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Benchtop NMR Coupling to Liquid Chromatography

Structural Analysis in Liquid Adsorption Chromatography (LAC)

In this application note, we continue our discussion and illustrations of the coupling of benchtop NMR to liquid chromatography. Previously, we demonstrated how to setup benchtop NMR for coupling to existing HPLC system for applications of size exclusion chromatography (SEC), and gave some example applications and approaches for data analysis. [Link] We now consider a different separation mode known as liquid adsorption chromatography (LAC), commonly applied for the separation of small molecules. The main distinction for NMR detection is that the analytes are typically completely separated, so that ^{1}H spectra can be acquired on essentially pure compounds against a solvent background of the continuously flowing eluent. Some published articles on benchtop LAC-NMR outline some of the approaches used for sensitivity improvement, as well as the development of more advanced detection and processing methods.[1-4]

Background

The most common mode used in LAC is the so-called reverse-phase, and this is the basis of discussion in this note. Here, small molecules are separated according to how strongly they interact with a more-or-less hydrophobic stationary phase; the interaction is essentially an energetic process, which distinguishes this mode from SEC, which is primarily an entropic process. The most common stationary phase consists of silica particles coated with an organic 'bonded phase' (**Figure 1**). The bonded phase is created by covalently attaching aliphatic chains to the silica particle surface, which both covers up unwanted silica interaction sites and provides a hydrophobic surface 'phase' for the desired interaction. The silica particles (~5 μm) are packed into separation columns under high pressure to produce a stable stationary phase with high surface area. The columns are readily available on the market and can be purchased ready-made with varying bonded phases to adjust their selectivity relative to the analyte. The mobile phase consists typically of two miscible solvents, one polar (often H_2O with buffer) and one organic (e.g. acetonitrile, methanol, THF, etc). The binary mobile phase is pumped through the column under high pressure and the dissolved analyte mixture is injected as a plug at the head of the column. The molecule's interaction with the column determines its retention within the column, and the elution is generally in the order of decreasing polarity. As the organic solvent component competes with the analyte for interaction on the stationary phase, one adjusts the mobile phase composition to fine-tune the analyte retention. The goal is to separate all the analytes into completely resolved peaks within the shortest possible time.

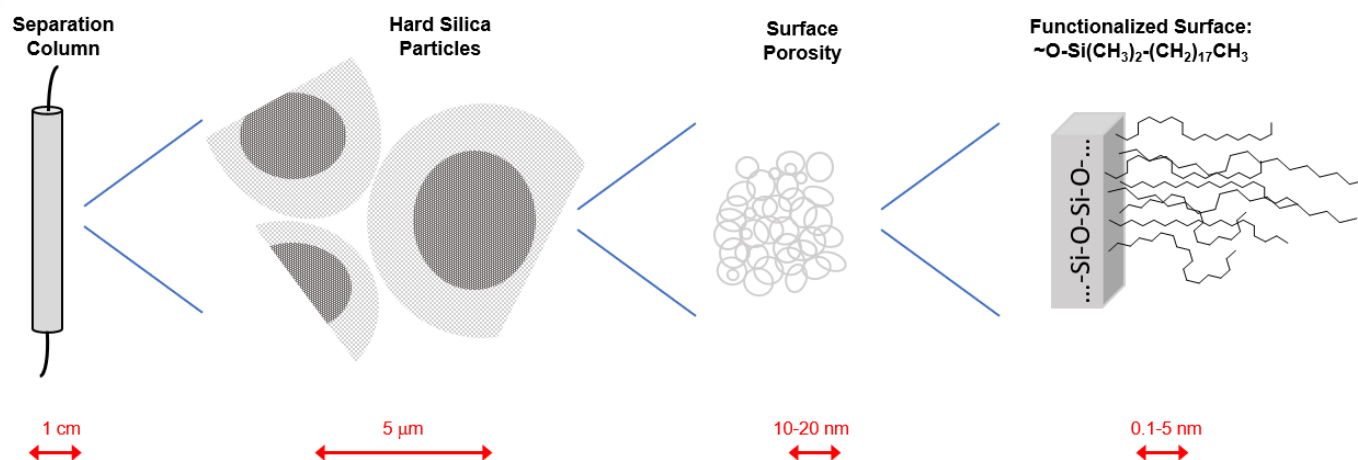


Figure 1. Length scales involved in LAC separation: separation column (1 cm), stationary phase silica particles (5 µm), surface porosity (10-20 nm), and chemical structure of bonded phase (0.1-5 nm).

