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Analytical Methods

Quality assurance of histamine analysis in fresh and canned fish

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ABSTRACT

Histamine determination is relevant for fish safety, quality and trade. Recently a study by the European Union (EU) compared the Codex and the EU mandated methods for the analysis of histamine and observed that they underestimated and overestimated the results, respectively. To solve this problem, a simple and efficient procedure for the extraction and quantification of histamine by ion-pair HPLC method with post-column derivatization and fluorimetric detection is proposed. It was optimized and validated for the analysis of histamine in fish. The method attended the performance criteria established by Commission Decision 2002/657/CE. The method was also submitted to proficiency testing; uncertainty was calculated; and the stability of solutions and standards was investigated. There was no matrix effect. The LOD, LOQ, CCα and CCβ were fit for the purpose. The method was successfully used in the analyses of freshwater fish and fresh and canned tuna.

1. Introduction

Histamine is a biogenic amine present in a large number of foods. It is also an endogenous substance which occurs naturally in the human body, playing important physiological functions related to gut mucosal immune responses, gastric acid secretion and neuromodulation (Gloria, 2005; Smolinska, Jutel, Crameri, & O'Mahony, 2014). However, high levels of histamine in foods can cause poisoning. Histamine or scombroid poisoning is the leading cause of foodborne illness associated with the consumption of fish containing high levels of histamine by itself or in the presence of other biogenic amines (e.g., putrescine and cadaverine) which can potentiate histamine's toxic effect. It is usually a mild illness with a variety of symptoms including rashes, nausea, vomiting, diarrhea, flushing, swelling of the face and tongue, sweating, headache, dizziness, palpitation, oral burning, metallic taste and hypotension. However, life-threatening cases have been reported. The severity of the symptoms varies depending on the amount of histamine ingested and the individual's sensitivity to histamine (Bulushi, Poole, Deeth, & Dykes, 2009; CDC, 2010; Chen, Lee, Hwang, Chiou, & Tsai, 2011; D'Aloia et al., 2011; FAO, 2015; Feng, Teuber, & Gershwin, 2015; Smolinska, Jutel, Crameri, & O'Mahony, 2014; Stratta & Badino, 2012; Yesudhason et al., 2013).

Nowadays, an increased consumption of fish has been recommended due to its health promoting properties. However, some fish can lead to histamine poisoning if its quality is not assured. Histamine can build up in fish species that contain high levels of free histidine in their tissue, including tuna and other pelagic species, which account for significant global fish production. When

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these fish are subjected to temperature above 4 °C during and/or after capture including storage, handling and processing, bacterial decarboxylation of histidine can lead to histamine formation. The sensory characteristics of the affected fish may appear satisfactory as high histamine levels may not be accompanied by other signs of spoilage. Therefore, the risk of histamine poisoning has attracted the attention of the international community which is concerned with public health and safety issues, as well as with the global fish trade (Bucher & Calello, 2013; D'Aloia et al., 2011; Demoncheaux et al., 2012; Feng, Teuber, & Gershwin, 2015; Mahmoudi & Norian, 2014; Tortorella et al., 2014; Wilson, Musto, & Ghali, 2012; Yesudhason et al., 2013).

In 1996, the United States Food and Drug Administration (FDA) implemented the seafood Hazard Analysis and Critical Control Point (HACCP) program to prevent seafood processing hazards that could lead to foodborne illness. A hazard action level of 50 mg/kg was set for scombroid or scombroid-like fish at the port. Codex Alimentarius limits histamine in fish and fish products to 200 mg/kg. Brazil and Mercosur limit histamine to 100 mg/kg (Brasil, 1997; FAO, 2012; FDA, 2011). European regulation limits histamine content in fish and products, from species associated with a high amount of histidine, to 100 and 200 mg/kg, and the decision making is based in a three-class attributes sampling plan (n = 9, c = 2, m = 100 and M = 200 mg/kg). It also specifies that a high performance liquid chromatography (HPLC) method must be used and two methods were suggested for histamine analyses which were based on derivatization of the extracted amines prior to RP-HPLC and ultra-violet (UV) detection (EC, 2005; EU, 2014).

