Separation and Characterization of Anthocyanins of *Monarda fistulosa* by High-Performance Liquid Chromatography

A. J. Davies

Department of Food Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

G. Mazza*

Crop Utilization Laboratory, Agriculture Canada Research Station, Morden, Manitoba, Canada R0G 1J0

Anthocyanins and other flavonoids in the petals of *Monarda fistulosa* were separated on a reversed-phase column by high-performance liquid chromatography, equipped with photodiode array and variable-wavelength detectors. Detection at 280 and 510 nm revealed the presence of 16 discrete peaks including five anthocyanin pigments. The major anthocyanidin derivatives and the presence of acylation could be distinguished on the basis of retention times and UV–visible spectra. Auxiliary analytical techniques used for pigment characterization included acid hydrolysis, paper and thin-layer chromatography, and paper electrophoresis. With the combination of these techniques, peak assignments for the major anthocyanins and other flavonoids of *M. fistulosa* and their relative concentrations were confirmed. The total anthocyanin concentration was 214.8 mg/100 g of fresh petals. The main anthocyanin was pelargonidin 3,5-diglucoside acylated with coumaric and malonic acids, which accounted for 81% of the total anthocyanin content and 17% of the total flavonoid content. Other flavonoids included flavone and apigenin 7-*O*-glucosides, 5-hydroxyflavone, and a dihydroxyflavone 8-*C*-glucoside.

INTRODUCTION

Monarda fistulosa L. or bee balm is an erect perennial native to the Canadian prairies. Plants are very floriferous and produce white, purple, red, or pink blooms. While the anthocyanins and other phenolics of *M. fistulosa* L. flowers have not been characterized, the major anthocyanins of *Monarda didyma* have been reported as a polyacylated anthocyanin containing coumaric and malonic acids (Kondo et al., 1985). The acylated anthocyanins are of interest as it has been demonstrated that anthocyanins with acyl group substitution exhibit increased stability (Brouillard, 1981; Sapers et al., 1981; Teh and Francis, 1988). Due to this enhanced degree of stability of acylated anthocyanins, it is of interest to investigate the composition of the anthocyanins of *M. fistulosa* L., as it is hypothesized that the flavonoid composition of *M. fistulosa* will resemble that of *M. didyma*.

The objectives of the present study were twofold: 1, to isolate and characterize the anthocyanins and other flavonoids of *M. fistulosa* L. using a combination of spectrophotometry, high-performance liquid chromatography (HPLC), and column, paper, and thin-layer chromatography techniques; 2, to use the polyacylated anthocyanins of *M. fistulosa* L. in subsequent investigations to study the enhanced stability of these acylated anthocyanins and further elucidate the complexation mechanisms responsible for the enhanced stability of these compounds.

MATERIALS AND METHODS

The petals of *M. fistulosa* L. were collected in July 1990 from a plantation at Agriculture Canada Research Station, Morden, Manitoba, immediately frozen in liquid nitrogen, subsequently freeze-dried, and stored at −20 °C until extraction.

Isolation and Purification of Anthocyanins and Other Flavonoids. Freeze-dried petals (5 g, 20 g fresh weight equivalent) were extracted overnight in the dark with 400 mL of methanol–acetic acid–water (10:1:9 MAW). The extract was filtered under vacuum through a Whatman No. 2 filter paper and the residue washed with 300 mL of 10:1:9 MAW. The extract was concentrated to dryness, in a Büchi rotary evaporator at 30 °C. Since malonated pigments may decompose slowly in solution, they were routinely stored at 0 °C as solids, after evaporation to dryness. The dried pigment extract was redissolved in MAW (10:1:9) just prior to high-performance liquid chromatography and filtered (0.45-μm Millipore filter). This extract was denoted MAW crude extract [methanol–acetic acid–water (10:1:9) crude extract].

The concentrated crude extract, after dissolution in MAW, was passed through a Sephadex LH20 column (30 × 800 mm) and eluted with MAW (5:1:14). The separated bands were collected, concentrated to dryness, redissolved, and analyzed by HPLC. If further purification was necessary, the pigment fractions were applied to a second Sephadex LH20 column and eluted with MAW (5:1:14).

