Reversed-Phase Ion-Pair Chromatography of Anthocyanins in Red Wines

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Abstract

A reversed-phase ion-pair chromatographic method has been developed for the separation of anthocyanins from red wines. Separation of anthocyanin monoglucosides can be achieved with a gradient mode elution using tetrabutylammonium hydrogen sulfate as a counter-ion, phosphoric acid, and methanol–acetonitrile–water in the mobile phase (pH = 2). This technique allows a relatively fast separation and identification of different anthocyanins in one run without prior treatment of the wine or derivatization of the compounds; it also avoids both the use of low pH (below 2) and, consequently, the degradation of the column. The order of elution of anthocyanin monoglucosides does not change in this mode of chromatography; they elute in the order of their polarity, as is typical in reversed-phase chromatography (delphinidin, cyanidin, petunidin, peonidin, and malvidin monoglucoside). Therefore, this characteristic profile is useful for the identification of anthocyanins of *Vitis vinifera* in red wines.

Introduction

Anthocyanin pigments have been characterized by different chromatographic techniques. However, high-performance liquid chromatography (HPLC) has been more frequently applied for the analysis of these compounds. Basically similar LC methods and examples of HPLC methods that can be used for determination of anthocyanins from different matrices have been reported in the literature by many analysts (1–24). In most cases, the reported separations were carried out with reversed-phase chromatography, C_{18} bonded-phase columns, and particle diameters between 3 and 10 µm. HPLC analysis using columns with smaller particles (e.g., 3 µm) permits faster separations than columns that have larger particles. Gradient elution seems ideal for separating anthocyanins, which are structurally very similar. Most of the solvent systems used in analytical HPLC include binary gradient elution with methanol or acetonitrile as organic modifiers. Ternary gradient elution was also reported for the separation of complex mixtures of anthocyanins in different varieties of grapes (25). It has also been reported (1) that alkylamines as mobile phase additives and butylamine (0.122M) in the mobile phase with gradient elution provided better resolution and less retention of red wine anthocyanins. In particular, the nature of the organic modifier in the mobile phase had a dramatic influence on the separation of anthocyanin compounds.

Solvent systems for the HPLC analysis of anthocyanins always include an acid to ensure that the anthocyanins are in the red flavylium cation form. Formic acid up to 10% (w/v) is most commonly used with reversed-phase columns, which corresponds to pH values of about 1.9 or even lower (4). Acids such as acetic acid (10,18,20,22), phosphoric acid (3,13,14,17), formic acid (4,7–9,15,16,19,21,23), trifluoroacetic acid, and perchloric acid (1,5,6) have also been used (2,24). Below pH 3.2, anthocyanins exist as two distinct interconvertible forms: the red flavylium cation and the colorless carbinol base. According to the data of Wulf and Nagel (8), an anthocyanin is 96% in the flavylium form at pH 1.5 but only 67% in that form at pH 2.5; therefore, low pH eluant is vital to the separation of anthocyanins. Eluants with pH values of 2.5 result in broad peaks and poor separation.

Producers of reversed-phase supports usually do not recommend using eluants that have pH values less than 2 because the octadecylsilyl (ODS) groups may be hydrolyzed from the silica backbone. However, many works have been published using mobile phases with low pH, and those authors claim that the life of the column is not affected (3,19). This problem was circumvented here by the use of reversed-phase ion-pair chromatography, which could separate the anthocyanins under mild conditions of pH with very nicely shaped anthocyanin peaks.

Experimental

Chemicals and reagents

HPLC-grade acetonitrile and methanol were obtained from Mallinckrodt Specialty Chemicals (Paris, Kentucky). Phosphoric acid was obtained from Merck–Mexico (Naucalpan de Juárez, Mexico). Tetrabutylammonium hydrogen sulfate was obtained

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from Aldrich Chemical (Milwaukee, WI), and grape skin extract (Enocyanin) was obtained from SEFCAL (Saint Julien de Peyrolas, France).

Instrumentation

HPLC was performed with two Waters Associates model 6000 A pumps (Millipore, Milford, MA) and a 680 automated gradient controller. A Rheodyne (Cotati, CA) injector model 7725 with a 20-µL loop and a Waters 746 integrator were also used. The analytical column was µBondapak C_{18} (Waters Associates) (300 × 3.9-mm i.d.) packed with 10-µm particles. The detector was an ultraviolet–visible (UV–vis) Waters 484 tunable absorbance detector.

