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Development of a fast and selective separation method to determine histamine in tuna fish samples using capillary zone electrophoresis

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A B S T R A C T

This paper reports on the development of a fast and selective separation method by capillary zone electrophoresis (CZE) for the determination of histamine in tuna fish samples. The background electrolyte was composed of 60 mmol L^{-1} hydroxyisobutyric acid and 10 mmol L^{-1} sodium hydroxide at pH 3.3. The internal standard used was imidazole. Separations were performed in a fused uncoated silica capillary (32 cm total length, 8.5 cm effective length and 50 μm internal diameter) with direct UV detection at 210 nm. The samples and standards were injected hydrodynamically (50 mbar, 3 s) from the outlet capillary end (nearest to the detector) and the electrophoretic system was operated under normal polarity and constant voltage conditions of 30 kV (positive polarity on the injection side). The migration time of histamine in the proposed method was only 0.34 min. The method was then validated and different tuna fish samples were analyzed. Good linearity ($R^2 > 0.999$), a limit of detection 0.14 mg L^{-1}, intra-day precision better than 3.5% (peak area of sample), and recovery in the range of 94–108% were obtained. The results of the histamine concentration determined in the samples by the CZE method were compared with the LC–MS/MS method.

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1. Introduction

Histamine is a biogenic amine present in various levels in many foods, such as cheese, vegetables, fish, and others. It forms in food by decarboxylation of the amino acid histidine catalyzed by L-histidine decarboxylase in the presence of decarboxylase-positive microorganisms, and by conditions that allow bacterial growth and decarboxylase activity. Free histidine can be found naturally in foods or may be liberated by proteolysis during processing or storage. Therefore, high concentrations of histamine in foods are related to microbial fermentation. Thus, histamine can be used as an indicator of hygienic food quality [1]. Furthermore, foods containing high levels of histamine are related to food-borne illness. In case of fish consumption, according to the Food and Drug Administration (FDA), histamine levels above 200 mg kg^{-1} can cause the development of an illness called scombroid poisoning. Some symptoms of this illness include tingling or burning in or around the mouth or throat, rash or hives on the upper body, drop in the blood pressure, headache, dizziness, itching of the skin, nausea, vomiting, diarrhea, asthmatic-like constriction of the air passage, heart palpitation, and respiratory distress [2,3]. In United States, the FDA regulates a maximum limit of 50 mg kg^{-1} (5 mg per 100 g) of histamine for fresh and canned fish [2]. In Brazil, the Brazilian Ministry of Agriculture and Livestock (Portuguese acronym MAPA) has established a maximum limit of 100 mg kg^{-1} of histamine in the muscles of fresh and frozen fish, as well as for canned fish [4–6]. Papers have reported that in addition to the conditions of hygiene, other factors of processing methods for fish, including their storage temperature and storage time, influence in the histamine concentration [7–11].

Several methods were proposed in the literature for the determination of histamine in fish samples. These include high-performance liquid chromatography with fluorescence or UV detection and the use of derivatization [12], gas chromatography with flame ionizing detector [13], ultra-performance liquid chromatography with UV detection [14], high-performance liquid chromatography with fluorescence detection using the pre-column derivatization procedure [15], high-performance liquid chromatography with fluorescence and UV detection [16], ion-exchange chromatography with conductivity detection [17], capillary electrophoresis and high-performance liquid chromatography with UV detection [18], and liquid chromatography–tandem mass spectrometry using a solid phase extraction procedure [19]. In the most of these methods, the separation time of

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the histamine varies over a wide range (3–12 min), and in some cases, is too long. The complexity of the sample matrix may contribute to these long separation times observed due to the appearance of the interfering peaks in the separation. In some cases, sample preparation (clean up) procedures are used in order to reduce these interference effects. However, these effects can be minimized while developing the method of analysis. Capillary electrophoresis (CE) is a separation technique that has the potential to allow the selection of appropriate conditions for the separation method in order to minimize the number of peaks that can interfere in the separation of the analytes. Likewise, the "cleaning of the electropherogram" may contribute to rapid separations with a duration less than 1 min, which increases the instrumental throughput of the analysis.

