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Effect of lead accumulation in maize leaves on their chemical images created by a flow-through electronic tongue

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ABSTRACT

A flow-through electronic tongue based on miniaturized ion-selective electrode array was used for the classification of the maize leaf samples (*Zea mays*) exposed to media containing Pb(II) ions. The system provided a good recognition of the extracts from the plant leaves treated with solutions of varying concentrations of $Pb(NO_3)_2$. Additionally, samples derived from specific segments of the maize leaf, representing different developmental stages of cells, were also discriminated. The presented results showed that the developed sensor array combined with Partial Least Squares-Discriminant Analysis (PLS-DA) technique allowed to recognize the plant samples on the basis of the changes in the ionic composition of the leaf homogenates.

1. Introduction

In recent years the interest in adaptation of plants growing in contaminated areas has received increased attention. Great concern is put especially on plants that evolved a mechanism of tolerance to high concentrations of heavy metals, useful for the biological soil cleaning—phytoremediation—considered as the cheapest and the most eco-friendly method of cleaning the environment [1,2]. Plants suitable for phytoremediation (called hyperaccumulators) should accumulate, translocate and tolerate high amounts of heavy metals in their tissues [3]. They should also exhibit rapid growth and large increase of biomass, have an extensive root system and be easily removable from the soil [4]. The efficiency of the phytoremediation, especially phytoextraction, is determined by the bioconcentration factor i.e. the ratio of the concentration of a particular contaminant in a biological tissue to its concentration in the environment. For all hyperaccumulators the bioconcentration factor is greater than 1 [5,6]. Therefore, many works were focused on physiology of plants accumulating large quantities of heavy metals, which by spontaneous succession inhabit contaminated areas [7,8]. On the other hand, genetic engineering research led to the development of plants adapted for heavy metals accumulation [9,10]. Many of the most productive crops in agriculture, such as maize and sorghum, are the key model system for gene discovery relating to biomass yield and quality in the bioenergy grasses [11].

Certain plants may also be used as environmental markers for bioindication, a method enabling to assess the environmental conditions, especially pollution, through their impact on living organisms. Various plant species are characterized by a specific tolerance range with respect to selected ecological factors, e.g. the soil pollution by heavy metals [12,13]. The visible symptoms of plant growth allow to evaluate the environmental conditions; however, the laboratory analysis of plant material provides a quantitative information on the degree of soil or air pollution.

Currently, the accumulation of heavy metals in plant materials is typically analyzed using UV-visible spectrophotometry and high performance liquid chromatography (HPLC). Size-exclusion chromatography (SEC) coupled with ICP and ESI mass spectrometry has been applied for the analysis of lead species in *Arabidopsis thaliana* exposed to Pb(II) stress [14]. Usually, these approaches are based on quite expensive equipment and involve complicated sample preparation procedures. On the other hand, the electronic tongue system can become an alternative tool for bioindication research. This device provides fast, automatic identification and classification of complex liquid samples, involving an array of non-specific sensors and a pattern recognition block based on chemometric techniques. Usually it is not used for selective analyte detection, but for the recognition of characteristic properties of food, pharmaceuticals and biotechnological samples [15–19].

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Table 1
Components used for the preparation of ion-selective electrodes.

Electrode type	Lipophilic salt (20–50 mol% vs ionophore)	Ionophore (1 wt%)	Plasticizer (65–66 wt%)	Polymer (31–33 wt%)	Internal filling solution
K^+	KTFPB	Valinomycin	DOS	PVC	0.01 M KCl
Na^+	KTPClPB	Na ionophore X	DOS		0.01 M NaCl
Pb^{2+}	KTFPB	Pb ionophore IV	DOS		0.01 M $PbCl_2$
Cation-selective	KTFPB[a]		DOS		0.01 M KCl
Anion-selective	TDMAC[b]		o-NPOE		0.01 M NaCl
Cl^-	Ag/AgCl planar electrode				

[a] 1 wt%.
[b] 3.5 wt%.

