Evaluation of biogenic amines in fish sauce by derivatization with 3,5-dinitrobenzoyl chloride and micellar liquid chromatography

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Abstract

A simple, selective and sensitive method to quantify the biogenic amines cadaverine, 2-phenylethyl-amine, histamine and spermidine has been developed. The analytes were derivatized with 3,5-dinitrobenzoyl chloride and separated by micellar liquid chromatography. This is a practical technique for the selective determination and quantification of biogenic amines in fish sauce. Optimization of chromatographic conditions was made by an interpretative model, and the separation conditions were: C18 column (125 mm x 4.6 mm, 5 μm particle size), UV detection set at 260 nm, and a mobile phase of 0.15 mol/L, sodium dodecyl sulfate (SDS), pH 7. Validation was performed following the United States Food and Drug Administration (FDA) guidelines using spiked samples. Under these conditions, validation parameters were: linearity (0.5–500 mg mL1, $r^2 > 0.9990$), limits of detection (in the 158–375 ng/mL, range); intra and inter-day precision (relative standard deviation < 3.2% and 4.2%) and accuracy (in the range of 88.6–103.7% and 94.2–101.5%), respectively, and variations were lower than 4%. The proposed method was successfully applied to the monitorization of biogenic amines formation in unsalted and salted fish sauce samples. The suggested methodology was found useful in routine analysis of biogenic amines in fish sauce.

Keywords: Anchovy sauce; Cadaverine; Food analysis; Food composition; Food safety; Histamine; Micellar mobile phase; Phenylethylamine; Spermidine

Highlights

► Biogenic amines from spoilage of anchovy sauce were analyzed.

► Methodology was fast, easy, accurate, precise, robust, reliable and non-polluting.

► The experimental procedure was expedited by the use of micellar media.

► Method validated following US Food and Drug Administration guidelines.

► Method was applied to compare anchovy sauce storage with and without salting.

1. Introduction

Cadaverine (CA; log Po/w = - 0.44; pKa = 10.5/10.93), 2-phenylethylamine (2-PE; log Po/w = 1.43; pKa = 9.84), histamine (HI; log Po/w = -0.97; pKa = 5.9/9.7) and spermidine (SD; log Po/w = - 1.28; pKa = 8.25/9.86/10.9) are biogenic amines (Fig. 1), which can be found in foods either as natural products or after fermentation, decomposition or putrefaction processes [1-3]). CA is largely responsible for the foul odor of putrefying flesh, and also contributes to the odor of bad breath and bacterial vaginosis. It is also found in semen and some microalgae. SD can be found in a wide variety of organisms and tissues, and it is an essential growth factor in some bacteria. 2-PE is a monoamine alkaloid which can be present in many foods such as chocolate, especially after microbial fermentation. HI is a biogenic amine involved in local immune responses, neurotransmission and chemotaxis of white blood cells. The consumption of an excess of biogenic amines, known as histaminic intoxication, is mainly related to heart disease (hypotension and palpitation) and headache. The toxin effects of biogenic amines also affect the gastrointestinal system, provoking nausea, vomiting, diarrhea, abdominal pain and indigestion and skin, causing rash, redness, itching, burning, urticaria, edema and local inflammation [4].

