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Fractionation and Identification of Some Low Molecular Weight Grape Seed Phenolics

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Grape seeds were extracted in order to isolate low molecular weight phenolics. These were fractionated by analytical and semipreparative HPLC, Sephadex LH-20 chromatography, and analytical thin-layer chromatography. Ten compounds were separated and identified: gallic acid, (+)-catechin, (−)-epicatechin, dimer procyanidins B1, B2, B3, and B4, procyanidin C1 (trimer), procyanidin B2 gallate, (−)-epicatechin gallate.

The high phenolic content of grape seed is of critical interest as these compounds form a large proportion of wine tannins (Singleton and Draper, 1964; Glories, 1978; Singleton, 1980; Oszmianski et al., 1986). They are involved in quality aspects as they affect the color and the flavor of wines (Rossi and Singleton, 1966; Glories, 1978, 1982) and some may be involved in oxidative browning of grapes (Romeyer, 1984; Oszmianski et al., 1985). Although it is known that grape seed is rich in condensed tannins (Glories, 1978), the consistent role of lower molecular weight phenolics, which are also present in grape seeds, in some wine characteristics has been demonstrated recently (Glories, 1982, 1986; Dournel, 1985). As a consequence, it was of interest to improve knowledge of this latter part of grape seed tannins. This is the purpose of the present work.

MATERIALS AND METHODS

Plant Material. Determinations were carried out in seeds from grapes cv. Carignane, picked during 1986 vintage in the INRA vineyards at the Chapitre Experimental Station near Montpellier (France), after veraison (ca. 15° Brix). This stage of the evolution of the fruit was chosen as the initial point of the study, which will be extended to the whole maturation period, as the phenolics level in grape seeds from unripe fruit is known to be higher than in fully ripe grapes (Su and Singleton, 1969; Czochanska et al., 1979; Singleton, 1980; Romeyer et al., 1986). Seeds were removed from the grapes, immediately frozen in liquid nitrogen, and crushed under liquid nitrogen in a ball grinder. A very fine powder was obtained and used immediately for the extraction of phenolic compounds.

Preparation of the Phenolic Extract. A 30-g portion of grape seed powder were extracted twice by stirring for 15 min at +4 °C with 100 mL of 70% acetone in water in the presence of 2000 ppm SO_2 to avoid oxidation. The mixture was filtered on sintered glass, and the filtrate was treated with chloroform (100 mL) in order to eliminate acetone and compounds other than phenolics. The aqueous phase containing phenolics was recovered; rinsing water (30 mL) of chloroformic phase was added (Jerumanis, 1985). Crystallized NaCl was then added to saturation point in order to precipitate the more polymerized tannins, thus enabling further fractionation of low molecular weight phenolics (Michaud et al., 1971). The preparation was filtered on Buchner filter and the filtrate extracted with ethyl acetate (3 × 90 mL). The EtOAc was concentrated under vacuum to approximately 20 mL, and 100 mL of chloroform was added to precipitate oligomer tannins. A filtration was made, the filtrate was discarded, and the precipitate was recovered to be redissolved in 96% EtOH. Referred to as "grape seed phenolic extract", it was subsequently fractionated by different chromatographic patterns.

Fractionation of Phenolic Compounds. *Fractionation of Acidic and Neutral Phenolic Compounds.* This was carried out according to Salagoity-Auguste and Bertrand (1984).

Semipreparative Fractionation. Gel Chromatography. Fractionation of compounds located in the grape seed phenolic