Several procedures are available for the analytical detection and determination of histamine and other biogenic amines in foods. HPLC with different types of detectors has been the most widely used method. Due to histamine's high polarity and in order to produce UV–visible or fluorescent products, some methods apply pre-column derivatization and reversed phase chromatography; and others, ion-pair liquid chromatography together with post-column derivatization. The current official method for histamine analysis (AOAC, 2012) is based on methanolic extraction, purification by ion-exchange column, derivatization with *o*-phthalaldehyde and measurement in a fluorimeter. Although advantageous due to relative simplicity and no requirement of sophisticated equipments, studies indicated that this method has a tendency to underestimate the histamine content in fish. On the other hand, the EU mandated method shows a tendency to overestimate the content of histamine in fish. Furthermore it is laborious and time consuming as derivatization is undertaken prior to the chromatographic separation (EU, 2014; Fernandes & Gloria, 2015; Önal, Tekkeli, & Önal, 2013)

It is clear that a robust, selective and precise method which can be used to determine histamine is needed for quality, safety and trade purposes. Thus, the objective of this study was to optimize and validate an ion-pair chromatographic method with fluorescence detection to quantify histamine in fish.

2. Materials and methods

2.1. Samples and reagents

Fresh tuna steaks (n = 117) were obtained from warehouses located at the Southeastern coast of Brazil. Freshwater fishes filets of Nile tilapia – *Oreochromis niloticus* (15 samples) and rainbow trout – *Oncorhynchus mykiss* (3 samples) were obtained from warehouses near fish farms within the state of Minas Gerais, Brazil. The fresh samples were frozen, kept under dry ice during transportation and stored at −80 °C until analysis. Canned tuna samples (n = 92) were obtained from local stores and kept at room temperature until analysis. Reference material (canned fish with histamine) from Food Analysis Performance Assessment Scheme – FAPAS® were also used.

Histamine (HIM) dihydrochloride, putrescine (PUT) dihydrochloride, spermidine (SPD) trihydrochloride, spermine (SPM) tetrahydrochloride, agmatine (AGM) sulfate, cadaverine (CAD) dihydrochloride, serotonin (SRT) hydrochloride, tyramine (TYM), tryptamine (TRM), 2-phenylethylamine (PHM) dihydrochloride standard and *o*-phthalaldehyde (OPA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Analytical grade reagents were used, except for solvents used in HPLC (acetonitrile and methanol), which were of chromatographic grade. The aqueous and organic solvents were filtered through 0.45 μm of pore size membranes (Millipore Corp., Milford, MA, USA). Ultrapure water was obtained from Milli-Q Plus system (Millipore Corp., Milford, MA, USA).

2.2. Instrumentation and analytical conditions

The amines were separated by ion-pair reverse phase HPLC and detected fluorimetrically after post-column derivatization with OPA. The HPLC system (Prominence) consisted of three pumps (two LC-20AD, used to elute the mobile phase, and one LC-10AD to deliver the derivatization reagent), an oven (CTO-10ASVP), a spectrofluorimetric detector (RF-10AXL), an auto-injector (SIL-20AHT), a system controller (CBM-20A), and an acquisition data software LC Solution®, all from Shimadzu (Kyoto, Japan). Separation was performed in a reverse phase Nova-Pak® C_{18} column (300 × 3.9 mm id, 4 μm) with a Nova-Pak® C_{18} guard-pak insert (Water, Milford, MA, USA). The mobile phase consisted of 0.2 mol/L sodium acetate and 15 mmol/L 1-octanesulfonic acid sodium salt solution (A), adjusted to pH 4.9 with acetic acid, and acetonitrile (B). The flow rate was set at 0.5 mL/min and the elution was carried out in gradient mode, set as: time (min)/%B: 0.01/5; 21/21; 22/2; 29/2; 30/40; 34/40; 35/5; 50/5 (adapted from Silva, Sabaini, Evangelista, & Glória, 2011).

The post-column derivatization reagent was delivered at 0.3 mL/min and consisted of 1.5 mL Brij-35, 1.5 mL mercaptoethanol and 0.2 g OPA dissolved in a 500 mL solution of 25 g of boric acid and 22 g of potassium hydroxide (pH adjusted to 10.5 with potassium hydroxide). The column and the post-column reaction apparatus were kept at 22 ± 1 °C. Detection was performed at 340 and 450 nm (excitation and emission wavelengths, respectively) (Silva et al., 2011).

