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Journal of Chromatography A, 1084 (2005) 134–144
JOURNAL OF CHROMATOGRAPHY A
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Simultaneous femtomole determination of cysteine, reduced and oxidized glutathione, and phytochelatin in maize (*Zea mays* L.) kernels using high-performance liquid chromatography with electrochemical detection

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Available online 24 June 2005

Abstract

Thiol compounds such as cysteine (Cys), reduced (GSH) and oxidized (GSSG) gluathione, and phytochelatins (PCs) play an important role in heavy metal detoxification in plants. These thiols are biological active compounds whose function is elimination of oxidative stress in plant cells. The aim of our work was to optimise sensitive and rapid method of high-performance liquid chromatography coupled with electrochemical detector (HPLC-ED) for determination of the abovementioned thiol compounds in maize (*Zea mays* L.) kernels. New approach for evaluation of HPLC-ED parameters is described. The most suitable isocratic mobile phase for the separation and detection of Cys, GSH, GSSG and PC_2 consisted of methanol (MeOH) and trifluoroacetic acid (TFA). In addition, the influence of concentrations of TFA and ratio of MeOH:TFA on chromatographic separation and detection of the thiol compounds were studied. The mobile phase consisting from methanol and 0.05% (v/v) TFA in ratio 97:3 (%; v/v) was found the most suitable for the thiol compounds determination. Optimal flow rate of the mobile phase was 0.18 ml min^{-1} and the column and detector temperature 35 °C. Hydrodynamic voltammograms of all studied compounds was obtained due to the selection of the most effective working electrodes potentials. Two most effective detection potentials were selected: 780 mV for the GSSG and PC_2 and 680 mV for determination of Cys and GSH. The optimised HPLC-ED method was capable to determine femtomole levels of studied compounds. The detection limits (3 S/N) of the studied thiol compounds were for cysteine 112.8 fmol, GSH 63.5 fmol, GSSG 112.2 fmol and PC_2 2.53 pmol per injection (5 μl). The optimised HPLC-ED method was applied to study of the influence of different cadmium concentrations (0, 10 and 100 μM Cd) on content of Cys, GSH, GSSG and PC_2 in maize kernels. According to the increasing time of Cd treatment, content of GSH, GSSG and PC_2 in maize kernels increased but content of Cys decreased. Decreasing Cys concentration probably relates with the increasing GSH and phytochelatins synthesis.

Keywords: Maize (*Zea mays*); Kernels; Cadmium; Heavy metals; High-performance liquid chromatography; Electrochemical detection; Method evaluation

1. Introduction

Higher organisms have a number of protection mechanisms against various toxic compounds [1–11]. The compounds come into agro-ecosystem especially from fertilizers, pesticides and industrial toxic products containing heavy metals and other undesirable substances [12]. The study of plant response to heavy metals stress is especially important for the understanding of many biological processes [4,7,13–15]. Plants respond to the presence of heavy metals by the production of cysteine-rich peptides such as glutathione and phytochelatins [2,13,16,17]. Phytochelatins (PCs), small peptides consists of 4–23 amino acids, par-

Abbreviations: Cys, cysteine; GSH, reduced glutathione; GSSG, oxidized glutathione; PC_2, phytochelatin

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doi:10.1016/j.chroma.2005.06.019

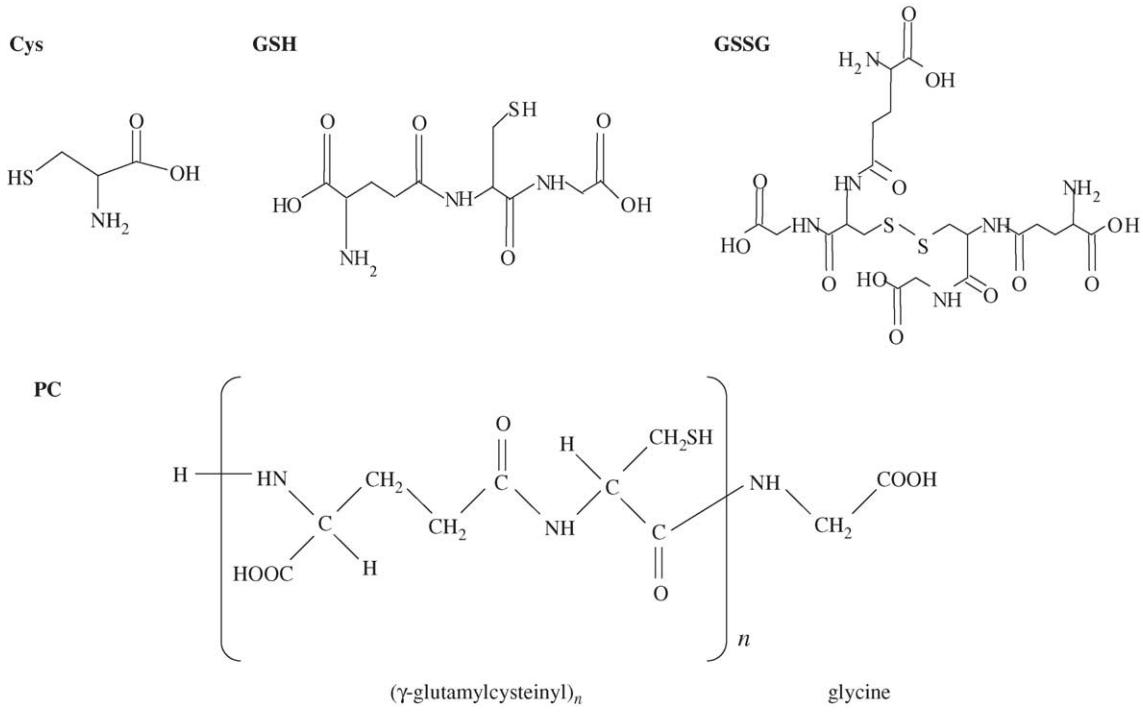

Fig. 1. Chemical formulas of cysteine (Cys), reduced glutathione (GSH), oxidized glutathione (GSSG) and phytochelatin (PC).

ticipate in the detoxification of heavy metals, because they have an ability to transport heavy metal ions to vacuole [17], where an immediate toxicity do not menace yet. PCs have a basic formula $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2$–11) [17] and with the presented heavy metals (M) form M-PC complexes, in which the metal is bind via SH group of cysteine unit [17,18] (Fig. 1). PCs are synthesized from glutathione, which is catalysed by PC synthase (γ-glutamylcysteine dipeptidyltranspeptidase, EC 2.3.2.15) activated by an increased concentration of the heavy metal (Cd, Cu, Hg, As or Pb) in a plant cytoplasm [2]. Reduced glutathione (GSH) itself plays the important role in cell protection against heavy metals, and reactive oxygen species (ROS) that are able to oxidize GSH to GSSG (oxidized glutathione; disulfide glutathione) [19]. The GSH:GSSG ratio was found as an indicator of cell damage and some diseases [19,20].

