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Rapid and Ultrasensitive Method for Determination of Phytochelatin$_2$ using High Performance Liquid Chromatography with Electrochemical Detection

Dedicated to UNEP – Lead and Cadmium activities

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Plants respond to heavy metal toxicity in a variety of different ways including synthesis of phytochelatins. The synthesis of phytochelatins is catalyzed by γ-Glu-Cys dipeptidyl transpeptidase named as phytochelatin synthase (PCS). The main aim of this study was to optimize high performance liquid chromatography coupled with electrochemical detector for determination of phytochelatin$_2$. The optimized procedure was subsequently used for determination of the mentioned molecules with special attention aimed at the possibility to determine PC_2 after activation of PCS in the tobacco BY-2 cells treated with different concentrations of cadmium(II) ions. The optimized conditions were as follows. Both the detector and the column were thermostated at 30 °C. Mobile phase consisted of A: trifluoroacetic acid (80 mM) and B: 100% methanol. Compounds were eluted by the following linear increasing gradient: 0-1 min (3 % of B), 1→12 min (20 % of B), 12→15 min (98 % of B), 15→20 min (98 % of B). Flow rate of the mobile phase was 1 ml min^{-1}. Detection was carried out at applied potential 900 mV postponed on four electrodes. Time of one analysis was 15 minutes. The estimated detection limit for PC_2 was 17 nM. In addition, the recoveries were from 93 to 98 %. Good precision was obtained with %C.V.s ranging from 5.3 to 7.5 % in the intra-assay. The inter-assay %C.V.s ranged from 8.9 to 10.2 %. Overall recoveries of were from 102 to 106 % (n = 30). Accuracy (%Bias) was about ±5 %. Further, the attention was aimed at determination of PC_2 in BY-2 tobacco cells treated

with cadmium(II) ions (0, 5, 10, 25, 50 and 100 μM). It clearly follow from the results obtained that the content of PC_2 enhanced with increasing concentration of the substrate and with the applied concentration of cadmium(II) ions.

Keywords: Cadmium, glutathione, phytochelatin, high performance liquid chromatography, electrochemical detector, tobacco BY-2 cell suspension

1. INTRODUCTION

Due to increasing anthropogenic activities, amounts of undesirable compounds in the environment have been increasing. The undesirable compounds may disperse in the atmosphere and/or dissolves in the ground water and consequently contaminate ecosystems and food chain – extensive pollution of the environment [1,2]. The heavy metals are one of the most toxic groups of these undesirable compounds that threaten both plants and animals [3-5]. Plants respond to heavy metal toxicity in a variety of different ways such as immobilization, exclusion, chelation and compartmentalization of the metal ions, and the expression of more general stress response mechanisms as synthesis ethylene and stress peptides [6]. One of the most studied plant stress peptides are phytochelatins (PC; a basic formula $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n = 2 to 11)) [6-9] participating in the detoxification of heavy metals, because they have an ability to bind heavy metal ions via –SH groups of cysteine units and consequently transport them to vacuole [6,8-11], where an immediate toxicity do not menace yet. Moreover, it was shown that PC-mediated heavy metal detoxification is not restricted to plants and some fungi but extends to animals by demonstrating that the ce-pcs-l gene of the nematode worm *Caenorhabditis elegans* encodes a functional PC synthase whose activity is critical for heavy metal tolerance in the intact organism [12,13].

The synthesis of phytochelatins proceeds from glutathione by transferring γ-Glu-Cys moiety from a donor to an acceptor molecule. Particularly, the reaction involved the transpeptidation of the γ-Glu-Cys moiety of GSH onto initially a second GSH molecule to form PC_2 or, in later stages of the incubation, onto a PC molecule to produce an $n + 1$ oligomer (Fig. 1). The reaction is catalyzed by γ-Glu-Cys dipeptidyl transpeptidase (EC 2.3.2.15, Fig. 1), which has been named as phytochelatin synthase (PCS). *In vitro* the activity of the partially purified enzyme is active only in the presence of metal ions. Cadmium is the best activator of PCS followed by Ag, Bi, Pb, Zn, Cu, Hg, and Au cations [6].

The great importance of plant thiols and their contents can be expected in the field of phytoremediation technologies [14,15], in which transgenic lines of *populus* [16], *brassica* [17], tobacco[18] and *arabidopsis* [19] have been prepared. The interest in the determination of phytochelatins and other thiol peptides and proteins (glutathione, metallothioneins, metalloenzymes) by classical electrochemical methods has been increasing [20-34]. Differential pulse and cyclic voltammetry belong to the most used electrochemical techniques for the mentioned purposes [35-40]. In addition recently chronopotentiometric analysis with constant current has been used for a sensitive determination of thiols [41-47]. As we mentioned above, the electrochemical method are sensitive and, if we are coupled them with effective separation method such as high performance liquid

chromatography (HPLC) [48-62], capillary electrophoresis [63,64] and/or capillary liquid chromatography [65] are also selective. In the case of thiols determination in flow system, carbon electrodes were used markedly as working electrodes [33,66-68]. Boron-doped diamond carbon electrodes can be also used for detection of thiols [69].