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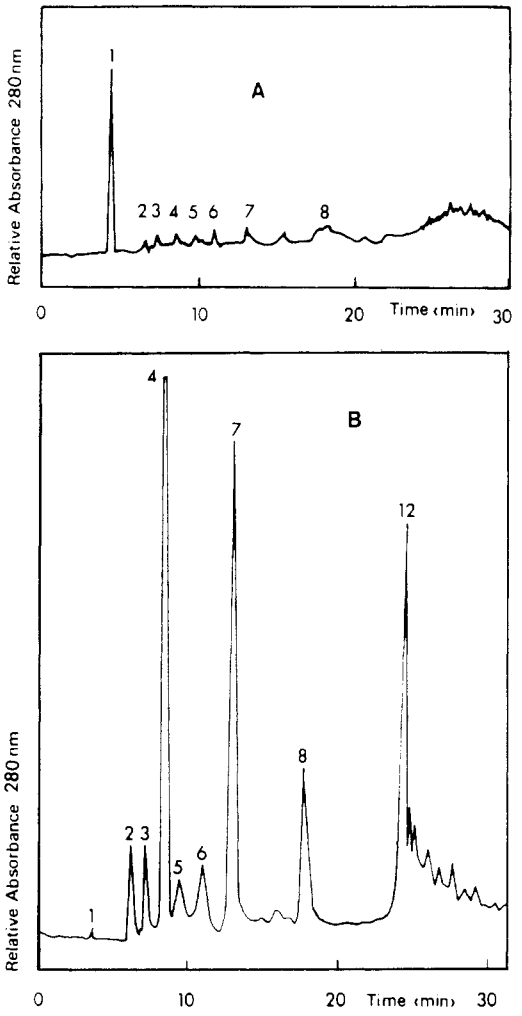

Figure 1. HPLC of acidic (A) and neutral (B) phenolics in grape seed phenolic extract. Peaks: 1, gallic acid; 2, procyanidin B1; 3, procyanidin B3; 4, (+)-catechin; 5, procyanidin B4; 6, procyanidin B2; 7, (−)-epicatechin; 8, procyanidin B2 gallate; 12, (−)-epicatechin gallate.

extract was carried out on a column (L = 40 cm, i.d. = 2.5 cm) filled with Sephadex LH-20 suspended in 96% EtOH (Lea et al., 1979; Outtrup, 1981; Asano et al., 1984; Delcour et al., 1984). Elution was carried out with the same solvent at a flow rate of 2 mL/min. Fifty 20-mL fractions were collected. Detection was at the column outlet at 280 nm. (+)-Catechin and (−)-epicatechin fractionation was carried out on Sephadex LH-20 in solution in water using a mixture of CH_3COOH and water with an acetic acid gradient from 0 to 25%.

Semipreparative HPLC. Fractionation of phenolics from the grape seed phenolic extract was carried out under the following conditions: Varian 5500 equipment; UV detection at 280 nm with a Varian UV-200 variable-wavelength detector; RP-18 spheri 5 column (L = 250 mm, i.d. = 7 mm), Brownlee Laboratories Inc. Santa Clara, CA; precolumn of the same make and of the same packing. Elution was made with a mixture of CH_3CN–H_2O (pH 2.6) (by addition of H_3PO_4) as follows: 89% water–11% CH_3CN at the beginning of run, 100% CH_3CN at the end of run (30 min).

Analytical Fractionation. This fractionation was carried out by HPLC under conditions similar to those of semipreparative HPLC fractionation except for the dimensions of the column (internal diameter 4.6 mm) and flow rate 1 mL/min.

Identification of Phenolic Compounds. The following means of identification were used.

Spiking the Retention Times under the Same Conditions with the Corresponding Standards. Commercially available standards were purchased from Fluka (gallic acid, (+)-catechin, (−)-epicatechin). Procyanidins B1, B2, B3, and B4 were donated by Prof. Jerumanis (University of Leuwen, Belgium), Procyanidin C1 (trimer) by Dr. Timberlake (University of Bristol, U.K.), and

