Identification and Determination of Amino Acid Ethyl Esters in Wines by Capillary Gas Chromatography–Mass Spectrometry

Tomas Herraiz and Cornelius S. Ough*

Department of Viticulture and Enology, University of California, Davis, California 95616-8749

Sixteen amino acid ethyl esters were identified in wines by GC–MS and 13 of them quantified in wine and an additional 2 in sherry using an improved extraction and derivatization technique. The amounts found in the wines indicated that these esters significantly contribute to the pool of basic compounds found in wines. Proline ethyl ester was found in the highest amounts. The other amino acid esters found in excess of 1 mg/L were alanine ethyl ester, glycine ethyl ester, and γ-aminobutyric ethyl ester.

INTRODUCTION

Numerous studies have been carried out to evaluate wine composition. As a result, hundreds of compounds, mainly neutral and acid compounds, have been successfully identified by GC–MS in wines from different cultivars (Schreier, 1979; Williams et al., 1981; Etievant and Bayonove, 1983; Di Stefano, 1985; Versini et al., 1985; Baumes et al., 1986; Rapp and Mandery, 1986; Herraiz et al., 1991). Basic volatile compounds in wines were reported by Ough et al. (1981) as being aliphatic amines.

Amino acids and ammonia present in grape juice are the main nitrogen sources for yeast growth during the fermentation. Although significant amounts of some amino acids are taken up during the fermentation (Jones and Pierce, 1964), some amounts remain in the wines at the end of the fermentation (Sanders and Ough, 1985; Ough et al., 1991). Arginine is a precursor of urea and for ethyl carbamate in wines (Ough et al., 1988, 1990).

Esterification of organic acid either during the fermentation (Suomalainen and Lehtonen, 1979) or chemically during wine aging (Onishi et al., 1977; Cantagrel and Carles, 1978) is well-known. Studies checking the possibility of esterification of amino acid are needed. Peppard and Halsey (1981) detected valine, leucine, and isoleucine ethyl esters in beers, and 3 years later Heresztyn (1984) found some amino acid ethyl esters in wine using a similar procedure of isolation with exchange resin. Despite these results, no more has been done about these compounds.

On the other hand, in a recent work looking for new salty compounds, Tamura et al. (1989) reported the taste of glycine ethyl ester hydrochloride as well as other amino acid ethyl esters as salty, sour, and light umami (like monosodium glutamate). These enhanced the saltiness of sodium chloride. Thus, these compounds may have an important sensory consideration.

This research elucidates the importance of these compounds as the main basic compounds in wine with contents in the range of several milligrams per liter and reports an improved method for their analysis. Further studies need to be done to determine their sensory relevance and their mechanism of formation in beverages.

EXPERIMENTAL PROCEDURES

Samples Analyzed. Ten wines made in the Department of Viticulture and Enology (University of California, Davis) in the vintage of 1990 were studied for their amino acid ester composition. Six wines, three sauvignon blanc and three grenache, were obtained by fermenting with *Saccharomyces cerevisiae* (Montrachet), *S. cerevisiae* (Lallemand 71B), and *Saccharomyces bayanus* (Prise de Mousse) active dry yeasts. Chardonnay, muscat blanc, and cabernet sauvignon wines were fermented with *S. cerevisiae* (Montrachet). The muscat blanc fermentation was stopped by adding a brandy at 17.6° Brix, ending with a final 10.7° Brix. A wine from tinta madeira grapes fermented similarly was fortified with brandy at 16° Brix reaching a final 4.3° Brix. In addition, two old Spanish sherries and an old Spanish brandy from sherry, made in the 1930s and two old commercial Californian sherries were also studied.

Isolation Procedure. Two methods were used to isolate ethyl esters of the amino acid.

Method A. Four hundred milliliters of the wine to be analyzed was adjusted to pH 4.5 with 1.5 M NaOH and passed slowly down a cation-exchange resin column (70 × 16.5 mm) of Spectra/Gel 50×8 150–300 μm (Spectrum). Before the wine was loaded, the resin was previously washed with distilled water (100 mL), ethanol (50 mL), distilled water (100 mL), 2 M HCl (100 mL), distilled water (100 mL), 1.5 M NaOH (100 mL), and distilled water (200 mL) until the pH of the eluate was ca. 5, as measured with pH indicator strips (American Scientific Products). To remove interfering retained wine components, the resin was washed with 200 mL of distilled water. The esters of amino acids were recovered with 100 mL of a sodium chloride saturated solution and subsequently by 200 mL of a saturated solution of sodium chloride adjusted to pH 10.5 with Na_2CO_3 (Heresztyn, 1984). The pH of the eluate was controlled by adding 2 M HCl to avoid basic conditions.

Method B. Four hundred milliliters of wine at pH 3–4 was concentrated in a rotoevaporator at 40 °C and 80 mbar to remove ethanol. The concentrated sample (ca. 200 mL) was adjusted to pH 4.5 and method A was followed as indicated above.

Extraction of Ethyl Esters of Amino Acids. The eluates from the resin columns containing the esters of amino acids (300 mL) were adjusted to pH 9.5 with 1.5 M NaOH and immediately extracted by shaking four times with 100 mL of CH_2Cl_2 for 1.5 min in a 500-mL separatory funnel. The methylene chloride extracts were concentrated in a rotoevaporator (32 °C and 650 mbar) to 10 mL, transferred to a test tube, evaporated with a gentle nitrogen stream to 1 mL, and then transferred to a reaction vial with a Pasteur pipet to be concentrated to a final volume of 150 μL.

Formation of Trifluoroacetyl Derivatives (TFA-AA). The esters of amino acids in the reactivial were added, with 1.8 mL of trifluoroacetic anhydride (Aldrich), and the vials were closed with Teflon septa screw caps and heated in an oven at 150 °C for 5 min to synthesize the *N*-trifluoroacetyl derivatives of the amino acid esters (Blau and King, 1978). The excess of reagent was gently removed with a stream of nitrogen until the initial volume of 150 μL was obtained, and 1 mL of CH_2Cl_2 was added. The final sample was transferred to an appropriate autosampler vial for gas chromatography.

