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Electromembrane extraction of amino acids from body fluids followed by capillary electrophoresis with capacitively coupled contactless conductivity detection

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ABSTRACT

Electromembrane extraction (EME) proved to be a simple and rapid pretreatment method for analysis of amino acids and related compounds in body fluid samples. Body fluids were acidified to the final concentration of 2.5 M acetic acid and served as donor solutions. Amino acids, present as cations in the donor solutions, migrated through a supported liquid membrane (SLM) composed of 1-ethyl-2-nitrobenzene/bis-(2-ethylhexyl)phosphonic acid (85:15 (v/v)) into the lumen of a porous polypropylene hollow fiber (HF) on application of electric field. The HF was filled with 2.5 M acetic acid serving as the acceptor solution. Matrix components in body fluids were efficiently retained on the SLM and did not interfere with subsequent analysis. Capillary electrophoresis with capacitively coupled contactless conductivity detection was used for determination of 17 underivatized amino acids in background electrolyte solution consisting of 2.5 M acetic acid. Parameters of EME, such as composition of SLM, pH and composition of donor and acceptor solution, agitation speed, extraction voltage, and extraction time were studied in detail. At optimized conditions, repeatability of migration times and peak areas of 17 amino acids was better than 0.3% and 13%, respectively, calibration curves were linear in a range of two orders of magnitude (r^2 = 0.9968–0.9993) and limits of detection ranged from 0.15 to 10 μM. Endogenous concentrations of 12 amino acids were determined in EME treated human serum, plasma, and whole blood. The method was also suitable for simple and rapid pretreatment and determination of elevated concentrations of selected amino acids, which are markers of severe inborn metabolic disorders.

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1. Introduction

Liquid phase microextraction (LPME) has gained significant attention as an alternative method for pretreatment and preconcentration of complex samples in recent years [1]. Main advantages of LPME are low impact on environment due to reduced amounts of organic solvents involved in the extraction process, small volumes of acceptor and donor solutions, possibility to be semi-automated, and to apply single-use disposable extraction units. Thus, LPME has potential to offer sample pretreatment procedures that are faster, easier, and less expensive, yet provide accurate and precise data with acceptable limits of detection. These days, LPME is mainly performed in form of single drop microextraction (SDME) [2,3], hollow fiber-liquid phase microextraction (HF-LPME) [4], dispersive liquid–liquid microextraction (DLLME) [5,6] and solidified floating organic drop microextraction (SFODME) [7]. Although these methods proved to be efficient in pretreatment and/or preconcentration of complex samples, several limitations have been observed and described [8]. Main drawbacks of the presented methods are relatively low stability of the hanging drop in SDME, long extraction times in HF-LPME and limited choice of organic solvents and tedious manual handling in DLLME and SFODME. Moreover, an initial clean-up of biological samples must be performed prior to the preconcentration step in DLLME and SFODME [8].

As an alternative method to HF-LPME, electromembrane extraction (EME) was presented recently [9]. It ensures significantly higher extraction speed at comparable extraction efficiencies. EME is based on electrically driven transport of charged species from aqueous solutions (donor) across a supported liquid membrane (SLM), formed as a thin layer of a water immiscible organic solvent on a supporting material, into another aqueous solution (acceptor). Main advantages are simple experimental setup, negligible consumption of organic solvents and minimal costs of the supporting material resulting into single-use disposable extraction units and therefore no sample carryover. Moreover, EME offers the possibility for tuning the extraction selectivity by choosing the composition of

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SLM and the composition and pH of donor and acceptor solutions. Until now, EME has been used mainly for extraction of compounds of pharmaceutical relevance [9,10], nevertheless, applications to other groups of analytes, such as inorganic ions [11,12], phosphonic acids (as degradation products of nerve agents) [13] and chlorophenols [14] were demonstrated. Recently, it has been shown that EME can be efficiently used also for extraction of small biochemical species. Balchen at al. were the first to report EME of short oligopeptides from standard solutions [15,16] and plasma samples [17]. A complete overview of EME applications can be found in recent reviews [18–20].

Amino acids are basic structural units of peptides and larger biochemicals in human bodies and concentrations of free amino acids in body fluids are important indicators of intermediary metabolism. Analytical methods for their determination in body fluids are mostly high-performance liquid chromatography (HPLC) and gas chromatography (GC) [21–23], which usually involve labour-intensive sample pretreatment step(s) in order to remove proteins and other bulk components from sample matrices [24–26]. Moreover, optical detection of amino acids at physiological concentrations requires their derivatization, which renders them amenable to fluorescent measurements. This further increases the total pretreatment time and may introduce additional errors in quantitative analysis. On the other hand, underivatized amino acids, which are present in aqueous solutions mostly in the form of ionic species, are well suited for conductivity detection, as was recently demonstrated in HPLC and capillary electrophoresis (CE) combined with axial capacitively coupled contactless conductivity detection (C^4D) [27,28]. CE has often been used as an alternative to HPLC and GC in analysis of amino acids [29–31] due to its low sample and reagent consumption, short analysis times and excellent separation efficiency. Combination of CE with C^4D is a well established analytical scheme [32–35] and its applications to analyses of amino acids in complex samples were described in several publications [28,36,37]. However, the use of CE for analysis of body fluids requires also extensive sample pretreatment, such as whole blood centrifugation, protein precipitation and subsequent centrifugation of the precipitated proteins [36,37]. Pretreatment of amino acids from complex samples can also be performed in downscaled format using LPME. However, the fact that amino acids are zwitterionic and majority of amino acids are highly hydrophilic species makes their extraction into organic solvents and back extraction into aqueous donor solutions rather difficult [38,39]. To achieve efficient recovery of amino acids, extraction conditions must be specifically altered. Amino acids were, for example, successfully extracted after neutralization by suitable derivatization agent [38], extraction with room temperature ionic liquids [40] and extraction with macrocyclic compounds, such as crown-ethers, which form stable hydrophobic “host–guest” complexes [39]. These procedures are, however, highly compound-selective and only a limited number of amino acids can be extracted at a time.

