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Short communication

Nuclear magnetic resonance monitoring of centrifugal partition chromatography in pH-zone-refining mode

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Abstract

The coupling of centrifugal partition chromatography to NMR spectroscopy as detection and analysis method is investigated. Separation of a test mixture of three N-2,4-dinitrophenyl amino acids was performed in pH-zone-refining elution mode. The NMR and chromatography instrumentation was very similar to the one of the LC–NMR technique.

Keywords: Centrifugal partition chromatography; Nuclear magnetic resonance spectrometry; Detection, LC; Amino acids

1. Introduction

We here report the first coupling of centrifugal partition chromatography (CPC) [1] with NMR spectroscopy. A CPC apparatus is a liquid–liquid extractor in which the liquid mobile phase percolates through a liquid stationary phase that is held in place by means of the centrifugal force (Fig. 1a,b). It can be used in a way that is very similar to HPLC using the same basic components including pump, injector, and detectors. Various detection schemes are possible, using UV–Vis spectroscopy, evaporating light scattering detection (ELSD) [2], or mass spectrometry [3]. The CPC technique has the ability to separate very polar and fragile compounds. There is no irreversible adsorption or degradation of material on a solid support. Problems related to saturation of a solid-state stationary phase are avoided. CPC is therefore appropriate for preparative separations. The experience that is reported here uses CPC in the pH-zone-refining mode [4–6]. In this mode the products are not eluted as separate blocks having each a gaussian concentration profile, but as contiguous blocks of constant concentration distributed according to their acid–base and hydrophobicity properties. In preparative operating conditions it is highly difficult to monitor the separation by means of a UV detector [6]. The latter is generally saturated due to the high concentration of the products. Moreover, there are no peaks to look at, but only a set of nearly unseparated plateaus which bring little or no information about the separation. For all compounds, only the beginning and the end of the outflow are clearly visible. Transitions between products are more accurately observed using an in-line pH measurement. The choice of NMR as a detection and analysis method is thus adequate, even at modest field strength, because sensitivity is not a

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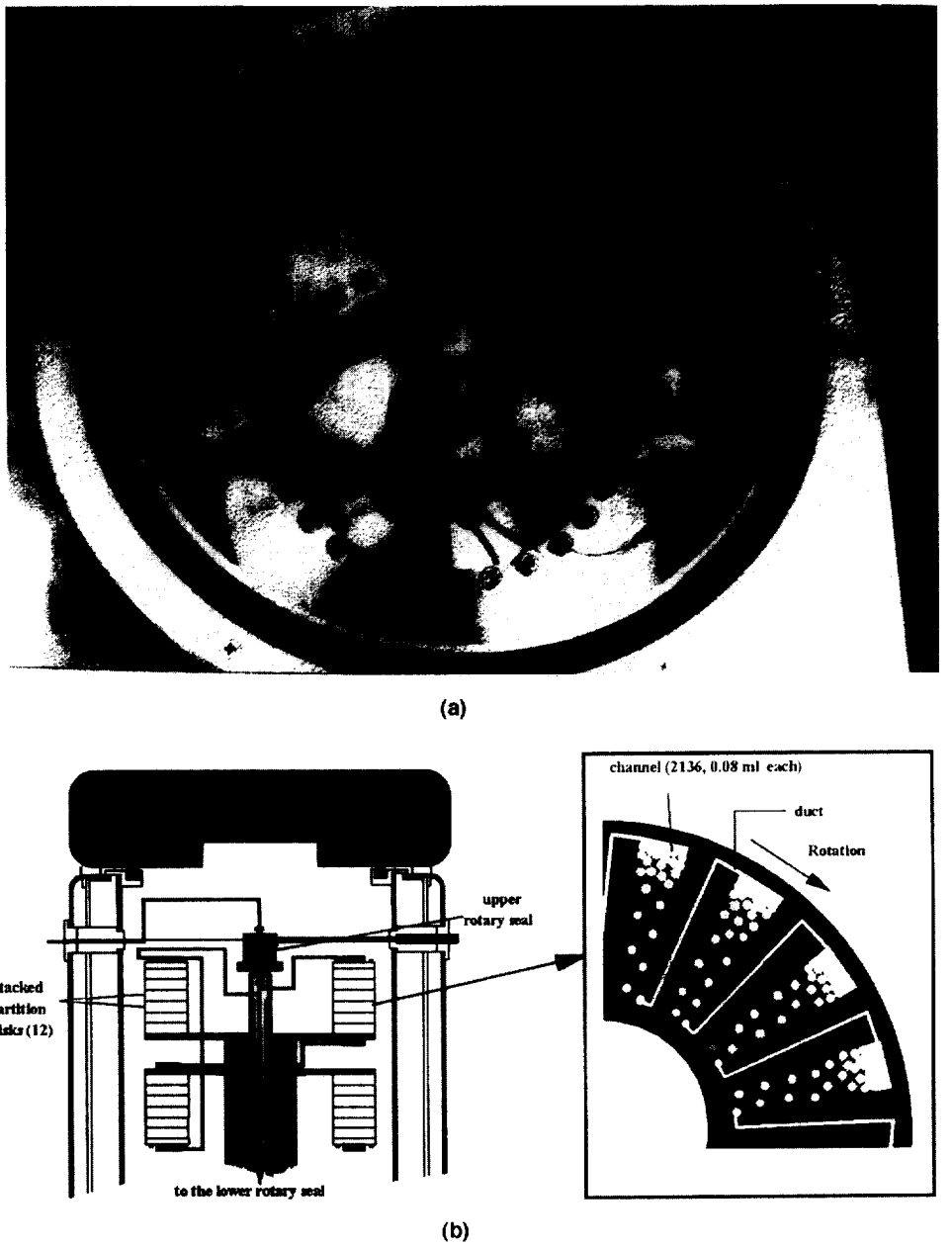

