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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 auraptenol 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 antiproliferative 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, crystallization, 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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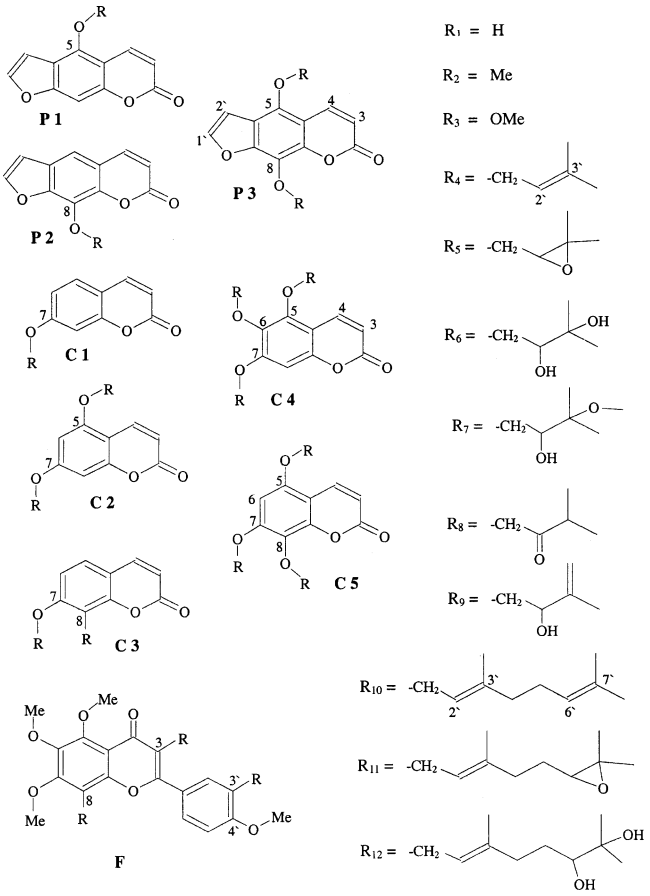

Figure 1. Molecular structures of lime and grapefruit OHC.

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 (Pharma-Tech 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_2O or hexane/EtOH/H_2O) 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_{18}-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^+_{TMS} 332), pabulenol (M^+_{TMS} 358), oxypeucedanin hydrate (M^+_{2TMS} 448) and oxypeucedanin methanolate (M^+_{TMS} 390).

Table 1. CCC Conditions Lime (Key Lime Type A/Persian Lime)[a]

fraction	elution	subsequent CCC solvent system	main constituents	minor constuents
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-*O*-methylscutellarein, heptamethoxyflavone
2	KL 20 mg PL10 mg	hexane/ethyl acetate/methanol/water 7:3:7:3 head to tail (H>T)	cnidilin, imperatorin	phellopterin
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-isopentenyloxy-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-geranyloxycoumarin, auraptene
coil retentate	KL 500 mg PL 400 mg		terpenes	

[a] The residues of Key lime type A (KL, 2.0 g) and Persian lime (PL, 2.2 g) were preseparated 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[a]
1	10 mg	meranzin hydrate
2	5 mg	marmin
3	15 mg	epoxybergamottin hydrate, auraptenol
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[b]
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

[a] 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). [b] 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 μL 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_2 atmosphere with 5 mg of palladium on charcoal for 2 h at room temperature. The solution was then filtered over silica gel and reeluted 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 $CDCl_3$ or C_6D_6 at room temperature. J-modulated ^{13}C NMR experiments were performed for the assignment of the ^1H 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 heptamethhoxyflavone, tetra-*O*-methylscutellarein, tangeritin, and nobiletin. Italian cold-pressed lemon oil was employed as standard for oxypeucedanin, imperatorin, phellopterin, 8-geranyloxypsoralen, 7-isopentenyloxycoumarin, and 5-geranyloxy-7-methoxycoumarin.

