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Capillary electrochromatographic separation of aromatic amino acids possessing peptides using porphyrin derivatives as the inner wall modifiers

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Abstract

Two different porphyrin derivatives (H_2TPP(m-OPh)$_4$ and Rh(III)TPP(m-OPh)$_4$) were investigated with respect to their capability to help resolution of five model aromatic peptides in capillary electrophoresis/open tubular capillary electrochromatography. Though the main separation mechanism was preferentially based on the ionic properties of the separated analytes, involvement of particularly H_2TPP(m-OPh)$_4$–peptide interactions at alkaline pH (8.0) was clearly demonstrated. In combination with Tris–phosphate buffer, a speed up of the separation was observed at pH 2.25 (particularly if Rh(III)TPP(m-OPh)$_4$ was used as capillary coating); in spite of the speed up of the separation the selectivity of the system was sufficient and resulted in a complete separation of the five model peptides. It can be expected that Rh(III)TPP(m-OPh)$_4$ capillary coating in combination with Tris–phosphate buffer can be generally used for a considerable speeding up of lengthy separations of peptides in acidic media with some decrease in the separation power of the system.

Keywords: Capillary electrochromatography, open-tubular; Capillary coating; Peptides; Aromatic amino acids; Porphyrins

1. Introduction

Coating of the inner capillary wall during capillary electrophoresis can affect the separation in two ways, namely: (i) by the modification of the endoosmotic flow and (ii) by modifying the selectivity of the separation if there exists an affinity between the separated solutes and the capillary coating.

The speed of the endoosmotic flow depends on the ζ-potential and understandably is higher at higher pH values [1–3]. If the endoosmotic flow is too slow (at pH values around 2.0), which typically occurs in the separation of peptides and proteins, low plate counts and poor migration reproducibility of the slowly moving peaks is the result. In other words there is a category of capillary separations in which it would be desirable to increase the speed of the endoosmotic flow [5,6]. There are basically two ways to handle this problem: (i) by increasing the ζ-potential or (ii) by joining the separation column to an extra-column generator [7] of the endoosmotic flow. Modifying the ζ-potential, mostly by using smart background electrolyte composition [8–10] has certain drawbacks similar to those like elevated column temperature [2,11,12]. The other alternative applicable in particular for capillary electrochromatography is based

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on joining the separation system with an additional column the role of which is to drag the background electrolyte through the separation bed ([4], for review, see Ref. [13]). Also this approach has some drawbacks the main one of which is joining the two (separation and electroosmotic flow generating) capillaries. Of course, this can be overcome (particularly in electrochromatography) by using partial filling of the separation column. Basically two modes can be used: (i) the packed separation column can be combined with an open flow generating column section [14,15], or (ii) the flow generating section can also be packed (though with a different packing the role of which is similar to the previously mentioned open section of the column) leaving a free space for the detector window (for review, see Ref. [16]).

On the other hand, one can speculate about coating of the inner capillary wall in such way that the coating used will increase the charge of the capillary wall at low pH values thereby increasing the speed of analysis. Except a short notice from our laboratory [17], to our best knowledge, no other report regarding this possibility has been published yet. Of course, this will shorten the time interval allowed for separation and the emerging peaks have smaller differences in migration times which may affect negatively the values of the separation factors [18].

Coating of the capillary wall can concomitantly bring about additional affinity to the separated solutes as we have shown with nucleoside mono-, di-, and triphosphates used as separated entities and sapphyrin-coated capillary [19] the result of which could be a better separation of analytes investigated, and possibly altered selectivity of such a system if the affinity between the separated analytes and capillary coating is strong enough.

In this communication we have investigated the properties of two different capillary coatings (porphyrin derivatives) for the separation of aromatic amino acids possessing tripeptides.