So far, only a few studies on plant material were carried out using the electronic tongue (ET) or electronic nose (EN) devices. EN based on quartz crystal microbalance smell sensor array was applied for volatile compounds analysis, enabling the quality control of ginseng Malaysian plant extracts (*Eurycoma longifolia*). The system replaced the previously used analytical procedures, including GC–MS, which reduced the time and costs of the analysis [20]. Chrysanthemum leaves were distinguished by EN in terms of their resistance to western flower thrips (WFT, *Franklinella occidentalis*), a vector of serious viral diseases of plants. The results indicated correct classification of the samples treated with WFT and the efficiency of EN for the resistance screening [21]. An electronic nose was also used to discriminate different varieties of the common-species Valerians (*Valeriana officinalis* and *Valeriana wallichii*), dispalying different therapeutic effects [22]. The system has proven to be a fast tool to differentiate not only both species, but also to separate specimens of different origin or the plants belonging to the same variety.

An electronic tongue based on an array of ion-selective electrodes was reported to recognize the type of plant metabolism as well as to estimate the plant development and light intensity during growth. Since the latter influences the nutritional values of the plants the system could be suitable for the estimation of their quality [23]. Another example is the application of the commercially available ET (Alpha M.O.S, France) for the quality control of white chrysanthemum, whose samples were effectively discriminated according to their grade, brand and adulteration [24].

The objective of this work was to test the ability of the potentiometric electronic tongue to recognize the maize (*Zea mays*) leaves treated with solutions of varying concentrations of Pb(II) ions. Maize was chosen as a model plant, since it represents a type of metabolism responsible for about 25–30% of global terrestrial productivity [25]. Moreover, maize plants are more resistant to lead treatment that other species and the inhibitory effect on photosynthesis was more pronounced in the younger tissues of basal parts of the leaves as compared with the top segments [26]. A flow-through array of miniaturized ion-selective electrodes (ISEs) was applied to study the ionic composition of homogenates from the leaf material. The analysis was performed to discriminate maize leaves treated with solutions of different Pb(II) ions concentrations. Finally, the classification of maize leaf segments representing various developmental stages of cells, exhibiting different metabolic activities and thus accumulating different amounts of Pb(II) was attempted.

2. Experimental

2.1. Membrane materials and ISEs preparation

All inorganic salts, 1-morpholinoethanesulfonic acid (MES), sulfuric acid and sodium hydroxide were of analytical grade and were obtained from Fluka. The working solutions of salts (0.1 M) were prepared in redistilled water. The polymeric membrane components: high-molecular-weight polyvinylchloride (PVC); plasticizers: bis(2-ethylhexyl) sebacate (DOS), o-nitrophenyl octyl ether (o-NPOE); lipophilic salts: potassium tetrakis[3,5-bis(trifluoromethyl)phenyl]-borate (KTFPB), potassium tetrakis(4-chlorophenyl)borate (KTPClPB), tridodecylmethylammonium chloride (TDMAC); ionophores: 4-tert-butylcalix[4]arene-tetraacetic acid tetraethyl ester (sodium ionophore X), valinomycin (potassium ionophore I), p-tert-butylcalix[4]arene-tetrakis(N,N-dimethylthioacetamide) (lead ionophore IV) were purchased from Fluka.

The membranes contained the appropriate ionophores (1 wt%), 20–50 mol% (vs ionophore) lipophilic salt, 65–66 wt% plasticizer, and 31–33 wt% high-molecular-weight PVC (Table 1). The membrane components (200 mg in total) were dissolved in 2 ml of THF. The method of membranes preparation was the same as that for the standard ISEs and was described in the previous reports [27,28].

A detailed architecture of the miniaturized ion-selective electrodes compatible with a single flow-through module was presented in [29], whereas the design of the modular flow-cell system is a subject of a polish patent application [30]. The flow-through sensor array consisted of 9 miniaturized electrodes selective towards: sodium, potassium and lead ions, as well as electrodes exhibiting anion- and cation-selectivity according to the Hofmeister pattern (i.e. containing only an appropriate ion-exchanger in the membrane). Two electrode specimens were prepared for each membrane composition (except for the anion-selective electrode). A planar Ag/AgCl electrode has been added, which resulted in a 10-electrode flow-through array. The components of the internal filling solutions were listed in Table 1. Solution of the following composition: 0.001 M NaCl, 0.001 M KCl, 0.001 M $PbCl_2$ was used for the conditioning of the electrodes. The constructed ISEs were preconditioned for at least 24 h. A scheme of the experimental procedure and the experimental set-up are shown in Fig. 1.