In this context, the aim of this study was to develop a fast and selective method for histamine determination using capillary zone electrophoresis (CZE) with UV detection. The proposed method was applied in the analysis of tuna fish samples and the results were compared with the LC–MS/MS method.

2. Experimental section

2.1. Chemicals and solutions

α-Hydroxyisobutyric acid (HIBA) purchased from Sigma-Aldrich (Sao Paulo, SP, Brazil) and sodium hydroxide purchased from Tedia Brazil (Rio de Janeiro, RJ, Brazil) were used in the background electrolyte (BGE). Ethanol (EtOH, content 99%) acquired from Synth (Diadema, SP, Brazil) was used to prepare the tuna fish samples. The standards of histamine and imidazole (internal standard, IS) were purchased from Sigma-Aldrich (Sao Paulo, SP, Brazil) and MERCK (Darmstadt, Germany), respectively. The standard stock solution of histamine (600 mg L^{-1}) was prepared in EtOH. Imidazole (30 mg L^{-1}) was prepared in deionized water. The calibration solutions of histamine were prepared by diluting the stock solution with EtOH, and were diluted in the ratio of 1:1 with IS solution before the injection for CZE (IS injected—15 mg L^{-1}).

2.2. Preparation of the sample and the CE system

Six samples of tuna fish were purchased at the local market: three samples of canned fish, one sample of fresh fish, one sample of old fish and one sample in the form of sushi. The canned samples were analyzed immediately after opening. The fresh fish and sushi samples were purchased and analyzed immediately. One sample of fish (frozen tuna fish) was analyzed after storage for three months in a freezer at −15 °C. About 10 g of the samples were weighed in a flask (volume ~150 mL) and 25 mL of EtOH was added. The samples were triturated using a processor (Philips-Wallita) for 1 min (for the formation of a homogeneous emulsion). A portion of the emulsion was inserted in a flask and centrifuged at 10,000 rpm for 3 min. An aliquot of the centrifuged solution was removed, diluted at 1:1 with the IS solution and injected in the CE instrument. Some samples were diluted with EtOH 5 or 10 times before dilution with the IS solution due to high levels of ions present in the matrix. The samples were prepared and injected in triplicate.

The experiments to optimize the separation were conducted on a CE system (7100 Capillary Electrophoresis System, Agilent Technologies, Palo Alto, United States) equipped with a diode array detector set at 210 nm, a temperature control device (set at 25 °C) and data treatment software (HP ChemStation). Samples and standards were injected hydrodynamically (50 mbar, 3 s) from the outlet capillary end (nearest to the detector) and the electrophoretic system was operated under normal polarity and constant voltage conditions of 30 kV (positive polarity on the injection side). For all experiments, a fused-silica capillary obtained from Polymicro Technologies (Phoenix, United States) measuring 32 cm (8.5 cm effective length) × 50 μm internal diameter × 375 μm outside diameter was used. The BGE was composed of HIBA 60 mmol L^{-1} and sodium hydroxide 10 mmol L^{-1} at pH 3.3 prepared in deionized water (Milli-Q, Millipore, Bedford, MA, United States) with a resistivity of 18.2 M Ω cm. Between runs, the capillary was rinsed for 30 s with the BGE.

