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par

Carolina Lyrio Tenorio Correia

Pour obtenir le grade de
Docteur
Spécialité : Chimie analytique

Le marquage des peptides avec des métaux et détection par MS et l'optimisation des procédures de l'extraction de métalloprotéin dans les échantillons biologiques à des fins de protéomique

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Thèse en co-tutelle dirigée par Dirk Schaumlöffel et Tatiana D. Saint'Pierre
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Para Deus
Para meus pais, Claudio e Tania, que me deram vida e força
Para Boris, meu marido, meu apoio, minha metade.

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Resumo

Tenorio-Daussat, Carolina L.; Schaumlöffel, Dirk; Saint'Pierre, Tatiana D. Rotulagem de peptídeos com metais usando detecção MS e otimização de procedimentos de extração de metaloproteínas em amostras biológicas para fins proteômicos. Rio de Janeiro, 2014, 162 p. Tese de Doutorado – Departamento de Química, Pontifícia Universidade Católica do Rio de Janeiro e Instituto de Ciências Analíticas e Físico-Química Ambiental e Materiais (IPREM), Universidade de Pau e dos Países do Adour.

Esta tese de doutorado é resultado de uma cotutela entre a PUC-Rio (Brasil) e a UPPA (França)

Método de identificação e quantificação de peptídeos, através da otimização de estratégias para a marcação de peptídeos com metais e subsequente separação por nano-HPLC-UV, MALDI MS. Primeiramente, peptídeos foram marcados com 3 diferentes metais lantanídeos usando um reagente funcional NHS-DOTA. Os resultados demonstraram que a reação de derivatização usando o reagente quelante DOTA foi eficiente para peptídeos simples e misturas dos mesmos, verificada através do MALDI MS a partir da relação m/z. A aplicação do método otimizado em uma amostra complexa (Cyt C digest) mostrou resultados comparáveis aos obtidos com os peptídeos-padrão. Em paralelo, análises ambientais foram realizadas pela otimização de um procedimento de extração de metalotioneína em bílis de peixe, uma vez que esta matriz tem sido reportada como um biomarcador ambiental de contaminação por metal. Diferentes procedimentos (variando o tempo de centrifugação e a temperatura de banho-maria) e agentes de redução (DTT, β-mercaptoethanol and TCEP) foram aplicadas para a extração de metalotioneína em bílis e fígado de peixe *(Oreochromis niloticus.* Análises espectrofotométricas foram realizadas a fim de quantificar os extratos de MT, e gel SDS-PAGE foi usado para avaliação qualitativa dos diferentes procedimentos usados. Cada procedimento foi avaliado estatisticamente. Metodologia de superfície de resposta foi aplicada para amostras de bílis, a fim de avaliar a resposta desta matriz. Em um contexto ambiental, concentrações de MT biliar foi mais baixa que MT do fígado, no entanto, a primeira mostrou-se mais adequada para um monitoramento ambiental em relação a exposição de xenobióticos que podem afetar a expressão proteomica e metalotmica de uma matriz ambiental. Além disso, num procedimento de exposição a metais em laboratório, alguns metais mostraram-se significativamente importantes para avaliação de contaminação a partir da quantificação de MT, segundo tratamento dos dados pela técnica de rede neural.

Palvras-chave

Peptídeos; nano-HPLC-ICP-MS; MALDI MS; SDS-PAGE; MT; bílis de peixe.

Resumé

Tenorio-Daussat, Carolina L.; Schaumlöffel, Dirk; Saint'Pierre, Tatiana D. Le marquage des peptides avec des métaux et détection par MS et l'optimisation des procédures de l'extraction de métalloprotéines dans les échantillons biologiques à des fins de protéomique. Rio de Janeiro, 2014, 162 p. Thèse de Doctorat – Departement de Chimie, Pontifícia Universidade Católica do Rio de Janeiro et Institut des Sciences Analytiques et Physico-Chimie pour l'Énvironnement et les Matériaux (IPREM), Université de Pau et des Pays de l'Adour.

Cette thèse de doctorat est résultat d'une cotutelle entre la PUC-Rio (Brésil) et l'UPPA (France).

Ce travail a développé une nouvelle méthode pour l'identification et la quantification des peptides, par l'optimisation de certaines stratégies disponibles appropriées pour le marquage des peptides avec des métaux lanthanide, une séparation par nano-HPLC et détection UV, et suivi par MALDI MS. Tout d'abord, les peptides ont été marqués avec les trois métaux lanthanides différents et un réactif fonctionnel - DOTA. Les résultats montrent que la réaction de transformation en dérivé à l'aide du réactif chélateur DOTA-NHS-ester a été efficace pour des peptides individuels et des mélanges de peptides, vérifiées à partir de la relation m/z obtenue par MALDI MS. L'application optimisée d'un complexe (Cytochrome C digest) a montré des résultats comparables à ceux obtenus avec des peptides modèles. En parallèle, nous avons effectué l'optimisation pour la purification de métalloprotéine dans la bile de poisson, qui est signalée entant que biomarqueurs de contamination métallique de l'environnement. Des procédures différentes (différents moments de centrifugation et différentes températures de traitement thermique) et les agents (DTT, β-mercaptoéthanol et TCEP) réduisant ont été apliqués pour purifier les MT isolées de la bile et du foie des poissons (Oreochromis niloticus). Des analyses spectrophotométriques ont été utilisées pour quantifier les échantillons de MT, et le gel SDS-PAGE a été utilisé pour évaluer qualitativement les différents résultats de la procédure. Chaque procédure a en suíte été évaluée statistiquement, une méhtode des surfaces de réponse a été appliquée. Les MT de la bile semblent être plus adéquate pour la surveillance de l'environnement en ce qui concerne l'exposition récente à des xénobiotiques qui peuvent influer sur l'expression protéomique et metalloproteomique de cette matrice biologique. Une procédure d'exposition à des métaux dans le laboratoire a montré que les métaux étaient significativement importante pour l'évaluation de la contamination à partir de la quantification de MT, selon le traitement de données par une techinique de réseau neural.

Mots clefs

Peptides; nano-HPLC-ICP-MS; MALDI MS; SDS-PAGE; MT; bile de poisson.

Abstract

Tenorio-Daussat, Carolina L.; Schaumlöffel, Dirk; Saint'Pierre, Tatiana D. Peptide labeling with metals using MS detection and optimization of metalloprotein extraction procedures in biological samples with proteomic purposes. Rio de Janeiro, 2014, 162 p. PhD Thesis – Departement of Chemistry, Pontifícia Universidade Católica do Rio de Janeiro and Institut des Sciences Analytiques et de Physico-Chimie pour L'Environnement et les Matériaux (IPREM), Université de Pau et des Pays de l'Adour.

This PhD thesis is a Cotutelle between the PUC-Rio (Brazil) and the UPPA (France).

This work developed a new method for the identification and quantification of peptides, by optimizing some of the available strategies suitable for labeling peptides with lanthanide metals with subsequent separation by nano-HPLC with UV detection, matrix-assisted laser desorption ionization-mass spectrometry (MALDI MS). First, peptides were labeled with the three different lanthanide metals using a functional DOTA-based reagent. The results demonstrate that the derivatization reaction using the chelating reagent DOTA-NHS-ester was effective for single peptides and peptide mixtures, verified from the m/z relation obtained by MALDI MS. The application of the optimized method in a more complex matrix (Cytochrome C digest) showed results comparable to those obtained with model peptides. In parallel, environmental analyses were conducted, by performing the standardization of metalloprotein purification in fish bile, since this matrix has been reported as a biomarker for environmental metal contamination. Different procedures (varying centrifugation times and heat-treatment temperatures) and reducing agents (DTT, β-mercaptoethanol and TCEP) were applied to purify MT isolated from fish (Oreochromis niloticus) bile and liver. Spectrophotometrical analyses were used to quantify the resulting MT samples, and SDS-PAGE gels were used to qualitatively assess the different procedure results. Each procedure was then statistically evaluated. A response surface methodology was applied for bile samples, in order to further evaluate the responses for this matrix. In an environmental context, biliary MT was lower than liver MT, and, bile MT seems to be more adequate in environmental monitoring scopes regarding recent exposure to xenobiotics that may affect the proteomic and metalloproteomic expression of this biological matrix. A procedure for exposure to metals in the laboratory showed that some metals are significantly important for the assessment of contamination from the quantification of MT, according to the data processing by atifical neural network (ANN).

Keywords

Peptides; nano-HPLC-ICP-MS; MALDI MS; SDS-PAGE; MT; fish bile.

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Acronyms List

1D SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis in 1 dimension
2D SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis in 2 dimensions
ACN	Acetronitrile
BSA	Bovine serum albumin
CE	Capillary electrophoresis
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOE	Design of experiments
DOTA	1,4,7,10-tetraazacyclodode- cane-1,4,7,10-tetraacetic acid
DTNB	5-(3-Carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid
DTPA	Diethylene triamine pentaacetic acid
DTPAA	Diethylenetriaminepentaacetic acid anhydride
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
ESI	Electrospray ionization
FIA	Flow injection analysis
FT-ICR	FourrierTtransform Ion Cyclotron Resonance
FT-IR	Fourrier Transformion Infra-red
GSH	Glutathione
HFBA	Heptafluorobutyric acid
ICP MS	Inductively coupled plasma mass spectrometry
MAL DOTA	Maleimidocysteineamido-DOTA
MALDI TOF MS	Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry
MMTS	S-methyl methanethiosulfonate
MT	Metalothionein
MW	Mass Weigth
NANO HPLC	Nano High Performance/Pressure Liquide Chromatography
NHS DOTA	N-terminus amino groups DOTA

PES	Protein Express Signature
RNA	Ribonucleic acid
TCEP	Tris(2-carboxyethyl)phosphine
TEAA	Triethylammonium acetate
TFA	Trifluoroethanoic acid
UV-VIS	Ultraviolet–visible
α-CHCA	α-cyano-4-hydroxycinnamic acid

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Contextualization

This thesis was developed in co-supervision between the Pontificia Catholic University of Rio de Janeiro (PUC-Rio) and Université de Pau et des Pays de l'Adour (UPPA).

Between 2011 and 2012, part of the work was developed in the city of Pau in France, under the supervision of Professor Dirk Schaumlöffel in his laboratory, IPREM-LCABIE, at the aforementioned university. The studies with complexation and derivatization of peptides with model lanthanide metals using the DOTA chelating reagent, was carried out basically using techniques nano-HPLC (DIONEX) and ICP-MS (Agilent), both belonging to LCABIE. The identification of peptides cited was conducted by a MALDI TOF MS in a partner laboratory under the supervision of Professor Andreas Tholey, located in the city of Kiel in Germany.

After the period "sandwich" the work continued in Brazil, in LABSPECTRO and Bioanalytical laboratories under the supervision of Professor Tatiana Saint'Pierre and co-supervision of Dr. Rachel Ann Hauser-Davis. Before the focus of the study was the derivatization and labeling of biomolecules with lanthanide metals and their identification from mass spectrometry, here in Brazil the goal was no longer the study of pseudo-metal biomolecules. The continuity of the work done in France was not possible in Brazil because there was no access to the same techniques used in France. This was also the main reason for granting the scholarship, a novel approach to study for Brazil.

Back in Brazil, molecules that are naturally linked to metals, providing to use them as potential biomarkers of environmental contamination, in addition, the possible choice of certain metals likewise be used as biomarkers were explored. Moreover, in this second stage of doctoral research, real samples, acquired from producers of fish, was used for the purpose of exposure to metals and study of possible changes in bile and liver of the fish studied, especially related to metallothionein, biomolecules that are severely induced by the environmental contamination with metals. For the analysis of metalloproteins and metal fish

studied were used techniques such as UV-Vis spectrophotometry (Hamilton), SEC-HPLC (Shimadzu), ICP-MS (PerkinElmer), FT-IR (PerkinElmer), ESI-MS (Waters), MALDI-MS (Bruker), and SDS-PAGE (GE). With the exception of the molecular mass spectrometric techniques, ESI and MALDI, all analyzes were performed at PUC-Rio. These other techniques were carried out in partnership with the Universities of Campinas (UNICAMP) and of Espírito Santo (UFES), respectively.

1. Introduction

1.1. Proteomics and metallomics

The genomics field aims to determine the entire DNA sequence of a certain organism. This area has contributed greatly to the better understanding of life in the last few decades. A limitation of this field, however, is that DNA sequencing data do not provide enough information regarding protein expression in a specific tissue or cell.[1; 2]

In the post-genomic era, the emergence of proteomics,[3] described as the separation, identification, and characterization of proteins present in a biological sample, is directly related to the need to investigate the control of gene expression and its impact on cellular metabolism. In this regard, proteomic studies can generate important information, such as which proteins are expressed[4], their expression levels, when they are expressed, the presence of post-translational modifications, protein responses expressed by cells in different conditions or experimental treatments and molecular differences between strains of cells and gene interactions.

The term 'proteome' was first used by Wasinger *et al.* in 1995, [5] meaning the total protein complement expressed by a genome. The proteome of a cell or a body fluid is denominated as the population of complete set of proteins being at a certain time point and under determined conditions within biological matrices. In opposition to the genome, the proteome varies from cell to cell and is very dynamic, since proteins are constantly synthesized or degraded in the biological environment, and, thus the composition of the proteome is highly flexible, meaning that the presence of particular proteins, as well as the amount of a

specific protein that can be altered depending on the biological state of the organism, is also flexible.[6]

Proteome investigations represent a new way to understand metabolic processes and cell machinery. Proteins have many functions within the cell, including gene regulation, signal relaying within and between cells, and metabolic process regulation. The knowledge of the proteome composition, as well as of the in-depth characterization of isolated proteins and the identification of proteins with altered expression profiles has a great impact on a variety of applications in medical, pharmaceutical and environmental research, for example:

- Development of novel cell types (*e.g.* bacteria or eukaryotic cells) which can be used for the sustainable bioproduction of high quality chemicals;

- Identification of potential targets in cells for the development of novel drugs;

- Identification of novel biomarkers for the early detection of diseases and the development of strategies for their treatment;

- Identification of biomarkers for protection of environment.

Furthermore, the protein structure (amino acid sequence) encoded in the genome can be altered by the covalent attachment of other present chemical groups to diverse chemical functionalities in the amino acid sequence - posttranslational modifications, which further alter protein properties in the proteome, allowing for distinct regulation of biological processes within cells. Thus, the knowledge regarding the proteins present in a proteome (identification), the amounts of a particular protein within this mixture in different biological conditions, *e.g.* healthy and diseased states (relative or absolute quantification), and the knowledge on the presence of posttranslational modifications (characterization) is the basis for the understanding of the fundamental processes of life. However, the acquisition of this knowledge is interfered by divers factors: (i) the proteome is usually a very complex mixture of hundreds up to hundreds of thousands of proteins; (ii) protein expression is characterized by a very high dynamic range, ranging from only a few protein copies per cell to hundreds of thousands of copies; (iii) the analysis of regulatory proteins (which are frequently present in low abundance) alongside proteins present in high abundance is very challenging.

With the advancement of genomic and proteomic studies, the role of metals in the various functions of proteins and enzymes has been highlighted. To discover the behavior and functions of the complexes formed by metals with biomolecules, such as proteins, has been, increasingly, a challenge of interest for various fields of science. The study of set of biomolecules which include metalloenzymes and metalloproteins that bind or interact with metals in a cell, organism or tissue, is part of the relatively recent field named metallomics, [7; 8] and the set of metals present in a biological system, in their different ways, is defined as the metallome.[9;10] Metallomics is directly associated with genomics and proteomics, since the synthesis and metabolic functions of a wide range genes and proteins do not occur in the absence of metals,[7] and the identification and elucidation of the biochemical or physiological function of the metallome in a biological system are the research targets in this field. Thus, speciation analyses, the identification/quantification of one or more individual chemical species in a sample, for metallobiomolecules, can be referred to as metallomics, an analogy to the genomics and proteomics. In other words, metallomics can be considered as a subset of speciation analyses.[7; 8; 10;11]

1.2.
Protein and metalloprotein biomarkers applied to environmental biomonitoring studies

1.2.1.
Biomarkers

Biomarkers are biological changes that may be related to exposure to or the toxic effects of chemicals.[109] Biomarkers represent measures of biological changes that may indicate the presence of contaminants and provide a means of interpretation of environmental levels of pollutants, being of extreme importance in environmental biomonitoring studies.[110] This original definition was modified by Adams, specifically with aquatic organisms in mind. He included characteristics of organisms, populations, or communities that respond to changes in the environment in measurable ways.[111] Later, Depledge added behavioral

responses, latency and genetic diversity to this definition.[112] Based on these authors, three types of biomarkers were then proposed, in an attempt to classify organism responses as biomarkers of exposure, effect and susceptibility.[112] As more biomarkers have been characterized and identified, it has become apparent that this ternary definition has significant overlap, since some biomarkers can be used in each of these capacities. As a more recent review of the biomarker paradigm has been described, an effect resulting from stressor exposure may be defined as an early adaptive non-pathogenic event or as a more serious altered functional event, depending on the mechanism of action of the stressor, the toxicokinetics, Biomarkers of exposure and effect may, thus, often be combined into a single classification with susceptibility occurring along any stage.[113]

The main concept of the biomarker approach in the evaluation of adverse effects or stress is based on the hypothesis that the effects of these disturbances are typically expressed at lower levels of biological organization before they arise at higher levels, such as populations, communities or ecosystems.[111] These initial effects are observed primarily at the molecular level with the induction of cellular defense systems and may provide answers on organism adaptation after exposure to the contaminant. However, if these defense processes are flawed, harm can occur at higher levels, such as histological or tissue damage. If these processes are permanently affected or modified during vulnerable development periods, organism reproduction or even survival may be affected, leading to changes in the population and, possibly, at community organization.[114] Therefore, in biological processes, the effects at higher hierarchical levels are always preceded by earlier changes (Figure 1), providing signals recognized as biomarkers of effects on levels of posterior responses.[115]

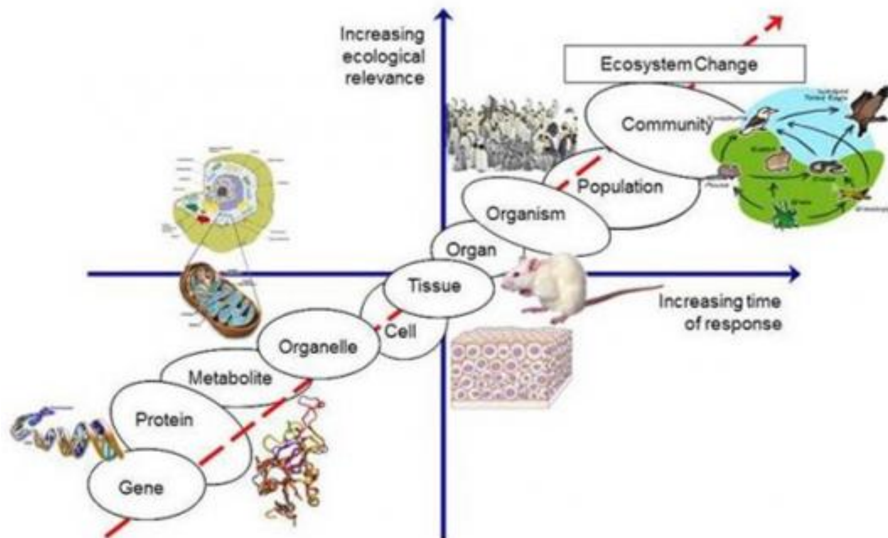


Figure 1. Scheme of the levels of responses of biological systems. From Bayne (1985).[115]

The study and the use of biomarkers often increases the possibility of identifying the cause of certain toxic effects and can also provide information regarding the bioavailability of pollutants and their potential environmental damage. However, these biomarkers can vary greatly in their specificity.[116] Such variations may reflect, for example, a biological response to external stimuli and / or contamination, resulting in different protein expressions or the redistribution of specific proteins within biological fluids or cells.[117; 118]

With the recent growth of genomics and proteomics-based new technologies the development and application of biomarkers has been the subject of intense research interest in this area. Proteomic techniques have allowed deeper investigations into environmental and biological issues, enabling the study of thousands of proteins. Until now, however, these techniques have benefited primarily well-characterized species such as humans, mice and yeast. However, in an environmental context, the species of interest is, many times, not well-characterized in proteomic or genomic aspects. Thus, new databases are of interest in proteomics, especially because these techniques are valuable in the identification of altered proteins after exposure to environmental pollutants, which may possibly come to be used as new and more comprehensive biomarkers,

since no prior knowledge regarding the biomarker relationship with pollutant toxic mechanisms is not necessary.[119]

1.2.2. Biomolecules in fish used as biomarkers in an environmental context

Proteomics applied to environmental ecotoxicology studies and environmental monitoring has as one of its goals to discover and identify novel candidate biomarkers of response to environmental contaminants.[119; 120] These biomarkers may be a set of genes or proteins expressed simultaneously, which could potentially provide information in several diverse areas, such as: (i) in characterizing functions of genes and gene products with similar profiles or regulatory mechanisms in common,[121] (ii) which can be used to classify compounds with similar modes of action, analyzing their toxicological "fingerprints",[122] and (iii) indicating different stress levels by integrating general and specific markers in a single assay. Thus, the use of proteomic techniques is becoming increasingly useful in the discovery of such biomarkers in the fields of ecotoxicology.[119; 123] Such studies can contribute greatly to the understanding of cellular responses to altered environmental conditions. The development of molecular and genetic biomarkers of increasing sensitivity may still further develop by revealing exposures that are presently unknown or merely speculated.[117]

Proteomics, thus, has been a tool increasingly used in the study and discovery of biomarkers in ecotoxicological and environmental contexts and thereby has contributed to the understanding of organism responses to changes in the environment.[123; 124; 125] This type of analysis allows the isolation of sets of proteins within the proteome that are specifically modified by different stressors, either biological, physical or chemical. These sets of responses to specific pollutants that modify protein expression are used as protein biomarkers and are termed "protein expression signatures" (PES),[126] and they not only allow the discovery of new protein biomarkers, but also provide insight into the hidden mechanisms of contaminant toxicity.

There is currently a growing number of studies applying a comparative proteomic 2-DE-based approach to marine pollution biomonitoring using fish as a model organisms in both laboratory and field experiments.[127] This has led to the establishment of "PES" following exposure to a variety of environmental contaminants including polychlorinated biphenyls (PCBs),[128] polyaromatic hydrocarbons (PAHs),[127] crude oil and metals.[129] Several research groups have pioneered the proteomic approach in environmental toxicology, but this field is still at a relatively early stage of its development.

Various biomolecules have been used as biomarkers of environmental exposure.[119] The antioxidant metalloenzymes superoxide dismutases, which bind to copper, iron, zinc, manganese and nickel are fairly well expressed in situations of oxidative stress in various organisms, including marine organisms [130]. Biotransformation enzymes, metallothioneins (MTs),[131;132;133] acetylcholinesterase (AChE) activity,[136;137] cytochrome P450 activities (responsible for the biotransformation of several xenobiotic compounds),[134] and morphological, haematological, histological and immunological parameters [139; 140] are also often used as measures of changes in organisms.

An interesting, biomolecule metallothionein (MT), has been extensively used in marine organisms as a biomarker of metal contamination. MT are proteins that bind to metals through cysteine residues present in high amount (approximately 30 % of MT amino acids) containing thiol groups, have molecular weight between 6 and 7 kDa, often appear in the form of dimers and are thermally stable.[135; 136] These molecules are capable of binding metal ions forming metal -thiol areas [137] and have an important role in cell regulation of metabolically important metals such as Cu and Zn. They also bind to non-essential elements such as Ag, Cd and Hg , thus presenting an additional role in reducing the toxicity of these metals in contaminated environments.[144; 145; 146; 147] The detoxification mechanism by these small molecules occurs through transcriptional activation of their genes by metals, leading to an increased synthesis of these proteins and their subsequent binding to free metals.[138]

Studies using MT for environmental monitory are abundant regarding the aquatic environment and are usually conducted by the analyses of liver tissue [139; 140], although studies using muscle,[141; 142] kidney [143; 144] and gills also exist.[145; 146] Liver measurements, however, are still the most employed, since this is the main

detoxifying organ of the body and is a validated organ regarding exposure to environmental contaminants.[147;148;149;150] An alternative way to evaluate contaminant effects on the proteomic or metalloproteomic of fish in environmental monitoring studies has been proposed, by using fish bile.[18; 151]

1.2.3. Proteomics in fish biomarker identification

In the last decade, proteomic technologies have been increasingly used in fish biology research. These organisms are good models for this type of study, because they live in different environments and must adapt to environmental parameters and different stresses, which can usually be easily reproduced under controlled laboratory conditions, if necessary.[152] Fish are recognized as bioindicators of environmental change, including environmental contamination by different compounds.[164; 165; 166] Moreover, they are also important links between the environment, possible contaminants and human populations through aquaculture and consumption.[164; 165] In view of this, studies of exposure to environmental contaminants and their subsequent responses are of great relevance.

Proteomics have been applied primarily to investigate the physiology, developmental biology and impact of contaminants in these organisms, with global analyses of cellular signaling routes involved in physiological mechanisms related to growth, reproduction, disease and stress (including stress linked to environmental contamination). Studies as a way to quickly identify new proteins and homologs of known proteins that play important role in other animal groups have also been also conducted. However, the lack of genetic information for most fish species has been a big problem for a more general application of the currently proteomic technologies available.[153]

Several studies focusing on proteomics in fish have been published, such as the study by Martin et al. regarding the effects of lack of food on the protein profiles of trout livers compared with control animals, in which differentially expressed proteins were observed.[154] More recently, Papakostas performed a

proteomic study investigating the differences in the proteome of the growth phase of salmonids, investigating development as a fundamental aspect of the biology of this species and showed that this can be affected by environmental parameters.[155] Other studies using proteomic techniques aimed at the exposure of organisms to contaminants in the laboratory, providing information about possible reactions of certain contaminants and suggesting new biomarkers.[156; 157]

In this context, an interesting study examined the exposure of rainbow trout to sublethal doses of cadmium, carbon tetrachloride and pyrene and β-naphthoflavone for some days, and verified that the redox condition and metabolism of oxidative species was affected in the fish, in addition to increased expression of antioxidant and a large number of proteins involved in oxidative stress, the latter, particularly by β-naphthoflavone.[156] Another study with rainbow trout exposed to sublethal doses of zinc found observed the induction of the expression of the beta-chain of C3-1 protein, which plays an important role in immune response and imunoregulatory functions,[158] which may indicate that the induction of this protein by the metal in question has a stimulating effect on the immune system of this species.

The approach to the analysis of differential proteomic expression in complex field situations has also been studied. This approach can pinpoint which genes, proteins or metabolites are more interesting to study in the laboratory, based on the answers found in the real environment.[157] Field studies in proteomics are still scarce, however some studies have been published outlining the differences in gene expression of sampled fish from contaminated and uncontaminated estuaries in eastern England and metabolic differences and protein in the liver of sole with and without tumors.[159] In this study, 56 proteins were up-regulated and 20 down-regulated in tumor tissues, and 12 of these proteins exhibit the potential to act as biomarkers relative to neoplastic lesions. Some recognized environmental contaminants such as cyanotoxins, specifically microcystins, have also been studied in the context of fish proteomics. One study identified 17 differentially expressed proteins in the livers of fish exposed to these toxins for just two hours, including proteins involved in cellular structures, signal transduction within cells, regulation of enzymes and oxidative stress, such as methyltransferases and B - tubulin, among others.[160] These altered proteins corroborate studies on the toxic effects of these contaminants and their modes of action, e.g., causing cell disruption.

These studies dealt with non-model organisms, making a connection between traditional and emerging proteomic studies within the field of ecotoxicology. Using sampled environmental organisms, attempting to discover biomarkers in environmental sentinels based on "omics" techniques, these types of research have the potential to be applied as rapid screening methods for disease classification and may in the end provide a mechanistic understanding of the effects of environmental stressors on the health of exposed organisms. Observing these different examples, it is clear that protein modifications can useful markers in environmental exposure to contaminants, and opportunities to study protein modifications are increasingly becoming important in ecotoxicological studies and environmental monitoring programs.[161]

1.2.4.
Bile as a bioindicator matrix

This biological matrix is a validated bioindicator regarding environmental contaminants, since it also excretes exogenous substances from blood and liver that were not excreted by the kidneys, such as metals and several organic compounds.[18; 19; 151; 162]

It is known that some environmental contaminants lead to morphological changes in various organs, especially the liver. Based on this, Morozov noted that a change in bile is also possible to be found.[163] Based on the aforementioned approaches, it is important to analyze this matrix as a potential bioindicator of environmental contamination, both in relation to the presence of metals and in the proteomic characterization of aspects that may possibly identify proteins of interest in an environmental context.

Bile or bile juice is a fluid made by the liver and stored in the gallbladder, and operates mainly on fat digestion (through the action of pancreatic lipase, an enzyme produced by the pancreas), in certain microorganisms to avoid decomposition of some foods and on the absorption of nutrients in the diet to pass through the intestine, besides also acting as a means of excretion of endogenous and exogenous substances.[164] Environmental studies have shown that many chemicals foreign to the body are excreted from the liver into fish bile

and then expelled from the body, such as the hydrocarbons and their metabolites.[165; 166; 167; 168] Bile is also a route for drug elimination. The factors influencing such elimination include chemical structure, molecular size and polarity, as well as the characteristics of the liver, such as specific transport active sites in the membranes of liver cells.[169; 170]

Studies also indicate that bile is also an excretion route for metals.[182; 183; 184] In laboratory studies with fish rainbow trout (*Oncorhynchus mykiss*) Cu, Hg and Pb were excreted in bile,[171] indicating that bil is the preferred route of excretion for Cu has, according to Grossel *et al.*[172] In the case of other elements such as zinc, excretion occurs equally either through the intestine or bile.[173] Ballatori shows bile as a route for disposal and transport of mercury, also demonstrating a close relationship of this metal to GSH present in bile.[174]

Studies with human bile on the other hand have identified several differentially expressed proteins in healthy patients and patients with tumors of the biliary tract such as cholangiocarcinoma, cholestasis (reduction or interruption of the flow of bile) and biliary stenosis.[175] Interestingly, studies regarding bile as a potential biomarker for contaminants are still very little explored. With the exception of the human species, there are few or almost no reports about bile in a pathologic context as a disease biomarker, or on identification of differentially expressed proteins in different environmental situations.

1.3.
Analytical techniques used in proteomic and metallomic studies

Analytical chemistry methods have an immense heaviness on approximately all fields of biomedical, biochemical and biotechnological research and are, therefore, indispensable for both the generation of fundamental knowledge as well as for the opening of novel applications in various fields of life sciences. Despite significant methodological and technical improvements achieved in the past, there is still a tremendous need for improvement of established methods as well as for the introduction of alternative approaches. In particular, protein analytics, both the analytics of single proteins (classical protein analytics) and the investigation of complex protein mixtures as found in living

cells (proteomics) is still a great challenge for bioanalytics.[12] Several analytical techniques have been extensively applied in this area, especially in protein quantification. Before applying analytical techniques to this problem, however, adequate sample preparation must be conducted.