High-Performance Liquid Chromatography. The HPLC equipment used for separation and characterization of petal pigments consisted of an LKB liquid chromatograph system (LKB-Produkter, Bromme, Sweden) equipped with a Model 2156 solvent conditioner, two Model 2150 pumps, a Model 2152 controller, and a Rheodine 7125 injector valve with a 100-μL loop and an Ultropac prepared column (250 × 4.6 mm) of Spheri 10-RP18 (10 μm) (Brownlee Labs, Santa Clara, CA). A Pharmacia Model 2141 variable-wavelength detector and an LKB Model 2140 photodiode array detector were interfaced with an IBM personal computer, Nelson data acquisition interface (900 Series), and a Canon A-1210 color printer.

The following solvent system and elution profiles were used for the separation of flavonoids by HPLC: solvent A, formic acid–water (5:95 w/v); solvent B, methanol; elution profile 0–10 min, 17–22% B (linear gradient); 10–12 min, 22–27% B; 12–33 min, 27–37% B; 33–39 min, 37–55% B; 39–49 min, 55–59% B; 49–54 min, 59–65% B; 54–55 min, 65–71% B. The injection volume was 100 μL with a solvent flow rate of 1.0 mL/min, and the column pressure was 50–60 bar. All separations were performed at 22 ± 1 °C, and all solvents were of HPLC grade filtered through a 0.45-μm Millipore filter before use. The retention times, peak area, and area percent were calculated with an IBM personal computer equipped with Model 2600 chromatography software, revision 3.1 (Nelson Analytical, Inc., Cupertino, CA). The UV maxima were determined from the spectrum

* Author to whom correspondence should be addressed.

0021-8561/92/1440-1341$03.00/0 Published 1992 by the American Chemical Society

Table I. Retention Times (t_R), UV-Vis Maxima (λ_{max}), Capacity Factors (k'), and Relative Retention Times (α), Peak Areas, and Area Percents of *Monarda* Petal Pigments on a Spheri 10-RP18 Column Using Methanol–Formic Acid–Water as Eluent[a]

peak	t_R	λ_{max},[b] nm	k'	α	peak area (×10⁵) 280 nm	peak area (×10⁵) 510 nm	area % 280 nm	area % 510 nm
1	4.9	295, 320	0.1		0.7		1.6	
				1.7				
2	6.2	307	1.2		1.0		2.1	
				1.1				
3	6.6	240, 295, 322	1.3		2.4		5.0	
				1.0				
4	8.2	290	1.3		3.2		6.6	
				3.6				
5	16.4	306	4.7		0.9		1.8	
				1.4				
6	22.0	280	6.7		0.4		0.8	
				1.1				
7	24.0	262, 330	7.4		0.7		1.5	
				1.2				
8	29.0	235, 290 sh, 325	9.1		5.6		11.6	
				1.1				
9	30.8	279, 313, 513	9.7		0.3	0.3	0.8	2.8
				1.1				
10	32.4	264, 325	10.3		14.7		30.8	
				1.0				
11	34.0	273, 312, 511	10.8		0.9	1.6	1.9	14.9
				1.1				
12	38.1	280, 313, 511	12.3		2.1	0.1	4.3	0.9
				1.0				
13	39.3	280, 320, 511	12.7		8.0	8.6	16.7	81.4
				1.1				
14	41.7	280, 315, 511	13.5		3.5	0.1	7.2	0.9
				1.1				
15	45.3	265, 325	14.8		0.9		1.8	
				1.0				
16	46.4	265, 333	15.2		0.3		0.7	

[a] Flow rate, 1.0 mL/min; t_0 = 2.87. [b] sh, shoulder.

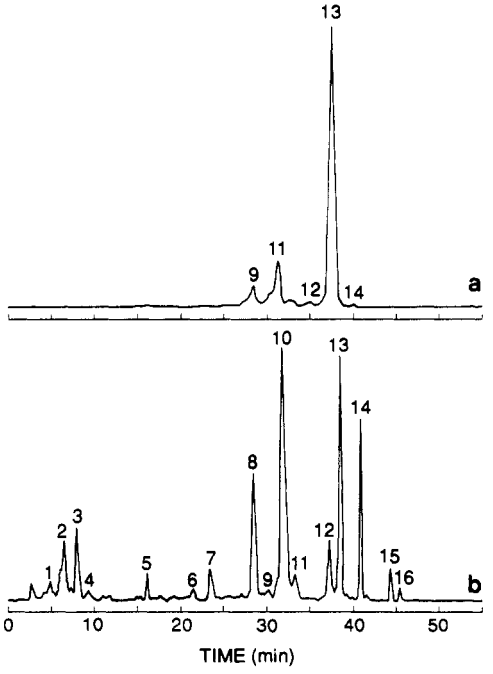

Figure 1. HPLC chromatograms of methanolic extract of *M. fistulosa* L. petals monitored at 510 (a) and 280 nm (b). See Table V for identification of peaks.

