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 and ^{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.

		Monoisotopic m/z values				
Peptide	Sequence	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.

Usually in proteomics, sample preparation includes cleaning steps, such as desalting and concentration of the sample to enhance detection in molecular MS, but unfortunately, it 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. It can be 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. A 100 fold molar excess is commonly used in labeling techniques, which is a “necessary evil”,[203] and, thus, some peptide loss that may have occurred, thereby impairing the complexation. According to Gregorius,[176] 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. However, the use of metal tags offers a number of potential advantages, justifying these efforts.

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, such as nano HPLC and ICP-MS couldn't be realized neither. However, most likely there has been some loss or error in preparing the reaction of derivatization and complexation of the peptides in question, since these same peptides were derivatized and complexed in a work by Gregorius.

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]

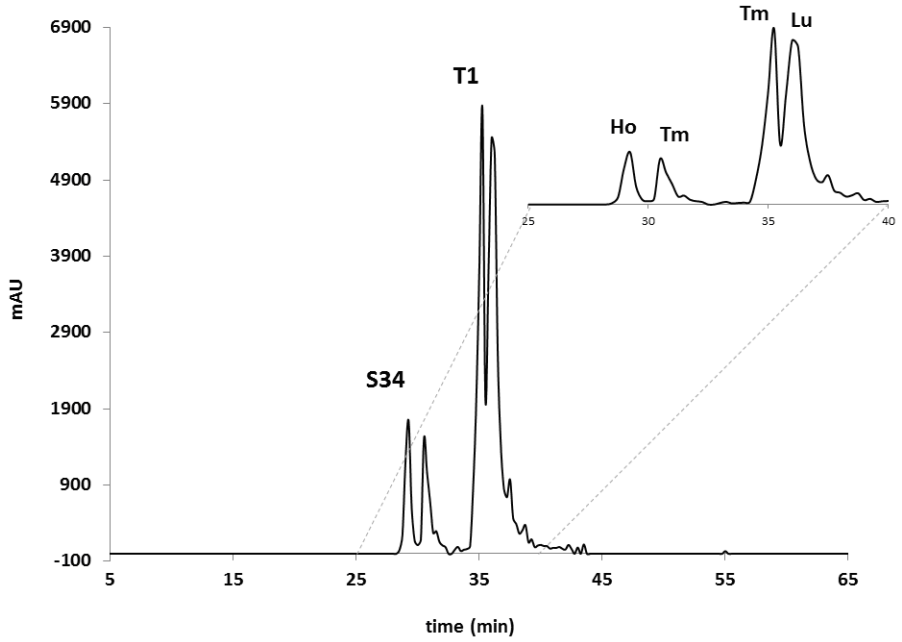

Figure 15. Peptides S34 and T1 identified in the mixture labelled solution (S34, S35, S36, M6, and T1) with Lu, Tm and Ho-NHS-DOTA.

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).

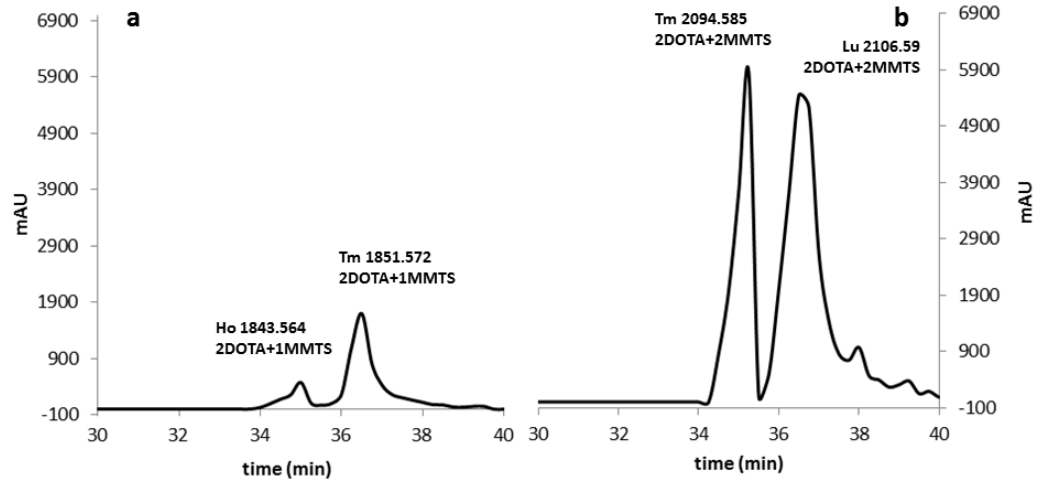

Figure 16. Peptides S36 (a) and T1 (b), respectively, with Lu, Ho and Tm-NHS-DOTA, eluted separately by nano-HPLC UV.

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.

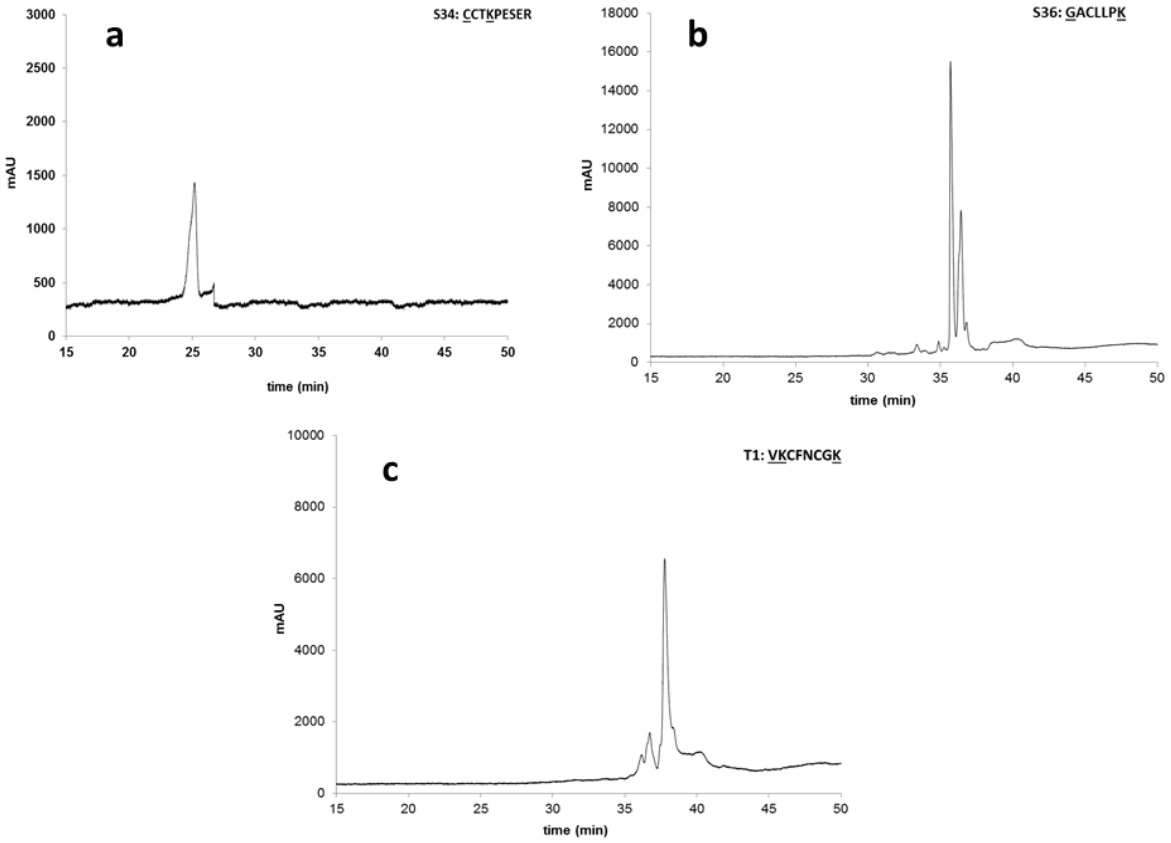

Figure 17. Nano-HPLC UV separation of the single peptides a (S34), b (S36) and c (T1), respectively, using element Tm for the complexation.

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 available, 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.

