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Electrochemical Analysis of Lead Toxicosis in Vultures

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Lead as one part of heavy metals group pose a detrimental risk to human health and the environment due to its toxicity, even exposure to minuscule quantities can be life threatening. Levels of lead are not stable in the environment and vary according to industrial production, urbanization, climate changes and many other factors. It is not surprising that rapid, sensitive and simple analytical determination of metal ions in environment is very important. Electrochemical techniques are one of the best methods for detecting metals due to their low cost, high sensitivity and portability. The aim of this study was to compare various microwave digestion procedures for lead(II) ions isolation in vultures samples of vultures available in very small quantities (5-20 mg) followed by differential pulse voltammetry as a detection method. We found that the combination of (65%, *w*/*w*) HNO_3 + (30%, *w*/*w*) H_2O_2 is advantageous for the resulting electrochemical response. To determine the effect of mineralization on the height of the lead(II) ions peak, eight different mineralization programs were tested. It clearly follows from the results obtained that 200 W power for 30 min. is the most optimal for mineralisation

of vultures' samples. In the following part of the study, the influence of four different locations of samples in the rotor of used microwave system was investigated. Under the optimized conditions, samples of blood and eggs from vultures poisoned by lead(II) ions were prepared and electrochemically analysed. Three types of working electrodes as hanging mercury drop electrode, carbon paste electrode and screen printed electrode made of multi-walled nanotubes (MWNTs) were employed for this purpose. All three electrodes were sensitive enough to be used for analysis of real samples. Based on the results obtained it clearly follows that the level of lead(II) ions in intoxicated vultures ranged from 1 to 5 μg/ml of blood. In addition, it was found that the lead toxicosis was diagnosed in all vultures properly. Besides lead, cadmium was determined electrochemically and Ca, Mg, Fe, Zn and Cu spectrometrically. Besides the content of lead(II) and cadmium(II) ions, we determined metallothionein as a protein connected with protection of vertebrates against metal ions using differential pulse Brdicka reaction. The level of MT was determined as 1.82 ± 0.31 μg/mg of proteins in the intoxicated vultures compared with the control group 0.71 ± 0.19 μg/mg of proteins, which means more than a 250% increase of MT levels.

Keywords: automated electrochemical detection; bioremediation; differential pulse voltammetry; heavy metal; diagnosis of toxicosis; metallothionein; spectrophotometry

1. INTRODUCTION

Accumulation and bioaccumulation of toxic substances is known from various trophic levels [1-10]. There can be determined the highest concentrations of these pollutants in predators as a top of the food chain (Fig. 1). Lead as member of the heavy metals group pose a detrimental risk to human health and the environment due to its toxicity because even exposure to minuscule quantities can be life threatening [11,12]. Levels of lead are not stable in the environment and vary according to industrial production, urbanization, climate changes and many other factors [13]. The levels of lead in the environment vary between 4 and 20 mg/g of dust. Uncontaminated waters contain lead in concentrations ranging from 0.001 to 0.06 mg/l. In soils, levels of lead reach 5 to 30 mg per kg of soil. The concentration of lead is not clearly known in sea ecosystems, but much higher content compared to soil can be expected [14]. The toxicity of lead in humans mainly arises from its mimicking action of occupying the calcium binding sites on numerous calcium-dependent proteins in cells resulting in the corresponding impairment of physiological functions [15-17]. Lead is still of concern for possible adverse effects to wild populations. Raptors are frequently reported dying from lead(II) ions exposure through ingestion of lead-contaminated sources including lead projectiles, fragments from the ammunition and others [1,18-20]. Lead poisoning has been also reported in 31 avian species other than waterfowl [21]. Lead(II) ions influence and modify the renal function and structure of vertebrates, bone, central nervous system, hematopoietic system and have adverse biochemical, histopathological, neuropsychological, teratogenic and reproductive effects [21-23]. Lead rapidly fluctuates between red blood cells and plasma (95% of lead are bound by erythrocytes). In addition, it is well know that lead is re-distributed to bones, where these ions can persist for a long time from months to years [24]. Excretion of lead from the body is extremely slow (biological half-life estimates of 10 years) [24,25]. Reference levels of lead(II) ions determined in a non-exposed Amazon Tribe did not exceed 0.02 ppb [26]. Moreover, heavy metal ions including lead(II) can cause marked changes in the level of reactive

oxygen species, which can damage biological membranes and alter biochemical pathways. Compounds rich in thiols (reduced glutathione and enzymes connected with this tripeptide, metallothionein, and others) can scavenge radicals [27-30]. Moreover, these compounds can complex metal ions, which do not pose a threat. Based on this, heavy metal chelators are used for treatment of metal ions intoxication [31].

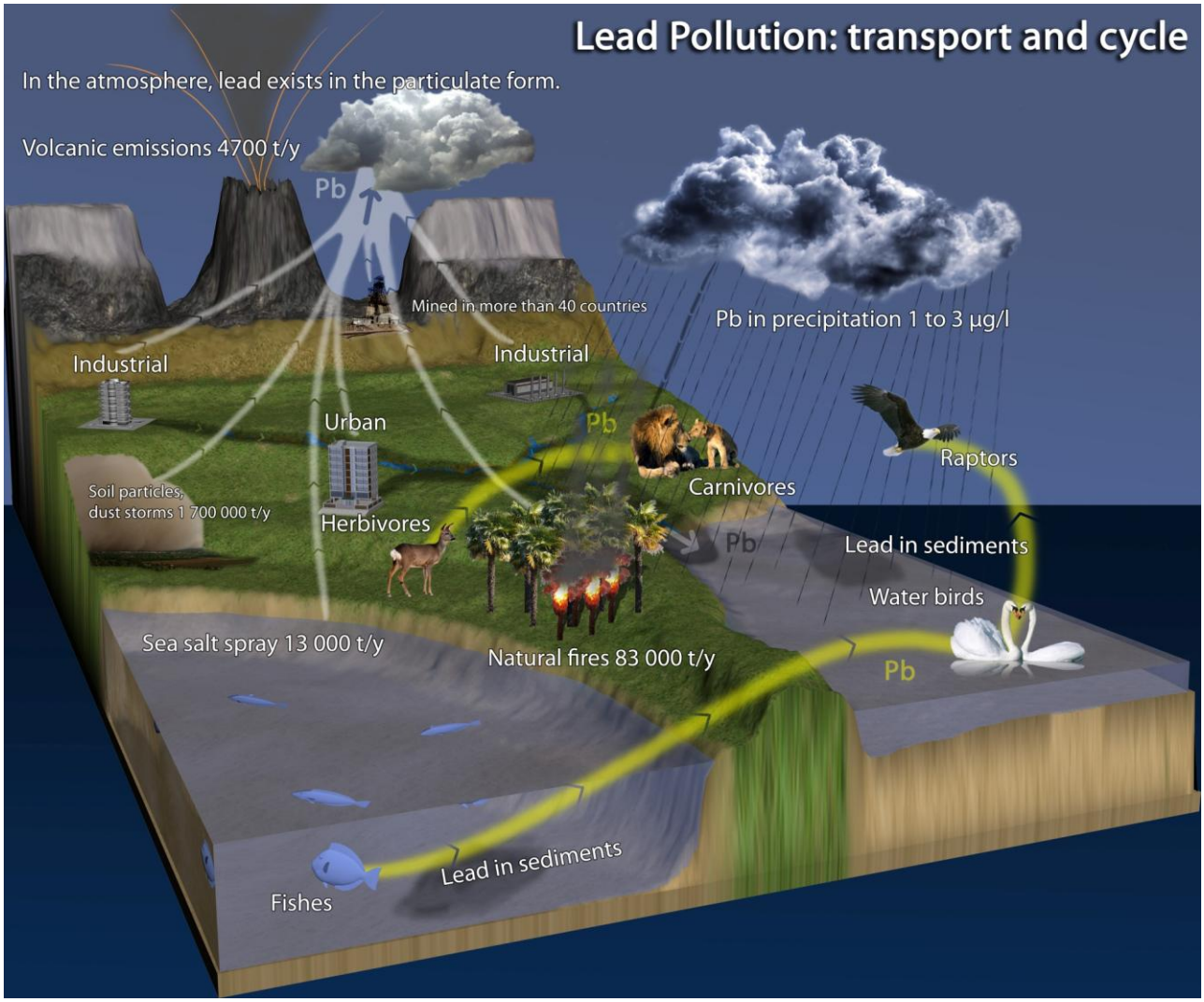

Figure 1. Lead pollution - transport and cycle. **Mining:** Mining of lead-containing rocks is carried out in more than 40 countries. The biggest miners are China (30 %) and Australia (22 %). More than 7 million tonnes of lead is used in industry annually. **Industrial:** Lead is used in batteries (78%), Pb compounds (8%), Pb sheets (5%), Pb ammunition (2%), alloys (2%), cable sheathing (1.2%), petrol additives (less than 1%). **Biota:** In biosphere, lead is toxic mostly for birds. The level of lead in the liver considered to be lethal to water birds is 5.0 ppm or more (10-14 µg/g expressed as dry weight). For some sensitive species of birds, reduced survival has been reported at lead doses of 75-150 ppm body weight, while reproduction was affected at dietary levels of 50 ppm. It was also reported that concentration of lead in various species in the areas polluted with these ions is 120-1500 mg/kg dry weight (DW) for plants, 170-2000 mg/kg DW for oligochaetes, and 1100 mg/kg DW for some insect species. **Transport:** Lead is not transported in the environment over long distances. **Atmosphere:** Non-organic compounds

of lead (form of $PbSO_4$ and $PbCO_3$) exist primarily in the particulate form in the atmosphere, which are likely to receive during the dust storm. Lead gets in concentrations 1 to 3 mg/l in the form of rains on the surface. From the landscape lead is also collected into waterways. **Rivers and lakes:** Rivers are important transport media for heavy metals on a national and regional scale. The speciation of lead in the aquatic environment is influenced by many factors, such as: pH, salinity, sorption and biotransformation processes. Lead is typically present in acidic aquatic environments as $PbSO_4$, $PbCl_4$, ionic lead, cationic forms of lead hydroxide and ordinary hydroxide $Pb(OH)_2$. Through these compounds, thousands of tonnes of lead come to sees annually. **Agriculture, soil:** In soil, lead is generally not very mobile. The lead content in uncontaminated soils of remote areas is generally within the range of 10-30 mg Pb/kg. Soil particles in dust storms are the biggest source of emissions of lead. By this way approximately 1.7 million tonnes of lead is released into the environment annually. A major source of lead emissions into the environment is released in natural fires 83,000 tonnes, sea salt spray 13,000 tonnes and volcanic emissions 4,700 tonnes. **Ocean:** Lead is accumulated in the world seas. Lead speciation in seawater is a function of chloride concentration and the primary species are $PbCl_3$> $PbCO_3$> $PbCl_2$> $PbCl^+$ > and $Pb(OH)^+$. Global, deep-sea ocean currents are (with varying strength) connected to one big, dynamic system, the so-called thermohaline circulation or "global conveyer belt", which transports enormous water masses through the Atlantic Ocean, the Southern Ocean around Antarctica, and the Pacific Ocean. This system is probably responsible for the movement of large quantities of heavy metals in the ocean environment. Adapted according to UNEP Lead and Cadmium activities [32].

