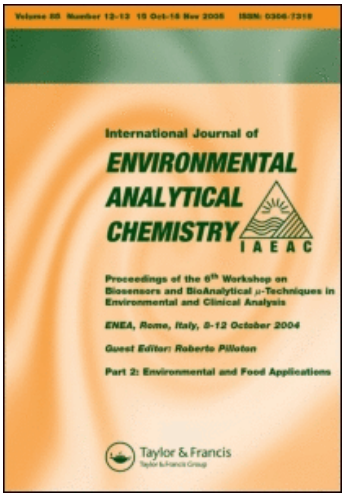

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Capillary Zone Electrophoresis of Phytochelatins and their Precursors Using On-Line Derivatization with N-Ethylmaleimide

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CAPILLARY ZONE ELECTROPHORESIS OF PHYTOCHELATINS AND THEIR PRECURSORS USING ON-LINE DERIVATIZATION WITH N-ETHYLMALEIMIDE

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A new procedure using capillary zone electrophoresis (CZE) combined with on-line derivatization of phytochelatins (PCs) and their precursors glutathione and γ-glutamylcysteine (γ-EC) is presented. PCs are metal binding thiol rich polypeptides which in plants and fungi are involved in heavy metal sequestration. The induction of the synthesis of PCs and their precursors can change depending on the presence and concentration of different heavy metals. Qualitative and quantitative analysis of thiolic peptides may provide useful information about the response of organisms to increased heavy metal concentrations in the environment. Therefore, the determination of these compounds from plant extracts requires sensitive and selective analytical methods. The peptides are derivatized during the electrophoretic run by N-ethylmaleimide immediately injected after the plant extracts. The method is characterized by high linearity and an increased sensitivity compared to the underivatized thiols. The automation of the PC determination with high sensitivity, combined to the advantages of CZE such as simplicity, high speed and small sample requirements, distinguish the new procedure.

Keywords: CZE; on-line derivatization; phytochelatins; glutathione; plant extracts

INTRODUCTION

Heavy metal pollution of the biosphere is a major environmental problem. Different organisms have developed various mechanisms by which metal ions entering their tissues and cells are immediately complexed and inactivated. In plants, phytochelatins (PCs) represent the main heavy metal-binding biomolecules and play a significant role in metal homeostasis[1]. The most widespread PCs in plants and fungi are linear polymers of γ-glutamylcysteine (γ-EC) units with a C-terminal glycine residue showing the central structure $(\gamma\text{-EC})_n$-glycine (n = 2–11, $(\gamma\text{-EC})_n$G, PC_{2-11}).

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The constitutive metal activated enzyme γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin -synthase) catalyzes the transfer of the γ-glutamylcysteine dipeptide moiety of glutathione (GSH) to an acceptor GSH molecule or to an already existing PC molecule. The precursor molecule glutathione is formed in plants from γ-EC and glycine in an ATP dependent process. In contrast to the PCs, cysteine rich metallothioneins are primary gene products[2].

The investigation of heavy metal binding thiolic peptides needs sensitive methods for their separation and detection. PCs can be analyzed preferentially by reversed-phase HPLC coupled to different detection methods, for example post-column derivatization with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB , Ellmans reagent)[3] and with maleimides[4] or monobromobimane using a pre-column derivatization procedure[5,6].

High separation efficiency, small sample requirements, and the speed of the method are remarkable features characterizing capillary electrophoresis (CE) for sensitive biological samples in complex matrices.

In the last years, new applications of capillary electrophoresis to the analysis of metalloproteins and metal binding peptides as well as metallothioneins have been published. With specialized electrophoretic techniques such as affinity capillary electrophoresis (ACE), micellar electrokinetic capillary chromatography (MECC), capillary isotachophoresis (CITP) or capillary isoelectric focusing (CIEF), such compounds from animal or plant tissues were not only quantified, but structural and metal binding studies as well as stability and enzymatic activity studies were also reported[7,8].

The simple and rapid CZE was applied to quantify glutathione and the oxidized product glutathione disulfide (GSSG) to determine the ratio of GSH/GSSG, indicating the response to oxidative stress[9,10]. The lack of sensitivity in capillary electrophoresis has been overcome by various detection techniques, such as electrochemical detection with a special solid copper electrode[11], glassy carbon electrode[12], or mercury/gold amalgam electrode[13]. A sensitive and specific determination of GSH and other small thiol peptides by CZE was based on UV detection after pre-column derivatization of the thiol function with N-(1-pyrenyl)maleimide (NPM), complementary to the HPLC methods[14].

