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Analysis of ethereal extracts of wines and other alcoholic beverages by high-performance liquid chromatography with microbore columns

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Abstract

The ethereal extracts of wines, beer and vermouth were analysed by high-performance liquid chromatography. The following three characteristic peaks were first identified using GC–MS and then quantitatively determined: 5-hydroxymethyl-2-furaldehyde, 2-(4-hydroxyphenyl)ethanol and 3-(2-hydroxyethyl)indole. © 1997 Elsevier Science B.V.

Keywords: Wine; Beer; Vermouth; 5-Hydroxymethyl-2-furaldehyde; 2-(4-Hydroxyphenyl)ethanol; 3-(2-Hydroxyethyl)indole

1. Introduction

Previous works [1,2] analysed the non-volatile phenolic acid fraction of wines (cinnamic and benzoic acids) applying the purification method reported in Fig. 1. Given the complexity of the matrix [3], determination of the acid compounds, after eliminating ethanol, required sample extraction (pH 8.0) with diethyl ether in order to separate the neutral and basic substances from the acidic ones. A previous study [2] showed that injecting this extract in high-performance liquid chromatography (HPLC) gave a chromatogram in which three characteristic peaks were present. This work will present the identification of the compounds corresponding to these three peaks, together with their quantitative determination.

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2. Experimental

2.1. High-performance liquid chromatography

A Jasco two-pump (PU-980) gradient HPLC system was used with a high-pressure Gilson dynamics mixer 811 C (1.5 ml). A Jasco Model 100 VA variable-wavelength UV detector equipped with a 1-μl flow cell was used. The injection valve was a Valco C6W with a 20-μl sample loop. Two Phoenix 20 (Fison) syringe pumps (master and slave) interfaced to an external computer (IBM) for remote control operations and a rapid scanning UV–Vis detector (micro UV–Vis 20) were used. The injection valve was a Rheodyne 7520 with a 1.0-μl sample loop.

Two columns were used. Column A, a Restek (Bellofonte, PA, USA) silcosteel tube (250 mm×1.0 mm I.D.), and column B, a stainless steel tube (165

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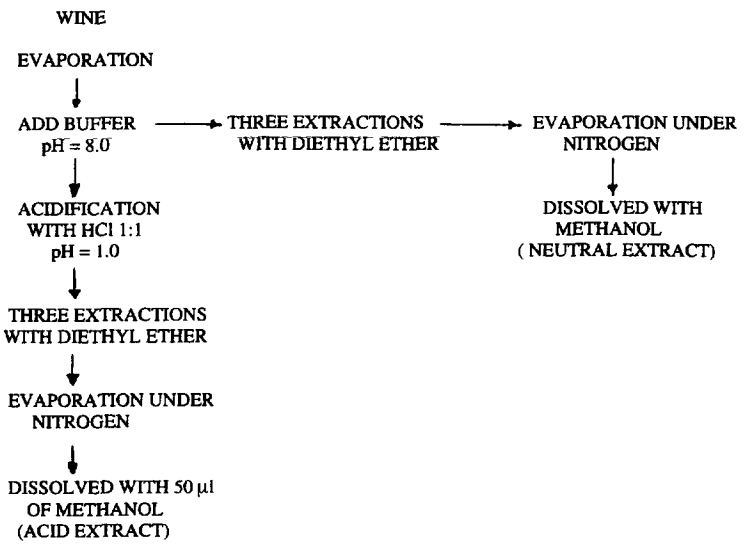

Fig. 1. Sample treatment for wine extraction.

mm×4.6 mm I.D.), were slurry packed in the laboratory with Spherisorb ODS2 (5 μm) obtained from Phase Separations (Norwalk, CT, USA) [4].

2.2. Gas chromatography–mass spectrometry

A Hewlett–Packard 5970 HSD was used with a SE-54 capillary column (25 m×0.15 mm I.D., d_f= 0.12 μm). Initial temperature was of 60°C for 1 min, then programmed to 280°C over 15 min and finally maintained at 280°C for 40 min. The carrier gas was helium and a split–splitless injection was employed (closed for 10 min, then opened at a split ratio of 10:1); transfer line and injection temperature was 280°C.

