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Electrochemical determination of Ag-ions in environment waters and their action on plant embryos

Radka Mikelova [a,b], Jiri Baloun [c], Jitka Petrlova [a], Vojtech Adam [a,d], Ladislav Havel [c], Jiri Petrek [c], Ales Horna [e], Rene Kizek [a,*]

[a] Department of Chemistry and Biochemistry, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-613 00 Brno, Czech Republic
[b] Department of Theoretical and Physical Chemistry, Faculty of Science, Masaryk University, Kotlarska 37, CZ-611 37 Brno, Czech Republic
[c] Department of Plant Biology, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-613 00 Brno, Czech Republic
[d] Department of Analytical Chemistry, Faculty of Science, Masaryk University, Kotlarska 37, CZ-611 37 Brno, Czech Republic
[e] Department of Food Engineering and Chemistry, Faculty of Technology, Tomas Bata University, CZ-762 72 Zlin, Czech Republic

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Abstract

We utilized liquid chromatography coupled with electrochemical detector (HPLC–ED) for analyzing of silver ions. The optimization of basic chromatographic parameters has been done. The detection limit (3 S/N) obtained were 20 nmol/dm^3. Influence of different interferences (anions and cations) on current response of silver ions has been described. Moreover, we used HPLC–ED to analyze waters of different purity including photographic emulsion, which naturally contained silver ions. We found out that content of silver ions in the emulsion was 1.57×0.03 mmol/dm^3. Moreover, we investigated influence of silver ions on early somatic embryos of Blue Spruce. We were interested in the issue how much silver ions can embryos uptake during four days long treatment. For this purpose, we used optimized HPLC–ED technique. The content increased with increasing treatment time and applied concentration. We also studied how silver ions can influence thiols content in the treated embryos. For these purposes we used adsorptive transfer stripping voltammetry in connection with differential pulse voltammetry — Brdicka reaction. It clearly follows from the obtained results that content of thiols increased with increasing treatment time and applied concentration.

Keywords: Glassy carbon electrode; Carbon paste electrode; Silver ions; Somatic embryos; Liquid chromatography; Brdicka reaction

1. Introduction

The impacts of silver ions in the environment have been intensively studying. The existence of International researches organisation such as Silver Coalition (1991–1995) and The Silver Council (from 1996 up to present; http://www.silvercouncil.org/html/default.htm) proves this fact. These organisations also establish International Conference on Transport, Fate and Effects of Silver in the Environment [1].

Silvers ions come to the environment, first of all, from industry [2]. The highest amounts of silver ions have been used in photographical industry (about 40% from the worldwide usage), following electrochemistry, medicine and others. Because silver is both a valuable resource and a danger waste, most processing facilities recover it using various techniques and equipment. Combinations of these techniques can recover between 90 and 99% of the silver on site. Silver that remains in solution is discharged in the wastewater from the facility [3,4]. In water silver (I) exists as various inorganic and organic compounds. Most of these are nonsoluble, but hydrated silver ions, Ag^+, may be also present in surface waters due to competing equilibria and kinetics, which, in turn, are dependent on the conditions of the water such as pH, hardness and many others. This concern relates to the fact that Ag^+ has been shown to be highly toxic to aquatic life, while other species of silver(I) are much less toxic [5–12]. On the other hand, the mechanism of the toxic effect is still unclear in spite of that Ag(I) has been used for centuries as an antimicrobial agent that could affect more than 650 microbes.

* Corresponding author. Fax: +420 5 4521 1128.
E-mail address: kizek@sci.muni.cz (R. Kizek).

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The commonly used techniques for the silver ions determination in biological samples are atomic absorption spectrometry, inductively coupled plasma mass spectrometry, stripping voltammetry and others [4,13–17]. An exception to this is the use of potentiometry or ion selective electrodes [18–22]. A very sensitive technique for determination of silver ions based on PVC-membrane ion-selective bulk optode has been published [23]. Besides that, there have been shown number of different modifications working electrode to determine silver ions sensitively [24–32].

In this work, we utilized flow and stationary electrochemical techniques for determining of silver ions and for observing their influence on early somatic embryos of Blue Spruce. Primarily we focused on optimization of determination of silver ions by flow injection analysis with electrochemical detection, which has not been used for this purpose yet. After that we adopted the optimized technique on determination of silver ions in different waters and spruce embryos treated by 0, 250, 500 and 1000 μmol/dm^3 $AgNO_3$ for four days, where we also determined content of thiols.

2. Experimental

2.1. Chemicals

Reduced glutathione (GSH), *S*-nitrosoglutathione (GSNO), silver nitrate and sodium acetate were purchased from Sigma Aldrich (St. Louis, USA). Phytochelatin (γ-Glu-Cys)$_2$-Gly (PC_2) was synthesized in Clonestar Biotech; purity over 90% (Brno, Czech Republic). Acetic acid was purchased from Fluka (USA). All reagents used were ACS purity. Stock standard solutions were prepared by ACS water (Sigma-Aldrich, USA) and stored in the dark at the temperature of −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, Mass., USA) prior to HPLC analysis. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by the personal computer program (MultiLab Pilot; Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14/3 mol/dm^3 KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany).

2.2. Stationary electrochemical measurement

Electrochemical measurements were performed using an AUTOLAB analyser (EcoChemie, The Netherlands). The three-electrode system consisted of the carbon paste working electrode, an Ag/AgCl/3 mol/dm^3 KCl reference electrode and a carbon counter electrode. The differential pulse voltammetry parameters were as follows: initial potential −0.4 V, end potential 0.8 V, modulation amplitude 25 mV and step potential 0.5 mV. All experiments were carried out at laboratory temperature. Acetate buffer (0.2 mol/dm^3, pH 5.0) was used as the supporting electrolyte. The raw data were treated using the Savitzky and Golay filter (level 2) and the moving average baseline correction (peak width 0.03) of the GPES software. The carbon paste was made of 70% graphite powder (Aldrich, USA) and 30% mineral oil (Sigma-Aldrich, USA; free of DNase, RNase and protease). The carbon paste was housed in a Teflon body having a 2.5 mm diameter of active disk surface. The electrode surface was polished before each determination with a soft filter paper prior to measurement; for more details see our previous published papers [33,34].

