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On-line electrodialysis–capillary zone electrophoresis–mass spectrometry of inositol phosphates in complex matrices

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Abstract

Electrodialysis has been coupled to capillary zone electrophoresis–mass spectrometry for on-line clean-up and analyte concentration. Two different electrodialysis devices have been developed, each with its specific features. The first device consisting of a donor and an acceptor compartment, separated by a membrane with a cut-off of M_r 30 000, offers selectivity based on molecular mass, shape and charge. The second device consists of three compartments, separated by membranes with a cut-off of M_r 500 and 30 000, respectively. In addition to selectivity this device enables analyte enrichment between the two membranes. The developed methods have been applied to the determination of inositol phosphates in fermentation broth and in blood plasma. © 1997 Elsevier Science B.V.

Keywords: Electrodialysis; Sample handling; Inositol phosphates; Carbohydrates

1. Introduction

For more than a decade a great deal of research has been done in the field of capillary electrophoresis (CE) and interest is still growing. Also, industrial laboratories invest in CE equipment as a complementary technique to high-performance liquid chromatography (HPLC) and gas chromatography (GC). The determination of analytes in real-life samples is complicated and requires a sample pretreatment prior to CE separation. Several methods have been developed for sample clean-up and/or concentration [1,2]. Off-line precipitation methods, (ultra)filtration, centrifugation [3], liquid–liquid extraction (LLE) [4], solid-phase extraction (SPE) and supported liquid membranes (SLM) [5] have been combined with CE. Furthermore, SPE [6,7], SLM [5], (micro)dialysis [8–12], capillary isotachophoresis (cITP) [13] and liquid–liquid electroextraction [14] have been coupled on-line to the CE capillary. Even direct injection of biological fluids, e.g., urine or plasma, into CE capillaries appeared to be possible, combined with micellar electrokinetic chromatography (MEKC) [2].

Inositol phosphates (IPs) are phosphorylated carbohydrates with interesting characteristics, especially in the field of biochemistry. IPs do not have any UV absorbent or fluorescent properties which hamper sensitive detection. Besides, IPs are strongly negatively charged over the whole pH range. Capillary zone electrophoresis (CZE) appeared to be an appropriate separation technique for these compounds [3] and concentration detection limits were improved by using mass spectrometric (MS) detection via an electrospray interface (ESI) [15]. Until recently, IPs in complex matrices have been pretreated using anion-exchange SPE [16], centrifugation [3], ultrafiltration [15] or Fe(III)-loaded stationary phases [17]

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all of which are rather laborious, time consuming or difficult to automate.

Electrodialysis is a sample pretreatment technique which can be used for sample clean-up and analyte enrichment [18]. By superimposing an electric driving force on a concentration gradient the dialysis process is accelerated and the selectivity is enhanced. In general, an electrodialysis set-up contains a donor and an acceptor phase, separated by a membrane over which a voltage is applied. The phases are flowing or stagnant. Electrodialysis has been used in the field of biotechnology [19–21] and for neutralization of alkaline [22–24] or acidic [25] samples prior to separation. Since 1990, several researchers have described an electrodialysis cell, enabling sample purification and analyte concentration, combined with HPLC [18,26]. Recently, we developed an electrodialysis device suitable for the on-line coupling to CE–(indirect) UV absorbance detection [27]. The present paper describes the coupling of electrodialysis to CZE–MS for on-line sample clean-up and analyte enrichment. Two different electrodialysis devices have been developed and are discussed.

2. Experimental

2.1. Chemicals

All inositol phosphates, e.g., inositol monophosphate (2-IP1) as dicyclohexylammonium salt and inositol bis-(1,2-IP2), tris-(1,2,6-IP3), tetrakis-(1,2,5,6-IP4), hexakisphosphate (IP6) as sodium salts, were kindly provided by Perstorp Pharma (Perstorp, Sweden). Adenosine-5′-triphosphate (disodium salt hydrate, 98%) was from Janssen (Beerse, Belgium). Acetic acid and methanol were obtained from Baker (Deventer, Netherlands). Ammonium acetate (analytical-reagent grade) was purchased from Merck (Darmstadt, Germany). For the preparation of the stock solutions of the analytes and buffer solutions, deionized water was used (Milli-Q system, Millipore, Bedford, MA, USA). For the polyacrylamide coating, 3-(trimethoxysilyl)-propylmethacrylate, 98% (Janssen), tetramethylethylenediamine (TEMED) and ammonium persulphate (Bio-Rad, Richmond, CA, USA) and acrylamide (Merck–Schuchardt, Hohenbrunn, Germany) were used.

