Quantitative Fractionation of Grape Proanthocyanidins According to Their Degree of Polymerization

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A method was developed for the fractionation of grape (seed or skin) proanthocyanidins according to their degree of polymerization. After precipitation in chloroform/methanol (75:25, v/v), the grape proanthocyanidins were deposited onto an inert glass powder column and sequentially dissolved in several fractions by increasing proportions of methanol in the solvent. Each fraction from each proanthocyanidin source was quantified and characterized after acidic degradation with phenylmethanethiol (i.e., thiolysis). The comparison of data from total extract and successive fractions showed that a quantitative separation was achieved so that estimation of polymer size distribution in relation to other compositional characteristics (proportions of prodelphinidin units, galloylation rate) was thus possible. Mean degree of polymerization of separated proanthocyanidins ranged increasingly from 4.7 to 17.4 in seed (8.1 for total extract) and from 9.3 to 73.8 in skin (34.9 for total extract). The method proposed is very interesting for the study of grape proanthocyanidins according to their degree of polymerization because it gives both qualitative and quantitative information especially on the highly polymerized forms, which were not fractionated by previous techniques.

Keywords: *Grape; proanthocyanidins; tannins; fractionation; thiolysis; polydispersity*

INTRODUCTION

Proanthocyanidins (condensed tannins) are oligomeric and polymeric flavan-3-ols based on various constitutive units. In grape, the main units are (+)-catechin, (−)-epicatechin, (−)-epicatechin-*O*-gallate, and (−)-epigallocatechin. They are linked to each other by C4−C8 or C4−C6 B-type bonds (Figure 1). Besides their positive role in human health (Masquelier, 1988; Ricardo da Silva et al., 1991a), proanthocyanidins play an important part in wine and in plant-derived foods in general. Thus, they are responsible for many organoleptic features such as color stability (Haslam, 1980), astringency, and bitterness (Haslam, 1974; Arnold and Noble, 1978; Gacon et al., 1996; Kallithrata et al., 1997).

Methods based on acid-catalyzed degradation in the presence of nucleophiles (e.g., phenylmethanethiol) allow determination of the constitutive units and mean degree of polymerization of tannin extracts (Thompson et al., 1972; Hemingway et al., 1983; Rigaud et al., 1991; Matthews et al., 1997). However, they only give access to an average composition and provide no information on polymer size distribution. Nevertheless, such a parameter has to be considered in the study of the organoleptic or pharmacological properties of proanthocyanidins, which largely depend on their structure and especially on their degree of polymerization (Haslam, 1974; Rigaud et al., 1993).

Several methods have been developed to separate oligomeric and polymeric proanthocyanidins on a molecular weight basis. On the one hand, many chromatographic separations of proanthocyanidins were attempted: gel permeation chromatography (Bae et al., 1994), affinity chromatography (Oh and Hoff, 1979), fractionation on C_{18} Sep-Pak cartridges (Sun et al., 1998), chromatography on Sephadex LH-20 (Lea and Timberlake, 1974; Boukharta et al., 1988), Sephadex G-25 (Somers, 1966; McMurrough and McDowell, 1978; Cacho and Castells, 1991), and Fractogel TSK HW-40 (Derdelinckx and Jerumanis, 1984; Ricardo da Silva et al., 1991b), normal-phase TLC (Lea, 1978) or HPLC (Rigaud et al., 1993), and cyano-bonded normal-phase HPLC (Wilson, 1981). These methods generally allow a good separation of tannin oligomers (from dimers up to heptamers), but as the degree of polymerization increases, the resolution becomes poor, and neither characterization nor quantification of main populations into the highly polymerized fraction is achieved. In addition, irreversible adsorption can occur during chromatographic separations, particularly for polymeric species

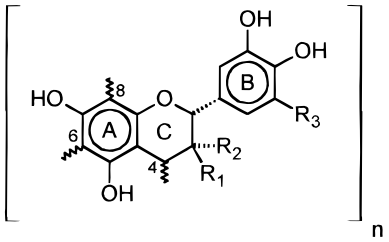

Figure 1. General scheme of grape proanthocyanidins: *n*, degree of polymerization; (4, 6, 8), carbon positions for C4−C6 or C4−C8 interflavanic linkages; (A, B, C), ring labels. Main constitutive units are as follows: (R1 = OH, R2 = H, R3 = H), (+)-catechin; (R1 = H, R2 = OH, R3 = H), (−)-epicatechin; (R1 = H, R2 = *O*-galloyl, R3 = H), (−)-epicatechin-3-*O*-gallate; (R1 = H, R2 = OH, R3 = OH), (−)-epigallocatechin.

