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Analytical approach to the metallomic of Nile tilapia (*Oreochromis niloticus*) liver tissue by SRXRF and FAAS after 2D-PAGE separation: Preliminary results

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ABSTRACT

An investigation was made into calcium, iron and zinc in protein spots in samples of Nile tilapia (*Oreochromis niloticus*) liver tissue obtained after protein separation by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and subsequent qualitative and quantitative evaluation by synchrotron radiation X-ray fluorescence (SRXRF) and Flame Atomic Absorption Spectrometry (FAAS). An analysis of the fluorescence spectra indicated the presence of calcium, iron and zinc in 12, 6 and 8 liver protein spots, respectively. The metal ions found were distributed mainly in proteins with a molar mass of less than 40.00 kDa and more than 12.00 kDa, with p*I* in the range of 4.70–9.40. The only exception was a spot presenting protein with a molar mass of 10.10 kDa. In addition to calcium, iron and zinc, sulfur and phosphorus – which are non-metals that may be part of the protein structure, were also detected. After microwave-assisted acid mineralization of the proteins spots, a FAAS estimation of the concentration of calcium, iron and zinc ions bound to these proteins indicated a range of 1.08–5.80 mg g^{-1}, 2.02–8.03 mg g^{-1} and 1.60–8.55 mg g^{-1}, respectively.

1. Introduction

Inorganic elements make up only 4% of animal body tissue, but their role as structural elements is essential in many vital processes. The main roles of these elements can be described as functional and structural. From the functional standpoint, they play a catalyzing role in enzymatic systems by binding their ions to substrates, thereby favoring various reactions, especially in the mediation of oxidation–reduction reactions, or redox reactions, through reversible changes in the oxidation state of the metal ion. Structurally, they stand out for their role as integrators of the body's organic compounds, such as iron in hemoglobin and cobalt in vitamin B12 [1–3]. Because their proportions and quantities in animal body tissue are variable, inorganic elements are classified as macroelements (elements the body needs in large quantities) and microelements (elements it requires in smaller amounts). Twenty-five of the chemical elements in the periodic table can be classified as essential. However, in practical terms, the macroelements considered essential are calcium, phosphorus, magnesium, potassium, sodium, chlorine and sulfur, while the essential microelements are iron, iodine, selenium, cobalt, manganese, zinc and copper [4–7].

Metalloproteins and metal ions bound to proteins represent a large portion of the total number of proteins. It is estimated that approximately 40% of all proteins and enzymes require the presence of a metal ion for their biological activity [8]. Metalloproteins are considered different from metal-bound proteins. Metalloproteins constitute a group of proteins that contain a metal cofactor incorporated by means of specific bonds, and are characterized by the high affinity of the metal–protein interaction. Metal-bound proteins, on the other hand, comprise a group of proteins which incorporate metal ions through nonspecific bonds, and are characterized by the low affinity of the metal–protein bond, which is therefore easily broken [9,10]. Monovalent ions such as sodium and potassium are weakly bound to proteins, while magnesium and calcium are moderately bound. Among the metals strongly bound to proteins, the most frequent are transition metals such as iron, copper, zinc, manganese, molybdenum and cobalt, which, due to their properties (density, small atomic radius, electromagnetic interaction and electrostatic forces), are bound to most of the metalloproteins [11,12].

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In recent years, studies related with the identity and localization of a metal or metalloid in a cell or tissue type, are termed the metallome. The study of a metallome, the interactions and functional connections of metal ions with genes, proteins, metabolites and other biomolecules within an organism or ecosystem is then termed metallomics. The metallomic allowed the integration of traditional analytical studies with inorganic and biochemical studies [13–15]. Thus, the emerging techniques that are available or being developed, should not just be restricted to analytical chemistry, bioanalytical chemistry, biochemical characterization and species analysis, but can and should be used to help increasing our understanding of the biological and environmental problems related to metal ions [16].

