Quantitative Analysis of Polymeric Procyanidins (Tannins) from Grape (*Vitis vinifera*) Seeds by Reverse Phase High-Performance Liquid Chromatography

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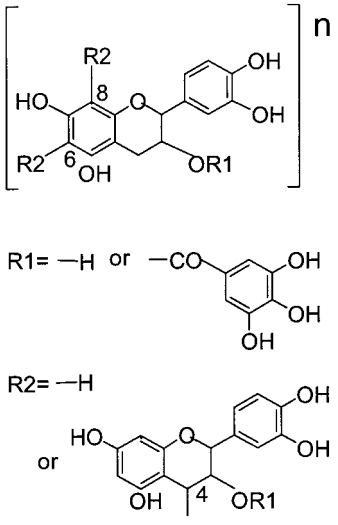
A reverse phase C_{18} HPLC method with potential for high automated throughput has been developed for the quantitative analysis of polymeric procyanidins (tannins) in grape seed extracts. Chromatography gave rise to 13 distinct UV-absorbing peaks with good baseline separation. The UV-absorbing peak eluting last is distinct and therefore easily quantified. Biochemical analyses including ultrafiltration, protein precipitation, and Sephadex LH20 chromatography combined with electrospray mass spectrometric analyses establish that this peak predominantly contains polymeric procyanidins. The polymers, which appear to be galloylated to various degrees and seem to fragment in a characteristic manner during electrospray mass spectrometry, are well separated from catechins and procyanidin oligomers of up to 4 units. The recovery of polymeric grape seed tannins with this HPLC method was 86%, which is similar to the 89% recovery achieved with commercial quebracho tannins. The concentration of tannins in seeds from ripe *Vitis vinifera* cv. Shiraz grapes ranged from 1360 to 2830 mg/kg of berries.

Keywords: *Proanthocyanidin; liquid chromatography electrospray mass spectrometry; Sephadex LH20 chromatography; ultrafiltration; protein precipitation; PVPP; Shiraz; Pinot Noir*

INTRODUCTION

Procyanidins constitute the major class of phenolic compounds in grape seeds and chiefly comprise flavan-3-ol polymers with an average degree of polymerization (DP) ranging from 2 to >15 and an average molecular mass ranging from 578 to >5000 Da (see structure;



1−*3*). Grape seed polymeric procyanidins (tannins) are extracted during the latter stages of wine-making and are believed to contribute to color stability and organoleptic properties of wine (*4*−*6*). It is not clear, however, what the relative impact of monomers, oligomers (here defined as DP ≤ 4), or polymers (here defined as DP ≥ 5) is in this regard and, indeed, how specific viticultural and oenological circumstances contribute to the modulation of the relative abundance of these various species. Knowledge in this area would be greatly assisted by a facile method for the rapid measurement of monomers, oligomers, and polymers of the seed and this could in turn lead to better informed decision-making about viticultural and wine-making practices.

Reverse phase high-performance liquid chromatography is an effective and accurate technique for the analysis of catechins and oligomeric procyanidins (e.g., refs *5*−*9*). However, reports on the use of this technique in the analysis of polymeric procyanidins are few and have not been complemented by the more recent technique of electrospray ionization technology so elegantly pursued by Cheynier and colleagues for the characterization of a variety of proanthocyanidins (e.g., *10*−*13*). In previous studies of grape seed composition using HPLC, a very broad UV-absorbing peak eluting late in the chromatogram was thought to represent a mixture of polymeric polyphenolic compounds (*4*, *14*−*17*). The broad nature of the suspected polymeric peak in those studies makes it hard to quantify. In this study we describe a method whereby a distinct and easy to quantify peak comprising mostly, if not exclusively, polymers is clearly resolved from previously interfering

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monomers and oligomers. The technique has been employed to determine levels of procyanidin polymers in seeds from grapes grown in commercial Australian vineyards.

