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Determination of biogenic amines in cheese using HPLC technique and direct derivatization of acid extract

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Abstract

Cheeses are among those high-protein-containing foodstuffs in which enzymatic and microbial activities cause the formation of biogenic amines from amino acids decarboxylation. Most of the methods for amine determination in this products involve acid extraction followed by a liquid–liquid purification step to selectively separate amines and amino acids. In this work a simple direct derivatization of the acidic extract with dansyl chloride (DCl) was applied. After an extraction step with diethyl ether, the amines were separated on a Kromasil C18 column using a water–acetonitrile elution gradient. Taking into consideration the recovery and repeatability data we can conclude that, when free amino acid determination is not of concern, the direct DCl derivatization procedure can be applied also for cheese samples with high free amino acid contents.

Keywords: Cheese; Biogenic amine; High performance liquid chromatography; Direct derivatization

1. Introduction

Biogenic amines are nitrogen compounds of biological importance in vegetable, microbial and animal cells. Since they are formed mainly by microbial decarboxylation of amino acids and transamination of aldehydes and ketones, in all foods that contain proteins or free amino acids and are subject to conditions enabling microbial or biochemical activity, biogenic amines can be expected (Silla-Santos, 1996). Cheeses are among those high-protein-containing foodstuffs in which enzymatic and microbial activities cause the formation of amino acids and biogenic amines (Laleye, Simatd, Gosselin, Lee, & Giroux, 1987). In fact, during cheese ripening, degradation of casein occurs leading to the accumulation of free amino acids that can be converted into biogenic amines by the activity of bacterial decarboxylases (Halasz, Barath, Simon-Sarkadi, & Holzhapeel, 1994).

The interest in amine determination is due to their ability to have a direct or indirect effect on the human vascular and nervous system. Indeed a large amount of the biogenic amines can cause rash, headache, nausea, hypo- or hypertension, cardiac palpitation, intracerebral haemorrhage, and anaphylactic shock, especially if alcohols or monoamine oxidase inhibitors (MAO-Is) are ingested at the same time (Lange, Thomas, & Wittmann, 2002; Stratton, Hutkins, & Taylors, 1991; Vinci & Antonelli, 2002). Moreover, in the specific case of the cheese, it is possible to use the determination of biogenic amines as a parameter of hygienic quality in cheese-making (Antila, Antila, Mattila, & Hakkarainen, 1984; Mah, Han, Oh, Kim, & Hwang, 2002; Marino, Maifreni, Moret, & Rondinini, 2000) or as an indicator of the degree of proteolysis and the typicalness of some particular cheeses (Celano, Cafarchia, Buja, & Tiecco, 1992; Innocente & D'Agostin, 2002).

Abbreviations: HPLC, high performance liquid chromatography; DCl, dansyl chloride; TRYP, Tryptamine; 2-PHE, 2-phenylethylamine; PUT, putrescine; CAD, cadaverine; HIS, histamine; TYR, tyramine; SPD, spermidine; SPM, spermine; IS, internal standard.

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Several analytical techniques including capillary electrophoresis (CE), thin-layer chromatography (TLC) (Celano et al., 1992; Perez-Martin, Franco, Molist, & Gallardo, 1987; Shalaby, 1995; Voight, Eitenmiller, Koehler, & Hamdy, 1974), gas chromatography (GC) (Laleye et al., 1987; Perez-Martin et al., 1987), ion exchange chromatography (Standara, Vesela, & Drdak, 2000) and high performance liquid chromatography (HPLC) (Mah et al., 2002; Marino et al., 2000; Moret & Conte, 1996; Novella-Rodrguez, Veciana-Nogués, Izquierdo-Pulido, & Vidal-Carou, 2003; Özogul, Taylor, Quantick, & Özogul, 2002) have been proposed for the determination of biogenic amine in various foods. Among the cited techniques, reversed-phase (RP)-HPLC is considered the most suitable one (Moret & Conte, 1996). To ensure adequate sensitivity, a derivatization step is generally performed before injection. Derivatization with dansyl chloride (DCl) has the advantage to allow UV detection (254 nm) and rapid elution time (using simple gradient elution program consisting of water and acetonitrile or methanol). *O*-phthaldialdehyde (OPA) derivatization represents a good choice when simultaneous determination of free amine and amino acids is of concern, but requires spectrofluorometric detection and longer elution time (Moret, Smela, Populin, & Conte, 2005).

