Future Developments

Looking to the future, evolutionary development can be expected because the separating power of multidimension liquid chromatography is greatly increased over single-dimension liquid chromatography. The simultaneous improvements on both the software and the chromatographic apparatus will lead to a system capable of automatically developing analytical methods for a wide range of analytes in many different matrices. Other desirable aspects are a reasonably short analysis time and flexible operating conditions.

The thrust of multidimensional research will most probably be to improve the combination of separation methods, including coupling to alternative separation techniques.

See also: **II/Chromatography: Thin-layer (Planar):** Modes of Development: Conventional. **Electrophoresis:** Two-Dimensional Electrophoresis. **III/Chiral Separations:** Liquid Chromatography. **Pharmaceuticals:** Chiral Separations: Liquid Chromatography.

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Normal Phase Chromatography: Mechanisms

See **II / CHROMATOGRAPHY: LIQUID / Mechanisms: Normal Phase**

Nuclear Magnetic Resonance Detectors

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Introduction

In many fields of chemistry, biology, pharmacy and medicine, progress is often limited by the ability to resolve complex analytical problems. To this end analytical techniques have been developed in recent decades dealing with an integrated approach to the separation of mixtures together with structural elucidation of unknown compounds. High performance liquid chromatography (HPLC), gel permeation chromatography (GPC) and supercritical fluid chromatography (SFC), as well as the capillary separation techniques capillary HPLC (CHPLC), capillary electrophoresis (CE) and capillary electrochromatography (CEC) are the most powerful techniques

within the group of chromatographic separation methods. Nuclear magnetic resonance (NMR) spectroscopy, in particular, is useful because of its powerful stereochemical information content but it has the disadvantage of lower sensitivity in comparison to other methods, e.g. mass spectrometry (MS).

The combination of chromatographic separation techniques with NMR spectroscopy is one of the most powerful and time-saving methods for the separation and structural elucidation of unknown compounds and mixtures. Especially for the structure elucidation of light- and oxygen-sensitive substances, for example, hop bitter acids and carotenoid stereoisomers, online liquid chromatography (LC)-NMR has important advantages. Here, structure elucidation with LC-MS is not possible, because the carotenoid isomers exhibit the same fragmentation pattern. Using a classical method with offline separation, enrichment and transfer to a NMR sample tube, the isolated substances would be isomerized. A closed-loop LC-NMR flow-through system solves this problem. Online LC-NMR also allows the continuous registration of time changes as they appear in the chromatographic run. Unequivocal structural assignment of unknown chromatographic peaks is possible by two-dimensional stopped-flow LC-NMR experiments.

NMR Flow Cell Design

Figure 1 shows the design of NMR cells employed for various coupling techniques. For online HPLC-NMR and GPC-NMR coupling, a vertically oriented flow cell with a directly fixed double-saddle Helmholtz coil is used (Figure 1A). The whole arrangement is centred in the glass dewar of a conventional probe body in which a thermocouple is inserted, allowing temperature-dependent measurements to be made. The internal diameter of the glass tube is 2, 3 or 4 mm, resulting in detection volumes of 60, 120 and 180 μL. The glass walls of the flow cell are parallel within the length of the proton detection coil, and taper at both ends to fit polytetrafluoroethylene (PTFE) tubing (i.d. 0.25 mm). PTFE and glass tubing are connected by shrink-fit tubing. Inverse continuous-flow probes contain an additional coaxial coil (tuned to the ^{13}C resonance frequency) surrounding the ^{1}H detection coil for heteronuclear $^{1}H/^{13}C$ shift-correlated experiments. This design leads to optimal NMR resolution values with a typical line width of chloroform at the height of the ^{13}C satellites of 9–12 Hz, allowing the determination of coupling constants of 1 Hz in continuous-flow NMR spectra. The disadvantage of this design is the high detection volume, leading to a degraded chromatographic resolution. For analytical HPLC columns (250×4.6 mm i.d.) the plate height is increased for solutes with capacity factors less than 2.5 at detection volumes higher than 48 μL.

The probe design employed for SFC-NMR coupling is shown in Figure 1B. The inner glass tube of the original LC-NMR probe is substituted with a sapphire tube (o.d. 5 mm, i.d. 3 mm, detection volume 120 μL) and the polyetheretherketone (PEEK) capillaries used in the LC-NMR probe are replaced by Titan tubings. A double-tuned proton deuterium coil is directly fixed to the sapphire flow cell. The whole arrangement is centred in the glass dewar of

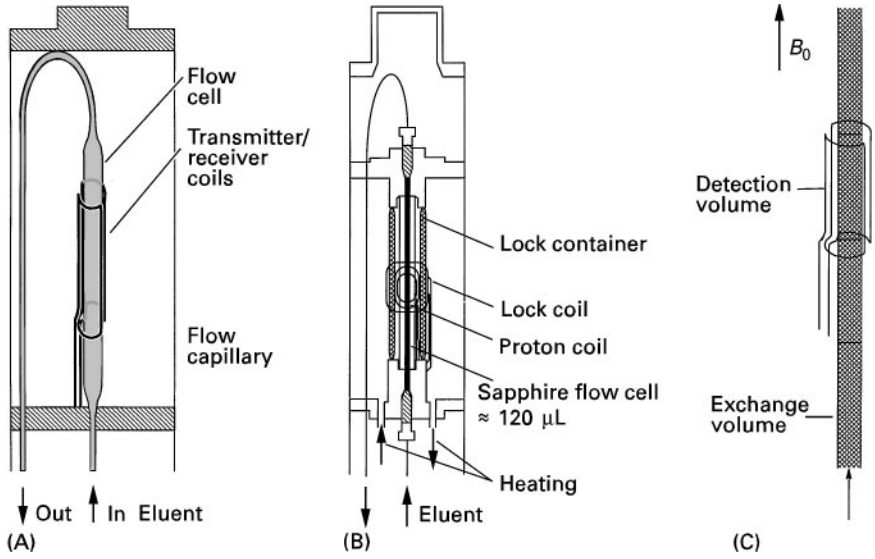

Figure 1 Design of NMR flow cell for: (A) HPLC-NMR and GPC-NMR experiments; (B) SFC-NMR experiments; (C) capillary NMR experiments.

a conventional probe body, in which a thermocouple is inserted, allowing temperature-controlled experiments.

Figure 1C shows the schematic diagram of the capillary NMR detection probe. Here a fused silica capillary is directly inserted within the NMR radio-frequency coil of a 2.0 mm microprobe. Within the area of the NMR detection coil the polyimide coating of the capillary is removed; either capillaries with an i.d. of 180 μm or bubble cell types with an increased i.d. of 220 μm are used.

Experimental Set-up

For online LC-NMR, GPC-NMR and SFC-NMR experiments, the chromatographic separation system consists of either HPLC or SFC pumps together with an injection valve, a separation column (250 × 4.6 mm i.d.) and an ultraviolet (UV) or refractive index (RI) detector. The system is located at a distance of 2.0 m from an unshielded cryomagnet (**Figure 2**). With shielded cryomagnets, the chromatographic separation system can be located at a distance of about 30 cm. The outlet of the UV (RI) detector is connected by stainless steel capillary tubing (0.25 mm i.d.) to a switching valve. The valve is used to trap the desired peak in the NMR flow cell for stopped-flow experiments. For online experiments with continuous registration of NMR spectra in distinct time intervals (1–8 s), the switching valve is open for a continuous flow through the probe to waste. Instead of the switching valve, a Bruker peak sample unit (BPSU) can be used. This technique is advisable when long NMR times are expected. Desired peaks from a separation can be stored in small capillary loops with the help of the peak sample unit. After complete separation, every single peak can be transferred into the probe and the desired stopped-flow experiment can be conducted.

In SFC-NMR experiments the outlet of the high pressure SFC probe is connected to a back-pressure regulator to guarantee supercritical conditions in the detection cell.

