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# Evaluation and comparison of tailor-made stationary phases based on spherical silica-based beads for capillary electrochromatography via peptide separation analysis

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**Abstract**

Small cyclic peptides have been employed to elucidate the performance of novel sorbents as stationary phases in capillary electrochromatography (CEC). In this paper chain length dependencies for ordinary liquid chromatographic sorbents are reported together with findings acquired on beads specifically designed to suit CEC. The latter, tailor-made, spherical, porous silica exhibits a distinguished surface modification to meet the criteria anticipated to enhance performance profiles in CEC. With well-characterised peptides resembling the analytes, probing of the CEC system in a systematic manner (predominantly via the organic modifier content of the background electrolyte (BE)) reveals insight into the complex interplay occurring in such analytical systems at the molecular and sub-molecular level in particular upon various modes of interaction.


*Keywords:* Stationary phases; Electrochromatography; Peptide separation analysis

## 1. Introduction

Capillary electrochromatography (CEC) is a capillary technique originally designed to feast on the benefits from two well-established separation techniques, namely high performance capillary electrophoresis (HPCE) and high performance liquid chromatography (HPLC), widely employed to characterise and separate bio-molecules such as peptides and proteins. Combining the selectivity potential of HPLC and the efficiency features of HPCE, originating from the plug-like flow profile of the latter was an intriguing promise stimulating the pioneers [1–6] of this potentially superior separation technique. The vision of accumulating the power to simultaneously separate charged, polar, and neutral, soluble compounds originating from a sample almost not limited in its complexity was created in the process. Subsequently research activity was increased and accelerated with respect to all aspects of the separation tool [7–12]. Immediate, triumphant and comprehensive success, however, remained elusive and consequently fundamental issues had to be addressed [13–22]. Obviously knowledge of fundamental concepts is important for the prediction and optimisation of analyses performed by utilisation of any separation tool. In spite of the vast amount of accessible knowledge from investigations into the parental experimental platforms CEC appears to suffer from a lack of systematic examinations and as a consequence from an insufficient arsenal of theoretical consideration peaking in the absence of a reliable migration/retention mechanism.

A considerable amount of investigation has been performed to overcome the lack of theoretical knowledge [13,14,20,23–27] but to date it has proven intangible to fill the gaps comprehensively. In particular the conduct of stationary as well as mobile phases in addition to the even more complex behaviour of solutes—especially regarding bio-molecules—under applied electric field conditions remain major fundamental uncertainties in CEC. The general trends with respect to the phenomenon of electroosmotic flow (EOF) have early been examined in much detail and for small neutral, hydrophobic molecules the likewise is true. The chromatographic behaviour of charged analytes, however, provided considerable difficulties for the scientific community. Intensive investigations into this field and respectable results soon identified charged analytes in

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particular charged bio-molecules as prime targets to demonstrate the separation capabilities of CEC. The successful demonstration of the orthogonality of the separation technique as compared to its parental experimental platforms boosted the prospects of CEC in a previously unknown way. Currently much effort is devoted to the achievement of peptide and protein separations on sorbents so manifold in their kind almost matching the resourcefulness of the analytes targeted.

The peptides selected for these investigations represent small cyclic peptides as model analytes omitting structural reorganisation upon electrochromatographic conduct to monitor the propensities of the sorbents under scrutiny. The synthetic peptides hence act as probes to investigate the response of the entire system to alterations of the background electrolyte (BE), which typically represents the preferred choice to vary separation parameters in the quest for improvements to a given separation. Despite the fact that in the present study one parameter only is subject to change, namely the acetonitrile (ACN) content in the BE, the complexity of the CEC separation tool expands nonetheless in its infamous, yet anticipated, style. Neglecting any bearing on the probes with respect to the variations introduced to the system as a whole, the response monitored by the elution profiles reveals insight into interfacial properties, which are subtle but nevertheless meaningful regarding mechanisms of migration/retention. Taking the gathered results into consideration may, however, facilitate the selection and advance the design of novel stationary phases to be applied to this extraordinary separation tool.

## 2. Experimental

The synthesis procedures for obtaining the base sorbent (MH OH) of the investigations performed on the (MH OH + MH RP-$n$ = MH X) materials are described elsewhere [28].

The procedures for slurry-packing of the capillary columns are available [29] and not repeated here. Detailing named experimental sections has therefore subsequently been omitted.

### 2.1. Chemicals and reagents

The solvents, buffer salts as well as thiourea and uracil and other reagents were provided by Merck KGaA, Darmstadt, Germany and were of gradient grade purity and p.a. quality, respectively. The organic modifier utilised in these investigations was ACN. The buffer was prepared as 100 mM stock solution from ammonium acetate and glacial acetic acid to yield pH 4.8 and stored at −20 °C in 10 ml aliquots. The BE was prepared by mixing the organic modulator and the buffer to result in the desired phase ratio. Sonication occurred in a Bandelin Sonorex TK 52, Berlin, Germany to ensure appropriate degassing of the BE prior to dosage and utilisation.

Water was deionised and purified utilising a MilliQ-system by Water Millipore, Eschborn, Germany.

### 2.2. The sample

The selected peptides desmopressin, oxytocin, and carbetocin are provided by Ferring AB, Malmö, Sweden (now Copenhagen, Denmark). These peptides are potent pharmaceutical substances. Desmopressin (1-Desamino-[D-Arg8]; dDAVP) represents a synthetic structural analogue of the antidiuretic hormone arginine vasopressin and is utilised for treatment of, e.g., nocturnal enuresis. Oxytocin is a natural occurring hormone. Its use as drug includes, e.g., induction and enhancement of labour at the time of parturition. Carbetocin epitomises an oxytocin-analogue specific for modulation of uterine contraction. The respective sequences representing the formal structures of these synthetic peptides are:

desmopressin (D)

Mpa*-Tyr-Phe-Gln-Asn-Cys*-Pro-DArg-Gly-$NH_2$

oxytocin (O)

H-Cys*-Tyr-Ile-Gln-Asn-Cys*-Pro-Leu-Gly-$NH_2$

carbetocin (C)

Bua*-Tyr(OMe)-Ile-Gln-Asn-Cys*-Pro-Leu-Gly-$NH_2$

where the cyclisation via the di-sulfide bridges are indicated by stars (*), Mpa is merkaptopropionic acid, Bua is butyric acid, and the p$K_a$ values are p$K_a$ = 11.47 for desmopressin and p$K_a$ = 8.80 for oxytocin, respectively.

The EOF velocity is monitored with uracil or thiourea, which are expected to exhibit non-interactive conduct in the CEC system under all employed experimental conditions.

