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Evaluation and standardization of different purification procedures for fish bile and liver metallothionein quantification by spectrophotometry and SDS-PAGE analyses

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ABSTRACT

Fish bile metallothioneins (MT) have been recently reported as biomarkers for environmental metal contamination; however, no studies regarding standardizations for their purification are available. Therefore, different procedures (varying centrifugation times and heat-treatment temperatures) and reducing agents (DTT, β-mercaptoethanol and TCEP) were applied to purify MT isolated from fish (*Oreochromis niloticus*) bile and liver. Liver was also analyzed, since these two organs are intrinsically connected and show the same trend regarding MT expression. Spectrophotometrical analyses were used to quantify the resulting MT samples, and SDS-PAGE gels were used to qualitatively assess the different procedure results. Each procedure was then statistically evaluated and a multivariate statistical analysis was then applied. A response surface methodology was also applied for bile samples, in order to further evaluate the responses for this matrix. Heat treatment effectively removes most undesired proteins from the samples, however results indicate that temperatures above 70 °C are not efficient since they also remove MTs from both bile and liver samples. Our results also indicate that the centrifugation times described in the literature can be decreased in order to analyze more samples in the same timeframe, of importance in environmental monitoring contexts where samples are usually numerous. In an environmental context, biliary MT was lower than liver MT, as expected, since liver accumulates MT with slower detoxification rates than bile, which is released from the gallbladder during feeding, and then diluted by water. Therefore, bile MT seems to be more adequate in environmental monitoring scopes regarding recent exposure to xenobiotics that may affect the proteomic and metalloproteomic expression of this biological matrix.

1. Introduction

Metalloproteins are proteins that contain one or more metallic ions in their structure, either directly connected to the peptide chain or inserted in a non-proteic molecule covalently linked to the peptide chain. Metallothioneins (MT) are a specific class of metalloproteins, first reported in 1957 in equine tissue [1]. Three years later, Kagi and Valee [2] described the term metallothioneins for the first time, suggesting their ability to bind to metal ions. Since then, the research area associated to metal-bound proteins has advanced considerably, and, for several decades, MT have been used as biomarkers in environmental monitoring studies, since they show differential expression in situations of environmental contamination by metals, specifically due to their direct relationship between the metal levels present in the environment and the concentrations found in animal tissues [3].

Studies using MT for this purpose are abundant regarding the aquatic environment and are usually conducted by the analyses of liver tissue [4,5], although studies using muscle [6,7], kidney [8,9] and gills also exist [10,11]. Liver measurements, however, are still the most employed, since this is the main detoxifying organ of the body and is a validated organ regarding exposure to environmental contaminants [12–15]. An alternative way to evaluate contaminant effects on the proteomic or metalloproteomic of fish in environmental monitoring studies has been proposed, by using fish bile [16,17]. This biological matrix is a validated biomarker

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regarding environmental contaminants, since it also excretes exogenous substances from blood and liver that were not excreted by the kidneys, such as metals and several organic compounds [16,18]. However, this option has not been explored enough in this context and studies are still scarce.

MT purification processes take into account several different factors. One of the main factors is the choice of the reducing agent, responsible for avoiding protein oxidation and commonly used to reduce the disulfide bonds, since MT are rich in cysteine groups. The most commonly used reagent in this case is β-mercaptoethanol [19–22]. However, other reagents are also able of carrying out this same function, such as DTT (ditiotreithol) and TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) [23]. The structures of these three compounds and their reactions when used as reducing agents are displayed in Fig. 1. 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 [24,25]. This agent selectively and completely reduces even the most stable water-soluble alkyl disulfides over a wider pH range (1.5–8.5) than DTT (pH between 6.5 and 9.0) and B-mercaptoethanol (between 5.0 and 8.5) [26].

Besides the reducing reagent, the procedures applied during purification also influence MT purification, such as centrifugation times and the temperatures used throughout the process, if thermal extraction is applied. All these factors must be evaluated before standardizing any purification method. Some studies [13,27,28] have used similar protocols for MT extraction from several organs, including liver, with slight modifications regarding these factors, however procedures for fish bile have not yet been analytically evaluated and standardized.