2.3. Comparative method by LC–MS/MS

The comparative method, using the LC–MS/MS analysis, was performed on a chromatographic equipment consisting of a high-performance liquid chromatography system (Agilent Technologies, Germany). The separation was performed on a Synergi Polar-RP 80A C-18 column (150 mm, 2.0 mm ID, 4 μm particle size) purchased from Phenomenex. The runs were performed by isocratic mode using a mobile phase composed of 95% solvent A (H_2O + 0.1% formic acid) and 5% solvent B (95:5 acetonitrile/H_2O). The flow rate was set at 200 μL min^{-1}. In all instances, the injection volume was 5.0 μL. The column temperature was set at 30 °C. The LC system was coupled with a mass spectrometer system consisting of a hybrid triplequadrupole/linear ion trap mass spectrometer Q Trap 3200 (Applied Biosystems/MDS Sciex, Concord, Canada). The Analyst software version 1.5.1 was used for the LC–MS/MS system control and data analysis. The mass spectrometry was tuned in to the negative and positive modes by the infusion of polypropylene glycol solution. The experiments were performed using the TurboIonSprayTM source (electrospray-ESI) in positive ion mode. The capillary needle was maintained at 4500 V. The MS/MS parameters were curtain gas, 10 psi; temperature, 450 °C; gas 1, 45 psi; gas 2, 45 psi; CAD gas, medium. Other parameters of the mass spectrometer for the cone and collision energy were precursor ion (mz 112.1), fragment quantitative transition (68.1), declustering potential (21), entrance potential (7), collision cell entrance potential (10), collision energy (27 and 19), and collision cell exit potential (4). The histamine was monitored and quantified using multiple reaction monitoring (MRM). The optimization of the mass spectrometer was performed by the direct infusion of an aqueous solution containing the analyte investigated. The tuna fish samples injected in LC–MS/MS needed clean-up before the injections. An aliquot of the centrifuged solution (same solution of the sample centrifuged prepared for CZE–UV analysis) was removed and diluted with a solution of trifluoroacetic acid (final concentration 10 mmol L^{-1}) and deionized water. After the dilution, 1.0 mL of the sample was inserted in a flask containing 0.25 mL of chloroform. This mixture was agitated and centrifuged, and the aliquot of the supernatant was reserved for LC–MS/MS analysis. The same procedure (partition with chloroform) was performed with the standards of the analytical curve.

3. Results and discussion

3.1. Choosing BGE components and internal standard

The chemical structure of histamine (Fig. 1-a) shows an imidazole ring capable of absorbing UV radiation, allowing the direct detection of the analyte in the CE instrument with a UV detector. Another structural characteristic of histamine in relation to acid–base dissociation constants is the existence of two values of pK_a, 6.0 and 9.8 [20], which confers to this molecule the ability to contain two positive charges, depending on the pH of the medium, as shown

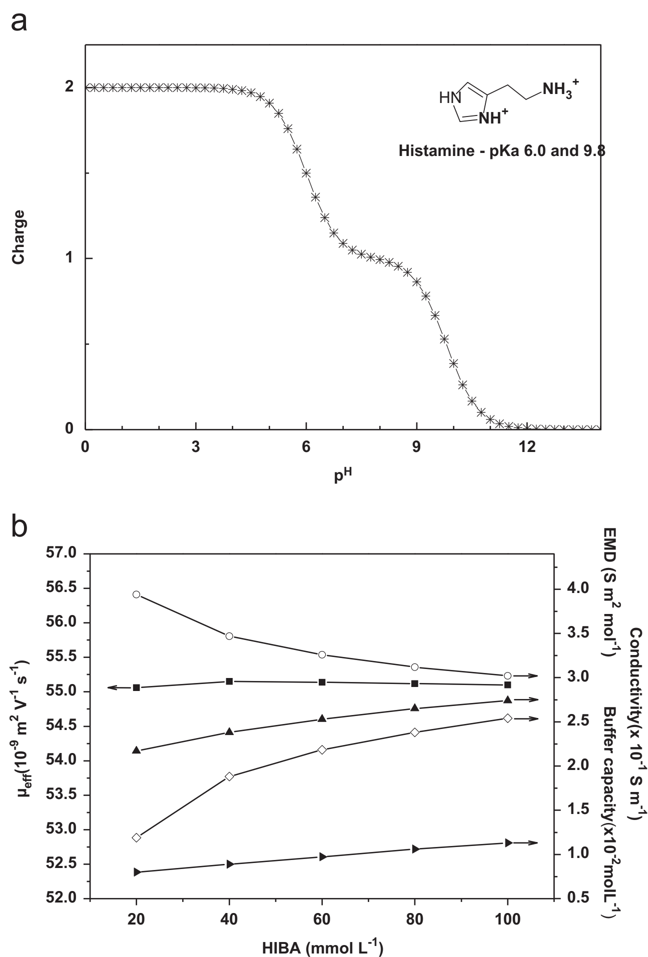

Fig. 1. Charge distribution of histamine (a), and optimization of BGE composition using the Peakmaster® software with fixed sodium concentration of 10 mmol L^{-1} and varying the concentration of HIBA of 20–100 mmol L^{-1} (b). Legends: (■) μ_{eff}; (○) pH; (◇) buffer capacity; (►) conductivity; (▲) EMD.