Recently, number of analytical approaches and methods for the determination of biological active compounds has been developed such as fluorimetry [21], capillary electrophoresis with UV detection [22–24], high-performance liquid chromatography (HPLC) with UV [21,25–27] and mass spectrometry detections [28–30]. The most commonly used method for separation and detection of thiol compounds (such as GSH and PCs) in biological samples is HPLC-UV–vis, where the derivatization is necessary for reaching of lower detection limits. Thiol compounds have been also analysed by electrochemical methods such as cyclic voltammetry (CV), differential pulse voltammetry (DPV), chronopotentiometric stripping analysis (CPSA) and adsorptive transfer stripping analysis [31,32] due to presence of the electroactive –SH and/or –SS– groups. The abovementioned electrochemical methods are very sensitive, rapid and low cost. Selectivity of electrochemical methods in complicated matrix increases by using of effective separation techniques (liquid chromatography, dialysis, capillary electrophoresis, etc.). One of the new analytical methods is high-performance liquid chromatography coupled with electrochemical detector (HPLC-ED) [33–36].

In this work, we optimised high-performance liquid chromatography with electrochemical detection (HPLC-ED) method for highly sensitive, rapid and selective separation and detection of cysteine, oxidized and reduced glutathione and phytochelatin in maize kernels. In this work a new method for evaluation of HPLC-ED parameters is described. Moreover, we studied detoxification mechanisms by optimised HPLC-ED method in the maize kernels exposed by three different cadmium concentrations (0, 10 and 100 $\mu mol\,l^{-1}$) during 5 days-long experiments.

2. Experimental

2.1. Chemicals

Cysteine (Cys), reduced (GSH) and oxidized (GSSG) glutathione and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Phytochelatin $(\gamma\text{-Glu-Cys})_2\text{-Gly}$ (PC_2) was synthesized in Clonestar Biotech; purity over 90% (Brno, Czech Republic). HPLC-grade acetonitrile (>99.9%; v/v) and methanol (>99.9%; v/v) from Merck (Darmstadt, Germany) were used. Other chemicals were purchased from Sigma–Aldrich. The stock standard

solutions of thiol compounds (Cys, GSH, GSSG and PC_2) at 10 μg ml^{-1} were prepared by ACS water (Sigma–Aldrich) and stored in the dark at −20 °C. Working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through a 0.45 μm Nylon filter discs (Millipore, Billerica, MA, USA) prior to HPLC analysis. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by personal computer program (MultiLab Pilot; Weilheim, Germany). The pH-electrode (SenTix H, pH 0-14/0-100 °C/3 mol l^{-1} KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany).

2.2. Plant material and cultivation

Kernels of maize (*Zea mays* L.) cv. Vegas (Danisco, Ref. No. 03UB416, Denmark) were cultivated in plastic photo-trays (260 mm × 220 mm). Glass platters (260 mm × 50 mm × 2 mm, 5 per tray) were situated about 1 cm over solution level. The platters were covered with strips of a filter paper (250 mm × 90 mm) the longer edges of which were inserted into cultivation solutions containing 0, 10 and 100 μmol l^{-1} cadmium concentration (as $CdCl_2$) to be continuously wetted with the cultivation solution. Weighed individual maize kernels were placed on the filter paper (10 pieces on each glass platter). The experiments were conducted for 5 days at 25 °C in the dark of a thermostatic box (Model TER-5/1, Chirana, Brno, Czech Republic) in duplicates.

Each experimental day we collected two sets (10 kernels each) of maize kernels from each photo-tray containing different cadmium concentrations (0, 10 and 100 μmol l^{-1}). The collected kernels were used for determination of dry weight and content of the thiol compounds.

2.3. Sample preparation for dry weight analysis

Ten maize kernels were placed into oven (KBC, Warszawa, Poland) heated on 105 °C and dried to constant weight. Dried kernels were weighed on Sartorius R160P balances (Sartorius, Goettingen, Germany).

2.4. Sample preparation for HPLC-ED determination

Maize kernels (approximately 0.2 g) were frozen by liquid nitrogen in a test-tube to disrupt the cells. Frozen kernels were spread in mortar and then exactly 1000 μl of 0.2 M phosphate buffer (pH 7.2) was added to the obtained powder. The mixture was homogenised by shaking on Vortex-2 Genie (Scientific Industries, New York, USA) at 4 °C for time of 30 min. The homogenate was centrifuged (14,000 × *g*) for 30 min at 4 °C using a Universal 32 R centrifuge (Hettich-Zentrifugen, Tuttlingen, Germany). The supernatant was filtered through a membrane filter (0.45 μm nylon filter disk, Millipore) before injection on the reversed-phase HPLC column [37–40].

2.5. Photographical documentation

The images of maize kernels were taken at the beginning of experiments and in certain intervals according to the goal of the experiments using an Olympus C-4040 ZOOM (Olympus, Tokyo, Japan) with Olympus SZH 10 lens (zoom 0.7). Obtained pictures were transferred to personal computer by data interface.

2.6. High-performance liquid chromatography with CoulArray electrochemical detector

An HPLC-ED system consisted of two solvent delivery pumps operating in range of 0.001–9.999 ml min^{-1} (Model 582, ESA, Chelmsford, MA, USA), a Zorbax XDB reversed-phase column (150.0 mm × 2.1 mm, 5 μm particle size; Agilent, Palo Alto, CA, USA) and a CoulArray electrochemical detector (Model 5600A, ESA). The electrochemical detector includes two low volume flow-through analytical cells (Model 6210, ESA). Each analytical cell is consisted of four carbon porous working electrodes, palladium electrodes as reference electrodes and auxiliary electrodes. The detector and the column were thermostated. Temperatures under 30 °C were reached by air-conditioner (ET9 Italy). The sample (5 μl) was injected manually. Thiosalycilic acid (TSA) was used as internal standard for the thiol compounds determination [41]. For other chromatographic conditions see Section 3.

2.7. Accuracy, precision and recovery

Accuracy, precision and recovery of thiol compounds were evaluated with homogenates (maize kernels) spiked with standards. Before extraction it was carried out, that 100 μl thiol compounds standards (concentrations varying from 1 to 100 ng ml^{-1}), 100 μl water and 100 μl TSA were added to maize kernel sample. Precision (RSD, %) of intra-day assay was performed in six homogenates. Inter-day precision was determined by analysing six homogenates over a 5-day period. Homogenates were assayed blindly and thiol compounds concentrations were derived from the calibration curves. Accuracy was evaluated by comparing the estimated concentration with the known concentrations of thiol compounds. Calculation of accuracy (bias, %), precision (RSD, %) and recovery was expressed according to [42,43].

Accuracy (bias, %) was expressed as

$$\frac{\text{measured value} - \text{true value}}{\text{true value}} \times 100,$$

precision (RSD, %) was expressed as

$$\frac{\text{standard deviation}}{\text{mean}} \times 100$$

and recovery as

$$\frac{\text{response of analyte spiked into matrix (processed)}}{\text{response of pure standard analyte (unprocessed)}} \times 100.$$

2.8. Statistical analysis

STATGRAPHICS (Statistical Graphics Corp., USA) was used for statistical analyses. Results are expressed as mean ± SD unless noted otherwise. Value of $p < 0.05$ was considered significant.

