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High-performance liquid chromatographic evaluation of biogenic amines in foods
An analysis of different methods of sample preparation in relation to food characteristics

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Abstract

Biogenic amines are compounds formed by amino acid decarboxylation in fermented foods. Most of the methods for amine determination involve acid extraction followed by a liquid–liquid purification step. The different parameters which can influence amine recoveries are considered; experience with different foods such as cheese, fish and meat preserves are reported and for each of them the optimized analytical procedure is described. Data concerning recovery and repeatability of the method are also reported and the various factors that influence amine extraction are discussed. The possibility of applying direct derivatization without any other purification step is also considered.

Keywords: Food analysis; Sample preparation; Amines

1. Introduction

Biogenic amines are substances that can occur in plants and in fermented foods: these compounds are produced by a number of microorganisms, as a result of amino acid decarboxylation reactions. These reactions are catalyzed by exogenous or endogenous enzymes that are specific for each amino acid and that have pyridoxal-5-phosphate as a co-factor. By means of decarboxylation reactions, tyrosine produces tyramine, histidine yields histamine and arginine leads to putrescine, by means of an intermediate state represented by ornithine. Cadaverine is derived from lysine, tryptamine from tryptophan and 2-phenylethylamine is derived from phenylalanine [1]. Furthermore, putrescine is also an intermediate of a metabolic pathway that leads to spermidine and spermine.

A number of chromatographic methods have been proposed for the quantitative determination of amines: thin-layer chromatography [2], ion-exchange chromatography [3,4], gas chromatography with a packed column [5] and several gas chromatographic techniques [6,7].

Reversed-phase HPLC [8] is usually considered the most suitable technique for this analytical purpose. All the cited methods involve two main steps, i.e. amine extraction from the matrix, including purification of the raw extract, and determination of these amines.

The first phase is the most critical in terms of obtaining an adequate recovery for each amine. The

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extraction of amines from a solid matrix can be carried out with water, at room temperature [9,10], or higher temperatures [8], so that only free amines are extracted, or in an acid medium, with hydrochloric acid (HCl) [8,11,12], perchloric acid ($HClO_4$) [13–16] or with trichloroacetic acid (TCA) [17–21], so that amines linked to other matrix components can also be extracted. Several organic solvents, such as methanol [22–24], acetone [25], acetonitrile–$HClO_4$ [26] or dichloromethane–$HClO_4$ [27] can be used. Some authors [22,28] have compared different acidification methods, obtaining different results.

An extract purification step may follow, however, the degree of purity needed is a function of the final analytical technique. The extract purification treatment can be divided into two main groups: (1) column chromatography with alumina or ion-exchange resins and (2) liquid–liquid extraction with organic solvents.

When the first method is applied, an initial pH adjustment is required, followed by activation, washing and elution steps that are time consuming [8,10,14,23].

When a liquid–liquid extraction is applied, the raw extract is saturated wih a salt, then the pH is adjusted to an alkaline value and an extraction with an organic solvent (butanol, butanol–chloroform, etc.) is carried out to obtain a solution of amines free of amino acids [13,17,22,23,29,30,31].

The choice of salt for saturation is important: Taylor et al. [22] tested a number of different salts but did not discuss the combined effects of salts and pH.

All the parameters which influence amine recovery under conditions where a purification step with an organic solvent of the acid extract follows, prior to derivatization with dansyl chloride and HPLC analysis on a reversed-phase column, will be discussed in this paper. Optimized methods of sample preparation for different foods (cheese, meat and fish) will be given.

2. Experimental

2.1. Chemicals

Tryptamine (Tryp), 2-phenylethylamine (2-Phe), putrescine (Put), cadaverine (Cad), histamine (His), tyramine (Tyr), spermidine (Spd), spermine (Spm), 1,7-diamminoeptane (1,7-Dh) and dansyl chloride (DCl) were purchased from Fluka (Buchs, Switzerland).

For HPLC analysis, Acetonitrile Super Gradient Lab Scan (Dese Lab, Piombino Dese, Padova, Italy) and water purified with a Milli-Q system (Millipore, Badford, MA, USA) were used throughout.

All other reagents and solvents were supplied by Carlo Erba (Milan, Italy).

2.2. Standard solution

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 the required volume with water.

