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High performance capillary electrophoresis separation of low molecular weight compounds in biological and environmental samples

Doctoral Dissertation

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……..
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DEDICATION

This dissertation is dedicated to my parents. I would not be who I am today without them.

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ABSTRACT

This dissertation thesis deals with the separation of small ions in biological and environmental samples using capillary zone electrophoresis (CZE) with capacitively coupled contactless conductivity detection (C^4D). Analysis of these samples has its own difficulties, because of the usually limited sample amount, the low analyte concentrations and the complex sample matrices. CZE can successfully handle the low sample volumes but sample pretreatment or preconcentration is usually required.

First, electrodialysis (ED) was used for rapid pretreatment of inorganic cations in biological samples that contain large amounts of high molecular weight compounds that can be adsorbed on the capillary wall and distort the separation. Combination of ED with CZE greatly improved the analytical performance, because the high molecular weight compounds could be efficiently removed from the small cations of interest. In µED system the volume of sample needed was as low as 1 µL per analytical run, which kept the analyses possible even in case the amount of biological sample is limited. Using the developed system, the analysis of a single drop of whole blood was possible.

Second, open tubular (OT) ion exchange precolumns were for the first time used for in-line sample clean-up and direct injection of biological samples into CZE. OT ion exchange precolumns were used as similar in function (remove proteins) but different in principle (ion exchange removal) and showed some advantages over previous ED system. Preparation of these precolumns was simple, fast and inexpensive. They can therefore be used as disposable sample pretreatment units which can be simply discarded after each use. The in-line sample pretreatment method was demonstrated on determination of small inorganic cations in blood serum and blood plasma samples.

Third, electromembrane extraction (EME) and CZE-C^4D was applied to rapid and sensitive determination of perchlorate in various samples of drinking water and environmental samples. Porous polypropylene hollow fibre impregnated with 1-heptanol acted as a suported liquid membrane (SLM), perchlorate was transported and preconcentrated in the fibre lumen upon application of an electric field. High sensitivity of perchlorate determination and its baseline separation from major inorganic anions was achieved in optimized CZE-C^4D conditions. Perchlorate could be detected in spiked tap water down to a concentration of 1µg/L, which is 15 times lower than the US Environmental Protection Agency (US EPA) recommended limit for drinking water.

The proposed method compares well with routinely used methods, with much shorter running times, unprecedented simplicity and reduced costs.

Keywords: Capillary zone electrophoresis (CZE), capacitively coupled contactless conductivity detection (C^4D), ion exchange (IE), electrodialysis (ED), electromembrane extraction (EME).

ABSTRAKT

Tato disertační práce se zabývá separací malých iontů pomocí kapilární zónové elektroforézy (CZE) s bezkontaktní konduktometrickou detekcí (C^4D) v biologických a environmentálních vzorcích. Analýza těchto typů vzorků je obtížná především proto, že jejich objem je omezený, analyty se v nich vyskytují v nízkých koncentracích a vzorky dále obsahují složitou matrici. CZE si je schopna poradit s omezeným objemem, ale pro citlivé stanovení je často potřeba úprava a zakoncentrování vzorku.

V první části této disertační práce byla pro úpravu biologických vzorků použita elektrodialýza (ED). Biologické vzorky obsahují velké množství vysokomolekulárních látek, které se lehce vážou na stěny křemenné kapiláry a způsobují zhoršení separace. Kombinací elektrodialýzy a CZE je možné tyto problémy eliminovat, protože vysokomolekulární látky jsou odděleny od malých iontů a nevstupují do separační kapiláry. Při použití mikroelektrodialýzy (μED) je možné navíc redukovat potřebné množství vzorku na ca. 1 μL, což umožňuje analýzu i takových vzorků, u kterých je množství limitováno. Bylo například možné stanovit malé kationty z jedné kapky krve.

Ve druhé části byly vůbec poprvé použity otevřené kapilární iontově výměnné kolonky pro in-line úpravu spojenou s přímým dávkováním do CZE systému. Otevřené kapilární iontově výměnné kolonky splnily podobnou funkci – odstranily ze vzorku vysokomolekulární látky, principem bylo ovšem navázání těchto rušivých látek na stěny kolonky pomocí iontově výměnného mechnismu. Výroba kolonek je jednoduchá, časově nenáročná a levná. Kolonky mohou být proto použity jednorázově a po jednom použití vyhozeny. In-line úprava vzorků byla využita pro stanovení malých kationtů ve vzorcích krevního séra a plasmy.

Ve třetí části této práce byla vyvinuta metoda elektromembránové extrakce (EME) spojená s CZE-C^4D pro citlivé stanovení chloristanu ve vzorcích pitné vody a dalších environmentálních vzorcích. Porézní polypropylénové duté vlákno bylo naimpregnováno 1-heptanolem a sloužilo jako kapalná membrána (SLM), přes kterou docházelo k selektivnímu transportu a zakoncentrování chloristanu za pomocí elektrického proudu. V optimalizovaném CZE systému bylo dosaženo separace chloristanu od majoritních iontů přítomných ve vzorcích. Chloristan byl stanoven ve vzorcích pitné vody až do detekčního limitu, který odpovídal 1μg/L, což odpovídá koncentraci 15x nižší než je doporučená mezní hodnota pro chloristan v pitné vodě dle

agentury EPA. Vyvinutá metoda je srovnatelná s rutinními analytickými metodami a vyznačuje se mnohem kratší dobou analýzy, jednoduchostí a malými náklady.

Klíčová slova: Kapilární zónová elektroforéza (CZE), bezkontaktní konduktometrická detekce (C^4D), iontová výměna (IE), elektrodialýza (ED), electromembránová extrakce (EME).

LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

PUBLICATIONS

1. **Thi Kieu Oanh Doan**, Pavel Kubáň, Petr Kubáň, Isaac Kipkoech Kiplagat, Petr Boček

   Analysis of inorganic cations in biological samples by the combination of micro-electrodialysis and capillary electrophoresis with capacitively coupled contactless conductivity detection.

   Electrophoresis. 32 (2011), 464-471.

2. Isaac Kipkoech Kiplagat, **Thi Kieu Oanh Doan**, Pavel Kubáň, Petr Kubáň, Petr Boček

   Use of disposable open tubular ion exchange pre-columns for in-line clean-up of serum and plasma samples prior to capillary electrophoretic analysis of inorganic cations.

   Journal of Chromatography A. 1218 (2011), 856-859.

3. Isaac Kipkoech Kiplagat, **Thi Kieu Oanh Doan**, Pavel Kubáň, Petr Boček

   Trace determination of perchlorate using electromembrane extraction and capillary electrophoresis with capacitively coupled contactless conductivity detection.

   Electrophoresis 32 (2011), 3008–3015.

CONFERENCE PRESENTATIONS

1. Kubáň, P., Langová, L., **Kieu Oanh Doan Thi**, Gebauer, P., Kubáň, P., Boček, P.

   Electric field enhanced transport across phase boundaries and membranes for sample pretreatment in bioanalysis.

   17th International Symposium on Electro- and Liquid Phase-Separation Technologies, ITP 2010, Baltimore, MD, USA, 2010. Invited oral presentation.

2. Kubáň, P., Strieglerová, L., **Kieu Oanh Doan Thi**, Kiplagat, I.K., Boček, P.

   Electromembrane extraction across supported liquid membranes combined with capillary electrophoresis for pretreatment and analysis of biological samples.

   18th International Symposium on Electro- and Liquid Phase-Separation Technologies, ITP 2011, Tbilisi, Georgia, 2011. Oral presentation.

1. INTRODUCTION

Modern analytical chemistry has been dramatically changed by the incorporation and development of novel physicochemical methods, by the recognition of chemical compounds and by the measurement of electrical, magnetic or optical properties.

Capillary zone electrophoresis (CZE) is a modern analytical technique with much promise based on how simple CZE actually is, and how quickly the analytical result can be obtained. CZE with capacitively coupled contactless conductivity detection (C^4D) is especially promising for the analysis of small inorganic ions and biochemical species.

However, analysis of small inorganic ions in biological samples by CZE has its own difficulties, because of the usually limited sample amount, the low analyte concentrations and the complex sample matrices. CZE can successfully handle the low sample volumes and in some instances even the low concentrations, however, the influence of matrix compounds on CZE separation performance presents a major limitation. Thus, separation of small molecules from the macromolecular matrix or clean-up of the macromolecular matrix from the sample is often required prior to a CZE analysis of real samples.

In environmental samples, the concentration of the pollutant ions is typically very low, even lower than the limit of detection of the commonly used analytical methods. The sample matrix composition on the other hand is not very difficult. Therefore, a clean-up step is often not necessary (especially in water samples), but preconcentration is often needed.

To help solve some of the issues in the separation of low molecular weight compounds in biological and environmental samples by CZE, various novel sample pre-treatment and pre-concentration methods were developed in this thesis.

2. THE GOALS OF THE THESIS

The goals of this thesis were:

1 - development and optimization of a suitable sample clean-up procedure for the determination of small ions in biological samples based on (off-line) electrodialysis and microelectrodialysis prior to CZE-C^4D analysis.

2 - development and optimization of an in-line clean-up procedure based on disposable open tubular ion exchange pre-columns for clean-up of biological samples prior to determination of small ions by CZE-C^4D.

3 - development and optimization of an electromembrane extraction procedure and apparatus for preconcentration and trace analysis of perchlorate by CZE-C^4D.

3. THEORETICAL BACKGROUND AND LITERATURE OVERVIEW

3.1. CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) encompasses a family of related separation techniques that use narrow-bore fused-silica (FS) capillaries and can be used to separate a complex mixture of large and small molecules. High electric field strengths are used to separate molecules based on differences in charge and size. Sample introduction is accomplished by immersing the end of the capillary into a sample vial and applying pressure, vacuum or voltage. Depending on the separation system the technology of CE can be segmented into several separation techniques, such as capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE) or capillary electrochromatography (CEC) [1]. There are numerous other sub-types of CE but for the purpose of the focus of this project, CZE will be discussed.

3.1.1. Basic instrumentation

The basic CZE instrumentation is shown schematically in Figure 1.

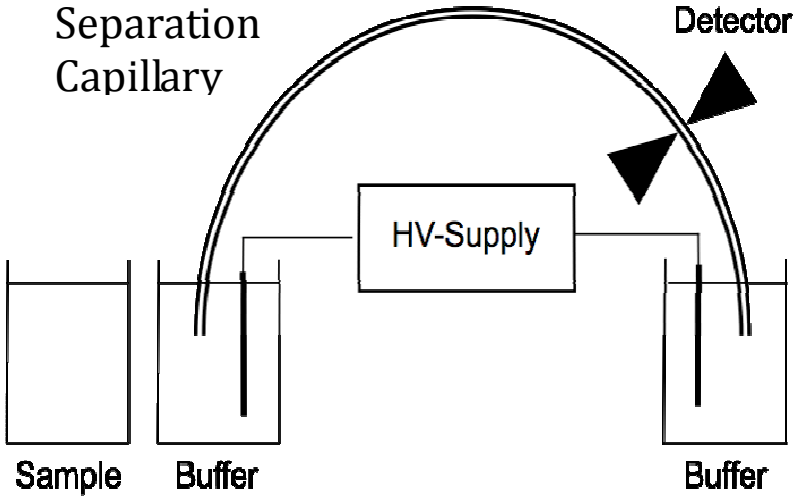


Figure 1. The schematic of a CZE system.

It includes a high voltage (HV) power supply capable to provide the potential in the range ±30 kV, a polyimide-coated FS capillary having inner diameter usually from 25 μm up to 100 μm, two separation electrolyte vials filled with background electrolyte (BGE) that can accommodate both the capillary ends and the platinum electrodes, and a detector. The data from detector are collected and evaluated usually with use of a personal computer.

3.1.2. Introduction to the CZE theory

Differences in solute velocity in an electric field make it possible for electrophoresis separation. Once the ions in the field are charged, the electric force received causes their acceleration; however, this force will be counteracted by the friction force of the surrounding media, which will result in a constant velocity of a particular ion. The velocity of an ion can be given by:

$$v_i = \mu_i E \qquad (1)$$

where v_i : ion velocity
μ_i : electrophoretic mobility of a species i
E : applied electric field

In this formula, the applied electric field, E, is simply a function of the applied voltage and capillary length (volts/cm). Based on the characteristic of a given ion and using the standard tables, the electrophoretic mobility, μ_i, is usually found as a physical constant. The electrophoretic mobility is determined by the electric force that the particle experiences, balanced by its frictional force through the medium. For a spherical ion the electrophoretic mobility can be given by :

$$\mu_i = z_i e / 6\pi\eta r_i \qquad (2)$$

where z_i : ion charge
e : electron charge in Coulombs
η : solution viscosity
r_i : ion radius

From this equation it is shown that the charge (z_i) and size (r_i) of each species influence its electrophoretic mobility. Electrophoretic mobility is high for small, highly charged species, but low for those with large sizes and low charge. Also, the migration velocity of a charged species depends on other factors, i.e. ambient temperature and electroosmotic flow.

Electroosmotic flow (EOF)

The main phenomenon accompanying all electrophoretic separations in the fused silica capillary is an electroosmotic flow (EOF). Electroosmotic flow is best described as the movement of liquid relative to a stationary charged surface under an applied electric field. This movement is generated at the solid-liquid interface of the internal wall of the capillary (in CZE). As per the illustration in Figure 2, in a fused silica capillary, the ionization of silanol groups gives rise to a negatively charged surface (SiO^-), which affects the distribution of nearby ions in solution. To maintain the charge balance, an excess of positive ions (counter-ions) is attracted to the surface, forming an electrical double layer whilst ions of like charge (coions) are repelled. Essentially the counter-ions are arranged in two regions, including the fixed layer at the surface (Stern layer) and the diffused layer that extends into the bulk of the solution (Gouy-Chapman layer) [2,3].

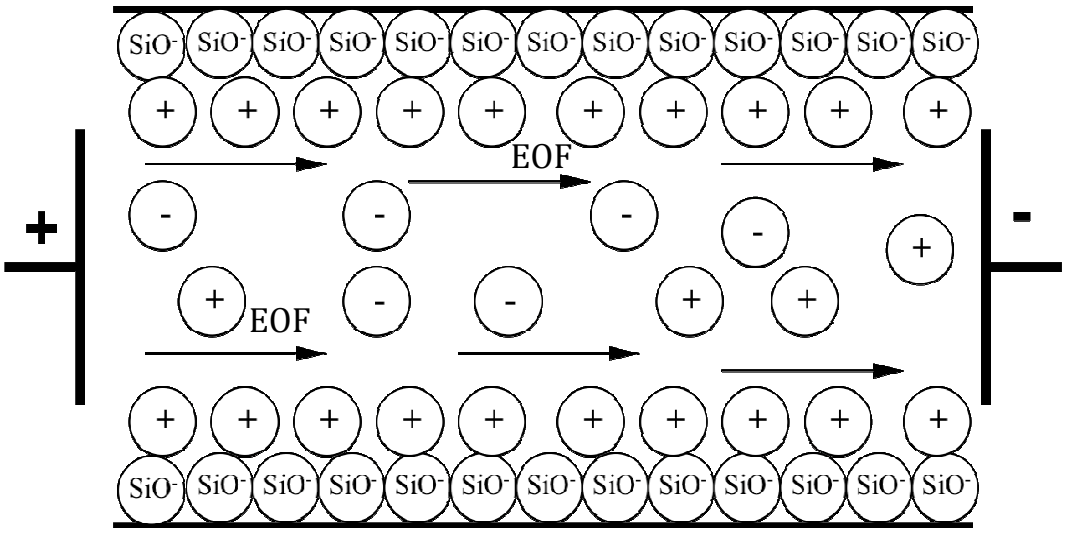


Figure 2. Internal surface of a fuse silica capillary filled with electrolytes.

The solvated cationic species under the influence of an electric field are electrically driven towards the negative electrode (cathode) and as they migrate toward the cathode, these solvated cationic species drag solvent molecules along, which generates electroosmotic flow. Consequently, in bare FS capillary with negatively charged capillary surface the EOF flow is from the anode to the cathode. Under special circumstances, with sufficiently high EOF, cations, neutrals, and anions will move in the same direction and hence positively and negatively charged species can be simultaneously determined in one single run, which is otherwise not feasible.

The EOF through the capillary has a characteristic flat profile, as shown in Figure 3A, in contrast to the parabolic profile typical for pressure-driven systems (in all chromatographic techniques) (Figure 3B). The flat EOF profile has a great advantage in that all solutes experience the same delivering velocity induced by EOF regardless of their cross-sectional position inside the capillary. Sharp peaks of high efficiency thus will be generated and the analytes migrate as narrow bands.

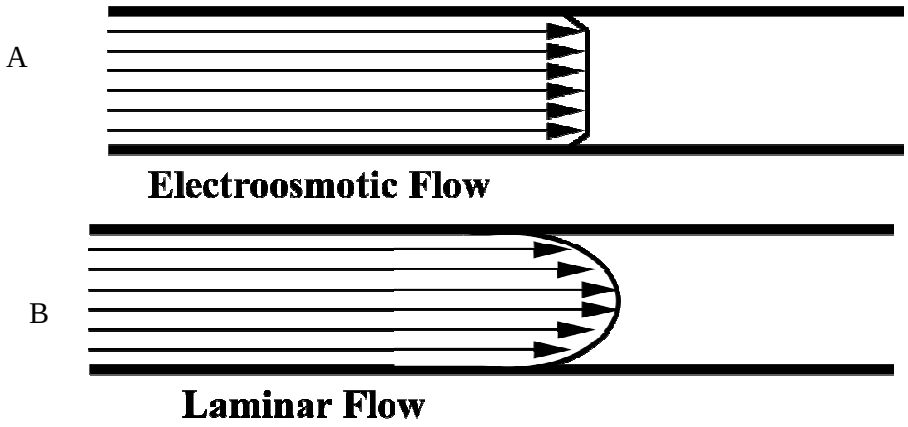


Figure 3. Comparison of a flow profile in A - capillary electrophoresis ; B - liquid chromatography.

The magnitude of the EOF can be derived in terms of velocity or mobility (vEOF and μEOF) in open tubes (for CE) according to Smoluchowski's equations

$$v_{EOF} = \frac{\varepsilon\zeta}{\eta} E \qquad (3)$$

$$\mu_{EOF} = \frac{\varepsilon\zeta}{\eta} \qquad (4)$$

where ε : dielectric constant of the electrolyte

η : the electrolyte viscosity

E : the electric field applied

ζ : the zeta potential of the wall

EOF has important benefits for CZE separations; however provided that it is properly controlled. By changing the corresponding experimental parameters such as electrolyte concentration, ionic strength, pH, temperature, surface characteristics, and co-solvents used, variables affecting EOF, i.e. the solution viscosity, dielectric constant and zeta potential can be modified and thus bring some detectable changes in the EOF. One of the methods to significantly modify the EOF is by coating of the capillary wall. Dynamic coatings (that is, separation electrolyte additives) or covalent coatings are the two most common means to modify the EOF. They can increase, decrease, or reverse the surface charge and thus the EOF. The compounds used for dynamic capillary wall coating (so called EOF modifiers) are usually cationic surfactants having a long alkyl chain and an ammonia group, such as cetyltrimethylammonium bromide (CTAB), tetradecylammonium bromide (TTAB), diethylenetriamine (DETA), etc. and are typically used in the analysis of anions. Further information about controlling EOF can be found in sevaral comprehensive books [1, 4, 5, 6, 7] and reviews [8, 9, 10].

3.1.3. Injection techniques in CZE

All capillary separation techniques including CZE have certain constraints on the amount of material that can be injected. The injection volume must be kept quite small because the total internal volume of a 50 cm long FS capillary with 50µm or 25µm ID is in the nanoliters (nL) range (981nL for 50 cm capillary, 50 µm ID). On the other hand sufficient injection volume is necessary to achieve acceptable limits of detection. There are two main modes of injection in capillary electrophoresis (CZE): hydrodynamic injection and electrokinetic injection.

Hydrodynamic injection

Hydrodynamic injection is accomplished by: elevating the capillary at the sample (inlet) end permitting sample introduction by siphoning, applying external pressure on the inlet vial with the sample, applying a vacuum on the outlet electrolyte vial or injecting by syringe and employing a splitter to reduce volume introduced. The volume of sample injected per unit time is determined by the Poiseuille equation :

$$Volume = \frac{\Delta P d^4 \pi t}{128 \eta L} \qquad (5)$$

where ΔP : the pressure difference across the capillary

d : the capillary internal diameter

t : the time

η : the separation electrolyte viscosity

L: the total length of the capillary.

For siphoning injection, the pressure differential, ΔP, in equation above is given by

$$\Delta P = \rho g \Delta h \qquad (6)$$

where ρ : the separation electrolyte density

g : the gravitational constant

Δh : the height difference between the liquid levels in the sample vial and in the detector-side separation electrolyte vial.

A typical siphoning injection is obtained by raising the sample vial 5 to 10 cm above the outlet vial for 10 to 30 seconds, depending on the conditions. Siphoning is typically used in systems without pressure injection capabilities.

Electrokinetic injection

Electrokinetic injection, which is also known as electromigration injection is performed by 2 main actions in the following sequence: (i) replacement of the injection-end vial with the sample vial and (ii) application of a high voltage (usually 3 to 5 times lower than that used for separation). In this injection process, analyte enters the capillary due to both migration and the pumping action of the EOF. The quantity loaded is dependent on the electrophoretic mobility of the individual solutes, which is a unique property of electrokinetic injection. As a result there are variations in quantity of loaded ions depending on their electrophoretic mobility and discrimination occurs for various ionic species. The quantity (Q) of an analyte injected is calculated by:

$$Q = \frac{(\mu_e + \mu_{EOF})V\pi r^2 Ct}{L} \qquad (7)$$

where μ_e : the electrophoretic mobility of the analyte

μ_{EOF} : the EOF mobility

V : the voltage

r : the capillary radius

C : the concentration of the analyte

t: the time

L : the capillary total length

Equation (7) illustrates that sample loading is dependent on three factors: (i) the EOF, (ii) the sample concentration, and (iii) the analyte mobility. The electrokinetic injection is generally not as reproducible as its hydrodynamic counterpart. The advantage of electrokinetic injection is that this process requires no additional instrumentation, is advantageous when viscous media or gels are employed in the capillary and when hydrodynamic injection is ineffective. Electrokinetic injection is very simple, but it has limitations for quantitative analysis.

