amounts of material, e.g. column chromatography using relatively cheap stationary phases (silica, alumina, polyamide or XAD ion exchange resins), flash chromatography or CCC. Size exclusion chromatography (SEC) is also becoming increasingly popular as a first purification step. Subsequent chromatographic steps on smaller quantities can be performed with more expensive column packings and equipment. Semipreparative HPLC is often reserved for final purification.

The combination of different preparative chromatography techniques for the isolation of various aromatic compounds from *Inulanthera nuda* (Asteraceae) is presented in **Figure 9**. A first liquid–liquid partition of the methanolic extract, dissolved in water, gave an enriched butanol extract which was further separated by CPC, affording 13 fractions. One of these fractions yielded (**20**) after recrystallization while the other constituents were further separated by MPLC or SEC. The final purification of flavonoid glycoside (**25**), for example, required a combination of SEC on Sephadex LH-20, followed by MPLC and semipreparative HPLC.

Conclusions

The introduction of modern liquid chromatographic methods has revolutionized the science of separation of natural products of plant origin. These new methods allow faster separations and facilitate the resolution of complex mixtures. As shown, techniques such as HPLC can be used both at the analytical and preparative level. At the analytical level and in combination with sophisticated detectors, structural information can be obtained online, while on the preparative scale closely related compounds can be successfully isolated.

The actual separation method or methods depend(s) on a number of factors relevant to the separation problem, but a judicial choice of strategy enables most targets to be reached. New methods and improvements are continually being introduced, with the result that the number of combinations available is steadily expanding, hopefully leading to a progressive simplification of the ever more complex separation problems that are being undertaken.

See also: **II/Chromatography: Liquid:** Detectors: Mass Spectrometry; Large-Scale Liquid Chromatography. **Extraction:** Solid-Phase Extraction. **III/Flash Chromatography. Medium-Pressure Liquid Chromatography. Natural Products:** High-Speed Countercurrent Chromatography; Thin-Layer (Planar) Chromatography. **Pigments:** Thin-Layer (Planar) Chromatography.

Further Reading

Bidlingmeyer BA (1987) *Preparative Liquid Chromatography*. Amsterdam: Elsevier.

Hostettmann K, Wolfender J-L and Rodriguez S (1997) Rapid detection and subsequent isolation of bioactive constituents of crude plant extracts. *Planta Medica* 63: 2.

Hostettmann K, Marston A and Hostettmann M (1998) *Preparative Chromatography Techniques*, *Applications in Natural Product Isolation*. Berlin: Springer.

Kingston DGI (1979) High performance liquid chromatography of natural products. *Journal of Natural Products* 42: 237.

Marston A and Hostettmann K (1991) Modern separation methods. *Natural Product Reports* 8: 391.

Marston A and Hostettmann K (1994) Counter-current chromatography as a preparative tool – application and perspectives. *Journal of Chromatography A* 658: 315.

Wolfender J-L, Maillard M and Hostettmann K (1994). Thermospray liquid chromatography–mass spectrometry in phytochemical analysis. *Phytochemical Analysis* 5: 153.

Liquid Chromatography–Nuclear Magnetic Resonance

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Hyphenation of chromatographic and spectroscopic methods is important in analytical chemistry and is of great value in modern natural product analysis. Gas chromatography–mass spectrometry (GC-MS) has been used for many years to analyse volatile compounds and derivatives of nonvolatile natural products. The development of liquid chromatography–mass spectrometry (LC-MS) extended the scope of MS coupling techniques to allow analysis of nonvolatile compounds without derivatization. Nuclear magnetic resonance (NMR) is less sensitive than MS but represents the most informative and most universal analytical technique for natural products. Thus, using a NMR spectrometer in coupling methods does not simply mean adding another detector but represents a new dimension in analytical natural product chemistry.

The combination of NMR and chromatographic, or electrophoretic, separation methods was made

possible by the introduction of high field spectrometers, with an increased dynamic receiver range, the development of suitable continuous-flow cell probes and solvent suppression techniques. High performance liquid chromatography (HPLC)-NMR was first described by Watanabe in 1979 and it is now an established method in the field of natural product research. A number of examples, mainly of plant natural products, are reviewed in this article, demonstrating the advantages and limitation of HPLC-NMR.

Methodology

Sample Preparation

As sensitivity of HPLC-NMR is currently in the microgram or even nanogram scale, the amount of tissue investigated can be dramatically reduced as compared with that required for conventional isolation of natural products. Small scale extractions using 0.5–1 g of dried plant tissue have been described as being sufficient to record HPLC-^{1}H NMR spectra with an excellent signal-to-noise ratio. Natural products which are present in living tissue in only trace amounts (pg g^{-1} tissue) are now amenable to NMR analysis without isolation, either directly in the crude extract, or after employing simple work-up steps.

The procedures for sample preparation are essentially the same as those for normal analytical HPLC. Since extracts of biological tissues are normally complex mixtures of various substances covering a broad range of polarity, including both lipophilic and hydrophilic components, pre-purification or fractionation of the crude extract can often improve the chromatographic resolution. Enrichment of the desired natural product prevents overloading of the column by unwanted components and enhances the concentration of analytes above the detection limit. Due to the use of a NMR spectrometer as detector, deuterated solvents are strongly recommended for injecting the analyte into the chromatographic system.

HPLC

There are only a few special requirements for HPLC combined online with NMR. A pulsation-free HPLC pump to provide proper gradient formation and efficient solvent mixing should be used. The first detector cell (usually UV), which in HPLC-NMR is no longer at the end of the process, should be as small as possible to reduce peak broadening to a minimum. In general, reversed-phase chromatography is used for most HPLC-NMR applications in natural product chemistry. Water and protonated organic solvents cause resonances in the NMR spectrum. These eluent signals might overlap with those of the analyte and thus prevent adequate spectrum evaluation. To minimize the intensity of solvent resonances, and to improve the detection limit, deuterated solvents are utilized. In practice, fully deuterated water (99% D_2O) is used in combination with nondeuterated HPLC-NMR-grade acetonitrile or methanol. The phenomenon of peak broadening, often occurring in longer isocratic HPLC runs, reduces the fraction of the peak transferred to the flow cell. To compensate for this broadening, solvent gradients are recommended for elution. Addition of trifluoroacetic acid or phosphoric acid also contributes to peak focusing and does not cause additional signals in the ^{1}H NMR spectrum. Due to the implicit requirements of NMR methodology (solvent suppression, lock solvent), reversed-phase gradients cannot begin below a minimum concentration of 1% of the organic component and are not useful when exceeding about 95%. Flow rates between 0.6 and 1.0 mL min^{-1}, usually employed in analytical HPLC, are also convenient under HPLC-NMR conditions. However, adaptation of flow rate to the particular HPLC-NMR mode (continuous-flow, stopped flow) is required. Recent developments, allowing the use of microbore and capillary columns, which require lower flow rates and consume smaller amounts of solvents, permit the economical use of completely deuterated eluents. In general, the highest sample amount possible should be injected to reduce measuring time. Even column overloading, and partial peak overlap in the UV trace, may be acceptable to some extent because only a fraction of the desired peak is located in the active volume of the flow cell during spectrum acquisition. It is important to note that the quality of chromatographic separation determines the success of the NMR measurement and, thus, should be executed as carefully as possible.

