Phytochemistry, Vol 23, No 11, pp 2523–2526, 1984
Printed in Great Britain

0031–9422/84 $3 00 + 0 00
Pergamon Press Ltd

HIGH PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF FLAVONOID CHEMICAL MARKERS IN PETALS FROM *GERBERA* FLOWERS AS AN ADJUNCT FOR CULTIVAR AND GERMPLASM IDENTIFICATION

SAM ASEN

Florist and Nursery Crops Laboratory, Horticultural Science Institute, Beltsville Agricultural Research Center, U S Department of Agriculture, Beltsville, MD 20705, U S A

(*Received* 14 *March* 1984)

Key Word Index—*Gerbera jamesonii*, Compositae, chemosystematics, flavonoid malonylglucosides

Abstract—Flavonoids present in petals from *Gerbera* flowers were resolved and quantitated by high pressure liquid chromatography (HPLC) The anthocyanins isolated from 18 cultivars, ranging in color from orange through lavender, were pelargonidin and cyanidin 3-malonylglucosides accompanied by smaller amounts of pelargonidin and cyanidin 3-glucosides Related flavonoid copigments were apigenin and luteolin 4′-glucosides and 7-glucosides, apigenin 7-malonylglucoside, kaempferol and quercetin 3-glucosides, 4′-glucosides and 3-malonylglucosides Both qualitative and quantitative differences in these flavonoid chemical markers distinguished cultivars with very similar colors Malonyl esters of anthocyanins are easily degraded by HCl and conventional extraction and purification procedures were adjusted to preserve their natural state

INTRODUCTION

The importance of flavonoid chemical markers to plant taxonomy at species and higher plant orders is well documented Their usefulness now has been extended to establish the identity of parental origin of natural hybrids [1, 2] and for cultivar identification [3–5] Although rich in carotenoids, this study was initiated to identify flavonoids in petals of gerbera flowers, to develop HPLC procedures for their resolution and quantitation, and to determine their usefulness for distinguishing cultivars with very similar colors

RESULTS AND DISCUSSION

Glycosides of pelargonidin and cyanidin previously were reported in petals of red *Gerbera jamesonii* Bolus [6] The major anthocyanins now identified from gerbera flower petals, ranging in color from orange through lavender, were pelargonidin and cyanidin 3-malonylglucosides accompanied by smaller amounts of pelargonidin and cyanidin 3-glucosides The HPLC resolution of these four naturally occurring anthocyanins is shown on the top of Fig 1

Anthocyanins whose sugars are acylated with malonic acid, when extracted with MeOH–HCl, form methyl esters that are easily deacylated and the general use of this solvent may account for the few reported species in which they occur [7] Their natural occurrence may be greater than previously thought The initial conversion of pelargonidin and cyanidin 3-malonylglucosides, in 1% HCl–MeOH, to methyl esters is shown in the bottom of Fig 1 Similar results have been reported Awobanin, isolated from blue-colored petals of *Commelina cummunis*, previously was identified as delphinidin 3-*p*-coumarylglucoside-5-glucoside [8, 9] Goto *et al* [10] now report that this anthocyanin is delphinidin 3-*p*-coumarylglucoside-5-malonylglucoside and it is converted to awabanin and malonylawabanin methyl ester in the presence of 1% HCl–MeOH The anthocyanin and flavone present in the blue pigment from *Centaurea cyanus*, previously were identified as cyanidin 3,5-diglucoside [11–13] and apigenin 4′-glucoside-7-glucuronide [14] Tamura *et al* [15] now report these compounds to be cyanidin 3-succinylglucoside-5-glucoside and apigenin 4′-malonylglucoside-7-glucuronide They concluded that the initial identification was due to the easily hydrolysed succinic and malonic acid half esters in MeOH–HCl Their identification was based on decomposing the blue pigment in TFA–HOAc–MeCN–H_2O (0 5 6 3 7 9 85 3) at room temperature for 0 5 hr and subsequent resolution by HPLC

