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CHEMICALLY-SENSITIVE detection in **H P L C**

Benchtop NMR spectrometer as online HPLC detector combined with sinusoidal sample injection

Picture: KIT / Mr. Markus Breig

Dr. Carlo Botha

studied chemistry at Stellenbosch University (SU) and Karlsruhe Institute of Technology (KIT). In 2021, he completed his doctoral studies where he was working on method development for chemical characterization on the topic of size-exclusion chromatography (SEC) hyphenated to a benchtop nuclear magnetic resonance (NMR) spectrometer in the research group of Prof. Manfred Wilhelm at KIT. During his doctorate, he was funded by Deutsche Forschungsgemeinschaft (DFG) in the collaborative research center project (SFB) 1176 Molecular Structuring of Soft Matter and later by the AiF 'Industrielle Gemeinschaftsforschung' (iGF) 19925N as part of industrial-academic cooperation between the rubber industry and KIT.

Hyphenating HPLC with a benchtop NMR spectrometer provides a cost-effective and powerful method for the identification, quantification, and characterization of analyte mixtures.

Overview

For efficient sample analysis nuclear magnetic resonance (NMR) spectroscopy and high-performance liquid chromatography (HPLC) have been combined. By hyphenating these methods, analytes can be simultaneously separated and spectrally analyzed in a single measurement. To substantially increase the sensitivity of the hyphenated analysis, a Fourier transformation HPLC injection method was developed.

To ensure the quality of a consumer product, detailed knowledge of its molecular composition is mandatory. Both nuclear magnetic resonance (NMR) spectroscopy and high-performance liquid chromatography (HPLC) are each powerful instrumental methods valuable for the analysis of food products, pharmaceuticals, cosmetics, and polymers [1–4]. The umbrella term "HPLC" comprises size exclusion chromatography (SEC), liquid adsorption chromatography (LAC), and liquid chromatography under critical conditions (LC-CC). The appropriate HPLC method is chosen according to the analytical question at hand in combination with the sample and solvent composition [3].

Using NMR spectroscopy information on the local chemical environment is obtained, which can be used for the structural elucidation of molecules [3].

