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# Optimization of a free separation of 30 free amino acids and peptides by capillary zone electrophoresis with indirect absorbance detection: a potential for quantification in physiological fluids

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**Abstract**

This report describes a rapid, single-run procedure, based on the optimization of capillary electrophoresis (CE) and indirect absorbance detection capabilities, which was developed for the separation and quantification of 30 underivatized physiological amino acids and peptides, usually present in biological fluids. *p*-Aminosalicylic acid buffered with sodium carbonate at pH 10.2±0.1 was used as the running electrolyte. Electrophoresis, carried out in a capillary (87 cm×75 μm) at 15 kV potential (normal polarity), separated the examined compounds within 30 min. Limits of detection ranged from 1.93 to 20.08 μmol/l (median 6.71 μmol/l). The method was linear within the 50–200 μmol/l concentration range (*r* ranged from 0.684 to 0.989, median $r=0.934$). Within run migration times precision was good (median C.V.=0.7%). Less favorable within run peak area precision (median C.V.=6.6%) was obtained. The analytical procedure presented was successfully tested for separation and quantification of amino acids in physiological fluids, such as plasma or supernatant of macrophage cultures. Sample preparations require only a protein precipitation and dilution step. 

*Keywords:* Amino acids; Peptides

## 1. Introduction

The free amino acids present in biological fluids are good indicators in various metabolic disorders [1–4]. There are numerous methods for their determination employing various techniques such as gas chromatography, high pressure liquid or ion-exchange chromatography [5–11], separating usually 20 to 30 or an even greater number of free amino acids and related compounds.

Since capillary electrophoresis (CE) was introduced as a new technology offering rapid separation of various ionic and/or ionizable compounds with low sample and solvent consumption, there has been attempts to use it for amino acid investigations [12–17]. Much of the early development of CE involved derivatized amino acids as test solutes [18]. However, it is still a challenging problem because of the large number of free amino acids in physiological fluids and the similarities of their electrochemical characteristics complicate separation by CE. Detection could be an additional problem, since amino acids generally possess very limited chromophores. Different reagents for amino acid derivatization have

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been used [10,12–14,19]. The reported CE methods usually separate 20 or fewer amino acids in a single run [10,12,14,15,20–25]. However, the derivatization process can be very time consuming and requires considerable additional work, as well as changing the native electrophoretic mobility of the analyte [23].

Recently there have been attempts to analyze native amino acids employing CE [12,21–24]. Derivatization procedures prior to CE could be avoided through the use of various strategies for amino acid detection, such as direct or indirect UV absorption, indirect fluorescence, electrochemical detection [14,15,17,21]. Moreover, it has been reported that the optimization of capillary electrophoresis–electrospray mass spectrometry allows quantification of 20 natural amino acids without any derivatization [21]. A similar number of amino acid peaks has been separated using indirect absorbance detection with *p*-aminosalicylic acid as the carrier buffer and a good background absorbance provider [23].

This report describes a rapid, single-run procedure, based on the optimization of CE and indirect absorbance detection capabilities, developed for the separation and quantification of 30 underivatized physiological amino acids and peptides, usually present in biological fluids. The analytical procedure presented is sufficiently sensitive to be used for the determination of some free amino acids in biological fluids. We have successfully tested it for amino acid separation and quantification in plasma and supernatant of macrophage cultures.

## 2. Experimental

### 2.1. Capillary electrophoresis system

Separations were performed on a P/ACE 5010 capillary electrophoresis system (Beckman, Palo Alto CA, USA) equipped with P/ACE system software controlled by an IBM computer. Data analysis was performed on the P/ACE system. Indirect absorbance at 254 nm was measured. Uncoated fused-silica capillary tubing (Beckman PN 338454) 87 cm total length (80.5 cm to detector) and 75 μm I.D. was used. The capillary was assembled in the cartridge with a 100×800 μm aperture and the detection window was located 6.5 cm from the capillary outlet.

### 2.2. Capillary electrophoresis procedures

Normal polarity and an aqueous running electrolyte, consisting of 8 mmol/l *p*-aminosalicylic acid and 2 mmol/l sodium carbonate at pH 10.2±0.1 were used for separations. A constant voltage of 15 kV was applied at a temperature of 20 °C. Under these conditions a current of 7.5±0.2 μA was encountered.

Samples were introduced into the capillary using 0.5 p.s.i. injection for 10 s (approximatively a 112-nl volume injected). Before each run the capillary was reconditioned with running buffer for 1 min. Between runs the capillary was washed with sodium hydroxide solution (0.1 mol/l) for 1 min, followed by water for 1 min. All procedures were automatically controlled by the P/ACE system.

In addition, to select the optimal CE conditions for separations, the effects of running electrolyte composition as well as different voltages and temperatures were examined. In these experiments we used running electrolytes with different concentrations of PAS (8 and 10 mmol/l) and $Na_2CO_3$ at alkaline pH ranging from 10.1 to 10.7 pH units. Separations were carried out for 30–50 min at voltages ranging from 10 to 15 kV at temperatures of 19, 20, and 21 °C.

### 2.3. Chemicals and standards

All chemicals were of analytical grade. *p*-Aminosalicylic acid as the sodium salt (PAS, MW=211.2) was obtained from Serva (Feinbiochemica, Heidelberg/NewYork). Sodium carbonate ($Na_2CO_3$) and sodium hydroxide (NaOH) were purchased from Merck (Darmstadt, Germany). Individual amino acids were from several suppliers. L-alanine (Ala), L-arginine·HCl (Arg), L-aspartic acid (Asp), L-glutamic acid (Glu), glycine (Gly), L-histidine·HCl (His), L-isoleucine (Ile), L-leucine (Leu), L-lysine (Lys), L-methionine (Met), L-phenylalanine (Phe), L-proline (Pro), L-serine (Ser), L-threonine (Thr), L-tyrosine (Tyr), L-valine (Val), L-cystine (Cyss), L-glutamine (Gln), L-asparagine (Asn), DL-tryptophane (Trp), β-alanine (bALA), γ-amino butyric acid (GABA), DL-homocystine (hCyss), DL-δ-hydroxy-

lysine·HCL (HyLys), L-cystine (Cyss), L-ornithine·HCL (Orn) and 3-methylhistidine (3-MeHis) were obtain from Serva (Heidelberg, Germany). DL-homocysteine (hCys), L-citrulline (Cit) and L-cysteine·HCl (Cys) were obtained from Fluka (Switzerland) and Merck (Darmstadt, Germany), respectively. L-carnosine and L-anserine were purchased from Serva (Heidelberg, Germany), while oxidized gluthathione hydrate (GSSG) and reduced gluthathione (GSH) were products of ICN Biomedicals (USA).

Solutions were prepared with water obtained from the Milli-Q water purification system (Waters, Millipore, Milford, MA, USA). The running electrolytes were daily prepared from individual stock solutions of 100 mmol/l PAS and 10 mmol/l $Na_2CO_3$ and the pH adjusted to the desired value with $Na_2CO_3$ or NaOH if necessary. Usually no pH adjustment with NaOH was used.

For calibrations, individual stock solutions of the examined amino acids and peptides were prepared in Milli-Q water at the concentration of approximately 10 mmol/l. Also the mixture of 17 amino acids (Beckman, PN 338088, amino acid standard for hydrolyzate analysis, ''STD'') was used. It contains Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val, Cys and ammonia in concentrations of 2.50 μmol/ml per constituent except for Cys (1.25 μmol/ml). It was diluted to 1 mmol/l concentration (solution STD). The other amino acid stock mixture consisting of Orn, GABA, Cit, hCyss, HyLys, bALA, GSSG and carnosine at 1 mmol/l concentration each was prepared from the individual 10 mmol/l stock solutions (solution S1). The third mixture consisting of Trp, Gln, Asn, hCys and GSH at 1 mmol/l concentration each (solution S2) was also prepared daily from the individual 10 mmol/l stock solutions. Working solutions (I) at 50, 100, 150, 200 μmol/l concentrations of individual compounds were prepared daily by mixing solutions STD, S1 and S2.

