Simultaneous Quantification of Primary, Secondary Amino Acids, and Biogenic Amines in Musts and Wines Using OPA/3-MPA/FMOC-Cl Fluorescent Derivatives

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ABSTRACT: Free amino acids and biogenic amines occur in musts and wines and affect the course of fermentation in musts and the quality of wines. Each sample was analyzed in 138 min and each analysis involved quantification of 23 amino acids and 10 amines. The detection limit for proline was 27.3 mg/L. The mean detection limit for amino acids was 0.58 mg/L and for amines 0.28 mg/L. Precision was 0.5 to 19.2% for wine samples and accuracy by the standard addition method was 99.8% (recovery value). In all, 33 wines and 39 must samples were analyzed. In wines, ethanolamine, histamine, ethylamine, and putrescine amounted to 93% of the amine content.

Keywords: *o*-phthalaldehyde, amino acids, amines, wine, must

Introduction

QUANTIFICATION OF FREE AMINO ACIDS, PRIMARY and secondary, and of biogenic amines in wines has great enological importance since these compounds are directly related to their quality (Trioli and Paronetto 1992; Herbert and others 2000a).

Free amino acids, which are essential nutrients for yeasts, directly affect the fermentation (Sablayrolles and others 1996; Bisson 1999) and the profile of fermentative and post-fermentative compounds of wines (Bosso 1996; Ayesterán and others 1998). Among the post-fermentative compounds, biogenic amines (Cabanis 1985) and ethyl carbamate (Stoewsand and others 1996) are the most important because of their relationship to free amino acids and because of their toxicological significance.

Biogenic amines are compounds that affect human health (Bauza and others 1995b) and may be indicators of unhygienic production conditions (Rivas-Gonzalo and others 1983). They can be directly formed from certain amino acids by decarboxylation (Bauza and others 1995b). Ethyl carbamate can be produced by the reaction of urea or citrulline with ethanol (Stevens and Ough 1993). Urea is formed during amino acid metabolism in the urea cycle, in which arginine, ornithine, and citrulline are intermediates (Monteiro and Bisson 1991; An and Ough 1993; Liu and others 1994). Therefore the reliable quantification of arginine, ornithine, citrulline, and ethanolamine, a compound that co-elutes with methionine in some analytical techniques (Herbert and others 2000b), or the amines histamine, tyramine, and β-phenylethylamine is of great interest (Radler and Fäth 1991).

However, routine quantification of these substances is not used in the quality control of wines mainly due to analytical difficulties. The analytical methods developed so far quantify only primary and secondary amino acids (Casoli and Colagrande 1982; Soufleros and Bertrand 1998), or only biogenic amines or compounds of both groups, but leave out some important substances (Bauza and others 1995a; Lehtonen 1996; Mafra and others 1999).

Quantification of free amino acids and biogenic amines in wine matrices confronts 2 main types of problems: first, the low levels of these compounds present in wines, biogenic amines having the lowest concentrations; and second, the interference effects caused by the complexity of the matrices.

Pre-column derivatization, with formation of fluorescent derivatives, allows a significant gain in the sensitivity and the elimination of much interference due to matrix complexity (Lindroth and Mopper 1979).

In the preparation of fluorescent derivatives of free amino acids and biogenic amines, one of the most studied reagents is *o*-phthalaldehyde (OPA), together with different alkylthiols such as ethanethiol (Hill and others 1979), *tert*-butylthiol (Qu and others 1998), several N-acyl-L-cysteines (Fitznar and others 1999; Molnár-Perl and Vasanits 1999), 2-mercaptoethanol (Dorresteijn and others 1996; Griffin and others 1988; Soufleros and Bertrand 1998), and 3-mercaptopropionic acid (3-MPA) (Graser and others 1985; Fiorino and others 1989; Molnár-Perl and Vasanits 1999). For each reagent it is necessary to compromise between the higher sensitivity of some and the higher stability and selectivity of others. However, OPA has the disadvantage of not reacting with secondary amino acids such as proline (Cooper and others 1984). In most cases this is a considerable drawback in the study of the wines because this amino acid is a major constituent and can be used as one of the elements of characterization (Castino and others 1981; Usseglio-Tomasset 1981). To bypass this obstacle, another compound, 9-fluorenylmethyl chloroformate (FMOC-Cl) has been studied; it reacts not only with primary amino acids and amines (Gustavsson and Betnér 1990; Bauza and others 1995a) but also with secondary amino acids (Fernandez-Trapiella 1990; Kirschbaum and others 1994). Using both reagents, OPA and FMOC-Cl, allows the simultaneous quantification of primary and secondary amino acids and of biogenic amines (Heems and others 1998).

The separation of the fluorescent derivatives of amino acids and biogenic amines is normally a difficult task due to their chemical similarity, their different concentrations in the same matrix (sometimes by a factor of 100), and the great number of compounds to be analyzed.

Reliable quantification of free amino acids and biogenic amines demands great selectivity and high resolution by the chromatographic method.

