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Analysis of proanthocyanidins by high-performance gel permeation chromatography

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Abstract

A high-performance gel permeation chromatography method was developed for the analysis of proanthocyanidins. The isocratic method consisted of two porous polystyrene–divinylbenzene columns (300×7.5 mm each, 5 μm, 100 and 500 Å individual pore size) and a mobile phase consisting of *N*,*N*-dimethylformamide containing 1% (v/v) acetic acid, 5% (v/v) water and 0.15 *M* lithium chloride. The flow-rate was maintained at 1 ml/min, with a column temperature of 60 °C and with detection at 280 nm. The method was used to analyze proanthocyanidin fractions of increasing molecular mass and from different plant tissues. The average molecular mass of proanthocyanidin fractions as determined by acid catalysis in the presence of phloroglucinol, related well with their gel permeation chromatography column retention, yet significant differences in the retention properties between individual plant tissue isolates existed. Proanthocyanidin compositional differences between isolates may explain these differences. A second-order calibration curve was generated from fractionated grape seed proanthocyanidins and this curve was used to analyze grape seed proanthocyanidins isolated from grapes harvested at extremes of maturity.
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1. Introduction

Proanthocyanidins are polymeric plant natural products composed of flavonoid flavan-3-ol subunits. A large degree of proanthocyanidin structural variation exists, with variation due to different subunits, interflavonoid bond position and branching, and modifications with non-flavonoid substituents [1]. In addition to this, proanthocyanidins can also vary considerably in molecular mass distribution.

Proanthocyanidins, because of their astringent properties, have importance in many foods and beverages, and contribute significantly to our dietary phenolic intake. Recent evidence suggests that they may have health benefits [2]. Astringency perception and the health benefits are considered to be dependent upon molecular mass [2,3].

Several chromatographic approaches have been developed for obtaining molecular mass information for proanthocyanidins. Cleavage techniques rely on the conversion of proanthocyanidins into their con-

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stitutive subunits via acid catalysis in the presence of an excess nucleophile [4,5]. Subsequent analysis by reversed-phase high-performance liquid chromatography (HPLC) of the subunit products can provide mean degree of polymerization (mDP). Also, with the additional knowledge of subunit composition, the mass-average molecular mass can be obtained. These methods however are unable to provide mass distribution information. Chromatographic methods that are used to analyze intact proanthocyanidins exist. Normal-phase and reversed-phase HPLC methods can be used to separate proanthocyanidins [6–8]. Again however, these methods are restricted in their ability to provide complete molecular mass distribution information.

Gel permeation chromatography (GPC) has been used as a chromatographic technique since the 1960s for polymer analysis, and is in common use as a fast and reliable means for gathering molecular mass distribution information. GPC methods have been developed for proanthocyanidin analysis. Early methods have relied on pre-derivatization of proanthocyanidins prior to analysis to eliminate interaction between phenolic functional groups and GPC packing material [9]. More recently, direct analysis of Douglas fir proanthocyanidins has been shown to be possible as well [10]. The analysis of a wider range of proanthocyanidins (i.e., composition and molecular mass variability) could help to determine the full utility of GPC.

The purpose of this investigation was three-fold: (1) develop a GPC method for the direct analysis of proanthocyanidins, (2) investigate the relationship between the average molecular mass of proanthocyanidins determined by acid-catalyzed cleavage in the presence of excess phloroglucinol and the average molecular mass as determined by the GPC method, and (3) determine the relationship between the retention properties of fractionated proanthocyanidins obtained from different plant material.

2. Experimental

2.1. Chemicals and materials

Acetone, acetonitrile, dichloromethane, *N*,*N*-dimethylformamide and methanol were HPLC grade and purchased from Fisher Scientific (Santa Clara, CA, USA). Also purchased from Fisher Scientific was glacial acetic acid, lithium chloride and sodium acetate. Formic acid was purchased from Fluka (Milwaukee, WI, USA). Phloroglucinol and 3′-indoleacrylic acid (IAA) were purchased from Sigma (St Louis, MO, USA). Rutin, (+)-catechin, (−)-epicatechin, gallic acid and trifluoroacetic acid were obtained from Aldrich (St Louis, MO, USA). Toyopearl HW-40F column packing material was obtained from Supelco (St Louis, MO, USA). Sephadex LH-20 was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Malvidin-3-glucoside was obtained from Polyphenols Labs (Sandness, Norway). The distilled or reverse-osmosis water used in all solutions was purified to HPLC grade using a Millipore Milli-Q water system (Bedford, MA, USA).

