4. Fergus, I. F. 1954. Manganese toxicity in an acid soil. Queensland J. Agr. Sci. 11:15–27.
5. Geraldson, C. M., G. R. Klacan, and O. A. Lorenz. 1973. Plant analysis as an aid in fertilizing vegetable crops. p. 365–379. In: L. M. Walsh and J. D. Beaton (eds.) Soil testing and plant analysis (rev. ed.). Soil Sci. Soc. of Amer., Madison, Wisc.
6. Hale, J. B. and S. G. Heintze. 1946. Manganese toxicity affecting crops on acid soil. Nature 157:554.
7. Jackson, M. L. 1958. Soil chemical analysis. Prentice-Hall, Englewood Cliffs, N.J.
8. Jackson, T. L., H. H. Rampton, and J. McDermid. 1964. The effect of lime and phosphorus on the yield and phosphorus content of legumes in western Oregon. Ore. Agr. Expt. Sta. Tech. Bul. 83.
9. Jackson, T. L., D. T. Westermann, and D. P. Moore. 1966. The effect of chloride and lime on the manganese uptake by bush beans and sweet corn. Soil Sci. Soc. Amer. Proc. 30:70–73.
10. Lathwell, D. J. and M. Peech. 1964. Interpretation of chemical tests. N.Y. Agr. Expt. Sta. Bul. 995.
11. Longnecker, D. and F. G. Merkle. 1952. Influence of placement of lime compounds on root development and soil characteristics. Soil Sci. 73:71–74.
12. Magistad, O. C. 1925. The Al content of the soil solution and its relation to soil reaction and plant growth. Soil Sci. 20:181–225.
13. Mansour, N. S. and G. Crabtree. 1979. Vegetable crops. p. 56–70. In: B. D. Brewster (ed.) Oregon weed control handbook. Oregon State University Extension Service, Corvallis.
14. Martin, E. W., J. Vlamis, and N. W. Stice. 1953. Field correction of calcium deficiency on a serpentine soil. Agron. J. 45:204–208.
15. Mortensen, W. P., A. S. Baker, and P. Dermanis. 1964. Effects of cutting frequency of orchard grass and nitrogen rate on yield, plant nutrient composition and removal. Agron. J. 56:316–320.
16. Volk, G. M. 1955. Factors determining the effect of various fertilizer materials on acidity in the soil profile. Fla. State Hort. Soc. Proc. 68:220–226.
17. Wear, J. I. 1956. Effect on soil pH and calcium on uptake of zinc by plants. Soil Sci. 81:311–315.
18. Wright, K. E. 1943. Internal precipitation of phosphorus in relation to aluminum toxicity. Plant Physiol. 18:708–712.

J. Amer. Soc. Hort. Sci. 107(5):744–750. 1982.

Identification of Flavonoid Chemical Markers in Roses and their High Pressure Liquid Chromatographic Resolution and Quantitation for Cultivar Identification[1]

S. Asen[2]

Florist and Nursery Crops Laboratory, Agricultural Research Service, Beltsville, MD 20705

Additional index words. chemotaxonomy, fingerprinting, quercetin glycosides, kaempferol glycosides, anthocyanins, *Rosa* sp

Abstract. **Flavonoids in flowers of 'White Masterpiece', 'Bridal Pink', and 'Samantha' roses (*Rosa* spp) were either kaempferol or quercetin 3-glycosides. They were the glucoside, xyloside, arabinoside, rhamnoside, glucuronide, rutinoside, a rhamnosylglucoside (not rutinose or neohesperidose), an acylated form of the rhamnosylglucoside, and galloylglucoside. Kaempferol 4'-glucoside, an important marker derived almost exclusively from old Scotch roses (*R. foetida* and *R. spinossisima*) was present only in 'White Masterpiece'. Anthocyanins from 'Bridal Pink' and 'Samantha' were either cyanidin or pelargonidin 3,5-diglucosides along with traces of the related 3-glucoside. Parameters for the resolution and the quantitation of these flavanoids, by high pressure liquid chromatography, were established. Uniform sampling techniques were developed because of the quantitative differences in the flavonoid distribution in a rose petal. Changes in the rate of fertilizer application and daylength affected only the concentration of the flavonoids, and the ratio of each compound to the total remained fairly constant.**

Flavonoids, ubiquitous in higher plants, have been widely used as taxonomic chemical markers. Plants usually contain complex mixtures of these compounds and column or thin-layer chromatographic (TLC) analyses of the various derivatives is complex, slow, and difficult to quantitate. High pressure liquid chromatography (HPLC) has been found to be applicable to the analysis of complex natural mixtures of flavonoids for rapid screening and "fingerprinting" purposes (1, 2, 18, 19, 21). The use of these chemical markers as an adjunct to the classical methods presently used for plant identification will provide a more positive identification of new cultivars, particularly those protected by the plant patent law. This study was initiated to identify flavonoid chemical markers present in roses and to develop proper sampling techniques and HPLC procedures for their resolution and quantitation.

[1]Received for publication Nov. 6, 1981.

The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

[2]Physiologist, Florist and Nursery Crops Laboratory, Horticultural Science Institute, Beltsville Agricultural Research Center. The author gratefully acknowledges the assistance of P. Semeniuk and P. S. Budin.

[3]Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Dept. of Agriculture and does not imply its approval to the exclusion of other products or venders that may also be suitable.