In Figure 2, a separation of a mixture of alkyl esters of *para*-hydroxybenzoic acid (parabens) on C_{18}-bonded columns is used to illustrate a typical LAC chromatogram. With increasing alkyl length of the ester, the elution time also increases, owing to the more hydrophobic character of the molecule and therefore stronger interaction with the column's stationary phase. A mass-proportional detector is used for detecting each paraben as it elutes, and we find under the separation conditions that they are all completely separated, or resolved to baseline. To identify each compound, however, requires prior knowledge of their precise elution time when separated under the same conditions (or, alternatively, relative to a known standard's elution time). To determine each analyte's concentration in the mixture, the mass-proportional detector must also be calibrated against known standards, so that the peak area yields the mass of analyte.

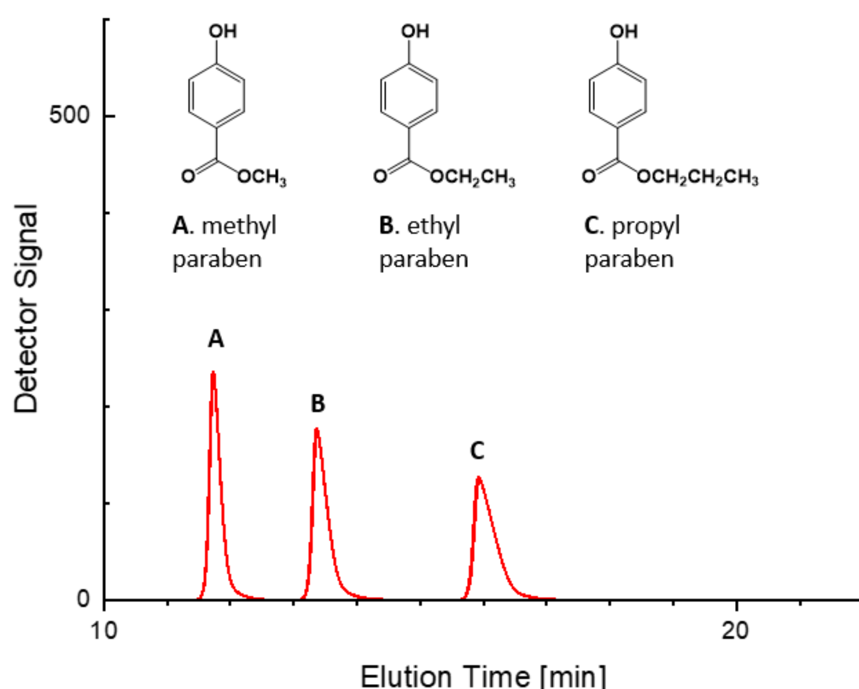


Figure 2. In LAC (here, reverse phase chromatography), small molecules are separated by the extent of interaction with column's stationary phase, typically consisting of alkyl chains (e.g. C_{18}) bonded to silica. The molecules elute in order of increasing hydrophobicity (or decreasing polarity). In this example, a mixture of methyl-, ethyl-, and propyl- *para*-hydroxybenzoic acids (called parabens) is separated in H_2O/acetone eluent, eluting in the order of increasing alkyl length of the ester. In conventional practice, each molecule is identified based on its elution time, by comparing elution times with known standards; the concentration is determined with a mass proportional detector.

