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Determination of phenolic acids in wine by high-performance liquid chromatography with a microbore column

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Abstract

A method for the extraction and separation of the non-volatile phenolic acids of wine is described. The extracts are analysed by HPLC with a microbore column and UV detection. The free phenolic acids and depsides in different wine samples were identified and determined.

1. Introduction

In a previous study [1], free phenolic acids in wine were separated and identified by HPLC after solid-phase extraction. Some compounds with retention times near those of phenolic acids were still present. In this work, an improved purification method was developed to obtain a better separation of the non-volatile phenolic compounds of wine. The fractions obtained were analysed by HPLC, using a microbore column (250 mm × 1.1 mm I.D.) and a rapid-scanning UV detector with a data system, for the identification of the characteristic peaks. The free phenolic acids were determined. The identities of the hydroxycinnamic acid–tartaric acid esters or depsides (esters of tartaric acid with cinnamic, caffeic, ferulic and *p*-coumaric acid) [2–4] were established through alkaline hydrolysis of the esters followed by HPLC of the free phenolic acids of the hydrolysate.

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2. Experimental

2.1. Apparatus

Two Phoenix 20 (Fisons) syringe pumps (master and slave) were interfaced to an external computer (IBM) for remote control operations. A rapid-scanning UV–Vis detector (Micro UVIS 20) was used. The injection valve was a Rheodyne Model 7520 with a 1.0-μl sample loop. The columns (250 mm × 1.1 mm I.D. and 500 mm × 1.1 mm I.D.) were slurry-packed in the laboratory with Spherisorb ODS_2 (5 μm) obtained from Phase Separations (Norwalk, CT, USA) [5].

2.2. Reagents

Distilled water was stored in glass, filtered and passed through a Norganic system cartridge (Millipore, Bedford, MA, USA).

The solvents methanol (HPLC grade), phosphoric acid and diethyl ether (RPE) (peroxides were eliminated by filtration through a CN

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disposable extraction column) were obtained from Carlo Erba (Milan, Italy). Polyethylene glycol 20 000 (PEG) and phenolic acids were purchased from Fluka (Buchs, Switzerland). All the cinnamic acids were in the *trans* form.

Phosphate buffer (pH 2.4) was prepared by adding 1.15 g of ammonium dihydrogenphosphate to 0.2 ml of phosphoric acid and diluting to 1 l with distilled water. The pH was monitored and more phosphoric acid was added if needed. This stock solution was filtered through a 0.45-μm Millipore filter and stored at 4°C. A 70.23-g amount of sodium perchlorate was added to this buffer solution. Phosphate buffer (pH 8.0) was prepared by mixing 47.5 ml of 0.1 *M* Na_2HPO_4 and 2.5 ml of 0.1 *M* KH_2PO_4. This stock solution was then filtered through a 0.45-μm Millipore filter and stored at 4°C.

A 3-ml Sep-Pak C_{18} cartridge was obtained from Waters (Milford, MA, USA) and a Bakerbond (quaternary amine) and a 3-ml CN cartridge from J.T. Baker (Phillipsburg, NJ, USA). The Sep-Pak C_{18} and CN cartridges were washed with methanol and stored in methanol and the Bakerbond cartridge with 1–2 column volumes of methanol, water, 0.1 *M* HCl and water.

2.3. Standards

A 2-mg amount of gallic acid (**1**) and 1 mg of 3,4-dihydroxybenzoic acid (**2**), vanillic acid (**3**), caffeic acid (**4**), syringic acid (**5**), *p*-coumaric acid (**6**) and ferulic acid (**7**) were each dissolved in 1 ml of methanol. The following amounts of the acids were transferred into a screw-capped vial and diluted to 900 μl with methanol: **1** 15, **2** 100, **3** 25, **4** 20, **5** 20, **6** 15 and **7** 25 μl. These solutions were stored at −20°C in the dark for no longer than 2 months.

