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Simultaneous Determination of Four Biogenic and Three Volatile Amines in Anchovy by Ultra-High-Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry

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ABSTRACT: A new method has been developed and validated for the simultaneous determination of four biogenic (putrescine, cadaverine, histamine, and tyramine) and three volatile amines (trimethylamine, triethylamine, and tripropylamine) in anchovy. Separation and determination of the selected compounds were carried out by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), using an electrospray ionization source (ESI) in positive ion mode. Chromatographic separation was carried out using an aqueous solution of formic acid (0.1%) and methanol as mobile phase in gradient mode. The method was validated, and mean recoveries were evaluated at three concentration levels (75, 150, and 250 μg/kg), ranging from 70 to 110% at the three levels assayed. Intra- and interday precision, expressed as relative standard deviation (RSD), were lower than 15% and 20%, respectively. Limits of quantitation (LOQs) were 25 μg/kg for all cases, except for that of TMA, which was set at 60 μg/kg. The developed procedure was applied to determine the target compounds in anchovy samples stored during 7 days at 4 °C, observing the increasing in the concentration of these compounds at longer storage time.

KEYWORDS: *volatile amine, biogenic amine, fish, UHPLC-MS/MS*

INTRODUCTION

Seafood is known to be easily deteriorated during processing and/or storage because of the action of different factors, such as microorganisms, endogenous enzyme activity, nonenzymatic lipid oxidation, and browning.[1,2]

During cold storage of seafood, some chemical changes, such as the formation of volatile amines (VAs) (i.e., trimethylamine (TMA), triethylamine (TEA), and tripropylamine (TPA)), have been produced, and they can affect the organoleptic properties of these products.[3] In this sense, the determination of the total volatile base-nitrogen (TVB-N) is frequently used to measure fish freshness.[4]

Moreover, during storage or processing steps, biogenic amines (BAs) such as putrescine (PUT), cadaverine (CAD), tyramine (TYR), and histamine (HIS), can also be formed, because of decarboxylation of precursor amino acids. These substances have a toxicological effect on human health.[5] In general, the content of BAs has been used as chemical indicators of seafood quality[6] and several parameters have been proposed. For instance, the sum of HIS, TYR, PUT, and CAD has been used as a biogenic amine index (BAI) to evaluate the hygienic quality of seafood.[7] Despite of the use of these indexes, only HIS content is regulated in seafood. Thus, the European Union (EU) has established an acceptable level of 100 mg/kg of HIS for fish species of the families Scombridae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae, and Scombresocidae (Commission Regulation (EC), No. 2073/2005)[8] to protect public health. For the rest of amines, the European and Spanish legislation establish the use of sensory analysis to determine the freshness of the fish and the use of chemical or microbiological analysis when the sensory evaluation is not definitive (Commission Regulation (EC), No. 2074/2005).[9]

Therefore, the analysis of BAs and VAs in fish tissues is of interest because of their toxicological risk and their use as food quality indicators. For that purpose, common extraction procedures such as solid–liquid extraction, using several acids such as perchloric acid,[10] trifluoroacetic acid,[11] and hydrochloric acid,[12] have been used for the extraction of BAs from seafood. For the extraction of VAs, acid extraction has also been used.[13] In addition, solid-phase microextraction (SPME) has been utilized,[14,15] but it has some disadvantages, such as limited duration of fiber and lack of reproducibility.

Usually, high performance liquid chromatography (HPLC) coupled to several detectors, such as ultraviolet (UV)[16] or fluorescence (FL),[17] has been described for the determination and quantitation of BAs in food matrices. However, a derivatization step is necessary for the suitable detection of these compounds,[18] which usually involves several problems, such as instability, reagent interferences, repeatability, and long preparation time. Therefore, these conventional detection techniques have been replaced by mass spectrometry (MS), bearing in mind that it often permits unambiguous BAs identification in food matrices.[19,20] Moreover, due to its high

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Table 1. Retention Time Windows (RTWs) and MS/MS Parameters of the Selected Amines

compd	abbr	RTW (min)	cone voltage (V)	quantitation transition[a]	confirmation transition[a]
histamine	HIS	1.01−1.24	25	112.0 > 95.2 (15)	112.0 > 68.1 (20)
cadaverine	CAD	1.02−1.15	20	103.1 > 86.1 (8)	103.1 > 69.1 (15)
putrescine	PUT	1.01−1.17	20	89.1 > 72.1 (8)	
trimethylamine	TMA	1.09−1.18	30	60.2 > 44.4 (15)	
triethylamine	TEA	1.36−1.44	35	102.1 > 74.2 (15)	102.1 > 58.3 (20)
tyramine	TYR	1.90−2.02	20	137.9 > 121.1 (20)	137.9 > 77.1 (25)
tripropylamine	TPA	3.98−4.31	30	144.4 > 102.4 (20)	144.4 > 60.3 (20)

