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# Electrochemical determination of lead and glutathione in a plant cell culture

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**Abstract**

In this work we established differential pulse anodic stripping voltammetry (DPASV) as the tool for analysis of lead in the plant cell culture. For the cultivation procedure, lead in Pb(II)-ethylenediaminetetraacetic acid (Pb-EDTA) chelate has been used. The detection limit of lead was found at 500 pM in phosphate buffer (pH 5.5), and 100 nM in prepared cells intracellular extract (20 pg Pb(II)/mg cells). For determination of cysteine-rich peptides, voltammetry in differential mode (DPV) in cobalt(III)-containing ammonia buffer (Brdicka reaction) was used. In this short communication, we present suitable voltammetric techniques for the physiological study of lead and thiols in plant cell culture.


*Keywords:* Determination of lead; Pb(II)-EDTA; Voltammetry; Mercury electrode; Brdicka reaction; Glutathione; Metallothioneins

## 1. Introduction

In recent years there has been increasing interest in determining the concentration levels of essential and heavy metals in various biological systems. The determination of metal concentration in biological materials is very important for finding environmental contamination and for studying physiological and biochemical processes in organisms. Essential metals such as zinc, copper, etc., are necessary for many physiological processes and heavy metals (e.g. cadmium, lead) are potentially highly toxic. Plants are able to minimize damage from exposure by elevated levels of essential and nonessential metals by the production of metallothioneins (MTs), glutathione (GSH), and phytochelatins (PCs) [1].

Several methods have been described for lead determination at low concentration, although spectroscopic [2,3], and electrochemical techniques have been the most frequently employed [4–7]. The advantages of voltammetric lead determination are firstly, economic efficiency, secondly, simplicity, and thirdly that the sample is not destroyed, as it is in atomic spectroscopy.

For the analysis of Cys-containing peptides (MTs, PCs, GSH) voltammetric and chronopotentiometric methods (reviewed in Refs. [8,9]), have been used. Electrochemical determination of GSH was suggested by several authors [10–13]. An electrochemical analyser can also be used in connection with liquid chromatography [14]. High sensitivity has been found using electrochemical determination of Cys-containing peptides and proteins based on catalytic processes which proceed at very negative potentials on mercury electrodes. For measurement of these catalytic signals, an adsorptive transfer stripping technique can be used in combination with constant current chronopotentiometric analysis (AdTS CPSA) [15–17]. For the determination of low content of peptides, voltammetry in differential mode (DPV) in a cobalt(III)-containing solution has been used—this technique was first described by Brdicka [18]. Recently our working

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group applied the Brdicka procedure for the sensitive determination of glutathione S-transferase [19].

In this work, we use voltammetry for determination of lead and thiols in the plant cell culture. The intracellular concentration of lead was determined by differential pulse anodic stripping voltammetry (DPASV). The SH-content (thiols) was determined by DPV in connection with the Brdicka procedure. The calibration procedure was performed with different concentrations of a reduced form of glutathione (GSH).

## 2. Experimental

### 2.1. Apparatus

Electrochemical measurements were carried out with an AUTOLAB electrochemical instrument (EcoChemie, Netherlands) in connection with the VA-Stand 663 (Metrohm, Switzerland). A three-electrode system was used including working electrode—HMDE (surface area 0.4 $mm^2$), reference electrode (Ag/AgCl, 3 M KCl), and auxiliary electrode (Pt wire). The raw data were smoothed using level 4 of the Savitzky and Golay filter [20] of the GPES software (EcoChemie). All experiments were carried out at laboratory temperature.

### 2.2. Reagents

All reagents of ACS purity were purchased from Sigma Aldrich (St. Louis, USA). We used 0.1 M phosphate buffer ($NaH_2PO_4 + Na_2HPO_4$), 0.2 M acetate buffer ($CH_3COOH + CH_3COONa$), and Britton–Robinson buffer (0.04 M $H_3PO_4$ + 0.04 M $CH_3COOH$ + 0.04 M $H_3BO_3$ + 0.2 M NaOH) as supporting electrolytes. For deproteinization and neutralization, 37% hydrochloric acid and NaOH were used. Solutions were prepared using deionized ACS water (Sigma). The pH value was adjusted (Pracitronic, MV870, Germany) by mixing buffer components, and the pH was verified before each electrochemical measurement.

