Metallomics Study of Lead-Protein Interactions in Albumen by Electrochemical and Electrophoretic Methods

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Lead(II) ions represent still threat to living organisms due to high burden of these ions in environment. The aim of this study was to design and perform experiments studying the interaction of lead(II) ions and proteins contained in egg albumen. For this purpose, we used electrochemical and electrophoretic techniques. We focused on a very detailed study of the interaction of lead(II) ions with the egg albumen proteins. Firstly, egg albumen was diluted with acetate buffer in the following ratios buffer:albumen – 1:0.5; 1:1; 1:2 and 1:4. Additions of different concentrations of lead(II) ions into the diluted egg albumen were 0; 1; 5; 10; 25; 50; 100; 150; 200; 250; 500; 750 and 1,000 nM. Mixtures were then placed on thermoblock at 37 °C for 15, 30, 45 and 60 min. Primarily, we used differential pulse anodic stripping voltammetry to analyse above prepared mixtures. It clearly follows from the obtained experimental data that there is a very strong interactions of lead(II) ions and biomolecules contained in egg albumen. Rapid increase in the concentration of lead(II) ions with a linear trend was

detected in the mostly diluted egg albumen (lower concentration of total protein). At higher concentrations and longer time of interactions, metal ions bind tightly or intercalate into biomolecules presented in egg albumen. Besides the effect of lead(II) ions and albumen concentrations, we aimed at the effect of various times of incubation. The obtained results were linearly plotted and the slopes of these straight lines were compared. All slopes steep decreased with the increasing time of interaction and content of egg albumen proteins (15 min interaction, the ratio of 1:4 and the slope 0.06; the ratio of 1:2 and the slope 0.038; the ratio of 1:1 and the slope 0.009; the ratio of 1:0.5 and the slope 0.0085). Moreover, it is evident a clear time-dependence of binding lead(II) ions to biomolecules. At the ratio of 1:0.5 there was observed the bond of app. 0.06 ng Pb per min. into biomolecules of egg albumen, at the ratio of 1:1 there was observed the bond of app. 0.08 ng Pb per min. into biomolecules of egg albumen, at the ratio of 1:2 there was observed the bond of 0.3 ng Pb per min. into biomolecules of egg albumen and at the ratio of 1:4 there was observed the bond of 0.55 ng Pb per min. to biomolecules of egg albumen. Probably, in the case of lower concentrations of albumen proteins, there is a change in the structure of these proteins. The samples obtained by incubation of various concentrations of lead(II) ions with egg albumen protein were also analysed by chip capillary electrophoresis under non-reducing conditions to study the influence of lead(II) ions on the protein profiles of egg albumen. We determined changes in the content of the selected proteins as lysozyme, flavoprotein, ovalbumin, ovomucoid, avidin and ovotransferrin and some unidentified as marked according to their molecular masses 55, 95, 110, 117, 123, 132 and 170 kDa. Based on determined protein profiles (as slopes of signal changes depending on the applied lead(II) concentration) at various dilutions it is clearly evident that the most significant changes are detected at ovotransferrin, avidin, lysozyme and ovomucoid. In addition, we employed optimized methods for studying of lead(II) induced complexes in died embryo of vulture. Contents of lead(II) ions, reduced and oxidised glutathione, metallothionein and zinc(II) ions were determined in albumen, yolk, liver, kidney, brain and bone obtained from the embryo. The highest lead contents were observed in liver and kidney app. 5 µg/g tissues. The highest contents of GSH and GSSH were found in brain tissue and in albumen, which can be associated with the needs to protect neural system against reactive oxygen radicals. The highest content of MT was determined in albumen (more than 12 µg/g) followed by yolk and kidney, and the lowest in liver. This is very interesting result, which shows a highly active defence of the embryo against lead(II) ions. The level of zinc(II) ions was highest in organs with maximum biochemical activity (liver and kidney). The results bring new and unique point of view in metallomics research and may provide new insights into mechanisms during embryonic development.

1. INTRODUCTION

Lead(II) ions represent still threat to living organisms due to high burden of these ions in environment. Adverse effects on brain tissue and neurotoxicity of these ions are most discussed [1,2]. However, there are still missing some data about the effects of lead(II) ions on an organism including relationships between intoxication of female parent and foetus. The transport of these ions is carried out with placenta, but the exact mechanism is not clear.

It can be assumed that a numerous of both small and large molecules with different biochemical mechanisms can participate in the process [3,4].

An egg and developing embryo can be considered as a suitable model for studying of the transport of lead(II) ions by placenta. There exists few information about trace mineral metabolism in the developing avian embryo begins with the formation of the egg, which are summarized in the following paper [5].

The attention of researchers is mainly paid to the area of monitoring of lead(II) ions in the environment [6]. These studies are performed in the areas with considerable burden of these ions [7-9]. Besides areas, monitoring of intoxication of living organisms is also aim of various researchers. Acute intoxication by lead(II) ions leading to their dying was found in Passeriformes species. The high levels of lead, as detected in the passerines (4.80-12.74 mg/kg) at the onset of mortality and at the follow-up sampling of the free-range chicken eggs (25.02-35.21 mg/kg in shells, 0.41-1.36 mg/kg in yolks and 0.40-0.75 mg/kg in the albumins) [10]. Trampel et al. determined the concentration of lead in blood, eggs (yolk, albumen and shell), and tissues (liver, kidney, muscle, and ovary) from 5 selected chickens over a period of 9 days. Blood lead levels ranged from less than 50 to 760 ppb. Lead contamination of the yolks varied from less than 20 to 400 ppb, and shells were found to contain up to 450 ppb lead. Albumen contained no detectable amount. Lead content of the egg yolks strongly correlated with blood lead levels.

Deposition of lead in the shells did not correlate well with blood lead levels. Mean tissue lead accumulation was highest in kidneys (1,360 ppb), with livers ranking second (500 ppb) and ovarian tissue third (320 ppb). Muscle contained the lowest level of lead (280 ppb) [11]. The two levels of Pb consistently resulted in increases in the Pb content of blood, kidney, liver, and gizzard, whereas only 20 mg Pb/kg per day of dose additionally increased the Pb in femoral muscle. More Pb was deposited in the kidney and liver after Pb exposure than in the gizzard or femoral muscle. Lead residues in yolk and eggshell from Pb-dosed ducks were significantly higher than in controls; however, Pb in albumen was generally low and was not influenced by Pb treatment [12]. Metal concentrations in fledgling feathers represent in part metals sequestered in the egg by females and accumulation from food brought back to chicks by parents, and thus may be a measure of local metal acquisition. There were significant interspecific differences in lead in eggs, and lead and cadmium in fledgling feathers. Herring gulls had the most lead in eggs (up to means of 6,740 ng/g, dry weight), whereas the terns had the least (mean of 318 ng/g, dry weight). Lead concentrations were high in fledgling feathers (up to 4,090 ng/g, dry weight) in some populations of all species [13].

The experiments with hens treated with various toxic compounds revealed that there decreased egg production and egg weight, and increased percentage embryonic mortality [14]. Based on these mutual effects, complexation of lead(II) ions must be also taken into account. Ketola et al. found consumption of alewife and other thiaminase containing fishes by cormorants on Lake Ontario did not appear to significantly impair the levels of thiamine in their eggs. However, they found that the concentration of thiamine in eggs was inversely related to lead concentration according to the equation: $T = -3.142 \, Pb + 16.25$. This relationship may reflect the known ability of thiamine to chelate lead and increase its excretion [15].

Analytical methods and instruments for detection of lead(II) ions have been reviewed several times [16-20]. To detect lead(II) ions in various types of samples, atomic absorption spectrometry [21]

and electrothermal and flame atomic absorption spectrometry (ETAAS-FAAS) with Zeeman-effect background correction [22,23] are commonly used.

