

Analysis of Proanthocyanidin Cleavage Products Following Acid-Catalysis in the Presence of Excess Phloroglucinol

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The analysis of proanthocyanidin cleavage products after acid-catalysis in the presence of excess phloroglucinol was investigated. In the developed analytical method, a solution of 0.1 N HCl in methanol, containing 50 g/L phloroglucinol and 10 g/L ascorbic acid was prepared. The proanthocyanidin of interest was reacted in this solution (5 g/L) at 50 °C for 20 min, and afterward combined with 5 volumes of 40 mM aqueous sodium acetate before analysis by reversed-phase HPLC using an aqueous acetic acid and methanol gradient. This procedure was used to investigate the composition of proanthocyanidins isolated from the seed and skin tissue of *Vitis vinifera* L. berries. The results compared favorably to results obtained when benzyl mercaptan was used as the trapping nucleophile, indicating that phloroglucinol is an effective reagent for this analysis.

Keywords: Proanthocyanidin; tannin; flavan-3-ol; molar absorptivity; *Vitis vinifera*; grape; phloroglucinol; benzyl mercaptan; thiolysis; yield

INTRODUCTION

Proanthocyanidins are polymeric flavonoid compounds composed of flavan-3-ol subunits, and are widely distributed in the plant kingdom (Figure 1). These compounds are present in grapes, *Vitis vinifera* (1), and are primarily responsible for the astringent properties of red wine (2). As part of a long-term research effort to understand the role that proanthocyanidin structures play in red wine astringency quality, chromatographic methods are being developed for their analysis.

Chromatographic methods used for proanthocyanidin analysis can be divided into those that are used to analyze intact proanthocyanidins (3–5), and those that are used to analyze the proanthocyanidins following acid-catalyzed cleavage (6, 7). Analyzing intact proanthocyanidins provides information on their number average molecular weight (M_n) and weight average molecular weight (M_w) while providing distribution information. Analyzing proanthocyanidins after acid-catalyzed cleavage provides information on their subunit composition as well as the interflavonoid bond location.

It is possible to determine the subunit composition of proanthocyanidins because of the relative ease with which the interflavonoid bond is cleaved [(Figure 2), 8]; and therefore, under acidic conditions, proanthocyanidins become depolymerized, releasing terminal subunits as flavan-3-ol monomers and extension subunits as electrophilic flavan-3-ol intermediates. The electrophilic intermediates can be trapped by a nucleophilic reagent to generate analyzable adducts.

The two most commonly used nucleophilic reagents, phloroglucinol and benzyl mercaptan, were first used in the 1960s and early 1970s (9–11), and have both been used with success. When the quantitative conversion of proanthocyanidins into their constitutive subunits is

desired however, previous reports have suggested that benzyl mercaptan is the preferred reagent (7, 12). Unfortunately, because of its unpleasant odor, benzyl mercaptan usage is limited to laboratories with specialized fume hood equipment.

The use of phloroglucinol as a trapping reagent has several potential advantages over the use of benzyl mercaptan: (1) phloroglucinol is odorless and therefore has no special handling requirements, and (2) there is more selectivity in the formation of 3,4-trans adducts from 2,3-trans flavan-3-ol extension subunits (13).

From previous investigations, it is evident that the conditions for phloroglucinol adduct formation are not ideal. The purpose of this investigation was to determine whether the phloroglucinol assay could be used as a benzyl mercaptan substitute under conditions where maximal conversion of proanthocyanidins into their constitutive subunit adducts was desired.

MATERIALS AND METHODS

Chemicals. All chromatographic solvents were HPLC grade and were purchased from BDH (Kilsyth, Vic., Australia). The following were purchased from Sigma (Castle Hill, NSW, Australia): (+)-catechin, (–)-epicatechin, (–)-epicatechin-3-O-gallate, ascorbic acid, and sodium borohydride. Phloroglucinol, benzyl mercaptan, and sodium acetate were purchased from Aldrich (Castle Hill, NSW, Australia).

Isolation of proanthocyanidins. Grape berries (*Vitis vinifera* L., cvs. Chardonnay and Shiraz) grown on the Waite Campus at the University of Adelaide were used as the source for proanthocyanidins. The fruit was harvested at commercial maturity. The seeds and skins were separated from the berry mesocarp, rinsed with distilled-deionized water, and the intact tissues (i.e., whole seed or skin) were extracted separately in covered Erlenmeyer flasks with 2:1 acetone/water at room temperature for 24 h. To minimize proanthocyanidin oxidation, solutions were sparged with nitrogen and the extraction was carried out in the dark. Following extraction, the extract was concentrated under reduced pressure at 35 °C to remove acetone, and then lyophilized to a dry powder.

