Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1052 (2004) 211–215
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Short communication

Rapid liquid chromatography–ultraviolet determination of organic acids and phenolic compounds in red wine and must

Zohar Kerem[a,*], Ben-ami Bravdo[b], Oded Shoseyov[b], Yizhar Tugendhaft[a,b]

[a] *Institute of Biochemistry, Food Science and Nutrition, The Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel*
[b] *Department of Horticulture, The Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel*

Received 6 January 2004; received in revised form 24 August 2004; accepted 24 August 2004

Abstract

A reversed-phase liquid chromatography–ultraviolet (LC–UV) method is proposed for the rapid simultaneous analysis of the main carboxylic acids and polyphenols in must and wine. Good resolution was obtained for citric, tartaric, malic, lactic, acetic, caffeic, ellagic and gallic acids, (−)-epicatechin, quercetin and resveratrol. A novel silica-based column containing ether-linked phenyl groups, with polar end-capping and suitable for low-pH aqueous mobile phases was used and found to be superior to others tested. The method employed a mixture of 0.2% TFA in water and acetonitrile as eluents, showed linearity and precision, and was applied to samples of must and wine.

Keywords: Must; Wine; Carboxylic acids; Organic acids; Polyphenols

1. Introduction

The profile and concentration levels of organic acids, mainly tartaric, malic and lactic acids are important parameters for must and wine during processing and in the final product. The contents of organic acids in must and wine influence not only the balance of the flavor, but also the chemical stability and pH, and thus the quality of the wine [1,2]. Therefore, it is important to be able to quantify organic acids that are present in must or wine for quality and process control.

Many analytical methods have been, and are still being used to detect and quantify organic acids and polyphenols in wine. Liquid chromatography (LC) is the most widely employed technique for the analysis of individual components. Organic acid detection and quantification can be achieved by several methods. Some use columns packed with ion-exclusion resin, thus requiring the removal of polyphenols from the sample prior to analysis. Elution systems are usually isocratic, with an acidified aqueous solvent [3–10]. Typical elution times are from 5 min [6] to 20 min [5]. Sample preparation is time-consuming and may decrease the reliability of the results. Other methods, which make use of reversed-phase columns, are of limited applicability to wine due to its alcohol content [6,11] or the number of acids resolved [12].

Polyphenols contribute color (anthocyanins), astringency (tannins), antioxidant activity and other health benefits to wine [1,2,13–15]. Their levels also influence the activity of important bacteria in the wine, such as *Oenococcus oeni* [16]. The ability to identify and control polyphenols content in wine during its processing and evolution in the winery is a valuable asset for the winemaker.

For the separation of polyphenols, both normal-phase and reversed-phase columns are available for use [17,18]. For RP columns, elution systems are usually binary. Isocratic, ternary and quaternary systems have also been described. Run times are long, generally from 30 to 80 min [6,13,18–23], thus limiting the number of analyses that can be carried out

* Corresponding author. Fax: +972 8 9476189.
E-mail address: kerem@agri.huji.ac.il (Z. Kerem).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.08.105

in a given period. A rapid method has been published [2], but is suitable only for the polyphenolics in wine. A recently developed method [11] offers separation and determination of organic acids and phenolic compounds on a C18 column, but it is time-consuming, taking 80 min, and does not tolerate alcohol in the sample, making it unsuitable for wine analyses. Here, we report the rapid quantification of both polyphenols and organic acids in red grape must and wine using a novel polar reversed-phase phenyl end-capped column adapted to aqueous low-pH solvent, high flow and rapid analyses.

2. Experimental

2.1. Apparatus and LC conditions

The LC analyses were carried out using a Spectra HPLC system with Chromquest software (version 2.51), a pump (p4000), an autosampler (AS3000), and a diode-array detector (UV6000LP) (Thermo Separation Products, San Jose, CA, USA). The separation was carried out on a Synergi^{TM} Polar-RP^{TM} column (250 mm × 4.6 mm i.d.) (Phenomenex, New York, NY, USA) with a guard column (Phenomenex polymerX^{TM}, 1.0 mm × 4.6 mm i.d.). Other columns used for comparison were:

1. Supelcogel C-610H (30 cm × 7.8 mm) 9 μm particle size (Supelco No. 59320, Sigma, St. Louis, MO, USA);
2. Phenomenex Luna C-18 (250 mm × 4.6 mm, 5 μm);
3. Phenomenex Synergi^{TM} Hydro-RP^{TM} (250 mm × 4.6 mm, 5 μm);
4. Agilent Zorbax SB-Aq (250 mm × 4.6 mm, 5 μm) (Agilent, Palo Alto, CA, USA).

