

Article

Analysis of Cadmium-Phytochelatin 2 Complexes Using Flow Injection Analysis Coupled with Electrochemical Detection Mass Spectrometry

Miguel Angel Merlos Rodrigo^{1,2,3}, Ondrej Zitka^{1,2,3,4}, Marketa Kominkova¹, Vojtech Adam^{2,3}, Miroslava Beklova⁴ and Rene Kizek^{1,2,3*}

¹Department of Chemistry and Biochemistry, and ²Lead and Cadmium Initiatives, United Nation Environment Program, Faculty of Agronomy, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic

³Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic

⁴Department of Veterinary Ecology and Environmental Protection, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences, Palackeho 1-3, CZ-612 42 Brno, Czech Republic

*E-mail: kizek@sci.muni.cz

Received: 22 November 2012 / Accepted: 15 October 2012 / Published: 1 April 2013

In this study, *in vitro* synthesized Cd-phytochelatin (PC2) complexes were characterized using flow injection analysis/high performance liquid chromatography with CoulArray or Coulochem electrochemical detector of PC2 and Cd-PC2 complexes. Method of the formation of complex by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) was confirmed. Higher pH had lowered effect for Cd(II) detection but PC2 detection suffers much in task of sensitivity and for further analysis we thus used the Britton-Robinson buffer at pH 2 as electrolyte. Higher temperature than 40 °C positively influenced the interaction between Cd and thiol groups of PC2 by Coulochem III. The time did not affect the forming of complex. The concentration had bigger effect on the formation of complex than time of incubation. The biggest change was, with regards to concentration, observed under applied concentration 400 µg/ml and where the slope was the most decreasing which means the decreasing the amount of PC2 and increasing of amount of complex. In the case of the influence of time, 400 µg/ml reported the most decreasing trend after 8 hour of incubation. Subsequently we proved the formation Cd-PC2 complex using MALDI-TOF-MS and these results indicated that the optimum conditions for the determination of PC2 utilizing HCCA as matrix was a laser intensity of 43.3 µJ and the concentration of the matrix was 15 mg/ml in 70% methanol (v/v). The obtained data show that the flow injection analysis coupled with electrochemical detection Coulochem III or/and Coularray in combination with MALDI-TOF is a reliable and fast method for the determination of these complexes.

Keywords: Flow Injection Analysis; Electrochemical Detection; Phytochelatin; Cadmium; Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry

1. INTRODUCTION

Heavy metals, such as cadmium (Cd), are potentially toxic to all organisms. It is not surprising that organisms have protective mechanisms to resist, at least partially, the adverse effect of metals. Cd is a non-essential heavy metal ubiquitously dispersed in the environment by natural and anthropogenic activities [1-3]. Biological systems respond to potentially toxic levels of metal ions mainly by chelation and sequestration of the excess of the element. Plants respond to Cd toxicity of different ways such as immobilization, exclusion, chelation and compartmentalization of the metal ions, and the expression of more general stress response mechanisms as stress peptides [4-6].

Phytochelatin (PC) are enzymatically synthesized peptides produced in a wide variety of plant species including monocots, dicots, gymnosperms, and algae as heavy metal binding ligands, such as cadmium. PCs form a family of structures with increasing repetitions of the -Glu-Cys dipeptide units followed by a terminal Gly, (γ -Glu-Cys) $_n$ -Gly or (γ -EC) $_n$ -Gly, where n ranges from 2–11 [4,7-9]. PCs are synthesized from reduced glutathione (GSH) by the constitutive enzyme PC synthase, activated by a variety of metal ions, among which Cd is the most effective [10-16].

PC are usually analysed by using a method developed by Grill et al. [17]. In addition to this method, Doring et al. used pre-column derivatization, high-performance liquid chromatography and fluorescence-detection for analysis of PC in plant [18]. In order to determine the Cd-PC complexes, different methodologies have been used in recent years. Techniques, such as high performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS) and high performance liquid chromatography electrospray mass spectrometry (HPLC-ESI-MS) are analytical instruments to study metal speciation in plants. They were applied in phytoremediation research as well as in the understanding of metal accumulation mechanism. Chen et al. characterized in vitro synthesized Cd-PC complexes and in vivo Cd-PC complexes in Cd-stressed plants, using size exclusion chromatography (SEC) ICP-MS and ESI-MS/MS [19]. Nano-electrospray ionization tandem mass spectrometry and capillary liquid chromatography/electrospray ionization tandem mass spectrometry method was used for the analysis of Cd-PC complexes in plant tissue culture [20]. Liquid chromatography coupled with on line chemical vapour generation and atomic fluorescence spectrometric detection [21], and inductively coupled plasma-mass spectrometry (ICP-MS) [22,23], were methods used for the determination of Cd-PC complexes.

The innovation of flow injection analysis (FIA) for the analysis of peptides or proteins with a sensitivity of low concentrations has greatly enhanced our capability to analyse metallic complexes, including the interaction between a peptide and a metal ion [24]. So far, few studies of the use of flow injection analysis in the analysis of PCs have been reported [24], but the direct analysis of PC-Cd complexes has not been explored. Therefore, the main aim of this study was to optimize flow injection analysis/high performance liquid chromatography coupled with CoulArray or Coulochem electrochemical detector for analysis of PC₂ and Cd-PC₂ complexes synthesized in vitro. The results were further evaluated by MALDI-TOF.

2. EXPERIMENTAL PART

2.1 Chemicals

Phytochelatin2 (PC2) (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) from Sigma-Aldrich (St. Louis, USA) was used. Other chemicals (H₃PO₄, CH₃COOH, H₃BO₃, NaOH and CdCl₂) were purchased from Sigma-Aldrich in ACS purity unless noted otherwise. Stock standard solutions of the PC2 (1 mg/ml) were prepared with ACS water 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 μ m Nylon filter discs (Millipore, Billerica, Mass., USA) prior to FIA-ED analysis. The pH value was measured using InoLab pH 730 WTW (Weilheim, Germany).

