Methyl and Ethyl Amino Acid Esters in Wine

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Amino acid esters were isolated from wines by a cation-exchange resin technique and subsequently identified and quantified by GC and GC–MS. Thirteen methyl and ethyl esters of amino acids were detected in ten wines, four of which were microbiologically spoiled. The results indicated that these compounds were unlikely to be products of microbial spoilage and were probably normal yeast metabolites produced during the course of fermentation. Furthermore, a comparison of the quantities present in wine, a beer and a port, suggests higher concentrations of these esters are obtained with greater sugar attenuation during fermentation. The levels present in wine suggest these amino acid esters were major basic components of this beverage.

As part of an investigation into microbiological spoilage of wine, work has focused on the nature of yeast or bacterially derived mousiness in wine (Craig and Heresztyn, 1984; Strauss and Heresztyn, 1984). Tucknott (1977) examined this taint in detail, and although he found at least one basic compound of interest, his results were inconclusive. Further investigation of the basic components of such tainted wines is continuing in this laboratory.

During the course of this work, thirteen amino acid esters were identified by GC–MS and eight of these were subsequently quantified. Further studies were conducted to determine the naturally occurring levels of these compounds in a range of fermented beverages. This paper reports, for the first time, the occurrence of methyl and ethyl esters of amino acids in wines and expands the number of amino acid ethyl esters now characterized in alcoholic beverages.

EXPERIMENTAL SECTION

Beverages Examined. Portions (400 mL) of the juice of two varieties of *Vitis vinifera*, Pedro Ximenez (1) and Shiraz (2), were adjusted to 10% v/v EtOH with redistilled 95% v/v EtOH (1A and 2A, respectively) and stored at 25 °C for 26 days. A mousy wine (3) was prepared on a small scale by fermenting a quantity of the same Pedro juice (1) at 25 °C with *Brettanomyces intermedius* (AWRI yeast culture no. 175). Other spoiled wines analyzed were a dry red (Shiraz) (4), a dry white (Chenin Blanc) (5), and a third dry white (6). Sound wines examined included a semisweet white (7), a dry white (8), a rosé (9), a ruby port (10), dry red red (Shiraz) (11), and a champagne (12). A commercial ale (13) was included for comparison.

Extraction of Amino Acid Esters from Beverages. *Method A.* The sample to be analyzed (400 mL) was passed through a column (70 × 16.5 mm) of Dowex 50W strongly acidic cation-exchange resin in the hydrogen ion form. The resin was washed with 10% v/v EtOH (150 mL) followed by distilled water (100 mL) to remove interfering acidic and neutral wine components adsorbed to the resin. The amino acid esters were subsequently eluted with saturated NaCl solution (30 mL), followed by a saturated NaCl solution (170 mL) adjusted to pH 10.5 with Na_2CO_3. The combined eluate from the resin was adjusted to pH 9.5 with Na_2CO_3 and then continuously extracted with Freon F11 for 24 h. This Freon F11 extract (ca. 200 mL) was concentrated to 50 μL in a specially calibrated pointed flask by distillation at a 35 °C bath temperature through a column (1 × 14 cm) of Fenske's helices. The amino acid esters contained in the extract were separated, identified, and estimated by GC and GC–MS.

Method B. In several trial experiments, wine (200–850 mL) was saturated with NaCl, adjusted to pH 2.0 with dilute H_2SO_4, and continuously extracted with Freon F11 for between 2 and 11 days. During the longer extractions, the solvent was changed 6–7 times to further facilitate the removal of neutral and acidic wine components. The wine was then adjusted to pH 8.5–11 with 8 M NaOH and continuously extracted with Freon F11 for a further 24 h. This basic Freon F11 extract was concentrated to ca. 200 μL as described in method A.

Quantitative Determination of Amino Acid Esters. Mixtures of known amounts of the authentic methyl and ethyl amino acid esters were dissolved in 10% v/v aqueous EtOH saturated with potassium hydrogen tartrate and passed down the resin. The esters were eluted, extracted, and analyzed as described in method A. Duplicate 5-μL injections were made into the GC, and the peak height and peak area of each ester were measured by a reporting integrator. Quantitative determinations were based on comparison of components in the beverage extracts with those in extracts of standard solutions passed down the resin. In this way, recoveries of esters from the resin were accounted for.