2.3. Reagents

Distilled water was stored in glass, filtered and passed through a Norganic system cartridge (Millipore, Bedford, MA, USA). Methanol and acetonitrile RS for HPLC, trifluoroacetic acid (TFA) were from Carlo Erba. Ammonium dihydrogen phosphate, disodium hydrogen phosphate dihydrate, potassium dihydrogen phosphate, ortho-phosphoric acid 85%, hydrochloric acid 37%, ethyl acetate, diethyl ether (RPE Carlo Erba). The ether had to be free of peroxides, which were eliminated by passing the ether through a CN cartridge just before use. A grain of Carbowax 20 000 was also added to saturate it. This operation reduces solute loss during evaporation under nitrogen in the test tube. The buffer at pH 8 was prepared by mixing 47.5 ml of a solution of 0.1 *M* Na_2HPO_4 and 2.5 ml of 0.1 *M* KH_2PO_4. Buffer solutions were stored at 4°C.

The cartridge was a Waters 3 ml C_{18}-ODS Sep-Pak. Before use, the cartridge must be activated by washing several times with methanol and then left in methanol.

2.4. Beverages

The white wines used were the Italian Corvo, Pinot Grigio, Frascati Superiore, Fontana Candida, Verdicchio, Cerveteri, Cirò, Salice Salentino and Robola. The red wines used were the Italian Corvo and Valpolicella and Port. Beer brands were Peroni (Nastro Azzurro), Dreher, Heineken, Kronenbourg and Heninger. The vermouth was red and white Martini.

2.5. Standards

(I) 2-(4-Hydroxyphenyl)ethanol, (II) 3-(2-hydroxyethyl)indole, (III) 5-hydroxymethyl-2-furaldehyde or 5-(hydroxymethyl)furfural (HMF), (IV)

phenethyl alcohol (Fluka, Buchs, Switzerland). Each substance (1 mg) was dissolved in 1 ml of methanol. A 10-μl volume was taken from III and diluted with 390 μl of methanol. To prepare the mixture of the four substances, the following volumes of solution were transferred to a test tube: a 300-μl diluted solution of 5-(hydroxymethyl)furfural, a 100-μl solution of 2-(4-hydroxyphenyl)ethanol, a 50-μl solution of 3-(2-hydroxyethyl)indole and a 50-μl solution of phenethyl alcohol. This standard solution contained: 18 mg/l of III, 250 mg/l of I, 120 mg/l of II and 120 mg/l of IV.

The chromatogram of the standard mixture is shown in Fig. 2.

2.6. Sample purification by HPLC

A 10-μl sample of beer extract was injected into the Jasco liquid chromatograph with column B at a flow-rate of 0.7 ml/min. The mobile phase was A=acetonitrile, B=distilled water with the following gradient: 0 min, A=10% for 10′; subsequently from 10–100% in 2 min. Each collected fraction of peaks no. 1 and no. 2 (30–40 ml acetonitrile–water mixture), after evaporation of acetonitrile under vacuum (below 30°C in a Rotavaporator), was extracted with 3×5 ml portions of diethyl ether. The collected organic layers were dried over sodium sulphate and concentrated under nitrogen, and the residue was dissolved in 50 μl of methanol.

The recoveries were measured by adding 50 μl of standard solution containing 300 μl of diluted solution I, 50 μl of solution II, 50 μl of solution III and 250 μl of methanol and 1 ml of buffer at pH 8.0 to 1 ml of aqueous solution containing 12% of ethanol. The ethanol was evaporated and the procedure described in Fig. 1 was carried out for the wines.

Recovery tests were carried out for the three purified substances in the neutral extract by using various solvents. Methylene chloride gave recoveries below 50%, whereas ethyl acetate extracted the third peak above 80% but not the first two. Peroxide free diethyl ether turned out to be the best solvent, obtaining ~80% recovery for all substances.

Direct extraction of compounds from wine was also tried, without eliminating ethanol, but 5-hydroxymethyl-2-furaldehyde was not recovered at all.

In the present study a mobile phase based on a methanol–water mixture with the addition of 10 μl of trifluoroacetic acid for every 100 ml of solvent (pH 2.9) was used.