Materials and Methods

Fertilizer and light treatments. 'White Masterpiece', 'Bridal Pink', and 'Samantha' roses were grown in 2-gal black plastic

containers under standard greenhouse cultural practices at 24°/16°C (day/night temperatures). Fertilizer and light treatments were started Nov. 2, 1980, and were as follows:

A) Full fertilizer—once a week application of Peters[3] (20%N–8.6%P–16.6%K) soluble fertilizer using a GEWA injector adjusted to a rate of 1–20.
B) 1/2 fertilizer—same as A, but at a rate of 1–40.
C) Full fertilizer plus light—same as A with 4 hrs supplemental light (2200-0200 HR daily) from incandescent lamps (0.216 Klx).
D) 1/2 fertilizer plus light—same as B and supplemental light as C.

Flavonoid isolation and identification. Petals from each cv were dried at 40°C in a forced-air oven and then ground to pass a 40-mesh screen. 'Bridal Pink' and 'Samantha' tissue, which contained anthocyanins, were extracted with 1% HCl in MeOH, followed by hot MeOH until free of flavonoids. 'White Masterpiece' tissue was extracted only with hot MeOH. Flavonoids in each extract were initially banded on a 25 × 400-mm column of purified polyvinylpyrrolidone (PVP) by methods previously described (2). Compounds present in each band were resolved and purified either by HPLC on a 7.8 × 300-mm column of Bondapak C_{18} (octadecyltrimethylchlorosilane chemically bonded to 10 μm porasil packing) or by preparative layer chromatography (PLC) on 2-mm celulose plates (Table 1).

Purified anthocyanins, from 'Bridal Pink' and 'Samantha' roses, were partially hydrolyzed by refluxing in EtOH:2N HCl (1:1) at 80°C for 30 min. Samples were removed at 5 min intervals for chromatographic examination for any intermediate compounds formed. Complete hydrolysis was accomplished at 100° for an additional 90 min. The anthocyanidin was extracted with iso-amyl alcohol and identified by co-chromatography with authentic standards. For solvents and comparable Rf values, refer to Harborne (13, p. 17, 31). The sugar moiety in the aqueous residue was determined by methods previously described (4).

Table 1. Flavonols from 'White Masterpiece', 'Bridal Pink', and 'Samantha' roses.

Bands eluted from PVP	Final resolution or purification	Absorption band no.	[z]Products of partial and complete hydrolysis	Identified as:
White Masterpiece or Bridal Pink		*(Fig. 1)*		
1	PLC, H_2O	1	Km3-glu; Km; glu; rha	Km3-rutinoside
2	HPLC, 20% CH_3CN in 2% HOAc	9	Km3-glu; Km; glu; rha	Km3-rhamnosylglucoside (acylated)
3	HPLC, 20% CH_3CN in 2% HOAc	4	Km; glu	Km3-glucoside
		8	Km; rha	Km3-rhamnoside
4	PLC, 15% HOAc	6	Km; xyl	Km3-xyloside
		5	Km; glu	[x]Km4'-glucoside
5	HPLC, 20% CH_3CN in 2% HOAc	7	Km; arab	Km3-arabinoside
6	HPLC, 20% CH_3CN in 2% HOAc	3	[y]Km; glucuronic acid	Km3-glucuronide
7	Band removed from PVP column and compound extracted in soxhlet with MeOH for 1 wk	2	Km3-glu; Km; glu; gallic acid	Km3-galloylglucoside
Samantha		*(Fig. 2)*		
1	HPCL, 30% CH_3CN in 2% HOAc	5	rQu3-glu; Qu; glu; rha	Qu3-rhamnosylglucoside
		11	Qu3-glu; Qu; glu; rha	Qu3-rhamnosylglucoside (acylated)
2	HPLC, 25% CH_3CN in 2% HOAc	1	Qu3-glu; Qu; glu; rha	Qu3-rutinoside
3	PLC, Me_2CO:H_2O:HOAc (30 87 3)	6	Qu; arab	Qu3-arabinoside
4	PLC, 2-PrOH:H_2O:HOAc (22 76 2)	7	Km; glu	Km3-glucoside
		10	Km; rha	Km3-rhamnoside
5	PLC, 15% HOAc	2	Qu; glu	Qu3-glucoside
		7	Qu; rha	Qu3-rhamnoside
6		4	Qu; xyl	Qu3-xyloside
7	Band removed from PVP column and compound extracted in soxhlet with MeOH for 1 wk.	3	Qu3-glu; Qu; glu; gallic acid	Qu3-galloylglucoside

[z]Abbreviations: Km = kaempferol; Qu = quercetin; glu = glucose; rha = rhamnose; xyl = xylose; arab = arabinose.
[y]Hydrolyzed with β-glucuronidase.
[x]Present only in 'White Masterpiece'.

Table 2. Typical distribution of flavonoids within a 'White Masterpiece' rose petal[z].

Kaempferol glycosides	Flavonoid concn (μ g/15-mm disk) Section of petal sampled[y] 1	2	3	4	5
3-rutinoside	4.7	4.0	4.2	4.3	2.5
3-galloylglucoside	6.4	7.4	14.3	7.2	8.6
3-glucuronide	20.8	22.3	68.5	21.6	35.8
3-glucoside	51.2	57.0	80.0	53.8	67.9
4'-glucoside	42.8	49.2	42.7	51.8	29.9
3-xyloside	14.8	16.8	26.0	16.1	23.9
3-arabinoside	4.1	5.2	11.1	5.0	6.0
3-rhamnoside	84.2	69.3	73.9	66.6	79.2
3-rhamnosylglucoside (acylated)	4.8	6.1	11.7	6.5	5.0
TOTAL	233.8	237.3	332.4	232.9	258.8

[z]Bud stage #6 (see ref. 3), 5th petal, sampled Jan. 15, 1981.
[y]A 15-mm disk: section 1 = apex; section 2 = equidistant from center to right edge; section 3 = base; section 4 = equidistant from center to left edge; section 5 = center.