Fig. 1. (a) Photograph of the upper part of the HPCPC apparatus, showing the upper part of the column and the rotary seal. (b) Schematic description of the HPCPC apparatus.

limiting factor. Hyphenation of CPC with NMR combines the power of separation of CPC with a maximum structural information by NMR. NMR detection allows the measurement of the relative concentrations of the eluted compounds as a function of elution time. Subsequently, it provides an easy and reliable way to validate the theoretical model of pH-zone-refining CPC separation [7].

2. Experimental

2.1. CPC apparatus

The separation was performed using a HPCPC Sanki Series 1000 column (Tokyo, Japan) (Fig. 1a,b). The column is a stacked circular partition disk rotor which contains 2136 channels with a total internal volume of around 240 ml. The column is connected to the injector and the detector through two high-pressure rotary seals. A four-port valve, included with the HPCPC, allows it to be operated in either the ascending or descending mode. The HPCPC was connected to a Bruker (Rheinstetten, Germany) LC22C pump. A Bruker LC313E UV detector was inserted between the column and the NMR probe (see below). The latter is fed through a 3 m long polyether ether ketone (PEEK) tubing (0.25 mm I.D.). The sample was injected through a Rheodyne model 7125 manual injector, equipped with a 10 ml loop.

2.2. Reagents

Methyl *tert.*-butyl ether (MtBE) was analytical grade (SDS, 13124 Peypin-France), D_2O, trifluoroacetic acid (TFA) and N-2,4-dinitrophenyl (DNP) amino acids were obtained from Sigma (St. Louis, MO, USA).

2.3. Preparation of solvent phases and sample solution

The biphasic system was prepared as following. MtBE and D_2O were thoroughly equilibrated and the two phases separated. The upper organic phase was acidified with TFA at the concentration of 13 m*M*, and used as mobile phase. The lower aqueous phase was basified with ammonia (22 m*M*) used as retainer. The sample is a mixture of DNP N-protected amino-acids: leucine, alanine and aspartic acid (0.46, 0.41 and 0.55 mmol, respectively), dissolved in 9.5 ml of stationary phase and 0.5 ml of MtBE. It is biphasic.

2.4. Separation procedure

The column was first filled with the stationary phase. Then, the sample was injected, the rotation speed was brought to 800 rpm, and finally the mobile phase was pumped into the column in ascending mode at a flow-rate of 2 ml/min, resulting in 70 bar backpressure. The beginning and the end of the separation were checked by UV absorbance measurement at 300 nm.

