

## Nonvolatiles of Commercial Lime and Grapefruit Oils Separated by High-Speed Countercurrent Chromatography

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The nonvolatile fractions of cold-pressed peel oils of Key and Persian lime as well as grapefruit were separated by high-speed countercurrent chromatography (HS-CCC). In addition to the isolation of the main coumarins, psoralens and polymethoxyflavones, a number of minor constituents were enriched and successfully characterized by GC–MS and HPLC–UV. 5,7,8-Trimethoxycoumarin and the cyclical acetals of oxypeucedanin hydrate with citral were determined as new nonvolatile trace constituents of lime oils and confirmed by NMR spectroscopy. The citral oxypeucedaninyl acetals were found particularly in Key lime oil type A, which as a result of the juice–oil contact, is exposed to acidic conditions during industrial processing. Some of the confirmed minor constituents, such as pabulenol, isooxypeucedanin, and oxypeucedanin methanolate in lime as well as auraptanol in grapefruit, may have been generated by hydrolysis-sensitive precursors during CCC separation or their respective industrial processing techniques.

**KEYWORDS:** Cold-pressed; Key lime oil; Persian lime oil; grapefruit oil; high-speed countercurrent chromatography; 5,7,8-trimethoxycoumarin; neral oxypeucedaninyl acetal; geranial oxypeucedaninyl acetal; oxypeucedanin methanolate

### INTRODUCTION

In comparison to other citrus oils, cold-pressed lime oils possess a very high content of nonvolatiles, mainly substituted coumarins and psoralens (**Figure 1**). Depending on variety, provenance, and industrial pretreatment, these may add up to nearly 10% of the oil (1) and at room temperature or below an amorphous to crystalline precipitation forms, mainly citropten and isopimpinellin (2).

The content of nonvolatiles in grapefruit oil is also high (up to over 4%). Again mainly coumarins and psoralens but also polymethoxyflavones were detected (1) (**Figure 1**).

The multiple pharmaceutical and antioxidative properties of the citrus oxygenated heterocyclic compounds (OHC) have repeatedly been the subject of analytical investigations (3). Psoralens, despite their phototoxicity (4), are used as agents against psoriasis (5, 6). Some coumarins were identified as inhibitors of tumor promotion (7, 8). Polymethoxyflavones exhibited cholesterol-lowering properties (9) and showed anti-proliferative activities against human cancer cell lines (10).

The UV activity of the coumarins, psoralens (11), and polymethoxyflavones is routinely employed for their analytical detection and identification. Additionally, HPLC constitutes the most suitable separation method; both normal phase as well as reversed phase modes were employed (2, 11–13). Semipreparative HPLC, column chromatography on silica gel, TLC, crystal-

lization, or a combination were employed for separation and isolation purposes (14).

High-speed countercurrent chromatography (HS-CCC) represents an universal preparative chromatographic method that permits both normal as well as reversed phase operation (15, 16). On the basis of the distribution of compounds between two immiscible liquids, this liquid–liquid chromatographic method offers the following advantages: (1) no adsorption and therefore complete recovery of the chromatographed sample, (2) simple technology (low-pressure method), and (3) low cost of operation (use of technical grade solvents).

HS-CCC has already encountered widespread application in the separation of natural products (17) and was used among others for the investigation of anthocyanins in red cabbage, black currant (18), and red wine or red grape skin (19). It was successfully employed for the isolation of black tea polyphenols and pigments (20, 21) and of flavone derivatives from licorice (22). HS-CCC was recently employed for the analysis of coumarins in lemon oil (23).

More than a decade ago, we performed investigations on the lime nonvolatiles within the scope of a doctoral thesis (14). This research was however only published as a subchapter of the thesis, as the results were not projected onto lime oils of different geographic origin, harvest years, and differing industrial production methods. Drawing on the considerable advantages of HS-CCC as far as time and investigated amounts are concerned, we employed this method for the in-depth investigation of various lime oils. The results obtained earlier by semipreparative

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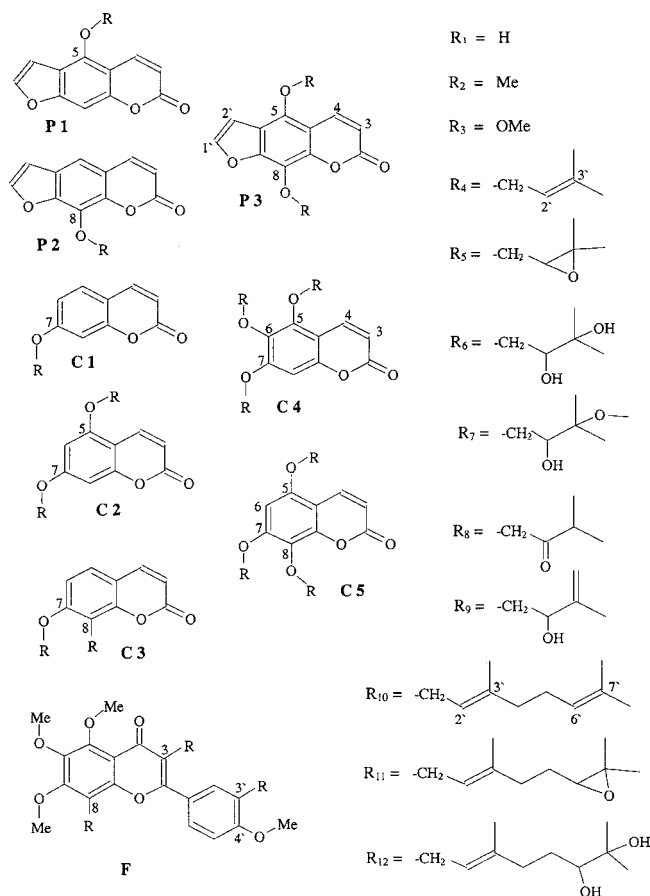


Figure 1. Molecular structures of lime and grapefruit OHC.

HPLC constituted a suitable starting point, and a number of lime trace constituents could again be confirmed.

It was the aim of the present work to elucidate the conditions for the preparative isolation of the main nonvolatiles of lime and grapefruit with HS-CCC and to enrich and characterize new minor constituents. The simple isolation and high purification achieved with this method also permits the usage of these compounds as analytical standards and the investigation of their pharmaceutical activity.

## MATERIALS AND METHODS

**Sample Preparation.** Commercially available cold-pressed oils of Key lime (*Citrus aurantifolia* Swingle) type A from Mexico (three samples), Persian lime (*Citrus latifolia* Tanaka) from Brazil (three samples), and white grapefruit (*Citrus paradisi*) from Florida (two samples) were investigated. The oils were derived from the harvests of 2002 and 2003.

Various processing methods are employed for the production of cold-pressed commercial lime oils (24). Key lime oils type B and Persian lime oils are obtained by a gentle processing method: The peels are grated, the oil is washed out from the oil glands with water, and, after finishing, the resulting oil–water emulsion is separated by centrifugation. As neither thermal nor acidic influences are present, this method yields oils that closely resemble the native composition of the oil glands. In the case of Key Lime type A, the entire fruit is screw-pressed to yield a fruit/oil/juice mixture. After separation of the peel residues, the oil is retained from the oil/juice/pulp emulsion via finisher and centrifuge. The different production methods—oil contact with the acidic media of the juice or without oil/acid contact—have a significant impact on the composition of the respective citrus oils and result in compositions that are considered as genuine for each of the commercial products, Persian lime oil, Key lime oil type A, and Key lime oil type B (24). Commercial grapefruit oils are mainly produced during juice production with the FMC technology. This method is very gentle and also widely

avoids contact between fruit juice and peel oil. Thermal as well as acidic influences are excluded (25).

Starting from the raw cold-pressed oils, the nonvolatiles of the lime oils were enriched by distilling off 90% of the volatiles in a short distance evaporator at 110 °C (200–300 Pa, heat contact approximately 15 min). The resulting dark green (lime) and reddish brown (grapefruit) highly viscous residues were chromatographed by HS-CCC. The precipitate from one sample of winterized Key lime oil type B (Mexico) was used without further separation for direct GC–MS analysis of the citral oxypeucedaninyl acetals.

**High-Speed Countercurrent Chromatography (HS-CCC).** A high-speed countercurrent chromatograph model CCC-1000 (Pharmatech Research Corp., Baltimore, USA) was equipped with three preparative coils, connected in series (diameter of tubing 2.6 mm, total volume 850 mL). The separations were performed at a revolution speed of 950 rpm.

Solvent systems with varying ratios (hexane/EtOAc/MeOH/H<sub>2</sub>O or hexane/EtOH/H<sub>2</sub>O) were used (flow rate: 3.5 mL/min; pumps: two Knauer HPLC pumps; Knauer GmbH, Berlin, Germany). All solvents for CCC were technical grade solvents. Elution was monitored with a Knauer variable UV detector at 310 nm, and 15 mL fractions were collected with a Pharmacia LKB fraction collector (Pharmacia, Upsala, Sweden). The amount of citrus oil nonvolatiles injected varied from 0.7 to 3 g. The highly viscous to solid samples were suspended in 25 mL of a 1:1 mixture of light and heavy phase, which was completely injected into the system by loop injection. CCC runs were employed in normal-phase (tail-to-head, T>H) and reversed-phase (head-to-tail, H>T) modes. For CCC conditions, see **Tables 1** and **2**.

**HPLC with Diode Array Detection (HPLC-DAD).** A Beckman System Gold with 126AA solvent module, diode array detector 168 (Beckman Coulter Inc., USA), and a Rheodyne 7125 injector was employed for HPLC analysis. System control, peak integration, and quantification were performed by Beckman System Gold Nouveau software. A C<sub>18</sub>-Spherisorb ODS2 column (250 × 4 mm, particle size 5 μm, Waters Corp., Milford, USA) was used. HPLC-grade acetonitrile (A) and water (B) with a gradient profile of 30% A changing to 40% during 5 min, kept for 40 min, were employed as mobile phase (constant flow 1.0 mL/min). Injection volume for each run was 10 μL of a 2–5 mg sample in acetonitrile/water (1:1). The runs were monitored by UV detection at 310 nm; the characterization of the single peaks was confirmed by the UV spectra recorded between 200 and 600 nm (13) as well as by comparing the HPLC retention times.

**Gas Chromatography–Mass Spectrometry.** The CCC fractions were analyzed by GC–MS employing a gas chromatograph Trace GC (Thermo Finnigan, Austin, USA) with a DB-5 column (15 m × 0.25 mm i.d. fused silica capillary, 0.5 μm; Restek Corp., Bellefonte, USA). Helium was the carrier gas with a constant flow of 1.0 mL/min with vacuum compensation. Injector temperature was held at 260 °C, and a split ratio of 1:20 was chosen. The oven temperature program: 50 °C held for 1 min, then rising at 10 °C/min to 320 °C was kept for 5 min. The column was connected directly to the ion source (240 °C) of a Trace DSQ. The electron impact (EI) mass spectra were recorded at 70 eV in an *m/z* range of 35 to 450 mass units (or 35 to 500, if necessary). The temperature of the transfer line was 280 °C.

