Journal of Chromatography, 591 (1992) 175–180
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 776

Improvement of extraction procedure for biogenic amines in foods and their high-performance liquid chromatographic determination

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(First received August 1st, 1991; revised manuscript received September 27th, 1991)

ABSTRACT

A high-performance liquid chromatography method is described for the simultaneous determination of the biogenic amines tryptamine, 2-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine in cheese. The optimization of the procedure for the extraction of amines from the matrix is described. The separation of dansyl derivatives of the amines was achieved by reversed-phase liquid chromatography with gradient elution, followed by UV detection at 254 nm. The mobile phase was acetonitrile–0.01 *M* phosphate buffer (pH 7)–water. Under these conditions, rapid elution of the amines in less than 13 min was obtained. Validation of the method included calibration experiments, addition of standard amines for the determination of amine recoveries and repeatability tests.

INTRODUCTION

The formation of biogenic amines, primarily as a consequence of microbial decarboxylation of specific amino acids, is a problem related to proteolytic processes, taking place during the production of fermented food such as cheese, fish products, sausages, wine and beer [1,2]. Biogenic amines are vasoactive products; the consumption of large amounts of these substances may cause problems to some consumers such as headache, nausea, hypo- or hypertension, cardiac palpitation and, in severe cases, intracerebral haemorrhage and death [3–5]. Cheese represents an ideal environment for amine production, but it is a complex matrix for amine determination.

All the analytical methods employed for amines involve two steps: extraction and determination. The extraction of different amines from the matrix represents a very critical step, often resulting in very low analytical recoveries. This problem arises from their different chemical structures. In this case it is necessary to find a compromise in order to obtain a satisfactory recovery for each analyte amine. Many different solvents have been used for the extraction of amines from cheese, such as perchloric acid [6,7], trichloroacetic acid [8], hydrochloric acid [9–11], methanol and other organic solvents [6,12].

The determination of biogenic amines has been carried out with different chromatographic methods: thin-layer chromatography [13–15], gas chromatography [16–18] and high-performance liquid chromatography (HPLC) [19–26], mostly with the last method owing to its high resolution and sensitivity.

In this work we developed an extraction proce-

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dure and a rapid HPLC method for the simultaneous determination of eight biogenic amines in cheese: histamine, tyramine, tryptamine, 2-phenylethylamine, putrescine, cadaverine, spermidine and spermine.

EXPERIMENTAL

Reagents and samples

Tryptamine (Try), 2-phenylethylamine (2-Phe), putrescine (Put), cadaverine (Cad), histamine (His), tyramine (Tyr), spermidine (Spd), spermine (Spm), 1,7-diaminoheptane (1,7-Dh) and dansyl chloride (>99%) were obtained from Fluka (Buchs, Switzerland). Amines, except for 1,7-diaminoheptane, were purchased as hydrochloride salts, and the results were corrected on the basis of their purity and referred to the free base. Orthophosphoric acid (85%) and anhydrous sodium carbonate were obtained from Merck (Darmstadt, Germany). HPLC-grade solvents (Carlo Erba, Milan, Italy) and water purified with a Milli-Q system (Millipore, Bedford, MA, USA) were used throughout.

Parmigiano Reggiano cheese samples were obtained from commercial sources.

Standard solutions

A stock standard solution was prepared by adding an accurately weighed amount of each amine (*ca.* 100 mg) to a 100 ml volumetric flask and diluting to volume with water. Five working standard solutions at different concentrations were prepared from the stock solution, adding a fixed and known amount of internal standard before dilution. Fresh dilute standard solutions must be prepared weekly because some amines are subject to decomposition.

TABLE I

HPLC ELUTION PROGRAMME FOR AMINE ANALYSIS

A = acetonitrile; B = phosphate buffer (pH 7); C = water. Flow-rate, 0.8 ml/min.

Time (min)	A (%)	B (%)	C (%)
0.0	65	35	0
1.0	65	35	0
5.0	80	20	0
5.1	80	0	20
6.0	90	0	10

High-performance liquid chromatography

HPLC determinations were performed with a Varian (Palo Alto, CA, USA) Model 9010 liquid chromatograph and a Rheodyne Model 7161 manual injector with a 10-μl loop. The detector was a Varian Model 9050 UV–VIS spectrophotometer set at 254 nm. An IBM Personal System/2 Model 30 286 computer and an Epson LX-400 printer were used for data aquisition and registration.