2.2. Instrumentation

An Agilent 1100 HPLC instrument (Palo Alto, CA, USA) which consisted of a vacuum degasser, pump, autosampler, column oven and diode array detector was used for all analyses. GPC data was analyzed using Agilent GPC software coupled with the standard Chemstation software.

2.2.1. Gel permeation chromatography

The high-performance GPC method used to analyze proanthocyanidins consisted of 2 PLgel (300×7.5 mm, 5 μm, 500 (effective molecular mass range of up to 4000 using polystyrene standards) by 100 Å (effective molecular mass range of 500–30 000 using polystyrene standards)) columns connected in series and protected by a guard column containing the same material (50×7.5 mm, 5 μm), all purchased from Polymer Labs (Amherst, MA, USA). The sample injection amount was typically 40 μg. The isocratic method utilized a mobile phase consisting of *N*,*N*-dimethylformamide containing 1% (v/v) glacial acetic acid, 5% (v/v) water and 0.15 *M* lithium chloride. The flow-rate was maintained at 1 ml/min with a column temperature of 60 °C and elution monitored at 280 nm. Calibration curves were constructed using fractionated proanthocyanidins, by correlating their average molecular mass (determined

by acid catalysis) with their cumulative mass distribution at 50%.

2.2.2. Reversed-phase HPLC of proanthocyanidin cleavage products

The reversed-phase HPLC method used to analyze the proanthocyanidins following acid catalysis in the presence of excess phloroglucinol consisted of two Chromolith RP-18e (100×4.6 mm) columns connected in series and protected by a guard column (Purospher STAR RP-18e, 4×4 mm, 5 μm), all purchased from EM Science (Gibbstown, NJ, USA). The method utilized a binary gradient with water containing 1% (v/v) aqueous acetic acid (mobile phase A) and acetonitrile containing 1% (v/v) acetic acid (mobile phase B). Eluting peaks were monitored at 280 nm, and the elution conditions were as follows: column temperature 30 °C; 3.0 ml/min; 3% B for 4 min, a linear gradient from 3% to 18% B in 10 min, and 80% B for 2 min. The column was washed with 3% B for 2 min before the next injection. This HPLC method was used over a previously published method [4] because of its reduced run time (from 70 min to 18 min), in addition to an improvement in sample resolution.

2.3. Experimental procedure

2.3.1. Hop proanthocyanidin isolation and fractionation

Hop cones (the female inflorescences of *Humulus lupulus* L. var. Willamette) were harvested in September 2000 at the United States Department of Agriculture—Oregon State University experimental hop yard, Corvallis, OR, USA. Hop cones were kiln-dried, baled, and stored at −15 °C until needed. Cones (50 g, 13% moisture) were extracted with 3×1000 ml dichloromethane (1 h with occasional stirring) to remove pigments, lipids (and other lipid-soluble material), and then air-dried in a fume hood overnight. The dry hops were ground in a Wiley mill to pass 20 mesh (840 μm), then extracted 3× with 800 ml of water–acetone (3:7; 2 h with continuous stirring). Extracts were pooled and rotary evaporated under vacuum at 35 °C to remove acetone. The resulting extract (~1000 ml) was washed with 800 ml hexane, followed by 400 ml dichloromethane, to remove additional lipid-soluble material, then rotary evaporated to remove organic solvents.

The washed extract was applied to a Sephadex LH-20 column (40×300 mm) equilibrated with water–methanol (MeOH) (3:1). The column was washed with water–MeOH (3:1) (400 ml), water–MeOH (1:1) (1040 ml), and water–MeOH (1:4) (400 ml) to elute glycosides and other materials and then catechin–epicatechin monomers and dimers. (Fractions were monitored using two-dimensional thin layer chromatography (TLC) on cellulose plates developed first with *tert*.-butanol–water–acetic acid (3:1:1), dried, then developed in the second dimension with water–acetic acid (47:3), then visualized with vanillin–HCl reagent.) Finally, water–acetone (3:7) (500 ml) was used to elute the proanthocyanidin polymers. Fractions containing all proanthocyanidins collected after the bulk of monomers and dimers had been eluted were pooled and rotary evaporated to remove organic solvents, then freeze dried to yield 1.940 g of crude proanthocyanidin.

