

Real-Time 2D NMR Identification of Analytes Undergoing Continuous Chromatographic Separation

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Abstract: We have recently proposed and demonstrated an approach that enables the acquisition of multidimensional nuclear magnetic resonance (NMR) spectra within a single scan. A promising application opened up by this new accelerated form of data acquisition concerns the possibility of monitoring in real time the chemical nature of analytes subject to a continuous flow. The present paper illustrates such potential, with the real-time acquisition of a series of 2D ^1H NMR spectra arising from a mixture of compounds subject to a continuous liquid chromatography (LC) separation. This real-time 2D NMR identification of chemicals eluted minutes apart under usual LC–NMR conditions differs from the way in which LC–2D NMR has hitherto been carried out, which relies on stopped-flow modes of operations whereby fractions are first collected and then subject to individual, aliquot-by-aliquot analyses. The real-time LC–2D NMR experiment hereby introduced can be implemented in a straightforward manner using modern commercial LC–NMR hardware, thus opening up immediate possibilities in high-throughput characterizations of complex molecules.

Introduction

Contemporary drug discovery relies on efficiently separating and identifying mixtures of pharmacologically active compounds. Best suited for the resolution of such complex mixtures is HPLC, a procedure which though simple and efficient yields little insight into the chemical nature of the compounds being separated. This has led to the development of so-called hyphenated methods, whereby the resolving power of chromatography is interfaced with the structural insight provided by a variety of sophisticated methods of detection.¹ Chief among the physical techniques underlying these detectors have been UV, IR, MS, and NMR spectroscopies. When considering their interphasing to an LC setup the first three of these methods possess unambiguous advantages over NMR, thanks to their superior sensitivities and small limits of detection. NMR spectroscopy, on the other hand, has long stood in a class of its own as the preeminent tool for identifying the structure of organic and biochemical molecules, including pharmaceuticals and natural products. Further, continuous hardware advances have increased the sensitivity of NMR to the point where sub-milligram amounts of medium-weight molecules can be routinely analyzed.² During the past decade LC–NMR and LC–NMR–MS hardware has thus become commercially available from all main NMR spectrometer manufacturers, and these procedures are becoming increasingly routine in pharmaceutical laboratories worldwide.

On considering what kind of NMR procedures to implement on analytes subject to LC separation, two main options are

available. One of these entails collecting a series of 1D spectra, usually involving ^1H NMR observation, as the analytes become eluted from the column. Since these NMR experiments are relatively fast they can be directly executed as chemicals eluted out of the column; this results in a retention-time-resolved NMR progression that becomes available as soon as the LC run has concluded. Alternatively the NMR analysis can be carried out in stop-flow mode, whereby fractions which have eluted from the LC column are individually collected and then inserted one by one into the NMR spectrometer for their individual analysis. Sacrifices are made by this stopped-flow procedure on two main accounts: one is the need for a more expensive, usually robot-driven, hardware setup; the second is the involvement of a much longer analysis time that precludes real-time visualization of the LC separation. Such a stop-flow mode is still often preferred over the on-line procedure because it enables the implementation of 2D homo- and heteronuclear NMR analyses on the eluted analytes. 2D NMR spectroscopy in turn opens up new vistas toward the elucidation of the chemical and structural characteristics of organic and pharmaceutical molecules that are simply unavailable in conventional 1D NMR. This often makes the penalties associated with the stop-flow operation worth paying.

The reason eluted analytes need to spend longer times inside the spectrometer when 2D NMR experiments are to be recorded resides in the overall design of this form of spectroscopy. Indeed regardless of the kind of 2D experiment involved in the LC analysis, all such sequences will collect their data by relying on the well-known paradigm³ preparation/excitation–evolution (t_1)–mixing–detection (t_2), whereby an evolution time t_1 is in charge of encoding an initial interaction frequency Ω_1 ,

