Hyphenated NMR Methods in Natural Products Research, Part 1: Direct Hyphenation

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Abstract

This review describes the current status of the development of techniques combining liquid chromatography and nuclear magnetic resonance and their use in the context of natural products. HPLC-NMR methods have a rapidly growing impact on natural products research by enabling structure determination of natural products directly from small amounts of extracts, i.e., prior to the investment in an often lengthy and laborious preparative-scale isolation process. This speeds up extract dereplication and helps to avoid re-isolation of already known extract constituents.

Direct HPLC-NMR hyphenation techniques, defined as methods in which NMR spectra are recorded using the HPLC eluate, are described. The recently developed indirect HPLC-NMR method employing an automated solid-phase extraction interface between HPLC and NMR, which replaces the chromatographic solvent with a different solvent for NMR data acquisition, is described in the second part of this review.

Key words

Nuclear magnetic resonance · liquid chromatography · LC-NMR · HPLC-NMR · hyphenated techniques · dereplication

Introduction

Natural products are usually extracted as complex mixtures containing many constituents present in widely different concentrations and representing a broad spectrum of physical and chemical properties. Initially, the composition of extracts is often completely unknown. For these reasons, isolation and structure elucidation of individual constituents from such mixtures pose a considerable challenge, requiring a lot of time, labor and costs.

The reason for undertaking an isolation study is very often some sort of biological activity, observed in a bioassay or anticipated from traditional uses. However, a positive pharmacological response by itself is not sufficient to qualify an extract for fractionation work, nor is its absence sufficient to disqualify it (because of inherent difficulties with assaying mixtures and the possibility of detecting false positive or false negative results). Even if a genuine pharmacological response resides in a single and well-defined constituent, it may not merit purification because the constituent is already known, trivial, or otherwise unwanted. Thus, the problem of isolation of pharmacologically active natural products quickly becomes chemical in nature, not only because one must decide about the methods of fractionation and purification (which depend on the chemical properties of the constituent in question), but also because it is desirable to know, as early in the fractionation process as possible, what is being purified. For these reasons, a rapid establishment of a bridge between a confirmed or anticipated biological activity and chemical structure (or at least a general chemical nature) of extract constituents present is a central issue in modern natural products research. The library approach [1], [2] for the discovery of drugs from natural sources also calls for use of targeted isolation procedures that necessitate early access to reliable structural information about extract constituents.

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In fact, seeking information about the chemical nature or identity of compounds present in an extract prior to their isolation is as old as the chemistry of natural products itself, and organoleptic tests and various color reactions have been commonly used in the past. Since identification of compounds directly from a complex mixture can normally be achieved only for the most abundant constituents, there is a need for combining a separation method with a method giving insight into the chemical structure. Hyphenated methods are thus defined as methods combining two or more analytical techniques (usually a separation and a spectroscopic technique) into one integrated technique described by use of a hyphen, as in HPLC-NMR. Although a modern hyphenated technique implies a computer-controlled on-line process, long-known combinations of paper chromatography or TLC with compound-class specific spray reagents may be regarded as classical hyphenated techniques. The introduction of photodiode-array (PDA) detectors providing UV spectra of compounds eluted from chromatographic columns constituted a great advantage compared to single-wavelength UV-VIS detectors, but structural information provided by electronic spectra is quite limited. During recent decades, MS methods combined with GC and more recently with LC (usually implemented as HPLC) had a great impact on natural products research. However, GC-MS is restricted to volatile constituents, and the ionization techniques used in LC-MS usually give information about the molecular ion and the basic fragments only. Thus, only NMR spectroscopy with its broad repertoire of highly sophisticated 2D experiments can be used for making definitive claims about chemical structures of more complex natural products, both new and already described. A brief overview of main hyphenated techniques used in natural products research is shown in Table **1**.

In this article, an overview of theory and applications of hyphenated NMR spectroscopic methods pertinent to or used in the context of natural products is given. Applications not related to natural products, such as those concerned with pharmaceutical analysis or drug metabolism, are not mentioned unless they describe important technical advancements. Part 1 of this review deals with direct HPLC-NMR methods, i.e., methods in which the HPLC system is connected directly to a flow-cell of the NMR spectrometer operating in continuous-flow or stopped-flow mode. The loop-storage technique, which still involves acquisition of NMR data in the solvent used for chromatography, is also described**.** The recently introduced HPLC-SPE-NMR technique is described in Part 2. Because the vast majority of LC methods hyphenated with NMR spectroscopy are standard HPLC methods, the term HPLC-NMR is used rather than LC-NMR.