2.2. Plant sample preparation

The maize plants (*Zea mays* L. Oleńka) were grown on vermiculite in a growth chamber under a 14 h photoperiod and a day/night regime 24/22 °C, at an irradiance 350 μmol photons $m^{-2}\,s^{-1}$ (medium light). Plants were fertilized with Knop's solution containing ($g\,l^{-1}$) 0.8 $CaNO_3$ $4H_2O$; 0.2 KNO_3; 0.2 KH_2PO_4; 0.2 $MgSO_4$ $7H_2O$; 0.028 EDTA-Fe enriched with A–Z microelements nutrient (Hoagland and Arnon 1939). Leaves were harvested from 3–4-week-old plants. Fourth maize leaves (~60 cm length and ~1.5 g weight) from different plants and repeated for different seedlings under identical growth conditions were used in the experiments.

Excised leaves of maize plants were detached and placed with their cut ends into a glass beaker containing either water (control sample—C), 5 mM $Pb(NO_3)_2$ (LPb) or 10 mM $Pb(NO_3)_2$ (HPb).

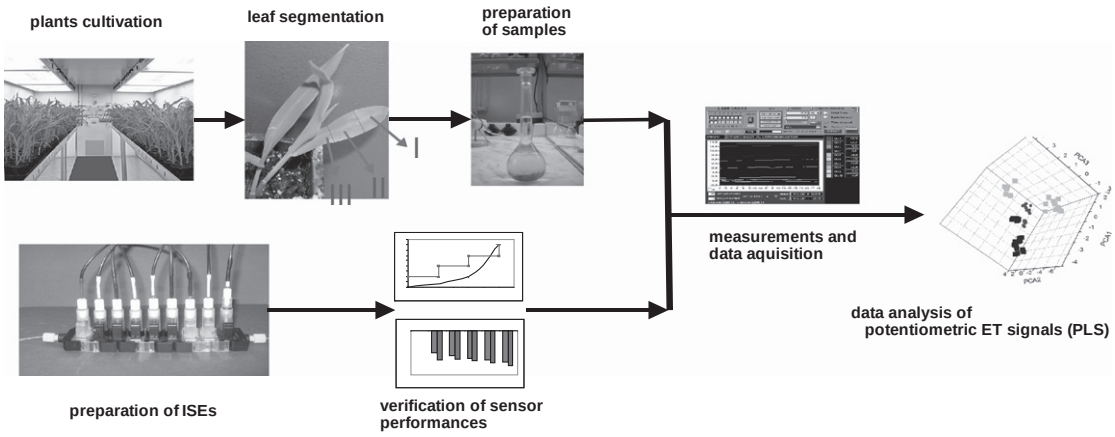

Fig. 1. Scheme of the experimental procedure and the experimental set-up.

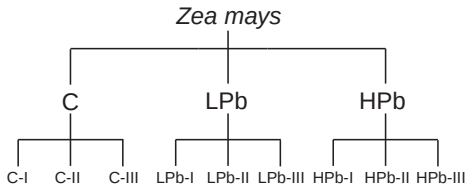

Fig. 2. Scheme of the maize leaf samples (C, control; LPb, 5 mM Pb(II); HPb, 10 mM Pb(II); tip (I), middle (II) and basal (III) parts of the leaf).

Table 2
Operating parameters for ICP-OES measurements.

Parameter	
Incident power [W]	1000
Viewing mode	Radial
Observation height above coil (mm)	5
Plasma gas flow rate (dm^3/min)	16
Auxiliary gas flow rate (dm^3/min)	0.5
Nebulizer gas flow rate (dm^3/min)	0.5
Wavelength (nm)	Pb 220.353
	Pb 316.999

After 24 h of continuous exposure to weak light (about 30 μmol m^{-2} s^{-1}) at room temperature and 80% relative humidity, leaves were divided into three parts: tip (I), middle (II) and basal (III) (Fig. 2). This procedure is convenient and commonly applied in biological studies to compare the response of plant tissues of different developmental stages and metabolic activities of cells to various abiotic stresses [31]. In this way 9 types of samples were obtained. Such samples were subsequently frozen in liquid nitrogen, lyophilized in a freeze-dryer (ALPHA 1–2/ LD) and stored at 4 °C.

