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Analytical Methods

Simultaneous determination of eight underivatised biogenic amines in fish by solid phase extraction and liquid chromatography–tandem mass spectrometry

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ABSTRACT

Biogenic amines on fish tissue are formed as a result of bacterial contamination and spoilage during storage. A new method based on liquid chromatography (LC) and tandem mass spectrometry (MS/MS) using a triple quadrupole (QqQ) analyser was developed for the analysis of eight biogenic amines (cadaverine, histamine, phenylethylamine, putrescine, spermine, spermidine, tyramine and tryptamine) in fish tissues. Sample preparation was performed by extraction with trichloroacetic acid 5% and solid phase extraction clean up with STRATA X cartridge. The MS/MS method was validated and compared with a method based on the analysis of dansyl derivatives by LC and fluorescence detector (FD). MS/MS achieved higher sensitivity (from 0.02 mg kg^{-1} for spermidine and phenylethylamine to 0.2 mg kg^{-1} for spermine) when compared to FD (from 1 mg kg^{-1} for putrescine and tyramine to 4 mg kg^{-1} for histamine); MS/MS method showed higher precision too, with intraday relative standard deviations (RSDs) from 1% to 4% with respect to those obtained with FD method (from 3% to 17%).

Recovery study was conducted at two different fortification levels and the average ranged from 71% to 93% for all of the studied compounds with RSDs lower than 18%. Matrix-matched standards were used to counteract matrix effect observed in MS/MS determination. The applicability of the method was demonstrated by the analysis of biogenic amines in fish obtained from commercials of Valencia.

1. Introduction

Biogenic amines are organic bases found in high-protein foods such as fish (scombroid), meat, fermented products (cheese, wine or beer), and spoiled foods as a result of a microbial contamination that exhibits amino acid decarboxylase activities (Shalaby, 1996). The composition of biogenic amines gives information about the hygienic conditions of raw material and manufacturing practices (Al Bulushi, Poole, Deeth, & Dykes, 2009). High levels of biogenic amines in food constitute a potential public concern due to their physiological and toxicological effects. The most frequent food-borne intoxication caused by biogenic amines involves histamine. Histamine poisoning is caused by ingesting seafood, mainly scombroid fish such as mackerel (*Scomber Japonicus*), tuna (*Thunnus* spp.), mahimahi (*Coryphaena hippurus*), sardine (*Sardina pilchardus*) and bluefish (*Pomatomus saltratrix*). Scombroid or histamine fish poisoning is a type of food poisoning with symptoms and treatment similar to those associated with seafood allergies (Al Bulushi et al., 2009; Hungerford, 2010). Tryptamine and tyramine have toxic effects in human beings such as blood pressure increase causing hypertension. Putrescine and cadaverine are known to enhance histamine toxicity by inhibiting histamine metabolising enzymes (monoamine or diamine oxidase and histamine methyl transferase). Putrescine, spermine, spermidine and cadaverine may react with nitrite and form carcinogenic nitrosamines (Al Bulushi et al., 2009).

The unique amine content regulated in seafood is histamine. To protect public health, the US Food and Drug Administration has established a guideline level of 50 mg kg^{-1}; as histamine is generally not uniformly distributed, it can be found 500 mg kg^{-1} in other parts of the fish (FDA, 1995). The European Union has established an acceptable level of 100 mg kg^{-1} of histamine for fish species of the families *Scombridae*, *Clupeidae*, *Engraulidae*, *Coryfenidae*, *Pomatomidae* and *Scombresocidae* (Commission Regulation (EC), No. 2073/2005). Furthermore, most of the biogenic amines are heat and acid stable and they cannot be destroyed by cooking, freezing or processing such as canning and smoking (FDA, 1998). Thus, the analysis of biogenic amines in fish tissues is of interest because of their toxicological risk and also their use as indicator of food quality.

Due to their low volatility and lack of chromophores, biogenic amines are mostly analysed by liquid chromatography (LC) with pre and post column derivatisation and UV–visible or fluorescence detection (Onal, 2007). The most common reagents used for the preparation of fluorescent derivatives are *o*-phthalaldehyde (OPA)

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(Moret, Smela, Populin, & Conte, 2005; Salazar, Smith, & Harris, 2000), benzoyl chloride (Balamatsia, Paleologos, Kontominas, & Savvaidis, 2006), dansyl chloride (Gosetti, Mazzucco, Gianotti, Polati, & Gennaro, 2007; Innocente, Biasutti, Padovese, & Moret, 2006; Mazzucco et al., 2010; Proestos, Loukatos, & Komaitis, 2008) and 6-aminoquinolyl-*N*-hydrosysuccinimidyl carbamate (AQC) (Pena-Gallego, Hernandez-Orte, Cacho, & Ferreira, 2009). Other newly used derivatives are 3-(4-chlorobenzoyl)-quinoline-2-carboxaldehyde (Zhang, Wang, Guo, & Zhang, 2008) and succinimidylferrocenyl propionate (Bomke, Seiwert, Dudek, Effkemann, & Karst, 2009). OPA has the disadvantage that it reacts only with primary amines. On the contrary, dansyl chloride forms derivatives, both with primary and secondary amines, and also their reaction products are more stable than those formed with OPA (Onal, 2007). However, derivation reactions are time consuming and increase the risk of low recovery, analyte loss and contamination.

