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Short communication

Analysis of phenolic constituents of biological interest in red wines by high-performance liquid chromatography ☆

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Abstract

We describe a reversed-phase HPLC method that uses gradient elution and diode array detection to determine four biologically active phenolic constituents of red wines: gallic acid, *trans*-resveratrol, quercetin and rutin. The method permits direct injection without sample pre-treatment. ODS Hypersil served as the stationary phase; the gradient was formed by acetic acid, methanol, and water. Each analysis required an equilibration period of 10 min and a run time of 50 min for completion. Previously, total phenols were analysed according to the Folin–Ciocalteu method, using gallic acid as the standard, and the results are given as gallic acid equivalent.

Keywords: Wine; Food analysis; Phenolic compounds; Gallic acid; Resveratrol; Quercetin; Rutin

1. Introduction

It is of great interest to evaluate the phenolic constituents of red wines because flavonoids and phenolic acids are widely distributed in higher plants and form part of the human diet. Common foods of plant origin contain a variety of hydroxylated flavonoids and other phenolics in amounts ranging from traces to several grams per kilogram [1]. Flavonoids and other plant phenolics have been reported to have multiple biological effects such as antioxidant activity, anti-inflammatory action, inhibition of platelet aggregation, and antimicrobial activities [2,3]. Grapes and wines contain large amounts of phenolic compounds, mostly flavonoids at high concentrations of 1000–1800 mg/l [4]. A large part of the phenolics in wines may act as antioxidants [5]. Some of these compounds may act selectively at very low concentrations to inhibit ex vivo low-density lipoproteins (LDLs) oxidation in vitro [6,7].

Reduced mortality from coronary heart disease (CHD) among moderate consumers of alcohol is a well-established epidemiologic phenomenon [8]. There is some evidence that those who regularly drink wine may have lower coronary heart disease mortality than those whose preference lies with other alcoholic beverages. This latter possibility has created great interest in constituents found in wine that may be responsible for these putative effects. It has been suggested that the moderate intake of wine provides protection against CHD because the anti-

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oxidant properties of the phenolic compounds of wine delay the onset of atherogenesis and regulate thrombotic tendencies. The ''French paradox'' (apparent compatibility of a high fat diet with a low incidence of CHD) has been attributed to the regular consumption of red wine [9]. The constituents of red wine have always been of interest due to the French paradox and the potential contributory role of the flavonoid constituents to decreasing coronary heart disease [10]. Recent interest in these phenolic constituents of red wine, including gallic acid, *trans*-resveratrol, quercetin and rutin has been stimulated by the potential beneficial effects on health.

Some recent studies have determined polyphenolic compounds in wines principally using chromatographic techniques, with detection being carried out by spectrophotometry [11–13] or fluorimetry [14,15].

In the present study, separation of the phenolic components, gallic acid, *trans*-resveratrol, quercetin and its glucoside rutin, was determined using reversed-phase liquid chromatography. Detection was performed using photodiode array detection. The method permitted the identification of the phenols in different types of wines by direct injection, without any prior purification of the sample. Previously, total phenols were analysed according to the Folin–Ciocalteu method, using gallic acid as the standard, and the results are given as gallic acid equivalents (GAEs).

2. Experimental

2.1. Apparatus

Absorption spectra were recorded on a Perkin-Elmer (Beaconsfield, UK) Lambda 16 UV–Vis spectrophotometer. The liquid chromatograph was from Merck–Hitachi (Darmstadt, Germany) and was equipped with a diode-array detector (L-4500, Merck), a biocompatible intelligent pump (Merck L-6220), an eluent mixing chamber, a manual injector with 20-μl loop and a chromatographic data processing software (Model D-6500, Hitachi). The operating conditions were at room temperature.

2.2. Chemicals and standards

Standard substances of *trans*-resveratrol, quercetin and rutin were purchased from Sigma (St. Louis, MO, USA). Gallic acid was obtained from Merck (Darmstadt, Germany). Solvents used for chromatography were acetic acid and methanol of high-performance liquid chromatography (HPLC) ultra gradient grade supplied by Merck and deionised water. Methanol of Uvasol grade from Merck was used for preparing standard solutions. 0.45-μm pore size membrane filters from Millipore were used for filtration of the mobile phase and the samples.

2.3. Wine samples

A group of commercially available red wines from different Spanish regions was analysed. Samples were opened, protected against sunlight and stored at 4°C. Analyses were carried out within a few days. The samples were filtered through a 0.45-μm membrane Millipore chromatographic filter.

2.4. Procedure

2.4.1. Colorimetric determination of polyphenols content

The concentration of wine phenolics was estimated by analysing for total phenol by the Folin–Ciocalteu procedure and expressing results in micrograms per milliliter or molar equivalents of gallic acid, a naturally occurring polyphenol. A calibration curve was prepared using concentrations of gallic acid ranging from 0 to 500 mg/l.

