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Development of a comprehensive analytical method for phosphate metabolites in plants by ion chromatography coupled with tandem mass spectrometry

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Abstract

This paper describes the development of a practical method for the analysis of phosphorus compounds with a focus on sugar phosphates from the model higher plant *Arabidopsis thaliana* by ion chromatography coupled to electrospray ionization tandem mass spectrometry (IC–ESI-MS–MS). After the analytical separation, the potassium hydroxide eluent was converted to water with an anion suppressor allowing the effluent from the IC to be connected to the mass spectrometer directly. In the optimized method, 17 phosphorous compounds (adenosine diphosphate (ADP), fructose 1,6-bisphosphate, fructose 2,6-bisphosphate, fructose 6-phosphate, galactose 1-phosphate, glucose 1-phosphate, glucose 1,6-bisphosphate, glucose 6-phosphate, mannose 6-phosphate, phosphoenol pyrvate, 3-phosphoglyceric acid, ribulose 1,5-bisphosphate, ribulose 5-phosphate, ribose 5-phosphate, sucrose 6-phosophate and uridine 5′-diphosphate-glucose (UDPG)) were determined. The linearity of response for these phosphorous compounds over the concentration range of 0 and 10 μM was better than 0.9993 in all cases. The minimum detection limit was between 0.01 and 2.50 μM for a 25 μL injection, and recovery rates for standard addition to the sample were within the range from 93% to 110%.

Keywords: Sugar phosphate; Ion chromatography; Electrospray mass spectrometry; *Arabidopsis*

1. Introduction

The understanding of whole metabolic patterns in both wild and genetically modified organisms (metabolomics) is becoming important for understanding the biological function of a genome. Phosphorous, an essential element, is indispensable to life-activity, such as inheritance, metabolism and energy conversion in animals and plants. The inorganic phosphate concentration in soil is present at micromolar concentrations. A plant develops various functions to use phosphate efficiently such as efflux (or extraction) of organic acid from root, accumulation of phosphate to vacuoles, and activation of phosphate uptake. Although it is well known that plants control the phosphate concentration in the cell by regulating metabolism of phosphate, the mechanisms are not known well. To examine the dynamics of phosphate metabolism, it is necessary to analyze the dynamic state of sugar phosphates, which play a central role in phosphate metabolism.

Phosphate-related metabolites can be measured simultaneously by recently developed analytical techniques, including nuclear magnetic resonance (NMR) [1–4], liquid chromatography (LC) [5–7], liquid chromatography coupled with mass spectrometry (LC–MS) [8,9], gas chromatography coupled with mass spectrometry (GC–MS) [10,11],

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capillary electrophoresis coupled with mass spectrometry (CE–MS) [12] or anion-exchange with pulsed amperometric detection (HPAEC-PAD) [13–16]. Although these methods are generally successful in determining the concentration of metabolites in simple materials such as cultured cells or yeast, there are still some problems. For example, the sensitivities for phosphorus compounds were low, derivatization procedures were necessary for each compound, or the resolution among some metabolites was poor. The unknown compounds that interfere with the determination of sugar phosphates are present at higher concentrations in a whole plant than in cultured cells or single cell organisms. It is therefore important to choose an analytical technique that can provide high sensitivity and high resolution for a whole plant sample. In the previous study, we described the method for sugar phosphates in plant by HPAEC-PAD with titanium dioxide column [17]. Some unknown compounds were removed by a titanium dioxide with in-line, which is known to be highly selective for phosphate compounds, and the reliability of determination was improved. It is well known that an anion-exchange separation is the most suitable separation mode for anionic metabolites like sugar phosphates, and PAD with a gold electrode has high selectivity for sugar species. However, even when the titanium dioxide pre-treatment column is used with HPAEC-PAD, some sugar phosphates present at low concentration cannot be determined due to interference from unknown matrix compounds. In this study, the combination of an ion chromatography (IC) and MS–MS was examined to improve selectivity and sensitivity for sugar phosphate determination in a whole plant sample.