A feasible experimental set-up for online capillary LC-NMR coupling is outlined in **Figure 3**. The separation device for either pressure or electroosmotic flow-driven separations is located at a distance of 2 m from the cryomagnet. Separation is performed on a packed fused silica capillary which is directly fixed in a microprobe. For capillary HPLC separations a T-piece in conjunction with a restriction column is used for flow rate adjustment of the eluent. The HPLC pump, the injection device and the packed separation capillary are connected by fused silica capillaries.

LC-NMR Coupling

The coupling of LC-NMR requires the adjustment of both analytical systems. The flow of the mobile

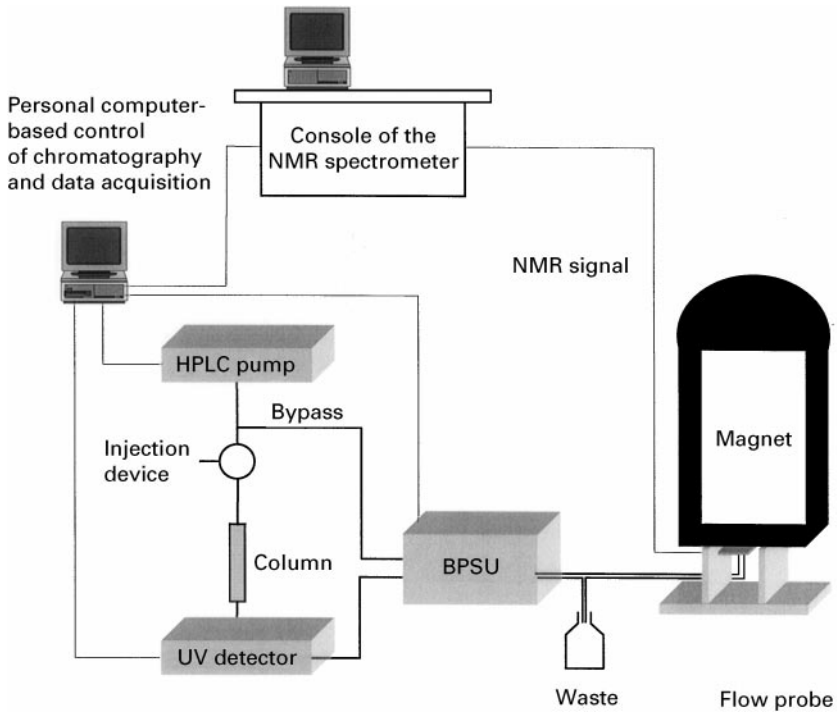

Figure 2 Experimental arrangement for HPLC-NMR, solid-phase extraction (SPE)-HPLC-NMR, GPC-NMR and SFC-NMR experiments. BPSU, Bruker peak sampling unit; fine lines, electronic junction; bold lines, capillary junctions.

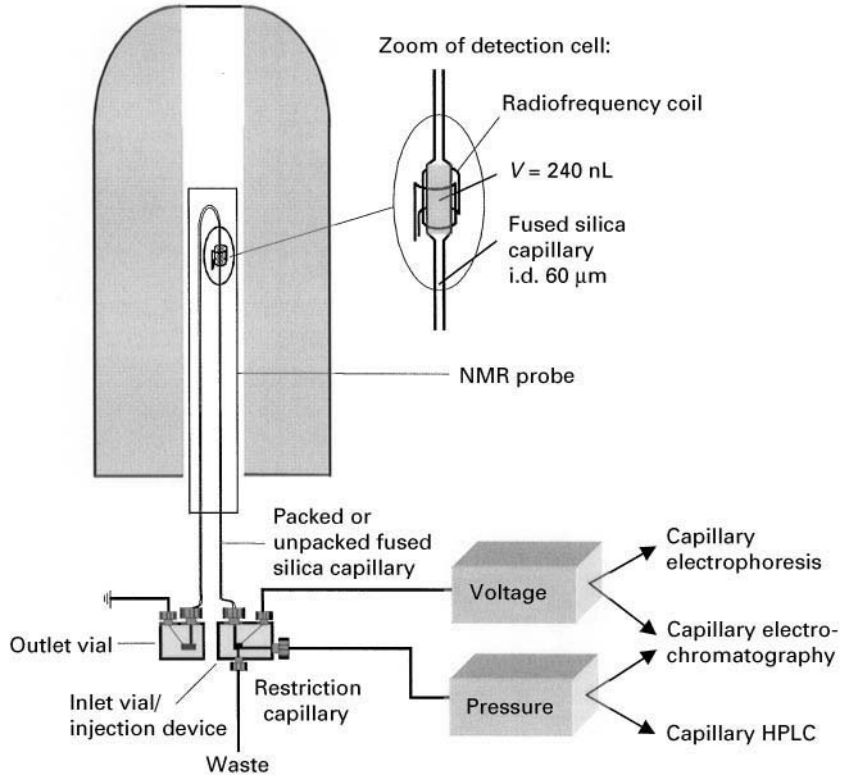

Figure 3 Experimental arrangement for coupling a capillary separation technique with NMR spectroscopy.

phase leads to a restricted exposure period τ for the nuclei in the flow cell. The time τ is defined as the proportion of the detection volume to the flow rate. This is the reason for a shorter transverse relaxation time T_2, which includes larger NMR signals. On the other hand the equilibrium state will be reached in a shorter time due to the permanent-flow nonexcited nuclei than only through the longitudinal relaxation time T_1. This allows a quicker repeat time rate for exposure of a spectrum and therefore greater sensitivity.

LC-NMR spectroscopy is a relatively insensitive method requiring sufficient sample concentration in the NMR flow cell. However, the separation column should not be overloaded, because then separation may be impossible. Most HPLC separations are performed with reversed-phase materials using binary solvent mixtures such as acetonitrile–water, acetone–water or methanol–water as mobile phases. The choice of the mobile phase should be suited to the NMR spectroscopy. An obvious advantage is to obtain a small number of solvent signals in the NMR spectrum, because the solvent signals may obscure the sample spectra. Generally, the chromatographic conditions in HPLC-NMR experiments are the same as in conventional chromatography, but water is replaced by deuterated water. The use of deuterated organic solvents is generally too expensive. For proper adjustment of the receiver gain of the NMR instrument, the solvent signals should be reduced to the height of the sample by applying a solvent suppression technique. Efficient solvent suppression an be performed, for example, by applying a NOESY-type presaturation scheme. With residence times in the order of 5 s and acquisition times of about 1 s, sufficient presaturation time is left even in the continuous-flow mode. Modern techniques like WET or WATERGATE sequences are based on a selective dephasing of the solvent signals using a magnetic field gradient.

Another problem is solvent purity. Most HPLC solvents have small amounts of impurities, often stabilizer additives. These additives have no UV activity and do not affect the chromatographic results, but will be detected in the NMR spectrum.

There exist in principle two general methods for carrying out LC-NMR: continuous-flow and stopped-flow experiments.

