# Rapid Fractionation of Grape Seed Proanthocyanidins

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A rapid method that permits separation of grape seed proanthocyanidins according to their polymerization degrees has been developed. This method was based on liquid/liquid extraction and relative solubility of these compounds in different solvents (water, ethyle acetate, methanol, and chloroform). The different fractions obtained were then analyzed by various HPLC techniques (normal phase, reversed phase after thiolysis, and gel permeation) to determine their mean degree of polymerization (DP) and molecular weight profiles. Results show that this method can be used on a preparative scale to separate these polymers according to their sizes.

**Keywords:** *Grape; seed; proanthocyanidin; size; molecular weight; fractionation*

## INTRODUCTION

Proanthocyanindins, also commonly called condensed tannins (Figure 1), are present in grape solid parts (seeds and skins) (*1*, *2*). During the wine-making process they are extracted and contribute to the wine taste and color (*3*). One important sensorial property conferred to wine is astringency, which is defined by the drying and puckering sensation felt in the mouth (*4*, *5*). This phenomenon is generally explained by a precipitation of salivary proteins by tannins (*6*, *7*). One important parameter influencing this phenomenon is the exact or mean polymerization degree of these polymers (*8*, *9*). The degree of polymerization (DP) may be determined quite easily by HPLC analysis after thioacidolysis (*10*−*12*), by gel permeation chromatography (*13*, *14*), or by NMR analysis (*15*, *16*). The affinity of tannins toward proteins in simple solutions increases in vitro with increasing DP (*17*−*19*). To conduct further research of such phenomena, it is necessary to have some method that allows separation of tannins according to their size for analytical and preparative purposes. Concerning the analytical aspect, convenient separation can be achieved by normal-phase TLC or HPLC (*8*, *20*). For preparative purposes, gel chromatography with different gels such as Sephadex G25, LH20, or more recently TSK HW40 (*21*, *22*) has been used. Unfortunately, only oligomers up to five are easily separated with these methods, and irreversible adsorption often occurs which limits the life of these expensive gels.

A dissolution procedure that use methanol and chloroform has been recently proposed (*23*), but the glass powder used was not specified and does not seem to be commercially available. In this paper we propose an alternative procedure that uses binary mixtures of chloroform and methanol in order to achieve successive precipitations (*3*). It has good yield and uses only solvents and glass filters, so it can be easily reproduced in other laboratories.

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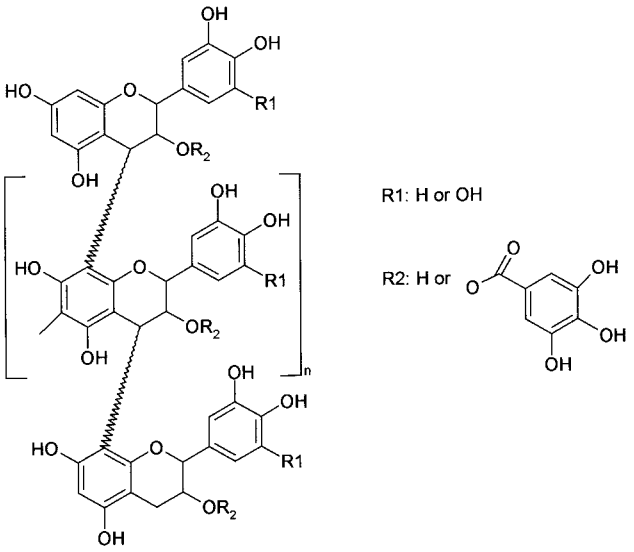


**Figure 1.** General proanthocyanidin structure in *Vitis vinifera* berries.

## MATERIALS AND METHODS

**Solvents and Reagents.** All solvents (HPLC grade) and reagents were from Fisher Scientific (Leicestershire, UK). Distilled water was purified in a milli-Q water purification system (Millipore, Bedford, MA).

**Tannin Crude Extract (TCE).** Grapes (*Vitis vinifera* var. merlot) were manually harvested at technological maturity (September 21, 2000). Seeds and skins were separated manually and frozen at −18 °C until used.

Portions (50 g) of seeds were extracted 2 times successively in acetone/water (7:3) under nitrogen with mechanical agitation for 12 h. The organic solvent was then evaporated, and the aqueous solution was freeze-dried to give 2.1 g and 1.3 g of crude extract, respectively. These extracts were blended to give the crude tannin extract (TCE).

**Oligomer/Polymer Fractionation.** The TCE powder was dissolved in 400 mL of aqueous solution to reach 10 g/L. This solution was extracted three times with ethyle acetate (v/v). The organic phase (EA) contained mainly oligomers, while the aqueous fraction (W) contained the polymers.