Only one paper reports on the successful CZE separation of phytochelatins in an untreated fused-silica capillary[15]. The rapid assay of PCs (n = 4–9) in crude extracts of microalgal cells was performed using an acid electrolyte (pH 3.5) and reversed polarity of the electrodes. Under these conditions only the very acidic phytochelatins possess enough negative charge to migrate to the anode and to the detection window, respectively. The majority of zwitterionic compounds in the extracts bear a positive charge and do not migrate to the detector cell. The main advantage of this technique is the pre-separation of the acidic phytochelatins

from other proteins and peptides by using a negative voltage. However, the analysis of smaller PCs (n= 2, 3) and the thiolic precursors GSH, γ-EC and cysteine seems to be difficult due to their insufficient negative charge under these operating conditions. No reports have been published concerning the simultaneous CZE analysis of PCs and their precursor molecules.

The aim of our studies is the development of a specific, sensitive and simple assay for phytochelatins and the thiol precursors from plant materials by CZE. To improve the selectivity and sensitivity of detection derivatization techniques for thiolic peptides, different reagents such as monobromobimane (mBBr), monobromotrimethylammoniobimane (qBBr)[5,6], sodium nitrite $NaNO_2$[16], 2-chloro-1-methylpyridiniumiodide[17] and maleimides[14] have been checked. However, applications of these specific derivatization techniques in CZE are limited. Unfortunately mBBr and qBBr are sensitive to light. Furthermore, long reaction times are needed. Nitrosothiols and qBBr-derivatives are unstable at a pH higher than 8, which was necessary to resolve the thiol compounds of interest. Another disadvantage of a pre-column derivatization is the dilution of the sample in the reaction vessel. To overcome this problem, "on-line" derivatization techniques have been developed, which realize the derivatization of the sample in the capillary. The so-called "sandwich" method was applied to analyze amino acids. Here the sample zone is located between two zones of the derivatization reagent[18,19]. A Cu(II)-coated capillary has been introduced for the determination of di-, tri-, tetra- and pentaglycine by CE coupled to electrochemical detection[20]. The characterization of single cells was performed by several workers using the front end of the capillary as a chamber to lyse the cells and derivatize the contents with naphthalene-2,3-dicarboxaldehyde[21,22]. All these methods reduce the dilution of the samples, which are often found in trace levels only. In addition, these techniques makes the sample handling easier and allow the automation of the whole analytical process.

In this paper we present a new simple and sensitive CZE method for thiol peptides involving on-line derivatization using N-ethylmaleimide. The suitability of the method for the analysis of PCs and their precursors in tomato cell culture extracts after heavy metal exposition with and without prior sample preparation, is demonstrated.

EXPERIMENTAL

Chemicals

Glutathione, γ-glutamylcysteine, cysteine, cysteinylglycine and N-ethylmaleimide (NEM) were purchased from Sigma (Germany), Tris-buffer from Merck

(Germany), and sodium hydroxide and $CdCl_2$ from Fluka (Germany). Phytochelatins (n = 2, 3, 4) were a gift from Prof. Dr. Zenk (University of Munich).

Stock solutions of the standards were prepared by dissolving appropriate amounts in distilled water of pH 1.8 (trifluoroacetic acid, Sigma) or in acetonitrile (Merck) in the case of N-ethylmaleimide. At the beginning of each working day, the pH of the Tris-buffer was adjusted to 8.3 with HCl (Riedel de-Häen, Germany).

CZE procedure

CZE was performed using a Hewlett Packard HP^{3D} system equipped with a diode-array-detector (DAD) and the corresponding Chemstation hard- and software allowing the analyses of the electropherograms.

The capillary used was a 47.5 cm untreated fused silica SUPELCO FS 50 CE tube (363 µm o.d., 50 µm i.d.) with the detector window at 39 cm.

The temperature of the capillary was maintained at 20 °C by an air conditioning system. Separations of the peptides were performed at 30 kV and positive polarity. Samples were applied hydrodynamically for 3–10 sec at a pressure of 50 mbar. The peptide derivatives were monitored at 200 nm.

Prior to each run the capillary was flushed for 1 min with 1 M NaOH followed by 0.1 M NaOH, distilled water and operating buffer (0.1 M Tris/HCl, pH 8.3).