*Synthesis of Neral Oxypeucedaninyl Acetal (**Figure 3**)*. 1.5 g of lime preseparation fraction 1 (polar compounds), dissolved in 2 mL of CH_2Cl_2, 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_2O (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_{max}(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)**[g] RI (GC DB-5)	UV_{max} (nm) MS (*m/z*)
heraclenol[a]	Rt, UV	**5.7**	217, 249, 304
8-(2′,3′-dihydroxy-isopentyloxy)-psoralen	RI, MS	2670	286 (0, M^+), 203 (12), 202 (100), 174 (29), 145 (7), 89 (16)
(P2: R_6)			
oxypeucedanin hydrate	Rt, UV	**6.4**	222, 250, 260, 311
5-(2′,3′-dihydroxy-isopentyloxy)-psoralen	RI, MS	2739	304 (11, M^+), 203 (17), 202 (100), 174 (28), 145 (10),
(P1: R_6)			59 (41), 43 (15)
			[TMS-di-ether: 448 (8, M^+), 403 (12), 274 (94), 201 (9),
			131 (100), 103 (13), 73 (63), 59 (2), 45 (3)]
herniarin	Rt, UV	**8.4**	200, 320
7-methoxycoumarin	RI, MS	1732	176 (85, M^+), 148 (96), 133 (100), 77 (22), 63 (19), 51 (22)
(C1: R_6)			
5,7,8-trimethoxycoumarin[a,b]	Rt, UV, ^{1}H NMR	**8.7**	206, 260, 326
(C4: 5 R_2, 7 R_2, 8 R_2)	RI, MS	2107	236 (80, M^+), 222 (13), 221 (75), 194 (11), 193 (100),
			165 (8), 150 (20), 69 (7)
xanthotoxin[a]	Rt, UV	**9.7**	219, 248, 301
8-methoxypsoralen	RI, MS	2040	216 (100, M^+), 201 (31), 188 (11), 173 (44), 145 (22),
(P2: R_2)			89 (27), 63 (15)
citropten	Rt, UV	**10.3**	207, 248, 327
5,7-dimethoxycoumarin	RI, MS,	1986	206 (94, M^+), 178 (100), 163 (75), 135 (44), 77 (15)
(C2: 5 R_2, 7 R_2)			
pabulenol[a,c]	Rt, UV	**10.5**	222, 250, 311
5-(2′-hydroxy-3′-methyl-but-3′-enyloxy)-psoralen	RI, MS	2596	286 (13, M^+), 203 (12), 202 (100), 174 (50), 145 (11),
(P1: R_8)			89 (9), 41 (7)
			[TMS-ether: 358 (19, M^+), 343 (2), 287 (13), 274 (39),
			259 (10), 201 (6), 157 (61), 143 (27), 73 (100), 45 (4)]
oxypeucedanin methanolate[a,b,c]	UV, ^{1}H NMR	**10.9**	223, 270, 313
5-(2′-hydroxy-3′-methoxyisopentyloxy)-psoralen	MS	2760	318 (6, M^+), 202 (45), 174 (13), 145 (12), 89 (10), 73 (100),
(P1: 5 R_7)			43 (14)
			[TMS-ether: 390 (10, M^+), 345 (3), 274 (9), 201 (7), 157 (4),
			145 (4), 103 (8), 89 (5), 73 (100), 45 (4)]
isopimpinellin	Rt, UV	**11.0**	223, 248, 269, 314
5,8-dimethoxypsoralen	RI, MS,	2240	246 (95, M^+), 231 (100), 203 (16), 188 (21), 175 (23),
(P3: 5 R_2, 8 R_2)			160 (15), 147 (11), 76 (13), 66 (12)
