Capillary electromigration strategies in inositol phosphate analysis

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Introduction

1.1. General introduction

Electromigration comprises all techniques in which an electric field is applied to initiate movement of charged particles. Electromigration technology originates from the 19th century and was not used for analytical purposes until the beginning of the 20th century. In analytical chemistry, electromigration can play a role in the analysis of samples containing a large number of (unknown) ions. It may be applied in:

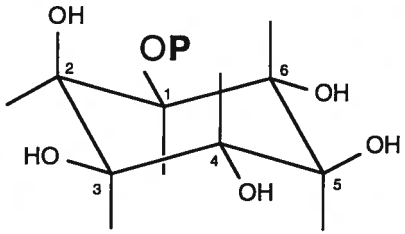
- the analytical separation,
- the sample pretreatment and
- the detection.

Furthermore, electromigration can be carried out to transfer the analytes from the sample pretreatment module to the separation system, e.g. electrokinetic transfer, and/or to the detection device, e.g. electrospray interface. Electromigration has been performed in gels, in free solution, in combination with membranes and even in the gas phase. Since the beginning of the 1980s, there has been a tendency towards the miniaturization of electromigration technologies to the capillary format, *viz.* capillary electrophoresis, enabling the application of extremely high voltages. In addition to speed, the miniaturization permits higher separation efficiencies, the analysis of extremely low sample volumes, i.e. single cell analysis, and the reduction of the waste of (organic) chemicals.

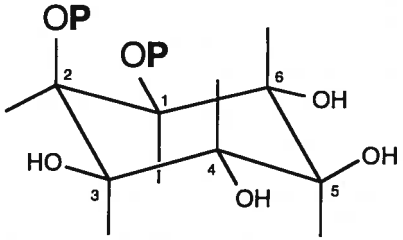
The aim of this Ph.D. study was the development of capillary electromigration technology for analytical purposes. After fifteen years of development, capillary electrophoresis has found widespread application in several research areas. However, analysis in complex matrices and in particular trace analysis may still be rather laborious (off-line) or complicated. Therefore, in this thesis attention is focused on capillary electrophore-

and derivatives have been chosen as model compounds. Inositol phosphates are negatively charged carbohydrates with very interesting characteristics which have been investigated in many research fields, such as biochemistry, pharmacochemistry, agricultural science, etc. Although a great deal of research has been done to discover the exact role of all inositol phosphates, still the investigation is far from finished. Therefore, more selective, more sensitive and faster analytical methods are desired by many researchers.

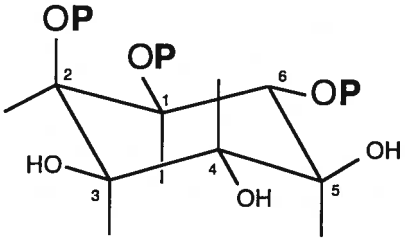
Capillary zone electrophoresis (CZE) (Chapter 1.2) has been combined with indirect UV absorbance detection for fermentation monitoring (Chapter 1.3 and Chapter 2). For structure elucidation and to improve the detection limits, CZE has been coupled to electrospray ionization-mass spectrometry (ESI-MS) (Chapter 1.3 and Chapter 3). Furthermore, CZE separation has been described in combination with very selective (off-line) immobilized metal affinity chromatography (IMAC) for bioanalytical purposes (Chapter 4). The second part of the thesis describes the development of an electromigration-membrane-based sample pretreatment (Chapter 1.4), i.e. electrodialysis, which has been coupled on-line to CZE for sample purification and analyte enrichment (Chapter 5-7). Finally, Chapter 8 discusses the results obtained and the perspectives of the developed methods.



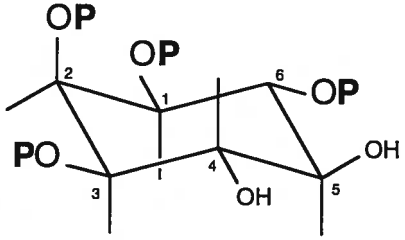
monophosphate (1-IP_1)



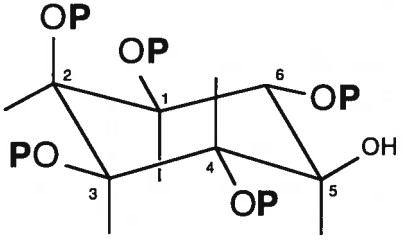
bisphosphate (1,2-IP_2)



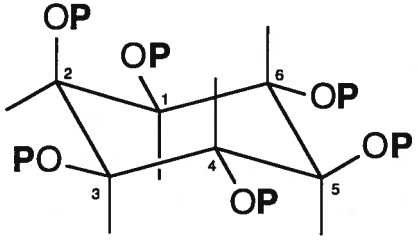
trisphosphate (1,2,6-IP_3)



tetrakisphosphate (1,2,3,6-IP_4)



pentakisphosphate (1,2,3,4,6-IP_5)



hexakisphosphate (IP_6)

Introduction

Electrophoresis is defined as the migration of charged compounds in an electric field. Electrophoresis originates from the 19th century when Kohlrausch (1897) derived the basic equations for electromigration of ions in solution [1]. Not until the beginning of the 20th century it became an analytical technique which was performed in slab gels for, among others, the separation of proteins. In the field of biochemistry slab gel electrophoresis is still a very useful tool.

Hjertén (1967) was the first who described electrophoresis in an open quartz tube of 1-3 mm inner diameter [2]. In 1974, Virtanen and coworkers performed electrophoresis in glass and PTFE capillaries with an inner diameter of 200-500 µm [3]. Capillary electrophoresis was finally introduced by Jorgenson and Lukacs who used glass (1981) [4] and fused-silica (1983) [5] capillaries with an inner diameter of 75 µm. Due to the high resistance in narrow-bore tubes, Joule heating is reduced and the produced heat can be effectively eliminated. Therefore, extremely high voltages, i.e. ca. 30 kV, can be applied between the capillary ends and consequently the analysis times are substantially reduced.

Electrophoresis

When an ion is placed in an electric field the force on the ion can be descibed as

$$F_e = q\,E \qquad (1)$$

where F_e is the electric or accelerating force, q is the charge of the ion, which is equal to the product of the charge number (z_i) and the elemental charge (e_o), and E is the electric field strength (V/m). The retarding frictional force F_f which is caused by the microenvironment of the ion acts on the migrating species in opposite direction according to Stokes law:

$$F_f = 6\,\pi\,\eta\,r\,v \qquad (2)$$

in which η is the dynamic viscosity of the solution and v is the electrophoretic velocity of the ion. The viscosity of a solution drops exponentially with increasing temperature

[1]. The Stokes radius or the hydrodynamic radius r of the ion represents the radius of the hydrated form of the ion [1]. The ion reaches a constant velocity v when the electric force F_e equals the frictional force F_f:

$$v = \frac{q E}{6 \pi \eta r} \tag{3}$$

Particles with a small Stokes radius r and/or a high charge q migrate with the highest velocity. However, in aqueous solution two more forces act on the particle due to the presence of oppositely charged particles, forming an ionic atmosphere. Owing to these so called electrophoretic and relaxation effects the effective velocity of an ion may be decreased [1, 6]. Therefore, the effective electrophoretic mobility μ_{ep} of an ion can be approached by:

$$\mu_{ep} = \frac{Q_{eff}}{6 \pi \eta R} \tag{4}$$

in which the theoretical charge q and the hydrodynamic radius r are replaced by the (smaller) effective charge Q_{eff} and the total radius R, respectively, taking into account the atmosphere of counterions. Each ion has its specific electrophoretic mobility μ_{ep} and can therefore be separated from other ions in an electric field provided that the electric field strength is high enough.

Electroosmosis

At the inner side of the wall fused-silica capillaries contain silanol groups with a pKa value of ca. 2. An electric double layer is formed consisting of tightly adsorbed cations to the negatively charged silanol groups (monomolecular Stern layer) and a diffuse part with an excess of cations (nanometer-layer). In the centre of the capillary the amount of cations is equal to the amount of anions. By applying an electric field the diffuse part of the electric double layer with the excess of cations causes a uniform plug-like flow in the direction of the cathode, which is called the electroosmotic flow (EOF). The elec-

(η) according to:

$$\mu_{eo} = \frac{\varepsilon \zeta}{\eta} \qquad (5)$$

The zeta-potential, which is the electric potential at the interface between the rigid and the diffuse double layer, is affected by the ionic strength and the pH of the electrophoresis medium. The diffuse double layer β is decreased with increasing ionic strength as more negative charges on the surface are balanced by counterions [1]. Furthermore, the EOF can be deliberately reduced or suppressed by coating the capillary wall with a polymer. A variety of static as well as dynamic coating procedures has been described so far [1].

Electrophoresis and electroosmosis

Compounds that are affected by both electrophoresis and electroosmosis, which may take place in opposite directions, have an apparent mobility μ_{app} being the sum of μ_{ep} and μ_{eo}. As all ions in the electric field undergo the same effect of the EOF, capillary electrophoretic separation of analytes is based on the differences in electrophoretic mobilities (μ_{ep}).

Separation efficiency and resolution

In capillary electrophoresis extremely high efficiencies can be obtained up to millions of theoretical plates, mainly due to the plug-like flow profile. In addition, if compared to liquid chromatography in a packed column, the multiple path term or Eddy diffusion in the Van Deemter equation can be eliminated in CE due to the use of open capillaries. Besides, the mass transfer term for equilibration between the mobile and stationary phases can be knocked out. Thus, band broadening is mainly caused by longitudinal diffusion. The efficiency, derived from Einstein's equation ($\sigma^2 = 2Dt$) and the plate height ($H = \sigma^2/x$), can be calculated with:

$$N = \frac{(\mu_{ep} + \mu_{eo})\, V}{2D} \qquad (6)$$

in which N is the number of theoretical plates, V refers to the applied voltage and D to the diffusion coefficient. Best efficiencies can be achieved at a high voltage for compounds having a low diffusion coefficient, e.g. macromolecules. As a consequence of the high efficiencies achieved in CE, the separation resolution R between two analyte peaks is quite high as well. It can be calculated as follows:

$$R = \frac{\sqrt{N}}{4} \frac{\Delta v}{\bar{v}} \tag{7}$$

where Δv is equal to the difference of the velocities of analyte 1 and 2, and $\bar{v}$ is the mean velocity. Equation 8 results from combining Eq. (6) and (7):

$$R = 0.177 \, (\mu_1 - \mu_2) \sqrt{\frac{V}{D \, (\bar{\mu} + \mu_{eo})}} \tag{8}$$

in which μ_1, μ_2 and $\bar{\mu}$ are the electrophoretic mobilities of the two analytes and the mean electrophoretic mobility, respectively. Equation 8 clearly demonstrates that if the EOF has the same direction as the electrophoretic migration the resolution of two peaks decreases with the magnitude of the EOF. Best resolution is achieved if $\bar{\mu}$ is nearly equal to $-\mu_{eo}$ but at the cost of time [1].

Dispersive processes

Ideally, an analyte is introduced into the CE capillary and detected as a sharp zone. However, during electromigration the analyte zone spreads as a function of time caused by longitudinal diffusion ($\sigma^2_{dif} = 2Dt$). Other sources of band broadening that may occur in CE are given below [7, 8]:

$$\sigma^2_{tot} = \sigma^2_{inj} + \sigma^2_{dif} + \sigma^2_{therm} + \sigma^2_{ads} + \sigma^2_{em} + \ldots\ldots \tag{9}$$

in which σ^2_{tot} is the total variance of all sources of band broadening, e.g. injection, diffusion, Joule heating, adsorption and electromigration dispersion, respectively. To minimize band broadening a maximum injection zone length (l) of 1% of the total cap-

analytes to the capillary wall usually causes severe peak tailing. Electromigration dispersion may occur if the mobilities of the background electrolyte and the analyte are mismatched, leading to peak fronting or peak tailing. Electromigration dispersion is most obvious in indirect detection systems (see Chapter 1.3.).

Injection modes

Injection in CE is carried out in the electrokinetic or in the hydrodynamic mode. For hydrodynamic injection the capillary inlet is dipped into a sample solution and a pressure difference is applied over the capillary. The injected volume can be calculated with Eq. 10 [1]:

$$\text{volume} = \frac{\Delta p\, \pi\, r^4\, t}{8\, \eta\, L_t} \qquad (10)$$

where Δp is the pressure difference between the capillary ends, r is the capillary inner radius, t is the injection time and L_t is the total capillary length. The injected sample zone is representative for the composition of the original sample. For electrokinetic injection the capillary is dipped in the sample solution and a voltage is applied between the capillary ends. The amount of analyte (Q) injected is:

$$Q = (\mu_{ep} + \mu_{eo})\, E\, \frac{\kappa_b}{\kappa_s}\, t\, \pi\, r^2\, C \qquad (11)$$

in which E is the electric field strength, κ_b / κ_s is the ratio of conductivities of the buffer and the sample and C is the analyte concentration. As all ions have different electrophoretic mobilities discrimination occurs during electrokinetic injection in favour of the ions with the highest apparent mobility (μ_{app}).

Experimental set-up

Nowadays, a capillary electrophoresis (CE) set-up consists of a fused-silica capillary (C) with an inner diameter of 10-200 µm (Fig. 2). The capillary, filled with a buffer solution, is positioned in two buffer vials (B) containing the anode and the cathode. By applying a high voltage over the capillary ions migrate to the cathode or the anode. Detection is performed at the capillary outlet. Simultaneously, the detection signal is amplified and monitored. For safety reasons, the buffer vial containing the high voltage electrode is positioned in a plexiglass box.

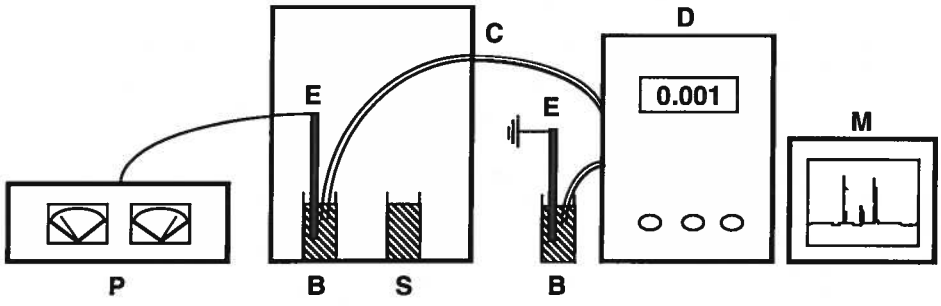


Fig. 2. Experimental set-up for capillary electrophoresis. P = power supply, E = electrode, B = buffer vial, S = sample vial, C = fused-silica capillary, D = detector, M = monitor.

Capillary electrophoretic modes

Capillary electrophoresis (CE) represents a group of electrophoretic techniques, all performed in a narrow-bore capillary. There are six different modes of CE, i.e. capillary zone electrophoresis (CZE), capillary isotachophoresis (CITP), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF) and (pseudo / pressurized) capillary electrochromatography (p)CEC). The differences are mainly based on the composition of the electrophoresis medium. As can be seen in Table I a distinction can be made between continuous buffer systems and discontinuous buffer systems. In continuous buffer systems the composition of the elec-

modes in CE offer different selectivity.

Recently, capillary electrophoresis has been miniaturized to the chip-format [9, 10]. With lithographic techniques borrowed from microelectronics industry micrometer-channels are etched in glass or quartz devices.

Table I. Different modes of capillary electrophoresis.

CE mode	buffer system	principle
CZE	continuous	one background electrolyte
MEKC	continuous	micelles in buffer
CGE	continuous	viscous gel
(p)CEC	continuous	packed capillaries
CITP	discontinuous	leading/terminating ion
CIEF	discontinuous	pH gradient

1.3. Detection in capillary electrophoresis

Introduction

For the coupling to capillary electrophoresis, conventional detectors used in liquid chromatography (LC) have been miniaturized. Besides, several interfaces have been developed to overcome the incompatibility of some detectors in relation to the high voltage capillary electrophoresis system. Quite a number of detection methods, each with its specific characteristics, has been successfully combined with capillary electrophoresis so far (Table II). Very selective detection methods, i.e. laser induced fluorescence (LIF) and radiometric detection, as well as (nearly) universal detection methods, such as conductivity detection and refractive index (RI) detection, have been reported. As shown in Table II the best limit of detection (LOD) can be achieved for radiometric detection. For structure elucidation fluorescence line narrowing (FLN), nuclear magnetic resonance (NMR) spectrometry and mass spectrometry (MS) have been coupled to capillary electrophoresis.

Indirect detection

Not all analytes lend themselves to the conventional detection methods that require specific physicochemical properties. These analytes can be derivatized to enhance their detectability. However, derivatization procedures are often rather laborious and may be quite problematic in trace analysis. Indirect detection methods can be considered an interesting alternative [42, 43]. For indirect UV absorbance detection, a chromophore having the same charge as the analyte of interest is added to the electrophoresis buffer which creates a high background signal. The zone of the analyte is revealed by a decrease of the UV absorbance due to charge displacement of the chromophore (Fig. 3). The charge displacement mechanism that takes place in indirect detection systems combined with CE can be described as [42, 43]:

$$C_{LOD} = \frac{C_c}{DR \times TR} \qquad (12)$$

where C_{LOD} is the concentration limit of detection, C_c is the concentration of the chro-

detection method	LOD (M)	ref.
UV absorbance	10^{-6}	11
laser-induced fluorescence	10^{-8}-10^{-9}	13-16
(pulsed) amperometric detection	10^{-6}-10^{-7}	17-20
potentiometry	5.10^{-8}	21, 22
(suppressed) conductivity	10^{-7}-10^{-8}	23-25
radiometric detection	$0.33.10^{-12}$	26, 27
Raman spectrometry	10^{-3}, 10^{-6}*	28
chemiluminescence	5.10^{-9}	29, 30
flame photometric detection	10^{-3}-10^{-4}	31
inductively coupled plasma OES/MS**	10^{-7}-10^{-9}	32, 35
refractive index detection	10^{-5}	36
fluorescence line narrowing	?	37
nuclear magnetic resonance spectrometry	35.10^{-3}	38-40
mass spectrometry	10^{-6}-10^{-8}***	41

* 10^{-6} after isotachophoretic preconcentration
** OES/MS: optical emission spectrometry/mass spectrometry
*** depending on the type of interface

transfer ratio. By adding a chromophore to the electrophoresis buffer the signal (S) as well as the baseline noise (N) is increased. In general, the resulting decrease of S/N or the dynamic reserve leads to higher detection limits. The transfer ratio (TR) is defined as the number of moles of the chromophore displaced by one mole of analyte ion and can be calculated with [44, 45] :

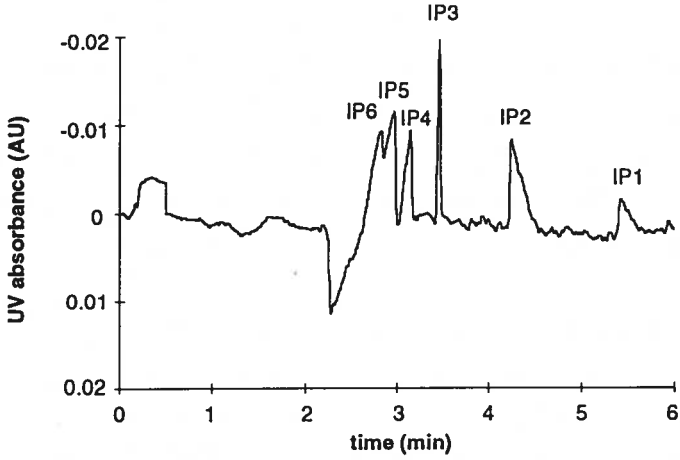


Fig. 3. Electropherogram using indirect UV absorbance detection: the chromophore (1-naphtol-3,6-disulfonic acid) is displaced by the inositol phosphates. The detector polarity was reversed [50].

$$TR = \frac{z_A}{z_B} \cdot \frac{\mu_B}{\mu_A} \cdot \frac{(\mu_A + \mu_C)}{(\mu_B + \mu_C)} \qquad (13),$$

derived from the Kohlrausch regulating function in which z_A and z_B are the charges on the analyte and the chromophore and μ_A, μ_B and μ_C are the electrophoretic mobilities of the analyte, the chromophore and the counterion, respectively. Several researchers [44, 45] have determined the transfer ratios experimentally and compared to the calculated values. Eq. 13 was shown to be valid for simple electrolyte systems that contain two components, namely the chromophore and its corresponding counterion. Best sensitivity can be achieved for analytes having an electrophoretic mobility μ_A close to that of the chromophore μ_B and by maximizing z_A/z_B and the molar absorptivity (ε) of the chromophore.

Due to differences in electrophoretic mobility between the analyte and the background electrolyte, electromigration dispersion often occurs in indirect detection systems where the chromophore concentration C_C is low compared to the relatively high analyte con-

mophore at the pH used. The analytes with a higher (IP4, IP5 and IP6) or lower (IP1, IP2) electrophoretic mobility show peak fronting or peak tailing, respectively. A shift of the symmetrical peak shape towards another inositol phosphate can be induced with the same chromophore by changing the pH of the buffer solution or by choosing another chromophore. Generally, electromigration dispersion is considered to be negligible when the concentration of the solute ions is two orders of magnitude lower than that of the background electrolyte [46].

Indirect UV absorbance detection systems have been developed for the determination of, among others, inorganic cations [47, 48] and anions [48], alkyl sulphates [49], inositol phosphates [50] and fatty acids [51]. Concentration detection limits achieved are in the micro- to millimolar range. Indirect fluorometry [52, 53] or indirect amperometric detection [54] can be performed by adding a fluorophoric or an electroactive compound, respectively, to the electrophoresis buffer.

Mass spectrometry

The high separation efficiency of capillary electrophoresis has been successfully combined with the high selectivity of mass spectrometric detection [41, 55, 56]. Moreover, mass spectrometry enables structure elucidation of unknown compounds. In order to transfer analytes after capillary electrophoretic separation into the mass spectrometer without affecting the separation efficiency, a number of interfaces has been developed to overcome several incompatibilities.

In general, CE is applied to polar analytes in an electrophoresis medium with similar polarity. For the coupling of CE to MS, ions in solution must be transformed into ions in the gas phase [57]. Consequently, the volatility of the electrophoresis medium should be enhanced. Electrophoresis buffers usually used in CE like phosphate, TRIS and borate are not very favourable for CE-MS. Instead, more volatile electrolytes like ammonium acetate, formate and carbonate buffers are used in CE-MS, with the addition of organic modifier. Furthermore, the electroosmotic flow (EOF) in CE is less than 1 µl/min and can be close to zero in coated fused-silica capillaries. Dependent on the type of interface that is used this flow rate must be adapted to higher values.

In CE, fused-silica capillaries are used with an inner diameter of ca. 10-100 µm. As the recommended injection volume is ca. 1 % of the total capillary volume the loadability is rather low (pl-nl) which leads to relatively high concentration detection limits, i.e. ca.

10^{-6} M. Many researchers have tried to improve the sensitivity in CE-MS, either by on-line concentrating techniques [58] or by improving the efficiency of the electrospray ionization or by using alternative types of mass spectrometers, i.e. ion trap MS [59], Fourier transform ion cyclotron resonance (FTICR) MS [60] or a position and time resolved ion counting (PATRIC) detector [61].
In 1987, Olivares et al [62] first described the on-line combination of CE and MS via an electrospray (ESP) interface. Other CE-MS-interfaces that have been successfully used are pneumatically assisted electrospray or ion spray (ISP) [63-65] and continuous-flow fast atom bombardment (CF-FAB) [61]. Desorption methods such as plasma desorption (PD) ionization and matrix assisted laser desorption ionization (MALDI) have been combined with CE after fraction collection of the separated analyte zones [66, 67].

Electrospray / ion spray ionization
A schematic representation of an electrospray interface is shown in Fig. 4. A high voltage (ca. 3 kV) is applied between the CE capillary outlet and an orifice, the sampling capillary, leading to the mass spectrometric system. In the positive ionization mode some positive ions in the liquid will drift toward the liquid surface and some negative ions drift away from it [57]. The accumulated positive charge at the surface leads to destabilization. The surface is drawn out downfield such that a liquid cone forms (Taylor cone). At a sufficiently high electric field E_{onset}, which is among others determined by the surface tension [57] of the liquid, a liquid filament is emitted from the Taylor cone tip. The liquid filament becomes instable at some distance from the cone and produces very small droplets, enriched with positive ions. By solvent evaporation the volume of the droplets is reduced which leads to droplets with a higher charge density. Fission of the small droplets is the result. Gas-phase ion production has also been described as ion evaporation where gas-phase ions are evaporated from the highly charged droplets [41]. The electrospray device can be considered as a special type of electrolytic cell in which the ion transport does not occur through uninterrupted solution but as charged droplets and later as ions in the gas phase [57].
Electrospray ionization is a soft ionization technique, implying minimal fragmentation of the parent ion, even for macromolecular compounds ($M_r > 10^6$) [64]. Multiple charging can be so extensive that ion *m/z* values are always less than about 2500. This finding

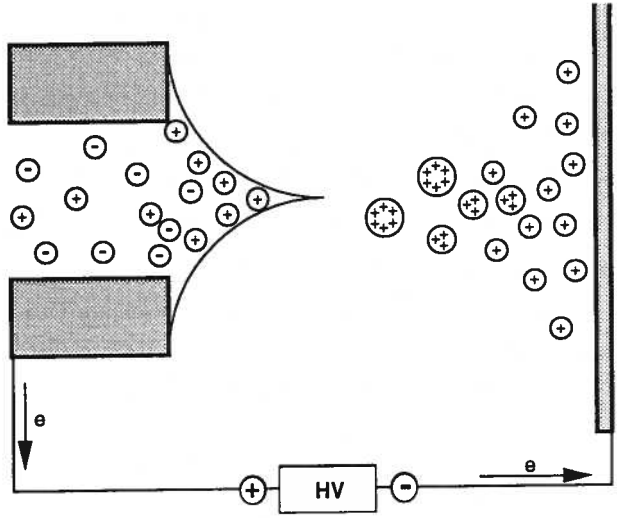


Fig. 4. Schematic representation of the electrospray process [57].

to molecular mass determination [41]. For CE-MS several types of electrospray interfaces have been developed as can be seen in Fig. 5. Fig 5A shows an electrospray interface with a coaxial sheath-liquid [41, 55, 56]. The CE capillary outlet is inserted into the stainless-steel needle, filled with sheath-liquid, to establish electrical contact. For a stable electrospray the EOF, generated in the fused-silica capillary, is not sufficiently high. Therefore, a sheath-flow is used at 1-2 µl/min which consists of ca. 99 % organic modifier, i.e. methanol. To generate a stable electrospray a high voltage is applied at the stainless-steel needle while keeping the sampling capillary at ground potential. Van der Hoeven et al [68] described a custom-made electrospray interface that fits in a thermospray source in order to heat the sampling capillary and the ion source.

Next to the sheath-flow designs, sheathless electrospray configurations have been developed in order to improve the detection limits; the ionic and neutral species, added in the sheath-flow, compete for available charge in the ESI process thus lowering the maximum analyte sensitivity obtainable [41]. To establish electrical contact a thin film of gold (Fig. 5B) is vapor deposited on the outer surface of the fused-silica capillary outlet [69]. The gold-coated capillaries are tapered at the outlet (20 µm i.d.) which allows electrospray operation at flow rates of 20-200 nl/min [70, 71]. Furthermore, electrical contact can be established by inserting a narrow metal (gold) wire (Fig. 5C) into

the capillary outlet [72] or via a microdialysis device [73] (Fig. 5D). With the microdialysis device approximately an order of magnitude improvement in detection limits has been achieved [73].

