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Determination of carboxylic acids, sugars, glycerol and ethanol in wine and grape must by ion-exchange high-performance liquid chromatography with refractive index detection

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ABSTRACT

Method to determine the major carboxylic acids, sugars, glycerol and ethanol in wine and grape must was developed using an ion-exchange column and refractive index detector. A solid-phase extraction method with a strong anion exchanger was used to determine these compounds in sweet wines and in grape musts. With this method it is possible to determine malic acid in sweet wines and in grape musts without interference from sugars. This high-performance liquid chromatographic method was compared with standard methods of analysis. There was good agreement in the accuracy and precision of the compared methods.

INTRODUCTION

Chromatographic methods are considered to be valid alternatives to enzymatic methods for the determination of carboxylic acids in wine as all the carboxylic acids can be determined chromatographically with a shorter analysis time and with similar accuracy [1,2].

Of the various high-performance liquid chromatographic (HPLC) techniques, reversed-phase HPLC [1–7] and ionic-exchange HPLC [1,8–13] are the most often used. The latter has several advantages over the reversed-phase methods, such as the possibility of determining the carboxylic acids simultaneously with fructose, glucose, glycerol and ethanol when a refractive index (RI) detector is used. The sensitivity of this method is not very high, but it is sufficient to determine these compounds in wine samples.

When ion-exchange HPLC is used to determine these compounds, poor resolution between glucose, malic acid and fructose is obtained. When the concentration of the two sugars is high, it is impossible to determine malic acid correctly. For this reason, various workers [1,11] have applied this method to dry wine samples only using IR detection, with which it is possible to determine malic acid. However, the identification and determination of organic acids in grape musts is impossible with this method because the high concentration of sugars requires a dilution of the sample before injection, and the carboxylic acids cannot be detected at such low concentrations.

The different behaviour of sugars, glycerol and ethanol compared with carboxylic acids allows the separation of these compounds using a solid-phase extraction by ion exchange. In this work, LC-SAX tubes (quaternary amine bonded silica with strong anion exchange) (Supelco) were used to separate the sample into two fractions, neutral and acidic. Once this separation has been carried out, it is possible to determine the carboxylic acids in wine or grape must independently of the concentration of sugars.

This separation method gives a determination of

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malic acid which is more similar to that obtained by enzymatic methods than to that obtained by the direct HPLC method without extraction.

EXPERIMENTAL

High-performance liquid chromatography

The samples were analysed using a Model LC-9A Shimadzu pump, a Shimadzu RID-6A detector and an ION-300 (300 × 7.8 mm) column containing a cation-exchange polymer in the ionic hydrogen form, with a GC-801 ion guard column (Interaction). The column was operated at 74°C using 0.013 *M* sulphuric acid as the mobile phase and a flow-rate of 0.6 ml/min.

Standard solution

The experiment was carried out with a standard solution of the main carboxylic acids found in wine and grape must (tartaric, lactic, malic, acetic, citric and succinic acids, Aldrich Chemicals) and glucose, fructose, glycerol and ethanol (R.A. quality, Merck) at concentrations of 2.5 g/l, except for ethanol, which was prepared at 5% (v/v).

Grape must and wines

Various red and white grape musts and wines from the Tarragona region of Spain were used. All the samples were supplied by the Institut Calatà de la Vinya i del Vi, where all the standard analyses were performed. The grape must samples were stored at 2–4°C after sterile filtration.

Sample preparation procedures

Three simple procedures depending on the products to be determined, were followed before the injection of the samples into the chromatograph.

Procedure A. All samples were filtered with a 0.45-μm nylon membrane (mSI Micron Separations).

Procedure B. To prolong the life of the column, it is necessary to remove the phenolic compounds in the red wines, and grape musts. This is achieved using a C_{18} Sep-Pak cartridge (Waters Assoc.). When a white grape must or wine is analysed, only dilution of the sample is required to obtain good results. The C_{18} Sep-Pak cartridge was activated by a recommended method [14] with 1 ml of methanol–water (10:90) then 1 ml of the wine sample was passed through the Sep-Pak cartridge, followed by 1.5 ml of 0.005–0.0025 *M* sulphuric acid.