Histamine was identified by comparison of the retention time in the samples with that of standard solutions in hydrochloric acid 0.1 mol/L and by adding known amounts of histamine to samples and following area increase. Standard solutions were analyzed along with samples. All analyses were performed in triplicate and the average results were used.

2.3. Preparation of standard solutions for the calibration curves

Stock solutions of histamine and of the other nine bioactive amines were prepared by dissolving the accurately weighed amine (as a free base) in 0.1 mol/L aqueous hydrochloric acid solution. The concentrations of the working solutions of amines were prepared immediately before use by diluting the stock solutions with the same hydrochloric acid solution to a concentration of 100 μg/mL.

Calibration curves were prepared in solvent and in a fish sample extract which did not contain detectable levels of histamine. Six concentrations were prepared by spiking appropriate amounts of the working solution of histamine. The concentrations of the calibration curve were 0.2, 10, 20, 30, 40 and 50 μg/mL of histamine which corresponded to 1, 50, 100, 150, 200 and 250 mg/kg in fish samples.

2.4. Optimization of the sample preparation step

The fish samples were quartered and ground using a food processer. After homogenization, 5 g samples were weighted and placed into centrifuge tubes containing 7 mL of 5% trichloroacetic acid. Then, the samples were fortified with histamine dihydrochloride standard solution to obtain the concentration of 150 mg/kg. The tube was vortex mixed and centrifuged. After centrifugation, the supernatant was filtered through qualitative paper. The acid extraction step was repeated twice, the filtrates were combined and the volume was brought up to 25 mL in calibrated volumetric flasks. The extracts were filtered through qualitative filter paper and 0.45 μm pore size membrane filter (Millipore Corp., Milford, MA, USA) prior to injection into the HPLC system (Silva et al., 2011).

A full factorial design was used to screen the main factors that could affect the recovery of histamine from fish. The variables investigated were vortexing time (X_1 = 20, 70 and 120 s), centrifugation time (X_2 = 3, 12 and 21 min), relative centrifugal force (X_3 = 1500, 11,250 and 21,000*g*) and centrifugation temperature (X_4 = 0, 3 and 6 °C). Sixteen tests were assembled (2^4) in duplicate with five replicates at the central point. The results were submitted to analysis of variance (ANOVA) at 5% probability using ADX do SAS, version 9.1.

2.5. Histamine quantification

The quantification of histamine was performed by interpolation in an external calibration curve. The concentration found in the sample was multiplied by a correction factor, which was calculated based on the recovery of the method.

The analysis was performed in triplicate and a sample spiked with histamine was used to evaluate recovery and to calculate the correction factor by dividing the content found by the content added multiplied by 100. For this, 0.75 mg of histamine was added to 5 g sample to reach a concentration of 150 mg/kg (which corresponds to the intermediate point of the calibration curve, 30 μg/mL).

2.6. Method validation

The validation process was carried out according to directive 2002/657/CE (EC, 2002) as recommended by the European Community concerning the performance of analytical methods and results interpretation. The analyzed parameters were linearity, matrix effect, accuracy, precision, specificity, trueness, ruggedness, limits of quantification and detection, detection capability (CCβ), and decision limit (CCα).

Linearity was assessed by six-point calibration curves in triplicate in three consecutive days. The curves were constructed by plotting the peak area of histamine versus the concentration and by means of linear regression (Ordinary Least Square Method) the equations and the correlation coefficient were determined. The linear range evaluated was from 0.2 to 50 μg/mL.

To investigate the existence of matrix effect, the slopes and intercepts of the linear equations for the constructed histamine calibration curves, in the solvent and in the fish matrix, were compared by Student *t*-test at 5% probability.

Accuracy and precision were evaluated by determining recoveries of histamine in a set of samples of identical matrices fortified with the analyte to yield concentrations equivalent to 0.5, 1.0 and 1.5 times the limit established for histamine (100 mg/kg). Each level was analyzed in six replicates. Each set of 18 samples was repeated three times at three different days with different analysts. The concentration of histamine in each sample was calculated and the mean concentration, the standard deviation and the coefficient of variation (%) of the fortified samples were calculated.

To verify the specificity of the method, an appropriate number of representative blank samples (n = 20) was analyzed and checked for interferences (signals, peaks, ion traces) in the region of interest where the target analyte was expected to elute. The method's ability to separate histamine from the other bioactive amines which might also be present in the fish samples was investigated. So, standard solutions of nine amines (tyramine, serotonin, 2-phenylethylamine, tryptamine, putrescine, cadaverine, agmatine, spermidine, and spermine) at 30 μg/mL were injected individually and altogether with histamine to verify the occurrence of interference or coelution.

