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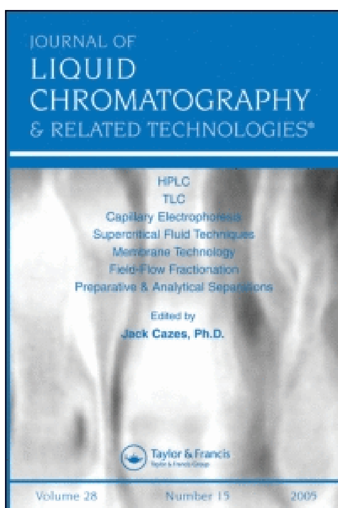

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AN IMPROVED HPLC METHOD FOR THE ANALYSIS OF ORGANIC ACIDS, CARBOHYDRATES, AND ALCOHOLS IN GRAPE MUSTS AND WINES

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ABSTRACT

An improved high performance liquid chromatography (HPLC) method for the analysis of the main organic compounds in musts and wines is presented. A column packed with hydrogen sulfonated divinyl benzene-styrene copolymer and two detectors connected in series were used (UV at 210 nm and RI, respectively). The addition of acetonitrile (6%) to the mobile phase (0.045*N* H_2SO_4) allowed the simultaneous separation and quantification of several organic acids, glucose, fructose, glycerol, and ethanol. Direct injection and sample clean-up with a SAX cartridge was tested and compared. The SAX fractionation gave satisfactory results, however the direct injection of diluted wine (1:20) provided the best precision (CV ≤ 2.1%) and accuracy of analysis. Statistical analysis (paired t-test) disclosed significant differences only for glucose, fructose, α-ketoglutaric, and pyruvic acid. The role of column temperature for the analysis of vitamin C (ascorbic acid) was also investigated. Vitamin C underwent thermal degradation during analysis with column temperature ≥ 30°C.

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INTRODUCTION

The determination of organic acids and alcohols is important for many disciplines, including food science,[1] biotechnology,[2] biochemistry,[3] and biomedicine.[4] The microbial metabolism of carbohydrate results in the production of these compounds. In particular, in the wine industry the analysis of sugars, organic acids, glycerol, and ethanol is commonly required for the quality evaluation and characterisation of grapes, musts and wines.[5, 6]

A HPLC method using column packed with hydrogen sulfonated divinyl benzene-styrene copolymer has been proposed for the simultaneous determination of carbohydrates, organic acids, glycerol, and ethanol in musts and wines.[7-12] Despite the fact that this HPLC method provided results comparable to those obtained with enzymatic or colorimetric assays,[9, 10] a poor resolution between several compounds was often observed. For example, tartaric and malic acid partially coeluted with fructose,[8, 9] while succinic and shikimic acids showed the same retention time.[10, 13] Also, lactic and fumaric acid were only partially resolved and the use of dual columns and/or UV–RI detectors connected in series only partially overcame these difficulties.[9, 13] Therefore, a preparative sample clean-up using SPE techniques (C_{18}, NH_2 or SAX cartridges) or ion exchange column has also been investigated.[8, 13, 15, 16] Recently, the use of acetonitrile as organic modifier for the analysis of sugars, furanic compounds, and ascorbic acid has been proposed for the study of the Maillard reactions.[1]

The aim of this work was to improve the HPLC analysis of the major organic compounds found in grapes, musts, and wines, including pyruvic and α-ketoglutaric acids. Two sample preparation procedures, direct injection and fractionation with SAX cartridges, respectively, were compared and validated in terms of precision and accuracy. The effect of the temperature of analysis on the HPLC determination of ascorbic acid was also investigated.

EXPERIMENTAL

Standard Solutions

Carbohydrates and organic acids commonly found in must and wine were used for this study (Table 1). Standards were purchased from commercial source (Sigma, Milano, Italy) and analysed in the concentration range typical of musts and wines (Table 1). The calibration curves, relating the absorbance to the concentration of the standard, were fitted by least square linear regression analysis using Statistica 5.0 software (StatSoft™, Tulsa, OK).

