JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Rapid Assay for Analyzing Biogenic Amines in Cheese: Matrix Solid-Phase Dispersion Followed by Liquid Chromatography−Electrospray−Tandem Mass Spectrometry

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A new rapid and sensitive method based on matrix solid-phase dispersion (MSPD) followed by liquid chromatography−electrospray−tandem mass spectrometry was devised for the determination of biogenic amines at trace levels in cheese samples. The method required 0.25 g of sample, CN-bonded silica as a dispersant sorbent, and a formic acid aqueous solution/methanol mixture as an eluting solvent. Extraction recoveries from soft cheese products were calculated in the 98 ± 4−110 ± 6% range. A procedure based on solid-phase extraction was also evaluated for the extraction of these compounds in cheese. Chromatographic separation was performed using a C18 column with an aqueous ammonium acetate/methanol mixture as the mobile phase under gradient conditions. The method was validated in terms of detection limits (LOD), quantitation limits (LOQ), linearity, recovery, precision, and trueness. Results in the 0.05−0.25 mg kg^{-1} range were obtained for the LOD of histamine, tyramine, and β-phenylethylamine in soft cheese samples. Linearity was established over 2 orders of magnitude. Excellent precision in terms of intra-day repeatability was calculated (RSD% < 5). The applicability of the method to the determination of biogenic amines in cheese products was demonstrated.

KEYWORDS: Biogenic amines; matrix solid phase dispersion; liquid chromatography−tandem mass spectrometry

1. INTRODUCTION

Biogenic amines (BAs) are synthesized through biological pathways, such as the decarboxylation of amino acids. Decarboxylation is also the primary source of BAs in fermented products, such as wine, and especially in meat and fish (*1*, *2*). In addition, various factors possibly influencing BA formation are hygienic conditions of raw materials and manufacturing practices. Consequently, the level of BAs in a food product is often considered as a marker of spoilage during storage and, therefore, a quality index (*3*, *4*).

The toxicological importance of biogenic amines is lower in comparison with acknowledged carcinogens such as heterocyclic aromatic amines or *N*-nitroso compounds (*2*). However, in allergenic individuals or people being administered monoamine oxidase inhibitors, negative health effects after intake of relatively low levels of BA can be expected (*2*).

A number of separation methods based on gas chromatography (*5*), high-performance liquid chromatography (HPLC) with both a fluorescence and mass spectrometry detector (*6*−*9*, *10*, *11*), capillary electrophoresis (*12*−*14*), micellar electrokinetic capillary chromatography (*15*−*17*), and micellar liquid chromatography (*18*) have been described in the literature for their ability to separate the derivatives of biogenic amines. For all of the above-mentioned techniques, the derivatization step, which usually precedes the chromatographic run, is performed off-line and includes an extraction step with an organic solvent prior to separation. This step increases the risk of low recovery, analyte loss and contamination, and involves long analysis time. Concerning sample treatment, solvent extraction and solid-phase extraction (SPE) have been proposed for the purification of BA samples (*19*) as well as for the preconcentration of their derivatives after off-line derivatization (*10*).

The results reported in this paper demonstrate the development of a rapid sample preparation method using matrix solid-phase dispersion (MSPD) to the extraction of biogenic amines from soft and hard cheese samples followed by HPLC−electrospray−tandem mass spectrometry (ESI−MS/MS) determination without any derivatization step. MSPD is a new extraction technique suitable for solid samples that combines homogenization, analyte extraction, and purification in one step. This approach has been successfully applied to the analysis of several residues in foods, such as pesticides, drugs, and mycotoxins (*20*, *21*). To our knowledge, this technique has not been used for the extraction of biogenic amines from foods. A comparison with a solvent extraction procedure followed by a purification with SPE was carried out. The method was validated, and its application to BA analyses in aged cheese samples was demonstrated.

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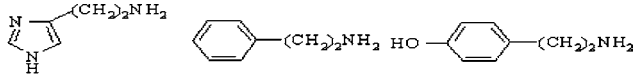
10.1021/jf048037g CCC: $30.25
Published on Web 04/19/2005



Histamine β-Phenylethylamine Tyramine

Figure 1. Chemical structures of biogenic amines investigated.