MATERIALS AND METHODS

Chemical Standards. The chemicals used were of the highest purity commercially available. Gallic acid and catechin standards were obtained from Fluka Chemie AG (Buchs, Switzerland) and epicatechin, epicatechin gallate, and polyvinylpolypyrrolidone (PVPP) from Sigma-Aldrich (Sydney, Australia). The commercial quebracho tannin (Quebracho UNITAN ATO) was a gift from Redox Chemicals Pty. Ltd., South Australia.

Preparative Sephadex LH20 Chromatography. Sephadex LH20 gel (Pharmacia Biotech AB, Uppsala, Sweden), preswollen in 60% (v/v) methanol and 0.2% (v/v) formic acid, was slurry-packed into a glass column (3.5 × 16 cm); prior to sample loading, the column was equilibrated with Milli-Q water. The ethanol extracts of grape seeds were loaded onto the column, and the column was washed sequentially with 2.3 L of 60% (v/v) methanol plus 0.2% (v/v) formic acid and 2.3 L of Milli-Q water at a flow rate of 0.3−0.5 mL/min. Polymeric procyanidins were eluted with 2.3 L of 60% (v/v) acetone plus 0.2% (v/v) formic acid. Acetone was evaporated with a rotary vacuum evaporator at 35 °C. The residual sample was lyophilized and further dried in a desiccator at room temperature for >24 h. The purity of seed tannin was examined by HPLC analysis (see below).

Source of Grape Samples. For HPLC method development, healthy *Vitis vinifera* cv. Shiraz, Pinot Noir, and Muscat of Alexandria grapes from the 1997 vintage with total soluble solids of between 22.7 and 24.7 °Brix were harvested from commercial vineyards in Australia and stored at −20 °C prior to extraction.

Extraction. Seeds were manually separated from ~100 berries and homogenized in a high-speed homogenizer (Ultraturrax T25, Janke & Kunkel GmbH & Co.) with 20 mL of 70% (v/v) aqueous ethanol and extracted with occasional manual shaking at room temperature for 1 h. The slurry was centrifuged (12000*g*, 5 °C, 40 min), and the precipitate was re-extracted twice with the same solvent for 30 min (2 × 20 mL). Supernatants were pooled, and the volume was adjusted such that 100 mL corresponded to the extract from 6 g of seeds.

HPLC Analysis. The HPLC apparatus used were standard Waters instruments with diode array detectors. Samples (20 μL), diluted with Milli-Q water (80 μL), were loaded onto a 4.6 × 250 mm C_{18} column (Exsil 100 5 μ ODS, Activon, Sydney, Australia) with a C_{18} guard cartridge with the same packing material equilibrated in solvent A [0.2% (v/v) phosphoric acid]. Phenolic compounds were eluted by a gradient of solvent B [82% (v/v) acetonitrile, 0.04% (v/v) phosphoric acid] from 0 to 15% solvent B in the first 15 min, from 15 to 16% from 15 to 40 min, from 16 to 17% from 40 to 45 min, from 17 to 43% from 45 to 48 min, from 43 to 52% from 48 to 49 min, held isocratic at 52% from 49 to 56 min, reduced from 52 to 43% from 56 to 57 min, reduced from 43 to 17% from 57 to 58 min, and reduced from 17 to 0% from 58 to 60 min. Peaks were detected at 280 nm and identified by comparison to retention times of standards and as described under Results and Discussion. The concentration of the polymer peak was quantified by comparison to the peak area of a purified grape seed polymeric procyanidin fraction. The fraction used as a standard was the acetone eluate obtained by preparative LH20 chromatography (see above). One hundred milligrams of the polymeric procyanidin standard was equivalent to 108 ± 2 mg of catechin or 97 ± 8 mg of gallic acid—when assessed by the Folin−Ciocateau method (*18*).

Recovery during HPLC Analysis A seed polymeric procyanidin standard prepared by preparative LH20 chromatography (see above, 100 mg/L) was injected onto the HPLC column, and samples were run either "through the column" (as described above) or "through a bypass" (the column was replaced by a short piece of stainless steel tubing). The eluting solvent for seed polymeric procyanidins run through a bypass was 42:58 acetonitrile/solvent A—the equivalent solvent under which the polymeric fraction elutes when run through the column. The ratio of the peak area obtained through the column to that obtained through a bypass was used as an indication of the recovery of the polymeric procyanidin. A preparation of commercial quebracho tannin solution (500 mg/L) was also tested.