Mazereeuw et al [74] developed a sheathless microelectrospray interface with a tapered capillary without electrical contact at the capillary outlet. Also with this set-up (Fig. 5E) a stable electrospray could be obtained at a flow rate equal to the EOF generated in the fused-silica capillary. Comparison with a conventional sheath-flow electrospray showed an improvement of sensitivity of approximately 1 order of magnitude with the microspray.

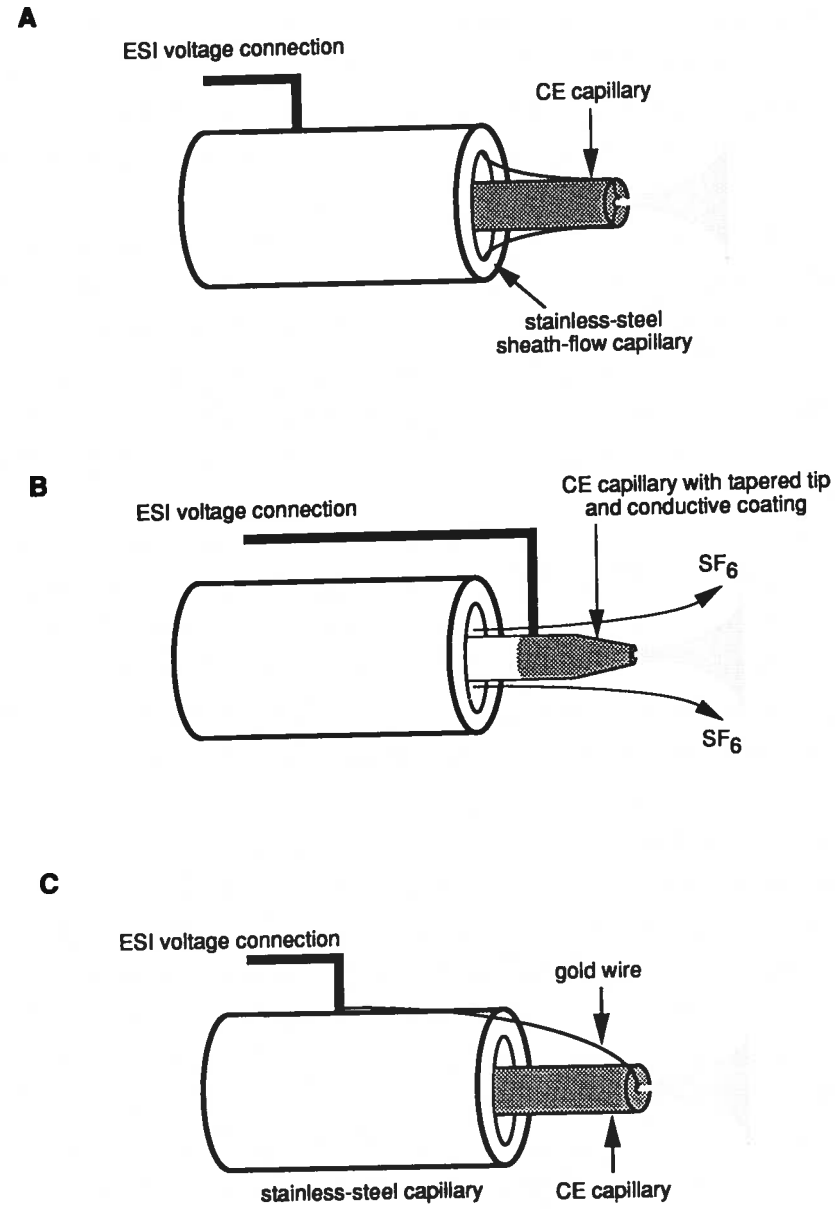
Obviously, there is a trend towards miniaturization of the electrospray interface, also called micro- or nanospray device, because of the higher electrospray efficiencies obtained with low flow rates and small droplet sizes [71]. Recently, several researchers [75-77] developed electrospray interfaces, fabricated on glass microchips. It should be stated that all sheathless ESP interfaces are dependent on the EOF generated in the fused-silica capillary, implying that coated capillaries where the EOF is nearly zero are not compatible.

Besides, pneumatically assisted electrospray or ion spray (ISP) has been used to interface CE and MS [55, 63]. By applying a nebulizing gas ISP enables stable electrospray operation at flow rates up to 1 ml/min. Because of the very efficient desolvation ISP can produce charged droplets and mass spectra at lower voltages than electrospray. Two types of ISP interfaces have been descibed, both using a makeup liquid: a coaxial sheath-flow configuration [78] and a liquid junction design [41].

Continuous-flow fast atom bombardment

Ionization by fast atom bombardment (FAB) is accomplished by directing a beam of highly energetic fast moving neutral atoms (Ar, Xe, Cs) onto a probe tip where the sample, dissolved in glycerol, is deposited [79]. The sample molecules are detached as a dense gas which can be ionized in the plasma just above the sample surface. As continuous-flow fast atom bombardment (CF-FAB) requires a flow-rate of 5 µl/min, the flow-rate in the CE capillary is not sufficient. In order to increase the flow-rate and to establish electrical contact a sheath-flow interface [41] and a liquid junction interface [61] have been used. Thus, the FAB matrix containing glycerol is delivered.

(ca. 10 µm) can be used, which limits the loadability of the system.



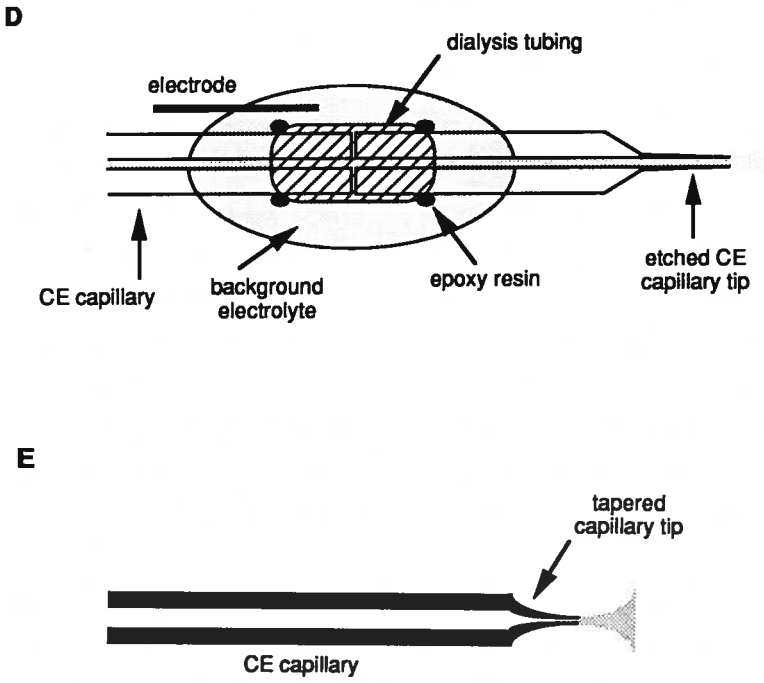


Fig. 5. Electrospray interfaces: A. ESP interface with coaxial sheath-liquid [41], B. sheathless ESP design with gold-coated tapered capillary end [69-71], C. sheathless ESP with narrow metal wire inserted into the capillary outlet [72], D. sheathless ESP with microdialysis device for electrical contact [73] and E. sheathless ESP without electrical contact [74].

Next to the coupling of capillary zone electrophoresis (CZE) to mass spectrometric detection [80], other modes of capillary electrophoresis appeared to be compatible with MS, after some modifications of the experimental set-up. Other CE modes that have been described in literature in combination with MS are capillary isotachophoresis (CITP) [81, 82], CITP-CZE [58], micellar electrokinetic chromatography (MEKC) [83-86], chiral (cyclodextrin-based) capillary electrophoretic separation [87, 88], capillary isoelectric focusing (CIEF) [89-92], (pressurized or pseudo) capillary electrochromatography (p-CEC) [93-95] and capillary gel electrophoresis (CGE) [96].

If analytes must be determined in a complex matrix, sample pretreatment is required prior to capillary electrophoresis (CE) in order to prevent adsorption of sample constituents to the fused-silica capillary wall, to avoid clogging of the CE capillary, to reduce the sample conductivity or to improve the concentration detection limit. Direct injection of a complex sample into the CE capillary without any sample pretreatment is rather rare. In general, the sample matrix affects the resolution as well as the quantification in CE. In MEKC, however, this effect is less dramatic than in CZE as the surfactants in the MEKC buffer solution solubilize proteins [97-99]. Complex matrices can be found in the field of bioanalysis (blood plasma, urine, tissues), food analysis (milk, fruit juices) and in industrial processes (fermentation broth, liquor from pulp and paper industry).

In general, on-line sample pretreatment techniques are preferred over off-line methods. On-line sample pretreatment is less laborious and can be automated more easily. Furthermore, in on-line (automated) methods the reproducibility is usually higher due to a reduced risk of analyte loss. Sample pretreatment techniques that have been combined with CE [100] in the *off-line mode* are dilution [101, 102], (ultra)filtration [103-105], centrifugation [104, 106], liquid-liquid extraction [61], solid phase extraction [107, 108], immobilized metal affinity chromatography [109] and protein precipitation [110, 111]. In the *on-line mode*, (affinity-based) solid phase extraction [112-115], (micro)column liquid chromatography [116, 117], membrane preconcentration [118], supported liquid membranes [119-121], stacking / field amplification [122-125], capillary isotachophoresis [126], (micro)dialysis [127-131], liquid-liquid electroextraction [132, 133] and electrodialysis [134-136] have been described in literature. The on-line sample pretreatment methods can be divided in three categories: I. electromigration-based, II. membrane-based, or III. electromigration-membrane-based sample preparation.

I. electromigration-based sample preparation

Stacking/field amplification

In order to improve the concentration detection limits in CE stacking or field amplification can be performed [1, 122-125]. Stacking / field amplification is based on conductivity differences between the sample and the CE buffer, i.e. a low-conductive sample in comparison to the CE buffer. As a result, the local electric field strength over the sample zone is high during electrokinetic injection and analytes form a sharp zone at the front boundary where they experience a sudden decrease of the local electric field strength in the CE buffer. Chien and Burgi injected a large plug of sample hydrodynamically after which the high voltage was applied. The large water plug was removed from the CE capillary using polarity switching or by dynamically coating the capillary to suppress the EOF [122]. For negative species a 100-fold improvement of the detection limit has been achieved.

Although field amplification cannot be performed in most matrices because of their complexity, in combination with liquid-liquid extraction (LLE) or solid phase extraction (SPE) stacking of the (evaporated and reconstituted) extract is very attractive [61].

Capillary isotachophoresis

Originally, capillary isotachophoresis (CITP) was used as a separation technique, based on differences in electrophoretic mobilities according to the Kohlrausch regulating function [137]. The analyte is sandwiched between a leading ion and a terminating ion with high and low electrophoretic mobility, respectively. During CITP the analyte concentration is adapted to the leading ion concentration. Only cations or anions can be pretreated.

For concentrating purposes, CITP can be coupled to CZE, either in the dual-capillary mode [138] or in the single-capillary mode [61, 139-142]. In the single-capillary mode, CITP-CZE has also been described as a transient process, i.e. transient isotachophoresis [143, 144]. The loadability of the single-capillary set-up is limited to the total volume of the used capillary, corresponding to several microliters [126]. By extending the fused-silica capillary, larger volumes up to 21 µl can be pretreated but at the cost of time (2.5 h) [145]. The effectiveness and speed of ITP as a clean-up step depends on the sample

(plasma) after deproteination [138].

Liquid-liquid electroextraction

Liquid-liquid electroextraction originates from chemical engineering where it has been used to enhance mass transfer through the liquid-liquid interface [132]. With this technique charged compounds can be rapidly extracted from large volumes of organic solvents due to the applied electric field strength.

Liquid-liquid electroextraction has been combined with capillary electrophoresis for analyte enrichment [132, 133]. Electroextraction is based on the conductivity difference between an organic phase, containing the analyte, and the CE buffer. By the application of an extremely high electric field over the organic phase the analyte is electrically extracted from the organic phase into the aqueous phase in the fused-silica capillary. Prior to electroextraction the analyte, which is usually dissolved in an aqueous sample, must be transferred to an organic phase via LLE or SPE. Furthermore, the pH of the organic extract must be adjusted in order to ionize the analyte. After electroextraction, which takes about 10 min, CITP is performed for 1-2 min followed by zone electrophoresis. Liquid-liquid electroextraction is a very powerful concentrating technique. For a 300-µl sample detection limits ranged from 10^{-9} mol/l (salbutamol and terbutaline) to 5.10^{-10} mol/l (neostigmine and propantheline) to 10^{-10} mol/l (crystal violet) using conventional UV absorbance detection [132].

II. membrane-based sample preparation

Membrane preconcentration

Membrane preconcentration-capillary electrophoresis (MPC-CE) is similar to solid phase extraction-CE. However, in MPC-CE the bed volume of the adsorptive phase is reduced which is advantageous with respect to CE performance [118, 146]. At the CE inlet a cartridge is positioned containing a membrane impregnated with a suitable stationary phase. Sample volumes of 1-150 µl can be introduced into the capillary. MPC-CE can be used for analyte preconcentration and for sample clean-up. Interfering matrix constituents are eluted from the phase before the analytes and washed through the CE capillary before electrophoresis. A C_8-silica-based membrane was used for the analysis of proteins and haloperidol metabolites were determined in urine [118].

Supported liquid membranes

Supported liquid membranes (SLM) enable sample clean-up and analyte enrichment [119-121]. The SLM set-up consists of a donor and an acceptor channel, separated by a porous membrane, immersed in organic solvent.

Pálmarsdóttir et al [119, 120] have coupled SLM to CE for bioanalytical purposes. The sample is pumped through the donor channel: the uncharged analyte molecules diffuse through the membrane liquid into the stagnant acceptor phase. Due to a change in pH value the analytes are ionized in the acceptor channel and do not diffuse back into the (organic) membrane. After enrichment the acceptor phase is transferred to the CZE capillary. The method was applied to the determination of (enantiomeric) bambuterol in plasma. SLM pretreatment took ca. 50 min. In combination with a double stacking method SLM gave detection limits in the low nM range.

Kuban et al [121] described the presence of an ion-pairing reagent, methyltrioctylammonium chloride, in the membrane phase in order to apply SLM-CZE to metallo-cyanides in waste solutions or environmental samples. A suitable anion, the perchlorate ion, in the acceptor phase formed a more stable ion-pair with methyltrioctylammonium ion than the metallocyanides, thus liberating the analytes. Preconcentration factors achieved were 50-600 at a preconcentration time of 120 min.

(Micro)dialysis

Dialysis has been coupled to CE for sample purification. The driving force in dialysis is a concentration gradient. A dialysis device consists of a donor and an acceptor compartment, separated by a membrane. Compounds with a molecular mass below the membrane cut-off value diffuse into the acceptor compartment (dialysate); the retentate contains compounds which are larger than the membrane pores. Thus, the selectivity of the technique is determined by the membrane cut-off value.

Dialysis can be performed in the static as well as in the dynamic mode. In the static mode all phases are stagnant whereas in the dynamic mode at least one phase is flowing. Kuban et al [129] described dynamic dialysis-CZE via a rotary injection valve with a donor and acceptor flow rate of 3.0 and 0.5 ml/min, respectively. Other researchers coupled a microdialysis probe to CZE via a microinjection valve [127, 128] or a flow-gated interface [131] using sampling flow rates of 1 µl/min and 79 nl/min, respec-

(micro)dialysis is not the method of choice.

III. electromigration-membrane-based sample preparation

Electrodialysis

Electrodialysis originates from the beginning of this century and became a more popular technique in the forties after the development of stable and selective membranes with low electric resistance [117]. A large variety of membranes is synthesized nowadays, all having specific properties, dependent on structural factors [147]. The organic (polymer) membranes belong to the most important class of materials. The membranes used in the field of electrodialysis can be divided into two groups, namely ion-exchange membranes and porous membranes.

Ion-exchange membranes allow the transfer of either anions or cations. Anion-exchange membranes contain positively charged groups attached to a polymer, for example those derived from quaternary ammonium salts. Cations are repelled from the membrane because of the fixed charge [147]. The opposite accounts for cation-exchange membranes which contain negatively charged groups, e.g. sulfonic or carboxylic acid. Bipolar membranes consist of a cation-exchange membrane, an anion-exchange membrane and an intermediate layer between the two membranes (filled with water).

Porous membranes, also used in (ultra)filtration, are often made of polymers such as polysulfone, cellulose derivatives and polyacrylonitrile [147]. In general, this type of membranes is characterized by the so called cut-off value, defined as the molecular mass which is 90 % rejected by the membrane. Unfortunately, it is not possible to predict the behaviour of an analyte by only one parameter. Other factors also play a role such as the shape and the flexibility of the molecule as well as its interaction with the membrane material. In addition, polarization phenomena and membrane fouling may occur, reducing the analyte flux in time [147].

The principle of electrodialysis is depicted in Fig. 6. Electrically charged membranes are used to remove ions from an aqueous solution. Several cation- and anion-exchange membranes are positioned in alternating pattern between a cathode and an anode. By pumping a feed solution (sodium chloride) through the cell pairs and applying a voltage, the positively charged sodium ions migrate to the cathode, the negatively charged chloride ions migrate to the anode and the neutral compounds are not affected by the

electric field. The sodium ions cannot pass the anion-exchange membranes and the chloride ions cannot pass the cation-exchange membranes. The result is an increase (concentrate) or a decrease (diluate) in the ion concentration in alternating compartments.

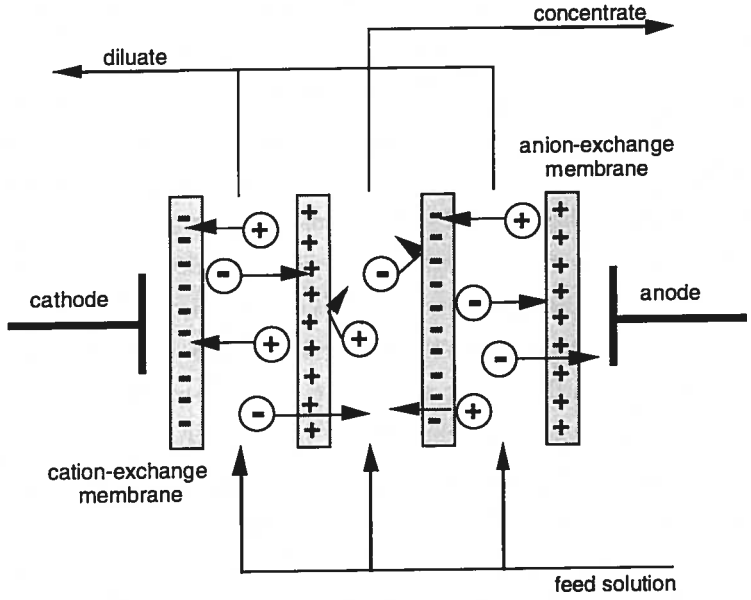


Fig. 6. Principle of electrodialysis [147].

Simultaneously, electrolysis occurs at the electrodes according to:

anode: $2H_2O \rightarrow 4H^+ + 4e + O_2$

cathode: $4H_2O + 4e \rightarrow 4OH^- + 2H_2$

Hydrogen and hydroxyl ions generated at the anode and cathode, respectively, will

the electrode compartments.

For commercial applications the number of cell pairs can be increased up to 20 [148] or more. Electrodialysis has often been used in biotechnological processes for the isolation/purification of organic acids like lactic acid [148], acetic acid [149], succinic acid, amino acid, citric acid and phosphoric acid [150]. Ottosen et al [151] described the electrodialytic remediation of soil which was polluted with copper from wood industry. Furthermore, electrodialysis has been used for the demineralization of surface and ground water [152], the generation of sodium hydroxide and hydrochloric acid from sodium chloride [153], neutralization of acidic [154] or alkaline [155, 156] samples and for the enrichment of copper, lithium and sodium ions [157] or vinegar [158]. Electrodialysis has been performed in the constant current [154, 156, 159] or in the constant voltage mode [148, 157]. Milliliters to liters of sample can be electrodialyzed per minute. The separation principle in this type of electrodialysis is based on the Donnan exclusion mechanism [147] due to the presence of non-porous ion-exchange membranes. After electrodialysis **off-line** analysis of the effluent (acceptor phase) takes place using titration [150], gas chromatography [149], ion chromatography [156, 159], liquid chromatography [148, 158] or atomic absorbance spectrophotometry [151].

Not until 1990, electrodialysis was coupled **on-line** to liquid chromatography (LC) [117, 160] and to ion-chromatography [161]. The electrodialysis cell developed by Haddad et al [161] is depicted in Fig. 7. It was constructed as a series of perspex blocks held together with screws to form a three-compartment cell separated by cation-exchange membranes. The electrodes were inserted into the electrode compartments. The sample compartment contained 300 µl. The device was used to neutralize alkaline samples which were pumped through the donor compartment. The outlet of the sample compartment was connected to a switching valve for direct injection of the neutralized sample into the ion chromatography system. A NaOH solution of 1 M could be neutralized in 3 min.

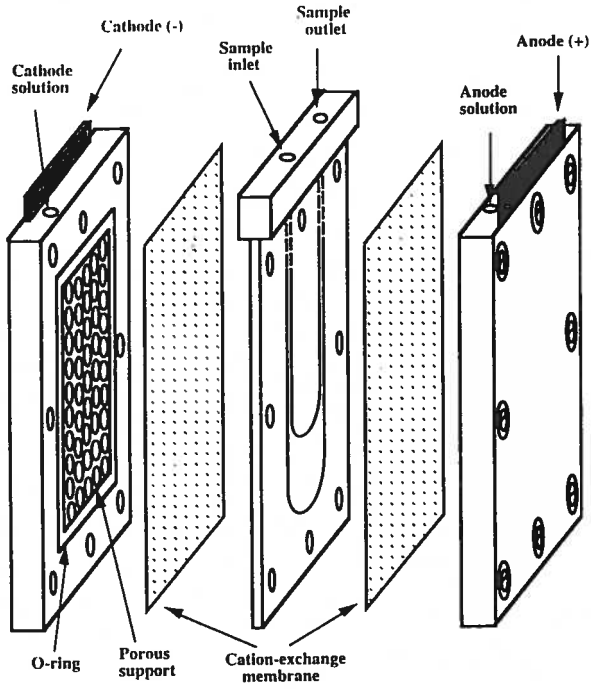


Fig. 7. Flow-through electrodialysis device for neutralization of strongly alkaline samples coupled on-line to ion-chromatography [161].

The electrodialytic unit coupled to the LC system is shown in Fig. 8 [117, 160]. It contains two ion-exchange membranes (3), to shield the electrodes and prevent electrochemical degradation of the analytes, and a molecular mass cut-off (1, 3.5, 15 kDa) membrane (5). The device consists of four compartments: a donor compartment (50 µl), an acceptor compartment (50 µl) and two electrode compartments (1). Electrodialysis was performed in the dynamic mode; the donor phase was flowing whereas the acceptor phase was stagnant. A potential of 7.5-10 V was applied. After electrodialysis the acceptor phase was transferred to the LC system via a switching valve. Debets et al [117] obtained a ten- to twenty-fold enrichment of ephedrine (in blood plasma) within 20 min of electrodialysis time whereas Groenewegen et al [160] achieved a 7-10-fold enrichment of anilines and chlorinated phenoxy acids from surface water samples within 15 min.

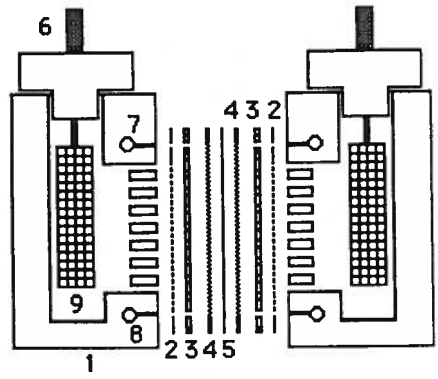


Fig. 8. Electrodialysis module for sample purification and analyte enrichment coupled on-line tc liquid chromatography [117, 160]. 1 = electrode vessel, 2 = PTFE spacer, 3 = ion-exchange membrane, 4 = PTFE spacer, 5 = cellulose acetate membrane, 6 = electrode connection, 7 = flow inlet, 8 = flow outlet, 9= platinum electrode.

In 1997, the on-line coupling of electrodialysis to capillary electrophoresis (CE) was first described by Buscher et al [134-136]. The aim of electrodialysis prior to CE was sample purification and selective analyte enrichment. Some of the electrodialysis devices described before allow analyte enrichment together with the background ions For the combination with CE, however, this is not very favourable as an increased sample conductivity would deteriorate the analyte enrichment. Besides, the maximum enrichment factor obtained with electrodialysis was only 20 [117]. The experimental set-up for the coupling of electrodialysis to CE has been modified drastically [Chapters 5-7].

5. Analysis of inositol phosphates and derivatives

tereoisomerism and nomenclature

here are nine possible stereoisomeric forms of hexahydroxycyclohexane, commonly alled inositols, of which some occur naturally and others have been synthesized [162]. he isomer which is most widely distributed in nature has been called myo-inositol, nce it was isolated from muscle (Greek μυσ, μυοs). The phosphorylated myo-inosi-ols have been investigated in this study. Their structure is shown in Fig. 1.

unction and physicochemical characteristics

iositol phosphates are very important compounds in several research areas. In the eld of biochemistry 1,4,5-inositol trisphosphate (IP3) is well-known as second mes-enger in signal transduction processes [163-165]. The same accounts for inositol mono-hosphate (IP1) and inositol bisphosphate (IP2). The 1,2,6-IP3 isomer has been under ivestigation because of interesting pharmacological characteristics. Phytic acid (IP6) found in grains and seeds and has been studied extensively in agricultural and food cience [166]. Although a lot of research has been done to discover the exact role of iositol phosphates, still the investigation is far from finished. Therefore, more effi-ent, selective, sensitive and faster analytical methods are desired by many research-rs.

s can be seen from Fig. 1 inositol phosphates are very polar carbohydrates with one to x phosphate groups. Even at low pH inositol phosphates are negatively charged. With espect to the detection characteristics, no chromophoric or fluorophoric groups are resent in the molecular structure. The low reactivity of the hydroxyl groups limits the pplicability of derivatization reactions for fluorescence detection. Although pulsed mperometric detection of inositol can be performed at high pH electrochemical detec-on of inositol phosphates is not very favourable as a result of sterical hindrance by the hosphate groups. Inositol phosphates have very strong metal-complexing properties. omplexes of inositol phosphates with Fe^{3+}, Al^{3+}, Zn^{2+} and Ca^{2+} ions are well-known [67-169].

eparation and detection of inositol phosphates

spectrometry (GC-EI-MS) of inositol phosphates was carried out after pertinently silylation [170]. Besides, mass spectrometric detection of inositol phosphates has bee performed with continuous-flow fast atom bombardment (CF-FAB) [170]. In GC-E MS the detection limit of inositol-1-phosphate (IP1) was 0.1 pmol, whereas in CF-FA detection limits achieved were about 10 nmol for inositol-1,4-bisphosphate (IP2) an inositol-1,4,5-trisphosphate (IP3).

Nuclear magnetic resonance (^{31}P-NMR) of inositol phosphates has been described b Mernissi-Arifi and coworkers [171] whereas Johnson et al [172] focused their attentic on 2D-^{31}P-NMR.

