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A new method for the determination of biogenic amines in cheese by LC with evaporative light scattering detector

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ABSTRACT

This paper presents a new LC method with evaporative light scattering detection (ELSD), for the separation and determination of the biogenic amines (histamine, spermidine, spermine, tyramine, putrescine and β-phenylethylamine) which are commonly present in cheese, as their presence and relative amounts give useful information about freshness, level of maturing, quality of storage and cheese authentication. The LC–ELSD method is validated by comparison of the results with those obtained through LC–UV determination, based on a pre-column dansyl chloride derivatisation step. The obtained data demonstrate that both methods can be interchangeably used for biogenic amines determination in cheese. The new LC–ELSD method shows good precision and permits to achieve, for standard solutions, limit of detection (LOD) values ranging from 1.4 to 3.6 mg L^{-1} and limit of quantitation (LOQ) values ranging from 3.6 to 9.3 mg L^{-1}. The whole methodology, comprehensive of the homogenization–extraction process and LC–ELSD analysis, has been applied in the analysis of a typical Calabria (Southern Italy) POD cheese, known as Caciocavallo Silano. The most aboundant amine found was histamine, followed, in decreasing order, by tyramine, spermine, putrescine, β-phenylethylamine and spermidine, for a total amount of 127 mg kg^{-1}. This value does not represent a possible risk for consumer health, according to the toxicity levels reported in literature and regarded as acceptable.

1. Introduction

Bioactive amines are nitrogenous compounds that can be classified by their formation pathway and biological functions. During the polyamine biosynthesis, they are formed by the activity of endogenous decarboxylase enzymes, naturally occurring in food, and play a variety of biological roles [1,2]; on the contrary, biogenic amines (BAs) are detrimental to health and originate in foods from decarboxylation of the corresponding amino acid and transamination of aldehydes and ketones by the activity of exogenous enzymes released by various microorganisms [3–6]. Therefore, BAs may be of endogenous origin at low concentrations even in non-fermented foods such as fruits, vegetables, meat, milk and fish, while high concentrations of these compounds can be found as a consequence of microbial activity in foods such as wine, fermented meat and fish products, cheese and fermented vegetables [2–4]. Since the consumption of food containing high concentrations of BAs may cause toxic reactions in susceptible individuals [2,3,6,7], in recent years considerable research has been undertaken to evaluate the presence of these compounds in various fermented, seasoned or conserved foodstuffs. Among foods commonly associated with the presence of BAs, particular attention has been devoted to cheese. Many factors have been found to affect the production of biogenic amines in cheeses, including the presence of microorganisms decarboxylating free amino acids [8–12], the presence of spoiling microorganisms [13], the synergistic effects between microorganisms and the availability of free amino acids produced as an outcome of proteolysis levels [14–17]. Several extrinsic processing factors may also play an important role, namely, pH, salt-in-moisture levels and ripening temperature [18,19]. Because the content of BAs is influenced by ripening and quality of storage conditions, interest in the presence and amount of BA in food is always increasing, not only because of the potential toxicity but also because the content of BAs is an useful parameter to evaluate the extent of ripening and the quality of storage [11,20,21].

Different analytical methods have been used to determine BAs in foods. Since most amines show neither natural UV absorption nor fluorescence, most methods require that amines should be derivatized before detection. Different chemical regents have been used for the amine analysis, such as dansyl chloride [22–27], dabsyl chloride [28–30], o-phthlaldehyde [31–34], fluorescein isothiocyanate

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[35–37], dichlorotriazinylamino-fluorescein [38], benzoyl chloride [39], 6-aminoquinolyl-N-hydroxysuccinimidyl arbamate [40,41], 9-fluorenylmethylchloroformate [42] and phenylisothiocyanate [43]. For the separation of BAs, various chromatographic techniques such as thin-layer chromatography, gas chromatography, LC as well as capillary electrophoretic methods are used [22,44–50]. LC methods with electrochemical detection have been also employed [51–53] as well as UPLC techniques [54–56]. Mass spectrometry detection with or without a previous derivatisation step [23,57–59] have been also developed to quantify BAs. However, a severe matrix effect has been reported either for reversed-phase with electrospray ionization (ESI) LC–MS/MS method or for hydrophilic interaction liquid chromatography (HILIC) LC–MS/MS method with atmospheric pressure chemical ionization (APCI). This effect is overcome by applying the standard addition method for quantification of each amine in cheese samples [57,58].