Ultrafiltration. A regenerated cellulose ultrafiltration membrane (Microcon 3, Amicon Inc., Beverly, MA) of 3000 nominal molecular weight cutoff (NMWCO) was used. Acidified aqueous samples [containing 0.2% (v/v) formic acid] of grape seed extracts were centrifuged (12000*g*, 5 min) to remove solids. The supernatant of 0.5 mL was placed in each cell and centrifuged (10000*g*, 45 min) until the retentate volume was reduced to ~0.1 mL. The first filtrate was directly used for HPLC analysis. The volume of the retentate was adjusted to 0.5 mL by the addition of 0.4 mL of 0.2% (v/v) formic acid. After gentle mixing, samples were further centrifuged as described above. The ultrafiltration was conducted three times, and after adjustment to its original volume, the final retentate was analyzed by HPLC.

PVPP Assays. A 0.9 × 7 cm glass column was packed with 1.2 g of cleaned and dry PVPP powder (Sigma-Aldrich, Sydney, Australia). Approximately 12 mL of the prepared seed extracts of Shiraz grapes was loaded and filtered under vacuum. Two milliliters of the filtrate was collected after the first 200 μL had been discarded. Samples of the PVPP-treated seed extracts and the untreated controls were analyzed by HPLC.

BSA Assays. Approximately 40 mL of 70% ethanol extracts of Shiraz grape seeds was dried in a rotary evaporator at 45 °C, redissolved in 4 mL of 12.5% (v/v) ethanol, and centrifuged (10000*g*, 5 °C, 40 min). The concentration of polyphenolic compounds in the supernatant was adjusted to ~8000 mg/L gallic acid equivalents and added to an equal volume of 8000 mg/L BSA (Sigma Pty. Ltd.) in 12.5% (v/v) ethanol, and the mixture was shaken thoroughly. An equal volume of the seed preparation and 12.5% (v/v) ethanol was mixed and used as controls. Both treatments and controls were centrifuged (12000*g*, 10 min, room temperature) after an incubation at −7 °C for 4 h. The supernatants (3 mL) were collected, and 10% (v/v) trichloroacetic acid was added (0.3 mL) prior to HPLC analysis in triplicate.

Liquid Chromatography Electrospray Mass Spectrometry (LC-ESI/MS) Analysis. An extract of Shiraz grape seed phenolic compounds was concentrated at 45 °C using a rotary vacuum evaporator before analysis on a mass spectrometer (API-300, PE Sciex, Thornhill, ON, Canada). The extract was loaded through a 5 μL sample loop onto an HPLC C_{18} column (Nova-Pak, 60 Å, 4 μm, 2 × 150 mm, Waters). The separation was carried out using a flow rate of 100 μL/min and a gradient the same as that described above except that 0.2% (v/v) formic acid replaced 0.2% (v/v) phosphoric acid to improve the signal-to-noise ratio. The eluant was split (1:4) postcolumn by a tee, at a flow rate of 20 μL/min to the mass spectrometer and 80 μL/min to a UV detector (HP1100, Hewlett-Packard) monitoring at 280 nm.

The mass spectrometer was operated under positive ion mode and scanned from *m*/*z* 250 to 3000 with a mass step size of 0.2 Da and a dwell time of 0.5 ms. The curtain (nitrogen) and nebulizer (air) gases were set at 8 and 10 units, respectively. The ion spray voltage was 5500 V, and the orifice potential was 40 V. Mass spectral data were processed using Bio-Multiview software 1.2 v 3 (PE Sciex).