2. Materials and methods

2.1. Chemical materials

Deionized water (18 MΩ) from a Milli-RO/Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare eluent and all standards. Metabolites for standards were purchased from Sigma (St. Louis, MO, USA). The following standards were selected in this study: adenosine diphosphate (ADP), adenosine monophosphate (AMP), erythrose 4-phosphate (Ery 4-P), fructose 1,6-bisphosphate (Frc 1,6-P), fructose 2,6-bisphospate (Frc 2,6-P), fructose 6-phosphate (Frc 6-P), galactose 1-phosphate (Gal 1-P), glucose 1-phosphate (Glc 1-P), glucose 1,6-bisphosphate (Glc 1,6-P), glucose 6-phosphate (Glc 6-P), mannose 6-phosphate (Man 6-P), phosphoenol pyrvate (PEP), 3-phosphoglyceric acid (3-PGA), ribulose 1,5-bisphosphate (Rbu 1,5-P), ribulose 5-phosphate (Rbu 5-P), ribose 5-phosphate (Rib 5-P), sucrose 6-phosphate (Suc 6-P), and uridine 5′-diphosphate-glucose (UDPG). The stock solutions (0.5 mM each) of the target analytes were made up in deionized water and then stored in a refrigerator for further use. Working standard solutions were prepared daily by appropriate dilution of the stock solution.

2.2. Plant material

Seeds of *Arabidopsis thaliana* (L.) Heynh. cv. Columbia wild-type were sterilized by a 5-min treatment in 70% ethanol and 10 min in a solution of 1% Triton-X and 0.4% sodium hypochlorite. The seeds were washed four times with autoclaved deionized water, and then spread on 0.2% gellum gum plates containing Murashige and Skoog nutrient solution. Plates were placed at 4 °C for 2 days, and then they were grown in a growth cabinet continuously illuminated (light level 50 μmol m^{-2} s^{-1}) at 22 ± 1 °C for 8 weeks until analyzed.

2.3. Plant extraction

Phosphorus compounds were extracted in accordance with the previous study [17]. A single whole *Arabidopsis* plant was frozen in liquid nitrogen and lyophilized for 4 h in a freeze dryer (Freezone 1L, Labconco, Kansas City, MO, USA), and stored at −80 °C. The sample was crushed into small pieces and placed in a 2.0 mL microcentrifuge tube. Boiling water (0.5 mL) was added and the extract was then immediately irradiated with 600 W of microwave for 15 s. After cooling on ice, the extract was centrifuged at 20 400 × *g* at 4 °C for 10 min. The supernatant was filtered through a 0.45 μm filter (Ekicrodisc AcroLC, Gelman Sciences, Japan) and applied to the ion chromatograph (IC) coupled with MS–MS detection.

2.4. IC–MS–MS analysis

An ICS-2000 ion chromatography system (Dionex Corp., Sunnyvale, CA, USA), which includes an eluent generator and conductivity detector, was used in this study. Dionex Chromeleon software version 6.6 was used for data processing. Dionex IonPac AS11-HC (4 mm × 250 mm) and AG11-HC (4 mm × 50 mm) columns packed with anion-exchange resin were used as the separation columns, because both sugar phosphates and sugar bisphosphates elute with potassium hydroxide eluent. Dionex ASRS-ULTRA II suppressor was placed between analytical column and conductivity cell. Deionized water was supplied to the suppressor for continuous regeneration of the ion-exchange sites via electrolysis, using 130 mA current. A 25 μL injection was made with a Dionex AS50 autosampler and the samples were cooled in the sample tray at 4 °C during analysis. The analysis was performed at 30 °C with the flow rate set at 1.0 mL min^{-1}. The eluent was generated as potassium hydroxide solution by the eluent generator. The eluent concentration was 5 mM from 0 to 5 min after 5 mM re-equilibration for 10 min, increased to 50 mM for next 25 min, and then held 50 mM for 5 min. The eluent from the conductivity cell was split 1:4 before coupling to the mass spectrom-