In order to evaluate trueness, six replicates of a reference material were analyzed and the concentration of histamine present in each replicate was determined, as well as the mean, the standard deviation and the coefficient of variation (%). The trueness was set by dividing the detected mean concentration by the certified value (measured as concentration) and multiplied by 100, to express the result as a percentage.

In order to verify ruggedness, variations in some analytical parameters were introduced in the method and the influences in the responses were evaluated. The following factors were selected: the analyst (n = 3), the reagent's source (n = 2) and standard's solution batch (n = 3). These factors were modified and possible factors that could affect the results were identified.

The limits of detection and quantification were based on the noise at the retention time of histamine of 20 independent sample blanks and expressed as the analyte concentration corresponding to mean sample blank value +3*s* and 10*s*, respectively.

To determine decision limit (CCα), 20 blank materials fortified with histamine at the limit established for histamine (100 mg/kg) were analyzed. It was calculated as the concentration at the permitted limit plus 1.64 times the corresponding standard deviation (α = 5%). To determine the detection capability (CCβ), 20 blank materials fortified with histamine at the decision limit were analyzed. CCβ was calculated as the value of the decision limit plus 1.64 times the corresponding standard deviation (β = 5%).

2.7. Proficiency testing

The determination of the reproducibility of the method by the single laboratory studies (in-house validation) concept also requires participation in proficiency tests (EC, 2002). Two proficiency tests provided by FAPAS® were undertaken. A total of 209 laboratories participated in the two tests, 98 in the first and 111 in the second. Each laboratory received a reference materials consisting of canned tuna samples for the determination of histamine.

2.8. Stability of standard solutions and mobile phases

The stability of standard solutions and mobile phases used in the determination of histamine were also evaluated. The following solutions had their stability evaluated: sodium acetate:octanesulfonic acid sodium salt solution (mobile phase A), solution of KOH/H_3BO_3 (derivatization buffer), 5% trichloroacetic acid (extracting acid) and histamine standard solution. The stability of the mobile phase A and solution of KOH/H_3BO_3 was established by means of pH. The stability of the trichloroacetic acid was established by means of the recovery percentage obtained in the fish analysis. The stability of the standard solution was established by comparing the instrumental response generated by a stored standard solution with the instrumental response produced by a recently made standard solution. The means were compared by the Student *t*-test (p = 0.05).

2.9. Determination of the uncertainty of the method

The uncertainty of the method was calculated according to Brasil (2011). For quality assurance purposes, in analytical chemistry, an expanded uncertainty (U) should be used. U provides an interval within which the value of the analyte concentration is believed to lie within a higher level of confidence (p = 95%). U was obtained by multiplying $u_c(y)$, the combined standard uncertainty, by a coverage factor k according to Eq. (1). The combined uncertainty $u_c(y)$ was calculated from the square root of the sum of several independent parameters, such as, the uncertainty of the intermediate precision $u(Pi)$; the recovery uncertainty $u(C_{rec})$; the calibration curve uncertainty $u(C_{cal})$; the correction factor of the calibration curve uncertainty $u(fc_{cc})$; the mass uncertainty $u(m)$; and the volume uncertainty $u(V)$ (Eq. (2)).

$$U = k * u_c(y) \quad (1)$$

$$u_c(y) = \sqrt{u^2(Pi) + u^2(C_{rec}) + u^2(C_{cal}) + u^2(fc_{cc}) + u^2(m) + u^2(V)} \quad (2)$$

2.10. Application of the method

The validated method was used to determine the concentration of histamine in fresh and canned tuna and in freshwater fish as described previously.

3. Results and discussion

3.1. Conditions for HPLC-fluorescence

Most of the histamine separation methods applied to fish use reverse phase HPLC with detection approaches based on pre-column or post-column derivatization to produce fluorescent products or strong chromophores. Considering the high polarity of histamine's molecule and, consequently, the poor interaction with reverse phase chromatographic groups, other separation-based methods, such as ion-pair chromatography, must be used. Therefore, the counter ion 1-octanesulfonic acid sodium salt solution was added to the mobile phase to enhance analyte interaction with the column and improve separation from matrix interferences (Cinquina et al., 2004; Fernandes & Gloria, 2015; Izquierdo-Pulido, Vidal-Carou, & Mariné-Font, 1993).

