Polyphenols Newly Extracted in Red Wine from Southwestern France by Centrifugal Partition Chromatography

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Polyphenols from the ethyl acetate extracts of red wine were successfully fractionated using a four-step process (solvent extraction, ion-exchange column chromatography, centrifugal partition chromatography, and semipreparative HPLC), which resulted in the isolation of 22 compounds belonging to different classes of polyphenols (stilbenes, cinnamic acids, flavonoids). Five of them are red wine constituents reported for the first time. The newly isolated compounds include resveratrol dimers, dihydroflavonols, and a cinnamic derivative.

Keywords: *Red wine; stilbenes; dihydroflavonols; centrifugal partition chromatography*

INTRODUCTION

Coronary heart diseases (CHD) have been associated with a high intake of saturated fat in the diet, but an exception seems to exist in France. The French paradox (*1*, *2*) has been attributed to the regular consumption of wine. It has been hypothesized that the phenolic substances of red wine might be responsible for these potential beneficial effects by their antioxidant and antiinflammatory properties (*3*−*5*). Indeed, they inhibit the oxidation of human low-density lipoproteins (*6*) and the aggregation of platelets (*7*−*9*) and produce endothelial nitric oxide-dependent vasorelaxation (*10*), which may in turn explain the decrease in CHD observed among regular and moderate wine drinkers. Moreover, wine polyphenols have been reported to have anticarcinogenic properties, delaying tumor onset in transgenic mice (*11*). Recently, *trans*-resveratrol was shown to have cancer chemopreventive activity in assays on three major stages of carcinogenesis (*12*).

Stilbenes occur naturally in various families of plants (*13*), but grapes and related products are considered the most important dietary sources of these substances (*14*). Beside *trans*- and *cis*-resveratrol, three stilbene glucosides have been characterized in wine: piceid, in its two isomeric forms *cis*- and *trans*- (*15*, *16*), and *trans*-astringin (*17*, *18*). Recently, novel stilbene derivatives were isolated from a commercial Riesling wine, such as resveratrol-2-C-glucosides and glucosides of resveratrol dimers (*19*). Moreover, as yet unreported stilbenes may be present in wine, such as *cis*- and *trans*-resveratroloside, resveratrol *trans*-dehydrodimer and its 11- and 11′-*O*-glucosides, which have been recently characterized in *Vitis vinifera* cell suspension cultures (*20*, *21*). Other phenolic substances in wine such as phenolic acids and flavan-3-ols are present in high amounts in red wine (*22*), but dihydroflavonols (flavanonols) appear to be rare in plants and food, and little is known about their occurrence in wine.

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Fractionation of polyphenols from natural sources is usually performed using column chromatography and HPLC. Although these methods are useful for analyses, they are not suitable for large-scale separation because they are time-consuming and use large amounts of solvents. Centrifugal partition chromatography has the advantage over other chromatographic techniques of not using a solid support matrix and therefore eliminating the irreversible adsorption of the sample. The aim of this work was to apply this procedure to a red wine extract to isolate and identify polyphenolic compounds, especially stilbenes, of potential biological activities.

MATERIALS AND METHODS

Apparatus. The centrifugal partition chromatograph apparatus (Sanki, model LLB-M) consisted of a centrifuge with a column of 230 mL capacity. The solvents were pumped into the column rotating at 1000 rpm with HPLC pumps (Bischoff, model 2250) at a flow-rate of 3 mL/min. A UV absorbance detector (Waters, model 480) was used at 280 nm, and fractions were collected with a fraction collector (LKB 2111 MultiRac model).

Solvents. Solvents were purchased in HPLC-grade quality or redistilled before use. The solvent system for centrifugal partition chromatography was H_2O/EtOH/hexane/EtOAc in the ratio 3/3/4/5 (v/v) or 7/2/1/8 (v/v) (Delaunay, J. C., and Castagnino, C., unpublished results). In each experiment, the resulting lower layer was used as the stationary phase for the ascendant mode.

