HPLC DETERMINATION OF AGMATINE AND OTHER AMINES IN WINE

DETERMINATION PAR HPLC DE L'AGMATINE ET DES AUTRES AMINES DANS LE VIN

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Abstract : An optimised HPLC analysis is described for the determination by dansylation of the following 11 biogenic amines in wine: agmatine, cadaverine, ethanolamine, histamine, methylamine, 2-phenylethylamine, spermine, spermidine, putrescine, tryptamine and tyramine. Seven amines were found in red and white wines produced in Southern Italy, being present at levels ranging from not detectable to 10.97 mg/L. The most abundant amine resulted ethanolamine, while the polyamine present at the highest concentration was agmatine with maximum levels of 9.92 mg/L. Total biogenic amines content was higher in the red wines.

Résumé : Nous décrivons une analyse optimisée pour la détermination par dansilation des 11 amines biogènes suivants : agmatine, cadavérine, éthanolamine, histamine, méthylamine, 2-phényléthylamine, spermine, spermidine, putrescine, tryptamine et tyramine. Sept amines ont été détectés dans les vins rouge et blanc produits dans le sud de l'Italie. Leur quantité varie du niveau non détectable à 10.97 mg/L. L'amine le plus abondamment présent est l'éthanolamine, alors que le polyamine présent en plus grande concentration est l'agmatine, atteignant un maximum de 9.92 mg/L. Le vin rouge contient un nombre total d'amines biogènes plus élevé.

Keywords: biogenic amines, HPLC analysis, wine

Mots clés : amines biogènes, analyse par HPLC, vin

INTRODUCTION

Biogenic amines are organic nitrogenous compounds of low molecular weight that can represent a serious health hazard for humans and animals when present in food in significative amounts. Therefore these compounds have been suggested as an index of quality or poor manufacturing practices in foodstuffs (GLORIA *et al.*, 1998; MORET and CONTE, 1996). These moieties are mainly produced from microbial decarboxylation of the corresponding amino acids. In wines it has been often reported that biogenic amines increases after malolactic fermentation, red wines usually being richer in amines than white wines (IÑIGUEZ CRESPO and VAZQUEZ LASA, 1994). Strains of *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* genera resulted able to produce histamine and also other amines (ARENA and MANCA DE NADRA, 2001; GUERRINI *et al.*, 2002; LONVAUD-FUNEL, 2001).

In wines, amines occur as odourless salts, but at the pH prevailing in the mouth amines are partly liberated and their flavour becomes apparent, affecting the sensory characteristics of the wine (ACHILLI *et al.*,1994; LEHTONEN, 1996). Furthermore, in alcoholic beverages it is important to take into account the synergic effect between the amines and ethanol (BUSTO *et al.*, 1997).

Generally the most investigated amines in wines are hystamine, tyramine, phenylethylamine, putrescine and cadaverine (LEHTONEN, 1996). Methylamine and ethanolamine seems to be abundant in wines, but they have been little investigated (BUSTO *et al.*, 1995; IÑIGUEZ CRESPO and VAZQUEZ LASA, 1994; Soleas *et al.*., 1999).

Natural polyamines, such as agmatine, cadaverine and putrescine are generally considered as ubiquitous bioregulators of numerous cell functions, being involved in cell growth, division and differentiation processes. On the other hand, some authors have suggested a synergic relationship between these polyamines and histamine with a consequent negative effect on the human health (FERNANDES and FERREIRA, 2000). In wines the presence of putrescine and cadaverine is well documented, while relatively few information are available about the determination and the role of agmatine, even if noticeable amounts of agmatine have been

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found in wines and in other alcoholic beverages (BAUZA *et al.*, 1995; GLORIA *et al.*, 1998; IZQUIERDO-PULIDO *et al.*, 1996).

The concentration of biogenic amines has been reported to range from a few mg/L to about 50 mg/L, depending on the quality and vine variety (BUSTO *et al.*, 1995); however only few data are available on the biogenic amines content in Italian wines (ACHILLI *et al.*, 1994; ARLORIO *et al.*, 1999; VECCHIO *et al.*, 1989).

