Separations coupled with NMR detection

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Understanding contaminant fate requires knowledge of the mechanisms by which a compound is degraded, including the rate at which it is transformed as well as the structures of the various products produced. This need to identify and characterize contaminant-transformation products at the molecular level introduces a number of unique analytical challenges. Because of the high information content provided by nuclear magnetic resonance (NMR) spectroscopy, it is an attractive method for the study of contaminant transformation, especially when combined with analysis by mass spectrometry (MS). The versatility of high-performance liquid chromatography (HPLC)-NMR makes it a robust and efficient method for the identification of components of complex mixtures, such as those encountered in the study of environmental contaminants. The role of HPLC-NMR as a tool for environmental analyses is discussed and its utility is illustrated by examples from our work to understand the transformation of the fluoroquinolone antibiotic, ciprofloxacin. Recent technological advances to improve sensitivity, such as the coupling of sample concentration by capillary (cITP), allow NMR spectroscopy to play an increasingly important role in studies of the fate of emerging contaminants.

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1. Introduction

Evaluation of the environmental risks posed by a contaminant requires knowledge of its concentration, chemical and physical properties, persistence, and ultimate fate. Ideally, as contaminants degrade, compounds of reduced toxicity or activity result. However, as was learned with DDT (1,1,1-trichloro-2, 2-bis-(p-chlorophenyl)ethane) and DDE (1,1 - dichloro - 2,2 - bis(4’ - chlorophenyl)ethylene), the environmental risk posed by a contaminant is not necessarily reduced by transformation of the parent compound. Significant scientific effort has been devoted to understanding the environmental transformation mechanisms of a variety of industrial and agricultural chemicals. Recently there has been an increased concern about pharmaceutical and personal care products as environmental contaminants, generating interest in the modes of introduction of these chemicals into the environment, the mechanisms by which they are attenuated, their eventual fate and their effects on impacted ecosystems [1-6].

In order to make a realistic assessment of the impact that a contaminant may have in the environment, the mechanisms by which it is degraded must be elucidated, including the rate at which the compound is transformed as well as the structures of the various products produced. The evaluation of the subsequent fate of contaminant-transformation products is also an important component of environmental risk assessment. Even though the parent compound may not be persistent under the relevant environmental conditions, the transformation products formed may lengthen or even enhance the environmental effect of a contaminant. These needs to identify and to characterize at the molecular level contaminant-transformation products introduce a number of unique analytical challenges. Typically, transformation products are new chemical entities, for which standards are not available. Furthermore, not only must the structures of the new compounds be elucidated, the experiments that provide the necessary structural information must also be performed on complex mixtures containing the transformation products at low levels. These analytical constraints require...
sensitive, selective characterization methods that provide a wealth of structural information.

NMR has a significant advantage over many other analytical methods in that a vast amount of structural information can be gained in a single analysis while conserving the sample for subsequent interrogation with other techniques. Although NMR is less sensitive than several other popular structure-elucidation techniques (e.g., MS), the information obtained from the NMR spectrum can be sufficient to identify an unknown analyte or environmental contaminant [7]. In addition, two-dimensional (2D) NMR experiments can be used to probe atom connectivity and arrangement when additional information is required for structure elucidation. In this article, we will discuss NMR as a tool for environmental analyses and will examine the role of HPLC-NMR in conjunction with MS in studies of emerging contaminant fate.

The transformation of the fluoroquinolone antibiotic, ciprofloxacin, will be used to illustrate the application of HPLC-NMR for structure elucidation in studies of contaminant transformation. Finally, we will close with a brief discussion of technological developments on the horizon that have the potential to improve greatly the sensitivity of NMR spectroscopy and enhance its use as a tool for environmental analyses.

