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Combination of SPE and fluorescent detection of AQC-derivatives for the determination at sub-mg/L levels of biogenic amines in dairy products

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ABSTRACT

Biogenic amines (BAs) are compounds generated by decarboxylation of their amino acid precursors. Their intake, even at low concentrations, can lead to several types of health problems in sensitive individuals. As they can be easily formed in fermented dairy products, their quantitative determination is very relevant. In the present paper, a method for the quantitative determination of four biogenic amines in different dairy products has been developed, validated and applied to 37 samples of milk, 23 of yogurt, and 14 of kefir. Amines were selectively extracted using solid phase extraction, subsequently derivatizatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and further determined by High Performance Liquid Chromatography with fluorescence detection. The method's sensitivity was highly satisfactory, with limits of detection lower than 0.2 mg/L. Optimal linearity and repeatability were also achieved. BAs were not detected in most of the milk samples, but they were found frequently at high levels in yogurt and kefir samples, reaching values of up to 79 mg/kg total BAs in kefir samples. Levels measured should not be a cause for concern for the population at large, but should be known by BAs-sensitive individuals.

1. Introduction

Biogenic amines (BAs) are low-molecular-weight nitrogenous compounds formed by enzymatic decarboxylation of their precursor amino acids (Linares et al., 2011; Zhang et al., 2019) or by amination and transamination of aldehydes and ketones (McCabe et al., 2003; Pluta-Kubica et al., 2020). The presence of BAs in food and beverages is brought about by microbial enzymes inherent to the raw material, or by enzymes from spoilage microorganisms (Özogul & Özogul, 2020).

The presence of BA in food constitutes a public health concern due to their physiological and toxicological effects (Ruiz-Capillas & Herrero, 2019). Although these compounds are involved in beneficial metabolic functions in humans including the immune system, the regulation of body temperature, and the growth and renewal of organs (Papageorgiou et al., 2018), the presence of high amounts of certain BAs can lead to cases of food poisoning (Schirone et al., 2016). BAs such as histamine (HIM), tyramine (TYM), putrescine (PUT), cadaverine (CAD), spermidine (SPD), and spermine (SPM) are involved in toxicological processes, since they can cause headache, accelerated heart rate, urticaria, nausea, allergies, and blood pressure alterations (Alvarez & Moreno-Arribas, 2014). TYM, PUT, and CAD produce synergistic cytotoxic effects together with HIM due to the inhibition of the detoxifying enzymes,

Abbreviations: AQC, 6-aminoquinolyl-*N*-hydroxyuccinimidyl carbamate; BAs, biogenic amines; BNZ-Cl, benzoyl chloride; CAD, cadaverine; DBS-Cl, dabsyl chloride; DNS-Cl, dansyl chloride; EDTA, Ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HCl, hydrochloric acid; HIM, histamine; IS, internal standard; LC, liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; MeOH, methanol; ND, non-detected; NSLAB, non-starter lactic acid bacteria; OPA, o-phthalaldehyde; PUT, putrescine; PVDF, polyvinylidene fluoride; RP-HPLC, Reverse Phase-High Performance Liquid Chromatography; RSD, relative standard deviation; SLAB, starter lactic acid bacteria; SPD, spermidine; SPE, solid phase extraction; SPM, spermine; TBA, total biogenic amines; THF, Tetrahydrofuran; TYM, tyramine; UHT, ultra-heat treated milk.

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which can increase the amines' cytotoxicity at their usual concentrations in food (del Rio et al., 2017; Palomino-Vasco et al., 2019). Sensitive individuals may also be toxicologically affected by ingestion of low concentrations of BAs (Bodmer et al., 1999). The individual toxicological threshold can vary from a few mg/kg in a sensitive person to approximately-one hundred mg/kg in a healthy person (Gardini et al., 2016).

HIM, TYM, PUT and CAD are the most common BAs present in dairy foods (Linares et al., 2012). Their occurrence is due to non-starter lactic acid bacteria (NSLAB) naturally occurring in milk, to bacteria added to the milk as a starter (SLAB), or to undesired bacteria caused by contamination from poor processing hygiene (Ladero et al., 2017; Moniente, García-Gonzalo, Ontañón, Pagán, & Botello-Morte, 2021).

Fermented dairy products contain variable amounts of BAs. Ripened cheeses are the products most usually associated with high concentrations of BAs as a result of microbial activity (Schirone et al., 2016), reaching quantities of 1,844.5 mg/kg of CAD (Andiç et al., 2010; Papageorgiou et al., 2018) and 2,500 mg/kg of HIM (Bodmer et al., 1999; Moniente, García-Gonzalo, Ontañón, Pagán, & Botello-Morte, 2021). Other dairy foods such as milk, yogurt, kefir, and unripened cheeses have lower concentrations of BAs, ranging from few milligrams to tens of milligrams per kg (Linares et al., 2011; Spano et al., 2010). In spite of these lower amounts, analytical controls to ensure safe levels and to improve manufacturing processes and practices are essential, particularly because of the expected notable increase of consumption rates of dairy products in the near future (Papageorgiou et al., 2018).

Due to the great variability of BAs concentration in dairy foods, as well as to the diversity of the toxicological threshold in healthy people as compared to those who are sensitive to BAs, it is important to have sensitive-enough analytical methods to supply consumers with safe dairy products – eventually certified as BAs-free.

Dairy products are considered as complex matrices with high percentages of proteins and fats that make it difficult to determine some analytes, for example BAs. Solid-liquid (S-L) or liquid–liquid (L-L) extraction methods depends on the dairy products texture, have been widely used for the determination of BAs in dairy products. However, these techniques have several disadvantages, such as slowness, high amounts of harmful organic solvents, low repeatability, a large sample volume … Solid phase extraction (SPE) is a sample preparation technique which is free of these disadvantages. Moreover, different cleaning steps can be applied obtaining very clean extracts but, surprisingly it has been only occasionally used for the analysis of BAs in dairy products.

Several instrumental techniques have been used to quantify BAs in dairy products (thin layer chromatography, capillary electrophoresis, enzyme-linked immunosorbent assay (ELISA), biosensor), but liquid chromatography (LC) is undoubtedly the most widely used. Moreover, derivatization reactions tend to be applied prior to LC in order to improve separation and detection of BAs (Moniente et al., 2022).