2.2. Preparation of the samples

A fermentation process was carried out on a laboratory scale. Phytic acid was added to the fermentation broth which contained yeast cells. Samples of 0.5 ml were taken from the mixture.

Blank human blood plasma was obtained from the University Hospital Leiden (Leiden, Netherlands). The plasma was spiked with IP2 and IP3 to a concentration of 10 μM.

2.3. Electrodialysis device

The electrodialysis device (EDD) has been described in detail elsewhere [27] and is shown in Fig. 1. It consists of a donor (5) and an acceptor (2) compartment, separated by a membrane (3) with a cut-off of M_r 30 000 and a diameter of 14 mm (Amicon, Danvers, MA, USA). The donor compartment is filled with sample solution, the acceptor compartment with Milli-Q water (both 0.5 ml). The cathode (6) was positioned in the donor compartment through a septum (7) which prevented leakage of sample fluid. For the same reason, a silicone O-ring (4) was used between the donor compartment and the membrane. During electrodialysis the fused-silica capillary (1) was positioned in the acceptor compartment. For a few seconds a voltage of −27 kV was applied over the EDD and the capillary. After electrodialysis, the fused-silica capillary inlet was placed in a buffer vial containing electrophoresis buffer and the cathode. CZE was performed by applying a voltage of −30 kV at the cathode.

2.4. Concentrating electrodialysis device

The concentrating electrodialysis device (CEDD) consists of three compartments and is depicted in Fig. 2. The three compartments (1, 2, 3) are bores with a diameter of 2 mm in ($2\times2\times2$ cm^3) cubes of perspex. Between compartments 1 and 2 a membrane (4) is positioned with a cut-off of M_r 30 000. Compartments 2 and 3 are separated by a membrane (5) with a cut-off of M_r 500. Both membranes were

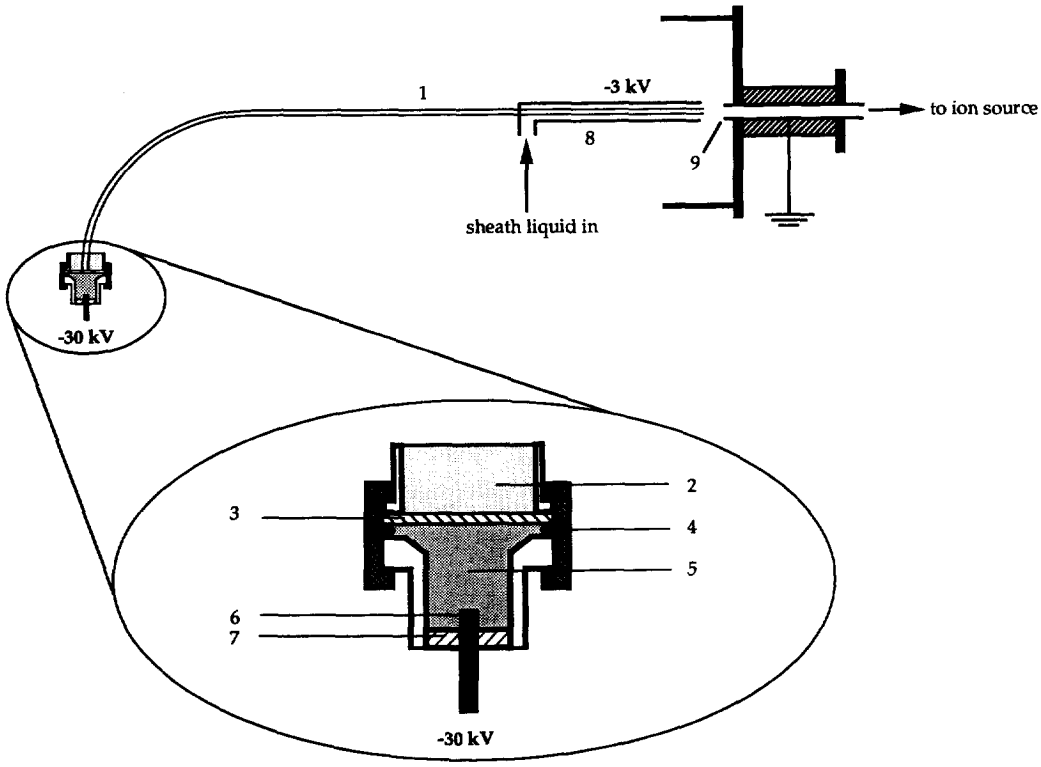

Fig. 1. EDD coupled to CE–ESI-MS system. 1=Fused-silica capillary, 2=acceptor compartment, 3=membrane, 4=O-ring, 5=donor compartment, 6=cathode, 7=septum, 8=stainless-steel needle, 9=sampling capillary.

