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Journal of Chromatography A, 990 (2003) 159–167
JOURNAL OF CHROMATOGRAPHY A
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Open tubular capillary electrochromatography of underivatized amino acids using Rh(III) tetrakis(phenoxyphenyl)porphyrinate as wall modifier

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Received 3 June 2002; received in revised form 23 September 2002; accepted 2 October 2002

Abstract

The separation of 17 ''common'' underivatized amino acids was attempted by open tubular capillary electrochromatography (OT-CEC) in fused-silica capillaries coated with Rh(III) tetrakis(phenoxyphenyl)porphyrinate (Rh(III)TPP(*m*-OPh)$_4$OAc) using sodium phosphate and Tris–phosphate buffers as background electrolytes (BGEs). The OT-CEC separation of amino acids was compared with that obtained by capillary zone electrophoresis in bare fused-silica capillaries using the same BGEs. The amino acids were not derivatized and the UV-absorption detection was set at 200 nm. Depending on the experimental conditions at least 15 amino acids were separated. The best separations were obtained in a Rh(III)TPP(*m*-OPh)$_4$OAc-coated capillary in 50 m*M* Tris–100 m*M* phosphate buffer at pH 2.25. Separation of the critical triplet Val–Ile–Leu was always at least indicated being better at higher BGE concentrations. Regarding the sensitivity of the method, lower concentration limits of detection (LODs) in the coated capillary were obtained for Thr, Gly, Tyr, and Val; the other amino acids exhibited lower LODs in the uncoated capillary. The separation of acidic amino acids was not achieved.

Keywords: Electrochromatography; Capillary column; Amino acids; Porphyrins; Metalloporphyrin

1. Introduction

Both chromatographic and electromigration techniques have been widely applied to the separation of common amino acids [1–3]. In view of the fact that most of these analytes are devoid of a strong chromophore which to a considerable extent precludes direct measurement of UV absorbance changes, it is not surprising that a lot of attention has been paid to their derivatization in order to enlarge the possibilities of their detection. A number of derivatization reactions directed towards the modification of amino groups became common over the years: 5-dimethylaminonaphthalene-1-sulfonyl chloride (Dansyl chloride, Dns-Cl), 9-fluorenylmethylchloroformate (FMOC), 2,4-dinitrofluorobenzene (DNFB), *o*-phthaldialdehyde (OPA), naphthalene-2,3-dicarboxaldehyde (NDA) and

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0021-9673/02/$ – see front matter
doi:10.1016/S0021-9673(02)01655-2

phenylisothiocyanate (PTC) being the most commonly used derivatization reagents (for reviews, see Refs. [2–4]). In capillary electromigration methods all the above-mentioned derivatization reactions can be exploited, as in this category of separations on column UV absorbance detection remains the most commonly used approach for monitoring the separated analytes.

The above derivatization reactions not only introduce a particular chromophore into the molecule (thereby increasing the sensitivity of detection) but they simultaneously alter the physico-chemical properties of the parent compounds (amino acids) making them less hydrophilic and suitable for different electrokinetic separation modes. Regarding the more recent detection techniques, laser induced fluorescence (using OPA or FITC derivatives) is to be mentioned first because of the high sensitivity of this approach [5–10].

Another option, electrochemical detection (ECD), generally offers high sensitivity and selectivity of analytes prone to reduction/oxidation reactions which, under routinely used conditions, is not the case with common amino acids. This fact also calls for pre-separation derivatization, typically with OPA or NDA [11,12]. Metal oxide (25% CuO) modified carbon paste electrodes can be used for ECD, however, the reported sensitivity is rather low [13]. Better results were obtained by exploiting Cu complexation of the underivatized common amino acids at the Cu electrode [14].

Another approach for the detection of underivatized common amino acids regards chemiluminiscence detection by making use of a tris(2,2′-bipyridine)ruthenium(III) ($Ru(bpy)_3^{3+}$) complex which was reported suitable for capillary electrophoresis (CE); however the limit of detection (LOD) varied greatly with different amino acids [15]. In a more recent paper Lee et al. [16] exploited stacking of large volumes of samples in CE which allowed acceptable LODs to be reached without derivatization, however, with protein and non-protein amino acids possessing aromatic moiety in their molecule only.

