Determination of Plant Thiols by Liquid Chromatography Coupled with Coulometric and Amperometric Detection in Lettuce Treated by Lead(II) Ions

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Abstract
The main aim of this paper is to utilize high performance liquid chromatography with electrochemical detection for determination of thiols content in plants tissues of lettuce treated with lead(II) ions (0, 0.5 and 1 mM). We used two HPLC-ED instruments: HPLC coupled with one channel amperometric detector and HPLC coupled with twelve channel coulometric detector to detect simultaneously twelve thiols. The detection limits of thiols measured by CoulArray detector were about two magnitudes lower in comparison to those measured by Coulochem III detector and were from tens to hundreds pM. Under the optimal conditions, we utilized HPLC-CoulArray detector for analysis of tissues from lettuce plants. In addition, distribution and accumulation of lead ions with high spatial resolution was monitored using laser induced breakdown spectroscopy.

Keywords: Thiols, Liquid chromatography, Coulometric detection, Amperometric detection, Heavy metal, Laser induced breakdown spectroscopy

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1. Introduction

Pollution of environment by both organic and inorganic toxic compounds, especially by salts of heavy metals ions including lead(II) salts, can be considered as a concern not only for developing but also for developed countries [1]. Thus, decontamination and remediation of the polluted environment by using modern, non-destructive and environment-friendly technologies is one of the most important tasks of United Nations Environment Programme [2–5]. One of such technologies is called phytoremediation, which describes the treatment of environmental problems through the use of plants [6–11]. Research on this field is mainly focused on acquiring of plants with the highest remediation efficiency, it means plants with both considerable resistance against toxic effects of heavy metal ions and ability to hyperaccumulate these ions. Thiols as glutathione and phytochelatins are the most important compounds maintaining the homeostasis of heavy metal ions inside the plant cell [12–15].

To evaluate ability of a plant to synthesise thiols, the precise analytical instruments with low detection limits are needed. Recently, a number of analytical approaches and methods for the determination of biologically active thiol compounds have been developed [16], including both stationary (for example voltammetry, chronopotentiometry, polarography) [17–21] and flow techniques [22] (like liquid chromatography (LC) [23–27], gas chromatography (GC) and capillary electrophoresis (CE)) [28–30]. With LC systems, there are numerous detection methods, including UV detection, mass spectrometry, and electrochemical detection. Each method has its advantages and limitations and may serve a particular need in analysis.

However, to consider whether the specific plant species is able to or is not able to remediate the polluted environment, level of thiols in plant tissues is not the only criterion. Likewise, the distribution of metal ions in the plant tissues

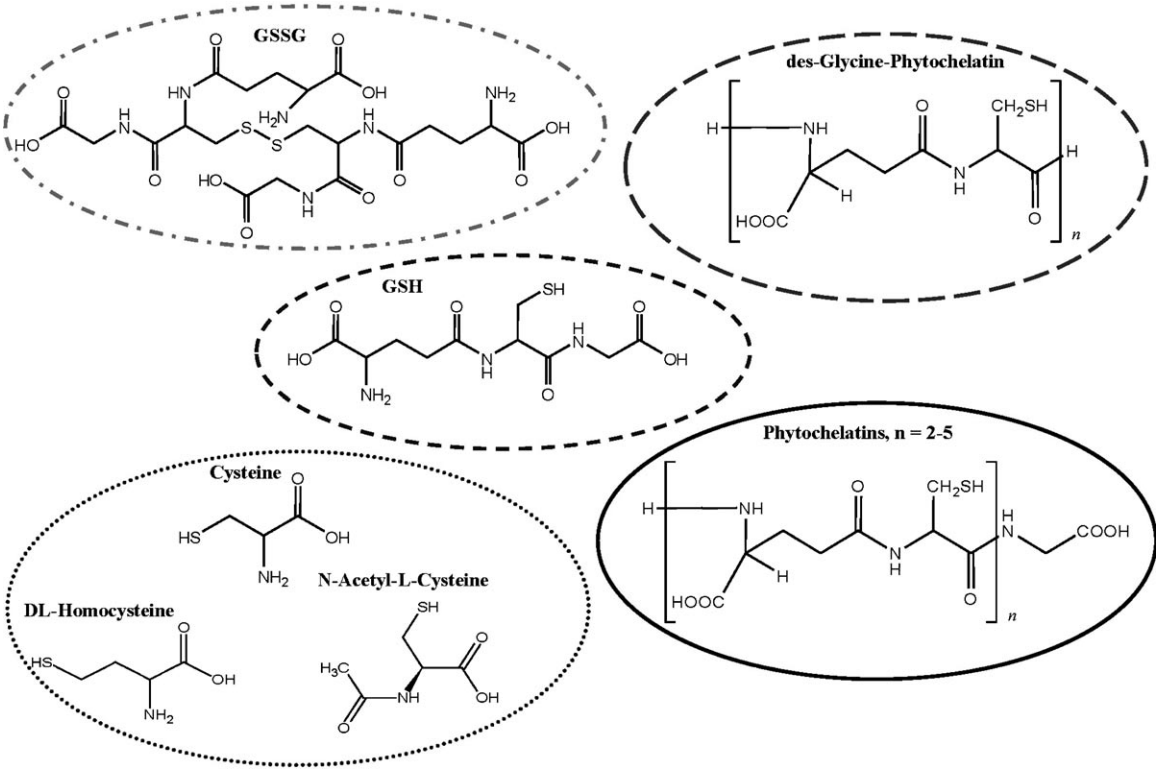

Fig. 1. Chemical structures of cysteine (Cys); reduced (GSH) and oxidized (GSSG) glutathione; homocysteine (H-cysteine); *N*-acetylcysteine (NAcCysteine); cystine; phytochelatins: des-glycine-phytochelatin (Des-gly-PC) and phytochelatins.

and organs must be analysed because of different capability of different plant tissues to detoxify metal ions. The diagnostic techniques enabling three-dimensional monitoring of distribution of elements within different plant tissues and organs include mainly X-ray imaging methods [31 – 33] [34]. Although the X-ray radiation based methods are widely used in laboratories, these methods have some disadvantages, especially limitation in utilization for in-situ analysis only. Due to this fact, we have been focusing on the realization of spatially-resolved spectrochemical analysis by utilizing laser-ablation based techniques. Laser induced breakdown spectroscopy (LIBS) is a type of atomic emission spectroscopy which utilises a highly energetic laser pulse as the excitation source and is able to provide high spatial-distribution of metal ions in different types of materials [35, 36].

The main aim of this paper is to utilize HPLC with electrochemical detection (ED) for monitoring of thiols content in plants tissues of lettuce treated with lead(II) ions, respectively. Two HPLC-ED instruments: HPLC coupled with one channel amperometric detector and HPLC coupled with twelve channel coulometric detector were used for simultaneous detection of twelve thiols (cysteine (Cys); reduced (GSH) and oxidized (GSSG) glutathione; homocysteine (H-cysteine); *N*-acetylcysteine (NAcCysteine); cystine; phytochelatins: des-Glycine-Phytochelatin (Des-gly-PC), phytochelatin$_2$ (PC_2), phytochelatin$_3$ (PC_3), phytochelatin$_4$ (PC_4) and phytochelatin$_5$ (PC_5), Fig. 1). The results obtained by using these LC techniques are compared. In addition, distribution and accumulation of lead ions with high spatial resolution was monitored using laser induced breakdown spectroscopy.

