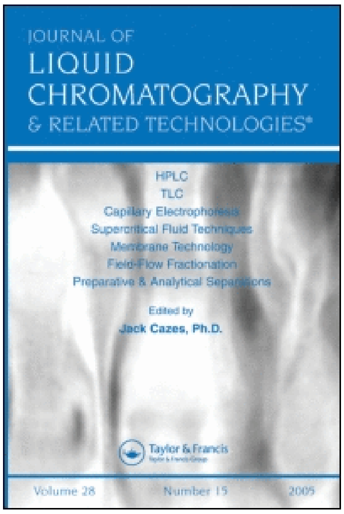

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SIMULTANEOUS DETERMINATION OF ORGANIC ACIDS, SUGARS, AND ALCOHOLS IN MUSTS AND WINES BY AN IMPROVED ION-EXCLUSION HPLC METHOD

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ABSTRACT

An HPLC method for the simultaneous quantification of the main carboxylic acids (including citric, tartaric, malic, shikimic, succinic, lactic, fumaric, and acetic acids), sugars (glucose and fructose), and alcohols (glycerol and ethanol) of musts and wine is proposed. Using a cation exchange column, improved separation of coeluting compounds (e.g., fructose vs. malic acid and succinic vs. shikimic acid) was achieved by moderate addition of tetarhydrofuran (THF) (4%) and *n*-propyl alcohol (*n*-PA) (5%) as organic modifier in the mobile phase. A factorial experiment was carried out for optimize acidity and temperature of elution.

The method showed good linearity and precision and, as an application, was successfully applied to a must and a wine.

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INTRODUCTION

In enology, determination of macro compounds, such as carboxylic acids, sugars, and alcohols, is required for the quality evaluation and characterization of musts and wines.

For simultaneous separation of several above mentioned compounds, methods based on ion-exclusion liquid chromatography performed with hydrogen sulfonated polystyrene (PS)–divynylbenzene (DVB) polymeric columns, has been proposed.[1,2] In this technique, because of the selective column exclusion of ionized species, the elution order of the different acids is substantially related to their pKa, the stronger being eluted before the weaker.[3] Furthermore, for neutral or non polar analytes, hydrophobic interactions with stationary phase can occur,[4] thus driving to a modification of predictable elution times and higher band broadening and tailing.

This fact is reflected by the poor resolution between compounds like malic acid and fructose[5] or lactic and fumaric acids.[5] Moreover, succinic and shikimic acids are totally unresolved.[1,2,5]

Addition of organic modifiers (typically acetonitrile) in the mobile phase only partially overcame these problems. Recently, shikimic acid levels in wines, has been proposed as a useful parameter for testing on the cultivar authenticity of grapes.[6]

Tetrahydrofuran (THF) is an organic modifier, whose ability in reducing peak tailing and enhancing performances of PS–DVB based columns, is recognized for reversed phase chromatography.[7,8,9] Due to its swelling properties, THF can improve sample peak shape because analyte diffusion is less hindered in the swollen polymer.[7,8,10] An alternative mechanism was also proposed, which includes the blocking of polymer micropores by THF, resulting in the reduction of the most hindered diffusion phenomena.[8]

The aim of the present work was to improve the selectivity of a PS–DVB resin based column for the separation of organic acids and sugars, naturally occurring in musts and wines, by using THF as an organic modifier. Tetrahydrofuran was initially tested for its ability in promoting succinic and shikimic separation. In a successive step, conditions for the analysis of carboxylic acids, sugars, glycerol, and ethanol were optimized.

EXPERIMENTAL

Standard Solutions and Samples

Stock solutions of organic acids, sugars, and alcohols (Sigma chemicals) were prepared in redistilled water at concentration ranges commonly found in musts and wines (Table 1).

