Int. J. Electrochem. Sci., 7 **(2012)** 5893 - 5909

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

Automated Electrochemical Detection of Iron Ions in Erythrocytes from MeLiM Minipigs Suffering from Melanoma

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Received: 10 May 2012 / *Accepted:* 7 June 2012 / *Published:* 1 July 2012

Modern analytical methods allow the detection of compounds of interest in biological samples in very low concentrations. However, some of these compounds are electro-active or provide electro-active complexes after derivatization. In this case, electrochemical detection is very useful. Stationary electrochemical analysers have a plenty of advantages, such as low costs, high sensitivity and repeatability. On the other hand, non-automated injection of samples belongs to the main disadvantages of electrochemical techniques. Due to this fact, we focused on testing of new electrochemical system enabling us to inject units of µl of a sample and cooled sample-rack area. The analyser can be used for detection and quantification of a wide range of compounds including metals, peptides, proteins, nucleic acids and drugs. To test the possibilities of suggested system, concentration of iron in blood samples of MeLiM minipigs was measured. Primarily, we optimized the following experimental conditions as supporting electrolyte 0.1 M $KBrO_3$ + 0.3 M NaOH + 0.01 M TEA of pH 11, and deposition potential of 0 V. Moreover, preparation of erythrocytes was optimized. Changes in iron content of control group (10 µg Fe ions/ml) and group of animals suffering from melanoma progression (3.5 µg Fe ions/ml) were determined.

Keywords: electrochemical detection; automation; voltammetry; iron; biological sample; metallomics

1. INTRODUCTION

Laboratory systems, which require minimized human operations, are significantly preferred in the clinical laboratories. Especially levels of biochemically significant ions, such as K^+, Na^+, Cl^-, and Ca^{2+} are automatically monitored [1]. Automation of laboratory operations is focused especially on accurate and precise sampling, on which automated connection with end analyser variously modified in the direction of improvement of selectivity of analysis is coupled [2-8]. Electrochemical methods using the mercury electrode or solid electrodes as a detection electrode are difficult to automatize [9,10]. Development of an automatic system for differential pulse amperometry (DPA) with electrochemical deposition represents one of the most important steps in automation of these techniques. The system consists of a microcomputer directed by home-made software, connected to a commercial potentiostat through a data acquisition board. System was applied for selenium determination using gold electrode and flow injection analysis (FIA) [11].

There are following possibilities of automation in electrochemical detection in laboratory arrangement: a) sample is transported together with supporting electrolyte into detection cell; larger volume of sample is necessary in this case. After analysis, washing process follows and procedure is repeated [12]; b) sample is transferred into detection cell by a transfer arm; in this case, demands on sample volume are not so high. After analysis, washing process follows and procedure is repeated [12]; c) sample is dosed in strictly defined volume, electrodes are positioned into the sample by transfer arm. After analysis, electrodes are regenerated and procedure can be repeated [13]; d) sample is placed into flowing supporting electrolyte. After analysis, electrodes are electrochemically regenerated and procedure can be repeated (FIA) [14]; e) sample is dosed into flowing supporting electrolyte as capillary electrophoresis or high performance liquid chromatography, individual components of a sample are separated and subsequently electrochemically analysed [14,15]. Recently, we described technologically available system for fully automated detection of thiol compounds with reproducible dosing of very low volume of sample (units of microlitres), which may be used in detection and quantification of selected thiol compounds [12]. Interesting system was introduced as a prototype of apparatus for operating with solid electrodes. Automatic voltammetry was established in 24-well microtiter plates. The assay used a movable assembly of a pencil rod working, an Ag/AgCl reference and a Pt counter electrodes for concentration-dependent current generation. A computer was in command of electrode (z) and microtiter plate (x, y) positioning and timed potentiostat operation. Synchronization of these actions supported sequential approach of all wells and subsequent execution of electrode treatment procedures at defined intervals in a measuring cycle [13]. Specialized analyses can be subsequently performed using the miniaturized platform of lab on a chip technology [16]. Recently, DNA sensor chip based on chronocoulometric measurement was realized [17].