2.2 Flow injection analysis coupled with electrochemical detector Coulochem III

The instrument for flow injection analysis with electrochemical detection (FIA-ED) consisted of solvent delivery pump operating within the range from 0.001 to 9.999 ml/min (Model 582 ESA Inc., Chelmsford, MA, USA), a guard cell (Model 5020 ESA, USA), a reaction coil (1 m) and/or a chromatographic column Zorbax eclipse AAA C18 (150 \times 4.6; 3.5 μ m particles, Agilent Technologies, USA), UV-VIS (Model 528, ESA Inc., Chelmsford, MA, USA) and an electrochemical detector. The electrochemical detector includes one low volume flow-through analytical cell (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 (20 μ l) was injected using autosampler (Model 542, ESA Inc., Chelmsford, MA, USA). The data obtained were treated with Clarity software (Version 3.0.04.444, Data Apex, Czech Republic). The experiments were carried out at room temperature. Guard cell potential was set as 0 V. A glassy carbon electrode was polished mechanically by 0.1 μ m of alumina (ESA Inc., Chelmsford, MA, USA) and sonicated at room temperature for 5 min using a Sonorex Digital 10 P Sonicator (Bandelin, Berlin, Germany) at 40 W. Flow rate of mobile phase was 1 ml/min. Hydrodynamic voltammograms were analyzed in range 100 – 1000 mV with 100 mV steps.

2.3 Flow injection analysis coupled with electrochemical detector Coularray

FIA-ED system consisted of two solvent delivery pumps operating within the range from 0.001 to 9.999 ml/min (Model 582 ESA Inc., Chelmsford, MA), a reaction coil (1 m) and a CoulArray electrochemical detector (Model 5600A, ESA Inc., Chelmsford, MA, USA). The electrochemical detector includes three flow cells (Model 6210, ESA Inc., Chelmsford, MA, 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. The sample (20 μ l) was injected using autosampler (Model 542 HPLC, ESA Inc., Chelmsford, MA, USA). Flow rate of mobile phase was 1 ml/min. Hydrodynamic voltammograms were analyzed in range 100 – 1000 mV with 100 mV steps.

2.4 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

The mass spectrometry experiments were performed on a MALDI-TOF mass spectrometer Bruker Ultraflex extreme (Bruker Daltonik GmbH, Germany) equipped with a laser operating at wavelength of 355 nm with an accelerating voltage of 25 kV, cooled with nitrogen and a maximum energy of 43.2 μJ with repetition rate 2000 Hz in linear and positive mode, and with software for data acquisition and processing of mass spectra flexControl version 3.4 and flexAnalysis version 2.2. The matrix used in the MALDI method was α -cyano-4-hydroxycinnamic acid (HCCA) (Bruker). The matrix was prepared in 70% methanol. Mixture was thoroughly vortexed and ultrasonicated using Bandelin 152 Sonorex Digital 10P ultrasonic bath (Bandelin electronic GmbH, Germany) to 2 minutes 50% of intensity at room temperature. Working standard solutions were prepared daily by dilution of the stock solutions. The sample solutions were TA30 (30% acetonitrile, 0.1% trifluoroacetic acid solution). The solutions for analysis were mixed in ratio of 1:1 (matrix/substance). After obtaining a homogeneous solution, 1 μl was applied on the MTP ground target plate (Bruker) and dried under atmospheric pressure and room temperature. A mixture of peptide calibrations standard (Bruker) was used to externally calibrate the instrument.

Samples preparation crystallization method for MALDI-TOF was done in three different ways: Dried Droplet (DD), Thin Layer (TL) and Double Layer (DL). For DD, a saturated matrix solution was prepared. This matrix solution was mixed in equal volumes with the sample solution (1:1). The sample solution was TA30. The mixture was pipetted on the target (1 μl) and dried at room temperature. The preparation yielded relatively large crystals on the target surface as well as regions without matrix or analyte. For TL, the matrix was prepared on the target to form a thin layer of very small and homogenous crystals. After spotting this solution spread on the target and evaporated very fast. The thin matrix layer remained on the surface of the target. The sample was applied on top of this thin layer. After the sample was dried, the analyte molecules remained on top of the matrix. One way to combine of DD and TL preparation is the DL method. Here, a thin layer of matrix was prepared as described above and on top of that thin layer a normal dried droplet was used.

PC2 (250 ng/ μl) and CdCl₂ (500 ng/ μl) in ACS water incubated for 1 h at 20 °C were used for Cd-PC2 complexes formation. The MS spectra were typically acquired by averaging 20 sub spectra from a total of 500 shots of the laser (Smartbeam 2. Version: 1_0_38.5) with an energy of 43.2 μJ (50%).

3. RESULTS AND DISCUSSION

3.1 Optimization of FIA-ED for PC2 and Cd-PC complex determination

Our experiments were focused on studying of change of electrochemical profile based on hydrodynamic voltammogram (HDV) generated by HPLC-ED using two different electrochemical detectors. Primarily, we optimized a detection using electrochemical detector Coulochem III in flow injection system. During electrochemical analysis the pH is the most influencing factor with regards to the observed oxidizing or reducing maxima or even of the sensitivity of detection. Therefore we tested pH in wide range using Britton-Robinson buffer as the mobile phase, which covers pH 2, 3, 4, 5, 6, 7

and 8. The results obtained from the analysis of individual samples of free PC2 and free Cd(II) are shown in Fig. 1A and B. We presented the maxima of the HDV of PC2 as 900 mV (inset in Fig. 1A) and Cd(II) as 600 mV (inset in Fig. 1B). For better understanding the development of the HDV based on change of pH the border and middle values are displayed as well. It clearly follows from the results obtained that the highest response for PC2 was obtained at pH 2. The pH has been further increasing the signal has been decreasing. In the case of Cd(II), the influence of increasing pH was marginal. The pH 2 provides only about 30% lower signal than the highest tested pH 8. Change of pH across the tested spectrum from 2 to 8 much more negatively affected the PC2 detection (up to 95% decrease) in comparison to Cd(II) (only 30% increase). Therefore it is obvious that higher pH has lower effect for Cd(II) detection but PC2 detection suffers much in task of sensitivity and for further analysis we thus used the Britton-Robinson buffer at pH 2 as an electrolyte.