For detection of BAs in foods, LC–ELSD could represent a useful alternative. ELSD response is based on the amount of light scattered by analyte particles created by evaporation of a solvent as it passes through a light beam. Therefore, the resulting signal corresponds to all compounds present in the sample which do not evaporate or decompose during evaporation of the solvent or mobile phase [60,61]. ELS detectors are especially attractive for determining non-volatile compounds not absorbing above 200 nm as their responses are independent of their optical characteristics. It follows that no chromophores need to be present in target molecules and no derivatization is required during the analytical process, avoiding the drawbacks of this analytical step (dependence on experimental parameters, incompleteness of derivatization reaction, prolonged analysis time, additional cost for derivatization system and reagents). Moreover, ELS detectors are more affordable than mass spectrometers and compatible with a broad range of solvents and gradient elution. Food applications of ELSD are mostly related with carbohydrate and lipids analysis [62–70]. Only in 1999, Sadain and Koropchak [71] applied condensation nucleation light scattering detection (CNLSD) for determination of biogenic amine in fish samples while to the best of the authors' knowledge, no LC–ELSD methods have been proposed for analytical determination of BAs in cheese. The aim of the present study is the application of a chromatographic method using ELS detector for quantitative evaluation of biogenic amines in an Italian POD cheese (Caciocavallo Silano). Method validation will be also presented as well as comparison of the obtained results with LC–UV with pre-column derivatization using dansyl-chloride.

2. Materials and methods

2.1. Samples

Three Caciocavallo Silano cheese samples were purchased at a local retail. Caciocavallo Silano obtained the Protected Designation of Origin (PDO) in 1996 (EC No. 1263/96) recently amended by EC No. 1204/03 [72,73]. PDO requires that cheese is produced in a defined area under a specific standard of identity. In particular, Caciocavallo Silano is produced in selected areas of the regions Calabria, Basilicata, Campania, Puglia and Molise (Southern Italy). The cheese is oval shaped (weight 1.5–2.5 kg) and it must be ripened for at least 1 month before the PDO logo is applied on its rind. Cheese samples considered in this study were ripened for 1 month and produced by the same cheesemaking plant near Cosenza (Calabria) applying the same standardized technological process. For BAs determination, cheeses 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 (ca. 3 mm of diameter) and were mixed and homogenized thoroughly into pools.

2.2. Chemicals

The bioactive amines spermine (SPM, tetrahydrochloride), spermidine (SPD, trihydrochloride), putrescine (PUT, dihydrochloride), histamine (HIM, dihydrochloride), tyramine (TYR, hydrochloride), phenylethylamine (PHE, hydrochloride), were purchased from Sigma–Aldrich (Milford, MA, USA). Dansyl-chloride was also acquired from Sigma–Aldrich (Milford, MA, USA) as well as hydrochloric acid (37%), ammonia (30%), trifluoroacetic acid 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 C18 cartridrges (0.5 g) were obtained from Supelco Inc. (Bellefonte, PA, USA).

2.3. Amine standard solutions

For LC–UV experiments, an individual standard solution of about 1.0 mg mL^{-1} of each amine was prepared in purified water and stored in darkness at 4 ± 1 °C. To perform calibration experiments, ten standard solutions containing all the amines were obtained with different aliquots of each water solution, all diluted to 25 mL with HCl 0.1 M. The final amine concentrations injected were 0.8, 2.0, 4.0, 5.0, 10.0, 16.0, 25.0, 50.0, 75.0, 100.0 mg L^{-1}.

For LC–ELSD analysis, different individual amine standard solutions (TYR 2.53, PUT 2.86, HIS 2.53, PHE 3.15, SPD 3.15 and SPM 2.11 mg mL^{-1}) were prepared in purified water in relation with the different detector responses. Ten standard solutions mixtures of all the amines were obtained by pooling different aliquots of each individual standard solution and reaching the final volume of 10 mL with purified water. Different concentration ranges were analysed for each amine. In particular the concentrations were: TYR 6.0–253.0, PUT 9.5–286.0, HIS 6.5–280.0, PHE 9.3–315.0, SPD 2.0–315.0 and SPM 5.8–211.0 mg L^{-1}.