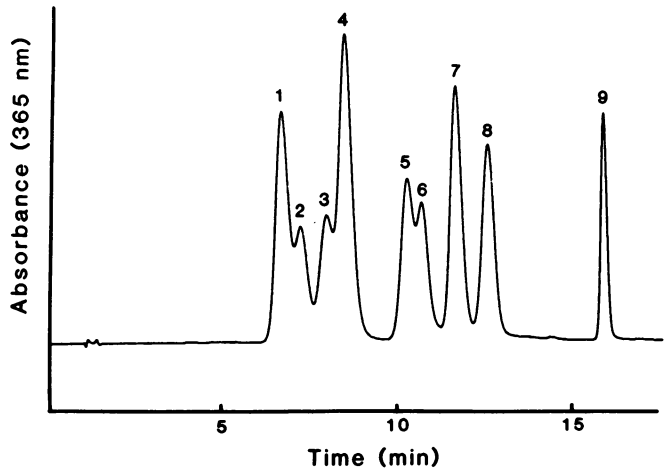

Fig. 1. HPLC separation of the flavonoid standards found in 'White Masterpiece' and 'Bridal Pink' roses. Column, radial pak A (C_{18}) with RCSS; solvents, 1% TEAP (pump A) and CH_3CN (pump B); gradient, non-linear (Waters #8) of 20–50% CH_3CN over a 20-min period at a flow rate of 3.0 ml/min with detection at 365 nm. 1 = kaempferol 3-rutinoside, 2 = kaempferol 3-galloylglucoside, 3 = kaempferol 3-glucuronide, 4 = kaempferol 3-glucoside, 5 = kaempferol 4'-glucoside, 6 = kaempferol 3-xyloside, 7 = kaempferol 3-arabinoside, 8 = kaempferol 3-rhamnoside, 9 = kaempferol 3-rhamnosylglucoside (acylated).

Procedures used for the acid hydrolysis of the flavonols were the same as those used for the anthocyanins, except that the initial temperature was 65°C and the aglycones were extracted with EtOAc. Flavonols, and their hydrolysate products, were compared to authentic standards and they were identified by spectral and chromatographic procedures. For comparable Rf and absorption spectra refer to Harborne (13, p. 69) and Mabry et. al (14), respectively. Authentic standards of kaempferol 3-rhamnosylglucoside, kaempferol 3-rhamnosylglucoside (acylated), kaempferol 3-galloylglucoside and the quercetin anologs were not available. Kaempferol 3-glucuronide (4 mg in 5 ml H_2O), difficult to acid hydrolyze, was hydrolyzed with an equal weight of β-glucuronidase for 17 hr at 37°.

The presence of an acyl moiety was determined by base hydrolysis with 1N NaOH for 30 min at room temperature in an atmosphere of N_2. After acidification the solution was extracted with Et_2O and the acyl moiety examined chromatographically and spectrophotometrically. Gallic acid was determined chromatographically (11) and by HPLC with 3% CH_3CN in 2% HOAc at a flow rate of 2.5 ml/min with detection at 280 nm.

Flavonoid resolution and quantitation by HPLC. Samples were taken from fresh flowers and they consisted of eight 15-mm disks. A disk, from sections 2 and 4 (Table 2), was taken from each of petals 4 to 7 from partially opened flowers. Maturity of flowers corresponded to stage 6 (3). Tissue for anthocyanin analysis was extracted by blending in 50 ml 1% HCl-MeOH. A separate but duplicate sample for flavonol analysis was extracted by blending in 50 ml MeOH. Extracts were filtered and reduced to dryness under reduced pressure at 40°C. The dried extracts were taken up in 1 ml of a solution 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). Anthocyanins present in 'Samantha' and 'Bridal Pink' roses were resolved and quantitated isocratically with a solvent containing 7% HOAc, 8.75% CH_3CN and 1.5% H_3PO_3 at a flow rate of 2.5 ml/min and detection at 546 nm. Separation of all known anthocyanins in roses was accomplished by gradient elution using a modification of the procedure of Strack et al (21). Flavonols present in 'Bridal Pink' or 'White Masterpiece' roses were resolved and quantitated by gradient elution with 20–50% CH_3CN in ca 1% TEAP (triethylamine buffered to pH 3 with H_3PO_3) using a non-linear gradient (Waters #8) over a 20 min period at a flow rate of 3.0 ml/min with detection at 365 nm. Two solvent systems were needed to resolve and quantitate the flavonols in Samantha roses. The first was 20–50% CH_3CN in ca 1% TEAP using a non-linear gradient (Waters #7) over a 25 min period at a flow rate of 3.0 ml/min. These parameters did not resolve kaempferol 3-glucoside and quercetin 3-rhamnoside (Fig. 2, band 7). Quercetin 3-rhamnoside was resolved and quantitated with 22–50% tetrahydrofuran (THF) in ca 1% TEAP using a non-linear gradient (Waters #8) over a 25 min period at a flow rate of 2.0 ml/min.