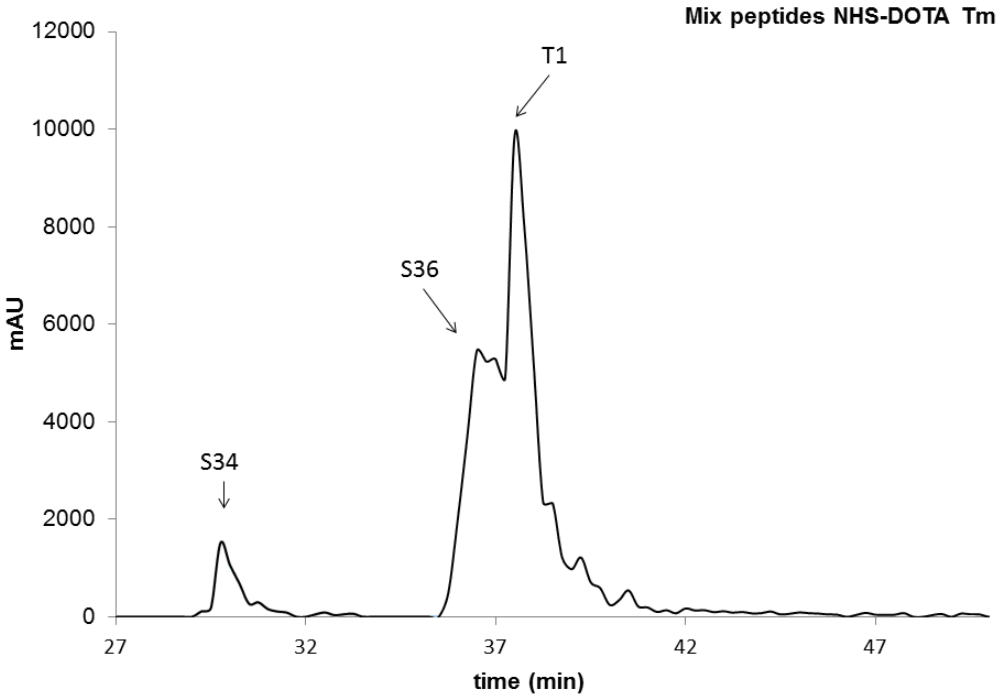

Figure 18. Peptide mixture (S34, S36 and T1) NHS-DOTA complexed with Tm and separated by nano-HPLC UV.

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]

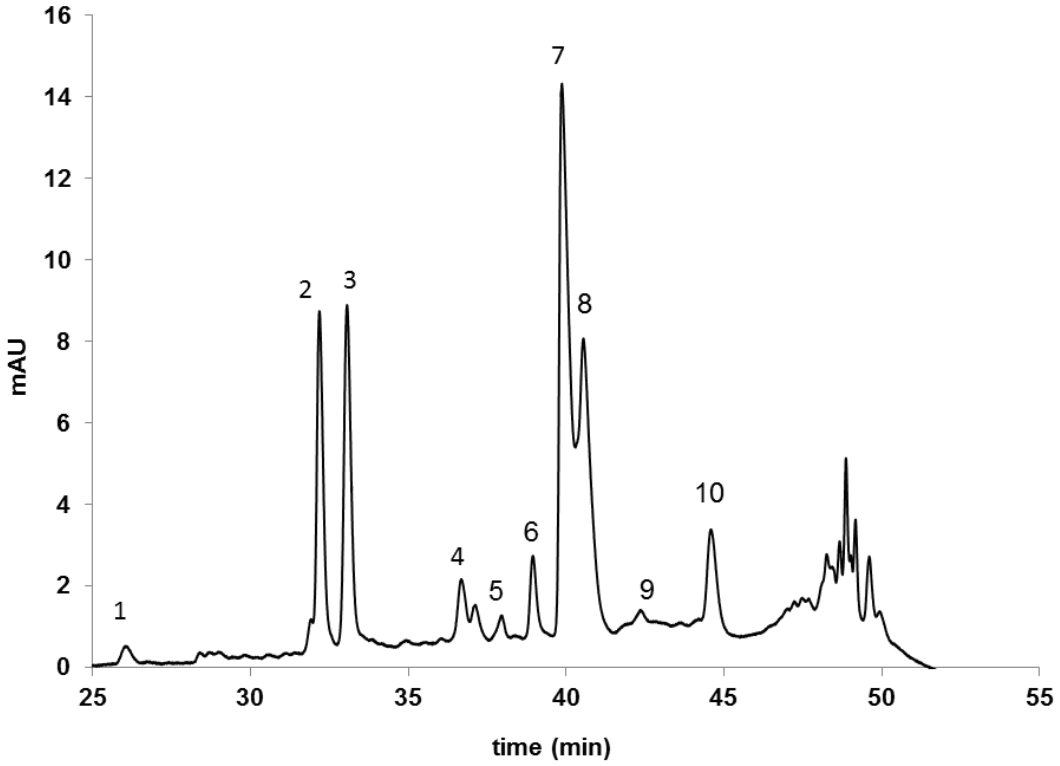

Figure 19. Cytochrome C complexed with Tm-DOTA-NHS-ester and separated by nano-HPLC UV.

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).

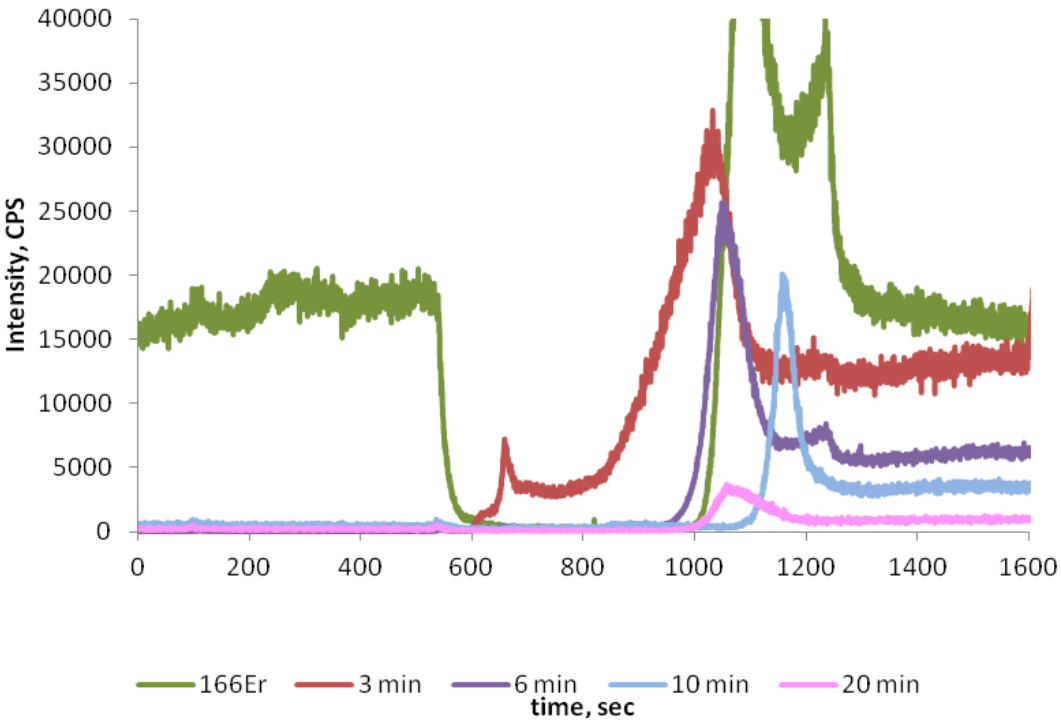

Figure 20. Tm-NHS-DOTA mixture peptide (S35, S36 and T1) pre-washing graph.

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.

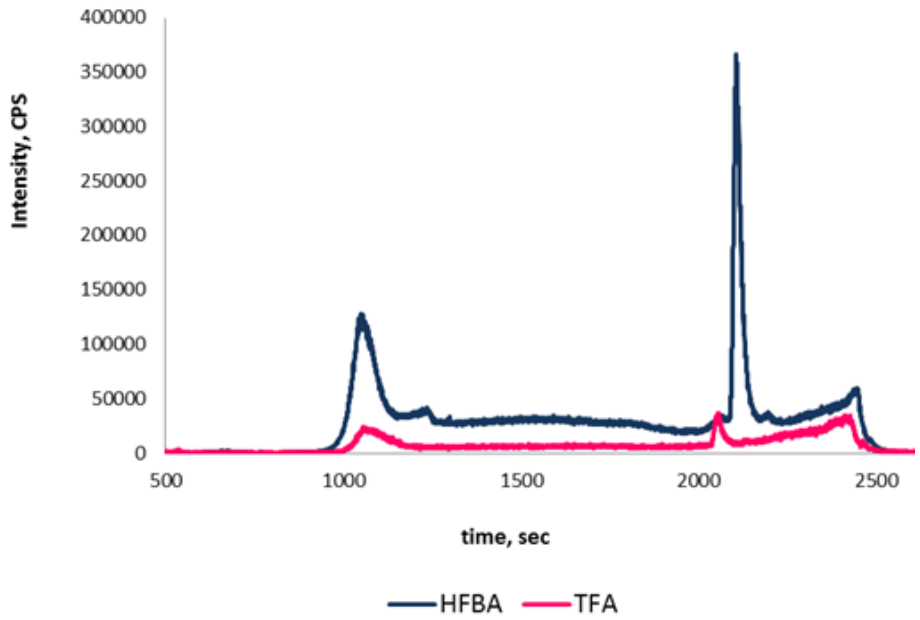

Figure 21. Tm NHS-DOTA-labeled peptide Hy analyzed with HFBA (blue) and with TFA (pink) in the loading buffer.