For the purpose of analyzing nutritionally relevant amino acids, direct UV detection (without derivatization) was used by Klampfl et al. [17]; the LOD values varied between 10 and 50 ppm (low ppm for amino acids possessing an aromatic residue). The method is obviously not extremely sensitive, however, it appears to be sufficient for the purpose for which it was used. Generally speaking the problem of direct UV detection of amino acids during CE separation stems from the fact that these analytes are only weak chromophores at short wavelengths (~185 nm) where the absorbance of their carboxyl group can be exploited. This absorbance wavelength lies at the ultimate limit of wavelengths that the operator can select with commercially available CE devices (190–200 nm), not to speak of the generally known problems with detecting delicate absorbance changes of the light beam passing the tiny capillary detector window. (Other reports relevant to the UV detection of underivatized common amino acids can be found in Refs. [18–24].) The last possibility for detection of underivatized amino acids is indirect detection: Arce et al. [25] used calixarenes as background probes for this purpose.

Recently, capillary electrophoresis electrospray ionization mass spectrometry (CE–ESI-MS) has rapidly developed as a powerful analytical tool for charged species. The ESI mode has proven to be sensitive, versatile and relatively easy to use in combination with CE. Few studies applied to amino acid analysis have been performed by CE–ESI-MS. Garcia and Henion reported a gel-filled CE–MS method for the determination of Dansyl-amino acids [26], while Lu et al. determined four amino acids and one tripeptide by CE–ESI-MS [27]. Soga and Heiger applied CE–ESI-MS as a method for determination of 19 underivatized amino acids and several physiological amino acids [28]. The same mode of detection was presented in the separation of 20 underivatized amino acids in single run. The method was also used for quantification of amino acids in child plasma sera [29].

All the above results of the separation of amino acids refer to CE methods. Only a limited number of reports is available on the capillary electrochromatography (CEC) of these analytes (for a review, see Ref. [30]). Briefly, separations of amino acids by CEC are performed either on classical C_{18}-modified packings or using new monolithic columns, e.g. CEC in packed columns with on-line electrospray ionization and time-of-flight mass spectrometry detection was used for the separation of phenylthiohydantoin

(PTH)-amino acid derivatives [31] and monolithic, cross-linked polyacrylamide-based columns were employed in the analysis of dansylated amino acids [32]. To the best of our knowledge no reports are available on amino acid separation by open tubular CEC (OT-CEC).

Iodorhodium(III)tetramethylchiroporphyrin was presented as a potential reagent for chiral selection and analysis of amino compounds. It affords a 1:1 adduct with (*R*)- or (*S*)-2-aminopropanol, and it binds *rac*-2-aminopropanol with an enantiomeric excess of 69% in favor of the (*R*)-enantiomer at the thermodynamic equilibrium after several days [33].

The aims of this work were: (i) to reveal whether modification of the inner capillary wall by an appropriate modifier, Rh(III)tetrakis(phenoxyphenyl)porphyrinate (Rh(III)TPP(*m*-OPh)$_4$OAc) can bring about better selectivity and thus make OT-CEC a useful approach for amino acid separation, and (ii) to reveal whether or not non-covalent interaction of the amino acids with the capillary wall modifier can help to improve the UV detectability of the analytes involved. The reasons for selection of particular metalloporphyrin derivatives are as follows: firstly, bulky phenoxy-phenyl periphery substitution of the porphyrin core provides sufficient lipophilicity of this particular derivative which in turn gives long-term stability of capillary coating modification. The rationale why a metallocomplex with the ability to form axial ligand binding was applied is based on the concept of the multiple binding mode of modifier enabling efficient separation. Here we have anticipated that combination of $\pi-\pi$ stacking, and hydrophobic interaction together with axial ligand binding (for carboxylate and amino groups of analytes) would increase the selectivity of the separation as revealed with aromatic amino acids and a model mixture of tripeptides in our previous papers [34,35].

2. Experimental

2.1. Instrumentation

All experiments were performed using a SpectraPhoresis 500 (SP 500) 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 an on-line, variable-wavelength UV–Vis detector.

The separations were run in two types of capillaries. Capillary zone electrophoretic (CZE) separations were performed in an uncoated fused-silica capillary, 50 μm I.D.×375 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA) fitted into the SP 500 cartridge. The total capillary length was 42.5 cm, and the effective length (to the detector) was 35.0 cm. OT-CEC separations were carried out in the fused-silica capillary modified with porphyrin derivative Rh(III)TPP(*m*-OPh)$_4$OAc (for the formula of the porphyrin derivative see Fig. 1). Other characteristics of the modified capillary were the same as specified above. The capillary modified by this procedure withstood at least 30 consecutive runs.

