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Validation and comparison of analytical methods for the determination of histamine in tuna fish samples

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Abstract

Histamine is a degradation product of the bacterial decarboxylation of the amino acid histidine, which is present in large amounts in fish tissues of the Clupeidae and Scombridae families and its presence is an indicator of good manufacturing practices and of the state of preservation of some food. A capillary electrophoresis and high-performance liquid chromatography method with diode arrays detection (HPLC–DAD) were compared and validated: no laborious pre-treatment, no clean up and no derivatization was necessary. In both techniques the correlation coefficient exceeded 0.999 for all the analyses carried out during validation. The limit of detection and the limit of quantitation are 1 and 2 mg/kg, respectively, for high-performance liquid chromatography and 0.5 and 1 mg/kg, respectively, for capillary electrophoresis. Good recoveries were observed for the histamine under investigation at all spiking levels and average recoveries were higher than 92% with the relative standard deviation less than 4% for high-performance liquid chromatography and average recoveries were higher than 85% with the relative standard deviation less than 3% for capillary electrophoresis.
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1. Introduction

Histamine is a product of the microbial degradation of the amino acid histidine due to the action of histidine decarboxylase. The formation of high levels of histamine correlates strongly with the number of microorganisms present in histidine rich foods (vegetables, fermented foods and certain fish species).

The bacterial species that contain this enzyme are very numerous: *Enterobacteriaceae*, *Pseudomonas*, *Clostridium perfrigens* and *Vibrio* spp. However, the microorganism with the strongest histidine activity is *Morganella morganii*, the bacterium mainly responsible for high concentrations of histamine [1]. The presence of histamine in these foods is of great importance as it acts as an indicator of the state of deterioration of the product and is thus a potential public health hazard.

The Decree Law of 30 December 1992, no. 531 [2], incorporating Directive 95/76/EC, as amended by Ministerial Decree of 31 December 1996 [3] adding the fish species *Engraulidae* and *Coryphaenidae*, states that: "nine samples must be taken for each batch, for each of which: (i) the mean content must not exceed 100 mg/kg; (ii) two samples may have a content of greater than 100 mg/kg but less than 200 mg/kg; and (iii) no sample must have a content exceeding 200 mg/kg. Furthermore, the fish of these families that have been subjected to an enzymatic maturation process in brine may display histamine contents that must not however exceed twice the above values".

The ingestion of 70–1000 mg per single meal can lead to "scombroid poisoning" which can lead to death in very sensitive subjects [4,5].

Over the years a number of different analytical methods [6–9] have been proposed mainly using liquid chromatography for histamine determination which may be divided into two classes.

(a) Histamine analysis as such: These are generally simple procedures with short analysis times although the chromatographic aspects are fraught with problems of sensitivity.
(b) Histamine analysis by means of pre- or post-column derivativization with fluorimetric detection: The formation of a fluorescent compound is accompanied by good sensitivity and specificity, long analysis times,

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non reproducibility and problems of derivativization stability.

Side by side with classical analytical techniques, an increasing number of methods involve the use of capillary electrophoresis [1,7,10,11].

The aim of the present work is to make a comparative evaluation and to validate according to ISO/IEC 17025 standards a method for determining histamine in sample of oil-preserved tuna fish using different chromatographic techniques: high-performance liquid chromatography method with diode arrays detection (HPLC–DAD) and capillary electrophoresis with diode array detection (CE–DAD).

2. Experimental

2.1. Chemicals

2.1.1. HPLC chemicals

The solvents used for HPLC (methanol and acetonitrile) were supplied by J.T. Baker (Deventer, Holland). The reference standard histamine dichlorohydrate (99% pure) was supplied by Sigma–Aldrich (Poole, UK). The chromatographic reference standard 1,1-dimethylbiguanide hydrochloride was supplied by Sigma–Aldrich.