### 2.3. Cell culture and treatment

The embryogenic culture of the Norway spruce (*Picea abies* (L.) Karst., clone 2/32) was maintained on modified semisolid "LP" medium (pH 5.7–5.8). Details of cultivation and medium composition are described in Ref. [21]. Plant cells were exposed (48 h) by a complex of lead with ethylenediaminetetraacetic acid (Pb-EDTA). A stock solution of Pb-EDTA was prepared from $Pb(NO_3)_2$ with EDTA in molar ratio 1:1 at 50 °C (1 h).

### 2.4. Sample preparation

At the end of the cultivation process, cells were washed with 1 M EDTA in phosphate buffer (pH 7.0) and centrifuged for 5 min, 3000 × *g* (Eppendorf 5402, USA). Cells (200 mg) denuded of cultivation medium and transferred to test tubes were frozen in liquid nitrogen to destroy the cell wall and cytoplasm membrane. Then the cells were added to 1 ml of phosphate buffer (pH 7.0), homogenated by shaking on vortex (15 min, Scientific Industries, Vortex-2 Genie, USA), and sonicated (5 min, 200 W, Bandelin, Germany). The acquired homogenate was centrifuged (14 000 × *g*, 30 min, 4 °C). For the determination of GSH concentration measurements were performed in supernatant. The supernatant (100 μl) was added to 1 ml of Brdicka supporting electrolyte. In the case of lead determination, supernatant (1 ml) was mixed with HCl (37%) for 30 min. After mixing, the deproteinized solution was neutralized by 13 M NaOH and added to 1 ml of supporting electrolyte (0.1 M phosphate buffer, pH 5.5).

### 2.5. DPASV measurement of lead

Samples were deoxygenated for 2 min by purging with water-saturated argon (99.999%) prior to measurements. The lead was deposited at HMDE at a potential of − 0.6 V with time accumulation 60 s. During deposition the process solution was stirred (1450 rpm). The anodic scan was initialized at − 0.6 V and stopped at 0 V. The step potential was 5 mV, modulation amplitude 50 mV, and time interval 0.24 s.

### 2.6. DPV measurement by Brdicka procedure

The supporting electrolyte (1 M $NH_4Cl$ + 1 M $NH_4OH$ + 0.6 mM $Co(NH_3)_6Cl_3$, pH 9.5) was prepared from ACS Chemicals (Sigma). The measurement was performed with a negative scan from − 0.9 to − 1.7 V with the following settings: step potential 2.5 mV, pulse amplitude 25 mV, time interval 0.5 s. To keep the concentration of the ammonia buffer constant, the supporting electrolyte was not deoxygenated.

## 3. Results and discussion

### 3.1. Determination of lead

Several analytical methods are suitable for the detection of lead in plant material. Taking into account speed, sensitivity, cost and time-consuming measurement, we can claim that DPASV is the most applicable method for lead detection. For determination of lead concentration in the plant cell culture treated by Pb-EDTA chelate [22] (Fig. 1A), we used DPASV in connection with HMDE. It is known that lead applied in inorganic form (nitrate, chloride, etc.) is precipitated by components present in "LP" cultivation medium. For this reason a Pb-EDTA chelate is necessary for experiments dealing with plant cell culture. Meagher [23] published that EDTA added to Pb(II)