Although the histamine imidazole ring can be analyzed by UV detection (Cinquina et al., 2004; Shakila, Vasundhara, & Kumudavally, 2001), fluorescence after post-column derivatization was employed to increase method selectivity and sensitivity for histamine. Furthermore, it allows detection and identification of the other amines that do not have chromophores (i.e. putrescine, cadaverine, spermine and spermidine). *o*-Phthalaldehyde was used as derivatization reagent due to its high selectivity for amines when compared to other reagents such as dansyl chloride and fluorescamine (Izquierdo-Pulido et al., 1993; Khuhawar & Qureshi, 2001; Lavizzari, Veciana-Nogués, Bover-Cid, Mariné-Font, & Vidal-Carou, 2006).

3.2. Optimization of the extraction method

For extraction of histamine and other bioactive amines, trichloroacetic acid was preferred over methanol and perchloric acid, because of the specificity of methanol for aromatic amines and of the danger of dealing with perchloric acid, which is explosive (Fernandes & Gloria, 2015).

During optimization of sample preparation, recoveries varied from 74.5% to 87.4% (Table 1). The variables that affected significantly histamine's recovery from fish were centrifugation time (37%) and centrifugation temperature (34%) as well as the interaction between them (25%), at a level of confidence of 95%, as can be seen in the Pareto chart (Fig. 1). By adding these parameters 96% of the total sum of square was obtained. Centrifugation time and centrifugation temperature affected the recovery negatively. Higher recoveries were obtained with 3 min of centrifugation and temperature of 0 °C. Therefore, after optimization, the established conditions for the extraction of histamine from fish samples were: vortexing time – 70 s, centrifugation time – 3 min, centrifugation force – 11,250×*g* and centrifugation temperature – 0 °C.

Table 1
Recovery of histamine during extraction using a full factorial design with four independent variables and five replicates in the central point.

Treatment	Vortexing time (s)	Centrifugation time (min)	Relative centrifugal force (*g*)	Centrifugation temperature (°C)	Recovery (%)
1	20	3	1500	0	82.7
2	120	3	1500	0	81.5
3	20	21	1500	0	87.4
4	120	21	1500	0	76.0
5	20	3	21,000	0	78.5
6	120	3	21,000	0	77.1
7	20	21	21,000	0	78.1
8	120	21	21,000	0	81.7
9	20	3	1500	6	85.4
10	120	3	1500	6	80.5
11	20	21	1500	6	86.7
12	120	21	1500	6	79.9
13	20	3	21,000	6	80.0
14	120	3	21,000	6	80.7
15	20	21	21,000	6	77.1
16	120	21	21,000	6	78.1
17	70	12	11,250	3	75.9
18	70	12	11,250	3	74.5
19	70	12	11,250	3	80.5
20	70	12	11,250	3	77.5
21	70	12	11,250	3	76.6

3.3. Method validation

The calibration curves for histamine in the solvent and in the matrix were linear within the range of 0.2–50.0 μg/mL, with a regression coefficient higher than 0.98. A typical standard curve was y = 45,0841x + 41,343 (Fig. 2).

In order to investigate the existence of matrix effect, the slopes and the intercepts of the calibration curves in the solvent and in the matrix were compared and no significant difference ($p < 0.05$) was observed. This result shows that the matrix had no effect on the histamine calibration curve. According to Chiu et al. (2010), simple separation methods such as dilution and protein precipitation are sufficient to minimize matrix effect. Since these are the steps used in sample preparation, matrix effect was minimized. These results confirm the absence of matrix effect, hence, calibration curves constructed using mobile phase were used.

The data relative to precision and accuracy of the method are presented in Table 2. The average accuracy (n = 18) determined in three different concentration levels was 92.1%. The coefficient of variation of repeatability (CVr) ranged from 2.3% to 2.6% and the CV of reproducibility (CV_R) ranged from 5.0% to 6.2%. These results were lower than the limits established at Brasil (2011) (CV_r 4.9% and CV_R 7.3%), which confirms the applicability of the method in the selected range.