2. Experimental

2.1. Chemicals and pH Measurements

Cysteine (Cys), reduced (GSH) and oxidized (GSSG) glutathione, homocysteine, *N*-acetylcysteine (NAcCysteine), cystine and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, USA). Phytochelatins: (γ-Glu-Cys) des-glycine-phytochelatin (des-gly-PC), (γ-Glu-Cys)$_2$-Gly phytochelatin$_2$ (PC_2), (γ-Glu-Cys)$_3$-Gly phytochelatin$_3$ (PC_3), (γ-Glu-Cys)$_4$-Gly phytochelatin$_4$ (PC_4) and (γ-Glu-Cys)$_5$-Gly phytochelatin$_5$ (PC_5) were synthesized in Clonestar Biotech (Brno, Czech Republic) with a purity above 90 %. HPLC-grade acetonitrile ($>99.9\%$; *v*/*v*) and methanol ($>99.9\%$; *v*/*v*) 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 (500 μg mL^{-1}) were prepared with ACS water (Sigma-Aldrich, USA) and stored in dark at $-20\,^{\circ}C$. Working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through 0.45 μm Nylon filter discs (Millipore, Billerice, MA, 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/3 mol L^{-1} KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany).

2.2. Plant Material and Cultivation

Lettuce seeds (*Lactuca sativa L.* var. *capitata* L.) were germinated on wet filter paper in special vessels at 23 ± 2 °C in dark. After seven days, lettuce seedlings were placed into vessels containing tap water and cultivated in Versatile Environmental Test Chamber (MLR-350 H, Sanyo, Japan) for eight days with 14 h long daylight per day (maximal light intensity was about 100 μE m^{-2} s^{-1}) at 23.5–25 °C and humidity 71–78%. Further, Pb-EDTA was added to the cultivation solution at final concentrations of 0, 0.5 and 1 mM. Plants grown without Pb-EDTA were used as a control. The lettuce plants placed in the vessels that contained tap water with addition of Pb-EDTA (0, 0.5 and 1 mM) were grown for 5 days. Four plants each were harvested at certain time intervals (24, 72, and 120 h) during the experiment, and their roots were rinsed three times in distilled water and 0.5 M EDTA. Further, each harvested plant was divided into leaves and root. Fresh weight of the samples was measured immediately after the rinsing by using a Sartorius scale.

2.3. Preparation of Plant Tissues for Thiols Determination

Weighed plant tissues (approximately 0.2 g) were transferred to a test-tube. Then, liquid nitrogen was added to the test-tube, and the samples were frozen to disrupt the cells. The frozen sample was transferred to mortar and spread for 1 min. Then exactly 1000 μL of 0.2 M phosphate buffer (pH 7.2) was added to mortar, and the sample was spread for 5 min. The homogenate was transferred to a new test-tube. The mixture was homogenised by shaking on a Vortex–2 Genie (Scientific Industries, New York, USA) at 4 °C for 30 min. The homogenate was centrifuged (14000 *g*) for 30 min at 4 °C using a Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, Tuttlingen, Germany). The supernatant was filtered through a membrane filter (0.45 μm Nylon filter disk, Millipore, Billerica, Mass., USA) prior to analysis.

2.4. Flow Injection Analysis/High Performance Liquid Chromatography Coupled with Coulochem III (Amperometric) or CoulArray (Coulometric) Detector

2.4.1. Coulochem III

The instrument for flow injection analysis with electrochemical detection (FIA-ED) and/or high performance liquid chromatography with electrochemical detection (HPLC-ED) consisted of solvent delivery pump operating in range of 0.001–9.999 mL min^{-1} (Model 582 ESA Inc., Chelmsford, MA, USA), a guard cell (Model 5020 ESA, USA), a reaction coil (1 m) and/or a chromatographic column Metachem Polaris C18A reverse-phase column (150.0 × 2.1 mm, 5 μm particle size; Varian Inc., CA, USA), and an electrochemical detector. The electrochemical detector includes one low volume flow-through analytical cells (Model 5040, ESA, USA), which is consisted of glassy carbon working electrode, hydrogen-palladium electrode as reference electrode and auxiliary electrode, and Coulochem III as a control module. The sample (5 μL) was injected using autosampler (Model 540 Microtiter HPLC, ESA, USA). The data obtained were treated by CSW 32 software (DataApex, Prague, Czech Republic). The experiments were carried out at room temperature. Guard cell potential was set as 0 V.

2.4.2. CoulArray

FIA/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), a reaction coil (1 m)/Metachem Polaris C18A reverse-phase column (150.0 × 2.1 mm, 5 μm particle size; Varian Inc., CA, USA) and a CoulArray electrochemical detector (Model 5600A, ESA, USA). The electrochemical detector includes three flow cells (Model 6210, ESA, USA). Each cell consists of four working carbon porous electrodes, each one with auxiliary and dry H/Pd reference electrodes. Both the detector and the reaction coil/column were thermostated. Temperature was optimized, see in "Results and Discussion" section. The sample (5 μL) was injected using autosampler (Model 540 HPLC, ESA, USA).

2.5. Laser Induced Breakdown Spectroscopy

To realize the measurements with high-spatial resolution, the sample holder with the investigated species was placed to the stage with precision movements (2 μm in *x*, *y* and *z* direction) inside the ablation chamber (Tescan, Czech Republic). The single-shot LIBS analysis was performed in air under atmospheric pressure. The ablation spot was targeted and controlled for each shot by a CCD camera placed outside of the chamber. The LIBS microplasma was created using the second harmonic (532 nm) of a Nd:YAG laser system (Quantel, Brilliant B). The laser pulse width was ~5 ns and the beam diameter 8 mm. The energy of the laser pulse was 10 mJ (at the sample). The laser-induced plasma was produced by focusing the laser beam with a 16 mm focal-length glass doublet (Sill Optics). Imaging system consisting of two quartz objectives was used to collect the LIBS microplasma radiation. Subsequently, the radiation was transported by a 3 m fibre optic system onto the entrance slit of the 0.32 m monochromator (Jobin Yvon TRIAX 320). In this study the grating 2400 g/mm of the monochromator and 50 μm entrance slit were used. The

dispersed spectrum of the plasma radiation was detected by an ICCD camera (Jobin Yvon Horiba). The time-resolved measurements were realized triggering the camera by the Q-switch signal of the laser. The detector was gated 1 μs after the Q-switch signal and the observation window was 10 μs. The lead-content within the leaf was detected by monitoring the 283.31 nm Pb(I) line in the created microplasmas

2.6. Accuracy, Precision and Recovery

Accuracy, precision and recovery of the thiols were evaluated with homogenates (lettuce tissues samples) spiked with the standards. Before extraction, 100 μL of the thiols standards, 100 μL of the water and 100 μL of thiosalicylic acid (TSA, used as internal standard for the thiols determination [37]) were added to lettuce tissues samples. Homogenates were assayed blindly and thiols concentrations were derived from the calibration curves. Accuracy was evaluated by comparing estimated concentrations with known concentrations of thiols. Calculation of accuracy (%Bias), precision (%C. V.) and recovery of this intraday assay was carried out as indicated by Causon [38] and Bugianesi et al. [39].

2.7. Descriptive Statistics

STATGRAPHICS (Statistical Graphics Corp, USA) was used for statistical analyses. Results are expressed as mean $\pm$ *SD* unless noted otherwise.