The separations were run at 15 kV (with electric current in the range 30–78 μA, depending on the background electrolyte (BGE) composition) at 20 °C and the analysis time was up to 30 min; detection wavelength was set at 200 nm.

An uncoated fused-silica capillary was conditioned sequentially by washing it for 5 min with water, 10 min with 1 *M* NaOH, 5 min with water and finally 5 min with the running buffer. For reconditioning the capillary was washed with the running buffer after each run for 5 min.

The modified capillaries were conditioned by

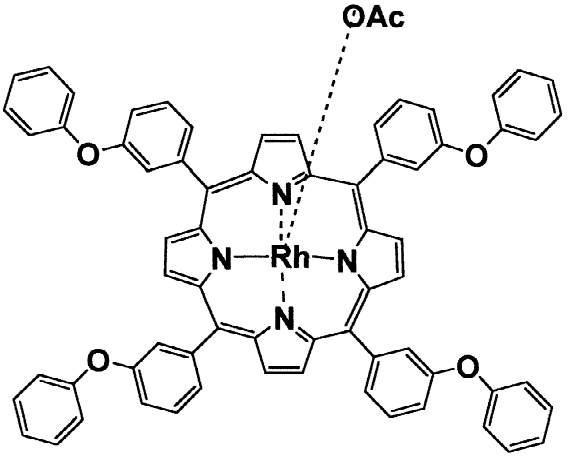

Fig. 1. Structure of Rh(III)5,10,15,20-tetrakis(*m*-phenoxyphenyl)-porphyrinate (Rh(III)TPP(*m*-OPh)$_4$OAc) used as the modifier of the fused-silica capillary.

sequentially washing them with the running buffer for 10 min.

2.2. Reagents

Sodium dihydrogenphosphate, sodium hydrogenphosphate, sodium hydroxide and phosphoric acid (all of analytical grade) were obtained from Lachema (Brno, Czech Republic). Amino acids alanine, arginine, cystein, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine were purchased from Sigma (St. Louis, MO, USA) and were of at least 97% purity grade.

The porphyrin derivative, Rh(III)TPP(*m*-OPh)$_4$OAc, was synthesized in our laboratory (unpublished results) and characterized by elemental analysis, ^{1}H NMR, MS and IR spectrometry.

Samples of amino acids for analysis were prepared by dissolving an appropriate amount (2.0–2.4 mg) of each solute in 200 μl of Milli-Q water and stored below 4 °C. Tyrosine was prepared by dissolving 6.2 mg in 600 μl of 0.1 *M* sodium phosphate buffer, sonicated and filtered. The supernatant was used for further experiments. The volumes of injected stock solutions of amino acids were in the range of 3–10 nl. All samples were injected hydrodynamically, pressure 75 mbar, injection time 5–15 s.

2.3. Coating of the capillary wall

A new fused-silica capillary was sequentially washed with water, 1 *M* NaOH and water, each step for 5 min. Next it was washed with methanol, dichloromethane and dried in a stream of air. The capillary was then filled with the solution of the porphyrin derivative, Rh(III)TPP(*m*-OPh)$_4$OAc, in dichloromethane (2 mg/ml) and deposited 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 the running buffer for 5 min. To stabilize and equilibrate the coating the capillary was run in the sodium phosphate BGE at 20 kV for 20 min; this procedure was repeated until the baseline drift was eliminated.

2.4. Background electrolytes

The following BGEs were used both for CZE and OT-CEC experiments: 50 m*M* sodium–100 m*M* phosphate, pH 2.25; 50 m*M* Tris–100 m*M* phosphate, pH 2.25 or 100 m*M* Tris–200 m*M* phosphate, pH 2.25; 100 m*M* sodium phosphate buffers with pH 2.5, 3.8, 5.5, 7.0, 8.6 and 10.0, respectively (100 m*M* phosphate, pH adjusted by the addition of appropriate amount of 1 *M* NaOH) were used to investigate the influence of pH on the separation pattern of the tested amino acids mixture.