NMR

HPLC-NMR probes do not make use of conventional removable NMR tubes but contain a continuous-flow cell, fitted to the HPLC via a polyetheretherketone (PEEK) transfer capillary. The capillary connection should be as short as possible, otherwise the stray field of the NMR magnet has to be considered. As a compromise, the HPLC is usually positioned slightly outside the 5 mT line (corresponding to about 1.5 m for a 500 MHz magnet) of the stray field. A valve interface between the HPLC detector and the NMR probe allows selection of different modes, like continuous-flow, stopped-flow and storage

mode. The active volume of the flow cells is between 40 and 240 μL in size. In continuous-flow mode, the detector volume and the flow rate determine the residence time of the sample in the flow cell and thus have a significant impact on sensitivity. Capillary flow cells with a detection volume in the order of 50–900 nL have been developed for microbore and capillary HPLC. Since there is no sample rotation it is possible to fit the radiofrequency coils directly on the glass body of the flow cell. This arrangement affords an optimal filling factor and, consequently, results in extraordinarily high sensitivity of the HPLC-NMR probes.

Commercially available HPLC-NMR probes are designed as inverse detection probes and, therefore, are most efficient for acquiring ^{1}H NMR spectra. **Figure 1** shows ^{1}H NMR spectra recorded in MeCN-D_2O with and without solvent suppression. In the nonsuppressed spectrum only the eluent signals are visible. To visualize the resonances of the analyte, suppression of the solvent signals is necessary. This can be accomplished by presaturation or by the 'water suppression enhanced through T1 effects' (WET) sequence. WET is more efficient in continuous-flow measurement because it requires shorter delays in comparison with the presaturation technique. However, even most efficiently suppressed solvent signals cover a certain part of the spectrum and may overlay some of the resonances of the analyte. This general drawback of HPLC-NMR can be reduced by running the same sample again in another eluent system having different chemical shift values, e.g. using acetonitrile (δ 2.0)–D_2O in the first and methanol (δ 3.2)–D_2O in a second run. The sensitivity of HPLC-NMR significantly depends on the operation mode and a number of further factors discussed above and by other authors. For the stopped-flow technique, which is the most sensitive mode, the detection limit in routine analysis (500 MHz; 120 μL flow cell) is below 1 μg in reasonable times. Using a capillary column and a nanolitre flow cell, the detection limit is now in the nanogram range. The stopped-flow technique is also suitable for acquiring homonuclear correlation spectra (COSY, TOCSY, NOESY and ROESY) of samples below 10 μg. Moreover, gradient-assisted inverse-detected heteronuclear correlation spectroscopy (GHSQC and GHMBC)

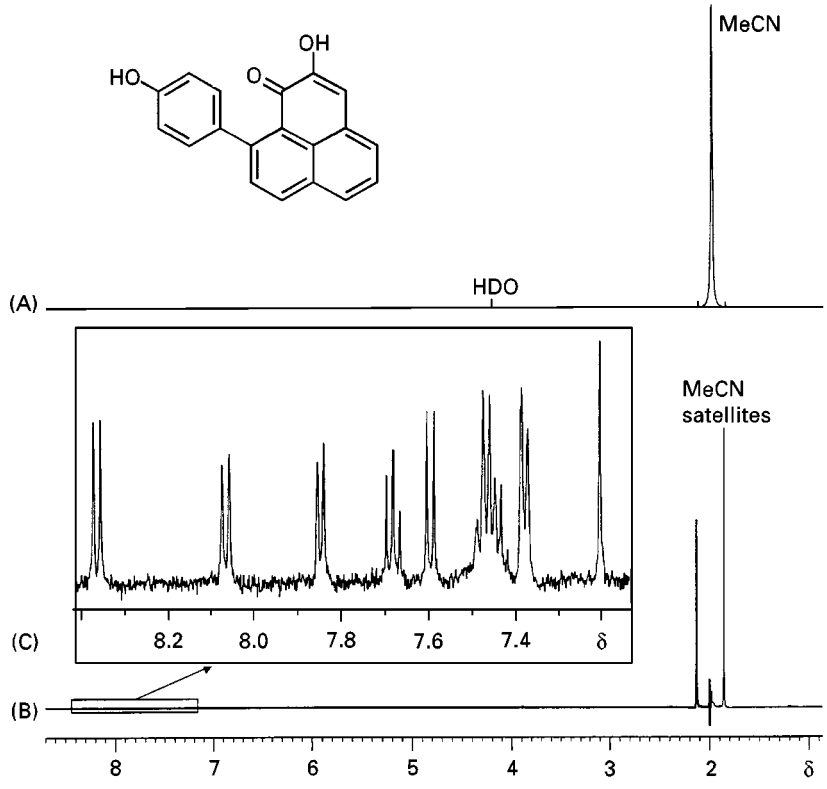

Figure 1 Stopped-flow HPLC-^{1}H NMR spectra of an aromatic natural product measured in MeCN-D_2O. Spectrum (A), which was acquired without solvent suppression, exhibits the large signal of nondeuterated MeCN, small satellites of MeCN and the signal of HDO. Resonances of the analyte are not visible. Spectrum (B) was acquired using solvent suppression by double pre-saturation of MeCN and HDO. Spectrum (C) is an enlargement of (B), showing the well-resolved resonances in the aromatic part of the spectrum.

is also possible under HPLC-NMR conditions. The measurement in stopped-flow mode requires an accurate determination of the transfer time between the first detector, usually an UV or diode array detector, and the active volume of the flow cell. This is to ensure that the HPLC pump stops just at the moment when the top of the peak is in the magnet. Synchronization of HPLC and NMR is also required in fully automated mode that enables measurement of several peaks without further interaction of the operator.

The continuous-flow mode is much less sensitive than the stopped-flow technique but provides the opportunity to scan an extract rapidly for interesting natural products. Since the NMR resonances of the solvent depend on the composition ratio of the eluent, gradient elution requires continuous adaptation of the solvent suppression frequency to the moving signal. This suppression frequency is determined for each increment by the so-called scout scan prior to the WET sequence. In continuous-flow spectra the retention times (y axis) are plotted versus the chemical shifts (x axis). The extraction of traces from these unusual two-dimensional spectra yield one-dimensional ^{1}H spectra of the desired increment that can collectively be outlined as stacked plots.