2.2.1. Adsorptive transfer stripping technique (AdTS) coupled with differential pulse voltammetry (DPV) Brdicka reaction

Electrochemical measurements were performed using an AUTOLAB analyser (EcoChemie, The Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The three-electrode system consisted of hanging mercury drop electrode as working electrode, an Ag/AgCl/3 mol/dm^3 KCl reference electrode and a carbon counter electrode. The Brdicka supporting electrolyte containing 1 mmol/dm^3 $Co(NH_3)_6Cl_3$ and 1 mol/dm^3 ammonia buffer (NH_3(aq) + NH_4Cl, pH = 9.6) was used; surface-active agent was not added. AdTS DPV Brdicka reaction parameters were as follows: an initial potential of −0.6 V, an end potential of −1.6 V, a modulation time of 0.057 s, a time interval of 0.2 s, a step potential of 1.05 mV/s, and a modulation amplitude of 250 mV, E_{ads} = 0 V. Temperature of supporting electrolyte was 0 °C [35].

2.3. Flow electrochemical measurement

An FIA–ED and/or HPLC–ED system consisted of solvent delivery pump operating in range of 0.001–9.999 mL/min (Model 583 ESA Inc., Chelmsford, MA, USA), a guard cell (Model 5020 ESA, USA), a reaction coil (1 m) and/or a chromatographic column (Polaris C18-A, 4.6 mm, 5 μm particle size), and an electrochemical detector. The electrochemical detector (ED) includes one low volume flow-through analytical cells (Model 5040, ESA, USA), which is consisted of glassy carbon working electrode, palladium electrode as reference electrode and auxiliary carbon electrode, and Coulochem III as a control module. The sample (5 μL) was injected manually. The obtained data were treated by CSW 32 software. The experiments were carried out at room temperature. Guard cell potential was 0 V.

A glassy carbon electrode was polished mechanically by 0.1 μm of alumina (ESA Inc., USA) and sonicated at the laboratory temperature for 5 min using a Sonorex Digital 10 P Sonicator (Bandelin, Berlin, Germany) at 40 W.

2.4. Used water

Different water samples (ACS water, Milli Q water — 18 MΩ, Distilled water — Aqua osmotic system 1 μS, Tap water from the city of Brno, Ponávka stream, Puddle from the city of Brno) were filtered through a 0.45 μm Nylon filter discs (Millipore, Billerica, Mass., USA) prior to HPLC analysis. Photographic emulsion was bought in Tesco store.

2.5. Plant material and cultivation conditions

Early somatic embryos (ESEs) clone of the Blue Spruce (*Picea pungens* Engelm.) designated as PE 14 were used in our

experiments. The cultures were maintained on a semisolid (Gelrite Gellan Gum, Merck, Germany) half-strength LP medium [36] with modifications [37]. The concentration of 2,4-dichlorofenoxyacetic acid and N^6-benzyladenine was 4.4 and 9 μmol/dm^3, respectively. The pH value was adjusted to 5.7–5.8 before autoclaving (121 °C, 100 kPa, 20 min). The organic part of the medium, excluding saccharose, was sterilized by filtration through a 0.2 μm polyethylensulfone membrane (Whatman, Puradisc 25 AS). Ten ESEs clusters were cultivated in one plastic Petri dish (100 mm in diameter) containing 30 mL of the medium. Sub-cultivation of stock cultures was made at 2-week intervals. The stock and experimental cultures were maintained in a cultivation box in the dark at a temperature of 23±2 °C.

2.6. Ag–EDTA LP modified medium

Cultivation medium was modified with an addition of silver chelate (Ag–EDTA) in concentrations of 0, 250, 500 and 1000 μmol/dm^3. A stock solution of Ag–EDTA was prepared by mixing AgNO3 with ethylene diamine tetra-acetic acid (EDTA) in a 1:1 molar ratio and stirred at 50 °C for 1 h. The filter-sterilized Ag–EDTA complex was added to the autoclaved culture medium.

2.7. Sample preparation

2.7.1. Preparation of ESEs samples for silver determination

Samples (ESEs) were washed with 1 mol/dm^3 EDTA in 0.2 mol/dm^3 phosphate buffer (pH 7.0) to remove their cultivation media, and centrifuged for 5 min at 3000 ×*g* (Eppendorf 5402, USA). Then they were transferred to a test tube and frozen by immersion in liquid nitrogen to disrupt the ESE. Due to disrupting of the cell membranes the cytoplasm has been obtained. The cytoplasm was added to 1 mL of 0.2 mol/dm^3 phosphate buffer (pH 7.0), homogenised by vortexing for 15 min (Scientific Industries, Vortex-2 Genie, USA), and finally sonicated for 5 min at 200 W (Bandelin, Germany). The homogenate was then centrifuged at 14,000 ×*g*, 30 min at 4 °C (Eppendorf 5402, USA). The supernatant (1 mL) was mixed with HCl (35%) for 30 min. After mixing, the deproteinized solution was neutralized by 13 mol/dm^3 NaOH. The samples were filtered through a 0.45 μm Nylon filter discs (Millipore, Billerica, Mass., USA) prior to HPLC analysis.

2.7.2. Preparation of ESEs samples for thiols determination

ESEs were washed with 1 mol/dm^3 EDTA in 0.2 mol/dm^3 phosphate buffer (pH 7.0) and centrifuged for 5 min at 3000 ×*g* (Eppendorf 5402, USA). ESEs (about 20 mg) washed of their cultivation media were frozen three times in liquid nitrogen to disrupt the cells and then transferred to a test tube. The frozen samples were homogenised by vortexing on a Vortex-2 Genie for 5 min at 4 °C (Scientific Industries, USA) and sonicated using a Bandelin Sonopuls HD 2070 for 10 s at 7 W (Germany). The homogenate was centrifuged (14 000 ×*g*) for 15 min at 4 °C using a Universal 32 R centrifuge (Hettich-Zentrifugen, Germany). The supernatant was filtered through a 0.45 μm Nylon filter discs (Millipore, Billerica, Mass., USA) before electrochemical analysis using stationary electrochemical system.