The aim of this work was to develop an analytical procedure that can accurately quantify free amino acids, with special attention to ornithine, citrulline, arginine, GABA, proline, methionine, and some biogenic amines such as histamine, a compound that normally presents some difficulties in its quantification, because of the low response factor of its derivative, the low levels found in wines, and the difficulty of separating it from other substances.

Materials and Methods

Standard solutions

Free amino acids, aspartate (Asp), glutamate (Glu), asparagine (Asn), glutamine (Gln), alanine (Ala), arginine (Arg), histidine (His), glycine (Gly), β-phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp) γ-aminobutyric acid (GABA), serine (Ser), lysine (Lys), threonine (Thr), methionine (Met), leucine (Leu), isoleucine (Ile), valine (Val), proline (Pro) and two intermediates of the urea cycle, ornithine (Orn) and citrulline (Cit), were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The 10 biogenic amines, ethanolamine (ETA), methylamine (METILA), ethylamine (ETILA), histamine (HISTA), tyramine (TIR), β-phenylethylamine (FEN), tryptamine (TRY), isoamlamine (ISO), cadaverine (CAD), and putrescine (PUT) were all supplied by Fluka Chemie (Buchs, Switzerland). The internal standard, norvaline (Nva), used in the quantification of primary amino acids and amines was from Hewlett-Packard (Waldbronn, Germany). Standard solutions were prepared from a stock solution by dilution with 0.1 M HCl. The average concentration of amino acids in the standards ranged from 1.1 to 11 mg/L, except for arginine (4.53 to 45.29 mg/L) and for proline (25.44 to 254.4 mg/L). Standard amine concentrations ranged from 0.15 to 4.20 mg/L. The concentration of norvaline (internal standard) in all standards was 5.86 mg/L.

Chemicals and supplies

All the necessary reagents for buffer preparation were analytical grade; chromatographic solvents were gradient grade, and all the derivatization reagents were fluorescence grade. Water was distilled, deionized (minimum resistivity of 18 MΩcm^{-1}, Milli-Q, Millipore system), and filtered (0.45 µm filter). Chromatographic solvents were Lichrosolv from Merck (Darmstadt, Germany). Eluents for chromatography were filtered through a 0.45-µm filter. Samples were filtered likewise.

OPA, 97% purity, was purchased from Aldrich-Chemie (Steinheim, Germany). FMOC-Cl, ≥ 99% purity, was from Fluka.

Sample preparation

Musts were centrifuged for 10 min at 4,000 rpm and then diluted (1:4 v/v) with internal standard solution (Nva) in 0.1 M HCl. This solution was again filtered through a disposable 0.45-µm filter (Teknochroma, Madrid, Spain) before it was injected onto the chromatographic column. Wine samples were prepared in a similar way, but excluding the initial step of centrifugation.

Chromatographic equipment

The HPLC chromatographic system employed was a Merck Hitachi model L-6200A, with a fluorescence detector Merck-Hitachi F-1080, an autosampler Merck-Hitachi L-7250 Lachrom, and interface D-7000 (Merck-Hitachi, Tokyo, Japan). The chromatographic column was Merck LichroCART®250-4 Superspher®100 RP-18 endcapped (25 cm x 4.6 mm; 5 µm particles) mounted in an oven 7981 from Jones Chromatography (Mid Glamorgan, Wales, U.K.), at 42 °C.

Analytical conditions

Mobile phases and flow gradient. The chromatographic separation was made using a binary gradient elution (Table 1). Mobile phase A was a 20 mM sodium acetate solution, with 0.018% (v/v) triethylamine, 0.3% (v/v) tetrahydrofurane, and 0.010% (v/v) of a 4% (m/v) solution of EDTA. The pH was adjusted to 7.20 with a 0.1% (v/v) solution of acetic acid. Mobile phase B was a solution with 20% (v/v) of a sodium acetate solution (100 mM, pH 6.0), 40% (v/v) of acetonitrile, 40% (v/v) of methanol, and 0.018% (v/v) triethylamine. The injected volume was 20 µL.

Detection. Excitation/emission wavelengths were respectively 340/450 nm for primary amino acids and 237/340 nm for secondary amino acids. The latter was used to enhance the sensitivity of proline detection. The change in wavelengths was made 115 min after the first injection.

Quantification

Using the conditions described above, the elution of all the compounds in a standard and in a must sample is shown in Figure 1 and 2, respectively. The internal standard method was used for quantification of the primary amino acid and amines, and the external standard method was used in the quantification of proline.