Eleven flavonoid copigments were isolated from gerbera flower petals and they consisted of five flavones and six flavonols One flavone and two flavonols were acylated with malonic acid as were the major anthocyanins (Table 1) The HPLC resolution of these chemical markers is shown in Fig 2 Not all compounds were resolved with tetrahydrofuran (THF) (bands 4 and 9) but the resolution obtained from a second solvent system (MeCN) provided the information necessary for the quantitation of all 11 compounds

The flavonoid composition of gerbera flower petals, from cultivars with very similar orange-red or yellow colors is shown in Table 2 Luteolin 4′-glucoside, luteolin 7-glucoside and quercetin 3-malonylglucoside were isolated from cultivars not included in this survey Significant differences between cultivars having very similar colors were both qualitative and quantitative Even at the more severe 1% significant level test, cultivars were distinguished from each other on the basis of the significant difference of usually more than one flavonoid chemical marker The orange-red cultivars S-39 and S-4, and the

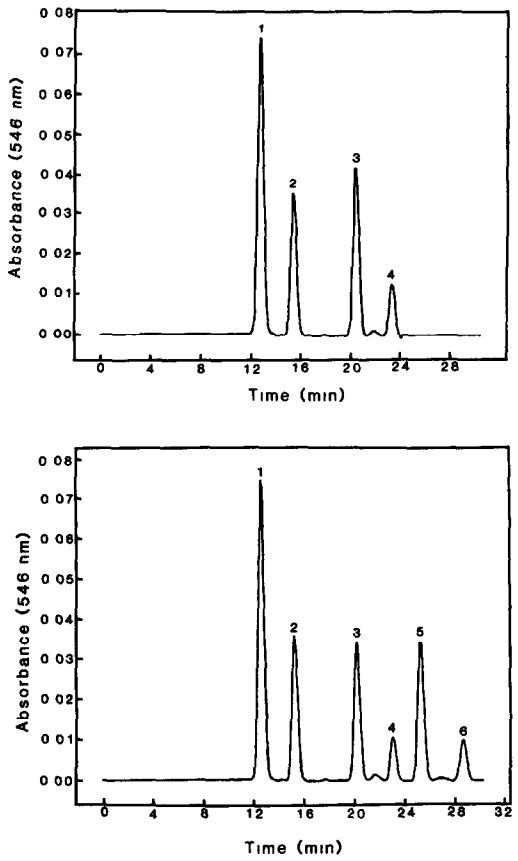

Fig 1 Top HPLC resolution of naturally occurring anthocyanins in petals from gerbera flowers Purified anthocyanin standards were taken up in 10% HOAc–MeOH 1 hr prior to injection Column = radial pak A (C_{18}) with radial compression separating system, solvents = 1 5% H_3PO_4 (pump A) and 20% HOAc, 25% MeCN in 1 5% H_3PO_4 (pump B), elution program = 25–75% B (linear gradient) in 40 min, flow rate = 2 ml/min, detection = adsorption at 546 nm 1 = cyanidin 3-glucoside, 2 = pelargonidin 3-glucoside, 3 = cyanidin 3-malonylglucoside, 4 = pelargonidin 3-malonylglucoside Bottom Purified anthocyanin standards made up in 1% HCl–MeOH 1 hr prior to injection 5 = conversion product from 3 cyanidin 3-malonylglucoside methyl ester, 6 = conversion product from 4 pelargonidin 3-malonylglucoside methyl ester

yellow cultivars Ceres-2000 and S-133 were the only comparisons with one significant flavonoid chemical marker. The amount of kaempferol 3-malonylglucoside was significantly greater in S-39 than S-4 and quercetin 4'-glucoside was detected in Ceres-2000 and not in S-133

Physiological and morphological attributes have been the primary criteria for differentiating cultivars These characteristics alone have not proven satisfactory, particularly when describing new cultivars protected by plant patent laws Flower color is one of these important characteristics, but many flower colors cannot be described adequately or satisfactory related to color charts Natural compounds usually responsible for flower colors are flavonoids [16] but little or no use has been made of these chemical markers to aid in positive cultivar identification Petal flavonoids are important because their

Table 1 Chromatographic data for flavonoids acylated with malonic acid, and their deacylated forms, from gerbera flower petals*