Procedure C. To separate the neutral compounds from the acidic compounds, a 3-ml Supelclean LC-SAX (strong anion exchange) tube (Supelco) was used. First, the tube was conditioned with 5 ml of Milli-Q water and then 0.5 ml of sample diluted 1:2, previously adjusted to pH 7–8 with 10 *M* sodium hydroxide solution, was slowly passed through the extraction tube. To obtain a good recovery of the neutral compounds, it is necessary to elute these compounds with 1 ml of water if a wine sample is analysed, and 2 ml of water if a grape must is analysed. The final volumes obtained are 1.5 ml for the wine samples and 2.5 ml for the grape must samples.

To elute the acidic compounds, two 0.5-ml aliquots of 0.5 *M* sulphuric acid were passed through the tube. Two small aliquots of eluent generally elute the compounds of interest more efficiently than one larger aliquot.

To determine the sugars in grape must samples, only a 1:50 dilution is necessary to determine glucose and fructose.

Chemical methods used as comparisons with HPLC

Citric acid, L-malic acid, glucose, fructose and glycerol were determined enzymatically using the Boehringer UV method [15]. Tartaric acid was determined by titration [16].

RESULTS AND DISCUSSION

In a previous paper [13] the optimization of an ion-exchange HPLC separation with an RI detector for the determination of the major carboxylic acids, sugars, glycerol and ethanol in wine samples with various chemometric methods was reported. The best chromatographic conditions obtained with an ION-300 column were: flow-rates, 0.6 ml/min; mobile phase 0.013 *M* sulphuric acid; and column temperature, 74°C.

Fig. 1 shows the chromatogram obtained for a standard mixture using these conditions. Good resolution between the different peaks was obtained, except for the glucose, malic acid and fructose peaks. In samples with high sugar concentrations (sweet wines and grape musts), the malic acid peak was completely masked by the high concentration

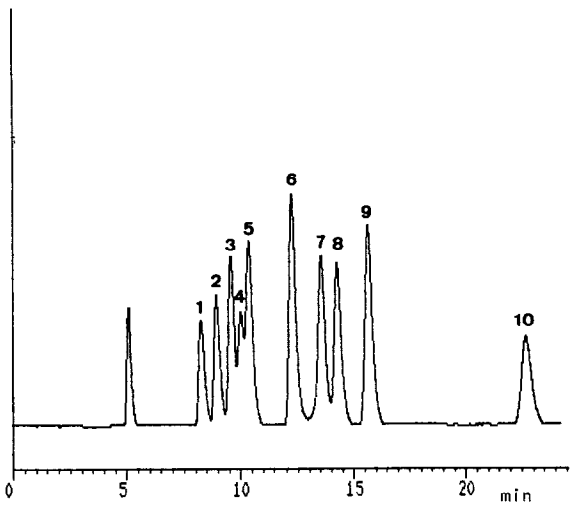

Fig. 1. Chromatogram of a standard solution. Peaks: 1 = Citric acid; 2 = tartaric acid; 3 = glucose; 4 = malic acid; 5 = fructose; 6 = succinic acid; 7 = lactic acid; 8 = glycerol; 9 = acetic acid; 10 = ethanol.

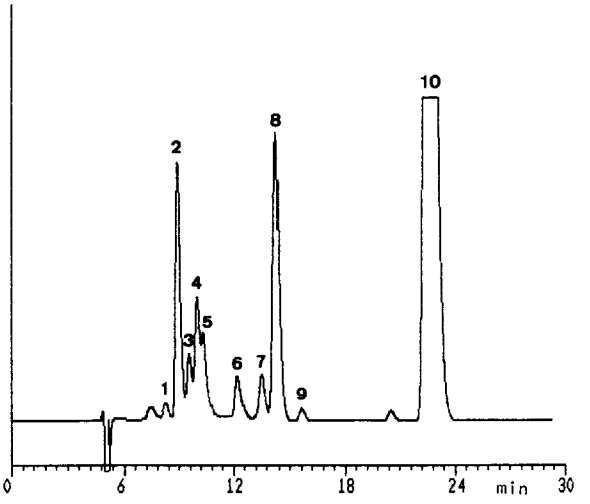

Fig. 2. Chromatogram of wine without SAX treatment. Peaks as in Fig. 1.

of glucose and fructose. However, when a grape must was analysed, the dilution of the sample which was necessary to determine the sugars made it impossible to determine carboxylic acids in the same injection. The determination of tartaric and citric acids can be achieved without dilution of the must, but the determination of malic acid is impossible.