Based on the above, the present work involved a qualitative and quantitative investigation of calcium, iron and zinc in protein spots of Nile tilapia (*Oreochromis niloticus*) liver samples by SRXRF and FAAS, after separation by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The results presented in this paper represent the beginning of the metallomic study of Nile tilapia fish.

2. Experimental

2.1. Equipment

In addition to the glassware normally used in a laboratory of analytical chemistry, the following equipment and accessories were used in this work: a Thermolyne M-37600 vortex mixer (Dubuque, USA), a Mettler AE200 analytical balance (Bedford, USA), GE SDS-PAGE electrophoresis vat (Uppsala, Sweden), a ThermoSpectronic GENESYS 6 UV–Vis spectrophotometer (Rochester, USA), a Quimis Q225M shaking table (Diadema, Brazil), a GE Healthcare Life Sciences Ettan™ Dalt Six 2D-PAGE electrophoresis system (Uppsala, Sweden), an Amersham Biosciences EPS1001 isoelectric focusing system (Uppsala, Sweden), a scanner (GE Healthcare), a BioAgency Bio-Spin-R refrigerated ultracentrifuge (São Paulo, Brazil), a Shimadzu AA-6800 atomic absorption spectrometer (Tokyo, Japan), and a Provecto Analítica DGT 100 Plus microwave oven (Campinas, SP, Brazil).

2.2. Reagents and solutions

All the solutions used in this work were prepared with ultrapure water (18.2 MΩ cm^{-1}) purified in an Elga PURELAB Ultra Ionic system. Analytical grade acetic (J. T. Backer) and phosphoric (Mallinckodt) acids were used throughout for the preparation of buffer solutions. All the organic solvents used (e.g., ethanol, acetone, methanol) were of analytical grade and were supplied by Merck. The solutions used in the electrophoretic separations and protein standards were prepared with reagents of analytical purity from Amersham Biosciences. Coomassie Blue G-250 (J. T. Backer) and bovine serum albumin (Merck) were used for the determinations of total protein. All the solutions were stored in polypropylene and/or glass flasks, and the solutions used for the electrophoretic separations were kept under refrigeration at 4 °C (e.g.) or frozen at −80 °C.

All the flasks used for storing buffer solutions, extracting solutions and protein standards were washed in nitric acid 10% (v/v) for 24 h, rinsed in ultrapure water and airjet-dried with pure air prior to use.

2.3. Sample collection and preparation

The liver samples were taken from Nile tilapia (*Oreochromis niloticus*) juveniles bred in the Laboratory of Nutrition of Aquatic Organisms of the Faculty of Veterinary Medicine and Zootechny/UNESP/SP. At the end of the feeding program, the fish were anesthetized with a benzocaine solution (100 mg L^{-1}), after which they were killed and the liver removed. The liver samples were then transferred to 15 mL polypropylene flasks and frozen at −80 °C.

2.4. Preparation of samples for electrophoresis

2.4.1. Protein extraction from the samples

The liver samples collected and stored as described above were subsequently macerated in deionized water, using a mortar and pestle. The extracts containing liver protein were then separated from the solid part, centrifuged at 13,000 rpm in an ultracentrifuge cooled to 4 °C, transferred to Eppendorf® tubes and later used for the quantification of protein content and for electrophoretic runs.

2.4.2. Protein precipitation and resolubilization

The proteins in the liver extracts were precipitated by mixing the samples with ice-cold solution of acetone 80% (v/v) in a proportion of 1:4 (sample:acetone). The protein precipitate was then centrifuged at 10,000 rpm in a refrigerated ultracentrifuge for 10 min and the supernatant was removed. After that, it was washed again twice with the same solution used for the precipitation. The albumin was also removed from the liver samples using a GE Healthcare RPN6300 Albumin and IgG Removal Kit, since these samples contained high concentrations of albumin. After these procedures, part of the protein precipitates was resolubilized in Tris–HCl buffer at 1 mol L^{-1} (pH 6.8) for the quantification of the total protein content and the remaining protein precipitates was resolubilized in buffer containing urea 7 mol L^{-1}, thiourea 2 mol L^{-1}, CHAPS 2% (m/v), ampholytes 0.5% (v/v), pH 3–10, and bromophenol blue 0.002%, as well as 2.8 mg of DTT. This mixture was used in the electrophoretic separations [17].