As a promising spectrometric techniques for this purpose, laser induced breakdown spectroscopy (LIBS) and laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) can be considered due to the ability to determine high-resolution mapping of accumulation and distribution of heavy metals [24-32]. Electrochemical ones are among the very sensitive analytical methods available for detection of heavy metal ions [6,33-42]. The classic instrument consists of a potentiostat/galvanostat with an electrochemical cell including three electrodes (working, reference and auxiliary).

However the current trend of analytical techniques is to miniaturize the whole instrument due to the many advantages of small devices including portability, low costs and less demands on service and operations, sufficient sensitivity and selectivity [43,44]. To miniaturize whole instrument, nano-based materials are used [43-59]. Besides the electrodes, the potentiostat controlling the electrode system also has to be miniaturized, portable and easy-to-use.

The aim of this study was to design and perform experiments studying the interaction of lead(II) ions and proteins contained in egg albumen. For this purpose, we used electrochemical and electrophoretic techniques. In addition, we employed optimized methods for studying of lead(II) induced complexes in died embryo of vulture. The results bring new and unique point of view in metallomics research and may provide new insights into mechanisms during embryonic development [60].

2. EXPERIMENTAL PART

2.1 Biological materials

Cinereous vultures (*Aegypius monachus*) were accidentally exposed to lead contamination in their aviaries, ZOO, Prague, Czech Republic. Water and food was applied according to standard protocols.

Vulture embryo died in the egg coming from an intoxicated female vulture was removed. Autopsy was performed and subsequently various tissues were obtained. Samples were carefully prepared to avoid external contamination and stored at -25 °C in freezer (Lieber) until chemical analysis. For controlled experiment, hens' eggs were used.

2.2 Chemicals and materials

Stock solutions of 1 mg/ml of Pb(II) were prepared by dissolving appropriate amount of lead nitrate. Working standard solutions were prepared daily by diluting the stock solutions. All other chemicals used were purchased from Sigma Aldrich (USA) unless noted otherwise. Acetate buffer of pH 5 was prepared by 0.2 M acetic acid and 0.2 M sodium acetate and diluted with water and used as a

supporting electrolyte. The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic).

The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.3 Differential pulse voltammetry Brdicka reaction for metallothionein determination

Differential pulse voltammetric measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder (4 °C). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and glassy carbon electrode was auxiliary. For data processing GPES 4.9 supplied by EcoChemie was employed.

The analysed samples were deoxygenated prior to measurements by purging with argon (99.999 %), saturated with water for 120 s. Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer (NH_3(aq) + NH_4Cl, pH = 9.6) was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: initial potential of –0.7 V, end potential of –1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude -250 mV, E_{ads} = 0 V, volume of injected sample: 20 µl (100 × diluted sample with 0.1 M phosphate buffer pH 7.0). All experiments were carried out at temperature 4 °C employing thermostat Julabo F25 (Labortechnik GmbH, Germany).0.01 g of samples in 500 µl of phosphate buffer (0.2 M, pH 6.9) was mechanically disintegrated using the Ultra-Turrax T8 homogenizer (Ika, Germany) placed in ice bath for 3 min. at 25 000 rpm. The samples of blood serum and the cell homogenate were kept at 99 °C in a thermomixer (Eppendorf 5430, Germany) for 15 min with shaking. The denatured homogenates were centrifuged at 4 °C, 15 000 × g for 30 min. (Eppendorf 5402, Germany). Heat treatment effectively denatures and removes thermolabile and high molecular mass proteins out from the samples [61]. The prepared samples were used for metallothionein analyses.

2.4 Electrochemical determination of lead(II) ions using hanging mercury drop electrode

Electrochemical analyser (Metrohm AG, Switzerland) was used for determination of Pb(II) [62]. The analyser (797 VA Computrace from Metrohm, Herisau, Switzerland) employs a conventional three-electrode configuration with a hanging mercury drop electrode (HMDE) working electrode: 0.4 mm^2, Ag/AgCl/3MKCl as reference electrode, and a platinum auxiliary electrode. The following setup assembled of automated voltammetric analysis is supplied by Metrohm. A sample changer (Metrohm 813 Compact Autosampler) performs the sequential analysis of up to 18 samples in plastic test tubes. For the addition of standard solutions and reagents, two automatic dispensers (Metrohm 765 Dosimat) are used, while two peristaltic pumps (Metrohm 772 Pump Unit, controlled by Metrohm 731 Relay

Box) are employed for transferring the rinsing solution in the cell and for removing solutions from the voltammetric cell.

Differential pulse voltammetric measurements were carried out under the following parameters: deoxygenating with argon 60 s; deposition potential -1.3 V; time of deposition 240 s; start potential -1.3 V; end potential 0.15 V; pulse amplitude 0.025 V; pulse time 0.04 s; step potential 5.035 mV; time of step potential 0.3 s.

*2.5 Capillary chip electrophoresi*s

Analyses on an automated microfluidic Experion electrophoresis system (Bio-Rad, USA) were carried out according to the manufacturer's instructions with supplied chemicals (Experion Pro260 analysis kit, Bio-Rad).

A sample (4 μl) was mixed with 2 μl of non-reducing sample buffer, and after 4 min boiling, 84 μl of water was added. After the priming of the chip with the gel and gel-staining solution in the diluted priming station sample, the mixture (6 μl) was loaded into the sample wells. The Pro260 Ladder included in the kit was used as a standard. For operation and standard data analysis Experion software v. 3.10 (Bio-Rad, USA) was used.

2.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS_PAGE)

The electrophoresis was performed according to Laemmli [63] using a Mini Protean Tetra apparatus with gel dimension of 8.3 × 7.3 cm (Bio-Rad, USA). First 15 % (m/V) running, then 5 % (m/V) stacking gel was poured. The gels were prepared from 30 % (m/V) acrylamide stock solution with 1 % (m/V) bisacrylamide. The polymerization of the running or stacking gels was carried out at room temperature for 45 min or 30 min, respectively. Prior to analysis the samples were mixed with non-reduction sample buffer in a 2:1 ratio. The samples were boiled for 2 min, and then 4 μl of the sample was loaded onto a gel. For determination of the molecular mass, the protein ladder "Precision plus protein standards" from Biorad was used. The electrophoresis was run at 150 V for 1 h (Power Basic, Biorad USA) in tris-glycine buffer (0.025 M Trizma-base, 0.19 M glycine and 3.5 mM SDS, pH = 8.3). Silver staining of the gels was performed according to Oakley et al. [64].

2.7 Preparation of samples for determination of lead(II) ions

To prepare the samples microwave digestion were used according to recently published papers [65,66]. Briefly, the mineralization of samples took place in a microwave system Multiwave3000 (Anton-Paar GmbH, Austria).

A sample (10 mg of egg sample) was placed into glass vials MG5 and 700 μl of nitric acid (65 %, *w/w*) and 300 μl of hydrogen peroxide (30 %, *w/w*) were added. Prepared samples were sealed and placed into the rotor 64MG5 (Anton-Paar GmbH, Austria). Rotor with the samples was inserted into the microwave system and the microwave digestion was carried out under various conditions to

optimize them as power (50, 100, 150 and/or 200 W) and time of mineralization (15 and/or 30 min.), maximum temperature 80 °C.

Using each possible combination of power: time five samples was mineralized. Sample preparation for subsequent electrochemical measurements was as follows: 100 μl mineralized sample was pipetted into Eppendorf tubes with 900 μl acetate buffer (pH = 5.00). A blank digestion was simultaneously carried out in the same way.

2.8 Automated spectrometric measurements for determination of zinc(II) ions

For determination of minerals, automated spectrophotometer BS–400 (Mindray, China), which consists of cuvette space (tempered to 37±0.1 °C), reagent space with carousel for reagents and preparation of samples (tempered to 4±1 °C) and optical detector with the tungsten halogen lamp as source of radiation, was used. Transfer of samples and reagents is provided by robotic arm with dosing needle. Content of cuvettes is mixed by automatic stirrer immediately after reagent or sample in volume of 2-45 μl addition. Contamination of reagents and samples is minimized due to the rising of dosing needle as well as stirrer by MilliQ water. For detection itself, following wavelengths could be used: 340, 380, 412, 450, 505, 546, 570, 605, 660, 700, 740, and 800 nm. Into plastic cuvettes, volume of 200 μl of kit for determination of zinc supplied by Greiner Company was pipetted (Greiner, Germany).