Standards. *trans*-Resveratrol, (+)-catechin, (−)-epicatechin, caffeic acid, vanillic acid, syringic acid, taxifoliol, myricetin, and quercetin were provided by Sigma (France). Tyrosol, isorhamnetin, and isoquercitrin were purchased from Extrasynthese (France). *trans*- and *cis*-piceid and pallidol were obtained from grape cell cultures and characterized as already described (*20*, *21*). *cis*-resveratrol was obtained by enzymatic hydrolysis of *cis*-piceid with a β-glucosidase (EC 3.2.1.21, Sigma) as follows: 10 mg of enzyme was mixed with 10 mg of compound in 10 mL of H_2O adjusted to pH 6 with NaOH (0.1 N). The mixture was incubated at 25 °C overnight (15 h) and extracted twice with EtOAc. *cis*-Resveratrol was purified by semipreparative HPLC.

Preparation and Fractionation of Red Wine Extract. Blended red wine (4 L) from southwestern France (A. O. C.

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Bergerac, 2000 vintage) was concentrated in vacuo and extracted four times with EtOAc. The concentrated EtOAc residue was then chromatographed over a 1.5 × 60 cm cation-exchange resin column (DOWEX, Sigma), rinsed with distilled water (4 L), and eluted with 75% (v/v) aqueous MeOH (2 L) to yield 1.320 g of solid powder after lyophilization. For further fractionation, this residue was submitted to two successive steps of centrifugal partition chromatography (CPC). In a first run, it was dissolved in 8 mL of the lower layer of the solvent mixture and fractionated using the solvent system in the ratio 3/3/4/5 (v/v). The ascendant mode yielded four major fractions (A−D), and the descendant mode did not show good separation, as suggested by TLC plates. Thus, these fractions were combined, lyophilized (fraction X, 993 mg) and submitted to a second run of CPC with solvents in the ratio 7/2/1/8 (v/v). The descendant mode made it possible to fractionate X into 6 fractions (E−J), and the descendant mode yielded a mixture fraction Y (232 mg).

Analysis of Fractions. Monitoring of the collected fractions was achieved by TLC on Polygram silica gel 0.2 mm with fluorescent indicator UV_{254} (Macherey-Nagel) in the mixture $CHCl_3$/MeOH/HCOOH, 85/15/3 (v/v). Revelation of TLC plates was done by spraying anisaldehyde reagent.

Isolation and Identification of Polyphenols. Final purifications of each fraction (A−J) were achieved by semi-preparative HPLC with a 4 × 250 mm Ultrasep RP18 reverse phase column (4 μm) (Bischoff) at room temperature using the solvents: H_2O/TFA, 97.5/2.5 (v/v) (A) and ACN/solvent A, 80/20 (v/v) (B) with gradient system as described earlier (*20*) and detection at 280 nm. ^{1}H NMR spectra were recorded at 303 K in the Fourier transform mode at 500.13 MHz on a Bruker AMX 500 spectrometer equipped with a broad band 20-mm probe, using a spectral width of 20 ppm and TMS as internal standard. Chemical shifts were expressed as ppm relative to the CD_3OD (3.3 ppm) resonance. Mass spectra were recorded on a VG Autospec-Q in the FAB^+ mode.

RESULTS AND DISCUSSION

We have developed a procedure in which CPC was used as the key step in the preparative scale separation of polyphenols from red wine (Figure 1). However, because of the very diverse nature of the phenolic compounds in the red wine extract, we found extensive chromatographic band overlapping in the reverse mode of the first CPC run (fraction X). Thus, this fraction was submitted to a second CPC procedure with the solvent system in a different ratio. In this way, 22 compounds belonging to different classes of polyphenols (stilbenes, cinnamic acid derivatives, and flavonoids) were obtained in pure form (Table 1). Among them, five are red wine constituents reported here for the first time.

Stilbenes. Six resveratrol derivatives were isolated in several fractions of the two CPC runs. During the first CPC run, *trans*- and *cis*-resveratrol (40 mg) were identified in fraction B by HPLC comparison with authentic standards and according to their ^{1}H NMR data (*23*). *Trans*-resveratrol was first isolated in wine by Siemann and Creasy (*24*) and appears to be involved in the health benefits associated with moderate wine consumption.

