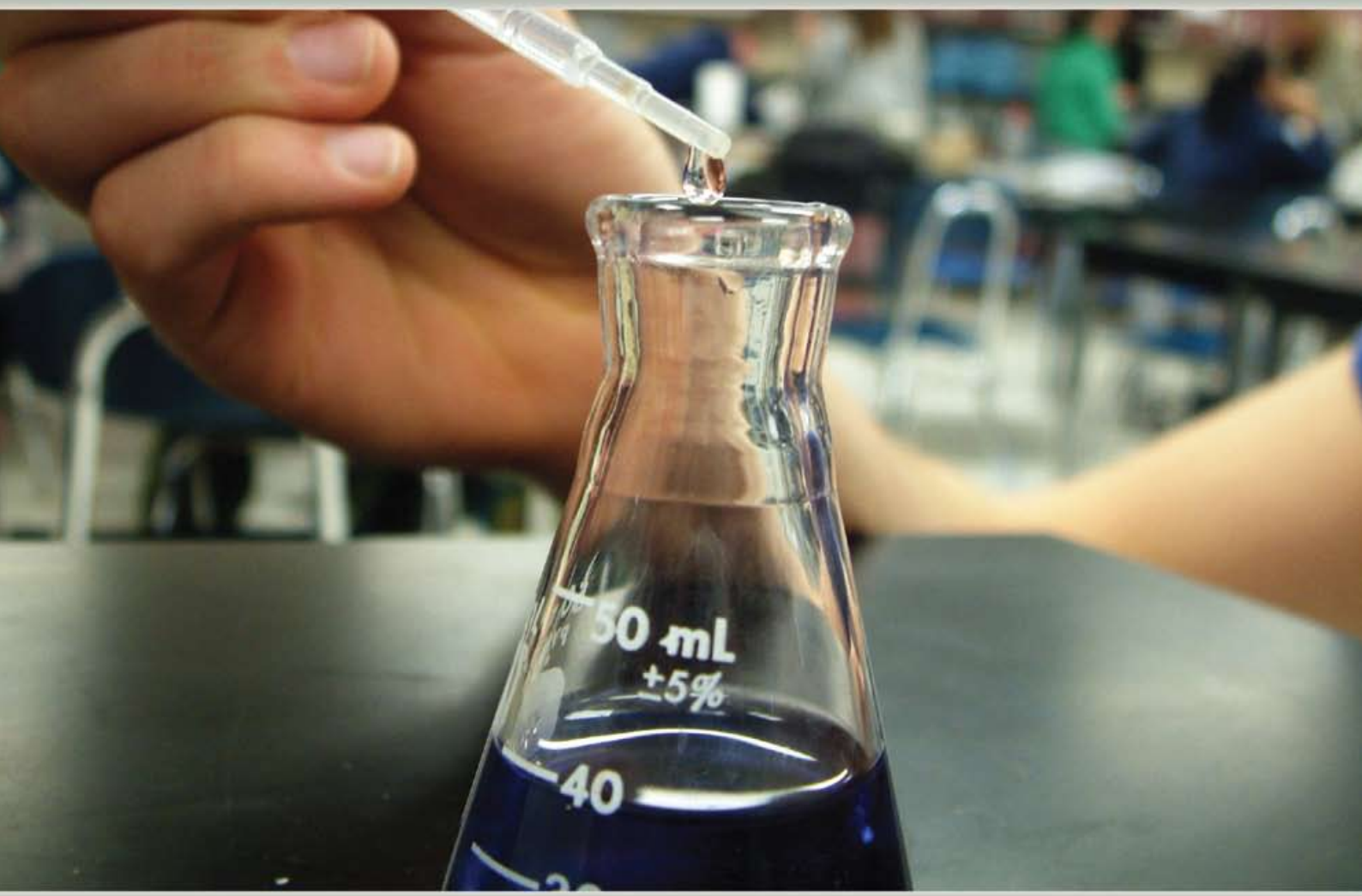

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Point of View

Are there available nowadays significant numbers of analytical methods for speciation in environmental studies? Who is able to handle speciation analysis, university or commercial laboratories or both? How many laboratories?