bergapten	Rt, UV	**11.3**	222, 249, 268, 312
5-methoxypsoralen	RI, MS	2062	216 (100, M^+), 201 (32), 188 (17), 173 (70), 145 (32),
(P1: R_2)			89 (13), 63 (6)
heraclenin[a]	Rt, UV	**11.6**	200, 216, 248, 300
8-(2′,3′-epoxy-isopentyloxy)-psoralen	RI, MS	2456	286 (12, M^+), 215 (9), 202 (100), 174 (26), 145 (7), 89 (13),
(P2: R_5)			85 (35), 59 (18)
oxypeucedanin	Rt, UV	**13.2**	221, 250, 308
5-(2′,3′-epoxy-isopentyloxy)-psoralen	RI, MS	2483	286 (14, M^+), 202 (16), 174 (13), 173 (15), 145 (26), 89 (24),
(P1: R_5)			85 (92), 59 (100), 57 (40), 41 (33)
nobiletin[a,b,e]	Rt, UV	**12.2**	208, 250, 270, 333
3′,4′,5,6,7,8-hexamethoxyflavone	RI, MS	3392	402 (20, M^+), 388 (20), 387 (100), 344 (17), 326 (11),
(F: 3 R_1, 3′ R_3, 8 R_3)			197 (33), 182 (24), 83 (20)
tetra-*O*-methylscutellarein[a,b,e]	Rt, UV	**13.3**	198, 266, 322
4′,5,6,7-tetramethoxyflavone	RI, MS	3186	342 (11, M^+), 328 (16), 327 (100), 284 (16), 167 (10),
(F: 3 R_1, 3′ R_1, 8 R_1)			132 (5), 89 (3), 69 (4)
3,3′,4′,5,6,7,8-heptamethoxyflavone[a,b,e]	Rt, UV	**13.5**	253, 271, 342
(F: 3 R_3, 3′ R_1, 8 R_1)	RI, MS	3375	432 (31, M^+), 431 (21), 417 (100), 401 (13), 387 (11),
			359 (11), 197 (14), 165 (16)
isooxypeucedanin[a,b,c]	Rt, UV	**14.0**	221, 250, 265, 309
5-(isopentyl-2′-on)-psoralen	RI, MS	2525	286 (100, M^+), 216 (33), 215 (39), 202 (33), 201 (39),
(P1: R_8)			187 (71), 157 (28), 145 (36), 89 (23), 71 (48), 43 (74)
7-isopentenyloxycoumarin	Rt, UV	**19.2**	202, 244, 322
(C1: R_4)	RI, MS	2130	230 (3, M^+), 162 (100), 134 (63), 133 (9), 105 (12), 77 (11),
			69 (44),41 (27)
imperatorin	Rt, UV	**19.3**	220, 249, 301
8-isopentenyloxypsoralen	RI, MS	2356	270 (0.1, M^+), 203 (12), 202 (100), 174 (26), 90 (10), 89 (19),
(P2: R_8)			69 (27), 68 (7), 67 (9), 41 (45)
			[hydrogenated: 276 (12, M^+), 206 (78), 178 (5), 164 (100),
			149 (4), 91 (4), 77 (5), 43 (2)]
phellopterin[a]	Rt, UV	**20.9**	224, 242, 270, 314
5-methoxy-8-isopentenyloxypsoralen		decomp	[232 (100), 217 (82), 189 (17), 160 (13), 69 (15), 41 (21)]
(P3: 5 R_2, 8 R_4)			[hydrogenated: 306 (11, M^+), 236 (60), 194 (100), 179 (11),
			165 (5), 77 (4), 57 (3), 43 (5)]
osthol[a,b,d]	Rt, UV	**20.9**	203, 257, 322
7-methoxy-8-isopentenylcoumarin	RI, MS	2143	244 (100, M^+), 229 (67), 213 (44), 201 (68), 189 (62),
(C3: 7 R_2, 8 R_4)			187 (30), 186 (26), 159 (32), 131 (45)