Different chemical derivatizing reagents have been used for BAs determination in dairy products: dansyl chloride (DNS-Cl), o-phthalaldehyde (OPA), ninhydrin, benzoyl chloride (BNZ-Cl), fluorescamine, dabsyl chloride (DBS-Cl), fluorescein isothiocyanate, phenyl isothiocyanate, and fluorenyl 9-methylchloroformate (Korös et al., 2008; Moniente et al., 2022; Önal, 2007). A reagent seldom used for dairy products as an alternative to the most common chemicals is 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC). It has nevertheless been used in pre-column derivatization for the analysis of primary and secondary amino acids and BAs (Korös et al., 2008). With AQC, highly stable derivatives are obtained, which produce intense fluorescent signals (Moniente et al., 2022).

The main objective of the present study was to develop and validate a sensitive (limit of detection lower than 1 mg/L) and reliable reverse phase-high performance liquid chromatography (RP-HPLC) method using a combination of SPE and AQC for the simultaneous determination of HIM, TYM, PUT, and CAD in dairy products. The developed method was validated and applied in the present study to commercial and non-commercial milk, yogurt, and kefir samples from Spanish sources in order to assess the occurrence and relevance of those analytes in these kinds of products.

2. Experiments

2.1. Chemicals, reagents, and standard solutions

Histamine (dihydrochloride) (≥99 %), tyramine (hydrochloride) (≥98 %), putrescine (dihydrochloride) (≥98 %), cadaverine (dihydrochloride) (≥98 %), the internal standard (IS) 1,7-diaminoheptane (≥98 %), ethylenediaminetetraacetic acid (EDTA) (≥98 %) and tetrahydrofuran (THF) (≥99.9 %), were used and purchased from Sigma Chemicals (St. Louis, MO, USA).

Standard solutions of the four BAs (1,000 mg/L) were prepared by dissolving the pure compounds in Milli-Q water. For the IS, a solution of 3,000 mg/L of 1,7-diaminoheptane was prepared with Milli-Q water. These solutions were stored at 4 °C until they were used. The AccQ Fluor reagent kit, consisting of AQC reagent, acetonitrile for dissolution of the reagent powder, and 0.2 mM sodium borate buffer (pH 8.8) for derivatization was supplied by Waters (Milford, MA, USA).

The reagents and HPLC solvents (methanol and hexane) used in this study were of chromatographic grade and were obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained from Milli-Q System (Millipore Corp., Milford, MA, USA). Acetic acid (CH_3COOH), phosphoric acid (H_3PO_4), calcium chloride ($CaCl_2$), sodium acetate ($C_2H_3NaO_2$), hydrochloric acid (HCl) and sodium hydroxide (NaOH) were obtained from Panreac (Barcelona, Spain).

2.2. Proposed method

A total of 5 mL of the milk sample or 10 mL of a 1:1 yogurt:water dilution are mixed with 5 mL of 2 % acetic acid in a 15 mL plastic centrifuge tube and vortexed for 30 s. The tube is then centrifuged at 4000 *g* at 4 °C for 15 min to separate lipids and proteins from the aqueous phase. 2.4 mL of the clear supernatant are then taken and percolated through 30 mg of Oasis MCX SPE cartridges from Waters, previously conditioned by passing 2 mL of MeOH followed by 2 mL of Milli-Q water. The cartridges are then dried under vacuum, washed up with 2 mL of hexane to remove residual fats, dried again and washed first with 2 mL of 10 mM H_3PO_4:MeOH (90:10) solution followed by 1 mL of Milli-Q water, and then with 2 mL of a 10 mM $CaCl_2$:MeOH (70:30) solution also followed by 1 mL of Milli-Q water. The analytes are subsequently eluted with 1.2 mL of 100 mM NaOH: MeOH (65:35) in a vial already containing 100 μL of 1.2 M HCl. Next, 40 μL of IS was added to 960 μL of the extract and then they are filtered through 0.20 μm nylon filters (Sartorius, Goettingen, Germany).

For derivatization, 20 μL of standard or sample are first buffered with 50 μL of a solution containing 0.2 M of sodium borate at pH 8.8 and 5 mM of disodium EDTA. Subsequently, 30 μL of the AQC solution are added to perform the derivatization reaction at 55 °C for 10 min.

2.3. Chromatographic conditions

The separation of the AQC-derivatives of the amines was carried out in a reversed-phase Luna C_{18} chromatographic column (25 cm × 4.6 mm, 5 μm) (Phenomenex, Torrance, CA, USA) preceded by a 20 mm × 4.6 mm precolumn and kept at 65 °C. The HPLC system was a 1220 Infinity LC device from Agilent Technologies (Santa Clara, CA, USA) coupled to a Prostar 363 fluorescence detector from Varian (Sunnyvale, CA, USA) set at excitation wavelength of 250 nm and emission wavelength of 395 nm. Data acquisition and processing were carried out with Chrom-Card software. The eluents were 50 mM sodium acetate in 1 % THF in Milli-Q adjusted to pH 6.6 by the addition of acetic acid (A) and MeOH (B) according to the methodology described by Mayer et al. (2010), with some modifications. Injection volume was 5 μL. The elution

program consisted of a gradient system with a flow rate of 1 mL/min using the following optimized gradient: from 15 to 80 % of eluent B linearly during 25 min, increasing to 100 % B within 1 min, and holding at 100 % B for 5 min. Subsequently, decreasing to 15 % B over 1 min, and holding at 15 % B for 5 min.

2.4. Method validation

Method development and validation were carried out using whole cow's milk and Milli-Q water. Limit of detection (LOD), limit of quantitation (LOQ), linearity, repeatability, and stability were assessed. LOD and LOQ were estimated as the concentration that gave a signal-to-noise ratio of 3 and 10, respectively. They were calculated in water and milk spiked with low amounts of BA.

Calibration curves were prepared by adding increasing amounts (five points) of the stock solution containing the four analytes to milk and ultrapure water, in a range from 1 to 20 mg/L. t-tests were performed to analyze the effects of the matrix by comparing the slopes of the calibration curves for the different samples.

The method's repeatability was studied by analyzing unspiked and spiked milk samples in triplicate.

2.5. Analysis of dairy samples

Seventy-four Spanish dairy samples (37 milk samples, 23 yogurt samples, and 14 kefir samples) were analyzed. Some of them were available in Spanish retail stores, whereas others were supplied by dairy product manufacturers. Information regarding milk, yogurt, and kefir samples is listed in Table 1. Samples were kept at −18 °C until their analysis.

The quantification of BA was carried out by using a sample-dependent response factor estimated by the analysis of the own sample spiked with known amounts (5 mg/L) of HIM, TYM, PUT, and CAD. The increase in the area of the measured peak was used to calculate the response factor.