3. Results and discussion

As demonstrated in Fig. 2, separation of underivatized common amino acids by CZE can be obtained in strongly acidic BGEs at pH 2.25. Though the pH of the BGE is decisive, slight variations in the profile of separated amino acids were observed using two BGEs of different composition, i.e. Tris–phosphate (Fig. 2A) and sodium phosphate (Fig. 2B). Under the conditions selected Lys was always the first amino acid to appear in front of the detector window, while a fused peak of cysteine and cystine was last. As in any separation of complex amino acid mixtures, there were a critical triplet and a critical pair that were difficult to separate (Val–Ile–Leu, and Thr–Trp). By increasing of the pH of the BGE to less acid, neutral and alkaline values the separation worsened and at pH 5.5 failed completely (data not shown).

The influence of the BGE composition (at pH 2.25) is evident by comparing Fig. 2A and B. Sodium phosphate BGE (Fig. 2B) offers better peak shapes, plate number and resolution compared to Tris–phosphate BGE (Fig. 2A). The other advantages of sodium phosphate BGE over Tris–phosphate are a complete separation of serine from the combined peak of Val–Ile–Leu, better separation of methionine from glutamine and a complete separation of proline from tyrosine. On the other hand threonine and tryptophan are at least partially separated in Tris–phosphate BGE while in sodium phosphate BGE these two amino acids comigrate and form a single fused peak (Table 1). However, in Tris–phosphate the tryptophan peak is only incom-

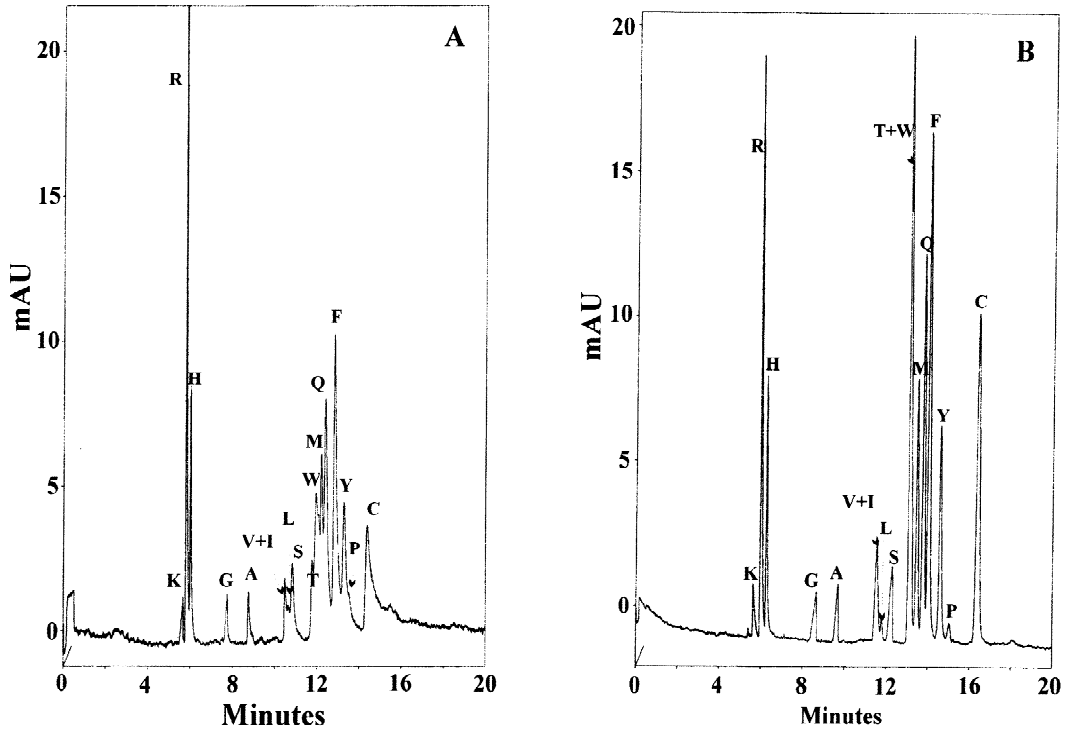

Fig. 2. CZE separation of the 17 ''common'' underivatized amino acids in uncoated fused-silica in acid (pH 2.25) BGEs: (A) 50 m*M* Tris–100 m*M* phosphate, and (B) 50 m*M* sodium–100 m*M* phosphate, 15 kV, capillary 42.5 cm (35.0 cm to the detector)×50 μm I.D.×375 μm O.D. Single letter symbols for amino acids are used for the description of individual peaks.

pletely resolved from the adjacent peaks of threonine (preceding) and methionine (migrating later). It is also noticeable that in sodium phosphate BGE the separation (as judged from the migration time of the cystine-cysteine peak) was 2 min longer which obviously helped achieve better resolution of some adjacent peaks.