Porphyrins represent a class of naturally occurring macrocyclic compounds, which contain four pyrrole rings linked via methine bridges in their molecules [20]. These macrocycles can possess various peripheral substituents and they have been exploited in many fields of analytical chemistry (for review, see Ref. [21]). Porphyrins have also found numerous applications in HPLC as the stationary phases for separation of low molecular peptides [22,24]. Protoporphyrin showed a cumulative binding affinity for small peptides containing amino acids that individually exhibited strong interaction with a given stationary phase [22]. In the studies on separation of peptides with metallated tetraphenylporphyrin (TPP) silicas, it was shown for several peptides containing histidine and phenylalanine residues, that retention decreases with increasing distance between these two amino acids in the peptide chain [24]. The separation of several dipeptides and tripeptides of tyrosine and several insulins was investigated with unmetallated and metallated Zn(II) and Cu(II)TPP [25]. Greater retention factors were obtained on metalled column, which confirmed the coordinative interaction between the metallic centre of porphyrin and the investigated peptide [23]. 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(II)TPP column, which indicated that the differences in the retention of tripeptides also depends on the conformation of the molecule [23].

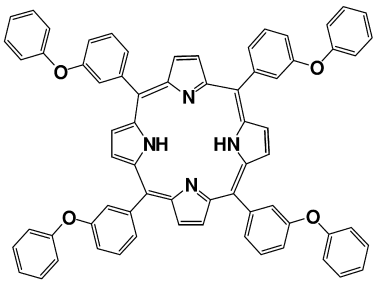
2. Experimental

2.1. Instrumentation

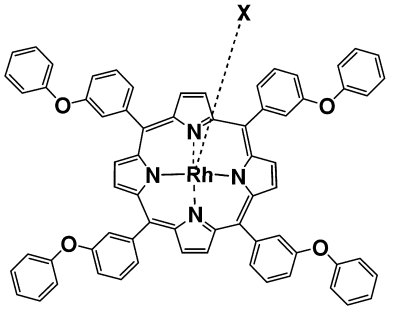
All experiments were done with SpectraPhoresis 504 apparatus from Thermo Separation Products (TSP, Riviera Beach, FL, USA), controlled with PC 1000 Version 2.6 software (supported on OS2 2.1), equipped with the on-line, variable-wavelength detector set to 200 nm.

The separations were run in three types of capillaries. The first type was an untreated fused-silica capillary, I.D. 50 μm, O.D. 375 μm fitted into the TSP cartridge. The total capillary length was 42.5 cm (34.5 cm to the detector). The second one was a capillary modified with H_2TPP(m-OPh)$_4$ porphyrin derivative (43.2 cm/35.3 to the detector), and the third one was Rh(III)TPP(m-OPh)4 porphyrin derivative modified capillary (43 cm/35.5 cm to the detector) (for the formula of porphyrin derivatives, see Fig. 1). Other characteristics of the modified capillaries were the same as specified above.

The separations were routinely run at 10 and 15



5, 10, 15, 20 tetrakis(m-phenoxyphenyl)porphyrin



5, 10, 15, 20-tetrakis(m-phenoxyphenyl)porphyrinate Rh(III)X$^-$

Fig. 1. Structures of porphyrin used as capillary coatings.

kV (20–80 μA according to the buffer composition and applied voltage) and the analysis time was up to 30 min; detection was done at 200 nm.

An uncoated silica capillary was conditioned by sequentially washing it for 5 min with water, 10 min with 1 mol/l NaOH, 5 min with water and finally 5 min with the running buffer. This procedure was also used to recondition the capillary after each run.

The modified capillaries were conditioned by sequentially washing with the running buffer for 10 min.

2.2. Reagents

Potassium dihydrogen phosphate, potassium hydrogen diphosphate, sodium hydroxide and phosphoric acid were obtained from Lachema (Brno, Czech Republic) and were of p.a. purity. Tripeptides alanine–alanine–alanine, glycine–glycine–phenylalanine, tyrosine–glycine–glycine, tris(phenylalanine) and tris(tyrosine) were purchased from Sigma (St Louis, MO, USA) and were of 97% purity grade.

The porphyrin derivatives were synthesized in the laboratory of V. Král (unpublished results) and characterized by ^{1}H NMR, MS and IR.

Samples of peptides for analysis were prepared by dissolving the appropriate amount (0.4–2.6) mg of each solute in 200 μl of Milli-Q water and stored below 4 °C. Tris(phenylalanine) was prepared by dissolving of 3.6 mg in 400 μl of Milli-Q water, sonicated and filtered. The supernatant was used for further experiments. The final sample comprised 5 μl of Gly–Gly–Phe, 3 μl of Tyr–Gly–Gly, 7 μl $(Tyr)_3$, 7 μl $(Ala)_3$ and 50 μl of $(Phe)_3$. All samples were injected electrokinetically at 5 kV for 2 s.