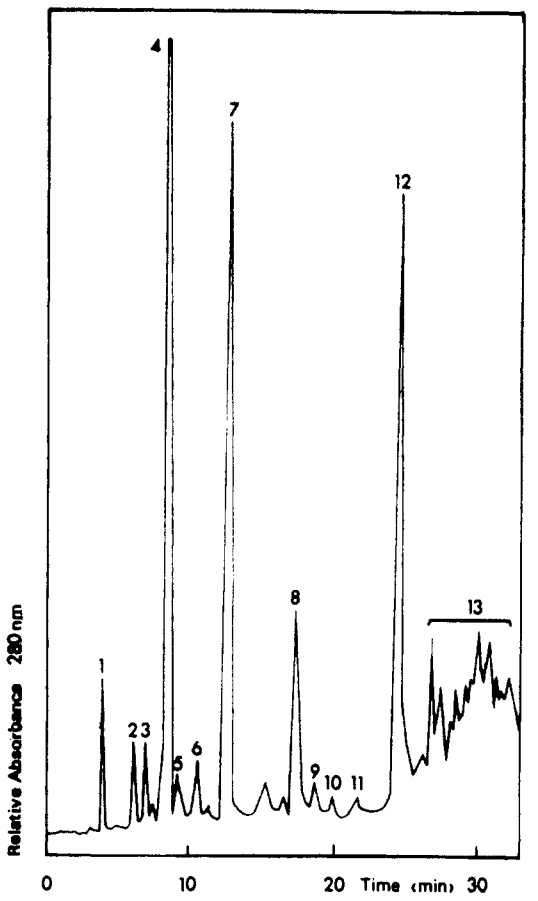

Figure 2. Analytical HPLC of grape seed phenolic extract. Peaks: 1, gallic acid; 2, procyanidin B1; 3, procyanidin B3; 4, (+)-catechin; 5, procyanidin B4; 6, procyanidin B2; 7, (−)-epicatechin; 8, procyanidin B2 gallate; 9, procyanidin C1 (trimer); 10, unknown; 11, unknown; 12, (−)-epicatechin gallate; 13, unknown.

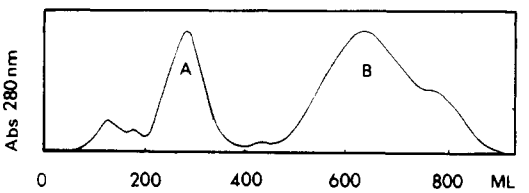

Figure 3. Sephadex LH-20 fractionation of phenolics from grape seed phenolic extract (solvent 96% EtOH). Peaks: A, (+)-catechin + (−)-epicatechin; B, other phenolics.

(−)-epicatechin gallate, (+)-gallocatechin, and (−)-epigallocatechin gallate by Dr. Saijo, Vegetable and Ornamental Crops Research Station, Ano, Mie, Japan.

Enzymic hydrolysis was carried out with Drum Pectinase 263 (Gist-Brocades) at 1 mg/mL of phenolic extract in an acetate buffer solution (0.1 M, pH 5.0) for 16 h at 35 °C. This enzymic preparation contained high esterase activity checked according to Dubourdieu et al. (1983, 1984) and Koh (1985).

Identification of procyanidins according to their degree of polymerization was effected by thin-layer chromatography under the conditions used by Wilson (1981).

RESULTS AND DISCUSSION

Representative chromatograms of acidic and neutral phenolic compounds of grape seed extract are shown in Figure 1. The major component in the acidic fraction was gallic acid (peak 1). All the other compounds were mainly located in the neutral extract. Gallic acid was located in peak 1 of the analytical HPLC chromatogram of whole grape seed extract (Figure 2).

Figure 3 shows the chromatogram obtained from the first Sephadex LH-20 chromatographic analysis. Two

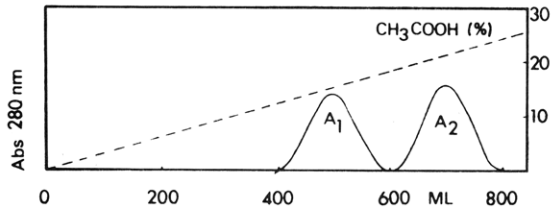

Figure 4. Sephadex LH-20 chromatography of peak A of Figure 3 (solvent CH_3COOH–water). Peaks: A1, (–)-epicatechin; A2, (+)-catechin.