Quantitative Determination of Amino Acid Ethyl Esters. Following the above isolation procedure A, 400-mL solutions (15 v/v, ethanol, pH 3.5 with tartaric acid) of ethyl esters of amino

* Author to whom correspondence should be addressed.

0021-8561/92/1440-1015$03.00/0

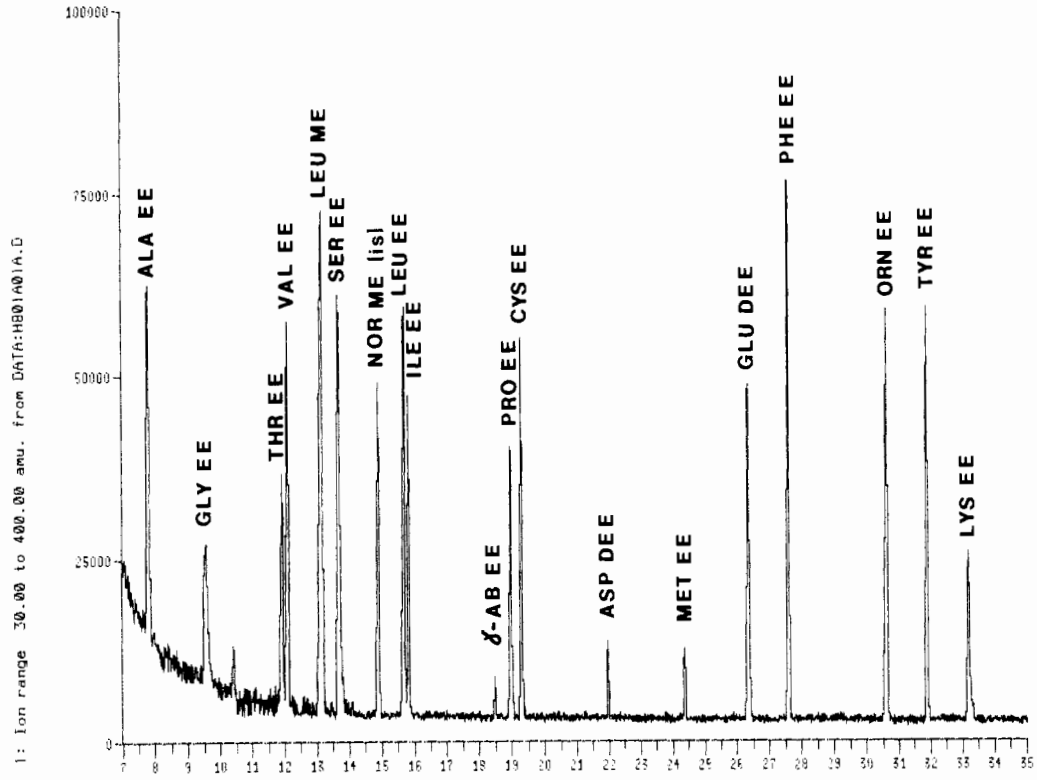


Figure 1. Total ion chromatogram obtained by GC–MS of trifluoroacetyl derivatives of an amino acid ester mixture. Conditions: 30 m × 0.25 mm (i.d.) OV-101 glass capillary column; temperature, 40 °C 2-min hold, programmed to 75 °C at 15 °C/min, and then to 200 °C at 4 °C/min; injection temperature and transfer line, 280 °C; carrier gas, helium; mass spectrometer at 70 eV.

Table I. Identification of Amino Acid Esters in Wines by GC–MS

amino acid ester	RRT[a]	isolation method[b]	evidence for assignment[c]	trifluoroacetyl amino acid ethyl ester mass spectra	sample identification[d]
alanine ethyl ester (Ala-EE)	0.516	A, B	RT, MS	140 (100), 69 (19), 141 (19), 70 (10), 45 (7), 92 (5)	all
glycine ethyl ester (Gly-EE)	0.630	A, B	RT, MS	126 (100), 127 (49), 69 (37), 78 (21), 58 (13), 154 (3)	14
threonine ethyl ester (Thr-EE)	0.80	A, B	RT, MS	152 (100), 153 (88), 69 (79), 57 (31), 84 (23), 41 (10)	13
valine ethyl ester (Val-EE)	0.809	A, B	RT, MS	168 (100), 55 (70), 153 (56), 69 (26), 114 (17), 43 (17)	all
isoleucine methyl ester (Ile-ME)	0.880			153 (100), 69 (86), 41 (52), 182 (41), 185 (34), 126 (12)	
leucine methyl ester (Leu-ME)	0.883			69 (100), 182 (69),140 (49), 43 (40),153 (36), 185 (73)	
serine ethyl ester (Ser-EE)	0.913	A, B	RT, MS	138 (100), 139 (87), 69 (74), 110 (19), 170 (9), 252 (5)	11 (wines)
norleucine methyl ester (Nor-ME) (IS)	1.000		RT, MS	69 (100), 182 (91), 41 (40), 126 (31), 153 (23), 114 (12)	
leucine ethyl ester (Leu-EE)	1.057	A, B	RT, MS	69 (100), 182 (54), 140 (53), 43 (40), 41 (26), 153 (18)	all
isoleucine ethyl ester (Ile-EE)	1.065	A, B	RT, MS	69 (100), 153 (83), 182 (70), 41 (48), 199 (36), 126 (14)	all
γ-aminobutyric ethyl ester (γ-AB-EE)	1.237	A, B	RT, MS	69 (100), 182 (93), 88 (92), 126 (75), 154 (47), 60 (29)	14
proline ethyl ester (Pro-EE)	1.277	A, B	RT, MS	166 (100), 69 (24), 41 (12), 96 (9), 71 (5), 239 (41)	all
cysteine ethyl ester (Cys-EE)	1.293			69 (100), 131 (60), 268 (45), 170 (40), 140 (40), 138 (26)	
aspartic acid diethyl ester (Asp-DEE)	1.472	A, B	RT, MS	212 (100), 140 (76), 170 (40), 139 (32), 69 (29), 166 (28)	4 (sherries)
methionine ethyl ester (Met-EE)	1.641	A, B	RT, MS	61 (100), 153 (73), 199 (66), 69 (33), 166 (22), 273 (13)	8
glutamic acid diethyl ester (Glu-DEE)	1.781	A, B	RT, MS	152 (100), 180 (72), 226 (63), 69 (17), 57 (11), 153 (10)	4 (sherries)
phenylalanine ethyl ester (Phe-EE)	1.865	A, B	RT, MS	91 (100), 176 (54), 131 (29), 148 (17), 69 (16), 216 (10)	13
ornithine ethyl ester (Orn-EE)	2.07	A, B	RT, MS	166 (100), 69 (23), 167 (10), 126 (9), 41 (8), 306 (6)	12
tyrosine ethyl ester (Tyr-EE)	2.156	A, B	RT, MS	203 (100), 288 (73), 69 (49), 243 (31), 260 (26), 328 (9)	14
lysine ethyl ester (Lys-EE)	2.247	A, B	RT, MS	180 (100), 69 (20), 67 (18), 126 (15), 153 (7), 293 (3)	12
hydroxyproline ethyl ester (OH-Pro-EE)	1.787	A	MS (tentative)	278 (100), 181 (86), 69 (91), 39 (29)	4 (sherries)

