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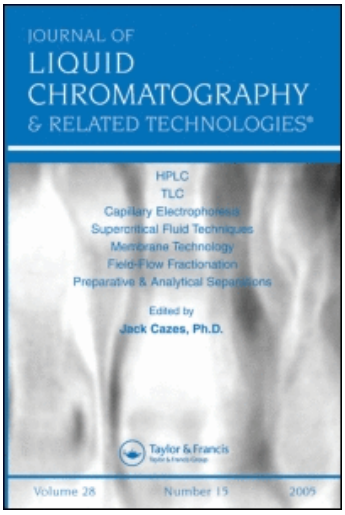

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Normal Phase HPLC/FT-IR Detection and Identification of β-Cypermethrins by the Flow-Through Cell Method: Advantages and Limitations

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Abstract: The capabilities and limitations of on-line FT-IR spectroscopic detection during normal phase HPLC separation have been examined and evaluated as applied to a practical analytical problem, using a commercial IR micro flow-through cell as an interface in conjunction with an up-to-date high speed, high sensitivity FT-IR spectrometer, and dedicated data processing utilities. It has been shown that the usual chromatographic mobile phases containing polar modifiers can be used in such measurements, although the technique has definite limitations depending on the spectroscopic properties of the mobile phases and the analytes in question. In case of analytes having strong IR features in regions clear from mobile phase absorption, IR chromatograms are suitable for major component detection, whereas narrow-region chemigram-type detection tuned to selected absorption bands of the target compound is more successful than Gram-Schmidt reconstructed chromatograms generated from

[1]Taken in part from the thesis of K.I. which will be submitted to the Department of Applied and Environmental Chemistry, Szeged University, in partial fulfilment of the Ph.D. degree.

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full-range IR spectra. Quantitative determination of the two major diastereomers in the β-cypermethrin sample using the "carbonyl chemigram" has shown good linearity in the concentration range from 0.3 to 4.0 mg/mL. The value of 1.0 mg/mL proved to be the identification limit still allowing spectral discrimination of diastereomers, while the detection limit for the β-cypermethrins examined was 0.3 mg/mL in most mobile phases, but was estimated to be 0.1 mg/mL in acetonitrile/*n*-hexane (AN/*n*HEX).

Keywords: HPLC/FT-IR, cypermethrin, normal phase chromatography, infrared chemigram, flow-through cell method

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INTRODUCTION

Direct interfacing of liquid chromatography with FT-IR spectroscopy is not a new idea: the first such measurements were reported in the middle of the 1970s on utilization of flow cell techniques.[1] This combination of methods promised reliable analyte identification in addition to component separation, but as it could be expected, strong absorption of the solvent or eluent proved to be a major restriction, limiting the potentials of the method concerning both identification and detection limits. An alternative approach free from this limitation, the eluent elimination (or solvent elimination) technique appeared shortly afterwards,[2] providing the possibility of full-range IR spectral identification and, simultaneously, reaching much more acceptable detection limits (in the low nanogram range). Several review papers are available in recent literature that cover both of these LC/IR approaches, [3–6] nevertheless, there are apparently *no cited data* (e.g., concentrations) characteristic of the attained detection limits with the flow cell technique on analytical columns. While the solvent elimination method has gone through some development during the years, with several variations of it becoming commercially available, the principal limitations of the flow cell IR spectroscopic detection precluded the latter from becoming a widely applied method.

Since the measurement techniques of FT-IR spectroscopy improved significantly during the past decades, it seems timely to evaluate the current possibilities: to attempt to apply the HPLC/FT-IR coupled technique to a practical analytical problem using up-to-date high speed, high sensitivity instrumentation, and dedicated data processing methods. While the papers concerning the normal phase HPLC–{flow cell}–FT-IR method have shown only attempts that apply IR-compatible solvents, rarely defining the volume of the IR cell,[1,7–9] our approach is based on the use of conventional mobile phase mixtures applied in normal phase HPLC, and an IR flow-through cell with a volume compatible with that of a usual UV detector in an analytical liquid chromatograph.

EXPERIMENTAL

All solvents used in the HPLC/FT-IR measurements and for making sample solutions (acetonitrile, dichloromethane, 2-propanol, and tetrahydrofuran) were of HPLC grade (Sigma Aldrich Co., USA). The sample solutions were made by dissolving the appropriate amounts of an industrial product of β-cypermethrin (a commercially available insecticide belonging to the family of pyrethroids, obtained from Agro-Chemie Kft., Budapest, Hungary) consisting of diastereomers, in a 20/80% CH_2Cl_2/*n*-hexane mixture to get a series of 0.1, 0.3, 0.5, 1.0, 2.0, and 4.0 mg/mL samples in amounts of 5 mL each.