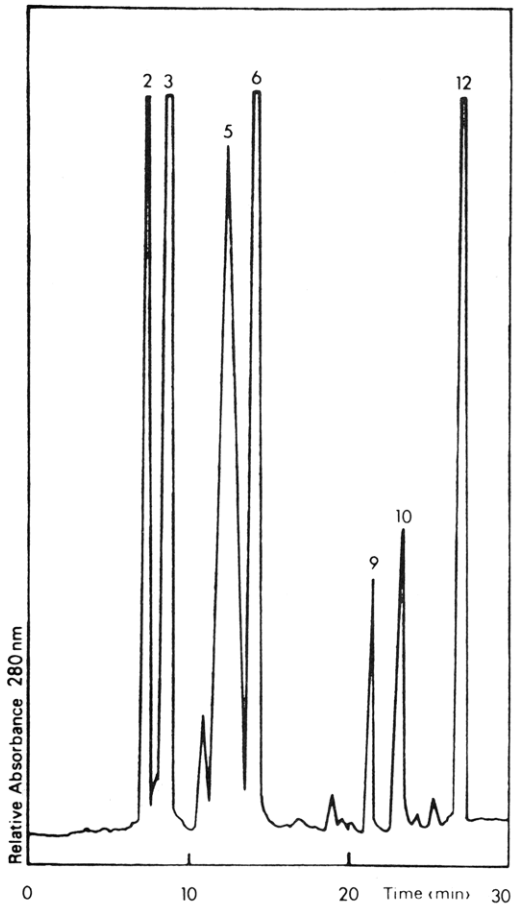

Figure 5. Semipreparative HPLC fractionation of phenolics of peak B from Sephadex LH-20 chromatography. Peaks: 2, procyanidin B1; 3, procyanidin B3; 5, procyanidin B4; 6, procyanidin B2; 9, procyanidin C1 (trimer); 10, unknown; 12, (–)-epicatechin gallate.

peaks were fractionated. Analysis of peak A using further Sephadex fractionation (Figure 4) gave good separation of (+)-catechin (peak A2) and (–)-epicatechin (peak A1). Semipreparative HPLC of each peak indicated a high degree of purity as (+)-catechin and (–)-epicatechin were the only compounds. Good resolution of the two phenolics was achieved with analytical HPLC (Figure 2) as they are located in peaks 4 and 7.

In agreement with previous results (Oszmianski et al., 1986) Figure 2 indicated good fractionation of dimer procyanidins (B group). However, in order to improve fractionation both quantitatively and qualitatively, semipreparative HPLC of phenolics present in peak B of Figure 3 was performed. As shown in Figure 5 a number of compounds were successfully separated. Procyanidins B1 (peak 2), B3 (peak 3), B4 (peak 5), and B2 (peak 6) were identified with reference to the corresponding standards. In addition, each peak in the group was collected as a single fraction at column outlet and examined by TLC (Figure 6). Fractions from peaks 2–6 gave a single spot (R_f 0.65)

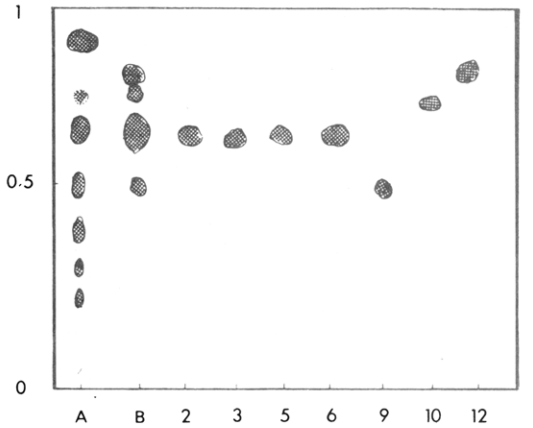

Figure 6. Silica thin-layer chromatography in one dimension with toluene–acetone–formic acid (30:60:10) as solvent. Key: A, grape seed phenolic extract; B, eluate of peak B from Sephadex LH-20 chromatography; 2, 3, 5, 6, 9, 10, and 12, eluates from corresponding peaks of peak B HPLC fractionation.