Table 1
IC–MS–MS condition

Ion chromatograph	Dionex ICS-2000
Column	IonPac AG11-HC (4.0 mm × 50 mm), AS11-HC (4.0 mm × 250 mm)
Column temperature	30 °C
Eluent	5–50 mM potassium hydroxide (5–25 min) gradient
Flow rate	1.0 mL min^{-1}
Suppressor	ASRS-ULTRA II (external water mode/130 mA)
Detector	Conductivity
Injection volume	25 μL
Mass spectrometry	Applied Biosystems Q Trap LC–MS–MS system
Ion source	TurboIonSpray (ESI)
Mode	Multiple reaction monitoring (MRM) mode
Polarity	Negative
Heater gas temperature	500 °C
Ion source voltage	−4300 eV
Curtain gas (nitrogen)	138 kPa
Collision gas (nitrogen)	5[a]
Nebulizer gas	414 kPa
Heater gas	483 kPa

[a] Collision gas parameter is the setting value of the instrument.

eter in order to ionize efficiently in the electrospray inlet.

A Q Trap LC–MS–MS system (Applied Biosystems/MDS Sciex, Concord, Ont., Canada) was used as MS–MS system, equipped with a TurboIonSpray source (electrospray ionisation (ESI)). The atmospheric pressure-negative ionization mode was used and the mass analyzer was operated in the multiple reaction monitoring (MRM) mode. The quadrupole operated at unit resolution. Detailed MS–MS settings are presented in Table 1. The mass spectrometer was operated using Analyst software version 1.4. Suitable transitions from precursor to product ions (MRM transitions) were identified with automatic tune function of the Analyst software.

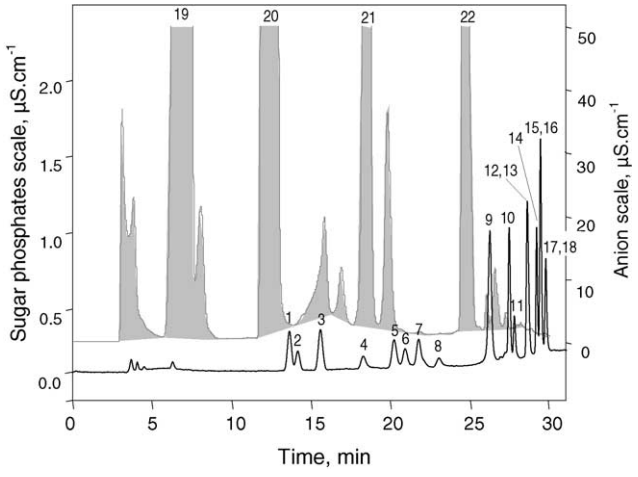

Fig. 1. Chromatogram of phosphates compound standards and anion in *Arabidopsis* extract analyzed by IC. Peaks of 10 μM phosphate compound standards are shown as 1–3 and 5–17, and peaks of anion in *Arabidopsis* extract are shown as 18–22. Analytical method is shown in a part of ion chromatography of Table 1. Peaks: (1) Gal 1-P; (2) Glc 1-P; (3) Suc 6-P; (4) carbonate; (5) Glc 6-P; (6) Man 6-P; (7) Frc 6-P; (8) Rib 5-P; (9) AMP; (10) Rbu 5-P; (11) 3-PGA; (12) UDPG; (13) PEP; (14) Glc 1,6-P; (15) ADP; (16) Frc 2,6-P; (17) Frc 1,6-P; (18) Rbu 1,5-P; (19) chloride; (20) nitrate; (21) sulfate; (22) phosphate.

