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Application of direct *on-line* coupling of HPLC and SFC with ^{1}H NMR spectroscopy for the investigation of monomeric acrylates

Klaus Albert[1], Ulrich Braumann[1], Roman Streck[2], Manfred Spraul[3], Rainer Ecker[4]

[1] Universität Tübingen, Institut für Organische Chemie, Auf der Morgenstelle 18, D-72076 Tübingen, Germany
[2] Dow Deutschland Inc., Industriestr. 1, D-77836 Rheinmünster, Germany
[3] Bruker Analytische Meßtechnik, Silberstreifen, D-76287 Rheinstetten, Germany
[4] Hewlett Packard GmbH, Ermlis-Allee, D-76337 Waldbronn, Germany

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Abstract. The separation of monomeric acrylates was perfomed with High Performance Liquid Chromatography (HPLC) and Supercritical Fluid Chromatography (SFC). A direct on-line structural assignment of all compounds by continuous-flow ^{1}H NMR spectroscopy is possible with both separation techniques. The direct SFC-NMR coupling offers the advantage that the recorded continuous-flow ^{1}H NMR spectrum is not obscured by solvent signals.

A Introduction

Acrylates are one of the most important classes of organic compounds used in the manufacturing of polymers and copolymers. Therefore, they are of extreme importance in a lot of industrial processes [1]. New developments in modern photopolymers, latices, coatings etc. necessitate the development of reliable quality control systems for the characterisation of the starting monomeric acrylates. Within hyphenated methods, the direct coupling of a separation technique together with NMR spectroscopy is one of the most powerful techniques for unequivocal structural assignment. Whereas HPLC-NMR is already starting to become an established hyphenated technique [2–13], the newly developed SFC-NMR-technique [14, 15] has to demonstrate its practical value.

Lately, chromatography with supercritical fluids, such as carbondioxide, has become more and more important. On one hand, the improvement of the technical equipment makes this separation technique again interesting. On the other hand, CO_2 is an environmentally friendly solvent, which can replace other more critical solvents.

From the viewpoint of chromatography, SFC is a technique which combines the efficiency and speed of the GC and the universal applicability of the HPLC. From the viewpoint of the NMR, coupling of SFC and ^{1}H NMR is interesting because all the problems related to large background signals arising from the HPLC-solvents do not exist.

Therefore it is interesting to check if the acquisition of NMR-spectra under such exotic conditions (concerning pressure, temperature, flowrate and viscosity) is possible.

Here the state-of-the-art of separation science with NMR detection is demonstrated and the results obtained by HPLC-NMR- and SFC-NMR-coupling are compared. Acrylates, acrylic acid, and chemically related acids were chosen as model system. A similar system was used for both separation techniques.

B Experimental

1 Demands of NMR for HPLC-separation

The method [16] suggested for the separation of acids and acrylates had to be modified according to the NMR requirements.

Methanol with phosphoric acid used as eluent in [16] produces two relatively broad signals, one of which appears in the area where ester protons are expected. Interference caused by this signal would create a large distortion in the spectrum of the sample. A complete substitution of methanol by its deuterated form was not practicable due to economic reasons (> 5–10 DM/ml). A partial substitution of the methanol would only minimally reduce the intensity of solvent signals so that the actual value of the substitution would not be proportional to the increased cost.

Two separate methods for the resolution of free acids and esters were provided. The separation, however, should be performed in one run and, when possible, without the aid of an ensuing solvent gradient to prevent any aggravation of the solvent suppression caused by drifting signals. Therefore it would be advantageous to use standard eluents as acetonitrile (ACN)/H_2O for the separation and to separate both substance classes without a gradient.

Water can be completely substituted by the relatively inexpensive D_2O so that this solvent signal is sufficiently reduced, making a signal suppression unnecessary and

Dedicated to Professor Dr. H. Kriegsmann on the occasion of his 70th birthday

Correspondence to: K. Albert

concealing only a small area in the spectrum (approx. 0.1 ppm).

The addition of phosphoric acid presents no problem for NMR, except for the introduction of protons which leads to an increased intensity of the water-signal. In special cases, where signals very close to those of water have to be observed, this may be overcome by using D_3PO_4.

2 *HPLC-method*

A first separation was achieved with an eluent of acetonitrile/water 20/80 with 0.01 mol/l of phosphoric acid on a LiChrosorb RP select B column which could be further optimised. A separation of the acrylates was also possible without the addition of phosphoric acid. Using a 75/25 eluent, methacrylic acid and methacrylate were incompletely separated, while at 50/50 a distinct split between the peaks is recognisable.

The separation was carried out using acetonitrile/water 40/60 with phosphoric acid. In this case, as before methacrylic acid and methacrylate were not baseline separated, however, the butyl-acrylate shifted to unacceptably high k-values ($k > 10$). The separation is shown in Fig. 1. The elution of the butylacrylates with a reasonable k′ value is only possible using a gradient, which can be retried later. The addition of phosphoric acid had no effect on the elution profile of the acrylate.