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.

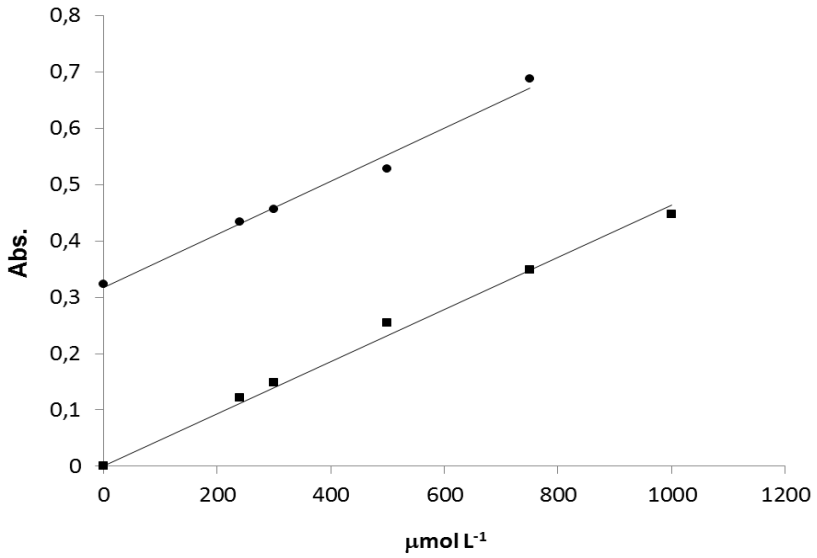

Figure 22. Analytical curves for metallothionein quantification: (■) GSH standards, I= 0.0005 C, R^2= 0.9943. (•) MT standard addition in bile samples, I= 0.0005 C + 0.3167, R^2= 0.9864.

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 it 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]

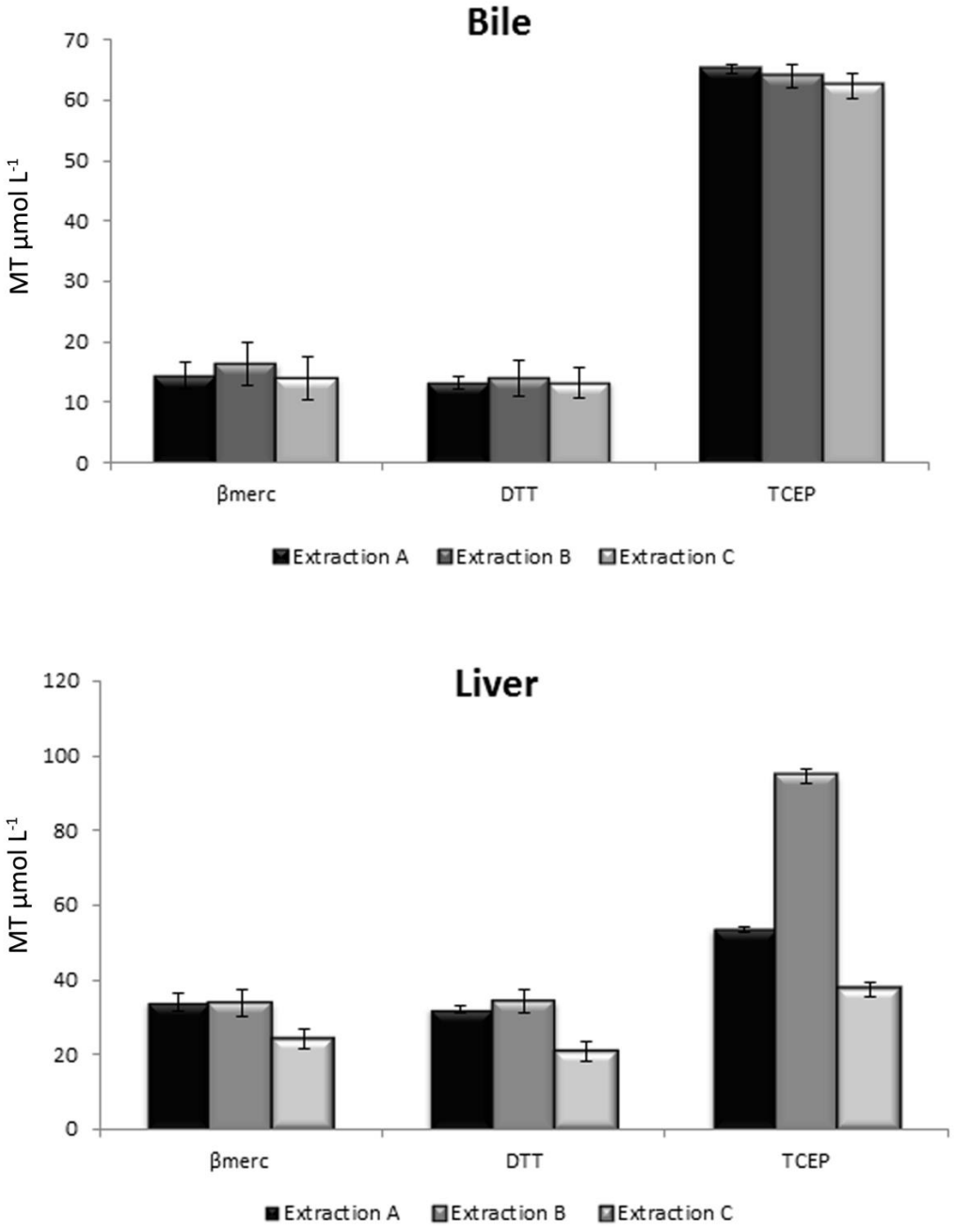

Figure 23. Metallothionein concentrations in bile and liver (expressed in μmol L^{-1}) for each of the tested purification procedures and reagents.

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.

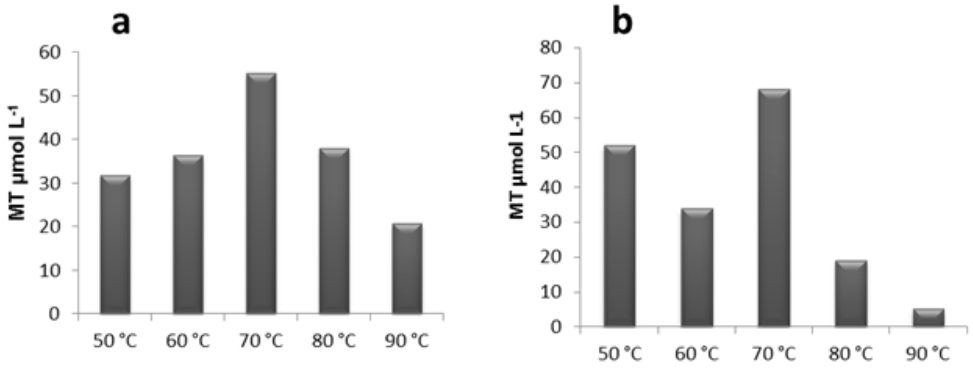

Figure 24. MT concentrations bile (a) and liver (b) at different water bath temperature conditions.

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.

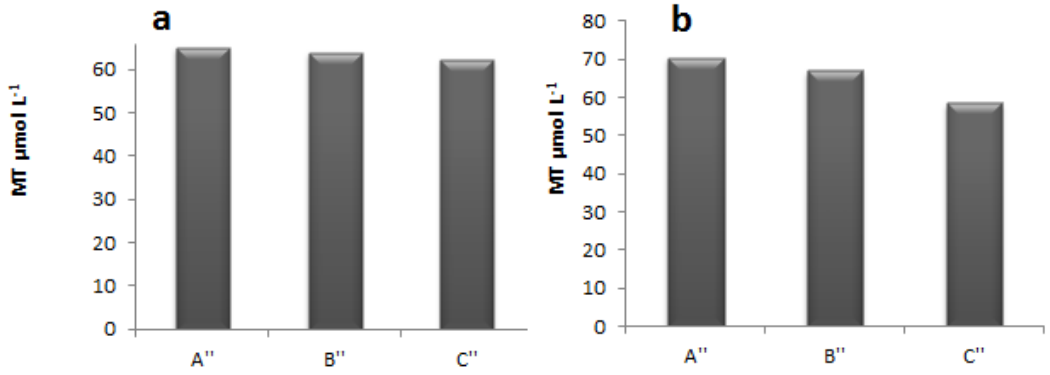

Figure 25. Concentration of MT in bile (a) and liver (b) at different centrifugation times.

