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Determination of organic acids in grape musts, wines and vinegars by high-performance liquid chromatography

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ABSTRACT

A method for the determination of tartaric, malic, shikimic, lactic, acetic, citric, succinic, citramalic and fumaric acid in musts, wines and vinegars is proposed, based on high-performance liquid chromatography using two 3-μm C_{18} columns (250 × 4.6 mm I.D.) in series, providing an efficiency between 50 000 and 80 000 theoretical plates/m. The mobile phase is phosphate buffer (pH 2.35) to which is added a small amount of methanol (3%) as polar modifier. The relative standard deviations were <6% and no effect of sample preparation (filtration and passage through Sep-Pak C_{18}) on the results was observed.

INTRODUCTION

Organic acids account for a significant fraction of musts, wines and vinegars. Their origins are diverse, the most important being biosynthesis by the vine, metabolic pathways related to sugar fermentation, malolactic fermentation and ethanol oxidation (in the case of acetic acid in vinegars). Organic acids affect stability, colour [1,2] and flavour of the final product.

Each acid may be determined enzymatically or spectrophotometrically after separating it from the other components [3], but there is no official method of analysis of this type for some of them, such as shikimic acid, fumaric acid and citramalic acid.

Proposals have been made for analysis by gas chromatography (GC) in the form of esters [4–6], by direct injection (acetic acid) [7] and by oxidation of lactic acid to acetaldehyde for headspace analysis [8]. For ion chromatographic analysis, it is necessary to separate sugars and polyalcohols previously in order to prevent them from interfering with the determination of some of the acids [9,10], or to limit the analysis to acids that separate well [3,11] or to adopt double detection of multiple peaks by means of UV and refractive index methods [12–14]. However, it would be very difficult to apply this technique to the determination of organic acids in musts or sweet wines because of the high concentration of sugars present.

Reversed-phase high-performance liquid chromatographic (RP-HPLC) analysis has also been described, but owing to the complexity of the oenological substrates, pretreatment of the sample is essential. With C_8 and C_{18} columns, polyphenols and anthocyanins must be separated previously, as they elute at the end of the

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chromatogram and would interfere in subsequent analyses. Activated charcoal [15], ion-exchange resins [16], mixed procedures [17] and Sep-Pak C_{18} cartridges [3,18,19] have been recommended for this step.

In this paper, a method for the determination of tartaric, malic, shikimic, lactic, acetic, citric, succinic, citramalic and fumaric acid by RP-HPLC is described, applicable to musts, wines and vinegars, based on the passage of the initial sample through Sep-Pak C_{18} cartridges and the use of two 250 × 4.6 mm I.D. columns in series with a 3-μm C_{18} packing. The method permits the determination of all the acids mentioned in a single run because of the high efficiency achieved with this system of columns, and with no problems due to the sugars present or to the anthocyanins and polyphenols, which are eliminated in sample preparation.

EXPERIMENTAL

Reagents

All the chemicals used were of analytical-reagent grade. Tartaric, malic, lactic, acetic, citric, succinic, fumaric and phosphoric acid and diammonium hydrogenphosphate were supplied by Merck (Darmstadt, Germany), shikimic and citramalic acid by Fluka (Buchs, Switzerland) and methanol by Panreac (Barcelona, Spain). Water purified using a Milli-Q system (Millipore, Bedford, MA, USA) was used.

Sample preparation

The samples were filtered through 0.2-μm pore-size membranes (Dynagard, 0.8 cm^2; Microgon, Laguna Hills, CA, USA). They were then fractionated using Sep-Pak C_{18} Classic cartridges (Millipore) that had previously been conditioned by means of succesive washes with 3 ml of methanol and 10 ml of water, drying the cartridge with air after each wash. A 0.5-ml volume of the filtered sample was passed through the cartridge and the retained acids were eluted with aliquots of phosphoric acid (5%) up to a final volume of 2 ml; in this way the sample was diluted fourfold.