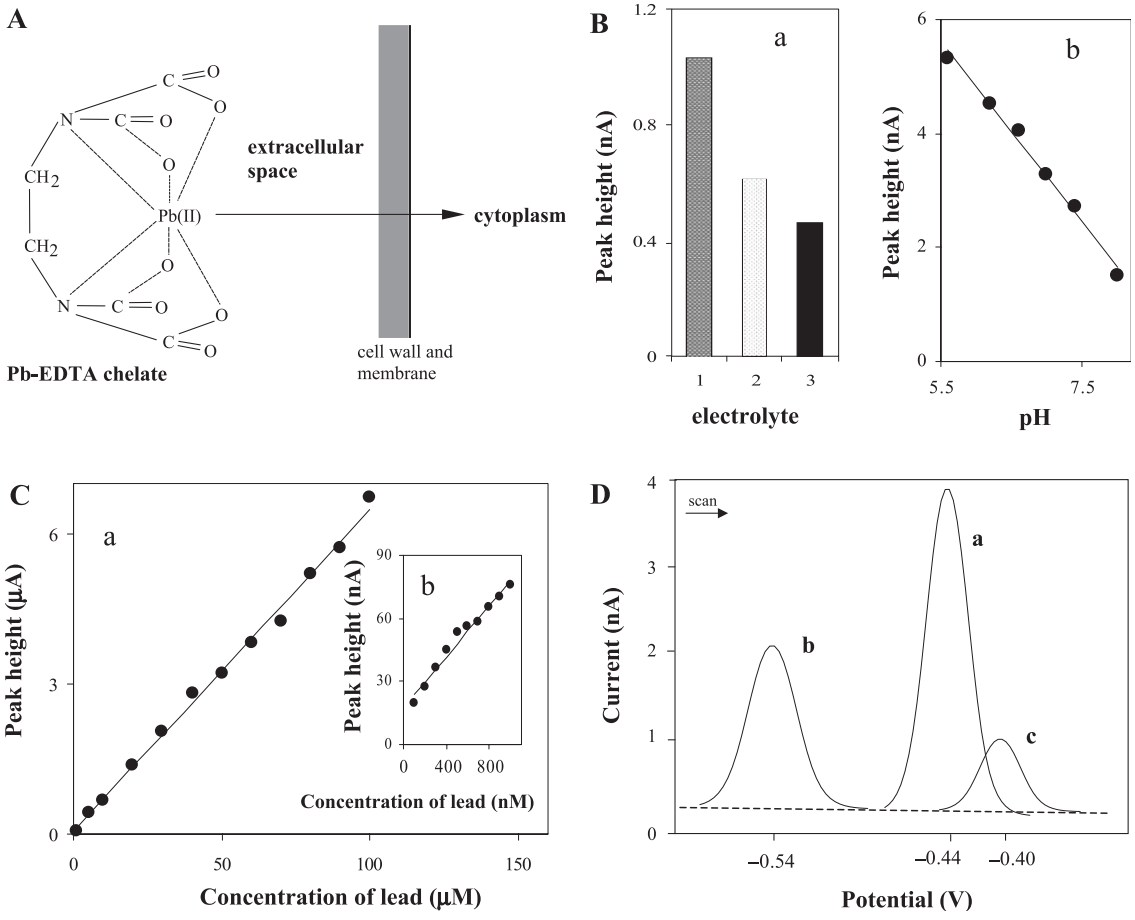


Fig. 1. (A) Scheme of lead(II) ethylenediaminetetraacetic acid (Pb-EDTA) chelate, and its transport from extracellular space to cytoplasm. (B) Plot of (a): Peak heights of 1 nM $Pb(NO_3)_2$ in different supporting electrolytes: 1—phosphate buffer (pH 7.0); 2—acetate buffer (pH 5.0); 3—Britton–Robinson buffer (pH 3.0). Plot of (b): dependence of 10 nM $Pb(NO_3)_2$ peak height on pH of 0.1 M phosphate buffer. (C) Calibration curves of lead in intracellular extract prepared from Pb(II)-untreated spruce cells. Calibration plots were measured by standard addition of $Pb(NO_3)_2$ in concentration range: (a) 1 to 100 µM, and (b) 100 nM to 1 µM. (D) DPAS-voltammograms of: (a) 10 nM $Pb(NO_3)_2$ detected in the 0.1 M phosphate buffer (pH 5.5); (b) Pb(II)-ions measured in the intracellular extract prepared from spruce cells treated 48 h by 500 µM of Pb-EDTA chelate; and (c) 100 µM $Pb(NO_3)_2$ added to "LP" cultivation medium (without agar). Dotted line—supporting electrolyte. For sample preparation and DPASV parameters see Experimental.