In order to improve the separation, the coating of

Table 1
Comparison of delicate differences in amino acids migration order in OT-CEC separations in a Rh(III)TPP(*m*-OPh)$_4$OAc-coated capillary and in CZE separations in the uncoated fused-silica capillary, in 50 m*M* sodium–100 m*M* phosphate or 50 m*M* Tris–100 m*M* phosphate BGEs, pH 2.25

Separation mode/capillary	BGE	Amino acids migration order (migration time)
OT-CEC/coated	Tris–phosphate	Thr (10.088), Trp (10.244), Met (10.414), Gln (10.576), Phe (10.924), Tyr (11.287)
OT-CEC/coated	Sodium phosphate	(Thr+Trp) (12.911), Met (13.139), Gln (13.462), Phe (13.736), Tyr (14.351)
CZE/uncoated	Tris–phosphate	Thr (11.748), Trp (11.925), Met (12.181), Gln (12.373), Phe (12.814), (Tyr+Pro) (13.400)
CZE/uncoated	Sodium phosphate	(Thr+Trp) (13.085), Met (13.446), Gln (13.753), Phe (14.007), Tyr (14.575)

Amino acids in parenthesis represent fused peaks, numbers in parenthesis at individual amino acids represent migration times of the particular solute in minutes.

the inner surface of the capillary with porphyrin derivative, Rh(III)TPP(*m*-OPh)$_4$OAc, was introduced using both sodium phosphate and Tris–phosphate buffers as BGEs (Fig. 3A,B). Of the four variations investigated in detail (i.e. sodium phosphate and Tris–phosphate BGEs, coated and uncoated capillary), the fastest separation, lasting only 12.5 min, was using Tris–phosphate and Rh(III)TPP(*m*-OPh)$_4$OAc coating; the resolution, particularly of tryptophan, methionine and glutamine was slightly better than in the uncoated capillary. Moreover in this system cysteine (Cys) was clearly separated from cystine ((Cys)$_2$). The best separation of the main peaks was achieved using sodium phosphate BGE, however, in this case the system was not capable of separating the Val–Ile–Leu triplet or threonine from tryptophan. Separation of the critical triplet Val–Ile–Leu was in both types of BGEs (Tris–phosphate and sodium phosphate) always at least indicated and was considerably better if the concentrations of the BGEs were increased (100 m*M* sodium or Tris, 200 m*M* phosphate), both in Rh(III)TPP(*m*-OPh)$_4$OAc-coated and in uncoated capillaries (Fig. 4). Much better resolution, however, was obtained at the beginning of the electropherogram (Lys–Arg–His triplet). On the other hand all separations run in the sodium phosphate BGE suffered from undesirable background noise. The order in which the individual amino acids came in front of the detector window was the same both in uncoated and in coated capillaries. Attempts to obtain better resolution by increasing the pH of the BGE and using coated capillaries did not offer acceptable results (data not presented).

Separation of acidic amino acids (glutamic and aspartic) was not possible in any of the systems used as they provided very dispersed peaks and ruined the separation of the other amino acids. Regarding the concentration LODs, better values for Thr, Gly, Tyr and Val were obtained in the Rh(III)TPP(*m*-OPh)$_4$OAc-coated capillary, while the other amino acids exhibited better LODs if an uncoated capillary