OPA reagent

The OPA reagent was made by dissolving 51.2 mg of *o*-phthalaldehyde in 1.5 mL ethanol. After this, 80 µL of 3-mercaptopropionic acid was added and 1750 mg of KSCN and then made up to a final volume of 50 mL with borate buffer

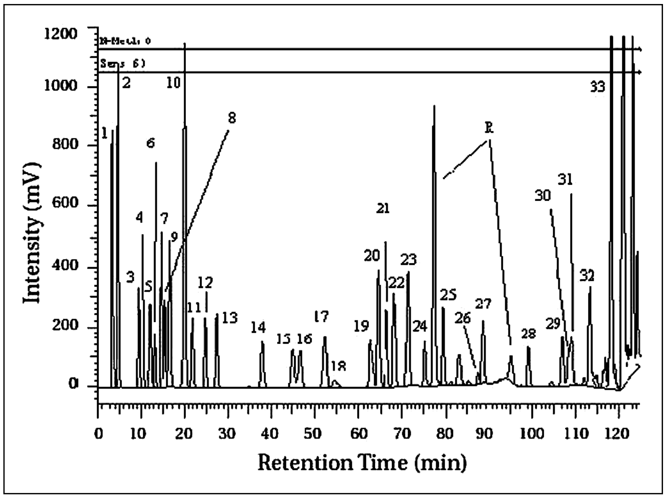

Figure 1–Chromatogram of a standard solution (S2). Legend: 1-Asp, 2-Glu, 3-Asn, 4-Ser, 5-Gln, 6-His, 7-Gly, 8-Thr, 9-Cit, 10-Arg, 11-Ala, 12-GABA, 13-Tyr, 14-ETA, 15-Val, 16-Met, 17-Nva, 18-HISTA, 19-Trp, 20-METILA, 21-Phe, 22-Ile, 23-Leu, 24-Orn, 25-Lys, 26-TIRA, 27-TIRA, 28-PUT, 29-CAD, 30-FEN, 31-TRY, 32-ISO, 33-Pro, R-reagent peaks present in the blanks.

(0.4 M, pH 10.4). This reagent was prepared in 2-mL aliquots and stored at -15 °C. Under these conditions it was stable for 2 mo. Fresh OPA reagent was prepared daily.

FMOC-Cl reagent

The FMOC-Cl reagent was made by dissolving FMOC-Cl in acetonitrile to a concentration of 2.5 g/L and stored at -15 °C. Under these conditions it was stable for several months.

Derivatization procedure

First Injection

Two hundred µL of OPA reagent was added to 200 µL borate buffer (0.4 M, pH 10.6). After this, 40 µL of diluted sample was added and the reaction mixture allowed to stand for 2.5 min in a clean auto-sampler vial. Then 20 µL was injected.

Second Injection

Fifteen µL of diluted sample was added to 420 µL borate buffer (0.4 M, pH 10.6). Then 15 µL of FMOC-CL reagent was added and the reaction mixture allowed to stand for 2 min in a clean auto-sampler vial. Then 20 µL was injected. The second injection was made 112 min after the first.

Waste disposal

In this work, whenever possible, we have recovered the residues produced in order to reuse certain substances, diminish the toxicity of others, and properly store those residues.The volume of waste eluents produced by each analysis was 102.6 mL. Of these, 51.9% was mobile phase A, and 48.1% mobile phase B. The eluent waste composition was, on average, 2.72 g/L of sodium acetate, 1.56 mL/L of tetrahydrofuran, 180 µL/L of triethylamine, 19.2% (v/v) of acetonitrile, 19.2% (v/v) of methanol, maximum 1.86 mg/L of OPA, and maximally 0.34 mg/L of FMOC-Cl.

The number of injections made per liter of eluent waste produced was 9. The procedure adopted with waste eluents, 3-mercaptopropionic acid residues, OPA reagent residues, and FMOC-Cl reagent residues is summarized in Table 2.

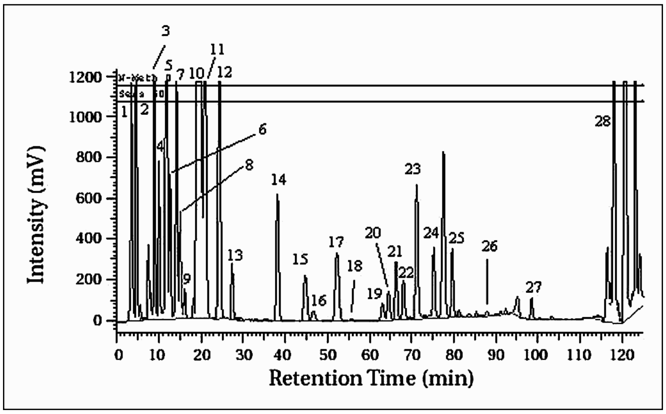

Figure 2–Chromatogram of a must sample at the beginning of alcoholic fermentation. Legend: 1-Asp, 2-Glu, 3-Asn, 4-Ser, 5-Gln, 6-His, 7-Gly, 8-Thr, 9-Cit, 10-Arg, 11-Ala, 12-GABA, 13-Tyr, 14-ETA, 15-Val, 16-Met, 17-Nva, 18-HISTA, 19-Trp, 20-METILA, 21-Phe, 22-Ile, 23-Leu, 24-Orn, 25-Lys, 26-TIRA, 27-PUT, 28-Pro.

