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Review

Liquid chromatography–nuclear magnetic resonance spectroscopy

Klaus Albert*

Universität Tübingen, Institut für Organische Chemie, Auf der Morgenstelle 18, D-72076 Tübingen, Germany

Dedicated to my wife Heidi Albert on the occasion of her 50th birthday

Abstract

A general overview of the experimental set-up for performing analytical-scale and nanoliter-scale liquid chromatography–^{1}H nuclear magnetic resonance spectroscopy (LC–^{1}H-NMR) experiments is given. The high power of combining LC with ^{1}H-NMR spectroscopy is demonstrated by two examples, where NMR acquisition was performed either in the continuous-flow mode on the analytical scale or in the stopped-flow mode on the nanoliter scale. Current developments employing the on-line coupling of capillary as well as supercritical fluid separation methods with ^{1}H-NMR spectroscopy together with LC–^{13}C-NMR spectroscopy are discussed.

Keywords: Nuclear magnetic resonance spectroscopy; Reviews; Detection, LC; Vitamins; Kitols

Contents

1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy is the most powerful technique for the structural elucidation of unknown compounds. This is the reason why the hyphenation between chromatographic separation techniques together with NMR spectroscopy is becoming increasingly popular.

The first on-line coupling experiments have con-

*Tel.: +49-7071-29-75335; fax: +49-7071-29-5875.
E-mail address: klaus.albert@uni-tuebingen.de (K. Albert)

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ducted at academia in the late 1970s [1–3], but the acceptance of the ''exotic'' hyphenation technique was stimulated by powerful applications in the 1980s and the early 1990s [4–18]. At the beginning of the new millennium, liquid chromatography (LC)–NMR is a well established analytical tool in all industrial laboratories and is now considered to play an active role in analytical laboratories even at conservative universities [19–21,34].

Why did it take nearly 20 years to establish LC–NMR as an equal partner to LC–mass spectrometry (MS)? The main reason is the ignorance of both classical chromatographers and NMR spectroscopists regarding the methodology and needs of their mutual techniques. Furthermore, especially in academia, it is the thinking in claims to maintain the benefice, similar to the priests in middle ages.

2. Design of NMR probes

In a conventional routine NMR investigation of an unknown peak in a high-performance liquid chromatography (HPLC) separation, the solvent or solvent mixture of the collected fraction is evaporated, the compound is redissolved in a deuterated solvent, and the solution is transferred to a 5-mm NMR tube. NMR acquisition is performed while the NMR tube is rotated to eliminate magnetic field inhomogenities (Fig. 1).

This design for the acquisition of routine NMR spectra is inherited from the early 1950s, when ^{1}H-NMR spectra were recorded with iron magnets and continuous-wave excitation. Today, cryomagnets with extreme high magnetic field homogeneity are used, employing the pulse Fourier transform technique introduced by the Nobel Laureate Ernst [22]. With 600–900 MHz NMR spectrometers applying modern highly sophisticated two-dimensional gradient pulse techniques, the NMR tube is not rotated any more because rotational side bands cause severe distortions in the two-dimensional NMR spectrum.

The first continuous-flow NMR probes for cryomagnets, introduced in the early 1980s [4,5], presented a clear contradiction to the existing dogma of acquiring high-resolution NMR spectra. By fixing a U-type glass tube in the dewar of a NMR probe body, the central symmetry of the magnetic field in the *z*-direction of the cryomagnet is broken and the rotation of the glass tube is not possible (Fig. 1). But even the first applications of this design showed an excellent NMR resolution approaching classical values with rotation of the NMR tube [4,5]. With the dramatically improved homogeneity of the current generation of cryomagnets, the registration of continuous-flow ^{1}H-NMR spectra with high resolution is not a problem.

But problems arise with the registration of ^{1}H-NMR spectra under routine HPLC conditions in the reversed-phase mode. Here, binary solvent mixtures, containing protonated solvents such as water, CH_3CN and CH_3OH, are typically used. For the optimal adjustment of the receiver gain of the NMR instrument the proton solvent signals have to be suppressed using a nuclear Overhauser effect spectroscopy (NOESY)-type presaturation technique [15,16].

The continuous-flow NMR probe design for routine applications employs detection volumes between 40 and 120 μl, much larger than conventional UV detection volumes, which are on the order of 8 μl. These extremely large detection volumes are necessary for two reasons [10]. First, in flowing systems there is a distinct residence time, τ, of the nuclei within the NMR detection cell. This residence time is defined by the ratio of the detection volume to the employed flow-rate. If τ is below 5 s, flow-induced NMR signal line broadening will contribute to the NMR signal half width and reduce the NMR spectral resolution. Second, NMR spectroscopy is a volume-sensitive detection technique and requires the maximization of NMR-active nuclei by extending the detection volume. Due to the diffusion process within a chromatographic separation, the concentration within later eluting peaks is reduced by a factor of at least 3. NMR detection of diluted solutions can only be performed with very high detection volumes in the ml range (10–20 mm NMR tubes). The employment of these immense detection volumes would differ sharply with conventional chromatography, wherein analytical columns only allow for detection volumes between 5 to 10 μl. The