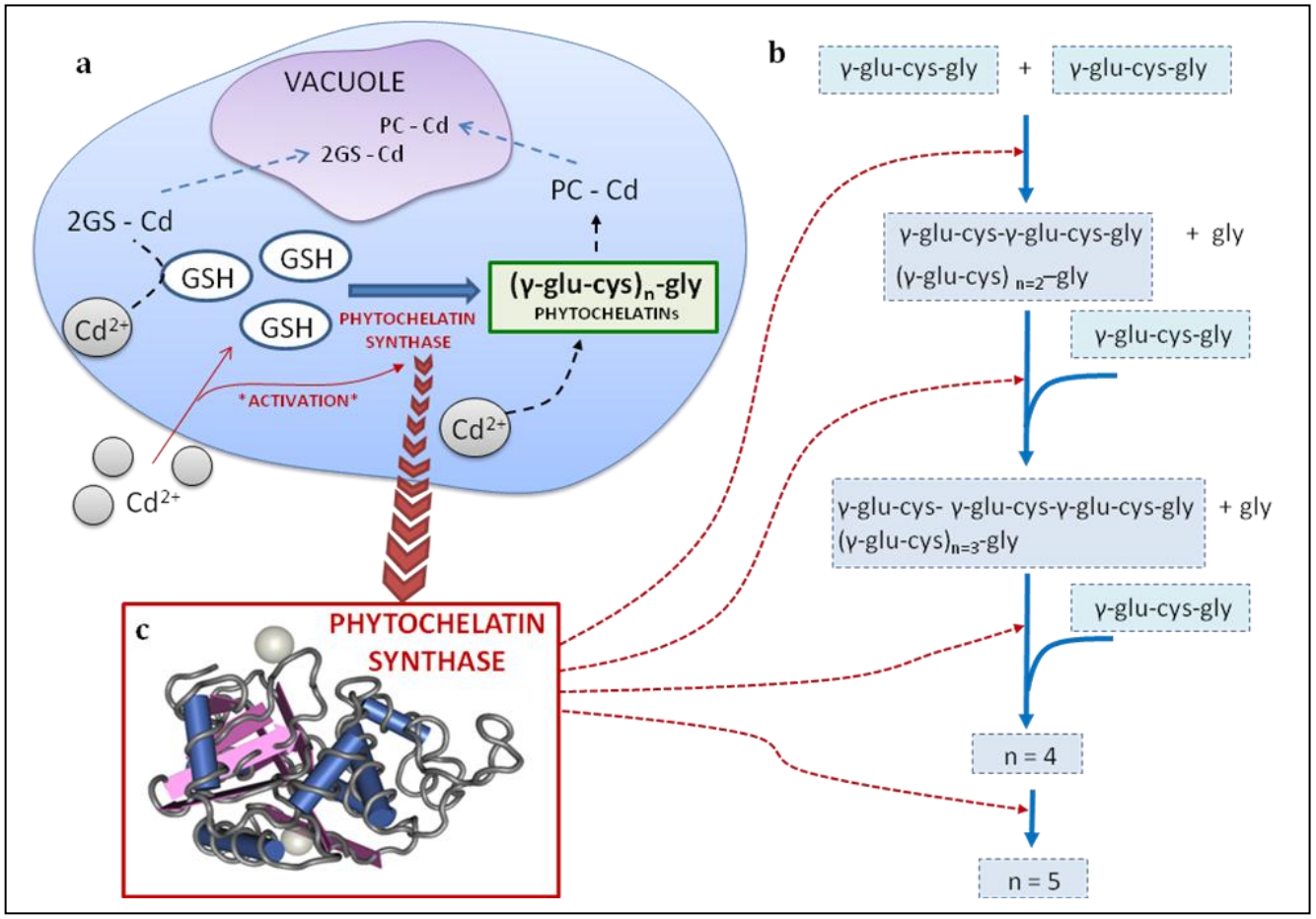

Figure 1. (**a**) Glutathione serves as a precursor of phytochelatins, (**b**) which are composed of two or more repeating gamma-glutamylcysteine units with a terminal glycine residue; (gamma-glutamylcysteine)$_n$-gly, where n = 2 to 11. (**c**) The enzyme responsible for the synthesis of these peptides is known as phytochelatin synthase (glutathione gamma-glutamylcysteinyltransferase or gamma-glutamylcysteine dipeptidyl transpeptidase), which is a constitutive enzyme that is activated by cadmium and other metal ions. The structure of the enzyme was done according to Vivares et al. [70].

The main aim of this study was to optimize high performance liquid chromatography coupled with electrochemical detector for determination of phytochelatin$_2$. The optimized procedure was subsequently used for determination of the mentioned molecules with special attention aimed at the possibility to determine PC_2 after activation of PCS in the tobacco BY-2 cells treated with different concentrations of cadmium(II) ions.