Chromatographic method

The equipment consisted of an SEC-4 solvent chamber (Perkin-Elmer, Norwalk, CT, USA), a Series 10 pump (Perkin-Elmer), a Model 7125 injection valve (Rheodyne, Cotati, CA, USA), an LC-90 variable-wavelength UV–Vis detector (Perkin-Elmer), a 450-MT2 data processing system (Kontron Instruments, Milan, Italy) and a MicropH 2001 pH meter (Crison Instruments, Barcelona, Spain).

Samples were injected through a 6-μl loop into a system consisting of two 250 × 4.6 mm I.D. columns in series (3-μm ODS-2; Symta, Madrid, Spain) and a 20 × 2 mm I.D. guard column (Pellicular C_{18}; Alltech, Deerfield, IL, USA). The mobile phase was 0.02 *M* diammonium hydrogenphosphate–methanol (97:3) (adjusted to pH 2.35 with phosphoric acid) at a flow-rate of 0.5 ml/min at room temperature and a working pressure of 3200–3500 p.s.i. (1 p.s.i. = 6894.76 Pa). Detection was by means of measurement of UV absorption at 210 nm.

Data treatment

Data were processed by means of the BMDP statistical package [20], using linear regression analysis (BMDP1R program) on a VAX 9200 computer.

RESULTS AND DISCUSSION

Preparation of samples

The volume of the initial sample and the volume of the elute collected from the Sep-Pak cartridges were calculated so as to elute the maximum amount of acids with minimum dilution of the original sample. Under these conditions, polyphenols and anthocyanins are retained in the cartridge.

Chromatographic separation

The variation of the capacity factor (k') of the different acids as a function of the mobile phase pH is shown in Fig. 1. It was considered desirable to give priority to the separation of tartaric acid, as this is the most significant acid among those deriving from grapes and because there is no enzymatic method for its measurement. At

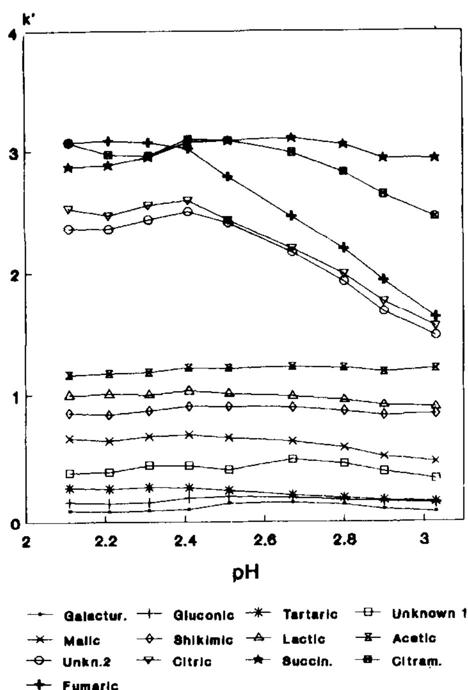

Fig. 1. Plots of capacity factors of organic acids *versus* eluent pH.

pH <2.4, tartaric acid separates well from gluconic and galacturonic acid and most other polar compounds of the sample, but it is not advisable to use much lower pH values so as not to shorten the life of the columns. Two unidentified compounds, unknown 1 and unknown 2, have been included in Fig. 1 in order to separate them and prevent them from being superimposed on the peaks of some of the remaining acids. Separation is good in all instances, with the exception of succinic, citramalic and fumaric acid, which elute very close together, although this can be improved by adding a polar modifier (3% methanol) to the mobile phase. As this is a difficult separation it is extremely important to adjust the mobile phase pH exactly.

The use of two columns in series (50 cm total length) with a packing of small particles (3 μm) ensures that the flow-rate does not exceed 0.5 ml/min so as not to subject the system to excessive pressure. Under these conditions, the efficiency achieved ranges from 50 000 to 80 000 theoretical plates/m for lactic and citric acid, respectively. In the literature efficiencies of 40 000 theoretical plates/m have been reported for succinic acid [15] and 17 000 theoretical plates/m for lactic acid [16]. Hence the use of the system of columns described makes it possible to double the reported efficiency and provides the necessary resolution to be able to determine all the acids of interest in a single run, with no interferences with each other or with other compounds in the sample. This makes it possible to achieve greater accuracy in quantitative analysis.