3. Results and Discussion

Phytoremediation technologies could be utilized for depolluting of soils with higher content of heavy metals, but the

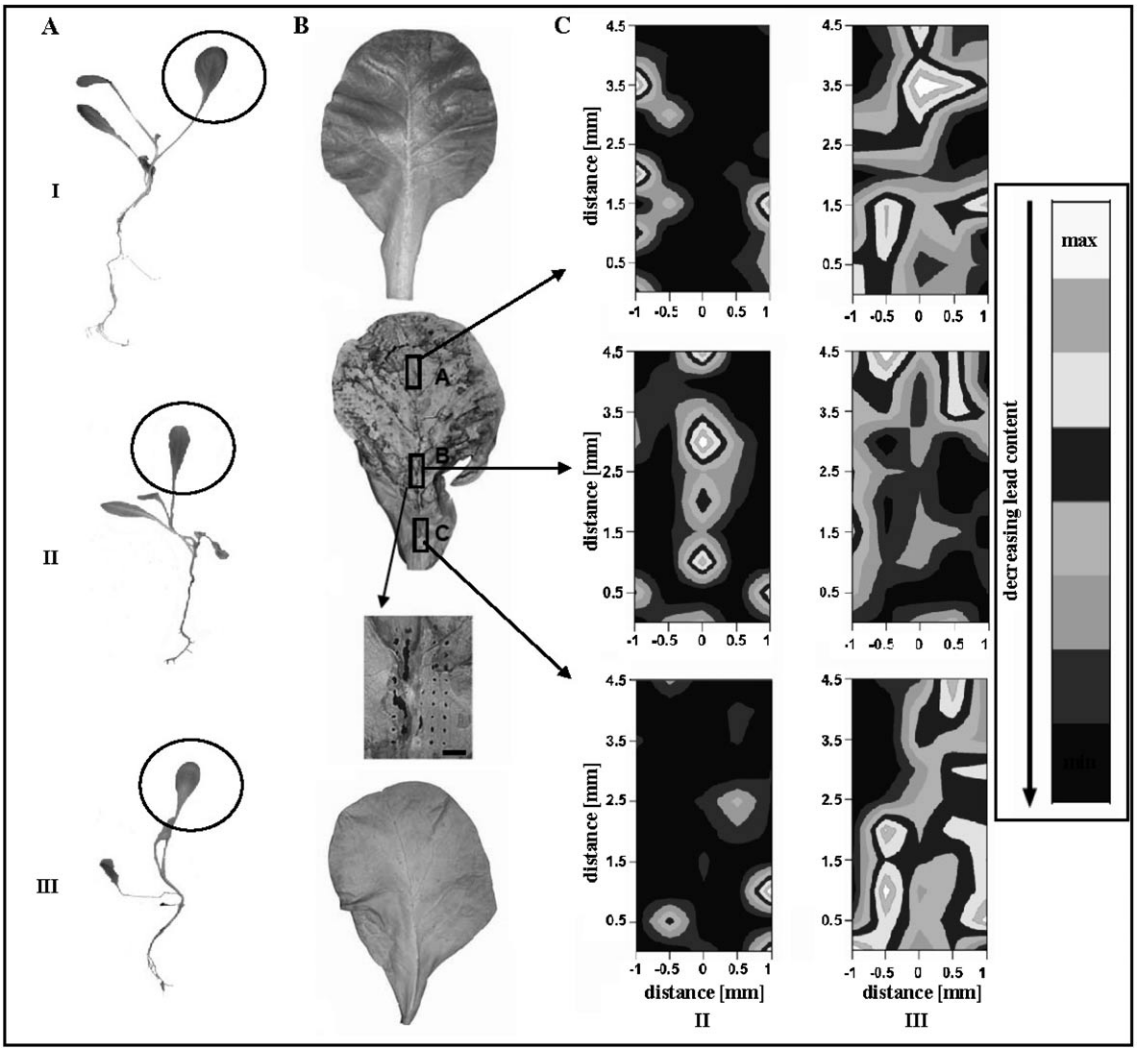

Fig. 2. Photography of whole plant of lettuce (*Lactuca sativa L.* var. *capitata* L.) (A) and its leaf (B) treated with 0 – control (I), b) 0.5 mM (II) and c) 1 mM (III) Pb-EDTA. The LIBS analysis was performed in the areas indicated by rectangles A, B and C in all three samples. In the insertion the typical LIBS and ablation pattern is shown. The length of the bar in the insertion is 1 mm. The maps of the lead accumulation in the selected areas (A, B and C) of the Pb-EDTA treated samples by 0.5 mM Pb-EDTA (II) 1 mM Pb-EDTA (III) including scale showing concentration of lead(II) ions (C).

suitability of single plant species for such purposes needs to be tested. Lettuce can be considered as one the promising plant specie for phytoremediation purposes due to its relative short growing season, higher production of biomass and well development. However, to evaluate phytoremediation capacity of this plant specie properly, two different points of view have to be studied: ability of a plant to degrade or store the pollutant × response of a plant on the presence of the pollutant. In the present paper we treated lettuce plants with Pb-EDTA 0, 0.5 and 1 mM for 120 hours. As a source of lead ions we used lead chelate (Pb-EDTA), because the lead from salts such as nitrite, chloride or acetate partly precipitates and the final concentration of lead ions is in doubt [40, 41]. The plants treated with Pb-EDTA shown well observable growth depression, the content of chlorophyll was decreased and necrotic changes on the leaves, especially on the margins of leaf blades, were observed. The fresh weight of the plants treated with 0.5 mM and/or 1 mM decreased for more than 45% and/or 55%, respectively, at the very end of the treatment compared to control plants (Fig. 2A, B).

3.1. Amperometric Detection – Coulochem III Detector

Typical hydrodynamic voltammograms (HDVs) of the thiols measured in the presence of trifluoroacetic acid (TFA):methanol (97:3, v/v, pH 1.42) are shown in Figure 3. The thiols were measured within the potential range from 300 to 900 mV (per 100 mV) using FIA-ED. The profiles of HDVs very depended on the structure of molecules analysed. Signals of the thiols with free −SH group like Cys and GSH enhanced most rapidly (Fig. 3A). On the other hand the signals of thiols with less accessible electroactive −SH or S−S groups like phytochelatins enhanced more gradually until we applied potential of 600 mV, from which we observed the sharp increase in the height of the signals (Fig. 3A). It clearly follows from the results obtained that