In the separation experiments, the following four solvent mixtures were applied as mobile phase: CH_2Cl_2/*n*-hexane (DCM/*n*HEX) (35/65 v/v%), tetrahydrofuran/*n*-hexane (THF/*n*HEX) (2/98 v/v%), 2-propanol/*n*-hexane (IPA/*n*HEX) (0.1/99.9 v/v%), and acetonitrile–saturated *n*-hexane (2.0 v/v%, at 23°C). The concentration of modifiers had been optimized with the aim that the retention times should stay below 10 min and be approximately the same for the second chromatographic peak in all experiments, while keeping the flow rate constant at 2 mL/min.

For separations, a DuPont ZORBAX-Sil column (4.6 × 250 mm^2, 5 μm) was applied, which was washed and equilibrated with the mobile phase used (before the measurements, at every change of mobile phase). Injections were made by a VICI (Valco Instruments Co. Inc.) injector handled by a remote control unit; the injection volume was 20 μL. The pump was an ISCO model 2350. The chromatographic and IR spectrometric parts of the system were interfaced by means of a commercial flow-through cell (Spectra-Tech Inc., USA) mounted in the normal focus of the sample compartment of the FT-IR spectrometer. The 10.8-μL flow-through cell was equipped with NaCl windows and a stainless steal inlet tube with inner and outer diameters usually applied in analytical chromatographs (0.01 in. i.d., and 0.0625 in. o.d.).

The IR absorption spectra of the separated compounds were detected by a Nicolet Magna 750 FT-IR spectrometer, which was equipped with a fast, high-sensitivity liquid-N_2-cooled MCT-A detector operating in the 4000–650 cm^{-1} spectral region. To obtain the highest throughput of energy, i.e., the highest detector signal, the intensity of the interferogram was maximized by changing the standard aperture size setting from 6 to 26. Spectra were typically detected at 8 or 16 cm^{-1} spectral resolution, accumulating (co-adding and averaging) 16 or 32 interferogram scans per spectrum. Working in the "real-time" type data collection regime (at mirror velocity of 5.0632 cm/sec, with maximum displacement of the moving mirror in the interferometer around 1.25 and 0.0625 mm), this resulted in 2.35 and 3.22-sec time resolution, respectively, in the resulting infrared "chromatogram".

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The task of measuring the series of IR spectra with the appropriate time resolution was controlled by the *Series* program module of the OMNIC™ software package (Thermo Nicolet Inc.). As part of the on-line measurements, each sample spectrum was rationed against a 256-scan background spectrum of the actual mobile phase (recorded under flowing circumstances immediately before the LC-IR run), giving the absorbance spectra of the analyte instantly (thus, there was no need to make any spectral subtraction after the measurement). The *Series* software could be used, also, to create real-time *Chemigrams* (i.e., chromatograms based on integrated IR absorption intensities in user-defined wave number regions characteristic of certain chemical functional groups) and to do the so-called Gram–Schmidt reconstruction,[10] producing GSR traces that can be viewed as full-IR-range chromatograms.

For reference, conventional normal phase HPLC separation of the β-cypermethrin sample was also performed using isocratic elution with CH_2Cl_2/*n*-hexane (25/75 v/v%) mobile phase at a flow rate of 2 mL/min on the same DuPont ZORBAX-Sil column as in the HPLC/FT-IR measurements. The Spectra Focus HPLC system (Thermoseparation Products, CA, USA) used consisted of a Spectra P2000 pump, a Rheodyne injector, and a Spectra UV2000 detector. The injection volume was 20 μL, and the UV absorption was detected at 240 nm.

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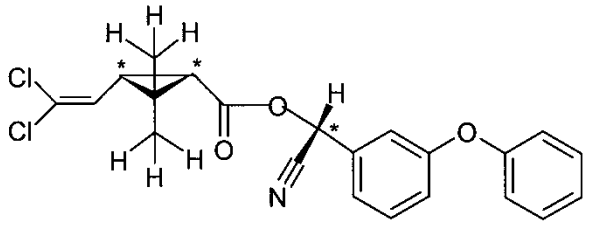
RESULTS AND DISCUSSION

