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Analysis of pigmented high-molecular-mass grape phenolics using ion-pair, normal-phase high-performance liquid chromatography

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Abstract

A normal-phase LC method has been developed to analyze high-molecular-mass grape phenolic compounds. Samples are prepared by first isolating phenolics using C_{18}-SPE. The analytical method uses a silica column and gradient elution with mobile phases of methylene chloride, methanol, formic acid and heptanesulfonic acid. This separation enables the analysis of these compounds from grape and wine samples in the presence of anthocyanins without extensive purification. Based on the elution order of proanthocyanidins and anthocyanins, phenolics elute in order of increasing molecular mass. Currently, it is not possible to identify all of the components separated in the chromatogram. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Phenolic compounds; Tannins; Anthocyanins; Proanthocyanidins

1. Introduction

Much of the visual and flavor properties of red wine are due to pigmented and colorless phenolic material with a molecular mass in excess of 600 [1–3]. Because of this, there is considerable interest in understanding the nature and fate of these compounds.

It is well established that the high-molecular-mass phenolic material in red wine is initially composed of proanthocyanidins extracted from the grape berry [4,5]. These compounds are important in providing red wine with bitterness and astringency [2]. Because of their general reactivity, proanthocyanidins change in size during production and aging, and can also react with anthocyanins to produce pigmented proanthocyanidin products [6,7]. They therefore become increasingly important as the visual component in red wine as it ages [8]. The structure and formation of these high-molecular-mass phenolics formed in wine remain in large part unknown. Due to their importance as visual and flavor components, however, there is considerable interest in developing improved methods for their analysis.

Chromatographic methods have been developed for the analysis of grape phenolics. Reversed-phase liquid chromatographic methods can provide specific information on various classes of phenolics in red wine [9–12], but are limited in their ability to analyze high-molecular-mass material. Gel permeation chromatography (GPC) methods have been developed for the molecular mass estimation of proanthocyanidins, but suffer from poor resolution and may require prior derivatization of the proan-

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thocyanidins before analysis [13,14]. Size-exclusion chromatography (SEC) methods exist, but are limited by poor resolution and recovery [15,16]. A promising normal-phase liquid chromatography (LC) method developed for the analysis of proanthocyanidins requires no prior derivatization, and separates in order of increasing molecular mass [17]. This method, however, fails when the proanthocyanidin material is present with anthocyanins, and therefore, is not useful for analyzing samples such as skin extracts of red grapes or wine. This failure is caused by the anthocyanins co-eluting with the proanthocyanidins, and thus interferes with their detection.

The method presented here is a modification of the normal-phase LC method that enables the analysis of proanthocyanidins in the presence of anthocyanins. The method also provides information on what appears to be high-molecular-mass pigmented material that heretofore has been analyzed colorimetrically [18].

2. Experimental

2.1. Chemicals

All chromatographic solvents and phosphoric acid were LC grade and were purchased from Fisher Scientific (Santa Clara, CA, USA). LC grade heptanesulfonic acid was purchased from Alltech (Deerfield, IL, USA). Benzyl mercaptan, pyruvic acid and quercetin were purchased from Aldrich (Milwaukee, WI, USA). Epicatechin and quercetin-3β-D-rutinoside were purchased from Sigma (St. Louis, MO, USA). Malvidin-3,5-diglucoside was purchased from Pflatz and Bauer (Waterbury, CN, USA). Glacial acetic acid, ammonium acetate, caffeine, formic acid, sodium acetate and trifluoroacetic acid (TFA) were purchased from Fisher Scientific. Malvidin-3-glucoside and its 6″ coumarate ester were isolated from the skins of *Vitis vinifera* cf. Cabernet Sauvignon harvested at commercial maturity, and were characterized by their absorption spectra, relative abundance, and retention times. Epicatechin-3-*O*-gallate was isolated from the seeds of *Vitis vinifera* cf. Cabernet Sauvignon harvested when berries were still green, and was characterized by its UV absorption, mass and ^{1}H nuclear magnetic resonance (NMR) spectra. Cacao bean proanthocyanidin extract was prepared as previously described [17].

2.2. Preparation of skin and seed extracts

Cabernet Sauvignon grapes were harvested either when green (for seed extracts) or when commercially ripe (for skin extracts). Previous work indicated that proanthocyanidins are more easily extracted from seeds selected from green berries. For skin extracts, commercially mature grapes were selected because they contain a maximal amount of potential interferences (i.e., anthocyanins and flavonols). In both cases, the tissue of interest was separated from the remaining berry. The seeds and skins were then extracted separately with 66% (v/v) aqueous acetone for 24 h, concentrated under reduced pressure at 35°C to remove acetone, and lyophilized to a dry powder.