The specificity of the method was verified by analyzing the chromatograms obtained during the analysis. The retention time of the histamine peak was 32.5 min ($k = 5.5$), and no significant interference was detected at the same retention time of the analyte

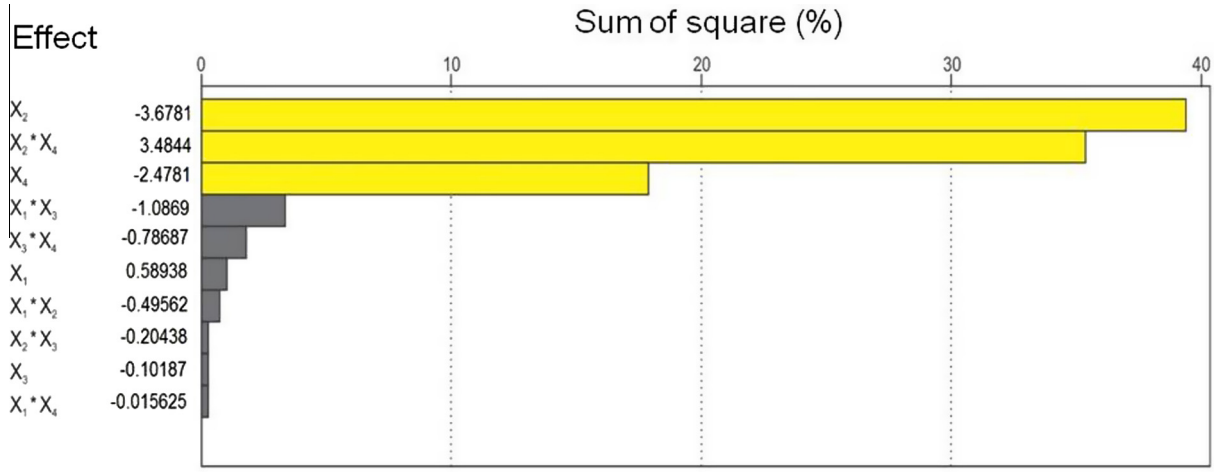

Fig. 1. Pareto chart showing the effect of each tested variable and their interactions (X_1 = vortexing time; X_2 = centrifugation time; X_3 = centrifugal force; X_4 = centrifugation temperature).

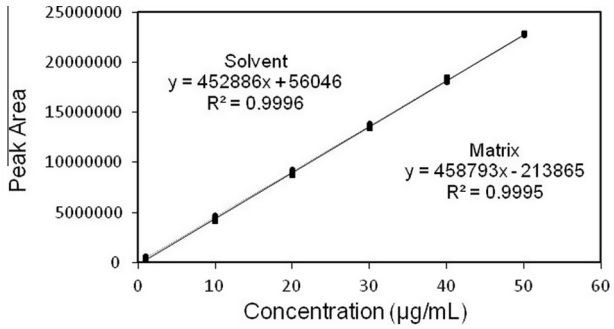

Fig. 2. Calibration curve of histamine solution in solvent (. . .) and in the fish matrix (- - -).

Table 2
Precision and accuracy results for histamine by HPLC.

Nominal concentration (µg/mL)	Average concentration (µg/mL ± sd)	Precision (%)		Accuracy (%)
		CV_r	CV_R	
10	9.03 ± 0.53	2.3	6.1	89.3
20	18.58 ± 1.14	2.6	6.2	93.2
30	27.96 ± 1.40	2.3	5.0	93.9

n = 18; sd – standard deviation; CV_r – coefficient of variation of repeatability; CV_R – coefficient of variation of reproducibility.

in the analyzed blank samples (n = 20). Standard solutions of the nine amines (tyramine, serotonin, 2-phenylethylamine, tryptamine, putrescine, cadaverine, agmatine, spermidine, and spermine), which can be present simultaneously in fish and other food matrices, were injected separately and all together with histamine to verify the occurrence of interference or coelution. There was no interference from the other nine bioactive amines. Therefore, the method was suitable to separate and identify histamine. Fig. 3 shows a typical chromatogram of an extracted fish sample spiked with standard solutions of histamine and other nine bioactive amines.

Trueness was assessed analyzing the reference material, which contained 126.7 mg of histamine per kg of sample. The histamine average concentration (n = 6) found was 118.17 ± 4.05 mg/kg. The coefficient of variation calculated was 3.4% and the accuracy was 93.3%.

