HPLC-NMR — a Powerful Tool for the Identification of Non-Volatiles in Lemon Peel Oils

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Citrus peel oils are of great importance for the flavor and fragrance industry because they are widely used in perfumes, beverages, food and cosmetic products.[1-4] Many publications cover the analysis of the volatile substances of peel oils.[5] Nevertheless, the non-volatile ingredients seem to play an important role in citrus oils, too. These substances act as odor fixatives and therefore influence the olfactory properties.[6,7] Non-volatiles found in lemon oils are mainly coumarins, psoralens, carotenoids, fatty acids and sterols.[8] The two first-mentioned classes of substances form a large part of the non-volatile residue. The presence of psoralens can lead to the formation of sediments in citrus oils, which can cause problems during their application and therefore have to be removed.[9] Coumarins and psoralens can be used as marker substances for the characterization or for detecting the adulteration of oils.[6,8,10-16] Besides their biological properties, they also seem to be important for the stability of citrus oils.[17-19]

In the past, analyses of the non-volatile substances have mainly been carried out by HPLC with UV or mass spectrometric detection.[11,16] This publication describes the analysis of a residue of a cold pressed (CP) lemon oil by HPLC-NMR. Sixteen coumarins and psoralens were identified. The structure of one heretofore unknown psoralen was elucidated.

The Coupling of HPLC with NMR

NMR spectroscopy is the most powerful spectroscopic method with which to determine the structures of unknown molecules. The drawback of this technique is the low sensitivity compared with other spectroscopic methods, like mass spectrometry, UV or infrared spectroscopy. With the availability of higher magnetic fields, development of special flow probes with enhanced sensitivity and improved methods for solvent suppression it was possible to couple NMR spectroscopy successfully with high performance liquid chromatography. First studies date back to the end of the 1970s and beginning of the 1980s.[20-22] Meanwhile, the technique is available at a routine level.

An HPLC-NMR system consists of two sub-units. The "main" detector system is an NMR spectrometer unit, which includes a magnet with a special LC-NMR flow probe, an RF console and a host computer. The second sub-unit of the HPLC-NMR system is an HPLC system, which is responsible for the sample cleanup. The sub-unit consists of a pump, usually a UV or RI detector, an injection valve, possibly a peak collector and a computer. This computer controls the HPLC system and communicates with the NMR unit in order to synchronize experiment-specific operations like starting or stopping the HPLC pump, switching valves or sending trigger pulses to initiate NMR acquisition.

Numerous review articles about HPLC-NMR have been published describing the technique and several applications.[23-28] For example, HPLC-NMR was successfully used for the analysis of pharmaceuticals, drug metabolites and polymers, as well as crude plant extracts.[29-33]

The use of non-deuterated solvents like acetonitrile or methanol in HPLC-NMR runs requires efficient methods to suppress the ^{1}H-NMR resonances of these liquids. This can be accomplished by using special pulse sequences like the WET pulse sequence (F-1).[34] The advantage of the WET sequence is its short duration (approximately 100 msec) compared with other sequences used for solvent suppression. This favors its usage in NMR experiments, which have to be repeated quickly in order to collect as many scans as possible.

Due to the construction of the flow cell NMR probe it is impossible to spin the sample, which results in a poorer line width compared with standard NMR measurements. Therefore, small (long-range) couplings usually cannot be resolved. On-line HPLC-NMR separations can be conducted in three different

^{1}H NMR spectra of peak 2 from a HPLC-NMR run in the stopped-flow mode

F-1

top: without suppression of the solvent signals
bottom: spectrum with WET pulse sequence applied, recorded with 512 scans (NMR signals of the analyte (citropten 2) can now be seen clearly)
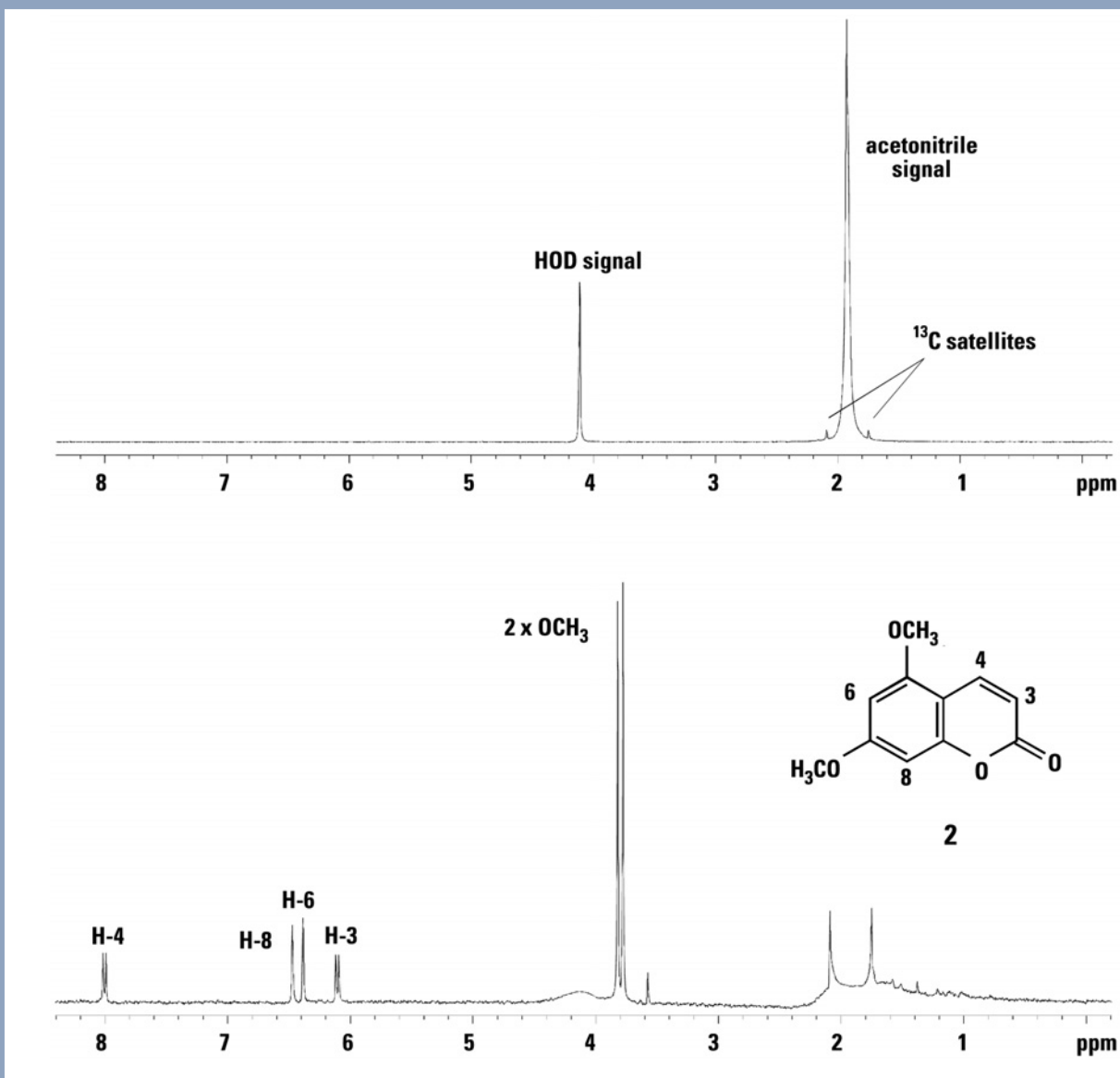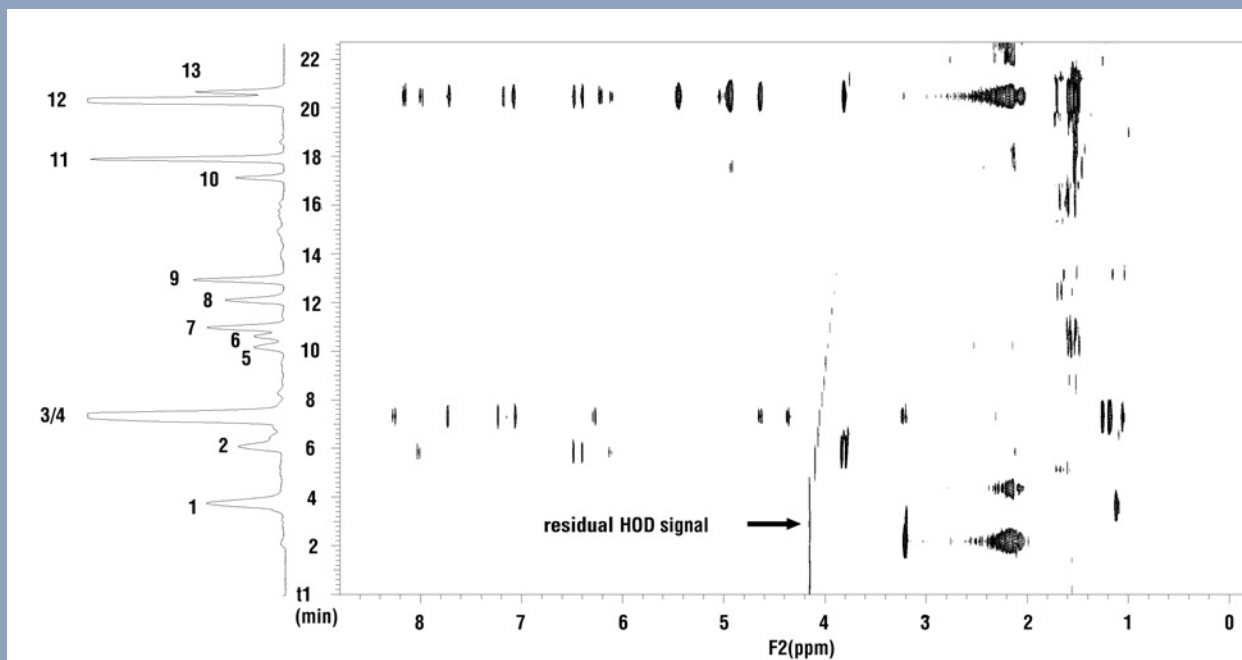

