

Hyphenated NMR Methods in Natural Products Research, Part 2: HPLC-SPE-NMR and Other New Trends in NMR Hyphenation

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Abstract

This review describes the principles and performance of a novel and highly promising hyphenated technique, HPLC-SPE-NMR, which is based on post-column analyte trapping by solid-phase extraction. The analytes are subsequently eluted from the SPE cartridges using deuterated solvents. This indirect HPLC-NMR hyphenation offers numerous advantages compared to direct HPLC-NMR methods. Multiple trapping leads to a dramatic increase of analyte amounts available for NMR, enabling acquisition of high-quality 2D NMR data within a short time. Other

new developments, including combination of solenoidal coil capillary flow-probes with microflow HPLC, are also discussed. Fast extract dereplication using these techniques enables focusing of isolation efforts on truly novel and promising natural products, based on precise structural data obtained with crude extracts or fractions.

Key words

Nuclear magnetic resonance · liquid chromatography · LC-NMR · HPLC-SPE-NMR · hyphenated techniques · microcoils · cryoproboscopes

Introduction

In the first part of this review [1], the use of direct NMR hyphenation in natural products research has been discussed. The direct HPLC-NMR methods were defined as methods in which the NMR data acquisition is performed on the HPLC column eluate [1]. This implies either a direct connection between the NMR spectrometer and the HPLC apparatus, or use of storage capillaries in which the HPLC eluate is temporarily stored prior to acquisition of NMR data.

Many useful results related to natural products have been obtained using direct HPLC-NMR methods [1] and the technique has been reviewed at various stages of its development [2], [3], [4], [5], [6], [7], [8], [9], [10], [11], [12], [13], [14], [15], [16], [17], [18]. Pharmaceutical, biomedical and other applications of NMR hyphenation have also been extensively reviewed [19], [20], [21],

[22], [23], [24], [25], [26], [27], [28], [29], [30]. However, the direct combination of state-of-the-art separation technology with the most powerful and versatile tool for structure elucidation of organic molecules in solution suffers from various practical problems. In short, these practical limitations are due to somewhat contradictory requirements of HPLC and NMR with respect to solvents and time-scale of the experiment, and thus compromises are necessary in order to hyphenate these two techniques. For example, ¹H-NMR spectra are preferably acquired in deuterated solvents, but use of deuterated solvents for HPLC is expensive and the selection is limited. Some useful HPLC eluents, such as buffers with high ionic strengths, are directly undesirable in direct HPLC-NMR hyphenation. Gradient elution of HPLC columns, required in most separations, implies constantly changing magnetic susceptibility and field homogeneity in the NMR flow-probe, not to mention variations of analyte chemical shifts with solvent composition.

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On the other hand, the limited sensitivity of NMR detection means that HPLC-NMR experiments are usually acquired in the stopped-flow mode [1]. This can result in difficulties caused by diffusion and can limit the possibility of obtaining 2D NMR data to one peak per chromatogram. The latter difficulty is circumvented by use of capillary loops for temporary storage of chromatographic peaks [1]; however, only a few studies relying on the loop storage technique have been reported so far [1]. To sum up, the outcome of a direct HPLC-NMR experiment is largely determined by chromatography, both in terms of the solvent used and because analyte from one injection only is available for NMR.

The recently introduced automated HPLC-SPE-NMR technique makes use of an entirely different approach to HPLC-NMR. Here, chromatographic peaks eluted from a reversed-phase HPLC column are passed one by one through small solid-phase extraction columns (SPE cartridges) in order to remove the analyte from the HPLC mobile phase. The SPE cartridges are subsequently dried with nitrogen gas, and the analytes desorbed with a deuterated solvent for NMR spectroscopy. The whole process is carried out automatically under software control (Fig. 1).

Early Combinations of Post-Column SPE with HPLC-NMR

Solid-phase extraction is a standard method of sample preparation and analyte concentration *prior* to HPLC analysis [31]. Such analyte pre-concentration by SPE can also be combined on-line with HPLC-NMR analysis; for example, de Koning et al. used an SPE-HPLC-NMR experiment for the analysis of hop bitter acids [32].