Analytical grade reagents (potassium dihydrogen phosphate, potassium hydrogen phosphate trihydrate, perchloric acid) were supplied by Merck (Darmstadt, Germany) and 1-decanesulfonic acid sodium salt (98% pure) by Sigma–Aldrich. The water used was obtained using the Milli-Q purification system of Millipore (Milan, Italy).

2.1.2. CE chemicals

The reference standard histamine dihydrochloride (99% purity) was supplied by Sigma–Aldrich. The chromatographic reference standard 1,1-dimethylbiguanide hydrochloride was supplied by Sigma–Aldrich.

The analytical grade reagents (sodium dihydrogenphosphate monohydrate and sodium hydroxide were supplied by J.T. Baker (Deventer, The Netherlands), phosphoric acid, hydrochloric acid and hydroxyethyl cellulose by Sigma–Aldrich. The water used was obtained using the Milli-Q purification system of Millipore.

2.2. Preparation of standard solutions

2.2.1. Preparation of standard solutions for HPLC and CE

The histamine stock solution containing 0.09 mol/l was prepared by dissolving 165.6 mg in 100 ml of a 0.1 mol/l solution of HCl.

The stock solution of 1,1-dimethylbiguanide hydrochloride was prepared by dissolving 128.2 mg in 100 ml of a 0.1 mol/l solution of HCl.

The stock solutions were stable for 6 months at +4 °C.

The working solutions at a concentration of 2–5–10–15–30 mg/l were obtained by suitably diluting the histamine stock solution in HCl 0.1 mol/l. The 1,1-dimethylbiguanide hydrochloride (chromatographic reference standard) was added to all the working solutions so as to obtain a final concentration of about 5 mg/l. The working solutions were prepared fresh every day.

2.3. Extracting solutions

The extraction solvent used for HPLC method was a solution of $HClO_4$ 1 mol/l and that for a CE method was a solution of 0.1 mol/l HCl.

2.4. Eluents

2.4.1. HPLC eluents

The buffer solution used was prepared by weighing out 1.70 g of KH_2PO_4, 2.85 g of $K_2HPO_4{\cdot}3H_2O$ and 0.49 g of $C_{10}H_{21}O_3SNa$ in 1 l water, obtaining a final pH of 6.9. The buffer solution was prepared at the time of use. Eluent A: 85% of buffer solution and 15% of methanol. Eluent B: acetonitrile.

2.4.2. CE running buffer

A phosphate buffer 50 mmol/l at pH 2.5 was used: it was prepared by weighing out suitable amounts of sodium dihydrogenphosphate monohydrate $NaH_2PO_4{\cdot}H_2O$ and adding H_3PO_4 at 85%. 0.05% hydroxyethylcellulose was added to the running buffer: before use, the solution was placed in an ultrasound bath for 15 min. The buffer solution remained stable for 1 month.

2.5. Equipment and conditions

2.5.1. HPLC equipment and conditions

The analyses were run on a HPLC–DAD equipped with a Series 200 autosampler (Perkin-Elmer, Shelton, CT, USA). The separations were performed under isocratic conditions using a mobile phase composed of 85% eluent A and 15% eluent B using a Luna $C_{18}(2)$ column, 250 mm × 4.6 mm i.d., particle diameter 5 μm, pore diameter 100 Å coupled with a Security Guard $C_{18}(2)$, 4 mm × 3 mm i.d. (Phenomenex, Torrance, CA, USA). The flow rate was 1 ml/min, the injection volume 20 μl and the diode array detector was positioned at a wavelength of 214 nm.

2.5.2. CE equipment and conditions

The tests were performed on a P/ACE MDQ (Beckmann Coulter, Fullerton, CA, USA) using an uncoated silica capillary 50 cm × 75 μm i.d. supplied by Beckmann Coulter. The capillary length between inlet and detector was 40 cm. Before use the new capillary was conditioned for 15 min with 0.5 mol/l NaOH, for 10 min with water and lastly with running buffer for 10 min. The electrophoretic method involves filling the capillary with buffer for 2 min, injection at a pressure of 0.5 psi for 5 s (1 psi = 6894.76 Pa), separating using a current of 30 μA for 13 min followed by rinsing of

the capillary with 0.5 mol/l NaOH for 2 min and with water for 2 min. The temperature of the capillary was maintained at +23 °C with the DAD system positioned at a wavelength of 214 nm.