Subsequently, 20 μl of measured sample was added. Absorbance was measured for 10 min. at λ = 678 nm for determination of zinc. For element content estimation, values of absorbance of reagent and absorbance of sample after 10 min of incubation were used. Values were subtracted and results recalculated in accordance with calibration curve for given element.

2.9 Mathematical treatment of data and estimation of detection limits

Mathematical analysis of the data and their graphical interpretation was realized by software Matlab (version 7.11.). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). The detection limits (3 signal/noise, S/N) were calculated according to Long and Winefordner [67], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

2.10 Accuracy, precision and recovery

Accuracy, precision and recovery of heavy metals were evaluated with homogenates (tissue extract) spiked with standards. Before extraction, 100 μl lead(II) ions standard, 100 μl water were added to samples. Homogenates were assayed blindly and heavy metals concentrations were derived from the calibration curves. Accuracy was evaluated by comparing estimated concentrations with known concentrations of heavy metals compounds. Calculation of accuracy (%Bias), precision (%C.V.), root mean square error (RMS error) and recovery was carried out as indicated by Causon [68].

3. RESULTS AND DISCUSSION

3.1 Electrochemical determination of lead(II) ions

Lead(II) ions still represent a considerable threat in areas with heavy mining industry. It is not surprising that these ions must be carefully monitored in the environment. Electrochemical determination of lead using mercury drop electrode has been greatly used for various purposes including attention to the perspective of automation steps [6]. In this study we focused on new directions in the use of electrochemistry, especially in the field of metal ion interactions with biomolecules.

Very high concentrations of toxic heavy metals cause significant changes in organisms already at the cellular level, but based on them it is not easy to determine their whole-body effect. Biologically important manifestations at the cellular level (no sign of acute toxicity), however, can be studied only under very low concentrations of pollutant. For this purpose it is necessary to have appropriate analytical tools that are sufficiently sensitive and robust (miniaturization) [62]. Detailed study requires very demanding experiments that without these basic conditions cannot be realized. Our experimental determination was performed in a fully automated laboratory system (the exact dosage of the sample, electrolyte replacement and cleaning of automated electrochemical cell), which was described in our previous papers [6,62,66].

3.2 Electrochemical study of lead(II) ions interactions with albumen proteins

Typical calibration dependence of peak height on concentration of lead(II) ions measured by differential pulse anodic stripping voltammetry (DPASV) in the presence of 0.2 M acetate buffer (pH 5.0) is shown in Fig. 1A. The peak of lead(II) ions was detected at 0.45 V (inset in Fig. 1A). The obtained dependence was linear with R^2 higher than 0.99 with relative standard deviation below 3 %. The limit of detection determined by dilution of the stock solution at 240 s accumulation time was 1 nM. The limit of quantification was determined as 5 nM. The results obtained are comparable with our previous paper [6].

It was also designed a simple experiment to verify our capabilities in the field of interaction of lead(II) ions with biomolecules (very complex biological matrix of egg albumen). Hen egg albumen was diluted to a total protein concentration of 10 mg/ml. The following concentrations of lead(II) ions (1, 3, 4, 6, 7, 8 and 9 μM) were added into egg albumen. The peak of lead(II) ions were detected and the dependence of its height on concentration of these ions is shown Fig. 1B. The dependence was also linear with R^2 higher than 0.99 but with relative standard deviation higher than 8 %. The presence of egg albumen caused higher standard deviations compared to standard and also caused decrease of detected peak for more than 40 %.

The experiment showed that the suggested experimental model could be used to study the lead-protein interactions.
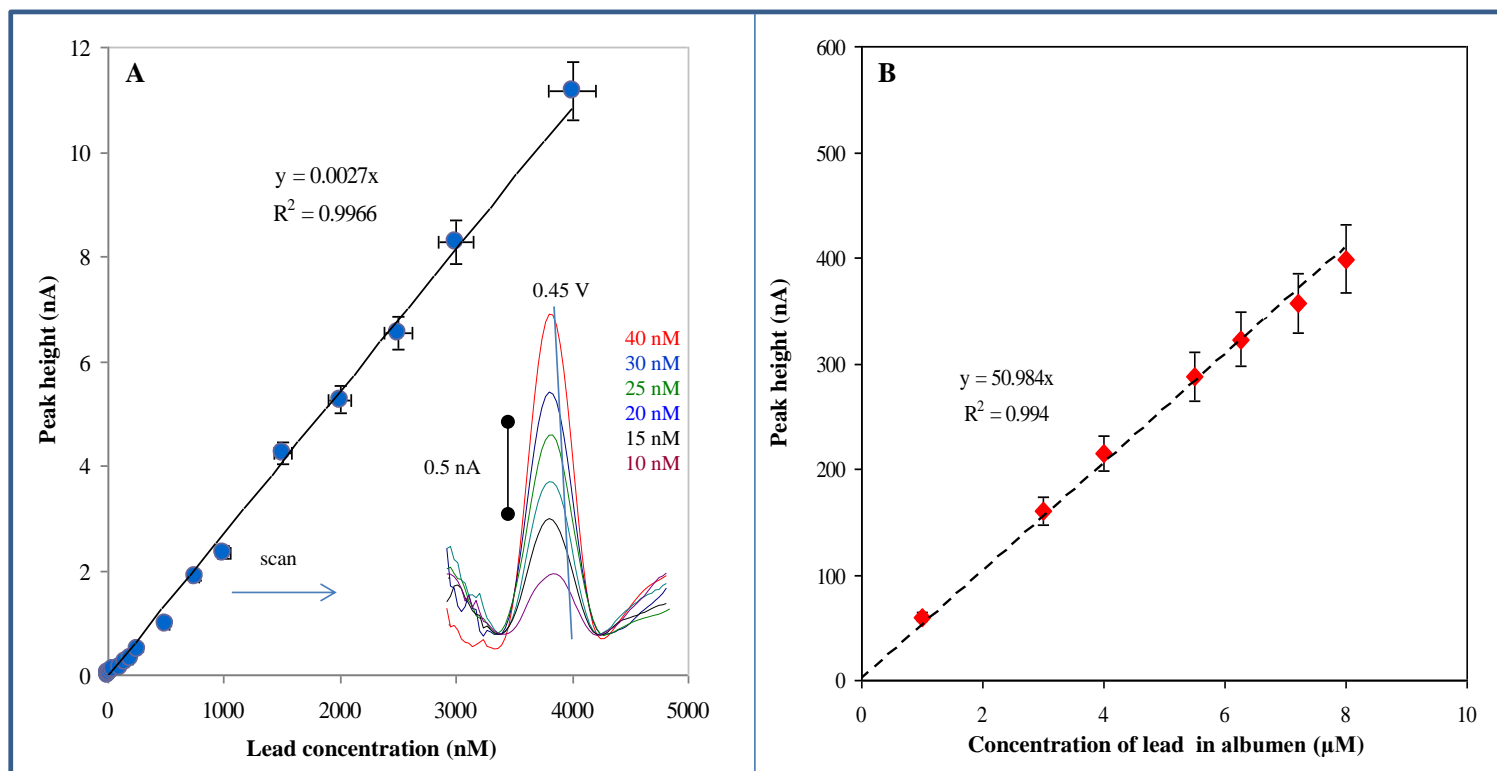
Figure 1. (**A**) Typical dependence of peak height on various concentrations of lead(II) ions measured by DPASV in the presence of 0.2 M acetate buffer (pH 5.0); in inset: DP voltammograms of lead(II) ions. (**B**) The effect of 10 mg/ml albumen on various concentrations of lead(II) ions (1, 3, 4, 6, 7, 8 and 9 µM). Differential pulse voltammetric measurements were carried out under the following parameters: deoxygenating with argon 60 s; deposition potential -1.3 V; time of deposition 240 s; start potential -1.3 V; end potential 0.15 V; pulse amplitude 0.025 V; pulse time 0.04 s; step potential 5.035 mV; time of step potential 0.3 s.

