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On-line coupling of a clean-up device with supported liquid membrane to capillary electrophoresis for direct injection and analysis of serum and plasma samples

Pavel Kubáň*, Petr Boček

Institute of Analytical Chemistry of the Academy of Sciences of the Czech Republic, v.v.i., Veveří 97, CZ-60200 Brno, Czech Republic

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ABSTRACT

A simple sample clean-up device with planar supported liquid membrane (SLM) was developed and coupled on-line to capillary electrophoresis (CE) for direct injection of human body fluids. Donor and acceptor compartments of the device were filled with diluted body fluid and deionized water, respectively, and the two solutions were separated by a thin SLM. Analytes of interest were selectively transported from the donor solution through the SLM into the acceptor solution by diffusion whereas interfering matrix components were efficiently retained on the SLM. Equilibrium between the concentrations of analytes at the SLM was obtained typically in 5 min. Then a CE separation capillary was inserted into the acceptor compartment to firmly touch the SLM and the pretreated sample was hydrodynamically injected into the capillary. The analytical procedure was demonstrated by rapid pretreatment, on-line injection, and CE determination of selected amino acids in human serum and plasma samples. 1-Ethyl-2-nitrobenezene and bis(2-ethylhexyl) phosphate (15%, v/v) was used as the selective SLM for clean-up of the body fluids and 0.5 M acetic acid was used as a background electrolyte solution for CE analysis of the pretreated amino acids. Concentrations of amino acids on acceptor side of the SLM reached 40–58% of their original concentrations in donor solution after 5 min equilibration time and then remained constant proving that equilibrium was achieved at the SLM. Injection of the pretreated samples was highly repeatable with RSD values of peak areas 2.4–8.4% and 3.4–10.5% for standard solutions and real samples, respectively. Limits of detection between 0.75 and 2.5 μM were achieved, corresponding to 3.75–12.5 μM in 1:4 diluted real samples, which ensure sensitive determination of most amino acids in the body fluids. The developed method is fast, simple, efficient, cheap and selective and may be applied to determination of a wide range of analytes in various samples with complex matrices.

1. Introduction

Body fluids are complex biological samples and their analysis is a non trivial task. High molecular mass compounds in body fluids adhere to the inner surface of an analytical column (separation capillary in CE or separation column in HPLC and/or GC) and often result in irreversible poisoning of the column. This consequently evokes serious changes in separation efficiency and usually leads to peak broadening and detection interferences [1,2]. Qualitative and quantitative analyses of directly injected biological samples are therefore performed only scarcely [3,4] and various sample pretreatment procedures are normally applied prior to their injection in order to remove interfering matrix components.

Traditionally, pretreatment of biological samples was performed in an off-line fashion using stand-alone pretreatment techniques, such as liquid–liquid extraction [5], solid phase extraction [6] and protein precipitation with subsequent centrifugation [7]. More recently, alternative sample pretreatment techniques, such as ultrafiltration [8], electrodialysis [9], liquid phase microextraction (LPME) [10,11] and solid phase microextraction [12,13] were introduced. These pretreatment techniques exhibit minimum environmental impact due to their reduced use of organic solvents. They also speed-up the pretreatment procedures, require small volumes of biological samples and reduce the overall costs of an analysis. However, these techniques are usually performed off-line and their on-line and/or in-line coupling to separation techniques are reported less frequently [9,12,14–18].

One of the perspective pretreatment techniques is LPME using supported liquid membranes (SLMs), which was first described by Audunsson [19]. The method has gained significant attention in subsequent years and has become a basis for further development of various sample pretreatment methods (e.g. hollow fibre-liquid phase microextraction (HF-LPME) [11], and electromembrane extraction (EME) [20]). All these methods are based on the same

* Corresponding author. Tel.: +420 532290140; fax: +420 541212113.
E-mail address: kuban@iach.cz (P. Kubáň).

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fundamental principle, i.e. extractions of analytes across thin SLMs. A porous inert supporting material (usually polypropylene (PP) or polytetrafluoroethylene (PTFE)) is impregnated with water immiscible organic solvent to form the SLM and separates two compartments filled with donor and acceptor solutions. The donor solution is usually aqueous sample to be pretreated and the acceptor solution is either aqueous or organic solution. The SLM creates a selective barrier that enables transfer of analytes from donor to acceptor solution, whereas interfering matrix components are retained on the SLM. When the LPME is finished, the acceptor solution is injected into an analytical system. This simple experimental set-up can be used for simultaneous sample clean-up and preconcentration and has been widely used in many application areas [11,21]. LPME using SLMs is primarily attractive due to its negligible costs, low consumption of organic solvents, simple handling, and applicability to most analytical techniques including HPLC, GC, CE and MS [11]. Although LPMEs with SLMs are mostly performed off-line, several authors have demonstrated that on-line and/or in-line coupling of the pretreatment technique with various analytical methods is feasible.