2.4. Extraction of BAs and solid phase extraction (SPE)

The extraction of BAs and SPE have been accomplished as already reported [57] with some modifications. 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 by vortex (40 Hz for 40 min) and then centrifuged at 8500 rpm for 25 min. The solution was then filtered with a syringe filter (0.20 μm), collected in a plastic vial and purified by SPE on a C18 sorbent, previously conditioned with 2.0 mL of CH_3OH and then with 2.0 mL of ultrapure water alkalinized with NH_4OH to pH 11.0. Four milliliters of the sample, brought to pH 11.0 for NH_4OH, were loaded onto the C18-packed sorbent. The cartridge, containing the sorbed analytes, was washed with 2.0 mL of ultrapure water and dried with nitrogen gas for 5 min. Two milliliters (two times) of CH_3OH were used as eluting solution, dried up with nitrogen gas and the residue re-dissolved in a plastic test tube with 1.0 mL of HCl 0.1 M or 800 μL of ultrapure water for LC–UV and LC–ELSD analysis, respectively.

LC–UV recovery experiments were performed by adding, before the extraction procedure, 1.0 mL of a standard solution 16.0 mg L^{-1} of each amine to cheese sample (5.0 g), while for LC–ELSD recovery experiments, the same amount of cheese was spiked with 80 μL of an amine standard solution containing different concentration of each amine (TYR 115.0, PUT 191.0, HIS 133.0, PHE 186.0, SPM 117.0 and SPD 189.0 mg L^{-1}).

2.5. Dansyl chloride derivatisation of the biogenic amines

Dansylation of BAs followed the procedure of Chiacchirini et al. [27] with the modification of the derivatization temperature and time which were 60 °C for 30 min instead of 25 °C for 20 min [25,57]. 1.0 mL of acid standard solution (or acid sample extract spiked with BAs or acid sample extract) was added of 200 μL of NaOH 2.0 M, 300 μL of saturated $NaHCO_3$ solution and 2.0 mL of dansyl-chloride solution (10.0 mg mL^{-1} in acetone prepared just before use). After the reaction time, the excess of dansyl-chloride was removed by adding 100 μL of NH_4OH 25% (v/v). After filtration with 0.45 μm syringe filters, a volume aliquot of 20 μL was injected for LC–UV analysis.

2.6. Instrumentations

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 UV detector (UV-2075, Jasco Inc., Easton, MD, USA) and with an ELS detector (1200 Series, Agilent Tech., Lexington, MA, USA). Data were collected and analysed with an integrator Jasco-Borwin1. For LC–UV analysis, a reverse-phase C18 column (250 mm × 4.6 mm I.D., 5 μm) (Supelco Inc., Bellefonte, PA, USA) equipped with a C18 guard-pak (10 mm × 4.6 mm I.D., 5 μm) were 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 (SIELC Technologies, Prospect Heights, IL, USA). A microprocessor pH meter (Hanna Istruments, Eboli (SA), Italy), equipped with a combined glass-calomel electrode, was employed for pH measurements. A centrifuge (Thermo Scientific, Milan, Italy) was used for the pretreatment of the cheese samples.

2.7. Chromatographic conditions and calibration

For LC–UV analysis, two solvent reservoirs containing (A) purified water and (B) acetonitrile were used to separate all the amines with an LC elution programme which began with 3 min of isocratic programme A–B 50:50 (v/v) reaching after 20 min A–B 10:90 (v/v). Then 3 min of isocratic elution were carried out and further 4 min were necessary to restore again the starting conditions (A–B 50:50, v/v). Flow was kept constant at 1.2 mL min^{-1}, for a total analysis time of 30 min and a time interval of 10 min between two successive injections was applied. Detection was accomplished at 254 nm.