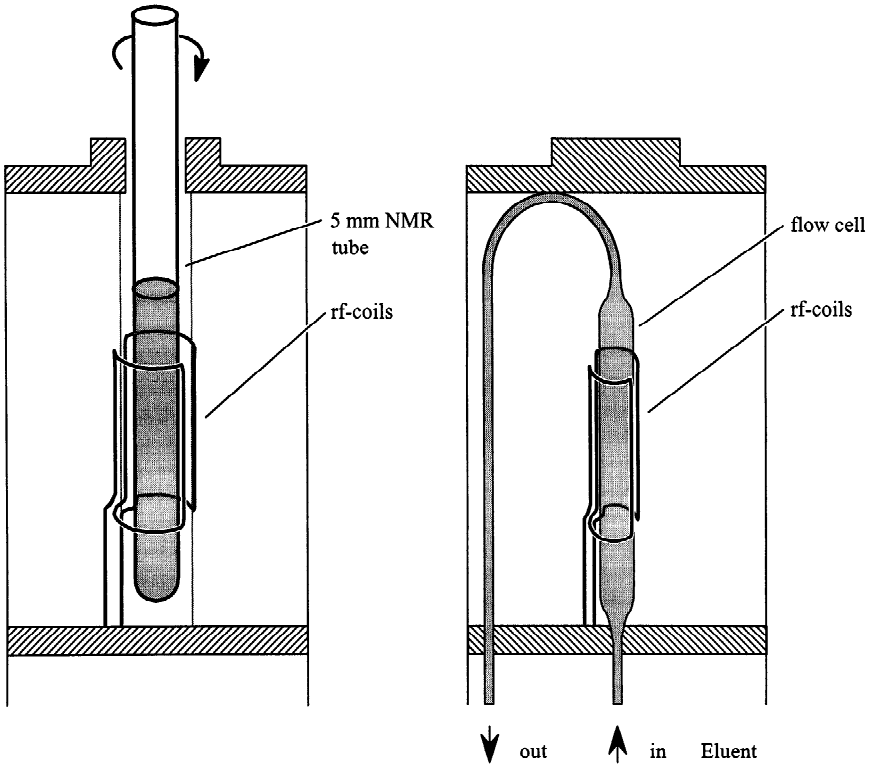

Fig. 1. Geometry of conventional and continuous-flow NMR probes.

presently employed design is a compromise between the needs of chromatography and the needs of NMR.

3. Experimental set-up for analytical LC–NMR experiments

What are the benefits of on-line HPLC–NMR coupling compared to traditional off-line approaches?

One advantage of real-world practical application is the easy set-up of the instrument configuration (Fig. 2). With the newly available shielded cryomagnets, the HPLC instrument fixed on a moveable table can be positioned at a distance of 30–50 cm from the cryomagnet. With conventional cryomagnets, the distance must be 1.5 to 2 m. The continuous-flow probe is inserted into the room temperature bore of the cryomagnet instead of the NMR probe employed for routine operation. The adjustment of field homogeneity (shimming) is a problem for inexperienced operators if no correction values (shim values) are known for a new probe. Here, the help of a service engineer is often needed when the LC–NMR system is installed, but the adjusted shim values can be stored on disk and used as starting values whenever the flow probe is inserted. Then, fine tuning of field homogeneity takes 30 min. The HPLC instrument and continuous-flow NMR probe are connected by stainless steel capillaries (0.25 mm I.D.). In many practical applications, the peak concentration is too low for continuous-flow NMR detection and stopped-flow NMR acquisition has to be performed. To capture the peak maximum in the flow cell after stopping the HPLC pump, a valve is inserted between the HPLC instrument and the flow cell. For the proper timing of stopped-flow acquisitions, the dead time between the passage of a peak through the detector of the HPLC instrument (UV or refractive index) and the NMR flow cell has to be carefully determined. Elaborate fully automated LC–NMR systems employ peak sampling units wherein the

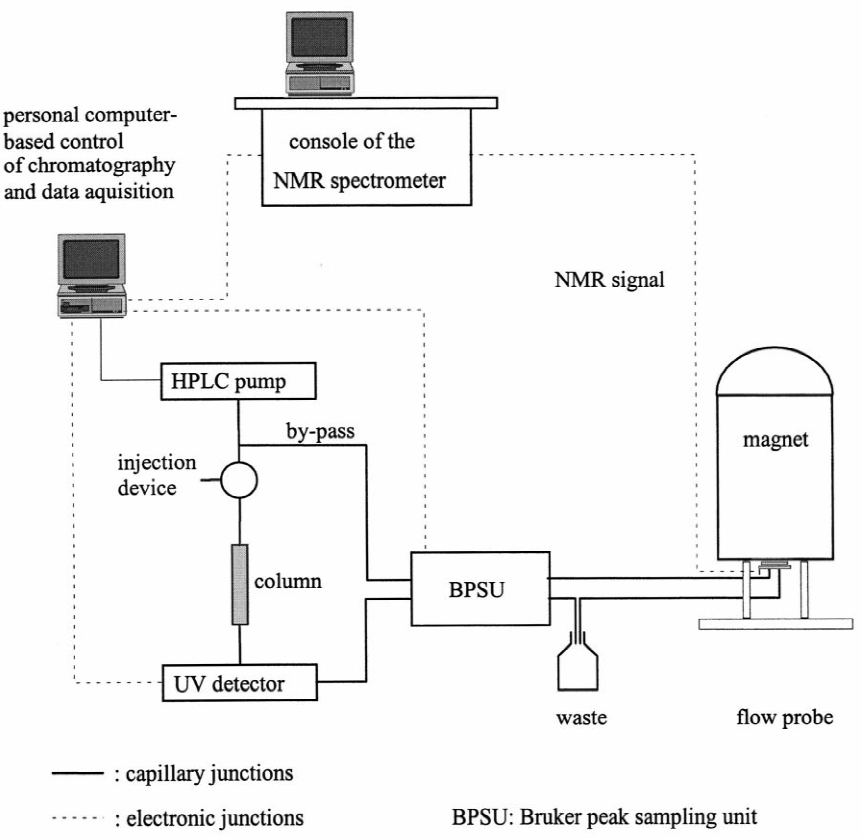

Fig. 2. Instrument configuration for analytical LC–NMR experiments.

separated peak is stored within a loop and NMR registration is performed by transferring the contents of the loops one after the other to the flow cell.