The column was a reversed-phase Spherisorb 3S TG (15 cm × 4.6 mm I.D.; particle size 3 μm), with a Spherisorb 5 ODS-2 guard column (Phase Separations, Queensferry, UK). The three solvent reservoirs contained the following eluents: (A) acetonitrile, (B) 0.01 M dipotassium hydrogenphosphate buffer solution, adjusted to pH 7 with orthophosphoric acid (85%), and (C) water. The elution programme consisted of the gradient system shown in Table I, with a flow-rate of 0.8 ml/min. Before use, the eluents were filtered through a 0.22-μm Durapore filter (Millipore) and degassed under vacuum. The eluted dansylamines were detected by monitoring the UV absorbance at 254 nm.

Amine extraction

A modification of the procedure developed by Voigt *et al.* [11] and Lovenburg and Engelman [10] was used for the extraction of all the investigated amines. A 10-g amount of ground cheese, accurately weighed into a centrifuge tube, was homogenized with 20 ml of 0.1 *M* hydrochloric acid containing a known amount of 1,7-diaminoheptane (internal standard) in a Politron homogenizer (Kinematica, Lucerne, Switzerland) at speed 5 for 1 min. The cheese slurry obtained with this step was centrifuged at about 14 000 *g* for 20 min at 4°C with a Cryofuge 20-3 centrifuge, rotor No. 8780 (Heraeus, Karlsruhe Germany). The aqueous layer was collected and the residue was re-extracted with the same procedure.

The two aqueous extracts were combined and, when necessary, filtered through a paper filter. A portion (10 ml) of this extract was saturated with solid sodium carbonate [27] (pH > 12), then an equal volume of *n*-butanol–chloroform 1:1 (v/v) was added and the mixture was blended with a horizontal orbital motion blender for 30 min. After decanting, the upper organic phase was removed, 1 ml of it was introduced into a screw-capped test-tube and two drops of 1 *M* hydrochloric acid were added.

The solution was then evaporated to dryness at ambient temperature with a Uniequip System (Uniequip, Martinsried, Munich, Germany), consisting of a centrifuge (Univapo 100 H) and a refrigerated aspirator (Uniject II). The residue was finally dissolved in 1 ml of 0.1 *M* hydrochloric acid prior to the derivatization step.

Derivative preparation

Other minor modifications to the analytical procedure [19] were introduced for the derivative preparation. A 0.5-ml volume of saturated $NaHCO_3$ solution and 1 ml of dansyl chloride reagent (5 mg/ml) were added in the same test-tube to the sample obtained from previous steps. The test-tube was sealed and the mixture was thoroughly mixed with a Vortex mixer (Tecno Vetro, Monza, Italy) for 20–30 s. The reaction mixture was then left for 1 h at 40°C, with occasional shaking. Finally, instead of extracting the mixture with several portions of diethyl ether and evaporating the combined ether extracts to dryness [19], the mixture was directly evaporated at 40°C with the Uniequip system and the residue was dissolved in 1 ml of acetonitrile for HPLC analysis.

It is assumed that the amine conversion to dansyl derivatives is complete (100%); unfortunately, at present analytical techniques for independently validating this assumption are not available.

RESULTS AND DISCUSSION

Chromatographic conditions

Different solvent mixtures and gradient programmes were tried in order to reduce the analysis time while maintaining a good resolution of all the amine peaks. The achievement of a good resolution of all the eluted peaks was made difficult by the presence of interfering peaks, probably related to the excess of dansyl chloride.

Initially, good results were obtained using as the mobile phase a mixture of acetonitrile and water with a solvent gradient. It was observed that, after a few injections, visible deterioration of the column efficiency occurred, manifested by the appearance of peak broadening and shoulders on the peaks. The replacement of water with 0.01 *M* phosphate buffer solution (pH 7) re-established the optimum column efficiency.

The solvent gradient procedure employed is reported in Table I. In order to avoid precipitation of phosphate buffer salt in the chromatographic system, occurring at acetonitrile concentrations higher than 80%, the HPLC elution computer program provides an exchange between the two reservoirs of phosphate buffer and water. It was observed that it was possible to use a solvent gradient of acetonitrile and water by washing the column every 10–20 injections with phosphate buffer for about 10 min to regenerate the column efficiency. Under these conditions all the amines were eluted in less than 13 min.

Optimization of extraction procedure

According to Voigt *et al.*'s method [11], cheese is extracted with a 0.1 *M* HCl, centrifuged and the aqueous extract is saturated with NaCl, adjusted to pH 10 with Na_2CO_3 and finally extracted with *n*-butanol. Using this procedure, some amines, in particular Put, Cad, Spd and Spm, were not extracted in detectable amounts. In order to obtain a satisfactory extraction recovery for each investigated compound, two extractions of a cheese sample with 0.1 *M* HCl were performed and the pH of the extract was increased above 12 with solid sodium carbonate.