3 *HPLC-NMR-coupling*

The coupling was performed under the chromatographic conditions described using a BRUKER AMX 600 spectrometer. The HPLC-equipment was positioned at a distance of 2 m beside the 11 T magnet. To perform the coupling, a ^1H inverse probe head with a cell volume of 120 µl was used. The probe head was shimmed with Humptest solution (3% $CHCl_3$ in Acetone-d_6) followed by the eluents. The signal line-width of chloroform at the height of the ^{13}C-satellites of 13.5 Hz/23 Hz is shown in Fig. 2.

During *on-line* experiments the flow was reduced to 0.3 ml/min, to increase the residence time in the flow cell and thereby the possible number of scans for one volume. For the presaturation of the acetonitrile signal, a NOESY-type sequence with presaturation was applied during relaxation of 0.6 s and mixing time of 30 ms 8 scans/spectrum were acquired with 8K datapoints and a sweep width of 8478 Hz, yielding an acquisition time of 0.483 s and therefore a time resolution of 10 s/row. During a total running time of 42 min 50 s, 256 spectra were acquired.

A Bischoff HPLC 2200 system was used with ACN/H_2O 40/60 and 0.01 mol/l H_3PO_4 as eluent and a flow rate of 0.5 ml/min (0.3 ml). A 5.7% (m/v) mixture of approx. equimolar amounts of methylacrylate, ethylacrylate, methylmethacrylate, methacrylic acid, diacrylic acid dissolved in eluent was injected as sample via a Rheodyne valve with an external 20 µl loop on a Merck LiChrosorb RP Select B, 250 × 4.6 mm column, UV detection was performed with a HP 1050 at 254 nm.

A valuable UV chromatograph does not exist for these experiments. In contrast to the 400 MHz apparatus, the clearly stronger stray field of the 600 MHz instrument appears to have disturbed the data acquisition. A test must be performed to determine if this effect is transferred by the UV detector or by the computer.

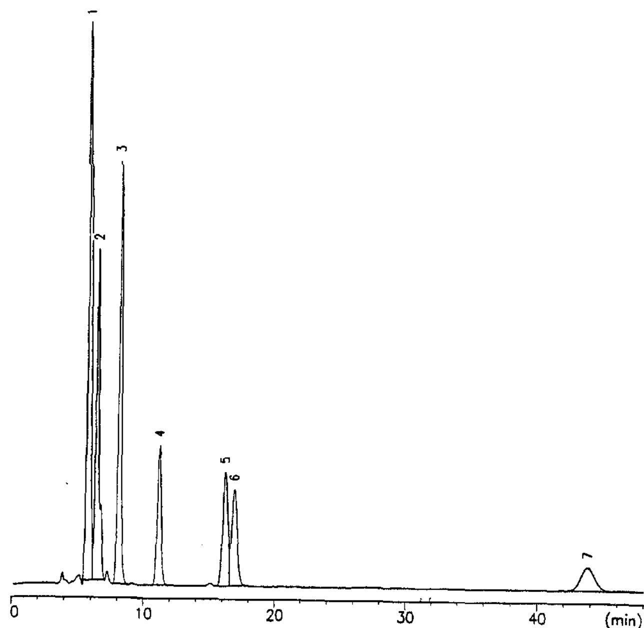

Fig. 1. UV-Chromatogram of the separation of acrylic acids, esters and related compounds using 0.5 ml/min of acetonitrile/water 40/60 with 0.01 mol/l phosphoric acid. *1* acrylic acid, *2* diacrylic acid, *3* methacrylic acid, *4* methyl acrylate, *5* ethyl acrylate, *6* methyl methacrylate, *7* butyl acrylate

4 *Demands of NMR for SFC*

In HPLC solvent gradients are applied to adjust the eluting power of the solvent which causes changes in the position of the solvent signals in the NMR spectrum. Because normally the solvent signals have to be suppressed, the main problem is the adjustment of the presaturation technique. Sample signals are not affected.

Applying a gradient in the SFC normally means to use a pressure gradient. It can be observed, that changing the pressure generally leads to a drift of all NMR signals. Higher density shifts the signals to a higher field. This has to be considered when the acquisition time for an NMR-spectrum is set up. To avoid considerable line broadening or in the worst case a destruction of the NMR-resolution, the drift during the acquisition has to be lower than the observed line width. If a step gradient is used, those problems only occur during the pressure-ramp.

The addition of modifier in the order of 1 to 5% in a typical SFC-separation leads to a solvent consumption of 1 ml or less. This allows the use of nearly any modifier in its fully deuterated form with a negligible increase of cost.