modes of operation: on-flow, stopped-flow and peak collection.

On-flow mode: In this mode the NMR spectrometer is continuously acquiring data from analytes after HPLC separation. On the other hand this mode has some drawbacks. It is the most insensitive one because only a limited number of scans (usually four to eight) can be collected for a ^{1}H-NMR spectrum. This mode is therefore restricted to one-dimensional ^{1}H-NMR experiments. Nevertheless, it is useful for obtaining NMR spectra of the main components and getting a quick survey of the different classes of sub-

stances in the sample by identifying their basic structure elements. The ^{1}H-NMR spectra collected in the run can be displayed in a pseudo two-dimensional HPLC-NMR chromatogram (F-2). The vertical axis in this contour plot is the time axis; the horizontal axis represents the frequency scale of the ^{1}H-NMR spectrum. The UV trace is plotted along the vertical axis.

Stopped-flow mode: HPLC-NMR operation in the stopped-flow mode requires a UV detector in the line between the HPLC column and the NMR probe to monitor signal peaks of the analytes. When a peak maximum is detected, the pump is stopped after a delay time, which is needed to transfer the substance into the NMR probe. Acquisition can then be started

Pseudo 2D HPLC-NMR contour plot of a lemon oil residue in the on-flow mode (the LC-UV spectrum is plotted along the y-axis) **F-2**

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to measure one- and even two-dimensional NMR data. The stopped-flow mode is much more sensitive than the on-flow mode, because a larger number of scans can be collected. The hold time of the pump is therefore determined by the time needed for the execution of the NMR experiment(s). Furthermore, it is possible to automatically acquire NMR data on a large number of peaks in the LC chromatogram, since the dedicated LC-NMR software of the HPLC and NMR host computers are able to synchronize the necessary operations including switching the valves, detection of the UV peak maxima and starting as well as stopping the NMR acquisition.

Peak/loop collection mode: The first step of this mode is to store the chromatographic peaks of interest in the loops of a peak collector. In the second step the contents of the loops are transferred sequentially into the NMR probe by the HPLC pump, followed by the NMR data acquisition. The advantage of the loop collection mode compared with the stopped-flow mode arises from the collection of the peaks without stopping the flow, thus avoiding diffusion effects in the HPLC column, which can deteriorate the chromatographic performance. It is therefore possible to do the post-chromatographic NMR data acquisition on every collected peak without any time restrictions.

Experiment Notes

A lemon oil, processed by sfumatrice extractors, was obtained from Misitano & Stracuzzi SPA, Messina, Italy.[35] D_2O (99%) was purchased from Deutero GmbH, Kastellaun, Germany, and acetonitrile

(HPLC-NMR grade) was bought from Riedel-de Haën, Seelze, Germany.

Sample cleanup: The volatile substances of the CP oil were separated at first by a film evaporator (2 mbar, 50°C) and then by molecular distillation (0.5 mbar, 60°C). The residue was dissolved in ethanol and freed from waxes by freezing-out (winterization; 5 days, -28°C). Ethanol was removed; the residue was dissolved in a 75:25 mixture of acetonitrile and D_2O, filtered through a membrane filter (pore diameter 0.45 μm) and subjected to HPLC analysis without further purification. The HPLC chromatogram of the residue is shown in F-3 (bottom).

To enrich parts of the chromatogram, the residue was dissolved in cyclohexane and extracted with a 70:30 methanol/water mixture. This mixture was then extracted twice with cyclohexane. The methanol/water extract was freed from the solvent in vacuo and the resulting residue was fractionated by HPLC in a preparative scale. HPLC analysis yielded the chromatogram displayed in F-3 (top).

Equipment: An [UNITY]INOVA 400 MHz NMR spectrometer (Varian, Palo Alto, CA) was used, controlled by VNMR 6.1C software. The probe employed was a $^1H\{^{13}C/^{15}N\}$ PFG triple resonance indirect detection microflow LC-NMR probe (IFC probe) with a detection volume of 60 μl.

Bottom: HPLC chromatogram of a sfumatrice lemon oil residue

top: HPLC chromatogram of a fraction resulting from preparative HPLC (UV detection, detection wavelength 270 nm; for numbering of the compounds refer to F-1 and F-2)

