Separation of Grape and Wine Proanthocyanidins According to Their Degree of Polymerization

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A method was developed for the fractionation of grape and wine proanthocyanidins according to their degree of polymerization. The grape and wine proanthocyanidins were fractionated on C_{18} Sep-Pak cartridges into three fractions by different organic solvents. The combination of TLC, analytic HPLC, and degradation with toluene-α-thiol confirmed that these three fractions contained, respectively, monomeric flavan-3-ols (catechins), oligomeric proanthocyanidins, and polymeric proanthocyanidins. The mean degrees of polymerization for oligomeric and polymeric proanthocyanidins in red wine were, respectively, 4.8 and 22.1, and those in the seed extract, 9.8 and 31.5. The method proposed is very interesting for the study of grape and wine proanthocyanidins according to their degree of polymerization, and a further quantification is also possible.

Keywords: *Grape; wine; catechins; proanthocyanidins; C_{18} Sep-Pak cartridge; fractionation*

INTRODUCTION

Proanthocyanidins (PA) play a very important role in enology. They can be beneficial or harmful to wine quality according to their chemical properties in various aspects: astringency and bitterness (Arnold and Noble, 1978; Arnold et al., 1980; Haslam, 1974; Singleton, 1992), haze formation and interactions with proteins (Jouve et al., 1989; Oh and Hoff, 1986; Powers et al., 1988; Ricardo da Silva et al., 1991b; Singleton, 1992; Yokotsuka and Singleton, 1987), oxidation and browning (Cheynier and Ricardo da Silva, 1991; Cheynier et al., 1988; Lee and Jaworski, 1988; Oszmianski et al., 1985), color stability (Timberlake and Bridle, 1976; Singleton and Trousdale, 1992), and aging behavior (Haslam, 1980). Several studies have considered that grape and wine PA may play a positive role in human health, in particular their effects on arteriosclerosis (Masquelier, 1982, 1988) and their radical-scavenging ability (Ricardo da Silva et al., 1991c).

However, all of these properties largely depend on their structures, on their levels, and especially on their degree of polymerization (DP) (Haslam, 1974; Haslam and Lilley, 1988; Masquelier, 1988; Okuda et al., 1985; Porter and Woodruffe, 1984; Rigaud et al., 1993; Robichaud and Noble, 1990).

Many methods have been proposed to separate PA according to their DP. The technique of thin-layer chromatography (TLC) with a silica phase permits the separation of oligomeric PA up to the heptamers (Lea, 1978). This method can be used only for a qualitative analysis. Column chromatographies on Sephadex G-25 (McMurrough and McDowell, 1978; Michaud and Margail, 1977; Somers, 1966), BSA−Sepharose CL-4B (Oh and Hoff, 1979), Sephadex LH-20 (Boukharta et al., 1988; Lea and Timberlake, 1974), Fractogel TSK-HW 40 (s) (Ricardo da Silva et al., 1991d), Fractogel TSK 50 (f) (Meirelles et al., 1992), and normal phase HPLC (Rigaud et al., 1993; Prieur et al., 1994) were also employed to separate PA. The main shortcomings of these techniques are that they are very delicate, which makes difficult their use for routine analysis.

Salagoïty-Auguste and Bertrand (1984) and Jaworski and Lee (1987) separated grape phenolics into acidic and neutral groups using a C_{18} Sep-Pak cartridge. More recently, Oszmianski et al. (1988) and Oszmianski and Lee (1990) were successful in separating neutral phenolic compounds other than anthocyanins using the same cartridge. This method was then improved by Revilla et al. (1991). However, none of these methods were aimed at the separation of PA on the basis of their DP.

This paper describes an improved method used for the separation of grape and wine PA on the basis of their DP using C_{18} Sep-Pak cartridges. A subsequent paper will present the application of this method for quantification of total flavan-3-ols in each fraction.