Among them, the protocol proposed by Erk et al. [27] for MT purification has been adapted and applied for the analyses of these proteins in several organisms, such as fish [17,29] and mussels [13,28,30,31]. This protocol has also been used in recent novel studies regarding fish bile MT with good results, demonstrating that bile MT are a potential biomarker for metal contamination [17]. However, again, there is a lack of studies regarding the effects of different purification procedures in this biological matrix.

It has been previously verified in our laboratory that this protocol also purifies other heat-stable proteins, including metal-bound proteins. Thus, due to the lack of studies and the importance of assessing differences in purification procedures that may significantly modify MT purification and quantification, the aim of the present study is to evaluate and standardize the currently applied thermal-extraction method for the purification and quantification of fish bile MT using different reagents and multivariate statistical analyses.

The selected species for this aim was the Nile Tilapia (*Oreochromis niloticus*). The choice of fish species is important, since several species may present characteristics that may not be ideal for future environmental contamination studies, such as migratory behavior patterns. The Nile Tilapia (*Oreochromis niloticus*) is a good choice in this regard, since it a resistant species, easily adaptable to laboratory conditions and validated as a bioindicator species regarding environmental contamination, including metals [32–35]. Also, Tilapia have been almost completely genetically sequenced, which is of great importance in proteomic studies, making protein identification and differential protein expression analyses, for example, much easier [36].

Additionally, in this context, it has also been observed in previous studies in our laboratory that bile MT follow the same trend as liver MT, of higher MT levels in organisms exposed to metals [17], indicating that MT in fish bile are an interesting potential biomarker regarding metal contamination. This shows the importance of protocol standardization for MT extraction, since different responses concerning different purification protocols lead to inconsistencies in MT level results. This is an important factor to take into account in the context of environmental monitoring and risk-assessment studies, demonstrating the importance of obtaining reproducible and comparable results regarding MT purification and quantification, since these inconsistencies may negatively affect decision-making policies regarding environmental contamination.

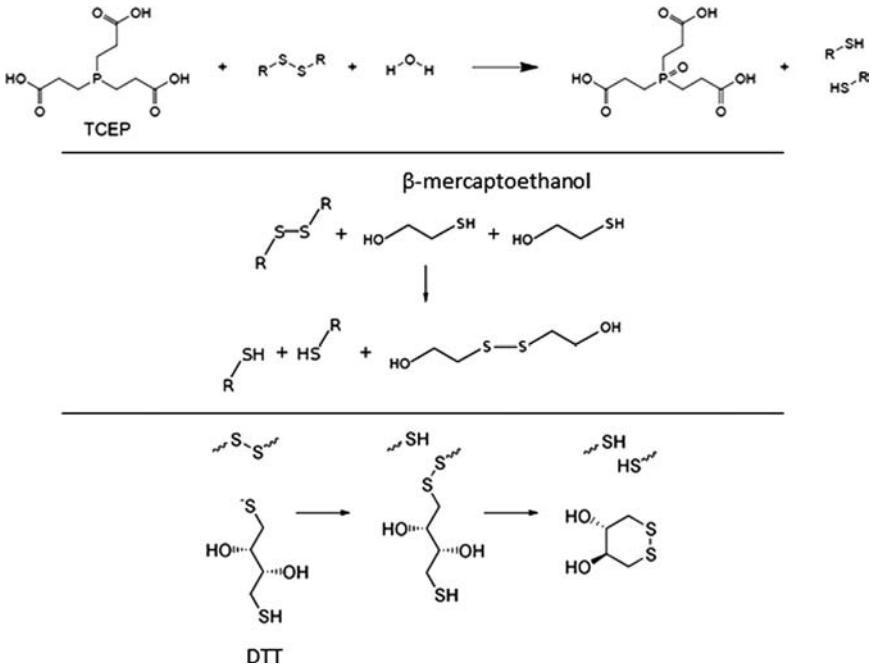

Fig. 1. TCEP, β-mercaptoethanol and DTT structures and their reactions when used as reducing agents.

2. Material and methods

2.1. Sample collection

Bile and liver samples were obtained from freshly caught Tilapia specimens (*Oreochromis niloticus*) obtained at a local fish market. Biometrical data was collected and the fish were dissected and liver and bile were removed, the latter by direct puncture of the gallbladder with a 5.0 mL syringe. Bile volume and color and liver weight were recorded. Samples for both bile and liver were pooled ($n=10$) and were then stored in sterile microtubes (bile) and polypropylene tubes (liver) at −80 °C until needed.