Table 1. Chromatographic Parameters, Concentration Ranges, Detection System, and Method Validation for Standard Organic Acids, Sugars, and Alcohols

No.	Compound	Rt (min)	Detection System	Retention Factor	Range (mg/L)	Intercept	Slope	r^2 ($n = 5$)	LOD (mg/L)	Repeatability RSD % ($n = 5$)	Reproducibility RSD % ($n = 5$)
1	Citric acid	8.95	UV	0.26	500–10	1,326	2,298	0.999	0.32	1.46	4.74
2	Tartaric acid	10.05	UV	0.42	3,500–100	12,919	3,835	0.999	0.20	1.43	2.03
3	Glucose[a]	10.47	RI	0.48	25–0.5	35,681	996,766	0.997	0.02	2.52	3.07
4	Malic acid	10.98	UV	0.55	2,000–50	−1,973	2,109	0.999	0.40	0.40	1.38
5	Fructose[a]	11.53	RI	0.63	25–0.5	14,462	982,102	0.998	0.03	2.13	2.71
6	Shikimic acid	13.07	UV	0.84	60–1	8,296	120,507	0.989	0.08	1.06	1.36
7	Succinic acid	13.57	UV	0.91	500–10	6,219	1,099	0.995	0.47	0.47	3.61
8	Lactic acid	14.55	UV	1.05	4,000–10	7,040	1,513	0.995	0.80	1.54	3.01
9	Glycerol[a]	15.39	RI	1.17	10–0.3	31,063	768,023	0.988	0.003	0.98	1.43
10	Fumaric acid	15.42	UV	1.17	10–0.2	3,401	1,160,768	0.999	0.01	0.22	1.09
11	Acetic acid	17.67	UV	1.49	500–10	−1,280	1,278	0.991	1.02	2.67	3.25
12	Ethanol[a]	23.68	RI	2.34	80–2	−1,942	45,645	0.999	0.15	2.85	3.62

[a] Expressed in g/L.

A white grape must (cv Trebbiano), a white wine (cv Chardonnay), and a red wine (cv Sangiovese) furnished by CATEV (Tebano, Italy), were also tested. Actual samples were diluted (up to five times for white must and wine, 10 times for red wine) and filtered (0.22 μm) before direct injection.

HPLC

A Jasco high performance liquid chromatography (HPLC) system (Jasco Inc., Tokyo, Japan) was used, equipped with a pump (PU 980), a variable wavelenght detector (UV 970) set at 215 nm, and connected in series with a refractive index detector (RI 830), a 20 μL loop (Rheodyne Inc., Cotati, CA).

The column was a Aminex HPX 87H (300 × 7.8 mm) (Bio-Rad Laboratories, Hercules, CA), thermostated at 30°C.

The conditions tested were: 0.005–0.05 N H_3PO_4, with 0–10% THF and 0–5% *n*-propyl alcohol (*n*-PA). The flow rate was 0.45 mL/min. Chromatograms acquisition and processing were carried out with Borwin 5.0 software (JMBS Developments, Grenoble, France).

Identification, Calibration, and Calculation

Peaks in actual samples were identified on the basis of retention times and spiking technique, while quantification was performed through an external standard calibration curve.

Linearity was obtained with peak areas of five solutions derived by sequentially diluting a concentrated standard solution. The concentration ranges of these solutions are shown in Table 1.

Repeatability of the method was determined by calculating the RSD % of the analyte concentration of five repeated HPLC runs of a standard solution containing each compound at the level commonly found in wines.

Reproducibility was evaluated by injecting, in triplicate, standard solutions over a period of 15 working days. Standard samples were quantified on five days randomly chosen in this working period.

The same calibration runs were used for detection limits, the latter being the concentration showing a signal at thrice the noise.

RESULTS AND DISCUSSION

With the goal to test the ability of THF to promote the separation through succinic and shikimic acid, increasing percentages of this organic modifier were added to the mobile phase.

In Table 2 are reported both the retention and separation factors, as affected by these changes.