3. Results and discussion

The studied thiol compounds (cysteine, oxidized and reduced glutathione and phytochelatin) are of great importance to the detoxification of heavy metals in plants [2,17,18,44]. Recently, we studied the basic electrochemical behaviour of Cys, GSH, GSSG and PC_2 on hanging mercury drop electrode by cyclic voltammetry [39,45]. The obtained data were consequently used to optimise high-performance liquid chromatography coupled with CoulArray electrochemical detector (HPLC-ED) method for the separation and determination of the abovementioned thiol compounds.

3.1. HPLC-ED optimisation

3.1.1. Influence of CoulArray detector working electrodes applied potential

Primarily, optimal potentials of CoulArray detector working electrodes had to be found to achieve the most sensitive determination of the studied compounds. In the beginning of our experiment, a Zorbax XDB reversed-phase column (150.0 mm × 2.1 mm, 5 μm particle size) and isocratic mobile phase consisting from 92% 0.05% (aq) (v/v) TFA (solvent A) and 8% acetonitrile (ACN) (solvent B) were used for chromatographic separation of the thiol compounds [40]. Flow rate of the mobile phase was 0.2 ml min^{-1}. Column and detector temperature was set on 35 °C. The potentials 80, 180, 280, 380, 480, 580, 680 and 780 mV were applied on porous graphite working electrodes. We assumed that the highest responses can be obtained at 600 mV for GSH and at 750 mV for GSSG, respectively [35,46,47]. Full scan of all studied thiol compounds is shown in Fig. 2A. The resulting hydrodynamic voltammograms, i.e. the dependence of current response on the cell detector applied potential (Fig. 2B), correspond to the beginning of sigmoidal curves. A potential obtained from the limit diffusion current area or from the point of the highest current difference and the smallest potential difference is the most suitable for the electrochemical detection of the studied substances [26]. We selected the potential from the point of the highest current difference and the smallest potential difference for measurements of thiols (Fig. 2B). The selected potentials were: 780 mV for the GSSG and PC_2 and 680 mV for determination of Cys and GSH. The most suitable analytical potential for simultaneous determination of Cys, GSH, GSSG and PC_2 on one working electrode is 680 mV (Fig. 2A and B).

3.1.2. Selection of mobile phase

Composition of mobile phase is very important for chromatographic analysis because significantly influences the retention, height and character of analytical signal [27,40]. An important requirement for effective electrochemical determination of the studied substances is the presence of electrolytes [27,46]. Recently, we published that suitable inorganic part of mobile phase (electrolyte) for organic compounds determination is TFA [40]. In addition, ACN is often used as organic part of mobile phases in liquid chromatography [48–51]. Other organic solvents (i.e. methanol) are used less often [52]. Mobile phase consisted from ACN and 0.05% (aq) (v/v) TFA in ratio 97:3 was selected on the basis of our recent experiments [40]. Well-separated and symmetric signals of individual thiol compounds are shown in Fig. 3A. From the obtained results it follows that the detection of PC_2 was not as sensitive as in the case of the other compounds (Fig. 3A). That is why we studied the influence of another organic part of mobile phase on separation and electrochemical response of the studied thiols. According to the published experiment results [52,53], we selected methanol in the same ratio (97:3). The electrochemical response was about 20% higher in case of Cys, GSH and GSSG in comparison with the signals obtained using of mobile phase consisted from ACN and 0.05% (aq) (v/v) TFA in ratio 3:97. Moreover, the signal of PC_2 increased more than 300 % (Fig. 3A). Because of the change of the mobile phase composition, alterations in retention times also appeared (Fig. 3A). Observed signals increase probably relates with better miscibility of mobile phase parts and solubility of studied substances. From that reason we selected mobile phase consisted from 0.05% (aq) TFA and methanol (97:3) as the most suitable for the chromatographic separation and electrochemical determination of the studied thiols.

3.1.3. Evaluation method for HPLC-ED parameters

Several authors have described evaluation of obtained results to achieve the best separation and detection conditions [42,48,52,54,55]. Our evaluation of HPLC parameters is based on new approach. We defined following factors to select the optimal parameters for the separation and detection of all studied thiol compounds in our work. We calculated the factor of peak height in percents ($H_{\%,i,j}$) and factor of peak width in the middle of the peak height ($W_{1/2}$) in percents ($W_{1/2,\%,i,j}$) for individual compounds i (i = Cys, GSH, GSSG or PC_2) and individual tested values j of the studied parameter (i.e. TFA concentration or temperature) according to the equation $H_{\%,i,j} = (H_{i,j}/H_{i,j,\mathrm{MAX}}) \times 100$, where $H_{i,j}$ is peak height of the compound i at tested value j of the studied parameter and $H_{i,j,\mathrm{MAX}}$ is maximal value of $H_{i,j}$ from whole range of tested values j of the studied parameter, and according to the equation $W_{1/2,\%,i,j} = (W_{1/2,i,j}/W_{1/2,i,j,\mathrm{MAX}}) \times 100$, where $W_{1/2,i,j}$ is width in the middle of the peak height ($W_{1/2}$) of the compound i at tested value j of the studied parameter and $W_{1/2,i,j,\mathrm{MAX}}$ is maximal value of $W_{1/2,i,j}$ from whole range of tested values j of the studied parameter. The average

