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CHEMOTAXONOMIC STUDY OF NEUTRAL COUMARINS IN ROOTS OF *CITRUS* AND *PONCIRUS* BY THIN-LAYER, GAS–LIQUID AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSES

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SUMMARY

Eight neutral coumarins were isolated from the roots of 11 *Citrus* and *Poncirus* cultivars by thin-layer chromatography (TLC). These coumarins included seselin, xanthyletin, poncitrin, osthol, suberosin and xanthoxyletin. The two others were only tentatively identified. The eight were completely resolved by gas–liquid chromatography (GLC) with two different liquid phases. With limited studies these eight were not however, completely resolved by TLC or high-performance liquid chromatography (HPLC). Values from HPLC supported the quantitative values obtained by GLC. Profiles of these eight coumarins were intrinsic to *Citrus* and *Poncirus* species, with hybrids having profiles characteristic of both genera.

INTRODUCTION

Chemotaxonomic marker compounds are being used increasingly in citrus horticulture to better define the parentage of some important cultivars and to help predict the characteristics of new hybrids prior to fruit development[1]. Diseases, cold hardiness and fruit yields of *Citrus* are often associated with specific rootstocks; hence knowledge of compounds found in certain rootstocks and not in others would be useful. In previous publications of our laboratory chemotaxonomy was used in delineating closely related species within the genus *Citrus*[2,3]. Coumarins are among the compounds which have served as chemotaxonomic markers for other workers[4]. In an earlier study on lipids associated with mycorrhizal fungus and citrus roots[5], we found about 60% of the extractable root material to be coumarins. Thus, these extracts seemed to be excellent sources of material for a chemotaxonomic study on *Citrus* rootstocks.

Seselin[6] and xanthyletin[7] are the only coumarins reported to be constituents of *Citrus* roots. Another coumarin, poncitrin, was isolated from the roots of *Poncirus*

trifoliata and appeared to be the major coumarin[8]. Use of gas–liquid chromatography (GLC) for analyzing coumarins has been limited[9–12] due primarily to the need to derivatize coumarins that contain hydroxyls or other polar groups and to the fact that many coumarins are heat and/or acid sensitive. Use of high-performance liquid chromatography (HPLC) on coumarins has been even more limited[13,14].

The current study reports the quantitation of 8 neutral coumarins from *Citrus* and *Poncirus* root extracts. One purpose of this study was to devise a GLC system that would allow all 8 coumarins to be quantified and to verify this quantification by HPLC. The second purpose was to use this GLC method to analyze the coumarins present in the roots of 6 *Citrus* rootstock varieties, *Poncirus trifoliata* and 4 *Citrus* × *Poncirus* hybrids for chemotaxonomic purposes.

METHODS*

Isolation of coumarins and structure determinations

Two- to six-month-old seedlings of 6 rootstocks were obtained from the nursery at the U.S. Horticultural Field Station, Orlando, FL, U.S.A. Two-year-old seedlings and roots from a 9-year-old seedling grapefruit tree were from the budwood liner plot and variety grove respectively, at Whitmore Foundation Farm, Leesburg, FL, U.S.A. The roots were excised, air dried and extracted for lipids with Folch reagent[5]. Individual coumarins were isolated by sequential preparative thin-layer chromatography (TLC) with chloroform[5] and cyclohexane–ethyl acetate (4:1)[6]. Purity of all fractions was determined by GLC. Portions of the 8 coumarins were hydrogenated for 1 h at room temperature and 60 p.s.i., with chloroform as solvent and 1% Pd/C as catalyst. Hydrogenated products were purified by TLC with cyclohexane–ethyl acetate (4:1). The UV spectra of natural and hydrogenated coumarins were determined in ethanol. Mass spectra were determined by gas chromatography–mass spectrometry (GC–MS) of a mixture of the 8 coumarins and a mixture of their hydrogenated derivatives. The mixtures were injected onto a 1.83 m × 2 mm, 1% SP-1000 glass column coupled with a VG Micromass 7070F mass spectrometer operated under 70 eV at 200°C.

Chromatographic analyses

TLC. Migration distances of the isolated root and reference coumarins were determined on 250-μm, 20 × 20 cm, nonactivated silica gel G plates developed with chloroform and with cyclohexane–ethyl acetate (4:1).