[a] Relative retention times to trifluoroacetyl norleucine methyl ester (IS) in a capillary column OV-101. Chromatographic conditions as under Experimental Procedures. [b] Isolation method used as explained under Experimental Procedures. [c] Assignment by agreement with retention time of pure standard (RT) and by agreement with mass spectra obtained from pure standards in the same conditions (MS). [d] Samples of wine, sherry, or brandy in which the compounds have been identified.

acids in 0.25, 0.5, 1.5, and 3 mg/L, except for glycine ethyl ester, alanine ethyl ester, and proline ethyl ester with 0.5, 1.0, 3.0, and 6 mg/L, were also analyzed. To obtain the calibration curves needed for quantification, norleucine methyl ester (2.53 mg/L) was added as an internal standard. Norleucine methyl ester (2.53 mg/L) was also added as an internal standard to the wines. In addition, solutions of 50 mg/L of each amino acid and 250 mg/L of proline in ethanol 15% v/v buffered at pH 3.5 with tartaric acid were also studied to check the possibility of artifact formation during the procedure.

Gas Chromatography–Mass Spectrometry. Analytical GC–MS was performed with a Hewlett-Packard 5870 gas chro-

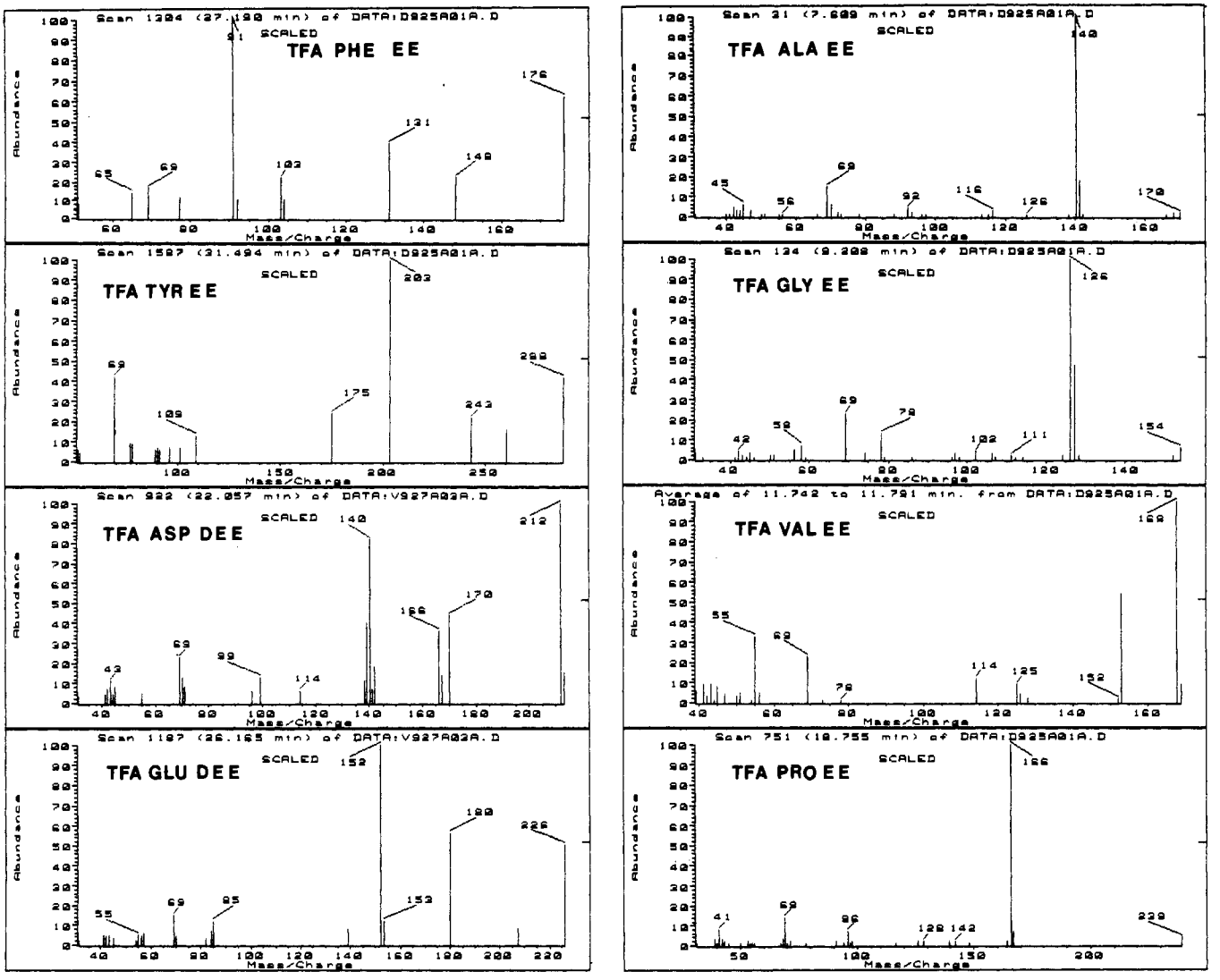


Figure 2. Mass spectra of some amino acid ethyl esters as trifluoroacetyl derivatives identified in wine.

matograph coupled to a mass spectrometer 5970 provided with an autosampler. A capillary column, methyl silicone fluid (25 m × 0.25 mm i.d.), was used in the following chromatographic conditions: 40 °C hold, 2 min, and then subsequently programmed at 15 °C/min to 75 °C, 4 °C/min to 200 °C, and 15 °C/min to 230 °C using an injector temperature at 280 °C and a transfer line at 280 °C. The mass spectra were obtained by EI at 70 eV scanning from m/z 30 to 400 or by single ion (SIM) every 1.16 s. Chromatographic injection was performed using helium as carrier gas.

