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Comparison of the chromatographic behavior of monolithic capillary columns in capillary electrochromatography and nano-high-performance liquid chromatography

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Abstract

Porous monoliths based on *N*,*N*-dimethylacrylamide (DMAA) or methacrylamide (MAA) were prepared inside fused silica capillaries as stationary phases for nano-chromatography. The columns were characterized in terms of flow rate and backpressure and showed, e.g. differences as a function of the salt concentration added to the polymerization mixture. When the columns were investigated for the separation of uncharged (polar hydroxylated aromatic compounds) and charged (amino acids) analytes under pressure driven conditions (pLC), differences to the previously observed behavior under voltage driven conditions (CEC) were observed. Whereas the non-charged analytes showed similar behavior in both cases—thus, corroborating the previous assumption of a mainly chromatographic separation mode driven by hydrophilic interactions in CEC—the charged amino acids did not. Assuming that the separation was governed by chromatographic phenomena in the pLC mode and by both chromatographic and electrophoretic effects in the CEC mode, the experiments allowed deconvoluting the two contributions. In particular, the charged amino acids appeared to interact with the stationary phases mainly by electrostatic interactions modified by some hydrophilic effects.

Keywords: Stationary phases, LC; Monoliths; Polar hydroxylated aromatic compounds; Amino acids

1. Introduction

The miniaturization of chromatographic separation systems both in the pressure (pLC) and the voltage (CEC) driven mode promises to open new possibilities in analytical chemistry. Probably, the most important advantages of such micro- and nano-LC systems (using 100–200 and 25–75 μm i.d. columns, respectively) are the option for parallel processing (e.g. in high throughput screening) and the possibility to inject extremely small sample volumes. In addition and especially in combination with MS detection, higher mass sensitivities and lower detection limits can often be attained. Finally, the considerably smaller elution volumes of the miniaturized systems lead to lower solvent consumption.

Whether pressure or voltage driven micro/nano-LC is the more attractive variant, remains to date an open question. Pressure driven micro/nano-LC, at least until now, has the advantage that the adaptation of conventional HPLC methods is more straightforward than in the case of CEC. This is especially the case for charged analytes. On the other hand is the application of an electrical field as mobile phase driving force more easily achieved in a miniaturized system than that of pressure. In the end, the two techniques may even prove to be complementary and combinations of them may be ideally suited for the separation of complex samples containing both charged and non-charged analytes.

Few authors have to date addressed the question of what to expect in terms of efficiency (plate numbers) and separation power from both types of miniaturization approaches. Some papers approach the question from a theoretical point of view and their predictions concerning the possible additions to the observed plate heights (van Deemter curves) depend to a large extent on the initial assumptions [1–5]. A general presumption seems to be that due to the predominance of a plug flow profile in CEC (at least as long as there is no significant double layer overlap) versus a parabolic flow profile in pLC, CEC tends to result in higher plate

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numbers under otherwise equal conditions. Moreover, since the electroosmotic flow (EOF) in CEC is independent of the particle size, smaller particles, and therefore, inherently more efficient columns can be used in CEC compared to micro/nano-pLC, if the problem of column packing is neglected for the time being.

Aside from these theoretical considerations, a number of experimental results have been published in regard to a direct comparison of voltage and pressure driven LC. Alexander et al. [6] have assembled a modular system that could be operated in the CEC or nano-pLC mode. With this instrument, gradient elution was possible in both modes. In the case of a packed capillary column (75 μm i.d.) the authors obtained plate numbers of 100,000 N/m in the CEC-mode compared to approximately 65,000 N/m in the case of nano-pLC (reversed phase chromatography conditions). Wen et al. [7] compared van Deemter plots recorded for both nano-pLC and CEC. Three kinds of reversed-phase materials (differing in particle size and porosity), as well as one ion-exchange packing material were compared. The authors came to the conclusion that band-spreading due to bed non-uniformities and mass transfer resistances was consistently greater when the mobile phase was driven by pressure rather than by the EOF. A so-called attenuation factor was proposed corresponding to the ratio of the van Deemter coefficients in CEC and nano-pLC, to indicate the favorable influence of the EOF on the various contributions to the overall band spreading.