2.2. Sample processing and purification procedures

MT extraction was based on the thermal-extraction procedure proposed by Erk et al. [27], recently applied by our group for fish bile analyses [17]. This protocol uses DTT as the reducing agent and centrifugation times of 1 h and then another 30 min, with thermal extraction temperature of 70 °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-fosphine) 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*g* for different pre-established times at 4 °C. 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*g* for varying times at 4 °C and the final supernatants containing MT in the purified sub-samples were separated and frozen at −80 °C until analysis.

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. The choice for using spectrophotometric quantification is considered more adequate than other available techniques when the aim is to screen several environmental samples at a time, since it is, for example, quicker than voltammetric analyses and is also simpler and less costly. Because of this, this technique has been used for several decades as a simple quantification tool for MT analyses in ecotoxicological and environmental monitoring studies [37,38]. 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 [39], and incubated for 30 min. The samples were then centrifuged at 3000*g* for 5 min and the supernatant absorbance was evaluated at 412 nm using a SpectraMax (Hamilton, USA) microplate reader. MT concentrations were estimated by using reduced glutathione (GSH) as a standard for the analytical curve (0–1000 μmol L^{-1}) from a 10 mmol L^{-1} stock solution, as described by Viarengo et al. [40]. MT content was then estimated by assuming the relationship of 1 mol MT = 20 mols GSH, as described by Kagi for fish [41]. To establish recovery percentages by standards addition, and provide accuracy to the spectrophotometrical 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.

2.4. 1D SDS-PAGE analyses

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 quantified by the Lowry method, modified by Peterson using Bovine serum Albumin (BSA) as standards [42].

Polyacrilamide gels (15%) were prepared according to Laemmli et al. [43]. Aliquots of both bile and liver MT extracts containing 50 μg of total protein were applied to each lane, along with the molecular weight standards. Gels were run, in triplicate, for approximately 2 h 30 min, at 45 mA/gel. Gels were then stained using the silver stain method as described by Heukeshoven and Dernick [44]. The molecular weights of the protein bands and spots were determined using molecular weight standards (Biorad Precision Plus ProteinTM Dual Color Standards). Gels were scanned using an ImageScanner II (GE Healthcare, Uppsala, Sweden) with the densitometer operating at 300 dpi resolution. Image-Master 2D Platinum 6.0 software (GeneBio, Geneva, Switzerland) was employed for gel imaging analysis. Optic densitometry using SDS-PAGE gels was not conducted for MT quantification, since this method is not as efficient as spectrophotometric analyses, as discussed elsewhere [17].

2.5. 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 [45]. In the present study, 10 measurement repeats were conducted to ensure the validity of the calculations.

2.6. 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.

3. Results and discussion

3.1. Spectrophotometric analyses

With the standard addition of a MT-I purified standard 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,

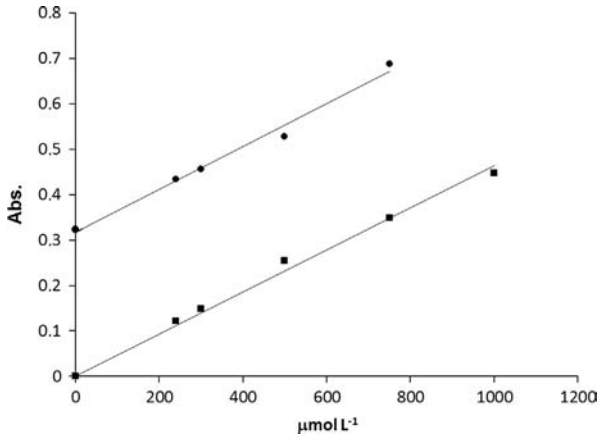

Fig. 2. Analytical curves for metallothionein quantification: (■) GSH standards, $I = 0.0005C$, $R^2 = 0.9943$. (●) MT standard addition in bile sample, $I = 0.0005C + 0.3167$, $R^2 = 0.9864$.

Table 1
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[a]	60	70	30
C	75	80	45

[a] Protocol established by Erk et al. [27].