Amino Acid Analysis. A Hewlett-Packard HP 1090 liquid chromatograph with an autosampler and a fluorescence detector HP 1046a was used. The *o*-phthaldehyde of primary amino acid and the 9-fluorenyl chloroformate for secondary amino acids were formed and analyzed in an ODS-Hypersil (C18), 5 μm, 20 × 2.1 mm, column following basically the Hewlett-Packard Amino Quant Series II method (1990).

Reference Compounds. Most of the ethyl esters of amino acids were available as commercial products in their hydrochloride salts: Glycine ethyl ester hydrochloride, L-tyrosine ethyl ester hydrochloride, L-cysteine ethyl ester hydrochloride, DL-serine ethyl ester hydrochloride, L-lysine ethyl ester dihydrochloride, and L-isoleucine methyl hydrochloride were from United States Biochemical Corp. L-Alanine ethyl ester hydrochloride, L-valine ethyl ester hydrochloride, L-methionine ethyl ester hydrochloride, L-phenylalanine ethyl ester hydrochloride, and L-leucine methyl ester hydrochloride were from Aldrich. D,L-Norleucine methyl ester hydrochloride, L-glutamic acid diethyl ester hydrochloride, and L-leucine ethyl ester hydrochloride from Sigma.

L-Threonine ethyl ester, L-isoleucine ethyl ester, L-aspartic acid diethyl ester, γ-aminobutyric ethyl ester, L-proline ethyl ester, and L-ornithine ethyl ester were synthesized from the corresponding amino acids. Thus, 2.5–3.0 g of L-α-amino acid hydrochloride was dissolved in 30 mL of 4 M HCl in absolute ethanol, and 20 mL of benzene was added. The mixture was maintained in reflux for 3 h at 72–82 °C with a Dean-Stark collector to remove water produced in the reaction (Dymicky et al., 1971). After concentration in high vacuum at low temperature, the residue was stirred in diethyl ether and concentrated again in high vacuum at low temperature. The compounds obtained were pure as shown by chromatographic and mass spectra analysis.

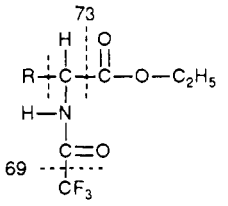
RESULTS AND DISCUSSION

To accomplish the chromatographic analysis, trifluoroacetyl (TFA) derivatives from amino acid ethyl esters were synthesized. A total ion chromatogram obtained by GC–MS of a standard solution of the amino acid ethyl esters after derivatization is shown in Figure 1. Our own spectral library was created with the spectra obtained from reference compounds. Identification in wines was carried out by taking into account the GC retention time and by comparison of the mass spectra obtained with the reference compounds.

Table I contains relative retention times (RRT) to TFA-Nor-ME as an internal standard (IS) and the MS fragments found for the various amino acid esters found in the wines. Sixteen amino acid esters were identified in wines by the two procedures of isolation used. Ala-EE, Val-EE, Leu-EE, Ile-EE, and Pro-EE were identified in all wines studied.

Gly-EE, γ-AB-EE, Thr-EE, Ser-EE, Phe-EE, Tyr-EE, and Lys-EE were identified in almost all samples, whereas Met-EE was identified in eight wines and the two diethyl esters, Asp-DEE and Glu-DEE, were only detected in sherries. Identification of Cys-EE was not made. This was probably due to its oxidation during the isolation process or previously in the wine. On the other hand, no methyl ester was found, as was previously by Heresztyn (1984). However, a tentative identification in sherry of OH-Pro-EE as its TFA derivative was found. The spectrum matches that of the amino acid ester reported by Biemann et al. (1961).

Biemann et al. (1961) reported that an important mass spectra peak of the amino acid esters, 73, is due to the breaking bond corresponding to $COOC_2H_5$, leaving the rest as a characteristic ion of these compounds. Breaks in the lateral chain can also be produced. Trifluoroacetyl derivatives follow this behavior, and in addition they produce ion 69 as a characteristic peak corresponding to (CF_3) fragment.



The mass spectra of some of the ethyl esters of amino acids found in wine samples are shown in Figure 2. TFA-Ala-EE, TFA-Gly-EE, TFA-Val-EE, and TFA-Pro-EE exhibit peak bases at 140, 126, 168, and 166, respectively, corresponding to the fragment $(MW - 73)^+$. Mass 69 and other mass peaks corresponding to lateral chain are also produced. TFA-Phe-EE and TFA-Tyr-EE show 216 and 328 corresponding to loss of 73, but the peak bases are respectively due to 91 $(C_6H_5CH_2)^+$ and 203 $(CF_3COOC_6H_4CH_2)^+$. TFA-Asp-DEE presents 212 $(MW - 73)^+$, whereas TFA-Glu-DEE has 226 $(MW - 73)^+$ and the base peak for the loss of another 73 from the carboxyl of the lateral chain. Another example of breaking in the lateral chain is TFA-Met-EE with base peak 61 corresponding to $(CH_3SCH_2)^+$ (Table I). Other TFA amino acid ethyl esters follow patterns similar to those presented in Figure 2.