Markus Matz

studied food chemistry at Karlsruhe Institute of Technology (KIT) in Karlsruhe, Germany. In his master's thesis, he developed a new liquid chromatographic injection method using periodic excitation. In 2020, he started his PhD at the Institute for Chemical Technology and Polymer Chemistry at KIT. Its research topic focused on HPLC-NMR hyphenation and the new liquid chromatographic injection method and is under the supervision of Prof. Dr. Manfred Wilhelm.
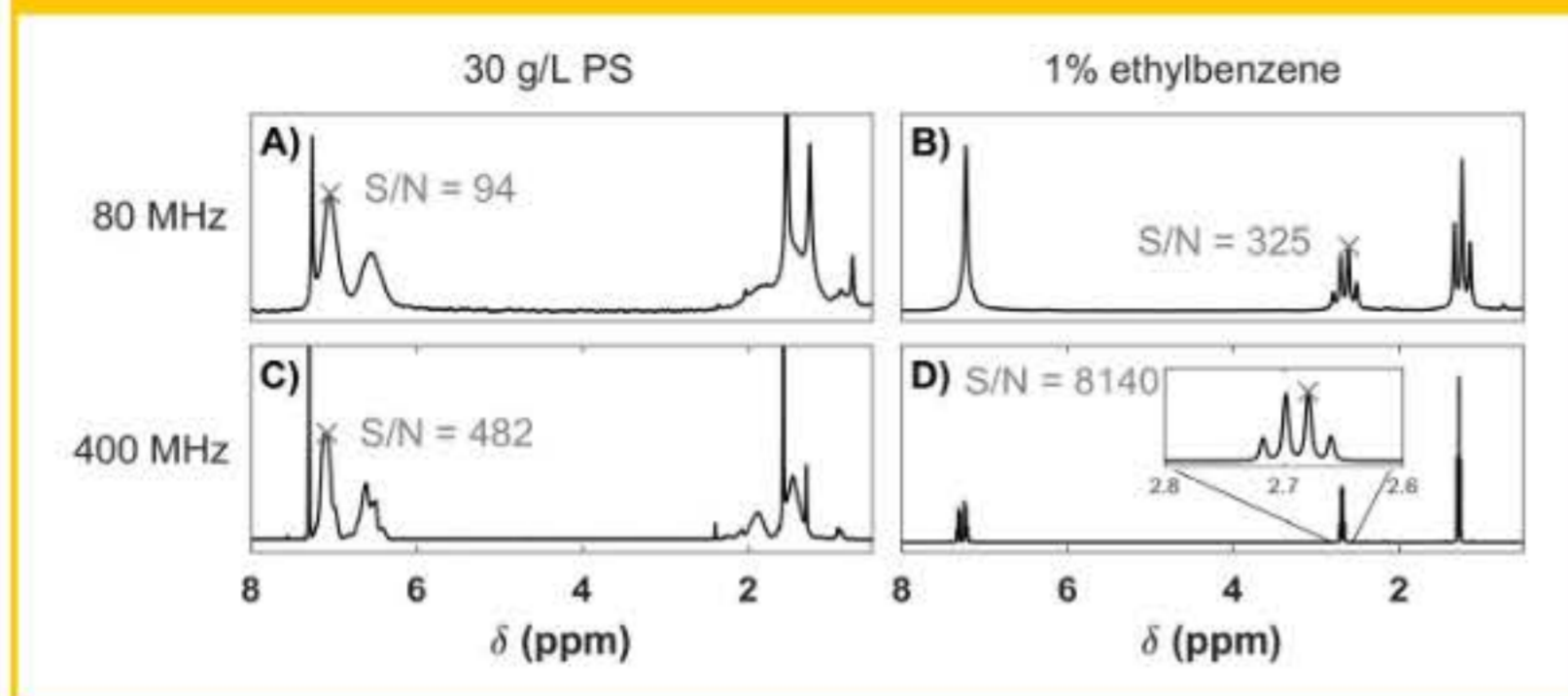
Fig.1: Comparison of an 80 MHz 1H-only benchtop NMR spectrometer from Magritek (A, B) with a 400 MHz NMR spectrometer from Bruker (C, D). The measured samples were 30 g/L polystyrene (PS) in deuterated chloroform (CDCl$_3$, A, C) and 1% ethylbenzene in CDCl$_3$ (B, D). Each spectrum was recorded with 4 scans. Due to the dispersion of the isotropic chemical shift of the polystyrene signals, the signal-to-noise ratio (S/N) of the aromatic proton signal at 80 MHz (A) is only about a factor 5 lower than that at 400 MHz (C), whereas for ethylbenzene (B, D) the resolved quartet at 2.7 ppm of the methylene protons of ethylbenzene is a factor 25 lower.

Fig. 2: Online HPLC-benchtop NMR setup. The eluate is directly passed through the NMR and is measured on-flow [5].

As the 1H NMR signal is directly proportional to the amount of ^{1}H nuclei, quantitative information is directly accessible. However, the spectral resolution in NMR spectroscopy is often insufficient to resolve all components of analyte mixtures due to the abundance of overlapping signals, increasing the difficulty of qualitative and quantitative analysis. The selectivity, however, can be significantly increased by adding HPLC as a separation technique prior to NMR detection [5].