Applications

Identification of Natural Products

An increasing number of applications of HPLC-NMR are devoted to the identification and structure elucidation of natural products. The main part of these investigations covers plant natural products as novel biologically active components for pharmacological and agricultural preparations. The aims and methodology of a number of these investigations are discussed in the following paragraphs. **Table 1** summarizes the classes of plant natural products and the plant families that have been analysed by hyphenated HPLC-NMR techniques.

Extracts equivalent to 250 mg of dried leaves of *Zaluzania grayana* (Asteraceae) were used for HPLC separation with direct measurement of ^{1}H NMR spectra in the online mode. Overlapping peaks could be separated more efficiently by the stopped-flow mode, collected in a sampling unit and analysed later by ^{1}H and 2D COSY measurements. These investigations provided information on the structure of two known and a novel sesquiterpene lactone of taxonomic relevance. Using a microsampling technique, glandular trichomes from the leaf surface of *Scalesia* species (Asteraceae) were collected. A sample combined from several species of the genus was used for online HPLC-NMR analysis. Flavones and sesquiterpene lactones were identified by comparison with spectra of authentic reference compounds or literature data.

Unstable and structurally closely related bitter acids from dried female flowers of *Humulus lupulus* were extracted by supercritical carbon dioxide and analysed by HPLC-NMR in the stopped-flow mode without any degradation. Using an acetonitrile–D_2O eluent containing H_3PO_4, well-resolved spectra of α- and β-hop acids were obtained despite column overloading (2.5 mg of extract was loaded on to an analytical reversed-phase column).

The online analysis of a CH_2Cl_2 extract of *Swertia calycina* (Gentianaceae) provided ^{1}H NMR spectra of all major constituents. Extraction of single traces from the 2D plot allowed a precise assignment of their specific resonances. Approximately 0.05 μmol per peak was needed to obtain a ^{1}H NMR spectrum in the online mode using a 500 MHz NMR instrument. To improve the quality of the ^{1}H spectra and to measure a 2D COSY spectrum of one of the components, sweroside, the same extract was investigated in the stopped-flow mode. The detection limit for a ^{1}H NMR spectrum could be lowered by a factor of about 100 under stopped-flow conditions but longer acquisition times were required in comparison with the continuous-flow mode. In the case of the more complex methanol extract of another Gentianaceae species, *Gentiana ottonis*, clear ^{1}H NMR spectra of secoiridoids, flavones and xanthones were only obtained in the stopped-flow mode. LC-UV and LC-MS data were also needed for full identification of compounds from both species.

Complementary HPLC-NMR and HPLC-MS studies were also performed on crude extracts and bioactive fractions from *Monetes engleri*. On-flow experiments indicated two major prenylated flavanone components in the CH_2Cl_2 extract of this plant but did not monitor minor components. A bioactive fraction obtained by medium pressure liquid chromatography (MPLC), containing these components, was subjected to stopped-flow HPLC-NMR analysis. ^{1}H NMR, 1D TOCSY, 2D NOESY and gradient-enhanced inverse ^{1}H, ^{13}C correlation experiments (GHSQC, GHMBC; **Figure 2**) were recorded from the enriched sample in a total acquisition time of 9.6 h. The WET sequence was used to suppress the eluent signals of the residual HDO resonance, the resonances of MeCN and its two ^{13}C satellites, and those of the propionitrile impurities of MeCN. The constitution of monotesone A, a new prenylated flavanone, was elucidated online using the strategy described. However, determination of the absolute configuration was only possible after isolation.

Table 1 Identification of plant natural products by HPLC-NMR

Species	*Family*	*Natural products*	*HPLC*	*NMR*	*Reference*
Zaluzania grayana	Asteraceae	Sesquiterpene lactones	RP-18, MeCN-D_2O, UV	500 MHz; online: ^1H stopped-flow (peak sampling): ^1H, 2D-COSY	Spring *et al.* (1995) *Phytochemistry* 39: 609
Scalesia species[a]	Asteraceae	Flavanones, sesquiterpene lactones	RP-18, MeCN-D_2O, MeOH-D_2O, UV	500 MHz; online: ^1H	Spring *et al.* (1997) *Phytochemistry* 46: 1369
Humulus lupulus	Moraceae	Lupulones	RP-18, MeCN-D_2O, UV	400 MHz; stopped-flow: ^1H	Hötzel *et al.* (1996) *Chromatographia* 42: 499
Swertia calycina	Gentianaceae	Naphthoquinones, secoiridoids, xanthones	RP-18, MeCN-D_2O, UV	500 MHz; online: ^1H; stopped-flow: ^1H, 2D-COSY	Wolfender *et al.* (1997) *Phytochem. Anal.* 8: 97
Gentiana ottonis	Gentianaceae	Flavones, secoiridoids, xanthones	RP-18, MeCN-D_2O, UV	500 MHz; stopped-flow: ^1H	
Monotes engleri	Dipterocarpaceae	Flavanones	RP-18, MeCN-D_2O, UV	500 MHz; online: ^1H; stopped-flow: ^1H, 1D TOCSY, 2D NOESY, GHSQC, GHMBC	Garo *et al.* (1998) *Helv. Chim. Acta* 81: 754
Lisianthius seemannii	Gentianaceae	Secoiridoid dimer glycosides	RP-18, MeCN-D_2O, UV-DAD	500 MHz; stopped-flow: ^1H	Rodriguez *et al.* (1998) *Helv. Chim. Acta* 81: 1393
Vernonia fastigiata	Asteraceae	Sesquiterpene lactones	RP-18, MeCN-D_2O MeOH-D_2O, UV	500 MHz; online: ^1H; stopped-flow: ^1H, 1D selective NOESY, 2D COSY, 2D NOESY	Vogler *et al.* (1998) *J. Natl. Prod.* 61: 175
Terminalia macroptera	Combretaceae	Sapogenines	RP-18, MeCN-D_2O, MeOH-D_2O, UV	500 MHz; online: ^1H; stopped-flow: ^1H, 1D selective NOESY, 2D COSY, 2D TOCSY, 2D NOESY	Vogler *et al.* (1998) *Natural Product Analysis*, Braunschweig/Wiesbaden: Vieweg, p. 143
Rubia tinctorum	Rubiaceae	Anthraquinones	RP-18, MeCN-D_2O, UV	500 MHz; stopped-flow: ^1H	Schneider *et al.* (1998) *Natural Product Analysis,* Braunschweig/Wiesbaden: Vieweg, p. 137
Taxus baccata	Taxaceae	Taxanes	RP-18, MeCN-D_2O, UV	500 MHz; stopped-flow: ^1H	
Taxus canadensis *Taxus chinensis* var. *mairei* *Taxus* × *media*	Taxaceae	Taxanes	RP-18, MeCN-D_2O, UV	500 MHz; stopped-flow: ^1H, 2D TOCSY	Schneider *et al.* (1998) *Phytochem. Anal.* 9: 237
Ancistrocladus guinensis	Ancistrocladaceae	Naphthylisoquinolines	RP-18, MeCN-D_2O, UV	600 MHz; online: ^1H; stopped-flow: ^1H, 2D-TOCSY, 2D ROESY	Bringmann *et al.* (1998) *Anal. Chem.* 70: 2805