To realize an on-column derivatization reaction, sample injection was followed by the hydrodynamic injection of 50 mM N-ethylmaleimide (NEM) for 1 to 3 sec. The addition of NEM to the thiol function takes place when N-ethylmaleimide passes the thiol peptide sample plug due to its greater mobility in the electric field.

The migration order of the substrates was determined by single injection of standards.

Sample preparation

A tomato cell culture from *Lycopersicon esculentum*[23] established in our work group was incubated with 150 µM cadmium chloride for 24 hours. Approximately 200 mg of the cells were extracted with 0.1 N HCl at a 1:3 ratio.

To purify the tomato cell extracts from other charged compounds, which can disturb the separation considerably, a filtration step prior to electrophoresis was carried out. The cell extracts were placed into a MICROSEP™ concentrator

equipped with a 1 kDa OMEGA™ membrane (Filtron, Germany) and centrifuged for 3 hours at 5000 rpm.

The filtrates containing the thiol peptides were immediately analyzed by CZE using on-column derivatization with N-ethylmaleimide without further sample preparation.

RESULTS AND DISCUSSION

We succeeded in developing a simple and sensitive CZE method for analyzing the thiol peptides phytochelatins, GSH and γ-EC as well as cysteine using an on-column derivatization with N-ethylmaleimide.

Maleimides react with the thiol group in an addition reaction providing very stable, UV absorbing and/or fluorescent active products. The addition of maleimide to the thiol group proceeds rapidly and quantitatively. This was confirmed by off-line derivatization of the standards with NEM followed by the analysis of the derivatives with CZE. The derivatization of the thiols was completed after a reaction time of only 30 seconds.

Under the given operating conditions, NEM possesses the greater electrophoretic mobility and can react with the thiol peptides while passing them in the electrophoretic run.

Figure 1 provides a representative electropherogram of a derivatized standard mixture of all thiols tested. The standard solution has a concentration of 250 μg/mL for each peptide. All peaks are fully resolved and the separation is completed within 6 minutes. The optimum operating buffer was found to be 0.1 M Tris/HCl, pH 8.3. Lower buffer concentration increases the electroosmotic flow resulting in a poorer resolution of the thiol peptides. Due to derivatization with NEM the migration times of the standards are reduced by 0.5 min compared with an underivatized sample (data not shown). Furthermore no peaks are observed at the migration time of the underivatized thiols. From this we conclude, that the derivatization reaction is entired. Since the surplus NEM elutes after 2 minutes it does not interfere with the CZE analysis of the derivatized compounds.

However, it is necessary to flush the capillary before every analysis as described in the experimental section to remove the adsorbed NEM from the capillary wall. A significant difference is the higher extinction coefficient of the derivatives also observed by Piccoli et al.[9]. Because of derivatization the sensitivity of the method increases 2–3 fold for the precursors (Figure 2). Due to the higher number of functional thiol groups the sensitivity of detection of the phyto-