Liquid chromatography (LC) coupled to mass spectrometry (MS) offers a great potential because it has the advantage that a relatively easy sample preparation is enough and no derivatisation process is required. LC with tandem mass spectrometry (MS/MS) can be a useful approach for an unequivocal confirmation of the studied analytes, and it is considered an important surveillance tool in monitoring biogenic amines in complex matrix. Only a few published methods propose a direct analysis of biogenic amines avoiding derivatisation step. Histamine, tyramine and phenylethylamine have been analysed in cheese by liquid chromatography with tandem mass spectrometry (LC–MS/MS) previous matrix solid phase dispersion (Calbiani et al., 2005). In another study, MS/MS method was compared with UV for the analysis of six amines in a typical Piedmont cheese (Gosetti et al., 2007). Gianotti et al. (2008) proposed a hydrophilic interaction MS/MS method using a hybrid triple quadrupole/linear ion trap to determine seven biogenic amines in cheese. Eight amines in wine were analysed using a LC coupled to an ion trap mass spectrometer without pre-treatment of the sample (Millán, Sampedro, Unceta, Goicolea, & Barrio, 2007). According to our knowledge, no published articles are reported in the literature about the direct analysis of these eight underivatised biogenic amines in fish.

The aim of this work was to set up a new analytical method for analysing eight underivatised biogenic amines in fish tissues using a solid phase extraction followed by LC–MS/MS analysis and to compare this method with that based on dansyl-derivatives analysed with fluorescence detection (FD). The proposed method was validated and its application to the analysis of biogenic amines in commercial fish samples was demonstrated.

2. Experimental

2.1. Materials and standards

Biogenic amines, i.e. spermine tetrahydrochloride ($C_{10}H_{26}N_4 \cdot 4HCl$, >98%, CAS No. 306-67-2), spermidine trihydrochloride ($C_7H_{17}N_3$ 3HCl, >98%, CAS No. 334-50-9), cadaverine dihydrochloride ($C_5H_{14}N_2$ 2HCl, >98%, CAS No. 1476-39-7), putrescine dihydrochloride ($C_4H_{12}N_2$ 2HCl, >98%, CAS No. 333-93-7), histamine dihydrochloride ($C_5H_9N_3 \cdot 2HCl$, >99%, CAS No. 56-92-8), tyramine hydrochloride ($C_8H_{11}NO \cdot HCl$, >98%, CAS No. 60-19-5), 2-phenylethylamine hydrochloride ($C_8H_{11}N \cdot HCl$, >98%, CAS No. 156-28-5), tryptamine hydrochloride ($C_{10}H_{12}N_2 \cdot HCl$, >99%, CAS No. 343-94-2), internal standard i.e. 1,7-diaminoheptane ($C_7H_{18}N_2$, >98%, CAS No. 646-19-5) and dansyl chloride ($C_{12}H_{12}ClNO_2S$, >99%, CAS No. 605-65-2) were supplied by Sigma–Aldrich (Madrid, Spain).

Individual stock solutions were prepared by dissolving 10 mg of each compound in 10 mL of HCl 0.1 M (Merck Darmstadt, Germany) and stored in glass-stopper bottles at 4 °C. Standard working solutions, at various concentrations, were daily prepared by appropriate dilution of different aliquots of the stock solutions with water. HPLC-grade acetonitrile, methanol and acetone were supplied by Merck (Darmstadt, Germany). HPLC-grade ammonium formiate 97% was supplied by Sigma Aldrich (Madrid). HPLC-grade formic acid 98–100%, sodium hydrogen carbonate (>99%), and sodium hydroxide were from Scharlau (Barcelona, Spain). Deionised water (<18 MΩ cm resistivity) was obtained from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). Ammonium hydroxide 28% was supplied by Prolabo (Barcelona, Spain). All the solvents and solutions were filtered through a 13 mm/0.45 μm (Whatman No. 1 paper) supplied by Análisis Vínicos (Tomelloso, Spain). Trichloroacetic acid (TCA) for analysis was from Panreac Química SA (Barcelona, Spain) and acetic acid glacial 100% from BDH Laboratory Supplies Poole (England). Conical tubes (50 mL, PP) for sample storage were purchased from DELTALAB (Barcelona, Spain). Cartridges Select HLB 6 mL/200 mg were acquired from Supelco (Madrid, Spain), Strata™ X 33 μm Polymeric Sorbent, Strata C18 and Strata C18-U 200 mg/6 mL were acquired from Phenomenex (Madrid, Spain). Chromabond® SPE Vacuum Manifold with 12 ports from MACHEREY–NAGEL GmbH & Co. KG (Düren, Germany) was used for solid phase extraction (SPE) process.