Continuous-flow Experiments

With this mode of operation the output of the chromatographic separation is recorded at the same time as the ^{1}H NMR spectra. In most cases the flow

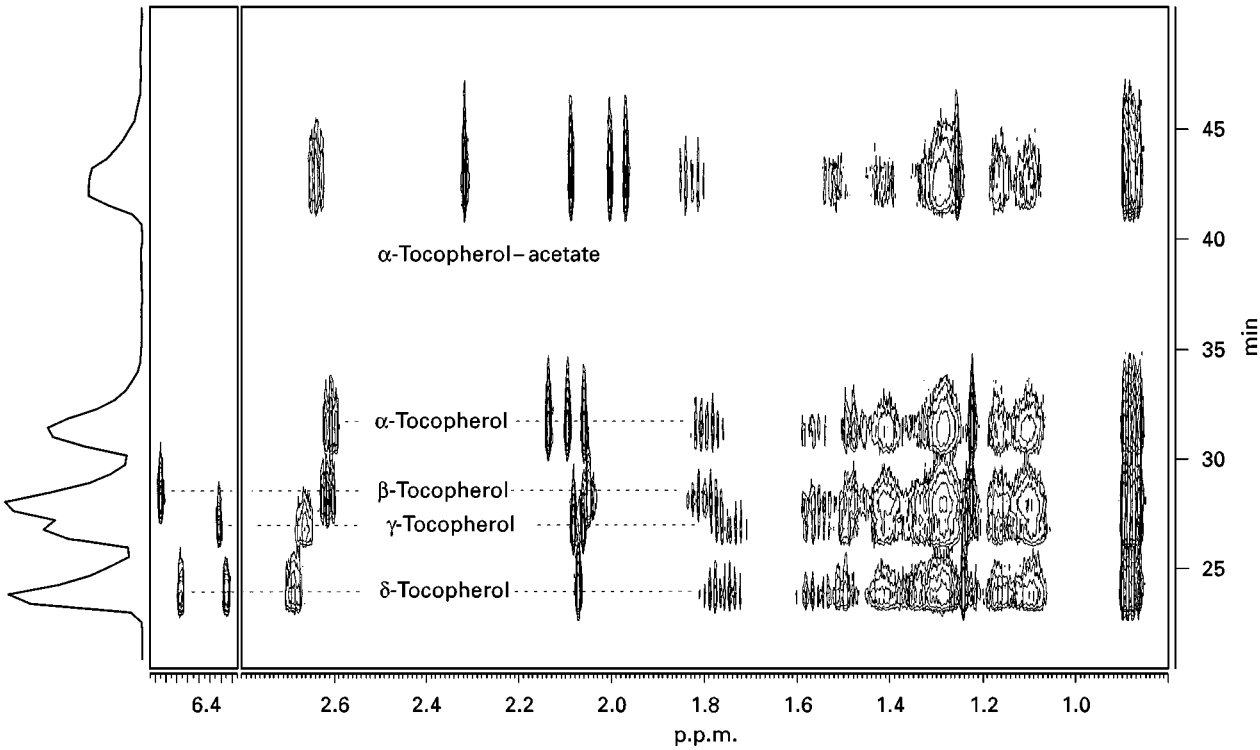

Figure 4 Contour plot (600 MHz) of the separation of tocopherol isomers.

rate of the mobile phase is decreased to yield an NMR scan accumulation of one chromatographic peak. The acquisition of online continuous-flow NMR spectra results in a two-dimensional contour plot of ^{1}H NMR signals of the separated compounds (x axis = ^{1}H chemical shift) versus retention time (y axis). **Figure 4** shows the contour plot of the separation of tocopherol isomers on a C_{30} stationary phase in methanol together with 2% CD_3OD. A time interval of 1 s was applied to presaturate the methyl group signal of the solvent. A total of 128 experiments with a time resolution of 27.3 were recorded at a flow rate of 0.3 mL min^{-1}. The chromatogram along the y axis was reconstructed by co-addition of all proton resonances between 0.8 and 1.9 p.p.m. The resolution of this ^{1}H NMR chromatogram suffers from the small number of 128 data points, but the separation of all five tocopherol isomers is readily apparent.

The structure of the separated compounds can easily be assigned by the methyl group ^{1}H NMR signals between 2.0 and 2.2 p.p.m. and by the aromatic resonances between 6.4 and 6.5 p.p.m. (**Figure 5**). For instance, the continuous-flow ^{1}H NMR spectrum of δ-tocopherol shows one signal for the methyl group at C_8 and two aromatic resonances with a small splitting due to the meta coupling between H_5 and H_7. Thus, unequivocal structural assignment of all different isomers is possible by considering chemical shifts, coupling constants and integration ratios.

Stopped-flow Experiments

In continuous-flow experiments only a short time is available for accumulation of the ^{1}H NMR spectrum. Thus, this spectroscopic technique can only be used for high concentrations of the sample, since otherwise the signal to noise ratio is too low. The sample is transferred through the flow cell analogous to the continuous-flow experiment, but in the stopped-flow mode the valves of the sample unit switch and the chromatographic run is stopped as soon as the maximum of the peak reaches the flow cell (indicated by UV detector). At this point conventional NMR experiments can be performed and, therefore, the NMR experimental time can be adjusted to the sample concentration. Another advantage of the stopped-flow experiment is the possibility of multidimensional NMR spectra.

In **Figure 6** a correlation spectroscopy (COSY) stopped-flow spectrum of the olefinic protons of the carotenoid all-*trans* zeaxanthin is shown, recorded on a Bruker AMX 400 spectrometer. The number of experiments in F1 dimension was 216, the number of accumulated transients was 256.

Due to the centrosymmetric molecule, the corresponding protons are identical. By means of the cross-peaks in the coupling system, the structure can be elucidated. Proton H_{11} couples with two other protons, H_{10} and H_{12}, while proton H_{15} couples with

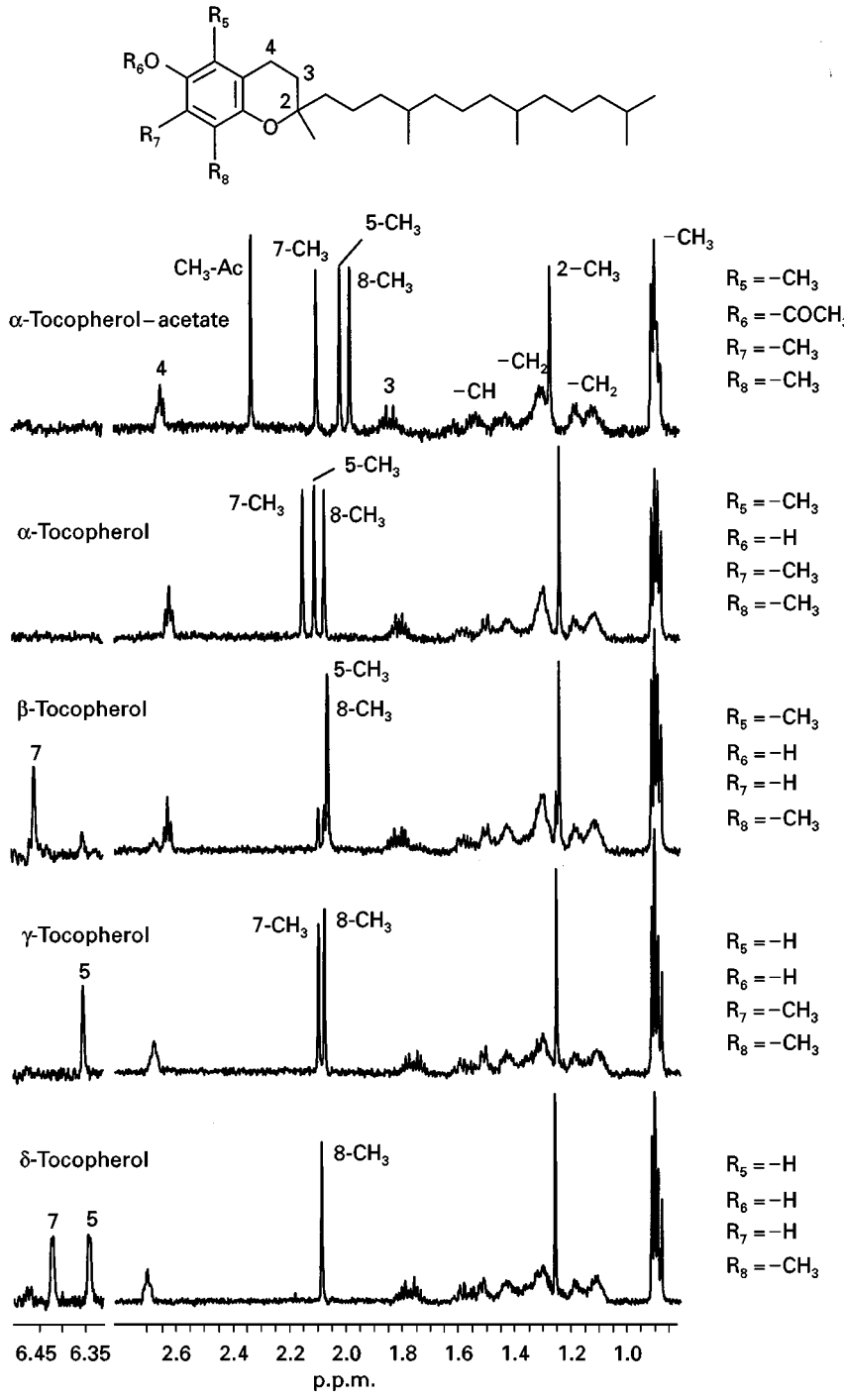

Figure 5 Continuous-flow ^{1}H NMR spectra (600 MHz) of tocopherol isomers.

proton H_{14}. The cross-peak at 6.1 p.p.m. can be assigned to the proton H_7 and H_8.