3. Results

This study aimed to identify the three major peaks present in HPLC chromatograms of the neutral ether

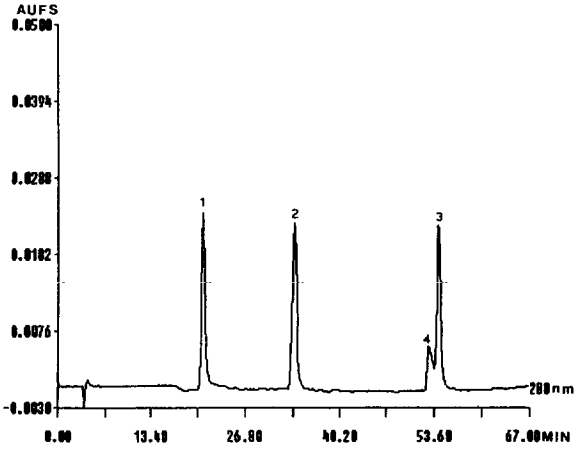

Fig. 2. RP-HPLC chromatogram of standards mixture. Peaks: 1=5-hydroxymethyl-2-furaldeide, 2=2-(4-hydroxyphenyl)-ethanol, 3=3-(2-hydroxyethyl)indole, 4=phenethyl alcohol. Column A; flow-rate, 40 μl/min; mobile phase: A=0.01% TFA in methanol, B=0.01% TFA in distilled water; gradient: 0 min, 0% A; 67 min, 56% A; 70 min, 100% A. (Phoenix 20).

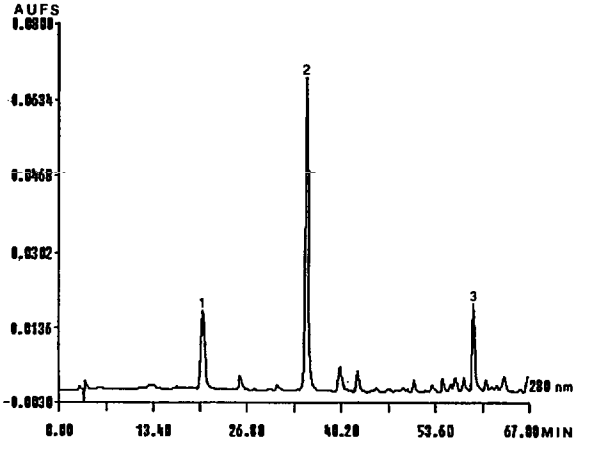

Fig. 3. Chromatogram of white wine. Conditions and peaks as in Fig. 2.

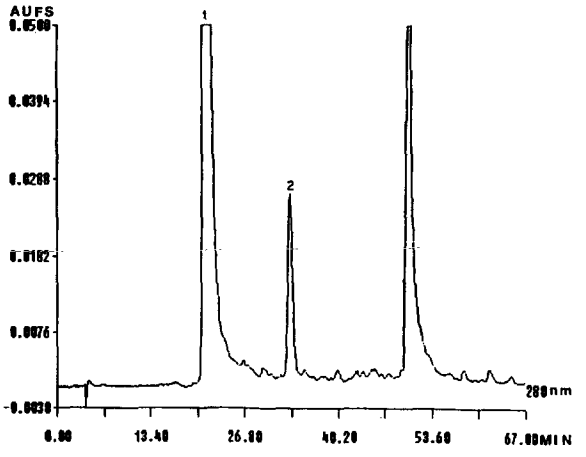

Fig. 4. Chromatogram of red vermouth. Conditions and peaks as in Fig. 2.

extracts obtained from white (Fig. 3) and red wines, beer and vermouth (Fig. 4). These extracts were analysed by GC–MS and Fig. 5 shows the chromatogram obtained from a Peroni (Nastro Azzurro) beer. Using the mass spectra library, the peaks present in the chromatogram were first identified; among all these compounds only three substances were found with UV absorption around a wavelength of about 280 nm. The peaks in the chromatogram of Fig. 5, with a retention time of 8.75 and 25.18 min, were thus identified as phenethyl alcohol (or benzene ethanol) and 3-(2-hydroxyethyl)indole (Figs. 6 and 7). These two substances were later injected in HPLC, using the same conditions as for the beer and wine ether extracts. 3-(2-Hydroxyethyl)indole was seen to correspond to peak 3 of these chromatograms. To confirm peak identification, based on retention times, comparisons were also made of the UV spectrum of the unknown peak of the neutral extract and of the pure standard. This double comparison showed that peak 3 was not pure but contained also a second substance that turned out to be phenethyl alcohol. This substance emerged in HPLC with a retention time that was very close to that of peak 3, but it absorbed very little at a wavelength of 280 nm.

