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Liquid Chromatography/Mass Spectrometry and Liquid Chromatography/Nuclear Magnetic Resonance as Complementary Analytical Techniques for Unambiguous Identification of Polymethoxylated Flavones in Residues from Molecular Distillation of Orange Peel Oils (*Citrus sinensis*)

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Liquid chromatography/mass spectrometry and liquid chromatography/nuclear magnetic resonance techniques with ultraviolet/diode array detection were used as complementary analytical tools for the reliable identification of polymethoxylated flavones in residues from molecular distillation of cold-pressed peel oils of *Citrus sinensis*. After development of a liquid chromatographic separation procedure, the presence of several polymethoxy flavones such as sinensetin, nobiletin, tangeretin, quercetogetin, heptamethoxyflavone, and other derivatives was unambiguously confirmed. In addition, proceranone, an acetylated tetranortriterpenoid with limonoid structure, was identified for the first time in citrus.

KEYWORDS: LC/NMR; LC/MS; *Citrus sinensis*; polymethoxylated flavones; polymethoxyflavones; tetranortriterpene; limonoid; proceranone

INTRODUCTION

Cold-pressed citrus oils are of high importance in the food industry (*1*, *2*). Apart from sensory aspects, potential health-promoting effects are associated with constituents such as flavonoid derivatives (*3*, *4*). Among such compounds, polymethoxylated flavones show antimutagenic as well as antitumor properties and therefore may possess chemopreventive potential (*5*, *6*). The composition of polymethoxylated flavones can significantly differ between different *Citrus* species and varieties (*7*−*11*). Therefore, a rapid and unambiguous assignment of these constituents in such extracts is necessary. Recently, some liquid chromatography/mass spectrometry (LC/MS) studies were performed on flavonoids from *Citrus* (*12*−*14*). In those studies, polymethoxylated flavones were normally identified by comparing MS and ultraviolet (UV) spectra obtained from LC/MS experiments with commercially available standard materials or previously isolated reference materials, which is quite time-consuming, and also with published data, which implies a certain tentativeness.

The hyphenated liquid chromatography/nuclear magnetic resonance (LC/NMR) technique as a complementary tool now speeds up evaluation of the compounds of interest by providing structural information from their NMR spectra. A rising number of publications on LC/NMR studies on natural extracts and ongoing technical developments indicate the increasing importance of this analytical tool (*15*−*17*). Recently, derivatives of coumarins and furocoumarins in distilled lemon peel oil were analyzed using LC/MS and LC/NMR (*18*). A high-performance liquid chromatography (HPLC) separation procedure was developed, which included detection and characterization of the polymethoxylated flavones by MS and NMR. The method was tested using distillation residues of *Citrus sinensis*, which should be enriched in such flavones.

MATERIAL AND METHODS

Plant Material. After pressing orange fruits (*C. sinensis*), the oil layer was separated from the juice phase. The oil was filtered and centrifuged. Subsequently, the oil was chilled thereby resulting in precipitation of waxes. After filtration, the cold-pressed oil was subjected to molecular distillation to produce an almost colorless oil. Residues from this molecular distillation were taken for further analysis.

HPLC/MS Analyses. About 1 mg of the residue was dissolved in 1 mL of acetonitrile (LAB-Scan, Dublin, Ireland), and 5 μL of the sample solution was injected without further purification.

LC/MS experiments were performed on a system consisting of a ThermoQuest LCQ mass spectrometer with an atmospheric pressure chemical ionization (APCI) interface and a Hewlett-Packard HP 1100 HPLC system (degasser, pump, column oven, and photodiode array detector at 210 nm) at 50 °C. APCI experiments were carried out in the positive mode. Nitrogen was used as the sheath gas.

The gradient starting with 65% H_2O (containing 0.01% formic acid v/v, Fluka, Buchs, Switzerland) and 35% acetonitrile (LAB-Scan) was changed to 100% acetonitrile within 55 min at a flow rate of 0.2 mL/min. A Varian OmniSpher C18 column (125 mm × 2 mm, particle