Results

Flavonoids from 'White Masterpiece.' Nine flavonols (Table 1) were identified or characterized, and all but one had absorption spectra characteristic of kaempferol 3-substituted compounds (14). The one compound (Fig. 1, No. 5) lacking all the characteristic 3-substituted spectra had a λ_{max}(MeOH) of 363 nm (band 1) and 267 (band 2). A marked decrease in adsorption of band 1, on the addition of NaOMe, indicated a 4'-substitution. Changes in Rf before and after base hydrolysis of the compound isolated from PVP (band 2) are indicative of the presence of an acyl moiety. This acyl moiety was not a typical cinnamic acid. Spectral examination revealed no apparent UV absorption. This compound (Fig. 1, No. 9) is an acylated kaempferol 3-rhamnosylglucoside, but not a rutinoside (rhamnose 1 → 6 glucose) or a neohesperidoside (rhamnose 1 → 2 glucose). These three kaempferol 3-rhamnosylglucoside isomers were resolved by HPLC

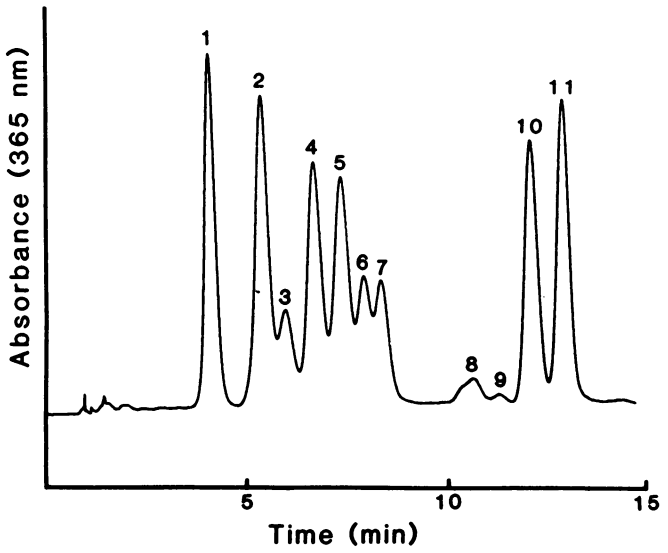

Fig. 2. HPLC separation of the flavonoid standards found in 'Samantha' roses. Column, radial pak A (C_{18}) with RCSS; solvents, 1% TEAP (pump A) and CH_3CN (pump B); gradient, non-linear (Waters #7) of 20–50% CH_3CN over a 25-min period at a flow rate of 3.0 ml/min with detection at 365 nm. 1 = quercetin 3-rutinoside, 2 = quercetin 3-glucoside, 3 = quercetin 3-galloylglucoside, 4 = quercetin 3-xyloside, 5 = quercetin 3-rhamnosylglucoside, 6 = quercetin 3-arabinoside, 7 = quercetin 3-rhamnoside + kaempferol 3-glucoside, 8 = contaminate, 9 = contaminate, 10 = kaempferol 3-rhamnoside, 11 = quercetin 3-rhamnosylglucoside (acylated).

with 20–50% CH_3CN in ca 1% TEAP using a non-linear gradient (Waters #7) over a 15-min period at a flow rate of 3 ml/min. Recently, Seshadri and Vydeeswaran (17) identified a kaempferol 3-rhamnosylglucoside (rhamnose 1 → 3 glucose) from *Rungia repens* flowers. The compound from band 2 may be an acylated form of this kaempferol 3-rhamnosylglucoside.

The flavonol responsible for band 2, Fig. 1, exhibited a stronger affinity for PVP than the other kaempferol glycosides and was retained on the column. This band was cut from the PVP column and the compound extracted in a soxhlet with MeOH for 1 wk. Chromatographic and HPLC examination of the acyl moiety was indistinguishable from an authentic sample of gallic acid and the strong affinity of this flavonol for PVP is consistent with the findings of Collins et al. (11) for flavonol glycoside gallates. Atypical of simple kaempferol 3-glycosides, in MeOH this compound exhibited absorption at 265–275 nm due to gallic acid (11), a strong absorption band at 300 nm with the addition of H_3BO_3/NaOAc, and the reduction to a shoulder of the 302-nm band obtained with $AlCl_3$, on the addition of HCl. The UV absorption spectra, in MeOH, of this compound was simulated with a solution containing a 3.6×10^{-5}M each of kaempferol 3-glucoside and gallic acid.

Flavonoids from 'Bridal Pink'. Four anthocyanins were isolated and they were present primarily in band 1 from PVP. They were resolved and purified by PLC with HOAc:HCl:H_2O (15-3-82 v/v) and n-BuOH:HOAc:H_2O (6-1-2 v/v). The major anthocyanin was cyanidin 3,5-diglucoside. The minor anthocyanin was pelargonidin 3,5-diglucoside. Two other anthocyanins were present in trace amounts and they were cyanidin 3-glucoside and pelargonidin 3-glucoside.

Eight flavonols were present and they were the same kaempferol 3-glycosides as those from 'White Masterpiece' roses. Kaempferol 4'-glucoside was not detected.

Flavonoids from 'Samantha'. Two anthocyanins, eluted primarily in band 1 from PVP, were isolated. The major one was cyanidin 3,5-diglucoside. The second anthocyanin, present in trace amounts, was cyanidin 3-glucoside.

Ten flavonols (Table 1) were identified or characterized. Two had adsorption spectra characteristic of kaempferol 3-substituted compounds and 8 were those of quercetin 3-substituted compounds. Compound 11, Fig. 2, is apparently the quercetin analog of kaempferol 3-rhamnosylglucoside (acylated) isolated from

