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Short communication

# Separation and identification of vitamin A acetate isomers by supercritical fluid chromatography–$^1$H NMR coupling

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**Abstract**

Unlike HPLC–NMR coupling, the combination of supercritical fluid chromatography (SFC) with NMR spectroscopy is not yet a routine method, but the feasibility of this coupling technique has already been demonstrated with synthetic mixtures. In this paper the first real life application is presented and the potential of this new method is shown. A mixture of five *cis/trans* isomers of vitamin A acetate is separated by SFC using supercritical $CO_2$ as eluent. Therefore no solvent signal suppression is necessary and the unrestricted observation of the whole spectral range is possible, contrary to the HPLC separation in *n*-heptane, where in the aliphatic region almost 2 ppm of the $^1$H NMR spectrum are affected by the suppression technique.

*Keywords:* Nuclear magnetic resonance spectrometry; Vitamins

## 1. Introduction

Within the last few years the coupling of high-performance liquid chromatography (HPLC) to $^1$H nuclear magnetic resonance (NMR) spectroscopy has become a routine method [1–7]. The advantage of the coupled chromatographic–NMR spectroscopic technique as against the conventional off-line technique is the considerable saving of time. Whereas in the off-line mode the different fractions have to be collected and prepared for the NMR experiment, separation and detection are performed more or less simultaneously in the on-line mode. However, the remaining spectral distortions arising from the suppression of the signals of non-deuterated proton containing HPLC solvents is a drawback of the technique. In this respect supercritical $CO_2$ represents an interesting alternative eluent to the conventional HPLC eluents. In addition to the absence of protons it offers advantages in the chromatographic process, i.e., high speed of analysis, good solvation of nonpolar to medium polar substances, and good compatibility with practically all HPLC and GC detectors. Using supercritical $CO_2$ as solvent in $^1$H NMR spectroscopy no solvent signal suppression is necessary and the unrestricted observation of the whole spectral range is possible, similar to $^1$H NMR spectroscopy in deuterated solvents.

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The possibility of coupling supercritical fluid separation techniques with $^1$H NMR spectroscopy has already been described [8–11] and the principal feasibility of the coupling technique was demonstrated with synthetic mixtures. Here we wish to present a first example of a real-life application that vividly demonstrates the potential of the new coupling technique. Vitamin A and its derivatives are essential for the human organism and play an important role in medical, pharmaceutical and food technologies [12–14]. *Cis* and *trans* isomers of the numerous conjugated double bonds are possible and isomerisation occurs readily. The biological activities of these isomers differ markedly, making their characterization in biological systems or technical products highly desirable.

The conventional method for the separation of structural isomers is HPLC [14,15]. But quite often the identification of the chromatographic peaks is not trivial. Characterization of the separated compounds by UV spectroscopy is quite uncertain as the small differences in the spectra of stereoisomers are difficult to correlate with stereochemical structures. Therefore, reference substances or at least reference spectra are needed (and very often unobtainable). Mass spectrometry cannot help in this case, since stereoisomers (in contrast to constitutional isomers) do not differ in their fragmentation patterns. High-resolution $^1$H NMR spectroscopy on the other hand provides the necessary stereochemical information for the identification of isomers and can often allow a deduction of the structural elements of unknown substances. The *cis/trans* isomers of vitamin A acetate are thus an ideal system for the demonstration of the capabilities of the NMR detection technique. The use of HPLC–$^1$H NMR coupling for structural assignment of vitamin A acetate isomers has been reported previously using *n*-heptane as mobile phase [16]. Because of its strong proton signals *n*-heptane is not the favoured solvent for HPLC–NMR coupling experiments from the NMR spectroscopic point of view.

## 2. Experimental

### 2.1. samples

A mixture of thermally isomerized vitamin A acetate was obtained from BASF (Ludwigshafen, Germany).

### 2.2. Chromatographic conditions

A Vydac Peptide and Protein $C_{18}$ column (250× 4.6 mm) was used. The separation was carried out with 130 bar under isobaric conditions at 60°C. With a flow-rate of 0.65 ml/min the vitamin A acetate *cis/trans* isomers could be eluted within 25 min.