Fig. 2 shows the chromatogram of a standard mixture of all the acids in aqueous solution at concentrations close to those in which they are usually found in wines, and Figs. 3, 4 and 5 show the chromatograms obtained under the conditions described for a grape must, a wine and a wine vinegar, respectively.

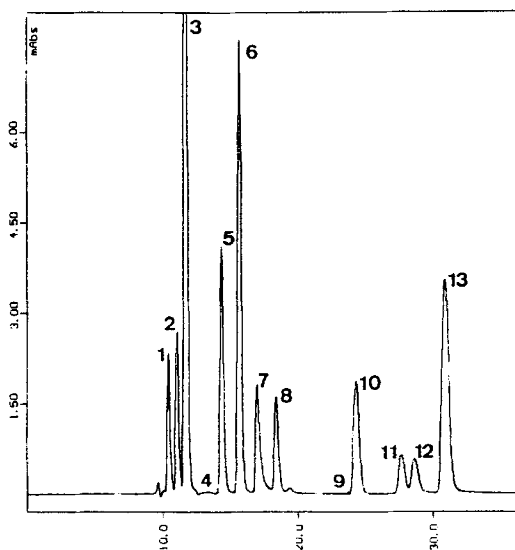

Fig. 2. Chromatogram of a synthetic solution of organic acids. Isocratic elution using two 3-μm ODS-2 columns (250 × 4.6 mm I.D.). Mobile phase, 0.02 *M* diammonium hydrogenphosphate–phosphoric acid (adjusted to pH 2.35) containing 3% of methanol as polar modifier; flow-rate, 0.5 ml/min. Peaks of acids: 1 = galacturonic (1.620 g/l); 2 = gluconic (0.801 g/l); 3 = tartaric (2.029 g/l); 4 = unknown 1; 5 = malic (1.002 g/l); 6 = shikimic (31.6 mg/l); 7 = lactic (1.016 g/l); 8 = acetic (0.710 g/l); 9 = unknown 2; 10 = citric (0.545 g/l); 11 = succinic (0.420 g/l); 12 = citramalic (0.437 g/l); 13 = fumaric (11.3 mg/l).

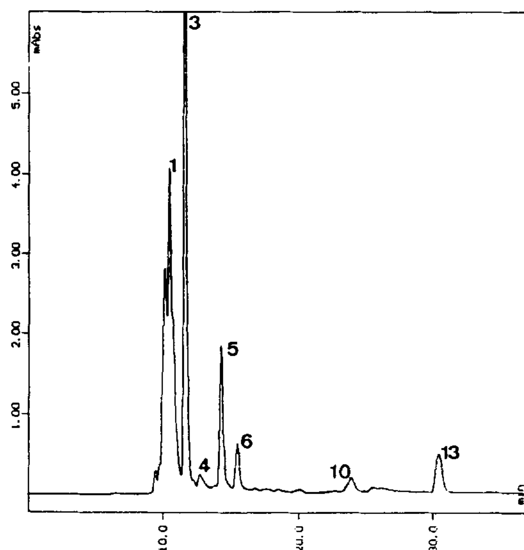

Fig. 3. Chromatogram of an Airén grape must. Chromatographic conditions and peak identification as in Fig. 2.

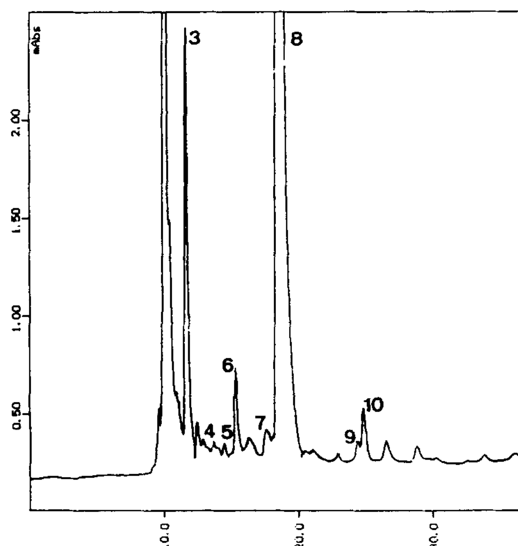

Fig. 5. Chromatogram of a commercial table wine vinegar. Chromatographic conditions and peak identification as in Fig. 2.