1.3.1. Sample preparation for proteomic and metallomic analyses

With the increase in the technological advances in proteomic techniques, sample preparation procedures have become a fundamental and critical step in order to obtain relevant data.[13] Due to the great variety of protein present in biological samples, the optimum sample preparation procedure for a given sample must be determined empirically. Ideally, the process should result in the complete denaturation, reduction and dissolution of the proteins in the sample. Some additional steps can also be used to further better sample quality. Considerations of this kind are crucial to obtain relevant results from proteomic experiments, and some specialists propose that the proteomic field has become limited due to lack of significant advances in sample preparation techniques.[14] Kozuka-Hata suggests an experimental flow (figure 2) for advance in proteomic studies by mass spectrometry from two standard methodologies used as sample preparation steps.[15]

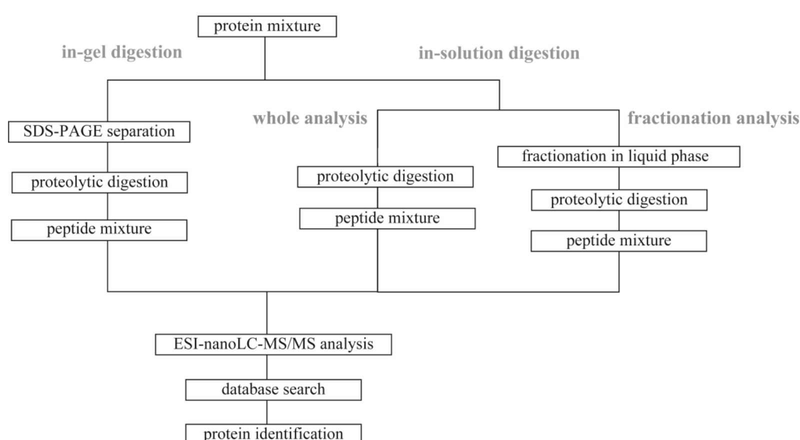


Figure 2. Scheme of experimental workflow for mass spectrometry-based proteomics. Adapted from Kozuka-Hata (2013).[15]

Basically, two major strategies for converting proteins extracted from biological material to peptides suitable for mass spectrometry (MS)-based proteome analysis are available. The first involves protein solubilization with detergents, their separation by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and the digestion of the gel-trapped proteins ('in-gel' digestion).[13; 16] The second method uses no detergents, comprising protein extraction with strong chaotropic reagents (i.e, urea and thiourea), protein precipitation and their digestion under denaturing conditions ('in-solution' digestion). This second approach can be followed by two-dimensional peptide separation, for example, in the multidimensional protein identification technology strategy.[17]

The great heterogeneity of proteins and interfering contaminants makes the global protein extraction, dissolution and simultaneous release of all proteins a major challenge for any sample source. The incorporation of membrane proteins and the formation of complexes with other proteins or nucleic acids complicate the process significantly. Minimizing these effects by optimizing the extraction protocol is a challenge that usually has to be repeated for each new sample source and analytical purposes. Several studies regarding sample "clean-up" for biological matrices have been reported, that applied ultra-centrifugation, adding commercial solutions for lipid removal, filtration for salt removal, protein precipitation and fractionation by adding different solvents such as acetone and dichloromethane for the removal of proteins present in such high quantitates that they may be considered an interferent,, such as albumin.[18; 19]

Basically, sample preparation for proteomic studies is composed of three steps: (i) contaminant removal or inactivation. This can be performed by using protease inhibitors, by conducting salt removal by dialysis, gel filtration or precipitation and by conducting the removal of nucleic acids, which increase the viscosity of the sample and can cause spots, besides obstructing gel pores. Recently, Wisniewski *et al.*[13] conducted a novel approach in detergent removal from membrane proteins by using a filter assisted sample preparation technique, where the use of strong detergents is used to "clean" the proteome; (ii) enrichment of the peptides of interest (modified or not), and, (iii) sample pre-fractioning through chromatography and/or electrophoresis.[20; 21; 22] The sample pH must be carefully monitored, heat must be avoided (especially in samples containing urea) and care to avoid proteolytic degradation is paramount (using

proteinase inhibitors or working on ice).[23] Protein dissolution is also critical, since possible interferences by unwanted protein precipitation may occur.[24]

1.3.2. Analytical methods used in protein quantification and characterization

Protein identification and quantification can be achieved either in the level of proteolytic peptides (shotgun or bottom-up approach) or in the level of intact proteins (top-down approach). In bottom-up approaches, protein identification relies on peptide fragment fingerprinting, in other words, the analysis of the MS/MS spectra of the peptides. In top-down approaches, after the separation of intact proteins, they can be identified either after proteolytic digestion by peptide mass fingerprinting analysis or by a combination of the latter with MS/MS data.[25; 26] Stimulated by the establishment of novel experimental and (bio)informatics approaches, such as the development of soft ionization techniques for molecular mass spectrometry (MS) (i.e. electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) for the analysis of biopolymers, peptide sequencing by tandem mass spectrometry (MS/MS), the development of database driven identification of proteins, differential two dimensional gel electrophoresis (DIGE) and multidimensional chromatographic separations of complex peptide and protein mixtures),[27] significant progress has now been achieved in the field of protein identification as well as in the qualitative analysis of posttranslational modifications. The acquisition of this type of data is also possible by using hyphenated (coupled) techniques. These techniques combine a high resolution separation technique with sensitive element- or molecule-specific detection.[28]

Despite the progress made in different fields of protein and proteome analytics in the past, there is still the need for further improvement of these techniques. Both for classical protein analytics and for proteomics, protein quantification and the quantification of the degree of possible posttranslational modifications [27] is still a challenge: quantification, both absolute and relative (differential), is still characterized by low accuracies; furthermore, in most

methods, the number of samples analyzed by multiplexing is still restricted. Thus, the development of novel approaches for the separation of proteins on level of intact proteins, the development of novel separation schemes for separation in shotgun proteomics and novel instrumental improvements on the side of mass spectrometry bear significant potential for further increase of confidence of identification and for the sensitivity, which is necessary for the coverage of complex proteomes. In fact, integrated elemental and molecular MS approaches could eventually prove to be the most useful in the field of proteomics.[29]

Up to now, most of the available quantitative proteomics research is based on relative approaches, this means comparing protein expression between two (or more) organism states.[30] Relative quantification of proteins based on imaging techniques (e.g. after protein labelling with dyes, fluorophores or radioactive substances) have good general performance, but require high resolution protein separations (typically using 2D gels) and do not give information regarding the identity of the measured protein. On the other hand, MS techniques may overcome such limitations, and so MS derived techniques have been increasingly used for this purpose. The major steps of these analyses involve sample preparation, the application of well-suited separation methods, mass spectrometric identification, characterization and quantification followed by data analysis and interpretation with bioinformatics tools.[13; 20; 31]

Mass spectrometry (MS) has experienced consistent instrumental improvements. Due to these important developments and their associated applications the current role and potential of MS is huge for proteomics-based investigations.[32] In fact, MS today is not just an established tool for structural proteomics research, but it is more than ever at the the first stage of functional proteomics.[33] So far, however, many efforts launched to proteomics have focused on developing methodologies that promote the efficient identification (and perhaps structural characterization) of a large number of proteins, while quantification studies are, so far, comparatively few.[32]

For the differential quantification of protein expression in two or more biological samples, labelling with stable isotopes can be used.[34; 35] The labelled peptide (or proteins) in most cases share equal or, at least, similar physicochemical properties, allowing for a simultaneous separation of differentially labelled proteomes. The peptides originating from the different

biological states can be distinguished by their different masses in MS analysis; their ratio, and hence the ratio of the proteins in the biological matrix to be investigated, can be read out by comparison of the corresponding signal intensities of the light and heavy isotope signals in the MS spectra. In other approaches, *e.g.* iTRAQ,[36] relative quantification can be performed by using signals of reporter ions derived upon release from the labelling reagent in MS/MS analysis. This method also shows the potential for a medium multiplexing capacity (an assay that simultaneously measures multiple analytes - dozens or more - in a single run). The introduction of (isotope) labels in the peptides or proteins can be achieved either by chemical modification,[37] by means of enzymatic methods,[38] or by methods allowing for an *in vivo* labelling, as SILAC (Stable Isotope Labeling by/with amino acids in cell culture)[39] or proteome metabolic labelling.[40] The latter approaches are restricted to organisms allowing for supply with labelled precursors; thus the application for human samples is restricted; furthermore, these methods provide only minor capacities for multiplexing. First approaches for label free quantifications (*e.g.* by spectral counting, by Liu *et al.*)[41] have been introduced very recently.

Regarding metallomic studies, there is a significant difference in the number of discoveries in this field when compared to strictly metallomic studies. The late advent of this field is due to several singular questions that should be considered when analyzing metalloproteins. These include the absence of any polymerase chain reaction similar to PCR in the genomics field, the occurrence of post-translational changes and, finally, the low concentration of the trace-elements present in biological tissues (usually $< 50\ \mu g\ g^{-1}$) and the high complexity of these matrices.[42] These factors mean that the analyses of metals bonded to biomolecules is very difficult and challenging. Because of this, metallomic analyses require sophisticated multidimensional analytical approaches. The continuous development of techniques that mix atomic spectrometry and biochemical or proteomic techniques, such as gel electrophoresis, capillary chromatography and multidimensional nanoflow, and the development of strategies for the complementary application of elements and specific techniques for detecting molecules, have led to new possibilities in this field of research.[43] For example, mass spectrometry with approached such as coupling with inductively coupled plasma (ICP-MS), electrospray (ESI-MS) and laser assisted ionization and desorption (MALDI-MS) are now routinely used,

alongside 1 or 2D protein separation by gel electrophoresis, in order to quickly and accurately identify the metalloid component of individual proteins. By applying these techniques, large volumes of data can be collected.[44]

Element mass spectrometry with inductively coupled plasma ionization (ICP-MS) is a very interesting alternative for absolute peptide and protein quantification in metallomics. This technique is highly sensitive, allows for multielement and multi-isotope detection, has a large dynamic range, and the ionization process is almost compound- and matrix independent.

The chemical derivatization of several types of biomolecules with different functional groups, isotopes, metals, or radionuclides has been a widely used in bioanalysis, allowing for sensitive and specific detection of the analytes.[45; 46; 47] Within this field an emerging new trend is the labelling of biomolecules, such as peptides and proteins which do not naturally contain an ICP-MS-detectable element, with heteroatom tags in order to make them visible and quantifiable by ICP-MS.[48;49] In 1992, the derivatization of sulfhydryl groups with p-chloromercuribenzoate was described. With the view to quantify cysteine residues in metallothionein via the ^{202}Hg signal in HPLC-ICP-MS.[50] For ultra-sensitive detection, the most suitable elements are metals, such as lanthanides, with high ionization efficiency, low background and no ICP-MS interferences, as recently described by Ahrends and co-workers.[51] Since a direct covalent bond of a lanthanide to a protein is not possible, metal complexes with strong chelators, such as DOTA derivatives or DTPA, can be alternatively used as tags binding to functional groups such as amino or sulfhydryl groups in peptides and proteins.[52; 53; 54] Lanthanide-DOTA tags have been recently applied to quantify entire proteins in flow injection (FIA) ICP-MS,[51] and first results on tagged peptide and protein analyses with LC-ICP-MS are available.[55] Most importantly, they showed complete tagging of all sulfhydryl groups even in complex protein mixtures when the reagent was applied in an excess of 20 times. Amino groups in peptides can be also derivatized with DTPA, in a reaction with its bicyclic anhydride (DTPAA).[52; 56] Other recent preliminary study used lanthanide-DTPA tags and ICP-MS for absolute peptide quantification, which requires a highly specific, efficient and ideally complete, reaction.[57]

1.3.2.1.

Total metal determination by ICP-MS and protein-bound metal determination by hyphenated systems coupled to ICP-MS detection

Inductively coupled plasma mass spectrometry (ICP-MS) was developed in the 1980s. This technique combine the easy sample introduction and rapid sample analysis of the ICP with the accurate and low limits of detection of a quadrupole mass spectrometer, thus obtaining an isotopic and elemental analyzer in a single instrument. The resulting instrument is capable of performing multielemental analysis, often at the ng L^{-1} level.[58]

The ICP-MS technique can accept mainly liquid samples, but solid samples can be introduced into the ICP through any specific accessory. Aqueous samples are usually introduced pneumatically by means of a peristaltic pump that takes the sample to a nebulizer operated with argon at high speed, forming a fine mist. The aerosol then passes into a mist chamber where larger droplets are removed through a drain.[59] Typically, only 2% of the original solution is aspirated through the spray chamber.[60] This process is necessary to produce droplets that are small enough to be vaporized in the plasma torch.

Once the sample solution passes through the nebulizer and is partially desolvated, the aerosol in movement towards the torch blends with more argon gas and reaches the plasma. The initial plasma is formed by ionization of argon, then occurs the transfer energy to this gas, partially ionized, through a coupling coil, which is used for transmission of radio frequency, in order to heat it.[59] The hot plasma removes any solvent and causes the atomization of the sample, followed by excitation and ionization.

The atomization/ionization occurs at atmospheric pressure; therefore, the interface between the ICP and the MS becomes crucial to create a vacuum environment for the MS system. The ions pass through a small hole in a vacuum system, where a supersonic jet is formed and the ions are then passed to the MS system at high speeds, expanding in the vacuum system.[59] The vacuum is needed so that the ions are free to move without collision with the air molecules. In the first stage of MS, the ions are removed from the plasma by an extraction

pump system. An ion beam is produced and focused and then taken to the mass separator and subsequently to the detector.[61]

The use of ICP-MS has increased significantly within the area of proteomics. One of advantages of the ICP is its capability to generate, as an ion source, monoatomic positive ions from most elements, besides being multielemental. Thus the elements can be identified based on their atomic mass spectra without the isotope peaks that complicate the mass spectra of the parent ions of biopolymers. Another advantage is the independence of the signal intensity of the molecular environment of each element. This allows the possibility of using inorganic elemental standards for the protein analysis.[62]

As the ICP-MS detector information is elemental, only the heteroatoms are in fact measured, while the interest may lie in the molecules (e.g. peptides). Thus, for proteomic and metallomic analyses, the elemental detector should be coupled to a previous separation (e.g. HPLC, capillary electrophoresis (CE) or 2D gel electrophoresis) of the element-containing biomolecule. Such instrumental approaches, mainly HPLC-ICP-MS, have been widely developed in the last decade within the particular field of trace element speciation in biological materials.[63; 64] In this context, several elements can be identified in the same chromatographic peak. In HPLC-ICP-MS approaches all peptides relevant to quantification must be separated before entering the MS, because all structural information is lost in the plasma, on like molecular MS. Consequently, separation of the biomolecules and information on their stoichiometry obtained by parallel molecular MS is mandatory to accurately quantify them via the element signal.

The increasing use of ICP-MS for biochemical applications has been inseparably linked with progress in three major areas. This included (i) advances in non-metal detection, (ii) increase in detection limits of ICP mass spectrometers allowing the coupling of capillary and planar electrophoretic techniques, and (iii) easier access to stable metal isotopes.[11]

The current state of the art of these techniques for the bio-inorganic speciation analysis is shown in Figure 3.

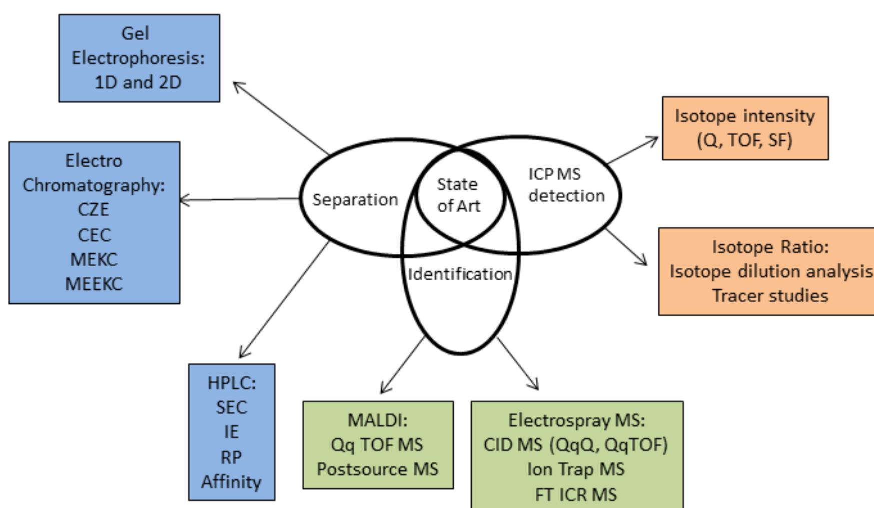


Figure 3. Scheme of hyphenated systems using ICP-MS detection.

For most elements, ICP-MS is a routine detection technique in chromatography and has been discussed elsewhere.[28; 62; 65] However, some biologically important elements, such as sulfur and phosphorus, are not efficiently ionized in the ICP. They also suffer from several polyatomic interferences. The problem of the low ionization efficiency also happens with halogens, especially fluorine. Also, some vital transition elements, such as iron or vanadium, show interferences by polyatomic ions [66]. Polyatomic interferences can be removed by using a high resolution mass spectrometer or a collision/ reaction cell.[67] The latter offers two approaches to the resolution of interferences: (i) by collision/ reaction of the interfering ions with gases such as H_2, He or Xe, or (ii) by reaction of the ion of interest with oxygen.[68]

1.3.2.2. Ultraviolet-visible molecular absorption and its applications in proteomics

Ultraviolet-visible molecular absorption spectroscopy is routinely used as an auxiliary technique for the characterization and identification of the proteome and metallome. This is a simple and quick method, indicated for the

determination of naturally colored analytes, or those that acquire color adjusted by the use of derivatization, absorbing light at certain wavelengths which can then be analyzed by a spectrophotometer.[69]

Molecular absorption spectrophotometry involves the energy absorbed in the ultraviolet-visible (UV-Vis) region by promoting the transition of valence electrons. This technique is directly related to the Lambert-Beer law, an empirical relationship that relates the optical absorption of light to the properties of the solution which it traverses. This law says that there is an exponential relationship between the light transmission through a substance and the concentration of said substance, as well as between the transmission and the length of the body that the light passes through. The concentration (c) of the substance can be deduced from the amount of light transmitted, through the equation $A = \varepsilon\, l\, c$, where A is the absorbance, ε is the molar absorptivity of the species and l is the optical path traveled by the light. Since protein molecules are able to absorb energy, the quantification of protein concentrations in solutions can be performed using this colorimetric technique.[70]

Some colorimetric methods commonly used in protein quantification are:

- The Biuret method (sensitivity - 0.5 to 10 mg mL^{-1})

The reagent of biuret is a compound formed by heating urea at 180 °C. When it is placed in the presence of a copper sulfate solution in an alkaline medium a blue compound is formed. The color is due to the formation of a complex between cupric ions and four adjacent nitrogen atoms. This type of reaction also occurs with peptides containing at least two peptide bonds and with proteins in general. Substances containing two carbonyl groups attached directly or via a nitrogen atom also appear blue in the copper sulfate alkaline solution. The colored product of the reaction has maximum absorption at 540 nm.[71; 72]

- Folin-Lowry method (sensitivity - 0.1 to 0.3 mg mL^{-1})

In alkaline conditions a divalent copper ion is capable of forming a complex with peptide bonds and is reduced to a monovalent ion (the biuret reaction). The monovalent copper ion alongside some the lateral chains of some protein amino acids (tyrosine, tryptophan, cysteine, histidine and asparagine) lead to the reduction of acid components present in the Folin reagent, amplifying

the color first obtained by the biuret reaction. In this method, the maximum absorption occurs at 650-750 nm.[73]

- BCA method (sensitivity - 0.1 to 0.5 mg mL^{-1})

This method is also known as bicinchoninic acid method. This procedure is applied for analysis in microplates. Unlike the Folin-Lowry colorimetric reagent, bicinchoninic acid is more stable under alkaline conditions.[74] BCA follows the principle of the Folin-Lowry assay, reacting with the complexes between copper ions and the peptides to produce a purple color which absorbs strongly at 562 nm. One advantage is that, as the BCA reagent is more stable under alkaline conditions, it can be added to the copper solution to allow a one-step procedure, making it faster than the Folin-Lowry technique.[75; 76]

- Bradford method (sensitivity – 0.06 to 0.3 mg mL^{-1})

When at acidic pH, the anionic dye Coomassie Blue forms complexes with proteins which contain basic and/or aromatic amino acids. The interaction between the protein and the dye causes the change in the wavelength of maximum absorption of the dye (465 nm - free dye) to 595 nm (protein complexed dye).[77]

- Ellman's reaction for the determination of free thiols (sensitivity – 50 to 1000 μmol L^{-1})

It works well for small peptides and proteins synthesized using standard solid phase synthetic methods. Peptides from these syntheses are usually in their reduced form, and are usually stable to oxidation in acidic solutions. Free thiol can be determined in solutions collected from chromatographic separations or from reconstituted lyophilized samples. This protocol has been used for peptides with a single Cys residue present and lacking tryptophan. The technique should be feasible for multiple Cys residues.[236]

There is, therefore, no universal quantification method. It is recommended to set the method to be used taking into account (i) the sensitivity required (dependent on protein concentration), (ii) the presence/absence of certain amino acids, because of the operating mechanism, i.e specificity, of each method, (iii) the nature and concentration of non-protein substances (potential interferents)

(iv) protein solubility under the method conditions (v) ease and reproducibility; (vi) speed and cost.

The choice for using spectrophotometric quantification is considered more adequate than other available techniques when the aim is to screen several environmental samples at a time, since it is, for example, quicker than voltammetric analyses and is also simpler and less costly. Because of this, this technique has been used for several decades as a simple quantification tool for MT analyses in ecotoxicological and environmental monitoring studies.[183; 184]

1.3.2.3.
One- and two-dimensional gel electrophoresis

There is still no technique able to separate all the proteins of an organism. A widely used technique with efficient resolution is one- and two-dimensional gel electrophoresis. Gel electrophoresis has several advantages, such as being cheap, and, especially robust against impurities, that interfere, mainly, in protein digestion. In-solution digestion is easily automated and minimizes sample processing, but the proteome may not be completely solubilized and digestion may be impeded by interfering substances, making gel electrophoresis an interesting alternative.[17]

The polyacrylamide gel, introduced in 1959, is the most commonly used matrix in electrophoresis to separate the proteins of a biological sample. The gel is a matrix consisting of acrylamide cross-linked with N, N-methyl bisacrylamide. Gel porosity can be chosen, to better adapt to the sample to be separated. The usual percentages are 5%, 7.5%, 10%, 12.5% and 15%. The higher acrylamide concentration, the smaller the pores of the resulting gel. Ammonium persulfate is used to generate free radicals and tetramethylethylenediamine (TEMED) is the catalyst that assists in the transfer of the electron of the free radical. The use of SDS in the gel system (SDS-PAGE) is so that the separation depends only on the molecular weight of the protein, rather than its form and native charge. SDS-PAGE was first described by Laemmli in 1970.[78] SDS is an anionic detergent which interacts with the peptide chains of the protein, denaturing them and

forming negatively charged SDS-protein complexes (Figure 4). By applying an electrical current, all proteins, now with the same charge, migrate toward the positive electrode and are separated only by differences in their molar masses. The smallest proteins migrate more quickly, while larger proteins find it more difficult to pass through the gel mesh and, thus, move more slowly. SDS-PAGE electrophoresis is a robust technique that has a wide range of application.[79] It is nowadays mainly used routinely to (i) estimate protein size, (ii) establish protein purity, (iii) quantify proteins, (iv) compare protein content of different samples; (v) analyze the size and number of protein subunits.[20; 24]

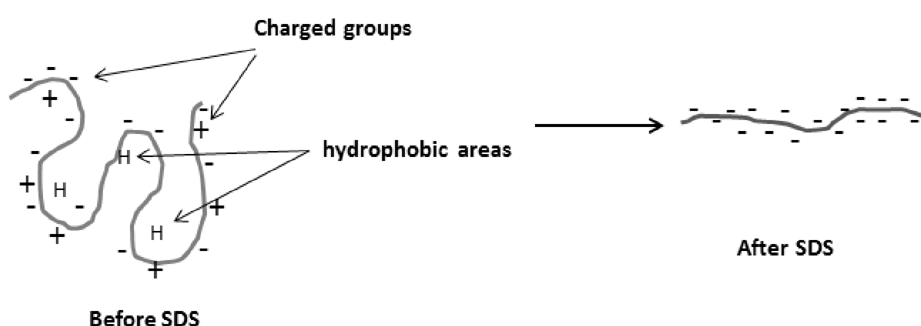


Figure 4. Scheme of protein modification with SDS in gel electrophoresis.

One-dimensional (1D) SDS gel electrophoresis in denaturing conditions (1D SDS-PAGE) consists in the migration of the solubilized proteins in a discontinuous electrophoresis system consisting of two polyacrylamide gels with different porosities and buffered at different pH, see Figure 5. The extracted proteins are solubilized, heated to 100 °C in a buffered Tris- HCl solution, pH 6.8, containing excess SDS and a reducing agent of the thiol group generally β-mercaptoethanol or DTT. SDS charged the proteins negatively and denatures them, aided by warming around the polypeptide backbone. The reducing agent reduces the disulfide bridges of the proteins that will thus unroll into the individual polypeptide subunits. Gels are bonded and polymerized in a vertical system - sandwich - between two glass plates. The sample is placed in the first gel (stacking gel) in a buffered Tris-HCl pH 6.8 solution, with wider crosslinking. Under these conditions, the presence of tricine in the buffer solution will allow protein concentration in the stacking gel which will then be separated according

to their molecular weights in the separation gel, buffered at pH 8.8 with much smaller cross-linking. pH 8.8 leads to an increase in the speed of migration of tricine ions, which will then migrate faster than the proteins, which are then not entrapped in a thin layer and are now separated according to their size by migration towards the positive electrode and, at the end of the run, they may be compared to a standard solution of known molecular weight.

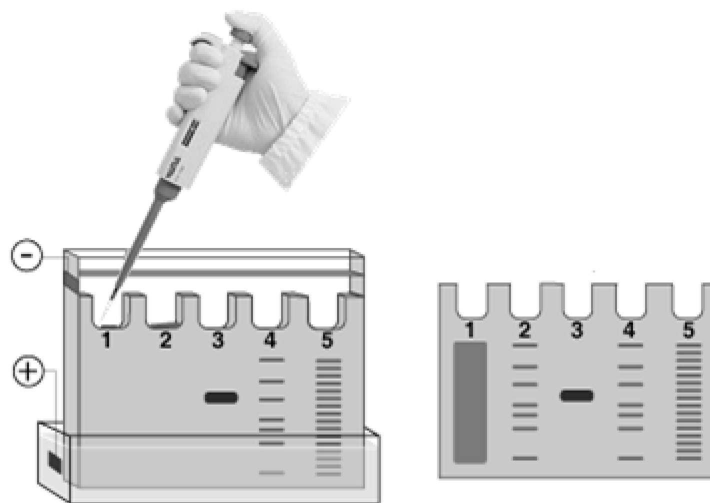


Figure 5. Example schematic of protein bands separated by 1D electrophoresis, where each trace in numerical columns represent protein bands of decreasing molecular weight.

Two-dimensional gel electrophoresis in denaturing conditions (2D SDS-PAGE) was introduced in the 1970s and perfected by O'Farrell.[80] Today this technique allows the resolution of up to 5000 proteins and continues to be the more direct way to map the proteome of an organism.[81; 82] In this technique, proteins are separated by two steps, through two of their physicochemical properties, isoelectric point (pI) and molecular weight (MW) (Figure 6). To separate proteins by pI, they must first pass through isoelectric focusing (IEF). A pH gradient, in which the charged proteins move to their pI, where no charge is present, is formed. Prior to the development of commercially available immobilized pH gradients backed on plastic strips, two-dimensional electrophoresis was performed using a discontinuous system, which did not allow for high reproducibility of IEF gels. With the development of these immobilized gradients, electrophoresis became extremely reproducible, and is still the most

used to perform protein separation. After IEF, the strips are equilibrated in a solution DTT-containing to break the disulfide bridges (SS bonds) present in proteins, with SDS facilitating access to their inner parts, due to the elimination of protein tertiary structure, and iodoacetamide to prevent reoxidation of thiol groups (alkylation). After IEF, the proteins are separated on SDS-PAGE gels in the same manner as 1D SDS-PAGE.

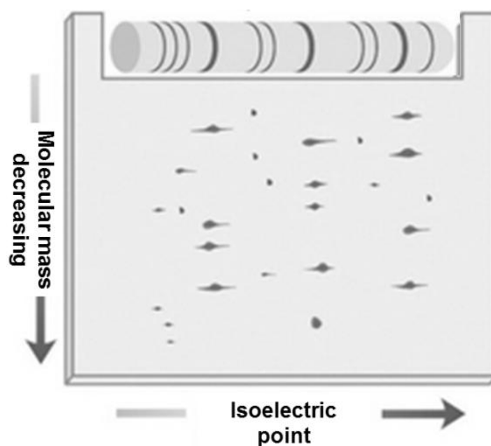


Figure 6. Example schematic of protein spots separated by two-dimensional gel electrophoresis, after the isoelectric focusing.

The final result, after gel staining, is a protein profile in which each spot corresponds to a set of few proteins, facilitating subsequent analysis by mass spectrometry. However, before MS analyses, the different proteins in the gel must be revealed, by staining with different methods. The choice of dye or fluorescent agent will determine the sensitivity of the technique. The most commonly used staining methods are reported in Table 1 with their respective detection limits and corresponding linearity ranges.

- Comassie blue: although less sensitive than other methods, this method is perfectly adequate for comparative studies, due to its linear response.

- Silver staining: this method is 100 times more sensitive than Coomassie blue staining, however, due to a large number of steps and reagents, may become more costly. Under certain concentrations the response becomes non-

linear and incompatible with mass spectrometric analysis by MALDI and ESI, as reported by Gevaert *et al.*[83]

- Fluorescence staining: currently a very sensitive staining, with a wider range of linearity, however, uses a fluorescent scanner, which is extremely expensive.

Other types of staining have also been developed in order to increase sensitivity and compatibility with mass spectrometry but have not been widely applied.[83; 84]

Table 1. Main protein staining methods after one- or two-dimensional gel electrophoresis.[84]

Staining method	Limits of detection (ng)	Linearity range (order of magnitude)
Coomassie blue	50 - 100	1 - 1,3
$AgNO_3$	1	2
Sypro® Orange, Ruby (Fluorescence)	1 - 8	>3
Deep purple ® (Fluorescence)	1	4

The analysis method of gel staining evolved thanks to the combined progress of computers and imaging analyses. The first stage of analysis is gel scanning, i.e., the transformation of the image in an experimental numerical information usable by the computer. The scanned image is then handled by specific software. The relevant information is then obtained from the differential and comparative analysis between proteins present in the gels. After choosing which protein bands or spots are of interest, they are excised, digested (usually by trypsin) and analyzed by MS.

1.3.2.4.