2.3. Potentiometric measurements and data analysis

Lyophilized plant material (0.2 g of dry weight) was mixed with 0.3 ml of 96% H_2SO_4 and 10 ml of 30% hydrogen peroxide, and digested in microwave generator (Plazmotronika, Poland) [32]. The process was conducted under the following conditions: cycle time – 30 min, pressure limit – 18 atm; 10 min of 70% heating, 2 min of cooling, 10 min of 100% heating, 8 min of cooling. Homogeneous and clear sample solutions (homogenates) were obtained after the mineralization process. 3 replicates for each of 9 types of lyophilized samples were analyzed.

All measurements were carried out in the flow-through mode (one channel system, sample volume: 10 ml, flow rate: 3.5 ml/min) with cells of the following type: Ag, AgCl; KCl 3 M|CH_3COOLi 1 M|sample solution||membrane||internal filling solution; AgCl, Ag.

Potentiometric multiplexer (EMF 16 Interface, Lawson Labs Inc., Malvern, USA) was used for the EMF measurements. To verify the performances of the sensors, potentiometric selectivity coefficients were determined by the Separate Solution Method (SSM) using 0.1 M solutions of corresponding salts [33], containing 0.005 M MES. Data analysis was performed in MatLab (The MathWorks, Inc., Natick, USA) and Origin (Microcal Software, Inc, Northampton, USA) software. Chemical images of samples of 9 classes (C-I, C-II, C-III, LPb-I, LPb-II, LPb-III, HPb-I, HPb-I and HPb-III, see Fig. 2) were processed using Partial Least Squares-Discriminant Analysis (PLS-DA). Autoscaling as preprocessing method and SIMPLS regression algorithm were applied. For cross-validation venetian blinds (10 splits, maximum 4 LVs) were used.

2.4. ICP-OES measurements

Lyophilized plant material (0.2 g of dry weight) was mixed with 5 ml of 65% HNO_3 and 3 ml of 30% hydrogen peroxide, and digested in a microwave generator (Berghof, Germany) [34]. The process was conducted under following conditions: first step: time 5 min, temperature 145 °C, pressure 50 bar, power 70%; second step: time 10 min, temperature 190 °C, pressure 50 bar, power 90%. After the mineralization process, homogeneous and clear sample solutions were obtained. 3 replicates for each of 9 types of lyophilized samples were analyzed.

The determination of the total amount of Pb in plant extracts was carried out with an inductively coupled plasma (ICP) spectrometer (Integra XL, GBC, Australia) using radially viewed mode. The ICP-OES operating conditions are given in Table 2. The analysis of lead content in LPb and HPb samples was performed after 40 fold dilution of the leaf homogenates.

3. Results and discussion

The flow-through sensor array was used to record the EMF signals in plant sample extracts prepared according to the procedure described in the experimental section. The obtained measurement data were processed with PLS-DA procedure. PLS-DA established the correlation of data matrix with target matrix [35]. The data matrix containing chemical images of