Triphyophyllum peltatum	Dioncophyllaceae	Naphthylisoquinolines	RP-18, MeCN-D_2O, UV	600 MHz; online: ^1H; stopped-flow: 2D TOCSY, 2D ROESY	Bringmann *et al.* (1998) *Natural Product Analysis,* Braunschweig/Wiesbaden: Vieweg, p. 147
Ancistrocladus likoko	Ancistrocladaceae	Naphthylisoquinolines	RP-18, MeCN-D_2O, UV	600 MHz; stopped-flow: ^1H, 2D TOCSY, 2D ROESY	Bringmann *et al.* (1999) *Magn. Res. Chem.* 37: 98
Dioncophyllum thollonii	Dioncophyllaceae	Naphthylisoquinolines, tetralones	RP-18, MeCN-D_2O, UV	600 MHz; online: ^1H; ^1H, 2D ROESY, ^1H time slice	Bringmann *et al.* (1999) *J. Chromatogr. A* 837: 267
Habropetalum dawei	Dioncophyllaceae	Naphthylisoquinolines, isoquinolines	RP-18, MeCN-D_2O, UV, CD	600 MHz; online: ^1H; stopped-flow: ^1H, 2D TOCSY, 2D ROESY	Bringmann *et al.* (1999) *Anal. Chem.* 71: 2678
Orophea enneandra	Annonaceae	Lignans, tocopherols, polyacetylene	RP-18, MeCN-D_2O, UV	500 MHz; online: ^1H	Cavin *et al.* (1998) *J. Natl. Prod.* 61: 1497
Torreya jackii	Taxaceae	Lignans	RP-18, MeCN-D_2O, UV	500 MHz; stopped-flow: ^1H	Zhao *et al.* (1999) *J. Chromatogr. A* 837:83
Senecio vulgaris[b]	Asteraceae	Pyrrolizidines	RP-18, MeCN-D_2O, UV	500 MHz; online: ^1H; stopped-flow: ^1H, 2D COSY	Wolfender *et al.* (1998) *Current Organic Chemistry* 1: 575
Cordia linnaei	Boraginaceae	Meroterpenoid, naphthoquinones	RP-18, MeCN-D_2O, UV	500 MHz; online: ^1H stopped-flow: ^1H	Ioset *et al.* (1999) *Phytochem. Anal.* 10: 137
Anigozanthos flavidus	Haemodoraceae	Phenylphenalenones, stilbenes	RP-18, MeCN-D_2O, UV	500 MHz; stopped-flow: ^1H, 2D TOCSY	Schneider *et al.* (1998) *Natural Product Analysis*, Braunschweig/Wiesbaden: Vieweg, p. 137; Hölscher and Schneider (1999) *Phytochemistry* 50: 155

[a] Combined sample of several species of the genus; [b] and other *Senecio* species.

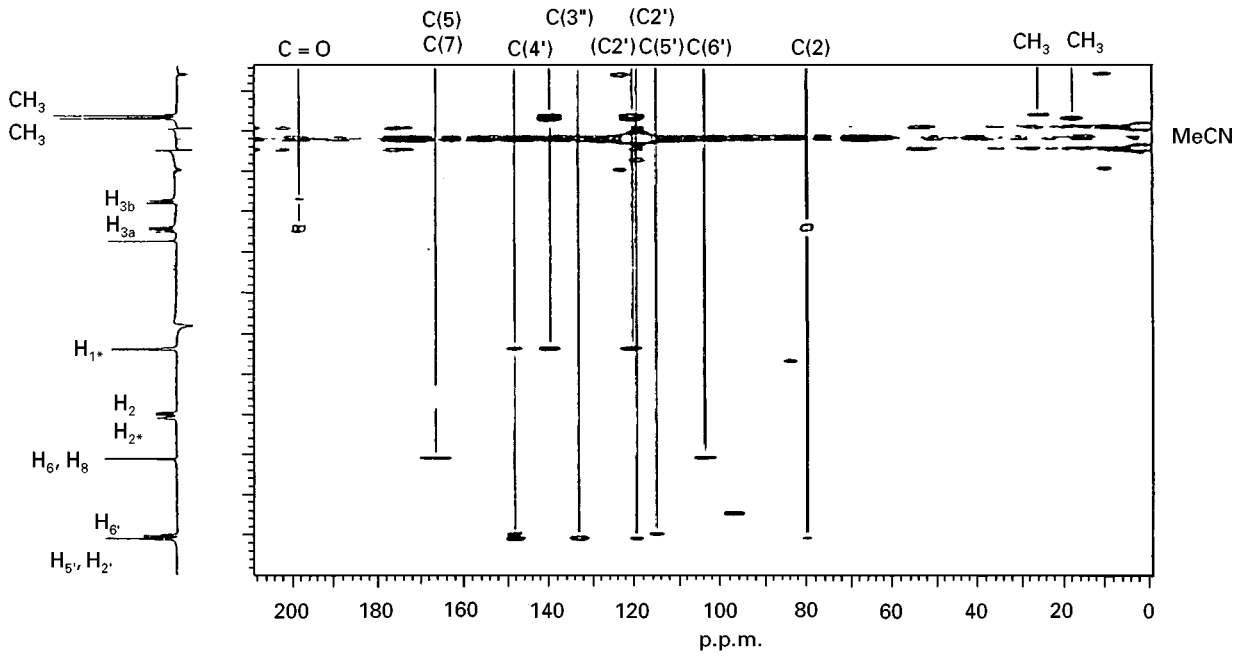

Figure 2 GHMBC spectrum of a prenylated flavanone acquired under HPLC-NMR conditions in 6 h 20 min. 1 mg of fraction injected. Reprinted with permission from Garo E, Wolfender JL, Hostettman K *et al.* (1998) Prenylated flavonones from *Monofes egleri*: on-line structure elucidation by LC/UV/NMR. *Helvetica Chimica Acta* 81: 754.

HPLC-NMR was shown as being the method of choice to assign the structure of a rapidly isomerizing dimeric secoiridoid glucoside carrying a (*Z*)-*p*-coumaroyl unit found in aerial parts of *Lisianthium seemannii* (Gentianaceae). The stopped-flow ^{1}H NMR spectrum of this unstable isomer was very similar to that measured for the isomerization product, as far as the monoterpenoic and glycosidic parts of the molecule were concerned. However, the resonances corresponding to the coumaroyl moieties exhibited signals of an (*E*)-double bond for the stable isomer and (*Z*)-double bond for the unstable one.