2.3. Fractionation and characterization of extracts

The lyophilized extracts were dissolved in methanol (approx. 30 g/l). The concentrated solutions were filtered through 0.45-μm, 13 mm PTFE syringe-tip filters (Millipore, Bedford, MA, USA) into LC vials, and were then fractionated using semi-preparative LC. The column was a LiChrospher Si-60 (particle size 10 μm, 250×10 mm I.D.), protected by a guard column (10×10 mm) containing the same material (EM Science, Gibbstown, NJ, USA). The method utilized a binary gradient with a flow-rate of 4.5 ml/min (mobile phase composition and gradient identical to the analytical method described below). Eluting peaks were monitored at 280 nm. Five fractions were collected: 0–20, 20–30, 30–40, 40–50 and 50–65 min. The collected samples were then concentrated under reduced pressure at 35°C to approx. 2 ml.

The proanthocyanidins contained in collected fractions were characterized by reinjection on an analytical column (method described below), and by acid-catalyzed thiolysis. For thiolysis, samples were prepared as follows: 800 μl of the concentrated solution was placed into a 1.8-ml LC vial. To this was added 800 μl of a 0.4 *M* HCl solution in

methanol, containing 12% (v/v) benzyl mercaptan. The LC vial was capped and placed in a 55°C water bath for 7 min. After thiolysis, 200 μl of a 1.6 *M* solution of ammonium acetate in methanol was added to reduce the acidity, thus minimizing epimerization reactions.

The thiolyzed material was cooled and analyzed by reversed-phase LC within 12 h. The column was a LiChrospher RP-18 (particle size 5 μm, 250×4 mm I.D.), protected by a guard column (10×4 mm) containing the same material (EM Science). The method used a binary gradient with mobile phases containing (A) 150 m*M* phosphoric acid, pH 1.5 and (B) methanol. The elution conditions were as follows: flow-rate, 1 ml/min; linear gradients, from 20 to 70% B in 35 min, 90% B for 5 min followed by re-equilibration of the column with 20% B for 5 min. Identification of eluting peaks was made by comparing with standards prepared from grape seed and skin extracts, using a method previously described for bark proanthocyanidin thiolysis [19]. Characterization of standards was made by UV–Vis, mass spectrometry (MS) and NMR spectroscopy. All properties of the standards were consistent with previously published data [20–22].

2.4. Preparation of anthocyanin derivative

A malvidin-3-glucoside–pyruvic acid adduct was prepared as follows: 2 g of the skin extract was placed into a 100-ml round bottom flask with 25 ml glacial acetic acid and 500 mg sodium acetate. A 2-g amount of pyruvic acid was then added and the resulting solution was stirred at room temperature for four days.

Afterwards, the crude mixture was partially purified using Toyopearl TSK (HW 40-F) size-exclusion resin (TosoHaas, Montgomeryville, PA, USA). To do so, a column (20×2 cm) was first equilibrated with 3% (v/v) aqueous formic acid. The crude anthocyanin mixture was applied to the column, and the modified anthocyanins were eluted with 3% (v/v) aqueous formic acid.

The modified anthocyanins were concentrated using a C_{18} solid-phase extraction (SPE) column (Alltech). To do so, the column was first activated with 3% (v/v) formic acid in methanol and then 3% aqueous formic acid. The fractions were applied and then eluted with 3% (v/v) formic acid in methanol. The methanol solution containing the modified anthocyanins was concentrated by rotary evaporation under reduced pressure at 35°C, and then diluted in a minimum amount of water for LC purification.

Final purification of the malvidin-3-glucoside–pyruvic acid adduct was performed using reversed-phase LC with a semi-preparative LiChrospher RP-18 (particle size 10 μm, 250×10 mm) column protected by a guard column (10×10 mm) containing the same material (EM Science). The method used a binary gradient with mobile phases containing (A) 0.5% (v/v) aqueous TFA and (B) methanol. The elution conditions were as follows: 3 ml/min and with a linear gradient from 20 to 60% B over 30 min. The adduct was collected and concentrated under reduced pressure at 35°C to remove methanol, and then lyophilized to a dry powder. For structural confirmation, spectral properties [UV–Vis, fast atom bombardment (FAB) MS and ^{1}H-NMR] of the purified anthocyanin was compared with previously published data [23].