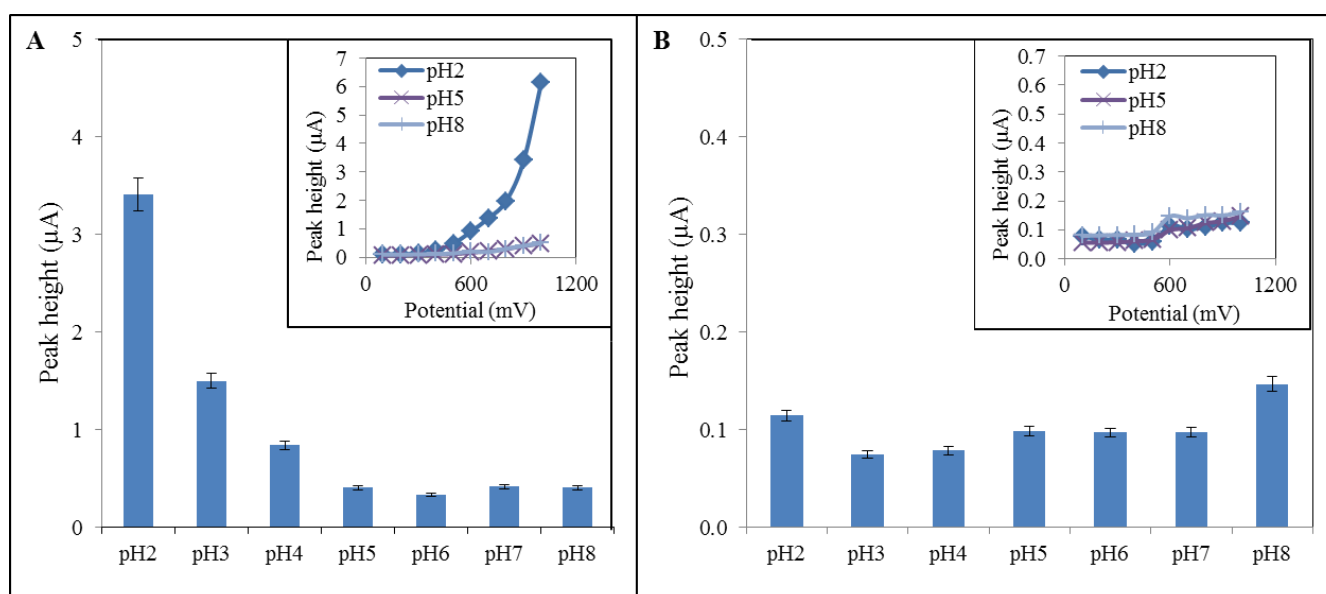


Figure 1. The obtained signal represented by peak height from FIA-ED determination using coulochem III electrochemical detector for (A) PC-2 and Cd(II) (B). In insets: the hydrodynamic voltammograms (HDV) obtained for pH 2, 5 and 8 for analysis of (A) PC-2 and (B) Cd(II).

3.2 Electrochemical studying complexes by Coulochem III

Further, we analysed the mixtures of PC2 and Cd(II) under applied concentration 100 µg/ml each. Therefore, we studied the influence of the incubation temperature (10, 20, 30 and 40 °C) and time of incubation (0, 2, 4, 6 and 8 h) on the forming of the complex during mixing in vortex under 600 rpm. Then, all variants were analyzed by Coulochem III for obtaining the HDVs (Fig. 2). Due to the optimal signal at 600 mV for Cd(II), we monitored the HDVs in this applied potential (Figs. 2A, B, C and D). There is also shown the change of profile of each HDV in greater details (Figs. 2Aa, Bb, Cc and Dd) for 0, 4, 6 and 8 hour at each temperature. It clearly follows from the results obtained that incubation time did not affect the interaction. With regards to applied temperature there is shown that 10-30°C caused signals lower than 1 µA. However, under the 40°C the intensity of signal was app. 1.5

μA , i.e. for 50 % higher. It clearly follows that higher temperature positively influenced the interaction between Cd and thiol groups of PC-2.