2.3. Coating of the capillary wall

A new fused-silica capillary was sequentially washed with water, 1 mol/l NaOH and water, each step for 5 min. Next it was washed with methanol, dichloromethane and dried in an air stream. Then the capillary was filled with the solution of porphyrin derivative in dichloromethane (2 mg/ml) and placed in a vacuum oven where it was dried for 1.5 h at 65 °C. After this procedure, the capillary was washed with water and running buffer for 5 min each. To stabilize the coating, the capillary was run in the separation buffer at 20 kV for a period of 30 min, and this procedure was repeated until the baseline drift was eliminated.

2.4. Buffer systems

The first electrolyte tested was phosphate buffer at the concentration 100 mmol/l at pH values 2.5, 3.82, 5.5, 7.0, 8.0 and 10.0. In addition, 50 mmol/l Tris–100 mmol/l phosphoric acid buffer at pH 2.25 and 8.0 was used as well. Separations were done in both the unmodified and porphyrin derivative-modified capillaries and the results were compared.

3. Results and discussion

The experimental work was focused on the separation of peptides containing aromatic residues in their sequence. The influence of pH of the run buffer, the composition of the background electrolyte and

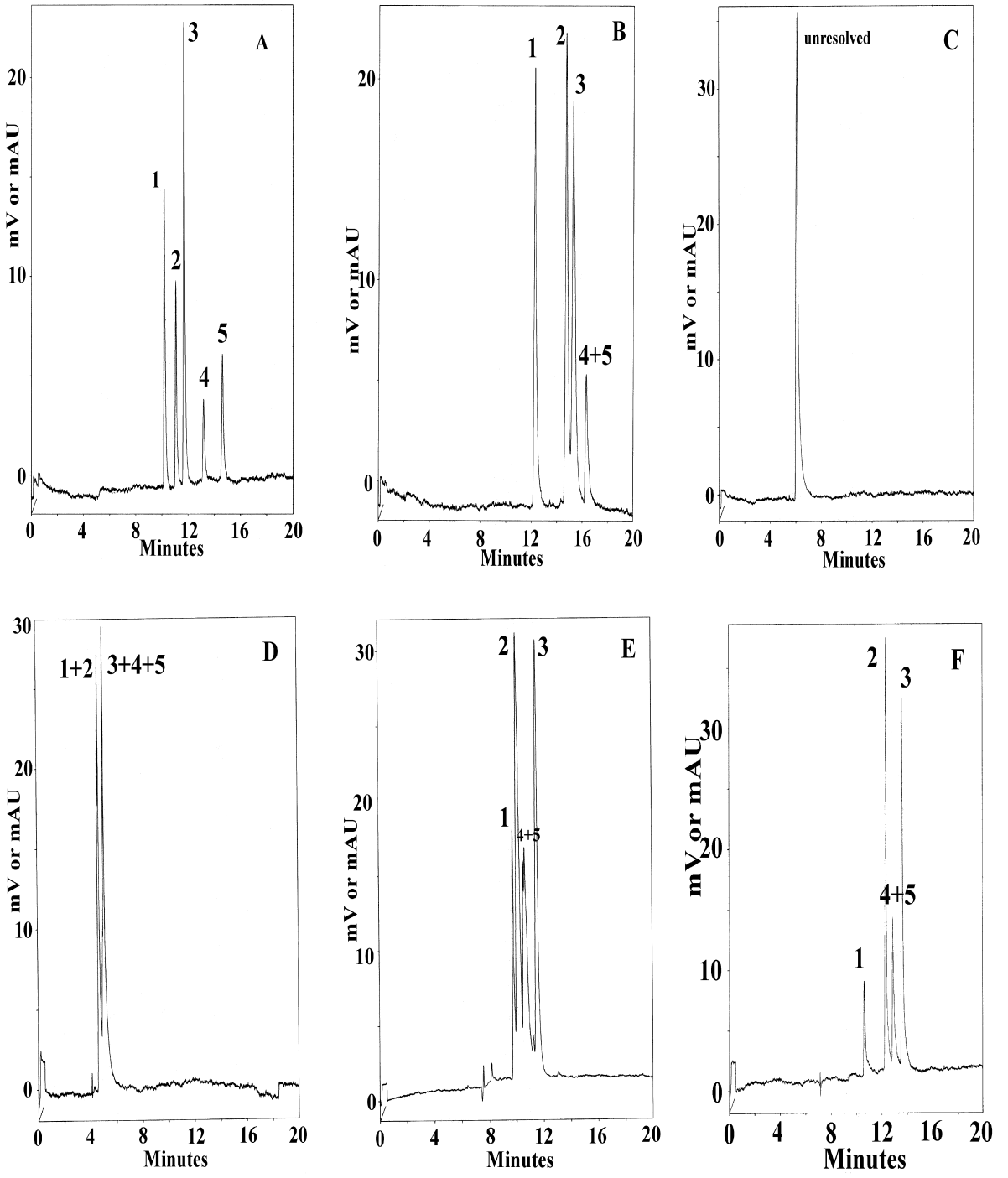


Fig. 2. The influence of pH of the background electrolyte on the separation of the model mixtures. Phosphate buffer 100 mmol/l, 15 kV (10 kV at high pH values), 40–80 μA, hydrodynamic injection for 10 s, detection at 200 nm. (A) pH 2.5, (B) pH 3.82, (C) pH 5.5, (D) pH 7.0, (E) pH 8.0, (F) pH 10.05, uncoated capillary (42.5 cm/34.5 cm to the detector). Peak identification: 1, Ala–Ala–Ala; 2, Gly–Gly–Phe; 3, Tyr–Gly–Gly; 4, Phe–Phe–Phe; 5, Tyr–Tyr–Tyr.

the role of porphyrin derivatives modified capillaries were investigated.