1.2 Vultures and lead

Vultures are predators, which scavenge on dead animals [33]. It is clear with respect to their feeding habits that they accumulate high concentration of toxic compounds including metal ions. Although some raptors may regurgitate shots effectively, part of the lead(II) contained in shot or bullet fragments is rapidly dissolved and absorbed after ingestion due to their low gastrointestinal pH and often results in death [34]. Pyrenean bearded vulture population shows a relatively low burden by chronic exposition of lead(II) ions. Mean concentration of lead(II) was determined as 4.7 μg/g dry weight of bones [34]. Elevated lead in the tissues of raptors, especially those that scavenge, is a common occurrence, and lead poisoning appears to be a significant problem in the ongoing recovery effort for California condors (*Gymnogyps californianus*). Elevated blood lead levels have been found in released birds, and a number of birds have died of lead poisoning. In earlier work, turkey vultures (*Cathartes aura*) were dosed with lead shot but found to be a poor model for lead poisoning. Further, four Andean condors (*Vultur gryphus*) were dosed with lead shots and found to be quite sensitive, as two of the birds died and the other two exhibit signs of lead poisoning within 50 days. All lead-responsive parameters were affected, and regurgitation of ingested shots occurred only once [35]. In another paper, insights into the sublethal effects of lead contamination on Egyptian vultures were done (*Neophron percnopterus*) [36]. An approach was done on the comparison of two populations (Canary Islands and Iberian Peninsula) differing in exposures to the ingestion of lead ammunition. Blood lead levels were higher in the island population (Canary Islands range: 5.10-1780 μg/l; Iberian Peninsula range: 5.60-217.30 μg/l showing clear seasonal trends, peaking during the hunting season. Moreover, males were more susceptible to lead accumulation than females. Bone lead concentration increased

with age, reflecting a bioaccumulation effect [36]. Clinical, electromyographic, and pathologic findings characteristic of lead toxicosis were detected in a turkey vulture (*Cathartes aura*). The bird had generally lower motor neuron dysfunction that progressed over 5 days. Electromyography revealed diffuse denervation potentials and a presumed decrement in the sciatic-tibial nerve conduction velocity. Histopathological examination of peripheral nerves obtained at necropsy revealed changes that could be compatible with lead-induced neuropathy [37].

1.3 Analytical determination of lead

Rapid, sensitive and simple analytical determination of metal ions in environment is very important. Nowadays, recent trends in macro, micro and nanomaterial based tools and strategies for heavy metal detection are summarized in Chemical Reviews [38]. Usually, the presence of trace amounts of heavy metals in environmental samples is determined by spectrophotometric techniques as atomic absorption spectrometry (AAS) and inductively coupled plasma-mass spectrometry (ICP-MS) [24,39-45]. Such laboratory equipment needs highly qualified operators, expensive chemicals and other laboratory interments [24]. Spectroscopic methods are, thus, expensive and their availability is limited. They are not well suited for *in situ* measurements and require complicated instrumentation. Electrochemical techniques on the other hand are one of the best methods for detecting metals due to their low cost, high sensitivity and portability. Mercury-based electrodes (hanging mercury drop electrodes (HMDE)), have traditionally been used because of their advantages, such as high sensitivity, reproducibility, purity of the surface, high hydrogen overpotential, and possibility of amalgam formation. Electrochemical techniques including sensors and biosensors can be also fully automated keeping good sensitivity, selectivity and possibility for the simultaneous determination of several metal ions [46-60].

Lead detection is connected with miniaturization of electrodes and potentiostat. Enhancement of selectivity and sensitivity can be reached a) by interactions of lead(II) ions with biological part (peptides, proteins, nucleic acids) and b) by chemical modification of electrode surface [61,62]. Summary of electrode modifications used for determination of lead(II) ions is shown in Table 1. It was developed as a method for the simultaneous detection of cadmium, lead, copper and mercury, using differential pulse and square wave anodic stripping voltammetry (DPASV and SWASV) at a graphite-polyurethane composite electrode with SBA-15 silica organo-functionalized with 2-benzothiazolethiol as a bulk modifier [63]. Bismuth-coated screen-printed electrodes with square wave voltammetric response is highly linear over the 10-100 ppb (2 min accumulation) with detection limit 0.3 ppb (10 min accumulation) [64]. A novel type of renovated silver ring electrode (RSRE), as a mercury-free sensor, for anodic stripping voltammetry (ASV) of lead(II) ions was suggested with detection limit 0.5 nM [65]. Chemically modified carbon nanotubes can be also used for sensitive voltammetric determination of metal ions. Cysteine-modified nanotubes enabled us to detect 15 ppb of lead(II) [66]. Detection limit of lead(II) ions at carbon nanotubes modified by Amberlite IR-120 was 21 nM. A recently published paper describing 5,5,dithiobis(2-nitrobenzoic acid) (DTBA) and a number of alkali, alkaline earth, transition and heavy metal ions showed a strong affinity of DTBA for lead ions

with detection limit as 0.6 nM [67]. A bimetallic Hg-Bi/single-walled carbon nanotubes composite electrode gave repeatable responses for lead(II) ions over three orders of magnitude with detection limit as low as 0.12 ng/ml [68]. Glassy carbon electrodes modified with nafion or poly(3-thiopheneacetate) and subsequently plated with mercury were used for lead speciation studies in buffered (0.1 M Tris, pH 8) sodium chloride (0.6 M) solutions. As complexing agents there were studied nitrilotriacetic acid, ethylenediamine, oxalic acid and the well known electrode 'foulant' humic acid. Most measurements were performed by square wave anodic stripping voltammetry [69]. The complex formed by lead and 4,5-dihydroxy-3-(p-sulphophenylazo)-2,7-naphthalene disulphonic acid trisodium salt (SPADNS) was able to be adsorbed on the hanging mercury drop electrode (HMDE) [70]. Mercury monolayer carbon fibre electrode was used for ultrasensitive determination of lead(II) ions with calculated detection limit as 80 fM for lead(II) ions. Investigation of the nature of the analytical signal revealed that the high sensitivity is due to ionization of Pb atoms in the mercury layer, which catalyses the oxidation of atomic hydrogen adsorbed at the Hg layer [71].

Table 1. Summary of electrode modifications used for determination of lead(II) ions.

Electrode	Detection limit (ng/ml)	Ref.
Antimony film microelectrode	642	[61]
Bismuth-coated screen-Printed Electrodes	3,000	[64]
Renovated silver ring electrode	0.104	[65]
Carbon screen printed electrodes doped by bismuth	1	[72]
Carbon nanotubes modified cysteine	15,000	[66]
Bismuth disk electrode	0.031	[73]
Mercury monolayer carbon fibre electrode	0.000017	[71]
Carbon nanotubes modified Amberlite IR-120	4	[74]
Bimetallic Hg-Bi/single-walled carbon nanotubes composite electrode	0.12	[68]
5,5,dithiobis(2-nitrobenzoic acid) modifier	0.124	[67]
Mercury film electrode	0.08	[75]
Bismuth modified carbon nanotubes-poly(sodium 4-styrenesulfonate) composite film electrode	40	[76]
Chitosan modified electrode	3.4	[77]
Bismuth bulk electrode	93	[78]
Ordered mesoporous carbon nanoparticles modified polyaniline electrode	0.828	[79]
Nafion-graphene mercury film electrode	1.5	[80]
4,5-dihydroxy-3-(p-sulphophenylazo)-2,7-naphthalene disulphonic acid trisodium salt modified hanging mercury drop electrode	0.1	[70]
Silver chip	13,500	[81]
Edge plane pyrolytic graphite	200	[82]
Montmorillonite-calcium modified carbon paste electrode	300	[83]
Mercaptobenzothiazole onto hanging mercury drop electrode	0.017	[84]
Carbon paste electrode modified by thiol-functionalized kaolinite	12	[85]
Screen-printed carbon electrodes (SPCEs), without chemical modification	2.5	[86]

*There were selected papers from 2000-2011 with keywords as lead and electrochemical analysis.

A calibration-free method using portable instrumentation for field screening of lead in whole blood was described in another paper. Whole blood (2.5 μg/l) was diluted first with distilled water (5.0 μg/l) and then with a matrix-modifying solution (17.5 μg/l) containing 0.50 M HCl, 600 mg/l Hg-II 5% *v*/*v* Triton X-100 and 100 μg/l v μg/l of cadmium(II), the latter being used for performance check [87]. Novel solid phase microextraction (SPME) technique using a hollow fibre-supported sol-gel combined with multi-walled carbon nanotubes, coupled with differential pulse anodic stripping voltammetry was employed for the simultaneous extraction and determination of lead, cadmium and copper in rice [88]. Multivariate calibration was applied to voltammograms acquired and measured at in-house printed carbon-ink screen-printed electrodes by the highly sensitive electrochemical method of differential pulse anodic stripping voltammetry [89]. Screen-printed carbon electrodes (SPCEs) and carbon paste electrodes (CPEs) were prepared as mercury-free electrochemical sensors for the determination of trace metal ions in aqueous solutions. SPCEs were coated with conducting polymer layers of either polyaniline (PANI), or polyaniline-poly(2,2'-dithiodianiline) (PANI-PDTDA) [90]. Carbon screen printed electrodes doped by bismuth seem to be also versatile for determination of trace lead vapours with detection limit of 1 ng of lead [72]. A simple method for anodic stripping voltammetric determination of lead using carbon paste electrode modified with biomolecular chitosan, is described in the paper [91]. A novel reflection-based localized surface plasmon resonance fibre-optic probe has been developed to determine lead(II) ion concentration. Monoclonal antibody as the detecting probe containing massive amino groups to capture Pb(II)-chelate complexes was immobilized onto gold nanoparticle-modified optical fibre [92].

1.4. Biosensors for heavy metals determination

Generally, biosensors for heavy metals detection can be divided into two groups based on their biological part as i) protein biosensors and ii) biosensors based on nucleic acids. Biosensors using nucleic acids are a new and rapidly developing branch. The very first publication on this issue was focused on lead(II) ions [93]. Protein biosensors can be divided into enzymatic and affinity ones, which include antibodies-based biosensors.

Many families of enzymes as oxidases, dehydrogenases, phosphatases, kinases and ureases have been used for heavy metals detection. The detection is based on activation or inhibition of enzyme activity.

Detected heavy metal ion activates the enzyme in case that the ion is a part of the enzyme structure, or inhibits it when the ion is able to bind to an active centre of the employed enzyme and, thus, inactivates it [94]. Biosensors for heavy metal ions determination based on inhibition of enzyme activity use more than one enzyme compared to those based on activation. Oxidases and dehydrogenases belong to the most commonly used enzymes in these types of biosensors. These enzymes are immobilised due to reticulate gelatinous film or affinity interaction with a special membrane. Furthermore, the biosensor consisting of an enzymatic system including L-lactate dehydrogenase and L-lactate oxidase as the substance non-sensitive for heavy metals - was developed and showed to be a good choice for various metal ions detection.

The detection limit for lead(II) ions was 50 μM [94,95]. This system was optimized by Fenouh et al., who obtained a twice lower detection limit as compared with the previous study [96].

The wide spectrum of metal-binding proteins from naturally occurring to artificial ones, prepared using protein engineering, which is mostly specific for one metal ion, is used for non-enzymatic or affinity based biosensors employed for heavy metal ions determination. Heavy metal binding proteins as SmtA metallothionein, MerR regulation protein, MerP periplasmatic protein and phytochelatin AC20 are mostly used for biosensors construction for different heavy metal ions including lead(II) ions in a wide concentration range from fM to mM. These biosensors have good sensitivity and selectivity, and also acceptable stability time (approximately 2 weeks) besides wide concentration intervals [97]. A biosensor based on synthetic phytochelatin and electrochemical capacity detection was successfully used for lead(II)ions determination in concentration from 100 fM to 10 mM. The biological component was regenerated in EDTA with 15 days stability [98].

Moreover, phytochelatin 2 was used for lead(II) ions determination [12]. Immunodetection belongs to other methods for metal ion determination, which in comparing with traditional detection methods bring some advantages such as high sensitivity and selectivity.

Such biosensors are theoretically useful for other metal complexes, where it is possible to prepare the antibody [94]. Monoclonal antibodies were prepared for EDTA complex with lead(II) ions [99].

The aim of this study was to compare various microwave digestion procedures for lead(II) ions isolation followed by differential pulse voltammetry as a detection method. The optimal procedure was utilized for determination of lead(II) ions in blood and eggs of vultures poisoned by these toxic ions. Moreover, we tested several types of working electrodes for this purpose.