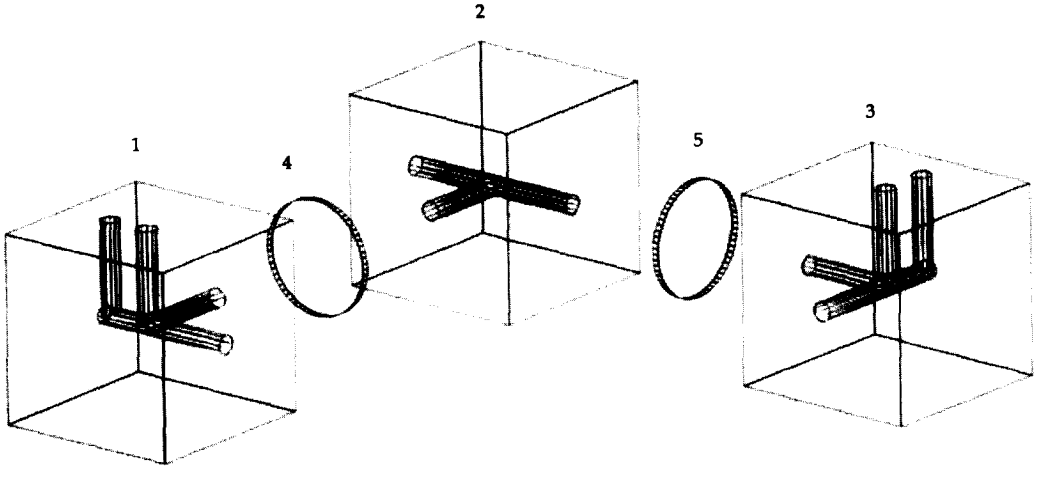

Fig. 2. CEDD. 1, 2, 3=Compartment 1, 2, 3; 4=membrane (cut-off M_r 30 000); 5=membrane (cut-off M_r 500).

made of regenerated cellulose, obtained from Amicon. Before electrodialysis, compartment 1 was filled with sample solution (ca. 50 μl) and compartments 2 and 3 with electrophoresis buffer, consisting of 10 m*M* ammonium acetate pH 4.8. The platinum cathode was placed in compartment 1, the anode in compartment 3. During electrodialysis a voltage of ca. −300 V was applied at the cathode whereas the anode was grounded. After electrodialysis (ca. 10 min) the fused-silica capillary was positioned on the membrane in compartment 2. The cathode was placed in compartment 3 and electrokinetic injection was performed into the fused-silica capillary. Subsequently, the capillary inlet was placed in a buffer vial and CZE was performed at −30 kV. Concentrating electrodialysis–CZE has been combined with UV absorbance detection and with MS.

2.5. Capillary zone electrophoresis–UV absorbance detection

A laboratory-built set-up was used for the CZE–UV absorbance experiments. The high-voltage power supply was from Spellman (1000R, Plainview, NY, USA). The platinum electrodes were positioned in the inlet buffer vial (cathode) and in the outlet buffer vial (anode). The fused-silica capillary came from S.G.E. (Ringwood, Victoria, Australia) (0.8 m×100 μm I.D.) and was coated with polyacrylamide according to the procedure described by Hjertén [28]. At 0.25 m before the capillary outlet UV absorbance detection was performed at 214 nm (CE-adapted-Spectroflow 773, Kratos Analytical Instruments, Ramsey, NJ, USA). The electrophoresis buffer consisted of 10 m*M* ammonium acetate pH 4.8. CZE was performed at −25 kV after electrokinetic injection (−10 kV) or after electrodialysis using the CEDD. For safety reasons, the buffer vial containing the high voltage electrode was positioned in a plexiglass box.

2.6. Capillary zone electrophoresis–electrospray ionization-mass spectrometry

The CZE experiments were performed using a laboratory-built set-up (Fig. 1). The high-voltage power supply was purchased from Spellman. The fused-silica capillaries (S.G.E.) were coated with polyacrylamide [28]. The length of the fused-silica capillaries (75 μm I.D.×190 μm O.D.) was ca. 0.6 m. The electrophoresis buffer consisted of 10 m*M* ammonium acetate pH 4.8. During CZE a voltage of −30 kV was applied to the platinum cathode which was positioned at the capillary inlet. Before each run, the capillary was rinsed with electrophoresis buffer using a syringe at the capillary inlet.