As demonstrated in Fig. 2 all peptides of this set were completely separated in bare capillary at pH 2.5 in 0.1 mol/l phosphate buffer. The separation occurred between 10.17 and 14.61 min of running time. As expected with increasing pH the separation disimproved and failed completely at pH 5.5. Further increase in pH resulted in partial restoring of the separation. On the alkaline side, the best separation was obtained at pH 8.0, however, separation of $(Tyr)_3$ and $(Phe)_3$ was only indicated. Further increase in pH to 10.0 did not resolve the latter two peptides, which emerged as a single peak. What is noticeable is the change in the sequence in which the peptides appeared before the detector's window. The first two peaks referring to $(Ala)_3$ and Gly–Gly–Phe were eluted in this order both in the acid and alkaline background electrolytes. However, the remaining three eluted in the sequence Tyr–Gly–Gly, $(Phe)_3$ and $(Tyr)_3$ in the acid media while in the alkaline buffer their order of elution was $(Tyr)_3$, $(Phe)_3$ and Tyr–Gly–Gly which eluted last.

All the above-described results refer to phosphate as a background electrolyte. Using Tris–phosphate buffer yielded nearly a complete separation of the model peptide mixture at the acid pH (Fig. 3) (Gly–Gly–Phe was only partially separated from Tyr–Gly–Gly, peaks 2 and 3), however the overall running time was considerably longer (15.05–20.36 min) which would have been expected not to distort the separation. In alkaline buffer (e.g. pH 8.0), the set offered only three closely moving peaks (data not shown).

In order to demonstrate the possible involvement of electrochromatographic separation, two types of capillary wall modifiers, namely $H_2TPP(m\text{-}OPh)_4$ and its rhodium complex were used (Fig. 4). Modification of the capillary wall by $H_2TPP(m\text{-}OPh)_4$ did not change the overall running time (Fig. 4B). However, the selectivity of separating Gly–Gly–Phe and Tyr–Gly–Gly was lower, though nearly baseline separation was obtained. It is feasible to conclude that some interaction between the coating and the investigated solutes occurred. If the Rh(III)TPP(m-$OPh)_4$ complex instead of uncomplexed $H_2TPP(m\text{-}OPh)_4$) was used (Fig. 4C) the speed of the run increased, the separation of the critical pair (Gly–

