

Determination of 1,2,6-inositol trisphosphate (derivatives) in plasma using iron(III)-loaded adsorbents and capillary zone electrophoresis–(indirect) UV detection

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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.

Keywords: Capillary electrophoresis; Adsorbents; Sample pretreatment; Inositol phosphates

1. 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 isotachopheresis (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 separation of inositol

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phosphates is very efficient but a higher sensitivity is still required.

Inositol phosphates are known to exhibit strong complexing properties with numerous metal ions like Fe^{3+} , Al^{3+} , Ca^{2+} , Cd^{2+} , Zn^{2+} , etc. [10–14]. Until now, metal-loaded 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.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade. Iron nitrate and acetic acid were obtained from J.T. Baker (Deventer, Netherlands). Ammonium acetate, ethylenediaminetetraacetic acid (EDTA), sodium hydroxide, sodium dodecylsulfate (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-glycolmethacrylate gel with 40–63 μm particle size (Lachema, Brno, Czech Republic) and 0.5 ml IDA-Sepharose (Pharmacia, Uppsala, Sweden). Hydroxypropyl-

methylcellulose (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.

2.2. 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.

2.3. Electrophoresis

The collected fractions were analyzed using

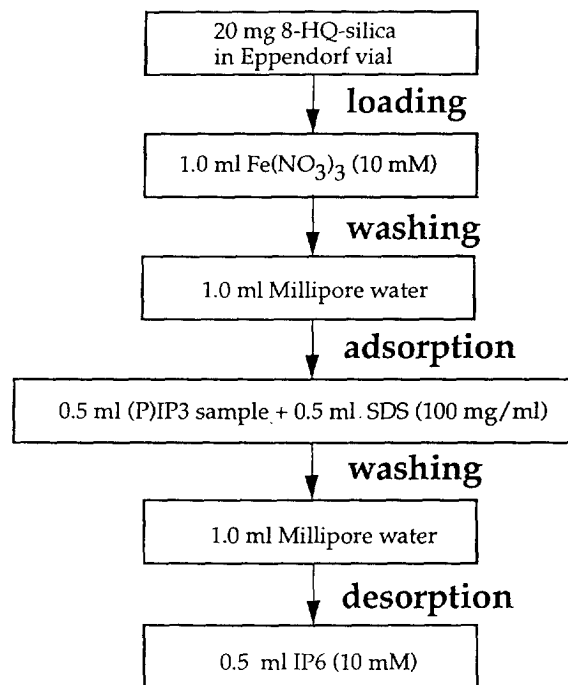


Fig. 1. Procedure for the pretreatment of (P)IP₃ samples.

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 ($\lambda = 214$ nm). For UV detection (PIP3) the electrophoresis medium consisted of 10 mM ammonium acetate buffer pH 5 and 0.01% HPMC to reduce the electroosmotic flow. For the indirect detection of IP3 a buffer was prepared composed of 0.5 mM NDSA, 30 mM acetic acid pH 3 and 0.01% HPMC [8]. In both systems a voltage of -25 kV was applied over a fused-silica capillary (SGE, Ringwood, Vic., Australia) of 0.57 m (0.50 m to the detector). After rinsing the capillary (75 μ m I.D., 375 μ m 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.

3. Results and discussion

3.1. 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; 5×6 mm I.D.) with iron(III) (2 ml; 10 mM), the adsorption of analyte (0.5 ml; 200 μ M) 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 no significant improvement was shown.

3.2. 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 analysis with UV detection because of the high ionic strength of the obtained sample, leading to enhanced Joule heating, changes in the local electric field strength and consequently peak distortion. Furthermore, the high concentration of nitrate ions interferes with the analyte in the electropherogram. Therefore, another mechanism for the desorption of analyte was examined, which was based on the displacement of analyte by a high concentration of a competing compound that complexes with iron(III). In that case, only the analyte is desorbed whereas iron(III) remains on the sorbent. EDTA, inositol hexakisphosphate (IP6), 8-hydroxyquinoline sulfate (8-HQS) and phosphoric acid all complex with iron(III). The analyte recoveries mounted to 88% using IP6 (0.5 ml, 10 mM) and 25% using EDTA (0.5 ml, 10 mM). 8-HQS and phosphoric acid were not effective at all. As the pH of the IP6 solution was 11, the effect of hydroxyl ions was investigated by adding 0.01 M sodium hydroxide to the adsorbent. The analyte appeared to be selectively displaced by IP6 ions and not by hydroxyl ions. Although the displacement mechanism is not yet completely understood, the

association constant of the displacer plays a predominant role.