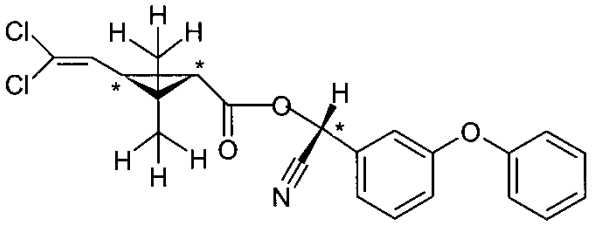
Comparison of IR Detection Regimes

Prior to the HPLC/FT-IR measurements, the composition of the β-cypermethrin sample used as a test material in this work has been checked by conventional HPLC analysis. A β-cypermethrin molecule contains three centers of asymmetry. Due to this, the sample is a mixture of eight stereoisomers, corresponding to four pairs of enantiomers (mirror image pairs), of which two pairs are present in the sample in much greater abundance than the other two. These two stereoisomers are shown in Figure 1 (only one enantiomer is given for each pair), with the centers of asymmetry indicated by asterisks. In the chromatogram, when an isotropic stationary phase is used, only the diastreomers will be distinguished from each other, while the enantiomers will have the same retention times. Hence, there should be two large and two small chromatographic peaks in the chromatogram, as observed indeed with UV detection (see Figure 2). The two major peaks eluted at 13.84 and 18.74 min correspond in Figure 1 to structures 1 and 2, respectively. According to the peak areas, their mass ratio is approximately 40/60. The two minor peaks seen in Figure 2 are due to the remaining two enantiomer pairs of β-cypermethrin. It is expected that in the HPLC/FT-IR measurement only the two major components will be observed.



Compound **1:**

(R)-α-cyano-3-phenoxybenzyl (1S)-*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate



Compound **2:**

(R)-α-cyano-3-phenoxybenzyl (1S)-*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate

Figure 1. Chemical structures and official IUPAC names of the two major components of β-cypermethrin diastereomers, designated as 1 and 2, present in the sample mixture in a 2 : 3 mass ratio.

To illustrate the pre-conditions of IR spectroscopic detection of β-cypermethrins in the flow-through cell, the solid phase IR spectrum of the stereoisomer mixture is presented in Figure 3, together with the absorption spectra of the four mobile phase mixtures used. Note that those spectral regions where the absorption of the mobile phases exceeds 2 absorbance units are useless for analyte detection (however, due to the principle of Fourier-transform IR measurement, these regions also included in the measurement). Comparison of the IR spectrum of the analyte mixture in Figure 3(a) to those of the mobile phase mixtures in Figure 3(b–e) makes it clear that the absorption bands above $1500\,cm^{-1}$ (especially the strong νC=O stretching band near $1740\,cm^{-1}$) are the most suitable for quantitative detection, because they are free from solvent interference. The strongest band of the analyte spectrum located at $1478\,cm^{-1}$ is useless for our purposes as it

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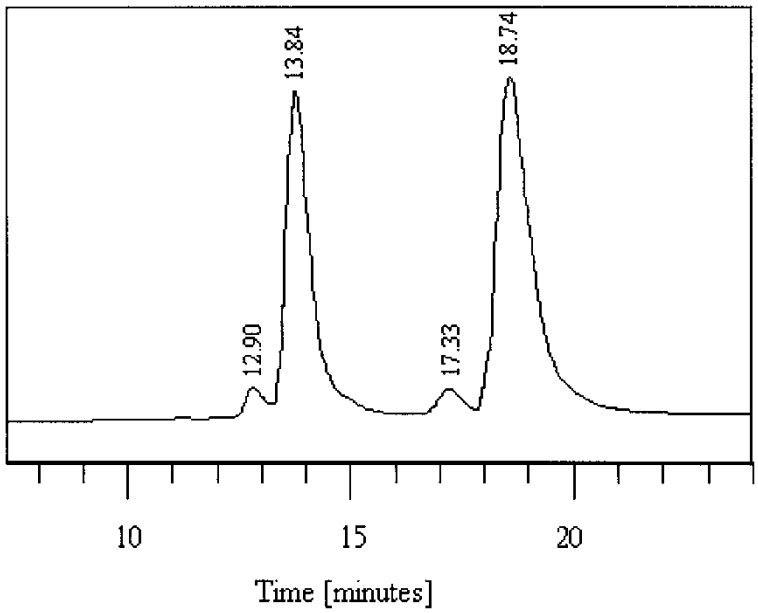

Figure 2. Normal phase HPLC chromatogram of the commercial β-cypermethrin product obtained with UV detection. The first major peak (at 13.84 min) corresponds to the cis structure (compound 1), while the second major peak (at 18.74 min) to the trans structure (compound 2).