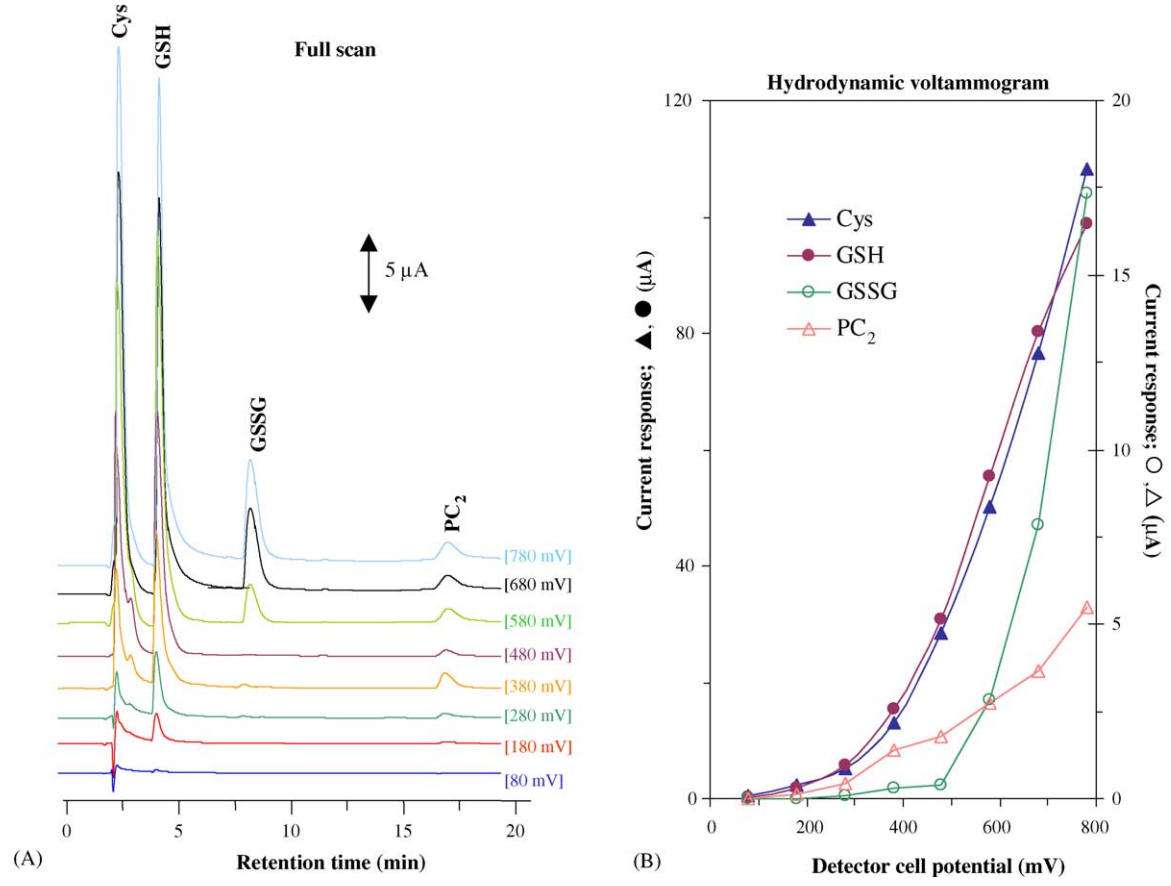

Fig. 2. HPLC-ED full scan chromatogram of Cys, GSH, GSSG and PC_2 (A). Dependence of current response of the thiol compounds on detector electrode potential (80, 180, 280, 380, 480, 580, 680 and 780 mV)—hydrodynamic voltammogram (B). HPLC-ED parameters—chromatographic column: Zorbax XDB reversed-phase column (150.0 mm × 2.1 mm, 5 μm particle size); flow rate of 0.2 ml min^{-1}; isocratic conditions: acetonitrile and 0.05% trifluoroacetic acid (8:92) (%; v/v); column and detector temperature: 35 °C; thiols concentrations: 10 μg ml^{-1}; 5 μl samples was injected.

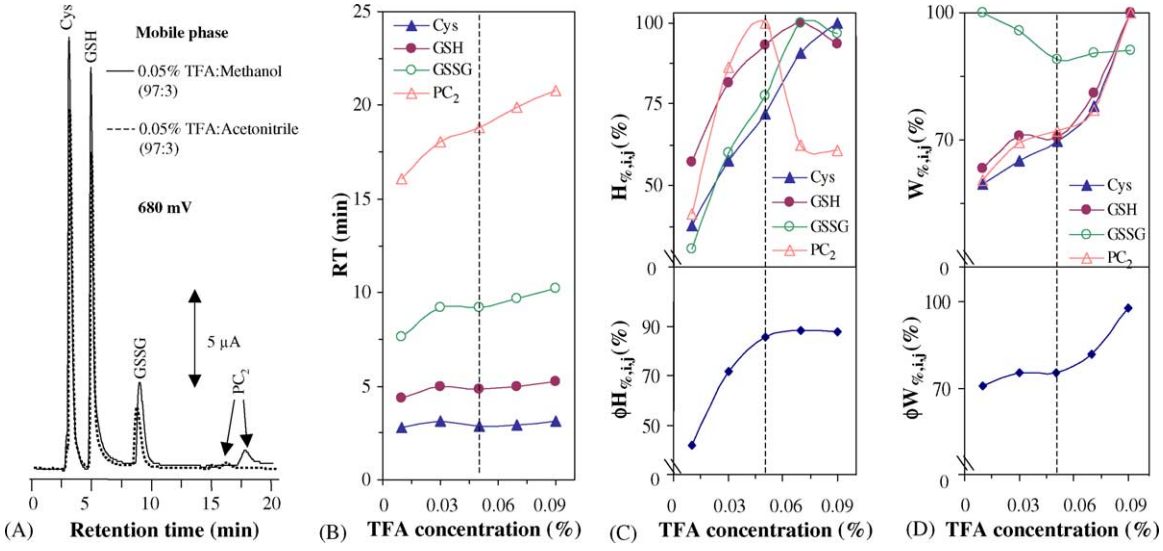

Fig. 3. Chromatograms of the studied thiol compounds; dashed line—isocratic conditions: acetonitrile and 0.05% trifluoroacetic acid (3:97) (%; v/v) and continuous line—isocratic conditions: methanol and 0.05% trifluoroacetic acid (3:97) (%; v/v) (A). Influence of different concentration of trifluoroacetic acid (0.01%, 0.03%, 0.05%, 0.07% and 0.09%) on retention time (B), peak height factor ($H_{\%,i,j}$) and average peak height ($\phi H_{\%,i,j}$) (C), width at half peak height ($W_{1/2,\%,i,j}$) and average width at half peak height ($\phi W_{1/2,\%,i,j}$) (D) of the thiol compounds; mobile phase—methanol:TFA. HPLC-ED parameters—detector electrode potential: 680 mV. Other chromatographic conditions see Fig. 2.

peak height in percent ($\phi H_{\%,i,j}$) and the average $W_{1/2}$ in percent ($\phi W_{1/2,\%,i,j}$) were consequently calculated from values of $H_{\%,i,j}$ and $W_{1/2,\%,i,j}$ for the value j of studied parameter according to the equations

$$\phi H_{\%,i,j} = \frac{\sum_{i=\mathrm{Cys,GSH,GSSG,PC_2};\, j=\mathrm{const.}} H_{\%,i,j}}{\text{number of studied compounds } (i)}$$

$$\phi W_{\%,i,j} = \frac{\sum_{i=\mathrm{Cys,GSH,GSSG,PC_2};\, j=\mathrm{const.}} W_{\%,i,j}}{\text{number of studied compounds } (i)}.$$

The value j was selected as optimal when calculated $\phi H_{\%,i,j}$ achieved the highest value and $\phi W_{1/2,\%,i,j}$ the lowest value from computed $\phi H_{\%,i,j}$ and $\phi W_{1/2,\%,i,j}$ of all values j. These parameters were applied for the chromatographic separation of thiol compounds.

3.1.4. Effect of trifluoroacetic acid concentration

Inorganic part of mobile phase also significantly influences process of individual thiol compounds separation [27,40]. Changes in retention times of Cys, GSH, GSSG and PC_2 according to different TFA concentration are shown in Fig. 3B. Trifluoroacetic acid concentration changes (from 0.01% to 0.09%) did not influence retention times of Cys and GSH (deviation about 0.35 and 0.9 min, respectively). On the contrary, the retention times of GSSG and PC_2 increased about 2.55 and 4.70 min, respectively (Fig. 3B). All studied compounds are well separated at all TFA concentrations. Concentration of TFA also affected peak height, peak width ($W_{1/2}$) and peak area (Fig. 3C and D). As it can be seen from the figures, height of Cys current response does not have a local maximum in studied TFA concentration interval and with increasing TFA concentration increased. The highest signals of GSH, GSSG and PC_2 were obtained at TFA concentrations equal to 0.07%, 0.07% and 0.05% TFA, respectively. The signal of PC_2 decreased more than 35% at higher concentrations of TFA 0.07% and 0.09% in comparison with the signal at the TFA concentration 0.05% (Fig. 3C). Moreover, peaks of Cys, GSH and PC_2 are broad and poorly resolved at higher concentrations of TFA 0.07% and 0.09% (Fig. 4D). On the contrary peak width of GSSG did not change markedly with increasing TFA concentration (about 10%), see in Fig. 3D.