NMR is also affordable for medium-sized laboratories

In recent years, NMR spectroscopy has undergone a development in which permanent magnets and modern electronics are combined to circumvent the technically and financially demanding cooling of superconducting magnets by nitrogen and helium [4]. NMR spectrometers based on permanent magnets have a maximum ^{1}H Larmor frequency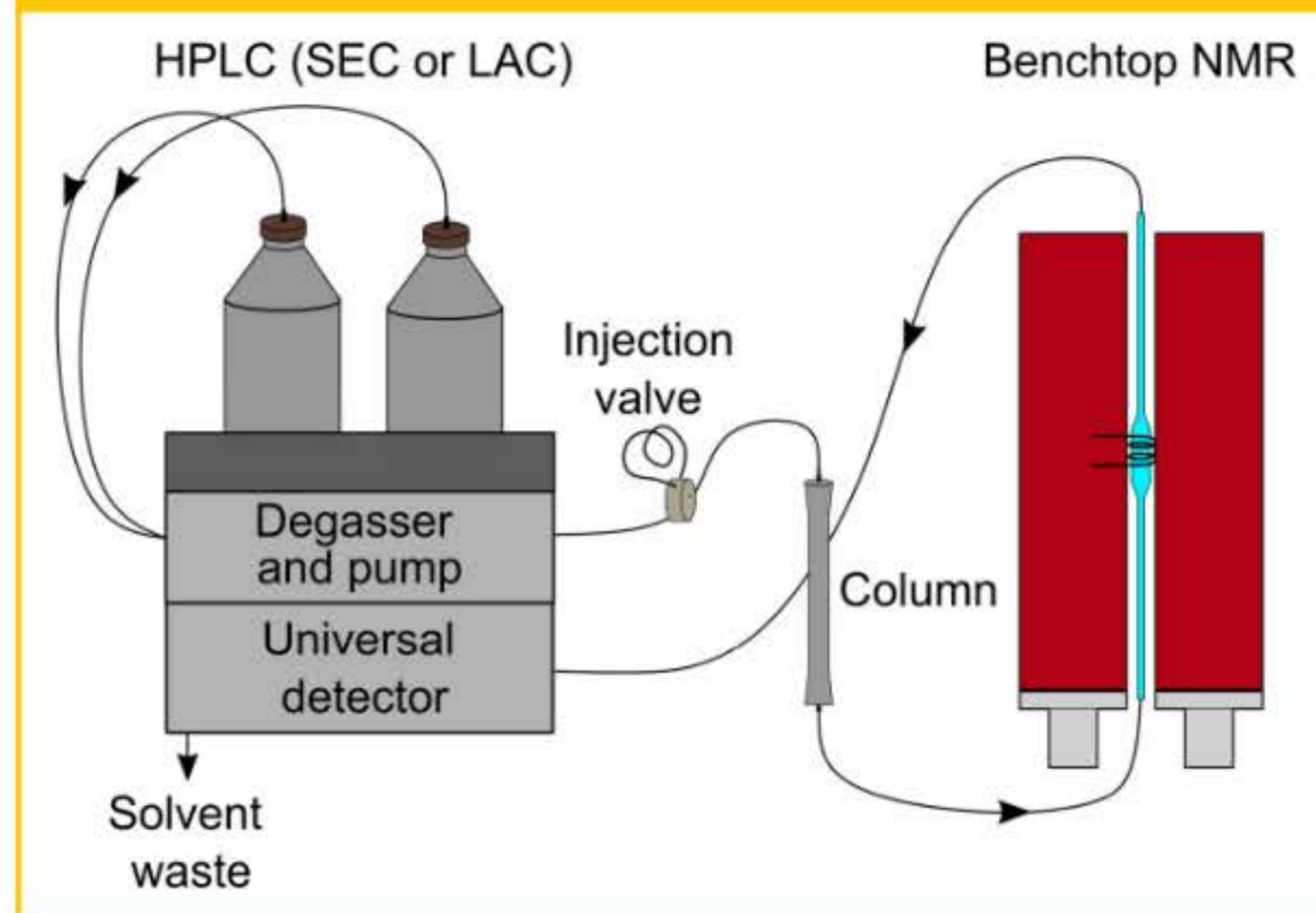 of ~100 MHz due to the composition of the permanent magnets and are thus referred to as low-field NMR spectrometers [4]. Compared to high-field NMR, low-field NMR (also known as benchtop NMR) is smaller in size (~ 60 cm × 60 cm × 60 cm, ca. 50 – 100 kg), and has lower investment costs with little to no maintenance costs. The signal-to-noise ratio (S/N) of an NMR spectrum scales with the magnetic field strength raised to the power of 1.5 [5]. Thus, benchtop NMRs are generally less sensitive than their high-field counterparts. However, the sensitivity difference