Trifluoroacetic acid (TFA, 0.2% in water), pH 1.9 (solvent A) and acetonitrile (solvent B) were used. Elution started with an isocratic step of solvent A only, for the first 7 min, at 1.5 ml/min. Then, a linear gradient was applied from 0 to 4% B during the next 3 min, and up to 50% B in the next 20 min; 10 min of equilibration were required before the next injection. Other parameters were as follows: injection volume, 10 μl; column temperature, 30 °C; detection wavelength, 210 and 280 nm; and UV spectra, 200–600 nm range.

2.2. Chemicals and standards

All standards were purchased from Sigma (St Louis, MO, USA): acetic acid, caffeic acid, citric acid, ellagic acid, (−)-epicatechin, formic acid, gallic acid, lactic acid, L-malic acid, oxalic acid, quercetin, resveratrol and tartaric acid. The individual organic acid standards were dissolved in water or 12% ethanol in deionized water. Stock solutions contained 20 mg/ml tartaric acid or 8 mg/ml of the other acids. Standards of phenolic compounds were dissolved in the same solution to 0.4 mg/ml, and were injected at four concentrations to determine individual retention times and calibration curves. Solutions were kept at 4 °C and used to optimize the LC separation conditions. The solutions were also applied to observe the between-day stability of retention times and peak areas. Stock solutions and diluted (up to 100 times) standards were injected to evaluate linearity and detection limits. All solutions were filtered through a PTFE 0.45-μm-membrane filter before LC analysis, and the mobile-phase solvents were degassed before use.

Must and wine samples were prepared from Cabernet Sauvignon (*Vitis vinifera* L.) grapes, harvested from three blocks in a single vineyard, and taken to the laboratory for vinification. At crushing, 20-ml aliquots of must from each replicate were sampled and frozen at −20 °C for later analyses. Samples from all replicates were taken for vinification. After 14 days of ethanolic fermentation and maceration and 14 days of malo-lactic fermentation, wines were filtered through paper filter (Whatman No. 1 filter paper, Whatman, Maidstone Kent, UK) and kept in the refrigerator. Commercially produced wines were purchased at a local liquor store.

2.3. Sample preparation

All standards and samples were filtered through a PTFE 0.45-μm-membrane filter prior to analysis by LC. For the analysis of carboxylic acids on a C-610H column, phenolics were removed as previously described: 0.5 ml polyvinylpolypyrrolidone (PVPP; Polyclar VT) was added to a 1.5-ml sample, vortexed for 30 s, and centrifuged at 15,000 rpm for 6 min. This procedure was repeated until samples were clear. Clear samples were filtered through Strata® C18-E 100 mg/1 ml cartridge (Phenomenex, Torrance, CA, USA), and analyzed according to manufacturer directions (Sigma).

3. Results and discussion

3.1. Method development

An analytical method that combines the analysis of both organic acids and phenolics in must and wine, using a reversed-phase LC column, was developed. To date, the separation of organic acids requires the use of specially designed ion-exchange columns [8–12], while the separation of phenolics is conveniently performed on RP columns. Moreover, the use of ion-exchange columns requires the preceding removal of phenolics from the sample. Using Synergi 4μ Polar-RP^{TM} column, the separation of seven organic acids and six wine phenolics in a single LC run was achieved. This column is an ether-linked phenyl phase with hydrophilic end-capping designed specifically to maximize retention and selectivity for both polar and aromatic analytes.