20 μl of a 30% (m/v) solution of thermally isomerized vitamin A acetate in $C^2HCl_3$ was injected. The exact determination of the injected amount is not possible, as a timesliced injection technique was used.

### 2.3. Supercritical fluid chromatography (SFC)–NMR coupling

The experimental setup has been already described [9,10]. A Hewlett-Packard supercritical fluid chromatograph G1205A was coupled to a Bruker ARX 400 spectrometer (9.4 T) operating at a $^1$H frequency of 400.13 MHz. The spectrometer was equipped with a specially designed, pressure-proof probe head with a cell volume of 120 μl (Bruker). The NMR flow cell was connected between the column outlet and a back pressure regulator.

#### 2.3.1. $^1$H NMR parameters

For the on-line measurements 16 transients with 4 K complex data points and a spectral width of 3632 Hz were recorded per retention time increment. A relaxation delay of 0.5 s and an acquisition time of 0.564 s per transient were used. The pulse angle was set to 40°. During the separation 128 Free Induction Decays (FIDs) were acquired within 37 min. The temperature of the NMR flow cell was adjusted to 35°C.

## 3. Results and discussion

In the results of the SFC–NMR coupling experiment presented here, the Fourier transformed FIDs are depicted as rows of a two-dimensional plot of $^1$H NMR chemical shift versus retention times (contour plot). The time interval between 10 min and 24 min is shown in the contour plot in Fig. 1.

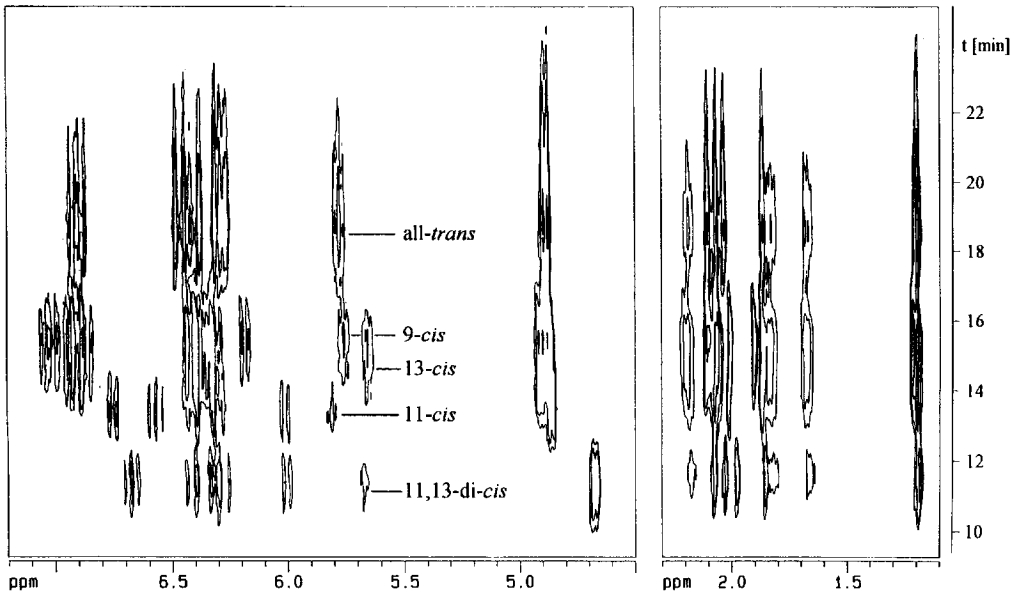


Fig. 1. Contour plot of the $^1$H NMR spectra (400 MHz) collected on-line during the SFC separation of vitamin A acetate isomers.

Four groups of $^1$H NMR signals clearly separated on the elution time axis can be distinguished. The $^1$H NMR spectra of these four fractions were generated by coadding the FIDs within each fraction to improve the signal-to-noise ratio.