As mentioned, with 0.01 N phosphoric acid as eluent, the two acids were unresolved and proved to be unaffected by any change in acidic strength. Also, the addiction of 5% of THF had no influence on separation factor, while the contemporary presence of *n*-PA led to separate succinic and shikimic acid. Addition of alcohols in the mobile phase was early reported to enhance peak shape of aliphatic carboxylic acids, by increasing the hydrophilicity of the PS–DVB surface.[11]

Table 2 show that percentages up to 4% of THF in 5% *n*-PA, gradually augmented the separation factor. For higher contents of organic modifier, the two acids were less resolved.

According to Ells et al.[8] the highest increase of analyte diffusion is associated with low amounts of THF (e.g. 2%), while limited additional effects are related to changing to 10% THF.

Moreover, the swelling properties of THF could drive to unacceptable backpressures (as we experienced with THF percentages higher than 5%) and resin collapse.

Due to these considerations, for the successive method optimization, the eluent contained 5% *n*-PA and 4% THF.

Eluent acidic strength and operative temperature are the main parameters affecting ion-exclusion chromatography. A factorial experiment was carried out

Table 2. Retention and Separation Factors of Shikimic and Succinic Acids as Affected by Changes in THF and *n*-PA Percentages in the Mobile Phase (Chromatographic Conditions: Column Aminex HPX 87H at 30°C, Flow 0.45 mL/min, Eluent 0.01 N H_3PO_4, Detection @215 nm)

Organic Modifier		Retention Factor		
THF (%)	*n*-PA (%)	Shikimic Acid	Succinic Acid	Separation Factor
0	0	1.14	1.12	0.99
5	0	1.02	1.05	1.03
0	5	1.10	1.12	1.03
1	5	1.06	1.11	1.05
2	5	0.95	1.00	1.05
3	5	0.92	0.98	1.07
4	5	0.87	0.94	1.09
6	5	0.92	0.99	1.07
10	5	0.90	0.96	1.07

with two factors at four levels (0.001; 0.005; 0.01; 0.5 N H_3PO_4 for eluent acidity and 22; 30; 40; 50°C for column temperature).

From the 16 runs we carried out, some general considerations can be made: (i) the lesser the eluent acidity, the lesser is the exploitability of pKa differences through acids, then a higher acidity tends to lead to better separations; (ii) column temperature greatly affects fumaric acid and fructose retention times, the