**Monomer Removal from Oligomers: Fraction F6.** The EA solution was evaporated to dryness (30 °C) and redissolved in 120 mL of $H_2O$. This solution was then applied to the SPE

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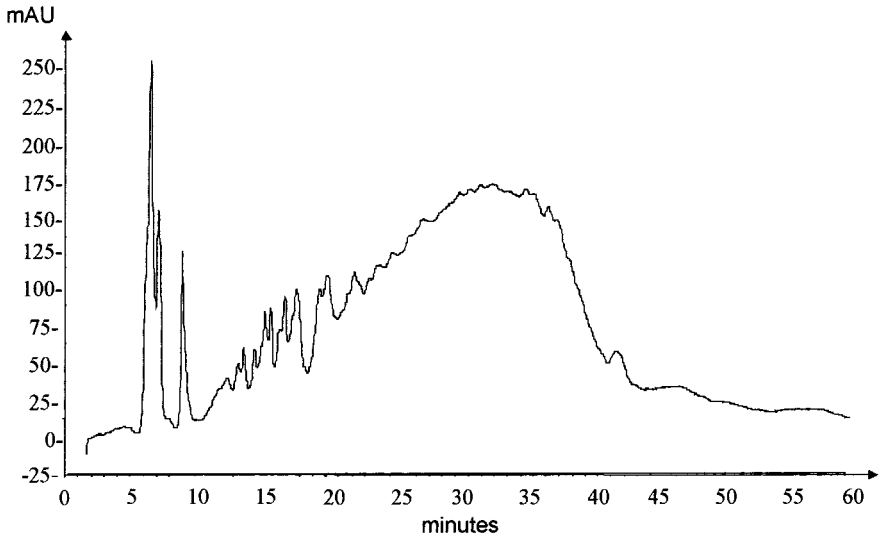


**Figure 2.** Normal-phase HPLC analysis of the TCE extract from grape seeds.

**Table 1. Determination of the Monomeric Composition of the Fractions by HPLC after Thiolysis, and Determination of the Degree of Polymerization (DP) by Thiolysis and GPC**

| | (−)epicatechin-gallate % | (+)catechin % | (−)epicatechin % | DPn (thiolysis) | DPn (GPC) |
|---|---|---|---|---|---|
| TCE | 13.4 ± 0.1 | 24.2 ± 0.0 | 62.2 ± 0.1 | 3.6 ± 0.1 | 3.4 ± 0.3 |
| fraction 1 | 19.2 ± 2 | 16.0 ± 0.4 | 64.4 ± 0.6 | 12.2 ± 0.5 | 9.4 ± 0.1 |
| fraction 2 | 16.2 ± 2.6 | 19.0 ± 0.1 | 64.8 ± 2.4 | 9.8 ± 0.0 | 8.3 ± 0.1 |
| fraction 3 | 15.4 ± 0.5 | 21.1 ± 0.2 | 63.4 ± 0.7 | 8.2 ± 0.3 | 6.9 ± 0.2 |
| fraction 4 | 16.0 ± 0.7 | 22.5 ± 0.2 | 61.4 ± 0.9 | 6.7 ± 0.1 | 5.3 ± 0.1 |
| fraction 5 | 10.9 ± 0.5 | 28.7 ± 0.4 | 60. ± 0.2 | 4.3 ± 0.1 | 3.8 ± 0.2 |
| fraction 6 | 17.7 ± 0.0 | 29.8 ± 0.5 | 52.4 ± 0.4 | 3.1 ± 0.1 | 3.4 ± 0.1 |

column (ENVI 18, 10 g, Supelco ref 57138) by applying 40 mL on each column. The monomers were then removed by eluting 120 mL of diethylether. The oligomers were finally recovered by 60 mL of MeOH. This methanol extract was then evaporated to dryness, redissolved in minimum water, and freeze-dried to give fraction F6. The efficiency of monomer removal was checked by thin-layer chromatography (Silica 60 F254, HPTLC, Merck ref 5556) with ethyl acetate/formic acid (98:2, v/v).

**Polymer Fractionation: Fractions F1 to F5.** The W solution was evaporated to dryness and redissolved in 200 mL of MeOH. Chloroform (200 mL) was added. The precipitate formed was collected by filtration on glass filters (Klab, Fisher Scientific ref A70.912.224). The filtrate was retained for the next steps while the filters were washed with minimum MeOH to recover the precipitate. This methanolic extract was then evaporated, redissolved in water, and freeze-dried to give fraction F1. This process was then repeated by adding successively 100, 167, and 133 mL of chloroform to the filtrate each time (representing 60, 70, and 75% of chloroform for the total volume). This results in F2, F3, and F4 for the precipitates and F5 for the last filtrate. All these solutions were evaporated, redissolved in water, freeze-dried, and kept at −18 °C until further analysis.