Spectrophotometric Analysis. Grape seed extracts (1 mL) were heated for 40 min at 95 °C with 6 mL of acid reagent [70% (v/v) concentrated HCl in butanol] in the presence of 0.2 mL of ferric reagent [2% (w/v) $NH_4Fe(SO_4)_2 \cdot 12H_2O$ (*23*)] followed by spectrophotometry at 520 nm. The quantity of procyanidin in grape seed extracts was determined from a standard curve constructed with purified grape seed polymeric procyanidin standard (see HPLC Analysis) subjected to the acid degradation procedure.

RESULTS AND DISCUSSION

HPLC Mobile Phase Development. In early trials we evaluated published HPLC methods (*4*, *15*, *17*, *19*) for separation of the phenolic compounds in extracts of seeds from Shiraz or Muscat of Alexandria grapes. The separation of compounds was in general similar to that showed by Guyot et al. (*12*). Thus, catechin, epicatechin, and epicatechin gallate appeared as small peaks on the shoulder of a broad hump. This broad hump eluted over a 30 min period and was thought to represent polymeric procyanidins (*16*). Following extensive testing and influenced by reports in the literature (see, e.g., refs *15* and *18*), we devised a method with a novel sequence of gradients.

The performance of the system is illustrated in Figure 1a. A well-resolved chromatogram with near baseline separation between most peaks was observed when a grape seed extract was analyzed. The sharp definition of peak 13, the *procyanidin polymer peak* (see below), was critically dependent on the inclusion of the isocratic segment six (49−56 min) at relatively high acetonitrile concentration. Gallic acid, catechin, epicatechin, and epicatechin gallate, represented by peaks 1, 7, 10, and 12, respectively, were identified by comparison of their spectral characteristics and retention times with those of commercial standards and by LC-ESI/MS (data not shown). The identities of peaks 2−6, 8, and 9 were assigned by LC-ESI/MS (see below) and are given in the caption to Figure 1. Peak 13 had the same retention time as the major peak for commercial quebracho tannin and a grape seed polymeric procyanidin fraction prepared by Sephadex LH20 chromatography (see below). This suggested that peak 13 represents grape seed polymeric procyanidins.

Confirmation of the Polymeric and Polyphenolic Nature of the *Procyanidin Polymer Peak* (Peak 13). We subjected grape seed extracts to a number of standard manipulations. First, we compared the HPLC chromatograms of nonfined and PVPP-fined seed extracts. Essentially all of the peaks, including the entire peak 13, were absent from the chromatogram of the PVPP-treated sample (data not shown), consistent with all substances represented by peak 13 being phenolic in nature (20).

Second, grape seed extract was submitted to fractionation using a 3000 NMWCO ultrafiltration membrane (Figure 1). Comparison of the chromatograms for the unfractionated extract (Figure 1a) and retentate (Figure 1b) showed that only peak 13 was common to the two chromatograms. Conversely, the chromatogram obtained for the filtrate was virtually identical to that of the whole extract, except for a >80% reduction in the area associated with peak 13 (compare parts a and c of Figure 1). The most likely explanation is that a large proportion, if not all, of the material associated with peak 13 is polymeric in nature and cannot pass through the ultrafiltration membrane, whereas gallic acid, the catechins, and procyanidin oligomers (peaks 1−12) all passed through the filter without a noticeable loss. It is possible that a small amount of polymeric material was formed in the filtrate following its passage through the ultrafiltration membrane and subsequent storage prior to HPLC analyses or that lower polymers (4 < DP < 8) were only partly retained by the 3000 NMWCO ultrafiltration membrane.

Sephadex LH20 gel filtration chromatography is commonly used for the separation of both condensed and hydrolyzable tannins from simple phenolic compounds on a preparative scale (e.g., refs *15* and *21*). Catechins and oligomeric procyanidins are eluted from the Sephadex LH20 column by 60% (v/v) methanol, whereas proanthocyanidin polymers are eluted by 60% (v/v) acetone (*15*). An HPLC analysis of the methanol eluate of cv. Muscat of Alexandria grape seed extracts is shown in Figure 2a. Catechin, epicatechin, and procyanidin dimers were the dominant components in the methanol eluants with virtually no peak 13 material. Conversely, only one clear peak eluted in the position of peak 13 was obtained when the acetone eluate was analyzed (Figure 2b). Later, LC-ESI/MS analysis of the acetone eluant gave results (data not shown) similar to those obtained when material in peak 13 from whole grape seed extracts was analyzed.