The HPLC-SPE-NMR technique, where SPE is used *after* the HPLC separation, has its roots in the early phase-system switching (PSS) technique developed to solve mobile-phase and flow-rate incompatibilities in HPLC-MS. Kokkonen and colleagues were apparently the first to use post-column addition of water to the HPLC eluate in order to diminish the eluting power of the mobile phase and withhold the analyte on a trapping column prior to desorption to the MS interface [33]. Silica-based C_8 material was used in this HPLC-SPE-MS experiment both in the analytical and in the trapping columns [33]. With respect to NMR applications, Wilcox and Phelan reported already in 1986 a procedure consisting of a reversed-phase HPLC separation, collection of HPLC fractions using an automatic fraction collector, dilution of the frac-

tions with water, solid-phase extraction of the diluted fractions using C_{18} SPE cartridges, and elution of the analyte from the cartridges for 1H -NMR at 250 MHz [34]. This off-line sequence of HPLC, SPE and NMR operations can be regarded as a prototype of modern HPLC-SPE-NMR experiments.

Griffiths and Horton [35] applied the concept of analyte concentration on a trapping column after water dilution of analytical column eluate [33] in connection with on-line NMR analysis. The analytes were desorbed from the trapping column with a back-flush of CD_3CN , but without prior drying of the trapping column [35]. Griffiths and Horton demonstrated that column trapping of chromatographic peaks leads to increased solute concentrations resulting in increased signal-to-noise ratios in the NMR spectra, showed the advantage of the use of deuterated solvents for analyte back-flush, and pointed out the similarity of their concept to the already introduced loop storage system [35]. The system of Griffiths and Horton can be regarded as an immediate forerunner of the state-of-the-art HPLC-SPE-NMR installations (Fig. 1). Later, Nyberg et al. used SPE-NMR hyphenation, transferring analytes from SPE cartridges with a system of switching valves to the NMR flow-probe with CD_3OD , whereas the separation of sample constituents (*Capsicum chinense* fruit extract or *Quillaja saponaria* saponin fraction) was performed off-line [36], [37].

Advantages of HPLC-SPE-NMR Hyphenation

The signal-to-noise ratio in NMR spectra increases linearly with analyte amount but only with the square root of acquisition time. This emphasizes the importance of maximizing analyte amounts in the sensitive region of the NMR flow-cell. The sensitivity of detection in direct HPLC-NMR experiments will also depend on the ratio between the active volume of the NMR flow-cell and the peak elution volume (flow rate \times peak width). Thus, in the majority of stopped-flow HPLC-NMR experiments performed with conventional columns with internal diameter of 4.6 mm or larger [1], only a small fraction of the analyte eluted from the column contributes to the NMR signal. For example, it can be calculated that for a Gaussian chromatographic peak with a standard deviation of 0.2 min (and hence the base-width of 0.8 min), eluted at a flow rate of 1 mL/min, the maximal percentage of the analyte present in the sensitive region of an NMR flow-probe is 23.6%, 11.9% or 6.0% for flow-probes with sensitive volumes of 120, 60

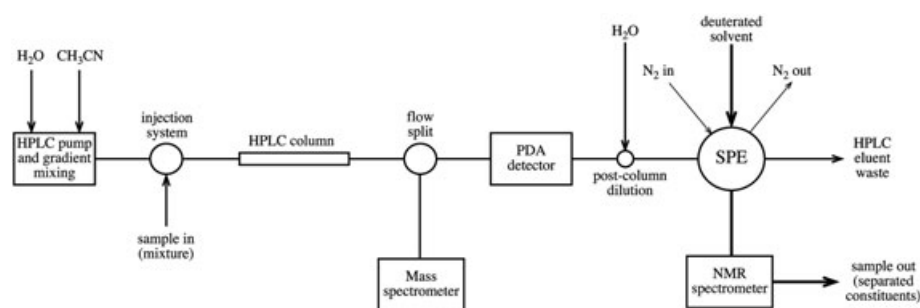


Fig. 1 Schematic description of an HPLC-SPE-NMR system. After separation of the mixture components on a reversed-phase HPLC column with non-deuterated eluents, the chromatogram is recorded using a photodiode array detector, a mass spectrometer, or both. The HPLC column eluate is then diluted with water and the analytes present in the chromatographic peaks are trapped on individual solid-phase extraction (SPE) cartridges. The trapping is governed by signals from the PDA or the MS detector. The SPE cartridges are then dried with a stream of nitrogen gas, and the analytes eluted with a deuterated solvent into the NMR flow-probe.