2.5. Red wine sample preparation

Red wines were prepared as follows: ethanol was removed from the wine by placing 5 ml into a 50-ml pear-shaped flask and evaporating under reduced pressure at 35°C. After ethanol removal, the flask contents was transferred to a centrifuge tube followed by 5 ml of a 0.1 *M* caffeine solution. The mixture was centrifuged briefly to settle the precipitate. The supernatant was applied to a C_{18}-SPE column (1 g, Alltech), previously activated with methanol followed by water. SPE was used to remove organic acids, residual sugars, and other compounds insoluble in the organic mobile phase, and also to convert from aqueous to methanolic solvents, minimizing peak broadening. The settled precipitate in the centrifuge tube was resuspended in water, centrifuged and the supernatant applied to the SPE column. The applied sample was washed with 18 ml of water and then eluted with 18 ml of methanol into a 50-ml pear shaped flask. The precipitate was also dissolved in methanol and placed in the pear shaped flask. The methanol was evaporated under reduced pressure and reconstituted in 2 ml methanol. The final samples were filtered through

0.45-μm, 13 mm PTFE syringe-tip filters (Millipore) into LC vials.

2.6. Analytical high-performance liquid chromatography (HPLC) conditions

The column was a LiChrospher Si-60 (particle size 5 μm, 250×4 mm I.D.), protected by a guard column (10×4 mm) containing the same material (EM Science). The method used a binary gradient with mobile phases containing methylene chloride–methanol–formic acid–water: (A) 0:97:2:1 and (B) 83:14:2:1 both containing 20 m*M* heptanesulfonic acid. The elution conditions were as follows: 0.75 ml/min, linear gradients from 0 to 60% A in 50 min, from 60 to 100% A in 5 min, and 100% A for 10 min. The column was re-equilibrated with B for 10 min before the next injection. Eluting peaks were monitored at 280, 365 and 520 nm.

3. Results and discussion

This method was developed for the analysis of high-molecular-mass phenolics in red grapes and wine. The primary goal was to be able to routinely analyze high-molecular-mass phenolics in the presence of anthocyanins, with a minimum of sample preparation. A previously published method [17] fulfilled many of the requirements, namely ease of sample preparation, and the prediction of approximate molecular masses, but it failed in the presence of anthocyanins (Fig. 1a), because of the co-elution of these two components.

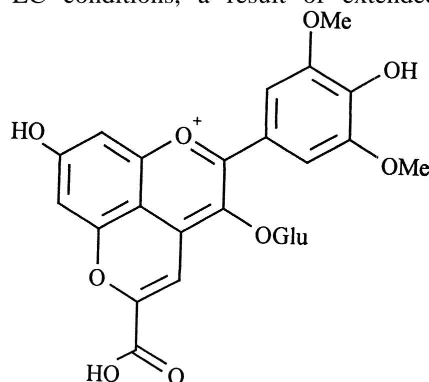
3.1. The effect of ion-pair reagent on interferences

It seemed likely that the charged flavylium form (Fig. 2) increased the polarity of the anthocyanins, resulting in strong adsorption onto the silica packing material. To reduce this polar interaction, 20 m*M* heptanesulfonic acid was added to the mobile phase as an ion-pair reagent (Fig. 1b).

To determine the anthocyanin contamination of high-molecular-mass phenolics, their fractions were collected by semi-preparative LC, and were analyzed by reversed-phase LC [14]. Based on the levels of malvidin-3-glucoside, 96% of the anthocyanins elute before 20 min, with the remaining 4% eluting before 30 min, confirming that the ion-pair reagent eliminates the additional interaction between the anthocyanins and silica.

Anthocyanins were also co-injected with a proanthocyanidin extract (Fig. 3) isolated from cacao beans (discussion of cacao bean proanthocyanidins below). The major anthocyanin peaks present in *Vitis vinifera* cf. Cabernet Sauvignon, malvidin-3-glucoside (compound **1**, Fig. 3), its 6″ acetate ester (**2**), and its 6″ coumaric acid ester (**3**), all eluted before the proanthocyanidin dimer, indicating that the major interferences had been eliminated. Another anthocyanin, malvidin-3,5-diglucoside (**4**), is not present in *Vitis vinifera*, but is present in other *Vitis* spp. The additional glucose residue increases column interaction and caused this compound to coelute with the proanthocyanidins.