If the method is applied to samples with high sugar contents, it is necessary to separate glucose and fructose from the malic acid before chromatographic determination. This separation is possible using a Supelclean LC-SAX (strong anion exchange) column to remove the acidic fraction (containing citric acid, tartaric acid, malic acid, succinic acid, lactic acid and acetic acid) from the neutral fraction (containing glucose, fructose, glycerol and ethanol). The use of a Sep-Pak cartridge to eliminate the coloured compounds is recommended by several workers [3,17] when red wines are analysed.

The volume of sample and the volume and concentration of the sulphuric acids passed through the cartridge to obtain a good recovery of the compounds were optimized. The volumes of sample and sulphuric acid studied were between 0.5 and 1.5 ml and the concentration of sulphuric acid was between 0.005 and 0.0025 *M*. The best results were obtained when the volume of sample was 1 ml, followed by 1.5 ml of 0.0025 *M* sulphuric acid.

Table I shows the results obtained for a red wine spiked with different standard solutions to determine the recovery values and the influence of the concentration on these values. A good recovery was obtained (>90%) and changes in the concentration of sulphuric acid did not affect the recovery.

A study of the repeatability of the method and its reproducibility between days was performed. The results obtained are given in Table II. The repeatability study under these conditions gave relative standard deviations ranging from 0.58 to 5.15% and reproducibilities between 1.13 and 5.62%.

The linearity of the response of the HPLC method was evaluated using a range of concentrations of each standard component. All components had a good linear response over the studied concentration range and can be quantified by an external standard. The linearity ranges were: citric acid, 0.05–1 g/l; tartaric acid, 0.1–6 g/l; malic acid, 0.05–6 g/l; succinic acid, 0.05–2.5 g/l; lactic acid, 0.1–5 g/l; acetic acid, 0.05–2 g/l; glucose and fructose, 0.1–4 g/l; glycerol, 0.2–10 g/l; and ethanol, 0.5–15% (v/v).

The chromatogram obtained for a wine with a low sugar content without SAX treatment can be seen in Fig. 2. The peaks corresponding to citric, tartaric, succinic, lactic and acetic acids, glycerol and ethanol have a good resolution and can be determined, but poor resolution was obtained with this column for glucose, malic acid and fructose.

TABLE I

RECOVERY STUDY OF SEP-PAK TREATMENT FOR A RED WINE

Compound	Concentration (g/l)				
	In wine	Added	Calculated	Found	Recovery (%)
Citric acid	0.145	0.155	0.300	0.295	99.5
	0.145	0.305	0.450	0.435	96.6
	0.145	0.510	0.655	0.590	90.4
Tartaric acid	2.780	1.200	3.980	4.045	101.6
	2.780	2.405	5.185	5.275	101.8
	2.780	4.005	6.785	6.725	99.1
Malic acid	1.515	0.605	2.120	2.135	100.8
	1.515	2.425	3.940	3.950	100.4
	1.515	4.040	5.555	5.395	97.1
Succinic acid	0.875	0.460	1.335	1.280	96.0
	0.875	0.925	1.800	1.720	95.7
	0.875	1.540	2.415	2.285	94.7
Lactic acid	1.625	0.505	2.130	2.295	107.8
	1.625	0.950	2.575	2.630	102.3
	1.625	1.895	3.520	3.430	97.4
Acetic acid	0.455	0.200	0.655	0.660	100.9
	0.455	0.620	1.075	0.995	92.6
	0.455	1.035	1.490	1.425	95.8
Glucose	1.175	0.605	1.780	1.770	99.5
	1.175	1.215	2.390	2.400	100.5
	1.175	2.025	3.200	3.150	98.5
Fructose	0.300	0.605	0.905	0.975	108.1
	0.300	1.210	1.515	1.680	111.0
	0.300	2.020	2.320	2.465	106.4
Glycerol	8.785	1.810	10.595	10.870	102.6
	8.785	3.620	12.405	12.675	102.2
	8.785	6.300	15.085	13.945	92.5
Ethanol[a]	14.021	1.000	15.021	15.312	101.9
	14.021	2.500	16.521	16.734	101.3
	14.021	4.000	18.021	18.453	102.4