In this contribution, rapid and simple approach to the above stated problems is demonstrated. 17 essential amino acids and related compounds in acidified human body fluids are electroextracted as cations through a tailor made SLM anchored in pores of a polypropylene (PP) hollow fiber (HF) using the action of electric field. The cationic amino acids are then trapped in acidic acceptor solution, which is placed in a lumen of the HF, and is instantly used for injection into CE with C^4D. Interfering compounds from body fluid matrices, such as proteins and cellular matter, are efficiently retained on the SLM and do not enter the acceptor solution. Analyses of amino acids in serum, plasma, whole blood, and urine of healthy individuals are demonstrated as well as analyses of body fluids with elevated concentrations of particular amino acids, which are markers of inborn metabolic disorders.

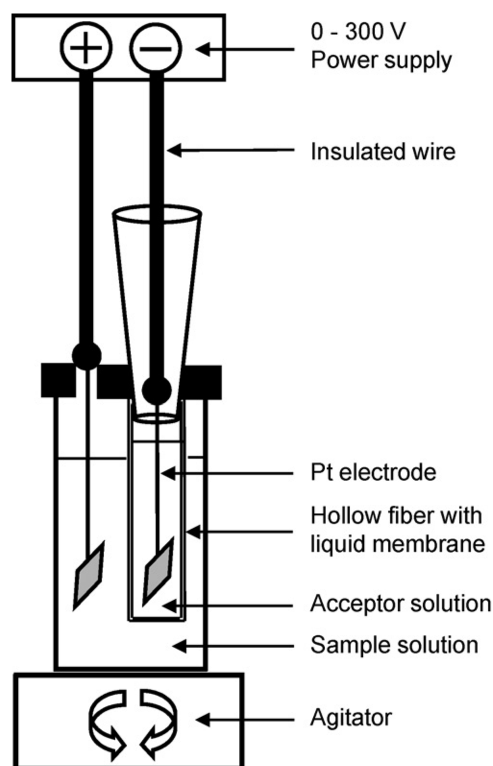

Fig. 1. Schematic drawing of the EME system for pretreatment of amino acids from body fluids.

2. Materials and methods

2.1. Instrumentation

2.1.1. Electromembrane extraction system

The equipment used for EME of amino acids is illustrated in Fig. 1. Donor compartment was a 1 mL glass vial (C4015-96, outer dimensions 8 mm × 40 mm, National Scientific, Rockwood, TN, USA) with a plastic cap. Two holes were punched through the cap in order to accommodate two 0.5 mm platinum electrodes (99.95%, Advent, Oxford, England). Due to a short distance between the two electrodes, cathode was insulated using a 200 μL plastic pipette tip to prevent possible short circuit with anode. 900 μL of a donor solution was filled into the glass vial and the vial was attached to a MS1 Minishaker (IKA Works Inc., Wilmington, NC, USA) with a 0–2500 rpm variable agitation speed. The porous PP HF used was Accurel PP 300/1200 (Membrana, Wuppertal, Germany) fiber with wall thickness of 300 μm and an internal diameter of 1200 μm. It served as a support for liquid membranes and as a compartment for acceptor solutions. Extraction units were 3 cm long HFs, which were pressed and heat sealed at the bottom resulting into internal volume of ca. 30 μL. Before EME, the HF was impregnated for 10 s with an organic solvent to form a SLM and then the lumen was filled with 20 μL of an acceptor solution. Excessive organic solvent was wiped off the HF using a lint-free tissue. The open end of the HF was then pulled on the pipette tip acting as the leading tube for cathode. Finally, the HF, attached to the pipette tip, was placed into the donor solution, cathode was immersed into the HF and anode was led through the second hole in the cap directly into the donor solution. The electrodes were connected to a power supply model ES 0300-0.45 (Delta Elektronika BV, Zierikzee, The Netherlands) with a programmable voltage in the range 0–300 V and maximum current of 450 mA. Electric current in the EME system was continuously monitored using an M-3800 (Metex, Seoul,

Korea) digital multimetre. After the extraction was completed the acceptor solution was transferred to a plastic microvial for CE analysis and the HF was discarded. All EME experiments were performed at ambient temperature of 22 ± 2 °C.

2.1.2. Capillary electrophoresis system

A 7100 CE instrument (Agilent, Waldbronn, Germany) with built-in C^4D [41] was operated at a potential of +30 kV applied at the injection side of the separation capillary for all runs. Separation capillary used was a fused-silica (FS) capillary (25 μm ID, 375 μm OD, 50 cm total length and 35 cm effective length, Polymicro Technologies, Phoenix, AZ, USA). Before use, new capillary was flushed at 950 mbar for 10 min each with 1 M HCl, deionized (DI) water and finally with a background electrolyte (BGE) solution. Between two CE runs, separation capillary was rinsed with BGE solution for 2.5 min. Injection of standard solutions and real samples was carried out hydrodynamically by application of 50 mbar for 20 s, which represents less than 1% (2.1 nL) of the total capillary volume. All CE–C^4D measurements were performed at 25 °C. The CE system was controlled and data were acquired by ChemStation CE software.