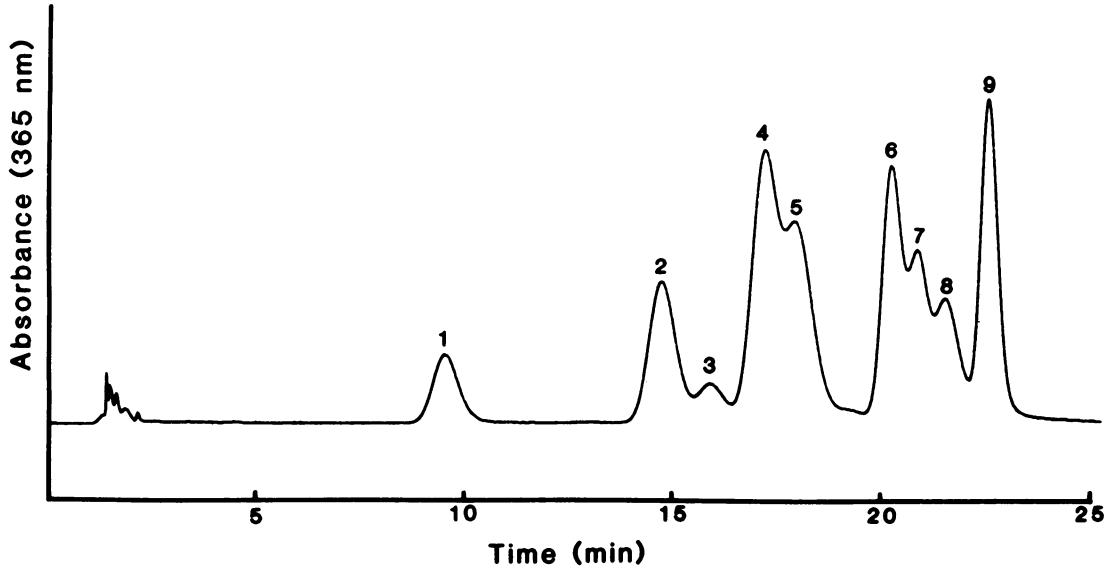

Fig. 3. HPLC separation of the flavonoid standards found in 'Samantha' roses. Column, radial pak A (C_{18}) with RCSS; solvents 1% TEAP (pump A) and THF (pump B); gradient, non-linear (Waters #8) of 20–50% THF over a 25-min period at a flow rate of 2.0 ml/min with detection at 365 nm. 1 = quercetin 3-rutinoside, 2 = quercetin 3-glucoside, 3 = contaminate, 4 = quercetin 3-rhamnosylglucoside + kaempferol 3-glucoside, 5 = quercetin 3-xyloside, 6 = quercetin 3-rhamnoside, 7 = quercetin 3-arabinoside, 8 = quercetin 3-galloylglucoside, 9 = quercetin 3-rhamnosylglucoside (acylated) + kaempferol 3-rhamnoside.

'Bridal Pink' or 'White Masterpiece'. The structure of these compounds will be determined when more material is available. After base hydrolysis compound 11, Fig. 2, reverted to compound 5, Fig. 2. Quercetin 3-galloylglucoside (compound 3, Fig. 2) also exhibited the atypical spectral characteristics and a strong affinity for PVP as did kaempferol 3-galloylglucoside isolated from 'White Masterpiece' or 'Bridal Pink'. The UV absorption spectra in MeOH of quercetin 3-galloylglucoside was simulated with a solution containing 3.6×10^{-5}M each of quercetin 3-glucoside and gallic acid.

HPLC resolution of rose anthocyanins. Although roses exhibit a wide range of colors, only 6 anthocyanins are known to occur in the petals (12). They are the 3,5-diglucosides and the 3-glucosides of pelargonidin, peonidin and cyanidin. These 6 anthocyanins can be resolved with a single solvent system (Fig. 4) by using a modification of the procedure of Strack et al (21).

HPLC resolution of rose flavonols. Parameters for the resolution of the kaempferol glycosides present in 'Bridal Pink' or 'White Masterpiece' roses with a single solvent system are shown in Fig. 1. 'Samantha' roses, which contain both kaempferol and quercetin glycosides, required the use of 2 solvent systems for the quantitation of all compounds present (Figs. 2, 3). The only compounds not resolved with CH_3CN were quercetin 3-rhamnoside and kaempferol 3-glucoside. Since quercetin 3-rhamnoside was resolved with THF, each compound can be quantitated simply by subtracting the value for quercetin 3-rhamnoside from that of quercetin 3-rhamnoside plus kaempferol 3-glucoside.

Distribution of flavonoids within a rose petal. All flavonols characterized or identified from 'White Masterpiece' roses were present in each section of the petal examined, but their distribution was not uniform (Table 2). There was a greater accumulation of most compounds at the base, and variations also were evident at the center and apex. The first 10 petals showed the same distribution pattern for all 3 cultivars examined. Visual observations of 'Bridal Pink' petals showed a decrease in anthocyanins at the base and a slight accumulation in the peripheral zones. Except for a slight decrease at the base, 'Samantha' roses exhibited a uniform distribution of anthocyanins within petals. For sampling, the most uniform distribution of flavonoids was from tissue equidistant from the center of the petal to the left and right edges (Table 2, sections 2, 4).

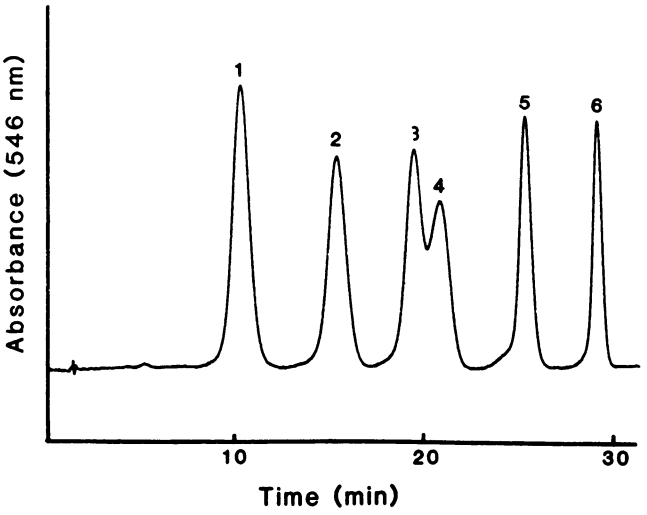

Fig. 4. HPLC separation of the known anthocyanin standards found in roses. Column, radial pak A (C_{18}) with RCSS; solvents, 1.5% H_3PO_4 (pump A) and 20% HOAc, 25% CH_3CN in 1.5% H_3PO_3 (pump B); gradient, non-linear (Waters #8) 25–100% of solvent (pump B) over a 45-min period at a flow rate of 2.5 ml/min with detection at 546 nm. 1 = cyanidin 3,5-diglucoside, 2 = pelargonidin 3,5-diglucoside, 3 = cyanidin 3-glucoside, 4 = peonidin 3,5-diglucoside, 5 = pelargonidin 3-glucoside, 6 = peonidin 3-glucoside.