1.1 Electrochemical detection of iron ions

Iron is one of most common metals in biological matrixes. Iron is an important element in the environment, because plays crucial and essential role in numerous biological systems. The presence of iron, alone or in complexes, has beneficial or deleterious effects on the properties of many substances

and subsequently various biological systems, especially due to its occurrence in two states – as divalent and trivalent ions with possibility to be involved in oxidation-reduction systems and due to chelatation properties of iron ions. The main iron functions are as follows: haemoglobin synthesis, oxidative processes in tissues, structural function in myoglobin, essential part of haemoproteins including ironsulphur proteins, and many co-factors involved in enzyme activities, such as cytochromes containing iron-porphyrin system, and catalases and peroxidases [18-20].

Detection and quantification of iron in the clinical, environmental, industrial, and pharmaceutical samples is very frequent and important. For iron detection, voltammetry, chromatography, spectrophotometric methods, capillary electrophoresis, controlled potential coulometry and potentiometry are commonly used [21,22]. Electrochemical methods have extraordinary position among analytical methods used for iron detection because of their sensitivity, simplicity, low cost and easy-to-use. Electroanalytical determination of iron ions demands on the application of some complexing agents [23-25]. These complexes provide sufficiently sensitive and selective signals on working electrodes. The summary of the most interesting ones follows. Determination of iron ions in various types of waters is of great interest. Fe(III) complexes with succinic acid, a ligand naturally present in seawater, were investigated in the work of Cmuk et al. [26]. A long-standing problem associated with voltammetric determination of iron and sulphide in reduced natural waters has been the nature of the presumed analyte responsible for a reduction peak at -1.1 V vs. Ag/AgCl. Cyclic voltammetry at the Hg electrode was used to study solutions with different Fe(II) to sulphide rates in chloride and acetate electrolytes. The results indicate that the -1.1 V peak can be assigned to reduction of Fe(II) or its labile complexes in FeS layers that partially cover the Hg electrode. Hg electrodes covered with FeS act like FeS solid electrodes over a very wide potential range (-0.35 to -1.9 V). The dominant mechanism involves attachment at the Hg surface of FeS nanoparticles, which are generated quickly in initially supersaturated mixtures of Fe(II) and S(-II). In a narrow deposition potential range, roughly -0.56 to -0.70 V, FeS layers were produced additionally by replacement of preformed HgS [27]. The chemical speciation of iron in seawater was determined by cathodic stripping voltammetry using 2,3-dihydroxynaphthalene (DHN) as adsorptive and competing ligand. The method was applied to samples from the ocean surface waters [28,29]. The determination of electrochemically labile iron in estuarine and coastal seawater was based on differential pulse anodic stripping voltammetry at a rotating silver-alloy disk electrode [30]. The electrochemical behaviour of iron ions in the presence of another complexing agent 1-ethyl-3-methylimidazolium bis(trifluoromethyl-sulphonyl)imide ([Emim](+)Ntf2(-)) and mixtures with Cl^- was studied with the aim of investigating the applicability of ionic liquids [31]. An adsorptive stripping voltammetry method for the iron ions determination was based on the adsorptive collection of a complex of iron ions with 1-(2-pyridylazo)-2- naphthol on a bismuth-coated glassy carbon electrode [32]. Sensitive and selective adsorptive stripping procedure for simultaneous determination of iron, copper and cadmium is based on the adsorptive accumulation of thymolphthalexone complexes of these elements onto a hanging mercury drop electrode surface followed by the reduction of adsorbed species by voltammetric scan using differential pulse modulation [33]. In addition, determination of complexation of iron(III) with natural organic complexing ligands in seawater using cathodic stripping voltammetry was presented to determine the extent of iron complexation by natural organic ligands in seawater.

Catalytic cathodic stripping voltammetry was used to take advantage of ligand competition between the added ligand, 1-nitroso-2-napthol (NN), and natural ligands present in seawater. The conditional stability constant for the complexation of iron by NN was calibrated for salinities between 1 and 36 using ligand competition with EDTA [34,35]. Determination of iron in seawater using cathodic stripping voltammetry preceded by adsorptive collection with the hanging mercury drop electrode was introduced too [36].