Another anthocyanin investigated was the pyruvic acid adduct of malvidin-3-glucoside (**5**), which has been identified in red wine [23–25]. This compound eluted much later than would be predicted by molecular mass alone, apparently due to a higher proportion of the polar flavylium form present under LC conditions, a result of extended conjugation.

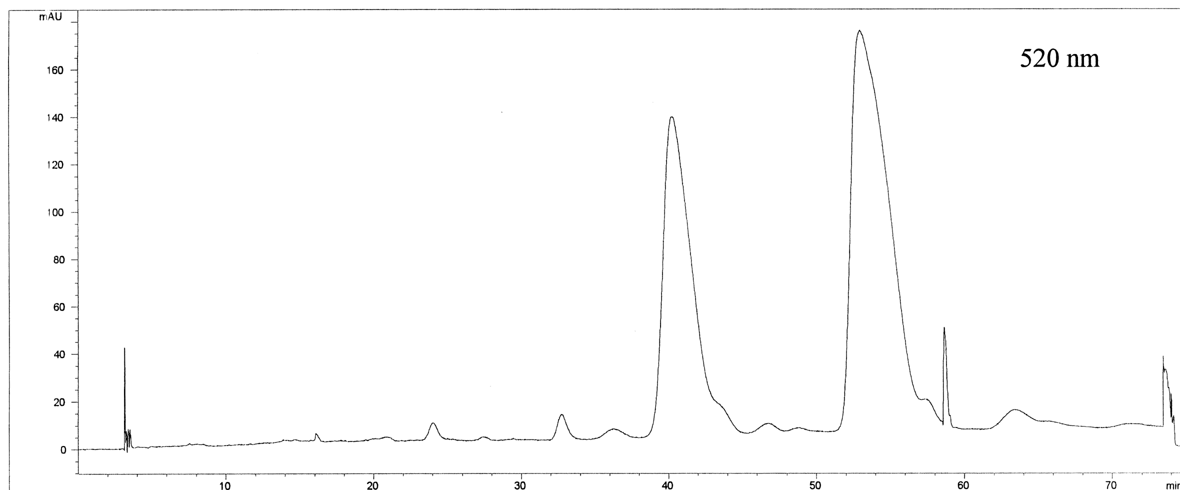


(5)

Attempts to suppress the additional interaction by increasing ion-pair concentration were unsuccessful. This class of compounds is not expected to be present in grape tissue extracts, but may be present in red wine.

Finally, two flavonols were selected based on their adsorption properties – quercetin-3β-D-rutinoside (**6**) and its aglycone quercetin (**7**). Quercetin did not coelute with the proanthocyanidins, but quercetin-

a).



b).

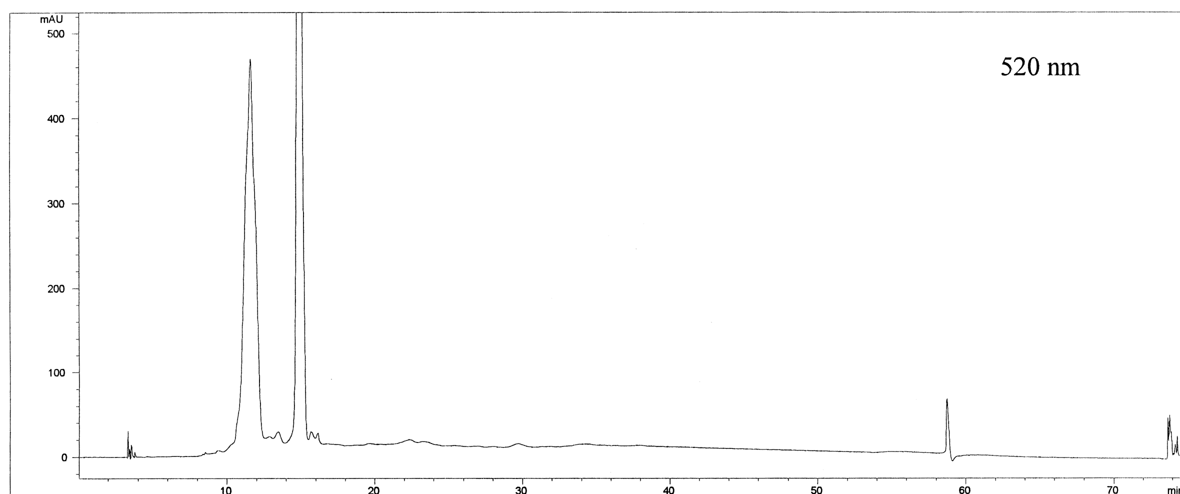


Fig. 1. Separation at 520 nm of red wine (Cabernet Sauvignon) extract before, (a), and after, (b), the addition of 20 m*M* heptanesulfonic acid to the mobile phase. In (a), the two broad peaks at approx. 40 and 55 min are the anthocyanins before heptanesulfonate addition; and in (b), the two peaks at approx. 12 and 15 min are the same anthocyanins after heptanesulfonate addition.

