Determination of Anthocyanidins in Berries and Red Wine by High-Performance Liquid Chromatography

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A high-performance liquid chromatographic (HPLC) method for the determination of anthocyanidins from berries and red wine is described. Delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin contents of bilberry (*Vaccinium myrtillus*), black currant (*Ribes nigrum*), strawberry (*Fragaria ananassa* cv. Jonsok), and a Cabernet sauvignon (*Vitis vinifera*) red wine were determined. The aglycon forms of the anthocyanins present in the samples were revealed by acid hydrolysis. A reversed phase analytical column was employed to separate the anthocyanidins before identification by diode array detection. The suitability of the method was tested by determining the recovery (95−102% as aglycons and 69−104% from glycosides) for each anthocyanidin. Method repeatability was tested by charting the total aglycon content of two samples over a period of 14 analyses and determining the coefficients of variation (1.41% for bilberry and 2.56% for in-house reference material). The method developed proved thus to be effective for reliable determination of anthocyanidins from freeze-dried berry samples and red wine. The total anthocyanidin content of the tested samples was as follows: in-house reference material, 447 ± 8 mg/100 g; strawberry, 23.8 ± 0.4 mg/100 g; black currant, 135 ± 3 mg/100 g; bilberry, 360 ± 3 mg/100 g; and Cabernet sauvignon red wine, 26.1 ± 0.1 mg/100 mL.

Keywords: *Anthocyanidins; HPLC; diode array; berries; red wine; Vaccinium myrtillus; Ribes nigrum; Fragaria ananassa; Vitis vinifera*

INTRODUCTION

Anthocyanins are phenolic plant metabolites belonging to the flavonoid family. They are responsible for most red and blue colors in fruits, berries, and flowers. An anthocyanin molecule constitutes an aglycon or anthocyanidin body, which in its natural state is glycosylated by one or more different sugars. Differences between the aglycon bodies are due to the number of hydroxyl groups and the degree of methylation of these groups. The six anthocyanidins present in edible plants are presented in Figure 1 (*1*).

Anthocyanins are potent antioxidants (*2*−*6*). The use of anthocyanin-containing foods as part of a healthy diet may be beneficial for the health (*7*). Therefore, practical, accurate, and reliable methods to determine the anthocyanin content of foodstuffs are needed. Anthocyanins have also received increasing attention as natural food colorants. As food is processed some destruction of the original color always occurs. These natural pigments can be used to replace the loss (*8*). Due to their antioxidant properties, anthocyanins may also enhance the shelf life of the food they have been added to.

The use of HPLC separation combined with diode array detection (DAD) has become the method of choice for the qualitative and quantitative analysis of anthocyanins (*1*, *9*). Most research has been done on the qualitative anthocyanin characterization of fruits and berries (*10*−*13*). Many authors have reported the anthocyanin content of the samples quantified using one glucoside as reference compound (*14*−*18*). The relative aglycon content based on total anthocyanin peak area has been presented in some papers (*19*, *20*). Many of these methods combine techniques to identify the sugar and acid components attached to the anthocyanidin, too. Laborious sample treatment procedures may be involved, such as concentration (*10*, *12*, *15*, *16*, *18*, *21*) liquid−liquid extraction (*15*, *16*, *21*), and solid phase extraction (*10*, *12*). A simple and thoroughly validated method to accurately quantify anthocyanin aglycons in plant-based material is still lacking. It is suspected that the substitution pattern on the B-ring is the key factor in the antioxidative behavior of anthocyanins (*6*). Therefore, the specific anthocyanidin content of foods and beverages is valuable information in the assessment of their nutritional value.

In this paper an HPLC method using DAD for the qualitative and quantitative analysis of the six anthocyanidins present in fruits and berries is described. Samples chosen for method development were freeze-dried berries and red wine, all known to be rich in anthocyanins.

MATERIALS AND METHODS

Standards. Delphinidin, cyanidin, pelargonidin, peonidin, malvidin, the 3-glucoside forms of pelargonidin, peonidin, and malvidin, and cyanidin-3-galactoside were obtained from Roth, Germany, as HPLC purity grade chloride salts. The standard used to ensure the retention time and spectrum of petunidin was produced by Polyphenols AS. The aglycons were used for calibration, and a series of dilutions was prepared in 1.2 M HCl in methanol at concentrations from 0.2 to 45 mg/L (delphinidin, cyanidin, and pelargonidin) or from 0.4 to 25 mg/L (peonidin and malvidin). For recovery tests the glycosides were dissolved into 0.1 M HCl in methanol. Standard solutions were stored in darkness at 4 °C and remained stable over 3 months.