2. EXPERIMENTAL PART

2.1 Biological materials

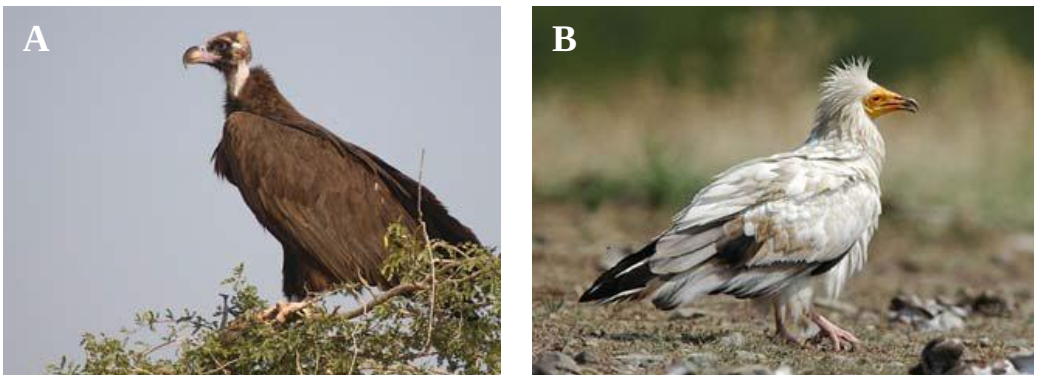

Figure 2. (**A**) Cinereous vultures (*Aegypius monachus*). (**B**) Egyptian vultures (*Neophron percnopterus*).

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

Other characteristics of these birds were: five females and five males, age ranging from 8 to 36 years, body weight from 7 to 11.5 kg in *Aegypius monachus* and from 1.6 to 2.2 kg in *Neophron percnopterus*. Lead non-exposed birds (i.e., four captive *Cinereous vultures*) were used as controls. Samples were carefully prepared to avoid external contamination and stored at -25 °C in freezer (Lieber) until chemical analysis. Photos of both avian species are shown in Fig. 2A and B.

2.2 Chemicals and materials

Stock solutions of 1mg/ml of Pb(II) were prepared by dissolving appropriate amount of lead nitrate (in water and diluted to 1000 ml volumetric flask). Working standard solutions were prepared daily by diluting the stock solutions. All other chemicals used were purchased from Sigma Aldrich 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. All used electrodes were from Metrohm. 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 [100]. 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) [46]. The analyser (757 VA Computrace from Metrohm, 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. All used electrodes were from Metrohm. 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 0.15V; pulse amplitude 0.025V; pulse time 0.04s; step potential 5.035 mV; time of step potential 0.3 s.

2.5 Electrochemical determination of lead(II) ions using carbon paste electrode

Differential pulse voltammetric measurements were performed using a CH-instrument (CH-Instruments, USA) using a plastic cell with three electrodes. The three electrode system consisted of carbon working electrode, an Ag/AgCl/3 M KCl reference electrode and a platinum counter electrode. Carbon paste electrode (CPE) was made of MWNTs (5 %, *w*/*w*) and carbon powder (65 %, *w*/*w*, 2 μm) and 30% mineral oil (*m*/*w*) (Sigma-Aldrich; free of DNase, RNase, and protease). This paste was housed in a Teflon body having a 2.5-mm-diameter disk surface. Prior to measurements, the electrode surface was renewed by polishing with a soft filter paper in preparation for measurement of a sample volume of 5 μl [101-105]. DPV conditions: time of accumulation 60 s, conditioning potential -1.15 V, modulation time 0.055 s, interval time 0.2 s. Initial potential -1.2 V, end potential 0.1 V, step potential 10 mV, modulation amplitude 25 mV.

2.6 Electrochemical determination of lead(II) ions screen printed electrode made of carbon multi-walled nanotubes powder

Differential pulse voltammetric measurements were performed using a CH-instrument (CH-Instruments, USA) using a plastic cell with three electrodes. The three electrode system consisted of carbon working electrode, an Ag/AgCl/3 M KCl reference electrode and a platinum counter electrode. The preparation of the working electrode is shown in Results and Discussion section. Surface of the

electrodes was studied using scanning electron microscopy (Tescan, Czech Republic). DPV conditions: time of accumulation 60 s, conditioning potential -1.1 V, modulation time 0.057 s, interval time 0.2 s. Initial potential -1.2 V, end potential 0.1 V, step potential 10 mV, modulation amplitude 25 mV.

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

To prepare the samples microwave digestion was used according to recently published papers [48,106]. Briefly, the mineralization of samples took place in a microwave system Multiwave3000 (Anton-Paar GmbH, Austria). A sample (10 mg of egg sample and/or 10 µl of avian blood) 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.), and maximum temperature 80 °C. Using each possible combination of power:time five samples were mineralized. Sample preparation for subsequent electrochemical measurements was as follows: 100 µl mineralized sample were 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

Spectrometric measurements were carried using an automated chemical analyser BS-200 (Mindray, China). Reagents and samples were placed on cooled sample holder (4 °C) and automatically pipetted directly into plastic cuvettes. Incubation proceeded at 37°C. The mixture was then stirred. The washing steps by distilled water (18 mΩ) were done in the midst of the pipetting. Apparatus was operated using software BS-200 (Mindray, China).

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 otherwise stated (EXCEL®). The detection limits (3 signal/noise, S/N) were calculated according to Long and Winefordner [107], 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 of vultures. 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 [108].

2.11 MEDUSA program

MEDUSA program (make equilibrium diagrams using sophisticated algorithms) () was used for the construction of a distribution diagram of different lead chemical forms present in the basic electrolyte. The basic parameters, including equilibrium constants that are necessary for the calculation of distribution diagrams are in the program database [32,109]. The program author is Ignasi Puigdomenech from the Inorganic Chemistry of Royal Institute of Technology, Stockholm, Sweden. The MEDUSA program is the freeware and is available on http:\\www.kemi.kth.se/medusa.

3. RESULTS AND DISCUSSION

Voltammetric techniques are known to show unique advantages in terms of both economic (relatively reasonable cost of equipment and very low operating costs, tens of euros per year) and excellent analytical features and advantages for the determination of low levels of metals in the buffer system and different matrices from samples of water to the biological samples [49]. The validation of the automated electrochemical methods for metals analysis was published in 2007 [49] and further developed for biological applications in our work published in 2010 [46].

3.1 Automated electrochemical analysis of lead ions at HMDE

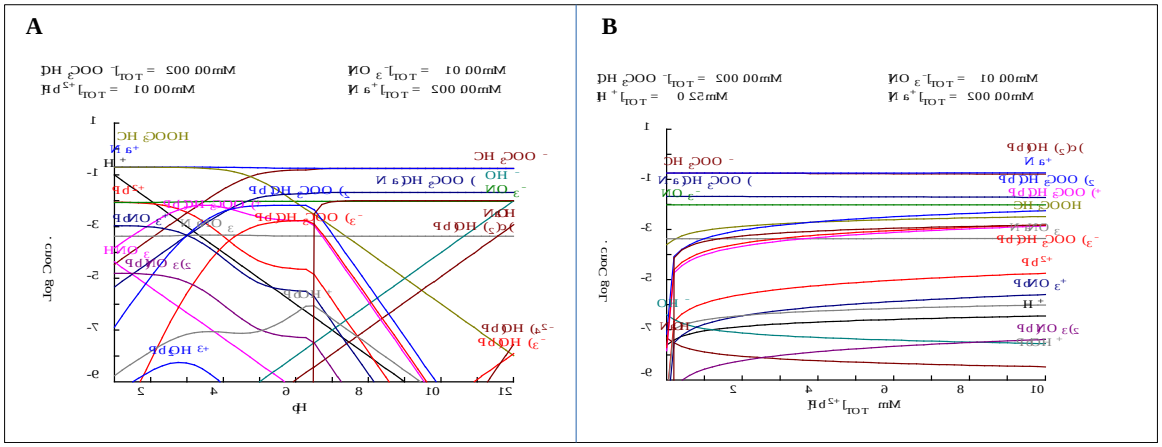

Figure 3. The distribution diagram calculated by MEDUSA. (A) Effect of pH (1-12) caused formation of complexes (10 mM lead(II) ions, 0.2 M sodium acetate). (B) Effect of concentration of lead(II) ions on formation of complexes in the presence of sodium acetate (1 nM – 10 mM, 0.2 M sodium acetate, pH 3.6).

We recently published papers discussing the problem of determination of selected heavy metals (Cu, Zn, Cd, Pb) at HMDE [48,109]. More and more it appears that the emergence of various complex compounds in the analytical determination plays a very important role. In the information environment of the MEDUSA program there was designed a simple diagram of the distribution of lead(II) ions under various conditions. As it is shown in Fig. 3A, there is a significant distribution of changes at pH 6. The changes due to formation of various lead complexes in acetate buffer are shown in Fig. 3B. The distribution diagram shows that analysis is appropriate at pH 6.

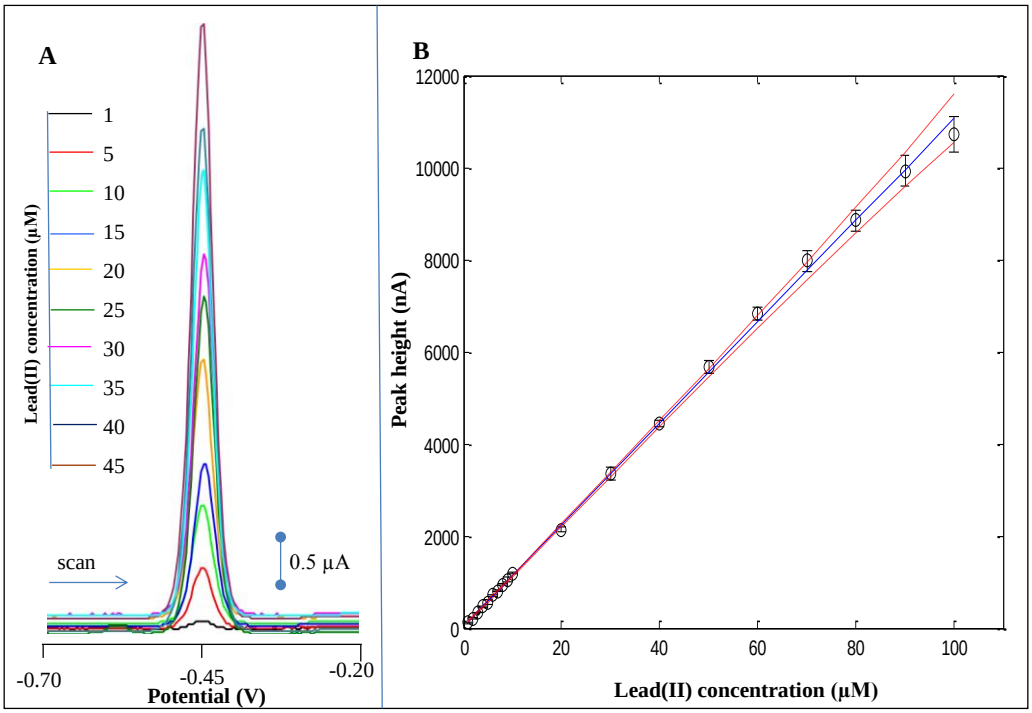

Figure 4. Electrochemical automatic detection of lead(II) ions. (**A**) DP voltammograms of lead(II) ions (1, 5, 10, 15, 20, 25, 30, 35, 40 and 45 µM) measured in the presence of 0.2 M acetate buffer pH 5.00. (**B**) Dependence of height of redox signals of lead(II) on concentration of these ions (10 nM – 100 µM). The following parameters were mathematically processed: mean, standard deviation, linear regression of average heights of measured peaks and exponential confidence interval (n = 10). Supporting electrolyte: 0.2 M acetate buffer pH 5.00, temperature of electrolyte 20 °C, 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.

The classical anodic stripping method for lead determination is commonly carried in the presence of acetate buffer pH 5.0. The suggested procedure uses 240 s deposition time for accumulation of lead ions under formation of various amalgams Pb/Hg, Pb(Hg)/Pb(II). It is known that we can detect ultra low concentration of the target molecule using this procedure. In our fully automated experiment, we determined the influence of concentration of lead(II) ions on their peak

height (concentration range from 10 nM to 100 µM, time of accumulation 240 s, potential of accumulation -1.3 V). Typical DP voltammograms of lead(II) ions with a very well developed and separated signal PbHg amalgam reduction throughout the tested concentration range are shown in Fig. 4A. The interday precision was 0.68% (n = 10) and intraday 1.1% (n = 10). The calibration line was strictly linear with R^2 = 0.9991 (y = 96.7x + 259.2, RMS error156.3, RSD 1.6%). Complexes of lead amalgam on HMDE probably show different thermodynamic properties and subsequently very complicated redox reactions proceed on the working electrode. Changes in complexes on the surface of HMDE influence electrochemical detection of lead(II) ions. The limit of detection (LOD) for lead(II) ions was 0.1 ng/ml and limit of quantification (LOQ) 0.3 ng/ml (Fig 4B).