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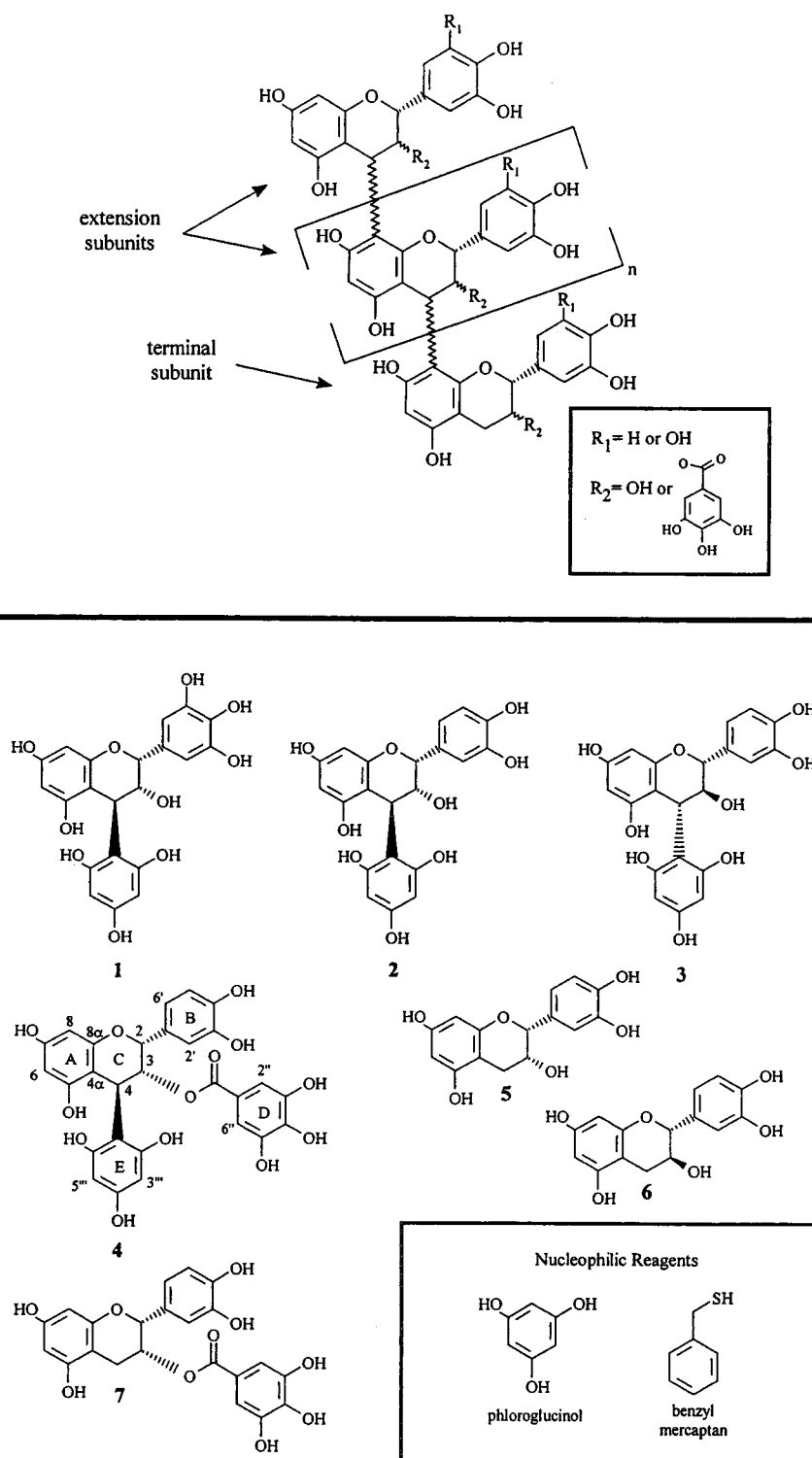


Figure 1. Generalized proanthocyanidin structure and subunit products found in *Vitis vinifera* berries following acid-catalysis in the presence of phloroglucinol.

The crude proanthocyanidins were purified using Toyopearl TSK HW 40-F size exclusion media (Sigma). The column (270 × 28 mm) was equilibrated with 1:1 MeOH/water containing 0.1% v/v trifluoroacetic acid. The proanthocyanidin powder was dissolved in a minimum amount of this mobile phase and then applied to the column. The column was then rinsed with 5 column volumes of the mobile phase to remove carbohydrate and low-molecular-weight flavan-3-ol monomer material. The proanthocyanidins were then eluted with 3 column volumes of 2:1 acetone/water containing 0.1% v/v trifluoroacetic acid. The eluent was concentrated under reduced pressure at 35 °C to remove acetone, and then lyophilized to a dry powder.

Isolation and Characterization of Proanthocyanidin Cleavage Products. With the exception of catechin-(4 α →2)-phloroglucinol, isolated flavan-3-ol-phloroglucinol adducts were prepared from grape proanthocyanidins (Chardonnay skin proanthocyanidins) using the optimized procedure described below.