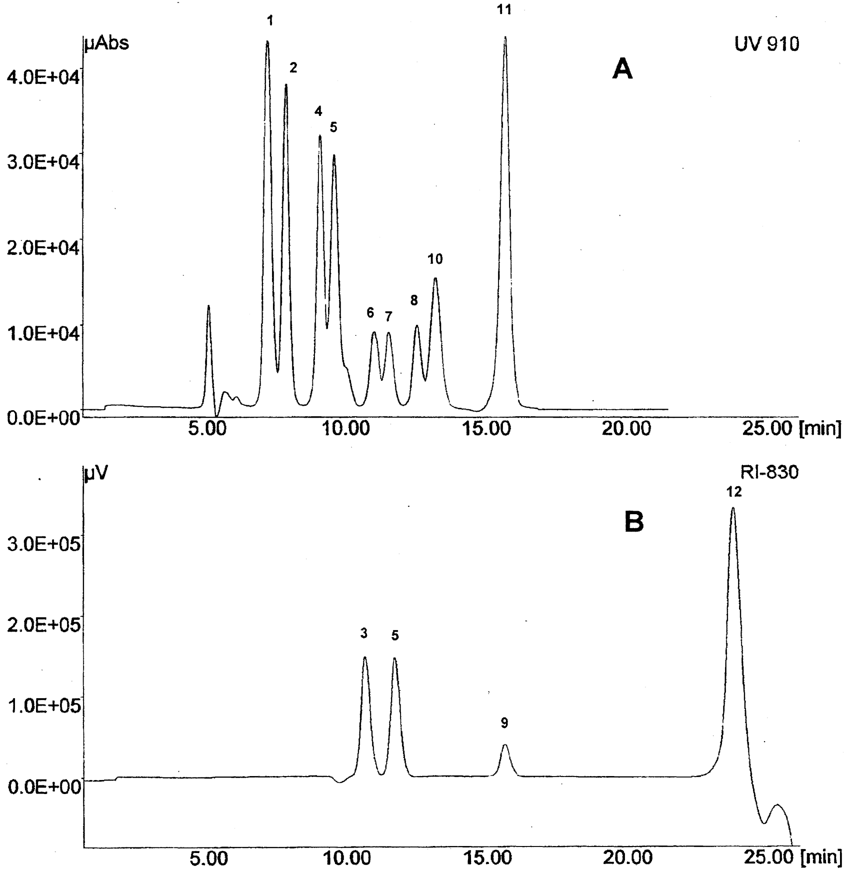

Figure 1. Chromatograms of standard mixtures. A: UV detection of (1) citric acid, (2) tartaric acid, (4) malic acid, (5) fructose, (6) shikimic acid, (7) succinic acid, (8) lactic acid, (10) fumaric acid, (11) acetic acid. B: RI detection of (3) glucose, (5) fructose, (9) glycerol, (12) ethanol. Chromatographic conditions: eluent 0.01 N $H_3PO_4 + 4\%$ THF + 5% *n*-PA; flow 0.45 mL/min; column temperature 30°C; UV detection @215 nm.

coelution of which with, respectively, lactic and malic acids can be adequately resolved; (iii) in our conditions, separation between succinic and shikimic acids are unaffected by any change in acidity or column temperature.

The best conditions were obtained with 5% *n*-PA and 4% THF in 0.01 N H_3PO_4 at 30°C (Fig. 1).

In the concentration ranges we tested, this method showed a good linearity and suitable limits of detections (Table 1). Method precision, evaluated through intraday (repeatability) and interday (reproducibility) essays was good (Table 1).

To test the method applicability to actual samples, a grape white must and wine and a red wine were analyzed. Samples were diluted according with sugars and polyphenols concentrations (five times for white must and wine, 10 times for red wine), filtered at 0.2 µm, and directly injected without any further

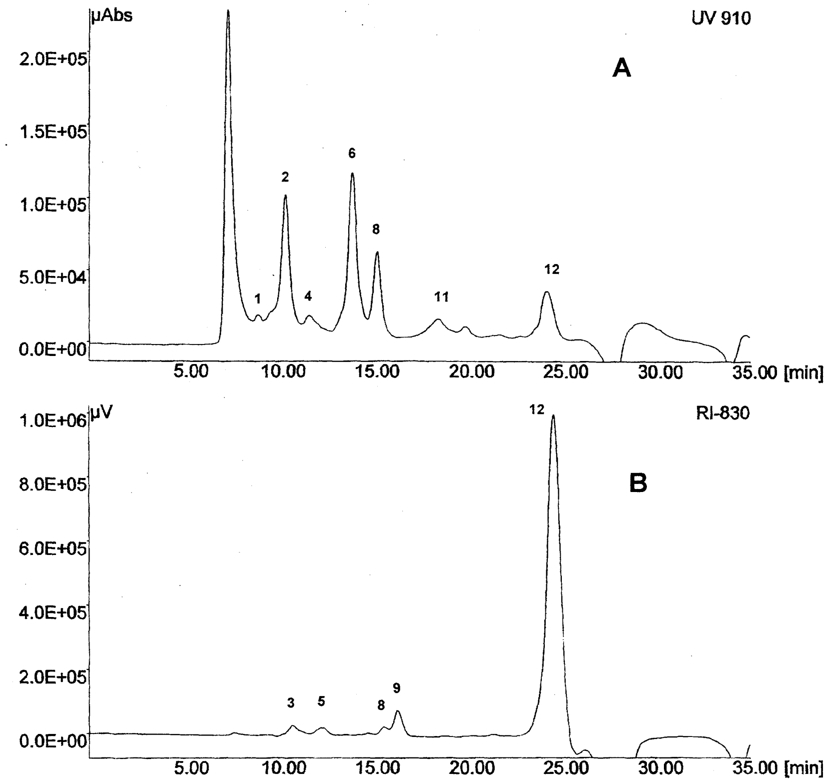

Figure 2. Chromatograms of white wine (cv Chardonnay). For peak identification and chromatographic condition see Table 1 and Fig. 1.

pre-treatment. In Figs. 2 and 3 are shown the chromatograms obtained for the white wine and must.