To fractionate, crude proanthocyanidins (1.75 g) were first dissolved in MeOH–water–formic acid (498.75:498.75:2.5) (90 ml), then applied to a Sephadex LH-20 column (40×450 mm) equilibrated with MeOH–water–formic acid (498.75:498.75:2.5). The column was then washed sequentially using the solvent systems and volumes described in Table 1. Fractions (~15 ml) were collected and monitored by two-dimensional TLC and by ESI-MS, then pooled

Table 1
Solvent systems and elution volumes used to fractionate proanthocyanidins

Solvent system[a]	Volume of eluent (ml)		
	Hop	Seed	Skin
60% (v/v) methanol	800	400	400
75% (v/v) methanol	800	400	400
90% (v/v) methanol	800	500	500
10% (v/v) acetone, 80% (v/v) methanol	600	800	800
20% (v/v) acetone, 65% (v/v) methanol	600	500	500
30% (v/v) acetone, 40% (v/v) methanol	600	500	500
60% (v/v) acetone	800	400	800

[a] Solvents applied sequentially from top to bottom with the balance within each solvent system consisting of water. In addition, solvent–water mixtures contained 0.2% (v/v) formic acid, and all solvents were sparged with helium prior to chromatography.

into eight parts (see Results and discussion). The pooled fractions were rotary evaporated, freeze-dried, dissolved in MeOH, and stored at −15 °C.

2.3.2. Grape proanthocyanidin isolation and fractionation

Vitis vinifera L. cv. Pinot noir grape berries grown during the 2001 harvest season in the Willamette Valley, OR, USA were used as the source material for grape seed and skin proanthocyanidins. A random sample (~10 kg) was collected approximately 4 weeks after fruit set (grape-early) and also at commercial harvest (grape-late). Grape berries were kept at 4 °C until processed (within 4 days). Grape processing and proanthocyanidin isolation and purification has been previously described [4]. The grape-early isolate served as the source material for the fractions described below.

For fractionation, grape skin or seed proanthocyanidin samples (2 g each) were dissolved in 50 ml of MeOH–water–formic acid (498.75:498.75:2.5) and quantitatively applied to a Sephadex LH-20 column (40×450 mm) equilibrated with MeOH–water–formic acid (498.75:498.75:2.5). The column was then washed sequentially using the solvent systems and volumes described in Table 1. Eluting proanthocyanidin fractions (~15 ml) were collected into test tubes. Fractions were pooled based upon a combination of ESI-MS (useful for lower-molecular-mass fractions) and visual inspection of eluting material. The pooled fractions were rotary evaporated, lyophilized to dryness, and stored at −20 °C.

3. Results and discussion

The focus of this investigation was the analysis of proanthocyanidin fractions varying in composition as well as molecular mass distribution to provide more information on the utility of high-performance GPC for the analysis of unmodified proanthocyanidins.

3.1. Characterization of proanthocyanidin fractions

Proanthocyanidins were isolated from different plant tissues and then fractionated using Sephadex LH-20 chromatographic media. Fractions were characterized by acid catalysis in the presence of excess phloroglucinol to produce information on subunit composition, conversion yield and mean degree of polymerization (mDP, Table 2). The conversion yields for the conversion of proanthocyanidins into known proanthocyanidin subunits ranged from 60 to 77% (m/m) for most proanthocyanidin fractions, with two falling below this range and one above. These results are consistent with previous results using this analysis [4].

The proanthocyanidins differed significantly from each other in composition. Hop proanthocyanidins contained prodelphinidins as well as procyanidin subunits, but did not contain galloylated subunits. Hop proanthocyanidins also contained the highest proportion of catechin extension subunits. Grape seed proanthocyanidins contained a significant amount of galloylation but did not contain prodelphinidin subunits. Grape skin proanthocyanidins contained a combination of galloylation (albeit low) and had similar amounts of prodelphinidins as hop proanthocyanidins. For grape proanthocyanidins, compositional information is in general agreement with previously published data [11,12]. For hop proanthocyanidins, limited compositional information exists [13].

There were clear trends within each of the proanthocyanidin isolates. The fractionation technique used clearly was effective in the division of the isolates into multiple fractions having different mDP. For all isolates, the mDP increased with retention on the Sephadex LH-20 packed column. Within each fraction as well, the proportion of galloylation and prodelphinidin content increased with mDP, consistent with previous work using this separation technique [14,15].

