Rapid Method for Complete Chemical Characterization of Simple and Acylated Anthocyanins by High-Performance Liquid Chromatography and Capillary Gas-Liquid Chromatography†

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A rapid method for complete chemical characterization of anthocyanins has been developed. The method uses a single acid hydrolysis of anthocyanins in methanol-2 N HCl to effectively cleave the intact pigments into stable aglycons, sugars, and acyl groups. The anthocyanidins and phenolic acids are characterized by reversed-phase high-performance liquid chromatography (RP-HPLC) in a single analytical run using retention times and UV-vis absorption spectra. The aliphatic acylating acids and the sugars are characterized by capillary gas-liquid chromatography (GLC) analysis of trimethylsilyl derivatives of sugars and aliphatic acids and methyl esters of aliphatic acids. This procedure, which is fast and reliable, requires minimal sample size and should be useful in the characterization of anthocyanins.

INTRODUCTION

With the current trend away from synthetic colors, the use of anthocyanins in foods, pharmaceuticals, and cosmetics is expected to increase (Francis, 1989; Mazza and Miniati, 1993). Most anthocyanins, however, do not have the same color stability under processing conditions as their synthetic counterparts, and as a result, they have limited applications. In recent years it has been found that anthocyanins containing two or more aromatic acyl groups are stable in weakly acidic and neutral aqueous solutions (Saito et al., 1985; Mazza and Miniati, 1993). In these pigments, color stability appears to increase with increasing content of organic acid (cinnamic and malonic acids) and also increased substitution of the aglycons.

In the search for stable, safe, and economical anthocyanin pigments, suitable as natural food colors, the availability of a rapid and reliable method of analysis would be most desirable. Available analytical methods for anthocyanins have been reviewed by Francis (1982), Jackman et al. (1987), Gross (1987), and Strack and Wray (1989).

Because of the instability of cinnamic acids during aqueous acid hydrolysis (Harborne, 1967), the phenolic acylation groups have normally been characterized after a separate alkali hydrolysis and separation of the acids from the alkaline hydrolysate (Harborne, 1967; Francis, 1982; Markham, 1982). These identification techniques for anthocyanidins and phenolic acids are lengthy and require a relatively large sample size of pure anthocyanin. Similarly, the detection and identification of aliphatic acylation in anthocyanin molecules is difficult by the traditional methods (Anderson et al., 1970; Bloom and Geissman, 1973; Fong et al., 1974; Wulf and Nagel, 1978; Krause and Strack, 1979; Goto et al., 1983).

Electrophoresis in pH 4.4 buffer has been used to detect dicarboxylic acylation (Harborne, 1986; Harborne and Boardley, 1985; Davies and Mazza, 1992). However, this technique, which is based on the zwitterion status of anthocyanin molecules, cannot distinguish different dicarboxylic acids. High-performance liquid chromatography has proven to be a useful tool for the characterization of anthocyanins (Strack et al., 1980; Vande Casteele et al., 1983; Mazza, 1986; Hong and Wrolstad, 1990a,b). However, identification of anthocyanins based solely on retention time and spectral characteristics of intact anthocyanins from HPLC requires authentic standard samples which are often difficult to obtain. Likewise, nuclear magnetic resonance (NMR) spectroscopy and fast atom bombardment mass spectrometry (FAB-MS), which have been found particularly useful in the characterization of acylated anthocyanins carrying malonyl substitution (Bridle et al., 1984; Takeda et al., 1986), are very expensive techniques and not readily available.

The purpose of this study was to develop a procedure for the preparation of an anthocyanin hydrolysate suitable for characterization of the structural units of anthocyanin molecules and to develop the analytical conditions for the complete chemical characterization of simple and acylated anthocyanins by HPLC and GLC.

MATERIALS AND METHODS

Chemicals. Phenolic acid standards were from commercial sources: Sinapic acid was from Calbiochem (Los Angeles, CA); syringic, vanillic, *p*-hydroxybenzoic, protocatechuic, and caffeic acids were from Sigma Chemical Co. (St. Louis, MO); ferulic acid was from Eastman Organic Chemicals (Rochester, NY); gallic acid was from Fisher Scientific Co. (Fair Lawn, NJ); and *p*-hydroxycinnamic acid was from J. T. Baker Chemical Co. (Phillipsburg, NJ).

Aliphatic acids were from the following sources: oxalic and succinic acids were from J. T. Baker; malonic acid was from Aldrich Chemical Co. (Milwaukee, WI); *l*-malic acid was from Metheson Coleman & Bell (Norwood, OH); acetic acid in the form of sodium acetate and glacial acetic acid (environmental grade) were from Anachemia Science (Montréal, PQ).

Anthocyanidin standards were from two sources: authentic compounds and fruit pigments of known composition. Pelargonidin chloride, ideain (cyanidin 3-galactoside) chloride, and malvidin chloride were from Sarsynthèse (Merignac, France). Peonidin, delphinidin, and petunidin as well as cyanidin and malvidin were extracted from lowbush blueberries (*Vaccinium angustifolium* Ait.), which are known to contain the 3-glucosides, 3-galactosides, and 3-arabinosides of these anthocyanidins (Francis et al., 1966; Mazza and Miniati, 1993).