2.4. Preparation of wine samples

After evaporation of the alcohol under vacuum below 30°C, to 1 ml of red or 2 ml of white wine was added a solution of phosphate buffer (pH 8) (1.0 ml for red and 1.5 ml for white wine). This solution was extracted with 3 × 1.5 ml of diethyl ether and the extracts were collected, dried over anhydrous sodium sulphate and evaporated under nitrogen (below 30°C in the dark). The residue was dissolved in 50 μl of methanol (alkaline extract). The remaining solution of wine was acidified with 6 *M* HCl to about pH 1.0. This solution was extracted with 3 × 1.5 ml of diethyl ether saturated with PEG [6]. The extract, dried over anhydrous sodium sulphate, was divided into two parts and evaporated under nitrogen. One residue sample was dissolved in 50 μl of methanol (acid extract); the second residue was dissolved in 2 ml of water and passed through the strong anion-exchange cartridge. The trapped compounds were washed with water and eluted with 14 ml of phosphate buffer (pH 2.4)–sodium perchlorate. This solution was passed through a Sep-Pak C_{18} cartridge. The acidic compounds were recovered by elution with 2 ml of methanol. The solution was dried and evaporated as above and the residue was dissolved in 50 μl of methanol (anionic extract). These extraction procedures are outlined in Fig. 1.

The solutions were stored in 1-ml screw-

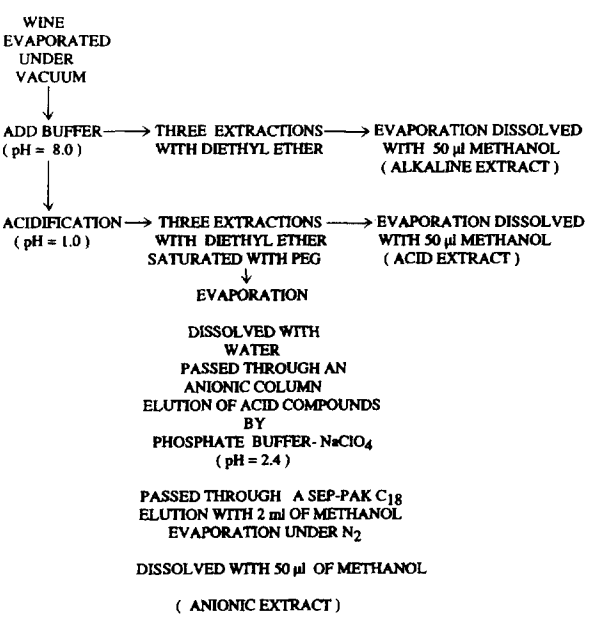

Fig. 1. Sample treatment for extraction of non-volatile compounds from wine.

Table 1
Recovery of standard acids added to 1 ml of red wine (mean of seven determinations)

No.	Acid	Recovery (%)	R.S.D. (%)
1	Gallic	75.6	3.5
2	3-4-Dihydroxy-benzoic	88.6	11.5
3	Vanillic	83.1	9.0
4	Syringic	85.3	10.2
5	Caffeic	85.1	8.9
7	*p*-Coumaric	84.1	9.6
8	Ferulic	80.4	8.5

capped vials (conical bottom) at −20°C in a freezer.

2.5. Hydrolysis of depsides

To 2-ml wine samples were added 2 ml of 2 *M* NaOH solution after evaporation of ethanol as described. The mixture was allowed to stand at room temperature, in the dark, for 48 h under nitrogen [2,7]. The alkaline solution was acidified to pH 1.0 and the free phenolic acids were extracted as described for the acid extract.

2.6. Recoveries

The recoveries were measured as outlined in Fig. 1 for acid extracts. Six samples of Corvo red wine and six of the same wine with 250 μl of the standard mixture added, were analysed (Table 1).

3. Results and discussion

Fig. 2 shows the chromatogram of cinnamic and benzoic acid standards. At 280 nm, all seven peaks are detected, whereas at 320 nm, only the peaks of caffeic, *p*-coumaric and ferulic acids are detected. In nature the cinnamic acids are present as *trans* isomers, but on exposure to UV radiation or daylight there is a gradual formation of *cis* isomers [8]. Figs. 3 and 4 show the chromatograms of the acids extracted from red and white wine, respectively, with detection at