**Identification of the Components in GC–MS.** The compounds were identified by comparing mass spectra and the corresponding retention indices with data of standards, literature, and the NIST MS library. In some cases, geranyl- and isopentenyl-substituted compounds decomposed in the GC injection port or during GC. This resulted in GC peaks of decomposition products (i.e., cnidilin) or broad peaks (i.e., bergamottin). Nevertheless, these decomposition mass spectra are provided in **Tables 3** and **4** as they give important information on the respective coumarin or psoralen skeleton and the number of methoxy-substituents at the coumarin/psoralen ring (14).

**Derivatizations.** To obtain further information, the coumarin derivatives or CCC fractions partly were submitted to derivatization reactions. Trimethylsilylations of the hydroxyl groups stabilized the OH compounds and resulted in TMS ethers with typical mass spectra: Auraptenol ( $M^+_{\text{TMS}}$  332), pabulenol ( $M^+_{\text{TMS}}$  358), oxypeucedanin hydrate ( $M^+_{\text{2TMS}}$  448) and oxypeucedanin methanolate ( $M^+_{\text{TMS}}$  390).

**Table 1.** CCC Conditions Lime (Key Lime Type A/Persian Lime)<sup>a</sup>

fraction	elution	subsequent CCC solvent system	main constituents	minor constituents
1	KL 350 mg PL 500 mg	hexane/ethyl acetate/methanol/water 6:4:5:5 head to tail (H>T)	herniarin, isopimpinellin, citropten, bergapten, oxypeucedanin	oxypeucedanin hydrate, heraclenol, heraclenin, xanthotoxin, 5,7,8-trimethoxycoumarin, pabulenol, isooxypeucedanin, nobiletin, oxypeucedanin methanolate, tetra- <i>O</i> -methylscutellarein, heptamethoxyflavone phellopterin
2	KL 20 mg PL 10 mg	hexane/ethyl acetate/methanol/water 7:3:7:3 head to tail (H>T)	cnidilin, imperatorin	
3	KL 80 mg PL 40 mg	hexane/ethyl acetate/methanol/water 7:3:7:3 head to tail (H>T)	8-geranyloxy-psoralen	5-isopentenyl-7-methoxycoumarin, isoimperatorin, osthol
4	KL 70 mg PL 50 mg	hexane/ethyl acetate/methanol/water 8:2:8:2 head to tail (H>T)	5-geranyloxy-8-methoxy- psoralen	neral oxypeucedaninyl acetal (2 isomers), geranial oxypeucedaninyl acetal (2 isomers)
5	KL 980 mg PL 1.2 g	hexane/ethanol/water 10:8:2 head to tail (H>T)	bergamottin, 5-geranyloxy- 7-methoxycoumarin	6,7-dimethoxy-5-geranyloxy-coumarin, auraptene
coil retentate	KL 500 mg PL 400 mg		terpenes	

<sup>a</sup> The residues of Key lime type A (KL, 2.0 g) and Persian lime (PL, 2.2 g) were pre-separated with a CCC solvent system of hexane/ethanol/water 10:8:2 in head to tail (H>T) mode.

**Table 2.** CCC Conditions Grapefruit

fraction	elution	constituents
		System 1 <sup>a</sup>
1	10 mg	meranzin hydrate
2	5 mg	marmin
3	15 mg	epoxybergamottin hydrate, auraptene
4	20 mg	nobiletin
5	40 mg	meranzin, isomeranzin, heptamethoxyflavone
6	15 mg	tangeritin
coil retentate	1.3 g	epoxyauraptene, epoxybergamottin/osthol, auraptene/bergamottin, terpenes
		System 2 <sup>b</sup>
1	1.0 g	terpenes, nonpolar, no UV absorption at 310 nm
2	400 mg	auraptene, bergamottin
3	50 mg	nootkatone
4	20 mg	osthol
5	330 mg	epoxybergamottin
6	35 mg	epoxyauraptene, 2 hydroxypentamethoxyflavones hydroxyhexamethoxyflavone
coil retentate	110 mg	polar coumarins, polymethoxyflavones

<sup>a</sup> Residue of grapefruit oil (1.5 g) was separated with a CCC solvent system of hexane/ethyl acetate/methanol/water 6:4:5:5 in head-to-tail mode (H>T). <sup>b</sup> Residue of grapefruit oil (2.0 g) was separated with a CCC solvent system of hexane/ethyl acetate/methanol/water 6:4:6:4 in tail-to-head mode (T>H).

For the stabilization and identification of the Claisen rearrangement product of geranyloxy-dimethoxycoumarin, the fraction was trimethylsilylated, resulting in a TMS ether ( $M^+_{TMS}$  430).

Hydration of double bonds in unsaturated side chains reduces fragmentation and therefore allows the visualization of the molecular peak of the compounds. Additionally, the number of double bonds in the side chain (isopentenyl + 2H or geranyl + 4H) and the presence of the psoralen (+ 4H) can be deduced from the mass spectra of the hydrogenated psoralens. In this manner, phellopterin + 6H ( $M^+$  306), cnidilin + 6H ( $M^+$  306), imperatorin + 6H ( $M^+$  276), and 5-geranyloxy-8-methoxypsoralen + 8H ( $M^+$  376) were confirmed. Mass spectra of the hydrogenated compounds are given in **Table 3**.

**Trimethylsilylations.** Approximately 5 mg of the substances or fractions were treated with 10–30  $\mu$ L of MSTFA and heated to 56 °C for 3 h in a reaction vial. The reaction mixture was then directly submitted to GC–MS analysis.

**Hydrations.** Approximately 5 mg of the substances or fractions were dissolved in 20 mL of hexane and stirred under H<sub>2</sub> atmosphere with 5 mg of palladium on charcoal for 2 h at room temperature. The solution was then filtered over silica gel and re-eluted with diethyl ether. The ether phase was submitted to GC–MS.

**NMR Analyses.** NMR experiments were performed on a 250 MHz Bruker ARX spectrometer in CDCl<sub>3</sub> or C<sub>6</sub>D<sub>6</sub> at room temperature. J-modulated <sup>13</sup>C NMR experiments were performed for the assignment of the <sup>1</sup>H signals of the citral oxypeucedaninyl acetals.

**Chemicals.** MSTFA was purchased from Macherey & Nagel (Düren, Germany), *p*-toluene-sulfonic acid from Merck (Darmstadt, Germany), isopimpinellin and bergamottin from Carl Roth GmbH (Karlsruhe, Germany), citropten and xanthotoxin from Fluka (Neu-Ulm, Germany), bergapten and palladium on charcoal from Sigma-Aldrich (Steinheim, Germany). Other oxygen heterocyclic compounds constitute well-known nonvolatiles of citrus oils. Native citrus oils (2) were therefore used as standards: Cold-pressed orange oil from Florida was used for heptamethoxyflavone, tetra-*O*-methylscutellarein, tangeritin, and nobiletin. Italian cold-pressed lemon oil was employed as standard for oxypeucedanin, imperatorin, phellopterin, 8-geranyloxy-psoralen, 7-isopentenyl-7-methoxycoumarin, and 5-geranyloxy-7-methoxycoumarin.

**Synthesis of Neral Oxypeucedaninyl Acetal (Figure 3).** 1.5 g of lime pre-separation fraction 1 (polar compounds), dissolved in 2 mL of CH<sub>2</sub>-Cl<sub>2</sub>, was used as a source for oxypeucedanin and mixed with 1 mL of neral (99%, isolated from Litsea cubeba oil by distillation). A total of 5 mg of *p*-toluene-sulfonic acid was added, and the mixture was homogenized ultrasonically. After 3 days at room temperature, the solvent was evaporated under reduced pressure, and the residue was chromatographed via HS-CCC (solvent system: hexane/EtOAc/MeOH/H<sub>2</sub>O (8:2:8:2), H>T (4.5 mL/min)). After elution of the polar constituents of the starting material the less polar acetals eluted in a broad peak (4–5 h) with traces of their geranial analogues.

**Neral Oxypeucedaninyl Acetal:** (diastereomer a, RI 3377 and b, RI 3388): UV<sub>max</sub>(diastereomer b), 196, 250, 307 nm; GC–MS (diastere-

Table 3. Oxygen Heterocyclic Compounds Identified in Lime Oil Residues after HS-CCC