3.2 Microwave digestion of samples for determination of lead(II) ions

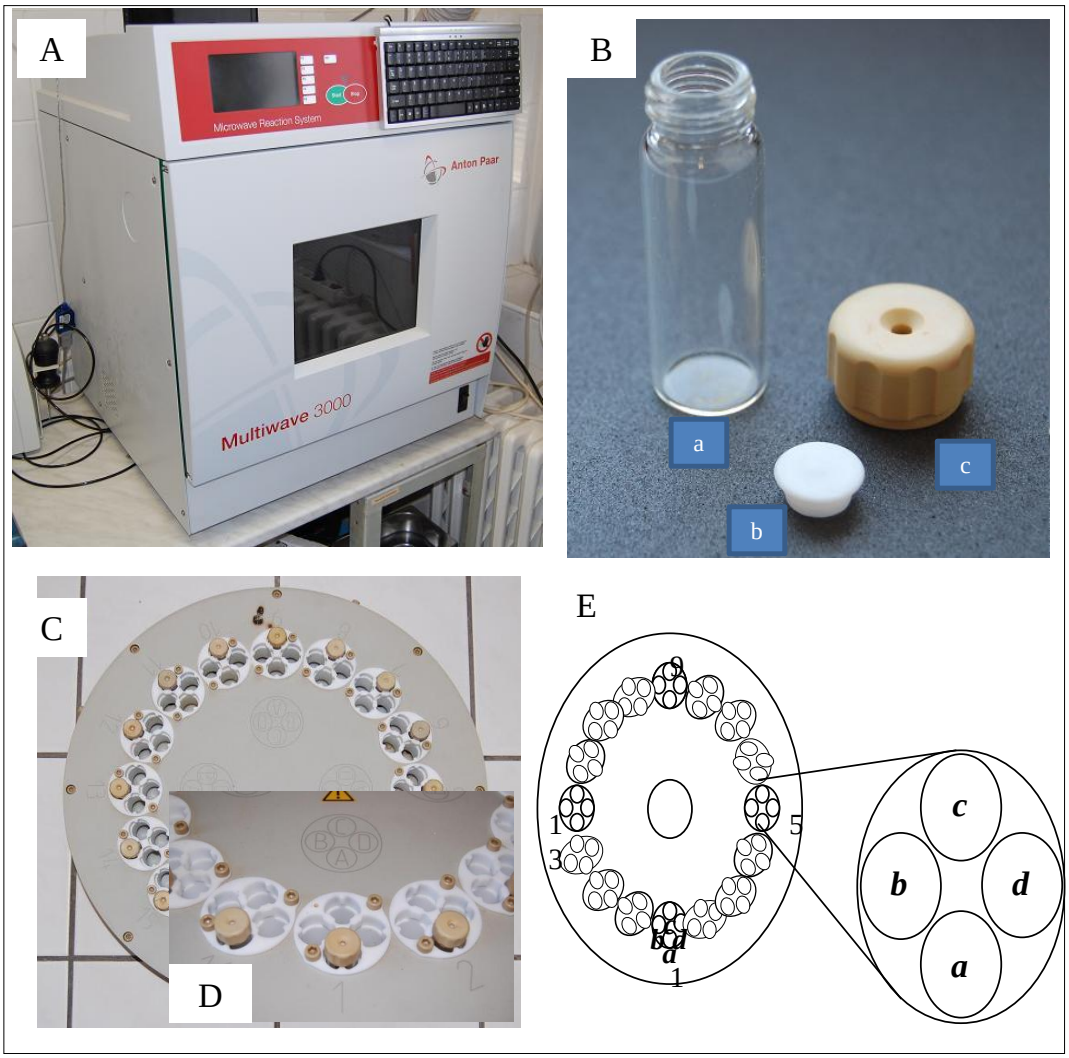

Figure 5. (**A**) Laboratory Microwave system Anton Paar 3000. (**B**) (a) Glass vial with (b) disposable PTFE seal and (c) screw cap. (**C**) Rotor 64MG5 with samples in positions ***a***. (**D**) Detail of one group for samples in rotor 64MG5. (**E**) Scheme and identification of positions in one group in rotor 64MG5.

The objective of the present study was to develop a high throughput digestion using microwave for lead(II) determination (Fig. 5A) in vultures samples available in very small quantities (5-20 mg). A commercially available 64-position rotor (Anton-Parr) accommodating disposable glass vials, was used as platform for the digestion. The disposable glass vials were closed with two special polytetrafluoroethylene (PFTE) seals in polyetheretherketone (PEEK) screw caps allowing temperatures up to 200 °C and pressures up to 20 bars (Fig. 5B) [106]. The arrangement of individual positions in the rotor is shown in Figs. 5C, D and E. Course microwave decomposition is controlled by the integrated program. Record of the mineralization (temperature, pressure, power) is transferred after completion of the program via RS232 to Excel®.

Sample preparation for analysis is a very critical step. In this study we focused on the possibility of preparing very small quantities of a rare biological sample for detection of lead. Typical effects of additions of nitric acid and/or nitric acid and hydrogen peroxide are shown in Fig. 6Aa and Fig. 6Ab.

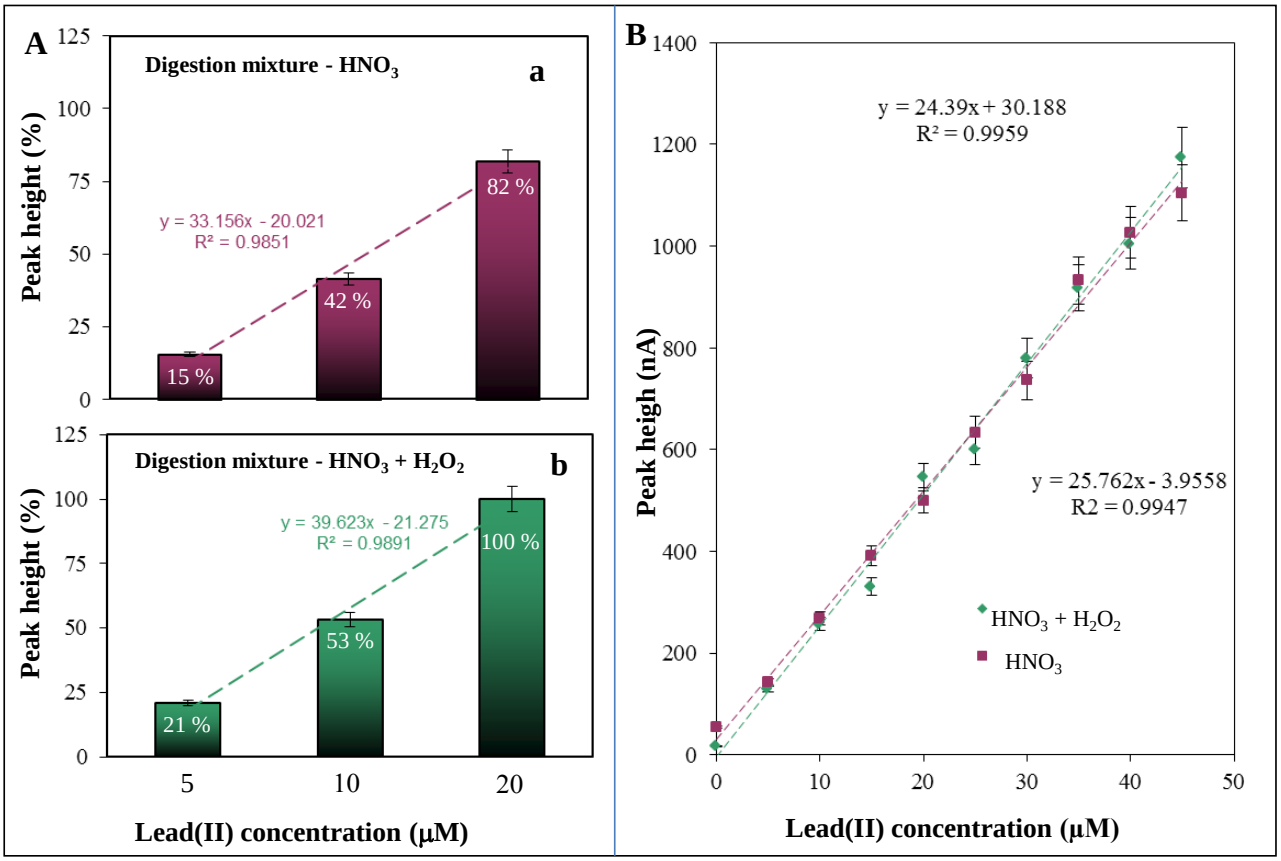

Figure 6. (**A**) Changes in the height of lead(II) ions peak after their addition to a) (65%, *w*/*w*) HNO_3, b) (65%, *w*/*w*) HNO_3 + (30%, *w*/*w*) H_2O_2. The highest response was used as 100 %. Concentrations of lead(II) ions were 5, 10 and 20 µM. (**B**) Dependence of lead(II) ions peak height on the their applied concentration in a) (65%, *w*/*w*) HNO_3, b) (65%, *w*/*w*) HNO_3 + (30%, *w*/*w*) H_2O_2. The conditions of mineralisation: length 30 min, power 80 W, glass vials. Other experimental conditions see in Caption of Figure 4.

Three concentrations of lead(II) ions were tested (5, 10 and 20 μM) at 15 replicates. The experiment was realized accordingly: i) 10 mg egg shells of vultures; ii) mineralisation in HNO_3 and/or HNO_3 + H_2O_2; iii) 100 μl of mineralised sample was added to 900 μl acetate buffer with 5, 10 and 20 μM lead(II) ions; iv) electrochemical analysis; v) the highest measured signal was selected as 100 %. Higher electrochemical responses were determined in the case of using (65%, *w*/*w*) HNO_3 + (30%, *w*/*w*) H_2O_2 (Fig. 6). The height of lead(II) ions peak increased with the increasing concentration of these ions accordingly 5 (21 %), 10 (53 %) and 20 (100 %) μM after mineralisation by combination of nitric acid and hydrogen peroxide. The equation of the straight line was y = 39.623x - 21.275; R^2= 0.9891; (n = 10), R.S.D. 2.9%. In the sample mineralised by nitric acid only, lead(II) ions peak increased also with the increasing applied concentration of lead(II) ions as follows: 5 (15 %), 10 (42 %) and 20 (82 %) μM. The equation of the straight line was y = 33.156x - 20.021; R^2= 0.9851; (n = 10), R.S.D. 2.9%. We found that the combination (65%, *w*/*w*) HNO_3 + (30%, *w*/*w*) H_2O_2 is advantageous for the resulting electrochemical response. The reasons for this behaviour are not too obvious, but there can be probably formed some more electroactive lead complexes in the presence of nitric acid and hydrogen peroxide compared to nitric acid only. These phenomena will be further investigated. Typical dependence of the height of the lead(II) ions peak on their concentration is shown in Fig 6B. The calibration dependence was prepared in mineralized egg shells of vultures prepared according to procedure shown in Fig. 6A. The first mineralization mixture contained 1000 μl of HNO_3 (65%, *w*/*w*) only. The second one contained 700 μl of HNO_3 (65%, *w*/*w*) + 300 μl H_2O_2 (30%, *w*/*w*). The samples were further mineralized in glass vials using microwave digestion at 80 W for 30 min. The measured calibration dependences for both mixtures are shown in Fig. 6B, a) y = 24.39x + 30.188; R^2= 0.9959; (n = 3), R.S.D. 3.7%; b)y = 25.762x - 3.956; R^2= 0.9947; (n = 3), R.S.D. 4.1%.