Gas Chromatography and Mass Spectrometry. Analytical GC was performed on a SP-1000 glass SCOT column (96 m × 0.5 mm i.d.). The column was operated isothermally at 50 °C for 10 min, programmed at 1 °C/min to 180 °C, and held at this temperature for 20 min.

For GC–MS, a SP-1000 glass SCOT column (105 m × 0.5 mm i.d.) was again used. The column was initially held at 60 °C for 10 min, programmed at 1 °C/min to 180 °C, and held at this temperature for 20 min. Electron impact mass spectra were taken at 70 eV, scanning from m/z 30 to 350 every second, with a 0.1-s delay between successive scans.

Reference Compounds. Most of these were available commercially as their hydrochloride salts. Ethyl esters of isoleucine and proline were prepared by refluxing the corresponding amino acid with an EtOH–benzene mixture under acidic conditions by using a Deane and Stark apparatus to remove water formed in the reaction. The hydrochloride salts of these two amino acid esters were prepared by the method of Boissonnas et al. (1956). Ethyl isoleucine hydrochloride was obtained as a solid after freeze-drying, while ethyl proline hydrochloride remained as a viscous syrup (Dymicky et al., 1971). Infrared spectra of the hydrochloride salts were consistent with the assigned structures, and mass spectra of both the free esters and their salts were identical with those published (Biemann et al., 1961).

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Table I. Amino Acid Esters Found in Beverages

amino acid ester	RRT[a]	evidence for assignment[b]	literature references: alcoholic beverages	literature references: MS data	method of isolation[f]	samples in which compound was detected
ethyl alanine[g]	0.60	A, B		d, e	A, B	3–13
ethyl glycine	0.75	A		e	A	4, 7, 8
methyl valine	0.80	A		d	A, B	3, 4, 8, 9, 12
ethyl α-aminobutyric acid	0.81	C		e	A, B	3
ethyl valine[g]	0.92	A, B	c	d, e	A, B	3–13
methyl isoleucine[g]	1.11	A		d	A, B	3–5, 7–9, 12
ethyl leucine[g]	1.17	A, B	c	d, e	A, B	3–13
ethyl isoleucine[g]	1.22	A, B	c	d, e	A, B	3–13
ethyl proline[g]	1.28	A, B		e	A, B	3–13
diethyl aspartic acid	2.42	C		e	A	3, 7, 8, 12
ethyl methionine[g]	2.54	A, B		e	A, B	3–9, 11–13
diethyl glutamic acid	2.71	A		e	A, B	3, 8
ethyl phenylalanine[g]	2.97	A, B		e	A, B	3–9, 11–13

[a] Retention time relative to ethyl octanoate. [b] A = the mass spectrum and retention time were identical with those of the authentic compound recorded under the same conditions; B = identification confirmed by peak enhancement with the authentic compound, on the SP-1000 column; C = tentative assignment based on comparison with published mass spectral data only. [c] Esters found in other alcoholic beverages (Peppard and Halsey, 1981). [d] Andersson et al. (1962). [e] Biemann et al. (1961). [f] Methods A and B are described under Experimental Section. [g] Quantitation studies were carried out on these compounds.

RESULTS AND DISCUSSION

The resin technique adopted from Peppard and Halsey (1980, 1981), when modified (method A), was found to be more selective for basic components of wine than the continuous extraction method (B). Large quantities of polar neutral compounds such as isoamyl alcohol, ethyl lactate, γ-butyrolactone, the isomeric 2,3-butanediols, and 2-phenylethanol were effectively diminished by successive washings of the resin prior to elution of the more strongly held amino acid esters. Attempts to eliminate the interfering wine components by method B were unsuccessful and excessive solvent extraction at the lower pH led to diminished recoveries of amino acid esters. In order to avoid CO_2 formation in the resin bed by the reaction of excess hydrogen ions and Na_2CO_3, a small quantity of saturated NaCl solution was first passed down the resin. This solution removed any excess hydrogen ions and also displaced some of the amino acid esters. The residual esters were subsequently eluted by the NaCl–Na_2CO_3 solution.