All the methods used for amine determination involve two steps: amine extraction from the matrix and analytical determination. Depending on the complexity of food matrix and the natural amount of free amino acids that can compete with the derivatizing agent, a further purification step can be needed prior to analytical determination.

Most of the methods employed for amines determination in cheese provide a preliminary extraction of the solid matrix in an acid medium. The raw extract is then saturated with a salt, adjusted to an alkaline pH, and partitioned with an organic solvent (butanol, butanol/chloroform) able to selectively extract free amines letting free amino acids in the aqueous layer. Since different amines show different optimum of pH for extraction, in order to assure satisfactory recoveries and reproducibility a strict control of this parameter is needed (Moret & Conte, 1996).

Direct derivatization of the acidic extract was successfully used by a number of authors (Mah et al., 2002; Moret et al., 2005; Vinci & Antonelli, 2002) for food matrices with relatively low free amino acid content such as meat, fish and vegetables (Moret & Conte, 1996). The aim of this work was to verify the applicability of direct derivatization of the acidic extract for the determination of biogenic amines in cheese with different degree of proteolysis.

2. Material and methods

2.1. Chemicals

Tryptamine (TRYP), 2-phenylethylamine (2-PHE), putrescine (PUT), cadaverine (CAD), histamine (HIS), tyramine (TYR), spermidine (SPD), spermine (SPM), 1,7-diaminoheptane (internal standard, IS) and prolina were purchased from Fluka (Buchs, Switzerland); hydrochloric acid, sodium bicarbonate, and diethyl ether from Carlo Erba (Milan, Italy), acetonitrile for HPLC from Merck; ultrapure water was obtained with a Milli-Q system (Millipore).

2.2. Preparation of standard solutions

An amount of 50 mg of each amine was accurately weighed in a 50 mL volumetric flask, added with 50 mg of 1,7-diaminoheptane as IS and diluted to the required volume with purified water to obtain a stock standard solution of amines. For the derivatization 1 mL of DCl reagent (5 mg/mL) was added to a 1 mL aliquot of the diluted standard solution (1:100). The reaction mixture was then left for 60 min at 40 °C (Mietz & Karmas, 1977) or for 15 min at room temperature in darkness (Vinci & Antonelli, 2002), with occasional shaking. In order to eliminate the excess of DCl the mixture was treated with 200 μL of a L-proline solution (100 mg/mL), vortexed for 1 min and left to react in the dark for 15 min at room temperature (Antolini, Franciosini, Floridi, & Floridi, 1999). The sample was then extracted twice with 1 mL aliquot of diethyl ether. The combined extracts were dried under nitrogen flow and the residue was re-dissolved in acetonitrile for injection.

2.3. Preparation of sample solution

A 10 g amount of ground cheese was weighed directly in a centrifuge tube, added with 20 mL of 0.1 M hydrochloric acid containing a known amount of 1,7-diaminoheptane (IS) and then homogenized in a Politron homogenizer (Kinematica, Lucerne, Switzerland) for 2 min. The cheese slurry obtained was centrifuged at 12,000*g* for 20 min at 4 °C, the aqueous layer was collected and the residue was re-extracted using the same procedure. The two aqueous extracts were combined and diluted to 50 mL with HCl 0.1 M. For derivative preparation 0.5 mL of saturated $NaHCO_3$ solution and 1 mL of DCl reagent (10 or 20 mg/mL) were added to a 1 mL aliquot of the diluted extract. The reaction mixture, that included many CO_2 bubbles, was then left for 60 min at 40 °C and then treated as previously described for standard solution.