4. LC–NMR on the analytical scale

Many applications have been published in recent years [23–37] dealing with the successful employment of LC–NMR in the fields of environmental [27,28], pharmaceutical [17,30,36], natural product [23–26,31–33,37], biomedical [18,29] and polymer research [35]. In particular, the research groups of Dorn, Nicholson, Preiss, Wilkins and Wilson have developed major new applications in the hyphenation of HPLC and NMR. The following two examples from our laboratory in Tübingen are intended to emphasize the extreme advantages of on-line HPLC–NMR coupling.

Carotenoids are essential nutrients for the human body. They have enormous potential as antioxidants and are very important in vision. Because the biological activity of *cis*/*trans* isomers of essential carotenoids is dependent upon the isomeric conformation, structure determination of the stereochemistry is of high importance. HPLC–MS coupling cannot be used for this application because the fragmentation pattern of different stereoisomers is the same. Thus, HPLC–^{1}H-NMR coupling is the method of choice for the solution of this problem.

Vitamin E plays an important role due to its ability to scavenge free radicals. The structures of its main isomers together with its main synthetic derivative, α-tocopherol acetate, are depicted in Fig. 3. These five compounds are well separated with an analytical C_{30} column (250×4.6 mm) employing a mobile phase composition of methanol–[$^{2}H_{4}$]methanol (98:2) [33]. A continuous ^{1}H-NMR monitoring of

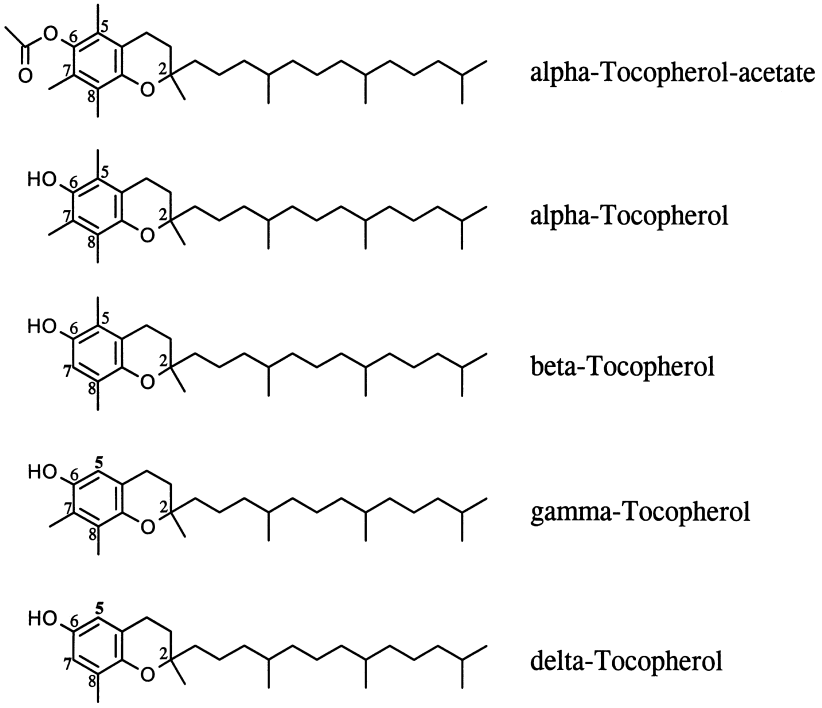

Fig. 3. Structures of tocopherol isomers.

the separation was possible with a flow-rate of 0.3 ml/min by injecting 25 μl of a mixture containing 500 μg of each compound. Continuous-flow data were accumulated on a 600 MHz spectrometer into 8 K data points over a sweep width of 7200 Hz. Sixteen transients were coadded for one interferogram, defining a time resolution of 27.3 s. In total, 128 interferograms (free induction decays) have been recorded over the entire separation. The Fourier transformation of each interferogram results in a typical high-resolution ^{1}H-NMR spectrum, representing the sum of all peaks within an elution volume of 137 μl. Fig. 4 shows the bird's eye view of the HPLC separation. Here, the signal summits of all spectra are displayed as a contour plot of the ^{1}H-NMR signals within the chemical shift ranges 0.9–2.7 ppm and 6.3–6.5 ppm against retention times between 0 and 50 min. The relative intensity of the sum of all protons contained within the time slice of 27.3 s can be displayed versus the retention time, as is shown in Fig. 4 on the left vertical axis. This ^{1}H-NMR chromatogram corresponds to the total ion chromatogram in an HPLC–MS separation, where the sum of all masses is registered versus the retention time. The resolution of the ^{1}H-NMR chromatogram is reduced due to the small number of only 128 data points over a 1 h separation. Nevertheless, even with a resolution of 30 s/data point, the separation of all five peaks is clearly visible in the reconstructed one-dimensional ^{1}H-NMR chromatogram. More information can be obtained from the two-dimensional contour plot. Due to the different ^{1}H-NMR shifts of γ- and β-tocopherol, these compounds can be easily identified despite their coelution in the separation. The continuous-flow ^{1}H-NMR spectra recorded at the peak maxima are displayed in Fig. 5. For instance, β- and γ-tocopherol are easily assigned due to their different pattern in the aromatic region. β-Tocopherol displays only one signal for H-7 at 6.42 ppm, whereas in the ^{1}H-NMR spectrum of γ-tocopherol the signal for H-5 at 6.33 ppm is readily seen.