3. Results and discussion

In the present study, an analytical method for the quantification of BAs in dairy products has been optimized and validated. The SPE and the derivatization procedures are based on the method previously developed by Peña-Gallego et al. (2009) for the analysis of BAs in wine, with some modifications. As dairy products have physicochemical properties quite different from those of wine, the different steps of the method were reoptimized and further validated. The method was subsequently applied to the analysis of milk, yogurt, and kefir samples from Spain, in order to evaluate the presence of BAs in those kinds of products.

3.1. Optimization of the analytical method

3.1.1. Breakthrough volumes

The breakthrough volume is the maximum volume of sample which can be introduced into the SPE sorbent without appreciable losses of analytes (Bielicka-daszkiewicz & Voelkel, 2009). Breakthrough volumes were determined by percolating through the SPE cartridge a 6-mL milk sample spiked with 20 mg/L of each BAs. The percolate was collected in 10 consecutive 600-μL fractions, which were filtered, derivatized and analyzed. Results can be seen in Fig. 1. The most retained BAs was TYM, which only could be detected in the 10th fraction, corresponding to the last 600 μL of the 6 mL percolated. HYM was also strongly retained and was only detected in the 9th and 10th fractions. By contrast PUT was the BAs most weakly retained in the SPE device, and was already detected in the 4th fraction, so that its breakthrough volume is around 2,400 μL. Consequently, 2.4 mL was chosen as the maximum volume of sample to be loaded in the cartridge.

3.1.2. Washing steps

Although a large part of fat is removed in the initial centrifugation of the acidified sample, the elimination was not enough to avoid an evident turbidity of the final extracts. For this reason, a washing step with hexane was included in the procedure to completely eliminate fats and avoid possible interferences. For this experiment, 2.4 mL aliquots of whole milk samples spiked with 10 mg/L of BAs were loaded in the SPE cartridges. Increasing volumes of hexane (0, 2, 4, 6, and 8 mL) were then applied, and analytes were further eluted, derivatized and analyzed. These experiments were carried out in duplicate. Results revealed that a washing volume of 2 mL was sufficient to obtain clean extracts without turbidity. As shown in Fig. 2, the washing with hexane did not caused appreciable losses of analytes up to 6 mL, however, the imprecision increased significantly at volumes above 4 mL. Therefore, 2 mL of hexane was retained as the optimal volume.

The three hydro-methanolic washing solutions proposed by Peña-Gallego et al. (2009) were also studied. The first is an acid solution (10 mM H_3PO4: MeOH [70:30]), the second is a neutral solution (10 mM $CaCl_2$: MeOH [70:30]) and the third is an alkaline solution (10 mM NaOH: MeOH [70:30]). For this, five aliquots of a 10 mg/L BAs spiked whole milk sample were loaded in the SPE cartridge and washed with hexane. In one of the aliquots no aqueous washing solutions (acid, neutral, and basic) were applied, in the second the acid washing was omitted, in the third, it was the neutral washing omitted, in the fourth the acid, and in the fifth, the three washings were included. Results are shown in Fig. 3. As can be seen, only in the experiments in which the alkaline washing was omitted (blue bars in the figure), the levels of BAs recovered were not significantly smaller than those measured in the reference without any washing. Therefore, the basic washing solution was thus not applied to the final procedure.

3.1.3. Filtration

Four different filters (0.22 μm Polyvinylidene Fluoride (PVDF), 0.2 μm nylon and 10 k and 3 k ultrafiltration filters) were considered for the filtration of the eluted sample. The resulting chromatograms (available in Supplementary Material) revealed that the 10 k and 3 k ultrafiltration filters introduced a range of impurities interfering with the chromatographic peaks of the BAs, and that PVDF filters retained significant amounts of all the analytes. The best results were obtained with the 0.2 μm nylon filters, which were selected for the procedure.

3.1.4. Derivatization reaction

In order to obtain the best yield of the derivatization reaction, the influence of the pH of the extract previous to the reaction was optimized. For that, five standard solutions containing 5 mg/L of each BAs, and pH in the range 1 to 12 (1, 5, 7, 10 and 12) were prepared, filtered and derivatized with the addition of 20 μL of the AQC reagent. Results are shown in Fig. 4 and reveal that HIM and TYM were best derivatized at pH 10, while PUT and CAD, showed maxima signals at pH 12.

Derivatization time was also studied by varying reaction times between 10 and 60 min, as can be seen in Fig. 5. The result obtained for HIM showed a higher signal at 10 min compared to the other times we assessed (20, 30 and 60 min). Similar results occurred for the other three biogenic BAs (TYM, PUT and CAD): a greater signal appeared after 10 min of derivatization compared to other time intervals we studied (although these differences were not significant). Derivatization of BAs with AQC at 55 °C for 10 min was the optimal condition, confirming the manufacturer (Waters) recommendation.

Some reproducibility problems were found with the signal of the derivatized samples; we therefore surmised that the temperature of the extracts after the derivatization process might be exerting an influence on their stability. To test this hypothesis, we stored extracts after derivatization at room temperature, refrigeration temperature (4 °C), and freezing temperature (−18 °C) for 5 min to decrease their temperature, after which they were analyzed. Based on absolute areas, the signals shown for all four BAs in the assay at refrigeration and freezing

Table 1
Characterization of milk, yogurt, and kefir samples regarding type of dairy product, milk source, fat content, heat treatment, biogenic amine (histamine, tyramine, putrescine and cadaverine), contents quantified by HPLC, and total biogenic amines (TBA).