compound systematic name structure (Figure 1)	identification	Rt (HPLC) <sup>a</sup> RI (GC DB-5)	UV <sub>max</sub> (nm) MS ( <i>m/z</i> )
<b>heraclenol<sup>a</sup></b> 8-(2',3'-dihydroxy-isopentyl-oxo)-psoralen (P2: R <sub>6</sub> )	Rt, UV RI, MS	<b>5.7</b> 2670	217, 249, 304 286 (0, M <sup>+</sup> ), 203 (12), 202 (100), 174 (29), 145 (7), 89 (16)
<b>oxypeucedanin hydrate</b> 5-(2',3'-dihydroxy-isopentyl-oxo)-psoralen (P1: R <sub>6</sub> )	Rt, UV RI, MS	<b>6.4</b> 2739	222, 250, 260, 311 304 (11, M <sup>+</sup> ), 203 (17), 202 (100), 174 (28), 145 (10), 59 (41), 43 (15) [TMS-di-ether: 448 (8, M <sup>+</sup> ), 403 (12), 274 (94), 201 (9), 131 (100), 103 (13), 73 (63), 59 (2), 45 (3)]
<b>herniarin</b> 7-methoxycoumarin (C1: R <sub>6</sub> )	Rt, UV RI, MS	<b>8.4</b> 1732	200, 320 176 (85, M <sup>+</sup> ), 148 (96), 133 (100), 77 (22), 63 (19), 51 (22)
<b>5,7,8-trimethoxycoumarin<sup>a,b</sup></b> (C4: 5 R <sub>2</sub> , 7 R <sub>2</sub> , 8 R <sub>2</sub> )	Rt, UV, <sup>1</sup> H NMR RI, MS	<b>8.7</b> 2107	206, 260, 326 236 (80, M <sup>+</sup> ), 222 (13), 221 (75), 194 (11), 193 (100), 165 (8), 150 (20), 69 (7)
<b>xanthotoxin<sup>a</sup></b> 8-methoxypsoralen (P2: R <sub>2</sub> )	Rt, UV RI, MS	<b>9.7</b> 2040	219, 248, 301 216 (100, M <sup>+</sup> ), 201 (31), 188 (11), 173 (44), 145 (22), 89 (27), 63 (15)
<b>citropten</b> 5,7-dimethoxycoumarin (C2: 5 R <sub>2</sub> , 7 R <sub>2</sub> )	Rt, UV RI, MS,	<b>10.3</b> 1986	207, 248, 327 206 (94, M <sup>+</sup> ), 178 (100), 163 (75), 135 (44), 77 (15)
<b>pabulenol<sup>a,c</sup></b> 5-(2'-hydroxy-3'-methyl-but-3'-enyl-oxo)-psoralen (P1: R <sub>6</sub> )	Rt, UV RI, MS	<b>10.5</b> 2596	222, 250, 311 286 (13, M <sup>+</sup> ), 203 (12), 202 (100), 174 (50), 145 (11), 89 (9), 41 (7) [TMS-ether: 358 (19, M <sup>+</sup> ), 343 (2), 287 (13), 274 (39), 259 (10), 201 (6), 157 (61), 143 (27), 73 (100), 45 (4)]
<b>oxypeucedanin methanolate<sup>a,b,c</sup></b> 5-(2'-hydroxy-3'-methoxyisopentyl-oxo)-psoralen (P1: 5 R <sub>7</sub> )	UV, <sup>1</sup> H NMR MS	<b>10.9</b> 2760	223, 270, 313 318 (6, M <sup>+</sup> ), 202 (45), 174 (13), 145 (12), 89 (10), 73 (100), 43 (14) [TMS-ether: 390 (10, M <sup>+</sup> ), 345 (3), 274 (9), 201 (7), 157 (4), 145 (4), 103 (8), 89 (5), 73 (100), 45 (4)]
<b>isopimpinellin</b> 5,8-dimethoxypsoralen (P3: 5 R <sub>2</sub> , 8 R <sub>2</sub> )	Rt, UV RI, MS,	<b>11.0</b> 2240	223, 248, 269, 314 246 (95, M <sup>+</sup> ), 231 (100), 203 (16), 188 (21), 175 (23), 160 (15), 147 (11), 76 (13), 66 (12)
<b>bergapten</b> 5-methoxypsoralen (P1: R <sub>2</sub> )	Rt, UV RI, MS	<b>11.3</b> 2062	222, 249, 268, 312 216 (100, M <sup>+</sup> ), 201 (32), 188 (17), 173 (70), 145 (32), 89 (13), 63 (6)
<b>heraclenin<sup>a</sup></b> 8-(2',3'-epoxy-isopentyl-oxo)-psoralen (P2: R <sub>5</sub> )	Rt, UV RI, MS	<b>11.6</b> 2456	200, 216, 248, 300 286 (12, M <sup>+</sup> ), 215 (9), 202 (100), 174 (26), 145 (7), 89 (13), 85 (35), 59 (18)
<b>oxypeucedanin</b> 5-(2',3'-epoxy-isopentyl-oxo)-psoralen (P1: R <sub>5</sub> )	Rt, UV RI, MS	<b>13.2</b> 2483	221, 250, 308 286 (14, M <sup>+</sup> ), 202 (16), 174 (13), 173 (15), 145 (26), 89 (24), 85 (92), 59 (100), 57 (40), 41 (33)
<b>nobiletin<sup>a,b,e</sup></b> 3',4',5,6,7,8-hexamethoxyflavone (F: 3 R <sub>1</sub> , 3' R <sub>3</sub> , 8 R <sub>3</sub> )	Rt, UV RI, MS	<b>12.2</b> 3392	208, 250, 270, 333 402 (20, M <sup>+</sup> ), 388 (20), 387 (100), 344 (17), 326 (11), 197 (33), 182 (24), 83 (20)
<b>tetra-O-methylscutellarein<sup>a,b,e</sup></b> 4',5,6,7-tetramethoxyflavone (F: 3 R <sub>1</sub> , 3' R <sub>1</sub> , 8 R <sub>1</sub> )	Rt, UV RI, MS	<b>13.3</b> 3186	198, 266, 322 342 (11, M <sup>+</sup> ), 328 (16), 327 (100), 284 (16), 167 (10), 132 (5), 89 (3), 69 (4)
<b>3,3',4',5,6,7,8-heptamethoxyflavone<sup>a,b,e</sup></b> (F: 3 R <sub>3</sub> , 3' R <sub>1</sub> , 8 R <sub>1</sub> )	Rt, UV RI, MS	<b>13.5</b> 3375	253, 271, 342 432 (31, M <sup>+</sup> ), 431 (21), 417 (100), 401 (13), 387 (11), 359 (11), 197 (14), 165 (16)
<b>isooxypeucedanin<sup>a,b,c</sup></b> 5-(isopentyl-2'-on)-psoralen (P1: R <sub>6</sub> )	Rt, UV RI, MS	<b>14.0</b> 2525	221, 250, 265, 309 286 (100, M <sup>+</sup> ), 216 (33), 215 (39), 202 (33), 201 (39), 187 (71), 157 (28), 145 (36), 89 (23), 71 (48), 43 (74)
<b>7-isopentenylloxycoumarin</b> (C1: R <sub>4</sub> )	Rt, UV RI, MS	<b>19.2</b> 2130	202, 244, 322 230 (3, M <sup>+</sup> ), 162 (100), 134 (63), 133 (9), 105 (12), 77 (11), 69 (44), 41 (27)
<b>imperatorin</b> 8-isopentenylloxypsoralen (P2: R <sub>6</sub> )	Rt, UV RI, MS	<b>19.3</b> 2356	220, 249, 301 270 (0.1, M <sup>+</sup> ), 203 (12), 202 (100), 174 (26), 90 (10), 89 (19), 69 (27), 68 (7), 67 (9), 41 (45) [hydrogenated: 276 (12, M <sup>+</sup> ), 206 (78), 178 (5), 164 (100), 149 (4), 91 (4), 77 (5), 43 (2)]
<b>phellopterin<sup>a</sup></b> 5-methoxy-8-isopentenylloxypsoralen (P3: 5 R <sub>2</sub> , 8 R <sub>4</sub> )	Rt, UV	<b>20.9</b> decomp	224, 242, 270, 314 [232 (100), 217 (82), 189 (17), 160 (13), 69 (15), 41 (21)] [hydrogenated: 306 (11, M <sup>+</sup> ), 236 (60), 194 (100), 179 (11), 165 (5), 77 (4), 57 (3), 43 (5)]
<b>osthol<sup>a,b,d</sup></b> 7-methoxy-8-isopentenylcoumarin (C3: 7 R <sub>2</sub> , 8 R <sub>4</sub> )	Rt, UV RI, MS	<b>20.9</b> 2143	203, 257, 322 244 (100, M <sup>+</sup> ), 229 (67), 213 (44), 201 (68), 189 (62), 187 (30), 186 (26), 159 (32), 131 (45)



Table 3. (Continued)

compound systematic name structure (Figure 1)	identification	Rt (HPLC) <sup>g</sup> RI (GC DB-5)	UV <sub>max</sub> (nm) MS ( <i>m/z</i> )
<b>5-isopentenyl-7-methoxycoumarin</b> (C2: 5 R <sub>4</sub> , 7 R <sub>2</sub> )	Rt, UV RI, MS	<b>21.6</b> 2351	208, 249, 326 260 (7, M <sup>+</sup> ), 193 (7), 192 (100), 164 (46), 163 (14), 149 (11), 135 (18), 69 (66), 41 (35)
<b>cnidilin</b> 5-isopentenyl-8-methoxypsoralen (P3: 5 R <sub>4</sub> , 8 R <sub>2</sub> )	Rt, UV	<b>22.2</b> decomp	224, 250, 269, 312 [232 (100), 217 (49), 189 (4), 175 (4), 160 (5), 69 (41), 41 (19)] [hydrogenated: 306 (46, M <sup>+</sup> ), 236 (60), 221 (25), 194 (100), 179 (16), 151 (8), 91 (7), 77 (6), 43 (15)]
<b>isoimperatorin</b> 5-isopentenyl-8-methoxypsoralen (P1: R <sub>4</sub> )	Rt, UV RI, MS	<b>22.6</b> 2394	202, 222, 252, 310 270 (1, M <sup>+</sup> ), 255 (1), 203 (5), 202 (58), 174 (16), 145 (9), 89 (18), 69 (100), 67 (11), 41 (82)
<b>8-geranyloxypsoralen</b> (P2: R <sub>10</sub> )	Rt, UV RI, MS	<b>28.6</b> 2865	197, 218, 248, 300 338 (-, M <sup>+</sup> ), 203 (30), 202 (100), 174 (20), 136 (21), 93 (13), 69 (15), 41 (10)
<b>6,7-dimethoxy-5-geranyloxycoumarin</b> <sup>a,b,f</sup> (C4: 5 R <sub>10</sub> , 6 R <sub>2</sub> , 7 R <sub>2</sub> )	Rt, UV MS	<b>28.6</b> decomp	204, 322 [Claisen: 358 (16, M <sup>+</sup> ), 289 (21), 275 (26), 259 (31), 236 (52), 235 (100), 123 (61), 122 (40), 41 (42)] [TMS-ether: 430 (10, M <sup>+</sup> ), 374 (5), 361 (25), 347 (13), 307 (65), 331 (10), 277 (9), 123 (12), 73 (100), 41 (18)]
<b>auraptene</b> <sup>a,b,d</sup> 7-geranyloxycoumarin (C1: R <sub>10</sub> )	RI, MS	<b>28.6</b> 2634	202, 322 298 (-, M <sup>+</sup> ), 163 (63), 162 (73), 137 (23), 136 (35), 134 (39), 93 (34), 81 (44), 69 (100), 41 (36)
<b>5-geranyloxy-8-methoxypsoralen</b> (P3: 5 R <sub>10</sub> , 8 R <sub>2</sub> )	Rt, UV RI, MS	<b>29.3</b> decomp	222, 249, 267, 310 [368 (-, M <sup>+</sup> ), 233 (13), 232 (100), 217 (35), 189 (4), 95 (11), 81 (23), 69 (52), 41 (19)] [hydrogenated: 376 (28, M <sup>+</sup> ), 236 (100), 221 (19), 194 (76), 179 (9), 85 (7), 71 (8), 57 (12), 43 (16)]
<b>neral oxypeucedaninyl acetal</b> <sup>a,b</sup> (diastereomer a) (Figure 3)	RI, MS	<b>29.5</b> 3377	196, 250, 307 438 (10, M <sup>+</sup> ), 368 (15), 287 (25), 245 (8), 203 (30), 202 (65), 201 (23), 95 (50), 69 (100), 41 (55)
<b>neral oxypeucedaninyl acetal</b> <sup>a,b</sup> (diastereomer b) (Figure 3)	<sup>1</sup> H NMR, <sup>13</sup> C NMR RI, MS	<b>29.5</b> 3388	196, 250, 307 438 (10, M <sup>+</sup> ), 368 (15), 287 (25), 245 (8), 203 (30), 202 (65), 201 (23), 95 (50), 69 (100), 41 (55)
<b>geranial oxypeucedaninyl acetal</b> <sup>a,b</sup> (diastereomer a) (Figure 3)	<sup>1</sup> H NMR, <sup>13</sup> C NMR RI, MS	<b>29.5</b> 3430	196, 250, 307 438 (3, M <sup>+</sup> ), 368 (5), 315 (9), 287 (20), 245 (10), 203 (20), 202 (40), 201 (18), 95 (20), 69 (100), 41 (45)
<b>geranial oxypeucedaninyl acetal</b> <sup>a,b</sup> (diastereomer b) (Figure 3)	RI, MS	<b>29.5</b> 3448	196, 250, 307 438 (3, M <sup>+</sup> ), 368 (5), 315 (9), 287 (20), 245 (10), 203 (20), 202 (40), 201 (18), 95 (20), 69 (100), 41 (45)
<b>bergamottin</b> 5-geranyloxypsoralen (P1: R <sub>10</sub> )	Rt, UV	<b>29.8</b> decomp	220, 250, 308 [Claisen: 338 (18, M <sup>+</sup> ), 269 (17), 255 (22), 227 (30), 215 (79), 171 (34), 123 (63), 115 (42), 69 (75), 41 (100)]
<b>5-geranyloxy-7-methoxycoumarin</b> (C2: 5 R <sub>10</sub> , 7 R <sub>2</sub> )	Rt, UV RI, MS	<b>30.1</b> 2862	207, 248, 322 328 (3, M <sup>+</sup> ), 193 (38), 192 (88), 164 (19), 135 (11), 81 (35), 69 (100), 41 (33)