Audunsson has developed an SLM-based pretreatment device coupled on-line to flow injection analysis (FIA) [19]. Basic set-up of this device was later used by Jönsson and coworkers who have demonstrated that, with few alterations, this instrumentation can be coupled on-line also to HPLC [22–24], GC [22,25], and atomic absorption spectrometry [26]. The system required additional external pumps to force the flows of the donor and the acceptor solutions through the device. Moreover, to replace the SLM, the extraction device had to be dismantled completely, which was quite complicated. To avoid the device dismantling, regeneration of the originally inserted SLM was used, which was time-consuming and could be a source of sample carry-over errors. Other authors have continued to use this approach in FIA [27–29], and extended the application field to microfluidic platforms [30–32] and to electroextraction assisted microfluidic platforms [33]. In the pretreatment devices, planar [25,31,34] as well as tubular [23,30,32,35] SLMs were used. Several reviews covering the major aspects of coupling SLM-based pretreatment devices to various flow-through analytical techniques and practical applications of the method are available [15–17,36].

Pretreatment devices using SLMs with the design originally introduced by Audunsson [19] were on-line coupled to CE [35,37]. The use of a complex pumping system and low flexibility for membrane replacement were the main limitations of these hyphenated systems. Moreover, a mismatch between the volume of pretreated sample and usual injection volumes in CE had to be overcome by a tedious and time consuming large-volume injection and double-stacking procedure [35,37]. Two alternatives for in-line coupling of a SLM directly to CE were presented by Valcárcel and coworkers [38–40]. Holes were cut out in an Eppendorf vial, which was then wrapped up in a planar PTFE membrane. The membrane was impregnated with organic solvent to form a SLM and the vial was filled with acceptor solution. Then the extraction assembly was placed in a conventional glass vial of a commercial CE instrument filled with a donor solution for extraction and injection [38,39]. Nozal et al. have also shown that efficient sample pretreatment can be achieved using a separation capillary of a commercial CE instrument coupled in-line to HF SLMs [40]. The HF was heat-sealed to the outer surface of two FS capillaries (64 and 0.5 cm long) and the distance between the two capillaries defined the extraction cell volume. Labor intensive procedure and low reproducibility of the manual fabrication of the extraction devices were mentioned as the main drawbacks of these arrangements [38–40] and the authors have preferred their repeated use and conditioning between two analytical runs. This, of course, increased the total pretreatment time and may be source of possible sample carry-over problems.

It has been shown, that coupling SLM to CE is a promising alternative for analysis of biological samples [35,37–40]. The on-line and/or in-line coupling to CE was, however, not fully solved until these days and many presented devices exhibit similar drawbacks, such as necessity of external instrumentation, low reproducibility, elaborateness, and possible sample carry-over. This work presents a simple pretreatment device with SLMs coupled on-line to CE for rapid pretreatment and analysis of human body fluids. Handling of the proposed device is simple and reproducible and was demonstrated by manual manipulation in this contribution. Planar SLM was screwed between two PTFE blocks with donor and acceptor solutions and was discarded after each use. Pretreated body fluids were injected hydrodynamically from the acceptor chamber by positioning the separation capillary directly onto the SLM surface. This ensured very short extraction times since equilibrium between the concentrations on both sides of the SLM was established rapidly. The method was applied to determination of amino acids in human serum and plasma and may be extended to a wide range of major and minor analytes since only 1:4 dilution of the original body fluids was necessary and the extraction selectivity could be fine-tuned by selection of a proper SLM.

2. Materials and methods

2.1. Instrumentation

2.1.1. Sample clean-up device

Fig. 1 shows the sample clean-up device and its connection to a CE system. It consists of two PTFE parts, screw and bolt type, with axial open hole channels of internal volume 50 μL (Link chambers, Harvard Apparatus, Holiston, MA, USA), separated by a polypropylene membrane (Accurel PP 1E R/P, Membrana, Wuppertal, Germany) with 100 μm thickness (porosity and pore size was not specified). The membrane was cut into 11 mm-radius circles, which exactly fitted into the inner part of the link chamber. Before sample treatment, the membrane was placed into one link chamber and was impregnated with 2.5 μL organic solvent to form a supported liquid membrane (SLM) in its centre. The organic solvent was let to soak into the membrane pores (this usually took ca. 5 s and the SLM became opaque) and the two link chambers were screwed together. Next, the donor chamber was filled with 50 μL of a body fluid using one micropipette and the acceptor chamber was filled with the same volume (50 μL) of an acceptor solution using a second micropipette. Measurement of the extraction time (typically 5 min) was initiated on filling the acceptor chamber with acceptor solution (see Fig. 1A). Simultaneously, the clean-up device was positioned into a holder, which was placed 30 cm (Δh) above the detection end of the separation capillary (C). The donor and acceptor solutions were held inside the chambers by capillary forces (the internal diameter of the chamber is approx. 3 mm) and no liquid losses were observed from internal chambers during up to 15 min. After the required equilibration time elapsed, sample injection was performed by inserting the separation capillary into the acceptor chamber to firmly touch the SLM. The capillary touches the SLM due to a slight tension created by the capillary bend (see Fig. 1B) and there is no gap between the capillary injection end and the SLM. After 60 s (injection time), the separation capillary was removed from the acceptor chamber, was placed into the vial with background electrolyte solution (E1) and high voltage power supply (HV) was switched on (see Fig. 1C). The clean-up device was then unscrewed, used SLM was discarded, internal channels of the link chambers were flushed with deionized (DI) water, dried out and immediately used for next sample clean-up. All clean-up experiments and sample injections were performed at ambient temperature of 25 ± 2 °C.