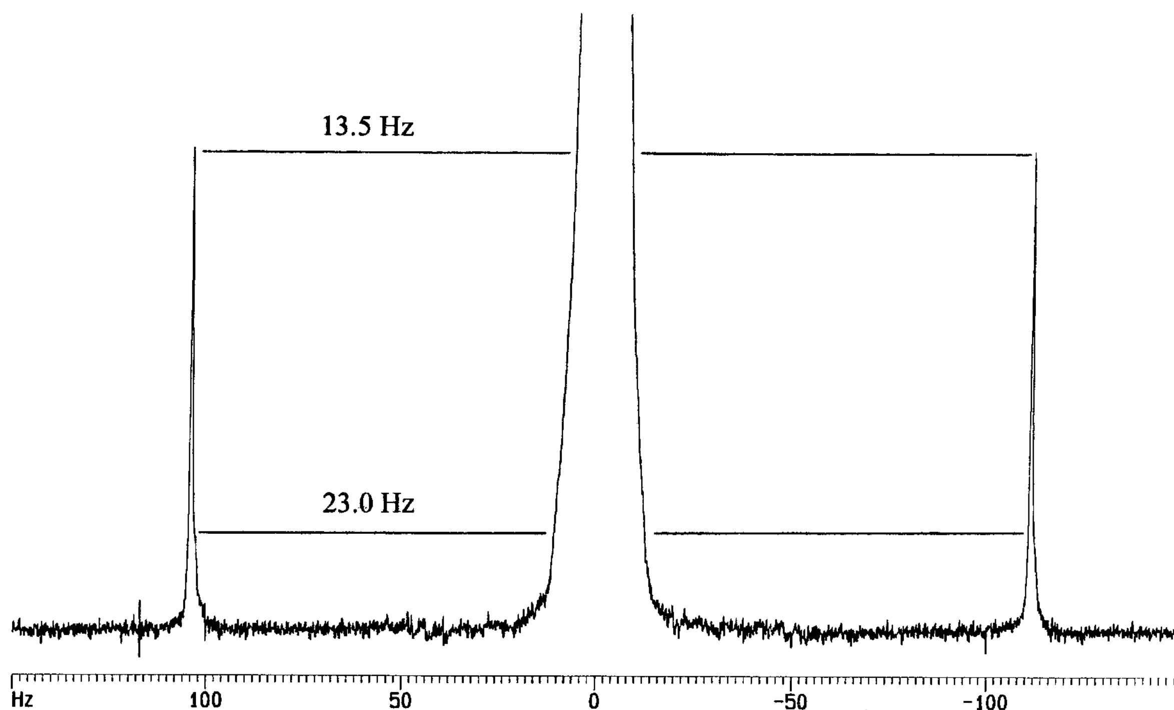

Fig. 2. Characterisation of the LC-probe of the 600 MHz instrument. Humptest solution (3% chloroform in acetone-d_6) under static sample conditions, linewidth 13.5/22.5 Hz at the height of the ^{13}C-satellites

5 Development of SFC-separation

To separate the acrylates by SFC they were eluted with pure CO_2 with a modifier content of 1% methanol to improve the peak shape. During the SFC-NMR coupling methanol was replaced by fully deuterated methanol, to avoid the need of special presaturation techniques in NMR-experiments.

At first a linear gradient was used to determine the adequate solvent strength for each compound. After coarse determination of the conditions, a step gradient was developed which eluted all compounds during two isobaric periods.

6 SFC-NMR coupling

A Hewlett-Packard Supercritical Fluid Chromatograph G1205A equipped with a HP 1050 diode array detector operating at 254 nm was used.

The separation was carried out on a 250 × 4.6 mm Spherisorb S5CN column with CO_2/Methanol-d_4 99/1 (v/v) at a flowrate of 1 ml/min. The following pressure gradient was used: 80 bar for 7 min, with 20 bar/min to 100 bar for 10 min. The oven temperature was set to 60 °C, whereas the NMR-cell was kept at 312.7 K.

The sample contained approximately equimolar amounts of methylacrylate, methylmethacrylate, ethylacrylate and butylacrylate. A precise amount of sample cannot be given, as the injection system allows no determination of the amount of sample actually injected.

The NMR-detection was carried out on a BRUKER AMX 400, using the 2D-NMR routines of the BRUKER software UXNMR. A special pressure proof selective probe with 120 µl cell volume was used. No solvent presaturation was applied. As the relaxation times are prolongated in the supercritical state, the observation pulse had to be reduced to avoid saturation of the sample. With a delay of relaxation of 0.5 s it was optimised to a 34° pulse to obtain maximum intensity of the NMR signal. Eight transitions per spectrum were recorded with a size of 2 K and a sweep width of 3623.2 Hz yielding an acquisition time of 0.2826 s and a time resolution of 6.4 s. During a total running time of 13.55 min, 128 spectra were acquired. To avoid disturbances of the lock signal during the pressure step affecting the stability of the spectrometer, field-frequency stabilisation was not used during the separation.

The spectra were calibrated on the methyl signal of the methanol which was set at 3.3 ppm. This calibration is relatively arbitrary, as the signal is dependent on temperature and pressure of the eluent.

C Results and discussion

1 HPLC

In Fig. 3 contour plot of the separation is shown. Each of the five components are clearly recognisable, the resolution of the spectrum is very good, and all signals appear in the contour plot. The remaining water or HDO signal causes little disturbance. The signal of the diacrylic acid at 4.43 ppm can still be observed.

