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On-Line Fourier Transform Infrared Spectrometric Detection in Gradient Capillary Liquid Chromatography Using Nanoliter-Flow Cells

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A capillary liquid chromatographic system has been successfully interfaced with a mid-IR Fourier transform infrared (FT-IR) spectrometer. Spectra were recorded on-line using a micromachined transmission CaF_2 cell (internal volume of 7.5 nL) that was placed on a dedicated beam condenser attached to the spectrometer. Linear gradients were run from (50:50) to (35:65) water (0.05% TFA)/acetonitrile in 15 min for the separation of standard solutions of four nitrophenols (4-nitrophenol, 3-methyl-4-nitrophenol, 2,4-dinitrophenol, and 2-nitrophenol) in a reversed phase system, providing limits of detection between 35 and 94 ng on-column. The changing background absorption due to gradient elution was successfully corrected by using a dedicated algorithm implemented in Matlab. When this chemometric data treatment was used, highly characteristic analyte spectra could be recorded as indicated by correlation coefficients between 89 and 95.8%, obtained when comparing mid-IR spectra of standard solutions and the spectra extracted from the chromatogram.

Liquid chromatography (LC) is a versatile separation technique with applications in a variety of fields extending from environmental analysis to life science. Coupled with Fourier transform infrared (FT-IR) spectrometry it can be used for both qualitative and quantitative determinations of a wide range of analytes because of the specificity of the molecular information provided. FT-IR detection in LC techniques may be achieved either on-line using flow cells for transmission measurements or off-line after solvent elimination by measuring dry residues. Early attempts to couple FT-IR spectrometry with LC almost exclusively concentrated on off-line techniques. The motivation for aiming at these in LC-FT-IR emerged from the generally agreed assessment that the dominating absorption from the mobile phase had to be physically eliminated for accurate measurement of the infrared spectrum of the separated analytes. Especially when considering gradient techniques, solvent elimination was believed to be mandatory.[1] Access to the complete infrared spectrum of the analyte and comparability of the thus obtained spectra with existing databases were additional arguments in favor of the off-line approach. Finally, recording of a chromatogram as a trace consisting of separated deposits opened the possibility to average as many scans as desired when measuring the infrared spectrum of a given deposit holding promise to reach lower detection limits. Several concepts for achieving reliable off-line detection have been published. Their realization into commercially available products, however, has been scarce. Only in the field of size exclusion chromatography and polymer analysis has this hyphenation gained relevance for routine analysis.

In comparison to the off-line approach, on-line coupling between LC and FT-IR is experimentally much easier as it can be achieved using standard flow cells for measurement of liquids. However, two challenges can be identified: (i) as the mobile phases employed in LC absorb strongly in the mid-infrared, their accurate compensation is crucial to obtain characteristic analyte spectra and (ii) FT-IR detection in LC is characterized by a significantly lower sensitivity as compared to other more commonly used detectors such as UV−vis spectrometry or mass spectrometry (MS). Recent advances in instrumentation used for liquid chromatography and infrared spectrometry, however, require reassessing the potential of the on-line approach in LC-IR. In the case of on-line isocratic LC-FT-IR systems correction for mobile phase absorption can be carried out by subtracting the spectra of the eluent recorded at the beginning of the run or immediately before elution of the analytes of interest from the spectra when the analyte elutes. When using the gradient technique accurate background correction presents important difficulties[1] because of existing changes in intensity and shape of the eluent absorption bands, which may be up to several orders of magnitude more intense than the absorption because of the analytes. Different chemometric techniques have been proposed to overcome this problem. In 2006 István et al.[2] proposed a method named objective subtraction of solvent spectrum with iterative use of PARAFAC and PARAFAC2 (OSSS-IU-PARAFAC and OSSS-IU-PARAFAC2) which yielded promising results when analyzing isocratic LC-IR data sets. Recently, new developments in the

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(1) Griffiths, P. R.; De Haseth, J. A. *Fourier Transform Infrared Spectrometry*, 2nd ed.; Wiley: New York, 2007.
(2) István, K.; Rajkó, R.; Keresztury, G. *J. Chromatogr. A* **2006**, *1104*, 154–163.

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Table 1. Calibration Features of On-Line LC-FT-IR Determination of 4-NP, 3m4-NP, 2,4-dNP, and 2-NP Using Height Values of the Calculated Chromatographic Peaks

	analytical signal[1] (y)							
	A(wavenumber) (cm^{-1})	baseline correction (cm^{-1})	$y^1 = (a \pm s_a) + (b \pm s_b)C$ (ng μL^{-1})	R^2	concentration range (ng μL^{-1})	LOD[2] (ng)	LOI[3] (ng)	RSD[4] %
4-NP	A(1334)+A(1338)+A(1342)	A(1315)	$(0.3 \pm 0.3)10^{-3} + (0.0643 \pm 0.0009)10^{-3}C$	0.9992	65−695	35	65	4.6
3m4-NP	A(1269)+A(1311)	A(1292)	$(0.1 \pm 0.1)10^{-3} + (0.0109 \pm 0.0003)10^{-3}C$	0.997	150−890	50	150	6.8
2,4-dNP	A(1342)+A(1346)+ A(1350)+A(1354)	A(1365)	$(0.3 \pm 0.4)10^{-3} + (0.0296 \pm 0.0010)10^{-3}C$	0.995	85−350	80	125	5.7
2NP	A(1331)+A(1335)+A(1338)	A(2075)	$(-0.1 \pm 0.3)10^{-3} + (0.0174 \pm 0.0008)10^{-3}C$	0.995	175−695	94	175	6.2