Table 1–Elution gradient used in the separation of amino acids and amines

Time (min)	Flow (mL/min)	% Eluent B
0	0.7	5
2	0.7	10
20	0.7	23
20.5	0.9	23
40	0.9	23
45	0.9	25
45.5	0.8	25
52.5	0.8	25
53	0.9	25
53.5	0.7	25
70	0.7	50
70.5	0.4	50
85	0.4	57
85.5	0.6	57
105	0.6	60
110	0.7	80
110.5	0.5	80
117	0.5	80
120	1.2	100
135	1.2	100
136	0.7	5
138	0.7	5

Results and Discussion

Validation of the methodology

Our methodology enables the analysis of a large number of compounds in a reliable way. The duration of the chromatographic analysis was 138 min and the fluorescence response was linear over the concentration range of the standards.

Detection limit (Table 3) was, on average, 0.58 mg/L for amino acids (except for arginine; 2.95 mg/L), 0.28 mg/L for amines, and 27.3 mg/L for proline based on the calibration curves. Although the detection limits obtained for amines are slightly higher than those obtained with some other methods (Mafra and others 1999), they are still appropriate for the analysis required.

The repeatability of 7 injections ranged from 0.6% to 11.6% (relative % standard deviation–RSD) for standard solutions (with an average concentration of amino acids of 2.75 mg/L and an average amine concentration of 1.4 mg/L) and from 0.5% to 19.2% for wine samples (Table 3). Tryptophan was the amino acid with the highest RSD in standards (11.1%) and glutamine in wines (19.2%). Of the amines studied, putrescine was the amine with the highest RSD in standards (11.6%), and tryptamine in wines (16.0%). Accuracy (Table 3) was calculated by the recovery obtained when a known amount of a standard was added to a wine sample. Six independent experiments produced an average recovery of 99.8% (coefficient of variation 11.1%). The minimum obtained was for isoamylamine (81%) and the maximum for histamine (138%).

The lengthy period required for the analysis of each sample is a compromise between the need for sufficient chromatographic separation between all the compounds and the need for a rapid analytical method. In a previously described method (Herbert and others 2000b) for amino acid analysis there were some important compounds co-eluting, and/or not included in the method's scope. To avoid these problems it was necessary to extend the analysis time in order to correctly quantify all the 23 amino acids and 10 amines so that it is possible to analyze with this method, including citrulline and ornithine.

Table 2–Procedure adopted in the analytical residues recovery and treatment

Residue	Procedure
Waste eluents	Peroxide test (Perex-test art.1.16206 Merck) if necessary, peroxides were deactivated with Kit Perex (art.1.16207 Merck) purification by fractional distillation the residue of the distilation was neutralized with a few drops of diluted HCl
3-mercaptopropionic acid	All the material and residues were oxidized for several hours with a solution of sodium hypochlorite
OPA reagent **FMOC-Cl**	Same as for 3-mercaptopropionic acid reagent neutralization by addition of an excess of methanol

Table 3–Validation parameters obtained for the analytical method

Compound	Repeatability[a] (RSD%)		Recovery[b] (%)	Detection limit[c] (mg/L)
	Standard	Red wine		
Asp	2.5	0.5	124	0.47
Glu	0.6	0.8	102	0.93
Asn	0.8	4.4	101	0.53
Ser	2.1	2.0	102	0.75
Gln	6.7	19.2	94	0.21
His	1.2	6.5	100	0.21
Gly	5.7	4.8	109	0.35
Thr	1.6	5.4	107	0.28
Cit	0.7	8.4	101	0.88
Arg	0.6	4.9	97	2.95
Ala	4.4	4.3	106	2.07
GABA	9.3	14.2	97	0.18
Tyr	1.6	2.2	101	0.44
ETA	8.7	3.2	98	0.21
Val	2.3	3.1	106	0.66
Met	0.8	3.9	102	0.84
Nva	3.8	5.5	98	-
HISTA	4.1	7.3	138	0.81
Trp	11.1	9.6	92	0.67
METILA	7.8	8.4	82	0.1
Phe	1.1	4.0	103	0.9
Ile	1.8	3.6	103	0.35
Leu	1.6	3.6	103	0.61
Orn	1.8	14.9	89	0.1
Lys	2.7	7.7	94	0.26
ETILA	4.3	7.3	85	0.24
TIRA	3.5	6.6	91	0.10
PUT	11.6	12.2	105	0.20
CAD	5.5	12.7	87	0.17
FEN	8.5	16.0	90	0.35
TRY	4.2	8.5	94	0.47
ISO	2.8	5.2	81	0.12
Pro	3.9	6.5	110	27.3

[a] Relative standard deviation of 7 injections (average concentration of amino acids of 2.75 mg/L and average amine concentration of 1.4 mg/L, for the standard solution

[b] Calculated as the ratio of the difference between the amount obtained after the addition and the amount before the addition, and the amount added, in percent.

[c] Calculated by the calibration curve.