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and the acquisition time t_2 encodes a final coupling Ω_2 . This final time t_2 refers to the physical data acquisition time usually involved in 1D Fourier spectroscopy, while the initial time t_1 is a variable parameter inside the NMR pulse sequence, of which numerous independent values are needed for a proper Fourier decoding of the Ω_1 interactions. As each of these t_1 increments corresponds in essence to an independent 1D NMR experiment, it follows that long residence times in the NMR coil (and thus off-flow operation) are needed even when dealing with systems possessing abundant signal-to-noise ratios. Very recently, however, we have proposed an alternative yet general route to acquiring nD NMR data which, unlike the traditional multidimensional NMR scheme, can be implemented within a single scan.^{4,5} This approach is based on replacing the indirect-domain temporal encoding of conventional nD NMR by a spatial one, imparted on the whole sample at once with the aid of oscillating magnetic field gradients applied in synchrony with a series of spatially selective excitation or refocusing pulses. Ample details describing the principles of this new technique as well as its practical implementation have been reported in recent publications, and will thus not be repeated here. The purpose of the present contribution is to exemplify what might eventually become an important application of these ultrafast NMR techniques; namely, the on-line characterization of analytes flowing out of a chromatographic column via real-time 2D NMR methods.

Materials and Methods

The LC–2D NMR results described in this work were measured using a Bruker Avance 500 MHz NMR spectrometer. As no LC–NMR facility was available on this particular instrument, a setup was constructed capable of recreating the typical conditions associated with LC procedures in commercial apparatuses. Toward this end, a 5 mm triple-resonance inverse Bruker probehead endowed with a longitudinal z -axis gradient was opened and modified to enable LC–NMR operation following the guidelines described by Barjat et al. for a similar Varian NMR probe.⁶ The main provision entailed in this modification is the installation of a cell centered at the position of the spins' irradiation/detection radio frequency (rf) coil insert, endowed with suitable in- and out-flow connections that couple to the chromatographic and to the waste collection systems, respectively (Figure 1). The flow cell in our study was made from of a commercial 3 mm outer diameter (OD) NMR tube cut to a 7 cm overall length and glued with epoxy to in- and out-flow connections machined out of Teflon. To implement the injection and ejection of chemicals through this flow cell, $1/16$ " OD tubing assemblies were employed coming through the probe's thermocouple aperture on its way to the coil and out through the upper bore of the magnet on its way out of the coil. All connections within these tubing assemblies were implemented using conventional HPLC plastic couplers. As pointed out by Barjat et al. in their original work, the overall NMR performance of the resulting setup is very satisfactory, leading in our case to $6.1 \mu\text{s } \pi/2$ pulses, $G_z \approx 60 \text{ G/cm}$ maximum z -field gradients, and half-widths of less than 3.5 Hz under routine lock-less flow operation. Further, no significant penalties were introduced by this setup into the quality of the chromatographic separation.

As the goal of the system that we set up was to test the performance of ultrafast 2D NMR experiments under conventional LC NMR conditions rather than to implement an actual chemical analysis on an unknown sample, a pressurized silica-based glass column was chosen

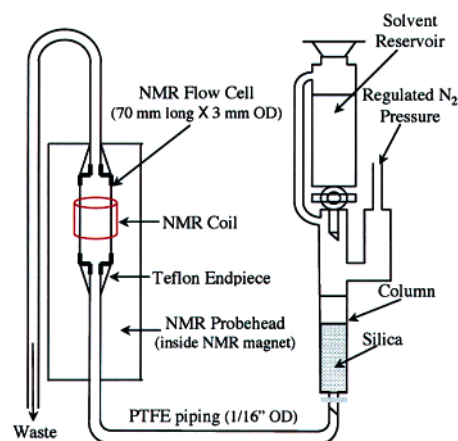


Figure 1. Scheme of the experimental setup employed to corroborate the possibility of carrying out real-time 2D NMR characterizations during the course of a chromatographic separation. Pointed out are the main additions made to our standard NMR spectroscopy hardware for the sake of recreating conventional on-flow LC–NMR conditions.

over more sophisticated HPLC-based alternatives for the sake of recreating the chromatographic conditions. Silica gel (10 cm) 230–400 mesh was thus loaded on a 2.5 cm OD column, and the eluent flowed through the whole system at a rate of ca. 6 mL/min using a regulated pressure source set at ~ 0.17 bar. Given the $100 \mu\text{L}$ active volume of the flow cell that was constructed, this implied that residence times of ca. 1 s characterized the passage of chemicals through the length of the rf coil; this residence time is typical of that in commercial LC–NMR systems and, being much longer than the usual mixing times or gradient-echo times involved in J-based ultrafast 2D NMR experiments (~ 30 ms and $300 \mu\text{s}$, respectively), had no effects on the actual outcome of the latter.