3.1.4. Selection of a separation system in CZE

The selection of the separation system and in particular the selection of background electrolyte (BGE) is of paramount importance, because it affects both the separation and repeatability of CZE analysis. There are few rules for the selection of a suitable BGE. First the BGE should have an appropriate buffer capacity in the pH range of choice and low mobility to minimize current generation. The mobilities of the BGE co-ion should match as closely as possible the mobility of the analyte(s) as it is important for minimizing the band distortion. These premises are fully valid in CZE with UV-detection, for instance, but for CZE-C^4D, they are not completely feasible. With C^4D (or conductivity detection in general) the measured signal is the difference in conductance of the analyte and the BGE co-ion. Thus, to maximize the sensitivity this difference should be high, on the other hand, to get symmetrical peaks the difference should be low. Therefore in CZE-C^4D, a compromise is needed in the BGE selection process. In CZE-C^4D, the separation is usually accomplished in a BGE consisting of a low conductance (and hence mobility) co-ion at relatively high concentration. Therefore the peaks detected in C^4D are not symmetrical but rather triangular. The high concentration of BGE co-ion asures that the separation efficiency is maintained. There are other factors however, that contribute to the success of the CZE separation and good repeatability, for instance the capillary type, capillary conditioning and even the quality of the power supply. They all affect the overall reproducibility of CZE separation, as shown in detail in Table 1.

Table 1. Factors affecting migration time reproducibility [11]

Factor	Cause/effect	Solution
Temprature change	- Change viscosity and EOF	- Thermostat capillary
Adsorption to capillary walls	- Change EOF - Caused by BGE, additive or samples adsorption	- Condition capillary and allow sufficient equilibration time
Hysteresis of wall charge	- Caused by conditioning capillary at high (or low) pH and employing a low (or high) pH running BGE	- Avoid pH differences - Allow sufficient equilibration time
Changes in BGE composition	- pH changes due to electrolysis - BGE evaporation - Conditioning waste flushed into outler vial	- Replenish BGE - Cap BGE vial and cool carousel - Used separate vial to collect wash solution
BGE vial not level	- Non-reproducible laminar flow	- Level liquid in vials
Different silanol content of silica batches	- Different wall charge and variations in EOF	- Measure EOF and normalize of necessary
Variations in applied voltage	- Proportional changes in migration time	- Not user accessible

3.1.5. Detection in CE

According to Heiger [11], due to the small dimensions of the separation capillary, CZE cannot be used for “trace” analysis even though only nanoliter volumes of samples are required and mass detection limits are excellent. The sample needs to contain relatively concentrated analyte solutions or pre-concentration is often necessary. To address the challenge of low sample injection volumes in CE, a number of detection methods have been modified for use with CZE. Many of the detection methods are similar to the ones employed in liquid column chromatography and UV-Visible detection is by-far the most common one. In the Table 2 below, the theoretically achievable mass detection limits for CZE are overviewed, accompanied by their respective concentration detection limits and list of advantages/disadvantages. As the major topic of this dissertation thesis was the analysis of small ions (anions and cations) only a few relevant detection methods will be further discussed in detail.

Table 2: Methods of detection for CE adopted from Ewing´s review [12].

Method	Mass detection limit (moles)	Concentration detection limit (molar)*	Advantages/disadvantages
UV-Vis absorption	$10^{-13} – 10^{-16}$	$10^{-5} – 10^{-8}$	- Universal - Diode array offers spectral information
Fluorescence	$10^{-15} – 10^{-17}$	$10^{-7} – 10^{-9}$	- Sensitive - Usually requires sample derivatization
Laser-induced fluorescence	$10^{-18} – 10^{-20}$	$10^{-14} – 10^{-16}$	- Extremely sensitive - Usually requires sample derivatization - Expensive
Amperometry	$10^{-18} – 10^{-19}$	$10^{-10} – 10^{-11}$	- Sensitive - Selective but useful only for electroactive analytes - Requires special electronics and capillary modification
Conductivity	$10^{-15} – 10^{-16}$	$10^{-7} – 10^{-8}$	- Universal - Requires special electronics and capillary modification
Mass spectrometry	$10^{-16} – 10^{-17}$	$10^{-8} – 10^{-9}$	- Sensitive and offers structural information - Interface between CE and MS complicated
Other	Radioactivity, thermal lens, refractive index, raman *assuming 10 nL injection volume		

UV detection

UV-Visible absorption detection is the most commonly used method, due to its nearly universal detection nature [11]. When fused-silica capillaries are used, the detection from below 200 nm up to the wavelengths in the visible spectrum range can be used. It is an on column detection technique, e.g. the signal is measured through the transparent walls of the separation capillary (it keeps the high separation efficiency observed in CZE). There is no zone broadening as a result of dead-volume or component mixing when the optical window is directly made on the capillary. As with all optical detectors, the width of the detection region should be small relative to the solute zone width to maintain high resolution. This is best accomplished with a slit

designed for specific capillary dimensions. Since peaks in CZE are typically 2 to 5 mm wide, slit lengths should be maximally one third this amount.

In short, in UV detection mode, the detection design is critical due to the short optical path length and the optical beam should be tightly focused directly into the capillary to obtain maximum light throughput at the slit and to minimize stray light reaching the detector. These aspects are important to both sensitivity and linear detection range.

Conductivity detection

Because of the fact that conductivity detection (CD) can respond to all charged species, it is the most universal of all types of electrochemical detection. The charged ions are all the time present in the background electrolyte; hence a baseline signal (and noise) is always present at the detector. The difference between CD and potentiometric or amperometric detection is that the detector measures the conductance of the solution between the two electrodes instead of relying on electrochemical reactions on the surface of the electrode. The conductivity detector consists of two inert, typically platinum, electrodes across which a high frequency AC potential is applied. An AC voltage is used instead of a DC voltage in order to avoid current limitation and electrolysis reactions on the surface of the electrodes. Under the AC voltage, a double layer is formed at the electrode surfaces, in which charges in the electrode are balanced by ions of opposite sign in the adherent solution. Such double layers behave like electronic capacitors, which are transparent to AC currents and voltages. As described by the Ohm's law, there is a correlation between conductivity and current, so the difference in conductance between the analyte and the background co-ion(s) will create a current signal that will be measured. Both contact and contactless mode can be implemented in conductivity detection as the detection is performed with or without galvanic contact of electrodes and the electrolyte solution, respectively. As a double layer originating from coulombic attraction of charges can be established through an insulating layer [13], it has been shown that conventional contact conductivity and capacitively coupled contactless conductivity (C^4D) detections result in no fundamental difference. However, the required operating frequency of these 2 modes differs greatly: it is necessary to have one of around 1 kHz to work in contact mode, whilst in contactless method higher frequencies of several hundreds of kHz are typically applied. Since detection sensitivity strongly depends on background conductivity, it is

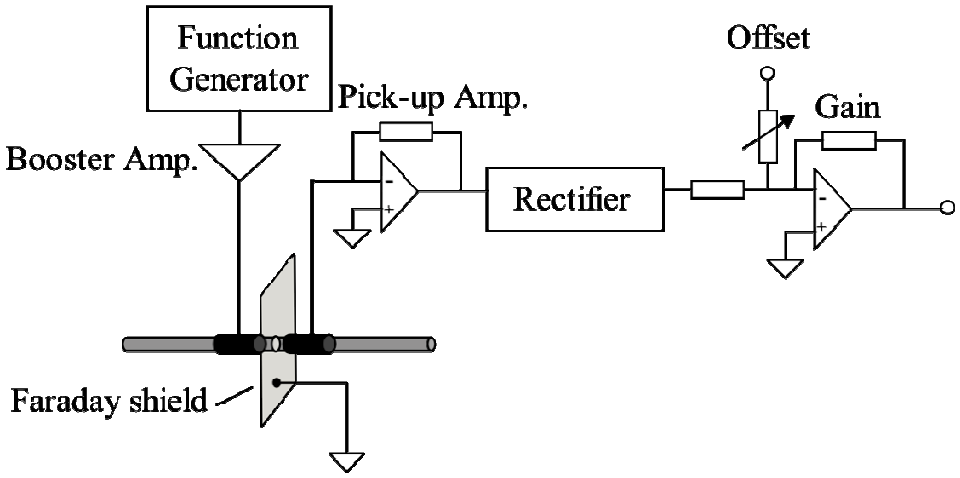
recommended to select BGEs of low conductivity for this mode of detection. Conductivity detection favours the determination of poor or non-UV absorbing charged species of relatively high specific conductivity, such as inorganic ions, amino acids etc. The details on principles and applications of conductivity detection can be found in several review articles [14, 15, 16, 17, 18, 19, 20, 21].

Capacitively coupled contactless conductivity detection (C^4D)

The contactless conductivity detection based on an AC voltage capacitively coupled to the detection cell, is more easily implemented and a more robust alternative to use in CZE. In this mode the electrodes are not in direct contact with the electrolyte solutions. First reports on C^4D in electrophoretic separations were published in the early 1980s by Gaš et al. [22, 23], who used a radial C^4D cell for the isotachophoretic determination of small anions. The cell was constructed from 4 thin wires, which were placed perpendicularly around the circumference of the separation tubing for capacitive coupling of the signal into and out of the solution. A significant contribution to C^4D was made independently by Zemann et al. [24] and da Silva and do Lago [25] by introduction of an axial arrangement of C^4D (as illustrated in Figure 4A).

Two electrodes of a few millimeter lengths, namely actuator and pickup electrodes, made from conductive silver varnish or short metallic tubes, which are separated by a gap of typically 1 mm, are placed side by side around the capillary. C^4D cells can be readily made for capillaries of the standard 365 μm outer diameter. Since the two sensing electrodes themselves can couple with each other to give a stray capacitance, leading to an additional background noise, which is not preferable for detection, they are normally separated by a Faradaic shielding to minimize their direct capacitive coupling. The Faraday shield is typically made of a thin copper foil, in which a hole is drilled to let the capillary pass. The two external electrodes form two capacitors (C) with the solution inside the capillary.

(A)



(B)

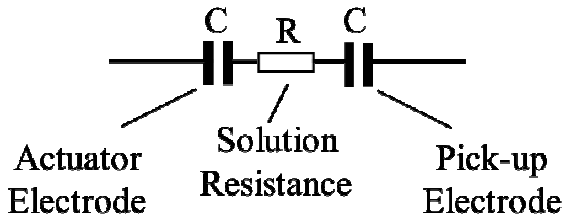


Figure 4. Schematic drawing of C^4D in an axial arrangement.
(A) Schematic drawing of the electronic circuitry; (B) Simplified circuitry

A simplified equivalent circuitry of a conventional contactless conductivity cell, as shown in Figure 4B, can be represented by an arrangement of two double layer capacitances C connected to the solution resistance R. An AC excitation voltage with high frequency of several hundreds of kHz is applied at the actuator electrode. The current (I) passing through such a circuitry is dependent on the applied alternative voltage (V) and frequency (f) as expressed by the following equation:

$$I = \frac{V}{\sqrt{R^2 + \left(\frac{1}{2\pi f C}\right)^2}} \qquad (8)$$

where I: the current

V: the voltage

R: the solution resistance

f: frequency

C: capacitance

According to equation (8), at low frequencies, the current is limited by the double layer capacitances. For the higher frequencies, the current is determined only by the solution resistance, not by the capacitance at the electrodes, resulting in a plateau value of current (I). The fact that the two electrodes are taken out of the solution leads to an increased separation of the charges, resulting in a greater distance between the two plates of a capacitor. This translates into a smaller capacitance, and hence higher required operating frequency. In practice, frequencies higher than 100 kHz are employed, and the value of 300 kHz is oftten recommended [26]. The AC current signal, which is picked up at the second electrode, first has to be transformed into a voltage with a feedback resistor and then rectified to obtain a recordable DC signal that varies with conductivity changes. Typically, the background signal should be suppressed electronically ("offset" or "zeroed") before amplifying the measured signal to obtain the best resolution of the analog-to-digital converter. More details on fundamental aspects of C^4D can be found in the papers by Kubáň and Hauser [26, 27, 28, 29].

The simplicity of the design of the axial cell and its electronic circuitry has led to a strong resurgence of interest in C^4D in the last few years, as documented by numerous papers [22 - 30, 31]. Its universality allows the detection of small inorganic ions as well as organic and biochemical species and applications of C^4D have been extended to a large range of organic and biochemical analytes. Due to its robustness, minimal maintenance demands and low cost, the popularity of this detector has been steadily growing. The CZE methods developed in this thesis used the capacitively coupled conductivity detection C^4D, which is highly sensitive for the detection of low molecular weight ions.

3.1.6. Aplication of CE-C^4D in the separation of small anions and cations

As described earlier, the use of C^4D for the determination of inorganic ions is very attractive. First, most of the inorganic ions are only detectable by indirect UV detection with the standard optical detectors in conventional electrophoresis instruments. The indirect UV detection detection mode is not particularly sensitive. Secondly the high difference in conductance between the (highly conductive) analyte and (weakly conductive) separation electrolyte co-ions – a typical scenario in CZE with C^4D - results in high detection sensitivity [29]. Third, a typical background electrolyte

used with C^4D can be used for analysis of both anions and cations only by switching the polarity, without need to change the separation electrolyte composition. It also can be used for the simultaneous determination.

A commonly used separation electrolyte solution is composed of 2-(N morpholino)ethanesulfonic acid (MES) and histidine (HIS). The application area of separation electrolyte base on MES/HIS is very wide. It was applied to the determination of anions [32, 33, 34], cations [32, 35, 36, 37, 38, 39], and the simultaneous determination of up to 12 anions and cations by using dual opposite-end injection [40].

In this work minor and major ionic analytes in human blood serum and urine were quantified in two different separation electrolyte solutions, which were used respectively for the separation of anions and cations [41].

3.1.7. Current problems of CE analysis of small ions

Pertaining problems that are frequently discussed in conjunction with CE analysis of real samples are its low concentration detection sensitivity and limited robustness to various matrices. Matrix interferences are usually caused by high molecular weight compounds that tend to adsorb to the inner capillary wall, which leads to irreproducible changes of EOF, analyte loss, need for frequent capillary rinses and equilibration. Additionally, the matrices containing high concentrations of small ions (i.e. in concentrated salt solutions, brines, sea water, etc.) are also of concern, because they increase the sample conductivity, causing problems in analysis and preconcentration of the samples. Often the high amounts of a particular ion cause co-migration of its peak with compounds of interest. Separation of small molecules from the macromolecular matrix and vice versa is thus often required prior to CE analyses of real samples, particularly for samples of biological origin [42].

In this thesis, the target analytes were small inorganic ions, which are found in biological samples as well as in environmental samples and are important constituents of these samples, for instance in clinical analysis. Direct CZE analysis of complex samples is usually not possible. In this work several sample pretreatment methods were combined with CZE-C^4D. These include sample clean-up/preconcentration using electrodialysis ED or micro-electrodialysis (μED) and ion exchange (IE). With focus in science shifting towards green analytical methods, efforts were recently made to

miniaturize the sample clean-up methods like liquid–liquid extraction. Because of the concern for the environmental safety, the minimization of use of the hazardous organic solvents is one of the key focuses of these initiatives. On the other hand, cost, time reduction, ease of automation, possibility of online coupling, high-throughput capability, and the small amounts of matrix available are major incentives that have also motivated scientists. Therefore in the final part, the use of electromembrane extraction (EME) was applied to the preconcentration of selected anions from the environmental samples. These three preconcentration techniques represent novel approaches in sample pretreatment for determination of small inorganic ions by CZE.

3.2. ELECTRODIALYSIS

3.2.1 Electrodialysis: some theoretical considerations

Electrodialysis (ED) is used to transport dissolved ions from one solution to another solution through a membrane under the influence of an applied electric potential [43]. This is done in a so called electrodialysis cell. The cell consists of a donor compartment and an acceptor compartment. The compartments are separated by a membrane. Two electrodes are placed in the donor and acceptor compartments. The membrane can be porous or non-porous [44] and acts as a size-selective or size/charge-selective barrier, respectively. When the electrodialysis is running, the direct current field affects the flow of dissociated ions in water solution in such way that cations move towards the cathode and on their way pass through the membrane. At the same time the anions are drawn to the anode and pass through the membrane in the opposite direction.

It can be approximately said that the principle of separation in ED is virtually the same as in electrophoresis, because the analytes are separated in an applied electric field. The most significant difference between CE and ED, apart from the lower voltages used in ED and different geometrical arrangement of the device, is that a separation membrane is used in ED to help separate the analytes of interest from the bulk matrix components. The molecules or ions larger than the pores of the membrane will not penetrate the membrane and will remain in the donor compartment. The small ions of interest will thus be separated from the bulk matrix (large ions). Compared to conventional dialysis arrangement without use of electric field the analytes of interest can be preconcentrated.

3.2.2. Applications of electrodialysis

The typical industrial application of electrodialysis has been the desalination of sea or brackish water [45, 46], removal of high concentrations of small ions or desalination of sugar syrups [47, 48]. ED can also be applied in chemical industry for separation of inorganic and organic solutions, purification of organic substances, e.g. amino acids, waste water treatment, recycling of chemical substances, in food industry for demineralization of milk whey, wine stabilization and desalination of fruit juices [49]. It can also be used in demineralization of biological solutions without affecting the quality of proteins and hydrocarbons.

The applications of ED in analytical chemistry was first described in a series of papers by Tsunakawa [50, 51, 52], in which various pharmaceutical compounds from model tablet formulations were electrodialyzed through a cellulose-type membrane. Although these initial articles provide a wide range of data on various compounds of pharmaceutical interest, only one compound at the time was analyzed using a simple flow injection analysis system.

Coupling of ED to another selective separation method, such as chromatography or electrophoresis would be a logical solution to multianalyte analysis and indeed in early 1990s, ED has been used as a sample pre-treatment technique for other separation methods. ED device using a sheet of planar cation exchange membrane was applied in determination of inorganic anions in sodium hydroxide solutions by ion chromatography (IC) [53, 54, 55], since such samples cannot be analyzed directly by IC. Okamoto et al. [56, 57] performed IC determination of inorganic cations and anions in strongly basic and alkaline solutions after ED pretreatment with tubular ion-exchange membranes. On-line coupling of ED to HPLC was described by Debets et al. [58, 59], Groenewegen et al. [60] and Brewster et al. [61]. In a series of papers, Buscher et al. [62, 63, 64] have used ED coupled to CE for pretreatment of inositol phosphate containing complex samples. In their arrangement, high voltage power supply for CE was used during the ED process and voltages up to 25 kV were used during the pretreatment step in order to move the ions of interest through the dialysis membrane directly into the separation capillary. As the electric current is reduced significantly in the separation capillaries with small internal diameters no membrane fouling or disruption was observed even at the kV levels and good preconcentration factors were achieved. A simple preconcentration system for CE analyses of proteins based on ED

sample pretreatment using hollow fibers with defined molecular weight cut-off values has shown by Wu et al. [65, 66]. The sample was inserted into the fibre lumen. On application of ED voltage, ions from the sample started to migrate through the fiber. Proteins with high molecular weight were retained and preconcentrated on the walls inside the fiber lumen, while low molecular weight ions migrated though the fiber into the surrounding electrolyte solution. The preconcentrated proteins in the fiber lumen were then electrophoretically transferred into the separation capillary and determined by CE. A reversed process was used for determination of small inorganic anions in complex samples – small ions could migrate inside the lumen, while high molecular weight compounds were selectively retained on the fiber walls and did not enter the lumen during the ED step [67]. Combination of ED sample pretreatment with CE analysis was also presented for protein preconcentration by Wang et al. [68] and by Liu and Pawliszyn [69]. The above examples demonstrate that it is principally possible to use ED as an efficient pretreatment technique for CE.

3.2.3. Intended use of ED in pretreatment of biological samples

In the first part of the thesis, our intention was to show that the combination of these progressive separation techniques (ED and CZE) may prove useful in fast pretreatment of various biological samples with rather complicated matrices (blood, blood plasma, blood serum, urine). We demonstrate this on the ED pretreatment of various body-fluid samples in analysis of small inorganic cations. We demonstrate that the adsorption of proteins present in blood and urine samples (such as HSA) on the capillary wall can be eliminated using a simple ED step prior to CZE analysis. The developed technique may have an impact on non-invasive analysis of biological samples, such as fingerstick blood from infants or elderly people, as only a single droplet of sample is needed.

3.3. ION-EXCHANGE (pre-column)

3.3.1. Ion-exchange: some theoretical considerations

Ion exchange is a physico-chemical process in which a solid material adsorbs ions from a liquid and, in exchange, discharges an equivalent amount of identically charged ions to the liquid. Ion exchange is based on the principle that the higher the valence (ionic charge), the more strongly the ions are bonded to an ion exchanger. Ion exchangers can only exchange a certain quantity of ions. When the exchange capacity is exhausted, the ion exchanger can be regenerated. This utilises the fact that ion exchange depends not only on the valence of the ions involved, but also on their concentration. So a large number of ions with a low valence can displace ions with a higher valence. In regeneration therefore, the exhausted ion exchanger is converted back to its original form by a high concentration of the orginal counterions. In the case of cation exchangers this is done with acids, and in the case of anion exchangers with bases. More details on fundamental aspects of ion exchange can be found in sevaral comprehensive books [70, 71, 72].

An ion exchanger consists of a matrix with permanently bonded ions and oppositely charged counterions. The counterions are exchanged with the ions being removed from the solution as show in Figure 5. Ion exchangers maybe natural material (such as zeolites) or synthetic resin (such as polystyrene, polyacrylate or poly(butadiene-maleic acid) (PBMA), epoxy resin, melamine resin, methyl methacrylate, etc.).

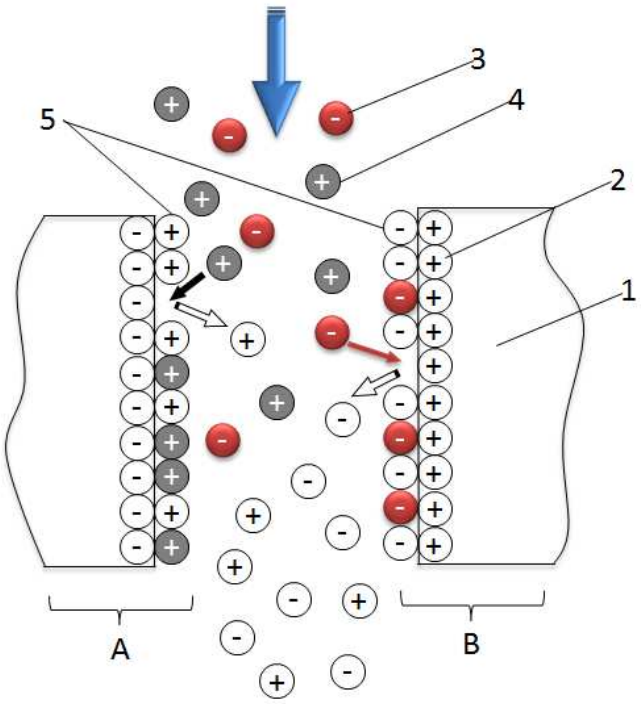


Figure 5. Fundamental principle of ion exchange

A: Cation exchanger; B: Anion exchanger

1- matrix, 2- permanently bonded ion, 3- anions, 4- cations, 5- counterions

3.3.2. Applications of ion exchange

Ion exchange is widely used in the various industries: food and beverage [73, 74], hydrometallurgical, chemical and petrochemical [75, 76], pharmaceutical, semiconductor, power nuclear, as well as to treat sugars and sweeteners, ground and potable water, for metal finishing and softening of industrial water [77]. The most typical example of application is preparation of high purity water for power engineering, electronic and nuclear industries; i.e. polymeric or mineralic insoluble ion exchangers are widely used for water softening, water purification, water decontamination, etc [78, 79, 80].