MATERIALS AND METHODS

Materials. (+)-Catechin, (−)-epicatechin, and toluene-α-thiol (benzyl mercaptan) were purchased from Fluka AG (Buchs, Switzerland). (−)-Epicatechin 3-*O*-gallate was obtained from Extrasynthèse (Genay, France). The precoated silica plates (DC-Fertigplatten kieselgel 60, layer thickness = 0.25 mm, particle size = 5−40 μm) were furnished by Merck (Darmstadt, Germany). Procyanidins B_1, B_2, B_3, B_4, B_1 3-*O*-gallate, B_2 3-*O*-gallate, and B_2 3′-*O*-gallate, trimer C_1, and trimer T_2 were isolated and purified from methanolic extract of grape seeds, in our laboratory, by Fractogel TSK HW-40 (F) and semipreparative HPLC, according to the method described earlier (Ricardo da Silva et al., 1991d). The C_{18} Sep-Pak cartridges were purchased from Waters Associates (Bedford, MA). The benzyl thioethers of catechin, epicatechin, and epicatechin 3-*O*-gallate obtained by thiolysis of polymeric PA from grape seeds were isolated and purified by semipreparative HPLC using a μBondapak C_{18} column (300 × 7.8 mm).

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S0021-8561(97)00753-X CCC: $15.00
Published on Web 02/26/1998

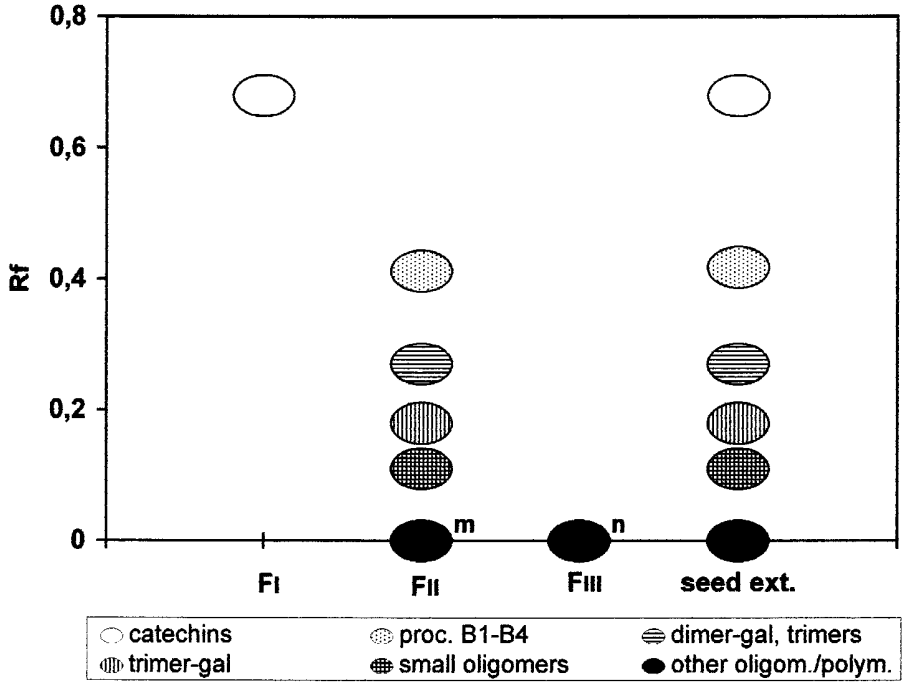

Figure 1. Silica TLC in one dimension of different seed PA fractions isolated from C_{18} Sep-Pak cartridges.

Harvest of Grapes and Extraction of Phenolic Compounds. Grapes (*Vitis vinifera* cv. Tinta Miúda) were sampled at harvest maturity in 1994 from vineyards of the INIA-Estação Vitivinícola Nacional (Dois Portos, Portugal). The Tinta Miúda red wines were obtained by fermentation on skins. The extraction of total polyphenols of different parts of grapes was performed according to the method described by Bourzeix et al. (1986).

Fractionation of Proanthocyanidins According to Their DP. The medium was dealcoholized by rotary evaporation at <30 °C and adjusted to pH 7.0 with NaOH solution and/or with phosphate buffer (pH 7.0). This sample was then passed through the two preconditioned neutral Sep-Pak cartridges connected in series: the superior one is tC_{18} Sep-Pak and the inferior is C_{18} Sep-Pak. Elution was carried out with 10 mL of H_2O adjusted to pH 7.0 to eliminate phenolic acids. After the cartridges were dried with N_2, elutions were carried out first with 25 mL of ethyl acetate to elute catechins and oligomeric PA, acompanied by some other small phenolic molecules [fractions (F) I and II], and then with 10 mL of methanol to elute the polymeric PA and anthocyanins (in the cases of red wine or red grape skin extract) (FIII). For the separation of catechins from oligomeric PA, FI+II was evaporated to dryness under vacuum at 25 °C, dissolved in distilled water, and then redeposited onto the same connected cartridges preconditioned with distilled water. After the cartridges were dried with N_2, separation of catechins and oligomeric PA was realized by sequential elution with 25 mL of diethyl ether (FI) and then with 10 mL of methanol (FII).