2.3. Equipment

1. Varian Liquid chromatograph (Palo Alto, CA, USA) Model 9010 equipped with a Rheodyne Model 7161 manual injector with a 10 μL loop.
2. Varian UV–VIS spectrophotometer detector Model 9050 UV–VIS set at 254 nm
3. In-line degasser ERC-3612 (ERMA, Tokyo, Japan)
4. Polytron Homogenizer (Kinematica, Luzerny, Switzerland)
5. Ultracentrifuge Cryofuge (Model 20.3, Heraeus, Christ Karlrhue, Germany), equipped with a Model No. 8780 rotor
6. Vortex mixer (Tecnovetro, Monza, Italy)
7. Blender (Model 711, Vetrotecnica, Milan, Italy)
8. Crison automatic titrator: Micro TT 2050 module, Crison high alkalinity electrode cat. No. 52-04 (Aella, Barcelona, Spain), automatic burette Micro Bur 2030 9-ml volume ($\pm$ 0.002 ml), shaker, sampling system suitable for working with 15 samples, Citizen printer (Crison Maselli, Carpi, Modena, Italy)
9. Uniequip system (Uniequip, Martinsried, Munich, Germany) for sample concentration

2.4. Chromatographic conditions

Tha column was a reversed-phase Spherisorb 3S TG, 15 cm $\times$ 1.6 mm I.D.; particle size 3 μm (Phase Separation, Queensberry, UK)

Table 1
HPLC elution profile programme for amine analysis

Time (min)	A (%)	B (%)
0	65	35
1	65	35
5	80	20
6	90	10

A = acetonitrile; B = water; flow-rate 0.8 ml/min.

The mobile phase consisted of acetonitrile and water; the gradient elution program is reported in Table 1.

2.5. Sample preparation

A precisely weighed 10 g sample was homogenized with a Polytron homogenizer in an acidic medium: if cheese is to be analyzed, two extractions with 20 ml of 0.1 *M* HCl were carried out, whereas for fish or meat products, three extractions with 15 ml of 5% trichloroacetic acid (TCA) were necessary. 1,7-diaminopentane (1,7-De) was used as the internal standard.

The organic extracts were saturated with NaCl and the pH was adjusted to 11.5 with an automatic titrator. An extraction with organic solvent (butanol for cheese and butanol–chloroform for fish and meat products) was then performed. This was carried out either in a test tube on 5 ml of acid extract, with three portions of 5 ml each (Vortex agitation) or in a separator funnel, with blender agitation, for 30 min.

The derivatization was then performed in a test tube as follows: 1 ml organic extract was dried under vacuum (UNIEQUIP), after 2 drops of 1 *M* HCl had been added. Then 1 ml of 0.1 *M* HCl, 0.5 μl saturated solution of $NaHCO_3$ and 1 ml dansyl chloride solution (5 mg/ml) were added. The reaction vessel was incubated at 40°C for 1 h, then the solution was dried under vacuum, acetonitrile was added and HPLC injection followed.

3. Results and discussion

As already discussed in Section 1, most of the methods available in the literature for biogenic amine determination involve an acid extraction from a solid matrix, after the saturation and alkalinization of the extract, a liquid–liquid partition with an organic solvent follows.

The selective extraction of single amines from the acid solution is a process influenced by many factors such as: the type of acid, the type of organic solvent, the salt used for saturation, the pH at which amine extraction (liquid–liquid partition with the organic solvent) is carried out, the time and the type of stirring, etc.

3.1. Choice of acid

Some authors have compared the extraction capability of several acids for different amines, but did not obtained the same results. The choice of acid has to be related to the characteristics of the matrix to be analyzed.

On the basis of our experience, 0.1 *M* HCl appears to be a good choice for the analysis of cheese, however, it is not a suitable acid for fish or meat products.

In the latter case, difficulties related to sample turbidity and the presence of poorly-resolved interfering peaks were observed. In the case of fish and meat product samples, 5% TCA represented a better choice. It has been used by a number of authors [17–19] because of its capacity to precipitate proteins.

3.2. Influence of pH

pH is the key parameter that almost certainly exerts the greatest influence on the partition equilibrium of amines between the two phases (aqueous and organic).

Amine recoveries versus pH of the extract in the liquid–liquid partition step are reported in Fig. 1. Fig. 1a refers to a standard solution of amines in 5% TCA, extracted with butanol–chloroform (1:1, v/v), while Fig. 1b–c report the recoveries of the same standard solution in 0.1 *M* HCl, extracted with *n*-butanol and butanol–chloroform (1:1, v/v).