Ion exchange of proteins is possible because most proteins bear nonzero net electrostatic charges at all pHs except at pH = pI (isoelectric point). At a pH > pI of a given protein, that protein becomes negatively charged (an anion), at the pH < pI of that same protein, it becomes positively charged (a cation).

3.3.3. Intended use of ion-exchange precolumns for protein removal prior to CZE analysis of small ions in biological samples

The abundance of proteins in biological samples is advantageous for those who study proteins; but when analyzing non-proteinaceous material in those samples, several problems occur and need to be solved. Typical problems involve adsorption on the capillary walls during capillary electrophoresis, and/or obscuring the peaks of the non-proteinaceous analytes of interest. It is thus required to remove all the proteins first in order to facilitate the analysis of non-proteinaceous material. In practice, there are many ways of protein removal, such as filtration, centrifugation, precipitation and solid-phase extraction; however they are conducted in the form of an isolated step, which is time consuming and adds complexity to the analytical procedure. Consequently, combining the proteins removal and analysis into a single process seems to be far simpler and advantageous. One practical way of doing the procedure in a single step is to adopt the idea of Kubáň et al [115], who developed a simple procedure for coating an open tubular cation exchange column, which consists of multiple layers of poly(butadiene-maleic acid) (PBMA) coated onto the walls of a fused silica capillary. Hence, as the inner diameter of the capillary is similar to that used in CZE separation, it is possible to connect a short piece of such an ion exchange capillary to an electrophoresis capillary and use it as a disposable on-line pretreatment unit. The PBMA coating will help to retain the proteins and alow the analytes migrate through the ion exchange capillary and subsequently be separated by CZE and then detected by a contactless conductivity detector. The feasibility of this idea was tested in the second part of the experimental work.

3.4. ELECTROMEMBRANE EXTRACTION (EME)

3.4.1. EME: some theoretical considerations

Electromembrane extraction (EME), introduced recently by Pedersen-Bjergaard and Rasmussen [81] combines the concept of hollow-fiber liquid-phase microextraction (HF-LPME) and electroextraction [82, 83, 84]) to achieve the objectives of low sample and solvent consumption. 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) [85]. It resembles the SLM preconcentration with adding of the value of an electrically driven transport across the membrane. This combination offers a highly selective sample preparation method using simple equipment and gains a high degree of enrichment within a short period of time.

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. This method is desirable because of reduced extraction time, particularly regarding small sample volumes.

3.4.2. Applications of EME

Extraction of both acidic and basic compounds is possible and EME has a future use in miniaturised analytical systems [86]. EME provides very rapid extractions and requires only a small amount of solvents [87]. Moreover, EME offers the possibility for tuning the extraction selectivity by choosing the composition of 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 [88], nevertheless, applications to other groups of analytes, such as inorganic ions [89, 90], phosphonic acids (as degradation products of nerve agents) [91] and chlorophenols [92] 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 [93, 94] and plasma samples [95].

3.4.3. Intended use of EME in preconcentration of perchlorate

Perchlorate is soluble and very mobile in aqueous systems and is non-reactive with other components in water. Therefore, perchlorate can persist for several years in ground and surface water [96] and there are several human health and ecological concerns that can arise even at its relatively low concentrations [97]. The widespread presence of perchlorate in the environment has recently become increasingly evident since perchlorate has been proven to bio-accumulate in plants such as lettuce and other broadleaf vegetables [98, 99]. Its occurrence in rain and snow [100], breast and dairy milk [101, 102], soil and drinking water [103] has also been documented. Perchlorate inhibits uptake of iodide to the thyroid gland [104] and completely discharges stored

iodide [105]. The United States Environmental Protection Agency (US EPA) has recently issued an Interim Drinking Water Health Advisory, determining that a level of 15 µg/L of perchlorate is protective of all sub-populations [106]. However, no strict regulatory levels have been set. Perchlorate has also been added to the US EPA Drinking Water Contaminant Candidate List [107] and "listed for regulation" [108].

Urbansky has reviewed several analytical methods applied to the analysis of perchlorate such as spectrophotometry, electrochemistry, capillary electrophoresis (CE), ion chromatography (IC), and mass spectrometry (MS) [109]. The most frequently used method for measurement of oxyhalide anions is IC with conductivity detection (IC-CD) [110, 111]. US EPA method 314.0, based on IC-CD, is commonly used for perchlorate analysis, and typically has a reporting limit of 4 µg/L. Several CE methods enable detection of perchlorate at or just below (5 µg/L) the US EPA recommended limit in drinking water [112, 113, 114], further improvement of sensitivity is essential. This can be readily achieved by off-line combination of CE with sample pretreatment techniques, which are commonly used for trace analysis. Thus, using EME in preconcentration prior to CZE is great idea for determination of trace perchlorate. The feasibility of this idea was tested in the third part of the experimental work.

4. MATERIALS AND ANALYTICAL METHODS

4.1. CHEMICALS

4.1.1. Standards

All chemicals were of reagent grade and deionized (DI) water with resistivity higher than 18 MΩ.cm was used throughout. Stock solutions of cations (250 mM) were prepared from the corresponding chloride salts except for the magnesium stock solution, which was prepared from magnesium sulfate. Stock solutions of anions (10 mM) were prepared from NaCl, $NaNO_3$, $NaNO_2$, Mg_2SO_4 and $KClO_4$. All chemicals were purchased from Pliva-Lachema, Brno, Czech Republic and Fluka, Buchs, Switzerland. All multi-ion standard sample solutions were then freshly prepared from these stock solutions and were diluted with DI water or 5 mM acetic acid. Human serum albumin (HSA, MW = 66478 Da) was purchased from Sigma, Steinheim, Germany and working solutions were prepared daily in DI water.

4.1.2. Separation electrolytes

Background electrolyte solutions for CZE measurements were prepared daily from stock solutions of L-arginine (L-Arg) (100 mM), maleic acid (100 mM) and 18-crown-6 (20 mM or 250 mM), acetic acid (1 M), L-histidine (L-His) (100 mM). The exact composition of the BGEs are given in the results and discussion part. All chemicals for electrolyte preparations were purchased from Pliva-Lachema, Brno, Czech Republic Sigma, Steinheim, Germany and Fluka, Buchs, Switzerland.

4.1.3. Other solutions

Electrolyte solutions for electrodialytic sample pretreatment were prepared daily from concentrated acetic or hydrochloric acid or weighted directly from pure chemicals (L-Arg and maleic acid). The final pH-values of all electrolyte solutions were measured using a pH-meter model 537 (WTW, Weilheim, Germany). Organic solvents for EME extractions were obtained from Sigma or Fluka and were of highest available purity; the solvents were used without any further purification. For precolumn preparation, PBMA

prepolymer (42% solids in aq. solution, Polysciences, Eppelheim, Germany) and AIBN (Acros, Geel, Belgium) were used.

4.1.4. Real samples

Real samples of human serum and human plasma were purchased as lyophilized powder from Sigma and were prepared according to manufacturers' instructions. Fingerstick whole blood and human urine was collected from volunteers.

The real samples were injected either directly without any pretreatment or were first processed in the ED/μED system and then injected into the CZE system. Untreated real samples were diluted with DI water 1:10 or 1:20 prior to the direct injection into the CZE system and 1:8 or 1:10 prior to the ED pretreatment. In case of μED system, the real samples were always diluted 1:80 with DI water. All real samples were stored in deep freezer at less than -20 °C. The dialysis membranes were stored in DI water for overnight storage or in sodium azide solution for long term storage. Human serum and plasma were stored in deep freezer at less than -20 °C, whole blood was always taken fresh at the time of analyses. The dialysis membranes were kept in DI water for overnight storage or in sodium azide solution for long term storage.

Water samples used in third part of the research were tap water, bottled water and various environmental samples. Perchlorate was not present or was present below the method LOD in all water samples and was therefore spiked at ≤ 15 μg/L. Tap water was sampled from the water-conduit at the Institute of Analytical Chemistry after 10 L of water were dispensed from the tap. Snow samples were collected from an open ground in Brno on a snowy day. Rain samples were collected during a rain event in Brno. Surface water was obtained from a local stream. Bottled water samples (Dobrá voda, Horský pramen) were purchased from a local supermarket. All samples were stored at 4°C until analysis and were allowed to warm up to the ambient temperature before EME.

4.2. INSTRUMENTATION

4.2.1. Electrodialytic systems

In our experiments, we used two electrodialytic systems, a commercial ED system and a microdialytical (μED) system.

4.2.1.1. Commerical electrodialytic system

Figure 6 shows the schematic of the commercial ED system. It consisted of a polymethylmethacrylate (PMMA) tank Electroprep^{TM} (Harvard Apparatus, Holiston, MA, USA), ED units (internal volume 50 μL), link chambers (internal volume 50 μL), union (internal volume 245 μL) and open-ended caps all made of polytetrafluoroethylene (PTFE) (Harvard Apparatus). Dialysis membranes with 500 Da molecular weight cut-off (MWCO) value (Harvard Apparatus) made of cellulose acetate were used with this appartus in all experiments. A power supply for electrodialytic sample pretreatment was a Scie-Plas MPSU-200/100 (Scie-Plas Limited, Southam, Warwickshire, United Kingdom) operated at a variable electric potential (25 - 200 V) and maximum current of 100 mA. The power supply was connected to two connectors on the top of a removable PMMA lid of the tank, which further extended to two platinum electrodes located on opposite walls inside the tank. The electric connection was interrupted when the lid was removed from the tank, thus protecting the operator from potential injury. Typical electric potential of the commercial ED system was 50 - 200 V, which generated currents in the range 0.2 - 5 mA. The membrane area was approximately 0.8 mm^2, leading to current densities in the range 0.25 mA/mm^2 - 6.25 mA/mm^2. The tank was divided into two compartments by a separation bar with a hole drilled approximately in the centre of the bar, which was sealed with a rubber o-ring to accommodate one of the ED units. All ED experiments were performed at ambient temperature.

Fig. 6A shows a schematic drawing of the whole system. The ED tank was filled with approximately 750 mL of an ED electrolyte solution, which was distributed evenly into both ED compartments through the hole in the separation bar, filling the tank almost until the upper end of the separation bar. Subsequently, a set of ED units, membranes, link chambers and open-ended caps with donor and acceptor solution was placed into the hole in the bar separating the two compartments of the ED tank. Fig 6B

shows a detail of the membrane arrangement with two ED units and three ED membranes separating the donor and acceptor solutions, which was used for standard electrodialytic procedures. This set-up usually consists of a donor unit with internal chamber, which is filled with standard solution or real sample and is closed by a dialysis membrane (membrane #1) and an open-ended cap (cap #1) on the right side. Second membrane (membrane #2), a union and an acceptor unit are screwed to the left side of the donor unit. In this case the membrane #2 is the size-selective barrier between the donor and acceptor solutions. The union and the acceptor unit are then filled with acceptor solution (usually the electrolyte solution used for ED) and the acceptor unit is finally closed on the left side with a dialysis membrane (membrane #3) and an open-ended cap (cap #2).

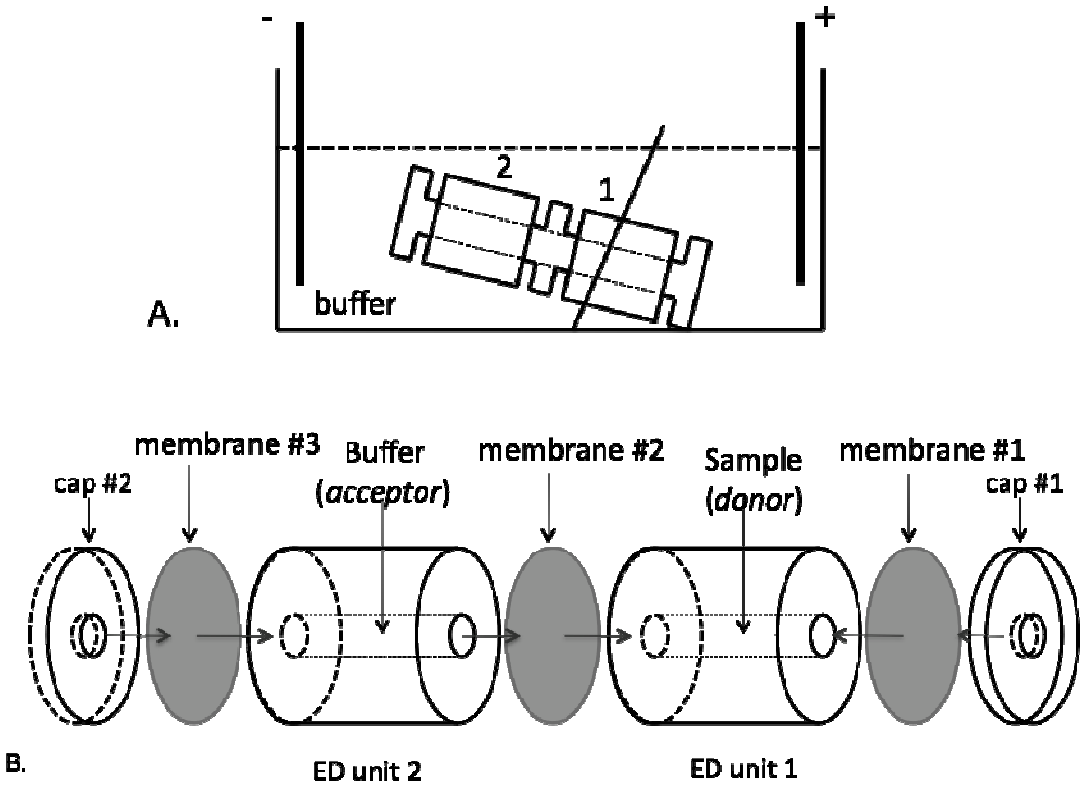


Figure 6. A. Schematic drawing of the commercial ED system, a – separation bar, b – open-ended cap #1, c – rubber o-ring, d – donor unit, e – union, f – acceptor unit, g – open-ended cap #2. B. Schematic drawing of the membrane arrangement in a commercial ED system with two ED units and three membranes.

The union and the acceptor unit could be replaced by a single link chamber or a set of several link chambers in order to change the volume of the acceptor side of the ED system. The system can also be used in an arrangement when one ED unit, two membranes and two open-ended caps are used for electrodialytic pretreatment of a sample, which is placed in the internal chamber of the donor ED unit. This arrangement was used in the initial optimization of the ED system.

4.2.1.2. Micro-electrodialytic (μED) system

The μED system is shown in Figure 7. It is simpler than the commercial ED system and it consisted of two polytetrafluoroethylene link chambers (internal volume 50 μL) (Harvard Apparatus, Holiston, MA, USA), separated by a cellulose acetate dialysis membrane (Harvard Apparatus) with 500 Da MWCO. Two Pt electrodes of the μED system were directly inserted into the donor and acceptor compartments, each filled with 50 μL of donor and acceptor solution, respectively. The μED system was operated at 15 V by using ES 0300-0.45 power supply (Delta Elektronika BV, Zierikzee, The Netherlands, 0 – 300 V variable voltage and maximum current of 450 mA). The electric potential of the μED system generated currents of approximately 100 μA, leading to current densities around 125 μA/mm^2. A 1 μL sample aliquot was diluted 1:80 with DI water and 50 μL of the diluted solution was introduced into the donor chamber of the unit using a micropipette. The acceptor chamber was then filled with the same volume (50 μL) of an acceptor solution using a second micropipette. The donor and acceptor solutions are held inside the chambers by capillary forces as the internal diameter of the chamber is only 1 mm. No liquid loss from internal ED chambers was observed during μED pretreatment. All μED experiments were performed at ambient temperature.

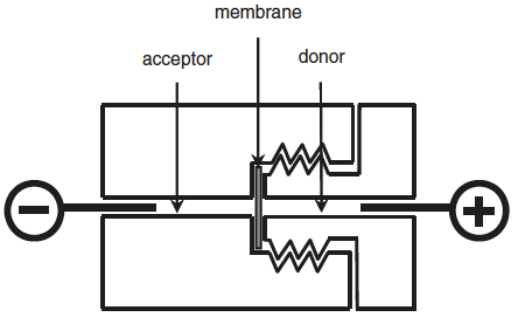


Figure 7. Schematic drawing of the micro-electrodialytic system.

4.2.2. The system with disposable open tubular ion exchange pre-columns

Preparation of sample clean-up precolumns

Preparation of poly(butadiene-maleic acid) (PBMA) based OT columns was described previously [115]. A thin layer of PBMA prepolymer mixed with 5% azobisisobutyronitrile (AIBN) was coated onto a 1 m long FS capillary (75 μm ID, 375 μm OD, Microquartz GmbH, Munich, Germany) and crosslinked at 160 °C for 15 min. The coating procedure was repeated n-times to create a n-layered precolumn. Preparation of these layers takes relatively short time. For a 4-layer PBMA capillary it takes about 2-3 hours. The prepared column was eventually cut into pieces of various lengths (3-12 cm). PBMA columns are cheap to prepare: 100 cm of fused silica capillary costs no more than € 5. Because a single analysis requires a 5-6 cm disposable pre-column which averages 30 cts per piece, they cost much less than instrumentations earlier described.

The pre-columns were connected to the analytical capillary through a low dead volume union (P-772, Upchurch Scientific, Oak Harbor, WA, USA). A sketch of the connection between the OT sample clean-up precolumn and the analytical capillary is shown in Figure 8.

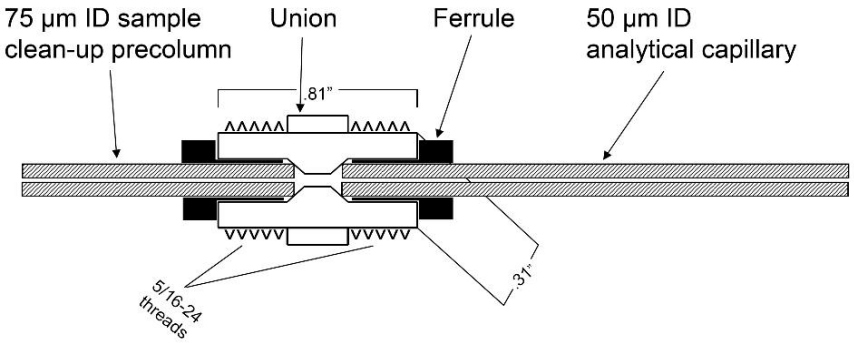


Figure 8. Interfacing of the clean-up precolumn and the analytical capillary using the low dead volume union.

4.2.3. Electromembrane extraction system

The EME system is shown in Figure 9. It consisted of a 4 mL glass vial (Beckman, Fullerton, CA, USA) with a screw-cap. Two holes were drilled through the cap in order to accommodate two 200 µL pipette tips that acted as leading channels for two 0.5 mm platinum electrodes (99.95%, Advent, Oxford, England). The two pipette tips insulated the two platinum electrodes and thereby avoided possible short circuit of a power supply. 3 cm length of a polypropylene (PP) hollow fibre (HF) (Accurel PP 300/1200, Membrana, Wuppertal, Germany; wall thickness of 300 µm and internal diameter of 1200 µm) was used as a single use extraction unit and was firmly pulled on the pipette tip acting as a leading channel for anode. The HF extraction units were pressed and heat sealed at the bottom resulting into approximately 30 µL internal volume.

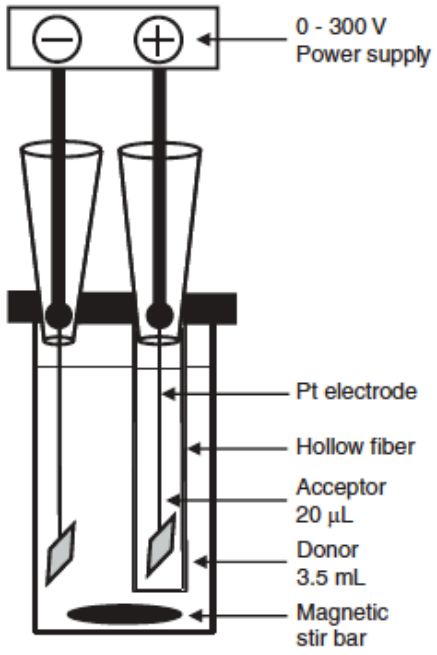


Figure 9. The electromembrane extraction system

Before EME, the fibre was dipped for a given time (impregnation time, usually 10 sec) into an organic solvent and then the lumen was filled with 20 µL of an acceptor solution. A 50 µL Hamilton syringe was used to fill the fibre lumen with acceptor solution and to aspirate the solution from the lumen after EME. A magnetic stir bar was

placed to the glass vial to ensure constant stirring of the donor solution (3.5 mL). Magnetic stirrer used was MR-Hei Standard (Heidolph, Schwabach, Germany) with 0 – 1400 rpm variable stirring rate. The EME system was operated at various voltages by using ES 0300-0.45 power supply (Delta Elektronika BV, Zierikzee, The Netherlands, 0 – 300 V variable voltage and maximum current of 450 mA). The electric potential of the EME system generated currents of approximately 10 – 100 μA, which were continuously monitored using M-3800 (Metex, Seoul, Korea) digital multimeter. All EME experiments were performed at ambient temperature of 22 ± 2 °C.

4.2.4. Capillary zone electrophoretic system

FS capillary pretreatment

The separation capillaries were preconditioned with 1 M NaOH for 10 min, deionized water for 10 min and with respective separation electrolyte solution for 10 min. Between two successive injections, the capillary was flushed with separation electrolyte solution for 2 min. When the separation capillary was contaminated by adsorption of high molecular weight compounds from untreated real samples, the preconditioning procedure was repeated.

The capillary electrophoretic systems, used for different parts of research, have different configurations and are described below.

4.2.4.1 CZE system combined with ED system and IE pre-columns

CZE system combined with the ED system and IE pre-columns

A purpose-built CZE instrument consisting of a box made from Perspex with two compartments for injection and detection was employed for all electrophoretic runs of experiments with the ED system and IE pre-columns. 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 + 15 kV applied at the injection side of the separation capillary for all runs. The separation capillaries used were fused-silica (FS) capillaries (50 μm ID, 375 μm OD, Polymicro Technologies, Phoenix, AZ, USA). With commercial ED system, the FS capillary had 50 cm total length and various effective lengths. With μED system, the capillary also had a total length of 50 cm, but

the effective length was 42 cm. With the IE system, the capillary had total length of 47 cm, and the effective length was 40 cm.