Principles of HPLC-NMR

Today, NMR spectroscopy is certainly the most powerful and versatile technique for structure elucidation of natural products. Its advantage as a HPLC detector resides not only in the fact that full structural and stereochemical information can be obtained (by use of 2D NMR), but also because it is a highly non-selective detection technique (^{1}H-NMR spectroscopy will detect any hydrogen-containing compound present in the HPLC eluate in a sufficient amount regardless of its structure). The interest in combining separation methods with ^{1}H-NMR spectroscopy arose

Table **1** Overview of main hyphenated techniques used in natural products research

Hyphenated technique	Principal advantages	Principal limitations
LC-PDA	Rapid, easy to use and cheap	Structural information provided by UV spectra very limited
GC-MS	Excellent resolving power of GC, high information content of electron-impact mass spectra (EI-MS)	Restricted to volatile samples; best applicable to known compounds for which EI-MS fingerprints are available for automatic (unsupervised) structure determination
LC-MS	Excellent resolving power of LC methods, applicable to non-volatile constituents	Structural information provided by electrospray ionization MS limited (extendable by MS/MS and high-resolution MS); ionizability of various compounds vary greatly
LC-NMR	Excellent resolving power of LC methods; highly non-selective; full structural information in principle available	Lower sensitivity compared to other LC detectors; automatic (unsupervised) structure elucidation still in infancy

already at the end of the 1970 s, when the capabilities and potential of this spectroscopic technique had become well recognized [3], [4], [5], [6], [7]. The initial trials used iron magnets, but the advantage of superconducting solenoid magnets was quickly recognized [8], [9]. However, it took almost two decades before the HPLC-NMR combination started to provide competitive results. In addition to the need for appropriately designed flow-cells [10], the principal issues in relation to the use of NMR spectrometers as detectors for liquid chromatography are sensitivity and solvent choice.

NMR is a comparatively insensitive spectroscopic technique, a fact that originates from the small difference between energy levels of atomic nuclei, which leaves only a small population excess of spins occupying the ground state at thermal equilibrium and giving rise to the NMR signal. However, already early iron-magnet spectrometers exhibited sensitivity comparable to that of refractive index (RI) detectors [4], and the sensitivity of commercial NMR instruments improves continuously [11], [12], [13]. Under ideal conditions, the signal-to-noise ratio in NMR is proportional to the 7/4 power of the magnetic field strength. While HPLC-NMR applications at 800 MHz have been reported [14], use of ultra-high field strengths is associated with excessive costs. On the other hand, small-volume (30 – 120 μL detection volume) flow-probes in current use offer a mass sensitivity (signal-to-noise ratio per mass unit) that is much greater than that of traditional NMR probes. Using a 600 MHz magnet, the limit of ^{1}H detection of a compound with MW 500 is roughly 100 ng [15], but micrograms would be required for routine structure elucidation work. The sensitivity of NMR detection increases three- or fourfold by use of probes with cryogenically cooled receiver coils and preamplifiers [12], [16]; the sensitivity gain achieved by cooling these electronic components to about 20 K is due to the reduced electronic noise. Thus, use of cryogenic probes enables NMR

spectra to be obtained with 3 – 4 times less material during a given data acquisition period, or to reduce the data acquisition time by a factor of 9 – 16 with the same sample size. Therefore, the current capability of obtaining good quality 1D ¹H-NMR spectra with sub-microgram amounts and to record ¹H-detected 2D NMR spectra with microgram quantities is very sufficient for natural products applications, especially when one considers that the method is fully non-destructive and analytical HPLC columns can usually be loaded with several hundred micrograms of extracts. A further decrease of detection limit is expected as a result of continued technology improvements.

An important issue is the solvent used for chromatographic separation, because the problem is not just to detect a given amount of material but also to do so in the dilute solution emerging from an HPLC column. Since most solvents contain hydrogen, they will give rise to an NMR signal much stronger than that of the analyte. Thus, a dynamic range problem of detecting small analyte resonances in the presence of $10^3 - 10^6$ times stronger solvent resonances arises, and it is necessary to apply pulse sequences that suppress solvent resonances. Most commonly used solvent-peak suppression pulse sequences are the standard 1D NOESY sequence applying soft pulses on solvent resonances during mixing time and relaxation delay for 1D spectra, and the pulsed field-gradient-based WET sequence (water suppression enhanced through T_1 effects) for 1D and 2D spectra [17]. It must be emphasized that solvent peak suppression methods only greatly reduce the intensity of solvent resonances, rather than removing them completely. The larger the initial solvent peak, the more difficult it is to suppress it. Moreover, the suppression brings about distortion of the spectral region where the solvent resonances were present, and distorts or removes nearest analyte resonances. In order to avoid problems with very strong and difficult or impossible to suppress solvent resonances, deuterated solvents are frequently used for chromatographic separations. This applies especially to water, which gives a relatively broad and difficult to suppress signal. Therefore, mixtures of D_2O and CH_3CN (usually not deuterated because of excessive cost) are the most frequently used solvents for reversed-phase (C_{18}) elution in HPLC-NMR, in gradient as well as isocratic mode. Because deuterated solvents usually contain 99.8 atom % of deuterium, suppression of the residual solvent signal will still be necessary. Since 1.1 % of the ¹H resonance of CH_3CN consists of $^{13}CH_3CN$, solvent suppression will be further improved by applying ^{13}C decoupling that collapses ^{13}C-satellites in the ¹H-NMR spectra.