In order to identify peaks 1 and 2 of chromatograms by HPLC, several extractions were necessary on about 600 ml of beer to have a more concentrated neutral extract. Beer was chosen because it contains more of the substances analysed than wine. HPLC was carried out on this extract, collecting peaks 1 and 2 (Fig. 8) separately. The two extracts were reinjected into HPLC to check their purity and to obtain the UV spectra. The extract containing peak 2 proved to be pure and in a considerable quantity (~1

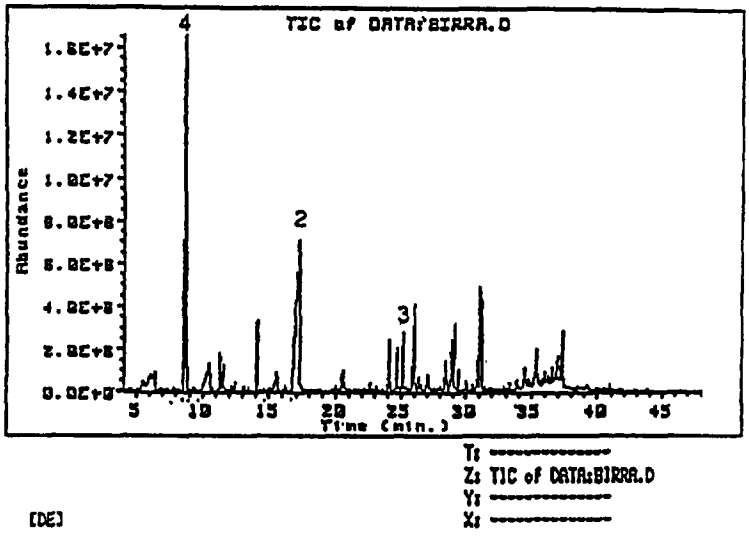

Fig. 5. GC–MS total ion chromatogram of beer sample. Column SE-54 (25 m×0.15 mm), d_f=0.12 μm; carrier gas, helium; temperature program: 60°C for 1 min, from 60–280°C in 15.7 min and 40 min at 280°C.