3.3 Effect of incubation time, temperature and concentration of lead(II) ions

In the following experiments, we focused on a very detailed study of the interaction of lead(II) ions with the egg albumen proteins. The egg albumen is about two-third of the total eggs weight out its shell, with nearly 92 % of the weight coming from water. The remaining weight of the egg albumen comes from protein, trace minerals, fatty material, vitamins and glucose. In 40 grams of an egg, there is app. 5 g of proteins, 0.3 g of carbohydrates and 60 mg sodium. Ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), ovoglobulin G2 and G3 (8%), ovomucin (3.5%), lysozyme (3.4%), ovoinhibitor (1.5%), ovoglycoprotein (1%), flavoprotein (0.8%), ovomacroglobulin (0.5%), avidin (0.05%) and cystatin 0.05% (total 95.8% listed) belong to the most commonly occurred proteins in eggs. It is obvious from this list that such a complex matrix must interact with lead(I) ions and form numerous complexes. Experimental plan how to study these interactions was as follows. Firstly, egg albumen was diluted with acetate buffer in the following ratios buffer:albumen – 1:0.5; 1:1; 1:2 and 1:4. Additions of different concentrations of lead(II) ions into the diluted egg albumen were 0; 1; 5; 10;

25; 50; 100; 150; 200; 250; 500; 750 and 1,000 nM. Mixtures were then placed on thermoblock at 37 °C for 15, 30, 45 and 60 min.

Primarily, we used DPASV to analyse above prepared mixtures. The results obtained are shown in Fig. 2. The changes in peak height with the increasing time of incubation are shown in Fig. 2A (15 min.), Fig. 2B (30 min.), Fig. 2C (45 min.) and Fig. 2D (60 min.). All dependencies were linearly plotted to better describe the apparent changes. It clearly follows from the obtained experimental data that there is a very strong interactions of lead(II) ions and biomolecules contained in egg albumen. Rapid increase in the concentration of lead(II) ions with a linear trend was detected in the mostly diluted egg albumen (lower concentration of total protein, Fig. 2A). Nevertheless, peak height of the same concentration measured in the presence of pure supporting electrolyte was app. 15 % higher compared to those determined in this solution. The peak height decreased with the increasing content of proteins in the mixture.
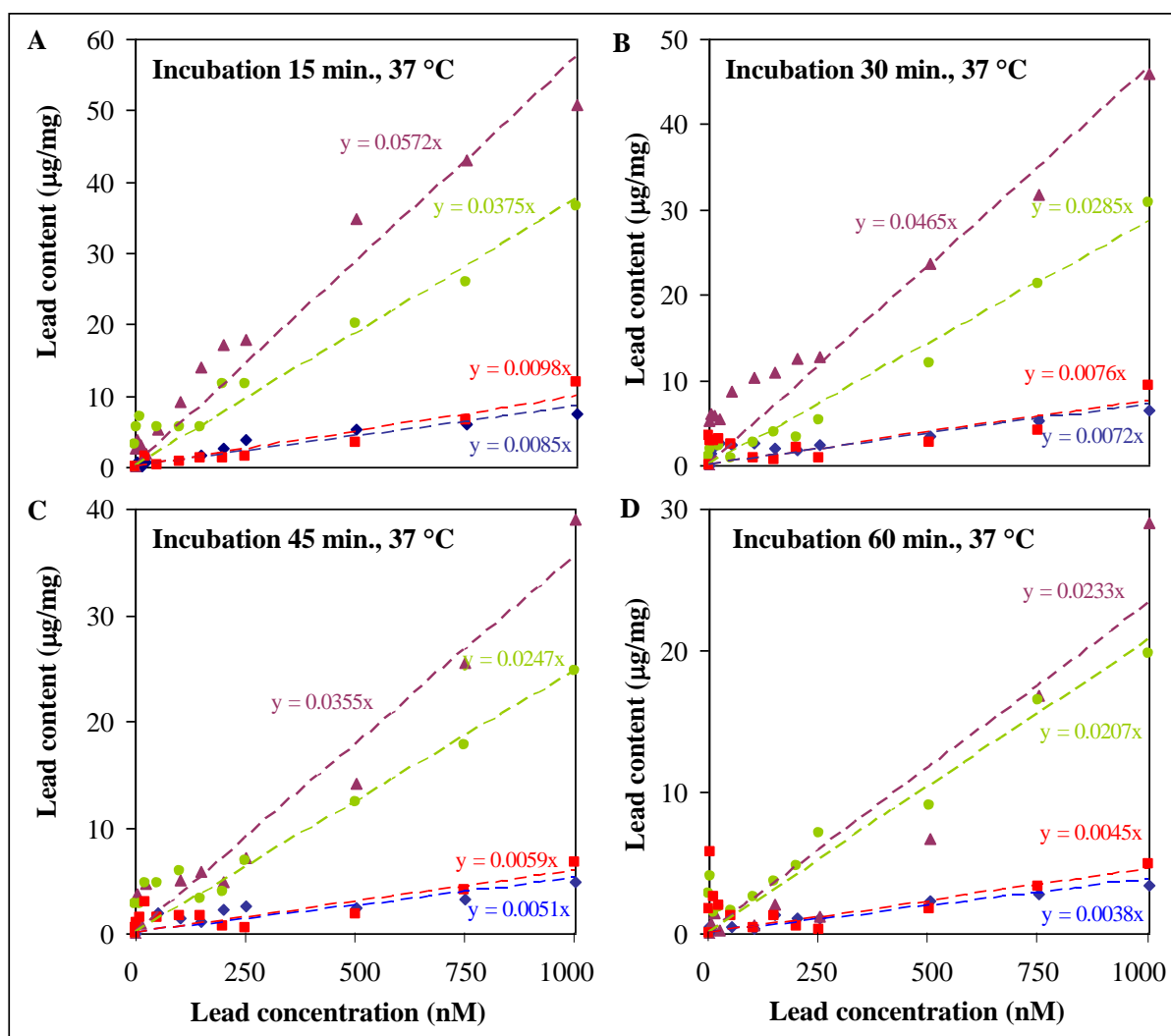
Figure 2. Content of lead related to the total content proteins. Ratios of albumen to acetate buffer (1:0.5 -♦-; 1:1 -■-; 1:2 -●-; 1:4 -▲-) and the applied concentrations of lead(II) ions (0; 1; 5; 10; 25; 50; 100; 150; 200; 250; 500; 750 and 1,000 nM). These mixtures were incubated for (**A**) 15, (**B**) 30, (**C**) 45 and (**D**) 60 min. at 37 °C (thermoblock, 400 rpm). DPASV conditions are shown in caption of Figure 1.

The study of interaction with lead(II) ions egg (1:2) leads to a decrease in the peak of lead(II) ions for more than 20 %.

Further increasing the concentration of protein content, however, causes a dramatic reduction in the detected signal for more than 80 %. In Figs. 2A, B and C, there is observable non-linear part within concentration of lead(II) ions from 1 to 250 nM. In this area it is probably the most significant interactions between the protein and lead(II) ions. At higher concentrations and longer time of interactions, metal ions bind tightly or intercalate into biomolecules presented in egg albumen.

This hypothesis is clearly confirmed by the obtained experimental data and by other *in vivo* study, in which cadmium and lead levels in feathers of mated pairs of common terns (*Sterna hirundo*) and in their eggs was determined if metal levels in eggs correlated with female levels, and whether there were intrapair and intermetal correlations. Eggs had significantly lower lead levels (89 ng/g) and cadmium levels (4.0 ng/g) than adult feathers (500 and 50 ng/g respectively). Adult females had higher metal levels than males. Cadmium and lead levels were correlated across families for females, males and eggs.