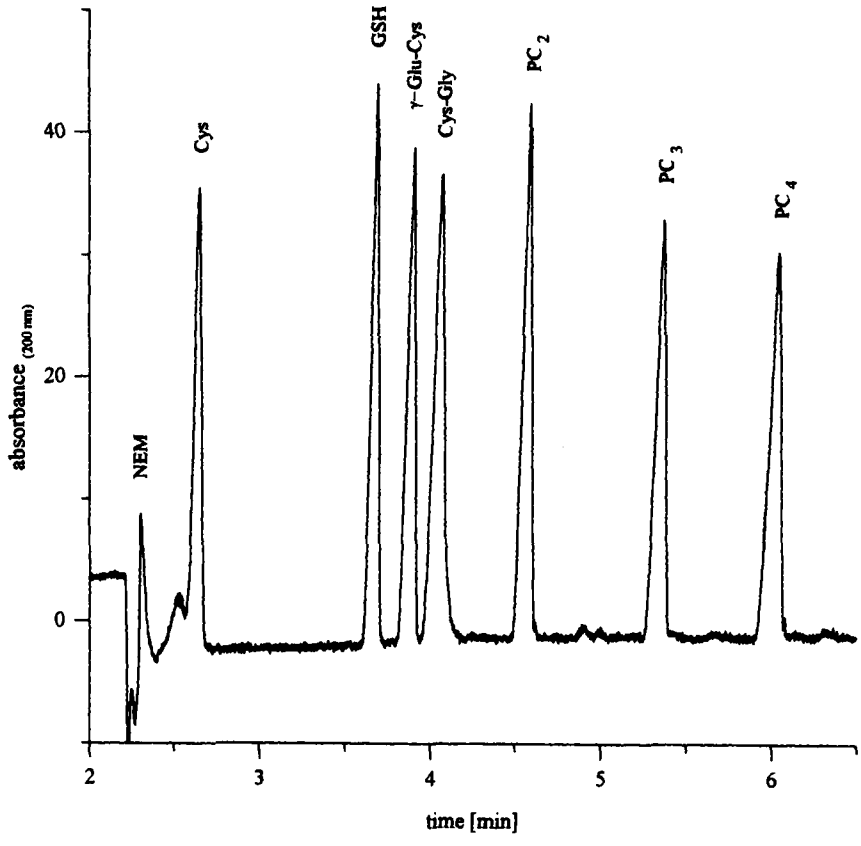

FIGURE 1 Separation of a thiol peptide standard mixture using on-line derivatization with N-ethylmaleimide (NEM). The separation was achieved in a 47.5 cm (total length) untreated fused silica SUPELCO FS 50 CE tube (363 μm o.d., 50 μm i.d.) with the detection window at 39 cm. Separation conditions: buffer: 0.1M Tris/HCl, pH 8.3; sample concentration for each compound: 250 μg/mL; detection: 200 nm. The injection of the sample was performed hydrodynamically (50 mbar, 3 sec) followed by the NEM injection (50 mbar, 2 sec)

chelatins rises even more than this factor. The detection limits of the standards are presented in Table I. Applying the newly introduced Z-shaped high sensitive detector cell of the given equipment, we found an additional remarkable increase of sensitivity (about 10-fold, data not shown) without any loss of resolution power. Regarding all features the CZE method overcomes the usual disadvantage of low sensitivity and can be used for trace determinations of phytochelatins.

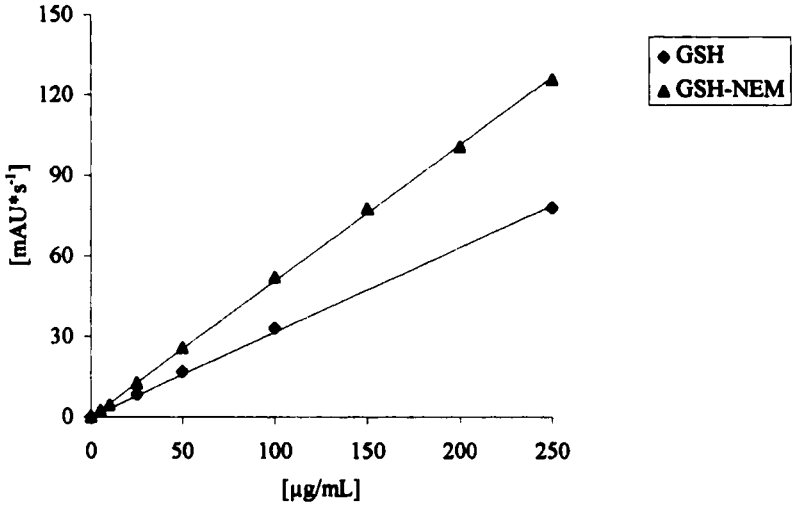

FIGURE 2 Increase of absorbance by the derivatization of glutathione with N-ethylmaleimide

TABLE I Detection limits of the thiol standard compounds

Thiol compound	*Detection limit [µM]*
Cysteine	130
Cysteinylglycine	105
γ-Glutamylcysteine	62
Glutathione	46
PC_2	29
PC 3	19
PC_4	17

The reproducibility was tested for various standard concentrations of cysteine, glutathione, γ-glutamylcysteine, cysteinylglycine, and PCs. Relative standard deviations determined on the basis of five injections are depicted in Table II, as well as the plate numbers of the separation. The method has a satisfactory reproducibility with a 0.5 – 4.7 % standard deviation of peak area. The migration times show standard deviations of 0.25 – 2.5 % over the whole range of calibration.