contaminated soils gave rise to a more than 100-fold increase in the uptake and transport of the Pb-EDTA into plants. Starting our experiments we optimized DPASV measurements in different supporting electrolytes (Fig. 1B(a)). Changing the pH of the phosphate buffer, we found the best current response of 10 nM $Pb(NO_3)_2$ at pH 5.5 (Fig. 1B(b)). At this medium, the lead concentration dependence on anodic current was performed with a detection limit of 500 pM of Pb(II) (not shown). Under more complex conditions, e.g. in cells that were not treated by Pb-EDTA (see Experimental) we obtained a calibration curve in the range 1–100 µM Pb (Fig. 1C(a)) with $R^2 = 0.9959$ and 100–1000 nM Pb (inset in Fig. 1C) with $R^2 = 0.9726$, with higher detection limit (100 nM Pb(II), 20 pg Pb(II)/mg cells). The DPASV can be used for determination of lead in modified "LP" cultivation medium (see Experimental), where we obtained calibration dependence in the Pb(II) concentration range of 100–1000 nM in limit detection 100 nM Pb (not shown). The stripping peak of lead appeared at about −0.44 V in the phosphate buffer (pH 5.5), in the sample of plant cells at −0.54 V, and in the cultivation medium at potential −0.40 V (Fig. 1D).

### 3.2. Determination of thiols

The determination of substances containing –SH groups was provided by a modified Brdicka procedure [19,24] for analysis in plant cells. The Brdicka procedure can be performed in a supernatant which is either heat-treated (not shown) or in native stage. In the case of thermostable peptides and proteins (including MTs), a heat treatment is usually included in the purification procedure [9,15,25]. A heat-treatment before analysis is also important due to fact that the Brdicka procedure is not specific to certain types of peptides and proteins containing –SH groups. The DP-voltammogram of 30 µM GSH (L-γ-glutamyl-L-cysteinyl-glycine; formula is in Fig. 2B) is shown in Fig. 2A. We can