Chromatographic conditions

The HPLC-grade solvents were filtered through a 0.45-µm filter and degassed in an ultrasonic bath. The anthocyanins were monitored at 520 nm. The flow rate in all analyses was 1.0 mL/min. In order to separate the anthocyanins in a single step, it was necessary to use the following gradient elution: 90% A to 50% A in 45 min by the linear solvent gradient, curve 6, of the 680 automated gradient controller. Mobile phase A was 0.0684% H_3PO_4 and tetrabutylammonium hydrogen sulfate (0.0018M) in acetonitrile–methanol (1:1). Mobile phase B was 0.0684% H_3PO_4 and tetrabutylammonium hydrogen sulfate (0.0018M) in water. The pH values of solutions A and B were 1.87 and 2.01, respectively.

Samples

A 0.3% grape skin extract (Enocyanin) solution was prepared in mobile phase A, and 20 µL was injected in order to obtain the characteristic profile of anthocyanins. The red wine samples that were analyzed are in Table I. All the wine samples were analyzed directly; 20 µL of each sample was injected. No cleaning step was necessary prior to analysis.

Table I. Red Wine Samples Studied

Sample	Region	Harvest year	Vine
1 (Spain)	La Rioja	1989	not specified
2 (France)	Bordeaux	1992	not specified
3 (France)	Loire	1992	not specified
4 (USA)	California	1988	Cabernet Sauvignon
5 (Chile)	Burgundy	1992	not specified
6 (Chile)	V. del Maipo	1993	Cabernet
7 (Mexico)	V. de Calafia, B.C.	1992	not indicated
8 (Mexico)	V. de Gpe., B.C.	not specified	not specified
9 (Mexico)	V. de Calafia, B.C.	1987	not specified
10 (Mexico)	V. de Gpe., B.C.	not specified	not specified
11 (Mexico)	V. de Calafia, B.C.	not specified	not specified
12 (Mexico)	Tijuana, B.C.	not specified	Cabernet Sauvignon
13 (Mexico)	Parras, Coahuila	not specified	not specified
14 (Mexico)	Cienegas, Coahuila	not specified	not specified
15 (Mexico)	Sn. J. del Rio, Qro.	not specified	Cabernet Sauvignon

Results and Discussion

For the relative identification of anthocyanin monoglucosides in the red wine samples, grape skin extract (Enocyanin) was used. Figure 1 illustrates the chromatographic separation of the anthocyanin pigments from Enocyanin using ion-pair chromatography. The following anthocyanins were separated: delphynidin-3-monoglucoside (peak 1), cyanidin-3-monoglucoside (peak 2), petunidin-3-monoglucoside (peak 3), peonidin-3-monoglucoside (peak 4), and the major *V. vinifera* anthocyanin (7,8), malvidin-3-monoglucoside (peak 5). Excellent separation and sharp peaks of the main anthocyanin pigments were obtained, and the elution order was in order of polarity, as was the elution obtained by reversed-phase chromatography (7,12).

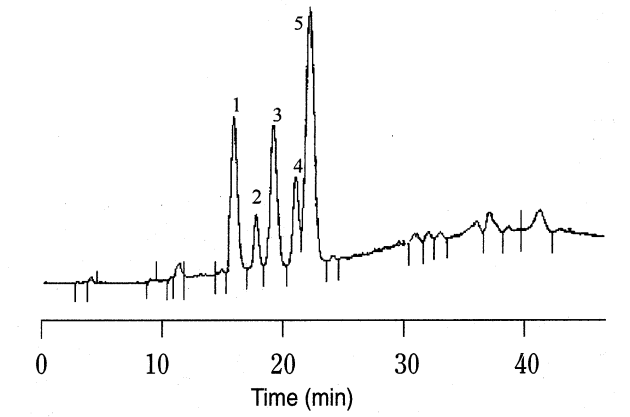

Figure 1. HPLC ion-pair analysis of Enocyanin. Peaks: 1, Delphynidin-3-monoglucoside; 2, cyanidin-3-monoglucoside; 3, petunidin-3-monoglucoside; 4, peonidin-3-monoglucoside; and 5, malvidin-3-monoglucoside. Gradient elution is described in the text.