Standard solutions of the reference compounds as hydrochloride salts in 10% aqueous ethanolic tartrate buffer were used to optimize this resin method. The recovery of amino acid esters eluted with the basified NaCl solution was improved by an order of magnitude, compared to elution with saturated aqueous NaCl solution only. This technique was adequate for many of the amino acid esters but was not uniformly applicable. For example, ethyl serine, ethyl lysine, and ethyl cysteine could not be extracted from basified aqueous solutions with Freon F11, although slightly better extraction was achieved with chloroform. Some of the amino acid esters found in wine, in particular ethyl glycine, were difficult to recover owing to high water solubility. Other problems resulted from the use of excessive alkaline conditions, which hydrolyzed all the esters, and ethyl alanine, ethyl glycine, and ethyl proline did not chromatograph as well as other esters. The resin bed volume appeared to be critical in the analysis, and recoveries of all amino acid esters were severely affected, with the higher molecular weight esters being most influenced by this parameter. Experiments with standard solutions showed that the recovery of individual bases ranged from 20 to 70% and decreased when resin bed volumes larger than the one employed were used.

Table I contains details of the esters identified in this work and the samples in which they were found. The mass spectra of these esters and the reference compounds agreed with those published (Biemann et al., 1961; Andersson et al., 1962). All samples that had undergone fermentation contained at least five of the esters. Only in the grape juice samples **1**, **1A**, **2**, and **2A** were no esterified amino acids detected. These results suggest that the esters were formed by the action of yeast during fermentation and not by acid catalyzed reactions of free amino acids with alcohols. Nor were they artifacts of the workup procedure. Twelve esters were detected in sample **3** and eight in sample **5**. These two wines were analyzed by continuous solvent extraction (B) as well as by the resin method (A), precluding the possibility of these esters being artifacts produced on the cation-exchange resin (Harding et al., 1977; Peppard and Halsey, 1981).

Table II. Levels of Amino Acid Esters Found in Wine[a]

amino acid ester	range, μg/L	median, μg/L
ethyl alanine	160–16 800	1200
ethyl valine	60–470	250
methyl isoleucine	trace–8	5
ethyl leucine	250–3320	700
ethyl isoleucine	100–500	220
ethyl proline	1300–15 000	3650
ethyl methionine	50–480	150
ethyl phenylalanine	250–1600	385

[a] These figures exclude wine **6**, which had been ion-exchanged at the winery and therefore had much lower quantities of amino acid esters. The port and the beer were also excluded since they were two notable exceptions in total amino acid ester content.

Eight amino acid esters were quantified in seven of the beverages examined, and the results are given in Table II. Their concentrations ranged from trace amounts as determined by MS ion searching, to 16 800 μg/L for ethyl alanine in sample **3**. Apart from samples **6**, **10**, and **13**, all wines analyzed exhibited a similar trend in the levels of individual esters. This pattern is illustrated by the median figures in Table II. For example, ethyl valine, ethyl isoleucine, and ethyl methionine were generally present in lower quantities than the other ethyl esters, while ethyl proline was present in quantities greater than all the other esters collectively. A similar trend was observed in the amino acid levels reported for musts (Ough and Tabacman, 1979; Ough and Bell, 1980; Cantagrel et al., 1982). The experimental *Brettanomyces* fermentation (**3**) was a notable exception. The ethyl alanine concentration in this

sample was 14 times that of the median figure. Amino acid analysis of grape juices was beyond the scope of this work, but in view of the alanine levels reported for must, the comparatively high concentration of ethyl alanine in sample 3 was surprising. Apart from the large ethyl proline concentration in the champagne (15 000 μg/L), the levels of other amino acid esters in this wine were well within the ranges presented in Table II, suggesting that yeast autolysis may not be a significant contributing factor to the presence of these compounds in wines. The concentration of methyl isoleucine found in wines ranged from trace quantities up to 8 μg/L in the champagne (12).