2. Environmental analyses using off-line NMR spectroscopy

Studies of contaminant fate can be conducted in situ by using NMR to monitor an isotope that has a relatively high natural abundance and intrinsic sensitivity, such as $^{19}$F [8, 9], $^{31}$P [10] or a specifically enriched nucleus such as $^{13}$C [11]. An advantage of this approach is that NMR analysis can be performed for complex samples without requiring a chromatographic separation. The specificity inherent in labeled compounds allows them to be used as stable-isotope tracers and can simplify the analysis, especially for complex sample matrices. Changes in the NMR spectrum, because of the loss of the parent contaminant and the sequential formation of transformation products, can be evaluated in situ with respect to the quantity and the variety of products generated. The chemical shift ranges of $^{19}$F, $^{31}$P and $^{13}$C are extensive therefore, the chemical shifts of labeled transformation products are often significantly different from those of the parent molecule and the resulting chemical shift differences can be diagnostic of the structural changes induced by the transformation reaction. While stable-isotope-tracer experiments can provide information about the rate of attenuation of the parent analyte and the variety of transformation products formed, they typically do not provide sufficient information for the complete structure elucidation of previously unidentified transformation products.

Experiments utilizing stable-isotope labels can also take advantage of the power of NMR spectroscopy as a quantitative technique, allowing for the determination of mass balance in in situ experiments. Unlike UV-visible absorption spectroscopy and MS, where quantitative analysis is possible only by comparing the analyte signal with the response obtained for a standard of the analyte, quantitative analysis can be performed with NMR using any convenient structurally unrelated reference compound containing the nucleus of interest [12]. The area of each NMR resonance is proportional only to the molar concentration of the analyte and the number of nuclei giving rise to the resonance. Quantitative analysis can therefore be performed with NMR for new or unknown analytes present in complex mixtures, as long as there are resolved signals for the analyte and the reference compound.

Unless the contaminant fortuitously contains a nucleus, such as $^{19}$F or $^{31}$P, as part of its molecular structure, a specific isotopic label, such as $^{13}$C, must be incorporated into the molecule synthetically, often at great expense. By contrast, all organic molecules contain hydrogen, making $^1$H NMR an attractive alternative. Compared with experiments detecting isotopes, such as $^{19}$F, $^{31}$P or specifically-labeled $^{13}$C, $^1$H NMR analysis of complex mixtures can be more difficult because of the narrow chemical shift range and ubiquitous presence of this nucleus. Despite these challenges, $^1$H NMR has been used to analyze mixtures of known environmental contaminants and transformation products produced by well-characterized pathways. For example, Suzuki et al. used $^1$H NMR to detect a number of low molecular-weight, water-soluble, organic compounds extracted from atmospheric particles [13]. $^1$H NMR was also used by Jensen et al. to study the formation of chloramines in wastewater treatment by examining the reaction of chlorine with the nitrogen of organic amides [14].

Because environmental transformation processes typically produce multiple products, often with closely related structures, the resulting mixture may be too complex for in situ analysis by NMR. Chromatographic separation and isolation of the analyte as a pure component can permit detailed structural analysis with NMR. For example, Haroune et al. monitored the formation of the major hydroxylated derivative of 2-amino-benzothiazole generated by *Rhodococcus rhodochrous* using both HPLC-UV and in situ $^1$H-NMR [15]. The structure of the purified metabolite was elucidated by $^1$H NMR experiments and through $^1$H-$^1$N long-range heteronuclear chemical shift correlation. However, it can be both difficult and time consuming to obtain pure compounds by chromatographic separation and fraction collection. In addition, co-isolation of contaminants
and degradation of the compound of interest are common problems faced in off-line, tube-based NMR analysis of isolated compounds. Many of these problems can be avoided and the structure-elucidation process facilitated by direct coupling of the chromatographic separation with on-line NMR detection.

3. Analysis of environmental samples with HPLC-NMR

Directly coupled chromatographic separations with NMR detection permit the on-line identification and structure elucidation of components in a complex mixture. HPLC-NMR has increased in popularity in recent years, largely because of the introduction of robust commercially available flow probes [16]. HPLC-NMR has been used extensively for the structure elucidation of natural products, drug degradation products and metabolites [16,17]. This technique can similarly be applied for the investigation of environmental transformations of organic contaminants. Table 1 summarizes published applications of HPLC-NMR for the analysis of environmental contaminants.