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]

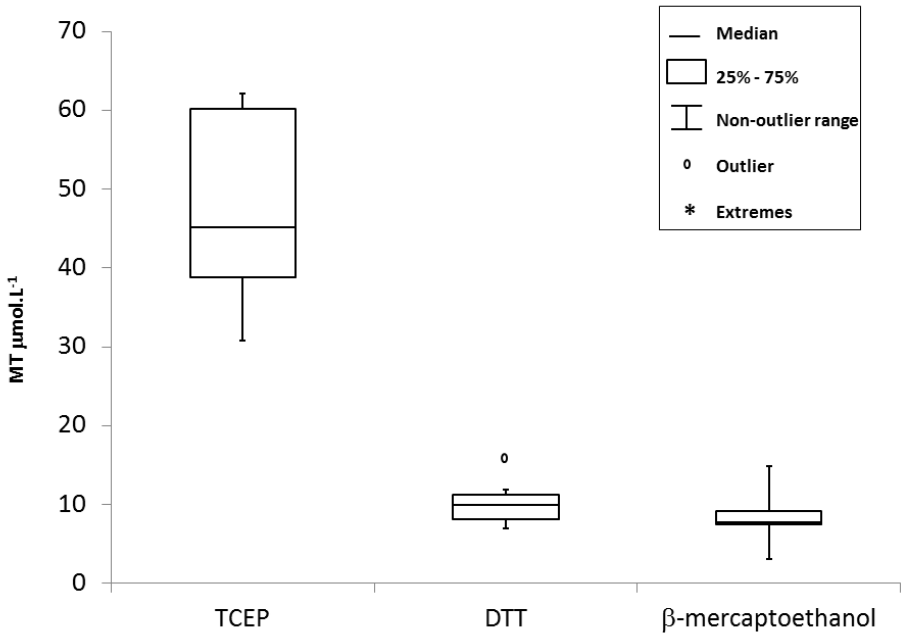

Figure 26. Reagent box-plot chart data after 4^3 multivariate statistical analyses with TCEP, DTT and β-mercaptoethanol.

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.

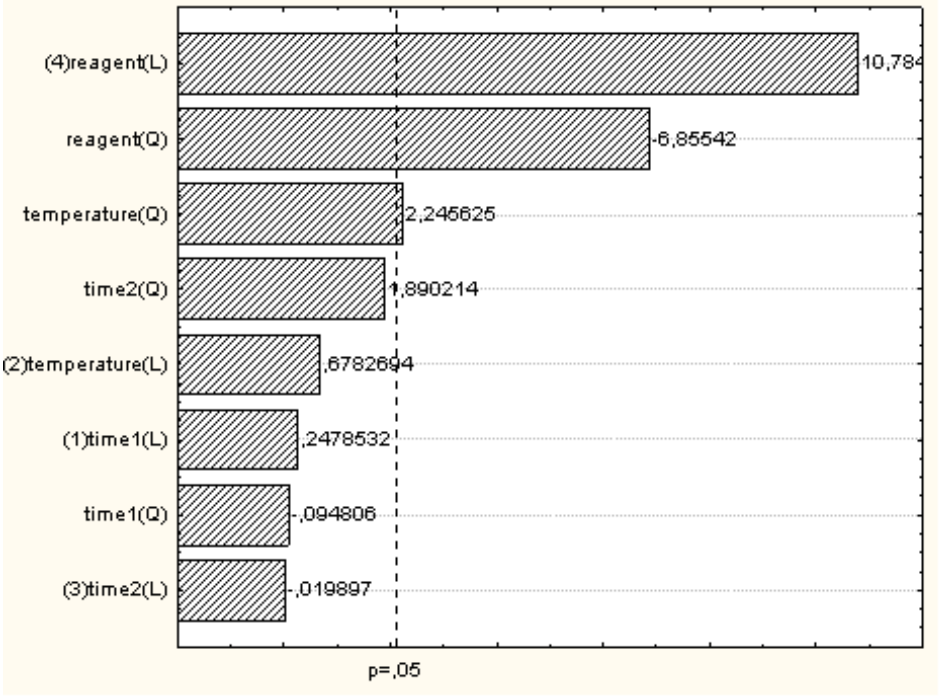

Figure 27. Pareto Chart of Standardized Effects regarding the studied factors for bile samples.

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%.

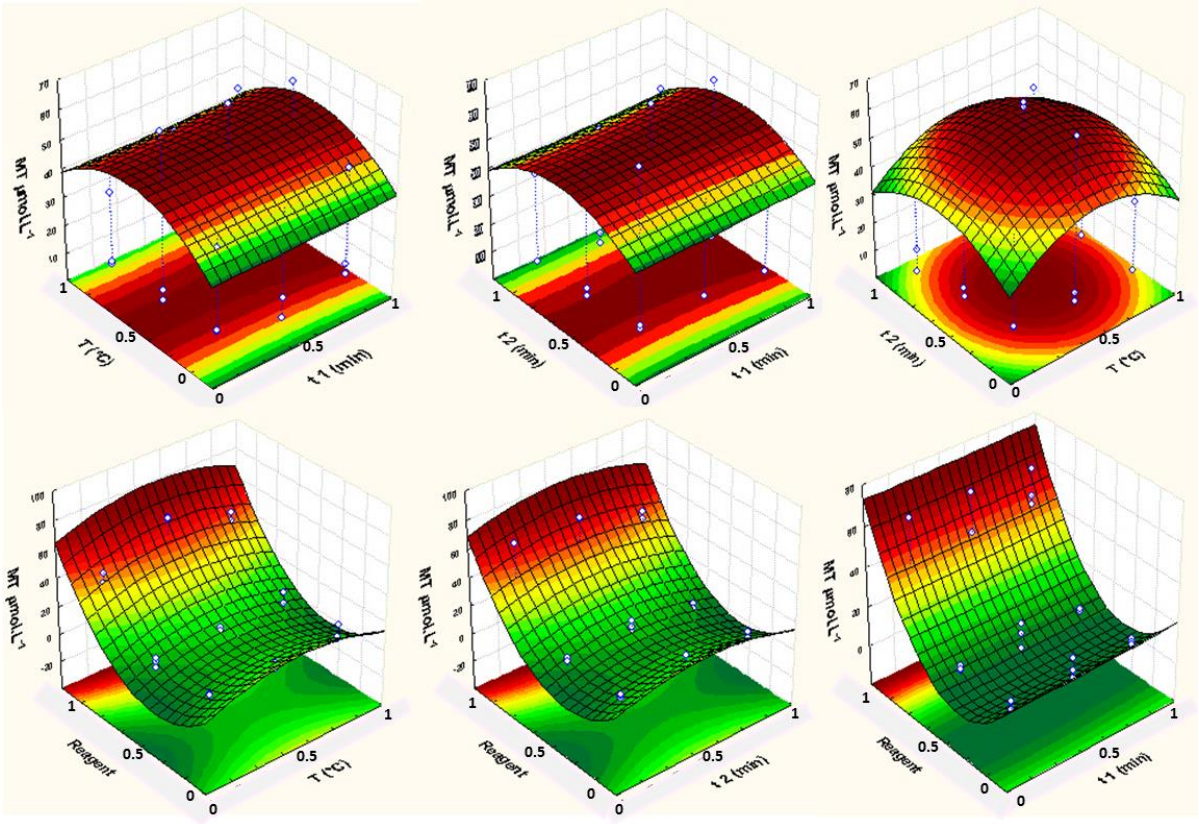

Figure 28. Response surface charts for each of the studied factors for tilapia bile MT samples.

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.