Figure 7 shows a stack plot of various zeaxanthin stereoisomers. Due to the *cis*-arrangements, the centrosymmetry of the molecule is repeated. Therefore, different resonance frequencies result for the shielded and unshielded protons. In Figure 7 the ^{1}H NMR spectra of all-*trans*, 13-*cis* and 9-*cis* zeaxanthin are depicted.

The assignment of *cis*/*trans* is adapted from the Karplus equation in general, with the difference that the coupling constants are available. In most cases the difference in the chemical shift values in systems with conjugated olefinic protons is more informative. They

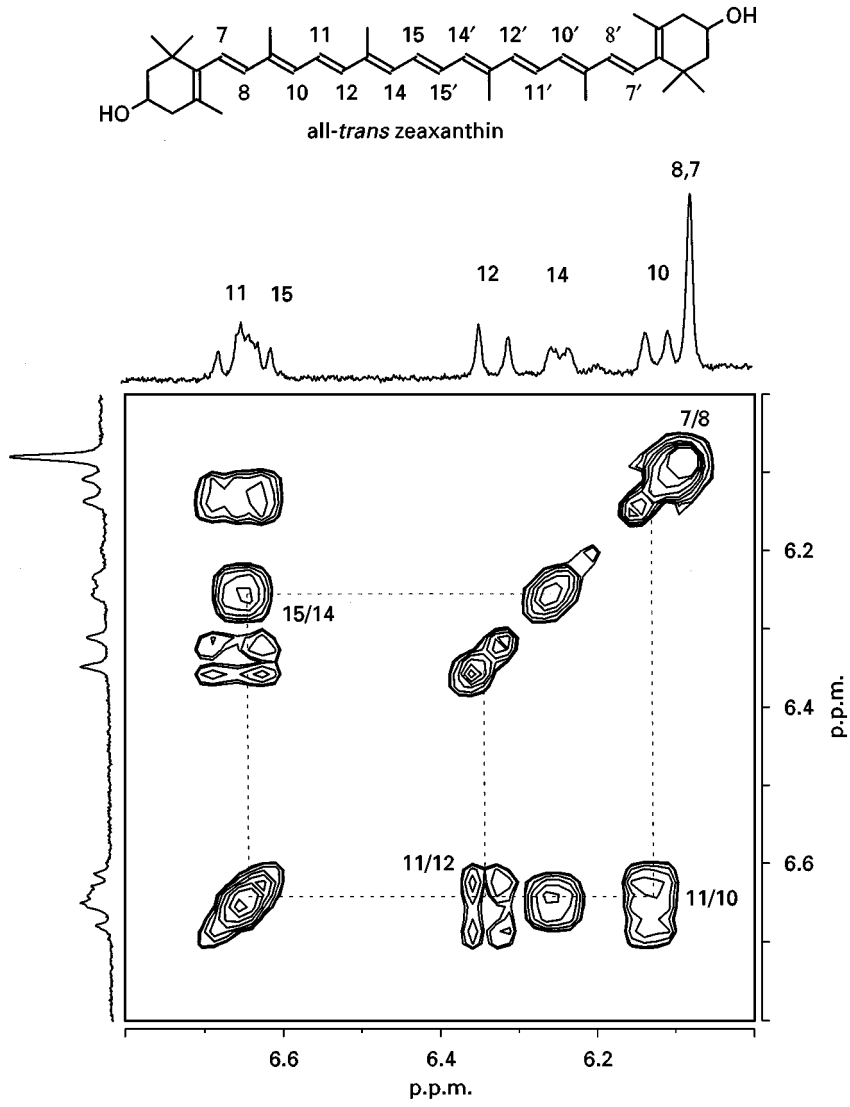

Figure 6 COSY stopped-flow spectrum (400 MHz) of all-*trans* zeaxanthin.

can amount up to 0.6 p.p.m. Looking at the protons of the *cis*-bonding, the outer protons experience a shift to higher field, while the inner protons shift to lower field. As shown in Figure 7, the protons H_{12} and H'_{15} in 13-*cis* zeaxanthin shift to lower field and proton H_{14} to higher field. The protons H_{10} and H'_{15} also show a small influence. The other protons have the same chemical shift values as the all-*trans* zeaxanthin and the *cis*-bonding has no further influence. Analogous effects could be explained for the 9-*cis* zeaxanthin. Here the *cis*-bonding has no effect on the shielded protons and only the unshielded ones are affected.

SPE-LC-NMR Coupling

In order to enhance the sample concentration injected onto the column for HPLC separation, solid-phase extraction (SPE) can be used. In online SPE, the sample is injected onto a cartridge that replaces the loop of the injection device of the chromatographic system. By switching the valve, the enriched sample is transferred directly onto the separation column. The advantage of online SPE-LC coupling is the direct enrichment of analytes on a cartridge in the HPLC system excluding light and oxygen.

A new online SPE-LC technique in which both the trace enrichment and the separation are performed on one single short HPLC column (10–20 mm in length) is called the SSC (single short column) approach. By performing online sample enrichment before LC-NMR analysis, analyte detectability may be significantly improved. The hyphenation of this approach with NMR spectroscopy can reduce the effective analysis time necessary for separation and structure elucidation. As in conventional LC-NMR coupling,