[1] The analytical signal (y) is the sum of single point baseline corrected absorbance values (A) at the wavenumbers indicated between brackets. [2] LOD: Limit of detection in ng calculated for an injection volume of 1 μL as the 3-fold standard deviation of the method (s_{x0}) according to ISO 8466-1. [3] LOI: Limit of identification estimated as the lowest concentrations providing: (i) recognizable spectra of the analyte and (ii) a signal-to-noise ratio of chromatographic signal higher than 3. [4] RSD is the relative standard deviation in % and was calculated from five repeated measurements of standard solutions with a concentration equal to the LOD of each analyte.

area of chemometric background correction have taken place. Quintás et al. developed univariate[3] and multivariate[4] methods to perform eluent subtraction in continuous liquid flow systems under isocratic and gradient conditions. For accurate background correction these methods used a data set that was recorded from a gradient experiment without injecting an analyte. By matching characteristic absorption bands of the eluents in the chromatographic run with those of the reference data set, it was possible to select or to calculate an appropriate background spectrum for recovering the analyte spectra. The proposed methods have been successfully tested on on-line reversed phase LC-FT-IR data sets using (H_2O:CH_3CN) [3−5] and (H_2O:CH_3OH)[6] mobile phases for the determination of analytes in the low mg mL^{-1} concentration range. Ongoing research seeks to extend the use of the aforementioned chemometric background correction approach to other eluent systems for both normal and reversed phase LC.

Previous studies in isocratic on-line LC-IR provided detection limits in the mg mL^{-1} range using attenuated total internal reflectance infrared microspectroscopy,[7,8] or a conventional internal reflection element mounted in a flow cell,[9] as well as standard IR flow cells.[3−6,10−14] Whereas these reports show the usefulness of on-line IR detection for the analysis of sugars, organic acids, and peroxides (explosives), an increase in sensitivity is needed to broaden the problem solving capabilities of this hyphenation. When assessing the instrumental constraints of on-line LC-IR several reasons can be identified being responsible for the apparent low sensitivity of on-line FT-IR detection as compared to UV−vis spectrometric detection in LC using 4.6 mm columns. The fact that absorptivities of molecules in the infrared are generally significantly smaller than in the UV−vis range sets a physical limitation. Furthermore, the maximum optical path of a flow cell used for on-line detection in the IR range is determined by the absorption of the mobile phase, being in the range of a few micrometers when using standard FT-IR spectrometers. This is contrary to the UV−vis range where the used solvents are generally transparent. This consideration defines also the typical volume of an IR flow cell to be used. Considering an optical path of 25 μm as reported by Vonach et al., a flow cell volume of 10 μL could be estimated. This is significantly smaller than the volume of a peak eluting from a 4.6 mm separation column, which in the paper of Vonach was in the order of 250−400 μL at a flow rate of 500 μL min^{-1}. Considering only the differences in optical path, a factor of 400 in terms of sensitivity is lost when compared to a 1 cm flow cell which is the standard in UV−vis detection. In the case of infrared detection, the time required to record and store an infrared spectrum also needs to be taken into account when recording a transient signal such as a chromatographic peak. Vonach et al. achieved a recording rate of 19 spectra per minute, which corresponded to a volume of 26.3 μL per spectrum. In this case the cell volume and the time required to record a single spectrum were appropriate to follow the elution of a chromatographic peak without facing dilution of the peak maximum because of instrumental constraints. The achieved average limit of detection (LOD) calculated from the sugars and organic acids analyzed, and estimated as the 3-fold standard deviation of the method s_{x0}, which was calculated according to ISO 8466-1, was 0.2 mg mL^{-1}. The corresponding absolute LOD was therefore 4 μg as 20 μL of sample had to be injected.

After sample injection in liquid chromatography, radial dispersion causes the sample to distribute over the entire cross-section

(3) Quintás, G.; Lendl, B.; Garrigues, S.; de la Guardia, M. *J. Chromatogr. A* **2008**, *1190*, 102–109.
(4) Quintás, G.; Kuligowski, J.; Lendl, B. Private communication.
(5) Kuligowski, J.; Quintás, G.; Garrigues, S.; de la Guardia, M. *Talanta,* **2008**, *77*, 779–785.
(6) Kuligowski, J.; Quintás, G.; Garrigues, S.; de la Guardia, M. *Anal. Chim. Acta* **2008**, *624* (2), 278–285.
(7) Patterson, B. B.; Danielson, N. D.; Sommer, A. *J. Anal. Chem.* **2003**, *75*, 1418–1424.
(8) Patterson, B. B.; Danielson, N. D.; Sommer, A. *J. Anal. Chem.* **2004**, *76*, 3826–3832.
(9) Edelmann, A.; Diewok, J.; Rodriguez-Baena, J.; Lendl, B. *Anal. Bioanal. Chem.* **2003**, *376*, 92–97.
(10) Vonach, R.; Lendl, B.; Kellner, R. *J. Chromatogr. A* **1998**, *824*, 159.
(11) Edelmann, A.; Ruzicka, I.; Frank, J.; Lendl, B.; Schrenk, W.; Gornik, E.; Strasser, G. *J. Chromatogr. A* **2001**, *934*, 123–128.
(12) Vonach, R.; Lendl, B.; Kellner, R. *Anal. Chem.* **1997**, *69*, 4286.
(13) Schulte, L.; Rasmus, E.; Quintás, G.; Lendl, B.; Karst, U. *Anal. Chem.* **2006**, *78* (23), 8150–8155.
(14) Kuligowski, J.; Breivogel, A.; Quintás, G.; Garrigues, S.; de la Guardia, M. *Anal. Bioanal. Chem.* **2008**, *392*, 1383–1389.