Distribution of flavonoids among petals. The concentration of each flavonol in the first 10 petals of 'White Masterpiece' rose was not always constant (Table 3). Kaempferol 3-glucuronide and kaempferol 3-rhamnoside showed the greatest variation and the concentration of each decreased with increased petal number. Flavonols in 'Bridal Pink' and 'Samantha' rose petals showed similar variations. The concentration of anthocyanins in the first 10 petals of 'Bridal Pink' and 'Samantha' rose petals was fairly constant. For uniformity, tissue selected for sampling consisted of eight 15-mm disks taken equidistant from the center to the left and right edges of petals 4–7.

Environmental effect on flavonoid composition. Both qualitative and quantitative changes in the flavonoid composition of plants have been reported, due to variations in daylength, light

Table 3. Distribution of flavonoids in sections 2 and 4[z] from the first 10 petals of 'White Masterpiece' rose.

	Flavonoid concn (μ g/2-15-mm disks[y])									
	Petal No.									
Kaempferol glycosides	1	2	3	4	5	6	7	8	9	10
3-rutinoside	8.6	8.4	8.2	8.0	7.6	8.5	8.5	8.7	8.9	8.7
3-galloylglucoside	22.9	22.9	22.5	23.5	23.0	23.9	22.7	24.4	23.0	23.8
3-glucuronide	105.7	103.3	97.1	89.3	81.4	77.2	75.8	72.4	66.9	64.5
3-glucoside	181.6	179.6	178.0	183.3	175.1	176.5	166.9	169.8	165.5	163.0
4'-glucoside	89.4	93.0	105.0	109.4	122.7	142.0	139.0	151.6	156.4	149.7
3-xyloside	61.1	56.6	56.3	57.5	54.7	53.0	50.4	52.9	51.2	49.5
3-arabinoside	16.0	15.5	16.4	16.4	17.1	17.0	17.7	18.6	17.1	17.0
3-rhamnoside	247.0	230.7	212.7	202.1	175.3	157.1	148.7	145.4	137.7	134.4
3-rhamnosylglucoside (acylated)	15.0	16.6	17.7	16.0	17.9	15.3	14.9	13.8	14.2	14.4
TOTAL	747.3	726.6	703.9	705.5	674.8	670.5	638.6	657.6	640.9	625.0

[z]See footnote Table 1.
[y]Average of 5 flowers sampled Jan. 16–26, 1981.

intensities, mineral nutrition, and herbicide applications (5, 6, 8, 9, 10, 15, 16, 20). The effect of variations in daylength and mineral nutrition on flavonoids in 'White Masterpiece', 'Bridal Pink', and 'Samantha' roses is shown in Table 4. The general effect of reducing the amount of fertilizer applied was to increase the concentration of flavonoids. The effect was most pronounced with 'Bridal Pink' and 'Samantha' and least effective with 'White Masterpiece'. The effect of 4 hr of supplemental light at each fertilizer treatment was more variable. The additional light at the full fertilizer treatment was most effective in decreasing the concentration of the flavonoids in 'Bridal Pink' and 'White Masterpiece' and almost ineffective with 'Samantha'. Additional light with the one-half fertilizer treatment had a slight effect in decreasing flavonoid concentration in 'Samantha' and 'Bridal Pink', except for a slight increase in pelargonidin 3,5-diglucoside, the minor anthocyanin present in 'Bridal Pink'. In contrast, one-third of the flavonols in 'White Masterpiece' showed an increase. Regardless of effect, it has been demonstrated that the concentration of flavonoids in rose flowers varies with changes in fertilizer and light treatments. Although concentrations varied with treatment, the percentage of the total for each compound remained fairly constant as shown for 'Samantha' (Table 5). Similar effects were evident for 'Bridal Pink' and 'White Masterpiece'.

Table 5. Effect of fertilizer and light on the composition of each flavonoid isolated from 'Samantha' roses (April 23–May 1, 1981).

	Flavonoid distribution (% of total)			
Flavonoids[z]	Full fert.[y]	1/2 fert.	Full fert. & light	1/2 fert. & light
Qu 3-rutinoside	1.1	1.0	1.1	1.1
Qu 3-glucoside	0.6	0.7	0.7	0.7
Qu 3-galloylglucoside	5.0	4.3	3.9	4.2
Qu 3-xyloside	1.2	1.2	1.2	1.2
Qu 3-rhamnosylglucoside	0.7	0.7	0.6	0.6
Qu 3-rhamnosylglucoside (acylated)	1.2	1.2	1.1	1.1
Qu 3-arabinoside	1.5	1.7	1.6	1.6
Qu 3-rhamnoside	9.6	7.9	10.5	8.5
Km 3-glucoside	0.9	1.0	0.8	0.7
Km 3-rhamnoside	0.7	0.7	0.7	0.6
Cy 3,5-diglucoside	76.9	78.9	77.1	79.1
Cy 3-glucoside	0.6	0.7	0.7	0.6

[z]Abbreviation: Km = kaempferol; Qu = quercetin; Cy = cyanidin.
[y]See methods for treatments.

