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Capillary electrochromatographic study of the interactions of porphyrin derivatives with amino acids and oligopeptides

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Abstract

Open-tubular capillary electrochromatography (OT-CEC) was used to study the interactions of synthetic (metallo)porphyrin derivatives (immobilized by physical adsorption to the fused-silica capillary wall) with three aromatic amino acids (phenylalanine, tyrosine, tryptophan), three aliphatic amino acids (β-alanine, proline, valine) and two oligopeptides (diglycine, triglycine). The effective mobilities of amino acids and peptides measured in OT-CEC mode in the acid and alkaline background electrolytes (BGEs) were compared with those obtained by capillary zone electrophoresis (CZE) in the bare fused-silica capillary in the same BGEs. In this way the influence of the peripheral substituents and the character of the central metal atom in porphyrin derivatives on the interactions with amino acids and peptides in the acid and alkaline media was investigated. Three types of noncovalent interactions, axial ligation to the central metal atom, π–π stacking and electrostatic repulsion seem to take part in the interactions of analyzed amino acids and peptides with porphyrin derivatives, resulting in a better separation of these analytes by OT-CEC than by CZE.

Keywords: Capillary electrochromatography, open-tubular; Noncovalent interactions; Capillary modification; Porphyrins; Amino acids; Peptides

1. Introduction

Capillary electrochromatography (CEC), a hybrid method utilizing both electrokinetic and chromatographic separation principles, is recently one of the most intensively developing capillary separation techniques [1–3]. The focus of recent CEC applications [4,5] has concentrated on the use of packed, monolithic and coated capillaries for the separation of biologically important species including amino acids [6–8] and peptides [9–11]. Separation of the enantiomers of several amino acids has been achieved using molecularly imprinted polymers [12] as the stationary phase material. The polymer was prepared on-column and L-phenylalanine was used as the print molecule. Polyacrylamide gels have been used in CEC as the stationary phase to separate dansylated amino acids [13]. UV-initiated acrylate-based both negatively and positively charged polymeric monoliths [14] were used as stationary phases for CEC separation of minute amounts of phenyl-

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thiohydantoin (PTH)-labeled amino acids, native peptides, and amino acids and peptides labeled with naphthalene-2,3-dicarboxyaldehyde (NDA).

Adsorption to the capillary wall was usually considered as a troublesome effect in capillary electrophoresis (CE), especially for the separation of peptides and proteins [11,15]. On the other hand Liu et al. presented as advantageous strongly adsorbed cetyltrimethylammonium bromide (CTAB) and lysozyme as possible stationary phases for CEC separation of amino acid enantiomers [16]. This approach, i.e. different adsorption of analytes to the etched large surface area [17] and/or chemically modified fused-silica capillaries [18] and to physically adsorbed stationary phases [19] represents the separation principle of open tubular capillary electrochromatography (OT-CEC), which is also applied to the separation of amino acids and peptides and to the investigation of the interactions of these and other analytes with ligands immobilized on the capillary wall.

Porphyrins, a class of naturally occurring macrocyclic compounds, play a very important role in the metabolism of living organisms. The tetrapyrrolic porphyrin ring is very stable and exhibits aromatic character. The porphyrin complexes with transition metal ions are very stable; almost all metals form complexes 1:1 (for more details see Ref. [20]). In addition to many other applications, porphyrins and their analogues are broadly exploited in analytical chemistry, including the field of separation techniques [21].

In one of their first studies, Kibbey and Meyerhoff have shown that zinc-tetraphenyl-porphyrin (Zn-TPP) retained imidazoles and histidine via strong cation–solute interaction [22]. This has implied the application of porphyrin stationary phases for the chromatographic separation of amino acids [23,24]. Various metalloprotoporphyrins [24] and metallotetraphenyl-porphyrins [25] covalently linked to the silica supports were presented as immobilized metal-ion affinity chromatography (IMAC) stationary phases for separation of amino acids and peptides. The double mechanism of retention has been observed including the coordinative interactions via the metallic center of porphyrin and $\pi-\pi$ interactions between π-electrons of macrocyclus of porphyrin and π-electrons of solute.

Porphyrin stationary phases were also employed for the separation of low-molecular-mass peptides [23,25]. Protoporphyrin IX, providing less hydrophobic phase than TPP when used in metallated stationary phase, showed a cumulative binding affinity for small peptides containing amino acids that individually exhibit strong interactions with a given stationary phase [23]. In other studies on separation of peptides with metallated TPP silicas, it was shown for several peptides containing histidine and phenylalanine, that retention decreases with increasing distance between these amino acids in the peptide molecule [25]. The separation of several dipeptides and tripeptides of tyrosine and several insulins was investigated with unmetallated and with Zn(II)- and Cu(II)-metallated TPP [26]. Greater retention factors obtained on metallated columns than on the unmetallated one confirm the coordinative interaction between the metallic center of porphyrin and peptide. It was found that the diversification of retention of tripeptides containing Tyr or Phe was greater than for individual Tyr and Phe, obtained on the same Zn-TPP column with 1% acetonitrile addition to the phosphate eluent [24]. It was indicated that the differences in the retention of tripeptides also depend on the structural conformation of the molecule.