TLC. Commercial silica gel plates (DC-Fertigplatten kieselgel 60) were utilized to control the DP of PA in each fraction obtained from C_{18} Sep-Pak cartridges. The chromatography was carried out using an ascending elution with toluene/acetone/acetic acid (3:3:1, v/v/v), according to the method earlier reported (Lea et al., 1979). A solution of 10% (w/v) vanillin in concentrated hydrochloric acid was used for detection.

Analytic HPLC. HPLC analysis was also employed to control the composition of each fraction obtained from C_{18} Sep-Pak cartridges. The HPLC apparatus was a Hewlett-Packard 1050, equipped with a quaternary pump, a UV−visible detector coupled to a data processing computer (Millennium 2010), a thermostat controlling the column temperature, and a manual injection valve. The column (250 × 4 mm) was a cartridge of 4-μm Superspher 100 RP18 (Merck). The mobile phase flow rate was fixed at 1.0 mL/min throughout the study. The detection was at 280 nm, and the column temperature was 30 °C. Two gradient elutions from water (A) to water/acetic acid (90:10, v/v) (B) were used: (catechins) 0−5 min, 10−80% B; 5−29 min, 80−100% B, 16 min with 100% B; (procyanidins) 0−40 min, 10−70% B; 40−55 min, 70−85% B; 55−74 min, 85−100% B.

Degradation of PA with Toluene-α-thiol. Acid-catalyzed degradation of PA in each fraction obtained from C_{18} Sep-Pak cartridges with toluene-α-thiol was carried out in sealed glass ampules in acidified methanol as described earlier (Prieur et al., 1994). The hydrolyzed solution was then analyzed by HPLC, to calculate the mean DP (mDP) of PA. The HPLC apparatus used was the same as that for analytic HPLC. The elution conditions were as follows: column, Lichrospher 100 RP-18 (5 μm, 250 × 4 mm) (Merck); flow rate, 1.0 mL/min; column temperature, 30 °C; solvent A, water/formic acid (98:2, v/v); solvent B, acetonitrile/water/formic acid (80:18:2, v/v/v); elution with linear gradient, 5−30% of B in 40 min, 30−50% of B in 20 min, 50−80% of B in 10 min, 80−100% of B in 5 min; detection wavelength, 280 nm. Calibration curves were established with (+)-catechin, (−)-epicatechin, (−)-epicatechin 3-*O*-gallate, and corresponding benzyl thioether standards. The latter were prepared by semipreparative HPLC as recently reported (Prieur et al., 1994).

RESULTS AND DISCUSSION

The wine or grape extracts could be separated on C_{18} Sep-Pak cartridges into three fractions by different organic solvents as described under Materials and Methods. Catechins and PA compositions in each fraction were verified by TLC, analytic HPLC, and acid-catalyzed degradation in the presence of toluene-α-thiol.

The TLC chromatograms of fractions isolated from grape seed extract are shown in Figure 1. The R_f values observed were compared with those already reported (Ricardo da Silva et al., 1991d).

It has been shown that the flavanols in FI are only catechins. The flavanols in FII consist of low molecular weight PA (dimers, trimers, tetramers, etc.), namely oligomeric PA. The FIII contains neither catechins nor