Sample Code	Type of Dairy Product	Milk Source	Fat content	Heat Treatment	Histamine[a]	Tyramine[a]	Putrescine[a]	Cadaverine[a]	TBA[a]
M1	Milk	Cow	Whole	UHT	<LOD	<LOD	<LOD	<LOD	–
M2	Milk	Cow	Whole	UHT	<LOD	<LOD	<LOD	<LOD	–
M3	Milk	Cow	Whole	UHT	<LOD	<LOD	<LOD	<LOD	–
M4	Milk	Cow	Whole	UHT	<LOD	<LOD	<LOD	<LOD	–
M5	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	–
M6	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	–
M7	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	–
M8	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	–
M9	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	–
M10	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	–
M11	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	–
M12	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	–
M13	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	–
M14	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	–
M15	Milk	Cow	Whole	Pasteurization	<LOD	<LOD	<LOD	<LOD	–
M16	Milk	Cow	Semi-skimmed	UHT	<LOQ	<LOD	<LOQ	<LOD	–
M17	Milk	Cow	Semi-skimmed	UHT	<LOD	<LOD	<LOD	<LOD	–
M18	Milk	Cow	Semi-skimmed	UHT	<LOD	<LOD	<LOD	<LOD	–
M19	Milk	Cow	Semi-skimmed	UHT	<LOD	<LOD	<LOD	<LOQ	–
M20	Milk	Cow	Skimmed	UHT	<LOD	<LOD	<LOD	<LOQ	–
M21	Milk	Cow	Skimmed	UHT	<LOD	<LOD	<LOD	<LOD	–
M22	Milk	Cow	Skimmed	UHT	6.239	9.129	7.054	17.690	40.112
M23	Milk	Cow	Skimmed	UHT	<LOD	<LOD	<LOD	<LOD	–
M24	Milk	Cow	–	–	<LOD	<LOD	<LOD	<LOD	–
M25	Milk	Cow	–	–	<LOD	<LOD	<LOD	<LOD	–
M26	Milk	Sheep	Semi-skimmed	UHT	<LOD	<LOQ	<LOD	<LOQ	–
M27	Milk	Sheep	Semi-skimmed	UHT	<LOD	0.690	<LOD	1.060	1.750
M28	Milk	Sheep	Semi-skimmed	UHT	<LOD	<LOD	<LOD	<LOD	–
M29	Milk	Sheep	–	Pasteurization	<LOD	<LOD	<LOD	<LOD	–
M30	Milk	Sheep	–	Pasteurization	<LOD	<LOD	<LOD	<LOD	–
M31	Milk	Sheep	–	–	<LOD	0.807	<LOD	<LOD	0.807
M32	Milk	Goat	Semi-skimmed	UHT	<LOD	<LOD	<LOD	1.303	1.303
M33	Milk	Goat	Semi-skimmed	UHT	<LOD	<LOD	<LOD	0.519	0.519
M34	Milk	Goat	Semi-skimmed	UHT	<LOD	<LOD	<LOD	<LOQ	–
M35	Milk	Goat	Semi-skimmed	UHT	<LOD	<LOD	<LOD	<LOD	–
M36	Milk	Goat	Semi-skimmed	UHT	<LOD	<LOD	<LOD	<LOD	–
M37	Milk	Goat	–	–	<LOD	0.860	<LOD	<LOD	0.860
Y1	Yogurt	Cow	–	–	<LOD	<LOD	<LOD	<LOD	–
Y2	Yogurt	Cow	–	–	0.819	1.071	2.734	5.440	10.064
Y3	Yogurt	Cow	–	–	<LOD	<LOD	<LOD	<LOD	–
Y4	Yogurt	Cow	–	–	3.054	<LOD	0.418	<LOD	3.472
Y5	Yogurt	Cow	–	–	<LOD	<LOD	<LOD	<LOD	–
Y6	Yogurt	Cow	–	–	<LOD	<LOQ	<LOD	<LOD	–
Y7	Yogurt	Cow	–	–	<LOD	<LOD	<LOD	<LOD	–
Y8	Yogurt	Cow	–	–	<LOD	<LOD	<LOD	<LOD	–
Y9	Yogurt	Cow	–	–	<LOD	<LOD	<LOD	<LOD	–
Y10	Yogurt	Cow	–	–	<LOD	<LOD	1.704	<LOD	1.704
Y11	Yogurt	Cow	–	–	<LOD	<LOD	2.449	<LOQ	2.449
Y12	Yogurt	Cow	–	–	<LOD	<LOD	<LOD	<LOD	–
Y13	Yogurt	Cow	–	–	<LOD	<LOD	<LOD	<LOD	–
Y14	Yogurt	Cow	–	–	<LOD	<LOD	<LOD	<LOD	–
Y15	Yogurt	Cow	–	–	<LOD	<LOD	<LOD	<LOD	–
Y16	Yogurt	Cow	–	–	<LOD	<LOD	0.985	<LOD	0.985
Y17	Yogurt	Sheep	–	–	1.153	<LOD	<LOD	<LOD	1.153
Y18	Yogurt	Sheep	–	–	17.160	<LOD	8.955	<LOD	26.115
Y19	Yogurt	Sheep	–	–	8.064	<LOD	27.013	<LOD	35.077
Y20	Yogurt	Goat	–	–	<LOD	<LOD	<LOD	<LOD	–
Y21	Yogurt	Goat	–	–	<LOQ	2.108	0.891	<LOQ	3.266
Y22	Yogurt	Goat and Sheep	–	–	<LOQ	<LOQ	<LOD	<LOD	0.637
Y23	Yogurt	Goat and Sheep	–	–	<LOD	<LOD	<LOD	<LOD	–
K1	Kefir	Cow	–	–	<LOD	<LOQ	0.878	1.214	2.092
K2	Kefir	Cow	–	–	0.831	2.679	5.945	6.492	15.947
K3	Kefir	Cow	–	–	1.211	2.142	7.003	9.169	19.525
K4	Kefir	Cow	–	–	<LOD	1.579	14.055	64.032	79.666
K5	Kefir	Cow	–	–	<LOD	<LOD	0.452	<LOD	0.452
K6	Kefir	Cow	–	–	<LOD	2.598	10.970	9.420	22.988
K7	Kefir	Cow	–	–	<LOD	3.678	3.588	3.746	11.012
K8	Kefir	Cow	–	–	<LOD	<LOD	<LOD	<LOD	–
K9	Kefir	Cow	–	–	<LOD	<LOD	<LOD	<LOD	
K10	Kefir	Cow	–	–	<LOD	2.830	<LOD	<LOD	2.830
K11	Kefir	Goat	–	–	<LOD	<LOD	3.717	24.221	27.938
K12	Kefir	Goat	–	–	<LOD	0.850	2.799	<LOD	3.649
K13	Kefir	Goat	–	–	<LOD	<LOD	<LOD	<LOD	–
K14	Kefir	Goat	–	–	<LOD	<LOD	7.870	<LOD	7.870

LOD: Limit of detection; LOQ: Limit of quantification.
[a] mg/L for milk and mg/kg for yogurt and kefir.