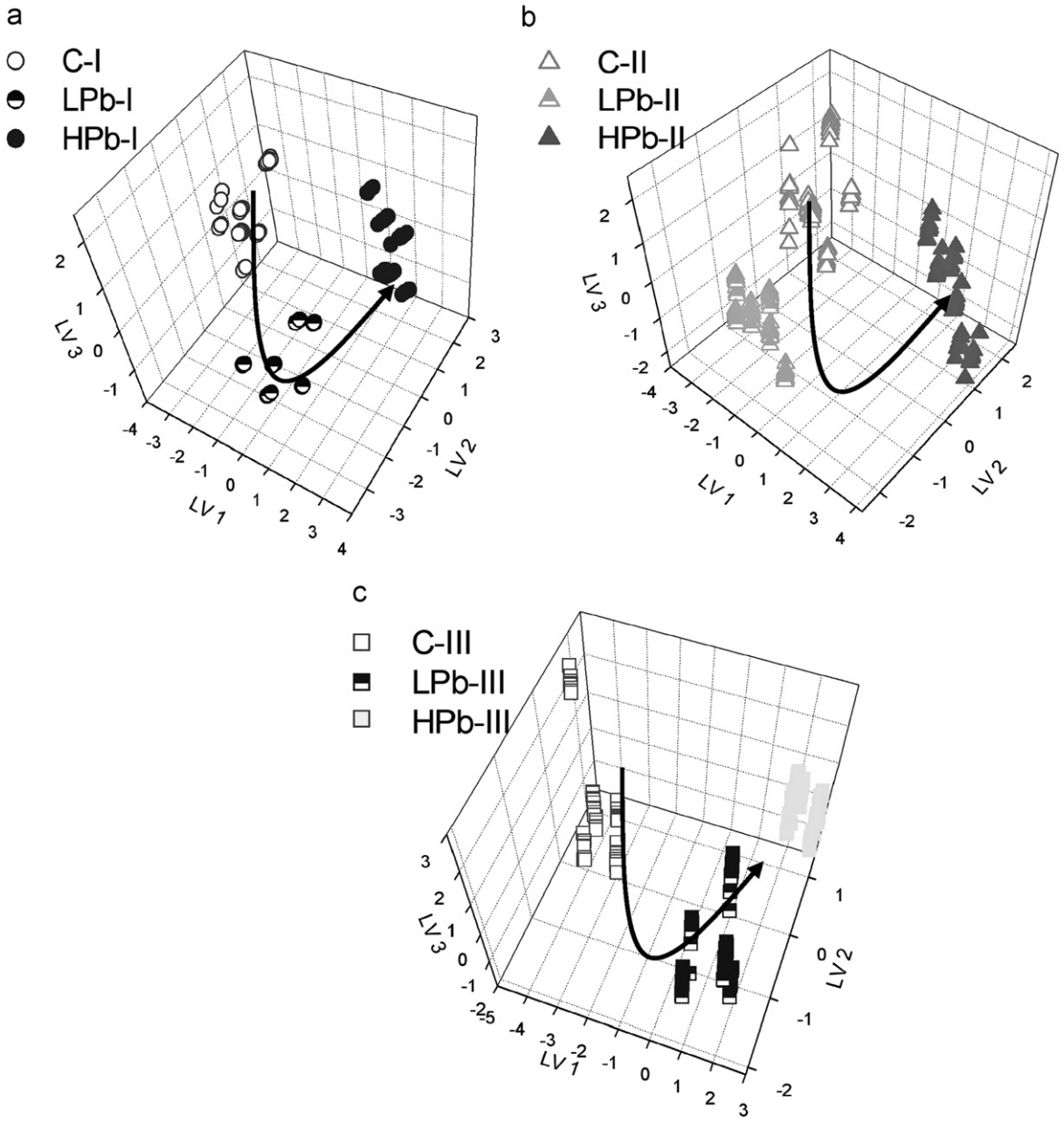

Fig. 3. 3D-PLS plots of chemical images of the samples from the control (C) leaves and exposed to 5 mM Pb(II) solution (LPb) and 10 mM Pb(II) solution (HPb): (a) tip (I), (b) middle (II) and (c) basal (III) parts of the leaves.

samples and its corresponding target matrix was constructed. The values in every row of the data matrix characterized the investigated samples; they were formed by the steady-state responses of the sensors. The data matrix was correlated with the target matrix, which contained the coded information about sample classification. The resulting chemical images of samples obtained by processing of the data by PLS-DA are presented in Figs. 3–5.

First of all, the PLS-DA processing of data matrices was performed for each group of plant samples exposed to the media containing different amounts of Pb(II) ions (samples marked as C, LPb, HPb). The samples were correctly classified according to the exposure to lead salt solutions of different concentrations; a good differentiation of control samples was also visible. Comparable PLS-plots were obtained for samples prepared from the respective segments of the leaves (Fig. 3). Moreover, in the case of all three parts of the leaves, chemical image exhibited the same change of the position in the pattern space related to the exposition towards Pb(II), which confirmed, that the chemical images reflected the toxic effect of that heavy metal.

According to the literature, heavy metals accumulate unevenly in various parts of plants. Pb ions from the soil solution are taken up by the roots, then they are transported within the xylem with transpiration stream from the roots to the stem and leaves. In addition, it was found that lead is deposited in the cell walls, and also in vascular system of both xylem and phloem [36]. Since the base of the leaf is more vascular in comparison with other segments, different ability of lead ions accumulation is considered for various parts of the leaf [37]. Moreover, the maize leaf represents natural developmental gradient in which the cells are arranged in a linear developmental array, with the youngest cells at the base and the eldest cells at the tip of the leaf. Therefore, different metabolic activities of the leaf cells are observed for various developmental stages altering the intensity of the metal uptake [38]. Such effect was confirmed by the PLS-DA results i.e. the homogenates derived from various parts of the