F-3
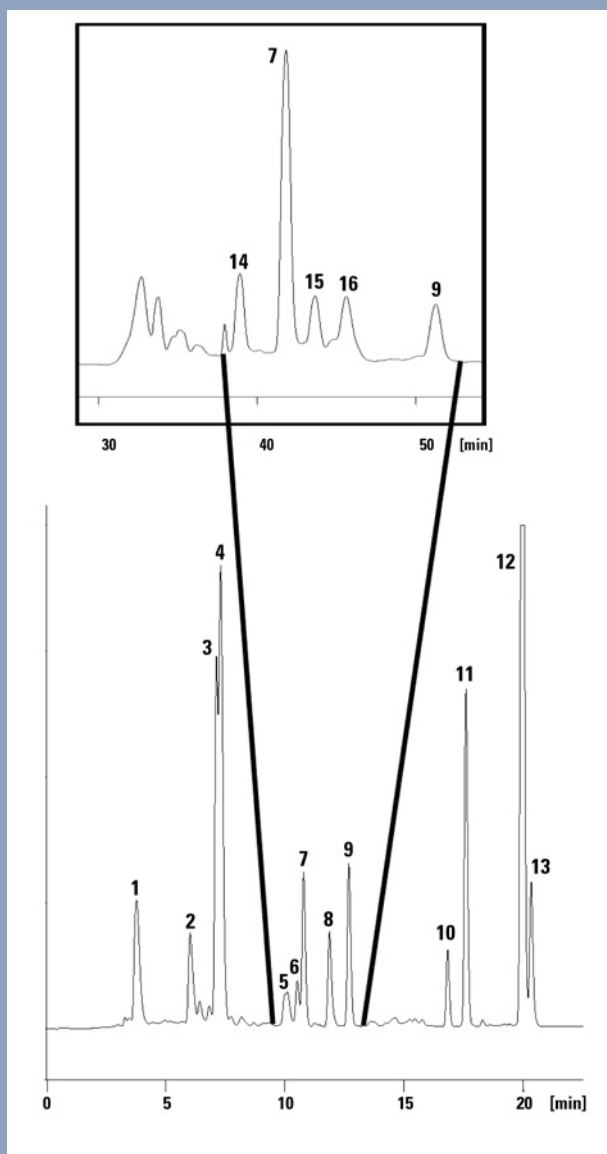
The probe was connected to a modular Varian HPLC system, comprising a ternary Varian Prostar 230 pump, an injection valve, a Varian ProStar 330 Photodiode Array Detector, a Varian ProStar 510 column oven and a Varian LC-NMR Analyte Collector with 15 loops. The chromatographic equipment was controlled by Varian Star LC Workstation software 5.5; the LC-NMR interface software (LC-2000) enables the system to perform programmable stopped-flow experiments as well as peak or fraction collection.

Chromatographic separations were carried out on an Omnispher C18 column (250 x 4.6 mm, particle size 5 µm) from Varian. The temperature of the LC column was maintained at 50°C. For the analysis of the residue, an acetonitrile/D_2O gradient (50:50 Æ 95:5 in 24 min) was used. To avoid tailing, trifluoroacetic acid (0.01% v/v) was added to the D_2O, resulting in a pH of 2.8. The analysis of the fraction resulting from preparative HPLC was carried out with a modified method (acetonitrile/D_2O; 50:50 Æ 0:100 in 25 min; 0:100 Æ 50:50 in 5 min, subsequently 30 min with a volume ratio of 50:50).

The flow rate was 1 ml/min for on-flow and stopped-flow experiments as well as for loop collection. Transferring the loop contents (120 µl) into the probe was done with a flow rate of 0.3 ml/min.

Ten mg of the residue was dissolved in 1 ml of a 75:25 mixture of acetonitrile and D_2O. For on-flow HPLC-NMR experiments, 100 µl of this solution were injected, which corresponds to 1 mg of the residue. In the loop collection mode, 50 µl of the solution was injected into the column which is equivalent to 0.5 mg of residue.

The use of D_2O permitted locking the spectrometer on deuterium. To remove the signals of the solvents (HOD, acetonitrile and its ^{13}C satellites), the WET pulse sequence was executed before each acquisition.[34] The acquisition time for on-flow experiments was 1 s, for stopped-flow we used 3 s with a relaxation delay (d1) of 2 s and a sweep width of 9000 Hz. All measurements were carried out at 20°C. A line broadening of 0.5 Hz was applied before Fourier transformation. The ^{1}H-NMR spectra were referenced to the chemical shift value of acetonitrile (1.95 ppm). DPFGSE-NOE experiments were carried out employing REBURP or RSNOB pulses and a mixing time of 800 msec. This pulse sequence was supplied by the Varian Applab, Darmstadt, Germany. [36-38]

Mass spectrometry was performed with a ThermoQuest LCQ, equipped with an ESI ion source and coupled with a Hewlett Packard HP 1100 HPLC system. For CID experiments helium was used as collision gas. ESI experiments were carried out in the positive mode. The electrospray voltage was 3.5 kV. Nitrogen was used as sheath gas. The chromatographic conditions were the same applied for the HPLC-NMR experiments except the flow rate, which was 0.4 ml/min. A Varian Omnispher C18 column (250 x 3 mm, particle size 5 µm) was utilized.

Results and Discussion

Chromatographic analysis of the residue was carried out under reversed phase conditions and yielded the chromatogram displayed in F-3 (bottom). Thirteen peaks were detected and analyzed by ^{1}H-NMR spectroscopy. As expected, the majority of these substances belong to the classes of coumarins and psoralens.

In the on-flow mode only the main components showed useful ^{1}H-NMR spectra (F-2). First examinations of the on-flow ^{1}H-NMR spectra enabled the classification of substance 2 as a coumarin and substances 4, 9 and 10 as psoralens. For more detailed analysis, the peaks were additionally isolated by peak collection and afterwards sequentially investigated by ^{1}H-NMR spectroscopy.
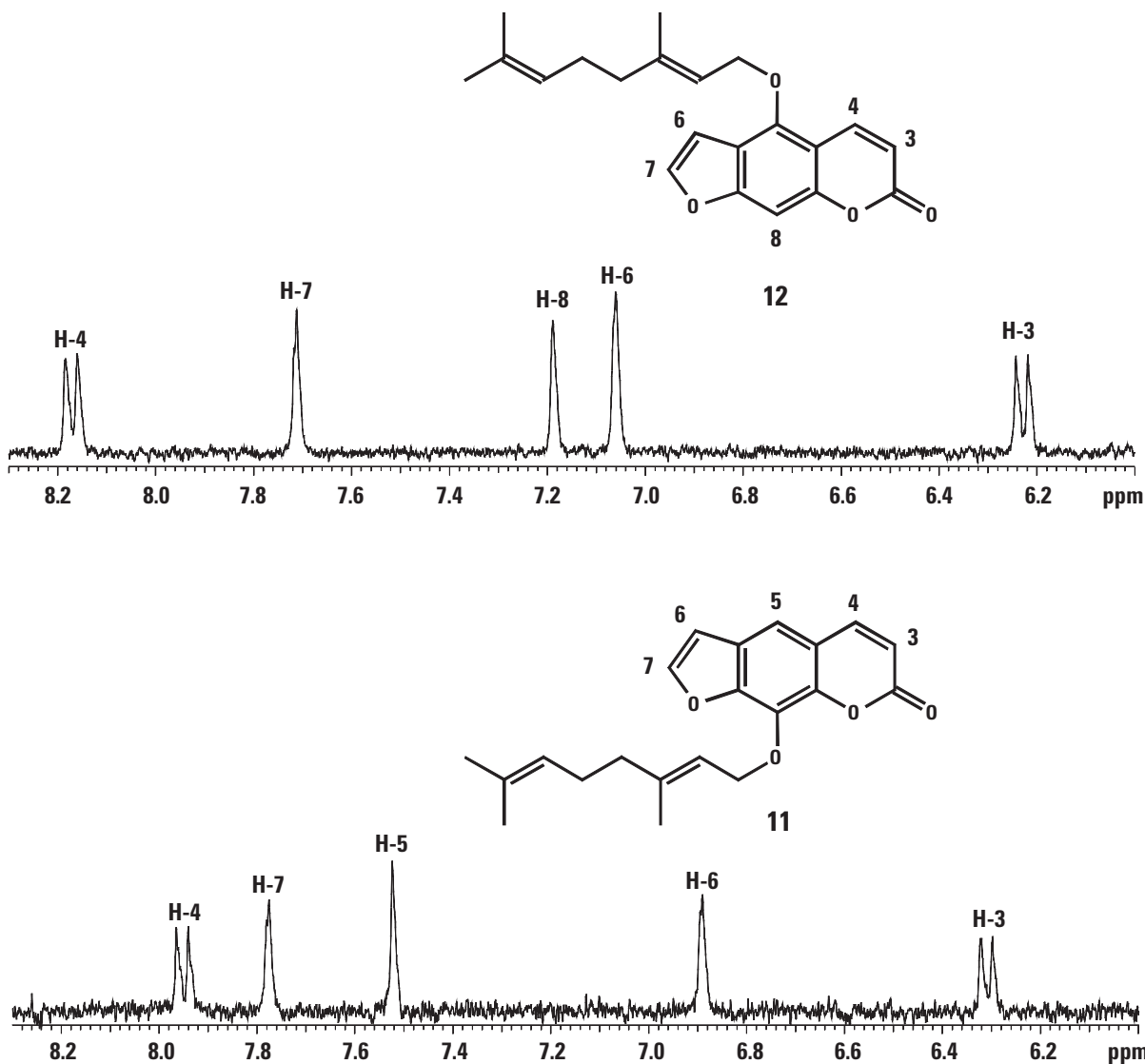
Coumarins and psoralens are relatively easy to identify by ^{1}H-NMR spectroscopy, since they show characteristic spin system patterns.[17] Two doublets at ~ 6.1 to 6.4 and ~7.5 to 8.3 ppm forming an AX spin system with a coupling constant of ~ 9.6 Hz strongly indicate the presence of an un-substituted pyrone ring, and thus can be assigned to the protons at positions 3 and 4 in the molecule. The signals of the hydrogen atoms of the furan ring of a psoralen can be found at 7.5 to 7.8 ppm and 6.7 to 7.2 ppm, respectively. They usually appear as doublets showing a coupling constant of ~ 2.2 Hz. With this information, compounds 1, 3, 4, 7, 8, 9, 10, 11, 12, 14 and 15 could be identified as psoralens and compounds 2, 13 and 16 as coumarins.