TABLE II Relative standard deviations of standard thiols (N = 5) and plate numbers (10 µg/mL)

Thiol compound	*Concentration [µg/mL]*	*Rel. standard deviation [%]*		*plate number*
		Peak area	*Migration time*	
Cysteine	10	3,95	0,25	25 600
	100	0,54	0,45	
Cysteinylglycine	10	4,00	0,44	38 800
	100	1,68	1,06	
γ-Glutamylcysteine	10	3,17	0,42	242 800
	100	0,84	1,38	
Glutathione	10	1,44	0,34	250 300
	100	0,98	1,27	
PC_2	10	2,42	0,44	264 900
	100	1,56	1,72	
PC_3	10	2,7	0,93	257 800
	100	1,27	2,11	
PC_4	10	4,67	1,16	176 300
	100	1,62	2,46	

Figure 3 shows calibration curves for glutathione and PC_4 obtained by injecting standard concentrations ranging from 5 – 250 µg/mL. A good linearity between peak area and concentration can be observed, with correlation coefficients ranging from 0.996 – 0.9996. A conspicuous aspect is the higher ascent of the calibration curve of PC_4 in contrast to that of glutathione. This indicates the increased derivatization capability of phytochelatins with higher chain length.

An example of the applicability of the method for the analysis of natural thiol peptides is shown in Figure 4. Electrophoresis was performed with an acidic extract (1:3 with 0.1 N HCl) of a cadmium incubated tomato cell culture from *Lycopersicon esculentum*. Comparing Figures 4a and 4b, remarkable differences between the analyses of the untreated and the filtered extracts were observed. First, the resolution of the precursors caused by the filtration step was achieved. So the identification of glutathione and γ-glutamylcysteine by standard addition and the quantification using calibration curves became possible. The concentration of cysteine was probably too low to be detected. Second, the unfiltered extracts exhibit a slight shift in migration times compared to the standard mixture

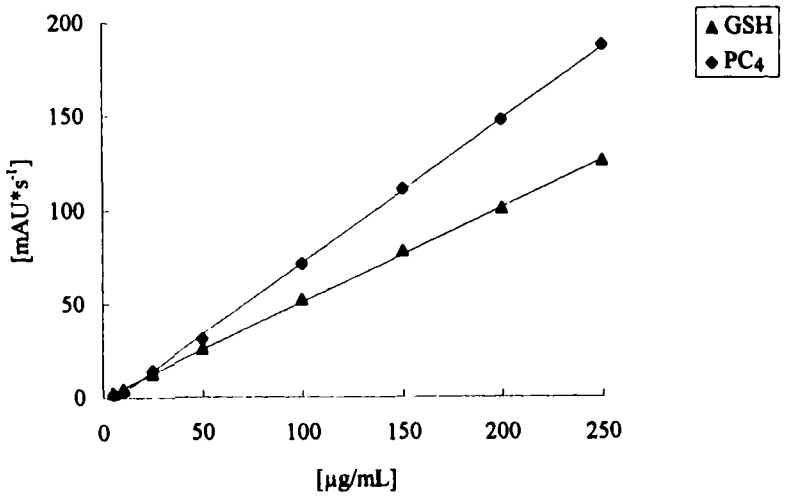

FIGURE 3 Calibration curves of standard thiols

(Figure 1) and to the purified sample. We assume that changes in EOF, the driving force in CZE, during the analytical run cause a slower migration of compounds. These changes may be caused by the complex nature of the crude plant extract, i.e., the chemical and biological background: the very acidic pH (0.9) of the extract, the adsorption of sample components to the capillary wall, changes in viscosity and dielectric constants compared to the aqueous standard mixture. However, the peak identity of the thiol peptides was confirmed by standard addition.

Nevertheless, filtration using the 1 kDa membrane may cause a loss of PC_{2-4} due to their higher molecular weight, which approaches 1 kDa. Addition of a standard mixture to the extract followed by filtration resulted in findings for PC_{2-4} of only 50 % and lower. However, the filtration step seems to be necessary for the quantitative determination of the precursor thiols, whereas PC_{2-4} can be quantified using the unfiltered extract.