These data were obtained by diluting two 5 ml portions of amine stock solution to 500 ml with 0.1 *M* HCl and 5% TCA.

50 ml portions of the solution thus obtained were then saturated with NaCl, the pH was adjusted to different values and then it was extracted as described in Section 2.5.

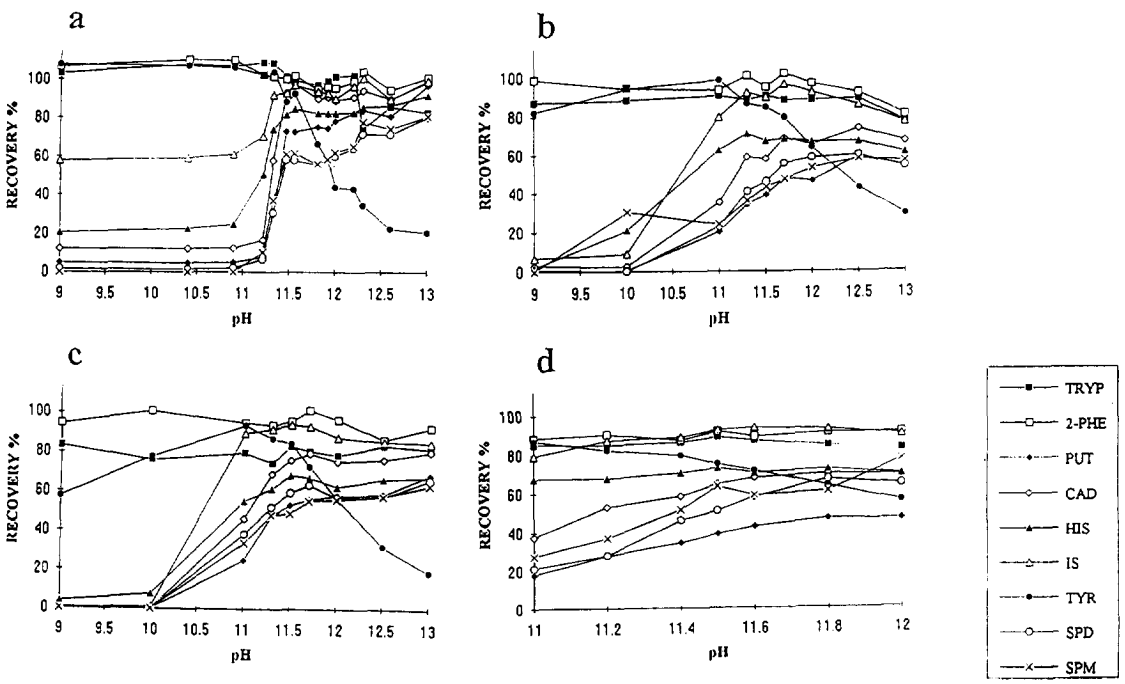

Fig. 1. Amine recovery trends as a function of pH: (a) from 5% TCA with butanol–chloroform (1:1, v/v); (b) and (c) from 0.1 *M* HCl with butanol–chloroform (1:1, v/v) respectively and (d) from a Grana Padano cheese extract (0.1 *M* HCl) with butanol.

Blank analysis was performed in previous work [32] for an acid solution without amines. No peaks with the same retention times as the amines were observed.

Recovery of each amine was calculated by comparing the chromatographic areas of the peaks with those obtained for a directly derivatized standard solution of the same concentration.

The graph in Fig. 1d shows the recoveries recorded for a sample of cheese with a low amine content, whose acid extract (0.1 *M* HCl) was spiked with a known amount of amine.

These data show that a strict control of pH in the extraction phase is absolutely essential to obtain reproducible data. The choice of optimum pH is related to the amines to be determined, as their behaviour changes at different pH, due to their different chemical structures. pH 11.5 represents a compromise situation which allows satisfactory recoveries for some amines, but above pH 11.5, Tyr recovery was drastically reduced, reaching minimum values at pH 12.5–13.0, which are values used by some authors to determine tyramine [13,33]. On the other hand, pH 10.0, as used by several authors [8,9,25,30] is the optimum for Tyr, but quite unsuitable for Put, Cad, Spm, Spd and His.