of the column. The dispersion in the column can be expressed by the standard deviation volume of the eluted peak (σ_v):

$$\sigma_v = \frac{\varepsilon l \pi r^2 (1 + k')}{n^{0.5}} \quad (1)$$

where k' is the capacity factor; r, l, and n are the radius, length, and efficiency of the column, respectively; and ε is the fraction of the mobile phase in the column that is available to the solute. When the internal diameter (ID) of column diminishes, the dilution is lowered resulting in an increased sensitivity of detection being that the concentration of the sample at the end of the column is inversely proportional to the square of the column diameter. Hence, according to the down-scaling factor in eq 2, a maximum 235-fold increase in sensitivity can theoretically be achieved by replacing a 4.6 mm ID column with a 300 μm ID capillary column:[15]

$$f = \frac{\mathrm{ID}^2_{\text{standard column}}}{\mathrm{ID}^2_{\text{capillary column}}} \quad (2)$$

It should be realized that this impressive gain in sensitivity is only achieved in case the same amount of sample can be injected onto the different columns. However, this is not always possible as the maximum allowable injection volume decreases with the column ID. Therefore, lower gains in concentration sensitivity are normally obtained because the increase in the chromatographic peak concentration by reduction of the column ID can be partially or fully compensated by the reduction in the injection volume.[16]

In the second edition of their book on Fourier Transform Infrared Spectrometry, published in 2007, Griffiths and de Haseth[1] estimated a limit of detection (LOD) of the order of 250 μg on-column for a typical reversed phase LC-FT-IR separation made using a 4.6 mm ID column and a flow-through cell with 10 μm

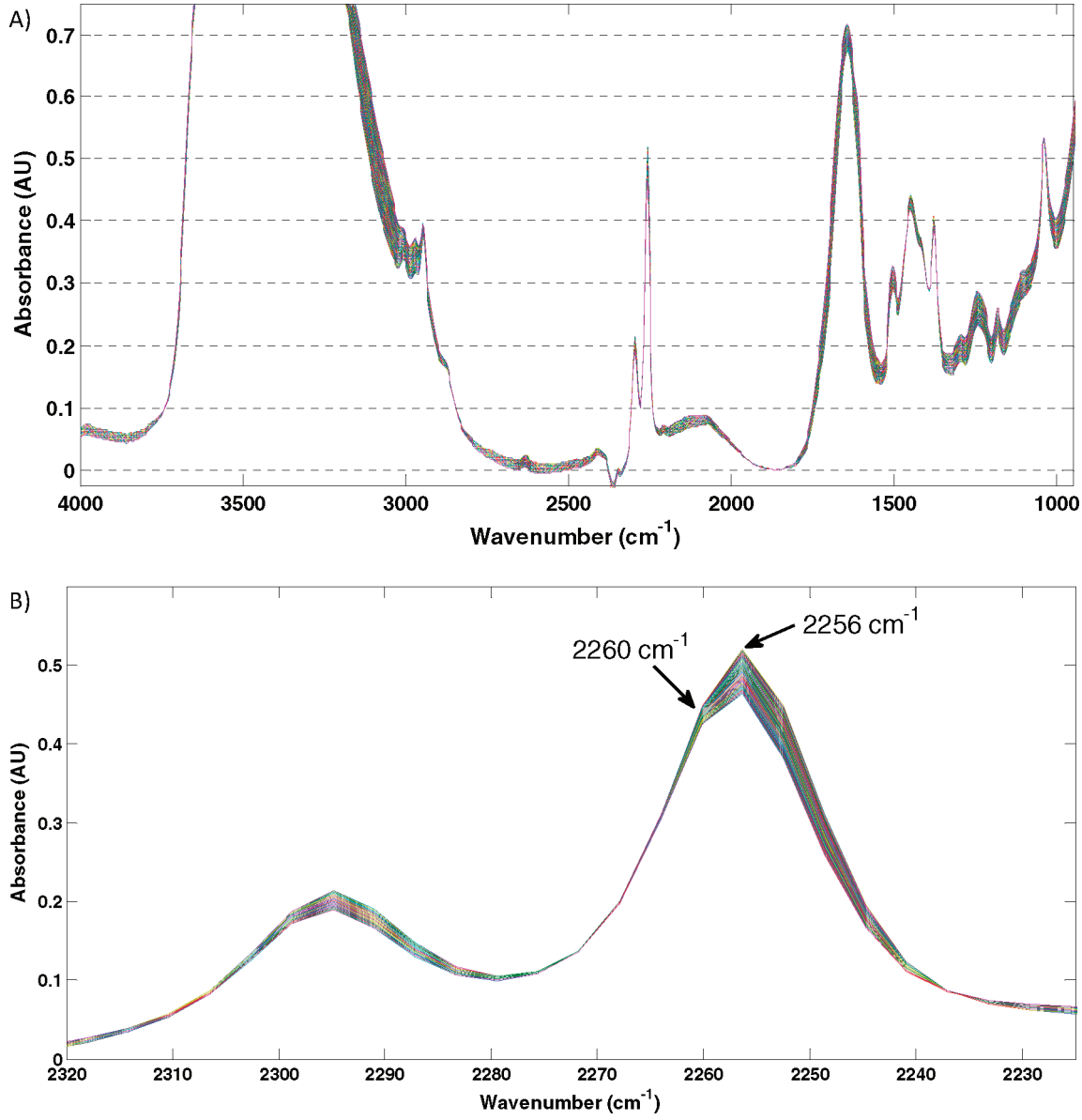

Figure 1. FT-IR spectra of water (0.05% TFA)/acetonitrile mixtures measured during a low-speed re-equilibration of the LC system after a gradient injection in the 4000−950 cm^{-1} (A) and in the 2320−2220 cm^{-1} region (B). Mobile phase composition ranged between (35:50) and (50:65) water (0.05% TFA):acetonitrile.