Proline quantification

In this analytical method, a second injection is performed during the running of the elution gradient. There is in the literature a methodology that performs, on the same sample volume, the reaction between OPA and the primary amino acids, and between FMOC and secondary amino acids, such as proline (Godel and others 1991). First, OPA is added to the sample and it reacts with primary amino acids; after this, FMOC reacts with the secondary amino acids. However, if this is performed with our elution gradient, the proline derivative co-elutes with the late amine peaks, and this makes impossible the quantification of these amines due to the change in the excitation/emission fluorescence wavelengths necessary to detect proline. To avoid this problem, we decided to make a second derivatization reaction further diluting the sample to diminish the interference of other amino acids and also to avoid overcharging the analytical column. We therefore made a second injection to quantify proline. Proline quantification is possible in these conditions because the derivatives of the primary amino acids elute first from the analytical column during the phase when the detector wavelengths are still adjusted to detect OPA derivatives. This procedure makes possible the analysis of proline, with good validation parameters as shown on Table 3 (recovery of 110%), however, with a small decrease in the lifetime of the analytical column.

Composition of OPA reagent

OPA needs the presence of a compound containing an SH-group to form the isoindole derivatives from amino acids. Several thiols have been used, depending on the objective of the reaction. In the derivatization of amino acids and amines, prior to HPLC analysis, we preferred 3-mercaptopropionic acid as the thiol group carrier due to the great stability of these derivatives when compared with those obtained from β-mercaptoethanol (Molnár-Perl and Vasanits 1999). Moreover, the OPA/3-MPA derivatives of amino acid and amines are more polar than OPA/MCE, thus requiring less organic solvent for chromatography. The molar ratio OPA/3-MPA used was 0.41.

Must and wine analysis

Tables 4 and 5 show free amino acid and amine contents of the 39 musts and 33 wines studied. Wines and musts analyzed were obtained from grapes from the Alentejo region. Musts were from the 1998 and 1999 harvests, and wines from 1997 and 1998, resulting from micro-vinifications (40 L for red wines and 20 L for white wines). Musts were kept at -15° C and wines were kept bottled until analysis.

Average total amino acid content for white musts was 1969 mg/L (Table 4), and for red musts 1867 mg/L. Average content of assimilable amino acids was similar in white and red musts. Proline represented, on average, 47% of total amino acids for all musts. Four amino acids, glutamine, histidine, threonine, and arginine, amounted to 55% of the average content of assimilable amino acids (total amino acids except proline).

In wines (Table 4), average total amino acid content was 1562 mg/L for white and 1522 mg/L for red. Average content of assimilable amino acid was higher in white wines (592 mg/L) than in red wines (332 mg/L). This difference was due mainly to a higher content (22.2% of the assimilable amino acids) of arginine in white wines than in red wines (11.2% of the assimilable amino acids).

The lower content of arginine in red wines does not seem to be due to a greater arginine metabolism during fermentation, because the average concentration of citrulline and ornithine in these wines is not higher than in white wines (Table 4); perhaps this is due to a higher content of arginine in white

Table 4–Free amino acid contents (mg/L) of 39 must and 33 wine samples

	White musts			Red musts			White wines			Red wines		
Amino acid	**average**	**max.**	**min.**	**average**	**max.**	**min.**	**average**	**max.**	**min.**	**average**	**max.**	**min.**
Asp	37.0	90.4	16.8	28.3	41.3	18.9	25.6	42.8	11.3	19.4	32.4	3.4
Glu	38.4	64.9	23.5	33.8	50.6	26.7	27.0	46.1	13.7	21.1	30.6	6.1
Asn	22.6	53.0	5.3	20.8	68.6	2.8	35.0	73.0	12.5	14.5	54.3	0.7
Ser	52.6	85.1	36.7	52.9	69.4	32.9	24.6	38.8	12.0	18.3	27.1	5.4
Gln	106.3	151.5	75.3	98.4	122.0	67.0	2.6	7.8	nd	0.8	4.3	nd
His	80.6	157.8	22.2	57.7	97.2	10.9	32.4	73.3	6.5	13.0	68.2	nd
Gly	6.3	33.5	nd	4.0	9.3	nd	25.5	48.8	10.1	18.7	40.7	nd
Thr	79.9	121.1	46.8	72.7	112.3	37.4	19.1	40.0	7.4	14.6	24.8	6.0
Cit	6.8	25.2	nd	4.1	18.7	nd	10.3	31.1	3.9	8.1	43.8	2.1
Arg	339.4	932.3	192.1	351.3	435.1	95.9	119.8	336.1	15.4	37.4	181.0	nd
Ala	63.8	196.4	nd	77.9	141.6	17.9	76.3	217.0	10.9	36.0	108.2	nd
GABA	54.0	118.1	21.5	43.1	110.3	20.2	38.3	99.4	6.9	23.6	105.7	4.5
Tyr	24.8	65.2	nd	18.8	45.9	nd	17.0	31.1	6.5	9.7	37.6	nd
Val	59.1	138.6	11.4	50.4	87.4	12.8	15.4	26.0	5.4	11.0	23.3	5.1
Met	8.9	24.5	nd	8.3	23.5	nd	5.1	11.9	<0.84	3.2	9.2	nd
Trp	6.4	17.8	nd	7.3	21.5	nd	8.1	21.6	3.4	7.7	27.2	3.4
Phe	28.2	85.5	4.5	13.4	15.0	nd	9.4	15.3	3.8	5.0	12.3	nd
Ile	34.0	106.5	4.7	30.0	71.3	4.8	10.1	18.6	4.2	6.8	12.4	2.2
Leu	51.0	107.0	9.3	42.8	81.6	5.0	32.3	58.9	nd	23.2	46.8	6.0
Orn	1.5	13.5	nd	1.1	3.6	nd	16.2	53.0	1.9	7.8	73.6	nd
Lys	12.4	25.3	3.9	9.6	15.5	0.7	42.0	81.6	17.2	32.5	59.0	7.8
Pro	855.5	1349	34.0	862.3	1296	612.2	969.9	1207	326.8	1189	1591	623.0
Ass.a.a.	1114	1893	679.8	1004	1257	424.9	592.0	1214	189.4	332.4	892.9	69.4
Total a.a.	1969	3242	777.4	1867	2398	1122	1562	2367	612.9	1522	2378	1077

