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(Normal-phase) capillary chromatography using acrylic polymer-based continuous beds

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Abstract

Microchromatographic separations of polar aromatic compounds (pyridine, 4-pyridylmethanol, 4-methoxyphenol, 2-naphthol, catechol, hydroquinone, resorcinol, 2,7-dihydroxynaphthalene) using continuous beds are described. The columns were prepared by a simple one-step in situ polymerization procedure: a solution of acrylic monomers, including the cross-linking agent piperazine diacrylamide, was polymerized in a fused-silica capillary pretreated with 3-(trimetoxysilyl)propyl methacrylate. The continuous bed formed contained a network of channels and was attached covalently to the wall of the silica capillary (100 μm I.D.) via its methacrylate groups. Therefore, the frit used in conventional, packed columns could be omitted. The separation mechanism is discussed, particularly with regard to whether the so-called aromatic adsorption to the matrix itself is involved, an interaction first described by Gelotte [1] (the ligands, isopropyl and sulfonate groups, are not required for separation). This discussion is relevant to the question of whether the separation technique described should be classified as normal-phase or adsorption chromatography.

The mobile phase from the HPLC pump was split via an open capillary to get a flow rate through the continuous bed of about 100 nl/min. The beds were tested up to a pressure of 150 bar (8.8 bar/cm).

A continuous bed synthesized at a relatively low molar fraction of the cross-linker in the monomer mixture (16.5%) and high total concentration of the monomers (31.9% (w/v)) afforded the highest efficiency for the separation of the polar organic compounds. Plate numbers up to 150 000 m^{-1} were obtained and the run-to-run reproducibility was high. The selectivity of the separations was adjusted by changing the composition of the mobile phase (hexane–ethanol–methanol). The sample was applied by a diffusion-based injection technique.

Keywords: Continuous beds; Normal-phase chromatography; Adsorption chromatography; Microchromatography; Capillary chromatography; Aromatic adsorption

1. Introduction

Normal-phase chromatography is employed in about 20% of all HPLC separations [2]. It has gained an important position in preparative scale applications because it permits the use of non-aqueous mobile phases with the property to solubilize solutes of low-polarity. However, in analytical applications normal-phase separations have not been widely accepted, mainly owing to the variable surface

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activity of bare silica HPLC adsorbents [3]. This difficulty can partially be overcome by using chemically bonded silica-based stationary phases [4]. Interestingly, cellulose beds can with advantage be employed for normal-phase capillary electrochromatography [5,6].

Continuous matrices synthesized from *water-soluble,* bio-compatible monomers have been employed successfully for hydrophobic-interaction chromatography [7,8], reversed-phase partition chromatography [9–12], ion-exchange chromatography [7,13–16], chiral separations [9,17], dye-affinity chromatography [18], chromatofocusing [19], immobilized liposome chromatography [20] and electrochromatography [12,21]. Also continuous beds synthesized from non-water soluble monomers have good chromatographic properties [22–30]. Crosslinked polar synthetic polymers in bead form were used more than fifteen years ago for normal-phase chromatography [31].

The main advantage of the method for the preparation of continuous beds for standard and capillary high-performance liquid chromatography and for capillary electrochromatography is that the synthesis of the adsorbent and the packing of the column are accomplished in one step. This approach does not require any special equipment, is simple, time- and cost-effective.

Many capillary column techniques permit fast, high-efficient on-line microanalyses. The continuous polymer beds have the additional advantage to be covalently attached to the wall of the fused-silica column tube and, therefore, they do not require supporting frits, which often cause band broadening and changes in back pressure and thereby the flow rate during the course of a chromatographic experiment. Upon electrochromatography the frits often generate bubbles, the presence of which cause a drop in current and the run must be interrupted.

The matrices of the continuous beds are synthesized from water-soluble and non-ionic monomers and, therefore, exhibit negligible electrostatic and hydrophobic interactions (no non-specific interactions) under high-polarity mobile phase conditions. The original objective of this study was to investigate whether continuous beds could be used for normal-phase chromatography under low-polarity mobile phase conditions. During the course of the investigation we found that the chromatographic behavior of some columns was typical for adsorption chromatography, which prompted us to deal with the separation mechanism, although to a very limited extent.