Table 2
Analyte-specific parameters, coefficients and MDL of phosphorous compounds by IC–MS–MS

Phosphate compound	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Correlation coefficient[a]	MDL[b] (μM)
ADP	426.0	79.0	1.0000	0.04
AMP	346.1	79.0	0.9993	0.68
Frc 1,6-P	338.9	79.0	0.9999	0.01
Frc 2,6-P	338.9	79.0	1.0000	0.02
Frc 6-P	259.0	97.0	0.9995	0.01
Gal 1-P	259.0	79.0	0.9999	0.01
Glc 1-P	259.0	79.0	0.9995	0.01
Glc 1,6-P	338.9	79.0	0.9997	0.01
Glc 6-P	259.0	97.0	0.9994	0.02
Man 6-P	259.0	97.0	0.9997	0.01
PEP	167.0	79.0	0.9999	0.04
3-PGA	185.0	79.0	0.9998	0.01
Rbu 1,5-P	309.0	79.0	0.9994	2.50
Rbu 5-P	228.9	79.0	0.9993	0.15
Rib 5-P	228.9	79.0	0.9993	0.03
Suc 6-P	421.1	79.0	0.9999	0.02
UDPG	565.1	97.0	1.0000	0.20

[a] Coefficient was calculated from calibration curve: 0, 0.01, 0.1, 1 and 10 μM standard.
[b] Minimum detection limit was calculated from 5× noise based on peak height.

3. Results and discussion

Most of phosphorus compounds can be detected by an electrical conductivity detector. However, high concentrations of anion peaks in a whole plant sample interfere with low concentrations of phosphorus compounds (Fig. 1).

Table 3
Recovery of standards added to *Arabidopsis* extract

Phosphate compound	Recovery (%)
ADP	106
AMP	105
Frc 1,6-P	95
Frc 2,6-P	96
Frc 6-P	97
Gal 1-P	97
Glc 1,6-P	106
Glc 1-P	100
Glc 6-P	95
Man 6-P	110
PEP	101
3-PGA	98
Rbu 1,5-P	96
Rbu 5-P	106
Rib 5-P	99
Suc 6-P	93
UDPG	98

Each 1 μM of phosphate compound standards were added to *Arabidopsis* extract.