HPLC-NMR analysis can be configured using two different modes of operation (on-flow or stop-flow) to detect the separated analytes. During an on-flow experiment, a series of NMR spectra is acquired rapidly as the HPLC eluent flows through the NMR probe. On-flow analysis is ideal when investigating concentrated samples for which NMR spectra can be measured without extensive signal averaging. However, most samples are not sufficiently concentrated to permit analysis by on-flow experiments. In addition, lengthy 2D NMR experiments, which provide the detailed information about spin connectivity required for complete structure elucidation, cannot be acquired in the limited time available for on-flow experiments. Therefore, stop-flow experiments, in which the analyte is held in the NMR detection probe, are used for extensive signal averaging or for the acquisition of 2D NMR spectra.

Stop-flow NMR experiments can be configured in several ways. The simplest approach halts the HPLC pump at the appropriate time, stopping the flow of mobile phase and trapping the peak of interest in the NMR probe. Although this approach is simple and effective, once the HPLC pump is stopped, band broadening processes compromise the separation of peaks remaining on the column. Therefore this stop-flow approach is used to detect a single chromatographic peak from each sample injection, presenting a problem for the analysis of mass-limited samples. An alternative approach traps fractions into one of several sample-collection loops. Many peaks can be collected from a single chromatogram into a series of sample loops and transferred one at a time to the NMR. A related strategy completely decouples the separation from NMR detection by trapping chromatographic peaks on solid-phase extraction cartridges, which can be subsequently eluted with deuterated solvents for NMR analysis [18,19].

The versatility of HPLC-NMR makes it a robust, efficient method for the identification of components of complex mixtures. HPLC-NMR has been used as the primary method of analysis in several studies of environmental contamination, including the groundwater at an ammunition plant [20–22], leachate water from an industrial waste site [22,23], and wastewater from a textile company [22,24]. These studies illustrate the capability of HPLC-NMR for the identification and the quantitation of contaminants. Levensen et al. have discussed the advantages and disadvantages of HPLC-NMR and HPLC-MS and the application of these techniques for the analysis of industrial environmental contaminants [22].

The selective, sensitive and rapid analysis provided by MS generally makes it the first choice from among the techniques for detecting the presence of contaminants and their transformation products. Tandem

<table>
<thead>
<tr>
<th>Environmental sample</th>
<th>Analyte(s)</th>
<th>Analysis techniques</th>
<th>Reference number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groundwater near a former ammunition plant</td>
<td>Nitroaromatic compounds (various)</td>
<td>HPLC-PDA and Continuous-flow HPLC-$^1$H NMR</td>
<td>[20]</td>
</tr>
<tr>
<td>Groundwater near a former ammunition plant</td>
<td>Phototransformation products of TNT</td>
<td>HPLC/Thermospray-MS, Continuous-flow HPLC-$^1$H NMR</td>
<td>[21]</td>
</tr>
<tr>
<td>Leachate from an industrial waste-disposal site</td>
<td>Aromatic carboxylic and sulfonic acids and other unidentified analytes</td>
<td>HPLC-PDA, Continuous-flow HPLC-$^1$H NMR</td>
<td>[23]</td>
</tr>
<tr>
<td>Aquatic effluent from a textile company</td>
<td>Various compounds, anthraquinone-type dyes</td>
<td>Stopped-flow HPLC-$^1$H NMR, HPLC-MS/MS</td>
<td>[24]</td>
</tr>
<tr>
<td>Laboratory-scale surface water transformation study</td>
<td>Ciprofloxacin (antibiotic), and transformation products</td>
<td>Stopped-flow HPLC-$^1$H NMR, HPLC-MS/MS</td>
<td>[27]</td>
</tr>
<tr>
<td>Development of LC-NMR method for analysis of polycyclic aromatic hydrocarbons in soil</td>
<td>16 polycyclic aromatic hydrocarbons that can be found in soils</td>
<td>GC-MS, HPLC-DAD, HPLC-Fluorescence and Continuous-flow HPLC-$^1$H NMR</td>
<td>[50]</td>
</tr>
</tbody>
</table>
mass spectrometry (MS/MS) is used to generate molecular ion fragments, which are derived from key structural features to create an overall picture of the intact molecule. However, especially when standards are not available, MS/MS cannot always provide sufficient information for complete elucidation of the structure of an analyte. In addition, MS can suffer from reduced sensitivity because of poor ionization inherent in the structure of the analyte or suppressed ionization resulting from matrix effects that arise from the complex natural organic matter found in many environmental samples [25,26]. As a result, the use of NMR and MS analysis in parallel [27] or, when possible, in tandem [28,29] is an extremely powerful approach for the investigation of contaminant fate.