Protein identification by mass spectrometry after separation by gel electrophoresis

In biology, the study of proteins involves the determination of their primary amino acid sequence. Edman degradation was, for a long time, chosen for the determination of the amino acid sequence using the automated sequencer marketed in 1967.[85]

Since the early 90s this method was overtaken by mass spectrometry, which currently is the most sensitive method for biomolecule characterization. Molecular mass spectrometry is now a standard technique for the identification and characterization of biomolecules. This growth was based on the development of two new ionization techniques in 1989, by Toichi Tanaka and John Bennett Fenn, respectively Matrix Assisted Laser Desorption - MALDI and electrospray Ionization - ESI. These techniques allow the transformation of biomolecules into ionized gas for the detection of their mass by mass spectrometry.

1.3.2.5.

MALDI mass spectrometry

Michael Karas and Franz Hillenkamp were responsible for the name and initial development of this technique in the late 1980s.[86] In the early 2000s, Tanaka pioneered the use of the technique for protein analyses,[87] and was one of the winners of the Nobel Prize in chemistry for developing desorption and ionization spectrometric methods for biological molecule analysis.

This technique involves radiation of a pulsed laser beam usually in the UV in a crystalline deposit containing an organic matrix and the sample (Figure 7). The analyte molecules are co-crystallized with the sample in a chemically inert matrix on stainless steel. A MALDI matrix is a small molecule capable of strongly absorbing the UV laser. The most commonly used matrices are α-cyano-4-hydroxycinnamic acid (α-CHCA) for the analysis of peptides and proteins, sinapinic acid, for the analysis of whole proteins, and 2,5-dihydrobenzoic acid (DHB), which is a polyvalent matrix. In order to facilitate ionization of the analyte molecules, the array is used in a molar excess of about 500x. This excess matrix

is needed since the crystals are the matrix that that absorb the UV laser light, with the sample being only, metaphorically, a built-in "impurity" in the crystal matrix. When the laser pulse impacts with the matrix in vacuum, the matrix molecules release their excess energy absorbed by sublimation and concomitant molecular fragmentation, however, if properly prepared, the sample is brought into the gas phase by a sublimation plume without fragmentation.[88] The process of ionization is not fully elucidated; the mechanism is complex and involves physical and chemical processes.

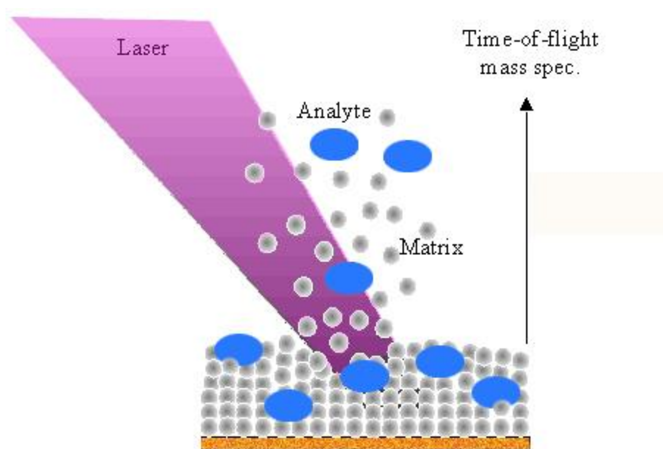


Figure 7. MALDI-MS scheme. Adapted from Fitzgerald (1995).[89]

The MALDI technique was first used with Time-of-Flight (ToF - Time of Flight) mass spectrometers analyzers. The ions are formed in the ionization source after a laser pulse, and are then accelerated by an electric potential. The ions then traverse the flight tube, which has a region free of electric field and arrive at the detector at characteristic times, according to their m/z. This period is roughly proportional to the square root of the mass-charge ratio (m/z) ion, and can be used to calculate the mass of the ion. Therefore, lighter ions have a shorter ToF than heavier ions (Figure 8). The fast analysis time (less than a millisecond) enables quick and simple analysis of different samples.[89]

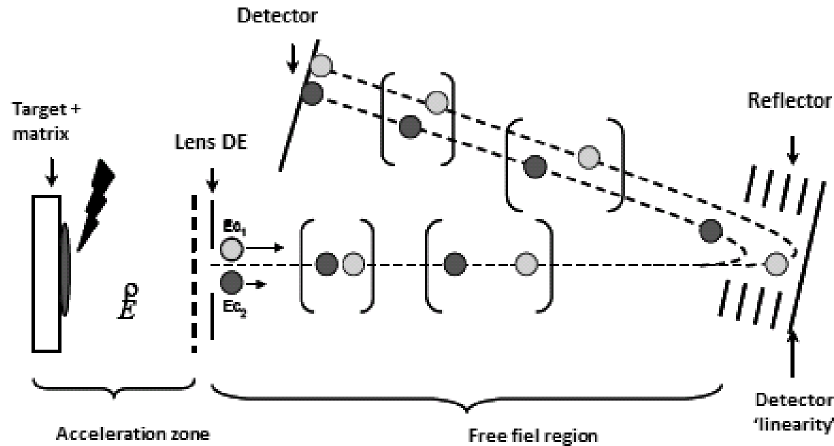


Figure 8. Schematic representation of the operating principle of a MALDI-TOF. Adapted from Horn (2004).[90]

The MALDI-TOF technique allows for the analysis in a wide mass range (unlimited in theory, however, what occurs in practice is up to 500 kDa) with good precision measurement (10 - 20 ppm), good resolution, good sensitivity, and a relatively simple and fast implementation, tolerant to salts and detergents commonly used in biology.[90]

1.3.2.6.
ESI MS

In a traditional approach the peptides are produced from a tryptic digestion, after gel electrophoresis, or directly from aqueous sample solutions. In cases where MALDI TOF MS analyses are not feasible, high performance liquid nanochromatography (nanoHPLC) coupled to an MS electrospray is an alternative analysis for the determination of peptides and identification of proteins from which they are derived.

The phenomenon of electrospray ionization was described by Dole in 1968,[91] but it was only in the 80s that this ionization technique was coupled to a quadrupole analyzer for the purpose of protein analysis.[92; 93] Because of its particularity in forming multicharged ions, ESI-MS allows the analysis of more heavy and complex molecules with a considerably "thin" (0.01%) mass

measurement accuracy. With this, the formation of ions at atmospheric pressure in the electrospray source allowed this technique to be coupled with liquid chromatography (LC-MS).[92] The general principle of mass spectrometry ionization electrospray is displayed in Figure 9 and detailed in the next paragraph.

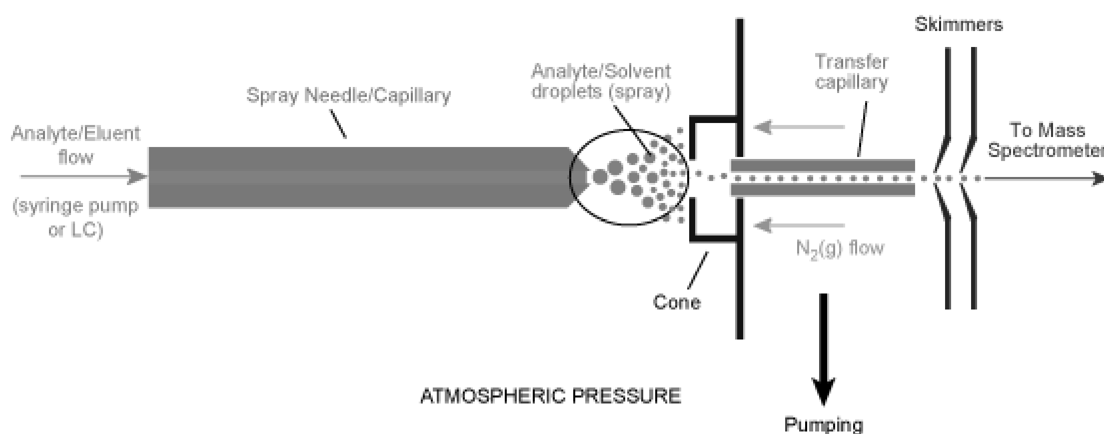


Figure 9. Ion formation in ESI. Adapted from Cole (1997).[94]

Electrospray ionization involves the formation of an electrostatic spray, from which charged droplets are generated and ions are released. The implementation of an electrospray source is relatively simple compared with other sources for mass spectrometry.[94] A source of high voltage (1000-7000 V) in contact with the solution containing electrolytes is required. Typically, the solution is pumped through a capillary (i.d. 50 to 100 mmol L^{-1}) with a flow rate of less than 10 L min^{-1}. In the case of less than 1 µL min^{-1} flow, the process is called nanoelectrospray.

All the source region is at atmospheric pressure. When a positive potential, for example, is applied to the solution, positive ions tend to move away to a less positive region, i.e., toward the counter electrode. Thus, the drop being formed at the tip of the capillary is enriched in positive ions. This type of charge separation is called electrophoretic process. As the charge density increases in the droplet, the electric field between the capillary and the electrode increases, causing the deformation of the drop. The drop takes the form of a cone which is called Taylor's cone.[95]

This cone-shaped drop remains "stuck" to the capillary until the charge density on the droplet surface and the increase in the repulsion between ions overcome the surface tension of the liquid, with the consequent release of small drops with high charge density. The frequency of this process depends on the magnitude of the electric field, the surface tension of the solvent and the conductivity of the solution. These small droplets formed are subjected to the same process several times until they form completely desolvated ions, and finally are transformed into gaseous form.[96] Once formed, the ion reaches the region of the capillary or sampling cone with a very small hole (of the order of a few micrometers).

From there, the system switches to the low pressure region. At the entrance, there is a set of lenses that lead ions to the mass analyzer according to their mass/charge ratio. Several types of analyzers compatible with electrospray ionization mode are marketed and the choice of mass analyzer used will be made according to the desired resolution, mass accuracy, sensitivity and ability to perform tandem mass spectrometry, MS/MS. In proteomic analyses, the following mass analyzers are commonly used:

- ion trap (IT);[97]
- orbitrap;[236]
- time of flight (TOF);[98]
- quadrupole (Q);[92]
- Fourrier transform ion cyclotron resonance (FT-ICR).[99]

These analyzers are used alone or in combination to create hybrid analyzers such as the Q-TOF type, benefiting the strongest points of each of the chosen analyzers.

1.3.2.7

Approaches to protein identification after MS analyses - databases and bioinformatics

The set of research strategies currently available for the automatic identification of proteins with mass spectrometry data was recently compiled by Hernandez *et al.*[100] Protein identification is performed using the values of the molecular weights of protein peptides obtained by mass spectrometry. In the case of MALDI, we use the monocharged signals comparing the experimentally obtained values with expected values stored in the database. All types of database sequences can theoretically be used, such as protein sequence banks, ESTs (Expressed Sequence Tag) banks and genomic libraries.[101] The proteomic analyses mostly use protein banks, which are usually "corrected" by selection and classification performed by specialists.

These databases are numerous, but the most known and used are the National Center for Biotechnology Information - NCBI and UniProt consortium banks. The NCBI database is bulky and sometimes redundant, but with the advantage of being relatively complete. It is accessible at http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi. The UniProt Consortium centralizes SwissProt, TrEMBL and PIR-P banks. These banks are accessible at http://www.expasy.uniprot.org/ site. The PIR-PSD (Protein Information Resource Protein Sequence Database) database is organized according to the classification by proteins families and super-families. This library contains functional annotations and structural and genetic bibliographic references. The SwissProt data bank is a library of less redundant protein sequences and is quite accessible. A special precaution is required to indicate the experimental nature or bioinformatics, functional information, as well as the confidence level given to this information.[102] When it comes to identifying proteins, the quality of the proteome present in the library varies depending on the organism. Thus, it is important to consult information related to multiple databases before using the data, to know the number of entries in the target organism, how these inputs were generated (by simple automatic or manual annotation prediction) and what is the status of the genome sequencing.[103] The knowledge of the genome of an organism is of great importance to enable the accurate identification of proteins by peptide

standards and, therefore, organisms that are not very well studied often do not have their proteins identified.

1.3.2.8.
Alternative methods for protein characterization

1.3.2.8.1.
Fourier-Transform infrared (FT-IR) vibrational spectroscopy in qualitative protein characterization.

Fourier-Transform infrared (FT-IR) vibrational spectroscopy is a measurement of the wavelength and intensity of infra-red radiation absorption of a sample. This is one of the oldest and most well-established experimental techniques for the analysis of the secondary structure of polypeptides and proteins. It is a non-destructive technique that requires little practice in sample preparation, and can be used under a wide variety of conditions.[104]

The vibrational spectrum of a molecule is determined by its three-dimensional structure and vibrational force field. An analysis of this spectrum in different regions, such as infra-red, can, therefore, provide information on the molecule structure and on the intramolecular and intermolecular interactions. A greater probing analysis of this spectrum is able to provide increasingly detailed information about the studied molecule.

While the three-dimensional structure and force field are determined solely by the vibrational frequencies of the molecule, the structure is, in general, not obtained directly from the spectrum. However, the atomic displacements in many of the vibrational modes of a large molecule are transferable between molecules. Therefore, in early peptides and proteins studies, efforts were mainly directed towards the identification of such characteristic frequencies and determination of their relationship to the molecule structure.[105]

Early protein studies by infrared spectroscopy were only qualitative. The position and number of bands were not accurately verified, and only

approximations regarding changes in the relative intensities of shoulders or amide I band shifts could be made.

Overlapping components in spectral profiles are common for complex samples such as proteins. The complexity of information contained in infrared spectra of proteins increases the difficulty of analysis, even with the increasing number of new methodologies for quantitative analysis of IR bands.[106]

The pioneering work in quantitative protein analysis by IR was performed by Susi and colleagues.[107] The work reported by Ruegg[108] included the analysis of the 1750-1300 cm^{-1} region of seven proteins, four of them with known X-ray structures. The information necessary to begin the quantification process was the number of tracks, the shape of the peaks, the peak frequencies and their allowed frequency range, and landmarks outside the absorption region where the total absorbance was initially assumed to be zero. Since the number of component bands and their "real" positions were not known, the number of bands and peaks at the positions of ribonuclease were based on previous studies on proteins and polypeptides with known conformations. The spectra were then resolved into Gaussian components with the aid of a computer program. Thus, even very complex spectra can be deconvoluted statistically into simples spectra for further analyses.

2.
Objectives

In order to better understanding of the work, it was separated in 2 parts.

2.1.
PART I: Peptide derivatization and complexation with metals

2.1.2.
General Objective

The aim of this part was to develop a new simple, sensitive, accurate and precise method for identifying and quantifying peptides based on simultaneous coupling of elemental and molecular mass spectrometry to multidimensional chromatographic separations.

2.1.2.1.
Specific objectives

- Development and optimization of suited strategies for the labeling of peptides with metal-chelators DOTA-(Lu, Tm and Ho).
- Elucidation, investigation, and optimization of analytical parameters for the (i) accurate and precise quantification of labelled peptides by ICP-MS and (ii) the molecular characterization of these peptides/proteins by MALDI MS.
- Separation of the labelled peptides and analyses by nano-HPLC with UV detection and MALDI MS.
- Comparison of the strategies developed with others established methods for quantification.

2.2.
PART II: Optimization of metalloprotein extraction procedures from environmental samples

2.2.1.
General objective

The aim of this part of the study was to evaluate and standardize the currently applied thermal-extraction method for the purification and quantification of fish bile MT using different reagents and multivariate statistical analyses in a proteomic/metalloproteomic context, since this protein has previously been reported as a potential biomarker in fish bile.

2.2.1.1.
Specific objectives

- Improvement of MT extraction procedure in fish bile and liver samples.
- Evaluation of the use of fish bile in the detection of metal contamination by comparisons with the same analysis in liver, in order to demonstrate fish bile potential as a biomarker by different analytical techniques.
- Qualitative and quantitative proteomic/metalloproteomic evaluation of bile and liver samples using different analytical techniques: SDS-PAGE, UV-Vis spectrophotometry, SEC-HPLC-ICP-MS, MALDI MS and ESI MS.
- Evaluation of biliary proteins in fish bile as potential biomarkers of environmental contamination using MT-induction responses in laboratory-exposed fish, using the statistical tools.
- Evaluation of clean-up effects in both fish bile and liver by vibrational spectroscopy analyses.

3. Methodology

3.1.
PART I: Peptide derivatization and complexation with metals

3.1.1.
Materials and reagents

The synthetic peptides used are describe below from their amino-acid sequences S35 (VLASSAR; $[M+H]^+$ = 703.41), S36 (GACLLPK; $[M+H]^+$ = 701.40), S34 (CCTKPESER); $[M+H]^+$ = 1052.45), M6 (SLLPAIVEL; $[M+H]^+$ = 954.59), and T1 (VKCFNCGK, $[M+H]^+$ = 898.43) were prepared by peptides in solid-phase (R. Pipkorn, DKFZ, Heidelberg, Germany). The reagent DOTA-NHS-ester was purchased from Chem Matech (Dijon, France), Figure 10. The other reagents as tris(2-carboxyethyl)phosphine (TCEP), triethylammonium bicarbonate buffer (TEAB), triethylammonium acetate buffer (TEAA), trifluoracetic acid (TFA), S-methyl methanethiosulfonate (MMTS), dimethyl sulfoxide (DMSO), acetonitrile (ACN), and the standard digest protein were purchased from Sigma-Aldrich (Taufkirchen, Germany), as lanthanide (III) - (holmium, lutetium, thulium) hexahydrate salts, as well Glu1-fibrinopeptide B and α-cyano-4-hydroxycinnamic acid (CHCA). The water purification system, Advantage A10 (Merck, Molsheim, France) was used to prepare deionized water (18.2 MΩ cm).

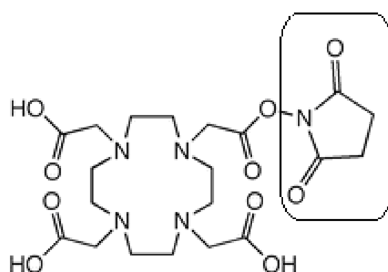


Figure 10. Structure of the DOTA derivatization reagent with the NHS-ester function for amine-specific (highlighted) labelling.

3.1.2.
Sample preparation

Basing on the procedure proposed by Gregorius *et al.*[176] was performed the derivatization reaction of the standard peptides and their mixture. Briefly, the labelling was performed by reacting the samples with an 100-fold molar excess regarding free NH_2-groups of NHS-DOTA, after TCEP-reduction (already available in 50 mM HEPES, pH 7 solution) at a 10nmol μL^{-1} concentration, as seen in section 3.1.2.1

With the view to validate the applicability of the metal-labelling procedure and peptide ID, the procedure was tested on a Cytochrome C digest (Dionex, Amsterdam).

To this end, after sample preparation and labelling, a nano-HPLC equipped with a C-18 pre-column coupled to a Probot microfaction collector was used to separate the mixtures prior to the MALDI TOF MS analyses.

3.1.2.1.
Labeling procedure

A preliminary reducing step was performed on all samples, for 1h at 60 °C using, per each cysteine residue, a threefold molar excess of TCEP solution.[176] After this, was performed the alkylation of the free cysteine residues with a sixfold molar excess of MMTS per thiol group for 10 minutes at room temperature.

All samples, singles peptide and mix peptide, were derivatized for 1h in 75% ACN, 25% 100 mM HEPES at pH 7.5 with a 100x molar excess of DOTA-NHS-ester (dissolved in a 10 mM DMSO water free solution) for each free amino group. This means that, besides the N-terminus, all the peptides were also derivatized in their lysine (K) portion (Figure 11), which has a free amino group.

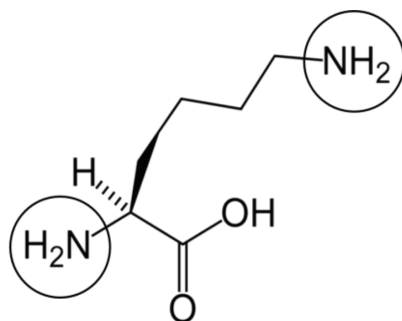


Figure 11. Amino acid lysine (K) with its 2 free amino groups.

Subsequently, holmium, thulium, and lutetium lanthanide salts were dissolved in 100 mM TEAA buffer (pH 5) from a 10 mM stock solution. The complexation of the labeled peptides with DOTA-NHS ester was conducted with a 10x molar excess of each lanthanide metal ion for each DOTA-NHS-ester used, with incubation at room temperature for 1 h.

The complexation was completed with sample vortexation. The final concentration of each peptide standard solution and the peptide mixture were, respectively, 5 μmol L^{-1} and 15 μmol L^{-1}. All treatments performed for peptides were also performed for the Cytochrome C digest.

3.1.2.2.
Sample preparation for MALDI TOF MS

Matrix-assisted laser desorption/ionization was used to evaluate the derivatization reaction efficiency for each sample, from the identification of their m/z values. Before the derivatization reaction with the unmodified samples, these were acidified with 10% TFA to pH 4. For simile of the response intensity of the single peptides, a 1 mL aliquot was spotted as a 50 fmol mL^{-1} CHCA solution in a 384-well Opti-TOF™ plate (Applied Biosystems, Darmstadt, Germany).

3.1.3.
Peptide separation by nano-Ion Pair-Reverse Phase-HPLC (nano-IP-RP-HPLC)

The labeled peptides were loaded onto a C-18 column trap before being eluted into the analytical column. At this stage, different run times were tested with the purpose of sample washing, to remove excess metals due to the complexing reaction. This was done in order to ensure the compromise between the removal of non-bound excess reagents and the minimum possible loss of peptides.

The wash time of the trap column refers to the time that the sample remains in the trap, while the mobile phase is eluted (3% ACN, 0.1% TFA), before sample injection. When the valve switched the clean sample is then eluted into the column (Figure 12).

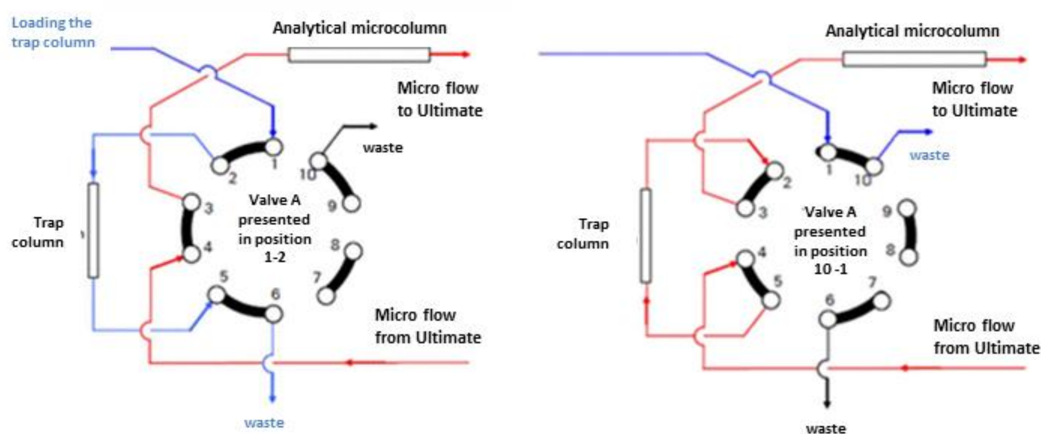


Figure 12. Preconcentration in the trap column and sample elution by nano-HPLC.

20 μL of the 15 μmol L^{-1} peptide solutions at were eluted by a 50 μL loop through a Acclaim PepMap100 C18 trap column (5 mm, 0.3 x 10mm; Dionex, Idstein, Germany) at a outflow of 30 μL min^{-1} in 0.1% TFA (aqueous) and 3% ACN during 6 minutes, optimized from prior tests performed on the nano-LC. We conducted tests with different times for choosing the better pre-cleaning time

taking into acocunt the ratio betwen the lowest metal background and approvable loss of peptides. The times studied were 3, 6, 10 and 20 minutes.

A separation column C18 (Acclaim PepMap100, 5 µm 75 µm, 150 mm, Dionex, Idstein, Germany) was used for eluted the peptides. The separation was accomplished with a outflow of 0.3 mL min^{-1} in 0.05% TFA (solution A) and 80% ACN, 0.04% TFA, 20% deionized water (v/v/v) (solution B) (Figure 13), 35 minute wash runs were conducted between sample injection. The detection with UV was realized at 214 nm.

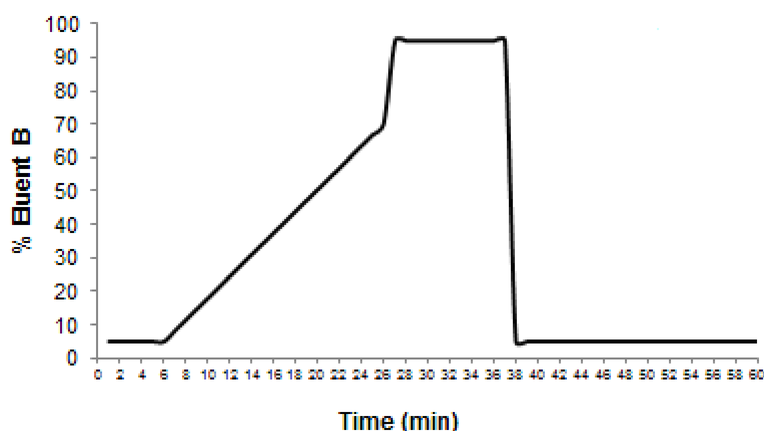


Figure 13. Eluent gradient for peptide mixture separation. Eluent A: 0.05% of TFA (aqueous) B: 0.04% TFA, 80% ACN, 20% and deionized water.

3.1.4.
Peptide analysis by MALDI-TOF-MS

This step was performed following the protocol proposed by Gregorius *et al.*[176] After elution, the peptides were mixed with the matrix solution (CHCA 3mg mL^{-1} in 0.1% TFA, 70% ACN, 5 nM Glu1-fibrinopeptide B) at a 1:3 ratio (v/v) and spotted each 20s in a range of 4-65 minutes. With a SCIEX AB TOF/TOFTM 5800 (Applied Biosystems, Darmstadt, Germany) the measurements were accomplished in the reflectron mode in positive ion.

From a U3000 nano-HPLC system (Dionex, Idstein, Germany) the mixture of the standard peptides was separated and collected on to the coupled to a Probot microfaction collector LC Packings (Amsterdam, The Netherlands).

3.1.5. Peptide analysis by nano-HPLC-ICP-MS

In this study an ICP-MS Agilent 7500ce (Tokyo, Japan) was used. Instrumental adjustment – RF power, nebulizer gas flow, and lens voltage – were tuned daily by controlling the signals of erbium (element added in form of a standard solution to eluents A and B). The final erbium concentration was at 40 µg L^{-1}. Typical ICP-MS performing conditions are in Table 2.

Table 2. Instrumental parameters of ICP-MS (Agilent 7500ce, using in LCABIE, UPPA).

ICP-MS Agilent 7500ce	
RF power	1500 W
Cones	Nickel
Sampling depth	7 mm
Nebulizer gas flow	0.7–1.13 L min^{-1}
Extraction lens	2.6 V
Monitored isotopes	^{166}Er

The nano-HPLC system was connected with the ICPMS instrument via a pneumatic nanonebulizer working in the nL min^{-1} range.[177; 178] The nebulizer used was employed as interface for a sheathless coupling of the nanoHPLC to the ICP-MS. The outlet capillary (20 mm inner diameter and 280 mm outer diameter) of the nanoHPLC column was introduced into the nebulizer and linked to the nebulizer capillary, without any dead volume.

The nanoflow nebulizer uses a hollow fused-silica capillary as nebulizer capillary. The small capillary tip (i.d. 20 µm, o.d. 90 µm) is centered in a 254-µm i.d. sapphire orifice allowing a stable and continuous nebulization of nanoliter flow rates of less than 500 nL min^{-1}. Nebulization characteristics can be optimized by adjusting the position of the capillary tip in the nebulizer orifice.

3.2.
PART II: Optimization of metalloprotein extraction procedures in environmental samples

3.2.1.
Fish Specimens

3.2.1.1.
Environmental samples for the optimization of metalloprotein extraction procedure

Nile Tilapia (*Oreochromis niloticus*) were purchased directly from certified farmers who sell fish for human consumption, in the south zone of Rio de Janeiro. Bile and liver were immediately removed, the latter by direct puncture of the gallbladder with a plastic 5.0 mL syringe. Liver was weighed and bile volume and color was recorded. Both organs were then stored at -80 °C until analysis in sterile polipropylene tubes. At the laboratory liver samples were freeze-dried (LioTop L101, São Paulo , Brazil), to facilitate sample manipulation.

3.2.1.2.
Laboratory exposure to metals

Tilapias purchased from a producer living in the municipality of Nova Iguaçu were subjected to sub lethal doses of 4 metals: Cd, Pb, Zn and Ni, while the control group was not exposed.

Exposure to toxic substances in the laboratory typically occurs within 24 to 96 hours. The tests are often conducted under static conditions, ie, without the renewal of water throughout the experiment, since this is simpler and less expensive compared to semi-static systems (partial replacement) or continuous-flow systems (continuous renewal). The static system is especially recommended when the test substance is proven stable in the environment, such as copper and other metals.[179]

Tanks of 500 L capacity containing dechlorinated water, pH 7.0, were prepared to receive the fish. Solutions containing Pb, Zn, Cd and Ni were used in sublethal doses according to the maximum permitted concentration of each element in brackish water, according to Resolution number 357 (March 17, 2005, CONAMA). The values of the final concentrations of each element are shown in Table 3.

Table 3. Concentrations of the solutions used in the laboratory exposure experiment according to the maximum permitted concentrations allowed by CONAMA Resolution 357 (2005).

Ni	0.025 mg L^{-1}
Zn	0.09 mg L^{-1}
Pb	0.01 mg L^{-1}
Cd	0.005 mg L^{-1}

The animals were transported in water tanks saturated with oxygen and transported to the laboratory. The fish were acclimated for 30 min and then transferred to the tanks. The experiment was static, without water renewal and with constant aeration, lasting 96 hours. This duration was based on previous experiments in the literature that indicate that this period is sufficient for protein modifications to occur in fish liver and other organs.[180] During this period, feeding was discontinued, to concentrate bile fluid, since when fish feed bile is diluted with water, which leads to low protein concentrations.[181] After 96 hours, fish were sacrificed by spinal dislocation. Fish were then weighed and measured and dissected. Bile and liver were immediately removed, the latter by direct puncture of the gallbladder with a plastic 5.0 mL syringe. Liver was weighed and bile volume and color was recorded. Both organs were then stored at -80 °C until analysis in sterile polipropylene tubes. At the laboratory liver samples were freeze-dried (LioTop L101, São Paulo, Brazil), to facilitate sample manipulation.

3.2.2.