2.8. Computer image analysis

We used a charge-coupled device (CCD) camera for observation of growth of spruce ESEs culture. The images of ESEs clusters were recorded at the beginning of the cultivation and in certain intervals according to the end of the cultivation. The data were converted to digital image in the Grab-IT (version 1.3) program. The area size of ESEs clusters in digital images was calculated by program Image-Pro Plus, (Sony, ver. 1.3). The data were processed in Microsoft Excel, version 2000 (Microsoft, Silicon Valley, USA).

2.9. Accuracy, precision and recovery

Accuracy, precision and recovery of silver ions were evaluated with filtrate (different water samples) and/or homogenate (plant embryos) spiked with standard. Before filtration and/or extraction it was carried out, that 100 μL silver ions standard and 100 μL water were added to water samples. Filtrates and/or homogenates were assayed blindly and silver ion concentration was derived from the calibration curve. Accuracy was evaluated by comparing the estimated concentration with the known concentrations of silver ions. Calculation of accuracy (%Bias), precision (%C.V.) and recovery was expressed according to [38–40].

2.10. Statistical analysis

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

3. Results and discussion

3.1. Stationary electrochemical system

A few authors described using glassy carbon electrode as working electrode due to determination of silver, but carbon paste electrode (CPE) has not been used yet. Thus, we utilized differential pulse voltammetry and CPE for silver ion determination. Electrochemical measurements were performed in the presence of 0.2 mol/dm^3 acetate buffer (pH 5.0) at room temperature. The obtained voltammograms, which are shown in Fig. 1A, were well reproducible (R.S.D. about 5%). We observed two signals called *peak 1* about 160 mV and *peak 2* about 190 mV (Fig. 1A). Due to obtaining higher current response and better sensitivity we selected *peak 2* instead of *peak 1* for constructing a calibration curve. The curve obtained in the range from 0 to 400 μmol/dm^3 of silver ions is shown in Fig. 1B. The height of *peak 2* was proportional to concentration of silver ions up to 300 μmol/dm^3, and then did not change much. This phenomenon probably relates with the saturation of

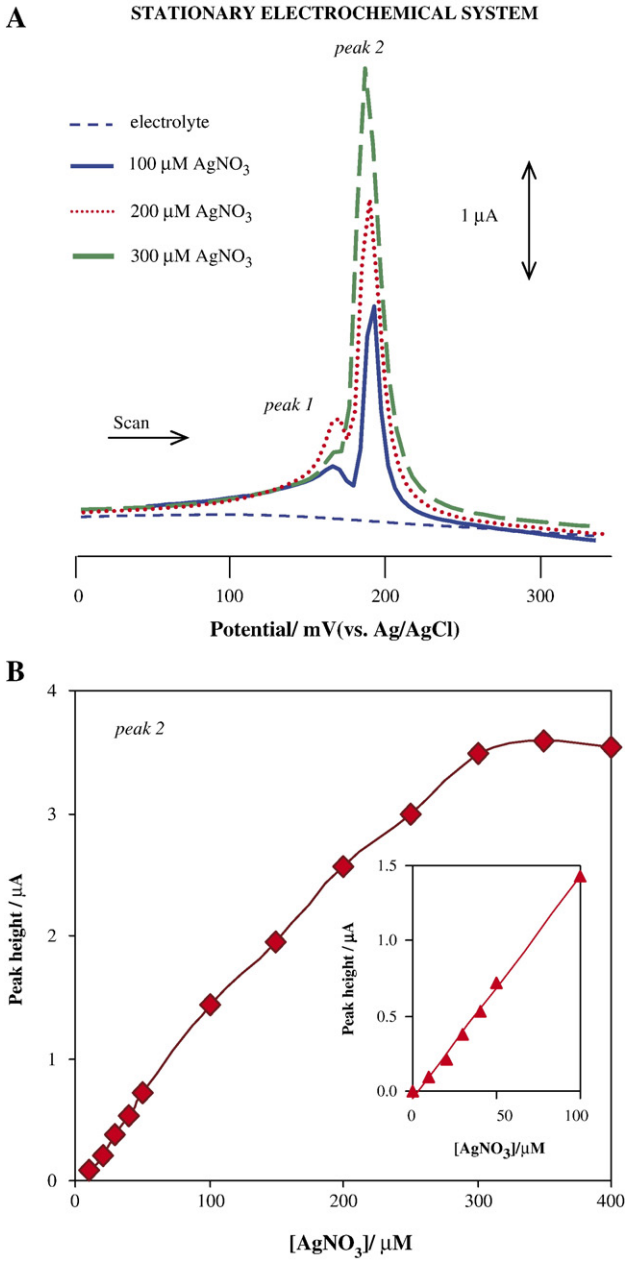

Fig. 1. Stationary electrochemical system. (A) DPV voltammograms of $AgNO_3$ measured on the surface of CPE, dashed line — supporting electrolyte (0.2 mol/dm^3 acetate buffer, pH 5.0). (B) Dependence of current response on silver ion concentrations (0–400 μmol/dm^3; in inset 0–100 μmol/dm^3). The differential pulse voltammetry parameters were as follows: initial potential −0.4 V, end potential 0.8 V, modulation amplitude 25 mV, step potential 0.5 mV and supporting electrolyte −0.2 mol/dm^3 acetate buffer (pH 5.0). All experiments were carried out at laboratory temperature.

the electrode surface. In addition, we obtained strictly linear calibration curve (y(peak height/μA) = 0.0147 × (silver concentration/μM) − 0.0448, R^2 = 0.997) in the range from 0 to 100 μM silver ions (inset in Fig. 1B). The detection limit obtained was 0.5 μmol/dm^3 (n = 3, R.S.D. 6.5%). In addition, the reproducibility of the results obtained with several carbon paste electrodes was about 95 ± 4% according to paste used for the filling the body of the electrode.