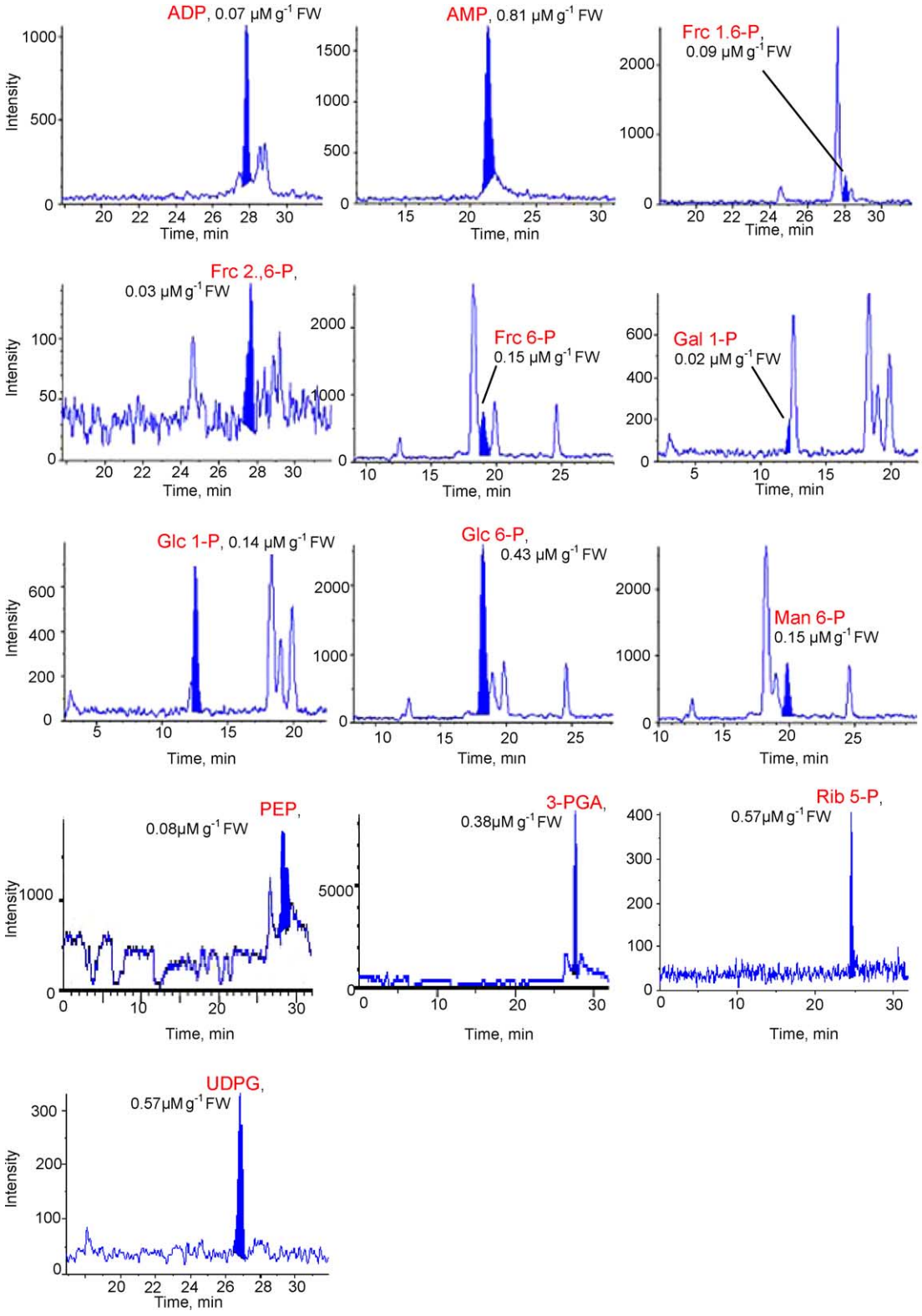

Fig. 2. Chromatogram of phosphates compounds in *Arabidopsis* extract analyzed by IC–MS–MS. Sample was extracted from *Arabidopsis* following the extraction method in Section 2. A 0.5 g of deionized water was added to 0.142 g fresh weight (FW) of *Arabidopsis*, and sample was diluted 10 times before IC–MS–MS analysis. Each concentration values in figure shows the amount in the diluted samples. Glc 1,6-P, Rbu 1,5-P, Rbu 5-P and Suc 6-P in this sample were not detected.

ESI-MS is effective tool for identification, especially for samples with complex matrixes. It is, however, necessary to separate sugar phosphates before detection by ESI-MS, because some sugar phosphates have the same mass-to-charge ratio (*m*/*z*). Anion-exchange separation is efficient for anionic species, even very similar compounds such as Gal 1-P and Glc 1-P. The IonPac AS11-HC column selected for this study has high resolution for anion species like sugar phosphates, and high capacity suitable for managing the highly ionic matrix of the whole plant extract sample.

In the anion-exchange separation, the potassium hydroxide (KOH) eluent is required to separate anions. The KOH effluent from the IC is not volatile and can foul and clog the inlet to the mass spectrometer. In addition, the ionic content of the eluent can suppress the ionization of target analytes during the electrospray process. The suppressor, which contains cation-exchange membranes, can desalt eluent by exchanging the K^+ for hydronium. The hydronium ion neutralizes the hydroxide ion from the eluent and the resulting water enters the mass spectrometer. Potassium hydroxide eluent was selected as an eluent because the AS11-HC phase is hydroxide selective. This means that many anionic analytes can be eluted with relatively low concentrations of hydroxide. The suppression capacity of the ASRS-ULTRA II suppressor (the desalting efficiency) is about 200 μeq. min^{-1}, meaning that the suppressor can neutralize about 200 mM hydroxide flowing at 1 mL min^{-1}.

In this study, 79 (PO_3^-) and 97 ($H_2PO_4^-$) fragments, which are derived from a phosphate group, were chosen as product ions on MS–MS detection, because they have high signal and these fragments clearly are derived from phosphorus compounds.