Less polar fractions of *Vernonia fastigiata* (Asteraceae) were investigated under continuous-flow conditions. In order to obtain information about signals hidden by suppressed peaks of the MeCN-D_2O eluent, HPLC-NMR spectra were measured a second time in MeOH-D_2O. The combination of both complementary spectra allowed the assignment of all proton resonances of the corresponding sesquiterpene lactones in just two HPLC-NMR runs (**Figure 3**). 2D COSY spectra of selected compounds from the more polar fractions were measured in the stopped-flow mode. 2D NOESY and a 1D selective NOESY have been employed to clarify stereochemical features. Due to the selective excitation technique in the 1D NOESY, no solvent suppression was required. A similar array of methods was reported for HPLC-NMR investigations on active triterpenoid sapogenines from *Terminalia macroptera* (Combretaceae).

An example of how natural products were identified by simply using HPLC-^{1}H NMR was described for anthraquinones from hairy root cultures of *Rubia tinctorum* (Rubiaceae). First, the HPLC-^{1}H NMR spectrum of a known anthraquinone, lucidine, was identified by comparison with an authentical standard and was then used to assign the structures of lucidin glycosides for which no reference compounds were available.

Partially purified extracts of *Taxus baccata* (Taxaceae) leaves were used in another example. Despite incomplete chromatographic separation, identification of two isomeric taxanes was clearly possible. Pre-purified extracts of only 0.5 mg air-dried needles of further *Taxus* species, *T. canadensis*, *T. chinensis* var. *mairei*, and *T.* × *media* cv. Hicksii, were subjected to stopped-flow HPLC-NMR by ^{1}H and 2D TOCSY analysis. Taxol® and several other neutral and basic taxanes were identified by means of comparison with spectra of reference compounds or were deduced from related compounds. Due to the use of different solvents in HPLC-NMR and conventional NMR spectroscopy, differences of chemical shifts have to be considered. Comparing spectra measured by HPLC-NMR in MeCN-D_2O with that of the same compound measured under conventional conditions in deuterochloroform indicated that chem-

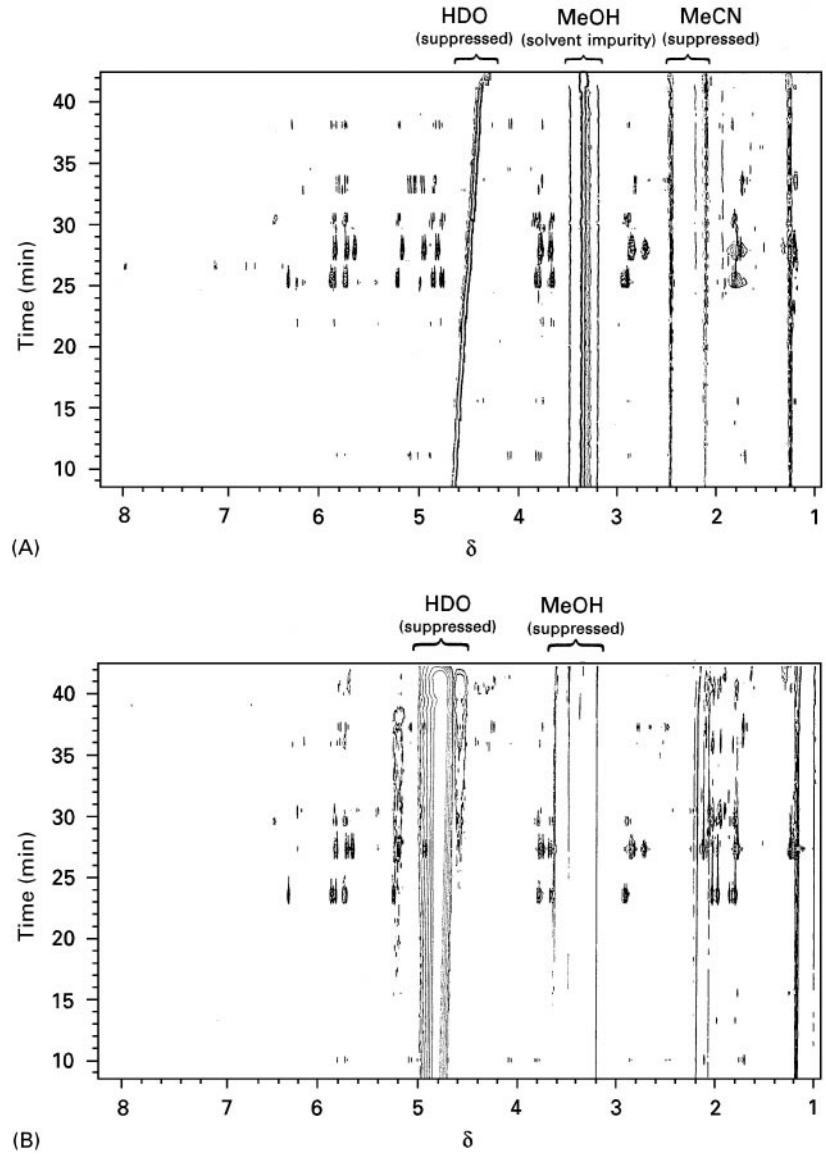

Figure 3 Comparison of 2D online HPLC-NMR plots of an extract of *Vernonia fastigiata* in (A) MeCN-D_2O and (B) MeOH-D_2O. Complementary HPLC-NMR eluents were used in order to provide information on signals hidden by suppressed resonances of each solvent. Adapted with permission from Vogler B, Conrad J, Hiller W, Klaiber I, Roos G and Sandor P (1998) Can LC-NMR serve as a tool for natural products elucidation? In: Schreier P, Herderich M, Humpf HU and Schwab W (eds) *Natural Product Analysis*, p. 143. Braunschweig/Wiesbaden: Vieweg.

ical shift differences did not exceed 0.2 p.p.m. However, due to the fact that some resonances were shifted upfield and others downfield, some signals appeared interchanged in sequence.