The aim of this paper was to introduce some porphyrin derivatives with different central metal atoms and peripheral substituents as physically adsorbed stationary phases used in OT-CEC, to investigate the interactions of these immobilized ligands with selected aliphatic and aromatic amino acids and peptides in acid and alkaline media and to evaluate how these interactions may influence the OT-CEC separations of these analytes.

2. Experimental

2.1. Instrumentation

All OT-CEC and CZE separations were performed in the home-made capillary electromigration device equipped with the UV-photometric detector operating at 206 nm [27]. The nonmodified, bare fused-silica (FS) capillaries (I.D. 50 μm, O.D. 200 μm, total length 30–31 cm, effective length 19–20 cm) were supplied by the Institute of Glass and Ceramic

Materials of the Czech Academy of Sciences (Prague, Czech Republic), the preparation of porphyrin-modified capillary is described below. Applied voltage was 7 or 10 kV, the electric current 30–40 μA, and the experiments were performed at the ambient laboratory temperature 22–24 °C. The samples were introduced in the capillary by pneumatically induced hydrodynamic flow, pressure 700 Pa was applied for 5–20 s.

2.2. Materials and methods

2.2.1. Chemicals

Tris(hydroxymethyl)aminomethane (Tris) was obtained from Serva (Heidelberg, Germany), phosphoric acid, β-alanine (BALA), proline, valine, phenylalanine, tryptophan, tyrosine, sodium tetraborate decahydrate, and thiourea were purchased from Lachema (Brno, Czech Republic), diglycine and triglycine were from Reanal (Budapest, Hungary). Porphyrin derivatives, 5,10,15,20-tetraphenylporphyrin (TPP)–**1**, Co(II)-phthalocyanine (Co-phthaloc.)–**2** and Rh(III)-5,10,15,20-tetrakis(m-phenoxyphenyl)porphyrinate (Rh-TPPP)–**3**, (see Fig. 1) were synthesized in our laboratory [28].

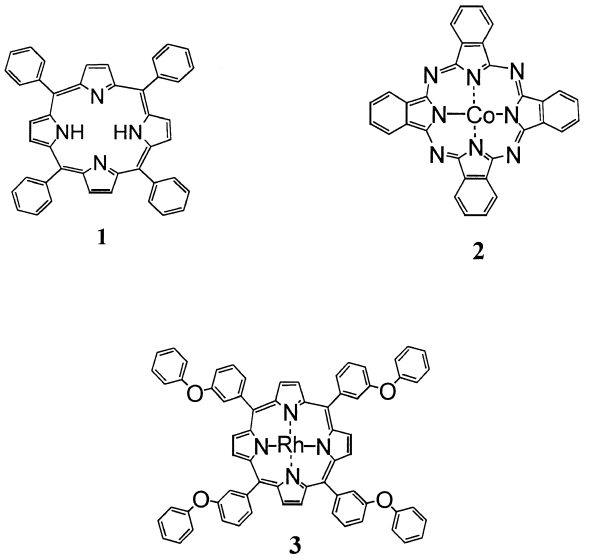

Fig. 1. Structures of porphyrin derivatives used as modifiers of the inner surface of the fused-silica capillary: **1**–5,10,15,20-tetraphenylporphyrin (TPP), **2**–Co(II) phthalocyanine (Co-phthaloc.), **3**–Rh(III)-5,10,15,20-tetrakis(m-phenoxyphenyl)porphyrinate (Rh-TPPP).

2.2.2. Modification of the inner capillary wall and capillary conditioning

Modification of the FS capillary is based on the physical adsorption of water- and water-buffer-insoluble porphyrin derivatives dissolved in organic solvent (dichloromethane) and flushed through the capillary.

The bare FS capillary was repeatedly (three times) washed with porphyrin derivative solution in dichloromethane in 1 mg/ml concentration for 10 min, followed by flushing it with air, methanol, water, and air again; each step for 5 min. Before each set of experiments the capillary was washed with the background electrolyte (BGE) for 10 min and then the blank run was performed to equilibrate the capillary under the high voltage (10 kV, 30 min).

The bare FS capillary was conditioned by sequential 5 min washing with water, 0.1 *M* sodium hydroxide, water and BGE. The porphyrin-modified capillary was washed with water and BGE, each step for 5 min. Both the unmodified and modified capillaries were washed with the BGE for 5 min between the runs.

2.2.3. Background electrolytes and sample solutions

Both CZE experiments in the bare FS capillary and OT-CEC experiments in porphyrin-modified FS capillaries were performed in two BGEs, in acid and alkaline pH region, respectively:

BGE I (acid): 0.1 mol/l phosphoric acid, 0.05 mol/l Tris, pH 2.1.

BGE II (alkaline): 0.05 mol/l disodium tetraborate, pH 9.7.

BGE solutions were prepared from the deionized water and filtered through 0.45 μm membrane filter (Millipore, Bedford, USA) prior to use.