Ass. a.a.–assimilable amino acids (all amino acids except proline)
Total a.a.–total amino acids
nd–not detected

musts than in red musts. Table 4 shows that average arginine content in white and red wines is similar. However, the maximum value for the arginine content found in a white must is approximately double that found in a red must. This difference should be studied by analyzing a larger number of musts and wines, complemented with an analysis of urea and ammonia in musts and wines.

Proline represented, on average, 70% of total amino acids. Five amino acids, arginine, alanine, γ-aminobutyric acid, leucine, and lysine, amounted to 50% of the average content in assimilable amino acids.

The values obtained for the concentrations of free amino acids in musts and wines are comparable in magnitude to those found in previous reports (Presa-Owens and others 1995; Spayd and Andersen-Bagge 1996).

In addition to this, the values obtained for the concentrations of citrulline (average 5.4 mg/L for musts and 9.2 mg/L for wines) and ornithine (average 1.3 mg/L for musts and 12 mg/L for wines) (Table 4) are in agreement with those found by other authors (Stevens and Ough 1993; Liu and others 1994).

Ethanolamine (Table 5) represented, on average, 59% (15.3 mg/L) of the total amines in musts, and ethanolamine, methylamine (17%) ethylamine (7%), and putrescine (14%) represented 97% of the average content of amines. The mean value for total biogenic amine (HISTA+TIRA+FEN+TRY) content in white musts was 4.2 mg/L and 1.8 mg/L for red musts. Tyramine and isoamylamine were not found in red musts, and β-phenylethylamine and tryptamine concentrations in musts were, on average, below the detection limit of the method.

Of the total amines found in wines (Table 5), ethanolamine represented, on average, 46%; and ethanolamine, histamine (23%), ethylamine (7%), and putrescine (17%) represented 93% of the average content of amines. The mean value for total biogenic amine (HISTA+TIRA+FEN+TRY) content in red wines was 9.8 mg/L, and 10.0 mg/L for white wines. Phenylethylamine and tryptamine were, on average, below the detection limits of the method in red wines, and β-phenylethylamine was undetectable in white wines.

These results are comparable to those obtained by other authors (Zee and others 1983) and those published by Vidal-Carou and others (1990) where histamine content in 226 wines and tyramine content in 186 wines were between "trace" and 34.25 mg/L and between "trace" and 7.8 mg/L, respectively.

Conclusions

THE NEW METHODOLOGY DESCRIBED ENABLES THE quantification of 21 free amino acids, 2 intermediates of the urea cycle, and 10 biogenic amines in musts and wines. The major features of this technique are: the possibility of simultaneous analysis of important compounds, which may interfere in wine quality; the reproducibility and accuracy obtained; the simplicity of the technique, which does not require a complex sample preparation; and the possibility of automation. Although it should be pointed out that the analysis is quite lengthy, a fact which is imposed by the problem of co-elution of some compounds, automation of the technique would overcome this difficulty, thus assuring an average of 8 determinations per day. In this paper the objective was centered on the development of a methodology that would quantify some important compounds belonging to the nitrogen fraction of the musts and wines. It was beyond the scope of this paper, to be treated in a future study dealing with the establishment of some important enological features, including: (a) the assessment of total assimilable amino acid content (and its variability in Alentejo wine) to be used

Table 5–Amines contents (mg/L) of 39 must and 33 wine samples

Amines	ETA	HISTA	METILA	ETILA	TIRA	PUT	CAD	FEN	TRY	ISO
n = 17					Red wines					
average	18.1	8.3	0.4	3.2	1.5	8.4	1.3	< 0.47	< 0.35	0.2
maximum	27.3	15.2	3.2	8.7	6.0	27.2	2.7	< 0.47	0.9	0.6
minimum	11.1	6.1	nd	0.4	nd	1.3	0.6	nd	< 0.35	nd
n = 16					White wines					
average	17.1	9.4	0.6	1.7	0.2	4.4	0.6	< 0.47	0.4	0.4
maximum	22.3	16.5	1.8	3.2	0.4	7.6	1.1	< 0.47	0.6	0.8
minimum	11.1	5.1	0.2	< 0.24	nd	1.0	< 0.17	nd	< 0.35	nd
n = 19					Red musts					
average	14.9	0.3	3.9	2.0	nd	4.3	0.3	<0.47	<0.35	nd
maximum	25.0	2.4	6.7	6.0	nd	8.2	1.3	0.5	0.7	nd
minimum	7.8	nd	2.1	nd	nd	1.1	nd	nd	nd	nd
n = 20					White musts					
average	15.7	nd	5.1	1.7	0.1	3.2	0.3	< 0.47	< 0.35	nd
maximum	26.2	nd	13.2	4.9	0.4	6.7	0.6	0.9	3.6	< 0.12
minimum	11.1	nd	1.3	nd	nd	nd	nd	nd	nd	nd