in Fig. 1-a. The choice of the BGE components was made in order to maintain the histamine with a maximum positive charge, for it to have maximum mobility in order to promote migration in the shortest time possible, and to exhibit a higher mobility for its separation from the sample matrix. The components selected for BGE composition were sodium as the co-ion and HIBA as the counter-ion. Both do not have chromophores groups in their chemical structure and consequently, do not contribute to background absorption in the UV. The choice of the concentrations of the BGE components, as well as the separation conditions were done with Peakmaster® software [21–23], setting the concentration of sodium at 10 mmol L^{-1} and varying the concentration of HIBA. In addition, to permit the BGE optimization using Peakmaster®, the electrophoretic mobility of histamine with maximum positive charge was measured by the Williams and Vigh [24] procedure employing acetone as the neutral marker and the obtained value of 64.8 $(\pm 1.5) \times 10^{-9}\, m^2\, V^{-1}\, s^{-1}$, $n=4$ (electrolyte: sodium 10 mmol L^{-1} and HIBA 60 mmol L^{-1}, ionic strength 10.6 mmol L^{-1}, pH 3.3) was introduced in the software. The results of optimization of BGE composition are shown in Fig. 1-b. It can be seen that with increasing concentration of HIBA, the effective mobility (μ_{eff}) of the histamine remains practically constant, with values close to $55 \times 10^{-9}\, m^2\, V^{-1}\, s^{-1}$; however, the value of the buffering capacity of the BGE increases significantly. At the same time a slight increase of the dispersion by electromigration (EMD) is observed to values below 3.0, as also a slight increase in conductivity. The concentration of HIBA selected for the BGE was 60 mmol L^{-1}, because in these conditions, the HIBA selected as a counter-ion showed a buffering capacity sufficient for the proposed method (21.5 mmol L^{-1}). Also, the sodium chosen as a co-ion guaranteed an EMD suitable for histamine due to it being a cationic species with effective mobility close to that of the histamine with maximum positive charge. Furthermore, in this condition the pH is around 3.3, which ensures a maximum positive charge density of the histamine, while the value of mobility of electroosmotic flow (EOF) on a fused uncoated silica capillary is practically negligible. This contributes to a better separation of histamine from the sample matrix. Significant values of EOF cathodic may decrease the resolution between histamine and interfering present in the sample matrix, as cationic compounds ionized containing chromophore groups. This occurs because of the addition of EOF mobility to the mobility of all other compounds of the separation. Moreover, the negligible EOF mobility does not influence the migration time of histamine, which may contribute to good accuracy results. Finally, a molecule that has structural characteristics similar to histamine that had to be chosen as the internal standard was the imidazole. It is detected in UV and in the used pH, it has high cationic mobility, besides being a synthetic molecule absent in the analyzed samples.

3.2. *Characteristics of the optimized method in the tuna fish samples*

The application of the method developed by CZE for the separation of histamine in tuna fish samples was performed, and the electropherogram obtained is shown in Fig. 2. It is possible to verify a good separation between the peak of histamine and the peaks of interference of the sample matrix in a very short duration of the separation (migration time of histamine −0.34 min). The two largest peaks observed in the electropherogram in the region described as the "matrix of sample" have been identified as being the peaks of creatinine (0.65 min) and histidine (0.75 min). These two interfering compounds are in the cationic form at the pH of the BGE of the proposed method and were also detected due to their absorptivity at the wavelength of histamine detection. These molecules are part of a group of nitrogen compounds that can be found in different types of tuna fish [25]. Thus, the separation conditions of the proposed method configure a fast and selective method for the separation and determination of the analyte of interest. This fact is mainly due to the high mobility of histamine and also due to the direct detection method employed, in which other compounds present in the sample matrix without chromophoric groups cannot interfere in the detection of the analyte. In addition, the extraction procedure with EtOH and the dilution employed reduced the load of species in the matrix, thereby also minimizing the ionic strength of the solution injection, thus contributing to the separation characteristics observed in the proposed method. The identification of histamine was performed by standard addition and also by comparison of the UV spectrum of the peak of histamine in a sample and a standard. Furthermore, the results of histamine concentration determined by the CZE–UV method in the tuna fish samples were compared with a method by LC–MS/MS.