According to the obtained values of the factor $\phi H_{\%,i,j}$ we were not able to select the most suitable TFA concentration for simultaneous determination of the thiol compounds (very low differences between the values were obtained at TFA concentration 0.05%, 0.07% and 0.09%, see Fig. 3C). On the other hand, we observed broadening of the thiol compounds signals at higher TFA concentration (above 0.05%) as you can see in Fig. 3D. On the basis of factor $\phi H_{\%,i,j}$ and $\phi W_{1/2,\%,i,j}$ values we selected concentration of TFA 0.05% as the most suitable for the simultaneous determination of all four thiols.

3.1.5. Effect of 0.05% TFA:methanol ratio

Influence of 0.05% TFA:methanol ratio in mobile phase was tested too. The tested ratios were 99:1, 97:3, 95:5 and 92:8 (0.05% TFA:methanol). The changing methanol amounts did not significantly influence the retention times of Cys, GSH and GSSG. In case of PC_2, we observed its

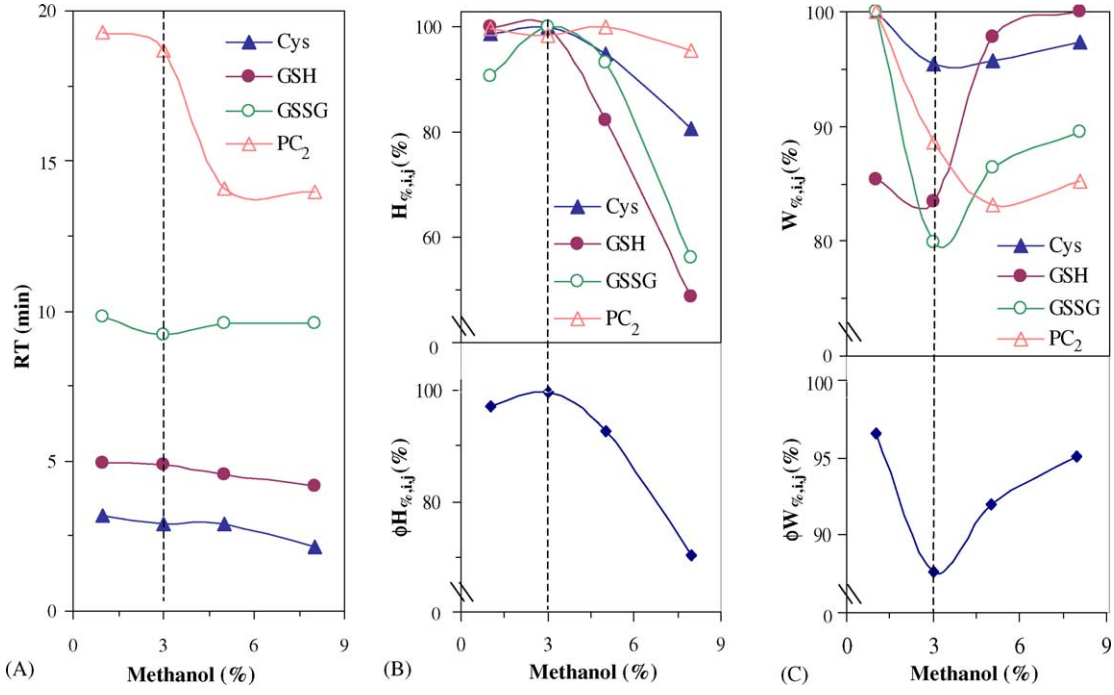

Fig. 4. Influence of different 0.05% TFA:methanol ratios (99:1, 97:3, 95:5 and 92:8) on retention time (A), peak height factor ($H_{\%,i,j}$) and average peak height ($\phi H_{\%,i,j}$) (B), width at half peak height ($W_{1/2,\%,i,j}$) and average width at half peak height ($\phi W_{1/2,\%,i,j}$) (C) of the thiol compounds. HPLC-ED parameters—isocratic conditions: methanol and 0.05% trifluoroacetic acid. Other chromatographic conditions see Fig. 3.

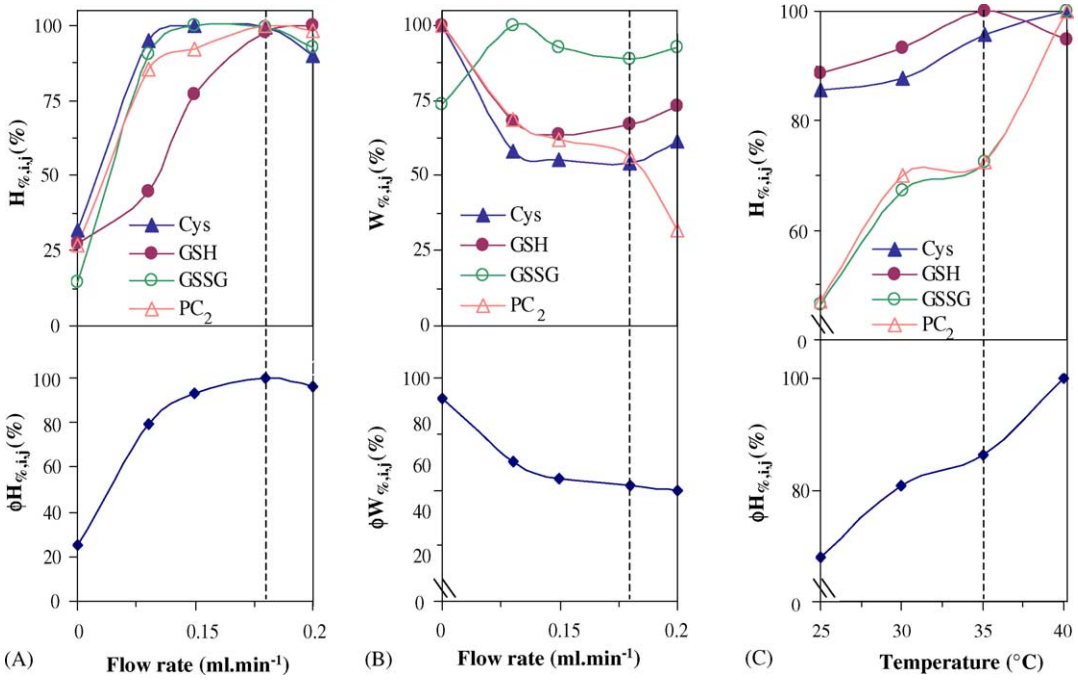

Fig. 5. Influence of different flow rates (0.10, 0.13, 0.15, 0.18 and 0.20 ml min^{-1}) of mobile phase consisted from 0.05% TFA:methanol in ratio 97:3 on peak height factor ($H_{\%,i,j}$) and average peak height ($\phi H_{\%,i,j}$) (A), width at half peak height ($W_{1/2,\%,i,j}$) and average width at half peak height ($\phi W_{1/2,\%,i,j}$) (B) of the thiol compounds. Influence of different column and detector temperatures (25, 30, 35 and 40 °C) on peak height factor ($H_{\%,i,j}$) and average peak height ($\phi H_{\%,i,j}$) (C). Other chromatographic conditions see Fig. 3.