The results from the separation of these proanthocyanidin isolates indicated that 18 fractions covering a large molecular mass range had been isolated. Additionally, compositional analysis of these proanthocyanidin fractions indicated that they varied significantly from each other. These fractions were used to investigate the relationship between molecular mass determination by acid catalysis and retention properties by high-performance GPC.

3.2. High-performance gel permeation chromatography

GPC has been successfully used for the separation

Table 2
Results from acid catalysis in the presence of excess phloroglucinol for proanthocyanidin fractions

Sample	EGC-P[a]	C-P	EC-P	C	ECG-P	EC	ECG	Yield[b]	mDP[c]	Est. M_r[d]	Log M_r
Hop 1	0.143[e]	0.215	0.398	0.212	–	0.031	–	0.49	4.1	1193	3.08
Hop 2	0.172	0.187	0.472	0.148	–	0.021	–	0.70	5.9	1726	3.24
Hop 3	0.199	0.162	0.517	0.107	–	0.015	–	0.63	8.2	2391	3.38
Hop 4	0.228	0.146	0.535	0.081	–	0.011	–	0.66	10.9	3184	3.50
Hop 5	0.262	0.133	0.537	0.060	–	0.008	–	0.67	14.6	4270	3.63
Hop 6	0.336	0.109	0.513	0.037	–	0.005	–	0.70	23.9	7023	3.85
Seed 1	–	0.146	0.344	0.385	0.009	0.108	0.007	0.71	2.0	582	2.76
Seed 2	–	0.105	0.726	0.043	0.114	0.012	0.038	0.73	11.1	3565	3.55
Seed 3	–	0.105	0.727	0.032	0.126	0.009	0.028	0.73	14.8	4714	3.67
Seed 4	–	0.105	0.733	0.024	0.131	0.008	0.023	0.68	18.7	5952	3.77
Seed 5	–	0.103	0.742	0.017	0.130	0.009	0.016	0.60	24.1	7570	3.88
Skin 1	0.119	0.025	0.583	0.249	0.011	0.013	–	0.57	3.8	1111	3.05
Skin 2	0.170	0.040	0.574	0.187	0.014	0.015	–	0.66	5.0	1450	3.16
Skin 3	0.196	0.038	0.619	0.119	0.019	0.010	–	0.72	7.8	2291	3.36
Skin 4	0.219	0.036	0.634	0.083	0.022	0.007	–	0.74	11.1	3276	3.52
Skin 5	0.241	0.031	0.644	0.055	0.022	0.007	–	0.74	16.0	4710	3.67
Skin 6	0.270	0.028	0.633	0.043	0.020	0.006	–	0.77	20.3	5990	3.78
Skin 7	0.344	0.024	0.591	0.022	0.015	0.003	–	0.86	39.0	11524	4.06
Seed-early[f]	–	0.085	0.700	0.052	0.121	0.014	0.026	0.94	10.7	3310	3.52
Seed-late[f]	–	0.124	0.637	0.098	0.089	0.052	0.064	0.81	4.9	1618	3.21

[a] Proportional composition of proanthocyanidins (in moles), and with the following subunit abbreviations: EGC-P, (−)-epigallocatechin extension subunit; C-P, (+)-catechin extension subunit; EC-P, (−)-epicatechin extension subunit; C, (+)-catechin terminal subunit; ECG-P, (−)-epicatechin-3-*O*-gallate extension subunit; EC, (−)-epicatechin terminal subunit; ECG, (−)-epicatechin-3-*O*-gallate terminal subunit.
[b] Conversion yield (m/m) in the conversion of proanthocyanidin fraction to known subunits.
[c] Mean degree of polymerization.
[d] Estimated average molecular mass based upon proportional composition and mDP.
[e] A minor component in hops (3–5 mol%) was consistent with the gallocatechin extension subunit and was included here.
[f] Proanthocyanidin isolates from grape seed collected at different stages of maturity.

of unmodified proanthocyanidins [10]. The method described here is essentially the same as the previously developed method [10] with small modifications as follows: Firstly, based upon expected column retention, it was observed that higher molecular mass proanthocyanidins were being excluded. It was thought that this might be due to proanthocyanidin self-aggregation. The addition of 0.15 *M* LiCl (with 5%, v/v, water) to the mobile phase resulted in the apparent elimination of self-aggregation and retention properties consistent with expectation. Secondly, a small addition of acetic acid was made to the mobile phase (1%, v/v) to reduce the potential for proanthocyanidin oxidation.