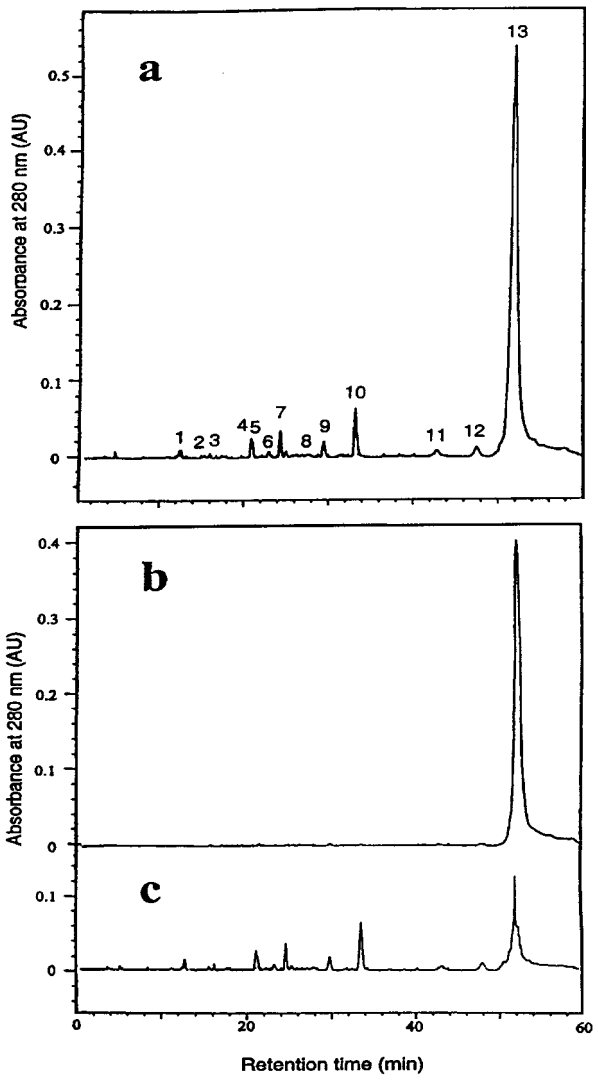

Figure 1. Effect of ultrafiltration on the phenolic composition of extracts of Shiraz grape seeds. The grape seed extract before ultrafiltration (a), the retentate after ultrafiltration through a 3000 Da NMWCO membrane (b), and the filtrate after ultrafiltration (c) were analyzed by RP-HPLC. The peaks were identified primarily by LC-ESI/MS as (1) gallic acid; (2) procyanidin trimer (P_3); (3) procyanidin tetramer (P_4); (4) procyanidin dimer (P_2); (5) procyanidin dimer (P_2); (6) procyanidin trimer (P_3); (7) catechin; (8) procyanidin dimer (P_2); (9) procyanidin dimer (P_2); (10) epicatechin; (11) monogalloylated procyanidin dimer (P_2G_1); (12) epicatechin gallate; and (13) polymeric procyanidins. The elution of procyanidin tetramers (P_4) as minor peaks at 21.5, 25.4, and 29.5 min was also observed.