When *n*-butanol–chloroform mixture (1:1, v/v) was used in place of *n*-butanol to accelerate subsequent solvent evaporation, no significant difference in amine recoveries was observed. Before evaporating to dryness, two drops of 0.1 *M* HCl were added to the organic extract in order to obtain amines in their ionic form (hydrochloride) to reduce their volatility. The addition of hydrochloric acid considerably improved the recovery of 2-Phe, but it reduced the recovery of Spd and Spm.

The whole procedure involves multiple steps, and for this reason the use of an internal standard (I.S.) is desirable. 1,7-Dh was chosen as the I.S. for several reasons: it has a retention time not corresponding to the other eluted components, it does not occur naturally in cheese samples and it gives good extraction recoveries.

Fig. 1 compares two different procedures: chromatogram A is for a cheese sample spiked with a known amount of each amine and extracted following the method of Voigt *et al.* [11], and chromatogram B was obtained for the same sample using the extraction method presented here. The improve-

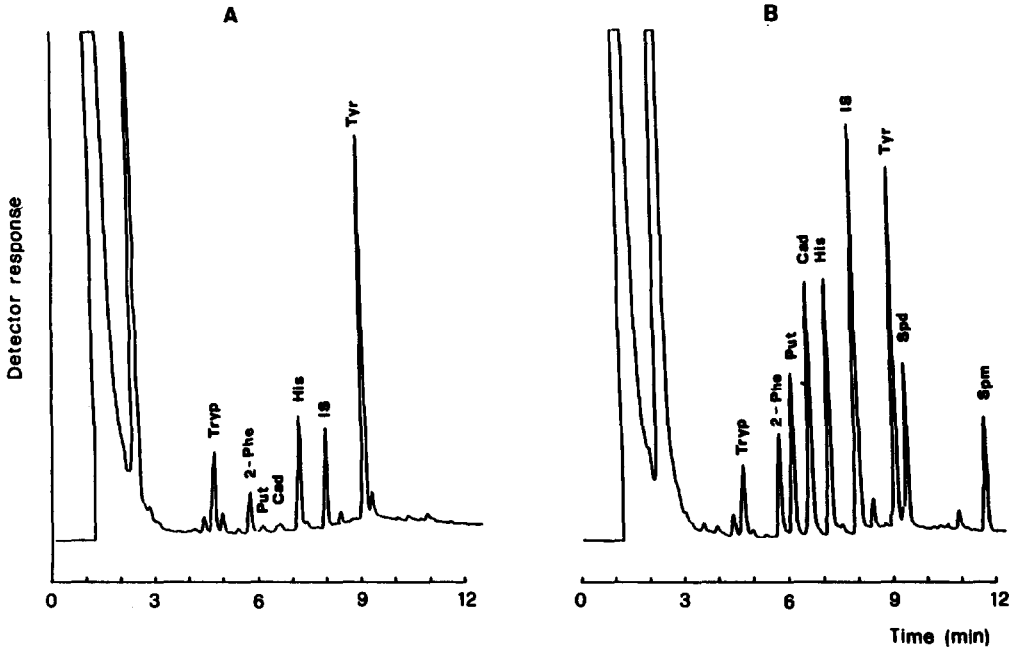

Fig. 1. Chromatograms of two aliquots of the same fortified cheese (see Table V) extracted following different procedures: (A) procedure of Voigt *et al.* [11]; (B) proposed procedure. For chromatographic conditions, see Experimental.

ment in amine recoveries with the present procedure is clear.

Linearity

In order to verify the linearity of the UV detector response at 254 nm for the working concentration of each amine, a portion of each of the five working standard solutions was derivatized and injected into the HPLC system. Calibration graphs were constructed by plotting the amine to I.S. peak-area ratios against the amine to I.S. weight ratios. Linear least-squares regression was used to calculate the slope, intercept and correlation coefficient (r^2). Data on linearity and detection limits are given in Table

TABLE II

DETECTION LIMIT AND LINEARITY

Detection limit is expressed as the amount of amine required to procedure a signal three times the background noise. Linearity data were obtained from a five-level calibration.