3.3. *Method validation*

The validation of the proposed method was performed using specifications described by the FDA [26], such as system suitability, repeatability, linearity, limit of detection, limit of quantification, and selectivity. The results obtained are shown in Table 1.

The number of plates for the separation of histamine was greater than 12,000, showing good results of peak symmetry and

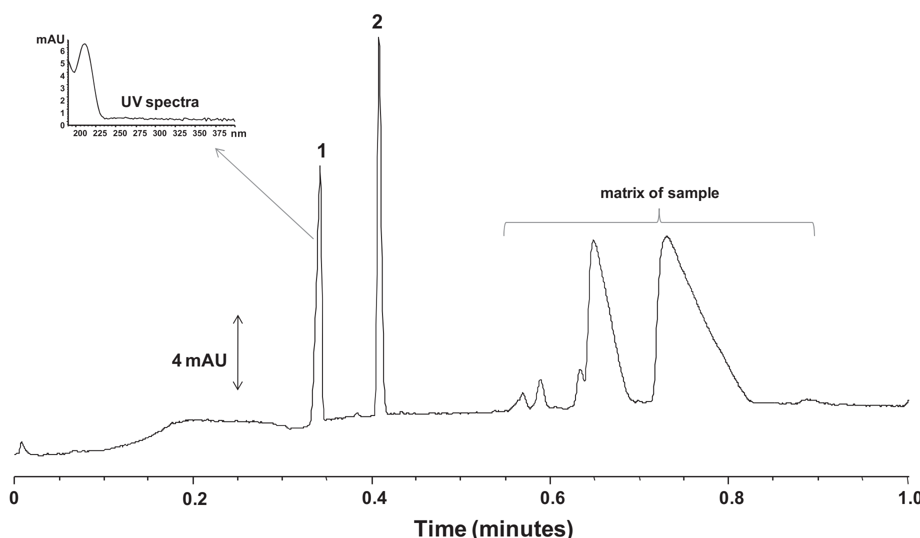

Fig. 2. Electropherogram typical of a tuna fish sample using the optimized method by CZE–UV. Peak legend: (1) histamine; (2) imidazole—IS. Experimental conditions: see Section 2.

Table 1
Analytical performance of the proposed CZE–UV method.

Parameter	*n*	Value
Number of plates (N)[a]	3	12,832
Resolution (R_s, histamine:imidazole)[a]	3	3.6
Peak symmetry[b]	3	1.1
Tailing factor (T_f)[b]	3	0.7
Instrumental precision—peak area corrected (RSD, %)	20	0.60 (0.90)
Instrumental precision—migration time (RSD, %)	20	0.74 (0.40)
Intra-day precision—peak area corrected (RSD, %)	8	2.6 (3.5)
Intra-day precision—migration time (RSD, %)	8	0.5 (1.3)
Inter-day precision—peak area corrected (RSD, %)	11	2.8
Inter-day precision—migration time (RSD, %)	11	0.72
Linearity—linear range (mg L^{-1})	7	1.0–102.0
Linearity—slope		0.0332
Slope standard deviation		0.0015
Linearity—intercept		0.0217
Intercept standard deviation		0.0033
Linearity—coefficient of regression, R^2		0.999
LOQ (mg L^{-1})		0.48 ± 0.07
LOD (mg L^{-1})		0.14 ± 0.02
F		72,228
Recovery (% at 5.1 mg L^{-1} level)[c]		104.9 ± 7.8
Recovery (% at 7.6 mg L^{-1} level)[c]		94.8 ± 2.1
Recovery (% at 15.3 mg L^{-1} level)[c]		107.6 ± 3.9
Recovery (% at 30.6 mg L^{-1} level)[c]		93.9 ± 6.5
Recovery (% at 50.9 mg L^{-1} level)[c]		101.4 ± 3.2

[a] $N=16(t_n/w_n)^2$ and $R_s=2(t_n-t_{n-1})/(w_n+w_{n-1})$, where t is the migration time (min) and w is the baseline peak width.
[b] Peak symmetry (data from HP ChemStation® software) and $T_f=(W_x/2f)$, where W_x is width of the peak determined at either 5% (0.05) from the baseline of the peak height and f the distance between peak maximum and peak front at W_x.
[c] Histamine 28.6 mg L^{-1} in the extraction solution of the tuna fish sample.

tailing factor. The resolution between histamine and imidazole was sufficient for a good separation.