The continuous signals observable at 1.27 and 2.5 ppm are due to impurities in the HPLC solvent

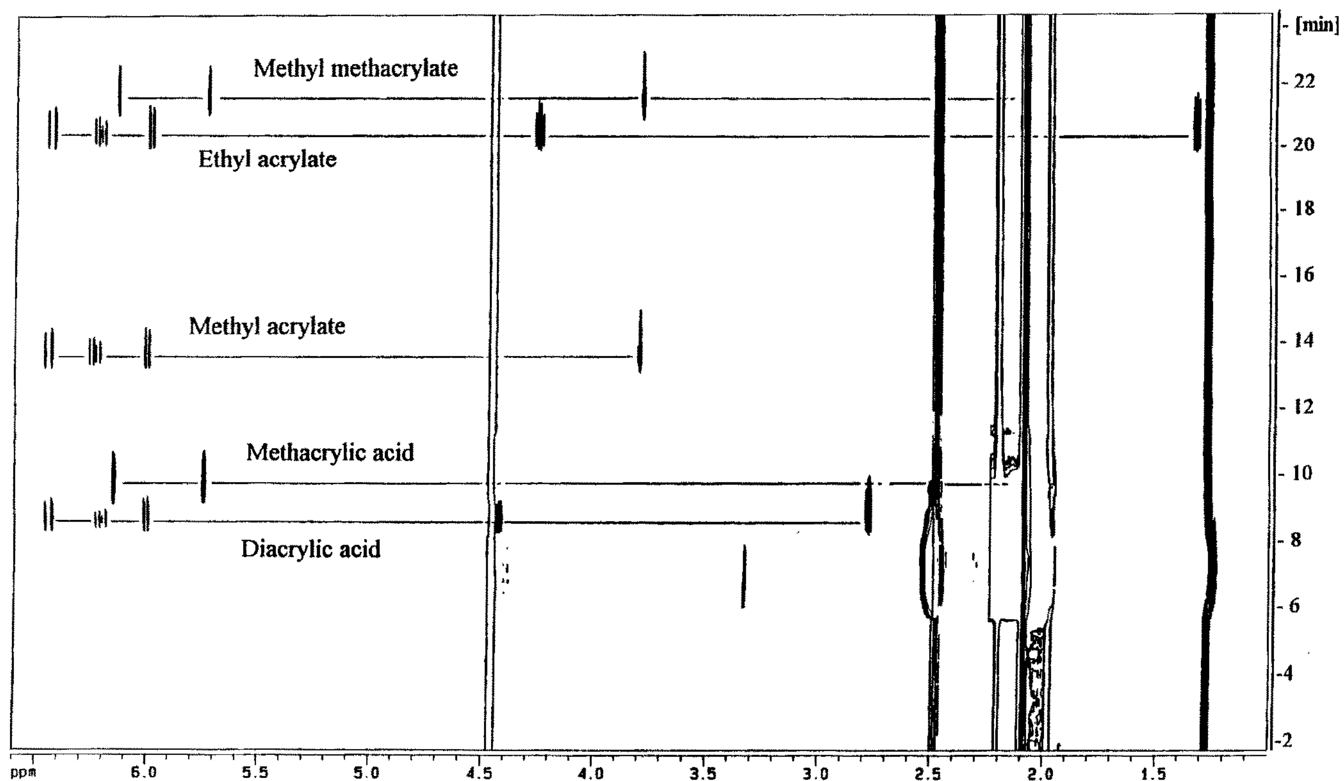

Fig. 3. Contour plot of the separation of 3 acrylates and 2 acids with HPLC

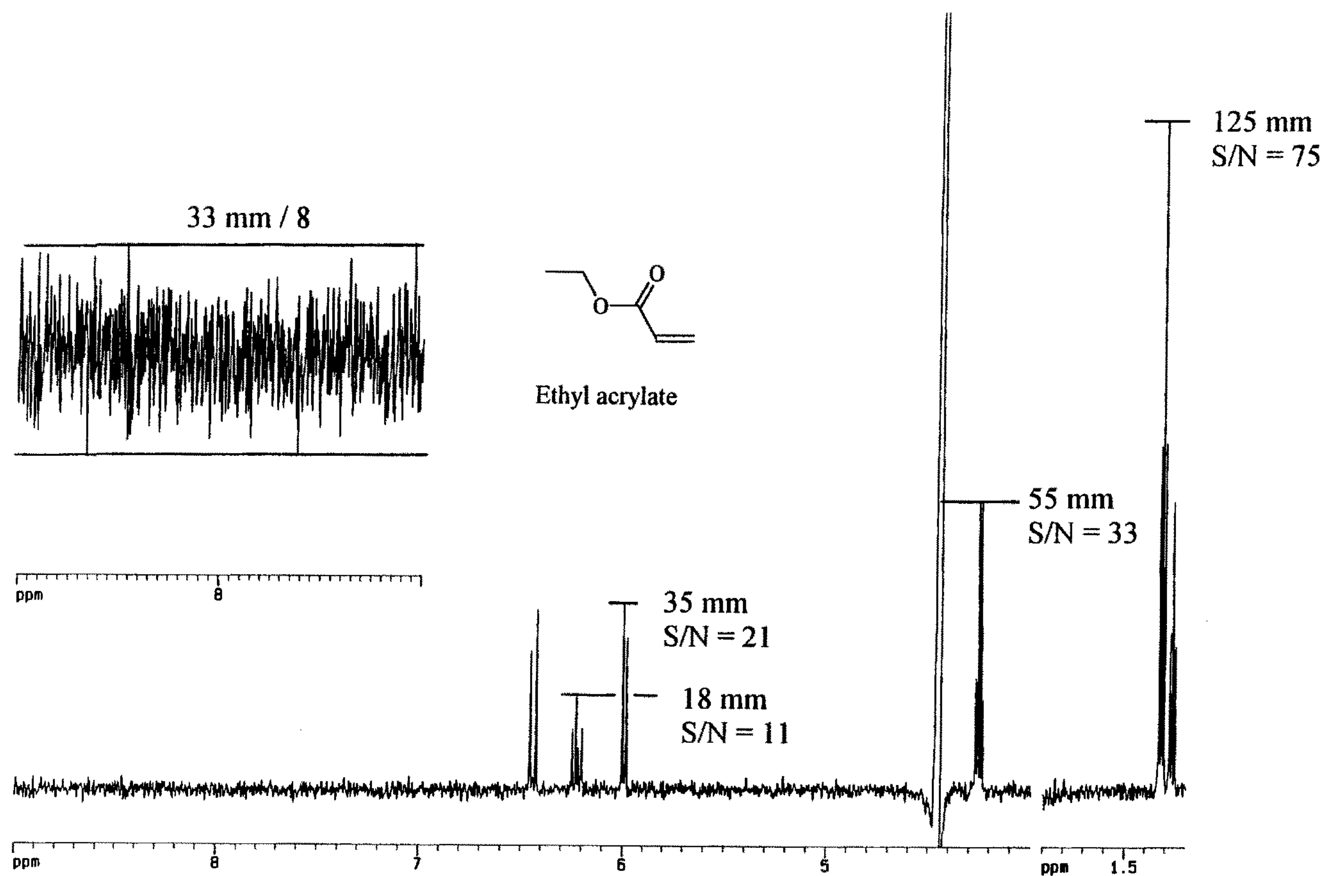

Fig. 4. Spectrum of ethyl acrylate extracted from the on-line coupling, number of scans 16

Table 1. Signal-to-noise values calculated for the 4. Peak (= ethylacrylate). Estimated limitations in detection under the assumption that a peak with S/N = 5–2 is still observable

Assignment [ppm]	6.25	6 + 6.45	4.25	1.35
1.99 μmol ethyl acrylate	Measured S/N-ratios			
Spectrum Nr. 113	11	21	33	75
Sum of rows 111–116	14	31	49	106
Assumed S/N-ratio of 5–2	Calculated detection limits			
[μmol]	0.71–0.28	0.32–0.13	0.2–0.80	0.094–0.038
[μg]	71–28	32–13	20–8	9.4–3.8