This method has several features that are desirable. Principle among these was that proanthocyanidins did not require prior derivatization, and the analysis provided full molecular mass distribution information with good reproducibility. In addition, the analysis times are reasonably short compared with other methods that are used for the analysis of intact proanthocyanidins. Finally, the separation of proanthocyanidins occurs under isocratic conditions and therefore, mobile phase recycling is possible.

The 18 isolated proanthocyanidin fractions described above were analyzed by GPC, and within each plant tissue the relationship between the elution time of the fraction and its molecular mass (determined by acid catalysis) was excellent, with an R^2 value exceeding 0.994 for the three proanthocyanidin sources (Table 3). Representative chromatograms for the grape skin proanthocyanidin fractions are overlaid in Fig. 1.

In addition to the analysis of proanthocyanidins, selected non-proanthocyanidin phenolics were ana-

Table 3
Regression equations for individual proanthocyanidin fractions

Sample	Regression equation	Correlation coefficient (R^2)
Hop proanthocyanidins	$y=-0.46(x)+9.30$	0.994
Grape seed proanthocyanidins	$y=-0.42(x)+8.64$	0.998
Grape skin proanthocyanidins	$y=-0.44(x)+9.09$	0.994
All fractions[a]	$y=-0.40(x)+8.54$	0.984

[a] Includes compounds **1**, **2**, **3**, **4**, and **5**.

lyzed to determine their retention properties relative to the proanthocyanidin fractions (Fig. 2). The phenolics analyzed were selected because of their potential presence in these plant tissues (i.e., potential impurities), and also because their structural variation might give some indication of how modifications in proanthocyanidin structure might affect their retention properties. The phenolics analyzed included rutin (**1**), malvidin-3-glucoside (**2**), (−)-epicatechin (**3**), (+)-catechin (**4**), and gallic acid (**5**). The analytical results indicate that the retention behavior of these phenolics is similar to the proanthocyanidins, and that modifications with these components should not have a dramatic effect on retention properties.

From Fig. 2, it can be seen that when all individual samples are overlaid, there is a very good relationship between molecular mass and retention time. For hop proanthocyanidins and grape skin proanthocyanidins, the combined regression equation has an R^2 value of 0.991. The R^2 value increases to 0.996 with the inclusion of the non-proanthocyanidin compounds. Yet, as shown in Fig. 2 the inclusion of all samples results in an R^2 value of 0.984. This is due to the anomalous behavior of the grape seed proanthocyanidins.

Inconsistent behavior between grape seed and skin proanthocyanidins has been observed when they are analyzed by normal-phase HPLC (i.e., their retention properties are not related to apparent molecular mass) [7]. This observation has been made with other proanthocyanidin isolates as well [16]. This could be due to a number of reasons including an error in molecular mass determination and adsorptive differ-

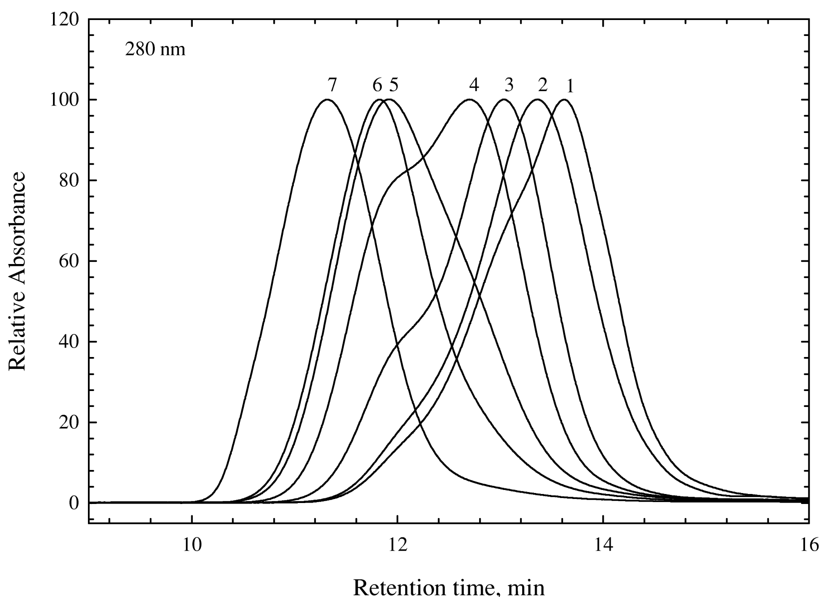

Fig. 1. Overlaid gel permeation chromatograms of fractionated grape skin proanthocyanidins.