Amine	Detection limit (ng)	Range of linearity (ng)	Slope	Intercept	Correlation coefficient (r^2)
Tryp	0.08	6.6–829	0.4430	0.0045	0.9999
2-Phe	0.06	6.1–736	0.5073	0.0039	0.9998
Put	0.02	3.7–463	1.3995	0.0131	0.9998
Cad	0.03	4.0–503	1.1755	0.0105	0.9998
His	0.04	8.0–1003	0.9639	0.0236	0.9997
Tyr	0.02	9.0–1130	0.8682	0.0501	0.9995
Spd	0.03	4.0–503	0.9843	0.0168	0.9990
Spm	0.05	4.1–512	0.8205	0.0069	0.9989

TABLE III

INSTRUMENTAL REPEATABILITY

Results are means of ten replicates of the same sample. $\bar{x}$ = Mean; S.D. = standard deviation; R.S.D. = relative standard deviation.

Amine	$\bar{x}^a$	S.D.	R.S.D. (%)
Tryp	2.110	0.004	0.2
2-Phe	1.949	0.008	0.4
Put	0.703	0.003	0.4
Cad	0.743	0.002	0.3
His	0.986	0.005	0.5
Tyr	1.003	0.003	0.3
Spd	0.845	0.011	1.3
Spm	1.084	0.011	1.0

[a] x = [(I.S. area/standard amine area) × (standard amine concentration/I.S. concentration)].

TABLE IV

ANALYTICAL REPEATABILITY

Results are means for ten cheese samples which underwent the whole analytical procedure. For abbreviations, see Table III.

Amine	$\bar{x}^a$	S.D.	R.S.D. (%)
Tryp	2.631	0.099	3.8
2-Phe	3.516	0.076	2.2
Put	0.388	0.036	9.3
Cad	0.933	0.060	6.4
His	5.719	0.188	3.3
Tyr	21.890	0.578	2.6
Spd	0.415	0.042	10.1
Spm	0.403	0.043	10.7

[a] x = [(amine area/I.S. area)/cheese weight] × 100.

II; a linear relationship with $r^2 > 0.999$ was always obtained. The concentration ranges are representative of amine concentrations detected in cheese samples.

Repeatability

A standard solution was derivatized and the mixture was injected ten times to evaluate the repeatability of the chromatographic system. Means ($\bar{x}$), standard deviations (S.D.) and relative standard deviations (R.S.D.) of the response factors are reported in Table III.

The repeatability of the whole method was examined on a Parmigiano Reggiano cheese sample with a low amine content. Ten aliquots of the same sample were extracted with 20 ml of 0.1 *M* HCl, containing a known amount of each amine, and analysed using the proposed procedure. Results are given in Table IV.

Recovery

The recovery of the method was determined by a standard addition technique. A sample of cheese was spiked with a known amount of amine standard

TABLE V

RECOVERY

Results are means for three cheese samples which underwent the whole analytical procedure.

Amine	Amount determined in sample (mg per 100 g)	Amount of standard added (mg per 100 g)	Amount found in sample (mg per 100 g)	Recovery (%)
Tryp	ND[a]	1.66	1.17	70.5
2-Phe	0.08	1.53	1.51	93.8
Put	0.03	0.93	0.50	52.1
Cad	0.01	1.01	0.78	76.5
His	0.45	2.01	2.46	100.1
Tyr	4.20	2.26	5.35	82.8
Spd	0.01	1.01	0.64	62.7
Spm	0.04	1.02	0.43	40.6
IS	ND	3.93	3.82	97.2

[a] ND = Not detectable.

solution in triplicate. The extraction recovery was determined by carrying out the proposed procedure. To calculate the percentage recovery, concentrations determined before and after standard addition were compared. Results calculated as means of three values, and expressed as milligrams of amine per 100 g of cheese sample, are summarized in Table V together with the percentage recoveries.

We observed that the acid extracts of cheese samples were not stable. Indeed, the recoveries of some amines, particularly Put, Cad, Spd and Spm, decreased considerably after only 3 days of storage in a freezer (−18°C).

CONCLUSIONS

The proposed method appears to be suitable for the rapid detection of a relatively large number of amines in cheese samples. Both a linear response of the detector with increasing analyte concentration and good repeatability of the results were obtained. The recoveries were good for all amines except for Put, Spd and Spm. However, a compromise between optimization of amine recovery and number of biogenic amines detected had to be made, owing to the differences in the partition coefficients of amines in the extraction medium. We expect this method also to be suitable for the detection of biogenic amines in other food matrices.

ACKNOWLEDGEMENTS

This work was carried out within the framework of the Ministero dell' Agricolture e delle Foreste (MAF)-application oriented project "Moderne Strategie Lattiero-Casearie" (No. 3). To support of MAF (Italy) is gratefully acknowledged.

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