Optimization of the HPLC gradient for purification of fraction E (28 mg) made it possible to isolate two compounds (1 mg each) whose mass spectra showed a molecular peak at *m/z* 455 $[M + H]^+$ for both, thus indicating resveratrol dimer structures. The ^{1}H NMR spectrum of compound **1** showed signals corresponding to pallidol, the symmetrical dimer of resveratrol (Figure 2): four aliphatic protons at δ 3.75 and 4.50 ppm (two large singlets) and two AA′BB′ systems characterized by two pairs of proton doublets at δ 6.68 and 6.95 ppm

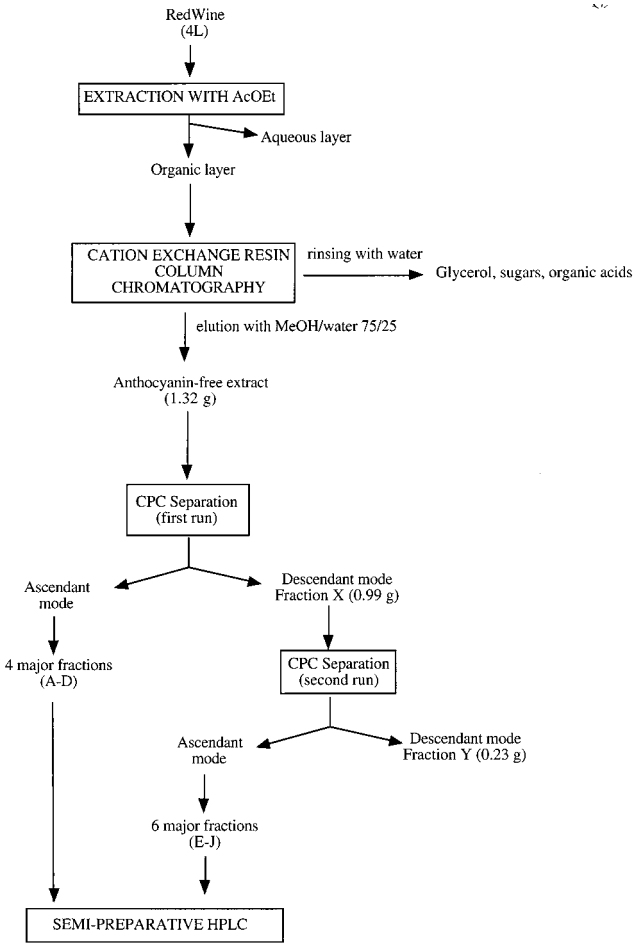

Figure 1. Four-step process for the extraction, fractionation, and purification of polyphenols from red wine.

(J = 8.5 Hz) corresponding to protons of the B and D rings, and by two pairs of proton doublets at δ 6.13 and 6.55 ppm (J = 2 Hz) corresponding to the A and C rings. These NMR data are identical to those published by Khan et al. (*25*) for pallidol, and its identity was confirmed by HPLC comparison with an authentic standard isolated from grape cell suspension cultures (*21*).

The ^{1}H NMR spectrum of compound **2** showed the presence of signals similar to those of **1**: a AA′BB′ system with signals at δ 7.21 and δ 6.78 (2 H, d, J = 8.6 Hz) corresponding to the protons of the phenyl ring C, a two meta-coupled proton system at δ 6.18 and 6.47 (d, J = 2.1 Hz, ring A), and two mutually coupled aliphatic protons (δ 4.22 and 3.70, d, J = 2.6 Hz) of the indane AB ring system. Additional resonances indicated the presence of two other benzene rings: the trisubstitued ring D (δ 6.16, 2 H, br m and δ 6.14, 1H, br m) and the *p*-disubstitued ring E (δ 6.96 and 6.70, 2 H, d, J = 8.5 Hz), together with an olefinic proton singlet at δ 6.28. Hence, compound **2** was identified as parthenocissin A (Figure 2) or one of its stereoisomers by comparison with ^{1}H NMR data reported by Tanaka et al. (*26*) and Adesanya et al. (*27*).