For LC–ELSD analysis, the mobile phase were composed by (A) acetonitrile/water 20/80 (v/v) mixture containing trifluoroacetic acid (0.05%, v/v) and (B) acetonitrile/water 20/80 containing trifluoroacetic acid (0.35%, v/v). The chromatographic separation was carried out using a linear binary gradient according to the following scheme: elution programme began with A–B 100:0 (v/v) reaching after 10 min A–B 70:30 (v/v) and further 8 min to reach A–B 40–60 (v/v). Then 2 min of isocratic elution were carried out and further 5 min to reach A–B 0:100 (v/v). Finally, 10 min were necessary to restore again the starting conditions (A–B 100:0, v/v). Flow was kept constant at 0.7 mL min^{-1}, for a total analysis time of 35 min and a time interval of 10 min between two successive injections was applied. The flow of nebulizer gas (N_2) was maintained at 3.5 bar and the drift tube temperature was set at 40 °C.

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. Data for calibration curves were collected for ten different concentrations and calibration graphs were constructed by plotting amine peak areas versus amine concentrations. Quantitative determination was accomplished by direct interpolation in the standard curves for each amine. In order to reassure repeatability, standards were run throughout the day and also between days.

3. Results and discussion

3.1. Optimization of LC–ELSD method

The main difficulty for the development of LC–ELSD analytical methods, is the restriction on the mobile phase volatility. Non-volatile modifiers, ion-pairing reagents, acids, bases and buffers cannot be used with ELSD. Therefore, a very useful part of the mobile phase chemistry is not compatible with ELSD, making quite difficult to convert a LC–UV method to a LC–ELSD method or to achieve efficient chromatographic separations for some type of analytes. Some acceptable volatile reagents are trifluoroacetic, heptafluorobutyric, nonafluoropentanoic, acetic and formic acid and their ammonium salts in low concentrations. Moreover, ELSD conditioning has to be carefully conducted and analytical parameters have to be changed as little as possible during the day once they are optimized and the instrument is conditioned.

In this study, different chromatographic conditions have been evaluated. Firstly, C18 and Silica columns, either isolated or in-line, were considered for separation. In all cases, no satisfactory retention of biogenic amines has been accomplished; moreover, changes in the composition of the mobile phase or in the elution program did not produce better results. The solution was offered by using an analytical column which is a reverse-phase column with embedded weak acidic ion-pairing groups, improving retention of basic compounds by cation-exchange mechanism. In this case, it was possible to separate all the amines in 35 min with a good resolution. Once the separation was achieved, ELSD parameters were to be considered for enhancing signal response. The fundamental principle of ELSD is that the column effluent is converted to an aerosol (nebulization of the chromatographic effluent) which is subsequently desolvated (evaporation of the mobile phase). Since analytes are generally much less volatile than LC solvents, conditions can be controlled so that the analyte does not evaporate, but remains as dry particles, while the mobile phase is removed. These analyte particles scatter the light which can be monitored to provide their detection. The intensity of the scattered light is proportional to the size of the solute particles, which is in turn determined by numerous experimental variables as well as the solute concentration; among them, the evaporator temperature is one of the most important [74,75]. The most productive approach to optimize the drift tube temperature is to tune the evaporation temperature to the lowest value which minimizes background scattering. As solutes in solid state scatter light more efficiently than in the liquid state, the response decreases when the temperature of the evaporator increases. As long as the temperature is not too high, the analyte remains as particles; an increase in temperature results in the formation of liquid particles, and a solute can vaporize partially, leading to a decrease of particle size [76]. In our case 30, 40, 50 and 60 °C were considered. Poor S/N ratio was obtained at 30 °C while a significant improvement was achieved at 40 °C. A further increase of the temperature did not result in any statistically relevant signal enhancement, so 40 °C was selected for the successive analyses.