### 2.3. The sorbents

The sorbent for the investigations on the chain length dependency is a material designed in the course of an EU-project (HPLC column as a reference column; EU-SMT4-CT96-2026) after a polyethoxysiloxane (PES) procedure first developed and used for synthesis in this laboratory [28]. The particle size distribution is very narrow and detailed as 3 μm average size. The base material after synthesis, sizing and post-treatment is essentially metal contamination free. While the base material is referred to as MH OH the silanised beads MH RP-$n$ are obtained by reacting the base material with $n$-alkyl dimethyl monochlorine silanes of varying chain length $n$ (with $n$ = 1–18).

The tailor-made sorbent was provided by Merck KGaA, Darmstadt, Germany. It consists of 3 μm sized porous silica-based beads. The research material is a derivative of the commercially available Purospher® STAR (with or without end-capping (e)) with the Merck internal code

Purospher® STAR RP-18(e) CEC. The end-capped specimen comprises a surface modification where the inner surface is exclusively derivatised with the non-polar ligand and additionally end-capped with the intention to minimise silanophilic activity, while the outer surface is thought to provide maximum EOF velocity. The synthesis is therefor designed to yield, in majority, isolated (cf. vicinal, geminal) silanol groups on the external surface and is consequently believed to predominantly exhibit primary silanol activity.

### 2.4. The apparatus

In these studies, a Hewlett-Packard (now Agilent Technologies) Model HP$^{3D}$CE capillary electrophoresis system, Waldbronn, Germany, modified to allow for pressurisation of the capillary ends to prevent cavity formation was utilised. Further the sample tray and capillary housing additionally holding the detection interface may be thermostated. UV-detection was performed at 220 nm. All runs were performed at ambient temperatures. The instrument is one of three prototypes customised on the bases of the original Model HP$^{3D}$CE.

### 2.5. Capillary columns

Fused silica capillaries (CS Chromatographie Service GmbH, Langerwehe, Germany) were sized to 33 cm with 100 μm inner and 360 μm outer diameter, respectively. The packed bed length was 8/25 cm held in place by frits created with a heating filament. The capillaries were manufactured (slurry-packed) utilising an in-house made packing device (workshop, Johannes Gutenberg-University, Mainz, Germany) and consecutively pre-conditioned with ACN and the desired BE prior to installation in the HP$^{3D}$CD capillary electrophoresis system. Further conditioning was performed according to Agilent recommendations for commercially available capillary columns before the system was considered operative.

### 2.6. Instrumental methods (operational modes)

Since a general tendency may be observed towards faster separations the short end approach was favoured, where packed beds of 8 cm loom in 33 cm capillaries, as compared to the commercially available columns comprising packed beds of 25 cm. The polarity of the applied voltage, which may be up to 30 kV, must be switched to yield 'negative' voltage, where the sign is only indicative of the reversed direction of EOF. Injection of the analytes was electrokinetic. In general the injection voltage is chosen to be lower than the operation voltage and this pattern was followed by injecting −10 kV for 1–3 s, hence the analytes enter the capillary via electroosmosis. Detection was performed with the instrument's diode array detector (DAD) allowing several wavelengths to be recorded. Detection was on-column through the detection window obtained by removal of the polyimid coating protecting the column from mechanical damage in immediate proximity to the outlet frit.

## 3. Results and discussion

Since the sorbents are of pre-eminent importance in acquiring the presented data-sets and their interpretation further to the respective solitary descriptions in the experimental section a few characterisation profiles are presented below ahead of the respective sections related to the two sorbent classes.

Table 1 surveys the calculated data for the pore structure and the carbon content from nitrogen sorption (isotherms) and elemental analysis, respectively. General inclinations observed for similar systematic synthesis procedures are decreasing specific surface area in tandem with increasing carbon content with increasing *n*-alkyl chain length. The highlighted results are indicative of deteriorations from the expected trends and assigned to synthesis related problems such as reactivity of the *n*-alkyl dimethyl monochlororine silanes temperature control, tetrahydrofurane (THF) treatment, etc.

Table 1
Survey of the values calculated for the pore structure from nitrogen sorption (isotherms) and the carbon content from elemental analysis with respect to the MH X sorbents

| Modification | Specific surface (BET), $a_S$ ($m^2$/g) | Specific porevolume (G), $v_p$ (ml/g) | Mean pore diameter (BJH), $p_d$ (nm) | Carbon content |
|---|---|---|---|---|
| Rehydroxylated | 302.8 | 1.06 | 13.3 | – |
| $C_1$ | 252.5 | 0.91 | 13.2 | 3.4 |
| $C_2$ | 249.8 | 0.88 | 11.6 | 4.8 |
| $C_3$ | 259.2 | 0.91 | 13.1 | 4.6 |
| $C_4$ | 276.0 | 0.93 | 11.5 | 4.0 |
| $C_5$ | 236.2 | 0.83 | 11.6 | 7.3 |
| $C_6$ | 230.3 | 0.81 | 11.5 | 8.3 |
| $C_8$ | 224.2 | 0.77 | 11.4 | 10.0 |
| $C_{10}$ | 234.7 | 0.80 | 11.4 | 9.2 |
| $C_{18}$ | 178.0 | 0.59 | 10.2 | 17.2 |

The highlighted rows display the sorbents which fail to fit expectable trends from a synthesis point of view. A similar pattern may be observed in Fig. 1A.

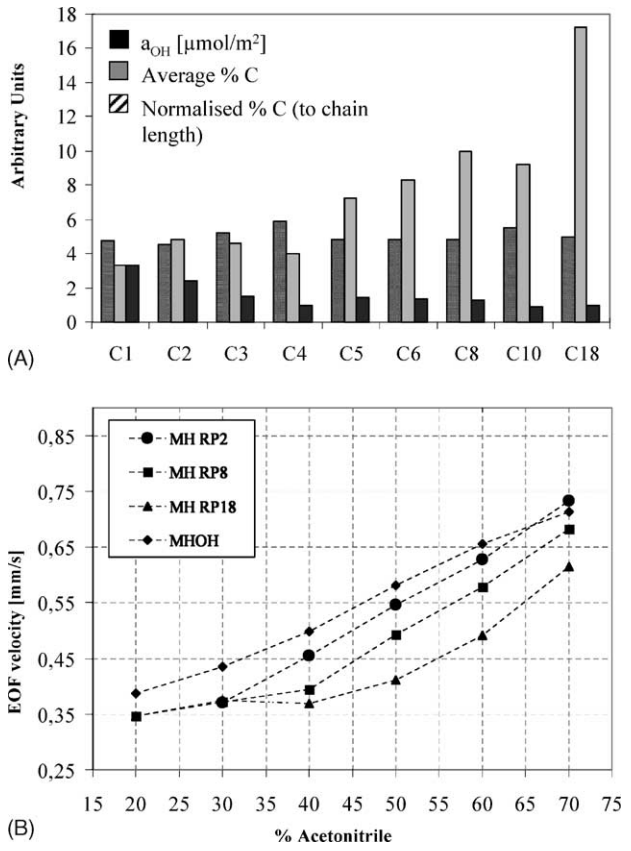


Fig. 1. (A) Silanol group density (the silanol group density $\alpha_{OH}$ was determined via the method developed by Molik and Matêjková, J. Chromatogr. 213 (1981) 33 and subsequently modified by du Fresne v. Hohenesche, Dissertation, Johannes Gutenberg-University 2002), average carbon content, and carbon content normalised to chain length for the rehydroxylated sorbent MH OH and the modified sorbents MH RP-*n* (MH X). (B) EOF velocity vs. ACN content in the background electrolyte featuring the columns modified with the various *n*-alkyl chains as indicated and the rehydroxylated sorbent (MH X). Operation conditions: capillaries 8.0 cm (33) × 100 μm; background electrolyte: $x/(100-x)$ (v/v) ACN/$NH_4Ac$, 7.5 mM, pH 4.8 injection: −10 kV 3 s; voltage: −15 kV; temperature: 20 °C; detection: 214 nm; EOF marker: uracil.

