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Determination of biogenic amines in different cheese samples by LC with evaporative light scattering detector

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ABSTRACT

The paper presents the application of liquid chromatography coupled with evaporative light scattering detector (LC-ELSD) for the determination of biogenic amines in different cheese samples, as their presence and relative amounts give useful information about freshness, level of ripening and quality of storage. Forty samples from different types of milk – hard-ripened, ripened and unripened – were considered. Results showed that the amine contents varied in relation to the manufacturing process, the highest concentration being in hard-ripened cheeses followed by ripened and then unripened. In hard-ripened cheeses amines were β-phenylethylamine (PHE) (69.8–136.6 mg kg^{-1}), tyramine (TYR) (19.7–147.1 mg kg^{-1}), spermidine (SPD) (nd–73.1 mg kg^{-1}), cadaverine (CAD) (nd–64.7 mg kg^{-1}), histamine (HIS) (17.6–48.2 mg kg^{-1}), spermine (SPM) (nd–47.4 mg kg^{-1}), putrescine (PUT) (nd–44.1 mg kg^{-1}) and agmatine (AGM) (nd–4.2 mg kg^{-1}); while in ripened cheese TYR (nd–116.7 mg kg^{-1}), PUT (nd–82.9 mg kg^{-1}), HIS (nd–57.7 mg kg^{-1}), PHE (nd–51.1 mg kg^{-1}), SPD (nd–31.5 mg kg^{-1}), CAD (nd–30.7 mg kg^{-1}), SPM (nd–26.9 mg kg^{-1}) and AGM (nd–4.8 mg kg^{-1}). On the basis of literature limits, in this study only hard ripened cheeses could represent a possible risk for consumers as they exceeded a proposed limit for PHE and total biogenic amines amount.

1. Introduction

Biogenic amines (BAs) are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones (Silla-Santos, 1996; Ten Brink et al., 1990). The decarboxylation process can proceed through two biochemical pathways which are activity of endogenous decarboxylase enzymes naturally occurring in food or activity of exogenous enzymes released by various microorganisms. So, in virtually all foods that contains proteins or free amino acids and subjected to conditions enabling microbial or biochemical activity BAs formation can be expected. However, the total amount of the different amines is strongly variable depending on the nature of food and the microorganisms involved (Halasz et al., 1994; Stratton et al., 1991; Ruiz-Capillas and Jimenez-Colmenero, 2004). The presence of BAs in non-fermented foods generally indicates inadequate or prolonged storage; on the other hand, their presence in fermented foods could be unavoidable due to the diffusion of decarboxylases among lactic acid bacteria.

Among foods, cheese is an ideal substrate for amine production as its manufacturing process involves the availability of free amino acids produced as an outcome of proteolysis levels, but also the possible presence of decarboxylase-positive microorganisms and the environmental conditions that allow their growth as well as the presence of suitable cofactors (pyridoxal phosphate) (Curtin and McSweeney, 2004; Bernardeau et al., 2008; Pereira et al., 2001; Pinho et al., 2004; Linares et al., 2011). Other factors affecting the production of BAs in cheeses include the presence of spoiling microorganisms and the synergistic effects between microorganisms (Silla-Santos, 1996; Marino et al., 2000).

In particular, it has been reported that increases in the amine content of cheese may be attributable to various micro-organisms possessing amino acid decarboxylase activity found among starter lactic acid bacteria, non-starter lactic acid bacteria and/or other spontaneous microflora (Halasz et al., 1994). However, it is difficult to find a straight correlation between microbial counts and BA content in cheese, because amine-producing abilities of different strains of various bacteria differ widely (Linares et al., 2011). Moreover several extrinsic processing factors may also play an important role, namely, pasteurization of milk, pH, salt-in-moisture levels and ripening temperature. In particular, the pH of cheese (5.0–6.5) is optimum for the activity of most decarboxylases and it has been found that the production of BAs

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is accelerated by high temperatures during production and manufacture of cheese and by the prolonged aging process (Gardini et al., 2001; Santos et al., 2003; Gennaro et al., 2003; Pinho et al., 2001; Bunková et al., 2010; Martuscelli et al., 2005; Marino et al., 2008; Novella-Rodrìguez et al., 2002a).

Although several BAs can play important roles in many human physiological functions (Kalac, 2009; Bardócz et al., 1995), their presence in foods is always undesirable because if absorbed at too high concentration, they may induce headaches, respiratory distress, heart palpitations, hypo- or hypertension and several allergenic disorders (Shalaby, 1996; Taylor, 1986; McCabe-Sellers et al., 2006; Jansen et al., 2003). These compounds can represent a serious health hazard for humans and animals when present in food in significant amounts, or ingested in the presence of potentiating factors, such as amine oxidase-inhibiting drugs, alcohol and gastrointestinal diseases (Stratton et al., 1991). Not all amines are equally toxic; HIS, TYR and PHE are of major concern (Shalaby, 1996). Cases of TYR intoxication have occurred subsequent to the consumption of cheese (Stratton et al., 1991; Taylor, 1985, 1986) and the term "cheese reaction" has been coined to refer to it (Silla-Santos, 1996). PUT and CAD may potentiate the toxic effects of HIS and TYR by inhibiting monoamine oxidase, diamine oxidase, and hydroxymethyl transferase (Bardócz et al., 1995; Straub et al., 1995).

As the presence of BAs has great impact on food quality and safety, during recent years different methods have been developed for their identification and quantitative determination. For the separation of BAs, various chromatographic techniques such as thin-layer chromatography, gas chromatography, liquid chromatography (LC) as well as capillary electrophoretic methods are used (Önal, 2007). Since many amines show neither natural UV absorption nor fluorescence, most LC methods require that amines should be derivatized before detection (Innocente et al., 2007; Soufleros et al., 2007; Krause et al., 1995; Özdestan and Üren, 2009; Chiacchierini et al., 2006; Busto et al., 1996; Lozanov et al., 2004; Köros et al., 2008; Pereira et al., 2008; Vandenabeele et al., 1998). LC methods with electrochemical detection have been also employed (Favaro et al., 2007) as well as ultra pressure liquid chromatography techniques (Latorre-Moratallaa et al., 2009; Dadáková et al., 2009; Mayer et al., 2010).

More recently, a new LC method with evaporative light scattering detector (ELSD) has been validated for BA determination in cheese (Restuccia et al., 2011), and the main advantage has been the elimination of the derivatization procedure drawbacks. In this regard, although LC methods coupled with mass spectrometry detection without a previous derivatization step (Gosetti et al., 2007; Gianotti et al., 2008; Saccani et al., 2005) have been developed to quantify BAs in cheese, a severe matrix effect has been reported. Moreover ELSD is more affordable than mass spectrometry, and is also compatible with a broad range of solvents and gradient elution.