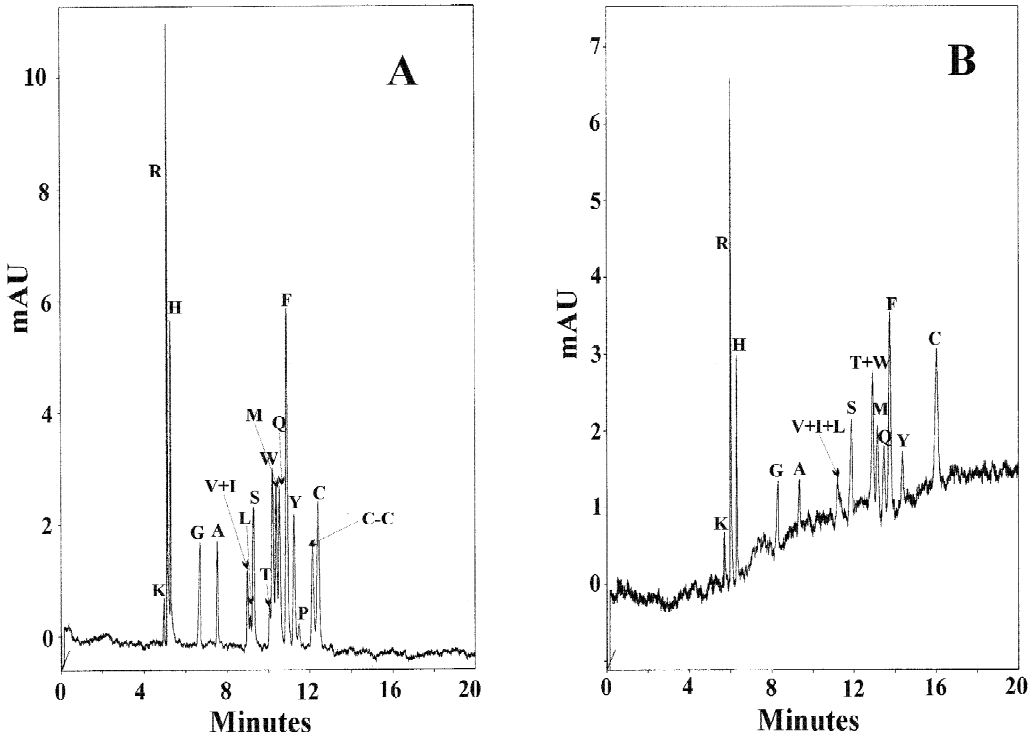

Fig. 3. Separation of the tested set of amino acids in Rh(III)TPP(*m*-OPh)$_4$OAc-coated capillary using (A) 50 m*M* Tris–100 m*M* phosphate, and (B) 50 m*M* sodium–100 m*M* phosphate buffers as BGEs. Separation conditions as in Fig. 2.

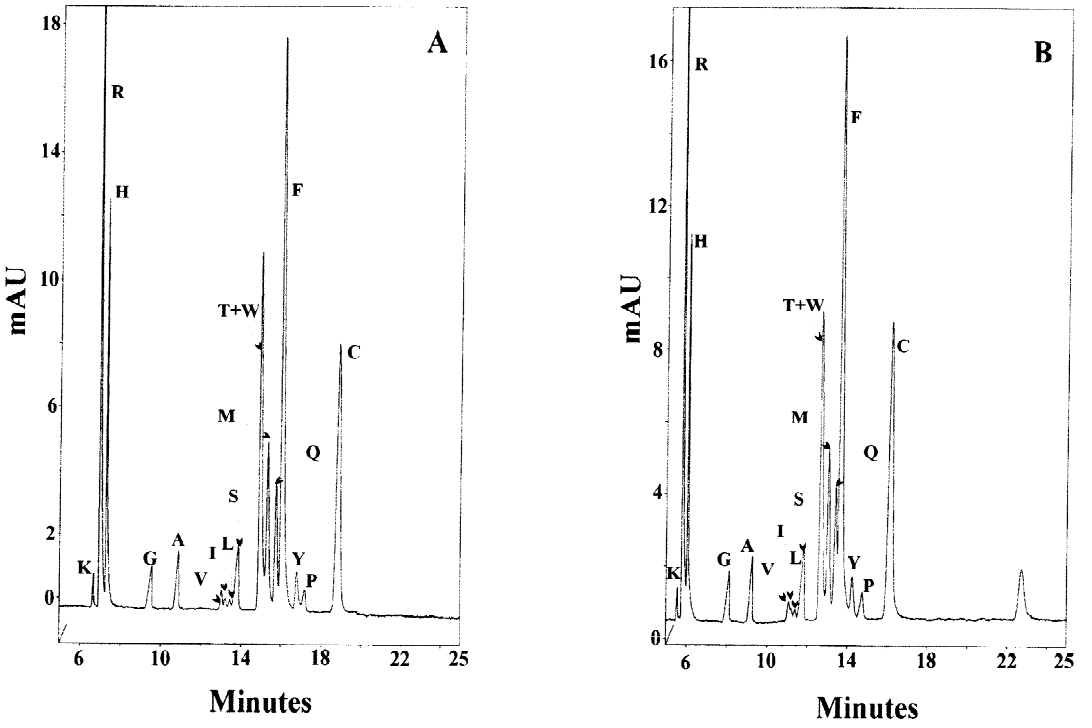

Fig. 4. Separation of the tested set of amino acids in (A) uncoated and (B) Rh(III)TPP(*m*-OPh)$_4$OAc-coated capillaries when 100 m*M* Tris–200 m*M* phosphoric acid was used as the BGE. Other conditions as in Fig. 2. The last peak in record B is an unidentified admixture.