Inositol-2-phosphate has been analyzed with high performance liquid chromatogr phy, using reversed phase material (C-8), combined with fluorometric detection aft derivatization with isatoic anhydride [173]. Furthermore, anion-exchange chromato raphy of inositol phosphates has been combined with chemically suppressed condu tivity detection [174], radiometric detection [175], post-column reaction detection, base on enzymatic hydrolysis of the phosphate esters and detection of the inorganic pho phate formed [176], post-column colorimetric detection [177] and amperometric dete tion of hexacyanoferrate(III) after enzymatic degradation of inositol phosphate usin an immobilized enzyme reactor [178]. Ion-pair chromatography of inositol phosphat has been combined with refractive index detection [179] and fluorometric detectic after complexation [167] which improved the detection limit down to ca. 10^{-8} M.

In 1992, Henshall et al [180] published the first paper on capillary zone electrophoresi indirect UV absorbance detection of inositol phosphates. Using phthalate and chr mate as chromophoric ions inositol mono-, bis-, tris- and hexakisphosphate were sep rated and detected. However, for routine analysis the method was said to be questio able because of the day-to-day variability. All inositol phosphates, IP1 to IP6, could b separated in less than 6 min using CZE-indirect UV detection with 1-naphtol-3, disulfonic acid as the chromophore [50]. The inter- and intra-day reproducibility of th method appeared to be sufficient for fermentation monitoring [Chapter 2]. In 1995, th coupling of CZE with electrospray ionization-mass spectrometry was described for th analysis of inositol phosphates and derivatives [80, Chapter 3]. Submicromolar dete tion limits could be obtained for IP2 and IP3. In 1994, Blatny and coworkers describe the analysis of inositol phosphates using capillary isotachophoresis and conductivit detection [181].

Sample pretreatment of inositol phosphates

As shown in Table III several sample pretreatment techniques have been applied for the determination of inositol phosphates in complex matrices. After purification, spectrophotometric [182, 183] or radiometric detection [184] was carried out or the samples were analyzed using ion-pair chromatography [185], capillary electrophoresis [50, 80, 108, 134-136] or capillary isotachophoresis [181].

Table. III. Sample pretreatment methods for inositol phosphates in various matrices.

sample pretreatment method	**matrix**	**ref.**
acid extraction	bean meal, soy bean	182
anion-exchange chromatography	bean meal	183
dialysis	cell lysates	184
microdialysis	fermentation mixture	185
dilution	enzymic hydrolysis mixture	181
centrifugation	fermentation broth	50
ultrafiltration	blood plasma	80
IMAC*	blood plasma	108
electrodialysis	fermentation broth, blood plasma	134-136

* immobilized metal affinity chromatography using Fe(III)-loaded adsorbents

Expected concentration levels

For pharmacokinetic and toxicologic research, bioanalysis of inositol phosphates must be performed. After administration of 1,2,6-IP3, the expected concentration levels of

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Chapter 2

Capillary zone electrophoresis-indirect UV detection

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Abstract

The potential of capillary zone electrophoresis for the fast monitoring of a fermentation process in which inositol phosphates are enzymatically hydrolyzed has been investigated. The developed analysis consists of capillary zone electrophoresis combined with indirect UV detection, using 1-naphtol-3,6-disulfonic acid as the chromophore. The total analysis of all six inositol phosphates covering a concentration range of 0-500 μM takes only 13 minutes.

Introduction

Specific inositol phosphates, e.g. 1,4,5-inositol trisphosphate, play an important role as a second messenger in signal transduction in the body [1]. Others, such as phytic acid, are found in grains and seeds [2] whereas specific isomers of inositol trisphosphate show several interesting pharmacological properties [3]. Because of their physicochemical characteristics, fast analysis of inositol phosphates has been a problem for many years. First, inositol phosphates are, depending on the number of phosphate groups, multiply negatively charged, even at low pH values. Second, because of the absence of chromophoric or fluorophoric groups in the molecule sensitive detection is rather complicated. For the separation of the compounds ion-pair [4,5] and ion-exchange chromatography [6,7] have been applied, as well as gas chromatography after derivatization of myo-inositol formed after enzymatic hydrolysis [8]. However, these separation methods are rather time consuming, caused by the equilibration times in ion chromatography or by the need of laborious derivatization procedures in the case of gas chromatography. Detection methods applied include refractive index detection [9], radiometric detection [10], colorimetric detection [11], fluorometry after complexation [3] and after derivatization [12], mass spectrometry [13], post-column reaction detection, based on enzymatic hydrolysis of the phosphate esters and detection of the inorganic phosphate formed [14], electrochemical detection of NADH after enzymatic oxidation of inositol

UV detection should be an attractive technique. Heinish et al [17] already described this method for IP1 (1- and 2-isomer), IP2, IP3 and IP6. However, IP4 and IP5 were not included and for a routine analysis the method was said to be questionable because of the day-to-day variation in migration time. Capillary zone electrophoresis, which is based on the charge and size of the molecules, appeared to be appropriate for the fast separation of the multiply charged inositol phosphates. It reduces the long analysis times as obtained with chromatographic systems while the loss of material due to adsorption onto the chromatographic support material is avoided.

Indirect detection methods [18] based on either UV, fluorescence or amperometric detection offer the advantage that no derivatization of the compounds is needed. Either a chromophore [19-21], a fluorophore [22-24] or an electrochemically active substance [25] is added to the buffer thus creating a constant, large background signal. Similar mobilities of the buffer constituent and the analyte are of major importance for the resulting peak shapes. The analyte signal is derived from the signal of the buffer constituent through displacement of the electrolyte by the analyte. A severe disadvantage of indirect detection is the increased noise level by the addition of the chromophore with its detection characteristics which leads to increased detection limits.

The present paper describes the determination of myo-inositol phosphates in fermentation broth with CZE and indirect UV detection using 1-naphtol-3,6-disulfonic acid as chromophore. The only clean-up step required consisted of centrifugation of the fermentation sample, the supernatant being directly injected in the CZE system.

Experimental

Chemicals

1-Naphtol-3,6-disulfonic acid (NDSA) was obtained from Janssen (Beerse, Belgium). Acetic acid p.a. was purchased from J. T. Baker (Deventer, The Netherlands). Both inositol monophosphate (2-IP1), as dicyclohexylammonium salt, and hydroxypropylmethylcellulose (HPMC), with a viscosity of 4000 cP for a 2% aqueous HPMC solution, came from Sigma (St. Louis, MO, USA). Inositol bis- (1,2-IP2), tris- (1,2,6-IP3), tetrakis- (1,2,5,6-IP4), pentakis- (1,2,4,5,6-IP5) and hexakisphosphate (IP6) were supplied as sodium salts by Perstorp Pharma (Perstorp, Sweden). For the preparation of the stock solutions of analytes and buffer solutions, deionized water was used

(Milli-Q system, Millipore, Bedford, MA, USA). Calibration curves were generated by spiked fermentation buffer with different concentrations of inositol phosphates. The buffer solution was filtered through a 0.2-µm Nylon acrodisc syringe filter (Gelman Sciences, Ann Arbor, MI, USA).

Electrophoresis

The experiments were performed on a P/ACE 2200 system (Beckman Instruments, Fullerton, USA), including a liquid thermostated (24 °C) capillary and a UV detector. The electrophoresis medium was prepared freshly every day and consisted of 0.5 mM NDSA, 30 mM acetic acid and 0.01 % HPMC to suppress the electroosmotic flow. The applied voltage was -30 kV generating a current of about 10 µA. Detection was performed at 214 nm with a data sampling rate of 5 Hz and a time constant of 0.5 s. For data collection and handling System Gold software, version 7.12 (Beckman) was used. This software did not integrate the large negative peak preceding the IP6 peak; integration was set to start at the IP6 peak base. Untreated fused-silica capillaries (75 µm I. D.) from S.G.E. (Ringwood, Victoria, Australia) with a total length of 0.57 m (0.50 m to the detector) were used. New capillaries were rinsed with deionized water and electrophoresis medium, each for 2 min. Before each injection the capillary was rinsed with electrophoresis medium for 2 min. Pressurized injection during 3 s, which corresponded to 34 nl, was applied.

Sample pretreatment

Samples taken from the fermentation broth containing yeast, buffer and inositol phosphates, were centrifuged for 5 min in an Eppendorf centrifuge 5415 (Eppendorf Geraetebau, Netheler & Hinz GmbH, Hamburg, Germany) at 11,000 rpm (4000 g). The supernatant was introduced into the capillary after 1:1 dilution with fermentation buffer.

Results and Discussion

Electrophoresis

Inositol phosphates have in general high electrophoretic mobilities due to the multiple charges of the phosphate groups. When electrophoresis takes place at pH values that [illegible] (EOF) under these condi-

low. As a consequence, the peaks are not sufficiently separated anymore to quantitate all peaks. Because the phosphate groups are negatively charged, this implies that without electroosmotic flow the analytes migrate in the direction of the anode. Since the electrophoretic velocity is the resultant of the electrophoretic mobility and the electroosmotic mobility the net result at the applied pH is rather low electrophoretic velocities leading to unacceptably long analysis times. Therefore, it has been decided to reverse the polarity of the system (negative inlet electrode, grounded outlet electrode). In that case it is necessary to suppress the electroosmotic flow as much as possible, which is realized by modifying the electrophoresis buffer. Although the stability of dynamically coated capillary walls is not as favourable as untreated capillaries, we obtained quite accceptable systems using HPMC which is demonstrated by the validation figures.

Due to the many phosphate groups of the inositol phosphates, the pH of the electrophoresis medium is a very critical parameter in the separation of inositol phosphates: 0.2 pH units deviation already induced considerable changes in the migration time. Thirty millimolar of acetic acid (pH 3.0) appeared to be adequate for this purpose. Migration times and relative standard deviations (R.S.D.) of all six inositol phosphates are shown in Table I. These are mean values from 15 measurements over the whole concentration range. The R.S.D. for all the compounds is less than 2.6 % allowing reliable peak

Table I. Migration times and relative standard deviation (R.S.D.) of the inositol phosphates.

compound	migration time (s)	R.S.D. (%)
IP1	315.5	0.4
IP2	240.2	1.6
IP3	205.3	0.9
IP4	187.0	2.0
IP5	176.7	2.5
IP6	166.4	1.4

identification. Because the effect of electrodispersion is more pronounced at higher concentrations, the migration times of the compounds that show fronting (IP4, IP5, IP6) tend to be slightly higher at increased concentrations, whereas the migration times of the compounds that show tailing (IP2, IP1) are reduced at higher concentrations.

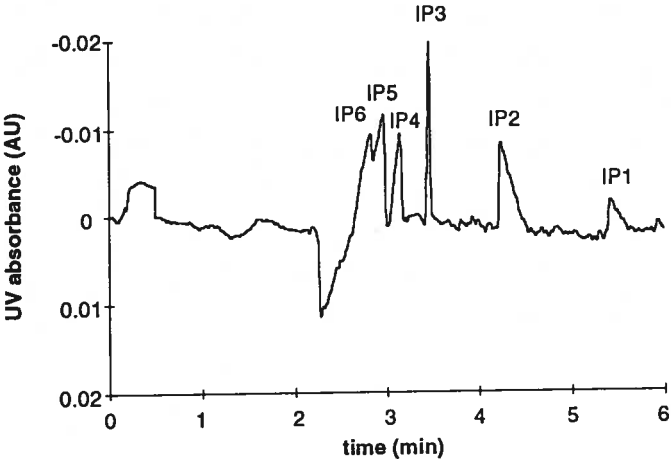


Fig. 1. Electropherogram of a standard mixture of all six inositol phosphates (1 = 340 μM IP6, 2 = 320 μM IP5, 3 = 160 μM IP4, 4 = 170 μM IP3, 5 = 220 μM IP2, 6 = 500 μM IP1). Conditions: applied voltage: -30 kV, current: 10 μA, λ = 214 nm, FS capillary: 75 μm i.d., electrophoresis buffer: 0.5 mM NDSA, 30 mM acetic acid, 0.01% HPMC.

Detection

With respect to indirect detection, NDSA has been chosen as the chromophore because its electrophoretic mobility matches closely with that of the most important analyte, IP3. The optimal matching has been obtained by adjustment of the pH of the electrophoresis buffer. As a consequence of the high optical background the noise of the baseline is considerably increased in comparison with direct UV detection. Fig. 1 presents the electropherogram of the inositol phosphates having analysis times of less than 6 min. Under these conditions the isomers are not separated. Although IP5 and IP6 are not

leads to fronting peak shapes. On the other hand, IP1 and IP2 have lower electrophoretic mobilities resulting in tailing peaks. This asymmetry of the peaks is caused by electrodispersion, deriving from local differences in conductivity and consequently differences in the local electric field strengths. This effect is more pronounced at concentrations of the analytes that are high in comparison with the chromophore [21]. IP3 is the only compound with a symmetrical peak shape. By adjusting the mobility of the chromophore to that of the analyte, electrodispersion of that particular compound is suppressed and the efficiency is improved [20]. In that way the limit of determination (LOD) of the analyte can be improved. Therefore, the LOD (defined as 10 times the noise) appeared to be lowest for IP3 (3.9 μM). The LODs for IP4 and IP6 amounted to 9.2 and 22.5 μM, respectively, applying an injection volume of 34 nl.
In a fermentation process, however, the concentrations of the main compounds of interest are in the 50-1000 μM range. For that reason the LOD of the compounds is not critical.

Quantitative aspects

Quantitative aspects have been examined by generating calibration curves for the compounds of interest. Therefore, fermentation buffer was spiked with concentrations of inositol phosphates up to 1 mM. Calibration curves for the inositol phosphates are linear in the 0-500 μM range. For concentrations above 500 μM being the concentration of the chromophore the peak area of the inositol phosphates does not increase linearly. By increasing the chromophore concentration, higher inositol phosphate concentrations can be determined. Unfortunately, the linear dynamic range only shifts to higher concentrations but is not expanded, while the LOD is still increased. Calibration plots for the inositol phosphates were thus made from 0-500 μM. The calibration plots of IP1 and IP6 have the lowest correlation coefficients of 0.993 and 0.986, respectively, which is caused by the least symmetric peak shapes. The correlation coefficients for the other inositol phosphates were higher: 0.998 (IP2), 0.996 (IP3), 0.997 (IP4) and 0.995 (IP5).
The developed analysis has been validated for the most important analytes in the fermentation mixture, IP2, IP3, IP4 and IP6. The intra-day and inter-day variability, expressed as imprecision (R.S.D., %), have been examined for different concentrations of inositol phosphates (Table II). The intra-day variability did not exceed 19.8 % (IP6), whereas the highest inter-day variability amounted to 7.8 % (IP6). As can be seen from

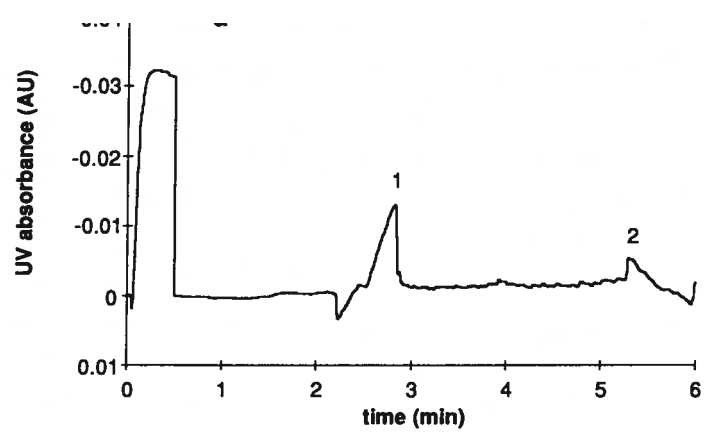
Table II, the developed method shows a good reproducibility. Although the intra-day variability for IP6 is relatively high, its value is accceptable for this application. Nevertheless, it indicates that every day a new calibration curve for the different analytes has to be constructed. The analytes for the calibration curves are dissolved in the fermentation buffer in order to simulate the real samples as much as possible thus increasing the accuracy of the analysis. Fermentation samples are diluted 1:1 with the fermentation buffer to avoid concentrations out of the linear range.

Table II. Intra-day and inter-day variability expressed as imprecision (R.S.D.) of the method.

compound	concentration (μM)	intra-day R.S.D (%)	n	inter-day R.S.D (%)
IP2	348	9.6	11	1.2
		14.5	8	
IP3	99	4.4	20	2.4
		5.7	25	
		6.1	16	
	395	3.9	6	2.5
		8.9	8	
IP4	192	13.6	20	1.4
		10.0	25	
		8.1	17	
	383	9.0	6	1.2
		12.7	8	
IP6	396	19.8	20	7.8
		19.8	26	
		13.1	19	

Fermentation monitoring

Fermentation monitoring, being the aim of the analysis, has been performed after a simple sample pretreatment of centrifugation which takes only 5 min. During the centrifugation the yeast is separated as a pellet from the inositol phosphates in the supernatant. Fig. 2 shows the electropherograms of the fermentation broth analyzed after 5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-0.02
-0.01
0
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1
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2
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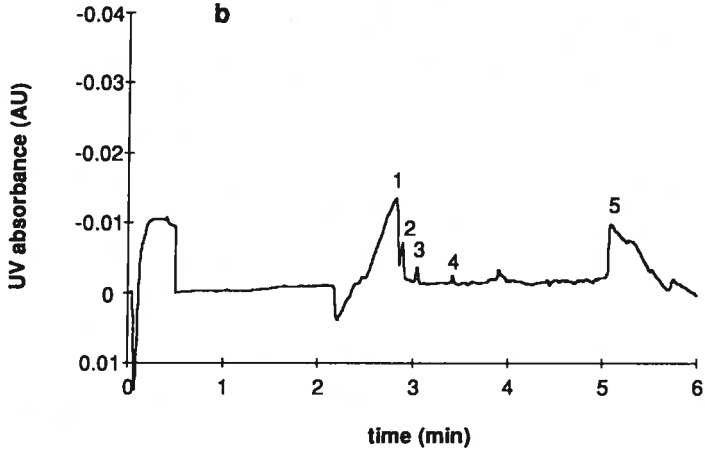
4
5
6
time (min)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UV absorbance (AU)
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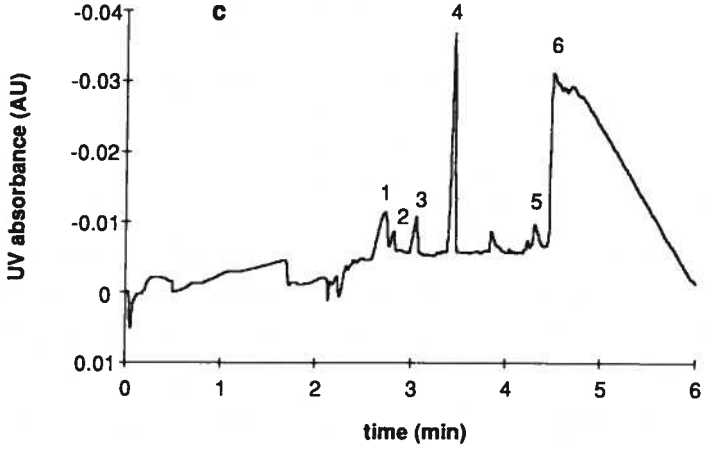
4
5
6
time (min)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UV absorbance (AU)
-0.04
-0.03
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4
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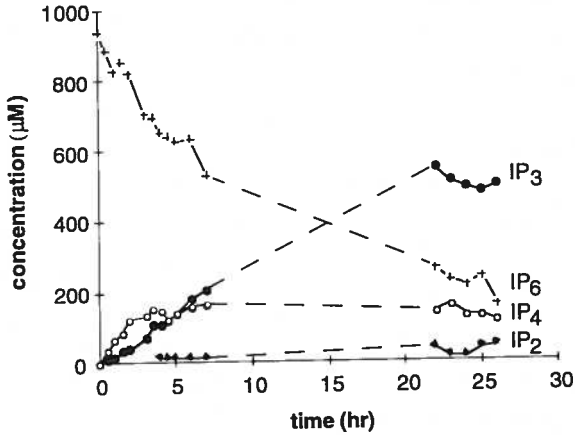
1
2
3
4
5
6
time (min)

Fig. 2. Electropherograms of a sample of the fermentation broth at 5 min (a), 60 min (b) and 22 h (c). Concentrations of the inositol phosphates: (a) 1 = 480 μM IP6, 2 = acetate, (b) 1 = 420 μM IP6, 2 = 30 μM IP5, 3 = 20 μM IP4, 4 = 8 μM IP3, 5 = phosphate + acetate, (c) 1 = 70 μM IP6, 2 = 20 μM IP5, 3 = 60 μM IP4, 4 = 300 μM IP3, 5 = 30 μM IP2, 6 = phosphate + acetate. For conditions see Fig. 1.

for quantification of the analytes. IP6, present at a high initial concentration (Fig. 2a), has been hydrolyzed into the other inositol phosphates and free phosphate after a few hours (Fig. 2b and 2c). The potential of monitoring the fermentation process is also demonstrated in Fig. 3. The more active the yeast, the faster phytic acid is hydrolyzed. During the hydrolysis a high amount of free phosphate has been formed. Phosphate has approximately the same electrophoretic mobility as IP1. For that reason IP1 can not be determined in the fermentation broth. At even higher phosphate concentrations, interference with IP2 also occurs. A minor drawback of the developed analysis is the off-line sample pretreatment. Instead of centrifugation which cannot easily be automated, dialysis but especially electrodialysis has to be explored as sample pretreatment. If a dialysis probe is positioned in the fermentation broth, a connection between the dialysis probe and a sample vial will allow the complete automation of the analytical method.



A method for monitoring the enzymatic hydrolysis of phytic acid has been developed. Fast separation and detection of all six inositol phosphates in deionized water has been achieved by using capillary zone electrophoresis with indirect UV detection. The mobility of the chromophore has to be adjusted to that of the compound to be quantified most accurately.

In the fermentation broth the high concentration of free phosphate masks IP1 thus preventing it from being detected. At long reaction times the high concentration of free phosphate formed during fermentation may also interfere with the detection of IP2.

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Chapter 3

Capillary zone electrophoresis-mass spectrometry

Chapter 3

Capillary zone electrophoresis - mass spectrometry

Abstract

Capillary zone electrophoresis (CZE) has been combined with mass spectrometric detection for the separation and determination of inositol phosphates (IPs). Apart from IP1 through IP6 (inositol mono- through hexakisphosphate), an IP3 derivative has been analyzed and identified. The detection limits achieved are in the low micromolar range corresponding to an injected amount of ca. 900 fmol. In addition, an IP3 spiked plasma sample was analyzed after sample pretreatment using ultrafiltration.

Introduction

Inositol phosphates (IPs) are important compounds in biochemistry (1,4,5-inositol trisphosphate) [1], agriculture (phytic acid) [2] and pharmaceutical science (1,2,6-inositol trisphosphate) [3]. From their chemical structures (Fig. 1A) it can be concluded that IPs are negatively charged and contain neither chromophores nor fluorophores. For many years, separation of IPs has been performed using liquid (ion-pair, ion-exchange) [4] and gas chromatography (after derivatization) [5]. However, capillary zone electrophoresis (CZE) appeared to be more appropriate and faster for the separation of IPs [6,7]. So far, CZE of IPs using indirect UV detection has been described. Indirect detection techniques, based on either UV absorbance, fluorescence or amperometric detection, have the disadvantage of increased noise levels and therefore relatively high detection limits. Next to CZE separation of IPs, capillary isotachophoresis (CITP) with conductivity detection has been described [8]. In CITP quantitation of low concentrations is rather limited because zone length instead of peak area or peak height of a compound is related to the amount in the sample volume injected. Sensitive detection of IPs is still problematic. Therefore, alternative detection methods have to be developed for their determination.

Until now, mass spectrometric detection of IPs has been performed using either continuous-flow fast atom bombardment (CF-FAB) or gas chromatography- electron im-

(GC-EI-MS).

On-line capillary electrophoresis-mass spectrometry (CE-MS) has been described first in 1987 by Olivares et al [10]. Since then, quite a number of publications on CE-MS have appeared. Thus far, three types of interfaces have been used for on-line CE-MS, i.e. CF-FAB [11], electrospray [12-16] and ionspray [17]. In addition, CE has been coupled with matrix-assisted laser desorption ionization (MALDI)-MS in the off-line mode [18].

This paper describes the mass spectrometric detection of IPs in the negative ionization mode after CZE separation, without the need of analyte derivatization. The developed method is also applicable for a synthesized IP3 derivative and its impurities with respect to both structure confirmation/elucidation and determination of the synthesis yield. A custom-made electrospray interface was used for the introduction of the column effluent into the mass spectrometer. In order to suppress electroosmotic flow, the capillary wall was coated with polyacrylamide [19].

Experimental

Chemicals

Acetic acid p.a. and methanol were obtained from J.T. Baker (Deventer, Holland). Ammonium acetate p.a. was purchased from Merck (Darmstadt, Germany). 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) and phenylacetate-IP3 (PIP3) (Fig.1B) as sodium salts were supplied by Perstorp Pharm (Perstorp, Sweden). For the preparation of the stock solutions of analytes and buffer solutions, deionized water was used (Milli-Q system, Millipore, Bedford, MA, USA). For the polyacrylamide coating, 3-(trimethoxysilyl)propylmethacrylate, 98 % (Janssen Beerse, Belgium), tetramethylethylenediamine (TEMED) and ammonium persulphate (Bio-Rad Laboratories, Richmond, USA) and acrylamide (Merck-Schuchardt Hohenbrunn, Germany) were used.

Procedures

Sample pretreatment

An amount of 500 µl of blank plasma, spiked to concentrations of either 20 or 200 µl IP3 was applied to AMICON sets (Amicon Corporation, Danvers, USA), consisting of

donor and acceptor compartment separated by filter with cut-off of M_r 30 000. Ultrafiltration was performed using an ultracentrifuge type JA-20 (Beckman, Fullerton, CA, USA) with fixed angle rotor (34°) at 2000 g for 30 min. The ultrafiltrate was injected into the CZE capillary.

Capillary coating

Before the fused-silica capillaries (BGB, Rothenfluh, Switzerland) were coated, they were rinsed for 5 min with 0.1 M sodium hydroxide p.a. (Merck), Milli-Q water and ethanol p.a. (Merck). Subsequently, the polyacrylamide coating procedure according to Hjertén [19] was performed.

Capillary zone electrophoresis

The experiments were performed using a programmable injection system and power supply (Prince, Lauerlabs, Emmen, The Netherlands). The electrophoresis buffer was prepared freshly every day and consisted of ammonium acetate (10 mM, pH 5)-methanol (90:10, v/v). The length of the fused-silica capillaries (100 µm I.D. and 170 µm O.D., unless stated otherwise) was 0.85 m. At the capillary inlet, a voltage of -28 kV and in conjunction, a pressure of 10 mbar was applied. Before each run, the capillary was rinsed with electrophoresis buffer for 2 min. Pressurized (200 mbar) sample injection was applied for 0.10 min, corresponding to 250 nl (75 µm I.D.) or 450 nl (100 µm I.D.)

Electrospray mass spectrometry

All experiments were carried out on a triple quadrupole mass spectrometer (Finnigan MAT TSQ-70) equipped with a custom-made electrospray interface that fitted in the thermospray source [20]. Most of the experiments were done in the negative ionization mode: the electrospray (ES) needle was kept at -3.5 kV with respect to the grounded heated sampling capillary. When operated in the positive ion mode, the ES needle was set at +3.5 kV. The sampling capillary and the ion source were kept at 175 respectively 150 °C. A slightly negative voltage was applied on the repeller for signal optimization of all ions. After removal of the polyimide layer at the capillary tip, the outlet of the fused-silica capillary was inserted into the stainless-steel needle assembly, slightly ahead of the needle tip. The sheath liquid consisted of ammonium acetate (100 mM, pH 5)-methanol (10:90, v/v) delivered at a flow rate of 1.2 µl/min by a Model 2400 syringe

The ^{31}P NMR spectrum of 2 mg phenylacetate-IP5, dissolved in 0.5 ml D_2O was obtained on a Bruker DMX-600 spectrometer (Karlsruhe, Germany).