A series of HPLC-NMR analyses have been carried out at 600 MHz on naphthylisoquinolines from two plant families, the Diocophyllaceae and the Ancistrocladaceae. ^1H spectra extracted from the pseudo-2D continuous-flow diagram obtained under isocratic HPLC conditions using crude leaf extracts of *Ancistrocladus guineënsis* showed the typical signal pattern of naphthylisoquinolines. An optimized nonlinear HPLC gradient was used for stopped-flow NMR analysis of a known alkaloid and two further closely related compounds. A 2D TOCSY of the HPLC fraction containing both compounds allowed the proton assignment of these isomers in a single NMR experiment. **Figure 4** shows 2D ROESY spectra of naphthylisoquinolines, the first example of the use of HPLC-NMR hyphenation to predict relative configuration. *Triphyophyllum peltatum* was analysed similarly. The major alkaloid of that species, dioncophylline A, was identified by ^1H, 2D TOCSY and 2D ROESY spectra. Additionally, two minor components

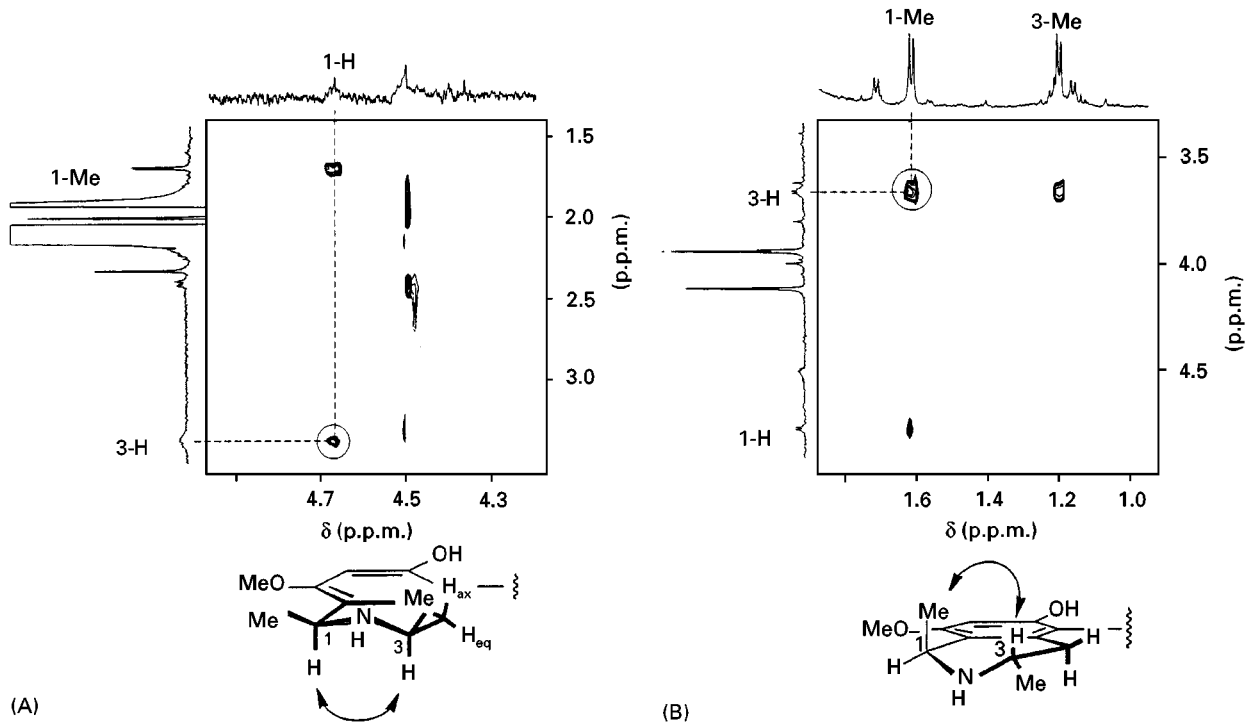

Figure 4 HPLC-NMR ROESY cross-peaks indicating the relative configuration at the isoquinoline moiety of diastereomeric naphthylisoquinoline alkaloids from *Ancistrocladus guineënsis*. (A) Peak eluting at $t_R = 21.05$ min exhibits a correlation between ^{3}H and ^{1}H. (B) Peak eluting at $t_R = 21.60$ min shows a correlation between 3-H and 1-Me. Reproduced with permission from Bringmann G, Günther C, Schlauer J. *et al.* (1998) HPLC-NMR on-line coupling including the ROESY technique: direct characterization of naphthylisoquinoline alkaloids in crude plant extracts. *Analytical Chemistry* 70: 2805.

were identified in the same plant using an array of analytical methods, including HPLC-NMR. The constitution and relative configuration of new naphthylisoquinoline alkaloids from *Ancistrocladus likoko*, with a 5,8′ coupling pattern, were also elucidated by application of 2D TOCSY and 2D ROESY experiments in the stopped-flow HPLC-NMR mode. Naphthylisoquinolines were also detected by online and stopped-flow HPLC-NMR techniques in *Dioncophyllum thollonii* (Dioncophyllaceae). Moreover, chromatographically unresolved diastereomeric tetralones with slightly different retention times were measured in a time-slice experiment. ^{1}H spectra were acquired in the stopped-flow mode at different positions of the chromatographic peak, and the diastereomers were distinguished by comparison of ^{1}H spectra of slices of pure and mixed components. The constitution and relative configuration of an isoquinoline and a naphthylisoquinoline from crude extracts of *Habropetalum dawei* (Dioncophyllaceae) were established by combined application of HPLC-NMR and HPLC-electrospray ionization (ESI)-MS-MS. Additional combinations with subsequent stopped-flow HPLC-circular dichroism (CD) experiments allowed deduction of the absolute configuration of these new metabolites.

While most HPLC-NMR studies make use of the stopped-flow option, either alone or after preliminary continuous-flow experiments, a variety of natural products from *Orophea enneandra* (Annonaceae) have been tentatively characterized by means of the continuous-flow technique without a subsequent stopped-flow run. Column overloading (2 mg) did not prevent proper separation of the components. The structures of three lignanes were identified by reference to literature data. In the cases of a tocopherol derivative and an unstable polyacetylene, targeted isolation and structure elucidation by complementary coupling techniques and conventional analytical methods were necessary.

A variety of lignanes were also identified from extracts of *Torreya jackii* (Taxaceae). Some of them were completely characterized by stopped-flow HPLC-^{1}H NMR while in other examples isolation was required to confirm the structures by conventional NMR spectroscopy. After HPLC-NMR measurements, individual lignanes were collected and subjected to MS, which was considered to be an indispensable tool for complete structure assignment.

A study on *Senecio vulgaris* (Asteraceae) has allowed identification of a variety of pyrrolizidine

alkaloids and differentiation of certain isomeric macrocyclic diesters of that type. These compounds adopt *cis-trans* configurations and are not distinguishable by LC-MS. This example demonstrated again that complementary measurements in MeCN-D_2O and MeOH-D_2O are necessary to observe all resonances. Information obtained from the continuous-flow ^1H spectrum (24 scans per increment; column overloading by 3 mg of extract) were shown to be comparable to those from a corresponding stopped-flow spectrum.