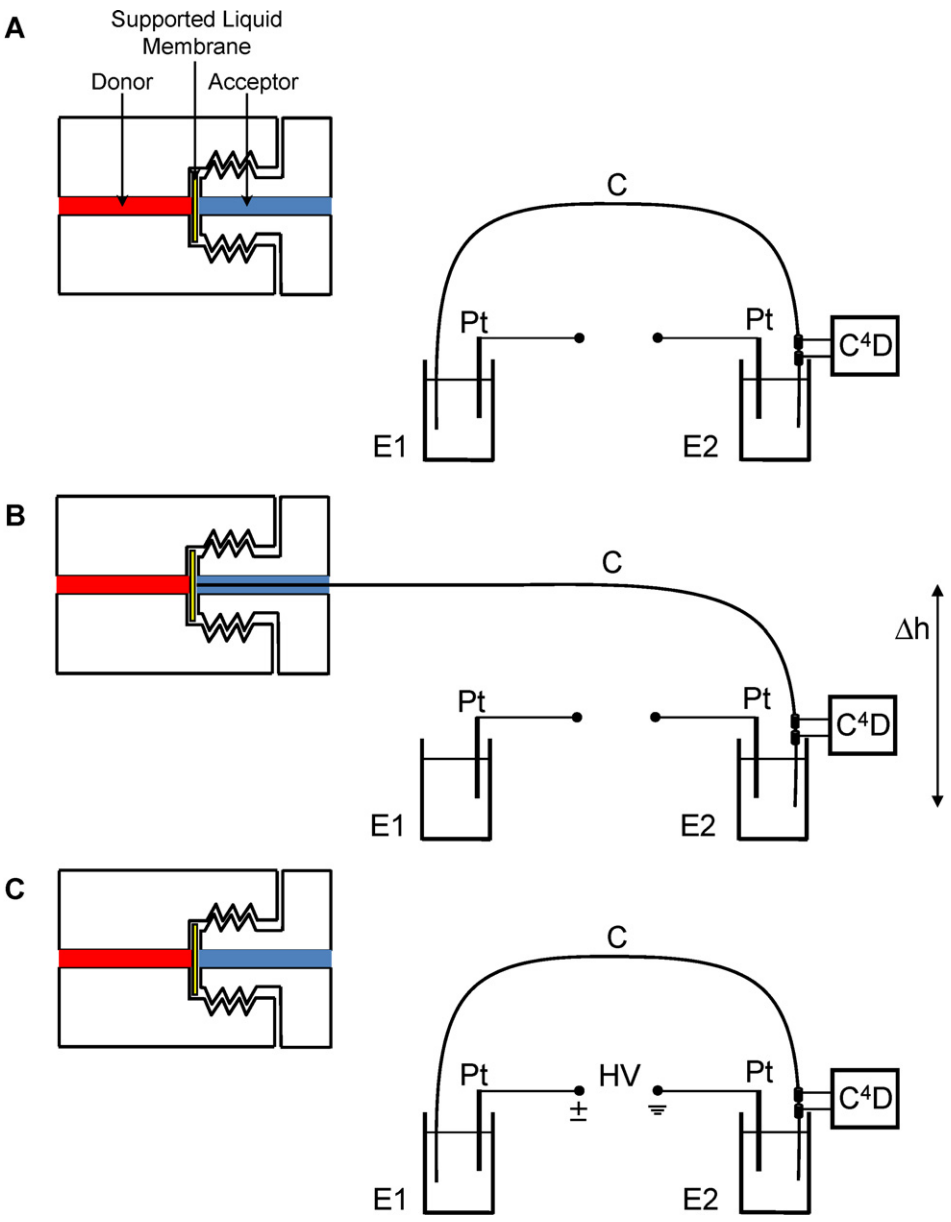

Fig. 1. Schematic drawing of the sample clean-up device for pretreatment of body fluids coupled on-line to CE. E1 and E2, vials with BGE solution; C, separation capillary; Pt, platinum electrodes; HV, high voltage power supply; C^4D, capacitively coupled contactless conductivity detector; Δh, height difference during sample injection. Analytical system during sample extraction (A), during sample injection (B) and during CE separation of the injected sample (C).