Many analytical methods based on different techniques, have been described for determining the biogenic amines content in wines, but high-performance liquid chromatography (HPLC) with ion-exchange or reversed-phase column is the one preferred, using either pre-column or post-column derivatization procedures with dansyl chloride (DnsCl) or o-phthalaldehyde (OPA) and UV or fluorimetric detectors for identification (Lehtonen, 1996).

In this paper a modification of a previous high performance liquid chromatography method applied to cheese (GALGANO *et al.*, 2001) has been described to assess the content of agmatine and other biogenic amines in wines produced in the Southern Italy.

MATERIALS AND METHODS

I - WINE SAMPLES

Five red wines (Aglianico of Vulture, from the Basilicata region) and five white wines (GRILLO, from the Sicilia region) were purchased at retail stores. Each wine was extracted and analysed twice.

II - STANDARD SOLUTIONS

Cadaverine (cad), ethanolamine (etam), histamine (his), methylamine (met), 2-phenylethylamine (2-phe), putrescine (put), spermidine (spd), spermine (spm), tryptamine (trypt), tyramine (tyr), were obtained from Fluka (Milano, Italy). Amines were purchased as hydrochloride salts of the highest available purity, and the analytical results were referred to the free base. A stock standard solution was prepared by accurately weighting about 25 mg of each amine in a 50 mL volumetric flask and bringing up to volume with bidistilled water. Agmatine (agm) (Fluka) was purchased as sulphate salt and the standard solution (about 25 mg/50 mL), was prepared separately utilising methanol as solvent. Five working standard solutions, containing all the amines at concentrations ranging from 0.2 to 20 mg/L, were then prepared from the stock solution.

III - DERIVATIZATION AND CONCENTRATION

Samples derivatization was performed modifying the method proposed by Galgano *et al.* (2001). An aliquot of 100 mL of sample was mixed with 5 mL of 1,7 diaminoheptane (Fluka) as internal standard (60 mg/mL), 200 mL of a saturated Na_2CO_3 solution and 400 mL of dansyl chloride (Fluka) solution (7.5 mg/mL dansyl chloride/acetone) and stirred for 2 min using a Vortex. The mixture was then transferred to an incubator and kept at 60 °C under agitation (195 strokes/min) for 5 min. The residual dansyl chloride was removed by adding 100 mL of a proline solution having a concentration of 100 mg/mL(Carlo Erba, Milano, Italy). The mixture, left for 15 min at 20 °C shielded from light, was then added with 500 ml of diethyl ether (Carlo Erba), and stirred twice for 1 min. The organic phase was subsequently recovered and evaporated under a stream of nitrogen, and the residue was solubilized in 300 mL of acetonitrile. The derivatized samples, stored at -20 °C until analysed, were filtered through a 0.22 mm PTFE filter (Alltech, Sedriano, Italy) immediately before the analysis.

IV - HPLC ANALYSIS

The chromatographic system consisted of a Varian (Leini, Italy) 9012 pump, a Varian 9050 UV/VIS detector and a personal computer running the chromatographic software STAR 4.5 (Varian). The chromatographic separations were carried out utilising either a C18 Spherisorb 3STG (3 mm, 150 x 4.6 mm, Waters, Milano, Italy), equipped with a Spherisorb 5 ODS-2 guard column (Waters), or a C18 Symmetry Shield RP18 (3.5 mm, 150 x 4.6 mm, Waters) equipped with a Sentry guard column (Waters). The analytes were eluted using acetonitrile and H2O as solvents (flow-rate 0.8 mL/min), and two different

Table I - HPLC elution program for biogenic amine analysis with the Simmetry Shield column.

Programme d'élution pour l'analyse HPLC des amines biogènes avec la colonne Simmetry Shield.

Time (min)	A (%)	B (%)
0.00	50	50
6.00	50	50
10.00	75	25
15.00	75	25
20.00	85	15
22.00	85	15
26.00	95	5
28.00	95	5
34.00	50	50
38.00	50	50

A=acetonitrile
B= H_2O

gradient profiles were utilised, according to the column chosen. All the solvents were of HPLC grade (Carlo Erba) and the injection volume was of 20 mL. Detection of the peaks was achieved at 254 nm and the amines were tentatively identified on the basis of their retention times and by spiking the samples with known amounts of standards.