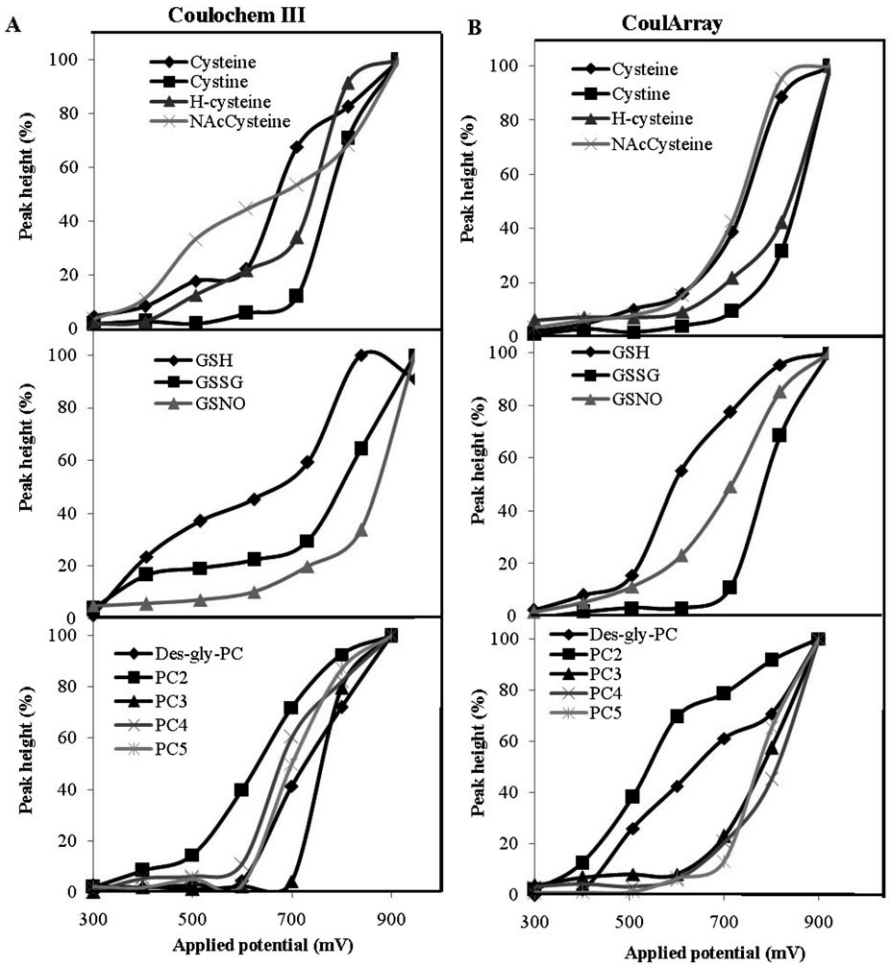

Fig. 3. Hydrodynamic voltammograms of analyzed thiols. The chromatographic conditions were the same for both detectors as follows: the concentration of Cys, Cystine, H-cysteine, NAcCysteine, GSH and GSSG was 10 μM; the concentration of Des-gly-PC, PC2, PC3, PC4 and PC5 was 25 μM; the mobile phase trifluoroacetic acid (TFA):methanol (97:3, v/v, pH 1.42) with flow rate of 0.7 mL/min; temperature and volume of the injected sample 4 °C and 10 μL, respectively; column and detector temperature 30 °C; length of reaction coil 1 m; the potential range 300 – 900 mV. The signals for CoulArray were evaluated as cumulative response – sum of the signals measured at all electrodes according to [43].

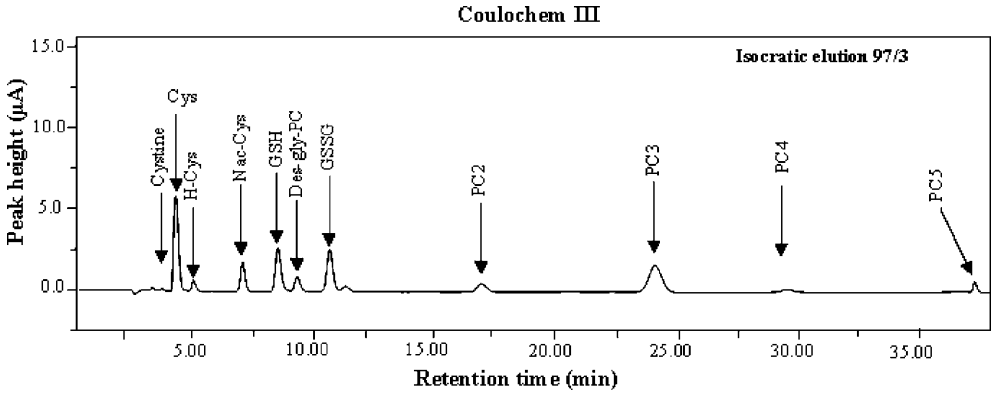

Fig. 4. Typical HPLC Coulochem III chromatogram of thiols standards. The applied potential was 900 mV. For other chromatographic conditions see in Fig. 3.

working electrode potential of 900 mV is the most suitable potential for simultaneous determination of the twelve compounds of interest.

Due to application of such high potential on working electrode, the electrode was polished electrochemically prior to analysis. Under this polishing the electrode can be used for more than 1000 analyses. Then, the glassy carbon

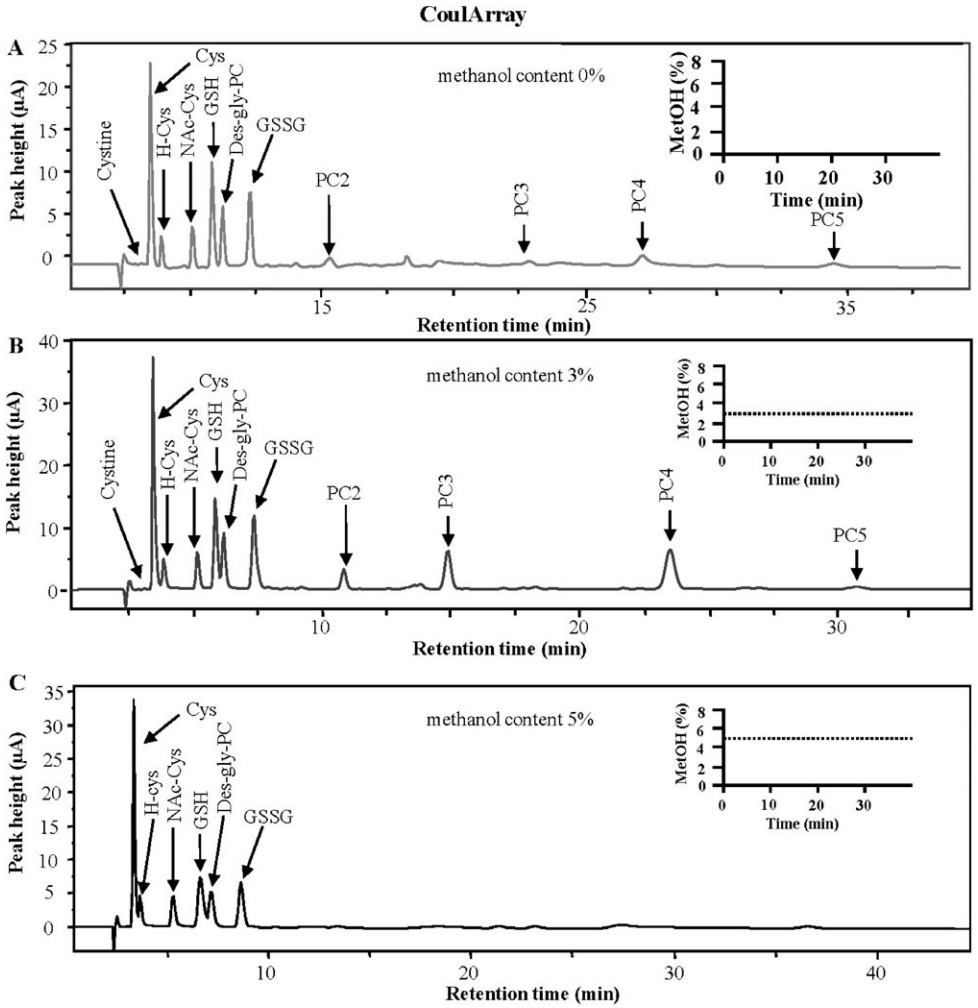

Fig. 5. Typical HPLC CoulArray chromatograms of thiols measured in mobile phase consist of 80 mM TFA with 0% (A), 3 % (B) and 5 % (C) of methanol. Flow rate 0.7 mL/min; temperature of the injected sample 4 °C; column and detector temperature 30 °C; applied potential 900 mV.

Table 1. Analytical parameters of Coulochem III analysis of the thiols ($n = 3$).