2.1.2. Electrophoretic system

A purpose-built CE instrument was employed for all electrophoretic runs. The separation voltage was provided by a high voltage power supply unit (Spellman CZE2000R Start Spellman, Pulborough, UK) and was operated at a potential of +20 kV applied at the injection side of the separation capillary for all runs. Separation capillaries used were FS capillaries (25 μm ID, 375 μm OD, 50 cm total length, 40 cm effective length, Polymicro Technologies, Phoenix, AZ, USA). Prior to CE measurements, separation capillaries were preconditioned by rinsing with 1 M NaOH for 5 min, DI water for 5 min and background electrolyte (BGE) solution for 5 min. Between two successive injections, the separation capillary was rinsed with fresh BGE solution for 1.5 min. Injection of standard solutions and real samples was carried out hydrodynamically by elevating the separation capillary to the sample clean-up device positioned in a height of 30 cm for 60 s, which represents less than 1.5% of total capillary volume. All CE experiments were performed at ambient temperature of 25 ± 2 °C.

2.1.3. Detection system

A capacitively coupled contactless conductivity detector (C^4D) used was developed and assembled at the Department of Physical Chemistry, Charles University and it operates at a frequency of 1.25 MHz [41]. Data were collected using a Panther-1000 (Ecom, Praha, Czech Republic) data acquisition system.

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 solution of 1.5 M Na^+ was prepared from NaCl (Pliva-Lachema, Brno, Czech Republic). Stock solutions of 10 mM creatinine (Crea), lysine (Lys), arginine (Arg), histidine (His), β-alanine (β-Ala), and ornithine (Orn) were prepared from pure chemicals (Sigma, Steinheim, Germany and Fluka, Buchs, Switzerland). Stock solution of 10 mM choline (Chol) was prepared from choline chloride (Sigma). Standard solutions for CE were prepared from these stock solutions and were diluted

with DI water. Standard donor solutions for on-line sample clean-up consisted of 19.25–75 mM Na^+ and various concentrations of amino acids and were diluted with DI water or various acetic acid solutions. Concentration of sodium corresponds to 1:1–1:7 diluted human serum samples; 1:1 and 1:4 dilutions were used for determination of amino acids in the body fluids (see next paragraph and Section 3.4). Organic solvents for liquid membranes were obtained from Sigma or Fluka and were of highest available purity. Bis(2-ethylhexyl) phosphate (DEHP) was purchased from Sigma. The solvents were used without any further purification. BGE solution for CE (0.5 M acetic acid) was prepared weekly from concentrated acetic acid (Fluka) and was kept at 4 °C.

Human plasma samples were purchased as lyophilized powders from Sigma and were prepared according to supplier's instructions. Human serum samples were obtained from volunteers at the Institute of Analytical Chemistry. Serum was prepared by centrifugation of whole blood at 6000 rpm for 10 min, immediately deep frozen and stored at −20 °C. All body fluids for sample clean-up were diluted 1:1 or 1:4 with DI water by mixing 25 μL (or 10 μL) of a particular body fluid with 25 μL (or 40 μL) of DI water.

2.3. Calculation of analyte transfer across the SLM

Transfer of analyte across the SLM was calculated according to the following equation [42]:

$$\text{Transfer}\,(\%) = \frac{n_{\text{a,final}}}{n_{\text{d,initial}}} \times 100 = \frac{V_{\text{a}}}{V_{\text{d}}} \times \frac{C_{\text{a,final}}}{C_{\text{d,initial}}} \times 100$$

where $n_{\text{a,final}}$ and $n_{\text{d,initial}}$ are the number of analyte moles finally present at the SLM in the acceptor solution and the number of analyte moles initially present in the donor solution, respectively. V_{a} is the volume of acceptor solution, V_{d} is the volume of donor solution, $C_{\text{a,final}}$ is the final concentration of analyte at the SLM in the acceptor solution and $C_{\text{d,initial}}$ is the initial analyte concentration in the donor solution.

3. Results and discussion

3.1. Selection of model analytes and CE conditions

To demonstrate the efficiency of the new on-line sample clean-up method, a set of seven rapidly migrating amino acids and related compounds (Chol, Crea, β-Ala, Orn, Lys, His, Arg) was selected in this experiment. These compounds are present in body fluids at concentrations significantly lower than the major inorganic cations, yet these concentrations are high enough to be easily accessed by CE with conventional detection techniques even in diluted body fluids. Sodium concentration ranges between 135 and 145 mM [43] and the seven amino acids are present at 0–220 μM [44] in serum and plasma samples of healthy individuals. Baseline separation of these analytes was achieved in acetic acid based BGE solutions recently [45,46] and we have adopted this BGE for our measurements. The samples were injected into the separation capillary by siphoning for 60 s from a height of 30 cm and CE-C^4D analysis of positively charged amino acids was achieved in 0.5 M acetic acid at +20 kV (see schemes in Fig. 1B and 1C, respectively).