Catechin-(4 α →2)-phloroglucinol (**3**) was prepared as follows. Taxifolin (500 mg) and sodium borohydride (250 mg) were stirred in absolute ethanol (100 mL) at room temperature for 1 h. Phloroglucinol (1.75 g) was dissolved in 0.1 N HCl (100 mL) and then combined with the taxifolin mixture and stirred for 30 min. Following reaction, 100 mL of water was added

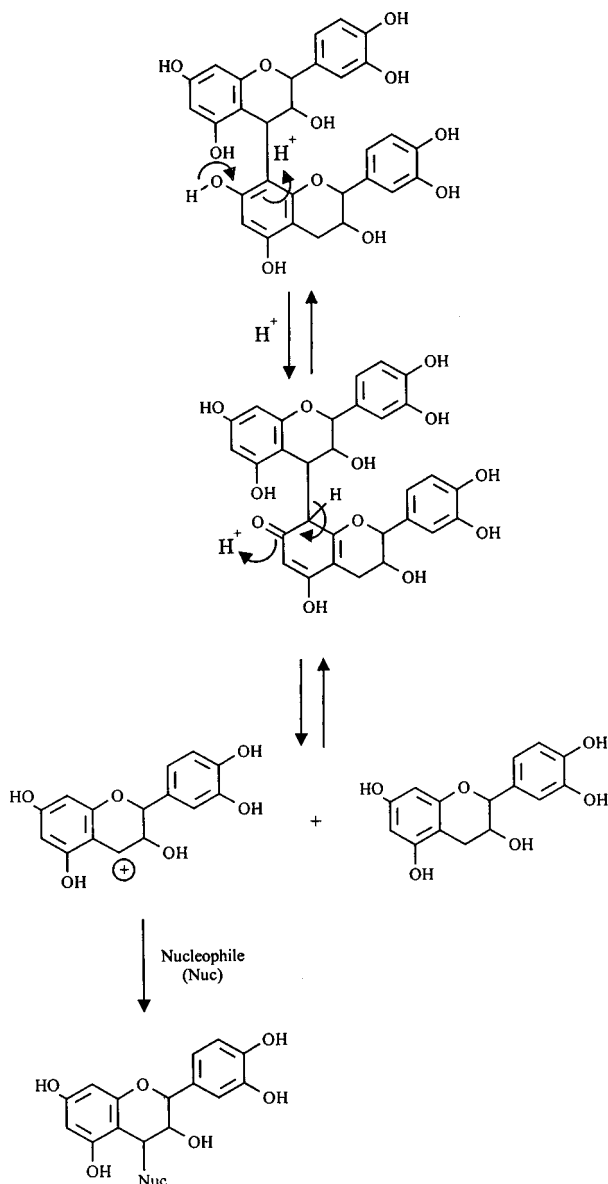


Figure 2. Hypothetical reaction mechanism explaining the acid-catalyzed cleavage of proanthocyanidins.

and the combined solutions were extracted with ethyl acetate (3×100 mL). The ethyl acetate was dried (Na_2SO_4), and evaporated under reduced pressure at $35^\circ C$. The crude catechin-($4\alpha \rightarrow 2$)-phloroglucinol was then purified by semi-preparative HPLC as described below, and lyophilized to a dry powder.

Phloroglucinol adducts were purified by preparative reversed-phase HPLC. The HPLC system consisted of a Waters (Rydalme, NSW, Australia) Prep Nova-Pak HR PrepLC column (particle size $6 \mu m$, 200×25 mm) equipped with a guard column containing the same material. The HPLC was run under isocratic conditions with a flow rate of 20 mL/min., and a mobile phase consisting of 5% v/v aqueous MeOH containing 0.1% v/v TFA. Isolated compounds were lyophilized to a dry powder. Preliminary identification based on the known composition of grape-based proanthocyanidins (14, 15), HPLC retention properties, and UV-Vis absorption spectra indicated that epigallocatechin-($4\beta \rightarrow 2$)-phloroglucinol (1), epicatechin-($4\beta \rightarrow 2$)-phloroglucinol (2), catechin-($4\alpha \rightarrow 2$)-phloroglucinol (3), and epicatechin-3-*O*-gallate-($4\beta \rightarrow 2$)-phloroglucinol (4) had been isolated. Phloroglucinol adducts 1, 2, and 3 were characterized by 1H NMR (600 MHz, Varian Inova) and LC ESI-MS (API-300, PE Sciex) operated in the positive mode (ion spray voltage, 5300 V; orifice potential, 50V). Comparison of the data with previously published values (16), identified 1, 2, and 3 as such.