Discussion

The identification of plants by their chemical constituents, as an adjunct to classical methods, offers an excellent objective means for positive identification. All inherent morphological manifestations of cultivar differences must ultimately have a biochemical difference, but not all biochemical differences are expressed morphologically. Thus, biochemical differences should be more numerous than morphological differences. The loss or modification of a single step in a biochemical pathway might occur without any significant morphological changes, and plants which are judged similar, on the basis of their morphological characteristics, might differ widely in their chemical constitution.

Among all plant chemical constituents, flavonoids have been more widely used as chemical markers than any other group of plant substances. The advantages, over most other low molecular weight constituents, are their universal distribution in vascular plants, considerable structural diversity, chemical stability, and the ease and rapidity of identification. The exceptional usefulness of flavonoids is that they are not actively concerned in cellular metabolic processes. Although the concentration of flavonoids can be influenced by the tissue sampled and changes in

Table 4. Effect of light and fertilizer on flavonoids in 'White Masterpiece', 'Bridal Pink' and 'Samantha' roses.

	Flavonoid concn (μ g/8-15mm disks[z])											
	White Masterpiece				Bridal Pink				Samantha			
Flavonoids[y]	Full fert.[x]	1/2 ert.	Full fert. & light	1/2 fert. & light	Full fert.	1/2 fert.	Full fert. & light	1/2 fert. & light	Full fert.	1/2 fert.	Full fert. & light	1/2 fert. & light
Km 3-rutinoside	17.2a	31.1b	13.3a	39.0b	43.7ab	59.8c	35.4a	48.7b	—	—	—	—
Qu 3-rutinoside	—	—	—	—	—	—	—	—	30.3a	37.2b	33.2a	38.6b
Km 3-galloylglucoside	65.1ab	69.0b	52.5a	91.7c	35.8a	45.4b	36.3a	40.2ab	—	—	—	—
Qu 3-galloylglucoside	—	—	—	—	—	—	—	—	141.5ab	156.7b	120.8a	154.7b
Km 3-glucuronide	224.1b	240.1b	167.1a	259.3b	88.8b	110.9c	69.2a	96.5bc	—	—	—	—
Km 3-glucoside	456.5b	593.1c	353.0a	702.8b	189.3b	268.8d	158.6a	229.9c	25.0a	35.1b	23.9a	26.7a
Qu 3-glucoside	—	—	—	—	—	—	—	—	17.8a	27.5b	21.9ac	25.5bc
Km 4'-glucoside	376.8b	490.6c	210.4a	650.8d	—	—	—	—	—	—	—	—
Km 3-xyloside	142.8ab	168.2bc	123.9a	186.5c	90.8b	103.6c	74.0a	90.3b	—	—	—	—
Qu 3-xyloside	—	—	—	—	—	—	—	—	32.5a	42.5bc	36.6ab	42.9c
Km 3-arabinoside	44.0b	51.1bc	32.4a	61.2c	57.7a	72.8b	50.9a	66.9b	—	—	—	—
Qu 3-arabinoside	—	—	—	—	—	—	—	—	43.4a	59.5b	50.2a	59.3b
Km 3-rhamnoside	580.5a	562.8a	565.4a	510.3a	307.2b	307.9b	236.4a	259.1ab	20.0a	23.6a	22.1a	20.6a
Qu 3-rhamnoside	—	—	—	—	—	—	—	—	269.3a	286.0ab	329.0b	311.3ab
Qu 3-rhamnosylglucoside	—	—	—	—	—	—	—	—	19.8a	29.3b	18.6a	20.5a
Km 3-rhamnosylglucoside(acylated)	55.8b	42.1ab	32.0a	51.4b	65.3b	48.4ab	42.0a	43.8a	—	—	—	—
Qu 3-rhamnosylglucoside(acylated)	—	—	—	—	—	—	—	—	33.9a	43.5a	34.1a	38.7a
Cy 3,5-diglucoside	—	—	—	—	45.4a	97.7b	49.9a	111.1b	2162.7a	2851.8c	2393.2ab	2883.0c
Cy 3-glucoside	—	—	—	—	1.4a	1.0a	1.2a	0.9a	16.8a	24.2b	21.3ab	22.0ab
Pg 3,5-diglucoside	—	—	—	—	12.9a	22.6b	12.0a	30.8c	—	—	—	—
Pg 3-glucoside	—	—	—	—	2.9a	1.7a	2.6a	1.8a	—	—	—	—

[z]Sections 2 & 4 (Table 1) from each of petals 4 to 7 sampled April 23–May 12, 1981, mean separation by Fisher's LSD method, 5% level. Avg for 4 flowers.
[y]Abbreviations: Km = kaempferol; Qu = quercetin; Cy = cyanidin; Pg = pelargonidin.
[x]Abbreviation: fert., fertilizer (see methods for treatments).

environment, any particular flavonoid constituent can be relied on to be present in more or less constant amounts when uniformly sampled from plants grown under the same environment.

Flavonoids isolated from 'White Masterpiece', 'Bridal Pink', and 'Samantha' roses were glycosides of pelargonidin and kaempferol and, in most cases, their cyanidin and quercetin analogs. Those not present in the 3 cultivars examined, but previously reported in roses, were quercetin 3-glucuronide (7) and quercetin 4'-glucoside (13). Trace amounts of kaempferol 3-rhamnosylglucoside were found in 'White Masterpiece', but were not reported. Chromatographic techniques most widely used for the resolution of flavonoids from plant extracts have been column, paper or thin-layer. Although good results have been obtained with these techniques, they are not easily adapted to quantitation and many closely related compounds cannot adequately be resolved. Differences between the flavonoid constitutents in plants are either qualitative or quantitative. Qualitative differences present no problem since a chemical marker present in one plant and not in another results in a positive identification. Unfortunately, most differences at the cultivar level have been quantitative. One of the advantages of using HPLC over conventional chromatographic methods is the ease of quantitation. Other advantages are shorter time for analysis, high sensitivity and resolution, and no derivatization or risk of thermal decomposition.