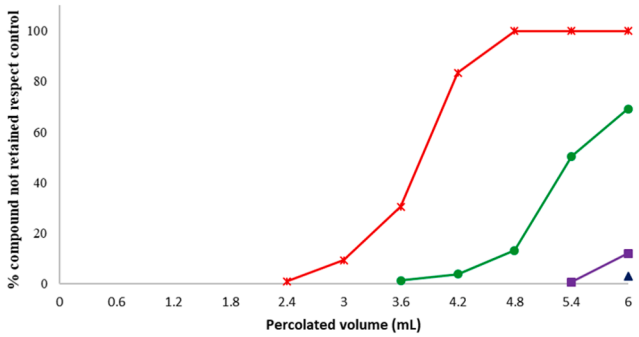

Fig. 1. Breakthrough volume of biogenic amines (putrescine, histamine, tyramine, and cadaverine) present in whole milk in the Solid Phase Extraction (SPE) cartridges used in the method.

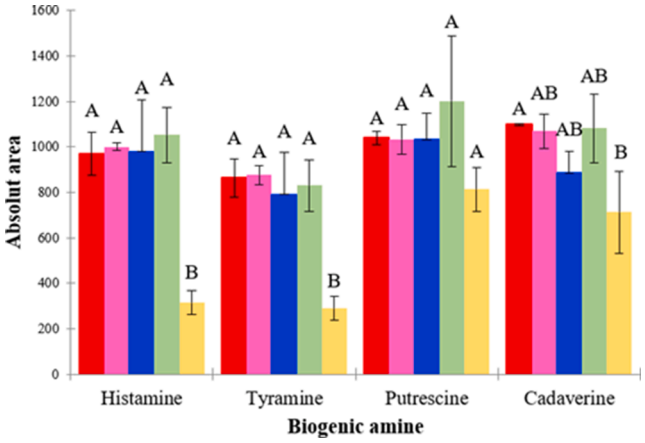

Fig. 2. Effect of different hexane washing up volumes (0, 2, 4, 6, and 8 mL) for the removal of fats from the SPE cartridge on the chromatographic area of biogenic amines analyzed by AQC-HPLC-FLD. Capital letters indicate significant differences ($p < 0.05$).

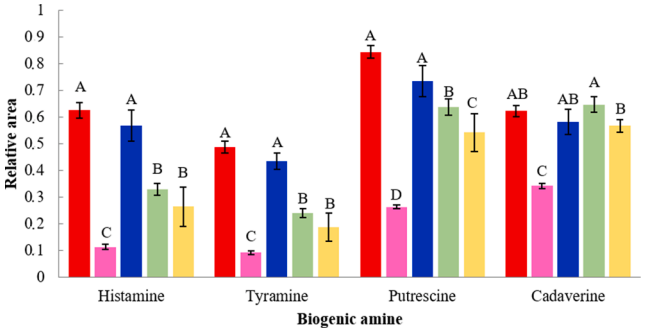

Fig. 3. Effect of different polar washing up procedures on the content of BAs retained in the SPE cartridge. : reference (no washing); , neutral and alkaline washings, , acid and neutral washing; , acid and alkaline washings, , all three washings. Capital letters indicate significant differences ($p < 0.05$).

temperatures are higher than those observed at room temperature (Fig. 6). Furthermore, results show better reproducibility at freezing temperature than at room temperature. The relative standard deviation (RSD) of samples stored at freezing temperature ranged from 2.19 % to 5 % for the different BAs, whereas the estimated values for samples stored at room temperature ranged from 3.23 % to 51.89 %. Samples were therefore kept at −18 °C for 5 min after the derivatization step.

3.2. Method validation

The chromatograms of spiked milk, yogurt, and kefir samples are given in Fig. 7. Repeatability of the experiment tested in milk samples is listed in Table 2. In the present study, mean repeatability of 6 milk samples was 9.34, 12.47, 2.68, and 6.36 % of HIM, TYM, PUT, and CAD, respectively. Good repeatability was also obtained in three samples of water spiked at 5 mg/L (0.27 %, 1.03 %, 7.84 %, 5.83 % of HIM, TYM, PUT, and CAD, respectively). HIM and TYM are two compounds with only one amine group, while PUT and CAD are diamine compounds. This structural reason could explain because the behaviour of HIM and TYM is more similar between them than with PUT and CAD. The presence of two amine groups in PUT and CAD can be an advantage for the derivatization reaction with lower competence with matrix components and perhaps this could explain the lower RSD in real samples.

LODs and LOQs of the four BAs are also shown in Table 2. LODs ranged from 0.12 to 0.2 mg/L, with lowest values for HIM and PUT, while LOQs were 0.4 mg/L for HIM and PUT, 0.667 mg/L for TYM, and 0.5 mg/L for CAD. These LOQs are much lower than those obtained in fermented milk by Costa et al. (2015) (LOQ up to 5.00 mg/L) and are also better than those provided by other analytical fluorescence RP-HPLC methods (LOD: 0.7–1.3 mg/kg and LOQ: 1.4–2.6 mg/kg in Sawilska-Rautenstrauch et al. (2010)), and even better than the few other methods using AQC reagent (LOD: 0.8–6.2 mg/kg and LOQ: 2.9–60.9 mg/k in Mayer et al. (2010); LOD: 0.5–4.4 mg/kg and LOQ: 1.6–14.5 mg/kg in Fiechter et al. (2013)). The improved sensitivity can be attributed to the combination of the SPE procedure with the fluorescence detection of the AQC derivatives. This increase in sensitivity is crucial since it can be suggested that dairy products with <1 mg/L of BA should be safe for consumers, including BAs-sensitive individuals (Rauscher-Gabernig et al., 2009).

The method's linearity was studied in water and in a milk sample spiked at different concentrations. For all BAs, our method showed adequate linearity in the range of concentration studied (1 to 20 mg/L) (Table 2). Determination coefficients (R^2) of calibration in milk samples ranged from 0.962 to 0.999.

Matrix effects were assessed by comparing the slopes of calibration curves obtained in water and in milk. Results revealed that the determination of HIM and TYM was free from matrix effects, but not the determination of CAD and PUT. Because of this, quantification was carried out by using a sample-dependent response factor. For that, each sample was analyzed unspiked and spiked with known amounts of BAs. The difference of signal between these samples was used to estimate the concentration of BAs in unspiked sample.