### 2.4. Biological samples

Pooled human plasma from healthy subjects and the supernatant of rat peritoneal macrophages cultivated for 24 h in serum-free medium (''Biorich 2''; Flow, Irvine, Scotland) were analyzed. The samples were stored at −20 °C until analysis (no more than 8 weeks) and deproteinized with acetone (100 μl biological sample plus 100 μl acetone) before analysis. Obtained supernatants (1 min centrifugation on Minifuge, Beckman) were five-fold diluted with Milli-Q water or solution with known concentrations of the examined compounds prior to the analysis. Also within run precision of the migration times and peak areas were determined on five-fold diluted deproteinized samples of the examined biofluids. Concentrations of the amino acids identified in these samples were calculated using means of the corresponding peak areas for the standard 100 μmol/l mixture, considering dilution factors.

### 2.5. Statistical evaluation of the method

Peak identification for each analyte was carried out by the injection of individual amino acids and/or by spiking samples with known standards. Migration times and peak areas were recorded.

Within- and between run precision of migration times and peak area measurements were determined within a period of 2 weeks. Values were expressed as means±standard deviation (SD) with coefficients of variation (C.V.). Non-parametric analysis was also performed (minimum, maximum, median). Linearity was assessed from the analysis of standard amino acid mixtures at concentrations ranging from 50 to 200 μmol/l. Linear regression coefficients ($r$), as well as slopes and intercepts for the calibration curves were determined ($n=33$).

## 3. Results

In order to obtain a single-run CE procedure for separating the large number of amino acids and peptides, normally presents in physiological fluids, we have tested various analytical conditions. The influence of running electrolyte composition (pH, ionic strength, concentrations of PAS and $Na_2CO_3$), as well as the temperature and voltage, on separation during CE was examined using a mixture of 30 compounds. At the very beginning we observed that supplementation of background (PAS) solution with $Na_2CO_3$ improved pH stability of the running electrolyte during CE. This was important and crucial in overcoming problems associated with reproducibility

of the separations, the precision of migration times and peak area measurements of the examined compounds. Here we present only the results obtained with running electrolyte containing both PAS and $Na_2CO_3$.

The running electrolyte was selected after experiments with various concentrations of PAS (8 and 10 mmol/l) and different pH (pH 10.15, 10.35, 10.7) at constant temperature (20 °C) and voltage (15 kV) (Figs. 1 and 2). It was found that the running electrolyte containing 8 mmol/l PAS in 2 mmol/l $Na_2CO_3$ at a pH of 10.15 provided the best separation of the examined compounds (Figs. 1a and 2c). Under these conditions we examined the effects of temperature (19, 20, 21 °C) (Fig. 3) and voltage (10, 11, 15 kV) (Fig. 4) alterations on amino acid and peptide separations. A temperature of 20 °C and 15 kV voltage were found to be optimal.

Under these optimal conditions migration times of the 33 compounds were determined by individual injection (Table 1). The results obtained indicate that the migration time of 3-MeHis was close to Phe, while anserine migrated near carnosine. We also noticed that Cyss and Cys had almost the same migration times. That may have been due to Cyss conversion to Cys during preparation. Therefore, 3-MeHis, Cyss and anserine were not included in the standard amino acid/peptide mixtures used in further examinations.

In order to test the interaction between the examined compounds in the mixture and to determine the precision of the measurements, we analyzed a standard calibration mixture (solution I) at 100 μmol/l concentration. Under the previously determined optimal conditions 29 peaks were separated from the mixture of 30 compounds. Namely, in the a single run, within less than 30 min, 25 amino acids (Arg, Lys, Orn, Pro, GABA, HyLys, β-Ala, Trp, Cit, Val, Phe, Ala, His, Met, Gln, Thr, Asn, Gly, Ser, Tyr, hCyss, hCys, Cys, Glu, Asp) and three peptides (carnosine, GSSG, GSH) were separated as individually measurable peaks, while Leu and Ile comigrated as a single peak (Fig. 5). However, we have to point out that the group of Phe, Ala, His, Met and Gln migrated within approximatively 35 s, while the migration times of Asn, Gly, Ser ranged within less than half a minute. For that reason, partial peak overlapping occurred occasionally, particularly if high concentrations were analysed. Considering only the well separated peaks (more than 50% of the areas separated), we determined the limit of detection and linearity (Table 1). The limits of detection (LODs), based on a signal-to-noise ratio of 3, ranged from 1.93 μmol/l for both Glu and GSSG to 20.08 μmol/l for Gly. However, median LODs for the examined compounds was 6.71 μmol/l. Linear regression coefficients ($r$), as well as slopes and intercepts for the calibration curves assessed for a 50–200 μmol/l concentration range are also presented in Table 1. Good linear relationships ($r > 0.900$) between the concentrations and peak areas were obtained for most of the compounds (Table 1).

In addition, within- and between-run precision of migration times and peak area measurements were determined (Table 2). There were good within-run precisions of migration times, with C.V. ranging between 0.1 and 1.0% (median 0.7%). However, higher C.V.s reaching 2% were obtained for between run migration times precision. Within run precision for peak area measurements had a C.V. around or less than 10%, except for Gly, Gln, Trp and Pro with C.V. 20.8, 18.4, 11.9 and 11.4%, respectively. For between run peak area precision the C.V. ranged from 3.2 to 23.5% (median 12.1%).

The applicability of the presented method for amino acid and peptide estimations in physiological fluids was tested on deproteinized samples of plasma and supernatant of macrophage cultures (Figs. 6 and 7). Compounds were identified by their migration times. Also peak identifications for each analyte were carried out by spiking samples with known standards of the examined compounds and the peaks with increased areas (height) were identified (electropherograms B in Figs. 6 and 7). In addition, we should point out that one big negative peak just before the systemic peak could be seen in the examined biofluids. These negative peaks are not presented in Figs. 6 and 7, because the absorbance of amino acid/peptide profiles were recorded in higher ranges. In the plasma samples Arg, Lys, Orn, Pro, Cit, Val, Phe, Ala, Met, Gln, Thr, Asn, Gly, Ser, Tyr, Cys(s), Glu and Asp were separated as individual peaks, while His/Met as well as Leu/Ile comigrated as single peaks (Fig. 6; Table 3). In the supernatant of cultivated macrophages we identified Lys, Orn, Pro, Val, Ala, Thr, Asn, Gly, Ser, Tyr, Cys(s), Glu