The aim of this work is application of an improved LC-ELSD method for quantitative determination of HIS, SPD, SPE, TYR, PUT, CAD, AGM and PEA in 40 commercial cheese samples with different technological characteristics (i.e. kind of milk, milk pasteurization and ripening times). In comparison with the previous study (Restuccia et al., 2011) two other amines have been added and LC parameters have been deeply modified to permit suitable quantitative determination. In particular CAD and AGM were considered: the first because it's one of the most abundant BAs present in cheese and the second because its LC–UV quantitative determination is very difficult to accomplish. Moreover as the first study was applied only to one cheese, the application to many samples which are expected to contain very different BAs amounts could assess the effectiveness of the method.

2. Materials and methods

2.1. Samples

Commercial cheese samples ($n = 40$) of different types were taken from local retail. Italian cheeses with Protected Designation of Origin (PDO) were chosen, as PDO requires that cheese be produced in a defined area under a specific standard of identity. However, other cheese samples without PDO were also considered. In particular, the following cheeses were analyzed: three Parmigiano Reggiano PDO ripened for 30 months (1a–c), two Parmigiano Reggiano PDO ripened for 24 months (2a–b), three Grana Padano PDO ripened for 22 months (3a–c), two grated Parmigiano Reggiano PDO (4a–b), two Grated Grana Padano PDO (5a–b), Provolone Valpadana PDO (6), Pecorino Romano PDO (7), Pecorino Crotonese (8), three ripened goat cheese (9a–c), Emmentaler Switzerland AOC (10), Asiago PDO (11), Taleggio PDO (12), Caciocavallo Silano PDO (13), Montasio PDO (14), Fontina PDO (15), Bel Paese (16), three Caciotta (17a–c), three unripened goat cheeses (18a–c), three cheese spreads (19a–c), three Mozzarella (20a–c), Mozzarella di Bufala Campana PDO (21), Robiola di Roccaverano PDO (22), and Ricotta Romana PDO (23).

Different cheese samples were evaluated in the study. Unripened and ripened cheeses were from raw and pasteurized milk, while hard-ripened cheeses were obtained only from raw milk. Ricotta Romana is an unripened whey cheese and is produced heating at 50–60 °C the whey separated from the ewe milk during the production of Pecorino Romano cheese. Unripened cheeses were manufactured from cow, water buffalo and goat milk; ripened cheeses were obtained from cow, ewe and goat milk and hard ripened cheeses were all from cow milk. Main characteristics of cheese samples are summarized in Table 1.

For BA determination, all cheese samples were cut in half, and a slice 2–3 cm thick was separated from each half. The outer section of each slice (1–2 cm) was removed and discarded; the remaining was reduced to small pieces of about 3 mm of diameter or grated (only extra-hard cheeses); then sample were mixed and homogenized thoroughly into pools. 5 g of the homogenized sample were then subjected to the extraction procedure. Only for samples 4a–b and 5a–b (already grated) and 19, 22 and 23 (slicing or grating were not possible to achieve), 5 g of cheese where directly subjected to extraction procedure.

As far as BAs extraction from cheese samples and SPE purification of the extracts is concerned, the applied protocol have already reported (Restuccia et al., 2011). Briefly, 20 mL of hydrochloric acid 0.1 M were added to about 5.0 g of cheese (or cheese spiked with standard solution), in a 50.0 mL test tube. The mixture was homogenized (vortex at 40 Hz for 40 min), centrifuged (12,000 × g for 25 min), filtered (syringe filter 0.20 μm), collected in a plastic vial and purified by SPE on a C_{18} sorbent (Loading: 4.0 mL of the sample; washing: 2.0 mL of water; Eluting: 2.0 mL (two times) of CH_3OH). The eluting solution, dried up with nitrogen gas and the residue re-dissolved in a plastic test tube with 800 μL of ultrapure water for LC-ELSD analysis. Recovery experiments were performed spiking, before the extraction procedure, samples 2a, 6 and 18b with different aliquots of a BAs standard mix in order to evaluate the method performances at three different levels of concentration. BAs amount added to cheeses were of the same order of magnitude of the supposed BAs concentration of each sample. In particular, sample 2a, sample 6 and sample 18b were spiked, respectively, with 1.0 mL, 0.6 mL and 0.15 mL of a standard solution mixture at concentration of 350 mg L^{-1}.

Table 1
Main characteristics of analyzed cheese samples.

		Raw material	Thermal treatment	Ripening (months)	Firmness	Geographic identification
1a–c	*Parmigiano Reggiano*	Cow milk	Raw	Hard ripened (30)	Extra-hard	PDO
2a–b	*Parmigiano Reggiano*	Cow milk	Raw	Hard ripened (24)	Extra-hard	PDO
3a–c	*Grana Padano*	Cow milk	Raw	Hard ripened (22)	Extra-hard	PDO
4a–b	*Parmigiano Reggiano*	Cow milk	Raw	Hard ripened (12)	Extra-hard (grated)	PDO
5a–b	*Grana Padano*	Cow milk	Raw	Hard ripened (12)	Extra-hard (grated)	PDO
6	*Provolone Valpadana*	Cow milk	Raw	Ripened (4)	Stretched curd	PDO
7	*Pecorino Romano*	Ewe milk	Pasteurized	Ripened (8)	Extra-hard	PDO
8	*Pecorino Crotonese*	Ewe milk	Pasteurized	Ripened (6)	Extra-hard	–
9a–c	*Ripened goat cheese*	Goat milk	Raw	Ripened (6)	Hard	–
10	*Emmentaler Switzerland*	Cow milk	Raw	Ripened (4)	Hard	AOC
11	*Asiago d'Allevo*	Cow milk	Raw	Ripened (6)	Hard	PDO
12	*Taleggio*	Cow milk	Raw	Ripened (1)	Soft	PDO
13	*Caciocavallo Silano*	Cow milk	Raw	Ripened (4)	Semi-hard	PDO
14	*Montasio*	Cow milk	Raw	Ripened (4)	Semi-hard	PDO
15	*Fontina*	Cow milk	Raw	Ripened (3)	Semi-hard	PDO
16	*Bel Paese*	Cow milk	Pasteurized	Ripened (1)	Soft	–
17a–c	*Caciotta*	Cow milk	Pasteurized	Ripened (1)	Soft	–
18a–c	*Unripened goat cheese*	Goat milk	Pasteurized	Unripened (–)	Soft	–
19a–c	*Cheese Spread*	Cow milk	Pasteurized	Unripened (–)	Soft	–
20a–c	*Mozzarella*	Cow milk	Raw	Unripened (–)	Stretched curd	–
21	*Mozzarella di Bufala Campana*	Water buffalo milk	Raw	Unripened (–)	Stretched curd	PDO
22	*Robiola di Roccaverano*	Goat milk	Raw	Unripened (–)	Soft	PDO
23	*Ricotta Romana*	Ewe milk whey	50–60 °c	Unripened (–)	Soft	PDO

PDO, Protected Designation of Origin; AOC, Appellation Origine Contrôlée.