Lead, but not cadmium, levels were correlated in females and their eggs [69]. The result therefore suggests that biomolecules (especially proteins) can be very effective and protective mechanism against the effects of heavy metals and the interactions with lead(II) ions predominate.

3.4 Rate of lead(II) ions binding into proteins contained in egg albumen

Time changes of interactions between proteins of egg albumen with lead(II) ions are shown in Fig. 3.

Data were obtained from those presented as slopes in Fig. 2. It is clearly evident that the interaction between biomolecules and lead(II) ions is very intense according to the obtained results (Figs. 3A, B, C and D). All slopes steep decreased with the increasing time of interaction and content of egg albumen proteins (15 min interaction, the ratio of 1:4 and the slope 0.06; the ratio of 1:2 and the slope 0.038; the ratio of 1:1 and the slope 0.009; the ratio of 1:0.5 and the slope 0.0085).

Moreover, it is evident a clear time-dependence of binding lead(II) ions to biomolecules. In all studied ratios, the slope change is linear with R^2 from 0.95 to 0.99. In the fact that the time dynamics of binding of lead(II) ions into the egg albumen proteins was investigated, an interesting phenomenon was found.

At the ratio of 1:0.5 there was observed the bond of 0.06 ng Pb per min. into biomolecules of egg albumen, at the ratio of 1:1, the bond of 0.08 ng Pb per min., at the ratio of 1:2 the bond of 0.3 ng Pb per min. and at the ratio of 1:4 the bond of 0.55 ng Pb per min. Most likely there is a change in the structure of these proteins in the case of lower concentrations of albumen proteins. A more open structure is then able to bind higher lead(II) ions concentrations compared to higher concentrations of proteins with more compact structure.
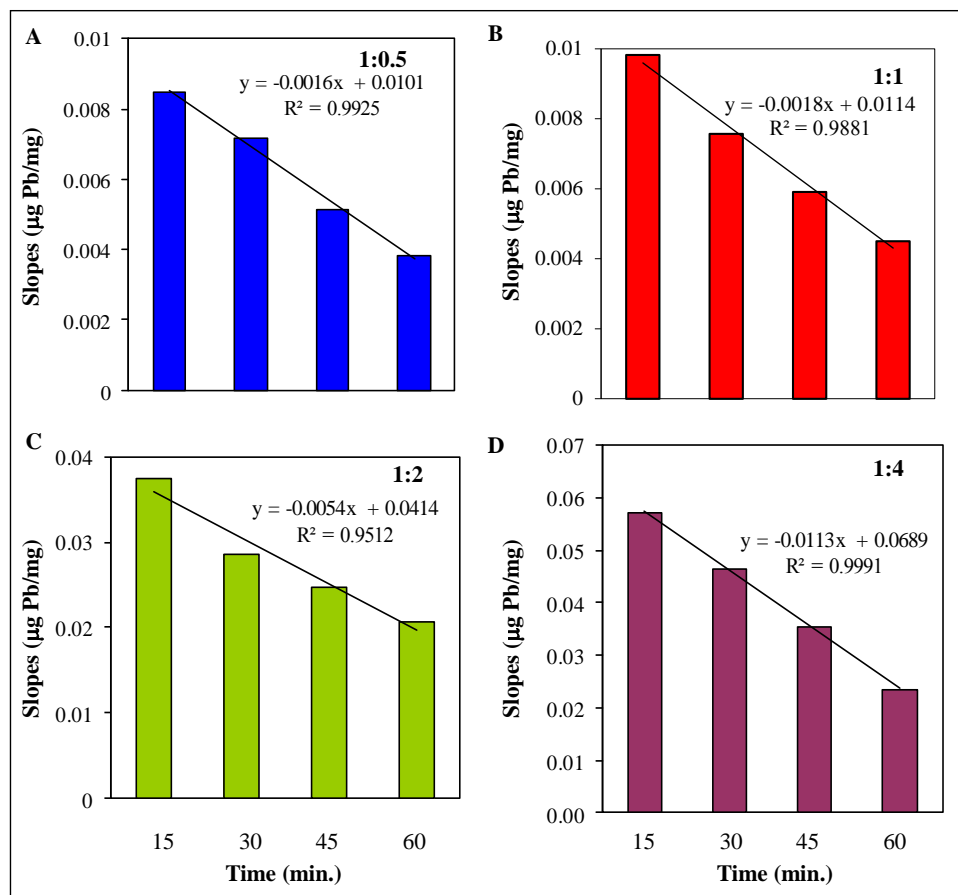
Figure 3. Dependence of slopes of linear plots shown in Figure 2 on the time of incubation for the following acetate buffer:albumen ratios − (**A**) 1:0.5; (**B**) 1:1; (**C**) 1:2 and (**D**) 1:4. Other experimental conditions are shown in captions of Figures 1 and 2.

*3.5 Study of changes in protein profiles of egg albumen after their interactions with lead(II) ions*

Proteome analysis is based on different technological platforms. The aim of the procedure is the revealing of role and function of individual proteins or groups of proteins [70]. The samples obtained by incubation of various concentrations of lead(II) ions with egg albumen protein prepared in Section 3.3 were analysed by chip capillary electrophoresis under non-reducing conditions to study the influence of lead(II) ions on the protein profiles of egg albumen. This method has been shown as a suitable for the studying of proteins and their interactions [71-76]. Experiments were carried out accordingly. A mixture of sample and buffer was incubated at 37 °C for 15 min. We have identified key proteins of albumen proteome such as lysozyme, flavoprotein, ovalbumin, ovomucoid, avidin and ovotransferrin being 75 % of all proteins in egg albumen. Typical electropherogram after 15 min interaction without the presence of lead(II) ions (Fig. 4A) and after addition of 1,000 nM lead(II) ions (Fig. 4B) are shown. Fig. 4C shows changes in protein profile determined by capillary chip electrophoresis. The picture reveals new peaks due to the addition of lead(II) ions on the size of 95 kDa and 123 kDa. Both the observed signals increased with the increasing applied concentration of