Analysis of the olefinic part of these spectra allows the assignment of the 11,13-di-*cis*, 11-*cis* and all-*trans* isomers to the fraction 1, 2 and 4, respectively. In order to emphasize the improvement in information content of the spectra in contrast to the spectra acquired in *n*-heptane, the aliphatic regions of the corresponding spectra are depicted in Fig. 2. The considerable and diagnostically important differences in the chemical shifts of the aliphatic protons of the *cis* and *trans* isomers are evident.

Closer inspection of the spectrum of the third fraction reveals that it contains two compounds. This is apparent from the two doublets at 4.9 ppm, characteristic of the methylene group at C-15, and can be proved by the signals of the aliphatic region shown in Fig. 2. There are two different signals of methyl groups at the C-1 atom of the ionone ring at 1.19 ppm. In the region around 2 ppm, seven methyl group-singuletts can be distinguished. This can be explained with partly overlapping signals of four remaining methyl groups of two nearly coeluting isomers. For a more detailed determination the time dependency of the intensities of the $^1$H NMR signals has been considered. All non-overlapping signals at the spectroscopic axis can be separated into two groups according to the different retention times of their intensity maxima, i.e., into signals belonging to an earlier and later eluting component.

This allows the assignment of the 13-cis (≠) and 9-cis isomer (*) respectively. The advantage of using SFC as the chromatographic coupling technique is depicted in Fig. 3. In the spectrum acquired during an HPLC–$^1$H NMR on-line run with *n*-heptane as solvent, the aliphatic region between 0.6 and 1.5 ppm is masked by the remaining signals of the *n*-heptane or distorted by the solvent suppression technique. Although the signals of the methyl groups at 1.7–1.8 ppm are observable, severe distortions from the presaturation of the solvent signals lead to deviations of the intensity ratios. The region thus affected amounts to almost 2 ppm. In contrast, no such distortions occur in the spectra collected on-line during the SFC separation. A signal linewidth of 1.7 Hz is observed, no extensive baseline correction or further data processing was necessary.

For further investigations of related compounds the ability to observe the aliphatic region of the spectrum may become more important. It allows the elucidation of the stereochemistry of these molecules

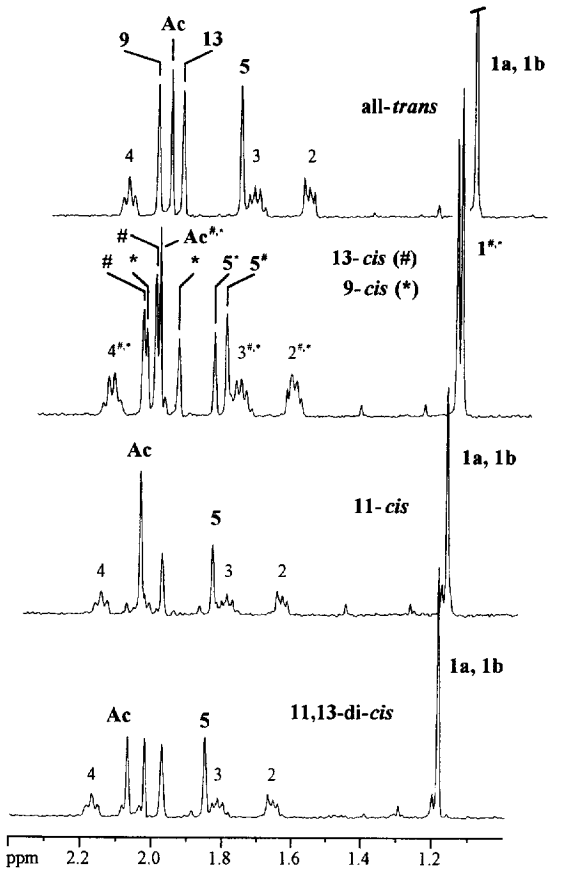


Fig. 2. Stacked plot of continuous-flow $^1$H NMR spectra (400 MHz) collected on-line during the separation. For enumeration see Fig. 3.