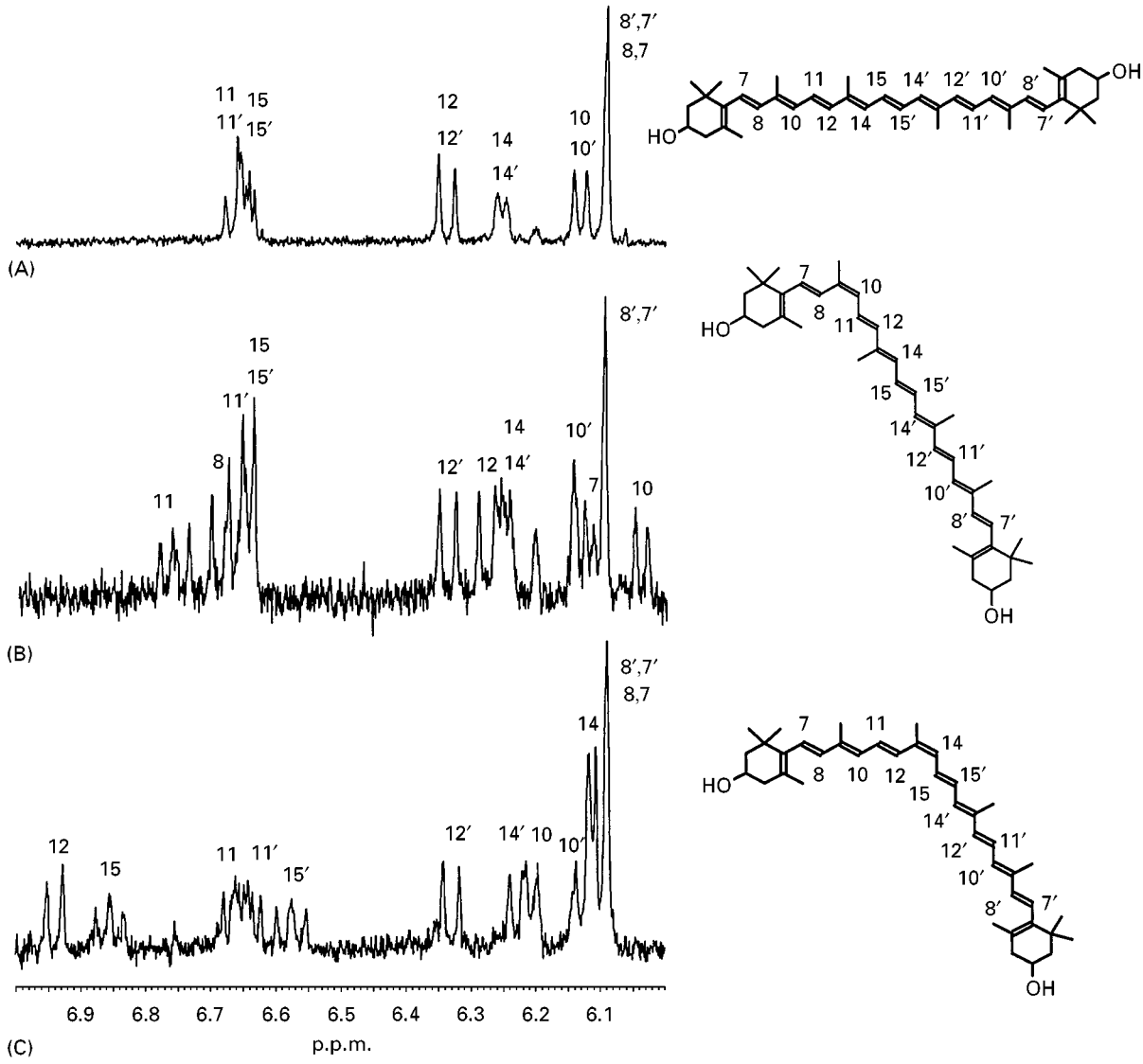

Figure 7 ^{1}H NMR spectra (600 MHz) of (A) all-*trans*, (B) 9-*cis* and (C) 13-*cis* zeaxanthin.

stopped-flow as well as continuous-flow experiments are possible. The higher concentration of sample is advantageous for continuous-flow LC-NMR experiments. For online SSC-NMR, 10 mL of a 5 μg mL^{-1} mixture was loaded on to a 12.5 × 4.6 mm i.d. C_{18} short column and separated on-column with the eluents (A) acetonitrile–0.05% phosphoric acid and (B) water–0.05% phosphoric acid 30 : 70 (v/v) at a reduced flow rate of 0.3 mL min^{-1} and transferred to the NMR spectrometer. **Figure 8** shows the contour plot of the separation of ibuprofen, fenoprofen and naproxen.

In the case of these drugs, the differences between the structures can be determined by the signals in the aromatic region, so the structure of the separated compounds can easily be assigned by the aromatic resonances. Whereas naproxen is a substituted naphthalene derivative, fenoprofen and ibuprofen both contain a phenyl ring which is meta-substituted in the case of fenoprofen and para-substituted in ibuprofen. For unambiguous structural elucidation, different rows could be extracted from the contour plot to obtain conventional ^{1}H NMR spectra, as shown in **Figure 9** for ibuprofen.

GPC-NMR Coupling

One of the most impressive advantages of continuous-flow ^{1}H NMR spectroscopy is the direct monitoring of the change in the microstructure of polymers and in the chemical composition of copolymers during GPC. In the case of synthetic polymers, the amount of

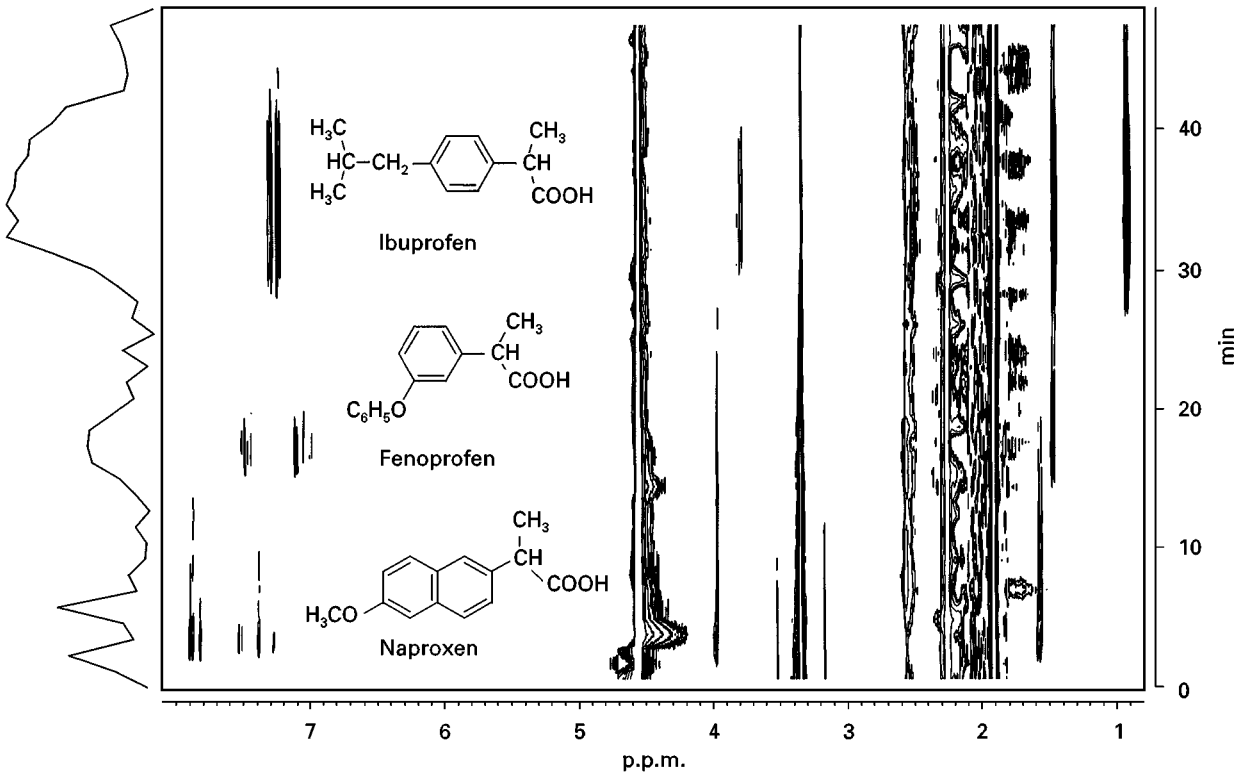

Figure 8 ^{1}H NMR chromatogram (contour plot, 400 MHz) of an SPE-HPLC separation of ibuprofen, fenoprofen and naproxen.

available sample is not limited and GPC is not sensitive to peak dispersion effects. A NMR flow cell with a detection volume of 120 μL with a 400 MHz spectrometer yields adequate signal-to-noise values within a reasonable resolution time of 8 s.

One example shows the possibilities of GPC-NMR coupling and is typical of a multitude of similar problems in the chemical industry. Two styrene-butylacrylate copolymers were synthesized under similar conditions, but the physical properties of the copolymers differed. Conventional polymer analysis failed to distinguish between the samples. In both cases the chemical composition and microstructure were identical.