3.3. Optimisation of mineralization process

To determine the effect of mineralization on the height of the lead(II) ions peak, eight different mineralization programs were tested. Single mineralization programs vary in length and power. The schemes of the programs are shown in Fig. 7A. All eight programs can be divided into two basic groups with the length of the main program 15 and 30 min. Each program begins and ends with the same ten-minute-long-step, beginning with the power of 50 W and ending with the power 0W. Microwave power varied from 50 to 200 W in the main part of the programs. Monitoring power and length of mineralization is critical with regard to the progress and effectiveness of the sample digestion. The system is used with great caution, bearing in mind that increased power, soaring temperatures can cause an exothermic reaction leading to an increase in pressure and rupture of Teflon cap (Fig. 5Bb). The effect of power used for a sample mineralisation with the subsequent electrochemical detection of lead(II) ions shows in Fig. 7B. The figure shows that the percentage height of the peaks relative to the maximum value corresponds to 200 W power and length of 30 min. It can be concluded that lower powers gave lower responses for more than 3 to 10 % after 15 min compared to 30 min lasting mineralisation. Comparing tested powers at the same time we found the intensity of the signal measured at 50 W decreased to 79 % of the highest measured signal (200 W, 30

min). When comparing 200 W and 50 W for 15 min lasting programs, decreasing the signal at 50 W for 85 % of those measured at 200 W was observed. It clearly follows from the results obtained that program 8, which uses 200 W for 30 min., is the most optimal for mineralisation of samples from vultures.

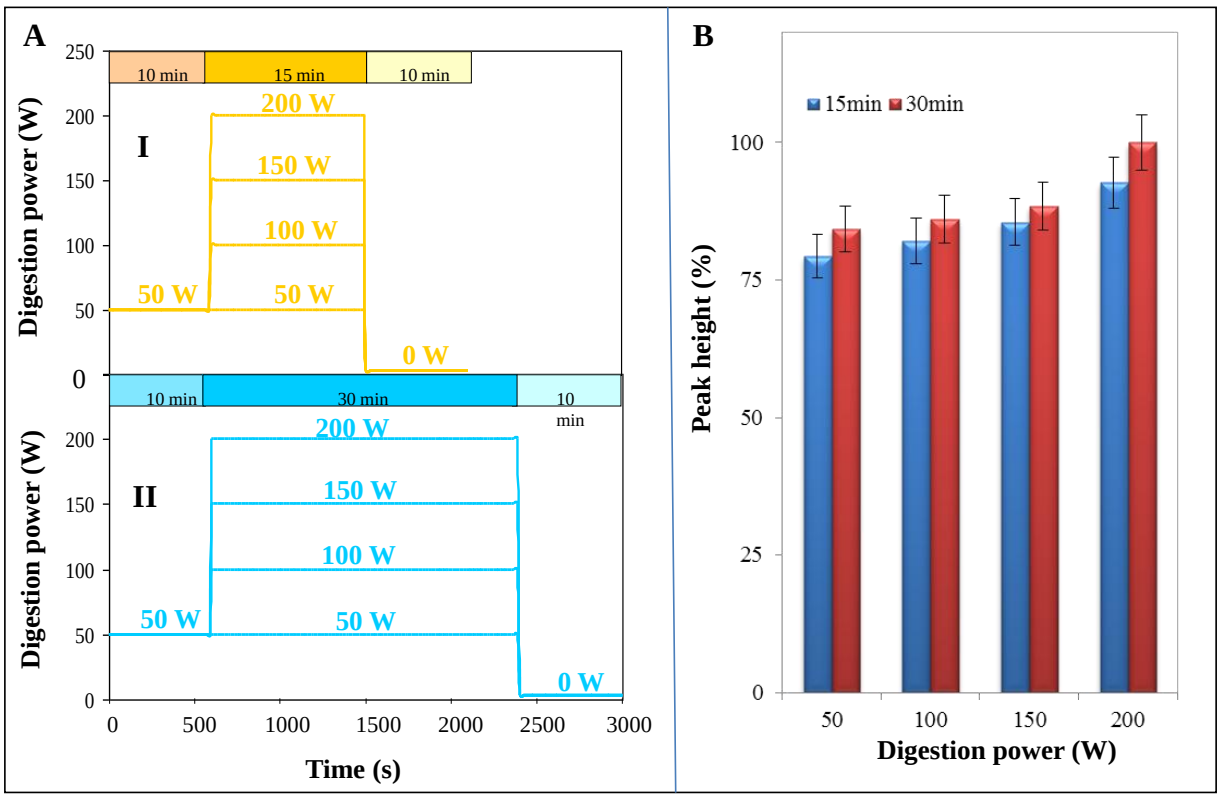

Figure 7. Digestion power as a function of time – digestion programme. (**A**) Four digestion programmes with 15 min long main period are presented in part I. Four digestion programmes with 30 min long main period are presented in part II. (**B**) Lead(II) ions peak height as a function of digestion power for eight digestion programmes. Peak height is presented as percentage of maximum value. Digestion mixture of (65%, *w*/*w*) HNO_3 + (30%, *w*/*w*) H_2O_2 was used. Other experimental conditions see in Caption of Figure 6.

In the following part of the study, the influence of different distribution of samples in the rotor 64MG5 microwave system Anton Paar Synthos 3000 was investigated (Fig. 8). The rotor consists of 16 groups of four positions, a total of 64 positions on the rotor for glass vials. Four variants for location of vials in the rotor 64MG5 were designed and labelled A-D as it is shown in Fig. 8. In addition, we have decided to deploy three types of samples in rotor, a vulture egg shell was the first sample, vulture shell with a standard addition of lead(II) ions (in the form of 100 µl of 2 µM solution of $Pb(NO_3)_2$) was the second type and pure water (milli-Q) represented third type of a sample. All samples were prepared in equal volumes (400 µl). The four types of layout samples are two basic approaches, namely the deployment uniformly (A, B, C) and irregularly (D) around the rotor. Distribution of A and B reflect an even distribution of samples, comparing the impact of inline blind

samples (milli-Q water). Distribution of C reflects the different distribution of samples within the same groups. Option D represents the uneven spread of samples around the perimeter of the rotor but occupying one place in rotor. Fig. 8 presents a comparison of the distribution of samples in terms of peak intensity of lead(II) ions for the samples with standard addition. Dependence of sample temperature on time during the digestion is shown in Figs. 8A-D for each type of samples layout. All four images represent the mineralization program at 100 W for 30 min. These conditions were selected for optimization of this step only. In all figures, the individual peaks are labelled according to the sample and the number that indicates the relative position of the sample in rotor. Microwave system Anton Paar 3000 measured during the course of mineralization temperature in all groups in the rotor. After the measurements, we obtained 16 temperature dependences (Fig. 8). Based on these records it is clear that during the mineralization of different samples temperature curves are different depending on the type of the sample. The samples of shells (the first group) have maximum temperature at the time approximately 450-500 s. Samples Sa2 and Sa3 (which are inside of the four samples on the rotor) have maximum temperature at 83 °C. Samples Sa1 and Sa4 (which are on the edges of the four samples on the rotor) have maximum temperature approximately 70 °C. Samples with the addition of lead have their maximum moved into the area from 650 to 800 s. Samples Pb2 and Pb3 show higher maximum temperature compared to Pb1 and Pb4 samples similarly to Sa samples.

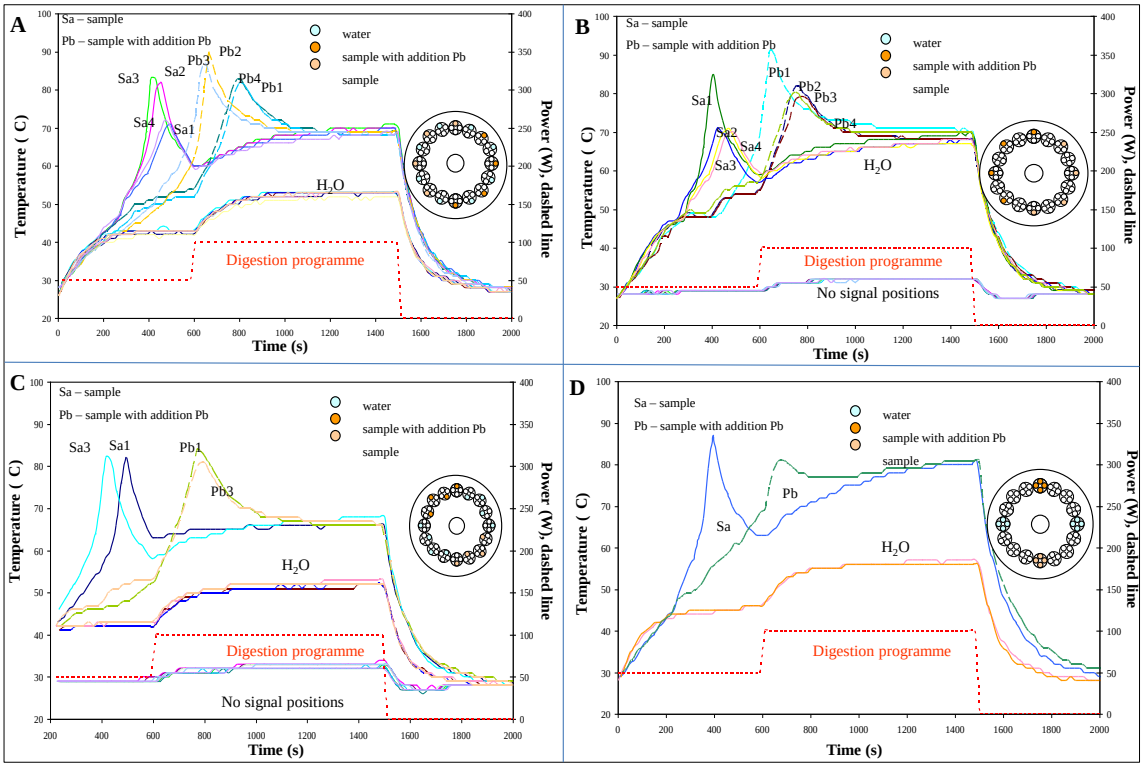

Figure 8. Samples temperature as a function of time during digestion process. Various locations of vulture egg samples, vulture egg samples with addition of Pb and vessels with water in 64MG5 rotor are presented (**A**); (**B**); (**C**) and (**D**) part of this figure. Applied digestion programme had three steps: i) 50 W 10 min, ii) 100 W 15 min., iii) 0 W 10 min. Other experimental conditions see in Caption of Figures 4 and 7.

Both types of samples reached the end of the main periods of temperatures app. 70 °C. Water samples as the third group reach maximum temperatures at 53 °C. The variants shown in Figs. 8A and 8B have similar time dependence. The difference is only in the records for an empty position, which is not in the variant shown in Fig. 8A. In variant shown in Fig. 8A, the curves differ only for samples Sa2 and Pb2. In the variant shown in Fig. 8C, there are again three sets of records, corresponding to three types of samples on the rotor. Contrary to expectations, there is a fourth group of records that corresponds to the empty positions in vessels. The microwave system is detecting the temperature in each vessel only in the positions *a*. Records shown in Fig. 8D are also divided into three groups. Temperature of samples (with or without additions of lead(II) ions) is similar for individual deployments at the end of the main part of the program (72 °C). When comparing the temperature at the end of the main part of the program in terms of different deployments of the position of samples, we determined an increasing line as follows: **C (66 °C) < A (67 °C) = B (67 °C) < D (80 °C)**. The experimental approach is shown in Fig. 8D has big difference in temperature at the end of the main part of the program and caused a complete occupancy of individual groups on the rotor and the uneven distribution of samples around the perimeter of the rotor. In the occupied vessels, there is a high concentration of mass and therefore the temperature at the same mineralization power increases.