2.2. Reagents, BGE solutions, standards and real samples

All chemicals were of reagent grade and DI water with resistivity higher than 18 MΩ cm was used throughout. Stock solutions of inorganic cations (150 mM Na^+, 20 mM K^+, 10 mM Ca^{2+} and 10 mM Mg^{2+}, Pliva-Lachema, Brno, Czech Republic) were prepared from their corresponding chloride salts. Stock solutions of amino acids 10 mM, Sigma, Steinheim, Germany and Fluka, Buchs, Switzerland: creatinine (Crea), lysine (Lys), arginine (Arg), histidine (His), glycine (Gly), alanine (Ala), valine (Val), isoleucine (Ile), leucine (Leu), threonine (Thr), asparagine (Asn), methionine (Met), tryptophan (Trp), citrulline (Cit), phenylalanine (Phe), proline (Pro), tyrosine (Tyr), β-alanine (β-Ala), and ornitine (Orn) were prepared from pure chemicals. Choline (Chol) was prepared from choline chloride. Standard solutions for CE were prepared from these stock solutions and were diluted with 0.5 M or 2.5 M acetic acid as stated. Standard donor solutions for EME consisted of 6 mM Na^+, 0.2 mM K^+, 0.08 mM Ca^{2+}, 0.04 mM Mg^{2+} and various concentrations of amino acids and were diluted with DI water or various acetic acid solutions. Gly, Thr, Asn, Cit and Tyr were always added in 4-fold higher concentrations than other 12 amino acids due to their lower EME efficiency. The concentrations of amino acids are therefore referred to as 50/200 μM (or other 1:4 ratios) in the text, where 50 μM is the concentration of the 12 amino acids and 200 μM is the concentration of Gly, Thr, Asn, Cit and Tyr. Concentrations of inorganic cations correspond to 25-fold diluted human serum samples; the 25-fold dilution was also used for determination of amino acids in all real samples (see next paragraph and Section 3.8). Organic solvents for EME were obtained from Sigma or Fluka and were of highest available purity. Bis(2-ethylhexyl)phosphonic acid (DEHP) was purchased from Sigma. The solvents were used without any further purification. BGE solutions for CE (0.5 M and 2.5 M acetic acid) were prepared weekly from concentrated acetic acid (Fluka) and were kept at 4 °C.

Human plasma and human serum samples were purchased as lyophilized powders from Sigma and were prepared according to supplier's instructions. Other real samples – human urine, serum, and whole blood – were obtained from volunteers at the Institute of Analytical Chemistry. Urine was sampled daily, stored at 4 °C and discarded at the end of the day. Serum was prepared by centrifugation of whole blood at 6000 rpm for 10 min, immediately deep frozen and stored at −20 °C. Whole blood was deep frozen immediately after withdrawal and stored at −20 °C. All body fluids for EME were diluted 25-fold by mixing 36 μL of a particular body fluid with 864 μL of 2.6 M acetic acid or DI water.

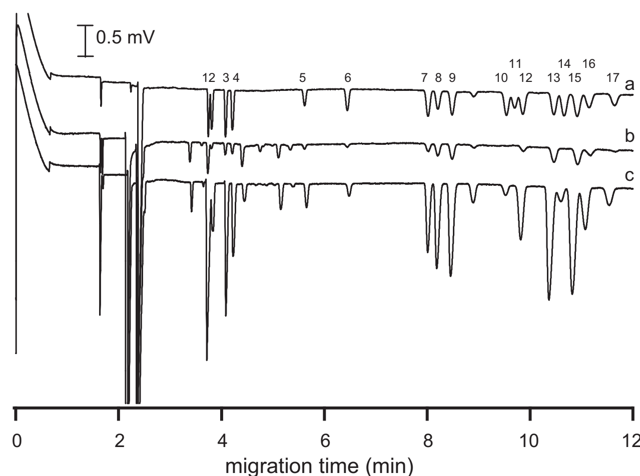

Fig. 2. (a) Determination of 17 amino acids (50 μM) in standard solution. (b) Determination of 17 amino acids (5/20 μM) after EME. (c) Determination of 17 amino acids (50/200 μM) after EME. EME conditions: liquid membrane: ENB + 15% (v/v) DEHP, impregnation time: 10 s, agitation: 500 rpm, extraction voltage: 50 V, extraction time: 10 min, acceptor solution: 2.5 M acetic acid. Donor solutions for EME were prepared in 2.5 M acetic acid with 6 mM Na^+, 0.2 mM K^+, 0.08 mM Ca^{2+}, 0.04 mM Mg^{2+}. CE conditions: BGE solution: 2.5 M acetic acid (pH 2.0), voltage: +30 kV, injection: 50 mbar for 20 s. Peak description: 1 – Crea, 2 – Lys, 3 – Arg, 4 – His, 5 – Gly, 6 – Ala, 7 – Val, 8 – Ile, 9 – Leu, 10 – Thr, 11 – Asn, 12 – Met, 13 – Trp, 14 – Cit, 15 – Phe, 16 – Pro, 17 – Tyr.