of each HPLC peak displayed on the IBM personal computer equipped with the Wavescan Spectral Detector program (LKB 2140-202). The capacity factor (k') was calculated by the equation (Kirkland, 1971)

$$k' = (t_r - t_0)/t_0$$

where t_r is the retention time of the compound, t_0 is the time of zero retention measured as the time of the nonretained solvent peak, and k' is the capacity factor of the compound. Fractions of selected HPLC peaks were collected, concentrated under reduced pressure by a rotary evaporator at 30 °C, and subjected to further analysis.

Paper Chromatography. R_f values of the purified pigments were obtained by descending chromatography using Whatman No. 1 paper and the following solvent systems (Francis, 1982; Mabry et al., 1970): 1-butanol–glacial acetic acid–water (BAW) 4:1:5, upper phase, aged 3 days; 1-butanol–2 N hydrochloric acid (Bu–HCl) 1:1, upper phase, paper equilibrated 24 h after spotting and before running, in tank containing aqueous phase of Bu–HCl mixture; concentrated hydrochloric acid in water (1% HCl) 3:97; water–glacial acetic acid–12 N hydrochloric acid (HOAC–HCl) 82:15:3; 15% glacial acetic acid–water (15% NOAC) 15:85.

Spectral Analysis. Purified anthocyanins were dissolved in methanolic 0.01% HCl; other flavonoids were dissolved in methanol. UV spectra of the anthocyanins and flavonoids were measured at 200–700 nm in a Beckman DU-50 spectrophotometer connected to an Epson RX-80 printer and an IBM personal computer equipped with a "peak pick" program (Beckman Quant 1 Soft-Pak, Beckman Instruments Inc., Scientific Instruments Division, Irvine, CA). Sodium methoxide (NaOMe), aluminum chloride ($AlCl_3$), aluminum chloride and hydrochloric acid ($AlCl_3$ + HCl), sodium acetate (NaOAc), and sodium acetate plus boric acid (NaOAc + H_3BO_3) shifts were recorded over the same wavelengths and interpreted as described by Mabry et al. (1970) and Markham (1982).

Hydrolysis of Flavonoids. Purified flavonoids were subjected to acid hydrolysis as described by Francis (1982). Two milliliters of 2 N HCl was added to 1–2 mg of flavonoid dissolved in a minimal amount of methanol. The aglycon and sugar portion of the flavonoid were obtained by heating the pigment–solvent mixture in a water bath at 100 °C for 1 h. After cooling, the aglycon was extracted with 3 × 2 mL of amyl alcohol and chromatographed against reference standards on Whatman No. 1 paper with Forestal (glacial acetic acid–concentrated hydrochloric acid–water 30:3:10) and formic acid. Prior to paper chromatography, the aqueous solution containing the sugar was washed with 10% di-*n*-octylmethylamine in chloroform as described by

Table II. Chromatographic Characteristics of *Monarda* Petal Pigments

peak	color[a] in vis	UV	UV/NH_3	R_f,[b] × 100 1% HCl (3:97)	BAW (4:1:5)	H_2O:HOAc:HCl (82:15:3)	BuOH:HCl (1:1)
1	inv	fl blue	fl blue	63	45	74	59
2	inv	fl blue	fl blue-gr	80	47, 63	65, 78	50, 87
3	inv	fl blue	green	72	48	74	76
6	inv	fl blue	green and purple	52	20, 51	8, 52	
7	inv	fl blue	y-gr	39	17	72	28
8	inv	yellow	fl blue	20	67		42
10	inv	fl blue	green	15	38	49	41
12	inv	yellow	pink	42	41		30
13	pink	pink	magenta	28	25	65	33
16	inv	fl blue		23			26
standards							
apigeninidin	org	yellow	pink	8	76	38	69
myricetin		yellow	yellow		40		
maringenin			pink		49	64	25
Pg 3,5-diglu	pink	yellow	pink	29	23	11, 60	8
quercitin		yellow	yellow		69	4	74
rutin	inv	y-gr	yellow	31	40	67	42

[a] Color: inv, invisible; fl blue, fluorescent blue; fl blue-gr, fluorescent blue-green; y-gr, yellow-green; org, orange. [b] Whatman No. 1 paper.