The displacement of analyte by IP6, being a cheap and non-toxic compound, has been investigated more thoroughly. The effect of using different concentrations of IP6 to the sorbent on the recoveries of PIP3 is shown in Fig. 2. Varying the IP6 concentration from 1 to 10 mM the recovery is increased to a maximum of 88% at 10 mM. At 20 mM IP6, analyte interference in the electropherogram becomes unacceptable. Moreover, it was observed that the effect of increasing the IP6 concentration in a standard solution, while keeping the analyte concentration constant, was a decrease of the PIP3 peak height caused by the higher conductivity of the sample. It is evident that the IP6 concentration present in the sample after desorption is lower than that added to the adsorbent. Nevertheless, this concentration difference can be neglected compared to the high IP6 concentration and, therefore, the recoveries obtained are related to analyte solutions with approximately the same IP6 concentrations. When ion-pair chromatography (IPC) instead of CZE would be combined with this sample pretreatment, the response factor probably remains the same while varying the IP6 concentration. As the collected fractions have an IP6 concentration of ca. 10 mM, transient isotachopheresis [24] could be considered with IP6 as the leading electrolyte. However, it must be concluded that transient isotachopheresis is very

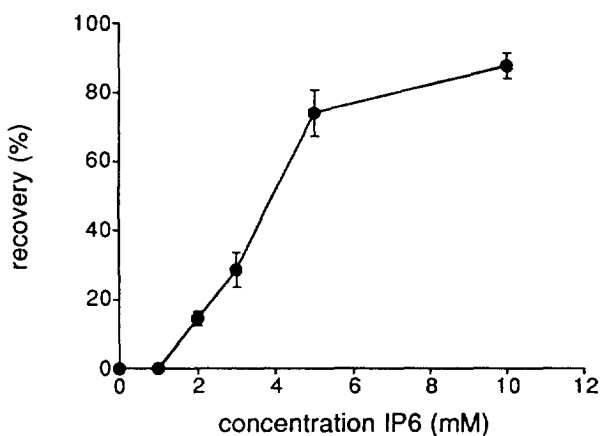


Fig. 2. Relation between the PIP3 sample recovery (%) and the IP6 desorption concentration (mM).

unlikely because the injected sample volume is low (50 nl) and the migration time does not change to any extent.

3.3. Choice of sorbent

So far, all experiments were performed using 8-HQ-silica as the adsorbent. Other adsorbents investigated were 8-HQ-glycolmethacrylategel [25,26], IDA-silica and IDA-Sepharose. The recoveries of PIP3 from water as well as from plasma using the different adsorbents are depicted in Fig. 3. For 8-HQ-silica, IDA-silica and IDA-Sepharose the recoveries of PIP3 in water are approximately the same (ca. 88%) whereas on 8-HQ-glycolmethacrylate gel 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-methacrylate gel (40–63 μm) compared with the other sorbents (5 μm), implying the presence of relatively deep pores within the particles through which the sample molecules diffuse in and out of very slowly [27]. Yet, this has not been investigated any further.