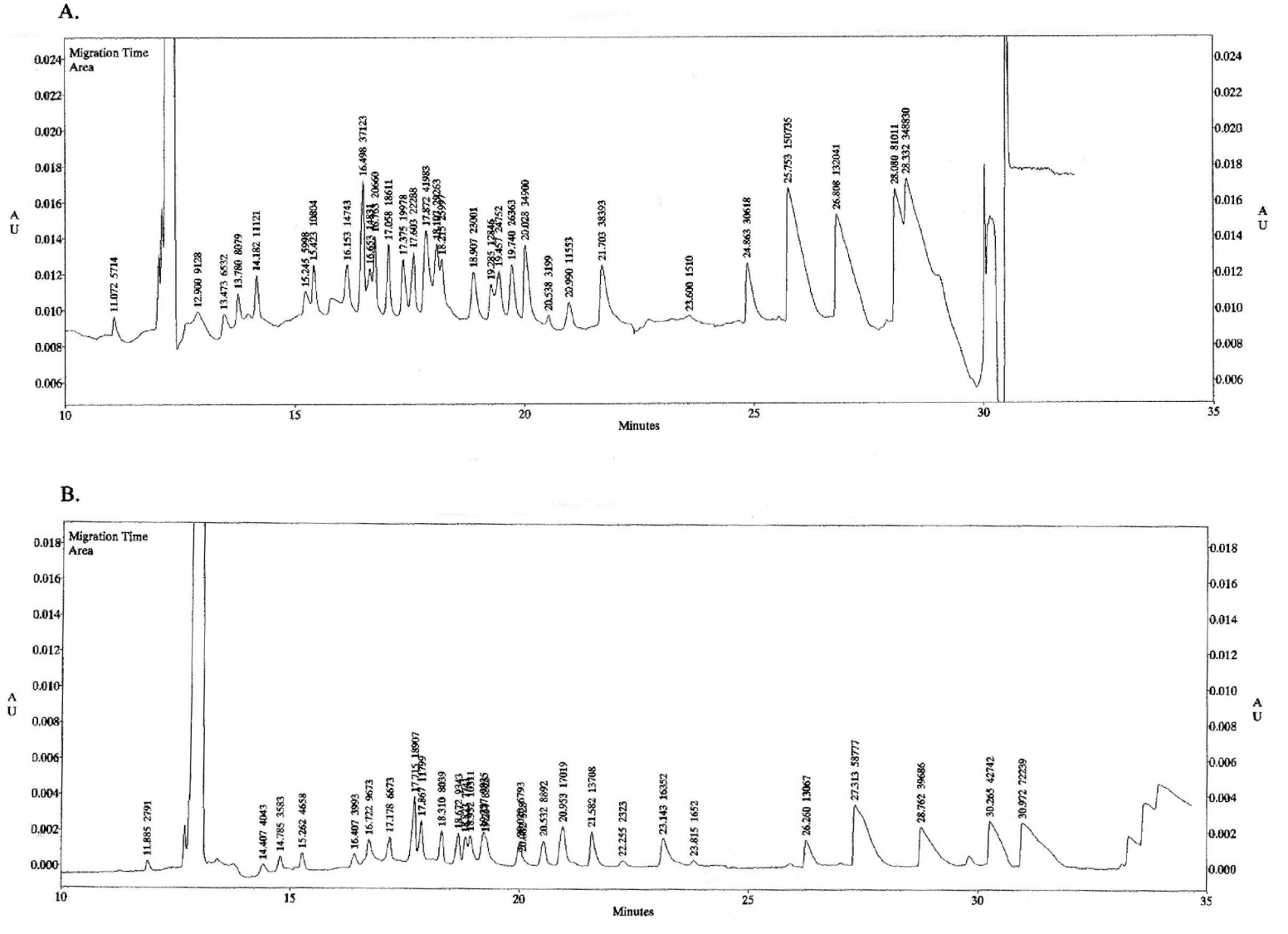


Fig. 1. Electropherograms of 30 amino acids and peptides in 8 mmol/l (A) and 10 mmol/l (B) PAS supplemented with $Na_2CO_3$ at pH 10.15 as running electrolyte. Concentrations of compounds: 100 μmol/l each. Voltage: 15 kV; temperature: 20 °C. Migration times and peak areas are presented. AU=indirect absorbance at 254 nm.

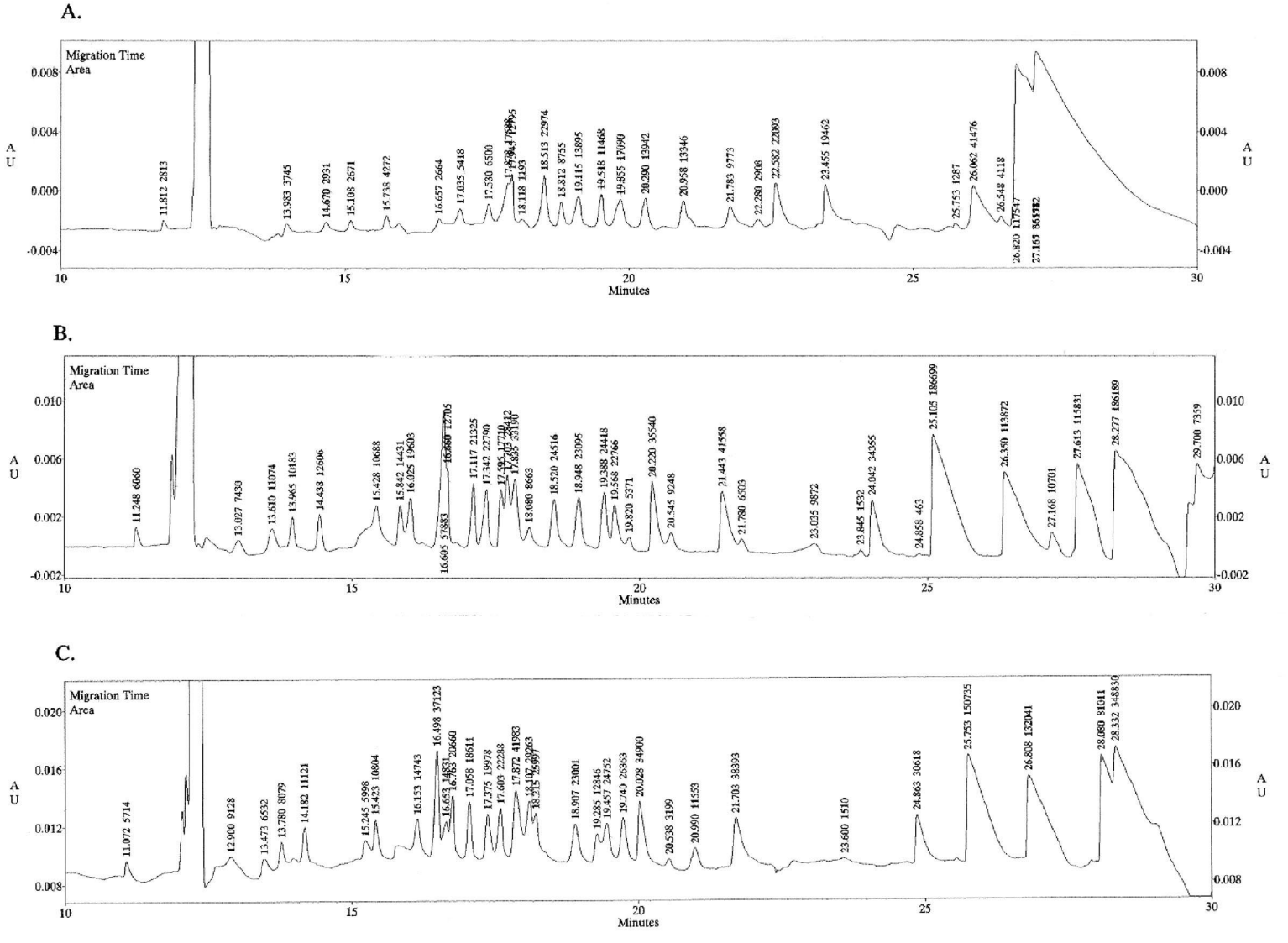


Fig. 2. Electropherograms of 30 amino acids and peptides in 8 mmol/l PAS adjusted to pH 10.15 (A), 10.35 (B), 10.70 (C) with $Na_2CO_3$. Concentrations of compounds: 100 μmol/l each. Voltage: 15 kV; temperature: 20 °C. Migration times and peak areas are presented. AU=indirect absorbance at 254 nm.