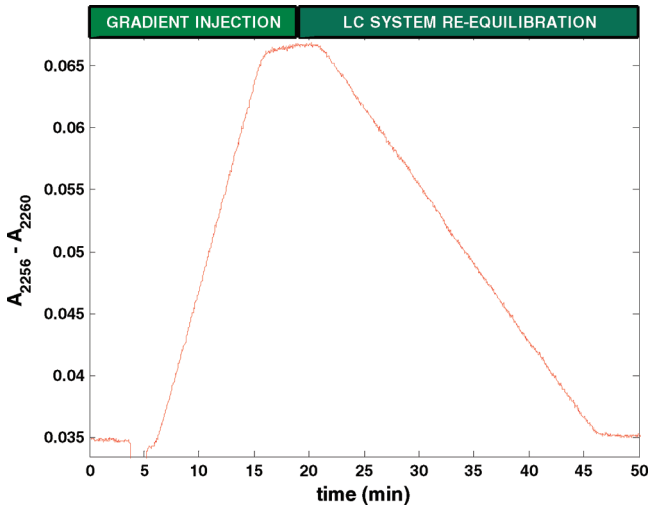

Figure 2. Change in the differential absorption at 2256 and 2260 cm^{-1} during a gradient LC run followed by the a slow re-equilibration of the system.

path length, 3 mm diameter, and a volume of 70 nL. According to their estimation, LODs of the order of 1 μg on-column can be expected using capillary columns with ID of the order of 300 μm.

Concerning on-line IR measurements in capillary LC, the lower flow rates (1−20 μL min^{-1}) used in capillary LC demand the use of low-volume flow cells because post-column dead volumes become critical as they can cause serious peak broadening and thus decrease sensitivity. Flow cells with nL-volumes were developed already in 2002 by Köhled et al.[17] and used to hyphenate capillary electrophoresis with FT-IR spectrometry. These micromachined flow cells had path lengths ranging from 8 to 50 μm with corresponding volumes of 2.4 to 15 nL, respectively.

This paper reports, for the first time, on-line FT-IR detection in capillary liquid chromatography employing gradient elution. It employs micromachined flow cells and chemometric background correction to extract the analyte spectra from the recorded chromatographic data set. The quality of the obtained analyte spectra is assessed by comparing the recovered spectra with reference spectra recorded in solution. A further purpose of this paper is to evaluate the advantages gained from miniaturization in terms of sensitivity of on-line LC-FT-IR. For this purpose four nitrophenols (4-nitrophenol (4-NP), 2,4-dinitrophenol (2,4-dNP), 2-nitrophenol, and 3-methyl-4-nitrophenol (3m4-NP)), listed as priority pollutants by the US Environmental Protection Agency (EPA), were selected as model compounds to be separated, quantified, and identified by reversed phase gradient LC using an acetonitrile:water mobile phase.

EXPERIMENTAL SECTION

Reagents. 4-Nitrophenol (4-NP), 2,4-dinitrophenol (2,4-dNP), 2-nitrophenol (2-NP) (PESTANAL, Fluka, Buchs, Switzerland), and 3-methyl-4-nitrophenol (3m4-NP) (98%, Sigma-Aldrich, Switzerland) standard solutions were prepared by dissolving an appropriate amount of each compound in a (95:5) water (0.05% TFA)/acetonitrile solution. Solutions were filtered (0.22 μm) prior to injection.

HPLC. The LC system consisted of a Dionex (Sunnyvale, CA, U.S.A.) UltiMate 3000 capillary LC system equipped an Acclaim PepMap C18 (300 μm ID × 15 cm, 3 μm, 100 Å) capillary column. Separations were carried out at 25 °C using a flow rate of 3 μL min^{-1} and an injection volume of 1 μL. Linear gradients were run from (50:50) to (35:65) water (0.05% TFA)/acetonitrile in 15 min. Standards with concentrations indicated in Table 1 were prepared, resulting in 65−890 ng on-column injections.

FT-IR. Infrared measurements were performed on a Bruker Equinox 55 (Bruker Optics, Ettlingen, Germany) FT-IR spectrometer equipped with a liquid nitrogen cooled mercury cadmium telluride (MCT) detector. To accurately focus the IR beam on the micromachined flow cell an in house built beam condenser was used which was attached to the spectrometer and placed in dry air purged Perspex housing. The parallel beam was taken out of the spectrometer and focused on the flow cell using an off axis paraboloid mirror with a focal length of 69 mm. The flow cell contained 1 mm thick CaF_2 windows. The flow cells were produced starting from CaF_2 windows with a diameter of 2.54 cm by using micromachining technology. On one window an epoxy-based photoresist SU-8 (Microchem Corp., Newton, MA) polymer was applied whose thickness determined the optical path. Then, using a photomask and UV light, the channel structure was formed. The top CaF_2 window was then used to close the structure. On one of the CaF_2 windows, a 200-nm-thick Ti layer was deposited by evaporation and patterned by a conventional liftoff technique. The Ti layer acted as an optical aperture strongly reducing IR radiation to pass the polymer which defines the flow channels. From the closed structure several micromachined flow cells could be obtained by cutting pieces of an area of 2 × 4 mm. The channel of the flow cells used in this work was 2 mm long with a cross section of 150 μm in width and an optical path of 25 μm. The volume of the flow cell was 7.5 nL. Detailed description and schematic views of both, the manifold and the construction of the flow cell, can be found in previous works.[17,18]

Special care was taken to reduce or eliminate peak broadening due to the connection of the LC system with the micromachined nL flow cell. The column outlet was connected using a zero dead volume connection (Dionex) to a 10 cm length untreated fused-silica capillary (ID 50 μm, OD 375 μm, Polymicro Technologies, Phoenix, AZ) which transferred the column eluate to the flow cell.