3. Results and discussion

3.1. Selection of BGE solution for determination of amino acids

Amino acids can be analyzed by CE–C^4D either as cations in strongly acidic BGE solutions [28,37] or as anions in strongly alkaline solutions [42]. Acidic BGE solutions consisting of acetic acid were adopted and optimized in this study. In order to minimize Joule heating and to increase separation efficiency, FS capillary with 25 μm ID was used for CE measurements. 2.5 M acetic acid was found suitable BGE solution for baseline separation of 17 amino acids. Note that CE separation of complete set of amino acids was not the goal of this study and is usually time demanding [36] or must be divided into three separate analyses using three different BGE solutions [37]. We therefore assume that selection of the 17 amino acids is sufficient to demonstrate the features of EME for analysis of body fluids. Fig. 2a shows separation of 50 μM of selected amino acids in 2.5 M acetic acid BGE solution (Fig. 2b and c shows determination of amino acids in EME treated standard solutions – see Section 3.7).

3.2. Selection of organic solvent for EME of amino acids

Several organic solvents were previously used for EME of small biochemical species [15,17,43]. These include 1-octanol and di-isobutylketone with addition of 8–15% DEHP, which acted as an ionic carrier. 1-Heptanol, 1-octanol, nitrophenyloctyl ether (NPOE), and 1-ethyl-2-nitrobenzene (ENB) were examined as SLM for EME of amino acids without and with addition of 8% DEHP. Acidic (100 mM acetic acid, pH ∼ 2.9) donor and acceptor solutions were used throughout. Low extraction efficiencies of amino acids were generally observed for all organic solvents without addition of the carrier. On the other hand, presence of DEHP in the SLM significantly increased the transfer of most amino acids into the acceptor solution and effect of various DEHP concentrations on EME

Table 1
Effect of donor solution matrix on extraction efficiency (% of recovery) of 17 amino acids at 50/200 μM. EME and CE conditions as for Fig. 2 except composition of donor solutions. $n = 3$, RSD ≤ 12%.

	DI water	50 mM acetic acid	2.5 M acetic acid
Crea	23.7	18.6	10.8
Lys	5.8	5.1	4.7
Arg	16.3	11.2	8.4
His	8.6	7.6	5.9
Gly	0.3	0.3	1.4
Ala	0.6	0.6	2.1
Val	3.0	3.4	5.7
Ile	8.0	9.0	10.0
Leu	7.9	8.4	9.6
Thr	0.2	0.2	0.5
Asn	n.d.	n.d.	0.1[a]
Met	n.d.	n.d.	5.5
Trp	12.3	12.3	10.6
Cit	n.d.	n.d.	0.6
Phe	9.0	9.7	10.1
Pro	3.2	3.6	7.5
Tyr	0.6	0.8	1.2

n.d. – not detected.
[a] RSD: 23.9%.

performance is described in detail in Section 3.4. Highest EME efficiency was achieved for 1-octanol and ENB (with 8% (v/v) DEHP), moreover, ENB showed to be more efficient than 1-octanol in eliminating inorganic cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) from entering the fiber lumen and was therefore used in all subsequent experiments.

3.3. Selection of donor and acceptor solution, stirring

Acidic donor and acceptor solutions were selected for EME in order to be compatible with acidic BGE solution used for CE–C^{4}D determination of amino acids. Donor solutions always consisted of 50/200 μM of amino acids and given concentrations of inorganic cations (see Section 2.2) and were prepared in three different matrices. DI water was used to prepare donor solutions at pH ∼ 5–6 and 50 mM acetic acid and 2.5 M acetic acid was used to lower the pH of donor solutions to 3.0 and 2.0, respectively. Acceptor solution was 2.5 M acetic acid, which facilitates the electric transfer of positively charged amino acids from the SLM into the acceptor solution. Taking into account quite diverse properties of amino acids (various pK_a values, hydrophobicity, net charge and polarity) it is not surprising that different EME behaviour was observed for the set of 17 amino acids. Extraction efficiencies at various donor conditions are summarized in Table 1. Highest extraction efficiency for Crea and basic amino acids (Lys, His, Arg) was observed for DI water. These analytes have p$K_{a(+1)}$ values between 4.8 and 9 and are therefore positively charged in DI water solutions and easily transferred to acceptor solution. The reason for their slightly reduced transfer in acidic solutions is not clear at the moment. Extraction efficiency of other amino acids (Gly, Ala, Val, Ile, Leu, Thr, Asn, Met, Cit, Pro, Tyr) increases or remains fairly constant (Trp, Phe) in acidic solutions. At near neutral pH of DI water, these amino acids are not or only poorly charged and their transfer due to electric field is limited. However, once they are protonized at lower pH, a significant increase in extraction efficiency is observed. Since highest extraction efficiency for most amino acids was obtained in 2.5 M acetic acid, we have selected this donor matrix for subsequent experiments. Note, however, that when extraction of a particular amino acid or specific group of amino acids is preferred, different donor conditions might be used which increase transfer rate of the target analyte(s).

Amino acids were analyzed as cations in CE–C^{4}D, thus acidic acceptor solutions were examined only. Similarly as for donor solutions, their compatibility with BGE solution was considered

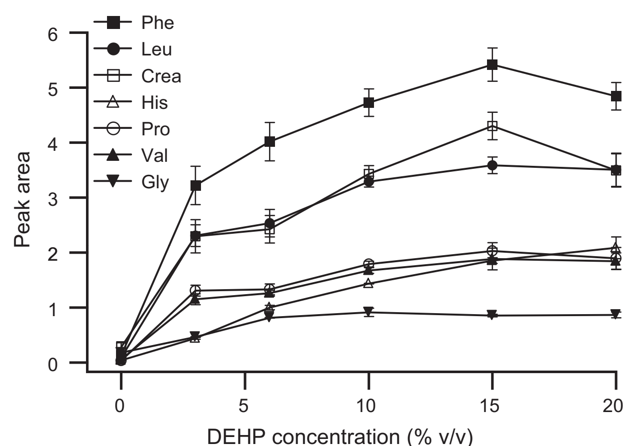

Fig. 3. Effect of DEHP concentration on extraction efficiency of selected amino acids. EME conditions as for Fig. 2, except DEHP concentrations. CE conditions as for Fig. 2.