To facilitate the recording of background-free ^1H NMR spectra, HPLC-grade carbon tetrachloride (Aldrich) was chosen as the mobile phase of our experiments. This nonprotonated solvent only required the suppression of a minor CHCl_3 impurity peak, implemented via low power presaturation pulses over the course of the recycling delay. Our reliance on this highly nonpolar eluent and on a silica column as the stationary phase implied that few chemicals could actually be subject to a chromatographic separation that would afford elution times comparable to those arising in a preparative HPLC–NMR separation. Three compounds that were found to fulfill these conditions and were chosen for this test analysis are shown in Figure 2; also shown in the figure are basic chromatographic and ^1H NMR parameters for the compounds, as well as typical single-scan 2D TOCSY ^1H NMR spectra⁷ collected for each of these compounds within our LC–NMR probehead. The actual chromatographic runs that were then implemented involved seeding 30 mg of each of these compounds dissolved in $750 \mu\text{L}$ of CCl_4 , leading to an average concentration for each of the chemicals being eluted throughout the LC–NMR cell of 15–20 mM.

As for the ultrafast 2D NMR experiments themselves, these were run using the scheme illustrated in Figure 3 and involving the following parameters: a conventional 30 ms long WALTZ-based mixing period, $N_1 = 44$ rectangular excitation pulses spread 5 kHz apart and lasting $200 \mu\text{s}$ each, $\gamma_e G_e = 137 \text{ kHz/cm}$, $\gamma_a G_a = 74 \text{ kHz/cm}$, $T_a = 225 \mu\text{s}$ plus a gradient switching time of $25 \mu\text{s}$, $5 \mu\text{s}$ physical dwell times, and $N_2 = 128$ cycles along the direct domain. Although such parameters afforded what in essence amounted to be an independent 2D ^1H NMR TOCSY spectrum per scan, the fact that minute-long retention times were involved was also exploited and eight phase-cycled scans separated by 4 s recycle delays were averaged for the sake of increasing the signal-to-noise ratio. This yielded a series of 2D NMR spectra separated by

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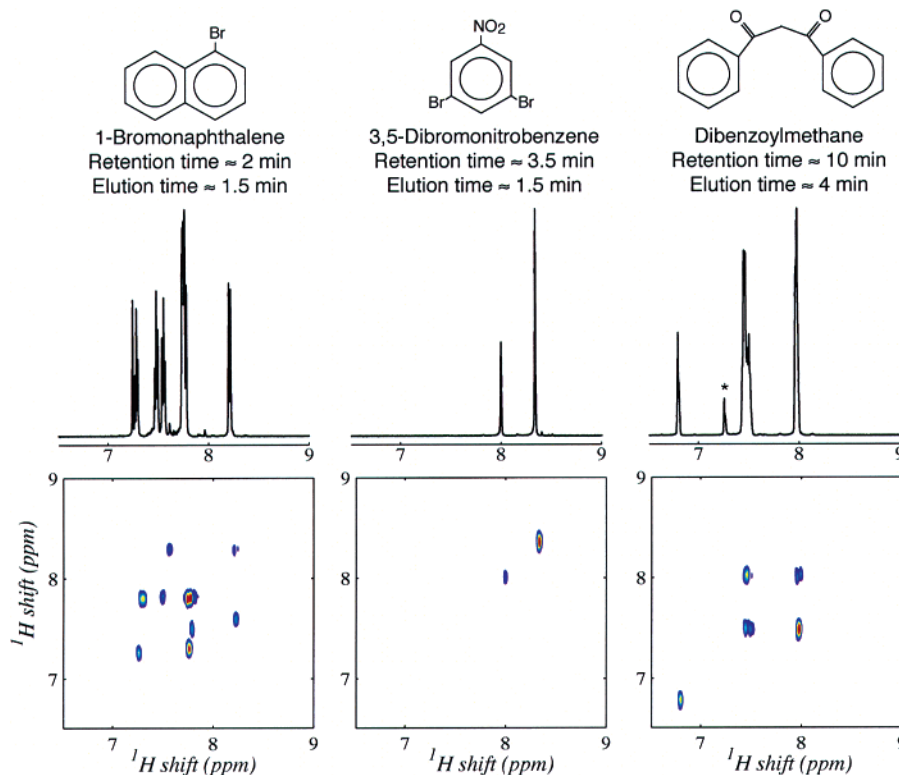