The scanner of the spectrometer was operated at a HeNe laser modulation frequency of 180 kHz. Twenty five scans were co-added for each spectrum using a spectral resolution of 8 cm^{-1} and a zero filling factor of 2, providing a scanning frequency of 25 scans s^{-1}, a spectra acquisition frequency of 29 spectra min^{-1}, and 4 cm^{-1} spectral data spacing. Every spectrum thus corresponded to a volume of approximately 100 nL. Reference FT-IR spectra of the four analytes were obtained from the measurement of nitrophenols standard solutions under stopped-flow conditions.

(15) Legido-Quicley, C.; Smith, N. W.; Mallet, D. *J. Chromatogr. A* **2002**, *976*, 11–18.
(16) Kranendijk, M.; Waterval, J. C. M.; Somsen, G. W.; Jong, G. J. *J. Sep. Sci.* **2005**, *28*, 1796–1802.
(17) Kölhed, M.; Hinsmann, P.; Svasek, P.; Johannes, F.; Karlberg, B.; Lendl, B. *Anal. Chem.* **2002**, *74*, 3843–3848.
(18) Kulka, S.; Quintás, G.; Lendl, B. *Vib. Spectrosc.* **2006**, *42*, 392–396.

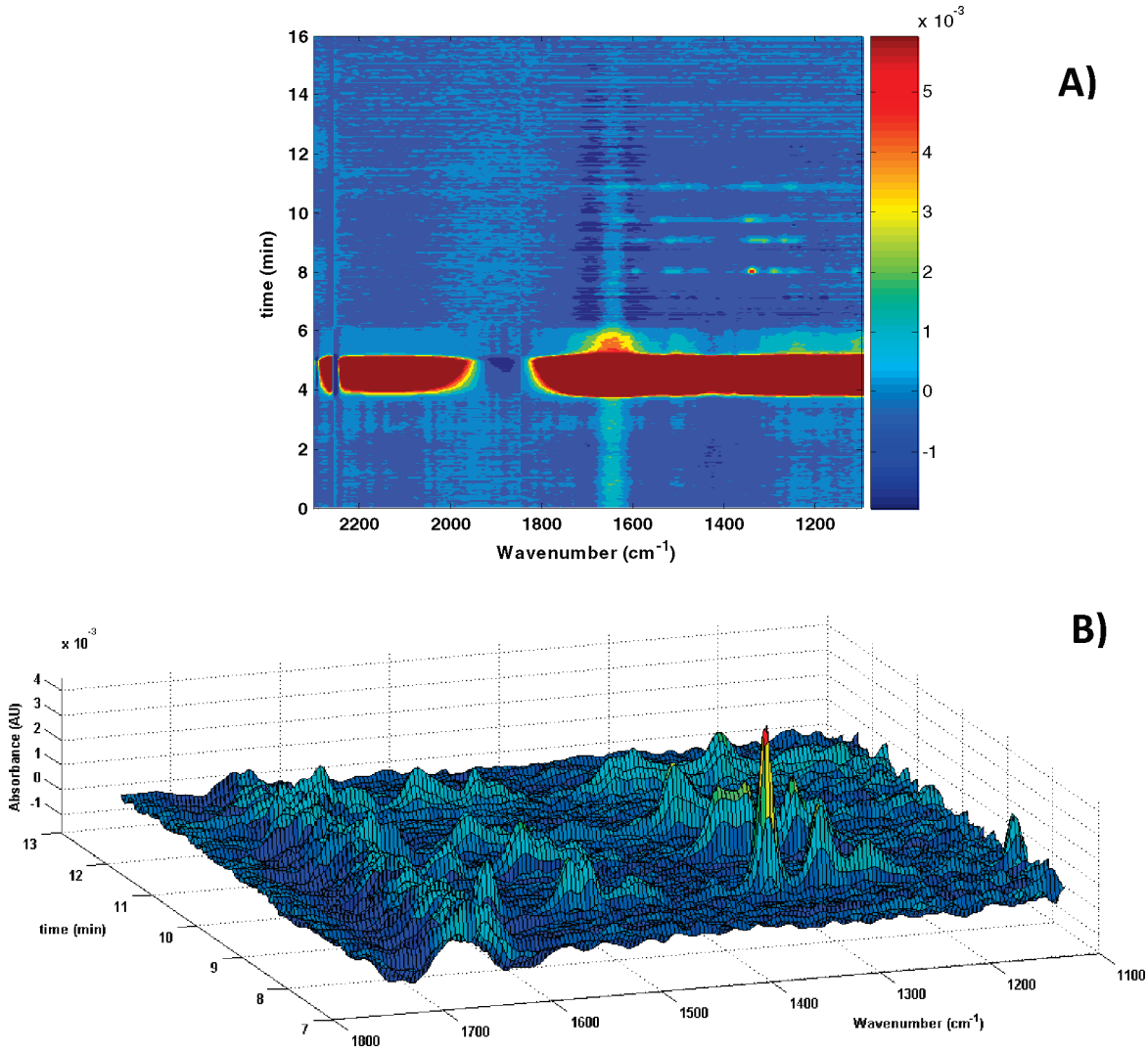

Figure 3. (A) Surface plot of background corrected spectra acquired upon injection of 1 μL of a standard mixture containing the four selected compounds 4-NP (270 ng μL^{-1}), 3m4-NP (230 ng μL^{-1}), 2,4-dNP (230 ng μL^{-1}), and 2-NP (230 ng μL^{-1}); (B) three dimensional close-up view of the time window in which the analytes elute.