The benefit of replacing the mass-proportional detector with ^1H NMR spectrometer is, of course, that compound identification no longer needs a calibration, but can be carried out directly using the ^1H

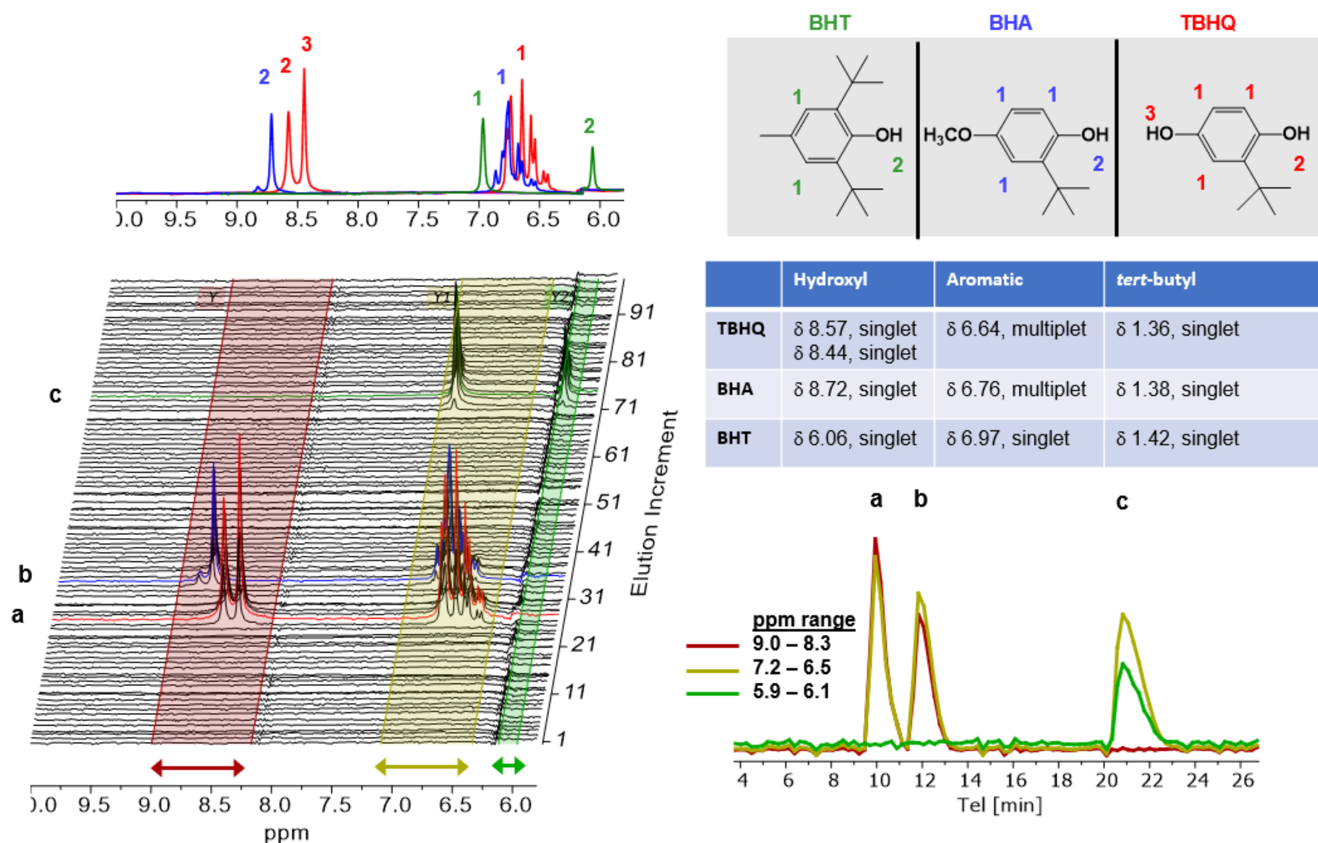
spectrum. In the simplest analysis using a 1D ^{1}H spectrum, an analytical workup would usually consist of an assignment of the chemical shift δ of each NMR signal to a proton in the molecular structure, a determination of the signal's multiplicity (i.e., number of participating protons), and a numerical integration of each signal to obtain relative proton abundance. Numerous other options may also be considered: as the analyte in the NMR flow cell has by definition been separated from other molecules and contaminants, any of the dozens of NMR pulse sequences and methods can be deployed for structural analysis. This goes well beyond the monitoring of an absorbance during elution.