2.2. Sample collection

Fresh and ungutted small hakes were purchased from a central fish market in Valencia. Samples were transported to the laboratory within one hour, packed in separate insulated polystyrene boxes with ice. Gutting and filleting was carried out in the laboratory manually. The average weight of the fish samples was 294.7 g with an average length of 37 cm. Fish muscles were cut with sterile knives to avoid post-harvesting contamination.

2.3. Sample extraction

For the analysis, a representative portion of fish tissues (50 g) was chopped into small pieces and finely ground with a blender to homogenise it before extraction. A total of 5 g of fresh/frozen fish sample was homogenised for one minute with 15 mL of TCA 5% using an Ultra-Turrax S 18N-10 G (IKA-Werke Gmbh & Co., Germany). The obtained homogenate was decanted into centrifuge tubes and spun at 2500*g* for 10 min at 4 °C. After removing the extracts, 10 mL of TCA 5% was added to the remaining solid and the process was repeated. Then, both extracts were combined and collected in a plastic vial.

For liquid chromatography and fluorescence detection (LC–FD), the derivatisation process with dansyl chloride was based on the method proposed by Rea, Cecchini, Stocchi, Loschi, & Ricciutelli (2005). Briefly, 1 mL of supernatant TCA extract was derivatised by adding 300 μL of $NaHCO_3$ (saturated solution), 200 μL of NaOH 2 N and 2 mL of dansyl chloride solution (10 mg/mL in acetone, daily prepared). The mixture was left under magnetic stirring in darkness for 45 min at 45 °C. Then, it was allowed to cool at room temperature, and 100 μL of NH_4OH 28% were added for neutralising the excess of dansyl chloride. Finally, 400 μL of acetone were added to the solution before LC–FD analysis.

For LC–MS/MS, the STRATA X cartridge were conditioned with 4 mL of methanol followed by 4 mL of Milli-Q water using a vacuum system. Then, 2 mL of the sample with a pH adjusted to 11 with NH_4OH 28% were passed through the cartridges at a flow rate of approximately 1 mL/min. After sample loading was complete, sample flasks and cartridges were rinsed with 2 mL of a mixture of MeOH/H_2O (5:95, v/v). Cartridges were, then, dried under vacuum for 5 min, to remove excess of water. Analytes were eluted from the STRATA X sorbents with 2 + 2 mL of a mixture methanol/

acetic acid (99:1, v/v). The eluting solutions were dried with nitrogen gas, the residue dissolved in 2 mL of HCl 0.1 M, filtered and injected in LC–MS/MS.

2.4. LC–FD analysis

LC–FD studies were performed using a LC-6A Liquid Chromatography system Shimadzu (Kyoto, Japan), made from a binary solvent pump, an injector Rheodyne Model 7125 (20 μL loop) and a Fluorescence Detector RF-10AXL Shimadzu (Columbia, MA, USA). The fluorescence excitation and emission wavelengths were 320 and 523 nm, respectively, using a sensitivity of 3.

The separation was achieved on an analytical column Gemini C_{18} (250 × 3.0 mm I.D., 5 μm) preceded by a Security Guard Cartridge C_{18} (4 × 3 mm I.D., 5 μm), both from Phenomenex (Torrance, CA, USA). The mobile phase for LC–FD analysis was water (A) and methanol/acetonitrile (70:30, v/v) (B), at a flow rate of 0.5 mL min^{-1}. The gradient program was: 0 min 70% B, 0–10 min 75% B, 10–20 min 90% B, 20–40 min 100% B. Finally, phase B was decreased to 70% from 40 to 45 min and held at 70% until the end of the run at 50 min. The injection volume was 20 μL.

2.5. LC–MS/MS analysis

The LC separation was performed using an Alliance 2695 HPLC separation module (Waters). In positive ion (PI) mode, a column Synergi Hydro (250 × 4.0 mm I.D., 5 μm) from Phenomenex (Torrance, CA, USA) was used. The mobile phase for LC–MS/MS analysis was ammonium formate 15 mM and formic acid in water (pH 3.3) (A) and methanol (B), at a flow rate of 0.5 mL min^{-1}.The gradient program was: 0 min 30% B, 0–15 min 90% B, 15–20 min 30% B, 20–25 min 30% B. The injection volume was 20 μL.