<sup>a</sup> Trace constituent. <sup>b</sup> Previously unknown in lime. <sup>c</sup> Artifact or transformation product during CCC. <sup>d</sup> Typical grapefruit components. <sup>e</sup> Typical orange components. <sup>f</sup> Tentatively. <sup>g</sup> Rt: HPLC retention time in minutes (conditions see HPLC with diode array detection).

omers a and b): (70 eV), *m/z* 69 (100%), 202 (65%), 41 (55%), 95 (50%), 55 (25%), 85 (25%), 287 (25%), 109 (20%), 135 (15%), 368 (15%), 438 (M<sup>+</sup>; 10%); <sup>1</sup>H NMR (diastereomer b) (250 MHz, CDCl<sub>3</sub>) δ 6.26 [d, 1 H, *J* = 9.7 Hz, H-C(3)], 8.17 [d, 1H, *J* = 9.7 Hz, H-C(4)], 7.16 [s, br, 1H, H-C(8)], 7.59 [d, 1H, *J* = 2.5 Hz, H-C(1'')], 6.95 [d, 1H, *J* = 2.5 Hz, H-C(2'')], 4.45–4.49 [m, 2H, H-C(1'')], 4.09 [m, br, 1H, *J* = 5.0 Hz, H-C(2'')], 1.41 [s, 3H, H-C(4'')], 1.29 [s, 3H, H-C(5'')], 5.69 [d, 1H, *J* = 7.5 Hz, H-C(1''')], 5.20 [m, br, 1H, *J* = 7.5 Hz, H-C(2''')], 2.14–2.20 [m, 2H, H-C(4''')], 2.14–2.20 [m, 2H, H-C(5''')], 5.12 [m, br, H-C(6''')], 1.60 [s, 3H, H-C(8''')], 1.68 [s, 3H, H-C(9''')], 1.76 [s, br, 3H, H-C(10''')], <sup>13</sup>C NMR diastereomer b (62.5 MHz, CDCl<sub>3</sub>) δ 161.1 [C-2], 112.9 [C-3], 139.2 [C-4], 107.0 [C-4a], 148.3 [C-5], 113.6 [C-6], 158.1 [C-7], 94.6 [C-8], 152.6 [C-8a], 145.1 [C-1'], 104.7 [C-2'], 72.0 [C-1''], 81.9 [C-2''], 79.2 [C-3''], 22.9 [C-4''], 25.9 [C-5''], 98.2 [C-1'''], 122.3 [C-2'''], 145.5 [C-3'''], 32.6 [C-4'''], 26.9 [C-5'''], 123.5 [C-6'''], 132.3 [C-7'''], 17.8 [C-8'''], 25.7 [C-9'''], 23.7 [C-10'''].

Synthesis of Geranial Oxypeucedaninyl Acetal (Figure 3). Analogous to the neral acetal, geranial (91%), isolated from Litsea cubeba oil by

distillation) was added to fraction 1 of the lime prepreparation to synthesize the geranial acetals of oxypeucedanin. The reaction mixture was chromatographed via CCC for isolation purposes. Due to the isomerization of geranial to neral under acidic conditions, the geranial acetals also contained some neral acetals.

Geranial Oxypeucedaninyl Acetal: (diastereomer a, RI 3430 and b, RI 3448): UV<sub>max</sub>(diastereomer a), 196, 250, 307 nm; GC-MS (diastereomers a and b): (70 eV), *m/z* 69 (100%), 41 (45%), 202 (40%), 55 (20%), 95 (20%), 287 (20%), 85 (15%), 109 (20%), 438 (M<sup>+</sup>; 3%); <sup>1</sup>H NMR (diastereomer a) (250 MHz, CDCl<sub>3</sub>) δ 6.28 [d, 1 H, *J* = 9.7 Hz, H-C(3)], 8.18 [dd, 1H, *J* = 9.7 Hz, *J* = 1.0 Hz, H-C(4)], 7.17 [s, br, 1H, H-C(8)], 7.59 [d, 1H, *J* = 2.4 Hz, H-C(1')], 6.96 [dd, 1H, *J* = 2.4 Hz, *J* = 1.0 Hz, H-C(2')], 4.45–4.50 [m, 2H, H-C(1'')], 4.11 [m, br, 1H, *J* = 5.0 Hz, H-C(2'')], 1.42 [s, 3H, H-C(4'')], 1.30 [s, 3H, H-C(5'')], 5.74 [d, 1H, *J* = 7.5 Hz, H-C(1''')], 5.21 [m, 1H, *J* = 7.5 Hz, *J* = 1.3 Hz, H-C(2''')], 2.01–2.10 [m, 2H, H-C(4''')], 2.01–2.10 [m, 2H, H-C(5''')], 5.07 [m, br, H-C(6''')], 1.58 [s, 3H, H-C(8''')], 1.66 [s, 3H, H-C(9''')], 1.78 [d, 3H, *J* = 1.3 Hz, H-C(10''')], <sup>13</sup>C NMR diastereomer a (62.5 MHz, CDCl<sub>3</sub>) δ 161.0

Table 4. Oxygen Heterocyclic Compounds Identified in Grapefruit Oil Residues after HS-CCC