faster elution (more than 5 min) with increasing amount of methanol in mobile phase (Fig. 4A). Heights and widths of the studied compounds peaks changed significantly with increasing methanol amounts (Fig. 4B and C). We obtained the highest peak current responses of the individual thiol compounds at the ratio 97:3 (0.05% TFA:methanol), see Fig. 4B. Width of the chromatographic peaks decreased (up to 3% methanol content), then markedly increased (Fig. 4C). On the basis of calculated factors ($\phi H_{\%,i,j}$ and $\phi W_{1/2,\%,i,j}$), the most effective mobile phase consisted from 3% of methanol and 97% of 0.05% TFA (Fig. 4B and C). Amount of organic part of mobile phase up to 10% using for separation of the biological active compounds was also published in [27,35,48–50,56].

3.1.6. Effect of mobile phase flow rate

Flow rate of mobile phase is also important experimental parameter [57]. An effect of different flow rates (0.10, 0.13, 0.15, 0.18 and 0.20 ml min^{-1}) onto peak height and peak width ($W_{1/2}$) was studied. As it can be seen from Fig. 5A and B, heights of individual chromatographic peaks increased and their width decreased with increasing flow rate of mobile phase (up to 0.18 ml min^{-1}). The most suitable flow rate was 0.18 ml min^{-1} according to evaluation of calculated factors $\phi H_{\%,i,j}$ and $\phi W_{1/2,\%,i,j}$ (Fig. 5A and B). Current response of the studied thiols decreased up to 85–95% at the highest mobile phase flow rate (0.20 ml min^{-1}) in comparison with the flow rate 0.18 ml min^{-1}. This phenomenon is probably caused by shorter time of pre-concentration of analyt on the working electrodes surfaces. On the contrary, decrease of the peak heights at lower flow rates of the mobile phase (0.10–0.15 ml min^{-1}) probably relates with saturation of active electrode surface (at given analyt concentration 10 μg ml^{-1}) (Fig. 5A and B) [58].

3.1.7. Temperature influence on HPLC-ED analysis

Temperature significantly influences chromatographic separation and detection of the studied thiol compounds. We studied the electrochemical behaviour of Cys, GSH, GSSG and PC_2 at temperatures 25, 30, 35 and 40 °C. Temperature on column and detector did not influence Cys and GSH signals (Fig. 5C). On the contrary, GSSG and PC_2 signals are significantly influenced by temperature (50% signal increase at 40 °C in comparison with 25 °C), see in Fig. 5C. Maximal signals heights were obtained at temperature 40 °C in case of Cys, GSSG and PC_2. On the contrary, GSH signal decreased and co-eluted with Cys signal (not shown). For that reason temperature 35 °C was selected as the optimal for simultaneous separation and detection of thiol compounds.

3.1.8. The most effective HPLC-ED parameters

The studied thiol compounds were well separated and detected (Cys—RT: 2.88 min, GSH—RT: 4.82 min, GSSG—RT: 9.23 min and PC_2—RT: 18.70 min) at the most suitable HPLC-ED conditions—mobile phase: 0.05% TFA:methanol in ratio 97:3; flow rate: 0.18 ml min^{-1}; column and detector temperature: 35 °C.

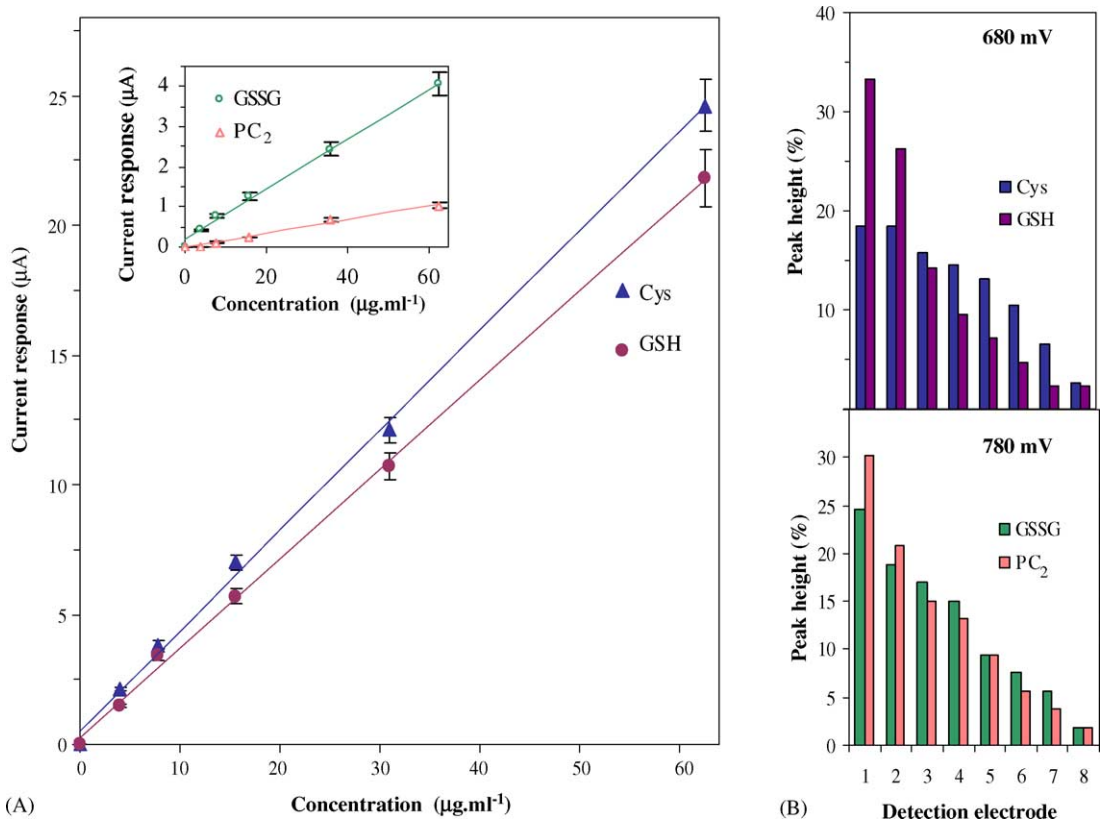

Fig. 6. Dependences of the current response of the Cys and GSH (A), GSSG and PC_2 (inset) on their concentrations obtained at full scan. Detector electrodes potentials at full scan: 80, 180, 280, 380, 480, 580, 680 and 780 mV. The current responses of the Cys, GSH, GSSG and PC_2 obtained at the individual electrodes of the CoulArray detector (B). Detector cell potentials at full scan was constant—680 mV for Cys and GSH, 780 mV for GSSG and PC_2. HPLC-ED parameters—chromatographic column: Zorbax XDB reversed-phase column (150.0 mm × 2.1 mm, 5 μm particle size); flow rate of 0.18 ml min^{-1}; isocratic conditions: 0.05% (%; v/v) trifluoroacetic acid and methanol (97:3); column and detector temperature: 35 °C; thiols concentrations: 10 μg ml^{-1}; 5 μl samples was injected.