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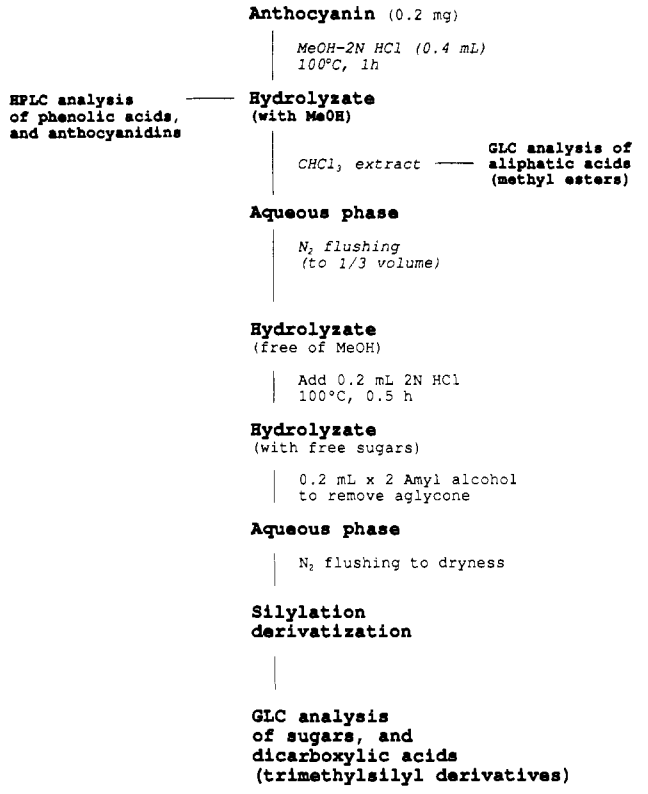

Figure 1. Schematic diagram for complete characterization of simple and acylated anthocyanins by HPLC and GLC.

The silylation reagent kit for analysis of sugars and dicarboxylic acids was from Pierce Chemical Co. (Rockford, IL).

Methanol and chloroform, which were free of ethanol, were of GLC grade, from Anachemia Science. All other chemicals used were of reagent or higher grade.

Preparation of Anthocyanin Hydrolysate for HPLC and GLC Analyses. A schematic diagram illustrating the preparation of samples for complete characterization of anthocyanins by HPLC and GLC is presented in Figure 1. Anthocyanin (ca. 0.2 mg) in dry form was solubilized in a minimal amount (0.4 mL) of methanol. An aliquot of the anthocyanin solution (10 μL) was transferred to 1 mL of 0.01% methanolic HCl (or other desired solvent) for recording the spectral characteristics (Markham, 1982) and for estimation of anthocyanin content in the methanolic solution. A calculated volume of the solution (containing ca. 0.2 mg of anthocyanin) was transferred into a 4-mL threaded vial, and the volume of the methanolic solution was adjusted to ca. 0.2 mL by adding methanol or removing excessive methanol by flushing the solution with a stream of nitrogen. To the same vial was added 0.2 mL of 2 N HCl, and the resulting anthocyanin solution was briefly flushed with nitrogen and immediately sealed with a Teflon-lined screw cap. The vial with the anthocyanin solution was then heated in a boiling water bath for up to 60 min (Markham, 1982). The hydrolysate was immediately cooled in ice water.

For HPLC analysis of phenolic acids and anthocyanidins, an aliquot (e.g., 30 μL) of the hydrolysate was taken and injected into the HPLC analytical column.

High-Performance Liquid Chromatography of Phenolic Acids and Anthocyanidins. The HPLC analysis was carried out on a reversed-phase C_{18} column, SuperPac Pep-S (5 μm, 4 × 250 mm), preceded by a guard cartridge (4 × 10 mm), both from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). The HPLC equipment consisted of a liquid chromatograph system (LKB Produkter, Bromme, Sweden) equipped with an LKB Model 2140 photodiode array (spectral) detector (detection wavelength from 190 through 370 nm), and a Pharmacia Model 2141 variable-wavelength detector for simultaneous detection at any two wavelengths from 190 to 600 nm.

A gradient solvent system was used for the separation of phenolic acids and anthocyanidins in a single HPLC run. Solvent A was formic acid–water (1:99 v/v), and solvent B was methanol. The elution profile was linear and as follows: 0–10 min, 17–22% B; 10–12 min, 22–27% B; 12–20 min, 27–31% B; 20–30 min, 31% B; 30–40 min, 31–38% B; 40–44 min, 38–47% B; 44–46 min, 47–60% B; 46–54 min, 60–65% B; 54–55 min, 65–17% B. The solvent flow rate was 0.9 mL/min and column pressure 30–34 bar. All separations were performed at 22 ± 1 °C, and all solvents were of HPLC grade filtered through a 0.45-μm Durapore filter (Millipore, Bedford, MA) before use.

Gas–Liquid Chromatography Analysis of Aliphatic Acylating Acids and Sugars. GLC analysis was carried out using a Varian Model 3400 gas chromatograph (Varian Associates, Palo Alto, CA) equipped with a flame ionization detector (FID) interfaced with a data system for chromatography data acquisition, processing, and quantitation. A 30 m × 0.25 mm i.d. fused silica capillary column packed with 1 μm J&W DB-1701 (J&W Scientific, Folsom, CA) was used. Two analysis programs were used. One program was for the detection of acetic and dicarboxylic acylation, and the other was for the detection of sugars and dicarboxylic acids. The same capillary column was used for both analyses.