Precisions were determined to evaluate the relative standard deviation (RSD) for the peak area corrected (ratio, area analyte/area IS) and the migration time of histamine using a standard solution of histamine 30 mg L^{-1} (results outside the brackets) and a sample of tuna fish (results inside the brackets). Instrumental precision and intra-day precision for the standard solution of histamine and sample showed RSD values less than 4%, with major values for the corrected peak area in the sample. The inter-day precisions performed just with the standard were less than 3%. The precisions were appropriate, especially considering the complexity of the sample matrix.

The calibration curve was prepared in duplicate and measured in duplicate showing good linearity (coefficient of determination >0.99) in a wide range of concentrations (1.0–100.0 mg L^{-1}). The linear model showed significance with $F>72{,}000$. Limits of detection (LOD) and quantification (LOQ) determined using the signal to noise ratios (S/N) of 3 and 10, respectively, were considered appropriate for the determination of histamine in the samples.

The selectivity of the proposed method was performed using the method of standard addition. The slopes of the curve of standard addition (0.0362 ± 0.0016; R^2 0.996) and the external calibration curve (0.0332 ± 0.0015; R^2 0.999) were compared. The t-test showed that the slopes are statistically equal ($t_{calc}=2.38$ and $t_{critic}=2.78$, at 95% confidence level). Proximity between the determination coefficients indicates that the method is suitably selective for the analysis of histamine in tuna fish samples. Also, the values obtained for the analysis of addition/recovery at five levels of concentration, with each measurement carried out in duplicate, showed good agreement with the reference values, indicating that the method is accurate.

3.4. Determination of histamine in the samples

The histamine concentration was determined in different samples of tuna fish, employing the optimized CZE–UV method and comparative method using LC–MS/MS. The results obtained are shown in Table 2. Statistical analysis using the paired t-test for the samples, applying the two methods, showed that the results are statistically equal, with a confidence level of 95%, because $t_{calc} < t_{critical}$ ($t_{calc}=0.37$; $t_{critical}=3.18$). According to Brazilian legislation only one of the samples analyzed had a concentration of histamine higher than allowed, while under United States law, four samples were above the maximum concentration allowed. The great difference between some of the values of the given histamine concentration in the samples showed that besides the type of fish processing, time and storage conditions also influence the levels of this marker, as reported in the literature [7–11].

Table 2
Results of the histamine concentration determined in the tuna fish samples by the CZE–UV method and LC–MS/MS method.

Sample	CZE–UV[a] (mg/100 g)	LC–MS/MS[b] (mg/100 g)
1—Canned tuna fish (grated)	<LOD	0.108 (± 0.010)
2—Canned tuna fish (solid light)	0.688 (± 0.047)	0.896 (± 0.054)
3—Canned tuna fish (solid in natura)	<LOD	0.082 (± 0.025)
4—Fresh tuna fish	5.15 (± 0.29)	5.21 (± 0.14)
5—Frozen tuna fish	143.4 (± 11.2)	150.2 (± 7.85)
6—Tuna fish (sushi)	0.609 (± 0.015)	0.639 (± 0.025)

[a] Data obtained in 210 nm; results expressed as mean with confidence limit of 95% for $n=3$.
[b] Data obtained in the MRM 112.1/68.1; results expressed as mean with confidence limit of 95% for $n=3$; range of analytical curve 10–1223 $\mu g\,L^{-1}$ to $n=7$, measured in triplicate; slope 191.06; intercept 1839.2; R^2 0.998; F-test 11,480; LOD 30 $\mu g\,L^{-1}$ and LOQ 102 $\mu g\,L^{-1}$.

4. Conclusions

The strategy of choosing the appropriate conditions of separation to develop a rapid method free of interferences may be employed in the development of new methods for the determination of other molecules in complex matrices, such as fish. The Peakmaster® software proved to be an important tool in the selection of the BGE components without the need for experiments. A simple and fast method was developed and could be used in the monitoring of histamine in tuna fish samples and also in other types of fish samples.

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