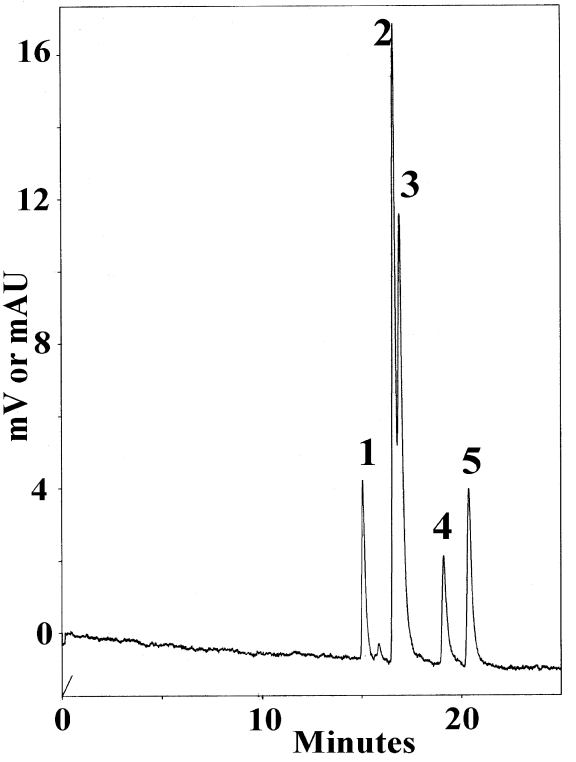


Fig. 3. The same separation as Fig. 2A effected in 50 mmol/l Tris–100 mmol/l phosphate at pH 2.25. Other conditions as in Fig. 2 except the run voltage (10 kV), uncoated capillary. Peak identification as in Fig. 2.

Gly–Phe and Tyr–Gly–Gly) was complete and the run time was slightly shorter (by roughly 1 min) when compared to the results obtained with the bare capillary (Fig. 2). If Tris–phosphate instead of phosphate was applied under otherwise unchanged conditions, the separation was nearly complete (Table 1), the sequence remained unchanged and the whole run lasted less than 9 min (a decrease in running time by 40%, Fig. 4D). This result supports the assumption that the leading separation mechanism involved is electrokinetic, with complementary interaction of the solutes in the Rh(III)TPP(m-$OPh)_4$ coated capillary. Concomitantly coating of the capillary with this complex leads to a considerable speed up of the electroosmotic flow which in acid media could be exploited very probably with other peptidic solutes as well. The interaction of the peptides with the capillary modifier is clearly demonstrated in Fig. 5. Here two partly resolved peaks were observed