lead(II) ions. It can be assumed that these signals are lead-induced proteins [77]. Ovalbumin, lysozyme and ovotransferrin are known to bind divalent heavy metals. Due to this fact one may expect the majority of their role in binding heavy metals.
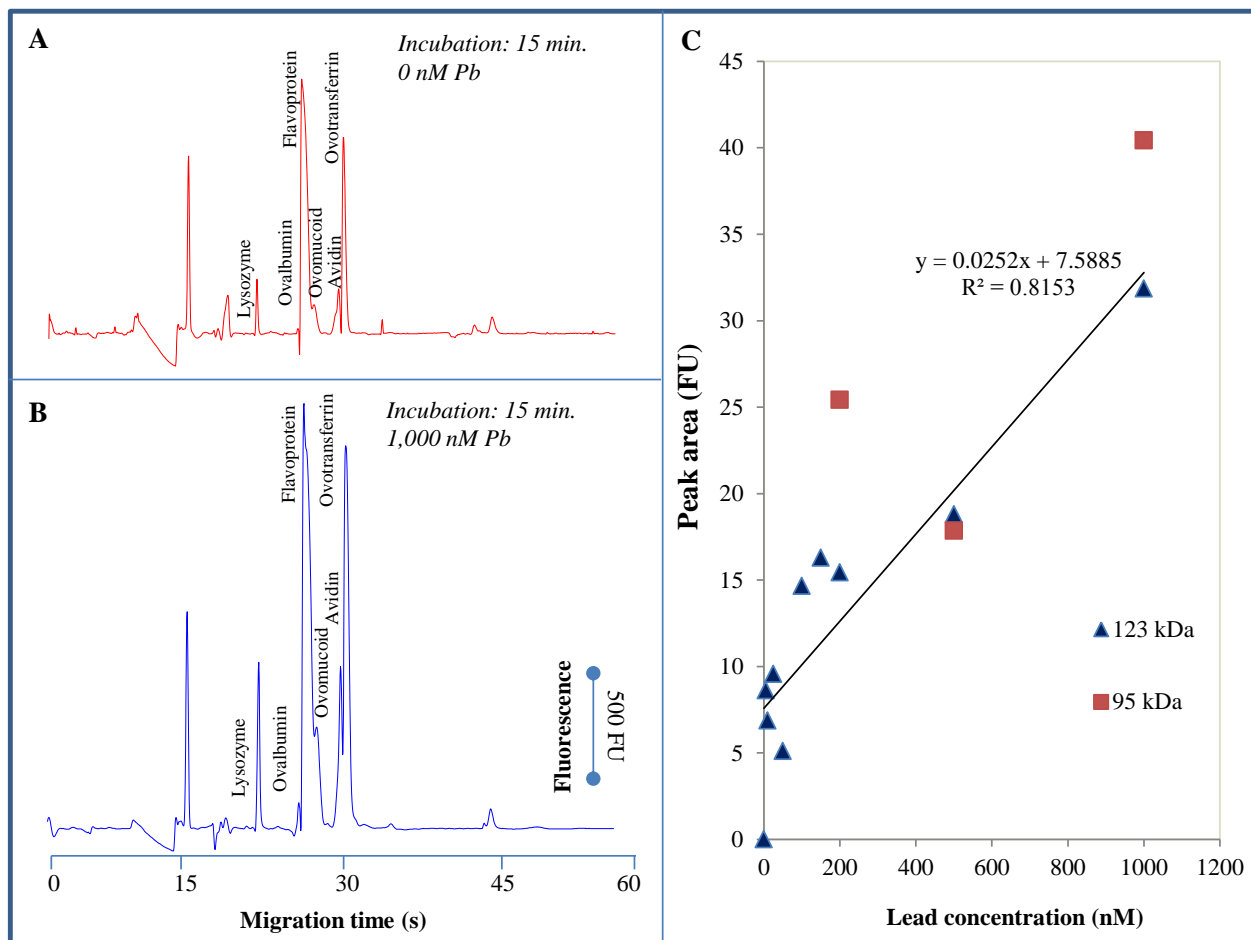
Figure 4. Capillary chip electrophoresis of egg albumen (**A**) without lead(II) ions and (**B**) after 15 min. long interaction with 1,000 nM lead(II) ions. (**C**) Changes of content of proteins with the following molecular mass 95 kDa and 125 kDa with the increasing concentration of lead(II) ions. Time of interaction 15 min at 37 °C in the presence of 0.2 M acetate buffer (pH 5).

Typical electrophoreograms of 1:0.5, 1:1, 1:2 and 1:4 mixtures with additions of various lead(II) ions concentrations are shown in Figs. 5A, B, C and D, respectively. It clearly follows from the results obtained that higher applied concentrations of lead(II) ions caused decrease of proteins with higher molecular masses. In addition, we found that some protein profiles were concentrated at more diluted samples (1:2 and 1:4) due to the presence of lead(II) ions (Figs. 5C and D). These results confirms hypothesis of higher rate of lead(II) ions binding into egg albumen proteins with the increasing concentration of lead(II) ions (Fig. 3).
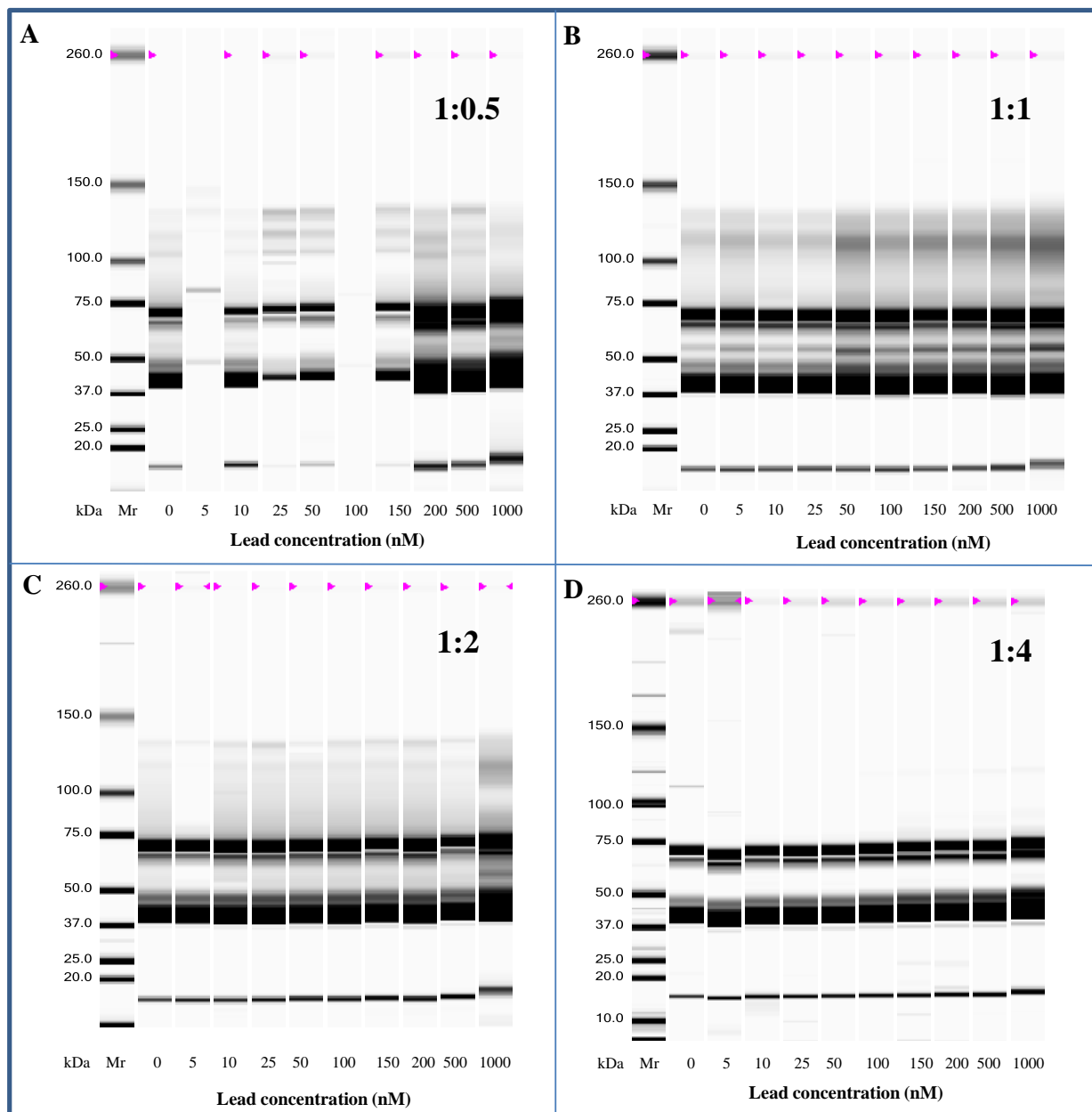
Figure 5. Electrophoreograms of various concentrations of lead(II) ions (0; 1; 5; 10; 25; 50; 100; 150; 200; 250; 500; 750 and 1,000 nM) added into egg albumen of the following dilutions with acetate buffer (**A**) 1:0.5; (**B**) 1:1; (**C**) 1:2 and (**D**) 1:4. Time of interaction 15 min at 37 °C in the presence of 0.2 M acetate buffer (pH 5).