2. MATERIAL AND METHODS

2.1. Chemicals and pH measurements

Reduced (GSH) and oxidized (GSSG) glutathione, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, USA). Phytochelatin$_2$ (PC_2) (γ-Glu-Cys)$_2$-Gly was synthesized in Clonestar Biotech (Brno, Czech Republic) with a purity above 90 %. HPLC-grade methanol (>99.9%; *v*/*v*) was from Merck (Dortmund, Germany) were used. Other chemicals were purchased from Sigma-Aldrich (St. Louis, USA) unless noted otherwise. Stock standard solutions of the thiols (1 mg.ml^{-1}) were prepared with ACS water (Sigma-Aldrich, USA) and stored in dark at -20 °C. Working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through 0.45 μm Nylon filter discs (Millipore, Billerica, Mass., USA) prior to HPLC analysis. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by software MultiLab Pilot; Weilheim, Germany. The pH-electrode (SenTix H, pH 0..14/0..100°C/3M KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany).

2.2. High performance liquid chromatography with electrochemical detection

HPLC-ED system consisted of two solvent delivery pumps operating in the range of 0.001-9.999 ml.min^{-1} (Model 582 ESA Inc., Chelmsford, MA), Zorbax eclipse AAA C18 (150 × 4.6; 3.5 μm particles, Agilent Technologies, USA) and a CoulArray electrochemical detector (Model 5600A, ESA, USA). The electrochemical detector includes one flow cells (Model 6210, ESA, USA). The cell consists of four analytical cells containing working carbon porous electrode, two auxiliary and two reference electrodes. The sample (20 μl) was injected using autosampler (Model 540 Microtiter HPLC, ESA, USA). Other experimental parameters were optimized.

2.3. Differential pulse voltammetry

Differential pulse voltammetric measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes. The three-electrode system consisted of hanging mercury drop electrode (HMDE) as working electrode, an Ag/AgCl/3 M KCl reference electrode and a glassy carbon auxiliary electrode. For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed. Acetate buffer (0.2 M CH_3COOH + 0.2 M CH_3COONa) was used as the supporting electrolyte. The measurements were performed at room temperature. The samples were deoxygenated prior to the measurements by purging with argon (99.999%) saturated with water for 10 min. Cadmium concentration was measured by a differential pulse adsorptive stripping voltammetric method. The anodic scan was initialised at −0.7 V and stopped at the −0.4 V. The cadmium was deposited on HMDE at potential −0.7 V with accumulation time of 180 s at room temperature. The solution was stirred (1450 rpm) during deposition process. Other parameters of method were: modulation time 0.02

s, interval time 0.1 s, step potential 1.05 mV scan rate 10.5 mVs^{-1}, modulation amplitude 49.5 mV [71,72].

2.4. Esterase assay

Cultivation medium of tobacco BY-2 cells was removed by centrifugation (360 g; 5 min; 20°C; centrifuge MR 22, Jouan, USA). The cells were washed twice in 50 mM potassium phosphate buffer (pH 8.7).

The washed BY-2 cells and harvested ESEs (100 – 200 mg, respectively) were mixed with extraction buffer (250 mM potassium phosphate, pH 8.7) to a final volume of 1 ml and homogenised using a Potter-Elvehjem homogenizator (Kavalier, Czech Republic) placed in an ice bath for 10 min. The redox state of the obtained solution was maintained by addition of 1 mM dithiothreitol (DTT). The homogenised samples were sonicated for 1 min in an ice bath using a Transsonic T310 sonicator (Czech Republic). The homogenate was centrifuged at 10 000 g; 15 min; 4°C (centrifuge MR 22, Jouan, USA). An aliquot (5 – 20 µl) of the supernatant was mixed with potassium phosphate buffer (995 – 980 µl, 1 M, pH 8.75). The reaction was started by the addition of FDA to final concentration of 5 µM. The final volume of the reaction mixture was 1 ml. As a blank sample an equal volume of extraction buffer was used.

After incubation (15 min, 45°C, dry block, Major Science, Taiwan), an aliquot (5 – 20 µl) of the reaction mixture was added to 5 mM potassium phosphate buffer (pH 8.7, 1980 – 1995 µl). The fluorescence ($\lambda_{excitation}$ 490 nm and $\lambda_{emission}$ 514 nm) was read immediately using a spectrofluorimetric detector RF-551 (Shimadzu, USA). A stock solution of FDA was prepared in acetone dried by anhydrous calcium chloride. The amount of acetone did not exceed 1% (*v*/*v*) in the reaction mixture. Esterase activity in international units (IU, one unit liberates one µmol of fluorescein per minute under specified conditions) was recalculated to relative units (100 % represents the highest activity measured in an experiment) [73,74].

2.5. Plant cell culture

The suspension culture of tobacco *Nicotiana tabacum* cv. BY-2 was grown in liquid Murashige and Skoog medium (MS medium Micro and Macro elements, Duchefa, The Netherlands) supplemented with sucrose (30 $g.l^{-1}$), KH_2PO_4 (0.2 $g.l^{-1}$), thiamine (1 $mg.l^{-1}$) and 2,4-dichlorophenoxyaetic acid (0.2 $mg.l^{-1}$) (all from Duchefa, Plant cell tested, The Netherlands) according to Nagata [75].