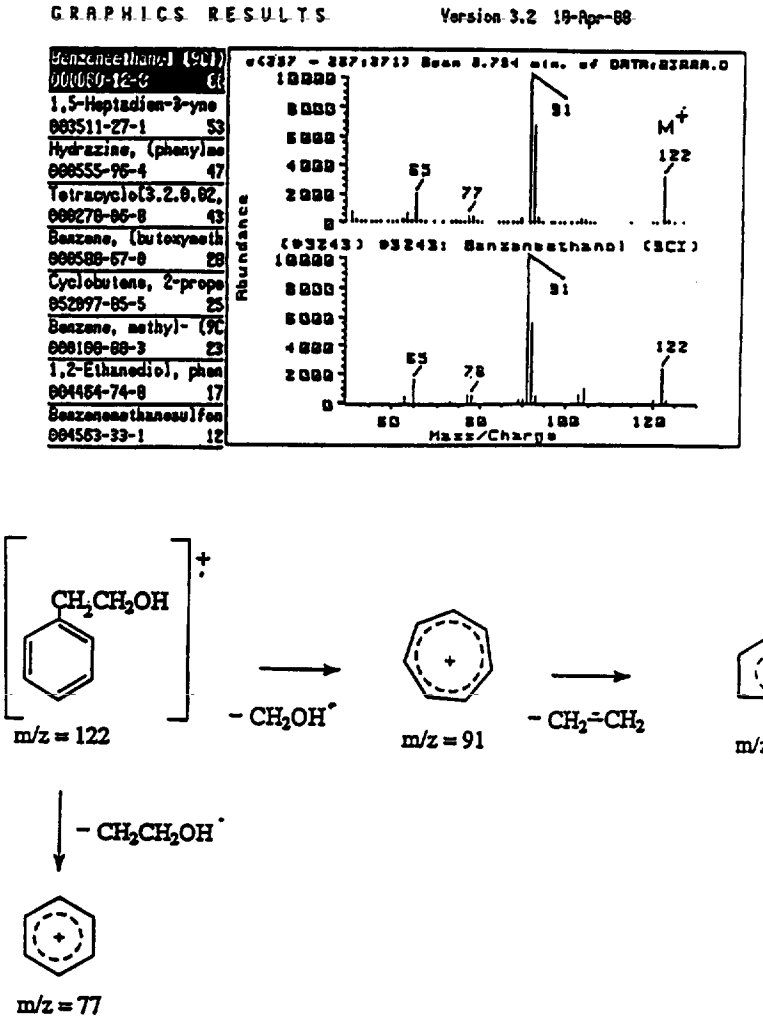

Fig. 6. GC–MS EI mass spectrum of peak No. 4.

mg/ml). This substance was reinjected in gas–mass. The gas–mass library was unable to identify the peak because it was not listed. The Eight Peak Index of mass spectra [5] was consulted, peak 2 was identified as 2-(4-hydroxyphenyl)ethanol (Fig. 9). As additional confirmation, the pure standard was injected into HPLC and coincident retention times and UV spectra were obtained.

The substance corresponding to peak 1 was obtained in a smaller quantity (~0.2 mg/ml) and it was not possible to identify it in gas–mass since it was either irreversibly retained in the column or decomposed. Examination of the UV spectrum revealed that substances with spectra similar to that of peak 1 contained a furanic ring. A study [6] reported the presence of furan-2-carboxylic acid, furfuril alcohol and furan-2-carboxaldehyde (furfural) and another study [7] reported the presence of furfuryl alcohol. When these compounds were injected in HPLC, under the same analytical conditions as for the wines, the retention times and spectra did not coincide with those of peak 1. Only the UV spectrum obtained from furan-2-carboxaldehyde was similar to that of peak 1.

Other works [8,9] mentioned the presence of 5-hydroxymethyl-2-furaldehyde (HMF) in wines that