3.2. Optimization of the on-line sample clean-up procedure

Transfer of the analytes from donor solution across the SLM into the acceptor solution is based on the same principle as described earlier for three phase SLM-LPME [15,19] and was examined using a standard solution of the seven amino acids at 50 μM prepared in 75 mM Na^+, which represents ca. 1:1 diluted human serum/plasma samples.

Briefly, a porous membrane, which is fully inert and allows no transfer of analytes at normal conditions, is impregnated with a water immiscible organic solvent to form a SLM, which separates donor and acceptor solutions. Analytes can then diffuse from donor solution through the SLM to acceptor solution depending on their distribution coefficients between organic and water phase and on the established concentration and pH gradient [16]. In SLM-LPME, the diffusive transfer is usually accelerated by convection, nevertheless, equilibrium is normally reached after 30–45 min and at certain conditions equilibration process may take up to several hours [11]. The complete acceptor solution is then on-line transferred to an analytical system [15,16] or is withdrawn and used for subsequent off-line injection [11].

The on-line arrangement of the sample clean-up device depicted in Fig. 1B allows the separation capillary to be inserted into the acceptor chamber and inject the pretreated sample directly from the surface of the SLM. Equilibrium between the donor solution on one side and the acceptor solution on the other side of the SLM is established rapidly (see Section 3.2.3) in the centre of the clean-up device and then is gradually extended to the whole acceptor chamber by diffusion. In other words, equilibrium is reached very fast at the SLM surface where the actual injection into the separation capillary takes place and the pretreatment time can thus be significantly reduced with no need for sample convection.

3.2.1. Composition of SLM

Selection of SLM is critical for efficient transfer of analytes from donor to acceptor solution. It has been shown that short peptides and amino acids can be transported through SLMs consisting of various organic solvents with addition of suitable ionic carriers [46–50]. These ionic carriers are prone to form ion pairs with charged amino acids, which can be then transferred easily through the organic layer. 15% (v/v) of DEHP was added to ENB in our previous study to increase the extraction efficiency of amino acids in EME [46] and addition of 15% (v/v) DEHP was also adopted in the actual experiments. 1-Octanol, 2-nitrophenyloctyl ether (NPOE) and 1-ethyl-2-nitrobenzene (ENB) were examined as suitable organic solvents. Two sets of on-line pretreatments were performed at 5 min equilibration time; one with pure organic solvents and the other one with the same organic solvents doped with 15% (v/v) of DEHP. The transfer of amino acids through the SLM formed by pure organic solvents was hindered for NPOE and ENB, however, approximately 30–40% of all amino acids was transferred to acceptor side of the SLM for 1-octanol. After addition of DEHP, transfer from donor to acceptor side of the SLM increased significantly for NPOE and ENB, whereas only a slight increase was observed for 1-octanol. Highest transfer of amino acids (40–50%) was observed for ENB with addition of DEHP (15%, v/v), which was also used in all subsequent experiments.

3.2.2. Donor and acceptor solutions

The effect of composition of donor and acceptor solution on transfer of amino acids to the acceptor side of the SLM was investigated. Acidic (10–500 mM acetic acid in DI water; amino acids are positively charged) and neutral conditions (DI water; amino acids are neutral or weakly positively charged) were examined for donor and acceptor solutions. Transfer through the SLM gradually decreased for increasing concentration of acetic acid in donor solution and, compared to DI water, 500 mM acetic acid gave approximately twofold lower transfer of the amino acids after 5 min equilibration time. Similar behaviour was also observed for acidified acceptor solutions. At low acetic acid concentration (10 mM) the results were comparable with DI water, whereas the transfer gradually decreased for increasing acetic acid concentrations. As observed, the pH gradients, which were investigated above and are often applied in LPME, has deteriorated the transfer of amino

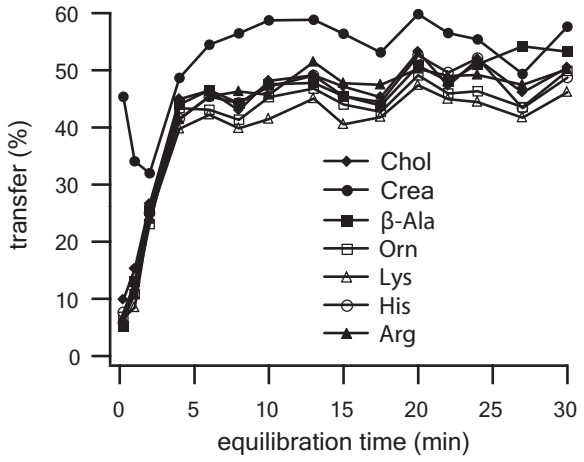

Fig. 2. Effect of equilibration time on transfer of amino acids through the SLM.