2. EXPERIMENTAL PROCEDURES

2.1 Chemicals. Histamine, tyramine, and β-phenylethylamine (≥99% purity) were from Sigma−Aldrich (Germany) (see **Figure 1**). Isotope-labeled standards (α-d_2 and β-d_2) histamine-d_4, (α-d_2 and β-d_2) tyramine-d_4, and (α-d_2 and β-d_2) β-phenylethylamine-d_4 (≥99% purity) were from CDN Isotopes (Pointe-Claire, Quebec, Canada). Ammonium acetate and HPLC-grade acetonitrile, methanol, and water were from Carlo Erba (Milan, Italy).

Stock standard solutions of biogenic amines and internal standards with concentrations of 0.5 mg/mL were prepared in 0.1 N HCl and stored in the dark at 4 °C. Working standard solutions were prepared daily by diluting stock solutions with HPLC-grade water.

2.2 Liquid Chromatography−Mass Spectrometry. An Alliance 2690 liquid chromatography (Waters, Milford, MA) equipped with a 120-vial capacity sample management system was used. Chromatographic separation was obtained using a Luna C18(2) (250 × 2.1 mm) (Phenomenex, Torrance, CA) column under gradient conditions. The mobile phase consisted of a mixture of 0.1% TFA (v/v) aqueous solution (eluent A) and methanol (eluent B) delivered at a flow rate of 0.2 mL min^{-1}. Gradient elution was as follows: solvent (B) was delivered by a linear gradient from 1 to 70% for 3 min, followed by an isocratic elution at 70% solvent B for 7 min before column re-equilibration (10 min). A Quattro LC triple quadrupole-mass spectrometer (Micromass, Manchester, U.K.), with a pneumatically assisted electrospray interface was used. Data acquisition was performed in positive-ion mode (ESI^+). Interface parameters were set as follows: capillary voltage, 2.9 kV; cone voltage, 15 V; extractor lens voltage, 3 V; source temperature, 100 °C; desolvation temperature, 240 °C; and rf lens, 0.1 V. The nebulizer and desolvation gases (nitrogen, 99.999% high purity) were delivered at 60 and 550 L hr^{-1}, respectively.

Experiments for optimization of ESI interface parameters were performed by directly infusing solutions into the ESI−MS system at a flow rate of 6 μL min^{-1}. Full-scan analyses were performed over the scan range of *m*/*z* 30−150 using a step size of 0.1 Da and a rate of 0.4 scans/s. When we operated in the MS/MS mode, product-ion scan mass spectra of protonated molecules were acquired in the *m*/*z* 30−150 range. Selected reaction monitoring (SRM) analyses were performed as follows: *m*/*z* 112 → 68 [collision energy (CE), 20 eV] and *m*/*z* 112 → 95 (CE, 14 eV) for histamine, *m*/*z* 116 → 99 (CE, 13 eV) for histamine-d_4, *m*/*z* 138 → 103 (CE, 21 eV) and *m*/*z* 138 → 121 (CE, 10 eV) for tyramine, *m*/*z* 142 → 125 (CE, 10 eV) for tyramine-d_4, *m*/*z* 122 → 77 (CE, 30 eV) and *m*/*z* 122 → 103 (CE, 22 eV) for β-phenylethylamine, *m*/*z* 126 → 109 (CE, 10 eV) for β-phenylethylamine-d_4. The dwell time and the interchannel delay were set at 0.25 and 0.01 s, respectively.

For data acquisition and processing, Masslynx version 4.0 software was used.

2.3 Sample Preparation. Soft creamy cheese was purchased in big trades. Sample treatment was carried out by applying MSPD and solvent extraction followed by SPE on C18 and CN silica supports.

Among hard cheese samples, Parmigiano-Reggiano cheese was considered. Hard cheese samples produced starting from the same raw milk and collected at different age ripening (12, 24, and 30 months) from one cheese factory were considered. Each sample was divided in three different parts (i.e., near rind, intermediate, and kernel), and each part was submitted for analysis. For this purpose, a representative portion of samples for each part (100 g) was chopped into small pieces and finely ground in a blender to homogeneity before extraction.