Although we can still consider speciation analysis to be a new approach, it has been carried out intensively for at least the last ten years. Clearly we have passed the initial stage, when it was necessary to show that the coupling between chromatography (more generally, separation techniques) and atomic spectrometry could be made robust (and efficient) and several types of interfaces were proposed. The second stage, when most of the publications about speciation analysis were addressed to applications has also been reached. Nowadays, there are a number of scientific meetings about speciation. In Brazil, we have a meeting (EspeQBrasil) that brings researchers from all parts of the country to discuss recent applications and developments in speciation. Even if we consider only environmental studies, new methods are necessary. The coupling between inductively coupled plasma mass spectrometry (or hydride generation atomic fluorescence) and chromatography (or capillary electrophoreses) is now commercially available, and at least one method for the speciation analysis of arsenic using liquid chromatography is included in an equipment operation manual. EVISA (the European Virtual Institute for Speciation Analysis, a web source for all those looking for information about analysis of chemical species) provides services for speciation analysis, acting as a broker between customers having speciation-related demands and partner laboratories offering solutions. Considering these prospects the answer to the first question is yes. Assuming that I know about the work that is under development in the universities, my answer to part of the second question is: Universities are able to handle speciation analysis for environmental studies. I do not personally know of commercial laboratories in Brazil that have carried out speciation analysis, but I know of one or two laboratories that have the equipment necessary for performing work involving speciation analysis. Nevertheless, I should like to point out that this limitation is not unique to Brazil. Recent information from EVISA indicates that there is a limited number of commercial laboratories that can handle a significant number of different analytical speciation methods (about 10 commercial labs worldwide). The explanation for this lies with the lack of demand for this type of analytical service. Since most regulations specify total concentration, most laboratories cannot survive on speciation analysis. Thus, nowadays the main question related to the extensive use of speciation analysis as a routine procedure is its role in regulation.

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Researcher IV
UNESP/CEA - Universidade Estadual Paulista / Centro de Estudos Ambientais

Point of View

Undergraduate Teaching in Analytical Chemistry: Must it be changed?

Brazilian Analytical Chemistry has been developing rapidly and intensely. This growth can be easily checked, for example, through the scientific papers published by Brazilian researchers in International Scientific Journals, as well as by the increase of attendees in the National Meeting on Analytical Chemistry (ENQA). Also, the publication of BrJAC and its rapid acceptance by the community is another factor that attests to a growing interest in the area of analytical chemistry.

On the other hand, there are an increasing number of chemicals registered annually, coupled to the discovery of new chemical compounds with different applicabilities in our daily lives. The chemical industry in Brazil is also growing, although the importation of chemical products has also increased, month after month, establishing records in the Brazilian trade balance.

In this context, analytical chemistry becomes increasingly important because it is responsible for the characterization of this variety of materials, products and compounds. Although there are excellent Universities in Brazil producing undergraduate chemists and chemical engineers, the teaching of analytical chemistry disciplines seems to have lagged, compared to current reality. Few of the chemistry departments or institutes responsible for teaching analytical chemistry disciplines have adequate infrastructure for scientific equipment. Analytical chemistry is an experimental science and, to fully learn it, the student needs to know well the new analytical concepts as well as to manipulate the instruments in the laboratory and also to perform chemical analyses of real samples. These lessons are extremely important because the basic chemical concepts of analytical chemistry are embedded in these techniques and methods. Today, spectrometric techniques such as Atomic Absorption Spectrometry (AAS, in its conventional and in high resolution modes), Optical Emission Spectrometry with Plasma Sources (ICP OES), Mass Spectrometry with Plasma Sources (ICP-MS) and chromatographic techniques such as Gas Chromatography coupled to Mass Spectrometry (GC-MS) and Liquid Chromatography coupled to Mass Spectrometry (LC-MS) are of relatively common use in modern laboratories that are active in quality control of products and processes. In addition, a series of new techniques, such as imaging techniques using X-rays, different modes for sample introduction in mass spectrometry and miniaturized electrochemical devices, for examples, have been developed and will soon be available in analytical laboratories. Thus, given the broad applicability and the increasing development of so-called instrumental methods of analysis, the Universities need to seek to equip their laboratories to allow students of undergraduate courses be directly involved with these modern techniques. This will reduce the gap between the teaching of analytical chemistry and the reality that the industrial sector presents today and will produce chemistry professionals compatible with the requirements of modern chemistry, necessary for their active participation in Brazilian technical and scientific development, which is a challenge that will have to be faced soon.

Thus I think that the Brazilian Universities should also engage in a process of change and modernization of the contents of the disciplines of analytical chemistry, as well as adapting the curriculum of undergraduate chemistry and chemical engineering courses, offering more knowledge about modern instrumental methods of analysis.

Ricardo Erthal Santelli
Full Professor of Analytical Chemistry at Rio de Janeiro Federal University

Events 2011 / 2012

ANALITICA LATIN AMERICA
2º CONGRESSO LATIN AMERICA
11th ANALITICA LATIN AMERICA
São Paulo, SP – September 20th to 22nd, 2011
http://www.analiticaexpo.com.br/

16º ENQA – 16º ENCONTRO NACIONAL DE QUÍMICA ANALÍTICA
Campos do Jordão, SP – October 23rd to 26th, 2011
http://www.usp.br/enqa2011

PBA 2011 – 23rd International Symposium on Pharmaceutical and Biomedical Analysis
João Pessoa, PB - October 9th to 12th, 2011
www.pba2011.com/

CBQ - 51º Congresso Brasileiro de Química
São Luís, MA – October 9th to 13rd, 2011
http://www.abq.org.br/cbq/

SBMM 2011 – XXIII Congresso da Sociedade Brasileira de Microscopia e Microanálise
Armação dos Búzios, RJ – October 15th to 18th, 2011
www.csbmm2011.com.br