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falls in the region of total absorption by *n*-hexane. Observation of the spectral features in the 1350–750 cm^{-1} region would also be of interest, since spectral differences distinguishing the separated diastereomers are expected to occur in this region of skeletal deformation vibrations.

The best HPLC/FT-IR chemigrams obtained with four different mobile phase mixtures utilizing the carbonyl absorption region are shown in Figure 4. Two of these chemigrams are compared with the corresponding GSR traces (full-IR chromatograms) in Figure 5. Comparison shows that during all HPLC/FT-IR measurements, the "carbonyl chemigrams" showed much better performance than the Gram–Schmidt reconstructions: the former gave the highest signal intensity at the chromatographic peaks when the wave number region was chosen to be 1760–1720 cm^{-1}. Both of the major β-cypermethrin diastereomers have one of their most intense IR absorption bands in this region, and, this is a region where neither *n*-hexane nor any of the mobile phase mixtures applied disturb detection of the analytes.

In contrast to this, it proved to be much harder to detect any signal in the GSR traces: even the highest concentration (4 mg/mL) samples showed detectable analyte signals (signal-to-noise ratios around 3) in only two of

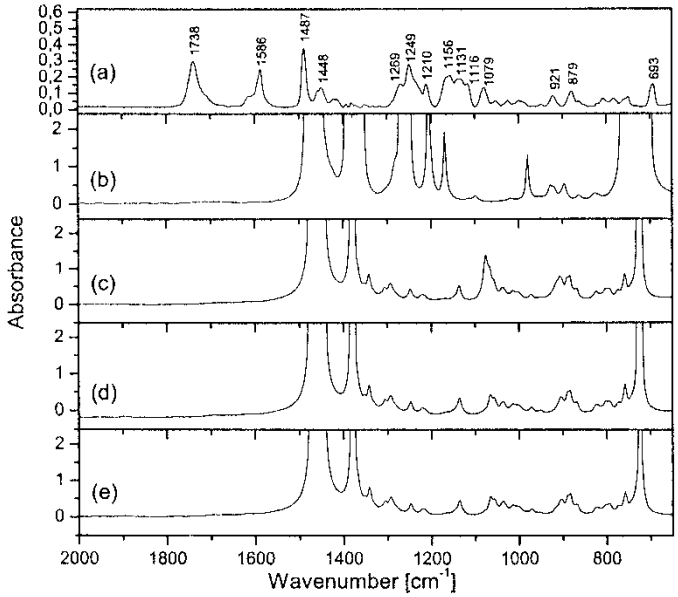

Figure 3. Comparison of the solid phase IR spectrum of the stereoisomer mixture (a) with the absorption spectra of four mobile phase mixtures: DCM/*n*HEX (b), THF/*n*HEX (c), IPA/*n*HEX (d), and AN/*n*HEX (e).

the applied mobile phase mixtures, THF/*n*HEX and acetonitrile/*n*-hexane (AN/*n*HEX). The reason for failure at lower sample concentrations was due to the fact that the intensity levels and signal-to-noise ratios (S/N) in GSR traces were inherently much lower than those in suitably chosen narrow-region chemigrams. While the maximum chemigram intensities for the 4 mg/mL sample varied between 0.018 and 0.029 (in absorbance units) depending on the mobile phase used (see Figure 4), the maximum GSR intensities were around 0.0018 and 0.0007 for the THF/*n*HEX and AN/*n*HEX mobile phases, respectively. As it is seen in Figure 4, the chemigram intensities and peak shapes responded to the properties of the mobile phases fairly well, with the peak intensities being comparable and remaining in the same magnitude. In case of the GSR traces [see Figures 5(b) and (d)], the detected peak intensities were not comparable on the relative intensity scale; the absolute peak intensities were determined not by the chromatographic but the spectroscopic behavior of the mobile phase (reflecting an average S/N in the whole spectrum, including the blanked-out regions). Namely, the same small change of absorption intensity of the sample ratioed against the total spectral intensity of the different mobile phases results in very different maximum signal levels on the relative intensity scale. Hence, the same concentrations of the analyte give incomparably