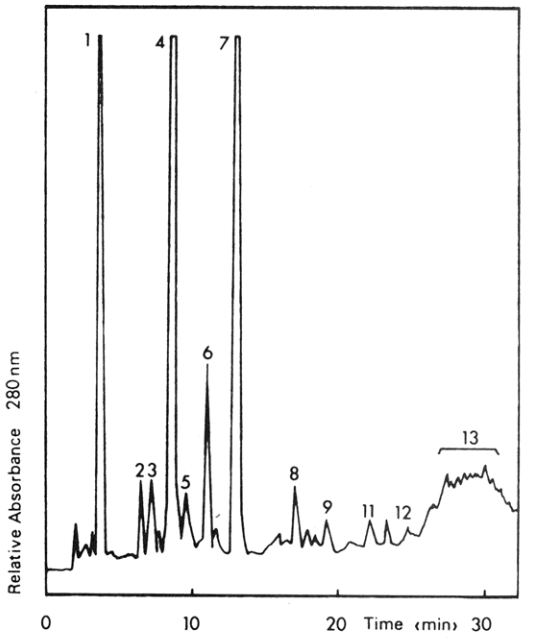

Figure 7. HPLC fractionation of phenolics from grape seed phenolic extract after enzymic hydrolysis (for control, see Figure 2).

corresponding to dimeric procyanidins according to Wilson (1981). Six levels of polymerization appeared in grape seed extract. Peak 9 was shown to be a trimeric procyanidin. This was confirmed by comparison with C1 standard.

As shown in Figure 2, a noticeable peak (peak 8) was present after (–)-epicatechin. Enzymic hydrolysis of grape seed extract indicated that after the Drum Pectinase effect for 16 h at 35 °C there was a dramatic decrease in this peak and a subsequent increase in procyanidin B2 and gallic acid (Figure 7). We therefore tentatively confirmed that this compound was procyanidin B2 gallate. A similar compound was previously found in grapes by Czochanska et al. (1979). Recently, Lee and Jaworski (1987) examined unknown phenolic compounds and suggested that they could be catechin–catechin–gallic acid isomers. These converging conclusions would tend to be in favor of the existence of such a compound in grape seed phenolics.

As shown in Figure 2, there is a high peak (peak 12) with a retention time of 25 min. This peak is present in all grape seed phenolic extracts. It is as large as (+)-catechin and (–)-epicatechin peaks in seeds from unripe grapes. It

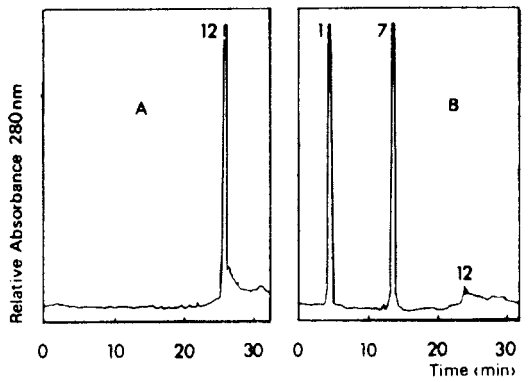

Figure 8. Analytical HPLC of peak 12 of Figure 2: A, before enzymic hydrolysis; B, after enzymic hydrolysis.

Table I. Area Percentages of the Phenolic Compounds Isolated from Grape Seeds

phenolic compound	area, %
gallic acid	1.6
procyanidin B1	1.2
procyanidin B3	2.0
(+)-catechin	32.5
procyanidin B4	1.1
procyanidin B2	1.5
(−)-epicatechin	19.0
procyanidin B2 gallate	7.4
procyanidin C1	1.2
(−)-epicatechin gallate	12.8

is much lower in seeds from mature grapes. Figures 7 (grape seed extract) and 8 (peak collected as a single fraction and analyzed by preparative HPLC) indicated disappearance of this peak after enzymic hydrolysis with, simultaneously, emergence of (−)-epicatechin and gallic acid. Cochromatography with the corresponding standard indicated the presence of (−)-epicatechin gallate. This finding confirms the presence of this ester in grape seed phenols (Singleton et al., 1966; Stonestreet, 1965; Milhé, 1969; Singleton and Esau, 1969; Ribéreau-Gayon and Milhé, 1970; Piretti et al., 1976; Czochanska et al., 1979; Foo and Porter, 1980; Lee and Jaworski, 1987). The presence of other gallic acid esters such as gallocatechin, (−)-epigallocatechin, and (−)-epigallocatechin gallate was not detected in our extracts.

The relative proportions of the different identified phenolic compounds are shown in Table I. Large differences in the concentrations were observed (+)-Catechin and (−)-epicatechin were the highest. (−)-Epicatechin gallate and procyanidin B2 gallate were in the third and fourth, considerable concentrations.