2.6. Sample preparation

Five grams of previously homogenized tuna fish were weighed out into a glass centrifuge test tube and 20 ml of 1 mol/L $HClO_4$ added for the HPLC method and 20 ml 0.1 mol/l di-HCl for the CE method; the mixture was vortex stirred for 1 min and then placed in an ultrasound bath for 15 min; centrifuged at +4 °C for 15 min at 4160 RCF×*g*, the supernatant was then drawn off and filtered through a Whatman filter into a 50 ml round-bottomed flask. This procedure was repeated twice. The extracts were then combined and the chromatographic reference standard (1,1-dimethylbiguanide hydrochloride) added so as

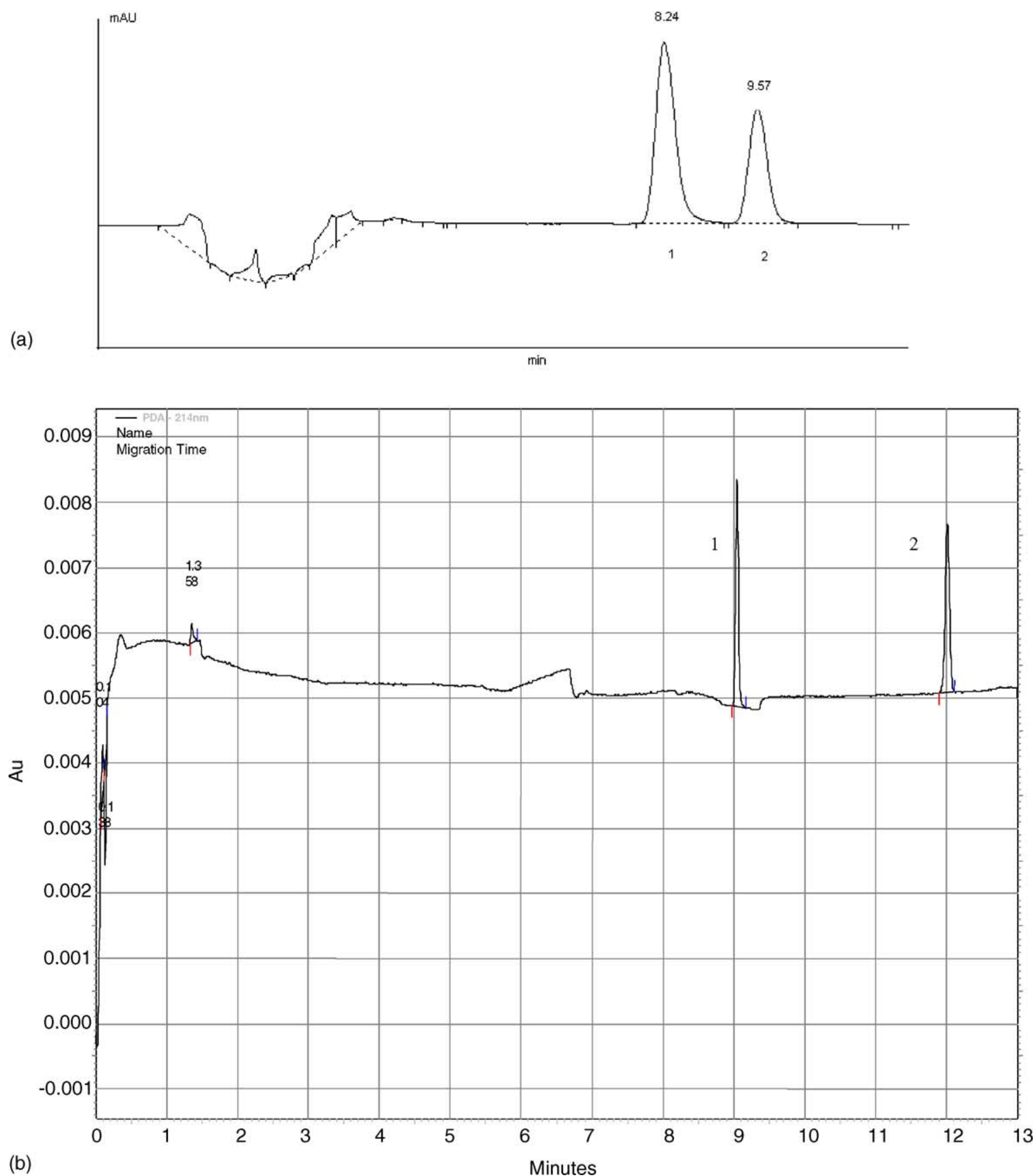