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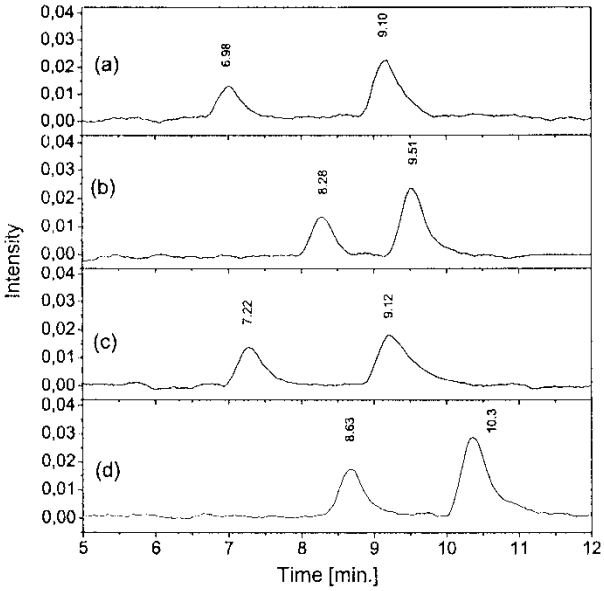

Figure 4. HPLC/FT-IR chemigrams of β-cypermethrin samples with 4 mg/mL injected concentrations obtained in the 1760–1720 cm^{-1} spectral region with four different mobile phase mixtures: DCM/*n*HEX (a), THF/*n*HEX (b), IPA/*n*HEX (c), and AN/*n*HEX (d).

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weaker peak intensities in the GSR traces, which explain the failure of measurements of the 4 mg/mL sample with dichloromethane/*n*-hexane (DCM/*n*HEX) and isopropanol/*n*-hexane (IPA/*n*HEX). Thus, we can conclude that the best choice of mobile phase for HPLC/FT-IR separation of β-cypermethrins is THF/*n*HEX or AN/*n*HEX considering the chromatographic aspects alone and utilizing the superior performance of the chemigram-type representation of the chromatogram.

Sample Identification and Detection Limits

Investigating the detection/identification limits, we observed that the 4, 2, and 1 mg/mL samples gave meaningful chemigrams at the spectral parameters of 8 cm^{-1} resolution and 16 accumulated scans per spectrum, while the 0.5 and 0.3 mg/mL samples, giving no peaks in the chemigram, required further changes in the measurement parameters. In the case of a signal changing in time (as in the chemigram), one has to be cautious with raising the number of accumulated scans per spectrum, since increasing the number of scans beyond a certain point does not improve the quality (S/N) of recorded

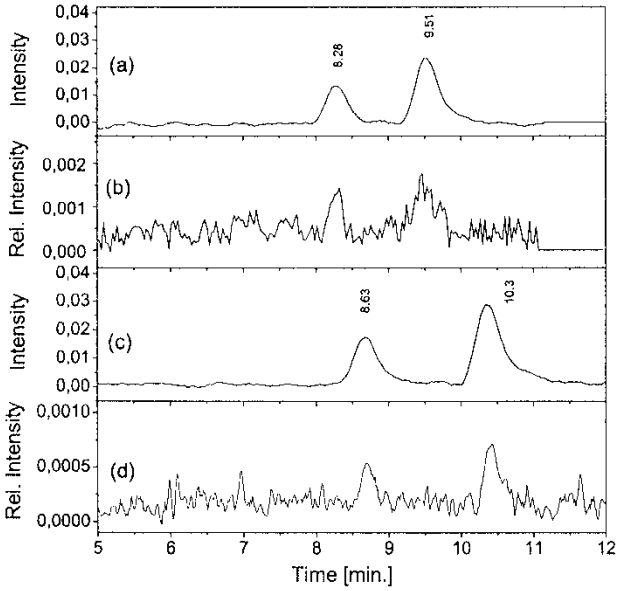

Figure 5. Comparison of recorded β-cypermethrin chemigrams with the corresponding Gram–Schmidt reconstructed chromatograms obtained with two mobile phase mixtures (4 mg/mL injected concentrations): chemigram for THF/*n*HEX (a), GSR trace for THF/*n*HEX (b), chemigram for AN/*n*HEX (c), and GSR trace for AN/*n*HEX (d).

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spectra any more,[11] on the contrary, it degrades time resolution and decreases the maximum peak intensity in the chemigram. The 32-scan spectral accumulation seemed to be effective regarding the appearance of chemigram peaks out of the noise ($S/N > 3$), but this was accompanied with loss of spectral information in the 1350–750 cm^{-1} region. Having no chance of identification, we opted for 16 cm^{-1} spectral resolution (instead of 8 cm^{-1}), to attain better time resolution in the chemigrams. As it is shown in Figure 6, chemigram peaks of β-cypermethrins could be detected for the 0.3 mg/mL samples with each of the four mobile phases (at 16 cm^{-1} spectral resolution with 32 accumulated scans per spectrum). Although the chemigrams obtained with THF/*n*HEX [Figure 6(b)] and IPA/*n*HEX [Figure 6(c)] could seem doubtful because of an unexpected noisy maximum around 6 min, checking the 1900–1500 cm^{-1} region of the spectra belonging to each data point on the time scale, we could observe the 1744 and 1588 cm^{-1} bands (characteristic for both of the diastereomers) only for the 8.3 and 9.5 min peaks in Figure 6(b) and for the 7.5 and 9.4 min peaks in Figure 6(c), thus the 6 min maxima proved to be only measurement noise.