Experimental Setup

The setup for benchtop LAC-NMR is identical to that described previously for SEC-NMR. [Link] Only the column and mobile phases are different. In a current setup a quaternary pump is used for low-pressure mixing of solvents from their reservoirs, if needed for setting up gradients during a separation. Like the SEC columns used in benchtop SEC-NMR, the LAC columns need to have a wider bore to maintain peak shape integrity at the detection cell as well as boost sensitivity with higher mass and volume loadings. The current setup uses reverse-phase columns with 10 mm diameter and H_2O/acetone as a mobile phase, as this approximates acetonitrile retention properties but with a better safety profile.

Benchtop LAC-NMR with 1D ^{1}H spectral acquisition

Two examples below illustrate how one might use the coupled benchtop spectrometer for structural identification of small molecules separated by LAC; in both cases, the mixtures were separated isocratically, i.e. without any change in solvent composition during the separation. The first example (**Figure 3**) demonstrates the separation and detection of a mixture of 3 phenolic antioxidants, BHT, BHA, and TBHQ. These molecules protect against oxidation through the labile hydroxyl proton and are commonly added to food products, a usage that is regulated by regulatory agencies such as The European Food Safety Authority, the Food and Drug Administration, etc. Here, a mixture of these compounds has been separated in H_2O/acetone = 20:80 (v/v) and monitored continuously using 1D spectral acquisition. The time resolution is ca. 14 sec (averaged over 4 scans), and a waterfall plot has been used to display the time-resolved spectra. The antioxidants elute in order of decreasing polarity, with TBHQ first (peak **a**), BHA second (peak **b**), and BHT third (peak **c**), and there is an accompanying overlay of spectral slices at the top. The elugram on the right has been obtained by numerically integrating over signals within a defined ppm range. Over the spectral range 5-9 ppm, we observe strong signals from the hydroxyl protons (singlets) and the aromatic protons (multiplets), and thus we can identify each compound using the chemical shift and multiplicity values shown in the table. Note that the aromatic multiplets of BHA and TBHQ have a very similar pattern, owing to the 1,2,4 proton substitution pattern, but the central peak in the TBHQ multiplet is shifted slightly, ca. -0.12 ppm (-9.6 Hz) relative to BHA. As the frequency scale (i.e. the ppm axis) is stable during separation, we are able to detect this small chemical shift difference by setting up the acquisition for longer acquisition times, so that the spectra are acquired with higher spectral resolution (correspondingly lower time resolution).



	Hydroxyl	Aromatic	*tert*-butyl
TBHQ	δ 8.57, singlet δ 8.44, singlet	δ 6.64, multiplet	δ 1.36, singlet
BHA	δ 8.72, singlet	δ 6.76, multiplet	δ 1.38, singlet
BHT	δ 6.06, singlet	δ 6.97, singlet	δ 1.42, singlet

Figure 3. Benchtop LAC-NMR of phenolic antioxidants, which elute in the order TBHQ (peak **a**), BHA (peak **b**), and BHT (peak **c**). ^1H spectra acquired in continuous flow are visualized in a waterfall plot, with an accompanying spectral overlay (top) and elugram (right). All aromatic and hydroxyl protons are spectrally resolved, thus can be structurally identified from proton chemical shift values and multiplicity.

In the second example (**Figure 4**), a mixture of *para*-hydroxy benzoic acids (commonly called parabens) are separated in H_2O/acetone = 40:60. Parabens with various length and type of ester groups are used as preservatives in cosmetic products because they prevent the growth of a wide range of spoilage microbes. Here, the experiment was setup for rapid acquisition of 1D spectra for improved sensitivity (ca. 1.4 s between spectra), and with solvent suppression (to minimize the influence of strong solvent signals of H_2O and acetone). With the contour plot visualization, we observe the spectrally-resolved separation of the two parabens: the methyl paraben elutes first (peak **a**), followed by the ethyl paraben (peak **b**). Both compounds have nearly identical splitting pattern and chemical shift of the two aromatic protons *ortho* and *meta* to the ester group. However, distinct differences are found in the ester group: in methyl paraben, the end methyl group shows a very strong singlet signal (**6**, δ 4.07 ppm) whereas in ethyl paraben we find a triplet signal (**3**, δ 1.48 ppm). These two analyte-specific signals uniquely identify the two compounds.[1]