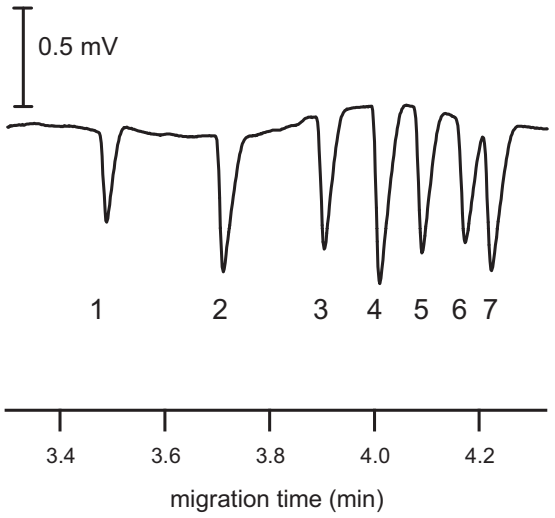

Fig. 3. CE-C^{4}D of selected amino acids (50 μM) in 75 mM Na^+ solution after on-line sample clean-up for 5 min. (1) Chol, (2) Crea, (3) β-Ala, (4) Orn, (5) Lys, (6) His and (7) Arg. CE conditions: BGE solution, 0.5 M acetic acid at pH 2.5, voltage, +20 kV, injection, 30 cm for 60 s.

acids through the SLM. One possible reason for this behaviour might be the competition of protons (from acetic acid) forming ion pairs with DEHP, which are transferred through the SLM. Acetic acid is partly dissociated in aqueous media and the presence of high concentrations of protons at the SLM surface shifts the balance of the equilibration process in favour of the major matrix ions, which are in significant excess compared to the analytes and are preferentially transferred through the SLM. This consequently results in slower or incomplete transfer of the amino acids. Another reason might be the fact that diffusion is monitored only in the very narrow band at the SLM where a local pH change formed due to the presence of DEHP might be sufficient for efficient transfer of analytes through the SLM. However a more complex study would be necessary to fully understand the transfer process. For these reasons and also in order to keep the clean-up system as simple as possible, DI water was selected for the donor and acceptor solutions.

3.2.3. Equilibration time

The speed of the equilibration process at the SLM was investigated and the results are depicted in Fig. 2. The clean-up device was filled with standard solution (donor chamber) and DI water (acceptor chamber) and was equilibrated for various times ranging from 15 s to 30 min. 15 s was the minimum equilibration time achieved due to manual manipulation with the clean-up device, micropipettes and separation capillary and 30 min was chosen as maximum since longer equilibration times were considered excessive. All amino acids (except Crea) exhibited gradual equilibration process at the SLM in the first 4 min. At 15 s, 4–10% of the original concentration of amino acids in donor solution was transferred to the SLM side of acceptor solution, which increased to 40–50% after 4 min of equilibration. Creatinine showed a slightly different pattern; the initial transfer after 15 s (almost 45%) was followed by a slight decrease within the first 2 min and then similar transfer rate as for the other analytes was observed. The reason for the different behaviour of creatinine in the initial 2 min is not known, but it may be explained by a former study on electrically induced transfer of amino acids across SLM composed of ENB/DEHP, where creatinine exhibited highest extraction efficiencies among all other amino acids [46]. At the equilibration time of 4 min flattening of the transfer curves can be observed for all analytes showing that the equilibrium was reached. No significant change in concentrations of amino acids was observed between 4 and 30 min. The total transfer was between 40 and 58% showing that approximately half of the initial concentration of amino acids in donor solution was transferred to the acceptor solution. 5 min equilibration time was selected, which ensures rapid pretreatment time and stable transfer efficiency as evidenced by the plateau regions of the curves. CE-C^{4}D analysis of a standard solution of 50 μM of the seven amino acids injected from the acceptor chamber after on-line sample clean-up for 5 min is depicted in Fig. 3.

In order to understand the transfer process more closely, concentrations of amino acids on the other side of the SLM (in donor chamber) were also analysed and it was found that concentrations of amino acid at the SLM surface decreased from 100% to 40–60% during the first 5 min of equilibration. This confirms the conclusion that the concentrations of analytes on both sides of the SLM reach equilibrium after relatively short time, are fairly constant and represent approximately 50% of the original donor concentration. Note, however, that this equilibrium is reached only at the SLM surface and in its closest vicinity. This is, however, not surprising considering the fact that the ratio of effective area of the SLM vs. volume of the clean-up device is small and diffusion in such device is rather slow.