Injection of standard solutions and real samples was carried out hydrodynamically by elevating the sample vial to a height of 25-30 cm for 5 – 30 s. The temperature during the experiments was 25 ºC ± 1 ºC.

Detection in CZE system with the ED system and IE pre-columns

A capacitively coupled contactless conductivity detector (C^4D) used was a modified version of an earlier design [116] and was described in a recent publication [117]. The C^4D consists of a detector cell, an external ac voltage source for excitation and an external detector circuitry for processing the cell current. The excitation voltage was provided by a circuitry based on a MAX038 oscillator (Maxim Integrated Products, Sunnyvale, CA, USA). The oscillator operated at various frequencies between 100 and 400 kHz and a voltage booster using a high voltage operational amplifier (PA91, Apex Microtechnology, Tucson, AZ, USA) produced an output of up to 360 V_{pp} (peak-to-peak). The detector cell contained a current-to-voltage converter (OPA655, Texas Instruments, Dallas, TX, USA) as a preamplifier. The detector was operated at 120 kHz and 300 V_{pp} in all experiments. Data were collected using a home written software and a 20 bit sigma-delta data acquisition card (Lawson Labs Inc., Malvern, PA, USA) or a Panther-1000 (Ecom, Praha, Czech Republic) data acquisition system connected to a Pentium I personal computer.

When combined with the commercial ED system, the peaks measured in 200 mM acetic acid solution were originally negative and were inverted in order to display positive peaks in the electropherograms.

4.2.4.2. CZE system combined with EME

A 7100 CZE instrument (Agilent, Waldbronn, Germany) was operated at a potential of – 30 kV applied at the injection side of the separation capillary for all runs of experiments with EME. Separation capillaries used were fused-silica (FS) capillaries (50 µm ID, 375 µm OD, 50 cm total length and 35 cm effective length, Polymicro Technologies, Phoenix, AZ, USA). A capacitively coupled contactless conductivity detector (C^4D) described by Gaš et al. in a recent publication [118] was used. Injection was carried out hydrodynamically by application of 50 mbar for 2 – 15 s, which

represents less than 2.6% of the total capillary volume (3.4 – 26 nL). All CZE-C^4D experiments were performed at 25 °C. The CZE system was controlled and data were acquired by ChemStation CE software.

5. RESULTS AND DISCUSSION

Sections of this dissertation have been reported in three scientific journals (Journal of Chromatography A, Electrophoresis). The results and discussion chapter is a summary of these three publications and the related results. This section has been arranged into three parts (5.1, 5.2, and 5.3), each of which deals with the corresponding scientific article.

5.1. ANALYSIS OF INORGANIC IONS IN BIOLOGICAL SAMPLES BY COMBINATION OF ED AND CZE-C^4D

Separation of small molecules from the macromolecular matrix and vice versa is often required prior to CZE analyses of real samples, particularly for samples of biological origin [see section 3.1.6 and 3.2.3]. Thus, ED and CZE were combined for rapid pretreatment and subsequent determination of inorganic cations in biological samples.

As described in section 4.2.2, we have used two ED systems, one is commercial ED unit (shown in Fig 6), another is a micro ED (μED) system (shown in Fig 7). The commercial unit was used in the beginning to test the parameters, gain understanding of the ED process and optimize it. Then the μED system was used later to get more advantages. In this section, 2 set of experiments are discussed, the commercial ED system and the μED system, respectively.

5.1.1. Selection of the BGE solution for initial experiments

In a CZE system combined with ED, the BGE solution should provide an adequate separation of selected cations and should also be fully compatible with acceptor solution used in the ED process. Tsunakawa [50] has shown that the best results concerning the efficiency and the recoveries of ED were obtained with weak acids or bases at relatively low concentrations. Acetic acid was found to be one of the best electrolyte solutions for ED [50]. Acetic acid can also be used for CZE-C^4D separation of cations and it thus seemed advantageous to use acetic acid as both the ED and BGE solution.

5.1.2 Combination of the commercial ED system and CZE-C^4D

5.1.2.1 Optimization of the commercial ED system

A BGE solution consisting of 200 mM acetic acid is suitable for simultaneous determination of inorganic cations and high molecular weight compounds, such as HSA and human blood proteins [50], which are present in cationic form in solutions at the electrolyte pH 2.7. Although the separation efficiency of acetic acid based BGE solutions is not adequate for baseline separation of all common inorganic cations in biological samples this electrolyte was used for ED system optimization. A standard solution of four inorganic cations (K^+, Ca^{2+}, Na^+ and Mg^{2+}) at 50 μM in DI water was electrodialyzed. Standard solutions (50 μL) before and after the ED process were analyzed using the CZE-C^4D system. The following parameters of the ED process were examined: ED voltage, ED time, and composition of electrolyte solution in the ED tank.

5.1.2.1.1. Optimization of ED voltage and time for small cation transfer

In the first experiment, the ion transfer through the 500 Da MWCO ED membrane was tested, by letting the samples migrate from the donor unit through the dialysis membrane into the ED solution (200 mM acetic acid) at the other side of the ED tank. The effect of ED voltage and time was examined in a set of 12 experiments with the voltage set to 50, 100 and 200 V and time to 30, 45, 60 and 120 s. For the voltage settings of 100 and 200 V, inorganic cations migrated rapidly from the donor unit through the dialysis membrane and into the surrounding ED electrolyte solution. After 30 s of ED at 200 V, only trace amounts of the inorganic cations could be detected in the donor solution, which could further be eliminated below the method's limits of detection when ED continued for another 30 s. At 100 V, the cations were detected at trace levels after 60 s ED time and complete removal from the donor solution was observed after 2 min. A slightly different situation was achieved for electrodialytic treatment at 50 V. Trace levels of all cations could be detected even after 5 min of ED showing that for the experimental setup used, the removal of cations from the donor solution is not linearly dependent on voltage and time. Based on these results, ED voltage of 200 V was used in all subsequent experiments. The current density was approximately 5 mA/mm^2, however, no excessive heating or membrane disruption was observed for 100 or more consecutive ED runs. Figure 10 shows the determination of the four inorganic cations in a donor solution, which was electrodialytically pretreated

at 200 V for 0, 30 and 60 s. Note that after 30 seconds of ED at 200 V there is still some discernable amount of all ions in the solution, but at 60s, all of the ions except calcium are fully transferred through the membrane. It was found that the calcium peak comes as an impurity in the 200 mM acetic acid solution used, as is described in a later section.

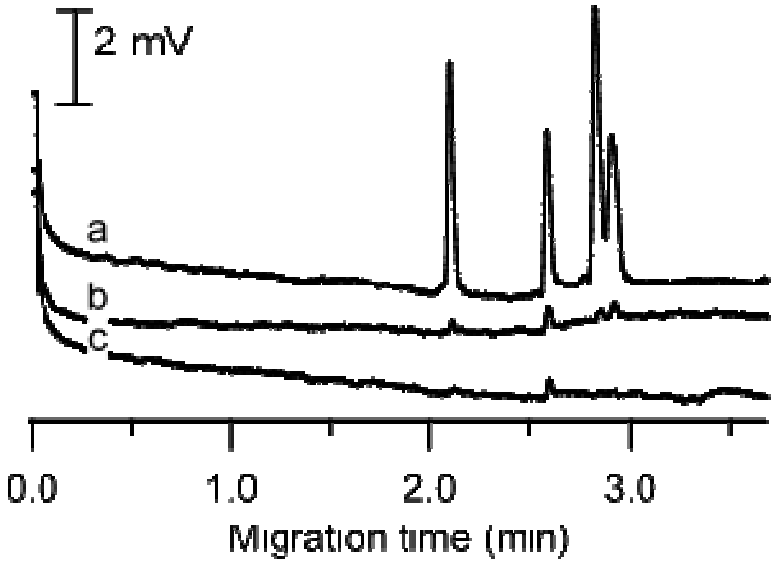


Figure 10. Effect of various ED times on concentration of four inorganic cations in donor ED unit. ED system contains one donor unit and two membranes, ED electrolyte: 200 mM acetic acid, pH 2.7, ED voltage: +200 V. a – standard solution of four cations (50 μM) without ED treatment, b – after 30 sec ED treatment, c – after 60 sec ED treatment. CZE conditions: $L_{tot/ef}$: 50/33 cm, BGE: 200 mM acetic acid, pH 2.7, separation voltage: +15 kV, hydrodynamic injection from 25 cm for 5 sec.

5.1.2.1.2. Study of the retention of model serum proteins on the ED membrane

As the main objective of this work was the analysis of small cations selectively separated from high molecular weight matrix in biological samples, the performance of the ED system was next examined for retention of high molecular weight compounds on the same ED membrane in the following experiments. A standard solution of 50 μM HSA was prepared and subjected to ED in the pretreatment system with one ED unit and two membranes. The standard solution of HSA was spiked with 50 μM K^+ and Ca^{2+} in order to easily control the ED process by simultaneously analyzing the inorganic cations in the donor compartment. Electrodialytic pretreatment of the standard solution was performed in ED electrolyte solution consisting of 200 mM acetic acid at 200 V and at three different times (0, 30, 60 s and 4 min). Inorganic cations migrated

quantitatively from the donor solution into the cathodic electrolyte compartment after 60 s of ED, as expected according to the results obtained in Fig. 10. The peak of HSA was reduced significantly after 30 s and further decrease of the HSA peak was observed after additional 30 s of ED. Finally, the HSA peak could not be detected in the donor solution for ED time of 4 min. The decrease of the HSA peak can be rationalized either as a migration of the protein out of the donor unit or by its adsorption on the membrane #2 (see Figure 6B). The first hypothesis was examined in an ED system with two ED units and three membranes. The donor unit was filled with a standard solution containing 50 μM HSA, K^+ and Ca^{2+}, the acceptor unit with 200 mM acetic acid and the standard solution was pretreated at 200 V for 3 – 10 min in 200 mM acetic acid. Two CZE-C^4D analyses were performed for each ED time, determining the content of inorganic ions and HSA in both the donor and the acceptor solutions. The concentration of all cations, including HSA, in the donor solution decreased gradually with ED time. If the HSA molecules migrated through the membrane interface (membrane #2) they should be detected in the acceptor solution. However, only inorganic cations were detected in the acceptor solution and no HSA peak was observed even after 10 min of ED. This observation confirmed that HSA did not migrate through the membrane into the acceptor unit but was retained on the membrane due to its considerable size.

In subsequent experiments, standard solution of 50 μM HSA was filled into the central donor unit, which was closed by two dialysis membranes (#1 and #2) and two caps. 200 mM acetic acid was used as ED electrolyte solution and the HSA solution was pretreated at 200 V for 4 min after which no HSA was detected in the donor solution (adsorption on the membrane #2). Then the polarity was reversed and the donor solution was further treated at – 200 V for 1, 2, 3 and 4 min. The peak of HSA was again detected in the donor solution after 1 min, whereas gradual peak decrease was observed after 2 and 3 min and no peak was detected after 4 min of ED with reversed polarity as it was gradually adsorbed at the dialysis membrane # 1 on the opposite side of the donor compartment. Thus HSA and very likely all other proteins of similar size do not cross the membrane interface. As has been shown, by reversing the voltage polarity, they can be transferred back to the donor solution after all small ions have been removed from the sample and later analyzed.

5.1.2.1.3. The optimization of ED solution composition

Because of the contamination from the concentrated acetic acid (200 mM) used as ED solution, in the next part the effect of the composition of the ED electrolyte solution was examined. The effect of decreasing the acetic acid concentration was studied with respect to the small cation transfer, but also with respect to the possible impurity migration into the donor solution. Concentration of acetic acid in the tank was varied (1, 5, 10, 100, and 200 mM) and ED of the standard solution of four cations was performed at 200 V for different times. For electrolyte solutions containing 200 and 100 mM acetic acid, the cations migrated rapidly from the donor solution into the cathodic electrolyte compartment whereas longer ED times were necessary for efficient removal of cations using 10 and 5 mM acetic acid solutions and almost no removal was observed for 1 mM acetic acid and ED times up to 10 min. Migration velocities of cations depend on the current flowing through the ED system and are highest for high concentrations of ED solution. On the other hand, too high concentration of acetic acid results in relatively high background concentrations of inorganic cationic impurities that penetrate from the anodic electrolyte compartment to the donor compartment. This contamination was noted in Fig. 10 (trace c) as a trace level peak of calcium. The contaminant peaks usually levelled off after 60 s of ED and further remained virtually constant, showing that the same amount of ions that entered the donor compartment through the membrane #1 was removed by ED through the membrane #2. Significant decrease of the contaminant peaks was observed for lower concentrations of acetic acid and no peaks were detected in the donor solution for concentrations of acetic acid lower or equal to 5 mM. Contamination of donor solution with inorganic cations was also observed when hydrochloric acid was used as ED electrolyte solution instead of acetic acid. For this reason, low concentrations of acetic acid were examined as potential electrolyte solutions for electrodialytic pretreatment of standard solutions and real samples in Section 5.1.2.5.

5.1.2.2 Electrodialysis of blank solutions

Electrodialytic pretreatment of blank solutions was performed at 200 V in a system with two ED units and three membranes. In this arrangement, the ED tank was filled with 1, 5, 10, 100 or 200 mM acetic acid, donor unit was filled with DI water and acceptor unit with corresponding acetic acid solution. CZE-C^4D analyses of inorganic

cations in both units were performed after 5 – 15 min of ED. No measurable peaks of inorganic cations were observed in the acceptor and donor solutions for concentrations of acetic acid lower or equal to 5 mM, the content of cationic impurities in both solutions gradually increased for higher concentrations of acetic acid. Similarly, the contamination in hydrochloric acid, when used as the ED electrolyte solutions, resulted in similar problem. The blank pretreatment was also performed using the solution of 12.5 mM maleic acid, 15 mM L-Arg and 3 mM 18-crown-6 as ED electrolyte solution with no measurable peaks of inorganic cations in both donor and acceptor solutions. Although the use of maleic acid/arginine based ED electrolyte solution is advantageous from the CZE separation point due to the absence of acceptor/donor solution contamination, fast ED process and absolute compatibility with subsequent CZE analysis, due to the considerable cost of such an ED solution, we have decided not to use it in further ED experiments and instead, we have used 5 mM acetic acid that provides a more cost effective compromise. On the other hand, use of maleic acid/arginine based electrolyte solution might be suitable for micro ED systems where the volume of ED electrolytes is significantly reduced and the above mentioned advantages might be accomplished.

5.1.2.3. Effect of sample pretreatment by the commercial ED system on CZE separation

Adsorption of high molecular weight compounds on the walls of FS capillaries is a well known and described phenomenon [119, 120]. For this reason, biological samples are usually pretreated before CE analysis in order to remove these matrix components. As one of the objectives of this work was to investigate the applicability of ED to avoid adsorption of high molecular weight compounds from biological samples in CZE, the amount of adsorption from samples of human urine, blood serum and blood plasma was investigated.

To allow identical initial conditions of the separation capillary for measurements of all samples, the capillary was equilibrated with 1 M NaOH, DI water and BGE solution prior to each set of experiments. Performance of the CE system was first investigated using a standard solution containing 5 mM of K^+, 150 mM of Na^+, 2.5 mM of Ca^{2+}, 1 mM of Mg^{2+} and HSA at a concentration of 80 g/L as a model protein. These concentration levels correspond to maximum concentrations of inorganic cations and to total protein concentration in human plasma samples [121]. The standard solution

diluted 1:20 with DI water was directly injected into the separation capillary 20 times and all cationic analytes were determined in BGE solution consisting of 200 mM acetic acid at pH 2.7. A gradual increase of migration times was observed for all species showing that adsorption of the positively charged protein on the capillary wall indeed significantly changes the surface properties of the separation capillary. The same set of measurements was performed with direct injection of real samples diluted 1:20 with DI water and, as expected, even more significant increase of migration times was observed for 20 consecutive injections of human plasma and serum. Slight changes of migration times of inorganic cations were also observed for 20 consecutive injections of untreated human urine. Human urine contains about two orders of magnitude lower concentration of proteins compared to human plasma [121] and therefore the amount of adsorbed protein on the capillary walls is expected to be significantly lower.

3 mM 18-crown-6 was added to the BGE solution in order to separate NH_4^+ and K^+ in urine sample. First, tenth and twentieth consecutive injection of the untreated human plasma determined by CZE-C^4D are depicted in Fig. 11A. The increase of migration times of each individual component can clearly be followed on the three traces; the peak labelled with an asterisk represents a cluster of unidentified blood plasma proteins. Quantitative aspects of the adsorption of matrix components onto the capillary inner surface were also examined and were evaluated as peak areas of individual peaks. The results, expressed as r.s.d. values of migration times and peak areas of all inorganic cations and blood proteins, are listed in the first part of Table 3. Note the unacceptably high RSD of migration times and peak areas for most of the samples.

The standard solution of inorganic cations and HSA and the three real samples were then pretreated in the ED system with two ED units and three dialysis membranes. ED electrolyte solution was 200 mM acetic acid, donor solutions were standard solution of inorganic cations and HSA, urine, serum and plasma samples diluted 1:20 with DI water, acceptor solution was 200 mM acetic acid and ED was performed at 200 V for 5 min. Acceptor solution was directly injected 20 times into the equilibrated capillary and analyzed by CZE-C^4D. Fig. 11B depicts three electropherograms for the first, tenth and twentieth consecutive injection of electrodialytically pretreated human plasma. Clearly, there is no visible change in migration times of the inorganic cations and no peak of serum proteins (they do not cross the dialysis membrane).

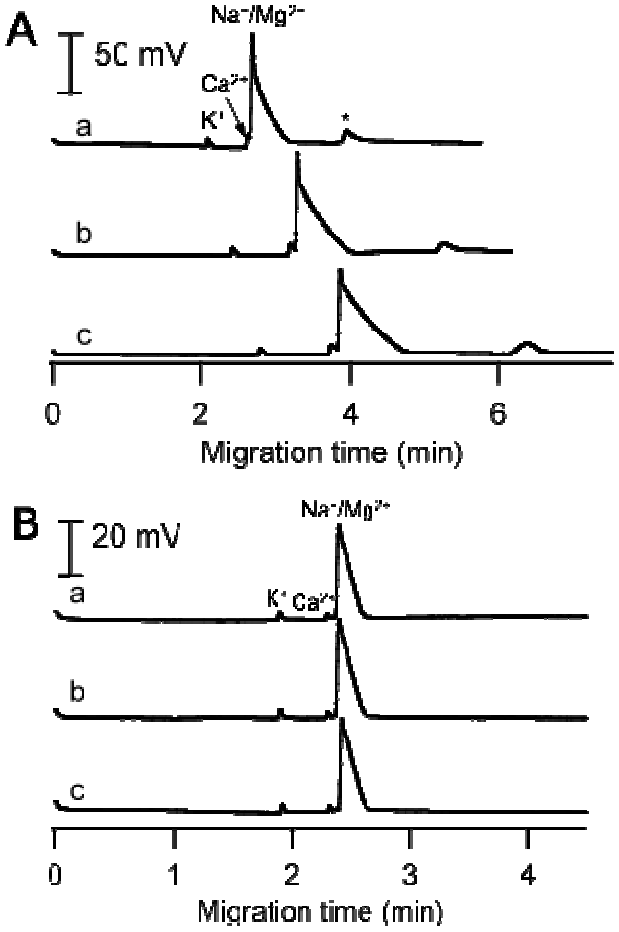


Figure 11. Effect of 20 consecutive analyses of human plasma sample on reproducibility of CZE measurements. A. Human plasma sample (diluted 1:20) injected directly into the separation capillary without any pretreatment. B. Human plasma sample (diluted 1:20) injected into the separation capillary after ED pretreatment for 5 min at +200 V. CZE conditions are same as in Fig. 10. a – 1st injection, b – 10th injection, c – 20th injection.

Repeatability measurements of migration times and peak areas of the inorganic cations in pretreated standard solution and real samples are summarized in the second part of Table 3. Excellent repeatability of migration times and peak areas was achieved for all solutions pretreated with the developed ED method showing that the solutions are free of interfering matrix components. Thus, direct injection of biological samples, especially untreated human plasma and serum, have adverse effect on the separation system performance due to the adsorption of proteinaceous matrix components onto the capillary wall, which deteriorate both the qualitative and quantitative results of the CZE measurements.

Table 3. Repeatability of CZE-C^4D measurements for a standard solution with high protein content and for real biological samples (diluted 1:20 with DI water) injected in the separation capillary untreated and after electrodialytic treatment, n = 20, r.s.d. values in %.

Untreated samples

	Standard solution		Urine		Plasma		Serum	
	M.T.	P.A.	M.T.	P.A.	M.T.	P.A.	M.T.	P.A.
NH_4^+	n.a.	n.a.	1.38	2.75	n.a.	n.a.	n.a.	n.a.
K^+	3.83	5.12	1.50	3.60	6.85	8.84	9.45	11.44
Ca^{2+}	5.00	12.91	1.70	7.54	8.33	12.59	12.29	14.94
Na^+/Mg^{2+}	5.35	8.26	1.86	3.85	8.50	10.99	13.56	19.87
Proteins*	8.02	6.98	n.a.	n.a.	11.11	21.95	18.45	26.81

Electrodialytically treated samples

	Standard solution		Urine		Plasma		Serum	
	M.T.	P.A.	M.T.	P.A.	M.T.	P.A.	M.T.	P.A.
NH_4^+	n.a.	n.a.	0.52	2.02	n.a.	n.a.	n.a.	n.a.
K^+	0.73	5.87	0.60	2.11	0.46	7.97	0.52	9.14
Ca^{2+}	0.89	5.38	0.69	6.58	0.46	4.63	0.70	9.13
Na^+/Mg^{2+}	0.97	3.59	0.76	2.44	0.52	3.45	0.75	3.49
Proteins*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

n.a. – not available
* – major protein peak detected after Na^+/Mg^{2+} peak

5.1.2.4. Optimization of BGE solution for CZE-C^4D of inorganic cations in real samples

In initial experiments that were performed to study the transfer of ions and retention of proteins on the ED membrane, it was found that in acetic acid based BGEs the separation efficiency of inorganic cations at concentration levels typical for biological samples was inadequate. An alternative BGE solution based on L-Arg and maleic acid was previously used in CZE-C^4D analyses of human body fluids by Kubáň et al. [122] . Its composition was optimized to allow full separation and quantification of inorganic cations in real samples. Its compatibility with acetic acid matrix, used as the ED acceptor solution, was also evaluated. Standard solution of 400 μM NH_4^+, 500 μM K^+, 2 mM Na^+ and 40 μM Ca^{2+} and Mg^{2+} in DI water was used for the optimization processes. BGE optimization was performed in two steps; in the first step, concentration of L-Arg and 18-crown-6 ether was kept constant at 15 and 1.5 mM, respectively, while concentration of maleic acid was changed from 11.5 to 13 mM. Resulting curves

showing dependency of migration times of all inorganic cations on concentration of maleic acid can be seen in Fig. 12A.