Sample solutions were prepared by dissolving 5 mg of each amino acid or oligopeptide in 1 ml of water or background electrolyte (BGE) and stored below 4 °C. Final concentration of tested solutes in the injected sample was in the range of 0.25–1.66 mg/ml. Thiourea in the concentration 1 mg/ml, dissolved in water, served as the marker of the electroosmotic flow (EOF).

3. Results and discussion

CZE separation of aromatic amino acids in unmodified, bare FS capillary, and OT-CEC separations of these amino acids in the FS capillaries modified with porphyrin derivatives **1**, **2** and **3**, respectively, in acid BGE, 0.1 mol/l phosphate/Tris, pH 2.1, are shown in Fig. 2. The effective electrophoretic mobilities obtained from CZE separations in the unmodified capillary were taken as the reference values. As can be seen, the OT-CEC separations of the tested analytes in all three types of porphyrin-modi-

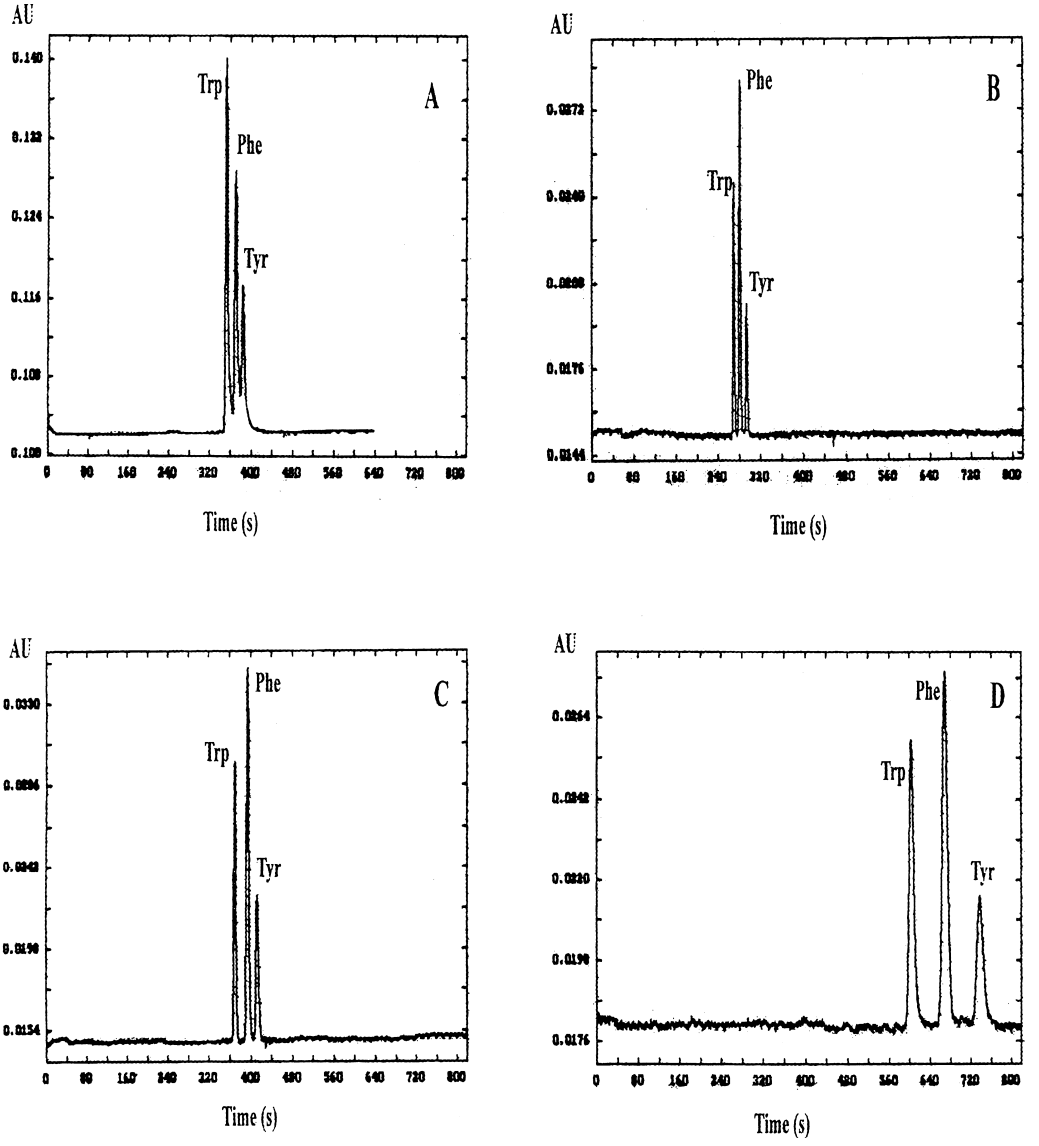

Fig. 2. CZE and OT-CEC separations of aromatic amino acids, Trp, Phe, and Tyr, in acid BGE, 0.1 mol/l phosphoric acid, 0.05 mol/l Tris, pH 2.1. Voltage 10 kV, current 45 μA, sample concentration 0.25 mg/ml, hydrodynamic sample injection: pressure 700 Pa, time 5 s, ambient temperature 23 °C. Other separation conditions are given in the section Materials and methods. AU=absorption at 206 nm. A) CZE separation in the bare FS capillary; B), C), D) OT-CEC separations in the FS capillaries modified with porphyrin derivatives **1**, **2**, and **3**, respectively.