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Table 1

Retention Times (Rt), Detection System, Response Factor, Calibration Curve and Limit of Detection (LOD)

No	Compound	Rt (min)	Detection System	Response Factor[a]	Calibration Curve Range	Intercept	Slope	r^2	SE	LOD
1	Citric acid (mg/L)	10.8	UV	0.10	5-250	5000	2195	0.999	526	1
-	Ascorbic acid	11.5	RI	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	Tartaric acid (mg/L)	11.8	UV	1.25	50-2500	59264	28983	0.998	9872	10
3	α-Chetoglutaric acid (mg/L)	12.3	UV	0.05	5-100	6767	9375	0.998	1025	1
4	Glucose (g/L)	12.5	RI	n.d.	0.5-20	-62877	1025305	0.999	112622	0.01
5	Malic acid (mg/L)	12.8	UV	1.00	40-2000	-56829	1624	0.995	687	12
6	Fructose (g/L)	13.4	RI	n.d.	0.5-20	-60473	1014949	0.999	102785	0.03
7	Pyruvic acid (mg/L)	14.4	UV	0.05	5-200	21987	16753	0.999	7294	1
8	Succinic acid (mg/L)	15.1	UV	0.50	20-1000	-1459	1291	0.995	2940	5
-	Shikimic acid	15.1	UV	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9	Fumaric acid	15.6	UV	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	Lactic acid (mg/L)	17.1	UV	1.00	10-2000	21751	1296	0.999	2024	3
11	Glycerol (mg/L)	18.8	RI	n.d.	60-1500	5677	775	0.999	1554	6
12	Acetic acid (mg/L)	20.1	UV	n.d.	10-300	3527	1152	0.998	4253	5
13	Ethanol (%, v/v)	30.0	RI	n.d.	2-20	29381	227493	0.999	49834	0.5

[a] Response factor = $1/[(conc._{ref}/area_{ref}) \times (area._x/conc._x)]$. n.d. = Not determined.

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Samples

Two white musts (cv. Trebbiano and Albana), a white (cv. Trebbiano) and a red (cv. Sangiovese) wine, respectively, were used. Samples were provided by CATEV (Centro Assistenza Tecnica Enologica e Viticola, Tebano, Italy) and stored at +4°C before analysis.

Sample Preparation

Two procedures were used: direct injection and sample clean-up with Bond Elute® SAX (strong anion exchange) cartridges (Varian, Harbor City, CA), respectively. Samples were diluted 1:20 with the mobile phase and filtered through a 0.22 μm cellulose-acetate membrane (Sigma F-0139, Sigma) before direct injection in HPLC. As alternative, the sample pre-treatment with SAX cartridges was tested. This procedure allowed the preliminary separation of the neutral from the acidic compounds. The cartridge (3 cc/ 500 mg) was conditioned with methanol (4 mL) and Milli-Q water (4 mL). Then the sample (0.5 mL), diluted 1:2 and adjusted to pH 9.0 with 1*N* NaOH, was passed through the cartridge. Neutral compounds (i.e., carbohydrates and alcohols) were recovered washing with Milli-Q water (1.5 mL), while acidic compounds were eluted with 1*N* HCl (2.5 mL). Each fraction was diluted with the mobile phase to a fixed final volume (5 mL), then filtered through a 0.22 μm cellulose-acetate membrane and injected in HPLC.

HPLC

The equipment used was a Jasco HPLC system (Jasco Inc, Tokyo, Japan) equipped with a pump (PU980), a variable wavelength detector (UV970), set at 210 nm, and connected in series with a refractive index detector (RI830). Samples were injected with a 20 μL loop using a 7125 valve (Rheodyne Inc, Cotati, CA). The separation was performed with an Aminex HPX-87H column (300×7.8 mm) protected with a pre-column (30×4.6 mm) filled with the same stationary phase (Bio-Rad Laboratories, Hercules, CA). Column and pre-column were thermostated at 45°C by a heater (Jones Chromatography, Mid Glamorgan, UK). The conditions tested were as follows: flow 0.5 mL/min, eluent 0.003–0.05*N* H_2SO_4 with 6% acetonitrile (v/v). Data acquisition and peak processing were performed with a Borwin 5.0 software (JMBS Developments, Grenoble, France).

Peak identification was based on retention times (Rt) and spiking technique, while peak quantification was based on the external standard method.

Method Validation

Precision (CV%) was calculated by five repeated analysis of a standard solution containing each analyte at the level commonly found in wines. The solution was tested using the direct injection and the sample clean-up technique, respectively. Accuracy (recovery %) was evaluated in triplicate using the white wine spiked with a known amount of each compound. Moreover, two white musts, a white and a red sweet wine were analysed in triplicate; each sample was subjected to both the pre-treatment procedures and quantitative results compared.

Statistical Analysis

The difference between results obtained by direct injection and sample clean-up with SAX cartridge was calculated. The Student's t-test for dependent samples was used to find the significant differences between methods at 5% level (Statistica 5.0, StatSoft™, Tulsa, OK).