[a]Collision energy (eV) is given in parentheses.

sensitivity, selectivity, and good linearity, HPLC coupled to MS/MS allows a reliable quantitation of compounds at trace levels.[21−23] Despite the fact that the application of ultra-high-performance liquid chromatography (UHPLC) improves sensitivity and resolution and reduces analysis time,[24] there are few published studies using UHPLC-MS/MS,[12] although a derivatization step is included.

On the other hand, for the determination of VAs, gas chromatography (GC) coupled to flame ionization detection (FID)[25] or MS,[14,26] has been also used. However, FID is not very suitable for confirmation purposes and MS presents some disadvantages, such as the need for a derivatization step. To overcome these problems, LC coupled to MS/MS has been used.[27,28] To our knowledge, there is not any published method for the determination of VAs by UHPLC-MS/MS, and in relation to the simultaneous determination of BAs and VAs, it is important to highlight that there is only one paper[29] that describes the determination of TMA, PUT, and CAD in muscle food by ion mobility spectrometry (IMS).

Therefore, the aim of the present work has been the development and validation of a new method for the simultaneous determination of BAs and VAs in fish, as well as the evolution of the content of these compounds in anchovies over 7 days when samples were stored at 4 °C. A solid−liquid extraction procedure and UHPLC-MS/MS determination without derivatization have been applied.

MATERIALS AND METHODS

Chemicals and Reagents. Commercial amine standards, putrescine (PUT), cadaverine (CAD), histamine (HIS), tyramine (TYR), trimethylamine (TMA), diethylamine (TEA), and tripropylamine (TPA), were obtained from Sigma-Aldrich (Steinheim, Germany). Stock standard solutions of individual compounds (with concentrations ranging from 200 to 300 mg/L) were prepared by exact weighing of the powder or liquid and dissolution in 50 mL of an aqueous solution of hydrochloric acid (HCl, 0.1 M), obtained from J.T. Baker (Deventer, Holland). These solutions were then stored at −18 °C in the dark. A multicompound working standard solution at a concentration of 10 mg/L of each compound was prepared by appropriate dilution of the stock solutions with ultrapure water acidified with HCl (0.1 M) and stored in screw-capped glass tubes at 4 °C in the dark.

HPLC analytical grade organic solvents (methanol and formic acid) were supplied by Sigma. Perchloric acid was obtained from Riedel-de-Haën (Seelze, Germany). Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA). Cellulose filters of 0.44 μm from Whatman (Maidstone, England) were also used.

Apparatus and Software. Chromatographic analyses were performed using an Acquity UPLC system (Waters, Milford, MA, USA), and separations were achieved using an Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm particle size) from Waters. Chromatographic separation was carried out using a gradient elution with methanol as eluent A and an aqueous solution of formic acid 0.1% (v/v) as eluent B.

The elution started at 10% of eluent A. This composition was kept during 2 min. Then, it was linearly increased up to 100% in 3 min. This composition was kept for 1.5 min and returned to the initial conditions in 0.5 min, followed by a re-equilibration step of 1.5 min, to give a total run time of 8.5 min. The injection volume was 5 μL, and the column temperature was maintained at 30 °C. The flow rate was set at 0.2 mL/min.

Mass spectrometry analysis was carried out using a Waters Acquity TQD tandem quadrupole mass spectrometer (Waters, Manchester, U.K.). The instrument was operated using electrospray ionization (ESI) in positive mode. Data acquisition was performed using MassLynx 4.0 software with a QuanLynx program (Waters). The capillary voltage and the extractor voltage were 3.5 kV and 3 V, respectively. The source temperature was 150 °C, and the desolvation temperature was set at 350 °C. The cone gas (nitrogen) and desolvation gas (also nitrogen) were set at flow rates of 80 and 600 L/h, respectively, and the collision-induced dissociation was performed using argon as the collision gas at the pressure of 4×10^{-3} mbar in the collision cell. The specific MS/MS parameters for the amines are shown in Table 1.