The tandem MS analyses were performed on a Micromass Quattro triple quadrupole mass spectrometer (Manchester, UK). Instrument control, data acquisition and evaluation were done with the Masslynx NT software (v. 3.4). The applied parameters were: radio frequency lens, 0.2 V; electrospray source block, 150 °C; low mass (LM) 1 resolution, 12.0; high mass (HM) 1 resolution, 12.0; LM 2 resolution, 12.0; HM 2 resolution, 12.0; multiplier 650 V; desolvation temperature: 350 °C; argon collision gas 2.5×10^{-3} mbar; cone nitrogen gas flow, 50 L h^{-1}; desolvation gas: 500 L h^{-1}. In PI mode, the extractor voltage was 3.0 V and capillary voltage 3.5 kV. Optimisation of electrospray (ESI) interface parameters were performance by directly infusing a standard solution into the LC–MS/MS system at a flow rate of 20 μL min^{-1}. Full-scan analyses and MS/MS product ion scan mass spectra of the selected ion were performed over a scan range of *m/z* 50–225 using a step size of 0.1 Da and a rate of 0.2 scan/s. The optimal quantification and confirmation transitions, their respective cone voltages, collision energies and time window are listed in Table 1. The dwell time and the interchannel delay were set at 0.2 and 0.01 s, respectively.

3. Results and discussion

3.1. Liquid chromatography–mass spectrometry

In order to develop the LC/MS/MS method, a mass characterisation study was firstly performed by direct infusion (flow rate 20.0 μL min^{-1}) of a solution of each amine. Mass spectrometry scans in PI mode were performed using ESI source ionisation. As shown in Table 1, for all biogenic amines the base peak of the mass spectrum and isolated precursor ion was a *m/z* signal that corresponds to the protonated molecule $[M+H]^+$. In MS/MS analysis, for the six of eight amines, i.e. cadaverine, histamine, phenylethylamine, putrescine, tyramine, tryptamine, the product ion was generated by the loss of a neutral fragment of ammonia, $[M+H\text{-}NH_3]^+$.

According to literature (Gosetti et al., 2007), for spermidine, the product ion was generated by the loss of 2 molecules of ammonia, giving *m/z* 112 $[M+H\text{-}2NH_3]^+$, while for spermine the fragmentation of the precursor ion *m/z* 203, produces an ion at *m/z* 112 $[M+H\text{-}(CH_2)_3N_2H_4\text{-}NH_3]^+$. The selected transitions (precursor ion/product ion) by setting two time windows, i.e. 0–8.5 min and 8.5–25 min, generated a peak for each biogenic amine. For optimising the ionisation of each molecule in the source and the relative signal, various mobile phases were tested. In particular, the solvents tested in combination with methanol were CH_3COOH solution 0.01% (pH 3.2) and HCOOH solution 0.01% (pH 3.1), obtaining in both cases too large and unintegrable peaks for all amines. By using a solution of $HCOONH_4$ 15 mM (pH 6.3), symmetric peaks for all biogenic amines were obtained, except for spermine and spermidine that have a tendency to give double peaks. Finally, the selected buffer solution of $HCOONH_4$/HCOOH 15 mM (pH 3.3) reached both the correct concentration of salt and the right value of pH necessary to give a right ionisation of these basic molecules.

3.2. Study of extraction conditions

Analysis of biogenic amines in fish tissues offers several drawbacks, as they have different chemical structures (aromatic, heterocyclic and aliphatic structures). In addition, the complexity of fish matrix can lead to some problems during the extraction.

In order to obtain a more rapid and sensitive method for analysing biogenic amines in fish, a SPE–LC–MS/MS method without derivatisation step was developed, and compared with a classical procedure based on dansylated derivatives analysis and fluorescence detection. Despite the recoveries obtained with LC–FD were good (>90%), the procedure was too time consuming and the obtained chromatograms contained various interferences.

For injecting into LC–MS/MS system a final clean eluate containing biogenic amines, various SPE cartridges were tested (C_{18}, C_{18}-U, HLB, Strata™ X 33 μm). In particular, SPE C_{18}, using water and methanol as washing and eluting solvent, respectively, provided low recoveries for polar compounds, especially for the

Table 1
MRM conditions used for LC/MS/MS analysis.

Amine	Precursor ion (m/z)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (V)	Dwell time (s)	Time window (min)
Spermine	203 $[M+H]^+$	112 $[M+H\text{-}(CH_2)_3N_2H_4\text{-}NH_3]^+$	10	10	0.2	0–85
Spermidine	146 $[M+H]^+$	112 $[M+H\text{-}2NH_3]^+$				
Cadaverine	103 $[M+H]^+$	86 $[M+H\text{-}NH_3]^+$				
Putrescine	89 $[M+H]^+$	72 $[M+H\text{-}NH_3]^+$				
Histamine	112 $[M+H]^+$	95 $[M+H\text{-}NH_3]^+$				
Tyramine	138 $[M+H]^+$	121 $[M+H\text{-}NH_3]^+$				
Phenylethylamine	122 $[M+H]^+$	105 $[M+H\text{-}NH_3]^+$	10	10	0.2	8.5–25
Tryptamine	161 $[M+H]^+$	144 $[M+H\text{-}NH_3]^+$				