Thiol	Retention time (min)	Regression equation; R^2	Concentration of thiol (μM)	Concentration of thiol (ng/mL)	LOD [a] (pg/mL)	LOD [a] (nM)	LOD [a] (fmol)*	LOQ [b] (pg/mL)	LOQ [b] (nM)	LOQ [b] (fmol)*	RSD [c] %
Cystine	3.8	$y = 14.303\,x - 12.218$; 0.9956	0.6–10	14–240	636	3	53	2121	9	177	4.5
Cys	4.3	$y = 68.027\,x + 269.01$; 0.9998	0.6–10	7–121	22	0.2	4	74	0.6	12	5.1
H-Cysteine	5.0	$y = 15.084\,x + 296.4$; 0.9979	0.6–10	60–1002	4872	5	97	16 240	16	324	6.3
NAcCysteine	6.5	$y = 14.249\,x + 261.57$; 0.9947	0.6–10	8–135	71	0.5	11	238	2	35	5.2
GSH	7.8	$y = 22.602\,x + 302.69$; 0.9983	0.6–10	74–1234	4625	4	75	15 417	12	250	4.9
Des-Gly-PC	9.0	$y = 13.772\,x + 258.88$; 0.9992	0.6–10	18–307	123	0.4	8	411	1	27	4.6
GSSG	10.5	$y = 29.968\,x + 413.57$; 0.9995	0.6–10	9–163	53	0.3	7	178	1	22	5.6
PC2	17.3	$y = 29.248\,x + 307.62$; 0.9998	0.6–10	46–770	3002	4	78	10 007	13	260	6
PC3	20.9	$y = 14.568\,x + 249.34$; 0.9987	0.6–10	15–250	80	0.3	6	266	1	21	5.9
PC4	24.1	$y = 4.6383\,x + 258.15$; 0.9963	0.6–10	37–613	2643	4	86	8810	14	288	5.7
PC5	29.3	$y = 18.468\,x + 290.72$; 0.9980	0.6–10	32–539	1600	3	59	5333	10	198	5.1

[a] Limit of detection evaluated as 3 *S/N*.
[b] Limit of quantification evaluated as 10 *S/N*.
[c] Relative standard deviation (%) for signals in certain regression equation.
* Per injection (10 μL).

electrode was polished mechanically by 0.1 μm of alumina (ESA Inc., USA) and sonicated at the room temperature for 15 min using a Sonorex Digital 10 P Sonicator (Bandelin, Berlin, Germany) at 40 W [40]. However, the chromatogram measured after mechanical polishing of the working electrode had different baseline. To solve the shift in baseline we employed program GPES 4.9 (EcoChemie, Netherlands). The chromatogram measured was exported to GPES and treated by smoothing and baseline correction. The typical mathematically processed chromatograms are shown in Figure 4.

HPLC coupled with Coulochem III detector does not enable us to use gradient elution to separate and subsequently analyze the target molecules. Therefore the analysis must be carried out in isocratic mode, which leads to relative longer total length of an analysis (more than 30 min., Fig. 4). In addition to this, the target molecules are measured at the surface of a glassy carbon electrode in wall jet arrangement. The main disadvantage of this arrangement bases in decreasing of the signals measured in the presence of a mobile phase with content of organic solvents higher than 5–10%. In spite of the mentioned disadvantages the peaks of thiols are well separated and developed with detection limits (3 *S/N*) from tenth to units of nM (Fig. 4, Table 1).

3.2. Coulometric Detection – CoulArray Detector

HPLC coupled with CoulArray detector was also utilized for measurement of the twelve thiols as Coulochem III instrument. CoulArray detector as multichannel detector consists of twelve working carbon porous electrode, whereas two auxiliary and two reference electrodes are connected with one working electrode. Electrochemical measurements are carried out on all working electrodes, which promotes near 100% electrochemical conversion of analytes below the designated potential of the detector. The shapes of the thiols HDVs measured using CoulArray detector are similar to those measured by Coulochem III detector. The working electrode potential of 900 mV is also the most suitable potential for simultaneous determination of the twelve compounds of interest using CoulArray detector (Fig. 3B).

As it was reported, presence of organic solvent in mobile phase can markedly influence both current response and separation of the target molecules [42, 43, 49, 50]. HPLC-ED chromatograms of the twelve thiols measured in the presence of mobile phase consisted of 80 mM TFA and 0, 3 or 5% methanol are shown in Figure 5. The mobile phase consists of 80 mM TFA only negatively influenced the chromatographic separation of the target molecules. The peaks are not well separated and the sensitivity is very poor (Fig. 5A). The content of methanol about 3% in the mobile phase was suitable for simultaneous determination of the twelve target molecules. The peaks were separated and well developed. Total length of the analysis was about 30 min. Repeatability of the analysis was good with standard deviation in the peak height up to 5% (Fig. 5B). However the portion of the organic solvent in mobile phase higher

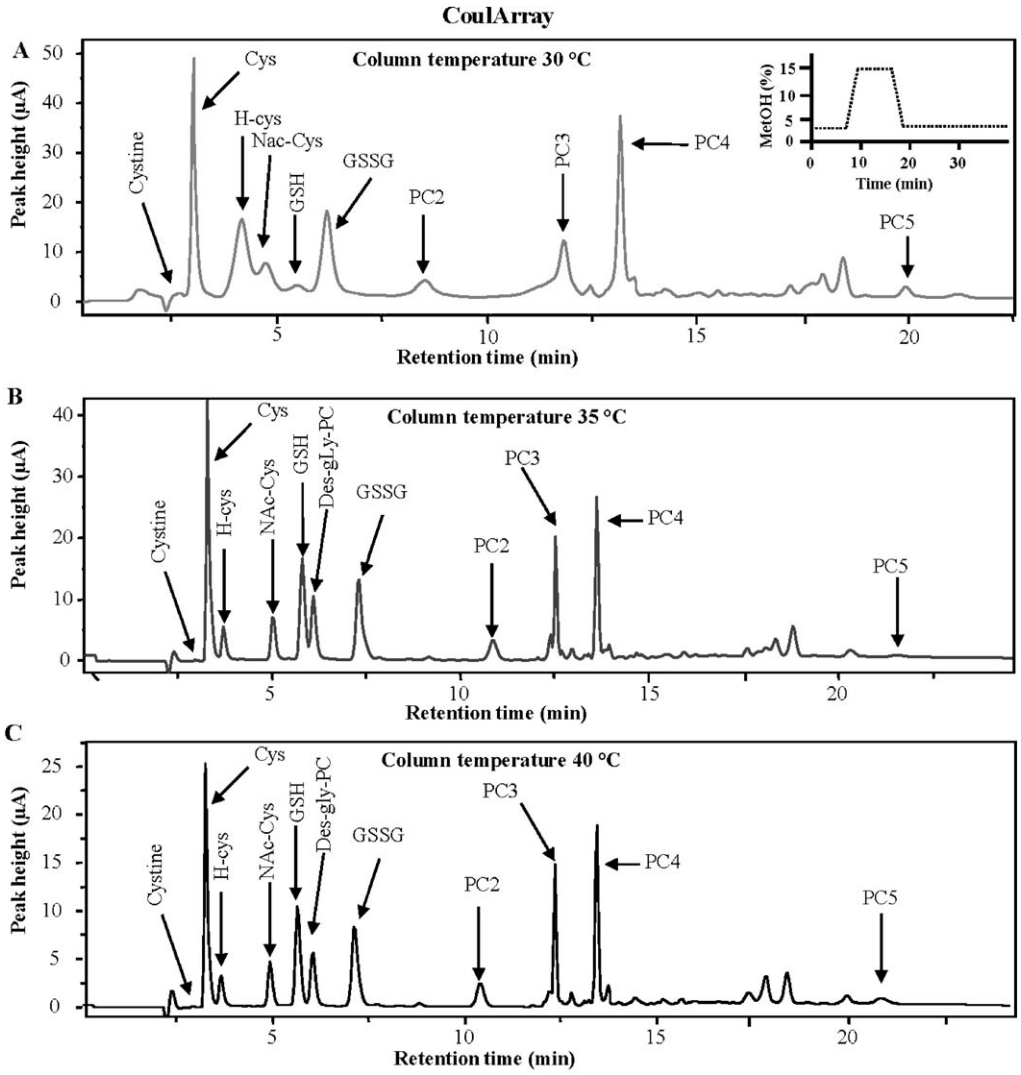

Fig. 6. Influence of gradient elution, and column and detector temperature on separation of the thiols, 30 °C (A), 35 °C (B) and 40 °C (C). Gradient profile for simultaneous thiol separation starting at 100:0 (80 mM TFA:methanol) kept constant for 9 min, then decreasing to 85:15 during one minute, and kept constant for 8 min, and finally increasing linearly up to 97:3 from 18 to 19 min. For other chromatographic conditions see in Fig. 5.

than 5% resulted in very low sensitivity to cystine, PC2, PC3, GSSG and Des-gly-PC signals (Figure 5C).