It must be stressed that HPLC separations should be carefully optimized for high column loading and for elution bands that are as sharp as possible. Broad and unsymmetrical peaks diminish the analyte concentration in the eluate and compromise the quality of NMR spectra. The sensitivity of continuous-flow HPLC-NMR experiments will also be limited by the residence time of the analyte in the NMR flow-cell. Short residence times reduce effective lifetimes of a particular excited spin state and increase NMR line-width, depending on a particular ratio between cell volume and flow rate [18], [19], [20]. In order to perform NMR data accumulations that require longer time, such as 2D NMR experiments or 1D experiments with small amounts of the analyte, the flow must be stopped when the chromatographic peak of interest

reaches the NMR cell. In the stopped-flow mode, compounds that still reside within the HPLC column will be broadened by diffusion, which can compromise NMR experiments with the remaining peaks. In order to avoid these problems, peak-storage devices for HPLC-NMR have been introduced. In the following paragraphs the use of various HPLC-NMR schemes to natural products is illustrated.

Continuous-Flow HPLC-NMR

As already mentioned, limited residence time of the analyte in the NMR flow-cell affects the spectral quality and limits the time available for the acquisition, which restricts the detectable amount of analyte to above 10 μg (compounds with MW 300 – 500), depending on experimental conditions. Nevertheless, on-flow experiments were the first HPLC-NMR experiments to be introduced and are still frequently used to gain an overview of the major metabolites present in an extract. They are rarely used as an isolated HPLC-NMR experiment but usually as an introductory experiment followed by stopped-flow investigations. In an early study (1994), Johnson et al. performed a model on-flow experiment with a mixture of limonoids using a 250×4.6 mm C_{18} column and a 500 MHz spectrometer equipped with a 60-μL flow-probe [21], and Spring et al. analyzed a crude dichloromethane extract of *Zaluzania grayana* (Asteraceae) using a similar set-up [22]. Hostettmann and Wolfender reported numerous on-flow HPLC-NMR experiments at 500 MHz [23], [24], [25], [26], [27], [28], [29], [30], [31]. These include analysis of a crude dichloromethane extract of *Swertia calycina* (Gentianaceae) leading to identification of three major extract constituents [23], identification of two main constituents in a crude dichloromethane extract of *Monotes engleri* (Dipterocarpaceae) [24], the study of an antioxidant fraction obtained from *Orophea enneandra* leaves (Annonaceae) [25], analysis of a crude dichloromethane extract of *Cordia linnaei* (Boraginaceae) [26], detection of isoflavanones in a crude dichloromethane extract of roots of *Erythrina vogelii* (Fabaceae) [27], investigation of decomposition products of hyperforin [28], analysis of tropane alkaloids from an alkaloid extract of *Erythroxylum vacciniifolium* (Erythroxylaceae) stem bark [29], and studies on dichloromethane and methanol extracts of *Potamogeton* (Potamogetonaceae) species [30], [31]. All these studies employed C_{18} columns and gradients of CH_3CN in D_2O. In some studies large (100×8 mm) columns were used, enabling column loadings of up to 10 mg extract, and eluted at very low flow rates (down to 0.1 mL/min) to increase analyte residence time in the 60-μL flow-cell used [27], [29], [30], [31]. Bringmann et al. used on-flow NMR typically operating in the isocratic mode with CH_3CN and D_2O mixtures at 0.7 – 0.8 mL/min, using a 250×4.6 mm column and a 120-μL flow-cell to detect naphthylisoquinoline alkaloids in crude plant extracts [32], [33], [34], [35]. Other continuous-flow studies reported in the literature include identification of constituents of a sesquiterpene lactone fraction from *Vernonia fastigiata* (Asteraceae) [36], use of the technique as guidance for pooling of separated glycolipid fractions [37], analysis of hop bitter acids extracted with supercritical CO_2 [38], and identification of ecdysteroids of *Silene otides* (Caryophyllaceae) [39]. Further examples include analysis of natural products presents in beverages [40], [41], analysis of *Streptomyces* metabolites in culture broth [42], analysis of caro-
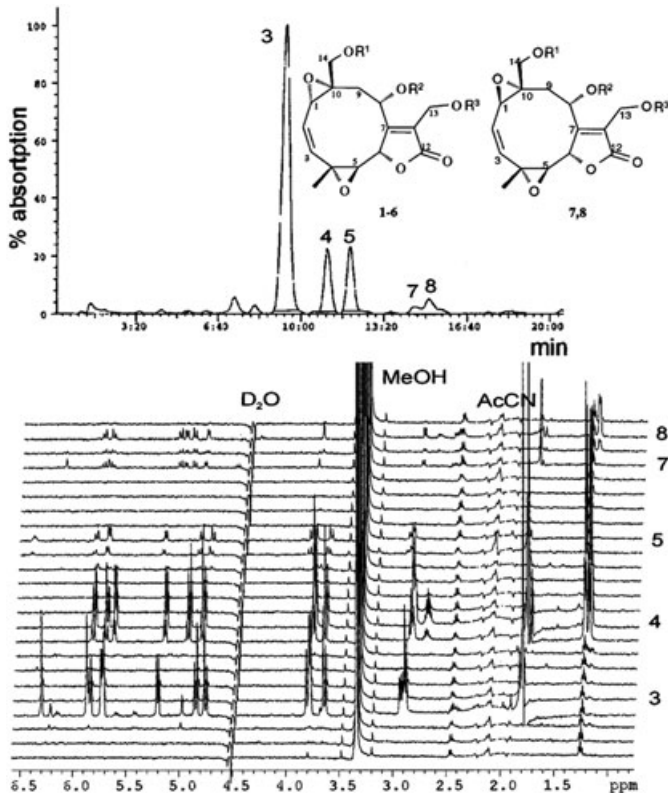
Fig. 1 *Bottom:* Continuous-flow HPLC-NMR analysis of a sesquiterpene lactone fraction from *Vernonia fastigata* (500 MHz, 65-µL flow-cell, C$_{18}$ column eluted with a CH$_3$CN gradient in D$_2$O at 0.9 mL/min, column loading 0.7 mg). *Top:* HPLC-UV chromatogram of the fraction. Reproduced from [36] by permission of the American Chemical Society.