Metallothionein (MT) purification from Tilapia liver and bile samples

MT extraction was based on the thermal-extraction procedure proposed by Erk *et al.*[182] and recently applied by our group for fish bile analyses.[18] This protocol uses DTT as the reducing agent and centrifugation times of 1 hour and then 30 minutes, with thermal extraction temperature of 70 ^{o}C. In the present standardization study, however, 100 µL of the pooled bile (n=10) and liver (n=10) purified MT supernatants were homogenized in three different solutions, containing either β-mercaptoethanol 0.01%, DTT (Ditiotreithol) 0.01% or TCEP (Tris-2-carboxyethyl-phosphine) 1% as reducing agents in Tris-HCl 20 mmol L^{-1} pH 8.6, with PMSF (phenylmethylsulphonylfluoride) 0.5 mmol L^{-1} added as an antiproteolytic agent. For liver samples, 100 mg of the pooled samples (from the same 10 fish used to obtain the bile samples) were homogenized in 2 mL of the same reducing solutions, using a glass rod. The samples were centrifuged at 20.000 x g for different pre-established times at 4 ºC. The supernatants were then carefully separated from the pellet and transferred to new sterile Eppendorf flasks and heated for 10 min at different pre-established temperatures. A second centrifugation was conducted at 20.000 x g for varying times at 4 ºC and the final supernatants containing MT in the purified sub-samples were separated and frozen at -80 ºC until analysis. An experimental planning was conducted to optimize and standardize MT purification from these samples. Three different protocols, A, B and C, were conducted with each of the reducing agents and varying centrifugation times and temperatures (Tables 4, 5 and 6).

Table 4. Description of each metallothionein purification procedure applied in the present study, with the first centrifugation step, the temperature and second centrifugation step indicated.

Purification procedure	**First centrifugation step (min)**	**Temperature (°C)**	**Second centrifugation step (min)**
A	45	60	15
B*	60	70	30
C	75	80	45

*Protocol established by Erk (2002).[182]

Table 5. Description of metallothionein purification procedure B applied in the second step of this study, with the first and second centrifugation steps fixed.

Purification procedure	**First centrifugation step (min)**	**Temperature (°C)**	**Second centrifugation step (min)**
B'	60	50	30
B'	60	60	30
B'	60	70	30
B'	60	80	30
B'	60	90	30

Table 6. Description of metallothionein purification procedure applied in the third step of this study, with the temperature step fixed.

Purification procedure	**First centrifugation step (min)**	**Temperature (°C)**	**Second centrifugation step (min)**
A''	45	70	15
B''	60	70	30
C''	75	70	45

The second stage of the study was to cross the parameters specifically for bile samples, using 4 factors with 3 levels each in a multivariate factor analysis. The description of each different MT reducing agent in conjunction with the different purification procedures conducted in the present study after the 4^3 multivariate statistical analysis is displayed in Table 7.

Table 7. Description of each different metallothionein reducing agent in conjunction with the different purification procedures conducted in the present study after a 4^3 multivariate statistical analysis.

Procedure code	t (min)	T (°C)	t (min)	Reagent
1	75	60	30	TCEP
2	75	60	45	DTT
3	60	80	30	DTT
4	45	70	15	TCEP
5	45	70	45	β-mercapEtOH
6	45	80	45	DTT
7	60	80	45	TCEP
8	75	60	30	β-mercapEtOH
9	75	80	45	TCEP
10	45	60	15	β-mercapEtOH
11	75	70	30	DTT
12	60	70	45	DTT
13	75	80	15	DTT
14	75	80	45	β-mercapEtOH
15	45	80	30	β-mercapEtOH
16	45	80	15	TCEP
17	60	60	45	β-mercapEtOH
18	75	70	30	TCEP
19	45	60	30	DTT
20	60	70	30	β-mercapEtOH
21	45	60	45	TCEP
22	60	60	15	TCEP
23	60	70	30	TCEP
24	60	60	15	DTT
25	45	70	15	DTT
26	75	70	15	β-mercapEtOH
27	60	80	30	β-mercapEtOH

3.2.3.
Metallothionein quantification by Ellman's assay

MT quantification was conducted by spectrophotometry applying Ellman's reaction. This is an indirect quantification assay, since it measures the sulfhydryl groups present in the sample and not the absolute concentration of MT.

After the application of the different purification procedures for both liver and bile samples, 50 µL of the purified sub-samples were treated with HCl 1 mol L^{-1} containing EDTA 4 mmol L^{-1} and NaCl 2 mol L^{-1} containing 0.43 mmol L^{-1} DTNB (5,5'-dithiobis-2-nitrobenzoic acid) buffered with 0.2 mol L^{-1} Na-phosphate, pH 8.0,[185] and incubated for 30 min. The samples were then centrifuged at 3000 x g for 5 min and the supernatant absorbance was evaluated at 412 nm using a SpectraMax (Hamilton, USA) microplate reader. In the color-forming reaction of Ellman's reagent with sulfhydryl groups (Figure 14), DTNB reacts with thiol groups, such as peptide cysteines, to form mixed S^{-2} (disulfide) and TNB, which make possible to measure the color at 412 nm. MT concentrations were approximate by using the standard, reduced glutathione (GSH), for the calibration curve (0 – 1000 µmol L^{-1}) from a 10 mmol L^{-1} stock solution, as described by Viarengo *et al.*, although this method to measure all thiols acids soluble, glutathione is more than 90% of the reactive thiol groups, being considered an appropriate standard.[186]

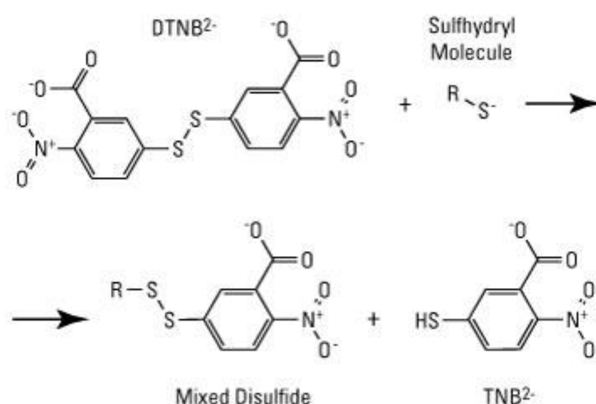


Figure 14. Color-forming reaction of Ellman's reagent with –SH groups.

The MT concentration was presumed by the relationship of 1 mol MT = 20 mols GSH, as reported by Kagi for fish.[187] To establish recovery percentages by standard addition, and provide accuracy to the spectrophotometric and SDS-PAGE analyses beyond thermal stability and molecular weight inherent to MT, we also used commercially available MT standards, namely, MT-I purified standard (Enzo sciences, USA) and compared the behavior and angular coefficients of both curves (GSH and MT) for further accuracy.

3.2.4. Figures of merit

The instrument limit of detection (LOD) and limit of quantification (LOQ) were estimated as 3 sd/S and (10 sd/S) * the dilution factor of the sample, respectively, where sd is the standard deviation for the blank measures (n=10) and S is the method sensitivity. The method limit of detection was estimated as the limit of detection multiplied by the dilution factor of the sample (1+3).

Repeatability tests were also conducted in restrictive conditions, using the same laboratory, analyst, instrument and, if possible, conducting the assays on the same day. For repeatability standard deviation calculations, 7 or more repeats are recommended.[188] In the present study, 10 measurement repeats were conducted to ensure the validity of the calculations.

3.2.5. Total protein quantification

Total protein concentrations in bile and liver samples were measured by the Lowry method modified by Peterson.[189] Analytical curve was prepared with serial dilutions of a stock solution of bovine serum albumin concentration of 2 mg mL^{-1} (BSA, Sigma-Aldrich) in a mass range from 0 to 50 mg BSA. Briefly, 0.1% copper sulfate solution, potassium sodium tartrate 0.2%, sodium carbonate 10% and NaOH and SDS, were mixed together, resulting in "Reagent A". "Reagent B", in turn, is a Follin-Ciocalteau solution (Sigma-Aldrich) diluted 5x with ultra-pure water. 400 μL of "reactive A" was added to every 10 μ L of sample – the original

bile and liver extracts and their respective purified extracts. Tubes were shaken by hand and allowed to rest for 10 min. 200 μL of reagent B were then added, with stirring. The tubes were then left resting for 30 minutes and the samples and calibration curve were measured at 750 nm, using a SpectraMax microplate reader (Hamilton, USA).

3.2.6.
1D and 2D gel electrophoresis

SDS-PAGE analyses were conducted in order to qualitatively assess differences in the different purification protocols. Total protein content of both liver and bile samples was determined by the Lowry method, modified by Peterson and the Bovine serum Albumin (BSA) was used as standard, as described above.

Polyacrilamide gels (15%) were prepared according to Laemmli *et al.*[78] Aliquots of both bile and liver MT extracts were applied to each lane, along with the molecular weight standards. Gels were run, in triplicate, for approximately 2h30min, at 45 mA/gel. Gels were then stained using the silver stain method, described below. We used the molecular weight standards (Biorad Precision Plus ProteinTM Dual Color Standards) for determine molecular weights of the protein bands and spots. Optic densitometry using SDS-PAGE gels was not conducted for MT quantification, since this method is not as efficient as spectrophotometric analyses, as discussed by Hauser-Davis.[19]

For 2D runs, individual bile samples, after clean-up were solubilized in a rehydration buffer containing urea 8 mmol L^{-1}, 2% CHAPS (w/v) DTT, 1% bromophenol blue, 0.002%, and 1.0% IPG buffer (specific for each pH range used). Samples were applied to Immobiline Dry Strips Strips (13 cm) (GE Healthcare®) and rehydrated passively on a specific tray (Reswelling Tray) for 10-20 h. First a pH range of 3-10 was used, and, when necessary, subsequent analyses were conducted at pH 4-7 for better resolution of the most abundant proteins. Isoelectric focusing was performed on an Ettan IPGphor 3 (GE Healthcare®). The applied focusing program was the same as recommended by GE Healthcare® for 13 cm strips (large gels) (Amersham-Biosciences, 2004) described below:

Step 1: 300 V for 30 min (extra step to remove any salts still present in the samples)

Step 2: 500 V for 1 h

Step 3: linear gradient from 500 V to 1000 V for 1 h

Step 4: linear gradient from 1000 V to 8000 V for 2h30min

Step 5: 8000 V for 30min

The total Vhs for each run was always 16650 VHS.

After isoelectric focusing, the strips were incubated under gentle agitation in an equilibration buffer (urea 6 mol L^{-1}, 30% glycerol (v/v), 2% SDS (w / v) and Tris-HCl 0.05 mol L^{-1}, pH 8.8) for 2 x 15 min, first with DTT (1% w/v) for protein reduction, and then with iodoacetamide (3% w/v) for protein alkylation. After this procedure, the strips were then transferred to a 15% SDS-PAGE gel, sealed with agarose (25 mmol L^{-1} Tris base, 192 mmol L^{-1} glycine, 0.1% SDS, 0,5% agarose, 0.002% bromophenol blue) and subjected to separation in the second dimension in a vertical Ruby SE600 GE Healthcare® system. The running program consisted of an initial step at 10 mA/gel for 15 minutes for slow and, therefore, more efficient protein migration from the strips to the gel, and a second step of 45 mA/gel for 3 h or until the blue magarose marker reached about 1 mm from the bottom of the gel. Molecular weight standards (ProteinTM Precision Plus Dual Color - Biorad) were used to estimate protein molecular weight.

3.2.7.
Gel staining after electrophoresis

3.2.7.1.
Coomassie blue G-250 staining

For Coomassie Blue G-250 staining, the gels were fixed for at least 30 minutes in a fixing solution (10% acetic acid, 40% ethanol). After fixation the gels were immersed in a Coomassie Blue G-250 solution (8% ammonium sulfate, 0.8% phosphoric acid, 0.08% Coomassie Blue G-250 and 20% methanol) for 24 hours following the protocol of Neuhoff and colleagues.[190]

3.2.7.2.
Silver nitrate staining

The gels stained with silver nitrate followed the protocol of Heukeshoven and Dernick,[191] where gels are first fixed with fixing solution (10% acetic acid/30% ethanol), sensitized in a 5% sodium thiosulfate/20% ethanol solution, washed with ultrapure water, reacted with nitrate silver (2.5%), and then washed again and revealed in a 3% sodium carbonate/0.05% formaldehyde solution. The development is then stopped with EDTA 1.5%, preventing further reduction of the silver ions in solution. The gels were then preserved in ethanol 25%/glycerol 5.3% (which prevents the gel from cracking during drying) for subsequent image analysis and mass spectrometry.

3.2.8.
Gel scanning for image analysis

The gels were scanned using the LabScan v.3.0 software (GE Healthcare®) on an ImageScanner Scanner II with densitometer operating at a 300 dpi resolution. The Image-Master 2D Platinum 6.0 (GeneBio, Geneva, Switzerland) software was used for gel image analysis. In the case of 2D gels, the analyses were conducted by a combination of automatic spot detection and manual detection.[192]

3.2.9.
Tryptic digestion of gel protein spots and bands for subsequent mass spectrometry analysis

Chosen protein spots were digested with trypsin, based on the protocol by Havlis and colleagues.[193] The spots were selected, removed from gels with the aid of a micropipette tip and cut into smaller 1 mm^2 pieces. Each spot was then transferred to Eppendorf tubes previously washed twice with methanol and ultra pure water. For the silver nitrate stained gels, a silver removal step was

necessary, conducted by a washing procedure with ultra-pure water and acetonitrile (twice for each spot).

1D gels also went through a reduction and alkylation process before trypsin digestion. The reduction was performed with a 65 mmol L^{-1} DTT solution at 56 °C for 30 minutes followed by alkylation in a 200 mmol L^{-1} iodoacetamide solution for 30 minutes at room temperature. The spots were then left on ice for 60 min in a 50 mmol L^{-1} NH_4HCO_3 solution of comprising 33 ng μL^{-1} trypsin (Promega, WI, USA). The proteins were then incubated at 58 °C for 30 min. 1 µl of 5% formic acid (v/v) was used to cease the reaction. The peptides were after extracted from the gels with 30 µL of a formic acid 5% (v/v) / 50% acetonitrile solution and vortexed for 40 seconds, then left in an ultrasonic water bath for 10 minutes, and vortexed again. After this extraction, the peptides were transferred to new Eppendorf tubes then stored at -80 °C until analysis.

3.2.10.
Mass spectrometry analyses for the fish bile sample

3.2.10.1.
MALDI-MS

The peptide mixture resulting from the tryptic digest from the fish bile sample was mixed at a 1:3 ratio with a saturated solution of α–cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.2% trifluoroacetic acid. 1.0 µL of this mixture was then pipetted onto the MALDI plate and dried at room temperature. The MALDI acquisition method was used in reflector type detection mode for ions with positive polarity. Successive laser shots were made on different sites of the plate containing the samples to obtain adequate signal strength.

3.2.10.2.
nESI-QTOF MS/MS

For nESI-QTOF MS/MS analysis, the peptides obtained by enzymatic digestion from fish bile sample were dried and solubilized in deionized water. An

aliquot (4.5 mL) of the resulting peptide mixture was separated using a C18 column RP-nanoUPLC (Waters BEH C18, 100 mm x 100 mm) (nanoAcquity, Waters) coupled to a Synapt HDMS mass spectrometer (Waters) with a nano-electrospray source at a flow rate of 1.0 mL min^{-1}. The gradient used was 2–90% acetonitrile in 0.1% (v/v) formic acid over 40 min. The instrument was operated using the Data Dependent Analysis (DDA), where the equipment acquires one spectrum per second. When multi-charged species were detected, the three most intense species were fragmented in the collision cell (collision energy set according to precursor's m/z and charge).

These two mentioned analyses were carried out in partnership with the University of Campinas (UNICAMP) and University of State of Espírito Santo (UFES).

3.2.11.
Database research and bioinformatics

For the MALDI MS results, the monoisotopic masses of each peptide were obtained and then inserted in the online program Mascot, MatrixScience using the SwissProt database and "Other Actinopterygii" as taxonomy, which allows the identification of proteins by their peptide maps (PMF - Peptide Mass Fingerprinting). Modifications for trypsin digestion were: carbamidomethylation of cysteine as a fixed modification and oxidation of methionine as a variable modification. Peptide charge: monoisotopic +1; Precursor tolerance; 75 ppm; MS/MS Fragment tolerance: ± 0.2 Da.

MS/MS search parameters defined as ± 0.1 Da peptide and fragment mass tolerance, carbamidomethylation of cysteine as a fixed modification, oxidation of methionine as a variable modification, and a maximum of one missed cleavage. The significance threshold was set at $P < 0.05$.

3.2.12. Sample preparation for metal determination by ICP-MS

100 μL of all liver and bile extracts, 100 μL of crude bile and 100 mg of crude liver were separated and transferred to 15mL sterile polypropylene tubes. 1.0 mL of sub-distilled HNO_3 (Merck, Germany) was added to each tube and allowed to rest for 24 hours. After this time, the tubes were heated on heating plate for 4 hours at 100 °C. After reaching ambient temperature, ultra-pure water was added to 10 mL.

Elemental detection was performed using a NexIon 300X (PerkinElmer, USA) inductively coupled plasma mass spectrometer equipped with a reaction and collision cell. The ICP-MS conditions are shown in Table 8. The following elements were analyzed: Cu, Pb, Cr, V, Fe, Co, Ni, Zn, As, Se, Cd, and Hg. The readings were obtained after external calibration with multielemental calibration solutions obtained by appropriate dilutions of a standard solution (Merck IV). Yttrium (Y) was used to compensate for matrix interferences as internal standard. The precision of the analytical procedure was verified using sample blanks and conducting the parallel analysis of a certified reference material (DORM -2 dog fish muscle tissue, National Research Council of Canada), see in annex 4, in triplicate. No fish bile certified reference material is available up to this date.

Direct analysis by ICP-MS was performed for all samples used in the metalloprotein extraction optimization process and for fish exposed to metals in the laboratory.

3.2.13. SEC-HPLC-ICP-MS analyses

Bile and liver samples from the different metallothionein extraction procedures were analyzed by SEC-HPLC-ICP-MS in order to investigate potential differences in the chromatographic and elemental profiles of each treatment. The SEC-HPLC-ICP-MS on-line coupling was performed by connecting the outlet of a Superdex 75 10/300 GL (GE Healthcare Bio-Science AB, Sweden) size exclusion chromatographic column coupled to a HPLC system

with quaternary pump, manual injector (20 µL loop) and UV detector (Shimadzu, Japan) to the ICP-MS nebulizer inlet by means of a 50-cm PEEK tubing. This system was assembled for elemental analysis subsequent to the protein separation in the chromatographic column. The analyses conditions are given in Table 8.

Six elements (Pb, Hg, Cd, Zn, Cu and Ni) known to bind with metallothioneins were analyzed. Pb, Hg, Cd were monitored to verify if any contamination by these metals was present in the samples, and Zn and Cu are known to be essential elements, both regulated by metallothioneins, while Ni, although still controversial due to being occasionally considered an essential and other times, a non-essential element (see Table 3), still influences MT induction and was, therefore, included in the analyses.

Table 8. Instrumental operating SEC-HPLC–ICP-MS conditions, using in LABSPECTRO – PUC-Rio.

SEC conditions	
Column	Superdex TM-75 (10 × 300 × 13 mm) (GE Healthcare, Uppsala, Sweden)
Effective resolution range	3–70 kDa
Exclusion limit	100 kDa
Mobile phase	Tris–HCl 0.02 mol L^{-1} (pH 7.4)
Flow rate	0.7 mL min^{-1}
Injection volume	20 µL
ICP-MS conditions – NexIon 300X	
Forward power	1100 W
Plasma gas flow rate	17.0 L min^{-1}
Auxiliary gas flow rate	1.2 L min^{-1}
Carrier gas flow rate	0.98 L min^{-1}
Sampling and skimmer cones	Pt
Dwell time	30 ms per isotope
Monitored isotopes	Pb, Hg, Cd, Zn, Cu, Ni for direct analyses also – Cr, V, Fe, Co, As, Se, Cd

3.2.14.

Fourier Transform vibrational spectroscopy in the infrared region (FT-IR) analyses – Qualitative clean-up effects of bile and liver samples

For FT-IR spectroscopy analysis, samples of the crude and extracted bile and liver samples were free-dried and then recorded on a Spectrum 2000 FT-IR spectrometer (PerkinElmer, USA). Sampling was conducted in KBr pellet to characterize the differences between the extracts and the crude samples by the presence of functional groups and the evaluation of the purity of the solid samples. Absorbance spectra of all samples were recorded between 4000 to 450 cm^{-1}.

In order to qualitatively compare the spectra from the crude, extracted and delipidized bile and liver samples, cholesterol standards (Sigma-Aldrich, USA) and the delipidation reagent (Cleanascite™ HC) were also analyzed by this technique, since cholesterol is a major bile constitutent and is removed by Cleanascite™ HC.[194; 195]

3.2.15.

Statistical analyses

For the purified sub-samples, the significant differences in the MT concentrations for both bile and liver samples purified by the different procedures were evaluated by applying the ANOVA test. A factor analysis was then conducted in order to summarize the information contained in the large number of variables into a smaller number of factors, to simplify the data. For bile samples, the Design & Analysis of Experiments (DOE) using response surface methodology (RSM) was also applied, since the main objective of this study is to standardize biliary MT purification procedures. Differences were considered significant when $p<0.05$. The statistical analyses were performed on Statistica 7 (StatSoft®) for Windows.

For the laboratory fish exposures to the different metals, the results of elemental quantification by ICP-MS and MT quantification were analyzed by Spearman's test, generalized linear models and neural networks. The first test,

the correlations (r) were identified as very weak when $0,00 < r < 0,19$; weak when $0,20 < r < 0,39$; moderate when $0,40 < r < 0,69$; strong when $0,70 < r < 0,89$; and very strong when $0,90 < r < 1,00$.[196] The second test, Artificial Neural Networks (ANN) was conducted in order to obtain correlations between the metals used in the fish exposure. This is a computational intelligence technique that consists of a set of basic processing units (neurons) connected by links that transmit signals from one neuron to another. These links have a numerical weight that represents the importance of each input neuron. Each neuron receives a number of input signals and always produces a single output signal that can be transmitted to other neurons.[197; 198]

These last mentioned statistical analyses were carried out in partnership with the Faculty of Statistics and School of Computer Science at the Federal University of Pará (UFPA).

4.

Results and Discussion

4.1.

PART I: Peptide derivatization and complexation with metals

In order to allow for sensitive and specific detection, the chemical derivatization of biomolecules with functional chelating groups, isotopes and metals is widely used in bioanalytical studies. In particular, derivatization reactions are much used in protein analysis for the identification and quantification of protein modifications.[57; 202]

The choice of these metals (Lu, Tm and Ho) occurred because they form thermodynamically and kinetically stable complexes with DOTA. Moreover, they are monoisotopic, with the exception of Lu, that has only two isotopes ($^{175}Lu/^{176}Lu$, natural abundance of 97.4 and 2.6%, respectively), which does not further complicate the isotopic pattern of the labelled peptides in molecular MS. Besides the fact that metals are rarely found in the environment, facilitating the study of optimization and analysis method.

In order to verify the effectiveness of the derivatization reaction in the present study, the labelled model peptides and their mixtures, were analyzed by MALDI MS. The monoisotopoic m/z values and the marked positions in the aminoacid sequence of peptides are shown in Table 9. The Table 9 also shows the m/z values of the possible combinations or combinations found in the MALDI MS analyses for the FPs (free peptides), LPs (labeled-peptides) and MLPs (metal-labeled peptides).

Table 9. Peptides derivatized with NHS-DOTA and their monoisotopic m/z values (derivatization and metal-lanthanide complexation with Lu^{3+}, Ho^{3+} and Tm^{3}). The peptide sequence marking locations are shown by an asterisk.

Peptide	Sequence	Monoisotopic m/z values				
		Underivat.	NHS-DOTA	Lu-NHS-DOTA	Tm-NHS-DOTA	Ho-NHS-DOTA
T1	V*K*CFNCGK*	898.428	2148.944	2664.696	2646.676	2634.664
S34	C*CTK*PESER	1144.426	1916.786	2260.621	2248.608	2240.600
S36	G*ACLLPK*	747.390	1519.75	1863.585	1851.572	1843.564
M6[3]	SLLPAIVEL	954.588	1340.768	1512.685	1506.678	1502.675
S35[3]	VLASSAR	703.410	1089.590	1261.508	1255.501	1251.497

NOTE: [1]A underlined letter C: reduced-SH with MMTS. [2] The underlined values refer to the m/z signals identified by MALDI MS. [3] M6 and S35 were not observed. The values displayed are the theoretical masses.

Usualy in proteomics sample preparation includes cleaning steps, such as desalting and concentration of the sample to enhance detection in molecular MS but unfortunately does not rule out losses in peptide or protein recovery.

Derivatization occurs when a covalent bond of the DOTA amino groups reacts with the free amines present in the peptide, transforming FPs into LPs. In a second step, the metal is complexed by a coordination bond with the ring system present in DOTA, resulting in MLPs. The derivatization with M6 and S35 may not have been effective since they were not observed by MALDI MS, mainly due to the excess amount of chelating reagent necessary for the reaction to occur when compared to the small amount of peptides. This represents a common problem for labeling reactions. The introduction of the metal-DOTA label via derivatization is accompanied by inherent disadvantages, i.e. problems occurring due to incomplete or unwanted side reactions, sample loss and the

costs and time needed. [176] However, the use of metal tags offers a number of potential advantages, justifying these efforts.

A 100 fold molar excess is used, which is a “necessary evil” in labeling techniques,[203] and, thus, some peptide loss that may have occurred, thereby impairing the complexation. Unfortunately the complexation for both peptides couldn’t be tested again after this time because the MALDI MS analysis were accomplished in Germany, and also how we don’t know exactly if the peptides were derivatized and complexed the other analysis like nano HPLC and ICP-MS couldn’t be realized neither. However, most likely has really been some loss or error in preparing the reaction of derivatization and complexation of the peptides in question, since there is literature the derivatization and complexation of these same peptides made by Gregorius *et al.*[176]

For the derivatized and complexed peptides S34, S36 and T1, the reaction with NHS-DOTA resulted in an increase in mass of 386.19 Da per free amino residue, and alkylation of cysteine residues with MMTS represents a mass increase of 91.975 Da. The results regarding S34, S36 and T1 are comparable, for all peptides and lanthanides investigated in this study, indicating the success of the derivatization.

For T1, the m/z signal 2148.944 indicates derivatization at three positions, in two lysine (K) groups and one terminal amino group, besides the reduction of the two cysteine groups present in this peptide. This indicates that T1 was completely alkylated and derivatized. However, complexation only occurred with Tm and Lu.

For S34, m/z values of 1916.786 for the derivatized peptide and 2248.608 and 2240.600 for MLPs Tm and Ho, respectively, were observed. A signal of about 5% of the most intense signal (MLP Ho) was also observed at 2260.621 for MLP Lu.

As for peptide S36, m/z signals were found for the derivatized and complexed forms with all three metals, respectively, as shown in Table 9. Figure 15 shows the nano-LC separation of Ho, Tm and Lu-labeled peptide mixture. The derivatization protocol was, thus, proven to be efficient and has already been successfully applied in other studies.[176]

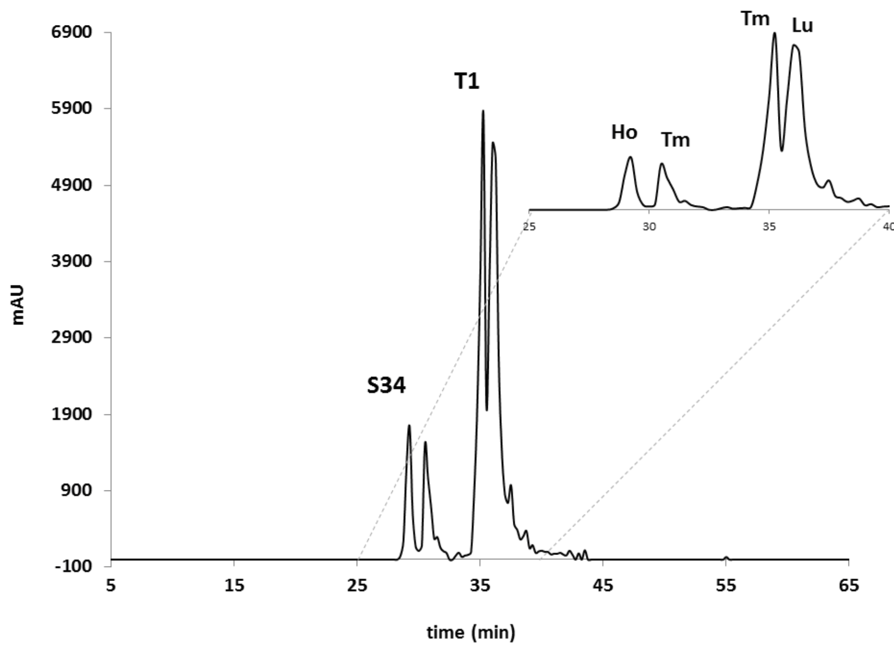


Figure 15. Peptides S34 and T1 identified in the mixture labelled solution (S34, S35, S36, M6, and T1) with Lu, Tm and Ho-NHS-DOTA.

According to the m/z values obtained by MALDI and the chromatogram data of the mixture, there is the possibility of T1 and S36 peak overlapping, which can also be verified by elution in nano-HPLC, of each peptide separately in which the elution time between these two peptides is very close (Figure 16).

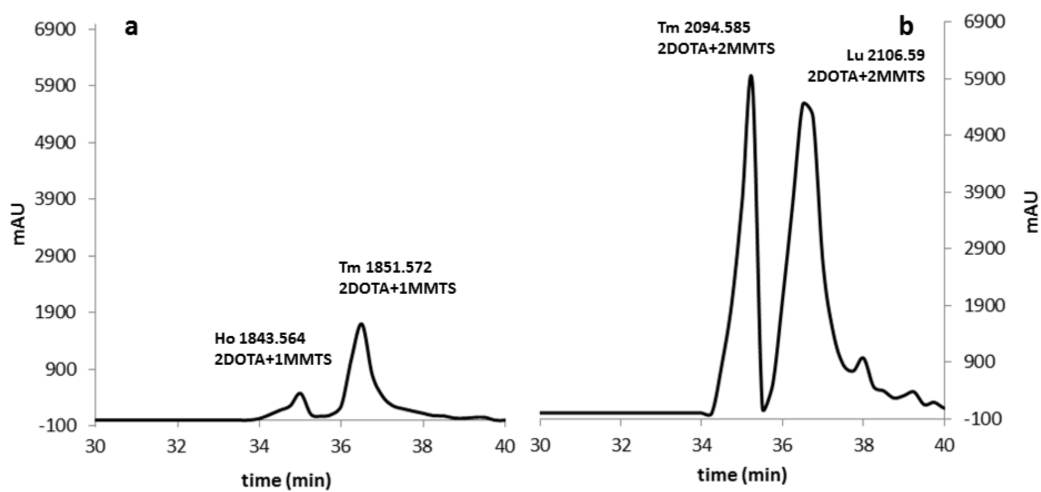


Figure 16. Peptides S36 (a) and T1 (b), respectively, with Lu, Ho and Tm-NHS-DOTA, eluted separately by nano-HPLC UV.

Tm was identified in all peptides and the mixtures peptides with all metals and with each metal separated. Thus, identification of the respective peaks for each element was possible from the chromatograms strict pH monitoring during complexation was conducted, as a way to ensure that the reaction did in fact occur.