2.2. Chemicals

BAs spermine (SPM, tetrahydrochloride), spermidine (SPD, trihydrochloride), PUT (dihydrochloride), HIS (dihydrochloride), TYR (hydrochloride), CAD (dihydrochloride), agmatine (AGM, sulfate) PHE (hydrochloride), were purchased by Sigma–Aldrich (Milford, MA, USA) as well as hydrochloric acid (37%), ammonia (30%), trifluoroacetic acid (TFA) and LC solvents (acetonitrile and methanol LC grade). Ultrapure water was obtained from Milli-Q System (Millipore Corp., Milford, MA, USA). Filters (0.45 μm and 0.20 μm) were purchased by Sigma–Aldrich (Milford, MA, USA). SPE C_{18} cartridges (0.5 g) were obtained from Supelco Inc. (Bellefonte, PA, USA).

2.3. Amine standard solutions and calibration

For LC-ELSD calibration, standard solutions of each amine were prepared in purified water at concentration of 3500 mg L^{-1}. Successively, ten standard solutions containing all the analytes were obtained by pooling different volumes of each standard solution and adjusting the final volume of 10 mL with purified water. Different concentration ranges were analyzed for each amine: PUT 8.0–350.0, CAD 7.0–350.0, HIS 6.7–350.0, AGM 2.0–350.0, TYR 4.1–350.0, PHE 7.8–350.0, SPD 4.3–350.0 and SPM 5.2–350.0 mg L^{-1}.

The identification of the amines was performed by comparing the retention times of peaks in the samples to those of standard solutions and by addition of the suspected amine to the samples. A calibration plot, reporting the peak area against standard concentration, was constructed for ten concentration levels and three independent replicates for each concentration level were performed. Quantitative determination was accomplished by direct interpolation in the standard curves for each amine. Fig. 1 shows a chromatogram of a standard mix of all the BAs, obtained by LC-ELSD.

2.4. Instrumentations and chromatographic conditions

LC analysis were performed with a Jasco PU-2080 instrument equipped with a Rheodyne 7725 injector with a 20 μL sample loop and a gradient pump (PU-2089 plus, Jasco Inc., Easton, MD, USA). The system was interfaced with an ELS detector (1200 Series, Agilent Tech., Lexington, MA, USA). Data were collected and analyzed with an integrator Jasco-Borwin1. For LC–UV analysis, a reverse-phase C_{18} column (250 mm × 4.6 mm I.D., 5 μm) (Supelco Inc., Bellefonte, PA, USA) equipped with a C_{18} guard-pak (10 mm × 4.6 mm I.D., 5 μm) was used (Supelco Inc., Bellefonte, PA, USA). For LC-ELSD determinations, a Primsep 200 column (SIELC Technologies, Prospect Heights, IL, USA) with Primsep 200 Guard Kit (10 mm × 4.6 mm I.D., 5 μm) was applied. This is a reverse-phase column with embedded weak acidic ion-pairing groups, improving retention of basic compounds by cation-exchange mechanism. A microprocessor pH meter (Hanna Instruments, Eboli (SA), Italy), equipped with a combined glass-calomel electrode, was employed for pH measurements. A centrifuge (Thermo Scientific, Milan, Italy) and a professional cheese grater GF2S (Europroject, Lecce, Italy) were used for the pre-treatment of the cheese samples.

3. Results and discussion

3.1. Method performances

Fig. 1 shows the chromatogram of a BAs standard solution with concentration of 150 mg L^{-1}, while in Fig. 2 the chromatogram of a cheese sample extract is depicted. As can be seen, good resolution of the eight BAs has been accomplished with a total run time of 40 min. ELSD parameters were optimized to obtain good S/N ratio for all amines, as already reported (Restuccia et al., 2011). In particular, as ELSD response is independent on optical characteristics of target molecules, it's possible to note that the signal of AGM is comparable with those of the other compounds, while using the UV detector AGM determination is very hard to accomplish as its UV response is very low.

On the other hand, the introduction of CAD and AGM made necessary to change chromatographic parameters for improving both retention and resolution of analytes. In fact, chromatographic conditions reported in our previous study showed to be unsuitable to separate PUT, CAD, HIS and AGM which appear as two peaks. Moreover, very small modifications of TFA concentration, mobile