An appealing characteristic of LC–ELSD is that, unlike UV detection (especially at low wavelengths) with gradient elution, the baseline might not drift; moreover the response factors towards different analytes are reasonably uniform at a given composition of the mobile phase. However, they change as the organic content of the mobile phase changes; increasing the organic content of the mobile phase the transport efficiency of the nebulizer increases allowing a greater number of particles to reach the detec-

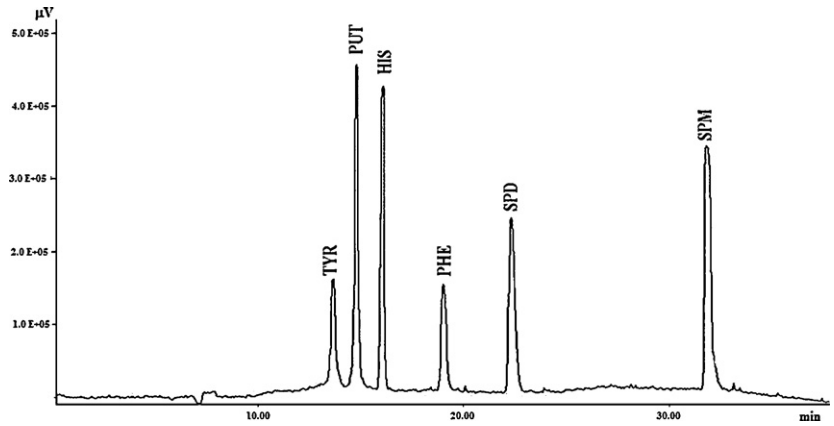

Fig. 1. Chromatogram of an amine standard solution obtained by LC–ELSD. Amine concentrations: TYR 115.0, PUT 191.0, HIS 133.0, PHE 186.0, SPM 117.0 and SPD 189.0 mg L^{-1}.

tion chamber, and also influencing the particle size distribution of the droplets. Some publications [77–79] have emphasized this limitation and some have attempted to correct for this behaviour with different approaches, such as additional calibration curves through the entire range of mobile phase composition [79] or mobile phase compensation [80]. However, all proposed strategies represent a tedious complication of the analytical set up. To face this problem, we decided to develop an elution gradient where the organic content of the mobile phase was not varied during the analysis. Proportions of water and acetonitrile in solvent A and B were in fact fixed to 80:20, while the retention and resolution were affected by the TFA concentration. Nevertheless, it must be underlined that the droplet distribution strongly depends not only on the mobile phase composition but also on mobile phase flow rate and carrier gas flow rate. This dependence is highly interactive, which makes the spray very hard to control and causes the droplets to change in size and number with time. To this regard, mobile phase flow rate and carrier gas flow rate were chosen properly to minimize this effect. Fig. 1 shows the chromatogram of an amine standard solution obtained with the optimized ELSD parameters.

3.2. Validation of LC–ELSD method

The accuracy of the new LC–ELSD method, developed for the simultaneous separation and determination of the six amines considered, was validated by comparison of the results with those obtained when LC–UV method, preceded by a dansyl chloride derivatisation step, was used.

For the two methods and for each analyte, a calibration plot, reporting the peak area against standard concentration, was constructed for ten concentration levels. Three independent replicates for each concentration level were performed. For each compound, applying the LC–UV method, linearity was observed into the considered concentration range, with good regression coefficients values. By contrast, for LC–ELSD method, a third order-polynomial fit was observed. The non-linear response by the ELSD is a consequence of the dependence of the efficiency of prevalent light-scattering processes (namely Rayleigh scattering, Mie scattering and reflection–refraction), on the average particle size [81]. It has been shown that low analyte concentrations lead to particles smaller than the optimal value, resulting in reduced response. Similarly, at high concentrations, particles larger than the optimal value are produced, so that less light is scattered per mass unit. These phenomena are responsible for the sigmoidal calibration curve profile typically observed for the ELSD.

Table 1 reports, for the two methods, the regression curve equation, the correlation coefficients. Limits of detection (LOD) and limits of quantitation (LOQ), for either standard solutions or cheese sample, are presented in Table 2. For standard solutions, LODs were calculated from the amount of amines required to give a signal-to-noise ratio of 3, while LOQs were obtained considering a signal-to-noise ratio of 10. Good LOQs values were obtained by LC–UV as compared with other studies [48,57]. As expected, it can be observed that LC–UV method offers higher sensitivity than the LC–ELSD method [61]. On the other hand, Sadain and Koropchak [71] showed that the 3σ detection limits for BA standard solutions were found to range from 8 to 20 ng mL^{-1} using LC–CNLSD. These values are very low and confirms that one of the main drawbacks of ELSD is the relatively low detectability (usually in the range of 0.1 μg mL^{-1}). Nevertheless, it is more than suitable in relation with concentrations of BAs generally found in cheese. Moreover, purification and pre-concentration steps, generally applied in food analysis irrespective of the detector coupled with LC, further increase the analytes concentration in the sample. This is confirmed by the values of LODs and LOQs, calculated in mg kg^{-1} of cheese, which are showed in Table 2. These values derived from standards including all handling steps during sample preparation and derivatization (for LC–UV analysis) [56].