Gusev et al. [8] compared column efficiencies recorded for a non-retained marker in CEC and nano-pLC using monolithic styrene divinylbenzene (Sty-DVB) based columns (75 μm i.d.). The van Deemter curve in the CEC-mode showed a nearly constant value of the plate height at flow velocities above 1 mm/s, whereas the plate height increased towards higher flow rates in the case of nano-pLC. This was surprising since monolithic columns are usually assumed to have a very small *C*-term [9]. The authors also observed differences in the plate height curves according to the procedure used for the preparation of the monolith. In this context, the use of a column derivatized with ammonium groups in the case of CEC and a similar, but underivatized one in the case of nano-pLC, may have been unfortunate, especially as the same group had previously demonstrated the influence of the stationary phase chemistry on the plate height [7]. Ericson et al. [10] compared continuous beds, namely a rigid acrylamide-based gel matrix, in the pressure and voltage driven mode. The authors reported plate heights below 10 μm in both cases. CEC was again slightly superior to nano-pLC in this regard, but in both cases the plate height was virtually independent of the flow velocities above 0.5 mm/s.

We have recently prepared a set of hydrophilic monolithic capillary columns intended primarily for CEC-applications [11]. The influence of various synthesis and chromatographic factors on the retention and separation of charged and non-charged analytes on these columns under CEC conditions has been investigated [12,13]. In order to deconvolute the contribution of the chromatographic and the electrophoretic contributions to the observed separations and thereby to improve the understanding of the underlying separation mechanism, in the present paper the columns were used in nano-pLC and the results compared to those previously obtained in CEC.

2. Materials and methods

2.1. Materials

Piperazine diacrylamide (PDA) was from BioRad (Hercules, CA, USA). Dimethyl formamide (DMF), ammonium acetate, the aromatic test compounds, *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), *N*,*N*-dimethylacrylamide (DMAA, >98%), methacrylamide (MAA), phosphate buffer (100 mM, pH 7.0), vinylsulfonic acid (VSA, as a 30% aqueous solution of its sodium salt), acetonitrile, and methanol were from Fluka (Buchs, Switzerland). All other chemicals, including 3-(trimethoxysilyl)-propyl methacrylate (bind-silane), and the amino acids were from Sigma (St. Louis, MO, USA). Chemicals were used without further purification. Ultrapure water (purification system: SG, Barsbüttel, Germany) was used throughout. The fused-silica capillaries (75 μm i.d., 360 μm o.d.) were from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Instrumentation

An HP3D instrument from Agilent (Waldbronn, Germany) equipped with pressure option (12 bar), diode array detector, and air-cooling was used for all CEC experiments. An Ultimate Capillary- and Nano-HPLC system from LC-packings (Amsterdam, The Netherlands) was used for the experiments in the pressure driven mode.

2.3. Calculations

The %*C* (g crosslinker/100 ml) and %*T* (g total monomer/100 ml) nomenclature suggested by Hjerten [14] was used for stationary phase characterization. The contribution of the (constant amounts of) VSA, i.e. the EOF-generating monomer, in the reaction mixture was not taken into account for these calculations. Plate height *H* and number *N* were calculated as follows from the retention times (t_r) and width at half height ($w_{0.5}$) of the recorded peaks:

$$N = \frac{L}{H} \approx 5.54 \left(\frac{t_r}{w_{0.5}} \right)^2$$

where *L* is the effective column length (injection to detection point in case of the CEC experiments).