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that interact strongly with adsorbent phases. On the other hand, a few methods based on liquid−liquid extraction (Yokotsuka et al., 1978; Kolodziej, 1985; Marston and Hostettmann, 1994) and on precipitation (Glories, 1978; Giner-Chavez et al., 1997) allow isolation of proanthocyanidin fractions from a crude polyphenolic extract. They are specific, quantitative, but the fractionation obtained is not sufficient to describe a tannin extract.

Finally, direct analysis of condensed tannins by ESI-MS without former fractionation (Cheynier et al., 1997) gives good results, because oligomers and even polymers are identified individually in the whole sample, but they are only qualitative so that the size distribution remains undetermined.

The purpose of this investigation was to develop a simple and quantitative method to achieve fractionation of proanthocyanidins allowing estimation of their size distribution.

MATERIALS AND METHODS

Materials and Thiolysis Standards. Organic solvents and phenylmethanethiol were respectively purchased from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). (+)-Catechin, (−)-epicatechin, and *p*-hydroxybenzoic acid methyl ester were purchased from Sigma (St. Louis, MO) and repurified by semipreparative reverse-phase HPLC. (−)-Epicatechin-3-*O*-gallate along with benzylthioether derivatives of (+)-catechin, (−)-epicatechin, (−)-epicatechin-3-*O*-gallate, and (−)-epigallocatechin were isolated from a thiolyzed extract of concentrated proanthocyanidins and purified by semipreparative HPLC as described earlier (Souquet et al., 1996).

Preparation of Grape Freeze-Dried Proanthocyanidins. Grape berries of *Vitis vinifera* cv. Cabernet franc were harvested at commercial maturity and stored at cold temperature before seeds and skins were recovered. Seeds were ground under liquid nitrogen and extracted with acetone/water (60:40, v/v), and the centrifugal supernatant was concentrated under vacuum at 30 °C to give the seed crude extract.

Before direct grinding in aqueous acetone, skins were washed with methanol to remove organic acids and phenolic compounds of low molecular weight. Extracting solvent was treated as for the seeds to give the skin crude extract.

Each extract was separately chromatographed at a preparative scale (column size = 35 × 8 cm) on Toyopearl TSK HW-50(F) gel from Tosoh Corp. (Tokyo, Japan) under the conditions already described by Souquet et al. (1996). After loading, the column was washed with 2 bed volumes of water. Residual monomers were eluted with 5 bed volumes of ethanol/water/trifluoracetic acid (55.00:44.05:0.05, v/v/v). The proanthocyanidin fraction was retrieved with 3 bed volumes of acetone/water (60:40, v/v).

After the organic solvent had been evaporated, seed or skin tannin powders were obtained by freeze-drying aqueous residues and kept at cold temperature. These powders were analyzed by reverse-phase HPLC-DAD (Sarni-Manchado et al., 1999) to check that they were not contaminated by monomeric constituants.

Column Fractional Dissolving Procedure. Fractionations were performed in triplicate for each sample on a 15 mL capacity column filled with 5 g of Pyrex glass microparticles (200−400 μm). The column was equilibrated with methanol/chloroform (25:75, v/v). Freeze-dried tannins were dissolved in methanol, and 1 mL of sample (10 mg) was used for fractionation. Tannins were massively precipitated on the top of the column by addition of 3 mL of chloroform before the methanol/chloroform elution step gradient given in Table 1 was applied. Each collected fraction was added with a known amount of *p*-hydroxybenzoic acid methyl ester as an internal standard for quantitation and then individually taken to dryness by evaporation under vacuum and finally redissolved in 1 mL of methanol.