Unlike Coulouchem instrument the CoulArray enable us to use gradient elution to detect the target molecules. If we applied rapid increase in content of the organic solvent in the mobile phase, the total length of the analysis was shortened to 20 min. However, the portion of the organic solvent in the mobile phase had not exceeded 15%, because it results in poor resolution of the peaks and very low sensitivity to most of the thiol signals. HPLC-ED chromatogram of the twelve target molecules is shown in Figure 6A. The signals were not well developed and the separation was poor. Therefore we increased flow rate of the mobile phase from up to 0.8 mL/min. This increase slightly improved the separation; however, we can not apply higher flow rates due to relative high pressure in the system (about 200 bars).

The influence of column temperature on the separation and detection of the target molecules was investigated (Figs. 6B, C). The higher temperature markedly improved the separation of the thiols. The signals were well separated and developed, and the sensitivity to cystine, PC2, PC3, GSSG and Des-gly-PC enhanced. Under the optimal conditions (gradient profile for simultaneous thiol separation starting at 100:0 (80 mM TFA:methanol) kept constant for 9 min, then decreasing to 85:15 during one minute, and kept constant for 8 min, and finally increasing linearly up to 97:3 from 18 to 19 min, mobile phase flow rate of 0.8 mL/min, and column temperature of 40 °C) we measured chromatogram of the thiols at their concentration of 200 or 500 nM (Figure 7). The signals of the thiols were well developed and separated. The calibration dependences were evaluated using the sum of the peak heights measured at the all working electrodes according to Potesil et al. [43]. The detection limits (3 *S*/*N*) were from tens to hundreds pM (Table 2).

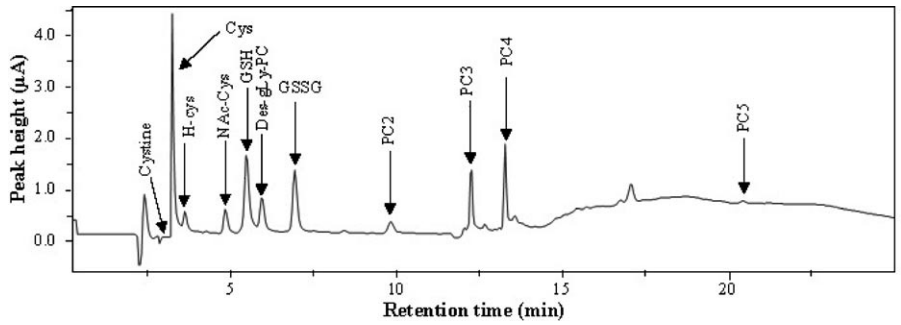

Fig. 7. HPLC CoulArray chromatogram of the thiols. Concentration of Cysteine, Cystine, H-cysteine, NAcCysteine, GSH and GSSG was 200 nM. Concentration of Des-gly-PC, PC2, PC3, PC4 and PC5 was 500 nM. For other chromatographic conditions see in Fig. 6.

3.3. Thiols Content – CoulArray Detector

The detection limits of thiols measured by CoulArray detector were about two magnitudes lower in comparison to those measured by Coulochem III detector. Moreover the total length analysis was about 10 minutes shorter in the case of CoulArray detector. Thus, we utilized HPLC coupled with CoulArray detector to determine thiols in tissues of lettuce plants treated with lead(II) ions. The typical chromatogram of the extract from leaves of lettuce treated with 0.5 mM Pb-EDTA in fifth day of the treatment is shown in Figure 8A. We determined the changes in content of the eight most important thiols involved in the plant cell protection against heavy metal ions effects. Recovery varied from 97 to 102% (Table 3). The changes in the content of cystine, Cys, Des-gly-PC, PC2, PC3, PC4, GSH and GSSG in leaves of plants treated with 0.5 mM Pb-EDTA with increasing time of the treatment is shown in Figure 8B. The content of Cys, PC2 and PC3 markedly increased after 72 h long exposition. Moreover the content of Des-gly-PC and GSH gradually increased during the treatment.

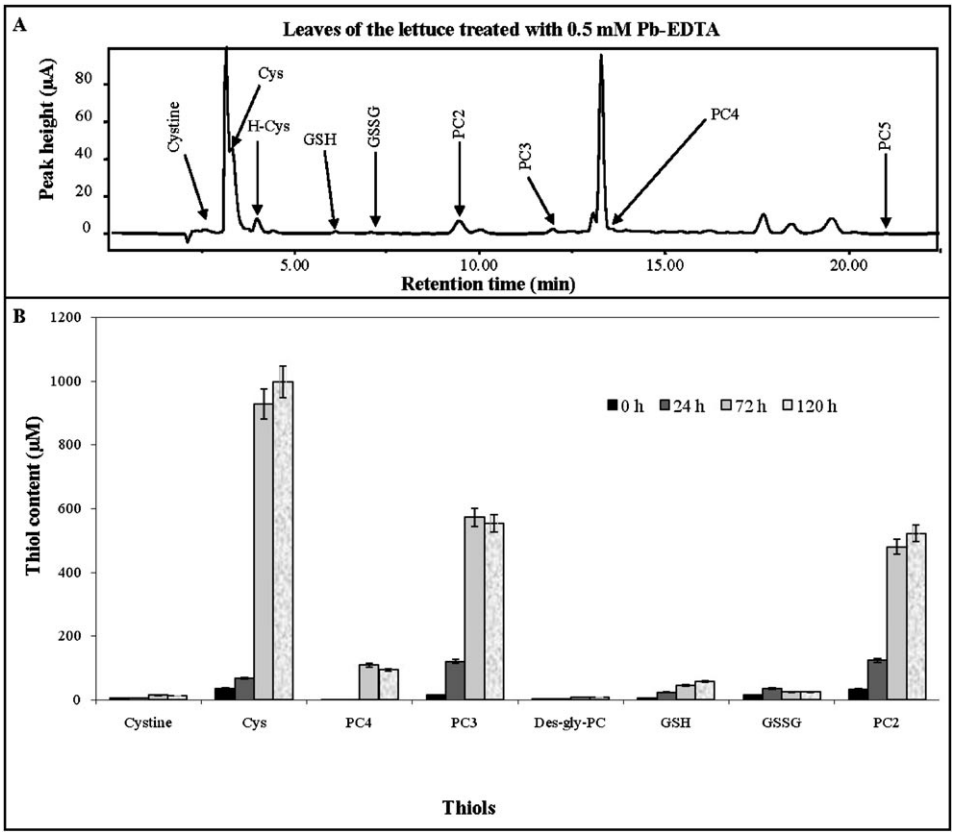

Fig. 8. HPLC CoulArray chromatogram of the extract from leaves of lettuce treated with 0.5 mM Pb-EDTA in fifth day of the treatment (A). Content of thiols in leaves of the lettuce plants treated with 0.5 mM Pb-EDTA. For other chromatographic conditions see in Fig. 6 and Sect. 2.1.

Table 2. Analytical parameters of CoulArray analysis of the thiols ($n = 3$).