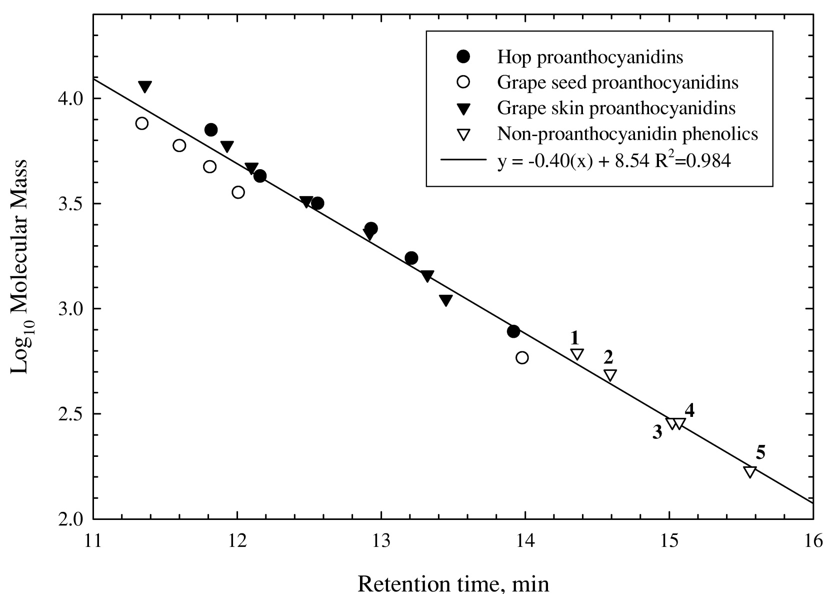

Fig. 2. Log_{10} molecular mass versus retention time for proanthocyanidin fractions and non-proanthocyanidin phenolics with a regression line for all of the components.

ences caused by differences in the number of sites that interact with the chromatographic media (chromatographic behavior). By analyzing these isolates using a non-adsorptive, GPC technique, it was speculated that additional information would be obtained that might help to explain this chromatographic inconsistency.

The results shown in Fig. 2 clearly indicate that the GPC behavior of grape seed proanthocyanidins is inconsistent with the other phenolics analyzed. These results are similar to those obtained by normal-phase HPLC. Assuming that GPC is a non-adsorptive technique, this indicates that the molecular size of grape seed proanthocyanidins is larger than grape skin and hop proanthocyanidins having the same molecular mass or that the molecular mass is underestimated. The mass conversion of grape seed proanthocyanidins however (discussion below) indicates that molecular size differences are the likely explanation. One possible reason for this could be that the increase in C-3 galloylation observed in grape seed proanthocyanidins (Table 2) results in a more extended conformation. This would explain the anomalous behavior when these samples are analyzed by normal-phase HPLC in that the more extended conformation of grape seed proanthocyanidins would provide more adsorptive sites for interaction.

As an example of the potential utility of this method, two grape seed isolates were analyzed by acid catalysis and GPC. The two samples selected were isolates from grapes: (1) harvested prior to véraison approximately 4 weeks after fruit set (grape seed-early), and (2) harvested at commercial maturity (grape seed-late). Compositional and average molecular mass data for these isolates are shown in Table 2. For GPC analysis, the grape seed fractions analyzed above were used to generate a second-order polynomial calibration curve. The results obtained from acid catalysis (Table 2) indicate that the two isolates are generally similar in composition but with very different calculated mDP. GPC analysis of these same samples provided additional distribution data (Fig. 3A). Assuming a constant composition for the two samples, the elution time corresponding to 50% mass elution is in good agreement with the mDP determined by acid catalysis (Fig. 3B). In addition to providing this detail, GPC provided additional information on the mass distribution not possible by acid catalysis. In this case, it is clear that proanthocyanidins isolated from commercially ripe grapes have a lower overall molecular mass distribution

