SHORT COMMUNICATION

Determination of selected wine phenolic constituents as possible markers for differentiation of wines

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Summary

A reversed-phase high performance liquid chromatography (HPLC) method for quantification of wine phenolic constituents (+)-catechin, (–)-epicatechin, hesperidin, rutin, quercetin and *trans*-resveratrol is described. Wine was analysed without any previous preparation except of microfiltration. The flavonoids were simultaneously detected at 256, 280 and 305 nm using a diode-array detector. The method was partly validated for white and red wine with satisfactory results, including detection limit and quantification limit (0.016–0.80 mg·l^{-1} and 0.02–1.34 mg·l^{-1}, respectively), linearity (coefficient of determination $r^2 > 0.999$), precision (maximum coefficient of variation 5.69%) and accuracy (recovery rate 77–110%). The present method is intended to be used for differentiation of wines.

Keywords

phenolics; wine; diode-array detector; HPLC

Phenolic compounds in grapes and wines are a diverse group of compounds with varying concentrations and composition. Their presence in wine significantly affects the flavour, stability and colour of red wines. Most of the wine phenolics come from grape skin, stems and seeds, with higher contents in red wine than in white wine [1]. Some of the compounds are formed during fermentation and aging process of wine [2].

Grape and wine phenolics comprise two main groups of nonflavonoids and flavonoids. Nonflavonoid resveratrol is synthetized in grape skin in response to microbial infection or stress, caused by the pathogens *Plasmospora viticola* or *Botrytis cinerea* [3, 4], or to abiotics such as UV light and mechanical injury [5]. Flavonoids are present free or in polymeric form with other flavonoids, saccharides, nonflavonoids or a combination of all [6]. The dominating flavonoids are flavanols, flavonols and anthocyanins. Flavanols (catechin and its epimer, epicatechin) may be found in grape seeds, stems and in the skin of immature grape as well as in ripe grapes. Flavanol monomers and oligomers (links of two to four monomers) contribute to bitterness, and their polymers to astringency of wine. Flavonols (quercetin, myricetin and kaempferol) are present in skin of grape. The most recognized flavonol is quercetin-3-glucoside, which is deglycosylated into its aglycone form during wine making and aging. Analogously to flavanols, quercetin glycosides are regarded as velvety astringents of red wine [7]. Anthocyanins are the main source of pigmentation in red wines. Because they are localized in the skin tissue of the majority of red grape cultivars, fermentation and maceration have a key effect on the amount of anthocyanins present in the final wine [8].

Numerous studies on the separation and quantification of individual monomeric and oligomeric flavonoids of wines by high-performance liquid chromatography with different detection systems have been recently published. The UV-VIS/photodiode-array detection-assisted methods that use direct injection of wine samples have been also reported [1, 3, 9–14].

This paper presents a HPLC method for determination of selected phenolic compounds that may characterize a specific profile of wines and be potential markers for differentiation of wines. Five flavonoids and one nonflavonoid were separated in a single liquid-chromatographic run. A multiwavelength diode-array detector was used for definite and accurate detection and quantification of individual phenolics.

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MATERIALS AND METHODS

Chemicals

Standards of (+)-catechin, quercetin (Sigma-Aldrich Chemie, Steinheim, Germany), (–)-epicatechin, hesperidin, rutin and *trans*-resveratrol (Sigma–Aldrich, St. Louis, Missouri, USA) were used. Methanol (LiChrosolv gradient grade; Merck, Darmstadt, Germany) and orthophosphoric acid (min. 85%; Lachema, Brno, Czech Republic) were used as mobile phase constituents. Water was purified in Rodem 6 water purification device (Ecotest, Zemné, Slovakia). Syringe filters of 0.45 μm pore size with a cellulose membrane (Agilent, Waldbronn, Germany) were used for filtration of wine samples.

All stock standard solutions were prepared in methanol at concentrations 500 mg·l^{-1} (catechin, epicatechin, rutin, hesperidin), 10 g·l^{-1} (*trans*-resveratrol) or 1 g·l^{-1} (quercetin). Calibration standards were made by an appropriate dilution of the stock solutions with methanol. Control standard solution was prepared with a concentration of 10 mg·l^{-1} of each component.

Wine samples

For method validation, one sort of white and red variety wine was used throughout all experiments. Wine samples were filtered through a 0.45 μm membrane filter prior to HPLC analysis.