3.2.4. Interferences

Concentrations of major inorganic cations and matrix components (such as proteins) in human blood samples are quite constant, however, in case of inborn or momentary diseases these concentrations vary and their effect on determination of amino acids was investigated. Concentration of Na^+ was varied among 19.25, 37.5 and 75 mM, whereas the concentration of amino acids (50 μM) was kept constant. No statistically significant change in concentrations (peak areas) of the pretreated amino acids was observed at the acceptor side of the SLM for the three different Na^+ concentrations, however, peak shapes were slightly dispersed for the lowest Na^+ concentration as can be seen in Fig. 4. We assume that the transient ITP stacking effect due to the broad zone of faster Na^+ cation is weakened for lower Na^+ concentrations and results in slightly dispersed peaks of amino acids. This means that there is no quantitative limitation of various Na^+ concentrations on determination of amino acids but since peak integration might be affected by the peak dispersion, the higher Na^+ concentration might be desirable and for this reason the real sample dilution should be minimal.

Blood proteins, such as human serum albumin (HSA, normal level of blood proteins is ca. 70 g/L), seriously interfere with CE analysis when blood samples are injected directly and their efficient elimination by SLM is of key importance. To investigate it, a set of model samples with various concentrations of

Table 1
Analytical parameters of the developed method for determination of amino acids, $n = 6$. Pretreatment and CE-C^{4}D conditions as for Fig. 3.

	Chol	Crea	β-Ala	Orn	Lys	His	Arg
RSD (%), MT	1.4	1.5	1.5	1.6	1.6	1.6	1.6
RSD (%), PA (5 min)	6.0	4.1	4.5	4.0	5.6	3.8	5.4
RSD (%), PA (12 min)	6.1	6.1	6.6	7.6	8.4	6.7	6.0
RSD (%), PA (19 min)	5.2	3.5	4.2	3.2	2.4	5.3	6.1
r^2	1.0000	0.9992	0.9999	0.9995	0.9999	0.9991	0.9995
LOD (μM)	2.5	1	1	0.75	1	1.5	1

MT, migration time; PA, peak area.

HSA (10, 20 and 40 g/L), and constant concentrations of Na^+ (75 mM) and of the seven amino acids (50 μM) was prepared. HSA is positively charged in acetic acid based BGEs and can also be sensitively determined using CE-C^{4}D [51]. A negative peak migrating faster than the seven amino acids was observed when standard solution of 50 μM HSA was injected. Concentrations (peak areas) of amino acids at the acceptor side of SLM were not affected by the presence of different HSA concentrations. Moreover, the peak of HSA was not observed in the electropherograms, demonstrating that the on-line clean-up process efficiently retained various concentrations of HSA.

3.3. Analytical performance

The method was validated and the results are summarized in Table 1. Repeatability of the measurements was calculated for six independent clean-ups of the standard solution and was around 1.5% and between 2.4 and 8.4%, for migration times and peak areas of the seven amino acids, respectively. Repeatability of peak areas for each clean-up procedure was examined at three different equilibration times (5, 12 and 19 min) and showed no significant variations. Concentrations of amino acids at the acceptor side of the SLM were fairly constant for all equilibration times. Absolute peak areas of the amino acids were slightly lower (85–95%) for 5 min equilibration time compared to those for 12 and 19 min, however, the difference was negligible. To maintain short pretreatment time, 5 min was used in all subsequent experiments. Linearity of the method was examined in the concentration range 5–100 μM of amino acids keeping the Na^+ concentration constant at 75 mM in all calibration solutions. Excellent linearity was achieved with correlation coefficients better than 0.9991. Limits of detection (LODs, based on 3 S/N criteria) were determined for the calibration solution with lowest concentration of amino acids and ranged from 0.75 to 2.5 μM. These LODs ensure sensitive determination of amino acids even in several-fold diluted real samples.

3.4. Applications to real samples

The on-line sample clean-up procedure was tested with human blood serum and plasma. First, serum and plasma samples were analysed at 1:1 dilution with DI water. At these conditions, deterioration of CE performance was observed as a consequence of matrix components adsorption onto the inner capillary wall. The complex blood matrix interacts very likely with the liquid membrane, leads to its partial depletion and penetrates into acceptor solution. Similar behaviour was also observed for EME using ENB and ENB/DEHP as SLM [42,46]. Moreover, the SLM is 2–3-fold thinner in the presented on-line arrangement compared to SLMs in [42,46], which significantly reduces the total volume of the organic phase between donor and acceptor solution and thus the SLM may be susceptible to depletion due to contact with minimally diluted real samples. In order to avoid the SLM depletion, serum and plasma samples were diluted 1:4 with DI water. At this dilution, performance of the analytical system was stable and demonstrated complete isolation of analytes from matrix components (Fig. 5). Six successive clean-ups of the 1:4 diluted serum and plasma samples were performed and the samples were on-line injected into the separation capillary. Repeatability of the measurements is summarized in Table 2. The RSD values of peak areas ranged between 3.4 and 10.5% and did not significantly differ from those for standard solutions.