Because of the lack of published information on 4, additional characterization was undertaken. The isolated compound had the following properties: white amorphous powder; mp, $189^\circ C$ dec; $[\alpha]_D^{20} +24.9$ (c 0.94, MeOH); UV-Vis: λ_{max} 279, ϵ_{279} 14846, ϵ_{280} 14766 (MeOH). The molecular formula was determined by HR-FAB MS (Kratos Concept ISQ). The measured mass (567.11450) was 1.11 ppm above the calculated mass for $C_{28}H_{23}O_{13}$ (567.11387), corresponding to the protonated 4 ion. Further characterization by 1H NMR (600 MHz, Varian Inova), ^{13}C NMR (150 MHz), and 2D NMR (1H - 1H COSY, HMQC, HMBC, NOESY, and ROESY) (Table 1) provided structural confirmation of 4.

Three flavan-3-ol monomers, (-)-epicatechin (5), (+)-catechin (6), and (-)-epicatechin-3-*O*-gallate (7), were identified by comparison with authentic standards.

Procedure using Phloroglucinol. A solution of 0.1 N HCl in MeOH, containing 50 g/L phloroglucinol and 10 g/L ascorbic acid was prepared. The proanthocyanidin of interest was reacted in this solution (5 g/L) at $50^\circ C$ for 20 min, and then combined with 5 volumes of 40 mM aqueous sodium acetate to stop the reaction.

Phloroglucinol adducts were analyzed by reversed-phase HPLC. The column was a Wakosil II 5C18 (particle size $5 \mu m$, 250×4.6 mm) purchased from SGE (Ringwood, Vic., Australia), protected by a guard column containing the same material. The method utilized a binary gradient with mobile phases containing 1% v/v aqueous acetic acid (mobile phase A) and MeOH (mobile phase B). Eluting peaks were monitored at 280 nm. The elution conditions were 1.0 mL/min; 5% B for 10 min, a linear gradient from 5 to 20% B in 20 min, a linear gradient from 20 to 40% B in 25 min. The column was then washed with 90% B for 10 min and reequilibrated with 5% B for 5 min before the next injection.

Proanthocyanidin cleavage products were estimated using their response factors relative to 6, which was used as the quantitative standard (Table 2). For compounds 2, 3 and 5, 6, their molar absorptivities were measured and then averaged. To calculate the apparent mean degree of polymerization (mDP), the sum of all subunits (flavan-3-ol monomer and phloroglucinol adduct, in moles) was divided by the sum of all flavan-3-ol monomers (in moles). To calculate conversion yield information, the mass of all subunits was summed (excluding the phloroglucinol portion of the phloroglucinol adducts) and then divided by the weighed mass of the proanthocyanidin reacted.

Procedure using Benzyl Mercaptan. The procedure used to convert proanthocyanidins into their benzyl mercaptan adducts has been previously described (17).

Benzyl mercaptan adducts were analyzed by reversed-phase HPLC. The column was a LiChrospher RP-18 (particle size $5 \mu m$, 250×3.2 mm), protected by a guard column containing the same material (Supelco; Castle Hill, NSW, Australia). The method used a binary gradient with mobile phases identical to those used for phloroglucinol adduct analysis. The elution conditions were 0.65 mL/min; a linear gradient from 20 to 70% B in 35 min. The column was then washed with 90% B for 5 min and then reequilibrated with 20% B for 5 min before the next injection.

For the quantitation of flavan-3-ol monomers and benzyl mercaptan adducts, 6 was also used as a quantitative standard. The response factors of the other products relative to 6 were used for their estimation as indicated by Kennedy et al. (17). With this procedure the benzyl mercaptan adducts were assumed to have the same molar absorptivities as their respective flavan-3-ol monomers. The mDP and yield information were obtained as described above.

RESULTS AND DISCUSSION

Formation of Phloroglucinol Adducts. From previous investigations, grape-based proanthocyanidins were considered to be a good model for this investigation because of their varied composition and molecular weight (14, 15). An initial analysis of the products

Table 1. ^1H and ^{13}C Assignments^a, HMBC and NOE Correlations for Compound **4**

