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).

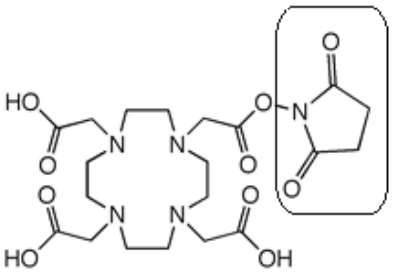

Figure 10. Structure of the DOTA derivatization reagent with the NHS-ester function for amine-specific (highlighted) labelling.

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.

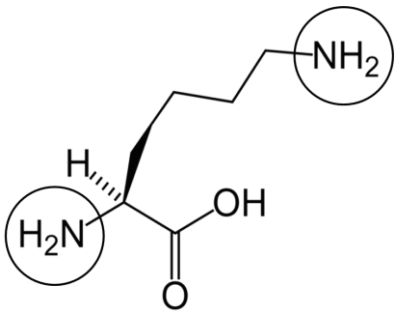

Figure 11. Amino acid lysine (K) with its 2 free amino groups.

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).

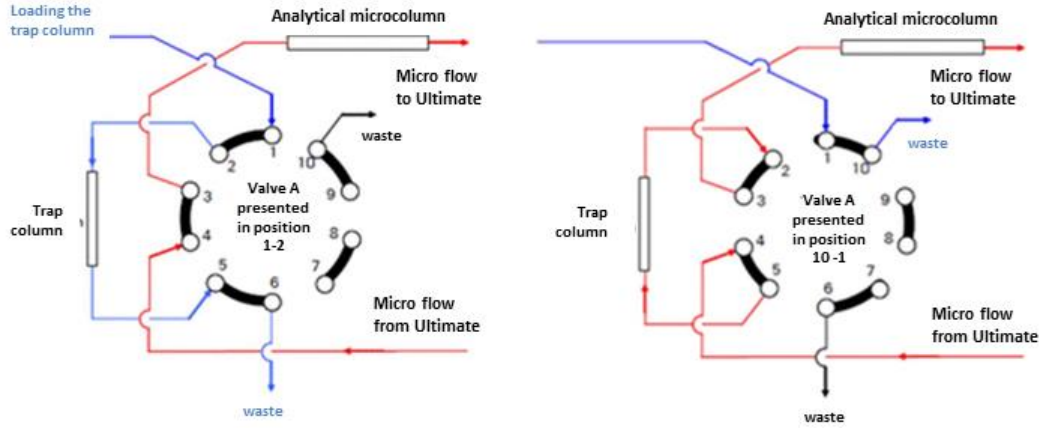

Figure 12. Preconcentration in the trap column and sample elution by nano-HPLC.

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.

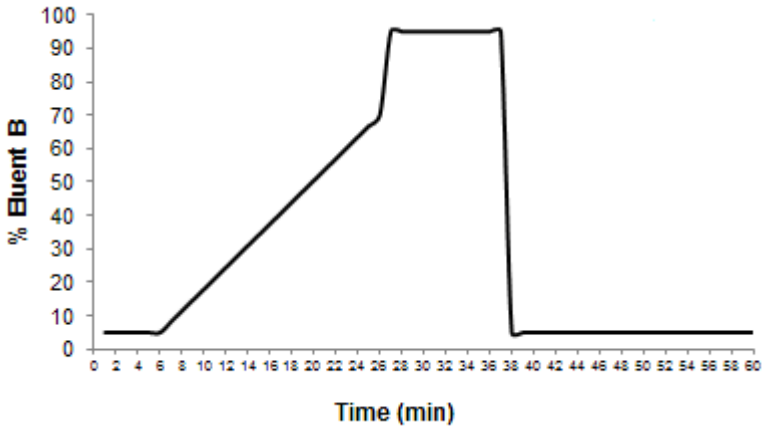

Figure 13. Eluent gradient for peptide mixture separation. Eluent A: 0.05% of TFA (aqueous) B: 0.04% TFA, 80% ACN, 20% and deionized water.

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]

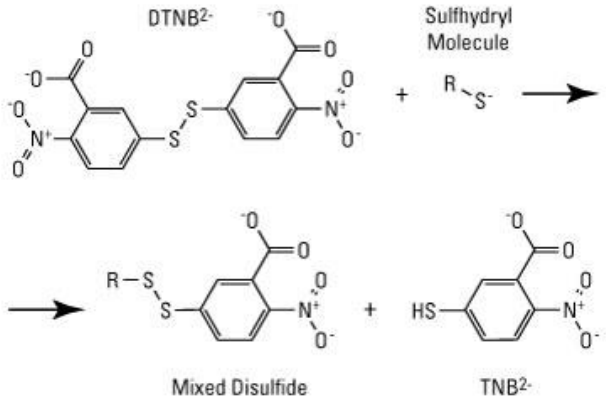

Figure 14. Color-forming reaction of Ellman's reagent with –SH groups.

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).