3.3. Application of the method for detection of the presence of biogenic amines in dairy products

The method was applied to the analysis of four BA (HIM, TYM, PUT, and CAD) in several dairy products. Concentrations of BAs determined in 37 milk, 23 yogurt and 14 kefir samples are shown in Table 1 together with the samples' characteristics. All but five milk samples (provided by a cheese manufacturing company that used them for the production of ripened cheese) were commercial, and were from cows, sheep, and goats, subjected to pasteurization or UHT processes, and containing different percentages of fat content (whole, semi-skimmed, and skimmed). Concentrations of total BAs (TBA) are also included in Table 1. The use of TBA as a measure, in conjunction with specific BAs contents, has been proposed to define tolerable levels for food safety purposes (Benkerroum, 2016).

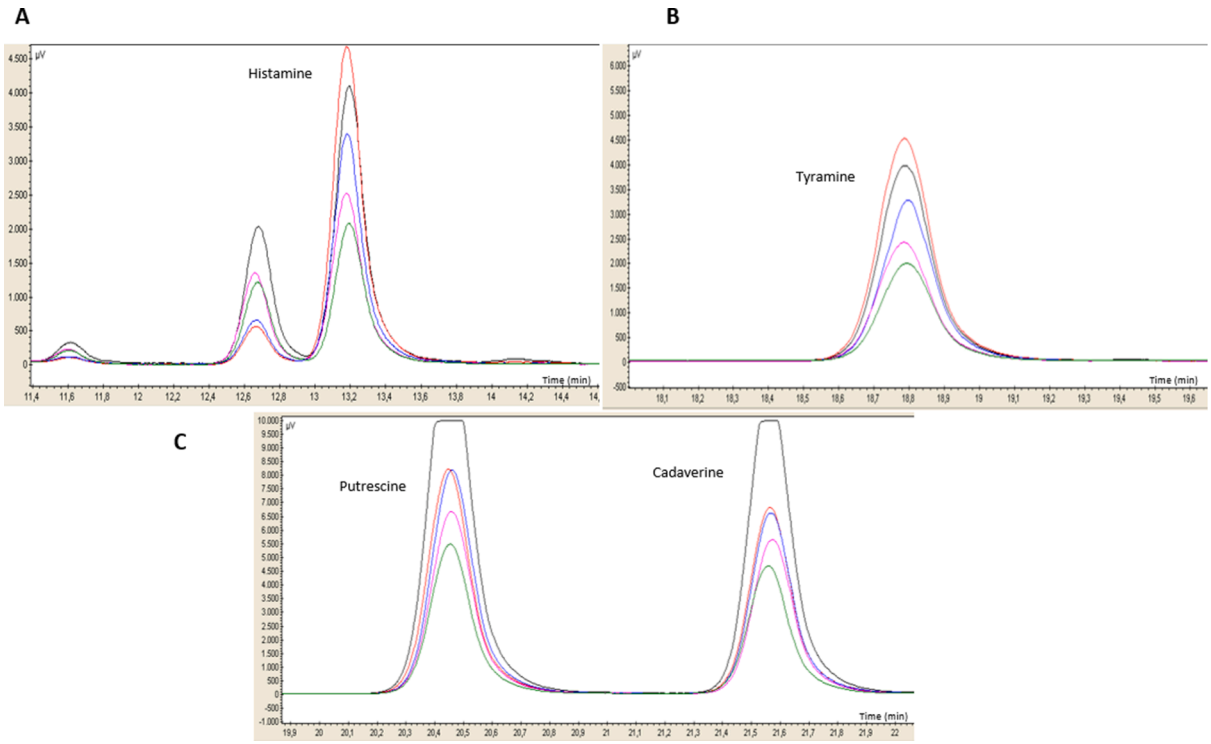

Fig. 4. HPLC chromatograms of the influence of the pH variation (1 ;5 ;7 ;10 ;12) on the signal of: (A) histamine, (B) tyramine, and (C) putrescine and cadaverine.

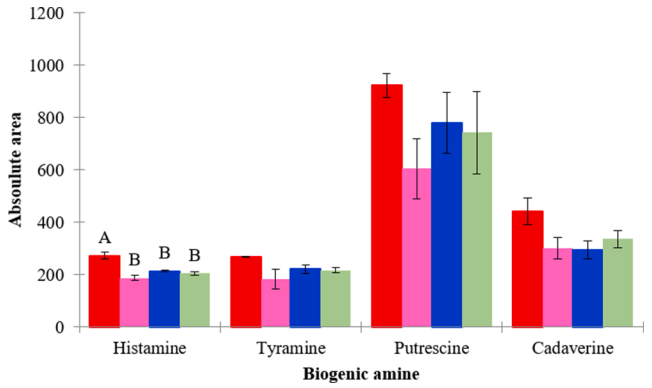

Fig. 5. Effect of derivatization time (10 , 20 , 30 , and 60 min) on the chromatographic area of biogenic amines in dairy products analysed by AQC-HPLC-FLD. Capital letters indicate significant differences ($p < 0.05$).

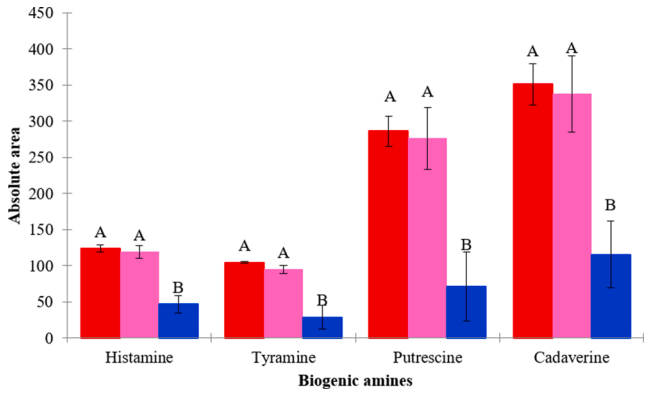

Fig. 6. Effect of different temperatures (freezing… , cooling , and room) on the chromatographic area of biogenic amines in dairy products analysed by AQC-HPLC-FLD. Capital letters indicate significant differences ($p < 0.05$).

Remarkably, BAs were not present in most of the milk samples. TYM and CAD were detected in 5 and 8 samples, respectively, and could quantified just in 4 cases each. Maxima levels of TYM were 9.13 mg/L and those of CAD 17.7 mg/L. HIM and PUT were found only in two samples and could be quantified only in one, reaching 6.24 and 7.05 mg/L, respectively. The highest TBA corresponds to sample M22, which reached 40.11 mg/L. No obvious relationship was observed between concentration of BAs and characteristics of milk, such as technological process, animal species, or brand. Some correlation studies were considered however, the number of samples with contents of BAs was very low to obtain good correlation results.