nd = not detected

by yeasts during fermentation; (b) the maximum content of arginine and related compounds interfering in the formation of ethyl carbamate (and, consequently, the precision of the ethyl carbamate content in older wines); and (c) the maximum content of biogenic amines in the wines of the Alentejo region.

References

An D, Ough C S. 1993. Urea excretion and uptake by wine yeasts as affected by various factors. Am J Enol Vitic 44(1):35-40.

Ayestaran B, Garrido J, Ancin C. 1998. Relation between fatty acid content and its evolution during fermentation and utilization of free amino acids in vacuum-filtered Viura must. J Agric Food Chem 46(1):42-48.

Bauza T, Blaise A, Daumas F, Cabanis J. 1995a. Determination of biogenic amines and their precursor amino acids in wines of the Vallée du Rhône by high-performance liquid chromatography with precolumn derivatization and fluorimetric detection. J Chrom A 707:373-379.

Bauza T, Blaise A, Teissedre P L, Cabanis J C, Kanny G, Moneret-Vautrin A, Daumas F. 1995b. Les amines biogènes du vin. Métabolisme et toxicité. Bull de L'OIV 767-768:42-67.

Bisson L F. 1999. Stuck and sluggish fermentations. Am J Enol Vitic 50(1):107-119.

Bosso A. 1996. Influenza dell'aggiunte di dosi crescenti di azoto ammoniacale ai mosti sulla composizione in sostanze volatili di origine fermentativa e sulle principali caratteristiche olfattive di alcuni vini bianchi. Riv Vitic Enol 3:3-28.

Cabanis JC. 1985. L'histamine et sa toxicité. Bull de L'OIV 656-657:1009-1015.

Casoli A, Colagrande O. 1982. Use of high-performance liquid chromatography for the determination of amino acids in sparkling wines. Am J Enol Vitic 33(3):135-139.

Castino M, Piracci A, Spera G. 1981. La prolina nei vini a D.O.C.: Colli Albani, Frascati, Marino, Velletri, Trebbiano e Merlot d'Aprilia, Cesanese del Piglio, Montepulciano d'Abruzzo, Verdicchio di Jesi. Annali dell'Istituto Sperimentale per L'Enologia Asti 12(520):340-352.

Cooper JDH, Lewis MT, Turnell DC. 1984. Pre-column *o*-phthaldialdehyde derivatization of amino acids and their separation using reverse-phase high-performance liquid chromatography I. Detection of the imino acids hydroxyproline and proline. J Chrom 285:484-489.

Dorresteijn R, Berwald L, Zomer G, Gooijer C, Wieten G, Beuvery E. 1996. Determination of amino acids using *o*-phthalaldehyde-2-mercaptoethanol derivatization. Effect of reactions conditions. J Chrom A 724:159-167.

Fernandez-Trapiella A C. 1990. Quantitative analysis of methionine, cysteine, and lysine in feeds by reverse-phase liquid chromatography using precolumn derivatization with 9-fluorenylmethyl chloroformate: preliminary study. J Assoc Off Anal Chem 73(6):935-939.

Fiorino A, Frigo G, Cucchetti E. 1989. Liquid chromatographic analysis of amino acids and imino acids in protein hydrolysates by post-column derivatization with *o*-phthalaldehyde and 3-mercaptopropionic acid. J Chrom 476: 83-92.

Fitznar H, Lobbes J, Kattner G. 1999. Determination of enantiomeric amino acids with high-performace liquid chromatography and pre-column derivatization with *o*-phthaldialdehyde and N-isobutyrylcysteine in seawater and fossil samples (mollusks). J Chrom A 832:123-132.

Godel H, Seitz P, Verhoef M. 1991. Automated amino acid analysis using combined OPA and FMOC-Cl pre-column derivatization. LC-GC Int 5(2):44-49.

Graser T, Godel H, Albers S, Földi P, Fürst P. 1985. An ultra-rapid and sensitive high-performance chromatographic method for determination of tissue and plasma free amino acids. Anal Biochem 151:142-152.

Griffin M, Leah J, Mould N, Compton G. 1988. Construction of an ion-exchange amino acid analyser kit for use with high-performance liquid chromatography apparatus. J Chrom 431:285-295.

Gustavsson B, Betnér I. 1990. Fully automated amino acid analysis for protein and peptide hydrolysates by precolumn derivatization with 9-fluorenylmethylchloroformate and 1-aminoadamantane. J Chrom 507: 67-77.