The magnitude of the influence of pH is well represented by the HPLC traces reproduced in Fig. 2, where the traces refer to the same amine solution in

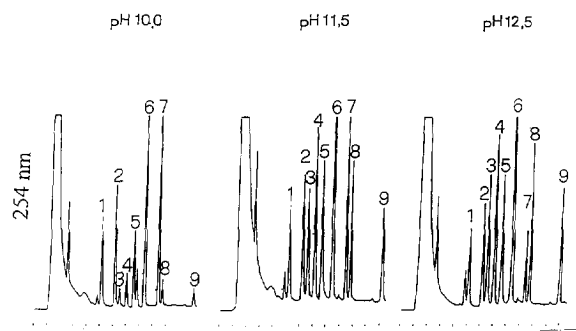

Fig. 2. Chromatograms of three aliquots of the same amine standard solution (5% TCA) extracted with butanol–cholorofrom (1:1, v/v) at three different pH values. Identification of the peaks: 1=Tryp, 2=2-Phe, 3=Put, 4=Cad, 5=His, 6=I.S. 8=Spd and 9=Spm.

5% TCA extracted with butanol–chloroform at three different pH values (10.0, 11.0 and 12.5).

3.3. Influence of the acid

Comparing the trends of recoveries obtained from standard solution of amines in 0.1 *M* HCl and 5% TCA represented in Fig. 1a–c, it can be possible noted that the maximum variation for Spm, Spd, Put and Cad occurs between pH 11.2 and 11.4 for 5% TCA (Fig. 1a). In this small range (0.2 pH units), the recovery of Cad rises from less than 20% to about 90%, whereas when 0.1 *M* HCl was used (Fig. 1b and Fig. 1c), similar modifications in Cad recovery were observed in the pH range between 10.0 and 11.0.

3.4. Influence of organic solvent

An examination of the recovery data for a standard solution of amine to variations of pH shows that different solvents [butanol or butanol–chloroform (1:1, v/v)] have different selectivity for different amines.

Better recoveries were obtained for Tryp and His when *n*-butanol was used at the pH of interest for amine extraction, but values were lower for Put, Cad, Spd and Spm.

The use of butanol–chloroform (1:1, v/v) was also more suitable considering the time needed for solvent evaporation, compared to pure butanol; nevertheless, for cheese, this solvent mixture often involves the formation of a gel which interferes with phase separation. Similar behaviour has been observed in non-food biological samples [32].

3.5. Salt effect

The presence of salt is very important as it allows amines to pass into the organic layer as in the absence of salts, unsatisfactory recoveries are obtained, even under optimum pH conditions.

A paper has appeared in the literature [24] which compares the use of several salts, and indicated that Na_2CO_3 was optimum for obtaining quantitative extractions. A method for cheese previously developed in our laboratory [30] used Na_2CO_3 to saturate the extract and render it alkali: recoveries calculated in this case were quite adequate for each amine; nevertheless, when this method was applied to fish or meat products, very low recoveries values for Put, Cad, Spm and Spd were obtained. The measurement of the pH of different food extracts after Na_2CO_3-saturation demonstrated a wide range of pH values reached under saturation conditions.

Table 1 reports pH values measured on a portion of salmon extract in 5% TCA, saturated with Na_2CO_3 at different temperatures (27°C and 22°C) to which 1 g NaCl had been added.

In our opinion, Na_2CO_3 saturation is difficult to standardize, as different amounts of solutes present in different matrices can influence the Na_2CO_3 solubility and, consequently, the pH reached at saturation. Furthermore, Na_2CO_3 solubility is highly influenced by temperature and differences of 5°C involve, as shown in Table 2, pH differences of 0.5 units. It is necessary to choose a salt that will not influence the pH. NaCl was suitable for this purpose.

3.6. Recovery

The recoveries obtained with the proposed method were determined by adjusting the pH of the extract from several foods to 11.5. Samples were spiked with a known amount of amines and were analyzed three times each. Recoveries were calculated by comparison of data obtained with and without spiking the samples. Data from these experiments and those concerning the standard solutions in TCA and HCl are reported in Table 3.