5- and 8-mono substitutions in psoralens are easy to differentiate: differences can be found in the pattern of the signal positions in the aromatic region of the ^{1}H-NMR spectrum — specifically, the shift values of the protons at positions 5, 6 and 8 differ significantly. The aromatic regions of the — ^{1}H-NMR spectra of 5-geranyloxypsoralen (bergamottin) 12 and 8-geranyloxypsoralen 11 are displayed in F-4. The signals of H-6 and H-8 are located at 7.063 and 7.190 ppm for bergamottin 12, whereas the signals of H-5 and H-6 can be found at 7.524 and 6.892 ppm for 8-geranyloxypsoralen 11 (see also F-5). Regarding this information, the substitution pattern of the substances 1, 4, 8, 12 and 13 could be determined to be a 5-monosubstitution and those of the compounds 11 and 14 were identified to be a 8-monosubstitution.

The substitution pattern of 5,8-disubstituted psoralens and 5,7-disubstituted coumarins were established by employing DPFGSE-NOE experiments.[36] For example, in the case of byakangelicol 3, two NOEs between

the methoxy group and the protons in positions 4 and 6 were detected (F-6), indicating the presence of the methoxy group in position 5. For the geranyloxy methoxycoumarin 13, NOEs were detected between H-6 of the aromatic system and H-1' of the geranyloxy side chain as well as between the methoxy group and H-8. The geranyloxy substituent is therefore situated at C-5 and the methoxy group at C-7.

It turned out that there were only a few kinds of substituents present in the (furo-)

¹H NMR spectra of bergamottin (= 5-geranyloxypssoralen) 12 (top) and 8-gerranyloxypsoralen 11 (bottom) acquired in the loop collection mode

F-4

coumarins (F-5 and F-7): a) methoxy groups in compounds 2 (citropten), 3 (byakangelicol), 7 (phellopterin), 13 (5-geranyloxy 7-methoxy coumarin) and 16 (5-isopentenyloxy 8-methoxy coumarin); b) isopentenyloxy substituents in compounds 7, 8 (isoimperatorin), 9 (5-isopentenyloxy 8-(2',3'-epoxy isopentenyloxy)-coumarin), 10 (cnidicin), 14 (imperatorin) and 15 (5-(2',3'-epoxy isopentenyloxy) 8-isopentenyloxy psoralen); c) geranyloxy substituents in compounds 11 (8-geranyloxypsoralen), 12 (bergamottin) and 13; d) 2,3-epoxy isopentenyloxy substituent in compounds 3, 4 (oxypeucedanin), 9 and 15; and e) 2,3-dihydroxy isopentanyloxy substituents in compounds 1 (oxypeucedanin hydrate), 9-hyd and 15-hyd, which easily could be characterized by ¹H-NMR spectros-copy. The signals of the methoxy groups of psoralens appeared downfield relative to those of the coumarins (4.1 ppm vs. 3.7 to 3.8 ppm, F-8 and F-9) and were unfortunately covered by the HOD signal in some cases. Nevertheless, their presence could be proved by the characteristic mass fragment of 232 (F-10) in the mass spectrum.

The typical features of the ¹H-NMR spectrum of an isoprenyloxy substituent are the doublet of H-1' at ~4.8 ppm, the triplet of the proton of the double bond at ~5.4 ppm and usually two singlets at ~1.6 and

F-5

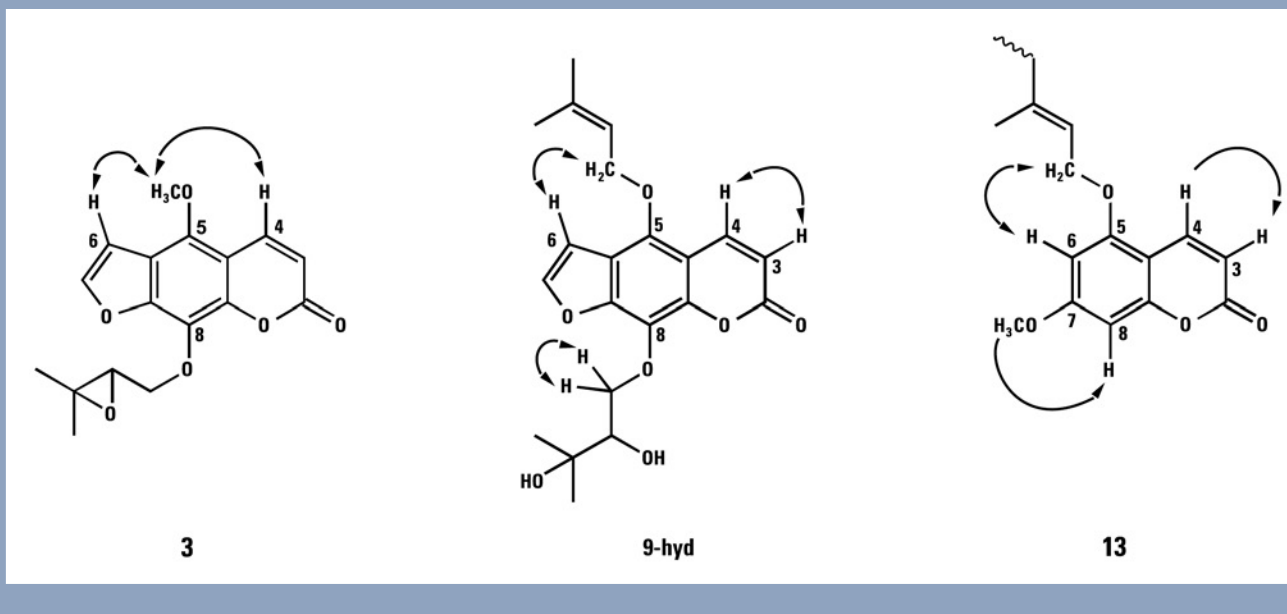**Psoralens identified in the residue of lemon peel oil**
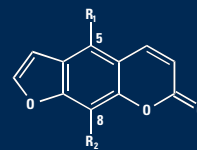
HPLC peak no. *	R_1	R_2	molecular formula	MW	common name
1	(structure)	H	$C_{16}H_{16}O_6$	304	oxypeucedanin hydrate
3	OCH_3	(structure)	$C_{17}H_{16}O_6$	316	byakangelicol
4	(structure)	H	$C_{16}H_{14}O_5$	286	oxypeucedanin
7	OCH_3	(structure)	$C_{17}H_{16}O_5$	300	phellopterin
8	(structure)	H	$C_{16}H_{14}O_4$	270	isoimperatorin
9	(structure)	(structure)	$C_{21}H_{22}O_6$	370	
9-hyd	(structure)	(structure)	$C_{21}H_{24}O_7$	388	
10	(structure)	(structure)	$C_{21}H_{22}O_5$	354	cnidicin
11	H	(structure)	$C_{21}H_{22}O_4$	338	
12	(structure)	H	$C_{21}H_{22}O_4$	338	bergamottin
14	H	(structure)	$C_{16}H_{14}O_4$	270	imperatorin
15	(structure)	(structure)	$C_{21}H_{22}O_6$	370	
15-hyd	(structure)	(structure)	$C_{21}H_{24}O_7$	388	