**Gel Permeation Chromatography (GPC).** Before GPC analysis, the compounds were acetylated as follows. A 50-mg aliquot of polymer was dissolved with ultrasonication in a mixture (1:1, v/v) of acetic anhydride and distilled pyridine. The reaction mixture was then kept in the dark for 72 h. It was then poured on melting ice at ambient temperature for 4 h. The precipitate formed was then collected by centrifugation (3500 rpm, 15 min) and rinsed three times with distilled water with the same precipitation procedure. The precipitate was then evaporated to dryness under vacuum at 30 °C. It was then washed three times with MeOH, redissolved in chloroform, and evaporated to dryness under vacuum at 30 °C.

The GPC analyses were then performed in the LCPO Laboratory (Pessac, France) on peracetate derivatives (*13*). The solvent used for HPLC was THF at 1 mL/min, and three TSK columns (2000H, 3000H, and 4000H) were connected in series. Detection was at 280 nm. The system was calibrated with 13 polystyrene standards (312−109 kDa).

**Thiolysis and HPLC Analysis.** The thiolysis reagent was prepared by dissolving 500 μL of benzyl mercaptan in 2 mL of HCl N. The final volume of this reagent was adjusted to 10 mL with MeOH. The reaction mixture (100 μL of reagent and 100 μL of tannin solution at 1 g/L) was placed in a sealed 8-mL Pyrex glass test tube and heated at 90 °C during 2 min (*23*). Before injecting the mixture for HPLC, 150 μL of water was added to avoid asymmetrical peaks (*10*). The HPLC conditions were adapted from Matthews et al. 1997 and were the following: flow rate, 1 mL/min; solvent A, water with 5% acetic acid; solvent B, MeOH with 5% acetic acid; gradient, 30% to 100% B in 35 min, 100% B during 5 min, and 100% to 30% B in 5 min; injection volume, 20 μL; detection at 280 nm; column thermostated at 30 °C.

## RESULTS AND DISCUSSION

In accordance with previous works, we made a tannin crude extract (TCE) of the seed in a mixture of acetone and water (7:3) under nitrogen. The HPLC normal phase analysis (Figure 2) and thiolysis show that this extract contains both monomers and polymers and has a mean polymerization degree of 3.6 ± 0.1. The composition in flavanol (estimated by reversed-phase HPLC after thiolysis) is given in the second line (TCE) of Table 1. The chromatogram and the composition obtained are in accordance with those of previous studies (*2*, *20*). The extract contains only procyanidins polymers with some galloylation on epicatechin units (13.4% of galloylation). This extract was perfectly soluble in MeOH and insoluble in chloroform, a typical property of tannin already used in our laboratory to precipitate polymerized tannin from red wine (*3*). We first tried to separate TCE directly by successive precipitations in chloroform.