A certain number of other compounds remain unknown. This is the case of peak 10 in Figure 5. Two compounds are in fact present (analytical HPLC) in this peak (Figure 9A). After enzymic hydrolysis (Figure 9B), one peak remained unchanged (10A) while the other disappeared to give gallic acid (Figure 9B), indicating another gallic acid ester structure. On the other hand, all the analytical HPLC patterns (Figure 2) show the presence of an unsatisfactorily separated group of peaks. This disappeared almost completely after enzymic hydrolysis (Figure 7). The compounds may also be gallic acid esters as polymeric complexes.

These results have undoubtedly a practical significance concerning the final quality of the wine as catechins and procyanidins contribute to astringency and bitterness (Rossi and Singleton, 1966; Singleton and Esau, 1969; Glories, 1978, 1982; Lea, 1978; Lea et al., 1979; Arnold et al., 1980; Ariga et al., 1981; Wilson, 1981). They also are

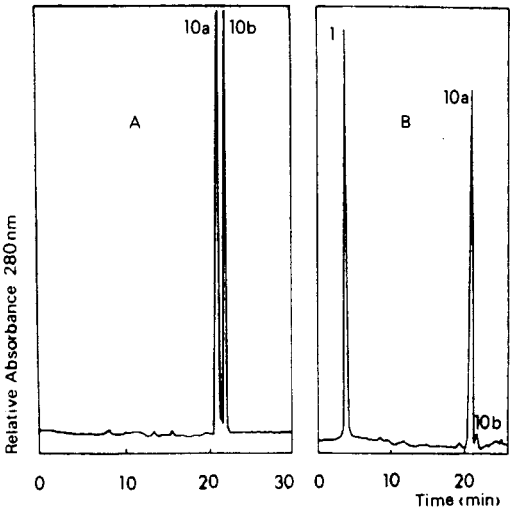

Figure 9. Analytical HPLC of peak 10 of Figure 5: A, before enzymic hydrolysis; B, after enzymic hydrolysis.

involved as (−)-epicatechin gallate in oxidation reactions (Wilson, 1981; Asano et al., 1984; Cheynier et al., 1988; Oszmianski, 1985, 1988) leading to browning and to a subsequent decrease of wine quality.

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Formation of Indole Glucosinolate Breakdown Products in Autolyzed, Steamed, and Cooked *Brassica* Vegetables

Bogdan A. Slominski and Lloyd D. Campbell*

The thermal decomposition of indole glucosinolates that occurs during cooking procedures and the autolysis of indole glucosinolates in raw *Brassica* vegetables were studied to evaluate the reported anticarcinogenic properties of these vegetables. Intact indole glucosinolates and the indole glucosinolate breakdown products, thiocyanate ion and indoleacetonitriles, were followed in raw (autolyzed), steamed (10 min), and cooked (40 min) samples of cabbage, cauliflower, broccoli, and Brussels sprouts. Heat treatment resulted in substantial decomposition of indole glucosinolates with thiocyanate ion and indoleacetonitriles accounting for 50 and 30%, respectively, of the degraded indoles. Autolysis, in contrast, resulted in the production of little or no indoleacetonitriles but with considerable production of thiocyanate ion and related compounds (i.e., indolemethanols). The consumption by humans of raw or variously cooked *Brassica* vegetables may result in different intakes of indole glucosinolate derived products, which ultimately could influence the anticarcinogenic properties of the vegetables.

The indole glucosinolates 3-indolylmethyl (glucobrassicin), 4-hydroxy-3-indolylmethyl (4-hydroxyglucobrassicin), and 4-methoxy-3-indolylmethyl (4-methoxyglucobrassicin) represent a significant proportion of the total glucosinolate content of cruciferous vegetables (Fenwick et al., 1983; Truscott et al., 1982). 3-Indolylmethyl glucosinolate has been shown to be the predominant indole glucosinolate in cabbage, broccoli, Brussels sprouts, and cauliflower (Heaney and Fenwick, 1980; Mithen et al., 1987). Virtanen (1965) demonstrated that, following rupture of the cells of plant material, 3-indolylmethyl glucosinolate is hydrolyzed by the endoge-

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