This example demonstrates that in a closed-loop separation–identification system, structural assignment of air- and UV-sensitive compounds can be performed ''on the fly'' during the chromatographic

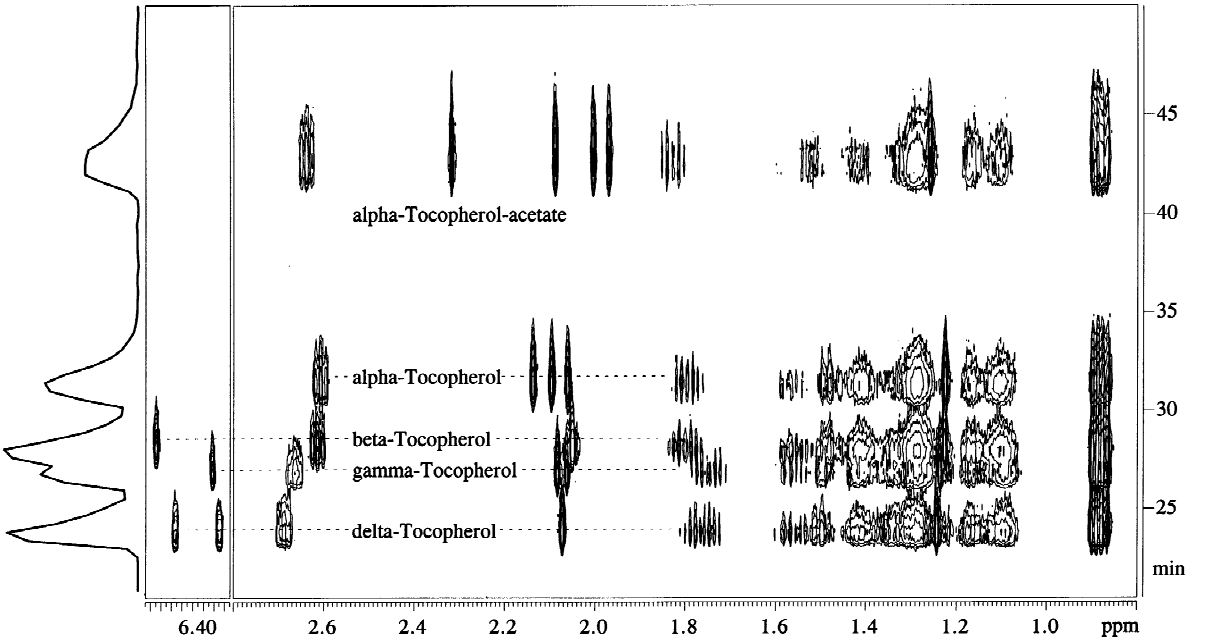

Fig. 4. Contour plot of a separation of tocopherol isomers.

separation. The disadvantage of this ''on the fly'' approach is the large amount of sample needed for the on-line NMR registration.

5. LC–NMR on the nanoliter scale

In many analytical problems, only a few micrograms, or even nanograms, of sample material are available. To fullfil the needs of modern analytical requirements at the beginning of the new millennium, LC–NMR probes on the nanoliter scale are currently under development [38–47]. Sweedler and co-workers pioneered the use of NMR microcoils with very small detection volumes of only few nanoliters [38–40]. By directly wrapping the wire of the NMR detection coil to a horizontally positioned fused-silica capillary they built a solenoidal coil arrangement for the registration of NMR spectra in the nanoliter scale. Thus they were able to perform the first on-line coupling experiments between capillary electrophoresis and ^{1}H-NMR spectroscopy. The design of Sweedler has the big advantage of a three-fold sensitivity versus conventionally employed double-saddle Helmholtz radio frequency (r.f.) coils. A further advantage is an excellent ''filling factor'' (a measure of the fraction of the r.f. coil volume occupied by the sample) due to the direct fixation of the r.f. coil to the capillary wall. But this design suffers from susceptibility induced line-broadening due to the direct wrapping of the coil to the capillary. To reduce the inherent line-broadening Sweedler inserted his microcoil into a matching fluid (fluorocarbon FC43) and obtained NMR spectra with a very good resolution. In a solenoidal measurement device the capillary is positioned perpendicular to the magnetic field of the cryomagnet. Thus in capillary electrophoresis (CE)–NMR and capillary electrochromatography (CEC)–NMR the direction of the electric current in the capillary is perpendicular to the magnetic field and the current-induced magnetic field results in a disturbance of the main magnetic field in the *z*-direction. Magnetic field inhomogeneities result in an increased NMR signal line width and reduced NMR resolution.