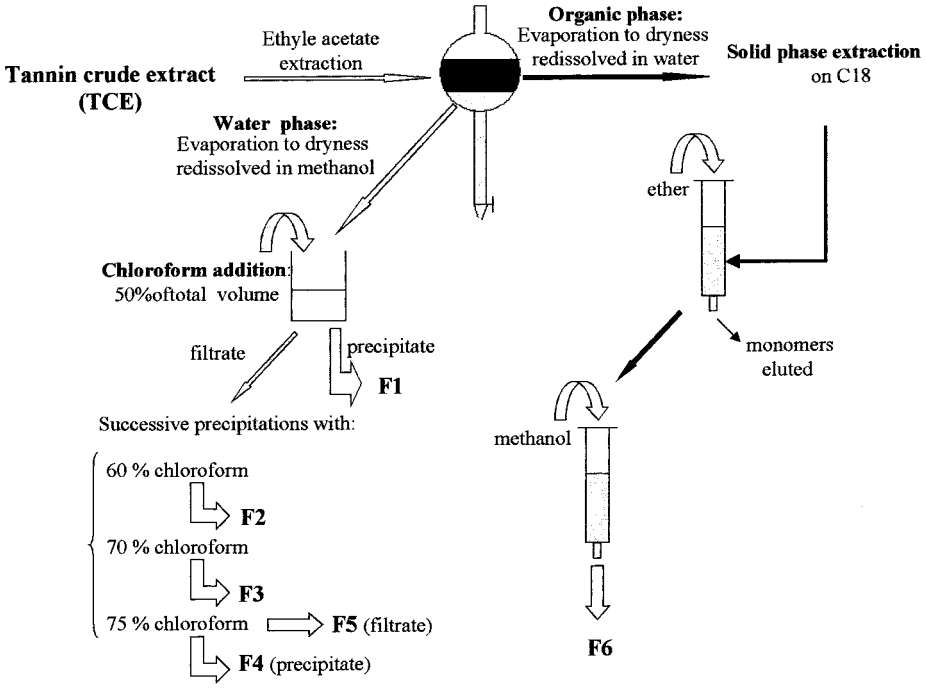


**Figure 3.** Extraction and purification procedure.

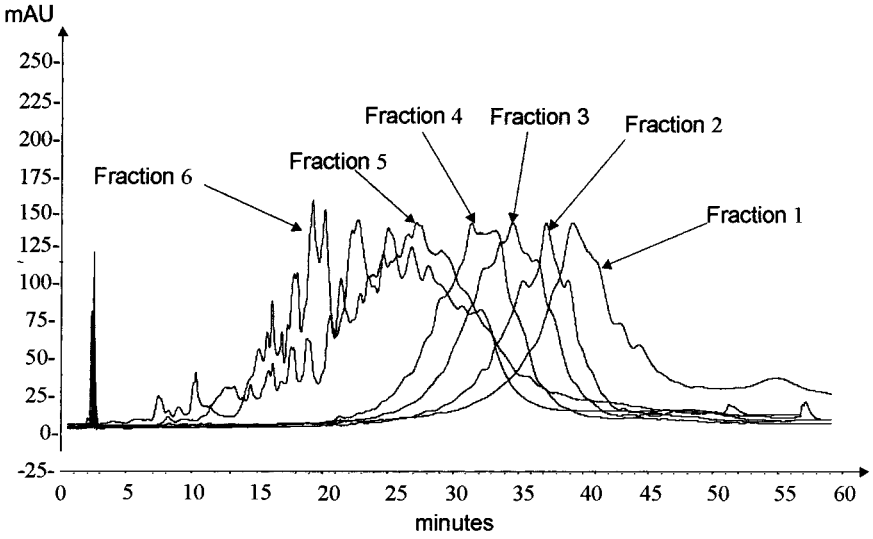


**Figure 4.** Normal-phase HPLC analysis of the different fractions obtained from the TCE grape seed extract.

The results were quite good (decreasing polymerization degree), except for the first fraction which did not have the highest polymerization degree and was quite polydisperse. We then decided to first extract the oligomers by ethyl acetate, a classic procedure in procyanidin chemistry (*24*). After monomer removal by solid-phase extraction on C18 with diethyl ether, we obtained an oligomeric fraction that we called F6. The remaining polymers were then dissolved in methanol and successively precipitated by adding increasing amounts of chloroform (see Materials and Methods section for details). Each fraction was then recovered by vacuum filtration (Büchner type) on glass filters. The entire purification procedure is summarized in Figure 3. The resulting fractions are then nicely ordered by decreasing mean molecular weight as seen on the normal-phase HPLC chromatogram (Figure 4). The degree of polymerization (DP) values obtained by GPC analysis after acetylation or directly on the fractions by thioacidolysis gave similar values and confirmed the results seen in normal-phase HPLC (Table 1). Indeed, these results confirmed that the fractions have decreasing DPs with increasing fraction numbers. However, the differences seen between the two techniques certainly come from the different calibration procedures, as observed by other authors (*2*, *14*).

Concerning the monomer composition in each fraction, it can be seen (Table 1) that it remains quite unchanged in the different molecular weight fractions. Epicatechin represents the most abundant monomer (around 60%) with a proportion of galloylation varying between 10 and 20% approximately. Catechin was less abundant in all fractions and was between 16 and 24%. These results, found in Merlot grapes, were close to those found in previous studies on Cabernet Franc (*23*) and Cabernet Sauvignon (*25*).

The yield of tannin obtained in each fraction, expressed in mass percentage, is given in Table 2. It can be seen that fractions F1, F5, and F6 together represent more than 75% of the recovered tannins. This corre-

**Table 2. Relative Weight of Each Fraction ($m_i/\Sigma m_i$)**

| fraction | mass distribution (in % of total) |
|---|---|
| 1 | 28.8 ± 3.7 |
| 2 | 5.2 ± 0.6 |
| 3 | 10.8 ± 1.1 |
| 4 | 4.2 ± 1.6 |
| 5 | 28.1 ± 5.5 |
| 6 | 22.8 ± 5 |

sponds to the more-polymerized and the oligomers which are indeed abundant as seen in Figure 1. The other fractions are, however, obtained in reasonable yields, especially if we consider the simplicity and preparative purpose of our method. Compared with the initial amount of tannin used (TCE), we obtained a 75% recovery (calculated in mass). Some losses may occur on the filters, and the state of hydration of the molecules may also vary.

## CONCLUSION

We have developed an easy and rapid procedure for the preparation of different molecular weight fractions of grape seed procyanidins. The compositions of the fractions and their degree of polymerization (DP) can be checked by GPC or normal-phase analysis. The fractions obtained have DP values varying between 3 and 13, approximately, and can be easily obtained in gram quantities. This fraction may be used in the future to study the different properties of these compounds (protein complexation and antioxidant properties).

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