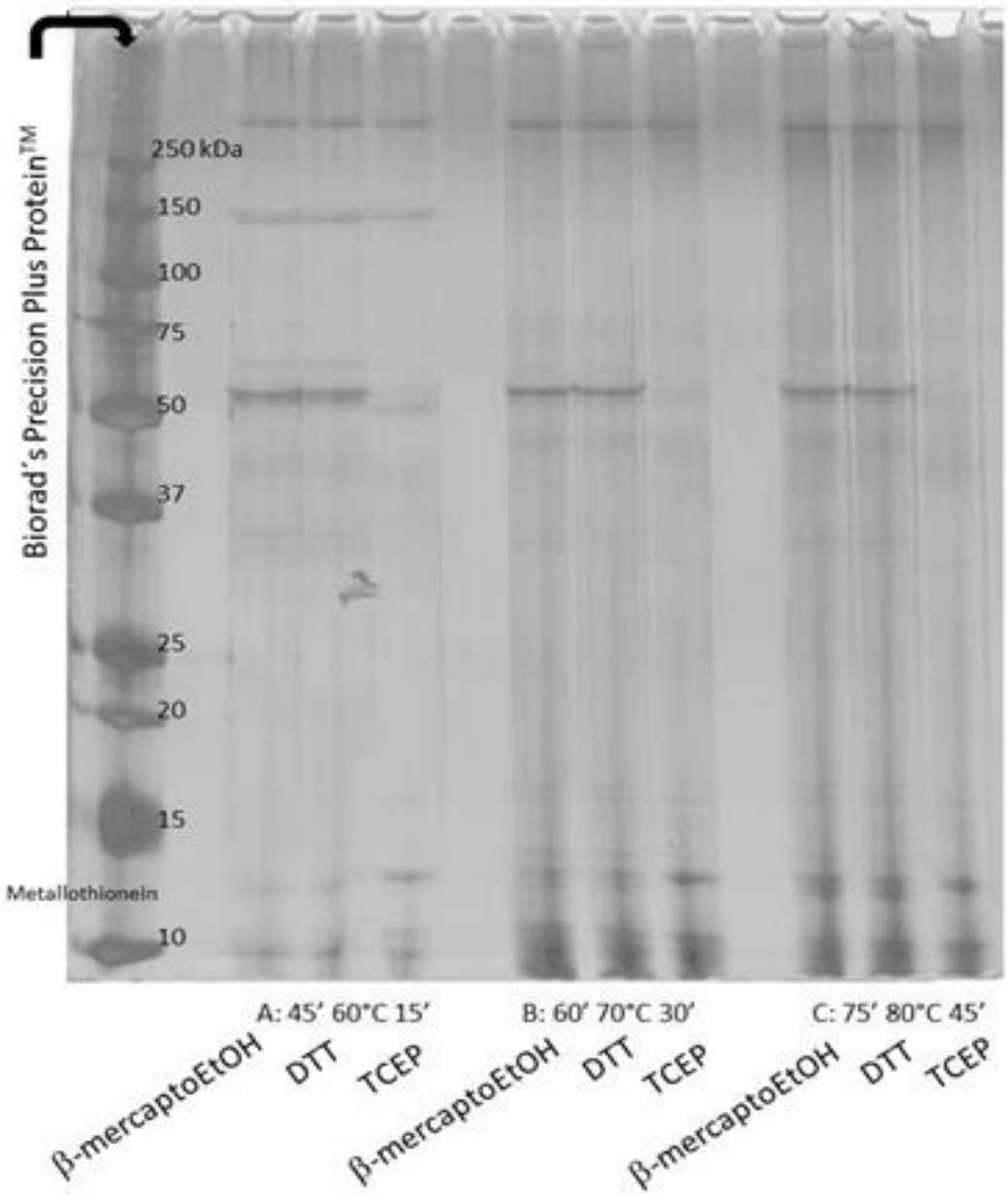

Figure 29. Qualitative SDS-PAGE gels for bile samples using the different extraction procedures and reagents analyzed in the present study.

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.

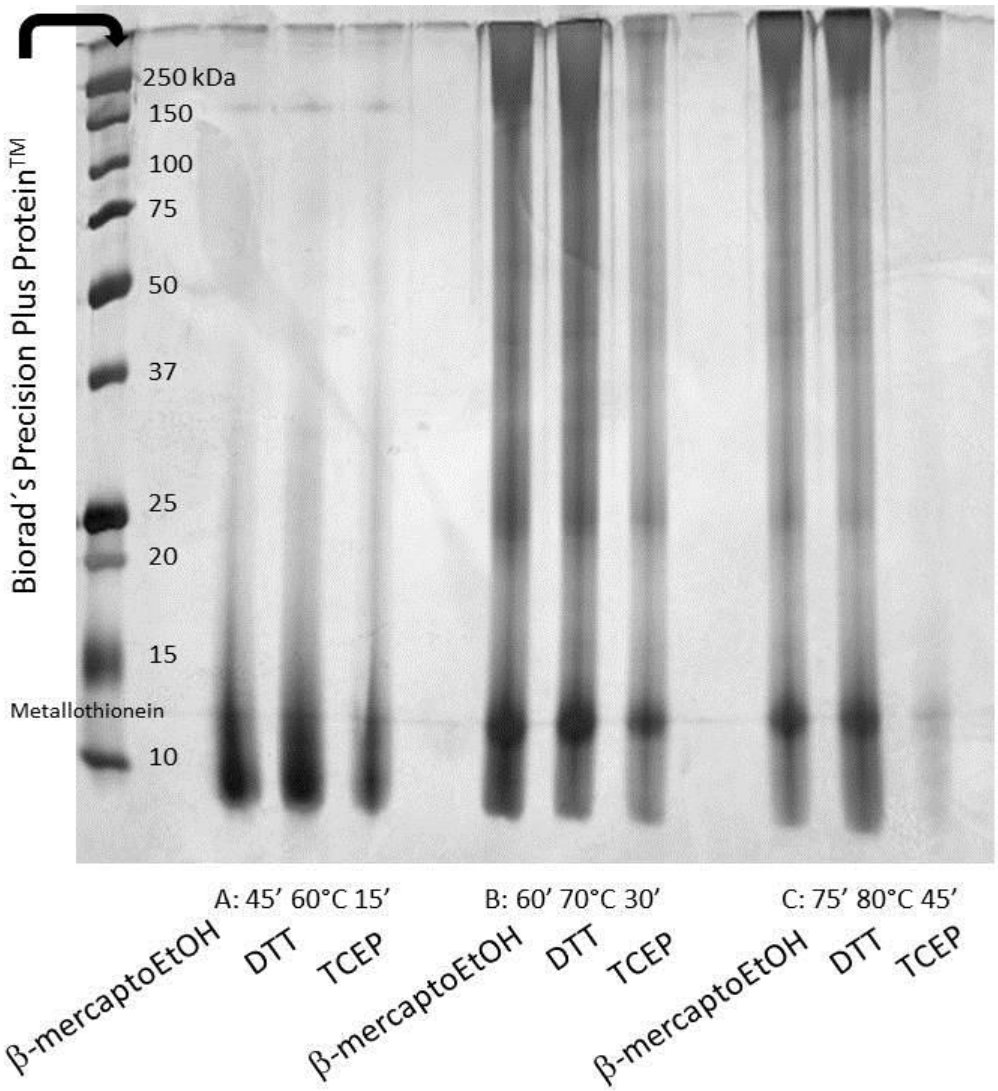

Figure 30. Qualitative SDS-PAGE gels for liver samples using the different extraction procedures and reagents analyzed in the present study.

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.

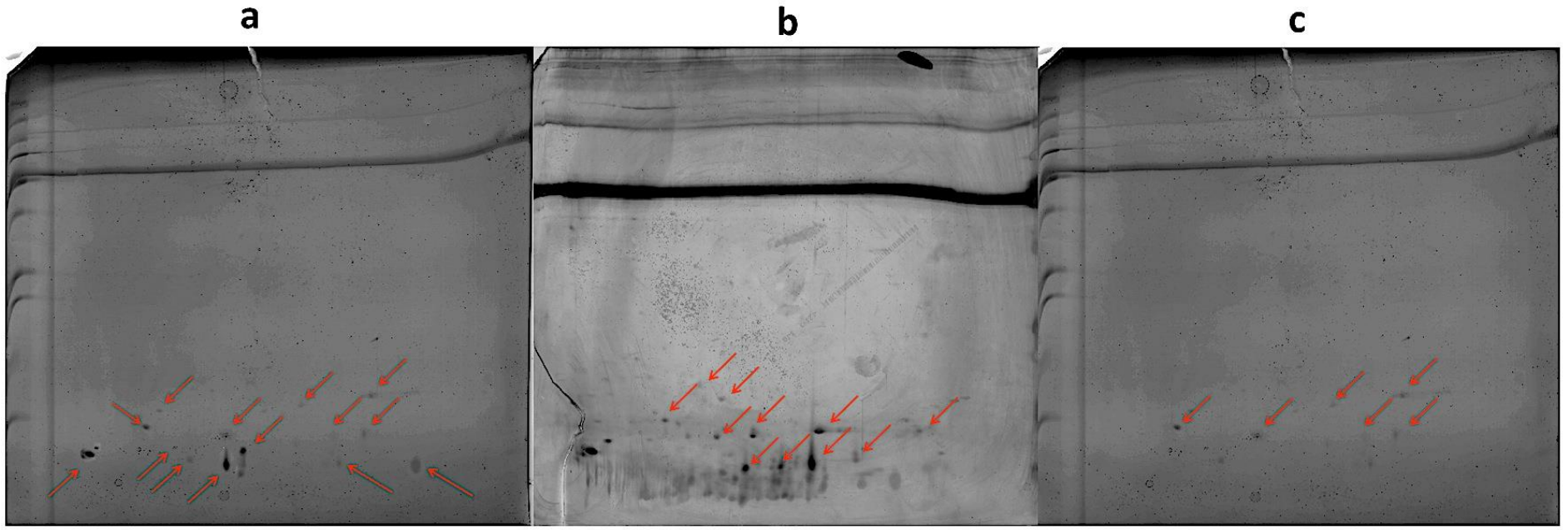

Figure 31. 2D Gels of an MT extracted bile sample - protocol B (60 minutes, 70 °C, 30 minutes) and reagents DTT (a) b-mercaptoethanol (b) and TCEP (c).