fied capillaries exhibit better separation (higher resolution) than the CZE separations in the unmodified capillary. The aliphatic amino acids were completely, base-line separated by CZE in the bare FS capillary, but their separation was also improved in the modified capillaries (electropherograms not shown). The typical separations of oligopeptides, diglycine (Gly2) and triglycine (Gly3), in the bare and modified FS capillaries in the acid BGE are shown in Fig. 3.

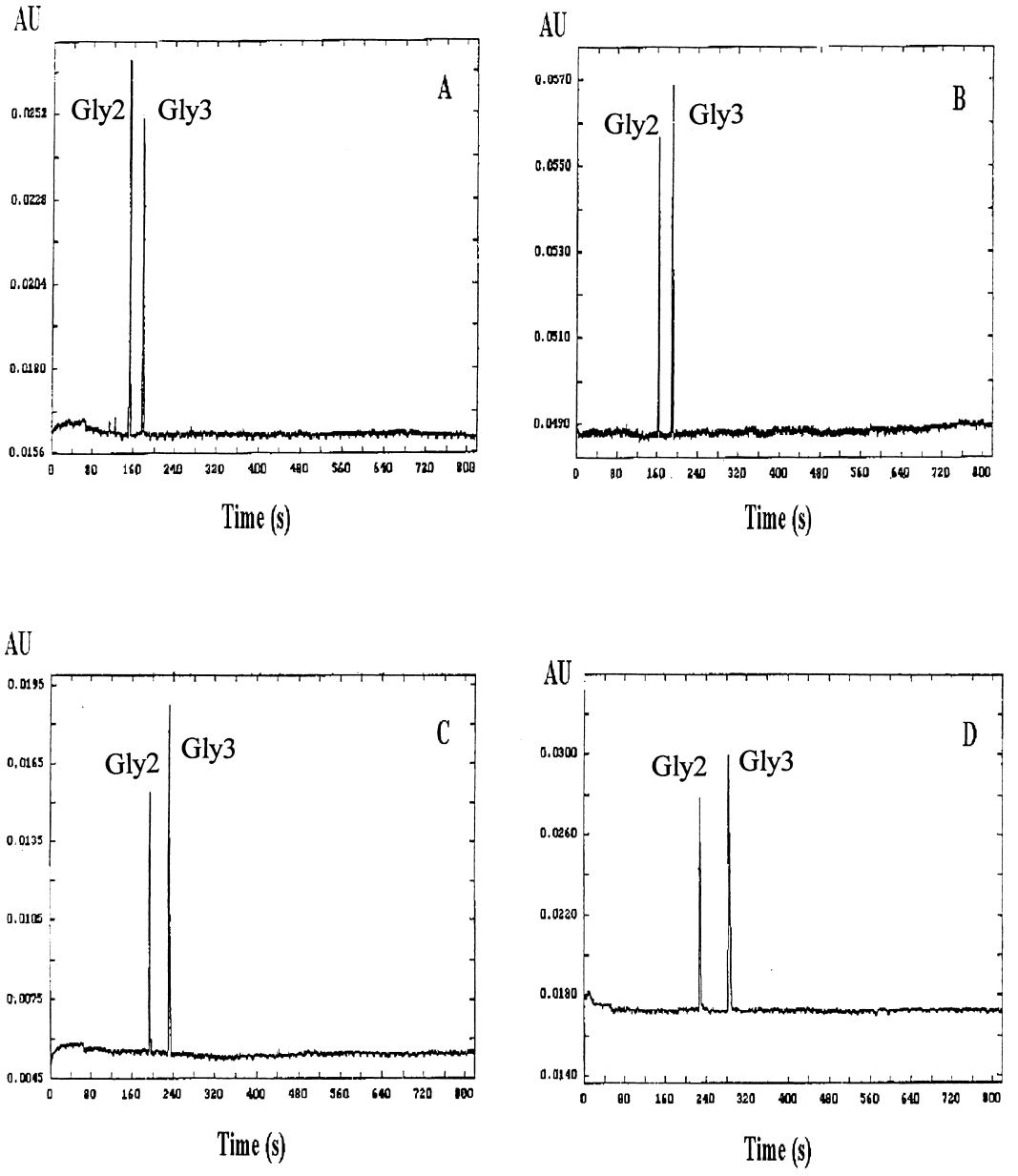

Fig. 3. CZE and OT-CEC separations of oligopeptides, diglycine (Gly2) and triglycine (Gly3) in acid BGE. Separation conditions as in Fig. 2. A) CZE separation in the bare FS capillary; B), C), D) OT-CEC separations in the FS capillaries modified with porphyrin derivatives **1**, **2**, and **3**, respectively.