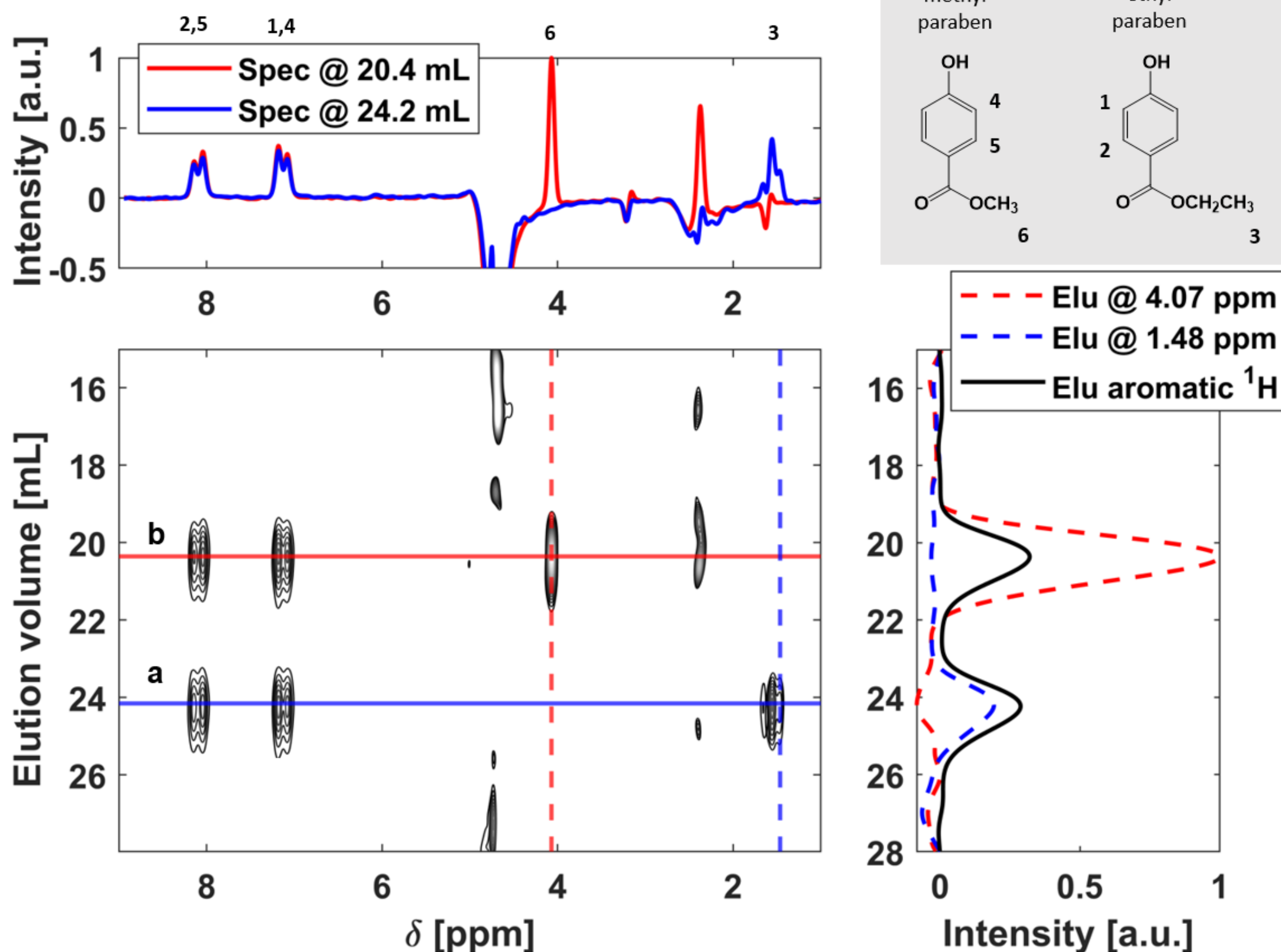


Figure 4. Benchtop LAC-NMR of *para*-hydroxybenzoic acids (parabens). 1D ^{1}H spectra acquired in continuous flow are visualized as a contour plot, with an accompanying spectral overlay (top) and elugram (right). Methyl paraben (peak **a**) can be distinguished from the ethyl paraben (peak **b**) by the chemical shift and splitting pattern of the ester's methyl group protons (**6**, δ 4.07 ppm; **3**, δ 1.48 ppm).[1]