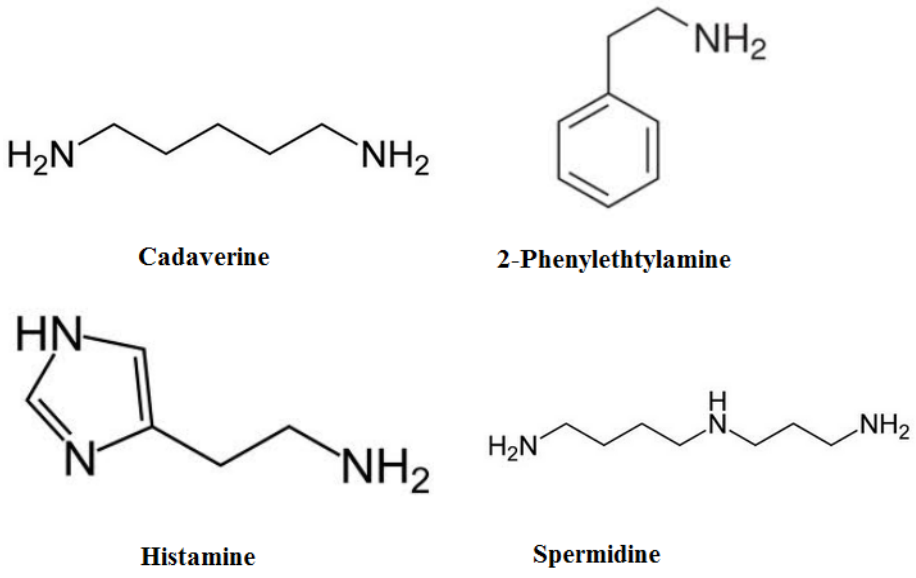


Fig. 1. Structure of the biogenic amines.

Biogenic amines can be found in a wide range of food, as alcoholic beverages, beef, chocolate, cheeses, fish, pork and poultry. These molecules can be considered as markers of microbial contamination and spoilage of fish derived products, such as fish flesh or fish sauce [2,4]. Biogenic amines are produced mainly by microbes, improper handling of the raw material, incorrect stocking conditions (if samples are not kept in a freezer at -18 ºC) samples, or manufacturing processes. Moreover, biogenic amines can also been directly produced by the activity of autolytic enzymes, and sometimes no correlation can be found between the amount of biogenic amines and the microbial counts. Indeed, this enzymatic activity produces substrate for microorganisms and encourages bacterial growth [5,6].

Thus the determination of these analytes is of the utmost importance to assure that fish sauce can be eaten without health risk [7,8]. The United States Food and Drug Administration (FDA) has established limits to prevent biogenic amines intoxication by intake of spoiled fish. The legal limit for HI has been set to 50 mg/mL [9].

Determination of biogenic amines can be performed by high performance liquid chromatography with UV detection (HPLC-UV) with derivatization using dabsyl chloride [10], dansyl chloride [11], benzoyl chloride [12] or 3,5-dinitrobenzoyl chloride [13]. Other authors propose methods based on HPLC-FLD (fluorescence detection) after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate [14] or ophthalaldehyde [15], or HPLC-ED (electrochemical detection) [16]. HPLC–MS (mass spectrometry detection) [17] has become a method of choice, but such instrumentation is usually not suitable for routine analysis due to financial reasons (purchasing cost and maintenance) [18,19].

The reagent 3,5-dinitrobenzoyl chloride (DNBZ-Cl) has been widely used as a chromophore to determine amines in food samples [20]. Derivatization reaction is quite fast (less than 5 min), quantitative and reproducible, and also, derivatives obtained are stable and show high sensitivity. In almost all approaches, the derivatized amines have to undergo extraction in a suitable organic solvent, evaporation to dryness and redissolution in order to preconcentrate and purify the analytes [13]. However, it introduces the risk of sample loss and contamination and also, increases the analysis time. Finally, chromatographic conditions result in either insufficient separation or prolonged analysis, which could take longer than an hour to perform [12,13,21,22]. These problems can be avoided by the use of micellar liquid chromatography (MLC), which allows direct injection of samples (after filtration), without

extraction and cleaning step. Moreover, they are less toxic, non-flammable, biodegradable and relatively inexpensive in comparison to aqueous–organic solvents. MLC has proved to be a useful technique in the determination of diverse groups of compounds in low time using mobile phases under isocratic program, by optimizing separation parameters [23,24] including food samples [25-27].

The aim of this work was to develop a rapid, simple and selective procedure for the determination of CA, 2-PE, HI and SD by MLC. Analytes were derivatized with a chromogen to improve sensitivity, and directly injected in the chromatographic system, avoiding extraction. The suggested methodology was validated in terms of linearity, sensitivity, limits of detection and quantification, accuracy, precision and recovery, following the FDA guideline [28]. Finally, the method was applied to the study of the anchovy sauce degradation by means of the determination of biogenic amines depending on storage treatment.