To investigate the ruggedness of the method, the following factors were selected: analyst, reagent brand and the lot of the standard solution. These factors were modified and they did not affect recovery, which remained within the range from 80% to 110% and CV lower than 5% (data not shown).

The detection limit (LOD) of the method for histamine in scombroid fish was 0.03 mg/kg and the quantification limit (LOD) was 0.09 mg/kg. CCα and CCβ were calculated and the values obtained were 102.61 mg/kg and 105.23 mg/kg, respectively. These values are adequate for the analysis of histamine in fish regarding the safety range established by regulatory agencies of several countries (100 mg/kg).

3.4. Proficiency testing

ISO 17025 establishes guidelines for the accreditation of laboratories that perform analytical services. This standard is recognized worldwide and, according to it, proficiency tests are required to assess the quality, accuracy and validity of the analytical results produced. Furthermore, ISO 17025 provides confidence in the competence of the laboratory personnel as well as the methods, equipments and reagents used and can identify nonconformities within the laboratory's quality system, allowing for improvements before results are affected (Wilder, 2015). In order to meet this standard and demonstrate the suitability of the developed method, the laboratory participated in two proficiency tests (ISO/IEC 17025, 2005).

The designed concentration of histamine for the samples obtained from the provider agency of the proficiency test (FAPAS®) were 311.1 mg/kg and 126.7 mg/kg and the results found were 311.8 mg/kg and 118.2 mg/kg, respectively. Thus, the z-score obtained for the first test was 0.0 and for the second test was −0.9. As these results are between ±2.0, method reproducibility was assured. This result indicates that the analytical data generated is accurate and reliable.

3.5. Stability of standard solutions and mobile phases

Sodium acetate:octanesulfonic acid sodium salt solution (mobile phase A) and solution of KOH/H_3BO_3 were stored for 30 days and the pH remained within the acceptable range. Trichloroacetic acid was also considered stable for 30 days, since the recovery obtained was kept within the acceptable range during this period. There was no significant difference ($p > 0.05$) comparing the instrumental response generated by a stored (160 days)