The suspension cultures (20 ml) were grown in 50 ml Erlenmeyer flasks at 27 °C with shaking at 135 rpm (ES-20, Biosan, Latvia). Subcultivation of culture was performed after 3 or 4 days by transferring 2 or 1 ml, respectively, of suspension culture into a fresh medium (total volume 20 ml). One day old suspension cultures were treated with 0, 5, 10, 25, 50 and 100 µM $Cd(NO_3)_2$ and were cultivated for three day in the medium at 27 °C with shaking at 135 rpm.

2.6. Preparation of BY-2 tobacco cells

At the end of three day long cultivation, app. 500 µg of BY-2 cells were harvested in 10 ml of the cultivation medium in all experimental groups. The media containing cells were centrifuged at 2000 rpm and 4 °C. The cells were further washed with 10 ml of 20 mM phosphate buffer (pH 7.5) for 10 min. and centrifuged for 15 min (2000 rpm, 4°C). Supernatant was removed and 200 µl of cells were homogenized were transferred to a test-tube, and liquid nitrogen was added. The samples were frozen to disrupt the cells. Then, 800 µl of 0.2 M phosphate buffer (pH 7.2) was added into the test tube. The mixture was prepared by hand-operated homogenizer ULTRA-TURRAX T8 (IKA, Germany) at 25,000 rpm for 3 minutes [76]. The homogenate was transferred to a new test-tube. The mixture was further homogenised by shaking on a Vortex–2 Genie (Scientific Industries, New York, USA) at 4 °C for 30 min. The homogenate was centrifuged (16 400 *g*) for 15 min at 4 °C using a Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, Tuttlingen, Germany). Prior to analysis the supernatant was filtered through a membrane filter (0.45 µm Nylon filter disk, Millipore, Billerica, Mass., USA) and used for determination of PC_2.

2.7. Sample preparation for cadmium determination

Maize kernels (approximately 0.2 g) were rinsed in 0.1 M ethylene diamine tetra-acetic acid (EDTA) and then were digested by an Multiwave 3000 microwave digestion furnace (Anton Paar, Germany). A three steps digestion procedure ((i) 120 s, 250 W; (ii) 120 s, 0 W (120 °C); (iii) 10 min 250 W (180 °C)) with addition of 5 ml 65% HNO_3 and 3 ml water was used. The clear digest was quantitatively transferred into a vessel and diluted up to 8 ml with water.

2.8. Accuracy, precision and recovery

Recovery of PC_2 were evaluated with homogenates (tobacco BY-2 cells) spiked with standard according to Causon [77]. Before extraction, 100 µl PC_2 was added to tobacco BY-2 cells. Precision (coefficient of variation; %C.V.) of intra-day assay was performed in 6 homogenates. Inter-day precision was determined by analysing six homogenates over a 5-day period. Homogenates were assayed blindly and PC_2 concentration was derived from the calibration curves. The spiking of PC_2 was determined as a standard measured without the presence of real sample. Accuracy was evaluated by comparing the estimated concentration with the known concentrations of thiol compounds. Calculation of accuracy (%Bias), precision (%C.V.) and recovery was expressed according to [77,78].

2.9. Descriptive statistics

Data were processed using MICROSOFT EXCEL® (USA). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). The detection limits (3 signal/noise, S/N)

were calculated according to Long and Winefordner [79], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

3. RESULTS AND DISCUSSION

Primarily we aimed at study of electrochemical behaviour of PC_2, as the main product of PCS-catalyzed reaction, at the carbon porous electrodes in flow system. Some results on electrochemical determination of PC_2 were published by Potesil et al. [54], Petrlova et al. [53] and Diopan et al. [48], however, they have not focused on the electrochemical behaviour this peptide.

3.1. Electrochemical behaviour of phytochelatin$_2$

Oxidation of sulphhydryl group was mainly studied at mercury electrodes, at which the electrolytic process forming Hg-S compounds occurs [10]. In the case of carbon paste electrodes and electrodes made of pyrolytic graphite the –SH moieties of PC_2 similarly to other thiols are oxidized according to the reaction $2RSH \rightarrow RSSR + 2H^+ + 2e^-$ [66,80].