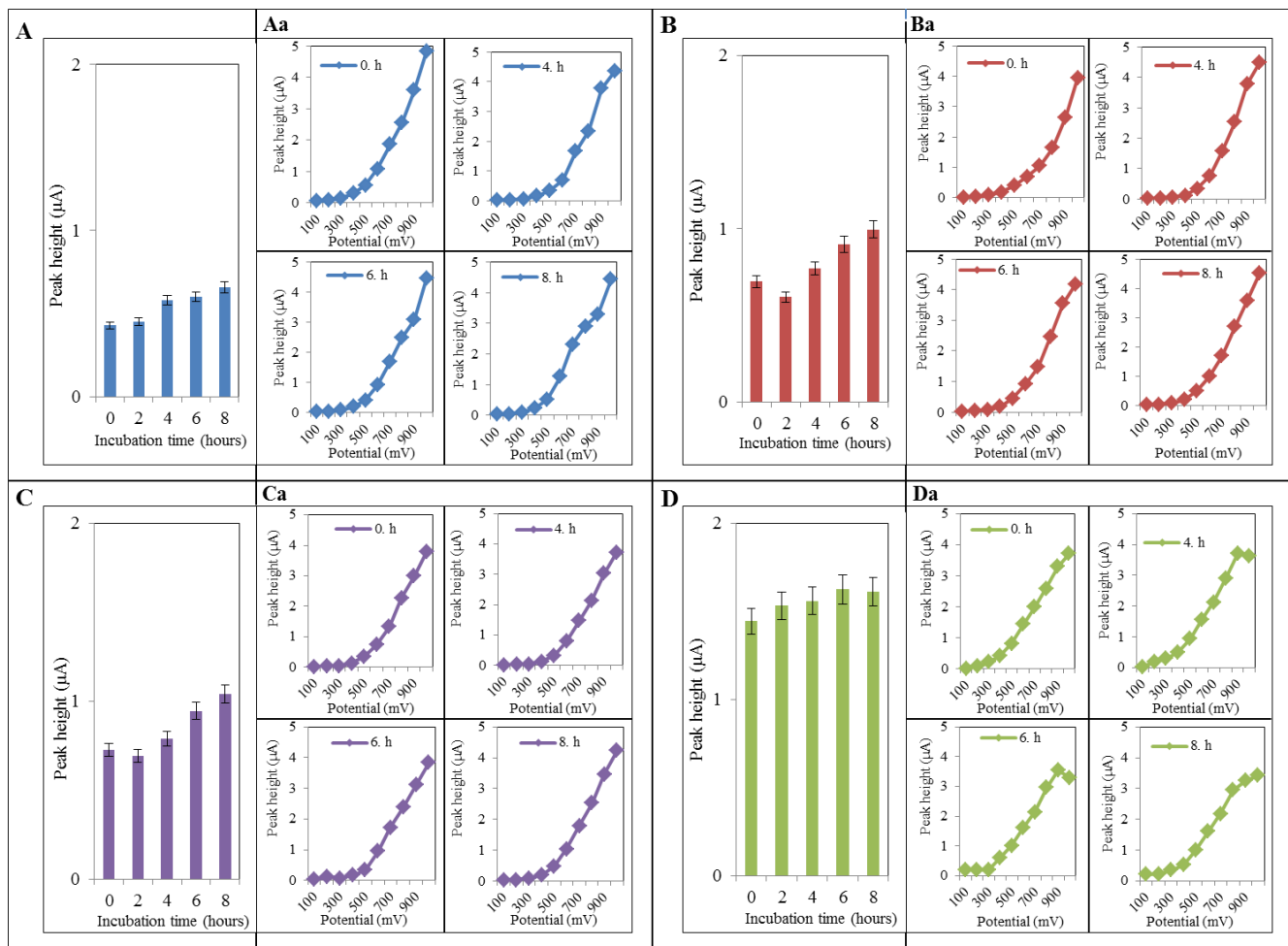


Figure 2. Signal intensities taken from hydrodynamic voltammograms of mixtures of PC2 (100 $\mu\text{g/ml}$) and CdCl_2 (100 $\mu\text{g/ml}$) under applied potential 600 mV in time of incubation 0, 2, 4, 6 and 8 hours under different temperatures as (A) 10 °C, (B) 20 °C, (C) 30 °C and (D) 40 °C. Real HDVs from the analysis for 0, 4, 6 and 8 hours for each tested temperature as (Aa) 10 °C, (Ba) 20 °C, (Ca) 30 °C and (Da) 40 °C.

3.3 Electrochemical studying of complexes by CoulArray

As we found the effect of temperature using Coulochem detector we attempted to use different detector for obtaining the HDV confirming the presence of complexes with different applied concentration. Coulometric detection enables to redox change more than 90% passing analyte in comparison with coulochem detection where less than 5% of passing analyte is detected [25]. Therefore Coularray as coulometric detector was used for this purpose. The various applied concentrations of PC2 were 100, 200, 300 and 400 $\mu\text{g/ml}$ mixed with Cd(II) 100 $\mu\text{g/ml}$. Analysis was carried out under temperature 20° C and for 0, 2, 4, 6 and/or 8 h. Same as in the previous case all variants were analysed for obtaining the HDVs and at 600mV we monitored the change of the intensity of electrochemical signal (Figs. 3A, B, C and D). There is also shown the change of profile of each

HDV in greater details in Figs. 3Aa, Bb, Cc and Dd for 100, 200, 300 and 400 µg/ml. It clearly follows from the results obtained that time did not affect the forming of complex as much as applied concentration. The higher intensity of creation complex is due to applied concentration of 100 µg/ml and no distinct change in HDV trend was observed under higher applied concentrations.