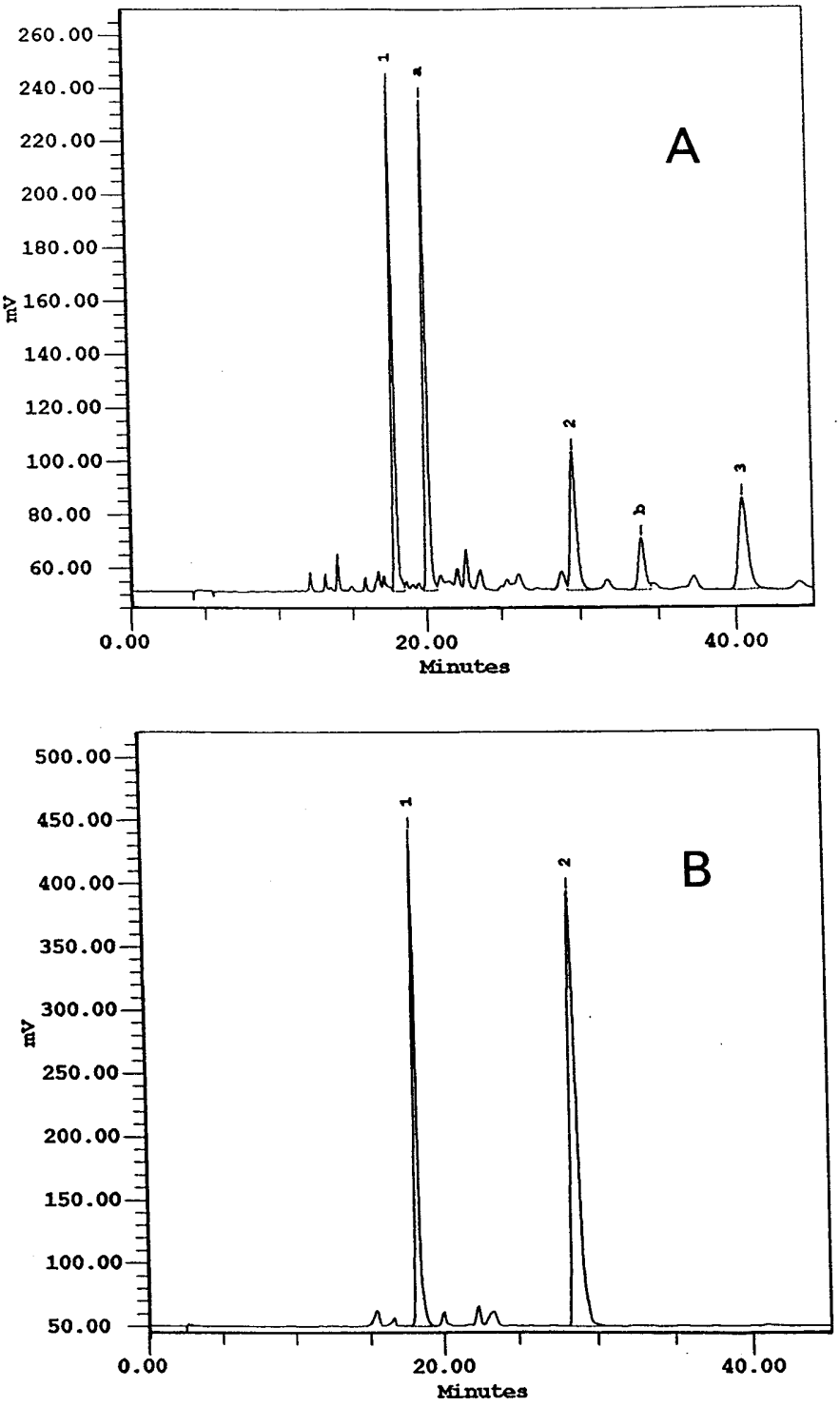

Figure 2. HPLC chromatograms recorded at 280 nm of the FI isolated from the red wine (A) and the grape seed extract (B) using C_{18} Sep-Pak cartridges. Peaks: 1, (+)-catechin; 2, (−)-epicatechin; 3, (−)-epicatechin 3-*O*-gallate; a and b, unknown.

oligomeric PA as presented in FII. It should be composed of more polymerized PA. Since procyanidin isomers have nearly the same R_f value (Ricardo da Silva et al., 1991d), we suppose that each spot in the TLC could contain several isomers with the same DP. Spots represented by **m** and **n** should be the mixture of more polymerized PA which did not migrate to the start. However, the mDP of the former should be lower than that of the later.

It is important to note that the TLC technique could give only a qualitative answer even though the chromatogram has shown a good separation among catechins, oligomeric PA, and polymeric PA. Confirmation of PA compositions in each fraction using other methods was undoubtedly necessary. For this reason, PA composition in each fraction was also verified by HPLC (Figures 2 and 3).