Another difference between the adsorbents becomes clear when plasma samples are pre-treated. Initially, 8-HQ-silica was supposed to be the more appropriate adsorbent as IDA occupies

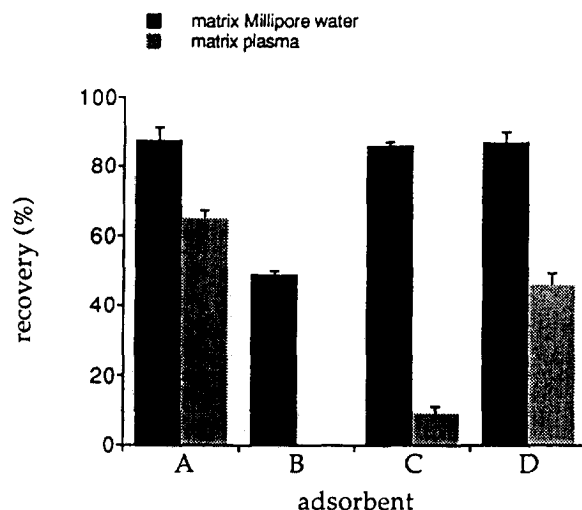


Fig. 3. Recoveries (%) obtained from water and plasma samples with different adsorbents. A = 8-HQ-silica, B = 8-HQ-glycolmethacrylategel. C = IDA-silica, D = IDA-Sepharose.

three positions in the metal sphere whereas 8-HQ only two. However, this difference has not been seen for standard solutions of PIP3. On the contrary, the 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 for plasma samples on IDA-Sepharose two approaches were used. First, the incubation 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].

3.4. 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 detection. The recovery was 88% (S.D. = 3.7%, $n = 5$). The excess of IP6 which is used for the displacement cannot be seen in the electropherogram because it is not a UV absorbing compound. The pretreatment of plasma samples, however, is much more complicated because of the matrix, containing ca. 70 mg/ml proteins, high concentrations of electrolytes (sodium, sulphates, phosphates, etc.) as well as fatty acids and lipids [28]. Besides, the very high and strong protein binding of IP3 derivatives in plasma, caused by electrostatic and hydrophobic interactions, must be substantially decreased. So far, several approaches have been applied in order to denature the proteins, such as organic solvents (methanol, acetonitrile), strong acids (e.g. perchloric acid), urea, ammonium sulfate

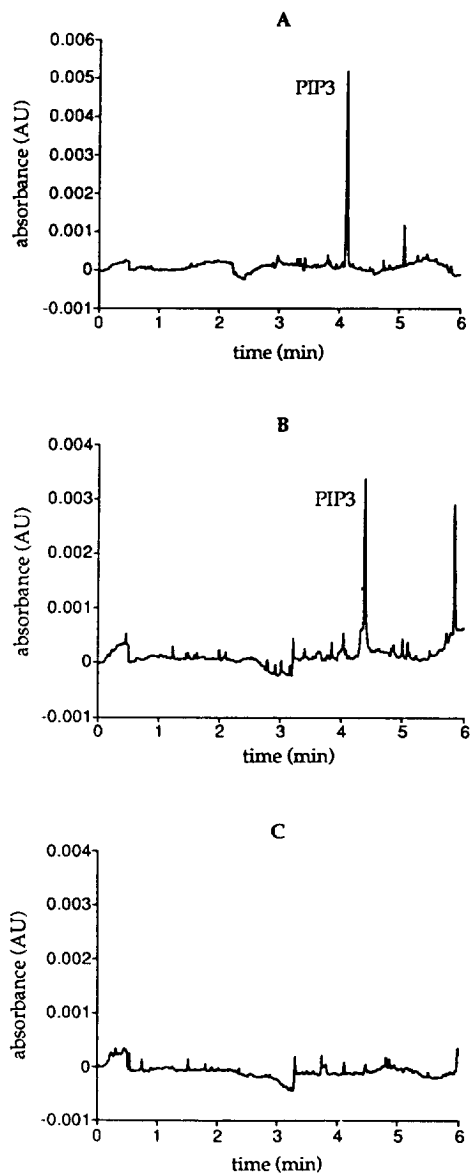


Fig. 4. Electropherograms of pretreated PIP3 samples in water (A), in plasma (B) and blank plasma (C). Conditions: UV detection at $\lambda = 214$ nm; CZE buffer: 10 mM ammonium acetate, pH 5, 0.01% HPMC.