Results and discussion

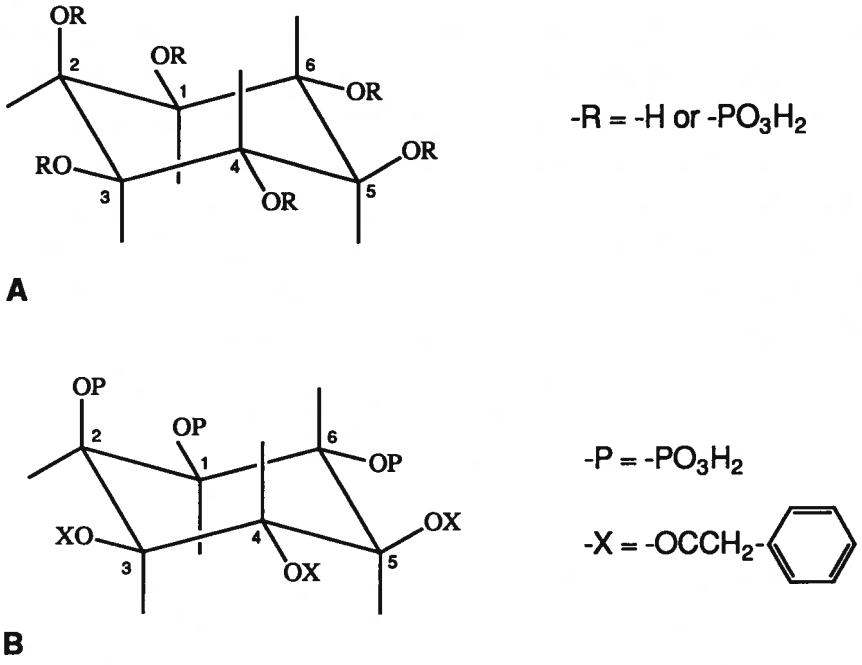
Capillary zone electrophoresis

Depending on the number of phosphate groups, IPs (Fig. 1) have multiple negative charges resulting in high electrophoretic mobilities in the direction of the anode. As the electroosmotic flow (EOF) is in the cathodic direction, net electrophoretic velocities are rather low, implying unacceptably long migration times. Therefore, the EOF must be suppressed using capillaries with either a static or dynamic coating of the wall. In first instance, hydroxypropylmethylcellulose (HPMC), a neutral hydrophilic polymer, was added to the electrophoresis buffer. However, this compound appeared to be incompatible with mass spectrometric detection because of contamination of the ion source. Moreover, the HPMC coating was destroyed when using an organic modifier, e.g. methanol or acetonitrile, as additive in the electrophoresis buffer. The polyacrylamide coating described by Hjertén [19] appeared to be a good alternative. This coating is static, which reduces the risk of ion source contamination. Furthermore, the coating is compatible with the use of organic modifiers in the electrophoresis buffer and the EOF is substantially reduced. As a consequence of EOF suppression, CZE of IPs has been performed with reversed polarity: the capillary inlet is at -28 kV and the outlet (ES needle) at -3.5 kV.

For the coupling of CZE and MS via an electrospray interface, a buffer must be chosen which is a compromise between aqueous (favourable for CZE of IPs) and non-aqueous (favourable for ES-MS). As CZE buffer, ammonium acetate was chosen, which was appropriate regarding the separation of IPs as well as the required volatility for ES-MS. In order to increase the buffer volatility even more, both methanol and isopropanol were examined as additives. At comparable modifier content, isopropanol gave longer migration times of the IPs than methanol, caused by its higher viscosity. Therefore, methanol was chosen as modifier.

Resuming, both the not completely suppressed EOF in the cathodic direction (capillary inlet) and the organic modifier as buffer additive lead to an increase of the migration times of the IPs. To compensate for this effect, pressure-assisted CZE can be performed.

:ompared with conventional CZE, this may lead to decreased efficiencies, caused by ıe hydrodynamic flow profile. Nevertheless, during all experiments a slight pressure ras applied in addition to the high voltage.



ig. 1. Chemical structure of inositol phosphates: inositol mono- (M_r 260), bis- (M_r 340), tris- M_r 420), tetrakis- (M_r 500), and hexakisphosphate (M_r 660) (A) and phenylacetate-IP3 (PIP3) M_r 774) (B) in the hydrated form.

lectrospray-mass spectrometry

ı order to investigate the electrospray performance and signal intensity as a function f the sheath liquid composition continuous-infusion-ES-MS experiments of PIP3 were arried out in the negative and positive ionization mode. The electrospray process is ıost stable at high organic modifier content, whereas the CZE separation of IPs is op- mal without any organic modifier in the buffer at all. The highest organic modifier

sion of PIP3 in the negative ionization mode. Although the spray performance appeare to be satisfactory, the IP3 derivative could not be detected at all. In the positive ioni zation mode with the same sheath liquid composition, however, a mass spectrum o PIP3 could be obtained. The spectrum mainly consisted of the $[M+H]^+$, $[M+NH_4]^+$ an $[M+Na]^+$ peaks (not shown). In the negative ionization mode, a mass spectrum coul not be acquired, unless the sheath liquid contained at least 90 % organic modifier. Th spectrum principally showed the $[M-H]^-$ peak of PIP3 and some impurities. Therefore all experiments have been performed in the negative ionization mode using a sheat liquid consisting of ammonium acetate (100 mM, pH 5)-methanol (10:90, v/v). In addi tion to the ES-MS experiments, ES-MS-MS of PIP3 in the negative ionization mode wa investigated. The observed loss of 98 u corresponds to the cleavage of one H_3PO_4 fror the IP3 derivative.

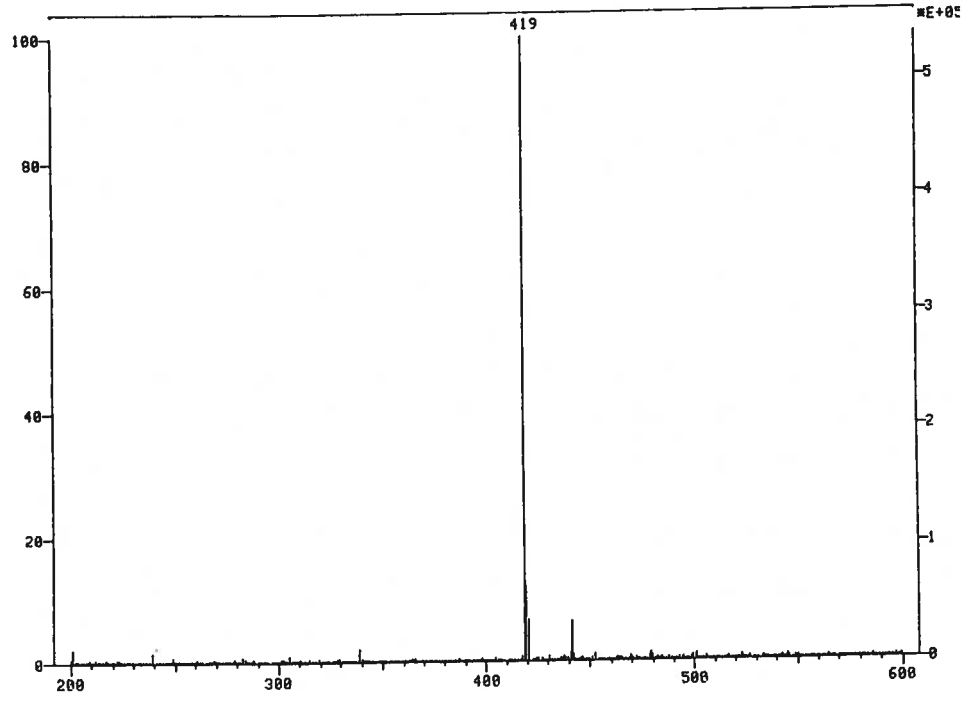


Fig. 2. Electrospray mass spectrum of 1,2,6-inositol trisphosphate in the continuous-infusic mode. Conditions: sheath liquid, ammonium acetate (100 mM, pH 5) and methanol (10:90, v), 2 µl/min; concentration IP3, 50 µM.

esides for PIP3, a mass spectrum for 50 µM IP3 has also been obtained in the continu-
us-infusion mode (Fig. 2). The main peak observed is $[M-H]^-$ (*m/z* 419). ES-MS-MS
xperiments of IP3 gave results comparable to those for PIP3: the loss of 98 u, corre-
ponding with H_3PO_4. Based on the results obtained during the continuous-infusion
xperiments the initial conditions in the CZE-ES-MS experiments could be readily cho-
en.

apillary zone electrophoresis-electrospray-mass spectrometry

Vhen the outlet of the CZE capillary was inserted in the electrospray needle and a high
oltage of -28 kV was applied, the voltage on the needle tip increased from -3.5 to
a. -4.2 kV. This phenomenon has also been observed by Perkins and Tomer [21], who
xplained it as a result of conductivity through the column. This increase of the ES
oltage was even more pronounced when samples with high conductivity (e.g. plasma)
ere analyzed. In that case, a total breakdown of the electrospray was observed which
ecessitated the use of lower voltages, e.g. -20 kV instead of -28 kV.
ased on the results obtained with the continuous-infusion ES-MS experiments of PIP3
multiple-ion detection (MID) procedure was designed. CZE-ES-MS of a concentrated
2 mM) and a 1:10 diluted solution of PIP3 was carried out. Fig. 3 shows that in the
oncentrated sample (Fig. 3A) more impurities than in the 1:10 diluted sample (Fig. 3B)
an be detected. The impurity with *m/z* 575 has come below the limit of detection in Fig.
B. By injecting the IP3 derivative and impurities at lower concentrations, the peak
hape of the analytes was substantially improved (Fig. 3B). Next to the impurities with
n/z 809 and 851, a PIP3 adduct has been detected. The ratios of the impurities and PIP3
dducts appear to differ depending on the concentration injected. Probably, the ioniza-
ion characteristics are dependent on the local conductivities. So far, the masses of the
P3 derivative and the impurities could be determined using CZE-ES-MS. In addition,
ZE-ES-MS-MS of PIP3 and some impurities has been performed for further structure
lucidation. Fig. 4 shows possible structures for some of the impurities. Structure pro-
osals are based on (i) the mass determination (MS), (ii) the presence of H_3PO_4 in the
nolecules (MS-MS) and (iii) on the migration times (electrophoretic mobilities) in the
nass electropherogram.

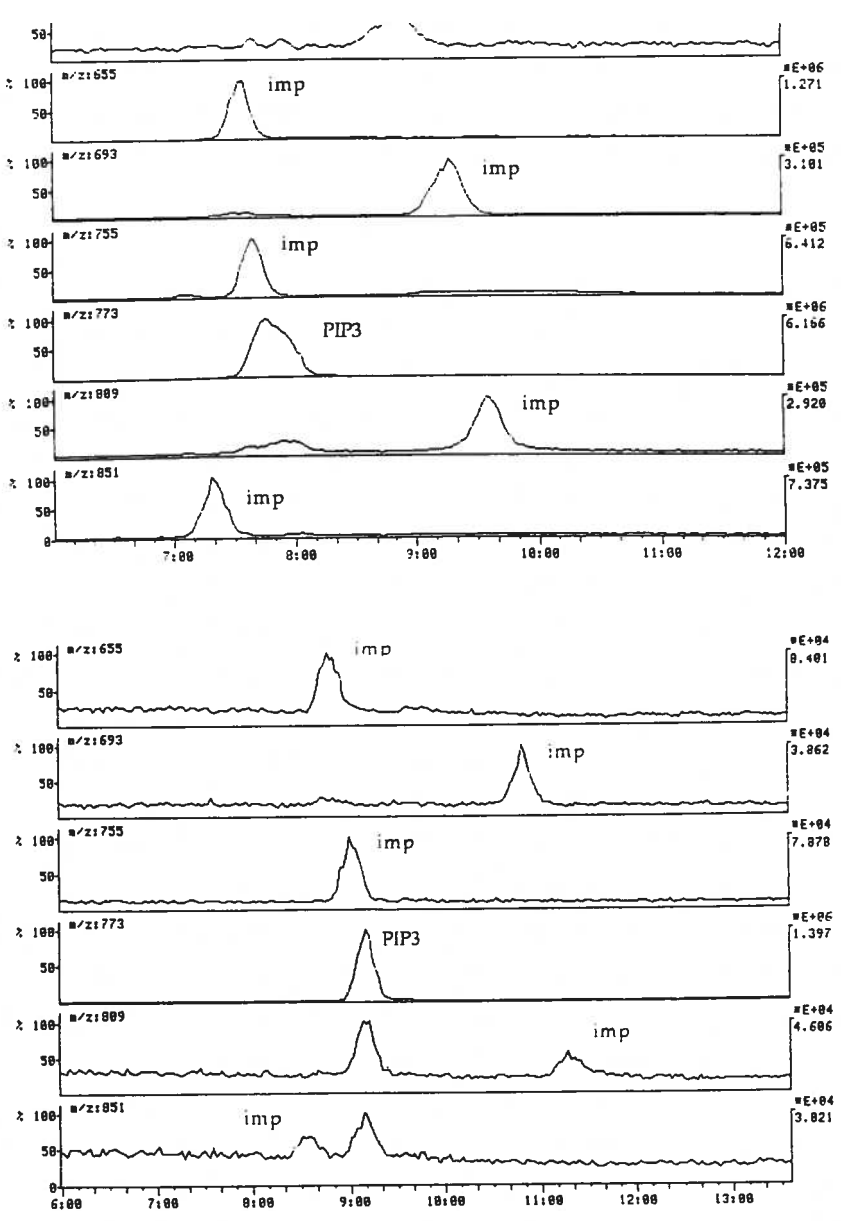


Fig. 3. Mass electropherogram of PIP3 and its impurities. Conditions: polyacrylamide-coate fused-silica capillary, 75 µm I.D., 150 µm O.D.; high-voltage capillary inlet, -28 kV; pressur 25 mbar; height difference between capillary inlet and ES needle, 5 cm; concentration IP3 de rivative, 2 mM (upper) resp. 200 µM (lower).

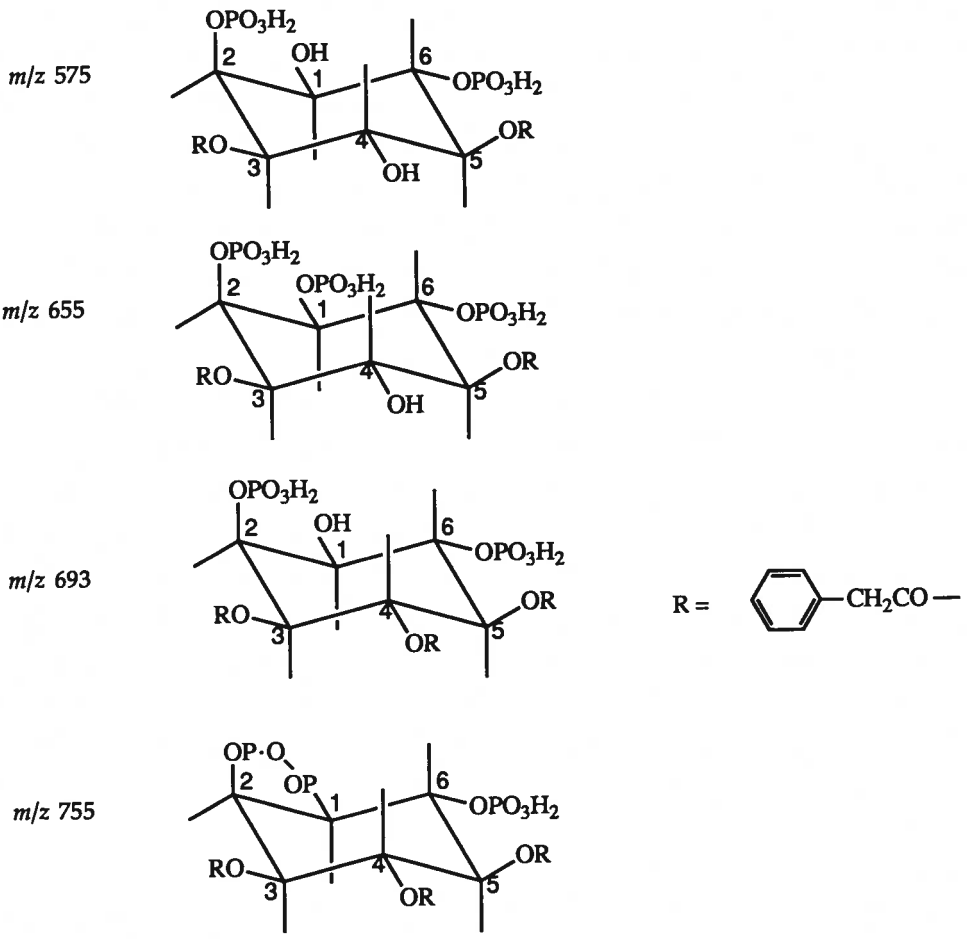


Fig. 4. Structure elucidation of impurities of PIP3.

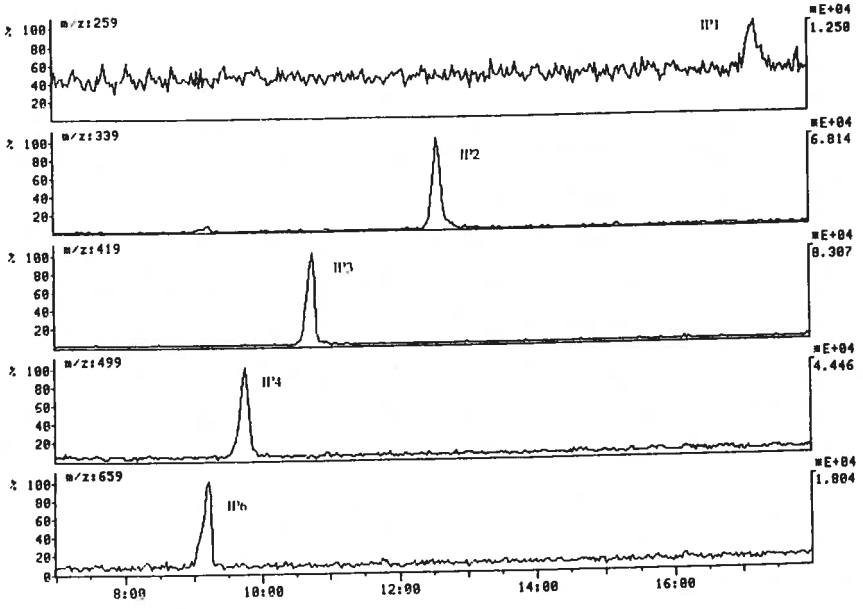
For the determination of the synthesis yield, the peak area of PIP3 was compared with the peak areas of the impurities. Using this parameter, the synthesis yield was 74-85 %, depending on the injected analyte concentration. One has to realize, however, that the IP3 derivative and the impurities do not have exactly the same response factors. Unfortunately, these factors cannot be determined as the analytes as such are not available. Nevertheless, assuming equal response factors the purity of PIP3 can be estimated. A complementary technique, NMR, has been performed to confirm the estimated purity.

and IP6, all at a concentration of ca. 20 µM, was analyzed using CZE-ES-MS. IP5, which is the first degradation product of IP6, was not present in the mixture because, in contrast to the other IPs, IP5 is rather unstable. The result is depicted in Fig. 5. The $[M-H]^-$ ions of IP1 to IP4 and IP6 have been detected. Most of the peaks are symmetrical. At higher concentrations, however, e.g. 200 µM, fronting (IP3-IP6) and tailing (IP1) peaks were more pronounced, which is the result of electromigration dispersion. Only IP2, with an electrophoretic mobility similar to the background electrolyte, had a symmetrical peak shape.

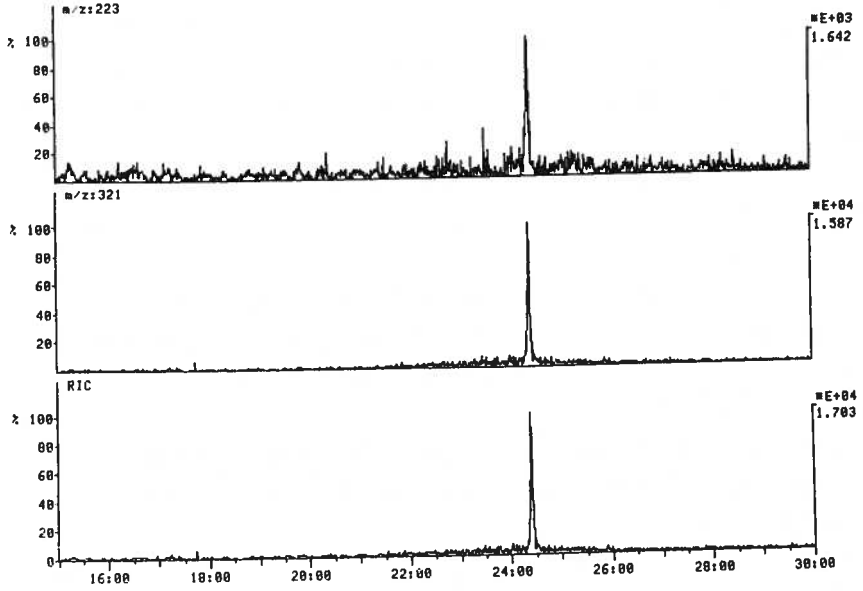
Qualitatively, the developed method appeared to be reproducible: during several days the same mass electropherogram has been obtained. Regarding the quantitative aspects, however, there are some deficiencies. Inter-day reproducibility with respect to the sensitivity is not completely satisfying, which can be overcome by using an internal standard. Furthermore, sensitivity differences between the IPs were observed: at a comparable concentration, the signal-to-noise ratios of the IPs differed substantially, implying different detection limits for the different IPs. Possibly, this is the consequence of working under MID conditions, considering the singly charged ions only. For IP2 and IP3, a concentration of 2 µM (absolute amount 0.9 pmol) could still be detected, whereas the detection limits for IP1, IP4 and IP6 are between 2 and 20 µM.

Bioanalytical aspects

Eventually, IP3 and its derivatives have to be determined in plasma and urine samples, requiring a sensitive determination method. Preliminary results show that the developed method can be used for the analysis of IP3 in plasma. After the plasma sample was spiked with IP3 to a concentration of 200 µM and pretreated by ultrafiltration, the ultrafiltrate was injected into the CZE capillary. Although the free fraction of IP3 in plasma is below 10 %, a mass electropherogram of the ultrafiltrate could be obtained by using CZE-MS-MS (Fig. 6). A loss of 98 and 196 u has been observed, which correlates with a subsequent loss of H_3PO_4 (twice). The developed method can be applied for the determination of IP3 in plasma, but IP3 concentrations in real-life samples will be in the nanomolar range. In order to improve the method for bioanalysis, the protein binding of IP3 must be decreased substantially to increase the recovery of the sample pretreatment. A second approach will be the application of a concentrating technique prior to CZE. For this purpose, isotachophoresis (ITP) can be combined with CZE [13,22-25].



. 5. Mass electropherogram of IP1, IP2, IP3, IP4, and IP6. Conditions; high-voltage capillary :t -28 kV; pressure, 10 mbar; concentrations inositol phosphates, 20 μM.



... of IP3 ... concentrated with ultrafiltration. Conditions: high-

The developed method, using CZE-ES-MS(-MS), appears to be applicable for the det mination of IPs and analogues without the need of derivatization. The structures c synthesized IP3 derivative and its impurities have been confirmed and elucidated. T yield of the IP3 synthesis could be well estimated.

Preliminary results show that the determination of IP3 in plasma can be perform after a sample pretreatment consisting of ultrafiltration. For the bioanalysis of real- samples, however, the sensitivity must be improved. Therefore, future research will devoted to improvement of the recovery of the sample pretreatment by breaking plasma protein-analyte bond. Furthermore, on-line concentrating techniques will considered.

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Chapter 4

Immobilized metal affinity chromatography-capillary zone electrophoresis

Chapter 4

Immobilized metal affinity chromatography-capillary zone electrophoresis

Abstract

A method for the determination of 1,2,6-inositol trisphosphate (IP3) and derivatives in plasma by capillary zone electrophoresis with (indirect) UV detection has been developed. The sample pretreatment is based on the selective isolation after complexation of inositol phosphates with iron(III) loaded on an adsorbent. Plasma protein denaturation was performed with sodium dodecyl sulfate. The selectivity of the method is demonstrated with the analysis of phenylacetate-IP3. The recoveries amount to 65 % and 88 % in plasma and in water, respectively.

Introduction

1,2,6-Inositol trisphosphate (1,2,6-IP3) and derivatives which have interesting pharmacological properties [1] have been investigated for pharmaceutical application. Therefore, an analytical method is required for the determination of 1,2,6-IP3, analogues and metabolites in plasma. Difficulties include the high protein binding fraction of the IP3 derivatives in plasma (> 99 %) by hydrophobic and electrostatic interactions and separation and sensitive detection of inositol phosphates.

Analysis of inositol phosphates has been a challenging task throughout the years. So far, inositol phosphates have been determined using ion-pair and ion-exchange chromatography, combined, among others, with suppressed conductivity detection [2], refractive index detection [3], radiometric detection [4] and fluorometry (after complexation) [1]. Furthermore, gas chromatography coupled to mass spectrometry has been applied after derivatization of the compounds [5]. Since 1992, several papers have been published dealing with the analysis of inositol phosphates based on capillary zone electrophoresis (CZE) and capillary isotachophoresis (CITP) combined with conductivity detection [6], indirect UV detection [7,8] and, a more sensitive detection technique, electrospray ionization-mass spectrometry (ESI-MS) [9]. The capillary electrophoretic

metal ions like Fe^{3+}, Al^{3+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, etc. [10-14]. Chiral resin, [illegible]
phases have been used for metal chelate affinity chromatography [15,16] and ligand exchange chromatography [17-19] of doxorubicin [20], phenols [21] and uracil derivatives [22]. It may be advantageous to use such selective sorbents for the isolation of 1,2,6-IP3 and derivatives from plasma in combination with a protein denaturation step. It has been established that the association of sodium dodecyl sulfate (SDS) with all proteins is accompanied by a drastic conformational change [23]. By the complexation of SDS all proteins are dissociated to their constituent polypeptide chains. Because the adsorbent is selective for iron(III) complexing compounds the adsorption of SDS can be neglected.

This paper describes a method for the analysis of 1,2,6-IP3 and a derivative, phenylacetate-IP3, using several iron(III)-loaded adsorbents in the plasma sample pretreatment prior to CZE with (indirect) UV detection.

Experimental

Chemicals

All chemicals were of analytical grade. Iron nitrate and acetic acid were obtained from J.T. Baker (Deventer, The Netherlands). Ammonium acetate, ethylenediaminetetraacetic acid (EDTA), sodium hydroxide, sodium dodecyl sulfate (SDS) and phosphoric acid were purchased from Merck (Darmstadt, Germany). 1,2,6-Inositol trisphosphate (IP3) and phenylacetate-IP3 (PIP3) were from Perstorp Regeno (Perstorp, Sweden). The amounts of column materials used were 20 mg 8-hydroxyquinoline(HQ)-silica and 20 mg iminodiacetic acid(IDA)-silica with 5 µm particle size (Serva, Heidelberg, Germany), 40 mg 8-HQ-glycolmethacrylategel with 40-63 µm particle size (Lachema, Brno, Czech Republic) and 0.5 ml IDA-Sepharose (Pharmacia, Uppsala, Sweden). Hydroxypropylmethylcellulose (HPMC) and phytic acid (IP6) were purchased from Sigma (St. Louis, MO, USA). 1-Naphtol-3,6-disulfonic acid (NDSA) came from Janssen (Beerse, Belgium) and 8-hydroxyquinoline-5-sulfonic acid (8-HQS) from Hopkins & Williams (London, UK). Blank human plasma, containing citrate for anticoagulation, was purchased from the Leiden University Hospital.