Background Correction. Background correction and data treatment were run under Matlab 7.0 from Mathworks (Natick, MA, 2004) using in-house written Matlab functions available from the authors. A modification of a recent approach named "univariate background correction based on the use of a reference spectra matrix" (UBC-RSM) was used for background correction. Detailed description of the basics of the UBC-RSM method can be found in a previous work.[3] The background correction process can be divided in five steps: Step 1 includes the measurement of the LC-FT-IR sample injection, **SM** (z,c), and a reference spectra matrix measured during the re-equilibration of the LC system after each gradient sample injection, **RSM** (r,c), where c is the number of variables (wavenumbers) and z and r are the number of spectra included in the **SM** and **RSM** matrices, respectively. Step 2 involves the calculation of a spectral parameter which is characteristic for the mobile phase composition for each spectrum included in the **SM** and the **RSM**. In this study, the parameter selected was a relative absorbance value (RW) defined as the difference in absorbance at two selected wavenumbers r_1 = 2256 cm^{-1} and r_2 = 2260 cm^{-1}. These two wavenumbers correspond to the stretching vibration of the C≡N bond in acetonitrile which is shifted to lower frequency because of hydrogen bonding with water. Therefore by focusing on this area of the spectrum the actual existing mobile phase composition can be determined.

$$RW_s = (y^s_{r_1} - y^s_{r_2}) \tag{3}$$

where $y^s_{r_1}$ and $y^s_{r_2}$ are the absorbance values at the wavenumbers r_1 and r_2 (cm^{-1}) measured in the spectra $s = (1, ..., z)$ for spectra included in the **SM** and $s = (1, ..., r)$ for the **RSM**. In Step 3, for each of the z spectra included in the **SM**, the most appropriate (the one with the closest RW value) background spectrum ($S_{\gamma,s}$, $s = 1, ..., z$) included in the **RSM** is located. Step 4 consists of the calculation of a correction factor (KF) which is determined for each sample spectrum. The objective of KF is to correct slight changes in the spectral intensity of the eluent during the run. KF is defined as the ratio between the absorbance values at the wavenumber φ of the sample spectrum s (y^s_φ) and its previously selected background spectrum $S_{\gamma,s}$ ($y^{S_{\gamma,s}}_\varphi$) (for $s = 1, ..., z$) using the following expression:

$$KF_s = (y_\phi^s / y_\phi^{s_{y,s}}) \quad (4)$$

being $\varphi = 2260\ cm^{-1}$ in this work. Step 5 includes the subtraction of the eluent background spectrum from the sample spectrum using the following expression:

$$\text{Corrected } S_s = S_s - KF_s \cdot S_{y,s} \quad (5)$$

where Corrected S_s is the background corrected sample spectrum s; S_s is the original sample spectrum; $S_{y,s}$ is the background spectrum included in the **RSM** and KF_S is the calculated correction factor for the sample spectrum S_s.

RESULTS AND DISCUSSION

FT-IR Spectra of the Mobile Phase-Flow Cell System. Figure 1 shows representative FT-IR spectra in the region between 4000 and 900 cm^{-1} of different eluent compositions between (50:50) and (35:65) water (0.05% TFA)/acetonitrile. The spectra show clearly distinguishable characteristic water and acetonitrile bands as described elsewhere.[19] Additionally, a set of SU-8 polymer absorption bands can be seen in the region 1540−1100 cm^{-1}. Negative bands in the region between 2420 and 2240 cm^{-1} arise because of changes in the CO_2(g) concentration during the measurements. Changes in intensity and shape of the absorption bands of the mobile phase components evidence the need for using an appropriate background correction.

The selection of the reference parameter for the selection of a background spectrum within the **RSM** is one of the most important steps of the UBC-RSM procedure. Figure 2 shows the change in the RW value during the injection of 1 μL of a standard solution containing 200 ng μL^{-1} of 4-NP, 2,4-dNP, 2-NP, and 3m4-NP (0−19 min), as well as during the re-equilibration of the LC system (19−50 min). Negative values between 4 and 5 min are caused by the elution of the sample solvent. This figure proves that the selected RW as reference parameter is characteristic of the mobile phase composition in the considered mixing ratios and is not influenced by the elution of the analytes. Hence, it can be used to identify and select appropriate eluent spectra within the **RSM** for the correction of the eluent absorption.

On-Line Gradient Capillary LC-FT-IR. Background corrected spectra acquired upon injection of 1 μL of a standard mixture containing the four selected compounds 4-NP (270 ng μL^{-1}), 3m4-NP (230 ng μL^{-1}), 2,4-dNP (230 ng μL^{-1}), and 2-NP (230 ng μL^{-1}) are presented in Figure 3. As shown, the sample solvent elution peak at 4 min and the elution windows of the analytes between 8 and 11 min can be easily identified. Figure 4 depicts FT-IR chromatograms extracted from data shown in Figure 3. As illustrated in this figure, all the analytes could be baseline resolved under the chromatographic gradient conditions with retention times of 8.03, 9.07, 9.78, and 10.76 min for 4-NP, 3m4-NP, 2,4-dNP, 2-NP, respectively. The presence of characteristic bands of the analytes (see Figure 5) allowed us to select different integration conditions for the extraction of their respective FT-IR chromatograms indicated in Table 1, thus maximizing the selectiv-