Solutions prepared of amino acids in ethanol (15% v/v) (pH 3–4) were passed down the resin following the two methods described under Experimental Procedures. No peak as a result of artifacts was found (Figure 3a,b). Previously, Jain et al. (1978) synthesized esters from amino acid and ethanol by employing strongly acid cation-exchange resins at room temperature. In this research, following Heresztyn's (1984) isolation method, chemical formation of these compounds between amino acid and ethanol was detected. To check if the formation was taking place in the resin, a solution of amino acids in water was passed through the cation resin in the acid form (ca. pH 3), washed with 500 mL of ethanol solution (10% v/v), and then eluted with sodium chloride saturated solution and sodium chloride saturated solution adjusted to pH 10.5. Several amino acid ethyl esters such as alanine, glycine, threonine, valine, serine, leucine, isoleucine, proline, methionine, phenylalanine, and tyrosine were formed as a result of chemical reaction in the resin (Figure 3c).

Consequently, modifications had to be introduced in the isolation method to be used in this research. The resin was washed with distilled water (200 mL) and adjusted to pH 5 before the wine sample (pH 4.5) was loaded, and absolutely no ethanol solution was passed down the resin to remove interfering acid and neutral compounds since ethyl esters could be formed in the resin by chemical reaction between ethanol and the amino acid already adsorbed on it. On the other hand, in isolation procedure B, ethanol was removed previously to the resin step so that no ethyl esters could be formed by chemical reaction. Following these two procedures no chemical derivatization was detected as shown in Figure 3a,b.

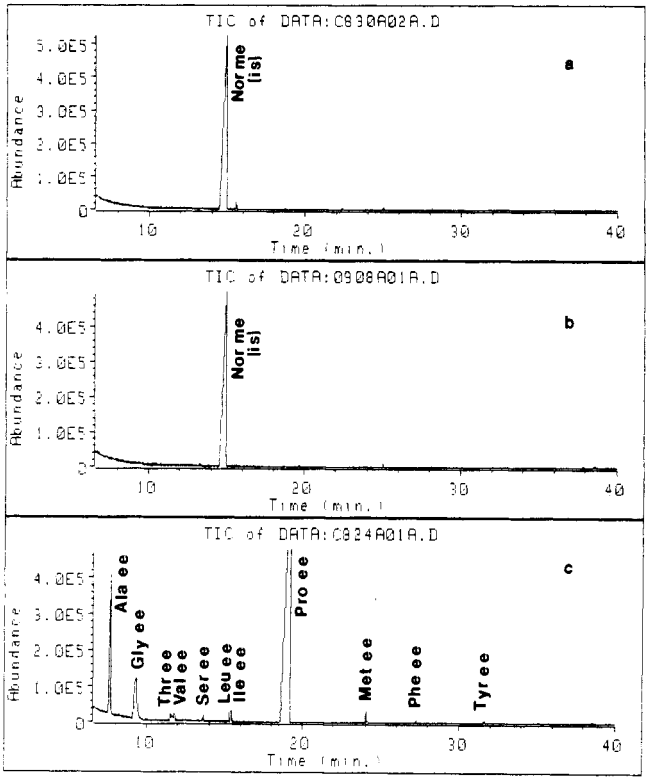


Figure 3. (a) 400 mL of a solution in ethanol 15% v/v, pH 3–4, of 50 mg/L of each amino acid and 250 mg/L of proline added with internal standard norleucine methyl ester (IS) adjusted to pH 4.5 and passed down the resin following the isolation procedure A. (b) Same ethanolic solution as in (a) using the isolation procedure B. (c) 400 mL of a solution in water of 50 mg/L of each amino acid and 250 mg/L of proline (pH 3.5) passed down the resin in pH ca. 3 and eluted with 500 mL of ethanol 10% v/v and then subsequently eluted as in isolation procedure A. Ethyl esters in the chromatograms are as TFA derivatives.

A total ion chromatogram obtained by GC–MS of a wine sample with the ethyl esters of amino acid detected as trifluoroacetyl derivatives is presented in Figure 4. A clear chromatogram without intefering peak and a fair separation could be obtained with the major peak being ethyl esters of amino acid. These compounds may be considered as important compounds in wine.

Quantitative analyses were carried out by GC–MS on the 15 samples of wines studied, and the concentrations obtained are included in Table II. Pro-EE, Gly-EE, Ala-EE, γ-AB-EE, and Leu-EE were the esters found in the highest amounts, most of them with more than 0.5 mg/L. Ethyl esters were generally found in higher amounts in the sherries, and Pro-EE and γ-AB-EE were determined in very high amounts in these samples (up to 28.9 and 16.6 mg/L, respectively). In addition, Glu-DEE and Asp-DEE were found in sherry in less than 0.6 mg/L but were not found in the rest of the wines. Ornithine ethyl ester was identified but not quantified because of difficulties of recovery, probably as a consequence of its instability during the process by formation of a lactam in basic conditions used in the isolation procedure as reported Biemann et al. (1961).