or 30 μL , respectively. If the peak-width is reduced four-fold (peak standard deviation 0.05 min and the base-width 0.2 min), the percentage of the analyte present at the center of the peak in the above-mentioned sensitive volumes increases to 77.0%, 45.2% and 23.6%, respectively. This emphasizes the importance of sharp peaks in direct HPLC-NMR experiments.

On the other hand, if the analyte can be trapped on an SPE cartridge quantitatively and eluted with about 30 μL of a solvent, most of the analyte will enter the NMR flow-cell and contribute to the NMR signal if a 30- μL flow-cell is used. In such a case, there will be an additional advantage of intrinsically higher mass sensitivity of the small-volume flow-cell. The only diminishing factor left will be the ratio between the sensitive volume and the total volume of the NMR cell (equal to about $1/2$). This focusing of the analyte band is the basis of the HPLC-SPE-NMR technique. Additional benefits come from the use of pure deuterated solvents for cartridge elution, which will render solvent suppression easier or even unnecessary. Last but not least, multiple peak trapping may give a very substantial increase of the analyte amount available for NMR.

Standard HPLC-SPE-NMR installations (Fig. 1) use 2×10 mm SPE trapping cartridges with a dead volume of about 30 μL that focus analytes eluted from standard HPLC columns into an NMR probe with a sensitive volume of 30 μL . Polystyrene- and/or divinylbenzene-based resins or C_{18} -bonded silica is normally used as the SPE material, and CD_3CN , CD_3OD or CDCl_3 as the eluting solvent. The prerequisites for successful operation of the HPLC-SPE-NMR system are 1) that the analyte is essentially quantitatively retained on the SPE cartridge, and 2) that it is quantitatively stripped from the cartridge as a sharp band, essentially with the solvent front. In order to increase affinity of compounds eluted from reversed-phase HPLC columns to the SPE material, dilution of the eluate with water is used prior to passage through the SPE cartridges, and the ratio of the post-column dilution is an adjustable parameter for optimization of HPLC-SPE-NMR experiments. Other post-column eluate modifications, such as pH adjustment, are in principle possible but have not yet been reported in conjunction with HPLC-SPE-NMR. Although a number of successful applications of the HPLC-SPE-NMR approach have been published (see below), quantitative studies of trapping and elution efficiency of various classes of natural products have yet to be reported.

Applications of HPLC-SPE-NMR

The first published study utilizing the automated HPLC-SPE-NMR technique described the identification of several flavonoids, rosmarinic acid and carvacrol in crude acetone extract of oregano using 1D ^1H -NMR spectra as well as TOCSY, HMQC and HMBC experiments [38]. The HPLC separation was performed on a 150×4.6 mm C_{18} column, analytes were trapped on a divinylbenzene-type polymer, and the spectra recorded with a cryogenic probe-head [38]. Other published studies describe identification of radical-scavenging flavonoids from extracts of *Rhaponticum carthamoides* (Asteraceae) separated on a 100×7.8 mm C_{18} column and employing polystyrene resin SPE cartridges [39], and structure determination of coumaric esters of iridoid glycosides from a pur-

ified fraction of *Harpagophytum procumbens* (Pedaliaceae), separated on a 250×2 mm C_{18} column and trapped on C_{18} -silica or poly(divinylbenzene) [40]. The latter work demonstrated the complexity of trapping efficiency and analyte recovery as a function of SPE stationary phase and eluting solvent [40].