and various concentrations of acetic acid (10, 100, 500, 1000 and 2500 mM) were tested. Note that the concentration of acetic acid in BGE solution for CE measurements was taken as the upper limit, since mismatch of the conductivity in sample zone and BGE solution might lead to spurious shapes of analyte peaks. Transfer rate of Crea and basic amino acids (Lys, His, Arg) remained fairly constant over the whole concentration range of acetic acid. This, again, can be rationalized by pH value of the acceptor solutions and pK_a values of the analytes which ensures their efficient protonation and transfer to acceptor solution over the whole pH range (2.0–3.4) of acceptor solutions. For the other 13 amino acids, extraction efficiency increased by a factor of 2–5 for 2.5 M compared to 10 mM acetic acid. This means that in order to increase their transfer from SLM into the acceptor solution, low pH, which ensures efficient protonation of amino acids, is advantageous.

Agitation of the donor solution was examined between 0 and 700 rpm. The EME system became unstable for 700 rpm and any higher agitation speeds. We assume that SLM was partly depleted at higher agitation speeds and electric current increased rapidly. Extraction efficiency increased by a factor of approximately 1.5–2 for agitated compared to stagnant EME system and agitation at 500 rpm was selected as optimum.

3.4. Addition of ionic carrier

As mentioned in Section 3.2, addition of an ionic carrier to the organic solvent is essential for better transfer of amino acids into acceptor solution. Effect of carrier concentration on amino acid transfer was examined in the range 0–20% (v/v) DEHP and is shown for selected amino acids in Fig. 3. Note that similar behaviour was observed also for the whole set of 17 amino acids (Lys and Arg followed the trace for His while other amino acids followed the traces of Leu and Phe) but they were not included in Fig. 3 for its clear presentation. Very limited transfer of amino acids was observed for pure ENB. Each gradual increase of DEHP concentration increased the transfer rate significantly and it reached its maximum for most analytes at 15% (v/v) DEHP. Higher concentrations of DEHP (20% and 30%, (v/v)) further increased electric current (to values higher than 100 μA) in the EME system, however, extraction efficiency of most amino acids remained constant or decreased, with the exception of basic amino acids (Lys, His, Arg), moreover, the EME system stability was compromised. 15% (v/v) DEHP was therefore selected as optimum concentration of carrier and was used for further optimization of the EME system.

Table 2
Analytical parameters of EME–CE–C^{4}D method for determination of amino acids, $n = 7$. EME and CE conditions as for Fig. 4.

	Crea	Lys	Arg	His	Gly	Ala	Val	Ile	Leu	Thr	Asn	Met	Trp	Cit	Phe	Pro	Tyr
RSD (%), MT	0.28	0.28	0.27	0.26	0.22	0.22	0.19	0.18	0.18	0.20	0.25	0.17	0.15	0.15	0.14	0.14	0.14
RSD (%), PA (50/200 μM)	10.8	6.1	6.6	5.2	8.0	4.1	6.0	9.6	8.7	10.9	[a]	6.9	12.0	9.6	11.1	6.8	6.0
RSD (%), PA (5/20 μM)	11.1	5.4	3.9	2.9	4.0	7.8	6.1	8.1	3.4	2.1	[a]	8.1	6.7	5.4	9.7	2.8	5.6
Absolute recovery (%) at 50/200 μM	10.8	4.7	8.4	5.9	1.4	2.1	5.7	10.0	9.6	0.5	[a]	5.5	10.6	0.6	10.1	7.5	1.2
r^2	0.9985	0.9977	0.9991	0.9989	0.9983	0.9976	0.9978	0.9983	0.9978	0.9987	[a]	0.9976	0.9981	0.9988	0.9987	0.9993	0.9968
LOD (μM)	0.15	0.75	0.25	0.5	10	3	0.5	0.5	0.35	10	[a]	1	0.35	10	0.35	1	10

MT – migration time.
PA – peak area.
[a] Results were not reproducible.

Table 3
Repeatability of peak areas of EME–CE–C^{4}D analysis and quantitative determination of the concentrations (μM) of amino acids in biological fluids, $n = 3$. EME and CE conditions as for Fig. 4.

	Crea	Lys	Arg	His	Gly	Ala	Val	Ile	Leu	Thr	Asn	Met	Trp	Cit	Phe	Pro	Tyr
RSD (%), serum	6.8	9.2	8.5	7.5	11.6	5.3	8.3	7.3	6.6	n.d.	n.d.	n.d.	5.4	n.d.	5.1	3.1	n.d.
RSD (%), plasma	10.2	7.3	3.5	4.4	6.1	4.8	3.2	4.7	4.3	n.d.	n.d.	n.d.	11.4	n.d.	6.6	1.2	n.d.
RSD (%), whole blood	10.2	10.3	12.5	10.3	8.2	3.0	7.9	9.6	11.4	n.d.	n.d.	n.d.	4.9	n.d.	7.8	9.5	n.d.
RSD (%), urine	12.7	[a]	5.0	4.5	1.0	13.0	8.3	12.8	7.6	n.d.	n.d.	n.d.	8.8	n.d.	6.9	n.d.	n.d.
Serum (μM)	53.3	108.8	67.4	86.6	323.6	503.8	299.7	92.3	156.8	n.d.	n.d.	n.d.	48.7	n.d.	105.0	246.8	n.d.
Plasma (μM)	90.6	83.1	53.9	78.3	221.8	400.0	296.3	96.9	127.9	n.d.	n.d.	n.d.	45.0	n.d.	41.7	236.5	n.d.
Whole blood (μM)	97.6	106.1	14.7	110.4	247.3	481.1	305.0	110.4	181.2	n.d.	n.d.	n.d.	77.3	n.d.	78.9	313.5	n.d.
Urine (μM)	17,500	[a]	78.8	556.4	539.0	133.6	57.9	19.4	32.8	n.d.	n.d.	n.d.	78.8	n.d.	51.1	n.d.	n.d.