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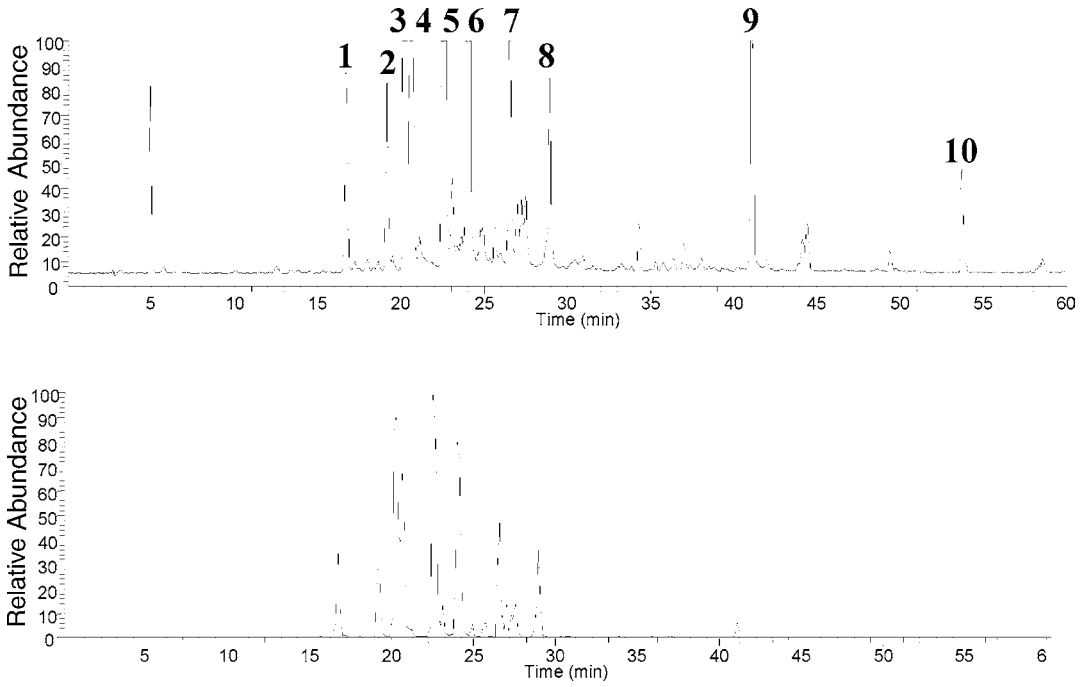

Figure 1. Chromatogram and total ion current (TIC) from the LC/MS experiment.

size 3 μm) was used for chromatographic separation. The water used (HPLC grade) was purified on a HP5 UV pure water system from TKA-Lab (Niederelbert, Germany).

HPLC/NMR Analyses. About 200 mg of the residue was dissolved in 2 mL of acetonitrile (LAB-Scan). Insoluble materials were filtered off (PTFE, 0.45 μm, Omnilab, Bremen, Germany), and 100 μL of the sample solution was injected.

LC/NMR experiments were performed on the Unity INOVA system using a $^1H\{^{13}C/^{15}N\}$PFG triple resonance indirect detection microflow LC/NMR probe (IFC probe) with a detection volume of 60 μL at 20 °C. The HPLC system consisted of a ternary Varian ProStar 230 pump, a Varian ProStar 330 photodiode array detector (210 nm), and a Varian ProStar 510 column oven (50 °C). Chromatographic separation was carried out using an OmniSpher C18 column (250 mm × 4.6 mm; particle size, 5 μm). The gradient starting with 85% D_2O (Deutero GmbH, Kastellaun, Germany), containing 0.01% TFA v/v (Fluka) and 15% acetonitrile (NMR Chromasolv, Sigma-Aldrich, Steinheim, Germany), was changed to 100% acetonitrile within 60 min at a flow rate of 0.2 mL/min. NMR experiments were run in the stop-flow mode. Solvent suppression was carried out by executing a WET pulse sequence before every acquisition. Chemical shifts were referenced relative to acetonitrile (1.93 ppm). NMR spectra were also recorded on a Varian Unity INOVA (400 MHz) spectrometer using $CDCl_3$ (Deutero GmbH) and TMS (Aldrich) as an internal standard.

Preparative HPLC. The preparative HPLC was performed with a Varian Prep Star, equipped with two SD1 pumps and a ProStar UV/vis detector (model 320, 225 nm). An isocratic mobile phase comprised of water (HPLC grade, see above, 30%) and methanol (p.a., Fluka, 70%) was used at a flow rate of 25 mL/min. The analytes were separated on a GROM Saphir 110 C18 column (150 mm × 20 mm, 5 μm). The column was kept at a constant temperature of 30 °C. The sample injection volume was 1 mL.