2. Experimental

2.1. Materials and equipment

Piperazine diacrylamide (PDA), *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) were from Bio-Rad (Hercules, CA, USA); methacrylamide (MA), a 30% aqueous solution of the sodium salt of vinylsulfonic acid (VSA), and 3-(trimethoxysilyl)propyl methacrylate (Bind Silane) from Fluka (Buchs, Switzerland). Isopropylacrylamide (IPA) was bought from Tokyo Chemical Industry (Tokyo, Japan) and 2-hydroxyethylmethacrylate (HEMA) from Sigma (St. Louis, MO, USA). Test compounds for the separation studies (see the legend to Fig. 2) were purchased from different companies.

The fused-silica capillaries (25, 50 and 100 μm I.D., 375 μm O.D.) were bought from MicroQuartz (Munich, Germany).

The chromatographic system consisted of a Model 2150 HPLC pump from LKB (Bromma, Sweden), a Model 200 UV detector from Linear Instruments (Reno, NV, USA) and a Model SP 4207 integrator from Spectra-Physics (San José, CA, USA). To obtain a constant operating pressure over the continuous bed, a 25 μm or 50 μm I.D. *open* fused-silica capillary was used to split the flow. Extracolumnal band broadening was eliminated by using on-column analyte loading and detection and by the omission of frits.

The flow rates were measured using a 10 μl graduated syringe (without piston) connected directly to the outlet of the capillary column and watching the movement of the meniscus [5,6].

2.2. The preparation of the continuous bed capillary columns

A 100 μm I.D. fused-silica capillary was pretreated with Bind Silane, as described in Ref.

[11,12], in order to attach covalently the methacryloyl groups. For the synthesis of the different beds varying amounts of the monomers, including PDA (the cross-linking agent) and ammonium sulfate (to induce hydrophobic interaction between the polymer chains formed) were dissolved in 1.0 ml of 50 m*M* sodium phosphate (pH 7) (the amounts are given in Table 1). Oxygen was removed from the monomer solution by purging with nitrogen for 3 min. To initiate the polymerization, 10 μl of 10% APS (w/v) and 10 μl of 10% TEMED (v/v) were added to 1 ml of this solution, which was then immediately sucked into the capillary. The polymerization proceeded overnight. The next day the column was coupled to the HPLC pump for washing with distilled water for 30 min (30–100 bar). During the washing procedure a detection window was made by burning off the polyimide coating by electrical heating [32]. This procedure also decomposed and washed out a narrow section of the gel inside the detection window [12]. The dimensions of the columns are given in Table 1.

2.3. Chromatographic conditions

The chromatographic experiments were carried out at ambient temperature under isocratic elution conditions. The mobile phases consisted of methanol or hexane–ethanol–methanol. The volume ratio between hexane and ethanol was kept constant at 85:15, whereas the concentration of methanol was varied [6]. Diffusion-based injection was accomplished by immersion of the inlet of the capillary column for 30 s into a vial containing the sample solution. Prior to the injection the short section at the inlet end of the capillary that contained buffer alone (no gel) was cut off to ensure close contact between the sample and the bed.

3. Results and discussion

3.1. Operation of the continuous beds under constant pressure

Conventional HPLC pumps do not produce constant flow rates on the μl/min level (or lower). To overcome this problem the flow delivered by the HPLC pump was split via an *open* capillary with an inner diameter of 25 or 50 μm, preferably smaller than that housing the continuous bed . The flow rate through the bed was about 100 nl/min. An open capillary as flow resistor/restrictor has the advantage over a packed capillary with a supporting frit that the risk of clogging is negligible, i.e. the back pressure over the open capillary is constant. Since the permeability of a continuous bed is high and does not seem to change during the course of several runs (probably because the bed is not compressed since it is anchored to the capillary wall and also because no frit to support the bed is required) the pressure over the capillary bed is constant, i.e. the columns operate in the constant pressure mode. We want to mention that the thermal pump recently introduced can, with advantage, be employed for capillary columns without splitting the eluent [33].