2.4. Synthesis protocols

The polymerization mixture for the monolithic capillary columns was prepared as follows. The reactants were weighted according to the desired %*T* and %*C* ration into 2.0 ml Eppendorf vials in the following order: (1) cross-linker (PDA), (2) functional monomers (DMAA/MAA and VSA), and (3) ammonium sulfate. The mixture was dissolved in 1 ml of aqueous buffer (if not indicated otherwise, 50 mM phosphate buffer pH 7.0), vigorously mixed, and ultrasonicated for 10 min. Ten microliters of a 10% (w/v) APS solution were added and the mixture was again briefly mixed, before 10 μl of a 10% (v/v) TEMED solution were finally added. Capillary preparation involved mainly a pretreatment with bind-silane according to a modification of the procedure suggested in [10]. For this purpose, the capillaries were flushed with 0.2 M NaOH and 0.2 M HCl for 30 min each, followed by water (30 min) and bind-silane (20 min). The latter was left overnight in the capillary, which was plugged with GC-septa. Immediately before the introduction of the polymerization mixture, the capillary was rinsed with methanol for 20 min followed by a brief wash with water. Then the polymerization mixture was quickly injected by plastic syringe. The capillaries were again plugged with GC-septa and the polymerization was allowed to proceed overnight. The next day approximately 2 cm were cut off at each capillary end and the capillary was inspected under the optical microscope. Capillaries that showed obvious inhomogeneities were discarded. The remaining columns were connected to an HPLC-pump (model 422, Kontron, Germany) and flushed with water for approximately 3 h in order to remove the ammonium sulfate and the unreacted monomers. Backpressures of up to 200 bar were regularly applied during this procedure. If necessary a detection window was created by pyrolyzing the monolith inside the capillary over a distance of approximately 0.5 cm, while continuously flushing the column with water.

2.5. Sample preparation

The aromatic analytes were dissolved in acetonitrile at a concentration of 2–5 mg/ml, and the test mixtures was then diluted to concentrations between 5 and 20 mM. Charged amino acids were prepared in the respective mobile phases at a concentration of 5 mg/ml and further diluted to concentrations between 1 and 5 mM.

2.6. Electrochromatography

Before each CEC experiment, the column was conditioned with the new mobile phase for 30–40 min by simultaneous application of pressure (11 bar) and voltage (7 kV), followed by 10 min of pure electrokinetic conditioning (25 kV). The mobile phases for the CEC experiments were prepared as follows, an aqueous stock solution of 100 mM ammonium acetate/4 M acetic acid was prepared. Then the proportions of (organic phase:water:stock solution) in the desired mobile phase were calculated as desired, e.g. in a way that the overall ionic strength of the mobile phase was kept constant when the organic phase content was varied. The ammonium acetate/acetic acid stock solution had a pH of 3.1. However, it should be noted that the apparent pH measured in the mobile phases deviated somewhat from this value. In particular, an apparent pH of 3.5 was measured for mobile phases containing 20 % organic solvent as well as 4.0 and 4.5 in mobile phases containing 40 and 60% of organic solvent, respectively. Injection was done electrokinetically at 7 kV (3 s), the temperature was 25 °C, and the detection wave length 200 nm. DMF was chosen as EOF-marker. The total capillary length for the CEC-experiments was 35.5 cm, corresponding to a separation length (inlet to detection window) of 27 cm.

2.7. Nano-pLC

For the nano-pLC experiments the tubing (25 μm i.d.) of the Ultimate Capillary and Nano-HPLC system connecting the solvent bottles to the injection valve and the calibrator cartridge (ULT-NAN-75) were adapted for the use in nano-flow columns (75 μm i.d.). The split ratio obtained was around 1:300. Sample injection by pressure was performed manually, using a four port injection valve with a 10 nl internal injection loop from Valco (Schenkon, Switzerland). If not otherwise mentioned, the length of the monolithic column in the nano-pLC experiments was 15 cm. A piece of fused silica capillary of 40 cm length and 25 μm diameter served as connecting tubing from the monolithic column to the detector cell. A U-Z View capillary flow cell (ULT-UZ-N10) with an illuminated volume of 3 nl was used with the UV-Vis detector of the system. Unless indicated otherwise a detection wavelength of 214 nm was used in the experiments. The data was processed with the Windows based software Ultichrome 3.1, delivered with the instrument. Mobile phases of varied compositions could be prepared in the mixing chamber of the nano-pLC system by drawing eluents from up to four supply bottles in the required proportions (bottle 1: acetonitrile, bottle 2: methanol, bottle 3: water, bottle 4: stock solution of 100 mM ammonium acetate, 5 M acetic acid in water, pH 2.95). Before each measurement and upon every change of the mobile phase, the stationary phase was conditioned until a stable baseline was obtained.