The identification of the Tm-labeled peptide peaks in the UV chromatogram can be verified from the elution of each single peptide, as seen in Figure 17.

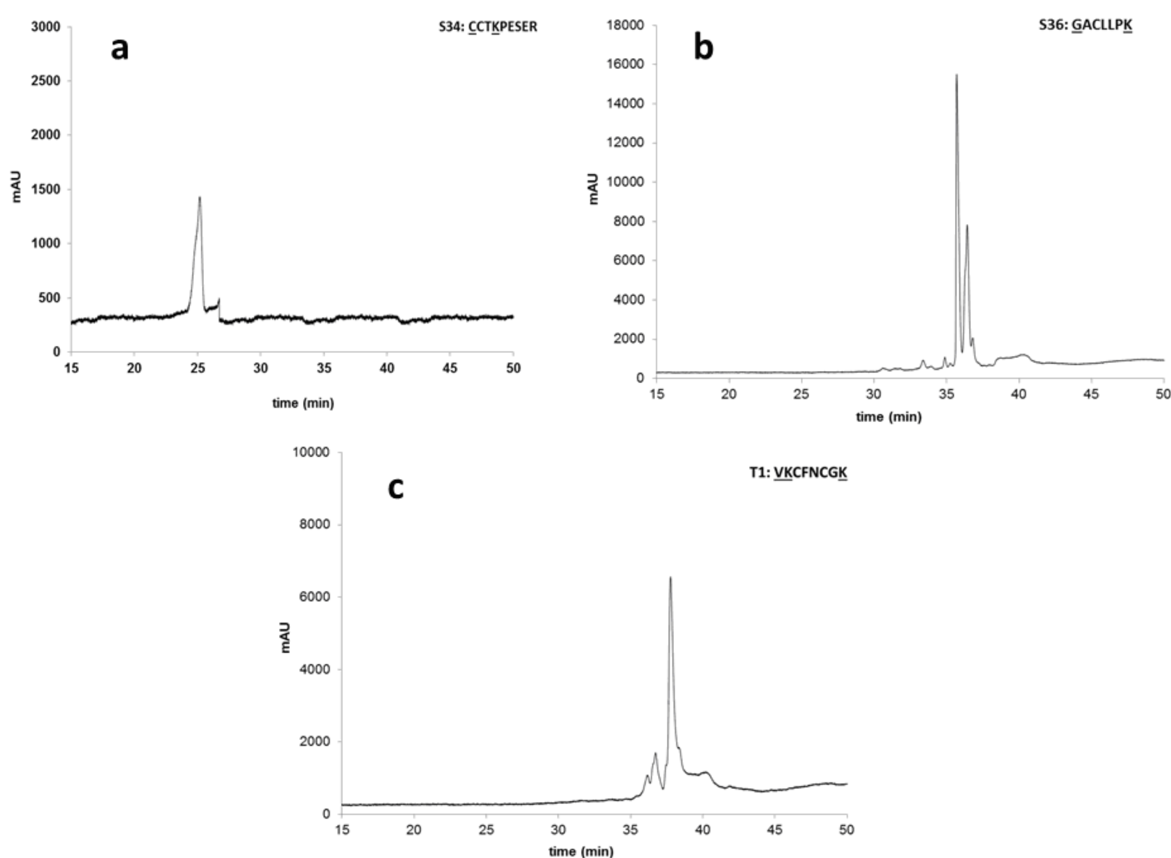


Figure 17. Nano-HPLC UV separation of the single peptides a (S34), b (S36) and c (T1), respectively, using element Tm for the complexation.

After complexation, the sample pH should be between 5 - 5.5, as was mentioned before about the monitoring pH during the reaction. Such monitoring helps control the derivatization reactions when no other methods for confirmation of the efficiency of reaction are avaiable, such as the use of identification by MALDI-MS. Thus, Tm was the element chosen for complexation in a mixture of the three peptides (T1, S34 and S36) and for the study of their separation efficiency by nano-HPLC (as shown in Figure 18), because of mentioned pH monitoring and the mass results obtained before by MALDI-MS.

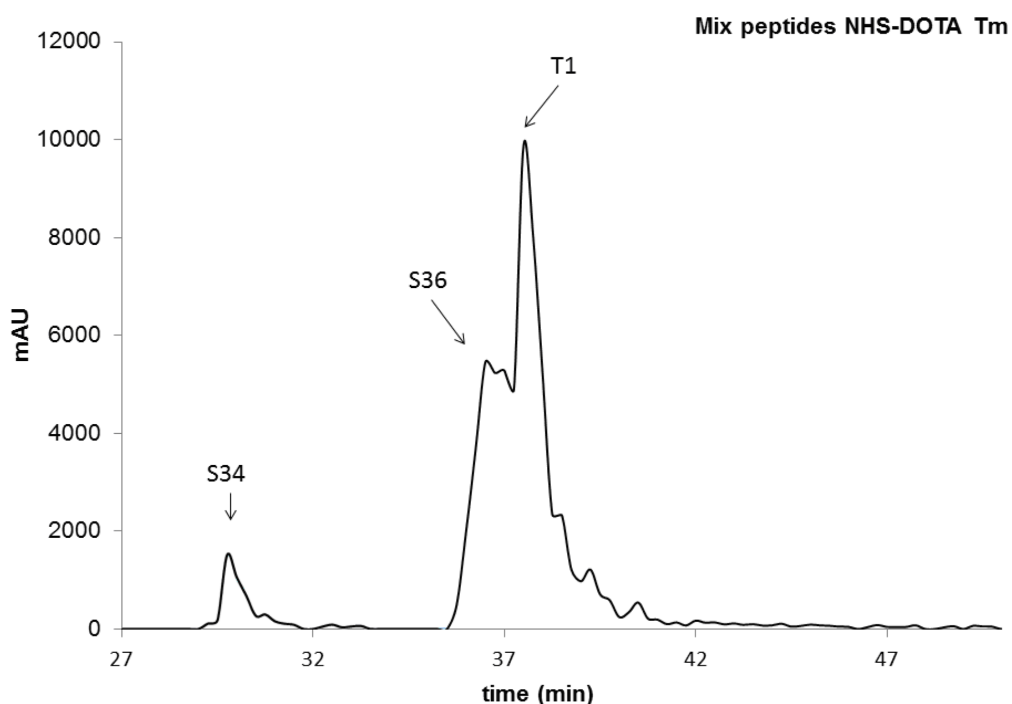


Figure 18. Peptide mixture (S34, S36 and T1) NHS-DOTA complexed with Tm and separated by nano-HPLC UV.

In order to demonstrate the applicability of the metal marking strategy and the identification of proteins in a complex mixture, we also analyzed a Cytochrome C (Cyt C) digest mixture with the DOTA, in order to verify correct peptide separation. Cyt C was chosen because it is a small protein, widely studied in the literature, is soluble in aqueous solutions and does not bind to oxygen.[204] Different experiments were carried out by nano-HPLC, applying different designs, such as different gradient percentages and run times, to verify

derivatization efficiency. Figure 19 shows the separation spectrum of Cyt C NHS-DOTA.

Due to technical issues, the Cyt C digest was not spotted and analyzed by MALDI to confirm the derivatization. However, we ensured the reaction occurred by verifying the pH, which was in accordance with the previous tests conducted with the other studied peptides. According to the manufacturer, the Cyt C digest would be showed by 12 peptides after trypsin digestion, with two of the twelve peptides being impossible to observe by MS and UV, due to adsorption to the vial surface. This is in accordance with Figure 19, in which only 10 peptides were observed. It would be interesting to analyze this digest by MALDI for identification of the separate peptides, in order to confirm the success of the derivatization procedure by an independent method.

The concern with excess metals in the samples was also analyzed. An excessive amount of metal transferred to the MS analysis may be harmful and may also cause equipment damage, in the form of unnecessary metal contamination. Thus, reducing the load of metal injected in the system contributes to the robustness of this analytical step. Because of this, we attempted to eliminate the excess unbound DOTA by a trap column since, due to the high DOTA excess, the background caused by excess metals can in this case be significantly high. It is noteworthy that no m/z signals of the metals were observed, meaning that there were no free metals in the samples after they eluted from the nano-HPLC, confirming that the washing step carried out by the trap column was effective in the metal removal.[205]

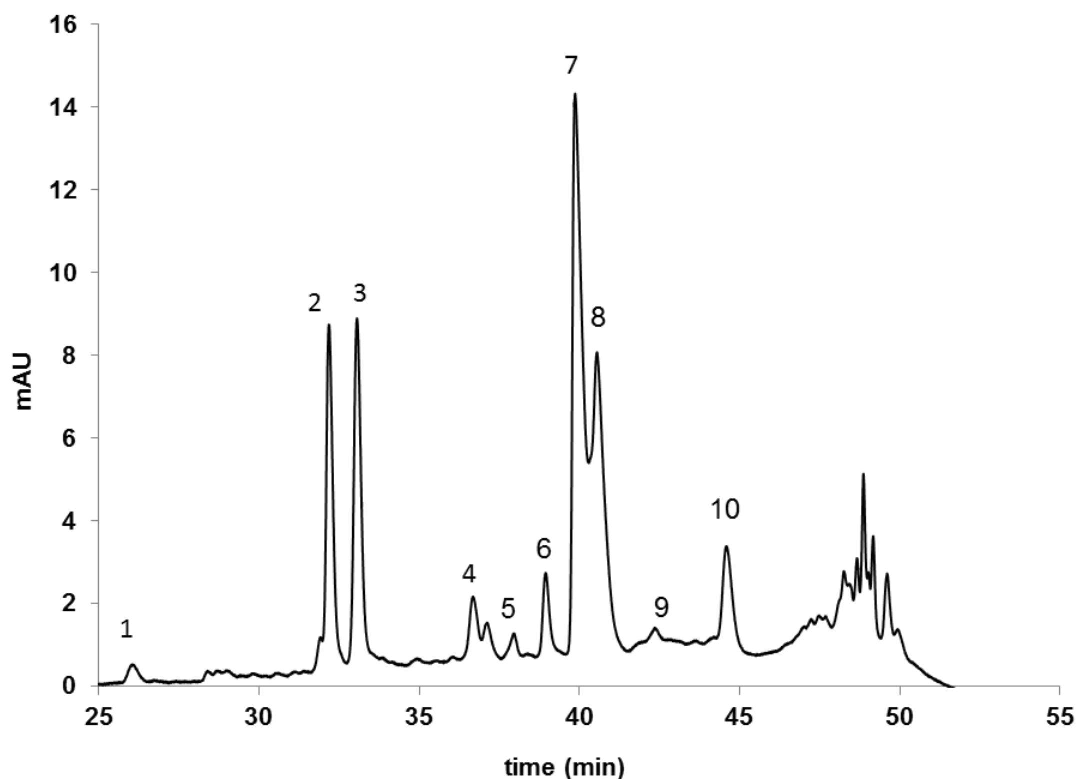


Figure 19. Cytochrome C complexed with Tm-DOTA-NHS-ester and separated by nano-HPLC UV.

4.1.1. Peptide analysis by nano-HPLC-ICP-MS

Due to the high excess used for derivatization with NHS-DOTA (100-fold over free amino groups), we expected a high background caused by the metal excess, especially for the nano-HPLC-ICP-MS coupling. Because of this, pre-washing studies were conducted with the hyphenated techniques, in order to minimize the high background values.

The peptides were trapped in the trap column and washed with the eluent until the valve release for peptide elution to the C18 column. With this we expected less metals in the sample and, consequently, lower ICP-MS background. For these pre-washing tests, we used one of the peptides labeled with ^{169}Tm NHS-DOTA mixture peptides (S34, S36 and T1) and monitored the

^{166}Er isotope added to eluent for monitoring the nebulization stability. Different pre-washing times were applied (3, 6, 10 and 20 minutes), taking into account that a short washing period may not be sufficient to remove all the excess metals, while a longer period, 20 min, could harm peptide detection, by causing peptide losses, where the level could be decrease by 80% compared to the 3 min flush. When was used 6 min, the peptide peak heights remained virtually the same as used 3 min, but in this case the peak was more narrow. Some losses were observed for the mix-peptide later 10 min washing time, while the other peptides in the mixture were still almost completely recovered. Also for the metal Tm, the background could be decreased compared to the minor time of washing. With this in mind, and assuming the best relationship between the possible peptide losses and a low ICP-MS background signal from the metals, a pre-wash period in the trap column of 6 minutes proved to be more efficient and was applied in all subsequent analyses (Figure 20).

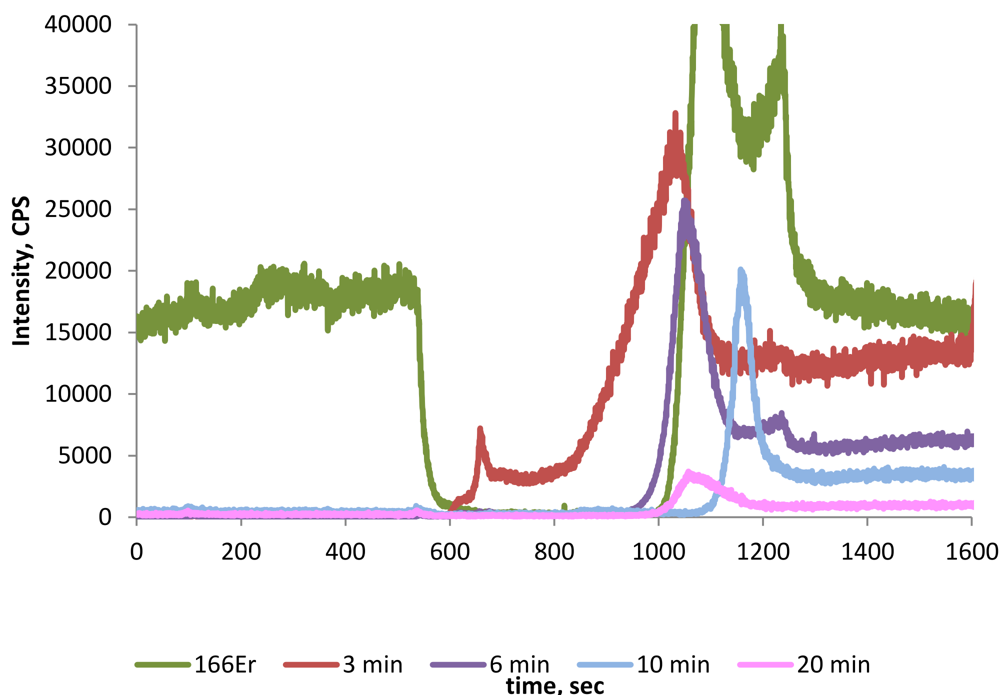


Figure 20. Tm-NHS-DOTA mixture peptide (S35, S36 and T1) pre-washing graph.

Previous studies have already reported chromatograms with a wide and intense profile in HPLC analyses with different columns and different types of reagents, resulting from excess metal.[206; 207; 208] In a study by Schaumlöffel *et al.*

using Lu-labeled peptides analyzed by nano-DTPA-LC-ICP-MS with no trap column, the peak of excess metal exceeded the most characteristic peak of a peptide by nearly 15 times.[57] This demonstrates that a high peak at the start of the analysis is likely to hamper the detection of peptides that elute at the same time, and would also affect the detection of subsequent peaks.

The use of another eluent (0.1% in HFBA (Heptafluorobutyric Acid) instead of TFA, was tested on a peptide with hydrophilic characteristics (sequence = Hy ESLSSSSEE) since RP-HPLC separation of such a peptide is usually not as efficient due to their retention in the column,[209] causing significant sample losses in approaches using pre-columns. A very common way to enhance the RP-HPLC separation is peptide derivatization to increase their hydrophobicity.[210; 211] Thus, the TFA solution was replaced by the more polar HFBA as loading buffer.

In order to improve the detection of the peptide, a Tm DOTA-NHS -labeled Hy peptide solution was tested by ICP-MS with the use of two buffers, TFA and HFBA, in order to better demonstrate the interaction of more polar solution with the peptide in question, shown in Figure 21, including demonstrating the possibility of trapping the Hy peptide in the trap column using the HFBA solution.

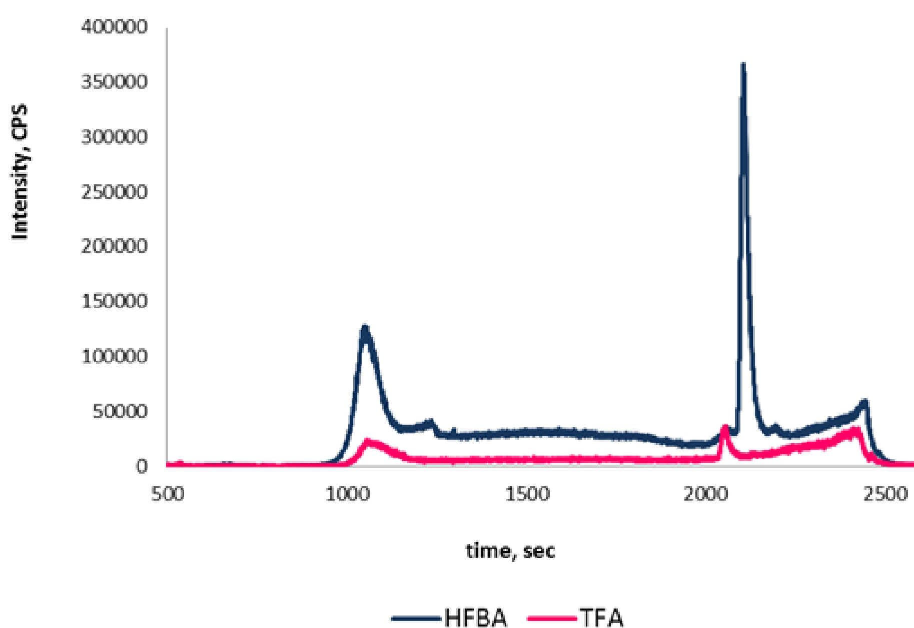


Figure 21. Tm NHS-DOTA-labeled peptide Hy analyzed with HFBA (blue) and with TFA (pink) in the loading buffer.

Care must be taken, however, when deciding to use HFBA for peptide mixtures, because, as shown by Holste *et al.*, when HFBA is mixed with hydrophobic peptides it may not be the best loading buffer choice. These authors, using a mixture of four peptides labeled with Mal-DOTA analyzed by using TFA 0.1% and HFBA 0.1%, respectively, showed that all peptides were separated using TFA, while applying HFBA resulted in a large overlap in retention times.[205]

4.2.
PART II: Optimization of metalloprotein extraction procedures from environmental samples

4.2.1.
Spectrophotometric analyses

With the standard addition of a MT-I purified standard in the fish bile and liver samples we obtained recovery percentages varying between 88.5 and 99.6%, indicating the appropriateness of the method. The angular coefficients of the GSH and the MT-I standard curves did not differ significantly, this result corroborated for the decision to use GSH as a standard for the analytical calibration, besides the fact that no matrix effect was observed. The R^2 for both curves were also very similar, of $R^2 = 0.9943$ for the GSH curve and $R^2 = 0.9864$ for the MT curve. Thus, we opted for using an external calibration with GSH as the standards throughout the study, since GSH is significantly cheaper and easier to obtain than MT standards. Both curves are displayed in Figure 22.

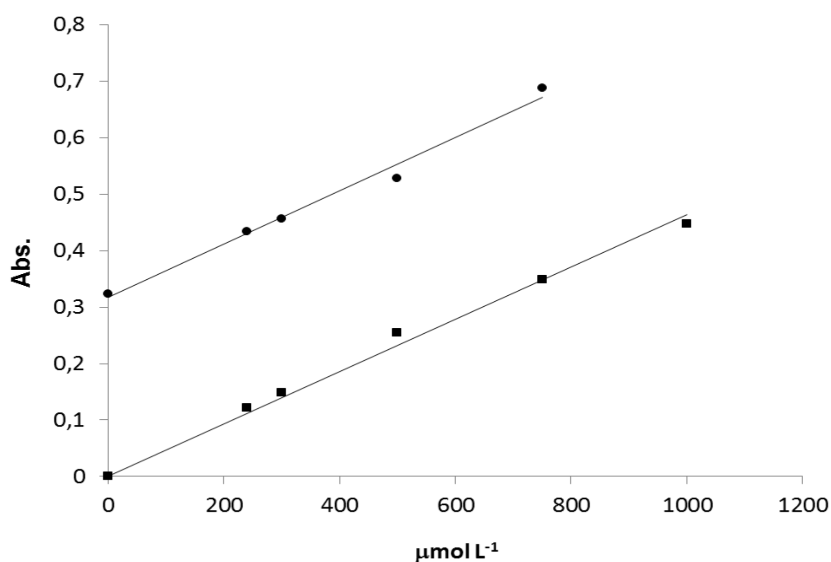


Figure 22. Analytical curves for metallothionein quantification: (■) GSH standards, I= 0.0005 C, R^2= 0.9943. (•) MT standard addition in bile samples, I= 0.0005 C + 0.3167, R^2= 0.9864.

The following figures of merit were calculated: Instrument LOD was 0.63 μmol L^{-1}, method LOD was 1.9 μmol L^{-1}, instrument LOQ was 2.1 μmol L^{-1} and method LOQ was 6.3 μmol L^{-1}. Repeatability standard deviation was 0.003 and the relative standard deviation was of 3.5%. No samples presented MT concentrations below the instrument or method LOQ or LOD.

Three different purification procedures, code-named A, B and C, where established, where the centrifugation times, the extraction temperature, and the different reagents reduction varied as a single factor. The choice of reducing reagents was based on literature that uses the extraction procedure of metallothionein. The most commonly used reagents are DTT and β-mercaptoethanol. However, in this work the efficiency of TCEP reduction reagent was also tested, which have been widely used in metalloproteomics analysis. This reducing reagent was used also in complexation and derivatization of peptides in this study (Part I). Another advantage of TCEP beyond the broader pH range is that its absorb less in UV than the others, and TCEP effectively keeps reducing conditions even at μM concentrations, all this also contributed for this work.

All analyses were conducted in triplicate according to the Table 4, and the results are in Annex 5.

No statistically significant difference between procedures A and B was observed for both bile and liver MT sub-samples ($p < 0.05$) when comparing MT quantification procedures, based on the information displayed in Table 4 (Figure 23). The extraction temperature of 70 ºC using TCEP as the reducing reagent (it has a wide range of pH 1.5 – 8.5 while β-mercaptoethanol has a range of pH 5.0 – 8.5 and the reducing power of DTT is limited to pH values above 6.5), however, were shown to be the most adequate for both matrices, with significant differences ($p < 0.05$) when compared to the other extraction temperatures and reducing agents. Comparing both organs, biliary MT was lower than liver MT, as expected, since liver accumulates MT with slower detoxification rates than bile, that is released from the gallbladder during feeding, and diluted by water.[212]

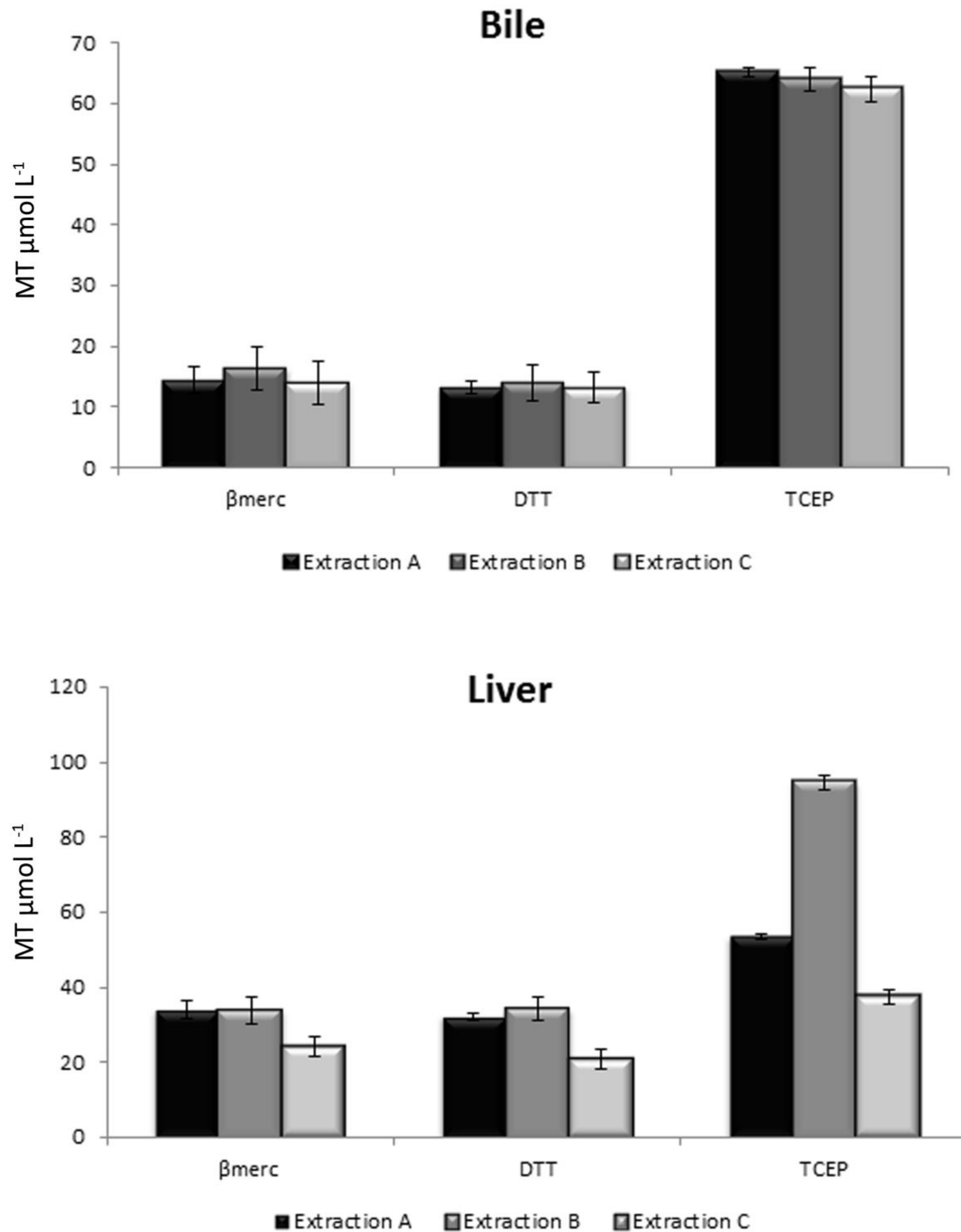


Figure 23. Metallothionein concentrations in bile and liver (expressed in μmol L^{-1}) for each of the tested purification procedures and reagents.

Following the experimental planning for the initial three protocols, we fixed the centrifugation times only to assess the influence of the water bath temperature, according to Table 5. 60 minutes for the first centrifugation and 30 minutes for the second centrifugation (extraction B centrifugation times) were chosen, since extraction B proved to be the most efficient when compared to the

others, even though no significant differences were observed. The reagent used was TCEP, as we concluded from the first extraction results that this is the most efficient among the three tested reagents. The results are in the Annex 6, and Figure 24 shows MT extraction results in bile and liver, with the different water bath temperatures.

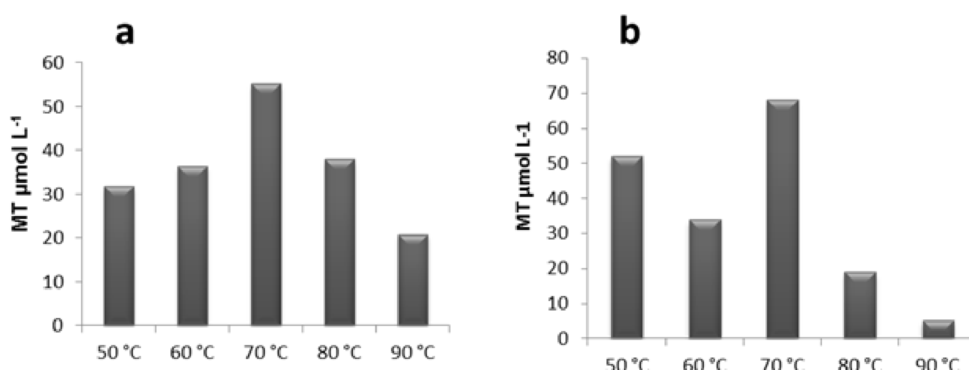


Figure 24. MT concentrations bile (a) and liver (b) at different water bath temperature conditions.

As can be seen by analyzing this data, which showed similar behavior for both bile and liver, the temperature profile resembles a Gaussian curve with a maximum point, corresponding to the optimum temperature for extraction. One reason for this behavior is that, despite being a thermostable protein, MT begins to denature at temperatures over 70 °C. Therefore, at higher temperatures, MT extraction is less effective. At lower temperatures, the extraction is also inefficient, possibly indicating that TCEP function may be impaired, since the better the temperature, the greater the number of interactions between the molecules of the reducing reagent and protein-containing thiol groups.

In the last MT extraction procedure (Table 6), the temperature of the bath was fixed (70 ^{o}C), as was the reducing reagent (TCEP) since we wanted to assess the influence of the first and second centrifugation times (Figure 25), the results are in the Annex 7.

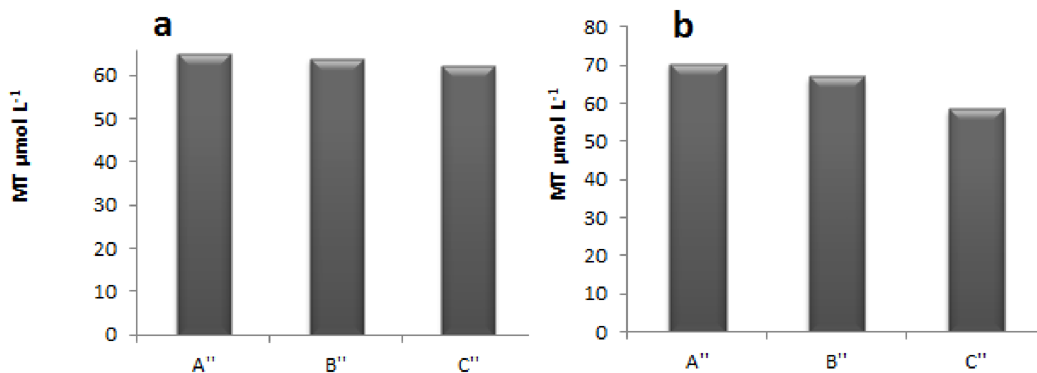


Figure 25. Concentration of MT in bile (a) and liver (b) at different centrifugation times.

As can be seen in Figure 26, although there is a difference between the extraction, it is not statistically significant.

These tests were conducted in order to identify possible differences between the extraction procedures and the reducing agents for both matrices, liver and bile. The second stage of the study was to cross the parameters specifically for bile samples, using 4 factors with 3 levels each in a multivariate factor analysis. The description of each different MT reducing agent in conjunction with the different purification procedures conducted in the present study after the 4^3 multivariate statistical analyses is displayed in Table 7.

Results demonstrated that TCEP showed significantly better extraction results than β-mercaptoethanol and DTT, which also showed higher relative deviations and the presence of outliers (Figure 26), which did not occur with TCEP.