The GLC program for acetic and dicarboxylic acids detection (HAc program) was as follows: column temperature at 35 °C for 2 min, followed by linear increase to 200 °C at 20 °C/min. Injector and detector temperatures were 250 °C. Helium was used as carrier gas (40 cm/s) with a split ratio of 25.

The GLC program for trimethylsilyl derivatives of sugars and dicarboxylic acids (sugar program) was as follows: column temperature starting from 120 °C (no hold time) and increased to 180 °C at a linear rate of 20 °C/min, followed by increase at 5 °C/min to 200 °C. All other conditions were the same as for the HAc program.

Preparation of Sample for GLC Analysis of Methyl Esters of Aliphatic Acids. The anthocyanin hydrolysate remaining after the portion for HPLC analysis had been taken was extracted with 100 μL of chloroform by vigorously shaking the mixture. After phase separation, an aliquot (30 μL) of the chloroform phase (bottom) was taken with an Eppendorf pipet or a Hamilton syringe and transferred to a 4-mL amber vial which contained preweighed anhydrous Na_2SO_4 (ca. 30 mg). The mixture was shaken a few times, and an aliquot (0.1–0.5 μL) of the chloroform extract was injected into the GLC capillary column and analyzed using the HAc program. This program permitted detection of acetic acid as both methyl acetate (major peak) and free acetic acid (minor peak) and detection of the dicarboxylic acids as dimethyl esters.

Preparation of Sample for Identification of Sugars and Dicarboxylic Acids by Silylation Derivatization. After the chloroform extract was taken for the analysis of methyl esters of aliphatic acids, the remaining anthocyanin hydrolysate was used for further analysis of sugars and confirmation of the dicarboxylic acids. For this purpose, the hydrolysate was flushed with a stream of nitrogen (to remove methanol) until less than one-third of the original volume was left in the vial. An additional 200 μL of 2 N HCl was then added to the vial, and the solution was again briefly flushed with nitrogen and sealed and heated at 100 °C for at least 30 min. After the hydrolysate was cooled to room temperature in cold water, it was extracted with amyl alcohol (0.2 mL × 2) to remove the aglycon. The aqueous (bottom) phase of the hydrolysate was then evaporated to dryness by a stream of nitrogen or by vacuum. The residual material contains the sugar(s) and the acylating dicarboxylic acid(s). The procedure of Sweeley et al. (1963) was used for silylation derivatization, with minor modification in that the volume of pyridine for sugar dissolution was reduced to 50 μL and the volumes of trimethylchlorosilane (TMS) and hexamethyldisilizane (HMDS) were reduced to 25 μL, respectively. Half a microliter of the sample was injected into the GLC and analyzed using the "sugar program" described previously. The chromatogram obtained was compared to that of standards for identification of sugar(s) and dicarboxylic acid(s).

Characterization of Anthocyanins from Red Grape Skins. Identification of anthocyanidins, sugars, and acylating phenolic and acetic acids of two anthocyanins from Cabernet Sauvignon grapes, which are known to contain acetylated and phenolic acid acylated anthocyanins (Wulf and Nagel, 1978), was carried out to demonstrate the application and effectiveness of the developed

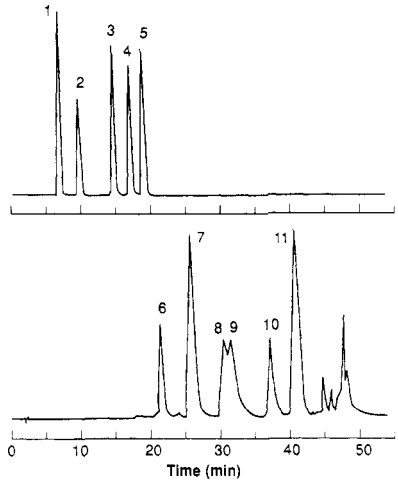

Figure 2. HPLC separation of the phenolic acids (top, 280 nm) and anthocyanidin standards (bottom, 525 nm). Peaks: 1, *p*-hydroxybenzoic acid; 2, caffeic acid; 3, *p*-coumaric acid; 4, ferulic acid; 5, sinapic acid; 6, delphinidin; 7, cyanidin; 8, petunidin; 9, pelargonidin; 10, peonidin; 11, malvidin. Retention times and UV spectra are given in Table I.

method for anthocyanin characterization. For this purpose, the grape pigments were extracted at room temperature with methanol–formic acid–water (10:0.2:9.8, MFW), and two of the over 20 anthocyanin peaks were accumulated by collecting the eluates from the HPLC column. The following elution profile was used: solvent A, 5% formic acid; solvent B, methanol; 0–17 min, 22% B; 10–54 min, 22–42% B (linear); 54–55 min, 42–75% B; 55–59 min, 75% B; 59–60 min, 75–22% B. All other conditions were the same as described under High-Performance Liquid Chromatography of Phenolic Acids and Anthocyanidins. The Cabernet Sauvignon grape skin material was donated by T. Fuleki, Horticultural Research Institute of Ontario, Vineland, ON.

Characterization of Anthocyanins from *Monarda* Flower Petals. Since the flower petals of Marshall's Delight *Monarda* contain anthocyanins acylated by both dicarboxylic and phenolic acids (Davies and Mazza, 1992), they were selected to demonstrate the application of the developed method for characterization of a diacylated anthocyanin. The procedure for the extraction and separation of one of the major anthocyanins of *Monarda* was as described previously (Davies and Mazza, 1992).