A literature comparison review of BAs results in dairy products is shown in Table 3. As can be seen, results reported here are consistent with those reported by other authors. In general, most authors report low or undetectable concentrations of BAs (Min et al., 2004; Novella-Rodríguez et al., 2000; Özdestan & Üren, 2010; Pekcici et al., 2021; Wu et al., 2015). In fact, previous reports on pasteurized milk showed levels generally not exceeding 1.07 mg/L HIM, 6.35 mg/L TYM, 1.4 mg/L PUT, and an almost unquantifiable value of 0.05 mg/L CAD (Pekcici et al., 2021), levels not raising a health concern with regard to BAs content (EFSA, 2011). However, Min et al. (2004) observed more than 18 mg/kg of CAD in commercial milk, which is a value very close to the one found in the Sample M22 of the present study.

A total of 23 yogurt samples were also analyzed. The incidence of BAs was higher in yogurt samples than in milk samples, not only in terms of number of samples, but also in terms of concentration. PUT and HIM were the most frequently occurring BAs: they were found in 8 and 7 samples, respectively. Meanwhile, TYM and CAD were also detected and quantified in 4 and 3 samples, respectively. HIM was quantified in 5 samples and ranged from 0.82 to 17.16 mg/kg. The concentration of PUT and TYM was higher than LOQ in all samples. PUT ranged from 0.418 to 27.013 mg/kg, and the range of TYM was lower: from 0.539 to

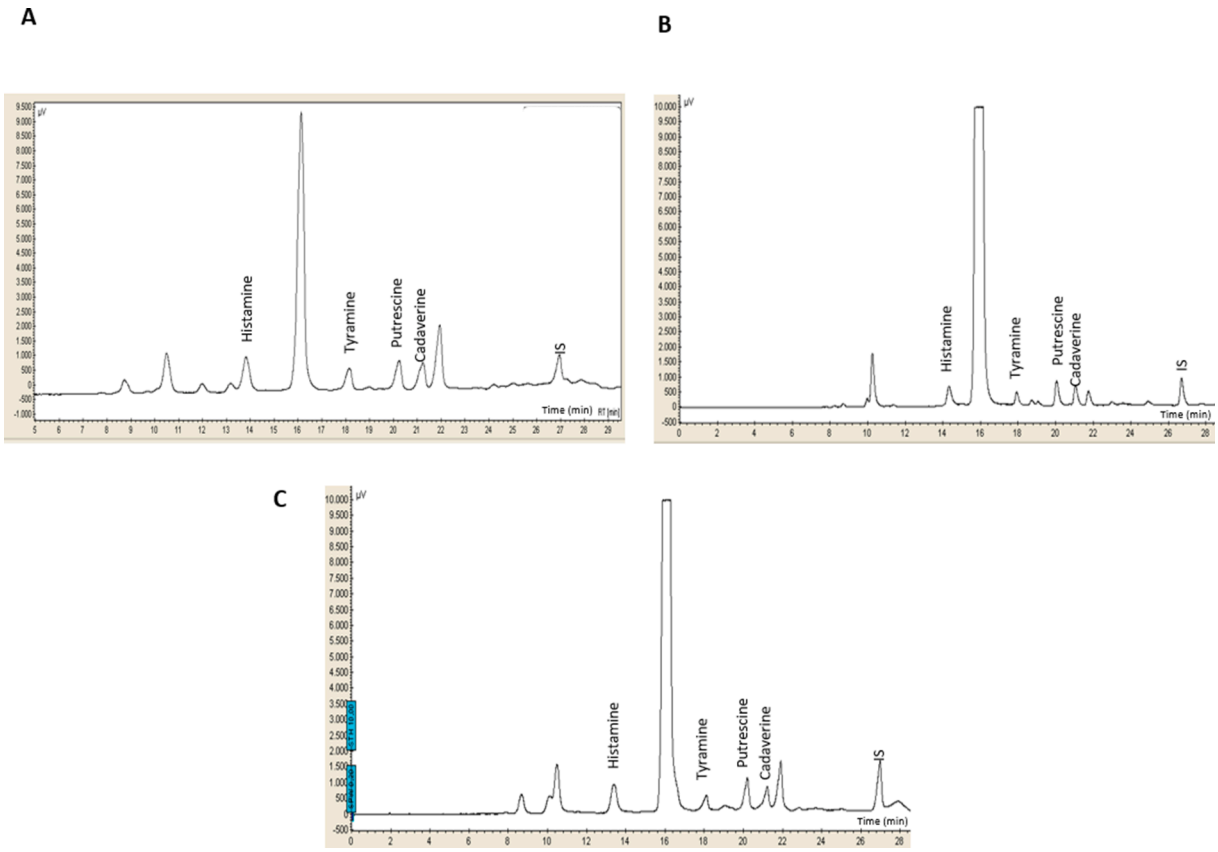

Fig. 7. HPLC chromatogram of spiked (A) milk, (B) yogurt, and kefir (C) samples. Peak identification: Histamine, Tyramine, Putrescine, Cadaverine, 1,7-diamino-heptane (IS).

Table 2
Figures of merit of the method developed in this study.

	Retention time (min)	Linear range (mg/L)	R^2	LOD (mg/L)	LOQ (mg/L)	Repeatability (RSD%) in milk	Repeatability (RSD%) in water
Histamine	13.67	1–20	0.998	0.12	0.400	9.34	0.27
Tyramine	19.23	1–20	0.999	0.2	0.667	12.47	1.03
Putrescine	20.91	1–20	0.962	0.12	0.400	2.68	7.84
Cadaverine	21.96	1–20	0.965	0.15	0.500	6.36	5.83

LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative Standard Deviation.

Table 3
Ranges of concentration of biogenic amines (histamine, tyramine, putrescine, and cadaverine) and of total biogenic amines (TBA) in dairy products (milk, yogurt and kefir) found in research literature.

	Histamine (mg/kg)	Tyramine (mg/kg)	Putrescine (mg/kg)	Cadaverine (mg/kg)	TBA (mg/kg)	Authors
Milk	ND-1.07	ND-6.35	ND-1.40	ND-18.52	ND-27.34	Bodmer et al., 1999; Min et al., 2004; Novella-Rodríguez & Veciana-Nogués et al., 2002; Pekcici et al., 2021
Yogurt	ND-65.18	ND-22.82	0.03–58.06	ND-34.53	ND-178.62	Bodmer et al., 1999; Min et al., 2004; Mayr & Schieberle, 2012; Bunkova et al., 2013; Adımcılar et al., 2017; Silva et al., 2019; Vieira et al., 2020; Pekcici et al., 2021
Kefir	ND-30.82	ND-12.8	0.3–47.69	ND −24.08	ND-115.69	Özdestan & Üren, 2010; Bunkova et al., 2013; Adımcılar et al., 2017; Pekcici et al., 2021

ND: non detected.