Table 1
Continuous beds

Column	Column lengths[a] with 100 μm I.D.(mm)	Composition of polymerization reaction mixture						Total concentration of monomers (% (w/v))	Molar fraction of PDA in mixture of monomers (%)	Flow rate at 70 bar in pure methanol (μl/min)	N/m for 2-naphthol
		IPA[b] (mg)	MA[b] (mg)	HEMA[b] (mg)	VSA[b] (30%) (μl)	PDA[b] (mg)	$(NH_4)_2SO_4$ (mg)				
I	171 (123)	100	75	–	–	125	50	22.2	26.7	4.8	8 700
II	175 (125)	300	50	–	–	125	10	31.9	16.5	0.22	95 000
III	174 (126)	–	–	180	5	150	50	23.9	35.8	1.8	36 000
IV	174 (123)	–	–	240	5	175	50	28.3	32.8	2.5	28 000

[a] Effective length from inlet end to detection window within parenthesis.
[b] IPA, *N*-Isopropylacrylamide; MA, Methacrylamide; HEMA, 2-Hydroxyethyl methacrylate; VSA, Vinylsulfonic acid; PDA, Piperazine diacrylamide.

3.2. Diffusion-based injection

The sample was applied by diffusion for 30 s. Since the analytes have different diffusion constants they diffuse in amounts which are not proportional to the concentrations in the sample toward the bed surface, where they are more or less strongly adsorbed. The Einstein formula ($\bar{x}^2 = 2Dt$) shows that the diffusional distances during 30 s in *free solution* are in the range of 0.3–0.4 mm for the aromatic compounds used in this investigation. However, adsorption and restricted diffusion makes the widths of the applied sample zones much narrower. In the subsequent exchange of the sample solution for the mobile phase there is, accordingly, an obvious risk that part of the sample zones becomes washed out in non-proportional amounts. For this reason, diffusion-based sample application does not give high reproducibility. For macromolecules with their small diffusion constants the application time must be 15–25 min. Consequently, the method is not generally applicable. It is of interest from the view point that it permits, at least theoretically, determination of diffusion constants. Whether this can be realized in practice remains to be shown.

3.3. The permeability of continuous beds

The permeability (flow resistance) of the continuous beds is very dependent on the composition of the polymerization reaction mixture [34]. Ammonium sulfate has an important role in determining the morphology of the continuous beds, since it induces hydrophobic interactions between the polymer chains formed during the polymerization and thus causes their aggregation (and precipitation) and the creation of the network of flow-channels in the beds. IPA, the polarity of which is lower than that of the other monomers, has a similar effect on the morphology of the continuous bed, which is illustrated in Table 1 for Columns I and II in terms of the size of the channels in the beds as reflected by their flow rates (4.8 and 0.22 μl/min, respectively, at 70 bar). Therefore, ammonium sulfate can be partly replaced by higher concentrations of IPA (Table 1, Column II). The mechanical stability of the continuous beds (and, accordingly, the back pressure) is highly dependent on the nature of the monomers and their relative and total concentrations [11,16,34]. The latter concentrations were in this study 22.2–31.9% (w/v) and the molar fractions of the cross-linking agent, PDA, in the mixture of monomers were 16.5–35.8% (Table 1).

The continuous beds, stabilized mechanically by covalent attachment to the capillary wall, were run at pressures up to 150 bar (which corresponds to a reduced pressure up to 8.8 bar/cm) without observable increase in flow resistance (experiments show that they stand even much higher pressures). No compression of the bed was observed at this pressure. Fig. 1a shows a linear relationship between flow rate and pressure (Columns I, III and IV). The lowest flow rate and highest flow resistance was observed for Column II (Table 1) which was prepared at a relatively low concentration of the cross-linking agent, PDA, (the molar fraction in the monomer mixture was 16.5%) and at a high concentration of the other monomers (31.9% (w/v)) (see Table 1). This less cross-linked bed gave the highest plate number (150 000/m for 4-methoxyphenol) indicating narrower flow channels. The bed corresponds to a high-quality packed column made up of small-diameter particles.

The less polar mobile phases with higher concentrations of hexane afforded higher flow rates at a given pressure, probably due to their lower viscosities (Fig. 1b).

3.4. (Normal-phase) chromatographic separations

Ternary mobile phases of hexane–ethanol–methanol were employed for isocratic elution in normal-phase micro HPLC. This mixture, which permits the preparation of a broad range of mobile phases of varying polarity and low-viscosity, has been employed successfully in normal-phase capillary electrochromatography [5,6].