6ª ESCOLA DE ELETROQUÍMICA 2011
São Paulo, SP – December 4th to 10th, 2011
http://sinquisp.org.br/acontece.php?id=183

BrMASS 2011 – IV Congresso Brasileiro de Espectrometria de Massas
Campinas, SP – December 10th to 13th, 2011
http://www.brmass.com.br/congresso/programacao/programacao.asp?cd=35&lang=1

POC 2012 – 14th International Union of Pure and Applied Chemistry Conference on Polymers and Organic Chemistry
Doha, Qatar – January 6th to 9th, 2012
www.poc2012.com

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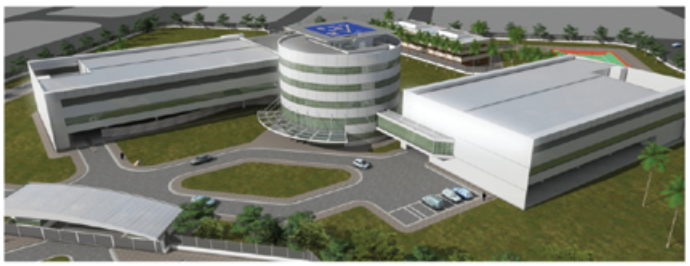
Centro Tecnológico Bromangol



Projeção da área externa do Laboratório Central Bromangol

Está em fase de implantação em Luanda - Angola, o Centro Tecnológico Bromangol. Esta obra vem com a missão de garantir segurança alimentar nos produtos importados e monitoramento da produção nacional.

Terá o compromisso de executar análises físico-químicas e microbiológicas referenciadas nos monitoramentos utilizados no Brasil e Comunidade Européia, visando o atendimento rigoroso de controle de qualidade e sanidade pública, além de prestar serviços analíticos, contando com experientes analistas, utilizando alta tecnologia e ciência.

A estratégia deste projeto é formar pessoal qualificado, disseminar o uso de alta tecnologia e contribuir com o crescimento científico do país, abrindo novas oportunidades de comércio entre os países, fomentado pelo controle de qualidade assegurado dos produtos e aumentar a visibilidade do país frente aos mercados internacionais, tornando-se competitivo comercialmente.

O Laboratório Central terá com 6.000m², com capacidade analítica acima de 30.000 análises/mês, divididas em testes físico-químicos e microbiológicos em alimentos, fármacos e brinquedos, além da possibilidade de expansão da capacidade analítica para até 40.000 análises/mês, em um único turno de trabalho.

Os laboratórios seguem desde sua construção os padrões de qualidade exigidos para a certificação ISO/IEC17025, sendo estruturados dentro de um sistema de qualidade totalmente rastreável, com os documentos e métodos prontos para a acreditação por órgão certificador, que pode ser o INMETRO no Brasil.

Com a futura acreditação do ISO/IEC17025, os laboratórios conquistarão a credibilidade e serão reconhecidos mundialmente pela competência técnica de laboratórios para a realização de ensaios analíticos.

Projeto Vale Omã

Fotos das áreas externa e interna do Laboratório Vale Oman

O projeto Omã compreende a construção de uma planta de pelotização, que será operada pela subsidiária da empresa, Vale Oman Pelletizing Company LLC (VOPC), com capacidade de produção de nove milhões de toneladas métricas de pelotas para redução direta, uma central de logística industrial com terminal marítimo e um centro de distribuição com a capacidade para a movimentação de até 40 milhões de toneladas de minério de ferro e pelotas por ano.

O Laboratório com mais de 1000m², responsável pela análise da matéria prima recebida e também da pelota acabada, está dividido em 5 áreas:

- Administrativa;
- Laboratório Metalúrgico;
- Laboratório Físico;
- Laboratório Químico;
- Piso Técnico.

A segunda etapa do fornecimento, foi a venda e instalação de todo o mobiliário técnico e equipamentos de Laboratório. Foram enviados para Omã técnicos brasileiros para supervisionar e instalar os equipamentos, e contratado no mercado local empresas terceiras para suporte desta disciplinas (marcenaria, mecânica, etc).

O know-how, aliado a experiência de mais de 50 anos projetando e construindo laboratórios, permitiu ao **Grupo Vidy** estar a frente deste projeto, oferecendo soluções inovadoras, na modalidade ***Turn-Key***, envolvendo até diversos equipamentos como mobiliários, capelas e equipamentos do laboratório.

Para obter mais informações sobre o **Grupo Vidy**, conhecer este e outros projetos realizados, principais obras, fazer downloads dos nossos catálogos técnicos com os nossos principais produtos, como bancadas, capelas e mobiliários, acesse nosso site.

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Certificada desde 2007, a Vidy é a única empresa de Engenharia de Laboratórios do Brasil com ISO 9001:2008, envolvendo: "Projeto, Construção Civil, Fabricação, Instalação de Laboratórios e Comercialização de Equipamentos e Acessórios para Laboratórios."

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