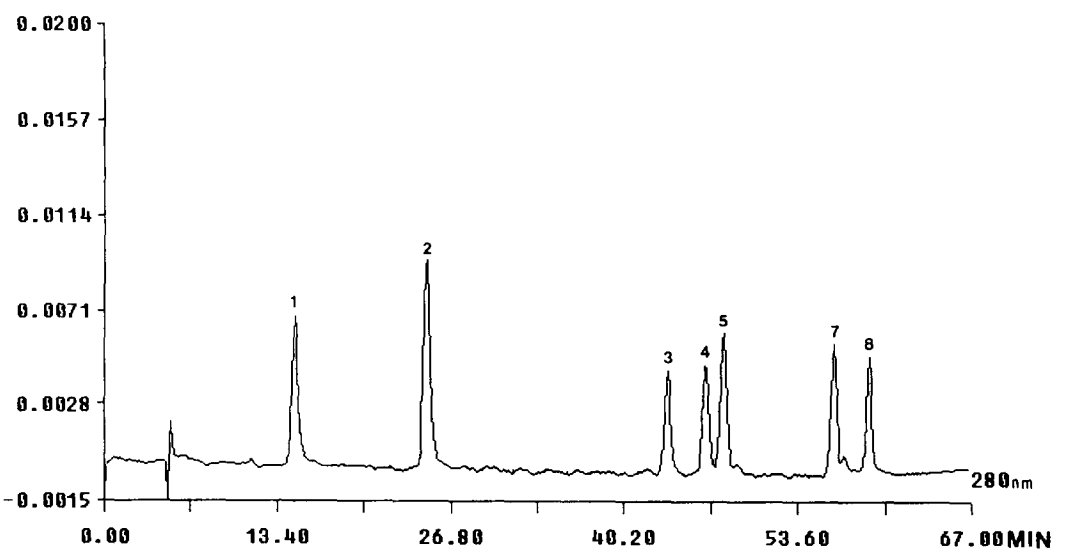

Fig. 2. Chromatogram of standard mixture (1 μl) detected at 280 nm. Peaks: 1 = gallic acid; 2 = 3,4-dihydroxybenzoic acid; 3 = vanillic acid; 4 = caffeic acid; 5 = syringic acid; 7 = *trans*-*p*-coumaric acid; 8 = ferulic acid. Column, ODS_2 (250 mm × 1.1 mm I.D.); flow-rate, 40 μl/min; mobile phase, A–methanol–phosphate buffer (pH 2.4) (95:5), B = phosphate buffer (pH 2.4)–distilled water (5:95), with the gradient 0 min, 100%B; 67 min, 56A%; 70 min, 100%A.

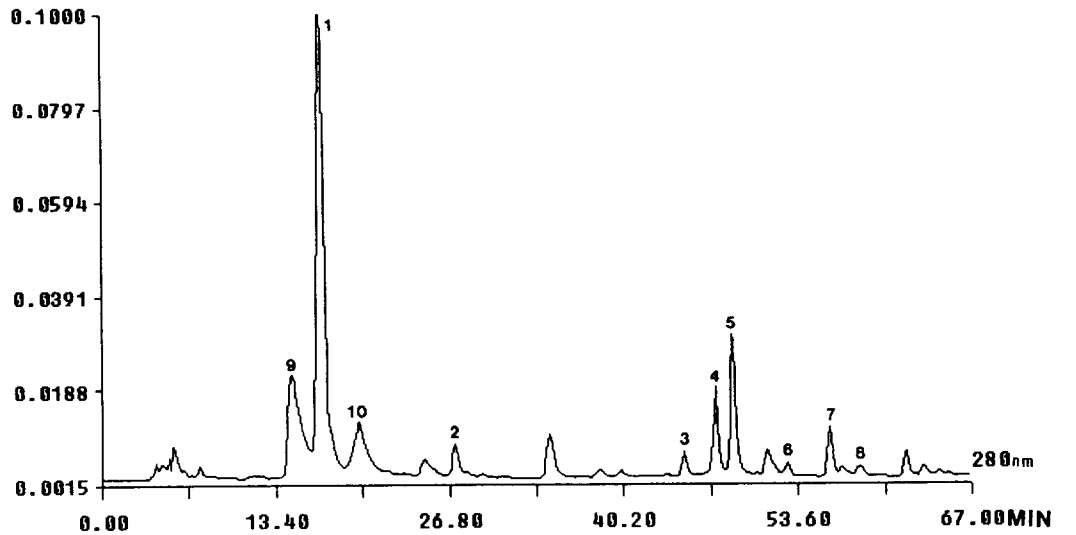

Fig. 3. Chromatogram of Corvo red wine (acid extract). Peaks as in Fig. 2; 6 = *cis*-*p*-coumaric acid; 9 = depside of caffeic acid; 10 = depside of *p*-coumaric or ferulic acid.