and a surfactant (sodium dodecylsulfate) added to the plasma sample. Another approach was the cleavage of proteins with a proteolytic enzyme (trypsin) at pH 8, incubated for several hours at 37°C. Subsequently, ion-pair solid-phase isolation (IP-SPI) was performed on a C_{18} column with tetrabutylammonium as a counterion or the

sample was analyzed directly by capillary zone electrophoresis with UV detection. Either the recovery or the reproducibility was too low. In contrast with the combination of IP-SPI and sodium dodecylsulfate (SDS) added to the plasma sample, the use of the iron(III)-loaded adsorbent was quite successful and allowed the presence of 1.4 g SDS/g protein in the sample [21]. Fig. 4B shows the electropherogram of a pretreated plasma sample containing 200 μM PIP3. Hardly any other compound adsorbs to the iron(III)-loaded sorbent, showing the selectivity of the method. The recovery mounted to 65% (S.D. = 2.2%, $n = 8$). Fig. 4C shows the electropherogram of pretreated blank plasma, demonstrating that no interfering peaks are present in the time window.

Next to PIP3, 1,2,6-IP3 has been pretreated using the developed method. However, IP3 cannot be detected with direct UV detection. Therefore, CZE was combined with indirect UV detection [8]. Inherent to this detection principle, the presence of 10 mM IP6 more or less interfered with the IP3 derivatives. The peak shapes were tailing, even at a higher pH where the mobilities of the analyte and chromophore match more closely. With this system only IP3 could be measured in the presence of a high IP6 concentration (Fig. 5). Although indirect UV detection will not be the detection method of choice, it has been used to check the sample pretreatment of IP3 in plasma. The recoveries obtained were 90% (S.D. = 7.2, $n = 2$) and 54% (S.D. = 2.8, $n = 4$) for IP3 in water and plasma, respectively, which are quite satisfying figures.

3.5. Quantitative aspects

The developed method has been investigated for its potential in quantitative analysis by determining the reproducibility, linearity and sensitivity. Although the method contains 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, $n = 8$) in water and plasma, respectively.

In order to examine the linearity of the meth-

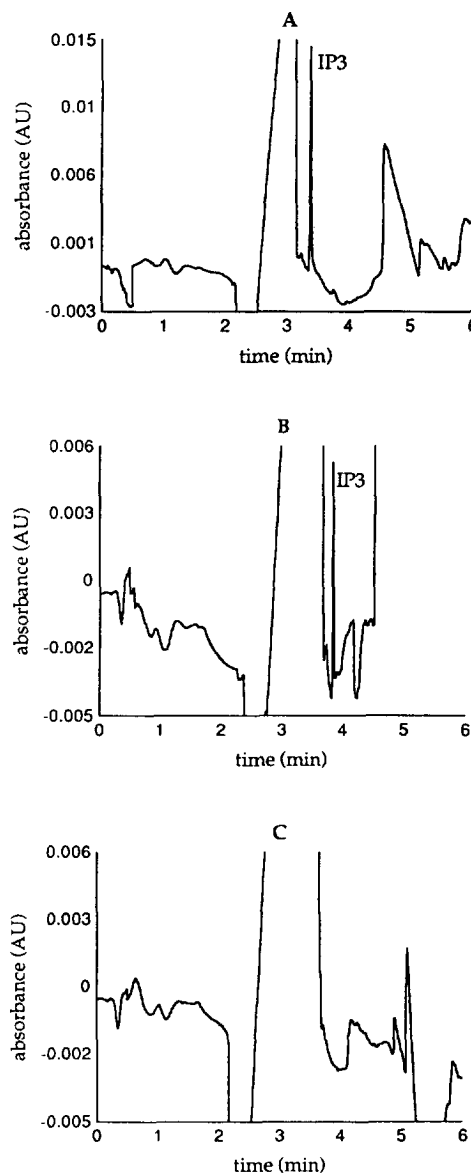


Fig. 5. Electropherograms of pretreated IP3 samples in water (A), in plasma (B) and blank plasma (C). Conditions: indirect UV detection at $\lambda = 214$ nm; CZE buffer: 0.5 mM NDSA, 30 mM acetic acid, pH 3, 0.01% HPMC.

od 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.