compound systematic name structure (Figure 1)	identification	R <sub>t</sub> HPLC (min) <sup>d</sup> RI <sub>GC</sub> (DB-5)	UV <sub>max</sub> (nm) MS ( <i>m/z</i> )
<b>meranzin hydrate</b> 7-methoxy-8-(2',3'-dihydroxy-isopentyl)-coumarin (C3: 7 R <sub>2</sub> , 8 R <sub>6</sub> )	Rt, UV RI, MS	<b>4.6</b> 2432	204, 258, 324 278 (0.3, M <sup>+</sup> ), 263 (2), 220 (24), 190 (20), 189 (27), 177 (100), 131 (22), 59 (9)
<b>auraptenol</b> <sup>a,b</sup> 7-methoxy-8-(2'-hydroxy-3'-methyl-but-3'-enyl)-coumarin (C3: 7 R <sub>2</sub> , 8 R <sub>9</sub> )	Rt, UV RI, MS	<b>8.3</b> 2284	204, 257, 324 260 (-, M <sup>+</sup> ), 191 (11), 190 (100), 189 (29), 175 (24), 161 (15), 160 (9), 131 (28), 103 (8), 77 (7) [TMS-ether: 332 (0.5, M <sup>+</sup> ), 317 (2), 262 (31), 247 (3), 189 (5), 143 (45), 131 (10), 73 (100), 45 (9)]
<b>marmin</b> <sup>a</sup> 7-(6',7'-dihydroxy-geranyloxy)-coumarin (C1: R <sub>12</sub> )	Rt, UV RI, MS	<b>8.6</b> 2985	202, 324 332 (0.6, M <sup>+</sup> ), 163 (67), 162 (100), 153 (65), 134 (49), 81 (76), 71 (71), 59 (42), 43 (60)
<b>citropten</b> <sup>a</sup> 5,7-dimethoxycoumarin (C2: 5 R <sub>2</sub> , 7 R <sub>2</sub> )	Rt, UV RI, MS	<b>10.3</b> 1986	207, 248, 327 206 (94, M <sup>+</sup> ), 178 (100), 163 (75), 135 (44), 77 (15)
<b>meranzin</b> 7-methoxy-8-(2',3'-epoxy-isopentyl)-coumarin (C3: 7 R <sub>2</sub> , 8 R <sub>5</sub> )	Rt, UV RI, MS	<b>10.5</b> 2255	202, 248, 321 260 (15, M <sup>+</sup> ), 217 (20), 202 (70), 189 (81), 187 (80), 159 (71), 131 (100), 103 (24), 77 (26)
<b>epoxybergamottin hydrate</b> <sup>a</sup> 5-(6',7'-dihydroxy-geranyloxy)-psoralen (P1: R <sub>12</sub> )	Rt, UV	<b>10.6</b> decomp	222, 252, 310
<b>isomeranzin</b> 7-methoxy-8-(isopentyl-2'-on)-coumarin (C3: 7 R <sub>2</sub> , 8 R <sub>8</sub> )	Rt, UV RI, MS	<b>11.1</b> 2243	202, 248, 320 260 (20, M <sup>+</sup> ), 191 (12), 190 (100), 189 (49), 175 (14), 161 (10), 131 (36), 71 (20), 43 (37)
<b>bergapten</b> <sup>a</sup> 5-methoxypsoralen (P1: R <sub>2</sub> )	Rt, UV RI, MS	<b>11.3</b> 2062	222, 249, 268, 312 216 (100, M <sup>+</sup> ), 201 (32), 188 (17), 173 (70), 145 (32), 89 (13), 63 (6)
<b>nobiletin</b> 3',4',5,6,7,8-hexamethoxyflavone (F: 3 R <sub>1</sub> , 3' R <sub>3</sub> , 8 R <sub>3</sub> )	Rt, UV RI, MS	<b>12.2</b> 3392	208, 250, 270, 333 402 (20, M <sup>+</sup> ), 388 (20), 387 (100), 344 (17), 326 (11), 197 (33), 182 (24), 83 (20)
<b>tetra-O-methylscutellarein</b> 4',5,6,7-tetramethoxyflavone (F: 3 R <sub>1</sub> , 3' R <sub>1</sub> , 8 R <sub>1</sub> )	Rt, UV RI, MS	<b>13.3</b> 3186	198, 266, 322 342 (11, M <sup>+</sup> ), 328 (16), 327 (100), 284 (16), 167 (10), 132 (5), 89 (3), 69 (4)
<b>3,3',4',5,6,7,8-heptamethoxyflavone</b> (F: 3 R <sub>3</sub> , 3' R <sub>1</sub> , 8 R <sub>1</sub> )	Rt, UV RI, MS	<b>13.5</b> 3375	253, 271, 342 432 (31, M <sup>+</sup> ), 431 (21), 417 (100), 401 (13), 387 (11), 359 (11), 197 (14), 165 (16)
<b>tangeritin</b> 4',5,6,7,8-pentamethoxyflavone (F: 3 R <sub>1</sub> , 3' R <sub>1</sub> , 8 R <sub>3</sub> )	Rt, UV RI, MS	<b>15.4</b> 3198	272, 322 372 (19, M <sup>+</sup> ), 358 (20), 357 (100), 314 (17), 296 (12), 197 (28), 182 (27), 83 (19)
<b>epoxyauraptene</b> 7-(6',7'-epoxy-geranyloxy)-coumarin (C1: R <sub>11</sub> )	Rt, UV RI, MS	<b>18.7</b> 2772	202, 322 314 (0.5, M <sup>+</sup> ), 163 (34), 162 (79), 153 (100), 135 (32), 134 (59), 81 (89), 71 (92), 43 (74)
<b>osthol</b> 7-methoxy-8-isopentenylcoumarin (C3: 7 R <sub>2</sub> , 8 R <sub>4</sub> )	Rt, UV RI, MS	<b>20.9</b> 2143	203, 257, 322 244 (100, M <sup>+</sup> ), 229 (67), 213 (44), 201 (68), 189 (62), 187 (30), 186 (26), 159 (32), 131 (45)
<b>epoxybergamottin</b> 5-(6',7'-epoxy-geranyloxy)-psoralen (P1: R <sub>11</sub> )	Rt, UV	<b>22.4</b> decomp	221, 250, 309 [354 (0.5 M <sup>+</sup> ), 202 (100), 174 (21), 153 (50), 135 (16), 81 (21), 71 (17)]
<b>auraptene</b> 7-geranyloxy coumarin (C1: R <sub>10</sub> )	Rt, UV RI, MS	<b>28.6</b> 2634	202, 322 298 (-, M <sup>+</sup> ), 163 (63), 162 (73), 137 (23), 136 (35), 134 (39), 93 (34), 81 (44), 69 (100), 41 (36)
<b>bergamottin</b> 5-geranyloxy psoralen (P1: R <sub>10</sub> )	Rt, UV	<b>29.8</b> decomp	220, 250, 308 [Claisen: 338 (18, M <sup>+</sup> ), 269 (17), 255 (22), 227 (30), 215 (79), 171 (34), 123 (63), 115 (42), 69 (75), 41 (100)]
<b>hydroxypentamethoxyflavone 1<sup>a-c</sup></b>	MS	nf <sup>e</sup> 3294	388 (44, M <sup>+</sup> ), 374 (20), 373 (100), 215 (14), 194 (13), 187 (24), 165 (33)
<b>hydroxypentamethoxyflavone 2<sup>a-c</sup></b>	MS	nf 3368	388 (54, M <sup>+</sup> ), 374 (19), 373 (100), 211 (24), 183 (25)
<b>hydroxyhexamethoxyflavone<sup>a-c</sup></b>	MS	nf 3383	418 (61, M <sup>+</sup> ), 404 (22), 403 (100), 209 (21), 180 (22), 165 (35)

<sup>a</sup> Trace constituent. <sup>b</sup> Previously unknown in grapefruit. <sup>c</sup> Unknown configuration, tentatively. <sup>d</sup> Rt: HPLC retention time in minutes (conditions see HPLC with diode array detection). <sup>e</sup> nf: not found.

[C-2], 113.0 [C-3], 139.1 [C-4], 107.1 [C-4a], 148.4 [C-5], 113.0 [C-6], 158.2 [C-7], 94.7 [C-8], 152.8 [C-8a], 145.2 [C-1'], 104.7 [C-2'], 72.0 [C-1''], 81.9 [C-2''], 79.2 [C-3''], 22.9 [C-4''], 24.6 [C-5''], 98.7 [C-1'''], 121.3 [C-2'''], 144.1 [C-3'''], 39.6 [C-4'''], 26.0 [C-5'''], 123.6 [C-6'''], 132.0 [C-7'''], 17.7 [C-8'''], 25.6 [C-9'''], 17.0 [C-10'''].

Oxypeucedanin-methanolate was synthesized from oxypeucedanin and methanol. 10 mg of oxypeucedanin (isolated from lime oil residues

by HS-CCC) was dissolved in 1 mL of MeOH, mixed with 2 drops of 2 N HCl, and kept at room temperature overnight. The solution was dried over sodium sulfate and submitted directly to GC-MS analysis. The solution contained oxypeucedanin methanolate.

RI 2760, UV<sub>max</sub>, 223, 270, 313 nm; GC-MS: (70 eV), *m/z* 73 (100%), 202 (45%), 43 (14%), 174 (13%), 145 (12%), 89 (10%), 318 (M<sup>+</sup>; 6%); <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 8.21 [d, 1 H, *J* = 9.75 Hz,

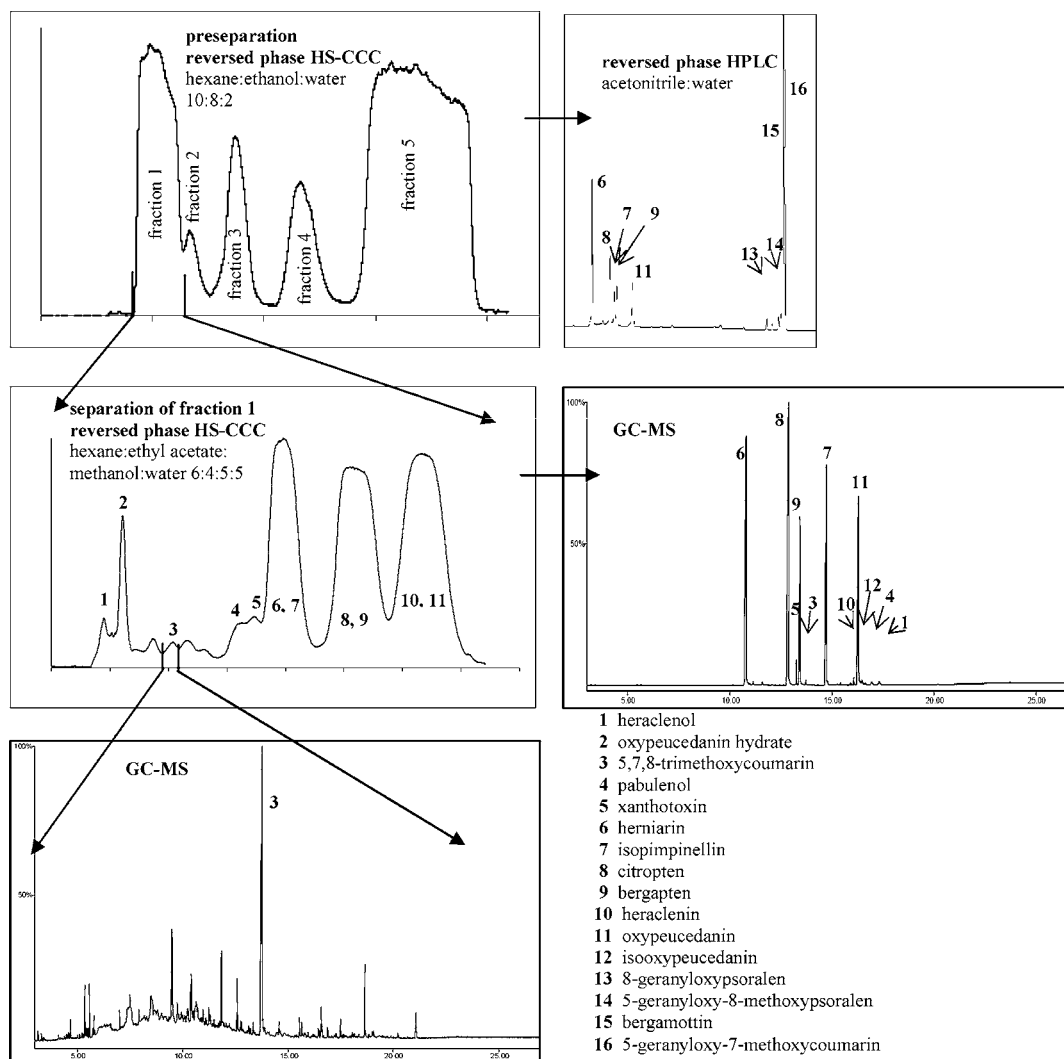


Figure 2. CCC isolation of 5,7,8-trimethoxycoumarin from Persian lime oil residue.

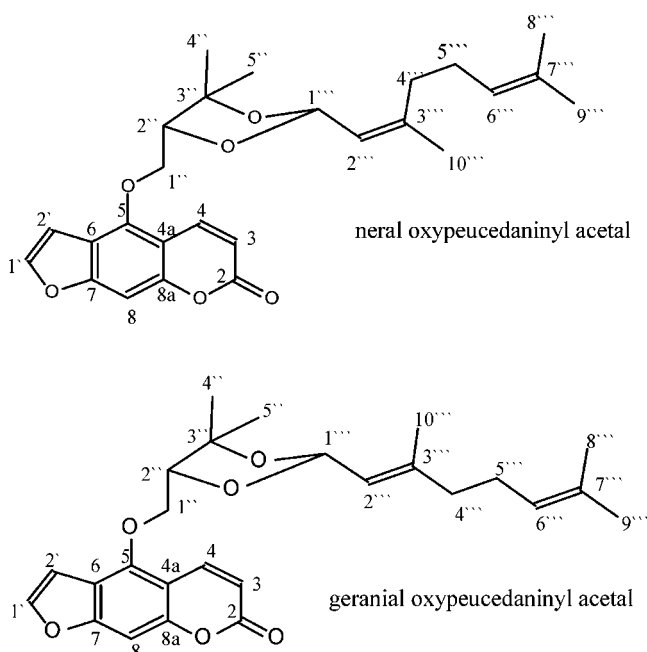


Figure 3. Neral oxypeucedaninyl acetal and geranial oxypeucedaninyl acetal.