RESULTS AND DISCUSSION

HPLC Separation of Acylating Phenolic Acids and Anthocyanidins. Figure 2 shows the HPLC chromatograms of the most commonly occurring acylating phenolic acids and anthocyanidins. The six anthocyanidins were separated on a reversed-phase column in the order of delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin, which is consistent with the elution sequence reported in the literature (Akavia and Strack, 1980; Hebrero et al., 1988). In the present study, however, the elution system was designed in such a way as to permit the anthocyanidins to be resolved on the later half of the chromatogram, allowing the phenolic acids to be eluted before any anthocyanidin and thus making it possible to simultaneously identify two components of anthocyanins.

In the profile for anthocyanidin standards (Figure 2, bottom), there are a few peaks which were eluted later than malvidin, the last eluting anthocyanidin. These peaks are believed to be decomposition products of anthocyanidin, as these peaks became larger and those of anthocyanidins smaller with time after hydrolysis. For the purpose of this study, it suffices to note that those peaks do not interfere with the identification of the anthocyanidins. The phenolic acids were clearly resolved in the first 20 min of the run and were well separated from the anthocyanidins. The actual and relative retention times of nine phenolic acids and six anthocyanidins are given in Table I.

Table I. Retention Times (t_R) and UV-Vis Maxima (λ_{max}) of the Most Commonly Occurring Acylating Phenolic Acids and Six Anthocyanidins in Plant Material[a]

phenolic acid/ anthocyanidin	t_R (min)	relative t_R ($t_{R\ acid}/t_{R\ p\text{-coumaric}}$)	λ_{max} (nm)
gallic acid	4.2	0.26	268
protocatechuic	5.6	0.36	255, 290
p-hydroxybenzoic	8.0	0.51	251
vanillic	10.0	0.63	256, 288
caffeic	10.7	0.68	232, 290 sh, 320
syringic	12.4	0.79	272
p-coumaric	15.7	1.00	291 sh, 305
ferulic	18.0	1.15	234, 294 sh, 320
sinapic	19.9	1.27	230, 320
delphinidin	22.5	1.43	240, 280
cyanidin	26.7	1.70	240 sh, 270
petunidin	31.5	2.01	242, 278
pelargonidin	32.5	2.07	264, 318
peonidin	38.2	2.43	248 sh, 270
malvidin	41.7	2.65	246 sh, 270

[a] The dead volume of the column (t_0) was 3.10 min. For chromatographic conditions, see Materials and Methods.

Stability of Phenolic Acids to Acid Hydrolysis Media. It is known that cinnamic acids are not stable in a hot acid medium (Harborne, 1967). Therefore, the acyl portion of anthocyanins has been traditionally determined by subjecting a separate anthocyanin sample to a mild alkali hydrolysis (Harborne, 1967; Markham, 1982; Francis, 1982), which, however, cannot yield anthocyanidins for identification as these are unstable in alkali media (Francis, 1982). Consequently, two separate hydrolysis procedures requiring longer time for analysis and a larger sample size of pure anthocyanins have traditionally been used. This is despite the fact that HPLC analysis requires little sample for detection of anthocyanins. Therefore, the key to a successful simultaneous identification of anthocyanidin and acylating phenolic acids is the conservation of phenolic acids during acid hydrolysis.

A series of acid hydrolysis media was tried in an attempt to find a system for maximum phenolic acid retention during hydrolysis. A mixture of methanol and 2 N HCl (1:1 v/v) was found to be the most suitable as it gave the highest recovery of the phenolic acids after hydrolysis. Other organic solvents, including ethanol, *tert*-butyl alcohol, and 2-propanol, were much less satisfactory, while hydrolysis in aqueous HCl (2 N) completely destroyed the cinnamic acids. The results of the hydrolysis conditions using the methanol–HCl and the aqueous HCl systems are presented in Figure 3 for five phenolic acids. Hydrolysis in the methanol–HCl system for 1 h at 100 °C yielded the original acids and several derivatives, which were identified as the methyl esters of phenolic acids. The concentration of the derivatives, on the basis of the peak areas at 280 nm, ranged from 35% to 70% of the original acids. Thus, the concentration of phenolic acids after hydrolysis was 30–65% of the original concentration. Hydrolysis for 1.5 h produced the same HPLC profile.

Because of the high reproducibility of the retention times and UV spectra, the derivative peaks may be used as supporting evidence for the presence of the original acids. The retention times of the methyl esters of the acids were 18.7, 22.3, 31.6, 35.6, and 38.2 min for the five phenolic acids, respectively, in the HPLC program used for the identification of phenolic acids and anthocyanidins. Analysis of methyl esters of phenolic acids has previously been recommended as a procedure for determination of phenolic acids in plants (Horvat and Senter, 1980).