GLC. An HP Model 7610A chromatograph, with a flame-ionization detector (FID) and injection heaters at 250°C and helium at 60 ml/min, was equipped with glass columns (1.83 m × 2 mm I.D.). These were packed with 100–120-mesh Gas-Chrom Q that had been coated with the following liquid phases and were operated isothermally at the indicated temperatures: (1) 3% SE-30 at 170°C, (2) 3% Dexsil 300 at 205°C, (3) 1% Dexsil at 180°C, (4) 1% OV-210 at 164°C, (5) 1% Carbowax 20M–terephthalic acid at 200°C, (6) 1% SP-1000 at 205°C and (7) 0.75% Silar 10C at 190°C.

* Mention of a trademark of proprietary product is for identification only and does not recommend its approval by the U.S. Department of Agriculture to the exclusion of others which may also be suitable.

HPLC. All analyses were performed on a Varian 5000 with a Vari-Chrom UV–VIS detector and a MicroPak MCH-10 (reversed phase, 10-μm bonded C_{18} at 14% load, 30 cm × 4 mm I.D.) column. The detector was operated at an absorbance range of 0.5, a band width of 16 and an optimum wavelength of 330 nm. The optimum elution programs were: (1) methanol–water (4:1), flow-rate held constant at 0.5 ml/min for 12 min, increased at 0.15 ml/min to 2.0 ml/min over a 10-min period; (2) acetonitrile–water (3:2), flow-rate held at 0.8 ml/min for 10 min, increased at 0.12 ml/min to 2.0 ml/min over a 10-min period; (3) hexane–tetrahydrofuran, constant flow of 0.5 ml/min; time (min)–% hexane program was as follows: 0–85%, 10–75%, 15–75%, 17–70%, 20–70%, 25–85%, 30–85%.

Quantitation. All rootstocks were analyzed for their neutral coumarin profiles by GLC with the 1% SP-1000 column. From 5 to 30 μg of sample in 2–3 μl chloroform were injected on-column at 205°C. Relative percentages were calculated with the aid of an Autolab Systems IV computing integrator. The means of 2 to 12 replicates of each rootstock analyzed in duplicate are reported. The GLC-determined coumarin profiles of the various rootstocks were compared with profiles obtained by HPLC at various wavelengths.

RESULTS AND DISCUSSION

Structures

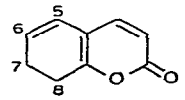
Two major fluorescent bands with R_F values of 0.43 and 0.33 were observed when the root lipid extracts were separated by TLC with chloroform. The major component of the 0.43 band was the angular seselin (I) (Table I) while the major component of the 0.33 band was the linear xanthyletin (II), as indicated by their UV spectra[6,7]. The mass spectra of these two compounds were nearly identical with *m/e* at 228 (molecular ion), 213 (base ion) and fragmentation ions at 185, 125 and 70; typical of non-substituted pyranocoumarins[15,16]. Upon hydrogenation of the compounds the 280–345 nm UV absorption maxima were lost. Molecular ions in the mass spectra of the hydrogenated coumarins were both at 232 while the base ions were at 217, in accordance with the characteristics of dihydropyranocoumarins[17].

Osthol (III) and suberosin (IV) isolated in this study are the biological precursors of the angular seselin and the linear xanthyletin, respectively[17,18]. Both III and IV gave very similar UV and mass spectra and were the same as spectra on a sample of osthol obtained from grapefruit peel oil[19] and the mass spectrum reported for suberosin[18]. These two prenyl-methoxy coumarins upon hydrogenation lost their 320-nm absorption bands. Retention times of isolated osthol were the same as for authentic osthol in all TLC, GLC and HPLC systems. Upon hydrogenation both isolated and authentic osthol gave the same retention times.

Xanthoxyletin (V) gave the same mass spectrum as reported[15,20] for monomethoxylated pyranocoumarins. Its UV spectrum was identical to that of an authentic sample and agreed with its reported spectrum[20]. Upon hydrogenation the coumarin showed enhanced UV absorption in the 230–238-nm area, a characteristic of hydrogenated methoxypyranocoumarins[8]. Retention times and volumes of isolated and authentic xanthoxyletin were the same on all TLC, GLC and HPLC systems used in this study. Their dihydro derivatives likewise gave the same chromatographic times and volumes. Coumarin VI had a mass spectrum nearly identical to that of