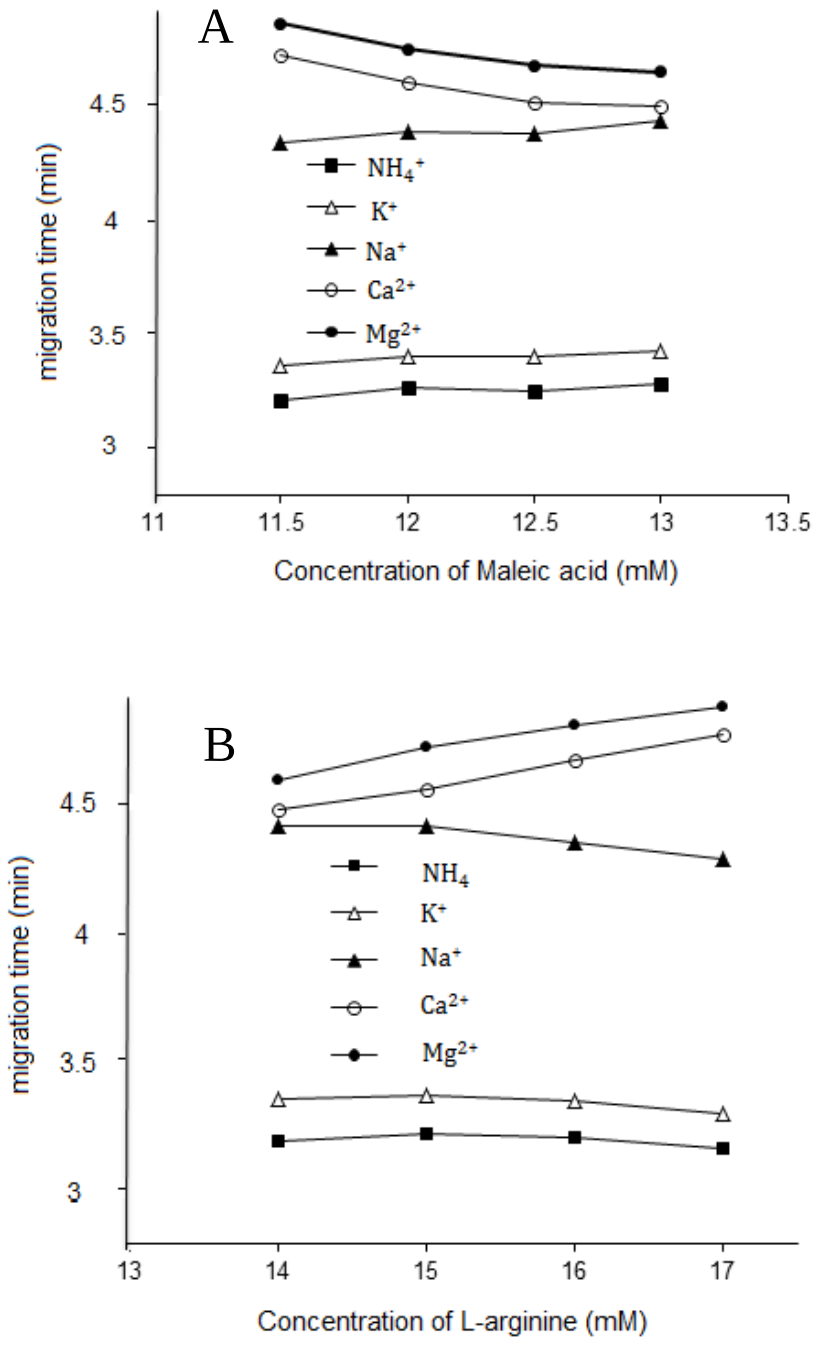


Figure 12. Optimization of the CZE conditions for determination of inorganic cations in biological fluids. A. BGE: 15 mM L-Arg, 1.5 mM 18-crown-6. B. BGE: 12.5 mM maleic acid, 1.5 mM 18-crown-6. Other CZE conditions: $L_{tot/ef}$: 50/42 cm, separation voltage: +15 kV, hydrodynamic injection from 25 cm for 30 sec, ion concentrations: NH_4^+, K^+ (400 µM), Na^+ (2 mM), Ca^{2+}, Mg^{2+} (40 µM).

Although separation of Na^+ and Ca^{2+} improves for lower concentrations of maleic acid, peak shapes of Ca^{2+} and Mg^{2+} deteriorated and quantitative determination of these two cations was not possible. For this reason 12.5 mM maleic acid was chosen as optimum and was also used in subsequent optimization, where concentration of L-Arg was investigated. In the second step, BGE solutions consisted of 12.5 mM maleic acid and 1.5 mM 18-crown-6 and concentration of L-Arg was changed from 14 to 17 mM. In this case, see Fig. 12B, improved separation efficiency of Na^+ and Ca^{2+} was achieved for higher L-Arg concentrations. Note however, that similar effect on peak shapes and separation efficiency of Ca^{2+} and Mg^{2+} was observed for higher concentrations of L-Arg and therefore optimum conditions for separation of all cations were: 12.5 mM maleic acid and 15 mM L-Arg at pH 5.5. The concentration of 18-crown-6 was increased to 3 mM in order to ensure baseline separation of K^+ and NH_4^+ in urine samples.

A typical separation of the five cations in this BGE solution is shown in Figure 13.

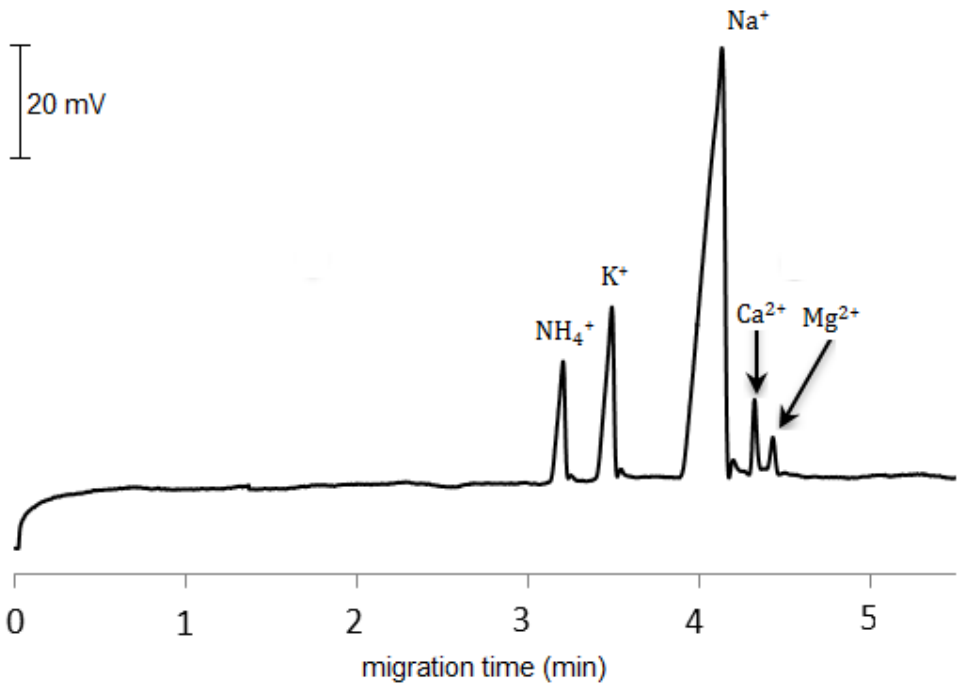


Figure 13. Separation of inorganic cations in the optimized BGE solution. Ion concentrations: NH_4^+, K^+ (400 µM), Na^+ (2 mM), Ca^{2+}, Mg^{2+} (40 µM), CZE conditions: $L_{tot/eff}$: 50/42 cm, separation voltage: + 15 kV, hydrodynamic injection from 25 cm for 30 sec, BGE: 12.5 mM maleic acid, 15 mM L-Arg and 3 mM 18-crown-6, pH 5.5.

5.1.2.5. Electrodialysis of real samples

For analysis of real samples the optimized BGE composed of 12.5 mM maleic acid and 15 mM L-Arg and 3 mM 18-crown-6 at pH 5.5 was used further. In the current experimental setup with 2 ED units and 3 membranes (Figure 6B), when ED is performed for longer time periods, the cations will migrate from the acceptor unit through the third dialysis membrane into the surrounding ED electrolyte solution. A compromise in ED time should be made to capture most of the analytes in the acceptor unit before they cross the third membrane and at the same time achieve a quantitative removal of these ions from the sample in the donor compartment. The best results would probably be achieved with long, narrow tubular acceptor unit (e.g. a short piece of a FS capillary), that would accommodate 100% of the analytes, however this arrangement was not possible in the commercial ED system.

In the following experiments, the donor unit was filled with standard solution or with real sample, ED tank and acceptor unit were filled with 5 mM acetic acid, and the system was treated at 200 V for 5 – 20 minutes. The donor to acceptor solution volume ratio was adjusted between 1:1 and 1:5 by using 1 – 5 link chambers as the acceptor unit resulting into final acceptor solution volume between 50 and 250 μL. The higher acceptor volume (i.e. longer migration path in acceptor unit) has ensured larger recoveries of both fast and slow migrating cations in the acceptor solution (results not shown) and therefore 5 link chambers were used as the acceptor unit in all subsequent experiments. CZE-C^4D analyses of donor and acceptor solutions were performed for each ED time and peak areas of the detected analytes were plotted against ED time. Fig. 14A and 14B show peak areas of the inorganic cations in acceptor solution for samples of human urine (diluted 1:8 with DI water) and human plasma (diluted 1:10 with DI water), respectively. ED times varied from 5 to 14 min. The peak areas of Ca^{2+} and Mg^{2+} in Fig. 14A were multiplied by a factor of 10 and peak areas of Na^+ in Fig. 14B were divided by a factor of 10 in order to display curves for all analytes in the same graph. Gradual decrease of peak areas of all analytes was observed in the donor solutions for increasing ED time. The graph for urine sample (Fig. 14A) showed patterns similar to those observed also when a standard solution with concentration levels of inorganic cations corresponding to urine sample was placed in the donor unit. The fastest cations under these experimental conditions (NH_4^+ and K^+) migrate quickly through the donor and acceptor unit and a gradual decrease of their peak areas in

acceptor solution is observed already after 5 min of ED as they start to migrate from the acceptor unit through the third membrane into the surrounding electrolyte. Na^+, Ca^{2+} and Mg^{2+}, which migrate significantly slower under these experimental conditions, increase their peak areas in the first 8 min of ED, reach their maximum at 8 min and then decrease, as they migrate out of the acceptor unit.

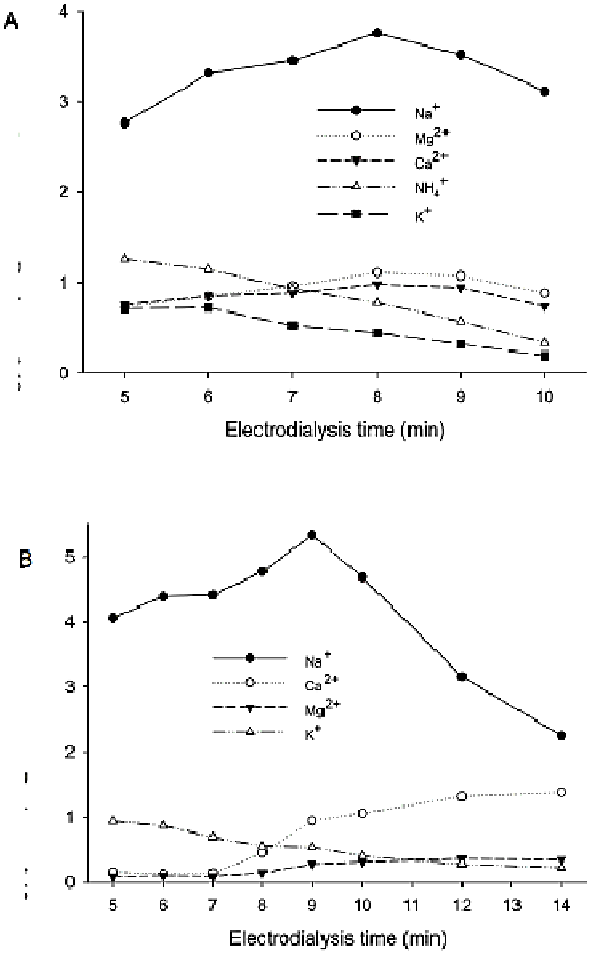


Figure 14. Effect of ED time on peak areas of inorganic cations in real samples. ED system with two units and three membranes, ED electrolyte: 5 mM acetic acid, pH 3.5, ED voltage: +200 V. A. ED treatment of human urine (diluted 1:8 with DI water); peak areas of Ca^{2+} and Mg^{2+} were multiplied by a factor of 10 to allow proper scaling. B. ED treatment of human serum (diluted 1:10 with DI water); peak areas of Na^+ were multiplied by a factor of 0.1 to allow proper scaling.

Similar behaviour was also observed for electrodialytic pretreatment of inorganic cations in human plasma sample, note however, that slightly different curves were obtained for some cations (Ca^{2+} and Mg^{2+} in Fig. 14B). Identical profiles as in Fig.

14B have been observed also when human serum sample and a standard solution with concentrations of inorganic cations corresponding to model plasma sample were placed in the donor unit. In Fig. 14B, peak areas of the fastest cation (K^+) gradually decrease for ED times longer than 5 min. Na^+ reaches its maximum at 9 min and then slowly decreases and finally, Ca^{2+} and Mg^{2+} reach their maximum at around 12 min.

The reason for this slightly different behaviour of Ca^{2+} and Mg^{2+} in urine and plasma/serum samples is thought to be due to the different composition of major and minor components in urine (major cations are K^+, NH_4^+ and Na^+) and plasma/serum (major cation is Na^+) samples. The results presented in Fig. 14 show that the ED process varies for different cations and partly depends also on their effective electrophoretic mobilities. ED time of 9 min was therefore selected as a suitable compromise for pretreatment of all real samples. Electropherograms for electrodialytically pretreated standard solution, human urine (diluted 1:8 with DI water), human serum and human plasma (both diluted 1:10 with DI water) samples are shown in Fig. 15.

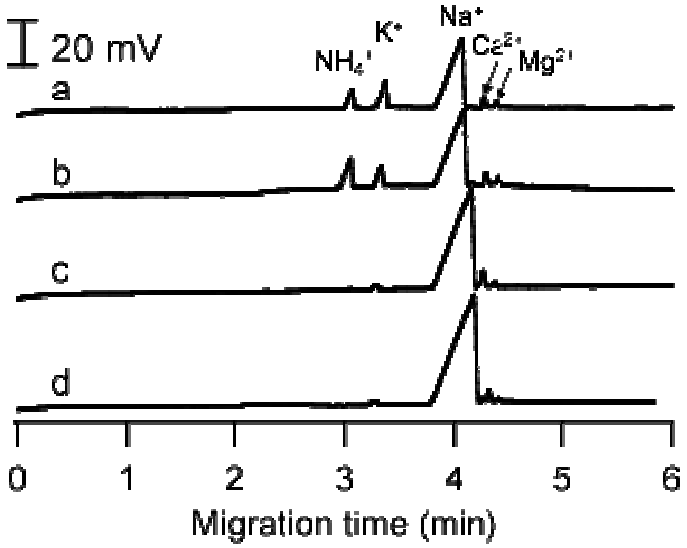


Figure 15. CZE-C^4D determination of inorganic cations in real samples after ED sample pretreatment. ED system with two units and three membranes, ED electrolyte: 5 mM acetic acid, pH 3.5, ED voltage: +200 V, ED time: 9 min. CZE conditions: $L_{tot/ef}$: 50/42 cm, BGE: 12.5 mM maleic acid, 15 mM L-Arg and 3 mM 18-crown-6, pH 5.5, separation voltage: +15 kV, hydrodynamic injection from 25 cm for 30 sec. a – standard solution (NH_4^+ (300 μM), K^+ (400 μM), Na^+ (2 mM), Ca^{2+} (50 μM), Mg^{2+} (30 μM)), b – human urine, c – human serum, d - human plasma.

5.1.2.6. Analytical performance

The analytical parameters of the CZE separation method with 12.5 mM maleic acid, 15 mM L-Arg and 3 mM 18-crown-6 BGE solution were evaluated. They are summarized in Table 4. RSD values for migration times and peak areas (n = 6) were measured for a standard solution consisting of 200 μM NH_4^+, 250 μM K^+, 1 mM Na^+, 100 μM Ca^{2+} and 50 μM Mg^{2+}, calibration curves were based on 5-point measurements. The limits of detection (LODs, based on 3 S/N criteria) were determined in standard solutions of cations with constant concentration of 2 mM Na^+. Sodium was added in excess since it is a dominant species in real samples that were later analyzed (urine, blood plasma, etc). Other cations are present in much lower concentrations, however, there was no adverse effect of high Na^+ concentration on the LODs of other minor cations.

Table 4. Analytical parameters of the developed CZE-C^4D method for determination of inorganic cations in biological fluids, n = 6

Ion	RSD(%) M.T.	RSD(%) P.A.	Calibration range (mM)	r^2	LOD (μM)
ammonium	0.36	1.18	0.04-0.4	0.9998	0.5
potassium	0.41	0.79	0.05-0.5	0.9998	0.66
sodium	0.4	1.31	0.2-2	0.9997	n.a.
calcium	0.42	2.46	0.02-0.2	0.9992	2
magnesium	0.43	1.18	0.01-0.1	0.9981	1

n.a. - not available, concentration of Na^+ was kept constant at 2mM in all LOD measurements

Repeatability of the electrodialytic pretreatment step was examined in the following experiments. A standard solution with concentrations of inorganic cations corresponding to the levels found in human blood plasma was prepared in DI water, diluted 1:10 with DI water, and subjected to 6 subsequent electrodialytic pretreatments. Similarly, 6 subsequent electrodialytic pretreatments were performed with raw human plasma, serum (both diluted 1:10 with DI water) and raw urine sample and in a standard solution containing concentrations of inorganic cations at the levels found in human urine (both diluted 1:8 with DI water). ED at 200 V for 9 min was used for all sample pretreatments and then the acceptor solution was subjected to CZE-C^4D analysis. Peak areas of the inorganic cations were evaluated and the resulting r.s.d. values are

summarized in Table 5. Very good repeatability was achieved for 6 independent pretreatments, which only slightly exceeded the repeatability of the CZE-C^4D method. The repeatability was usually 3 – 6%, slightly higher r.s.d. values were obtained for measurements of pretreated cations with low concentrations (up to 7.7%). For recovery measurements, a standard solution with concentrations of inorganic cations corresponding to the levels found in human urine (diluted 1:8 with DI water) was prepared and analyzed by CZE-C^4D; concentrations of the cations were calculated from corresponding calibration curves. Then the standard solution was pretreated in the ED system with two ED units and three dialysis membranes at optimized conditions and concentrations of the cations in the acceptor unit were again calculated and then compared with the original concentrations. The same set of measurements was also performed for raw urine sample (diluted 1:8 with DI water) without and with electrodialytic pretreatment. All measurements were performed as triplicates and the recovery results are also shown in Table 5. The recovery values (32 – 64%) for standard solution and real sample do not vary significantly, thus the selected ED conditions present a good compromise between extraction optimum for fast- and slow-migrating cations and quantification can be performed. The lower recovery values are not a problem in the measurements of real samples since the concentrations of inorganic cations in human body fluids are rather high, exceeding the method detection sensitivity by several orders of magnitude.

Table 5. Repeatability of peak areas and recovery values of electrodialytic pretreatment of standard solutions and biological fluid samples at optimized ED conditions. ED electrolyte solution: 5 mM acetic acid, ED voltage: + 200 V, ED time: 9 min, donor volume 50 µL, acceptor volume 250 µL. CZE-C^4D conditions as for Figure 15. Dilutions: plasma, serum and plasma-like standard solution 1:10, urine and urine-like standard solution 1:8 with DI water.

	Repeatablility (r.s.d.) values in %, n = 6				
	NH_4^+	K^+	Na^+	Ca^{2+}	Mg^{2+}
Standard solution (plasma)		7.62	4.81	5.89	6.62
Human plasma		7.34	3.84	5.62	7.74
Human serum		5.23	3.13	5.50	4.33
Standard solution (urine)	4.92	4.92	5.73	6.92	7.04
Human urine	6.01	4.28	3.32	6.06	5.71
	Recovery values in %, n = 3				
Standard solution (urine)	35.3	34.5	64.3	36.2	54.3
Human urine	32.3	32.1	63.9	49.9	57.2

5.1.2.7. The membrane lifetime

The lifetime of the dialysis membranes was also investigated by running ED of the real samples with the same set of membranes over one-week period and including one electrodialytic run of a test standard solution (5 inorganic cations at concentration levels corresponding to those found in human urine, diluted 1:8 with DI water) at the beginning and at the end of each working day. Peak areas of the analytes in the pretreated test solution were evaluated for the one-week period (n = 10) and the r.s.d. values were within the values shown in Table 4 and did not exceed 6.1%. This corresponds to approximately 100 runs performed on the same set of membranes. The membranes were therefore regularly replaced after one week.

5.1.3 Combination of the µED system and CZE-C^4D

In the previous part a commercial ED system was used and promising results were achieved. Unfortunately the commercial system has several disadvantages, such as high consumption of ED solution (750 mL), and need to make compromise between the ED time that is needed for transfer of the selected cations through the membrame and their escape to the surrounding media. This can be different for each type of real sample and makes this device rather difficult to use, requiring optimization for each sample type.

Therefore, in the next part a µED device was developed and tested. In this case, the device is composed of the ED unit only, without a bulky ED tank and the electrodialytical procedure takes place directly inside the unit. The µED system with 2 compartments and 1 membrane is shown in Figure 7, which is simpler and cheaper than the commercial ED system. Its construction brings several advantages, because the consumption of solutions is greatly reduced (expensive solutions can be used without incurring extra high cost) and as the Pt electrodes are inserted directly into the donor and acceptor compartments, there is no risk of the analyted to escape to the surrounding ED media as it was in the case of the commercial unit. On the other hand, one should be careful to avoid analyte decomposition due to the electrolysis on the electrodes. The BGE solution for CZE-C^4D separation of inorganic cations was the same as in the previous study: 12.5 mM maleic acid, 15 mM L-Arg and 3 mM 18-crown-6 at pH 5.5.