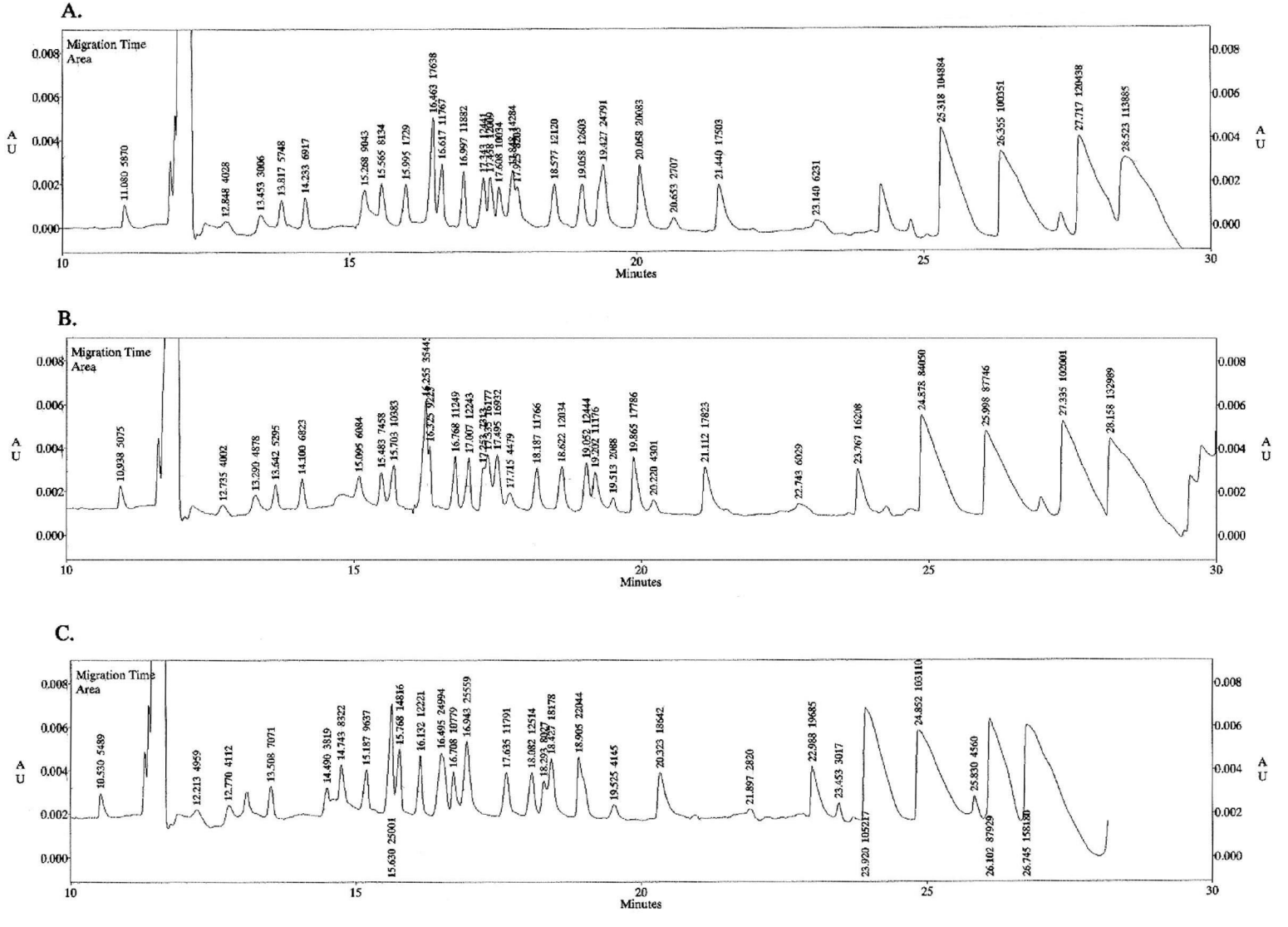


Fig. 3. Electropherograms of 30 amino acids and peptides in 8 mmol/l PAS–2 mmol/l $Na_2CO_3$, pH 10.15 and 19 °C (A), 20 °C (B) or 21 °C (C). Concentrations of compounds: 100 μmol/l each. Voltage: 15 kV; temperature: 20 °C. Migration times and peak areas are presented. AU=indirect absorbance at 254 nm.

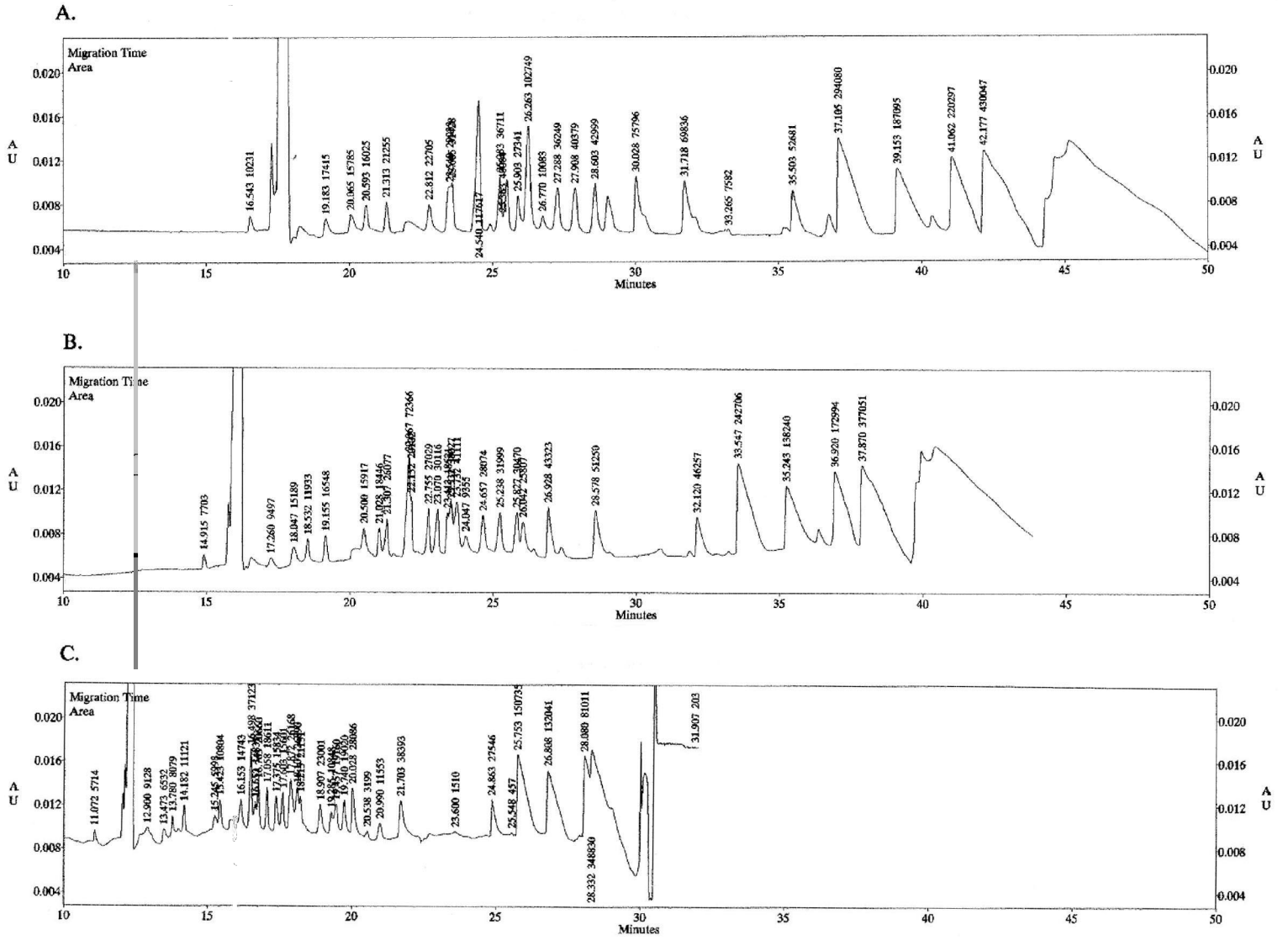


Fig. 4. Electropherograms of 30 amino acids and peptides in 8 mmol/l PAS–2 mmol/l $Na_2CO_3$ at pH 10.15, 20 °C and voltages 10 kV (A), 11 kV (B) and 15 kV (C). Concentrations of compounds: 100 μmol/l each. Voltage: 15 kV; temperature: 20 °C. Migration times and peak areas are presented. AU=indirect absorbance at 254 nm.