All experiments were carried out on a triple stage quadrupole mass spectrometer (Finnigan MAT TSQ-70, San Jose, CA, USA) equipped with a custom-made ESI [29]. The experiments were done with multiple ion detection (MID) in the negative ionization mode: the electrospray needle (8) was kept at −3 kV with respect to the grounded sampling capillary (9). The sampling capillary and the ion source were kept at 175°C and 150°C, respectively. A slightly negative voltage was applied to the repeller for signal optimization of all ions. The CZE capillary outlet was inserted into the stainless-steel needle assembly, slightly ahead of the needle tip. The sheath liquid consisted of 100 m*M* ammonium acetate–methanol (10:90, v/v) and was delivered at a flow-rate of 1–2 μl/min by a Model 2400 syringe pump (Harvard Apparatus, Edinbridge, UK).

3. Results and discussion

3.1. Electrodialysis–capillary zone electrophoresis–electrospray ionization mass spectrometry

The analysis of real-life samples using CE requires sample clean-up in order to remove interfering compounds. Therefore, sample pretreatment techniques common in liquid chromatography (LC) like protein precipitation, LLE and SPE have been combined with CE. Furthermore, purifying and concentrating techniques have been developed for or adapted to CE [1,2].

Electrodialysis is a sample pretreatment technique which offers selectivity based on molecular mass, shape and charge. For the on-line coupling of electrodialysis to CE a device has been developed which is shown in Fig. 1. In this configuration, the donor and the acceptor phase are stagnant. By applying a voltage over the membrane and the fused-

silica capillary, only ions smaller than the membrane cut-off and with appropriate charge and shape are introduced into the capillary. Neutral compounds, oppositely charged ions and compounds larger than the membrane pores are retained. As electrodialysis with this set-up takes only 10–20 s, molecular diffusion can be neglected.

As inositol phosphates are multiply negatively charged, the electrophoretic mobility in the direction of the anode is quite high. In order to increase the net velocity, the electroosmotic mobility in the direction of the cathode has been suppressed by a polyacrylamide coating at the capillary wall. Therefore, during electrodialysis and during CE the cathode is positioned at the capillary inlet, the anode at the capillary outlet.

First, a sample solution containing inositol phosphates in pure water was analyzed using electrodialysis–CZE–ESI-MS (Fig. 3). The electrodialysis time was 10 s. Inositol mono- (IP1, M_r=260), bis- (IP2, M_r=340), tris- (IP3, M_r=420), tetrakis- (IP4, M_r=500) and hexakisphosphate (IP6, M_r=660) were all present in the sample at a concentration of 10 μ*M*. Inositol pentakisphosphate (IP5, M_r=580) was present in the sample as a degradation product of IP6. Whereas inositol phosphates are multiply charged in aqueous solution (pH 4.8), in the gas phase they were detected as singly charged ions $[M-H]^-$. For some inositol phosphates also the doubly charged ion (IP3, IP4, IP6) and even the triply charged ion (IP6) could be detected (not shown). In Fig. 3 MS detection occurred in the MID mode, including only the predominant ion of each inositol phosphate, i.e., the singly charged ion of IP1–IP5 and the doubly charged ion (m/z=329) of IP6. All inositol phosphates were introduced from