3.2. Flow injection analysis coupled with electrochemical detector

3.2.1. Hydrodynamic voltammogram

After that we have studied basic behaviour of silver ions on the surface of CPE by stationary electrochemical system, we were interested in the issue on how silver ions behave on the surface of glassy carbon electrode coupled with flow injection analysis (FIA–ED, for more details, please, see Fig. 2 and/or Experimental section). Primarily we studied influence of different potentials on current response of silver ions in the presence of 0.2 mol/dm^3 acetate buffer (pH 5.0) as mobile phase. The obtained hydrodynamic voltammogram (HDV) is shown in Fig. 3A. If we determined silver ions on the surface of CPE by DPV, we obtained two peaks called *peak 1* about 160 mV and *peak 2* about 190 mV (Fig. 1). Thus, we expected the same behaviour in this case. We observed two peaks on the HDV obtained; first peak at potential of 120 mV and second one at 200 mV. The shift of the peak potentials in comparison with Stationary electrochemical measurements is due to using of different working electrode. In the following experiments we applied 200 mV as the most suitable potential for determination of silver ions by flow injection analysis coupled with glassy carbon electrode.

3.2.2. Effect of current R, flow rate, composition and pH of mobile phase, and time filter

In addition, we explored the effect of the current R on height of peak of 250 μmol/dm^3 $AgNO_3$. The obtained chromatograms are shown in Fig. 3Ba. The height of silver ions peak decreased steeply with the increasing current R (Fig. 3Ba,b). If we applied current 10 μA, silver ions peak height decreased about 80% in comparison with ones obtained at 1 μA. Thus, we choose current 1 μA as the most suitable due to highest current response obtained and well reproducibility with R.S.D. 1% (Fig. 3Bb).

Composition of mobile phase and its flow rate is one of the most important parameters of flow analysis with electrochemical detection which can influence a height of response of compounds of interest. Thus, we changed composition of the mobile phase (Britton Robinson (BR) buffer, Acetate buffer, Phosphate buffer and Borate buffer), and we found out that the highest response of silver ions was obtained in the presence of acetate buffer (inset in Fig. 3C). As we mentioned, we also attempted to study the influence of flow rate (from 0.1 to 1.6 mL/min) of the mobile phase on a current response of silver ions. A height of silver ions signal increased up to flow rate of 0.5 mL/min, and then did not change much (Fig. 3C). We also tested different pH of acetate buffer and their influence on current response of silver ions (Fig. 4A). The highest current response of silver ions was observed at pH 4.0. The response markedly decreased with increasing pH (decrease more than 30% at pH 5.5 in comparison with the highest response). In addition, data obtained from our flow analyser could be treated by time filter. If we treated data obtained by time filter, we found out that time filter of 2 s is the most suitable for determination of silver ions (Fig. 4B).

It clearly follows from the obtained results that the most suitable parameters for the determination of silver ions were as

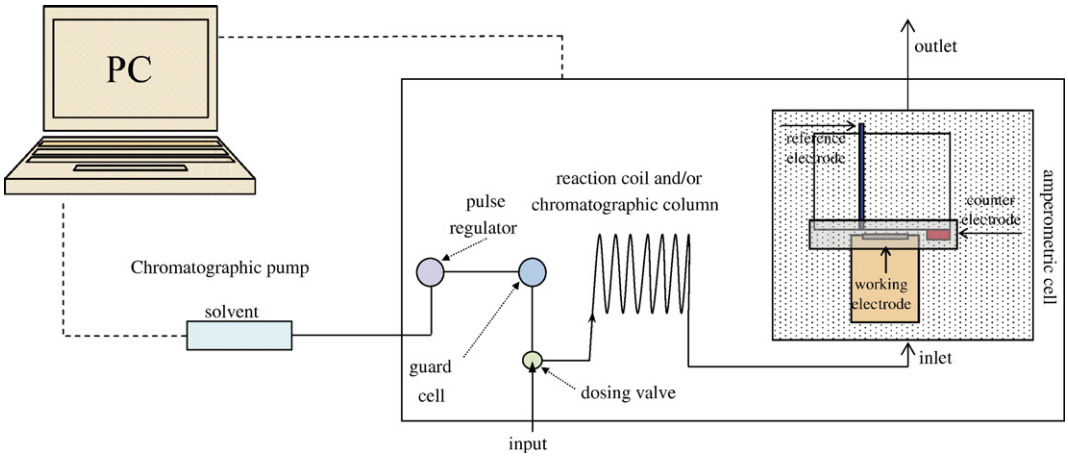

Fig. 2. Scheme of flow injection (FIA) and/or HPLC system with amperometric detection.

follows — flow rate: 0.5 mL/min, guard cell potential: 0 mV, working electrode potential: 200 mV, current *R*: 1 μA, time filter: 2 s. The optimal mobile phase was 0.2 mol/dm^3 acetate buffer (pH 4.0).

3.2.3. *Influence of methanol and silver ions concentration*

We have previously published that organic part of mobile phase (e.g. methanol, acetonitrile) negatively influence analysis of compounds of interest [33,40–45], but is needed, if we apply the optimized technique on analysis of real samples. Here, we investigated the influence of methanol content in mobile phase on silver ions current response. Methanol content up to 10% (*v*/*v*) did not influence the response much, and then the response decreased markedly (Fig. 4C). Thus, we choose mobile phase containing methanol and 0.2 mol/dm^3 acetate buffer pH 4.0 (10:90, *v*/*v*) as the most suitable mobile phase for determination of silver ions in real samples.

After that we optimized all chromatographic parameters above mentioned, we studied dependence of silver ion peak height on its concentration (0–1000 μmol/dm^3). The peak height is proportional to silver ion concentration, particularly; the height markedly increased up to 200 μmol/dm^3, and then increased more slightly (Fig. 4D). We obtained strictly linear calibration curve in the range of 0–31 μmol/dm^3 (*y*(peak height/