2.5. NMR spectroscopy and chromatographic software system

NMR data were acquired on a Bruker DRX500 spectrometer equipped with 4 mm inverse dual $^{1}H/^{13}C$ flow probe with triple axis gradients. The Noesy-presat-1D pulse sequence [8] was used in order to achieve the double presaturation on MtBE signals at 3.15 ppm and 1.1 ppm. Mixing time length was 50 ms, presaturation was applied during relaxation delay (2 s) and mixing time. Each spectrum was acquired with 64 scans using 32K time domain points and 12 000 Hz sweepwidth. Solvent suppression was automatically optimised to avoid Bloch Siegert shifts. The two largest peaks were picked and their frequencies used in the real experiment. Time slicing was achieved using CHROMSTAR software and BPSU-12 software. NMR stop-flow spectra were acquired with time intervals of 3 min between the stops. Water droplet decantation time was 2 min (see below).

3. Results and discussion

As expected from literature data, DNP-Leu, DNP-Ala, and DNP-Asp were successively eluted in this order from the column [4,5]. The spectra on Fig. 2 show the evolution of the ^{1}H-NMR spectrum of CPC outflow, in the region of the aromatic and α-protons. Droplets of aqueous stationary phase escaping from the column caused some random variations of spectral resolution. A higher column rotation speed would reduce this phenomenon but would also increase backpressure to unmanageable values. As a consequence the experiment was carried out on stop-flow mode, so that droplets are allowed to decant partially away from the NMR observation zone. Stopping the elution is not a severe drawback in CPC separation as diffusion from cell to cell is minimal