Subsequent reports described the identification of flavonoids and cardenolides from various parts of *Kanahia laniflora* (Apocynaceae) separated on a 150×4.6 mm C_{18} column and trapped on C_{18} SPE cartridges [41], and identification of 17 compounds, mainly isoflavonoids, present in a crude extract of *Smirnowia iranica* (Fabaceae) roots [42]. In the latter study, a linear accumulation of an isoflavan on C_{18} SPE material in seven repeated trapings was demonstrated [42], illustrating the advantages of the multiple trapping approach; similar multiple trapping with a coumarin present in the root extract of *Croton membranaceus* (Euphorbiaceae) showed that poly(divinylbenzene) SPE material is more effective than C_{18} material under the conditions used [43] (Fig. 2). Moreover, inversion recovery experiments showed that the relaxation times determined in CD_3CN , a commonly used solvent for cartridge elution, are much longer than those in CDCl_3 [43]. Thus, not only SPE trapping and elution parameters, but also NMR acquisition parameters should be carefully optimized on a case-to-case basis for optimal performance of hyphenated NMR experiments. Constituents of a phenolic fraction of olive oil [44], lignans from *Phyllanthus urinaria* (Euphorbiaceae) [45], and oxygenated diterpenes from *Rosmarinus officinalis* (Labiatae) [46] were also analyzed using HPLC-SPE-NMR.

At present, the HPLC-SPE-NMR technique has been demonstrated to work well with many natural products classes including flavonoids, flavonoid glycosides and other glycosides, iridoids, various groups of terpenoids, simple phenolics, lignans and other phenylpropanoids, cardenolides, and alkaloids [38], [39], [40], [41], [42], [43], [44], [45], [46], [47]. Moreover, applications related to water and soil analysis [48] and drug metabolism [49], [50] have been reported. Xu and Alexander used HPLC-SPE-NMR in conjunction with a 150×10 mm C_{18} column to concentrate trace constituents for NMR analysis; since SPE trapping necessitates post-column water addition to the column eluate, a high-capacity retention and mixing device was introduced in order to achieve a sufficient dilution ratio of the high eluent flow (5 mL/min) emerging from the column used [51].

Thus, even though the number of studies utilizing HPLC-SPE-NMR is still rather limited compared to those employing direct HPLC-NMR methods [1], its advantages are clearly apparent. The combined benefits of analyte concentration and accumulation and use of deuterated solvents enable acquisition of high quality 2D NMR data from multiple peaks, including minor peaks present in very complex chromatograms, without compromising the chromatographic separation process. Access to less-sensitive 2D NMR experiments such as NOESY/ROESY and availability of ^{13}C chemical shifts (from HSQC and HMBC experiments) allow rigorous structure elucidation of rather complex natural products directly from extracts and mixtures (Figs. 3 and 4). The large analyte amounts available by multiple trapping allow the recording of NMR spectra with excellent signal-to-noise ratios without solvent peak suppression, increasing the robustness of the experiment and eliminating possible suppression artifacts

(Fig. 5). The fact that chemical shift data are obtained in well-defined solvents (as opposed to mixed HPLC solvents) opens the possibility for the use of databases for computer-aided spectra interpretation.

For these reasons, HPLC-SPE-NMR is envisaged to be a prime future tool for extract dereplication. Cryogenically cooled probes result in a further sensitivity gain and were applied in several of the above-mentioned studies [38], [49]. The HPLC-SPE-NMR studies reported thus far have been performed using 400 MHz [38], [39], [45], [46], 500 MHz [49], [51] or 600 MHz magnets [40], [41], [42], [43], [44], [48], [50], but an increase in routinely available field