2.108 mg/kg. CAD was detected in a lower number of samples (3) and could only be quantified in one sample at a concentration of 5.44 mg/kg.

PUT concentrations found by most authors in yogurt samples have been very low: 0.6 mg/L (Min et al., 2004), 0.47 mg/L (Pekcici et al., 2021) or even undetectable (Vieira et al., 2020). However, Adımcılar et al. (2017) quantified levels of up to 47 mg/kg in commercial yogurts and 58 mg/kg in homemade yogurts. In the present study up to 9 mg/kg of PUT in Sample Y18 have been observed.

The concentrations of HIM and TYM found in previous studies were higher than those of the other two BAs, reaching values from 21.2 to 65.2 mg/kg for HIM (Min et al., 2004) and up to 22.82 mg/kg for TYM (Vieira et al., 2020). Pekcici et al. (2021) observed maxima HIM values of 6.97 mg/kg, which is clearly smaller than the concentration found in Y6 sample, 17 mg/kg HIM (Y6). This sample contained highest levels of

HIM and TYM, which could indicate an instance of bacterial contamination. Maxima values of TYM in this study (2.108 mg/kg in Y7) are smaller than those previously reported. The highest TBA was obtained in Sample Y19 (35.07 mg/kg), due to a high content of HIM and PUT (8.064 and 27.013, respectively). It is noteworthy that the two yogurt samples with the highest TBA were made exclusively from sheep milk. Similar results occurred with the cured cheeses made with sheep's milk, where there was a higher concentration of HIM than in those made with cow's milk (Botello-Morte et al., 2022).

A total of 14 kefir samples were also analyzed. BAs were detected in 11 samples. Kefir samples showed the highest TBA levels, yielding up to 79.66 mg/kg, double of the highest concentration of TBA in milk (40.11 mg/kg) and yogurt (35.07 mg/kg). Although HIM was quantified only in two at 0.831 and 1.211 mg/kg, TIM, PUT, and CAD were found in a greater number of samples (7, 10 and 7, respectively) and in a higher concentration (between 0.850 and 3.678 mg/kg, 0.452–14.05 mg/kg and 1.21–64.032 mg/kg, respectively). PUT was the BA most found in kefir, and was detected in all but in four samples (K8, K9, *K*10 and K13). The higher concentration of cadaverine could be associated, among other factors, with the use of a starter with the capacity to produce cadaverine or with an occasional lack of hygiene that produces contamination with bacteria that produce this metabolite. Synthesis of biogenic amines is a multifactorial process which depends, among others, on bacterial metabolic state, presence of decarboxylase genes, and environmental conditions. Therefore, higher levels of cadaverine in comparison to histamine could be associated, among others, with a high representation of lysine decarboxylase (e.g. cadA) genes in NSLAB, SLAB or bacterial contaminants, concomitant with environmental conditions (such as temperatures inducing oxidative stress or low pH leading to acid stress) that would increase their gene expression in kefir samples.

It is well-known that fermentation processes can cause an increase in BAs in the final product (Ladero et al., 2017). A higher degree of formation of HIM is found in yogurt (65.18 mg/kg) and in kefir (30.82 mg/kg) (Adımcılar et al., 2017) than in milk (1.07 mg/kg) (Pekcici et al., 2021). In our case, a low amount of HIM (ND-1.211) was observed in kefir, while a considerable concentration of CAD (ND-64.032 mg/kg) was found, exceeding values described by other authors (24.08 mg/kg in Adımcılar et al. (2017) and 2.2 mg/kg in Özdestan and Üren (2010).

Milk samples had a lower concentration of BAs than was the case in yogurt or kefir. Several reasons could explain this difference. One reason could lie in the different capacity of certain fermentative lactic acid strains for producing BAs; a further reason might be found in the different kinds of environmental microbiota present in equipment used in industry (Moniente et al., 2021). The lower concentration of BAs in milk could also be attributed to the possible use of different starter cultures that may also have different BAs production capacities (Mokhtar et al., 2012); moreover, the use of different milks with different concentrations of amino acid precursors during manufacture can likewise exert an influence on BAs content. Lastly, the type of processed food strongly affects the growth rate and the concentration of lactic acid microbiota present in the matrix during cold storage (Samelis et al., 2000).

Levels of BAs found in milk may be due to a lack of hygiene after the pasteurization process, which can lead to a bacterial proliferation capable of producing BAs. It is necessary to highlight the need to respect norms of good hygiene by properly cleaning and disinfecting food equipment throughout manufacturing. When such measures fail, high concentrations of BAs can emerge and cause a food risk for consumers. Moreover, if milk contaminated with BAs-producing microorganisms is used in the production of cheeses, BAs concentration can increase uncontrollably, even exceeding 2,500 mg/L of HIM in the case of ripened cheese (Maintz & Novak, 2007).

4. Conclusion

A new method for the determination of BAs in dairy products was validated in this study. The method is based on a SPE procedure followed by derivatization with AQC and analysis by HPLC with fluorescence detection. This methodology makes it possible to achieve detection limits lower than 0.2 mg/L, and quantification limits lower than 0.667 mg/L for the different amines, which is highly satisfactory. Mean repeatability is lower than 10 % of RSD, and the method is linear in an adequate range of concentrations.

We applied this method to the quantification of BAs in samples of Spanish milk, yogurt, and kefir. Our results show pronounced differences between milk and fermented products. While BA are just seldomly present in milk samples, the amount of BA in yogurt samples is greater, and is even greater and more frequent in kefir samples. Although these levels would not cause any harmful effect on most consumers, they should be taken into account by individuals who are sensitive to BAs.

CRediT authorship contribution statement

Marta Moniente: Investigation, Data curation, Writing – original draft, Writing – review & editing. **Laura Botello-Morte:** Writing – review & editing. **Diego García-Gonzalo:** Writing – review & editing. **Raquel Virto:** Project administration, Funding acquisition, Writing – review & editing. **Rafael Pagán:** Project administration, Funding acquisition, Writing – review & editing. **Vicente Ferreira:** Funding acquisition, Writing – review & editing. **Ignacio Ontañón:** Supervision, Investigation, Data curation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.112448.

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