A test mixture consisting of eight organic compounds (1–8, see the legend to Fig. 2) was used for the chromatographic experiments. Similar but not identical chromatograms were obtained with all four columns prepared for this study (Columns I–IV, see Table 1). For instance, there were some small differences in the selectivity factors (<20%). The elution order for the test compounds on the columns containing isopropyl ligands (Columns I and II) and

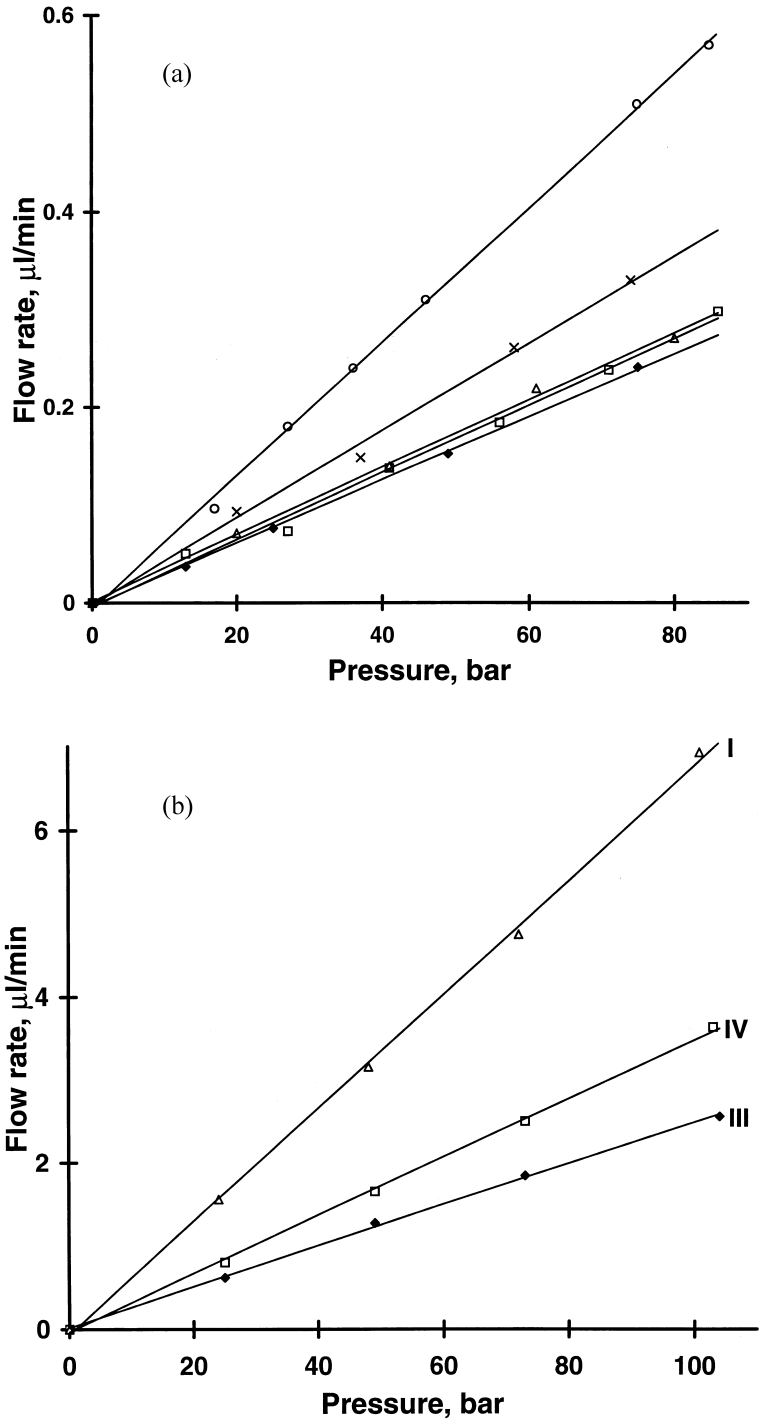

Fig. 1. Flow rate dependence of operational pressure. (a) Columns I, IV and III (for composition see Table 1) with pure methanol as mobile phase. (b) Column II with the following compositions of the mobile phase, hexane–ethanol–methanol (v/v): ○ 0:0:100, ×21.25:3.75:75, △ 43.5:7.5:50, □ 63.75:11.25:25, ♦ 85:15:0.