carbon number	chemical shift of carbon δ (ppm)	chemical shift of proton δ (ppm) ^b	HMBC correlation H–C	NOE correlation ^c
2	75.1	5.45, d (<1)	3,4,1',2',6'	3,2',6'
3	74.7	5.23, dd (1.8, <1)	4,4 α ,1',C=O,1'''	2,4,2',6'
4	33.9	4.61, d (1.8)	2,3,4 α ,5,1',1''',2''',6'''	3, 2'',6''
4 α	100.6	–	–	–
5	157.7	–	–	–
6	96.1	6.00, d (2.4)	4 α ,5,7,8	–
7	157.3	–	–	–
8	95.3	6.08, d (2.4)	4 α ,5,6,7	–
8 α	157.1	–	–	–
1'	131.0	–	–	–
2'	114.4	6.95, d (1.8)	2,1',3',4',6'	2,3,2'',6''
3'	145.1	–	–	–
4'	145.2	–	–	–
5'	115.4	6.72, d (8.4)	1',3',4'	6'
6'	118.7	6.77, dd (8.4,1.8)	2,2',3',4'	2,3,2',5',2'',6''
1''	120.9	–	–	–
2''	109.8	7.04, s	1'',3'',4'',6'',C=O	4,2',6'
3''	145.6	–	–	–
4''	138.8	–	–	–
5''	145.6	–	–	–
6''	109.8	7.04, s	1'',2'',3'',4'',C=O	4,2',6'
1'''	105.7	–	–	–
2'''	157.8 ^d	–	–	–
	157.7 ^e	–	–	–
3'''	96.4 ^d	5.96, brs	1''',2''',5''',6'''	–
	96.3 ^e	–	–	–
4'''	158.0 ^d	–	–	–
	157.9 ^e	–	–	–
5'''	96.4 ^d	5.96, brs	1''',2''',3''',6'''	–
	96.3 ^e	–	–	–
6'''	157.8 ^d	–	–	–
	157.7 ^e	–	–	–
C=O	166.9	–	–	–

^a Recorded in acetone- d_6 with acetone as internal standard. ^b J values are given in Hz in parentheses. ^c From NOESY and ROESY experiments. ^d Major rotamer. ^e Minor rotamer.

Table 2. Retention Properties, Molar Absorptivities, and Response Factors for the Major Grape Proanthocyanidin Cleavage Products

compound	retention factor (k)	molar absorptivity ^a (ϵ_{280})	relative molar response ^b	corrected relative mass response ^{b,c}
1	5.8	1344	0.34	0.32
2	9.4	4218	1.06	1.06
3	9.1	4218	1.06	1.06
4	13.4	14766	3.70	2.44
5	16.6	3988	1.00	1.00
6	13.1	3988	1.00	1.00
7	19.1	12611	3.16	2.07

^a In methanol. ^b Relative to **6**. ^c Not including the phloroglucinol (D-ring) moiety.

formed in the presence of excess phloroglucinol revealed that seven major products were formed (Figure 3), consistent with results obtained when using benzyl mercaptan (Figure 4).

Based primarily upon a previous investigation where reaction yield information was determined (14), standard reaction conditions were selected. These conditions were 0.1 N HCl in methanol. The principle deviation from the method of Prieur et al. (14) was that the reaction temperature was reduced from 90 to 50 °C because it was believed that the reported reaction time of 2 min would be difficult to control. Additionally, ascorbic acid (10 g/L) was added to improve conversion yield and reproducibility (as discussed below).

Phloroglucinol Addition. An examination of previous work that has utilized phloroglucinol as a nucleo-

phile reveals that a variety of conditions have been used (7, 12, 18). In contrast to methods that have utilized benzyl mercaptan (14, 19, 20), the phloroglucinol-based methods are deficient in the number of equivalents of phloroglucinol used in the reaction.

The effect of added phloroglucinol concentration on seed and skin proanthocyanidin isolates from Chardonnay grapes was investigated because these isolates had the lowest and highest nucleophile requirements based on mDP (determined by thiolysis), respectively. Increasing the amount of added phloroglucinol had a dramatic effect on the conversion of both grape seed (Figure 5A) and skin (Figure 5B) proanthocyanidins into their constitutive subunits. Consistent with predictions based on mDP, the seed proanthocyanidins approached maximal conversion sooner than the skin-based proanthocyanidins.

The amount of added phloroglucinol also affected the observed proanthocyanidin subunit composition. It can be seen from Figure 5 that the yields of flavan-3-ol monomers were close to their maximum values even at a low phloroglucinol equivalency. For the phloroglucinol adducts, **4** was closer to its 20 equivalent value than were **1**, **2**, or **3**, indicating that reported subunit composition could vary depending on reaction conditions and that if the purpose of cleaving proanthocyanidins is to determine subunit composition, then much higher phloroglucinol equivalents are required than have been used previously.