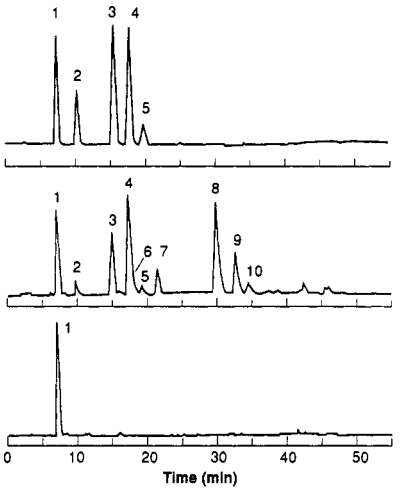

Figure 3. HPLC chromatograms of phenolic acids hydrolyzed under two conditions and recorded at 280 nm. (Top) Phenolic acids before acid hydrolysis; (middle) phenolic acids hydrolyzed at 100 °C for 1 h in MeOH–2 N HCl (1:1 v/v); (bottom) phenolic acids hydrolyzed at 100 °C for 1 h in aqueous 2 N HCl. Phenolic acid and its methyl ester: peaks 1 and 6, *p*-hydroxybenzoic acid; peaks 2 and 7, caffeic acid; peaks 3 and 8, *p*-coumaric acid; peaks 4 and 9, ferulic acid; peaks 5 and 10, sinapic acid.

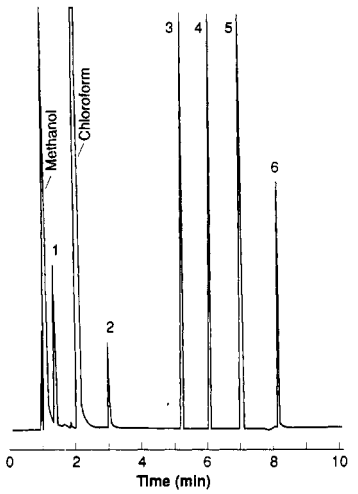

Figure 4. GLC chromatogram of aliphatic acids in the form of methyl esters. Separation conditions are described under Materials and Methods. Peaks: 1, acetic acid (methyl ester, t_R = 1.364 min); 2, acetic acid (in free form, 2.991 min); 3, oxalic acid (5.238 min); 4, malonic acid (6.113 min); 5, succinic acid (7.017 min); 6, malic acid (8.213 min).

A smaller proportion of methanol in the methanol–HCl system did not seem to reduce the concentration of the derivatives formed during hydrolysis. Significant disappearance of cinnamic acids (as judged by the darkening and HPLC analysis of the hydrolysate) was noted when the methanol proportion was less than 1:2 in the methanol–HCl system.

Gas–Liquid Chromatography (GLC) Detection of Aliphatic Acyl Groups in the Form of Methyl Esters. The GLC analysis conditions described offered a high resolution for the analysis of aliphatic acid acylation of anthocyanins. Of the four organic solvents tested (ethyl ether, carbon tetrachloride, chloroform, and dichloromethane), chloroform was found to be the most satisfactory solvent, as it possessed an adequate extracting capability and relatively safer handling characteristics. In Figure 4 is presented a GLC chromatogram of the chloroform extract of acid hydrolysate of the most commonly occurring dicarboxylic acids and acetic acid obtained by the described hydrolysis procedure. Chloroform has been used in the analysis of dicarboxylic acids in a different derivatization procedure (Alcock, 1965; Harmon and Doelle, 1969; Hautala and Weaver, 1969).

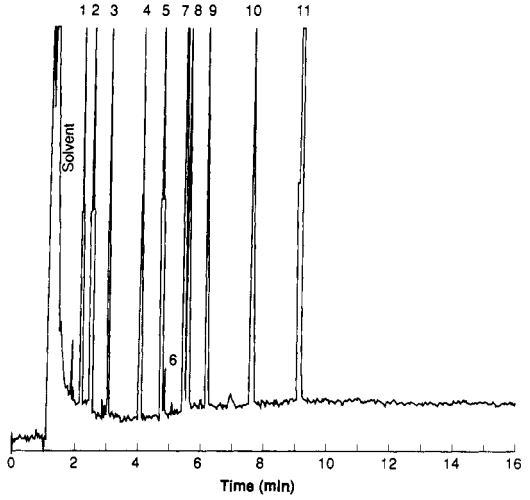

Figure 5. GLC chromatogram of dicarboxylic acids and sugars after silylation derivatization. Separation conditions are described under Materials and Methods. Peaks: 1, oxalic acid (t_R = 2.164 min); 2, malonic acid (2.470 min); 3, succinic acid (3.011 min); 4, malic acid (4.061 min); 5 and 7, rhamnose (4.716, 5.455 min); 6, 8, and 9, xylose (4.766, 5.581, 6.149 min); 10 and 11, glucose (7.587, 9.111 min).

The presence of acetic acid acylation is indicated by two peaks, methyl acetate and free acetic acid (peaks 1 and 2 in Figure 4), offering double evidence for the presence of acetic acid. It should be noted, however, a much smaller sample size than 0.2 mg of anthocyanin would produce a dilute methyl ester chloroform extract, which would require a relatively large injection volume into the GLC column, which in turn may cause the methyl acetate to appear as two peaks. However, a relatively large injection volume (e.g., 1 μL) was found to be necessary for the clear detection of the free acetic acid peak. This is because in the hydrolysis treatment the acetic acid released from acetylated anthocyanins is mostly converted into methyl acetate.

The identities of all the aliphatic acids in the form of methyl esters were confirmed by injection of authentic compounds. Also, the presence of acylating dicarboxylic acids was further confirmed by the trimethylsilylation derivatization procedure described in the following section.

Gas–Liquid Chromatography of Trimethylsilyl Derivatives of Sugars and Dicarboxylic Acids. GLC analysis of sugars after silylation (Sweeley et al., 1963; Markham, 1982) is one of the most reliable techniques for the detection of sugars and related polyhydroxy compounds.