Biogenic amines were extracted from hard cheese samples by applying MSPD using CN solid phase. Extraction recovery was calculated on the 24-month aged sample by spiking analytes at two different concentration levels (15 and 50 μg/g histamine; 10 and 50 μg/g tyramine and β-phenylethylamine) and performing three extractions and two LC analyses for each extract.

2.4 Matrix Solid-Phase Dispersion Procedure. CN-silica support was pretreated by washing 10 g with 20 mL of hexane, 20 mL of 2-propanol, and 20 mL of methanol. C18-solid phase was washed with 20 mL of 2-propanol and 20 mL of methanol prior to use. An aliquot of the sample (0.25 g) was placed into a mortar, and 1 g of the C18 or CN-silica sorbent (Supelco) previously treated was added and gently blended for 5 min using a pestle to obtain a homogeneous mixture. The homogenized sample was introduced into the cartridge and tamped to form a compact extraction layer (2 mL). Using both stationary phases, the MSPD column was eluted sequentially under slight pressure from a syringe plunger. In particular, the target analytes were eluted first with 4 mL of 0.1% (v/v) formic acid aqueous solution/methanol (20:80, v/v) and then with 4 mL (v/v) formic acid aqueous solution/methanol (90:10, v/v). The extract was collected in a vial and filtered (Nylon syringe-type filter, 0.2 μm porosity) before LC−MS analysis.

2.5 Solvent Extraction and SPE Procedure. A total of 1 g of homogenized sample was weighed into a polypropylene graduated conical tube with cap (15 mL), and 200 μL of 4 μg/mL internal standard solution (d_4-labeled internal standards in 0.1 N HCl) together with 10 mL of 0.1 N HCl were added. After the extract was mixed for 10 min on a vortex, it was centrifuged at 4800 rpm (1350*g*) for 15 min. The clarified solution was removed, and a final volume of 25 mL was obtained by adding HPLC-grade water. To improve analyte retention on SPE cartridge, the pH of the solution was increased to 9.5 pH units with ammonia. A cleanup procedure of 2 mL of the final extract was performed by SPE using both C18 and CN cartridges (300 mg, 6 mL) (Supelco). Cartridges were conditioned with 5 mL of methanol, and the 2 mL sample was then loaded and eluted first with 2 mL of 0.1% (v/v) formic acid aqueous solution/methanol (20:80, v/v) and then with 2 mL of (v/v) formic acid aqueous solution/methanol (90:10, v/v). SPE cleanup was performed on two extracts of each sample, and three replicated LC analyses were run for each extract.

2.6 Validation Procedure. Validation of the LC−ESI−MS/MS method was carried out following EURACHEM guidelines (*22*) on soft cheese matrix extracted by MSPD using CN solid phase.

The detection limit (y_D) and quantitation limit (y_Q) were preliminarly calculated as signals based on the mean blank ($\overline{y_b}$) and the standard deviation (s_b) of the blank signals as follows:

$$y_D = \overline{y_b} + 2ts_b \qquad y_Q = \overline{y_b} + 10s_b$$

where t is a constant of the t-Student distribution (one-sided) depending on the confidence level and the degrees of freedom ($\nu = n - 1$, n = number of measurements). A total of 10 blank measurements were performed to calculate $\overline{y_b}$ and s_b. An extract of fresh cheese in which the absence of biogenic amines had been previously verified was used as the blank to calculate matrix-matched LOD and LOQ. y_D and y_Q were converted from the signal domain to the concentration domain to estimate the limit of detection (LOD) and limit of quantitation (LOQ), respectively, using an appropriate calibration function. To satisfy basic requirements such as homoscedasticity and linearity, the Bartlett test and the Mandel's fitting test were performed at the 95% confidence level. In the case of evaluation of instrumental performance in terms of LOD and LOQ, blank analyses were performed using methanol/water (1:1, v/v).