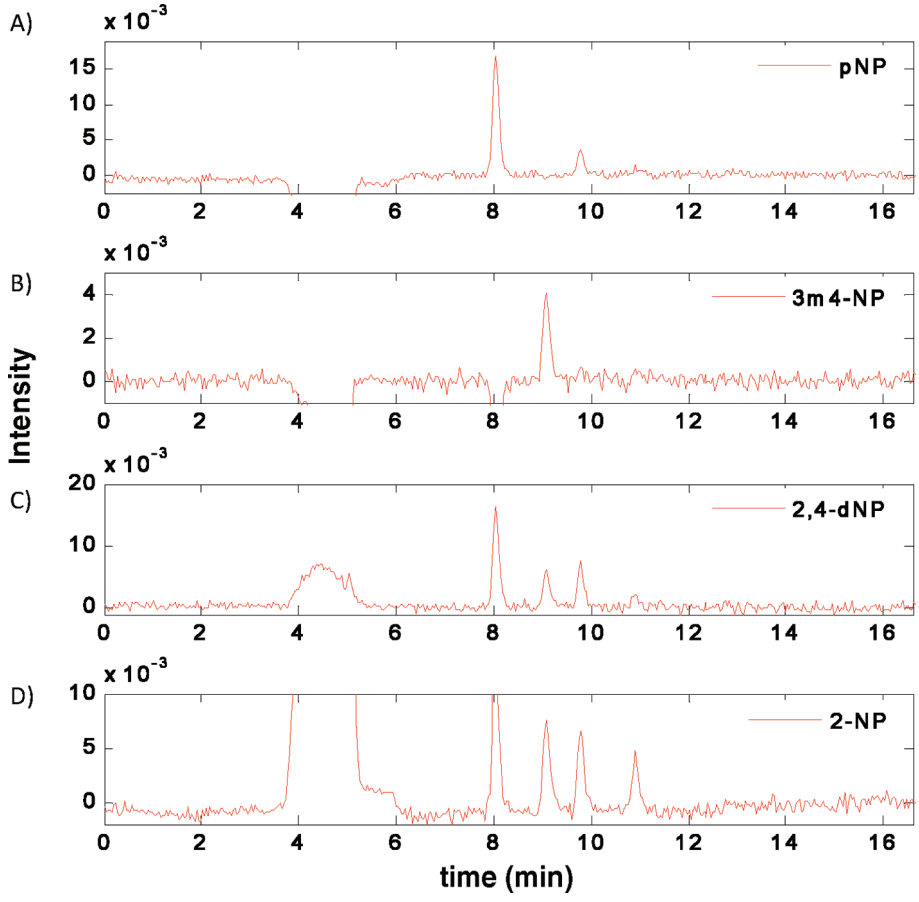

Figure 4. Extracted chromatograms from the injection of 1 μL of a single standard solution containing 270 ng μL^{-1} 4-NP (a) and 230 ng μL^{-1} of 3m4-NP (b), 2,4-dNP (c), and 2-NP (d).Note: FT-IR chromatograms extracted as indicated in Table 1.

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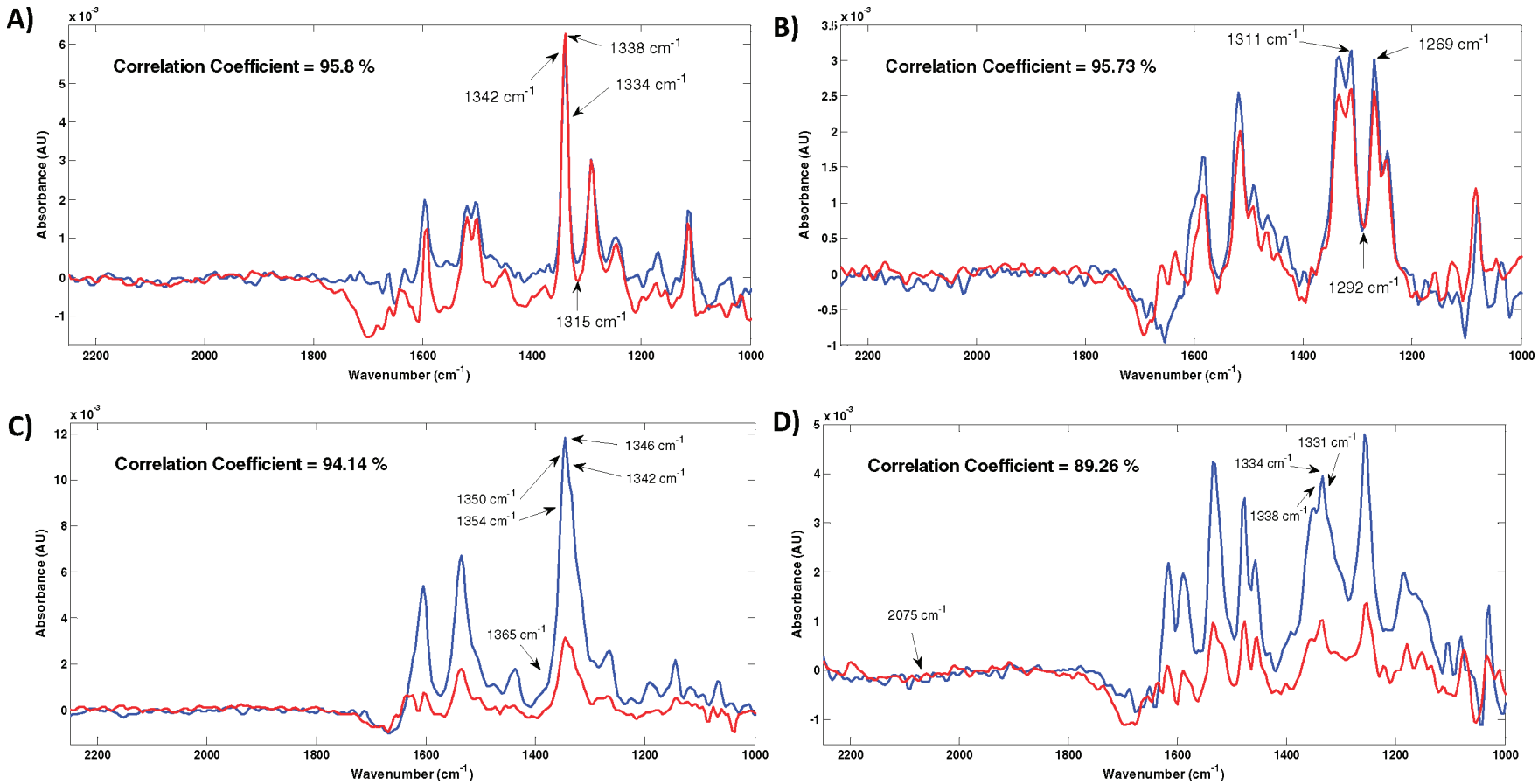