* see F-3

NOEs detected in byakangelicol 3, psoralen 9-hyd and 5-geranyloxy 7-methoxycoumarin 13

F-6

3   **9-hyd**   **13**

Coumarins identified in the residue of lemon peel oil
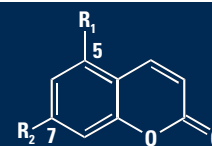
F-7

HPLC peak no. *	R_1	R_2	molecular formula	MW	common name
2	OCH$_3$	OCH$_3$	$C_{11}H_{14}O_4$	206	citropten
13	(geranyloxy)	OCH$_3$	$C_{20}H_{24}O_4$	328	
16	(prenyloxy)	OCH$_3$	$C_{15}H_{16}O_4$	260	

* see Figure 7

~1.5 ppm arising from the two methyl groups (F-11, top).

The ^{1}H-NMR spectrum of an geranyloxy substituent shows an additional signal of a double bond proton in the range of 4.8 to 5 ppm (F-8, F-9 and F-11, bottom). In the acetonitrile/D$_2$O solvent system the aliphatic protons H-4' and H-5' are usually covered by the acetonitrile signal (F-11, bottom).

Diastereotopic protons H-1'A, H-1'B as well as H-2' of an epoxy isopentenyloxy or a dihydroxy isopentanyloxy substituent form a characteristic ABX spinsystem in the ^{1}H-NMR spectrum (F-8 and F-12).

For the differentiation between the epoxides and their respective hydrates, the information about the molecular mass and mass fragments obtained from mass spectrometry is absolutely essential. For example, peak 1 could be assigned to oxypeucedanin hydrate by its molecular mass and retention behavior. The ^{1}H-NMR spectrum appears to be similar to oxypeucedanin 4, but shows some significant differences in the shift values of the protons of the side chain (F-8), especially for H-2', which resonates at 3.243 ppm in the case of the epoxide and at 3.738 ppm for the hydrate. It must be concluded that oxypeucedanin 4 seems to be unstable in aqueous acidic solutions, because ring opening obviously occurred during NMR measurement of peak 4 in loop

F-8

Spectroscopic data for psoralens found in the residue of lemon oil

peak no.	aromatic system NMR data *d [ppm], multiplicity, J [Hz]						R_1 / R_2 NMR data *d [ppm], multiplicity, J [Hz]	MS data $[M+H]^{+m}/_z$
	H-3	**H-4**	**H-5**	**H-6**	**H-7**	**H-8**		
1	6.264, d, 9.6	8.327, d, 9.8		7.111, d, 2.2	7.711, d, 2.2	7.199, s	R_1: H-1A: 4.599, J_{AB}=10.1, J_{AX}=2.3, 1 H H-1B: 4.299, J_{AB}=10.1, J_{BX}=8.0, 1 H H-2: 3.738, J_{AX}=2.3, J_{BX}=8.0, 1 H CH$_3$: 1.131, s, 3 H CH$_3$: 1.121, s, 3 H	305 202
3	6.250, d, 9.8	8.184, d, 9.8		7.143, d, 2.2	7.735, d, 2.2		R_1: H-1: 4.46-4.34, m, 2 H H-2: 3.23, t, 5.6, 1 H [a] CH$_3$: 1.250, s, 3 H CH$_3$: 1.184, s, 3 H R_2: OCH$_3$: [b]	317 232 202
4	6.288, d, 9.8	8.269, d. 9.8		7.064, d, 2.2	7.730, d, 2.2	7.244, s	R_1: H-1A: 4.646, J_{AB}=10.6, J_{AX}=3.9, 1 H H-1B: 4.361, J_{AB}=10.6, J_{BX}=7.0, 1 H H-2: 3.243, J_{AX}=3.9, J_{BX}=7.0, 1 H [a] CH$_3$: 1.251, s, 3 H CH$_3$: 1.186, s, 3 H	287 202
7	6.248, d, 9.8	8.186, d, 9.8		7.133, d, 2.2	7.739, d, 2.2		R_1: H-1: 4.738, d, 7.9, 2 H H-2: 5.443, t, 7.9, 1 H CH$_3$: 1.607, s, 3 H CH$_3$: 1.522, s, 3 H R_2: OCH$_3$: 4.138, s [b]	301 232 218 202
8	6.241, d. 9.9	8.183, d, 9.9		7.074, d, 2.2	7.718, d, 2.2	7.198	R_1: H-1: 4.896, d, 7.0, 2 H H-2: 5.461, t, 7.0, 1 H CH$_3$: 1.662, s, 3 H CH$_3$: 1.559, s, 3 H	202 [d]
9								371 302 286 202
9-hyd	6.266, d, 9.8	8.156, d, 9.8		7.044, d, 2.2	7.752, d, 2.2		R_1: H-1: 4.767, d, 7.4, 2 H H-2: 5.434, t, 7.4, 1 H CH$_3$: 1.632, s, 3 H CH$_3$: 1.490, s, 3 H R_2: H-1A: 4.413, J_{AB}=10.4, J_{AX}=2.4, 1 H H-1B: [b] H-2: 3.742, J_{AX}=2.4, J_{BX}=8.2, 1 H CH$_3$: 1.099, br s, 6 H	