All four figures, i.e. Figs. 8A-D, show differentiation of temperature dependences for sampled shells (Sa) and shell samples with the addition of lead (Pb). The amount of lead in the samples without any external addition of lead(II) ions is so small compared with the samples with the addition of lead(II) ions. Therefore, these Sa records can be considered as the temperature curves of records for sample matrix mineralization only. The increase in temperature of samples in records at 400 s could be caused by the accumulation of microwave energy that is not consumed for the decomposition of the sample, but is used only for its heating. Probably there is a change of use of energy earlier in samples without addition of lead(II) ions, because of lower content of lead, respectively lead compounds as standard addition. The temperature curves of samples with the addition of lead (Pb) have their maximum at temperatures between 650 and 800 s. Here is the temperature increase caused by adding power at the time of 600 s, i.e. the increasing power from 50 to 100W. The sample is not apparently able to "use" power supplied for the decomposition, but this increase in power is consumed for heating the sample. In the following time power supply (100 W) is constant and therefore the temperature is also constant.

Besides temperature curves, we also determined the effect of sample positions on the yield of lead(II) ions using the above optimized electrochemical method (Fig. 9). The highest electrochemical response was achieved using the distribution of samples according to model B. Only a few smaller peaks were obtained using variant A. The decrease in signal intensity probably related with the location of blank water samples between the real samples, which probably caused scattering of mineralization power. The distribution of samples according to the variant C caused a decrease of peak height to 90 %. This decline is probably related to the greater degree of disorderliness of sample deployment in the rotor, which means filling positions of the rotor in two concentric circles instead of one as it is shown in variants A and B. The distribution of samples according to the model D caused the decrease in the signal to 81% of the maximum value (B). In this case, there is already much greater dispersion of microwave power outside the filled position of the rotor (Fig. 9).

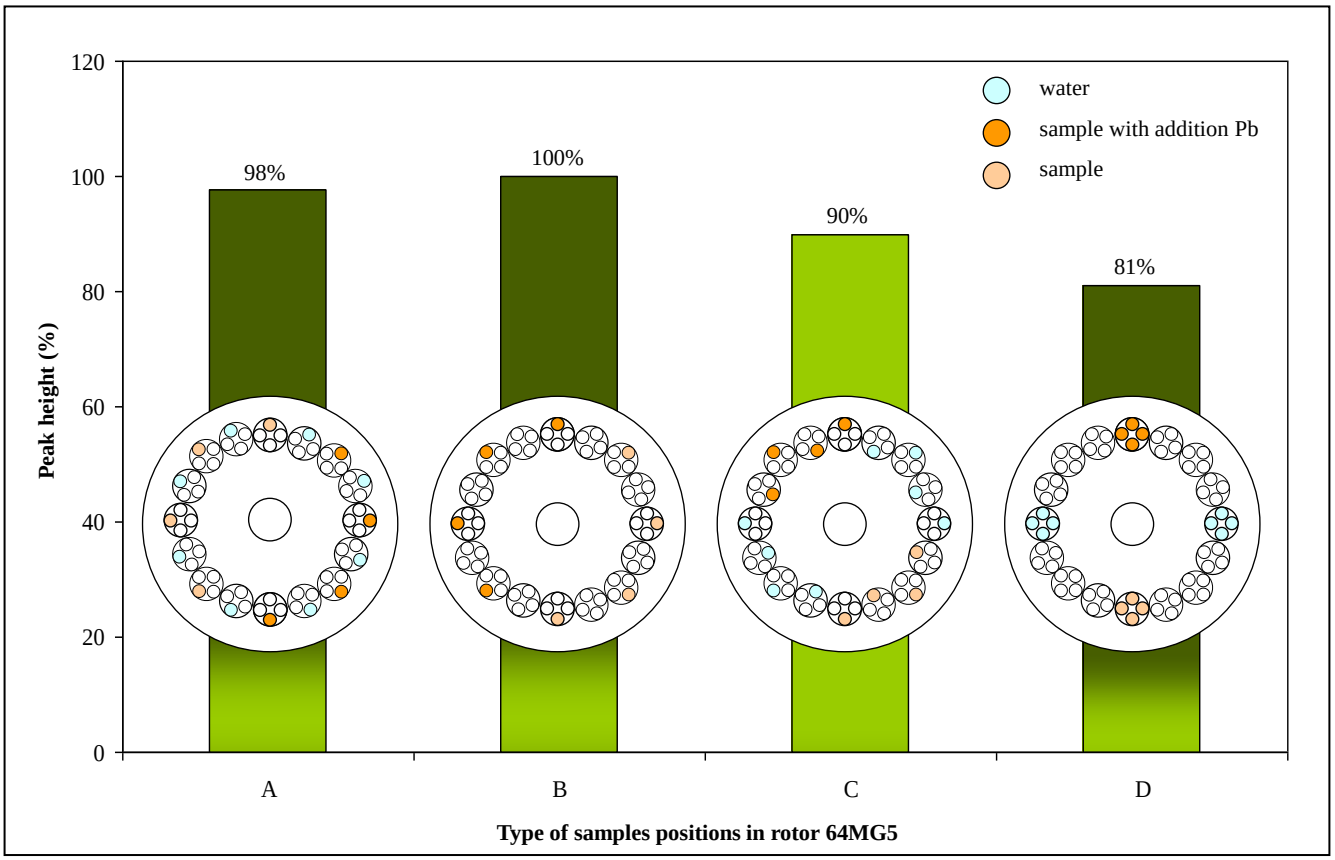

Figure 9. Peak height of lead(II) ions as a function of sample positions in rotor 64MG5. The lead(II) ions were determined in the samples of vulture eggs with external addition of 100 µl of 2 µM lead(II) ions. This sample was digested in the presence of (65%, *w*/*w*) HNO_3 + (30%, *w*/*w*) H_2O_2. Peak height is presented as percentage of maximum value. The applied digestion programme had three steps: i) 50 W 10 min, ii) 100 W 15 min., iii) 0 W 10 min. Electrochemical detection: 100 µl digested mixture + 900 µl acetate buffer. Other experimental conditions see in Caption of Figures 4 and 7.

3.4. The thick-film electrode fabrication

Besides lead(II) ions determination at HMDE, electrodes fabricated by us were also tested for the same purpose. The electrode schematically shown in Fig. 10A was fabricated on 0.65 mm thick alumina substrate by screen-printing using the standard thick film technology [x, y]. The conducting layer is made of cermet AgPdPt paste ESL 9562-G and the insulating covering layer is made of ESL 4917 cermet paste (ESL Electroscience, UK). Both layers were screen-printed and fired at 850 °C. The electrode active layer was fabricated using multi-walled nanotubes (MWNTs) based pastes as it is described below. The MWNTs based pastes were used for active electrode area fabrication to increase the active electrode area of standard thick-film electrodes. Moreover, there is a possibility of relatively easy chemical modification of the nanotubes to improve the sensitivity to investigated species [z1-z5]. There was prepared one paste composed from vehicle CV59 (resin dispersed in BCA) with appropriate thinners obtained from ESL Electroscience Company, UK. The MWNTs powder of purity higher than 95 %, 0.5 to 2 µm of length, wall size of 1 to 2 nm and 20 to 30 nm of diameter were obtained from

Sigma Aldrich. The MWNTs paste was mixed in the ratios shown in the Table 1 and then precisely homogenized.

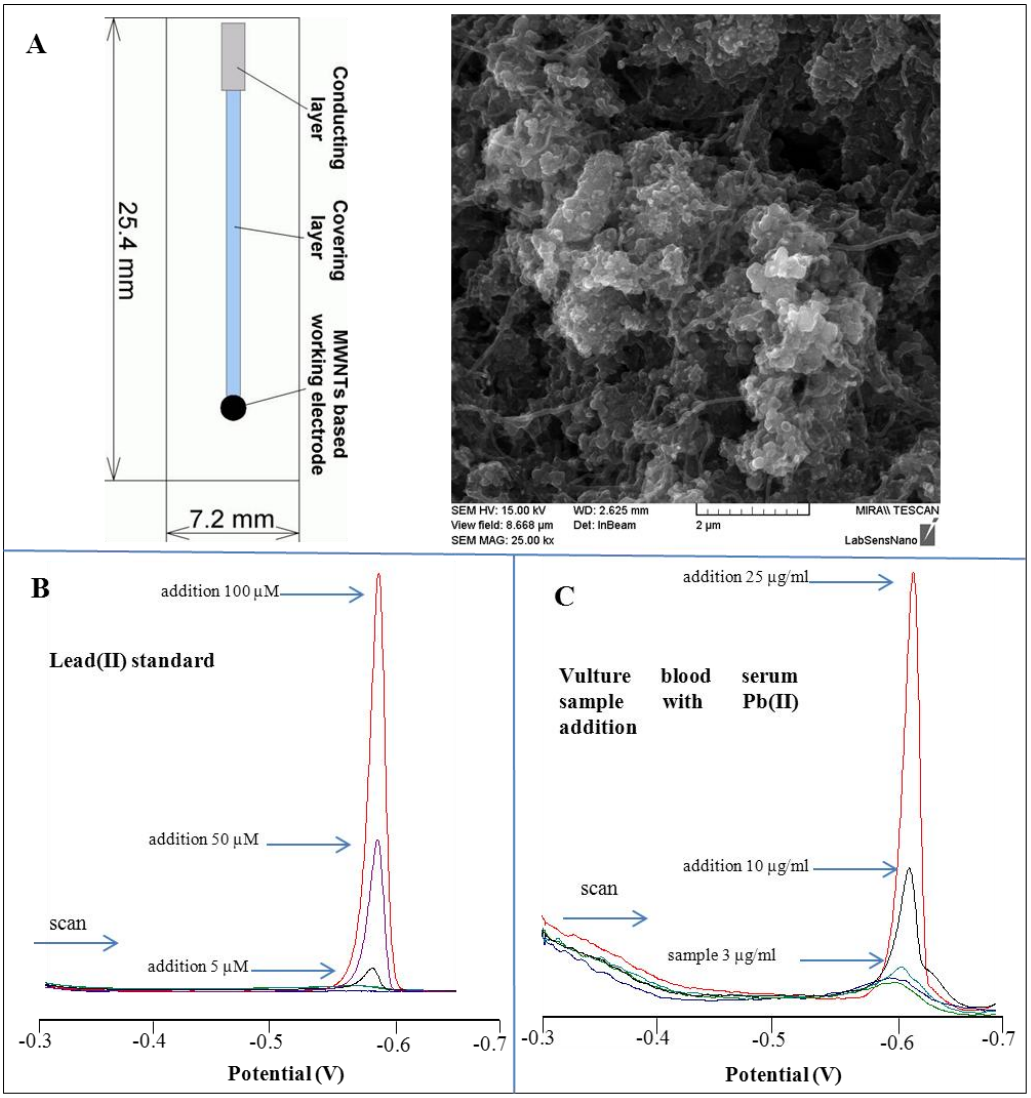

Figure 10. (**A**) Scheme of screen printed electrode made of carbon MWNTs powder of purity higher than 95 % (left). SEM picture of fabricated electrodes type MW1. Parameters of SEM analysis: 15 kV, In beam SE detector, 25,000 × magnified. Typical DP voltammograms of lead(II) ions measured on MWNTs electrode, (**B**) lead(II) ions standard, (C) mineralized blood serum of vultures with external additions of lead(II) ions. Parameters of electrochemical measurements: 0.2 M acetate buffer, pH 5.00.

The paste was screen-printed on the previously fabricated electrode substrate. Then the MW1 type electrodes were dried and cured at 150 °C for 30 minutes. The geometrical size of the fabricated working electrode is 4.5 mm^2. Fabricated electrodes were analysed using scanning electron microscopy (SEM). SEM picture is shown in Fig. 10A, where the surface of the electrode is clear. The fabricated electrode system called MW1 was subsequently used for detection of standard of lead(II) ions (Fig. 10B) and lead(II) ions in sample of vulture blood serum (Fig. 10C). It is very clear from the

figuresthat the fabricated screen-printed electrode is applicable for detection of lead(II) ions in both buffered and real samples of mineralised blood serum.

Table 1. Thick-film MWNTs based pastes preparation compound quantity.