Figure 5 presents a GLC chromatogram of trimethylsilyl derivatives of four standard dicarboxylic acids and three sugars. As can be noted, all standards were well separated by capillary GLC. For this to take place, however, a number of precautions must be taken. First, the dicarboxylic acids obtained from the anthocyanins by acid hydrolysis and converted to their methyl esters must be regenerated into their acid form so that silylation derivatization can be achieved. Otherwise, the methyl esters of the dicarboxylic acids will complicate the separation and identification of the acids in the form of trimethylsilyl derivatives by the sugar program. Moreover, the sugars obtained from the hydrolysate in MeOH–HCl are a mixture of free and methylated sugars (as confirmed by GC–MS, data not shown); direct use of silylation derivatization would produce at least two more peaks in the GLC

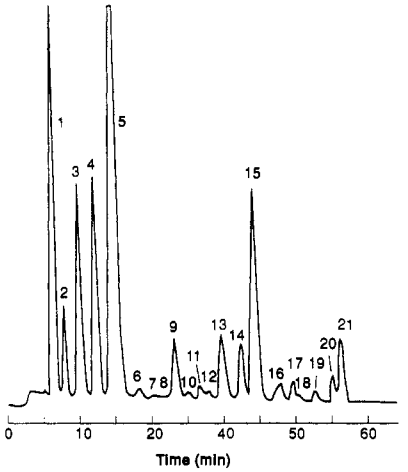

Figure 6. Typical HPLC chromatogram of a methanol–formic acid–water extract of Cabernet Sauvignon grape skin. Peaks 15 and 21 were collected from the HPLC column outlet and used to demonstrate the applicability of the method for anthocyanin characterization developed in the present study.

chromatogram for each sugar, which would complicate the identification of sugars, especially when more than one sugar exists in the anthocyanin molecule.

The regeneration of the methylated sugars and dicarboxylic acids was carried out by subjecting the hydrolysate of methanol–HCl to a hydrolysis treatment in aqueous HCl for 0.5 h at 100 °C under nitrogen. Before this treatment, methanol in the previous hydrolysate was removed by gently flushing the hydrolysate with nitrogen until more than two-thirds of the hydrolysate was removed.

After regeneration of the acids and sugars into their native forms and before silylation derivatization, the aglycons should be removed from the hydrolysate by extracting it with amyl alcohol. Trace amounts of aglycon left in the hydrolysate did not interfere with the above-described GLC analysis for sugars and dicarboxylic acids. Before hydrolysis in aqueous HCl, the sample was flushed with nitrogen to remove any oxygen since a nitrogen atmosphere would prevent excessive decomposition of the aglycons during hydrolysis, thus facilitating its removal by the amyl alcohol extraction.

Application of Acid Hydrolysis and HPLC–GLC Analysis for Identification of Acylation, Aglycons, and Sugars of Anthocyanins. Two acylated anthocyanins from Cabernet Sauvignon grape skins and one acylated anthocyanin from the flower petals of *Monarda fistulosa* L. were used to demonstrate the applicability of the method for complete chemical characterization of acylated anthocyanins.

Figure 6 shows the HPLC chromatogram of a methanol–formic acid–water extract of the grape skin material, which is very similar, in terms of the distribution of the anthocyanin peaks, to those reported by Wulf and Nagel (1978) and by Fuleki (1990), both of whom used the same cultivar in their studies. From the pattern of the peaks and their retention times, peak 15 corresponds to the acetylated malvidin 3-glucoside and peak 21 to malvidin 3-glucoside acylated with *p*-coumaric acid.

For the characterization of the grape anthocyanin represented by peak 21, 11 mL of solution with absorbance of 1 at maximum absorption wavelength was collected as a separate fraction from the HPLC column outlet, hydrolyzed in 0.4 mL of methanol–2 N HCl (1:1 v/v), and subjected to the HPLC/GLC analyses. The HPLC chromatograms for the phenolic acid and anthocyanidin fractions are shown in Figure 7. On the basis of the

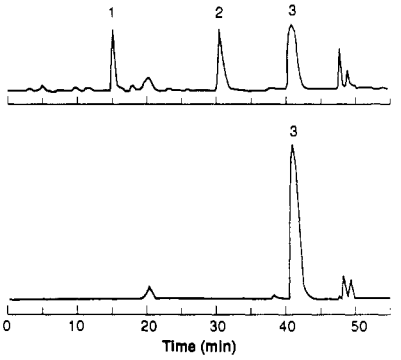

Figure 7. HPLC chromatograms for characterization of phenolic acid (top, recorded at 280 nm) and anthocyanidin (bottom, 525 nm), after MeOH–2 N HCl hydrolysis of peak 21 from the grape skin extract (see Figure 6). Peaks: 1, *p*-coumaric acid (t_R = 15.7 min); 2, methyl ester of *p*-coumaric acid (31.5 min); 3, malvidin (41.5 min).