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RESULTS AND DISCUSSION

Method selectivity can be accomplished by means of selective detection and/or more efficient separation system. To improve the selectivity glucose, fructose, glycerol, and ethanol were monitored with RI detector, while the organic acids were detected at 210 nm. Different elution condition were tested. The best separation was obtained at 45°C using 0.045*N* sulphuric acid containing 6% acetonitrile, with a flow rate of 0.5 mL/min. The low concentration of acetonitrile was chosen to affect the peak mobility and to resolve the peak coelution.

Figure 1 shows a typical chromatogram of a standard solution. The retention times and peak identification are reported in Table 1. Acetonitrile was detected with RI and gave a negative peak (22.5 min) which did not interfere with any compounds of interest. The separation of succinic from shikimic acid was lacking (Table 1). However, a successful separation of fumaric from lactic acid, and α-ketoglutaric from pyruvic acid, was achieved, respectively. It is important to note that the sulfuric acid concentration of the eluent greatly affects the retention time of α-ketoglutaric and pyruvic acids, respectively (data not shown). In particular, with a sulfuric acid concentration <0.045N the α-ketoglutaric and tartaric acids coeluted. This may cause a great overestimation of tartaric acid concentration due to the low response factor at 210 nm of α-ketoglutaric acid.

The linearity of response, expressed in terms of coefficient of determination (r^2), showed values of $r^2 \geq 0.995$, implying an excellent suitability of the

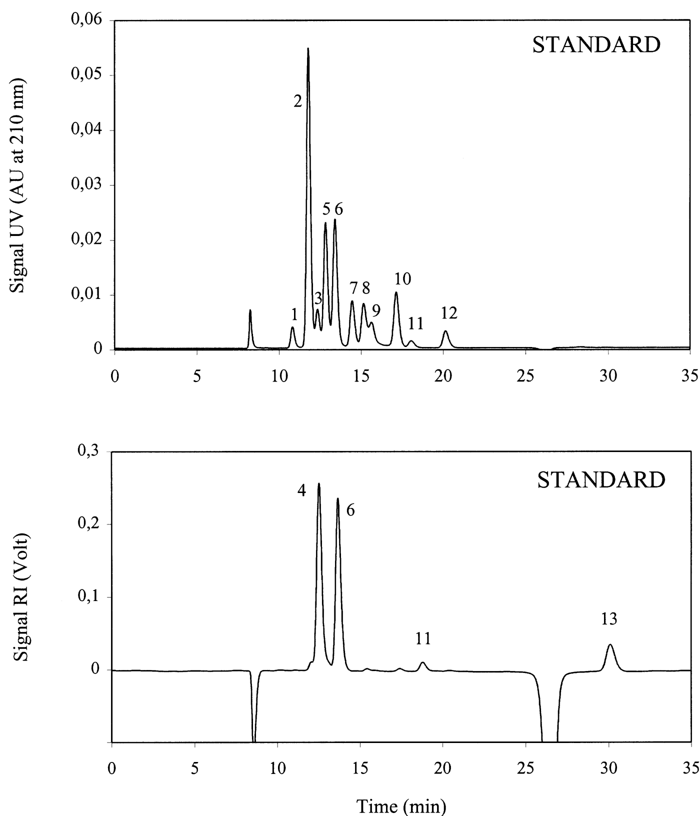

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Figure 1. Chromatograms of a standard mixture. Legend: UV detection (top), and refractive index detection (bottom). Peaks: (1) citric acid; (2) tartaric acid; (3) α-chetoglutaric acid; (4) glucose; (5) malic acid; (6) fructose; (7) pyruvic acid; (8) succinic acid; (9) fumaric acid; (10) lactic acid; (11) glycerol; (12) acetic acid; (13) ethanol. Chromatographic conditions as in materials and methods.

HPLC system (Table 1). According to Frayne,[9] the linearity of ethanol and glucose deteriorated above the concentration of 18% (v/v) and 37.5 g/L, respectively. The limits of detection (LOD: S/N = 3) ranged between 1–30 mg/L (Table 1), and were similar to values found in the literature.[6] Results demonstrated the best repeatability with direct injection, while sample clean-up with SAX affected, to a large extent, the determination of pyruvic and lactic acids (Table 2). The determination of lactic acid is particularly important in red wines during malo-lactic fermentation. Recoveries were satisfactory showing values from 93% to 101%.