is reduced by a factor of ~3 in favor of low-field NMR due to the different B0-field orientation and the related geometry of the detection coil [6]. We work with an 80 MHz benchtop NMR spectrometer from Magritek (Aachen, Germany), equipped with a 1H only probe head which gives another factor ~2 – 3 sensitivity increase compared to a dual channel probe head [7]. Figure 1 illustrates the sensitivity and selectivity difference between the 80 MHz benchtop NMR and a standard 400 MHz high field NMR from Bruker (Rheinstetten, Germany).
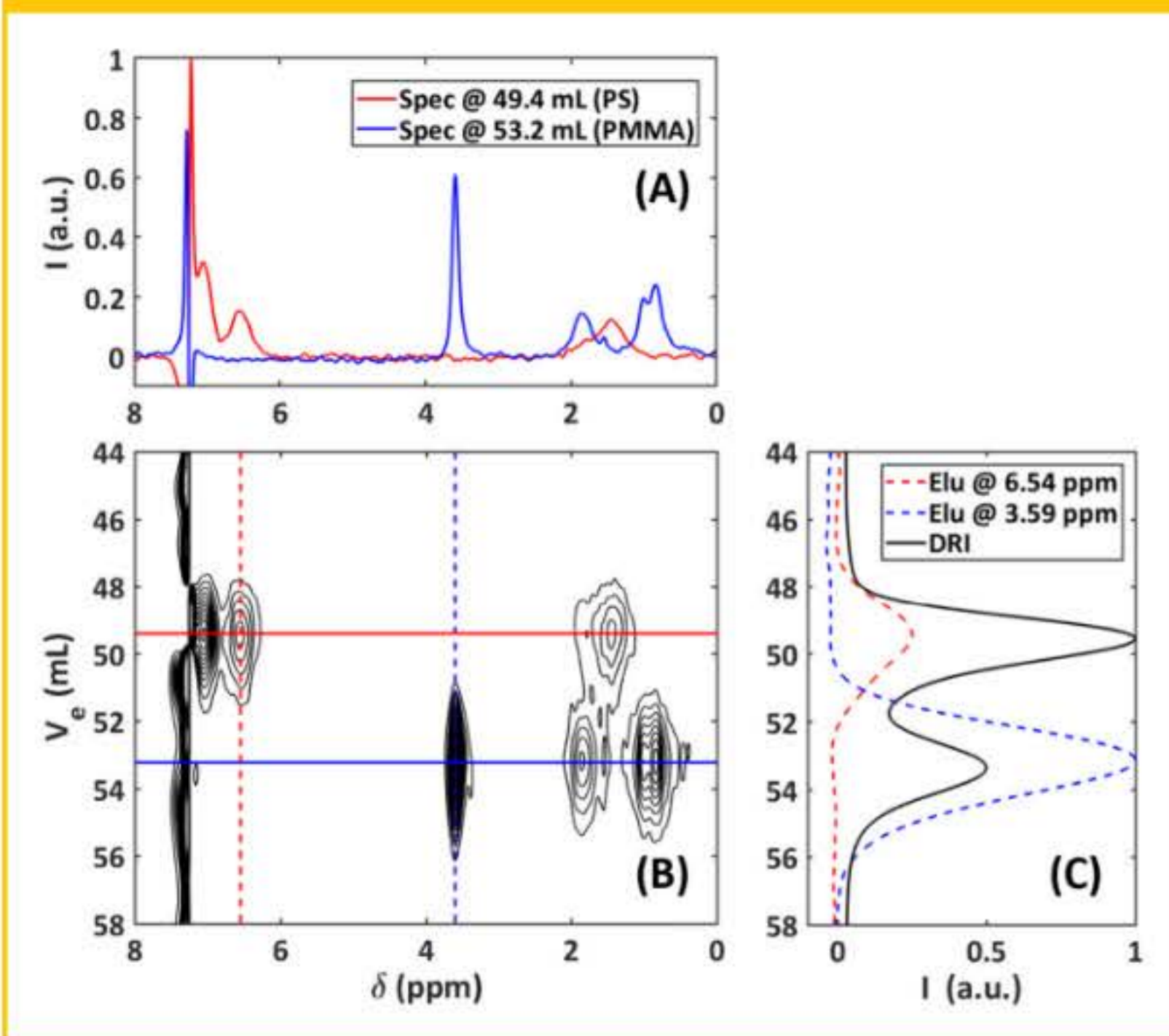
Fig. 3: 2D SEC-NMR correlation plot of a polystyrene (PS) and polymethylmethacrylate (PMMA) mixture in chloroform (CHCl₃). A total of 1 mg of this mixture was injected onto a semi-preparative SEC column (300 × 20 mm) at 1 mL/min. Solvent suppression was achieved by exploiting T1 effects combined with numerical methods. [Reproduced from Figure 13 of ref. [5] with permission from the Royal Society of Chemistry.]