RESULTS AND DISCUSSION

Chromatographic separation of the amines was initially attempted on the C18 Spherisorb 3STG column, using the analytical method proposed by GALGANO *et al.* (2001) for the analysis of biogenic amines in cheese samples, and using dansyl chloride as derivatising agent. The choice of this method was made taking into account the simplicity of the derivatisation step and the high stability of the dansyl derivates (KRAUSE *et al.*, 1995). Despite several attempts to optimise the gradient profile for the separation of the eleven amines investigated, only eight were well resolved; agmatine and methylamine showed the same retention time, while ethanolamine was not adequately retained by the column and was eluted with the solvent front.

In order to achieve complete separation of the amines, a slightly different column (Symmetry Shield RP18) was utilised. A peculiar characteristic of this end-capped column, is the presence of embedded polar groups, in close proximity of the silica backbone, that can further reduce the activity of the surface silanols of the column.

With an adequate modification (table I) of the elution gradient previously utilised, it was possible to improve noticeably the chromatographic separation, and all the eleven amines were completely separated. Figure 1 shows a typical chromatogram obtained using the Symmetry Shield RP18 column. All the analytes were well resolved and the other peaks appearing in the chromatogram could be ascribed to secondary products originated during the derivatization process.

The analytical characteristics of the proposed method are reported in table II. Linearity on a three-point calibration was assessed for each amine at concen-

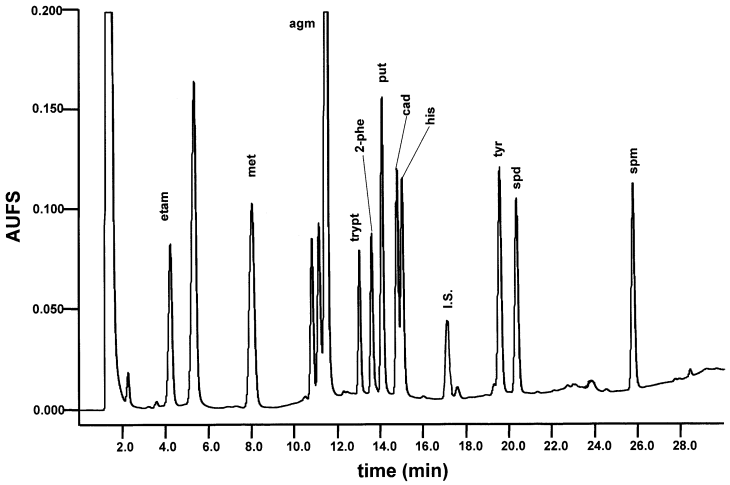

Fig.1 - Chromatographic separation of a biogenic amine standard solution with Simmetry Shield column.

etam= ethanolamine; met= methylamine; agm= agmatine; trypt= tryptamine; 2-phe =2-phenylethylamine; put= putrescine; cad= cadaverine; his= histamine; I.S.= internal standard; tyr= tyramine; spd= spermidine; spm= spermine.

Fig.1 Séparation par chromatographie des amines biogènes présents dans une solution standard avec la colonne Simmetry Shield .

etam= éthanolamine; met= méthylamine; agm= agmatine; trypt= tryptamine; 2-phe =2-phényléthylamine; put= putrescine; cad= cadavérine; his= histamine; I.S.= standard interne ; tyr= tyramine; spd= spermidine; spm= spermine.

Table II - Analytical characteristics of the HPLC method for the analysis of biogenic amines in wine.

Caractéristiques analytiques de la méthode HPLC pour l'analyse des amines biogènes du vin.

Biogenic amines	Linearity[a] (R_2)[b]	Overall recovery[c] (%)	Reproducibility[c] (CV %)
Agmatine	0.999	95 ± 2.8	3.1
Cadaverine	0.986	87 ± 3.7	4.2
Ethanolamine	0.990	98 ± 2.4	2.9
Histamine	0.966	99 ± 5.6	6.2
Methylamine	0.995	94 ± 6.2	5.0
2-Phenylethylamine	0.991	102 ± 7.4	4.3
Putrescine	0.984	94 ± 3.2	3.9
Spermidine	0.962	70 ± 2.1	4.2
Spermine	0.975	34 ± 1.5	4.8
Tryptamine	0.994	103 ± 7.5	3.6
Tyramine	0.989	97 ± 5.1	7.1

a Linearity was determined for amine concentrations ranging from 0.2 to 20 mg/L.
b Square of regression coefficient.c Average of six replicates of each of three concentrations. a La linéarité a été déterminée pour une concentration en amines variant de 0.2 à 20 mg/L. b Carré du coefficient de régression c Moyenne de six réplications de chacune des trois concentrations.