Heems D, Luck G, Fraudeau C, Vérette E. 1998. Fully automated precolumn derivatization, on-line dialysis, and high-performance liquid chromatographic analysis of amino acids in food, beverages, and feedstuff. J Chrom A 798:9-17.

Herbert P, Barros P, Alves A. 2000a. Detection of Port wine imitations by discriminant analysis using free amino acids profiles. Am J Enol Vitic 51(3):262-268.

Herbert P, Barros P, Ratola N, Alves A. 2000b. HPLC determination of amino acids in musts and Port wine using OPA/FMOC derivatives. J Food Sci 65(7):1130-1133.

Hill DW, Walters FH, Wilson TD, Stuart JD. 1979. High-performance liquid chromatographic determination of amino acids in the picomole range. Anal Chem 51(8):1338-1341.

Kirschbaum J, Luckas B, Beinert W-D. 1994. Pre-column derivatization of biogenic amines and amino acids with 9-fluorenylmethyl chloroformate and heptylamine. J Chrom A 661:193-199.

Lehtonen P. 1996. Determination of amines and amino acids in wines–A review. Am J Enol Vitic 47(2):127-133.

Lindroth P, Mopper K. 1979. High-performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with *o*-phthaldialdehyde. Anal Chem 51(11):1667-1674.

Liu S -Q, Pritchard G G, Hardman M J, Pilone G J. 1994. Citrulline production and ethyl carbamate (urethane) precursor formation from arginine degradation by wine lactic acid bacteria *Leuconostoc oenos* and *Lactobacillus buchneri*. Am J Enol Vitic 45(2):235-242.

Mafra I, Herbert P, Santos L, Barros P, Alves A. 1999. Evaluation of biogenic amines in some Portuguese quality wines by HPLC fluorescence detection of OPA derivatives. Am J Enol Vitic 50(1):128-132.

Molnár-Perl I, Vasanits A. 1999. Stability and characteristics of the *o*-phthaldialdehyde/3-mercaptopropionic acid and *o*-phthaldialdehyde/N-acetyl-L-cysteine reagents and their amino acid derivatives measured by high-performance liquid chromatography. J Chrom A 835:73-91.

Monteiro F F, Bisson L F. 1991. Amino acid utilization and urea formation during vinification fermentations. Am J Enol Vitic 42(3):199-208.

Presa-Owens C, Lamuela-Raventos RM, Buxaderas S, Torre-Boronat MC. 1995. Characterization of Macabeo, Xarello, and Parellada white wines from the Penedès region. II. Am J Enol Vitic 46(4):529-541.

Qu Y, Arckens L, Vandenbussche E, Geeraerts S. 1998. Simultaneous determination of total and extracellular concentrations of the amino acid neurotransmitters in cat visual cortex by microbore liquid chromatography and electrochemical detection. J Chrom A 798:19-26.

Radler F, F_th K-P. 1991. Histamine and other biogenic amines in wines. In: Rantz J, editor. Proceedings of the international symposium on nitrogen in grapes and wines. Davis, CA: American Society for Enology and Viticulture. P 185-195.

Rivas-Gonzalo J C, Santos-Hernandez J F, Marine-Font A. 1983. Study of the evolution of tyramine during the vinification process. J Food Sci 48:417-418, 429.

Sablayrolles J-M, Salmon J-M, Barre P. 1996.Carences nutritionnelles des moûts. Efficacité des ajoutes combinés dóxygène et d'azote ammoniacal. Rev. Française d'Œnol. 159 (Juin-Juillet):25-32.

Soufleros E, Bertrand A. 1998. Evaluation d'une méthode CLHP adaptée au dosage des acides aminés du vin. Vitis 37(1):3-53.

Spayd SE, Andersen-Bagge J. 1996. Free amino acid composition of grape juice from 12 *Vitis vinifera* cultivars in Washington. Am J Enol Vitic 47(4):389-402.
Stevens DF, Ough CS. 1993. Ethyl carbamate formation: reaction of urea and citrulline with ethanol in wine under low to normal temperature conditions. Am J Enol Vitic 44(3):309-312.
Stoewsand GS, Anderson JL, Munson L. 1996. Wine and the effect of body weight on ethyl carbamate-induced tumorigenesis in mice. J Wine Research 7(3):207-211.
Trioli G, Paronetto L. 1992. Relazioni tra componenti azotate dei mosti e qualità dei vini. Vigne Vini 19(1/2):29-36.
Usseglio-Tomasset L. 1981. Sulla validitá del dosaggio della prolina per il giudizio di genuinitá del vino. Riv Vitic Enol 34(3):120-121.
Vidal-Carou M C, Codony-Salcedo R, Mariné-Font A. 1990. Histamine and tyramine in Spanish wines: relationships with total sulfur dioxide level, volatile acidity, and malo-lactic fermentation intensity. Food Chem 35(3):217-227.
Zee JA, Simard RE, L'Heureux L, Tremblay J. 1983. Biogenic amines in wines. Am J Enol Vitic 34(1):6-9.

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