4. Structure elucidation of ciprofloxacin-transformation products

Antibiotics pose an environmental risk through their potential to increase the incidence of antibiotic resistance in non-target bacteria, as well as through negative impacts on algae and other microorganisms that form the foundation of the food web [30,31]. Studies of antibiotic fate must determine both the rate at which the antibiotic is attenuated and the identity of the transformation products produced, since many of the known metabolites and the degradation products of antibiotic drugs retain a significant degree of antimicrobial activity [32]. Furthermore, since a primary mode of entry of antibiotics into the environment is through human and agricultural waste streams, waters that receive these waste streams may experience an essentially continuous dose of contaminants, even though the antibiotics may be diluted and/or transformed further downstream. For the past two years, we have been engaged in a study of the fate and effects of fluoroquinolone antibiotics in aquatic ecosystems. A key component of this work has been the development of an analytical strategy employing HPLC-NMR and HPLC-MS/MS to study the environmentally relevant transformation pathways of the fluoroquinolones. Although this research is still work in progress, our preliminary results for a popularly prescribed fluoroquinolone antibiotic, ciprofloxacin, illustrate the power of HPLC-NMR for the study of contaminant-transformation processes.

Ciprofloxacin (Fig. 1A) is a broad-spectrum synthetic antibiotic often prescribed for the treatment of urinary tract and respiratory infections, including anthrax exposure [33]. Ciprofloxacin is prescribed in high doses (≥1000 mg/day) of which a large fraction (40–70%) is excreted unmetabolized. As a result, like many other pharmaceutical contaminants, ciprofloxacin can enter the environment through human-waste streams. Ciprofloxacin has been detected in hospital wastewater [34] and in trace amounts in surface waters [35]. Although little is known about the environmental fate of ciprofloxacin, its metabolic fate in humans has been extensively investigated and has also been studied in various fungi [36,37].

Our strategy for studying the fate of aquatic contaminants, such as ciprofloxacin, has been to employ both laboratory-scale studies (i.e. in 2-L flasks under carefully controlled conditions) and field simulations conducted in a “quasi-natural” setting using mesocosm environments (3.2 m by 1.4 m cylindrical, fiberglass tanks placed in a pond ecosystem) [38]. Laboratory-scale experiments using natural waters obtained from a protected pond or lake source are often conducted at concentrations higher than those expected in the environment. The high concentrations are necessary to provide sufficient material for structure-elucidation experiments using NMR and MS/MS. These laboratory-scale simulations provide preliminary information about the attenuation rate and give insight into the transformation pathways that may be observed during a larger-scale mesocosm experiment. Ultimately though, to achieve an accurate picture of its true environmental fate, the fate of the organic contaminant must be evaluated at concentrations similar to those expected in the environment. Once the structures of the transformation products are known from the laboratory-scale experiments, the transformation rate and the fate of an organic contaminant can be evaluated using mesocosm experiments conducted at environmentally realistic concentrations with analysis by HPLC-MS. An additional advantage of mesocosm experiments is that the environmental conditions can be manipulated to simulate aquatic ecosystems, ranging from those of an isolated pristine glacial lake to the receiving waters of a eutrophic agricultural pond [38]. This feature is important, as contaminant-transformation mechanisms may depend on the concentration and the type of aquatic natural organic matter (humic and fulvic acids), the presence of suspended particulates and the characteristics of the microbial community.