Quantitative analysis

In order to ascertain the precision of the method for quantitative analysis, all the acids in different concentrations were added to the same wine, each of the samples being injected in triplicate. The results are given in Table I. The relative standard deviations (R.S.D.s) are lower than 6% for all determinations within a relatively wide range of concentrations and the recoveries are satisfactory. These results suggest that, in order to achieve good quantitative accuracy, it is sufficient to inject a standard solution at the beginning and end of the working day.

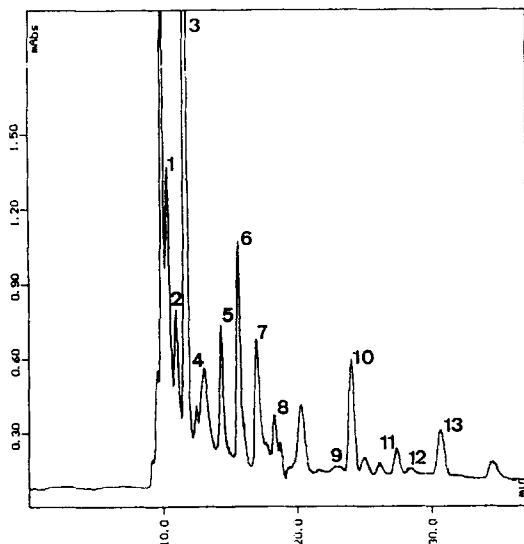

Fig. 4. Chromatogram of a red wine submitted to malolactic fermentation. Chromatographic conditions and peak identification as in Fig. 2.

Five different sample preparations of the same wine were also made up and analysed in triplicate on five different days. The R.S.D.s obtained for each acid on the same day (due to the chromatographic method) are *ca.* 3% and always lower than 6%, and those due to sample preparation (obtained on different days) are between 0.7% for shikimic acid and 5.7% for lactic acid, with a mean of 3.2%. These results, corresponding to fifteen determinations of each acid, demonstrate that there are no important differences due to the chromatographic method or to sample preparation.

Determination of organic acids in different substrates

The proposed method enabled data to be collected on the content of fixed acids in grape musts and wines from recent harvests in the Castilla-La Mancha (Spain) area and in commercial vinegars (Table II).