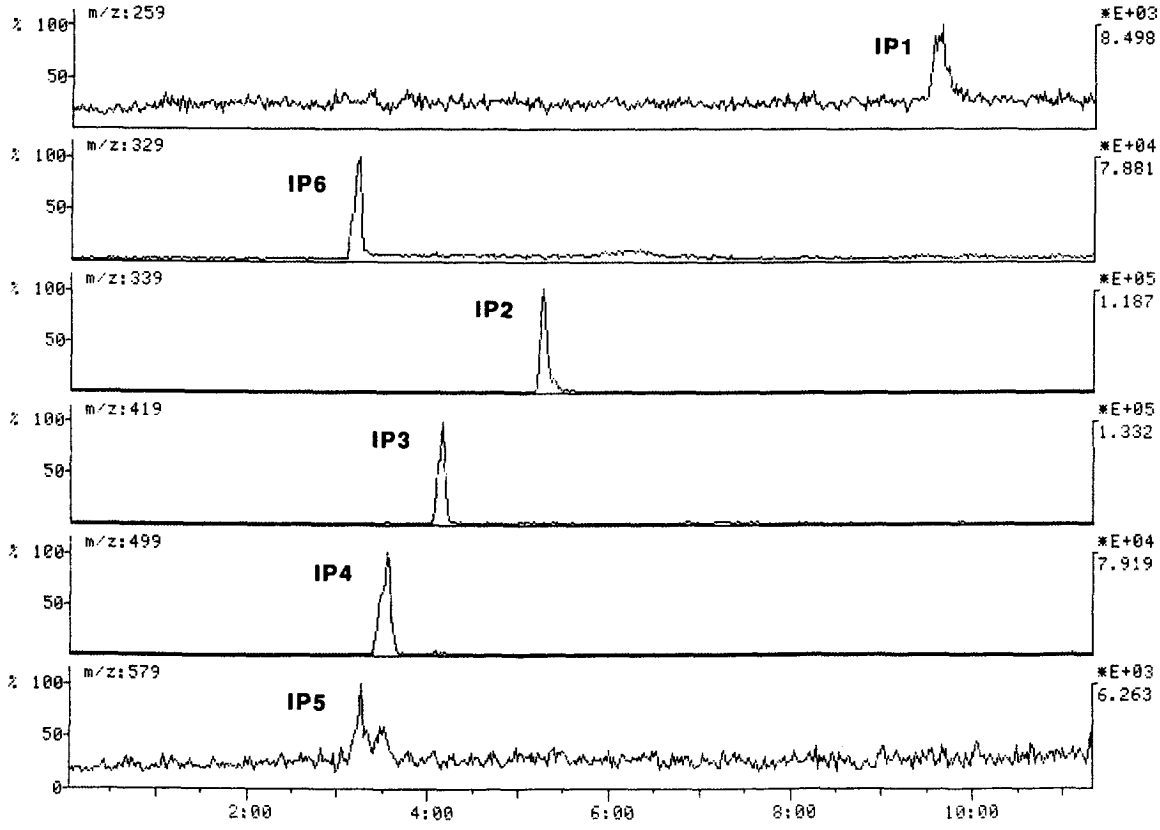

Fig. 3. Mass electropherogram of a standard solution of inositol phosphates (10 μ*M*) after 10 s electrodialysis. MS was performed in the MID mode. For further details see Section 2.6.

the donor compartment through the membrane into the fused-silica capillary. No high-molecular-mass compounds were present in the sample nor any other disturbing compounds. In several respects, electrodialysis is similar to electrokinetic injection [27]. Stacking of the analytes occurs as a consequence of the low-conductivity matrix. The amount of analyte (Q) introduced into the capillary during electrodialysis can be calculated by Eq. (1):

$$Q = \mu_{ep} V \pi R^2 C t / L \quad (1)$$

in which μ_{ep} is the electrophoretic mobility, V the voltage applied over the EDD and the fused-silica capillary, R the capillary radius, C the analyte concentration, t the electrodialysis time and L the total capillary length. Similar to electrokinetic injection, in electrodialysis discrimination of ions occurs in favour of the compounds with a higher μ_{ep}. Thus, the calculated introduced amount of inositol phosphates in Fig. 3 ranges from $4.6 \cdot 10^{-13}$ mol (IP1) to $14.0 \cdot 10^{-13}$ mol (IP6). However, during electrodialysis the high voltage is applied over the EDD and the fused-silica capillary whereas during electrokinetic injection the voltage is applied over the capillary. Therefore, the actual amount of analyte electrodialyzed into the capillary is somewhat lower than the calculated amount. Furthermore, Eq. (1) illustrates a linear relationship between the introduced amount of analyte Q and the electrodialysis time t, as we previously demonstrated experimentally [27]. Consequently, the peak width increases with a longer electrodialysis time which is reflected in the separation resolution and the efficiency of inositol phosphates. In Fig. 4 the separation resolution is plotted versus the electrodialysis time for IP2, IP3, IP4 and IP6. Best resolution was obtained at 5 s of electrodialysis time. At an electrodialysis time shorter than 5 s reproducibility becomes unacceptable. Therefore, a lower electrodialysis voltage could be applied in order to increase the resolution even more, but at the cost of sensitivity [27].