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Table 2

Precision (CV%), Recovery (%), Paired t-Test Comparision Between Direct Injection and Sample Clean-up with SAX Cartridge (n=12), and Sample Composition (Range)

Compound	Precision (n=5) Direct Injection	Precision (n=5) SAX	Recovery (n=5) Direct Injection	Recovery (n=5) SAX	t-test[a] *p*-Value	Samples Range
Citric acid (mg/L)	0.94	2.59	98.5	97.3	0.8703	7-29
Tartaric acid (mg/L)	0.65	1.29	99.5	98.4	0.1498	88-391
α-Chetoglutaric acid (mg/L)	0.76	2.16	94.3	92.9	0.0004	Trace
Glucose (g/L)	0.95	1.41	99.6	96.6	0.0058	0-6
Malic acid (mg/L)	1.06	1.45	99.4	99.5	0.0682	97-261
Fructose (g/L)	0.76	1.22	99.3	99.1	0.0288	0-6
Pyruvic acid (mg/L)	1.41	4.49	97.7	93.2	0.0400	Trace
Succinic acid (mg/L)	0.94	1.36	99.3	100.3	0.1162	170-191
Lactic acid (mg/L)	1.02	7.45	96.1	95.7	0.1412	38-59
Glycerol (mg/L)	1.27	3.99	99.7	99.7	0.4059	338-368
Acetic acid (mg/L)	2.12	2.57	98.9	101.2	0.4806	Trace
Ethanol (%, v/v)	0.88	0.97	99.6	98.7	0.1098	0-12

[a] Significant value with $P \leq 0.05$.

The proposed HPLC method allowed the simultaneous determination of many compounds of interest in wines (Figure 2). The major peaks were identified and quantified (Table 2) while additional compounds were also detected. Attempts to identify these peaks have so far been unsuccessful. According to the literature[9, 15] some unidentified phenolic compounds may interfere with acetic and malic acid determination. Malic acid showed a satisfactory resolution from fructose, therefore a reliable determination of these compounds in musts and sweet wines was achieved. Glucose does not absorb at UV 210 nm and its presence did not interfere with the UV detection of organic acids. A dilution factor of 1:20 for musts and wines was necessary to avoid the column overloading and to achieve the linearity of response. As expected, the paired t-test comparison between direct injection and sample clean-up with SAX cartridge disclosed a significant difference for glucose and fructose determination. Instead, the significant difference found for α-ketoglutaric and pyruvic acids was probably due to the low concentration of these compounds.

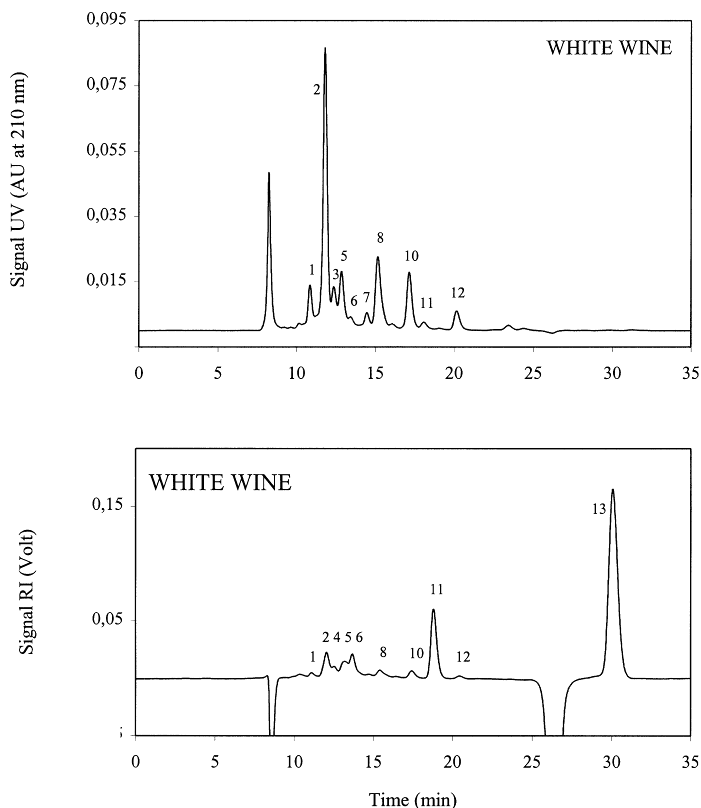

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Figure 2. Chromatograms of white wine (cv. Trebbiano) with direct injection. Legend: UV detection (top), and refractive index detection (bottom). Peaks: (1) citric acid; (2) tartaric acid; (3) α-chetoglutaric acid; (4) glucose; (5) malic acid; (6) fructose; (7) pyruvic acid; (8) succinic acid; (9) fumaric acid; (10) lactic acid; (11) glycerol; (12) acetic acid; (13) ethanol. Chromatographic conditions as in materials and methods.