To apply the optimized LC–UV and LC–ELSD methods in the analysis of Caciocavallo Silano sample, the recovery yield of each analyte was evaluated by spiking the cheese sample with known amounts of standard analytes and quantifying them. Since recovery depends on the concentration level of the analyte in the matrix [82], a previous qualitative and quantitative evaluation of BAs content was performed in cheese sample, and the native amounts were so evaluated. Standard analytes at concentrations comparable with those quantified were added and the sample was subjected to the whole treatment of SPE, dansylation, and LC–UV (or LC–ELSD) analysis. The recovery was evaluated for each amine by comparing the amount found after spiking (with respect to that initially estimated) and the amount added. For both methods, the recovery percentages were always >90% with relative standard deviations (RSD) <5%, indicating that the applied extraction and purification procedure did not produce any loss of analytes in the sample to be injected.

The repeatability (intra-day and inter-day analysis) was verified evaluating the relative standard deviations values for peak areas measured for six repeated analyses of the same cheese sample extract. For both methods, data reported in Table 3 indicate an acceptable precision for all BAs analysed [83].

3.3. Levels of BAs in Caciocavallo Silano POD cheese sample

In Figs. 2 and 3, the chromatograms of the cheese sample extracts analysed by LC–ELSD and LC–UV are depicted, while the BAs concentrations are reported in Table 3. As can be seen, data achieved by these techniques are comparable either in terms of

Table 1
Validation parameters for LC–ELSD and LC–UV methods.

BAs	LC–ELSD		LC–UV	
	Calibration curve equation	R^2	Calibration curve equation	R^2
TYR	$y = 282{,}430{,}000x^3 - 33{,}488{,}800x^2 + 17{,}099{,}300x - 43{,}580$	0.9975	$y = 8288.1x + 13{,}571$	0.9982
PUT	$y = 744{,}928{,}000x^3 - 166{,}427{,}000x^2 + 30{,}000{,}900x + 1{,}519{,}200$	0.9989	$y = 22{,}503x + 48{,}379$	0.9978
HIS	$y = -472{,}726{,}798x^3 + 219{,}507{,}442x^2 + 6{,}857{,}890\,x - 406{,}532$	0.9979	$y = 26{,}735x + 50{,}816$	0.9973
PHE	$y = -198{,}746{,}479\,x^3 + 125{,}896{,}837x^2 - 9{,}175{,}367\,x + 694{,}145$	0.9970	$y = 20{,}506x + 9387.9$	0.9987
SPD	$y = -359{,}940{,}090x^3 + 254{,}404{,}371x^2 - 7{,}466{,}202\,x + 182{,}757$	0.9986	$y = 20{,}506x + 9387.9$	0.9987
SPM	$y = -3{,}078{,}600{,}000x^3 + 1{,}167{,}700{,}000x^2 - 97{,}928{,}400\,x + 3{,}627{,}210$	0.9962	$y = 14{,}452x + 24{,}970$	0.9985

Table 2
Limits of detection (LOD) and quantitation (LOQ) for LC–ELSD and LC–UV methods expressed in mg L^{-1} and mg kg^{-1}.