Fig. 1. (a) Chromatogram of histamine standard (1) and 1,1-dimethylbiguanide standard (2) monitored at 214 nm; conditions: column Luna C_{18}(2) at +23 °C, eluents A–B (85:15, v/v) at a flow-rate of 1 ml/min, injection loop 20 µl. (b) Electropherogram of histamine standard (1) and 1,1-dimethylbiguanide standard (2) monitored at 214 nm; conditions: uncoated silica capillary 50 cm × 75 µm i.d.; run buffer, 50 mmol/l phosphate (pH 2.5) with 0.05% of hydroxyethylcellulose; pressure injection 0.5 psi for 5 s, current 30 µA (+ to −).

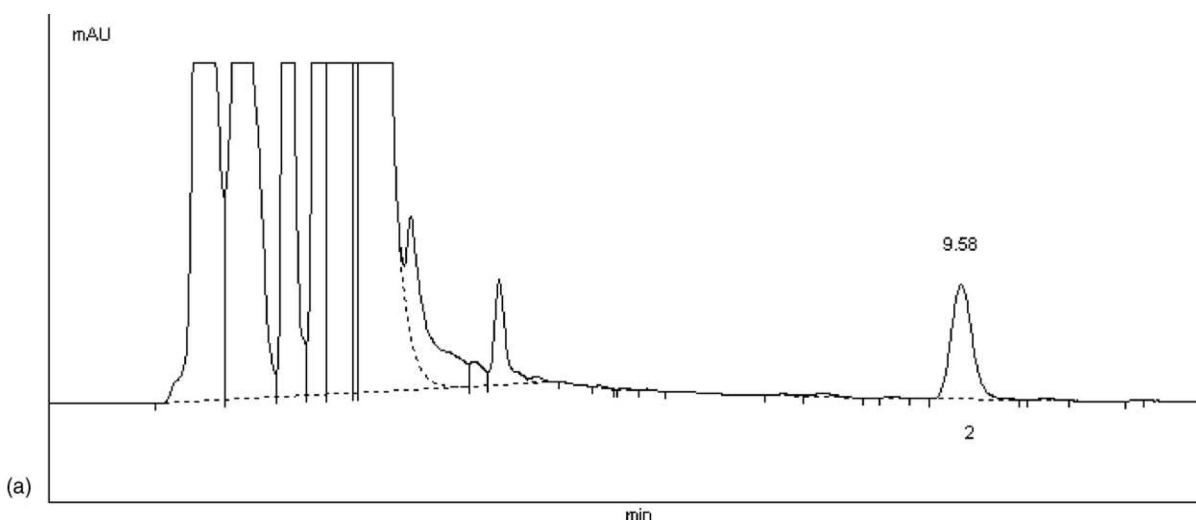
to obtain a concentration of about 5 mg/l, bringing up to volume with water. They were then filtered with a 0.45 μm PTFE and injected into the HPLC–DAD and the CE–DAD systems.