Table 1. Numbering of Collected Fractions[a] and Methanol/Chloroform Elution Step Gradient Related to Proanthocyanidin Source

	MeOH/$CHCl_3$ (v/v)	
fraction	seed source	skin source
I	25:75	25:75
II	30:70	30:70
III	35:65	35:65
IV	40:60	40:60
V	45:55	45:55
VI	50:50	50:50
VII	55:45	55:45
VIII	100:0	60:40
IX		65:35
X		70:30
XI		100:0

[a] Fraction volume = 10 mL.

Characterization and Quantitation of Proanthocyanidin Fractions. Each methanolic fraction was mixed with an equal volume of thiolysis reagent (5% solution of phenylmethanethiol in methanol containing 0.2 M HCl), stirred, and heated at 90 °C for 2 min. The thiolyzed solution was analyzed directly by HPLC (Souquet et al., 1996) with a Kontron (Milano, Italy) apparatus including a dual-wavelength detector model 430, an autosampler model 460, and a pump system model 325. The column used was a Nucleosil 120-3 μm C_{18} (125 × 4 mm) placed into an oven at 30 °C. Elution conditions were as follows: 0.8 mL/min flow rate, linear gradient from 15 to 75% acetonitrile/water/formic acid (80:18:2, v/v/v) in water/formic acid (98:2, v/v), UV detection at 280 nm. Compound identification and calibration curves (based on peak areas) were performed using thiolysis standards.

By thiolysis reaction, proanthocyanidin terminal units were released as flavan-3-ols (eventually 3-*O*-galloylated), whereas extension units were released as their benzylthioether derivatives. Molar amount of macromolecules in sample was equal to the sum (in moles) of terminal units released. Proanthocyanidin sample weight was obtained by summing the weights of all units released. Molar means for molecular weight (mMW_n, i.e., the ratio between weight and number of macromolecules in sample), degree of polymerization (mDP_n, estimated by dividing molar amounts of total units by molar amounts of terminal units), and molar percentages of galloylated or (−)-epigallocatechin units characterized each proanthocyanidin fraction.

RESULTS AND DISCUSSION

Condensed tannins extracted from grape materials (seeds and skins from *V. vinifera* cv. Cabernet franc) were first separated from phenolic monomers by low-pressure chromatography on Toyopearl TSK HW-50 (F).

The freeze-dried proanthocyanidin extracts were dissolved in methanol and submitted to a column fractionation dissolving procedure as described under Materials and Methods. Eight (respectively, 11) tannin fractions were obtained from 10 mg of seed (respectively, skin) proanthocyanidins.

The average composition of proanthocyanidins in total extract and in each collected fraction was determined using acid-catalyzed depolymerization in the presence of phenylmethanethiol (i.e., thiolysis), followed by reverse-phase HPLC analysis with UV detection. Proanthocyanidins in each sample were thus not only quantified but also characterized by their mean degree of polymerization (mDP_n) or molecular weight (mMW_n) and some percentages of constitutive units.

Fractionation Procedure. When mixing chloroform into a concentrated methanolic solution of proantho-

Table 2. Distribution and Compositional Data of Procyanidin Fractions from Grape Seeds (*V. vinifera* Cv. Cabernet franc)[a]

fraction	distribution n_i (%)	distribution w_i (%)	mMW_n	mDP_n	galloylated units (%)
total extract	100.0	100. 0	2601 ± 5	8.1 ± 0.0	20.4 ± 0.1
I	59.4 ± 3.2	34.4 ± 2.1	1507 ± 12	4.7 ± 0.0	21.1 ± 0.4
II	9.4 ± 0.9	10.3 ± 1.4	2848 ± 222	8.9 ± 0.7	20.1 ± 0.3
III	6.9 ± 1.0	9.5 ± 2.1	3563 ± 272	11.1 ± 0.9	20.4 ± 0.7
IV	7.1 ± 0.7	11.5 ± 1.7	4208 ± 243	13.2 ± 0.8	20.2 ± 0.1
V	5.6 ± 1.7	10.2 ± 2.9	4784 ± 196	15.0 ± 0.6	20.1 ± 0.5
VI	3.5 ± 2.0	7.2 ± 4.2	5339 ± 271	16.8 ± 0.6	18.4 ± 2.8
VII	5.6 ± 2.4	12.0 ± 5.6	5536 ± 193	17.4 ± 0.6	19.3 ± 0.5
VIII	1.2 ± 0.7	2.3 ± 1.3	5018 ± 351	15.7 ± 1.3	19.8 ± 3.1
∑ I−VIII	98.7 ± 1.5	97.6 ± 2.3			
μ_{I-VIII}			2571 ± 58	8.0 ± 0.2	20.7 ± 0.3
σ_{I-VIII}			548 ± 4	1.7 ± 0.0	0.3 ± 0.1