Our approach for capillary HPLC–NMR, CE–NMR and CEC–NMR consists of a capillary inserted into a 2.5 or 2.0 mm NMR microprobe equipped with a Helmholtz coil (Fig. 6) [41–47]. Despite the very bad ''filling factor'' this ''insert design'' has the

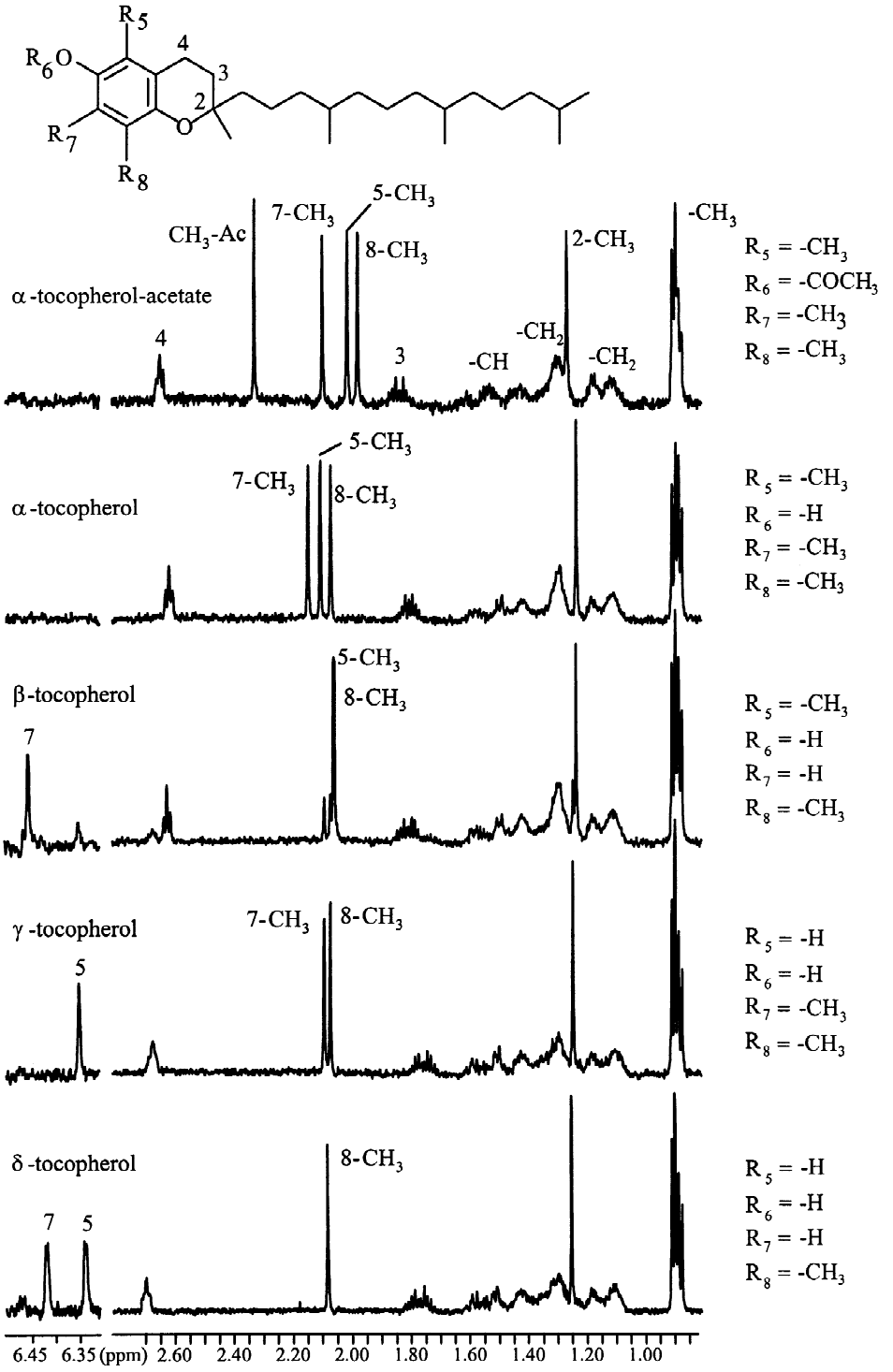

Fig. 5. Continuous-flow ^{1}H-NMR spectra of tocopherol isomers.