2.4. Apparatus and chromatographic conditions

HPLC determinations were performed with a Varian Model 230 Pro Star and Rheodyne Model 7725i injector with a 10 μL loop. The detector was a Varian Model 330 Pro Star UV–Vis spectrophotometer set at 254 nm. The column was a reversed-phase Kromasil KR 100-5 C18. The two solvent reservoirs contained the following eluents: (A) acetonitrile and (B) water. The elution programme consisted of a gradient system with a flow-rate of 0.8 mL/min (Moret & Conte, 1996; Moret et al., 2005).

2.5. Recovery studies

Recovery studies were performed with Parmigiano Reggiano cheese samples spiked with a 1 mL of a stock standard solution of amines.

3. Results

As different authors report different derivatization conditions, in order to verify the possibility to reduce reaction time and temperature, two reaction conditions, i.e. 40 °C for 60 min in thermostat (Moret et al., 2005) and room temperature for 15 min in the darkness (Vinci & Antonelli, 2002) were investigated using a standard solution of amines. Fig. 1 reports the area obtained for all the amines as means of six injections performed for each dansylation condition. Mean area values calculated from the data relative to the samples incubated at 40 °C for 60 min are considerably greater than those calculated from the data relative to the samples incubated at 20 °C for 15 min. Moreover, the poor repeatability of the values for reduced time and temperature of reaction could be explained considering that at 20 °C dansylation reactions occur but not in a quantitative way and so in repeated tests different dansylation degrees are achieved. Therefore incubation at 40 °C for 60 min has been preferred. Fig. 2 shows the chromatogram of a

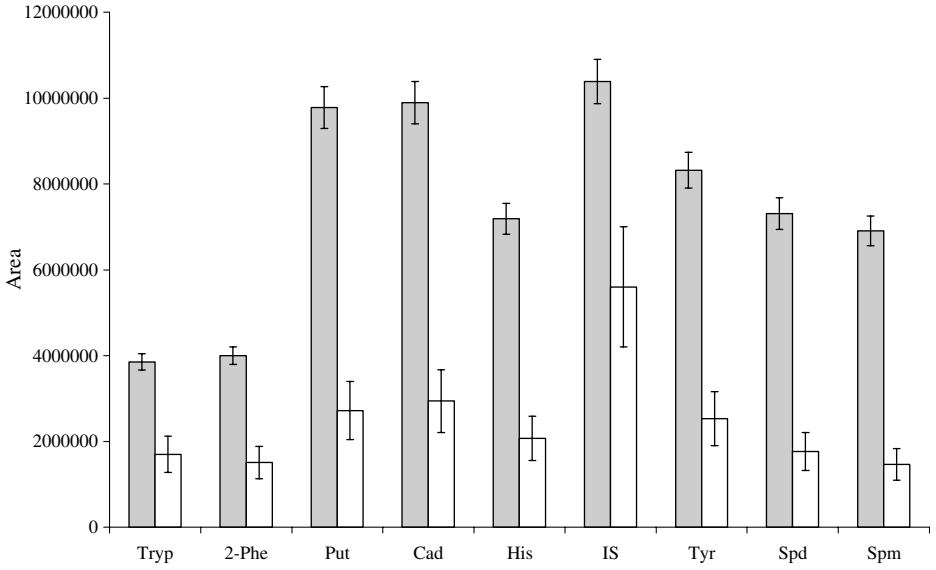

Fig. 1. Histogram relative to the biogenic amine areas of a standard solution derivatized with incubation at 40 °C for 60 min (▩) or at 20 °C for 15 min (□).