These two resveratrol dimers are red wine constituents reported for the first time. Pallidol was first isolated from *Cissus pallida* (*25*) and is a natural constituent of *V. vinifera*, as reported by Waffo-Téguo et al. (*21*) in grape cell suspension cultures. Furthermore, one of its glucoside has recently been reported at very low levels in a Riesling wine (*19*). Cyclooxygenase-1

Table 1. Time-Course Elution of Polyphenols from Red Wine in the Two CPC Runs

first run	fraction no.	elution time (min)	yield (mg)	main polyphenols
	A	0−69	108	ethylcoumarate
ascendant	B	70−102	80	*trans*- and *cis*-resveratrol
	C	103−162	101	caffeic acid, vanillic acid
	D	163−312	36	myricetin, quercetin, taxifoliol
descendant	X	0−45	993	not separated
second run	**fraction no.**	**elution time (min)**	**yield (mg)**	**main polyphenols**
	E	0−26	54	pallidol (**1**), parthenocissin (**2**)
	F	27−33	34	astilbin (**3**), syringic acid, tyrosol, isorhamnetin
ascendant	G	34−48	190	catechin, epicatechin
	H	49−69	200	dihydromyricetin-3-rhamnoside (**4**), *trans*- and *cis*-piceid
	I	70−122	123	quercetin 3-glucoside, myricetin 3-glucoside
	J	123−336	154	*trans-p*-coumaroyl 6″-glucoside (**5**)
descendant	Y	0−62	232	unidentified compounds

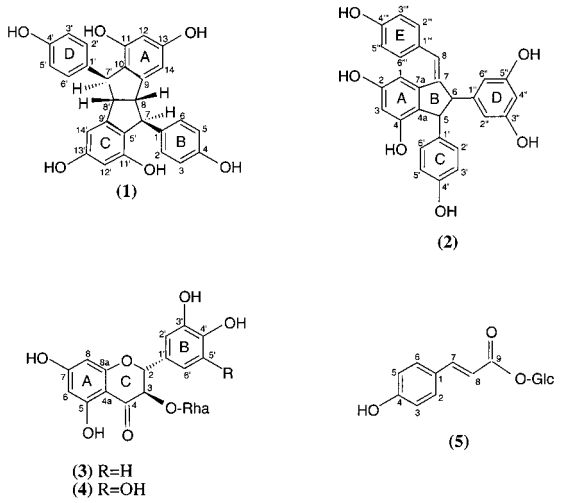

Figure 2. Structures of polyphenols newly isolated from red wine. **1**, pallidol; **2**, parthenocissin A; **3**, astilbin; **4**, dihydromyricetin 3-*O*-rhamnoside; **5**, *trans-p*-coumaroyl 6″-glucoside. Rha = rhamnopyranose; Glc = glucopyranose.

and -2 (COX-1 and -2) inhibitory activities have been studied for pallidol indicating that it was inactive against both enzyme isoforms (IC50 > 70 mg/mL) (*21*). Parthenocissin A was reported in stems of *Parthenocissus quinquefolia* (*26*) and *Cissus quadrangulariz* (*27*).

Two other stilbenes that have already been extracted from grapes and wine were isolated during the second run. These are *trans*- and *cis*-piceid (70 mg in fraction H) identified by HPLC comparison with authentic standards and according to their ^{1}H NMR data (*20*). These resveratrol glucosides are often the major stilbenes in wine and grape juice (*28*) and can reach concentrations superior to 20 mg/L (*18*). Concerning their biological activities, it has been shown that wine stilbenoids are potent constituents in inhibiting the development of DMBA-induced pre-neoplastic lesions in a mouse mammary organ culture assay, thus indicating a possible cancer chemopreventive activity (*29*).

Flavonols. Purification of fraction D (first run) by semipreparative HPLC yielded 4 mg of taxifoliol together with 24 mg of quercetin and 5 mg of myricetin. They were identified by HPLC comparison with authentic standards. In wine, quercetin usually shows a mean value of 25 mg/L, and its potential health effects have been reviewed (*30*). In the second run (fraction I), quercetin 3-*O*-*β*-glucopyranoside (isoquercitrin) (35 mg) and myricetin 3-*O*-*β*-glucopyranoside (30 mg) were identified by HPLC comparison with authentic standards and according to their ^{1}H NMR and FAB$^+$MS data (*m*/*z* 449 and 465) (*31*). In addition, isorhamnetin was isolated in fraction F.