Almost all of the analyzed samples (Table I) showed the characteristic profile of anthocyanin pigments. As examples of the anthocyanin pigment chromatographic profiles, Figures 2, 3, and 4 illustrate the direct analysis of the samples 2, 4, and 13, respectively. As expected, the characteristic profile of *V. vinifera* anthocyanins was obtained by using the proposed method. Peaks 6, 7, and 8 were not identified because it was not possible to get the standards. However, according to the literature (8,16), they are probably malvidin-3-monoglucoside–acetate, malvidin-3-monoglucoside–caffeoate, and malvidin-3-monoglucoside-*p*-coumarate, respectively.

Although the vine or grape variety was not indicated in some samples (samples 1–3,5,7–11,13, and 15), the anthocyanins chromatographic profile was quite similar. This chromatographic profile suggests that they were probably of the same grape variety.

Two samples showed alterations in the anthocyanins chromatographic profile. Figure 5 illustrates the analysis of sample 6, in which the

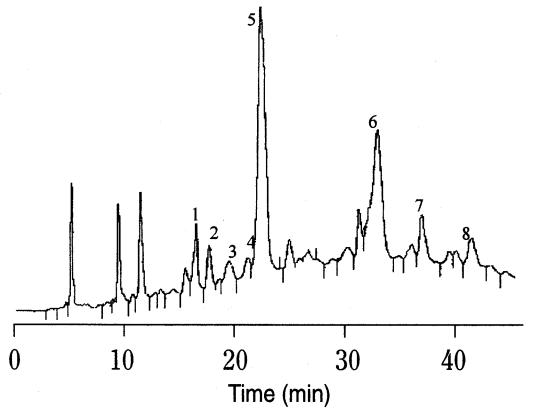

Figure 2. HPLC ion-pair analysis of sample 2. Peaks: 1, Delphynidin-3-monoglucoside; 2, cyanidin-3-monoglucoside; 3, petunidin-3-monoglucoside; 4, peonidin-3-monoglucoside; 5, malvidin-3-monoglucoside; 6, malvidin-3-monoglucoside acetate; 7, malvidin-3-monoglucoside caffeoate; 8, malvidin-3-monoglucoside-*p*-coumarate. Gradient elution in the text.

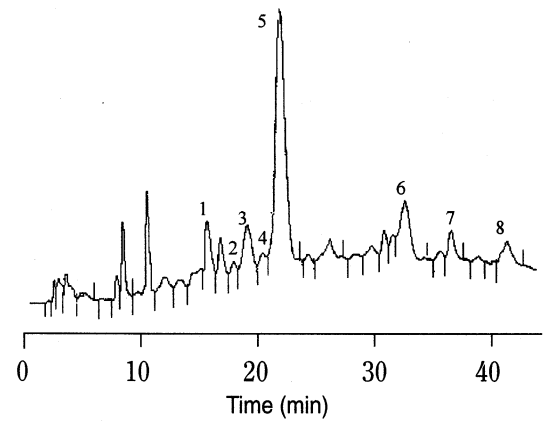

Figure 3. HPLC ion-pair analysis of sample 4. Identification of peaks is in Figure 2.

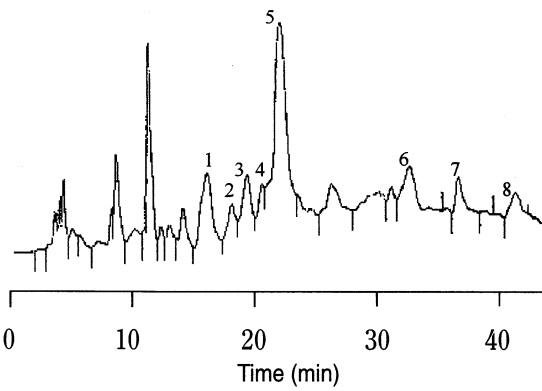

Figure 4. HPLC ion-pair analysis of sample 13. Identification of peaks is in Figure 2.

characteristic anthocyanin profile was identified. However, the total profile was quite different from that of the other wine samples, as is shown in the chromatogram. The largest peak (peak 9) was different than malvidin-3-monoglucoside (peak 5), which is characteristic of *V. vinifera*. It was not possible to identify peak 9, which was also present in sample 14 (Figure 6). In this case, the characteristic profile of anthocyanins from *V. vinifera* was missing, and only the unknown peak (peak 9) was present.