Sample pretreatment

The sample pretreatment was performed in Eppendorf vials (Fig. 1). Each step consisted of vortexing, centrifugation (Eppendorf centrifuge 5451, Eppendorf Geraetebau, Netheler und Hinz, Hamburg, Germany) for 10 min at 5000 g and removal of the supernatant from the pellet. The plasma sample was mixed with SDS (100 mg/ml plasma) for 3 min before it was added to the adsorbent.

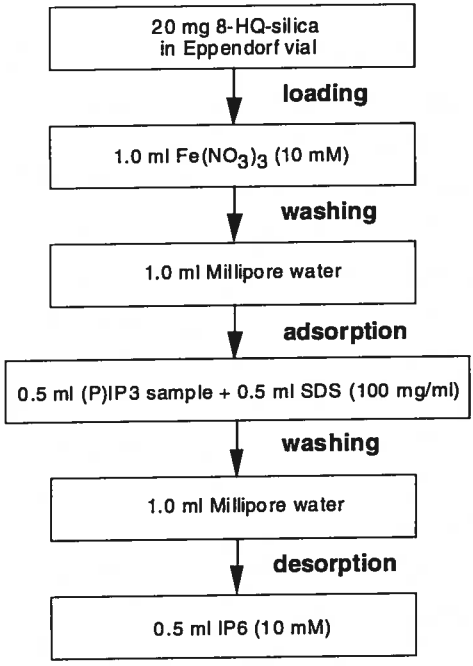

Fig. 1. Procedure for the pretreatment of (P)IP3 samples.

Electrophoresis

The collected fractions were analyzed using capillary zone electrophoresis (CZE) combined with (indirect) UV detection. CZE was performed on a P/ACE 2200 system (Beckman, Fullerton, CA, USA) equipped with a UV detector (λ = 214 nm). For UV detection (PIP3) the electrophoresis medium consisted of 10 mM ammonium acetate

silica capillary (SGE, Ringwood, Victoria, Australia) of 0.37 m (0.30 m to the detector). After rinsing the capillary (75 µm I.D., 375 µm O.D.) for 2 min with electrophoresis buffer pressurized injection was applied for 3 s, corresponding to about 50 nl. For data collection and data handling System Gold software, version 7.12 (Beckman) was used.

Results and discussion

Adsorption

The first sorbent investigated for the adsorption of PIP3, a UV absorbing IP3 analogue, was 8-HQ-silica. After loading an 8-HQ-silica hand-packed column (20 mg; 6 mm I.D.) with iron(III) (2 ml; 10 mM) the adsorption of analyte (0.5 ml; 200 µM) was only ca. 50 %. The same result was obtained when PIP3 was first incubated with iron(III) and subsequently added to the column. Presumably, both the complexation of 8-HQ-silica with iron(III) and the complexation of iron(III) with analyte need more time. Therefore, the whole procedure was performed in an Eppendorf vial, which allowed both complexations after another (Fig. 1). This approach resulted in 100 % adsorption of analyte to the iron(III)-loaded 8-HQ-silica. The non-specific binding of PIP3, determined as the analyte sorption on untreated 8-HQ-silica, was below the detection limit.

In order to get insight into the selectivity of the method, the effect of low pH on the adsorption was investigated. Inositol phosphates have multiple negative charges, even at low pH. At pH 3 (10 mM phosphate buffer), however, the analyte adsorption was insufficient, caused by the competing phosphate ions. By increasing the incubation time to 1 h the analyte adsorption could be improved to 100 % due to PIP3's high affinity for iron(III). As acetate ions do not complex with iron(III), pH adjustment with 30 mM acetic acid pH 3 instead of phosphate buffer did not affect the analyte adsorption. Nevertheless, all further experiments were performed without pH adjustment because it had not shown significant improvement.

Desorption

After the selective adsorption of PIP3 to the sorbent the desorption of analyte was investigated. Van der Vlis et al [20] desorbed doxorubicin from iron(III)-loaded HQ-silica with 1 M nitric acid. By lowering the pH substantially, 8-HQ is protonated and iron(III) desorbs together with the analyte. However, this approach is incompatible with CZE

alysis with UV detection because of the high ionic strength of the obtained sample, ıding to enhanced Joule heating, changes in the local electric field strength and con- ıquently peak distortion. Furthermore, the high concentration of nitrate ions inter- 'es with the analyte in the electropherogram. Therefore, another mechanism for the sorption of analyte was examined, which was based on the displacement of analyte a high concentration of a competing compound that complexes with iron(III). In ıt case, only the analyte is desorbed whereas iron(III) remains on the sorbent. EDTA, ɔsitol hexakisphosphate (IP6), 8-hydroxyquinoline sulfate (8-HQS) and phosphoric d all complex with iron(III). The analyte recoveries mounted to 88 % using IP6 (0.5 , 10 mM) and 25 % using EDTA (0.5 ml, 10 mM). 8-HQS and phosphoric acid were t effective at all. As the pH of the IP6 solution was 11, the effect of hydroxyl ions was 'estigated by adding 0.01 M sodium hydroxide to the adsorbent. The analyte ap- ared to be selectively displaced by IP6 ions and not by hydroxyl ions. Although the ıplacement mechanism is not yet completely understood, the association constant of ɔ displacer plays a predominant role.

e displacement of analyte by IP6, being a cheap and non-toxic compound, has been 'estigated more thoroughly. The effect of using different concentrations of IP6 to the bent on the recoveries of PIP3 is shown in Fig. 2. Varying the IP6 concentration from ɒ 10 mM the recovery is increased to a maximum of 88 % at 10 mM. At 20 mM IP6, alyte interference in the electropherogram becomes unacceptable. Moreover, it was ɜerved that the effect of increasing the IP6 concentration in a standard solution, while ɘping the analyte concentration constant, was a decrease of the PIP3 peak height ıɜed by the higher conductivity of the sample. It is evident that the IP6 concentration ɜent in the sample after desorption is lower than that added to the adsorbent. vertheless, this concentration difference can be neglected compared to the high IP6 ıcentration and, therefore, the recoveries obtained are related to analyte solutions th approximately the same IP6 concentrations. When ion-pair chromatography (IPC) tead of CZE would be combined with this sample pretreatment the response factor ɔbably remains the same while varying the IP6 concentration. As the collected frac- ns have an IP6 concentration of ca. 10 mM, transient isotachophoresis [24] could be ıɜidered with IP6 as the leading electrolyte. However, it must be concluded that nsient isotachophoresis is very unlikely because the injected sample volume is low

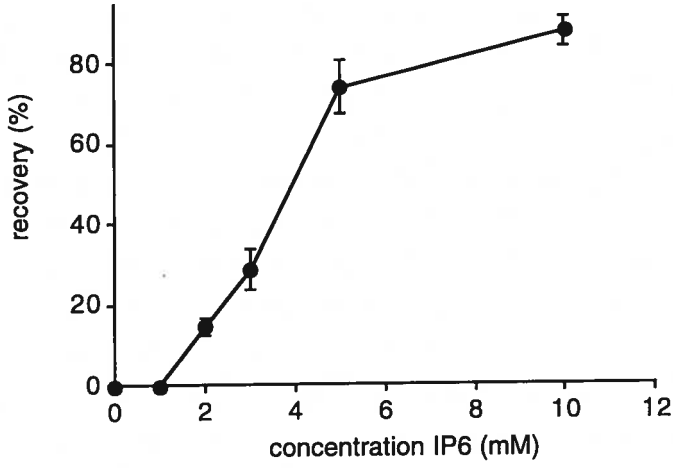

Fig. 2. Relation between the PIP3 sample recovery (%) and the IP6 desorption concentrati (mM).

Choice of sorbent

So far, all experiments were performed using 8-HQ-silica as the adsorbent. Oth adsorbents investigated were 8-HQ-glycolmethacrylategel [25,26], IDA-silica and ID Sepharose. The recoveries of PIP3 from water as well as from plasma using the diff ent adsorbents are depicted in Fig. 3. For 8-HQ-silica, IDA-silica and IDA-Sepharo the recoveries of PIP3 in water are approximately the same (ca. 88 %) whereas on 8-H glycolmethacrylategel the recovery appeared to be much lower (49 %, S.D. = 1.0 %, n 2). Presumably this is caused by the larger particle size of 8-HQ-methacrylategel (40- µm) compared with the other sorbents (5 µm), implying the presence of relatively de pores within the particles through which the sample molecules diffuse in and out very slowly [27]. Yet, this has not been investigated any further.

Another difference between the adsorbents becomes clear when plasma samples a pretreated. Initially, 8-HQ-silica was supposed to be the more appropriate adsorbent IDA occupies three positions in the metal sphere whereas 8-HQ only two. Howev this difference has not been seen for standard solutions of PIP3. On the contrary, t recovery of analyte in plasma pretreated on 8-HQ-silica is substantially higher (65 than on the IDA-sorbents (9 % and 46 %). In order to improve the performance f plasma samples on IDA-Sepharose two approaches were used. First, the incubati

time of the plasma sample with the sorbent was increased to 1 h in order to achieve equilibrium. Second, the capacity of the adsorbent was increased by using a higher volume (1.0 ml instead of 0.5 ml) of adsorbent. Unfortunately, neither of the approaches affected the performance of the adsorbent. Furthermore, the recovery of a standard solution of PIP3 with SDS was the same for IDA-Sepharose as for 8-HQ-silica. Thus, the difference must be caused by interactions between certain plasma constituents and the IDA-sorbent. Therefore, it was chosen to continue the experiments with 8-HQ-silica, which can be synthesized according to the method described by Shahwan and Jezorek [21].

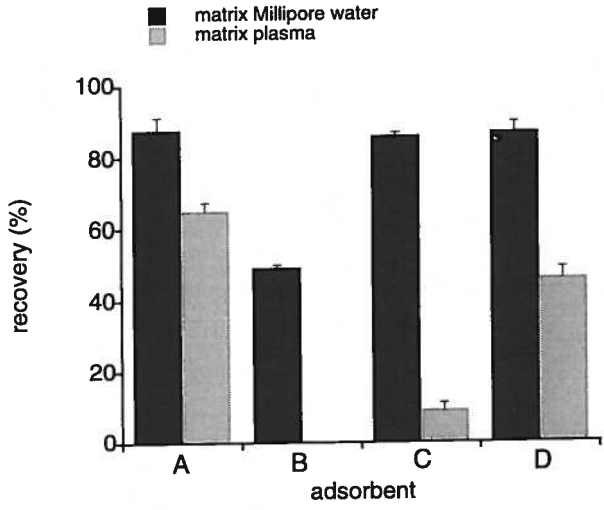

Fig. 3. Recoveries (%) obtained from water and plasma samples with different adsorbents. A = 8-HQ-silica, B = 8-HQ-glycolmethacrylategel, C = IDA-silica, D = IDA-Sepharose.

Application to plasma samples

In Fig. 4 the results are depicted as obtained with the developed procedure. Fig. 4A shows the electropherogram of an aqueous solution of PIP3. An amount of 200 nanomoles of PIP3 was pretreated and subsequently analyzed by CZE with UV detec-

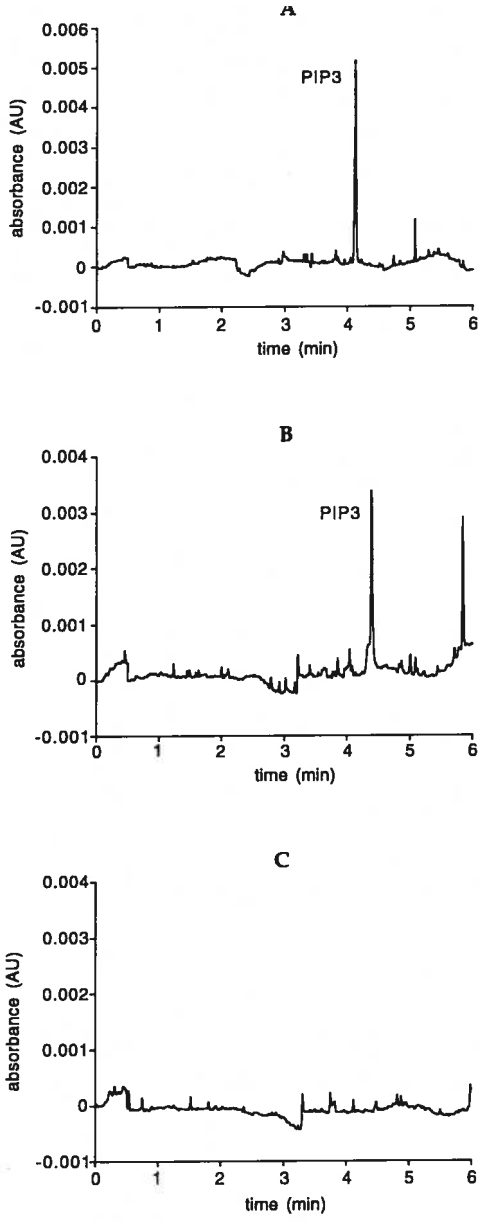

Fig. 4. Electropherograms of pretreated PIP3 samples in water (A), in plasma (B) and blank plasma (C). Conditions: UV detection at λ = 214 nm; CZE buffer: 10 mM ammonium acetate, pH 5, 0.01 % HPMC.

npound. The pretreatment of plasma samples, however, is much more complicated
:ause of the matrix, containing ca. 70 mg/ml proteins, high concentrations of electro-
es (sodium, sulphates, phosphates, etc.) as well as fatty acids and lipids [28]. Be-
es, the very high and strong protein binding of IP3 derivatives in plasma, caused by
ctrostatic and hydrophobic interactions, must be substantially decreased. So far, sev-
ıl approaches have been applied in order to denature the proteins, such as organic
vents (methanol, acetonitrile), strong acids (e.g. perchloric acid), urea, ammonium
lfate and a surfactant (sodium dodecyl sulfate) added to the plasma sample. Another
proach was the cleavage of proteins with a proteolytic enzyme (trypsin) at pH 8,
:ubated for several hours at 37 °C. Subsequently, ion-pair solid phase extraction (IP-
'E) was performed on a C_{18} column with tetrabutylammonium as a counterion or the
mple was analyzed directly by capillary zone electrophoresis with UV detection. Ei-
er the recovery or the reproducibility was too low. In contrast with the combination
IP-SPE and sodium dodecyl sulfate (SDS) added to the plasma sample, the use of the
)n(III)-loaded adsorbent was quite successful and allowed the presence of 1.4 g SDS/
protein in the sample [21]. Fig. 4B shows the electropherogram of a pretreated plasma
ımple containing 200 µM PIP3. Hardly any other compound adsorbs to the iron(III)-
aded sorbent, showing the selectivity of the method. The recovery mounted to 65 %
;.D. = 2.2 %, n = 8). Fig. 4C shows the electropherogram of pretreated blank plasma,
emonstrating that no interfering peaks are present in the time window.
lext to PIP3, 1,2,6-IP3 has been pretreated using the developed method. However, IP3
annot be detected with direct UV detection. Therefore, CZE was combined with indi-
ect UV detection [8]. Inherent to this detection principle, the presence of 10 mM IP6
ıore or less interfered with the IP3 derivatives. The peak shapes were tailing, even at a
ıigher pH where the mobilities of the analyte and chromophore match more closely.
Vith this system only IP3 could be measured in the presence of a high IP6 concentra-
ion (Fig. 5). Although indirect UV detection will not be the detection method of choice,
t has been used to check the sample pretreatment of IP3 in plasma. The recoveries
)btained were 90 % (S.D. = 7.2, n = 2) and 54 % (S.D. = 2.8, n = 4) for IP3 in water and
)lasma, respectively, which are quite satisfying figures.

Quantitative aspects

...ential in quantitative analysis

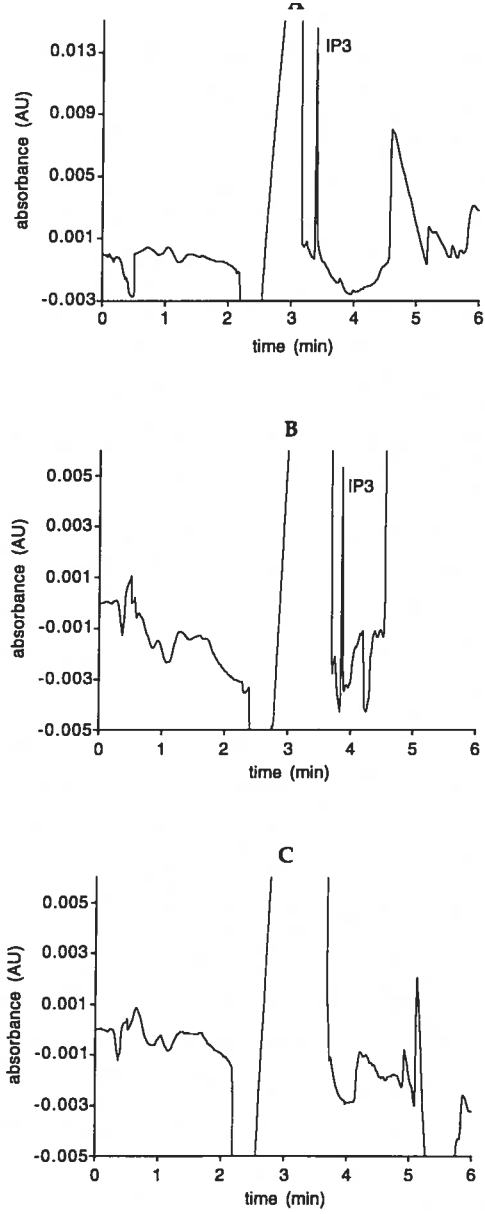

Fig. 5. Electropherograms of pretreated IP3 samples in water (A), in plasma (B) and blan plasma (C). Conditions: indirect UV detection at λ = 214 nm; CZE buffer: 0.5 mM NDSA, 3 mM acetic acid, pH 3, 0.01 % HPMC.

tains many manual steps the reproducibility appeared to be quite good. Using 10 mM IP6 for the desorption the PIP3 recoveries were 88 % (S.D. = 3.7 %, n = 5) and 65 % (S.D. = 2.2, n = 8) for analyte in water and plasma, respectively.

In order to examine the linearity of the method calibration plots were made in the concentration range 10-200 μM PIP3 in water or plasma. As the migration time of PIP3 varied only a few seconds during the day it was chosen to plot the peak height instead of the peak area versus the concentration. The correlation coefficients (r) were 0.999 and 0.996 for PIP3 in water and plasma, respectively, implying a good linearity in this concentration range without the use of an internal standard.

Because of the relatively low sensitivity of UV detection and especially indirect UV detection, the limit of detection of the developed method is rather high (ca. 10 μM). CZE coupled with electrospray mass spectrometry of inositol phosphates already showed an improvement of the sensitivity with one order of magnitude [9]. Thus, for real bioanalysis of inositol phosphates a concentrating technique [24,28-30] and/or a more sensitive detection method will be required.

Conclusions

The developed method can be used for the determination of 1,2,6-IP3 and derivatives in plasma. The advantage of the sample pretreatment is the selectivity enabling the analysis of highly protein-binding IP3 derivatives (PIP3). The method can be combined with both CZE and IPC. It shows good reproducibility and linearity without the use of an internal standard. As the method is rather laborious possibilities for automation will be investigated. A minor drawback of the method is the sensitivity, which is determined by the detection method used. Therefore, a concentrating technique and a more sensitive detection method are under investigation for the determination of inositol phosphates in real samples.

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Chapter 5

Electrodialysis-capillary zone electrophoresis

Chapter 5

Electrodialysis-capillary zone electrophoresis

Abstract

Electrodialysis (ED) has been coupled to capillary zone electrophoresis (CZE) as an on-line sample pretreatment technique. Analytes are introduced into the fused-silica capillary through a membrane with a molecular mass cut-off of 30 kDa. As reversed polarity is applied only small negatively charged ions are injected whereas positively charged and/or large compounds are retained. ED takes only ca. 20 s and is comparable with electrokinetic injection in several respects. ED-CZE has been applied to adenosine triphosphate in blood plasma and to inositol phosphates in fermentation broth.

Introduction

After fifteen years of development, capillary zone electrophoresis (CZE) has been introduced in many (routine) analytical laboratories. For clean samples, CZE without sample pretreatment works quite well. However, real-life samples require a laborious and time-consuming pretreatment before CZE can be performed. Most of these sample pretreatment techniques are performed off-line. However, an on-line liquid-liquid electroextraction-CZE method has been developed lately which can be performed e.g. after (off-line) solid-phase extraction (SPE) [1,2]. Furthermore, an on-line SPE-CZE procedure was developed by Strausbauch et al [3]. Unfortunately, the described procedures are not suitable for highly polar compounds such as inositol phosphates which are ionized over nearly the whole pH range.

Inositol phosphates are important compounds in several research areas [4,5]. These phosphorylated sugars are negatively charged, even at low pH. Besides, they do not contain any chromophoric/fluorophoric groups in the molecular structure which makes sensitive detection rather difficult. Analysis of inositol phosphates in complicated matrices, such as fermentation broth, blood plasma or tissue homogenate, requires a sample pretreatment before their separation. So far, samples containing inositol phosphates have been pretreated using anion-exchange SPE [6,7], ultrafiltration [8], centrifugation [9,10], or iron(III) loaded stationary phases [11], all being rather laborious, time-con-

the sample, dialysis as well as microdialysis have been combined with liquid chroma-
tography (LC) in the on-line mode [12-15]. Furthermore, several researchers coupled microdialysis on-line to capillary electrophoresis [16-19]. By applying a voltage over the dialysis membrane, analytes do not only diffuse but also selectively electromigrate through the membrane. This technique, which is called electrodialysis, has been used for biotechnological purposes [20-22], neutralization of samples [23-26], enrichment [27] and purification of bioanalytical [28] and environmental [28,29] samples. In comparison to dialysis, electrodialysis is much faster and more selective.

This paper describes the on-line coupling of electrodialysis to CZE for inositol phosphates. Electrodialysis has been compared with electrokinetic injection. As inositol phosphates cannot be detected with UV detection, adenosine triphosphate, with nearly the same electrophoretic mobility as inositol trisphosphate, has been used as a model compound during the development.

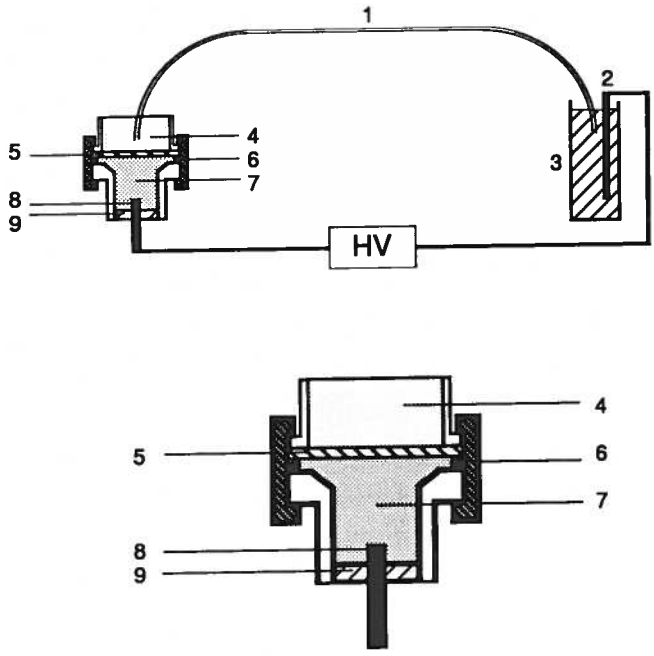
Experimental

Chemicals

Acetic acid p.a. was purchased from J.T. Baker (Deventer, Netherlands). Hydroxypropylmethylcellulose (HPMC), with a viscosity of 4000 cP for a 2 % aqueous HPMC solution, bovine albumin (fraction V, 98-99 %), inositol monophosphate (2-IP_1) as dicyclohexylammonium salt and phytic acid (IP_6) were provided by Sigma (St. Louis, MO, USA). Ammonium acetate p.a. came from Merck (Darmstadt, Germany). Adenosine-5'-triphosphate (disodium salt hydrate, 98 %) and 1-naphtol-3,6-disulfonic acid (NDSA) were from Janssen (Beerse, Belgium). Inositol bis- (1,2-IP_2), tris- (1,2,6-IP_3) and tetrakis- (1,2,5,6-IP_4) phosphate were supplied as sodium salts by Perstorp Pharma (Perstorp, Sweden). Blank plasma came from the University Hospital Leiden (Leiden, Netherlands). For the preparation of the stock solutions of the analytes and buffer solutions, deionized water was used (Milli-Q system, Millipore, Bedford, MA, USA). The buffer solution was filtered through a 0.2-μm Nylon acrodisc syringe filter (Gelman Sciences, Ann Arbor, MI, USA).

Equipment and procedure

The electrodialysis-capillary zone electrophoresis set-up is shown in Fig. 1. The system consisted of two parts: an electrodialysis device (EDD) and a custom-made capillary



g. 1. Electrodialysis device coupled to the CZE system. 1 = fused-silica capillary; 2 = anode; 3 buffer vial; 4 = acceptor compartment; 5 = membrane; 6 = O-ring; 7 = donor compartment; 8 cathode; 9 = septum.

ectrophoresis (CE) system. The EDD was a modified ultrafiltration device (Amicon, anvers, MA, USA) and consisted of a donor (7) and an acceptor (4) compartment oth 0.5 ml) separated by a membrane (5) with a molecular mass cut-off of 30 kDa and diameter of 16 mm (Amicon). A silicone O-ring (6) was positioned between the donor ompartment and the membrane to prevent sample leakage along the membrane. The onor compartment was filled with sample solution and sealed with a silicone septum) through which the platinum cathode (8) was positioned. The acceptor compartment as filled with Milli-Q water. The inlet of the fused-silica capillary (1) (SGE, Ringwood, ictoria, Australia) was positioned in the acceptor compartment, the outlet of the cap- lary in the buffer vial (3), together with the platinum anode (2). The electrophoresis

lary (75 µm I.D., 375 µm O.D., l = 0.75 m). After electrodialysis the EDD was replaced b
a buffer vial and CZE was started at -25 kV (90 µA). At 0.25 m before the capillar
outlet, UV detection (CE-adapted-Spectroflow 773, Kratos Analytical Instruments
Ramsey, NJ, USA) was performed at 200 nm, unless stated otherwise.

Inositol phosphates in fermentation broth

An amount of 1 ml of fermentation broth, used for the enzymatic hydrolysis of phyti
acid [10], was spiked to a concentration of 100 µM inositol hexakis-, tetrakis-, tris-, bis
and monophosphate. Samples were analyzed using electrodialysis (20 s, -25 kV) - CZE
For these experiments the same set-up was used as described above. The CZE buffe
consisted of 0.5 mM NDSA, 0.005 % HPMC and 30 mM acetic acid pH 3. During CZE
high voltage of -30 kV (35 µA) was applied. Indirect UV detection was performed a
214 nm.