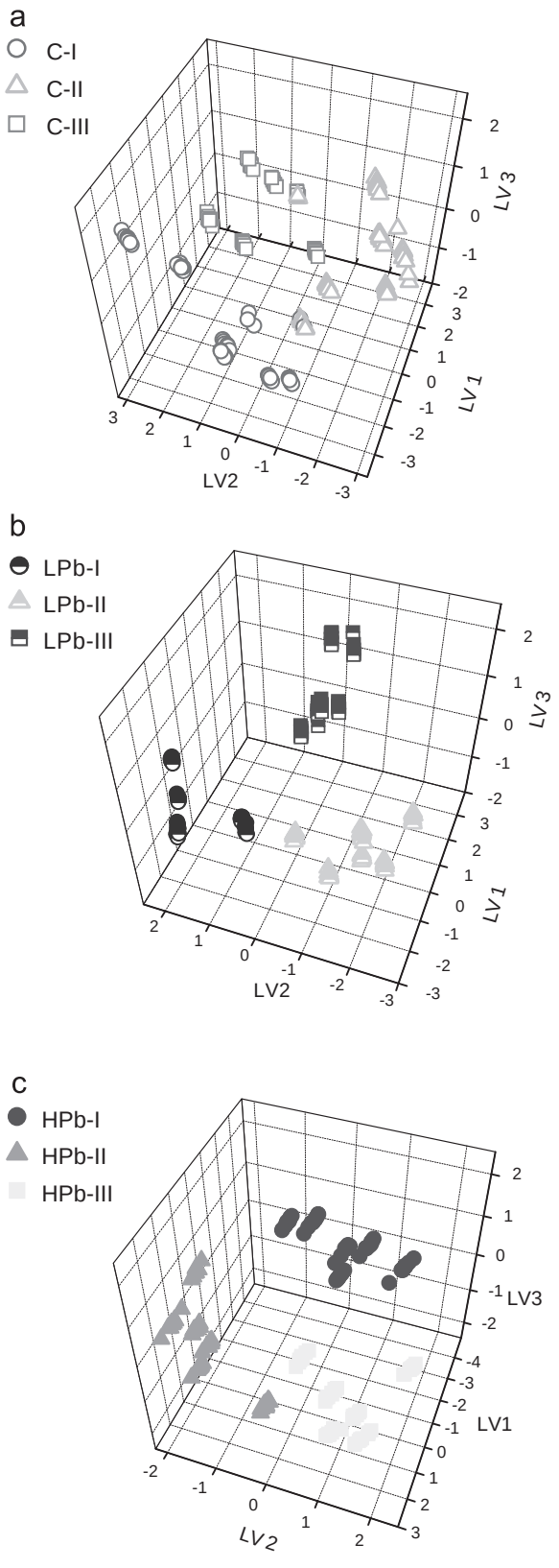

Fig. 4. 3D-PLS plots of chemical images of the samples from the tip (I), middle (II) and basal (III) parts of: (a) the control (C) leaves, (b) exposed to 5 mM Pb(II) solution (LPb) or (c) 10 mM Pb(II) solution (HPb).