3.2. Fermentation monitoring

Enzymatic hydrolysis of phytic acid was performed in order to produce 1,2,6-IP3. The fermentation broth contained yeast and buffer. Next to the main product several side products were formed, i.e., IP1, IP2, IP4 and IP5. In order to monitor the composition of the fermentation broth analysis is recommended every 30 min. Sample pretreatment before CE is required to prevent clogging of the capillary and/or adsorption of polluting constituents to the wall. Fig. 5 shows the mass electropherogram of IP2 to IP6 in fermentation broth after on-line electrodialysis. Within a few minutes, IP2 to IP6 are electrodialyzed, separated and detected. Although Fig. 5 demonstrates the potential of qualitative analysis the method also allows quantitative fermentation monitoring by adding an internal standard to the fermentation sample. Effects of membrane fouling as well as membrane memory effects were avoided by the use of disposable membranes. The device can be taken to pieces within 30 s, including the replacement of the membrane.

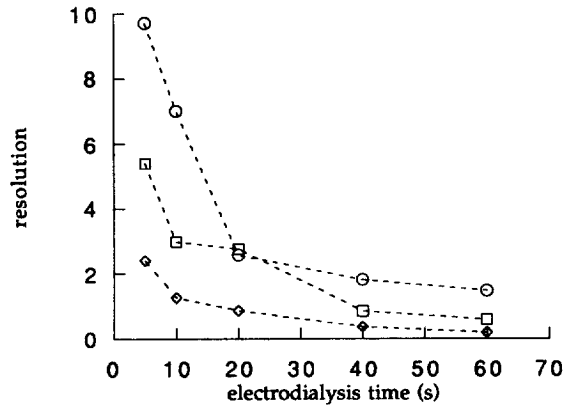

Fig. 4. Separation resolution (R_s) versus electrodialysis time for several inositol phosphates. – ○ – = R_s IP2–IP3, – □ – = R_s IP3–IP4 and – ◇ – = R_s IP4–IP6.

3.3. Concentrating electrodialysis device

The EDD as described above has many advantages as an on-line sample pretreatment technique: it is fast, selective, inexpensive and simple to use. Nevertheless, it lacks concentrating properties. The electrodialysis set-up, described by Debets [18] can be used for analyte enrichment before LC. However, this set-up is not suitable for CZE as it does not selectively concentrate the analytes but also matrix ions which are smaller than the membrane cut-off.