Gas Chromatography (GC)/MS Analysis. GC/MS experiments were performed using a MAT 8200 from Thermo Finnigan (70 eV ionization energy, EI mode) equipped with a DB-1 capillary column (30 m, film 0.25 μm, i.d. 0.25 mm). The column temperature was programmed from 80 to 280 °C at 4 °C/min, and helium was used as the carrier gas.

*5,6,7,3′,4′-Pentamethoxyflavone (**1**, Sinensetin).* ^{1}H NMR (400 MHz, D_2O/CH_3CN): 7.59 (dd, J = 2.1 and 8.7 Hz, H-6′), 7.46 (d, J = 1.9 Hz, H-2′), 7.08 (s, H-8), 7.06 (d, J = 8.8 Hz, H-5′), 6.64 (s, H-3), 3.90 (s, 3H), 3.85 (s, 3H), 3.82 (s, 3H), 3.79 (s, 3H), 3.77 (s, 3H).

*3,5,6,7,3′,4′-Hexamethoxyflavone (**2**, Quercetogetin).* ^{1}H NMR (400 MHz, D_2O/CH_3CN): 7.69 (dd, J = 2.0 and 8.6 Hz, H-6′), 7.63 (d, J = 1.9 Hz, H-2′), 7.06 (d, J = 8.6 Hz, H-5′), 6.99 (s, H-8), 3.87 (s, 3H), 3.82 (s, 3H), 3.82 (s, 3H), 3.81 (s, 3H), 3.76 (s, 3H), 3.66 (s, 3H).

*5,6,7,8,3′,4′-Hexamethoxyflavone (**3**, Nobiletin).* ^{1}H NMR (400 MHz, D_2O/CH_3CN): 7.62 (dd, J = 2.2 and 8.6 Hz, H-6′), 7.47 (d, J = 2.2 Hz, H-2′), 7.09 (d, J = 8.4 Hz, H-5′), 6.67 (s, H-3), 4.02 (s, 3H), 3.95 (s, 3H), 3.86 (s, 3H), 3.84 (s, 6H), 3.77 (s, 3H).

*5,6,7,4′-Tetramethoxyflavone (**4**, Tetra-O-methylscutellarein).* ^{1}H NMR (400 MHz, D_2O/CH_3CN): 7.92 (d, J = 9.0 Hz, H-2′, H-6′), 7.06 (d, J = 9.0 Hz, H-3′, H-5′), 7.05 (s, H-8), 6.60 (s, H-3), 3.90 (s, 3H), 3.81 (s, 3H), 3.80 (s, 3H), 3.71 (s, 3H). The NMR data of **4** in $CDCl_3$ are consistent with data from literature (*19*).

*3,5,6,7,8,3′,4′-Heptamethoxyflavone (**5**).* ^{1}H NMR (400 MHz, D_2O/CH_3CN): 7.72 (dd, J = 2.1, 8.6 Hz, H-6′), 7.66 (d, J = 2.1 Hz, H-2′), 7.08 (d, J = 8.6 Hz, H-5′), 3.99 (s, 3H), 3.90 (s, 3H), 3.83 (s, 9H), 3.79 (s, 3H), 3.70 (s, 3H).

*5,6,7,8,4′-Pentamethoxyflavone (**6**, Tangeretin).* ^{1}H NMR (400 MHz, D_2O/CH_3CN): 7.93 (dd, J = 8.7 Hz, H-2′, H-6′), 7.07 (d, J = 8.7 Hz, H-3′, H-5′), 6.63 (s, H-3), 4.01 (s, 3H), 3.93 (s, 3H), 3.83 (s, 3H), 3.81 (s, 3H), 3.76 (s, 3H).

*5-Hydroxy-6,7,8,3′,4′-pentamethoxyflavone (**7**, 5-Desmethylnobiletin).* ^{1}H NMR (400 MHz, D_2O/CH_3CN)*: 7.62 (dd, J = 2.2 and 8.5 Hz, H-6′), 7.46 (d, J = 2.2 Hz, H-2′), 7.07 (d, J = 8.5 Hz, H-5′), 6.73 (s, H-3), 3.89 (s, 3H), 3.84 (s, 3H), 3.83 (s, 3H), 3.79 (s, 3H). *One signal of a methoxy group was eliminated by HDO suppression.