HPLC equipment

The separation was performed using a liquid chromatography instrument Agilent Technologies 1100 Series equipped with a diode array detector (DAD), quartenary pump, degasser, column thermostat and autosampler (Agilent Technologies, Waldbronn, Germany). Chromatographic separations were performed on a Purospher STAR RP-18e column, 250 × 4.6 mm i.d., 5 μm (Merck) at 30 °C. The linear gradient programme was as follows: 0–1 min 100% B, 1–5 min 80% B, 5–10 min 75% B, 10–12 min 65% B, 12–18 min 50% B, 18–26 min 50% B, 26–28 min 100% B. Solvent A was 100% methanol and solvent B was the mixture of 0.01 mol·l^{-1} orthophosphoric acid + methanol (95 + 5, v + v). The flow rate of the mobile phase was 1.3 ml·min^{-1}. Samples of 20 μl were injected. Detection was carried out by DAD at 256, 280, and 305 nm.

Identification, quantification and statistics

The compounds were identified by the comparison of their retention times with those of pure standards. Phenolics were monitored with DAD in a range of 200–400 nm for match factor spectral analysis. Measured data were processed through a chromatographic programme Agilent ChemStation. For calibration, linear regression diagnostics was carried out by the Excel XP software (Microsoft, Redmont, Washington, USA) as well as one-factor analysis of variance (ANOVA) for assessment of difference between means. The statistical significance was considered at the value $P < 0.05$.

Method validation

For validation of the method, detection limit, quantification limit, calibration, linearity, precision and accuracy were assessed. The calculation of limit of detection (*LOD*) and limit of quantification (*LOQ*) was based on analysis of blank wine sample. The calibration measurements were carried out with standard solutions that covered a six-points linear range. The precision was evaluated as intra-day ($n = 10$) and inter-day repeatability within five days ($n = 5$), using control standard solution 10 mg·l^{-1}. Unfortified wines were also analysed for intra-day repeatability. The recovery rate as a measure of accuracy was assessed by addition of a known amount of the compound to a blank wine sample.

RESULTS AND DISCUSSION

Our former work had confirmed that the use of HPLC with a mobile phase containing methanol and 0.01 mol·l^{-1} orthophosphoric acid facilitated a good separation of fruit juice flavonoids [15]. In this study, the previously used C_6-phenyl stationary phase was replaced with C_{18}-reversed phase. Due to the complexity of the wine matrix, some minor modifications had to be carried out in the composition of the mobile phase. The linear gradient elution programme and the flow rate of the mobile phase were modified in order to achieve acceptable separation of the studied phenolics. These were finally well distinguished from the other wine matrix constituents.

Spectral analysis

Because of structural variations of the studied flavonoids and *trans*-resveratrol, absorption maxima were investigated in the range of 200–400 nm by recording the corresponding absorbance of standard compounds (Tab. 1). The acquired spectra were saved in the computer-aid library. On the basis of obtained spectra, the absorption maxima were selected as follows: 256 nm for rutin and quercetin, 280 nm for catechin, epicatechin and hesperidin, and 305 nm for *trans*-resveratrol. Satisfactory separation of all compounds was achieved

Tab. 1. Determined data on absorption maxima and retention times with standard solutions (10 mg·l^{-1}).

Phenolic compound	Mean retention time (n = 20) [min]	Absoption maximum [nm] / Absorbance [mAU]
Catechin	10.993 ± 0.096	280 / 8.00
Epicatechin	14.452 ± 0.075	280 / 15.32
Hesperidin	19.738 ± 0.065	284 / 38.67
Rutin	19.990 ± 0.085	256 / 30.56; 356 / 25.04
trans-Resveratrol	20.435 ± 0.082	216 / 133.35; 305 / 188.20
Quercetin	26.320 ± 0.219	256 / 23.75; 372 / 23.67

within 40 min including equilibration, needle washing and sample injection. Fig. 1 depicts typical chromatograms of unfortified wines at all detecting wavelengths.

Each compound was assigned a value between 0 and 1 000 for equivalence between the spectra of the compound peak and that of the pure standard from the computer-aid library. Accurate comparison of the spectra was accomplished via computer program Agilent ChemStation. Because the match factor of 1 000 (correlation factor = 1) describes identical spectra [16], the inner criterion for purity factor ≥ 990 was set up for acceptation of a pure compound.