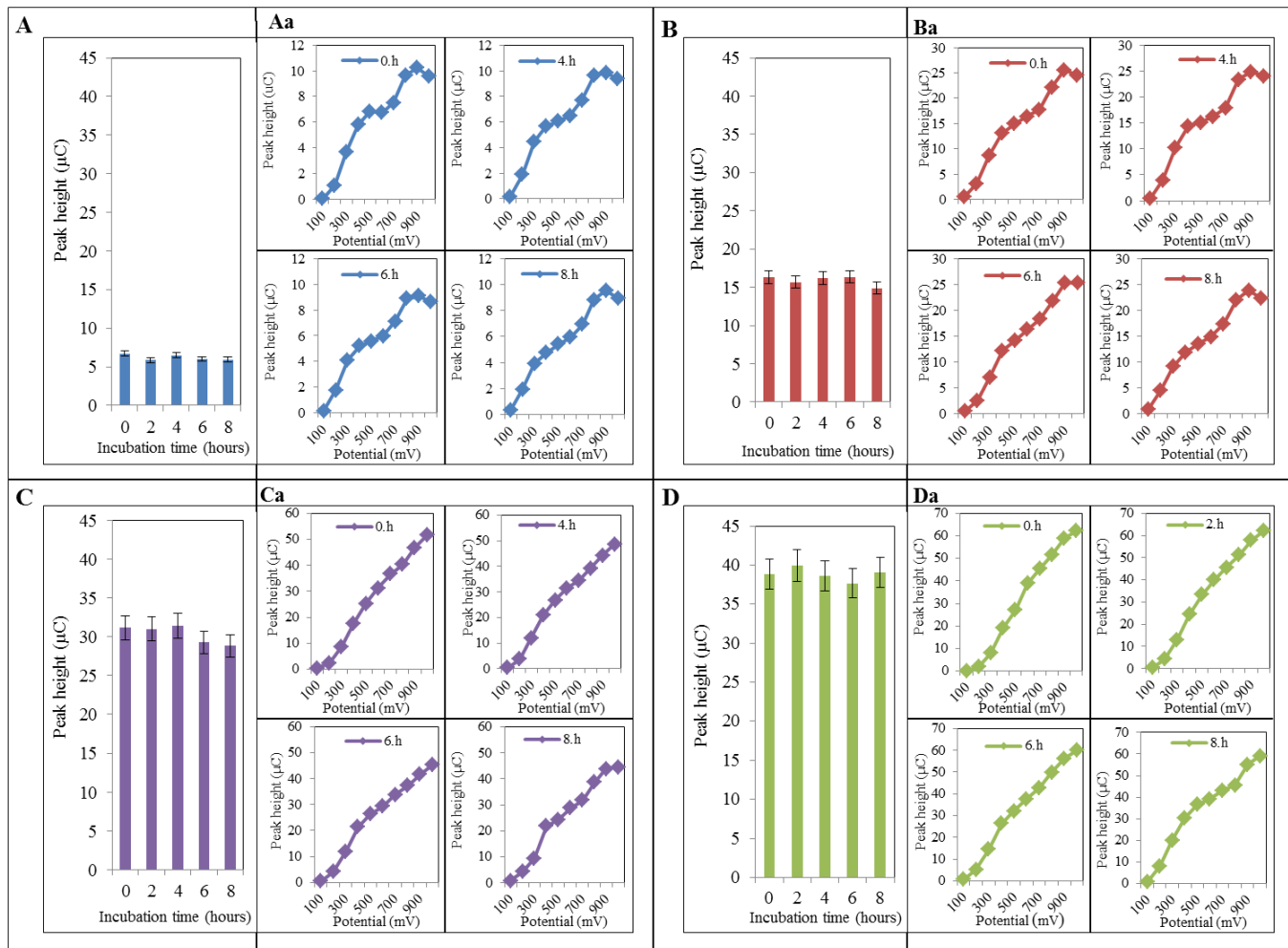


Figure 3. Signal intensities taken from hydrodynamic voltammograms of mixtures of PC2 and CdCl₂ (100 µg/ml) under applied potential 600 mV in time of incubation 0, 2, 4, 6 and 8 hours in various concentration of PC2 as (A) 100 µg/ml, (B) 200 µg/ml, (C) 300 µg/ml and (D) 400 µg/ml. Real HDVs from the analysis of interactions lasted for 0, 4, 6 and 8 hours for each applied concentrations of PC2 as (Aa) 100 µg/ml, (Ba) 200 µg/ml, (Ca) 300 µg/ml and (Da) 400 µg/ml

3.4 Influence of concentration and temperature

We decided to make a graphical expression of the real influence of the time of incubation and applied concentration, which is shown in Fig. 4. For more accurate counting we took an inflection points from the first peak visible in HDV which was appearing near to 500 mV. This approach is in accordance with the method of counting the HDV maxima [25]. Due to counting of inflection points we found that the biggest change is made as creation of disproportion between the peaks in 500 and 900 mV in HDV. In the 900 mV there is maximal response of PC2, which was reported before [26]

and the increasing of signal in lower potentials app. 500 mV was reported to be pointing to the metal-thiol complex formation [24]. Here, this can be considered as the formation of Cd-thiol complex. After making the difference between these two maxima we obtained the figures for temperature influence (Fig. 4A) and for influence of concentration (Fig. 4B). These figures coincidentally reported that due to affecting by selected factor (time, concentration) there is observed the change of the ratio which we expressed as the change of slope and we reported it in Fig. 4C, where it is obvious that concentration has bigger effect on the formation of complex than time of incubation. The biggest change is under applied concentration 400 $\mu\text{g/ml}$ and (Fig. 4A) where the slope is the most decreasing which means the decreasing the amount of PC2 and increasing of amount of complex. Other concentrations has slowly decreasing trend in order to applied concentrations from 100 to 300 $\mu\text{g/ml}$. In the case of the influence of time the difference between 100-300 $\mu\text{g/ml}$ was negligible but 400 $\mu\text{g/ml}$ reported the most decreasing trend after 8 hour of incubation.

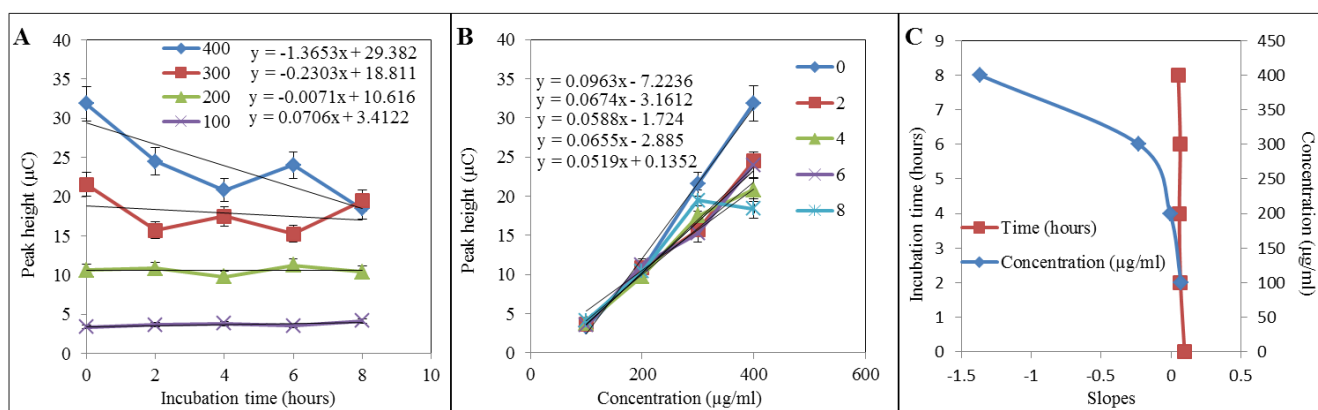


Figure 4. The peak height difference between 500 mV and 900 mV taken from HDV of mixture Cd(II) 100 $\mu\text{g/ml}$ and PC2 in concentration of: 400, 300, 200 a 100 $\mu\text{g/ml}$ with time of interaction 0, 2, 4, 6 a 8 hodini (A) Dependence of difference of the peak height on time. (B) Slopes from dependence of peak height for each variant of time of incubation. (C) Values of the slopes of the peak heights in dependence of the applied concentrations of PC2 and time of interactions

3.5 Study of the optimal conditions for the determination of PC2 and Cd-PC2 complexes in vitro in MALDI-TOF/TOF

We attempted to analyse the same sample mixture, as we used for electrochemical analysis, by mass spectrometer. During optimization we found number of factors as used matrix, on spot sample preparation approach and intensity of laser to be rather influencing of the subsequent MALDI analysis. Our results showed an increase in signal intensity (a.u) as a function of the intensity of the laser (%) in 5, 10 and 15 mg/ml of HCCA (Figs. 5A, B and C). The highest signal intensity (a.u) was obtained by using of concentration of 15 mg/ml HCCA. For the realization of this study, quantification the number of crystals by computer analysis of the matrix pictures was performed and showed that 1 μl of matrix and sample (1:1) had a mean of 3430 ± 80 (SE) crystals. The number of crystals was the highest and more homogeneous under application of 15 mg/ml HCCA than by application of lower concentrations.