Comparison of the effective mobilities of the amino acid and peptide analytes in the bare FS capillary and in the capillary modified with porphyrin derivative **1** (see Table 1 and Fig. 4) shows that the effective mobilities of all amino acids and peptides in the modified capillary are slightly higher than those in the nonmodified one. The reason of such increase may be the fact that under the acid conditions the porphyrin core of derivative **1** is protonated and thus the electrostatic repulsion of the positively charged porphyrin core and the analytes comes to the consideration. Nevertheless, the EOF is a little bit slower (as expected) due to the 'shielding' of silanol groups on the inner capillary surface by the adsorbed porphyrin derivative.

The capillary modified with porphyrin derivative **2** showed some interesting features. While the EOF is about 20% higher compared to the nonmodified capillary, the effective mobilities of all analytes are decreased. This decrease is significant (40–46%) mainly for aromatic amino acids and partially also for aliphatic amino acids proline and valine (see Table 2 and Fig. 4), whereas the mobility decrease of BALA and oligopeptides Gly2 and Gly3 is much smaller, probably due to the missing potential for hydrophobic interactions of the latter analytes.

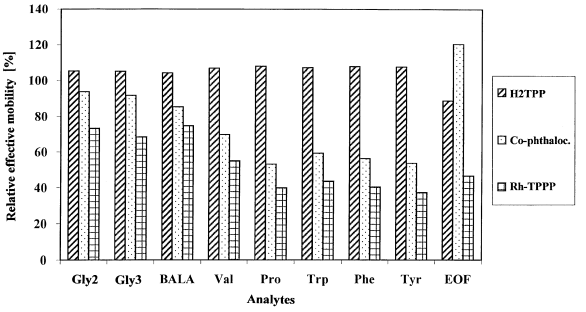

Fig. 4. Comparison of relative effective mobilities of separated amino acids and oligopeptides in acid Tris–phosphate (pH 2.1) BGE in the FS capillary modified with porphyrin derivatives **1**, **2**, and **3**, respectively. The mobilities are related to the mobility in the unmodified, bare FS capillary, which is set to be 100%.

The most interesting results were obtained in the separations of our analytes using the capillary with

Table 1
Effective mobilities of separated amino acids and oligopeptides in acid and alkaline BGEs in the unmodified (bare) FS capillary and in the capillary modified with porphyrin derivative **1**, and relative effective mobilities of these analytes in the capillary modified with derivative **1**

BGE	Effective mobility$\times 10^9$ [$m^2\ V^{-1}\ s^{-1}$]			Relative effective mobility (%)
	Analyte	m_{ef} (unmod)	m_{ef} (mod)	$m_{ef,rel}$ (mod)
0.1 *M* phosphate	Gly2	31.7	33.4	105.4
0.05 *M* Tris	Gly3	26.7	28.1	105.1
pH 2.1	BALA	38.9	40.6	104.3
	Val	20.5	21.9	107.0
	Pro	15.4	16.6	108.0
	Trp	17.4	18.6	107.3
	Phe	16.4	17.7	108.0
	Tyr	15.6	16.8	107.9
	EOF	4.1	3.6	88.9
0.05 *M* sodium	Gly2	−30.7	−25.1	81.6
tetraborate	Gly3	−25.5	−25.1	98.3
pH 9.7	BALA	−6.4	−6.7	104.3
	Val	−13.0	−11.7	90.2
	Pro	−3.0	−3.1	102.4
	Trp	−14.1	−15.3	109.0
	Phe	−16.1	−20.5	127.4
	Tyr	−19.6	−14.0	71.5
	EOF	53.7	28.4	53.0

m_{ef} (unmod)=effective mobility in unmodified capillary; m_{ef} (mod)=effective mobility in modified capillary, $m_{ef,rel}$ (mod)=relative effective mobility in modified capillary; $m_{ef,rel}$ (mod)=[m_{ef} (mod)/m_{ef} (unmod)]·100.

Table 2
Effective mobilities of separated amino acids and oligopeptides in acid and alkaline BGEs in the unmodified (bare) FS capillary and in the capillary modified with porphyrin derivative **2** and relative effective mobilities of these analytes in the capillary modified with derivative **2**

BGE	Effective mobility×10^9 [m^2 V^{-1} s^{-1}]			Relative effective mobility (%)
	Analyte	m_{ef} (unmod)	m_{ef} (mod)	$m_{ef,rel}$ (mod)
0.1 *M* phosphate	Gly2	31.7	29.7	93.8
0.05 *M* Tris	Gly3	26.7	24.5	91.8
pH 2.1	BALA	38.9	33.2	85.4
	Val	20.5	14.3	69.9
	Pro	15.4	8.2	53.3
	Trp	17.4	10.3	59.4
	Phe	16.4	9.3	56.5
	Tyr	15.6	8.4	53.9
	EOF	4.1	4.9	120.4
0.05 *M* sodium	Gly2	−30.7	−29.4	95.8
tetraborate	Gly3	−25.5	−25.1	98.3
pH 9.7	BALA	−6.4	−6.8	107.0
	Val	−13.0	−13.7	105.5
	Pro	−3.0	−3.1	103.0
	Trp	−14.1	−15.8	112.7
	Phe	−16.1	−20.6	127.8
	Tyr	−19.6	−21.6	110.4
	EOF	53.7	38.1	70.9

m_{ef} (unmod)=effective mobility in unmodified capillary; m_{ef} (mod)=effective mobility in modified capillary, $m_{ef,rel}$ (mod)=relative effective mobility in modified capillary; $m_{ef,rel}$ (mod)=[m_{ef} (mod)/m_{ef} (unmod)]·100.