[a] Column dissolving fractionation was performed on a 10 mg total extract in 1 mL of methanol. n_i, w_i = molar and weight relative frequency in percentage of total extract. mMW_n, mDP_n = molar means for molecular mass and degree of polymerization. μ_{I-VIII}, σ_{I-VIII} = means and standard deviations calculated from molar distribution. Values mentioned are means of three repetitions with their confidence interval at 5% significance level.

cyanidins (10−20 g/L), it remained limpid until the volumic concentration of the added solvent reached 30% for skin tannins and 45% for seed tannins. A haze then appeared, and the more chloroform was added, the more important the precipitate became. No further increase of the precipitate was observed at proportions >75%. It is now demonstrated that skin tannins are more polymerized molecules than seed tannins as the mDP_n ranges from 2 to 15 in seed (Prieur et al., 1994) and from 3 to 80 in skin, where 90% (weight percentage) of proanthocyanidins are larger than decamers (Souquet et al., 1996). As a consequence, because seed tannins began to precipitate at a higher chloroform proportion (45%) than skin tannins did (30%) and because precipitates increased in both situations as chloroform was added, chloroform/methanol was assumed to be a suitable solvent/nonsolvent system for the separation of proanthocyanidins according to their degree of polymerization, as proposed earlier by Glories (1978).

Given this, a fractionated precipitation of tannins was tried, but losses were encountered when supernatant was separated from precipitate (by filtration or centrifugation) so that such a method could not be quantitative. Nevertheless, the mDP_n estimated in consecutive supernatants decreased as expected, making this technique suitable for preparative purposes: tannins of higher mDP_n were less soluble in chloroform so that they were removed first when nonsolvent was added.

To improve proanthocyanidin recovery, the reverse technique was employed: tannins were first massively precipitated by addition of chloroform and then sequentially dissolved by increasing methanol concentration in the system. The fractionation was performed on a column filled with glass powder, which is rather inert as far as proanthocyanidins are concerned: the purpose was not to make a chromatographic separation but only a fractionated solubilization of condensed tannins.

Seed Tannins. Thiolysis of the total seed tannin extract released (+)-catechin, (−)-epicatechin, (−)-epicatechin-3-*O*-gallate, and the corresponding benzylthioethers, confirming that these tannins were procyanidins containing galloylated units in agreement with previous work on similar material (Prieur et al., 1994). The yield from thiolysis degradation, calculated as the ratio between the summed amounts of total released units and the initial extract weight, was 88%, which is rather good compared to values obtained by previous authors with the same method (Prieur et al., 1994; Matthews et al., 1997). The total extract was characterized by an mDP_n of 8.1, a galloylation rate of 20.4%, and an mMW_n of 2600, which are usual values for seed procyanidins. After fractionation, each collected sample was quantified relative to total extract and characterized (Table 2).

A complete recovery of the initial sample was achieved because the summed amounts of tannins in the eight fractions yielded, respectively, 98.7% in moles and 97.6% in weight of total sample processed, which was not different from 100% at a 5% significance level. As a result, neither loss nor hydrolysis of polymers occurred during the fractionation. The mDP_n increased significantly for each step from 4.7 (fraction I) to 17.4 (fraction VII) and slightly decreased to 15.7 in the last fraction, but not significantly. The galloylation rate remained constant (20%) in each fraction so that mMW_n varied in the same way as mDP_n. Moreover, for these procya-