tenoid mixtures using C$_{30}$ stationary phases, which have a high loading capacity [43], [44], investigation of a saponin fraction isolated from *Bacopa monniera* (Scrophulariaceae) [45], characterization of cardiac glycosides from *in vitro* cultured *Isoplexis* species (Scrophulariaceae) [46], analysis of cytotoxic fractions of *Stauranthus perforatus* (Rutaceae) [47], use of on-flow HPLC-NMR to guide isolation of sesquiterpene lactones from *Scalesia* and *Viguiera* species (Asteraceae) [48], [49], [50], and analysis of a mixture of saponins isolated from starfish [51], [52]. The latter study [52], apart from providing structures of a number of starfish saponins, emphasized the importance of relaxation times in HPLC-NMR measurements. Examples of on-flow HPLC-NMR data are shown in Figs. **1** and **2**.

In conclusion, on-flow measurements provide only preliminary information about extract or fraction components, normally restricted to major components. One advantage of on-flow HPLC-NMR experiments is that they do not require any specialized hardware apart from the NMR flow-cell. Because other solvents have a reputation of being difficult to wash out from HPLC-NMR systems, acetonitrile is widely used as the organic component of mobile phases, but examples of the use of acetone [44] and methanol [45], [50] have been reported. Cases reported in the literature usually employ D$_2$O-CH$_3$CN mixtures, using isocratic as well as gradient elution. In the latter case, the chemical shift of CH$_3$CN changes with the composition of the mobile phase, which may affect spectral resolution and quality of solvent suppression. One study employed a gradient of CD$_3$CN in D$_2$O [39]. In order to avoid potentially deteriorating effects of magnetic susceptibility changes of the HPLC eluate during gradient elution, post-column solvent mixing to obtain a constant solvent composition in the NMR flow-cell was proposed [53]. Quantitative analysis by on-flow NMR measurements has been discussed [54], [55].
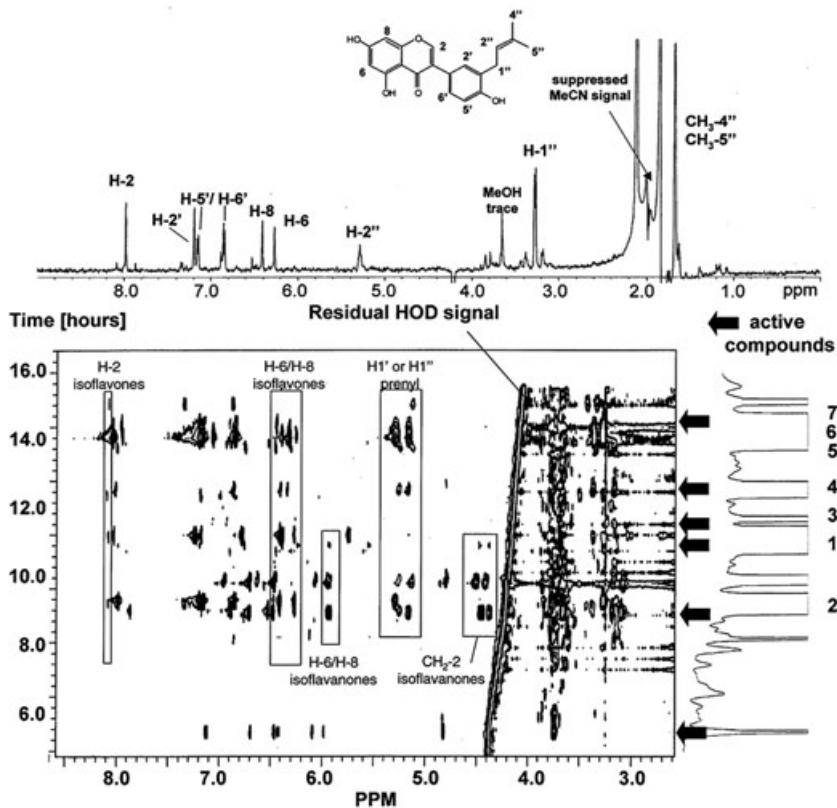