H-C(4), 6.26 [d, 1H,  $J = 9.75$  Hz, H-C(3)], 7.14 [s, br, 1H, H-C(8)], 7.57 [d, 1H,  $J = 2.25$  Hz, H-C(1')], 6.98 [d, 1H,  $J = 2.25$

Hz, H-C(2')], 3.25 [s, 3H, H-CH<sub>2</sub>-O], 2.33 [br, 1H, H-O], 1.27 [s, 3H, H-CH<sub>2</sub>], 1.22 [s, 3H, H-CH<sub>2</sub>], 4.55 [dd, 1H,  $J_{AB} = 10.0$  Hz,  $J_{AX} = 3.0$  Hz, H<sub>A</sub>-C(1'')], 4.37 [dd, 1H,  $J_{AB} = 10.0$  Hz,  $J_{BX} = 7.75$  Hz, H<sub>B</sub>-C(1'')], 3.92 [dd, 1H,  $J_{AX} = 3.0$  Hz,  $J_{BX} = 7.75$  Hz, H-C(2'')].

5,7,8-Trimethoxy-coumarin was identified by MS and <sup>1</sup>H NMR data. RI 2107, UV<sub>max</sub>, 206, 260, 326 nm; GC-MS: (70 eV),  $m/z$  193 (100%), 236 ( $M^+$ , 80%), 221 (75%), 150 (20%), 44 (14%), 165 (8%), 178 (5%); <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>)  $\delta$  7.96 [d, 1H,  $J = 9.75$  Hz, H-C(4)], 6.14 [d, 1H,  $J = 9.75$  Hz, H-C(3)], 6.32 [s, 1H, H-C(6)], 3.89 [s, 3H, H-CH<sub>2</sub>-O], 3.90 [s, 3H, H-CH<sub>2</sub>-O], 3.95 [s, 3H, H-CH<sub>2</sub>-O], <sup>1</sup>H NMR (250 MHz, C<sub>6</sub>D<sub>6</sub>)  $\delta$  7.49 [d, 1H,  $J = 9.75$  Hz, H-C(4)], 5.85 [d, 1H,  $J = 9.75$  Hz, H-C(3)], 5.72 [s, 1H, H-C(6)], 3.74 [s, 3H, H-CH<sub>2</sub>-O], 3.29 [s, 3H, H-CH<sub>2</sub>-O], 3.15 [s, 3H, H-CH<sub>2</sub>-O]. <sup>1</sup>H NMR data were collected in CDCl<sub>3</sub> and C<sub>6</sub>D<sub>6</sub> to ensure the substitution positions using the aromatic solvent induced shift [26].

**Syntheses of Pabulenol, Isooxypeucedanin, and Oxypeucedanin Hydrate.** 10 mg of oxypeucedanin (isolated from lime oil by HS-CCC) were dissolved in 1 mL of acetonitrile, mixed with 2 drops of 2 N HCl, and kept at room temperature overnight. The solution was dried over sodium sulfate and submitted directly to GC-MS analysis. For analytical data of pabulenol, isooxypeucedanin, and oxypeucedanin hydrate, and their derivatization products, see Table 3 (for derivatizations, see GC-MS).

## RESULTS AND DISCUSSION

**HS-CCC of Lime Oil OHC.** The raw cold-pressed peel oils of Persian and Key (type A) lime were reduced to 1/10 of their weight under vacuum to enrich the nonvolatiles to a minimum of 70% and then submitted to HS-CCC.

**Preseparation (Figure 2).** The high amount and considerable variety of the OHC in lime oils (Table 3) required preseparation of the residue. CCC with a solvent system of hexane/EtOH/H<sub>2</sub>O (10:8:2) was performed as reversed-phase (H>T) chromatography and resulted in five fractions. Fraction 1 represented the most polar lime OHC (approximately 20% of the residue) and included the methoxy constituents herniarin, isopimpinellin, citropten, and bergapten as well as the epoxide oxypeucedanin. Fraction 5 with the less polar main coumarins contained bergamottin and 5-geranyloxy-7-methoxycoumarin, together more than 50% of the lime oil OHC. Approximately 20–25% of the chromatographed sample remained on the CCC column due to its low polarity (terpenes) and were recovered by emptying the column.

The fractions 1–5 were then fractionated in subsequent CCC runs with individually optimized solvent systems of hexane/EtOAc/MeOH/H<sub>2</sub>O in varying ratios.

**Main CCC Separation.** Apart from the above-mentioned major compounds, a considerable number of minor constituents (Table 3) was detected. Starting with the polar compounds in fraction 1 (Figure 2), oxypeucedanin hydrate, traces of its 8-substituted isomer heraclenol and heraclenin were identified. Moreover, xanthotoxin, which was previously only found in Persian lime (26), was detected in both lime varieties. Additionally, we were able to confirm 5,7,8-trimethoxycoumarin by <sup>1</sup>H NMR comparison with the literature (27, 28) in both lime oil types. The 5,7,8-substitution pattern was elucidated by aromatic solvent induced shift (ASIS) of the proton signals (28). Pabulenol and oxypeucedanin methanolate could be identified as minor constituents of this fraction, but their possible formation during this separation process should be kept in mind. The identification of pabulenol and oxypeucedanin methanolate was based on their mass spectra and their synthesis from oxypeucedanin (see reactions of oxypeucedanin) as well as derivatizations with MSTFA. The <sup>1</sup>H NMR of the methanolate was in accordance with literature data (29). The compound was previously detected in Angelica roots (29, 30) and recently isolated from lemon oil by HS-CCC (21). The second fraction contained cnidilin, imperatorin, and traces of phellopterin. In the third fraction, 8-geranyloxy-psoralen, 5-isopentenyl-7-methoxycoumarin, isoimperatorin, and 7-isopentenyl-oxycoumarin could be identified. Unfortunately, the latter two coeluted under all tested CCC conditions.

Fraction 4 contained 5-geranyloxy-8-methoxypsoralen. Additionally, a group of four novel isomeric compounds was detected by GC (Figure 3), which turned out to be acetals of neral and geranial with oxypeucedanin hydrate (Figure 4). In lemon oil, the geranial oxypeucedaninyl acetal has already been detected and characterized (14).

Individual syntheses of the neral and geranial acetals from neral and geranial with oxypeucedanin permitted the unambiguous assignment and confirmation in GC, <sup>1</sup>H, and <sup>13</sup>C NMR. The <sup>1</sup>H NMR spectra turned out to be significant with regard to the psoralen skeleton, as the proton resonance of H(C4) ( $\delta = 8.17$ ) confirmed the 5-O substitution (31, 32).

The assignment of the side chain resonances was verified in the literature by synthesis of analogous model acetals from 3-methyl-(2,3-dihydroxy)-butyl acetate and citral. The resulting acetals were subsequently analyzed by 2D NMR (14). NMR comparison with the citral oxypeucedaninyl acetals permitted their definite assignment. In this context, the resonance of the acetalic protons in the 5-ring appears to be noteworthy as it shows a considerable low-field shift in the model compounds ( $\delta = 5.62$ ; 5.75) and the isolated acetals from citrus ( $\delta = 5.69$ ;

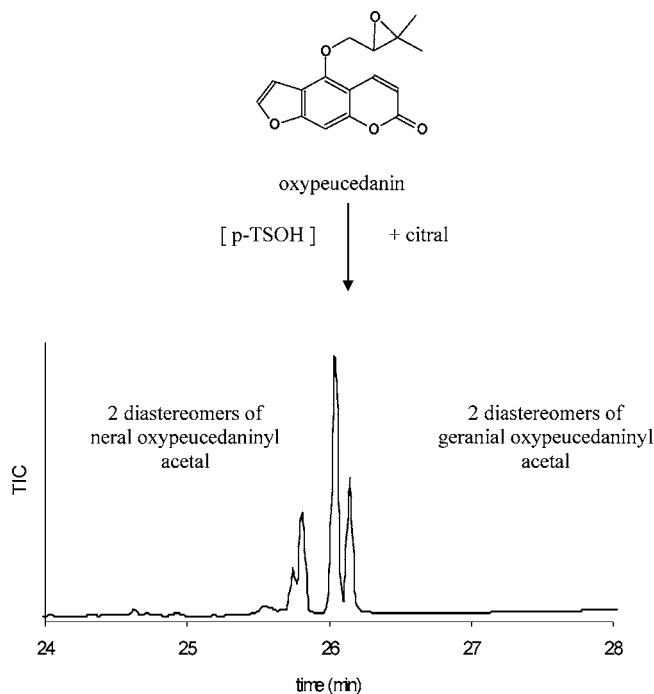


Figure 4. Formation of the citral oxypeucedaninyl acetals.

5.74). In the <sup>13</sup>C NMR 26 carbon signals were observed; the acetalic carbon resonance is situated in the extended range ( $\delta = 98.2$  and 98.7).