Method validation

Tab. 2 summarizes some data from the method validation. The calibration measurements and linearity evaluation were based on linear regression analysis. All responses were linear over the entire range tested ($r^2 > 0.999$).

The detection limit and quantification limit were estimated by successive dilution of the standard added to wine to achieve minimal concentration of particular phenolics that allowed the lowest signal be registered by the detector. The detection limits varied from 0.016 mg·l^{-1} (*trans*-resveratrol) to 0.80 mg·l^{-1} (catechin) and were comparable to those obtained by a conventional HPLC-DAD [17, 18].

The precision of analyses was determined as intra- and inter-day repeatability of ten and five replicate analyses characterized by coefficient of variation (*CV*). According to statistical analysis, there was no significant difference between intra- and inter-day repeatability ($P = 0.996444$).

CV values listed in Tab. 2 indicated conformity with the required criterion on precision, e.g. that maximum *CV* was 10% the for mass fraction ≥ 1000 μg·kg^{-1} [19].

The recovery rate was determined for each compound by spiking wine with a standard solution at two concentration levels. Tab. 3 shows the mean recoveries of phenolics from wines. When considering the recoveries with white and red wines independently, red wines showed a bit worse recovery but generally there was no constant difference in this parameter. The associated *CV* values were evidently higher for catechin, epicatechin and rutin (22–29%), which was probably due to a larger dispersion of the bulk data processed for both white and red wines. The recovery rates were comparable to those published with other methods [20, 21].

Tab. 2. Calibration data and repeatability of the HPLC separation of phenolic compounds.

Phenolic compound	Linearity range [mg·l^{-1}]	r^2	*LOD* [mg·l^{-1}]	*LOQ* [mg·l^{-1}]	Repeatability, *CV* [%]			
					Intra-day control standard (n = 10)	Inter-day control standard (n = 5)	Inter-day white wine (n = 5)	Inter-day red wine (n = 5)
Catechin	1–100	0.9998	0.80	0.97	1.60	0.78	3.02	5.69
Epicatechin	0.40–100	0.9999	0.25	0.36	1.30	0.69	2.30	3.63
Hesperidin	0.27–100	0.9999	0.20	0.27	1.10	2.04	nd	nd
Rutin	0.30–100	0.9999	0.10	0.26	2.05	1.77	nd	nd
trans-Resveratrol	0.020–20	1.0000	0.016	0.020	1.00	0.69	2.14	4.86
Quercetin	1.35–200	0.9999	0.68	1.34	1.49	0.72	nd	nd

r^2 – correlation coefficient, *LOD* – limit of detection, *LOQ* – limit of quantification, *CV* – coefficient of variation, nd – not detected.