Thiol	Retention time (min)	Regression equation; R^2	Concentration of thiol (μM)	Concentration of thiol (ng/mL)	LOD [a] (pg/mL)	LOD [a] (nM)	LOD [a] (fmol)*	LOQ [b] (pg/mL)	LOQ [b] (nM)	LOQ [b] (fmol)*	RSD [c] %
Cystine	2.8	$y = 0.4983\,x + 6.4169$; 0.9989	0.6–10	14–240	34	140	3	112	466	9	3.2
Cys	3.3	$y = 0.7606\,x + 14.773$; 0.9992	0.6–10	7–121	1	9	0.2	4	31	1	2.8
H-Cysteine	3.5	$y = 1.6025\,x + 0.3348$; 0.9963	0.6–10	60–1002	232	232	5	773	772	15	1.4
NAcCysteine	5.2	$y = 0.9221\,x + 14.961$; 0.9992	0.6–10	8–135	3	24	0.5	11	79	2	2.5
GSH	5.7	$y = 4.2751\,x - 1.7088$; 0.9987	0.6–10	74–1234	307	249	5	1024	829	17	1.9
Des-Gly-PC	6.8	$y = 0.3542\,x + 7.0765$; 0.9993	0.6–10	18–307	7	23	0.5	24	78	2	2
GSSG	7.3	$y = 3.658\,x + 24.664$; 0.9995	0.6–10	9–163	3	15	0.3	8	51	1	1.2
PC2	10.8	$y = 5.299\,x - 1.3425$; 0.9995	0.6–10	46–770	158	205	4	527	684	14	3.1
PC3	12.5	$y = 0.2212\,x + 3.4563$; 0.9997	0.6–10	15–250	6	24	0.5	20	80	2	3
PC4	12.9	$y = 2.0503\,x + 12.352$; 0.9994	0.6–10	37–613	148	242	5	493	805	16	2.9
PC5	13.6	$y = 0.5553\,x + 2.9283$; 0.9993	0.6–10	32–539	90	166	3	299	554	11	2.4

[a] Limit of detection evaluated as 3 *S/N*.
[b] Limit of quantification evaluated as 10 *S/N*.
[c] Relative standard deviation (%) for signals in certain regression equation.
* Per injection (10 μL).

3.4. Spatial-Distribution of Lead(II) Ions – LIBS

Based on the above-mentioned facts, we further aimed our attention on investigating of lettuce ability to accumulate lead ions and on studying of the plant stress response on the presence of this toxic pollutant. For our experiment, we used LIBS technique because of its ability to provide high spatial-distribution of metal ions including lead(II) ions in different types of materials including plant tissues. The laser-generated patterns consisting of precisely ablated micro-craters have been utilized for mapping the lead distribution on 4.5×2 mm^2 leaf sections of lettuce samples. Three sections were studied in each leaf from control and Pb-EDTA treated plants (Fig. 2B-II). Typical LIBS ablation patterns are shown in Figure 2B-II. For each of the analyzed spectra from different ablation crater on the sample the background was subtracted and the peak area of emission lines for Pb calculated. Analyzing the maps obtained in different areas of the Pb-EDTA treated samples we observed higher Pb content in the vein structure of the sample treated with 0.5 mM Pb-EDTA. The main vein – vascular bundle – is clearly observable in all investigated areas within this leaf (Fig. 2C). In the case of 1 mM Pb-EDTA treated sample the lead is spread more homogenously within the sample in the investigated area around the main vein; and there is only a slight increase of the lead signal on the main vein (see Fig. 2C – III). In spite of the fact that LIBS is not an absolute method [5] and in the case of investigation of plant tissues it is almost impossible to obtain standard samples that could be used for LIBS calibration [7], the increasing Pb content with higher dose of lead(II) ions was evident on the higher intensity (and area) of the monitored spectral lines. As a control test, we did not observe any Pb signal on the control (untreated) samples. Within a 1 mM Pb-EDTA treated leaf, the higher concentration of the pollutant was detected on the part near to the petiole. High lead(II) ions concentration was detected especially at surrounding of main vein – vascular bundle – which is formed by mechanical tissue with very thick-walled cells. This result may indicate the deposition of lead(II) ions in cell walls. We did not observe any significant difference between the maps from different parts (A, B and C) in the case of 0.5 mM Pb-EDTA treated sample.

Based on the results obtained it can be concluded that treated lettuce plant transports actively the lead(II) ions within the whole plant, where are detoxified especially by thiols, most of all glutathiones and phytochelatins, which can be considered as the main molecules synthesized as the response of the plant cell on the presence of a heavy metals ions.

4. Conclusions

Phytoremediation technologies belong to the most promising procedures in decontamination of heavy metal polluted environment. However, determination of not only molecules playing key role in heavy metal detoxification

Table 3. Recovery of the thiols spiked in a lettuce sample ($n = 5$).

Thiol	Homogenate (μM) [a]	Spiking (μM) [a]	Homogenate + spiking (μM) [a]	Recovery (%)
Cystine	13.8 ± 0.2 (1.4)	10.3 ± 0.3 (2.9)	23.5 ± 0.9 (3.8)	98
Cys [b]	927 ± 45 (4.8)	101 ± 3 (2.9)	1045 ± 52 (5.0)	98
PC4 [b]	108 ± 4 (3.7)	103 ± 4 (3.8)	215 ± 12 (5.6)	102
PC3 [b]	573 ± 25 (4.4)	105 ± 2 (1.9)	656 ± 29 (4.4)	97
Des-Gly-PC	8.4 ± 0.3 (3.6)	10.4 ± 0.1 (0.9)	18.3 ± 0.9 (4.9)	99
GSH	45.0 ± 1.9 (4.2)	10.1 ± 0.3 (2.9)	53.4 ± 1.8 (3.4)	97
GSSG	24.9 ± 1.1 (4.4)	10.3 ± 0.5 (4.9)	34.1 ± 1.1 (3.2)	97
PC2 [b]	479 ± 15 (3.1)	99 ± 3 (3.0)	580 ± 29 (5.0)	100

[a] Thiol concentration; expressed as mean ± S. D. (C. V.%).
[b] The sample was ten times diluted with ACS water prior to analysis.

processes, but also their precursors is needed to understand the processes taking place within a plant cell. Recently, the papers focused on simultaneous determination of these low molecular thiols using high performance liquid chromatography with electrochemical detection have been published [26, 42 – 48]. The eight channel coulometric detector has been proved very suitable for analysis of thiols; however, the comparison of amperometric and coulometric detector has not been done yet. Here, we compared both type of electrochemical detectors utilized for simultaneous determination of twelve biologically important thiols (cysteine (Cys); reduced (GSH) and oxidized (GSSG); homocysteine (H-cysteine); *N*-acetylcysteine (NAcCysteine); cystine; phytochelatins: des-Glycine-Phytochelatin (Des-gly-PC), phytochelatin$_2$ (PC_2), phytochelatin$_3$ (PC_3), phytochelatin$_4$ (PC_4) and phytochelatin$_5$ (PC_5)). In this work, we proposed chromatographic procedure suitable for analysis of real samples without derivatization of the target thiols. Moreover we successfully utilized LIBS for determination of high spatial-distribution of lead(II) ions within the leaf.