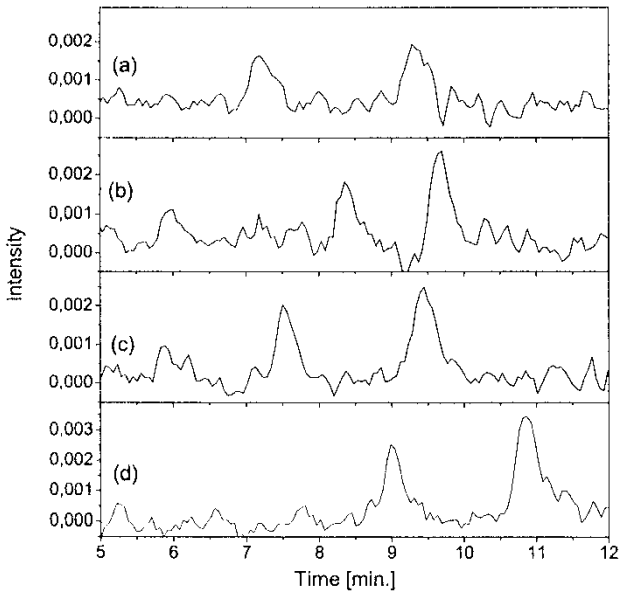

Figure 6. β-Cypermethrin chemigrams (1760–1720 cm^{-1} spectral region) for 0.3 mg/mL samples with DCM/*n*HEX (a), THF/*n*HEX (b), IPA/*n*HEX (c), and AN/*n*HEX (d).

Considering the noise levels of the chemigrams obtained with the 0.3 mg/mL samples, the 0.3 mg/mL concentration proved to be the general detection limit for the β-cypermethrins. At the same time, it is estimated that with the AN/*n*HEX mobile phase the detection limit may be at least 0.1 mg/mL, which has been proved by test measurements.

On the other hand, considering that with the 1 mg/mL samples we have been able to obtain not only chromatographic but spectral information as well, without loss of the 1350–750 cm^{-1} region (at 8 cm^{-1} spectral resolution with 16 accumulated scans per spectrum), we can establish that positive analyte identification (i.e., discrimination of diastereomers) can be made for 1 mg/mL samples.

Comparison of the IR spectra recovered from the two well separated chromatographic peaks of the 1 mg/mL samples for the four mobile phases (see Figures 7 and 8) reveals that the IR spectra of the two major components of the stereoisomer mixture show characteristic differences suitable for sample identification in the spectral region from 1300 to 1050 cm^{-1}. It is also clear that there may be definite differences between the mobile phase mixtures from the point of view of spectral identification. While the totally absorbing bands of *n*-hexane block the same spectral regions (from 1500 to 1300 cm^{-1} and below 800 cm^{-1}) and affect the spectra in the same way in

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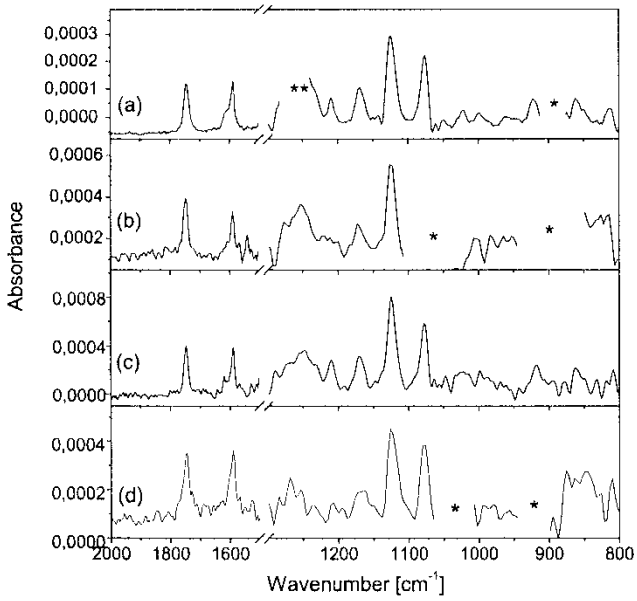