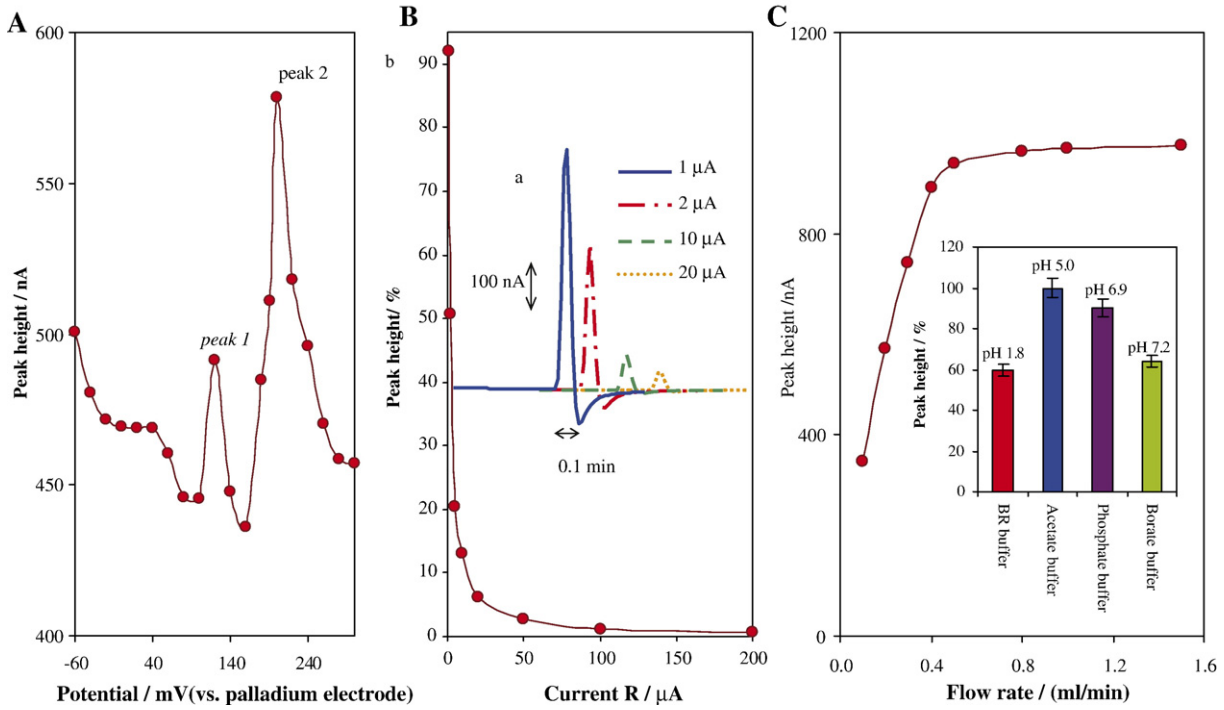

Fig. 3. Flow electrochemical measurement. Influence of (A) potential, (Bb) current *R*, (C) flow rate and (inset in C) composition of mobile phase on current response of silver ions. (Ba) FIA–ED chromatograms of silver ions measured at different currents *R* (1, 2, 10 and 20 μA). FIA–ED conditions were as follows: silver ion concentration of 250 μmol/dm^3; isocratic mobile phase — 0.2 mol/dm^3 acetate buffer, pH 5.0 (except inset in C); injected volume 5 μL; column and detector temperature 25 °C, guard cell potential 0 V.

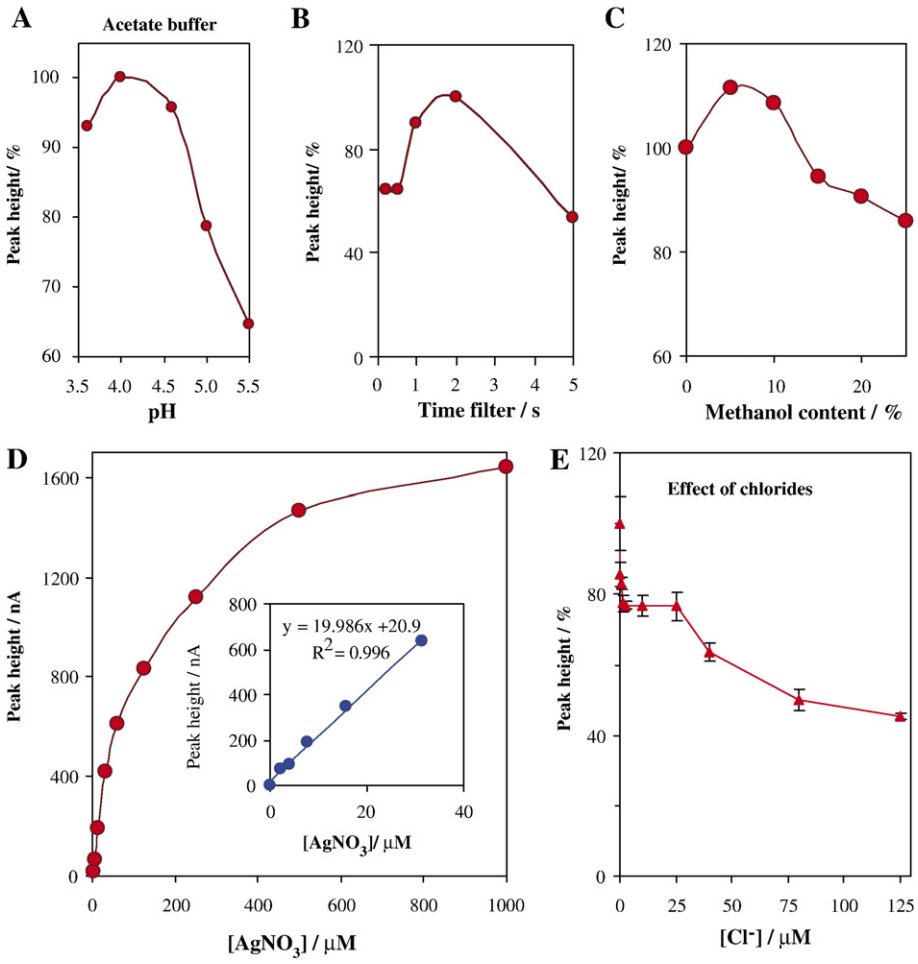

Fig. 4. Effect of (A) different pH values of 0.2 mol/dm^3 acetate buffer, (B) time filter and (C) methanol content on current responses of silver ions. (D) Dependence of current response on silver ion concentrations (0–1000 µmol/dm^3; in inset 0–31 µmol/dm^3). Effect of interferences. (E) Influence of different concentrations of chlorides, on current responses of silver ions. Chromatographic conditions were as follows — flow rate: 0.5 mL/min, guard cell potential: 0 mV, working electrode potential: 200 mV, current *R*: 1 µA, time filter: 2 s, guard cell potential: 0 V, chromatographic column Polaris C18-A, 4.6 mm, 5 µm particle size (except A and B), mobile phase: methanol and 0.2 mol/dm^3 acetate buffer pH 4.0 (10:90, *v*/*v*, except A, B and C).

nA) = 19.986 × (silver concentration/µM) + 20.9, R^2 = 0.996; inset in Fig. 4D). The detection limit (3 S/N) obtained were 20 nmol/dm^3, if we measured silver ions in the presence of ACS water (n = 3, R.S.D. about 5%).