	MW1	
	Quantity	Type
MWNTs powder	750 mg	MWCNTs (Sigma-Aldrich)
Vehicle	2.3 g	CV59 (ESL)
Thinner	800 ml	402 (ESL)

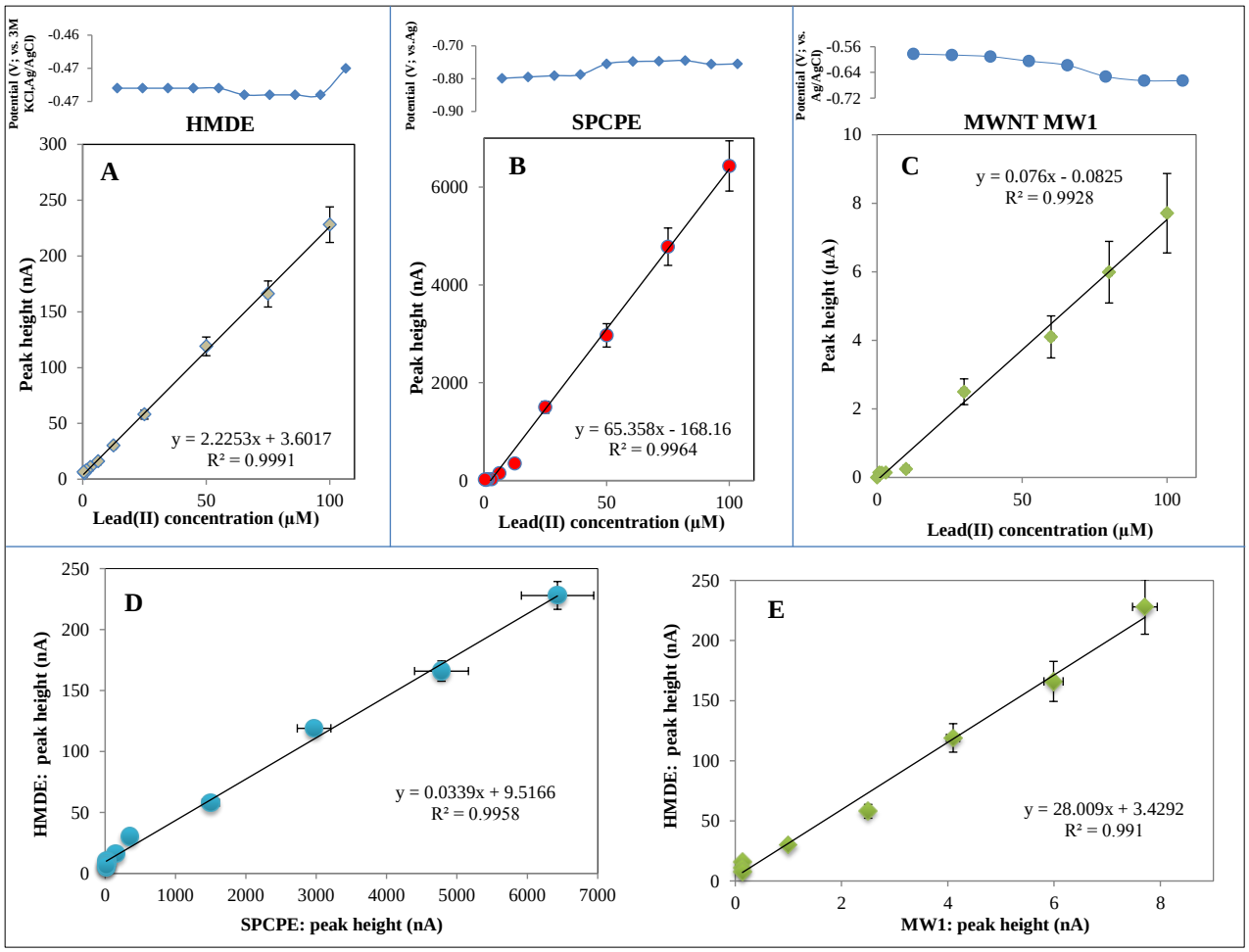

Figure 11. Dependencies of the lead(II) ions peak heights and potentials on their concentrations measured at (**A**) HMDE, (**B**) SPCEP, (**C**) MWNT MW1. Correlation dependencies of lead(II) ions peak heights measured at (**D**) HMDE/SPCPE and (**E**) HMDE/MWNT MW1. Other experimental conditions see in Caption of Figure 6.

Comparison of lead(II) ions detection by three different working electrodes is shown in Fig. 11. Use of HMDE is shown in Fig. 11A. We found that the peak potential was independent on concentration of the analyte concentration (-0.467±0.002 V, inset in Fig. 11A). The equation of the calibration dependence was y = 2.225x + 3.60; R^2= 0.9991; (n = 5). Carbon paste electrode (CPE) was made of MWNTs (5 %, *w*/*w*) and carbon powder (65 %, *w*/*w*, 2 μm) and 30% mineral oil (*m*/*w*) (Sigma-Aldrich; free of DNase, RNase, and protease). In the case of reduction scan, peak of lead(II) ions was measured at -0.81±0.04 V (inset in Fig. 11B). The increasing concentration of the analyte caused a shift of the peak potential to more positive values. Calibration dependence was strictly linear with the equation as y = 65.358x – 168.16; R^2= 0.9964; (n = 5, Fig. 11B). The screen printed electrode made of MWNTs called MW1 was also tested (Fig. 11C). In the case of reduction scan, peak of lead(II) ions was measured at -0.58±0.06 V (inset in Fig. 11C). The increasing concentration of the analyte caused a shift of the peak potential to more negative values. Calibration dependence was linear with the equation as y = 0.076x – 0.0825; R^2= 0.9928; (n = 5, Fig. 11C). Correlations between HMDE measurements and SPCPE and MW 1 measurements are shown in Figs. 11D and E. The correlations were as follows HMDE/SPCPE: y = 0.0339x + 9.5199; R^2= 0.9958 and HMDE/MWNT MW1: y = 28.009x + 3.4292; R^2= 0.991. Based on these results it can be concluded that results are in very good agreement and also demonstrate good potential of the carbon electrodes to be used for detection of lead(II) ions.

3.5. Determination of lead(II) ions in vultures accidentally poisoned by lead

Detailed description of the poisoning in vultures will be published elsewhere. Briefly, captive vultures were accidentally exposed to lead contamination in the Prague ZOO. The toxicosis developed after one month of returning the birds to their aviaries following old minium paint removal, because the paint dust and chips sanded off the steel aviary construction contaminated the soil. Levels of lead ranged from 2 to 10 mg/g liver. Summary of the content of the selected ions and compounds determined in blood sera from poisoned vultures is shown in Tab. 2. Using the methodology developed by us levels of lead(II) ions were electrochemically detected. Based on the results obtained it clearly follows that the level of lead(II) ions in intoxicated vultures ranged from 1 to 5 μg/ml of blood. In addition, it was found that the lead toxicosis was diagnosed in all vultures properly. However, the control group of birds also showed elevated levels of lead(II) ions (from 0.4 to 1.2 μg/ml blood). Besides lead(II) ions, content of cadmium(II) was also determined. Based on the results obtained it is obvious that the paint digested by vultures contained both lead(II) and cadmium(II) ions. Determined levels of cadmium ions were 0.4 to 1 μg/ml of blood in the poisoned vultures, which was more than 2.5 times higher compared to the control group (0.3 to 0.5 μg/ml blood). The correlation between the content of lead(II) and cadmium(II) ions in blood sera of vultures is shown in Fig. 12A. The dependence indicates a link between contents of both ions (R^2 = 0.4933) and also shows on the possibility that vultures were also poisoned by cadmium. In addition to these toxic elements, contents of Ca, Mg, Fe, Zn and Cu were further determined using spectrometry. The measured calibration

dependencies are shown in Figs. 12B, C, D, E and F for Ca, Mg, Fe, Zn and Cu, respectively. All calibration curves are strictly linear. The results of photometric analysis are summarized in Tab. 2.

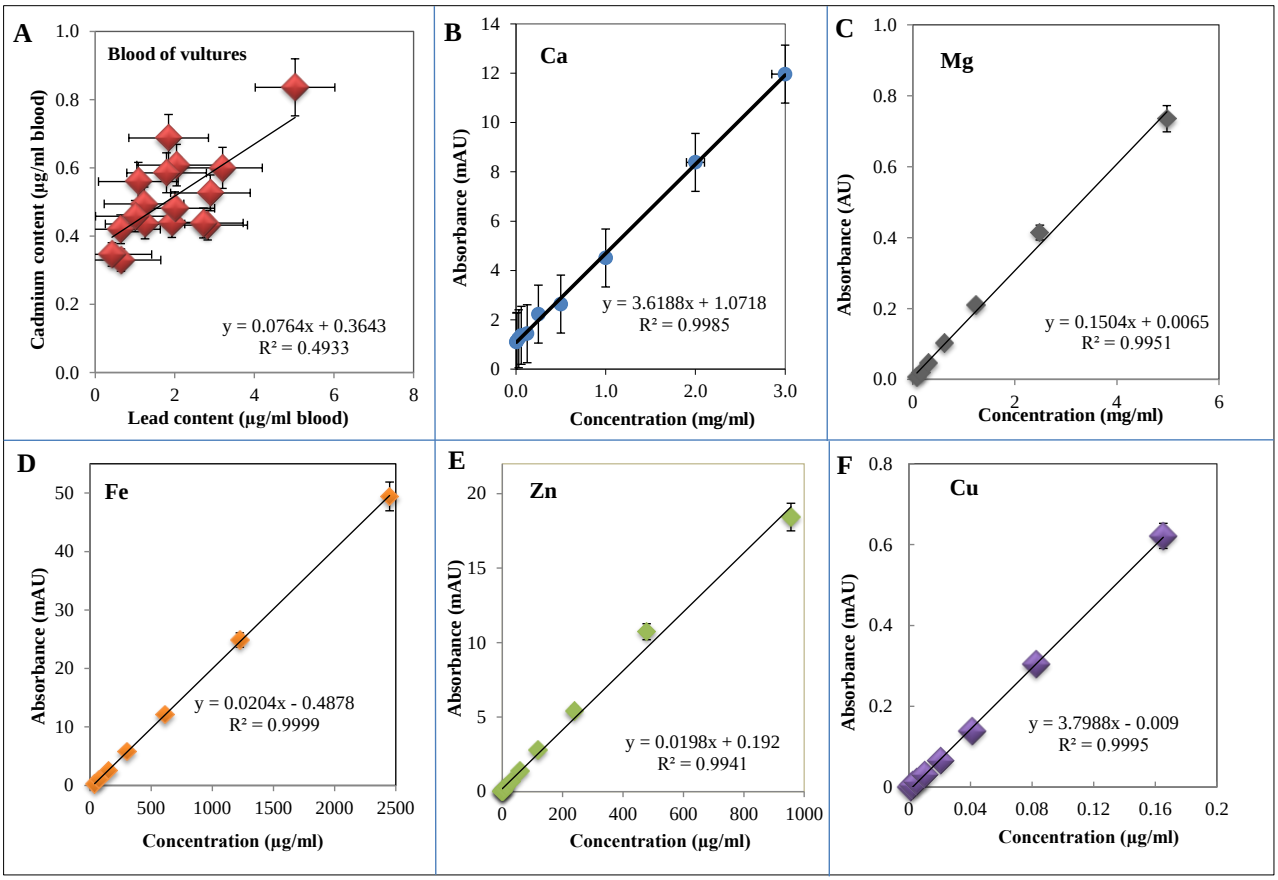

Figure 12. (**A**) Correlation between electrochemical detection of lead content and cadmium content in blood serum of vultures. Spectrometric detection of (**B**) Ca; (**C**) Mg; (**D**) Fe; (**E**) Zn and (**F**) Cu, the following volumes were injected 3, 3, 40, 10 and 10 µl, respectively. Other experimental conditions see in Caption of Figure 4.