n.d. – not detected.
[a] Comigration with peak of Crea.

3.5. Extraction voltage

Extraction voltage was varied between 0 and 100 V; 100 V was selected as maximum since unstable electric current and EME performance was observed at higher voltage settings. At 0 V, i.e., extraction due to diffusion, analytical signals below or slightly above the limit of detection (LOD) of the CE–C^{4}D method were observed for all 17 amino acids. Then, extraction efficiencies increased linearly between 0 and 50 V, which clearly shows that electric field is essential for the transfer of charged amino acids across the SLM and enhances the extraction process significantly. For voltages 50–100 V, extraction efficiencies remained fairly constant for most amino acids. For some analytes (Ala, Gly, Thr, Cit, Asn), gradual increase was observed also between 50 and 100 V, whereas for several other (Crea, Met, Trp, Phe), efficiency decrease was observed. Extraction voltage of 50 V was selected for subsequent experiments since the extraction efficiency was optimal for most analytes and stable EME performance was achieved.

3.6. Extraction time

Extraction time is the key parameter that determines total amount of ions transferred from donor to acceptor solution, provided the extraction voltage (or current) is constant during the whole extraction period. EME of amino acids was performed for 1–15 min and extraction efficiencies of single analytes were examined. In the first minute, the transfer of amino acids into acceptor solution was low and then linearly increased in the time period 3–15 min for all analytes. Further increase in extraction time resulted in slightly higher extraction efficiencies, however, unstable EME performance was observed for real samples at 15 min and any longer extraction times. Therefore, optimum extraction time of 10 min was selected. Moreover, in addition to 10 s impregnation time, 3 μL of liquid membrane was dispensed into the HF lumen prior to acceptor solution. This ensures better replenishment and stabilization of liquid membrane on the surface of HF and has previously been used in EME of real samples to improve the EME performance [44]. At these conditions, a stable EME performance was achieved for both standard solutions and real samples.

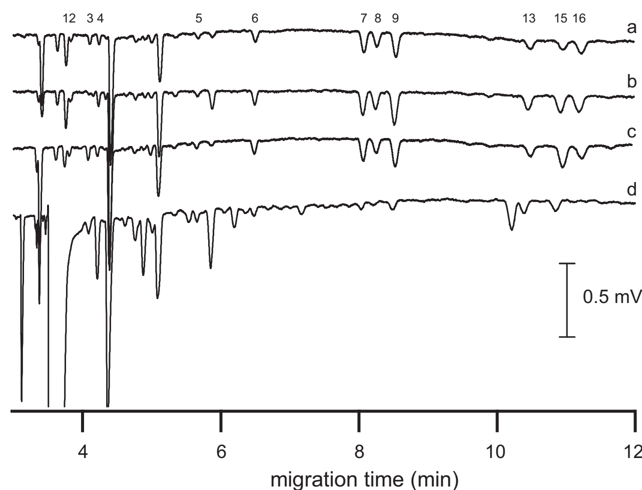

Fig. 4. Determination of amino acids in body fluids. (a) Plasma, (b) whole blood, (c) serum and (d) urine. EME conditions as for Fig. 2, except 3 μL of liquid membrane being dispensed into lumen of each HF prior to filling with acceptor solution. CE conditions and peak description as for Fig. 2. Body fluids were 25-fold diluted with 2.6 M acetic acid.

3.7. Analytical performance

Based on previously described experiments, following experimental conditions were selected for EME of amino acids: liquid membrane: ENB + 15% (v/v) DEHP, impregnation time: 10 s + addition of 3 μL of liquid membrane (ENB + 15% (v/v) DEHP) into the HF lumen, agitation speed: 500 rpm, extraction voltage: 50 V and extraction time: 10 min. The donor solution was 5/20 and 50/200 μM of 17 amino acids in 2.5 M acetic acid (with addition of