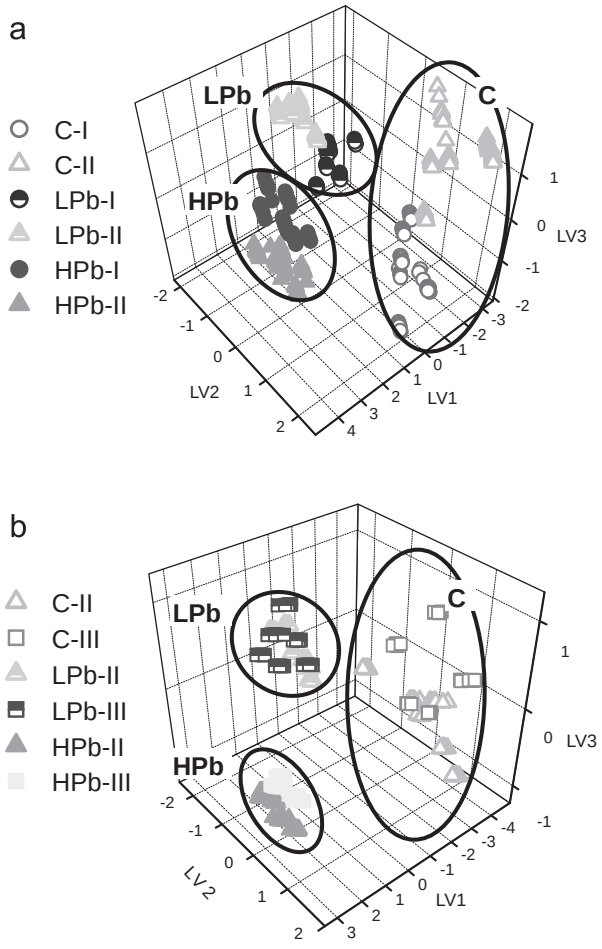

Fig. 5. 3D-PLS plots of chemical images of the samples derived from: (a) the tip (I) and middle (II), (b) middle (II) and basal (III) parts of maize leaves.

leaves (I, II and III) were characterized by different chemical images (Fig. 4). A good discrimination of the samples exposed to solutions containing Pb(II) salt was observed (the most clear recognition was obtained for respective HPb samples), whereas the differentiation of control samples (C) was not obvious. It proved, that the effect of Pb(II) uptake caused a distinction of leaf parts of LPb and HPb samples.

The classification results were compared with the total amount of lead in plant homogenates, provided by the ICP-OES technique. According to the expectations, the presence of the Pb(II) salt in the medium induced a ~1000 fold increase of lead concentration in leaf material, whereas the values found for HPb samples were on average 50% larger than those for LPb samples (Table 3). However, the differences in Pb content in LPb samples derived from various parts of the leaves were negligible. The latter result led us to the conclusion, that the changes in the ionic composition of the homogenates, and not the amount of accumulated Pb(II), was the basis of the efficient recognition of LPb samples presented in Fig. 4b. In other words, a simple determination of the lead content in leaf material (using ICP-OES or Pb^{2+}-selective electrode) would not be sufficient to differentiate LPb samples of various leaf parts. The same conclusion can be drawn in the case of I, II and III HPb samples, for which the differences in Pb concentration would correspond only to ~1 mV signal change of the Pb^{2+}-selective electrode (assuming theoretical electrode response). Nevertheless, the greatest amount of Pb was accumulated in the basal segments of the leaves. These results indicate that under higher Pb(II) concentration (HPb samples), the transport of heavy metal ions through the basal part of the maize leaf (with not differentiated plastids) to other segments is disturbed.

Table 3
Determination of the total amount of Pb in plant homogenates by ICP-OES technique.

Sample	Determined Pb content (mg/dm^3)
C (I)	0.12 ± 0.03
C (II)	0.07 ± 0.04
C (III)	0.04 ± 0.03
LPb (I)	121 ± 6
LPb (II)	126 ± 6
LPb (III)	124 ± 8
HPb (I)	170 ± 5
HPb (II)	184 ± 10
HPb (III)	195 ± 18

Mean values $\pm$ SD for 3 determinations.

Further classification capabilities of the potentiometric flow-through sensor array can be discussed on the basis of Fig. 5, where the chemical images of the C, LPb and HPb samples of various parts of leaves are collected on the same plot. First of all, it must be noticed that the samples treated in the same way occupy the same area in the pattern space on PLS plot i.e. the control samples were on the right side, whereas LPb and HPb samples were accordingly placed on the left side. Moreover, a clear distinction can be observed between the clusters representing middle and basal segments of the leaves (Fig. 5b compared to Fig. 5a). Such effect was caused by higher diversification of C, LPb and HPb samples in that case the effect of Pb(II) was most pronounced for chemical images of the middle and basal segments, since the lead ions accumulation in these parts of the leaves seems to be higher (therefore, the clusters LPb and HPb are more separated in Fig. 5b than in Fig. 5a).