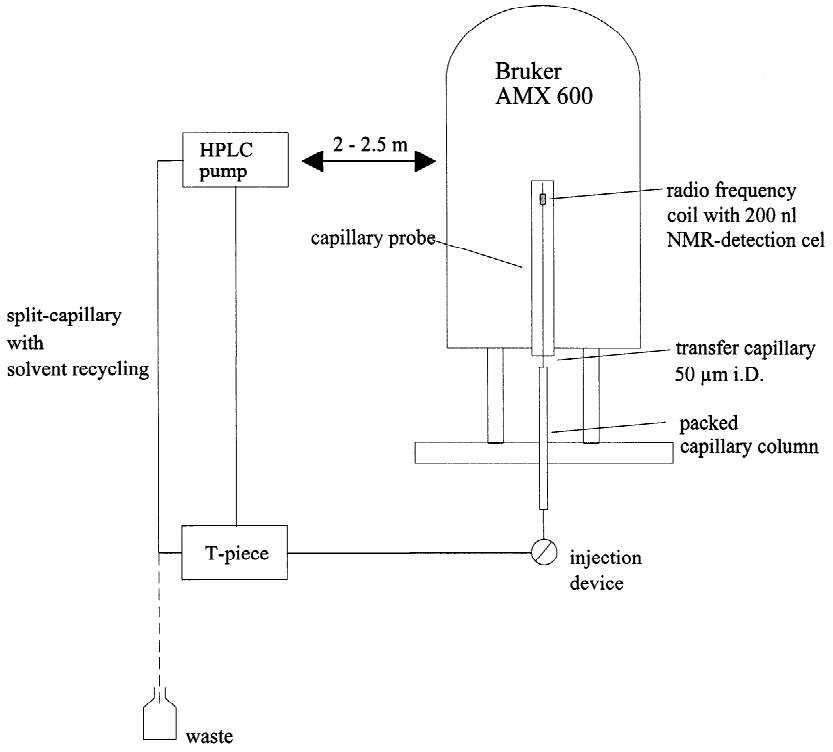

Fig. 6. Instrument configuration for LC–NMR experiments on the nanoliter scale.

big advantage that an easy exchange of the detection capillary is possible. In CE and CEC separations no additional field gradient in the *z*-direction of the cryomagnet is created enabling the acquisition of undisturbed NMR spectra.

For our first experiments we employed a capillary with an internal diameter of 315 μm, thus creating a detection volume of 900 nl. The flow-rate of the capillary HPLC–NMR separation was adjusted to 3 μl/min with the help of a T-piece inserted between the HPLC pump and the injection device. A fused-silica capillary of 70 cm overall length and an internal diameter of 315 μm was packed to a length of 12 cm with a C_{18} stationary phase at a distance from 50 cm from the inlet side employing sintered fused-silica gel frits. The polyimide coating was removed over the length of the NMR r.f. coil (12 mm) directly after the outlet frit of the capillary packing. Employing this design a separation of two dansylated amino acids (2 nmol each) could be monitored [42].

A further modification of the instrumental set-up was performed by placing the packed capillary separation column (150 mm×250 μm I.D., packed with a 3 μm C_{18} phase) directly below the cryomagnet [43–45]. With the help of a transfer capillary (400 mm×50 μm I.D.), the eluate was transferred to the detection capillary with an internal diameter of 180 μm. (Fig. 6). Here, a 2 mm microprobe was used, the NMR detection volume corresponded to 200 nl. With this design, 50 μg of a mixture of thermally isomerized retinyl acetate containing chemically labile kitols (retinyl acetate dimers) have been separated using fully deuterated acetonitrile as eluent [44]. By continuously monitoring the separation progress, the chromatographic peak maximum can be detected, and by the stopping the flow, one- and two-dimensional NMR acquisition of interesting peaks even at low concentrations can be performed. Fig. 7 shows the stopped-flow ^{1}H-NMR spectrum of a hitherto unknown kitol isomer recorded within an acquisition time of 320 s. Full structural assignment

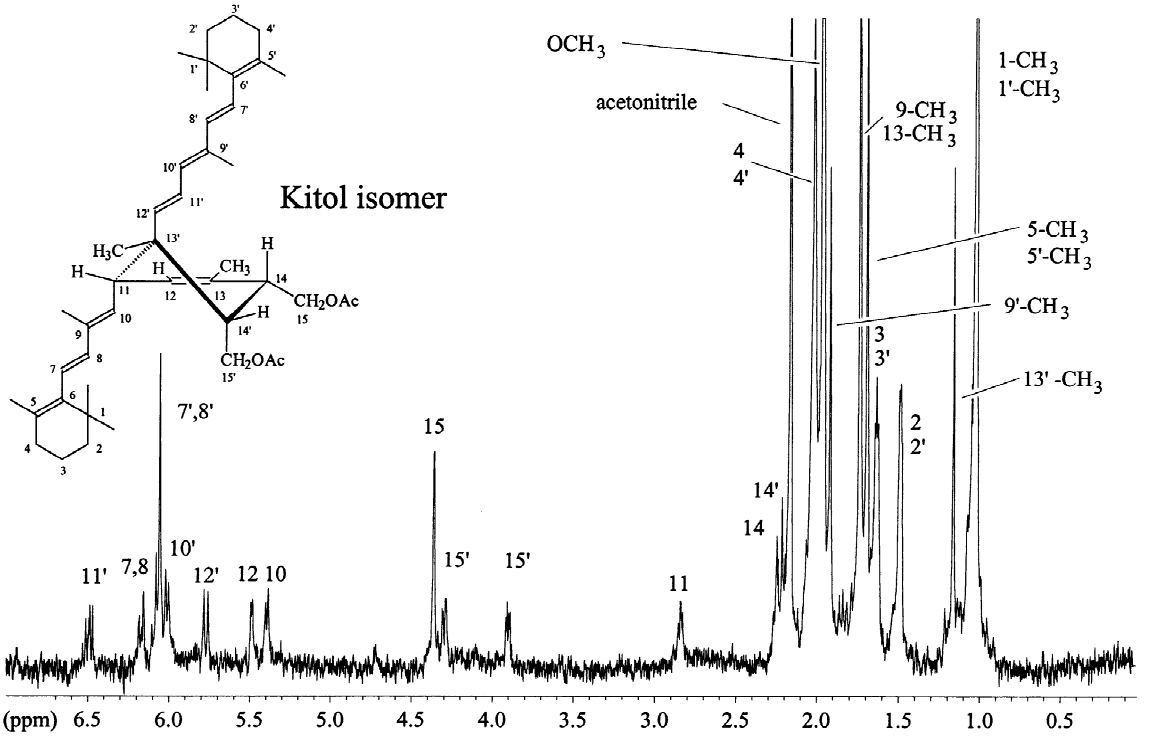

Fig. 7. Stopped-flow ^{1}H-NMR spectrum of a kitol isomer recorded in a detection volume of 200 nl.

is accomplished with the help of a two-dimensional total correlation spectroscopy (TOCSY) experiment recorded within 15 h (Fig. 8).