Fig. 2 *Bottom:* Continuous-flow HPLC-NMR contour plot obtained with a crude dichloromethane extract of *Erythrina vogelii* roots; horizontal and vertical axis represent ^{1}H-NMR chemical shifts and HPLC retention times, respectively (500 MHz, 60-µL flow-cell, 100×8 mm C$_{18}$ column eluted with a CH$_3$CN gradient in D$_2$O at 0.1 mL/min). *Top:* Slice though the contour plot representing ^{1}H-NMR spectrum of one extract component. Reproduced from [27] by permission of Elsevier.

Stopped-Flow Measurements

2D NMR experiments, necessary for definitive structural assignments except in the simplest cases, are not feasible under continuous-flow conditions because of insufficient sensitivity. While extremely slow elution rates [56] can compensate for this limitation, the usual solution is to acquire spectra under stopped-flow conditions. Therefore, nearly all continuous-flow studies mentioned above also report stopped-flow experiments, either in order to improve the quality of ^{1}H-NMR data or to perform 2D NMR experiments. It is perhaps fair to say that, in order to achieve uniquely useful HPLC-NMR data, stopped-flow conditions must be involved. These conditions also allow enough time for careful optimization of field homogeneity and acquisition parameters.

Most studies use UV absorption to select peaks for stopped-flow NMR acquisition, although MS signals can also be used. The analyte can be kept for a very long time in the NMR flow-cell without problems caused by diffusion, allowing acquisition of very time-consuming experiments. This is a function of the flow-probe design as well as the fact that peak elution volumes are usually larger than the total volume of the flow-cells used for HPLC-NMR, and thus thin tubing on both sides of the NMR flow-cell contains practically the same analyte concentrations as the cell. After all stopped-flow NMR experiments with a particular peak are completed, the flow may be restored, the next peak transferred to the NMR flow-cell, and so on. Diffusion-mediated band broadening of analytes still within the column may vary from very serious to insignificant, depending on the particular chromatographic conditions and the time period with stopped flow. Arguably, in the case of gradient elution, most of the analyte remaining at the column may be confined to a narrow region within the stationary phase at the column entrance, and sharp peaks may be eluted in spite of prolonged periods without flow. In other cases, stopped-flow conditions will result in a substantial decrease of concentration within elution bands and collapse closely spaced peaks, not only because of analyte diffusion but also because of diminishing solvent gradients across the column due to the diffusion of solvent molecules. In such cases one can acquire NMR data with different peaks from successive chromatographic runs (if no prohibitive limits to sample size exist). An analyte eluted as a large peak may contaminate the following, minor peaks because of the peak-broadening effect of the NMR flow-cell.
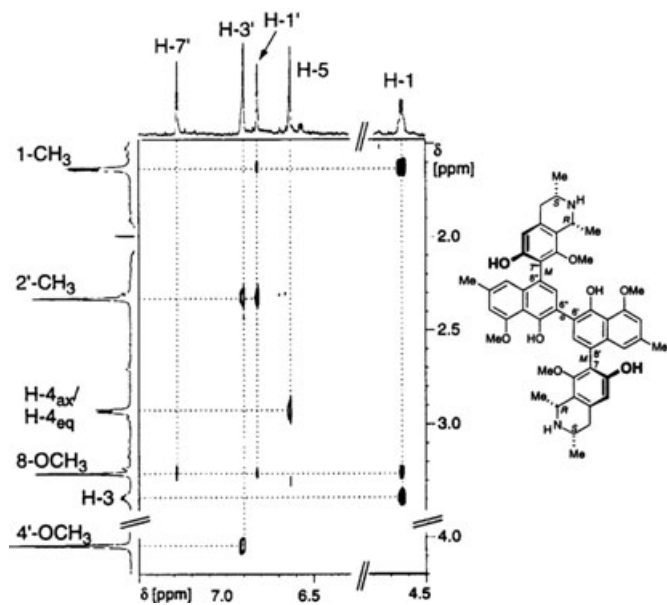
Numerous stopped-flow experiments with crude extracts or pre-purified fractions have been reported in the literature. The chromatographic conditions used are generally the same as in on-flow experiments, with CH_3CN-D_2O mixtures as eluents, various