The results presented below were generated in laboratory-scale experiments using relatively high

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**Figure 1.** Structure of fluoroquinolone antibiotic ciprofloxacin (A) and transformation product TP-330 (B).
concentrations of ciprofloxacin in pond water [38]. Even though the concentrations used in these laboratory simulations are higher than those observed in the environment, a sample-concentration step is still necessary for analysis using HPLC-NMR. Solid-phase extraction with C-18 cartridges was used to isolate and concentrate ciprofloxacin and its transformation products from the acidified samples [39,40]. Previously reported ciprofloxacin transformation products were identified using HPLC-UV/MS. Chromatographic peaks that could not be identified from the HPLC/MS results were the focus of further experiments using a Varian 600 MHz NMR spectrometer equipped with a HPLC flow-probe interfaced to a Varian 230 HPLC with a 330 photodiode array (PDA) UV-visible absorption detector. The NMR flow probe has an observe volume of 60 μL, although the total volume of the detection cell is 120 μL. As concentrations permitted, 2D NMR experiments were performed to facilitate the structure-elucidation process. The relative polarity (judged by chromatographic retention time), molecular weight, MS/MS fragmentation patterns along with the spectral information obtained from NMR experiments were used to arrive at transformation-product structures that are self-consistent with respect to all the available experimental results.

Preliminary analyses were performed for ciprofloxacin to provide baseline data for comparison with results obtained for the transformation products. A static 1H-NMR spectrum (Fig. 2) of ciprofloxacin (approx. 1000 ppm) was measured in a solution containing 79% 0.05% trifluoroacetic acid in D₂O and 21% CD₃CN, a solvent system similar to that used to separate the transformation products. The structure of ciprofloxacin is rather simple with respect to the number and the type of protons, so many of the resonances can be assigned by simple inspection of chemical shifts and relative intensities. Homonuclear spin-spin coupling is observed for the aliphatic protons in the cyclopropyl (H₆, H₇ and H₈) and piperazine rings (H₁₁ and H₁₂,2'). The doublets observed in the aromatic region result from spin coupling between the two aromatic protons (H₃ and H₄) and the fluorine atom. The MS/MS spectrum of ciprofloxacin ([M+H]⁺, 332 m/z) was acquired using a Quattro Ultima (Micromass, Manchester, UK) triple quadrupole instrument with an electrospray ionization (ESI) source. Significant fragments observed reflect the loss of [H₂O+HF] as well as numerous other combinations involving loss of water plus fragments of the piperazine ring and loss of the cyclopropyl group.

The transformation of ciprofloxacin (250 ppb) in pond water was initially studied in the laboratory under fluorescent lights cycled diurnally. The solution was monitored with HPLC-UV/MS to evaluate the attenuation rate and to monitor the formation of transformation products. After 126 hours, several

Figure 2. The static 600 MHz 1H-NMR spectrum of ciprofloxacin in D₂O/CD₃CN measured in a 5 mm NMR tube. The inset shows an expansion of the aromatic region of the spectrum.
transformation products could be observed in the HPLC-UV chromatogram, including TP-330 and TP-270, which will be used to illustrate the utility of HPLC-NMR in the structure-elucidation process.

Because of the low concentration at which TP-330 was present in the initial transformation reaction, it was necessary to perform an experiment at higher ciprofloxacin concentration (5000 ppm) to obtain sufficient TP-330 (Fig. 1B) for NMR analysis. Although a large fraction of the ciprofloxacin was not transformed (as revealed by the UV chromatogram), the amount of TP-330 present was adequate to obtain an HPLC-NMR spectrum in 21 minutes. Comparison of the $^1$H NMR spectra of ciprofloxacin (Fig. 2) and TP-330 (Fig. 3) reveals no significant changes in chemical shifts, coupling or number of aliphatic protons (0.5–6 ppm), indicating that the piperazine and the cyclopropyl rings remain intact in the transformation product. However, major differences are clearly observed in the aromatic region (7–10 ppm) of the spectrum. The loss of the $^{19}$F coupling observed for TP-330 must reflect the loss of fluorine. We hypothesized that the loss of fluorine resulted from substitution with a hydroxyl group to yield a product with a molecular ion of 330 (332 $^{19}$F $^{+}$). This structure was confirmed by tandem MS. Comparison of neutral losses in the MS/MS spectra of ciprofloxacin and TP-330 revealed that the signature ion indicative of the combined loss of H$_2$O and HF observed for ciprofloxacin was missing in the mass spectrum of TP-330. Once the structure of TP-330 was realized, a literature search for this compound revealed that it had been previously reported as both a ciprofloxacin metabolite of the brown rot fungus Gloeophyllum striatum and a photodegradation product of ciprofloxacin in aqueous solution [37,41]. However, in addition to TP-330, several transformation products that have not been reported in the literature, including TP-270, were also observed and are the subject of current investigation in our laboratory.