Flavanonols. The ^{1}H NMR spectrum for compound **3** (25 mg, fraction F) showed a signal corresponding to a rhamnose residue at typical shift δ 1.18 ppm (J 6.2 Hz). The large singlet at δ 4.06 ppm (J 1.5 Hz) indicated an α configuration of the anomeric proton of the sugar (H-1″). Identification as astilbin (dihydroquercetin 3-*O*-α-rhamnoside) (Figure 2) was confirmed by FAB$^+$MS data (*m*/*z* 451) and by comparison of the ^{1}H NMR shifts and coupling constants with literature data (*32*, *33*). Astilbin has been previously reported in white grape skin, white wine (*34*), grape pomace (*31*), and grape stems (*35*). Several biological activities have been found for astilbin: it decreases total liver cholesterol concentration in rats (*36*) and protects rat red blood cells against oxidative stress in vitro (*37*).

Comparison of the ^{1}H NMR spectrum of compound **4** (20 mg, fraction H) with that of astilbin showed the absence of the H5′ signal at δ 6.80 ppm. Furthermore, FAB$^+$MS spectrum showed a molecular ion at *m*/*z* 467, corresponding to an additional hydroxyl group. Hence, compound **4** was identified as dihydromyricetin 3-*O*-α-rhamnoside (Figure 2). This compound has been isolated from *Catha edulis* (*38*), but no reports concerning its biological properties have been published until now. These two flavanonols are reported for the first time in red wine.

Flavanols. In fraction G, two flavan-3-ols monomers, (+)-catechin and (−)-epicatechin (190 mg), were isolated. They were identified by HPLC comparison with authentic standards and according to their ^{1}H NMR data (*32*).

The ^{1}H NMR spectra of two other compounds in fraction H (5 mg each) showed signals for (+)-catechin and (−)-epicatechin, but their HPLC chromatograms both showed a single peak. Moreover, FAB$^+$MS gave a molecular peak at *m*/*z* 579 (positive ion FAB$^+$MS) for both compounds, thus indicating procyanidin dimers consisting of epicatechin and catechin units. However, the position of the interflavanoid linkage could not be determined by HMBC and HMQC experiments because of insufficient quantity of the substances.

Flavan-3-ols are abundant in grapes, especially (+)-catechin, which is the predominant monomeric form found in Californian and French red wines. Epicatechin, which is the major compound in the oligomeric and polymeric forms from grape skins and seeds, is typically found at a lower level than catechin in red wines (about 40−100 mg/L). Concerning procyanidin dimers, they are found at a mean total concentration of 275 mg/L in French red wines (*17*).

Phenolic Acid Derivatives. A group of rare *p*-coumaric esters was isolated in several fractions of the two CPC experiments. In the fraction A, a completely separated peak, gave 60 mg of *trans*-*p*-ethylcoumarate. Its ^{1}H NMR spectrum showed the presence of a *trans*-*p*-coumaroyl group, characterized by an AA′BB′ resonance system (δ 6.85 and 7.55 ppm, $J = 8.7$ Hz) and a pair of proton doublets (δ 6.35 and 7.65, $J = 15.9$ Hz) attributable to the *trans*-ethylenic protons. In addition, five aliphatic protons (a quadruplet for 2 H at δ 4.25 ppm and a triplet for 3 H at δ 1.35 ppm) indicated the presence of an ethyl residue. This compound has already been isolated and identified from white wine (*39*).

Compounds **5a** and **5b** (10 mg each) were obtained as a mixture in fraction K, but were well separated by the HPLC gradient program. The ^{1}H NMR spectra of these two compounds are identical: both include a glucopyranose unit linked by an ester linkage with a *p*-coumaroyl part via the C6″, and both show α- and β-configurations in solution (5.15 ppm, $J = 3.5$ Hz and 4.53 ppm, $J = 8$ Hz). These data are consistent with those described by Shimomura et al. (*40*) for *trans*-*p*-coumaroyl 6″ -glucoside (Figure 2). The latter was isolated from bark of *Prunus buergeriana* (*40*) and leaves of *Alsophila spinulosa*.

Four other phenolic acid derivatives previously reported in wine were isolated: these are caffeic and vanillic acids in fraction C and syringic acid and tyrosol in fraction F. Their identifications were performed by HPLC comparison with authentic standards and according to their ^{1}H NMR data (*42*). The results of this study show that centrifugal partition chromatography is a suitable method, particularly on the preparative scale, to separate from red wine several compounds belonging to different classes of polyphenols. Furthermore, it shortens the development time as compared to other classical chromatographic methods. Further investigations are in progress to monitor these newly extracted compounds in different types of wine.

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