In these two examples, the separation conditions, acquisition parameters, and data processing differed, but in each case the molecules were resolved both chromatographically (owing to the success of the LAC separation mode) and spectrally (thanks to analysis of the spectral signature of each molecule). We see here a strong capability of the benchtop LAC-NMR method for zeroing in on molecular, structural information of a pure analyte against a binary solvent background. In the case of parabens, advanced methods for acquisition and data processing, as well as chromatography optimization, has led to successful detection of these compounds at concentrations well below the legal limit of 4 g/L per paraben in mixtures.[1]

Benchtop LAC-NMR with 2D ^{1}H-^{1}H COSY acquisition

When coupling benchtop NMR to LAC separations, we are of course not limited to the acquisition of 1D spectra, and any number of different experiments may be carried out with due attention to the requirements of each pulse program, measurement duration, and parameter optimization for on-flow conditions. An important example was recently demonstrated for acquiring 2D homonuclear COSY spectra in continuous flow during LAC separation. In a COSY spectrum, data are acquired by a pulse sequence that increments an internal delay time (to allow for spin-spin coupling evolution), generating a 2D dataset which is Fourier-transformed in two dimensions. As the overall timespan of a COSY measurement can exceed several

hours, the measurement is generally unsuited for on-flow measurement. Ultimately, applying parameter optimization and a non-uniform sampling (NUS) schedule, the measurement time could be significantly reduced, remarkably in this case to a few minutes. With NUS, the number of spectral acquisitions in the indirect dimension was reduced from 100% to 37.5%, yielding a considerable time savings.

The technique is illustrated in **Figure 5**. Methyl and propyl paraben have been separated by LAC in the mobile phase H_2O/acetone = 40:60. During elution of the methyl paraben (peak **a**), the spectrometer started acquisition of a COSY spectrum during continuous flow just after the molecule eluted. The duration of the measurement was ca. 2 minutes and covered symmetrically the central portion of the peak, as shown by the red line. The COSY spectrum represents in that case only the pure molecule against its solvent background. We find in the methyl paraben spectrum that the 2 protons *meta* and *ortho* to the ester function (**1**, δ 8.12 ppm; **2**, δ 7.15 ppm) are split by each other (^3J =8.8 Hz), as known from the 1D spectrum. These are the characteristic cross peaks expected for a *para* substituted aromatic ring with two