Table 3. (Continued)

compound systematic name structure (Figure 1)	identification	Rt (HPLC)[g] RI (GC DB-5)	UV_{max} (nm) MS (*m*/*z*)
5-isopentenyloxy-7-methoxycoumarin	Rt, UV	**21.6**	208, 249, 326
(C2: 5 R_4, 7 R_2)	RI, MS	2351	260 (7, M^+), 193 (7), 192 (100), 164 (46), 163 (14), 149 (11), 135 (18), 69 (66), 41 (35)
cnidilin	Rt, UV	**22.2**	224, 250, 269, 312
5-isopentenyloxy-8-methoxypsoralen		decomp	[232 (100), 217 (49), 189 (4), 175 (4), 160 (5), 69 (41), 41 (19)]
(P3: 5 R_4, 8 R_2)			[hydrogenated: 306 (46, M^+), 236 (60), 221 (25), 194 (100), 179 (16), 151 (8), 91 (7), 77 (6), 43 (15)]
isoimperatorin	Rt, UV	**22.6**	202, 222, 252, 310
5-isopentenyloxypsoralen	RI, MS	2394	270 (1, M^+), 255 (1), 203 (5), 202 (58), 174 (16), 145 (9), 89 (18), 69 (100), 67 (11), 41 (82)
(P1: R_4)			
8-geranyloxypsoralen	Rt, UV	**28.6**	197, 218, 248, 300
(P2: R_{10})	RI, MS	2865	338 (-, M^+), 203 (30), 202 (100), 174 (20), 136 (21), 93 (13), 69 (15), 41 (10)
6,7-dimethoxy-5-geranyloxycoumarin[a,b,f]	Rt, UV	**28.6**	204, 322
(C4: 5 R_{10}, 6 R_2, 7 R_2)	MS	decomp	[Claisen: 358 (16, M^+), 289 (21), 275 (26), 259 (31), 236 (52), 235 (100), 123 (61), 122 (40), 41 (42)]
			[TMS-ether: 430 (10, M^+), 374 (5), 361 (25), 347 (13), 307 (65), 331 (10), 277 (9), 123 (12), 73 (100), 41 (18)]
auraptene[a,b,d]		**28.6**	202, 322
7-geranyloxycoumarin	RI, MS	2634	298 (-, M^+), 163 (63), 162 (73), 137 (23), 136 (35), 134 (39), 93 (34), 81 (44), 69 (100), 41 (36)
(C1: R_{10})			
5-geranyloxy-8-methoxypsoralen	Rt, UV	**29.3**	222, 249, 267, 310
(P3: 5 R_{10}, 8 R_2)	RI, MS	decomp	[368 (-, M^+), 233 (13), 232 (100), 217 (35),189 (4), 95 (11), 81 (23), 69 (52), 41 (19)]
			[hydrogenated: 376 (28, M^+), 236 (100), 221 (19), 194 (76), 179 (9), 85 (7), 71 (8), 57 (12), 43 (16)]
neral oxypeucedaninyl acetal[a,b]		**29.5**	196, 250, 307
(diastereomer a) (**Figure 3**)	RI, MS	3377	438 (10, M^+), 368 (15), 287 (25), 245 (8), 203 (30), 202 (65), 201 (23), 95 (50), 69 (100), 41 (55)
neral oxypeucedaninyl acetal[a,b]	^{1}H NMR, ^{13}C NMR	**29.5**	196, 250, 307
(diastereomer b) (**Figure 3**)	RI, MS	3388	438 (10, M^+), 368 (15), 287 (25), 245 (8), 203 (30), 202 (65), 201 (23), 95 (50), 69 (100), 41 (55)
geranial oxypeucedaninyl acetal[a,b]	^{1}H NMR, ^{13}C NMR	**29.5**	196, 250, 307
(diastereomer a) (**Figure 3**)	RI, MS	3430	438 (3, M^+), 368 (5), 315 (9), 287 (20), 245 (10), 203 (20), 202 (40), 201 (18), 95 (20), 69 (100), 41 (45)
geranial oxypeucedaninyl acetal[a,b]		**29.5**	196, 250, 307
(diastereomer b) (**Figure 3**)	RI, MS	3448	438 (3, M^+), 368 (5), 315 (9), 287 (20), 245 (10), 203 (20), 202 (40), 201 (18), 95 (20), 69 (100), 41 (45)
bergamottin	Rt, UV	**29.8**	220, 250, 308
5-geranyloxypsoralen		decomp	[Claisen: 338 (18, M^+), 269 (17), 255 (22), 227 (30), 215 (79), 171 (34), 123 (63), 115 (42), 69 (75), 41 (100)]
(P1: R_{10})			
5-geranyloxy-7-methoxycoumarin	Rt, UV	**30.1**	207, 248, 322
(C2: 5 R_{10}, 7 R_2)	RI, MS	2862	328 (3, M^+), 193 (38), 192 (88), 164 (19), 135 (11), 81 (35), 69 (100), 41 (33)

[a] Trace constituent. [b] Previously unknown in lime. [c] Artifact or transformation product during CCC. [d] Typical grapefruit components. [e] Typical orange components. [f] Tentatively. [g] 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^+; 10%); ^{1}H NMR (diastereomer b) (250 MHz, $CDCl_3$) 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‴)], ^{13}C NMR diastereomer b (62.5 MHz, $CDCl_3$) 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 preseparation 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_{max}(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^+; 3%); ^{1}H NMR (diastereomer a) (250 MHz, $CDCl_3$) 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‴)], ^{13}C NMR diastereomer a (62.5 MHz, $CDCl_3$) δ 161.0