TABLE I

ANALYSIS OF THE SAME WINE BEFORE AND AFTER ADDITION OF ACIDS IN INCREASING AMOUNTS

Acid	Concentration added (g/l)	Peak area (mV min) (mean ± S.D., $n = 3$)[b]	Regression line		Initial acid concentration in the wine (g/l)	Recovery (%)
			Slope	r^2		
Tartaric	0.000	52.70 ± 2.52 (4.8)	16.646	0.997	3.192	–
	0.253	58.28 ± 0.70 (1.2)				96.1
	0.505	60.99 ± 0.56 (0.9)				95.2
	1.214	73.36 ± 0.14 (0.2)				97.2
Malic	0.000	22.43 ± 0.12 (0.5)	10.241	0.970	2.212	–
	0.249	26.00 ± 0.11 (0.4)				100.4
	0.564	27.55 ± 0.12 (0.4)				99.3
	0.972	32.91 ± 0.18 (0.6)				93.8
Shikimic	0.00[a]	22.44 ± 0.08 (0.4)	0.755	0.968	30.72[a]	–
	5.00[a]	28.45 ± 0.38 (1.3)				106.3
	11.00[a]	30.68 ± 0.76 (2.5)				94.0
	18.00[a]	36.87 ± 0.59 (1.6)				101.4
Lactic	0.000	3.72 ± 0.20 (5.4)	6.024	0.994	0.640	–
	0.259	5.68 ± 0.18 (3.2)				96.6
	0.477	6.60 ± 0.21 (3.2)				96.9
	0.924	9.41 ± 0.02 (0.3)				103.2
Acetic	0.000	2.82 ± 0.14 (5.0)	7.896	0.988	0.393	–
	0.212	5.08 ± 0.11 (2.2)				103.7
	0.389	6.32 ± 0.00 (0.0)				98.6
	0.750	8.87 ± 0.28 (3.2)				98.4
Citric	0.000	13.85 ± 0.17 (1.2)	14.424	0.984	0.993	–
	0.183	17.85 ± 0.26 (1.4)				98.4
	0.368	19.24 ± 0.04 (0.2)				98.2
	0.786	25.64 ± 0.34 (1.3)				98.5
Succinic	0.000	4.13 ± 0.09 (2.2)	7.446	0.997	0.574	–
	0.214	5.97 ± 0.14 (2.4)				96.4
	0.395	7.35 ± 0.23 (3.1)				95.0
	0.803	10.16 ± 0.38 (3.8)				95.7
Citramalic	0.000	4.86 ± 0.14 (2.9)	11.239	0.995	0.462	–
	0.170	7.00 ± 0.28 (4.0)				93.0
	0.241	7.95 ± 0.09 (1.2)				99.1
	0.531	10.87 ± 0.20 (1.9)				87.5
Fumaric	0.00[a]	7.41 ± 0.22 (3.0)	1.500	0.988	5.23[a]	–
	2.76[a]	11.96 ± 0.25 (2.1)				94.2
	6.26[a]	18.31 ± 0.28 (1.5)				94.7
	10.79[a]	23.41 ± 0.06 (0.3)				93.4

[a] Concentrations in mg/l.
[b] R.S.D. (%) in parentheses.

CONCLUSIONS

The HPLC method proposed for the determination of organic acids in oenological substrates consists of the use of two 3-μm C_{18} columns in series, which provide an efficiency of between 50 000 and 80 000 theoretical plates/m. The mobile phase is phosphate buffer (pH 2.35) to

TABLE II

RANGES OF CONCENTRATION OF ORGANIC ACIDS IN DIFFERENT OENOLOGICAL SUBSTRATES

Acid[a]		Grape must		Wine		Wine vinegar ($n = 24$)
		White ($n = 11$)	Red ($n = 12$)	White ($n = 22$)	Red ($n = 47$)	
Tartaric	Max.	6.47	7.65	3.94	5.74	4.01
	Min.	3.94	4.07	1.77	2.60	0.13
Malic	Max.	2.04	2.91	2.64	3.13	0.93
	Min.	1.34	1.99	0.11	0.06	0.10
Shikimic[b]	Max.	18.29	22.25	30.90	40.59	38.07
	Min.	12.39	5.09	15.94	16.12	2.01
Lactic	Max.	–	–	2.98	4.89	1.80
	Min.	–	–	0.13	0.07	0.10
Acetic	Max.	–	–	1.50	1.44	44.46
	Min.	–	–	0.20	0.30	26.28
Citric	Max.	0.30	0.35	0.54	0.40	2.84
	Min.	0.20	0.25	0.21	0.17	0.06
Succinic	Max.	–	–	1.19	1.22	0.58
	Min.	–	–	0.27	0.48	0.03
Citramalic	Max.	–	–	0.31	0.34	–
	Min.	–	–	0.09	0.17	–
Fumaric[b]	Max.	7.49	10.69	6.22	12.56	2.46
	Min.	6.00	5.11	1.45	1.30	0.23

[a] Concentrations in g/l except where indicated.
[b] Concentrations in mg/l.

which is added a small amount of methanol (3%) as polar modifier.

The efficiency achieved with this system is much superior to those described previously and, as interferences are avoided, permits greater accuracy of the results. The R.S.D.s are <6% and no differences are observed in the results depending on sample preparation (filtration and passage through Sep-Pak C_{18}).

ACKNOWLEDGEMENT

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