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.

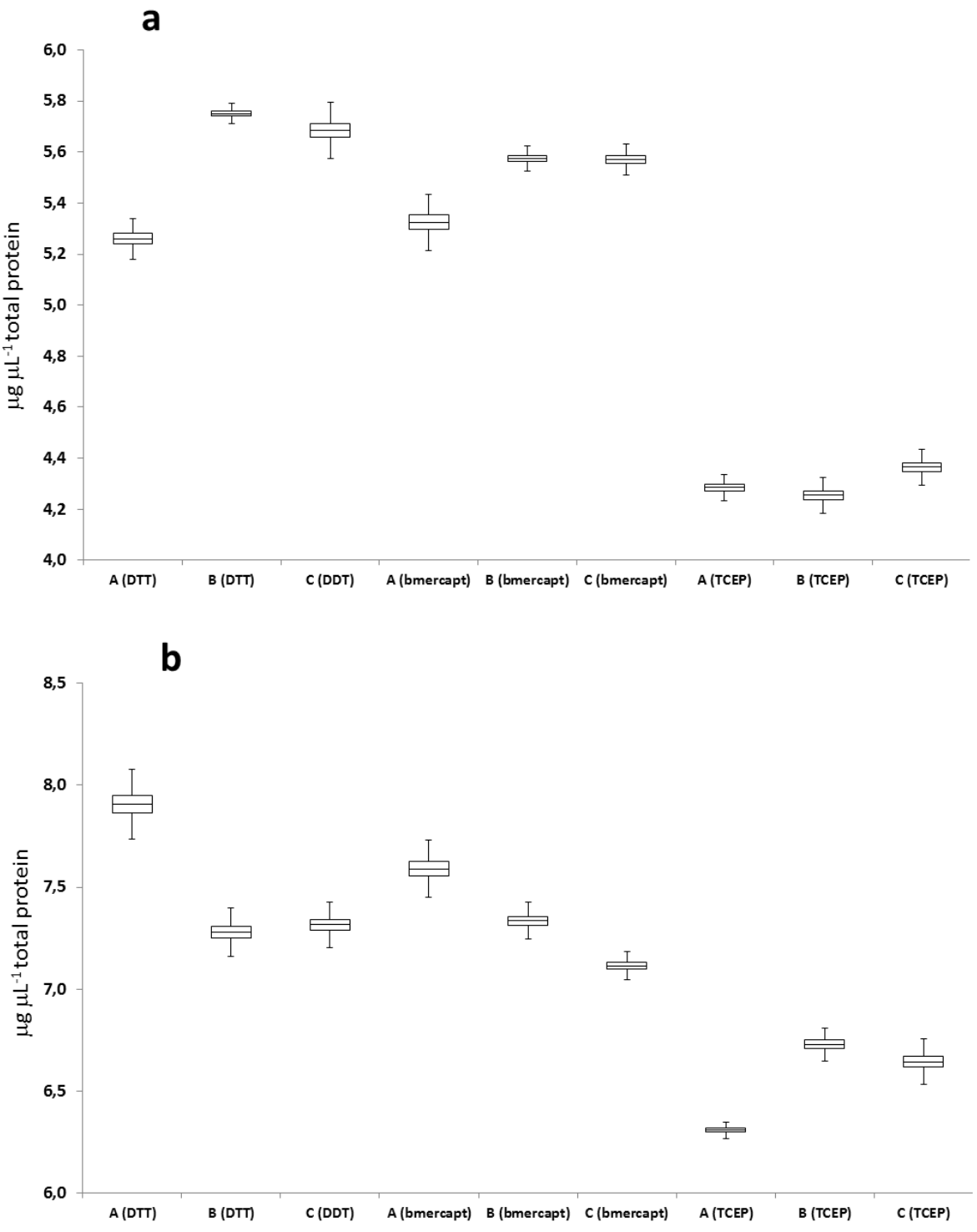

Figure 32. Box plot - measurement of total protein in bile (a) and liver (b) extraction with different procedures.

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.

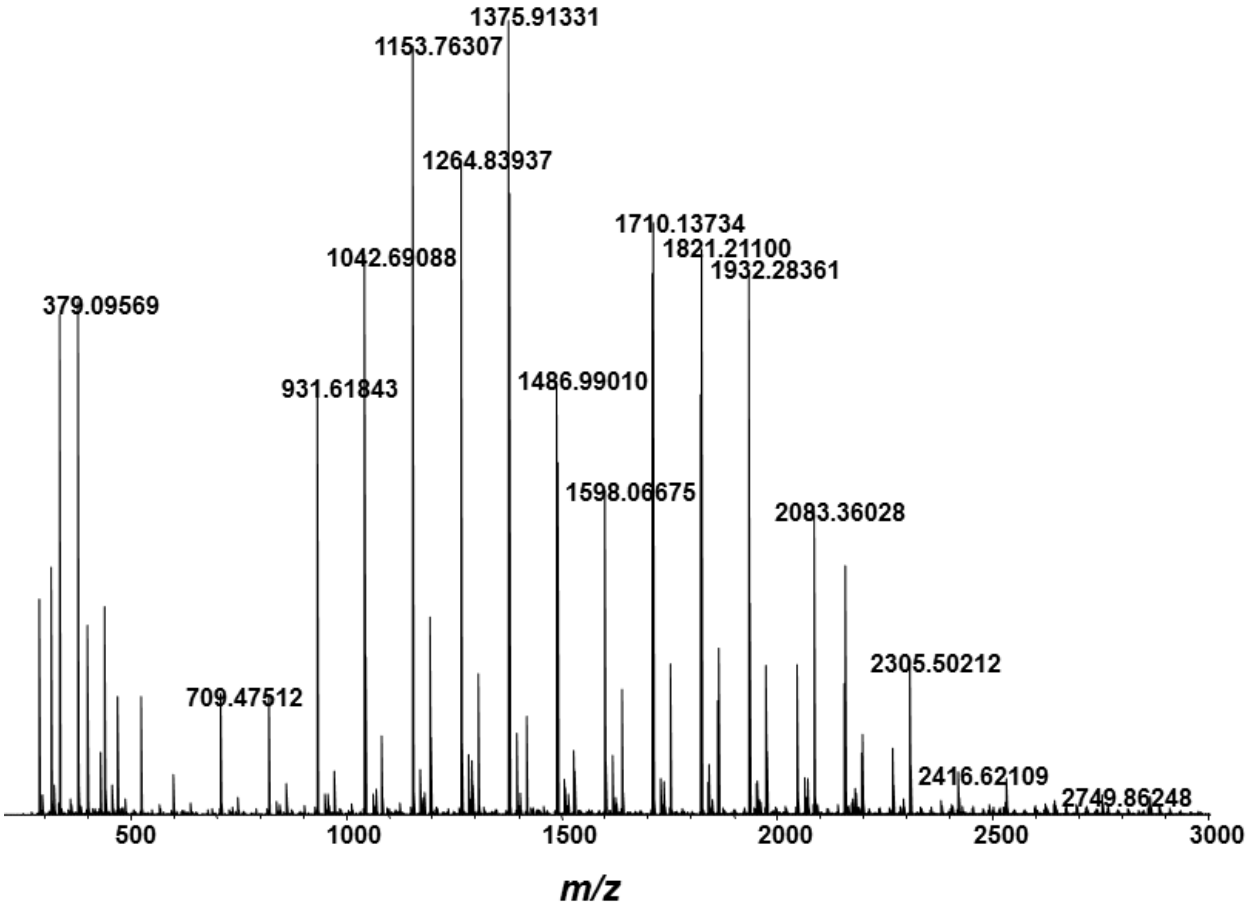

Figure 33. MALDI MS spectra of spot gel 2D trypinized where obtained the MT-B.

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.

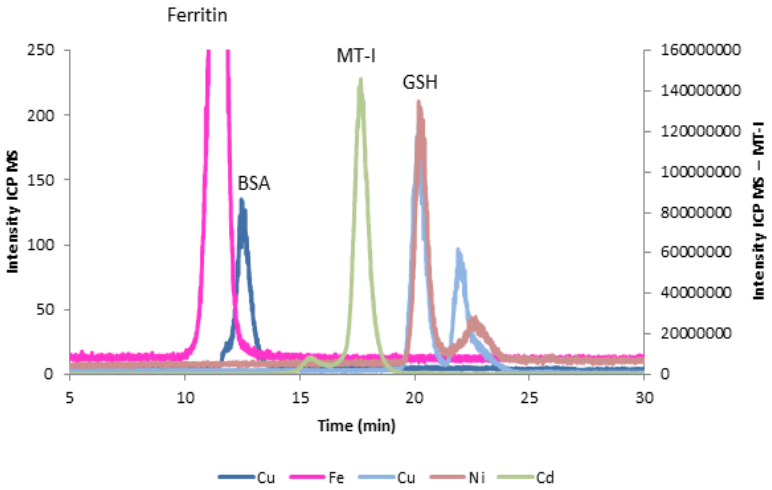

Figure 34. Standards used for column calibration in the SEC-HPLC-ICP-MS analyses.