Fig. 3 Part of ROESY spectrum of a dimeric naphthylisoquinoline alkaloid from a pre-purified extract of *Ancistrocladus griffithii*, acquired in the stopped-flow mode (600 MHz, 60-μL flow-probe, total acquisition time 17 h, 2 mg of the extract injected to a C_{18} column eluted with a gradient of CH_3CN in D_2O). Reproduced from [60] by permission of the American Chemical Society.
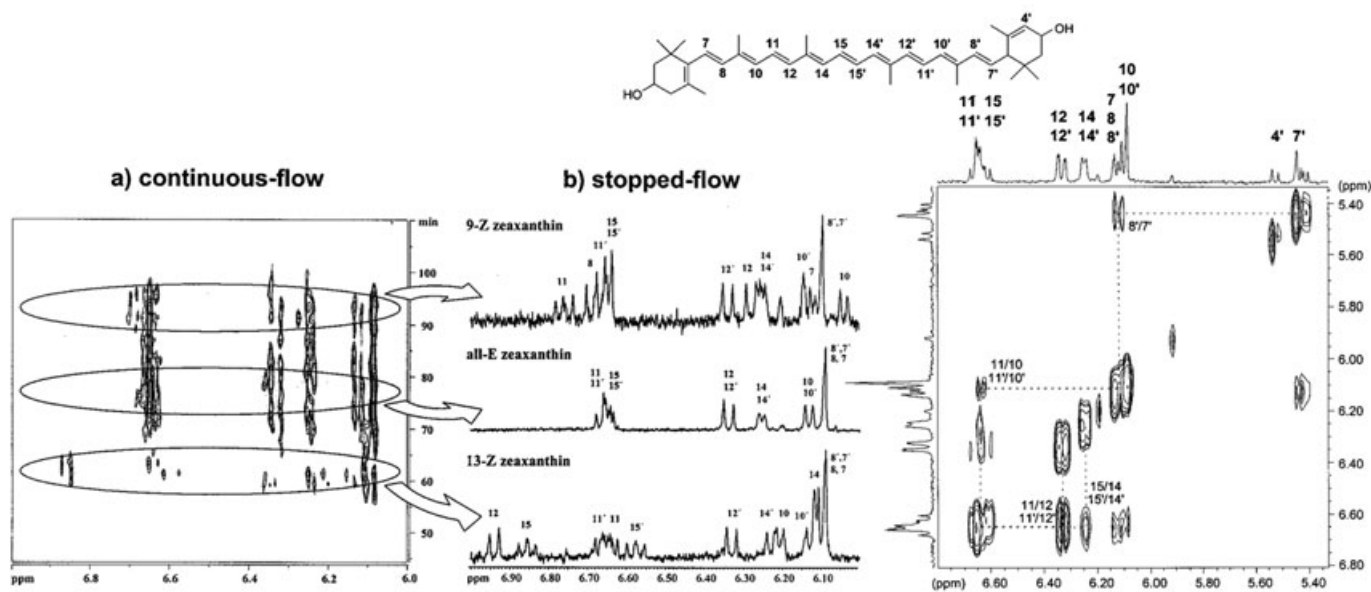