Centrifugations were performed in a high-volume centrifuge (Centronic, Barcelona, Spain). A kitchen blender (Sammic, Azkoitia, Spain) was used to process samples.

Sample Preparation. Five kilograms of anchovy (*Engraulis encrasicolus*) was bought from a fish market. The fish had been caught the night before they were purchased, and they were kept on ice until sample preparation (the same day of purchasing). Before the extraction procedure, the samples were washed with tap water and then with plenty of Milli-Q water. The flesh was triturated, and it was vacuum packed in plastic bags and stored at 4 °C.

Extraction Procedure. All fish samples were processed according to the following procedure:[30] 10 g of homogenized fish was weighed in a 50 mL polypropylene centrifuge tube, and 12 mL of 0.6 M perchloric acid was added. The mixture was centrifuged at 6000 rpm for 10 min. After that, the suspension was vacuum-filtered. The filtered was stored and the remaining residue was extracted again. The extracts were combined in 25 mL volumetric flasks, and the volume was completed with 0.6 M perchloric acid. Finally, the filtered solution was injected into the chromatographic system.

RESULTS AND DISCUSSION

The aim of this work has been the development of a method for the simultaneous determination of BAs (PUT, CAD, HIS, and TYR) and VAs (TMA, TEA, and TPA) in seafood, as well as the application of the proposed method to study the evolution of the content of these compounds in anchovy.

Optimization of the UHPLC−MS/MS Determination. Chromatographic and MS conditions were optimized to get short analysis time and suitable sensitivity. First, ESI-MS/MS parameters were optimized by direct infusion of a standard solution of 20 mg/L of each amine at a flow rate of 0.01 mL/min. Solutions were prepared in 5 mL of a mixture of an aqueous solution of water/methanol (50:50, v/v) with 50 μL of

Table 2. Validation Parameters of the Developed Method

		recovery[a]				
compd	linear range (μg/L)	75 μg/kg	150 μg/kg	250 μg/kg	interday precision[b]	LOQ (μg/kg)
HIS	10−750	95.8 (12.1)	71.7 (9.2)	104.8 (4.1)	11.3	25
CAD	10−750	96.0 (13.5)	71.4 (10.4)	108.4 (6.2)	12.9	25
PUT	10−750	86.0 (9.3)	82.9 (5.8)	95.2 (4.5)	9.2	25
TMA	25−750	76.3 (14.5)	81.9 (10.3)	88.2 (6.4)	18.4	60
TEA	10−750	102.4 (12.7)	90.4 (6.8)	92.9 (2.9)	11.2	25
TYR	10−750	96.9 (8.9)	108.2 (7.3)	104 (5.9)	9.6	25
TPA	10−750	107.3 (14.2)	106 (6.9)	99.2 (3.6)	17.0	25

[a]Intraday precision is given in parentheses (n = 4). [b]Spiked concentration: 150 μg/kg (n = 5).

formic acid, and they were injected into the ESI source. ESI in positive and negative ion modes was evaluated, observing that all amines were detectable in positive mode. Full scan MS and MS/MS spectra were acquired. First, cone voltage was optimized in single MS mode to obtain the most abundant precursor ion. The protonated molecule $[M + H]^+$ was the most intense ion for all the compounds. From the collision induced dissociation (CID) spectra, the collision voltage was optimized to monitor two product ions, selecting the most sensitive transition for quantitation, whereas another one was used for confirmation purposes. Two transitions were selected for CAD, HIS, TYR, TEA, and TPA, whereas for PUT and TMA, only one transition was monitored with good sensitivity, due to the low molecular mass of these compounds.[23,31] For some of the selected amines, such as CAD, HIS, TYR, and PUT (BAs), the most intense product ion was generated by the loss of ammonia, $[M + H - NH_3]^+$, which is a characteristic loss of this type of compounds, whereas, for VAs, the loss of hydrocarbon chains was observed. Table 1 shows the MS/MS transitions, as well as the cone voltages and collision energies optimized for each compound. Other parameters, such as desolvation and cone gas flow, source and desolvation temperature, and capillary voltages, were studied, selecting the optimum conditions indicated in the Materials and Methods.