HPLC analysis permits quantitative analysis of some simple flavanols—catechins and dimeric and trimeric procyanidins. The numbered peaks in Figures 2 and 3 correspond to those compounds that were identified by

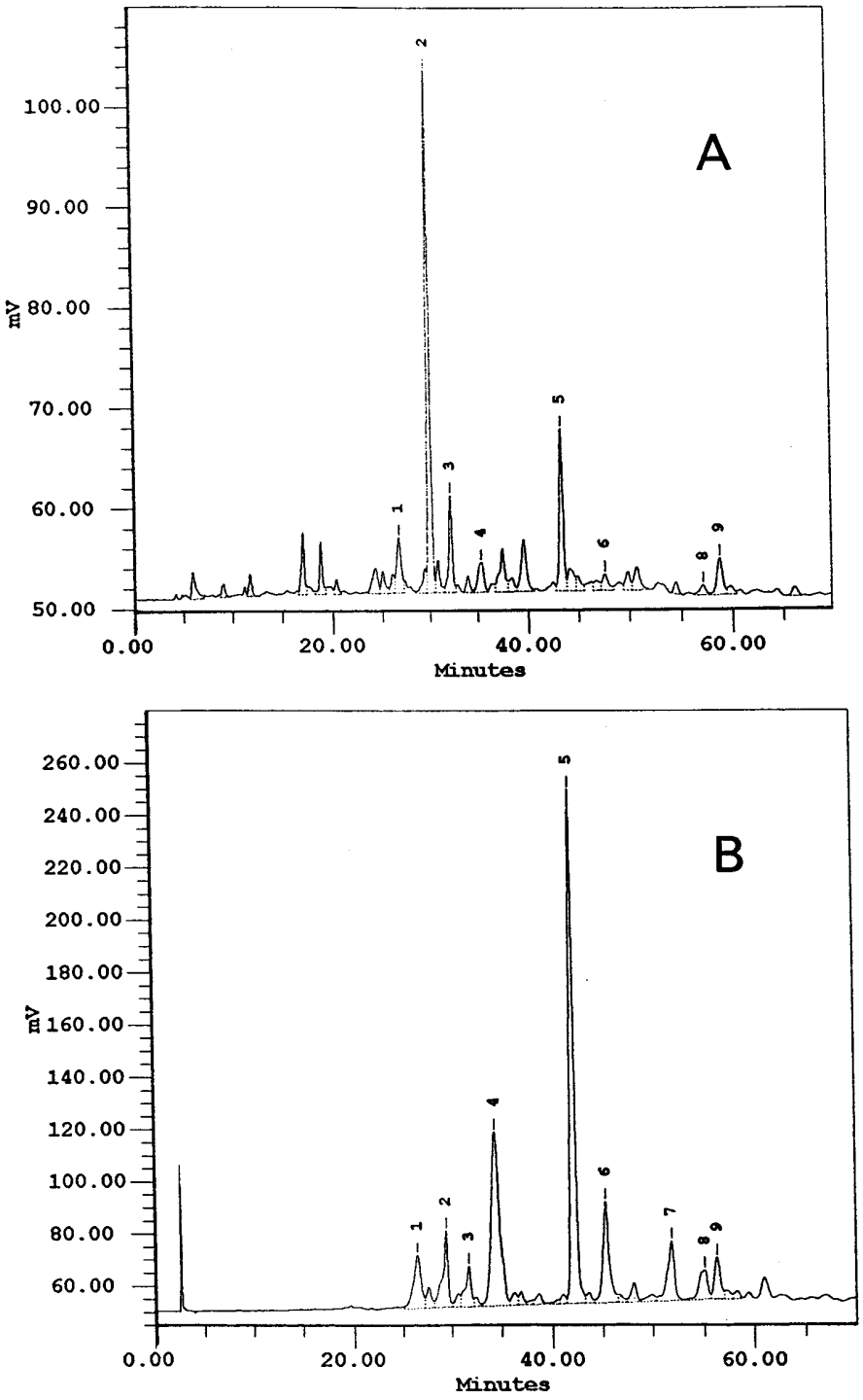

Figure 3. HPLC chromatograms recorded at 280 nm of the FII isolated from the red wine (A) and the grape seed extract (B) using C_{18} Sep-Pak cartridges. Peaks: 1, procyanidin B_3; 2, procyanidin B_1; 3, procyanidin trimer 2 (T_2); 4, procyanidin B_4; 5, procyanidin B_2; 6, procyanidin B_2 3-*O*-gallate; 7, procyanidin B_2 3′-*O*-gallate; 8, procyanidin B_1 3-*O*-gallate; 9, procyanidin trimer 1 (C_1).

injection of the standards and confirmed by toluene-α-thiolysis as described previously (Prieur et al., 1994).