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.

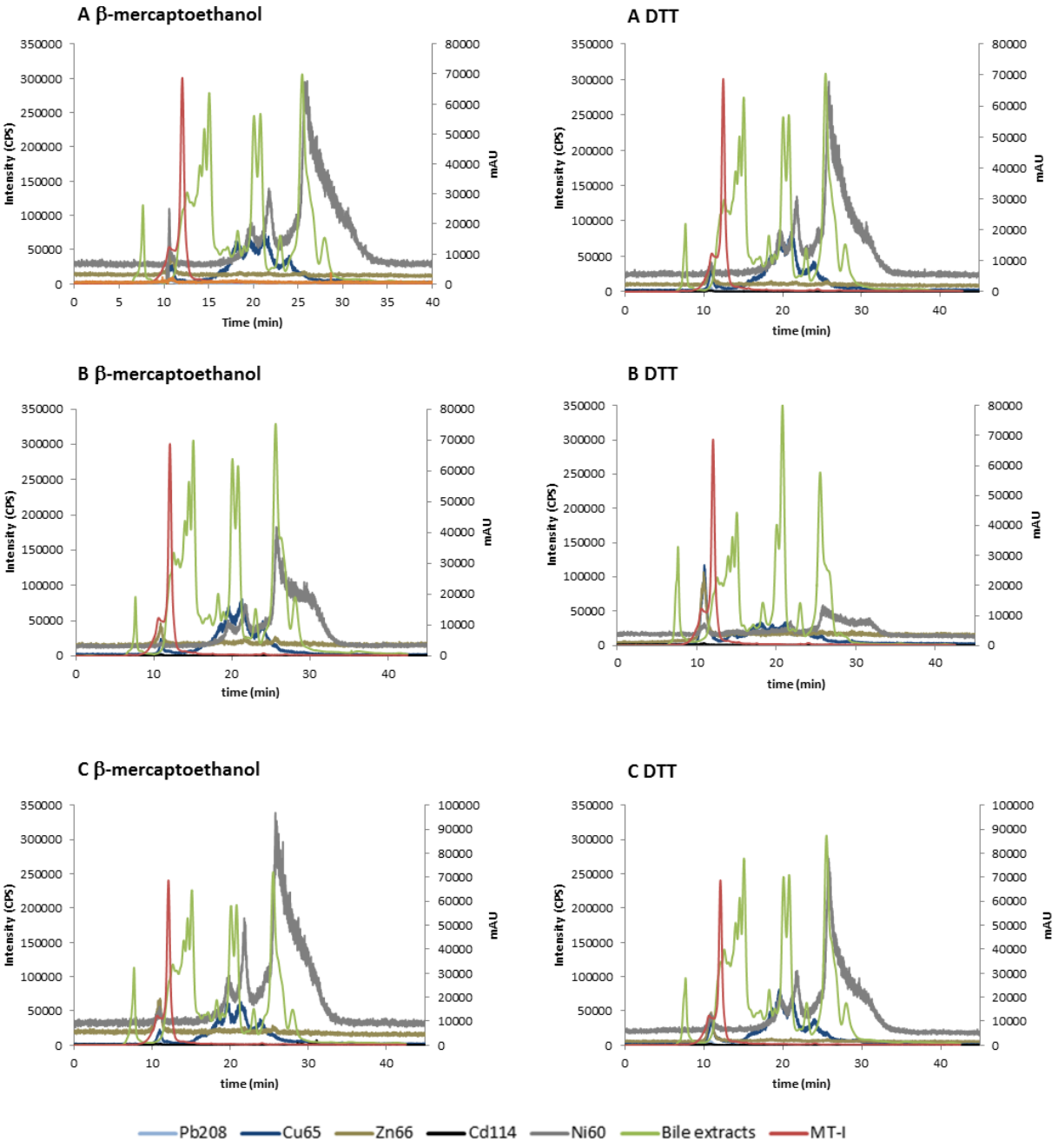

Figure 35. SEC-HPLC-UV-ICP-MS samples and MT-I standard for bile extracts.

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).

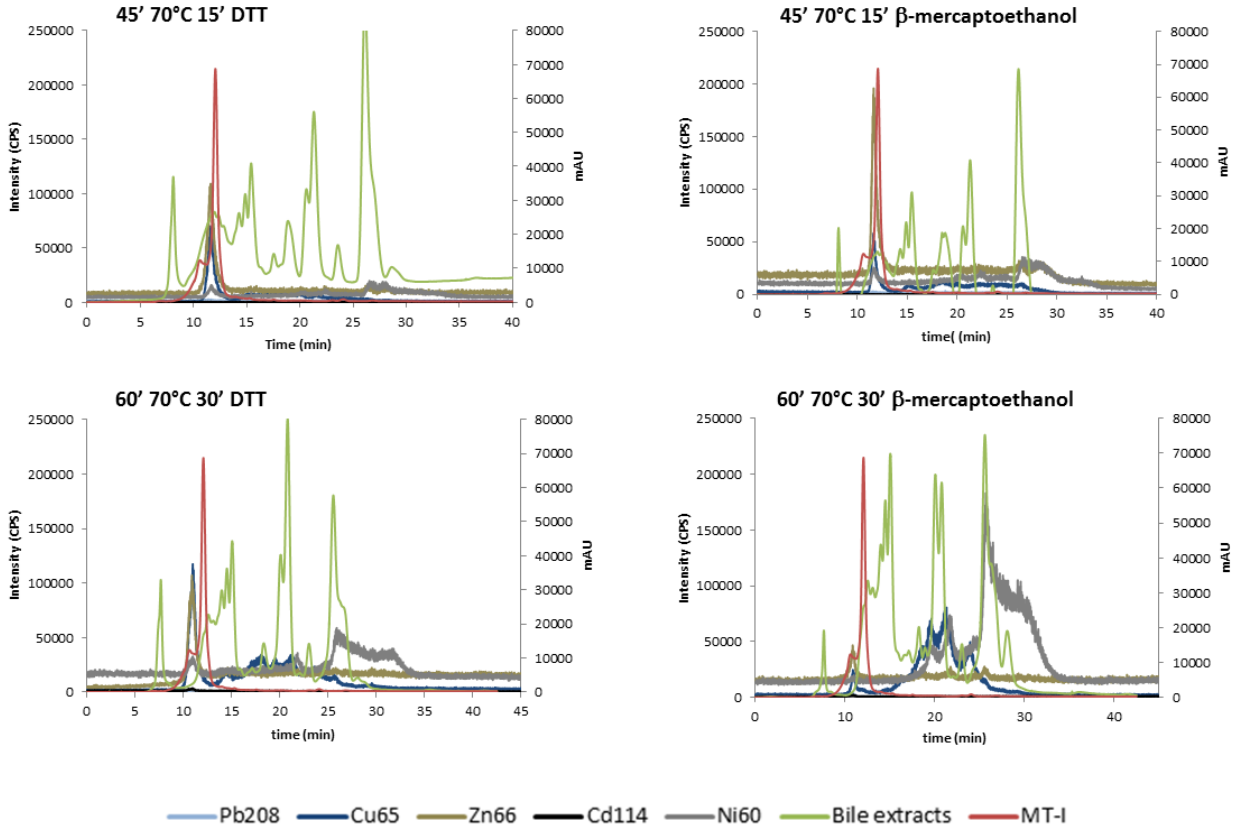

Figure 36. SEC-HPLC-UV-ICP-MS fish bile samples and MT-I standard for different extraction centrifugation times and the same temperature.