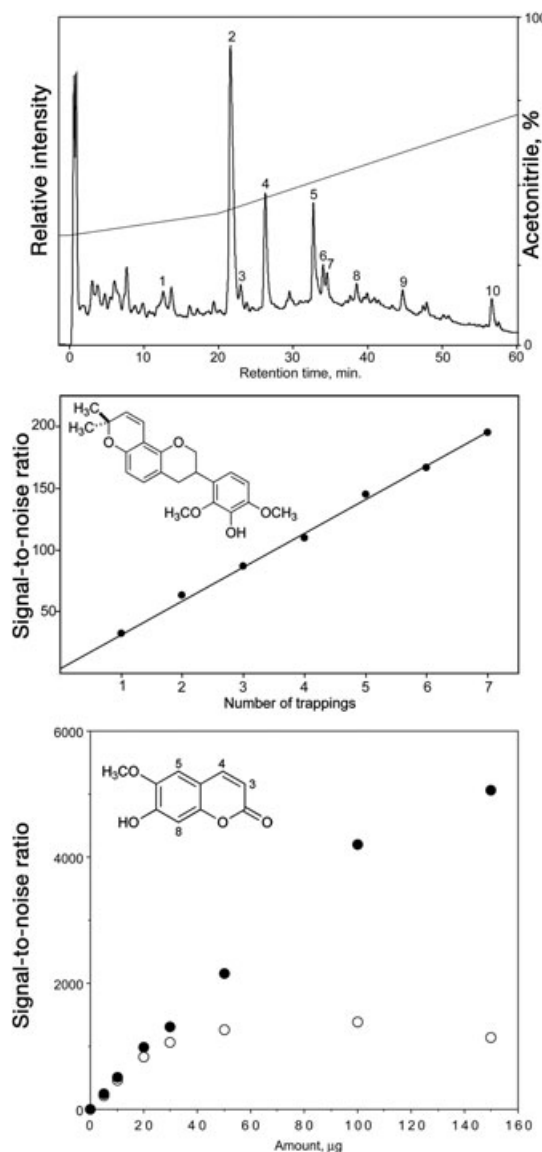


Fig. 2 Multiple trapping in HPLC-SPE-NMR experiments. Top: HPLC chromatogram of a crude extract of *Smirnovia iranica* roots (150×4.6 mm C₁₈ column eluted with CH₃CN gradient in H₂O). Center: Linear improvement of ¹H-NMR signal-to-noise ratio during seven trappings of the minor peak 10 from the above chromatogram on a 2×10 mm C₁₈ SPE cartridge. Bottom: Illustration of preferred trapping of a coumarin on poly(divinylbenzene) SPE cartridges (filled circles) as compared to C₁₈ SPE cartridges (open circles). All spectra were recorded at 600 MHz and in CD₃CN, using a 30-μL flow-probe. Reproduced from [42] and [43] by permission of the American Chemical Society and John Wiley and Sons, respectively.

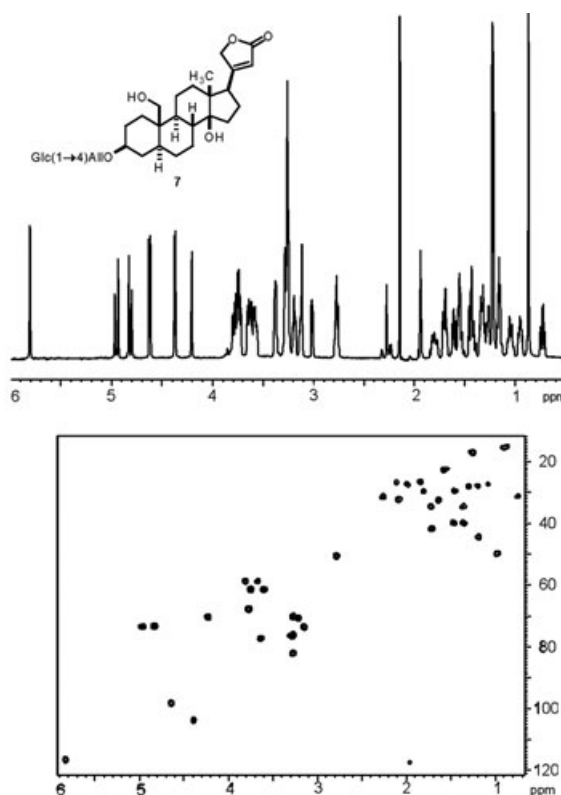


Fig. 3 1D ¹H-NMR spectrum (top) and HSQC spectrum (bottom) of a 5α-cardenolide present in *Kanahia laniflora*. The spectra were recorded in the HPLC-SPE-NMR mode following eight trappings on a 2×10 mm C₁₈ SPE cartridge (150×4.6 mm C₁₈ column eluted with CH₃CN gradient in H₂O, 600 MHz, 30-μL cell). Reproduced from [41] by permission of the American Chemical Society.