the inner surface covered with porphyrin derivative **3**. Effective mobilities were largely decreased not only in the case of aromatic amino acids but also for the aliphatic ones (see Table 3 and Fig. 4). Trying to rationalize and explain the behavior of the three modifiers we have to consider three factors. First is the protonation of the porphyrin core which can be achieved only in the derivative **1** in acid buffer and which causes the distortion of planar porphyrin structure [20]. Then the charge of this porphyrin derivative is +2 and thus there is a significant electrostatic repulsion influence on the separation of positively charged solutes. The second factor is the presence of the central metal atom. While the cobalt atom at the core of porphyrin derivative **2** is divalent making thus the complex electroneutral, the rhodium central atom in the derivative **3** is trivalent and the final complex charge is +1. Nevertheless, the best separation of aromatic amino acids was achieved in the capillary the inner surface of which was modified with derivative **3** (presented in Fig. 2D). Now we have to include the third factor, peripheral substituents, which brings additional effects. While the periphery of derivative **2** has got minimal possibility of changing the 'flat' structure, the derivative **3** can change the orientation of the phenol moiety due to the rotation around the etheric bond and this seems to have a fundamental effect on the separation. What is also clear from the Fig. 3 is that the oligopeptides are always well separated and the influence of the modifiers employed is significant mainly with derivative **3**.

The course of the separations in the modified capillaries is consistent with three interaction types mentioned above: π−π (hydrophobic) stacking, axial ligation to the central metal atom and electrostatic repulsion.

Separations run at the alkaline BGE also revealed several specificities. While in the experiment run in the unmodified capillary phenylalanine and tyrosine remained unseparated, these amino acids were at least partially resolved in capillaries modified with porphyrin derivatives **2** and **3**, and completely resolved in the capillary coated with derivative **1** (see

Table 3
Effective mobilities of separated amino acids and oligopeptides in acid and alkaline BGEs in the unmodified (bare) FS capillary and in the capillary modified with porphyrin derivative **3** and relative effective mobilities of these analytes in the capillary modified with derivative **3**

BGE	Effective mobility×10^9 [m^2 V^{-1} s^{-1}]			Relative effective mobility (%)
	Analyte	m_{ef} (unmod)	m_{ef} (mod)	$m_{ef,rel}$ (mod)
0.1 *M* phosphate	Gly2	31.7	23.2	73.4
0.05 *M* Tris	Gly3	26.7	18.3	68.5
pH 2.1	BALA	38.9	29.1	74.9
	Val	20.5	11.3	55.2
	Pro	15.4	6.1	40.0
	Trp	17.4	7.6	43.7
	Phe	16.4	6.7	40.5
	Tyr	15.6	5.9	37.5
	EOF	4.1	1.9	46.8
0.05 *M* sodium	Gly2	−30.7	−29.9	97.4
tetraborate	Gly3	−25.5	−24.6	96.6
pH 9.7	BALA	−6.4	−6.45	101.0
	Val	−13.0	−13.2	101.6
	Pro	−3.04	−3.0	98.7
	Trp	−14.1	−14.3	101.4
	Phe	−16.1	−16.3	101.0
	Tyr	−19.6	−19.5	99.9
	EOF	53.7	38.8	72.4

m_{ef} (unmod)=effective mobility in unmodified capillary; m_{ef} (mod)=effective mobility in modified capillary, $m_{ef,rel}$ (mod)=relative effective mobility in modified capillary; $m_{ef,rel}$ (mod)=[m_{ef} (mod)/m_{ef} (unmod)]·100.

Fig. 5). Even more noticeable is the fact that the separation order in the capillary with derivative **1** is changed to give the migration (retention) sequence Tyr, Trp, Phe, while in the other modified capillaries the migration (retention) sequence was Trp, Phe, Tyr. Comparing the electropherograms of oligopeptides obtained in experiments in the alkaline BGE in modified capillaries we could see (Fig. 6) that there was only minor influence of the character of the modifier on the separation, the changes in resulting migration (retention) times and resolution are influenced only by the changes of EOF (see Tables 1–3 and Fig. 7). One reason for that could be the nonaromatic character of these solutes. The most distinct change in the effective mobility was obtained with phenylalanine which showed about 27% increase in the capillaries modified with derivatives **1** and **2** and for tyrosine, the mobility of which was decreased by almost 30% in the capillary modified by derivative **1** (see Tables 1–3 and Fig. 7).