Flavonoids	R_f (×100) in solvent 15% HOAc	H_2O	BAW	PhOH
Apigenin				
7-malonylglucoside	25	47	69	67
7-glucoside	17	3	67	95
Kaempferol				
3-malonylglucoside	56	82	85	44
3-glucoside	46	13	82	69
Quercetin				
3-malonylglucoside	45	87	73	30
3-glucoside	32	9	70	49
Pelargonidin				
3-malonylglucoside	47	—	63	—
3-glucoside	42	—	44	—
Cyanidin				
3-malonylglucoside	41	—	44	—
3-glucoside	35	—	28	—

*Cellulose plates (250-μm layer), BAW = *n*-BuOH–HOAc–H_2O (6 1 2), PhOH = phenol–H_2O (73 27, w/v)

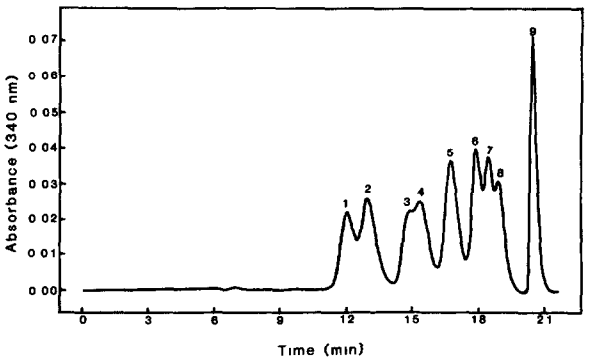

Fig 2 HPLC resolution of naturally occurring flavonoid copigments in petals from gerbera flowers Column = radial pak A (C_{18}) with radial compression separating system, solvents = 1% triethylamine buffered to pH 3 with H_3PO_4 (pump A) and tetrahydrofuran (pump B), elution program = 22–50% B (Waters gradient #9) in 20 min, flow rate = 2 0 ml/min, detection absorbance at 340 nm, 1 = luteolin 7-glucoside, 2 = quercetin 3-glucoside, 3 = quercetin 3-malonylglucoside, 4 = apigenin 7-glucoside + kaempferol 3-glucoside, 5 = apigenin 4'-glucoside, 6 = luteolin 4'-glucoside, 7 = kaempferol 3-malonylglucoside, 8 = apigenin 7-malonylglucoside, 9 = kaempferol 4'-glucoside + quercetin 4'-glucoside

similar phenotypic color expression This has been demonstrated with poinsettia bracts [3, 4], geranium florets [5] and now gerbera petals Although changes in environment can influence the biosynthesis of flavonoids, any particular flavonoid constituent can be relied on to be present in more or less constant amounts when tissue for analysis is uniformly sampled from plants grown under the same environment The HPLC resolution and quantitation of flavonoid chemical markers, as an adjunct to the

Table 2 Flavonoid concentration in dried petals from gerbera flowers sampled June 1983

	μg/100 mg dry wt																
Cultivar	Cy3-glc	Cy3-malonyl-glc	Pg3-glc	Pg3-malonyl-glc	Qu3-glc	Qu4'-glc	Qu3-malonyl-glc	Km3-glc	Km4'-glc	Km3-malonyl-glc	Ap4'-glc	Ap7-glc	Ap7-malonyl-glc	Lu4'-glc	Lu7-glc	Antho-cyanins (Total)	Flavonoid copigments (Total)
								Group I Orange-red									
Ceres-2000	34	323	4	40	ND	147	625	160	21	149	ND	ND	ND	ND	ND	401	1102
S-4	3	46	63	766	40	97	ND	307	ND	178	ND	ND	ND	ND	ND	878	622
S-39	1	8	67	879	42	170	ND	186	ND	433	ND	ND	ND	ND	ND	955	831
S-92	1	2	253	602	38	198	51	664	56	1103	27	ND	ND	ND	24	858	2161
LSD 5%	31	33	40	201	30	73		187		113						221	172
LSD 1%	52	53	65	301	51	112		277		171						332	258
								Group II Yellow									
Ceres-2000	ND	ND	ND	ND	23	74	77	341	20	1296	ND	ND	ND	ND	26	ND	1857
S-14	ND	ND	ND	ND	12	ND	ND	71	ND	285	ND	ND	ND	ND	ND	ND	368
S-103	ND	ND	ND	ND	58	ND	26	183	20	789	ND	ND	ND	ND	ND	ND	1076
S-133	ND	ND	ND	ND	23	ND	76	285	19	1057	ND	ND	ND	ND	17	ND	1477
S-196	ND	ND	ND	ND	59	211	ND	227	ND	941	984	18	199	ND	ND	ND	2639
SC-202	ND	ND	ND	ND	21	129	ND	130	152	348	668	61	278	ND	ND	ND	1787
LSD 5%					26	170		93	23	355							719
LSD 1%					35	253		138	37	525							1068