Increasing the concentration of PC2 from 3 to 30 pg per crystal, the intensity of signal was increased linearly ($y = 179.04x - 247.11$; $R^2 = 0.9349$) when the laser intensity was 28.8 μJ (30%), while the signal increased exponentially ($y = 678.77e^{0.122x}$; $R^2 = 0.9861$) when the laser intensity was 43.3 μJ (50%), under the same concentrations of PC2 (Figs. 5D and E). These results indicated that the optimum conditions for the determination of PC2 by utilizing HCCA as matrix was a laser intensity of 50% (43.3 μJ) and the concentration of the matrix more convenient for obtaining an appropriate signal was 15 mg/ml in methanol (70%, v/v).

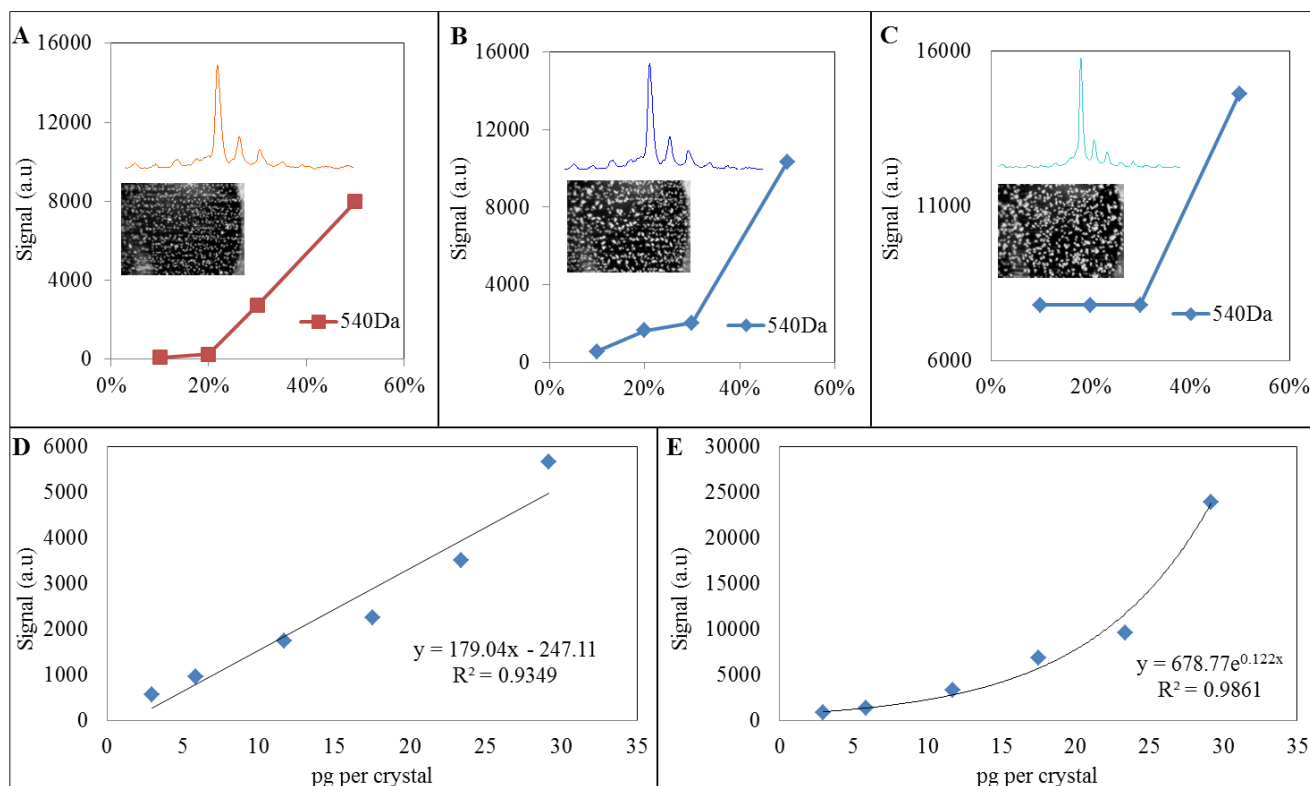


Figure 5. The signal intensity PC2 measured in the presence of different concentrations in cyano-4-hydroxycinnamic acid (HCCA) solution matrix as (A) 5, (B) 10 and (C) 15 mg/ml and different laser powers (%); in inset: spectra of PC2 (540 Da) measured at 50% laser power and the photos of spots in different concentration of HCCA with PC2 (100 ng/ μl in TA30). Dependences of signal intensity for PC2 in different concentrations 10, 20, 30, 60, 80 and 100 ng/ μl in TA30 measured under (D) 30 % and/or (E) 50% laser power.

Figure 6 shows the effect of methanol on PC2 when it used as samples solutions. Using of water and 20% methanol resulted in the spectra showed in Figs. 6A and B. The 70% methanol showed the loss of the signal (Fig. 6C), which interferes in the detection of PC2 signal and inhibits the formation of crystals. This result confirms that methanol at high concentrations (70%) cannot be used as the sample solution, but it is possible to use it in lower concentrations (up to 20%), because the intensity was similar to when the sample was water (Figs. 6A and B). However, the methanol 70% can be used in the HCCA because it facilitated the dilution of the matrix and homogeneous crystal formation and accurate for the determination of PC2 in MALDI-TOF. The spotting methods used for MALDI-TOF were dried droplet (DD), thin layer (TL) and double layer (DL). The quantification of

number of crystals in DD, TL and DL showed an average of 3430 ± 80 , 4600 ± 200 and 4270 ± 70 , respectively. The results showed that the three methods are reproducible for determination of PC2 as in Figs. 7A, B and C. However, DD was showed the highest intensity at the different concentrations of PC2 in both intensities of the laser as 28.8 and 43.3 μJ showed in Figs. 7D and E, respectively. DD spotting method was 64.5% higher than LT and 85 % than DL in 30 pg PC2 per crystal in 43.3 μJ . This method is surprisingly simple and provides good results for many different types of samples. Dried droplets were very stable and can be kept before running a MALDI-TOF experiment.