2.4.3. Determination of the total protein concentration

The total protein concentration in the liver protein extracts was determined following Bradford's method, using bovine serum albumin as standard. Analytical calibration curves were built in the range of 25–100 μg mL^{-1}, starting from a standard stock solution of 500 μg mL^{-1} of bovine serum albumin, with the same buffer used in the resolubilization of the proteins. Whenever necessary, the samples were diluted with the same buffer. 200 μL of properly diluted standard/sample and 2.5 mL of Bradford reagent were then mixed in plastic vessels. After 15 min of reaction, absorbance readings were taken at 595 nm [18,19].

2.4.4. Electrophoretic separations

Before starting the electrophoretic separations, the liver protein extracts were applied on 13-cm strips for isoelectric focusing, which contained the prefabricated gel with the immobilized ampholytes at pH 3–10 or 4–7. These strips were placed on a focusing tray, where they remained for 12 h at room temperature to be rehydrated with the protein extract. A mass of approximately 250 μg of protein was added to these strips (considering the protein concentration determined in the liver extract). Besides protein extract, about 900 μL of mineral oil was also placed on the strips. After this 12-h period, the rehydrated strips were placed in an isoelectric focusing unit for the first-dimension separation of 2D electrophoresis. After the strip rehydration step with protein extract, the first dimension of the electrophoretic process was performed in the isoelectric focusing unit, using the following program [15]: Step 1 – 500 V in 500 Vh; Step 2 – 1000 V in 800 Vh; Step 3 – 10,000 V in 11,300 Vh and Step 4 – 10,000 V in 3000 Vh (total of accumulated voltagem 15,600 Vh) [20].

The first-dimension separation program lasted on average 4.5 h. At the end of the isoelectric focusing step, the strip with the proteins separated as a function of the p*I* was equilibrated in two stages. The

first stage involved the use of 10 mL of solution containing urea 6 mol L^{-1}, SDS 2% (m/v), glycerol 30% (v/v), Tris–HCl 50 mmol L^{-1}, bromophenol blue 0.002% (m/v) and DTT 2% (m/v). The purpose of this stage, called reduction, is to keep the proteins in their reduced forms. A solution with a similar composition was used in the second stage, but the DTT was replaced by iodoacetamide 2.5% (m/v). The purpose of this stage, called alkylation, is to alkylate the thiol groups of the proteins, preventing them from reoxidizing during the electrophoretic process. Each of these stages lasted 15 min and was performed under mild shaking on a shaking table [17].

After equilibration of the strips, the second dimension of the electrophoretic process (SDS-PAGE) was performed. The strips containing liver proteins were placed in polyacrylamide gel 12.5%, previously prepared on 180 mm × 160 mm × 1.5 mm glass slides. The polyacrylamide gels were prepared from the following solutions: Acrylamide, N,N′-methylenebisacrylamide, Tris(hydroxymethyl) aminomethane, Sodium Dodecyl Sulfate (SDS), N,N′,N,N′-tetramethylenediamine (TEMED), Hydrochloric Acid (HCl) and Ammonium Persulfate. A sheet of filter paper was placed on the polyacrylamide gel, next to the strip, onto which was applied 10 μL of molar mass standard containing the proteins β-phosphorylase (97.00 kDa), albumin (66.00 kDa), ovoalbumin (45.00 kDa), carbon anhydrase (30.00 kDa), trypsin inhibitor (20.01 kDa), and α-lactalbumin (14.40 kDa). The strip and filter paper were sealed with a hot solution of agarose 0.5% (m/v) to ensure their contact with the polyacrylamide gel. The second dimension electrophoretic run was then performed in a 2D-PAGE electrophoresis unit, in two stages, using the following program [15,20,21]: Stage 1 – voltage = 90 V, electric current = 25 mA, power = 100 W, time = 0.5 h; Stage 2 – voltage = 250 V, electric current = 25 mA, power = 100 W, time = 5 h.