The molecular ion [M+H]$^+$ of TP-270 was determined to be 270 m/z, 62 mass units smaller than ciprofloxacin. Earlier on-line $^1$H-NMR analysis of TP-270 contained a number of compounds that co-eluted with this analyte. As a result, the transformation reaction was periodically monitored by HPLC-UV, allowing the sample to be extracted at a point in the reaction when TP-270 was the primary transformation product, yielding a relatively pure sample. However, because of the low concentration of TP-270 in this solution, isolation of sufficient quantities of TP-270 for HPLC-NMR analysis required SPE of 5 liters of sample.

Comparison of the $^1$H-NMR spectra (Fig. 4) of TP-270 measured in D$_2$O and CD$_3$CN with those of TP-330 and ciprofloxacin suggests that, while TP-270 still contains the cyclopropyl ring, the piperazine ring has been cleaved. In the aliphatic region the CH$_2$ peaks of the cyclopropyl ring are clearly visible; however, the CH resonance (4.5 ppm) appears as a shoulder on the HOD solvent peak. This dramatic 0.7-ppm downfield shift suggests that the cyclopropyl CH group in TP-270 is

![Figure 3](http://www.elsevier.com/locate/trac)
near a significant point of transformation. The assignment of the cyclopropyl CH chemical shift was confirmed by observation of a cross peak to the CH2 resonances in the 1H-1H correlation spectroscopy (COSY) spectrum. In addition, a prominent ethyl group was also observed in this spectrum (Fig. 4). This ethyl group is due to propionitrile (CH₃CH₂CN), a common acetonitrile impurity often observed in HPLC-NMR spectra of dilute analytes. The aromatic region of the TP-270 NMR spectrum (Fig. 4) shows distinct differences from the ciprofloxacacin spectrum (Fig. 2). In TP-270, the aromatic doublets are shifted downfield and have the same coupling constant, suggesting homonuclear coupling between these protons, which was confirmed by the presence of cross peaks in the COSY spectrum. The MS/MS spectrum of TP-270 supports the loss of both fluorine and the piperazine ring while preserving the cyclopropyl moiety.

The true complexity and diversity of products produced by ciprofloxacacin degradation would have been greatly underestimated if the transformation reaction had been monitored by in-situ ¹⁹F NMR. At least two of the major transformation products, TP-330 and TP-270, would not have been detected in such an experiment because they no longer contain fluorine. The application of in situ ¹H NMR would have generated many overlapping peaks because of the large number of transformation products generated. With resolution of the solution components by HPLC-NMR, simple comparison of the ¹H-NMR spectra of the transformation products with that of the parent pharmaceutical allowed the detection of subtle changes in molecular structure by evaluating changes in the number of resonances, chemical shifts and coupling constants. These spectral differences reflect the features of the parent contaminant that have been retained, lost or altered upon the formation of the transformation product. As demonstrated here, the combination of information provided by HPLC-NMR and HPLC-MS/MS together facilitate structure elucidation even without access to standards of the transformation products.

5. Future prospects: sensitivity enhancements

Even with the power of signal averaging, the quantity of analyte available is often too small to permit analysis by standard HPLC-NMR instrumentation. The NMR results reported here were measured with a 600 MHz (14 T) NMR spectrometer, a relatively high-field instrument. However, the state-of-the-art is currently 900 MHz with increasingly available access to 800 MHz instruments. Because NMR sensitivity increases with magnetic field scaled to the 7/4 power, performing measurements at higher fields translates directly into lower detection limits or reduced experiment times. The use of a cryo-flow probe, which has recently become commercially available, also provides enhanced
sensitivity with an increase in signal-to-noise up to a factor of four when compared with a conventional NMR detection probe [29,42,43]. The development of solenoidal microcoil probes with nanoliter to microliter detection volumes has greatly enhanced the mass limits of detection of NMR. These probes are especially useful for analysis of samples in conjunction with capillary HPLC or capillary electrophoresis [44–46]. Another recent innovation that shows promise for analysis of ionic environmental contaminants is the coupling of cITP for sample concentration and nanoliter microcoil NMR probes for on-line detection [47].