*5-Hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (**8**).* ^{1}H NMR (400 MHz, D_2O/CH_3CN)*: 7.75 (dd, J = 2.2 and 8.7 Hz, H-6′), 7.66 (d, J = 2.2 Hz, H-2′), 7.08 (d, J = 8.7 Hz, H-5′), 3.88 (s, 3H), 3.84 (s, 3H), 3.83 (s, 3H), 3.78 (s, 3H), 3.74 (s, 3H). *One signal of a methoxy group was eliminated by HDO suppression.

*Proceranone (**9**).* ^{1}H NMR (400 MHz, D_2O/CH_3CN): 7.37 (dd, J = 1.6 and 1.6 Hz, 1H) 7.26 (s, 1H), 6.55 (d, J = 12.3 Hz, 1H), 6.29 (d, J = 1.6 Hz, 1H), 5.72 (d, J = 12.3 Hz, 1H), 5.28 (dd, J = 1.6 and 3.3 Hz, 1H), 5.08 (dd, J = 2.4 and 3.3 Hz, 1H), 2.72 (dd, J = 7.1 and 10.9 Hz, 1H), 2.32−2.42 (m, 2H), 2.19 (ddd, J = 3.6, 7.4 and 15.3 Hz, 1H), 1.68−1.78 (m), 1.57−1.64 (m, 1H), 1.34 (s, 3H), 1.25 (s, 3H), 1.18 (s, 3H), 1.13 (s, 3H), 0.68 (s, 3H). The NMR data of **9** in $CDCl_3$ are consistent with data from literature (*20*, *21*).

*Linoleic Acid (**10**).* ^{1}H NMR (400 MHz, D_2O/CH_3CN)*: 5.22−5.34 (m, 4H), 2.73 (t, J = 6.2 Hz, 2H), 2.20 (t, J = 7.4 Hz, 2H), 1.48 (m, J = 6.9 Hz, 2H), 1.23 (bs, 14H), 0.82 (t, J = 6.6 Hz, 3H). *Signals from methylene groups adjacent to the double bonds were eliminated by acetonitrile suppression.

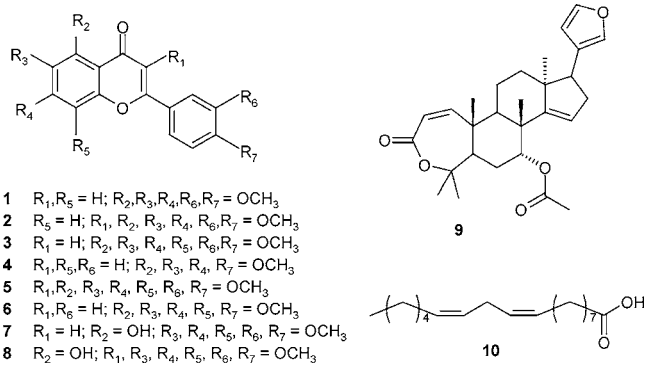

Figure 2. Identified compounds by LC/MS and LC/NMR experiments.

Table 1. APCI-MS and UV Data of Detected Polymethoxylated Flavones[a]

compd	t_R (min)	$[M + H]^+$ (*m*/*z*)	MS/MS (*m*/*z*)	UV λ_{max} (nm)
1	16.7	373	358, 343, 312	242, 266, 332
2	19.2	403	388, 373	243, 253, 266 (sh), 337
3	20.3	403	388, 373	250, 272, 335
4	20.6	343	328, 313, 282	268, 323
5	22.6	433	418, 403	255, 270, 343 (sh)
6	24.1	373	358, 343, 312	272, 324
7	26.4	389	374, 359, 328	255, 284, 343
ND	27.0	403	388, 373,	268, 333
ND	27.5	389	374, 359	257, 274, 339, 362
8	28.9	419	404, 389	259, 279, 348

[a] ND, not determined; sh, shoulder.