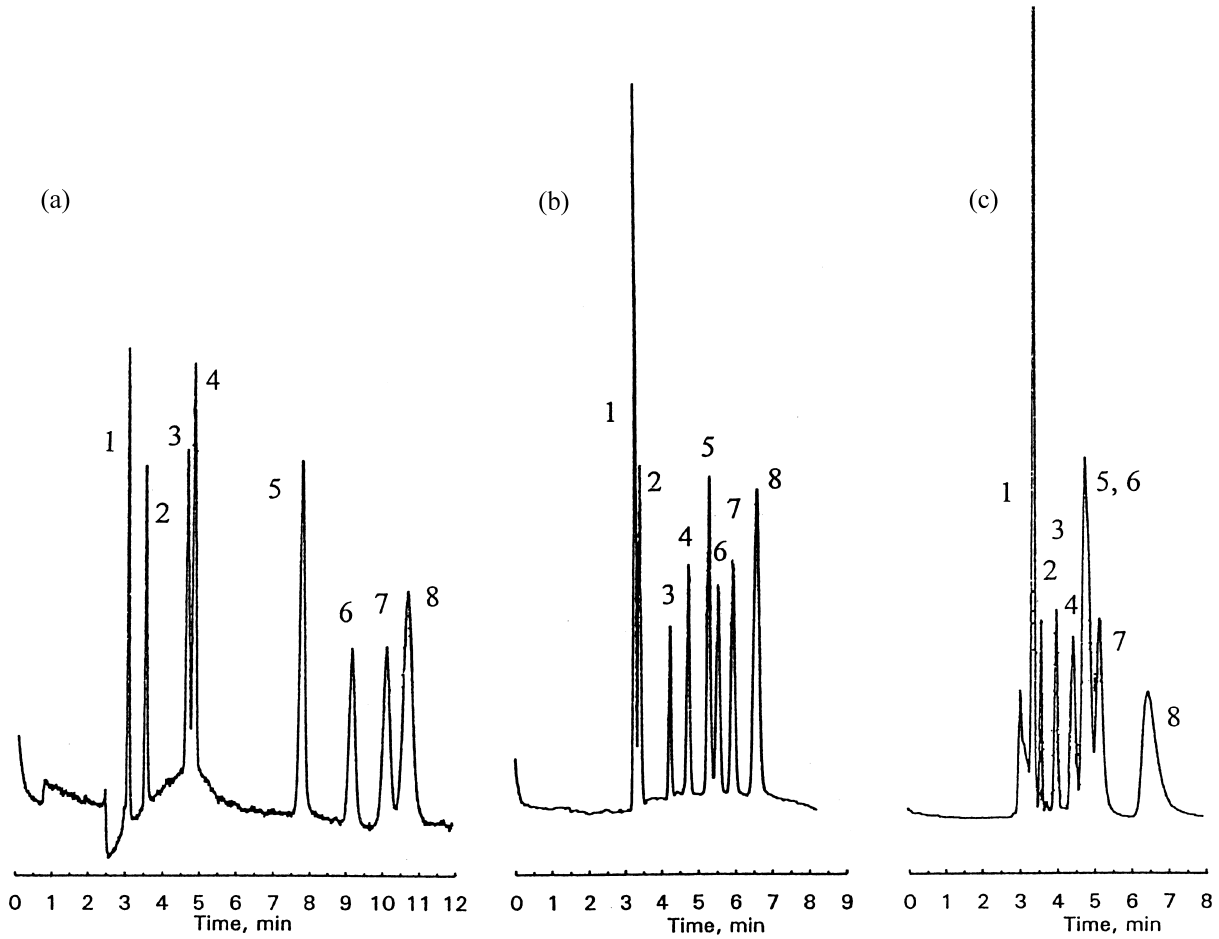

Fig. 2. Normal-phase capillary chromatography of polar aromatic compounds: ○ pyridine (1), △ 4-pyridylmethanol (2), □ 4-methoxyphenol (3), ◊ 2-naphthol (4), ● catechol (5), ▲ hydroquinone (6), ■ resorcinol (7), ♦ 2,7-dihydroxynaphthalene (8). UV detection at 220 nm. (a) Column II, mobile phase: hexane–ethanol–methanol, pressure: 62 bar; (b) Column II, mobile phase: pure methanol, pressure: 56 bar; (c) Column III, mobile phase: pure methanol, pressure: 5 bar. The compositions of the columns are given in Table 1.