Figure 7. IR spectra extracted from chromatographic peak #1 of the 1 mg/mL sample in case of the four mobile phases: DCM/*n*HEX (a), THF/*n*HEX (b), IPA/*n*HEX (c), and AN/*n*HEX (d).

all four cases, the differences in the conditions are determined primarily by the absorption bands of the modifiers that have commensurable intensities with those of the analyte. As it has been noted in a recent review,[5] the use of a small percentage of a polar modifier in the mobile phase may prohibit effective detection in certain regions. Others have claimed[11] that many modifiers added in small (less than 5%) amounts affect chromatographic separation without loss of additional spectral information. In our experimental set-up, the extra absorption bands due to a low concentration modifier should have been compensated during the measurement, but because of molecular interactions between the modifier and the analyte, they may get slightly distorted and appear in the spectrum as positive or negative disturbances in the spectrum of the separated analyte components. (The spectral intervals affected by this effect are blanked and marked with an asterisk in Figures 7 and 8.)

Figures 7(a) and 8(a) show that the 1230–1270 cm^{-1} band of the DCM modifier applied in greater proportion (35%) obscured this valuable part of the analyte spectrum (this region is marked with double asterisk), which is a definite drawback. The IPA/*n*HEX mobile phase cannot be regarded as a good choice either, because of the loss of spectral information in the 1050–1300 cm^{-1} region for the second chromatographic peak [see Figure 8(c)].

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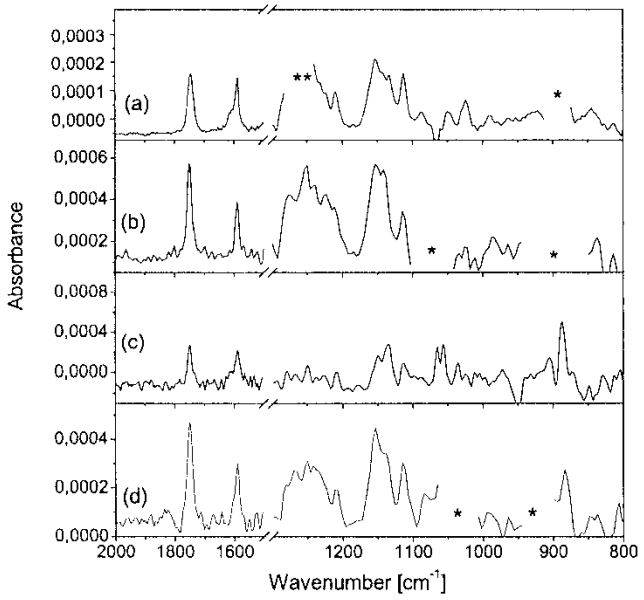

Figure 8. IR spectra extracted from chromatographic peak #2 of the 1 mg/mL sample in case of the four mobile phases: DCM/*n*HEX (a), THF/*n*HEX (b), IPA/*n*HEX (c), and AN/*n*HEX (d).

Measurements with THF/*n*HEX and AN/*n*HEX supplied the best quality spectra, thanks to the fact that the THF and AN bands affected only low information content spectral regions of the analyte below $1050\,cm^{-1}$ [marked with single asterisks in Figures 7(b) and (d), and Figure 8(b) and (d)], leaving the more characteristic parts of the spectra intact.

Concentration Dependence

Investigating the response of chemigram peak intensities (referring to IR absorption in the $1760-1720\,cm^{-1}$ spectral region) to change of concentration, we have found a fairly good linear relationship between the peak heights and concentrations in the concentration range from 0.3 to 4.0 mg/mL for all mobile phases, as it is shown in Figures 9 and 10. The correlation coefficients of linear regression varied between 0.9997 and 0.9940 for the first chemigram peak (compound 1, Figure 9), and between 0.9986 and 0.9928 for the second chemigram peak (compound 2, Figure 10).