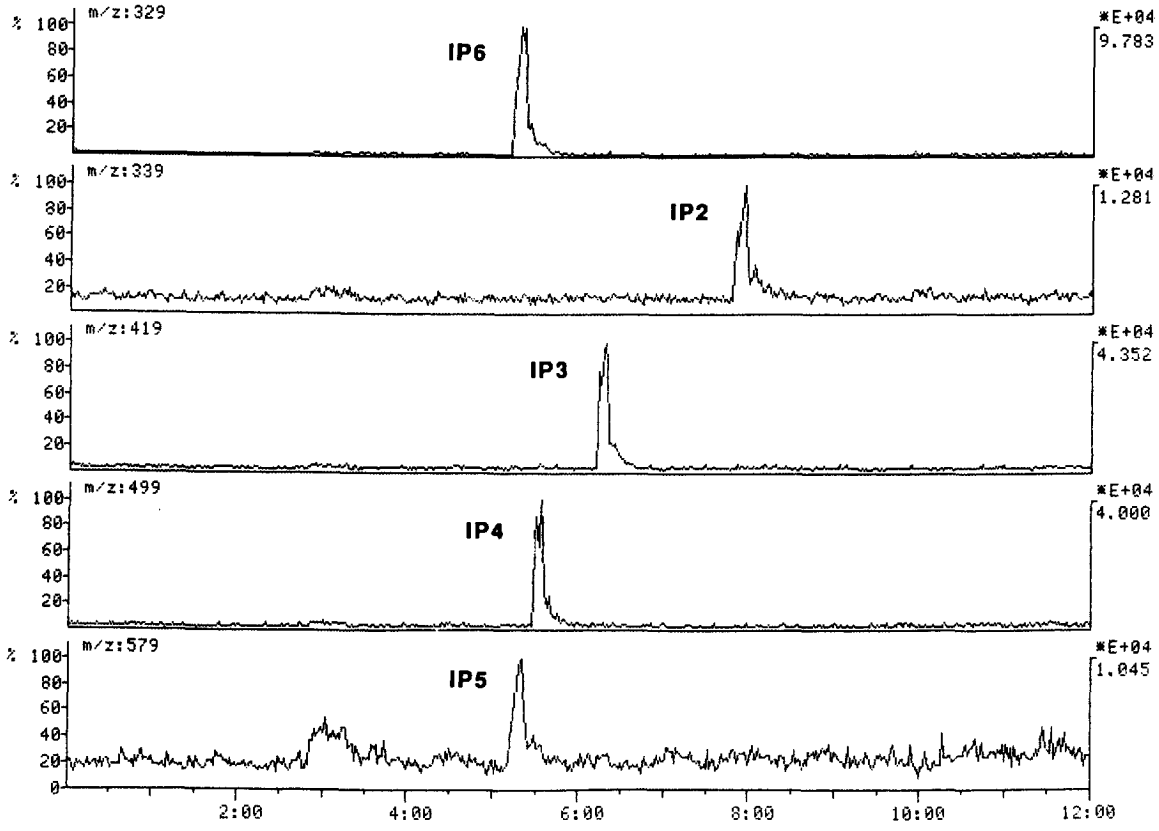

Fig. 5. Fermentation monitoring of inositol phosphates by electrodialysis–CZE–MS. Electrodialysis time: 10 s. MS was performed in the MID mode.

The analysis of real-life samples in CZE is rather problematic because of the high concentration of matrix ions which affect the local electric field strength. As a consequence, a lower amount of analyte is electrokinetically injected and the peak shape may be distorted, leading to higher detection limits. This is an inherent limitation in the analysis of real-life samples with CZE which is not overcome by the EDD described in Section 2.3.

In Fig. 2 an alternative electrodialysis device is depicted which enables selective concentration of the analyte and clean-up at the same time. During electrodialysis high-molecular-mass (>30 000) compounds are retained in the first compartment. By applying a negative voltage, negatively charged compounds with a molecular mass between 500 and 30 000 (analytes) migrate through the first membrane into the second compartment and are retained at the second membrane whereas those with a molecular mass <500 (matrix ions) migrate through the second membrane into the third compartment. Thus, negatively charged analytes can be separated from high- and low-molecular-mass compounds and from positively charged ions. Furthermore, analytes can be selectively concentrated in the second compartment. Diffusion of small neutral compounds through the membrane is dependent on the electrodialysis time.

In order to investigate the concentrating power of the device a standard solution of adenosine triphosphate (ATP) was added to the first compartment. The second and the third compartments were filled with electrophoresis buffer. Fig. 6 shows the electropherograms of ATP in electrophoresis buffer before (left) and after (right) electrodialysis. A concentration factor of about five has been achieved after 10 min of electrodialysis. During this experi-

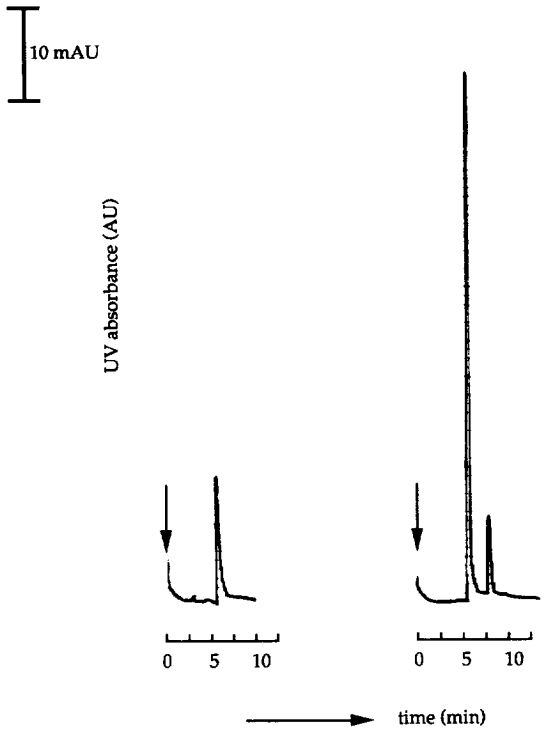

Fig. 6. Comparison between CZE (left) and concentrating electrodialysis–CZE (right) of a solution of ATP in 10 m*M* ammonium acetate. UV absorbance detection at 214 nm. Electrophoresis buffer: 10 m*M* ammonium acetate pH 4.8.

ment, all phases were stagnant. The enrichment factor will be improved by the use of a flowing donor phase.

3.4. EDD versus CEDD

Both the EDD and the CEDD have many advantages but also a few drawbacks. A comparison between the two devices can be made with respect to selectivity, electrodialysis time, enrichment and the minimum required sample volume. The selectivity of the sample pretreatment is, among others, dependent on the cut-off of the membrane(s). As in the CEDD two different membranes are used, a higher selectivity can be achieved than in the EDD. The electrodialysis time is shortest for the EDD, i.e., 10–20 s. Electrodialysis using the CEDD takes about 10 min. By trapping of the analyte on a membrane with very small pore size, enrichment can only be obtained with the CEDD. Furthermore, for the EDD a sample volume of 0.5 ml is required versus a volume of ca. 50 μl for the CEDD. Whether one should use the EDD or the CEDD is dependent on the application.