It can be noted that FI contains (+)-catechin and (−)-epicatechin. In the case of the red wine, (−)-epicatechin 3-*O*-gallate was also found in this fraction, but this compound is absent in the grape seed extract. In addition, some unknown peaks were present in the red wine FI fraction (Figure 2). These unknown peaks gave no coloration with vanillin−H_2SO_4, indicating that they were not flavanols. Therefore, the flavanols existing in this fraction are only catechins.

In FII, as shown in Figure 3, some already identified dimeric procyanidins (B_1, B_2, B_3, B_4, B_1 3-*O*-gallate, B_2 3-*O*-gallate, and B_2 3′-*O*-gallate) and trimeric procyanidins (C_1 and T_2) were found. The HPLC chromatogram was very similar to that obtained by another separation method used by Ricardo da Silva et al. (1990, 1991a). Furthermore, although traces of (+)-catechin

Table 1. Extraction Efficiency of Some Detectable Catechins and Procyanidins Using the Proposed Method

compd	amounts[a] (mg/L) known added quantities (standard)	wine (measured) concn	SD	CV[b]	wine + known added quantities (measured)	extraction efficiency (%)
(+)-catechin	31.0	44.8	1.6	3.5	74.6	96.4
(−)-epicatechin	29.8	26.6	1.2	4.4	55.2	96.0
procyanidin B_1	15.1	71.1	3.2	4.5	84.4	87.7
procyanidin B_2	33.4	28.3	2.3	8.2	59.2	92.6
procyanidin B_3	20.0	11.1	0.5	4.7	29.4	91.8
procyanidin B_4	31.5	12.5	0.1	1.0	42.1	94.2

[a] Means of four replications. [b] CV, coefficient of variation.

Table 2. Structural Composition (Percent in Moles) of Red Wine and Grape Seed PA Fraction[a]

fraction		terminal units Cat	Epicat	EpicatG	extension units Cat	Epicat	EpicatG	Epig
I	red wine	61.4	28.3	1.5	4.8	0	4.0	0
	grape seed	48.8	40.8	4.1	0.9	4.0	1.4	0
II	red wine	14.1	6.7	0.2	8.8	60.1	2.6	7.5
	grape seed	3.5	3.0	3.7	9.4	61.0	19.3	0
III	red wine	1.6	2.8	0.2	6.1	71.2	6.2	11.9
	grape seed	1.3	0.7	1.2	5.7	66.1	25.0	0

[a] Cat, Epicat, EpicatG, and Epig are the abbreviations of catechin, epicatechin, epicatechin gallate, and epigallocatechin units, respectively.

Table 3. Characteristics of Red Wine and Grape Seed PA Fractions

fraction	red wine mDP	aMW	cis:trans	% G	grape seed mDP	aMW	cis:trans	% G
I	1.1	328	0.5:1	5.5	1.1	328	1.0:1	5.5
II	4.8	1396	3.0:1	3.0	9.8	2881	6.7:1	23.0
III	22.1	6384	10.5:1	7.3	31.5	9123	13.3:1	26.2

and (−)-epicatechin, which were not revealed by TLC, were sometimes detected in FII by HPLC, they generally accounted for <2% (w/w) of total amounts.

As expected, for FIII, neither catechins nor dimeric and trimeric PA were detected by HPLC; FIII consisted of polymeric PA, which only could be eluted as a broad peak when the column was washed isocratically with an acidified methanol/water solvent. These results also indicate that a good separation of PA could be achieved by the proposed method.

It is worth noting that, in the case of red wine or red grape skin extract, anthocyanins were eluted only with methanol and hence present in FIII, which was confimed by HPLC analysis. Furthermore, it is also possible to separate anthocyanins from oligomeric PA using the proposed method.