3.2.4. Interferences

Hg(II) and Au(III) ions represent the most critical elements in the framework of interference studies within the electrochemistry of Ag(I) ions, but we attempted to study influence of more commonly occurring ions in waters. An interaction between silver and chloride ions is the well-known and described interaction of silver ions. Thus, we added different concentration of chloride ions to 250 µmol/dm^3 of silver ions, shaken the mixture for 2 min and measured by optimized HPLC–ED technique. The obtained dependence of silver ions peak height on chloride concentration is shown in Fig. 4E. If we added chlorides below 1 µmol/dm^3, we observed decrease of silver ion peak height about 15–20%, and then the height decreased more slightly. The phenomenon relates with an interaction proceeding between silver and chlorides ions: $Ag^+ + Cl^- \Rightarrow AgCl$. Moreover, we were interested in the issue how other ions can influence the silver ions current response. We found out that other tested anions and cations (concentration 10–30 µmol/dm^3) did not influence the response much (about

Table 1
Influence of different ions on height of current response silver ions, $AgNO_3$ (n = 3)

Interference (anion)	Concentration (µmol/dm^3)	Change of peak current (%)[a]	Interference (cation)	Concentration (µmol/dm^3)	Change of peak current (%)[a]
NO_3^-	20	−1.2	Cu^{2+}	10	−1.2
CO_3^{2-}	10	4.9	Na^+	20	4.9
SO_4^{2-}	10	−0.4	Zn^{2+}	10	−0.4
MoO_4^{2-}	10	0.8	NH_4^+	20	0.8
SO_4^{2-}	30	−1.8	Al^{3+}	20	−1.8
SO_4^{2-}	10	−5.7	K^+	20	−5.7
$Cr_2O_7^{2-}$	10	−1.0	K^+	20	−1.0
MnO_4^{2-}	10	−6.5	K^+	10	−6.5
SO_4^{2-}	20	−5.7	NH_4^+	20	−5.7
			Fe^{2+}	10	−5.7

[a] Concentration of silver ions was 250 µmol/dm^3 and its current response was 1120 nA, for other details, please, see Results and discussion section.

Table 2
Recovery of silver ions ($AgNO_3$) measured in the presence of different type of waters ($n=5$)

Compound of interest	Sample matrix	Filtrate (nA)[a]	Spiking (nA)[a,b]	Filtrate+spiking (nA)[a]	Recovery (%)
Silver ions	Milli Q water	nd[c]	935.1±22.1 (2.4)	905.5±23.5 (2.6)	97
	Distilled water			895.8±24.3 (2.7)	96
	Tap water			874.6±29.9 (3.4)	94
	Ponávka stream			860.2±31.5 (3.7)	92
	Puddle			834.1±33.8 (4.1)	89

[a] Silver ions current response; expressed as mean±S.D. (C.V.%).
[b] Silver ions current response (165 μmol/dm^3); expressed as mean±S.D. (C.V.%).
[c] Not detected.

7%, Table 1). It clearly follows from the obtained results that different combination of anions and cations influence the signal variously. A studying of the interaction is needed and will be published elsewhere.

3.3. High performance liquid chromatography coupled with electrochemical detector

3.3.1. A determination of content of silver ions in waters

We applied the HPLC–ED technique to analyze waters of different purities (ACS water, Milli Q water — 18 MΩ, Distilled water — Aqua osmotic system 1 μS, Tap water from the city of Brno, Ponávka stream, Puddle from the city of Brno). We added different concentrations of $AgNO_3$ to water samples and measured a current response of silver ions. The responses measured in the presence of ACS water were used as reference value (R.S.D. about 0.5%), because ACS water was the purest one. We found out that waters decreased the current response in the range of 3–11% in comparison with the signal obtained in the presence of ACS water (Table 2). The observed decrease relates with purity of the water samples.

It is a common knowledge that silver ions come to environment from, first of all, photographic industry [2]. Thus, we determined silver ions in commonly used photographic emulsion. We diluted (minimally 500×) the emulsion before HPLC–ED analysis by ACS water. We found out that content of silver ions in the emulsion was 1.57±0.03 mmol/dm^3. In addition, we attempted to simulate wasting of different types of waters (ACS, Tap water and Puddle) by photographic emulsion. If we compared signals of silver ions after addition of photographic emulsion to different waters, as we mentioned, we found out that silver ions signal decreased about 10% in comparison with the signal measured in the presence of ACS water (Table 3). The detection limits obtained in different matrices are shown in Table 3. We were able to determined tens of nmol/dm^3 of silver ions in different wasted waters.

3.4. Influence of silver ions on growth and thiols content in somatic embryos of Blue Spruce

Pollutants containing heavy metals and toxic organic substances enter the ecosystem especially from applications of fertilizers, pesticides and other industrial products [46,47]. Studies of plant responses to heavy metal stress are especially important for the understanding of a number of biological processes [48–52]. On the other hand, study of influence of heavy metals on a tree is rather difficult, sometimes, almost impossible. Thus, possibilities on how to simplify this kind of study are needed. Using cell cultures for the above mentioned purposes is one of these possibilities [53]. Thus, we investigated influence of silver ions on early somatic embryos of Blue Spruce clone PE 14. Photography of an early somatic embryo in bright field consisted from (a) embryonic group, (b) embryonic tubes and (c) embryonic suspensor is shown in Fig. 5A. The single embryos form clusters in the cultivation medium (Fig. 5B). The embryos were treated by silver ions (0, 250, 500 and 1000 μmol/dm^3) for four days. We aimed primarily on determination of growth of the embryos. Recently, we published that the most suitable technique for this purpose is image analysis (IA), where we observe increase in cluster area according to time, for more details see [53]. Thus we used IA for determination of the growth and found out that growth of the embryos treated by the highest concentration of silver was higher in comparison with control ones. Nevertheless, we observed decrease in the growth of the treated embryos with increasing time of treatment in comparison with control ones (Fig. 5C). Thus, we were interested in the issue how much silver ions can embryos uptake during four days long treatment. For this purpose, we used optimized HPLC–ED technique. The determined content of silver ions in treated ESEs with increasing time of treatment is shown in Fig. 5D. The content