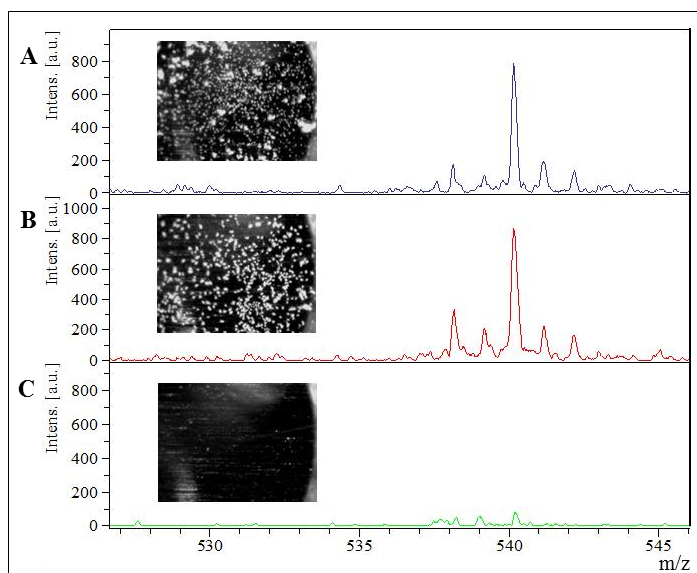


Figure 6. Spectra of 100 ng/ μl PC2 in (A) water, (B) methanol (20%, v/v) in TA30 and (C) methanol (70%, v/v).

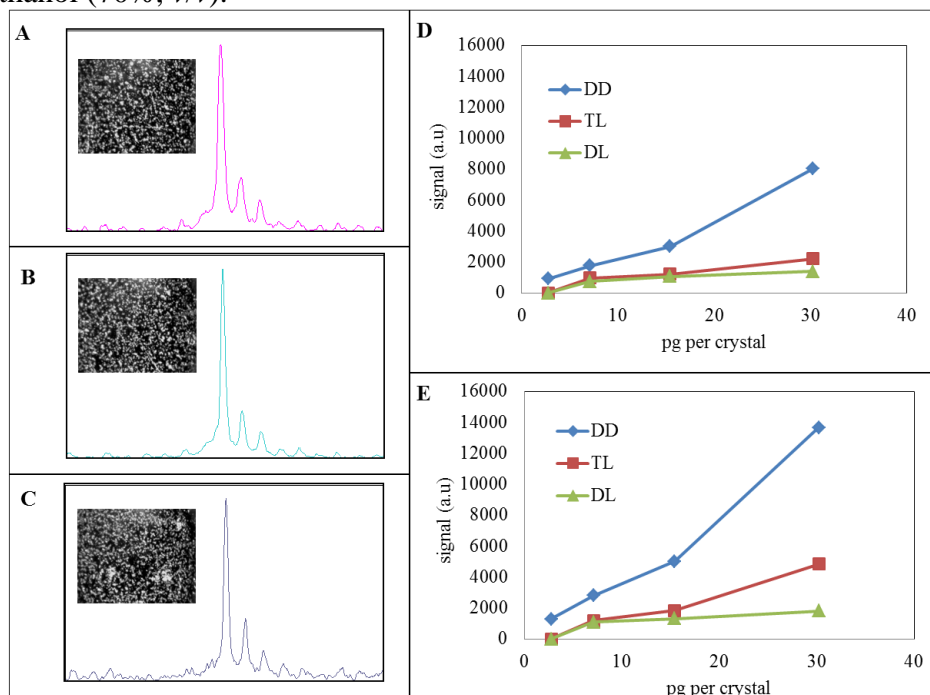


Figure 7. Spectra and crystal photos of different methods preparations (A) DD: Drop Dry, (B) TL: Thin Layer and (C) DL: Double Layer. Dependences of signal intensity of PC2 in different concentrations of PC2 in DD, TL and DL methods under (D) 30% and (E) 50% laser powers.

3.6 Determination of Cd-PC2 complexes *in vitro* in MALDI-TOF/TOF

These ideal conditions were used for determination of Cd-PC2 complex and to verify that Cd-PC2 complexes were formed. The main observed signals shown in Figure 8 were assigned as follows: $[\text{PC2} + \text{H}]^+$ (m/z 540.2), $[\text{M}_3 + \text{H}]^+$ (m/z 568.2) (correspond to matrix cluster trimer) for determination of PC2 (Fig. 8A), while PC2 incubation with Cd(II) (Fig. 8B) showed a new signal corresponding to complexes: $[\text{Cd-PC2} + \text{H}]^+$ (m/z 650.1). It is evident that a suggested intramolecular complex, Cd-PC2, was found. The presence of the observed mass of 650.1 Da confirms the abundance of intramolecular complexes.