aliphatic ones. Better results were obtained using methanol/acetic acid (98:2, v/v) as eluting solvent. SPE C_{18}-U cartridges, with a more polar sorbent as stationary phase, were used with methanol/acetic acid (98:2, v/v) as eluting solvent, leading to increased results but still not satisfactory for spermine and spermidine. The SPE HLB cartridges gave good results for phenylethylamine and tryptamine but low recoveries for putrescine, cadaverine and histamine. Finally, a STRATA X SPE cartridge was selected as the most efficient sorbent for purifying the extracts. Various solvents and combination of them were studied to choose the best eluting mixture by spiking fish samples with 5 mg kg^{-1} of each biogenic amine. Fig. 1 reports the areas of the recovered amines with STRATA X cartridges, testing methanol/acetonitrile (50:50, v/v), methanol/acetonitrile/acetic acid (49.5:49.5:1, v/v/v), methanol/acetonitrile/acetic acid (49:49:2, v/v/v), methanol/acetic acid (99/1, v/v), methanol/acetic acid (98:2, v/v). Low recoveries were obtained using methanol/acetonitrile without acid, whereas increased recoveries were obtained using 2% of acetic acid added to methanol/acetonitrile, especially for aliphatic amines as spermidine, putrescine and cadaverine. Finally, methanol with 1% and 2% of acetic acid as eluting solvents were studied; being methanol/acetic acid (99:1, v/v) the eluting mixture finally selected, because higher recoveries were obtained for some amines, especially tryptamine. In literature, the number of reports that propose SPE for the purification of biogenic amines is very scarce (Calbiani et al., 2005; Gianotti et al., 2008; Gosetti et al., 2007; Saaid et al., 2009) and most of them extract derivatised biogenic amines. Some of these articles proposed C_{18}, but these extraction procedures extract only a few of the biogenic amines studied in this article. Molins-Legua & Campins-Falcó (2005) reviewed different cleaning methods ranging from non-polar phases, such as C_{18} silica to more polar such as cyanopropylsilica (CN) but the study showed that none of these resins is ideal for the quantification of all amines in the same analysis.

Table 2 shows the recoveries obtained with the selected eluting mixture (methanol/acetic acid 99:1, v/v) calculated at two fortification levels (2.5 and 5 mg kg^{-1}). The percentages of recoveries ranged from 71% to 92% at 2.5 mg kg^{-1} with relative standard deviations (RSDs) below 18% and from 72% to 93% at 5 mg kg^{-1} with RSDs below 16%. Spermine was recovered with the lowest percentages (71–72%) probably due to its polar nature; in fact, although the stationary phase of STRATA X contains free nitrogen that forms hydrogen bond with unprotonated biogenic amines, the high hydrophilicity of spermine (log $P = -1.54$) gave us the lowest recovery, probably due to its partial dissolution in washing solvent.

3.3. Method validation

A classical method for analysing biogenic amines in fish tissues that uses a derivatisation process (dansylation) was studied for comparison with the new non-derivatisation method developed by our laboratory, based on SPE and LC–MS/MS. Dansyl chloride reacts with primary and secondary amino groups and it has been used extensively in the analysis of biogenic amines in various matrices, such as biological fluids (Mao, Chen, Qian, & Liu, 2009), food (Latorre-Moratalla et al., 2009; Mayer, Fiechter, & Fischer, 2010), and beverages (García-Villar, Hernández-Cassou, & Saurina, 2009; Pena-Gallego et al., 2009). The TCA extract from fish was derivatised and injected in reversed phase LC–FD. In addition, 1,7-diaminoheptane was used as internal standard. Due to the difficulty of obtaining blank samples, each sample was previously analysed for FD optimization studies. The amount of biogenic amine found was subtracted from spiked samples.

Table 2 gives an overview of the performance of the LC–MS/MS method considering the following parameters: precision, recoveries, limits of detection (LODs), and limits of quantification LOQs.