Figure 2. Relevant characteristics of the compounds chosen to illustrate the feasibility of carrying out real-time LC–2D NMR characterizations. Shown are the chromatographic properties of the compounds on being subject to the conditions described under Materials and Methods, as well as their conventional 1D ^1H and single-scan 2D TOCSY NMR spectra recorded in the chromatography's solvent. The conditions used for these 2D acquisitions were similar to those implemented during the chromatography run, except for the fact that they were recorded off-flow. Marked with an asterisk is a residual arising from a CHCl_3 impurity that was not presaturated; such a residual became noticeable also during the course of the chromatography runs owing to imperfections in the presaturation procedure upon flowing the sample.

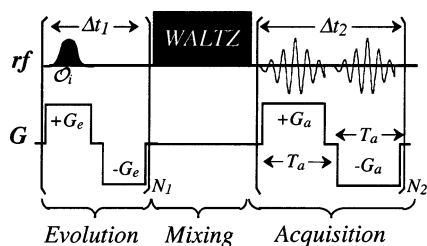


Figure 3. Generic scheme of the ultrafast 2D TOCSY NMR experiments carried out in this study. As described elsewhere in more detail, these single-scan acquisitions are based on an initial spatial encoding of the indirect evolution frequencies by a train of N_1 rf excitation pulses applied at frequency offset increments $\Delta O = |O_{i+1} - O_i|$, a conventional mixing period (in this case entailing a WALTZ sequence), and a final train of multiple (N_2) decoding gradient echoes during the course of the acquisition time t_2 .

a little over half a minute; 30 such 2D TOCSY spectra were collected consecutively over the course of a chromatographic run.

Results and Discussion

Figure 4 presents a typical series of results observed throughout the numerous experiments carried out for testing the feasibility of real-time LC–2D NMR characterizations. It displays a series of 2D TOCSY ^1H NMR spectra acquired within a 15 min time span, showing the characteristic patterns observed as the different analytes elute out of the chromatographic column and into the NMR rf coil. The time and spectral resolution of the resulting chromatogram are very encouraging, as they appear suitable from both an analytical and a structural perspective. In fact it was found that sufficient sensitivity was available to observe the 2D NMR spectra of these flowing compounds within

very few scans, essentially one. Still, averaging eight such scans increased the quality of the data without compromising the chromatographic separation and provided a smaller number of 2D NMR spectra that were thus easier to process. The details of this processing were essentially as those described elsewhere for conventional ultrafast 2D NMR experiments;⁵ such procedure essentially involved zero-filling and weighting of the data along both the indirect k/ν_1 and direct t_2 domains, a single-dimensional Fourier transform along the direct dimension, and subsequent contour plotting. As all chromatogram plots were carried out on data after these had been converted to magnitude-mode the overall processing could be easily automated, a feature we expect will facilitate further the applicability of this approach. For the purpose of the present study, all necessary processing and plotting routines were incorporated into a single Matlab 6.5 (The MathWorks Inc.) software program.

The main objective of the present study was to demonstrate the feasibility of exploiting ultrafast NMR's main feature, namely its decrease of nD NMR acquisition times by orders of magnitudes, in order to follow chromatographic separations in real time. It would have been our choice to test this possibility using commercial high-field equipment incorporating state-of-the-art HPLC columns, deuterated solvents, solvent-gradient pumps, better solvent suppression sequences, etc. Still, by choosing proper column-chromatography conditions, we believe that the more modest low-pressure, isocratic setup employed in the present study also conveyed a realistic feeling for the prospects opened up by this type of on-flow analysis. Particularly important in this respect was mimicking the actual elution