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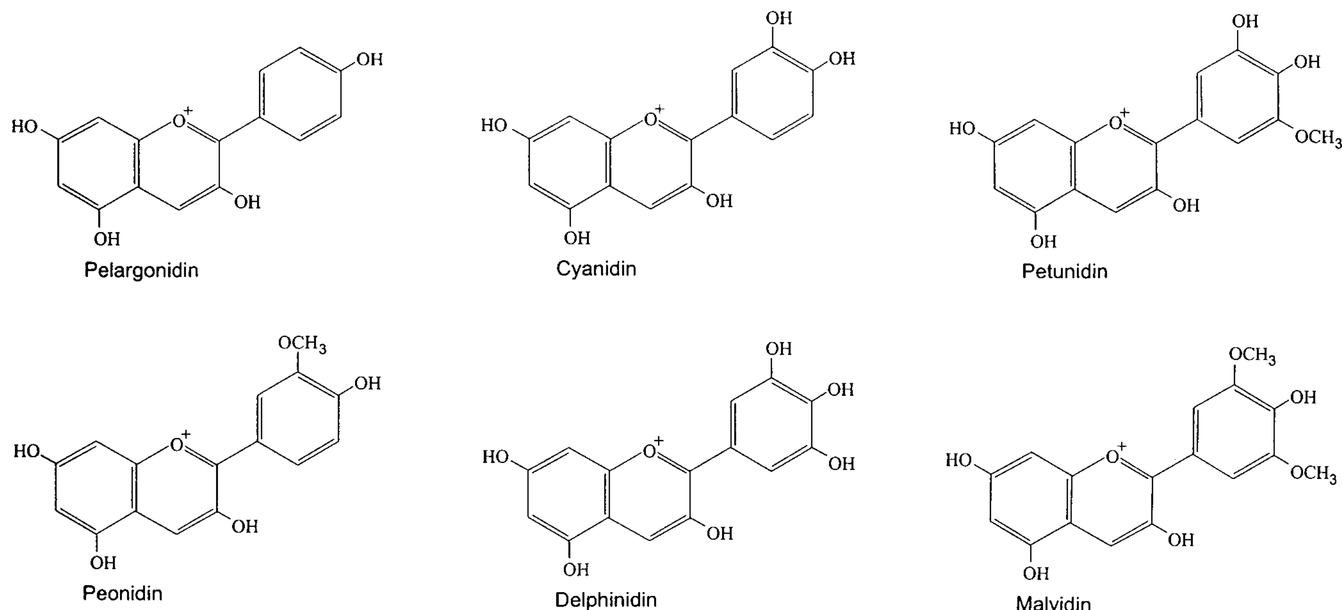

Figure 1. Six anthocyanidins found in fruits and berries.

Samples. Bilberry (*Vaccinium myrtillus*), black currant (*Ribes nigrum*), and strawberry (*Fragaria ananassa* cv. Jonsok) samples were collected in Finland. The red wine was Gato Negro Cabernet sauvignon (*Vitis vinifera*), vintage 2000, from Lontué Valley, Chile. An in-house reference material was also used in method development. It was prepared using 0.25 kg of fresh orange plus 0.5 kg of lemon plus 0.25 kg of cranberry plus 0.5 kg of black currant plus 0.5 kg of spinach plus 0.38 L of red wine plus 0.38 L of infused green tea. Tea was prepared according to the label instructions (3.8 g of tea was steeped at 90 °C in 380 mL of water; after 3 min, the tea leaves were removed). All samples, except wine, were homogenized, lyophilized, and stored at −18 °C.

Hydrolysis. Hyrolysis conditions were optimized using bilberry and red wine samples. The conditions of maximum aglycon yield were used to obtain the results for other samples and recovery tests. A dried sample of 0.5 g was weighed into a 70 mL screw-cap test tube. Depending on the anthocyanin concentration of the sample, 18−60 mL of 2 M HCl in methanol was added. The test tubes were sealed tightly, and hydrolysis was done at 90 °C in a water bath for 50 min. For red wine a combination of two hydrolyses gave best results: 5 mL of sample was pipetted into the test tube; 5 mL of methanol was added along with 3 or 5 mL of 12 M HCl. This yielded 2 and 4 M acid concentrations for hydrolysis, which was carried out under the same conditions as for dry samples. Depending on the volume after hydrolysis, the cooled samples were decanted into 25−100 mL flasks and brought up to the final volume with methanol. All samples were filtered into autosampler vials through a 0.2 μm PTFE filter.