Table 2 shows the linearity and minimum detection limit (MDL) of phosphorous compounds using IC–MS–MS. All correlation coefficient of phosphorous compounds in the concentration range of 0–10 μM were better than 0.9993. MDLs of phosphorus compounds in this analysis method were between 0.01 and 2.50 μM using a 25 μL injection. MDLs of AMP and Rbu 1,5-P are slightly higher than that of the others, because of low peak efficiency. Table 3 shows the recovery of standards. One micromolar of each phosphate compound standard was added to *Arabidopsis* extract. Recoveries of standards were within the range from 93% to 110%. Ery 4-P was detected as four peaks at 20.5, 22.0, 27.2 and 29.4 min (data not shown). Though they seem to be structural isomers, it was not confirmed in this study. These results show IC–MS–MS is reliable method for determination phosphate compound except Ery 4-P in extract from a whole plant sample.

Chromatograms of phosphate compounds in *Arabidopsis* extract analyzed by the optimized IC–MS–MS method are shown in Fig. 2. The sample was extracted from *Arabidopsis* following the extraction method in Section 2. A 0.5 g aliquot of deionized water was added to 0.142 g (fresh weight) of *Arabidopsis*, and the sample was diluted 1:10 before IC–MS–MS analysis. Glc 1,6-P and Suc 6-P in this sample were not detected.

4. Conclusion

We previously reported that sugar phosphates in a whole plant can be determined by the HPAEC-PAD with pre-treatment by titanium dioxide column [17]. Although HPAEC-PAD with the titanium dioxide column is a convenient and reliable method, it was difficult to determine some sugar phosphates in a whole plant because of interference of sample matrices. In this study, we have developed a simple method for the determination of phosphate compounds from *Arabidopsis* plant by using IC–MS–MS. IC–MS–MS method is superior not only in the MDL, but also in the respect that phosphate compounds detected by MS are scarcely interfered with by sample matrixes. Furthermore, IC–MS–MS has the advantage that phosphate (and other inorganic anions) is determined simultaneously with sugar phosphates by conductivity detector connected before MS. This method provides a practical protocol for determining phosphates compounds in complex matrices such as whole plant.

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References

[1] C. Foyer, C. Spencer, Planta 167 (1986) 376.
[2] M.J. Lauer, D.G. Blevins, H. Sierzputowska-Gracz, Plant Physiol. 89 (1989) 1331.
[3] S. Tu, J.R. Cavavaught, R.T. Boswell, Plant Pysiol. 93 (1990) 778.
[4] H.J. Vogel, P. Lundberg, K. Bagh, In Vitro Cell Dev. Biol. 35 (1999) 144.
[5] H. Ashihara, X. Li, T. Ukaji, Ann. Bot. 61 (1988) 225.
[6] M. Bhattacharya, L. Fuhrman, A. Ingram, K.W. Nickerson, T. Conway, Anal. Biochem. 232 (1995) 98.
[7] S. Sawada, R. Ono, T. Sato, S. Suzuki, O. Arakawa, Anal. Biochem. 314 (2003) 63.
[8] A. Buchholz, R. Takors, C. Wandrey, Anal. Biochem. 295 (2001) 129.
[9] V.V. Tolstikov, O. Fiehn, Anal. Biochem. 301 (2002) 298.
[10] U. Roessner, C. Wagner, J. Kopka, R.N. Trethewey, L. Willmitzer, Plant J. 23 (2000) 131.
[11] J. Chen, C. Yager, R. Reynolds, M. Palmieri, S. Segal, Clin. Chem. 48 (2002) 604.
[12] T. Soga, Y. Ueno, T. Nishioka, Anal. Chem. 74 (2002) 2233.
[13] T.S.M. Taha, T.L. Deits, Anal. Biochem. 219 (1994) 115.

[14] H.P. Smits, A. Cohen, T. Buttler, J. Nielsen, L. Olsson, Anal. Biochem. 261 (1998) 36.
[15] N.B.S. Jensen, K.V. Jokumsen, J. Villadsen, Biotechnol. Bioeng. 63 (1999) 356.
[16] E. Groussac, M. Oritz, J. Feancois, Enzyme Microb. Technol. 26 (2000) 715.
[17] Y. Sekiguchi, N. Mitsuhashi, Y. Inoue, H. Yagisawa, T. Mimura, J. Chromatogr. A 1039 (2004) 71.