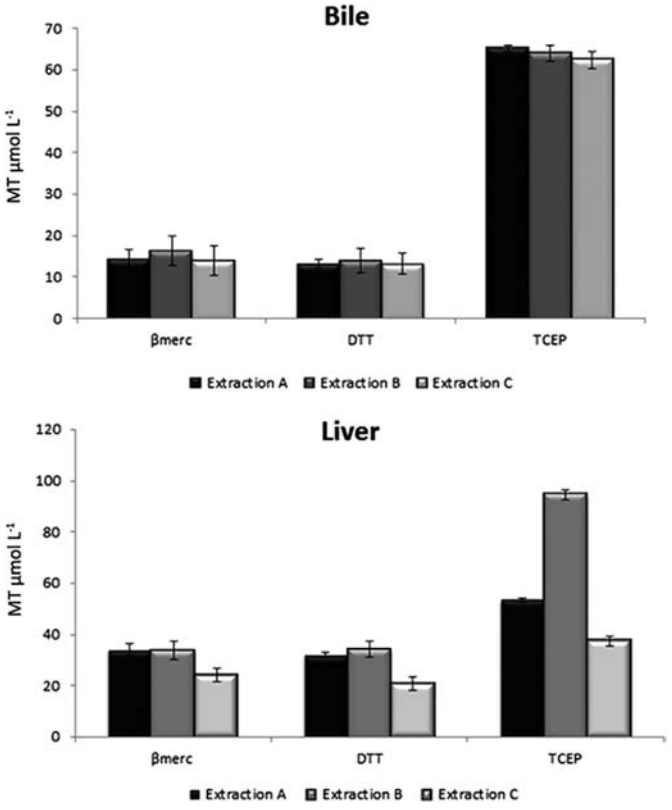

Fig. 3. Metallothionein concentrations in bile and liver (expressed in µmol L^{-1}) for each of the tested purification procedures and reagents.

thus corroborating these results. 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 GSH as the standards throughout the study, since GSH is significantly cheaper and easier to obtain than MT standards. Both curves are displayed in Fig. 2.

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 and the extraction temperature varied as a single factor, as can be seen in Table 1. All analyses were conducted in triplicate.

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

Table 2
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

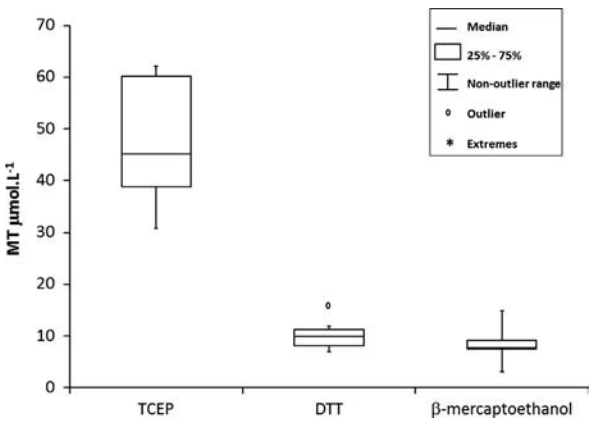

Fig. 4. Reagent box-plot chart data.

displayed in Table 1 (Fig. 3). The extraction temperature of 70 °C using TCEP as the reducing reagent, however, was 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 [46].

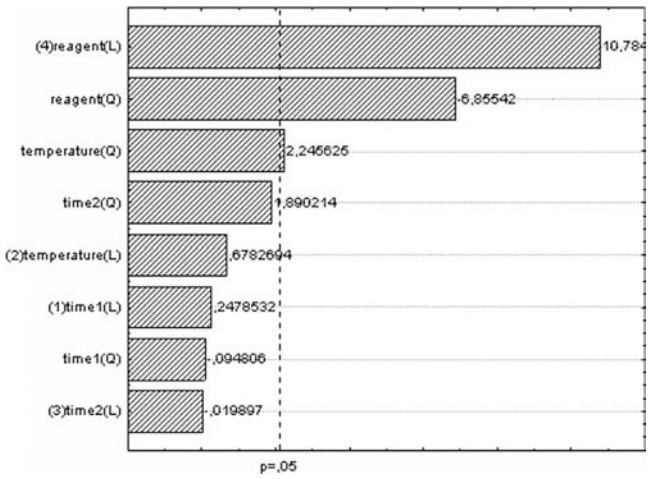

Fig. 5. Pareto Chart of standardized effects regarding the studied factors.