2. Materials and methods

2.1 Apparatus and methods

The pH of solutions was measured with a Crison GLP 22 (Crison Instruments, Barcelona, Spain) equipped with a combined Ag/AgCl/glass electrode. The balance used was a Mettler-Toledo AX105 Delta-Range (Mettler-Toledo, Greifensee, Switzerland). The vortex shaker and ultrasonication unit were from Selecta (Barcelona). The chromatographic system was an Agilent Technologies Series 1100 (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump, a thermostated autosampler and column compartment. A Kromasil C18 column (125 mm x 4.6 mm, 5 µm particle size) from Scharlab (Barcelona) was also used. Dead time was determined as the mean value of the first significant deviation from the baseline in the chromatograms of the analytes. The signal was acquired by a PC computer connected to the chromatograph through a HP Chemstation (Agilent Technologies).

133 *2.2 Chemical and reagents*

134

135 The biogenic amines CA, 2-PE, HI, SD, and 3,5-dinitrobenzoyl chloride (98% pure)
136 were purchased from Sigma–Aldrich (St. Louis, MO, USA). The surfactant sodium dodecyl
137 sulfate (SDS, 99% pure) was from Merck (Darmstadt, Germany); the organic solvents
138 acetonitrile, ethanol and propanol were from Scharlab, the buffer sodium dihydrogen
139 phosphate and HCl and NaOH were from Panreac (Barcelona). All solutions were prepared in
140 Simplicity ultrapure water (Millipore, S.A.S. Molsheim, France). Biogenic amine solutions
141 were filtered through 0.45 μm, 13 mm nylon membranes (Millex-HN, Millipore, Bedford,
142 MA, USA). The corresponding biogenic amines hydrochlorides were solved in 0.1 mol/L
143 HCl to provide a final concentration of 100 μg/mL.

144

145 *2.3 Derivatization of biogenic amines with 3,5-dinitrobenzoyl chloride*

146

147 As a derivatizing reagent 3,5-dinitrobenzoyl chloride (5 mmol/L) was solved in
148 acetonitrile. Aliquots (400 μL) of biogenic amine standards, 1 mol/L NaOH (1200 μL), 2-
149 propanol (700 μL) and 3,5-dinitrobenzoyl chloride (2100 μL) were mixed in a reaction tube.
150 After 3 min of shaking at 25 ºC, 1000 μL of a 2 mol/L HCl solution were added to stop the
151 reaction. Finally, after 1 min of shaking, derivatized biogenic amines were filtered and
152 injected into the chromatographic system. Under these conditions, the formed derivatives
153 were $(DNBZ)_2CA$, (DNBZ)(2-PE), $(DNBZ)_2HI$ and $(DNBZ)_3SD$ [13]. The fish sauce
154 medium does not affect the derivatization reaction, because the conditions were strongly
155 changed by the addition of organic alcohol and sodium hydroxide. Some matrix compounds
156 are precipitated in ethanol/NaOH media, and others are solubilized in the SDS-medium [13].

157

158 *2.4 Chromatographic conditions*

159

160 Derivatized biogenic amine separation was performed in a reversed-phase C18
161 column thermostated at 25ºC. The mobile phase was 0.15 mol/L SDS–NaH_2PO_4 0.01 mol/L
162 at pH 7. The flow rate, injection volume and UV wavelength were 1 mL/min, 20 mL and 260
163 nm, respectively. Samples were thermostated at 5 ºC. Under these conditions, the retention
164 times (min) for biogenic amines were 11.6, 14.9, 18.1 and 20.7 for CA, 2-PE, HI and SD,
165 respectively. Chromatographic signals were acquired and processed with an Agilent
166 ChemStation (Rev. B.01.03).