3. Results and discussion

A simple, rapid and specific method for determining histamine in tuna fish samples was validated using two techniques, HPLC–DAD and CE–DAD.

In both techniques the differences between the retention/migration times must not be greater than ±1%: in actual fact, the retention/migration time for histamine displayed an 10% average difference CE and 5% for HPLC; the difference was reduced to ±1% by introducing 1,1-dimethylbiguanide as chromatographic reference standard. 1,1-Dimethylbiguanide was chosen instead of diamminobenzoic chlorohydrate acid because it is a basic compound with characteristics more similar to histamine.

A chromatogram of histamine standard and 1,1-dimethylbiguanide standard monitored at 214 nm is shown in Fig. 1a.



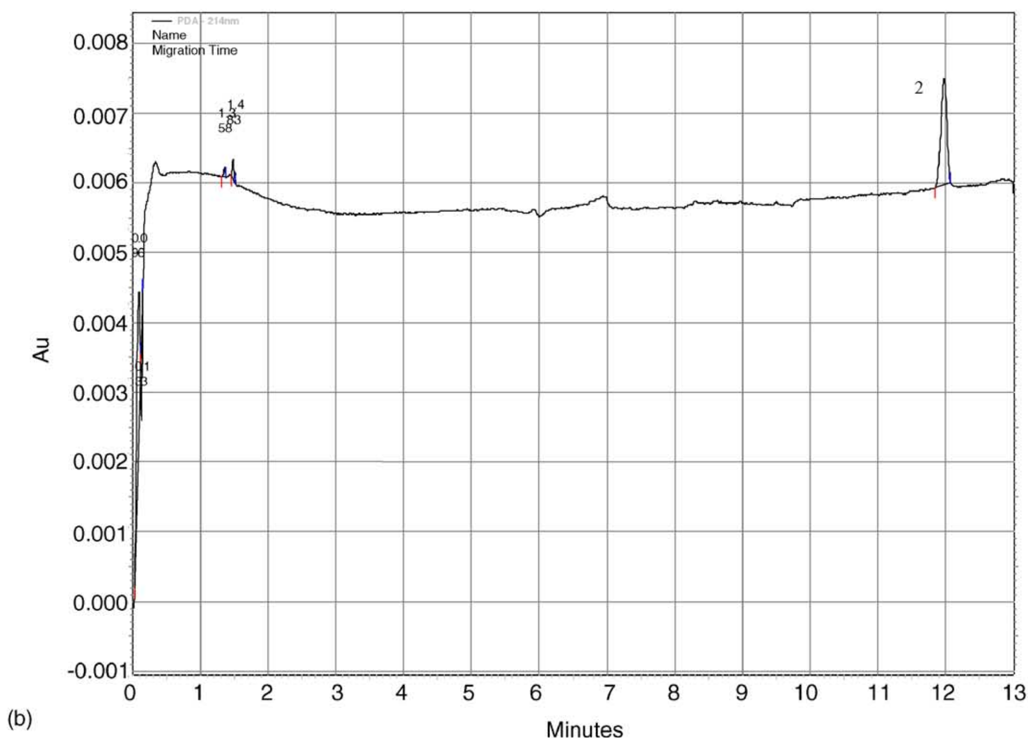
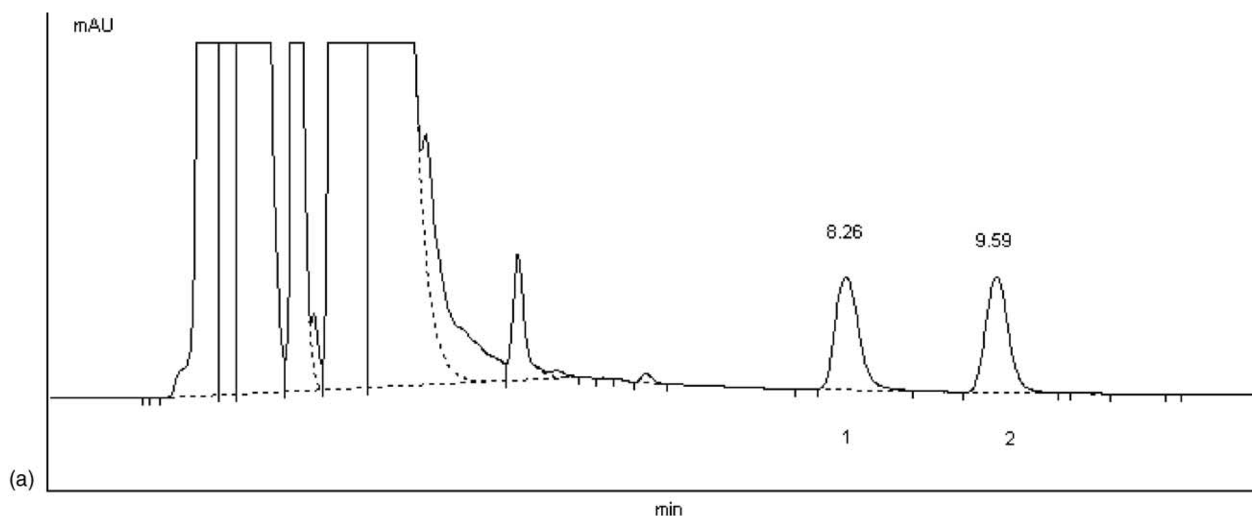