The separation of carboxylic acids required the use of a purely aqueous mobile phase. Indeed, the polar end-capping is designed to prevent 'phase collapse' when a purely aqueous eluent is used. The elution program allowed carboxylic acids and gallic acid to be resolved within only 6 min (Fig. 1a). To

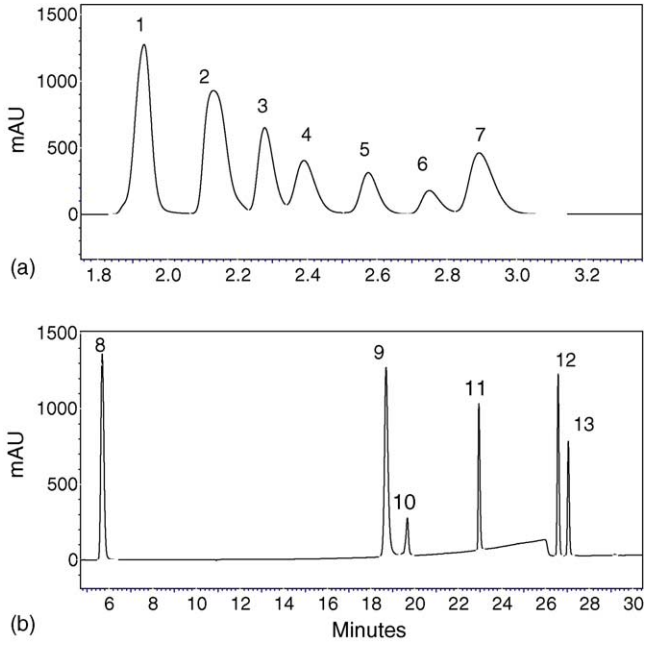

Fig. 1. LC–UV of mixed standards of (a) organic acids at 210 nm and (b) phenolics at 280 nm. For peak numbers, see Table 1; for LC conditions, see Section 2.

achieve resolution of all tested phenolics, a mild gradient of 2.3% acetonitrile per minute for 20 min was used. Using this gradient resulted in the elution of resveratrol and quercetin at 26.7 and 27.2 min, respectively (Fig. 1b). Resveratrol and quercetin are usually among the last phenolics to elute when wine phenolics are separated on RP columns [2,14]. Aromatic selectivity may be enhanced due to the π–π interactions between the aromatic rings of the analyte and the phenyl functional group of Synergi Polar-RP™.

Several other columns are available that combine hydrophobic resin with stability in pure aqueous eluents. Of these, Synergi Hydro-RP™ (Phenomenex) and Zorbax SB-Aq (Agilent) were tested for their ability to separate wine carboxylic acids, but showed lower resolution (data not shown). The separation of standards served to compare the proposed method with commonly used methods. Carboxylic acids were separated with an ion-exchange column (Supelcogel C-610H), according to manufacturer directions [5]. A relatively rapid elution of all standards was achieved, within 1 min only, using the Synergi Polar-RP™ column (Fig. 1), as compared to the common separation on a C-610H column (Fig. 2). The results obtained using Synergi Polar-RP™ column showed resolutions greater than 1.4 for all peaks, except for malic and formic acid which showed a value of 1.0. Integration of the areas under the curve (AUC) resulted in similar values for both chromatographic processes. The average peak base width in the proposed method was 5 s (Fig. 1). A linear correlation of the AUC to the amounts was observed over wide ranges for all acids (Table 1). These ranges are compatible with currently used methods, and with amounts in must and wine [4,6]. To evaluate the separation of wine phenolics using

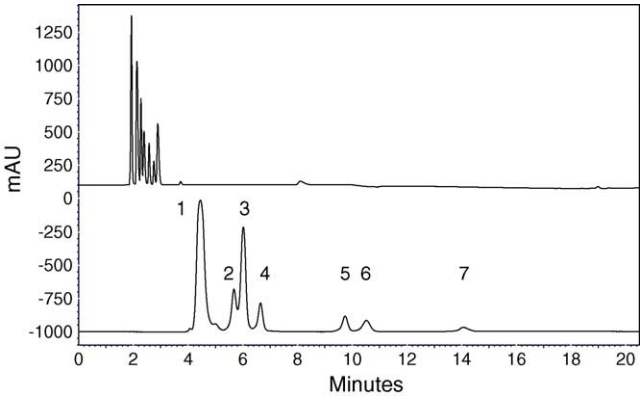

Fig. 2. LC–UV of mixed standards of organic acids at 210 nm, separated using (upper trace) Synergi™ Polar-RP™ column and (lower trace) Supelcogel C-610H column. For peak numbers, see Table 1; for LC conditions, see Section 2.