3. Results and discussion

3.1. Column characterization

When the efficiency (plate height) of the monolithic columns had previously been studied under electrochromatographic conditions [11,13] van Deemter curves had been recorded that showed little if any increase in plate

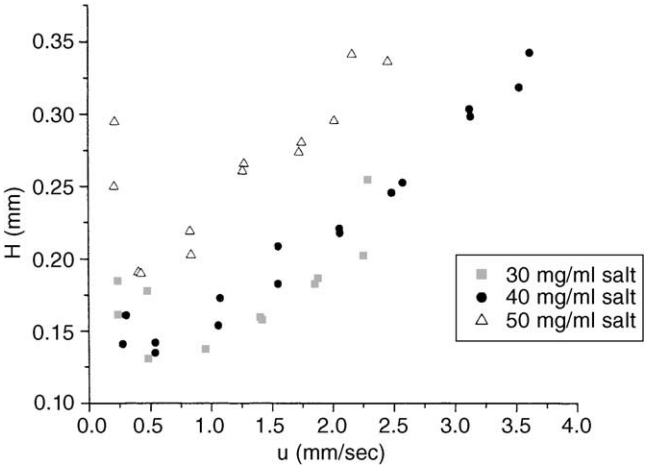

Fig. 1. van Deemter plots of DMF (non-retained tracer) on DMAA columns ($T = 15\%$, $C = 52\%$) prepared with 30, 40, 50 mg/ml ammonium sulfate in the polymerization mixture. Mobile phase, ACN:MeOH 4:6; column length, 15 cm.

height towards higher flow rates within the investigated range (approximately 0.1–1.0 mm/s), i.e. efficiencies that were largely determined by the A-term. The mobile phase composition seemed to be of greater importance than either the stationary phase chemistry or the average pore size of the stationary phase. A given mobile phase resulted in similar plate height functions for columns of different chemistry, e.g. MAA and DMAA based monoliths, but also for columns of different average pore size, e.g. DMAA monoliths prepared in the presence of different amounts of ammonium sulfate [13]. A change in the mobile phase composition, e.g. from an organic to a hydroorganic one, on the other hand, normally entailed a significant change in the plate height function. Hydro-organic mobile phases, for instance, consistently yielded lower plate heights than purely organic ones.

Fig. 1 compiles the van Deemter curves recorded under pressure driven conditions for DMAA monoliths prepared with 30, 40, and 50 mg/ml ammonium sulfate as pore forming (salting out) agent, respectively (all: $T = 15\%$, $C = 52\%$). These curves differ in a number of ways from those measured previously by us and others for similar capillary columns under CEC conditions. First of all—even taking possible extra column effects into account—with values between 120 and 180 μm, the A-term of the columns under pLC conditions is, roughly one order of magnitude larger than in the CEC mode, in which case the corresponding values were between 3 and 10 μm [11]. Furthermore, the plate height increases significantly at higher velocities, whereas in CEC generally only a small increase in plate height is observed towards higher flow rates. This seems to indicate a slower mass transfer (*C*-term of the van Deemter equation) in the monolithic column under pressure driven compared to electro-driven conditions. Similar results had previously been observed by Gusev et al. [8], albeit not by some other authors [10].

There also seems to be a significant influence of the salt concentration in the polymerization mixture on the plate height of the final monolith, since columns prepared with 30 mg/ml salt (presumably smallest pore size) consistently showed the lowest plate heights and the column prepared with 50 mg/ml (presumably largest pore size) the highest. In addition there seems to be a slight shift in the optimal linear flow rate, which increases from the column prepared in the presence of 50 mg/ml salt, to that prepared in the presence of 40 mg/ml and finally the column prepared in the presence of 30 mg/ml of salt. No explanation can at present be given for this repeatedly made observation. Differences were also observed when the linear flow velocities were recorded as a function of the volumetric flow rate for the three DMAA columns prepared with differing amounts of salt, respectively, when the backpressures created by the column were measured as a function of the volumetric flow rate. Under CEC-conditions, the flow rates (EOF) measured for a range of applied voltages were identical for all three columns [13]. This is obviously not the case in the pressure driven mode. In this case, the back pressure created at a given flow velocity by a column prepared in the presence of 30 mg/ml of salt is considerably higher than that caused by columns prepared with 40 and 50 mg/ml salt, Fig. 2a. However, this is not necessarily related to a corresponding change in porosity. In fact, the monolith prepared with 40 mg/ml of salt seems to have the highest porosity since for a given volumetric flow rate, this monoliths shows the highest linear flow rate, Fig. 2b.