The concentrations of ethyl valine (6 μg/L), ethyl leucine (36 μg/L), and ethyl isoleucine (6 μg/L) in the beer (13) were consistent with those reported by Peppard and Halsey (1981) for English beers. Methyl isoleucine was not detected at all. The median concentration of each amino acid ester in the wines examined ranged from 10 to 250 times the corresponding quantity found in beer. In particular, ethyl proline in the table wines ranged from 1300 to 15 000 μg/L while its level in the beer was only 14 μg/L. The large difference between the levels of individual amino acid esters in wines and beer was noteworthy considering the levels of the corresponding amino acids in musts and worts. For example, the concentration of proline reported in musts ranges from 300 to 2000 mg/L (Ough and Tabacman, 1979; Ough and Bell, 1980; Cantagrel et al., 1982), and that reported for worts ranges from 200 to 525 mg/L (MacLeod, 1977). As in the case of the unusually large ethyl alanine level in sample 3, the difference in relative concentrations of amino acid esters in beer and wines suggests that amino acid concentration in the fermentation starting materials may not necessarily influence the synthesis of the corresponding ester.

The port (10) contained lower concentrations of all the esters, with the ethyl esters of alanine, valine, leucine, and isoleucine all below 50 μg/L and ethyl proline at 850 μg/L. Ethyl methionine and ethyl phenylalanine were not detected at all. These figures are probably a reflection of the way in which ports are made. Fermentation is stopped prematurely to retain sugar levels, and the wine is then diluted in the fortification process. The comparison between beer, wine, and the port indicates a trend to higher concentrations of amino acid esters with greater sugar attenuation during fermentation. This further reflects the importance of yeast to the formation of these esters. Wine 6 contained an average of only ca. 5% of the amino acid ester levels represented by the median figures in Table II. This was explained by the fact that this wine had been cation-exchanged at the winery.

While there is no evidence for the mechanism of formation of these amino acid esters, it may be postulated that they are byproducts of yeast protein synthesis. During protein synthesis, the carboxyl group involved in binding the amino acid to the hydroxyl end of tRNA in the aminoacyl-tRNA molecule is susceptible to nucleophilic attack (Lehninger, 1972). A possible mechanism for the synthesis of these amino acid esters may be nucleophilic substitution upon this carboxyl group by ethanol and to a lesser extent by methanol, resulting in the appropriate amino acid ester and free tRNA. This mechanism would only account for esterification of amino acids at the amino end of the molecule and thus does not completely account for the diethyl esters of aspartate and glutamate.

CONCLUSION

This study was not intended to identify all amino acid esters in wine but to determine whether such compounds were normal constituents of fermented alcoholic beverages. Accordingly, amino acid esters additional to those found here would be expected to be found in wines if alternative specific isolation and analytical procedures were used. Other workers (Ough and Daudt, 1981; Ough et al., 1981) have analyzed basic extracts of grapes and wines by chemical derivatization and GC–MS. Both spoiled and sound wines were examined in this study, but in order to preserve the characteristic odor of the basic extracts, samples were analyzed without chemical derivatization. This may explain why these compounds have not been sighted in wine before.

There appeared to be no significant correlation between relative concentrations of amino acid esters found in sound or microbiologically spoiled wines. Furthermore, the levels of amino acid esters found in this study indicate that these compounds are major volatile bases of wines.

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Registry No. Ethyl alanine, 3082-75-5; ethyl glycine, 459-73-4; methyl valine, 4070-48-8; ethyl α-aminobutyric acid, 22621-37-0; ethyl valine, 17431-03-7; methyl isoleucine, 2577-46-0; ethyl leucine, 2743-60-4; ethyl isoleucine, 921-74-4; ethyl proline, 5817-26-5; diethyl aspartic acid, 13552-87-9; ethyl methionine, 3082-77-7; diethyl glutamic acid, 16450-41-2; ethyl phenylalanine, 3081-24-1; methanol, 67-56-1; ethanol, 64-17-5.

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