Table III. Spectral Characteristics of *M. fistulosa* L. Flavonoids and Authentic Standards in Methanol with the Addition of Shift Reagents

peak	λ_{max}, nm	NaOMe Δ band I	$AlCl_3$ Δ band I	$AlCl_3$/HCl Δ band I
1	269, 323 sh, 374	0		
2	309	+47	0	
3	204, 249, 332	+43	+5	-3
5	291	+7, +38[a]		
7	257, 320, 376	+38	+60	-4
8	267, 331	+57		
10	276, 296, 328, 258	+66		
16	268, 328	+45	+49	+2
standards				
rutin	256, 357	+50	+52	+43
myricetin	253, 375	+49	+76	+54
quercitin	254, 306 sh, 372	+33	+80	+51
naringenin	286	0	+52	+49
apigeninidin	265, 423, 482	+55	+48	+45

[a] Appearance of new peak at 330 nm upon addition of shift.

Francis (1982) and then dried under vacuum. The sugar was dissolved in a few drops of water and chromatographed on Whatman No. 1 paper with reference standards using the solvents 1-butanol–ethanol–water (BEW) 4:1:2.2 and 1-butanol–benzene–pyridine–water (BBPW) 5:1:3:3. For viewing of sugar paper chromatograms, aniline hydrogen phthalate sugar reagent was used (Markham, 1982). The position of the sugar in the flavonoid molecule was determined from spectral shifts (Markham, 1982; Mabry et al., 1970) and by comparing HPLC retention times of *Monarda* flavonoids with those of authentic compounds analyzed under the same conditions.

Acyl Hydrolysis. Acylated anthocyanins were subjected to cold alkali hydrolysis as described by Terahera et al. (1990). The acylated anthocyanin was dissolved in 2 mL of methanol, and 1 mL of 2 N NaOH was added. The solution was kept under nitrogen for 30 min at room temperature and then acidified with 1 mL of 2 N HCl and taken to dryness under vacuum. The residue was dissolved in methanol, and the liberated acid was extracted from the residue by the addition of 1 mL of ethyl ether. The acyl group was chromatographed on microcrystalline cellulose TLC plates and detected using glucose aniline as described by Takeda et al. (1986). Two solvents, EtOH–H_2O–NH_4OH (16:3:1) and EtOAc–HOAc–H_2O (3:1:1) were used in the TLC chromatography. Following hydrolysis of the acyl group, the deacylated pigment was subjected to HPLC, spectral analyses, and acid hydrolysis as described previously.

Paper Electrophoresis. Electrophoresis was conducted on Whatman No. 3 paper in acetate buffer, pH 4.4, for 2 h at 10 V cm^{-1}, 2 mA cm^{-1}. The equipment consisted of a Gelman electrophoresis unit (Gelman Instrument Co., Ann Arbor, MI) equipped with power supply (Gelman Model 38200) and an electrophoresis chamber (Gelman Model 51170). The papers were removed, dipped briefly in 1% aqueous HCl, dried, and viewed under UV.

Total Anthocyanins. Total anthocyanins were determined by the pH differential method of Wrolstad (1976) and calculated on the basis of the extinction coefficient (31 900) of the predominant polyacylated anthocyanin of *Monarda* in 0.01% HCl methanol.

RESULTS AND DISCUSSION

The HPLC retention data and UV–vis maxima in methanol–formic acid–water are presented in Table I. Typical chromatograms of the methanolic extract of *M. fistulosa* petals monitored at 280 and 510 nm are illustrated in Figure 1. It is known that the principal ultraviolet absorption for anthocyanins is at 270–280 nm, with absorbance in the visible region at 500–550 nm, while that of hydroxycinnamic acids is at 290–300 nm, and the absorbance of other flavonoids is at 310–370 nm. The retention times and spectral properties presented in Table I reveal that peaks 1, 2, 4, and 5 exhibit spectral characteristics of hydroxycinnamic acids and peaks 7, 8, 10, 15, and 16 exhibit characteristics of flavones, while peak 9 and 11–14 exhibit spectral characteristics of anthocyanins. From comparison to standard values reported in the literature (Mabry et al., 1970; Kondo et al., 1985; Velioglu and Mazza, 1991), peaks 9 and 11–13 correspond to acylated pelargonidin glycosides.