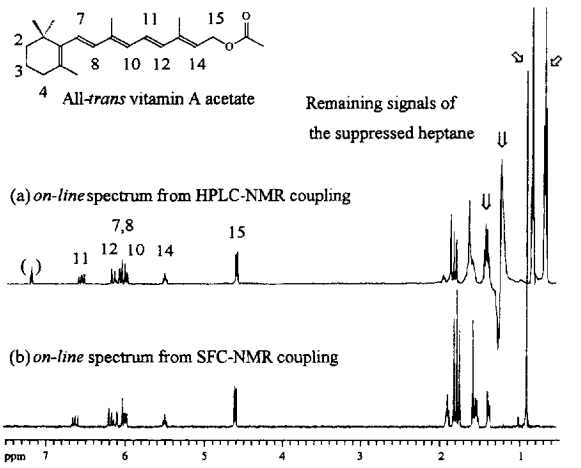


Fig. 3. Comparison of the on-line collected spectra (400 MHz) of all-*trans* vitamin A acetate from the (a) HPLC- and (b) SFC–$^1$H NMR coupling.

(e.g., by measuring NOE effects of the methyl groups). Furthermore, the reported increase of the $T_1$-relaxation times in supercritical fluids [9,10,17] should enlarge the observable NOE effects [18].

## 4. Conclusion

The potential of SFC–$^1$H NMR coupling has been vividly demonstrated by the above shown results. The separation of vitamin A acetate isomers is an excellent study case for comparing these two coupling methods. The advantage of directly coupling a chromatographic separation technique with $^1$H NMR spectroscopy is evident. Within 25 min all information necessary for the identification of the main compounds of the mixture can be collected. The two-dimensional chromatogram that can be obtained compensates for the loss in chromatographic resolution resulting from the high sample loading required. Although only four peaks can be seen on the chromatographic axis, the enhanced resolution provided by the second dimension allowed a complete interpretation of the $^1$H NMR spectra.

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## References

[1] E. Bayer, K. Albert, M. Nieder, E. Grom and T. Keller, J. Chromatogr., 186 (1979) 497.
[2] M. Spraul, M. Hofmann, P. Dvortsak, J.K. Nicholson and I.B. Wilson, Anal. Chem., 65 (1993) 327.
[3] H.C. Dorn, Anal. Chem., 56 (1984) 747A.
[4] D.A. Laude Jr. and C.L. Wilkins, Trends Anal. Chem., 5 (1986) 230.
[5] K. Albert, M. Kunst, E. Bayer, M. Spraul and W. Bermel, J. Chromatogr., 463 (1989) 355.
[6] K. Albert, J. Chromatogr. A, 703 (1995) 123.
[7] S.A. Korhammer and A. Bernreuther, Fresenius J. Anal. Chem., 354 (1996) 131

[8] L.A. Allen, T.E. Glass and H.C. Dorn, Anal. Chem., 60 (1988) 390.
[9] K. Albert, U. Braumann, L.-H. Tseng, G. Nicholson, E. Bayer, M. Spraul, M. Hofmann, C. Dowle and M. Chippendale, Anal. Chem., 66 (1994) 3042.
[10] K. Albert and U. Braumann, in D.L. Andrews and A.M.C. Davies (Editors), Frontiers in Analytical Chemistry, Royal Society of Chemistry, Cambridge, 1995, p. 86.
[11] U. Braumann, H. Händel, K. Albert, R. Ecker and M. Spraul, Anal. Chem., 67 (1995) 930.
[12] L. Zechmeister, Fortschritte Chem. Org. Naturst., 18 (1959) 224.
[13] O. Isler, Angew. Chem., 68 (1956) 547.
[14] M. Vecchi, G. Englert, R. Maurer and V. Meduna, Helv. Chim. Acta, 64 (1981) 2746.
[15] M. Vecchi, J. Vesely and G. Oesterhelt, J. Chromatogr., 83 (1973) 447
[16] K. Albert, G. Schlotterbeck, U. Braumann, H. Händel, M. Spraul and G. Krack, Angew. Chem., Int. Ed. Engl., 34 (1995) 1014.
[17] D. Lamb, D. Van der Velde and J.J. Jonas, J. Magn. Reson., 73 (1987) 345.
[18] D. Neuhaus, The Nuclear Overhauser Effect in Structural and Conformational Analysis, VCH, New York, 1989.