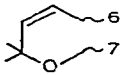
TABLE I

FOUND AND PROPOSED STRUCTURES OF NEUTRAL COUMARINS IN *CITRUS* AND *PONCIRUS* ROOTS



Coumarin

Substituents



Dimethylpyrano

$-CH_2-CH=C(CH_3)_2$ $-OCH_2CH=C(CH_3)CH_2CH_2CH=C(CH_3)_2$

$-C_5H_9$ $-OC_{10}H_{17}$ (geranyloxy-)

Code	*Common name*	*Position of substituents on coumarin ring*			
		5	*6*	*7*	*8*
I	Seselin*			Dimethylpyrano	
II	Xanthyletin*		Dimethylpyrano		
III	Osthol*			$-OCH_3$	$-C_5H_9$
IV	Suberosin*		$-C_5H_9$	$-OCH_3$	
V	Xanthoxyletin*	$-OCH_3$	Dimethylpyrano		
VIa	Braylin**		$-OCH_3$	Dimethylpyrano	
VIb	Luvangetin**		Dimethylpyrano		$-OCH_3$
VIc	Alloxanthoxyletin**	Dimethylpyrano		$-OCH_3$	
VII	Poncitrin*	$-OCH_3$	Dimethylpyrano		$-C_5H_9$
VIII	Geranyloxypyranocoumarin***		Dimethylpyrano		$-OC_{10}H_{17}$

* Found structure.

** Structure of VI is thought to be one of these three compounds.

*** Proposed structure.

xanthoxyletin. Three monomethoxylated pyranocoumarins besides xanthoxyletin (V) are reported to be present in *Rutaceae*[17] braylin (VIa), luvangetin (VIb) and alloxanthoxyletin (VIc). Until a standard is obtained for chromatographic comparison with VI, its structure remains uncertain.

Poncitrin (VII) was isolated as a minor component of the "xanthyletin" TLC band. Its UV and mass spectra were identical to reported values for poncitrin, and its hydrogenated derivative's UV spectrum was the same as reported for tetrahydroponcitrin[8].

The mass spectrum of the 8th coumarin (VIII) gave only a 4% ion at *m/e* 365. The presence of this weak anion may be a reflection of the facile fragmentation of an allylic aliphatic side chain [15,19] since the next ions were at 242 (92%), 227 (25%), 211 (100%) and 183 (49%). The molecule failed to produce an isolatable compound when subjected to hydrogenation under conditions used for the other 7 coumarins. Likewise, two geranyloxycoumarins found in grapefruit peel oil[19] —7-geranyloxycoumarin and bergamottin— failed to produce an isolatable compound under these hydrogenation–TLC conditions. The UV spectrum of VIII had a maximum at 301 nm. Structures which support this data are 5-methoxy-8-geranyloxy psoralen[21] and a

geranyloxy pyranocoumarin. The first structure was ruled out since an authentic sample of this compound gave a HPLC RRV (RRV = retention volume of coumarin/retention volume of seselin) of 1.08 while the corresponding RRV for VIII was 1.80 (Table II). Pyranocoumarins with geranyloxy substituents have not been reported in the literature. Thus, further studies need to be carried out on coumarin VIII before its structure can be established.

TABLE II

MIGRATION RATIOS OF NEUTRAL *CITRUS* AND *PONCIRUS* ROOT COUMARINS RELATIVE TO SESELIN ON TLC, GLC AND HPLC

Chromatographic system	R_F *or ret. vol. of seselin*	*Migration ratios*						
		II	*III*	*IV*	*V*	*VI*	*VII*	*VIII*
TLC								
Chloroform	R_F 0.43	0.83	0.98	0.88	0.86	0.95	0.83	0.95
Cyclohexane–ethyl acetate (4:1)	R_F 0.47	0.77	0.77	1.00	0.81	1.11	1.09	0.51
GLC								
1% Dexsil 300	180 ml	1.48	1.60	2.07	2.64	2.39	5.33	2.95
1% SP-1000	297 ml	1.48	1.33	1.81	1.99	2.15	2.37	2.65
HPLC								
Methanol–water	5.6 ml	1.00	1.00	1.29	1.10	1.25	1.67	1.35
Acetonitrile–water	5.6 ml	0.97	1.33	1.55	1.08	1.28	3.02	1.27
Hexane–tetrahydrofuran	4.5 ml	1.25	1.31	0.94	1.06	0.92	0.86	1.80

TLC. Various methods have been used to compare migration patterns of similar compounds in chromatography. In this study we found the simplest and most meaningful was a comparison of all migrations to that of seselin (I). This coumarin had the lowest molecular weight and was the most mobile of the eight neutral coumarins. By this method we were able to compare the structural influences of the eight coumarins on their migrations in all three chromatographic systems.