2.4.2. Chromatographic conditions

The separation was performed using an ODS Hypersil 5 μm column, 250 mm×4 mm I.D. as stationary phase preceded by a guard column of LiChrospher 100 RP-18, 5 μm, 4 mm×4 mm. Samples of 20 μl of wine or calibration standard were directly injected onto the column and eluted with a gradient comprising acetic acid (A), methanol (B) and water (C). Zero-time conditions were A–B–C (5:15:80) at a flow-rate of 0.4 ml/min. After 5 min, the pumps were adjusted to A–B–C (5:20:75) at a flow-rate of 0.5 ml/min, and at 30 min to A–B–C (5:45:50) at 0.5 ml/min until termination of

the run at 50 min. The system was equilibrated using the starting conditions for 10 min prior to injection of the next sample. Detection was routinely carried out by monitoring the absorbance signals at 280, 306 and 360 nm. At the end of each day, the column was washed with the zero-time solvent mixture. The wine samples, standard solutions and mobile phases were filtered by a 0.45-μm pore size membrane filter, and degassed before their use.

2.4.3. Calibration graphs

Standard calibration curves were established by plotting the area of peaks against different concentrations of phenolic compounds (varying from 0.5 to 25 μg/ml for gallic acid, quercetin and rutin, and from 0.1 to 15 μg/ml for *trans*-resveratrol). The optimum absorbance found for *trans*-resveratrol is in accordance with some recent accurate studies [16,17]. Standard solutions of *trans*-resveratrol were prepared in obscurity.

2.4.4. Evaluation of the peak purity and linearity

To check the peak purity, the eluates were monitored with a photodiode array detector (λ=200–400 nm). The three spectra corresponding to the upslope, apex and downslope of each peak were computer normalised and superimposed. Peaks were considered pure when there was exact coincidence between the three spectra (match factor ≥99.5).

The linearity of the detector responses for the prepared standards was assessed by means of a linear regression analysis regarding the amounts of each standard (measures in μg) introduced in the loop of the chromatographic system and the area of the corresponding peak on the chromatogram.

3. Results and discussion

3.1. Total polyphenols content

Red wines contain large amounts of phenolic compounds. The concentration of phenolics, estimated by analysing total phenols in wines, is presented in Table 2. The results show that the red wines contain high concentrations of phenolics. Similarity results were obtained when wines were distilled to remove alcohol by vacuum and diluted to the original concentration by distilled water at room temperature.

Recently much attention has been focused on the protective biochemical function of naturally occurring antioxidants in biological systems and on the mechanisms of their action. Most phenolic compounds, which occur widely in plants, are present in red wines. Our results confirm a variation in phenolic content among several red wines, from 1800 to 2300 mg/l phenolics. These results are in agreement with those published by others authors [4].

3.2. Spectroscopic identification and maximum wavelength of standard phenols

It is well known that most flavonols (quercetin and its glycoside rutin) exhibit two major absorption bands in the ultraviolet–visible region, Band I in the 320–385 nm range, and band II in the 250–285 nm. Gallic acid and *trans*-resveratrol present a maximum of absorbance at 280 and 306 nm, respectively. The λ_{max} and ϵ values in methanol at various wavelengths of the phenolic compounds studied in this work are presented in Table 1.

3.3. Analysis of phenolic compounds using photodiode array detection

Although normal-phase chromatography has been used for the separation of phenolic compounds, it is now generally agreed that reversed-phase HPLC is the method of choice for the separation of a wide variety of phenolic compounds. Generally, such separations are rapid and provide high resolution and sensitivity. Besides, the reversed-phase chromatography was selected because polyphenols are insoluble in water but soluble in alcohols. The stationary phase was ODS (C_{18}), which permitted greater

Table 1
Absorbance data for standard phenolic compounds

Phenolic compound	λ_{max} (nm)	$\epsilon_{\lambda_{max}}$ (M^{-1} cm^{-1})
Gallic acid	272	10 210
trans-Resveratrol	306	29 352
Quercetin	373	24 373
	256	23 797
Rutin	360	17 699
	259	21 153