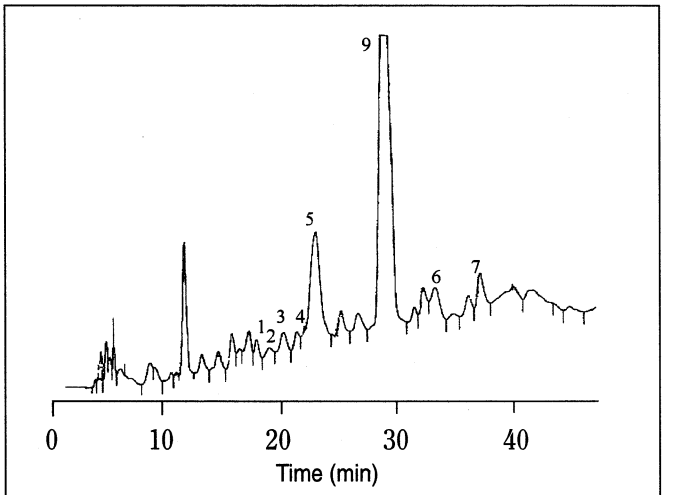

Figure 5. HPLC ion-pair analysis of sample 6. Identification of peaks is in Figure 2; peak 9 is unknown.

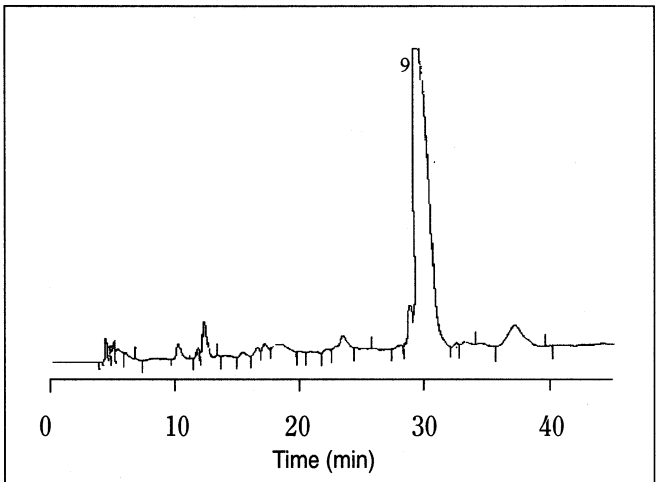

Figure 6. HPLC ion-pair analysis of sample 14. Peak 9 is unknown.

Conclusion

The proposed method used C_{18} ODS reversed-phase columns at a pH of 2 without causing degradation of the ODS bonded-phase on the column. At that pH, the flavylium form of anthocyanin was present, which allowed good separation and good sensitivity for detection.

Due to the large number of compounds present in red wine and the fact that the samples were not prefractionated, it was not possible for us to confirm the identity of each peak on the chromatogram. However, the compounds have been tentatively identified based on their elution order with those obtained by grape skin extract (delphinidin, cyanidin, petunidin, peonidin, and malvidin monoglucoside).

This method was used to analyze the anthocyanins of red

wines, giving a characteristic anthocyanins profile that allows for the identification of the grape variety. It is also possible to detect adulteration, admixes of other *Vitis*, or other sorts of adulteration (9).