Fig. 1 exemplifies in panels A and B some important features endorsing the understanding of the electrochromatographic demeanour of the home-made sorbents. Panel A reduces to summarise and visualise the propensities of Table 1 primarily influential to electrochromatograpic properties of sorbents in general. Panel B displays the EOF velocity versus alterated ACN content for selected sorbents as indicated.

The results presented below resemble a selection of the electropherograms (raw data) where they appear to be essential to the interpretation together with plots to detail general trends where applicable.

Fig. 2 shows a variety of electropherograms, which are acquired with altering ACN contents to the BEs on columns differently modified with respect to the chain length of the bonded moiety. Fig. 2A displays the analysis of the three peptides on the column packed with beads modified with *n*-octadecyl dimethyl monochlorine silane. Between 40 and 70% of ACN content in the BE baseline separations can be achieved, while at 30% carbetocin fails to elute within the selected time frame and desmopressin and oxtocin co-elute. The non-charged carbetocin which is also associated with the largest hydrophobicity coefficient [30] among the three elutes with the velocity of the EOF at high ACN contents in the BE indicating that hydrophobic interactions under these conditions are sufficiently masked to cause no net-retention for this late eluting probe. The charged analytes desmopressin and oxytocin are propelled well separated to the detection site ahead of the EOF by their respective electromigration velocity vectors, which is in the same direction as the respective vector for the EOF under the applied electric field conditions. While the carbetocin follows an elution pattern generally matching neutral hydrophobic analytes in CEC in the course of varying the ACN content the loss of the separation for desmopressin and oxytocin may be assigned to desmopressin 'feeling' the decreased ACN content and hence the increasingly pronounced presence of the bonded hydrophobic moiety. Oxytocin on the other hand is hardly sensitive to the rather drastic changes. At first glance the generalisation that one migration/retention mechanism may be promoted to govern over the others occurring in a CEC system is traumatised. By comparing the profiles obtained on the remaining sorbents supplying RP character the trend to be observed is phenomenally similar providing sound support for theoretical predictions [25]. The situation is much the same for the separations acquired on the reversed phase sorbents whereby reduced hydrophobicity of the bonded moiety may be gauged from the elution demeanour of cabetocin and desmopressin. Fig. 2B presents the performance of MH OH (indicating the rehydroxylated normal phase base sorbent) as separation device under the same experimental conditions. The performance is indeed decreased, 60% ACN content BE conditions resembling the sole conditions for a moderately decent separation. The general trend, however, may still be realised, indicating that some interactions proceeding in CEC systems may have been underestimated while others experience consideration beyond physical reason. Fig. 3 reveals the influence of the ACN content in the BE and the length of the *n*-alkyl chain of the sorbent on the retention coefficient $\kappa_{CEC}$. The $\kappa_{CEC}$ values derived for analytes in the CEC operational mode must not be confused with the retention coefficient as known from liquid chromatography. EOF marking experiments in HPCE and open tubular CEC may also be considered substantially different from the measurements determining the EOF in packed capillaries due to the open segments when UV detection is employed and to the entirely different phase ratios per se. With these precautionary notes one can turn to the analysis of the $\kappa_{CEC}$ versus % ACN plots. In panel A the desmopressin dependency is monitored. The positively charged analyte elutes ahead of the marker under all conditions investigated hence the negative values for $\kappa_{CEC}$. The response of $\kappa_{CEC}$ to increasing ACN content in the BE is more complex than expected. The data are generally in