Fig. 2. (a) Representative chromatogram of a blank tuna fish sample with 1,1-dimethylbiguanide standard (2). (b) Representative electropherogram of a blank tuna fish sample with 1,1-dimethylbiguanide (2) standard.

An electropherogram of histamine standard and 1,1-dimethylbiguanide standard monitored at 214 nm is shown in Fig. 1b. Under the HPLC conditions adopted, the analytes were fully separated in 12.50 min with symmetrical peaks. Retention times were 8.24 and 9.57 min, respectively. Under the CE conditions adopted, the analyses were completed in 13.00 min with symmetrical peaks. Migration times were 9.02 and 12.01 min, respectively.

Representative chromatogram and electropherogram of a blank tuna fish sample are reported in Fig. 2a and b.

In order to develop a CE method three different capillaries were tested: an untreated fused-silica capillary, an ECAP neutral capillary, and an ECAP amine capillary (Beckmann Coulter). As no relevant differences were detected, the silica capillary was chosen being the less expensive and the less fragile. Two different run buffer were chosen in differ-



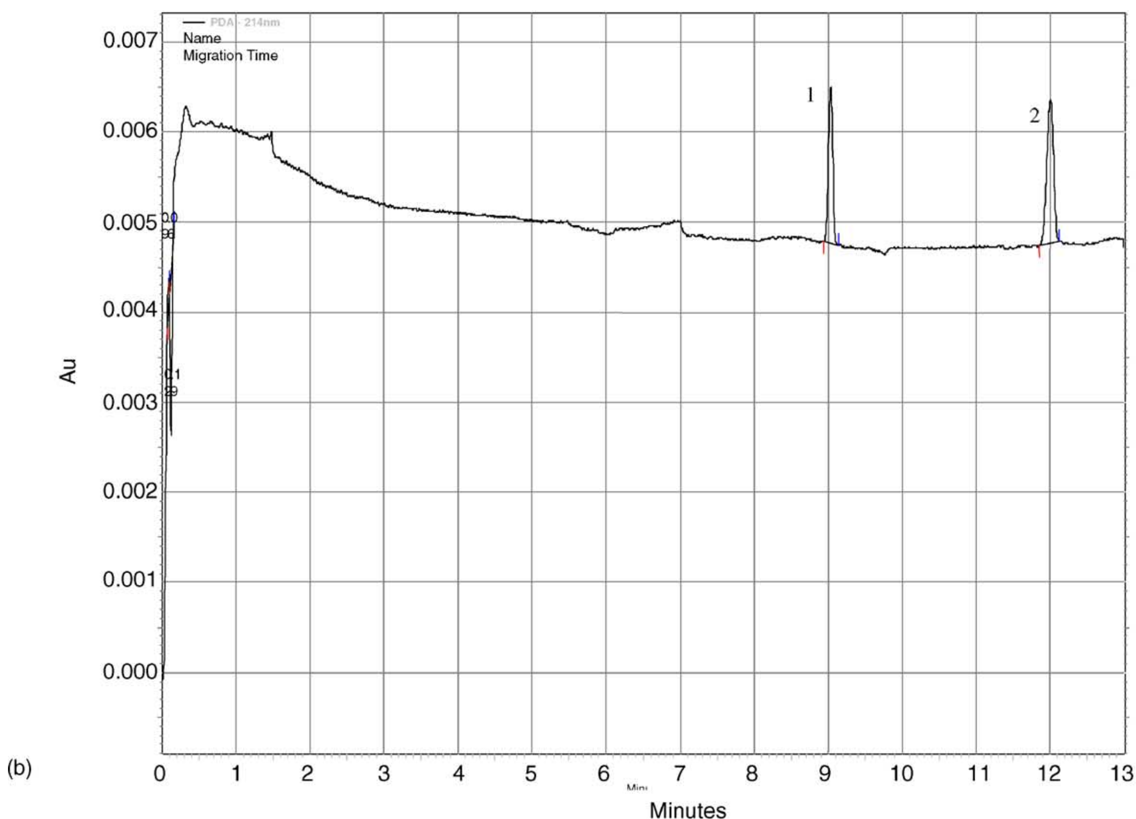


Fig. 3. (a) Representative chromatogram of a sample spiked with 100 mg/kg of histamine (1) with 1,1-dimethylbiguanide standard (2). (b) Representative electropherogram of a sample spiked with 100 mg/kg of histamine (1) with 1,1-dimethylbiguanide standard (2).

ent pH ranges: phosphate buffer (pH 2.2–2.5–3.0) for untreated fused-silica capillary and amine capillary; acetate buffer (pH 4.0–4.5–5.0) for neutral capillary and untreated fused-silica capillary. The phosphate buffer at pH 2.5 was chosen for it was the one giving the best results in terms of sensitivity.

In order to perform a dynamic coating both hydroxypropylmethylcellulose and hydroxyethylcellulose were added to the run buffer; adding the 0.05% of hydroxyethylcellulose gave the best results in terms of base line stability.