Table III - Biogenic amines content in red and white wine samples of Southern Italy.

Amines biogènes présents dans les vin rouge et blanc du sud de l'Italie.

Biogenic amines	Concentration (mg/L)	
	Red wines (n=5)	White wines (n=5)
Agmatine	9.92 ± 2.07	2.46 ± 1.16
Cadaverine	0.23 ± 0.40	nd
Ethanolamine	10.97 ± 4.71a	2.22 ± 0.97
Histamine	1.07 ± 0.31	nd
Methylamine	ndb	nd
2-Phenylethylamine	0.53 ± 0.25	nd
Putrescine	2.04 ± 0.87	2.15 ± 0.53
Spermidine	nd	nd
Spermine	nd	nd
Tryptamine	1.26 ± 0.82	nd
Tyramine	0.58 ± 0.22	nd
Total amines	26.02 ± 9.12	6.83 ± 1.46

Mean values ± standard deviation were calculated by using zero for nd.

a = standard deviation ; b = not detected

Moyenne +/- écart type ont été calculés en utilisant 0 pour la non détection.

a = écart type ; b = non détection

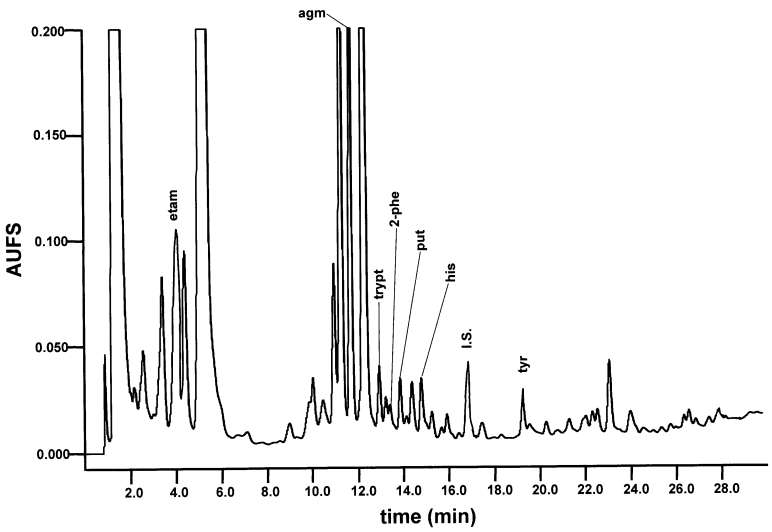

Fig.2. Chromatographic separation of biogenic amines in a wine sample.

etam= ethanolamine; agm=agmatine; trypt= tryptamine; 2-phe= 2-phenylethylamine; put= putrescine; his= histamine; I.S.= internal standard; tyr= tyramine.

Séparation par chromatographie des amines biogènes présents dans échantillon de vin.

etam= éthanolamine; agm= agmatine; trypt= tryptamine; 2-phe =2-phénylethylamine; put= putrescine; his= histamine; I.S.= standard interne; tyr= tyramine.

trations ranging from 0.2 to 20 mg/L. Percentages of amine recoveries were calculated extracting six times wine samples spiked with known amounts (6.12 mg/mL, 10.25 mg/mL and 14.45 mg/mL) of each amine.

Recoveries were good for all the amines, according to ROMERO *et al.* (2000), with the exception of spermidine and spermine, whose recoveries were 70 % and 34 %, respectively. These results indicate that the method is not suitable for the research of these two amines, especially spermine. However, it should be pointed out that spermidine and spermidine have been reported to be present in alcoholic beverages at very low concentrations, and that they contribute in a minimal amount to the total amine content (BAUZA *et al.*, 1995; BUIATTI *et al.*, 1995; IZQUIERDO-PULIDO *et al.*, 1996; ROMERO *et al.*, 2000).

The reproducibility of the analyses was tested by making 6 analysis on the same wine sample. The results showed that the method had a good reproducibility, with a CV ranging from 2.9 % (ethanolamine) to 7.1 % (tyramine).