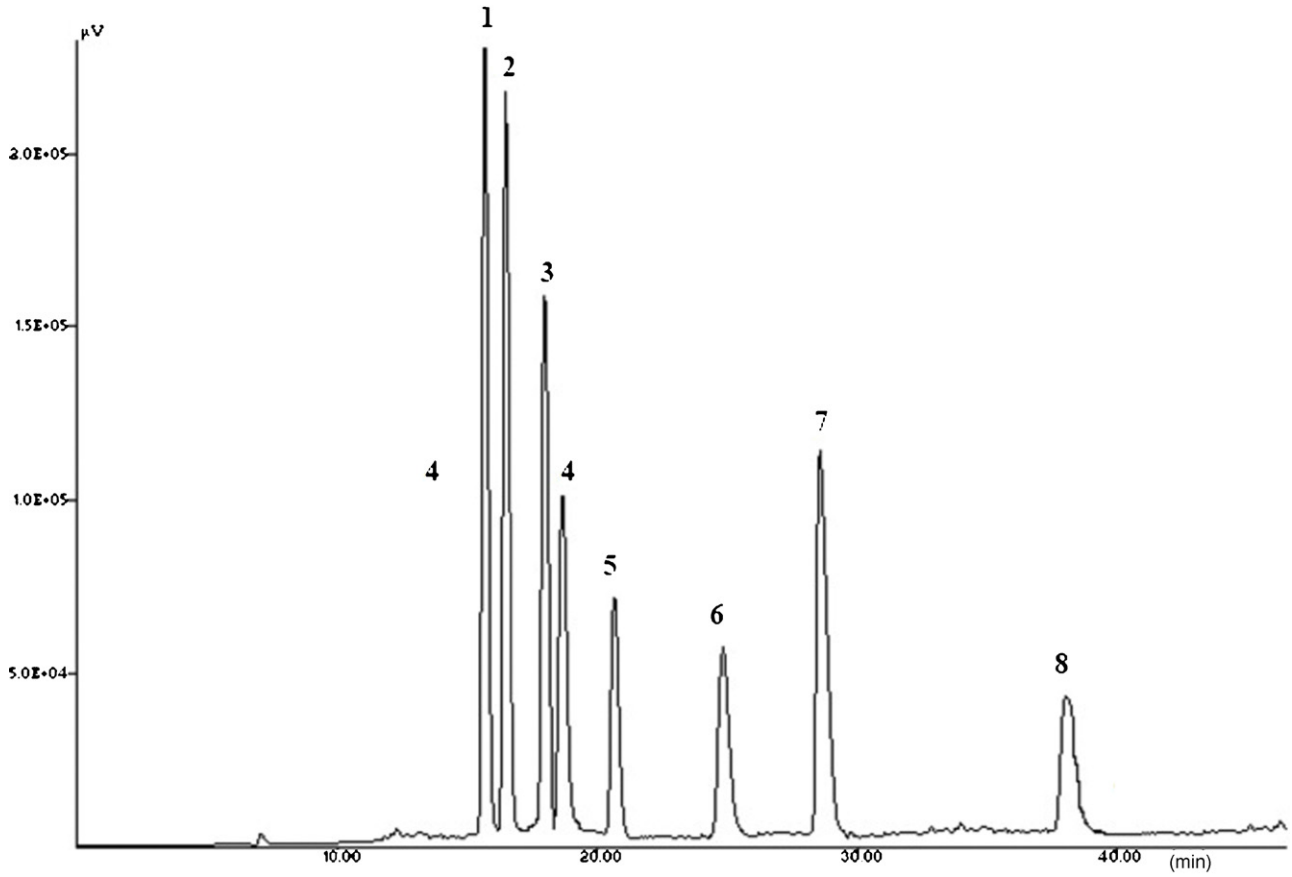

Fig. 1. Chromatogram of a BAs standard mix, obtained by LC-ELSD. The resolution was carried out under gradient conditions as specified in Table 2. The peak numbers in the chromatograms correspond to the following analytes: (1) PUT; (2) CAD; (3) HIS; (4) AGM; (5) TYR; (6) PHE; (7) SPD; (8) SPM. Biogenic amines concentrations 150 mg L^{-1}.

phase composition and gradient elution showed to produce dramatic effects chromatograms appearance. Complete resolution of BAs was finally achieved by increasing the TFA concentration in the mobile phase, the polarity of the mobile phase with higher water concentration and tuning the elution program with amines polarity (Table 2).

Tables 3 and 4 summarize the LC-ELSD method performances. As can be seen, good values of recovery, as well as intra- and inter-day repeatability are reported for all the BAs. Percentages of recovery at the three levels of concentration considered show that the whole procedure of extraction, purification and quantitative determination does not produce significant loss of analytes, even at low concentrations. Limit of detection (LOD) and limit of quantitation (LOQ) values referred to the sample expressed in mg kg^{-1}, are derived from LOD and LOQ values relative to standard solutions, considering all handling steps during sample preparation. As can be seen, the LC-ELSD method is sufficiently sensitive for BA determination in cheese. However, generally speaking, the method is known to possess low sensitivity compared with other LC techniques (Chiacchierini et al., 2006; Latorre-Moratallaa et al., 2009; Gosetti et al., 2007). This is an intrinsic limit of ELSD technology as detectability in the range of 0.1 μg mL^{-1} is considered the lower limit for this detector. It follows that, if analyte concentration is under this threshold, another detector should be preferred or a very severe pre-concentration step should be accomplished. However, referring to cheese analysis, the concentrations of BAs generally found in samples are sufficiently high to achieve suitable quantitative determination. As can be deduced from regression curve equations reported in Table 3, another disadvantage of ELSD is that the relation between concentration and detector response is fundamentally non-linear. Since they are not spectrometric detectors, light scattering detectors do not obey Beer's law, as scattering is independent of the particle's chemical properties and it is a function of multiple processes including Rayleigh scattering, Mie scattering, refraction and reflection. The absolute detector response is a mixture of all scattering types, although any one type may predominate in a given sample. Moreover, the size and shape of the particle, the number of particles, and the wavelength of the incident light all impact light scattering. Because light scattering efficiency changes exponentially with particle size, ELSD response curves are typically complex and often sigmoidal.

3.2. Levels of BAs in cheese samples

Total concentrations of BAs for the 40 cheese samples are reported in Fig. 3, where it is possible to note that the higher is the ripening period, the higher is the total amine content in cheese samples. This conclusion has been already reported by several authors (Bunková et al., 2010; Lapa-Guimaraes and Pickova, 2004; Novella-Rodrìguez et al., 2002b; Ordonez et al., 1997; Schneller et al., 1997) as the time of ripening is considered a critical factor for the BAs accumulation. The proteolysis occurring during cheese aging, in fact, may increase the release of amino acids from casein which can be successively decarboxylated by bacterial enzymes, giving rise to the accumulation of BAs. In many cases, the accumulation of BA has been attributed mainly to the activity of the non-starter microflora. However an indirect role of the starter lactic acid bacteria can be hypothesized as the peptidases released by the lysis of starter lactic acid bacteria could be essential to provide precursor amino acids (Valsamaki et al., 2000). In any case, prolonged ripening seems to increase BAs concentration, although wide variations of total BA contents were observed among ripened