3.2. Separation of sample analytes

A mixture of five non-charged but polar hydroxylated model compounds, namely hydrochinone monomethyl ether (HQMME), 2-naphthol, catechol, hydroquinone, and resorcine was subsequently separated under isocratic chromatographic conditions on a DMAA ($T = 29\%$, $C = 52\%$) column of 15 cm length, Fig. 3. The same set of polar aromatic compounds had previously been separated under electrochromatographic conditions on a similar DMAA column, albeit with a length of 27 cm and a total monomer concentration of $T = 15\%$ [13]. The crosslinker concentration was $C = 52\%$ in both cases. As expected, the elution order for these uncharged molecules was the same in both cases. The plate number achieved under nano-pLC conditions is approximately 10,000 N/m, i.e. in the same order of magnitude as for the CEC experiments [13].

While the separation of uncharged analytes hence seems to be governed in a rather similar manner by chromatographic effects in nano-pLC and CEC, differences must be expected for charged analytes, where electrophoretic effects play a role under voltage driven (CEC) conditions. It had previously been noted [12] that for charged amino acids, namely histidin (His), tryptophan (Trp), and phenylalanin (Phe) differences can be observed between the two modes. Not only did the amino acids tend to elute before the uncharged EOF marker DMF under CEC conditions, but there

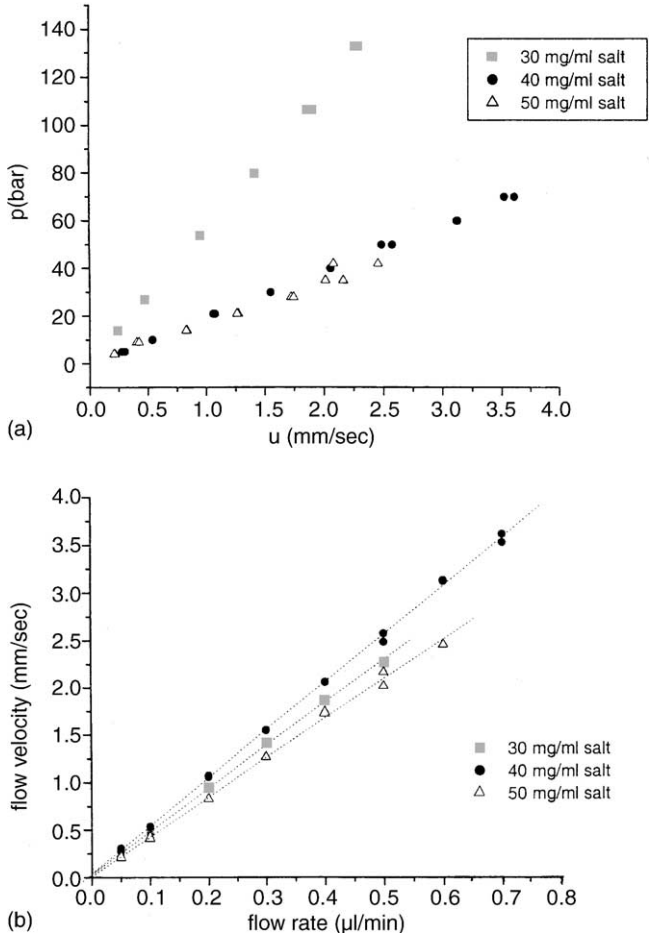

Fig. 2. (a) Plot of the linear flow velocity against the applied volumetric flow rate in three DMAA columns ($T = 15\%$, $C = 52\%$) prepared with 30, 40, 50 mg/ml ammonium sulfate. Mobile phase, ACN:MeOH 4:6; column length, 15 cm. (b) Backpressures generated by DMAA columns ($T = 15\%$, $C = 52\%$) prepared with 30, 40, 50 mg/ml ammonium sulfate for various linear flow velocities. Mobile phase, ACN:MeOH 4:6; column length, 15 cm.