Acylation is known to produce a 2-fold increase in the retention time of anthocyanins when using reversed phase HPLC (Takeda et al., 1986; Kim, 1989). Comparison of the retention data of peak 13 (Table I) to retention data of pelargonidin 3,5-diglucoside (t_r = 15.5 min), as determined by Velioglu and Mazza (1991) under identical experimental conditions, reveals a 2.5-fold difference in the retention times of pelargonidin 3,5-diglucoside and peak 13. Total anthocyanin content of *M. fistulosa* L. was 214.8 mg/100 g of fresh petals, and peak 13 accounted for approximately 81% of the total anthocyanins.

Comparison to selected flowers reveals that *Monarda* petals contain a slightly higher anthocyanin concentration than roselle (Du and Francis, 1973) but a considerably higher concentration than that of rose petals (Velioglu and Mazza, 1991). Although other flower sources containing pelargonidin as the primary anthocyanidin derivative have been characterized in recent years, the total anthocyanin concentrations have not been reported.

Table IV. Spectral Characteristics of *M. fistulosa* L. Anthocyanins in Methanolic 0.01% HCl Solution

peak	λ_{max}, nm	E_{440}/E_{vismax}, %	E_{358}/E_{vismax}, %	E_{UVmax}/E_{vismax}, %	E_{257}/E_{vismax}, %	E_{acyl}/E_{vismax}, %
12	273, 310, 507	28	32	128	118	92
13	283, 306, 506	20	13	179	98	144
standards						
pelargonidin	257, 359, 520	82	832	1014	1014	
Pg 3,5-diglu	265, 304, 511	22	31	71	68	

Ten of the 16 peaks indicated in Figure 1 were collected as separate fractions from the HPLC column, concentrated, and subsequently analyzed by paper chromatography as described under Materials and Methods. Absorption spectra of the anthocyanins and colorless flavonoids were obtained in the 200–700-nm range, and shift reagents were used to elucidate detailed structural information concerning the substitution patterns of the compounds (Mabry et al., 1970).

Table II presents the R_f values and colors of nine peaks before acid hydrolysis. Peaks 1–3 all exhibited a fluorescent blue color in UV light before and after exposure to NH_3. The chromatographic color and early elution time from the HPLC column suggest that peaks 1–3 are hydroxycinnamic acid derivatives. The principal absorbance in the 290-nm range (Table III) provides further evidence to support this supposition. The presence of a glucose molecule attached to *p*-coumaric acid of peak 2 was confirmed by chromatography against sugar standards. Peak 5 had the spectral characteristics of a flavonol glycoside; however, limited quantity prevented complete characterization of this peak. Peaks 6–8, 10, and 16 all exhibited fluorescent blue colors when exposed to UV light and changed to fluorescent yellow-green or blue-green upon exposure to NH_3 (Table II). On the basis of these colors and color changes, it is suggested that peaks 7, 8, 10, and 16 are glycosylated flavones (Mabry et al., 1970). Peak 6 appears to be a flavone; however, limited quantity prevented complete characterization.

The spectral characteristics of peaks 7, 8, 10, and 16 in the presence of sodium methoxide (Table III) indicated that these peaks are flavone glycosides with free 4′-hydroxyl substitution. The addition of a milder alkali shift provides evidence for the absence of free 7-hydroxyl groups on peaks 7 and 10 and differentiates the glycosyl site of these three peaks, leading to the identification of peaks 7 and 10 as flavone 7-*O*-glucosides and peak 8 as a dihydroxyflavone 8-*C*-glucoside. The shift behavior of peak 7 in the presence of boric acid indicates the absence of a dihydroxyl group on the A ring, whereas the shift observed in peak 8 indicates the presence of alkali-sensitive groups on the A ring. Peak 7 was assigned the structure of flavone 7-*O*-glucoside on the basis of this information. The absence of a band I shoulder following the addition of sodium acetate provides evidence for a free 7-hydroxyl group on peak 8. Following acid hydrolysis, the UV–vis spectra and the chromatographic behavior of the aglycon of peak 10 exhibited close similarity to those of apigenin, thus confirming the suggestion that peak 10 is an apigenin derivative. On the basis of the spectral information and chromatographic mobility, peak 8 was tentatively identified as 7,4′-dihydroxyflavone 8-*C*-glucoside and peak 10 was identified as apigenin 7-*O*-glycoside. On the basis of the magnitude of the shift and the appearance of two new peaks on the spectrum of peak 16 in the presence of $AlCl_3$ and HCl, it is suggested that peak 16 is a 5-hydroxyflavone (Table V).