Results and discussion

Electrodialysis

In dialysis, solutes diffuse from one compartment to another through a membrane as
result of a concentration gradient. This implies that equilibrium is reached if the con
centration gradient approaches zero. If, in conjunction with the concentration gradien
a voltage is applied over the membrane analyte ions do not only move by molecula
diffusion but also by electromigration. Thus, the process, called electrodialysis, become
much faster and more selective. Similar to dialysis, the electrodialysis set-up consists c
a donor and an acceptor compartment, separated by a membrane. By positioning th
cathode and the anode which are covered with ion-exchange membranes in the sepa
rate compartments, only the ions with the appropriate charge and with a molecula
mass smaller than the membrane cut-off value will migrate into the acceptor compart
ment. (Electro)dialysis can be performed in the static or in the dynamic mode. In th
static mode the donor and acceptor phase are stagnant whereas in the dynamic mod
at least one of the phases is moving. Consequently, enrichment of the sample solutio
by dialysis can be obtained only in the dynamic mode. By electrodialysis, howeve
analyte concentration can be achieved in the static as well as in the dynamic mode.
In general, samples containing macromolecular compounds next to the analyte are pre

ed using (ultra)filtration, centrifugation or (micro)dialysis prior to the separation. vever, a major drawback of these sample pretreatment techniques is their duration. hermore, on-line performance of (ultra)filtration or centrifugation to capillary zone trophoresis (CZE) is rather complicated. By using electrodialysis on-line to CZE, ple pretreatment and separation of the analytes becomes non-laborious and fast.

trodialysis-capillary zone electrophoresis

on-line electrodialysis-capillary zone electrophoresis (ED-CZE) set-up is shown in 1. The cathode is positioned in the donor compartment and the anode in the buffer . Whereas during electrokinetic injection of a sample the capillary inlet is placed in sample vial, in on-line ED-CZE the capillary inlet is positioned in the acceptor com- ment. In general, the acceptor compartment is filled with water which prevents tamination of the capillary inlet with impurities from the sample. For the perfor- ice of off-line ED(-CZE), the anode can be positioned in the acceptor compartment. trochemical reactions of the analytes which might occur under these conditions be avoided by covering the electrodes with ion-exchange membranes [28]. In the line ED-CZE set-up (Fig. 1), however, electrochemical reactions of the negative lytes do not take place at the anode before detection.

analysis of a sample using on-line ED-CZE is a two-step procedure. In the first , negative analytes smaller than the membrane cut-off value migrate from the (stag- t) donor compartment via the (stagnant) acceptor compartment into the fused-silica illary by applying a voltage over the whole system. At the same time, electromigration mall negative (to the anode) and positive (to the cathode) ions occurs. The EDD s not contribute significantly to the total electrical resistance of the system. This is wn by the current in the system which is constant, with or without the EDD. After introduction of analyte ions into the capillary, the EDD is removed and CZE is formed. As adenosine triphosphate (ATP) and inositol phosphates are multiply nega- ly charged at pH 5, the electrophoretic mobility is quite high. An electroosmotic w (EOF) in opposite direction would lead to a small net velocity. Therefore, the EOF uppressed by adding hydroxypropylmethylcellulose to the electrophoresis buffer l reversed polarity is applied during ED and CZE. The result is depicted in Fig. 2. e electropherograms of pure water (A) and an ATP standard solution (B) analyzed

analyte occurred.

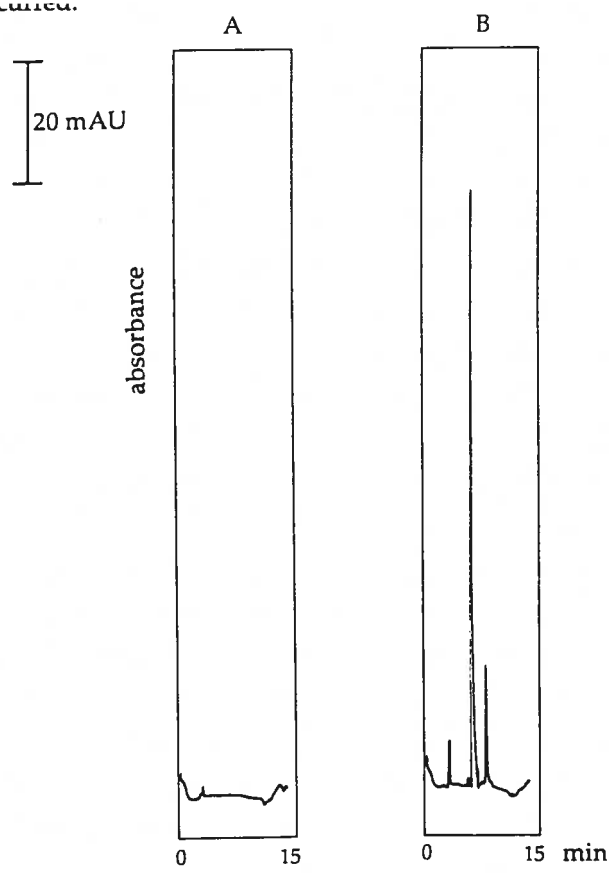


Fig. 2. Electropherograms of (A) pure water and (B) 50 μM ATP in water, analyzed by E CZE. Conditions: ED 20 s, -25 kV, CZE -25 kV; λ = 200 nm.

Characterization and optimization

In order to get more insight into the ED-CZE system the influence of a number of rameters was investigated. First, the capillary inlet position in the acceptor compa ment was examined. Best performance was achieved if the capillary inlet contacted membrane, independent of the location on the membrane.

In comparison to electrokinetic injection ED differs with respect to the voltage appli During ED a voltage is applied over the electrodialysis device (EDD) and the fus silica capillary, whereas during electrokinetic injection the voltage is applied over fused-silica capillary only. On the contrary, one of the similarities is the linear relati

ship between the injected/electrodialyzed amount of analyte and the applied voltage or electrodialysis time. In Fig. 3, the peak area of ATP obtained with ED is plotted as a function of time for different voltages. During these experiments the donor compartment contained 50 µM ATP in water, while the acceptor compartment was filled with pure water. Each data point consists of three measurements. For the lower curve (-5 kV) and for the middle curve (-15 kV), an almost linear relationship exists up to 60 s of electrodialysis time. For the upper curve (-25 kV), however, linearity does not hold above an electrodialysis time of 30 s. In the non-linear region the injected amount of analyte constitutes more than 1 % of the total capillary volume and leads to band broadening. Consequently, the peak width is significantly increased. Nevertheless, due to the low standard deviation also longer electrodialysis times can be used, which is favourable in determining low concentrations. In this chapter, all further experiments were done at an electrodialysis voltage of -25 kV during 20 s which appeared an optimum of the system with respect to time and peak width.

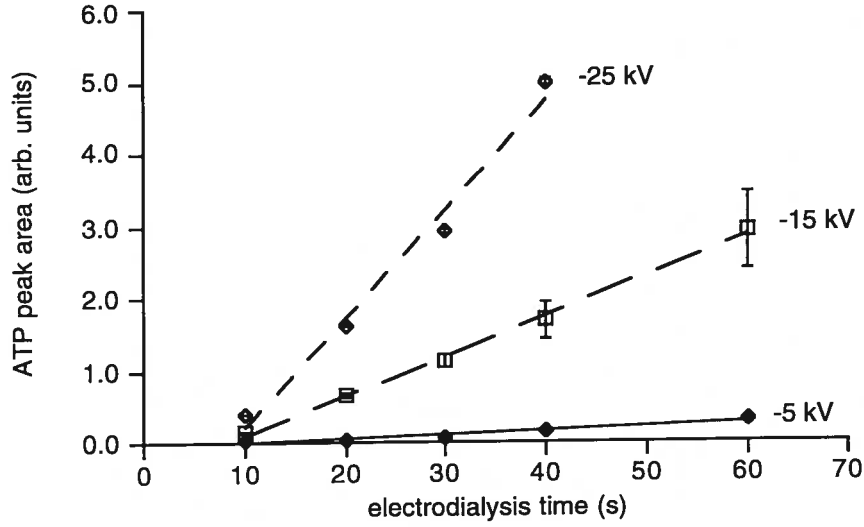


Fig. 3. ATP peak area as a function of the electrodialysis time for different voltages. Conditions: donor compartment: 50 µM ATP in water; acceptor compartment: water; CZE voltage: -25 kV;

with sample solutions, having the same ATP concentration but different ammonium acetate concentrations. The acceptor compartment was filled with pure water. Electrodialysis was performed by applying a voltage of -25 kV during 20 s after which CZE was carried out. Fig. 4 shows the relationship between the peak area of ATP and the ammonium acetate concentration in the sample. Each data point consists of three measurements. As a result of the difference in conductivity with respect to the sample zone field amplification or zone sharpening takes place, similar to the situation in electrokinetic injection of samples with a lower conductivity than that of the CZE buffer [30]. By raising the sample conductivity while keeping the ATP concentration constant, the peak area decreases, leading to a higher limit of detection (LOD). Only at a sample conductivity equal to that of the CZE buffer there is hardly any local difference in the electric field strength. Provided that the acceptor compartment contains pure water, the electrodialysis process is very similar to electrokinetic injection with respect to the sample conductivity.

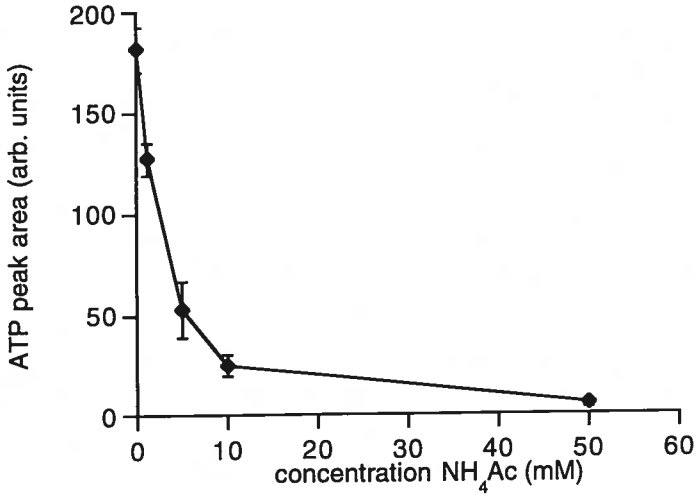
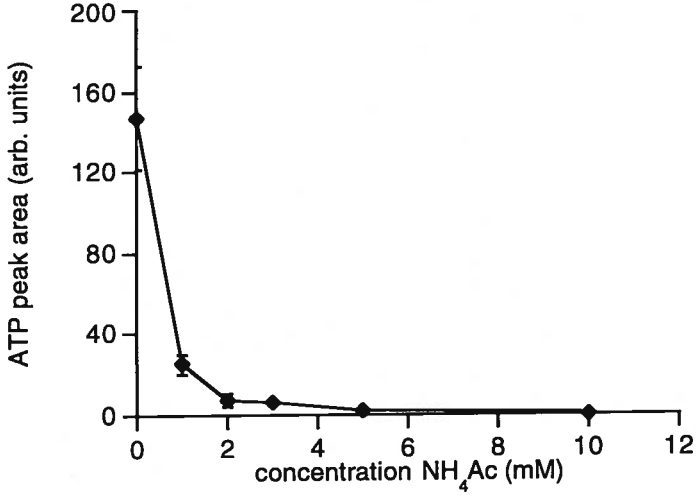


Fig. 4. Effect of the donor conductivity (NH_4Ac concentration) on the ATP peak area. Conditions: donor compartment: 100 µM ATP in different matrices; acceptor compartment: water; ED 20 s, -25 kV; CZE -25 kV; $\lambda = 200$ nm.

xt to the donor compartment, the composition of the acceptor compartment can play ignificant role during electrodialysis. As stated before, the electrodialysis process is ilar to electrokinetic injection if the acceptor compartment contains pure water. But, igher conductivity medium in the acceptor compartment affects the ED performance ATP drastically, which is shown in Fig. 5. Each data point consists of three measure-nts. During all these experiments the donor compartment contained 100 µM ATP in re water. A slight increase of the ammonium acetate concentration in the acceptor npartment resulted in a significant decrease of the peak area. At 10 mM ammonium etate, the ATP signal was even below the LOD. Possibly, analyte stacking occurs at capillary inlet if the acceptor compartment contains pure water. If, however, the ceptor compartment contains a higher conductivity medium than the donor com-rtment stacking might occur at the donor/acceptor interface, resulting in a higher)D. The low ionic strength of the acceptor may rise due to electrodialysis or ctromigration of electrolytes from the donor compartment or the CZE buffer. In or-r to maintain quantitative reproducibility the acceptor compartment is filled with sh water after every run. Furthermore, disposable membranes are used to prevent mbrane fouling.



electrodialysis, was investigated next. Therefore, the protein bovine albumin was add
to the analyte solution at a concentration of 50 mg/ml which is similar to the huma
serum albumin (HSA) concentration in blood plasma. The total concentration of pr
teins in plasma is ca. 70 mg/ml [31]. Using a sample solution of pH 6, bovine album
with a pI of 4.7 is negatively charged and will be introduced into the capillary witho
the use of a membrane. As the molecular mass of albumin (or HSA) is 67,000 and th
membrane molecular mass cut-off was 30 kDa, albumin was retained in the donor com
partment. In Fig. 6 the electropherograms are shown of a sample consisting of 10 µ
ATP and bovine albumin in water (left) and a blank solution containing only bovi
albumin in water (right), analyzed with ED-CZE. The acceptor compartment was fill
with pure water. Small peaks present in both electropherograms can be ascribed
impurities in the bovine albumin.

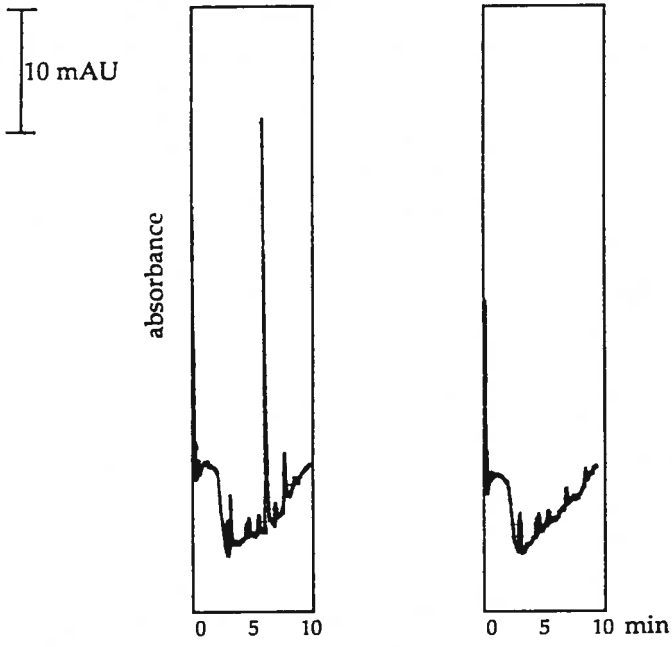


Fig. 6. Electropherograms of 10 µM ATP and 50 mg/ml albumin in water (left) and 50 mg/ albumin in water as the blank (right). Conditions: acceptor compartment: pure water; ED 20 -25 kV; CZE -25 kV; λ = 200 nm.

Quantitative aspects

In order to use ED-CZE in quantitative analysis the system was validated with respect to linearity and sensitivity. For the investigation of the linearity, standard solutions of ATP ranging from 0.5 to 50 μM were analyzed using ED-CZE. The method appeared to be linear over (at least) two decades with a correlation coefficient of 0.9993. Based on a signal-to-noise ratio of 3, a limit of detection (LOD) for ATP in water down to a concentration of 300 nM has been obtained. The LOD is significantly increased in other matrices than water, which is an inherent limitation in CZE. In order to lower the LOD in such situations, analytes can be selectively concentrated in the on-line mode by means of (transient) isotachophoresis [32-36] after electrodialysis.

Applications

I. Analysis of inositol phosphates in fermentation broth

The developed method has been applied to the determination of inositol phosphates in fermentation broth which requires a sample pretreatment in order to prevent capillary clogging and/or wall adsorption by yeast cells. Therefore, fermentation broth was spiked to a concentration of 100 μM inositol hexakis- (IP_6), tetrakis- (IP_4), tris- (IP_3), bis- (IP_2) and monophosphate (IP_1) and subsequently analyzed with ED-CZE. The result is depicted in Fig. 7. IP_6 and IP_4 could not be base-line separated due to the home-built instrumental set-up which complicated a further increase of the electric field strength. Nevertheless, it can be concluded that the electropherogram obtained by ED-CZE is very similar to the one obtained after centrifugation of the sample, followed by electrokinetic injection of the supernatant and CZE (not shown). However, off-line centrifugation-electrokinetic injection takes more than 5 min whereas on-line electrodialysis takes only 20 s.

II. Determination of ATP in plasma

Blank plasma was spiked with ATP to a concentration of 100 μM and analyzed with ED-CZE. Next to the high amount of proteins, plasma contains, among others, chloride, phosphate, sodium, carbohydrates, fatty acids and lipids [31]. In order to enhance the selectivity of the method, UV detection was performed at 259 nm. Fig. 8 shows the

result of the relatively high conductivity of plasma, analysis of a 1:10 diluted plasma sample showed a similar analyte signal.

As the sensitivity of the method is limited for high-conductivity samples, future research will be devoted to on-line, selectively concentrating techniques between ED and CZE. A minor disadvantage of the ED-CZE system is that samples containing analytes bound to macromolecular compounds, e.g. plasma proteins cannot be analyzed as such. In that case, an extra sample pretreatment step must be introduced to release the analyte from the macromolecular compounds.

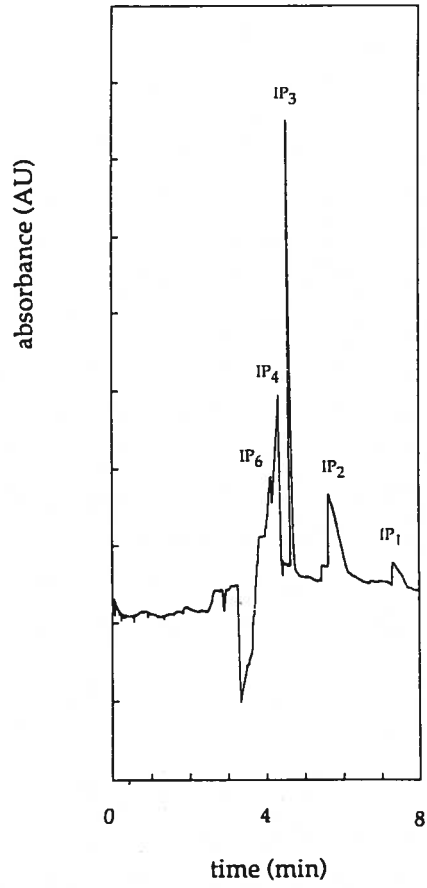
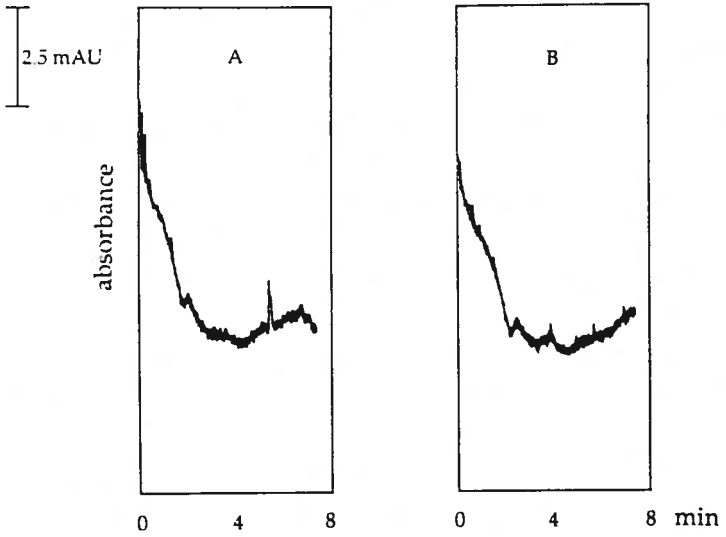


Fig. 7. Determination of inositol phosphates in fermentation broth by ED-CZE. Conditions: CZE buffer: 0.5 mM NDSA, 30 mM acetic acid pH 3, 0.005 % HPMC; indirect UV detection at 214 nm.



. 8. Bioanalysis of ATP by ED-CZE. A = 100 μM ATP in plasma, B = blank plasma. Condi-ns: acceptor compartment: pure water; ED 20 s, -25 kV; CZE -25 kV; λ = 259 nm.

nclusions

ctrodialysis has been coupled on-line to capillary zone electrophoresis. Macromo-ular compounds in the sample, like albumin or yeast cells, are effectively retained by membrane whereas small negatively charged ions are introduced into the capillary only ca. 20 s. Similar to electrokinetic injection, the sample conductivity has a nega-e effect on the amount of analyte electrodialyzed into the capillary. So far, bioanalysis n be performed only at relatively high analyte concentrations. Future research will be used on sensitivity enhancement in ED of high-conductivity samples.

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Chapter 6

Electrodialysis-capillary zone electrophoresis-mass spectrometry

Chapter 6

Electrodialysis-capillary zone electrophoresis-mass spectrometry

bstract

ectrodialysis has been coupled to capillary zone electrophoresis-mass spectrometry r on-line clean-up and analyte concentration. Two different electrodialysis devices ıve been developed, each with its specific features. The first device consisting of a ɔnor and an acceptor compartment, separated by a membrane with a cut-off of ı kDa, offers selectivity based on molecular mass, shape and charge. The second de-ce consists of three compartments, separated by membranes with a cut-off of 500 Da ıd 30 kDa, respectively. In addition to selectivity this device enables analyte enrich-ent between the two membranes. The developed methods have been applied to the ɛtermination of inositol phosphates in fermentation broth and in blood plasma.

ıtroduction

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

ıositol phosphates (IPs) are phosphorylated carbohydrates with interesting character-

to be an appropriate separation technique for these compounds [5] and concentrati
detection limits were improved by using mass spectrometric (MS) detection via a
electrospray interface (ESI) [15]. Until recently, IPs in complex matrices have been pr
treated using anion-exchange SPE [16], centrifugation [3], ultrafiltration [15] or Fe(III
loaded stationary phases [17] all of which are rather laborious, time consuming or di
ficult to automate.

Electrodialysis is a sample pretreatment technique which can be used for sample clea
up and analyte enrichment [18]. By superimposing an electric driving force on a co
centration gradient the dialysis process is accelerated and the selectivity is enhance
In general, an electrodialysis set-up contains a donor and an acceptor phase, separate
by a membrane over which a voltage is applied. The phases are flowing or stagnar
Electrodialysis has been used in the field of biotechnology [19-21] and for neutraliz
tion of alkaline [22-24] or acidic [25] samples prior to separation. Since 1990, sever
researchers described an electrodialysis cell, enabling sample purification and analy
concentration, combined with HPLC [18,26]. Recently, we developed an electrodialys
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 samp
clean-up and analyte enrichment. Two different electrodialysis devices have been d
veloped and are discussed.

Experimental

Chemicals

All inositol phosphates, e.g. inositol monophosphate (2-IP1) as dicyclohexylammoniu
salt and inositol bis- (1,2-IP2), tris- (1,2,6-IP3), tetrakis- (1,2,5,6-IP4), hexakisphospha
(IP6) as sodium salts, were kindly provided by Perstorp Pharma (Perstorp, Sweden
Adenosine-5'-triphosphate (disodium salt hydrate, 98 %) was from Janssen (Beers
Belgium). Acetic acid and methanol were obtained from Baker (Deventer, The Nethe
lands). Ammonium acetate p.a. was purchased from Merck (Darmstadt, Germany
For the preparation of the stock solutions of the analytes and buffer solutions, deio
ized water was used (Milli-Q system, Millipore, Bedford, MA, USA). For the polyacr
lamide coating, 3-(trimethoxysilyl)propylmethacrylate, 98 % (Janssen
tetramethylethylenediamine (TEMED) and ammonium persulphate (Bio-Rad, Ric

mond, CA, USA) and acrylamide (Merck-Schuchardt, Hohenbrunn, Germany) were used.

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, The Netherlands). The plasma was spiked with IP2 and IP3 to a concentration of 10 µM.

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 30 kDa 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.

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 (2x2x2 cm^3) cubes of perspex. Between compartments 1 and 2 a membrane (4) is positioned with a cut-off of 30 kDa. Compartments 2 and 3 are separated by a membrane (5) with a cut-off of 500 Da. Both membranes were 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 mM

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 mass spectrometry.

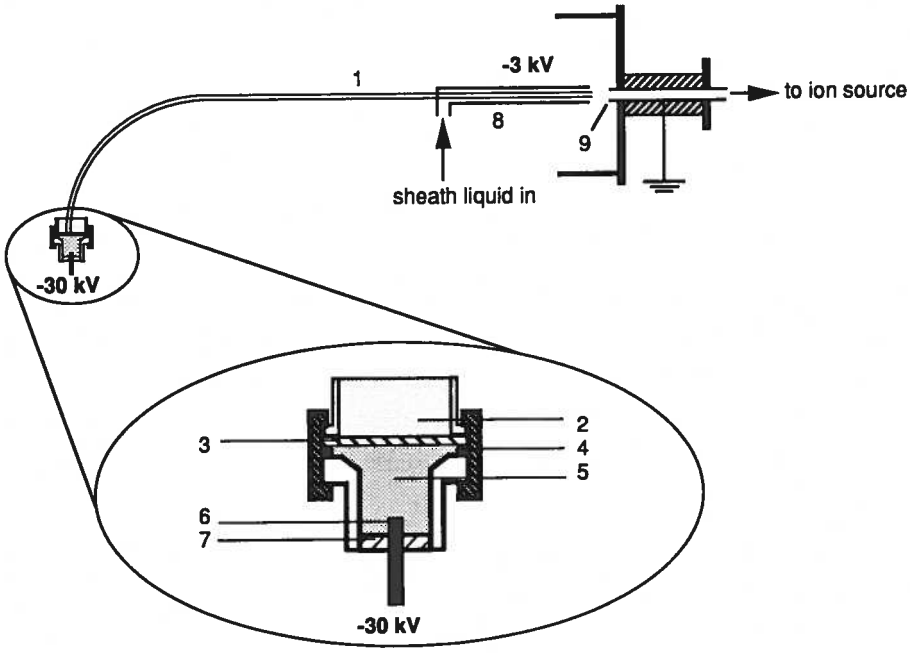
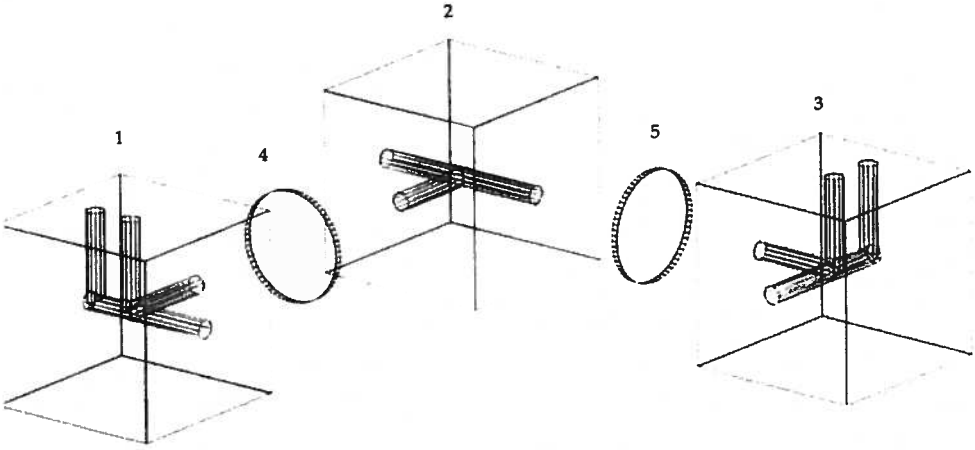


Fig. 1. EDD coupled to CZE-ESI-MS system. 1 = fused-silica capillary, 2 = acceptor compartment, 3 = membrane, 4 = O-ring, 5 = donor compartment, 6 = cathode, 7 = septum, 8 = stainless-steel needle, 9 = sampling capillary.