To sum it up, the linearity criterion does not exclude any of the tested mobile phase mixtures from usage, but the quality of the useful part of the spectra ($1800-1500$ and $1300-1050\,cm^{-1}$) clearly indicates that THF/*n*HEX

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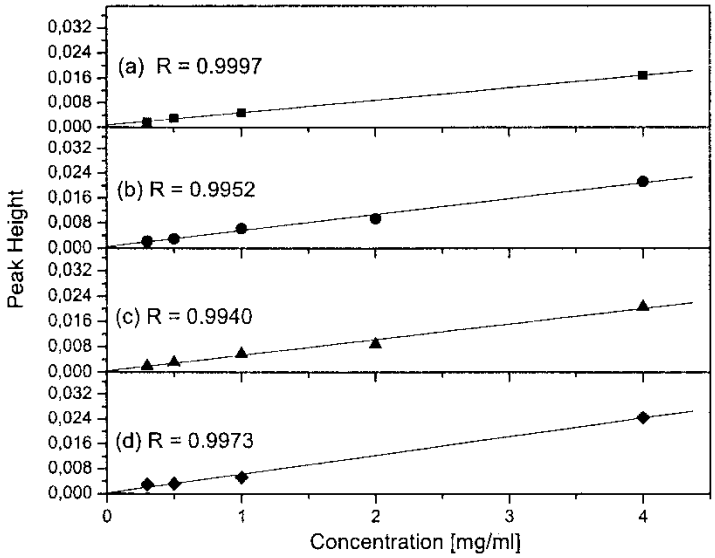

Figure 9. Concentration dependence of chemigram peak intensities (referring to IR absorption in the 1760–1720 cm^{-1} spectral region) for peak #1 obtained in case of DCM/*n*HEX (a), THF/*n*HEX (b), IPA/*n*HEX (c), and AN/*n*HEX (d) mobile phases. The correlation coefficients of the linear regression are shown on the plot.

is the best choice mobile phase when major component identification is also considered an important issue.

CONCLUSIONS

The experiments presented above have demonstrated that on-line coupling of analytical HPLC with a research grade FT-IR spectrometer (equipped with fast and high sensitivity IR detector and appropriate software for series measurements) by means of a micro flow-through cell, is a viable approach to major component detection and identification of organic chemical products. In contrast to the related main-stream literature, we have shown that the usual chromatographic mobile phases containing polar modifiers can be used in such measurements, although the technique has definite limitations depending on the spectroscopic properties of the mobile phase and the analytes in question.

It has been established, that in case of an analyte having strong IR absorption in a region clear from mobile phase absorption, IR chromatograms are suitable for major component detection using narrow-region chemigram-type detection tuned to spectral properties of the target compound. Attempts

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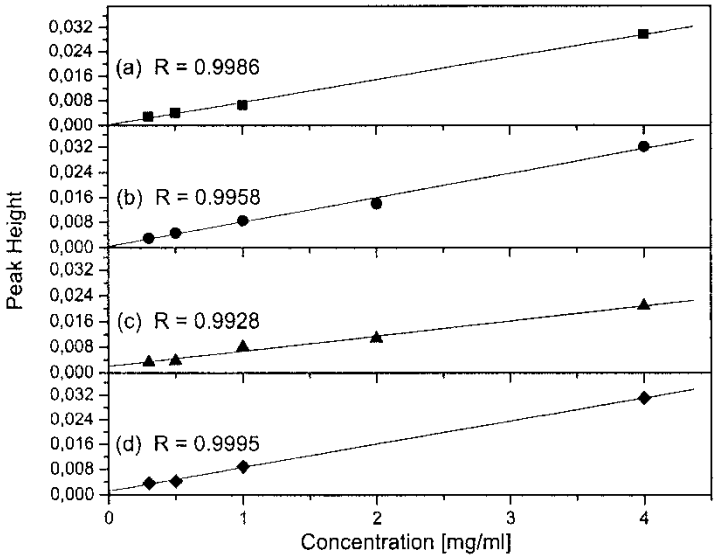

Figure 10. Concentration dependence of chemigram peak intensities (referring to IR absorption in the 1760–1720 cm^{-1} spectral region) for peak #2 obtained in case of DCM/*n*HEX (a), THF/*n*HEX (b), IPA/*n*HEX (c), and AN/*n*HEX (d) mobile phases. The correlation coefficients of the linear regression are shown on the plot.

to record Gram–Schmidt reconstructed chromatograms from the full-IR spectra (a highly successful method in GC/IR) were unsuccessful at concentration levels below 4 mg/mL of the analyte.

Quantitative determination of the two major diastereomers in the β-cypermethrin sample studied using the "carbonyl chemigram" has shown good linearity in the concentration range from 0.3 to 4.0 mg/mL. The value of 1.0 mg/mL proved to be the identification limit still allowing spectral discrimination of diastereomers, while the detection limit for the β-cypermethrins examined was 0.3 mg/mL in most mobile phases, but was estimated to be 0.1 mg/mL in acetonitrile/*n*-hexane.

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