RESULTS AND DISCUSSION

Solubles from distillation residues of cold-pressed orange peel oils (*C. sinensis*) in acetonitrile were analyzed. The chromatograms (UV detection at 210 nm) from the LC/MS and LC/NMR experiments are in good agreement since the same stationary phase was used. In **Figure 1**, the chromatogram and total ion current (TIC) of the LC/MS experiment are shown. The retention time interval between 16 and 30 min is occupied by different polymethoxyflavones. Compounds **1**−**6** are indicated as tetra-, penta-, hexa-, and heptamethoxy-substituted flavones due to the protonated molecular ions $[M + H]^+$ at *m*/*z* 343, 373, 403, and 433 in the positive APCI mode. Compounds **7** and **8** could be assigned as being monohydroxylated penta- and hexamethoxyflavones due to molecular weights of 388 and 418 (**Table 1**). Two further polymethoxyflavone derivatives could clearly be detected due to their protonated molecular ions $[M + H]^+$ at *m*/*z* 403 and 389 and were therefore designated as being hexamethoxy- and monohydroxylated pentamethoxyflavones, respectively.

The substitution pattern of the phenyl residues of the different flavones can easily be deduced by NMR. The para-substituted phenyl moieties of compounds **4** and **6** are assigned as a consequence of two doublets at about 7.9 and 7.1 ppm and a coupling constant of about 9.0 Hz. For both compounds, the typical singlet signal of the proton attached to C-3 is observed at about 6.6 ppm. Regarding the residual four methoxy groups, compound **6** could be unambiguously identified as tangeretin. Derivative **4** with three remaining methoxy groups in position C-5, C-6, and C-7 is confirmed as being tetra-*O*-methylscutellarein via its isolation by preparative HPLC and comparison with published data (*19*).

The phenyl residues of flavones **1**−**3** and **5** are indicated as being 1,3,4-substituted isomers by a double doublet at about 7.6 (**1**, **3**) or 7.7 ppm (**2**, **5**), a doublet around 7.5 (**1**, **3**) or 7.6 ppm (**2**, **5**), and a doublet at 7.1 ppm with coupling constants of 8.6 and 2.1 Hz. The absence of substitution for **1** and **3** at C-3 is evidenced by the presence of the signal at 6.6 ppm for the proton. Compound **3** is therefore unambiguously identified as nobiletin due to the remaining four methoxy groups. Comparing the chemical shifts of **1** with those of tetramethylscutellarein **4**, examination of the protons at the chromen-4-one system shows almost identical shifts thereby leading to the structure of sinensitin for **1**. For compound **5**, all protons at the chromen-4-one system are substituted by methoxy groups and considering the trisubstituted phenyl residue 3,5,6,7,8,3′,4′-heptamethoxyflavone could be confirmed. The LC/MS experi-