5.1.3.1 Optimization of the μED system of inorganic cations

Because of the different geometrical arrangement and solution volumes in the μED system compared to the commercial ED system, the used ED voltages, times and other parameters needed to be optimized. For this purpose a standard sample solution of four inorganic cations (60 μM K^+, 1.875 mM Na^+, 31 μM Ca^{2+} and 15 μM Mg^{2+}) was prepared in DI water as a donor solution. Following solutions were then tested as suitable acceptor solutions in the μED system: DI water, various concentrations of acetic acid and various concentrations of hydrochloric acid. DI water was not suitable for μED since the electric current in the system was very low even for highest available potential (300 V) and total transfer of cations from donor to acceptor solution was extremely slow. On the other hand, hydrochloric acid produced very high electric currents and excessive formation of air bubbles was observed in the donor and acceptor chambers resulting into unstable performance. Moreover, even very low concentrations of hydrochloric acid resulted into splitting peak shapes of inorganic cations in CZE-C^4D separations due to effect of highly conductive sample matrix [123]. Similarly to the previous system, acetic acid enabled suitable μED performance and best results were achieved at concentration of 5 mM. The ion transfer through the membrane was studied at 15 V and the results are shown in Fig. 16. It can be seen that it takes approximately 7 minutes for transferring about 80% of all cations present in the real samples to the acceptor solution, while at about 15 minutes around 100% of the ions are transferred into the acceptor solution. Thereafter the content of cations in the acceptor solution did not change. The effect of voltage on μED of the analytes was also examined and it has been found out that the μED can be further accelerated by higher voltages. However, higher applied voltages induced higher electric currents and the system performance became unstable, which resulted in irreproducible performance. For this reason, 15 V was maintained in all subsequent experiments, which yielded electric current of not more than 100 μA. At this current value, only firm bubble formation was observed in both μED chambers and stable μED system performance was achieved.

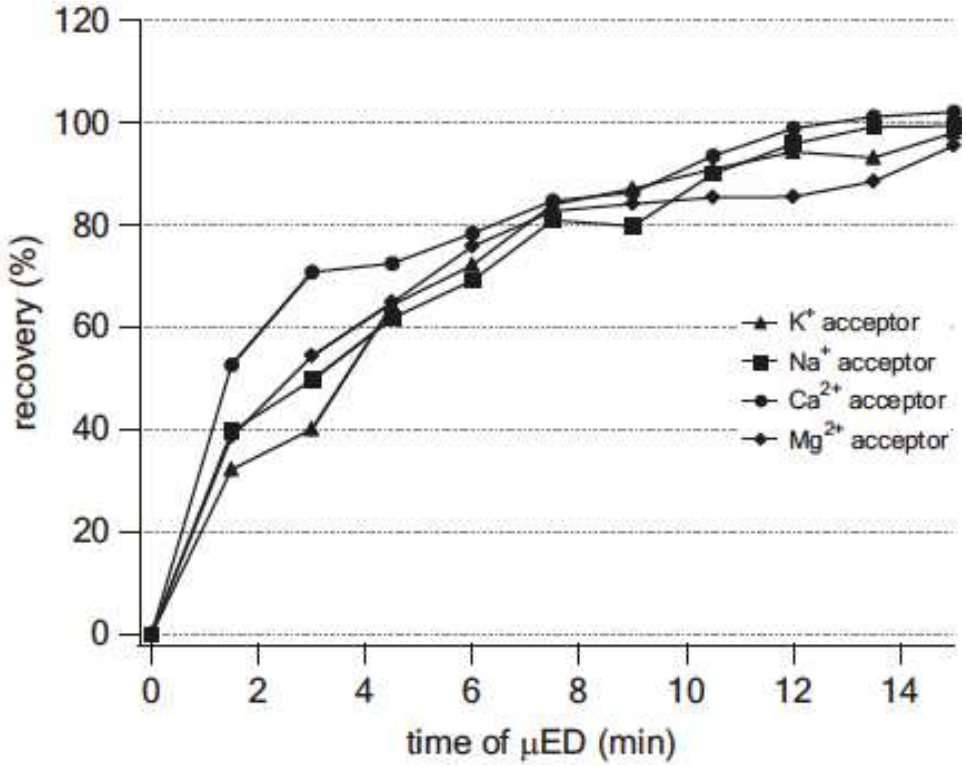


Figure 16. Effect of various ED times on recovery of four inorganic cations into the acceptor solution. μED system conditions: donor solution of four inorganic cations (60 μM K^+, 1.875 mM Na^+, 31 μM Ca^{2+} and 15 μM Mg^{2+} in DI water), acceptor solution: 5 mM acetic acid, pH 3.5, voltage: 15 V. CZE conditions $L_{tot/ef}$: 50/42 cm, separation voltage: +15 kV, hydrodynamic injection from 30 cm for 30 sec, BGE: 12.5 mM maleic acid, 15 mM L-Arg and 3 mM 18-crown-6, pH 5.5.

5.1.3.2. Effect of sample pretreatment by the μED system on CZE separation

Comparison of untreated sample injection and the pretreatment with the μED system.

The adverse effect of the adsorption of the high molecular weight compounds on the inner walls of a FS capillary and its influence on the performance of the CZE system was investigated as in the previous study. A standard sample solution was prepared containing 5 mM of K^+, 150 mM of Na^+, 2.5 mM of Ca^{2+}, 1 mM of Mg^{2+} and 80 g/L of HSA as a model protein corresponding to maximum concentrations of inorganic cations and to total protein concentration in human serum samples [121]. This standard sample solution diluted 1:80 with DI water was directly injected into the separation capillary 15 times and inorganic cations were separated by CZE-C^4D. A gradual increase of

migration times was observed for all species showing that adsorption of the protein on the capillary wall indeed significantly changes the surface properties of the separation capillary. The same set of measurements was performed with direct injection of human plasma, serum and whole blood samples diluted 1:80 with DI water and, as expected, even more significant effect on migration times of inorganic cations was observed for 15 consecutive injections. This is clearly seen in Figure 17 that shows the plot of relative migration times vs. the injection number for 80-fold diluted serum and whole blood samples. K^+ and Ca^{2+} were selected for graphical presentation only, however the behaviour of Na^+ and Mg^{2+} followed the same trends. The relative migration times were calculated by dividing the migration time of a selected analyte from all subsequent injections by the migration time of the same analyte from the first injection in the series. The increase in migration times was quite significant for both real samples (full symbols in Figure 17A and 17B, respectively). While a steady increase that followed approximately linear dependence was observed for the serum sample, a rather steep initial change in migration times after only a few injections, followed by a slow gradual increase of migration time, was observed for the whole blood. The different behaviour probably depends on the presence of red blood cells in the whole blood sample, however the adsorption phenomenon has not been studied in detail here, as the primary goal was to eliminate it and to achieve stable migration times of the analytes. Note also that adsorption of proteins and other high molecular weight compounds takes place even though, for example, HSA is uncharged at the pH of 5.5 and is brought to the separation capillary solely by EOF.

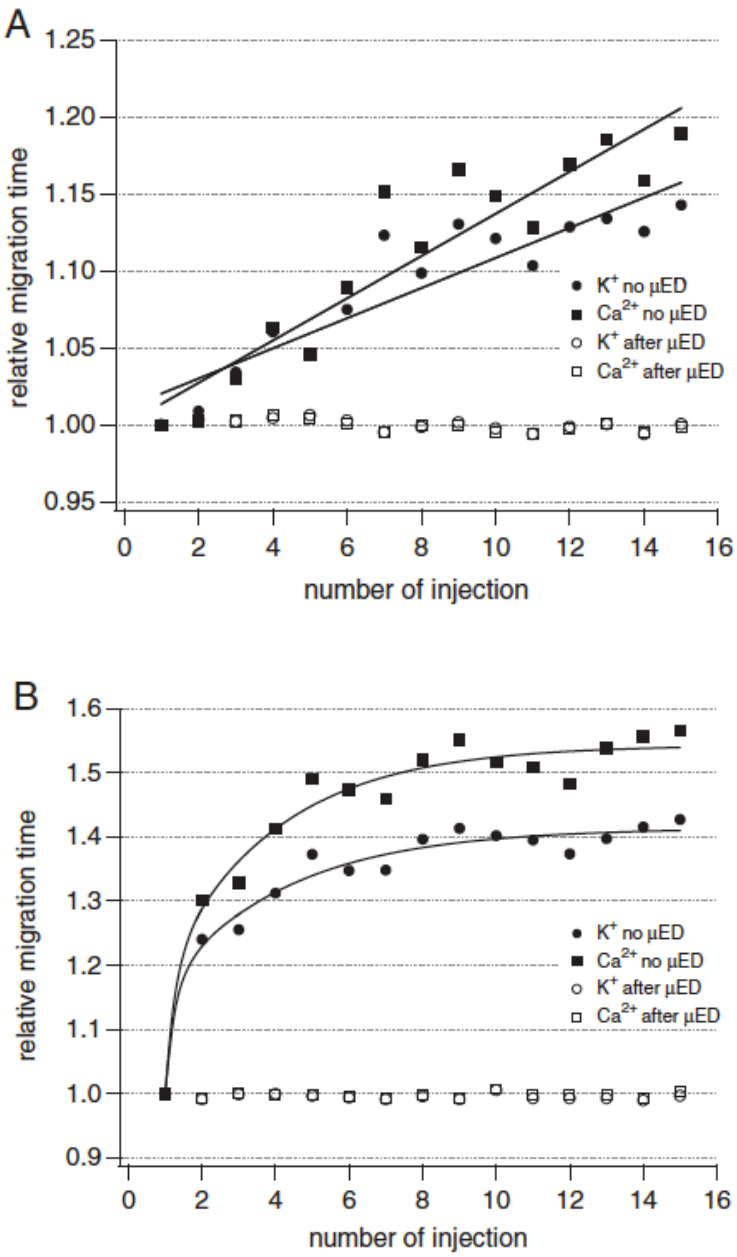


Figure 17. Effect of 15 consecutive analytes of untreated and pretreated human serum and whole blood sample on surface properties of a CZE separation capillary. A. Human serum (diluted 1:80) injected directly into the separation capillary without any pre-treatment (full symbols) and subjected to µED at 15 V for 15 min (empty symbols). B. Human whole blood (diluted 1:80) injected directly into the separation capillary without any pre-treatment (full symbols) and subjected to µED at 15 V for 15 min (empty symbols). CZE conditions as in Fig. 16.

Then, the retention of high molecular weight compounds by the dialysis membrane was studied. A standard sample solution of four inorganic cations and 50 μM HSA in DI water was subjected to μED. As expected, inorganic cations migrated through the ED membrane from the donor into the acceptor solution. For HSA peak, as determined in 200 mM acetic acid BGE solution, no peak was observed in the acceptor solution even after 15 min of μED. Obviously, HSA and very likely all other proteins of similar size do not cross the membrane.

Stability of CZE after μED was examined. Donor solutions were the standard sample solution of inorganic cations and HSA, serum, plasma and whole blood samples diluted 1:80 with DI water and acceptor solution was 5 mM acetic acid. μED was performed at 15 V for 15 min. Acceptor solution after μED was injected 15 times into the equilibrated CZE capillary and analyzed by CZE-C^4D. The results for human serum and whole blood samples are shown in Figure 17A and 17B, respectively (empty symbols). On contrary to untreated serum and whole blood samples, there is no visible change in relative migration times of the inorganic cations. The effect of protein adsorption on the capillary wall associated with direct injection into CZE capillary is completely eliminated by applying the simple micro-electrodialytic pretreatment step.

5.1.3.3 Electrodialysis of real samples using μED system

The optimized μED system conditions, e.g. 15 V for 15 minutes, were applied to analysis of cations in real samples. In these measurements, 1 μL of each sample was diluted 1:80 with DI water to yield ca. 80 μL of donor solution, out of which 50 μL were pipetted into the μED donor chamber. Fig. 18 shows series of electropherograms of a standard solution and a range of real samples electrodialytically pretreated in the μED system.

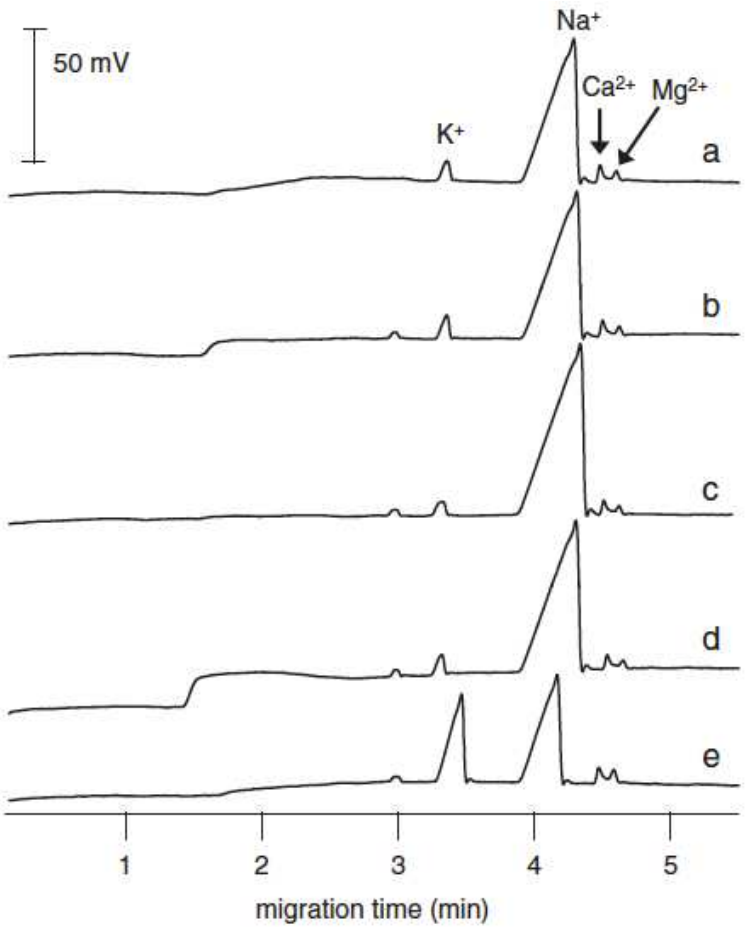


Figure 18. CZE-C^4D determination of inorganic cations in real samples after μED sample pretreatment. μED conditions: acceptor solution: 5 mM acetic acid, pH 3.5, voltage: 15 V, time: 15 min. CZE conditions: same as for Fig 16. (a) Standard solution (60 μM K^+, 1.875 mM Na^+, 31 μM Ca^{2+} and 15 μM Mg^{2+} in DI water); (b) standard solution after μED; (c) human plasma after μED; (d) human serum after μED; (e) human whole blood after μED; all real samples were diluted 1:80 with DI water.

5.1.3.4 Analytical performance

The analytical parameters of the CZE separation method with 12.5 mM maleic acid, 15 mM L-Arg and 3 mM 18-crown-6 BGE solution were described in section 5.1.2.6. In this section, we discuss the repeatability and recovery of the μED system.

Repeatability of the μED system at optimized conditions, e.g. 15 V for 15 min, was examined using a standard solution with concentrations of inorganic cations corresponding to the levels found in human serum diluted 1:80 with DI water, and subjected to 6 subsequent electrodialytic pretreatments. Similarly, 6 subsequent

microelectrodialytic pretreatments were performed with human serum, human plasma and whole blood samples (all diluted 1:80 with DI water) and then the acceptor solutions were subjected to CZE-C^4D analysis. Peak areas of the inorganic cations were evaluated and the resulting r.s.d. values are summarized in Table 6. The repeatability was usually 2 – 6%, slightly higher r.s.d. values were obtained for measurements of pretreated cations being present at low concentrations (up to 11.8%). Recovery values of cations through the dialysis membrane were also evaluated by comparing the measured peak area after μED with the peak areas in the original solution before μED for each cation [124]. For these measurements, the standard solution containing concentrations of inorganic cations corresponding to the levels found in human serum (diluted 1:80 with DI water) and human serum and plasma sample (diluted 1:80 with DI water) were used. Recovery results (96.3 – 110%, n = 6) are also summarized in Table 6 and do not differ significantly for standard solution and real samples. Thus the μED system can be used for analysis of both standards and real samples. Calculations of recovery values were not possible for whole blood samples since direct injection of whole blood results in strong adsorption of sample matrix during first three analyses and did not allow reproducible CE measurements.

Table 6. Repeatability and recovery values of electrodialytic pretreatment of standard solution and biological fluid sample in μED system.

	K^+	Na^+	Ca^{2+}	Mg^{2+}
Repeatablility (r.s.d.) values in %, n = 6				
Standard solution	2.0	2.8	3.8	5.2
Human plasma	3.6	2.3	6.8	8.2
Human serum	6.4	6.1	11.8	6.8
Whole blood	4.9	3.7	8.9	6.9
Recovery values in %, n = 6				
Standard solution	110.0	102.4	98.7	96.3
Human plasma	101.5	105.7	101.5	103.0
Human serum	98.2	99.2	99.1	98.4

μED aceptor solution: 5mM acetic acid, μED voltage: 15V, μED time: 15min, donor volume 50 μL, aceptor volume 50 μL. CZE-C^4D conditions as for Fig 16. Dilutions 1:80 with DI water.

The lifetime of the dialysis membrane in μED system was similar to the commercial ED system and was approximately 100 runs performed with one dialysis

membrane. Although no obvious membrane fouling was observed, the dialysis membrane was regularly replaced after 100 runs as a precaution.

5.2. USE OF DIPOSABLE OPEN TUBULAR ION EXCHANGE PRE-COLUMNS FOR IN-LINE CLEAN-UP OF BIOLOGICAL SAMPLES PRIOR TO CZE ANALYSIS OF INORGANIC CATIONS

In previous part, two electrodialysis systems were described for the pretreatment of biological samples such as blood plasma, blood serum, whole blood and urine. These systems could efficiently remove proteins from the samples and enhance the system performace by eliminating the protein adsorption on the capillary walls. The procedure was off-line, e.i. after the ED sample treatment, the unit was manually removed from the ED tank, disassembled, the acceptor solution was pipetted into an injection vial and analyzed by CZE. The μED system was much simpler, nevertheless, the manual sample pipetting and electrode removal still did not allow on-line or in-line coupling of the preconcentration to the CZE system. As in-line systems are advantageous, mainly because they eliminate the experimental errors associated with manual handling, in the ensuing part of the experimental work an in-line pretreatment method for analysis of inorganic cations in biological sample by CZE was developed. A simple concept for in-line clean-up of biological samples is presented using short disposable, open-tubular (OT) precolumn coupled to CZE capillary. The precolumn fulfils similar goal as the electrodialysis membrane, but using different principle. It uses adsorption of interfering compounds (proteins) from the injection plug onto the surface of the precolumn and thus allows stable CZE performance similar to previous ED systems. An advantage of the new system is easy handling of the capillaries, such as flushing with background electrolyte (BGE) and injecting samples and in-line interfacing: the injection and separation is done in a single step, greatly simplifying the whole analytical procedure. The coupling is sufficiently robust and simple to construct with a commercially available low dead volume union and induces no additional sample dispersion. The precolumn is easily connected/disconnected to/from CZE capillary and can be simply disposed of after each use. Performance characteristics of the proposed system are demonstrated on analysis of small inorganic cations in blood serum and plasma samples.

5.2.1. Evaluation of the system performance

The BGE solution used for determination of inorganic cations in biological fluid samples was optimized previously (section 5.1.2.4) and consisted of 15 mM L-arginine, 12.5 mM maleic acid and 3 mM 18-crown-6. The BGE solution was used to examine the effect of the connection between precolumn and analytical capillary and also of the PBMA ion-exchanger on CZE performance. The PBMA sample clean-up precolumn was connected to the analytical capillary through a P-772 union (as show in Fig 8). First, a 47 cm analytical capillary was used for separation of K^+, Na^+, Ca^{2+} and Mg^{2+} at concentrations corresponding to human serum diluted 1:80. Then, 6 cm of the analytical capillary was cut off using a capillary scriber and the two sections (6 and 41 cm) were connected through a P-772 union. Finally, 4 layer PBMA sample clean-up precolumn (6 cm) was connected to the 41 cm analytical capillary through a P-772 union. 7 measurements of the standard solution of the four cations were repeated for each separation system and several parameters were examined as summarized in Table 7. The separation of the four cations in the three separation systems is depicted in Fig. 19. Note, that no deterioration of the CZE performance was observed for the capillaries connected through the union compared to a single-piece analytical capillary, showing that the low dead volume union induces no measurable zone dispersion. Repeatability of migration times and peak areas was in the same range for all three separation systems (r.s.d. values 0.13 – 0.25% and 0.6 – 5.0%, respectively), and no effect was also observed for peak widths and separation efficiency. A slightly better resolution for separation of Na^+/Ca^{2+} and Ca^{2+}/Mg^{2+} pairs was achieved for PBMA precolumn connected to the analytical capillary, which is due to stronger interaction of Ca^{2+} and Mg^{2+} ions with the ion-exchanger of the precolumn [125], and results in their prolonged migration. However, even though interaction of Ca^{2+} and Mg^{2+} ions with the ion-exchanger was observed, the short migration path through the precolumn (6 cm) had no effect on peak shapes of the two ions as was evidenced from data in Table 7.

Table 7. Analytical parameters for analysis of a standard solution of four inorganic cations in a 47 cm long analytical capillary and a 41 cm long analytical capillary connected to 6 cm FS capillary and to 6 cm pre-column with 4 layers of PBMA, n = 5. CZE conditions as for Fig. 19.

Peak area, (r.s.d., %)				
	K^+	Na^+	Ca^{2+}	Mg^{2+}
AC	2.83	2.36	1.71	1.55
FS+AC	2.99	0.60	4.99	2.12
PBMA+AC	2.89	1.10	2.93	1.77
Migration time, (r.s.d., %)				
	K^+	Na^+	Ca^{2+}	Mg^{2+}
AC	0.13	0.12	0.20	0.23
FS+AC	0.18	0.18	0.24	0.25
PBMA+AC	0.24	0.20	0.22	0.22
Peak width (min)				
	K^+	Na^+	Ca^{2+}	Mg^{2+}
AC	0.105	0.327	0.082	0.080
FS+AC	0.108	0.329	0.082	0.077
PBMA+AC	0.107	0.330	0.080	0.071
N (theoretical plates/m)				
	K^+	Na^+	Ca^{2+}	Mg^{2+}
AC	49000	7100	114000	141000
FS+AC	49500	7200	113000	147000
PBMA+AC	50500	7200	115000	158000
Resolution				
	K^+/Na^+	Na^+/Ca^{2+}	Ca^{2+}/Mg^{2+}	
AC	3.58	0.79	1.74	
FS+AC	3.56	0.85	1.82	
PBMA+AC	3.56	1.01	1.93	

AC – 47 cm analytical fused silica capillary only
FS + AC – 6 cm fused silica capillary + 41 analytical fused silica capillary
PBMA + AC – 6 cm 4-layer PBMA capillary + 41 analytical fused silica capillary

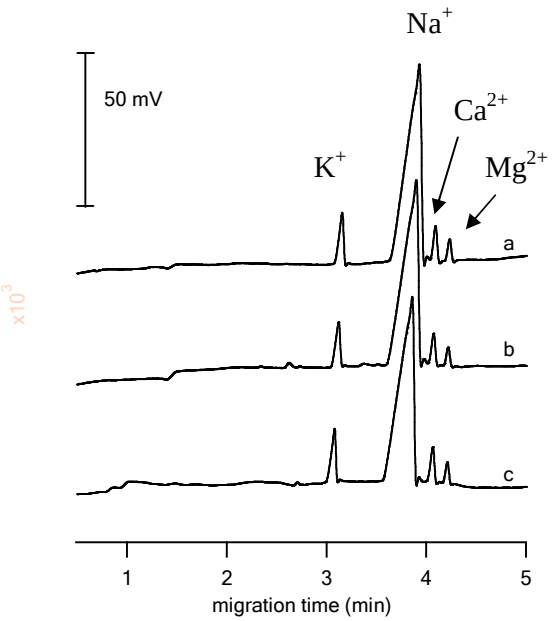


Figure 19. Electropherograms of inorganic cations in standard solution, 0.1 mM K^+, 1.8 mM Na^+, 0.05 mM Ca^{2+}, 0.025 mM Mg^{2+}, in a) 47 cm FS analytical capillary b) 6 cm FS + 41 cm FS analytical capillary c) 6 cm 4 layers of PMBA precolumn + 41 cm FS analytical capillary. CZE conditions: BGE solution, 15 mM L-arginine, 12.5 mM maleic acid, 3 mM 18-crown-6, pH 5.5; injection, hydrodynamic from 30 cm for 30 seconds, separation voltage + 15 kV.