Linearity was established over 2 orders of magnitude of concentration in matrix-matched solutions. For each analyte, the corresponding labeled internal standard was used to calculate the relative peak area. Six equispaced concentration levels were chosen, and three replicated injections were performed at each level. The homoscedasticity test was run, and the goodness of fit of the calibration curve was assessed by applying the lack-of-fit and Mandel's fitting tests (*23*). A t test was carried out to verify the significance of the intercept (confidence level of 95%).

Precision was calculated in terms of intra-day repeatability as RSD% at two concentration levels for the analysis of both standard solutions and sample extracts.

Trueness was evaluated by calculating the recovery function, which allows us to assess both constant and proportional systematic errors. For this purpose, first the calibration function of the fundamental analytical procedure was determined:

$$y = a_c + b_c x_c$$

The analytical calibration procedure was performed on a fresh cheese blank sample spiked at six equispaced concentration levels. The

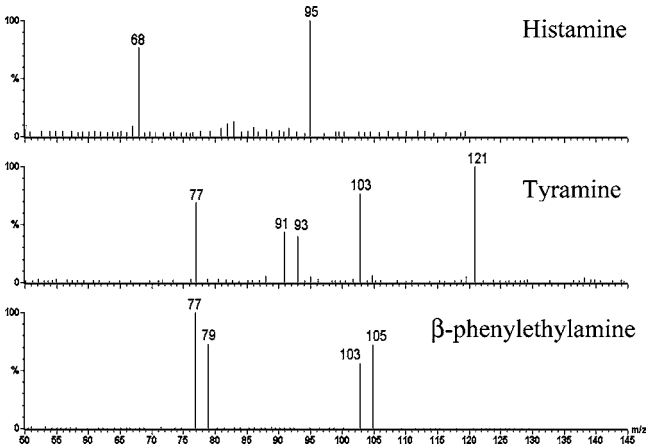


Figure 2. LC–ESI+–MS/MS product ion mass spectra of a standard solution of BAs (0.5 μg/mL).

analytical results x_f were then calculated using the measured signal values y_f and the analysis function, i.e., the calibration function solved for x

$$x_f = y_f - a_c/b_c$$

When the measured concentrations (x_f) were plotted versus the original calibration concentrations (x_c), the recovery curve, which is mathematically described by the recovery function (linear regression line), was calculated

$$x_f = a_f + b_f x_c$$

In the ideal case, i.e., absence of systematic errors of both kinds, the recovery function results in a line with the intercept $a_f = 0$ and the slope $b_f = 1$ as well as in a residual standard deviation that corresponds to the standard process deviation of the fundamental analytical procedure.

Histamine, tyramine, and β-phenylethylamine were used to calculate recovery in the cheese extracts. Recovery studies were carried out by spiking samples of soft cheese at two different concentration levels (15 and 50 μg/g histamine; 10 and 50 μg/g tyramine and β-phenylethylamine). Spiked samples was left to stand overnight at 4 °C. The sample was equilibrated to room temperature before following the extraction procedures described above.

All statistical analyses and tests were performed by using the statistical package SPSS version 9.0 for Windows (SPSS, Bologna, Italy).

3. RESULTS AND DISCUSSION

3.1 Mass Spectrometry and LC Separation. Under ESI+–MS conditions, mass spectra of all compounds showed abundant $[M + H]^+$ ions without fragmentation.

The product ion MS/MS spectra as obtained by low-energy collision-induced dissociation of protonated molecules showed fragmentation patterns dominated by the loss of ammonia, leading to product ions at *m*/*z* 95, *m*/*z* 121, and *m*/*z* 105 for histamine, tyramine, and β-phenylethylamine, respectively (**Figure 2**). Additional loss of 28 amu fragments accounted for the release of the ethyl moiety from the $[M + H-NH_3]^+$ ions. In addition, the peak at *m*/*z* 103 resulting from the elimination of a water molecule from the $[M + H-NH_3]^+$ fragment ion was visible in the MS/MS spectrum of tyramine.

Criteria for unambiguous identification of biogenic amines in real samples under SRM MS/MS conditions, taking into account that the low *m*/*z* value of the molecular ion of analytes could also correspond to that of naturally occurring constituents of cheese samples, included monitoring of two characteristic transitions for each contaminant for confirmation purposes.