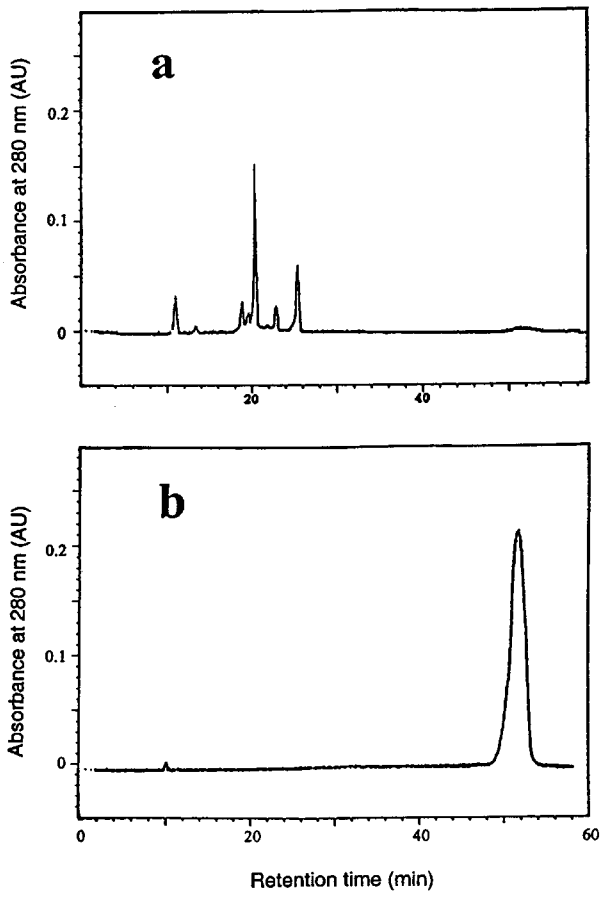

Figure 2. Separation of phenolic compounds in Muscat of Alexandria grape seed extracts by Sephadex LH20 low-pressure LC. Extracts were loaded on a Sephadex LH20 column as described in the text and eluted with (a) 60% (v/v) methanol and (b) 60% (v/v) acetone before HPLC analysis.

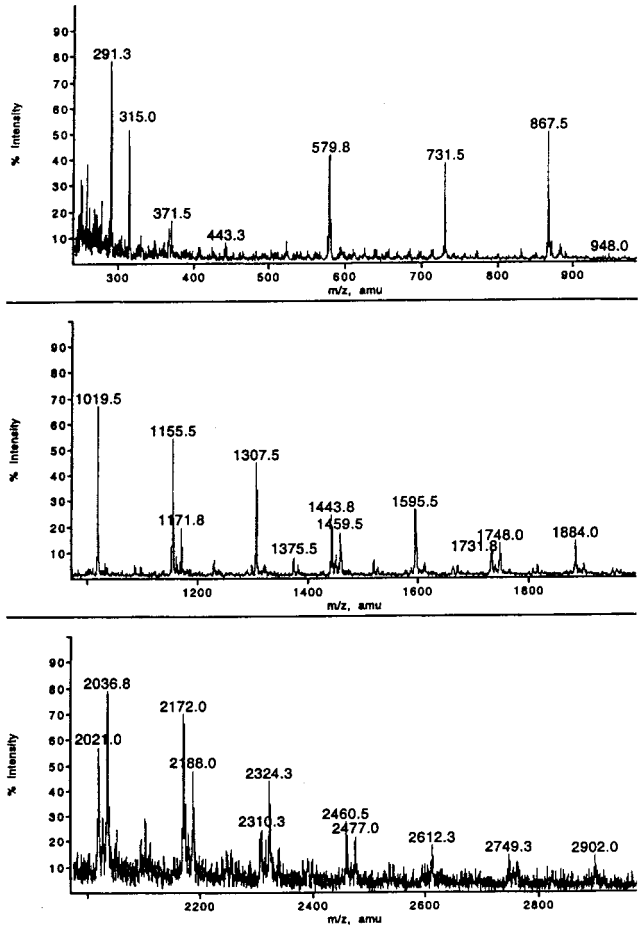

Figure 3. Spectral data for phenolic compounds in the *procyanidin polymer peak* of seed extracts determined by ESI/MS. The mass spectrum of the *procyanidin polymer peak* (peak 13, Figure 2a) is presented in three *m*/*z* ranges: 250−1000, 1000−2000, and 2000−3000. See also Table 1.