Table 1
Migration times, limits of detection (LODs) and linearities for the calibrations curves obtained for examined amino acids and peptides

| Compound | Migration time[a] (min) | LOD[b] (μmol/l) | Linearity[c] | | | |
|---|---|---|---|---|---|---|
| | | | *r* | Slope | Intercept | *n* |
| Arg | 11.15 | 8.82 | 0.973 | 61.59 | −290 | 33 |
| Lys | 12.81 | 5.22 | 0.933 | 123.5 | −4140 | 26 |
| Orn | 13.71 | 6.86 | 0.934 | 122.5 | −3974 | 32 |
| Pro | 13.79 | 6.88 | 0.933 | 119.5 | −2008 | 33 |
| GABA | 14.33 | 6.71 | 0.933 | 130.4 | −1070 | 33 |
| HyLys | 15.53 | 7.20 | 0.880 | 154.7 | −4370 | 33 |
| β-Ala | 15.69 | 6.72 | 0.927 | 167.6 | −849.6 | 33 |
| Anserine | 16.22 | 10.3 | n.q. | n.q. | n.q. | – |
| Carnosine | 16.29 | 5.13 | 0.971 | 214.7 | −4512 | 33 |
| Leu | 16.43 | n.q. | n.q. | n.q. | n.q. | – |
| .Ile | 16.49 | n.q. | n.q. | n.q. | n.q. | – |
| Leu/Ile | 16.48 | 8.83 | 0.985 | 556.03 | −10 049 | 25 |
| Trp | 16.59 | 7.2 | 0.957 | 129 | 129 | 33 |
| Cit | 16.77 | 2.12 | 0.909 | 226.4 | −466 | 33 |
| Val | 17.16 | 2.76 | 0.962 | 233.6 | −2493 | 33 |
| Phe | 17.48 | 3.99 | 0.860 | 319.2 | −8711 | 30 |
| Ala | 17.59 | 5.09 | 0.745 | 270.2 | −2154 | 27 |
| 3-MeHis | 17.66 | n.q. | n.q. | n.q. | n.q. | – |
| His | 17.87 | 9.24 | 0.684 | 152.5 | 31 422 | 27 |
| Met | 17.90 | 5.22 | 0.947 | 352 | −1059 | 27 |
| Gln | 18.22 | 6.80 | 0.927 | 438.8 | −13 858 | 27 |
| Thr | 18.71 | 13.07 | 0.955 | 251.7 | −999 | 33 |
| Asn | 19.47 | 20.08 | 0.947 | 206.4 | −1915 | 33 |
| Gly | 19.78 | 8.78 | 0.961 | 228.6 | −1919 | 25 |
| Ser | 19.85 | 9.18 | 0.932 | 262.3 | 1183 | 25 |
| Tyr | 20.46 | 3.22 | 0.953 | 342.7 | 792.2 | 33 |
| hCySS | 21.68 | 5.16 | 0.855 | 312.5 | 3417 | 33 |
| hCyS | 24.05 | n.q. | 0.931 | 238.8 | −15 959 | 8 |
| Cys–Cys | 24.53 | n.q. | n.q. | n.q. | n.q. | |
| Cys | 24.59 | 4.12 | 0.989 | 371.2 | −1487 | 33 |
| GSSG | 26.12 | 1.93 | 0.989 | 1501.1 | 6811.2 | 33 |
| Glu | 26.59 | 1.93 | 0.951 | 1237.7 | 7685.3 | 33 |
| GSH | 28.23 | 2.35 | 0.959 | 2406.2 | −527 340 | 33 |
| Asp | 29.09 | 2.18 | 0.812 | 1273.0 | 121 101 | 33 |

n.q.=not quantified.

[a] Migration times determined by individual injections of the examined compounds at 100 μmol/l concentration.

[b] LODs determined by the injection of amino acid/peptide mixture at 100 μmol/l concentrations of each compound, based on signal-to-noise ratio of 3.

[c] Calibration curves were obtained using the standard mixtures with increasing individual concentrations of the amino acids/peptides at 50 ($n$=7), 100 ($n$=12), 150 ($n$=7), 200 ($n$=7) μmol/l concentration levels. $n$=number of determination performed for assessment of the linearity.

and Asp as individual peaks while Leu/Ile and His/Met comigrated (Fig. 7). Also in that sample it could be observed that Trp and Cit coeluted, probably due to their high concentrations and/or overlapping with some unknown compound. Finally, individual concentrations of the compounds identified in the examined biofluids were determined, and within run precision of their migration times, peak areas and concentration levels were assessed (Table 3).

## 4. Discussion

The presented method permits separation of 30 underivatized physiological amino acids and peptides

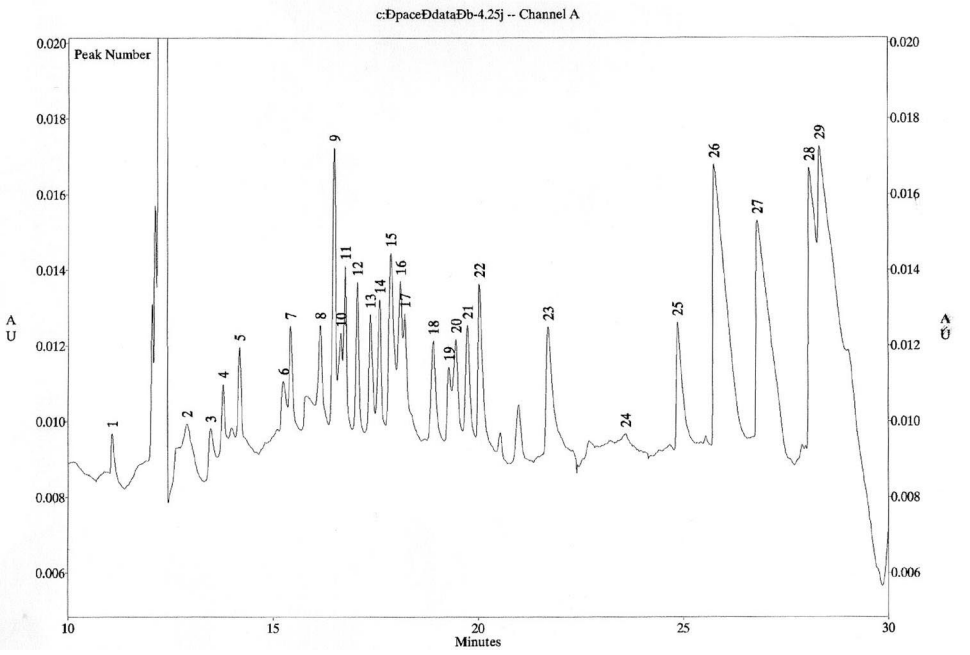


Fig. 5. Electropherogram of 30 amino acids and peptides in 8 mmol/l PAS–2 mmol/l $Na_2CO_3$ at pH 10.15; voltage: 15 kV; temperature: 20 °C. Concentrations of compounds 100 μmol/l each. AU (ordinate)=indirect absorbance at 254 nm. Migration order (peaks) (1) Arg, (2) Lys, (3) Orn, (4) Pro, (5) GABA, (6) HyLys, (7) αALA, (8) carnosine, (9) Leu/Ile, (10) Trp, (11) Cit, (12) Val, (13) Phe, (14) Ala, (15) His, (16) Met, (17) Gln, (18) Thr, (19) Asn, (20) Gly, (21) Ser, (22) Tyr, (23) hySS, (24) hCyS, (25) CyS(S), (26) GSSG, (27) Glu, (28) GSH, (29) Asp.

from the same sample in a single run by CE. In our study a greater number of amino acids was separated in a single run than with other CE methods [10,14,21–25]. Qualitatively it is comparable with some chromatographic procedures separating 20–40 amino acids in a single run [5,8,11]. Similarly to the other CE methods, the presented method is faster than chromatographic procedures and utilizes much smaller sample volumes. It is as fast as the other methods utilizing CE for amino acid separations usually taking less than 1 h [21–25]. In fact, under the CE conditions used here, separations of 30 compounds were achieved within 30 min, while the other reported CE methods separated not more than 20 amino acids within 30 min [21,23].

Use of indirect absorbance detection permitted us to analyze amino acids, both with and without UV absorbing properties, without prior derivatization. Recently, a CE method separating 14 non-protein and protein amino acids without derivatization has been reported [24], but in that case the authors monitored only those with aromatic moieties absorbing at 200 nm. Most other methods analyzed derivatized amino acids, qualitatively or semiquantitatively [13,14,17,26–28]. It was also reported that the labeling reaction was influenced by the total amino acid concentrations present in the sample and absolute quantification would be problematic [27].