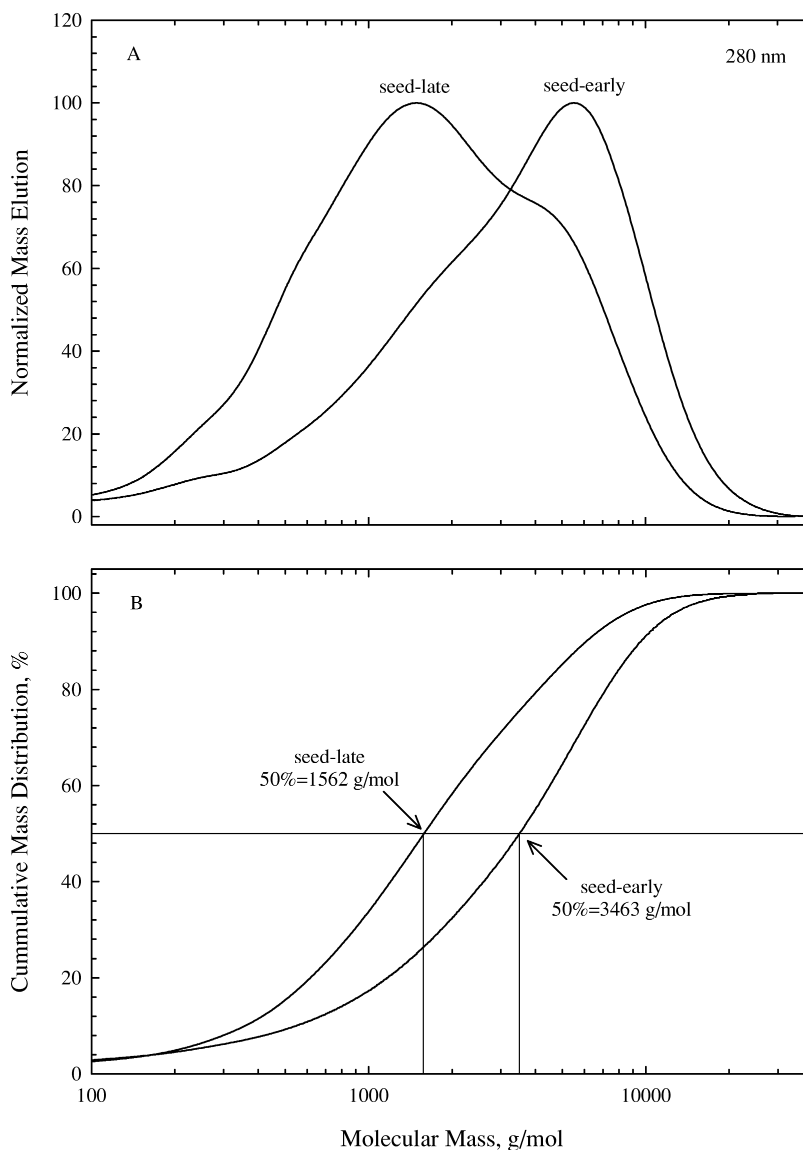

Fig. 3. (A) Normalized mass elution and (B) cumulative mass elution (%) versus molecular mass for purified grape seed proanthocyanidins isolated from grapes prior to véraison (seed-early) and at commercial harvest (seed-late).

than grapes harvested at an earlier time. Additionally, the high conversion yield of the grape seed proanthocyanidins (94%) provides good evidence that the inconsistent behavior of grape seed proanthocyanidins is due to size differences and not an incomplete assessment of molecular mass.

The importance of using appropriate molecular mass standards is confirmed here. It has been well established that molecular mass standards should have a similar chemical composition to the material under investigation. The results of this study indicate that even within the proanthocyanidin class of compounds, differences in hydrodynamic volumes exist. In the case of the grape seed proanthocyanidins, when molecular mass standards from grape seeds are used, the predicted molecular mass at 50% elution is

within 5% of the average molecular mass as determined by acid catalysis. If grape skin proanthocyanidin molecular mass standards are used to predict grape seed proanthocyanidin molecular masses, the calculated values exceed those determined by acid catalysis by 38–59%. Additional studies need to be conducted to provide confirmation for the effect of proanthocyanidin C-3 galloylation on hydrodynamic volume.

4. Conclusion

The results of this investigation indicate that there is a high degree of correlation between proanthocyanidin average molecular mass as determined by acid catalysis and GPC retention time. These two methods are also complementary in that they provide separate information but are ''linked'' by a common measurement.

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