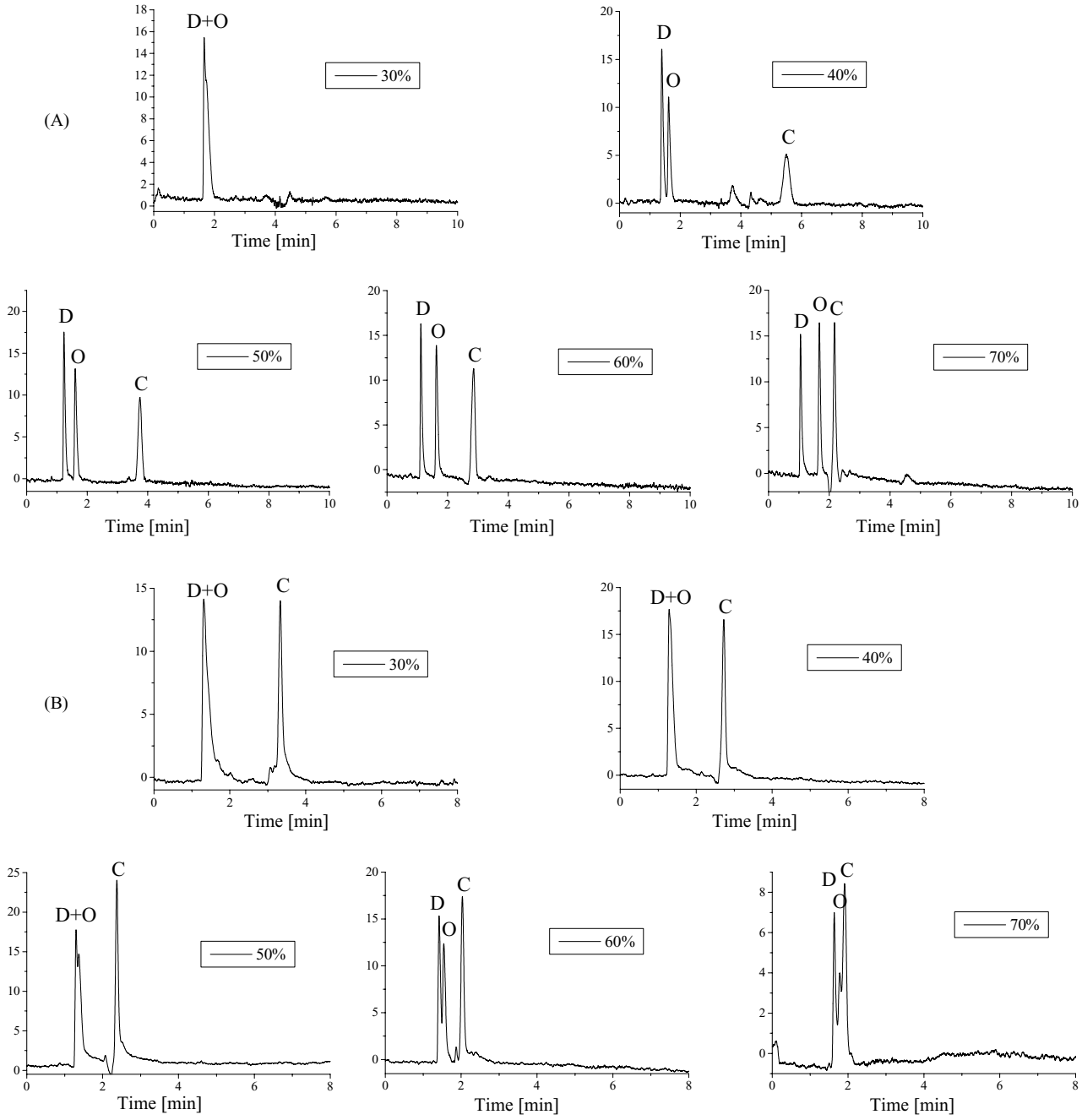


Fig. 2. (A) Elution profiles for desmopressin (D), oxytocin (O), and carbetocin (C) on a column packed with MH RP-18 at varying ACN contents in the background electrolyte. Operation conditions: capillary 8.0 cm (33) × 100 μm; background electrolyte: $x/(100-x)$ (v/v) ACN/$NH_4Ac$, 7.5 mM, pH 4.8 injection: −10 kV 3 s; voltage: −15 kV; temperature ambient; detection: 220 nm (intensities are in mAU); sample: desmopressin (D), oxytocin (O), and carbetocin (C). (B) Elution profiles for desmopressin (D), oxytocin (O), and carbetocin (C) on a column packed with MH OH at varying ACN contents in the background electrolyte, Operation conditions: same as in (A).

good agreement with the observation that increasing the ACN content decreases the $\kappa_{CEC}$ value but obviously there are exceptions. It is noteworthy that the sorbents comprising *n*-alkyl chains > $n = 5$ display very similar coefficients with a maximum at 40%. The sorbents modified with the C-1 to C-4 bonded phases generally display lesser values for $\kappa_{CEC}$. The slope for the same dependency on the rehydroxylated sorbent, however, is more pronounced and in particular for the high ACN contents very small $\kappa_{CEC}$ values are obtained. Desmopressin with its highly pronounced basicity experiences the highest charge under the employed experimental conditions. Under the influence of an electric field it will migrate and hence may only be moderately susceptible to hydrophobic interaction with the various bonded moieties presented. Only by incorporating the EOF into the consideration of the migrational conduct of desmopressin it

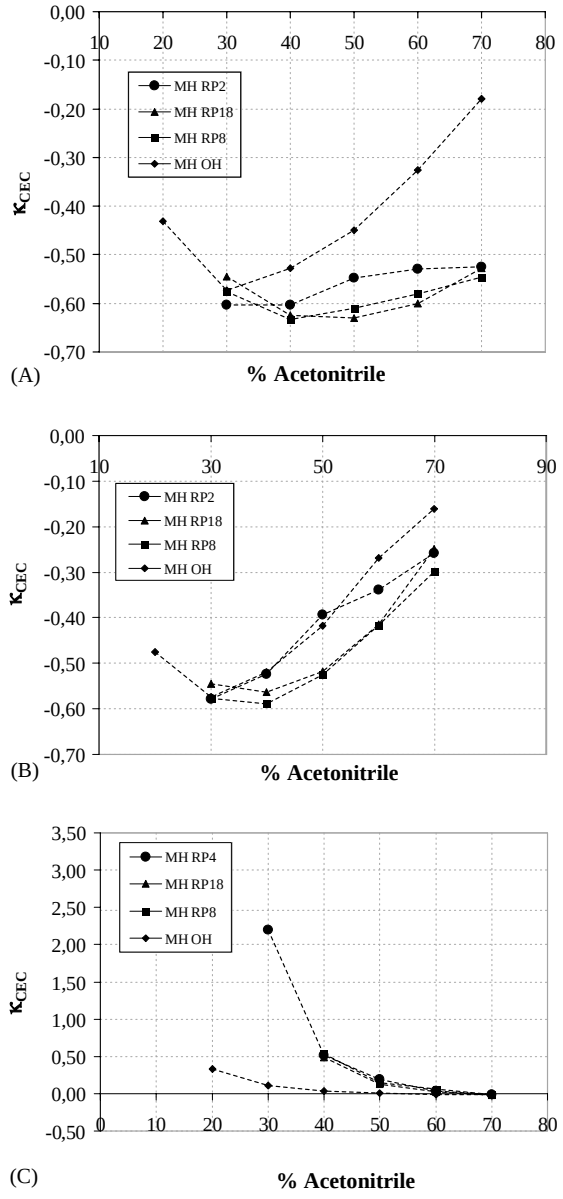


Fig. 3. Dependency of $\kappa_{CEC}$ vs. ACN content in the background electrolyte on the modified sorbents MH OH, MH RP-2 (4), MH RP-8, and MH RP-18 in Panel A, B and C, respectively, Dependency of $\kappa_{CEC}$ vs. ACN content for desmopressin (D). Operation conditions: capillary 8.0 cm (33) × 100 μm; background electrolyte: $x$/(100 − $x$) (v/v) ACN/$NH_4Ac$, 7.5 mM, pH 4.8 injection: −10 kV 3 s; voltage: −15 kV; temperature ambient; detection: 220 nm; sample: desmopressin (D), oxytocin (O), and carbetocin (C). Dependency of $\kappa_{CEC}$ vs. ACN content for oxytocin (O). Operation conditions: same as in (A). (C) Dependency of $\kappa_{CEC}$ vs. ACN content for carbetocin (C). Operation conditions: same as in (A).