Further, we determined changes in the content of the selected proteins as lysozyme, flavoprotein, ovalbumin, ovomucoid, avidin and ovotransferrin and some unidentified as marked according to their molecular masses 55, 95, 110, 117, 123, 132 and 170 kDa. We found that signal intensity of selected proteins enhanced with the increasing concentration of lead(II) ions (Fig. 6). Changes in protein profiles were linear with R^2 higher than 0.9 at lower dilutions of egg albumen proteins (1:4, 1:2). These changes were observed mainly for ovomucoid, ovotransferrin, avidin and lysozyme. In the case of ovalbumin coefficient of determination was 0.87. Peak increase was not linear

for flavoprotein and 55 kDa unidentified protein with coefficients of determination as 0.71 and 0.48, respectively. There was also a mobility shift of peaks towards higher molecular masses of app. 4 kDa. We also found new peaks of higher molecular mass (100 kDa), which arose probably due to changes in the structure of egg proteins due to the presence of lead(II) ions. These new peaks had coefficients of determination higher than 0.8. In the case of 1:0.5 dilution there was observed a dramatic change in protein profile identified as ovotransferrin (Fig. 6A). Ovotransferrin signal enhanced markedly with the increasing applied concentration of lead(II) ions. A similar enhancement of signal was observed for other proteins (lysozyme, avidin and ovoalbumin). For further dilutions of 1:1, 1:2 and 1:4, there were observed the most significant changes in protein profile mainly in two proteins as ovomucoid and ovotransferrin (Figs. 6B, C and D). These changes are not detectable for other proteins. The obtained data are summarized in Fig. 6E. Based on determined protein profiles (as slopes of signal changes depending on the applied lead(II) concentration) at various dilutions it is clearly evident that the most significant changes are detected at ovotransferrin, avidin, lysozyme and ovomucoid.
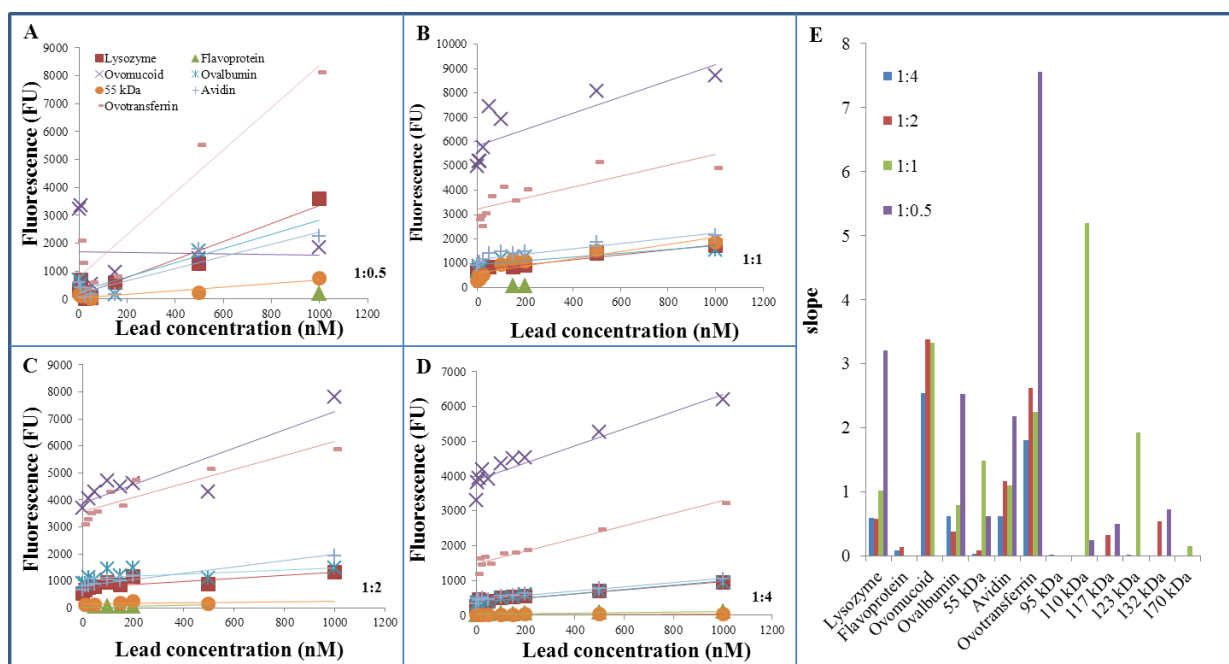
Figure 6. Changes of protein profiles of various concentrations of lead(II) ions (0; 1; 5; 10; 25; 50; 100; 150; 200; 250; 500; 750 and 1,000 nM) added into egg albumen of the following dilutions with acetate buffer (**A**) 1:0.5; (**B**) 1:1; (**C**) 1:2 and (**D**) 1:4. (**E**) Slopes of dependencies obtained in the previous figures. Time of interaction 15 min at 37 °C in the presence of 0.2 M acetate buffer (pH 5).

3.7 Lead(II) ions and their effects on vulture embryo

In our previously published paper we analysed blood samples obtained from vultures poisoned by lead(II) ions. The captive vultures were exposed to lead contamination in Prague ZOO (lead paint contained more than 20 mg/g of lead). In the cage, where they vultures are bred, in several birds there

were observed developed clinical signs of lead intoxication and after a few days these individuals died. Levels of lead were in the range 2-10 mg/g liver [6]. It was also found that one intoxicated female laid eggs, which were subsequently incubated. During the perching on the eggs, signs of acute intoxication of female vulture were evident and female died due to this. Eggs were collected and the foetus was subsequently analysed in detail in this study. Foetal autopsy was performed and single tissues (albumen, yolk, liver, kidney, brain, bone) were obtained. Contents of lead(II) ions, reduced and oxidised glutathione, metallothionein and zinc(II) ions were determined.

Lead(II) ions.

We focused primarily on analysing the content of lead(II) ions in these tissues. The results are shown in Fig. 7A. The highest lead contents were observed in liver and kidney app. 5 μg/g tissues. The burden of these tissues is expected in the view of their metabolic activity and the presence of the main detoxification mechanisms. On the other hand, content of lead in bone and brain was low, probably because of short-term exposure.
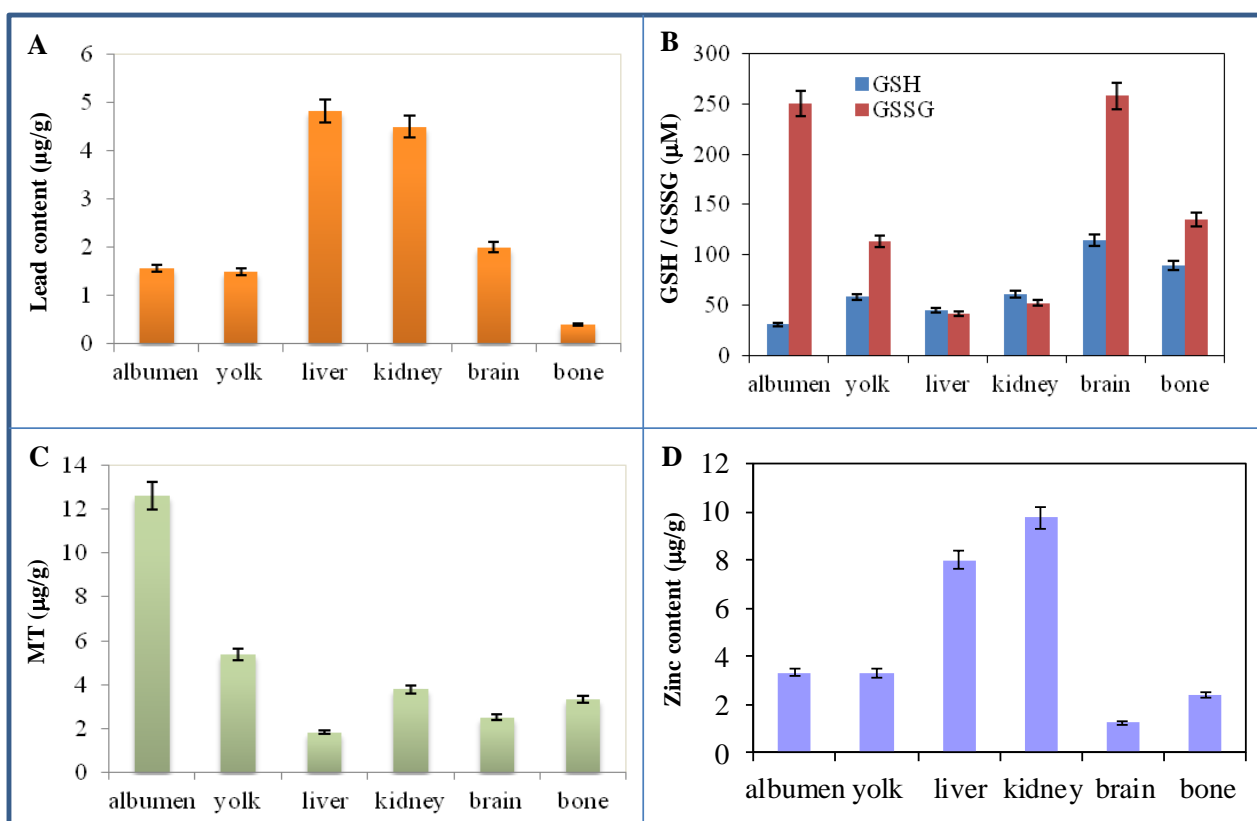
Reduced and oxidised glutathione.

