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POLYMERIC PROANTHOCYANIDINS FROM GRAPE SKINS

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Key Word Index—*Vitis vinifera*; Vitaceae; grape; flavan-3-ols; procyanidins; prodelphinidins; LC-MS; toluene-α-thiol derivatives.

Abstract—LC-mass spectrometric analysis of the degradation products released by thioacidolysis of a grape (*Vitis vinifera* var. Merlot) skin extract showed that catechin, epicatechin, epicatechin gallate and epigallocatechin were the major constitutive units of grape skin tannins. Gallocatechin and epigallocatechin gallate were also detected. Epicatechin represented 60% of the extension units, whereas 67% of the terminal units consisted of catechin. Six fractions were prepared from the skin tannin extract by normal phase and analysed by reverse-phase HPLC after thioacidolysis. The mean degree of polymerization (mDP) calculated for each fraction ranged from three (in fraction I) to 80 (in fraction VI), confirming that proanthocyanidins were eluted from the normal phase column in increasing order of M_r. All fractions contained prodelphinidins. The proportion of galloyllated units was low (3% to 6%) and independent of mDP. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Grape proanthocyanidins (condensed tannins) are responsible for some major wine organoleptic properties (astringency, browning and turbidity) and are involved in wine ageing processes [1, 2]. The structure of proanthocyanidin dimers and trimers has already been studied and elucidated in grape seeds [3, 4]. Some of them have also been reported in skins [5]. However, oligomers are usually present in lower quantities than higher M_r tannins in plants [6]. Also, astringency and bitterness of procyanidins are known to vary both with the degree of polymerization [7, 8] and with the extent of galloylation [8, 9]. The oligomeric and polymeric proanthocyanidins from grape seeds [10] were shown to be procyanidins, consisting of flavan-3-ol units ((+)-catechin (**1**), (−)-epicatechin (**3**) and epicatechin-3-*O*-gallate (**5**)) linked by C-4–C-8 or C-4–C-6 bonds. Epicatechin was the major component in the extended chain. The proportion of galloylated units varied from 13% to 23% as the mean degree of polymerization (mDP) increased.

The purpose of the present work was to study the oligomeric and polymeric proanthocyanidins in grape skin, because they may contribute significantly to wine tannin composition.

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RESULTS AND DISCUSSION

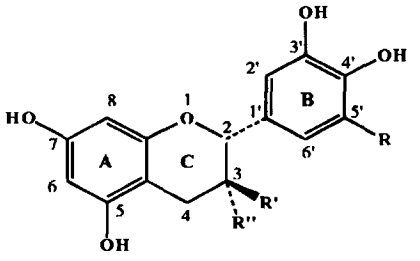
Condensed tannins extracted from grape skins (*Vitis vinifera* var. Merlot) were first separated from contaminating anthocyanins, phenolic acids and flavonols by low pressure chromatography on Toyopearl TSK HW-40 (F). The tannin extract was then submitted to normal phase HPLC, because this technique was shown to achieve elution of procyanidins according to their degree of polymerization [11]. Six tannin fractions (I–VI) were thus obtained. The average composition of proanthocyanidin oligomers and polymers in the extract and in each fraction was determined using acid-catalysed degradation in the presence of toluene-α-thiol [12, 13], followed by reverse-phase HPLC analysis with UV-visible detection as described earlier [14]. The degradation allows the distinction between terminal units (released as flavan-3-ols) and extension units (released as toluene-α-thiol derivatives), and, thus, gives access to mDP and to the respective amounts of each constitutive unit.