hydroxyethyl ligands (Columns III and IV) was the same, although these ligands differ in their polarities. These findings indicate that the solutes interact with the polyacrylamide-based matrix, maybe by 'aromatic adsorption' onto the matrix [1,35–42] (see section 'The Separation Mechanism'). The sulfonic acid groups in Columns III and IV are of little importance for the adsorption because of their low concentration (the VSA monomer was used in order to generate electroendosmotic flow in planned future CEC experiments). In fact, a continuous bed synthesized in the absence of isopropyl acrylamide and vinyl sulfonic acid separated the model analytes in the same order as did the bed prepared for the experiments in Fig. 2a and b. The width of the peaks was, however, much larger since the omittance of isopropyl acrylamide (see Table 1) gave a bed with a different morphology and thereby lower resolution (as soon as the concentration of one monomer is changed the concentration of the other monomers must also be changed to get a bed with optimum efficiency). The highest resolution was obtained with Column II (Fig. 2a and b). Column III (Fig. 2c) and Columns I and IV (chromatograms for these columns

are not shown herein) afforded considerably lower resolution. The catechol and hydroquinone peaks overlapped on Column III (peaks 5 and 6).

The above observation that the appearance of the chromatograms is relatively independent of the chemical structure of the bed (the polarity of the ligands) has the advantage that small variations in the bed composition that may occur between different batches do not affect significantly the reproducibility of the experiments.

3.5. The influence of the composition of the mobile phase on selectivity and retention

The possibility to change the selectivity by altering the mobile phase composition is demonstrated in Fig. 2. With pure methanol as mobile phase (Fig. 2b and c) peaks 3 and 4; and peaks 7 and 8 are much better resolved than with the less polar mobile phase hexane–ethanol–methanol 42.5:7.5:50 (v/v) (Fig. 2a), whereas the opposite is true for peaks 1 and 2; peaks 5 and 6; and peaks 6 and 7. A comparison of the chromatograms in Fig. 2a–c gives information on how to design the composition of an elution gradient to optimize the resolution.

Fig. 3 shows the influence of the mobile phase composition on the retention factors of the test compounds in an experiment on Column II (for its composition, see Table 1). The increase of the polarity of the mobile phase decreased the retention factors of the more retained compounds dramatically, as expected for normal-phase but not for reversed-phase chromatography experiments.

3.6. The efficiency of the separations

Column II (see Table 1) has a relatively high back pressure and, accordingly, relatively narrow channels. As expected theoretically, Columns I, IV and III, which are characterized by higher permeability (lower back-pressure) and therefore wider channels, had much lower plate numbers at similar flow rates. For instance, the theoretical plate numbers calculated for 2-naphthol (see Table 1) were 8700, 28 000,

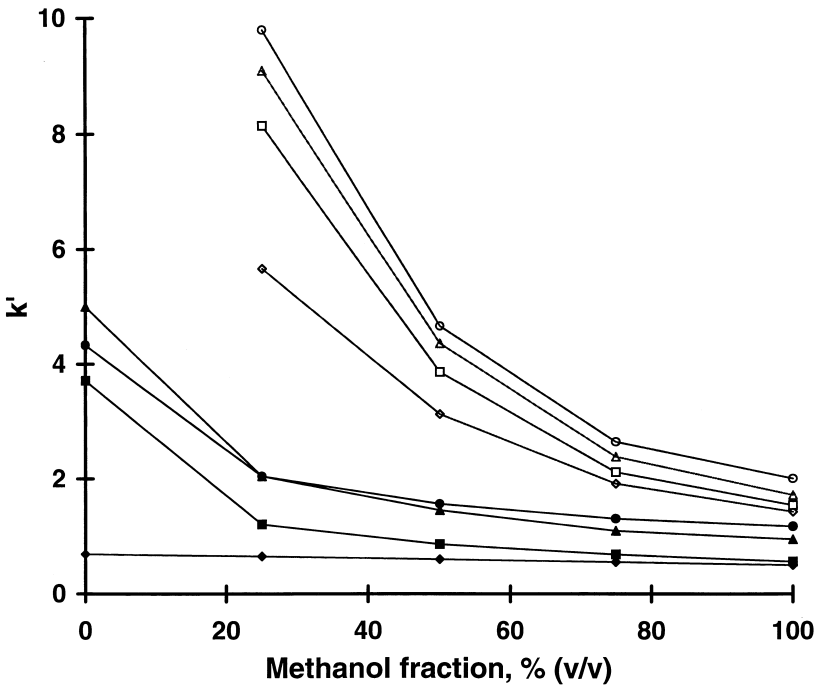

Fig. 3. Capacity factors of the same polar compounds as in Fig. 2 at different methanol concentrations in the hexane–ethanol–methanol phase (the hexane:ethanol (v/v) ratio was constant 85:15). For the composition of the bed, see Table 1, Column II.