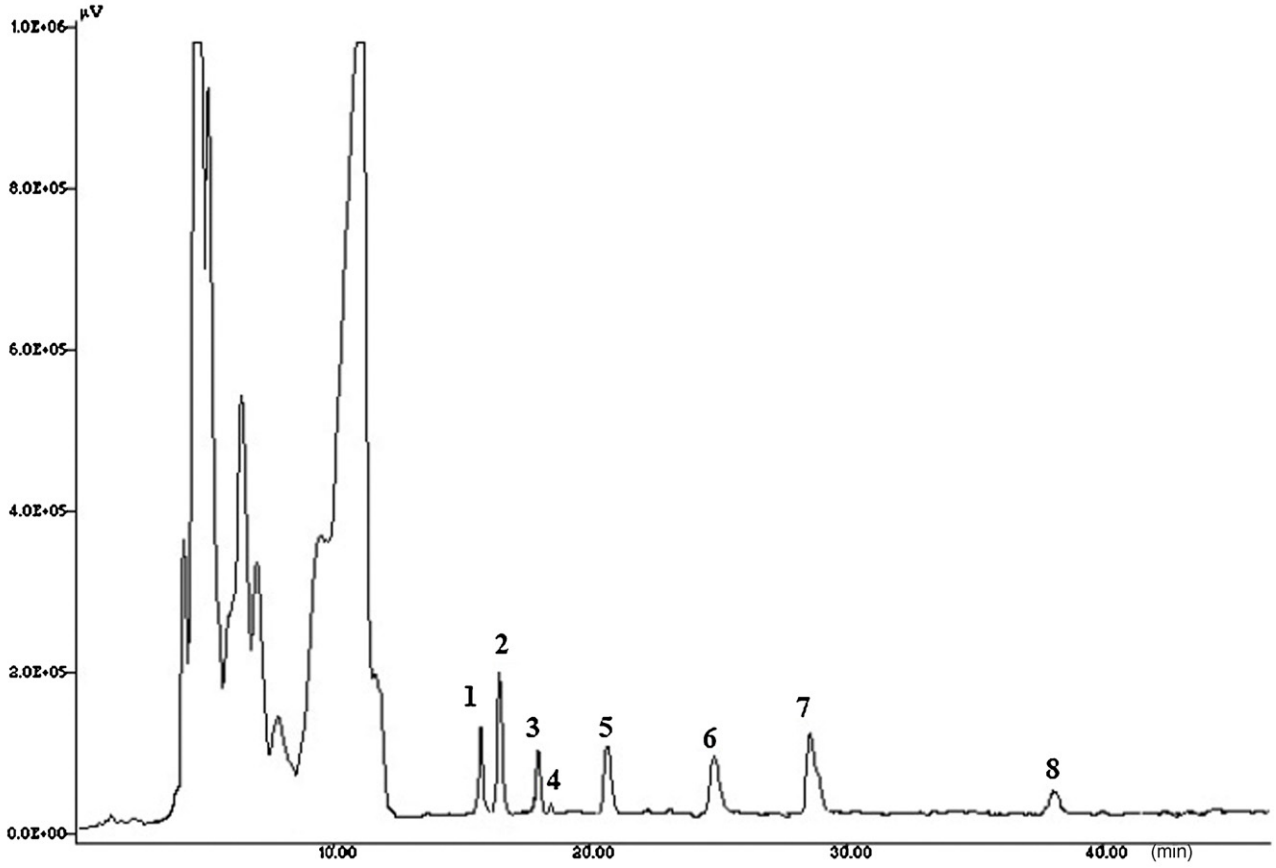

Fig. 2. Chromatogram of the extract of sample 1a obtained by LC-ELSD. The resolution was carried out under gradient conditions as specified in Table 2. The peak numbers in the chromatograms correspond to the following analytes: (1) PUT; (2) CAD; (3) HIS; (4) AGM; (5) TYR; (6) PHE; (7) SPD; (8) SPM.

cheeses, probably in relation with the intensity of the ripening process (Novella-Rodrìguez et al., 2003; Gosetti et al., 2007). On the other hand, also milk pasteurization seems to have influence on BAs total content. As can be seen, samples 7 and 8 are, respectively, 8 and 6 months ripened but contain similar amounts of BAs showed by samples 12 and 13 which are 1 and 4 months ripened, respectively. This result seems to indicate that also the heat treatment of the milk can play a role in the BAs formation. This is not surprising when considering that many decarboxylating bacteria do not survive pasteurization. As the use of milk of high bacteriological quality is critical for amine formation (Gennaro et al., 2003; Lanciotti et al., 2007), it follows that pasteurization can be also crucial because it reduces the growth of microorganisms that form amines. Many studies confirmed this hypothesis reporting that in the cheeses made from raw milk, the bacteria contents are higher than in those made from pasteurized milk (Gennaro et al., 2003; Ordonez et al., 1997; Schneller et al., 1997; Novella-Rodrìguez et al., 2002b). However, Ladero et al. (2011) studied the resistance of the most common species thought responsible for BA production in dairy products to pasteurization (63 °C for 30 min), concluding that many native, metabolically active BA producers could be found in pasteurized milk owing to their resistance to the thermal treatment. Although the pasteurization of milk reduces the BA content of cheese (Novella-Rodrìguez et al., 2004; Fernandez et al., 2007) other factors related with thermal treatment should be considered such as the slower rate of proteolysis observed in cheeses made from pasteurized milk (Lau et al., 1991) and the inactivation by heat of the cofactor for decarboxylase activity (pyridoxal phosphate) (Ladero et al., 2008). However, comparing sample 7 and 8 with sample 12 and 13 it should be also considered that they come from milks of different origin (ewe and cow). To this regard, few and contradictory literature data are available on the specific effect of the type of milk on the BA content of cheeses (Novella-Rodrìguez et al., 2002b; Ladero et al., 2008; Lanciotti et al., 2007). Comparing samples 9a–c and 11 made both from raw milk and with the same ripening time, similar BAs amounts were found although they are made from cow and goat milk. Conversely the comparison between samples 12 and 16 made both from bovine milk and 1 month ripened shows that sample 16, made from pasteurized milk, contains a much lower BAs concentration than sample 12. This suggests that, if the type of milk affects BAs formation, its influence is not so relevant in comparison with heat treatment of milk.

Table 2
Chromatographic conditions and detection parameters.

	t (min)	A^*–B^* (v/v)
Gradient elution	0	95:5
	10	70:30
	15	70:30
	25	20:80
	35	20:80
	40	95:5
Column	Primsep 200 (250 mm × 4.6 mm I.D., 5 µm) Primsep 200 Guard kit (10 mm × 4.6 mm I.D., 5 µm)	
Loop/Eluent flow	20 µL/0.7 mL min^{-1}	
Detection parameters	N_2 flow = 3.7 bar Drift tube temperature = 40 °C	

A^*: acetonitrile/water 5/95 (v/v) containing trifluoroacetic acid (0.06%, v/v); B^*: acetonitrile/water 5/95 (v/v) containing trifluoroacetic acid (0.45%, v/v).

Table 3
Method performances for extraction protocol and LC-ELSD analyses of BAs ($n = 5$).

BAs	Calibration curve equation	R^2	LOD		LOQ	
			mg L^{-1}	mg kg^{-1}	mg L^{-1}	mg kg^{-1}
PUT	$y = 3.71347 \cdot 10^6 x^3 + 1.27374 \cdot 10^6 x^2 + 9.26984 \cdot 10^7 x + 6.38911 \cdot 10^6$	0.9926	3.2	2.6	8.0	6.4
CAD	$y = -1.55234 \cdot 10^8 x^3 + 1.92028 \cdot 10^8 x^2 - 6.26289 \cdot 10^6 x + 1.54326 \cdot 10^6$	0.9974	2.8	2.2	7.0	5.6
HIS	$y = -3.70251 \cdot 10^8 x^3 + 2.00093 \cdot 10^8 x^2 - 5.87979 \cdot 10^6 x + 1.83023 \cdot 10^6$	0.9986	2.7	2.1	6.7	5.4
AGM	$y = -3.21786 \cdot 10^8 x^3 + 1.58103 \cdot 10^8 x^2 + 9.82437 \cdot 10^5 x + 1.36388 \cdot 10^6$	0.9934	1.0	0.8	2.5	2.0
TYR	$y = 2.95851 \cdot 10^7 x^3 + 3.23786 \cdot 10^7 x^2 + 8.44175 \cdot 10^6 x + 1.34095 \cdot 10^6$	0.9982	1.5	1.2	4.1	3.2
PHE	$y = -6.73601 \cdot 10^7 x^3 + 3.10211 \cdot 10^7 x^2 + 6.75341 \cdot 10^6 x + 8.32638 \cdot 10^5$	0.9938	2.8	2.2	7.8	6.2
SPD	$y = -1.81503 \cdot 10^8 x^3 + 1.6654 \cdot 10^8 x^2 - 1.00931 \cdot 10^7 x + 2.97413 \cdot 10^6$	0.9936	1.7	1.4	4.3	3.4
SPM	$y = -2.21522 \cdot 10^8 x^3 + 7.99269 \cdot 10^7 x^2 + 1.37881 \cdot 10^7 x + 1.14492 \cdot 10^6$	0.9955	1.8	1.4	5.2	4.2

PUT, putrescine; CAD, cadaverine; HIS, histamine; AGM, agmatine; TYR, tyramine; PHE, β-phenylethylamine; SPD, spermidine; SPM, spermine, LOD, limit of determination; LOQ, limit of quantitation.

Table 4
Values of recovery, intra- and inter-day repeatability, performed with LC-ELSD method. Data represent mean ± SD ($n = 5$).