The fractionation of PA on C_{18} Sep-Pak cartridges as described under Materials and Methods begins with an elution with ethyl acetate to isolate catechins and oligomeric PA (FI + FII) and then with methanol to isolate polymeric PA (FIII). The separation of FI from FII is obtained by a sequential elution with diethyl ether (FI) and then with methanol (FII). Neverthless, it is important to note that direct elution with diethyl ether, after phenolic acids are eliminated and the cartridges are dried, can also give a catechin fraction, identical to FI. In other words, the analytical process is much simpler in the case of quantifying only catechins. However, if a successive elution is carried out first with diethyl ether and then with ethyl acetate as described earlier (Sun et al., 1995), the ethyl acetate fraction obtained is different from FII: part of the small oligomeric PA, particularly dimers and trimers, were retained in the cartridges. This was confirmed by HPLC analysis. It is a reasonable presumption that, after elution of catechins with diethyl ether, some PA were more strongly associated with the stationary phase with the aid of diethyl ether molecules; stronger solvent, such as methanol, for example, will eluate not only oligomers but also polymeric forms. In consequence, for separation of oligomeric PA from catechins, utilization of the process as described is absolutely necessary.

The extraction efficiency of some detectable catechins and procyanidins was determined by analysis of the red wine with and without addition of known amounts of standard solution. The results are given in Table 1.

The recoveries of all tested catechins and procyanidins except procyanidin B_1 were relatively high, >91−96%; the recovery of procyanidin B_1 was a little lower, 87.7%, even though this value is generally acceptable. Furthermore, the highest coefficient of variation is that of procyanidin B_2, being 8.2%. It is evident from these results that the proposed separation method accompanied by HPLC analysis permits evaluation of some catechins and dimeric procyanidins with good recovery and repeatability.

In addition, the structural composition of PA in each fraction (FI, FII, and FIII) was determined according to the thioacidolysis method (Prieur et al., 1994). Compositional data of wine and grape seed PA fractions are presented in Table 2.

From the thioacidolysis data given in Table 2, some structural characteristics, that is mDP, cis:trans ratio, and percentage of galloylation (% G) of PA, could be calculated. These results, as well as average molecular weight (aMW) are presented in Table 3.

It would be not surprising to find from Table 3 that the mDP values of each fraction are very different: mDP of FIII > mDP of FII > mDP of FI.

The mDP values of FI obtained both from the red wine and from the grape seed extract are identical, being 1.1, indicating that the flavanols in FI exist essentially in monomeric forms. However, this value, not being exactly 1.0, also suggests the presence of traces of PA in FI.

For FII, on the other hand, the red wine and the grape seed extract do not give identical mDP values (4.8 and 9.8, respectively). These results could be explained by the different percentage distribution of oligomeric PA in the wine and the grape seed extract; the percentages of small oligomeric PA (dimers, trimers, etc.) in wine are much higher than those in grape seed extract. Furthermore, the DP of PA in FII ranges from 2 to at least 10.

Similar results were obtained for FIII; the mDP of the red wine fraction is only 22.1 as compared with 31.5 for the grape seed extract. It should be noted that the mDP value of 31.5 observed by us for the most polymerized fraction is higher than the 15.1 determined by Prieur et al. (1994), using a silica normal-phase HPLC, followed by thioacidolysis of each fraction. The explanation for this difference may be the fact that the fractionation by C_{18} Sep-Pak cartridges permitted recovery of highly polymerized PA, using methanol as the last solvent.

From Table 3, it can also be found that the cis:trans ratio and the percentage of galloylation, both for wine PA and for grape seed PA, increase as the mDP increases. These results agree with those observed by Prieur et al. (1994), who reported for the first time the estimation of the percentage of galloylation in polymeric PA.

CONCLUSION

The separation of PA into monomers, oligomers, and polymers by the C_{18} Sep-Pak cartridges was proved by combination of different techniques: TLC, HPLC, and thioacidolysis.

The proposed method is simple and easy to use, without major instrumentation. The present method could be applied routinely, especially for further quantitative analysis of some individual procyanidins (DP $\leq$ 3), using HPLC as described above, or total contents of flavan-3-ol in each fraction by colorimetric assays, even for the most polymerized fraction. This study was done on grapes and wine, although the application of this method is also possible for other plant tissues or beverages.

ACKNOWLEDGMENT

B.S. thanks the Fundação Oriente and Junta Nacional de Investigação Cientifica e Tecnológica (Portugal) for their grants.

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Received for review September 2, 1997. Revised manuscript received January 8, 1998. Accepted January 14, 1998.

JF970753D