Spectroscopic data for psoralens found in the residue of lemon oil

peak no.	aromatic system NMR data *d [ppm], multiplicity, J [Hz]						R_1 / R_2 NMR data *d [ppm], multiplicity, J [Hz]	MS data [M+H]$^{+m}$/$_z$
	H-3	**H-4**	**H-5**	**H-6**	**H-7**	**H-8**		
10	6.230, d, 9.8	8.122, d, 9.8		7.024, d, 2.0	7.730, d, 2.0		$R_1 = R_2$: H-1: 4.748, d, 6.4, 4 H H-2: 5.417, t, 6.4, 2 H CH$_3$: 1.615, s, 3 H CH$_3$: 1.577, s, 3 H CH$_3$: 1.482, s, 3 H CH$_3$: 1.452, s, 3 H	<u>355</u> 286 218
11	6.309, d, 9.8	7.953, d, 9.8	7.524, s	6.892, d, 2.0	7.778, d, 2.0		R_2: [e] H-1: 4.913, d, 7.1, 1 H H-2: 5.399, t, 7.1, 1 H H-4, H-5: [c]H-6: 4.833, m$_c$, 1 H CH$_3$: 1.495, br s, 6 H CH$_3$: 1.420, s, 3 H	<u>339</u> 202
12	6.231, d, 9.8	8.173, d, 9.8		7.063, d, 2.1	7.716, d, 2.1	7.190, s	R_1: [e] H-1: 4.9)9, d, 6.6, 2 H H-2: 5.420, t, 6.6, 1 H H-4, H-5: [c]H-6: 4.919, m$_c$, 1 H CH$_3$: 1.527, br s, 6 H CH$_3$: 1.460, s, 3 H	<u>339</u> 202
14	6.321, d, 9.7	7.966, d, 9.7	7.530, s	6.898, d, 2.1	7.783, d, 2.1		R_2: H-1: 4.884, d, 7.0, 2 H H-2: 5.445, t, 7.0, 1 H CH$_3$: 1.604, s, 3 H CH$_3$: 1.548, s, 3 H	202 [d]
15								<u>371</u> 302 286 202
15-hyd	6.297, d, 9.8	8.185, d, 9.8		7.079, d, 2.2	7.784, d, 2.2		R_1: H-1A: 4.388, J_{AB}=10.0, J_{AX}=2.2, 1 H H-1B: [b]H-2: 3.902, J_{AX}=2.2, J_{BX}=8.3, 1 H CH$_3$: 1.123, s, 3 H CH$_3$: 1.102, s, 3 H R_2: H-1: 4.801, d, 7.2, 2 H H-2: 5.467, t, 7.2, 1 H CH$_3$: 1.667, s, 3 H CH$_3$: 1.521, s, 3 H	

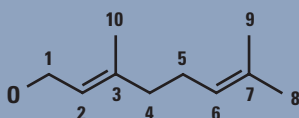* measured in D$_2$O/acetonitrile, refer]nced lo shift value of acetonitrile (1.95 ppm)

[a] partially covered by signal of acetonitrile impurity (Æ methanol)

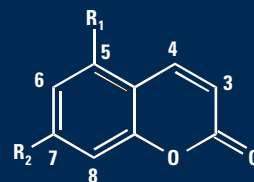[b] (partially) covered by signal of HOD

[c] covered-by signal of acetonitrile

[d] no molecular mass detected by HPLC-MS (ESI⁺)

[e] numbering of positions according to the rules for terpenoids:

F-9

**Spectroscopic data for coumarins found in the residue of lemon oil**

peak no.	aromatic system NMR data *d [ppm], multiplicity, J [Hz]				R_1 / R_2 NMR data *d [ppm], multiplicity, J [Hz]	MS data $[M+H]^{+m}/_z$
	H-3	**H-4**	**H-6**	**H-8**		
2	6.107, d, 9.7	8.009, d, 9.7	6.383, br s	6.470, br s	R_1: OCH$_3$: 3.815, s, 3 H R_2: OCH$_3$: 3.773, s, 3 H	207 192
13	6.100, d, 9.6	7.985, d, 9.6	6.363 br s	6.465 br s	R_1: [b] H-1: 4.607, d, 6.2, 2 H H-2: 5.385, t, 6.2, 1 H H-4, H-5: [a] H-6': 4.986, m$_c$, 1 H CH$_3$: 1.659, s, 3 H CH$_3$: 1.536, s, 3 H CH$_3$: 1.493, s, 3 H R_2: OCH$_3$: 3.772, s, 3 H	329 192
16	6.193, d, 9.6	8.085, d, 9.6	6.470, d, 1.8	6.561, d, 1.8	R_1: H-1: 4.664, d, 6.6, 2 H H-2: 5.496, t, 6.6, 1 H CH$_3$: 1.785, s, 3 H CH$_3$: 1.751, s, 3 H R_2: OCH$_3$: 3.860, s, 3 H	261 192

[a] covered by signal of acetonitrile

[b] numbering of positions according to the rules for terpenoids:

F-10
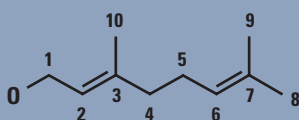
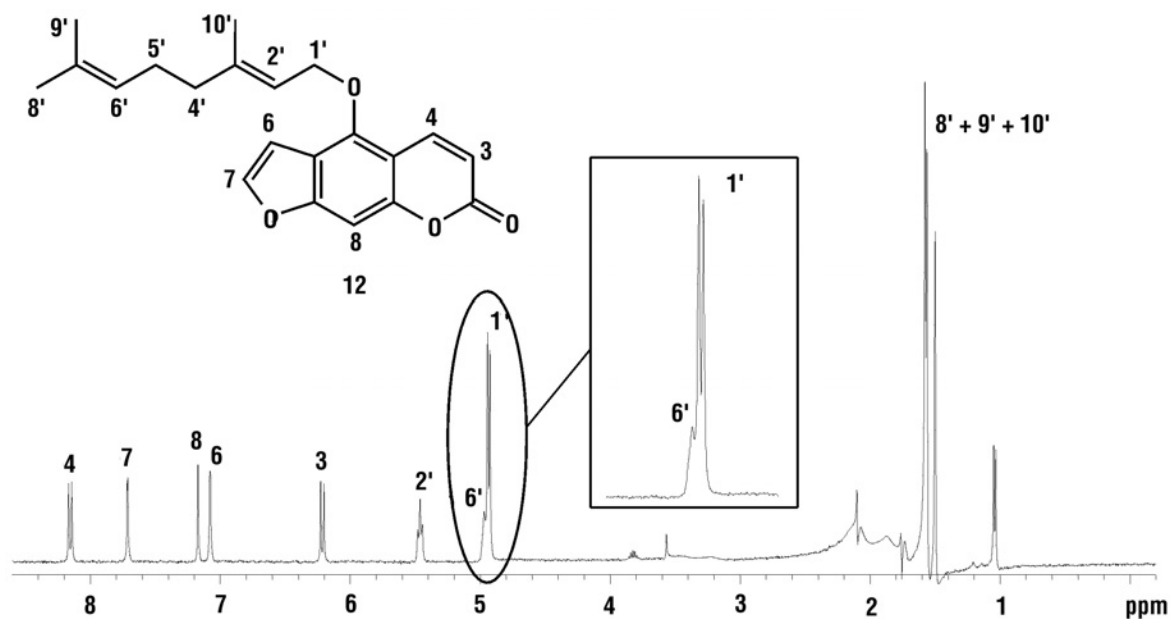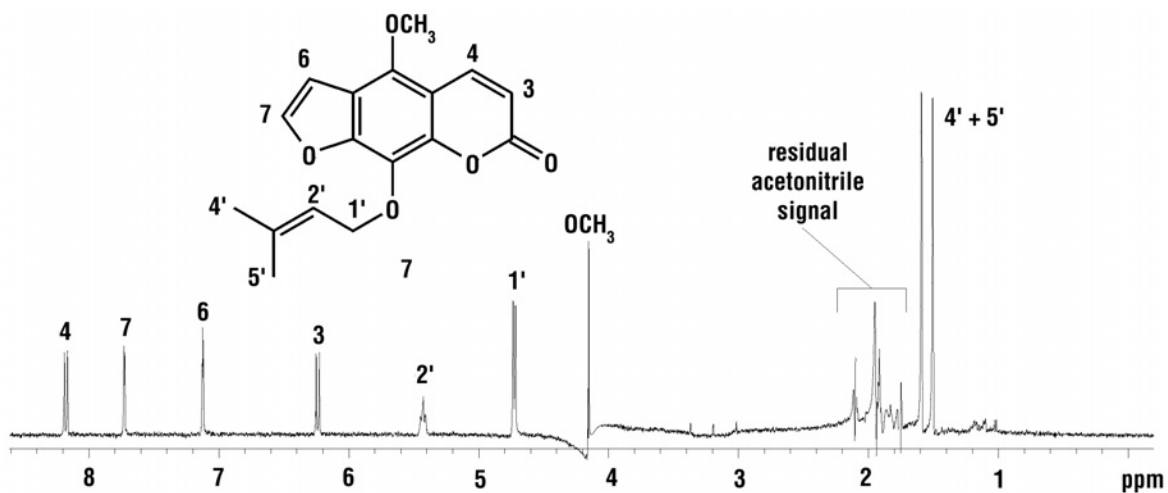**Important mass fragments in mass spectra of substituted psoralens**
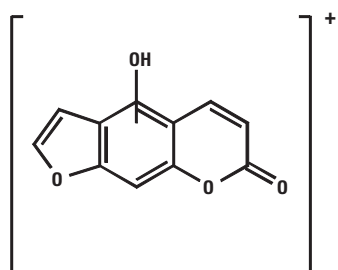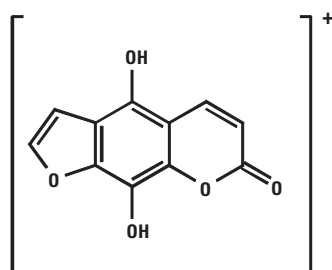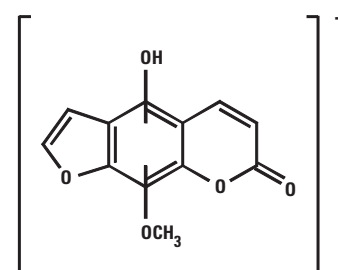
m/z = 202   m/z = 218   m/z = 232