Table 3. Distribution and Compositional Data of Proanthocyanidin Fractions from Grape Skins (*V. vinifera* Cv. Cabernet franc)[a]

fraction	distribution n_i (%)	distribution w_i (%)	mMW_n	mDP_n	galloylated units (%)	(−)-epigallocatechin units (%)
total extract	100.0	100. 0	10442 ± 178	34.9 ± 0.6	2.3 ± 0.0	40.9 ± 0.7
I	28.5 ± 1.7	7.6 ± 0.4	2771 ± 76	9.3 ± 0.3	2.9 ± 0.4	31.6 ± 0.4
II	6.6 ± 2.9	3.4 ± 1.3	5541 ± 401	18.5 ± 1.3	3.0 ± 0.3	33.5 ± 0.3
III	7.4 ± 1.5	4.3 ± 0.7	6129 ± 789	20.5 ± 2.7	2.6 ± 0.7	35.6 ± 0.7
IV	8.8 ± 1.7	6.9 ± 1.7	8103 ± 777	27.1 ± 2.6	2.8 ± 0.1	36.9 ± 0.1
V	9.1 ± 1.7	9.2 ± 0.9	10716 ± 1 369	35.8 ± 4.7	2.6 ± 0.5	38.3 ± 0.5
VI	9.1 ± 1.5	12.4 ± 1.2	14286 ± 1 844	47.7 ± 6.3	2.6 ± 2.8	39.5 ± 2.8
VII	6.0 ± 3.3	10.4 ± 4.7	18598 ± 6 561	62.1 ± 22.0	2.7 ± 2.8	40.0 ± 2.8
VIII	4.4 ± 0.3	7.5 ± 1.6	17726 ± 3 167	59.2 ± 10.8	2.9 ± 2.8	39.8 ± 2.8
IX	13.4 ± 4.5	28.0 ± 8.6	22081 ± 2 020	73.8 ± 6.9	2.5 ± 2.8	41.3 ± 2.8
X	1.2 ± 1.3	2.6 ± 2.8	21344 ± 1 353	71.2 ± 4.9	3.6 ± 0.5	34.3 ± 0.5
XI	0.4 ± 0.4	0.4 ± 0.3	15233 ± 10 405	50.9 ± 35.0	3.6 ± 3.1	32.1 ± 3.1
∑ I−XI	95.0 ± 6.1	92.7 ± 3.9				
μ_{I-XI}			10199 ± 58	34.1 ± 1.6	2.7 ± 0.3	36.3 ± 0.6
σ_{I-XI}			2690 ± 4	9.0 ± 1.3	0.1 ± 0.0	1.4 ± 0.1

[a] Column dissolving fractionation was performed on a 10 mg total extract in 1 mL of methanol. n_i, w_i = molar and weight relative frequency in percentage of total extract. mMW_n, mDP_n = molar means for molecular mass and degree of polymerization. μ_{I-VIII}, σ_{I-VIII} = means and standard deviations calculated from molar distribution. Values mentioned are means of three repetitions with their confidence interval at 5% significance level.

nidins and according to this fractionation procedure, the extent of galloylation seemed to be independent from mDP_n.

Although the estimated mDP_n of the total extract was 8.1, the more numerous procyanidins were oligomers with mDP_n 4.7 (60% in fraction I). Besides, the distribution not only was not centered on the mean but also was not unimodal, as shown in Table 2: procyanidins of mDP_n 4.7 and $mDP_n \sim 15-17$ represented the two major clusters. As a consequence, the estimated mDP_n of the total extract (8.1) actually represented a minor class in the procyanidin sample studied, showing that such a parameter taken from the whole tannin extract is not sufficient for a good characterization.

Means and standard deviations reported in Table 2 for mMW_n, mDP_n, and rate of galloylation were calculated from these parameters weighted by the relative frequency in which they occur in each fraction. Means of the three parameters calculated from distribution showed no difference from those estimated by thiolysis on total extract.

Skin Tannins. Thiolysis of the total skin tannin extract released the same compounds as those from seeds plus an additionnal one corresponding to the benzylthioether of (−)-epigallocatechin. As reported by Souquet et al. (1996), skin tannins are proanthocyanidins containing both procyanidin and prodelphinidin units.