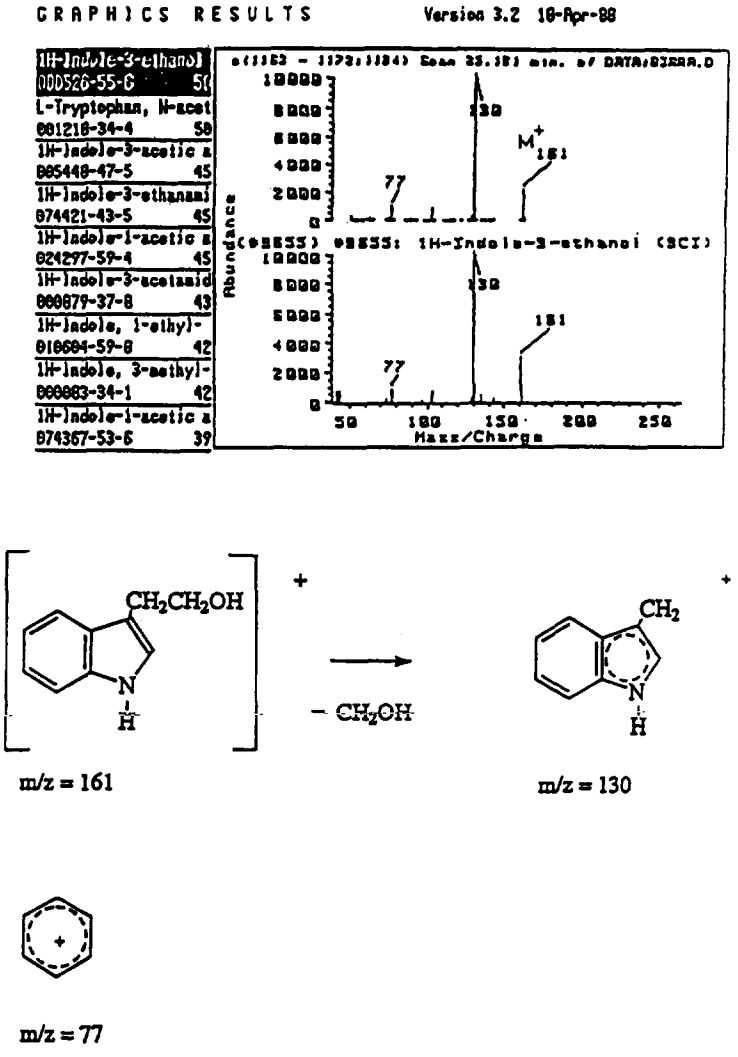

Fig. 7. GC–MS EI mass spectrum of peak No. 3.

underwent thermal treatment. These wines had been produced from concentrated musts, from musts desulfitated through heat, or from musts added with concentrated musts or containing inverted sugar. Even wines like Xeres or Malaga contain significant quantities of 5-hydroxymethyl-2-furaldehyde. A standard sample of 5-hydroxymethyl-2-furaldehyde was injected in HPLC and the retention time and UV spectrum coincided with the substance corresponding to peak 1.

3.1. Quantitative analysis

Table 1 reports quantitative data relative to the three substances present in wine, beer and vermouth. They are the mean values of $n=5$ determinations with a R.S.D. in a range of 6–10%. HMF is very important since it can provide useful information on: (1) must concentration via the elimination of water through heating [15] and generally any treatment involving a rise in temperature [9–14,16,17]; (2) any

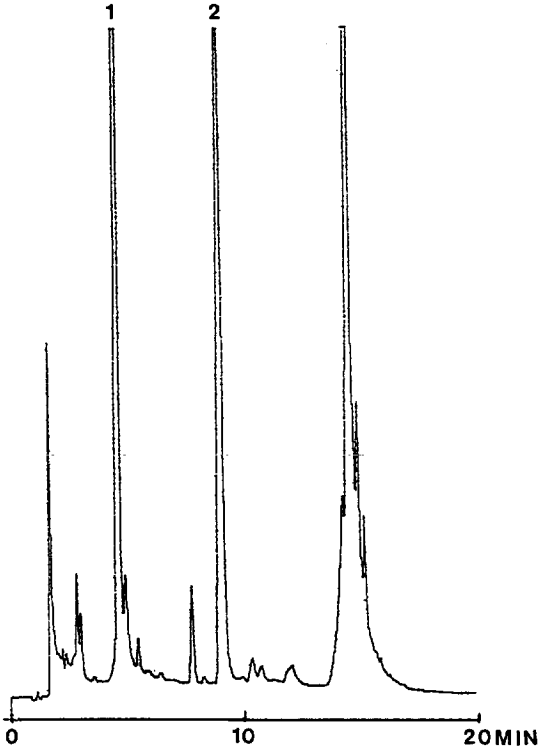

Fig. 8. HPLC chromatogram of beer sample. Flow-rate, 0.7 ml/min (Jasco pump). Mobile phase: A=acetonitrile, B=distilled water. Gradient: A=10% for 10 min, subsequently from 10–100% in 2 min. Peaks 1 and 2 were collected.