The formerly used HPLC method was performed on a ionic couple mobile phase and a C_{18} reversed-phase column which can be used in a narrow pH range 2–7 [12]. As the mobile phase pH is greater than 7, and the non protected silica dissolves at pH greater than 7, there is a strong counter-pression which shortens the column life. The use of a silica B column (Luna, C_{18}) with 17.5% carbon allows the use of mobile phases in a broad pH range (1–10).

The histamine stability was checked for different solvents; it is not stable in water, where it lasts for 1 week only, while it is stable in acidic conditions. Moreover, working in acidic conditions allows a better chromatographic peak resolution. Different solvents were used to extract the sample; HCl, $HClO_4$, methansulfonic acid. The best recovery was obtained using $HClO_4$.

In order to verify the specificity of the method, 20 blank tuna fish samples from different origins were analysed using both techniques. No interference was observed in the region of interest where the analytes were eluted, as is shown in the blank sample chromatogram and electropherogram. Representative chromatogram and electropherogram of a sample spiked with 100 mg/kg of histamine are reported in Fig. 3a and b, respectively. Quantification was carried out by comparison of the analyte peak areas versus an externally generated calibration curve. In both techniques five calibration standards were used dissolved in 0.1 mol/l HCl ranging from 2 to 30 mg/l to generate external calibration curves; the correlation coefficient exceeded 0.999 for all the analyses carried out during the validation procedure of the analytical method. The validation procedure included the determination of limit of detection (LOD), limit of quantification (LOQ), specificity, accuracy and precision for the quantitative confirmation method.