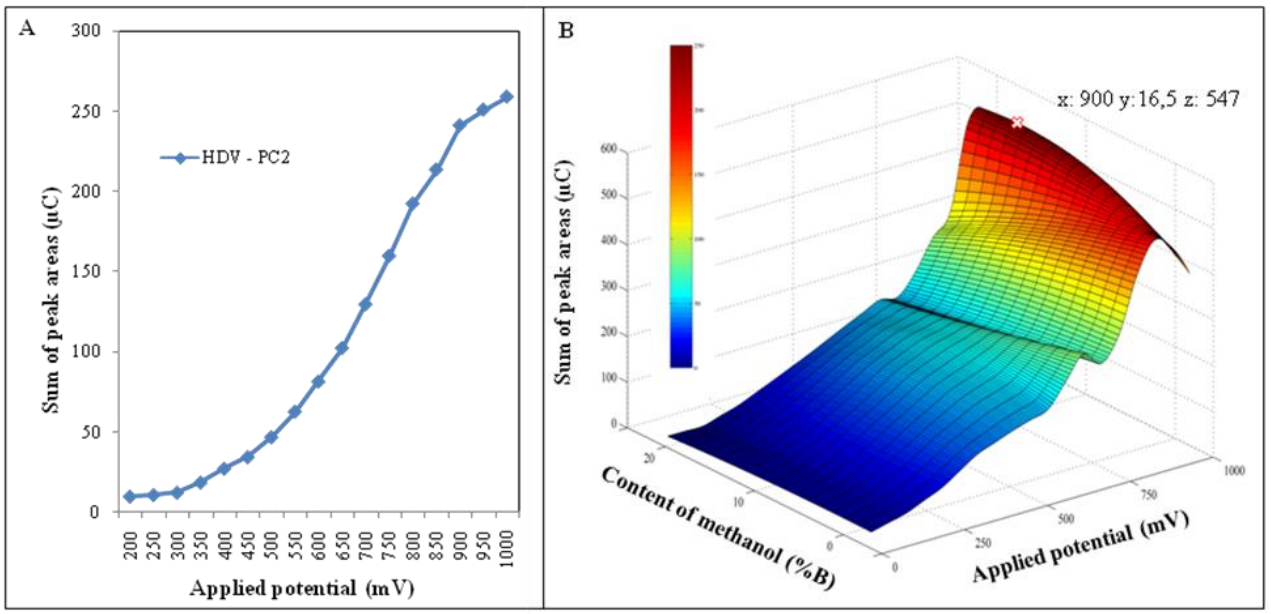

Figure 2. (**A**) Hydrodynamic voltammogram of dependence of applied potential on current response of PC_2. Increasing applied potential gives highest response in sum of peaks. (**B**) Three dimension graph of dependence of sum of peak areas on the changing content of methanol and applied potential. Higher potential as well as higher concentration of methanol gives the best response in sum of peak areas. The best response is in peak maximum at 900 mV and 16.5% of methanol (*v*/*v*). Experimental conditions. Mobile phase: trifluoracetic acid (80 mM) and methanol in content from 0 to 20% (*v*/*v*) per 5 % steps. Flow rate 1 ml.min^{-1}. Column and detector temperature: 30 °C, n = 5.

Primarily, we aimed at studying of electrochemical behaviour of PC_2 and at optimizing of the working electrode potential for detection of this peptide. As a mobile phase in our experiments, we used a combination of 80 mM solution of TFA/methanol ratio of 97 %/3 % (*v*/*v*) and its flow rate as 1 $ml.min^{-1}$ according to our previously published results [54]. To select a suitable potential, where the detector would provide the highest current response of the analyte, is used so-called hydrodynamic voltammogram (HDV). Its design is based on the cumulative current response of the substance, which usually produces a curve of sigmoidal character. In the obtained HDV, we are looking for an interval with the highest current and the lowest potential difference between the measured values. In our case, we applied one potential on all four working electrodes and sum the measured current responses.

The influence of the applied potential on current response of PC_2, which means HDV for PC_2, is shown in Fig. 2A. The potential has been postponed from 200 mV to 1000 mV per 50 mV steps. It clearly follows from the figure that current response enhanced with increasing applied potential. The sharpest increase was determined under 900 mV, which was selected as the most suitable potential for determination of PC_2 in the following experiment. Our result is in good agreement with paper from Zhang et al. [66].

Gradient elution with increasing content of an organic solvent is obviously used during the chromatographic separation on a reversed phase column. It is not surprised that these changing conditions can markedly influence current responses of the analytes. Therefore, we were interested in the issue how changing content of methanol from 0 to 20 % (*v*/*v*) per 5 % steps could influence HDVs of PC_2. HDVs were measured under same protocol as mentioned above. Based on the results obtained 3D graph of the dependence of sum of peak areas on the changing content of methanol and applied potential was constructed (Fig. 2B). The local maxima determined within the interval from 600 to 700 mV can be related to oxidation of better accessible PC_2 moieties. However, the highest signals were determined under the highest applied potentials and the highest content of methanol with the maximum at 900 mV and 16.5 % of methanol (*v*/*v*). These results can be of interest for those researchers, which need to have higher content of an organic solvent in mobile phase due to analysis of complex matrix.

3.2. Calibration and recovery

Due to the fact that reduced glutathione is a substrate for PCS and based on the above optimized conditions, we selected the following chromatographic parameters to detect GSH, GSSG and PC_2 simultaneously with the highest sensitivity for PC_2. Both the detector and the column were thermostated at 30 °C. Mobile phase consisted of A: trifluoroacetic acid (80 mM) and B: 100% methanol. Compounds were eluted by the following linear increasing gradient: 0-1 min (3 % of B), 1→12 min (20 % of B), 12→15 min (98 % of B), 15→20 min (98 % of B). Flow rate of the mobile phase was 1 ml min^{-1}. Detection was carried out at applied potential 900 mV set on four electrodes. Time of one analysis was 15 minutes, which is shorter than other published methods [48,53]. The chromatograms of GSH, GSSG and PC_2 standards measured on the first electrode are shown in Fig. 3A. It clearly follows from the results obtained that the signals enhanced with the increasing concentration of the thiol. However, sums of peak areas measured on all twelve electrodes were used

for determination of calibration curves for GSH, GSSG and PC_2 (Fig. 3B). The calibration curves were strictly linear within the concentration range from 0.05 – 100 µg/ml (GSH), 0.2 – 100 µg/ml (GSSG) and 0.1 – 100 µg/ml (PC_2). The ranges cover concentrations of the target molecules in real samples and are much larger and therefore versatile compared to Diopan et al. [48]. Moreover, we estimated detection limits as units or tens nM for thiols, which is lower compared to the same paper [48]. The other analytical parameters are shown in Tab. 1.