Table 3
Simulation of wasting of different types of waters by photographic emulsion, which contained naturally silver ions ($n=3$)

Sample matrix	Filtrate (mmol/dm^3)[a]	Spiking of photographic emulsion (mmol/dm^3)[a]	Filtrate+spiking (mmol/dm^3)[a]	Recovery (%)	pH[b]	LOD (nmol/dm^3)[c]
ACS water	nd[d]	1.57±0.03 (1.9)	1.57±0.03 (1.9)	100	7.50	20
Tap water			1.47±0.04 (2.6)	94	7.12	40
Ponávka stream			1.45±0.05 (3.4)	92	7.60	61
Puddle			1.46±0.07 (4.8)	93	7.33	67

[a] Silver ions concentration; expressed as mean±S.D. (C.V.%).
[b] pH of "filtrate+spike" solution.
[c] Limit of detection expressed as 3 S/N.
[d] Not detected.

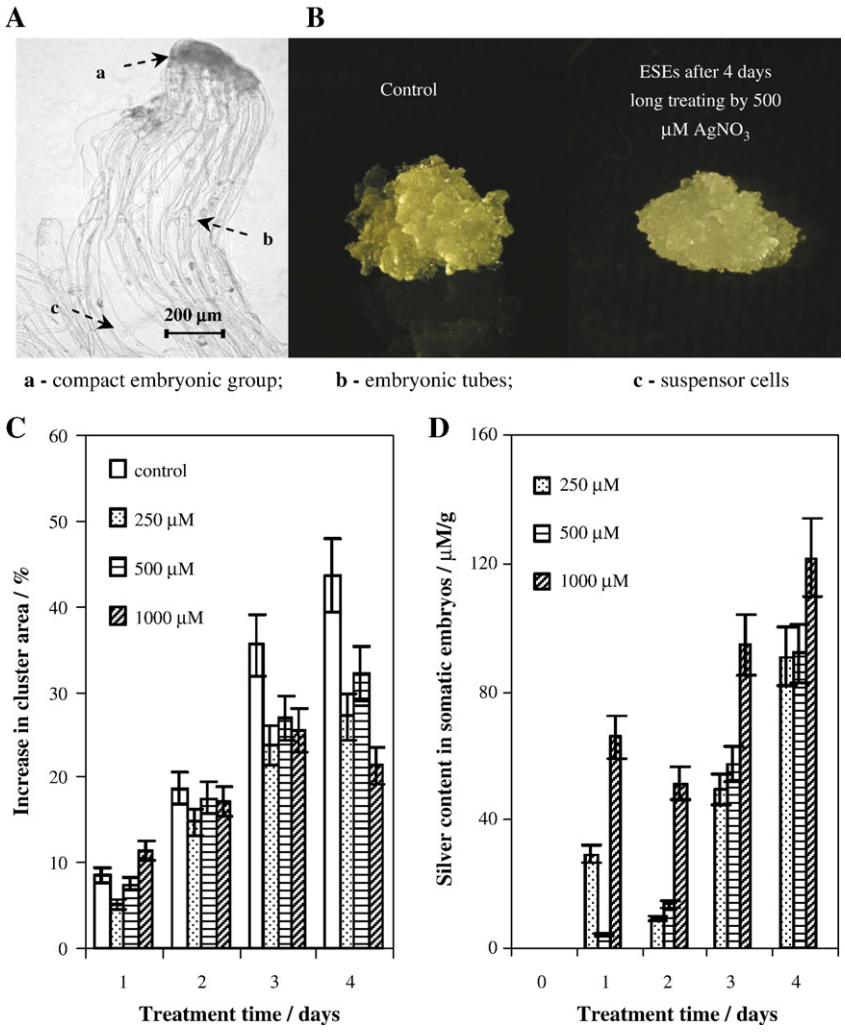

Fig. 5. ESEs culture of Blue Spruce clone PE 14. (A) Images of an early somatic embryo in bright field: (a) embryonic group, (b) embryonic tubes and (c) embryonic suspensor and (B) ESE clusters. ESEs (about 0.1 mg) were harvested by a scalpel and transferred on slide microscopic. The ESEs were spread and superimposed by cover glass. Then, the sample was placed to microscope (Olympus AX 70, Japan). The images were forty times magnified by digital camera Olympus 4040 and converted to digital image in the Grab-IT (version 1.3) program. Influence of $AgNO_3$ (0, 250, 500 and 1000 µmol/dm^3) on (C) growth of ESEs and (D) silver content. For other details see Fig. 4, and Experimental section.

increased with increasing treatment time and applied concentration (Fig. 5D). Recovery was checked for the compound of interest by addition of known amounts of working standard to homogenates (Table 4). Recovery of silver ions was from 96 to 97% (Table 4).

The presence of heavy metals in the plant cell activates protective mechanisms. They initiate the synthesis of cysteine-containing compounds (e.g. phytochelatins and glutathione) used in detoxification processes [40,50–57]. Phytochelatins (PCs) represent a group of short non-protein heavy metal-binding peptides with the general formula $(\gamma\text{-Glu-Cys})_n$Gly, where $n=2–11$. PCs are synthesized by enzyme PC synthase from different isoforms of glutathione: γ-Glu-Cys-Gly. Glutathione (GSH) is the most abundant non-protein thiol, and which plays important roles in antioxidant defence, protein folding and signal transduction [58–60]. Moreover, recently it was discovered that GSH is connected to the nitric oxide (NO) metabolism by means of *S*-nitrosoglutathione, which could serve as a donor of NO [61–64]. Thus, we wanted to know how silver ions can influence thiols content in the treated embryos. Recently we found out that Brdicka reaction could be used for sensitive determination of thiols [35]. On the base of these results, we attempted to use Brdicka reaction to determination of glutathione (GSH), *S*-nitrosoglutathione (GSNO), phytochelatin 2 (PC_2) and phytochelatin 5 (PC_5).