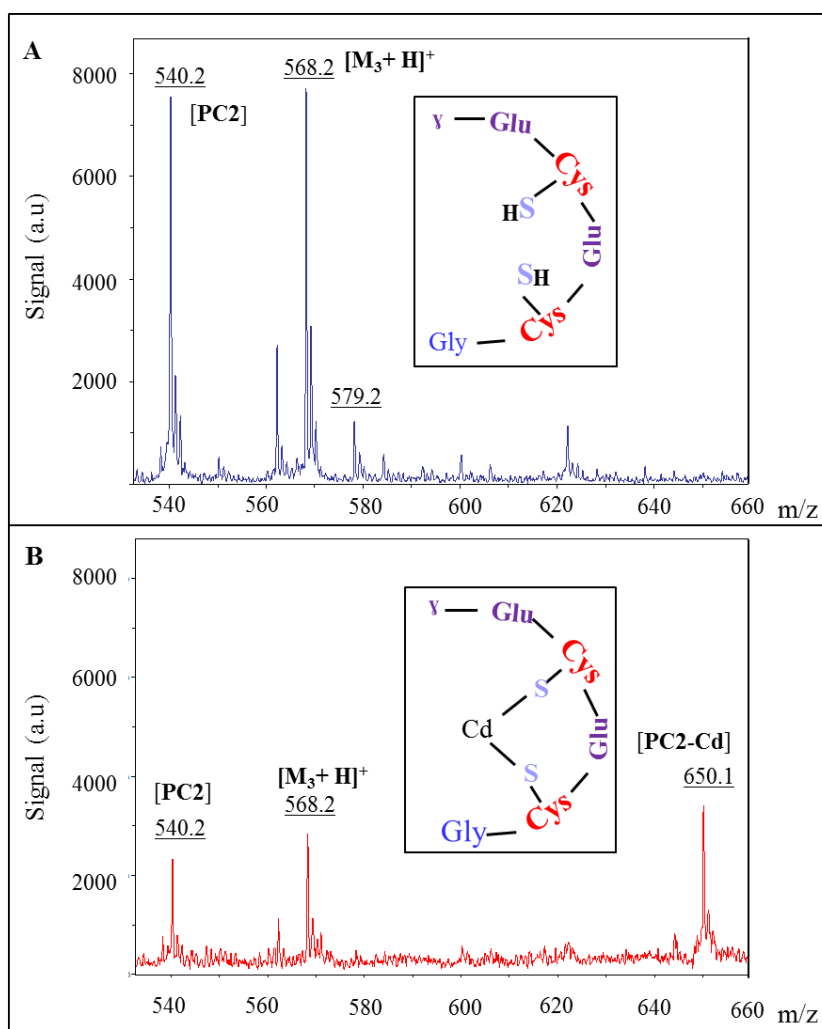


Figure 8. Mass spectra of (A) PC2 and (B) Cd-PC2 complexes in HCCA matrix by MALDI-TOF.

4. CONCLUSIONS

Studying of complexes among proteins and metals are of great interest for numerous branches. In this study, the obtained data show that the flow injection analysis coupled with electrochemical

detection Coulochem III or/and Coularray in combination with MALDI-TOF is a reliable and fast method for the determination of these complexes.

ACKNOWLEDGEMENTS

The work was supported by CEITEC CZ.1.05/1.1.00/02.0068 and MSMT 6215712402.

References

1. T. W. Lane and F. M. M. Morel, *Proc. Natl. Acad. Sci. U. S. A.*, 97 (2000) 4627.
2. J. L. Pan, J. A. Plant, N. Voulvoulis, C. J. Oates and C. Ihlenfeld, *Environ. Geochem. Health*, 32 (2010) 1.
3. G. J. Wagner, *Adv. Agron.*, 51 (1993) 173.
4. C. S. Cobbett, *Plant Physiol.*, 123 (2000) 825.
5. B. Semane, A. Cuypers, K. Smeets, F. Van Belleghem, N. Horemans, H. Schat and J. Vangronsveld, *Physiol. Plant.*, 129 (2007) 519.
6. B. M. Shanmugaraj, H. M. Chandra, B. Srinivasan and S. Ramalingam, *Int. J. Phytoremediat.*, 15 (2013) 206.
7. E. Grill, S. Löffler, E. L. Winnacker and M. H. Zenk, *Proc. Natl. Acad. Sci. U. S. A.*, 86 (1989) 6838.
8. R. Pal and J. P. N. Rai, *Appl. Biochem. Biotechnol.*, 160 (2010) 945.
9. W. E. Rauser, *Plant Physiol.*, 109 (1995) 1141.
10. M. H. Zenk, *Gene*, 179 (1996) 21.
11. D. Potesil, J. Petrlova, V. Adam, J. Vacek, B. Klejdus, J. Zehnalek, L. Trnkova, L. Havel and R. Kizek, *J. Chromatogr. A*, 1084 (2005) 134.
12. P. Babula, V. Adam, R. Opatrilova, J. Zehnalek, L. Havel and R. Kizek, *Environ. Chem. Lett.*, 6 (2008) 189.
13. V. Adam, I. Fabrik, V. Kohoutkova, P. Babula, J. Hubalek, R. Vrba, L. Trnkova and R. Kizek, *Int. J. Electrochem. Sci.*, 5 (2010) 429.
14. 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 and R. Kizek, *Sensors*, 7 (2007) 932.
15. D. Huska, O. Zitka, O. Krystofova, V. Adam, P. Babula, J. Zehnalek, K. Bartusek, M. Beklova, L. Havel and R. Kizek, *Int. J. Electrochem. Sci.*, 5 (2010) 1535.
16. A. Kleckerova, P. Sobrova, O. Krystofova, J. Sochor, O. Zitka, P. Babula, V. Adam, H. Docekalova and R. Kizek, *Int. J. Electrochem. Sci.*, 6 (2011) 6011.
17. E. Grill, E. L. Winnacker and M. H. Zenk, *Method Enzymol.*, 205 (1991) 333.
18. S. Doring, S. Korhammer, M. Oetken and B. Markert, *Fresenius J. Anal. Chem.*, 366 (2000) 316.
19. L. Q. Chen, Y. F. Guo, L. M. Yang and Q. Q. Wang, *J. Anal. At. Spectrom.*, 22 (2007) 1403.
20. T. Y. Yen, J. A. Villa and J. G. DeWitt, *J. Mass Spectrom.*, 34 (1999) 930.
21. E. Bramanti, D. Toncelli, E. Morelli, L. Lampugnani, R. Zamboni, K. E. Miller, J. Zemetra and A. D'Ulivo, *J. Chromatogr. A*, 1133 (2006) 195.
22. I. Leopold, D. Gunther, J. Schmidt and D. Neumann, *Phytochemistry*, 50 (1999) 1323.
23. B. B. M. Sadi, A. P. Vonderheide, J. M. Gong, J. I. Schroeder, J. R. Shann and J. A. Caruso, *J. Chromatogr. B*, 861 (2008) 123.
24. O. Zitka, M. A. Merlos, V. Adam, N. Ferrol, M. Pohanka, J. Hubalek, J. Zehnalek, L. Trnkova and R. Kizek, *J. Hazard. Mater.*, 203 (2012) 257.
25. J. Sochor, J. Dobes, O. Krystofova, B. R. Nedecky, P. Babula, M. Pohanka, T. Jurikova, O. Zitka, V. Adam, B. Klejdus and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2013) 5429.

26. O. Zitka, H. Skutkova, O. Krystofova, P. Sobrova, V. Adam, J. Zehnalek, L. Havel, M. Beklova, J. Hubalek, I. Provaznik and R. Kizek, *Int. J. Electrochem. Sci.*, 6 (2011) 1367.

© 2013 by ESG (www.electrochemsci.org)