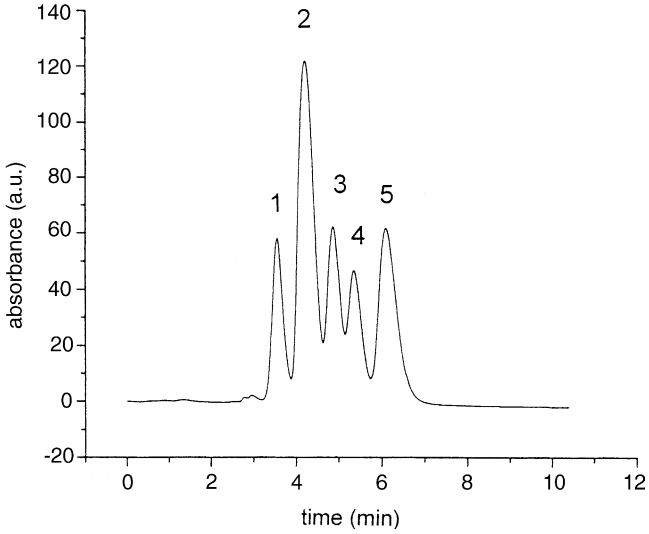

Fig. 3. Isocratic separation (pLC) of five hydroxylated aromatics on a DMAA monolith ($T = 29\%$, $C = 52\%$). Mobile phase, ACN:MeOH 6:4; flow rate, 0.3 µl/min; measured column backpressure, 23 bar. Peak identification: HQMME (1), 2-naphthol (2), catechol (3), hydroquinone (4), and resorcine (5).

were also changes in the elution order (His, Phe, Trp in CEC compared to Phe, Trp, His in nano-pLC). The subsequent investigation of the contribution of the various interaction mechanisms to the retention in CEC as a function of the mobile phase composition proved exceedingly difficult and no clear idea concerning the main interaction mechanism and possible modifications thereof could be developed.

A similar investigation of the influence of the mobile phase composition on the retention of the charged amino acids was undertaken here under nano-pLC conditions with the aim of deconvoluting the chromatographic and the electrophoretic contribution to the separation behavior in CEC. As before the two parameters that were varied in these experiments were the buffer salt concentration of the mobile phase and its organic solvent content.

The effect of an increase in the salt concentration for three organic solvent contents (15, 30, and 45%) is compiled in Fig. 4. In all cases, an increase in the salt concentration reduces the retention of the analytes. However, the elution order stayed the same, i.e. Phe, Trp, His. In CEC, His tended to overtake the other two analytes at higher salt concentration. A predominately ion-exchange type of interaction between the analytes and the (negatively charged) monolith could explain these differences. If we assume that histidin bears the highest charge density, this would explain the strong retention of this amino acid, which is observed both in CEC and in nano-pLC at low buffer salt concentration. While under nano-pLC conditions, retention becomes less strong as the salt concentration in the mobile phase increases, the amino acid histidin remains nevertheless always the most retained analyte. Under CEC conditions, on the other hand, the high charge density leads to a strong electrophoretic acceleration in the electric field, hence the tendency of His to elute in front of all other sample components in the higher salt buffers.

In the case of non-charged analytes retention on the DMAA and MAA monoliths had been ascribed to some form of hydrophilic or aromatic interaction [11,13]. That this interaction was also operative in the case of the charged amino acids is demonstrated by the fact that retention on MAA ($T = 15\%$, $C = 52\%$) columns was consistently stronger than on the corresponding DMAA columns, Fig. 4.