Quantification. HPLC analysis was performed on a Hewlett-Packard 1090M series II high-performance liquid chromatograph equipped with a Hewlett-Packard 1090M series II diode array detector. The HPLC pumps, autosampler, column oven, and diode array system were controlled by computer using a Hewlett-Packard 3D Chem Station program. The analytical column employed was a C-18 Inertsil ODS-3 (4.0×150 mm, particle size = 3 μm) column manufactured by GL Sciences, Inc. A C-18 guard column was installed before the analytical column. The temperature of the column oven was 35 °C. Gradient elution was performed using 10% formic acid (solution A) and 100% acetonitrile (solution B) as follows: linear gradient from 96% A/4% B to 85% A/15% B, 0−8 min; isocratic elution 85% A/15% B, 8−23 min; linear gradient from 85% A/15% B to 20% A/80% B, 23−24 min; isocratic elution 20% A/80% B, 24−27 min; linear gradient from 20% A/80% B to 96% A/4% B, 27−28 min; post-time 6 min before next injection. Flow rate was 0.8 mL/min. The injection volume was 10 μL for all samples and standard solutions. The wavelengths used for quantification were 530 nm for delphinidin, cyanidin, petunidin, peonidin, and malvidin and 510 nm for pelargonidin. For calibration the external standard method was used, and calibration curves were plotted for each standard compound on the basis of peak area. Petunidin was quantified using the calibration curve of cyanidin.

Method Validation. The identities of the sample peaks were confirmed by comparison to the retention time and UV spectra of the standard compounds. The purity of the peaks was also monitored using the diode array purity test system included in the software. To find out if the concentration of hydrochloric acid has a significant effect on peak spectrum, standard samples of 0.1 and 2.4 M HCl in methanol were prepared. The area, height, spectrum, and apex wavelength of the peaks were compared. Linearity and detection and quantification limits were tested using standards in 1.2 M HCl in methanol. Detection limits were defined as 3 times the height of the baseline noise acquired using the Chem Station software. Quantification limits were defined as 10 times this value. Recovery tests were made by spiking samples with glycoside forms of cyanidin, pelargonidin, peonidin, and malvidin before hydrolysis. The concentration of the fortification was obtained by calculating the concentration of the corresponding aglycon after complete hydrolysis of the glycoside. The concentration of fortification ranged between 50 and 500% of the original aglycon content of the sample. Recovery of the aglycons from blank samples was tested by subjecting delphinidin, cyanidin, pelargonidin, and peonidin standards to 2 and 4 M HCl hydrolyses. The repeatability of the method was studied by making identical hydrolysates of the in-house reference material and the bilberry sample and following the total aglycon content over 14 days.

RESULTS AND DISCUSSION

With regard to sample treatment before actual analysis, investigators must choose whether to analyze the intact anthocyanins in the sample or to release the anthocyanidin body from the sugar moiety. Due to the attached sugars and acids often acylating these sugars, the number of anthocyanins is 15−20 times greater than the number of aglycon forms (*1*). This results in a large number of peaks on the chromatogram and difficulties in identifying individual anthocyanins. To establish the definitive anthocyanidin composition and concentration in a sample, the most reliable method is to reveal the

Table 1. Anthocyanidin Content of Tested Samples and Results from Previous Studies