TCEP, in particular, is a potent reducing agent, versatile and practically odor-free. It has been applied broadly to protein studies and other research involving the reduction of disulfide bonds. It is also easily soluble in aqueous solutions. TCEP reduces disulfide bonds as effectively as DTT, but unlike this and other thiol-containing reducing agents, TCEP does not have to be removed before certain sulfhydryl-reactive cross-linking reactions.[108]

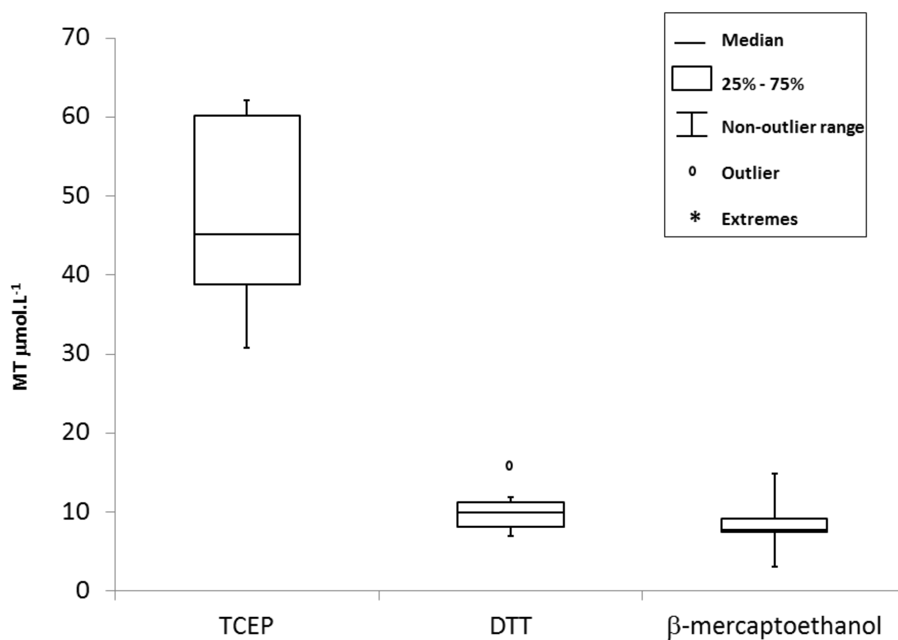


Figure 26. Reagent box-plot chart data after 4^3 multivariate statiscal analyses with TCEP, DTT and β-mercaptoethanol.

The results of a second ANOVA test at this stage showed that both temperature and the choice of the reducing agent are significant ($p<0.05$) factors for MT quantification, as shown in the Pareto Chart of Standardized Effects displayed in Figure 27, constructed from the procedures displayed in Table 7.

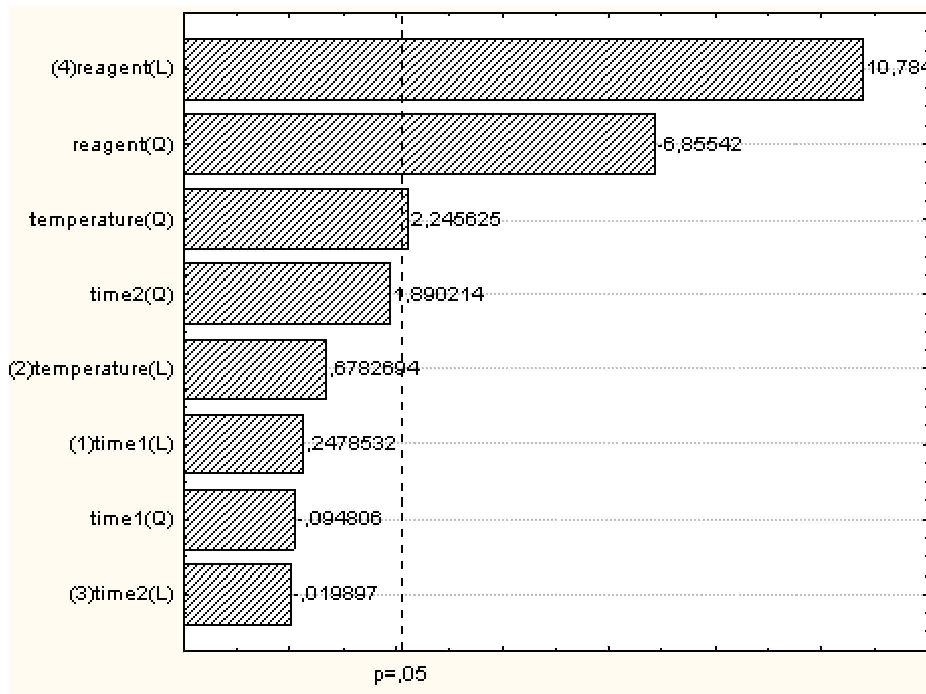


Figure 27. Pareto Chart of Standardized Effects regarding the studied factors for bile samples.

4.2.2.
Response surface methodology for bile samples

Response surface methodology (RSM) explores the relationships between several explanatory variables and one or more response variables.[213] The main idea of RSM is to use a sequence of designed experiments to obtain an optimal response. By analyzing the surface graphs (Figure 28) we observe that the best response for bile samples is given by using the combination of extraction procedures, temperature and reagents, consisting of 45 and 15 min centrifugations, as conducted in procedure A, and 70 ºC, as conducted in extraction B with TCEP 1%.

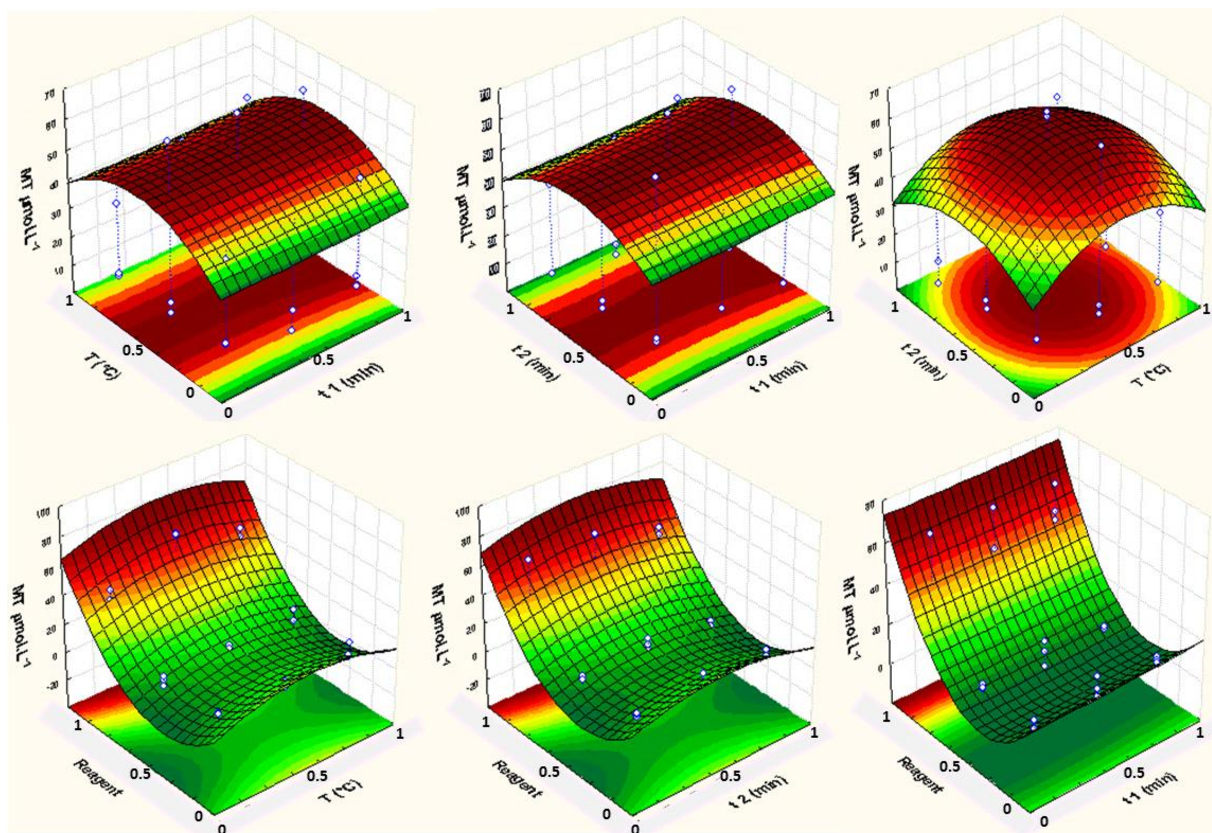


Figure 28. Response surface charts for each of the studied factors for tilapia bile MT samples.

This differs from the protocol found in the literature,[182] which uses 60 min during the first centrifugation, followed by a 70 ºC thermal-extraction and 30 min during the second centrifugation and uses β-mercaptoethanol as the reducing agent. The protocol established in the present study, therefore, is quicker and significantly more efficient for fish bile, and also corroborates previous reports indicating that TCEP is a powerful reducing agent, due to the increased extraction efficiency observed for bile and liver MT when compared to both DTT and β-mercaptoethanol. Also as is reported in the literature, TCEP selectively and completely reduces even the most stable water-soluble alkyl disulfides over a wide pH range. Compared to DTT, TCEP is more stable, more effective, and able to reduce disulfide bonds at lower pHs.[237]

TCEP, thus, may be used as a substitute for both of these reducing agents. Bile is also an easier biological matrix to analyze when compared to liver, since it is naturally present in liquid form, may be sampled without having to

sacrifice the animal, since bile duct cannulation is a possibility [172] and shows enormous potential regarding environmental monitoring of xenobiotic effects on the proteomic and metalloproteomic expression of this fluid.

4.3.
SDS-PAGE analysis

4.3.1.
1D-SDS-PAGE

The qualitative 1D SDS-PAGE analyses also demonstrated that better purification results are achieved when using TCEP as the reducing agent for both liver and bile, corroborating the statistical analyses described previously. Also, this reagent is efficient in a wider pH range than the other two reagents, more stable, odorless and non-toxic, as described previously, making it a better choice for this type of study. When using this reducing agent, MT extraction was more efficient and the final supernatant was purer, with less discernible protein bands in different molecular weights, than MT (14 kDa in tilapia, as described by Hauser-Davis, *et al.*)[18]

When comparing these electrophoretic qualitative results (Figure 29) with the spectrophotometric quantifications of bile MT, differences where observed: For bile samples, the spectrophotometric analyses showed no significantly statistical difference ($p<0.05$) for procedures A and B, while the SDS-PAGE analyses showed that protein bands at around 150 kDa disappeared in extraction procedures B and C. However, procedure A was more efficient regarding the exclusion of low molecular weight proteins. Bands above 250 kDa were present in all extraction procedures. Bands at around 50 kDa were present in all procedures except for those using TCEP, further confirming this reagents' efficiency. Weak bands between 50 and 75 kDa were present only in extraction A with DTT and β-mercaptoethanol and absent from the TCEP procedures and in extraction B and C with these reducing agents. Procedure C, even when using TCEP, was not as efficient, as seen by the slightly fainter bands on the SDS-

PAGE gels, probably due to the significantly higher temperature used in the process, which may severely denature proteins present in the sample, while procedures A and B (60 ºC and 70 ºC, respectively), showed stronger MT bands.

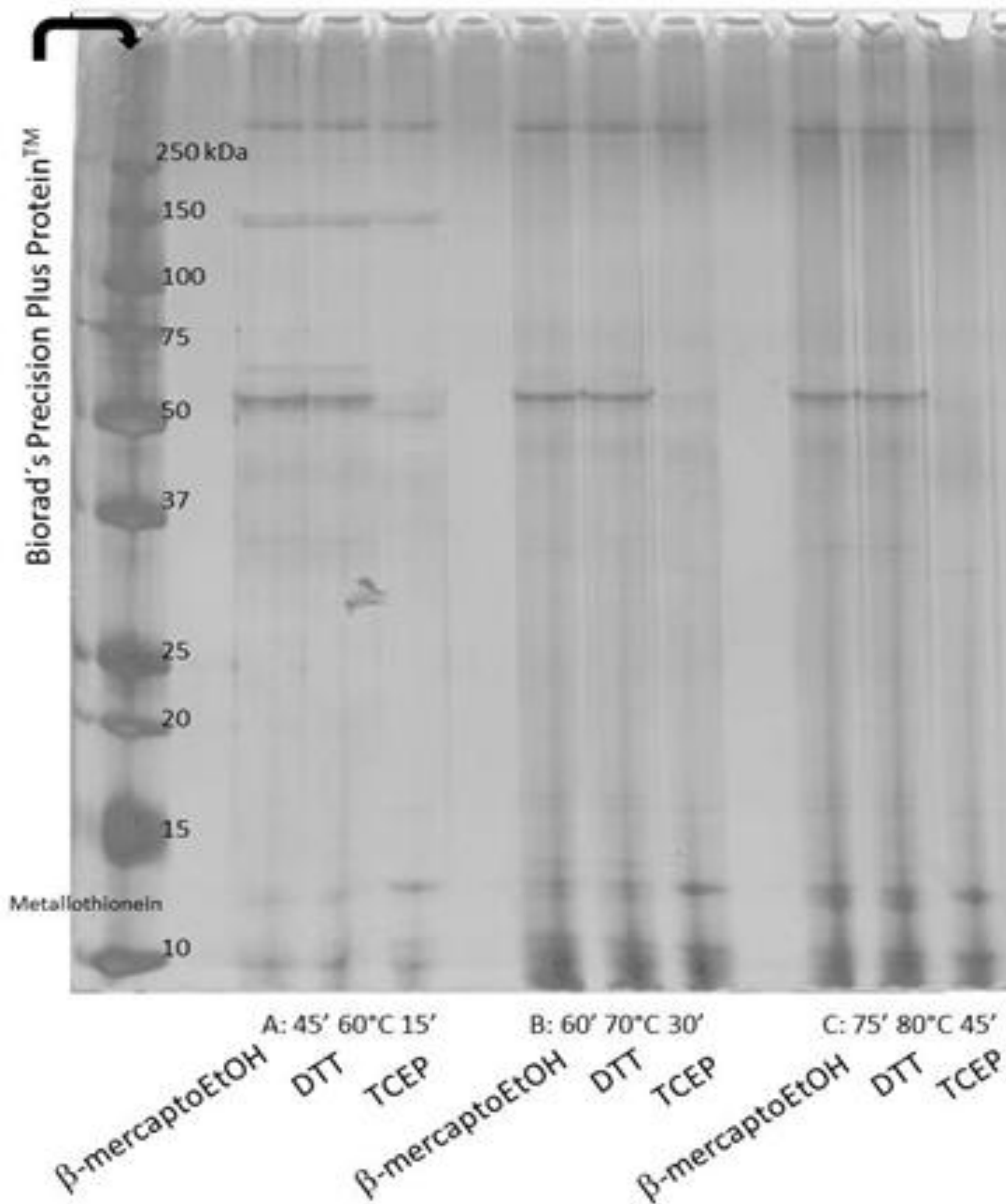


Figure 29. Qualitative SDS-PAGE gels for bile samples using the different extraction procedures and reagents analyzed in the present study.

For liver samples, protein bands at around 150 kDa also disappeared in extraction procedures B and C, indicating that the proteins present in this band denature in temperatures above 70 ºC. Weak bands between 25 and 20 kDa were present in extractions B and C with DTT and β-mercaptoethanol for both procedures, and even weaker bands were present in extraction B using TCEP, Figure 30. This band was absent from the TCEP procedure in extraction. Procedure C, even when using TCEP, was not as efficient for MT extraction, as seen by the fainter bands on the SDS-PAGE gels, also probably due to the higher temperature used in the process. Liver, however, probably due to being solid and more complex than bile, did not show such “clean” gels and distinct protein bands when compared to bile in the present study, further indicating that bile analyses are easier to conduct and show better results in this context.

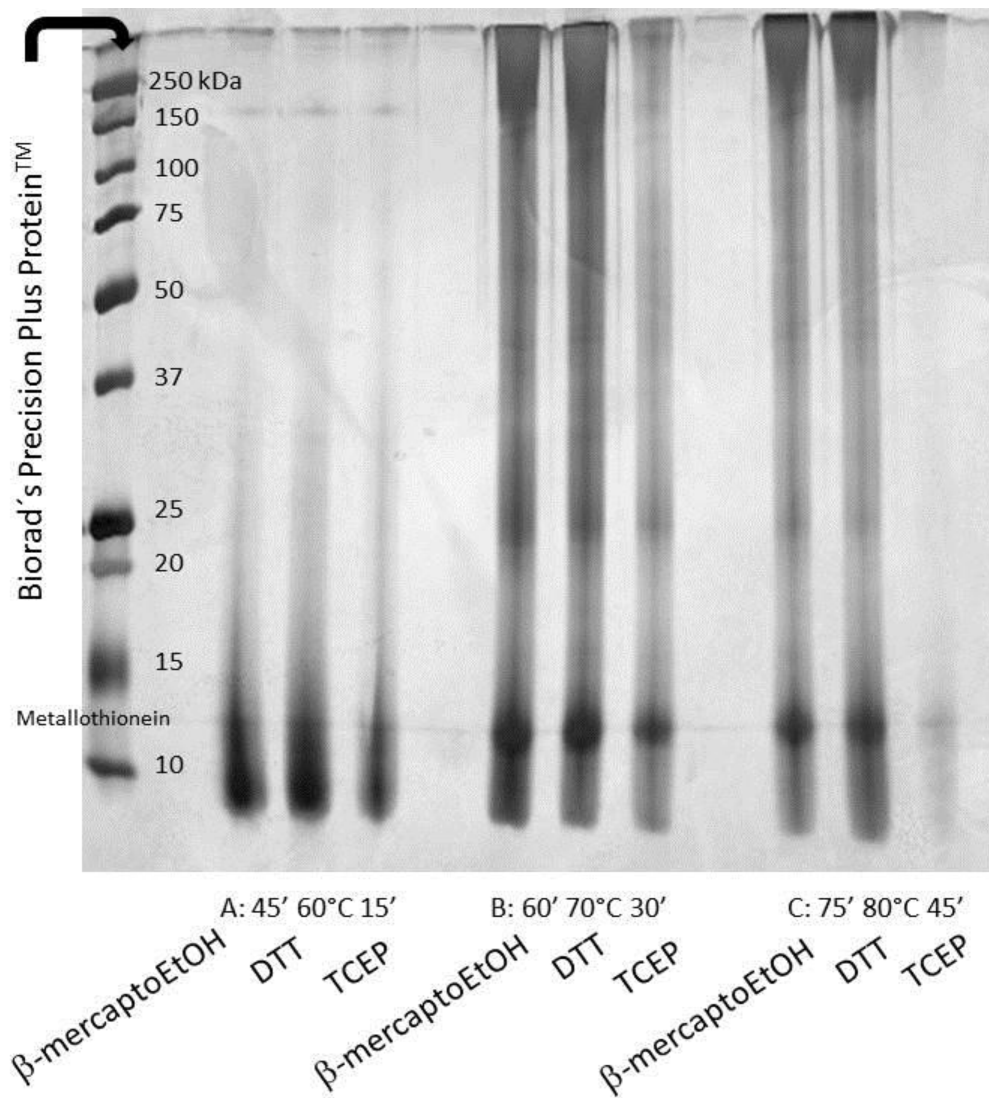


Figure 30. Qualitative SDS-PAGE gels for liver samples using the different extraction procedures and reagents analyzed in the present study.

4.3.2.
2D-SDS-PAGE

Samples submeted to 2D gels were run using the three extraction procedures (A, B and C) treated with the three reducing reagents (DTT, β-mercaptoethanol and TCEP), in order to identify qualitative differences between the different protocols after analysis by mass spectrometry for the identification of the proteins present in each spot. Significant differences regarding the reducing reagents were observed, with better, “cleaner” gels resulting from the samples treated with TCEP, with some spots present in the gels where DTT and β-mercaptoethanol were used and absent from the TCEP gels (figure 31). Some spots were selected, trypsinized and analyzed by mass spectrometry after trypsinization.

These results indicate that SDS-PAGE analyses are useful in corroborating the standardization results obtained by the spectrophotometric and statistical analyses regarding bile and liver MT. Furthermore, they aided in distinguishing certain characteristics that may not be observed in spectrophotometric analyses of the different purification processes, such as the presence of other proteins in the purified samples. In this regard, the presence or absence of other proteins in fish bile may be of interest in environmental monitoring contexts and proteomic studies, and may or may not interfere with other downstream applications, and are, therefore, of interest and should be further analyzed.

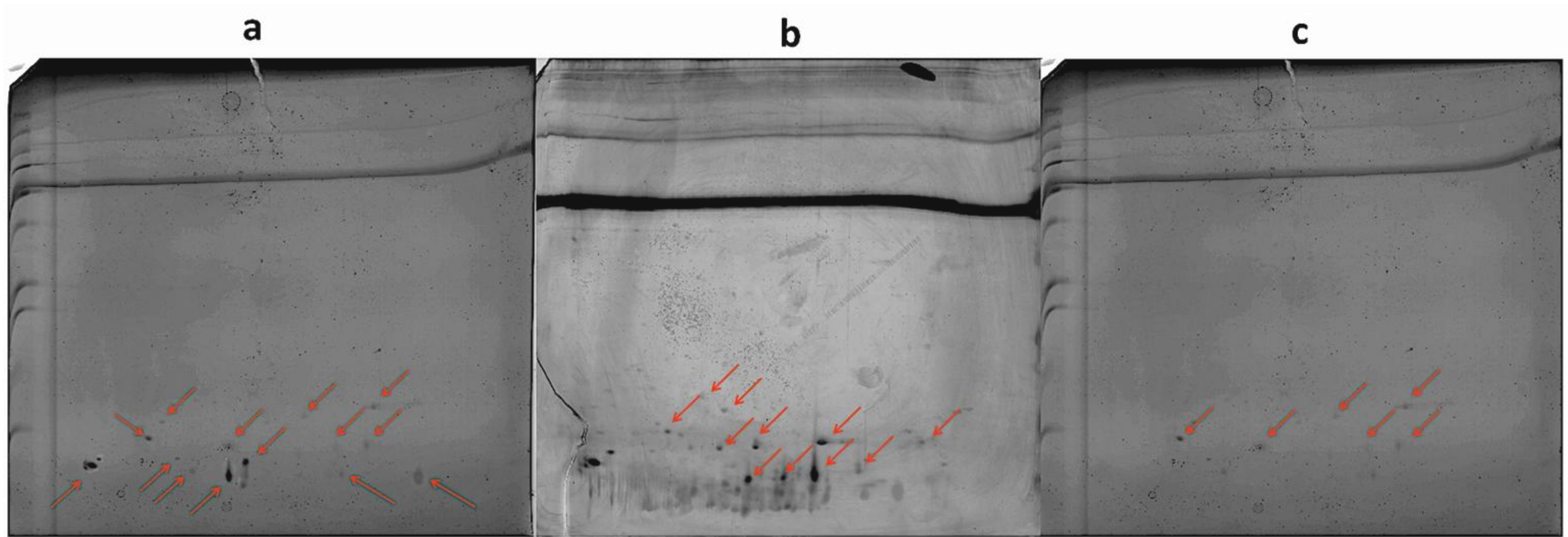


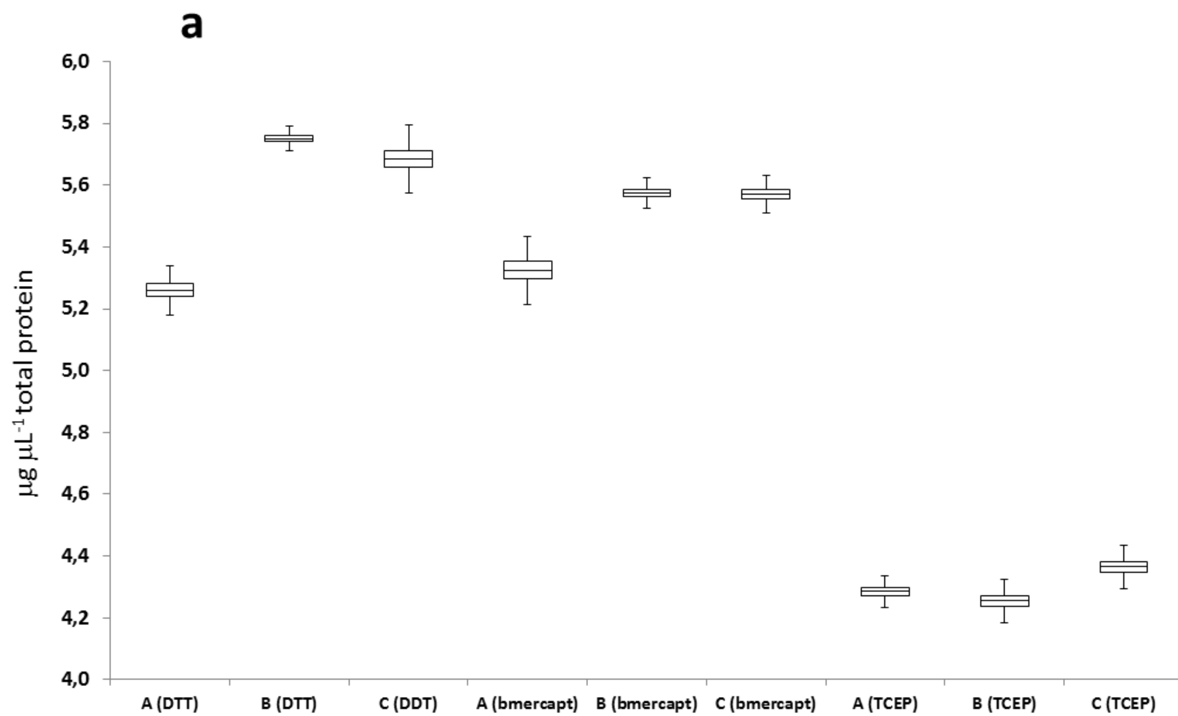
Figure 31. 2D Gels of an MT extracted bile sample - protocol B (60 minutes, 70 ° C, 30 minutes) and reagents DTT (a) b-mercaptoethanol (b) and TCEP (c).

4.4.
Total protein quantification

4.4.1.
Commercial fish samples

Samples acquired with the fish suppliers were used for the optimization tests. The level of total protein in these samples was determined due to the need to know the total protein concentration for gel preparation and, consequently, mass spectrometry analysis in order to identify the proteins present in the extracted samples. The Lowry method modified by Peterson was used and proved efficient and reproducible.

The quantification of total protein in extracts of liver and bile used to optimize the extraction protocol metallothionein proved consistent with the results obtained by SDS-PAGE, as in Figures 30, 31 and 32. The best extraction, as discussed above, was with the reagent TCEP since the 1D and 2D gels treated with the reagent proteins appear "cleaner", which indicates a better extraction of heat stable, rich in SH residues, proteins with the same characteristics as MT. Consequently, a more efficient extraction should lead to less total protein content in the samples (see Figure 32), leaving only MT-like proteins in the extracted samples.



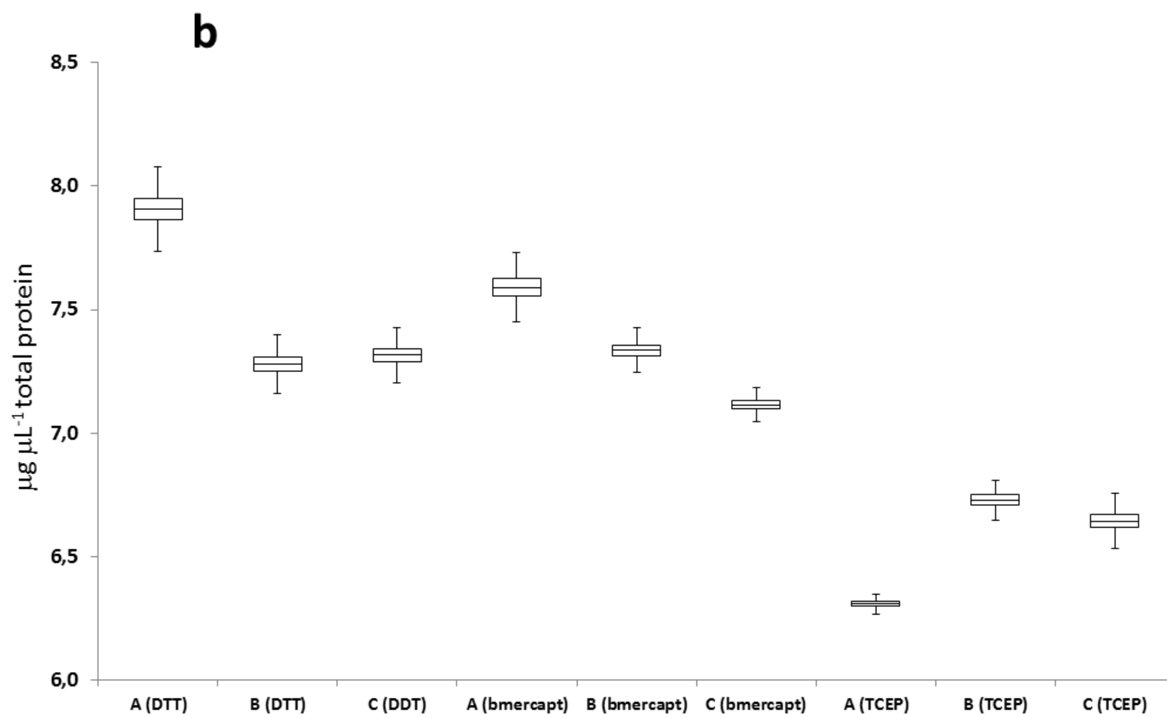


Figure 32. Box plot - measurement of total protein in bile (a) and liver (b) extraction with different procedures.

4.5.
Mass spectrometry analyses

In addition to the 1D and 2D gel bands and spots, extracted bile solutions were also delipidized by Cleanascite®, dessalted by Vivaspin® and trypsinized (in-solution digestion). However, after digestion and resuspension in 3% ACN and 0.1% FA, an inefficient trypsinization was observed for the extracted bile solutions and, therefore, these samples could not be analyzed by mass spectrometry.

Thus, the results shown (Table 10) and discussed refer to the 1D and 2D gel spots and bands for bile.

Table 10. Identified proteins from fish bile from bands and spots gels.

Protein
Protein phosphatase 3, catalytic subunit, alpha isozyme
Ferric uptake regulator [*Escherichia coli* O157:H7 str. EDL933]
Hemoglobin subunit beta-A-like isoform 1 [*Oreochromis niloticus*]
Parvalbumin beta-like protein [*Oreochromis niloticus*]
Metallothionein B [*Oncorhynchus mykiss*]

Phosphatases are enzymes responsible for the dephosphorilation of phosphoaminoacid residues, mainly phosphotyrosine and phosphoserine/treonine, which separates this class of proteins in the PTP (phosphatase tyrosine protein) and the PP (phosphoserine/treonine protein). Several members of the PP family, in particular the PPP (Phosphoprotein phosphatase) subfamily, exist as holoenzymes composed of a catalytic subunit associated to one or more regulating, which confers functional diversity to these proteins.[214] The protein phosphatase 3 (PPP 3), also known as Calcineurin or phosphatase 2B (PP2B), is a serine/treonine protein firmly regulated by Ca^{2+}, and plays a critical role in transduction pathways mediated by calcium.[215] Separation and characterization studies with phosphatases show the permanence of active

sites and structural elements at high temperatures (70 °C) [216; 217] indicating that these are also thermostable proteins, as what we obtained in our study.

