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Original Research Article

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-phenylethylamine, 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 × 4.6 mm, 5 μm particle size), UV detection set at 260 nm, and a mobile phase of 0.15 mol L^{-1} 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 μg mL^{-1}, $r^2 > 0.9990$), limits of detection (in the 158–375 ng mL^{-1} 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.

1. Introduction

Cadaverine (CA; $\log P_{o/w} = -0.44$; $pK_a = 10.5/10.93$), 2-phenylethylamine (2-PE; $\log P_{o/w} = 1.43$; $pK_a = 9.84$), histamine (HI; $\log P_{o/w} = -0.97$; $pK_a = 5.9/9.7$) and spermidine (SD; $\log P_{o/w} = -1.28$; $pK_a = 8.25/9.86/10.9$) are biogenic amines, which can be found in foods either as natural products or after fermentation, decomposition or putrefaction processes (Kimberly and Goldstein, 1981; Izquierdo-Pulido et al., 1996; Craig and Newton, 2004). 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 (Pons Sánchez-Cascado, 2004).

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 (Izquierdo-Pulido et al., 1996; Pons Sánchez-Cascado, 2004). 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 °C) 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 (Truelstrup Hansen et al., 1996; Muratore et al., 2007).

Thus the determination of these analytes is of the utmost importance to assure that fish sauce can be eaten without health risk (Yongsawatdigul et al., 2004; Rodtong et al., 2005). 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 μg mL^{-1} (Lehane and Olley, 2000).

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Determination of biogenic amines can be performed by high performance liquid chromatography with UV detection (HPLC-UV) with derivatization using dabsyl chloride (Ramos et al., 2009), dansyl chloride (Soufleros et al., 2007), benzoyl chloride (Paleologos et al., 2003) or 3,5-dinitrobenzoyl chloride (Kirschbaum et al., 2000). Other authors propose methods based on HPLC-FLD (fluorescence detection) after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Busto et al., 1996) or *o*-phthalaldehyde (Busto et al., 1997), or HPLC-ED (electrochemical detection) (Bose et al., 2004). HPLC–MS (mass spectrometry detection) (Forgó and Kiss, 2010) has become a method of choice, but such instrumentation is usually not suitable for routine analysis due to financial reasons (purchasing cost and maintenance) (Peris-Vicente et al., 2005, 2007).

The reagent 3,5-dinitrobenzoyl chloride (DNBZ-Cl) has been widely used as a chromophore to determine amines in food samples (Chin-Chen et al., 2011). 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 (Kirschbaum et al., 2000). 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 (Kirschbaum et al., 2000; Paleologos et al., 2003; Soylak et al., 2011a,b). 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 (Esteve-Romero et al., 2010; Ochoa-Aranda et al., 2011) including food samples (Rambla-Alegre et al., 2010a,b; Beltrán-Martinavarro et al., 2011).

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 guidelines (*FDA Guidance for Industry, 2001*). 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 instrumentation

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 × 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).

2.2. Chemicals and reagents

The biogenic amines CA, 2-PE, HI, SD, and 3,5-dinitrobenzoyl chloride (98% pure) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The surfactant sodium dodecyl sulfate (SDS, 99% pure) was from Merck (Darmstadt, Germany); the organic solvents acetonitrile, ethanol and propanol were from Scharlab, the buffer sodium dihydrogen phosphate and HCl and NaOH were from Panreac (Barcelona). All solutions were prepared in Simplicity ultrapure water (Millipore, S.A.S. Molsheim, France). Biogenic amine solutions were filtered through 0.45 μm, 13 mm nylon membranes (Millex-HN, Millipore, Bedford, MA, USA). The corresponding biogenic amines hydrochlorides were solved in 0.1 mol L^{-1} HCl to provide a final concentration of 100 μg mL^{-1}.

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

As a derivatizing reagent 3,5-dinitrobenzoyl chloride (5 mmol L^{-1}) was solved in acetonitrile. Aliquots (400 μL) of biogenic amine standards, 1 mol L^{-1} NaOH (1200 μL), 2-propanol (700 μL) and 3,5-dinitrobenzoyl chloride (2100 μL) were mixed in a reaction tube. After 3 min of shaking at 25 °C, 1000 μL of a 2 mol L^{-1} HCl solution were added to stop the reaction. Finally, after 1 min of shaking, derivatized biogenic amines were filtered and injected into the chromatographic system. Under these conditions, the formed derivatives were $(DNBZ)_2CA$, (DNBZ)(2-PE), $(DNBZ)_2HI$ and $(DNBZ)_3SD$ (Kirschbaum et al., 2000). The fish sauce medium does not affect the derivatization reaction, because the conditions were strongly changed by the addition of organic alcohol and sodium hydroxide. Some matrix compounds are precipitated in ethanol/NaOH media, and others are solubilized in the SDS-medium (Kirschbaum et al., 2000).

2.4. Chromatographic conditions

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

2.5. Sample preparation

Anchovy sauce samples (*Engraulidae* spp.) were obtained from a local market. A part of the anchovies was mixed with common salt in a relation of 75/25 (w/w) (a well-known treatment to avoid food spoilage) and another portion was untreated. In both cases, samples were stored in a fridge at 5 °C. For the analyses of the fish sauces, 1 g of each was mixed with 0.5 mL of ethanol and topped up to 10 mL with 0.1 mol L^{-1} SDS solution. The samples were stored in a glass vessel without vacuum package. In the case of spiking, the appropriate volume of biogenic amines standard solution (100 μg mL^{-1} of each analyte solved in 0.1 mol L^{-1} HCl) were spilt on 1 g of sample and vigorously shaken to favor homogenization and stored for one day in the fridge at 5 °C to favor the contact between analytes and the sample, and also solvent evaporation (Peris Vicente et al., 2004; Cano-Sancho et al.,