Besides analytical parameters of the optimized method, recovery is other important parameter. Therefore, we estimated recovery of PC2 determination in extract of BY-2 tobacco cells. Samples of tobacco BY-2 cells were prepared according to protocol mentioned in Material and Methods section with special attention to redox status of a sample because the fact that peptides rich in –SH moieties may undergo oxidation to form disulfide bridge during sample preparation [81,82]. The reduced forms of peptides such as GSH formed during sample preparation GSSG conjugates, thereby decreasing the concentration of the determined reduced peptides.

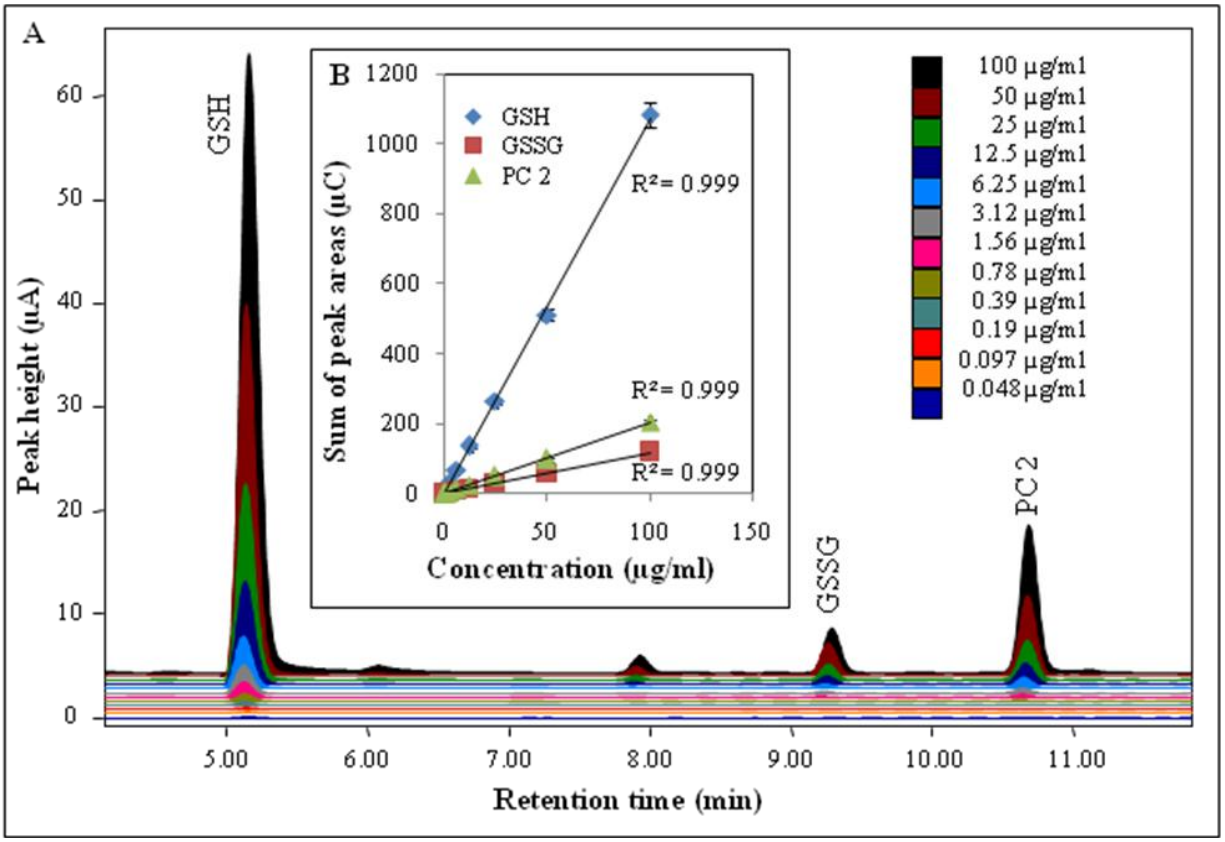

Figure 3. (**A**) Chromatograms of GSH, GSSG and PC_2 standards. Retention time is 5.13 min for GSH, 9.26 min for GSSG and 10.66 min for PC_2. With increasing concentration of single thiols their signal is also enhanced. (**B**) Calibration curves of GSH, GSSG and PC_2. In concentration range from 0.0488-100 µg/ml (GSH), 0.195-100 µg/ml (GSSG) and 0.097-100 µg/ml (PC_2) the curves are strictly linear with R^2 = 0.999. Experimental conditions. Mobile phase: trifluoroacetic acid (80 mM) and 100% methanol in linear increasing gradient elution 0-1 min (3 % of B), 1→12 min (20 % of B), 12→15 min (98 % of B), 15→20 min (98 % of B). Flow rate 1 $ml.min^{-1}$. Applied potential 900 mV. Column and detector temperature: 30 °C, n = 5.