The effect of an increase in the organic content of the mobile phase in nano-pLC, Fig. 5a, was very similar to the effect observed under CEC conditions, Fig. 5b. The retention of histidin increased with increasing acetonitrile content, the retention of tryptophan decreased, whereas the retention of phenylalanin was largely independent of the composition of the mobile phase. In previous experiments, His had shown the strongest electrostatic interactions of the three amino acids. For a given salt concentration, remaining electrostatic interactions are enforced by an increase in the organic

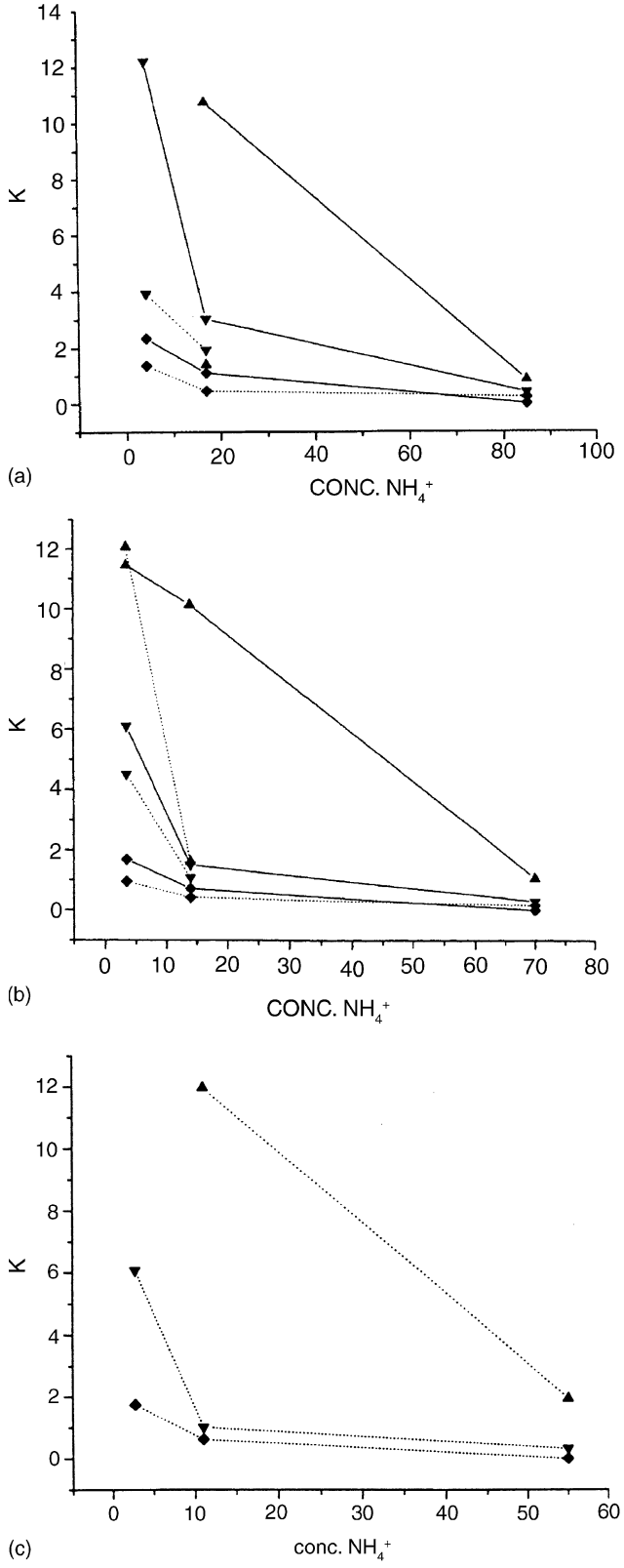

Fig. 4. Comparison of the retention factors of Phe (◆), Trp (▼), and His (▲) as a function of the ammonium acetate concentration (mM) in the mobile phase containing in addition: (a) 15% acetonitrile, (b) 30% acetonitrile, and (c) 45% acetonitrile. Stationary phases: MAA monolith (T = 15%, C = 52%) (solid lines); DMAA monolith (T = 15%, C = 52%) (dotted lines).