3β-D-rutinoside did. While quercetin-3β-D-rutinoside has not been reported in *Vitis vinifera*, other flavonol disaccharides have and therefore, would be expected to interfere. These limited data also suggest that retention properties are related to the number of hydrogen bond donors/acceptors and hydrophilicity.

Fig. 2. The anthocyanin equilibrium forms. At red wine pH (approx. 3.5), the flavylium form of malvidin-3-glucoside makes up approx. 10% of the equilibrium pool.

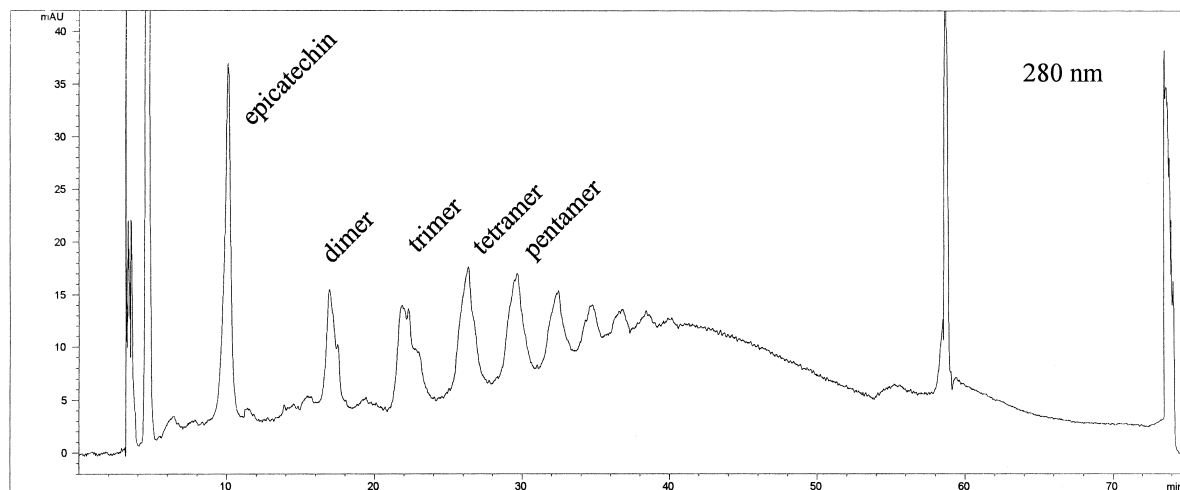
3.2. The effect of the ion-pair reagent on proanthocyanidin retention

Fig. 4a and b show the retention behavior of proanthocyanidins isolated from cacao beans before and after ion-pair reagent addition, indicating that the ion-pair reagent had very little effect on the resolution of proanthocyanidins.

The resolving ability of the method was investigated using fractions of grape seed and skin extracts collected by semi-preparative LC. When the fractions were reinjected on an analytical column, they eluted

Fig. 3. Plot of molecular mass vs. retention time for cacao bean-based proanthocyanidins (closed circles) and other phenolic compounds (see text for description). In order of increasing retention time, closed circles correspond to epicatechin, epicatechin dimer, trimer, tetramer, etc.

a).



b).