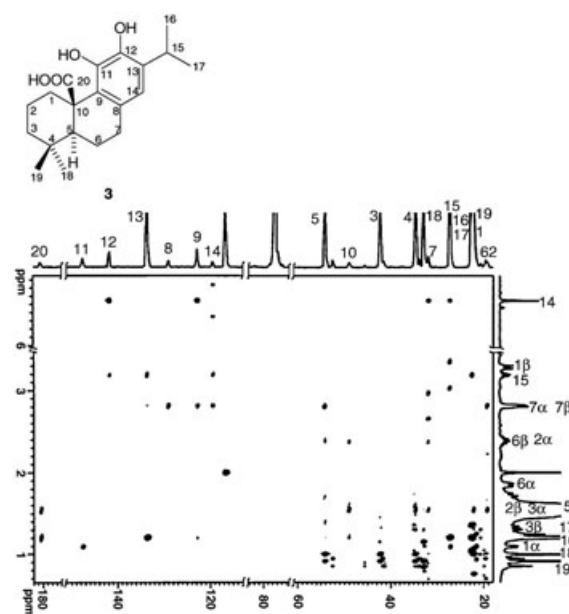


Fig. 4 HMBC spectrum recorded in the HPLC-SPE-NMR mode from an extract of *Rosmarinus officinalis* (Lamiaceae) following single trapping on a 2×10 mm polystyrene-type SPE cartridge (150×4.6 mm C₁₈ column eluted with CH₃CN gradient in H₂O, 400 MHz, 120-μL cell, cartridge eluted with CDCl₃). Reproduced from [46] by permission of Elsevier.

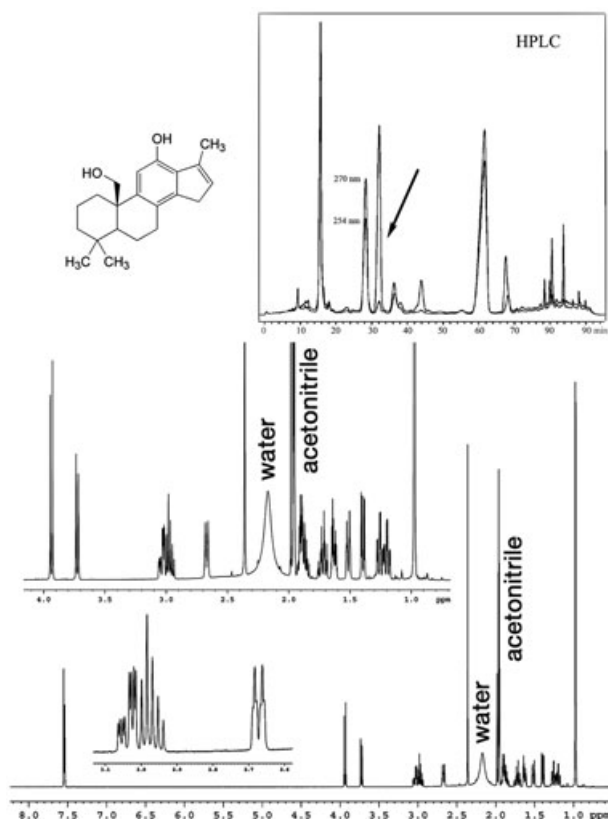


Fig. 5 600 MHz ^1H -NMR spectrum obtained in the HPLC-SPE-NMR mode of a diterpene present in the ethyl acetate extract of roots of *Perovskia atriplicifolia* (Lamiaceae). The crude extract (1.5 mg per injection) was separated on a 150×4.6 mm C_{18} column with CH_3CN gradient in H_2O . The arrow points to the peak analyzed (six trappings on a 2×10 mm C_{18} SPE cartridge, elution with CD_3CN , 30- μL cell NMR flow-cell, no solvent peak suppression). Unpublished work from the author's laboratory.

strengths is expected as the cost of high-field magnets drops. No systematic comparisons have yet been published, but 30- μL (active volume) flow-probes together with 2×10 mm SPE cartridges are believed to be preferable and were used in most of the aforementioned investigations [38], [40], [41], [42], [43], [44], [50].