The protein **ferric uptake regulator (Fur)**, present in the cell cytoplasm, acts as a global negative controlling element, employing Fe^{2+} as a cofactor to bind the operator of the repressed genes. This protein regulates the expression of several outer-membrane proteins including the iron transport operon [218]. This protein can be activated by elements such as cadmium, cobalt, copper and manganese. Although iron is essential for most organisms, high concentrations may be toxic due to hydroxyl radical formation.[219] Ferric uptake regulation can also control zinc homeostasis (see statistical results about elementary inter-correlations in the study of exposure in the section 4.7.1.) and is the subject of research on the pathogenesis of mycobacteria.[220] Conformational and thermodynamic stability studies with this protein have confirmed its thermo stability, possibly related to its low thermal capacity.[221]

Hemoglobin is the iron-containing oxygen-transport metalloprotein in the red blood cells of all vertebrates as well as the tissues of some invertebrates. Certain species use different molecules to bind to hemoglobin altering affinity for O_2, under unfavorable conditions. Fish, for example, use ATP (adenosine triphosphate) and GTP (Guanosine-5'-triphosphate). These bind to a phosphate "pocket" on the fish hemoglobin molecule, which stabilizes the tense state and therefore decreases oxygen affinity.[222] A **hemoglobin subunit beta**, specifically, is involved in the transport of oxygen from the gills to the various peripheral tissues.[223] This is also a thermo stable protein,[224] as we can verify in our study.

Parvalbumin (PV) is a calcium-binding albumin protein with low molecular weight (typically 9-11 kDa). It is a small protein containing EF-hand (helix- loop-helix structural domain) type calcium binding sites. It is involved in calcium signaling and localised in fast-contracting muscles, where its levels are highest, and in the brain and some endocrine tissues.[225] This is a widely studied protein because it is one of the main proteins that causes allergies to fish products.[226; 227] Studies also demonstrate the relevance of the thermostable capacity of this protein in different thermal treatment studies, through eletrophoresis studies using heated PVs.[228] With this affirmation, we can also mention as a promising and simple biochemical tool to differentiate closely related species.

Metallothioneins have a high content of cysteine residues that bind various heavy metals. Class I metallothioneins contain 2 metal-binding domains: four divalent ions are chelated within cluster A of the alpha domain and are coordinated via cysteinyl thiolate bridges to 11 cysteine ligands. Cluster B, the corresponding region within the beta domain, can ligate three divalent ions to 9 cysteines. In our study, this protein was identified at the 14 kDa region of the 2D gel and its spectrum is shown in the Figure 33, in the *Oncorhynchus mykiss* (rainbow trout) database. This protein may not have been identified in the *Oreochromis niloticus* database due to the fact that this species is not yet 100% coded in databases of protein identification programs. In addition to the presence of various isoforms,[229] some of these have not yet been identified. Also, the fact that trypsinization was derived from a dimer spot of this protein (14 kDa instead of 7 kDa), in which polymorphism may have occurred, may have caused minor conflicts in the protein identification.

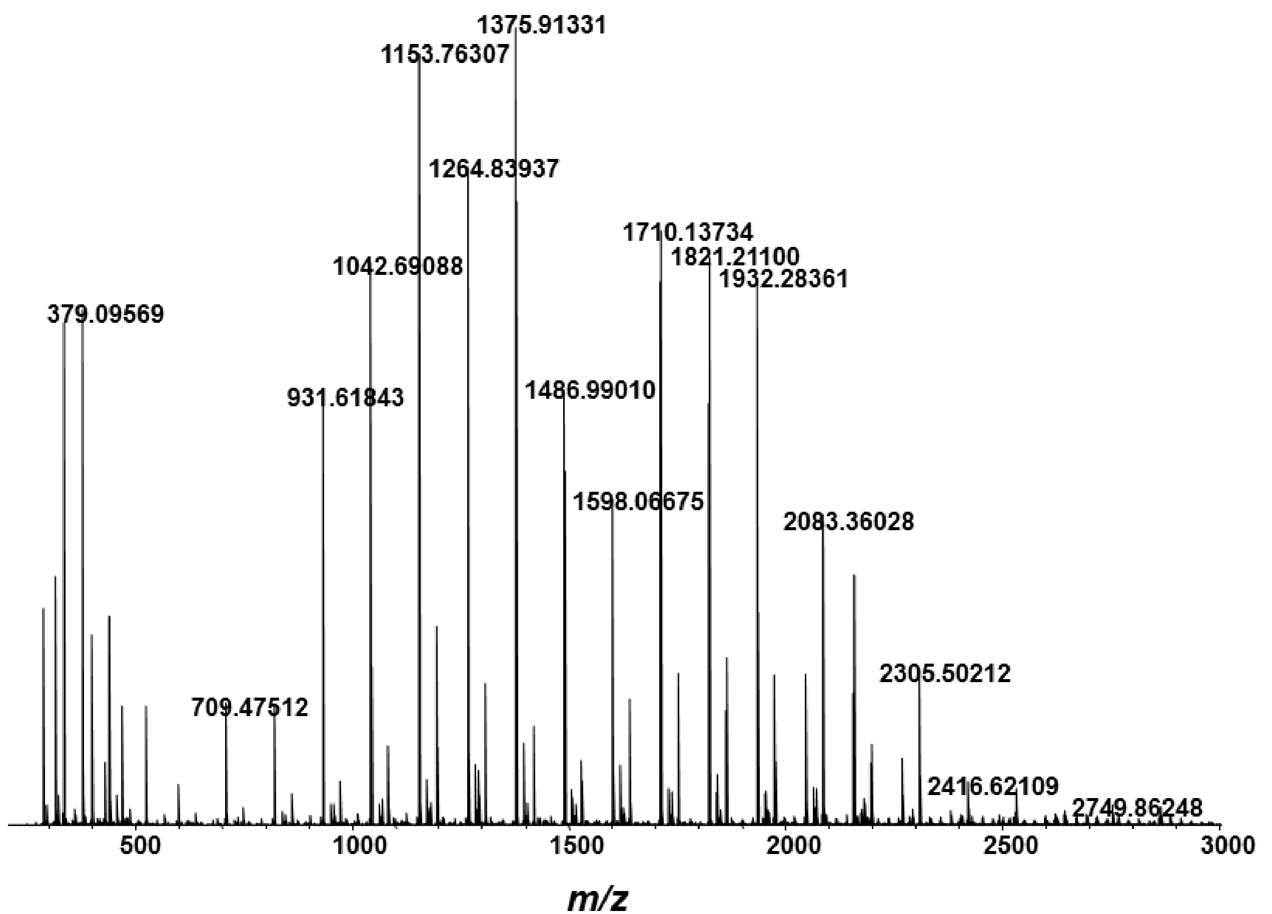


Figure 33. MALDI MS spectra of spot gel 2D trypinized where obtained the MT-B.

All the identified proteins are thermostable, which makes sense in the context of this study, since only thermostable proteins would be able to resist the high temperatures employed in the MT extraction procedures applied. The proteomic profile of fish bile after these procedures, thus, contains mainly these types of proteins/metalloproteins. The identification of other proteins besides the metallothionein bile extracts shows that in addition to being as previously described thermostable proteins are proteins which may also have strong potential biomarkers and/or indicators of biological changes in organisms studied.

4.6.
SEC-HPLC-ICP-MS analyses

The detection of metal compounds in a sample is the pre-requisite for any additional metallomic study regarding identification, characterization and role in biochemistry.[230] SEC-HPLC-ICP-MS analyses are a first recommended step for viewing metallothionein profiles, and are considered a valuable tool in detecting metalloproteins in general.[48] By coupling SEC-HPLC with ICP-MS, we can determine which elements are attached to these proteins, aiding in the characterization of metal contamination, if any, and also in the investigation of possible differences in the behavior of MT metal-binding profiles in different situations.

For this analysis, bile and liver extracts treated with the different procedures studied for metallothionein extraction were used, employing two of the different reagents involved: DTT and β-mercaptoethanol. TCEP was not tested because of a delay in importing this reagent and, when the reagents finally arrived, because of equipment problems. MT-I standard solutions (with the presence of Cd), BSA, GSH and ferritin were used for column calibration and control, figure 34.

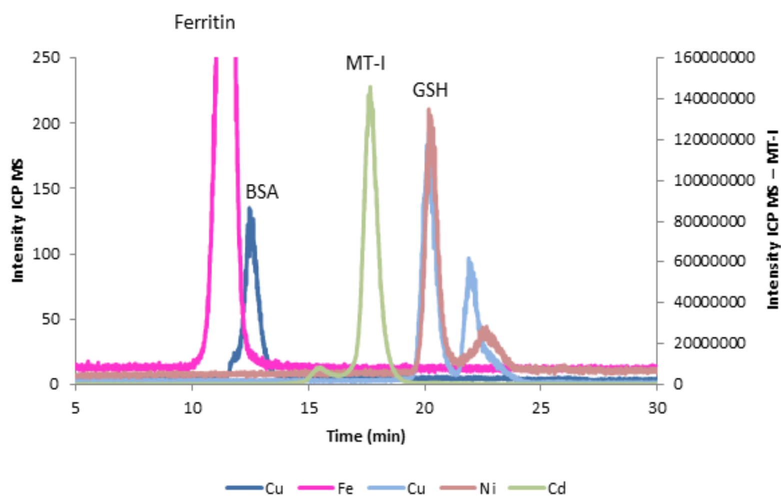


Figure 34. Standards used for column calibration in the SEC-HPLC-ICP-MS analyses.

The SEC-HPLC-UV chromatogram (Bile extracts and MT-I) and SEC-ICP-MS spectra (metalloproteins) obtained with the different extraction procedures (treated with DTT and β-mercaptoethanol (protocols A, B and C)) are displayed in figure 35.

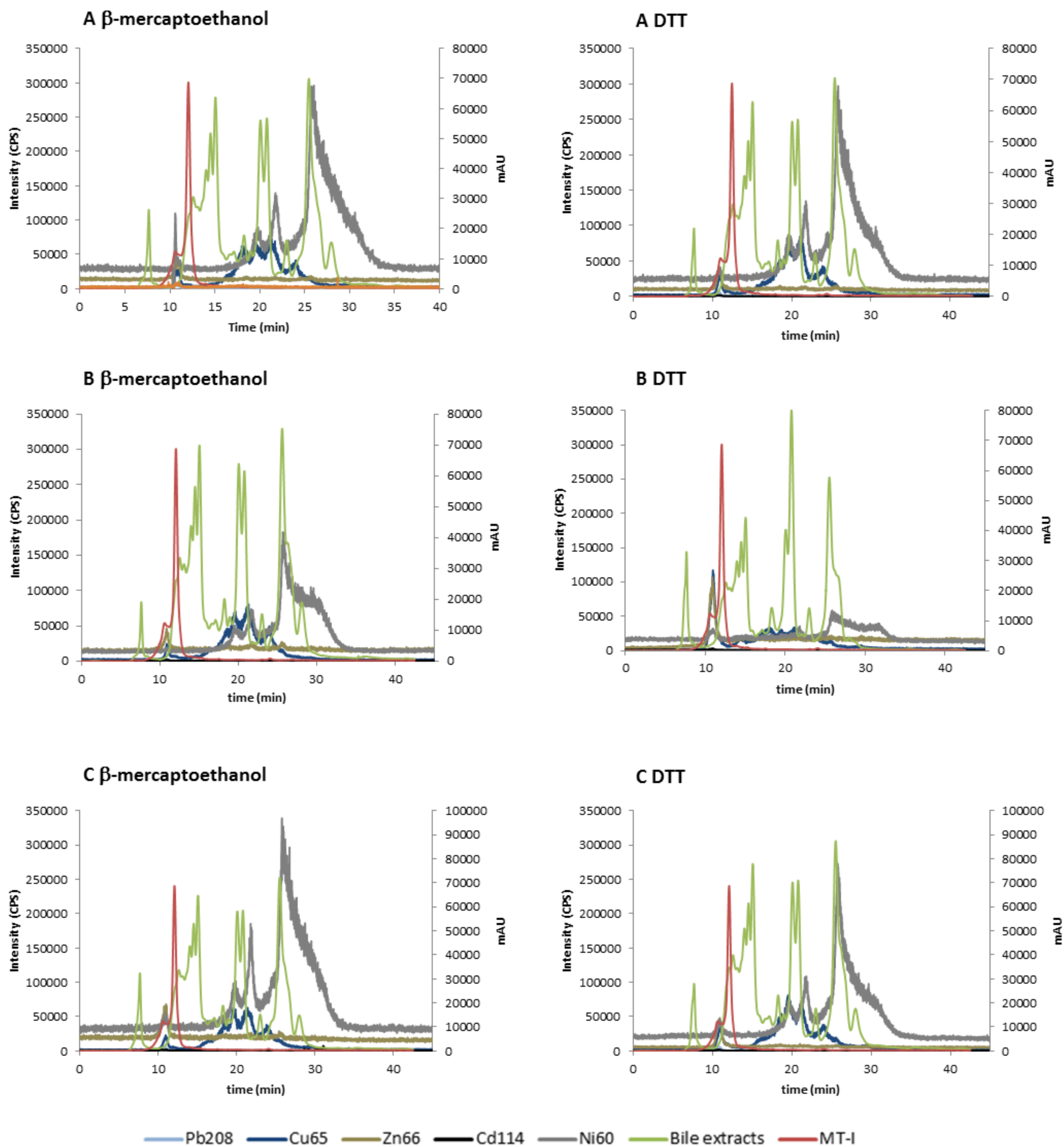


Figure 35. SEC-HPLC-UV-ICP-MS samples and MT-I standard for bile extracts.

All readings were conducted on the same day, in order to compare signal intensities, and thus, indirectly, concentrations. No significant changes were observed between the treatments, however, a small difference in the chromatographic profile when the sample is treated with β-mercaptoethanol (protocol B) was observed, influencing the MT peak and the zinc bound to this

protein, although the peak of this element was well defined in all measurements. The analyses were performed on the same day, thus enabling the comparison of the intensities between the two spectra.

The presence of Zn in the samples is due to the fact that this metal is an essential trace element required for certain metabolic processes, playing a role as a cofactor for several metalloproteins and enzymes in most living organisms, while the other factors analyzed in the study (Pb, Cd and Ni) are non-essential metals. In addition, zinc is also a metallothionein regulator.[231] These factors further corroborate the presence of basal MT levels in non-contaminated situations.

The rapid differentiation between MT bound or non-bound cadmium by this methodology is also very important, since Cd is a toxic element and a noteworthy environmental contaminant, especially in aquatic ecosystems, and, since MT also plays a role in the detoxification of both essential and non-essential elements, such as Cd, this is of interest in environmental contamination studies.

The next step was to conduct SEC-HPLC-ICP-MS analyses on samples from the 4^3 multivariate statistical planning of a same pool of bile samples extracted according to the previous experimental planning for spectrophotometric determinations. This stage used two reagents only, DTT and β-mercaptoethanol, since, unfortunately, TCEP was not available at the time. The same temperature was used (70 °C), with the different centrifugation times applied for procedures A (45 min and 15 min) and B (60 min and 30 min). In this case, it was possible to visualize that, in the lesser centrifugation times, the removal of metalloproteins from fish bile was more efficient, including the elimination of possible free metal ions (figure 36).

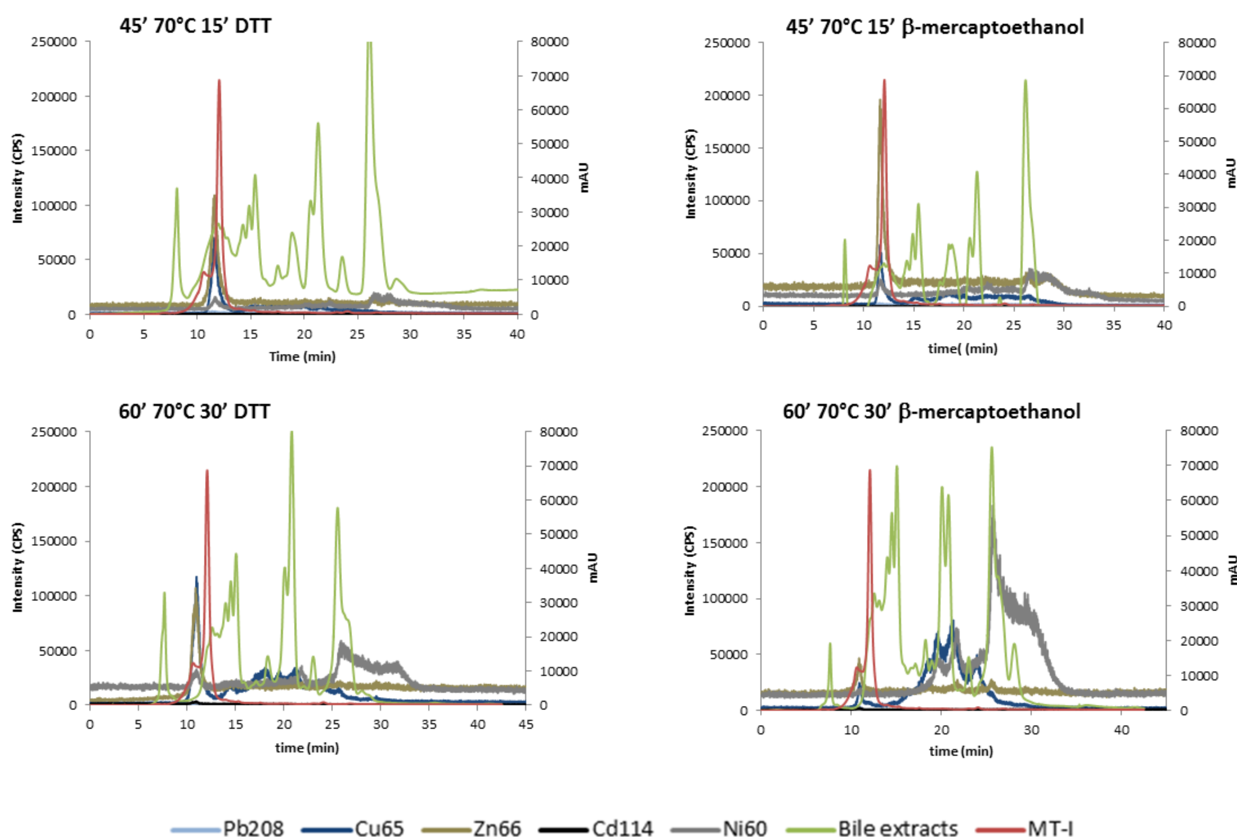


Figure 36. SEC-HPLC-UV-ICP-MS fish bile samples and MT-I standard for different extraction centrifugation times and the same temperature.

Significant differences where observed for the liver samples (see figure 37) when compared to the bile samples. Obviously, normal differences exist between both matrices, although some similarities are also observed since these organs are interconnected in the body.

Nickel was more present in bile when compared to liver, probably due to the fact that this matrix reflects recent exposure to contaminants more rapidly that liver, which takes more time to accumulate these xenobiotic. Also, this element is normally extracted by the kidneys and excreted by the urine, although excretion by other pathways, such as bile, is also possible, depending on the form of the element absorbed by the organism and the type of exposure the animal suffers.[18,232]

The presence of Cu and Cd was more accentuated in liver and these elements where present in other metalloproteins other than MT, in the DTT

extraction procedure, indicating that this reagent is not capable of eliminating thermostable metalloproteins bound to these metals. The presence of Cd in the matrices may indicate a remote contamination in the commercial, since this element is less present in bile. Cu, however, is an essential trace-element and is expected to appear in both bile and liver.

Zn is predominantly bound to MT in both matrices, but less pronounced in liver in samples treated with DTT.

MT peaks were also present in all the tests, indicating the presence of MT in the bile and liver samples, independently of metal exposure or not, confirming that this is a naturally occurring metalloprotein in these organisms. The presence of only one MT peak in the chromatograms indicates that size exclusion chromatography is not efficient in separating MT isoforms, as indicated previously.[233]

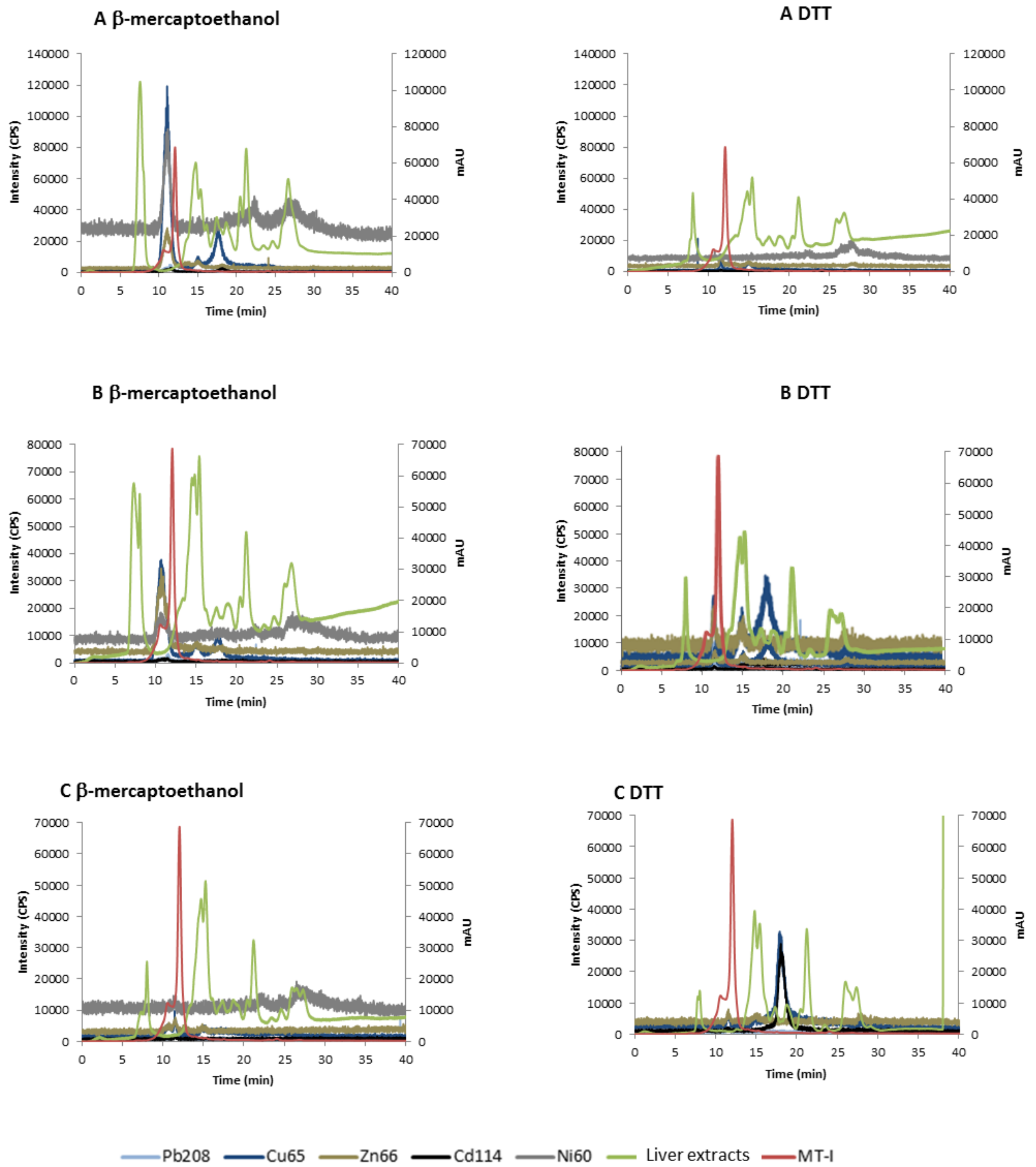


Figure 37. SEC-HPLC-UV-ICP-MS samples and MT-I standard for liver extracts.

4.7.
Statistical analyses for the laboratory fish exposures

Tests for exposure to metals were performed in the laboratory using tanks of 500 L capacity with 10 fish for each metal. At the end of the experiment, 96h, EDTA solution proportional to the amount of the excess metal in solution was added to the tanks to chelate metals, Pb, Zn, Cd and Ni, and facilitate the discharge of 2000 liters of water contaminated in this experiment.

4.7.1.
Spearman correlations and Artificial Neural Networks (ANN)

Not many studies exist that analyze fish bile with the specific aim of using this matrix as a bioindicator regarding metal contamination. In a study by Westerlund *et al.,*[234] oligoelements in fish bile were compared to hepatic concentrations. However, the author observed very low trace-element concentrations, and statistical analyses were not very efficient in determining correlations between these matrices. Thus, the authors indicated the need for more information regarding areas with better documented trace-element levels in lower levels of the trophic food web, as well as in water and sediments, in order to verify possible links between metal levels in the environment and levels in fish bile. Further studies were conducted by Hauser-Davis *et al.*, indicating that this matrix show potential in this regard.[151] In the present study, the experiments were conducted in the laboratory, exposing fish to one element at a time, with no complex mixtures, in order to further investigate the use of this matrix as a bioindicator for metal contamination in fish.

The first step in the statistical analyses was to conduct a correlation analysis, by the Spearman correlation test. We correlated the metals determined by ICP-MS (see in annex) in each exposed group for both bile and liver, in order to evaluate the interference of each metal exposure on other metals, essential and non-essential.

The following significant correlations were observed for bile in the control group: a strong positive correlation between Cu and Fe (rho = 0.857, p<0.025), a strong negative correlation between Se and Fe (rho = 0.810, p<0.025) and a very strong positive correlation between Se and Cu (rho = 0.905, p<0.025) (table 11). For the Ni-exposed group, no correlations were observed. No correlations were observed for MT concentrations and the determined elements in both cases, although many of the correlation values approached the limit imposed by p<0.05, which indicates the need for further studies.

Table 11. Significant Spearman correlations for bile in the control group.

Elements	Rho	Strength of the association	Signal
Fe/Cu	0.857	strong	+
Fe/Se	0.810	strong	+
Cu/Se	0.905	very strong	+

The following significant correlations were observed for liver in the control group (table 12): strong negative correlation between Ni and Pb (rho = -0.733, p<0.025), strong positive correlation between Zn and Cr (rho = 0.767, p<0.025), strong positive correlation between Fe and Ni (rho = 0.733, p<0.025) and strong positive correlation between Zn and Se (rho = 0.850, p<0.025): Regarding MT, the following significant correlations were observed: strong positive correlation between MT and Fe (rho = 0.850, p<0.025) and strong positive correlation between MT and Ni.

Table 12. Significant Spearman correlations for liver in the control group.

Elements	Rho	Strength of the association	Signal
Cr/Zn	0.767	strong	+
Fe/Ni	0.733	strong	+
Fe/MT	0.850	strong	+

Ni/Pb	0.733	strong	-
Ni/MT	0.833	strong	+
Zn/Se	0.850	strong	+

Regarding the Ni-exposed group, in liver the following significant correlations were observed (table 13): a strong negative correlation between Pb and Fe (rho = -0,817, p<0.025), a strong positive correlation between Co and V (rho = 0,800, p<0.025), a strong negative correlation between As and Fe (rho = -0,850, p<0.025), a strong negative correlation between Ni and Se (rho = -0.867, p<0.025), and a very strong positive correlation between Fe and Zn (rho = 0.900, p<0.025). No correlations were observed for MT concentrations and the determined elements in both cases, although many of the correlation values approached the limit imposed by p<0.05, which indicates the need for further studies.

Table 13. Significant Spearman correlations for liver in the Ni-exposed group.

Correlation	Rho	Force	Signal
Pb/Fe	0.817	strong	-
V/Co	0.800	strong	+
Fe/Zn	0.900	very strong	+
Fe/As	0.850	strong	-
Ni/Se	0.867	strong	-

For the Zn-exposed group for bile the following significant correlations were observed: very strong positive correlation between Pb and Ni (rho = 1.000, p<0.025), very strong positive correlation between Pb and MT (rho = 1.000, p<0.025), strong positive correlation between Ni and MT (rho = 0.810, p<0.025) and very strong negative correlation between Se and Pb (rho =1.000, p<0.025) see Table 14.

Table 14. Significant Spearman correlations for bile in the Zn-exposed group.

Elements	Rho	Strength of the association	Signal
Pb/Ni	1.000	very strong	+
Pb/MT	1.000	very strong	+
Ni/MT	0.810	strong	+
Se/Pb	1.000	very strong	-

The following significant correlations were observed for liver in Zn-exposed: strong positive correlation between Co and Pb (rho = -0.825, $p<0.025$), strong positive correlation between Cd and Pb (rho = 0,889, $p<0.025$), strong positive correlation between Co and Fe (rho = 0.830, $p<0.025$) and very strong positive correlation between Cd and Co (rho = 0.994, $p<0.025$). No correlations were observed for MT concentrations and the determined elements in both cases, although many of the correlation values approached the limit imposed by $p<0.05$, which indicates the need for further studies.

Table 15. Significant Spearman correlations for liver in the Zn-exposed group.

Correlation	Rho	Force	Signal
Pb/Co	0.825	strong	+
Pb/Cd	0.889	strong	+
Fe/Co	0.830	strong	+
Co/Cd	0.944	strong	+

The results obtained by the Spearman correlation analysis demonstrated certain variability in the data between the exposed and non-exposed groups. This limited the application of other statistical techniques. Thus, we chose to use an intelligence computational technique, Artificial Neural Networks (ANN), see figure 38, where variable data with low sample number are better analyzed. This technique naturally excludes missing or outlier variables, which is why some of

the determined elements were not integrated in this analysis, such as Pb, Co and Zn.

The results of the normalized importance of each variable for both bile and liver are displayed in table 16.

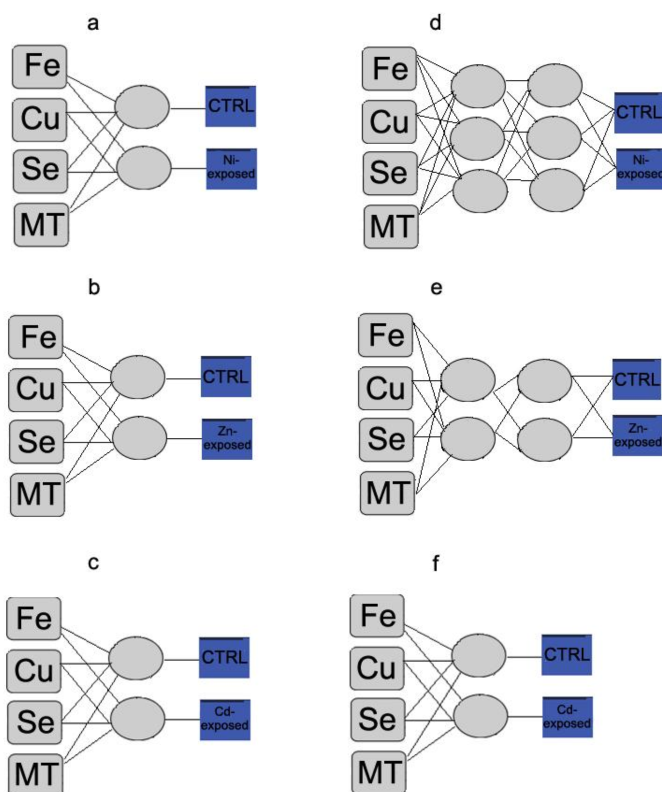


Figure 38: Architecture of ANN for classification as to metal exposure. (a, b and c) in bile samples, and (d, e and f) in liver samples.