167

168 *2.5 Sample preparation*

169

170 Anchovy sauce samples (*Engraulidae spp.*) were obtained from a local market. A part
171 of the anchovies was mixed with common salt in a relation of 75/25 (w/w) (a well-known
172 treatment to avoid food spoilage) and another portion was untreated. In both cases, samples
173 were stored in a fridge at 5 ºC. For the analyses of the fish sauces, 1 g of each was mixed with
174 0.5 mL of ethanol and topped up to 10 mL with 0.1 mol/L SDS solution. The samples were
175 stored in a glass vessel without vacuum package. In the case of spiking, the appropriate
176 volume of biogenic amines standard solution (100 μg/mL of each analyte solved in 0.1 mol/L
177 HCl) were spilt on 1 g of sample and vigorously shaken to favor homogenization and stored
178 for one day in the fridge at 5 ºC to favor the contact between analytes and the sample, and
179 also solvent evaporation [29,30]. Then the spiked sample was mixed with 0.5 mL of ethanol
180 and topped up to 10 mL with 0.1 mol/L SDS solution. An aliquot of the sample (400 mL) was
181 derivatized as explained in Section 2.3, filtered (13 mm nylon membranes, 0.45 μm porosity)
182 and directly injected into the chromatograph.

183

3. Results and discussion

3.1 Optimization strategy and mobile phase selectionn

SDS was selected as surfactant because of its low cost, high purity, low critic micellar concentration, high solubility in water, and low viscosity of its aqueous solution, then it is easy to remove from the chromatographic system [26].

In order to obtain reproducible retention times, pH of the mobile phase must remain constant. Basic pH was discarded to avoid damaging the column. Then the mobile phase was buffered at pH 7, which allows an adequate separation of the 3,5-dinitrobenzoyl biogenic amine derivatives [13] and also take care of the column.

Most of the studied biogenic amines are polar compounds, according to their low log Po/w, which means that using a C18 column and pure micellar mobile phases would provide an adequate retention time. Thus, initially, the SDS concentration effect was studied. Several mobile phases containing 0.05, 0.1 and 0.15 mol/L SDS were tested. At low SDS concentrations, the retention times were found too high, so the concentration 0.15 mol/L was taken for further studies. At high SDS concentration, the number of micelles is higher, then increasing the interaction of the analytes with the mobile phase and reducing the interaction with the stationary phase. As result, analytes elute quicker [31].

In a pure micellar mobile phase of SDS, the high retention time of compounds usually makes it necessary to add a small amount of organic solvent in order to decrease the retention times. In this case, different amounts of propanol were tested, however no significant differences were found in terms of resolution. Therefore, no organic solvent was added to the mobile phase, then reducing the production of toxic waste. After the optimization step, the selected mobile phase was 0.15 mol/L SDS–0.01 mol/L NaH_2PO_4 at pH 7. The retention times, retention factors, efficiencies and asymmetries of the biogenic amines under these conditions are shown in Table 1.

213 **Table 1**. Retention times (min) (Rt), retention factor (k), efficiencies (N) and assymetries (B/A) of the biogenic
214 amines obtained in the 0.15 mol/L sodium dodecylsulfate-0.01 mol/L NaH_2PO_4, mobile phase.

Biogenic amine	***t***	***k***	***N***	***B/A***
Cadaverine	11.7	11.8	3000	1.1
2-Phenylethylamine	14.9	15.6	6200	1.1
Histamine	18.1	19.0	1700	1.4
Spermidine	20.7	18.5	4200	0.8

215

216 *3.2 Method validation*

217

218 FDA guidelines were applied in the validation of this chromatographic method for the
219 determination of CA, 2-PE, HI and SD [28]. In all cases, the validation was performed using
220 spiked samples of both unsalted and salted anchovy sauce.

221 To test the selectivity, a blank of fresh sample was analyzed by the proposed
222 methodology. No peaks were found near the retention times of the analytes (Fig. 2A). Then, a
223 sample spiked with 10 mg/mL of CA, 2-PE, HI and SD was analyzed. The obtained
224 chromatogram shows that the analytes elute without overlapping between them or with other
225 compounds (Fig. 2B). The excess of reagent elutes at the front of the chromatogram and do
226 not interfere with the analysis.