The yield from thiolysis degradation on these proanthocyanidins was 67%, which is quite acceptable. This value, lower than encountered in seed, was due to possible contaminants in our sample or even to nonflavanic structures (e.g., anthocyanins) included inside the polymer but not identified in skin tannins yet. In addition, traces of (+)-gallocatechin, (−)-epigallocatechin, and (−)-epigallocatechin-3-*O*-gallate were identified as skin tannin units using LC/MS by Souquet et al. (1996), but neither these minor components nor probable rearrangement compounds (McGraw et al., 1993) were taken into account in the tannin quantification. Finally, the thiolysis conditions used were certainly suitable for seed tannins but not optimal for skin tannins.

The total extract was characterized by an mDP_n of 34.9, a galloylation rate of 2.3%, a percentage of (−)-epigallocatechin units of 40.9%, and an mMW_n of 10440, which is in agreement with values reported previously in *V. vinifera* cv. Merlot (Souquet et al., 1996). After fractionation, each of the 11 fractions was quantified relative to total extract and characterized (Table 3).

The recovery was 95.0 ± 6.1% in moles against 92.7 ± 3.9% in weight, suggesting that a specific loss of higher polymers might occur. The mDP_n increased significantly from 9.3 in fraction I to 47.7 in fraction VI and ranged from 50.9 to 73.8 in fractions VII−XI. For these fractions, the rather large confidence intervals did not allow us to conclude if a separation according to increasing mDP_n was effectively achieved or not. For a given mDP_n, tannins with numerous C4−C6 bonded chains may be less soluble than exclusively C4−C8-linked polymers. As a consequence, some branched proanthocyanidins with lower mDP_n could contaminate the pool of higher polymerized species based only on C4−C8 bonds. Unfortunately, this remains a hypothesis because thiolysis does not give any information concerning the type of linkage.

The galloylation rate was low and independent from mDP_n (2.7%). The percentage of (−)-epigallocatechin units slightly increased with mDP_n (from 31.6% in fraction I to 41.3% in fraction IX). These results confirmed those of Souquet et al. (1996) obtained by thiolysis following normal-phase HPLC separation of similar material.

The mean percentage of (−)-epigallocatechin units calculated from distribution (36.3%) is lower than estimated by thiolysis on total extract (40.9%). A specific loss (e.g., by adsorption on glass) of tannins with a high proportion of trihydroxylated B-rings could explain this result. It must be noticed that the same solvent mixture solubilized skin proanthocyanidins with mDP_n higher than seed procyanidins, showing that seed tannins were less soluble in chloroform than skin tannins, for a given mDP_n. This property could be related to the differences in tannin composition between both sources (galloylation rate, percentage of prodelphinidin units, extent of ramification), leading to molecules of rather different solubility.

As for seed procyanidins, the estimated mDP_n of the total extract (34.9) did not accurately characterize the proanthocyanidin sample because the distribution revealed at least three major clusters: 28.5% with mDP_n of 9.3 (oligomers in fraction I), 18.2% with mDP_n of 41.7 (polymers in fraction V pooled with fraction VI), and 14.6% with mDP_n of 72.5 (high polymers in fraction IX pooled with fraction X). Standard deviations calculated from distribution gave polydispersity indexes for mDP_n (9.0) and mMW_n (2690) higher than for seed procyanidins (Table 2), as expected.

CONCLUSION

The use of a column fractional dissolving procedure with methanol/chloroform solvent is a good way to achieve fractionation of condensed tannins according to their degree of polymerization. The technique developed permits the quantitative separation of proanthocyanidins for either analytical or preparative purpose. The procedure does not need heavy instrumentation and takes rather little time: elutions are as fast as chromatographic methods, and solvent evaporation, before further analyses (e.g., thiolyses), is really easy. The normal-phase HPLC separation achieved by Rigaud et al. (1991) is believed to be actually a combining of fractional dissolving and adsorption chromatography: the methanol/dichloromethane solvent used by these authors was a system likely to achieve differential solubilization of proanthocyanidins as methanol/chloroform does.

Finally, the molar distribution of proanthocyanidins in a sample gives access to the polydispersity of the mDP_n related to the extent of copolymerization (e.g., galloylation) into the flavanic chain: in our particular case, we demonstrate that there was little change in the tannin apparent structure over the molecular weight profile.

Such a knowledge could help us to understand either the organoleptic or the pharmacological properties of proanthocyanidins contained in plant foods and beverages.

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