Figure 5. Background corrected spectra of the analytes at their corresponding peak apex obtained from the injection of 1 μL of a standard solution containing 270 ng μL^{-1} 4-NP (a) and 230 ng μL^{-1} of 3m4-NP (b), 2,4-dNP (c), and 2-NP (d). Reference spectra (blue lines) are included in the figure for comparison. Note: The correlation coefficient between two spectra y_1 and y_2 is defined as the ratio from the covariance (Cov(y1, y2)) and the product of the two standard deviations s_{y1} and s_{y2}. According to this definition, a value of percentage of correlation coefficient of 100 indicates identical spectra.

ity and the sensitivity of the chromatographic signal. Table 1 also summarizes the main analytical features of the proposed hyphenation for the determination of the considered analytes.

Studies of linearity showed appropriate R^2 values, ranging from 0.995 to 0.9992, obtained for the four analytes by injecting two series of 5 standards at different specified concentration ranges for each analyte. Limits of detection (LODs) were calculated as the 3-fold standard deviation of the method s_{x0}, which was calculated according to ISO 8466-1. Limits of identification (LOI) were estimated as the lowest concentrations providing the following: (i) recognizable spectra of the analytes in the region between 1700 and 1050 cm^{-1} and (ii) a signal-to-noise ratio of chromatographic signal higher than 3. Multiplication of the relative LODs and LOIs by the injection volume results in absolute LOD and LOI expressed as ng analyte on-column. By using these criteria, the calculated LODs were 35, 50, 80, and 94 ng on-column for 4-NP, 3m4-NP, 2,4-dNP, and 2-NP, respectively. Estimated values of LOIs ranged between 65 and 175 for 4-NP and 2-NP, respectively. The relative standard deviation (RSD, $n = 5$) of the peak height values found from the repeated injections of a standard with a concentration of 175 ng μL^{-1} of each analyte were 4.6, 6.8, 5.7, and 6.2% for 4-NP, 3m4-NP, 2,4-dNP, and 2-NP, respectively.

Although it is difficult to establish a direct comparison of sensitivities obtained using different LC-FT-IR systems and analytes, the obtained LODs represent a significant improvement in the sensitivity compared to previous results reported in literature using on-line LC-FT-IR. For example, Schulte-Ladbeck et al.[13] reported limits of detection for triacetonetriperoxide (TATP) and hexamethylenetriperoxide diamine (HMTD) of 4440 and 2080 ng on-column using a LiChroSpher RP18 column (250 × 3 mm, 5 μm) and an isocratic mobile phase of acetonitrile/H_2O (75:25) for the separation and a CaF_2 flow cell with 25 μm path length for FT-IR detection. More recently, Kuligowski et al.[14] reported a limit of detection of glycolic acid by isocratic on-line LC-FT-IR in rapid scan acquisition mode of 680 ng on-column using a RP18 column (250 × 2 mm, 5 μm) and an isocratic mobile phase of acetonitrile/phosphate buffer (25 mM, pH 2.7) (3:97) for the separation and a CaF_2 flow cell with 14 μm path length and a volume of 1.7 μL for FT-IR detection. In short, limits of detection are dependent on the analyte but are typically in the range of 50 ng μL^{-1} by using micromachined nL-IR flow cells in capillary LC, and in the low μg μL^{-1} range or higher using standard flow cells.

A major interest of FT-IR detection in LC, compared to UV detection, is the possibility of both, detection and identification of the eluted analytes. FT-IR spectra of the four nitrophenols were extracted from the background corrected FT-IR chromatograms at selected retention times corresponding to their peak apex and were compared with reference spectra previously measured (Figure 5a–d). As shown, spectra extracted at the peak apex from the background corrected gradient chromatograms correlate well with the reference spectra. The calculated correlation coefficients at different concentrations indicated in the figure show that the selected conditions of measurement and background correction are appropriate for the identification and quantification of the analytes in the studied concentration ranges.

CONCLUSIONS

The present study shows the capabilities of on-line FT-IR detection in gradient capillary LC using micromachined nL-flow cells. Four model compounds could be separated and identified using an ACN:H_2O gradient with limits of detection in the concentration range of 35–94 ng μL^{-1}. Considering the injection volume of 1 μL, this corresponds to 35–94 ng on-column representing an increase in mass sensitivity by a factor of approximately 30 as compared to LC systems employing a 4.6 mm column. Despite using a gradient technique high quality analyte spectra could be extracted from the recorded data by using an advanced approach for background correction as evidenced by the achieved correlation coefficients from 89 to 96% in the spectral region from 1700 to 1050 cm^{-1} when injecting 230–270 ng of analytes. Because of the simplicity of the experimental approach, it may be expected that this new type of on-line hyphenation in LC IR may find its use also in routine laboratories as opposed to off-line detection.

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(19) Takamuku, T.; Tanata, M.; Yamaguchi, A.; Nishimoto, J. *J. Phys. Chem. B* **1998**, *102*, 8880–8888.