The relative retention times (RRT = $R_{F\text{coumarin}}/R_{F\text{seselin}}$) of the eight neutral coumarins on plates developed with chloroform fell into two ranges, 0.83–0.88 and 0.95–1.00 (Table II). For coumarins I to V and VII these ranges corresponded to pyranocoumarins and their biological precursers with the linear and angular structures respectively. With the cyclohexane–ethyl acetate system the addition of a methoxy or prenyl group onto the basic pyranocoumarin structures increased their RRT values from 0.04 to 0.33 RRT units. With cyclohexane–ethyl acetate coumarin VIII had an RRT of 0.51 while the RRT values of standards were: imperatorin, 0.64; phellopterin, 0.42; 5-methoxy-8-geranyloxy-psoralen, 0.65 and bergamottin, 1.26. The closeness of VIII's RRT to the RRT values of the first three standards indicate that the geranyloxy substituent of VIII was on carbon 8. The slight but distinct differences in the eight root coumarins' RRT values in the two TLC systems allowed them to be isolated by preparative TLC.

GLC. Previously we found that under temperature-programmed conditions with a 3% SP-1000 column, the eight coumarins were eluted after the fatty acid methyl esters[5]. The last coumarin (VIII), however, was unstable giving us erratic quantitative results. Chromatographic studies were conducted to maximize resolution

but minimize the time needed for the coumarins to be on the column. The eight coumarins were analyzed isothermally on a series of low-load columns of increasing polarity and at temperatures needed to elute the last coumarin within 20 min. Best resolutions were obtained on 1% SP-1000 and 1% Dexsil-300 columns.

Isoprenoid ethers such as imperatorin (8-prenyloxy-psoralen) are not stable under GLC conditions, undergoing rearrangement as well as degradation with heat[9]. Steck and Bailey[11] were able to elute 7-prenyloxy-coumarin and phellopterin (5-methoxy-8-prenyloxy-psoralen) but not imperatorin on a 5% SE-30 column. Under our improved conditions such as glass columns, low-load phases, on column injection and silanized supports, we were able to gas chromatograph such isoprenoid ethers as 7-geranyloxy-coumarin and imperatorin on 3% SE-30, 1% Dexsil-300 and 1% SP-1000 as symmetrical peaks in a reasonable length of time. We were, however, able to elute bergamottin (5-geranyloxy-psoralen) as a sharp, non-tailing peak only on the 1% Dexsil-300 column. There thus seems to be a difference in heat stability of 5- and 8-isoprenoid coumarin ethers on various GLC liquid phases. Our ability to elute VIII from both polar and non-polar phases lends support to the proposed 8-geranyloxy-pyranocoumarin structure for VIII.

HPLC. To the best of our knowledge there is no literature on HPLC of pyranocoumarins. The general recommended procedure with C_{18} columns (non-polar, partition chromatography) is to elute with various mixtures of 2 polar solvents, *e.g.*, water–methanol or water–acetonitrile. Resolution of angular seselin from linear xanthyletin (Table I) was the major objective in these HPLC experiments. This objective was not achieved with either of these two solvent systems, although other coumarins were separated (Table II). Baseline separation was obtained, however, for seselin (I) and xanthyletin (II) by using the non-conventional C_{18} column solvents hexane–tetrahydrofuran; the linear isomer was eluted after the angular isomer, as observed in GLC. Contrary to the sequence observed on GLC however, osthol (III) was eluted after its isomer suberosin (IV), the III/IV ratio of RRV being 1.39. The sequence of elution for the linear pyranocoumarins was VII > V > II, the reverse of what one would expect if the column separated these coumarins by adsorption chromatography as indicated by the separation of seselin and xanthyletin.