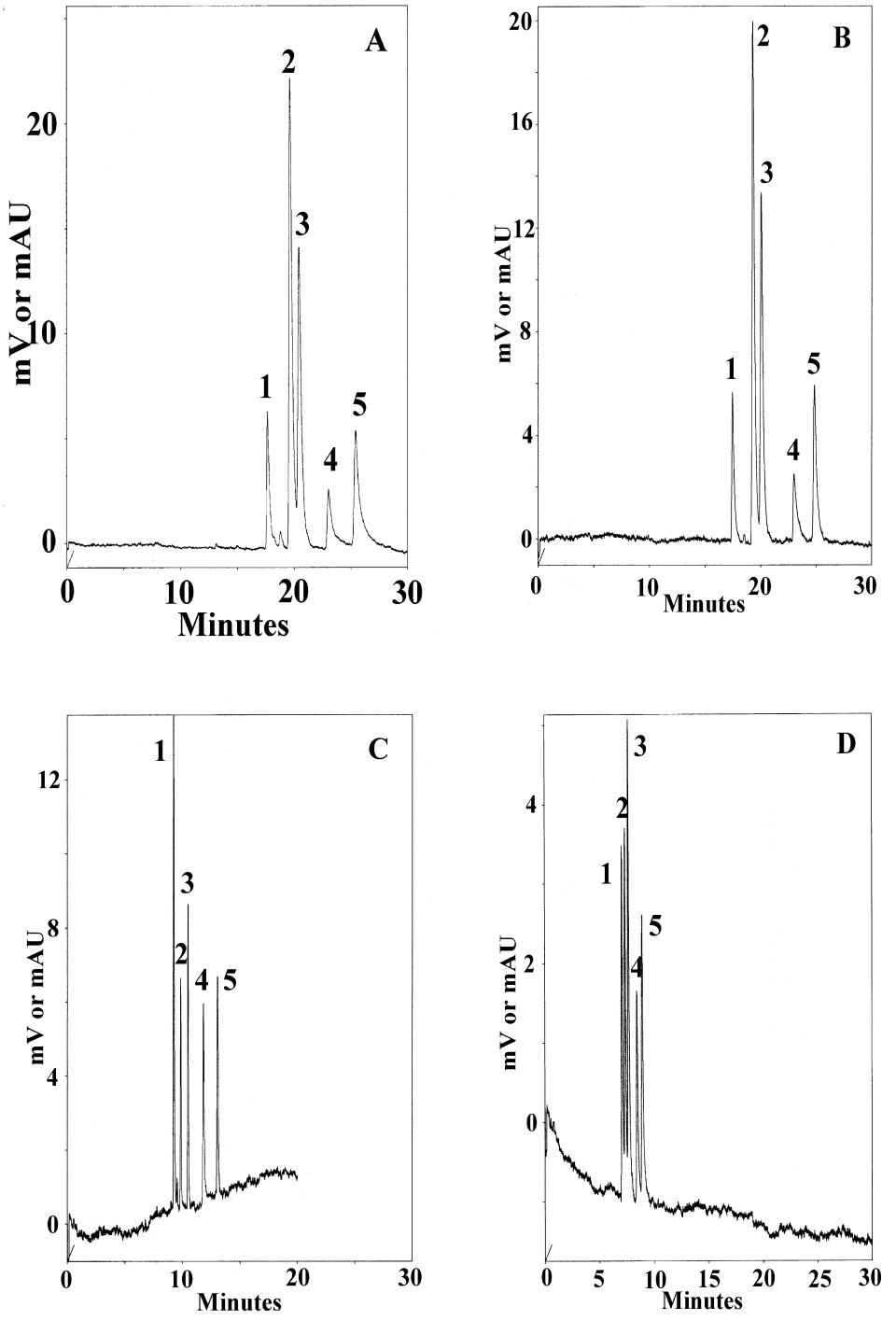


Fig. 4. (A) Separation of the test mixture at pH 2.5 in 100 mmol/l phosphate buffer in uncoated capillary (42.5 cm/34.5 cm to the detector), 10 kV. (B) The same as in (A) except that the capillary was coated with H_2TPP(m-OPh)$_4$ (43.2 cm/35.3 cm to the detector). (C) The same as in (A) Rh(III)TPP(m-OPh)$_4$ coated capillary, 15 kV (43 cm/35.5 to the detector). (D) As in (C), except 50 mmol/l Tris–100 mmol/l phosphate pH 2.25 was used as run buffer. Peak identification as in Fig. 2.

Table 1
Number of theoretical plates and resolution factors from the preceding peak for bare and Rh(III)TPP(m-OPh)$_4$ coated capillary in 0.05 Tris–0.1 *M* phosphate acid, pH 2.25

	Solute	Mig. time (min)	*N*	Resolution
Uncoated capillary	Ala–Ala–Ala	8.944	42 504	
	Gly–Gly–Phe	9.464	84 608	1.97
	Tyr–Gly–Gly	9.935	52 448	1.97
	Phe–Phe–Phe	11.232	119 917	5.28
	Tyr–Tyr–Tyr	12.042	100 664	3.07
Rh(III)TPP (m-OPh)$_4$ coated capillary	Ala–Ala–Ala	7.028	30 328	
	Gly–Gly–Phe	7.308	32 784	1.16
	Tyr–Gly–Gly	7.613	32 264	1.22
	Phe–Phe–Phe	8.407	43 392	3.06
	Tyr–Tyr–Tyr	8.880	43 896	1.76

while in a bare capillary at this pH (5.5) only a single peak was seen on the electropherogram (Fig. 2C).

In alkaline background electrolyte (pH 8.0) and uncoated capillary (phosphate) only an indicated resolution of (Tyr)$_3$ and (Phe)$_3$ was obtained (see Fig. 2E, results with Tris–phosphate were similar, not shown). Using a H_2TPP(m-OPh)$_4$ coated capillary offered a complete resolution of this critical pair (Fig. 6D), however, the separation was longer by about 30% running time. The sequence of separated peptides was the same in both coated and uncoated capillaries. (For stabilization of the capillary after coating during the first three runs in alkaline media, see Fig. 6A–C.) It appears likely that the improved separation was preferentially due to the slow-down of the endoosmotic flow though delicate interactions with the porphyrin coating cannot be excluded.