becomes apparent that its $\kappa_{CEC}$ value is modestly depending on both the bonded moiety and the ACN content in the BE to moderate the interaction between the ligand and the analyte. Panel B shows the dependencies for oxytocin and it is apparent that the slope increases sharply with increasing ACN percentage in the BE indicative of lower $\kappa_{CEC}$ values. It is therefor conclusive to assume that its transport features are influenced by hydrophobic interactions upon migration under electric field conditions. The individual curves for the sorbent under investigation are hence neighboured by altering sorbents when comparing the $\kappa_{CEC}$ values for the different analytes. This fact alone is symptomatic of a complex array of interactions prevailing in the CEC system under scrutiny despite the detail that of the tunable BE parameters only the ACN content is varied, which subsequently is believed to single-handedly govern the hydrophobic interaction analysed for the alteration of the hydrophobic ligand. Panel C finally displays the behaviour of carbetocin the neutral analyte of the sample. Apparently the ACN content must be very high to cause elution with the bulk flow. As the ACN content is decreased, however, the well-established curvature for neutral hydrophobic analytes emerges with grace. Surprisingly the rehydroxylated base material displays a similar slope for carbetocin albeit much less pronounced but the riddle to be solved is challenging enough as it stands. The $\kappa_{CEC}$ value for carbetocin exhibits a minor dependency on the *n*-alkylchain length only, since decreasing EOF velocity parallels increasing retention. These findings are in excellent agreement with other studies [31,32] signifying that column hydrophobicity and hydrophobic selectivity may vary significantly when operated under pressure driven and electroosmotic driven flow conditions. The obtained results moreover foster judgement towards substantially different contributions from molecular descriptors to retention in regard of hydrophobicity. The concept of hydrophobicity and the phenomenon of hydrophobic interaction certainly remain the same, but how they are to be associated with chromatographic retention in CEC may have to be reconsidered. Since it is redundant to discuss hydrophobicity and hydrophobic selectivity in the absence of RP-temperament, as is the case with the MH OH material the reason behind the (decreased) rate of migration/retention must be sought elsewhere. Many phenomena have been associated with contributions to mechanisms occurring under CEC conditions by various authors [13,14,33–40]. The only contributor that can be ruled out to be responsible for the observed elution of carbetocin with an increased positive $\kappa_{CEC}$ value so far is the RP-ligand. Therefore the assumption of the BE in co-operation with the total particle surface area individually being key contributors to the migration/retention characteristics of sorbents in general appears to be justified. The focus is on the general trends covering the whole range of hydrophobicity exhibited by the bonded moieties since the problems arising from the experimental error of the chemical modification has been discussed above. The electro-migration/retention conduct of carbetocin at 60% ACN content in the BE shows to be rather independent of the chain length of the bonded ligand except where there is no ligand to be found (on the rehydroxylated base-material) its elution is with the EOF. Desmopressin and oxytocin elute with increasing coefficients the sign only indicating elution ahead of the EOF marker. This result is in good agreement with the observed higher EOF velocity for the sorbents modified with the longer chain silanes. If the difference can

be related to an alteration of the ζ-potential the same mechanism accelerating the EOF should account for the faster transport of these analytes. Starting from the modification with $n > 6$ this trend fades, however, to give rise to relative independence of the $\kappa_{CEC}$ value from the chain length. It becomes apparent that the migration/retention of the carbetocin becomes increasingly dependent on the *n*-alkyl chain length nourishing the notion of hydrophobic interaction to occur. Starting from $n = 8$ under 30% ACN conditions carbetocin does not elute anymore within any provided time frame.

Migration of the charged peptides is less dramatic with respect to the slope of the dependency under scrutiny, but what is spectacular is the apparent 'loss' of molecular diversity and the wide range over which it is exhibited. Variation of the electrostatic interaction simultaneously is the key to 'reinstating' their molecular diversity and to exploring the nature of the interface when (these synthetic) peptides approach the surface [41].

Since the Purospher® STAR based material is available as research batches only the characterisation comprises solely the most significant discriminative property in comparison to the commercially available sorbent namely the silanol group density as can be grasped from Fig. 4A and the results from EOF velocity determinations as the corresponding

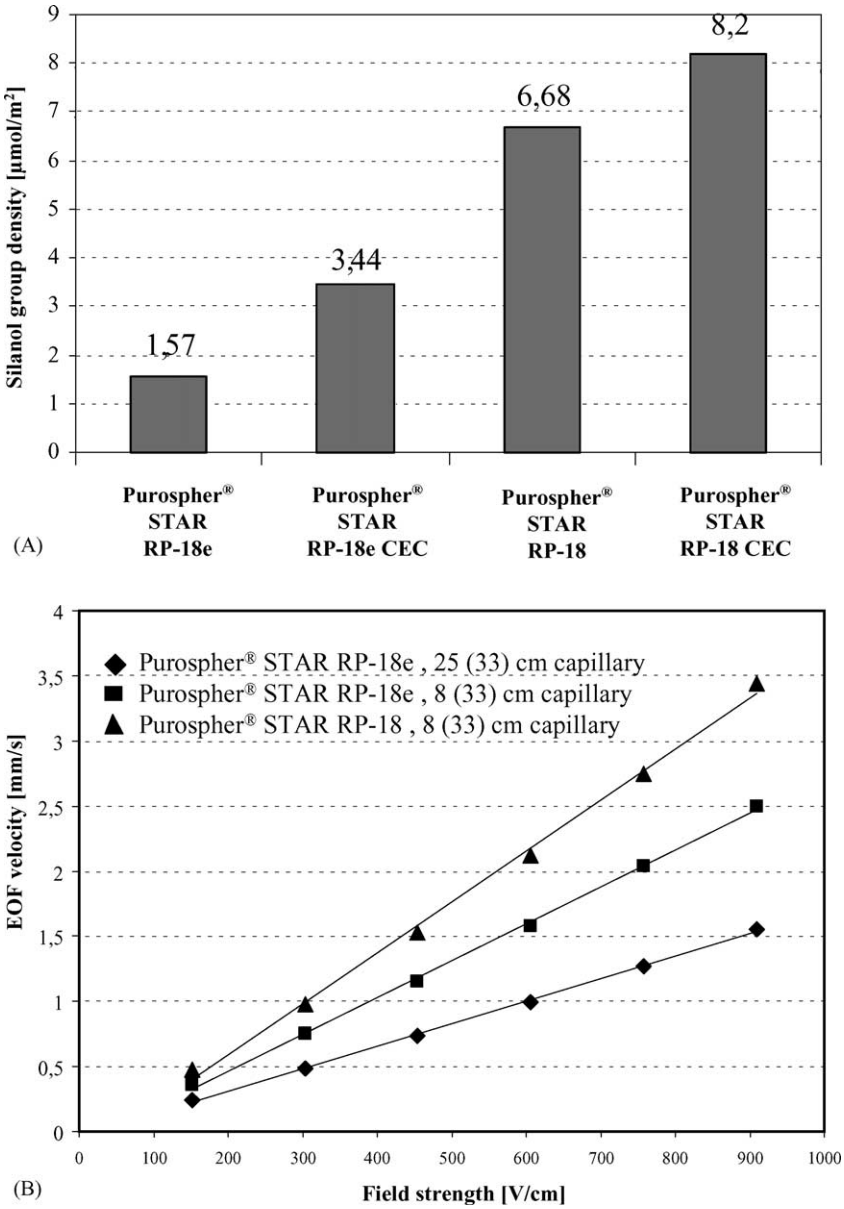


Fig. 4. (A) Silanol group density (the silanol group density $\alpha_{OH}$ was determined via the method developed by Molik and Matêjková, J. Chromatogr. 213 (1981) 33 and subsequently modified by du Fresne v. Hohenesche, Dissertation, Johannes Gutenberg-University 2002) for the various Purospher® STAR sorbents. (B) EOF velocity vs. electric field strength with the alterations of the sorbent as indicated. Operation conditions: capillary 8 (33) and 25 (33) cm, respectively; 100 μm i.d.; background electrolyte: 60/40 (v/v) ACN/$NH_4Ac$, 5 mM, pH 4.8 injection: −10 kV 1 s; temperature ambient; detection: 214 nm; EOF marker: uracil.

chromatographic characterisation in panel B. The obtained results are in excellent agreement with similar chromatographic characterisations [24] and trounce the expectations prior to the electrochromatographic application in view of the generated total EOF in particular with respect to the end-capped sorbent.