Thioacidolysis of the total skin tannin extract yielded (+)-catechin (**1**), (−)-epicatechin (**3**) and (−)-epicatechin-3-*O*-gallate (**5**), and the corresponding benzylthioethers (**6**, **8**, **10**), identified earlier after thioacidolysis of grape seed tannins [14], but also an additional compound, eluting earlier than the benzylthioethers released from procyanidins. Acid hydrolysis of this fraction, followed by HPLC analysis with UV-visible diode array detection, showed that both cyanidin and delphinidin were formed, indicating that

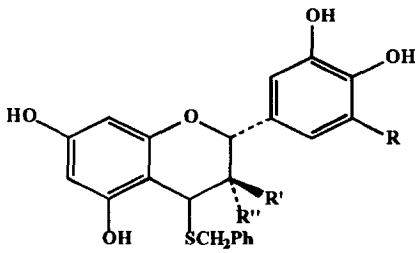
both procyanidins and prodelphinidins were present in grape skins.

LC mass spectrometric analysis, in the negative mode, confirmed the present of (+)-catechin (**1**) (R_t 5.5 min, $[M-H]^-$ m/z 289), (−)-epicatechin (**3**) (R_t 7.0 min, $[M-H]^-$ m/z 289) and (−)-epicatechin3-*O*-gallate (**5**) (R_t 9.3 min, $[M-H]^-$ m/z 441), and of the benzylthioethers of catechin (**6**) (R_t 15.4 and 16.1 min, $[M-H]^-$ m/z 411, $[(M\text{-}Ph\text{-}CH_2S)-H]^-$ m/z 287), epicatechin (**8**) (R_t 16.6 min, $[M-H]^-$ m/z 411, $[(M\text{-}Ph\text{-}CH_2\text{-}S)-H]^-$ m/z 287) and epicatechin3-*O*-gallate (**10**) (R_t 18.2 min, $[M-H]^-$ m/z 563), in the thiolysed extract. Note that two stereoisomers (4α and 4β) of benzylthiocatechin (**6**) but only one epicatechin benzylthioether (**8**) were detected, as noted by other authors [15–18]. The other epicatechin benzylthioether has been reported [17] but was not detected under our experimental conditions, due either to its low concentration or to coelution with one of its isomers.

Two unknown polar compounds (R_t 3.2 and 5.0 min), showing $[M-H]^-$ at m/z 305, and five late eluting compounds were detected in addition to grape seed tannin constituents. The former were present in trace amounts and identified, respectively, as (+)-gallocatechin (**2**) and (−)-epigallocatechin (**4**), by comparison of their R_ts, UV-visible spectra and mass spectra with those of standards. Among the latter, four compounds, eluting at 12.4, 13.4, 13.8 and 14.3 min, showed $[M-H]^-$ at m/z 427, with characteristic fragment ions $[(M\text{-}Ph\text{-}CH_2\text{-}S)-H]^-$ at m/z 303, suggesting that they were stereoisomeric (epi)gallocatechin benzylthioethers (**7**, **9**). The fifth one (R_t 16.1 min, $[M-H]^-$ m/z 579) is presumably a benzylthioether of (epi)gallocatechin-3-*O*-gallate (**11**). ^1H NMR analysis of the major (and least polar) compound at m/z 427 showed that it is 4-benzylthioepigallocatechin (**9**), confirming the presence of prodelphinidins in grape skin, although the actual conformation on C-4 remains to be established. According to Tanaka *et al.* [19], the low coupling constants on the heterocycle indicate a 2,3-*cis* and 3,4-*trans* configuration, although some exceptions have been reported in the case of procyanidin oligomers [20].



(1) R=H, R'=OH, R"=H
(2) R=OH, R'=OH, R"=H
(3) R=H, R'=H, R"=OH
(4) R=OH, R'=H, R"=OH
(5) R=H, R'=H, R"= O-galloyl



(6) R=H, R'=OH, R"=H
(7) R=OH, R'=OH, R"=H
(8) R=H, R'=H, R"=OH
(9) R=OH, R'=H, R"=OH
(10) R=H, R'=H, R"=O-galloyl
(11) R=OH, R'=H, R"=O-galloyl

Quantitative data obtained on the total extract and on each fraction collected from the normal-phase HPLC are presented in Table 1. With the exception of benzylthio-epigallocatechin, (epi)gallocatechin and their derivatives were below the detection level under the conditions of the HPLC-UV analysis and were therefore not quantified.