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

compound systematic name structure (Figure 1)	identification	Rt_{HPLC} (min)[d] RI_{GC} (DB-5)	UV_{max} (nm) MS (*m*/*z*)
meranzin hydrate	Rt, UV	**4.6**	204, 258, 324
7-methoxy-8-(2′,3′-dihydroxy-isopentyl)-coumarin	RI, MS	2432	278 (0.3, M^+), 263 (2), 220 (24), 190 (20),
(C3: 7 R_2, 8 R_6)			189 (27), 177 (100), 131 (22), 59 (9)
auraptenol[a,b]	Rt, UV	**8.3**	204, 257, 324
7-methoxy-8-(2′-hydroxy-3′-methyl-but-3′-enyl)-coumarin	RI, MS	2284	260 (-, M^+), 191 (11), 190 (100), 189 (29),
(C3: 7 R_2, 8 R_9)			175 (24), 161 (15), 160 (9), 131 (28), 103 (8), 77 (7)
			[TMS-ether: 332 (0.5, M^+), 317 (2), 262 (31),
			247 (3), 189 (5), 143 (45), 131 (10), 73 (100), 45 (9)]
marmin[a]	Rt, UV	**8.6**	202, 324
7-(6′,7′-dihydroxy-geranyloxy)-coumarin	RI, MS	2985	332 (0.6, M^+), 163 (67), 162 (100), 153 (65),
(C1: R_{12})			134 (49), 81 (76), 71 (71), 59 (42), 43 (60)
citropten[a]	Rt, UV	**10.3**	207, 248, 327
5,7-dimethoxycoumarin	RI, MS	1986	206 (94, M^+), 178 (100), 163 (75), 135 (44), 77 (15)
(C2: 5 R_2, 7 R_2)			
meranzin	Rt, UV	**10.5**	202, 248, 321
7-methoxy-8-(2′,3′-epoxy-isopentyl)-coumarin	RI, MS	2255	260 (15, M^+), 217 (20), 202 (70), 189 (81), 187 (80),
(C3: 7 R_2, 8 R_5)			159 (71), 131 (100), 103 (24), 77 (26)
epoxybergamottin hydrate[a]	Rt, UV	**10.6**	222, 252, 310
5-(6′,7′-dihydroxy-geranyloxy)-psoralen		decomp	
(P1: R_{12})			
isomeranzin	Rt, UV	**11.1**	202, 248, 320
7-methoxy-8-(isopentyl-2′-on)-coumarin	RI, MS	2243	260 (20, M^+), 191 (12), 190 (100), 189 (49),
(C3: 7 R_2, 8 R_8)			175 (14), 161 (10), 131 (36), 71 (20), 43 (37)
bergapten[a]	Rt, UV	**11.3**	222, 249, 268, 312
5-methoxypsoralen	RI, MS	2062	216 (100, M^+), 201 (32), 188 (17), 173 (70),
(P1: R_2)			145 (32), 89 (13), 63 (6)
nobiletin	Rt, UV	**12.2**	208, 250, 270, 333
3′,4′,5,6,7,8-hexamethoxyflavone	RI, MS,	3392	402 (20, M^+), 388 (20), 387 (100), 344 (17),
(F: 3 R_1, 3′ R_3, 8 R_3)			326 (11), 197 (33),182 (24), 83 (20)
tetra-*O*-methylscutellarein	Rt, UV	**13.3**	198, 266, 322
4′,5,6,7-tetramethoxyflavone	RI, MS	3186	342 (11, M^+), 328 (16), 327 (100), 284 (16),
(F: 3 R_1, 3′ R_1, 8 R_1)			167 (10), 132 (5), 89 (3), 69 (4)
3,3′,4′,5,6,7,8-heptamethoxyflavone	Rt, UV	**13.5**	253, 271, 342
(F: 3 R_3, 3′ R_1, 8 R_1)	RI, MS	3375	432 (31, M^+), 431 (21), 417 (100), 401 (13),
			387 (11), 359 (11), 197 (14), 165 (16)
tangeritin	Rt, UV	**15.4**	272, 322
4′,5,6,7,8-pentamethoxyflavone	RI, MS	3198	372 (19, M^+), 358 (20), 357 (100), 314 (17),
(F: 3 R_1, 3′ R_1, 8 R_3)			296 (12), 197 (28), 182 (27), 83 (19)
epoxyauraptene	Rt, UV	**18.7**	202, 322
7-(6′,7′-epoxy-geranyloxy)-coumarin	RI, MS	2772	314 (0.5, M^+), 163 (34), 162 (79), 153 (100),
(C1: R_{11})			135 (32), 134 (59), 81 (89), 71 (92), 43 (74)
osthol	Rt, UV	**20.9**	203, 257, 322
7-methoxy-8-isopentenylcoumarin	RI, MS	2143	244 (100, M^+), 229 (67), 213 (44), 201 (68),
(C3: 7 R_2, 8 R_4)			189 (62), 187 (30), 186 (26), 159 (32), 131 (45)
epoxybergamottin	Rt, UV	**22.4**	221, 250, 309
5-(6′,7′-epoxy-geranyloxy)-psoralen		decomp	[354 (0.5 M^+), 202 (100), 174 (21), 153 (50),
(P1: R_{11})			135 (16), 81 (21), 71 (17)]
auraptene	Rt, UV	**28.6**	202, 322
7-geranyloxycoumarin	RI, MS	2634	298 (–, M^+), 163 (63), 162 (73), 137 (23), 136 (35),
(C1: R_{10})			134 (39), 93 (34), 81 (44), 69 (100), 41 (36)
bergamottin	Rt, UV	**29.8**	220, 250, 308
5-geranyloxypsoralen		decomp	[Claisen: 338 (18, M^+), 269 (17), 255 (22), 227 (30),
(P1: R_{10})			215 (79), 171 (34), 123 (63), 115 (42), 69 (75), 41 (100)]
hydroxypentamethoxyflavone 1[a–c]		nf[e]	
	MS	3294	388 (44, M^+), 374 (20), 373 (100), 215 (14), 194 (13),
			187 (24), 165 (33)
hydroxypentamethoxyflavone 2[a–c]		nf	
	MS	3368	388 (54, M^+), 374 (19), 373 (100), 211 (24), 183 (25)
hydroxyhexamethoxyflavone[a–c]		nf	
	MS	3383	418 (61, M^+), 404 (22), 403 (100), 209 (21),
			180 (22), 165 (35)