Peaks 12 and 13 exhibited yellow and pink spots, when viewed in UV light, and pink spots following exposure to NH_3 (Table II). According to Markham (1982), these colors and color changes are indicative of anthocyanidin 3,5-diglucosides. The presence of aromatic organic acids attached to peaks 12 and 13 is evident by comparison of R_f values in four solvents to the literature values (Harborne, 1967; Takeda et al., 1986). The greater mobility of these two peaks as compared to that of the pelargonidin 3,5-diglucoside standard confirms the presence of acyl groups in these compounds (Table II). Characterization of peaks 9, 11, and 14 was not complete as these compounds could not be adequately separated from coeluting colorless flavonoids. Nonetheless, their chromatographic and spectral characteristics indicate that they are acylated pelargonidin glycosides.

Table V. Anthocyanins and Other Phenolics of *M. fistulosa* L. Cv. Marshalls Delight

peak[a]	compound
1, 3	hydroxycinnamic acids[b]
2	*p*-coumaric acid glucoside
5	flavonol glycoside[b]
7	flavone 7-*O*-glucoside
8	7,4′-dihydroxyflavone 8-*C*-glucoside
9, 11, 14	acylated pelargonidin glycosides[b]
10	apigenin 7-*O*-glucoside
12, 13	diacylated pelargonidin 3,5-diglucoside
16	5-hydroxyflavone

[a] Refers to peak number in Figure 1. [b] Tentative identification.

Peaks 12 and 13, however, were adequately purified and subjected to detailed characterization. Their UV–vis spectra in methanol acidified with HCl reveal maximum absorbance at 280 and 506 nm, an absorption peak at 306–311 nm, and the lack of a distinct shoulder in the 410–440-nm range, which are characteristic of acylated anthocyanins with a sugar residue at both the 3- and 5-OH positions (Harborne, 1967) (Table IV). From the similarities in retention times, spectral data, and chromatographic behavior both before and after hydrolysis, it is suggested that peaks 12 and 13 are both pelargonidin 3,5-diglycosides acylated to different degrees. The strong intensity of the UV absorbance of peak 13 at 306 nm as compared to the lower intensity of the UV absorbance in peak 12 suggests complex polyacylation of peak 13 by phenolic acids (Saito et al., 1985). The different degree of acylation is apparent from comparison of the E_{acyl}/E_{vismax} absorbance ratios (Table IV). As is the case with peaks 12 and 13, Saito and co-workers (Saito et al., 1985) observed the appearance of a smaller peak closely preceding the major peak and suggested that this close association may be the result of a hydrolysis reaction (loss of an unstable malonyl group) or an isomer differing in some slight structural detail from the major peak. It is suggested that peak 12 is acylated to a lower degree than peak 13 from the lower E_{acyl}/E_{vismax} ratio and the slightly earlier retention time.

Following acid hydrolysis of peaks 12 and 13, the aglycons and sugars were confirmed to be pelargonidin and glucose, respectively, by paper and thin-layer chromatography techniques. The presence of malonylation was confirmed only by the electrophoretic mobility of peak 13 toward the anode in pH 4.4 acetate buffer. It has been well established in the literature (Harborne and Boardley, 1985; Takeda et al., 1986) that zwitterionic anthocyanins can be clearly distinguished from cationic antho-

cyanins by their mobility toward the anode upon electrophoresis.

It is believed that malonic acid is attached to the C6 of glucose, as this represents the most widely occurring site of acyl attachment (Harborne, 1986; Borger and Barz, 1988). Evidence to support this was obtained by subjecting the pigment to spectral analysis following alkali hydrolysis. Since there was no change in the absorbance, it is evident that the acyl group cleaved from the molecule during alkali hydrolysis was attached to the sugar molecule of the anthocyanin (Markham, 1982).

In comparison to the findings of Kondo et al. (1985) regarding *M. didyma*, it has been established that several acylated forms of pelargonidin diglucoside also exist in *M. fistulosa* which possess similar UV–vis spectra and E_{440}/E_{510} ratios to those of donardaein isolated from *M. didyma*.