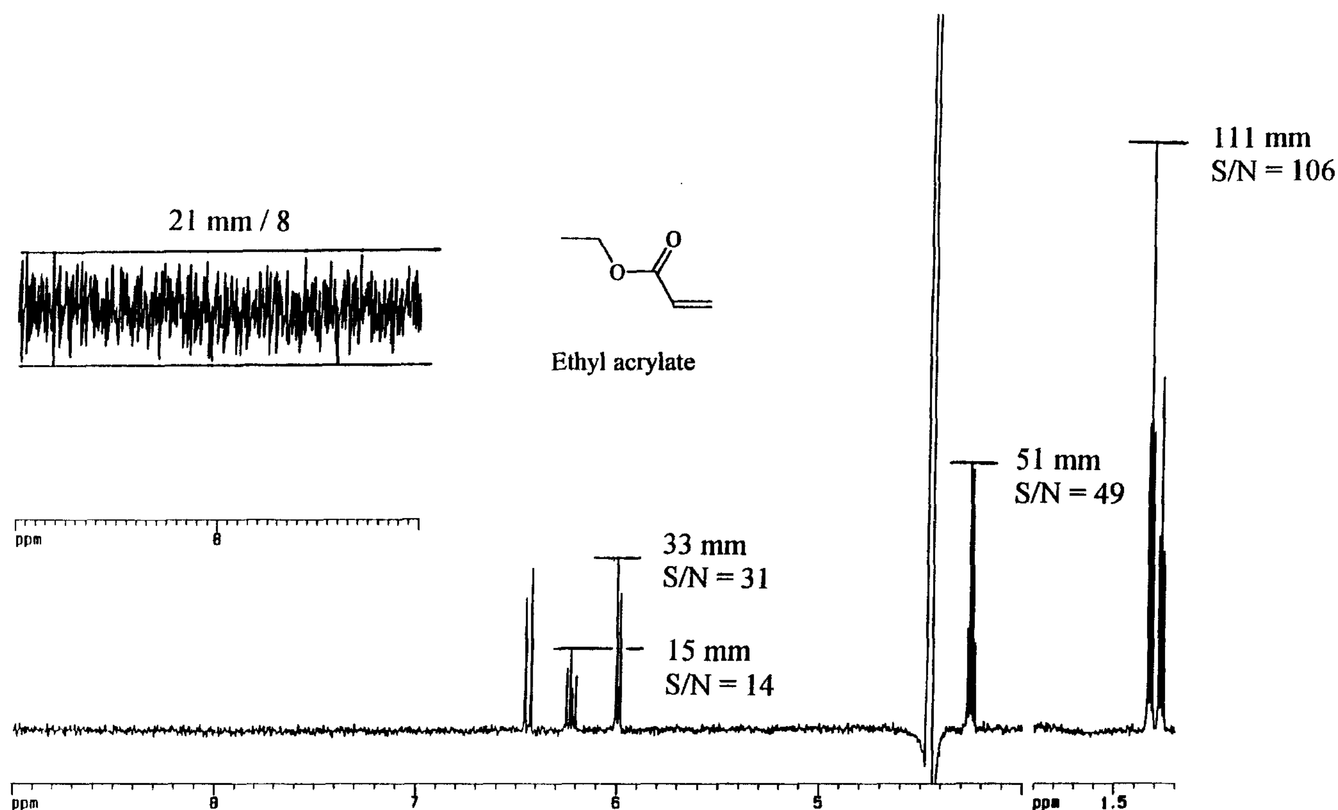

Fig. 5. Spectrum of ethyl acrylate created by summation of seven spectra containing signal intensity of the ethyl acrylate; seven spectra co-added yielding a total of 112 scans; increase of the signal/noise by the factor of approx. 45%

(probably diethylether), those at 2.1 and 4.45 ppm are caused by acetonitrile and HDO. The HPLC solvent of "grade" quality contains obvious amounts of impurities, as mainly UV permeability is chosen as purity criterion for HPLC solvents. The disturbance appearing with the passage of the dead volume through the NMR cell flow can be led back to the different compositions of sample solution and eluent. Therefore, the following should be carefully controlled:

a) Deuterated solvents should be used for the preparation of samples.

b) In contrast to conventional HPLC, where the sample is often dissolved in a weaker solvent than the eluent to achieve a concentration effect on the column head, for the HPLC-NMR coupling the solvent should have the same composition as the eluent.

Although the deteriorated acetonitrile suppression did not cause overmodulation (and thereby destruction of the spectrum), the changed composition of the solvent results in a base line disturbance to prevent correct representation of the contour plot.

Limitations in detection. Figure 4 shows the extracted spectrum of the ethyl acrylate. This compound was chosen because it shows signals with differing intensities as well as a normal broadening for a relatively late appearing peak.

The signal-to-noise ratios for the different signals are presented in Table 1 calculated from the composition of the sample reduced from 4.4% (m/V) (corresponding to an amount of 199 μg ethyl acrylate) to approximately 0.7%. All signals can still be detected (i.e. S/N ≈ 2). However, with a sample of about 0.1%, only a limited identification, i.e. the detection of the more intense signal at 1.35 ppm of the methyl group, is possible.

The flow rate can also be reduced without a significant influence on the separation quality, therefore, this can improve the sensitivity of the method.