Online and stopped-flow HPLC-NMR analysis of two minor isomeric meroterpenoid napthoquinones from *Cordia linnaei* (Boraginaceae) yielded ^1H NMR spectra exhibiting identical signals in the aromatic region. Differences were only found in methyl signals when MeCN-D_2O and MeOH-D_2O were used as complementary HPLC-NMR eluents. One of the isomers, cordiaquinone C, carried a senecioic acid moiety while the other, being a new compound, was found to contain a tigloyl substituent instead.

Extracts of *Anigozanthos flavidus* (Haemodoraceae), a plant family accumulating phenylphenalenones and stilbenes in the roots, were investigated using stopped-flow HPLC-NMR. Comparison with spectra of references and literature data was used to differentiate between known and novel compounds. A number of known compounds were identified without isolation, while others had to be isolated, especially when possessing regions poor in hydrogen atoms.

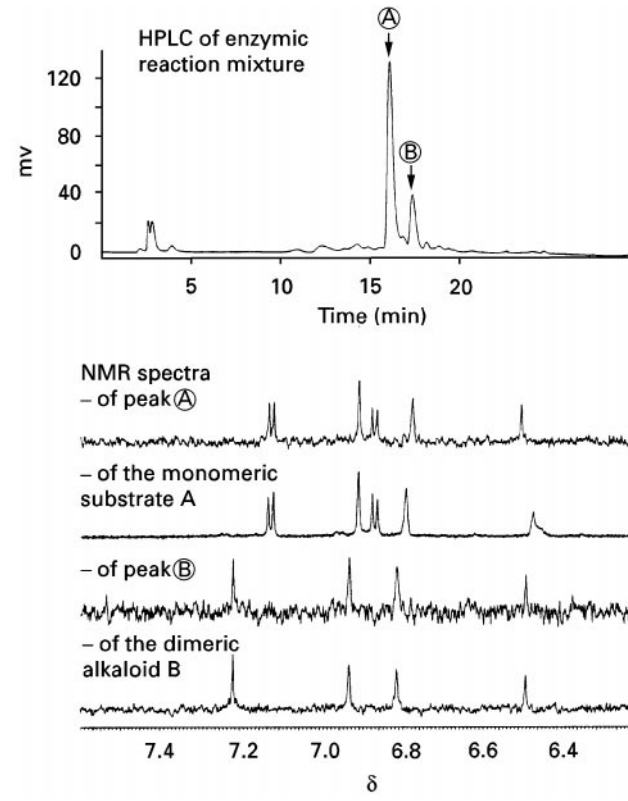

Figure 5 Stopped-flow HPLC-^1H NMR experiment confirming exclusive formation of a dimeric naphthylisoquinoline **B** from monomer **A** in the coupling reaction catalysed by peroxidase purified from *Ancistrocladus heyneanus*. Adapted with permission from Schlauer J, Rückert M, Herderich M *et al.* (1998) Characterization of enzymes from *Ancistrocladus* (Ancistrocladaceae) and *Triphyophyllum* (Dioncophyllaceae) catalyzing oxidative coupling of naphthylisoquinoline alkaloids. *Archives of Biochemistry and Biophysics* 350: 87.

Biosynthetic Applications

HPLC-NMR has been used in biosynthetic and enzymatic investigations of secondary plant products. Michellamines, representing dimeric naphthylisoquinoline alkaloids highly active against HIV, were formed biosynthetically by oxidative coupling of their inactive korupensamine monomers. This dimerization was catalysed by peroxidase preparations from three *Ancistrocladus* species (Ancistrocladaceae) and from *Triphyophyllum peltatum* (Dioncophyllaceae). The peroxidase was partially purified from *Ancistrocladus heyneanus* and characterized in more detail. The exclusive formation of a michelleamine from its monomeric korupensamine precursor, shown in **Figure 5**, was confirmed by HPLC-MS and stopped-flow HPLC-^1H NMR experiments.

Details of the phenylpropanoid metabolism preceding later steps of the biosynthesis of phenylphenalenones from *Anigozanthos preissii* (Haemodoraceae) were elucidated by Schmitt and Schneider using the stopped-flow HPLC-NMR technique. Incorporation of dihydrophenylpropanoids into phenylpropanoids in root cultures of that plant was proved by the coupling pattern in the HPLC-^1H NMR spectrum of *p*-coumaric acid biosynthesized from [2-^{13}C]dihydrocinnamic acid (**Figure 6**). A number of simple phenolics, which are supposed to be formed from phenylpropanoids, were also detected in these experiments by HPLC-^1H NMR spectroscopy in the stopped-flow mode.

Applications Related to Natural Products

In areas related to natural product research, Careri and Mangia have reviewed the analysis of natural food components by HPLC-NMR. Lindon *et al.* have published an overview on HPLC-NMR in biomedical applications, and a small number of investigations have shown that the analysis of amino acids and peptide mixtures is also possible by HPLC-NMR.

The first HPLC-NMR analysis of biological macromolecules, published by Rückert *et al.*, utilized the combination of ion exchange chromatographic

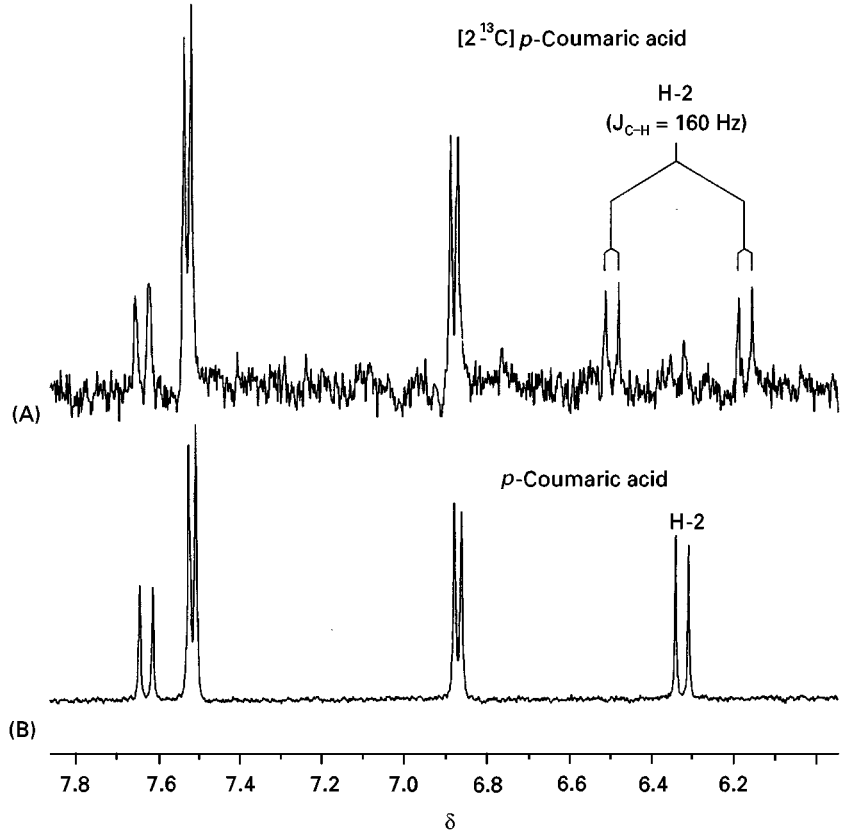

Figure 6 Stopped-flow HPLC-^{1}H NMR experiment confirming biosynthetic incorporation of ^{13}C label into C-2 of *p*-coumaric acid. ^{1}H NMR spectra of (A) [2-^{13}C]*p*-coumaric acid resulting from treatment of *Anigozanthos preissii* root cultures with [2-^{13}C]dihydrocinnamic acid and (B) nonlabelled reference.