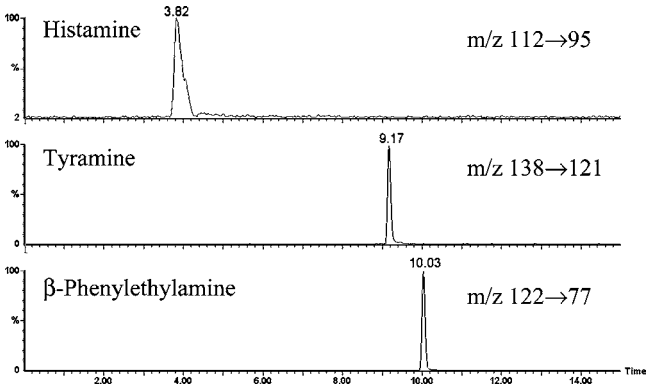


Figure 3. LC–ESI+–MS/MS extracted transition chromatograms of a standard solution of BAs (0.5 μg/mL).

Table 1. Average Recoveries (%)[a] and Standard Deviation Obtained by Using C18 and CN SPE and MSPD Extraction of Soft Cheese [Elution Solvents: MeOH/H_2O, 10:90 (v/v), and 0.1% HCOOH Followed by MeOH/H_2O, 80:20 (v/v), and 0.1% HCOOH]

analyte	concentration level (μg/g)	SPE		MSPD	
		C18	CN	C18	CN
hystamine	15	84 ± 16	93 ± 6	83 ± 6	110 ± 6
	50	86 ± 8	96 ± 4	88 ± 4	101 ± 2
tyramine	10	72 ± 3	90 ± 2	93 ± 9	103 ± 6
	50	73 ± 6	86 ± 2	89 ± 6	99 ± 3
β-phenylethylamine	10	80 ± 8	93 ± 9	97 ± 5	105 ± 5
	50	86 ± 16	97 ± 6	95 ± 2	98 ± 4

[a] $n = 6$.

Gradient elution under reversed-phase partition conditions allowed separation of the analytes in less than 10 min with high repeatability (RSD < 0.5% for all of the compounds, $n = 10$) (**Figure 3**).

3.2. Performance of the Solid-Phase Extraction and the Matrix Solid-Phase Dispersion Procedure. In the development of SPE and MSPD extraction procedures for soft cheese, elution solvents and polarity of solid-phase materials were adequately selected as reported in the Experimental Procedures.

Recoveries obtained by using C18 and CN solid phases are reported in **Table 1**. It can be observed that the best recoveries with the lowest standard deviation for all BAs were obtained using CN solid phase for both SPE and MSPD methods. The differences between the mean recoveries obtained with the C18 sorbent with respect to those obtained using the CN solid phase were statistically significant at a 95% confidence level ($p < 0.05$). In addition, MSPD extraction performed on CN-bonded silica provided the highest recovery values with good repeatability (RSD < 6%). After the LC–MS/MS SRM chromatograms of soft cheese extracts obtained by SPE or MSPD were acquired, no differences were observed between the two profiles. In particular, no interferences in correspondence to the transitions monitored for the analytes were detected. However, as for the matrix effects, comparable suppression effects were observed for all of the analytes when analyzed using both approaches, suggesting the presence of not detected coeluting matrix compounds in both extracts. In comparison with SPE, MSPD offers the advantage of avoiding long solid–liquid extraction procedures.

The application of SPE and MSPD extraction procedures to hard cheese samples provided different results. The SPE procedure exhibited high variability in the recovery values with RSD% up to 60%. Such variability was attributed to the enhanced protein precipitation observed for this kind of sample when the pH of the liquid extract was increased to pH 9.5.

Table 2. LOD and LOQ Values Calculated for BAs Using the LC–ESI–MS/MS Method

	matrix: soft cheese (mg/kg)[a]	
analyte	LOD	LOQ
hystamine	0.05	0.09
tyramine	0.06	0.13
β-phenylethylamine	0.25	0.55

[a] Referred to 1 g of sample extracted with 8 mL of solvent. See the Experimental Procedures.