[a] Trace constituent. [b] Previously unknown in grapefruit. [c] Unknown configuration, tentatively. [d] Rt: HPLC retention time in minutes (conditions see HPLC with diode array detection). [e] 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_{max}, 223, 270, 313 nm; GC−MS: (70 eV), *m*/*z* 73 (100%), 202 (45%), 43 (14%), 174 (13%), 145 (12%), 89 (10%), 318 (M^+; 6%); ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.21 [d, 1 H, J = 9,75 Hz,

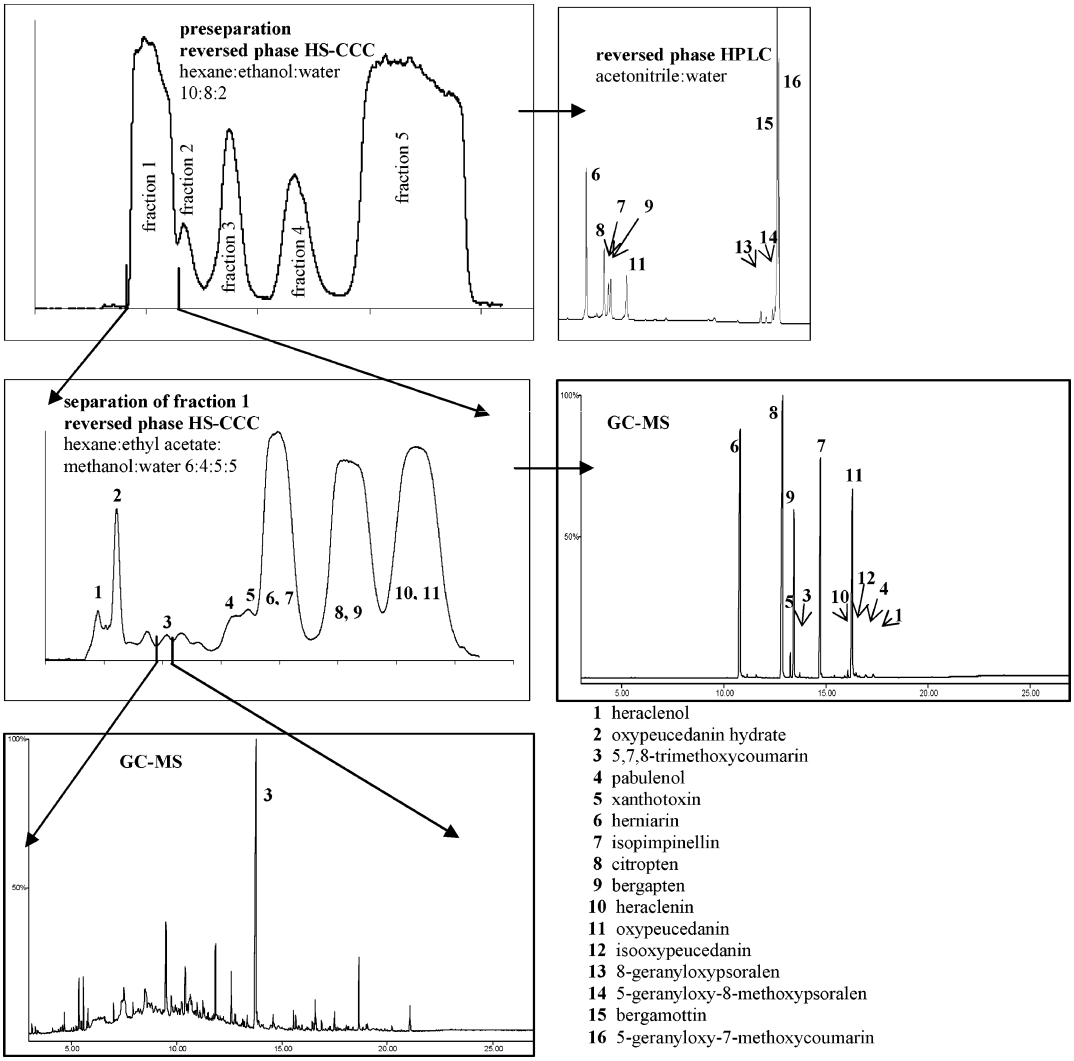

Figure 2. CCC isolation of 5,7,8-trimethoxycoumarin from Persian lime oil residue.