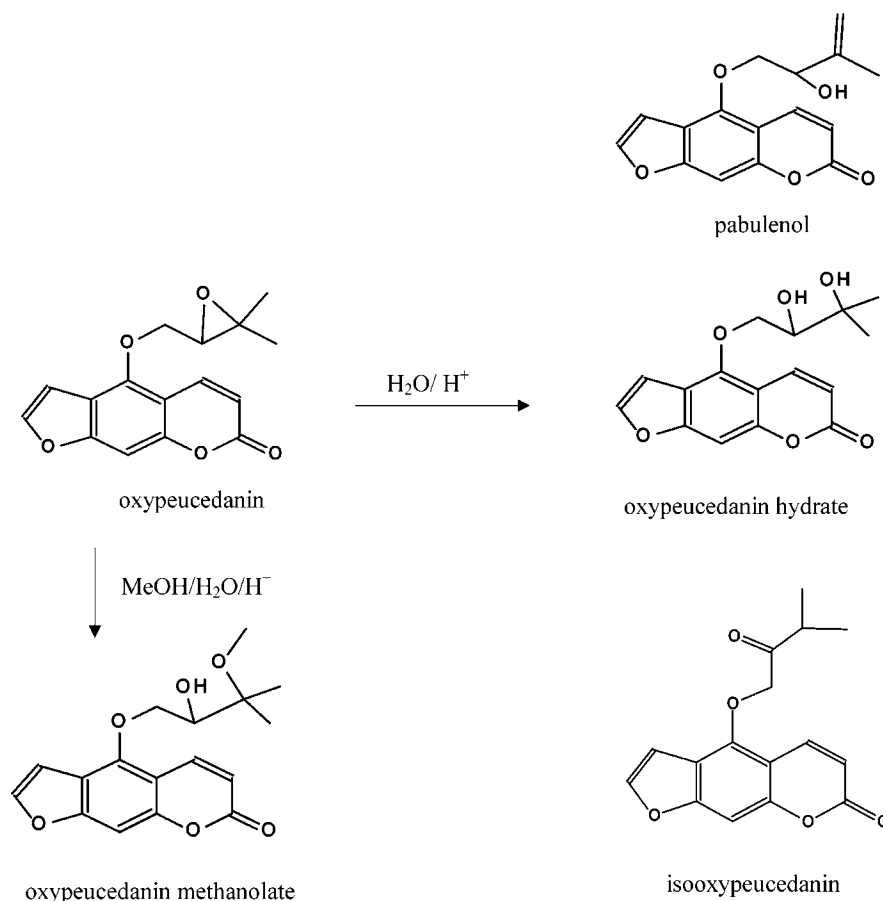
Fraction 5, nonpolar, showed bergamottin and 5-geranyloxy-7-methoxycoumarin as main constituents. An additional trace coumarin ( $M^+ 358$ ) was found in GC-MS of this fraction, which formed a TMS ether ( $M^+_{TMS} 430$ ) with MSTFA. From the MS cleavage pattern of the coumarin and its TMS ether the presence of a Claisen rearrangement product of a dimethoxygeranyloxy coumarin was deduced. It shows the same mass spectra as published for the 6,7-dimethoxy-5-geranyloxy coumarin derivatives (14). 6,7-Dimethoxy-5-geranyloxy coumarin was previously reported in lemon and lime oil; in the literature its structure and substitution pattern were determined by <sup>1</sup>H NMR and ASIS analyses (14).

**Reaction Products of Oxypeucedanin.** *Hydrolysis, Methanolysis, and Secondary Products (Figure 5).* The epoxide ring of oxypeucedanin can easily be opened by acidic hydrolysis leading to the 1,2-diol oxypeucedanin hydrate (33, 34). This well-established citrus oil constituent was confirmed in all lime oils investigated by CCC.

The industrial processing technique has a considerable impact on the content of oxypeucedanin in cold-pressed Key lime type A and Persian lime oils. Persian lime oil is produced by pressing only the lime peels without juice contact. On the other hand, the entire fruit is squeezed during the industrial production of Key lime oil type A. This results in the contact of the peel constituents with the acidic lime juice. Key lime oil type A, therefore, typically contains considerably less, sometimes even no, oxypeucedanin compared to Persian lime oil. As a result of the acidic hydrolysis of oxypeucedanin, it is transformed to a great extent into its hydrate, which is then partly lost due to its solubility during the ensuing rinsing process with water.

Apart from hydrolysis to diols, the formation of secondary products during the acid-catalyzed reaction of coumarins with side chain epoxides was reported. McHale described the 1,3-enolic compound 5-(6'-hydroxy-3',7'-dimethyl-2',7'-octadienyl-oxo)-psoralen, which resulted from epoxy-bergamottin (35). The formation of ketones, such as isomeranzin from 7-methoxy-8-





**Figure 5.** Hydrolysis and methanolysis of oxypeucedanin.

(2'-hydroxy-3'-methyl-3'-butenyl)-coumarin, was described by Stanley et al. (36). We were able to confirm the analogous conversions of oxypeucedanin in aqueous acetonitrile solution with hydrochloric acid (see Materials and Methods). Starting from oxypeucedanin, the side chain reactions led to pabulenol with enolic 2-hydroxy-3-methyl-3-butenyloxy structure and the side chain ketone isooxypeucedanin.

Pabulenol was found in the residues of Key lime oil type A and Persian lime oil. The side chain ketone isooxypeucedanin was confirmed in some of the CCC lime fractions as a trace—possibly the transformation products of the epoxy precursors due to the intensive water contact during CCC.

On the other hand, without the support and usage of the HS-CCC workup, which leads to an enhanced enrichment of minor constituents, these trace compounds would not be detectable at all. It has to be kept in mind that the methods required for their visualization may on the other hand implicate rearrangements or secondary transformation products. Rearrangement products, such as pabulenol, may therefore be derived from the industrial processing required for this specific oil (see sample preparation) or the rearrangement during the isolation and visualization of the minor constituents.

However, the methanol adduct mentioned before can be considered as reaction product of the chromatographic process and its limitations. The assumption that the novel oxypeucedanin methanolate, so far unknown in lime, only formed during chromatographic processes with methanol-containing solvent systems, was confirmed by its absence in separations with the solvent systems hexane/ethanol/water.

**Acetalization (Figure 4).** Direct conversion of citral (neral/geranial 1:1.25) with the epoxide oxypeucedanin (isolated from

lime oil) in the presence of catalytic amounts of *p*-toluene sulfonic acid led to the formation of the four stereoisomers in a ratio of neral *a*/neral *b*/geranial *a*/geranial *b* = 1:2:4:3, rather similar to the isomer ratio found in lime residues (approximately 1:2:6:3). The acetals therefore obviously derive from the lime oil components citral and oxypeucedanin.

To confirm the origin of the cyclical acetals (artifact formation during workup vs genuineness in commercial oils), the raw lime oils were winterized at  $-20\text{ }^\circ\text{C}$ . The resulting precipitates were investigated directly by GC-MS (SIM *m/e* 69, 202, 438) with regard to the acetals. Traces of the compounds could be confirmed in the precipitate of Persian lime and Key lime oil type B; both are produced analogously (no contact with the acidic juice, subsequent cold separation process for separation of the oil, see sample preparation). On the other hand, in the residues of Key lime type A, industrially produced with juice/water/oil contact, the acetal concentration was so high that they could already be directly detected in the GC-MS total ion current.

The acetals could therefore, irrespective of the CCC workup, be confirmed as new constituents of all cold-pressed commercial lime oils. Whereas they are only present as traces in Persian and Key lime oil type B, their significant increase in Key lime oil type A is the result of the oil's processing technique (see sample preparation).

**Unexpected Citrus OHC in Lime Oil.** It is often through a closer analytical look and the appropriate analytical method that the detection of trace compounds, which are generally not considered as characteristic for an individual citrus variety, becomes feasible (37, 38). In this context, a series of minor trace OHC (<1 ppm), were identified in the CCC fractions of

the commercial Persian and Key lime oils. These compounds are typical constituents of grapefruit (auraptene, osthol) or orange oil (the polymethoxyflavones tetra-*O*-methylscutellarein, nobiletin, and heptamethoxyflavone) (39). The assumption that these OHC derive from contamination with small amounts of grapefruit or orange oil during industrial production cannot be refuted totally, as no laboratory-produced oils were analyzed. The presence of these compounds in oils of different geographic origin, sources, and production method calls for further investigation.

**HS-CCC of Grapefruit Oil OHC (Table 4).** Analogously to lime, the grapefruit volatiles were removed prior to HS-CCC. No further pre-separation by HS-CCC was required due to the relatively small amount of grapefruit OHC in total. The polar grapefruit OHC were separated from the residue with a CCC solvent system hexane/EtOAc/MeOH/H<sub>2</sub>O (6:4:5:5) in reversed phase chromatography (H>T), resulting in six fractions (approximately 5 h).

The most polar components meranzin hydrate, marmin (36, 40), epoxybergamottin hydrate (34, 41), and auraptenol eluted successively. Auraptenol, which is new in grapefruit, was so far only reported as a constituent of orange oil (36). By transformation of auraptenol into its TMS ether with MSTFA, the presence of the hydroxy group was confirmed. UV and mass spectrometry showed the expected spectra (see Table 4).

The polar hydroxy compounds constitute hydrolysis products of the epoxides meranzin, epoxyauraptene, and epoxybergamottin. The following fractions contained nobiletin, tetra-*O*-methylscutellarein (39), meranzin, isomeranzin, and heptamethoxyflavone. Tangeritin finally eluted in the last fraction (approximately 4.5 h), with small amounts of citropten and bergapten.

The nonpolar part of the OHC remained on the stationary CCC phase. The coil content was cut into three fractions (epoxyauraptene, epoxybergamottin/osthol, and auraptene/bergamottin) by careful emptying of the coils.

To achieve optimum separation and analysis of the less polar OHC, normal phase conditions (T>H) with hexane/EtOAc/MeOH/H<sub>2</sub>O (6:4:6:4) were employed. With this method, terpenes and other nonpolar oil constituents, which were not completely removed by the prior evaporation, eluted first. In the second fraction, bergamottin immediately preceded the main constituent auraptene. Nootkatone was then eluted in an intermediate fraction, followed by osthol and epoxybergamottin.

In the last fraction, the main OHC component epoxyauraptene (7-epoxygeranyloxycoumarin) was detected. In the mass spectrum, it exhibited a fragmentation pattern that is typical for a monosubstituted coumarin (*m/e* = 162) with an 6',7'-epoxidized geranyloxy side chain (*m/z* 71, 153, and 135).

Three unknown grapefruit trace constituents have been detected in GC. Their retention indices and their MS cleavage patterns are very similar to polymethoxyflavones (39). MS data indicates the presence of two hydroxypentamethoxyflavones [*m/z* 373 (M<sup>+</sup> - 15, 100%), 388 (M<sup>+</sup>, 50–55%)] and one hydroxyhexamethoxyflavone [*m/z* 403 (M<sup>+</sup> - 15, 100%), 418 (M<sup>+</sup>, 60%)]. Hydroxypolymethoxyflavones were so far reported in the peel oils of orange, tangerine, and mandarin (42, 43).

The main constituents of the (T>H) column retentate consisted of the polar grapefruit OHC meranzin hydrate, meranzin, isomeranzin, the polymethoxyflavones and traces of citropten, bergapten as well as auraptenol.

To conclude, HS-CCC turned out to be an extremely useful tool for the workup of citrus oil nonvolatiles, as the present work illustrates for lime and grapefruit. The main constituents

could be largely separated and isolated on a semipreparative scale. CCC is, therefore, well suited for obtaining nonvolatile standards from citrus oil matrices. Additionally, the separation of minor components was also achieved; in fact, the extreme enrichment even permitted the characterization of so far unreported lime constituents. As a result of the intensive water contact, diols and other secondary products are formed from hydrolysis-sensitive compounds, such as epoxides. Together with the nonvolatiles, volatile citrus oil constituents also are chromatographed during CCC.