	present study					other studies	
sample	aglycon	recovery, %	relative content, %	aglycon content, mg/100 g of fw[b]	glucoside content, mg/100 g of fw[b]	relative content, %	glucoside content, mg/100 g of fw[b]
strawberry $n = 7$	cyanidin	96	8	2.1 ± 0.1	3.2	5 (*27*); 11 (*14*); 25 (*19*)	
	pelargonidin	79	92	21.7 ± 0.4	34.6	75 (*19*); 89 (*14*); 95 (*27*)	
	total			23.8 ± 0.4	37.8 ± 0.8		13 (*14*); 7–30 (*28*)
black currant $n = 4$	delphinidin		43	59 ± 2	87	44 (*31*); 47 (*20*); 49 (*30*); 53 (*29*); 55 (*14*)	
	cyanidin		57	76 ± 3	118	44 (*29*); 45 (*14*); 51 (*30*); 53 (*20*, *31*)	
	total			135 ± 3	205 ± 7		157 (*14*); 250 (*30*)
bilberry $n = 14$	delphinidin		35	126 ± 2	205	43 (*15*)	
	cyanidin		36	125 ± 2	215	31 (*15*)	
	petunidin		14	52 ± 1	81	14 (*15*)	
	peonidin		5	18.1 ± 0.4	30	7 (*15*)	
	malvidin		10	39 ± 1	58	5 (*15*)	
	total			360 ± 3	588 ± 8		370 (*15*); 648–698 (*32*)
reference[c] $n = 14$	delphinidin		43	193 ± 6			
	cyanidin	85	49	216 ± 6			
	petunidin		1	6.4 ± 0.6			
	peonidin	92	5	23 ± 1			
	malvidin	59	2	9 ± 1			
	total			447 ± 8			
red wine[d] $n = 2$	delphinidin		20	5.0 ± 0.1	8.2	10 (*33*)[e]	3 (*33*)[e]
	cyanidin	99	19	4.5 ± 0.1	7.8	0 (*33*)[e]	0.1 (*33*)[e]
	petunidin		6	1.49 ± 0.01	2.32	8 (*33*)[e]	2 (*33*)[e]
	peonidin	103	3	0.70 ± 0.01	1.15	4 (*33*)[e]	0.9 (*33*)[e]
	malvidin	67	52	14.4 ± 0.1	21.4	78 (*33*)[e]	9.7 (*35*); 20 (*33*)[e]
	total			26.1 ± 0.1	40.8 ± 0.4		10.7–62.0 (*34*); 26 (*33*)[e]; 31.9 (*36*)

[a] Average content ± standard deviation. [b] fw, fresh weight. [c] Results obtained in this study are given as dry weight. [d] Results obtained in this study are given as mg/100 mL ± standard error of mean. [e] Content at 48 days.

aglycon by acid hydrolysis. This reduces the number of peaks on the chromatogram to six. Hydrolysis with hydrochloric acid has become an accepted practice in the analysis of other flavonoids, too (*9*, *22*). In anthocyanidin analyses, the concentration of HCl in methanol ranges from 1 M (*11*) to 3 M (*13*) and analysis is carried out in a boiling water bath for between 20 (*13*) and 60 min (*11*). In the present study it was found that a 50 min hydrolysis in 2 M HCl at 90 °C was adequate for most samples and did not destroy the aglycons. For red wine with a high malvidin 3-glucoside content, hydrolysis in 4 M acid was needed to maximize the aglycon yield.

Because anthocyanins are unstable compounds, the availability of standards is poor and their price is high. For the determination of anthocyanins in the glycoside form, it is very difficult to obtain standards for every anthocyanin form present in the sample. Many authors have overcome this problem by choosing one reference glycoside for calibration. This is possible because the chromatographic responses of these compounds are rather similar at wavelengths around 530 nm (*14*, *16*, *17*, *23*). This reference compound may be extracted from a natural source (*11*, *17*, *18*). Anthocyanin contents have also been calculated using known molar extinction coefficients (*15*). In the present method the concentration and identity of the aglycons were assured by using commercial standards to quantify each anthocyanidin separately.

The structure of anthocyanins is pH dependent. Only at pH values below 2 is the red flavylium cation the dominant structure. When the pH is increased, the anthocyanin molecule first turns into a blue quinoidal base form and then to colorless carbinol pseudo-base and chalcone forms, thus complicating spectrometric detection (*1*, *24*). Attention must also be paid to the solvent system in which the sample is prepared. An aqueous buffer and acidified methanol both at the same pH result in different absorption characteristics (*25*). Therefore, the matrix of standard solutions should resemble that of samples as closely as possible. In this study the effect of acid concentration was inspected by comparing the spectrum and peak height and area of aglycons in 0.1 and 2.4 M HCl in methanol. The spectral properties were not affected by the difference in HCl concentrations. Therefore, standards in 1.2 M HCl in methanol were suitable for quantification of samples with an acid concentration between 1.2 and 1.5 M, as in this study. Successful spectrometric detection of anthocyanins and anthocyanidins requires a low pH in the mobile phase, too. As silica columns do not tolerate very low pH values, a common compromise is to use 5 (*15*, *26*) or 10% (*12*, *17*, *21*) formic acid in combination with methanol (*15*, *26*), acetonitrile (*12*, *21*), or both (*17*). Due to the wide polarity range of anthocyanidins, gradient elution is often chosen (*15*, *17*, *26*). The combination of 10% formic acid and acetonitrile chosen in the present study produced well-resolved chromatograms and did not deteriorate the column too rapidly.