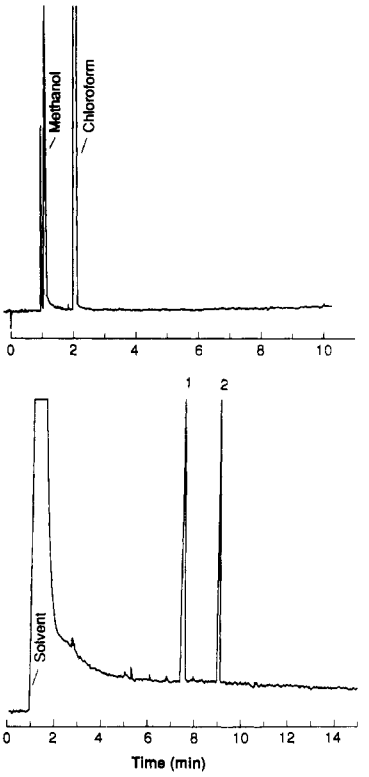

Figure 8. GLC chromatograms of the chloroform extract for aliphatic methyl esters (top) and the trimethylsilyl derivatives of the sugar fraction from peak 21 (in Figure 6) of the grape skin extract. Peaks: 1 and 2 (t_R = 7.580, 9.109 min, respectively), glucose, alpha and beta forms.

retention times and the UV spectra it can easily be seen (Table I) that the organic acid of this anthocyanin peak is *p*-coumaric acid and the aglycon is malvidin. The identity of the phenolic acid is further confirmed by the presence of its methyl ester peak and the UV spectrum (not shown).

The GLC chromatograms (Figure 8) further show that there are no aliphatic acids in the peak 21 anthocyanin; however, it is shown that by retention times glucose is the sugar moiety in this anthocyanin. These results, together with those from the HPLC analysis of the peak, demonstrate that the anthocyanin is a malvidin glucoside acylated by *p*-coumaric acid, which is in full agreement with the result of Wulf and Nagel (1978).

For anthocyanin represented by peak 15, 8 mL of anthocyanin solution with absorbance of 1 at 525 nm was

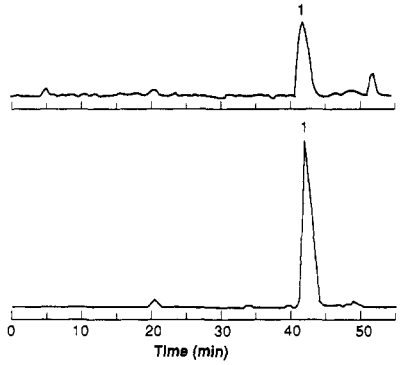

Figure 9. HPLC chromatogram for characterization of phenolic acid (top, recorded at 280 nm) and anthocyanidin (bottom, 525 nm), after MeOH–2 N HCl hydrolysis of peak 15 from the grape skin extract (see Figure 6). Peak: 1 (t_R = 42.0 min), malvidin.

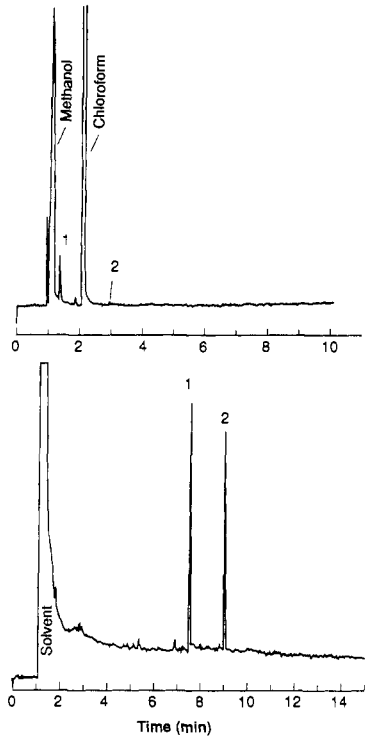

Figure 10. GLC chromatograms of the chloroform extract for aliphatic methyl esters (top) and the trimethylsilyl derivatives of sugar fraction from peak 15 (in Figure 6) of the grape skin extract. Peaks: 1 (t_R = 1.364 min), methyl acetate; 2 (2.990 min), free acetic acid. (Bottom) Peaks: 1 and 2 (t_R = 7.588, 9.109 min, respectively), glucose, alpha and beta forms.

used for the complete characterization. The HPLC chromatograms for the detection and characterization of the acyl group and the aglycon are given in Figure 9. On the basis of the retention time of the aglycon peak, it can easily be concluded that the anthocyanidin peak corresponds to that of malvidin. The UV spectra (data not shown) of the anthocyanidin confirmed this finding. Figure 10 (top) shows the GLC chromatogram of the aliphatic methyl esters extracted with chloroform from the acid hydrolysate. It clearly shows that there is a methyl acetate peak (peak 1 in Figure 10, top) and a small peak corresponding to free acetic acid (peak 2). The two large peaks in the lower chromatogram of Figure 10 correspond to the alpha and beta forms of glucose, respectively. These results demonstrate that the grape anthocyanin represented by peak 15 (Figure 6) is an acetylated malvidin glucoside, which is also consistent with the result of Wulf and Nagel (1978).

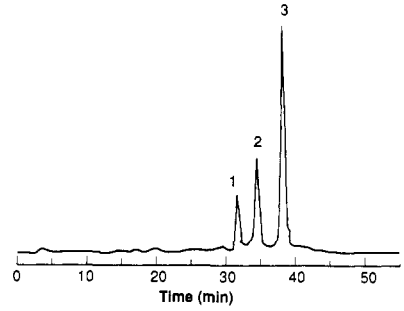

Figure 11. Typical HPLC chromatogram of a methanol–formic acid–water extract of *Monarda* flower petals. Peak 2 was collected from the HPLC column outlet and used to demonstrate the applicability of the method for anthocyanin characterization developed in the present study.