Solvent suppression ACN/D_2O with NOESY - sequence

remaining H_2O/HDO

remaining acetonitrile

*: ^{13}C-satellites intensity: 0.55% of main ACN-signal

Δt = 0.33 min

ppm 4.5 4.0 3.5 3.0 2.5 2.0

Fig. 6. Demonstration of the stability of the solvent suppression

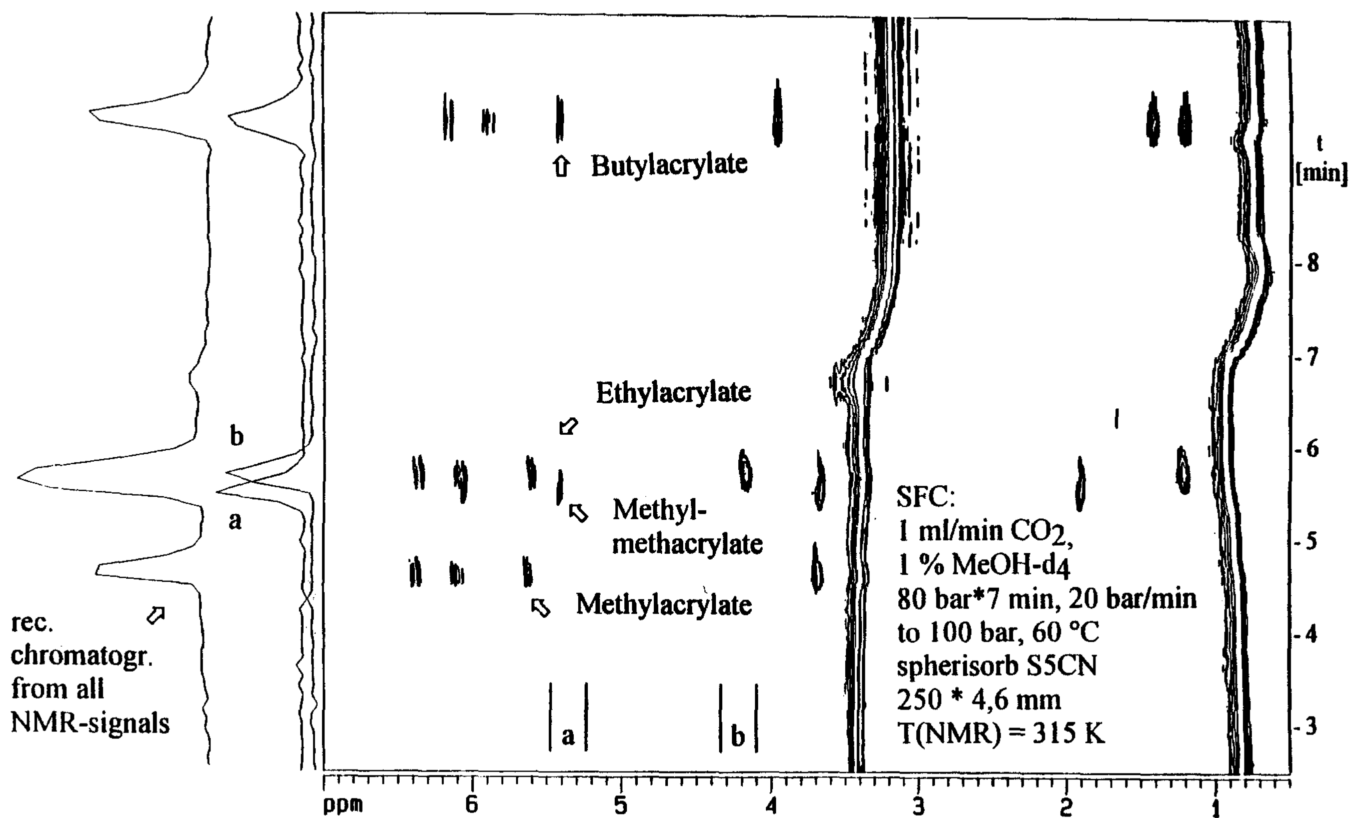

Fig. 7. Contour plot of the SFC-separation of the acrylates

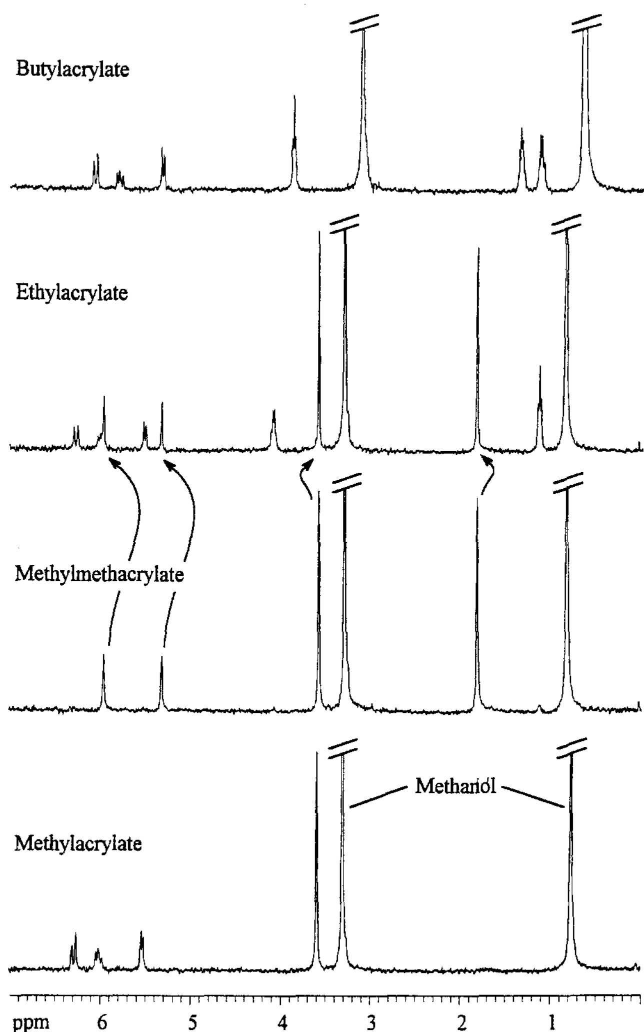

Fig. 8. Spectra extracted from the SFC on-line separation; corresponding to the peak maxima of each compound

The improvement of the signal, acquired by the summation of multiple rows, is shown in Fig. 5. With this procedure, the number of scans per spectrum (rows) can be increased after the acquisition, without deterioration of the time resolution. When the intensity of the signal is too weak, a corresponding number of rows can be later co-added. The following advantages arise with this supplementary summation:

a) The time dimension that contributes to a spectrum can be supplementarily determined.

b) With gradients, the chemical shift caused by the change of eluents can be equalised by removing the shift before the addition.

c) With intensive signals where the detection requires only a few scans, the advantage of the time resolution is retained.

In comparison to the results obtained with an AMX 400 equipped with a conventional lock system, the quality of the solvent suppression was clearly improved with the implementation of a digital lock of the 600 MHz instrument (BRUKER BOSS 1). The solvent suppression fluctuated from spectrum to spectrum by a factor of 2 to 10 with the formerly used AMX 400. The remaining acetonitrile signal stayed constant in the higher order of the ^{13}C satellites during the experiments (Fig. 6) with AMX 600.