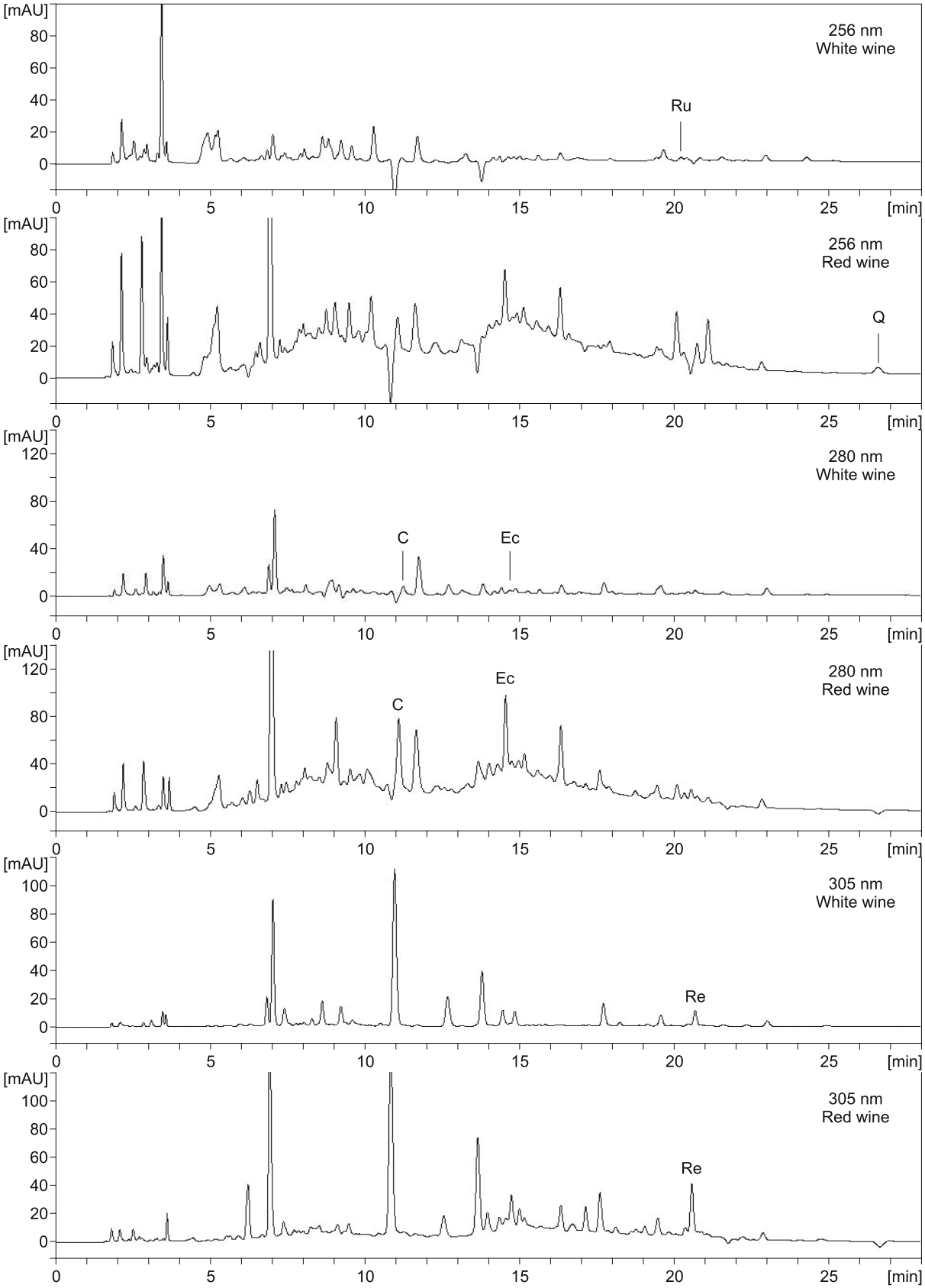

Fig. 1. Chromatographic profiles of unfortified white and red wine at 256, 280 and 305 nm.

The x-axis represents time, y-axis represents absorbance.
Ru – rutin, Q – quercetin, C – catechin, Ec – epicatechin, Re – *trans*-resveratrol.

Tab. 3. Recovery rates of phenolics from red wine and white wine at two spiking levels.

Compound	Catechin		Epicatechin		Hesperidin		Rutin		*trans*-Resveratrol		Quercetin	
Spiking level [mg·l^{-1}]	5	80	5	80	5	80	5	80	2	15	5	100
Mean recovery rate (n = 6) [%]	85	86	89	86	98	100	77	97	110	104	108	99
Coefficient of variation, *CV* [%]	28	27	24	22	9	2	29	4	7	1	5	1

CONCLUSIONS

Although a majority of HPLC methods published up to now affords separation of much more wine phenolics in one chromatographic analysis, this work is fitted only for a few of them. The exact aim of this preliminary study was to adjust the HPLC method to separate and quantify six wine phenolics that might be feasible component markers for wine recognition, along with other factors. As the present method is comparatively rapid, precise and accurate, it is promising to fit to this purpose.