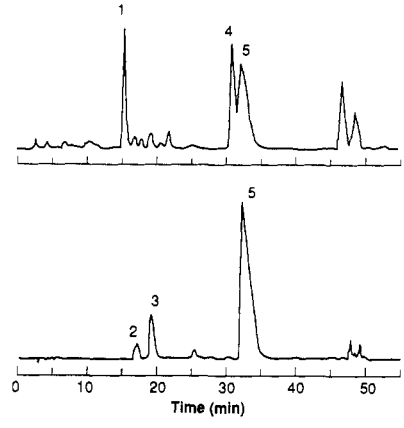

Figure 12. HPLC chromatograms for characterization of phenolic acid (top, recorded at 280 nm) and anthocyanidin (bottom, 525 nm), after MeOH–2 N HCl hydrolysis of peak 2 (in Figure 11) from *Monarda* flower petals. Peaks: 1 (t_R = 15.8 min), *p*-coumaric acid; 2 and 3 (17.1, 19.5 min, respectively), pelargonidin 3-glucoside and 5-glucoside, respectively; 4 (31.5 min), methyl ester of *p*-coumaric acid; 5 (32.5 min), pelargonidin.

Figure 11 shows a typical HPLC separation of the anthocyanins of *Monarda* flower petals extracted with methanol–formic acid–water (10:0.2:9.8). It is similar to that reported previously (Davies and Mazza, 1992). Peak 2 was collected and used to illustrate the applicability of the method for characterization of a diacylated anthocyanin.

The HPLC chromatograms (Figure 12) of the phenolic acid and anthocyanidin fractions of the second most abundant anthocyanin of *Monarda* show *p*-coumaric acid as the acylating acid and pelargonidin as the aglycon.

The GLC chromatogram by the HAc program (Figure 13, top) shows that malonic acid is also present and is detected in the form of the methyl ester of malonic acid (peak 1). In the analysis of samples after silylation, glucose was found as the only sugar, and the presence of malonic acid is confirmed by silylation derivatization (peak 1 in Figure 13, bottom). Therefore, it can be concluded that the anthocyanin from *Monarda* is a pelargonidin glucoside acylated by both malonic acid and *p*-coumaric acid. The characterization is in full agreement with previous results (Davies and Mazza, 1992).

Conclusions. A rapid procedure based on RP-HPLC and capillary GLC has been developed. The method uses a single acid hydrolysis step and is suitable for complete chemical characterization of anthocyanins. The best hydrolysis conditions were found to be in methanol–2 N HCl (1:1 v/v) under nitrogen. The anthocyanidins and phenolic acids can be identified by their retention times on HPLC and by spectral characteristics as recorded by

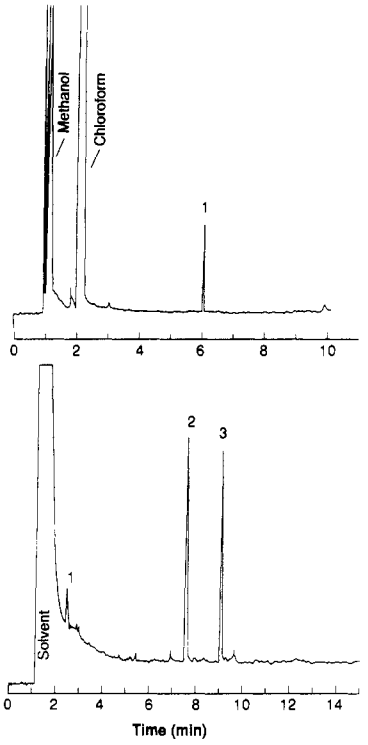

Figure 13. GLC chromatograms of the chloroform extract for aliphatic acid methyl ester(s) (top) and the trimethylsilyl derivatives of sugar fraction(s) from peak 2 (in Figure 11) of the anthocyanin from *Monarda* flower petals. (Top) Peak: 1 (t_R = 6.114 min), malonic acid. (Bottom) Peaks: 1 (2.472 min), malonic acid; 2 and 3 (7.585, 9.106 min, respectively), glucose, alpha and beta forms.

a spectral detector. Under the hydrolysis conditions used, 30–65% of the acylating phenolic acids can be retained as free acid and the remainder as methyl esters, which offer supporting evidence for phenolic acid identification. The acylating aliphatic acids and sugars can be identified from at least two pieces of evidence obtained by GLC analysis of their trimethylsilyl or methyl derivatives. By this procedure, 0.2 mg of anthocyanin is sufficient for the complete identification of acylating acids, anthocyanidin, and sugars of an anthocyanin. For characterization of the types of linkages among aglycons, sugars, and acids, stepwise hydrolysis of the anthocyanins will yield information on the structure of the pigments. The method reported in this paper is expected to be applicable using other RP-HPLC and capillary GLC columns under the same or similar analytical conditions and therefore should be useful and easily adaptable in other laboratoriers for the characterization of anthocyanins.

ACKNOWLEDGMENT

This work was supported in part by the Natural Sciences and Engineering Research Council of Canada in the form of an NSERC–Canadian Government Laboratory Visiting Fellowship to L.G.

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Received for review May 19, 1993. Revised manuscript received September 21, 1993. Accepted September 28, 1993.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts*, November 15, 1993.