The same standards were analysed at intervals over a 1-week period ($n = 5$) in order to determine the inter-day precision and five times the same day for intra-day precision. The best RSD values obtained with MRM acquisition ranged from 1% to 4% for run-to-run precision and from 2% to 18% for day-to-day precision. For FD, RSD ranged from 3% to 17% for run-to-run precision and from 5% to 19% for the day-to-day precision. LODs were calculated as the concentration of the analyte that gives a signal equal to the average background (S_{blank}) plus three times the standard deviation (s_{blank}) of the blank (LODs = $S_{blank} + 3s_{blank}$) and the LOQ were calculated as (LOQs = $S_{blank} + 10s_{blank}$). MS/MS achieved lower LODs (Table 2) calculated with the matrix (from 0.02 mg kg^{-1} for spermidine and phenylethylamine to 0.2 mg kg^{-1} for spermine) when compared to fluorescence detection (from 1 mg kg^{-1} for putrescine and tyramine to 4 mg kg^{-1} for histamine). These values are quite

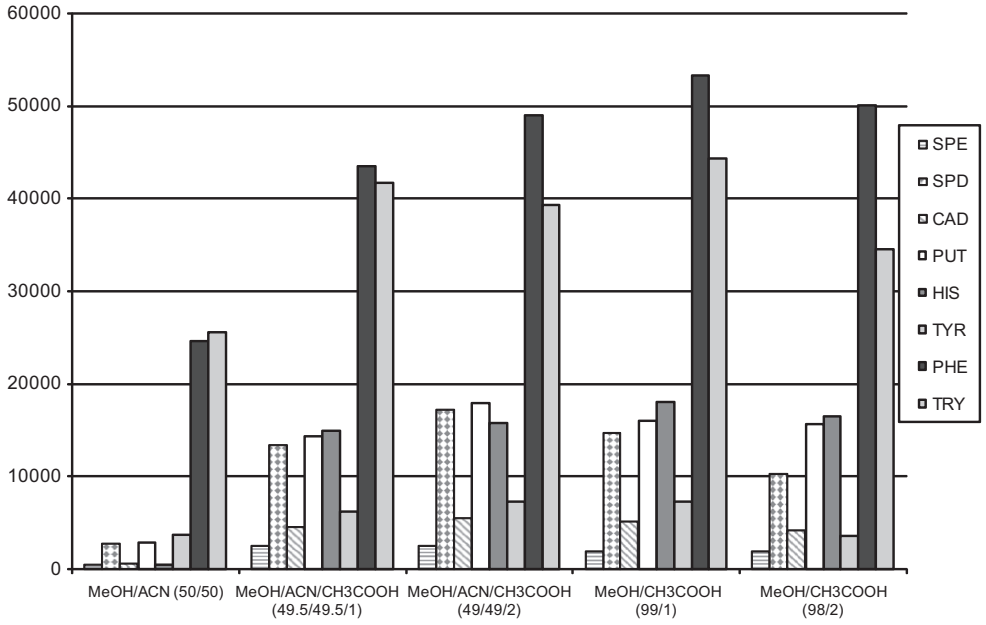

Fig. 1. Comparison of the absolute response obtained of biogenic amines using various eluting mixture with SPE STRATA X cartridges.

Table 2
Percent recovery and repeatability of LC/MS/MS method at two fortification levels (2.5 and 5 mg kg^{-1}) by using SPE STRATA X, limit of detection (LOD) and limit of quantification (LOQ) in the matrix, and precision of the method.

Amine	Recovery % ± RDSs		LOD mg kg^{-1}	LOQ mg kg^{-1}	RSD (%) intraday	RSD (%) interday
	2.5 mg kg^{-1}	5 mg kg^{-1}				
Spermine	71 ± 18	72 ± 16	0.25	0.75	4	18
Spermidine	80 ± 17	81 ± 7	0.02	0.07	3	7
Cadaverine	92 ± 4	90 ± 5	0.12	0.41	4	4
Putrescine	91 ± 1	93 ± 4	0.12	0.41	3	7
Histamine	89 ± 3	92 ± 3	0.05	0.15	1	2
Tyramine	91 ± 4	90 ± 5	0.12	0.45	4	15
Phenylethylamine	86 ± 15	88 ± 2	0.02	0.07	1	4
Tryptamine	79 ± 9	80 ± 1	0.05	0.15	4	8

Table 3
Evaluation of matrix effects: comparison of the calibration curves slopes and calculation of signal suppression/enhancement SSE (%) for the studied biogenic amines.

Amine	Slope		R^2		SSE (%)
	Solvent	Matrix-matched	Solvent	Matrix-matched	
Spermine	3085	2904	0.9991	0.9412	94
Spermidine	10361	13380	0.9995	0.9543	129
Cadaverine	6508	7634	0.9966	0.9734	117
Putrescine	9384	11805	0.9967	0.9893	126
Histamine	24042	26523	0.9973	0.9639	110
Tyramine	12687	15158	0.9955	0.9909	119
Phenylethylamine	46789	73714	0.9995	0.9718	157
Tryptamine	50873	70015	0.9984	0.9747	137

high, especially for histamine, which is the one of the most important amines present in fish due to its toxicological aspects.

The linearity of both methods for each amine assayed was studied by the linear calibration curve obtained from 6 points of different concentration. For MS/MS, all the calibration curves showed determination coefficients $R^2 > 0.9955$, whereas for FD lower R^2 were obtained (>0.9889).