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) (100 μm I.D., l = 0.8 m) and was coated with polyacrylamide according to the procedure described by Hjert n [28]. At 0.25 m before the capillary outlet UV absorbance detec-

on was performed at 214 nm (CE-adapted-Spectroflow 773, Kratos Analytical Instruents, Ramsey, NJ, USA). The electrophoresis buffer consisted of 10 mM ammonium cetate pH 4.8. CZE was performed at -25 kV after electrokinetic injection (-10 kV) or ter electrodialysis using the CEDD. For safety reasons, the buffer vial containing the gh voltage electrode was positioned in a plexiglass box.



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

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 mM 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 ioniza-

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 o 100 mM ammonium acetate-methanol (10:90, v/v) and was delivered at a flow rate o 1-2 µl/min by a Model 2400 syringe pump (Harvard Apparatus, Edinbridge, UK).

Results and discussion

Electrodialysis-capillary zone electrophoresis-electrospray ionization mass spectrometry

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

Electrodialysis is a sample pretreatment technique which offers selectivity based o 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 dono and the acceptor phase are stagnant. By applying a voltage over the membrane and th fused-silica capillary, only ions smaller than the membrane cut-off and with the appro priate charge and shape are introduced into the capillary. Neutral compounds, oppo sitely charged ions and compounds larger than the membrane pores are retained. A 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 i the direction of the anode is quite high. In order to increase the net velocity, the elec troosmotic mobility in the direction of the cathode has been suppressed by a polyacry lamide coating at the capillary wall. Therefore, during electrodialysis and during CI 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 analyzec using electrodialysis-CZE-ESI-MS (Fig. 3). The electrodialysis time was 10 s. Inosito 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 concentratio of 10 µM. Inositol pentakisphosphate (IP5, $M_r = 580$) was present in the sample as a degradation product of IP6. Whereas inositol phosphates are multiply charged in aque ous solution (pH 4.8), in the gas phase they were detected as singly charged ion

$[M-H]^-$. 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 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.

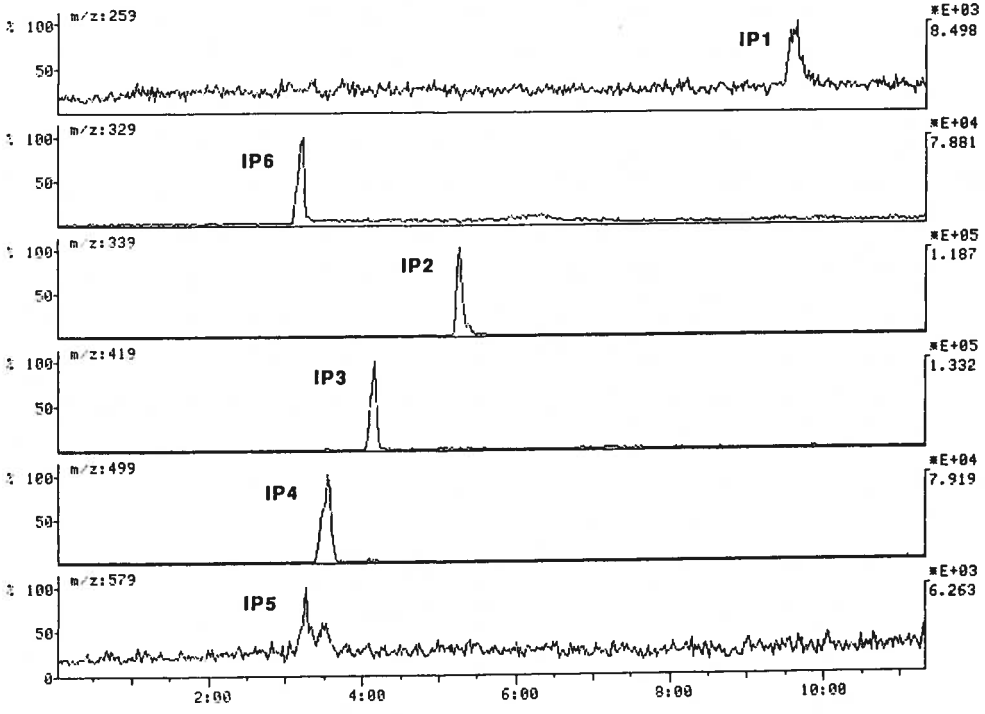


Fig. 3. Mass electropherogram of a standard solution of inositol phosphates (10 μM) after 10 s electrodialysis. MS was performed in the MID mode. For further details see experimental section.

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.

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 $4.6 .10^{-13}$ mol (IP1) to $14.0 .10^{-13}$ 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].

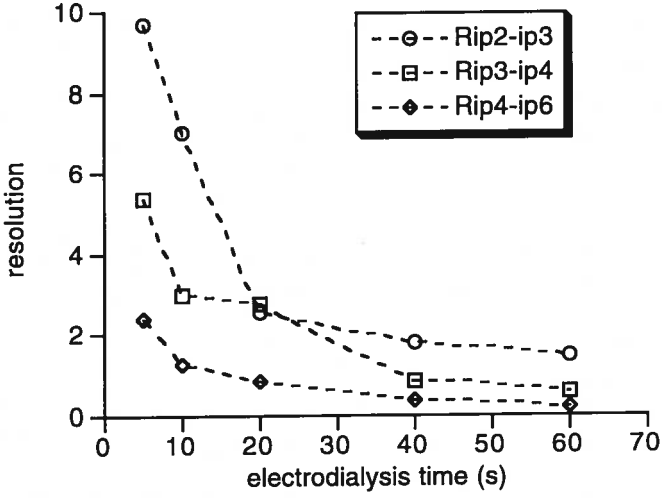
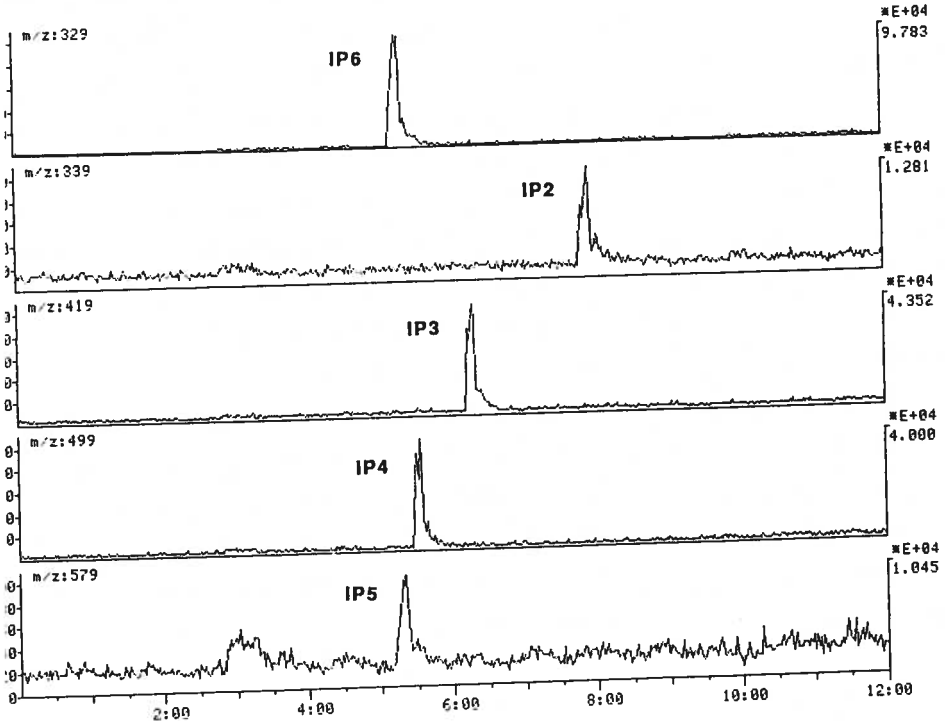


Fig. 4. Separation resolution (R_S) versus electrodialysis time for several inositol phosphates.

entation monitoring

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



The EDD as described above has many advantages as an on-line sample pretreatm technique: it is fast, selective, inexpensive and simple to use. Nevertheless, it lacks c centrating properties. The electrodialysis set-up, described by Debets et al [18] car used for analyte enrichment before LC. However, this set-up is not suitable for CZI it does not selectively concentrate the analytes but also matrix ions which are sma than the membrane cut-off. The analysis of real-life samples in CZE is rather probl atic because of the high concentration of matrix ions which affect the local electric fi strength. As a consequence, a lower amount of analyte is electrokinetically injected a the peak shape may be distorted, leading to higher detection limits. This is an inher limitation in the analysis of real-life samples with CZE which is not overcome by EDD described in the first part of this chapter.

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

In order to investigate the concentrating power of the device a standard solution adenosine triphosphate (ATP) was added to the first compartment. The second and t third compartment were filled with electrophoresis buffer. Fig. 6 shows the electr pherograms of ATP in electrophoresis buffer before (left) and after (right) electrodial sis. A concentration factor of about five has been achieved after 10 min of electrodial sis. During this experiment, all phases were stagnant. The enrichment factor will improved by the use of a flowing donor phase.

EDD versus CEDD

Both the EDD and the CEDD have many advantages but also a few drawbacks. A cor parison between the two devices can be made with respect to selectivity, electrodialys

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.

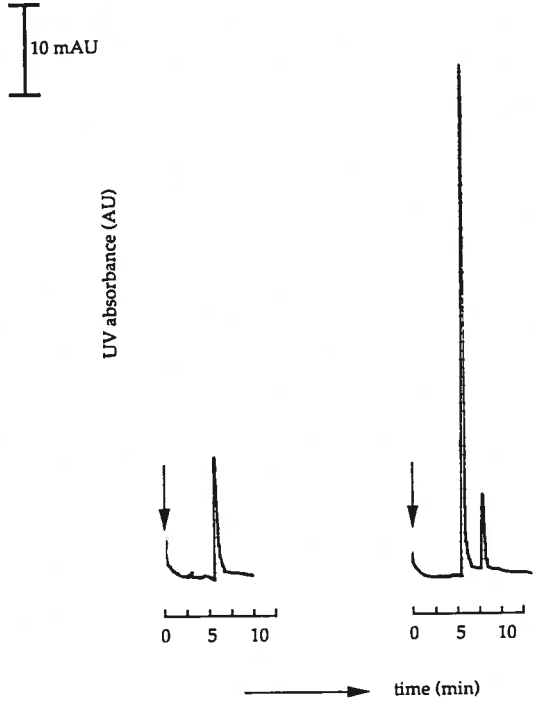


Fig. 6. Comparison between CZE (left) and concentrating electrodialysis-CZE (right) of a solution of ATP in 10 mM ammonium acetate. UV absorbance detection at 214 nm. Electrophoresis buffer: 10 mM ammonium acetate pH 4.8.

Determination of inositol phosphates in blood plasma

The CEDD has been applied to the determination of inositol phosphates in plasma

IP3 to a concentration of 10 µM. Compartments 2 and 3 were filled with electrophoresis buffer. During electrodialysis proteins like albumin, with a molecular mass of 67 000 and a pI value of 4.7, are retained at the first membrane. Most electrolytes in plasma migrate through the second membrane with a cut-off of 500 Da. Although the protonated molecules of IP2 and IP3 have a molecular mass smaller than the membrane cut-off ($M_{IP2} = 340$; $M_{IP3} = 420$), 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 µM (IP2) and 4 µM (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.

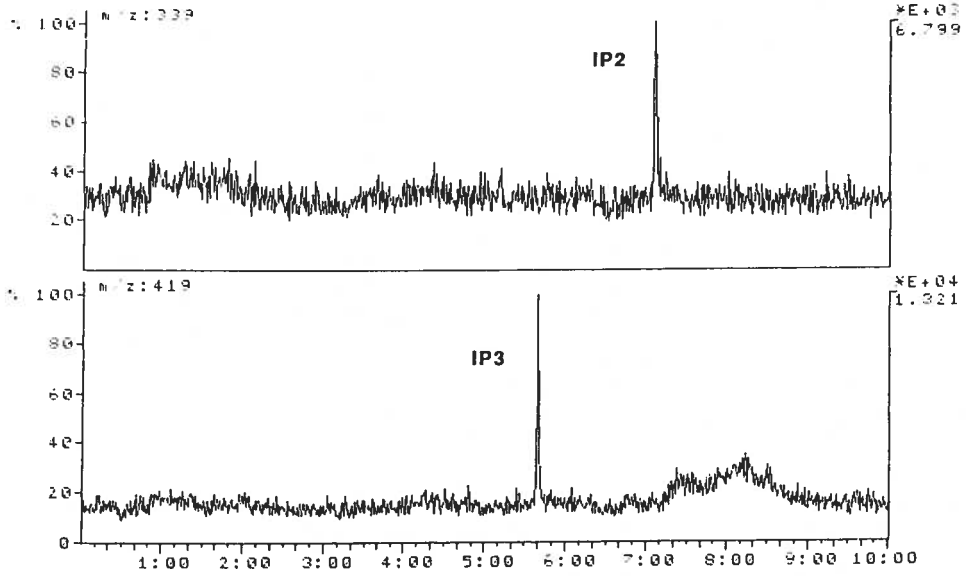


Fig. 7. Mass electropherogram of IP2 and IP3 in plasma after pretreatment with CEDD. MS was performed in the MID mode.

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

Acknowledgment

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Chapter 7

Concentrating electrodialysis-capillary zone electrophoresis

Chapter 7

Concentrating electrodialysis-capillary zone electrophoresis

Abstract

An electrodialysis device for on-line coupling to capillary electrophoresis has been developed. The device consists of three compartments that are separated by two membranes with cut-off values of 500 Da and 30 kDa, respectively. The selectivity of the method is based on charge, molecular mass and shape. A concentration factor of 40-50 has been achieved. Sample clean-up and analyte enrichment take only ca. 5 min. Optimization and characterization of the device have been performed and electrodialysis has been applied to the analysis of an inositol trisphosphate derivative in fermentation broth.

Introduction

Among the separation techniques, capillary electrophoresis (CE) is well known for its high efficiency and short analysis times. Furthermore, CE is selective and simple to use. With respect to environmental considerations, only very small amounts of chemicals and sample are needed. Unfortunately, CE has also certain drawbacks, i.e. the concentration sensitivity, caused by the limited loadability of the capillary and, in the case of spectrophotometric detection, the optical path length of the detection cell. Therefore, attention has been paid to enhancement of the loadability by stacking [1] or field amplification [2], capillary isotachophoresis (CITP) [3-6] and liquid-liquid electroextraction [7]. Besides, preconcentration of analytes has been achieved by the insertion of a small bed of a specific [8-9] or non-specific [10-12] adsorptive solid phase at the inlet of the CE capillary [13]. Investigation has also been focused on the improvement of the detection cell geometry [14] and the coupling of detection techniques to CE with very low concentration detection limits, such as laser induced fluorescence [15].

In general, if CE analysis is performed in a complex matrix, sample pretreatment is required in order to remove compounds that interfere with the characteristics of the analytes or that block the fused-silica capillary. Several sample pretreatment techniques [illegible] CE, either in the off-line or on-line mode, such as

membrane-preconcentration [24], CITP [25] and (concentrating) electrodialysis [26, 27]. Some of these techniques enable the combination of both sample clean-up and analyte enrichment, which is very favourable in the case of trace analysis in complex matrices.

Inositol phosphates (IPs) play a very important role in several research areas, among them, biochemistry [28]. IPs are multiply negatively charged sugars with one to six phosphate groups. Even at very low pH values, they are charged. Therefore, most sample preparation methods, based on neutralized analytes, are not suitable. Electrodialysis is a concentrating and purifying technique that can handle charged analytes.

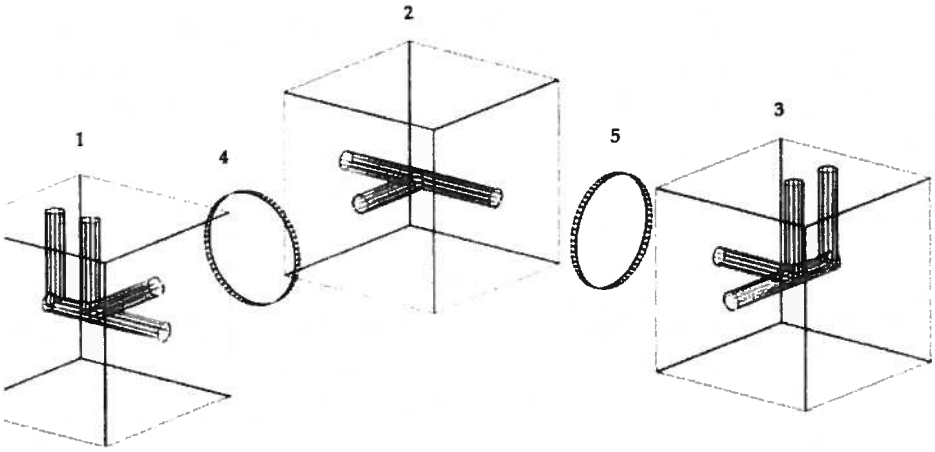
During electrodialysis analytes migrate from the donor compartment to the acceptor compartment by an electric driving force superimposed on a concentration gradient. Electrodialysis can be performed in the static as well as in the dynamic mode [29]. In the off-line mode, electrodialysis has been used for the enrichment of ions [30] and for the neutralization of acidic [31] or alkaline [32, 33] solutions. Furthermore, electrodialysis has been coupled on-line to ion chromatography [34] and to liquid chromatography [35].

Recently, we combined static (concentrating) electrodialysis with capillary electrophoresis [26, 27]. Until now, attention was mainly focused on the selectivity of the device. This paper describes the optimization and characterization of the device with respect to sensitivity. An inositol trisphosphate derivative has been used as a model compound.

Experimental

Chemicals

Ammonium acetate (analytical-reagent grade) was obtained from Merck (Darmstadt, Germany). Acetic acid came from J.T. Baker (Deventer, The Netherlands). Hydroxypropylmethylcellulose (HPMC), with a viscosity of 4000 cP for a 2 % aqueous HPMC solution was purchased from Sigma (St. Louis, MO, USA). Phenylacetate inositol trisphosphate (PIP3) was kindly provided by Perstorp Pharma (Perstorp, Sweden). For the preparation of the stock solutions of the analytes and buffer, deionized water was used (Milli-Q system, Millipore, Bedford, MA, USA). The buffer solution was filtered through a 0.2-μm nylon acrodisc syringe (Gelman Sciences, Ann Arbor, MI, USA). The electrophoresis buffer consisted of 10 mM ammonium acetate, pH 5, and 0.005 % HPMC.



1. Exploded view of the three-compartment-electrodialysis device (1, 2, 3) including two branes with cut-off values of 30 kDa (4) and 500 Da (5).

pment and procedures

laboratory-made electrodialysis device (Fig. 1) consists of three compartments (1, separated by two porous membranes, made of regenerated cellulose, with cut-off ues of 30 kDa (4) and 500 Da (5) (Amicon, Danvers, MA, USA), respectively. The partments are bores with a diameter of 2 mm in cubes of Perspex (2 x 2 x 2 cm^3). r to electrodialysis, the first compartment is filled with sample solution (ca. 50 µl), second and the third compartment with pure water and 10 mM ammonium acetate er, pH 5, respectively. All phases are stagnant. The platinum electrodes are posi- ed in the first (cathode) and the third (anode) compartment. Electrodialysis is per- ned by applying a voltage (150-600 V) over the electrodes for a few minutes. Subse- ntly, the fused-silica capillary (S.G.E., Ringwood, Victoria, Australia) (75 µm I.D., µm O.D., l_{tot} = 0.80 m, l_{det} = 0.55 m) is inserted through a septum onto the mem- ne in the second compartment and electrokinetic injection is carried out at -10 kV 5 s with the cathode in the third compartment and the anode (ground) in the outlet er vial. After injection, the capillary inlet is placed in a buffer vial and capillary e electrophoresis is performed at a voltage of -30 kV (Spellman 1000R, Plainview,

positioned in a plexiglass box. In between analyses, the EDD is taken to pieces and channels are rinsed with pure water. In ca. 1 min, the device is cleaned, filled with f solvents and, if necessary, provided with a new membrane.

Results and discussion

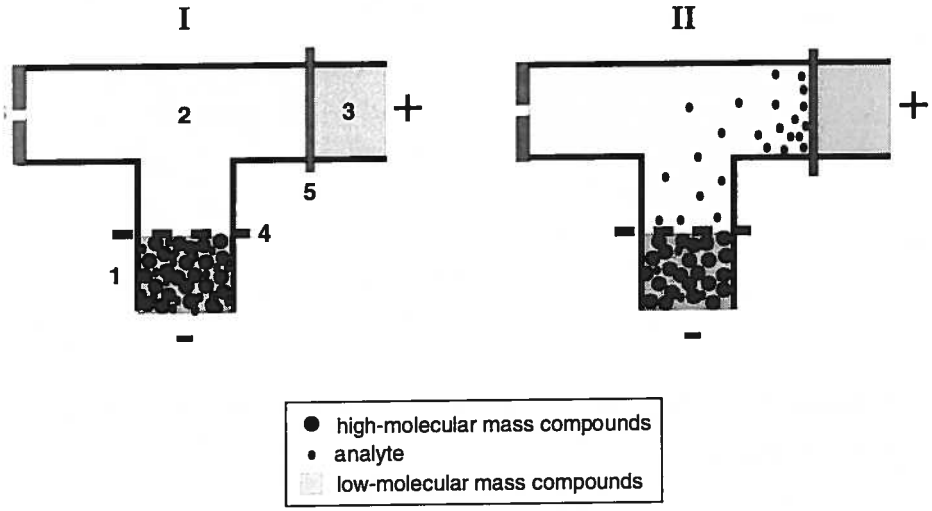
Electrodialysis

Commonly, electrodialysis is performed in a device, containing two compartments donor and acceptor compartment, which are separated by a membrane. The anode cathode are positioned in the electrode compartments [35]. Electrodialysis thus formed allows the separation of high- from low-molecular mass compounds, de mined by the membrane cut-off value. At the same time, positive ions can be separ from negative ions, which is determined by the polarity of the electrodes. Enrichn of ions can be achieved by the use of a flowing donor phase and a stagnant acce phase [29]. With such a device all ions having the same charge and a molecular r below the membrane cut-off value are concentrated in the acceptor compartment. ' method has been combined with ion chromatography [34] and with liquid chroma raphy [35].

Electrodialysis combined with CE requires another approach. Enrichment of the anal together with the low-molecular-mass background ions is not very favourable, as will also increase the sample's conductivity. Electrokinetic injection of high-condu ity samples is disadvantageous in CE as the sample conductivity affects the amou analyte injected. Whereas a poorly conductive sample leads to stacking/field amp cation (high local electric field strength), from a highly conductive sample, less ana is injected because of a decrease of the local electric field strength. This would dimi the concentrating effect on the analyte(s) achieved during electrodialysis. There selective analyte enrichment is needed.

So far, two devices have been developed for on-line coupling of electrodialysis to One device consists of two compartments and one membrane and allows sample cl up in only 10-20 s [26, 27]. The other device (Fig. 1) consists of three compartments two membranes with different cut-off values and enables not only sample purifica but also selective analyte enrichment [27]. A schematic representation of this electr alysis process is shown in Fig. 2. Before electrodialysis, the first compartment is f

h a sample solution, consisting of molecules with different molecular masses. The ond and third compartment are filled with water and electrophoresis buffer, respec- ely. By applying a voltage of ca. 600 V, ions with the appropriate charge, in this case ons, and a molecular mass smaller than the size of the membrane pores will migrate m the first to the second compartment. Anions with a molecular mass below the ond membrane cut-off value will eventually migrate into the third compartment, ereas anions larger than the size of the (second) membrane pores are retained at the mbrane. Thus, by a proper selection of the membranes, i.e. the size of the first mem- ne pores (cut-off: 2-100 kDa) being larger than the analyte and the second mem- ne pores (cut-off: 100 or 500 Da) being smaller than the analyte, the analyte can be arated from high-molecular-mass compounds (first compartment) as well as from -molecular-mass compounds (third compartment). Moreover, the device enables selective enrichment of analytes on the membrane in the second compartment [27]. ne pores of the second membrane are too large, the analyte will migrate through the mbrane together with the matrix ions. Consequently, no enrichment of analyte will ur. In this case, the sample is only purified from high-molecular-mass compounds l eventually loss of analyte occurs.



. 2. *Electrodialytic process. I = before electrodialysis, II = after electrodialysis.* 1, 2, 3 = com-

In order to achieve the best performance, several parameters have been investiga
that have an effect on the electrodialysis process. First, attention was focused on
influence of the applied electric field on analyte enrichment. Therefore, compartmer
was filled with a sample solution consisting of 10 µM PIP3 (M_r = 774.1) in electroph
sis buffer. Using an electrodialysis time of 5 min, the electrodialysis voltage was var
from 0 to 800 V. At 600 V, the best result was obtained. At a voltage higher than 60
too much heat was generated and gas bubbles were formed. If the applied voltage v
zero and, thus, pure dialysis was carried out for 5 min, no PIP3 could be detect
Therefore, the contribution of diffusive dialysis to the electrodialysis process in the f
5 min can be neglected.

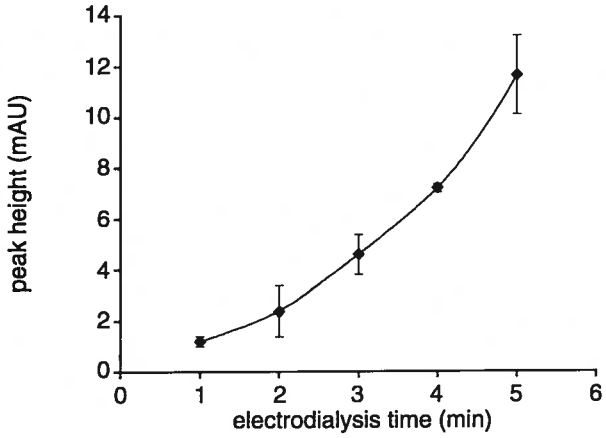


Fig. 3. Peak height of PIP3 versus electrodialysis time (n = 3). Electrodialysis voltage, 60
electrokinetic injection, 15 s, -10 kV; CZE, -30 kV.

Next, the electrodialysis time was optimized. Again, compartment 1 was filled with
µM PIP3 in electrophoresis buffer. The electrodialysis time was varied from 1 to 10 r
at a voltage of 600 V. From 1 to 5 min, the enrichment of PIP3 was improved, wh
resulted in a non-linear curve (Fig. 3, n = 3), whereas at an electrodialysis time of 8 o

min, too much heat was generated, leading to the formation of gas bubbles and a reduction of the current. Presumably, the non-linearity of the curve is also due to Joule heating, leading to a decrease in the viscosity and an increase in the electrophoretic mobility of PIP3. Obviously, the dialysis time has to be regulated accurately to achieve reproducible results.

In Fig. 4, two electropherograms are depicted, obtained after electrokinetic injection and capillary zone electrophoresis (CZE) of a solution containing 10 μM PIP3 in electrophoresis buffer, without (A) and with (B) electrodialysis pretreatment. In this figure, the enrichment is clearly shown by the enormous increase in peak height. The concentration factor for PIP3 was ca. 50 in only 5 min of electrodialysis time. Besides, two analyte impurities with higher electrophoretic mobilities than that of PIP3 can be distinguished in the electropherogram. The impurities were also concentrated in the electrodialysis device due to their molecular masses, 656 and 756, respectively, which were determined with CZE coupled to mass spectrometric detection [36]. The concentrating effect on the impurities was higher than on PIP3, due to their higher electrophoretic mobilities.