Table 16. Normalized importance for each variable for both bile and liver in the Ni-exposed group.

Matrix Element	Liver Normalized importance (%)	Bile Normalized importance (%)
Fe	6.8%	53.6%
Cu	79.9%	100%
Se	58.1%	66.2%
MT concentrations	100%	27.5%

Analyzing these results, we can observe that Fe and Cu show significantly higher normalized importance in bile when compared to liver. For Se, this difference between both matrices was low, but even so the normalized importance is still higher for bile. MT concentrations, however, presented higher normalized importance in liver. Thus, we can conclude that, in tilapia exposed to Ni, the Fe, Cu and Se concentrations in bile can be used instead of in liver to indicate environmental Ni contamination. Ni is a trace element that influences the amount of iron absorbed by the organism and may be important in helping make red blood cells. The exposure to Ni can cause a decrease in arterial oxygen pressure, and the Fe being one of the main responsible for the transport of hemoglobin in the blood (see section 4.5.), with a modification of the amount of the oxygen, suffers indirectly by such contamination in the organism.[235] Regarding the MT concentrations, these results indicate that better biomarker potential in liver when compared to bile. However, if the percentages obtained by the ANN are maintained constant, in a future study it may be possible to create a constant to take these differences into account.

Table 17. Normalized importance for each variable for both bile and liver in the Zn-exposed group.

Matrix Element	Liver Normalized importance (%)	Bile Normalized importance (%)
Fe	83.3%	18.1%
Cu	100%	100%
Se	0.7%	26.7%
MT concentrations	75.8%	32.9%

In the Zn-exposure group, Fe and Cu showed higher normalized importance in bile, the latter with 100%. Se showed low normalized importance in both matrices, however, even so, higher in bile. MT concentrations also showed higher normalized importance in liver, similarly to the Ni-exposed group, but with a lower percentage difference when compared to bile (table 17). Thus, we can conclude that, like in the Ni-exposed group, Fe, Cu and Se concentrations in bile may be used instead of in liver to indicate environmental Zn contamination. Zinc is a trace element that has several important functions, for example helps make

new cells and enzymes. The contamination with Zn can reduces the amount of copper that the organism can absorb.

Regarding MT concentrations, liver still showed higher importance, however, as in the previously discussed group, if the percentages obtained by the ANN are maintained constant, in a future study it may be possible to create a constant to take these differences into account.

4.8.
Fourier Transform vibrational spectroscopy in the infrared region (FT-IR) analyses – Qualitative clean-up effects in bile and liver samples

FT-IR analyses after bile and liver clean-up showed significant differences between the crude, purified and delipidized purified liver extract spectra.

Crude liver bands at 2925 and 2853 cm^{-1} (v-CH), 1659 cm^{-1} (v-C=O), and 1240, 1154, 1081 and 1025 cm^{-1} (v-P-O e v-C-C) showed significant intensity decreases in the purified extract sample, possibly due to the effects of protein extraction process by mechanical lysis, resulting in phospholipid and triacylglycerol removal.

Delipidation modified the relative transmittance of OH (ca. 3400 cm^{-1}) and CH (2925 cm^{-1}) stretching bands, expressed, mainly, in the different %T(CH/OH) ratios of 1.08 and 1.27, in the purified and delipidized extract, respectively. This agrees with lipid removal from the sample.

The main differences between crude and purified liver samples, concerning the 1200-980 cm^{-1} region (see figure 39), were in terms of relative intensities of bands, with few shifts observed.

However, upon delipidation, multi-peak fitting showed a very complex band composition probably due to lipids removal, which leads to a better resolution of other bands.

Similar effects were verified for the bile samples, although not as pronounced as in liver.

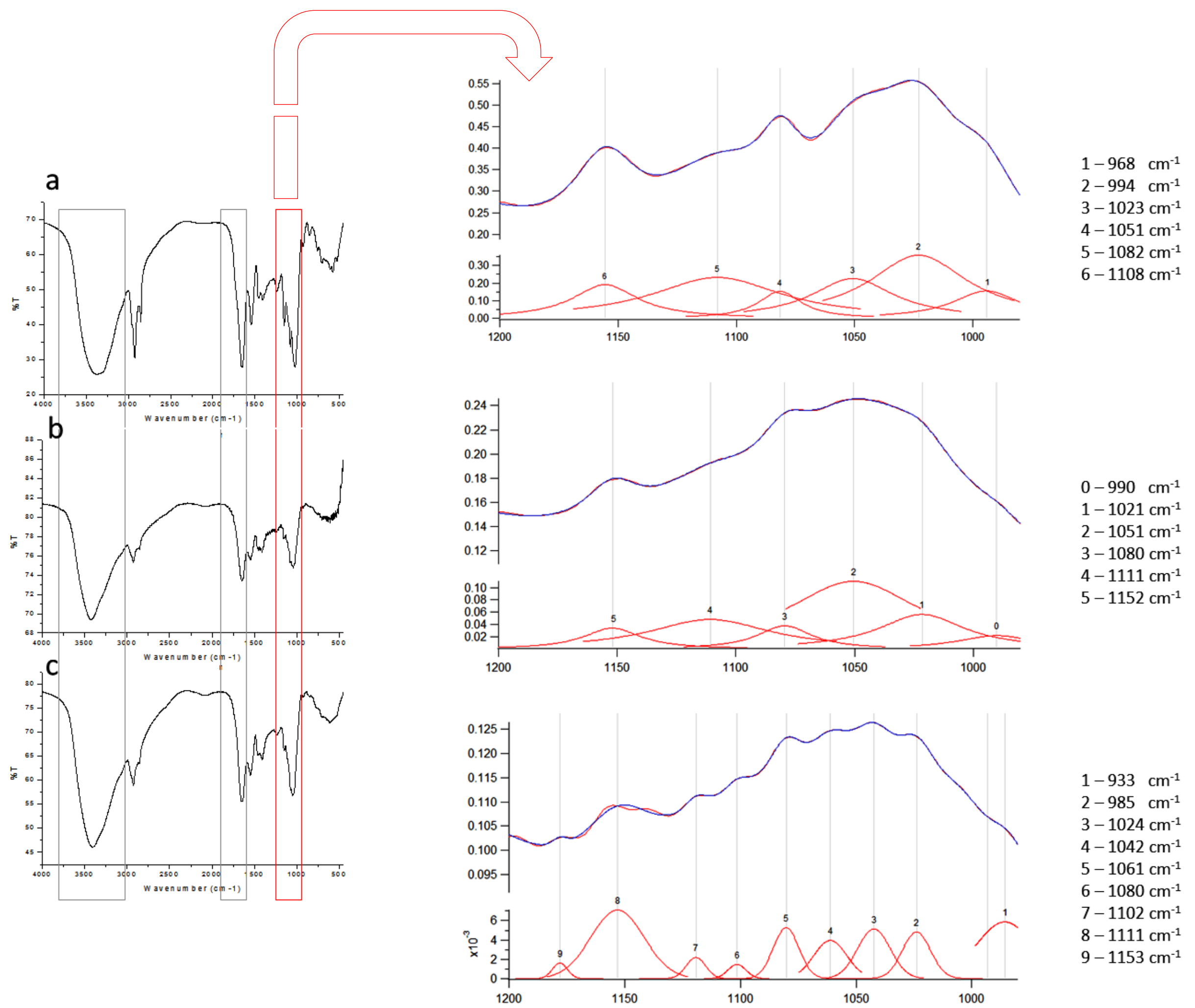


Figure 39. Infrared spectrum (a) crude liver, (b) purified liver and (c) delipidized liver; and on the right side of the figurethe deconvolution in the 1200 – 980 cm^{-1} region.

5.
Conclusions

5.1.
PART I: Peptide derivatization and complexation with metals

This work demonstrated that the derivatization reaction using the chelating reagent DOTA-NHS-ester was effective for labelling single peptides and peptide mixtures with metals, verified by MALDI TOF MS. Furthermore, an efficient separation method for all peptides, singly and in mixture, and also a Cyt C digest, was developed by nano-HPLC, using a trap column, necessary for the washing step prior to analytical column elution.

An implementation of this pre-column/pre-cleaning step in other chromatographic configurations is simple and significantly improves technique performance for other couplings, such as nano-HPLC-ICP-MS. The use of nano-LC-MALDI TOF MS was established for the analysis of peptides labeled with lanthanides, and is a first step for the application of mass spectrometry, both elemental and molecular, in protein analysis in quantitative proteomics. However, to achieve this aim it is necessary to conduct new developments in order to obtain better yields in the derivatization reactions, improve the separation systems and develop better bioinformatic techniques.

5.2.
PART II: Optimization of metalloprotein extraction procedures from environmental samples

5.2.1.
Optimization with commercial fish

The SDS-PAGE analyses revealed the existence of a protein band at approximately 14 kDa, strengthening the literature which reports that fish MT in liver are presented as dimers (7 kDa each).

Heat treatment effectively removes most undesired proteins from fish liver and bile samples, however results indicate that temperatures above 70 ºC are not the most efficient since they also remove MT from both matrices. Among the three analyzed reducing agents, TCEP was shown to be the most efficient, whereas DTT and β-mercaptoethanol showed similar results both in the spectrophotometric quantification and the qualitative SDS-PAGE analyses.

SDS-PAGE analyses were shown to be useful in corroborating the standardization results obtained by the spectrophotometric and statistical analyses regarding bile and liver MT. Furthermore, they aided in distinguishing certain characteristics that may not be observed in spectrophotometric analyses of the different purification processes, such as the presence or absence of other proteins in the purified samples. Our results also indicate that the centrifugation times are not as important in MT quantification as the choice of reducing agent, and that the centrifugation times described in the literature can be reduced in order to analyze more samples in the same timeframe with the same quantification response. Thus, the protocol established in the present study, therefore, is quicker and significantly more efficient for fish bile, and also corroborates previous reports indicating that TCEP is a powerful reducing agent.

The profiles obtained from the SEC-HPLC-ICPMS analyses showed no significant differences when comparing bile and liver, suggesting that both matrices would be able to provide the same information regarding MT induction, further corroborating bile potential as an interesting biomarker. However, MT

isoforms are not able to be separated by this technique. This technique also corroborated the SDS-PAGE analyses, indicating that the extraction procedure is not effective only for MT, since other thermo stable proteins are also extracted in the process and were also separated by size exclusion chromatography technique (SEC), alongside MT.

The results obtained in the present study regarding MT also allow us to consider FTIR spectroscopy as a promising tool for the analyses of biological samples submitted to different extraction and clean-up processes, since the efficiency of each procedure can be qualitatively evaluated, allowing the choice of the most efficient method. The studies involving the multi-peak fitting of the IR absorption bands result in more accurate analyses regarding these types of samples.

All these results are of extreme importance in an environmental monitoring context, where samples are usually very numerous and speed of analysis is of the essence, and, bile could be a validated alternative in this regard. In an environmental context, biliary MT was lower than liver MT, as expected, since liver accumulates MT with slower detoxification rates than bile, which is released from the gallbladder during feeding and diluted by water. Therefore, bile MT seem to be more adequate than liver MT in environmental monitoring contexts regarding recent exposure to xenobiotic that may affect the proteomic and metalloproteomic expression of this biological matrix.

5.2.2.
Analyses of the laboratory-exposed fish

For the laboratory-exposed fish, the Spearman correlation test indicated which are the most important elements to be analyzed for both matrices, bile and liver, allowing the choice of certain elements in bile instead of liver to be used as biomarkers for environmental contamination in this matrix.

Significant correlations between the concentrations of trace elements in bile in the control group were obtained for three essential elements (Fe, Cu and

Se). Furthermore, Cu and Se are elements that are normally also reported as being bound to MT, strengthening the proposition that MT are present in small amounts in the body even with no environmental MT inductor, such as xenobiotic. Moreover, in the Zn-exposed group, significant correlations were obtained for Pb, Ni, Se and MT concentrations, indicating that there was strong induction of MT as a consequence of Zn exposure. This is interesting since Zn is an essential trace-element. Although MT are known to regulate Zn and Cu in the body, the excess of these metals also seems to pose a significant risk to these organisms.

The statistical of artificial neural networks indicated that Fe, Cu and Se concentrations in bile were sufficient to validate their use as environmental contaminants instead of liver concentration in the Ni-exposed group, which may indicate a more recent contamination in the environment, since bile excretion in this case is more efficient than liver detoxification. MT concentrations, despite being lower in bile, can also be considered, since in many cases significant correlations with these elements were observed.

In the case of Zn-exposed group, Cu and Se again showed important values to validate their use in environmental monitoring contexts, even though the liver showed a normalized Cu importance of 100% of. In this case, the MT is not significantly induced in the liver as occurred during Ni-exposure. In addition, Fe showed higher normalized importance in bile when compared to liver, also indicating a possibly more efficient excretion of this metal by bile.

There is the possibility of not having to sacrifice the animal when using bile as a bioindicator, making this method more interesting and eligible for the primary and routine monitoring of trace elements in bile, especially in cases of recent contamination, since bile reflects localized and recent contamination better than liver, which is a better indicator of chronic contamination.

These results indicate that bile, hence, has great, unexplored, potential, and further studies can and should be conducted, both in-depth studies of the biomarkers analyzed in this study and in the search for new biomarkers present in this matrix.

5.3.
Future prospects

The search for potential biomarker targets and bioindicators in environmental samples for the investigation of responses found in the environment due to the contamination and/or exposure is a current trend. The modernization and advancement of several techniques have brought great progress for the studies in question, although the use of more established techniques is also of due importance. Based on these principles and seeking a more profound analyses of the subject present in the present study, of this little has been conducted in Brazil, we highlight some of the future prospects of this study.

The use of more effective methods of separating metallothioneins is an important point to be studied and elaborated on, with the proposed addition of new columns to separate by size exclusion. Furthermore, PUC-Rio has available a nanoACQUITY UPLC mass spectrometer, on which proteomic maps of bile and liver samples of fish exposed to metals can be investigated, which would allow for the verification of possible changes related to protein contamination and the identification of new thermostable proteins identified in these matrices. This may also lead to the discovery of other potential protein biomarkers of environmental contamination.

Regarding environmental contamination, another important purpose of this study was the analyses of metal interaction in exposed fish, with, in the future may come to include exposure to more than only one metal per group, which will lead to further studies regarding these contaminants and their interactions in the analysed specimens, as well as behavior of protein biomarkers regarding this contamination, as well as their detection and identification by MS techniques.

6.

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7.

Annex

Paper published and/or submitted

1. Evaluation and standardization of different purification procedures for fish bile and liver metallothionein quantification by spectrophotometry and SDS-PAGE analyses. Carolina Lyrio Tenório-Daussat, Marcia Carolina Martinho Resende, Roberta L. Ziolli, Rachel Ann Hauser-Davis, Dirk Schaumlöffel, Tatiana D. Saint'Pierre. **Talanta. V.120, p. 491-497, 2014. Impact factor: 3.733. Level A2 (CAPES).**
2. Peptide derivatization and complexation with Lanthanides-NHS-ester-DOTA by nano-IP-RP-HPLC-UV. Carolina Lyrio Tenório-Daussat; Rachel Ann Hauser-Davis; Tatiana D. Saint'Pierre; Dirk Schaumlöffel. **Submitted. Michrochemical Journal. Impact factor: 2.850. Level A2 (CAPES).**

Annex 1. ICP MS detection for liver control and elementar exposition.

Unit	μg/L										
Element	**Pb**	**V**	**Cr**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**As**	**Se**	**Cd**
MW	**208**	**51**	**53**	**57**	**59**	**60**	**65**	**66**	**75**	**82**	**114**
Ctrl 1	3,10	45,9	100	1954	85,8	313	6580	609	2,73	334	2,13
Ctrl 2	2,40	36,7	44,5	2020	25,3	255	786	96,9	1,97	265	< 0.001
Ctrl 3	3,39	48,6	40,5	1997	28,8	215	1932	145	1,89	250	< 0.001
Ctrl 4	4,45	28,5	69,5	1737	25,2	237	1171	218	2,34	352	8,63
Ctrl 5	1,22	57,2	86,7	2296	50,6	319	853	267	5,90	320	< 0.001
Ctrl 6	3,38	62,0	82,0	1575	27,8	251	1851	337	5,51	357	< 0.001
Ctrl 7	2,01	36,5	58,7	2225	25,3	312	875	163	8,34	275	< 0.001
Ctrl 8	4,40	24,0	70,4	909	17,4	182	1037	113	3,91	196	0,61
Ctrl 9	2,59	29,9	75,0	1516	28,5	219	681	161	5,09	308	< 0.001
Ni 2	2,95	86,5	121	2665	71,4	67,2	2516	330	6,93	519	1,46
Ni 3	2,16	22,8	237	5070	37,0	111	2568	376	2,16	294	0,62
Ni 4	4,26	26,0	34,7	3625	44,8	101	2490	348	1,43	384	< 0.001
Ni 5	4,17	29,4	48,2	3235	29,7	255	2613	343	3,61	232	0,63
Ni 6	4,76	23,3	63,2	1552	35,2	280	1926	54,3	8,21	239	21,0
Ni 9	8,47	62,0	92,1	1818	67,1	215	2523	225	7,12	336	5,39
Ni 10	11,1	56,4	107	1426	39,2	354	2975	279	7,43	192	3,08
Ni 11	7,38	31,1	59,6	2294	38,0	231	3097	283	6,01	221	2,08
Ni 12	7,92	20,8	74,1	1582	26,5	241	1556	194	3,69	263	1,98
Cd 1	3,59	83,3	55,5	1850	37,8	205	1522	246	6,10	492	23,2
Cd 2	4,40	41,6	39,4	1542	20,7	186	652	129	7,16	250	2,14
Cd 3	4,51	28,3	63,9	2329	36,4	205	2632	202	7,32	375	10,0

Cd 4	6,80	44,8	55,7	5237	39,5	281	1215	172	6,42	321	23,0
Cd 5	3,62	58,6	28,0	2093	33,4	208	1660	99,3	5,70	365	1,69
Cd 6	5,26	16,9	66,9	4936	41,8	244	868	183	6,90	438	13,0
Cd 7	9,77	41,2	100	1425	36,2	273	932	273	7,71	436	18,6
Cd 8	5,00	30,5	85,9	1743	26,3	205	815	157	2,79	376	1,68
Cd 9	9,20	81,8	162	1671	29,6	255	1743	203	4,40	501	3,20
Zn 1	5,15	42,5	81,4	2433	62,7	248	2601	279	6,89	341	< 0.001
Zn 2	1,41	51,8	42,5	1828	42,9	183	2494	222	2,41	457	< 0.001
Zn 3	0,61	17,0	< 0.001	3274	63,3	214	4014	172	6,18	496	0,45
Zn 4	1,04	26,2	7,06	1935	41,4	220	993	234	2,41	237	0,30
Zn 5	0,69	25,4	29,3	2044	47,2	163	2574	981	2,69	222	0,66
Zn 6	0,068	20,4	57,7	3210	52,4	344	1731	358	3,98	309	0,79
Zn 7	0,78	28,7	423	2059	48,6	330	2512	131	3,28	252	< 0.001
Zn 8	9,89	33,2	67,8	4832	104	321	3217	586	3,77	273	4,29
Zn 9	0,81	31,9	37,2	1978	70,0	257	2221	176	4,06	205	2,27
Pb 1	< 0.001	31,3	48,7	2750	25,7	199	851	334	3,99	246	< 0.001
Pb 2	13,1	10,8	45,4	1720	26,6	199	813	123	5,01	374	< 0.001
Pb 3	< 0.001	28,9	67,2	2163	19,4	202	1445	221	4,84	252	< 0.001

Annex 2. ICP MS detection for fish bile control and elementar exposition.

Unit	μg/L										
Element	**Pb**	**V**	**Cr**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**As**	**Se**	**Cd**
MW	**208**	**51**	**53**	**57**	**59**	**60**	**65**	**66**	**75**	**82**	**114**
Ctrl 1	< 0.001	< 0.001	< 0.001	855	< 0.001	1,20	63,8	124	< 0.001	74,9	< 0.001
Ctrl 2	< 0.001	< 0.001	< 0.001	1532	< 0.001	< 0.001	31,0	< 0.001	< 0.001	28,7	< 0.001
Ctrl 3	< 0.001	< 0.001	< 0.001	665	< 0.001	3,38	26,1	32,4	< 0.001	26,5	< 0.001
Ctrl 4	< 0.001	< 0.001	< 0.001	651	< 0.001	18,1	15,2	< 0.001	< 0.001	13,9	< 0.001
Ctrl 5	< 0.001	< 0.001	< 0.001	639	< 0.001	3,35	9,65	< 0.001	< 0.001	17,3	< 0.001
Ctrl 6	< 0.001	< 0.001	< 0.001	1897	< 0.001	20,7	43,4	132	< 0.001	75,7	< 0.001
Ctrl 8	< 0.001	< 0.001	< 0.001	690	< 0.001	< 0.001	18,3	< 0.001	< 0.001	15,5	< 0.001
Ctrl 9	< 0.001	< 0.001	64,7	777	< 0.001	3,08	38,1	16,1	< 0.001	47,8	< 0.001
Cd 1	< 0.001	< 0.001	1,28	656	< 0.001	2,78	22,3	24,0	< 0.001	35,6	< 0.001
Cd 2	0,44	< 0.001	< 0.001	700	< 0.001	3,97	14,1	4,28	< 0.001	25,2	< 0.001
Cd 3	< 0.001	< 0.001	< 0.001	695	< 0.001	6,58	30,0	< 0.001	< 0.001	37,4	< 0.001
Cd 4	< 0.001	< 0.001	< 0.001	719	< 0.001	2,35	56,0	35,0	< 0.001	57,0	< 0.001
Cd 5	< 0.001	< 0.001	< 0.001	796	< 0.001	9,52	63,0	< 0.001	< 0.001	55,1	< 0.001
Cd 7	11,6	< 0.001	< 0.001	683	< 0.001	11,4	105	76,0	< 0.001	14,5	< 0.001
Cd 8	< 0.001	< 0.001	< 0.001	716	< 0.001	1,90	25,5	< 0.001	< 0.001	34,1	< 0.001
Cd 9	< 0.001	< 0.001	< 0.001	844	< 0.001	7,87	38,9	< 0.001	< 0.001	60,6	< 0.001
Zn 1	< 0.001	< 0.001	< 0.001	735	< 0.001	14,0	15,8	< 0.001	< 0.001	47,2	< 0.001
Zn 2	4,40	< 0.001	2,11	924	< 0.001	17,2	61,0	293	< 0.001	22,6	< 0.001
Zn 3	0,32	< 0.001	< 0.001	818	< 0.001	4,04	88,2	66,0	< 0.001	46,9	< 0.001
Zn 4	< 0.001	< 0.001	< 0.001	721	0,18	5,30	79,2	< 0.001	< 0.001	61,8	< 0.001
Zn 5	< 0.001	< 0.001	< 0.001	689	< 0.001	7,79	136	147	< 0.001	31,0	< 0.001

Zn 6	3,55	< 0.001	< 0.001	758	< 0.001	5,46	124	18,8	< 0.001	38,3	< 0.001
Zn 7	< 0.001	< 0.001	< 0.001	656	< 0.001	3,79	55,8	30,0	< 0.001	28,6	< 0.001
Zn 8	< 0.001	< 0.001	< 0.001	1501	< 0.001	13,6	39,8	119	< 0.001	54,2	< 0.001
Zn 9	< 0.001	< 0.001	< 0.001	712	< 0.001	< 0.001	22,4	< 0.001	< 0.001	20,1	< 0.001
Pb 1	7,86	< 0.001	< 0.001	777	< 0.001	7,08	32,6	78,2	< 0.001	42,7	1,18
Pb 2	5,62	< 0.001	< 0.001	729	< 0.001	10,8	83,5	110	< 0.001	58,5	< 0.001
Ni 1	< 0.001	< 0.001	< 0.001	760	< 0.001	7,57	56,0	< 0.001	< 0.001	57,4	< 0.001
Ni 2	< 0.001	< 0.001	< 0.001	699	< 0.001	7,24	135	< 0.001	< 0.001	69,0	< 0.001
Ni 3	< 0.001	< 0.001	< 0.001	739	< 0.001	3,35	260	39,7	< 0.001	98,2	< 0.001
Ni 4	< 0.001	< 0.001	< 0.001	733	< 0.001	5,70	139	66,0	0,055	58,4	1,22
Ni 6	< 0.001	< 0.001	< 0.001	692	< 0.001	1,79	66,0	< 0.001	< 0.001	39,7	< 0.001
Ni 9	< 0.001	< 0.001	173	908	1,25	50,1	234	167	0,33	58,1	< 0.001
Ni 8	< 0.001	< 0.001	< 0.001	668	< 0.001	2,20	67,8	9,63	< 0.001	27,5	< 0.001
Ni 11	< 0.001	< 0.001	< 0.001	729	< 0.001	7,95	97,5	14,0	< 0.001	38,8	< 0.001

Annex 3. MT concentrations for fish bile and liver samples.

bile				liver			
Sample	μmol/L MT	Sample	μmol/L MT	Sample	μmol/L MT	Sample	μmol/L MT
Pb1	54,7	Ni1	19,5	Pb1	8,8	Ctrl9	3,2
	56,0		20,5		10,5		2,8
Pb2	65,2	Ni2	23,0	Pb2	5,7	Ni2	6,0
	77,3		22,2		6,2		7,2
Zn1	74,7	Ni3	33,0	Pb3	23,8	Ni3	17,5
	71,5		34,3		21,7		18,8
Zn2	72,0	Ni4	28,7	Zn1	9,8	Ni4	11,2
	70,0		29,0		11,2		12,0
Zn3	50,0	Ni5	29,2	Zn2	3,7	Ni5	7,2
	47,7		30,7		5,7		7,8
Zn4	39,7	Ni6	59,2	Zn3	13,0	Ni6	10,2
	41,3		63,3		12,0		11,0
Zn5	51,2	Ni7	23,5	Zn4	12,7	Ni9	10,5
	49,8		24,2		13,2		11,2
Zn6	54,0	Ni8	36,7	Zn5	29,3	Ni10	7,5
	68,2		37,3		28,3		8,8
Zn7	47,0	Cd1	64,7	Zn6	11,8	Ni11	7,8
	44,3		66,7		13,3		8,8
Zn8	91,2	Cd2	95,8	Zn7	4,5	Ni12	6,5
	88,8		97,2		4,0		7,2
Zn9	56,0	Cd3	14,0	Zn8	4,7	Cd1	2,7
	57,2		15,5		5,3		3,2
Ctrl1	5,8	Cd4	19,5	Zn9	12,3	Cd2	5,3
	7,2		20,7		12,8		6,3
Ctrl2	34,2	Cd5	22,8	Ctrl1	7,7	Cd3	4,7
	40,7		23,3		9,0		5,5
Ctrl3	52,3	Cd6	21,2	Ctrl2	7,0	Cd4	5,7
	53,5		21,0		7,2		7,2
Ctrl4	41,0	Cd7	76,5	Ctrl3	6,2	Cd5	5,0
	43,2		75,0		6,8		5,5
Ctrl5	41,0	Cd8	86,3	Ctrl4	3,7	Cd6	3,3
	72,2		87,3		4,0		3,5
Ctrl6	70,8			Ctrl5	7,2	Cd7	5,2
	74,0				7,8		5,3
Ctrl8	38,7			Ctrl6	3,3	Cd8	4,2
	40,7				5,0		5,8
Ctrl9	13,3			Ctrl7	10,8	Cd9	6,0
	14,3				12,0		7,2
				Ctrl8	1,3		
					1,7		

Annex 4. Metal concentrations for DORM-2 quantified by ICP-MS NexIon 300X - PerkinElmer.

Metal	DORM-2 mg kg^{-1}	
	Certified value	Quantified value
Cu	2.34 ± 0.16	2.18
Pb	0.065 ± 0.007	0.052
Cr	34.7 ± 5.5	33.2
Fe	142 ± 10	134
Co	0.182 ± 0.031	0.177
Ni	19.4 ± 3.1	18.2
Zn	25.6 ± 2.3	25.0
As	18.0 ± 1.1	16.3
Se	1.40 ± 0.09	1.25
Cd	0.043 ± 0.008	0.033
Hg	4.64 ± 0.26	3.99

Annex 5. Spectrophotometric results for metallothionein concentration of extraction procedures for bile and liver.

	Procedure	μmol/L MT			average value μmol/L MT
BILE					
β-mercaptoethanol	A	13,86	17,33	12,13	14,44
	B	19,73	13,81	15,78	16,44
	C	13,50	16,88	11,81	14,06
DTT	A	15,83	11,08	12,66	13,19
	B	13,38	16,73	11,71	13,94
	C	12,78	15,98	11,18	13,31
TCEP	A	50,10	62,63	43,84	52,19
	B	121,13	84,79	96,90	100,94
	C	36,06	45,08	31,55	37,56
LIVER					
b-mercaptoethanol	A	32,82	41,03	28,72	34,19
	B	32,70	40,88	28,61	34,06
	C	23,34	29,18	20,42	24,31
DTT	A	38,93	27,25	38,93	32,44
	B	33,06	41,33	28,93	34,44
	C	20,10	25,13	17,59	20,94
TCEP	A	51,42	64,28	44,99	53,56
	B	90,90	113,63	79,54	94,69
	C	36,06	45,08	31,55	37,56

Annex 6. Concentration of MT in bile and liver at different water bath temperature.

Temperature		BILE μmol/L MT		Average
50 °C	33,40	35,31	26,72	31,81
60 °C	38,26	40,45	30,61	36,44
70 °C	57,95	61,26	46,36	55,19
80 °C	39,83	42,11	31,87	37,94
90 °C	21,85	23,10	17,48	20,81
		LIVER μmol/L MT		
50 °C	45,19	52,98	57,65	51,94
60 °C	29,42	34,49	37,53	33,81
70 °C	59,21	69,42	75,55	68,06
80 °C	16,48	19,32	21,02	18,94
90 °C	4,40	5,16	5,62	5,06

Annex 7. Concentration of MT in bile and liver at different centrifugation times.

Procedure				
	BILE			
	μmol/L MT			Average
A''	78,30	62,64	54,81	65,25
B''	76,80	61,44	53,76	64,00
C''	74,85	59,88	52,40	62,38
	LIVER			
	μmol/L MT			
A''	69,67	73,89	67,56	70,38
B''	66,33	70,35	64,32	67,00
C''	58,04	61,56	56,28	58,63