These first 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 four factors with three 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 2.

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 (Fig. 4), which did not occur with TCEP.

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 Fig. 5, constructed from the procedures displayed in Table 2.

3.2. Response surface methodology for bile samples

Response surface methodology (RSM) explores the relationships between several explanatory variables and one or more response variables [47]. The main idea of RSM is to use a sequence of designed experiments to obtain an optimal response. By analyzing the surface graphs (Fig. 6) we observe that the best response for bile samples is given by using the combination of extraction procedures, temperature and reagents of extraction A, consisting of 45 and 15 min centrifugations, 70 °C, as conducted in extraction B with TCEP 1%.

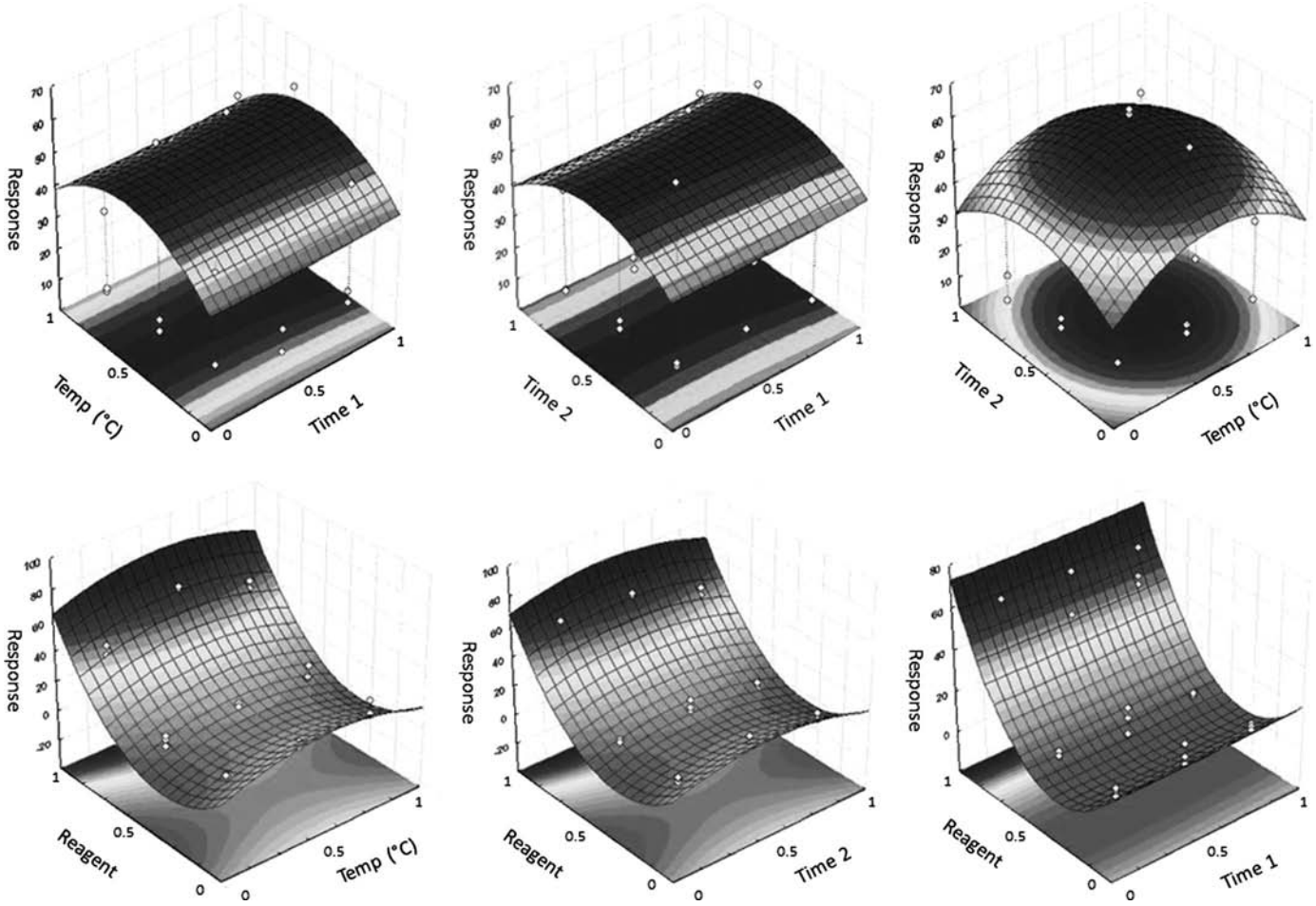

Fig. 6. Response surface charts for each of the studied factors for tilapia bile MT samples.