[a] Expressed as % (v/v).

They can be identified, although the accuracy of the determination is a function of the integration criteria used.

Fig. 3 shows the chromatogram obtained when the wine sample is passed through a SAX tube. In the region corresponding to glucose, malic acid and fructose, only one peak appears, corresponding to malic acid. At the end of the chromatogram a small peak appears corresponding to glycerol together with another peak corresponding to ethanol, showing a high concentration in the wine. The extraction of glycerol and ethanol is not quantitative with the solid-phase extraction.

Fig. 4 shows the neutral compounds after SAX extraction, when a higher fraction of glycerol and ethanol compounds appear. In the first zone there are three peaks, corresponding to neutral compounds. One possible identification was assigned to glucose, malic acid and fructose, assuming a poor extraction of the malic acid, but in accuracy study,

TABLE II

STUDY OF BETWEEN-DAY REPEATABILITY AND REPRODUCIBILITY OF SEP-PAK TREATMENT FOR A RED WINE

Compound	Repeatability (n = 10)		Reproducibility (n = 10)	
	Mean (g/l)	R.S.D. (%)	Mean (g/l)	R.S.D. (%)
Citric acid	0.16	2.22	0.16	2.30
Tartaric acid	2.64	0.91	2.73	2.81
Malic acid	1.38	2.07	1.43	3.36
Succinic acid	0.92	1.87	0.87	1.32
Lactic acid	1.65	0.58	1.64	1.63
Acetic acid	0.41	2.84	0.44	3.38
Glucose	1.08	3.84	1.16	4.31
Fructose	0.39	5.15	0.33	5.62
Glycerol	8.53	0.78	8.62	1.13
Ethanol[a]	13.84	0.96	13.91	1.43

[a] Expressed as % (v/v).

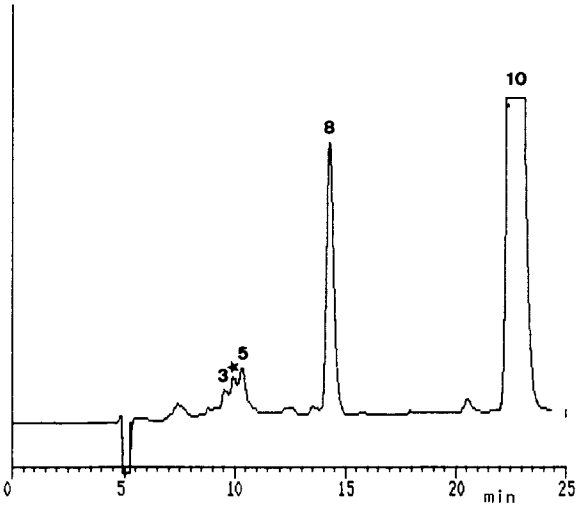

Fig. 4. Chromatogram of neutral fraction of wine sample after SAX treatment. ★ = Unknown peak; other peaks as in Fig. 1.

comparing the results obtained by various chromatographic methods and the enzymatic method, the peak appeared between glucose and fructose and was assigned to another unknown neutral compound.

When a grape must is analysed, only a dilution of the sample is required to determine the sugar compounds (Fig. 5). In this instance, only a small peak corresponding to the main acid present in grape must, tartaric acid, appears before the glucose and fructose peaks. If acidic compounds are analysed, the extraction of sugars with the SAX cartridge is required. Fig. 6 shows the chromatogram obtained after SAX treatment, when citric, tartaric and malic acids can be assigned and quantified.