BAs	LC–ELSD				LC–UV			
	LOD		LOQ		LOD		LOQ	
	(mg L^{-1})	(mg kg^{-1})	(mg L^{-1})	(mg kg^{-1})	(mg L^{-1})	(mg kg^{-1})	(mg L^{-1})	(mg kg^{-1})
TYR	1.9	1.5	5.1	4.1	0.08	0.4	0.25	1.3
PUT	3.6	2.8	9.3	7.4	0.03	0.2	0.10	0.5
HIS	2.1	1.7	5.7	4.6	0.06	0.3	0.17	0.9
PHE	3.0	2.4	8.7	7.0	0.07	0.4	0.21	1.1
SPD	1.4	1.1	3.6	2.9	0.09	0.5	0.26	1.3
SPM	1.9	2.3	5.5	4.4	0.06	0.3	0.20	1.0

Table 3
Values of recovery, repeatability and concentrations of BAs in Caciocavallo Silano cheese, performed with LC–ELSD and LC–UV methods.

BAs	LC–ELSD				LC–UV			
	Recovery (%)	Concentration (mg kg^{-1})	RSD intra-day	RSD inter-day	Recovery (%)	Concentration (mg kg^{-1})	RSD intra-day	RSD inter-day
TYR	104 ± 3	29 ± 2	0.6	1.5	105 ± 3	29 ± 2	0.1	0.3
PUT	96 ± 3	16 ± 2	0.8	1.7	100 ± 3	16 ± 2	0.1	0.2
HIS	100 ± 1	42 ± 3	0.7	1.0	98 ± 2	43 ± 2	0.2	0.3
PHE	102 ± 4	10 ± 1	1.2	2.8	100 ± 4	10 ± 1	0.2	0.3
SPD	96 ± 4	4 ± 1	1.5	2.6	95 ± 2	4 ± 1	0.1	0.2
SPM	91 ± 3	26 ± 3	0.9	2.0	90 ± 2	26 ± 2	0.2	0.3

mean concentrations or in terms of standard deviations, showing that both procedures can be used interchangeably for quantitative determination of BAs in cheese. The most abundant amine found in Caciocavallo Silano POD was HIS, followed, in decreasing order, by TYR, SPM, PUT, PHE and SPD for a total amount of 127 mg kg^{-1}. Although the estimation of the total toxic dose of individual biogenic amines is very difficult [1], Shalaby [3] and Valsamaki et al. [84] stated that the "safe" sum of histamine, tyramine, putrescine and cadaverine should not exceed significantly higher dose of 900 mg kg^{-1}. Although, at the moment, no legal upper limit for BAs in cheese has been established, the obtained data show that analysed cheese sample does not represent a possible risk for consumer health, keeping in mind that, for sensitive people and/or patients treated with monoamine oxidase inhibitors, the threshold for BAs toxicity could be much lower. This is of particular concern, because, other studies reported the presence of much higher amounts of BAs in cheeses [14,16,20,48,56–58,85]. Mayer et al. [56] applied an UPLC method to the analysis of 58 cheese samples as retailed in Austria, finding that the BAs contents varied to a great extent (reaching values of 1940 mg kg^{-1} in two samples), depending not only on the type of cheese (extra-hard, hard, semi-hard, blue-veined, mould-ripened, smear-ripened, and acid

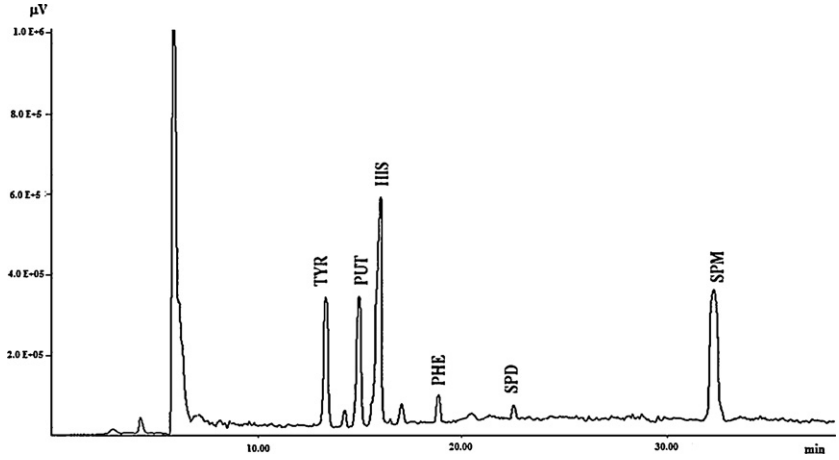

Fig. 2. Chromatogram of the Caciocavallo Silano POD cheese sample extract obtained by LC–ELSD.