This differs from the protocol found in the literature [27], which uses 60 min during the first centrifugation, followed by a 70 °C 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. TCEP, thus, may be used as a substitute for both 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 [48] and shows enormous potential regarding environmental monitoring of xenobiotic effects on the proteomic and metalloproteomic expression of this fluid.

3.3. 1D SDS-PAGE analyses

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. [17]).

When comparing these electrophoretic qualitative results (Fig. 7) 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 °C and 70 °C, respectively) showed stronger MT bands.

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 °C. 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. 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.

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.

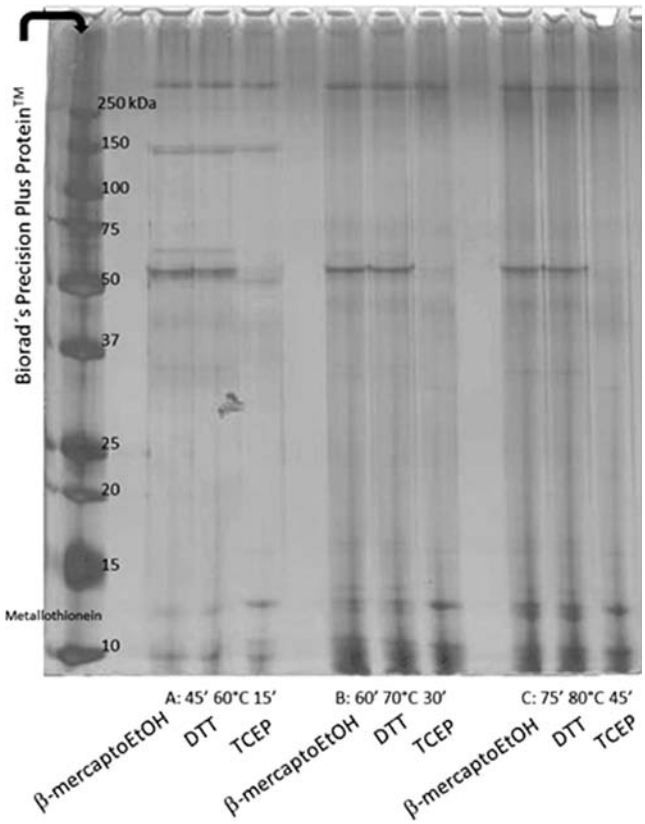

Fig. 7. Qualitative SDS-PAGE gels for bile samples using the different extraction procedures and reagents analyzed in the present study.

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.

4. Conclusions

Heat treatment effectively removes most undesired proteins from fish liver and bile samples, however results indicate that temperatures above 70 °C are not the most efficient since they also remove MT from both matrices. Among the three analyzed reducing agents, TCEP was shown to be the most efficient, whereas DTT and β-mercaptoethanol showed similar results both in the spectrophotometric quantification and the qualitative SDS-PAGE analyses. SDS-PAGE analyses were shown to be useful in corroborating the standardization results obtained by the spectrophotometric and statistical analyses regarding bile and liver MT. Furthermore, they aided in distinguishing certain characteristics that may not be observed in spectrophotometric analyses of the different purification processes, such as the presence or absence of other proteins in the purified samples. Our results also indicate that the centrifugation times are not as important in MT quantification as the choice of reducing agent, and that the centrifugation times described in the literature can be reduced in order to analyze more samples in the same timeframe with the same quantification response. This is of extreme importance in environmental monitoring contexts where samples are usually very numerous and speed of analysis is of the essence. In an environmental context, biliary MT was lower than

liver MT, as expected, since liver accumulates MT with slower detoxification rates than bile, which is released from the gallbladder during feeding and diluted by water. Therefore, bile MT seems to be more adequate than liver MT in environmental monitoring contexts regarding recent exposure to xenobiotics that may affect the proteomic and metalloproteomic expression of this biological matrix.

Acknowledgments

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