We have used PAS, already established to be one of the most suitable carrier buffers and background providers that have an effective mobility close to the mobilities of most amino acids at alkaline pH [23]. Utilizing PAS as a background provider, Lee and Lin [23] recommended metal cations as well as long-chain cationic surfactants as buffer additives either to decrease or to reverse the electroosmotic flow in order to improve the resolution of 17–19 amino acids in CE employing indirect absorbance detection. The CE method developed in the present study achieved satisfactory separations of 30 compounds without addition of any special electroosmotic modifiers, but by optimizing both running electrolyte (pH,

Table 2
Within- and between-run precision of the migration times and peak areas for the examined compounds in mixture

| Compound | Within-run precision ($n$=5) | | | | Between-run precision ($n$=5) | | | |
|---|---|---|---|---|---|---|---|---|
| | Migration time (min) | | Peak area[a] | | Migration time (min) | | Peak area | |
| | Mean±SD | C.V. | Mean±SD | C.V. | Mean±SD | C.V. | Mean±SD | C.V. |
| Arg | 11.22±0.07 | 0.6 | 5641±129 | 2.3 | 11.19±0.09 | 0.8 | 6138±370.1 | 6.0 |
| Lys | 13.03±0.07 | 0.5 | 8897±936 | 10.5 | 13.06±0.06 | 0.5 | 7183±1690 | 23.5 |
| Orn | 13.62±0.08 | 0.6 | 8472±688 | 8.1 | 14.19±0.97 | 6.9 | 7486±1121 | 15.0 |
| Pro | 13.91±0.08 | 0.6 | 9559±1087 | 11.4 | 13.89±0.12 | 0.9 | 9624±2194 | 22.8 |
| GABA | 14.35±0.10 | 0.8 | 11 612±770 | 6.6 | 14.29±.013 | 0.9 | 11 704±1395 | 11.9 |
| HyLys | 15.47±0.12 | 0.8 | 10 697±1156 | 10.8 | 15.38±.015 | 1.0 | 10 500±795 | 7.6 |
| β-Ala | 15.69±0.16 | 1.0 | 18 335±775 | 4.2 | 15.67±0.18 | 1.2 | 16 719±2418 | 14.5 |
| Carnosine | 16.26±0.05 | 0.3 | 16 776±1146 | 6.8 | 16.19±0.14 | 0.9 | 16 284±2212 | 13.6 |
| Leu/Ile | 16.69±0.11 | 0.7 | 44 178±520 | 1.2 | 16.60±0.17 | 1.0 | 44 391±3078 | 6.9 |
| Trp | 16.73±0.08 | 0.5 | 13 788±1649 | 11.9 | 16.65±0.15 | 0.9 | 13 434±1449 | 10.8 |
| Cit | 16.88±0.07 | 0.4 | 24 524±1020 | 4.2 | 16.83±0.16 | 0.9 | 23 038±1685 | 7.3 |
| Val | 17.26±0.11 | 0.6 | 21 045±715 | 3.4 | 17.17±0.16 | 0.9 | 21 498±3080 | 14.3 |
| Phe | 17.58±0.18 | 1.0 | 20 310±1962 | 9.7 | 17.50±0.20 | 1.1 | 24 751±5669 | 22.9 |
| Ala | 17.73±0.15 | 0.8 | 22 451±1673 | 7.5 | 17.67±0.18 | 1.0 | 24 110±3220 | 13.4 |
| His | 17.82±0.14 | 0.8 | 52 192±3456 | 6.6 | 17.70±0.04 | 0.2 | 52 068±6531 | 12.5 |
| Met | 17.95±0.09 | 0.5 | 34 459±1484 | 4.3 | 17.90±0.09 | 0.5 | 33 025±3470 | 10.5 |
| Gln | 18.15±0.12 | 0.7 | 27 119±5002 | 18.4 | 18.02±0.05 | 0.3 | 27 859±3418 | 12.3 |
| Thr | 18.98±0.05 | 0.2 | 22 092±126 | 5.6 | 18.89±0.18 | 0.9 | 23 071±728 | 3.2 |
| Asn | 19.49±0.03 | 0.1 | 20 550±317 | 1.5 | 19.35±0.22 | 1.2 | 17 389±3046 | 17.5 |
| Gly | 19.67±0.14 | 0.7 | 20 495±4270 | 20.8 | 19.57±0.22 | 1.1 | 19 439±3340 | 17.2 |
| Ser | 19.84±0.07 | 0.4 | 31 131±3334 | 10.7 | 19.71±0.21 | 1.1 | 26 966±2175 | 8.1 |
| Tyr | 20.34±0.19 | 1.0 | 37 605±3153 | 8.4 | 20.29±0.28 | 1.4 | 34 381±2152 | 6.3 |
| hCySS | 21.93±0.13 | 0.6 | 32 710±1887 | 5.8 | 21.84±0.28 | 1.3 | 34 738±3146 | 9.1 |
| CyS(S) | 25.03±0.10 | 0.4 | 36 154±1576 | 4.4 | 24.87±0.32 | 1.3 | 37 128±2786 | 7.5 |
| GSSG | 26.07±0.22 | 0.8 | 154 640±4923 | 3.2 | 26.00±0.35 | 1.4 | 159 192±5294 | 3.3 |
| Glu | 27.13±0.25 | 0.9 | 131 349±4199 | 3.2 | 27.03±0.47 | 1.7 | 137 211±9499 | 6.9 |
| GSH | 28.44±0.21 | 0.7 | 192 256±7124 | 3.7 | 28.31±0.50 | 1.8 | 189 399±25 419 | 13.4 |
| Asp | 29.14±0.28 | 1.0 | 264 898±23 550 | 8.9 | 29.05±0.55 | 1.9 | 264 898±23 550 | 14.4 |

Amino acids and peptides present at 100 μmol/l concentration each.
[a] Peak areas are given as P/ACE system readings.

concentration, ionic strength) and eletrophoretic conditions (voltage, temperature).

Considering that determination of p*I* is crucial for the optimization of amino acid capillary electrophoretic separations [24] and knowing that the majority of the native amino acids are isoelectric in the pH region between 6 and 7 [12] we used a running electrolyte at pH>10. Namely, at that alkaline pH, the carboxyl group is unprotonated and the α-amino group is near equilibrium as the conjugate base. We used pH 10.2, where the majority of the amino acids had negative net charges and could be separated as anionic forms due to mass-to-charge differences between them. The exception is arginine which, due to guanidinium ionization, has a positive net charge, at the alkaline pH employed. It is known that the level of electroosmotic flow is highly dependent on electrolyte pH while full ionization of silanol groups occurs at a pH above 9 [18]. Considering that, electroosmotic flow seemed to be strong under the conditions used in the present study. Other authors, employing indirect fluorescence for detection, also used an alkaline electrolyte at pH 9.7 and separated Arg, Pro, Leu, Ala, Phe, Ser, Tyr, Cys, Glu and Asp within 15 min at 15 kV [12]. It was reported that PAS could be used as the background electrolyte at a more alkaline pH (above 11). With or without electroosmotic flow modification with special long-