Further to the evidently successful display of the effect of end-capping in Fig. 4 the elution profiles of a standard test mixture are consulted to comprehensively demonstrate the benefits of such treatments for separations in CEC. The standard features thiourea (1) as EOF marker, atenolol (2) as cation, the analytes digitoxygenin (3), and diphenylsulfone (4) for the purpose of scrutinising silanophilic activity, and phenazine (5) to monitor the hydrophobicity of selected CEC sorbents. Fig. 5A shows the electropherogram acquired on the stationary phase Purospher® STAR RP-18 CEC, while Fig. 5B shows the trace for the same sample on the end-capped sorbent Purospher® STAR RP-18e CEC. The immediate impression of the end-capping becomes apparent by observing the enhanced resolution of 3 and 4.

With the selected peptides resembling analytes which are not expected to be highly sensitive to their respective environments as far as structural flexibility is concerned probing of the CEC system is believed to predominantly exhibit the characteristics of the forces provided by the interacting species, i.e., co- and counter ions of the BE, the bonded ligands and/or the provided surfaces. The peptides comprise of nine AAs with the cyclisation in place at sequence positions 1 and 6 increasing the rigidity of the peptide backbone for the residues featuring in the cycle. Peptides exhibiting short sequences are highly likely to exist in random coil structures and to display globular shape, hence in case of cyclic peptides the likelihood of existing in the described form is even more pronounced. Therefor the CEC system's response to altering the phase ratio of the BE may qualitatively be assigned to the inner and outer surface and the bulk solution in the separation capillary. The novel stationary phase may in return offer some insight into the migration/retention properties of the cyclic peptides. Fig. 6 displays the separation on the Purospher® STAR RP-18e CEC column. The elution profiles of these separations are particularly valuable when compared with the ones obtained on the MH RP-18 sorbent (cf. Fig. 2A). All profiles require considerably shorter recording times as expected with increased EOF velocities and decreased carbon content. At high ACN content the non-charged, hydrophobic peptide carbetocin elutes simultaneously with the EOF and at low ACN content in the BE the resolution for the charged eluites desmopressin and oxytocin degrades. Transformed into retention coefficient $\kappa_{CEC}$ versus ACN plots (Fig. 7) the plain trends complement those found for the MH X sorbents (cf. Fig. 3) screening a similar range.

### 3.1. Evaluation

The acquired results cultivate several evaluations.

The theoretical predictions [25] that massive hydrophobicity with respect to the bonded moiety is not essential to successful separations involving neutral/hydrophobic analytes is confirmed experimentally. The acquisition of model peptide separations on the native, rehydroxylated sorbent under the employed conditions indicates that separation features are operative which are not reflected in current models on migration and retention (Table 1, Figs. 1–3).

The importance of the surface charge of the sorbent has been shown to be overemphasised, since obviously masking the inner (!) surface by end-capping is not detrimental to achieving resolution. On the other hand, by comparing Figs. 2A and 6 and perhaps more transparent by consulting Fig. 7 the electrochromatographic conduct of the synthetic peptides exhibits peculiar similarities. However, by scrutinising Fig. 5 where the sole transformation (from panel A

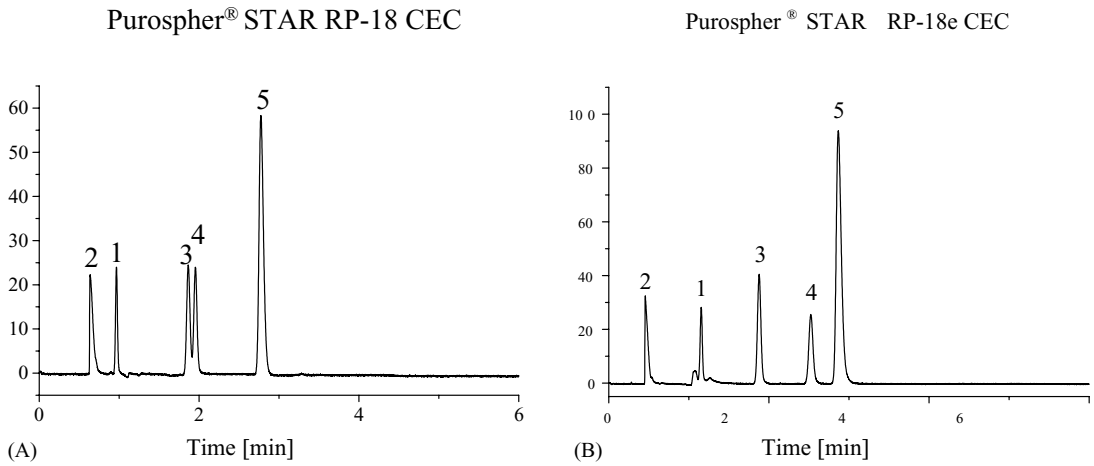


Fig. 5. Elution profiles of a test mixture utilised as standard in this laboratory. Standard run on (A) Purospher® STAR RP-18 CEC and (B) Purospher® STAR RP-18e CEC, respectively. The corollary of the end-capping is effectively monitored in the right hand panel. Operation conditions: capillary: 8 (33) cm × 100 μm; background electrolyte: 60/40 (v/v) ACN/$NH_4Ac$ 5 mM, pH 4.8; injection: −10 kV 1 s; voltage: −15 kV; temperature: ambient; detection: 220 nm (intensities are in mAU); sample: (1) thiourea, (2) atenolol, (3) digitoxigenin, (4) diphenylsulfone, (5) phenazine.