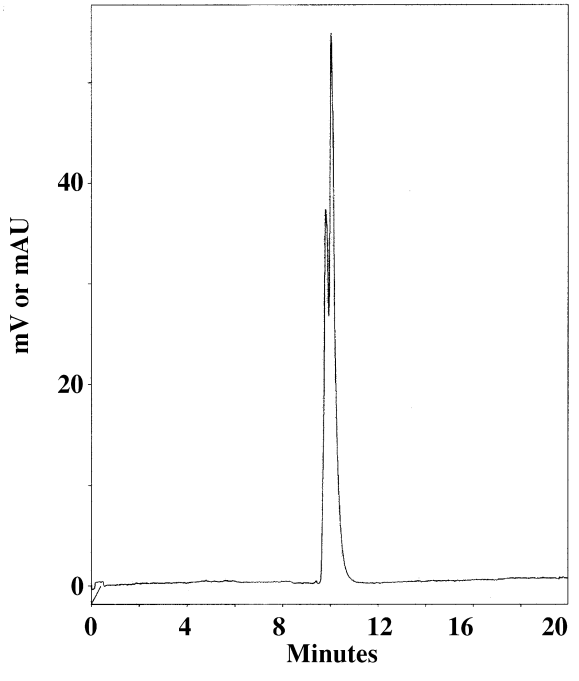


Fig. 5. Separation of the test mixture in H_2TPP(m-OPh)$_4$ coated capillary, 100 mmol/l phosphate, pH 5.5, 15 kV.

4. Conclusions

The conclusions of the presented results can be summarized as follows (Table 2):

1. There is no change in the sequence in which the peptides are brought to the detector whether bare capillary or porphyrin modified capillary is used. However, upon changing the pH of the background electrolyte, the order of elution of Tyr–Gly–Gly, (Phe)$_3$, and (Tyr)$_3$ at pH 2.5 is changed to (Tyr)$_3$, (Phe)$_3$ and Tyr–Gly–Gly if the pH is increased to 8.0 (runs in phosphate buffer).
2. Coating of the capillary wall with H_2TPP(m-OPh)$_4$ at acid pH results in a resolution similar to that obtained with the uncoated column. In alkaline pH, the resolution is complete, however,