Exemplary results of samples differentiation obtained from PLS-DA data processing (average, minimum, maximum and standard deviation values—y_{pred}) are presented on box-and-whiskers plots in Fig. 6. The target containing 3 values was constructed for basal parts of the maize leaves exposed to solutions of different lead(II) contents. The target matrix contained 3 columns determining the class membership of the samples. The samples described by a vector [1,0,0] belonged to the control samples (C). LPb samples were coded by vector [0,1,0], whereas HPb by [0,0,1]. After correlation of the data matrix with the target matrix, the prediction matrix (Y_{PRED}) containing prediction vectors (y_{pred}) was calculated (e.g. vector [0.98, 0.15, 0.23] indicating, that the sample was assigned to control sample C). It should be noticed that all calculated mean values are close to the target values i.e. they are close to zero or one respectively. In most cases, minimum and maximum values are slightly different from the average values, so the relative standard deviation is small, within the limits of 3–16%. Moreover, values close to "0" and "1" were obtained at appropriate outputs in the case of every vector. It shows, that the classification of the appropriate samples was correct, and the class mismatch did not occur. Therefore, all testing samples were properly recognized (100% of correct classifications), which proved appropriate sensitivity (100%) and specificity (100%) of the developed electronic tongue system.

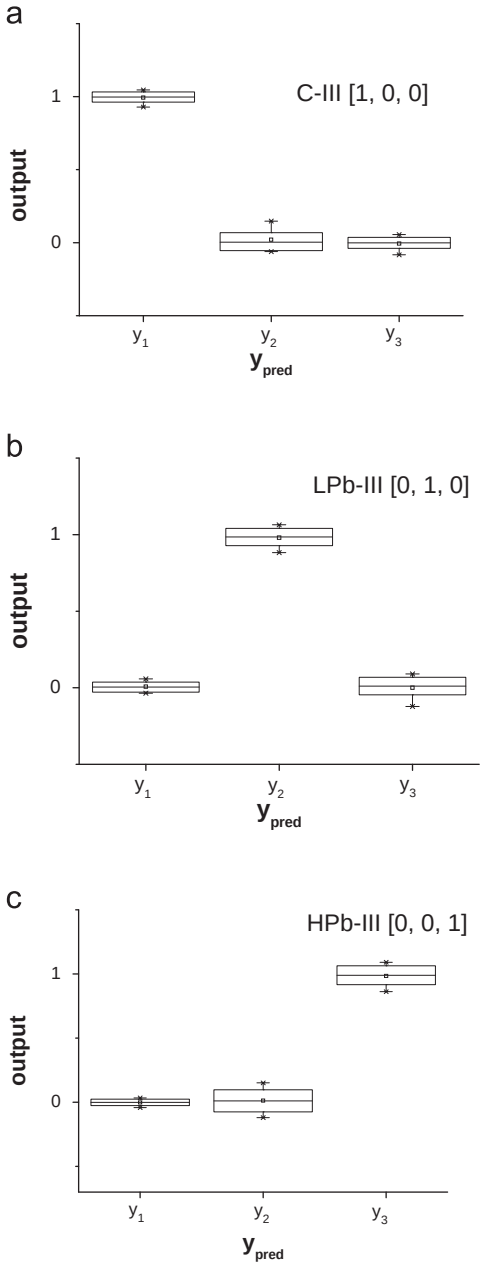

Fig. 6. y_{pred} values calculated for the samples derived from the basal parts of maize leaves exposed to solutions of different lead salt content: (a) control (C), (b) 5 mM Pb(II) (LPb), (c) 10 mM Pb(II) (HPb).

4. Conclusions

A flow-through potentiometric electronic tongue system was applied to recognize the treatment conditions of the maize leaves. The analysis of the ionic composition of the maize leaf homogenates provided clear differentiation of the control samples from those exposed to the lead(II) salt solutions. Moreover, distinct chemical images were obtained for samples exposed to media containing different Pb(II) concentrations as well as for samples derived from various parts of the leaves (exhibiting different metabolic activities and accumulating different amounts of Pb(II)). The determination of the total Pb content in extracts carried out by ICP-OES suggested that the efficient classification resulted from the effect of lead exposure on the ionic composition of the homogenates. The changes of the ionic composition were probably related to the lead(II) accumulation in leaves affecting the plant metabolism (detailed discussion on the effect of Pb(II) on maize metabolism can be found in [39]).

The results presented in this paper led us to the conclusion, that the developed electronic tongue system based on flow-through ISEs could be a potential tool for the estimation of the cultivation conditions of plants during e.g. bioindication or phytoremediation studies. Moreover, the recognition of the plant segments accumulating distinct or comparable amounts of Pb(II)

is also important from the point of view of biological studies. The differentiation of the chemical images of various parts of the leaves upon lead exposure indicates the alteration of their metabolism and could be useful to model the mechanism of the plant response to heavy metals.

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