3.1.9. Influence of thiol compounds concentration on their electrochemical response

We studied the sensitive determination of the thiol compounds by HPLC-ED at setting of all optimal method parameters. Dependences of electrochemical detector current response on individual thiols concentration were linear and RSDs were from 2.4% to 2.9% ($n = 5$). The calibration curves of Cys, GSH, GSSG and PC_2 obtained at full scan of eight channel CoulArray detector (potentials applied on individual electrodes were: 80, 180, 280, 380, 480, 580, 680 and 780 mV) are shown in Fig. 6A. The calibration curves were plotted from current responses obtained at potentials 680 mV for Cys and GSH and 780 mV for GSSG and PC_2, respectively. Equations of the calibration curves and values of R^2 obtained at detection potentials 680 mV/780 mV for Cys and GSH/GSSG and PC_2, respectively, are in Table 1. Detection limits were 112 fmol (Cys), 63.5 fmol (GSH), 112.2 fmol (GSSG) and 2.53 pmol (PC_2) per injection (5 μl) for the signal-to-noise ratio S/N = 3.

CoulArray detector operates in coulometry mode (that theoretically means 100% deposition of detected substance on working electrode surface) [59]. Therefore, we applied the same potential on all working electrodes (680 mV—Cys and GSH; 780 mV—GSSG and PC_2). We assumed that we obtained the electrochemical signal only on the first electrode. On the contrary, we observed only 20 up to 35% signals considering to the total sum of signals from all eight working electrodes. Obtained thiol compounds signals approximately linearly decreased with increasing placing of working electrodes (Fig. 6B). Influence of the different concentration of the studied thiols on their heights of the signals was also tested. We observed that the signals on the eighth electrode disappeared with decreased studied compounds concentrations (not shown). That is why we calculated sums of analytical signals obtained from all individual working electrodes for better quantification of the studied thiol compounds. Calibration curves plotted for the cumulative sums were linear (R^2 from 0.990 to 0.995). The relative standard deviations were about 7%. Therefore, it is possible to use this method for evaluation of HPLC-ED chromatograms of the studied compounds. Detection limits obtained due to the cumulative sum of individual thiol electrochemical signals were markedly lower about 86 up to 93% (Table 1) in comparison with the abovementioned detection limits. The evaluation method partially eliminates imperfect coulometric detection at full scan when detected compound is quantified only on

Table 1
Validation data for the determination of thiol compounds ($n = 5$) at 680 and 780 mV

Thiol compound	t_R[a] (min)	Regression equation	Single electrode response				Total electrode response		
			R^2[b]	LOD[c,e] (fmol)	LOQ[d,e] (pmol)	RSD[f] (%)	LOD[c,e] (fmol)	LOQ[d,e] (fmol)	RSD[f] (%)
Cys[g]	2.88	$y = 0.385x + 0.533$	0.9982	112.8	0.376	2.4	14.8	49.5	6.8
GSH[g]	4.82	$y = 0.344x + 0.256$	0.9989	63.5	0.212	2.7	4.5	15.1	7.2
GSSG[h]	9.23	$y = 0.063x + 0.18$	0.9948	112.2	0.374	2.6	10.5	35.3	7.3
PC_2[h]	18.70	$y = 0.017x - 0.015$	0.9918	2531.1	8.437	2.9	238.5	796.0	8.4

[a] Retention times in min.
[b] Regression coefficients.
[c] Limits of detection per column (3 S/N).
[d] Limits of quantification per column (10 S/N).
[e] Injection 5 μl.
[f] Relative standard deviations.
[g] Detection potential 680 mV.
[h] Detection potential 780 mV.

the basis of oxidative signal obtained from the one electrode (yield of coulometric detection on one electrode is approximately 30%; Fig. 6B). Coulometric deposition of 100% detected substance on working electrode surface is not probably able to achieve in flow system due to the continual flowing of mobile phase (shorter time of detected substance deposition in comparison with stationary electrochemical methods) [27,59].

3.2. Reproducibility, recovery, precision and accuracy

Recovery was checked for the compounds of interest by addition of known amounts of the Cys, GSH, GSSG and PC_2 working standards to homogenates (Table 2). Recoveries of Cys, GSH, GSSG and PC_2 were from 96% to 103% (Table 2). Reproducibility of the procedure was tested by analysing representative samples in six replicates during 5 days (Table 3). Good reproducibility was obtained for all thiol compounds in maize kernels samples, with RSDs ranging from 3.2% to 5.4% for lower concentration in the intra-assay; values from 3.9% to 5.5% resulted for higher amounts of thiol compounds. The inter-assay RSDs ranged from 3.6% to 5.8% for lower concentration and from 4.1% to 6.5% for higher amounts of Cys, GSH, GSSG and PC_2; overall recoveries of were from 94% to 100% ($n = 30$). Accuracy (bias, %) was about ±5%.

3.3. Application of the optimised HPLC-ED method for thiols determination in maize kernels

Optimised HPLC-ED method was subsequently applied for the determination of thiol compounds amounts (Cys, GSH, GSSG and PC_2) in maize kernels exposed by cadmium. It is known that plants treated by heavy metals can synthesize group of thiol compounds, above all glutathione and phytochelatins [2,13,17,18,44]. Maize kernels placed in photo-trays were exposed by three different cadmium concentrations (0, 10 and 100 μM $CdCl_2$) during 5 days long experiment. Every experimental day we collected two sets (10 kernels each) of maize kernels from each photo-tray containing different cadmium concentrations (0, 10 and 100 μmol l^{-1}). Higher concentration of cadmium stimulated germination of the maize kernels (not shown). Dependence of dry weight (DW) of the treated maize kernels on time of cultivation is shown in Fig. 7A. Observed DW decrease relates with respiration of organic compounds in the kernels. The respiration increases with rising cadmium concentration (Fig. 7A).

Preparation of the maize kernels samples for analysis of thiol compounds by HPLC-ED method is described in Section 2. Glutathione and PC_2 amounts increased with rising cultivation time and applied dose of cadmium. Content of the studied thiol compounds in maize kernels at fifth day of

Table 2
Precision and recovery of thiol compounds for maize kernels sample analysis ($n = 5$).