The structure of this very unstable kitol could only be determined by the application of the outlined micro-HPLC–NMR system. The use of fully deuterated eluents precludes the need to suppress NMR signals from the solvent. The extreme low solvent consumption of the capillary separation – only a few ml within one week of coupling activities – makes these experiments economically feasible. Moreover, the entire proton spectral range can be used for structure determination because, in contrast to HPLC grade solvents, deuterated solvents usually do not contain proton-containing impurities.

The latest ''insert'' capillary design employs ''bubble cells'' with detection volumes between 240 to 400 nl [46,47]. These ''bubble cells'' are created by rinsing the capillary with HF while heating the area of the detection window. With the help of theses bubble cells capillary HPLC–NMR separations of humulones [46], CE–NMR separations of amino acids [46] and an human urine extract [46] as well as CEC–NMR separations of alkylbenzoates [46] and paracetamol conjugates from an human extract [47] could be performed.

6. Trends and perspectives

6.1. Supercritical fluid chromatography–NMR

High-throughput separation techniques are expected to be widely employed for fast screening purposes in combinatorial chemistry, in natural product analysis and in drug metabolism research in the beginning of the new millennium. Besides capillary HPLC, CE and CEC, supercritical fluid chromatography (SFC) offers the big advantage of a very fast separation in a 10-min time frame due to the enhanced diffusion in the supercritical state. Because the mobile phase, supercritical carbon dioxide, does

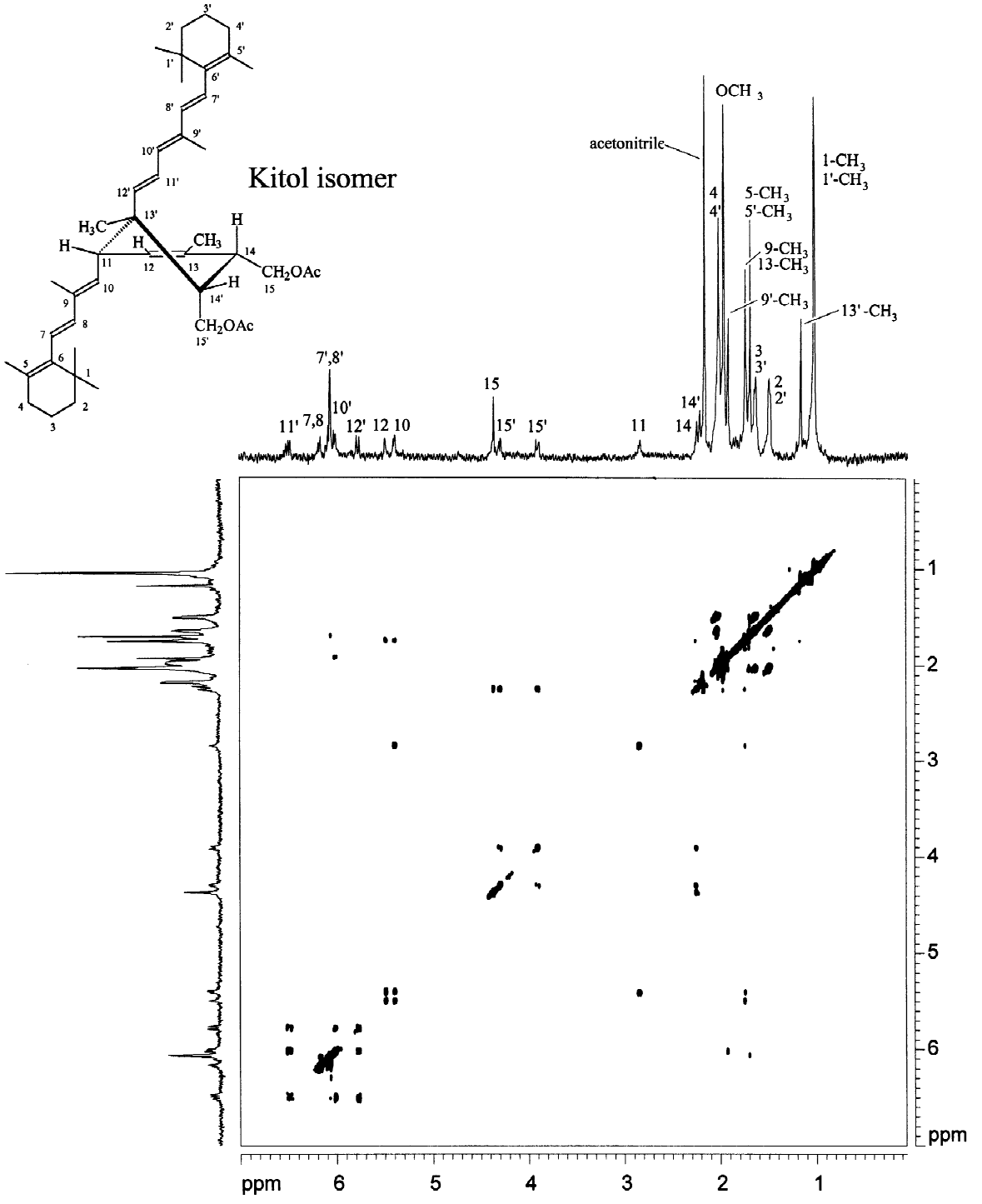

Fig. 8. Stopped-flow two-dimensional TOCSY NMR spectrum of a kitol isomer recorded in a detection volume of 200 nl.