When the grape seed extracts were combined with BSA in 12.5% (v/v) ethanol and held at −7 °C, the solution became turbid. The haze formed could be removed by centrifugation. HPLC analyses showed that the supernatant contained less of substances eluted as peak 13 (up to 80% reduction) compared to the samples without treatment with BSA (data not shown). No significant change in the areas of the other peaks was observed, and the protein solution alone did not give a significant peak under the same conditions. This result indicated that the substances represented by peak 13 had stronger affinity for BSA than did simple procyanidin compounds. Previous researchers have suggested that the affinity of grape seed procyanidins for protein is determined by the number of *o*-dihydroxyphenyl groups and increases with the DP and galloylation (*22*).

Recovery. The recovery for polymeric procyanidins in peak 13 was 86.4% ($n = 7$) for grape seed tannins. The recovery was 89% ($n = 4$) when commercial quebracho tannins were analyzed.

Qualitative Analyses of Seed Procyanidins Separated by HPLC. In-depth description of individual molecular species contained within the various peaks was attempted using LC-ESI/MS. Cheynier and colleagues have used LC-ESI/MS extensively to investigate procyanidins and have found that detection was most effective when the mass spectrometer was run in the negative ion mode despite the acidic nature of the HPLC mobile phase (*10*, *11*). With our solvent systems, however, we found that the total ion counts were higher in the positive ion mode and that the predominant occurrence of the procyanidins as singly charged species, rather than multiply charged species, as occurs in negative ion mode, simplified interpretation of the data.

Qualitative Analysis, Peaks 1—12. Assignments of the major peaks, which appeared in the first stage of the elution (Figure 1a), were confirmed by LC-ESI/MS analysis (data not shown). The designed mobile phase from 0 to 45 min eluted nearly all of the simple procyanidins (monomers and oligomers) so far reported in grape seeds by HPLC. No significant signals of procyanidin pentamers (*m*/*z* 1443) and higher DP procyanidins were determined in the eluant from 10 to 48 min during HPLC analysis. Importantly, the later peaks were not "surfing" on a large unresolved hump of mostly polymeric material.

Qualitative Analyses of the *Procyanidin Polymer Peak (Peak 13).* To characterize the material associated with peak 13 further, it was recovered from HPLC, concentrated, and subjected to LC-ES/MS analysis (Figure 3). The major signals distributed in the *m*/*z* range of 250−3000 are given (Table 1) and tentative assignments made. The major ions appear to be singly charged, although we did see some evidence of multiply charged species such as the signal at *m*/*z* 1375 (Figure 3) for the doubly charged species of a monogalloylated procyanidin of DP 9 (P_9G_1).

Table 1. Major *m/z* Signals Extracted from Peak 13 by LC-ESI/MS Analysis and Their Identities

m/z	identity [a]
291	P_1 [b]
443	P_1G_1
579	P_2
731	P_2G_1
867	P_3
1019	P_3G_1
1155	P_4
1171	P_3G_2
1307	P_4G_1
1443	P_5
1459	P_4G_2
1595	P_5G_1
1731	P_6
1748	P_5G_2
1884	P_6G_1
2021	P_7
2036	P_6G_2
2172	P_7G_1
2188	P_6G_3
2310	P_8
2324	P_7G_2
2460	P_8G_1
2477	P_7G_3
2612	P_8G_2
2749	P_9G_1
2902	P_9G_2

[a] Tentative assignment based on molecular weights. Note that all species tabulated were singly charged. [b] Abbreviations: P, procyanidin; P_2, procyanidin dimer, etc.; G, gallate; G_1, monogallate, etc.

Given the biochemical characterization of peak 13 above and the HPLC behavior of pure catechin/epicatechin, the relatively low *m/z* values of 291, 443, 579, 731, and 867 in the polymer peak required some further attention and interpretation. Their isotopic mass pattern indicates that these low molecular weight species are singly charged. Expansion of the base peak area of the spectra showed catechin, the procyanidin dimer, and procyanidin trimer all gave a parent peak (P) of $(M + H)^+$ and one (P + 1) or more isotopic peaks (P + *n*) but no fragments with $m/z \leq$ (P-2) (data not shown). For instance, catechin with a molecular weight of 290 gave the parent peak at *m/z* 291 and an isotopic peak at *m/z* 292 but no species of $m/z \leq 289$. However, when the *m/z* 291 region associated with a procyanidin dimer was expanded and compared to that of the *m/z* 291 region associated with catechin, additional species at *m/z* 289 and 287 were clearly observable (not shown). A similar pattern was observed when the expanded spectra of procyanidin dimer-like fragments derived from a procyanidin trimer were compared to the spectra of a dimer or similarly when trimer-like fragments from procyanidin tetramers were compared to a trimer. In regard to the species associated with peak 13, a similar situation exists and the data indicate that the presence of the lower molecular mass species in the mass spectrum extracted from peak 13 are consistent with their derivation from fragmentation of higher molecular mass species. We are not aware of this phenomenon being reported previously and can only speculate about the potential mode of formation.