Reaction Time. The reaction time was determined as a function of side product formation. Previous studies have indicated that of the products formed from proan-

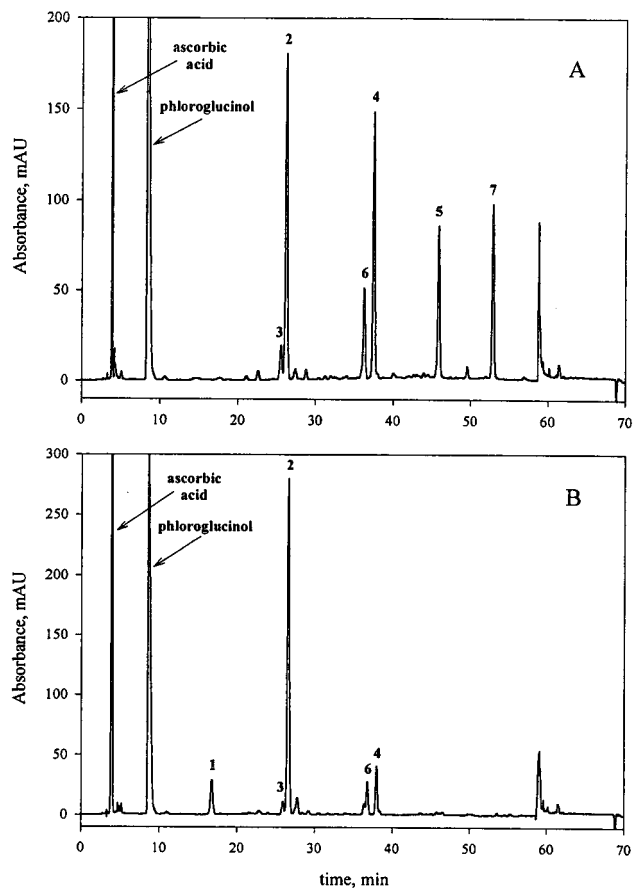


Figure 3. HPLC chromatogram of proanthocyanidin cleavage products from (A) Chardonnay grape seed and (B) Chardonnay grape skin following acid-catalysis in the presence of phloroglucinol.

thocyanidin cleavage the flavan-3-ol monomers are the least stable, undergoing C-2 epimerization. Of the flavan-3-ols found in grapes, Prieur et al. (14) found that 5 is the least stable. Based on this work, the stabilities of the proanthocyanidin cleavage products 2, 5, and 6 were investigated.

The products investigated all degraded following apparent zero-order kinetics. Compound 2 was the most stable ($t_{90} = 133$ min), consistent with its benzyl mercaptan analogue (14). Compounds 5 and 6 were much less stable ($t_{90} = 18$ and 30 min, respectively) than 2, also consistent with previous work. Based on these results, a maximum reaction time of 20 min was selected. For phloroglucinol adduct formation the extent of reaction after 20 min was approximately 90% of the maximal amount measured. Because of the larger proportion of proanthocyanidin extension subunits in the samples under investigation, and the small change that additional reaction time would have on their total concentration, it was decided that the reaction time would be limited by the degradation of 5.

The main products formed from 5 and 6 have the same retention factors (10.1), and based on LC-MS data (m/z 417), the products are consistent with the C-2 phloroglucinol adducts [(Figure 6), 21]. The amount of this degradation product formed could provide important information on the extent to which side reactions have occurred.

Solvent Comparison. On the basis of previous studies, a limited investigation into the effect of various solvents was undertaken. The more commonly used

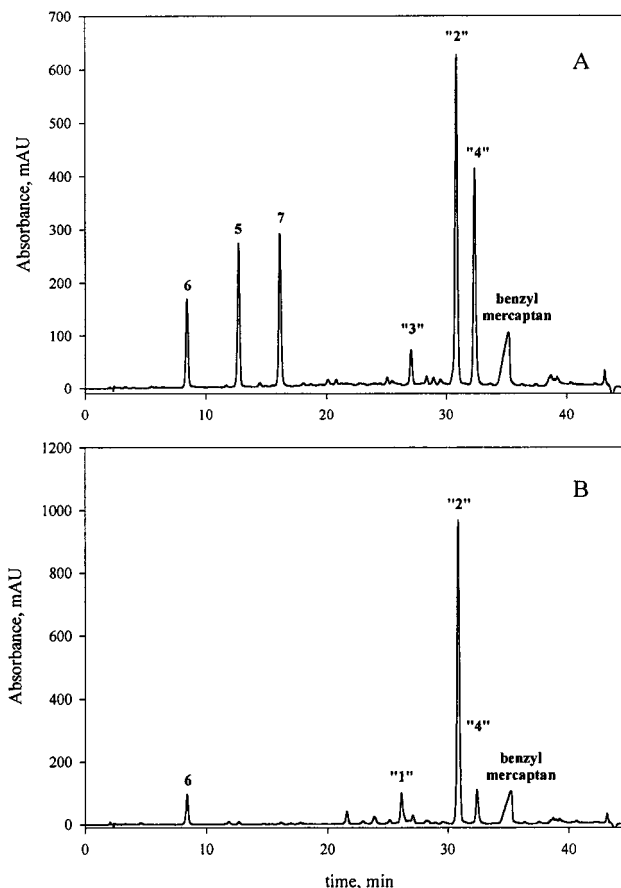


Figure 4. HPLC chromatogram of proanthocyanidin cleavage products from (A) Chardonnay grape seed and (B) Chardonnay grape skin following acid-catalyzed cleavage in the presence of benzyl mercaptan. The compounds "1", "2", "3", and "4", are the benzyl mercaptan equivalents of the phloroglucinol adducts 1, 2, 3, and 4, respectively.