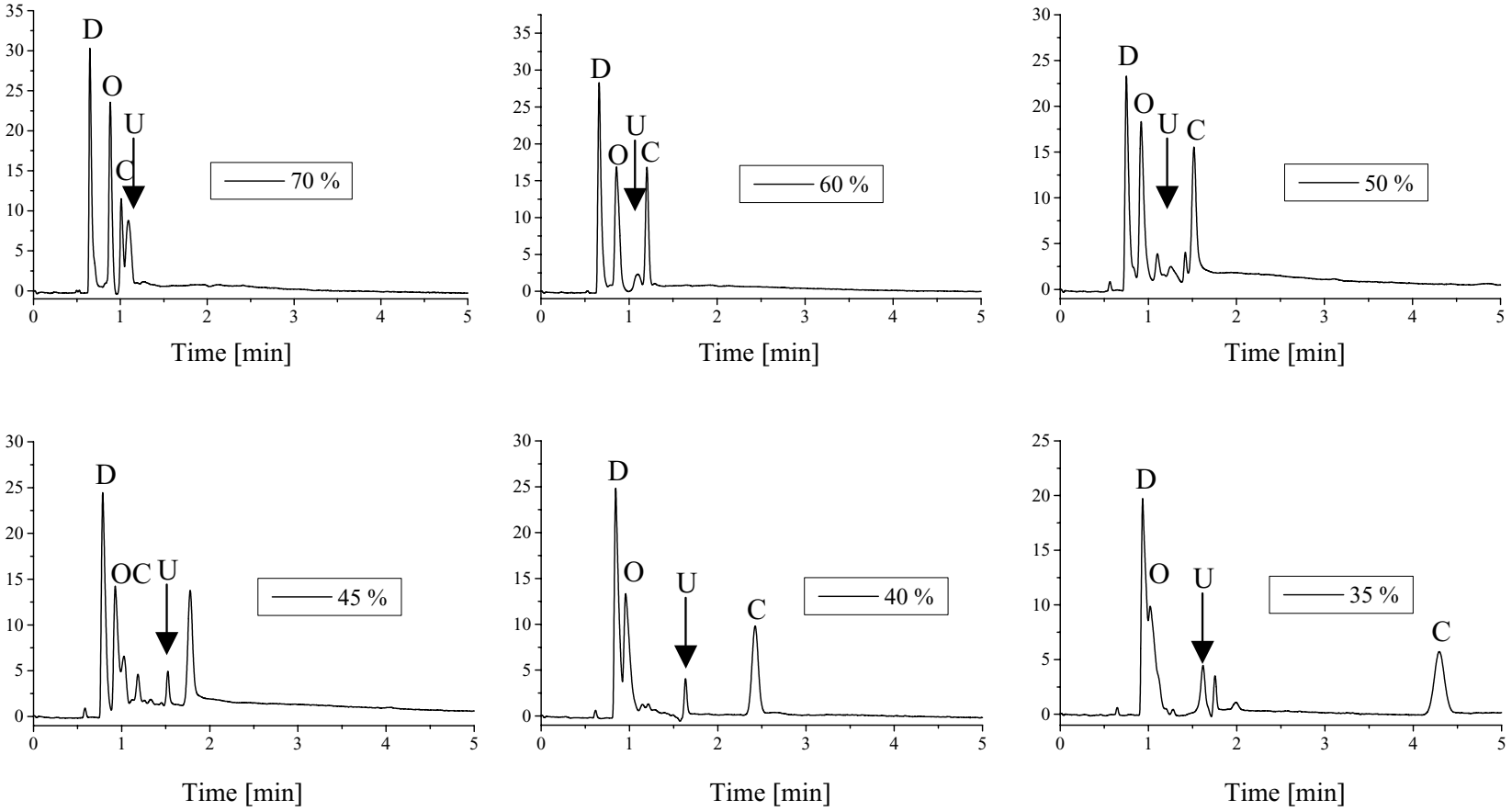


Fig. 6. Elution profile for desmopressin (D), oxytocin (O), and carbetocin (C) separated on a Purospher® STAR RP-18e CEC column. Operation conditions: capillary: 8 (33) cm × 100 μm; background electrolyte: 60/40 (v/v) ACN/$NH_4Ac$ 5 mM, pH 4.8; injection: −10 kV 3 s; voltage: −15 kV; temperature: ambient; detection: 220 nm (intensities are in mAU); sample: desmopressin (D), oxytocin (O), and carbetocin (C).

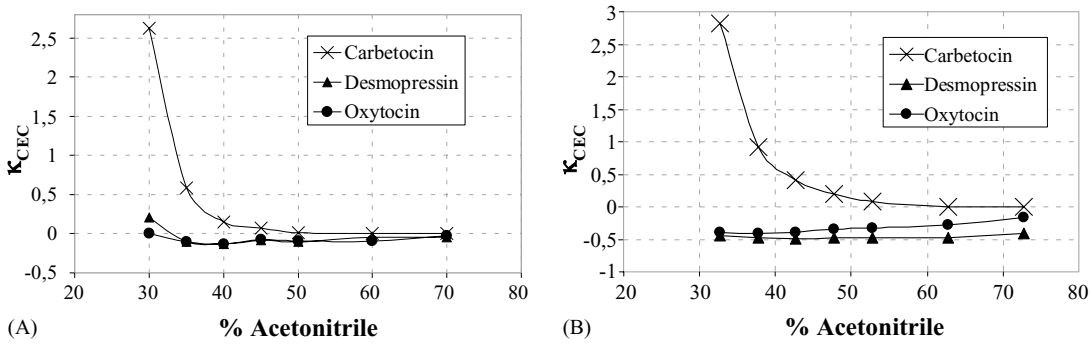


Fig. 7. $\kappa_{CEC}$ vs. ACN content in the background electrolyte featuring the columns (A) Purospher® STAR RP-18 CEC and (B) Purospher® STAR RP-18e CEC, respectively. Operation conditions: capillaries 8 (33) cm × 100 μm; background electrolyte: $x/(100-x)$ (v/v) ACN/$NH_4Ac$, 7.5 mM, pH 4.8 injection: −10 kV 3 s; voltage: −15 kV; temperature: ambient; detection: 220 nm; sample: desmopressin (D), oxytocin (O), and carbetocin (C).

to B) is represented by the end-capping, the state-of-affairs may be elucidated. Monitoring the effect of the end-capping by accumulating the improvement of the resolution for digitoxigenin and diphenylsufone, and decreased EOF as gauged by thiourea is insufficient. While the later detection of phenazine could be assigned to interaction with the end-capping species as well as the decrease of the EOF, the earlier (!) elution of atenolol exposes a frailty along general lines of mechanistic doctrines in CEC. This acceleration of the cationic species may be attributed to decreased electrostatic interaction with the masked deprotonated silanols, which may cause the net retention observed for this analyte in Fig. 5A. Conclusive electrochromatographic behaviour of the synthetic peptides desmopressin and oxytocin at very low and very high ACN contents in the BE emerges along the same line of evidence as well as the loss of molecular diversity, which may then be overcome by variation of the prevailing electrostatic interactions and the ζ-potential of the sorbent via the BE.

Finally, it materialises to be mandatory to adjust the synthesis and derivatisation of the sorbents to the separation of the target analytes—in this case peptides.