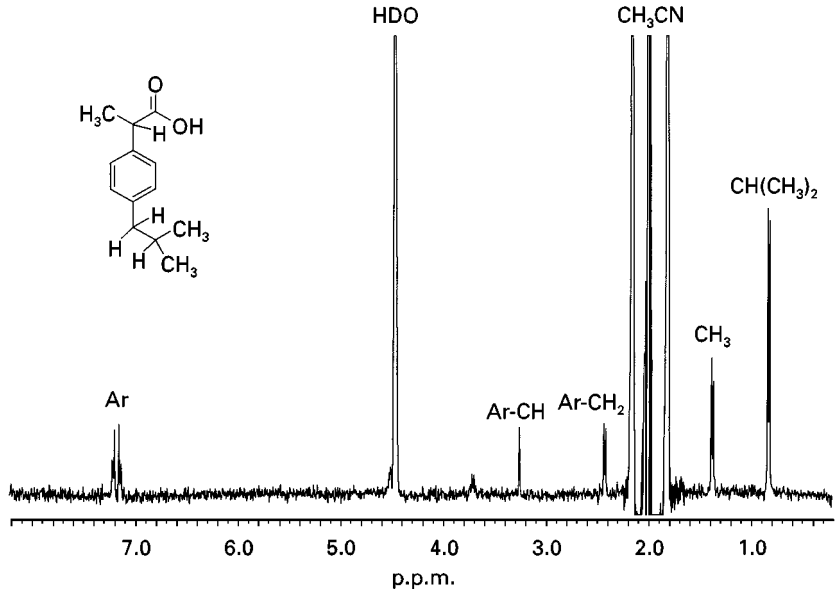

Figure 9 ^{1}H NMR spectrum of ibuprofen extracted from the SPE-HPLC-NMR run depicted in Figure 8.

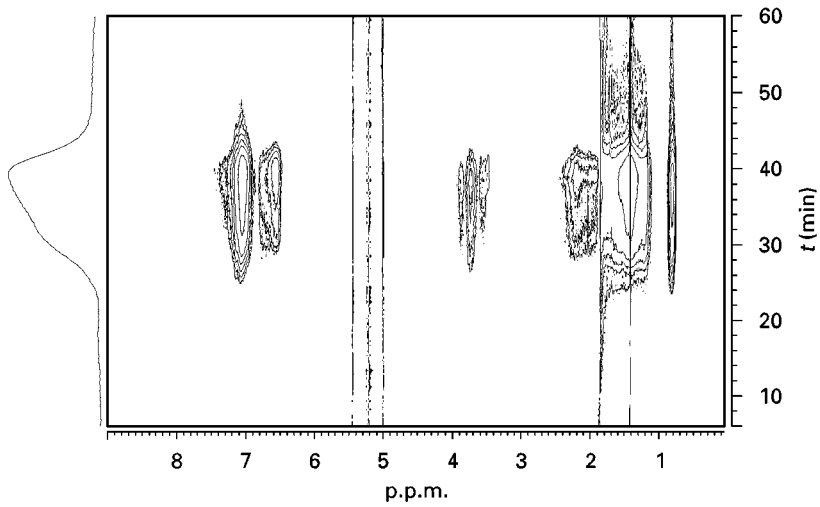

Figure 10 Stacked plot (400 MHz) of the GPC-NMR separation of a styrene-butylacrylate copolymer.

A GPC separation of 100 μL of a 7.5% copolymer solution was performed with a 250×40 mm GPC column using dichloromethane as eluent at a flow rate of 0.4 mL min^{-1}. Sixteen transients were co-added, defining a time resolution of 8.4 s. The Fourier-transformed spectrum results in a row in the two-dimensional plot of ^{1}H chemical shifts versus retention times (**Figure 10**).

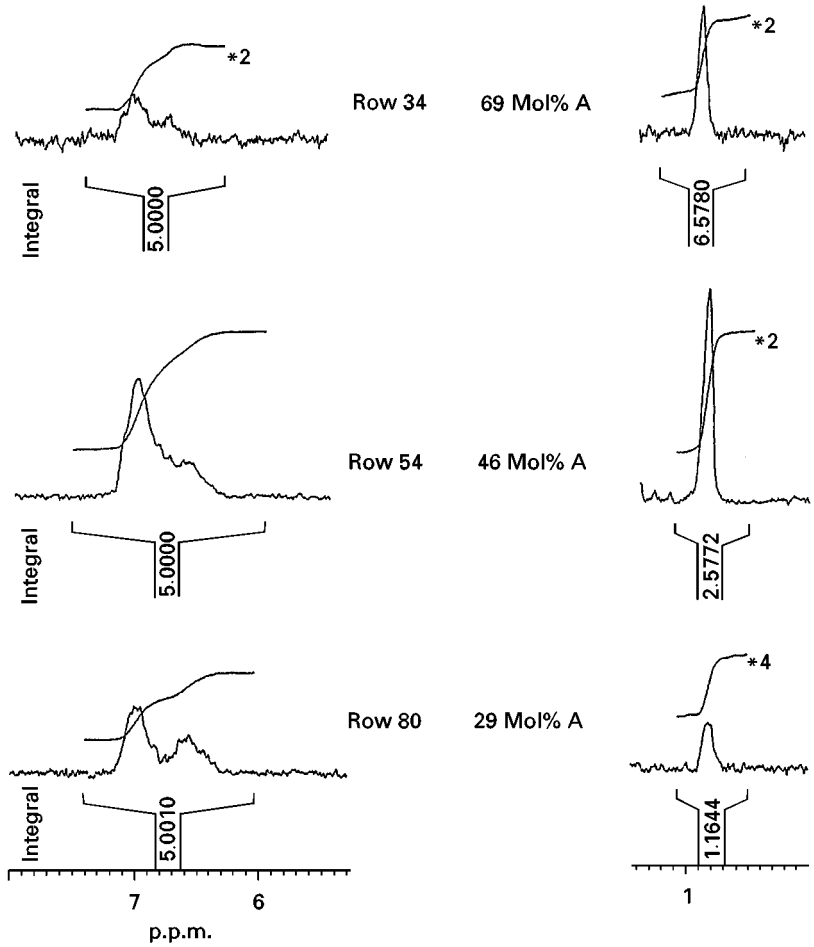

Figure 11 Selected rows of the GPC-NMR separation of a styrene-butylacrylate copolymer (Figure 10) showing signals from the aliphatic and aromatic spectral region.

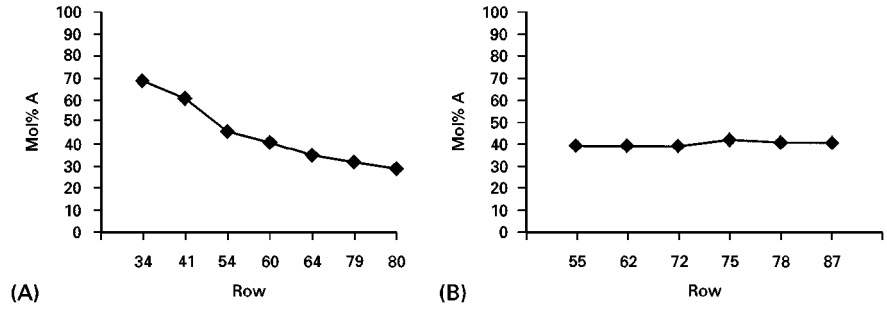

Figure 12 Styrene-butylacrylate copolymer composition versus GPC elution time. (A) Latex A; (B) Latex B.

The methyl group and oxymethylene signals of the acrylate (A) and the aromatic resonances of the styrene (S) units can be used in an online GPC-NMR run to derive information about the molecular weight dependence of the chemical composition.

Within one separation run, up to 128 rows were accumulated, resulting in an overall acquisition time of 42 min. Three selected rows are depicted in **Figure 11**, showing the varying intensities of the CH_3 signals of butylacrylate at 0.85 p.p.m. versus the aromatic signal of styrene at 7 p.p.m. for one copolymer sample.

Thus, the copolymer composition can be directly determined from the elution curves of both signals at any row of the chromatogram. The results from the GPC-NMR coupling for both samples are shown in **Figure 12**. The copolymers show a completely different behaviour in their dependence of the chemical composition on molecular weight.