The method was validated by determining the re-

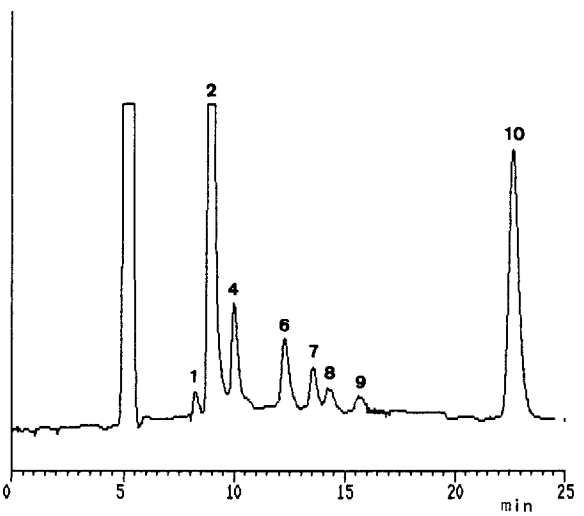

Fig. 3. Chromatogram of acidic fraction of wine sample after SAX treatment. Peaks as in Fig. 1.

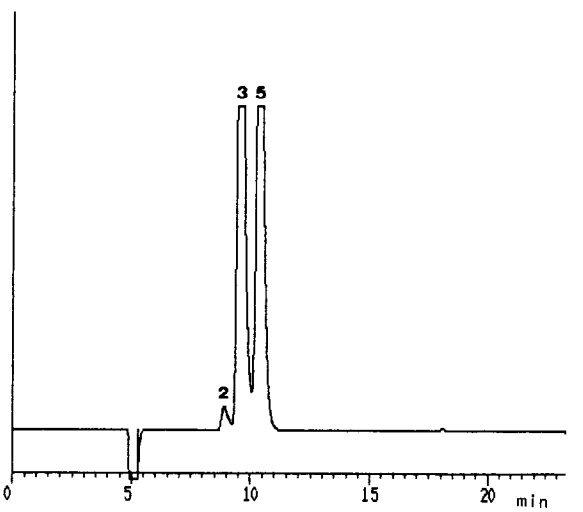

Fig. 5. Chromatogram of grape must diluted 1:50. Peaks as in Fig. 1.

TABLE III

STUDY OF BETWEEN-DAY REPEATABILITY AND REPRODUCIBILITY OF SAX TREATMENT FOR A WHITE WINE

Compound	Repeatability (n = 10)		Reproducibility (n = 10)	
	Mean (g/l)	R.S.D. (%)	Mean (g/l)	R.S.D. (%)
Citric acid	0.15	2.98	0.15	3.23
Tartaric acid	3.59	2.22	3.53	2.81
Malic acid	0.83	2.48	0.84	2.74
Succinic acid	0.73	3.10	0.72	3.61
Lactic acid	0.63	4.14	0.63	4.87
Acetic acid	0.18	3.51	0.17	3.82
Glucose	0.58	1.58	0.59	2.81
Fructose	0.68	4.11	0.68	4.60
Glycerol	4.95	2.57	4.98	2.98

peatability, reproducibility, recovery and accuracy. Table III shows the results obtained for repeatability and between-day reproducibility of the analytical method. The study of repeatability showed a relative standard deviation between 1.58 and 4.14% and a between-day reproducibility of 2.74–4.87%.

Recovery analysis after SAX treatment was performed on a white wine. This wine was spiked with various amounts of each component. For malic acid the results were compared with those of a non-spiked sample, deducting the amount added from the amount in the wine, because it was not possible to determine malic acid without SAX treatment due to the co-elution of an unknown peak. The results are shown in Table IV as a percentage of recovery. The recovery was greater than 88% for citric, tartaric and malic acids. The other three carboxylic