5.2.2. Optimization of the number of PBMA layers in the clean-up precolumn

As has been shown previously, proteins from biological samples adsorb on the separation capillary walls and cause increase in migration times of the analytes for each successive injection, rendering the quantitation difficult. It is thus necessary to remove them prior to the analysis. The amount of proteins that can be removed by the precolumn depends on its ion exchange capacity, which is in turn dictated by the number of PBMA layers deposited. Too high ion exchange capacity may have a detrimental effect on the separation of cationic analytes because they also interact with the cation exchanger sites. To optimize the precolumn composition and length, precolumns with 2, 4, 6, 8 and 10 PBMA layers were connected to analytical capillary and their effect on sample clean-up and CZE separation was examined; precolumn lengths varied between 3, 6 and 12 cm. A standard solution of the four inorganic cations and 1 g/L HSA was injected five-times into the separation system and final electropherograms were analyzed. Retention of HSA on the ion-exchanger of the

precolumns was estimated based on stability of migration times and peak areas of inorganic cations (section 5.1.2.3) and was low for all three lengths of precolumns with 2 layers of PBMA. Increased migration times and peak areas of cations were observed already after second (3 and 6 cm precolumn) and third (12 cm precolumn) injection when 2 layer PBMA precolumn were used as these did not have sufficient IE capacity. On the other hand, stable CZE performance was observed even after 5 successive injections for precolumns (6 cm) with 4 or more layers of PBMA. For precolumns with 8 and 10 layers of PBMA, a strong interaction between the ion-exchanger and Ca^{2+} and Mg^{2+} ions resulted in much longer migration times and seriously deteriorated peak shapes of the two cations and their quantitative determination was not possible. Even when using shorter precolumn (3 cm, 8 and 10 layers of PBMA) their peak shapes did not improve.

A 4 layer PBMA precolumns (6 cm) was therefore used in all subsequent experiments due to their good ability to retain proteins (such as HSA) and due to their faster preparation compared to clean-up precolumns with 6 PBMA layers. The precolumns can be used for at least five repetitive measurements of solutions with high protein concentration but can also be used as single-use disposable extraction units, when needed. If higher number of clean-ups per clean-up precolumn should be performed, longer precolumns or more PBMA layers (maximum 6) may also be applied.

5.2.3. Effect of 4 layer PBMA sample clean-up precolumn to CZE-C^4D

The performance of the 4 layer PBMA precolumn was tested on blood serum and plasma samples diluted 1:80 with DI water. The diluted samples were first injected directly into the separation capillary. The behaviour was similar to that observed in the previous system with ED unit (without ED pretreatment). Increased migration times and peak areas were observed for each subsequent injection as the proteins from the real samples were attached to the inner surface of the capillary (Figure 20, full symbols). The 4 layer PBMA sample clean-up precolumns (6 cm) were then tested with these real samples. The precolumn was coupled in-line to the analytical capillary and five direct injections of blood serum were performed. This procedure was repeated for three unique precolumns, resulting into 15 measurements of blood serum, and the same set of 15

measurements was then performed for blood plasma. Excellent stability of migration times for both real samples was achieved, as shown for blood serum in Figure 20 (open symbols).

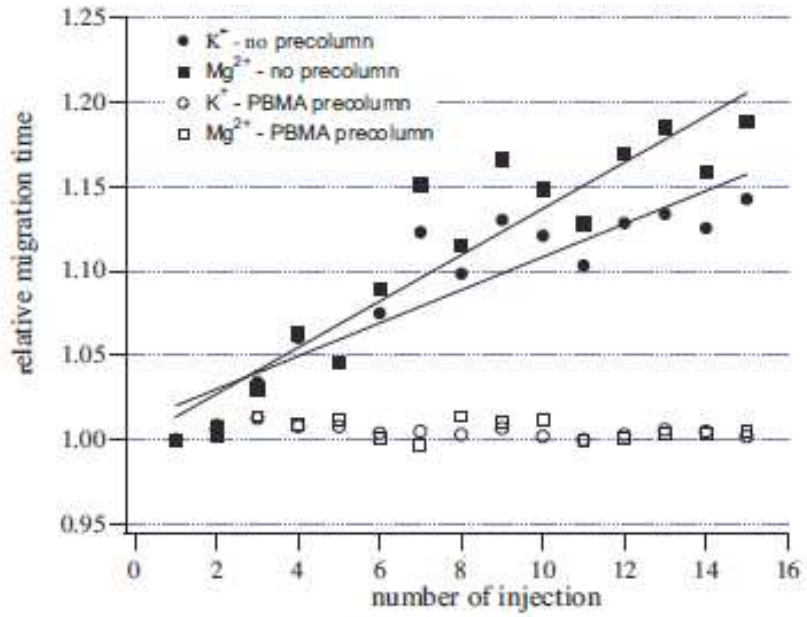


Figure 20. Effect of precolumn on migration times for direct injection of a serum sample (diluted 1:80 with DI water). 15 consecutive injections were performed without (full symbols) and with (open symbols) a 6 cm 4-layer PBMA precolumn. CE conditions are as in Fig. 19a and 19c.

Repeatability data for CZE-C^4D analyses of in-line cleaned-up real samples are summarized in Table 8 and electropherograms for direct injection of the standard solution, blood serum and blood plasma with the in-line coupled system are shown in Fig. 21.

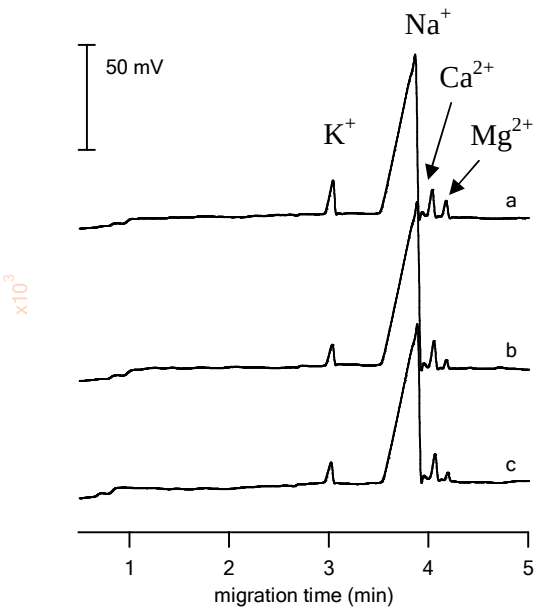


Figure 21. Direct injection of real samples using in-line sample clean-up, 6 cm 4- layer PBMA precolumn + 41 cm FS analytical capillary; a) standard solution, b) serum, c) plasma, Concentrations in standard solution and CZE conditions are as in Fig. 19c, real sample dilution: 1:80 with DI water.

5.2.4. Analytical performance

Three different 4 layer PBMA precolumns were cut (6 cm each) and were connected/disconnected/reconnected five times to the analytical capillary in order to examine the effect of the connection procedure. Migration times and peak areas were measured for the standard solution of the four cations and repeatability results are summarized in Table 8. From these results, the robustness of the connection can be seen from the r.s.d. values which were utmost 5.5% for peak areas and correspond to the values for a single clean-up precolumn connected to the analytical capillary as presented.

A slight deterioration of the repeatability of migration times, which was observed for repeated connection of the three unique precolumns, is very likely caused by slightly different lengths and column-to-column capacity factors of these precolumns and might be remedied by including an internal standard (such as Li^+) if necessary. However, as the composition of blood samples is almost constant and only the four cations migrate in this time window in the CZE measurement, identification of all peaks is straightforward and internal standard was not necessary in our subsequent measurements.

Table 8. Effect of connecting three various precolumns with 4 layers of PBMA to analytical capillary. CZE conditions as for Fig. 18.

Peak area, (r.s.d., %), n = 15				
	K^+	Na^+	Ca^{2+}	Mg^{2+}
Standard	3.67	5.48	3.19	2.7
Human serum	5.88	4.48	3.75	3.69
Human plasma	7.32	2.34	2.17	7.22
Migration time, (r.s.d., %), n = 15				
	K^+	Na^+	Ca^{2+}	Mg^{2+}
Standard	1.49	1.26	1.19	0.99
Human serum	1.4	1.2	1.33	1.25
Human plasma	0.74	0.96	1.01	1.07

All samples were diluted 1:80 with DI water

The performance of the sample clean-up precolumn coupled in-line to CZE was very good, showing that proteins and other high molecular weight compounds from real samples are retained by the ion exchanger in the precolumn and that this simple experimental set-up can be used for direct injection of biological samples in CZE. The sample matrix contains also anionic and neutral compounds but as the EOF is reduced in the BGE solution (1.9×10^{-8} m^2/Vs), most anionic compounds migrate in the opposite direction and do not enter the separation capillary. Neutral and slow anionic matrix compounds are dragged by the EOF through the precolumn and potentionally may penetrate into the separation capillary. Since no deterioration of CZE performance was observed, we assume that under the CZE conditions employed, these species are either eliminated by sorption onto the precolumn or they do not adsorb onto the capillary wall and thus have negligible effect on CZE performance.

5.3. TRACE DETERMINATION OF PERCHLORATE USING ELECTROMEMBRANE EXTRACTION AND CZE-C^4D

In previous two parts, the clean-up and removal of interferents from biological samples was a goal. In environmental samples, the cleanup is often not necessary (especially in water samples), but often preconcentration is needed. While the ED system could be used also for enrichment, the ED process is based on size difference and all ions of similar size are preconcentrated to a similar degree. Electrokinetic

migration across supported liquid membranes (SLM) can provide another degree of selectivity, as it may influence the amount of ions transferred based on the membrane and the membrane liquid properties. Thus, it may for instance allow selective preconcentration of some ions in the presence of large concentration of other ions. This is not possible with ED unless their migration is significantly different. In this section the results based on electrokinetic migration across a SLM followed by CZE with C^4D and its use in trace analysis of perchlorate in drinking water and environmental samples down to the sub-µg/L range are shown. This section concludes the range of novel sample pretreatment techniques that were developed in this thesis.

5.3.1. Choice of BGE solution for separation of perchlorate from other anions

A solution consisting of L-His and HAc has previously been used as BGE for the separation of small cations and anions, including perchlorate, in CZE-C^4D [113]. In this work, the BGE solution was simplified by eliminating 18-crown-6 and the BGE solution composition was 7.5 mM L-His and 40 mM HAc at pH 4.1. Modification/reversal of electroosmotic flow (EOF) was not necessary since the EOF magnitude is notably reduced at pH 4.1 and small anions migrate rapidly even against the EOF [113]. For standard solutions, it was possible to achieve complete separation of the 5 anions at 50 mg/L (chloride, sulfate, nitrate, nitrite, perchlorate), and most importantly, complete resolution of perchlorate in about 150 sec. The separation of a standard solution is shown in Figure 22a. The developed CZE method ensured high selectivity of the perchlorate determination in matrices with high excess of other inorganic anions, moreover, C^4D enabled highly sensitive determination of perchlorate due to its high specific conductivity and low background conductivity of the selected BGE solution. Capillary equilibration at the beginning of each working day was performed using five runs of a standard solution of the 5 anions to stabilize the migration times.

5.3.2. Selection of EME conditions

5.3.2.1. Choice of the organic solvent for EME

The properties of the organic solvent in the SLM are important for a practical EME system. A selective extraction system for anions was designed in our experiments by testing different organic solvents in combination with varied potential differences. Simple alcohols with different chain lengths, ranging from 1-pentanol to 1-decanol were tested. The main criterion for choosing a suitable organic solvent was the stability of the currents during EME and more importantly its extraction selectivity for perchlorate. 1-heptanol was found to be suitable because it showed excellent selectivity and adequate current stability while having acceptable electrical resistance to the applied voltage. Electric current was low enough to avoid bubble formation, liquid membrane depletion and excessive electrolysis.

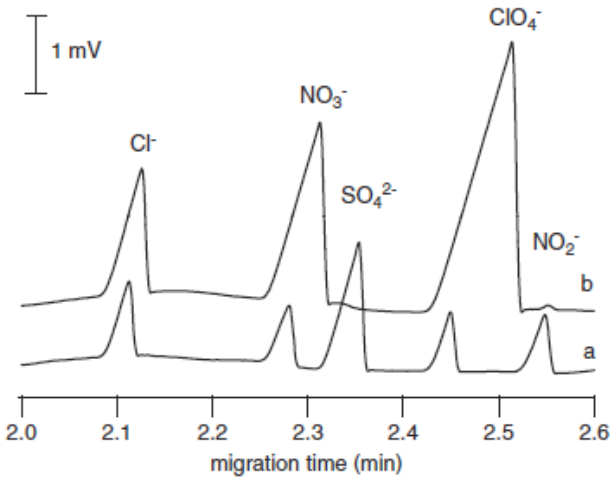


Figure 22. Electropherogram of a standard solution of the 5 anions in DI water. (a) without EME (b) after EME. Anion concentrations: Cl^- – 0.9 mg/L, NO_3^- – 1.5 mg/L, SO_4^{2-} – 2.4 mg/L, ClO_4^- – 2.5 mg/L and NO_2^- – 1.15 mg/L. EME conditions: liquid membrane: 1-heptanol, impregnation time: 10 s, agitation: 750 rpm, extraction voltage: 25 V, extraction time: 5 min, acceptor solution: 1 mM Tris. CZE conditions: BGE solution: 7.5 mM L-His, 40 mM HAc (pH 4.1), voltage: – 30 kV, injection: 50 mbar for (a) 7 sec and (b) 2 sec.

Fig. 22b shows an analysis of a standard solution of the 5 anions at 25 µM subjected to EME using 1-heptanol as the liquid membrane. The enrichment factors were 7.5, 18, 0.1, 40, 0.2, respectively, for the 5 anions. Note the favourable enrichment for perchlorate. For example, in comparison with higher alcohols, 1-heptanol had highest extraction efficiency and highest selectivity for perchlorate. On the other hand, it was difficult to perform EME with lower alcohols because of the excessive and unstable currents, which were obtained already at low electric potential settings (2 and 5 V for 1-pentanol and 1-hexanol, respectively).

5.3.2.2 Effect of extraction time

Standard solutions were extracted for different times between 1 and 10 minutes at 25 V. The extraction time profile of perchlorate and other 4 anions from an EME at the varying times are shown in Fig 23. Peaks of nitrate and sulfate partially or fully comigrated and for this reason were always treated as a single peak. The transfer of perchlorate with respect to the extraction times was examined and it was found that the optimum extraction yield is obtained after an enrichment time of 5 min where no change in perchlorate transfer happens.

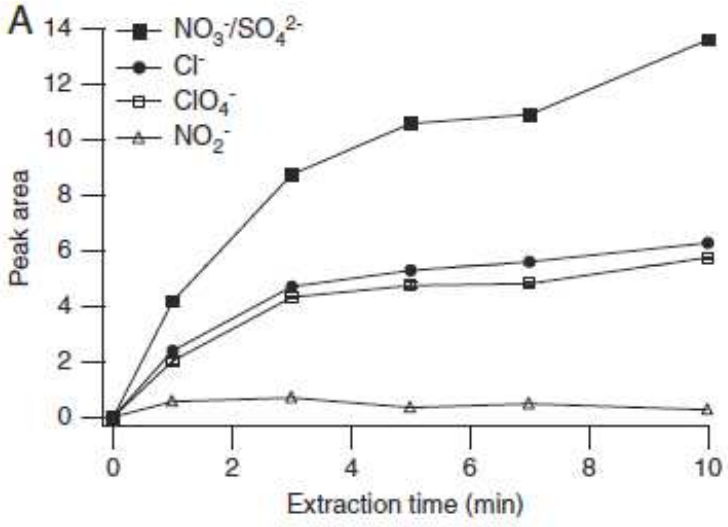


Figure 23. Effect of extraction time on EME of inorganic anions. EME and CZE conditions as for Fig. 22. Injection: 50 mbar for 7 sec. Concentrations of anions: chloride, nitrate, sulfate: 5 mg/L, nitrite, perchlorate: 100 µg/L. Peak areas of chloride and nitrate/sulfate were divided by a factor of 10.

The system entered steady-state conditions, and the gain in transfer became minimal. This trend was observed previously, for instance in the EME of drugs [86, 124]. A sudden increase in current occurred after approximately 8 min. The current became erratic, probably due to the integrity of the membrane being compromised, resulting from depletion of the organic solvent from the SLM. Increased current results in electrolysis at the electrodes and partial vaporization of the acceptor solution [86]. Therefore, 5 min was chosen as an optimum.

5.3.2.3 Effect of extraction voltage

The flux of analytes varies with applied potential [124] and Gjelstad et al. [126] presented a mathematical model of EME in which they showed that high initial flux of analytes can be obtained for high extraction voltages only and lowering the voltage results in slow extraction process. When sufficient time is allowed for the extraction, the same steady-state recoveries can be obtained. Different voltages were applied to the EME system ranging from 0 - 50 V as shown in Fig. 24.

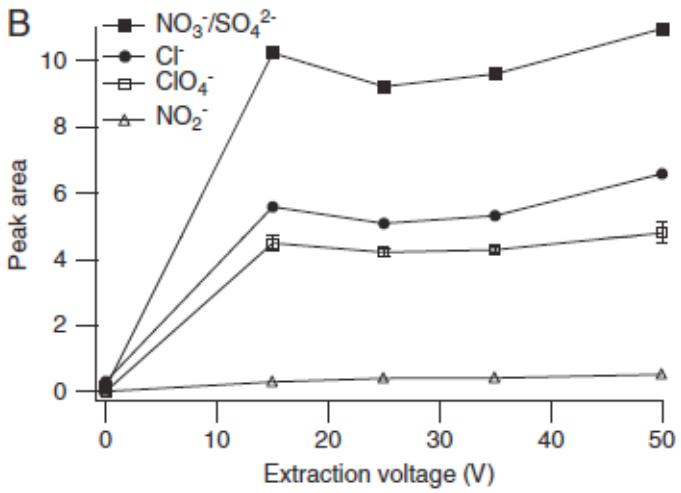


Figure 24. Effect of extraction voltage on EME of inorganic anions. EME and CZE conditions as for Fig. 22. Injection: 50 mbar for 7 sec. Concentrations of anions: chloride, nitrate, sulfate: 5 mg/L, nitrite, perchlorate: 100 µg/L. Peak areas of chloride and nitrate/sulfate were divided by a factor of 10.

Without the application of voltage, transfer of perchlorate into the acceptor phase was not observed. It therefore seems that passive diffusion was not forthcoming at these conditions in such a short time. On the other hand, on application of extraction voltage, the negatively charged perchlorate ions at the SLM interface rapidly migrate in the direction of the positively charged electrode and as their replenishment on the SLM surface is ensured by constant stirring of the donor solution, they can efficiently be transferred into the HF lumen in a short time. The extraction efficiency of perchlorate at 15 V, 25 V, 35 V and 50 V had insignificant difference in range of only 12%. Extraction voltage of 25 V was therefore selected for the rest of the study.

5.3.2.4 Effect of stirring

The volume of the donor solution was 175 times higher than the acceptor solution volume. Stirring of the donor solution during EME increases the physical movement of the analytes in the bulk donor phase and reduces the thickness of the stagnant layer at the interface between the donor phase and the SLM [127]. Stirring speeds between 0 and 1000 rpm were studied. The stirring is important as a 2-fold increase of the extraction efficiency was observed when stirring speed was changed from 0 rpm to 250 rpm. The extraction efficiency reached a plateau phase at 250 rpm and there was no significant change in efficiency even at 1000 rpm. A stirring rate of 750 rpm was chosen as a compromise.

5.3.2.5. Effect of donor and acceptor solution composition

Donor solutions were prepared in three various matrices. Neutral (DI water), acidic (1 mM HCl) and alkaline (1 mM Tris, 1 mM NaOH) solutions were used as matrices for EME of the 5 inorganic anions. Extraction efficiency of perchlorate was 2 – 10 fold worse in acidic and alkaline donor solutions compared to that prepared in DI water and for this reason DI water was used as diluent for standard solutions in subsequent EME measurements. The reduced extraction efficiencies at acidic and alkaline conditions can be rationalized by the presence of anions (Cl^- and OH^-) in the donor solutions that partly hinder the perchlorate transfer into the HF lumen.

The properties of acidic, neutral, and alkaline acceptors on extraction efficiency of anions in the EME system were also studied using standard solutions of the 5 anions. DI water was used as neutral acceptor, 1 mM HCl as acidic acceptor and 1 mM Tris and 1 mM NaOH as alkaline acceptors. Compared to 1 mM Tris, there was about three- and

two-times lower extraction efficiency for DI water and 1 mM HCl, respectively. The extraction efficiency for 1 mM NaOH and 1 mM Tris solutions was almost identical showing no significant difference between the two different alkaline acceptors. Different concentrations of Tris were also studied and there was no significant difference in extraction efficiency between 1, 2, 5 and 10 mM Tris acceptor solutions. 1 mM Tris solution was therefore chosen for further experiments. To study EME of blank solutions, DI water containing standard anions with no perchlorate was used. EME and subsequent CZE-C^4D analyses of standard solutions without (blank solution) and with addition of 15 µg/L of perchlorate are shown in Fig. 25.