After the run, which lasted approximately 5.5 h, the proteins were revealed using a colloidal Coomassie stain reagent consisting of a solution of ammonium sulfate 8% (m/v) phosphoric acid 1.6% (v/v), Coomassie blue G-250 0.08% (m/v) and methanol 25% (v/v) [16,17]. Before staining, the proteins were fixed for 1 h, using a solution containing acetic acid 10% (v/v) and ethanol 40% (v/v) [15,21]. The dye remained in contact with the gel for 72 h, after which it was removed in successive washes with deionized water. The resulting liver gels were then scanned.

2.5. Qualitative determination of calcium, iron and zinc by SRXRF in the samples' protein spots

The qualitative determinations of calcium, iron and zinc in the protein spots were performed on an X-ray fluorescence line with synchrotron radiation at the Brazilian National Laboratory of Synchrotron Light–LNLS, located in Campinas, SP, Brazil. The protein spots were removed from the gel with the tip of a pipette, dried for 20 min under an infrared lamp, and fixed on an aluminum platform. After establishing the analytical conditions, mapping was carried out by irradiating the spot for 200 s at two points. The spectra thus collected were processed using the AXIL program, which allows the variation of the intensity of the synchrotron radiation beam to be corrected, normalizing the area of the peaks of the chemical species detected by counting the argon peak. A fraction of polyacrylamide gel that had not protein spots was used as analytical blank [22].

2.6. Quantitative determination of calcium, iron and zinc by FAAS in the samples' protein spots

The calcium, iron and zinc in the protein spots were quantified by Flame Atomic Absorption Spectrometry after mineralizing the samples in a microwave oven. The calcium, iron and zinc determinations were carried out with a SHIMADZU AA-6800 atomic absorption spectrometer equipped with background absorption correction with a deuterium lamp and a self-reverse (SR) system. Hollow cathode lamps of calcium (wavelength = 422.7 nm), iron (wavelength = 248.3 nm) and zinc (wavelength = 213.9 nm) operating with a current of 10 mA and a spectral resolution of 0.5 nm were used. The analytical curves were prepared using Titrisol Merck standard solutions and as analytical blank was used the region of the gel where no protein spots appeared [23].

3. Results and discussion

3.1. Determination of the samples' total protein concentration

The total protein content in the extracts of the liver samples was determined under Section 2.4.3, in order to determine the best protein mass to be applied in the electrophoretic runs by 2D-PAGE, taking into account the resolution of the gel. The results of this determination indicated that extracts of the liver samples contained 5.25 ± 0.07 μg μL^{-1} of total protein. Based on these findings, volumes of 50 μL of liver samples were applied to the strips containing prefabricated gel for isoelectric focusing, in order to have a mass of approximately 250 μg of protein for each type of sample.

3.2. Optimization of the electrophoretic separations

The 2D-PAGE separation systems were tested to try to obtain high resolution electrophoretic gels, since the electrophoretic separation system in only one dimension would not show a good resolution, but instead, electrophoretic bands of a group of proteins with similar molar masses. In 2D-PAGE electrophoresis, each stain in the gel, commonly referred to as a "spot", represents most likely a single protein [15,16,21]. All the experiments on the muscle samples were performed in triplicate. Correlation analyses were also made between the gel repetitions, and the spots were counted. Fig. 1 shows the gel obtained for the liver samples with protein spots, in which calcium, iron and zinc were detected by SRXRF (highlighted with a circle).