The method was then utilised for the determination of the amine content in two groups of Southern Italian wines, red and white, consisting each of 5 different samples, for a total amount of 10 wines. In figure 2 the chromatogram of biogenic amines in a wine sample is shown.

Table III reports the mean biogenic amine content of the five red and the five white wine samples analysed in duplicate. The red wines had a much higher amine content, as reported also by other researches (IBE *et al.*, 1991; IÑIGUEZ CRESPO and VAZQUEZ LASA, 1994), with white wines showing the presence only of limited amounts of agmatine, ethanolamine and putrescine. In the red wine the most abundant amine was ethanolamine, as reported for some Spanish wines (BUSTO *et al.*, 1995), present at a concentration 5 to 10 times higher than that measured for the other amines, with the exception of agmatine, whose concentration was only slightly lower of that measured for ethanolamine.

Regarding polyamines, agmatine and putrescine significantly contributed to the total amine content both in red and white wines, representing the 38 % and the 8 % of the total amine in the red wines and the 36 % and 32 % in the white ones. These data confirmed what has been reported by BAUZA *et al.* (1995) and IZQUIERDO-PULIDO *et al.* (1996), who found high levels of these amines in alcoholic beverages, while cadaverine, spermine and spermidine were rarely present.

Histamine was not detected in white wines, while in the red wines its level was lower than the suggested limit for alcoholic beverages (2-10 mg/l) (IZQUIERDO-PULIDO *et al.*, 1996) and lower than that reported for other Italian wines (ACHILLI *et al.*, 1994; ARLORIO *et al.*, 1999). Tyramine levels exceeding 10 mg/L in beverages should be considered unsafe for patients taking monoamine oxidase inhibitors; in our case, tyramine was detected in small amounts only in red wines, and methylamine was not detected in all the samples. ACHILLI *et al.* (1994) reported the presence of high levels of tyramine in some Italian wines. High concentrations of methylamine have been found in some French and Canadian wines (BAUZA *et al.*, 1995; SOLEAS *et al.*, 1999), while in Italian wines this amine, as well as ethanolamine and agmatine, has never been investigated (ACHILLI *et al.*, 1994; ARLORIO *et al.*, 1999; VECCHIO *et al.*, 1989).

Generally putrescine and agmatine significantly contribute to the total amine content in alcoholic beverages (BAUZA *et al.*, 1995; GLORIA *et al.*, 1998; IZQUIERDO-PULIDO *et al.*, 1996), while the presence of the other amines seems to be dependent on the wine considered, thus on the winemaking process (BAUZA *et al.*, 1995; GLORIA *et al.*, 1998; VECCHIO *et al.*, 1989). It is also suggested that the formation of some amines, among them putrescine-tyramine in Cabernet–Sauvignon and 2-phenylethylamine-agmatine and histamine-spermidine in Pinot noir, is affected by the same factors (GLORIA *et al.*, 1998).

Even though the toxicological significance of biogenic amines in wine is still not well established, it is advisable to prevent any avoidable formation and accumulation in wine; therefore, the sources and critical control points for amine formation during wine manufacture should be determined in order to limit its formation and accumulation (GLORIA *et al.*, 1998).

CONCLUSIONS

Wine is an alcoholic beverage produced and consumed in large amounts in Italy, and the determination of any possible harmful level of moieties such as biogenic amines, is of the utmost importance, both from a commercial and an hygienic point of view.

In this paper a simple and rapid method for the extraction and quantification of several biogenic amines in wines was developed. The use of a solvent having a low boiling point (diethyl ether) and the need of only very limited amounts of sample and reagents make the utilisation of this method very easy, quick and economical compared to methods requiring the use of SPE cartridges (BUSTO *et al.*, 1995) or based on ion pair extraction (FERNANDES and FERREIRA, 2000).

The proposed method allows very good recoveries for most of the amine studied, especially for those oenologically more important, such as agmatine, an amine little investigated despite its possible major contribution to the total biogenic amine content. Only for spermidine and spermine unsatisfactory recoveries were recorded, 70 % and 34 % respectively; however, these amine are usually present in wine at very low levels.

The method has then been utilised for the analysis of Southern Italian wines, in which ethanolamine and agmatine showed to be present at the relatively highest levels; however in these wines, the content in histamine and in total biogenic amines was low and did not represent a possible toxicological threat for the human health.

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