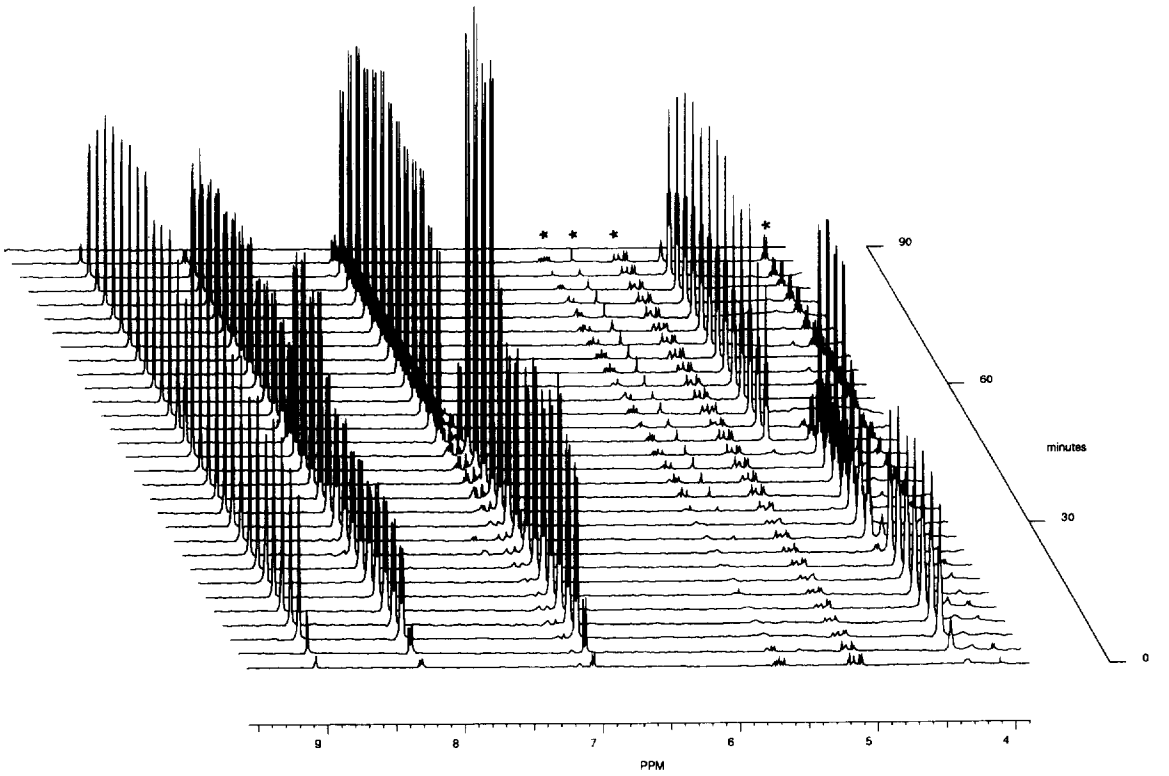

Fig. 2. Stacked plot of the ^{1}H-NMR spectra of HPCPC effluent, in the zone of aromatic and α-protons. Asterisks show positions of solvent impurities.

(Fig. 1b). However, small resolution variations are still noticeable, especially at the beginning of DNP-Ala elution. An on-flow analysis can performed if a third solvent like acetonitrile is injected via a mixing chamber placed after the column outlet. This makes it necessary to presaturate at a third frequency. Full traces corresponding to DNP-Leu, DNP-Ala, and DNP-Asp are drawn on Fig. 3a,b,c, respectively. The spectra shown in Fig. 3a,c do not show the γ- and δ-protons of DNP-Leu and the β-protons of DNP-Asp. These protons are either cleared by the solvent suppression sequence or are superimposed with the ^{13}C satellites of the MtBE resonances. The latter can be suppressed by an appropriate decoupling sequence applied to carbons. The distribution of the products as a function of the elution time was quantified by the value of the integral of the α-protons signals. The plots shown in Fig. 4 illustrate the quality of the separation and the features of CPC in pH-zone-refining mode. DNP-Ala and DNP-Leu are mono-acids and are eluted in their acidic neutral forms. Their concentrations are approximately those of the displacer (i.e., TFA) in the mobile phase. DNP-Asp is eluted partly in its monoanionic form, and partly in its diacidic form. If it was completely eluted in its monoanionic form, its behaviour would be the one of a monoacid, and its concentration would be the same as for DNP-Ala and DNP-Leu.

Conversely, if DNP-Asp was eluted in its diacidic form, its concentration would have only been half. The present situation is the intermediate one due to relative pK_a values of the components in the analysed mixture. Actual concentration of DNP-Asp is about two thirds of its maximum possible value, as already experimentally estimated [5,7].