Obviously, the success of HPLC-SPE-NMR experiment depends on trapping efficiency and very polar compounds are not expected to be trapped on reversed-phase materials. Also, very apolar compounds may be difficult to elute from the SPE cartridges with CD_3CN [50] and the use of other solvents such as CDCl_3 may be necessary. However, most classes of natural products appear to have suitable trapping and elution properties and even alkaloids eluted from HPLC columns with acid-containing eluents as charged species were trapped on polystyrene-type cartridges [47]. Moreover, an extension of the range of SPE materials used for HPLC-SPE-NMR experiments and further optimization of trapping parameters [52] as well as of flow-cell design can be envisaged.

Hyphenation of Other Techniques with HPLC-SPE-NMR

Standard HPLC-SPE-NMR installations include a photodiode array detector monitoring HPLC separation and triggering SPE

trappings (Fig. 1), and hence the technique is sometimes referred to as HPLC-UV-SPE-NMR or HPLC-DAD-SPE-NMR. Thus, UV spectra are available along with the NMR data.

Already in the case of direct HPLC-NMR hyphenations [1], most of reported studies are supported by mass spectrometry, either using a separate HPLC-MS installation, or by introducing a split in the effluent flow and acquiring MS data simultaneously with the NMR data (HPLC-NMR-MS). It is of course possible to combine MS on-line with HPLC-SPE-NMR [38], [40], [49] (Fig. 1). This extended hyphenation is useful not only by making MS or MS/MS data available simultaneously with the NMR data, but also because trapping of non-UV-absorbing constituents can be triggered by signals provided by MS (total ion or single ion monitoring). Furthermore, it can be mentioned that evaporative light scattering detection (ELS), already used in connection with stopped-flow HPLC-NMR [53], could be used to trigger trapping in a putative HPLC-ELS-SPE-NMR experiment.

In principle, other spectroscopic techniques such as CD [16], [54], [55], [56], or for special applications IR [57], might be hyphenated with HPLC-SPE-NMR. Chirality determination of natural products is especially important and CD spectra recorded with SPE-eluted material would benefit from increased concentration, use of pure solvents and the possibility of calibration of analyte concentration from the NMR response. Hyphenation of bioassays with HPLC-SPE-NMR is another exciting possibility [46].

Other New Trends in NMR Hyphenation

In an attempt to increase the sensitivity of NMR data acquisition, attention has been directed towards miniaturization of NMR coil design [58]. Small probe-volumes facilitate enclosing of a larger proportion of the total sample volume within the sensitive region of the probe (improved observe factor) and achieving maximal filling of the coil detection volume with the sample (improved filling factor). Moreover, the sensitivity per mass unit of miniaturized Helmholtz (saddle) coils used for vertical NMR tubes as well as vertical flow-cells used for direct HPLC-NMR hyphenation and for HPLC-SPE-NMR increases with reduction of coil length and diameter [59]. For these reasons, the 30- μL (sensitive volume) flow-probes used for HPLC-SPE-NMR experiments have a higher mass sensitivity than the 120- μL probes normally used for on-flow and stopped-flow HPLC-NMR.

Vertical capillary probes (with Helmholtz coils) having sensitive volumes down to 2.5 μL have already been used for some time instead of normal NMR tubes [58], [60]. However, additional sensitivity gain can be achieved with probes consisting of a solenoid wrapped around a horizontal capillary [58], [59], [61], [62]. The sensitivity of such probes is inversely proportional to the coil diameter and very short radiofrequency pulses at low power are possible with these microcoils, which can be as small as 50 μm in diameter.