separation with ^{1}H, 2D TOCSY and 2D NOESY spectroscopy to characterize small proteins in mixture. The authors expect that HPLC-NMR at very high field (750 and 800 MHz) and further enhancements in sensitivity should permit online experiments and heteronuclear 2D and 3D stopped-flow experiments in the future.

Summary and Future Developments

HPLC-NMR coupling has been developed into a valuable tool for natural product analysis. In general, the online technique is used to provide a rapid overview of the major components occurring in plants and other sources of natural products. The more sensitive stopped-flow method allows the detection and structure assignment of even minor components and enables the use of various homo- and heteronuclear correlation NMR experiments. However, unambiguous structure assignment of novel compounds of unexpected structural types requires information from other analytical methods, especially MS. Complete structure elucidation, together with stereochemical information, by multiple online combinations including NMR is possible but currently is rather the exception. Rapid development in analytical chemistry is expected to overcome present limitations of HPLC-NMR. The future scenario in a natural product laboratory could be an automated characterization of sources of natural products, starting with extraction and separation, followed by hyphenated instrumental analysis and finally computational structure elucidation. Additional combination with biological screening could avoid isolation of inactive compounds.

HPLC-NMR is an excellent approach to search for novel biologically active structures to be tested as new medicinal and agricultural agents, to identify known compounds without isolation, and to avoid unwanted re-isolation of known constituents from living organisms. Due to the large amount of structural information provided by NMR spectroscopy, its combination with HPLC and further spectroscopic techniques is also suitable when searching for new sources of rare natural products, for clarification of uncertain chemotaxonomic relationships and distribution of secondary compounds in various tissues. The introduction and routine application of capillary HPLC

and innovative fused capillary nanolitre flow cells in NMR probes, and further development in cryoprobe technology along with the use of improved processing procedures, will continue to enhance the sensitivity of HPLC-NMR coupling. As a microanalytical method, HPLC-NMR allows the detection of various groups of natural compounds and other biomolecules in the nanogram or even picogram range and, therefore, can contribute to the solution of problems of biochemical, physiological and chemoecological research.

See also: **II/Chromatography: Liquid:** Mechanisms: Reversed Phases; Nuclear Magnetic Resonance Detectors. **III/Medium-Pressure Liquid Chromatography. Natural Products:** Liquid Chromatography. **Terpenoids: Liquid Chromatography.**

Further Reading

Albert K (1995) On-line use of NMR detection in separation chemistry. *Journal of Chromatography A* 703: 123.

Albert K (1997) Supercritical fluid chromatography–proton nuclear magnetic resonance spectroscopy coupling. *Journal of Chromatography A* 785: 65.

Behnke B, Schlotterbeck G, Tallerek U *et al.* (1996) Capillary HPLC-NMR coupling: high resolution ^{1}H NMR spectroscopy in the nanoliter scale. *Analytical Chemistry* 68: 1110.

Careri M and Mangia A (1996) Multidimensional detection methods for separations and their application in food analysis. *Trends in Analytical Chemistry* 15: 538.

Lindon JC, Nicholson JK, Sidelmann UG and Wilson ID (1997) Directly coupled HPLC-NMR and its application to drug metabolism. *Drug Metabolism Reviews* 29: 705.

Pusecker K, Schewitz J, Gfrörer P *et al.* (1998) On line coupling of capillary electrochromatography, capillary electrophoresis, and capillary HPLC with nuclear magnetic resonance spectroscopy. *Analytical Chemistry* 70: 3280.

Rückert M, Wohlfarth M and Bringmann G (1999) Characterization of protein mixtures by ion-exchange liquid chromatography coupled on-line to NMR spectroscopy. *Journal of Chromatography A* 840: 131.

Seddon MJ, Spraul M, Wilson ID *et al.* (1994) Improvement in the characterization of minor drug metabolites from HPLC-NMR studies through the use of quantified maximum entropy processing of NMR spectra. *Journal of Pharmaceutical and Biomedical Analysis* 12: 419.

Smallcombe SH, Patt SL and Keiffer PA (1995) Wet solvent suppression and its application to LC NMR and high resolution NMR spectroscopy. *Journal of Magnetic Resonance Series A* 117: 295.

Schmitt B and Schneider B (1999) Dihydrocinnamic acids are involved in the biosynthesis of phenylphenalenones. *Phytochemistry* 52: 45.

Schreier P, Herderich M, Humpf HU and Schwab W (eds) (1998) *Natural Product Analysis*. Braunschweig/Wiesbaden: Vieweg.

Watanabe N, Niki E and Shimizu S (1979) An experiment on direct combination of high performance liquid chromatography with FT-NMR (LC-NMR). *Jeol News* 15A: 2.

Wolfender JL, Ndjoko K and Hostettmann K (1998) LC/NMR in natural products chemistry. *Current Organic Chemistry* 1: 575.

Wu N, Webb L, Peck TL and Sweedler JV (1995) On-line NMR detection of amino acids and peptides in microbore LC source. *Analytical Chemistry* 67: 3101.

Supercritical Fluid Chromatography

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The mild elution temperatures and the wide range of molecular masses it can accommodate makes supercritical fluid chromatography (SFC) particularly applicable to natural products. It is becoming the preferred method for the separation of enantiomers, and is especially useful for combined or hyphenated techniques. It forms a link between liquid chromatography (LC) and chromatography (GC), it has capabilities between the two and shares the instrumental set-ups of both, so both capillary column and packed column applications are recorded here.

The advantages and disadvantages of the method are debated elsewhere, but some of its strong points are indicated here. In all, 99% of supercritical fluid applications use supercritical carbon dioxide, since it has the great advantage that it is a nontoxic, nonflammable, pure, cheap mobile phase that presents no disposal problems. The greatest usefulness of SFC comes in connection with supercritical fluid extraction, which has received much attention for the isolation of natural products. If a substance can be extracted from plant or animal material with a supercritical fluid and some of the extract can be diverted to an online SFC, the course of the extraction can be followed very easily. Many of the applications of SFC recorded for natural products are of this type. The