An interesting observation is that large differences can occur in the recovery of each amine in comparison with those of a standard solution, dissolved

Table 2
Effect of temperature and sodium chloride on sodium bicarbonate solubility

Sample	*T* (°C)	pH
A	27	11.0
B	22	10.5
C	27	10.6

A, B = salmon extract (TCA 5%) saturated with Na_2CO_3 and Na_2CO_3 respectively; C = salmon extract added with 1 g NaCl, saturated with Na_2CO_3.

Table 3
Recovery

Amine	Standard solution	Salmon		Tuna		Salami		Standard solution	Grana cheese			Gorgonzola cheese
	TCA 5% Recovery	Recovery	R.S.D.	Recovery	R.S.D.	Recovery	R.S.D.	0.1 *M* HCl Recovery	Recovery	R.S.D.	Recovery	R.S.D.
Tryp	100	N.Q.		N.Q.		N.Q.		91	62	6.7	74	8.2
2-Phe	101	85	1.8	91	1.7	87	2.6	95	79	5.5	92	3.9
Put	86	65	2.8	68	4.1	2.9	40	40	2.5	44	2	
Cad	86	80	2.2	86	1.9	82	2.6	58	61	2.7	65	1
His	81	81	2.7	84	1.7	76	3.2	67	67	1.8	63	2.1
I.S.	82	88	2.9	99	3.7	91	3.7	90	80	6.2	88	0.3
Tyr	85	71	2.5	76	2.5	77	2.5	85	59	6	63	0.5
Spd	61	49	4.4	60	5	67	4.2	46	49	7.1	49	1.2
Spm	59	47	9.8	52	2.6	72	6	43	44	5.3	46	5.8

Results are the mean for three samples that underwent the whole analytical procedure.
N.Q. = not quantifiable for the presence of interfering peaks.

directly in acid solution. This fact could be due to the effect of some substance which passes from the matrix to the solution, affecting the partition rate of the amines.

3.7. Repeatability

The repeatability of the entire analytical procedure for a cheese sample as described in Section 2.5 was verified using a sample of Grana Padano cheese with an average amine content. Average amine content (*Xa*), standard deviation (S.D.) and R.S.D. of area values measured for eight replicate analyses are reported in Table 4.

3.8. Direct derivatization

The direct derivatization of the acid extract has allowed many authors [12,15,16,21,28] to obtain the simultaneous determination of free amino acids and biogenic amines using an HPLC–ion pair technique. However, the complete separation of these two classes of compounds takes a long time and critical separations often occur. In the chromatographic conditions used in this paper, free amino acids did not produce any interfering peaks, as they were all eluted within the first four minutes of the chromatographic run; nevertheless, some difficulties may be encountered with samples having high free amino acid content. The amount of dansyl chloride (DCl), even if generally employed in considerable excess, may not always be adequate to obtain a quantitative derivatization of the amino acids and amines. A larger amount of DCl can be used, but over certain levels (10–20 mg/ml), the HPLC trace becomes worse, intefering peaks appear and a sample dilution may be necessary. In these cases it would be necessary to know the free amino acid content before direct derivatization is applied.

In conclusion, this technique can be advantageous, mainly in terms of recoveries which, in practice, are quantitative; on the other hand, however, in samples with high concentrations of free amino acids, the necessity of a dilution leads to a lack of sensitivity.

Table 4
Analytical repeatability

Amine	*Xa*[a]	S.D.	R.S.D. (%)
Tryp	tr	–	–
2-Phe	tr	–	–
Put	0.323	0.020	6.1
Cad	0.282	0.013	4.8
His	0.341	0.205	6.1
Tyr	1.521	0.052	3.4
Spd	0.599	0.047	7.8
Spm	0.212	0.027	12.5

Results are the mean of eight cheese samples which underwent the whole analytical procedure.
Xa = mean; S.D. = standard deviation; R.S.D. = relative standard deviation.
[a] *Xa* = [(amine area/I.S.)/cheese weight] × 100.

4. Conclusions

In consideration of the results in this paper, some quantitative evaluations of amines in fermented foods should probably be corrected, as data often appear in the literature that have not been obtained under optimum pH conditions. This is an important consideration, as data concerning amine content are often related to the microbiological characteristics of the product, which is often the result of human action, as is the case of microbial starters for cheese, or meat preserves such as salami. As different microbial strains are able to produce different actions in terms of amine production, only a rigorous control of the analytical conditions used allows the possibility of formulating a correct microbial starter.

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