References

1. M. Drdák, P. Daucik, and J. Kubask. Analysis of anthocyanins in red wines by high-performance liquid chromatography using butylamines in the mobile phase. *J. Chromatogr.* **504:** 207–209 (1990).
2. H. S. Lee and V. Hong. Chromatographic analysis of anthocyanins. *J. Chromatogr.* **624:** 221–234 (1992).
3. R. Lamuela-Raventós and A.L. Waterhouse. A direct hplc separation of wine phenolics. *Am. J. Enol. Vitic.* **45:** 1–5 (1994).
4. J.P. Goiffon, M. Brun, and M.J. Bourrier. High-performance liquid chromatography of red fruit anthocyanins. *J. Chromatogr.* **537:** 101–21 (1991).
5. J. Bakker and C.F. Timberlake. The distribution of anthocyanins in grape skin extracts of port wine cultivars as determined by high-performance liquid chromatography. *J. Sci. Food Agric.* **36:** 1315–24 (1985).
6. P. Etiévant, P. Schlid, A. Bertrand, P. Symonds, and J.C. Bouvier. Varietal and geographic classification of french red wines in terms of pigments and flavonoid compounds. *J. Sci. Food Agric.* **42:** 39–54 (1988).
7. J.A. Fernández-López, V. Hidalgo, L. Almela, and J.M. López Roca. Quantitative changes in anthocyanin pigments of vitis vinifera cv monastrell during maturation. *J. Sci. Food Agric.* **58:** 153–55 (1992).
8. L.W. Wulf and C.W. Nagel. High-pressure liquid chromatography separation of anthocyanins of vitis vinifera. *Ant. J. Enol. Agric.* **29:** 42–49 (1978).
9. P. Symons. Compositional analyses for fruit juices-flavonoids, anthocyanins. Food authenticity, 2nd European symposium, Nantes, France, October 1993.
10. M.L. Hale, F.J. Francis, and I.S. Fagerson. Detection of enocyanin in cranberry juices cocktail by hplc anthocyanin profile. *J. Food Sci.* **51:** 1511–15 (1986).
11. F.J. Francis. Detection of enocyanin in cranberry juice cocktail by color and pigment profile. *J. Food Sci.* **50:** 1640–42 (1985).
12. V. Hong and R.E. Wrolstad. Characterization of anthocyanin-containing colorants and fruit juices by hplc/photodiode array detection. *J. Agric. Food Chem.* **38:** 698–708 (1990).
13. V. Hong and R.E. Wrolstad. Use of hplc separation/photodiode array detection for characterization of anthocyanin. *J. Agric. Food Chem.* **38:** 708–15 (1990).
14. A. Chandra, M.G. Nair, and A. Iezzoni. Evaluation and characterization of the anthocyanin pigments in tart cherries *(Prunus cerasus* L.). *J. Agric. Food Chem.* **40:** 967–69 (1992).
15. M. Williams and G. Hrazdina. High-pressure liquid chromatographic separation of 3-glucosides, 3,5-diglucosides, 3-(6-*O*-*p*-coumaryl)glucosides and 3-(6-*O*-*p*-coumaryl glucoside-5-glucosides of anthocyanindins. *J. Chromatogr.* **155:** 389–98 (1978).
16. M.L. Gonzales-San Jose, G. Santa-Maria, and C. Diez. Anthocyanins as parameters for differentiating wines by grape variety, wine region and wine-making methods. *J. Food Comp. Anal.* **3:** 54–66 (1990).
17. L. Gao and G. Mazza. Rapid method for complete chemical characterization of simple and acylated anthocyanins by high-performance liquid chromatography and capillary gas–liquid chromatography. *J. Agric. Food Chem.* **42:** 118–25 (1990).
18. K. Bronnum-Hansen and S. Honore Hansen. High-performance liquid chromatographic separation of anthocyanins of *Sambucus nigra* L. *J. Chromatogr.* **262:** 385–92 (1983).
19. A.L. Camire and F.M. Clydesdale. High-pressure liquid chromatography of cranberry anthocyanins. *J. Food Sci.* **44:** 926–27 (1979).
20. J. Kärppa, H. Kallio, I. Peltonen, and R. Linko. Anthocyanins of crowberry, empetrum nigrum coll. *J. Food Sci.* **49:** 634–36 (1984).
21. M. Wilkinson, J.G. Sweeny, and G.A. Iacobucci. High-pressure liquid chromatography of anthocyanidins. *J. Chromatogr.* **132:** 349–51 (1977).
22. M. Mayen, J. Merida, and M. Medina. Free anthocyanins and polymeric pigments during the fermentation and post-fermentation standing of musts form cabernet sauvignon and tempranillo grapes. *Am. J. Enol. Vitic.* **45:** 160–66 (1994).
23. A. Rommel, R.E. Wrolstad, and D.A. Heatherbell. Blackberry juice and wine: Processing and storage effects on anthocyanin composition, color and appearance. *J. Food Sci.* **57:** 385–91 (1992).
24. J. Oszmianski and J.C. Sapis. Anthocyanins in fruits of aronia melanocarpa (chokeberry). *J. Food Sci.* **53:** 1241–42 (1988).
25. J. Bakker and C.F. Timberlake. The distribution and content of anthocyanins in young port wines as determined by high-performance liquid chromatography. *J. Sci. Food Agric.* **36:** 1325–33 (1992).

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