From the evaluation of the effective mobilities of all analytes in the capillaries modified with porphyrin derivatives and comparing them with the effective mobilities obtained from CZE separations in the bare FS capillary we can summarize that:

(a) Separations in the acid BGE are relatively strongly affected by the presence and character of the central metal atom in the structure of the porphyrin derivative molecule. This influence is significant mainly in the separations of all aromatic amino acids and aliphatic amino acids proline and valine, while the separation of aliphatic amino acid BALA and oligopeptides diglycine and triglycine is less affected (see Tables 1–3 and Fig. 5).
(b) At acid pH the porphyrin derivative **3** with more flexible peripheral substituents is able to fit better the analyte conformation and thus improve the interactions with these solutes than the relatively rigid derivative **2**, which was proven by the larger decrease of the effective mobility of the analytes and improved separation profile

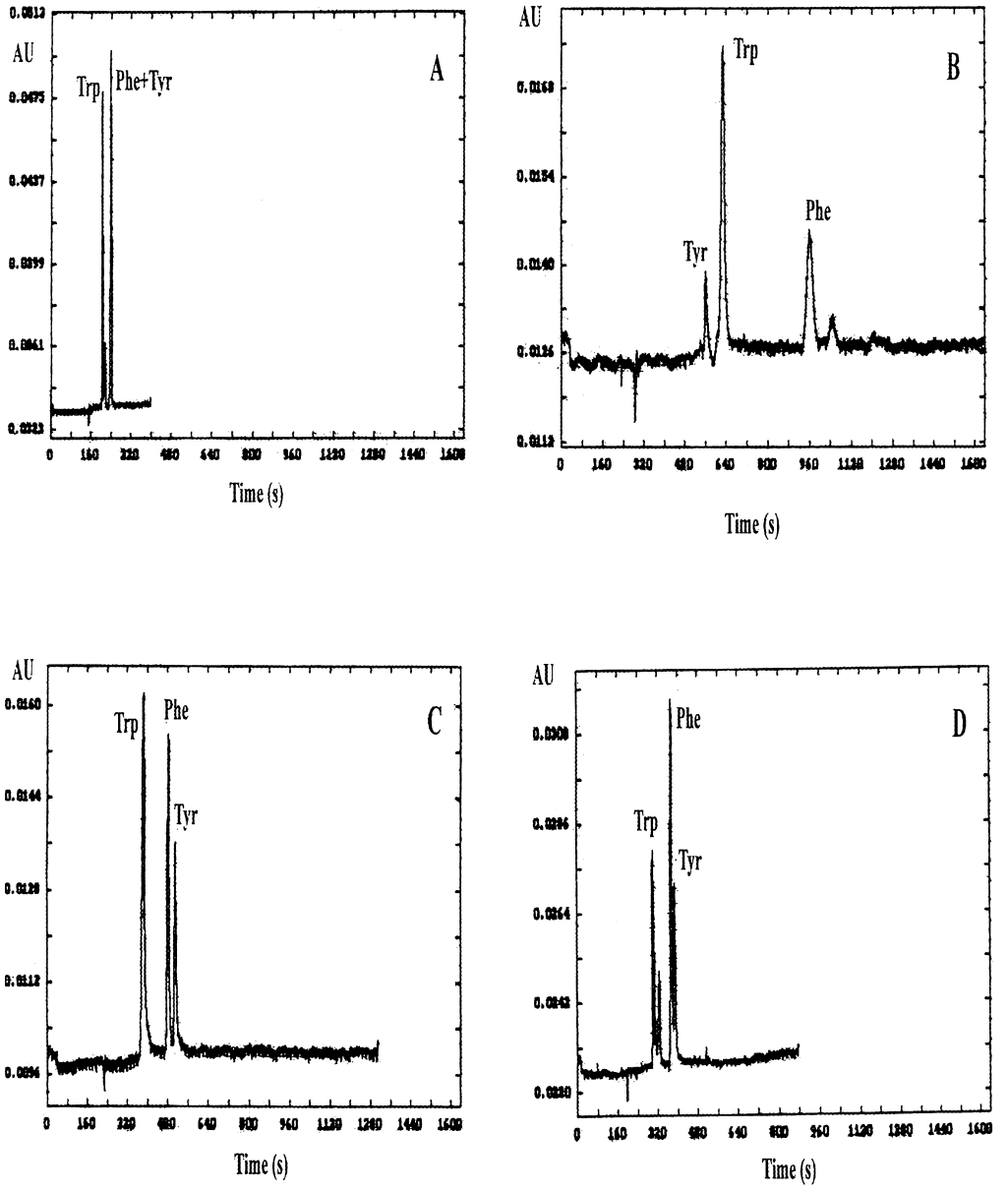

Fig. 5. CZE and OT-CEC separations of aromatic amino acids, Trp, Phe and Tyr, in alkaline BGE, 0.05 mol/l sodium tetraborate, pH 9.7. Voltage 7 kV, current 45 μA, Other separation conditions as in Fig. 2. A) CZE separation in the bare FS capillary; B), C), D) OT-CEC separations in the FS capillaries modified with porphyrin derivatives **1**, **2**, and **3**, respectively.

in the capillary modified with derivative **3** than in the capillary modified with derivative **2**.