Chemotaxonomy

Table III shows the neutral coumarin profiles of six *Citrus* taxa, *Poncirus trifoliata* and four *Citrus* × *Poncirus* hybrids, all used as rootstocks. As a group the *Citrus* rootstocks were essentially free of poncitrin (VII), had very little xanthoxyletin (II), from 1 to 2% osthol (III) and various amounts of the other five coumarins. Sweet and sour oranges differed noticeably in their (VI) and (VIII) percentages. Cleopatra mandarin differed from two oranges in relative abundance of (VI) and xanthyletin (II). Rough lemon and Rangpur lime differed from the above three citrus rootstocks by having lower percentages of seselin (I) and high values of both xanthyletin (II) and suberosin (IV). These rootstocks differed from each other in their percentages of these two coumarins. Grapefruit, like Cleopatra mandarin, had 13% (VI) and 1.9% osthol (III). This rootstock however, differed from all of the other five *Citrus* taxa by containing considerably higher percentages of xanthoxyletin (V) and VIII. The profile of *Poncirus trifoliata* contained 78.9% seselin (I), only 11% poncitrin (VII) and less than 4% each of the 6 other coumarins. Its low percentage of poncitrin was un-

TABLE III

RELATIVE PERCENTAGE OF NEUTRAL COUMARINS IN ROOTS OF *CITRUS*, *PONCIRUS* AND *CITRUS* × *PONCIRUS* BY GLC

No.	*Rootstock*	*Relative % in coumarin*							
		I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*
1	Sweet orange (*Citrus sinensis* [L.] Osb.)	67.9	13.8	1.1	4.4	—*	1.8	—	11.0
2	Sour orange (*C. aurantium* L.)	71.3	13.3	1.0	2.6	0.4	6.1	—	5.3
3	Cleopatra mandarin (*C. reticulata* Blanco)	68.9	7.0	1.9	4.0	0.5	13.5	—	4.2
4	Rough lemon (*C. limon* [L.] Burm. F.)	34.1	37.6	0.5	24.4	—	0.6	—	2.8
5	Rangpur lime (*C. reticulata* var. *austera* Swing)	37.1	49.1	0.6	10.1	0.5	0.6	0.1	1.9
6	Seedling grapefruit (*C. paradisi* Macf.)	41.9	20.7	1.9	—	4.8	13.2	—	17.5
7	Carrizo citrange (*Poncirus trifoliata* × *C. sinensis*)	54.7	22.8	0.3	1.5	5.0	1.0	7.4	7.3
8	Troyer citrange (*P. trifoliata* × *C. sinensis*)	57.5	19.6	0.3	1.2	4.6	0.3	7.7	8.8
9	Rusk citrange (*C. sinensis* × *P. trifoliata*)	43.9	27.7	0.2	0.9	7.0	0.1	11.5	8.7
10	Swingle citromelo (*C. paradisi* × *P. trifoliata*)	48.7	20.7	1.4	1.5	7.1	10.9	8.3	1.4
11	Trifoliata orange (*Poncirus trifoliata* [L.] Raf.)	78.9	3.3	0.1	1.1	4.0	—	11.0	1.6

* If present below 0.1 relative %.

expected in the light of a previous study[8]. The two citranges Carrizo and Troyer cannot readily be distinguished from each other in the grove; nor could we distinguish them, whether by a chemotaxonomic study of their leaf hydrocarbons[2] or of their root coumarins. Rusk, the third citrange, differed from the other two by having higher percentages of all three of the linear pyranocoumarins. Like the citranges, the citrumelo, "Swingle", showed its *Poncirus* parentage by the presence of 8% poncitrin (VII). The citrumelo reflected its grapefruit parentage by its 10.9% VI and 1.4% osthol (III). In general *Citrus* had higher amounts of the angular pyranocoumarin and its biological precurser osthol (III) while *Citrus* × *Poncirus* contained more linear pyranocoumarins. In agreement with previous studies[6,7], furanocoumarins, which are widely found in aerial parts of citrus, were not found in the roots.

In previous *Citrus* scion studies many cultivars were available[3,4]; however, in our study the number of rootstocks available was limited. With the exception of Carrizo-Troyer, these 11 rootstocks each had characteristic root neutral coumarin profiles. These coumarins appear to be confined to the roots of these trees. Two exceptions to this are the reports of osthol in the peel of fruit[17] and xanthyletin in the stem of souorange[6]. All eight of the coumarins were readily quantitated by GLC using a low phase-load and relatively low oven temperatures. Under these conditions the unstable VIII was eluted without decomposition. Our limited trials with HPLC could not resolve all eight coumarins on a single column. However we were able to resolve "critical pairs" of coumarins on a reversed-phase C_{18} column, using mixtures of two non-polar solvents. Placing coumarin VIII in a non-destructive chromatographic environment (HPLC) clearly showed that it was present in the roots and was not an artifact formed under GLC conditions.

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