TABLE IV

STUDY OF THE RECOVERY OF ACIDS AFTER SAX TREATMENT

Compound	Concentration (g/l)				
	In wine (g/l)[a]	Added	Calculated	Found	Recovery (%)
Citric acid	0.083	0	0.083	0.082	99.4
	0.083	0.083	0.166	0.161	97.0
	0.083	0.166	0.249	0.237	95.2
	0.083	0.416	0.499	0.466	93.3
Tartaric acid	1.742	0	1.742	1.666	95.6
	1.742	0.127	1.869	1.712	91.6
	1.742	0.254	1.996	1.842	92.3
	1.742	0.632	2.374	2.149	90.5
Malic acid[b]	–	0	–	0.343	–
	–	0.101	–	0.432	88.1
	–	0.202	–	0.531	93.1
	–	0.505	–	0.791	88.7
Succinic acid	0.372	0	0.372	0.337	90.6
	0.372	0.146	0.518	0.460	88.8
	0.372	0.293	0.665	0.600	90.2
	0.372	0.731	1.103	0.888	80.5
Lactic acid	0.531	0	0.531	0.548	103.1
	0.531	0.127	0.658	0.612	93.0
	0.531	0.255	0.786	0.704	89.6
	0.531	0.637	1.168	0.903	77.3
Acetic acid	0.180	0	0.180	0.177	98.3
	0.180	0.081	0.261	0.242	92.7
	0.180	0.162	0.342	0.303	88.6
	0.180	0.406	0.586	0.459	78.3

[a] Amount in wine diluted 1:2.

[b] See results and discussion for explanation.

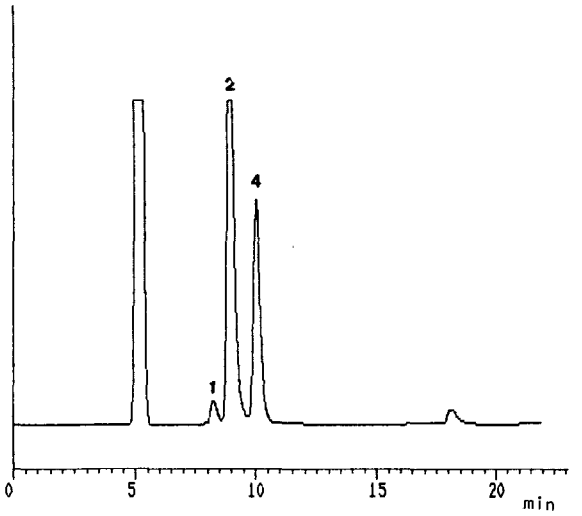

Fig. 6. Chromatogram of acidic fraction of grape must after SAX treatment. Peaks as in Fig. 1.

acids, succinic, acetic and lactic acid, have a poor recovery if their concentration is high, but this is not a problem in these wine samples and their recovery was good after SAX treatment. However, if these compounds are present in large concentrations, the determination must be carried out without SAX extraction (direct method) or dilution of the wine samples.

To determine the accuracy of the method the results obtained by the direct method (without SAX treatment), by the method with SAX treatment and by standard method [15,16] for wine and grape must were compared (Table V). Citric, tartaric and malic acids were not determined by the direct method because of the dilution necessary to quantify the sugars. A good accuracy was obtained for the two chromatographic methods, but this was less for malic acid. The high value obtained for malic acid in the direct method confirms the existence of another unknown peak with the same retention time. In this instance, the best results were obtained when the SAX treatment was used. If a grape must is analysed, only after SAX treatment is it possible to determine citric, malic and tartaric acids in the same injection and the results are similar to those obtained by the enzymatic method.

To confirm the better determination of malic acid after SAX treatment compared with the direct method, various wine and must samples were analysed and their results compared with those obtained by the enzymatic method. In the direct method the criteria of integration considered were the valley bases. The results can be seen in Table VI. This high value for malic acid in the direct method

TABLE V

RESULTS OBTAINED BY THE DIRECT METHOD WITHOUT SAX TREATMENT (A), WITH SAX TREATMENT (B) AND WITH THE STANDARD METHODS (C)

Compound	Concentration (g/l)		
	A	B	C
Wine			
Citric acid	0.15	0.15	0.15[b]
Tartaric acid	3.8	3.6	3.6[c]
Malic acid	1.15	0.83	0.80[b]
Succinic acid	0.81	0.73	–
Lactic acid	0.64	0.63	
Acetic acid	0.20	0.18	–
Glycerol	5.0	4.9	4.7[b]
Glucose	0.55	0.58	0.52[b]
Fructose	0.69	0.68	0.67[b]
Must			
Citric	ND[a]	0.28	0.24[b]
Tartaric acid	ND	4.9	4.8[c]
Malic acid	ND	3.33	3.39[b]
Glucose	133	122	129[b]
Fructose	93	86	90[b]

[a] ND = not determined by this method.
[b] Enzymatic method.
[c] Titration method.