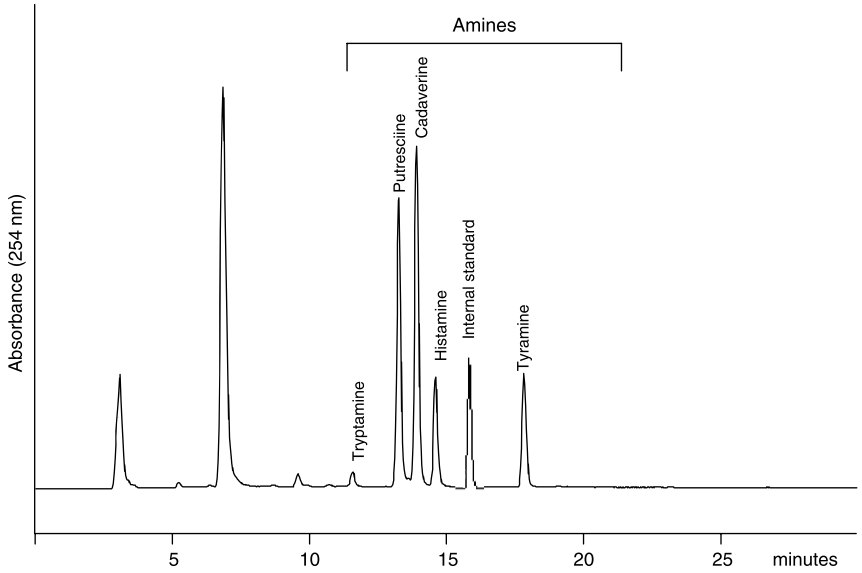

Fig. 2. HPLC chromatogram relative to a semi-hard Italian cheese sample.

cheese sample which underwent direct derivatization of the acid extract followed by proline addition and extraction with diethyl ether as described in Section 2.

The derivate so obtained can be also injected directly avoiding extraction with diethyl ether. Direct injection has the advantage of giving quantitative recoveries of all the researched amines (Moret & Conte, 1996). Even if the chromatographic conditions reported in this paper are used, the free amino acids that remain in sample are all eluted within the first minutes of the chromatographic run. When cheese samples with particularly high free amino acids are processed a sample dilution (that always leads to a lack of sensitivity) may be necessary in order to avoid interference problems for the first eluted amine peaks.

Table 1
Amine recoveries in Parmigiano Reggiano cheese. Derivatization procedure with 10 or 20 mg/mL amounts of dansyl chloride (DCl) are been employed

	DCl			DCl		
	10 mg/mL acetone			20 mg/mL acetone		
	Mean[a]	SD[b]	CV[c]	Mean	SD	CV
Tryptamine	67a[d]	4.9	7.4	72a	3.9	5.5
2-Phenylethylamine	62a	3.8	6.2	58a	3.5	6.0
Putrescine	98a	3.3	3.4	98a	3.9	4.0
Cadaverine	98a	5.3	5.4	95a	5.3	5.6
Histamine	72a	4.5	6.2	78a	1.6	2.1
Internal Standard	97a	3.7	3.8	95a	3.8	4.0
Tyramine	80a	4.9	6.1	84a	2.1	2.5
Spermidine	77a	5.3	6.9	79a	6.9	8.8
Spermine	67a	6.0	8.9	62a	5.9	9.5

[a] Means of six repetitions.
[b] SD, standard deviation.
[c] CV, coefficient of variation.
[d] Values followed by the same letter in the same line are not significantly different for $P \leqslant 0.05$ (Student's test).

For this reason an extraction step with diethyl ether (Antolini et al., 1999; Mietz & Karmas, 1977; Özogul et al., 2002) was introduced before injection. In this way only dansyl amines are extracted from the organic solvent, whilst dansyl amino acids remain within the aqueous layer. This simple extraction step requires about 10 min and enables to obtain HPLC trace free from amino acids as shown in Fig. 2.

If derivatization is carried out on a sample solution which contains amines and free amino acids (samples with a high proteolysis degree) the amount of DCl has to be in adequate excess in order to assure quantitative derivatization of biogenic amines.

To optimize the derivatization step, different aliquots of Parmigiano Reggiano cheese sample (seasoned for 24 months) characterized by a high free amino acids content were spiked with a known amount of amines and were then derivatized with increased amounts of dansyl chloride (10 and 20 mg/mL). Each sample was analyzed six times and recoveries were calculated by comparing the data obtained with and without spiking the samples. Data from these experiments are reported in Table 1. In order to verify whether there was a statistically significant difference between the means obtained with different concentration of DCl a Student's test was carried out. This showed that the differences cannot be considered statistically significant, therefore a DCl concentration of 10 mg/mL was already adequate to obtain a quantitative derivatization of the amino acids and the amines. Tryptamine, 2-phenylethylamine and spermine showed the lower recoveries (60–70%), while other amines had all recoveries about 70%.