the proposed method, standards were also chromatographed on a Phenomenex Luna C-18 column [14]. Linear correlation was observed for the selected standard polyphenols in both methods, showing much higher sensitivity to concentrations, ranging from nM to μM (Table 1). These ranges are well-suited to amounts found usually in must and wine [14]. It is thus suggested that our proposed combined method provides similar quantitation and recovery values as each of the two methods used separately.

3.2. Analytical performance

In a recent work, methanol, ethanol or acetone concentrations of 10% in the sample were shown to decrease the resolution, and to reduce t_R of the carboxylic acids [11]. In the proposed method, carboxylic acids were well resolved when authentic wine samples, containing 12% (v/v) ethanol were analyzed (Fig. 3). Resolution was kept even when samples of the same wine were fortified with ethanol up to 24% (v/v) (Fig. 3). This demonstrates the applicability of the proposed method to wines and fortified (desert) wines.

Table 1
Retention times and linear range of carboxylic acids and phenolic compounds in wine

No.	Common name	Retention time (min)	Linear range (mg/ml)
1	Oxalic acid	1.90	0.5–8
2	Tartaric acid	2.10	1.25–20
3	Formic acid	2.30	0.5–8
4	Malic acid	2.40	0.5–8
5	Lactic acid	2.60	0.5–8
6	Acetic acid	2.75	0.5–8
7	Citric acid	2.90	0.5–8
8	Gallic acid	5.90	0.025–0.4
9	Caffeic acid	18.90	0.025–0.4
10	(−)-Epicatechin	19.85	0.025–0.4
11	Ellagic acid	22.70	0.025–0.4
12	Resveratrol	26.70	0.025–0.4
13	Quercetin	27.25	0.025–0.4

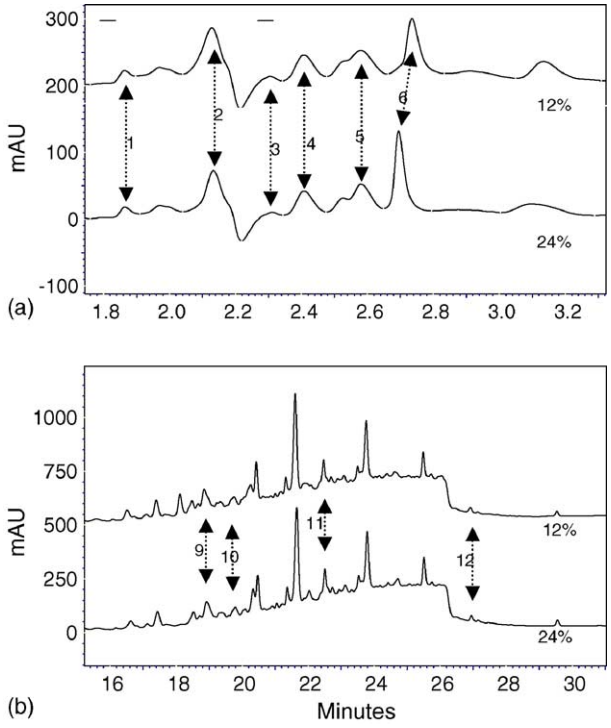

Fig. 3. LC–UV of wine samples containing either 12 or 24 (fortified) vol.% of ethanol: (a) organic acids at 210 nm and (b) phenolics at 280 nm. For peak numbers, see Table 1; for LC conditions, see Section 2.