Table 1. Analytical parameters of HPLC-ED used for detection of reduced (GSH) and oxidized (GSSG) glutathione and PC2.

Compounds[1]	Retention time	Regression equation	Linear dynamic range (μM)	Linear dynamic range (μg/ml)	$R^{2,2}$	LOD[3] (nM)	LOD (ng/ml)	LOD (fmol) per injection	LOQ [4] (nM)	LOQ (ng/ml)	LOQ (nmol) per injection	RSD[5] (%)
GSH	5.13	y = 10.71x - 0.154	0.159 - 325	0.0488 - 100	0.999	6	2	110	60	17	1	3.2
GSSG	9.26	y = 1.187x - 0.106	0.318 - 163	0.195 - 100	0.999	26	16	510	260	160	5	4.8
PC_2	10.66	y = 2.028x - 0.021	0.180 - 185	0.097 - 100	0.999	17	9	340	170	90	3	4.3

1…studied thiol compounds.
2…regression coefficients.
3…limits of detection of detector (3 S/N).
4… limits of quantification of detector (10 S/N).
5…relative standard deviations.

Table 2. Recovery of PC_2 for tobacco BY-2 cell suspension sample analysis (n = 5).

Compounds	Spiking (ng/ml)			Cell extract (ng/ml)			Cell extract + spiking (ng/ml)			Recovery (%)
PC_2	500	±	22	610	±	26	1086	±	47	98
	100	±	4	610	±	26	691	±	30	97
	50	±	2	610	±	26	613	±	26	93

Table 3. Intra- and inter-day precision and accuracy of the chromatographic method.

Compounds	Added (ug/ml)	Intra-day (n=8) C.V. (%)	Recovery (%)	Inter-day (n=3) C.V. (%)	Recovery (%)
PC_2	10	7.5	102	10.2	106
	50	6.2	105	8.9	104
	100	5.3	106	9.6	102

If the peptide contains more –SH moieties such as PC_2, there may occur intramolecular disulfide bonds [83]. For these reasons, we have added the reducing agent dithiothreitol (DTT) to homogenates. Except DTT, phosphine derivatives, dithionit or potassium borohydride can be also used to reduced thiols [35,81,84,85]. We added three various concentrations of PC_2 (20, 50 and 100 $ng.ml^{-1}$) to the cell extract. Then, the cells were prepared according to protocol and analysed by the optimized method. The recoveries were from 93 to 98 % (Tab. 2). Our recoveries are in good agreement with the results published by Petrlova et al. [53] and Potesil et al. [54]. Reproducibility of the procedure was tested by analysing representative samples in six replicates during 5 days (Tab. 3). Good precision was obtained with %C.V.s ranging from 5.3 to 7.5 % in the intra-assay. The inter-assay %C.V.s ranged from 8.9 to 10.2 %. Overall recoveries of were from 102 to 106 % (n = 30). Accuracy (%Bias) was about ±5 %.

3.3. Content of PC_2 in BY-2 tobacco cells treated with cadmium(II) ions

Experiment on BY-2 cells, which were exposed to different concentrations of 0, 5, 10, 25, 50 and 100 µM $Cd(NO_3)_2$. These cells were cultivated according to conditions shown in Material and Methods for three days. Primarily, the growth of the treated cells was estimated using esterase assay (Fig. 4A). It clearly follows from the results obtained that cadmium(II) ions had adverse effect on the growth of the cells, which enhanced with the increasing time of the treatment and applied concentration of toxic ions.

The growth of the cells treated with the highest concentration of cadmium(II) ions were depressed for more than 50 % compared to control cells (Fig. 4B). Further, differential pulse adsorptive stripping voltammetry was employed for determination of cadmium(II) ions accumulated by tobacco cells during the treatment. Concentration of accumulated cadmium(II) ions enhanced with the increasing time of the treatment and applied concentration, which can be closely related with poor growth of the cells.

In addition, defined amount of the cells were harvested from all experimental groups at the end of the treatment (3^{rd} day of the treatment) and homogenised according to protocol in Material and Methods section. We were interested in the issue whether we could determine changes in PC_2 content after activation of PCS by cadmium(II) ions. The protocol for activation of phytochelatin synthase activity was adopted from Nakazawa et al. [86] and modified. Reduced glutathione as a substrate of the enzyme reaction (0.1, 0.5, 1 and 5 mM) and cadmium(II) ions (50 µM $Cd(NO_3)_2$) as an activator of PCS were added to the cell suspension supernatant (100 µl). These mixtures were incubated at 35 °C for 30 min. The addition of 4 µl 5-sulfosalycilic acid (1 M) stopped the enzyme reaction. After the stopping the reaction, the content of PC_2 was immediately determined in the obtained samples using HPLC-ED. The results are shown in Fig. 4C. Content of PC2 in BY-2 tobacco cells treated with cadmium(II) ions is shown in inset in Fig. 4C. It clearly follow from the results obtained that the content of PC_2 enhanced with increasing concentration of the substrate and with the applied concentration of cadmium(II) ions, which were used for treatment of BY-2 tobacco cells. The highest concentration of PC2 (135 µg/ml) was determined in tobacco extract obtained from cells treated with 100 µM cadmium(II) ions and activated by 5 mM GSH for 30 min.