TABLE VI

DETERMINATION OF MALIC ACID IN WINE AND MUST SAMPLES BY THE DIRECT METHOD WITHOUT SAX TREATMENT (A), WITH SAX TREATMENT (B) AND WITH THE STANDARD METHODS (C)

Sample	Concentration (g/l)		
	A	B	C
White wine (René Barbier)	1.10	0.83	0.80
White wine (Colombard-Verdú)	3.01	2.69	2.84
White wine (Chardonnay-Batea)	3.05	2.78	2.84
Red wine (Garnatxa-Riudabella)	0.73	0.55	0.53
Red wine (Garnatxa-Riudabella)	0.86	0.62	0.58
White must (Chardonnay-Verdú)	ND[a]	3.33	3.74
Red must (Ull de llebre-Barberà)	ND	3.41	3.58

[a] ND = not determined by this method.

was noted by Frayne [1]; it is a neutral compound co-eluting with the malic acid peak.

CONCLUSIONS

Ion-exchange chromatography with RI detection is a good alternative method to determine carboxylic acids, sugars, glycerol and ethanol in wine samples, but if malic acid is to be determined, SAX treatment is required to obtain better results. The compounds were separated and quantified by this method in less than 25 min.

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REFERENCES

1 R. F. Frayne, *Am. J. Enol. Vitic.*, 37 (1986) 176.
2 R. M. Marcé, M. Calull, R. M. Marcé, F. Borrull and F. X. Rius, *Chromatographia*, 29 (1990) 54.
3 D. Tusseau and C. Benoit, *J. Chromatogr.*, 395 (1987) 323.
4 E. Mentasti, M. C. Gennaro, C. Sarzanini, C. Barochi and M. Savigliano, *J. Chromatogr.*, 322 (1985) 177.
5 M. C. Polo, F. Barahona and I. Cáceres, *Connais. Vigne Vin*, 3 (1986) 175.
6 F. Caccamo, G. Carfagnini, A. Di Corcia and R. Samperi, *J. Chromatogr.*, 362 (1986) 47.
7 R. M. Marcé, M. Calull, F. Borrull and F. X. Rius, *Am. J. Enol. Vitic.*, 41 (1990) 289.
8 A. Schneider, V. Gerbi and M. Redoglia, *Am. J. Enol. Vitic.*, 38 (1987) 151.
9 J. Haginaka, J. Wakai, H. Yadua and T. Nomura, *J. Chromatogr.*, 447 (1988) 373.
10 J. P. Goiffon, A. Blanchere and C. Reminiac, *Analusis*, 13 (1985) 218.
11 P. Pfeiffer and F. Radler, *Z. Lebensm.-Unters.-Forsch*, 181 (1985) 24.
12 J. D. McCord, E. Trousdale and D. D. Y. Ryu, *Am. J. Enol. Vitic.*, 35 (1984) 28.
13 M. Calull, E. López, R. M. Marcé, J. C. Olucha and F. Borrull, *J. Chromatogr.*, 589 (1991) 151.
14 D. Woo, L. Treat-Clemons and R. M. Patel, *Application Note*, Interaction Chemicals, 1989.
15 Boehringer Mannheim, *Methods of Enzymatic Food Analysis 82/83*, Gebr. Parcus, Munich, 1982.
16 *Official Methods of Analysis*, Association of Official Analytical Chemists, Washington DC, 13th ed., 1980, pp. 153–572.
17 R. Badoud and G. Pratz, *J. Chromatogr.*, 360 (1986) 119.