The response obtained with MS/MS was linear up to 1 mg/L. For those samples that presented levels higher than 5 mg kg^{-1}, successive dilutions were done. Other authors have also observed that LC–MS/MS shows a linear response in a narrow dynamic range (Gianotti et al., 2008; Gosetti et al., 2007).

LC–MS/MS was the most precise and sensitive method. LC–FD chromatograms presented interfering peaks, and furthermore, this type of detection required the extract to be derivatised before analysis, being time consuming and bringing time dependence in what respects to the derivatising reagent stability. The derivatisation process compromises both the sensibility and the repeatability of the results with respect to those obtained by underivatised method (MS/MS detection).

One of the main problems of MS/MS was that the presence of matrix components can affect the ionisation of the analytes, reducing or enhancing their response, when compared to standards in solvents, so the influence of the matrix response must be studied. Linearity and matrix effects were studied using standard solutions (prepared in 0.1 N HCl) and matrix-matched calibrations (prepared by adding the standard to the extract previously obtained by SPE). For matrix-matched calibration, fish extracts were analysed before adding the standard aliquot. Calibration curve was obtained subtracting in each concentration the amount of amine obtained from the blank extract. Table 3 shows the calibration curves obtained using six concentration levels, between 0.05 and 1 mg/L (equivalent to 0.25–5 mg kg^{-1} in fish tissues). The slope of the standard addition plot was compared with the slope of standard calibration plot to evaluate the matrix effects. The signal suppression/enhancement (SSE) was calculated according to Eq. (1) defined by Sulyok, Berthiller, Krska, and Schuhmacher (2006).

$$\mathrm{SSE} = (\text{slope matrix} - \text{matched calibration}/\text{slope standard calibration in solvent}) \times 100 \quad (1)$$

The results showed that every biogenic amine was influenced by the matrix and the extent was quite different. As it can be noted, a matrix effect was detected (enhancement of the signal is observed for all the amines except spermine) and must be corrected to obtain reliable results. The solution adopted to compensate matrix effect was the quantification with matrix-matched standards, which is up to now the most widely used method.

"Standard addition method" is usually used when totally blank samples are not available. However, this study proposes the use of a matched-matrix calibration. Fish extracts were analysed before being spiked with a known volume of the working standard. The amount of biogenic amines obtained was subtracted to each of the matrix-matched calibration points. The main advantage was

Table 4
Results of biogenic amines from fish samples (small hakel) of Valencia market. Analysis were performed in triplicate obtaining in all cases a RSDs <5%.

Amine	Sample 1 (mg kg^{-1})	Sample 2 (mg kg^{-1})	Sample 3 (mg kg^{-1})	Sample 4 (mg kg^{-1})	Sample 5 (mg kg^{-1})	Sample 6 (mg kg^{-1})	Sample 7 (mg kg^{-1})	Sample 8 (mg kg^{-1})
Spermine	5.16	2.35	nd	1.14	5.30	1.23	4.67	3.12
Spermidine	5.56	0.25	0.90	0.26	4.96	0.98	2.31	1.34
Cadaverine	nd[a]	0.67	1.13	0.74	1.19	0.45	0.76	0.56
Putrescine	0.45	0.43	2.00	nd	5.66	1.78	0.56	nd
Histamine	nd	0.69	0.73	0.77	nd	nd	0.81	0.92
Tyramine	nd	nd	0.92	0.82	nd	0.88	0.99	nd
Phenylethylamine	nd	0.76	0.90	0.69	0.43	0.47	0.77	0.92
Tryptamine	nd	1.10	0.97	0.71	nd	0.81	nd	0.75

[a] nd = <LOD (limit of detection).