280 nm. Peak 9 (Fig. 3) is the depside of caffeic acid (**9**) whereas peak 10 is the *p*-coumaric or ferulic depside that also absorbs at 350 nm.

The second purification step (anionic extract) (Fig. 5) was carried out only as a confirmatory test. Here the acids are better separated from the impurities and the depsides are not present. Some different solvents were tested and the best recoveries were obtained with diethyl ether (peroxide free) saturated with PEG.

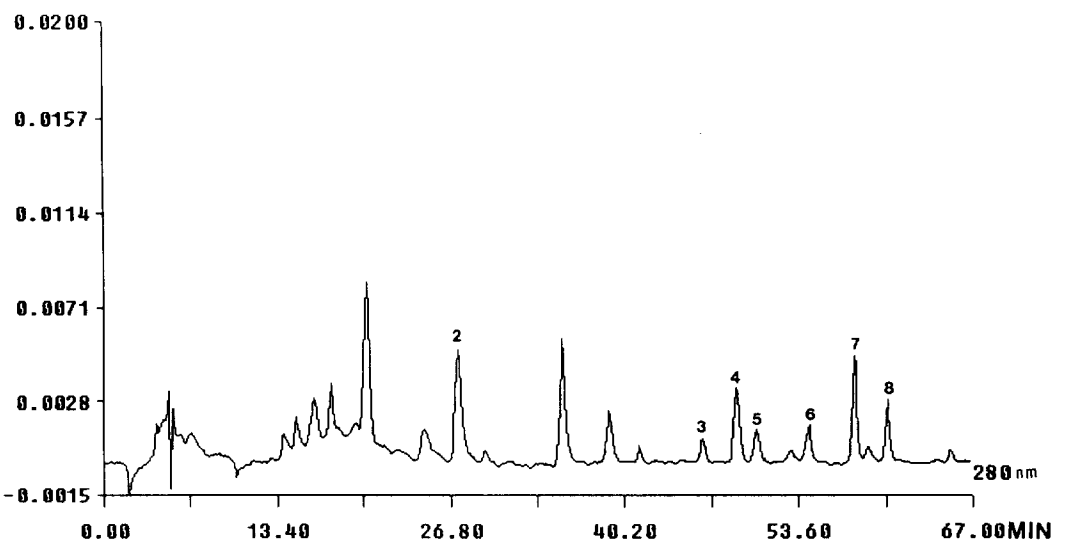

Fig. 4. Chromatogram of Corvo white wine (acid extract). Peaks as in Fig. 2.