To lend further support for the contention that the lower *m/z* species seen in the spectra extracted from the polymer peak (peak 13) were due to fragmentation of larger polymers, the filtrate from ultrafiltration through the 3000 NMWCO filter was subjected to LC-ESI/MS analysis. Major peaks at *m/z* 291 (P_1), 579 (P_2), 731 (P_2G_1), 867 (P_3), 1019 (P_3G_1), 1155 (P_4), and 1443 (P_5) were extracted from the first phase of the LC analysis (0−45 min). No significant signals with these *m/z* values were observed from peak 13 in the filtrate [except that at *m/z* 1443 (P_5)], presumably because the molecules previously giving rise to these signals from peak 13 were retained by the ultrafiltration membrane and hence of considerably higher molecular mass than their mass spectra indicated. This result supports the assertion above that *m/z* signals <1400 seen with peak 13 represented ion fragments from procyanidins with DP ≥ 4 rather than molecular ions.

All of the available evidence suggests that peak 13 contains little or no oligomeric procyanidins with DP ≤ 4 and that the devised HPLC method constitutes a reliable assay for the polymeric seed procyanidins.

HPLC Assay of Grape Seeds Sampled from Commercial Vineyards. The concentrations of polymeric procyanidins in grape seeds ranged from 33200 to 50700 mg/kg of seeds or from 1680 to 3190 mg/kg of grapes. The results obtained by HPLC analysis were linearly correlated ($r^2 = 0.96$) to the data obtained using the HCl−butanol assay (*23*), although the latter consistently gave higher values, presumably because it includes the measurement of procyanidin oligomers as well as polymers.

It is clear there is a large degree of variability in the content of seed procyanidin polymers in grapes of the same variety at practically identical sugar maturity. Given that at least half of the tannins in a red wine may be derived from seeds (*24*), prior knowledge of the potential polymer content of a particular grape lot clearly will enable wine-makers to exert greater choice in an informed manner when allocating particular grape lots for specific purposes during vintage.

Varietal Difference. Seeds from Shiraz grapes from eight different sites and Pinot Noir grapes from three different sites were analyzed using the HPLC method. The ratios of polymers to monomers (catechin plus epicatechin) were 8.9 ± 1.4 ($n = 9$) for Shiraz and 5.2 ± 1.9 ($n = 13$) for Pinot Noir, which are consistent with the findings of Thorngate and Singleton (*18*), who detected a ratio of polymeric flavan-3-ols to monomeric flavan-3-ols of <5 for Pinot Noir grapes grown in California. The contribution of catechins to the sensation of astringency and bitterness is different from that of polymeric procyanidins (*25*): the former was shown to be more bitter and less astringent than the latter [see also a recent review by Gawel (*26*)]. The difference in tannin-related taste between Shiraz and Pinot Noir wines might therefore be related, at least in part, to the difference in phenolic composition of their seeds. However, further evidence is needed to confirm this.

ABBREVIATIONS USED

DP, degree of polymerization; RP-HPLC, reverse phase high-performance liquid chromatography; G_1, monogallate; G_2 digallate, etc.; LC-ESI/MS, liquid chromatography electrospray mass spectrometry; NMWCO, nominal molecular weight cutoff; P, parent peak; P_1, procyanidin monomer (catechin or epicatechin); P_2, procyanidin dimer, etc.; PVPP, polyvinylpolypyrrolidone.

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