The signals, located only at 0.25 ppm next to the residue solvent signal, can still be easily observed by a complete substitution of H_2O by D_2O and a sufficient time for column equilibration.

Presaturation or any other method of solvent suppression is not necessary for this signal, as it yielded in no further increase of the receiver gain, and therefore in no improvement of sensitivity. Presaturation would additionally introduce disturbances around the water signal.

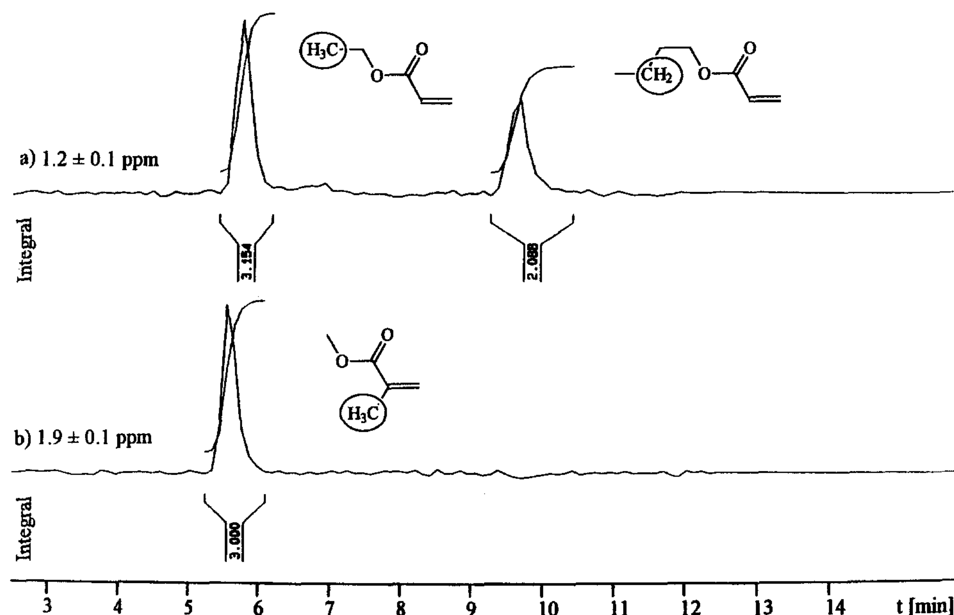

Fig. 9a, b. Chromatograms reconstructed from the NMR-intensities, in the region of **a** 1.2 ± 0.1 ppm for the methyl protons of the ethyl acrylate (and the methylene protons of the butyl acrylate), **b** 1.9 ± 0.1 ppm for the methyl protons of the methyl methacrylate

2 SFC

Figure 7 shows the contour plot of the separation. All four components are clearly visible and provide good *on-line* spectra. Figure 8 also shows the extracted spectra of the SFC-NMR run. Only in the spectrum of the butylacrylate the triplet of the methyl group is covered by the hydroxyproton of methanol at 0.8 ppm.

Ethylacrylate and methacrylate are nearly co-eluting. Using the second dimension, provided by the NMR, it is possible to separate the two components, as already hinted at in the reconstructed chromatogram in Fig. 7. In a chemical shift range of 1.9 ± 0.1 ppm only the methyl group at the double bond of the methylmethacrylate shows a signal, whereas in a range of 1.22 ± 0.1 ppm, only the terminal methyl group of the ethylacrylate shows a signal. The peak at 9.7 min arises from the methylene group of the butylacrylate which also has an NMR signal at 1.2 ppm. But as this compound is already separated by chromatography, it causes no interference for the integration.

In Fig. 9 the summation of all NMR-signals in each of the specified ranges are shown. They provide a reconstructed chromatogram for the methyl protons of the methylmethacrylate in the lower trace range (1.9 ppm) and the ethylacrylate in the upper trace range (1.2 ppm). The comparison of the two integrals, which both represent three protons, give a ratio of 0.951 (= 3.00/3.154) where 1.0 would be expected. The error of approximately 5% allows a good quantification. Also the value of 2.088 for the methylene protons of the butylacrylate which should be 2, shows a deviation of only 4.4%.

The experiment shows that it is possible to use proton NMR as a detector for SFC. The use of a modifier is also feasible, even if for some regions of the spectrum no information can be obtained. The background signals are very small compared to those normally arising from solvent signals during coupling of HPLC and NMR. No solvent suppression techniques are required.

One drawback of the experiments is, that for the coupling with NMR other modifiers should be used. Even with the use of fully deuterated modifiers, some information is lost. Methanol which is often used in SFC provides two relatively broad signals. Other modifiers, for example acetonitrile, would be more suitable for the NMR and cause the same cost as deuterated methanol.

The reconstruction of the chromatogram from intensities of the NMR-signals shows the possibilities of the second dimension provided by the NMR. The second peak, which consists actually of two compounds which are nearly co-eluting, can clearly be separated using the difference in the NMR spectra. Even for those compounds which are similar in the UV spectrum, they can be "baseline"-separated by the NMR-spectrum.

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