The gels of the liver samples showed good resolution and efficient protein separation. These gels displayed a wide variety of proteins with p*I* of 4.70–9.40 and molar mass of 12.70–38.90 kDa, and a single protein spot with a molar mass of less than 12.70 kDa appeared with greater intensity. The correlation analysis of the

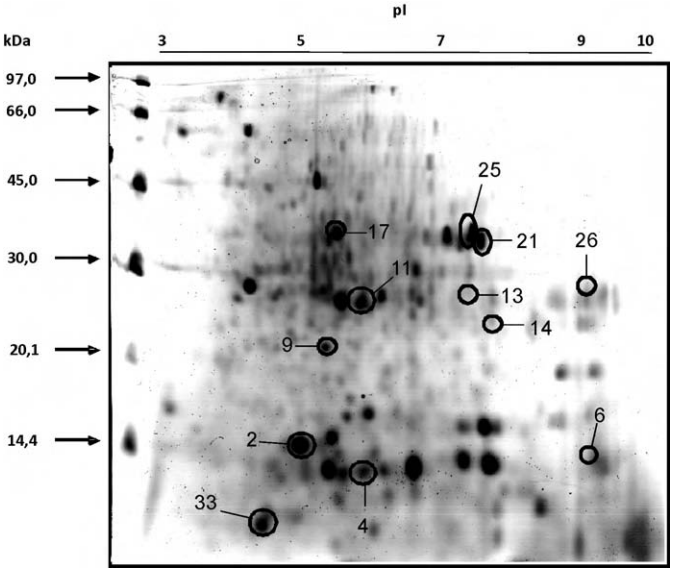

Fig. 1. Gel obtained for the liver samples with the protein spots which were detected calcium, iron and zinc by SRXRF highlighted with a circle.

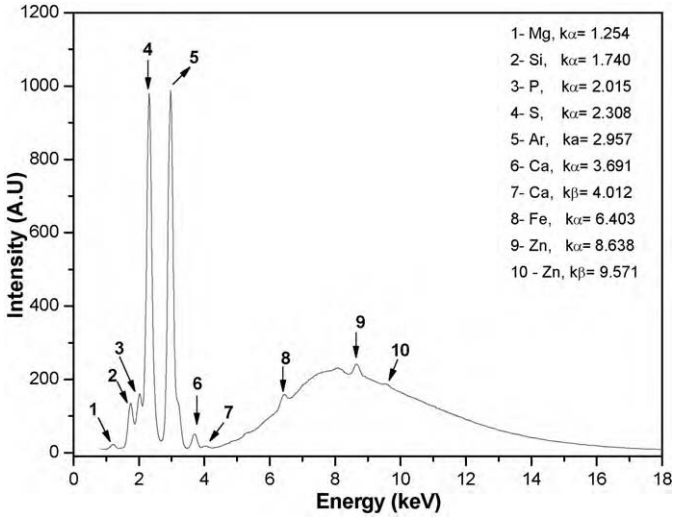

Fig. 2. Example of the SRXRF spectra obtained to one of the protein spots in the liver samples.

two gels showed that 86.4% of the proteins were present in both gels, representing about 436.3 protein spots. The average number of spots in these gels was 505, with a standard deviation of 12 spots.

3.3. Qualitative determination of calcium, iron and zinc by SRXRF in the protein spots in Nile tilapia liver samples

The protein-bound calcium, iron and zinc in the spots were evaluated qualitatively considering the sensitivity of the SRXRF technique and the possibility that the protein spots might contain low concentrations of metal ions [15,22]. Fig. 2 shows an example of fluorescence spectra obtained for one of the protein spots in the liver samples, while Fig. 3 shows a 3D image of the protein spots, in which calcium, iron and zinc were detected by SRXRF. As can be seen in the graphs in Fig. 2, the spectra exhibit intense continuous backgrounds. These backgrounds are due mostly to Compton scattering of the X-ray beams on the gel matrix, which may mask the signals of the element of interest [24,25]. Therefore, using the values of the normalized peak areas (after discounting the analytical blank, the region of the gel where no protein spots appeared) of the SRXRF spectra, it was possible to identify calcium (k_α = 3.621, K_β = 4.012), iron (k_α = 6.403) and zinc (k_α = 6.638, K_β = 9.571) bound to the proteins in the spots highlighted with a circle in Fig. 1. In