Thiol compound	Homogenate (pg)[a]	Spiking (pg)[a]	Homogenate + spiking (pg)[a]	Recovery (%)
Cysteine	50.0 ± 0.9 (1.8)	30.8 ± 0.9 (2.9)	78.4 ± 2.2 (2.8)	97
		299 ± 5 (1.7)	345.5 ± 10.7 (3.1)	99
GSH	50.1 ± 1.1 (2.2)	30.1 ± 0.6 (2.0)	81.0 ± 1.7 (2.1)	101
		305 ± 8 (2.6)	365.8 ± 12.4 (3.4)	103
GSSG	49.8 ± 0.9 (1.8)	28.9 ± 0.7 (2.4)	78.7 ± 2.3 (2.9)	100
		296 ± 4 (1.4)	338.9 ± 15.3 (4.5)	98
PC_2	50.0 ± 2.0 (4.0)	30.4 ± 1.4 (4.6)	77.2 ± 1.8 (2.3)	96
		295 ± 10 (3.4)	341.6 ± 13.3 (3.9)	99

[a] Thiol compound amounts per column; expressed as mean ± SD (RSD, %).

Table 3
Reproducibility and recovery of thiol compounds for maize kernels sample analysis

Thiol compound	Homogenate (pg)[a]	Spiking (pg)[a]	Homogenate + spiking (pg)[a]	Recovery (%)
Cysteine				
Intra-day ($n = 6$)	50.3 ± 1.1 (2.2)	30.3 ± 1.1 (3.6)	79.2 ± 2.5 (3.2)	98
		301 ± 7 (2.3)	350.5 ± 13.6 (3.9)	100
Inter-day ($n = 30$)	50.1 ± 1.5 (3.0)	30.1 ± 2.4 (8.0)	79.8 ± 3.8 (4.7)	99
		304 ± 28 (9.2)	352.8 ± 14.5 (4.1)	100
GSH				
Intra-day ($n = 6$)	49.6 ± 1.5 (3.0)	29.3 ± 1.3 (4.4)	78.8 ± 2.5 (3.2)	100
		300 ± 9 (3.0)	347.5 ± 14.2 (4.1)	99
Inter-day ($n = 30$)	49.4 ± 1.2 (2.4)	29.8 ± 1.4 (4.7)	77.9 ± 2.8 (3.6)	96
		297 ± 25 (8.4)	342.3 ± 22.2 (6.5)	98
GSSG				
Intra-day ($n = 6$)	50.6 ± 1.8 (3.6)	30.0 ± 1.8 (6.0)	76.0 ± 4.1 (5.4)	96
		295 ± 12 (4.1)	339.7 ± 14.2 (4.1)	98
Inter-day ($n = 30$)	50.7 ± 2.3 (4.5)	30.4 ± 2.0 (6.6)	76.2 ± 4.4 (5.8)	94
		301 ± 14 (4.7)	341.1 ± 12.6 (3.7)	97
PC_2				
Intra-day ($n = 6$)	49.6 ± 3.4 (6.9)	31.4 ± 1.0 (3.2)	78.6 ± 4.0 (5.1)	97
		306 ± 8 (2.6)	337.8 ± 18.6 (5.5)	95
Inter-day ($n = 30$)	49.9 ± 2.7 (5.5)	31.0 ± 1.2 (3.9)	79.3 ± 4.6 (5.8)	98
		302 ± 9.8 (3.2)	337.8 ± 21.2 (6.3)	96

[a] Thiol compound amounts per column; expressed as mean ± SD (RSD, %).

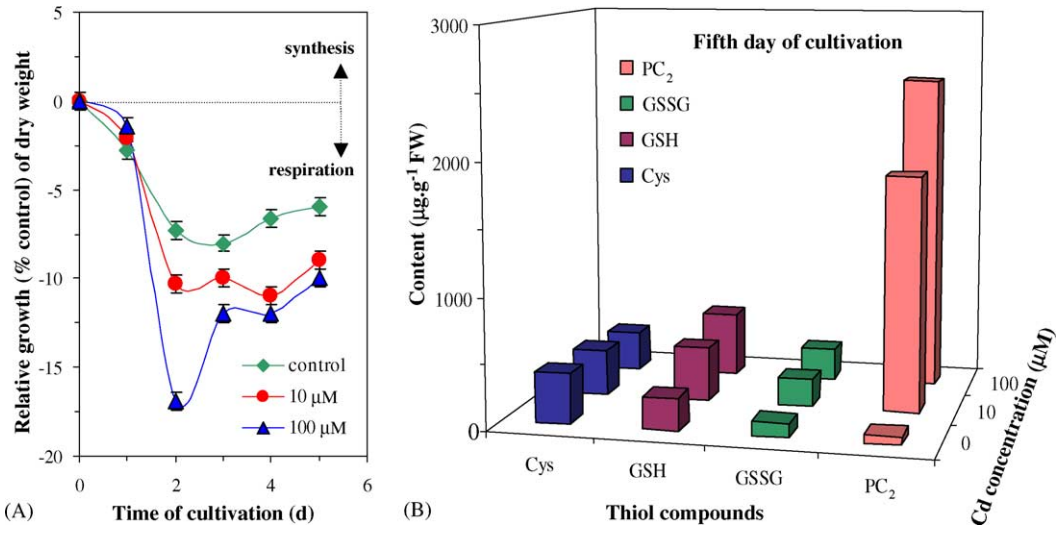

Fig. 7. Dependence of dry weight (DW) of the maize kernels exposed by three different cadmium concentrations (0, 10 and 100 µmol l^{-1}) on cultivation time (A). Influence of the three applied different cadmium concentrations on contents of thiol compounds (Cys, GSH, GSSG and PC_2) in treated maize kernels, fifth day of cultivation (B). Other chromatographic conditions see Fig. 6.

cultivation is shown in Fig. 7B. We observed the markedly increase of PC_2 amount according to the rising cadmium concentration in comparison with control. Concentration of phytochelatin, which is synthesized from GSH, is constant in heavy metal undamaged plant cells [13]. However, increasing cadmium concentration damages cell enzymatic apparatus, which leads to breakdown of this balance [60–64]. Concentrations of GSH and GSSG also increased with rising cadmium concentration. The concentration increase also relates with damage of cell enzymatic apparatus of plant cells (Fig. 7B) [63,64].

4. Conclusion

Analytical chemistry develops very intensively and brings new methods for biochemical and molecularly biological study of protein and peptides. In our work we optimised

HPLC-ED method for the determination of cysteine (Cys), reduced (GSH), oxidized (GSSG) glutathione and phytochelatin (PC_2). The studied thiol compounds were well separated and detected (Cys—RT: 2.88 min, GSH—RT: 4.82 min, GSSG—RT: 9.23 min and PC_2—RT: 18.70 min) at the most suitable HPLC-ED conditions – mobile phase: 0.05% TFA:methanol in ratio 97:3; flow rate: 0.18 ml min^{-1}; column and detector temperature: 35 °C. We applied our optimised method to study of defensive mechanisms in maize kernels exposed by cadmium.

Acknowledgements

This work was supported by grants: GA CR No. 525/04/P132, IGA MZLU No. 250061/2005 and INCHEMBIOL MSM 0021622412.

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