Benchtop NMR as HPLC detector

Benchtop NMRs typically have an external fluorine lock to compensate for temporary magnetic field fluctuations. This allows for measurement without the addition of deuterated solvents. Accordingly, HPLC-benchtop NMR measurements can be performed using standard HPLC solvents. Different HPLC-NMR setups are conceivable, but

the most efficient in terms of working load and measurement time per sample is the online approach [8]. Figure 2 depicts our online HPLC benchtop NMR setup [5].

The measurement time during an on-flow experiment is limited and corresponds to the residence time of the analyte within the NMR detection coil, which is ca. 8 s at a flow rate of 1 mL/min for our setup [5]. This low residence time, combined with on-flow effects and the use of protonated solvents, leads to a further decrease in the sensitivity of online HPLC NMR measurements. Therefore, to increase sensitivity, careful optimization of the parameters in both the NMR and HPLC dimensions is necessary. Additionally, preparative or semi-preparative HPLC columns should be used to increase the amount of analyzable analyte, and, if the chromatographic resolution is high enough, the flow rate could be reduced to increase the residence time within the NMR detection coil [5, 9].

In addition to the NMR detection, a chromatogram is recorded by a universal detector with which the NMR spectra can be correlated to obtain a two-dimensional (2D) spectrally resolved chromatogram, which is exemplified in the consecutive section [5, 9].

Polymer analysis using SEC-NMR hyphenation

Size-exclusion chromatography (SEC) is often the method of choice for the analysis of polymer samples [3]. In SEC, the molecules are separated according to their hydrodynamic volume in solution, which allows for a determination of the molecular weight distribution [3]. Figure 3 shows a 2D correlation plot of the SEC-NMR measurement of a mixture composed of polystyrene (PS) and polymethylmethacrylate (PMMA) [5].

Using NMR detection both polymers can be identified as PS and PMMA as well as the relative concentration determined [5]. With the optimized parameters for the SEC 60 MHz benchtop NMR hyphenation, the limit of detection (LOD) and limit of quantification (LOQ) (determined at S/N = 3 and 10, respectively) was ca. 0.1 mg and ca. 0.4 mg injected mass PS [10].

For pharmaceuticals and food analysis, liquid adsorption chromatography (LAC) is more often used to separate the compounds [2]. In LAC, the column dimensions and the sample injection volumes are typically lower compared to SEC, leading to even more reduced sensitivity. Currently, we investigate the hyphenation of LAC to an 80 MHz benchtop NMR. However, despite upgrading the field strength from 60 MHz to 80 MHz, the low sensitivity is still the major challenge. To overcome the sensitivity issue, a modified injection method has been developed that resulted in a substantially increased S/N.

Fourier transformation liquid chromatography: Factor 50 higher sensitivity

To counter the low sensitivity of the HPLC-benchtop NMR, an HPLC injection method referred to as Fourier transformation liquid chromatography (FT LC) has been developed [11]. Instead of injecting a small sample volume into the HPLC, in FT-LC the analyte is continuously introduced into the HPLC. The fundamental principle is shown in Figure 4 [11].