Fig. 4 *Left:* Continuous-flow HPLC-NMR analysis of a carotenoid mixture. *Middle:* Stopped-flow ^{1}H-NMR spectra obtained from the same mixture (600 MHz, 120-μL flow-cell, C_{30} column eluted with acetone-D_2O at 1 mL/min). *Right:* COSY spectrum recorded under stopped-flow conditions with a spinach extract (total acquisition time 25 h). Reproduced from [44] by permission of the American Chemical Society.
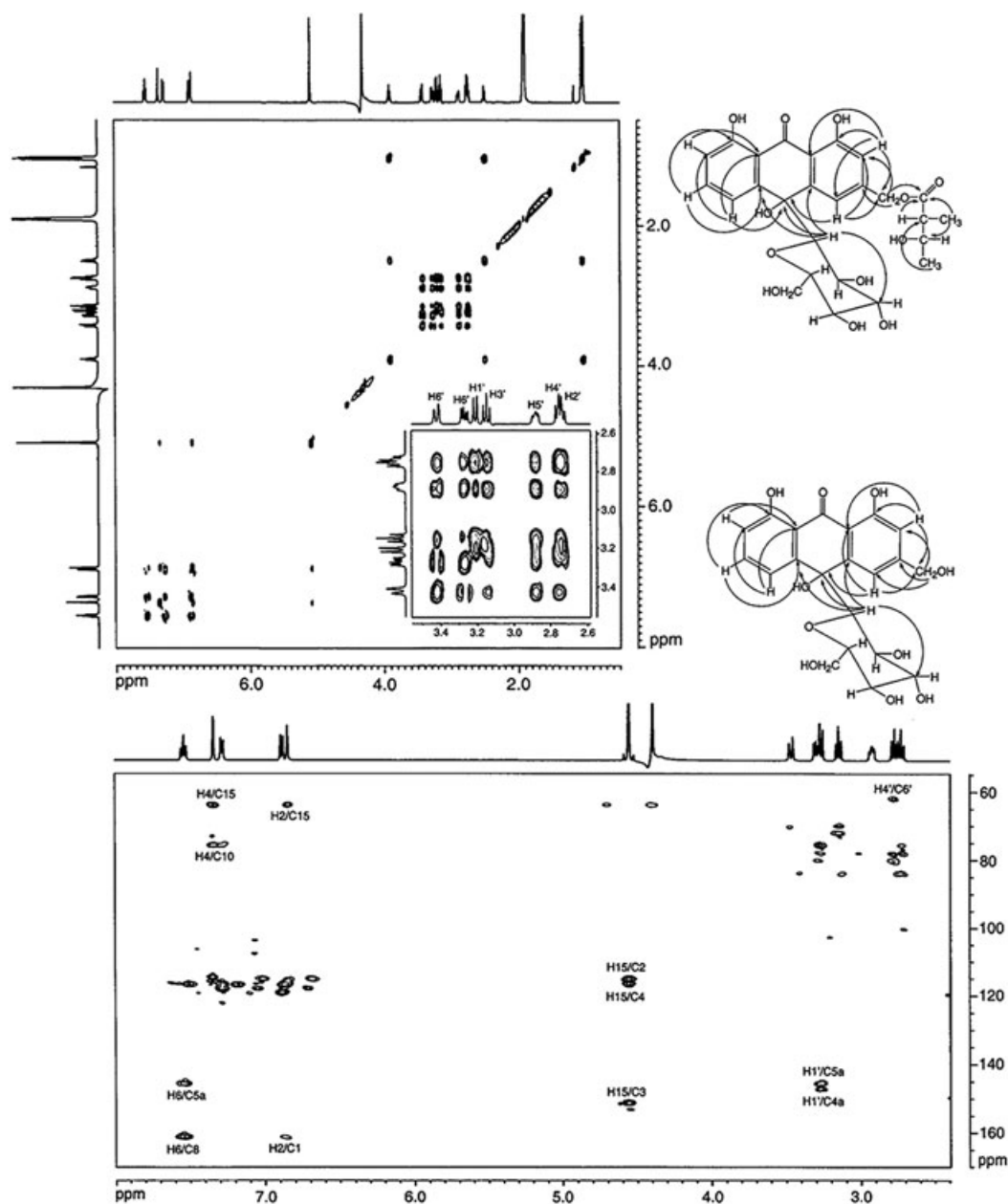
Fig. **5** Stopped-flow TOCSY (*top*) and HMBC (*bottom*) spectra acquired in the stopped-flow mode with *Aloe littoralis* leaf exudate (500 MHz, 120-μL cell, C_{18} column eluted with a CH_3CN gradient in D_2O, 1.6 mg mixture injected); because of the long data acquisition times, the spectra were acquired from separate injections. Reproduced from [79] by permission of John Wiley and Sons.

sizes of analytical columns (typically 250 × 4.6 mm), and column loadings usually close to 1 mg. Bringmann et al. exploited the power of 2D NMR experiments acquired in the stopped-flow mode [32], [33], [34], [35], [57], [58], [59], [60], [61]. An example of a 2D NMR through-space correlation obtained in the stopped-flow mode with a fractionated extract is shown in Fig. **3**. The previously mentioned C_{30} reversed-phase material was applied in stopped-flow investigations of samples containing flavonoids [62], [63] and carotenoids [43], [44], [64] (Fig. **4**). Other reported stopped-flow experiments deal with hop bitter acids [38], [65], starfish saponins [51], ecdysteroids of *Silene otites* (Caryophyllaceae) [39], [66], taxanes from *Taxus* species (Taxaceae) [67], metabolites from *Anigozanthos* species (Haemodoraceae) [68], [69], [70], lignans from *Torreya jackii* (Taxaceae) [71], extract of St. John's wort [72], *Agromyces* culture broth extract [73], a lignan glucoside from flax seed [74], flavonoids in *Sorocea bomplandii* (Moraceae) [75] and *Trifolium pratense* (Fabaceae) extract [76], protoberberine alkaloid fraction from *Corydalis* (Fumariaceae)

cell cultures [77], [78], major metabolites of *Aloe littoralis* (Asphodelacaea) [79] (Fig. **5**), flavonoid glycosides in transgenic tomatoes [80], sesame oil lignans [81], quinic acid derivatives from *Fagara zanthoxyloides* (Rutaceae) [82], betalain fraction from *Hylocereus polyrhizus* (Cactaceae) [83], phthalides from *Ligusticum chuanxiong* (Apiaceae) [84], and metabolites from several Chinese plants [85]. Moreover, various stopped-flow spectra were reported by the Hostettmann group [23], [24], [28], [29], [86], [87], [88], [89]. One study described use of a tetraethylammonium salt as a chromatographic ion-pairing agent, the signals of which had to be suppressed in stopped-flow HPLC-NMR experiments [89]. Selective (1D) versions of 2D correlation experiments, which can provide crucial information in a very short time, have so far been only rarely used in HPLC-NMR studies of natural products [84], [90].