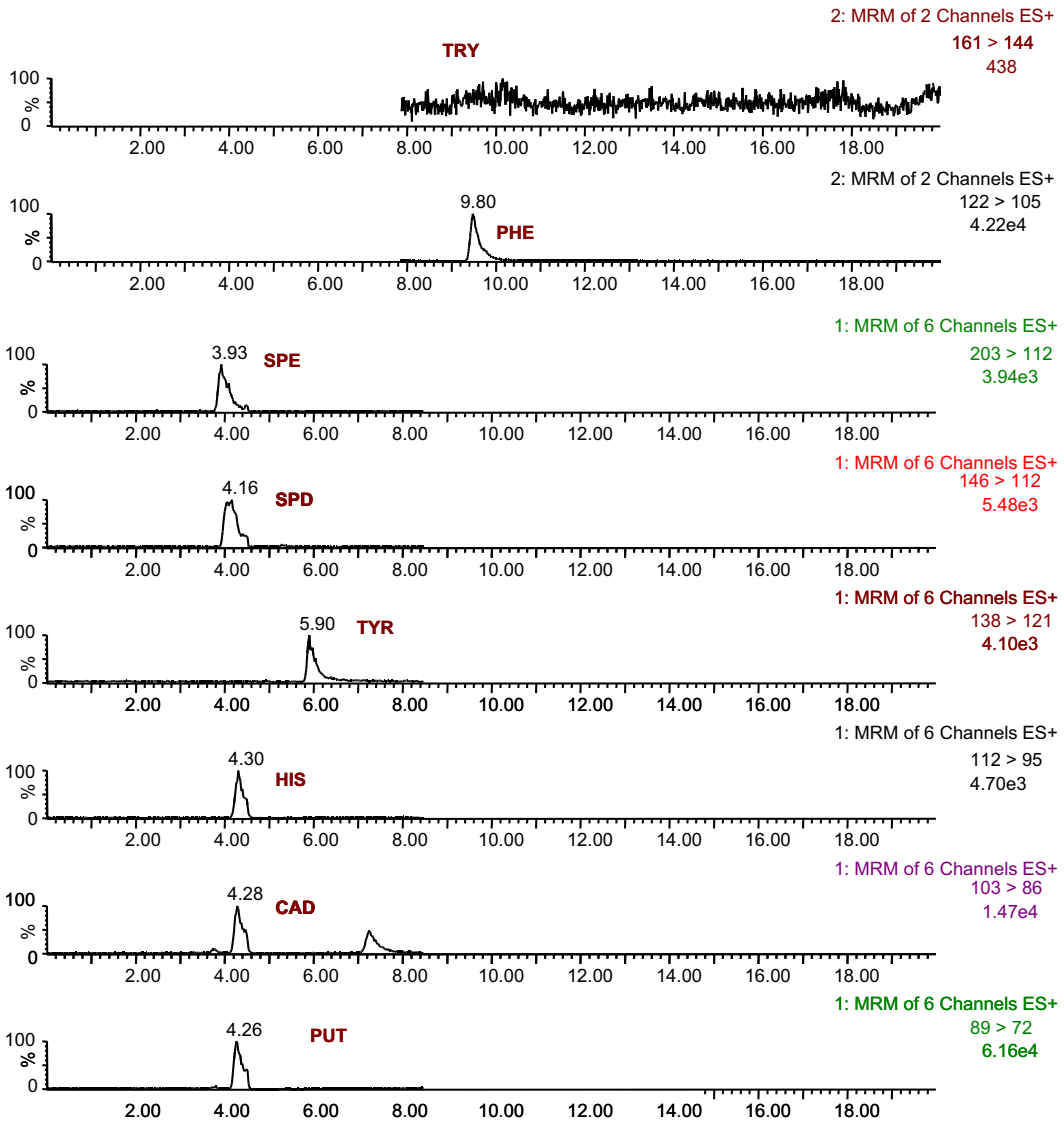

Fig. 2. LC/ESI(p)-MS/MS MRM chromatogram obtained from a sample of small hake (sample no. 7).

that, once calibration curve has been obtained, samples were analysed just once and not further additions are required.

3.4. Application to real samples

The developed technique was applied to 8 samples of small hake from different markets of Valencia to assess fish freshness. For quantification of biogenic amines in fish, correction of the obtained results with recoveries has been introduced (Table 4). Fig. 2 shows the chromatogram obtained from sample no. 7. Histamine was detected in 5 of 8 samples in a concentration range of 0.73–0.92 mg kg^{-1}, that is very far from the maximum level established by European Union (100 mg kg^{-1}) or FDA (50 mg kg^{-1}). Spermine was the amine found in 7 of 8 samples at highest levels, with an average concentration of 3.28 mg kg^{-1} and in a range of 1.14–5.16 mg kg^{-1}; spermidine was found in all 8 samples with an average concentration of 2.07 mg kg^{-1} in a range of 0.25–5.56 mg kg^{-1}. The other biogenic amines, i.e. cadaverine, putrescine, tyramine, phenylethylamine and tryptamine were detected in a concentration range of 0.43–5.66 mg kg^{-1}. Low levels of histamine were found in hake compared with the values reported by other authors for scombroid fish. We would like to underline that the analysis of biogenic amines, including histamine, to evaluate the quality of fish is considered a suitable indicator for these other fish species (hake, anchovies or salmon) too (Prester et al., 2009).

4. Conclusions

The selected method is a reliable, accurate, and sensitive alternative to the analysis of derivatised biogenic amines in fish tissues by LC and FD. A method based on a simple extraction with TCA at 5% and a clean-up step by means of SPE prior to analysis of the extract by LC–MS/MS was developed. The easiness-to-handle of these extraction methods was definitely in favour of the LC–MS/MS procedure, since the extraction was faster and involves less intermediate steps. This method also showed higher sensitivity and precision than the analysis of dansyl derivatives by FD. The presented method was applied to the analysis of fish samples from various commercials from Valencia. The detected levels of biogenic amines were considered not a risk for the consumer's health.

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