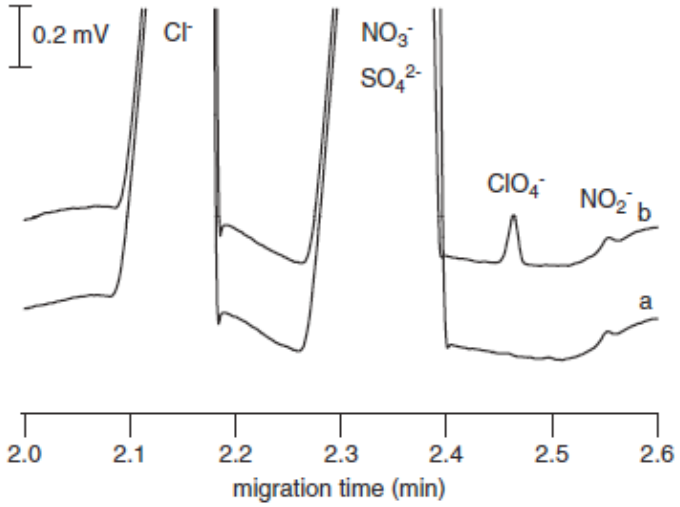


Figure 25. EME-CZE-C^4D of a standard solution of 5 mg/L chloride, nitrate, sulfate. (a) without perchlorate and (b) spiked with 15 µg/L of perchlorate. EME and CZE conditions as for Fig. 22. Injection: 50 mbar for 7 sec.

5.3.3. Analytical performance

The analytical characteristics of the EME-CZE-C^4D are summarized in Table 9. The linearity was calculated from six-level calibration curve in the range from 1 to 100 µg/L, including blank. The precisions of the curve, as indicated by the relative standard deviation (r.s.d.), were in the ranges of 0.06 – 0.2% for migration time and 1.3 – 8.7% for peak area (at concentration level of 15 µg/L of perchlorate, n = 7), while correlation coefficients (r^2) ranged from 0.9988 – 1.0000 for the standard and the real water samples. LODs, defined as three-times signal-to-noise ratio, were determined by consecutive dilution of perchlorate in the various water samples and all were below or

equal to 1 µg/L. Recoveries at 15 µg/L of perchlorate were lowest in snow and highest in bottled water sample and ranged from 95.9 to 106.7% in all different matrices.

Table 9. Analytical parameters of the developed EME-CZE-C4D method for determination of perchlorate (spiked at 15 µg/L) in various saples, n = 7, calibration range: 1 – 100 µg/L. EME and CZE-C^4D conditions as for Fig 23.

	STD	Rain	Snow	Potable 1	Potabe 2	Potable 3
RSD MT (%)	0.15	0.06	0.20	0.06	0.08	0.12
RSD PA (%)	1.3	8.7	3.8	5.9	3.3	6.5
Recovery (%)	98.9	103.8	95.9	106.7	102.8	106.5
r^2	1	0.999	0.999	0.999	0.999	0.999
LOD (µg/L)	0.3	0.25	0.3	0.3	0.35	1.0
LOQ (µg/L)	1.0	0.8	1.0	1.0	1.2	3.3

5.3.4. Analysis of drinking water and environmental samples

The objective of this study was to develop an analytical method for the analysis of trace perchlorate at low to sub-µg/L levels in drinking and environmental water samples; ideally the method would reduce the level of interfering matrix ions without loss of perchlorate in addition to speed, efficiency and reduced cost. To demonstrate the applicability of perchlorate analysis among other inorganic anions, EME-CZE-C^4D was applied to different samples; tap, bottled potable and surface water and to snow and rain samples. The samples were used without any pretreatment and were directly sampled into donor vial for EME.

Perchlorate was not detected in any of the samples. There was no perchlorate in untreated samples or perchlorate was present at concentrations below the LOD of the EME-CZE-C^4D method. When spiked with 15 µg/L of perchlorate, it was detected in all samples with recoveries ranging from 95.9 to 106.7% as summarized in Table 9. Electropherograms of selected EME treated samples spiked at the concentration of 15 µg/L of perchlorate are shown in Fig. 26. It can be seen that perchlorate, spiked at the advisory limit recommended by the US EPA, was detected in all EME treated samples,

although their matrix composition varies significantly. Slightly reduced sensitivity for perchlorate analysis was obtained for EME treated tap water.

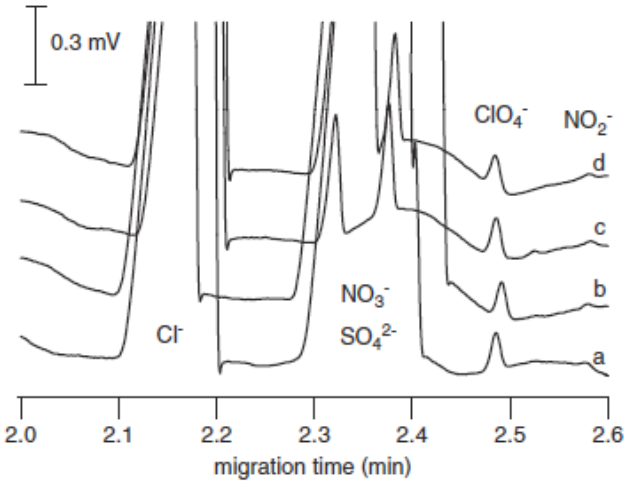


Figure 26. EME-CZE-C^4D of various real samples. (a) snow, (b) surface water, (c) Dobrá voda, (d) Horský pramen; all spiked with 15 µg/L of perchlorate. EME and CZE conditions as in Fig. 22. Injection: 50 mbar for 7 sec.

Apart from other anions, tap water in Brno district contains high concentration of nitrate (about 40 – 45 mg/L, which approaches its maximum allowable concentration for drinking water – 50 mg/L) and since nitrate is also preferentially transported across the SLM (see section 5.3.2.1), the transport of perchlorate was partially hindered. Relationship between matrix composition and the sensitivity of perchlorate analysis was, however, often reported for IC-CD [128] and also for hyphenated IC-MS/MS [116] systems. Despite the high nitrate content in the tap water, 15 µg/L of perchlorate can be easily detected in the EME treated tap water sample with LOD being 1 µg/L. Electropherograms of the EME treated neat tap water and the tap water spiked with 5, 15 and 50 µg/L of perchlorate are shown in Fig. 27.

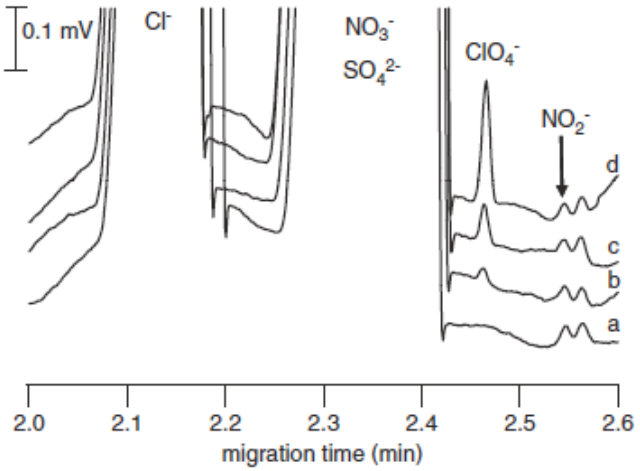


Figure 27. EME-CZE-C^4D of (a) neat tap water, (b) tap water spiked with 5 µg/L of perchlorate (c) tap water spiked with 15 µg/L of perchlorate, (d) tap water spiked with 50 µg/L of perchlorate. EME and CZE conditions as in Fig. 22. Injection: 50 mbar for 15 sec.

Determination of perchlorate at sub-µg/L concentrations in rain sample and bottled potable water is shown in Fig. 28A and 28B, respectively. No perchlorate was detected in EME treated neat samples as is evidenced in traces (a). After spiking the two samples with 1 and 5 µg/L of perchlorate and subsequent EME treatments, distinguished perchlorate peaks were observed in CZE-C^4D analyses, which were about 10- and 35-fold higher than the baseline noise – see traces (b) and (c), respectively.

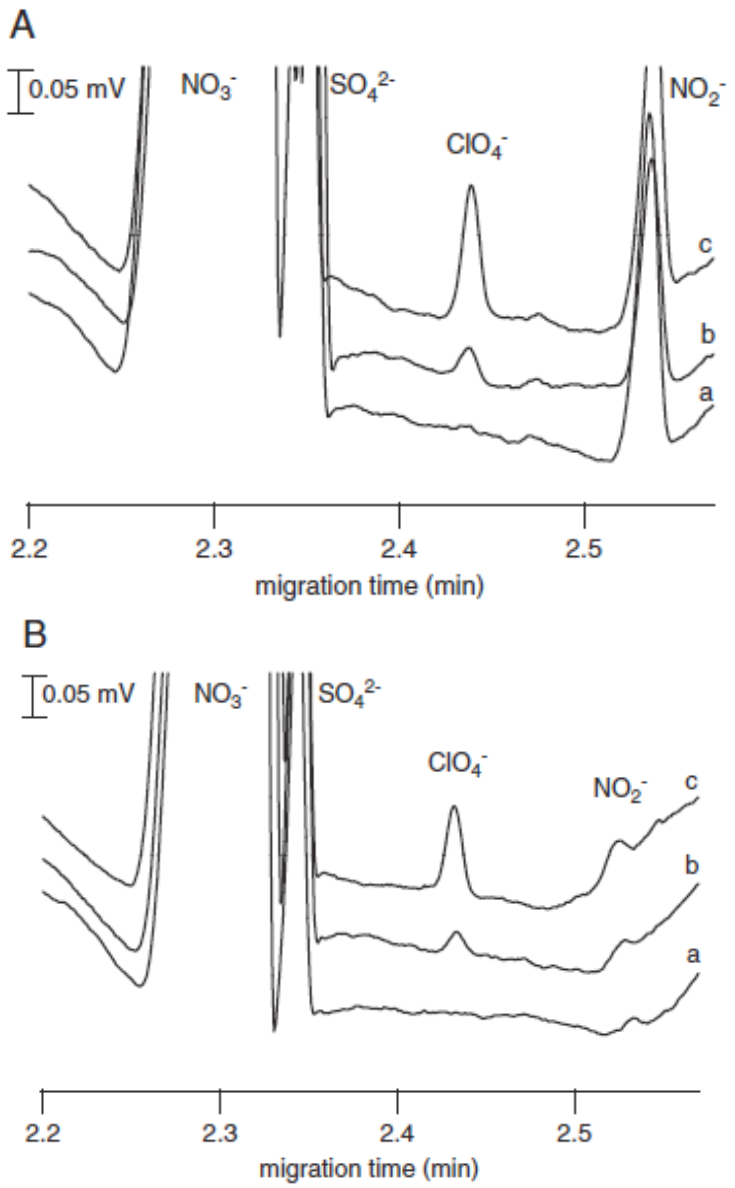


Figure 28. (A) EME-CZE-C^{4}D of rain water. (a) Neat rain water, (b) rain water spiked with 1 µg/L of perchlorate and (c) rain water spiked with 5 µg/L of perchlorate. (B) EME-CZE-C^{4}D of bottled water Horský pramen. (a) Neat Horský pramen, (b) Horský pramen spiked with 1 µg/L of perchlorate and (c) Horský pramen spiked with 5 µg/L of perchlorate. EME and CZE conditions as in Fig. 22. Injection: 50 mbar for 15 sec.

6. CONCLUSIONS

Three novel approaches for sample cleanup and preconcentration were developed for analysis inorganic ions in biological and environmental samples by CZE.

Both the commercial ED system and the μED system have demonstrated to be an effective method for pretreatment of raw human biological fluids and for subsequent analysis of the pretreated samples by CZE with C^4D. High molecular weight matrix compounds were retained in donor compartment of the ED system unit using a 500 Da MWCO membrane and potential serious deterioration of CZE performance caused by adsorption of matrix compounds from untreated real samples was thus efficiently eliminated. On the contrary, the small cations migrated through the dialysis membrane into the acceptor compartment, and could be analyzed by CZE-C^4D. The achieved excellent EOF stability of the CZE system enabled analyses of ED pretreated biological fluid samples. In the μED system used later the volume of real sample needed was as low as 1 μL per analytical run, which kept the analyses possible even in case the amount of biological sample is limited. The μED system does not suffer from the disadvantage of the commercial ED system , in which several hundreds mL of electrolyte solution to fill the ED tank and run the pre-treatment process are needed. One slight disadvantage of the μED system is that it is an off-line system.

OT ion exchange precolumns were for the first time used for in-line clean-up and direct injection of biological samples in CZE. OT ion exchange precolumns were used as a similar in function (remove proteins) but different in principle. In-line coupling present an added advantage over previous off-line commercial ED and μED systems. Preparation of the OT precolumns is simple, fast and inexpensive. They can therefore be used as disposable sample pretreatment units which can be simply discarded after each use. The in-line sample pretreatment method was demonstrated on determination of small inorganic cations in blood serum and plasma samples.

Trace analysis of perchlorate in various drinking water and environmental samples was feasible by a combination of EME and CZE-C^4D. Perchlorate was selectively preconcentrated into the lumen of a hollow fibre based on its electrically induced migration across a SLM. Solid particles (in environmental samples) and most inorganic anions were retained on the SLM and did not interfere with CE analysis. Presence of high concentrations of nitrate (close to its maximum allowable level) and

chloride in tap water sample slightly hindered the perchlorate transport as these ions also partially migrated into the lumen. Nevertheless, baseline separation of perchlorate and all co-extracted anions was achieved at optimized CZE-C^4D conditions and perchlorate could be detected in tap water down to a spiked concentration of 1 µg/L. This is 15 times lower than the US EPA regulatory requirement for drinking water. Sub-µg/L concentrations of spiked perchlorate could be detected in rain, snow, surface and bottled potable water samples. These samples have generally lower content of inorganic anions and therefore less influence on EME transport of perchlorate. Although the proposed method does not achieve sensitivity of extremely costly and complex hyphenated IC-MS/MS systems, it compares well with routinely used IC-CD and IC-MS systems, with much shorter running times, unprecedented simplicity and reduced costs.

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8. LIST OF ABBREVIATIONS AND SYMBOLS

BGE	background electrolyte
C^4D	capacitively coupled contactless conductivity detection
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CTAB	cetyltrimethylammonium bromide
CZE	capillary zone electrophoresis
DETA	diethylenetriamine
DI	deionized
ED	electrodialysis
EME	electromembrane extraction
EOF	electroosmotic flow
FS	fused-silica
HAc	acetic acid
HF	hollow fibre
HF-LPME	hollow-fiber liquid-phase microextraction
HIS	histidine
HPCE	High performance capillary electrophoresis
HPLC	High performance liquid chromatography
HSA	human serum albumin
IC	ion chromatography
IC-CD	ion chromatography-conductivity detection
ID	internal diameter
IE	ion exchange
L-Arg	L-arginine
LLE	liquid–liquid extraction
LOD	limit of detection
LPME	liquid phase microextraction
MEKC	micellar electrokinetic chromatography
MES	2-(N-morpholino)ethanesulfonic acid
MS	mass spectrometry

MWCO	molecular weight cut-off
OD	outer diameter
OT	open tubular
PBMA	poly(butadiene-maleic acid)
PMMA	polymethylmethacrylate
PP	polypropylene
PTFE	polytetrafluoroethylene
RSD	repeatability standard deviation
SLM	supported liquid membrane
SPE	solid phase extraction
TTAB	tetradecyltrimethylammonium bromide
US EPA	United States Environmental Protection Agency
UV-Vis	ultraviolet - visible
μED	micro-electrodialysis

C	concentration of the analyte
d	capillary internal diameter
E	applied electric field
e	electron charge in Coulombs
f	frequency
g	gravitational constant
I	current
L	length of the capillary
L_{eff}	Effect length of capillary
L_{tot}	total length of capillary
r_i	ion radius
t	time
V	voltage
z_i	ion charge
Δh	height difference
ΔP	pressure difference across the capillary
ε	dielectric constant of the electrolyte
ζ	zeta potential of the wall

η	solution viscosity
μ_e	electrophoretic mobility of the analyte
μ_{EOF}	EOF mobility
μ_i	electrophoretic mobility of a species I
v_i	ion velocity
ρ	separation electrolyte density

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$L_{tot/eff}$: 50/42 cm, separation voltage: + 15 kV, hydrodynamic injection from 25 cm for 30 sec, BGE: 12.5 mM maleic acid, 15 mM L-Arg and 3 mM 18-crown-6, pH 5.5.

Figure 14. Effect of ED time on peak areas of inorganic cations in real samples. ED system with two units and three membranes, ED electrolyte: 5 mM acetic acid, pH 3.5, ED voltage: +200 V. A. ED treatment of human urine (diluted 1:8 with DI water); peak areas of Ca^{2+} and Mg^{2+} were multiplied by a factor of 10 to allow proper scaling. B. ED treatment of human serum (diluted 1:10 with DI water); peak areas of Na^{+} were multiplied by a factor of 0.1 to allow proper scaling.

Figure 15. CZE-C^4D determination of inorganic cations in real samples after ED sample pretreatment. ED system with two units and three membranes, ED electrolyte: 5 mM acetic acid, pH 3.5, ED voltage: +200 V, ED time: 9 min. CZE conditions: $L_{tot/ef}$: 50/42 cm, BGE: 12.5 mM maleic acid, 15 mM L-Arg and 3 mM 18-crown-6, pH 5.5, separation voltage: +15 kV, hydrodynamic injection from 25 cm for 30 sec. a – standard solution (NH_4^+ (300 μM), K^+ (400 μM), Na^+ (2 mM), Ca^{2+} (50 μM), Mg^{2+} (30 μM)), b – human urine, c – human serum, d - human plasma.

Figure 16. Effect of various ED times on recovery of four inorganic cations into the acceptor solution. μED system conditions: donor solution of four inorganic cations (60 μM K^+, 1.875 mM Na^+, 31 μM Ca^{2+} and 15 μM Mg^{2+} in DI water), acceptor solution: 5 mM acetic acid, pH 3.5, voltage: 15 V. CZE conditions $L_{tot/ef}$: 50/42 cm, separation voltage: +15 kV, hydrodynamic injection from 30 cm for 30 sec, BGE: 12.5 mM maleic acid, 15 mM L-Arg and 3 mM 18-crown-6, pH 5.5.

Figure 17. Effect of 15 consecutive analytes of untreated and pretreated human serum and whole blood sample on surface properties of a CZE separation capillary. A. Human serum (diluted 1:80) injected directly into the separation capillary without any pre-treatment (full symbols) and subjected to μED at 15 V for 15 min (empty symbols). B. Human whole blood (diluted 1:80) injected directly into the separation capillary without any pre-treatment (full symbols) and subjected to μED at 15 V for 15 min (empty symbols). CZE conditions as in Fig. 16.

Figure 18. CZE-C^4D determination of inorganic cations in real samples after μED sample pretreatment. μED conditions: acceptor solution: 5 mM acetic acid, pH 3.5, voltage: 15 V, time: 15 min. CZE conditions: same as for Fig 16. (a) Standard solution (60 μM K^+, 1.875 mM Na^+, 31 μM Ca^{2+} and 15 μM Mg^{2+} in DI water); (b) standard solution after μED; (c) human plasma after μED; (d) human serum after μED; (e) human whole blood after μED; all real samples were diluted 1:80 with DI water.

Figure 19. Electropherograms of inorganic cations in standard solution, 0.1 mM K^+, 1.8 mM Na^+, 0.05 mM Ca^{2+}, 0.025 mM Mg^{2+}, in a) 47 cm FS analytical capillary b) 6 cm FS + 41 cm FS analytical capillary c) 6 cm 4 layers of PMBA precolumn + 41 cm FS analytical capillary. CZE conditions: BGE solution, 15 mM L-arginine, 12.5 mM maleic acid, 3 mM 18-crown-6, pH 5.5; injection, hydrodynamic from 30 cm for 30 seconds, separation voltage + 15 kV.

Figure 20. Effect of precolumn on migration times for direct injection of a serum sample (diluted 1:80 with DI water). 15 consecutive injections were performed without (full symbols) and with (open symbols) a 6 cm 4-layer PBMA precolumn. CZE conditions are as in Fig. 19a and 19c.

Figure 21. Direct injection of real samples using in-line sample clean-up, 6 cm 4- layer PBMA precolumn + 41 cm FS analytical capillary; a) standard solution, b) serum, c) plasma, Concentrations in standard solution and CZE conditions are as in Fig. 19c, real sample dilution: 1:80 with DI water.

Figure 22. Electropherogram of a standard solution of the 5 anions in DI water. (a) without EME (b) after EME. Anion concentrations: Cl^- – 0.9 mg/L, NO_3^- – 1.5 mg/L, SO_4^{2-} – 2.4 mg/L, ClO_4^- – 2.5 mg/L and NO_2^- – 1.15 mg/L. EME conditions: liquid membrane: 1-heptanol, impregnation time: 10 s, agitation: 750 rpm, extraction voltage: 25 V, extraction time: 5 min, acceptor solution: 1 mM Tris. CZE conditions: BGE solution: 7.5 mM L-His, 40 mM HAc (pH 4.1), voltage: – 30 kV, injection: 50 mbar for (a) 7 sec and (b) 2 sec.

Figure 23. Effect of extraction time on EME of inorganic anions. EME and CZE conditions as for Fig. 22. Injection: 50 mbar for 7 sec. Concentrations of anions: chloride, nitrate, sulfate: 5 mg/L, nitrite, perchlorate: 100 µg/L. Peak areas of chloride and nitrate/sulfate were divided by a factor of 10.

Figure 24. Effect of extraction voltage on EME of inorganic anions. EME and CZE conditions as for Fig. 22. Injection: 50 mbar for 7 sec. Concentrations of anions: chloride, nitrate, sulfate: 5 mg/L, nitrite, perchlorate: 100 µg/L. Peak areas of chloride and nitrate/sulfate were divided by a factor of 10.

Figure 25. EME-CZE-C^4D of a standard solution of 5 mg/L chloride, nitrate, sulfate. (a) without perchlorate and (b) spiked with 15 µg/L of perchlorate. EME and CZE conditions as for Fig. 22. Injection: 50 mbar for 7 sec.

Figure 26. EME-CZE-C^4D of various real samples. (a) snow, (b) surface water, (c) Dobrá voda, (d) Horský pramen; all spiked with 15 µg/L of perchlorate. EME and CZE conditions as in Fig. 22. Injection: 50 mbar for 7 sec.

Figure 27. EME-CZE-C^4D of (a) neat tap water, (b) tap water spiked with 5 µg/L of perchlorate (c) tap water spiked with 15 µg/L of perchlorate, (d) tap water spiked with 50 µg/L of perchlorate. EME and CZE conditions as in Fig. 22. Injection: 50 mbar for 15 sec.

Figure 28. (A) EME-CZE-C^4D of rain water. (a) Neat rain water, (b) rain water spiked with 1 µg/L of perchlorate and (c) rain water spiked with 5 µg/L of perchlorate. (B) EME-CZE-C^4D of bottled water Horský pramen. (a) Neat Horský pramen, (b) Horský pramen spiked with 1 µg/L of perchlorate and (c) Horský pramen spiked with 5 µg/L of perchlorate. EME and CZE conditions as in Fig. 22. Injection: 50 mbar for 15 sec.

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