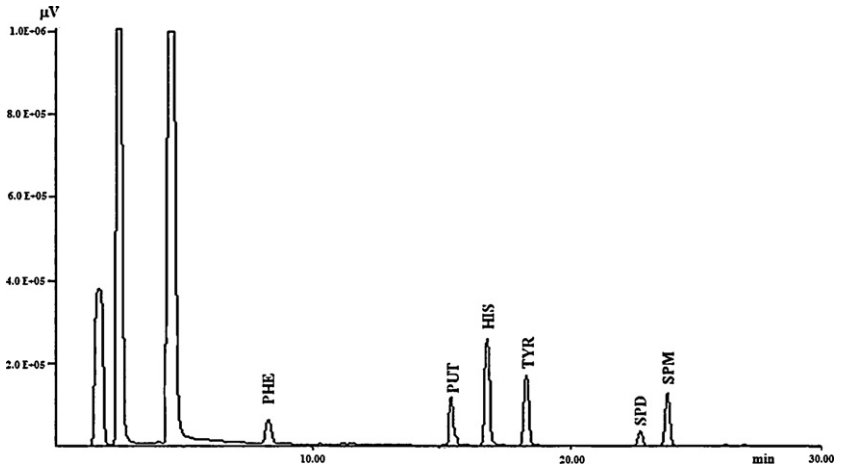

Fig. 3. Chromatogram of the Caciocavallo Silano POD cheese sample extract obtained by LC–UV.

curd cheese), but also within a certain cheese variety. Other results [57,58], obtained applying LC–MS/MS analysis to typical Piedmont (North-West Italy) cheeses (i.e. Toma Piemontese, Raschera and Castelmagno), also indicated that either the relative compositions or the total amounts in amines of the three cheeses investigated were quite different (1200–3200 mg kg^{-1}). Considering that cheese samples were similar in texture and taste and are produced in the same Italian region, author suggested to use the BAs profile for typicisation purposes. This aspect could be particularly interesting for Caciocavallo Silano POD because, while Caciocavallo Silano indicates a PDO cheese produced in a defined area under a specific standard of identity, there is no protection of the general designation "Caciocavallo" and cheeses of similar manufacture are marketed under this common name. In such situations, it could be of great concern to clarify the differences among these cheeses. To this regard, it is reported [8–12,14–17] that the accumulation of BAs in cheese is mainly attributed to the activity of the non-starter microflora with an indirect role of the starter lactic acid bacteria. However, the composition of the microflora of commercial Caciocavallo cheeses showed a high variability and no clear relationship was found with either the region of origin or the cheese denomination [86], implying that non direct correlation is possible for this kind of cheese between microflora and typicization. To this regard, other characteristics, such BAs relative composition, could be assessed to further differentiate the individual cheeses on the basis of the geographical origin.

4. Conclusions

The new chromatographic method here developed permits the determination of six biogenic amines without the need of the derivatization step with time-saving and with good values of recovery, precision, although LOD, and LOQ values (mg L^{-1}) are much higher than those obtained by LC–UV (about one order of magnitude). The use of ELSD permits to quantify any solute less volatile than the solvent. However, the droplet size (and thus the response) is highly dependent on the flow of the nebulizing gas, the temperature of the evaporating tube and the flow rate and on the composition and physical characteristics of the mobile phase. Therefore, working conditions should be optimized to ensure the highest possible detector sensitivity and should be reproduced rigorously each time. The selected parameters presented in this study were found to be a good compromise between sensitivity, resolution, time analysis and ELSD limitations which can make too laborious a quantitative analytical method. Once optimised and validated, the analytical procedure, after a suitable extraction of the analytes, has been successfully applied for the determination of BAs in Calabria POD cheese, where the total BAs content was relatively low in comparison with that reported for cheeses suspected for outbreaks of food poisoning. Nevertheless, strict monitoring of BAs content in cheese should accomplished not only for the hygienic significance of these compounds, but also for the possible exploitation of BAs as authentication markers of cheese.

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