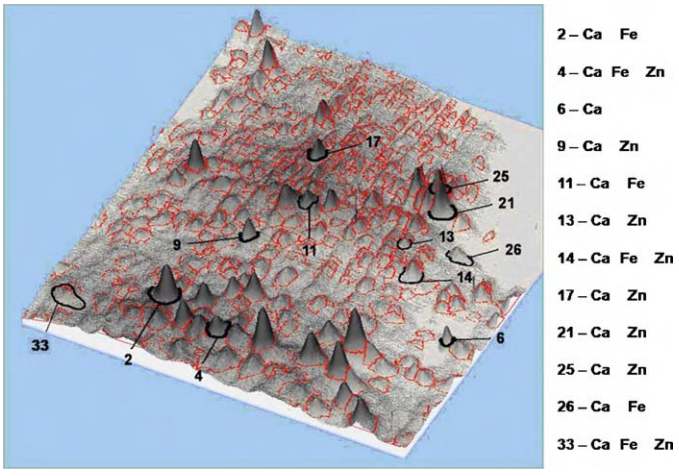

Fig. 3. Three-dimensional protein spots of liver sample contained calcium, iron and zinc, which highlighted with a circle.

Table 1
Molar mass (Mm) and isoelectric point (p*I*) of proteins of liver samples of Nile tilapia separated by 2D-PAGE with binding Ca, Fe and Zn.

Proteins	Mm (kDa)	p*I*	Metal ions
2F	13.90	5.20	Ca, Fe
4F	12.70	5.90	Ca, Fe, Zn
6F	13.60	9.30	Ca
9F	22.80	5.70	Ca, Zn
11F	28.80	5.90	Ca, Fe
13F	29.60	7.10	Ca, Zn
14F	25.90	7.30	Ca, Fe, Zn
17F	38.80	5.80	Ca, Zn
21F	37.70	7.40	Ca, Zn
25F	38.90	7.30	Ca, Zn
26F	29.90	9.40	Ca, Fe
33F	10.10	4.70	Ca, Fe, Zn

general, the fluorescence spectra showed a profile similar to the spectrum depicted in Fig. 2. As can be seen in the spectrum of Fig. 2, in addition to calcium, iron and zinc, sulfur and phosphorus, which are non-metals that may be part of the protein structure, were also detected. Another metal ion also detected was magnesium. The presence of metal ions in protein spots is acceptable, since a single metalloprotein or metal binding protein may have various active sites formed by atoms of different metal ions [26]. The high intensity peaks on the argon (k_α = 2.957) and silicon (k_α = 1.740) that appear in all spectra were expected because, in this case, the tape onto which the protein spot sample was attached contained silicon and argon is present in ambient air at a constant rate of 0.934% (v/v) [24–26].

Table 1 indicates the spots in which the presence of calcium, iron and zinc was identified. An analysis of the results presented in Table 1 suggests the presence of calcium, iron and zinc in 12, 6 and 8 liver protein spots, respectively. The metal ions found were distributed mainly in proteins with a molar mass of less than 40.00 kDa and more than 12.00 kDa, with p*I* in the range of 4.70–9.40, with the exception of one spot which contained protein with a molar mass of 10.10 kDa.

3.4. Quantitative determination of calcium, iron and zinc by FAAS in the protein spots in Nile tilapia liver samples

Having identified the presence of calcium, iron and zinc by SRXRF in the protein spots of liver samples by 2D-PAGE, these metal ions were quantified by FAAS after microwave-assisted acid mineralization of the spots. The concentration of metal ions in the protein spots was determined considering the estimated protein mass obtained by optical density using the ImageMaster 2D Platinum version 6.0 program. Table 2 lists the calcium, iron and zinc concentrations determined in the protein spots.