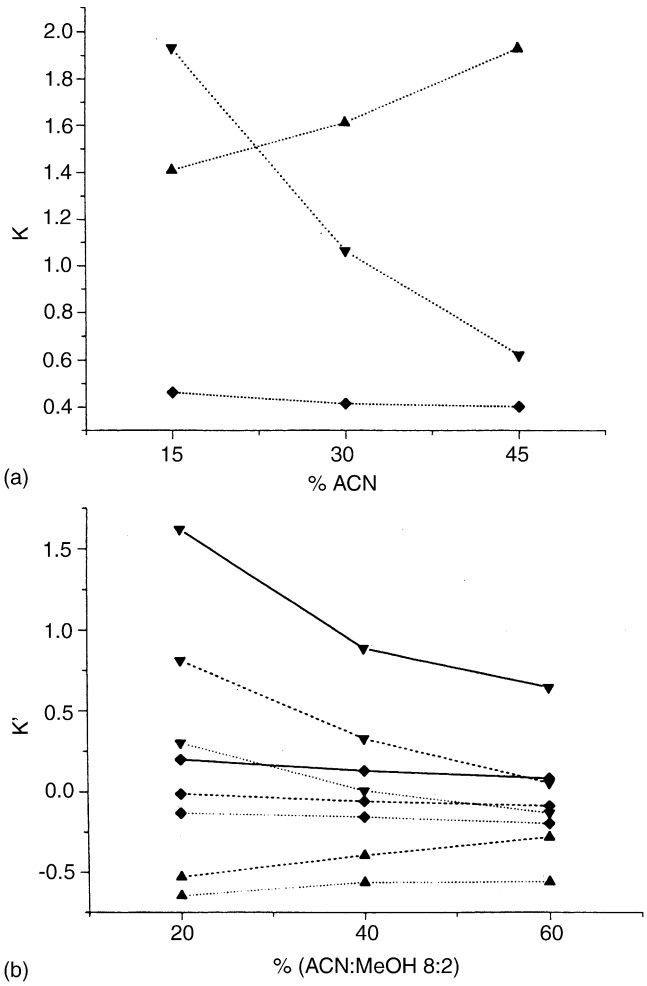

Fig. 5. (a) Comparison of the retention factors (pLC-mode) of Phe (◆), Trp (▼), and His (▲) as a function of the acetonitrile content in the mobile phase (aqueous phase 20 mM ammonium acetate/500 mM acetic acid, the overall electrolyte concentration hence decreases with increasing acetonitrile content). Stationary phase: DMAA monolith (T = 15%, C = 52%). (b) Comparison of the CEC retention factors (CEC-mode) of Phe (◆), Trp (▼), and His (▲) as a function of the organic solvent content in the mobile phase containing 4 mM (solid lines), 10 mM (dashed lines), or 20 mM (dotted lines) ammonium acetate. Stationary phase: DMAA monolith (T = 15%, C = 52%).

solvent content, hence the observed increase in retention with increasing organic solvent content in the case of histidin. If anything, the effect of the organic solvent on the retention of histidin seems to be more pronounced in nano-pLC than in CEC. Clearly under nano-pLC conditions the enforcement of the electrostatic interactions by the increasing organic solvent content cannot be compensated by electrophoretic acceleration as it is the case in CEC. Trp, on the other hand, showed the least pronounced electrostatic interaction. The fact that the retention of this amino acid decreases with increasing organic solvent content may even point to a hydrophobic (reversed phase) aspect to the interaction of this molecule with the monolith. In the case of Phe the various contributions to the retention/elution behavior are presumably able to balance each other, hence

the observed relative indifference to the composition of the mobile phase.

4. Conclusions

The comparison of the performance of monolithic stationary phases in the nano-pLC and the CEC mode enabled to some extent the deconvolution of the chromatographic and electrophoretic contributions to the CEC separations previously achieved with these stationary phases. In the case of non-charged analytes, this separation is chromatographic in nature. Judging from the elution order and the previously observed effects of a change in the mobile phase under CEC conditions, the interactions seem to be of hydrophilic. In the case of charged analytes, both electrostatic and hydrophilic interactions contribute to the retention.

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