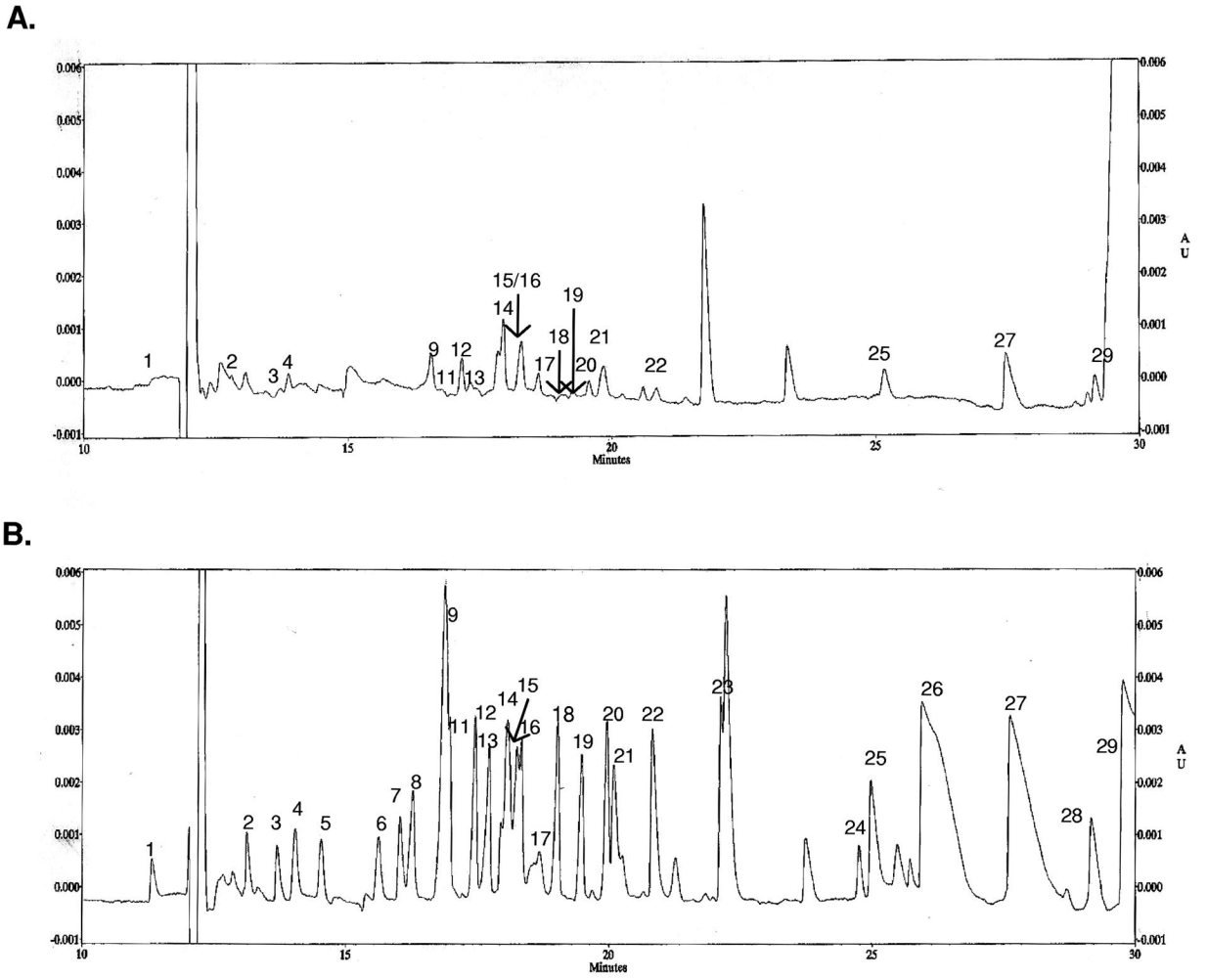


Fig. 6. Electropherograms of five-fold diluted deproteinized plasma samples without (A) and spiked with (B) 100 μmol/l standard amino acid/peptide mixture. Peak identification and CE parameters as in Fig. 5.

chain cationic surfactants and metal cations, this resulted in the separation of 17–19 underivatized amino acids in a single run [23]. In our experiments PAS was used as the background electrolyte, but we observed that during CE a fast electrolyte pH decrease below 10 occurred, affecting both separation and migration times of the examined compounds. Supplementation of the background electrolyte with $Na_2CO_3$ overcame the problems associated with reproducibilities of the results. Probably, $Na_2CO_3$ increased the buffering capacity of the running electrolyte [12] and thus, permitted both qualitative and quantitative standardization of the method in the present study.

We also examined the influence of some working parameters on the separation of the compounds and on their migration times. The results obtained in the present study indicated that both separation and migration times of the examined compounds were significantly influenced by the running electrolyte composition. In particular pH affected it. Thus higher pH increased migration times, probably by increasing electroosmotic flow. The slower migration, particularly of acidic compounds and those without net

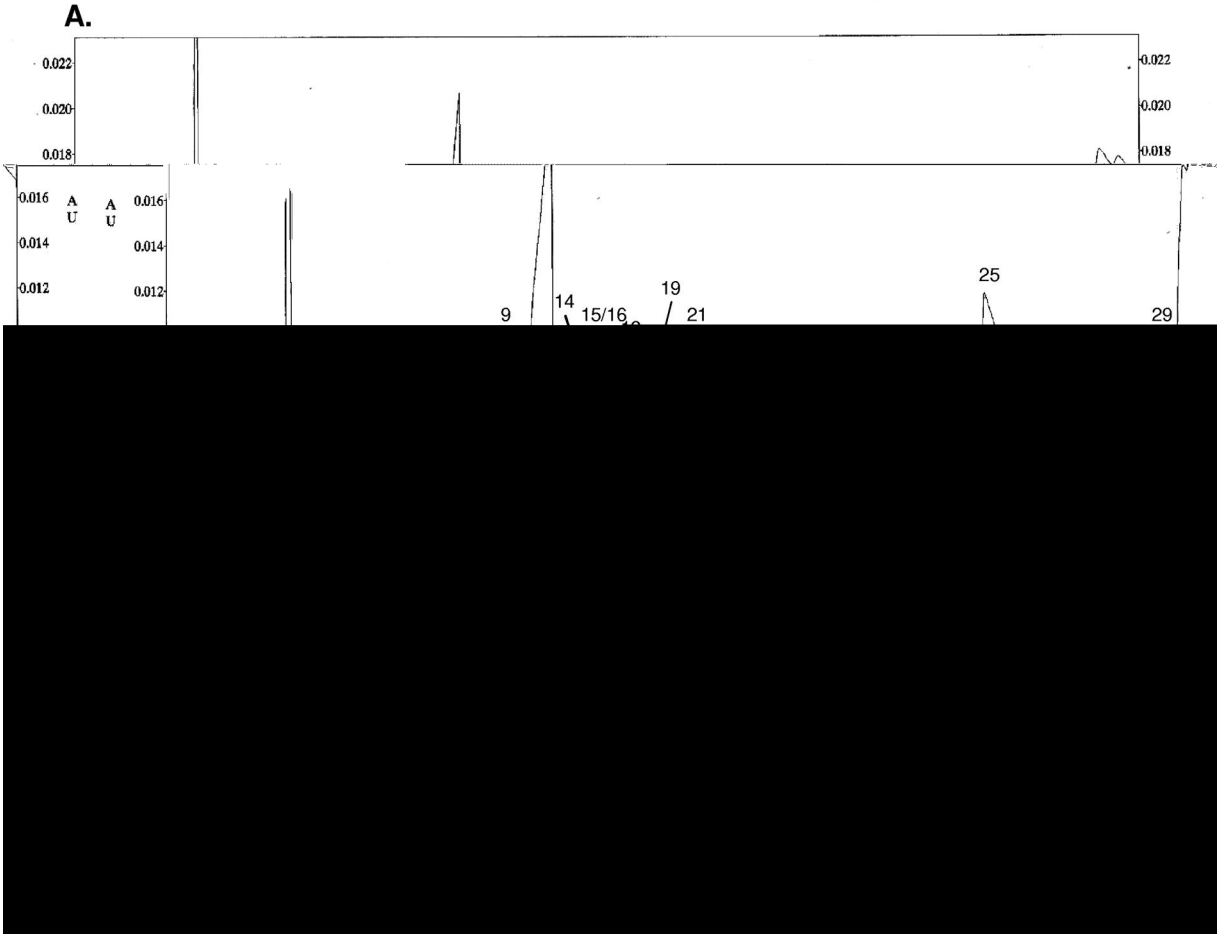


Fig. 7. Electropherograms of five-fold diluted deproteinized supernatant of macrophage cultures without (A) and spiked with (B) 25 μmol/l standard amino acid/peptide mixture. Peak identification and CE parameters as in Fig. 5.

charges on the molecules led to comigration of GSSG, GSH, Glu and Asp. Simultaneously, it improved separation of basic amino acids (Lys, Orn, Pro, GABA), but disturbed the separation between Leu/Ile, Trp and Cit. Lower pH improved the separation of the examined compounds and so pH $10.2\pm0.1$ was chosen as optimal. On the other hand, the concentration of background electrolyte, i.e. PAS concentration, at the same pH and $Na_2CO_3$ concentration did not significantly influence separation of the compounds, while it reduced the sensitivity of peak area response. Namely, the running electrolyte with 10 mmol/l PAS produced smaller peak areas for a standard mixture than the electrolyte with 8 mmol/l PAS level. The results obtained indicated that voltage had the greatest effect on the migration times. The effects were greater on acidic than on basic amino acids. Thus at 10 kV Arg migrated in 16.543 min and Asp in 42.177 min, while their migration times at 15 kV were 11.072 and 28.332 min, respectively. Although the effects of temperature on migration times were smaller than voltage effects, temperature changes could be used for fine adjustments and optimization of the separation conditions. Taking into consideration all the influences of the examined working parameters on CE of the examined compounds, the conditions presented at the beginning of this paper were chosen as optimal.