In addition to the simple, direct stop-flow experiments mentioned above, it is possible to stop the flow in regular intervals

covering a part or the whole chromatogram. This time-slice stopped-flow experiment is an extension of the on-flow experiment with a very slow elution rate and has the advantage of being independent of other methods for detecting the location of chromatographic peaks; it was used for the analysis of beer constituents [40].

Loop-Storage Technique

In order to avoid potential problems with diffusion and pump start/stop effects on chromatograms, a device was developed that directs individual chromatographic peaks into capillary loops, where they are sealed and can be subsequently transferred into the NMR flow-probe in an arbitrary order. Since the loops are made of capillaries and not cavities as the NMR flow-cells, only minimal peak broadening occurs. Moreover, the flow-cell can be extensively washed between transfers of individual peaks stored in the loops, and thus analyte cross-contamination can be avoided. Therefore, this HPLC-NMR experiment has most of the advantages and no drawbacks inherent to the stopped-flow experiment, perhaps with the exception of increasing chance of decomposition of unstable constituents. However, only very few applications of this technique to natural products have been reported. One example describes the analysis of an artificial mixture of aporphine alkaloids [91]. Loop-storage HPLC-NMR analysis (including 2D NMR) of acetylated *xylo*-oligosaccharides using chromatographic separation with a CH_3OH gradient in D_2O was also reported [92]. Other examples include analysis of liquid food samples [40], [41], extract of apple peel [90], and sesquiterpene lactones [22]. The loop-storage technique can be regarded as intermediate between direct and indirect HPLC-NMR methods; although acquisition of NMR data can be physically and chronologically disconnected from the HPLC separation, the NMR data are still obtained in the solvent used for HPLC.

Other Direct NMR Hyphenations

In principle, NMR can be hyphenated with liquid-chromatographic methods other than HPLC. However, such techniques are in their infancy and not widely used. Since supercritical fluid (SF) extraction and chromatography (SFC) employing CO_2 with various modifiers are broadly applicable within the natural products field, hyphenation of supercritical separations with NMR is of potential interest, especially because normal-phase separation mechanisms in SFC supplement those of reversed-phase HPLC. As CO_2 does not contain hydrogen, the need of solvent suppression is limited to possible modifiers. The first report on SFC-NMR hyphenation appeared already in 1988 [93] and the field was reviewed relatively recently [94]; genuine natural products applications have yet to be reported.

Since SFC is intermediate between gas and liquid chromatography, it should be mentioned that some early attempts of GC-NMR hyphenation have been made [95], [96], [97], [98]. However, since modern GC separations are performed practically exclusively with capillary columns, the amount of material is insufficient for NMR, in addition to the purely technical difficulties that exist [94].

Several studies explored the use of superheated D_2O as eluent for HPLC-NMR [99], [100]. Unlike supercritical water, which requires very high temperatures and is chemically aggressive, superheated water is a relatively inert eluent applicable for reversed-phase HPLC; stopped-flow applications to ginger extract on a C_{18} phase [101] and to vitamins on polystyrene-divinylbenzene [102] have been reported. NMR hyphenation with ion-exchange chromatography [103], centrifugal partition chromatography [104], gel permeation chromatography [105] and electro-driven separations [106] have been described.

Finally, it should be mentioned that although HPLC-NMR is capable of providing very detailed and unambiguous data on the molecular structure of natural products, it does not give any information about chirality. Therefore, hyphenation of HPLC with circular dichroism (HPLC-CD) is a useful supplement to HPLC-NMR [35], [59], [60], [61], [107], [108]. A further extension of enantiomer characterization in connection with HPLC-NMR is the use of chiral HPLC columns [107] and HPLC-NMR of Mosher-type esters [109], [110], [111]. The latter is an elegant way of combining the need of obtaining ^{1}H-NMR chemical shift data with purification of microgram amounts of Mosher-type derivatives.

New Trend: Indirect NMR Hyphenation

Since its introduction in the late 1970s, HPLC-NMR has been developed from a scientific curiosity to a reliable technique that constitutes a crucial step towards rapid structure determination of natural products directly from mixtures (crude extracts). However, the common feature of the methods described in this review is that the HPLC side determines to a large extent the performance of the NMR part. Thus, NMR data are acquired at the analyte concentration delivered in the elution band and in the solvent used for HPLC. This is not optimal from the point of view of NMR spectroscopy due to various problems mentioned above and limits the range of suitable HPLC eluents. Moreover, the chemical shift data obtained with mixed solvents of varying composition are not directly comparable with the legacy chemical shift data on natural products available in the literature. The recently introduced HPLC-SPE-NMR technique, where solvent change between HPLC and NMR is achieved by use of a solid-phase extraction (SPE) interface, creates a possibility of running reversed-phase HPLC separations as well as NMR acquisitions at optimal conditions and largely without mutual restrictions. Evidence accumulates that this new hyphenated technique offers dramatic improvements in the performance of structure elucidation of natural products directly from crude extracts [112], [113], [114], [115]. Detailed discussion of HPLC-SPE-NMR, together with presentation of other newest developments in NMR hyphenation, will be given in a forthcoming, second part of this review.

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