This example demonstrates the great time-saving nature of the hyphenation of chromatography with NMR spectroscopy. To yield the same information as in the online GPC-NMR run, 128 fractions of the GPC separation would have to be collected and 128 routine ^{1}H NMR spectra recorded. Whereas the GPC-NMR data were obtained within less than 1 h, offline separation and NMR examination would take at least 3 h.

SFC-NMR and SFE-NMR Coupling

A separation technique employing nonprotonated solvents is SFC, using CO_2. For SFC-NMR experiments a pressure-stable flow cell has been developed using a sapphire tube instead of glass (Figure 1B). At a temperature of 323 K and a pressure of 161 bar, high resolution continuous-flow NMR spectra in supercritical CO_2 can be obtained.

This is demonstrated in **Figure 13**, showing the ^{1}H NMR spectrum of ethylbenzene in supercritical CO_2. Often, SFC-NMR separations can be performed with a pressure gradient. Thus, different isomers of vitamin A acetate are easily separated (**Figure 14**).

Supercritical CO_2 can also be used for supercritical fluid extraction (SFE) purposes. As an example, **Figure 15** shows the ^{1}H NMR spectrum of piperin extracted from black pepper at a temperature of 370 K.

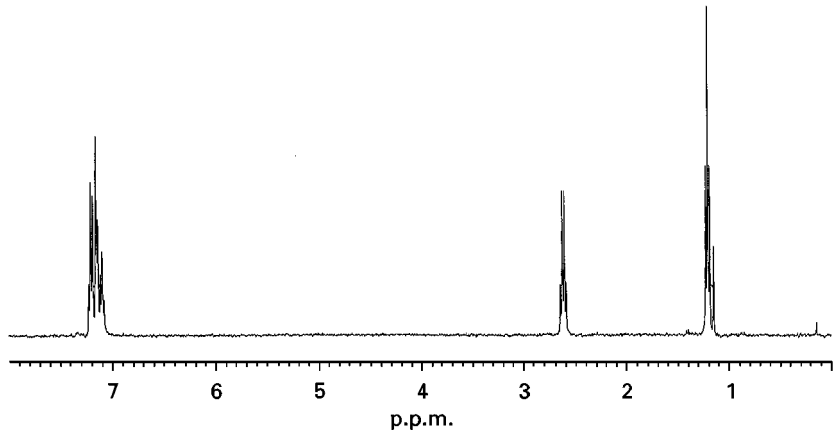

Figure 13 ^{1}H NMR spectrum (400 MHz) of ethylbenzene in supercritical CO_2.

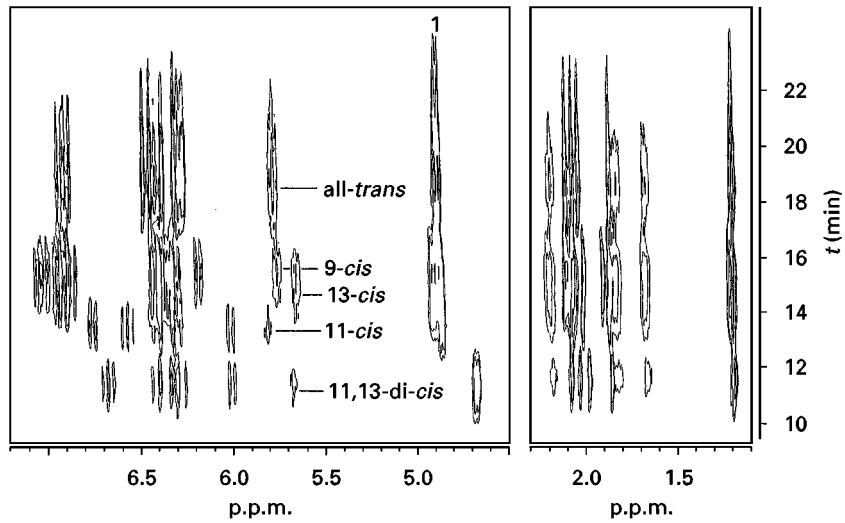

Figure 14 Contour plot (400 MHz) of the separation of vitamin A acetate stereoisomers in supercritical fluid CO_2. Reprinted from *Journal of Chromatography A*, 761, with permission from Elsevier Science.

Capillary Separations

The above examples clearly show the great advantage of combining efficient separation techniques with NMR detection for the solution of complex analytical problems. In all the applications described, analytical columns (250×4.6 mm) together with sample quantities in the μg range and HPLC analysis times of up to 30 min were employed.

However, there is an increasing need for high-throughput screening and analysis of mixtures containing unknown compounds in the growing field of biotechnology and genetic engineering. Here, only a few nanograms of biologically active mixtures of compounds may be available, and these must be fully characterized. This task can only be performed by miniaturizing the closed–loop separation identification system, combining capillary separation technique with nanolitre-scale NMR spectroscopy.

Whereas capillary techniques are already well developed in the field of separations, nanolitre NMR spectroscopy is still in its infancy. Various hardware approaches exist to record NMR spectra on the nanolitre scale. One is the use of a solenoid coil directly attached to the fused silica capillary. The other employed by our group is the application of a microprobe with a double-saddle Helmholtz microcoil. The capillary is fixed within the microcoil in the *z*-direction of the cryomagnet. This approach has advantages and disadvantages. Because the capillary is inserted within the coil, it can be easily exchanged to meet other separation problems but this design has the inherent disadvantages that the filling factor (the ratio of the coil versus the sample volume) is lower in

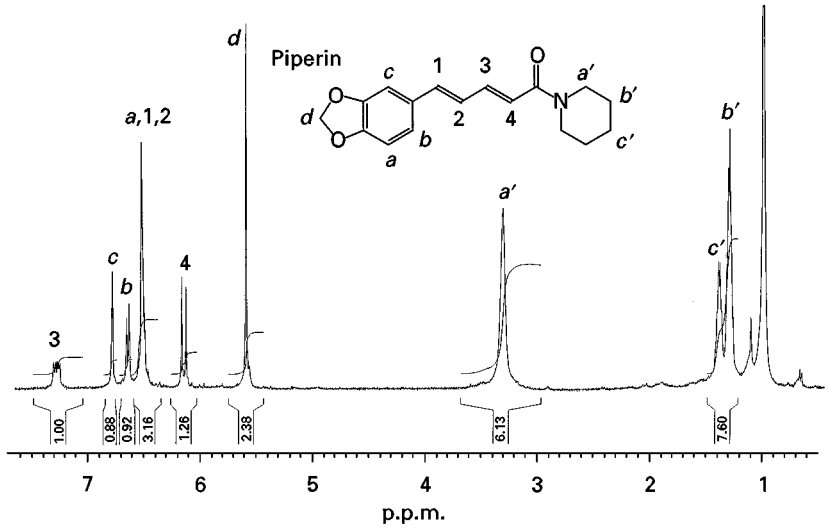

Figure 15 ^{1}H NMR spectrum (400 MHz) of piperin extracted from black pepper.