BAs	Recovery (%)[a]	Recovery (%)[b]	Recovery (%)[c]	RSD intra-day	RSD inter-day
PUT	98 ± 2	101 ± 3	96 ± 3	0.7	1.5
CAD	99 ± 4	95 ± 3	96 ± 3	1.0	1.9
HIS	103 ± 1	105 ± 1	100 ± 1	0.5	1.1
AGM	98 ± 3	99 ± 4	102 ± 4	0.8	1.0
TYR	95 ± 2	99 ± 4	96 ± 4	0.7	1.3
PHE	101 ± 4	98 ± 3	97 ± 3	1.3	2.2
SPD	96 ± 4	103 ± 2	100 ± 1	1.3	2.4
SPM	95 ± 2	99 ± 4	102 ± 1	1.0	2.1

PUT, putrescine; CAD, cadaverine; HIS, histamine; AGM, agmatine; TYR, tyramine; PHE, β-phenylethylamine; SPD, spermidine; SPM, spermine.
[a] Recovery sample 2a (high concentration). BAs added to 5 g of cheese: 1.0 mL of a standard mix 350 mg L^{-1}.
[b] Recovery sample 6 (medium concentration). BAs added to 5 g of cheese: 0.6 mL of a standard mix 350 mg L^{-1}.
[c] Recovery sample 18b (low concentration). BAs added to 5 g of cheese: 0.15 mL of a standard mix 350 mg L^{-1}.

Considering the BAs distribution in cheese samples (Table 5), a great variability is observed, not only among samples belonging to different classes, but also within the same class of cheeses as already confirmed by other studies (Novella-Rodrìguez et al., 2000, 2003; Mayer et al., 2010; Gosetti et al., 2007; Ordonez et al., 1997). Very small amounts or no BAs were found in unripened cheeses, suggesting that the presence of high quantities of amines in these type of cheeses should be considered as a consequence of a poor hygienic milk quality. Although the absence of ripening seems to play the key role in explaining the obtained results, other factors can produce synergistic effects. In fact, the use of pasteurized milk as for sample 18a–c, can further decrease the BA content. In this case, except for PHE, mainly polyamines are found which do not necessarily result from bacterial metabolism as they can be naturally found in foods (Bardócz et al., 1995). In case of samples 20a–c, 21 and 22, although made from raw milk, the total absence of BAs can be also explained by the manufacture process involving also unheated curd; this is considered another parameter negatively affecting the accumulation of BAs (Gennaro et al., 2003). The small amount of TYR and CAD found in cheese spread

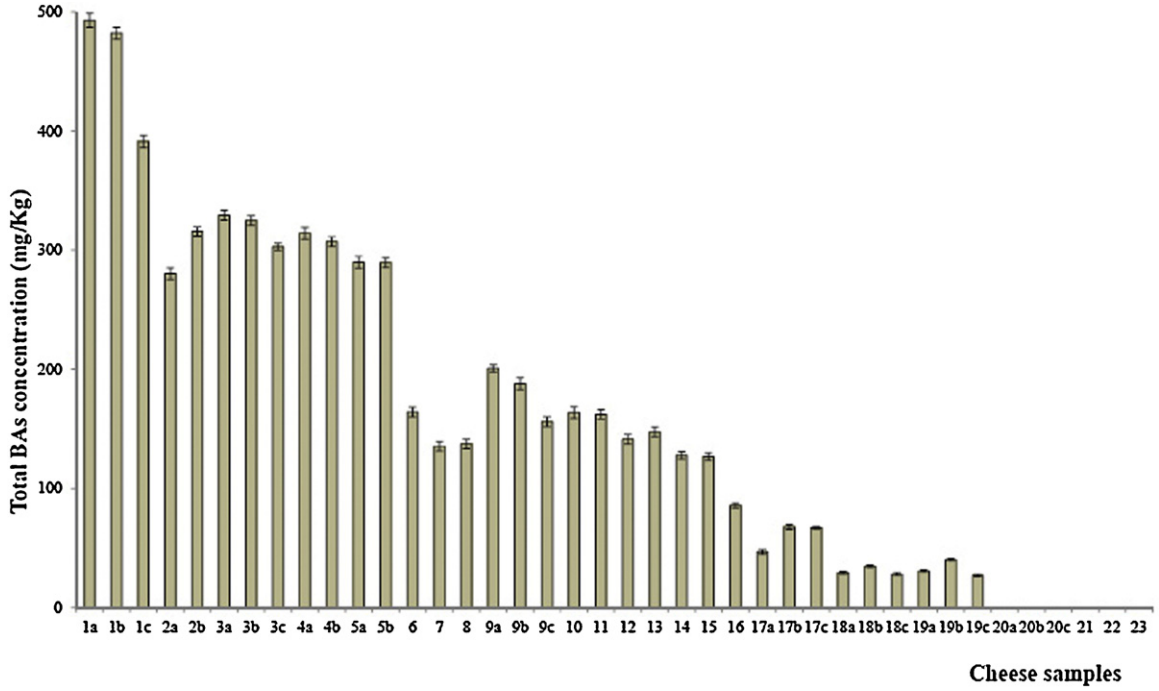

Fig. 3. Total concentration of BAs (mg kg^{-1}) determined by LC-ELSD in cheese samples. The SPE eluting solution was dried up with nitrogen gas and the residue re-dissolved with 800 μL of ultrapure water. Standard deviations derived from five analysis of each sample.

Table 5
Concentration of BAs (mg kg^{-1}) in cheese samples. Data represent mean ± SD (n = 5).