¹H-NMR spectra of peak 4 top: on-flow ¹H-NMR spectrum with oxypeucedanin 4 as main component; bottom: ¹H-NMR spectrum measured in loop collection mode with oxypeucedanin hydrate 1 as main component [peaks denoted with asterisks arise from oxypeucedanin 4; the signal of H-2 (3.243 ppm) of 4 is partially covered by the signal of methanol (impurity of acetonitrile)]. The signals of H-1′B (4.2-4.4 ppm) are damped due to the suppression of the HOD signal at ~4.1 ppm
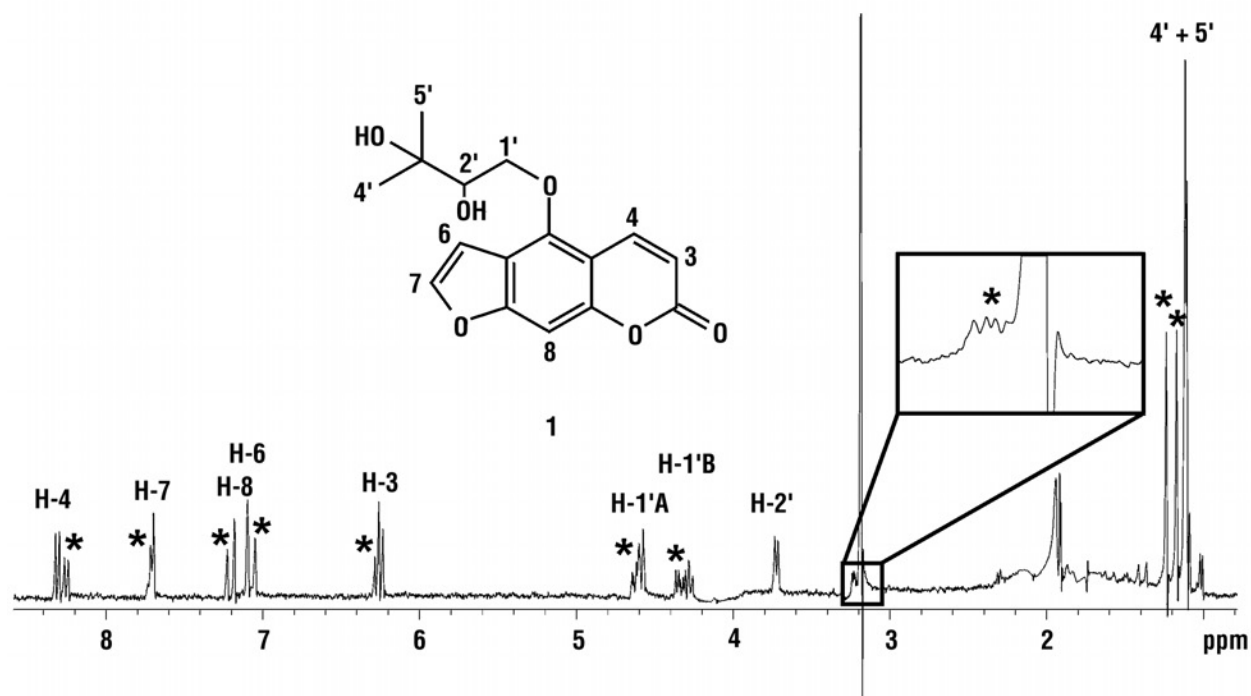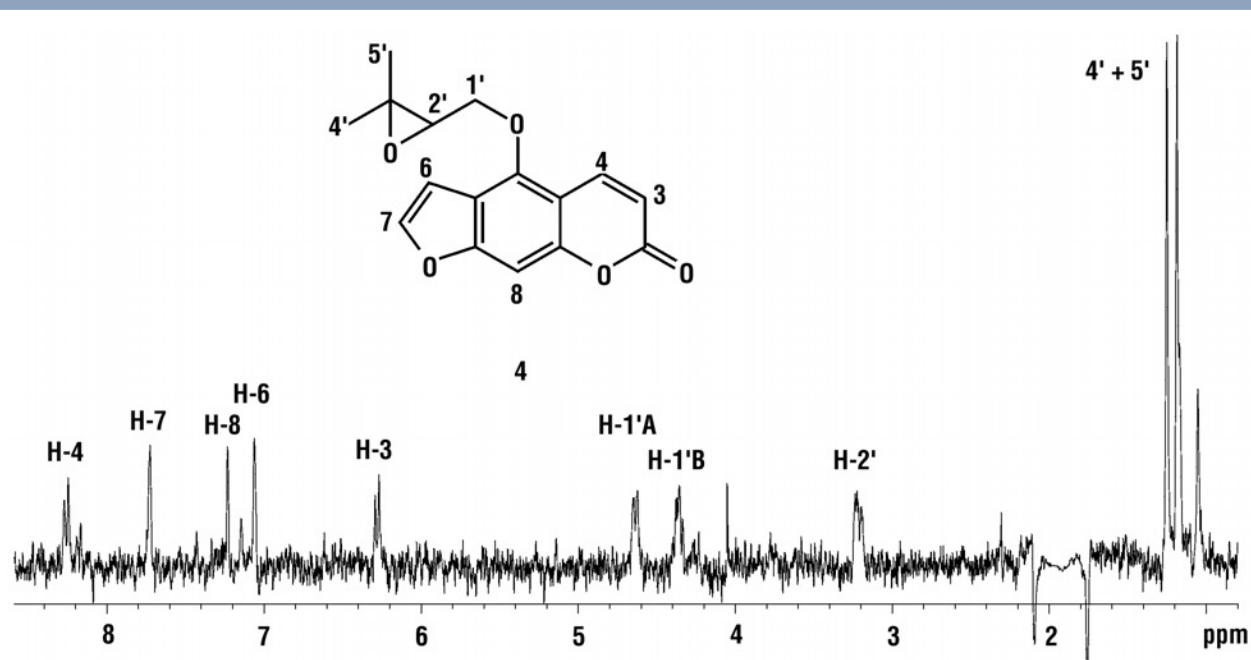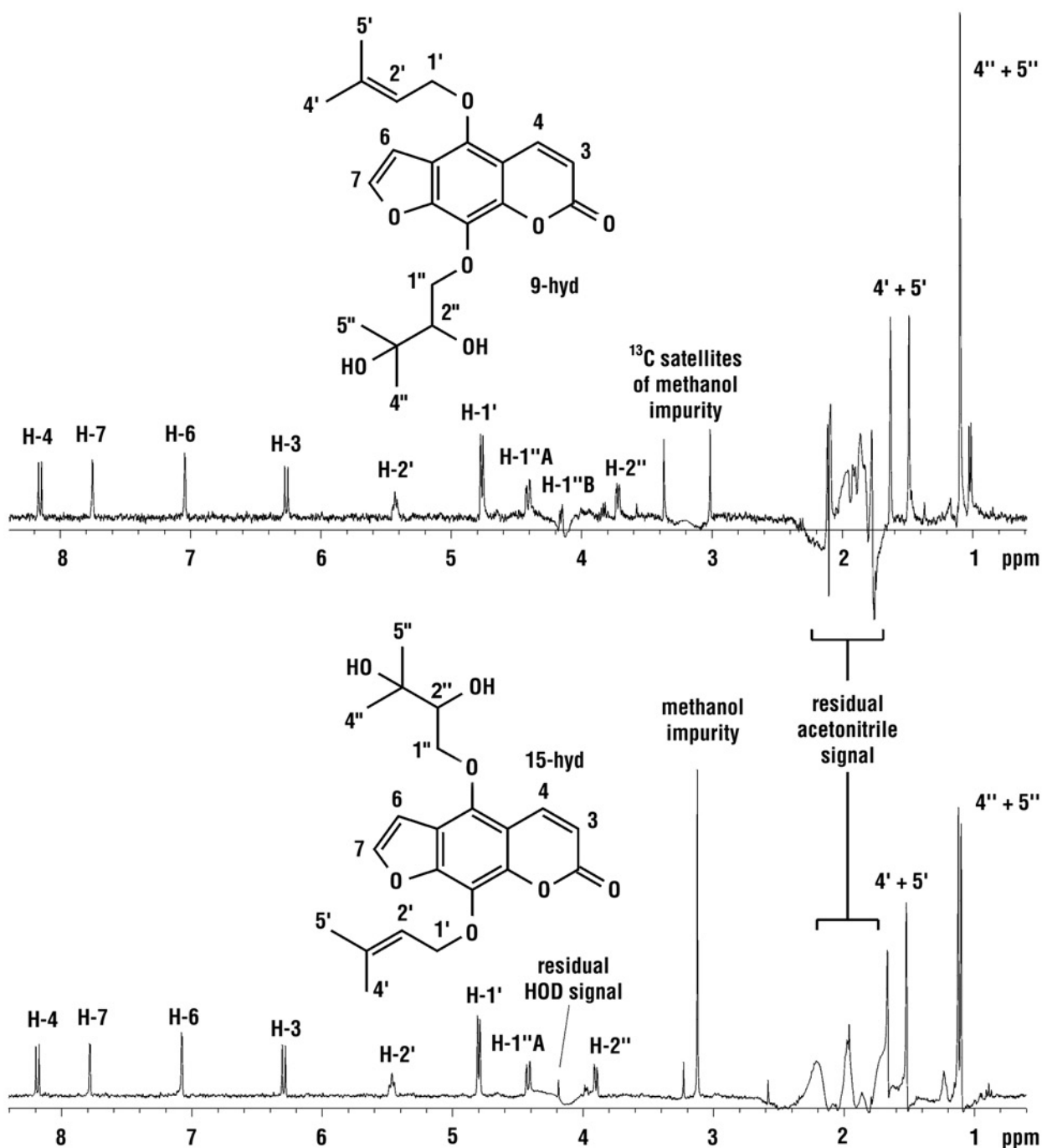
collection mode resulting in a spectrum showing a mixture of epoxide 4 as the minor component and its hydrate 1 (F-12, bottom). As opposed to this, the rapid measurement of peak 4 in the on-flow mode yielded a spectrum showing oxypeucedanin 4 as the major component (F-12, top). The ^{1}H-NMR spectrum of peak 3 also shows a mixture of the epoxide byakangelicol 3 and its hydrate. This seems to be a general problem when acquiring NMR data for lower concentrated and labile species in unsuitable environments such as epoxides in acidic aqueous solutions over a longer period of time. For example, the ^{1}H-NMR spectrum of peak 9 was acquired much later than the spectra of the peaks 3 and 4. Although the MS data of peak 9 clearly indicate the presence of the epoxide 9 by its molecular mass of 370, the signal position of H-2' (3.742 ppm, F-8) in the ^{1}H-NMR spectrum strongly points to the respective hydrate 9-hyd. No ^{1}H-NMR signals arising from the epoxide 9 could be detected implying a full hydrolysis of the epoxide.