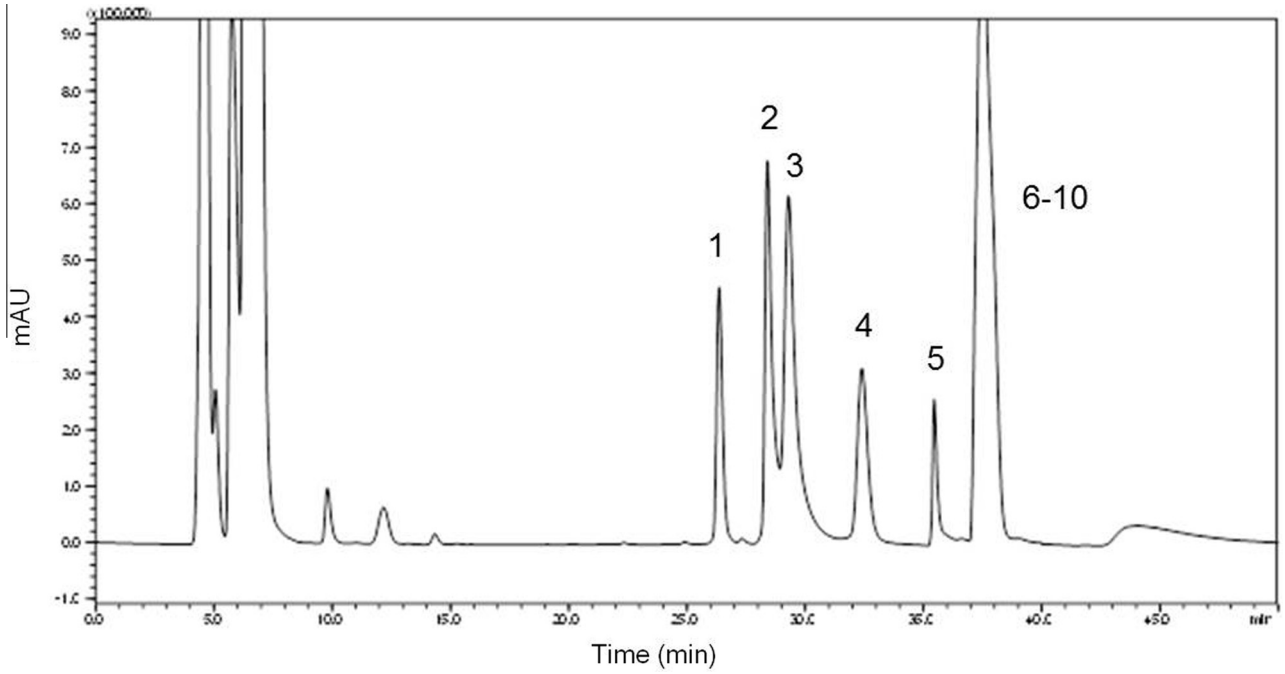

Fig. 3. Chromatogram of an extracted fish sample spiked with standards of ten amines. 1 = Tyramine; 2 = Putrescine; 3 = Cadaverine; 4 = Histamine; 5 = Serotonin; 6–10 (2-Phenylethylamine, Tryptamine, Agmatine, Spermidine, and Spermine). HPLC conditions are described in the text.

standard solution with the instrumental response provided by a recent standard solution. Therefore, the standard solution was considered stable for 160 days.

3.6. Uncertainty of the method

The concentration of 100 mg/kg of histamine in the sample was adopted as reference for the estimation of uncertainty of measurement. Calculations showed that the four major sources of uncertainty were intermediate precision, recovery, calibration curve and calibration curve correction factor. The combined standard uncertainty obtained was equal to 8.0 and the choice of the factor k was based on the level of confidence desired. For a confidence level of 95%, k is 2. Then, according to Eq. (2), the expanded standard uncertainty (U) was equal to 16.0 ($U = k \times u_c(y) = 2 \times 8.0 = 16.0$). Therefore, the result of histamine concentration (C_{HIM}) with expanded standard uncertainty of 16.0 and coverage probability of 95% and $k = 2$ was $C_{HIM} = (100.0 \pm 16.0)$ mg/kg.

3.7. Method application in the determination of histamine in fish

In order to demonstrate the effectiveness of the optimized and validated method, the levels of histamine were determined in different types of fresh fishes and canned tuna.

As indicated in Table 3, different types of canned tuna samples were found in the consumer market. Among the 92 samples analyzed, histamine was not detected (LOQ = 0.09 mg/kg) in 51 samples (55.4%); whereas levels varying from 0.45 to 83.73 mg/kg were detected in 41 samples (44.6%). These levels of histamine found in canned tuna were below all of the limits established (Brasil, 1997; EU, 2014; FAO, 2012; FDA, 2011).

Histamine was not detected in any of the fresh tuna from warehouses in the southeastern coast of Brazil. It was also not detected in freshwater fish fillets from warehouses near fish farms in the state of Minas Gerais, Brazil. These results suggest the high quality of the fresh fish available in the warehouses.

Table 3
Histamine levels in fresh tuna from the southeastern coast of Brazil, fresh water fish from farms in the state of Minas Gerais, Brazil, and canned tuna from the Brazilian consumer market.

Samples	Samples (n+/n)	Histamine levels (mg/kg)
Fresh fish (*total*)	0/135	nd
Tuna	0/117	nd
Tilapia	0/15	nd
Rainbow trout	0/3	nd
Canned tuna (*total*)	41/92	nd – 83.73
Solid in salt and water	10/22	nd – 81.43
Solid in oil	4/16	nd – 11.06
Grated in salt and water	4/12	nd – 2.06
Grated in oil	8/12	nd – 56.02
Grated with herbal sauce	0/9	nd
Grated with spicy tomato sauce	6/9	nd – 19.4
Grated with tomato sauce	9/12	nd – 83.73

n = number of samples analyzed; n+ = number of positive samples; nd = not detected (LOQ = 0.09 mg/kg).

4. Conclusion

A simple and efficient procedure for the extraction of histamine from tuna was optimized. An ion-pair HPLC method with post-column derivatization and fluorimetric detection was optimized and validated for the quantification of histamine in fresh and canned tuna. The optimized method showed to be fit for the purpose and the data generated by the laboratory is reliable for the assurance of the quality and safety of fish and useful for trade purposes. The method was used in the analyses of fresh and canned tuna, and freshwater fish. Histamine was not detected in any of the fresh samples of tuna and freshwater fish. On the other hand, it was detected in 44.6% of the canned tuna samples, but at levels below limits established.

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