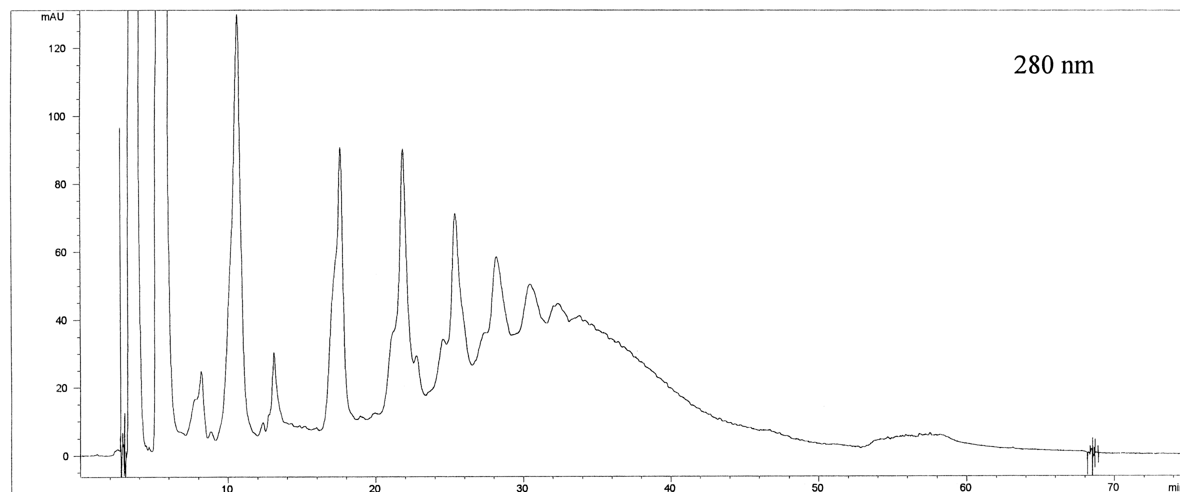


Fig. 4. LC chromatograms of cacao bean extract before, (a), and after, (b), the addition of 20 m*M* heptanesulfonic acid to the mobile phase.

later with each successive fraction, confirming that the properties of the eluting material changed consistently with retention time. Given the chromatographic conditions used, the UV spectra of eluting material, previously observed elution orders [17], and the phenolic composition of grape seed and skin [20,21], it is reasonable to infer that the eluting material consisted of proanthocyanidins of increasing size.

The collected fractions were also subjected to acid-catalyzed thiolysis, a standard method to determine the mean degree of polymerization (mDP) of

Table 1
The mean degree of polymerization for proanthocyanidin fractions collected at different times, and from different grape sources as determined by acid-catalyzed thiolysis

	Fraction No. (min)				
	0–20	20–30	30–40	40–50	50–65
Seed	1.3	6.0	11.3	8.3	10.3
Skin	–[a]	11.7	32.7	62.0	70.8

[a] None detected.

proanthocyanidins (Table 1). With the skin extract, later eluting fractions had higher mDP. While the mDP of the seed extract increased through the first three fractions, no increase was observed in later eluting fractions. It is difficult to explain this result, but it may be caused by different bonding in the larger sized fractions, bonds resistant to thiolytic cleavage.

3.3. Molecular mass approximation of grape and wine phenolics

A cacao bean-based proanthocyanidin extract was used to obtain molecular mass information on poorly resolved grape and wine phenolics (Fig. 4). Cacao bean proanthocyanidins are composed principally of epicatechin subunits [19] and therefore, the resolution of the oligomers is better than grape based proanthocyanidins which also contain other subunits [20,21]. While the subunit composition of cacao bean proanthocyanidins is different from grape based proanthocyanidins, they are very similar and preferred compared to the use of the polystyrene standards employed in GPC to approximate the molecular mass of peracetylated proanthocyanidins [13].

The degree of polymerization for grape seed extracts (determined by thiolysis) compared favorably with the degree of polymerization for cacao bean proanthocyanidins (visually determined from retention times) (Table 1). In addition, based on the retention time of (−)-epicatechin-3-*O*-gallate, (compound **8** in Fig. 3), cacao bean proanthocyanidins can be used to predict molecular mass when galloylated, grape-based, proanthocyanidins are present. By incorporating cacao bean proanthocyanidins into LC run sequences, information on phenolic size can be obtained from analytes having little to no resolution.

3.4. Method performance

In Fig. 5, the chromatography of a red wine extract is shown. Caffeine, used to improve recovery, elutes close to the solvent front in the chromatogram. The poor resolution of the high-molecular-mass wine phenolics, as seen in the 280 nm chromatogram (Fig. 5a), alludes to the complexity of the material present. In addition to the complexity of the grape based proanthocyanidins, wine is an acidic solution; and therefore, the proanthocyanidins readily undergo acid-catalyzed hydrolysis during storage and aging [26]. Given the plethora of potential nucleophiles in red wine (anthocyanins, flavan-3-ols, flavonols and glutathione, to name a few), it is expected that the proanthocyanidins rapidly become modified in wine to so many compounds that it is not possible to separate them.

In the 520 nm chromatogram it is possible to see broad, late eluting peaks, suggesting that it is possible to observe pigmented tannins in addition to the monomeric anthocyanins which elute very early in the chromatogram.