Furthermore, we performed experiments with N-(1-pyrenyl)-maleimide, a derivatization reagent which is characterized by an additional absorption at 314 nm and fluorescence activity (data not shown). Using the on-line technique we could not achieve a complete derivatization of a glutathione standard within the time of analysis, in spite of longer capillaries. Additionally, we observed a considerable increase in the number of peaks, a result of the high tendency of maleimide derivatives to hydrolyze[24,25]. The pre-column derivatization of thiol

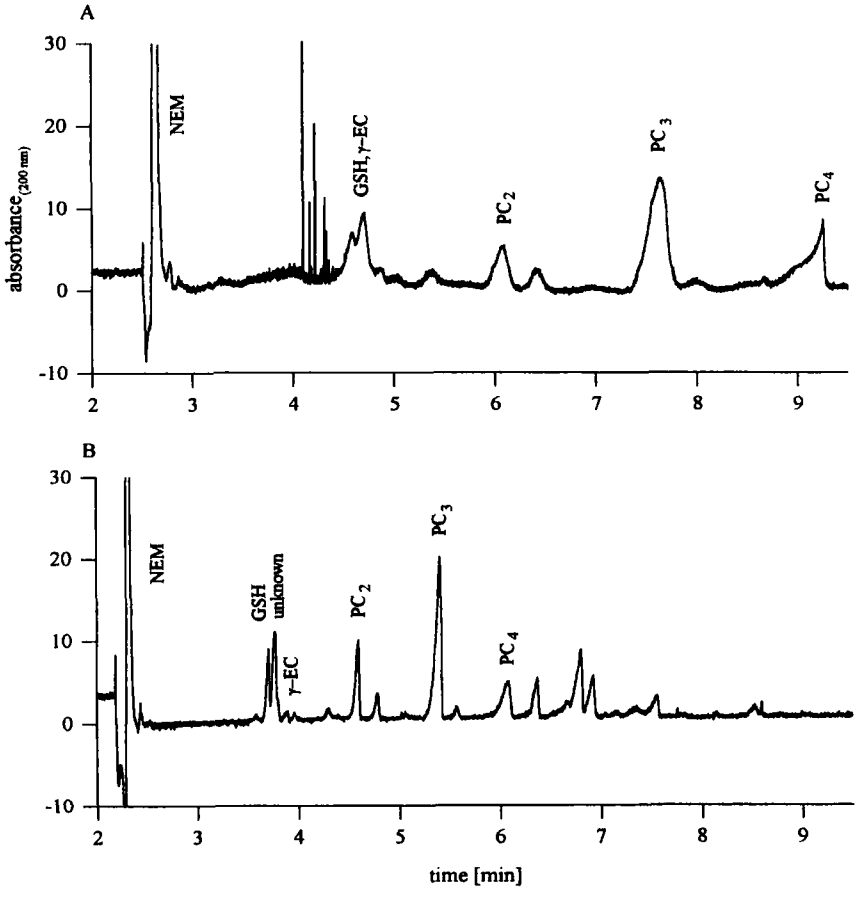

FIGURE 4 Separation of an extract of a cadmium incubated tomato cell culture of *Lycopersicon esculentum*using on-line derivatization with NEM. **A:** Separation of an untreated extract. **B:** Separation of a filtered extract (MICROSEP™, 1kDa, 5000 rpm). Separation conditions: see Fig. 1

peptides and the subsequent CZE analysis, however, should be a practical alternative if trace amounts of PCs have to be measured by the much more sensitive fluorescence detection. In conclusion, CZE is of great value as a method for analyzing thiol peptides. The presented method distinguishes itself by a complete and simultaneous separation of PC_{2-4} and their precursor molecules. It combines rapid analysis times and small sample requirements. On-column derivatization of the thiol peptides with NEM can partly compensate for the lack of sensitivity and lower the limit of detection. A further advantage of an on-line derivatization is

the automation. Subjective errors will be avoided and time to perform the derivatization reaction will be spared. Attention has to paid to the fact that sample parameters such as pK-values, stability and solubility of the analytes can influence the separation conditions. In forthcoming studies we will apply this rapid and sensitive method to investigate biochemical processes involved in the heavy metal stress response in water mosses[26,27] and aquatic hyphomycetes[28]. In this connection, qualitative and quantitative analyses of thiol peptides will be emphasized, because these compounds represent potential biomarkers in mosses. The method described should also allow the separation of other classes of phytochelatins, so-called iso-PCs[1].

CONCLUSIONS

The CZE method described here for the rapid and simple analysis of metal binding thiol peptides was demonstrated to be a suitable procedure for the simultaneous determination of PCs and their precursor molecules which are involved in heavy metal stress response in plants. Using the selective on-line derivatization of thiol peptides with N-ethylmaleimide a remarkable increase of sensitivity over the required detection range could be achieved. The method combines the advantages of capillary electrophoresis with the opportunity to automate the derivatization step.

The new developed CZE procedure has been successfully applied to the investigation of different plant species which were exposed to heavy metal stress.

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