36 000 and 95 000 per meter for Columns I, IV, III and II, respectively (flow rate: 0.27 μl/min, mobile phase: pure methanol). The drop in efficiency correlates with the permeability of the columns (see Table 1 and Fig. 1a) and can be explained by differences in the bed morphology: the denser beds had a higher hydrodynamic resistance but also a higher efficiency. Evidently, for low back pressure one has to sacrifice resolution. Peak 3 (corresponding to 4-methoxyphenol) in Figs. 2a, b and c had the following plate numbers per meter: 110 000, 150 000 and 100 000, respectively.

3.7. The separation mechanism

The two types of partition chromatography, reversed-phase and normal-phase chromatography, are distinguishable based upon the relative polarities of the mobile and stationary phases [43]. By definition, normal-phase chromatography means that the stationary phase is more polar than the mobile phase, i.e., the method presented herein could belong to that category of separation techniques when the eluent was hexane–ethanol–methanol (Fig. 2a). However, in the ideal normal-phase mode the analytes should be eluted in the order of increasing polarity (non-hydrophobicity, water-solubility), which is not the case in our investigations. The explanation of this strong deviation from the expected elution order in ideal normal-phase chromatography could be: (1) the organic solvent in the stationary phase has other chromatographic properties than in the mobile phase, in analogy to a proposed separation mechanism for molecular-sieve chromatography in aqueous solutions [44]; (2) the analytes adsorb onto the stationary phase (=the matrix) (in the above experiments the separations are similar in the presence and in the absence of isopropyl and sulfonate ligands); (3) partition occurs in parallel with alternative (2).

Experimental evidence for proposal (1) has never been presented for any separation method. Proposal (2) cannot be excluded since it is well-known that aromatic substances interact with many crosslinked polymers (gels). This interaction was discovered in 1960 by Gelotte when he chromatographed aromatic compounds on Sephadex columns and was called ‘aromatic adsorption’ [1]. This vague term has since then been retained, one reason being that the nature of the adsorption is still obscure in spite of the many attempts at finding the true interaction mechanism, although it seems to be generally accepted that conjugated π-electrons in the analytes often are responsible for the interaction [35–42]. Most reported experiments have been performed in aqueous solutions. In ethanol the interaction was less [38]. The same observation was made when the organic solvent (methanol) in the experiment shown in Fig. 2b was replaced by an aqueous buffer.

It is interesting to note that the same order of elution as that in the chromatogram in Fig. 2 was obtained in an experiment performed in the classical ‘aromatic adsorption mode’, i.e., in an aqueous medium (0.01 *M* sodium phosphate, pH 7.0) and on a bed without ligands [a bed synthesized as column II, but in the absence of isopropylacrylamide (and vinylsulphonic acid)]. Accordingly, the eluent need not be a mixed organic solvent, as in Fig. 2a, but can also consist of one solvent, for instance water (or methanol as in Fig. 2b and c). These features are not characteristic of partition, but rather of adsorption chromatography. Perhaps, therefore, it may be more relevant to employ the latter term for the separation technique described than normal-phase chromatography.

Commercial normal-phase packings have polar ligands, such as cyano, diol and amino groups [43]. The continuous beds described herein differ from these packings in that they function also in the absence of ligands. The ‘aromatic adsorption’ occurs not only in gels but also in polymer *solutions* which has been utilized for *electrophoretic* separation of solutes [45].

4. Conclusions

The selectivity and the retention factors for polar aromatic compounds changed very little when isopropyl ligands were replaced by hydroxyethyl ligands. Therefore, and also for other reasons, the retention mechanism seems to involve, at least partially, adsorption onto the polyacrylamide matrix itself. The retention factors of the test compounds increased with a decrease in the polarity of the mobile phase. The efficiency was greatly dependent on the conditions of the synthesis, i.e., the morpholo-

gy of the continuous bed. The beds were stable under an operating pressure up to at least 150 bar and afforded reproducible chromatograms.

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