Organisms protect themselves against metal ions via synthesis of thiols as reduced glutathione (GSH). GSH, a ubiquitous tripeptide thiol, is a vital intra- and extra-cellular protective antioxidant, which plays a number of key or crucial roles in the control of signalling processes, detoxifying of some xenobiotics and heavy metals as well as other functions. Glutathione is found almost exclusively in its reduced form; since the enzyme, which reverts it from its oxidized form (GSSG) called glutathione reductase, is constitutively active and inducible upon oxidative stress. In fact, the ratio of reduced to oxidized glutathione within cells is often used as a marker of cellular toxicity [78-81]. Therefore, we determined levels of both compounds. The highest contents of GSH and GSSH were found in brain tissue and in albumen (Fig. 7B). Brain content can be associated with the needs to protect neural system against reactive oxygen radicals. The lowest ones were determined in liver and kidney. This fact is in well agreement with the determination of lead(II) ions, because burden of these tissues can cause depletion of free GSH and thus GSSG, which are not detectable by HPLC-ED. Decrease of GSH levels with the increasing concentration of placental lead was determined also in women [82]. This fact can be related to triggering of some protective mechanisms already in placenta to defend embryo. The other authors found that intoxication of rats by lead caused the increasing of weights of liver, kidney, spleen and brain a the higher contents of glutathione in erythrocytes, liver and kidney were determined [83].

Metallothionein.

Metallothioneins (MTs) were discovered by Margoshes and Valee in 1957 as newly identified proteins isolated from a horse renal cortex tissue [84]. These proteins occur in whole animal kingdom with high degree of homology. Similar proteins are expressed by bacteria, fungi and even plants. MTs are low molecular mass (from 2 to 16 kDa) proteins with unique abundance of cysteine residues (more than 30 % from all aminoacids). Other interesting structural property is the lack of aromatic amino acids. However - as discovered recently – there is an exception: a group of certain yeast and bacterial species rarely containing histidine [85]. The main function of MTs in organism is a metal ion transport, maintenance of the oxidative-reducing conditions, regulation of gene expression and detoxification of metal ions [86-88]. It is well known that this protein binds heavy metal ions and its level is a good

indicator of heavy metal intoxication [68]. This study was based on published paper aimed at determination of metallothionein in biological samples [89-97]. The highest content of MT was determined in albumen (more than 12 µg/g) followed by yolk and kidney, and the lowest in liver (Fig. 7C). This is very interesting result, which shows a highly active defence of the embryo against lead(II) ions. In addition, it can be assumed that MT plays a role in antioxidant metabolism as it was demonstrated in the paper [98]. The authors found that rat hepatic apo-metallothionein and to a lesser extent Zn-MT inhibit Fe2+-dependent lipid peroxidation in suspensions of egg yolk lipoproteins. ZnCl2 or its combination with cysteine at corresponding concentrations activate lipid peroxidation [98]. The other paper shows that the interaction of Pt, Pd and Rh in the mixture seems to favour metal accumulation and MT induction in the liver but not the brain. These results indicate that induction of MT plays a protective role against these metal ions. Results may imply that MT has an important role as a tolerance mechanism against heavy metals toxicity [99].

Figure 7. Effect lead intoxication on vultures' embryo. Twenty days old foetus of lead poisoned female vulture was analysed. Content of (**A**) lead, (**B**) reduced (GSH) and oxidised (GSSG) glutathione, (**C**) metallothionein and (**D**) zinc ions. For more details see in Experimental section.

*Zinc(II) ions.*

The level of zinc(II) ions was highest in organs with maximum biochemical activity (liver and kidney, Fig. 7D). The role of essential elements as possible very simple tools to prevent the transport of toxic heavy metals should be further studied intensively and is supported by studies already carried out, where this effect is demonstrated [100]. An overview of the content of zinc, iron and copper is

described in detail in the work of Richards. Essential metals are contained in the yolk followed by albumen [5]. Richards also studied the binding copper, zinc and iron on vitellogenin, lipovitellin and phosvitin [5]. In addition, significant reduction in the concentration of mineral nutrients in the avian embryo during development was determined [5].

4. CONCLUSIONS

Metallomics research is intensively developing areas. Our knowledge in this field, however, is still not sufficient. The paper discussed a potential mechanism of proteins action contained in the egg on lead(II) ions poisoning. These ions move into the egg during development after intoxication of female. Accumulation in different parts of the newly developing embryo is not entirely clear. It is possible that lead(II) ions are accumulated in organs as liver and kidney. A very important role play thiol compounds such as metallothioneins (Fig. 8). All studied changes as essential elements contents and MT levels are very interesting and should be also carefully studied.
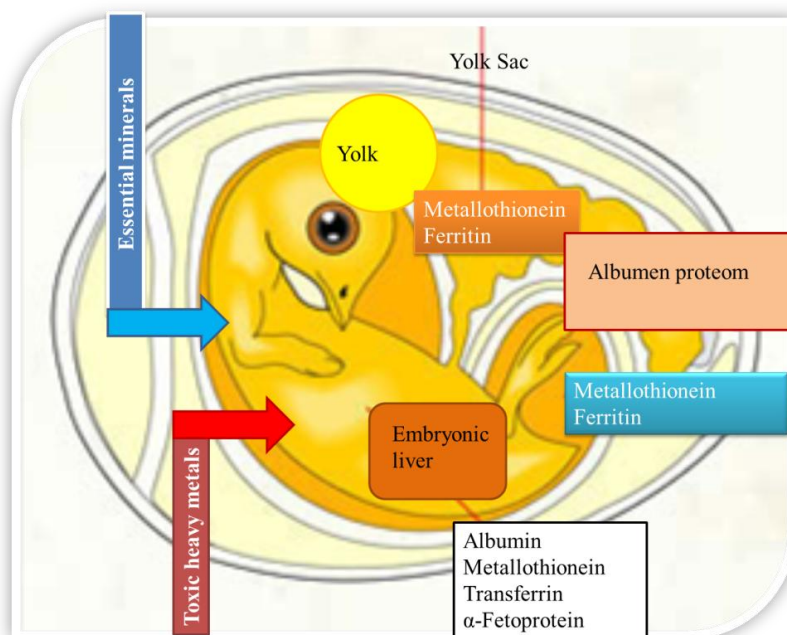
Figure 8. Scheme of lead effect in embryo. Proteins contained in the albumen interact with heavy metals and reduce their toxicity. The presence of these ions increases the synthesis of thiol compounds that are transported to other parts of the developing embryo. The scheme describes the mobilization of egg trace mineral stores in yolk and their transfer to embryonic liver. Metallothioneins probably play important role in inter-organ transport of these ions. Adopted according to Richards [5].

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