In any case it is important to remember that when DCl is employed in such considerable excess it is very important to provide its neutralization with the L-proline to avoid the appearance (Vallè, Malle, & Bouquelet, 1997) of an inter-

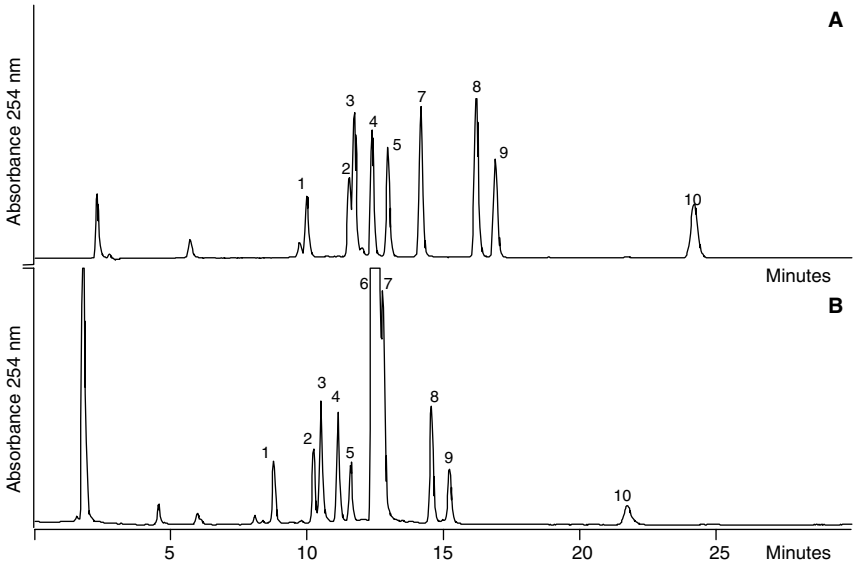

Fig. 3. HPLC chromatograms of a semi-hard Italian cheese derivatized with (A) and without (B) addition of L-proline solution: 1, Tryptamine; 2, 2-Phenylethylamine; 3, Putrescine; 4, Cadaverine; 5, Histamine; 6, Interfering peak of dansyl chloride; 7, 1,7-diaminoheptane (IS); 8, Tyramine; 9, Spermidine; 10, Spermine.

Table 2
Analytical repeatability

	μg/g of cheese		
	Mean[a]	SD[b]	CV[c]
Tryptamine	11.85	0.65	5.5
2-Phenylethylamine	9.51	0.43	4.5
Putrescine	75.87	1.51	2.0
Cadaverine	15.56	0.67	4.3
Histamine	28.55	0.78	2.7
Tyramine	29.89	0.99	3.3
Spermidine	7.71	0.18	2.4
Spermine	4.46	0.94	21.0

[a] Means of six repetitions.
[b] SD, standard deviation.
[c] CV, coefficient of variation.

fering peak (see Fig. 2). In particular, a 200 μL portion of a 100 mg/mL aqueous solution of L-proline was sufficient to neutralize the excess of DCl even when this was added at concentrations of 20 mg/mL (see Fig. 3).

The repeatability of the entire analytical procedure was verified using a sample of semi-hard Italian cheese with an average amine content. Average amine content, standard deviation and coefficient of variation measured for six repeated analyses are reported in Table 2.

4. Conclusions

In conclusion this technique can be considered advantageous in order to reduce the time of the analysis and to obtain good recoveries and repeatability since it does not provide the purification of the acid extract with an organic solvent that is considered a critical step that requires a strict control of the pH. Moreover the direct derivatization of the acid extract can also be used successfully for cheese samples with high concentrations of free amino acids without the use of sample dilution that causes a decrease of sensibility.

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