solvent systems include (in addition to methanol) ethanol (19, 20, 22) and dioxane/water solutions (7, 12). For this reaction, the solvent methanol has been reported to be preferred over the more commonly used ethanol because of the shorter reaction times necessary and improved solubility of the proanthocyanidins (23). Under the reaction conditions employed here, there was no difference between methanol and ethanol in the generation of phloroglucinol adducts of proanthocyanidin cleavage products. The combined solubility of proanthocyanidins, phloroglucinol, and ascorbic acid, however, were higher in methanol, and therefore, methanol was used.

The addition of water clearly affected the reaction. With a 20% addition of water to methanol, total phloroglucinol adduct formation was reduced by 13%. This reduction was similar for all products with the exception of 4, which was reduced by 63%. On the basis of these results, the aqueous dioxane solvent system was not investigated.

Method Performance. The success of this method was determined by its reproducibility and performance relative to the results obtained using the benzyl mercaptan-based method. The results of the analysis of replicated proanthocyanidin samples indicate that the phloroglucinol method has excellent reproducibility (Table 3). In limited studies, the addition of ascorbic acid to the reaction solution has improved the reproducibility of the method over reactions carried out without ascorbic acid (Table 4).

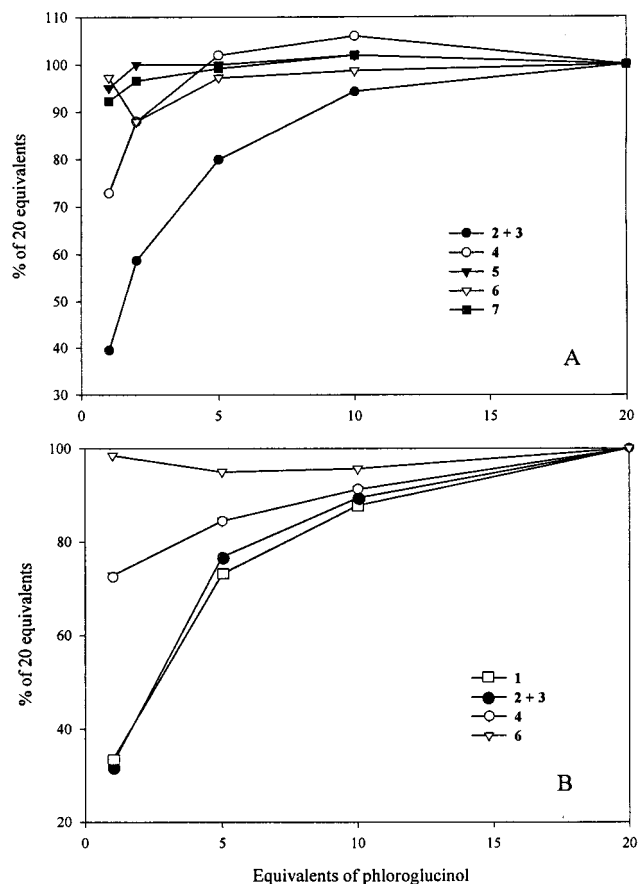


Figure 5. Effect of added phloroglucinol on the production of (A) Chardonnay grape seed and (B) Chardonnay grape skin proanthocyanidin cleavage products.

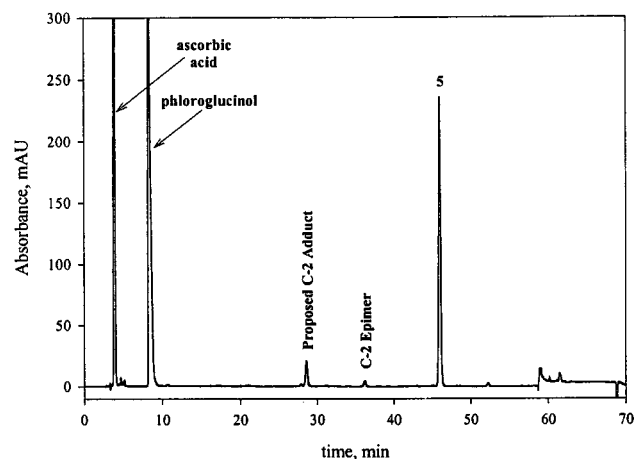


Figure 6. HPLC chromatogram of **5** and its major degradation products following acid-catalysis in the presence of 20 equivalents of phloroglucinol.