The fact, that the traditional Brdicka's Polarographic catalytic "double wave" of proteins containing cysteine (which are also thiol compounds) is formed of the partial superposition of three voltammetric catalytic maxima (designated A, B and C),which can be generated by one and the same cysteinyl residue in the

Table 4
Recovery of silver ions ($AgNO_3$) for spruce embryos sample analysis ($n=5$)

Compound of interest	Homogenate (µmol/dm^3) [a]	Spiking (µmol/dm^3) [a]	Homogenate+spiking (µmol/dm^3) [a]	Recovery (%)
Silver ions	94.5±7.9 (8.4)	25.4±1.0 (3.9) 98.5±3.4 (3.5)	116.8±8.4 (7.2) 185.6±14.7 (7.9)	97 96

[a] Expressed as mean±S.D. (C.V.%).

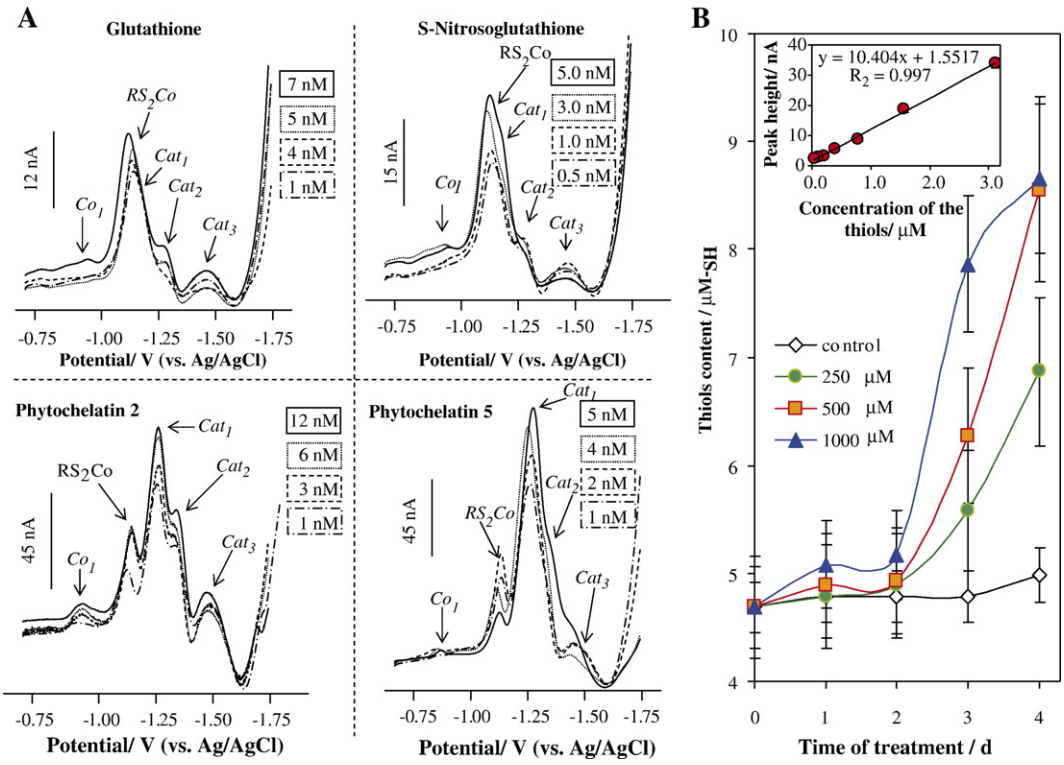

Fig. 6. Electrochemical determination of thiols. (A)Typical DPV voltammograms of different thiols (glutathione, *S*-nitrosoglutathione, phytochelatin 2 and phytochelatin 5) concentrations measured in the presence of supporting electrolyte contained 1 mmol/dm^3 $Co(NH_3)_6Cl_3$ and 1 mol/dm^3 $NH_3(aq)+NH_4Cl$, pH=9.6. (B) Influence of $AgNO_3$ (0, 250, 500 and 1000 µmol/dm^3) on thiols content in ESEs treated for four days; inset: the dependence of total content of thiols in the treated embryos on treatment time. AdTS DPV Brdicka reaction parameters were as follows: an initial potential of −0.6 V, an end potential of −1.6 V, a modulation time of 0.057 s, a time interval of 0.2 s, a step potential of 1.05 mV/s, a modulation amplitude of 250 mV, E_{ads}=0 V. Temperature of supporting electrolyte was 0 °C [35]. For other details see Experimental section.

protein, complexed with cobalt, was published for the first time already in 1967 [65] and since then it has been repeatedly confirmed [35,51,57,66–75]. DP voltammograms obtained are shown in Fig. 6A. We observed five thiols signals — Co_1, RS_2Co, Cat1, Cat2 and Cat3 during analysis of GSH, GSNO, PC_2 and PC_5 by modified AdTS DPV Brdicka reaction. Signals of Cat1, Cat2 and Cat3 correspond to the reduction of hydrogen at the mercury electrode [76]. Another signal, which is appeared at the potential about −1.0 V, relates with the reduction of the RS_2Co complex [76]. In addition the signal called Co_1 could result from reduction of $[Co(H_2O)_6]^{2+}$ [76]. It clearly follows from the figure that character of the mentioned thiol signals change with their different amount. We used signal sum of heights of Cat3 signals of the single thiols for determination of total content of thiols in the treated embryos. The height was proportional to their concentration: y(peak height/µA)=10.404(thiols concentration/µM)+1.5517, R^2=0.997 (inset in Fig. 6B). The dependence total content of thiols in the treated embryos on treatment time is shown in Fig. 6B. It clearly follows from the obtained results that content of thiols increased with increasing treatment time and applied concentration of silver ions. Content of thiols in untreated (control) embryos did not change much in comparison with the highest silver concentration, where content of thiols was almost two times higher. The concern relates to the fact that embryos protect themselves against toxic heavy metal from the beginning of the treatment.

4. Conclusion

A low cost and sensitive technique for determination of silver ions in environment is needed not only due to high toxicity of these ions but also due to investigating of silver effects on organisms. Here, we suggest the technique for determination of this compound of interest both in environmental samples and plant embryos. Moreover, we also observed effect of the silver ions on growth and thiols content at the treated embryos.

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