3.5. Determination of inositol phosphates in blood plasma

The CEDD has been applied to the determination of inositol phosphates in plasma samples. Plasma is a complex matrix which consists of, among other compounds, ca. 70 mg/ml proteins and a high concentration of sodium, chloride, phosphate and sulfate [30]. Before electrodialysis, compartment 1 was filled with plasma spiked with IP2 and IP3 to a concentration of 10 μ*M*. Compartments 2 and 3 were filled with electrophoresis buffer. During electrodialysis proteins such as albumin, with a molecular mass of 67 000 and a p*I* value of 4.7, are retained at the first membrane. Most electrolytes in plasma migrate through the second membrane with a cut-off of M_r 500. Although the protonated molecules of IP2 and IP3 have a molecular mass smaller than the membrane cut-off ($M_{rIP2}=340$; $M_{rIP3}=420$), these IPs are retained at the second membrane, probably due to their shape. The result is shown in Fig. 7. Both IP2 and IP3 have been isolated from the plasma matrix. Detection limits ($S/N=3$) were determined to be 7 μ*M* (IP2) and 4 μ*M* (IP3). Although for bioanalytical purposes the detection limits are not yet completely satisfying the enrichment factor may be improved, among others, by the use of a flowing donor phase.

4. Conclusions

Electrodialysis has been coupled on-line to CZE–ESI-MS for sample clean-up and concentration. Two different electrodialysis devices have been developed and discussed. The two-compartment device is recommended if the analyte concentration is not critical and the sample volume is large enough (>0.5 ml). If, however, the sample volume is limited and/or analyte enrichment is required the three-compartment device should be used. The developed methods have been applied to the analysis of inositol phosphates in fermentation broth and in plasma. Future research

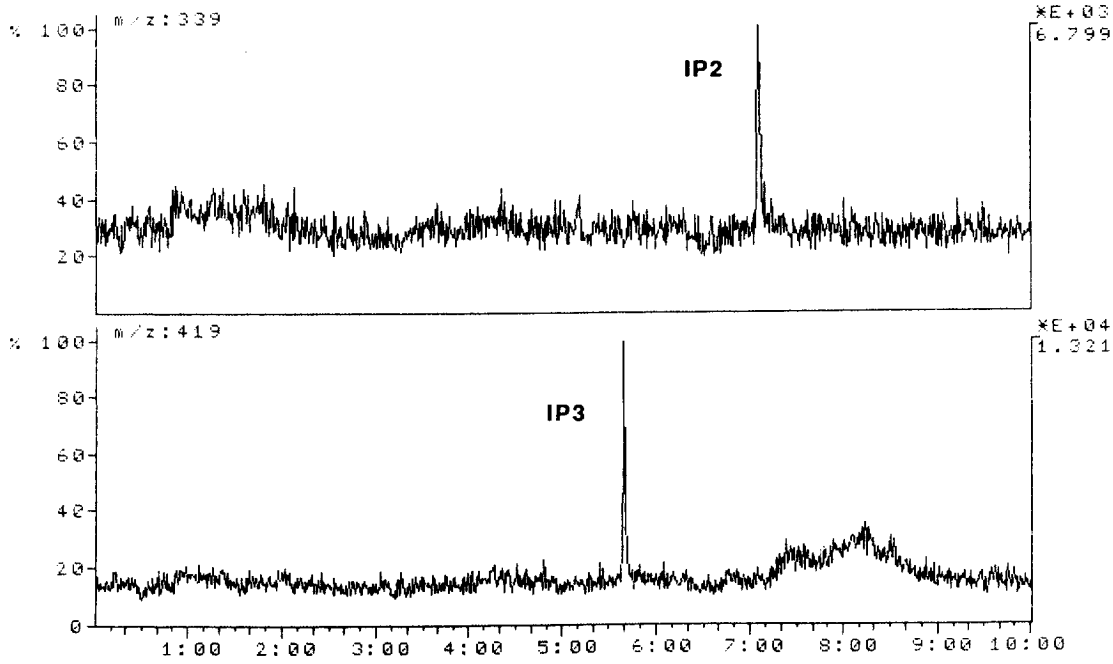

Fig. 7. Mass electropherogram of IP2 and IP3 in plasma after pretreatment with CEDD. MS was performed in the MID mode.

will be devoted to the characterization and optimization of the CEDD.

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