Code	Cheese	Biogenic amines (mg kg^{-1})							
		PUT	CAD	HIS	AGM	TYR	PHE	SPD	SPM
1a–c	a: *Parmigiano Reggiano PDO 30 months (Roncoscaglia)*	25.9 ± 2.3	64.7 ± 1.1	28.9 ± 2.4	2.8 ± 0.1	124.5 ± 4.1	136.6 ± 3.2	73.1 ± 1.5	36.7 ± 2.3
	b: *Parmigiano Reggiano PDO 30 months (Ambrosi)*	30.7 ± 2.1	50.2 ± 0.4	36.5 ± 3.1	3.5 ± 0.1	147.1 ± 2.8	101.1 ± 2.8	65.7 ± 2.4	47.4 ± 3.3
	c: *Parmigiano Reggiano PDO 30 months (Sma)*	19.1 ± 1.6	27.0 ± 0.9	38.4 ± 2.3	ND	100.5 ± 0.7	97.7 ± 3.6	70.4 ± 1.8	38.2 ± 2.1
2a–b	a: *Parmigiano Reggiano PDO 24 months (San Lorenzo)*	25.7 ± 1.3	55.1 ± 2.1	28.9 ± 2.3	4.2 ± 0.4	19.7 ± 2.2	135.2 ± 3.5	ND	11.5 ± 1.2
	b: *Parmigiano Reggiano PDO 24 months (Roncoscaglia)*	12.3 ± 2.0	24.7 ± 1.3	34.2 ± 1.3	2.8 ± 0.2	125.4 ± 3.1	97.2 ± 2.4	24.4 ± 1.6	12.0 ± 0.8
3a–c	a: *Grana Padano PDO 22 months (Ambrosi)*	ND	43.1 ± 1.4	23.9 ± 1.4	2.1 ± 0.6	129.1 ± 3.0	122.0 ± 2.1	3.1 ± 0.1	6.0 ± 0.7
	b: *Grana Padano PDO 22 months (Levoni)*	44.1 ± 16	ND	21.4 ± 1.1	ND	118.7 ± 2.4	128.4 ± 2.4	12.4 ± 1.1	ND
	c: *Grana Padano PDO22 months (Casearia Cantarelli)*	30.7 ± 0.8	27.4 ± 0.5	17.6 ± 0.6	ND	100.2 ± 31	107.7 ± 3.3	8.7 ± 0.7	10.5 ± 1.0
4a–b	a: *Grated Parmigiano Reggiano PDO 12 months (Fiorucci)*	27.3 ± 2.2	27.1 ± 1.8	48.2 ± 1.8	3.0 ± 0.4	107.4 ± 3.8	88.7 ± 1.4	ND	12.5 ± 1.1
	b: *Grated Parmigiano Reggiano PDO 12 months (Sma)*	30.4 ± 1.8	20.9 ± 1.2	33.1 ± 0.4	2.1 ± 0.1	122.1 ± 1.9	69.8 ± 2.1	20.1 ± 1.0	8.7 ± 0.6
5a–b	a: *Grated Grana Padano PDO 12 months (Sma)*	20.1 ± 1.6	17.3 ± 2.4	22.1 ± 0.8	ND	127.8 ± 2.5	87.9 ± 1.1	14.6 ± 0.9	ND
	b: *Grated Grana Padano PDO 12 months (CRAI)*	24.7 ± 0.9	ND	38.1 ± 0.8	2.3 ± 0.1	110.3 ± 2.6	95.0 ± 2.8	8.8 ± 0.2	10.3 ± 0.7
6	*Provolone Valpadana PDO (Auricchio)*	11.8 ± 1.2	21.1 ± 2.6	9.7 ± 1.2	2.2 ± 0.1	86.8 ± 3.8	ND	31.5 ± 3.6	ND
7	*Pecorino Romano PDO (Brunelli)*	ND	8.9 ± 0.5	ND	ND	116.7 ± 4.1	9.8 ± 0.9	ND	ND
8	*Pecorino Crotonese (Maiorano)*	16.6 ± 1.5	8.7 ± 0.2	19.1 ± 1.6	ND	53.1 ± 3.7	24.2 ± 2.6	15.7 ± 0.9	ND
9a–c	a: *Ripened goat cheese (La Casera)*	82.9 ± 3.1	16.3 ± 0.8	42.6 ± 2.9	3.9 ± 0.2	ND	26.1 ± 2.7	13.5 ± 1.3	15.5 ± 1.4
	b: *Ripened goat cheese (San Lorenzo)*	65.7 ± 2.4	10.9 ± 0.6	50.2 ± 0.7	ND	10.1 ± 1.8	15.1 ± 2.1	20.1 ± 0.8	15.8 ± 0.8
	c: *Ripened goat cheese (Occelli)*	45.4 ± 0.9	30.4 ± 0.3	33.9 ± 1.4	4.8 ± 0.1	6.3 ± 0.2	19.7 ± 3.0	8.8 ± 0.5	6.7 ± 0.4
10	*Emmentaler Switzerland AOC (Huguenin SA)*	28.1 ± 2.3	9.7 ± 0.2	37.4 ± 2.4	2.4 ± 0.1	16.7 ± 1.3	54.1 ± 3.6	ND	15.3 ± 1.3
11	*Asiago d'Allevo PDO (Basso)*	28.8 ± 1.5	7.4 ± 0.4	57.7 ± 3.7	3.8 ± 0.4	33.9 ± 2.7	ND	30.6 ± 2.7	ND
12	*Taleggio PDO (Conad)*	ND	12.6 ± 0.7	ND	ND	80.3 ± 3.9	8.8 ± 0.9	24.3 ± 2.5	15.3 ± 2.3
13	*Caciocavallo Silano PDO (Eurolatte)*	15.5 ± 1.6	20.5 ± 1.2	42.0 ± 3.4	ND	28.5 ± 2.3	10.2 ± 1.4	3.8 ± 0.8	26.9 ± 2.5
14	*Montasio PDO (APROLAV)*	50.3 ± 3.7	10.4 ± 1.0	16.8 ± 1.5	2.2 ± 0.1	8.1 ± 0.7	19.8 ± 1.5	13.9 ± 1.4	6.7 ± 0.8
15	*Fontina PDO (Dunoyer)*	8.6 ± 0.7	31.4 ± 1.7	10.4 ± 0.8	ND	71.2 ± 2.8	ND	5.1 ± 0.8	ND
16	*Bel Paese (Galbani)*	8.5 ± 0.7	24.6 ± 1.6	ND	ND	21.1 ± 2.5	15.4 ± 1.3	10.8 ± 1.2	5.3 ± 0.5
17a–c	a: *Caciotta (Grifo Latte)*	10.7 ± 2.5	ND	ND	ND	19.0 ± 1.8	6.2 ± 1.8	3.7 ± 0.6	7.1 ± 1.1
	b: *Caciotta (Brunelli)*	ND	30.7 ± 2.0	5.5 ± 0.5	ND	11.0 ± 1.2	7.7 ± 1.3	8.4 ± 0.9	4.4 ± 0.7
	c: *Caciotta (La Casara)*	7.6 ± 1.4	19.9 ± 1.4	5.9 ± 0.6	ND	10.6 ± 2.0	ND	12.7 ± 0.2	10.3 ± 0.4
18a–c	a: *Unripened goat cheese (Guffanti)*	ND	ND	ND	2.0 ± 0.3	ND	8.1 ± 0.5	4.8 ± 0.5	14.3 ± 1.3
	b: *Unripened goat cheese(Mantuanella)*	ND	6.1 ± 0.3	ND	ND	ND	9.2 ± 1.1	5.5 ± 0.1	13.7 ± 1.0
	c: *Unripened goat cheese (Di Nucci)*	ND	ND	ND	3.1 ± 0.3	3.3 ± 0.4	6.5 ± 0.3	6.2 ± 0.4	8.9 ± 0.9
19a–c	a: *Cheese Spread (Galbani)*	ND	5.8 ± 0.9	ND	ND	24.9 ± 1.6	ND	ND	ND
	b: *Cheese Spread (Parmareggio)*	ND	ND	ND	ND	40.2 ± 0.8	ND	ND	ND
	c: *Cheese Spread (Inalpi)*	ND	7.9 ± 1.1	ND	ND	19.1 ± 1.1	ND	ND	ND
20a–c	a: *Mozzarella (Vallelata)*	ND	ND	ND	ND	ND	ND	ND	ND
	b: *Mozzarella (Galbani)*	ND	ND	ND	ND	ND	ND	ND	ND
	c: *Mozzarella (Granarolo)*	ND	ND	ND	ND	ND	ND	ND	ND
21	*Mozzarella di Bufala Campana PDO (Granarolo)*	ND	ND	ND	ND	ND	ND	ND	ND
22	*Robiola di Roccaverano PDO (Arbiora)*	ND	ND	ND	ND	ND	ND	ND	ND
23	*Ricotta Romana PDO (Brunelli)*	ND	ND	ND	ND	ND	ND	ND	ND