Figure 3 shows the chromatograms of the ascorbic acid (vitamin C) analyzed at different column temperatures (25-60°C). A single peak was detected only with a column temperature of 25°C. The increased temperature of analysis generated an on–line degradation of vitamin C and the appearance of an early broad peak which represented the unresolved degradation product(s). Under these conditions of analysis, the determination of vitamin C was inaccurate. In addition, the degradation product(s) of ascorbic acid may interfere with the analysis of the close eluting compounds, such as α-ketoglutaric, citric, tartaric, malic acid, and glucose. The use of refractive index detector minimized

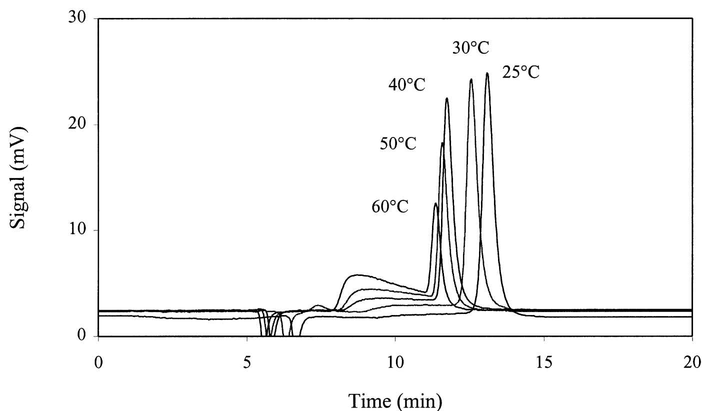

Figure 3. Chromatograms of ascorbic acid standard solutions at different temperature (25-60°C). Chromatographic conditions as in materials and methods.

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the background interference of ascorbic acid eventually present in the sample. However, concentration of ascorbic acid above 0.4 gr/L would be detected.

CONCLUSIONS

An HPLC method for the simultaneous analysis of sugars, organic acids, and alcohols was improved. The method validation was satisfactory, and the direct injection allowed decreasing the time and cost of analysis with no significant lack of accuracy. Wines showed a characteristic elution profile, however, unknown peaks were present and their identification need to be further studied. This HPLC method showed a great potential for the quality control and research in food science, as well as, in biotechnology and biomedicine. Particular attention should be paid when ascorbic acid is present in the sample as a major component or as interference. A column temperature ≤25°C should be selected to avoid the on-line degradation of vitamin C.

REFERENCES

1. J. P. Yuan, F. Chen, Food Chem., **64**, 423-427 (1999).

2. W. Koliander, C. Arnezeder, W. A. Hampel, Acta Biotechnol., **10**, 387-394 (1990).

3. A. M. Vogt, C. Ackermann, T. Noe, D. Jensen, W. Kübler, Biochem. Biophys. Res. Commun., **248**, 527-532 (1998).

4. P. Oefner, G. Bonn, G. Bartsch, J. Liq. Chromatogr., **8**, 1009-1023 (1985).

5. T. Fuleki, E. Pelayo, B. Palabay, J. AOAC, **76**, 591-600 (1993).

6. E. López-Tamames, M. A. Puig-Deu, E. Teixeira, S. Buxaderas, Am. J. Enol. Vitic., **47**, 193-198 (1996).

7. S. A. Kupina, Am. J. Enol. Vitic., **35**, 59-62 (1984).

8. J. D. McCord, E. Trousdale, D. D. Y. Ryu, Am. J. Enol. Vitic., **35**, 28-29 (1984).

9. R. F. Frayne, Am. J. Enol. Vitic., **37**, 281-287 (1986).

10. A. Schneider, V. Gerbi, M. Redoglia, Am. J. Enol. Vitic., **38**, 151-155 (1987).

11. E. Falqué-López, E. Fernández Gómez, J. Chromatogr. Sci., **34**, 254-257 (1996).

12. M. W. Dong, LC-GC, **16**, 1092-1097 (1998).

13. D. Tusseau, C. Benoit, J. Chromatogr., **395**, 323-333 (1987).

14. J. J. Hunter, J. H. Visser, O. T. De Villiers, Am. J. Enol. Vitic., **42**, 237-244 (1991).

15. M. Calull, R. M. Marcé, F. Borrull, J. Chromatogr., **590**, 215-222 (1992).

16. C. Da Porto, M. Munari, Ital. J. Food Sci., **4**, 35-40 (1989).

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