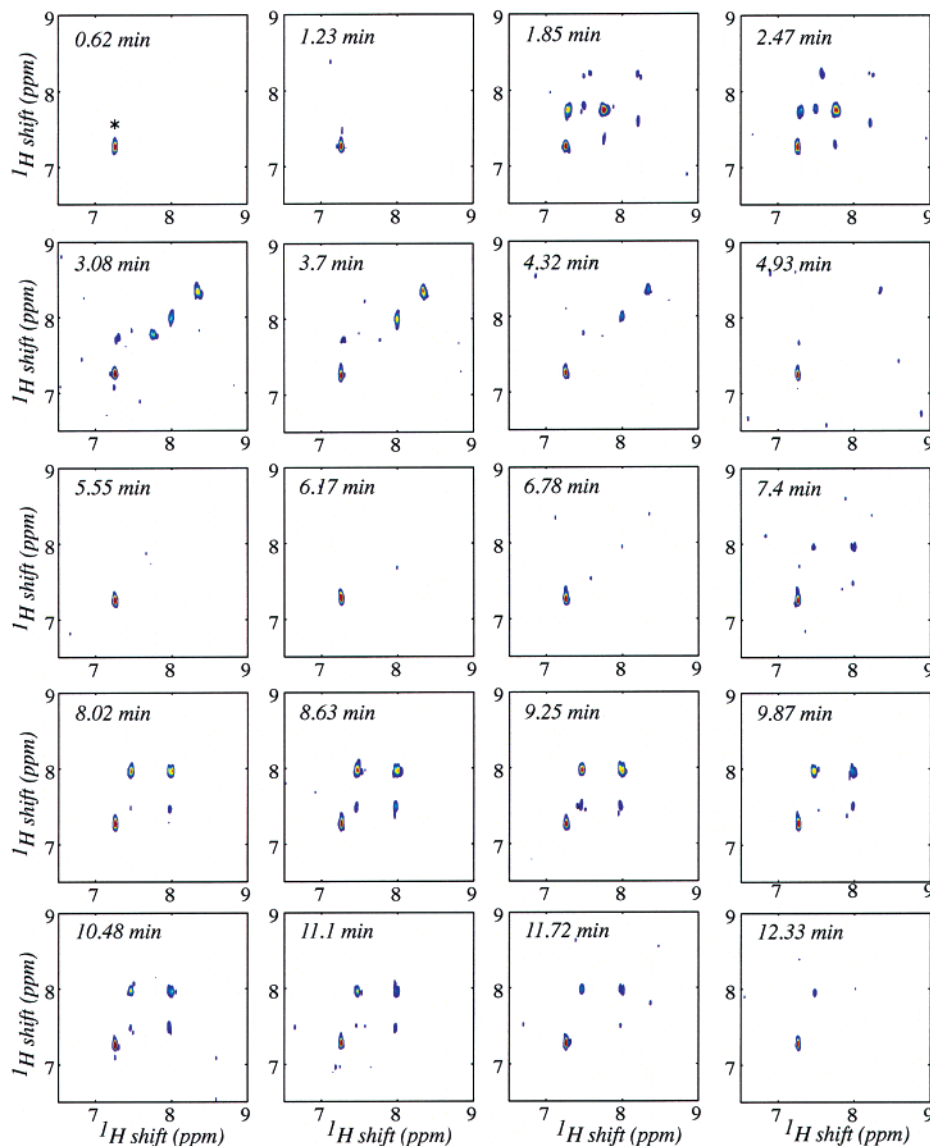


Figure 4. Real-time 2D TOCSY ^1H NMR spectra recorded on the mixture of compounds detailed in Figure 2. Chromatographic and spectroscopic parameters were as described in the main text; data were zero-filled to 128×128 matrices before their final Fourier processing and plotting. As spectra were recorded ca. 37 s apart, the times indicated in each frame correspond to the approximate minutes elapsed since the seeding of the sample. The peak arising in all frames at around 7.3 ppm (marked with an asterisk in the first spectrum) corresponds to an incompletely suppressed CHCl_3 impurity.

times, flow rates, and to some extent the analyte concentrations usually associated with commercial LC–NMR setups. Under such conditions, the application of the ultrafast LC–2D NMR protocol resulted in truly straightforward results, particularly when considering that the relatively long elution times of the compounds (~ 1 min) allowed for the averaging of a number of scans and thereby the implementation of phase cycling and other artifact-suppression schemes.⁸ Further sensitivity gains can also be expected from operating at higher fields than the one employed in this study or from using cryogenically cooled probes;⁹ given the minute-long time scales associated with the elution of analytes, the incorporation of additional field gradients enabling the acquisition of higher-dimensional ($\geq 3\text{D}$) NMR

spectra is also a possibility worth exploring.¹⁰ Research along these various avenues is currently in progress.

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