Flavonols present in 'White Masterpiece' and 'Bridal Pink' roses were kaempferol glycosides and they could be easily resolved by HPLC with a single solvent system. Samantha roses contained both kaempferol and quercetin glycosides and two solvent systems were required for the resolution and quantitation of all compounds. With roses that may contain a more complex mixture of kaempferol and quercetin glycosides than 'Samantha', slight modifications of the established HPLC parameters may be necessary for complete resolution.

Changes in the rate of fertilizer application and daylength affected the concentration of flavonoids in rose flowers, but the ratio of each compound to the total remained fairly constant. If this consistency were to be maintained over greater environmental changes, then the shape of the absorption profiles for the resolved flavonoids, obtained by HPLC, also could be used as an aid for positive identification.

The value of chemical characters, whether they be flavonoids or other plant constituents, as an aid for plant identification, has been demonstrated. The use of flavonoid chemical markers as an adjunct to the classical methods now being used will help resolve the difficulties of describing new rose cultivars, particularly those protected by the plant patent law and also will permit the positive identification of germplasm.

Literature Cited

1. Asen, S. 1977. Flavonoid chemical markers as an adjunct for cultivar identification. HortScience 12:447–448.
2. Asen, A. 1979. Flavonoid chemical markers in Poinsettia bracts. J. Amer. Soc. Hort. Sci. 104:223–226.
3. Asen, S. and M. Lieberman. 1963. Ethylene oxide experimentation aimed at cut flower longevity. Flor. Rev. 131:27–28.
4. Asen, S. and P. S. Budin. 1966. Cyanidin 3-arabinoside-5-glucoside, an anthocyanin with a new glycosidic pattern, from flowers of 'Red Wing' azaleas. Phytochemistry 5:1257–1261.
5. Asen, S., N. W. Stuart, and H. W. Siegelman. 1959. Effect of various concentrations of nitrogen, phosphorus and potassium on sepal color of *Hydrangea macrophylla*. Proc. Amer. Soc. Hort. Sci. 73:495–501.
6. Asen, S., L. L. Jansen, and J. L. Hilton. 1963. Effects of 2,2-dichloropropionic, trichloroacetic, and 2,3-dichlorisobutyric acids on synthesis of anthocyanin in flowers of *Salvia splendens* cv. America. Nature 198:185–186.
7. Asen, S., K. H. Norris,and R. N. Stewart. 1971. Effect of pH and concentration of the anthocyanain-flavonol co-pigment complex on the color of 'Better Times' roses. J. Amer. Soc. Hort. Sci. 96:770–773.
8. Ball, G. A., E. O. Beal, and E. A. Flecker. 1967. Variation in chromatographic spot pattern of two species of clonal plants grown under controlled environmental conditions. Brittonia 19:273–279.
9. Biran, I. and A. H. Halvey. 1974. Effects of varying light intensities and temperature treatments applied to whole plants, or locally to leaves or flower buds, on growth and pigmentation of 'Baccara' roses. Physiol. Plant. 31:175–179.
10. Blume, D. E. and J. W. McClure. 1978. C-glycosylflavone accumulation in sandoz 6706 treated barley seedlings is a photocontrolled response. Phytochemistry 17:1549–1551.
11. Collins, F. W., B. A. Bohm, and C. K. Wilkins. 1975. Flavonol glycoside gallates from *Tellima grandiflora*. Phytochemistry 14:1099–1102.
12. Harborne, J. B. 1961. The anthocyanins of roses. Ocurrence of peonin. Experientia 17:72–73.
13. Harborne, J. B. 1967. Comparative biochemistry of the flavonoids. Academic Press, New York.
14. Mabry, T. J., K. R. Markham, and M. B. Thomas. 1970. The systematic identification of flavonoids. Springer-Verlag, New York.
15. McClure, J. W. and R. E. Alston. 1964. Patterns of selected chemical components of *Spirodela oligorhiza* formed under various components of axenic culture. Nature 201:311–313.
16. Parks, C. R., S. S. Sandhu, and K. R. Montgomery. 1972. Floral pigmentation studies in the genus Gossypium IV. Effect of different growing environments on flavonoid pigmentation. Amer. J. Bot. 59:158–164.
17. Seshadri, T. R. and S. Vydeeswaran. 1972. Chrysoeriol glycosides and other flavonoids of *Rungia repens* flowers. Phytochemistry 11:803–806.
18. Stewart, R. N., S. Asen, D. R. Massie, and K. H. Norris. 1979. The identification of Poinsettia cultivars by high pressure liquid chromatographic analysis of their anthocyanin content. Biochem. System. Ecol. 7:281–287.
19. Stewart, R. N., S. Asen, D. R. Massie, and K. H. Norris. 1980. The identification of Poinsettia cultivars by high pressure liquid chromatographic analysis of their flavonol content. Biochem. System. Ecol. 8:119–125.
20. Strack, D., K. Fuisting, and G. Popovici. 1979. High-performance liquid chromatographic analysis of oat *(Avena sativa)* flavone derivatives. J. Chromatography 176:270–273.
21. Strack, D., N. Akavia, and H. Reznik. 1980. High performance liquid chromatographic identification of anthocyanins. Z. Naturforsch. 35C:533–538.