4. Conclusion

Coupling of CPC with NMR detection was suc-

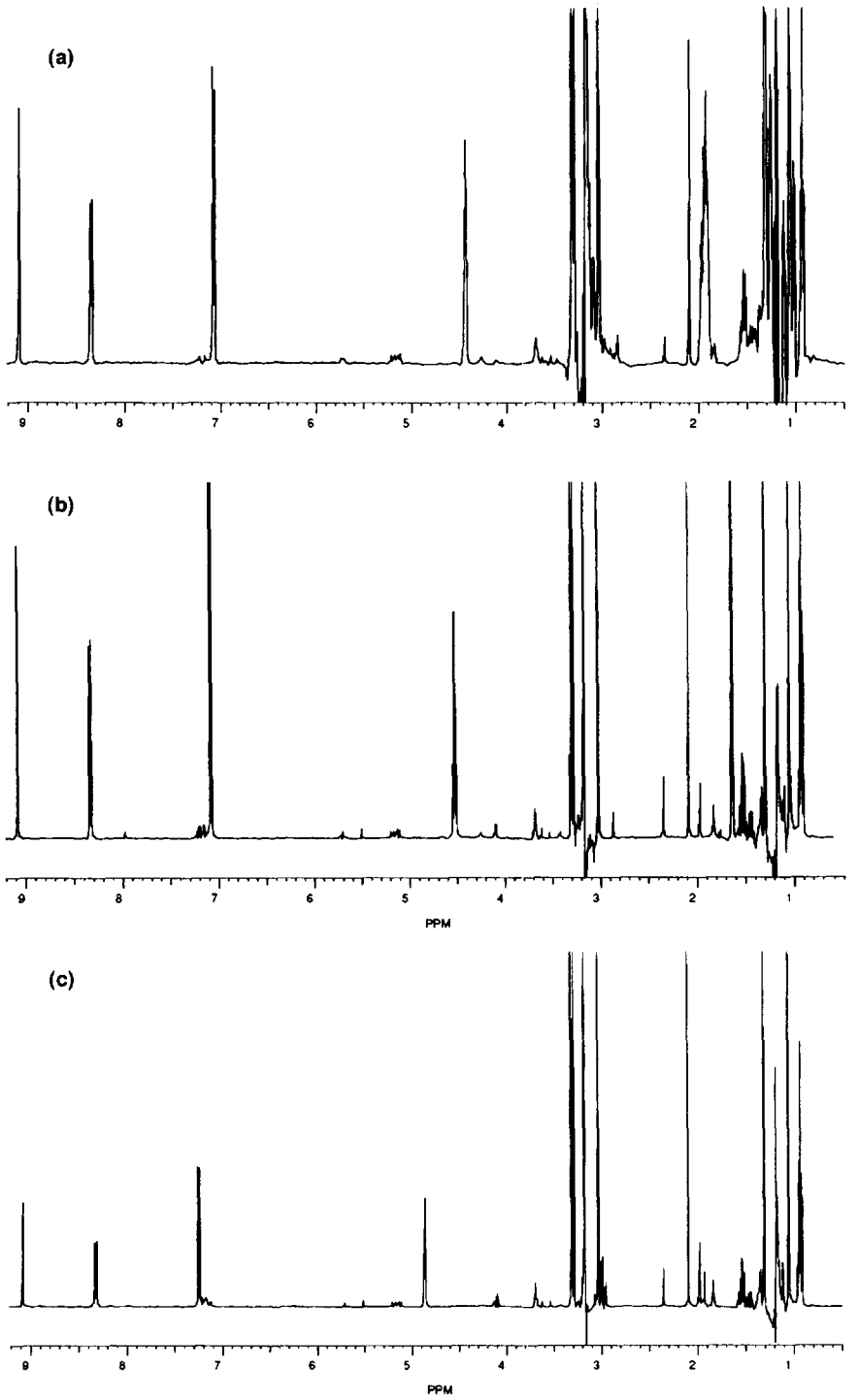

Fig. 3. (a), (b) and (c) full-width spectra of HPCPC effluent at elution times of 15 min (DNP-Leu), 45 min (DNP-Ala) and 75 min (DNP-Asp), respectively.

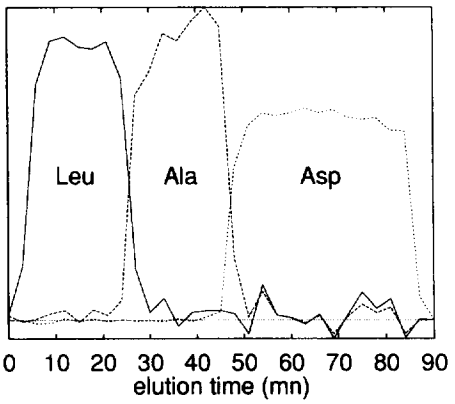

Fig. 4. Evolution of the relative concentrations of DNP-Leu, DNP-Ala, and DNP-Asp as a function of elution time. Concentrations were measured as the integral of the signals of the α-protons.

cessfully achieved using the presently available technology of HPLC–NMR instrumentation. The CPC–NMR technique provides simultaneously structural and quantitative measurements on the various separated compounds. The pH-zone-refining method used in this study leads to the clean preparative separation of three acidic compounds that could not be easily monitored by means of UV detection. This separation method yields products at concentrations that can be easily varied by the operator. Their high values make it possible to operate the NMR detection even at an intermediate field values. Minor troubles due to minute stationary phase leaking are overcome by operating in stop-flow mode. Also, on-flow injection of a third solvent as remixing agent was successfully tested. Further studies will involve NMR detection applied to CPC separation carried out in simple liquid–liquid extraction mode. In this mode, the elution profiles are totally similar to those observed in standard HPLC conditions. The sensitivity limitations of HPLC–NMR will be then encountered. In turn, triggering of fractions collection from UV chromatogram will be feasible.

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