## 4. Conclusions

The EOF velocity on the MH based materials is less differing than previously expected. As a consequence the reversed phase bonded moiety is considered to play a subordinate role only with respect to the EOF. This consideration may also hold true in view of the separation of charged and neutral compounds although judgement on migration/retention processes, and hence selectivity features, via EOF determination alone is not justified. Apparently RP modification is not a mandatory feature since the rehydroxylated MH OH has proven to be selective under conditions regularly applied to RP (electro)chromatography. In summary the native, rehydroxylated silica displays minor differences for charged analytes whereas neutral (hydrophobic) substances experience significantly decreased retention as would be expected. Comparison of the modified silicas, however, demonstrates altered migration/retention times while the $\kappa_{CEC}$ values taking the EOF velocity into account fail to display a similar trend.

All materials (incl. the Purospher® STAR sorbents) display increasing EOF velocities with rising ACN contents in the BE. The difference is, however, less pronounced at low ACN contents, while starting from 50% ACN the difference with respect to the generated EOF becomes increasingly prominent. With the high organic modifier contents the *n*-alkyl chains are superiorly solvated, which may contribute to the variations found with the differently modified sorbents. A systematic dependency of the EOF velocity on the *n*-alkyl chain length was not observed but the trend that the sorbents derived after modification with the shorter silanes generate larger EOF is evident. Sterical hindrance in general and spatial occupancy of the *n*-alkyl chain close to the surface generating the ζ-potential and the same chain stretching out in the bulk solution may be considered to have an impact but this is unwarranted until the nature of the interactions provided by the driving force—the electric field—is better understood.

It proves to be conclusive that in CEC utilisation of modified sorbents, i.e. reversed phase sorbents, is desirable but not necessary. When occupied with complex samples, however, care should be taken to select a sorbent, which provides sufficient selectivity for the model peptides to guarantee a wide range elution window for the eluites and hence modified beads may attest beneficial with respect to the detailed requirements.

## References

[1] V. Pretorius, B.J. Hopkins, J.D. Schieke, J. Chromatogr. 99 (1974) 23.
[2] J.W. Jorgenson, K.D. Lukacs, J. Chromatogr. 218 (1981) 209.
[3] J.H. Knox, I.H. Grant, Chromatographia 24 (1987) 135.
[4] J.H. Knox, I.H. Grant, Chromatographia 26 (1988) 329.
[5] J.H. Knox, I.H. Grant, Chromatographia 32 (1990) 317.
[6] T. Tsuda, LC-GC Eur. 5 (1992) 26.

[7] F. Svec, E.C. Peters, D. Sykora, J.M.J. Frechet, J. Chromatogr. A 887 (2000) 3.
[8] J.J. Pesek, M.T. Matyska, J. Chromatogr. A 887 (2000) 31.
[9] L.A. Colon, T.D. Maloney, A.M. Fermier, J. Chromatogr. A 887 (2000) 43.
[10] G. Choudhary, A. Apffel, H. Yin, W. Hancock, J. Chromatogr. A 887 (2000) 85.
[11] C.A. Rimmer, S.M. Piraino, J.G. Dorsey, J. Chromatogr. A 887 (2000) 115.
[12] M. Pursch, L.C. Sander, J. Chromatogr. A 887 (2000) 313.
[13] A.S. Rathore, Cs. Horváth, J. Chromatogr. A 743 (1996) 231.
[14] K. Walhagen, K.K. Unger, A.M. Olsson, M.T.W. Hearn, J. Chromatogr. A 853 (1999) 263.
[15] H. Yamamoto, J. Baumann, F. Erni, J. Chromatogr. 539 (1992) 313.
[16] M.M. Dittmann, K. Wienand, F. Bek, G.P. Rozing, LC-GC 13 (1995) 800.
[17] R.J. Boughtflower, T. Underwood, C.J. Paterson, Chromatographia 40 (1995) 329.
[18] C. Yan, R. Dadoo, H. Zhao, R.N. Zare, D.J. Rakestraw, Anal. Chem. 67 (1995) 2026.
[19] M.T. Dulay, C. Yan, D.J. Rakestraw, R.N. Zare, J. Chromatogr. A 725 (1996) 361.
[20] M.M. Dittmann, G.P. Rozing, J. Chromatogr. A 744 (1996) 63.
[21] N. Smith, M.B. Evans, J. Chromatogr. A 832 (1999) 41.
[22] M.M. Dittmann, G.P. Rozing, J. Microcol. Sep. 5 (1997) 399.
[23] M. Dittmann, G. Rozing, F. Bek, Int. Lab. 10 (1996) 26.
[24] H.S. Deary, V. Spikmans, N.W. Smith, F. Moffatt, S.A.C. Wren, K.P. Evans, J. Chromatogr. A 929 (2001) 123.
[25] B.A. Grimes, A.I. Liapis, J. Colloid Interf. Sci. 234 (2001) 223.
[26] B.A. Grimes, A.I. Liapis, J. Chromatogr. A 919 (2001) 157.
[27] A.I. Liapis, B.A. Grimes, K. Lacki, I. Neretnieks, J. Chromatogr. A 921 (2001) 135.
[28] V. Ehwald, Dissertation, Johannes Gutenberg-University Mainz, Germany, 1999.
[29] M. Huber, Dissertation, Johannes Gutenberg-University, Mainz, Germany, 2002.
[30] M.C.J. Wilce, M.I. Aguilar, M.T.W. Hearn, Anal. Chem. 67 (1995) 1210.
[31] J. Jiskra, C.A. Cramers, M. Byelik, H.A. Claessens, J. Chromatogr. A 862 (1999) 121.
[32] J. Jiskra, T. Jiang, H.A. Claessens, C.A. Cramers, J. Microcol. Sep. 12 (2000) 530.
[33] M.M. Robson, M.G. Cikalo, P. Myers, M.R. Euerby, K. D Bartle, J. Microcol. Sep. 9 (1997) 357.
[34] M.R. Euerby, C.M. Johnson, S.F. Smyth, N. Gillott, D.A. Barrett, P. N Shaw, J. Microcol. Sep. 11 (1999) 305.
[35] K. Schmeer, B. Behnke, E. Bayer, Anal. Chem. 67 (1995) 3656.
[36] K. Walhagen, Dissertation, University Leiden, Leiden, Netherlands, 2002.
[37] M.M. Dittman, K. Masuch, G.P. Rozing, J. Chromatogr. A 887 (2000) 209.
[38] K.K. Unger, M. Huber, K. Walhagen, T.P. Hennessy, M.T.W. Hearn, Anal. Chem. 74 (2002) 200 A.
[39] X. Huang, J. Zhang, Cs. Horváth, J. Chromatogr. A 858 (1999) 91.
[40] C. Yu, F. Svec, J.M.J. Fréchet, Electrophoresis 21 (2000) 120.
[41] T.P. Hennessy, R.I. Boysen, K.K. Unger, M.T.W. Hearn, in preparation.