Because the gradient program could be optimized for the six aglycons only, rapid elution and excellent resolution were achieved. Elution times were between 10 (delphinidin) and 20 (malvidin) min (Figure 2). The sample matrix did not have an effect on the chromatogram quality, and no unidentified peaks could be seen at the detection wavelengths. The chromatogram of a hydrolyzed bilberry sample is shown in Figure 3. The detector response was linear over the tested concentration ranges for all aglycons, and the coefficients of correlation were over 0.999. Detection limits were between 0.32 (pelargonidin) and 0.85 (malvidin) mg/L. Detection limits for anthocyanidin glycosides in the study by Versari et al. were between 1.7 and 3.2 mg/L. For example, for cyanidin 3-glycoside the detection limit was 1.9 mg/L, which corresponds to 1.2 mg/L of aglycon (*14*). Thus, an improvement in sensitivity was achieved

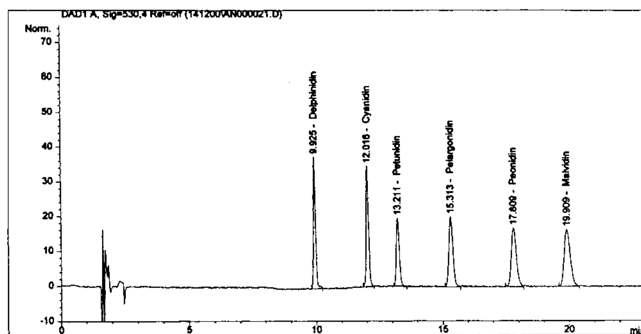

Figure 2. HPLC-DAD chromatogram of anthocyanidin standard mixture at 530 nm. Peaks (from left to right): delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin.

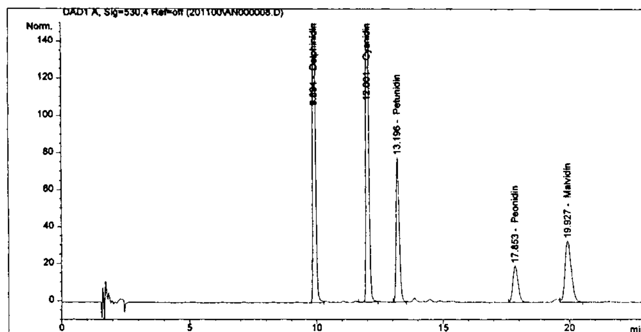

Figure 3. Chromatogram of a bilberry sample at 530 nm hydrolyzed with 2 M HCl in MeOH for 50 min at 90 °C. Peaks (from left to right): delphinidin, cyanidin, petunidin, peonidin, and malvidin.

by this method. Recovery tests were done in duplicate using in-house reference material, strawberry, and red wine samples. The recoveries of cyanidin, pelargonidin, and peonidin as glycosides hydrolyzed to aglycons were between 79 and 103% (Table 1). In a blank sample the recoveries were 95−104%. The recovery of malvidin 3-glucoside as malvidin was 59−67%, depending on the sample matrix. In a blank sample the recovery was 69%. The reason for the poor recovery of malvidin compared to the other antocyanidins is not known. It may be that it is more easily degraded than the others. A decrease in standard peak area was first observed in malvidin during this study. Also, the malvidin 3-glucoside standard used was not a certified standard material. The standard was roughly 6 months old at the time of use, and some degradation may already have occurred. Hence, it may be that the original concentration of the glucoside was not as high as expected in the recovery tests. The recovery of the aglycons subjected to 2 and 4 M HCl hydrolyses was 95−102% (malvidin not tested). It can be stated that the aglycons resisted the hydrolysis conditions well. No record of recovery tests was found in papers previously published on quantitative anthocyanin or anthocyanidin analyses. The repeatability of the method was tested over 14 days by following the total anthocyanidin content of the in-house reference material and bilberry samples. The coefficients of variation of identical hydrolysates were 1.41% for the bilberry sample and 2.56% for the reference material. The repeatability of the method presented by Versari et al. was inspected by studying the peak area of individual blueberry anthocyanin peaks over a period of 10 injections. The coefficient of variation was 1.9−4.9% (*14*).

The anthocyanidin contents of the tested samples are given in Table 1. The contents were not corrected for recovery. To enable comparison with earlier studies, the aglycon content of strawberry and black currant was converted to cyanidin 3-glucoside content and the aglycon content of bilberry and red wine to malvidin 3-glucoside content. Results from earlier studies are given in Table 1. The anthocyanidin composition and content can vary considerably depending on climatic factors, fruit ripeness, and storage time (*24*). The results obtained in this study are in agreement with previous research, especially when the possibility for great variation due to the reasons indicated above is taken into consideration.

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