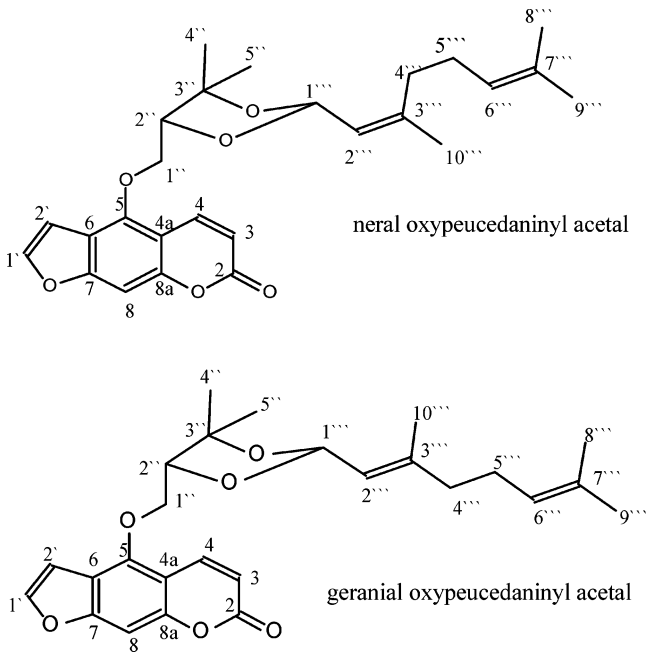

Figure 3. Neral oxypeucedaninyl acetal and geranial oxypeucedaninyl acetal.

H−C(4)], 6.26 [d, 1H, J = 9.75 Hz, H−C(3)], 7.14 [s, br, 1 H, H−C(8)], 7.57 [d, 1 H, J = 2.25 Hz, H−C(1′)], 6.98 [d, 1 H, J = 2.25 Hz, H−C(2′)], 3.25 [s, 3H, H−CH_2−O], 2.33 [br, 1 H, H−O], 1.27 [s, 3 H, H−CH_2], 1.22 [s, 3 H, H−CH_2], 4.55 [dd, 1H, J_{AB} = 10.0 Hz, J_{AX} = 3.0 Hz, H_A-C(1″)], 4.37 [dd, 1H, J_{AB} = 10.0 Hz, J_{BX} = 7.75 Hz, H_B-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 ^{1}H NMR data. RI 2107, UV_{max}, 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%); ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.96 [d, 1 H, J = 9,75 Hz, H−C(4)], 6.14 [d, 1H, J = 9.75 Hz, H−C(3)], 6.32 [s, 1 H, H−C(6)], 3.89 [s, 3 H, H−CH_2−O], 3.90 [s, 3 H, H−CH_2−O], 3.95 [s, 3 H, H−CH_2−O], ^{1}H NMR (250 MHz, C_6D_6) δ 7.49 [d, 1 H, J = 9,75 Hz, H−C(4)], 5.85 [d, 1H, J = 9.75 Hz, H−C(3)], 5.72 [s, 1 H, H−C(6)], 3.74 [s, 3 H, H−CH_2−O], 3.29 [s, 3 H, H−CH_2−O], 3.15 [s, 3 H, H−CH_2−O]. ^{1}H NMR data were collected in $CDCl_3$ and C_6D_6 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_2O (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_2O 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 ^{1}H NMR comparison with the literature (*27*, *28*) in both lime oil types. The 5,7,8-substition 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 ^{1}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-geranyloxypsoralen, 5-isopentenyloxy-7-methoxycoumarin, isoimperatorin, and 7-isopentenyloxycoumarin 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, ^{1}H, and ^{13}C NMR. The ^{1}H NMR spectra turned out to be significant with regard to the psoralen skeleton, as the proton resonance of H(C4) (δ = 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 (δ = 5.62; 5.75) and the isolated acetals from citrus (δ = 5.69; 5.74). In the ^{13}C NMR 26 carbon signals were observed; the acetalic carbon resonance is situated in the extended range (δ = 98.2 and 98.7).