A comparison of the phloroglucinol-based assay with the benzyl mercaptan-based assay indicates that the phloroglucinol-based assay is as effective as the less convenient benzyl mercaptan-based assay (Table 5). For seed proanthocyanidins, very similar subunit compositions, mDPs, and conversion yields were obtained from both methods. For the analysis of the skin proanthocyanidins, different subunit compositions were obtained. No readily apparent explanation for this difference could be determined.

The yield for the conversion of proanthocyanidins into their constitutive subunits, apparently low and varied

Table 3. Summary of Grape Proanthocyanidins Following Acid-Catalysis in the Presence of Phloroglucinol with Added Ascorbic Acid \pm Standard Deviation ($n = 5$)

compound	Shiraz skin	Shiraz seed	Chardonnay skin	Chardonnay seed
1	32.5 ^a \pm 0.1	^b	24.4 \pm 0.3	^b
2	51.5 \pm 0.1	53.2 \pm 0.5	64.1 \pm 0.1	45.8 \pm 0.3
3	3.2 \pm 0.1	7.7 \pm 0.1	2.6 \pm 0.4	5.0 \pm 0.1
4	4.6 \pm 0.1	16.3 \pm 0.1	2.5 \pm 0.1	10.5 \pm 0.1
5	^b	10.4 \pm 0.5	^b	18.7 \pm 0.3
6	8.2 \pm 0.1	8.5 \pm 0.2	6.4 \pm 0.1	10.5 \pm 0.1
7	^b	3.9 \pm 0.1	^b	7.4 \pm 0.1
mDP ^c	12.1 \pm 0.2	4.4 \pm 0.1	15.7 \pm 0.3	2.6 \pm 0.1
% yield ^d	56.4 \pm 1.1	62.2 \pm 2.1	80.1 \pm 2.5	70.6 \pm 1.2

^a Values are given in proportional composition (mole %). ^b Not observed. ^c Mean degree of polymerization. ^d Conversion yield into known proanthocyanidin subunits (by mass).

Table 4. Summary of Grape Proanthocyanidins Following Acid-Catalysis in the Presence of Phloroglucinol without Added Ascorbic Acid \pm Standard Deviation ($n = 5$)

compound	Shiraz skin	Chardonnay skin
1	31.7 ^a \pm 2.1	24.1 \pm 1.1
2	51.5 \pm 2.3	63.5 \pm 1.4
3	3.3 \pm 0.4	2.6 \pm 0.4
4	4.7 \pm 0.1	2.7 \pm 0.2
5	^b	^b
6	8.8 \pm 0.6	7.1 \pm 1.4
7	^b	^b
mDP ^c	11.4 \pm 0.7	14.5 \pm 2.4
% yield ^d	56.9 \pm 1.6	79.9 \pm 4.6

^a Values are given in proportional composition (mole %). ^b Not observed. ^c Mean degree of polymerization. ^d Conversion yield into known proanthocyanidin subunits (by mass).

Table 5. Summary of Grape Proanthocyanidins Following Acid-Catalysis in the Presence of Benzyl Mercaptan

compound	Shiraz skin	Shiraz seed	Chardonnay skin	Chardonnay seed
1	18.4 ^a	^b	6.9	^b
2	54.3	55.1	73.7	47.4
3	14.0	7.9	9.4	5.6
4	5.2	16.2	3.0	10.3
5	^b	9.3	^b	18.3
6	8.1	7.8	7.0	11.5
7	^b	3.6	^b	6.9
mDP ^c	12.3	4.8	14.3	2.7
% yield ^d	58.6	65.0	68.4	68.3

^a Values are given in proportional composition (mole %). ^b Not observed. ^c Mean degree of polymerization. ^d Conversion yield into known proanthocyanidin subunits (by mass).

(56.4–80.1%), may be an indication of the heterogeneity of the interflavonoid bond, and not indicative of a method weakness. In an initial study using the phloroglucinol method to monitor the development of grape seed proanthocyanidins in Shiraz grapes, the conversion yield varied considerably, but was related to fruit maturity (24). For proanthocyanidins isolated from pre-véraison grapes the conversion yield averaged 89%, indicating that the phloroglucinol method can be very effective in the conversion of proanthocyanidins into their constitutive subunits. After véraison however, the conversion yield declined steadily to a low of 69% at commercial maturity. This observation, along with others (compositional changes in flavan-3-ol monomers, increasing organic radical species, and changing seed coat color) suggests that proanthocyanidins are oxidized after véraison, and also

suggests that the conversion yield could be a meaningful parameter for proanthocyanidin characterization.

Given the performance of the phloroglucinol procedure, and the difficulties associated with benzyl mercaptan use, the present results provide a clear justification for using phloroglucinol in the acid-catalyzed cleavage studies of proanthocyanidins.

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Supporting Information Available: ^1H , ^{13}C , and 2D NMR data of epicatechin-3-*O*-gallate-(4 β -2)-phloroglucinol. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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