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REFERENCES

1. Basha, S. M. – Musingo, M. – Colova, V. S.: Compositional differences in the phenolics compounds of muscadine and bunch grape wines. African Journal of Biotechnology, *3*, 2004, pp. 523–528.
2. Vazquez, L. C. – Perez-Coello, M. S. – Cabezudo, M. D.: Effects of enzyme treatment and skin extraction on varietal volatiles in spanish wines made from chardonnay, muscat, airen, and macabeo grapes. Analytica Chimica Acta, *458*, 2002, pp. 39–44.
3. Careri, M. – Corradini, C. – Elviri, L. – Nicoletti, I. – Zagnoni, I.: Direct HPLC analysis of quercetin and trans-resveratrol in red wine, grape, and winemaking byproducts. Journal of Agricultural and Food Chemistry, *51*, 2003, pp. 5226–5231.
4. Feijóo, O. – Moreno, A. – Falqué, E.: Content of trans- and *cis*-resveratrol in Galician white and red wines. Journal of Food Composition and Analysis, *21*, 2008, pp. 608–613.
5. Adrian, M. – Jeandet, P. – Breuil, A. C. – Levite, D. – Deborg, S. – Bessis, R.: Assay of resveratrol and derivative stilbenes in wine by direct injection high performance liquid chromatography. American Journal of Enology and Viticulture, *51*, 2000, pp. 37–41.
6. Jackson, R. S.: Wine science: Principles and applications. 3rd ed. Burlington : Elsevier Academic Press, 2008. 789 pp. ISBN 0123736463.
7. Hufnagel, J. C. – Hofmann, T.: Orosensory-directed identification of astringent mouthfeel and bitter-tasting compounds in red wine. Journal of Agricultural and Food Chemistry, *56*, 2008, pp. 1376–1386.
8. Kennedy, J. A.: Grape and wine phenolics: Observations and recent findings. Ciencia e Investigación Agraria, *35*, 2008, pp. 107–120.
9. Revilla, E. – Ryan, J.-M.: Analysis of several phenolic compounds with potential antioxidant properties in grape extracts and wines by high-performance liquid chromatography-photodiode array detection without sample preparation. Journal of Chromatography A, *881*, 2000, pp. 46–469.
10. López, M. – Martínez, F. – del Valle, C. – Orte, C. – Miró, M.: Analysis of phenolic constituents of biological interest in red wines by high-performance liquid chromatography. Journal of Chromatography A, *922*, 2001, 359–363.
11. Russo, P. – Andreu-Navarro, A. – Aguilar-Caballos, M.-P. – Fernandez-Romero, J.-M. – Gomez-Hens, A.: Analytical innovations in the detection of phenolics in wines. Journal of Agricultural and Food Chemistry, *56*, 2008, pp. 1858–1865.
12. Bonerz, D. P. M. – Pour Nikfardjam, M. S. – Creasy, G. L.: A new RP-HPLC method for analysis of polyphenols, anthocyanins, and indole-3-acetic acid in wine. American Journal of Enology and Viticulture, *59*, 2008, pp. 106–109.
13. Darias-Martín, J. J. – Andrés-Lacueva, C. – Díaz-Romero, C. – Lamuela-Raventós, R. M.: Phenolic profile in varietal white wines made in the Canary Islands. European Food Research and Technology, *226*, 2008, pp. 871–876.
14. Weingerl, V. – Strlič, M. – Kočar, D.: Comparison of methods for determination of polyphenols in wine by HPLC-UV/VIS, LC/MS/MS and spectrophotometry. Acta Chimica Slovenica, *56*, 2009, pp. 698–703.
15. Belajová, E. – Suhaj, M.: Determination of phenolic constituents in citrus juices: Method of high performance liquid chromatography. Food Chemistry, *86*, 2004, pp. 339–343.
16. Enhanced diode-array detector sensitivity and automated peak purity control. Technical note. Publication number 5965–5900E. Waldbronn : Agilent Technologies, 1997. 8 pp.
17. del Álamo, M. – Casado, L. – Hernández, V. – Jiménez, J. J.: Determination of free molecular phenolics and catechins in wine by solid phase extraction on polymeric cartridges and liquid chromatography with diode array detection. Journal of Chromatography A, *1049*, 2004, pp, 97–105.
18. Goldberg, D. M. – Tsang, E. – Karumanchiri, A. – Diamandis, E. P. – Soleas, G. – Ng, E.: Method to

assay the concentrations of phenolic constituents of biological interest in wines. Analytical Chemistry, *68*, 1996, pp. 1688–1694.
19. Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal of the European Communities, L221, 2002, pp. 8–36.
20. Mattila, P. – Astola, J. – Kumpulainen, J.: Determination of flavonoids in plant material by HPLC with diode-array and electro-array detections. Journal of Agriculture and Food Chemistry, *48*, 2000, pp. 5834–5841.
21. Stenerson, K.: The Analysis of resveratrol in red wine by on-fibre derivatisation / SPME. Reporter Supelco Analytical, *39*, 2010, pp. 21–22.

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