Because of the satisfactory resolution between malic acid and fructose and the lack of UV absorption of glucose, the method proved to be suitable for simultaneous determination of acids and sugars, even with higher percentages of the latter (Fig. 3).

In wines, some unidentified compounds (probably phenols) were detected after 15 min of the run (Fig. 2). Their presence in high percentages could interfere with acetic acid determination, and may be partially overcome by an adequate sample dilution.

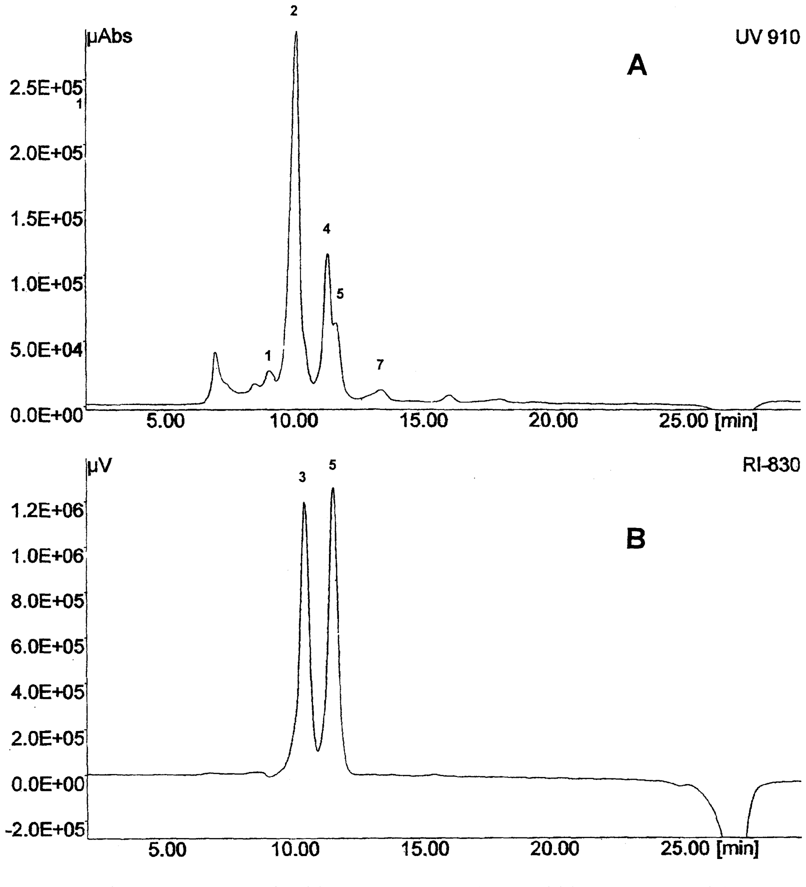

Figure 3. Chromatograms of white grape must (cv Trebbiano romagnolo). For peak identification and chromatographic condition see Table 1 and Fig. 1.

CONCLUSIONS

The simultaneous separation of the main acids, sugars, and alcohols of musts and wines, was obtained by an improved HPLC method with dual detection. Addition of 4% THF and 5% of *n*-PA to the mobile phase improved the separation of shikimic and succinic acids. To our knowledge, it is the first time that these acids are resolved by ion-exclusion chromatography. Optimized conditions for the quantification of the other principal compounds were obtained by varying eluent acidity and column temperature. Musts and wines were successfully analyzed through direct injection of diluted samples.

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