5.
Conclusions

5.1.
PART I: Peptide derivatization and complexation with metals

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

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

PUC-Rio - Certificação Digital Nº 1012265/CA

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

5.2.1.
Optimization with commercial fish

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

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

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

The profiles obtained from the SEC-HPLC-ICPMS analyses showed no significant differences when comparing bile and liver, suggesting that both

PUC-Rio - Certificação Digital Nº 1012265/CA

matrices would be able to provide the same information regarding MT induction, further corroborating bile potential as an interesting biomarker. However, MT isoforms are not able to be separated by this technique. This technique also corroborated the SDS-PAGE analyses, indicating that the extraction procedure is not effective only for MT, since other thermo stable proteins are also extracted in the process and were also separated by size exclusion chromatography technique (SEC), alongside MT.

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

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

PUC-Rio - Certificação Digital Nº 1012265/CA

5.2.2.
Analyses of the laboratory-exposed fish

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

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

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

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

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

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

PUC-Rio - Certificação Digital Nº 1012265/CA

5.3.
Future prospects

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

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

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

PUC-Rio - Certificação Digital Nº 1012265/CA