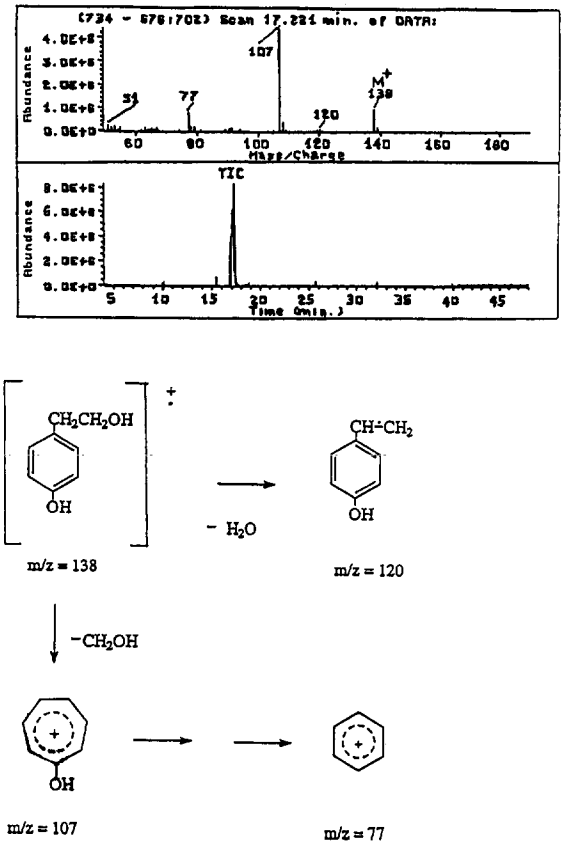

Fig. 9. GC–MS EI mass spectrum of peak No. 2.

addition of caramel [17]. Moreover, there is a relation between HMF and sulphur dioxide [11]. When sulphur dioxide is present in wine, it reacts with HMF and thus changes retention times and UV spectrum, which shows a considerable reduction of the peak absorbance at 280 nm.

The absence of HMF in wines may be due to two factors:

- the wine must was not concentrated by heating;
- the wine must was treated with bisulfite and thus HMF was transformed into a different molecule.

Observing the data in the table, it may be noted that peak 1 was not determined for some wines since it was either undetectable, as in the case of white Cerveteri, red Corvo and white Martini, or because the quantity detected was slight and covered by other impurities, as in the case of Salice Salentino. The strong presence of HMF in red Martini is mainly due to caramel addition.

Two home-made wines (red and white), produced by the authors, were also analysed. HMF turned out to be absent from both the red and white wines, confirming the fact that the wine and must had not been heated. Peak 2 was still much in evidence in both the white and red wines and is produced by yeasts during fermentation. 2-(4-Hydroxyphenyl)ethanol or tyrosol is defined as a 'bitter sapophore' [18]; it results from the deamination and decarboxylation of tyrosine and is an antioxidant. Peak 3, 3-(2-hydroxyethyl)indole, was not always present or was only present in minor quantities. Because its retention time is situated in a range where there are various overlapping impurities, its

Table 1
Quantitative determinations of substances in some alcoholic beverages

Samples	Substances[a] (mg/l)		
	1	2	3
Wines			
Cirò (b)	0.19	3.80	0.38
Cerveteri (b)	–	7.83	–
Salice Salentino(b)	–	8.92	0.76
Corvo (b)	0.54	6.63	–
Frascati superiore(b)	0.17	9.61	–
Pinot Grigio (b)	0.09	12.80	–
Valpolicella (r)	0.12	4.10	2.70
Corvo (r)	–	10.30	6.86
Porto (r)	0.10	1.60	1.50
Robola (b)	0.58	7.40	1.72
Beers			
Heninger	0.72	3.20	–
Heineken	3.97	7.41	0.49
Kronenburg	1.46	2.74	0.25
Nastro Azzurro	0.31	3.20	1.26
Dreher	1.16	5.26	1.06
Vermouth			
Martini bianco	–	15.10	–
Martini rosso	15.70	13.70	–

[a] 1=5-hydroxymethyl-2-furaldehyde, 2=2-(4-hydroxyphenyl)-ethanol, 3=3-(2-hydroxyethyl)indole.
b=white, r=red.

proper determination is not always possible. This was the case with white Cerveteri and white Corvo. Peak 3, 3-(2-hydroxyethyl)indole, was present in smaller non-determinable quantities in Frascati Superiore, Pinot Grigio and in Heninger beer; in red Martini it was not pure and therefore not determined. It is also present in white Martini but also in small quantities overlapped with impurities. In quantitative analysis of very complex matrices, particularly when the sample size is rather small, a UV spectrum of the peak must be carried out to check purity.

4. Conclusion

The proposed procedure allows analysis of the reported substances present in a very complex matrix, such as wines or other alcoholic beverages, by separating the acidic type substances from the neutral ones. In this way, it is possible to avoid an overlap of the peaks of the two fractions which would otherwise make detection impossible. The two separated fractions allow a simultaneous analysis of various groups of substances, reducing the time necessary for initial sample preparation.

An analysis of all these compounds can give some information on the treatment and conservation of wines.

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