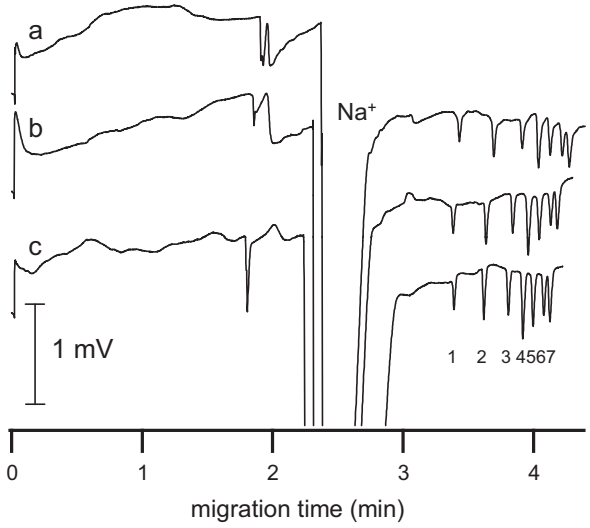

Fig. 4. CE-C^{4}D of selected amino acids (50 μM) in various concentrations of Na^+ after on-line sample clean-up for 5 min. Peak description and CE conditions as for Fig. 3. (a) 19.25 mM Na^+, (b) 37.5 mM Na^+ and (c) 75 mM Na^+.

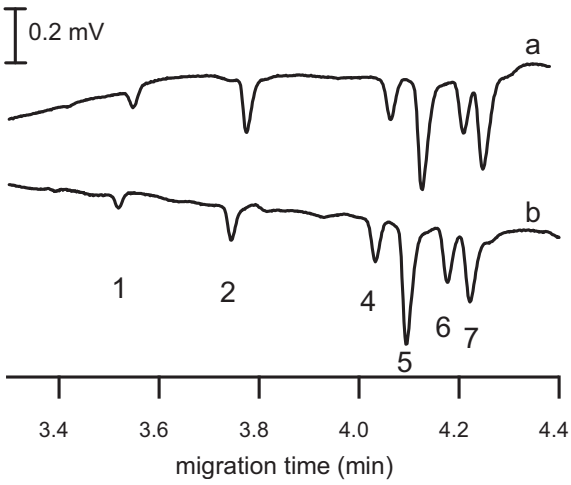

Fig. 5. CE-C^{4}D of selected amino acids in human serum and plasma after on-line sample clean-up for 5 min. Peak description and CE conditions as for Fig. 3. (a) Human serum and (b) human plasma. Body fluids were diluted 1:4 with DI water.

Table 2
Repeatability of the sample pretreatment method coupled to CE-C^4D and quantitative determination of the concentrations (μM) of amino acids in blood serum and plasma, $n = 3$. Pretreatment and CE-C^4D conditions as for Fig. 3.

	Chol	Crea	β-Ala	Orn	Lys	His	Arg
RSD (%), serum	7.3	8.5	n.d	6.3	8.9	10.5	9.9
RSD (%), plasma	7.9	5.0	n.d.	3.9	4.9	3.4	5.6
Serum (μM)	38.3	53.8	n.d.	26.2	132.9	68.7	113.8
Plasma (μM)	31.2	31.4	n.d.	32.8	139.2	60.8	59.8

n.d., not detected.

Concentrations of amino acids in serum and plasma samples, also summarized in Table 2, were calculated based on external calibration curves (derived as in Section 3.3, 4-point calibration using 30 mM Na^+ and 5–50 μM of amino acids in DI water) and were within the ranges of reference values given in [44,45,52].

4. Conclusions

A simple device was described, which can be coupled on-line to CE for rapid and efficient clean-up of samples with complex matrices. Body fluids, such as blood serum and plasma, were only diluted with DI water and sampled into a donor chamber, which was separated from an acceptor chamber by a thin SLM. This SLM was formed by impregnating PP planar membrane with water immiscible organic phase consisting of ENB and DEHP. Equilibrium between the donor and acceptor solution at both sides of the SLM was reached rapidly and proper selection of the liquid membrane enabled the analytes of interest to be transferred to the acceptor chamber, whereas interfering matrix compounds were retained on the SLM and did not interfere with subsequent CE determination. The ion transfer through the SLM is driven solely by diffusion and the sample is injected directly from the SLM surface. The method was demonstrated by the determination of a set of seven amino acids in blood serum and plasma and may be applied to a wide range of analytes ranging from major (in mM concentrations) to minor (in μM to sub-μM concentrations) blood components since only 1:4 dilution of the original sample is necessary. The method is environmentally friendly (uses only 2.5 μL organic solvent per clean-up), cheap (the membrane price is less than 0.01 € per clean-up), which renders it a single-use disposable membrane, the device can simply be assembled/disassembled and can therefore be applied in any analytical laboratory. Moreover, selectivity of the ion transfer from donor to acceptor chamber can be fine-tuned by proper selection of the SLM composition. The consumption of biological samples is only 10 μL per analysis.

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