In FT-LC, an oscillating sinusoidal analyte concentration profile is generated. Consequently, a sinusoidal chromatogram is obtained, which is evaluated using the Fourier transformation. Similar to the retention time in conventional liquid chromatography (LC), the sinusoidal chromatograms in FT-LC have an analyte-specific phase angle which is determined via Fourier transformation. Using this method, the S/N normalized to the square root of time is increased by a factor of 50 compared to the conventional injection method. Currently, we investigate the application of FT-LC to HPLC-NMR hyphenation to further increase the sensitivity of the benchtop NMR and suppress the solvent in the NMR spectra.
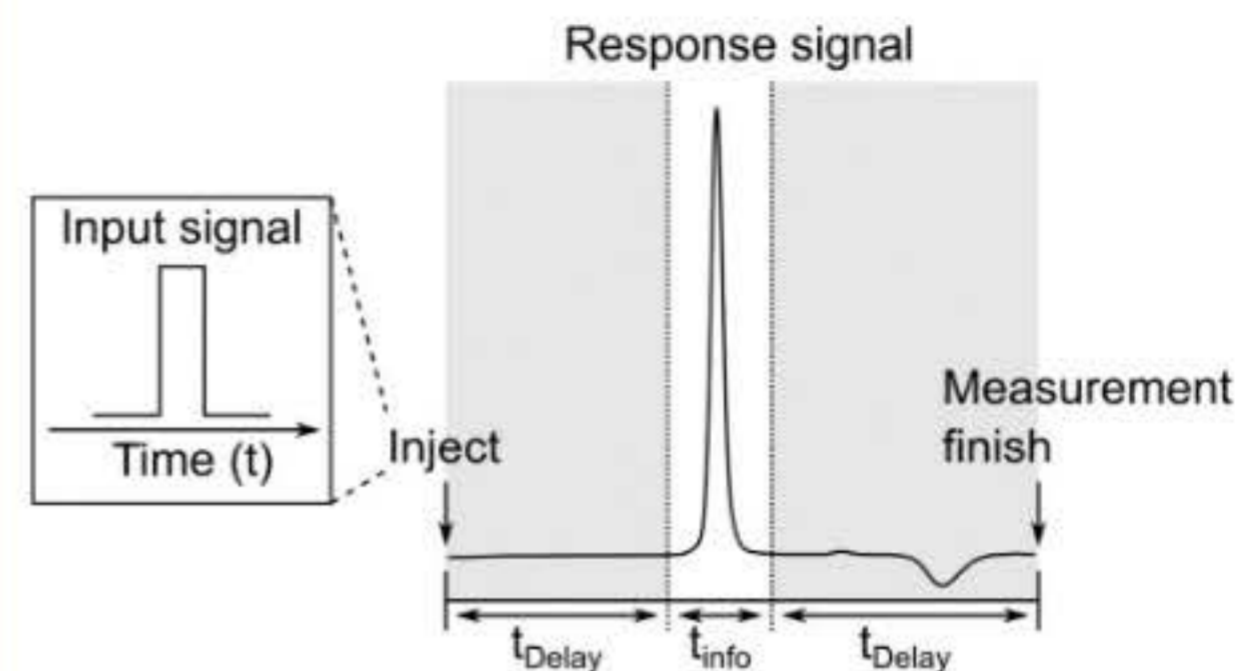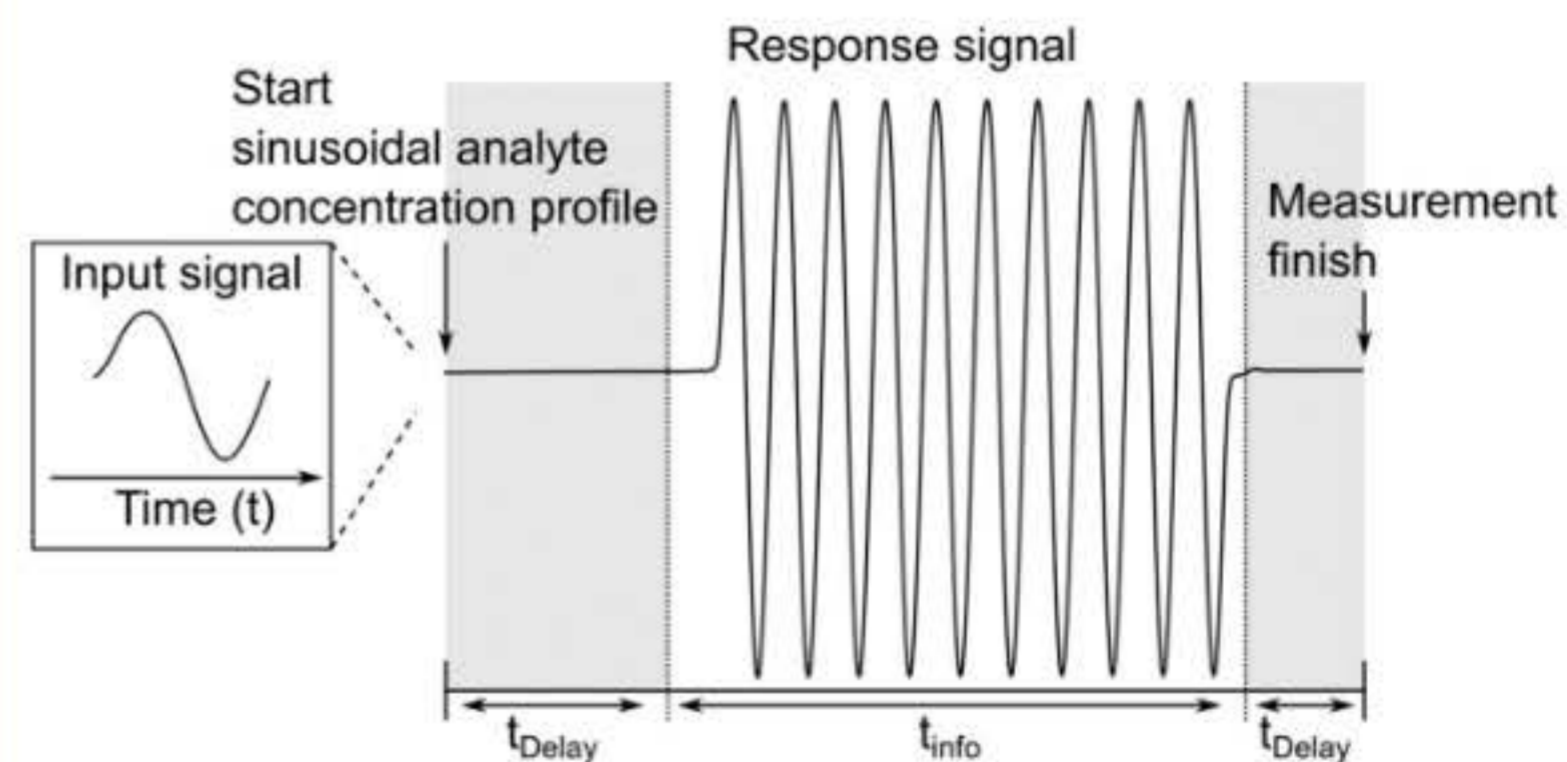

Fig. 4: Illustration of the Fourier transformation liquid chromatography (FT-LC) principle. A sinusoidal analyte concentration profile is continuously introduced into the HPLC, starting at 50% of the maximum concentration. The retention time of the molecules leads to an analyte-specific phase angle, which is evaluated using Fourier transformation. Using FT-LC, the S/N can be increased by a factor of 50. [Reproduced from Figure 1 of ref. [11] with permission from the Royal Society of Chemistry.]

Conclusion

Benchtop NMRs have become powerful instruments that are affordable also for smaller laboratories. If HPLC is hyphenated with a benchtop NMR, analytes can be simultaneously characterized and quantified. In HPLC-NMR hyphenation the lack of sensitivity is the main challenge, which can potentially be overcome using the new injection method called FT-LC.

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● Contact

Prof. Dr. Manfred Wilhelm
Karlsruhe Institute of Technology
Institute for Chemical Technology
and Polymer Chemistry, Karlsruhe,
Germany
manfred.wilhelm@kit.edu
https://www.itcp.kit.edu/wilhelm/
index.php

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