To separate the selected amines and to provide an overall optimum response for MS/MS detection, gradient elution was performed, using methanol and an aqueous solution of formic acid 0.1% (v/v) as mobile phase components. Using these conditions, good separation of the selected amines and good MS sensitivity were achieved. Other parameters, such as flow rate, injection volume, and column temperature were optimized to get a fast and reliable separation, selecting as optimum values 0.2 mL/min as flow rate, 30 °C as column temperature, and 5 μL as injection volume. Using these conditions, all the selected compounds were eluted with high sensitivity and selectivity in less than 8.5 min, including cleaning and re-equilibration steps. Retention time ranged from 1.05 to 4.22 min, and the analytes were distributed in three acquisition functions. Different dwell times (from 5 to 100 ms) were tested to obtain a sufficient number of data points across the peak. At least 15 data points for each peak were obtained, applying an interscan delay time of 20 ms and a dwell time of 15 ms for the first acquisition function, which implies the monitoring of four compounds (PUT, CAD, HIS, and TMA), 25 ms for the second function, monitoring two compounds (TEA and TYR), and 100 ms for the third function, which used TPA monitoring. Finally it must be indicated that although PUT, CAD, and HIS have the same retention time, the use of MS/MS allows the selective determination of these compounds, bearing in mind that they have different precursor and product ions.

Validation of the Proposed Method. The performance characteristics of the optimized method were established by a validation procedure, studying linearity, matrix effect, trueness, precision, and limits of quantitation.

The identification of the BAs and VAs was carried out by searching the appropriate retention time windows (RTWs) defined as the retention time ± three times the standard deviation of the retention time of ten samples, using the ions indicated in Table 1 for identification purposes. The selectivity of the method was evaluated by analyzing unspiked samples. The absence of any signal at the same elution time of the target compounds suggested there were no matrix interferences that give a false positive signal.

The matrix effect was evaluated, and several concentrations (from 10 to 750 μg/L) were analyzed in pure solvent (methanol), in extracted fish sample, and in extraction solution (perchloric acid). The slopes obtained in the calibration with matrix-matched standards were compared with those obtained with solvent based standards and extraction solution. It was observed that matrix significantly suppresses the response for all amines studied, although they were similar to those obtained using the extraction solution. Therefore, for quantitation purposes, a calibration was carried out in the extraction solution.

The linearity of the response was evaluated by injecting various concentrations of the selected BAs and VAs. The calibration functions obtained by plotting the peak area versus the concentration of the compound were linear for all cases from 10 to 750 μg/L, except for that for TMA, which was linear from 25 to 750 μg/L. The determination coefficient was higher than 0.995, and deviation of the residuals of each calibration point was always equal to or lower than 20%.

Trueness was estimated through recovery studies, evaluating three different fortification levels (75, 150, and 250 μg/kg). Four samples were spiked with the selected compounds at each fortification level, showing the obtained results in Table 2. Good results were obtained, and recoveries ranged from 76 to 107% at 75 μg/kg, from 71 to 108% at 150 μg/kg, and from 88 to 108% at 250 μg/kg.

Precision of the overall method was studied performing intraday (repeatability) and interday precision experiments. Intraday precision was evaluated at the three concentration levels of the recovery studies, performing four replicates at each level, whereas interday precision was evaluated at 150 μg/kg in five consecutive days. The obtained results are shown in Table 2. It can be observed that values, expressed as relative standard deviation (RSD) were always lower than 15% for all the amines and concentration levels assayed (75, 150, and 250 μg/kg), and

it was lower than 18% in interday studies, indicating the stability of the developed method.

Limits of quantitation (LOQs) were determined as the lowest concentration level that yielded a signal-to-noise (S/N) ratio of 10. LOQs were 25 μg/kg for the selected compounds except for the case of TMA (60 μg/kg). In relation to previous studies,[12,26,31,32] the validation parameters, including LOQs, obtained in this work were similar or even better, indicating the suitability of the proposed method for the simultaneous determination of BAs and VAs.

Sample Analysis. To evaluate the evolution of these compounds, anchovy samples were stored during 7 days at 4 °C. Samples were analyzed every 12 h (from 0 to 168 h). Samples were purchased from a fishmonger located in Granada (Spain). To ensure the quality of the results when the proposed method was applied, an internal quality control was carried out in every batch of samples. This internal quality control implies the analysis of the following: (i) a blank extract, that eliminated false positives caused by a contamination in the extraction procedure or by the presence of a interference; (ii) a reagent blank (obtained by performing the whole procedure without sample), which removed any possibility of false positive due to contamination in the instruments or reagents employed; (iii) a matrix-matched calibration to check linearity and sensitivity, and (iv) a spiked blank sample at 250 μg/kg to assess the extraction efficiency. Table 3 shows the obtained results. It must be indicated that the same samples should be diluted before the chromatographic analysis due to the high concentration detected for some compounds.