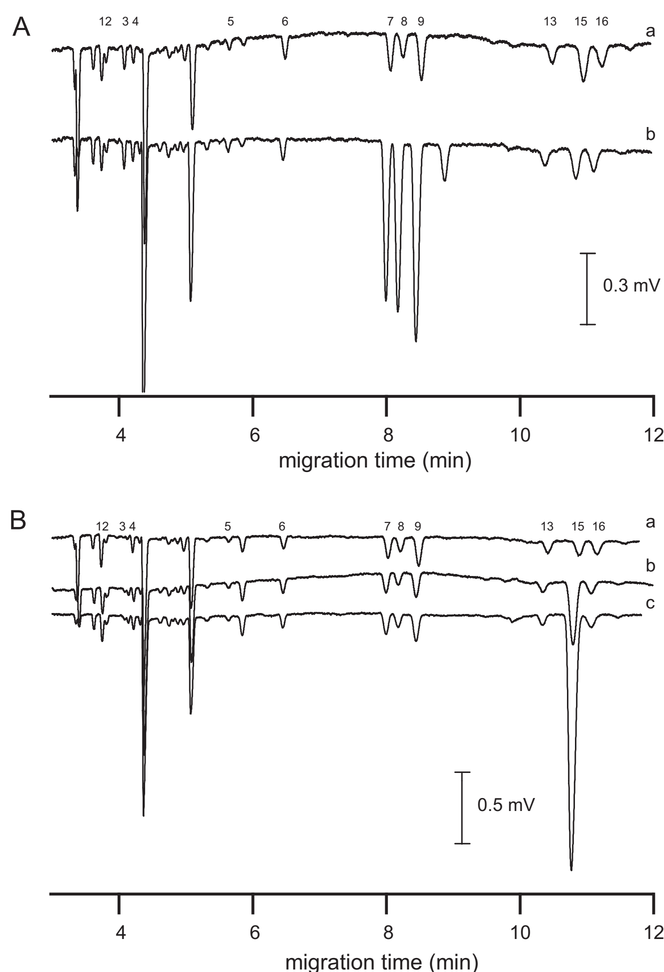

Fig. 5. (A) Determination of elevated concentrations of branched-chain amino acids in serum. (a) Unspiked serum, (b) serum spiked with 500 μM Val (7), Ile (8), Leu (9). (B) Determination of elevated concentrations of phenylalanine (15) in whole blood. (a) Unspiked whole blood, (b) whole blood spiked with 350 μM Phe and (c) whole blood spiked with 1500 μM Phe. EME and CE conditions as for Fig. 4. Body fluids were 25-fold diluted with 2.6 M acetic acid.

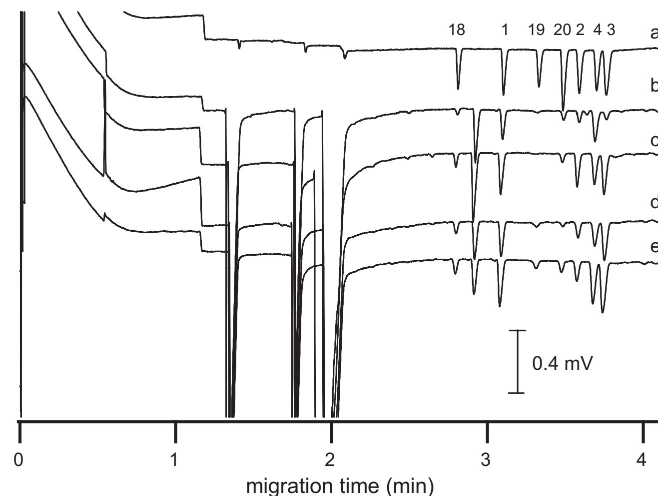

Fig. 6. Determination of fast amino acids and related compounds in body fluids. (a) Standard solution (25 μM) without EME, (b) whole blood, (c) plasma, (d) serum and (e) serum spiked with 5 μM of the analytes after EME. EME conditions as for Fig. 4 except acceptor solution: 0.5 M acetic acid. CE conditions as for Fig. 2 except BGE solution: 0.5 M acetic acid (pH 2.5). Peak description: 1 – Crea, 2 – Lys, 3 – Arg, 4 – His, 18 – Chol, 19 – β-Ala, 20 – Orn. Body fluids were 25-fold diluted with DI water.

inorganic cations, see Section 2.2) and acceptor solution was 2.5 M acetic acid. EME was repeated 7 times and each acceptor solution was analyzed three times in CE–C^{4}D system. Repeatability of the EME–CE–C^{4}D measurements, as RSD values of peak areas, is summarized in Table 2 and varies between 2.1 and 12%. Repeatability of migration times is also given in Table 2 and shows excellent stability of the CE–C^{4}D separation system with RSD values below 0.3%. Electropherograms for EME treated standard solutions at 5/20 and 50/200 μM are shown in Fig. 2b and c, respectively. The peaks migrating at 1.75–2.25 min (with no labels) are inorganic cations coextracted during the EME procedure and are well separated from all analytes. Electrokinetic transfer of inorganic cations through the SLM is significantly reduced based on experimentally determined concentrations of Na^+ in donor and acceptor solutions. For Na^+, which is the major inorganic cation in body fluids, the total transfer was calculated to be less than 0.5%. Calibration measurements of the EME method for amino acids were performed in concentration range from 1/4 to 100/400 μM at constant concentrations of inorganic matrix components (see Section 2.2). The calibration curves (correlation coefficients are summarized in Table 2) showed excellent linearity and were used for quantitative analyses of amino acids. LODs, at signal-to-noise ratio of 3, for EME treated solutions of amino acids are also summarized in Table 2 and range from 0.15 to 10 μM. These LODs allow for determination of most amino acids in real samples (for reference concentrations see e.g., [37]) at the selected dilution factor of 25. Absolute recoveries of amino acids, calculated according to [9], are also summarized in Table 2 and range from 0.5 to 10.8%. In order to improve extraction efficiency of particular amino acids, EME parameters (mainly SLM chemistry, acceptor/donor composition, etc.) may be changed, however, one has to be aware, that such action will alter EME performance also for other analytes and must be precisely optimized for each particular application. An example of fine-tuning EME–CE–C^{4}D conditions for determination of fast amino acids and related compounds is demonstrated in the last paragraph of Section 3.8.