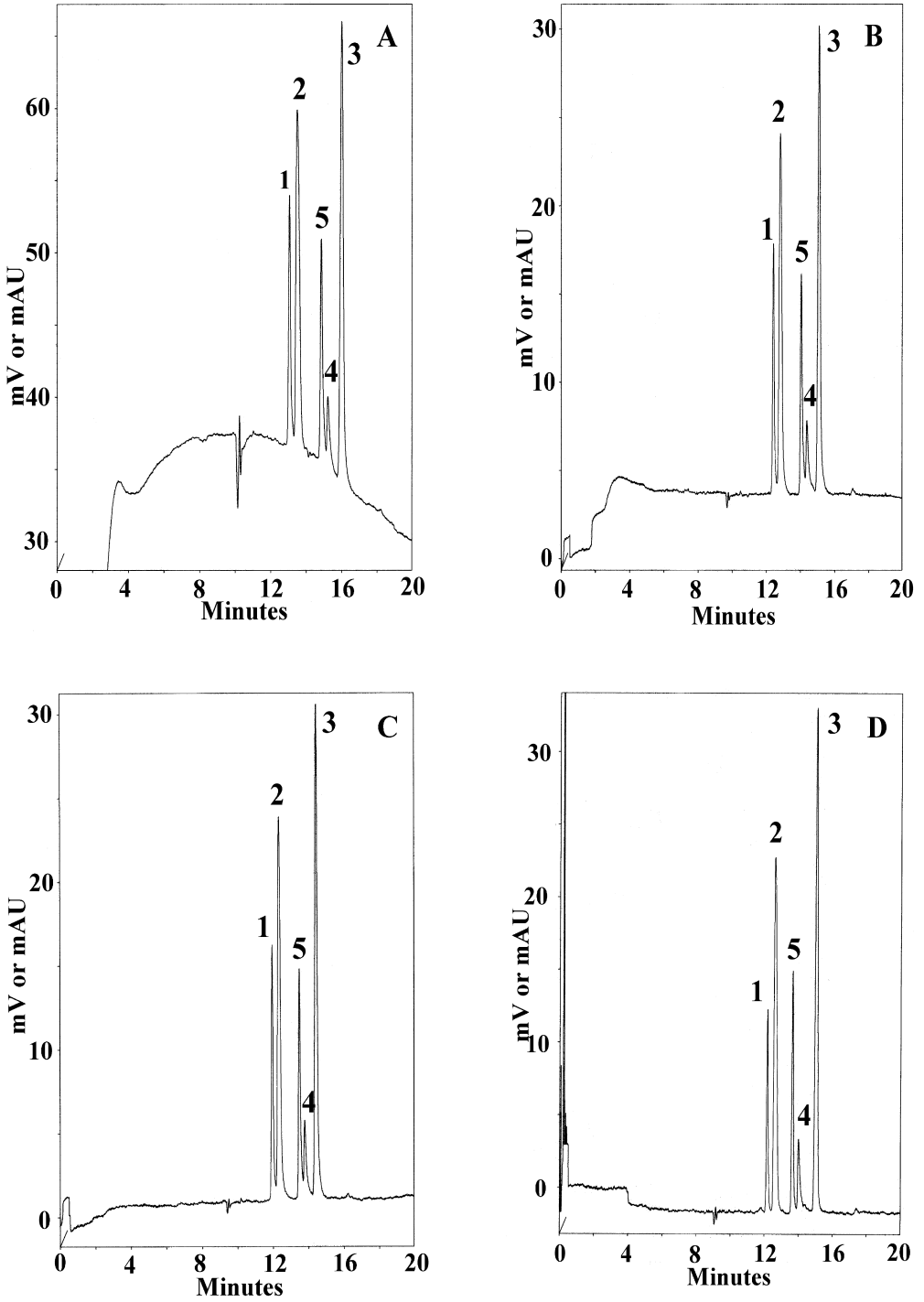


Fig. 6. Stepwise stabilization of the $H_2TPP(m\text{-}OPh)_4$ coated capillary in three subsequent runs A–C, 100 mmol/l phosphate, pH 8.0. (D) shows the result with a well stabilized capillary. Peak identification as in Fig. 2.

Table 2
Summary of the resolution and running times of the set of aromatic peptides

Coating/buffer	pH 2.5		pH 8.0	
	Resolution	Running time of the last peak (min)	Resolution	Running time of the last peak (min)
Uncoated/phosphate	Complete resolution	25.415 (10 kV)	Partially resolved, $(Tyr)_3$ and $(Phe)_3$ unresolved	11.501 (10 kV)
Uncoated/Tris–phosphate	Sufficient, Gly–Gly–Phe and Tyr–Gly–Gly partially resolved	20.365 (10 kV)	Poor resolution	6.744 (10 kV)
H_2TPP(m-OPh)$_4$/phosphate	Complete	24.817 (10 kV)	Sufficiently resolved	14.418 (10 kV)
H_2TPP(m-OPh)$_4$/Tris–phosphate	Sufficient, Gly–Gly–Phe and Tyr–Gly–Gly partially resolved	23.341 (10 kV)	Only three poorly resolved peaks obtained	8.253 (10 kV)
Rh(III)TPP(m-OPh)$_4$/phosphate	Completely resolved	13.022 (15 kV)	Not measured	
Rh(III)TPP(m-OPh)$_4$/Tris–phosphate	Completely resolved	8.88 (15 kV)	Not measured	

the run time is increased by about 30% in the coated capillary.

3. With Rh(III)TPP(m-OPh)$_4$ coating (at acid pH, i.e. pH 2.5), the resolution is always complete. If Tris–phosphate is used as background electrolyte, the whole run is completed within less than 9 min, which represents shortening by 40% compared to the bare capillary.

It is possible to conclude that both delicate interactions and ionic properties of the analytes are involved in the separation though the latter are clearly dominating. Coating of the capillary in particular with Rh(III)TPP(m-OPh)$_4$ can help (under proper pH conditions) resolution of otherwise poorly resolved peaks and can speed up the separation in acid buffers (Tris–phosphate).

Acknowledgements

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