Table 3. Amines Concentration (mg/kg) in Anchovy Samples Stored at 4 °C

	biogenic amines				volatile amines		
storage time (h)	HIS	CAD	PUT	TYR	TMA	TEA	TPA
0		0.17	0.12		3.68		
12		0.14	0.09		3.53		
24		0.18	0.11		4.94		
48		0.18	0.12	0.05	5.98		
72		0.18	0.11	0.06	6.40		
96		0.16	0.11	0.13	6.81		
120		0.19	0.17	0.82	19.46		
144	0.03	0.79	0.65	5.12	207.28		
168	0.04	3.67	1.37	22.30	287.66		

Initially, the anchovy sample only contained PUT, CAD, and TMA at low concentrations, but during the storage period, the concentration of other amines, such as TYR (detected after 48 h) and HIS (detected after 144 h), increased. On the other hand, it must be indicated that neither TEA nor TPA were detected during the storage time evaluated in this study, as was observed previously.[33] For all the detected amines, it can be observed that the concentration increased during storage time. However, the variation of the concentration depends on the monitored amine. For instance, HIS, which is the legislated amine, only appears after 144 h at low concentrations (0.03 mg/kg), whereas the final concentration of TYR was higher (from 0.05 to 22.30 mg/kg). Figure 1 shows the obtained results for TMA and BAI (the sum of PUT, CAD, HIS, and TYR). A high increase of the concentration of TMA during storage time (from 3.68 to 287.66 mg/kg) can be observed, especially after 120 h. The same trend was observed for BAI (from 0.29 to 27.4 mg/kg), which is usually used as a freshness indicator for anchovies, with a limit of acceptability set at 15 mg/kg.[33] According to the obtained results, the anchovies can be stored at 4 °C during 6 days, and after that, anchovies are not acceptable to be used for human consumption. Furthermore, it must be emphasized that, after 5 days, a fishy-odor was noted, indicating an increase in the concentration of amines.

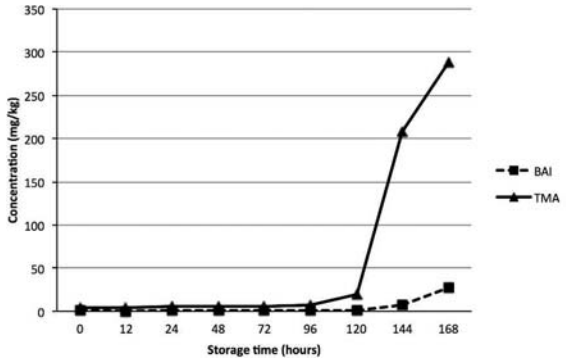

Figure 1. Variation of BAI and TMA concentration (mg/kg) during storage time.

The obtained results are similar to those observed by other authors, who indicate that, in general, BAs concentration increased after 5 days[34] or 1 week.[11] In relation to VAs, in other studies lower concentrations of TMA (<16 mg/kg) were observed,[34] although it must emphasized that fish was stored in ice. If fish was stored at room temperature, TMA concentration can be higher than 200 mg/kg after 2 days.[35] Furthermore, lower limits were obtained if storage temperature was lower (−20 °C), observing that TMA concentration was below 10 mg/kg after 76 days of storage.[26] Finally, in relation to the case of marinated fish (TMA > 20 mg/kg after 150 days at 4 °C),[36] the obtained TMA concentrations in this work are considerably higher.

In conclusion, this work presents a suitable method for the simultaneous determination of BAs and VAs by UHPLC-MS/MS. The selection of the UHPLC coupled to MS/MS allows a suitable and fast elution and detection of the target compounds in less than 8.5 min, avoiding the detection of interferences. Good validation parameters (linearity, trueness, precision, and quantitation limits) were obtained, indicating the reliability of the method for the simultaneous determination of these important indicators of fish freshness, simplifying the current analytical methods, which use derivatization steps or two different analytical methods to determine separately BAIs and VAs. Other amines, such as spermidine and spermine, which are used to determine the chemical quality index, could be included. Therefore, the proposed method can be applied in routine analysis. Moreover, the content of amines in anchovy was monitored at different storage times, observing that amines concentration increases during storage time, indicating that anchovies can be stored at 4 °C during 6 days without a significant increase of the amine content.

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Notes

The authors declare no competing financial interest.

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