not contain a proton signal, the coupling between SFC and ^{1}H-NMR spectroscopy would be the perfect fit for the combination of a separation and a spectroscopic technique. Allen et al. published the first pioneering SFC–NMR instrumental set-up and used SFC–NMR for the separation of fuel mixtures [48]. High-resolution ^{1}H-NMR spectra without any solvent signal disturbance could be obtained by a further development employing a pressure-stable sapphire flow-cell [49]. SFC–NMR separations of plastifiers, acrylates, vitamin A acetate isomers could be recorded with analytical packed columns [49–53]. A direct on-line monitoring of the supercritical fluid extraction (SFE) process of roasted coffee and of black pepper with ^{1}H-NMR spectroscopy was possible by using the stainless steel analytical column as an extraction cell [54].

Due to decreased viscosity in the supercritical compared to the liquid states the spin-lattice relaxation times, T_1, of the protons of the separated compounds are three- to five-fold increased. Under the applied measurement conditions in the supercritical state the continuous-flow ^{1}H-NMR spectra could not be quantified. Recently, we were able to obtain quantitative continuous-flow ^{1}H-NMR spectra under supercritical conditions by inserting a cartridge with immobilized free radicals (silica-immobilized nitroxide radicals) before the SFC probe to reduce T_1 dramatically [55]. Applying this technique with packed SFC capillaries will definitely extend the application power of the hyphenation of capillary separation techniques with ^{1}H-NMR spectroscopy.

6.2. LC–^{13}C-NMR

Structural assignment of unknown compounds is usually performed by the combined use of ^{1}H- and ^{13}C-NMR spectroscopy. Due to the low natural abundance of 1.1% of the ^{13}C isotope continuous-flow ^{13}C-NMR spectra are favorably recorded with ^{13}C-labeled compounds [56,57]. Recently Stevenson and Dorn introduced a technique to achieve the sensitivity increases needed to detect ^{13}C-NMR signals in natural abundance in the continuous-flow mode [58]. Allowing the transfer of spin polarization from the above mentioned immobilized free radicals (silica-immobilized nitroxide radicals) to the flowing analytes (dynamic nuclear polarization, DNP) ^{13}C-NMR spectra of the separation of a mixture of halogenated hydrocarbons could be obtained. An extension of the DNP approach to more wider applications would be of enormous interest and importance.

6.3. Combined use of HPLC–MS and HPLC–NMR together with solid-phase extraction

Due to the high stereochemical information content NMR spectroscopy (especially ^{1}H- and ^{13}C-NMR spectroscopy) gives the highest possible information content. But it is very difficult or even impossible to derive the correct structure of an unknown peak without the molecular mass information. Therefore LC–MS is an equal partner of LC–NMR. Newer ionization techniques such as atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) enable an easy and reliable hyphenation of either analytical HPLC, capillary HPLC, CE and CEC together with APCI-MS or ESI-MS [59–61].

Two strategies for the combined use of HPLC–MS and HPLC–NMR are used. By combining a mass spectrometer together with an NMR spectrometer and a HPLC system a split technique is used where 5% of the HPLC eluent is sent to the mass spectrometer [62]. Because of the use of deuterated solvents in HPLC–NMR the observed molecular ions will be increased by 1 for each exchangeable proton replaced with deuterium. One of the first applications of this approach is reported by Wilson et al. investigating the compounds of a plant extract [62]. The second approach is the parallel use of LC–NMR and LC–MS. Because of HPLC–APCI-MS detection limits in the 1-ng range the overall consumption of valuable compound is negligible. Thus HPLC–MS and HPLC–NMR proved to be extremely valuable tools for the separation and identification of tocotrienols from a palm-oil extract and of essential carotenoids in biological matrixes [63–65]. On-line solid-phase extraction (SPE) is often needed to concentrate a sample prior to HPLC–NMR analysis. By combining on-line SPE with HPLC, a direct and complete transfer of the investigated compound is possible [36,65].

7. Conclusions

Thus, the fundamental hyphenated techniques are established, but further optimization is needed. NMR sensitivity must be improved at least by a factor of three employing an improved special probe design. The application of liquid helium cooled cryoprobes or solenoidal coils with a susceptibility corrected wire are promising approaches.

From the chromatographic viewpoint, the application of on-line enrichment techniques [36] in capillary–NMR separations will also stimulate an increased use of this hyphenated technique in functional food analysis [37]. With the availability of high-sensitive capillary NMR probes, CEC–NMR separations will play a major part within hyphenated techniques [46,47].

Finally, wider applications of capillary separations with NMR spectroscopy and ESI- and APCI-MS will lead to a breakthrough in life science for the determination and high-throughput screening of drug candidates. This development as a ''millennium vision'' may help in the battle against more and more aggressive viral and bacterial agents by supporting the development of therapeutic new drugs without side effects.

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