In the 365 nm chromatogram the 20 min peak gives an indication of interfering flavonol behavior. Since the flavonols are expected to be monomers, albeit with glycoside conjugation, and dimers are expected to elute at 17 min (Fig. 3) this slow eluting peak constitutes an interference.

The repeatability and recovery of this method was determined in red wine by preparing and analyzing a red wine five times. Repeatability of high-molecular-mass phenolic quantitation is very good (approx. 5%), approaching LC reproducibility. The recovery of added high-molecular-mass phenolics was determined by adding known amounts (1 g/l) of grape seed or skin extract to a red wine and comparing concentration increases with the same level of seed or skin extract dissolved in methanol. The recovery of high-molecular-mass phenolics from added grape seed extract was excellent, with $>90\%$ of the added material recovered. The recovery of high-molecular-mass phenolics from skin extract is less (85%). The addition of caffeine to the sample before SPE has

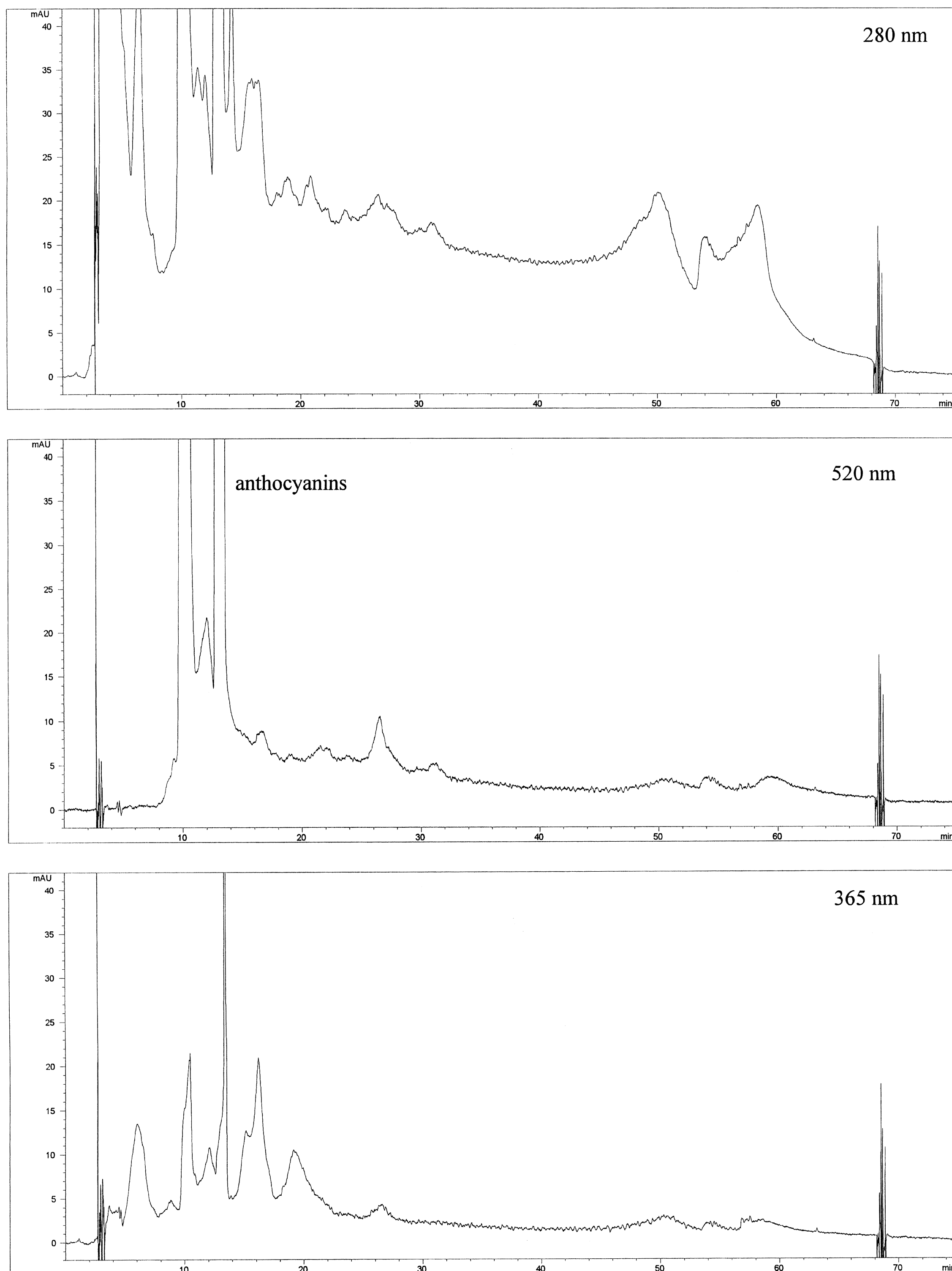


Fig. 5. LC of a 1998 Cabernet Sauvignon red wine extract.

been found to improve recovery by reducing SPE column overloading.