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]

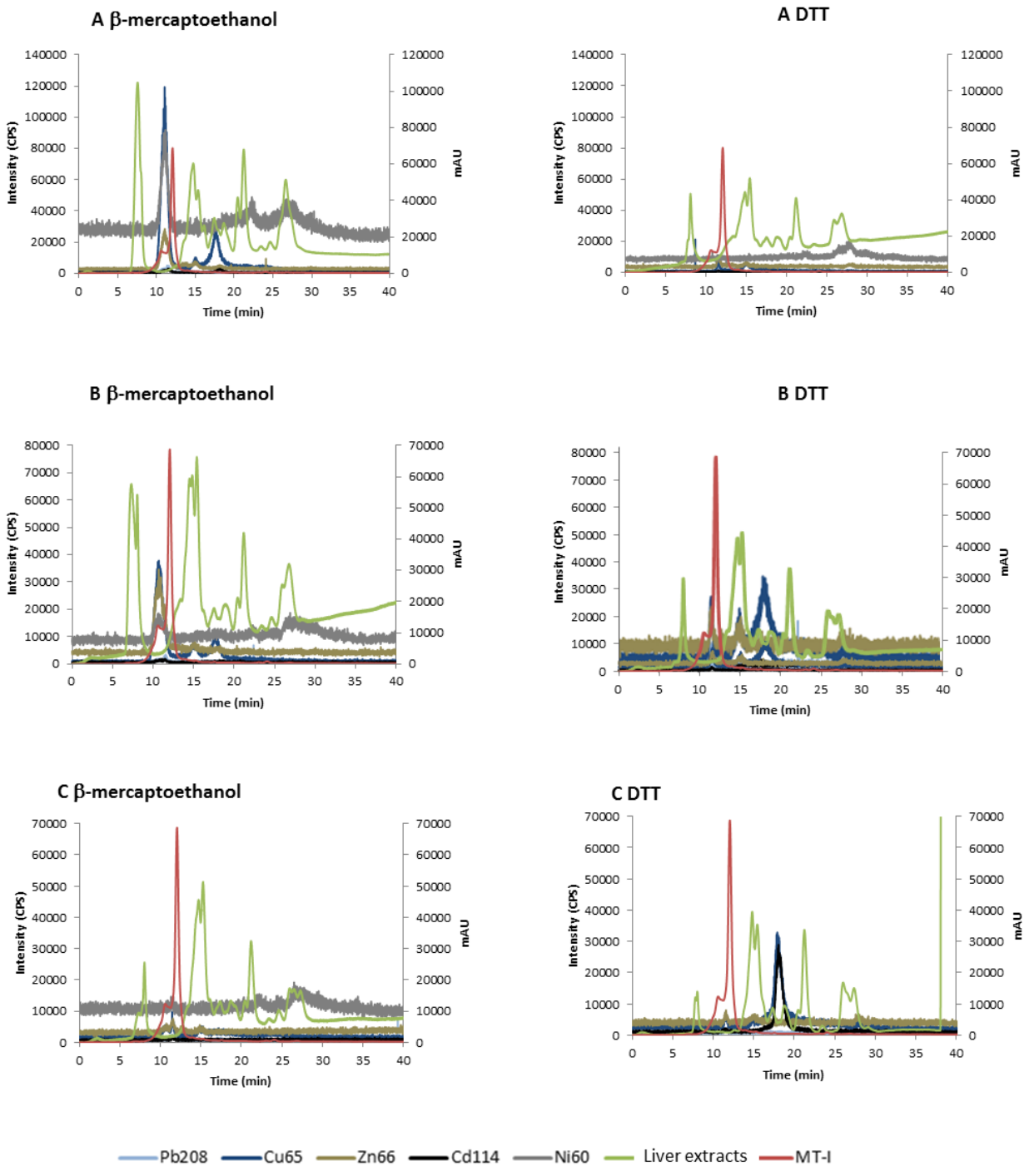

Figure 37. SEC-HPLC-UV-ICP-MS samples and MT-I standard for liver extracts.

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, 96 h, 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.

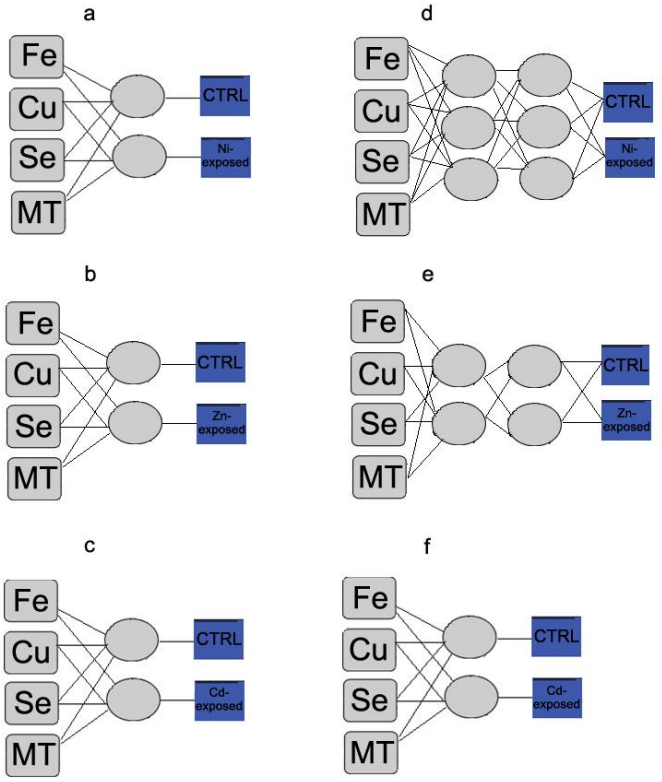

Figure 38: Architecture of ANN for classification as to metal exposure. (a, b and c) in bile samples, and (d, e and f) in liver samples.

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

Matrix	Liver	Bile
Element	Normalized importance (%)	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	Liver	Bile
Element	Normalized importance (%)	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.

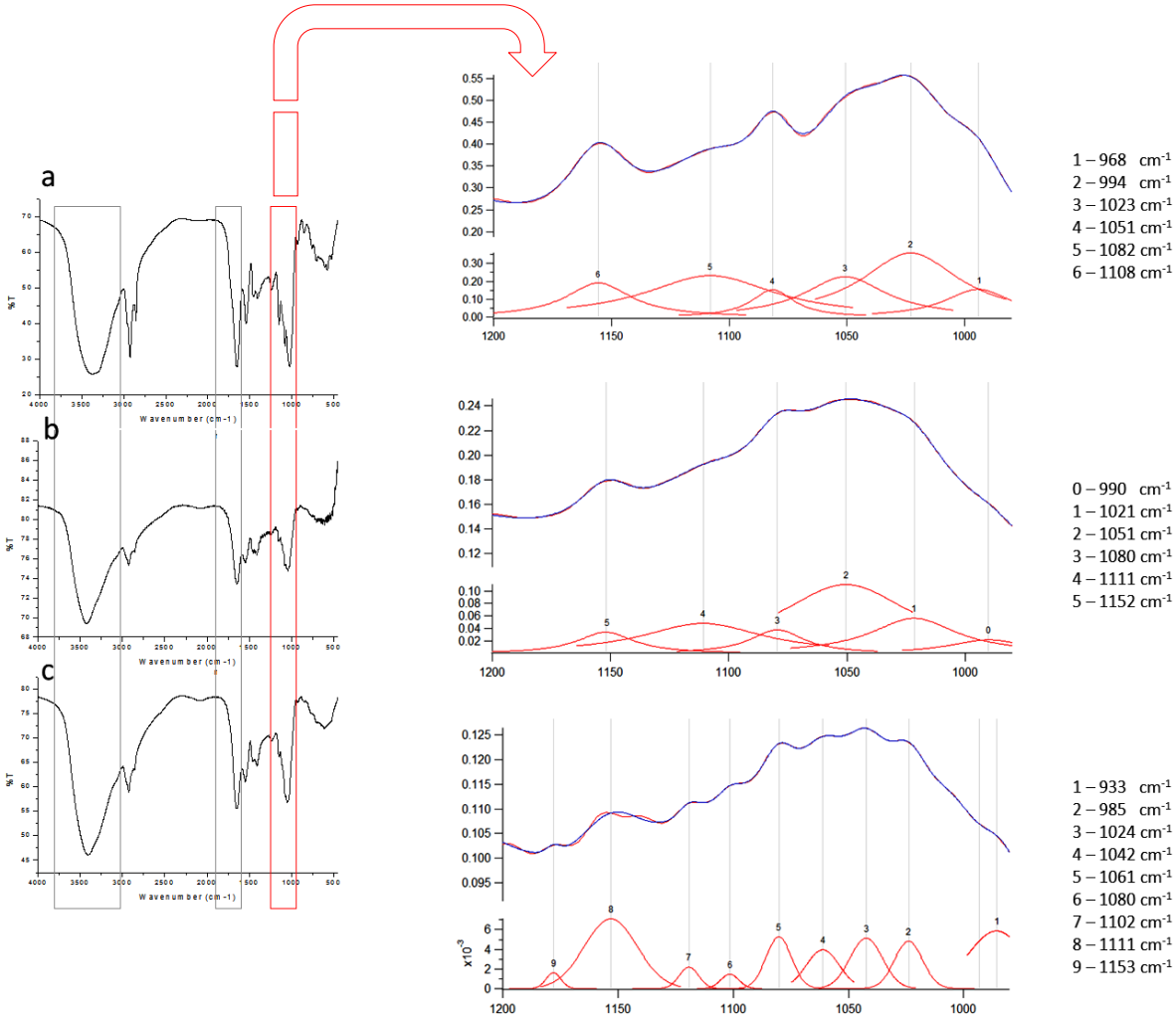

Figure 39. Infrared spectrum (a) crude liver, (b) purified liver and (c) delipidized liver; and on the right side of the figurethe deconvolution in the 1200 – 980 cm^{-1} region.