4. Conclusions

The developed method can be used for the determination of 1,2,6-IP3 and derivatives in plasma. The advantage of this 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.

References

- [1] H. Irth, M. Lamoree, G.J. de Jong, U.A.Th. Brinkman and R.W. Frei, *J. Chromatogr.*, 499 (1990) 617.
- [2] Dionex Application Note AN 65. 1990, Dionex, Sunnyvale, CA, USA.
- [3] B. Tangendjaja, K.A. Buckle and M.J. Wootton, *J. Chromatogr.*, 197 (1980) 274.
- [4] H. Binder, P.C. Weber and W. Siess, *Anal. Biochem.*, 148 (1985) 220.
- [5] W.R. Sherman, K.E. Ackerman, R.A. Berger, B.G. Gish and M. Zinbo, *Biomed. Environ. Mass Spectrom.*, 13 (1986) 333.
- [6] P. Blatny, F. Kvasnicka and E. Kenndler, *J. Chromatogr. A*, 679 (1994) 345.
- [7] A. Henshall, M.P. Harrold and J.M.Y. Tso, *J. Chromatogr.*, 608 (1992) 413.
- [8] B.A.P. Buscher, H. Irth, E. Andersson, U.R. Tjaden and J. van der Greef, *J. Chromatogr. A*, 678 (1994) 145.
- [9] B.A.P. Buscher, R.A.M. van der Hoeven, U.R. Tjaden, E. Andersson and J. van der Greef, *J. Chromatogr. A*, 712 (1995) 235.
- [10] R.H. Jackman and C.A. Black, *Soil Sci.*, 72 (1951) 179.
- [11] P. Vohra, G.A. Gray and F.H. Kratzer, *Proc. Soc. Exp. Biol. Med.*, 120 (1965) 447.
- [12] W.J. Evans and A.G. Pierce, *J. Food Sci.*, 47 (1982) 1014.
- [13] M. Cheryan, F.W. Anderson and F. Grynspan, *Cereal Chem.*, 60 (1983) 235.
- [14] E. Graf, *Phytic acid: Chemistry and Applications*, Pilatus Press, Minneapolis, 1986, p. 1.
- [15] J. Porath, *Trends Anal. Chem.*, 7 (1988) 254.
- [16] Z. El Rassi and Cs. Horvath, *J. Chromatogr.*, 359 (1986) 241.
- [17] R.W. Frei and K. Zech, *Selective Sample Handling and Detection in High-Performance Liquid Chromatography, Part A*, Elsevier, Amsterdam, 1988.
- [18] K.K. Unger, *Packings and Stationary Phases in Chromatographic Techniques*, Marcel Dekker, New York, NY, 1990.
- [19] V.A. Davankov and A.V. Semechkin, *J. Chromatogr.*, 141 (1977) 313.
- [20] E. van der Vlis, H. Irth, U.R. Tjaden and J. van der Greef, *Anal. Chim. Acta.*, 271 (1993) 69.
- [21] G.J. Shahwan and J.R. Jezorek, *J. Chromatogr.*, 256 (1983) 39.
- [22] G.-J. Krauss, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 419.
- [23] C. Tanford, *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, Wiley and Sons, New York, 1973.
- [24] F. Foret, E. Szoko and B.L. Karger, *Electrophoresis*, 14 (1993) 417.
- [25] Z. Slovak, S. Slovakova and M. Smrz, *Anal. Chim. Acta*, 87 (1976) 149.
- [26] Z. Slovak and S. Slovakova, *Fresenius' Z. Anal. Chem.*, 292 (1978) 213.
- [27] C.F. Poole and S.K. Poole, *Chromatography Today*, Elsevier, Amsterdam, 1991.
- [28] D.S. Stegehuis, Thesis, Leiden University, Leiden, Netherlands, 1992.
- [29] N.J. Reinhoud, U.R. Tjaden and J. van der Greef, *J. Chromatogr.*, 641 (1993) 155.
- [30] D. Kaniansky and J. Marak, *J. Chromatogr.*, 498 (1990) 191.