Table 3. Matrix-Matched Calibration Curves Established in Soft Cheese Extracts Using the LC–ESI–MS/MS Method[a]

analyte	concentration range (mg/kg)	homoscedasticity test[b]	Mandel's test[b]	$b_1 \pm s_{b1}$ [b]
hystamine	0.1–17	0.139	0.125	1.319 (±0.011)
tyramine	0.15–30	0.089	0.085	0.934 (±0.006)
β-phenylethylamine	0.5–50	0.158	0.091	0.123 (±0.004)

[a] Calibration function: $y = b_1x$. [b] Confidence level of 95%.

Applying the MSPD procedure resulted in a significant improvement of the precision with RSD% values of about 8% and recovery values ranging from 84 ± 10 to 117 ± 3%.

3.3. Calibration and Method Performances. To check the methods' performance, quality parameters such as detection limit, quantitation limit, linearity range, precision, trueness, and recovery were studied in the matrix (soft cheese).

Under the optimized LC−MS/MS conditions and operating in SRM mode, LOD and LOQ of BAs in fresh cheese samples were at levels in the 0.05−0.25 and 0.09−055 mg/kg range, respectively (**Table 2**).

Using external calibration, good linearity of the method was established over 2 orders of magnitude ($r^2 = 0.998-0.999$, $n = 18$) on the matrix. Homoscedasticity was verified over the range tested by means of the Bartlett test, whereas the application of the Mandel fitting test showed that a quadratic regression did not provide a better fitting than the linear one (**Table 3**).

Excellent precision in terms of intra- and inter-day repeatability was calculated, providing RSD% values in the 3−5% ($n = 5$) and 6−7% ($n = 15$) ranges, respectively (**Table 4**).

Finally, a calculation of the recovery function was performed to test trueness of the developed method and thus to ascertain the influence of the matrix in the determination of biogenic amines. For this purpose, spiked fresh cheese samples were analyzed. The intercept of the recovery function calculated from these data was compared with 0 by means of a *t* test. For all analytes, the *t* values calculated for the intercept (1.14, 1.971, and 1.26 for histamine, tyramine, and β-phenylethylamine, respectively) were not significantly different than the tabulated value at a 95% confidence level (2.11, $\nu = 17$), thus demonstrating that constant systematic errors are not present. Instead, the presence of proportional systematic errors was evidenced because slope values of the recovery functions were demonstrated to be significantly different from 1 ($p < 0.05$),. These findings suggest that to attain accuracy in the quantitative analysis of biogenic amines in cheese samples the use of the labeled internal standard method is recommended.

Table 4. Precision: Intra-Day and Between-Day Repeatability of the LC–MS/MS Method Calculated on Soft Cheese Matrix

		RSD%	
analyte	concentration level (mg/kg)	intra-day repeatability ($n = 5$)	between-day repeatability ($n = 15$)
hystamine	3	5.6	6.8
	15	3.5	7.3
tyramine	5	2.6	6.4
	30	4.7	6.9
β-phenylethylamine	3	4.5	6.2
	50	4.2	6.9

3.4 Determination of Biogenic Amines in Cheese Products Using the LC−ESI−MS/MS Method. The LC−ESI−MS/MS method developed and validated was then applied to the identification and determination of biogenic amines in different aged cheese samples treated by MSPD with the CN solid-phase extraction procedure.

With the aim of adding confidence to the identity of the analytes, two SRM transitions were monitored. In particular, the qualitative analysis of biogenic amines in the samples investigated was based on both the retention time and on the comparison of peak intensity ratios of the *m*/*z* 112 → 68 and *m*/*z* 112 → 95 transitions for histamine and *m*/*z* 138 → 103 and *m*/*z* 138 → 121 for tyramine. The peak height ratio differed by less than 1% from the expected response ratio, confirming the identity of these analytes in the cheese samples (**Figure 4**).

The MSPD procedure carried out on the hard cheese sample by using CN solid phase provided very good extraction recoveries ranging from 84 ± 5 to 90 ± 4% for histamine, from 101 ± 3 to 88 ± 3% for tyramine, and from 104 ± 6 to 99 ± 3% for β-phenylethylamine, with RSD values lower than 6%.