227 Calibration curves were constructed using the areas of the chromatographic peaks
228 (nine injections) obtained at seven different concentrations, in the 0.5–500 mg/ mL range
229 (Table 2). To study the variability of the calibration parameters, the curves were obtained for
230 5 days over a period of four months for a different set of standards. Results were similar in
231 both matrices (unsalted and salted anchovy sauce). The slope and intercept were determined
232 by the method of least square linear regression analysis and determination coefficients (r^2)
233 were always higher than 0.9990. Limits of detection for biogenic amines was calculated
234 following the 3s criterion, LOD was 3 times the standard desviation of the blank (the standard
235 desviation of the lower concentration calibration point; 9 replicates) divided by the sensitivity
236 (the slope of the calibration curve). Results are shown in Table 2. Limits of quantification
237 (LOQ) were defined as the lower concentration reached for the calibration curve (0.5 mg/mL)

238 in accordance with the FDA guidelines [28]. It is observed that the results obtained were
239 under the safety limits proposed by the FDA [9].
240
241 **Table 2**. Parameters of the calibration curves: slope, intercept, limit of detection (LOD, ng/mL) for the biogenic
242 amines studied.

Biogenic Amine	Slope [a]	Intercept [a]	LOD
Cadaverine	0.68 ± 0.05	- 0.03 ± 0.06	375
2-Phenylethylamine	0.75 ± 0.02	0.02 ± 0.03	307
Histamine	0.85 ± 0.03	- 0.04 ± 0.05	307
Spermidine	1.85 ± 0.05	0.08 ± 0.10	158

243 [a] average ± standard deviation of 9 measurements
244
245 The intra- and inter-day precision and accuracy of the analytical method were
246 determined by analysis of the spiked sample with the studied biogenic amines at three
247 different levels (Table 3). The intra-day values were determined by assaying each sample
248 nine times on the same day, and inter-day values was the average of nine measurements of
249 intra-day values taken on 5 days over a 4-month period. Intra- and inter-day precision, which
250 was defined as the percentage of relative standard deviation (RSD), were between 0.6% and
251 4.2%, respectively. Intra- and inter-day accuracy, which was defined as the percentage of
252 analyte recovery, were between 88.6% and 103.7%, respectively.
253
254 **Table 3.** Precision and accuracy values obtained for the studied biogenic amines.

Biogenic Amine	Intra-day precision[a] (RSD, %)			Intra-day accuracy[a] (%)			Inter-day precision[b] (RSD, %)			Inter-day accuracy[b] (%)		
	1	10	100	1	10	100	1	10	100	1	10	100
Cadaverine	1.7	1.6	0.8	89.8	93.5	99.0	1.7	0.8	0.8	94.6	97.5	98.6
2-Phenylethylamine	1.3	1.2	1.2	92.6	94.5	97.6	1.2	0.7	0.6	97.5	98.6	100.3
Histamine	3.2	2.4	1.3	103.7	102.5	99.5	4.2	3.5	1.1	98.6	100.6	101.5
Spermidine	2.2	2.0	1.2	88.6	95.6	101.6	2.0	1.0	1.0	94.2	96.2	97.7

255 [a]n=9; [b]n=5

The intra- and inter-day precision and accuracy of the analytical method were determined by analysis of the spiked sample with the studied biogenic amines at three different levels (Table 3). The intra-day values were determined by assaying each sample nine times on the same day, and inter-day values was the average of nine measurements of intra-day values taken on 5 days over a 4-month period. Intra- and inter-day precision, which was defined as the percentage of relative standard deviation (RSD), were between 0.6% and 4.2%, respectively. Intra- and inter-day accuracy, which was defined as the percentage of analyte recovery, were between 88.6% and 103.7%, respectively.