All fractions contained (+)-catechin, (−)-epicatechin and (−)-epicatechin-3-*O*-gallate as terminal units. Extension units consisted of (−)-epigallocatechin, in addition to the monomeric flavanols mentioned above. Thus, proanthocyanidins from grape skins were both procyanidins and prodelphinidins in all fractions. Epicatechin accounted for 60% of the constitutive units, whereas catechin was particularly abundant in terminal units. Note that only seven procyanidin dimers and trimers among about 30 found in seeds [3], have been identified in grape skins on the basis of their R_ts and thioacidolysis [5]. The major ones were epicatechin-(4$\beta \rightarrow$8)-catechin (B1) and epicatechin-(4$\beta \rightarrow$8)-epicatechin-(4$\beta \rightarrow$8)-catechin, which is in agreement with the distribution of catechin and epicatechin among terminal and other units observed in our study.

Different characteristics calculated from the results of thioacidolysis (*cis*:*trans* ratio, extent of galloylation, mDP and % of prodelphinidins) are shown in Table 2. The total extract showed a mDP of 33, with 3.95% of galloylated units and 31% of prodelphinidins (i.e. epigallocatechin units). The mDP increased from 3.4 in fraction I to 83.3 in the last one, indicating that normal phase HPLC separated grape skin proanthocyanidins in order of increasing M_r, as expected from previous work [10]. The values of mDP in skin condensed tannins were particularly high, compared with those determined earlier in seed tannins. Ratios of *cis*:*trans* increased from 3.3 to 30.3, signifying that the dominant stereochemistry in grape skin tannins was *cis*, although catechin was in larger amounts in terminal units. Except for fraction I, corresponding to lower mDP (<4) with 23.1% of catechin in terminal units, epicatechin units increased from 17.4% in fraction I to 23.6% in fraction II to reach *ca* 30% in the subsequent ones. The proportion of gallates was low in all fractions (<6%) and was not correlated with mDP, whereas in

Table 1. Composition of grape skin proanthocyanidin fractions determined by HPLC following thioacidolysis degradation

	Terminal units			Extension units			
Fraction	Cat	Ec	EcG	Cat	Ec	EcG	EGC
I	23.1	5.1	1.3	—	49.5	3.6	17.4
II	6.9	1.3	2.1	—	62.8	3.3	23.6
III	4.2	0.8	2.3	0.8	60.5	3.7	27.7
IV	2.1	0.3	0.1	1.8	61.3	2.6	31.8
V	1.3	0.2	0.1	2.5	62.9	3.3	28.7
VI	0.9	0.2	0.1	2.3	61.8	3.1	31.6
Total extract	2.05	0.5	0.5	2.4	60.1	3.45	31.0

Cat, Ec, EcG and EGC are the abbreviations for catechin, epicatechin, epicatechin gallate and epigallocatechin units. All amounts represent relative concentrations (in mol).

grape seeds it varied from 13 to 30% as the mDP increased from 2.3 to 15 [10].

The proportion of each fraction was evaluated from peak areas at 280 nm on the normal phase HPLC profile. Monomers, oligomers (from dimer to decamer) and polymers (mDP > 10) represented *ca* 2%, 8% and 90%, respectively, of total flavanols extracted from Merlot skins.

Thus, grape skin tannins were shown to differ from seed tannins primarily by the presence of prodelphinidins but also by their higher mDP and lower amounts of galloylated derivatives. Additionally, although grape seeds contain larger amounts of tannins, skin tannins are expected to be more easily extracted during wine-making, given their localization (in vacuolar liquid, bound to vacuolar membrane and to the cell wall) [21] and higher polarity (prodelphinidins). Therefore, the wine-making conditions known to influence tannin extraction kinetics (e.g. fermentation temperature or pomace contact duration) should modify the proanthocyanidin pool and may, consequently, alter wine quality.