3.8. *Applications to real samples*

Human serum, plasma, whole blood and urine were all diluted 25-fold with 2.6 M acetic acid (see Section 2.2) and were pretreated in the optimized EME system. Additional volume (3 μL) of ENB with 15% (v/v) DEHP was added to each extraction unit prior to acceptor solution (see Section 3.6) in order to stabilize SLM and achieve stable EME performance. Fig. 4 shows four CE–C^{4}D electropherograms of the body fluids after EME pretreatment. 12 amino acids were determined in the three blood samples. A major peak of creatinine (time 3.75 min) was observed in urine sample and did not allow determination of comigrating Lys, however, other 9 amino acids were determined in urine at the conditions employed. Repeatability of EME–CE–C^{4}D measurements of biological fluids expressed as RSD of peak areas is summarized in Table 3. The repeatability values range from 1 to 13% and do not significantly differ from those for standard solutions. Quantitative determination of amino acids in the body fluids was based on external calibration and is also summarized in Table 3. Concentrations determined for all identified amino acids were within the ranges for healthy individuals [37,45,46].

EME was further examined for pretreatment of body fluids with subsequent determination of specific amino acids, which are markers of severe inborn metabolic disorders. Fig. 5A shows EME–CE–C^{4}D determination of elevated concentrations of branched-chain amino acids (human serum of a healthy individ-

ual was spiked with 500 μM of Val, Ile, and Leu). Accumulation of these amino acids in body fluids is a typical symptom for individuals suffering from maple syrup urine disease (MSUD) and may lead to serious toxicity in human bodies. Trace (a) and (b) shows analyses of unspiked and spiked human serum sample, respectively. Clearly, the elevated concentrations of the three branched-chain amino acids result in significantly higher analytical signals for the corresponding peaks (7–9), whereas no visible difference in analytical signals is observed for the complete profile of other amino acids. Fig. 5B shows EME–CE–C^{4}D determination of elevated concentrations of phenylalanine in human whole blood (whole blood of a healthy individual was spiked with 350 and 1500 μM of Phe). Phenylalanine accumulates in body fluids of humans suffering from phenylketonuria (PKU) and leads to severe mental retardation. Trace (a) shows analysis of unspiked whole blood, and traces (b) and (c) show analyses of the same whole blood spiked with 350 μM Phe (trigger concentration for PKU neonatal screening tests) and 1500 μM Phe (severe mental retardation), respectively. The increased concentrations of Phe (peak 15) can again be clearly distinguished from its baseline level in unspiked whole blood, whereas the profile of other amino acids remains unchanged.

Fine-tuning of EME–CE–C^{4}D conditions for pretreatment and determination of fast migrating amino acids (β-Ala, Orn, Lys, His, Arg) and related compounds (Chol, Crea) is demonstrated in Fig. 6. At the CE–C^{4}D conditions optimized for determination of the set of 17 amino acids, some of the 7 compounds cannot be determined since they comigrate with other analytes in 2.5 M acetic acid BGE solution. Moreover, the selection of EME conditions (donor/acceptor composition) for the set of 17 amino acids partially hinders the transfer of Crea and basic amino acids into acceptor solution. Selection of proper EME and CE–C^{4}D conditions can improve extraction selectivity and separation of these analytes. Donor solutions were diluted with DI water (see Table 1) and acceptor solution was 0.5 M acetic acid. This acceptor composition was selected in order to match the concentration of acetic acid in BGE solution and has marginal effect on extraction efficiency of Crea, Lys, His and Arg as discussed in Section 3.3. Baseline separation of Chol, Crea, β-Ala, Orn, Lys, His and Arg in 0.5 M acetic acid BGE solution is depicted in Fig. 6, trace (a). All 7 analytes were then determined in body fluids as depicted for whole blood (b), plasma (c) and serum (d) sample. Trace (e) shows analysis of the same serum sample as in (d) spiked with the set of the 7 analytes after EME to confirm their identity. Significantly better separation efficiency was achieved due to selection of an optimized BGE solution and sensitivity enhancement of a factor 3–5 was achieved for Crea, Lys, His and Arg compared to the previously used EME–CE–C^{4}D conditions. This sensitivity improvement is evoked partially by different EME conditions and partially by using BGE solution with higher sensitivity. Fine-tuning of extraction and separation conditions might therefore be useful for specific applications when only a particular amino acid or a group of selected amino acids is of interest.

4. Conclusions

Electromembrane extraction proved to be a rapid and simple pretreatment method for analysis of amino acids in body fluids. Amino acids were extracted as cations from acidified body fluids into aqueous acidic solutions, which were then used for injection into CE. Extracted amino acids were detected using C^{4}D with no need for additional sample derivatization. Interferences from high molecular mass matrix components (proteins, red blood cells, etc.) and high concentrations of inorganic ions were eliminated by selection of suitable SLM and other EME conditions. The developed method was suitable for determination of endogenous concentrations of 12 out of 17 tested amino acids in blood and urine samples. Practical applicability of the developed EME–CE–C^{4}D method was also demonstrated on pretreatment and analysis of human whole blood and serum for determination of elevated concentrations of selected amino acids, which are markers of inborn metabolic disorders such as phenylketonuria and maple syrup urine disease. Since amino acids are analytes with significantly different physicochemical properties, EME of a complete set of amino acids at single extraction conditions is a difficult task. It might therefore be useful to optimize extraction and separation conditions for smaller groups of amino acids with similar properties. An example of such an approach was demonstrated by extraction and analysis of fast migrating amino acids and related compounds.

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