Abbreviations Cy = cyanidin, Pg = pelargonidin, Qu = quercetin, Km = kaempferol, Ap = apigenin, Lu = luteolin, Glc = glucoside, ND = not detected LSD values are based on duplicate analyses

excellent objective method to aid in the positive identification of cultivars as well as available germplasm

EXPERIMENTAL

Plant material Ceres-2000 cultivars were supplied by Ceres 2000, Inc, Winter Haven, Florida, and all other cultivars by Hartman's, Palmdale, Florida The plants were grown under standard greenhouse cultural practices at Beltsville, Maryland

Identification of petal flavonoids Petals from each cultivar were dried in a forced-air oven at 40°, and then ground to pass a 40-mesh screen Four anthocyanins were isolated but each was not present in all 18 cultivars examined Pelargonidin 3-malonylglucoside and pelargonidin 3-glucoside were isolated from the orange-red cultivars S-39 and S-92, and cyanidin 3-malonylglucoside and cyanidin 3-glucoside from the red-purple cultivars SC-201 and S-58 Carotenoids were first extracted with petrol and then the anthocyanins were extracted with 15% HOAc–MeOH The extracts were reduced to almost dryness at 40°, under red pres, taken up in a minimum volume of MeOH, filtered, and then placed on a column of polyvinypyrrolidone (PVP) made with H_2O Anthocyanins were eluted with 10% HOAc–MeOH and then passed through a cellulose column with EtOAc–HCOOH–H_2O (70 15 15) Final purification was by HPLC with 40% MeOH in 2% HOAc for the pelargonidin glucosides and 50% MeOH in 2% HOAc for the cyanidin glucosides

Flavonoid copigments were isolated from a yellow cultivar SC-202 because of the large supply of this tissue Carotenoids were first extracted with petrol and then the flavonoid copigments were extracted with hot MeOH The extracts were reduced to almost dryness at 40°, under red pres, taken up in 50% MeOH, filtered and then placed on a PVP column made with H_2O The flavonoid copigments were banded into 10 fractions by gradient elution with H_2O–MeOH (0–100% MeOH) Any remaining compounds were finally eluted with 10% HOAc–MeOH Flavonoids in each band were resolved and identified by procedures similar to those previously described [17] Quercetin 3-malonylglucoside, luteolin 4'-glucoside and luteolin 7-glucoside, not detected in the yellow cultivar SC-202, were isolated from red-purple cultivars S-58 and SC-201 The compounds in each band were resolved isocratically by HPLC with 20–24% MeCN in 2% HOAc or 46–50% MeOH in 2% HOAc Final purification of the isolated compounds was through polyamide (SC-6) with 10% HOAc–MeOH for those acylated with malonic acid and MeOH for all others Identification was by R_f with known standards, UV spectral analyses, and the products of controlled acid or base hydrolyses For comparable R_f and UV absorption spectra refer to Harborne [18] and Mabry *et al* [19] Malonic acid was determined by GC/MS by converting the free acid obtained from base hydrolysis with BF_3–MeOH to the dimethyl ester

Flavonoid HPLC resolution and quantitation Each sample consisted of 100 mg of dried petals free of carotenoids Anthocyanins were extracted by blending for 30 sec in 50 ml 10% HOAc–MeOH The tissue was then filtered and washed free of anthocyanins Flavonoid copigments were extracted with MeOH Each extract was reduced to dryness at 40°, under red pres, and taken up in a soln which contained the smallest percentage of the organic phase of the solvent used for HPLC analysis All extracts were passed through a 0 5 μm Millipore filter prior to analysis A Waters Associates HPLC model ALC/GPC 244 with system controller, data module, and radial compression separation system (RCSS) was used Resolution was accomplished with a Radial-Pak A cartridge (reverse phase permanently bonded octadecylsilane, particle size 10 μm) with a RCSS guard-pak C_{18} disposable precolumn insert