(c) While the EOF is decreased in the capillary modified with derivative **1** in the acid BGE, the effective mobilities of all analytes are increased in this capillary, which can be ascribed to the protonation of porphyrin tetrapyrrolic core under these conditions and from this fact the resulting

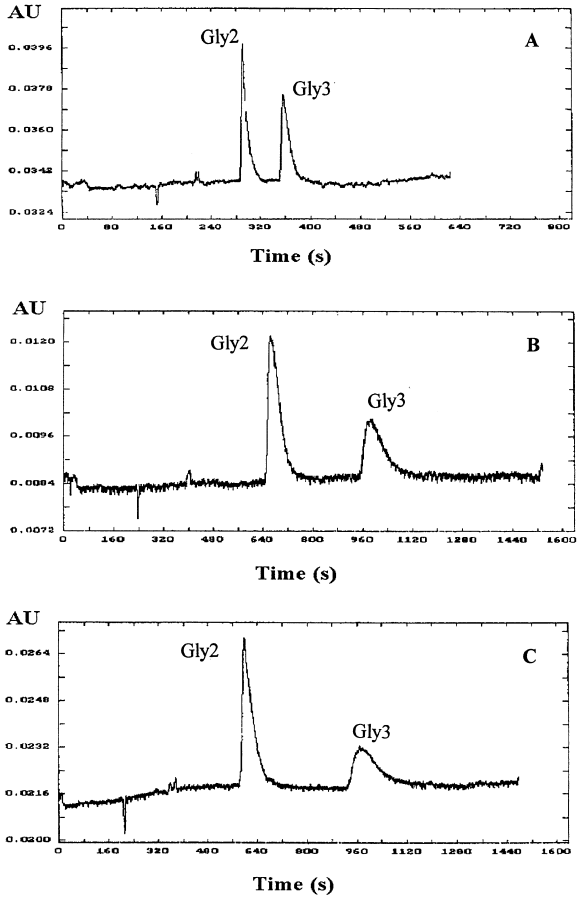

Fig. 6. CZE and OT-CEC separations of oligopeptides, diglycine (Gly2) and triglycine (Gly3), in alkaline BGE. Other separation conditions as in Fig. 2. A) CZE separation in the bare FS capillary; B), C) OT-CEC separations in the FS capillaries modified with porphyrin derivatives **2**, and **3**, respectively. (Remark: no separation of Gly2 and Gly3 was achieved in the capillary modified with derivative **1**, record not presented.)

repulsion of positively charged analytes from the capillary wall.

(d) The separations in alkaline BGE did not reveal much about the interactions of the used porphyrin derivative modifiers with the tested analytes. Mostly nonsignificant changes in the separations of the tested analytes might be ascribed to the fast EOF under which the influence of analyte interactions with the modified capillary wall on their separation is relatively limited.

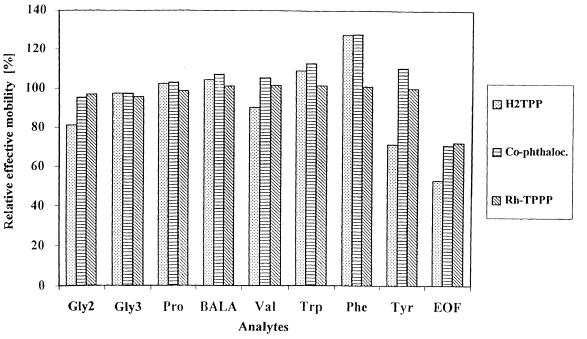

Fig. 7. Comparison of relative effective mobilities of separated amino acids and oligopeptides in alkaline sodium tetraborate (pH 9.7) BGE in the FS capillary modified with porphyrin derivatives **1**, **2**, and **3**, respectively. The mobilities are related to the mobility in the unmodified, bare FS capillary, which is set to be 100%.

4. Conclusion

We have shown a new possibility of using water and water-buffer insoluble porphyrin derivatives in CEC, specifically for their application as the modifiers of the inner surface of the FS capillaries. The modifiers can be attached to the capillary wall simply by the physical adsorption of porphyrin derivatives during flushing their solution in dichloromethane through the capillary.

The separation conditions in the acid BGE seem to be more appropriate than in the alkaline one because of the lower EOF and stronger interaction of tested analytes with the wall modifiers in the acid environment. Experiments carried out in the acid Tris–phosphate BGE revealed that there is a significant dependence of interactions of our tested amino acids and peptides with porphyrin derivatives on their peripheral substituents and on the central metal atom. The separation mechanism is based on the involvement of three noncovalent forces: $\pi-\pi$ (hydrophobic) stacking, electrostatic repulsion and axial ligation between the central metal atom of the modifier and the analyte.

In further experiments we would like to focus our attention on the separation of more complex mixtures of amino acids and peptides and on the design of specific capillary modifiers for various functional groups of biologically important species.

Acknowledgements

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