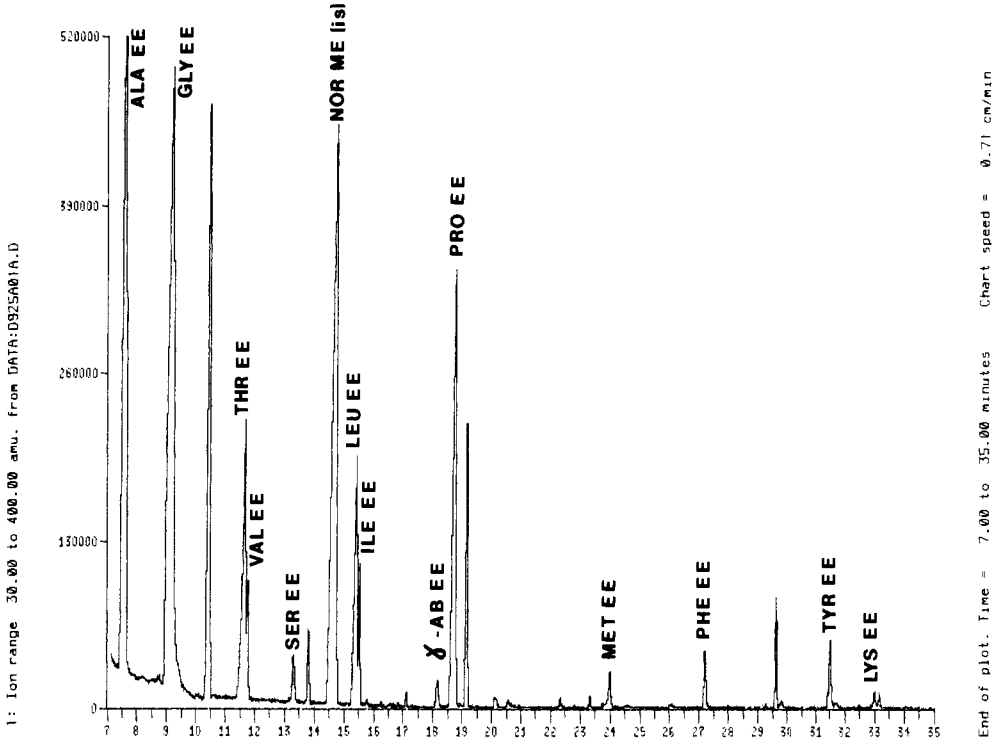


Figure 4. Total ion chromatogram obtained by GC–MS of trifluoroacetyl derivatives of the amino acid ethyl esters isolated from a wine sample. Chromatographic conditions were as in Figure 1.

Table II. Amino Acid Ethyl Ester Concentrations (Milligrams per Liter) in the Wines Analyzed

amino acid ethyl ester (AA-EE)	sauvignon blanc			grenache			tinta madeira	char-donnay	cabernet sauvignon	muscat blanc	Spanish flor sherry[b]	Californian flor sherry[b]	brandy from sherry	range	mean[c]
	A[a]	B	C	A	B	C									
Ala-EE	0.65	0.64	0.64	1.11	1.31	0.83	0.82	0.63	0.56	0.17	2.13	2.08	0.20	0.17–2.13	0.96
Gly-EE	1.2	0.72	0.96	3.37	2.10	3.29	0.83	1.67	1.32	0.21	0.90	1.02		0.21–3.37	1.46
Thr-EE	0.44	0.43	0.57	0.50	0.77	0.53	0.44	0.26	0.25	0.20	0.41	0.42		0.20–0.77	0.43
Val-EE	0.19	0.17	0.22	0.26	0.20	0.30	0.15	0.18	0.34	0.09	0.80	0.36	0.08	0.09–0.80	0.27
Ser-EE	0.33	0.35	0.39	0.40	0.45	0.45	0.45	0.30	0.26	0.01	0.19			0.01–0.45	0.32
Leu-EE	0.59	0.64	0.79	0.70	0.86	0.76	0.23	0.41	0.22	0.17	0.88	0.59	0.15	0.17–0.88	0.57
Ile-EE	0.31	0.30	0.37	0.56	0.45	0.61	0.28	0.34	0.67	0.16	0.96	0.49	0.17	0.16–0.96	0.46
γ-AB-EE	0.70	1.95	0.53	0.52	0.46	0.36	2.36	0.33		0.29	7.26	15.2	1.24	0.0–15.2	2.72
Pro-EE	4.13	1.61	3.25	2.79	1.81	2.76	1.38	8.88	10.68	1.83	16.60	29.8	0.67	1.38–29.8	7.12
Asp-DEE											0.58	0.32		0.0–0.58	0.45
Met-EE	0.04	0.07	0.06	0.15	0.19	0.21	0.03	0.04						0.0–0.21	0.10
Glu-DEE											0.22	0.19		0.0–0.22	0.21
Phe-EE	0.26	0.26	0.33	0.29	0.38	0.33	0.20	0.22	0.14	0.14	0.92	0.34		0.14–0.92	0.32
Tyr-EE	0.31	0.34	0.34	0.27	0.45	0.26	0.27	0.26	0.20	0.21	0.50	0.30		0.20–0.50	0.31
Lys-EE	0.48	0.51	0.45	0.57	0.70	0.59	0.44	0.51			0.77	0.63		0.0–0.77	0.56
total AA-EE	9.6	8.0	8.89	11.5	10.14	11.3	7.88	13.97	14.64	3.48	33.1	51.7	2.51	3.48–51.7	15.35
ethanol[d]	12.45	11.41	12.68	13.13	12.90	13.22	14.46	13.27	13.38	13.16	18.1	18.0	40.2		

[a] Yeast used for fermentation: A, *S. cerevisiae* (Montrachet); B, *S. cerevisiae* (Lallemand 71B); C, *S. bayanus* (Prise de Mousse). [b] Means of two different commercial samples of sherry. [c] Values obtained from data in which ethyl esters were determined. [d] v/v %.

The range of total concentrations of amino acid ethyl esters in wines, other than sherry, was from 3.5 to 14.6 mg/L, with the muscat blanc wine containing the lowest amount. As was indicated under Experimental Procedures, the making of this wine included the addition of brandy so that the fermentation was stopped at 10.7° Brix. In sherry wines, the total concentration range from 33.1 to 51.7 mg/L and lower concentrations of Pro-EE and γ-AB-EE were found in old sherries in comparison to the Californian sherries, but higher concentrations of other ethyl ester were detected. However, the old brandy contained very low concentrations of the esters detected with the exception of γ-AB-EE, probably as a consequence of the distillation process. Low amounts of amino acid ethyl esters would be expected in brandy and other distillate beverages.

In wines fermented with the same variety, sauvignon blanc, and three different yeasts, some differences in concentration were found in Pro-EE, γ-AB-EE, and Gly-EE, while in grenache wines, differences were found in the above compounds as well as in Ala-EE, Tyr-EE, and Thr-EE. The cabernet sauvignon wine analyzed had been made from a grape juice with medium to high nitrogen content. High amounts of Ile-EE, Pro-EE, and Val-EE were detected. Some further research needs to be carried out before discussion of the formation of these compounds as a result of the fermentation or as a result of the aging process.