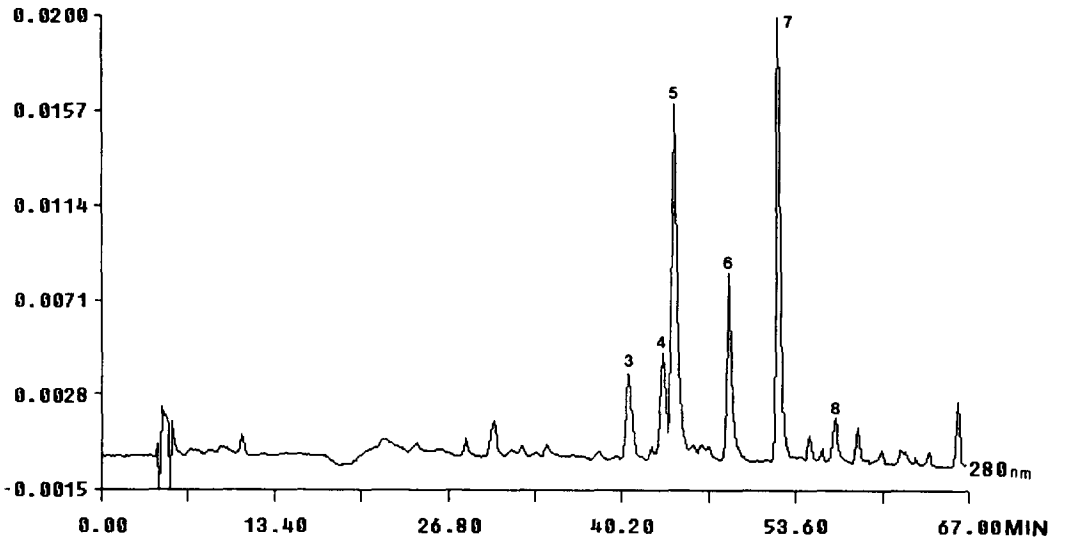

Fig. 5. Chromatograms of anionic extract of Corvo red wine detected at 280 nm. Peaks and conditions as in Fig. 1.

Recoveries at different pH (8–10) were also investigated by adding 2 ml of distilled water to 50 μl of phenolic acids solution and treating the samples as illustrated in Fig. 1 (acid extract). At pH 10, *trans*-caffeic acid is decomposed and *trans*-*p*-coumaric acid is partially converted into the *cis*-isomer.

To confirm the presence of cinnamic acid depsides, an alkaline hydrolysis of wine was carried out to obtain tartaric acid and free

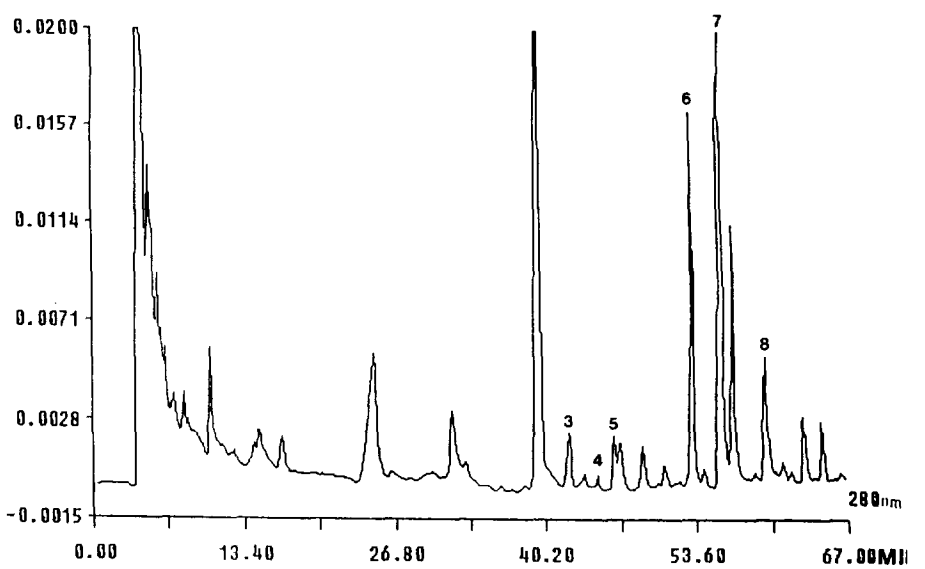

Fig. 6. Chromatogram of Corvo red wine after alkaline hydrolysis and acid extraction, with detection at 280 nm. Peaks and conditions as in Fig. 2.

phenolic acids. The caffeic acid is completely decomposed, because the wine samples were maintained in alkaline solution for a long time. Fig. 6 shows the chromatogram obtained after alkaline hydrolysis. Peaks 9 and 10, assigned to depsides in Fig. 3 are no longer present and there are large amounts of *cis*- and *trans*-*p*-coumaric and ferulic acid. The quantitative data are reported in Table 2 (average data from six determinations for each sample). In white wine, gallic acid is present in small amounts. For the determination of *p*-coumaric acid, the absorption coefficient at 280 nm was used, where the two forms (*trans* and *cis*) have the same absorption.