Anthocyanins were resolved and quantitated by gradient elution using a modification of the procedure of Strack *et al* [20] The following six parameters were used (1) pump A = 1 5% H_3PO_4, (2) pump B = 20% HOAc, 25% MeCN, in 1 5% H_3PO_4, (3) linear gradient = 25–75% B in 40 min, (4) flow rate = 2 ml/min *ca* 1500 psi, (5) detection = absorption at 546 nm, and (6) chart speed = 0 5 cm/min The resolution from two solvent systems was required to quantitate all 11 flavonoid copigments Parameters for the first system were (1) pump A = 1% triethylamine buffered to pH 3 with H_3PO_4 (TEAP), (2) pump B = tetrahydrofuran (THF), (3) gradient = 22–50% B (Waters #9) in 20 min, (4) flow rate = 2 0 ml/min *ca* 1200 psi, (5) detection = absorbance at 340 nm, (6) chart speed = 1 cm/min Parameters for the second system were (1) pump A = TEAP, (2) pump B = MeCN, (3) gradient = 20–35% B (Waters #8) in 25 min, (4) flow rate = 2 ml/min *ca* 800 psi, (5) detection = absorbance at 340 nm, (6) chart speed = 0 5 cm/min

Acknowledgements—The author gratefully acknowledges the technical assistance of P S Budin He is also indebted to B B Leonhardt, Organic Chemical Systhesis Laboratory, Agricultural Environmental Quality Institute, Beltsville, Maryland, for the GC/MS analysis of malonic acid Mention of a trademark, proprietary product, or vender does not constitute a guarantee or warranty of the product by the U S Department of Agriculture and does not imply its approval to the exclusion of other products or venders that also may be suitable

REFERENCES

1 Alston, R E and Turner, B L (1963) *Biochemical Systematics* Prentice-Hall, Englewood Cliffs
2 Williams, C A, Fronczyk, J H and Harborne, J B (1983) *Phytochemistry* **22**, 1953
3 Stewart, R N, Asen, S, Massie, D R and Norris, K H (1979) *Biochem Syst Ecol* **7**, 281
4 Stewart, R N, Asen, S, Massie, D R and Norris, K H (1980) *Biochem Syst Ecol* **8**, 119
5 Asen, S and Griesbach, R (1983) *J Am Soc Hortic Sci* **108**, 845
6 Richter, E (1974) *Z Pflanzenzucht* **71**, 33
7 Harborne, J B and Mabry, T J (1982) *The Flavonoids, Advances in Research* Chapman & Hall, New York
8 Kurodo, C (1936) *Bull Chem. Soc Jpn* **11**, 265
9 Takeda, K and Hayashi, K (1964) *Proc Jpn Acad* **40**, 510
10 Goto, T, Kondo, T, Tamura, H and Takase, S (1983) *Tetrahedron Letters* **24**, 4863
11 Bayer, E (1958) *Chem Ber* **91**, 1115
12 Hayashi, K, Saito, N and Mitsui, K (1961) *Proc Jpn Acad* **37**, 393, 485
13 Asen, S and Jurd, L (1967) *Phytochemistry* **6**, 577
14 Asen, S and Horowitz, R M (1974) *Phytochemistry* **13**, 1219
15 Tamura, H, Kondo, T, Kato, Y and Goto, T (1983) *Tetrahedron Letters* **24**, 5749
16 Asen, S, Stewart, R N and Norris, K H (1972) *Phytochemistry* **11**, 1139
17 Asen, S (1982) *J Am Soc Hortic Sci* **107**, 744
18 Harborne, J B (1967) *Comparative Biochemistry of the Flavonoids* Academic Press, New York
19 Mabry, T J, Markham, K R and Thomas, M B (1970) *The Systematic Identification of Flavonoids* Springer, New York
20 Strack, D, Akavia, N and Reznik, H (1980) *Z Naturforsch* **35c**, 533