Reproducibility of the analysis was studied by analyzing five samples of the same wine using five different resin columns. The standard deviation residual found for the esters analyzed by the five different columns range from 2.2 to 7.6 but for Met-EE was 12.6. The calibration curves obtained with four different concentrations, each using a different resin column, had determination coefficients (R^2) higher than 0.99, but Thr-EE and Ser-EE had 0.988 and

Table III. Amino Acid Concentrations (Milligrams per Liter) in the Wines Analyzed

amino acid (AA)	sauvignon blanc			grenache			tinta madeira	char-donnay	cabernet sauvignon	muscat blanc	Spanish flor sherry[b]	Californian flor sherry[b]	brandy from sherry	range	mean[c]
	A[a]	B	C	A	B	C									
alanine	16.3	34.2	16.1	18.7	36.7	7.0	37.9	13.2	3.0	3.8	34.0	30.2		3.0–37.9	20.9
glycine	13.3	18.0	11.0	8.0	24.2	4.5	17.0	4.0	1.8	2.3	22.3	24.0		1.8–24.0	12.5
threonine	3.8	5.3	4.4	2.0	9.2	2.5	10.8	2.3	0.7	1.7	12.6	9.7		0.7–12.6	5.4
valine	3.8	7.4	5.2	2.3	5.2	1.2	9.1	2.0	0.4	1.1	15.8	10.6		0.4–15.8	5.3
serine	3.6	5.1	4.3	2.1	7.8	1.9	8.7	2.3	1.6	2.3	12.1	9.3		1.6–12.1	5.1
leucine	13.2	23.3	16.8	5.3	35.7	4.6	66.7	5.6	1.2	2.0	28.5	18.6		1.2–66.7	18.4
isoleucine	2.9	4.0	4.8	1.2	2.6	1.3	6.3	1.7	0.6	0.7	14.1	5.4		0.6–14.1	3.8
γ-aminobutyric acid	9.1	33.0	5.9	14.8	18.0	6.0	61.6	3.2	2.9	1.9	12.0	23.3		1.9–61.6	16.0
proline	558	332	423	763	795	811	692	854	1200	425	621	703		332–1200	681
aspartic acid	10.2	15.1	13.2	4.9	13.7	3.9	21.7	4.0	2.1	2.6	224.7	20.5		2.1–24.7	11.4
methionine	26.0	27.6	25.7	35.6	41.5	39.4	42.7	17.5	17.0	11.8	27.8	28.7		11.8–41.5	28.4
glutamic acid	18.9	24.6	19.2	19.4	25.2	14.7	33.7	12.6	8.8	12.6	18.7	16.9		8.8–33.7	18.7
phenylalanine	7.2	11.2	10.7	2.0	5.0	1.9	5.9	3.3	0.7	1.6	17.3	9.4		0.7–17.3	6.3
tyrosine	6.5	12.2	9.1	3.4	7.0	2.3	8.1	2.7	1.0	2.2	13.0	10.5		1.0–13.0	6.5
lysine	19.5	32.8	23.3	4.6	21.0	7.1	15.3	6.6		4.6	33.8	18.6		0.0–33.8	17.0
asparagine	6.3	8.4	9.5	6.8	17.8	5.5	6.1	4.1		2.4	1.4	3.2		00–17.8	6.5
histidine	5.8	6.1	5.5	6.4	12.8	5.3	55.0	3.8	3.0	1.1	10.0	24.0		1.1–55.0	11.5
arginine	21.2	46.0	20.4	22.7		9.1	118.7	8.4		6.0	44.5	71.5		0.0–118.7	36.8
tryptophan	4.3	9.7	2.7	5.1	4.5	2.9	30.5	2.3	1.4	2.7	5.6	8.3		1.4–30.5	6.7
total AA[d]	712	585	593	887	1047	909	1037	935	1241	476	907	1123		476–1241	871

[a–c] As in Table II. [d] Total amino acid in wines for which ethyl esters were quantified.

0.986, respectively. The average error in the determination of the concentration was less than 8% but in the case of serine and methionine was 14 and 20%, respectively.

Although quantitative results were obtained by areas in the total ion GC–MS chromatogram, single ion obtained with the main ions of each trifluoroacetyl ester of amino acid provided no significant differences. Quantification with 69 ion could be possible, but some ethyl esters have a relatively small abundance of this ion (Table I).

Measurements of the amino acid content of the wines were carried out, and the results are given in Table III. The concentrations were in the ranges found previously for wines (Ough and Amerine, 1988). Although a total correlation between amino acid ethyl esters and amino acid in the wine cannot be found, sherries presented high concentrations of some amino acids as well as a high concentration of esters. On the other hand, the two wines with the highest proline contents were also the highest in contents of proline ester. In addition, the muscatel wine had the lowest ester content and also a low amino acid content. The highest amount of γ-AB-EE correlated with the highest concentration of the amino acid found in two wines—sauvignon blanc B and tinta madeira.

Pro-EE was found in the wines studied in the highest amounts but over a large concentration range. This is in agreement with the large concentration range of proline in juices and wines from ca. 200 to ca. 4000 mg/L reported by Ough and Tabacman (1979), Ough and Bell, (1980), and Huang and Ough (1989). On the other hand, taking into account the smaller concentration of other amino acids in the juice and wine in comparison to proline, ethyl esters other than proline ester are also in a significant concentration in wine. Concentrations obtained were in agreement with the only ones reported for these compounds in the literature (Heresztyn, 1984).

These compounds have rarely been reported before. The explanation may be that the isolation procedures are difficult and derivatization for GC separation is required. After derivatization, the best separation was obtained with a nonpolar column (methyl silicone). Although a GC–MS was used to quantify in this work, a GC with a FID detector also gave good peaks and could be used as easily to analyze these compounds. Some sensory interest is indicated.

ACKNOWLEDGMENT

T. Herraiz thanks the North Atlantic Treaty Organization (NATO) for supporting him with a fellowship.

LITERATURE CITED

Baumes, B.; Cordonnier, R.; Nitz, S.; Drawert, F. Identification and determination of volatile constituents in wines from different vine cultivars. *J. Sci. Food Agric.* **1986**, *37*, 927–943.

Biemann, K.; Seibl, J.; Gapp, F. Mass spectra of organic molecules. I. Ethyl esters of amino acids. *J. Am. Chem. Soc.* **1961**, *83*, 3795–3804.

Blau, K.; King, G. *Handbook of derivatives for chromatography*; Heyden: London, 1978; pp 133–134.