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References

[1] D. E. Salt, R. D. Smith, I. Raskin, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 643.
[2] V. Hooda, *J. Environ. Biol.* **2007**, *28*, 367.
[3] S. L. Wang, C. N. Mulligan, *J. Hazard. Mater.* **2006**, *138*, 459.
[4] R. Q. Zhang, C. F. Tang, S. Z. Wen, Y. G. Liu, K. L. Li, *J. Integr. Plant Biol.* **2006**, *48*, 1257.
[5] C. W. A. do Nascimento, B. S. Xing, *Sci. Agric.* **2006**, *63*, 299.
[6] S. Cherian, M. M. Oliveira, *Environ. Sci. Technol.* **2005**, *39*, 9377.
[7] K. Dercova, J. Makovnikova, G. Barancikova, J. Zuffa, *Chem. Listy* **2005**, *99*, 682.
[8] E. L. Arthur, P. J. Rice, P. J. Rice, T. A. Anderson, S. M. Baladi, K. L. D. Henderson, J. R. Coats, *Crit. Rev. Plant Sci.* **2005**, *24*, 109.
[9] M. N. V. Prasad, H. M. D. Freitas, *Electron. J. Biotechnol.* **2003**, *6*, 285.
[10] I. D. Pulford, C. Watson, *Environ. Int.* **2003**, *29*, 529.
[11] I. Raskin, R. D. Smith, D. E. Salt, *Curr. Opin. Biotechnol.* **1997**, *8*, 221.
[12] C. S. Cobbett, *Curr. Opin. Plant Biol.* **2000**, *3*, 211.
[13] C. Cobbett, P. Goldsbrough, *Annu. Rev. Plant Biol.* **2002**, *53*, 159.
[14] S. Clemens, *Planta* **2001**, *212*, 475.
[15] W. E. Rauser, *Annu. Rev. Biochem.* **1990**, *59*, 61.
[16] R. E. Hansen, H. Ostergaard, P. Norgaard, J. R. Winther, *Anal. Biochem.* **2007**, *363*, 77.
[17] A. K. M. Kafi, F. Yin, H. K. Shin, Y. S. Kwon, *Curr. Appl. Phys.* **2007**, *7*, 496.
[18] V. Adam, S. Krizkova, O. Zitka, L. Trnkova, J. Petrlova, M. Beklova, R. Kizek, *Electroanalysis* **2007**, *19*, 339.
[19] S. Krizkova, I. Fabrik, V. Adam, J. Kukacka, R. Prusa, G. J. Chavis, L. Trnkova, J. Strnadel, V. Horak, R. Kizek, *Sensors* **2008**, *8*, 3106.
[20] I. Fabrik, J. Kukacka, J. Baloun, I. Sotornik, V. Adam, R. Prusa, D. Vajtr, P. Babula, R. Kizek, *Electroanalysis* **2009**, *21*, 650.
[21] S. Krizkova, I. Fabrik, V. Adam, J. Kukacka, R. Prusa, L. Trnkova, J. Strnadel, V. Horak, R. Kizek, *Electroanalysis* **2009**, *21*, 640.
[22] R. Possari, R. F. Carvalhal, R. K. Mendes, L. T. Kubota, *Anal. Chim. Acta* **2006**, *575*, 172.
[23] E. Bramanti, L. D'Ulivo, C. Lomonte, M. Onor, R. Zamboni, G. Raspi, A. D'Ulivo, *Anal. Chim. Acta* **2006**, *579*, 38.
[24] S. K. Kawakami, M. Gledhill, E. P. Achterberg, *Trac – Trends Anal. Chem.* **2006**, *25*, 133.
[25] A. K. Sakhi, R. Blomhoff, T. E. Gundersen, *J. Chromatogr. A*. **2007**, *1142*, 178.
[26] V. Supalkova, D. Huska, V. Diopan, P. Hanustiak, O. Zitka, K. Stejskal, J. Baloun, J. Pikula, L. Havel, J. Zehnalek, V. Adam, L. Trnkova, M. Beklova, R. Kizek, *Sensors* **2007**, *7*, 932.
[27] M. Farkas, J. O. Berry, D. S. Aga, *Analyst* **2007**, *132*, 664.
[28] T. Revermann, S. Gotz, U. Karst, *Electrophoresis* **2007**, *28*, 1154.
[29] X. Yao, Y. T. Wang, G. Chen, *Biomed. Chromatogr.* **2007**, *21*, 520.
[30] V. Lavigne, A. Pons, D. Dubourdieu, *J. Chromatogr. A*. **2007**, *1139*, 130.
[31] K. H. A. Janssens, F. C. V. Adams, A. Rindby, *X-ray Fluorescence Analysis*, Wiley, Chichester, UK **2000**.
[32] S. Jorks, *X-ray Microscopy, Instrumentation and Biological Application*, Springer, Berlin **1987**.

[33] J. Kaiser, L. Reale, A. Ritucci, G. Tomassetti, A. Poma, L. Spano, A. Tucci, F. Flora, A. Lai, A. Faenov, T. Pikuz, L. Mancini, G. Tromba, F. Zanini, *Eur. Phys. J. D.* **2005**, *32*, 113.
[34] J. Kaiser, O. Samek, L. Reale, M. Liska, R. Malina, A. Ritucci, A. Poma, A. Tucci, F. Flora, A. Lai, L. Mancini, G. Tromba, F. Zanini, A. Faenov, T. Pikuz, G. Cinque, *Microsc. Res. Tech.* **2007**, *70*, 147.
[35] A. V. Miziolek, V. Palleschi, L. Schlechter, *Laser Induced Breakdown Spectroscopy*, Cambridge University Press, New York **2006**.
[36] J. Kaiser, M. Galiova, K. Novotny, R. Cervenka, L. Reale, J. Novotny, M. Liska, O. Samek, V. Kanicky, A. Hrdlicka, K. Stejskal, V. Adam, R. Kizek, *Spectrochim. Acta, Part B* **2009**, *64*, 67.
[37] E. Camera, M. Rinaldi, S. Briganti, M. Picardo, S. Fanali, *J. Chromatogr. B* **2001**, *757*, 69.
[38] R. Causon, *J. Chromatogr. B* **1997**, *689*, 175.
[39] R. Bugianesi, M. Serafini, F. Simone, D. Y. Wu, S. Meydani, A. Ferro-Luzzi, E. Azzini, G. Maiani, *Anal. Biochem.* **2000**, *284*, 296.
[40] R. Mikelova, J. Baloun, J. Petrlova, V. Adam, L. Havel, H. Petrek, A. Horna, R. Kizek, *Bioelectrochemistry* **2007**, *70*, 508.
[41] J. Vacek, J. Petrek, R. Kizek, L. Havel, B. Klejdus, L. Trnkova, F. Jelen, *Bioelectrochemistry* **2004**, *63*, 347.
[42] J. Petrlova, R. Mikelova, K. Stejskal, A. Kleckerova, O. Zitka, J. Petrek, L. Havel, J. Zehnalek, V. Adam, L. Trnkova, R. Kizek, *J. Sep. Sci.* **2006**, *29*, 1166.
[43] D. Potesil, J. Petrlova, V. Adam, J. Vacek, B. Klejdus, J. Zehnalek, L. Trnkova, L. Havel, R. Kizek, *J. Chromatogr. A* **2005**, *1084*, 134.
[44] P. C. White, N. S. Lawrence, J. Davis, R. G. Compton, *Electroanalysis* **2002**, *14*, 89.
[45] G. Noctor, C. H. Foyer, *Anal. Biochem.* **1998**, *264*, 98.
[46] E. Camera, M. Picardo, *J. Chromatogr. B* **2002**, *781*, 181.
[47] K. Stejskal, Z. Svobodova, I. Fabrik, V. Adam, M. Beklova, M. Rodina, R. Kizek, *J. Appl. Ichthyol.* **2008**, *24*, 519.
[48] A. K. Sakhi, K. M. Russnes, S. Smeland, R. Blomhoff, T. E. Gundersen, *J. Chromatogr. A* **2006**, *1104*, 179.
[49] B. Klejdus, J. Vacek, V. Adam, J. Zehnalek, R. Kizek, L. Trnkova, V. Kuban, *J. Chromatogr. B* **2004**, *806*, 101.
[50] D. Potesil, R. Mikelova, V. Adam, R. Kizek, R. Prusa, *Protein J.* **2006,** *25*, 23.