The results obtained confirmed that the presented CE conditions could be used for amino acid quantifi-

Table 3
Within run precision of the migration times, peak areas and concentrations of identified amino acid in plasma (A) and supernatant of cultivated macrophages (B)

| Compound | Migration time (min) | | Peak area[a] | | Concentration (μmol/l) | |
|---|---|---|---|---|---|---|
| (A) *Plasma* ($n$=3) | | | | | | |
| Arg | 11.50±0.05 | (0.5) | 381.0±37.3 | (9.8) | 65.7±6.61 | (9.8) |
| Lys | 12.98±0.06 | (0.5) | 2104.5±196.3 | (9.3) | 236.5±22.1 | (9.3) |
| Orn | 13.58±0.10 | (0.8) | 533.5±17.0 | (3.2) | 63.0±2.0 | (3.2) |
| Pro | 13.75±0.09 | (0.6) | 575.5±17.3 | (3.0) | 60.2±1.8 | (3.0) |
| Leu/Ile | 16.46±0.09 | (0.5) | 5809.0±134.7 | (2.3) | 263.0±6.1 | (2.3) |
| Cit | 16.61±0.08 | (0.5) | 683.0±42.0 | (6.1) | 27.9±1.7 | (6.2) |
| Val | 17.04±0.09 | (0.5) | 4001.0±167.3 | (4.2) | 190.1±7.9 | (4.2) |
| Phe | 17.26±0.04 | (0.3) | 851.5±83.0 | (9.7) | 41.9±4.1 | (9.7) |
| Ala | 17.70±0.17 | (0.9) | 7876.0±820.0 | (10.4) | 350.8±36.5 | (10.4) |
| His/Met | 18.26±0.16 | (0.9) | 5955.5±515.0 | (8.6) | 137.5±11.9 | (8.7) |
| Gln | 18.43±0.12 | (0.7) | 6556.5±581.7 | (8.9) | 241.8±21.5 | (8.9) |
| Thr | 18.77±0.19 | (0.9) | 1923.5±441.0 | (22.9) | 87.1±19.9 | (22.9) |
| Asn | 19.22±0.03 | (0.2) | 403.5±55.0 | (13.6) | 19.6±2.7 | (13.6) |
| Gly | 19.62±0.15 | (0.8) | 4856.0±424.7 | (8.7) | 236.9±20.7 | (8.8) |
| Ser | 19.68±0.07 | (0.3) | 2894.0±147.0 | (5.1) | 93.0±4.7 | (5.1) |
| Tyr | 20.75±0.06 | (0.3) | 1391.5±275.7 | (19.8) | 37.0±7.3 | (19.8) |
| Cys(s) | 25.02±0.09 | (0.4) | 3171.5±533.0 | (16.8) | 87.7±14.7 | (16.8) |
| Glu | 27.18±0.20 | (0.7) | 13 526.5±1041.0 | (7.7) | 102.98±7.9 | (7.7) |
| Asp | 28.84±0.21 | (0.7) | 2714.0±282.7 | (10.4) | 10.3±1.1 | (10.4) |
| (B) *Supernatant of cultivated macrophages* ($n$=3) | | | | | | |
| Lys | 12.77±0.11 | (0.9) | 1525.3±242.9 | (15.9) | 270.4±43.1 | (15.9) |
| Orn | 13.15±0.18 | (1.3) | 671.3±98.2 | (14.6) | 79.2±11.6 | (14.6) |
| Pro | 13.47±0.14 | (1.0) | 349.3±87.6 | (25.1) | 36.5±9.2 | (25.1) |
| Leu/Ile | 16.33±0.16 | (1.0) | 11 873.3±970.4 | (8.2) | 537.5±43.9 | (8.2) |
| Val | 17.44±0.04 | (0.2) | 3845±404.7 | (10.5) | 182.7±19.2 | (10.5) |
| Ala | 17.51±0.07 | (0.4) | 3724.0±1027.3 | (27.6) | 165.9±45.8 | (27.6) |
| His/Met | 17.97±0.12 | (0.6) | 4943.0±236.7 | (4.8) | 114.1±5.5 | (4.8) |
| Gln | 18.28±0.01 | (0.7) | 1005.7±582.9 | (57.9) | 37.1±21.5 | (57.9) |
| Thr | 18.75±0.10 | (0.5) | 6163.0±261.3 | (4.2) | 279.0±11.8 | (4.2) |
| Asn | 19.18±0.04 | (0.2) | 1245.0±90.2 | (7.2) | 60.6±4.4 | (7.2) |
| Gly | 19.56±0.28 | (1.5) | 2832.7±157.1 | (5.5) | 138.2±7.7 | (5.5) |
| Ser | 19.99±0.13 | (0.7) | 3322.7±182.9 | (5.5) | 106.7±5.9 | (5.5) |
| Tyr | 20.88±0.27 | (1.3) | 1241.0±305.3 | (24.6) | 33.0±8.1 | (24.6) |
| Cys(s) | 25.37±0.11 | (0.4) | 52 513.7±6246.9 | (11.9) | 339.6±40.4 | (11.9) |
| Glu | 26.87±0.49 | (1.8) | 4499.0±562.7 | (12.5) | 34.3±4.3 | (12.5) |
| Asp | 28.64±0.32 | (1.1) | 7830.0±2803.0 | (35.8) | 3.0±1.1 | (35.8) |

Values are presented as means±SD (C.V.). Deproteinized biofluid samples were five-fold diluted with water prior the analysis.
[a] Peak areas are given as P/ACE system readings.

cations in physiological fluids, such as plasma or supernatant from macrophage cultures. As the other ionizable compounds usually present in physiological fluids, particularly proteins, could influence CE of amino acids, we analyzed deproteinized samples. We found acetone to be the most suitable reagent among those tested (ethanol, sulphosalicylic acid). Namely, ethanol required higher reagent volumes for deproteinization than acetone did thereby increasing dilution of the samples and reducing the detection of certain compounds. Sample deproteinization with sulphosalicylic acid negatively influenced separation of the examined compounds during CE, probably by affecting the pH.

On the other hand, dilution of the samples with water seemed to be necessary for good separation of

the examined compounds. This was probably due to the high electrolyte content in the examined physiological fluids. The negative peak, that was observed prior to the systemic peak confirmed this. A similar negative peak could be observed when physiological sodium chloride solution was analyzed (data not presented). Thus, it is likely that the observed negative peak originated from sodium chloride, normally present in physiological fluids. The area of this negative peak decreased with dilution of the biofluid samples, so that following 15-fold dilution of the sample with water, it was eliminated from the electropherograms. However, such high dilution prevented precise quantification of the amino acids due to their low levels in the injected samples. Three- to five-fold dilution of deproteinized biofluids seemed to be a good compromise that eliminated the significant influence of the other ions on amino acid migration during CE and permitted their quantification. However, dilution of the sample with water significantly reduced the precision of some peak area measurements, due to their low levels. In spite of that, plasma amino acid concentrations determined by the presented CE method were comparable with the values obtained for plasma amino acids in healthy subjects determined by ion-exchange chromatographic methods [5,29]. Unfortunately, there was no possibility to compare the results obtained in the present study with other CE methods, since they lacked quantitative data.

Although much more work has to be done on further standardization before the presented method could be recommended as an alternative to amino acid analyzers, its simplicity and rapidity suggests that it may find a place among the other more powerful assays for determination of free amino acids in physiological fluids.

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