The LOD, calculated as the smallest concentration from which it is possible to deduce the presence of the analyte with reasonable statistical certainty (signal/noise, 5:1) was 1 mg/kg for HPLC and 0.5 mg/kg for CE. The LOQ, calculated as the smallest measured content of the identified analyte in a sample that may be quantified with a specified degree of accuracy ($n = 18$) and within-laboratory reproducibility, was 2 mg/kg for HPLC and 1 mg/kg for CE.

The histamine and 1,1-dimethylbiguanide stabilities were determined in solution: stability of stock standard solutions had been verified at room temperature (in darkness and light), at +4 and −20 °C for 12 weeks; the stock standard solutions were stable for 12 weeks in all stored conditions.

In order to evaluate the precision, accuracy and recoveries of the analytical method, the tuna fish samples were spiked at three different concentrations (50, 100 and 200 mg/kg) and then analysed. Inter-day precision and accuracy data on three different days for histamine in tuna fish samples in HPLC and CE analysis are shown in Table 1. The precision of the method was determined by calculating the relative standard deviation (R.S.D., %) for the repeated measurements. The accuracy of the method (trueness, %) was determined by assessing the agreement between the measured and nominal concentrations of analysed samples. For inter-day data, the overall precision ranged from 3.07 to 3.44 for HPLC, and 0.90 to 2.91 for CE. These values were considered very satisfactory. Recoveries are shown in Table 2. In both techniques, the recoveries were obtained by spiking tuna fish samples at three different concentrations (50, 100 and 200 mg/kg) and then by analysing the samples six times on three different days ($n = 18$). Good recoveries were observed for the histamine under investigation at all spiking levels and average recoveries were higher than 92% with the R.S.D. less than 4% for HPLC and average recoveries were higher than 85% with the R.S.D. less than 3% for CE.

Table 1
Inter-day precision and accuracy data for histamine in tuna fish samples in HPLC and CE analysis

Parameter	Spiked sample level (mg/kg)		
	50	100	200
HPLC			
Average (mg/kg)	46.46	95.46	199.42
S.D. (mg/kg)	1.51	3.29	6.12
Precision (R.S.D., %)	3.26	3.44	3.07
Trueness (%)	−7.08	−4.54	−0.29
n	18	18	18
CE			
Average (mg/kg)	42.74	86.26	176.42
S.D. (mg/kg)	1.25	1.01	1.59
Precision (R.S.D., %)	2.91	1.17	0.90
Trueness (%)	−14.52	−13.74	−11.79
n	18	18	18

Table 2
Recoveries for histamine in tuna fish samples in HPLC and CE analysis

Spiked level (mg/kg)	Tuna fish sample		
	Average recovery (%) ($n = 18$)	S.D.	R.S.D. (%)
HPLC			
50	92.92	3.03	3.26
100	95.46	3.29	3.44
200	99.71	3.06	3.07
CE			
50	85.48	2.49	2.91
100	86.26	1.01	1.17
200	88.21	0.79	0.90

4. Conclusions

Different analytical techniques (HPLC–DAD and CE–DAD) for determining histamine in tuna fish samples preserved in olive oil were compared and validated. Both techniques gave excellent results in terms of accuracy, precision, linear range and reproducibility. A fundamental contribution was made by the introduction of the chromatographic reference standard 1,1-dimethylbiguanide since in capillary electrophoresis the migration time is strongly dependent on the characteristics of the buffer used. For both techniques different columns, capillaries and batches of reagent were tested and the robustness of the method demonstrated.

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