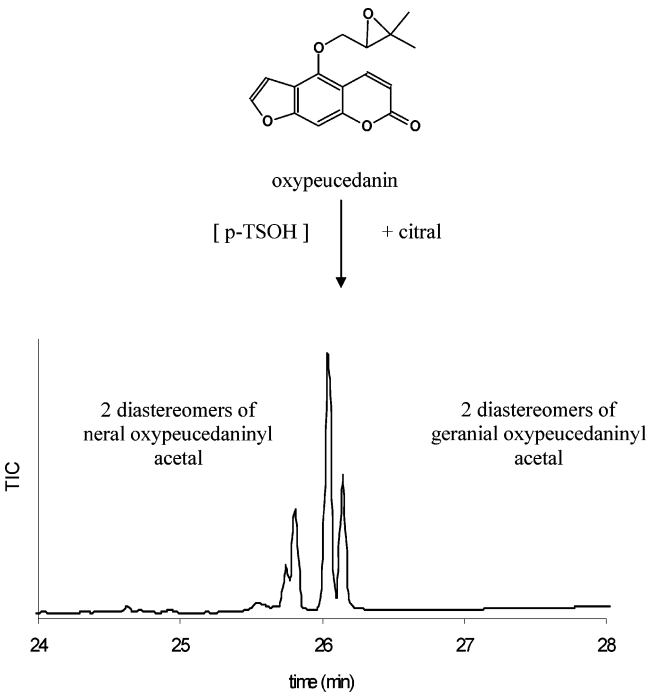

Figure 4. Formation of the citral oxypeucedaninyl acetals.

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 dimethoxy-geranyloxycoumarin was deduced. It shows the same mass spectra as published for the 6,7-dimethoxy-5-geranyloxycoumarin derivatives (*14*). 6,7-Dimethoxy-5-geranyloxycoumarin was previously reported in lemon and lime oil; in the literature its structure and substitution pattern were determined by ^{1}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′-octadienyloxy)-psoralen, which resulted from epoxy-bergamottin (*35*). The formation of ketones, such as isomeranzin from 7-methoxy-8-

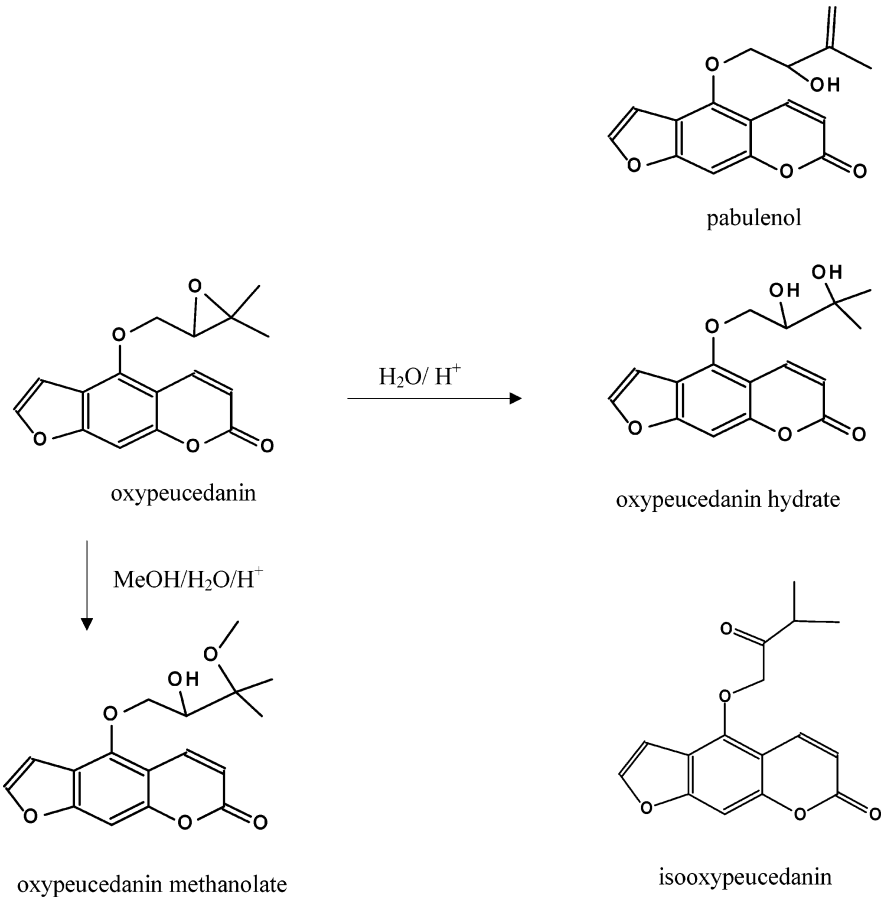

Figure 5. Hydrolysis and methanolysis of oxypeucedanin.

(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 °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 preseparation 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_2O (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_2O (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 ($M^+ - 15$, 100%), 388 (M^+, 50−55%)] and one hydroxyhexamethoxyflavone [*m*/*z* 403 ($M^+ - 15$, 100%), 418 (M^+, 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-trifluoro acetamide; OHC, oxygen heterocyclic compounds; PL, Persian lime; T>H, tail-to-head; TMS, trimethylsilyl.

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