In a cITP separation, the sample matrix containing the dilute, charged analytes is sandwiched behind a leading electrolyte (LE), which has a larger electrophoretic mobility and ahead of a trailing electrolyte (TE), which has a smaller electrophoretic mobility. Application of an electric field across the capillary not only separates the components of the sample based on their electrophoretic mobilities, but also concentrates the analytes by 2–3 orders of magnitude, greatly enhancing the NMR analysis [48,49]. Fig. 5 shows the basic instrumental configuration for cITP NMR. The cITP capillary can be threaded through a sleeve, around which the copper microcoil is wrapped. The bottle, through which the capillary passes, houses the microcoil in a fluorocarbon fluid with a magnetic susceptibility near that of copper; this is important for producing narrow NMR peaks. The LE and TE buffer solutions and high-voltage connections are made outside the superconducting magnet. Samples can be injected hydrodynamically or using a syringe pump to draw solutions into the capillary. In the typical mode of operation, the sample is allowed to focus just outside the NMR detection coil and is drawn into the observed region by electrophoretic flow. Fig. 6 shows how cITP can enhance the ability of NMR to detect small volumes of dilute analytes. Fig. 6 is a stacked plot of NMR spectra acquired in real time during the cITP separation as the LE, the focused analyte band and the TE sequentially pass through the NMR microcoil and are detected. In this separation, the LE was 160 mM tetramethylammonium acetate at a pH of 4.76. A 22 μL sample of 200 μM atenolol [4.4 nanomoles] in 50% TE and 50% D₂O was injected into the capillary. The TE was 10 mM acetic acid. Tertiary butyl alcohol (tBuOH) was added to all solutions at a concentration of 50 mM as an internal quantitation standard, chemical shift and line shape reference.

On application of the 10 kV separation voltage, the cITP band begins to focus outside the microcoil and slowly flows through the coil, allowing the detection of focused components. The rate at which the focused sample band moves depends on the electrophoretic mobility of the species as well as how effectively the capillary modifications reduce the electroosmotic flow profile of the capillary walls. Each spectrum was acquired in 26 s by coaddition of 16 scans using a 360 MHz Bruker AM NMR spectrometer and a 16-turn solenoidal microcoil probe. As shown in Fig. 6, the bottom two spectra contain the signals of only LE. The next two spectra contain resonances of the focused sample band. By the time the top spectrum was acquired, the sample had moved out of the detection

![Figure 5. A schematic of the instrumental configuration for on-line cITP-NMR.](image)

![Figure 6. A stacked plot showing the results of a cITP-NMR experiment to focus and to detect atenolol (resonances denoted with an A). Each spectrum was measured in 26 s with a Bruker 360 AM NMR spectrometer using identical NMR parameters. The atenolol band focused outside the NMR microcoil and flowed slowly through the active volume of the probe allowing detection of the 1H NMR spectrum of the cITP concentrated sample.](image)
region, leaving behind only the TE. In the middle spectrum containing the most intense peaks of the sample, the concentration of the atenolol was 58 mM, reflecting an increase of nearly 300-fold as a result of the cITP stacking.

The high information content of NMR experiments makes this an attractive technique for the analysis of contaminant-transformation processes, especially when coupled with a separation method. Because of the complementary nature of the results provided by each technique, the combination of NMR and MS/MS analysis is an especially powerful approach for elucidating the structure of new transformation products. Recent technological advances in probe technology and the continued advance to higher magnetic field strengths, both of which significantly improve sensitivity, will result in an increasingly important role for NMR spectroscopy in studies of the fate of emerging contaminants.

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