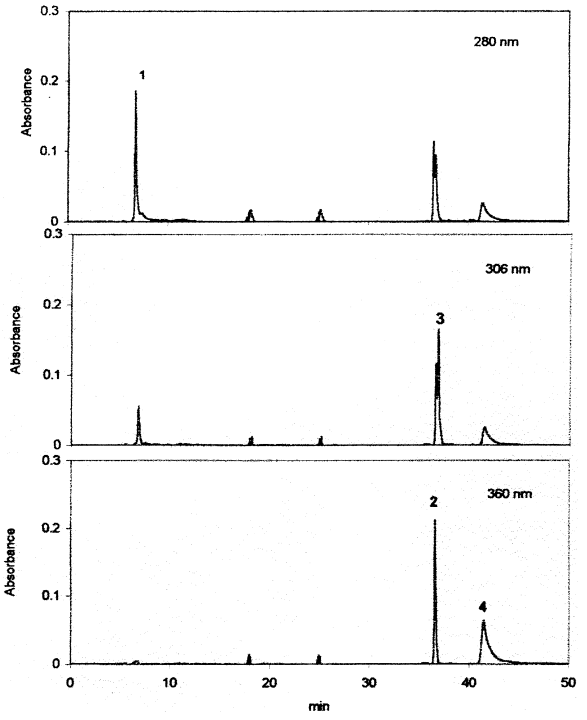

Fig. 1. Chromatographic profile using gradient elution. The peaks correspond to: 1, gallic acid (25 μg/ml) measured at 280 nm; 2, rutin (25 μg/ml) measured at 360 nm; *trans*-resveratrol (5 μg/ml) measured at 306 nm and 4, quercetin (25 μg/ml) measured at 360 nm.

retention. The organic solvent selected for preliminary experiments was methanol (MeOH) due to the high solubility of phenols in this solvent. The retention behaviour of phenols was studied in the presence of an acid, which prevented ionisation of the hydroxyl groups. An initial mobile phase containing methanol–5% aqueous acetic acid was selected. The chromatograph obtained using this programme is shown in Fig. 1. The elution order and the retention characteristics were: 1, gallic acid (t_R=6.7 min); 2, rutin (t_R=36.5 min); *trans*-resveratrol (t_R= 37.1 min); quercetin (t_R=41.5 min).

3.4. Analysis of wines

Once the chromatographic conditions for the separation had been studied, the procedure was applied to the determination of phenol components in wines. These studies were carried out using photodiode array detection. The identification of the different compounds was achieved by comparison of both retention time and the absorption spectra obtained for each eluted peak with those obtained for the standards. The concentrations of the components were calculated from the chromatogram peak areas (Table 2).

Five Spanish red wines were analysed for their concentration in phenolic compounds. A typical chromatogram is shown in Fig. 2 (monitored at 280 and 360 nm). As regards red wines, the concentration of these substances seem to vary considerably, since it depends on diverse factors, such as cultivar, climate and the vinification techniques [2,3]. Gallic acid was present in all analysed wines (Table 2) and the level of this compound in wines was similar to those described in a global survey [1]. Sample 2 contained a considerably level of *trans*-resveratrol. Quercetin and rutin were measurable in only one of five wines.

Table 2
Phenolic compound concentrations and phenol detected in several commercial red wines

Wine	Phenolics[a] (mg/l)	Gallic acid (μg/ml)	Rutin (μg/ml)	*trans*-Resveratrol (μg/ml)	Quercetin (μg/ml)
Sample 1	1857	53.3	ND	ND	ND
Sample 2	2315	46.2	ND	1.34	ND
Sample 3	1848	48.1	ND	ND	ND
Sample 4	2050	38.8	4.62	ND	ND
Sample 5	1950	27.7	ND	ND	4.66

ND, Not detected.
[a] Total phenols are expressed as gallic acid equivalents (GAEs).

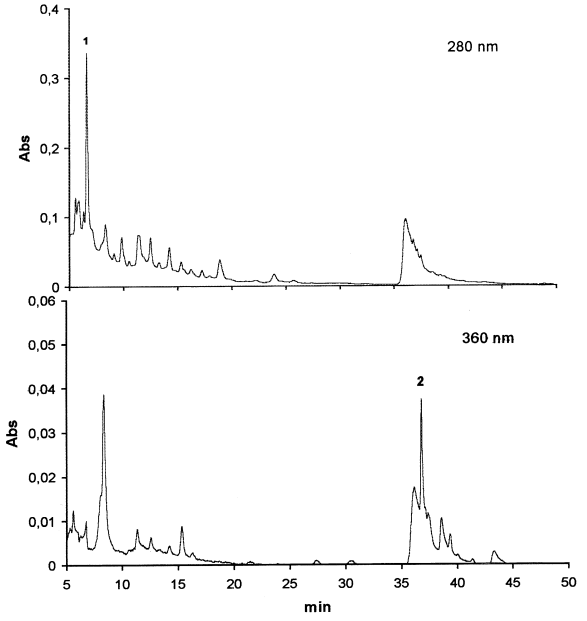

Fig. 2. Chromatogram for a red wine sample using gradient elution and photodiode array detection. The peaks correspond to: 1, gallic acid, measured at 280 nm, and 2, rutin, measured at 360 nm.

4. Conclusions

We have described a method for the analysis of four phenolic constituents of wine by HPLC of a 20-μl sample which is directly introduced without the need for prior preparatory procedures. Further investigation is required to determine the effects of geographical origin, ageing, climate and the vinification techniques. We expect this assay to also be suitable for the analysis of the phenolic compounds in various types of wines and other beverages such as beers and liqueurs. Furthermore, molar absorptivities over a range of commonly reported wavelengths were accurately determined for phenolic compounds.

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