Table 2
Calcium, iron and zinc concentrations and estimate protein masses determined in protein spots from liver samples.

Spots	Calcium (mg g^{-1})	Iron (mg g^{-1})	Zinc (mg g^{-1})	Protein mass (μg)
2F	5.80	8.03	–	5.10
4F	5.30	7.25	8.55	4.64
6F	5.62	–	–	4.93
9F	4.75	–	7.01	4.20
11F	2.70	3.70	–	10.55
13F	1.30	–	2.02	5.40
14F	1.70	2.02	2.23	4.73
17F	1.10	–	1.60	7.04
21F	2.05	–	1.66	12.80
25F	1.08	–	1.80	13.18
26F	1.45	3.62	–	5.06
33F	3.33	5.53	6.82	3.20

Table 3
Estimate of number of protein molecules containing calcium, iron or zinc and of the number of calcium, iron or zinc atoms by protein spot.

Spots	Protein molecules ($\times 10^{14}$)	Number of calcium atoms ($\times 10^{14}$)	Number of iron atoms ($\times 10^{14}$)	Number of zinc atoms ($\times 10^{14}$)
2F	2.21	4.45	4.42	–
4F	2.19	3.70	3.60	3.65
6F	2.18	4.16	–	–
9F	1.04	2.99	–	2.70
11F	2.20	4.30	4.21	–
13F	1.10	1.05	–	1.00
14F	1.10	1.21	1.05	0.97
17F	1.09	1.16	–	1.04
21F	2.04	3.94	–	1.96
25F	2.04	2.14	–	2.18
26F	1.02	1.10	2.01	–
33F	1.90	1.60	1.94	2.01

An analysis of the results in Table 2 indicates that the concentrations of calcium, iron and zinc found in the samples' protein spots ranged from 1.08 to 5.80 mg g^{-1}, 2.02 to 8.03 mg g^{-1} and 1.60 to 8.55 mg g^{-1}, respectively. However, the results in Table 2 offer little information because they do not indicate which proteins are involved. Therefore, the estimated protein masses and calcium, iron and zinc masses were converted into quantities in mol of protein molecules and calcium, iron and zinc atoms, which allowed for an estimation of how much calcium, iron or zinc atoms were present in each molecule of protein [25]. Table 3 presents the results of the estimated protein molecules and calcium, iron and zinc atoms. The results listed in Table 3 provided an idea of the proportion of calcium, iron or zinc to protein molecule. For example, with respect to spot 4F, it was estimated that each protein molecule may have from 1 to 2 atoms of calcium, iron or zinc. Based on this reasoning, it can be inferred in relation to other spots that, in general, the metal/protein ratio falls within the range of 1–2 calcium, iron or zinc atoms per protein molecule too, with the exception of spot 9F, which each protein molecule may have from 2 to 3 calcium or zinc atoms.

4. Conclusions

The use of 2D-PAGE electrophoresis as the initial stage in the evaluation of metalloproteins allowed for the fractionation of the proteins present in samples of Nile tilapia (*Oreochromis niloticus*) liver tissue. The good correlation obtained in the repetitions of the gels indicated that the total protein extraction procedures were efficient, preserving the metal–protein structure and enabling the calcium, iron and zinc in the protein spots to be mapped by SRXRF. The results obtained in the FAAS quantification of calcium, iron and zinc in the protein spots were consistent with the identification of these element by SRXRF and enabled proportions of 1–3 calcium, iron or zinc atoms per protein molecule to be estimated, which may be an indication that the fractionated biomolecules are metalloproteins. Future studies comprise the characterization of the twelve proteins in which were detected the presence of calcium, iron and zinc and also the development of a similar study for other metal ions and/or metalloids of interest in fish nutrition such as magnesium, manganese, cobalt and selenium. Furthermore, a metallomic analytical approach of Nile tilapia (*Oreochromis niloticus*) using muscle tissue and blood samples will be carried out.

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