Fig. 7 shows the chromatogram of red Corvo wine obtained after direct acid extraction (pH

Table 2
Phenolic acid concentrations (μg/l) in wine, vermouth and beer

Acid[a]	Corvo (red)	Chianti (red)	Salice Salentino (red)	Cerveteri (red)	Porto (white)	Vermouth (red Martini)	Verdicchio (white)
1	455.1	409.1	217.7	290.5	182.8	–	–
2	72.8	120.7	79.2	52.5	81.2	45.2	15.0
3	33.7	24.6	17.0	22.4	42.4	29.5	7.3
4	83.7	47.3	91.7	86.7	27.7		14.4
5	70.4	24.8	40.0	23.1	81.5	52.6	3.8
6	2.3	5.1	5.0	2.9	5.4	3.3	3.8
7	26.3	10.3	15.6	19.8	14.1	5.1	13.2
8	11.4	9.8	7.2	12.5	7.4	4.6	7.2

	Vernaccia San Gimignano (white)	Frascati Superiore (white)	Robola (white)	Pinot Grigio (white)	Cerveteri (white)	Corvo (white)	Moretti beer
1	–	–	–	–	–	–	–
2	51.3	24.2	64.9	12.6	14.1	61.0	
3	7.9	5.4	4.6	4.2	4.5	5.1	16.1
4	41.0	23.4	38.8	27.6	49.6	15.1	4.2
5	9.9	3.2	–	4.3	3.6	4.4	2.2
6	5.4	4.3	12.1	3.4	4.1	3.4	–
7	13.3	14.3	60.8	11.6	4.4	7.1	17.1
8	10.8	13.3	21.6	11.7	3.2	9.3	40.4

	Ciro (white)	Fontana Candida (white)	R.S.D. (%)[b]
1	–	–	2.0
2	10.9	21.0	3.1
3	5.7	4.6	4.8
4	24.3	12.7	2.9
5	3.0	3.1	5.4
6	3.9	4.6	10.4
7	2.6	8.9	6.1
8	6.7	8.3	5.8

[a] See Table 1
[b] $n = 6$

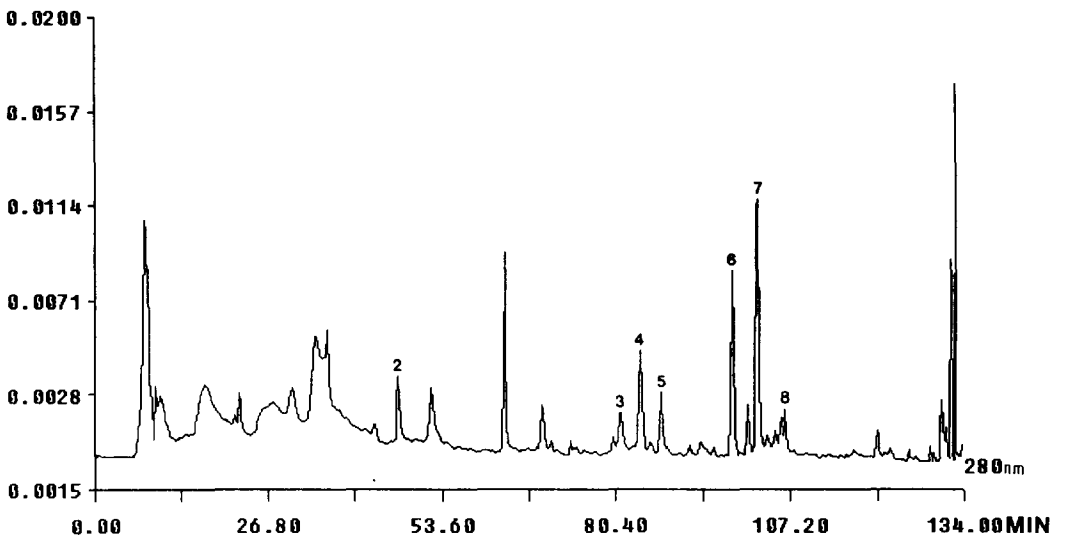

Fig. 7. Chromatogram of Corvo red wine after direct acid extraction (pH 1.0). Column 2 (500 mm × 1.1 mm I.D.). Conditions as in Fig. 2 except that the gradient was doubled in time.

1.0), utilizing a 500-mm microbore column. A good separation was obtained for all the compounds investigated.

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