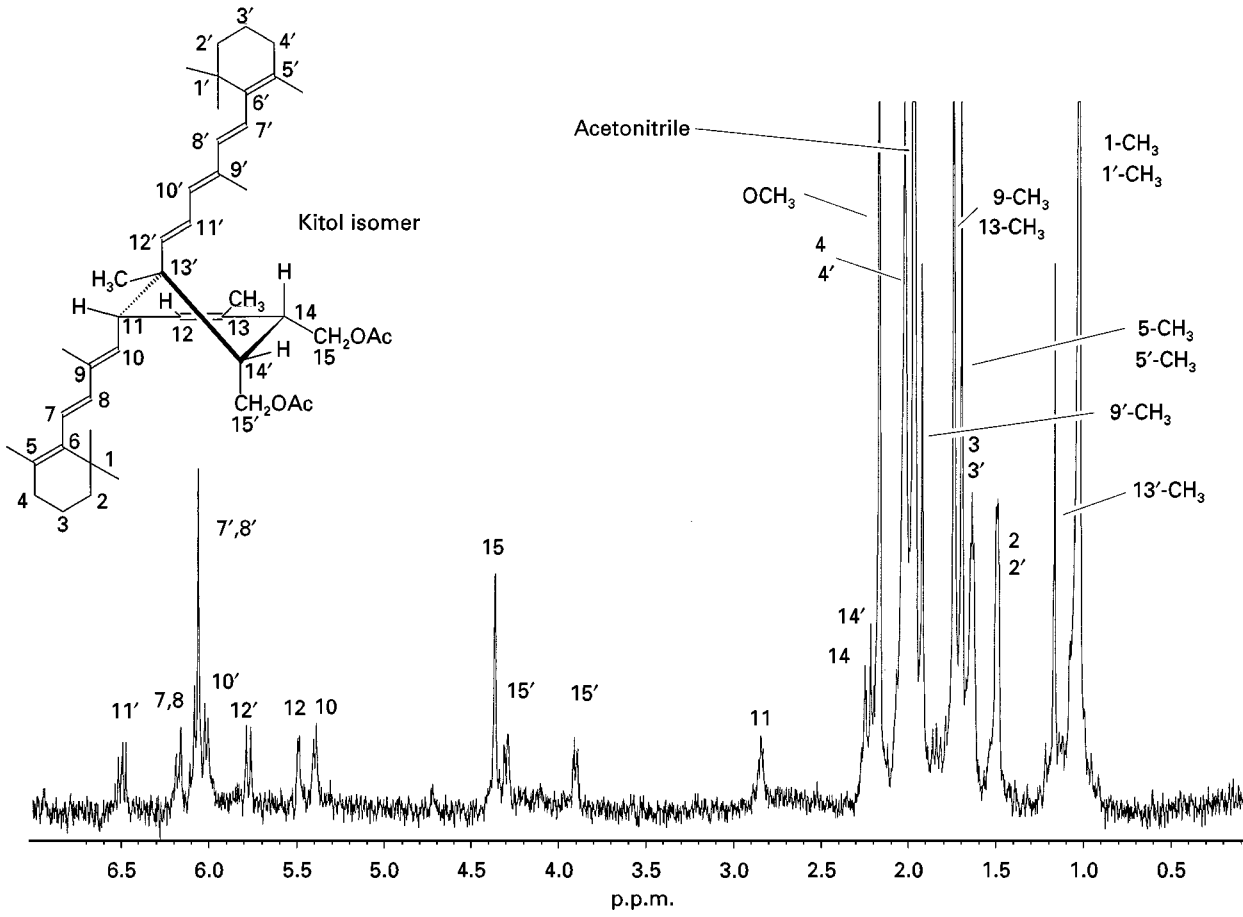

Figure 16 Stopped-flow capillary ^{1}H NMR spectrum of a vitamin A dimerization product recorded within a capillary (detection volume 200 nL).

comparison to a design where the coil is directly fixed to the capillary. Solenoid coils with a perpendicular orientation to the B_0 direction of the cryomagnet result in a threefold increase in sensitivity in comparison with the Helmholtz microcoil design; however, the currently developed approaches suffer from susceptibility-induced line broadening due to the solenoidal coil fixing to the fused silica capillary. This design has already been improved by the application of a susceptibility-matching fluid and shows potential for further optimization. On the other hand, the orientation of the capillary within the *z*-direction of the B_0 field has the tremendous advantage that the NMR signal half-width is not affected when electroosmotic flow-driven separation techniques (CE, CEC) are performed. Here, the induced magnetic field of the current within the capillary has no component in the *z*-direction of the cryomagnet. Thus, a vertically oriented Helmholtz microcoil is a feasible design, at least for capillary separations.

Coupling of capillary HPLC rather than conventional separation techniques with NMR has several distinct advantages. The reduced solvent consumption allows the use of the deuterated solvents, thus rendering elaborate solvent suppression unnecessary. If only a small amount of sample is available, higher concentrations of analyte in the detection cell are obtained when the column dimensions are small. Currently obtained sensitivity levels of this design are in the 500 ng range with acquisition times of some seconds.

One of the first practical results obtained by online capillary HPLC-NMR coupling was the structure elucidation of a vitamin A derivative. The structure of the dimers of vitamin A acetate, so-called kitols, was unknown for a long time because these compounds are sensitive to UV irradiation and to air. The classical procedure of isolation, removal of the extraction solvent and resolvation in a deuterated solvent resulted in many isomerization products. By combining a capillary separation together with NMR microcoil detection, structure elucidation of a previously unknown kitol was possible. **Figure 16** shows the capillary ^{1}H NMR spectrum of the unknown kitol, indicating that the resolution is sufficient to obtain all necessary coupling constants, whereas the hump

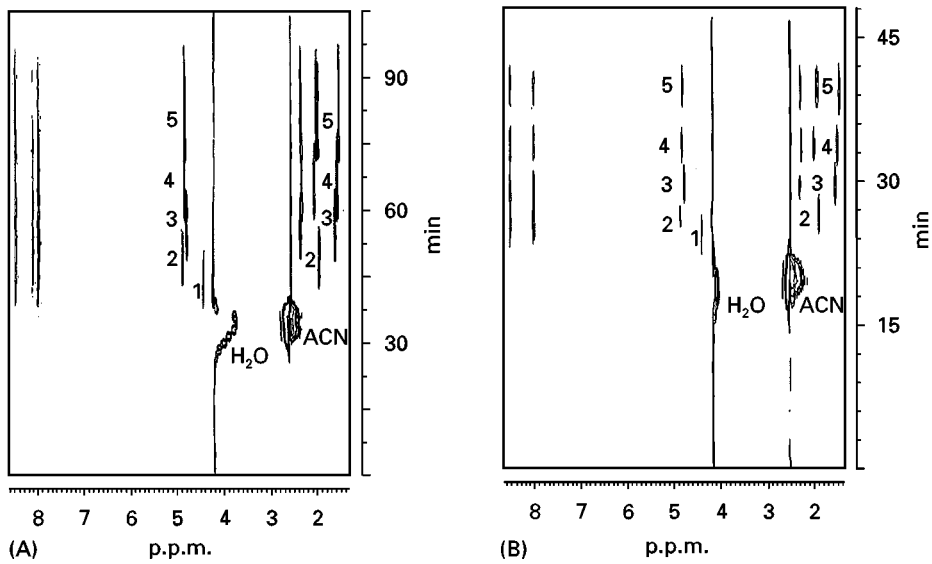

Figure 17 Contour plots of (A) CHPLC and (B) CEC separation of alkylbenzoates: 1, methyl; 2 ethyl; 3, propyl; 4, butyl; 5, pentyl benzoate; ACN, acetonitrile.

(signal line width) at the height of the ^{13}C signals must be further optimized.

A further example is the online CE-NMR and online CEC-NMR separation of alkylbenzoates. **Figure 17** shows the contour plot of the separation performed in the CHPLC and the CEC mode. It is evident from the CEC-NMR contour plot that all compounds are baseline-separated, resulting in distinct NMR rows in the two-dimensional display.

This example shows the great power of CHPLC, CE and CEC-NMR to derive unambiguous information of substances in complex organic molecules. The first steps towards a high-throughput separation system have already been made. For the successful performance of real-life applications, NMR sensitivity must be improved. If NMR probes with 1 ng sensitivity become available, an increasing number of capillary separations coupled with nanoscale NMR will be performed in many applications.

See also: **II / Chromatography: Supercritical Fluid:** Instrumentation. **Electrophoresis:** Detectors for Capillary Electrophoresis. **III/Carotenoid Pigments: Supercritical Fluid Chromatography. Gradient Polymer Chromatography: Liquid Chromatography. Natural Products:** Liquid Chromatography-Nuclear Magnetic Resonance. **Pigments:** Liquid Chromatography. **Polyethers: Liquid Chromatography.**

Further Reading

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Partition Chromatography (Liquid–Liquid)

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Introduction

Partition or liquid–liquid chromatography (LLC) is a powerful separation technique which has been successfully used for the separation and analysis of