The quantitative assay was performed using suitable solvent calibration curves with the use of the labeled internal standard of each analyte.

β-Phenylethylamine was not detected in all of the samples investigated (**Table 5**), whereas a significant increase of

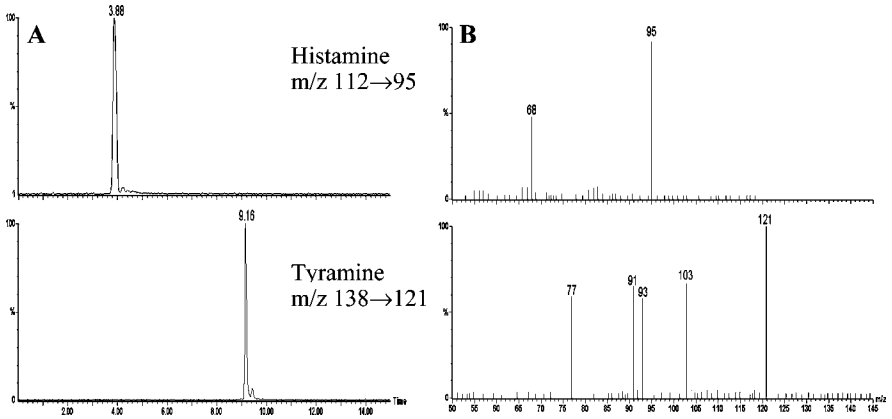


Figure 4. (A) LC–ESI$^+$–MS/MS extracted transition chromatograms of the BAs identified in a hard cheese sample extract (24-month aged; near rind part) and (B) corresponding LC–ESI$^+$–MS/MS product ion mass spectra.

Table 5. Determination of BAs in Hard Cheese Samples at Different Aging Periods

		analyte		
aging period	sample portion	hystamine (mg/kg)[a]	tyramine (mg/kg)[a]	β-phenylethylamine (mg/kg)[a]
12 months	kernel	5.1 ± 0.8	0.78 ± 0.08	nd[b]
	intermediate	4.5 ± 0.5	0.31 ± 0.08	nd
	near rind	4.3 ± 0.2	1.81 ± 0.02	nd
24 months	kernel	5.9 ± 0.6	1.21 ± 0.09	nd
	intermediate	7.4 ± 0.5	0.66 ± 0.02	nd
	near rind	8.9 ± 0.3	0.21 ± 0.03	nd
30 months	kernel	7.5 ± 0.5	1.9 ± 0.2	nd
	intermediate	8.9 ± 0.5	6.7 ± 1.7	nd
	near rind	12.3 ± 0.8	16.8 ± 1.4	nd

[a] $n = 6$. [b] nd = not detected.

histamine and tyramine levels was observed as a function of the aging. In particular, the intermediate and the near rind parts exhibited the highest increase with a significant difference in samples from 24 to 30 months. These findings would suggest that the concentration of biogenic amines increases during ripening and that the BA formation does not occur equally in different parts of the sample or that biogenic amines may diffuse based on water content.

4. CONCLUSIONS

This study demonstrates applicability of a simple MSPD extraction procedure followed by RP-HPLC under gradient conditions and ESI^+−MS/MS detection mode as a rapid and reliable method for qualitative and quantitative analysis of biogenic amines in cheese. With the proposed MSPD-based procedure, extraction and cleanup can be performed in a single step with good recoveries on complex matrixes such as hard cheese samples. Selectivity of the MS/MS technique allows unambiguous identification and accurate determination of BAs in a complex matrix. The evaluation of matrix effects by means of the recovery function indicates the absence of constant systematic errors, otherwise detectable only by using an labeled internal standard. Further, validation data demonstrate that this method is convenient for routine analysis of biogenic amines in cheese products, because detection and quantification limits in the low mg kg^{-1} were calculated and an excellent repeatability was proven.

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Received for review November 23, 2004. Revised manuscript received March 10, 2005. Accepted March 10, 2005. This work was financially supported by MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca, Italy), COFIN 2002 Project "Food safety. New analytical methods for the control of natural trace contaminants".

JF048037G