PUT, putrescine; CAD, cadaverine; HIS, histamine; AGM, agmatine; TYR, tyramine; PHE, β-phenylethylamine; SPD, spermidine; SPM, spermine. ND, not detectable.

probably derived from the cheese refuses used for their preparation. In case of Ricotta Romana no BAs were found. Although Gosetti et al. (2007) found a small amount of TYR and SPD in ricotta cheese no other literature data are available for confirmation. Moreover, no manufacture parameters were declared in the cited study regarding the ricotta sample analyzed, making impossible any direct comparison between data.

Hard-ripened cheeses were the products with higher BA content; they were all Parmesan cheeses which is a general term used to refer to hard ripened cheeses made from cow's raw milk, whose principal feature is represented by a grainy texture. Parmigiano Reggiano and Grana Padano are the names of the two PDO cheeses with long ripening time of at least 12 months. Besides the long ripening period, it should be underlined that the formation of biogenic amines in cheese is enhanced by temperature (mainly if >18 °C), pH (>5) and low salt content as demonstrated by different authors (Ten Brink et al., 1990; Joosten, 1988). Samples 1–5 present all the favorable conditions to the accumulation of biogenic amines (pH > 5.30 and sodium chloride concentration <3%). Surprisingly, all the analyzed cheeses

were characterized by an high amount of PHE and TYR. To this regard, Joosten (1988), Petridis and Steinhart (1996), Bover-Cid and Holzapfel (1999), Montel et al. (1999) and Gardini et al. (2001), observed that enterococci lead to accumulation of PHE and TYR at the same time being apparently not able to produce relevant amounts of PUT. Enterococci have been found in this kind of cheese (Coppola et al., 2000; Lazzi et al., 2004), although, they do not seem to be dominant and final conclusion cannot be drawn as the literature is lacking in the evaluation of the decarboxylating activity of Parmesan cheeses strains.

Considering ripened cheeses, once again, a wide distribution is observed among amines. TYR (nd–116.7 mg kg^{-1}) was present in most of the samples with higher concentrations, followed by PUT (nd–82.9 mg kg^{-1}), HIS (nd–57.7 mg kg^{-1}), PHE (nd–51.1 mg kg^{-1}), SPD (nd–31.5 mg kg^{-1}), CAD (nd–30.7 mg kg^{-1}), SPM (nd–26.9 mg kg^{-1}) and AGM (nd–4.8 mg kg^{-1}). SPD and SPM were found in most samples of ripened cheeses and, in general, SPD was the prevailing polyamine, confirming previous data reported in literature (Lapa-Guimaraes and Pickova, 2004; Novella-Rodrìguez et al., 2003; Lanciotti et al., 2007), while other studies report an opposite trend (Mayer et al., 2010; Gosetti et al., 2007). It has been reported that the levels of polyamines in unripened cheeses were higher than those in milks only reflecting the effect of the milk concentration (Novella-Rodrìguez et al., 2003). However, the higher contents observed in ripened cheeses could hardly be explained by a concentration effect only, supporting the idea that the production of polyamines throughout the ripening process can take place as well (Lapa-Guimaraes and Pickova, 2004).

Finally, in case of ripened goat cheese (samples 9a–c) major amines were PUT and HIS and low concentrations of TYR can be noted. Obtained data are in accordance with those obtained by Galgano et al. (2007) who studied four experimental lots of cheeses Semicotto Caprino, a traditional Italian goats' cheese. In Semicotto Caprino, results showed that all the BAs monitored, increased during ripening, the most abundant at each ripening time being PUT. HIS was not detected at 15 days (as in case of samples 18a–c), but it reached 104 mg kg^{-1} cheese at the end of ripening (60 days).

While no upper limit of BAs levels of cheese is in place in legislation, some limits have been posed only for HIS. European legislation (EC No. 2073/2005) limits HIS levels in fishery products to 200 mg kg^{-1} for fresh fish and up to 400 mg kg^{-1} for cured products while the US Food and Drug Administration consider it a danger to health if the HIS level is equal to 500 mg kg^{-1}. Moreover some authors have suggested other limits either for HIS alone (100 mg kg^{-1}) (2) or as a sum of HIS, TYR, PUT and CAD (900 mg kg^{-1}) (Shalaby, 1996; Valsamaki et al., 2000). Previous studies (Nout, 1994) considered that not all amines are equally toxic and HIS, TYR and PHE were considered of major concern, proposing for fermented foods 50–100 mg kg^{-1}, 100–800 mg kg^{-1} and 30 mg kg^{-1} for HIS, TYR and PHE, respectively, or a total of 100–200 mg kg^{-1}. As can be noted from reported data, none of the analyzed samples represent a possible risk for consumer health considering the suggested limits, except PHE concentrations found in hard-ripened cheese are far above 30 mg kg^{-1} as well as BAs total amount exceeding 200 mg kg^{-1} in this kind of cheese. On the basis of our results, hard ripened cheese made from raw milk should be avoided in sensitive people, also taking into account additional risk factors such as amine oxidase-inhibiting drugs, alcohol and gastrointestinal diseases which can play an important role in determining the threshold for BAs toxicity.

4. Conclusions

The LC-ELSD method proposed in this study permitted the quantitative determination of eight BAs in cheese samples avoiding the derivatization step with time-saving and with good values of recovery and sensitivity, even at low concentrations. Among cheeses, there were great differences in total concentrations of BAs, with the hard-ripened cheeses showing the greatest contents, followed by ripened and unripened cheeses. None of the analyzed samples represented a possible risk for consumer health according to the toxicity levels posed by legislation or reported in literature and regarded as acceptable. However, hard-ripened cheeses exceeded one of the suggested limit for PHE concentration and BAs total amount while very small amounts or no BAs were found in unripened cheeses irrespective of their characteristics.

This result confirms that BA total amount is mostly dependent on the ripening period and, to a lesser extent, on the milk heat treatment and type of milk. However, a great variability was observed in the distribution of BAs, not only among the different classes of cheese but also within the same type. Among different cheeses, these differences could be related with microbiological and hygienic features of raw materials, as well as with different handling and cheese-making practices, all these aspects affecting the conditions that might support growth and activity of certain groups of microorganisms directly involved in proteolytic degradation and/or BAs synthesis. These differences make impossible to draw a general conclusion also for POD cheeses which are supposed to be produced under strictly controlled manufacture process. Also in this case, small differences in quality of milk (generally raw) and cheese-making practice can produce significant differences in BAs distribution.

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