was used (Table 2). As expected, LODs of aromatic amino acids are lower than those of aliphatic amino acids. A relatively low LOD of aliphatic amino acid lysine was achieved probably due to the fastest migration of this analyte both in the initial water plug (concentration effect) and in the acid BGE (limited time for diffusion). In the cases where better detection limits were obtained with Rh(III)TPP(*m*-OPh)$_4$OAc-coated capillary it is feasible to assume that this change was similar to the mechanism reported in Ref. [14] for $Ru(bpy)_3^{3+}$ complexes, i.e. by the higher UV absorption of complexed analyte than of a free analyte.

4. Conclusions

(1) It has been shown that the 17 ''common'' underivatized amino acids can be separated by OT-CEC in the fused-silica capillary coated with porphyrine derivative, Rh(III)TPP(*m*-OPh)$_4$OAc, using acid BGEs (sodium phosphate or Tris–phosphate, pH 2.25).

(2) Separation of the Val–Ile–Leu triplet was always at least indicated being better when the cation of the BGE (sodium or Tris) was 100 m*M*, no matter whether Rh(III)TPP(*m*-OPh)$_4$OAc coating was used or not.

(3) The best separations were obtained either with uncoated capillary using 50 m*M* sodium–100 m*M* phosphate as BGE or using using 50 m*M* Tris–100 m*M* phosphate buffer and a Rh(III)TPP(*m*-OPh)$_4$OAc-coated capillary.

(4) The separations were always shorter than 20 min (the shortest runs were obtained with the Rh(III)TPP(*m*-OPh)$_4$OAc-coated capillary) and the concentration LODs ranged from 1350.0 to 0.59 μg/ml depending on the nature of the amino acids and the separation system used. Better detection limits for Thr, Gly, Tyr and Val were obtained with a coated capillary, while the others exhibited better values if an uncoated capillary was used.

Table 2
Comparison of concentration LODs of amino acids separated by OT-CEC in Rh(III)TPP(*m*-OPh)$_4$OAc-coated capillaries and by CZE in uncoated fused-silica capillaries, run in 50 m*M* Tris–100 m*M* phosphate BGE, pH 2.25

Amino acid	LOD (μg/ml) coated capillary	LOD (μg/ml) uncoated capillary	Comparison of LODs in coated and uncoated capillary[a]
Lys (K)	10.8	1.88	+
Arg (R)	5.4	0.94	+
His (H)	3.36	0.59	+
Gly (G)	116.3	121.8	–
Ser (S)	345.8	181.3	+
Ala (A)	172.3	128.0	+
Leu (L)	973	255	+
Thr (T)	114.5	480	–
Trp (W)	1.8	1.56	0
Phe (F)	34.8	3.0	+
Met (M)	20.8	13.6	+
Gln (Q)	40.0	26.3	+
Tyr (Y)	24.6	51.5	–
Pro (P)	1350	590	+
Cys (C)	118.0	61.8	+
Val (V)	58.4	122.5	–

[a] A ''plus'' (''minus'') sign indicates a higher (lower) detection limit in Rh(III)TPP(*m*-OPh)$_4$OAc-coated capillary, while a ''zero'' sign indicates no change in detection sensitivity.

(5) Coating of the capillary with Rh(III)TPP(*m*-OPh)$_4$OAc and running the separation in Tris–phosphate buffer at pH 2.25 offered a complete resolution of Cys and (Cys)$_2$ (these two amino acids formed a fused peak under all other conditions).

Acknowledgements

This work was supported by the Grant Agency of the Czech Republic, grant No. 203/02/1467, by grant No. 223400008 of the Ministry of Education of the Czech Republic and by the research project AVOZ4 055 905 of the Academy of Sciences of the Czech Republic.

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