#### ABBREVIATIONS USED

ASIS, aromatic solvent induced shift; CCC, countercurrent chromatography; DSQ, dual stage quadrupole; HS-CCC, high-speed countercurrent chromatography; H>T, head-to-tail; KL, Key lime; MSTFA, *N*-methyl-*N*-(trimethylsilyl)-2,2,2-trifluoroacetamide; OHC, oxygen heterocyclic compounds; PL, Persian lime; T>H, tail-to-head; TMS, trimethylsilyl.

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#### LITERATURE CITED

- Dugo, G.; Bartle, K. D.; Bonaccorsi, I.; Catalfamo, M.; Cotroneo, A.; Dugo, P.; Lamonica, G.; McNair, H.; Mondello, L.; Previti, P.; Stagno d'Alcontres, I.; Trozzi, A.; Verzera, A. Advanced analytical techniques for the analysis of citrus essential oils. Part 3. Oxygen heterocyclic compounds: HPLC, HPLC/MS, OPLC, SFC, fast HPLC analysis. *Essenze, Deriv. Agrum.* **1999**, *69*, 251–283.
- McHale, D.; Sheridan, J. B. The oxygen heterocyclic compounds of citrus peel oils. *J. Essent. Oil Res.* **1989**, *1*, 139–149.
- Benavente-Garcia, O.; Castillo, J.; Marin, F. R.; Ortuno, A.; Del Rio, J. A. Uses and properties of citrus flavonoids. *J. Agric. Food Chem.* **1997**, *45*, 4505–4515.
- Forlot, P. Cosmetic and cosmeceutic uses of bergamot oil. *Essenze, Deriv. Agrum.* **2000**, *70*, 3–7.
- Forlot, P. Perspectives de la bergamotte dans le domaine pharmaceutique. *Essenze, Deriv. Agrum.* **1998**, *68*, 45–56.
- Pendino, G. M.; Il bergamotto in terapia medica: Attualita e prospettive. *Essenze, Deriv. Agrum.* **1998**, *68*, 57–62.
- Miyake, Y.; Murakami, A.; Sugiyama, Y.; Isobe, M.; Koshimizu, K.; Ohigashi, H. Identification of coumarins from lemon fruit (*Citrus limon*) as inhibitors of in vitro tumor promotion and superoxide and nitric oxide generation. *J. Agric. Food Chem.* **1999**, *47*, 3151–3157.
- Takahashi, Y.; Inaba, N.; Kuwahara, S.; Kuki, W.; Yamane, K.; Murakami, A. Rapid and convenient method for preparing aurapten-enriched product from hassaku peel oil: implications for cancer-preventive food additives. *J. Agric. Food Chem.* **2002**, *50*, 3193–3196.
- Kurowska, E. M.; Manthey, J. A. Hypolipidemic effects and absorption of citrus polymethoxylated flavones in hamsters with diet-induced hypercholesterolemia. *J. Agric. Food Chem.* **2004**, *52*, 2879–2886.
- Manthey, J. A.; Guthrie, N. Antiproliferate activities of citrus flavonoids against six human cancer cell lines. *J. Agric. Food Chem.* **2002**, *50*, 5837–5843.
- Frerot, E.; Decorzant, E. Quantification of total furocoumarins in citrus oils by HPLC coupled with UV, fluorescence and mass detection. *J. Agric. Food Chem.* **2004**, *52*, 6879–6886.

- (12) Dugo, P.; Mondello, L.; Sebastiani, E.; Ottanà, R.; Errante, G.; Dugo, G. Identification of minor heterocyclic compounds of citrus essential oils by liquid chromatography-atmospheric pressure chemical ionisation mass spectrometry. *J. Liq. Chromatogr., Relat. Technol.* **1999**, *22*, 2991–3005.
- (13) Bonaccorsi, I. L.; McNair, H. M.; Brunner, L. A.; Dugo, P.; Dugo, G. Fast HPLC for the analysis of oxygen heterocyclic compounds of citrus essential oils. *J. Agric. Food Chem.* **1999**, *47*, 4237–4239.
- (14) Ziegler, H. Analytik der Cumarine des Zitronenöls, Thesis, University of Bayreuth, 1992.
- (15) Foucault, A. P. Countercurrent chromatography. *Anal. Chem.* **1991**, *63*, 569A–579A.
- (16) Sutherland, A.; Brown, L.; Forbes, S.; Games, G.; Hawes, D.; Hostettmann, K.; McKerrel, E. H.; Marston, A.; Wheatley, D.; Wood, P. Countercurrent chromatography (CCC) and its versatile application as an industrial purification & production process. *J. Liq. Chromatogr., Relat. Technol.* **1998**, *21*, 279–298.
- (17) Ito, Y. Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography. *J. Chromatogr. A* **2005**, *1065*, 145–168.
- (18) Degenhardt, A.; Knapp, H.; Winterhalter, P. Separation and purification of anthocyanins by high-speed countercurrent chromatography and screening for antioxidant activity. *J. Agric. Food Chem.* **2000**, *48*, 338–343.
- (19) Degenhardt, A.; Hofmann, S.; Knapp, H.; Winterhalter, P. Preparative isolation of anthocyanins by high-speed countercurrent chromatography and application of the color activity concept to red wine. *J. Agric. Food Chem.* **2000**, *48*, 5812–5818.
- (20) Degenhardt, A.; Engelhardt, U. H.; Wendt, A.-S.; Winterhalter, P. Isolation of black tea pigments using high-speed countercurrent chromatography and studies on properties of black tea polymers. *J. Agric. Food Chem.* **2000**, *48*, 5200–5205.
- (21) Degenhardt, A.; Engelhardt, U. H.; Lakenbrink, C.; Winterhalter, P. Preparative separation of polyphenols from tea by high-speed countercurrent chromatography. *J. Agric. Food Chem.* **2000**, *48*, 3425–3430.
- (22) Wang, Q.-E.; Lee, F. S.-C.; Wang, X. Isolation and purification of inflacoumarin a and licochalcone a from licorice by high-speed countercurrent chromatography. *J. Chromatogr. A* **2004**, *1048*, 51–57.
- (23) Körn, A.; Jerz, G.; Winterhalter, P. Isolierung von cumarinderivaten aus citrus limon mittels high-speed counter current chromatography (HSCCC). *Lebensmittelchemie* **2004**, *58*, 21.
- (24) Clark, B. C., Jr.; Chamblee, T. S. Acid-catalyzed reactions of citrus oils and other terpene-containing flavors. In *Off-Flavors in Foods and Beverages*; Charalambous, G., Ed.; Elsevier Science Publishers B. V.: Amsterdam, Netherlands, 1992; pp 229–285.
- (25) Simon, M. Flavouring preparation and some source materials. In *Flavourings*; Ziegler, E.; Ziegler, H., Eds.; Wiley-VCH: Weinheim, Germany, 1998; pp 146–164.
- (26) Nigg, H. N.; Nordby, H. E.; Beier, R. C.; Dillman, A.; Macias, C.; Hansen, R. C. Phototoxic coumarins in limes. *Food Chem. Toxicol.* **1993**, *31*, 331–335.
- (27) Kayser, O.; Kolidziej, H. Highly oxygenated coumarins from pelargonium sidoides. *Phytochemistry* **1995**, *39*, 1181–1185.
- (28) Dean, F. M.; Costa, A. M. B. S. R. C. S.; Harborne, J. B.; Smith, D. M. Leptodactylone, a yellow coumarin from leptodactylon and linanthus species. *Phytochemistry* **1978**, *17*, 505–509.
- (29) Mendez, J.; Castro-Pocheiro, J. Furocoumarins from angelica pachycarpa. *Phytochemistry* **1983**, *22*, 2599–2602.
- (30) Kaul, V. K.; Weyerstahl, P. Further evidence for the revised structure of angelicain. *Fitoterapia* **1987**, *58*, 129–132.
- (31) Ziegler, H.; Spiteller, G. Coumarins and psoralens from Sicilian lemon oil (*Citrus limon* (L.) Burm. f.). *Flavour Fragrance J.* **1992**, *7*, 129–139.
- (32) Sommer, H.; Bertram, H. J.; Krammer, G.; Kindel, G.; Kühnle, T.; Reinders, G.; Reiss, I.; Schmidt, C. O.; Schreiber, K.; Stumpe, W.; Werkhoff, P. HPLC NMR — a powerful tool for the identification of nonvolatiles in lemon peel oils. *Perfum. Flavor.* **2003**, *28*, 18–34.
- (33) Radford, T.; Olansky, A. D. A process for dewaxing citrus oils. U.S. Patent 5 362 714, 1994.
- (34) Dugo, P.; Mondello, L.; Lamonica, G.; Dugo, G. Characterization of cold-pressed Key lime and Persian lime oils by gas chromatography, gas chromatography/mass spectroscopy, high-performance liquid chromatography, and physicochemical indices. *J. Agric. Food Chem.* **1997**, *45*, 3608–3616.
- (35) McHale, D.; Sheridan, J. B. Detection of adulteration of cold-pressed bitter orange oil. *IXth International Congress of Essential Oils*, Singapore, 1983; Technical Paper Book 3, pp 43–48.
- (36) Stanley, W. L.; Jurd, L. Citrus coumarins. *J. Agric. Food Chem.* **1971**, *19*, 1106–1110.
- (37) Barrett, V. L.; Nelson, D. B. Characterization of coumarin and psoralen levels in California and Arizona citrus oils. In ACS Symposium Series 705: *Flavor Analysis*; Mussinan, C. J., Morello, M. J., Eds.; American Chemistry Society, Washington, DC, 1998, Chapter 20, 233–238.
- (38) Thomas, A. F.; Bassols, F. Occurrence of pyridines and other bases in orange oil. *J. Agric. Food Chem.* **1992**, *40*, 2236–2243.
- (39) Berahia, T.; Gaydou, E. M.; Cerrati, C.; Wallet, J.-C. Mass spectrometry of polymethoxylated flavones. *J. Agric. Food Chem.* **1994**, *42*, 1697–1700.
- (40) Fisher, J. F.; Trama, L. A. High-performance liquid chromatographic determination of some coumarins and psoralens found in citrus peel oils. *J. Agric. Food Chem.* **1979**, *27*, 1334–1337.
- (41) Tatum, J. H.; Berry, R. E. Coumarins and psoralens in grapefruit peel oil. *Phytochemistry* **1979**, *18*, 500–502.
- (42) Tatum, J. H.; Berry, R. E. Six new flavonoids from citrus. *Phytochemistry* **1972**, *11*, 2283–2288.
- (43) Iinuma, M.; Matsuura, S.; Kuroguchi, K.; Tanaka, T. Studies on the constituents of useful plants. V. Multisubstituted flavones in the fruit peel of *Citrus reticulata* and their examination by gas-liquid chromatography. *Chem. Pharm. Bull.* **1980**, *28*, 717–722.

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