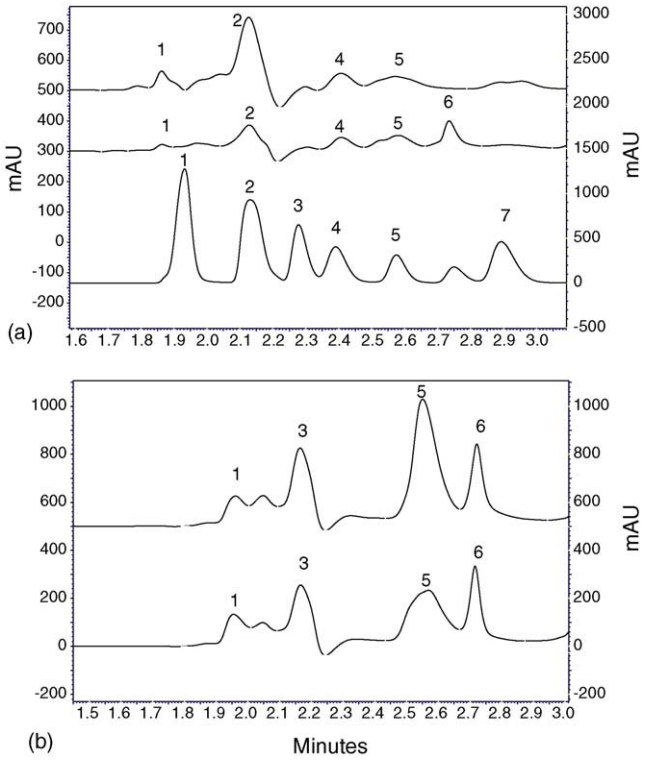

Fig. 4. LC–UV of organic acids at 210 nm in (a) (upper trace) must and (middle trace) wine samples and (lower trace) mixed standards; (b) either (upper trace) spoiled or (lower trace) characteristic Cabernet Sauvignon. For peak numbers, see Table 1; for LC conditions, see Section 2.

Injection volumes of 10–20 μl of wine are routine in the analyses of wines [6,7,14]. To further determine the robustness of the method, increasing volumes of wine or must samples were injected on the column. Similar resolution values were obtained for the tested organic acids, up to 20 μl injection volumes. Increasing the injection volume over 20 μl resulted in reduced resolution not only of carboxylic acids but also of wine phenolics. This might be due to close distribution coefficients and the competitive adsorption behavior of the latter.

The within-day repeatability ($n = 5$) and between-day precision ($n = 3$) of retention times were within 1.0% relative standard deviation (R.S.D.), respectively. The repeatability ($n = 5$) and between-day precision ($n = 3$) of peak area were within 5% R.S.D. The accuracy of the method was confirmed by spiking must or wine with known amounts of standards and comparing their AUC values with the calibration curves.

3.3. Applications

Must contains a variety of carboxylic acids, mainly tartaric and malic, which are essential for a high-quality end product. An analysis of carboxylic acids in must gives a good indication of the ripening stage of the grapes, which is essential to determining optimal harvest date. A sample chromatogram of must shows the levels of oxalic, tartaric and malic acids in must produced from Cabernet sauvignon (Fig. 4a). Later, one can follow fermentation development in the winery. Determination and quantification of malic and lactic acids during wine processing is required for setting the seized malo-lactic fermentation [3]. The resultant wine contains less tartaric and malic acids, and more lactic and (unfortunately) acetic acids (Fig. 4a). Thus, the suggested method allows also the detection of levels of carboxylic acids that may not be acceptable in wine. These may come from bad practice or from attempted taste correction, as was demonstrated in our lab by chance. While fermenting grape juice, we experienced a sluggish fermentation in one of the containers. Following our attempts to restart the fermentation, we were able to reach the correct pH value. Tasting revealed a problem, later analyzed by our LC–UV method to be lactic acid accumulation (Fig. 4b). To further assess the applicability of the method, several commercial wines were analyzed. An interesting example was obtained in a single sauvignon blanc wine (three bottles) that contained 8.07 g tartaric acid/l, while an average 4 g/l was detected in all other red and white wines tested, suggesting correction actions taken by the wine maker.