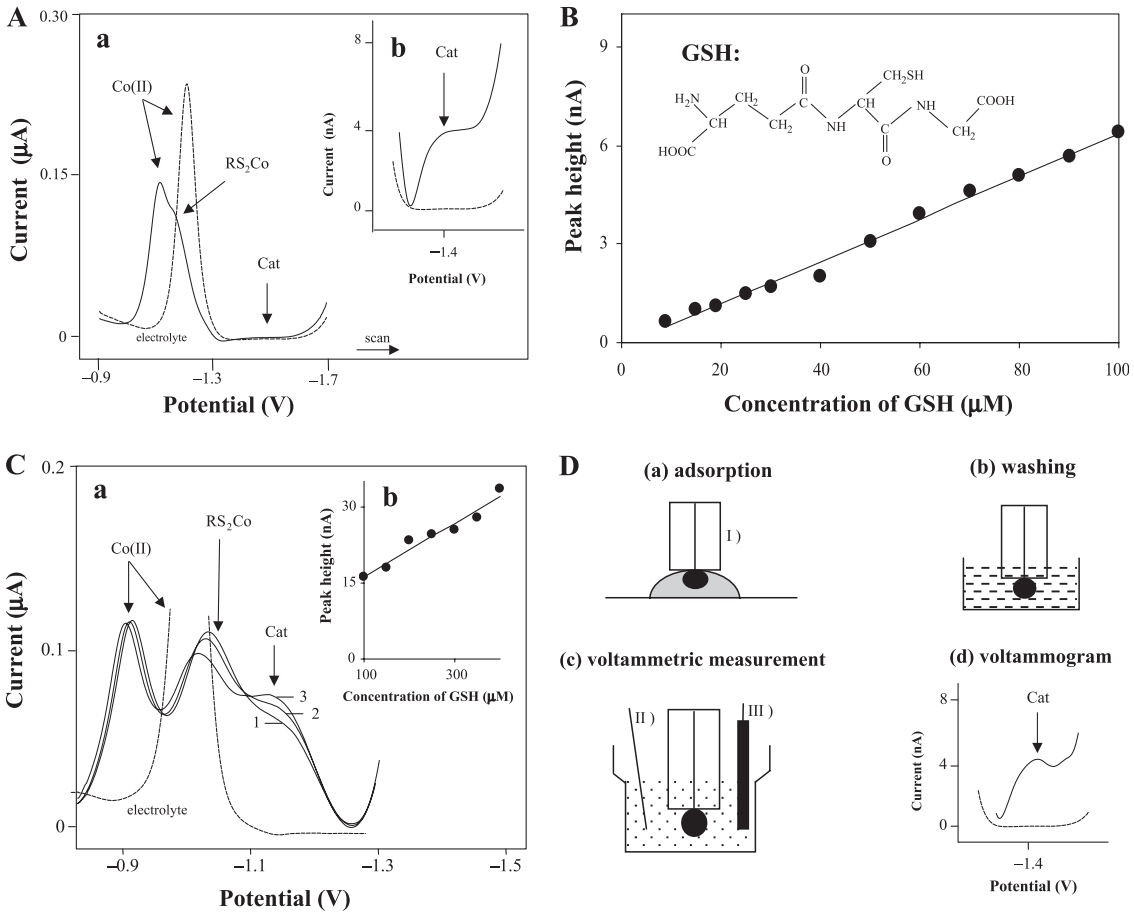


Fig. 2. (A) Voltammetric curves of 30 μM glutathione (GSH) recorded by differential pulse technique at HMDE. Plot (b) is inset of catalytic peak (Cat) from plot (a). (B) The formula and calibration plot of GSH in the concentration range 10–100 μM measured in 1 ml of Brdicka solution (1 M $NH_4Cl$ + 1 M $NH_4OH$ + 0.6 mM $Co(NH_3)_6Cl_3$, pH 9.5). (C) Voltammograms recorded with 100 μl of intracellular extract prepared from spruce Pb(II)-untreated cells added to 1 ml of Brdicka solution, after the addition of (1) 100, (2) 200, 3 (400) μM GSH. Plot b is the dependence of current response on the concentration of GSH (100–400 μM) which was added to spruce intracellular extract. (D) Scheme of experimental procedure of adsorption transfer stripping technique in combination with voltammetric measurement. (a) A drop of 100 μM GSH in ammonia buffer was adsorbed ($t_A$ = 60 s) onto HMDE; (b) the mercury drop was washed in water; (c) the measurement was performed in Brdicka solution (1 ml); and (d) resulting DP-voltammogram. Explanation: Cat—catalytic signal; Co(II)—reduction peak of cobalt; $RS_2Co$—complex GSH with Brdicka reagent; (I) HMDE, (II) auxiliary electrode, (III) reference electrode. For parameters of voltammetric measurement and sample preparation, see Experimental.

observe three electrochemical responses: reduction peak of Co(II) (about − 1.1 V), peak of $RS_2Co$ complex (about − 1.15 V), and catalytic peak Cat (about − 1.4 V) (Fig. 2A). In plants the concentration of GSH is thought to be between 3 and 10 mM, and is present in the major cellular compartments [26]. The calibration curve of GSH was measured as the dependence of peak height (Cat) on a concentration of GSH (Fig. 2B). We found the linear response at a range from 10 to 100 μM GSH with the $R^2$ value 0.9926 (Fig. 2B). In Fig. 2C current–potential curves are shown as measured in Pb(II)-untreated intracellular extract of spruce cells after the addition of different concentrations of GSH. It is obvious that the peak height of Cat signal proportionally increased with an increasing concentration of GSH. Recently, we showed that rabbit liver metallothionein ($M_r$ 6 kDa) [16] and glutathione S-transferase ($M_r$ 25 kDa) [19] can be determined by means of the adsorptive transfer stripping technique. This approach we applied to the determination of the small tripeptide of GSH ($M_r$ 0.3 kDa) and we found that GSH is adsorbed at HMDE and can be transferred to a supporting electrolyte where a DPV curve was obtained (Fig. 2D). We suppose that pulse voltammetric method in connection with the Brdicka procedure can be applied for the determination of thiols and/or generally cys-containing peptides and proteins in plant cell culture. More detailed studies will be published elsewhere.

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