EXPERIMENTAL

Materials. (+)-catechin and (−)-epicatechin were purchased from Sigma and repurified by prep. reverse-phase HPLC to eliminate contaminants. Epicatechin gallate and derivatives (**6**, **8–10**) were isolated from thioacidolysis of concentration skin extract following purification by semi-prep. HPLC. Cyanidin and delphinidin chlorides were from Extrasynthese (France).

Extraction and isolation of skin proanthocyanidins. Grape skins were extracted from 100 berries of *V. vinifera*, var. Merlot, harvested at commercial maturity. Grapes were frozen before separating skin, pulp and seeds. Skins were ground in 250 ml of Me_2CO–H_2O (3:2) and the mixt. filtered. A portion (20 ml) was taken to dryness by rotary evapn, dissolved in 2 ml of EtOH–H_2O–TFA (11:9:0.001). This extract was then chromatographed on Toyopearl HW-40(F)(TOSO-HAAS) (12 cm, 1 cm ID, 1 ml min^{-1}) to eliminate anthocyanins with 30 ml of EtOH–H_2O–TFA (11:9:0.001) and the tannin fr. eluted with Me_2CO–H_2O (3:2) (30 ml). Tannins were taken to dryness under vacuum, dissolved in MeOH and subjected to chromatography on normal phase HPLC using the elution condition described in ref. [10]. Fractions were collected every 10 min, except for fr. I (20 min). Frs were dissolved in MeOH for the thioacidolysis reaction.

Characterization of polymeric proanthocyanidins. Each fr. was placed in a glass ampoule with an equal vol. of reagent (5% soln of toluene-α-thiol in MeOH containing 0.2 M HCl). After sealing, the mixt. was shaken and heated at 60° for 10 min. The hydrolysed soln was then analysed directly by HPLC under the following conditions: flow rate 0.8 ml min^{-1}, temp. 30°, column, Nucleosil C18 3 μM (125 × 4 mm) (Macherey-Nagel), solvent A, H_2O 2% HCO_2H; solvent B, MeCN–H_2O–HCO_2H (40:9:1), elution with linear gradient from 85% to 35% of A in 15 min followed by washing the re-equilibration, detection UV 280 nm. The different frs were analysed in triplicate. Calibration curves were established from standards. Most standards were purified by semi-prep HPLC (250 × 10 mm). The elution conditions were the same as used for analytical HPLC, but the flow rate was 2 ml min^{-1}. Strong acid hydrolysis was performed as described in ref. [22].

^{1}H *NMR.* 400 mHz, 25° in CD_3OD: δ 3.80 (1H, *br d*, $J_{3,4}$ = 2 Hz, C-3-H), 3.92 (2H, *s*, CH_2-S-), 3.97 (1H, *d*, $J_{4,3}$ = 2 Hz, C-4-H), 5.11 (2H, *br s*, C-2-H), 5.84 (1H, *d*, J = 2 Hz, C-6-H), 5.90 (1H, *d*, J = 2 Hz, C-8-H),

Table 2. Characteristics of grape skin proanthocyanidin fractions and total extract

Fraction	I	II	III	IV	V	VI	Total extract
Ratio (*cis*:*trans*)	3.3	13.5	19	24.6	25.3	30.3	21.47
Galloylation (%)	4.9	5.4	6.0	2.7	3.4	3.2	3.95
mDP (thioacidolysis)	3.4	9.7	13.7	40.0	62.5	83.3	33
Prodelphinidins (%)	17.4	23.6	27.7	31.8	28.7	31.6	31

6.42 (2H, *s*, C-2′-H, C-6′-H), 7.15–7.38 (5H, *m*, ArH ($\underline{Ph}CH_2S$)).

Mass spectrometry. Analysis was performed on quadrupole instrument fitted with an electrospray source in the negative mode. Ion spray voltage: −4 kV: orifice voltage −60 V. Chromatographic separation was done on C18 Nucleosil (125 × 2 mm, 3 μM) with the same solvent as used in analytical HPLC, but the flow rate was 200 μl min^{-1}.

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