Robustness of the method was examined by replicate injections (n = 6) of a standard solution at a concentration of 10 mg/mL under small changes in the following chromatographic parameters: SDS concentration (0.145–0.155 mol/L), pH (6.9–7.1) and flow rate (0.95–1.05 mL/ min). Insignificant differences in peak areas and lower variability in retention times were observed (see Table 4.4). Results indicate that the selected factors remain unaffected by small variations in these parameters (less than 4%).

Stability studies indicated that degradation of biogenic amines derivatized with 3,5-dinitrobenzoyl chloride took place in 12 h. These results were confirmed by the displacement of the peaks in chromatograms. All solutions were kept at 5 ºC. The stock solutions of biogenic amines and 3,5-chloride were stable for three days in the fridge and four months kept in a freezer.

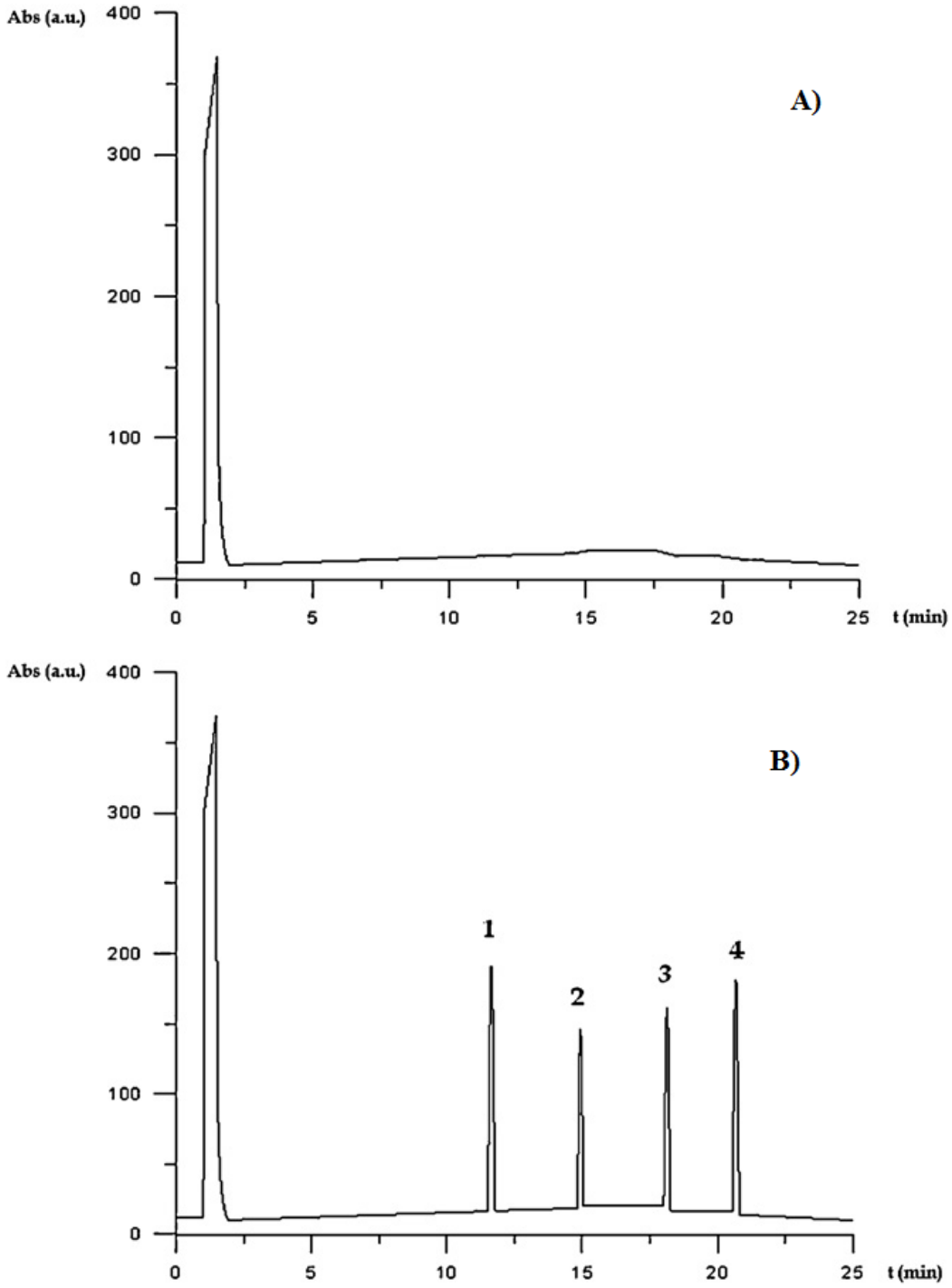


Fig. 2. Chromatogram obtained by the analysis of fresh unsalted anchovy sauce samples following the proposed methodology: (A) blank and (B) spiked with 10 mg/mL of (1) cadaverine, (2) 2-phenylethylamine, (3) histamine, and (4) spermidine.

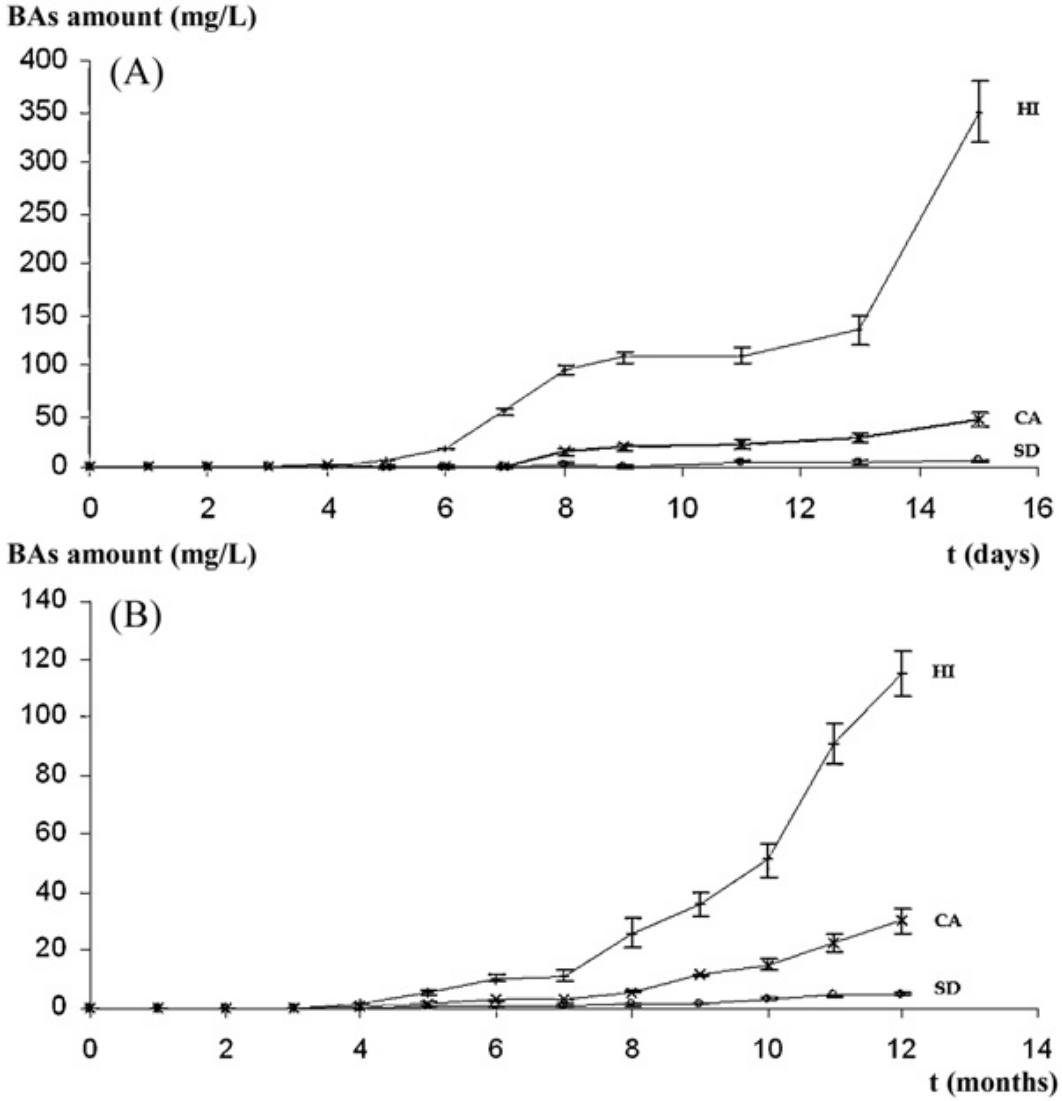
Table 4. Evaluation of the robustness of the method.