The superior performance of solenoidal microcoils for the identification of natural products has been demonstrated using direct analyte injection [63], [64], [65], [66]. However, the main potential of microcoils is in their combination with microflow liquid

chromatography, resulting in capillary HPLC-NMR [67], [68], [69], [70], [71], [72], [73], [74]. Stopped-flow capillary HPLC-NMR was recently used for the identification of isoflavonoids in plant material [75]. Use of microcoil NMR detectors together with microbore or capillary HPLC separations, which operate at considerably higher analyte concentrations in elution bands, is a very promising new development within HPLC-NMR that enables high-throughput applications [76]. Further improvements are expected from use of micromachined coils [77] and multiple coil probes [69], [78], [79]. Miniaturization, SPE interfaces as well as the already mentioned cryogenic probe technology [80], [81], [82] are expected to continue to improve the sensitivity of HPLC-NMR experiments. Both the cryoprobes and the microcoil probes show a capability of making direct observation of ^{13}C NMR spectra (Fig. 6) feasible with analyte amounts that can be achieved in the HPLC-NMR mode, especially when combined with SPE. In addition, microcoils facilitate NMR detection in electro-driven capillary separations [83], [84], [85], [86], [87].

Since analyte quantification is an important aspect of NMR hyphenation, use of the ERETIC method (Electronic Reference To access *In vivo* Concentrations), originally introduced for internal calibration of *in vivo* NMR spectra [88], is of interest. The method, which relies on the generation of a synthetic reference signal with defined frequency and amplitude, was subsequently adapted for solution studies [89], [90], [91], and can be used as a means of quantification of HPLC-NMR data.

Another new development is the use of ultra-fast (single scan) acquisition of 2D NMR data relying on spatial encoding of spin interactions [92], [93], [94]; although this approach suffers from serious sensitivity limitations, its applicability for macro-scale on-flow LC-NMR has been demonstrated [95].

Conclusions

The HPLC-SPE-NMR technique is a very powerful tool for on-line extract dereplication, enabling routine access to high-quality 2D NMR data without distortion of the HPLC separation of the remaining extract constituents. In contrast to direct HPLC-NMR ex-

periments, the amount of analyte available for NMR studies is not limited to that provided by a single injection to the HPLC column. The broad applicability of the technique to natural products has already been demonstrated, and the technique is envisaged to be an increasingly important analytical platform for natural products research. HPLC-SPE-NMR and other modern, hyphenated NMR techniques are expected to provide an increasing number of rigorously determined natural product structures directly from extracts and isolates, including compounds present in minor amounts. These developments will enable high-throughput extract analysis for the selection of those of extract constituents that deserve isolation and purification on a preparative scale, and in perspective on-line combinations of separation, automatic structure elucidation and bioassays.

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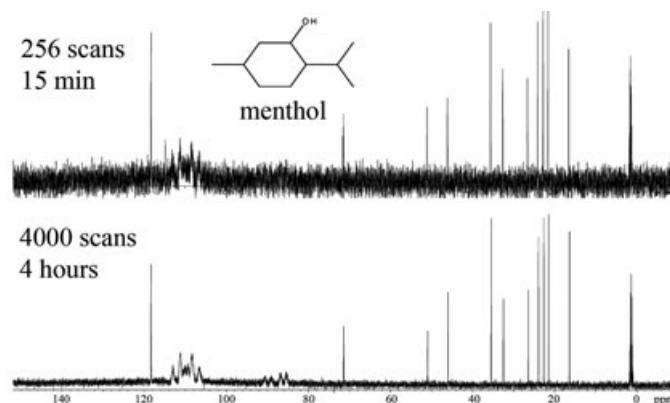


Fig. 6 Direct ^{13}C observation using solenoidal capillary probe (cell volume 5 μL , sensitive volume 1.5 μL , 330 μg of menthol, 150 MHz observation frequency); broad signals between 80–120 ppm are probe background. Reproduced from [71] by permission of the American Chemical Society.

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