4. Conclusions

A simple and rapid method was developed for the simultaneous determination of carboxylic acids and phenolic compounds in must and wine by LC. The capability to separate

wine carboxylic acids, in the presence of ethanol in the sample, using LC is the main advantage of the proposed method. This provides the potential to analyze must and wine samples in a single chromatographic run, eliminating the requirement for sample preparation. Seven non-phenolic acids and six common wine phenolics were eluted within 30 min, which is rapid enough for a quality control unit. Separating and analyzing polyphenols using LC is not new, thus calibrating additional standards may be used to detect additional wine polyphenols. Using a single rapid protocol may assist in developing quality criteria for the levels of both acids and phenolics in wine. The established method was successfully used to determine a variety of carboxylic acids and phenolic compounds in wine and grape must samples. The robustness and sensitivity of the method establishes a basis for its many potential applications.

Acknowledgements

This work was supported in part by a scholarship to Y.T. from the Molcho Center and by the Zigmond and Estel Lovenberg Fund. We wish to thank Mrs. P. Weinberg for her technical support.

References

[1] R.B. Boulton, V.L. Singleton, L.F. Bisson, R.E. Kunkee, Principles and Practices of Winemaking, Aspen publishers, Gaithersburg, MD, USA, 1998.
[2] M. Ibern-Gomez, C. Andres-Lacueva, R.M. Lamuela-Raventos, A.L. Waterhouse, Am. J. Enol. Vitic. 53 (2002) 218.
[3] F. Chinnici, U. Spinabelli, A. Amati, J. Liq. Chromatogr. Relat. Technol. 25 (2002) 2551.
[4] E. Lopez-Tamames, M.A. Puig-Deu, E. Teixeira, S. Buxaderas, Am. J. Enol. Vitic. 47 (1996) 193.
[5] Supelco, http://www.sigmaaldrich.com/Graphics/Supelco/objects/4600/4568.pdf.
[6] M.W. Dong, LC/GC: Mag. Sep. Sci. 16 (1998) 1092.
[7] T. Walker, J. Morris, R. Threlfall, G. Main, J. Agric. Food Chem. 51 (2003) 1543.
[8] T. Perez-Ruiz, C. Martinez-Lozano, V. Tomas, J. Martin, J. Chromatogr. A 1026 (2004) 57.
[9] A. Kotani, Y. Miyaguchi, E. Tomita, K. Takamura, F. Kusu, J. Agric. Food Chem. 52 (2004) 1440.
[10] A. Endelmann, J. Diewok, J.R. Baena, B. Lendl, Anal. Bioanal. Chem. 376 (2003) 92.
[11] G.H. Shui, L.P. Leong, J. Chromatogr. A 977 (2002) 89.
[12] M. Kordis-Krapez, V. Abram, M. Kac, S. Ferjancic, Food Technol. Biotechnol. 39 (2001) 93.
[13] H.M. Merken, G.R. Beecher, J. Agric. Food Chem. 48 (2000) 578.
[14] M. Lopez, F. Martinez, C. Del Valle, C. Orte, M. Miro, J. Chromatogr. A 922 (2001) 359.
[15] J. Kanner, T. Lapidot, Free Radic. Biol. Med. 31 (2001) 1388.
[16] N. Rozes, L. Arola, A. Bordons, Lett. Appl. Microbiol. 36 (2003) 337.
[17] G.J. Soleas, D.M. Goldberg, E. Ng, A. Karumanchiri, E. Tsang, E.P. Diamandis, Am. J. Enol. Vitic. 48 (1997) 169.
[18] D.M. Goldberg, E. Tsang, M. Levesque, G.J. Soleas, J. Liq. Chromatogr. Relat. Technol. 22 (1999) 1843.
[19] B. Baderschneider, D. Luthria, A.L. Waterhouse, P. Winterhalter, Vitis 38 (1999) 127.
[20] P. Jeandet, A.C. Breuil, M. Adrian, L.A. Weston, S. Debord, P. Meunier, G. Maume, R. Bessis, Anal. Chem. 69 (1997) 5172.
[21] D.M. Goldberg, G.J. Soleas, in: H. Sies (Ed.), Oxidants and Antioxidant, Pt. B, Academic Press, NY, USA, Methods Enzymol. 299 (1998) 122.
[22] D.M. Goldberg, E. Tsang, A. Karumanchiri, G.J. Soleas, Am. J. Enol. Vitic. 49 (1998) 142.
[23] H. Chen, Y.G. Zuo, Y.W. Deng, J. Chromatogr. A 913 (2001) 387.