CONCLUSIONS

The structural characteristics of the major flavonoids of *M. fistulosa* L. have been determined. It has been established that the flavonoid profile of *M. fistulosa* L. does contain several acylated forms of pelargonidin 3,5-diglucoside. These relatively rare plant phenolics will provide a unique opportunity to elucidate the copigmentation mechanism in flowers and food products in studies currently in progress in our laboratory.

ACKNOWLEDGMENT

We thank the Natural Science and Engineering Research Council of Canada for financial support.

LITERATURE CITED

Borger, G.; Barz, W. Malonated flavonol 3-glucosides in *Cicer Arietinum*. *Phytochemistry* **1988**, *27*, 3714.

Brouillard, R. Origin of the exceptional color stability of *Zebrina* anthocyanin. *Phytochemistry* **1981**, *20*, 143.

Du, C. T.; Francis, F. J. Anthocyanins of Roselle (*Hibiscus sabdariffa*, L.). *J. Food Sci.* **1973**, *38*, 810.

Francis, F. J. Analysis of anthocyanins. In *Anthocyanins as Food Colors*; Markakis, P., Ed.; Academic Press: New York, 1982; p 181.

Harborne, J. B. *Comparative Biochemistry of the Flavonoids*; Academic Press: New York, 1967.

Harborne, J. B. The natural distribution of anthocyanins acylated with aliphatic dicarboxylic acids. *Phytochemistry* **1986**, *25*, 1887.

Harborne, J. B.; Boardley, M. The widespread occurrence in nature of anthocyanins as zwitterions. *Z. Naturforsch.* **1985**, *40C*, 305.

Kim, H. J.; Nonaka, G. I.; Fujieda, K.; Uemoto, S. Anthocyanidin malonylglucosides in flowers of *Hibiscus syriacus*. *Phytochemistry* **1989**, *28*, 1503.

Kirkland, J. J. *Modern Practice of Liquid Chromatography*; Wiley-Interscience: New York, 1971; p 8.

Kondo, T.; Nakane, Y.; Tamura, H.; Goto, T. Structure of Monardein, a bis-malonylated anthocyanin isolated from golden balm, *Monarda didyma*. *Tetrahedron Lett.* **1985**, *26*, 5879.

Mabry, T. J.; Markham, K. R.; Thomas, M. B. *The Systematic identification of flavonoids*; Springer-Verlag: New York, 1970.

Markham, K. R. *Techniques of Flavonoid Identification*; Academic Press: New York, 1982.

Saito, N.; Abe, K.; Honda, T.; Timberlake, C.; Bridle, P. Acylated delphinidin glucosides and flavonols from *Clitoria ternatea*. *Phytochemistry* **1985**, *24*, 1583.

Sapers, G. M.; Taffer, I.; Ross, L. R. Functional properties of a food colorant prepared from red cabbage. *J. Food Sci.* **1981**, *46*, 105.

Takeda, K.; Harborne, J. B.; Self, R. Identification of malonated anthocyanins in the Liliacae and Labiatae. *Phytochemistry* **1986**, *25*, 2191.

Teh, L. S.; Francis, F. J. Stability of anthocyanins from *Zebrina pendula* and *Ipomoea tricolor* in a model beverage. *J. Food Sci.* **1988**, *53*, 1580.

Terahera, N.; Saito, N.; Honda, T.; Toki, K.; Osajima, Y. Acylated anthocyanins of *Clitoria ternatea* flowers and their acyl moieties. *Phytochemistry* **1990**, *29*, 949.

Velioglu, Y. S.; Mazza, G. Characterization of flavonoids in petals of *Rosa damascena* by HPLC and spectral analysis. *J. Agric. Food Chem.* **1991**, *39*, 463.

Wrolstad, R. E. Color and pigment analysis in fruit products. *Oreg., Agric. Exp. Stn. Bull.* **1976**, *No. 624*.

Received for review December 16, 1991. Revised manuscript received April 1, 1992. Accepted May 11, 1992.

Registry No. Hydroxycinnamic acid, 25429-38-3; *p*-coumaric acid glucoside, 14364-05-7; flavone 7-*O*-glucoside, 71802-05-6; 7,4′-dihydroxyflavone 8-*C*-glucoside, 3681-96-7; apigenin 7-*O*-glucoside, 578-74-5; 5-hydroxyflavone, 491-78-1.