Besides contents of lead(II) and cadmium(II) ions, we determined metallothionein using differential pulse Brdicka reaction [72,110,111]. It is well known that this protein binds heavy metal ions and its level is a good indicator of heavy metal intoxication [68]. Our study was based on already published papers aimed at determination of the metallothionein in biological samples [10,62,65,71,94-98]. In this study, different biological samples (blood serum, blood) of vultures intoxicated with lead ions were analysed. Preparation of samples was carried out by our established method (heat denaturation), followed by centrifugation and the sample was analysed using an automated electrochemical analyser (Fig. 13). Cat2 signal was used for quantification of metallothionein in low volumes samples (3, 5 and 10 µl). The obtained experimental data shows that under the sampling of 3 µl sample levels of MT as 45.1±9.4 µg/ml, median 44.1 µg/ml, max 66.7 µg/ml and min 20.2 µg/ml were determined. Under sampling of 5 µl of a sample, levels of MT as 69.6±9.3 µg/ml, median 68.3

µg/ml, max 99.3 µg/ml and min 40.0 µg/ml were determined. Under sampling 10 µl of a sample, levels of MT as 48.7±9.2 µg/ml, median 47.1 µg/ml, max 68.9 µg/ml and min 34.5 µg/ml.

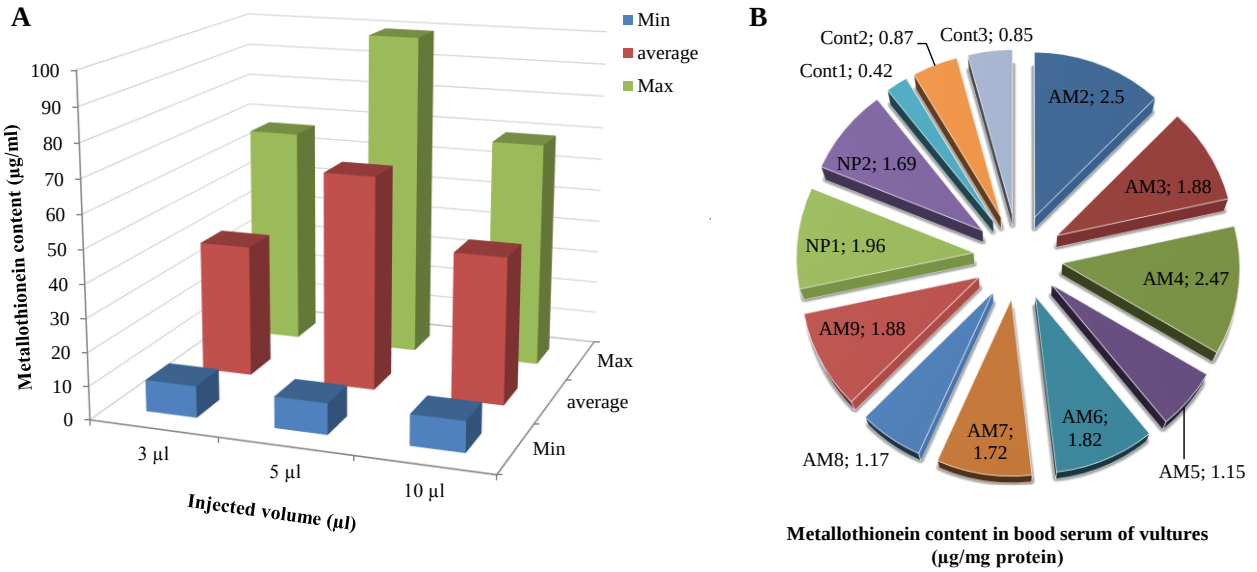

Figure 13. (**A**) Level of metallothionein determined in blood sera of vultures, the applied volume injections were as follows 3, 5 and 10 µl. (**B**) Distribution of metallothionein content in blood sera of vultures (µg/mg protein). Other experimental conditions see in Experimental part.

Table 2. Summary of determination of selected elements, total protein content and metallothionein in Cinereous vultures (*Aegypius monachus*) and Egyptian vultures (*Neophron percnopterus*), which were accidentally exposed to lead contamination. All samples are blood serum.

Samples	Pb[1] µg/ml	Cd[1] µg/ml	Cu[2] µg/ml	Fe[2] µg/ml	Ca[2] µg/ml	Mg[2] µg/ml	Zn[2] µg/ml	Protein[2] mg/ml	MT[3] µg /mg protein
AM1	1.08±0.04	0.97±0.01	0.05±0.01	0.19±0.02	25.66±1.11	4.68±0.22	0.08±0.01	25.72±0.85	2.50±0.01
AM2	5.02±0.03	0.84±0.02	0.04±0.01	0.19±0.02	8.53±0.12	3.90±0.09	0.07±0.01	27.56±0.99	1.88±0.03
AM3	3.02±0.02	0.86±0.01	0.03±0.01	0.52±0.02	8.66±0.015	4.93±0.07	0.09±0.01	26.34±0.48	2.47±0.05
AM4	1.93±0.03	0.85±0.02	0.03±0.01	0.45±0.02	10.50±0.02	4.57±0.03	0.05±0.01	62.51±0.33	1.15±0.04
AM5	1.25±0.02	0.44±0.01	0.04±0.01	0.18±0.02	7.44±0.03	4.26±0.03	0.63±0.02	26.73±0.41	1.82±0.03
AM6	2.05±0.03	0.61±0.01	0.04±0.01	0.28±0.02	9.08±0.03	4.81±0.02	0.34±0.02	29.29±0.01	1.72±0.02
AM7	1.84±0.02	0.69±0.01	0.03±0.01	0.32±0.01	12.43±0.05	4.64±0.02	0.24±0.01	39.07±0.03	1.37±0.02
AM8	1.79±0.01	0.59±0.01	0.03±0.01	0.23±0.01	6.33±0.02	3.52±0.01	0.46±0.01	43.45±0.88	1.17±0.02
AM9	2.83±0.02	0.43±0.01	0.03±0.01	nd	nd	nd	nd	44.45±0.88	1.88±0.02
NP1	2.72±0.02	0.44±0.01	0.03±0.01	nd	nd	nd	nd	33.85±0.68	1.96±0.04
NP2	2.89±0.01	0.53±0.01	0.03±0.01	nd	nd	nd	nd	38.85±0.78	1.69±0.03
Cont1	0.49±0.02	0.30±0.01	0.04±0.01	0.22±0.01	8.45±0.03	3.29±0.08	0.1±0.005	29.7±0.08	0.42±0.01
Cont2	1.23±0.02	0.49±0.01	0.03±0.01	0.19±0.01	9.15±0.04	3.08±0.01	0.30±0.01	27.87±0.01	0.87±0.01
Cont3	1.01±0.02	0.46±0.01	0.03±0.01	0.33±0.01	6.21±0.05	8.10±0.06	0.08±0.005	29.33±0.01	0.85±0.03

nd … not detected
AM … Cinereous vultures (*Aegypius monachus*)
NP … Egyptian vultures (*Neophron percnopterus*)
Cont … control Egyptian vultures (*Neophron percnopterus*)
[1] … electrochemical detection of Pb, Cd in blood samples
[2] … spectrometrical detection of Cu, Fe, Ca, Mg, Zn and total protein content
[3] … electrochemical detection of metallothionein (MT) by Brdicka reaction

Based on the results obtained it is clear that the sampling volume affects determined MT concentration to the maximum extent of 35 %. For our purposes sampling of 5 µl was used primarily due to increased sensitivity of the determination of rare specimens (detection of MT in young vultures, paper in preparation). MT levels are summarized in Table 2. In samples obtained from vultures intoxicated by lead(II) ions, the increase in MT levels above 1 µg/mg proteins was determined. The mean level was determined as 1.82 ± 0.31 µg/mg of proteins in these vultures compared with the control group 0.71 ± 0.19 µg/mg of proteins, which means more than 250% increase of MT levels.

In the paper published in Ecotoxicology, the authors observed levels of many metal ions including lead(II) ones in decayed vultures in Korea. Levels of lead(II) ions in dry weight varied from 1 to 34 µg/g [1]. Similar results were found from Primorye, Russia, where levels of lead(II) ions determined in the kidneys and liver were from 12 to 18 µg/g [112]. In this paper, it was found that distribution of lead in the tissues of vultures decreased in the following order: **liver > kidneys > feathers = heart > brain > muscles** with significant correlation of lead content kidneys-liver and kidneys-brain [112]. On average, the concentrations of heavy metals did not exceed critical levels and, therefore, could not cause death in the birds [112]. A comprehensive study was performed on diagnostics and treatment of lead toxicosis in falcons in Saudi Arabia. Lead toxicosis, which belongs to the most commonly diagnosed diseases of falcons, was found in 2.9 % of falcons included in the study [113]. Further, determined level of lead(II) ions in raptors ranged from 25 to 65 µg/l [113]. In the following study, Pyrenean bearded vulture blood was analysed and it was found that lead(II) ions content was up to 20 µg/l. The levels in organs were also low [34]. Level of lead(II) ions was approximately 20 µg/g in liver and kidneys in eagles from USA, Canada and Germany [114]. In addition, 118 *Haliaeetus albicilla* was collected between 1981 and 2004 in Sweden, from which both liver and kidney samples were taken [115,116]. A total of 22 % of all eagles examined had elevated (>6 µg/g DW) lead concentrations, indicating exposure to leaded ammunition, and 14 % of the individuals had either liver or kidney lead concentrations diagnostic of lethal lead poisoning (>20 µg /g DW). Lead concentrations in liver and kidney were significantly correlated. In individuals with lead levels <6 µg/g, concentrations were significantly higher in kidney than in liver; in individuals with lead levels >20 µg/g, concentrations were significantly higher in liver [115]. Lead levels leading to death of individuals are associated with biological and physiological factors of birds/raptors. In the case of controlled experiment with defined lead(II) doses, marked enhancement of lead(II) concentration in organs was found (more than 200 µg/g). All individuals from the experimental group treated with higher dose of lead (13-19 pellets) died within ten days. In the case of low dose (1-2 pellets), 50% individuals died within 20 days [21,117]. To evaluate toxicity of lead the ratios of hepatic Pb/bone Pb and kidney/bone Pb are commonly used. The use of this marker is rather complicated due to differences in blood perfusion rates within these tissues [118]. Moreover, it is well known that acute and chronic exposure to Pb can affect avian health by altering reproductive success, behaviour and immune response [119,120]. Concentration of lead(II) ions in avian blood is summarized in review by Buekers *et al.* [121].

The diagnosis of lead toxicosis in birds can by relatively straightforward in the presence of typical clinical signs (including ataxia, paralysis of the wings and legs, amaurosis, seizures, lethargy, anorexia, shredding of food, intermittent regurgitation, delayed crop emptying, progressive weight

loss, poor flight performance , and pastel green-coloured urates) together with clinical information gathered through the anamnesis [113]. Diagnostics is supported by radiographic detection of metallic pellets or fragments within the gastrointestinal tract. The radiographic absence of lead pellets or fragments within the gastrointestinal tract makes the diagnosis of lead toxicosis more difficult [122]. Electrochemical detection is also very convenient for detection of blood lead concentrations within several minutes, mainly by LeadCare system. The LeadCare System relies on electrochemistry and a sensor to detect lead concentrations in whole blood, particularly the red blood cells (RBCs). When whole blood is mixed with its reagent, containing 1% hydrochloric acid, RBCs are lysed and lead is released. Lead then collects on the sensor, and its concentration is measured and displayed in micrograms per decilitre (mg/dl). Whole blood samples were stored immediately in commercially available tubes containing lithium heparin anticoagulant (1.8 mg lithium heparin per 1 ml whole blood; Lithium Heparin Paediatric Tubes, Bibby Sterilin, Staffordshire, England). Heparinized blood (50 µl) was thoroughly mixed with the LeadCare System's treatment reagent and allowed to stand for 1 minute. From this sample, 50 µl was withdrawn and applied over the well within the sensor. The sensor was inserted into the analyser, and the blood lead concentration was displayed in 3 minutes. The electrochemical detection was used for diagnostics of lead toxicosis in falcons in Saudi Arabia [113].

4. CONCLUSIONS

Metal ions such as cadmium(II) and lead(II) are known to be extremely toxic in natural environments [123,124]. Their mobility is often connected both with natural and anthropogenic activities. As a result, metal ions significantly affect human health and wildlife species. It was found that lead can influence not only the physiology and health of individual organisms, but also the demographics and the distribution of species [12]. For understanding transport and cycles of toxic metals in environment, joint efforts under flag of international platforms as United Nations Environment Programme with Lead and Cadmium Activities is required [32].

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