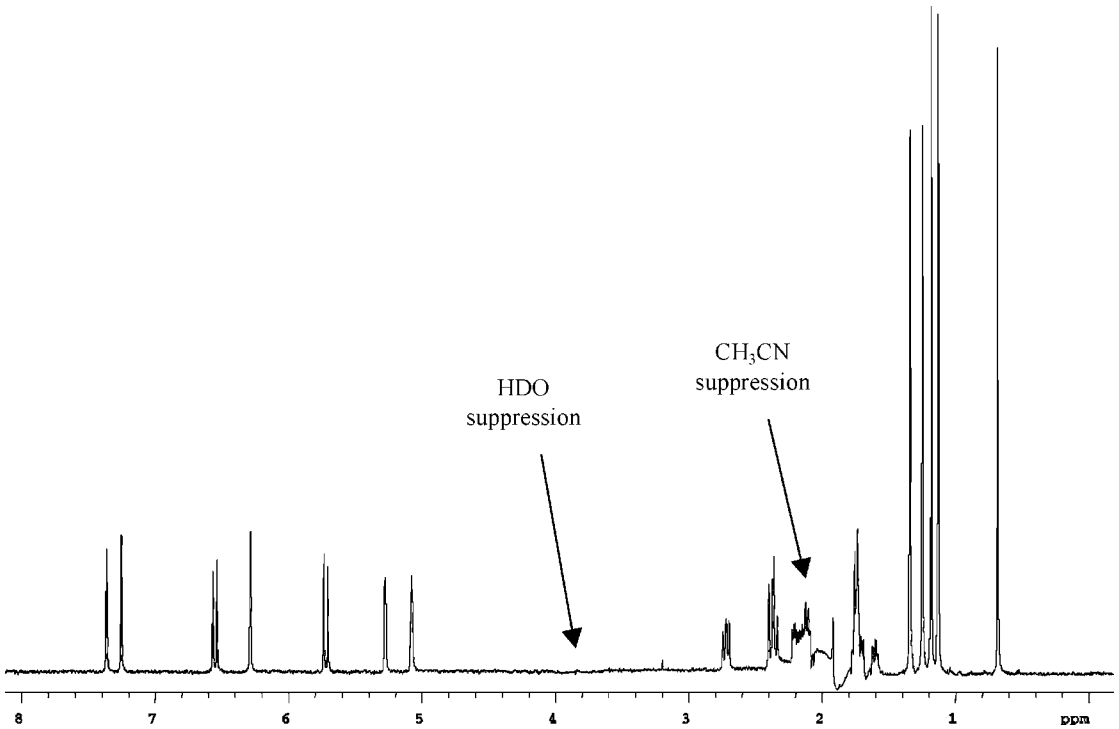

Figure 3. ^{1}H LC/NMR spectrum of proceranone **9**.

ment indicates compound **2** as hexamethoxyflavone. The missing signal in the ^{1}H NMR spectrum of **2** at about 6.6 ppm for the proton at C-3 results from substitution by a methoxy group. NMR data of compound **2** are identical with those of 3,5,6,7,3′,4′-hexamethoxyflavone, which we have recently isolated from clementine peel oils by preparative HPLC (*22*) and confirmed by comparison with published data (*23*).

Compounds **7** and **8** were indicated as monohydroxylated penta- and hexamethoxyflavones, which could also be confirmed by LC/NMR experiments. The 1,3,4-trisubstituted pattern of both compounds is reflected by the three signals of the aromatic protons as described above. The chemical shifts of these protons of constituent **8** are almost identical to those of **2** and **5**; therefore, three of six methoxy groups should be attached to C-3, C-3′, and C-4′. Because the exact position of the hydroxy group could not be determined by the LC/NMR measurements, comparison of LC/^{1}H NMR data with those of 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone showed complete identity. The latter has recently been detected in clementine peel oil residues (*22*), isolated, and compared with published data (*24*). Comparing the NMR spectrum of **7** with those of **1** and **3**, the proton attached to C-3 and the 1,3,4-substitution pattern with methoxy groups in C-3′ and C-4′ can easily be assigned. According to the literature, arrangement of the three remaining methoxy and one hydroxy groups for constituents isolated from citrus fruits is described for the hydroxy group at C-5 (*19*) or C-7 (*25*). Because the data from the ^{1}H NMR spectrum do not discriminate between the two derivatives, an HMBC experiment was performed in the loop collection mode. The protons of two methoxy groups attached to the chromen-4-one moiety in position C-6 and C-8 show correlations with aromatic carbon singlets at about 133 and 137 ppm. These values fit well with data of 5-hydroxy-6,7,8,3′,4′-hexamethoxyflavone (*19*).

The results are in good agreement with literature data from orange (*C. sinensis*; Rutaceae) (*10*, *26*) since six of eight reported structures of polymethoxyflavones were reidentified (**Figure 2**).

The LC/^{1}H NMR spectrum of compound **9** (**Figure 3**) did not show the typical pattern of a flavonoid structure. Instead, seven olefinic or methine protons attached to oxygenated carbon atoms in the region of 5.0−7.4 ppm and five methyl groups as singlets from 0.8 to 1.2 ppm were observed. In consideration of a proposed molecular ion $[M + H]^+$ at *m*/*z* 453, a higher terpene homologue was assumed. For structure elucidation, compound **9** was isolated by preparative HPLC and its ^{13}C spectrum in combination with the molecular mass of 452 indicates a molecular formula of $C_{28}H_{36}O_5$. The NMR data are consistent with reported data for proceranone obtained from *Carapa procera* (*21*).

By LC/UV detection, a further compound could be detected around 54 min, but efficient ionization in APCI could not be achieved. The NMR spectrum displays the typical pattern of an unsaturated fatty acid and linoleic acid **10** was tentatively assigned by analysis of the integrals. The LC peak was recovered and measured by GC/MS, and the structure of **10** could be confirmed.

Several polymethoxyflavones could be unambiguously identified due to their proton pattern or in comparison with known data from literature, especially in the given solvent system. This was possible without prior isolation and therefore resulted in accelerated evaluation of those derivatives. The characterization of components with low ionization effiency shows another strength of the LC/NMR technique. In contrast, no NMR data could be obtained from two polymethoxyflavones, which were detected by LC/MS, showing the lack of sensitivity of the LC/NMR method.

Proceranone, a tetranortripernoid with limonoid structure, was not described in citrus before and may have interesting healthful properties such as other limonoids (*27*).

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