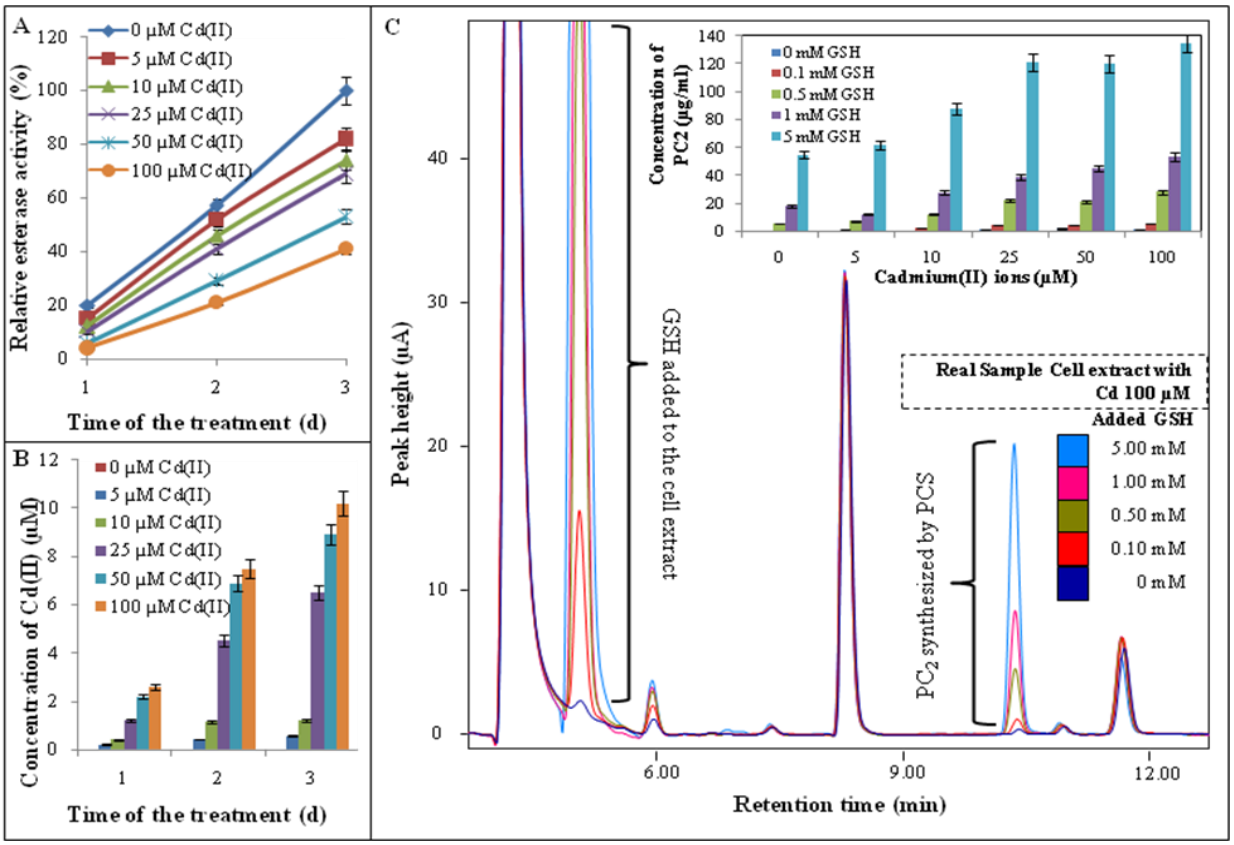

Figure 4. (**A**) Growth of tobacco BY-2 cells treated with cadmium(II) ions for three day estimated using esterase activity. (**B**) Content of cadmium(II) ions in tobacco BY-2 cells treated with cadmium(II) ions for three day determined by differential pulse adsorptive stripping voltammetry. (**C**) Chromatograms of extracts from cells treated with cadmium(II) ions (100 µM) and with subsequently added GSH in various concentrations. The signal of PC_2 enhances with increasing amount of GSH. The highest PC_2 synthesis is at GSH concentration 5 mM. (**Inset in C**) Content of PC2 in cells treated with cadmium(II) ions. Experimental conditions for HPLC-ED are same to those in Figure 3. The other experimental parameters are shown in Material and Methods section.

4. CONCLUSIONS

In recent years, electrochemical detectors using carbon-based working electrodes have been employed to determine a wide range of compounds from nucleic acids to proteins [87] and biologically important low molecular weight substances [45,88-95]. In this study, HPLC-ED was optimized to detect of femtomoles of PC_2. Due to the fact that phytochelatins are currently intensively studied in lower and higher plants and more recently also been identified in animals as a model organism the nematode *Caenorhabditis elegans* [10], HPLC-ED represents new tool that significantly contribute to the possibilities of analytical chemistry of metal-binding peptides.

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