In order to investigate the effect of the sample conductivity on analyte enrichment, several sample solutions, all containing 10 μM PIP3, were electrodialyzed. The sample conductivity was varied by changing the ammonium acetate concentration. As reference experiments, electrokinetic injection followed by CZE was performed with identical sample solutions. As has been described by several researchers, analyte stacking can be achieved during electrokinetic injection by the use of a poorly conductive matrix and a highly conductive electrophoresis buffer in the fused-silica capillary [37]. Using a CZE buffer of 10 mM of ammonium acetate, the effect of sample conductivity on the electrokinetic injection is shown in Fig. 5 (diamonds). A sample matrix of 10 mM ammonium acetate did not cause any stacking or dilution. However, a decrease of the conductivity in the sample leads to a higher peak height of PIP3, and vice versa.

A similar relationship between matrix conductivity and peak height was observed after electrodialysis-electrokinetic injection-CZE (Fig. 5, triangles). However, the PIP3 peak height in this curve is much higher, due to the concentrating effect of electrodialysis. Also, for this curve, a highly conductive sample solution appeared to be disadvantageous for PIP3 peak height. Acetate ions, which are smaller than the membrane pores,

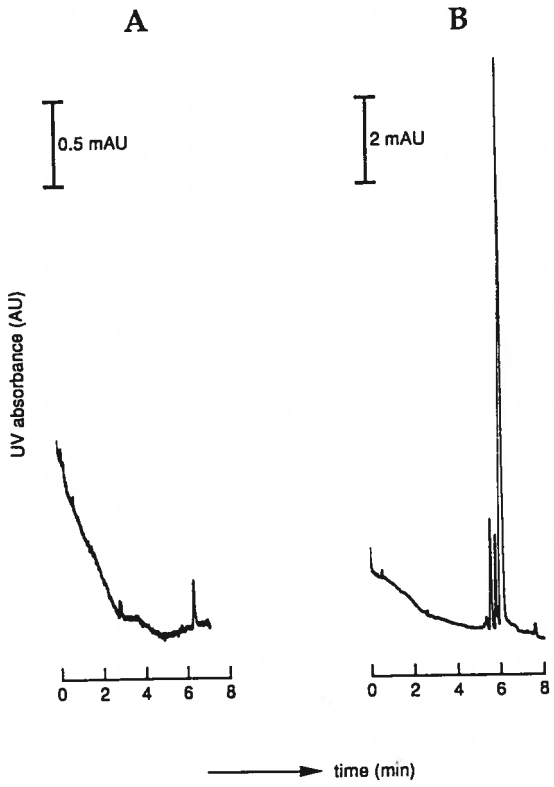


Fig. 4. Electropherograms of 10 μM PIP3 after electrokinetic injection-CZE without (A) and with (B) electrodialytic pretreatment. UV detection was at λ = 200 nm.

of the first compartment before electrodialysis. Consequently, the initial acetate concentration in the sample has an effect on the electrokinetic injection performed after electrodialysis. PIP3, however, has been selectively concentrated on the second membrane, as its molecular mass is larger than the pore size.

Furthermore, in Fig. 5, the enrichment curve (bullets) is constructed as the quotient of the electrodialysis curve and the electrokinetic curve. For PIP3 samples with an ammonium acetate concentration equal to or higher than 10 mM, the enrichment factor was more than 50. For a poorly conductive sample, however, the extra enrichment by electrodialysis, on top of the stacking achieved during electrokinetic injection, is rather low

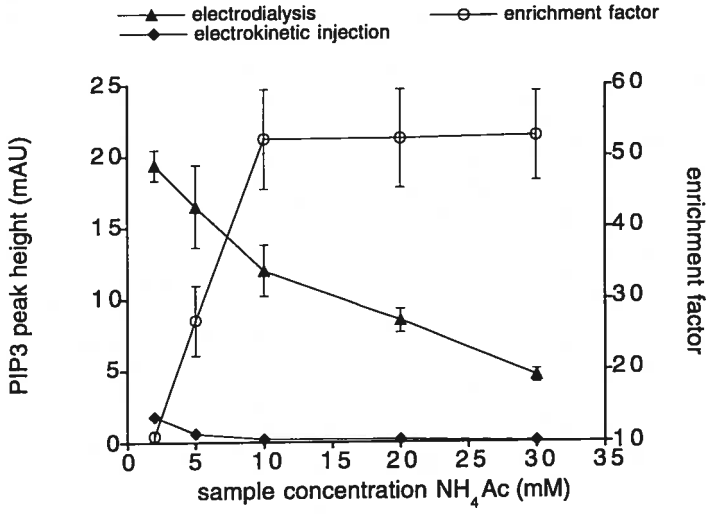


Fig. 5. Effect of the conductivity of the sample matrix (ammonium acetate concentration) on the peak height of 10 µM PIP3 (left axis) and the enrichment factor (right axis). A comparison between electrokinetic injection with (triangles) and without (diamonds) electrodialysis pretreatment is shown. The bullets indicate the enrichment factor of PIP3 as a function of sample conductivity.

This is probably the result of the buffer solution (10 mM ammonium acetate) in the third compartment, which increases the conductivity in the second compartment during electrodialysis, thus affecting electrokinetic injection after electrodialysis. Fig. 5 clearly demonstrates that sample pretreatment using electrodialysis and electrokinetic injection prior to CZE is influenced by the conductivity of the sample. Calibration of the system is recommended for accurate quantitative analysis.

Because the electrodialysis device has been developed for trace analysis in complex matrices, its performance was tested at the submicromolar level. Unexpectedly, the concentration factor of ca. 50 could not be achieved at PIP3 concentrations below 5 µM. This phenomenon appeared to be caused by adsorption of PIP3 to the Perspex electrodialysis device. By a competitive mechanism between PIP3 and another inositol phos-

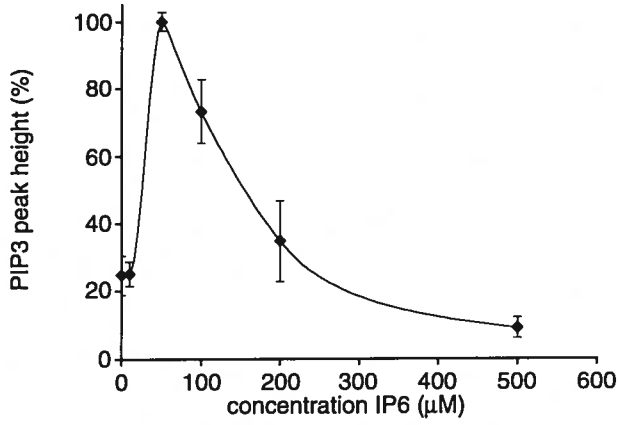


Fig. 6. PIP3 peak height after electrodialysis-electrokinetic injection-CZE versus the concentration of IP6 added to the sample (n = 3). PIP3 concentration, 1 µM in electrophoresis buffer.

sample (n = 3). The peak height of PIP3 is actually the result of two effects. First, IP6 reduces the wall adsorption of PIP3 during electrodialysis, leading to a higher PIP3 signal (Fig. 6). Second, IP6, having a molecular mass of 660, is also concentrated during electrodialysis. Due to this increase of the conductivity, IP6 has a negative effect on the electrokinetic injection of PIP3, performed after electrodialysis (Fig. 6). An optimum of both effects was obtained at 50 µM IP6.

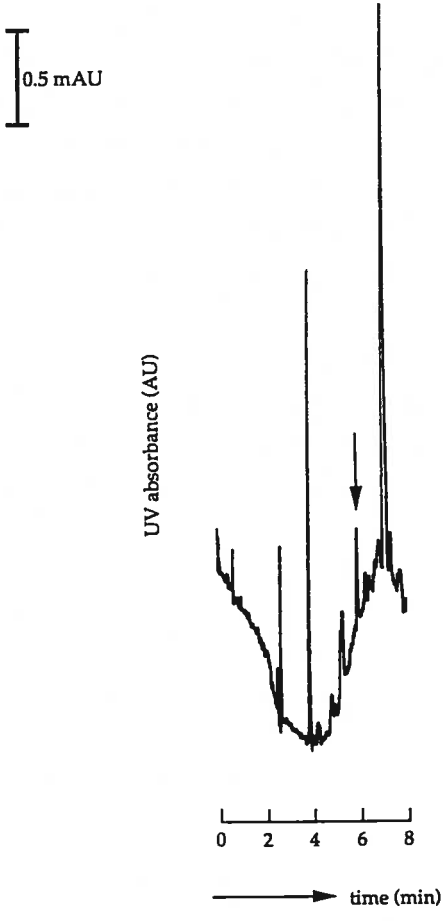
Quantitative aspects

For quantitative purposes, a comparison has been made between electrokinetic injection with and without electrodialysis pretreatment. The following parameters were compared: The reproducibility, expressed as the relative standard deviation (R.S.D.), the linearity, characterized by the correlation coefficient, and the concentration limit of detection (CLOD). All experiments were carried out for standard samples of PIP3 in electrophoresis buffer and for the electrodialysis experiments, 50 µM IP6 was added to the sample. The reproducibility of electrokinetic injection without electrodialysis was significantly higher (R.S.D. = 7.8, n = 5) than with electrodialysis (R.S.D. = 13.8, n = 5). In order to investigate the linearity of the method as well as the CLOD, a calibration

ot was constructed. It showed that, without electrodialysis pretreatment, the correla-
on coefficient was 0.996 in the concentration range 5-500 µM (n = 3). With electrodi-
ysis pretreatment, the correlation coefficient was 0.989 in the concentration range 0.1-
µM (n = 3). The CLOD was 100 nM with electrodialysis compared to 5 µM without
ectrodialysis.

etermination of IP3 derivative in a complex matrix

g. 7 demonstrates the usefulness of electrodialysis pretreatment for the analysis of
P3 in fermentation broth. The yeast cell matrix, containing high- as well as low-molecu-



500 nM and 50 µM, respectively. In this complex matrix, the addition of 50 µM IP6 ha the same effect as in a standard solution of PIP3. The cut-off values of the membrane were 30 kDa and 500 Da. Under these conditions, however, too many compounds fror the matrix were still being captured in the second compartment. Therefore, the sele tivity was improved by using a membrane with a cut-off value of 2 kDa. The result shown in Fig. 7. Only negatively charged compounds with a molecular mass betwee 500 and 2000 Da are trapped and concentrated. Thus, the selectivity of the sample pu rification can be modified without affecting the concentrating effect significantly.

Conclusions

An electrodialysis device has been developed and described for on-line coupling t capillary electrophoresis. It allows selective analyte enrichment and sample clean-up a the same time. The electrodialysis time was only 5 min and a concentration factor of 4(50 has been achieved. In addition to the speed, the developed technique is relativel cheap, uses a small amount of sample (ca. 50 µl), a small volume of other chemicals, i. electrophoresis buffer, and does not use any organic modifiers.

Acknowledgements

The authors wish to thank Henk Verpoorten for his skillful help in the development (the electrodialysis device. Perstorp Pharma is kindly acknowledged for support.

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Chapter 8

Conclusions and perspectives

Chapter 8

Conclusions and perspectives

Although research in the field of capillary electrophoresis (CE) is far from finished, it has obviously established its place outside the fundamental research laboratories. For (bio)analysis, CE has become a very attractive separation technique complementary to liquid chromatography (LC). So far, a large number of sample pretreatment and detection methods formerly applied in LC has been successfully adapted to the capillary format. In addition, new technologies have been developed and coupled (on-line) to CE.

Still, in the field of CE attention is focused on the development of techniques enabling trace analysis and/or analysis in complex matrices. Preferably, sample purification, analyte enrichment and detection are carried out in the on-line mode.

Electrodialysis coupled on-line to CE offers the advantage of both sample purification and analyte enrichment. Three-compartment electrodialysis is a very flexible technique that can be applied to almost any charged analyte, by a proper choice of the membrane pores and the electrode polarity. Besides, if combined with mass spectrometry (MS), structure elucidation can be carried out simultaneously. For example, the cell contents, i.e. peptides, in in-vitro studies can be selectively isolated, enriched and identified after the cells have been lysed.

With respect to CE-MS, future research will focus on a more efficient transfer of analytes from the CE capillary into the mass spectrometer in order to improve the sensitivity. Yet, several researchers have improved the detection limits about one order of magnitude by the development of miniaturized electrospray interfaces. Furthermore, the miniaturization of CE to the chip-format seems to be very promising as it offers the advantages of extremely small sample volumes, speed of analysis and, possibly, portable systems for analysis in the field. Speed is particularly important with respect to combinatorial chemistry and high-throughput screening of the synthesized compounds. However, the miniaturized CE systems lack sensitivity especially in combination with spectrophotometric detection devices. Consequently, another research topic will be sensitivity enhancement, e.g. based on enzymatic multiplication techniques [1].

Summary

In this thesis, attention is focused on the development of electromigration technology using inositol phosphates and derivatives as model compounds. Every chapter describes the development of one particular technique or the coupling of techniques.

In Chapter 1, the theory of capillary electrophoretic (CE) separation has been described (Chapter 1.2) as well as the detection methods and sample pretreatment techniques combined with CE so far. Special attention is paid to indirect UV absorbance detection, mass spectrometric detection (Chapter 1.3) and electrodialytic sample pretreatment (Chapter 1.4). Chapter 1.5 gives a brief review about the analysis of inositol phosphates and derivatives.

Chapter 2 describes capillary zone electrophoresis (CZE) using indirect UV absorbance detection to monitor inositol phosphates in fermentation broth. 1-Naphtol-3,6-disulfonic acid appeared to be an appropriate chromophore especially for inositol trisphosphate (IP3) owing to the similar electrophoretic mobilities. All inositol phosphates were separated with a total analysis time of only 13 min, the sample pretreatment (centrifugation) included.

The detection limits of inositol phosphates were improved at least one order of magnitude by coupling CZE to mass spectrometry (MS) via an electrospray interface (ESI) (Chapter 3). The electrophoresis buffer and the sheath liquid contained ammonium acetate and methanol, 90:10 and 10:90 (v/v), respectively. A stable electrospray was obtained in the negative ionization mode. The structure of a synthesized IP3 derivative and impurities could be confirmed and elucidated. Besides, the synthesis yield has been well estimated.

Chapter 4 describes the development of a very selective sample pretreatment for the determination of inositol phosphates in plasma. The sample pretreatment is based on the isolation after complexation of inositol phosphates with iron(III) loaded on an adsorbent. Sodium dodecylsulfate was added to the plasma samples to release the plasma proteins from the analytes. Desorption of the analyte from the adsorbent was achieved by displacement using a high concentration (10 mM) of phytic acid (IP6). The recoveries, determined with CZE-(indirect) UV detection, amounted to 65 % (S.D. = 2.2 %) and

UV absorbance detection (Chapter 5). The device consists of a donor (sample) and acceptor compartment separated by a porous membrane. By applying a high voltage analytes with the appropriate charge and a molecular mass smaller than the pores are electrodialyzed into the fused-silica capillary. Electrodialysis takes only 20 s and has been successfully applied to adenosine triphosphate in blood plasma and to inositol phosphates in fermentation broth.

Chapter 6 describes the coupling of electrodialysis to CZE-ESI-MS. Two different electrodialysis modules have been developed and discussed. One device consists of two compartments separated by one membrane (Chapter 5) whereas the other device contains three compartments and two membranes with different molecular mass cut-off values. Both modules offer selectivity based on charge, molecular mass and shape and, in addition, the three-compartment device permits analyte enrichment. The combination with MS is an excellent choice as inositol phosphates are not UV absorbent and (if required) structure information/elucidation of unknown compounds can be obtained.

The three-compartment electrodialysis device has been characterized and optimized for analyte enrichment (Chapter 7). By using membranes with molecular mass cut-off values of 30 kDa and 500 Da, respectively, for an IP3 derivative ($M_r = 774$) a concentration factor of ca. 50 could be achieved in only 5 min of electrodialysis time.

Finally, in Chapter 8 the developed techniques are briefly discussed and future research topics are mentioned, electromigration technology included.

Samenvatting

Het onderwerp van dit proefschrift is de ontwikkeling van capillaire electromigratietechnologie in de analytische chemie waarbij inositolfosfaten en derivaten als modelcomponenten fungeren. Ieder hoofdstuk beschrijft een bepaalde techniek of de koppeling van diverse technieken.

In Hoofdstuk 1 wordt de theorie van capillaire electroforetische scheiding beschreven (Hoofdstuk 1.2) en de tot nu toe daaraan gekopppelde monstervoorbewerking- en detectiemethoden. De aandacht is vooral gericht op indirecte UV absorptiedetectie, massaspectrometrische detectie (Hoofdstuk 1.3) en monstervoorbewerking gebaseerd op electrodialyse (Hoofdstuk 1.4). Hoofdstuk 1.5 geeft een kort overzicht van (de analysemethoden voor) inositolfosfaten en derivaten.

Hoofdstuk 2 behandelt de combinatie van capillaire zone electroforese (CZE) met indirecte UV absorptiedetectie voor het monitoren van inositolfosfaten tijdens een fermentatieproces. 1-Naftol-3,6-disulfonzuur bleek een zeer geschikte chromofoor, met name voor inositoltrisfosfaat (IP3), door de vergelijkbare electroforetische mobiliteiten. Met behulp van deze methode konden alle inositolfosfaten in minder dan 13 min van elkaar gescheiden worden, inclusief de monstervoorbewerking bestaande uit het centrifugeren van het fermentatiemonster.

Door CZE via een electrospray interface (ESI) met massaspectrometrie (MS) te koppelen (Hoofdstuk 3) konden de detectielimieten voor inositolfosfaten met ten minste een factor 10 verbeterd worden. De electroforesebuffer en de sheath vloeistof bestonden uit ammoniumacetaat en methanol, in de verhoudingen 90:10 en 10:90 (v/v). Een stabiele electrospray werd verkregen in de negatieve ionen mode. De structuur van een gesynthetiseerd IP3-derivaat en de bijbehorende onzuiverheden werden bevestigd en opgehelderd. Verder kon de synthese-opbrengst met een behoorlijke nauwkeurigheid bepaald worden.

Hoofdstuk 4 beschrijft de ontwikkeling van een zeer selectieve monstervoorbewerking voor de bepaling van inositolfosfaten in plasma. De monstervoorbewerking is gebaseerd op de isolatie na complexatie van inositolfosfaten met een ijzer(III) beladen adsorbent. Natriumdodecylsulfaat werd aan het monster toegevoegd om plasma-eiwitten vrij te [illegible] van de analieten. Desorptie van het IP3(-derivaat) van het adsorbent werd bereikt

65 % (S.D. = 2.2%) in plasma en 88 % (S.D. = 5.7 %) in water.

Een electrodialysemodule is ontwikkeld en on-line gekoppeld met CZE en indirecte UV absorptiedetectie (Hoofdstuk 5). De module bestaat uit een donor- (monster) en acceptorcompartiment die van elkaar gescheiden zijn door middel van een membraan. Door een hoog voltage aan te leggen migreren de analieten met de juiste lading en een molecuulmassa kleiner dan de membraanporiën vanuit het monstercompartiment door de membraan naar het fused-silica capillair. Electrodialyse duurt slechts ca. 20 s en is succesvol toegepast voor de bepaling van adenosinetrifosfaat in plasma en inositolfosfaten in een fermentatiemengsel.

Hoofdstuk 6 beschrijft de koppeling van electrodialyse met CZE-ESI-MS. Twee verschillende modules zijn ontwikkeld en worden bediscussieerd. De ene module bestaat uit twee compartimenten die worden gescheiden door een membraan (Hoofdstuk 5) terwijl de andere module uit drie compartimenten en twee membranen met verschillende poriegrootte bestaat. De selectiviteit van beide modules is gebaseerd op de lading, de molecuulmassa en de vorm van het molecuul. Bovendien kunnen analieten met behulp van het drie-compartimentenmodel geconcentreerd worden. De combinatie met MS is een zeer geschikte keus gezien het feit dat inositolfosfaten UV-licht niet absorberen en, indien vereist, structuurinformatie / structuuropheldering van onbekende verbindingen kan worden verkregen.

In Hoofdstuk 7 wordt de drie-compartimenten-electrodialysemodule gekarakteriseerd en geoptimaliseerd voor analietverrijking. Door membranen met een molecuulmassa cut-off waarde van 30 kDa en 500 Da te gebruiken kan voor een IP3-derivaat ($M_r = 774$) een concentreringsfactor van ca. 50 bereikt worden in slechts 5 min electrodialysetijd.

Tenslotte worden in Hoofdstuk 8 de ontwikkelde technieken kort bediscussieerd en wordt de aandacht gevestigd op toekomstig onderzoek waaronder electromigratietechnologie.

Curriculum vitae

Brigitte Agnes Peter Buscher werd geboren op 2 november 1967 in Weert. Na het behalen van het gymnasium β diploma aan het Bisschoppelijk College (Weert) in 1986, begon zij in september van dat jaar aan de opleiding OK-assistente, richting Anaesthesiologie, in het Academisch Ziekenhuis te Leiden. Deze opleiding werd in augustus 1989 met succes afgerond waarna zij Biomedische Wetenschappen studeerde aan de Rijksuniversiteit Leiden, in de faculteit der Geneeskunde. Na het behalen van de propaedeuse in augustus 1990 werd aangevangen met de bovenbouwstudie Bio-Farmaceutische Wetenschappen in de faculteit der Wiskunde en Natuurwetenschappen. Het onderzoek in het kader van de hoofdvakstage werd uitgevoerd binnen de Sectie Analytische Chemie van het Leiden/Amsterdam Center for Drug Research (LACDR) waarna in augustus 1993 het doctoraalexamen werd behaald. In oktober 1994 ontving zij de KNMP prijs.

Vanaf september 1993 tot en met augustus 1997 verrichtte zij onder leiding van dr. U.R. Tjaden en prof. dr. J. van der Greef promotie-onderzoek bij de Sectie Analytische Chemie van het LACDR. Het promotie-onderwerp was de ontwikkeling van electromigratiemethoden waaronder capillaire electroforese en daaraan gekoppelde monstervoorbewerking en detectiemethoden. De resultaten hiervan staan beschreven in dit proefschrift.

Sinds 1 oktober 1997 is zij als projectleider werkzaam bij TNO Voeding, Divisie Analytical Sciences, Bio-Farmaceutische Analyse, te Zeist.

Nawoord

Promotie-onderzoek is een uitermate uitdagende en soms zeer zware bezigheid. Inzet alleen is niet de sleutel tot succes. Allerlei andere factoren spelen een rol zoals creativiteit, zelfvertrouwen, doorzettingsvermogen en inzicht. In deze moeilijke periode heb ik voortdurend steun ondervonden van vele mensen om mij heen die ik graag zou willen bedanken. Allereerst wil ik alle collega-promovendi, analisten en stage-studenten noemen op de afdeling Analytische Chemie voor hun bijdrage aan de goede sfeer waardoor het afscheid veel te zwaar was. Een aantal van hen wil ik graag persoonlijk noemen: Mareike Lutz (Karlsson) met wie ik onvergetelijke (onderzoeks)ervaringen gedeeld heb, Maite Villaverde-Herraiz (Oosterkamp) vanwege haar Spaanse hartelijkheid, Erik van der Vlis en Aaike Oosterkamp (die Franse chansons wel kunnen waarderen) en Martin Mazereeuw, voor hun humoristische toevoeging aan de sfeer en de wetenschappelijke interesse, en uiteraard Bea Reeuwijk voor haar luisterend oor en de gezelligheid in het lab.

De wetenschappelijke staf heeft een essentiële bijdrage geleverd aan mijn wetenschappelijke, technische en met name persoonlijke ontwikkeling gedurende de afgelopen vier jaar.

I would like to acknowledge the pleasant cooperation with Eva Andersson, Sinnika Okkola and Lena Ramnemark from Perstorp Regeno, Perstorp, Sweden.

Tenslotte wil ik mijn familie bedanken en ben ik mijn naaste vrienden en vriendinnen erkentelijk voor hun belangstelling en begrip, ook als ik even geen tijd voor hen had. Marinus' steun en vermogen mij op te peppen tijdens mijn talloze dipjes zijn voor mij van onschatbare waarde geweest.

List of publications

B.A.P. Buscher, H. Irth, E.M. Andersson, U.R. Tjaden and J. van der Greef.
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B.A.P. Buscher, A.J.P. Hofte, U.R. Tjaden and J. van der Greef.
On-line electrodialysis-capillary zone electrophoresis-mass spectrometry of inositol phosphates in complex matrices.
J. Chromatogr. A, 777 (1997) 51

B.A.P. Buscher, U.R. Tjaden and J. van der Greef.
Three-compartment-electrodialysis device for on-line sample clean-up and enrichment prior to capillary electrophoresis.

Stellingen

behorende bij het proefschrift

'Capillary electromigration strategies in inositol phosphate analysis'

1. Voor de optimalisering van de combinatie van de 3-compartimenten-electrodialysemodule en capillaire electroforese met massaspectrometrie dient een compromis te worden bereikt tussen de buitendiameter van de capillairen in de electrodialysemodule en de binnendiameter van het scheidingscapillair.

   Dit proefschrift.

2. Voor de koppeling van capillaire electroforese met massaspectrometrie via een nano-electrospray interface is een (electroosmotische) flow noodzakelijk.

   Dit proefschrift.

3. Bij de bewering dat de 'high-throughput' microchip in farmacokinetische studies een belangrijke rol gaat spelen wordt het feit, dat niet de scheiding maar de monstervoorbewerking de snelheidsbepalende stap is, onvoldoende belicht.

   C. Henry, Anal. Chem., 69 (1997) 359A

4. De combinatie van membraan-preconcentrering met capillaire electroforese, waarbij de geëlueerde verontreinigingen tijdens de monstervoorbewerking via het scheidingscapillair worden afgevoerd, is weinig elegant.

   A.J. Tomlinson, L.M. Benson, S. Jameson, D.H. Johnson and S Naylor, J. Am. Soc. for Mass Spectrometry, 8 (1997) 15

5. Het gebruik van 'supported-liquid membranes' in combinatie met capillaire electroforese is slechts dan succesvol, indien de 'double-stacking' procedure voor analietverrijking overbodig wordt gemaakt.

   S. Pálmarsdóttir, E. Thordarson, L.-E. Edholm, J.Å. Jönsson and L. Mathiasson. Anal. Chem., 69 (1997) 1732

kernspinresonantiespectrometrie gaat een groot deel van de favoriete eigenschappen van capillaire electroforese verloren.

K. Albert, Angew. Chem. Int. Ed. Engl. 34 (1995) 641

7. Het gebruik van heptakis(2,6-di-O-methyl)-β-cyclodextrine bij chirale scheidingen met behulp van capillaire electroforese-electrospray ionisatie massaspectrometrie komt de robuustheid van het systeem niet ten goede.

   R.L. Sheppard, X. Tong, J. Cai and J.D. Henion, Anal. Chem., 67 (1995) 2054

8. Beschrijving van membraan-preconcentrering met een verrijkingsfactor 1 is geen verrijking van de literatuur.

   A.J. Tomlinson, L.M. Benson, N.A. Guzman and S. Naylor, J. Chromatogr. A, 744 (1996) 3

9. Bij het automatiseren dient men doel en middel goed uit elkaar te houden.

10. De oplossing voor het fileprobleem in de randstad is de aanleg van een uitgebreid metrosysteem.

11. De tijd die verspild wordt door te forensen per openbaar vervoer kan beperkt worden door deze slapend door te brengen.

12. Sport is onontbeerlijk voor een goede geestelijke en lichamelijke gezondheid.

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