4. Conclusion

This method can be used to separate high-molecular-mass phenolics in grapes and wine in the presence of anthocyanins, routinely and reproducibly. Based on the mDP of eluting grape proanthocyanidins, the elution order is from small to large material. The information provided by this method is novel and provides a new avenue to understand the nature of grape and wine phenolics.

While the identity of low-molecular-mass grape and wine phenolics can be deduced with the use of standards, the identity of material eluting later is not yet possible given current technology.

References

[1] T.C. Somers, Phytochemistry 10 (1971) 2175.
[2] A.C. Noble, in: R.L. Rouseff (Ed.), Bitterness in Foods and Beverages, Developments in Food Science, Vol. 25, Elsevier, 1990, p. 145, Ch. 8.
[3] V.L. Singleton, in: R.W. Hemingway (Ed.), Plant Polyphenols – Synthesis, Properties, Significance, Plenum Press, 1992, p. 859.
[4] R.S. Thompson, D. Jacques, E. Haslam, J. Chem. Soc. PI, (1972) 1387.
[5] L.Y. Foo, L.J. Porter, J. Sci. Food Agric. 32 (1981) 711.
[6] C. Dallas, J.M. Ricardo-da-Silva, O. Laureano, J. Sci. Food Agric. 70 (1996) 493.
[7] T. Escribano-Bailon, O. Dangles, R. Brouillard, Phytochemistry 41 (1996) 1583.
[8] T.C. Somers, Vitis 7 (1968) 303.
[9] J.-P. Roggero, Am. Lab. January (1997) 12D.
[10] A.G.H. Lea, J. Chromatogr. 194 (1980) 62.
[11] R.M. Lamuela-Raventos, A.L. Waterhouse, Am. J. Enol. Vitic. 45 (1994).
[12] A.L. Waterhouse, S.F. Price, J.D. McCord, Methods Enzymol. 299 (1999) 113.
[13] V.M. Williams, L.J. Porter, R.W. Hemingway, Phytochemistry 22 (1983) 569.
[14] Y.S. Bae, L.Y. Foo, J.J. Karchesy, Holzforschung 48 (1994) 4–6.
[15] K. Kantz, V.L. Singleton, Am. J. Enol. Vitic. 42 (1991) 309.
[16] J. Cacho, J.E. Castells, Am. J. Enol. Vitic. 42 (1991) 327.
[17] J. Rigaud, M.T. Escribano-Bailon, C. Prieur, J.-M. Souquet, V. Cheynier, J. Chromatogr. A 654 (1993) 255.
[18] T.C. Somers, M.E. Evans, J. Sci. Food Agric. 28 (1977) 279.
[19] L.J. Porter, Z. Ma, B.G. Chan, Phytochemistry 30 (1991) 1657.
[20] C. Prieur, J. Rigaud, V. Cheynier, M. Moutounet, Phytochemistry 36 (1994) 781.
[21] J.-M. Soquet, V. Cheynier, F. Brossaud, M. Moutounet, Phytochemistry 43 (1996) 509.
[22] S. Matthews, I. Mila, A. Scalbert, B. Pollet, C. Lapierre, C.L.M. Hervé du Penhoat, C. Rolando, D.M.X. Donelly, J. Agric. Food Chem. 45 (1997) 1195.
[23] H. Fulcrand, C. Benabdeljalil, J. Rigaud, V. Cheynier, M. Moutonet, Phytochemistry 47 (1998) 1401.
[24] J. Bakker, P. Bridle, T. Honda, N. Saito, H. Kuwano, N. Terahara, C.F. Timberlake, Phytochemistry 44 (1997) 1375.
[25] J. Bakker, C.F. Timberlake, J. Agric. Food Chem. 45 (1997) 35.
[26] R.W. Hemingway, G.W. McGraw, J. Wood Chem. Tech. 3 (1983) 421.