Cantagrel, R.; Carles, J. Analytical characterization of varietal wines and phenomena of aging. *Ann. Nutr. Aliment.* **1978**, *32*, 1073–1094.

Di Stefano, R. *Vignevini* **1985**, *12* (12), 39–46.

Dymicky, M.; Mellon, E. F.; Naghski, J. A general, highly efficient azeotropic method of esterification of amino acids. *Anal. Biochem.* **1971**, *41*, 487–491.

Etievant, P. X.; Bayonove, C. L. Aroma components of pomaces and wine from the variety muscat de Frontignan. *J. Sci. Food Agric.* **1983**, *34*, 393–403.

Heresztyn, T. Methyl and ethyl amino acid esters in wine. *J. Agric. Food Chem.* **1984**, *32*, 916–918.

Herraiz, T.; Reglero, G.; Martin-Alvarez, P.; Herraiz, M.; Cabezudo, M. D. Identification of aroma components of Spanish Verdejo wine. *J. Sci. Food Agric.* **1991**, *55*, 103–116.

Huang, Z.; Ough, C. S. Effect of vineyard locations, varieties, and rootstocks on the juice amino acid composition of several cultivars. *Am. J. Enol. Vitic.* **1989**, *40*, 135–139.

Jain, J. C.; Dev Choudhury, M. N.; Bajaj, K. L.; Mathur, N. K. Some applications of polystyrene sulfonic acid resins. In *Proceedings of the ion-exchange symposium*; Garde, G. T., Ed.; Central Salt and Marine Chemicals Research Institute: Bhavnagar, India, 1978; pp 242–245.

Jones, M.; Pierce, J. S. Absorption of amino acids from wort by yeast. *J. Inst. Brew.* **1964**, *70*, 307–315.

Onishi, M.; Guymon, J. F.; Crowell, E. A. Changes in some volatile constituents of brandy during aging. *Am. J. Enol. Vitic.* **1977**, *28*, 152–158.

Ough, C. S.; Amerine, M. A. *Methods for analysis of musts and wines*, 2nd ed.; Wiley: New York, 1988; pp 172–175.

Ough, C. S.; Bell, A. A. Effects of grape fertilization of grape vines on amino acid metabolism and higher alcohol formation during grape juice fermentation. *Am. J. Enol. Vitic.* **1980**, *31*, 122–123.

Ough, C. S.; Tabacman, A. Gas chromatographic determination of amino acid differences in Cabernet Sauvignon grapes and wines as affected by rootstocks. *Am. J. Enol. Vitic.* **1979**, *30*, 306–311.

Ough, C. S.; Daudt, C. E.; Crowell, E. A. Identification of new volatile amines in grapes and wines. *J. Agric. Food Chem.* **1981**, *20*, 938–941.

Ough, C. S.; Crowell, E. A.; Mooney, L. A. Formation of ethyl carbamate precursors during grape juice (Chardonnay) fermentation. I. Addition of amino acids, urea, ammonia: effects of fortification on intracellular and extracellular precursors. *Am. J. Enol. Vitic.* **1988**, *38*, 243–249.

Ough, C. S.; Stevens, D.; Sendovski, T.; Huang, Z.; An, D. Factors contributing to urea formation in commercially fermented wines. *Am. J. Enol. Vitic.* **1990**, *41*, 68–73.

Ough, C. S.; Huang, Z.; An, D.; Stevens, D Amino acid uptake by four commercial yeasts at two different temperatures of growth and fermentation: effect on urea excretion and reabsorption. *Am. J. Enol. Vitic.* **1991**, *42*, 26–40.

Peppard, T. L.; Halsey, S. A. Amino acid esters in beer. *J. Inst. Brew.* **1981**, *87*, 85–86.

Rapp, A.; Mandery, H. Wine aroma. *Experientia* **1986**, *42*, 873–883.

Sanders, E. M.; Ough, C. S. Determination of free amino acids in wine by HPLC. *Am. J. Enol. Vitic.* **1985**, *32*, 43–46.

Schreier, P. Flavor composition of wines: a review. *CRC Crit. Rev. Food Sci. Nutr.* **1979**, *12*, 59–111.

Suomalainen, H.; Lehtonen, M. The production of compounds by yeast. *J. Inst. Brew.* **1979**, *85*, 149–156.

Tamura, M.; Seki, T.; Kawasaski, Y.; Tada, M.; Kikuchi, E.; Okai, H. An enhancing effect on the saltiness of sodium chloride of added amino acids and their esters. *Agric. Biol. Chem.* **1989**, *53*, 1625–1633.

Versini, G. *Vignevini* **1985**, *12* (12), 57–67.

Williams, P. J.; Strauss, C. R.; Willson, B. Classification of the monoterpenenoid composition of muscat grapes. *Am. J. Enol. Vitic.* **1981**, *32*, 230–235.

Received for review November 13, 1991. Accepted March 3, 1992.

Registry No. Ala, 56-41-7; Gly, 56-40-6; Thr, 72-19-5; Val, 72-18-4; Ser, 56-45-1; Leu, 61-90-5; Ile, 73-32-5; βAB, 56-12-2; Pro, 147-85-3; Asp, 56-84-8; Met, 63-68-3; Glu, 56-86-0; Phe, 63-91-2; Tyr, 60-18-4; Lys, 56-87-1; Asp, 70-47-3; His, 71-00-1; Arg, 74-79-3; Trp, 73-22-3; Ala-EE, 3082-75-5; Gly-EE, 459-73-4; Thr-EE, 23926-51-4; Val-EE, 17431-03-7; Ser-EE, 4117-31-1; Leu-EE, 2743-60-4; Ile-EE, 921-74-4; γ-AB-EE, 5959-36-4; Pro-EE, 5817-26-5; Asp-DEE, 13552-87-9; Met-EE, 3082-77-7; Glu-DEE, 16450-41-2; Phe-EE, 3081-24-1; Tyr-EE, 949-67-7; Lys-EE, 4117-33-3; Orn-EE, 4189-46-2; Thr·HCl, 82650-07-5; Ile·HCl, 17694-98-3; γ-AB·HCl, 5959-35-3; Pro·HCl, 7776-34-3; Asp·HCl, 17585-59-0.