Peaks 5 and 6 could be assigned to the citral isomers neral 5 and geranial 6. The ^{1}H-NMR spectra showed aldehyde signals at 9.665 ppm (d, 8.5 Hz) and 9.786 ppm (d, 8.4 Hz), respectively. The signals of H-2 could be found at 5.787 and 5.758 ppm, appearing as doublets (8.5 Hz, 8.4 Hz.). The protons at C-6 resonate at 5.041 and 5.009 ppm (t, 7.2 and 6.8 Hz). As expected, the signals of the methyl groups could be found as singlets in the region of 1.48 – 1.47 ppm (5: 1.563 ppm, 2 CH_3 and 1.483 ppm, 1 CH_3; 6: 1.575 ppm, 2 CH_3 and 1.513 ppm, 1 CH_3). The remaining signals of the methylene groups were covered by the acetonitrile resonance.

Compounds 7 (phellopterin) and 9 were also found in the residue fraction resulting from preparative HPLC (F-3, top). In the analogous to the analysis of the residue, the ^{1}H-NMR spectrum only showed the resonances of the hydrate 9-hyd, whereas the mass spectrum indicated the presence of the epoxide 9. Additionally, imperatorin 14 and coumarin 16 could be identified. Interestingly, the UV spectra and molecular masses of psoralens 15 and 9 were identical. Since the ^{1}H-NMR spectra of both peaks 15 and 9 (F-13) were similar, two conclusions must be made on the basis of the spectroscopic data:
  a) Epoxide 15 must be a positional isomer of epoxide 9.
  b) Epoxide 15, which was originally present in the residue, was obviously hydrolyzed during the NMR measurement resulting in hydrate 15-hyd as an artifact.
The substitution pattern of 9-hyd was determined by NOE experiments. Amongst others, an NOE was detected between the methylene protons of the isoprenyloxy substituent and H-6 indicating a 5-substitution of this group (F-6). Because of the similarity of the spectral data of 9, 15, 9-hyd and 15-hyd it is evident, that in psoralen 15 (15-hyd) the isoprenyloxy group must be situated in 8-position and the 2,3-epoxy isopentenyloxy (2,3-dihydroxy isopentanyloxy) substituent in 5-position. To the best of our knowledge, psoralen 15 is not known in the literature so far.

Conclusion
The HPLC-NMR coupling proved to be a useful tool for the fast and reliable identification of the non-volatile constituents in a sfumatrice lemon peel oil. Sixteen substances were detected and their stuctures identified. It was shown that the residue of a sfumatrice expressed lemon oil consisted mainly of psoralens substituted in 5- and/or 8-position with isoprenyloxy or geranyloxy substituents and/or their epoxidized analogues.

Although the NMR spectrometer is a detector with low sensitivity, ^{1}H-NMR spectra of low concentrated compounds could be obtained with less than 7,000 scans on a 400 MHz NMR system. Caution is advised in the investigation of compounds, which are unstable in aqueous acidic media, since these species can decompose during NMR data acquisition.

In this case consideration of mass spectrometric data is an absolute must.

Acknowledgements

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