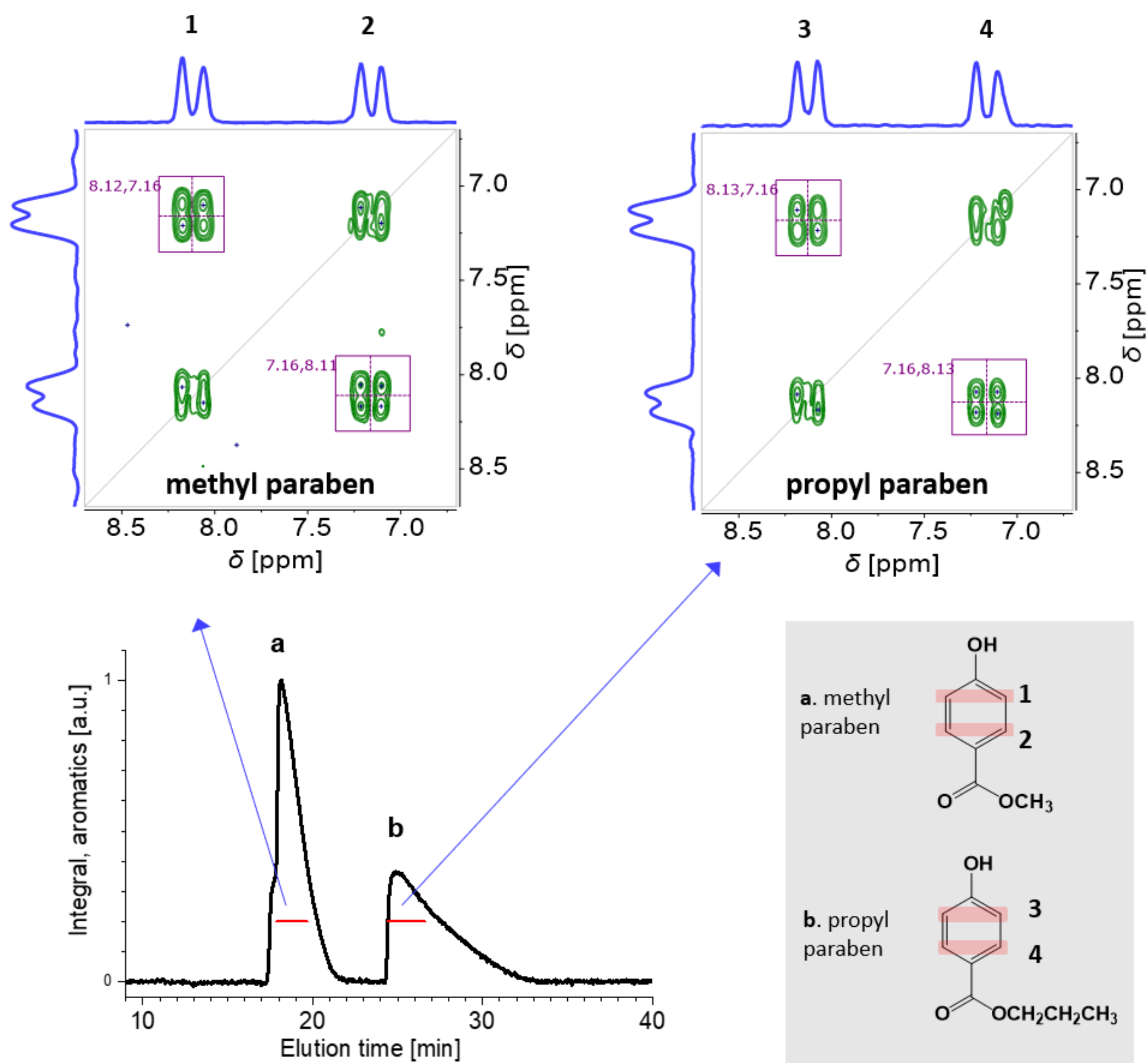


Figure 5. Benchtop LAC-NMR in which 2D COSY spectra are acquired across individual eluting peaks. In this example, COSY spectra of methyl paraben (peak **a**) and propyl paraben (peak **b**) have been acquired in continuous flow over a duration of 2 minutes as the peak elutes (red lines). Thus, the characteristic cross peak of the aromatic *meta* protons (**1**, δ 8.12 ppm, doublet, J=8.8 Hz) with the *ortho* protons (**2**, δ 7.15 ppm, doublet, J=8.8 Hz) in methyl paraben can be observed. This is reproduced during elution of the propyl paraben, which has lower concentration and additional band spreading.[2]

different substituents (i.e. methyl paraben's symmetry). This measurement was repeated during propyl paraben elution (peak **b**), but in this case the acquisition was timed for an earlier start due to the peak's asymmetric shape, to ensure that the peak maximum would be measured. Here we observe again nearly identical observations for these protons, with the characteristic cross peaks reflecting the known molecular structure.[2]

Conclusion

We have illustrated here some examples of structural analysis of small molecules using the benchtop ^{1}H-NMR spectrometer as an on-flow detector in LAC separations. These examples include on-flow acquisition of 1D spectra for detection of phenolic antioxidants and parabens, as well as 2D COSY spectra for investigation of spin-spin coupling.

For further information and discussion…

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