Biogenic amine	Changes in the parameters	Level	Retention time (min) (RSD, %)	Area (arbitrary unit) (RSD, %)
Cadaverine	Conc. SDS (mol L^{-1})	0.145–0.155	11.7±0.3 (2.6)	6.79±0.08 (1.2)
	pH	6.9–7.1	11.5±0.4 (3.5)	6.80±0.11 (1.6)
	Flow (mL min^{-1})	0.95-1.05	11.3±0.4 (3.5)	6.82±0.14 (2.1)
2-phenylethylamine	Conc. SDS (mol L^{-1})	0.145–0.155	14.7±0.2 (1.4)	7.53±0.12 (1.6)
	pH	6.9–7.1	14.6±0.3 (2.1)	7.49±0.13 (1.7)
	Flow (mL min^{-1})	0.95-1.05	15.0±0.6 (4.0)	7.50±0.18 (2.4)
Histamine	Conc. SDS (mol L^{-1})	0.145–0.155	18.1±0.4 (2.2)	8.5±0.2 (2.4)
	pH	6.9–7.1	17.8±0.6 (3.4)	8.4±0.3 (3.6)
	Flow (mL min^{-1})	0.95–1.05	18.2±0.5 (2.7)	8.7±0.18 (2.1)
Spermidine	Conc. SDS (mol L^{-1})	0.145–0.155	20.5±0.2 (1.0)	18.7±0.3 (1.6)
	pH	6.9–7.1	20.7±0.4 (1.9)	18.5±0.5 (2.7)
	Flow (mL min^{-1})	0.95-1.05	21.0±0.7 (3.3)	18.8±0.6 (3.2)

n = 6

3.3 Analysis of food samples

Unsalted and salted anchovy sauces (*Engraulidae spp.*) were analyzed at several times in order to evaluate the formation of biogenic amines. The studied interval time was different for unsalted (15 days) and salted anchovy sauces (1 year), because salted sauce was expected to undergo slower degradation. In both cases, the amount of CA, HI and SD increased at longer storage times. The 2-PE was not detected in the samples, as it only appears at high spoiled fish samples [32]. According to the safety level of biogenic amines, especially for HI, anchovy sauces can be considered inadequate for human consumption at 7 days for unsalted (Fig. 3A) and 10 months for salted (Fig. 3B) samples. The results clearly indicate that mixing with salt is an excellent way to prevent spoilage in anchovy sauces.



296

297 **Fig. 3.** Amount of cadaverine, histamine and spermidine in salted and unsalted anchovy sauce stored at 5 ºC,
298 and analyzed by the proposed methodology at several times. The samples were stored: (A) without treatment
299 and (B) mixed with salt 75/25 (w/w).

300

301 **4. Conclusions**

302

303 Results indicate that the proposed micellar liquid chromatography procedure can be
304 used for the analysis of cadaverine, 2-phenylethylamine, histamine and spermidine with
305 analysis times below 25 min. The method is sensitive enough for food quality control and
306 routine analyses. It is a simple, rapid, and effective method, and does not require an

extraction step. The reagent 3,5-dinitrobenzoyl chloride was found to be highly suitable for the analysis of biogenic amines using the proposed method due to its fast reaction, and therefore it is recommended to be used in pollution surveys and in the routine practice of food-quality control.

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