Various types of working electrodes are tested for determination of iron ions as nafion-coated glassy carbon electrodes, which were employed for pre-concentration and detection of Fe(II) and Fe(III) cations from aqueous solutions. The influence of the supporting electrolyte composition and of the redox state on the analyte partitioning within the Nafion coating were examined. Using the cyclic voltammetry, the ion-exchange voltammetric determination of iron was achieved from voltammetric peak currents relevant to the reversible redox process [37]. A thick-film graphite-containing electrode modified with calomel was suggested for determining iron(III) by stripping voltammetry, and pyrocatechol was found to be the most sensitive in determining iron(Ill) [38].

Besides standard conditions, electroanalytical techniques can be also used for analysis of iron ions under high temperatures and pressures. The cyclic voltammetry method using Mo electrode was applied for studying of Fe electrodeposition by dissolving the Fe_2O_3 or $FeCl_2$ in molten alkaline-earth and/or alkali metal halides under a constant temperature of 1123 K [39]. The voltammetry of microparticles was applied to characterize the electrocatalytic ability of solids towards the selected electrochemical processes. The variation of catalytic currents under fixed electrochemical conditions permits to obtain composition/time data to be fitted with solid state kinetic models. This methodology was applied to analyse the thermal decomposition of magnesiochromite (ferroan) and chromian-chloride standards and a South Africa iron chromite ore on the basis of the significant catalytic effect on the electrochemical oxygen evolution reaction in aqueous alkaline media at mineral-modified graphite electrodes [40].

1.2 Tools for iron detection in geology

The mining industry around the world produces wastes, which represent a serious environmental problem not only but also due to high toxic metal and iron sulphide content. Iron sulphide oxidation under weathering conditions provokes the main environmental problem of the mining industry called as the generation of Acid Rock Drainage. Paper shows the utilization of cyclic voltammetry using carbon paste electrodes as an alternative tool in the study of the oxidation capacity of iron sulphides and mining wastes [41]. Mineralogical discrimination of iron oxides in soils and sediments is not a trivial task, mainly because of their small grain size and low concentration. With mineral-magnetic techniques, highly magnetic ferrimagnetic spinels can be determined with a very low detection limit (similar to 10 ppm). Unfortunately, the magnetic signal of natural samples is often dominated by magnetite, and in particular the expression of weakly magnetic antiferromagnetic minerals is suppressed by this high signal. In contrast, electrochemical techniques, such as voltammetry of microparticles (VMP) are not affected by these differences in magnetic signal. VMP

makes use of the electrochemical law that iron(oxy)(hydr)oxides can be reductively dissolved at potentials that are specific for their mineralogy and reactivity [42]. VMP was also used for identification of iron oxides and oxy-hydroxides. This technique allowed distinguishing different mineral species, such as hematite, goethite, magnetite and maghemite, in both synthetic and natural samples. By measuring synthetic pigments at different temperatures, evidence was found of an electrochemical behavioural pattern, which allowed differentiating them. The current peak location (potential value) proved to vary according to the mineral species, grain size and crystallinity degree [43].

1.3 Interaction of iron ions with biological matrix

There are great possibilities in the field of iron determination complexed by peptides, proteins and other biologically important molecules [44,45]. The electrocatalytic iron release from *Pyrococcus furiosus* Erauso ferritin upon reduction with a series of electron mediators was studied by Tatur et al. The observed iron release rate as a function of mediator midpoint potentials was described by a two-step model, in which electron transfer from the mediator to ferritin was rate limiting at low driving force, and the proteins. The upper limit of the mediator potential, at which the reductive iron release activity of *P. furiosus* ferritin has been observed in the electrochemical cell is -47 mV [46]. The sample preparation and analytical methodology were described for detecting biologically produced iron(III)-binding ligands in laboratory cultures of coastal marine phytoplankton. The iron(Ill)-binding ligands from the culture media were purified by passage through a column packing with a hydrophobic absorbent. The concentrations and stability constants of the ligands were determined by adsorptive cathodic stripping voltammetry with competitive ligand equilibration [47]. In our study, we aimed our attention at suggestion and verification of fully automated electrochemical measuring system for iron ions determination. To test the possibilities of suggested system, concentration of iron in blood samples of MeLiM minipigs was measured.

2. EXPERIMENTAL PART

2.1 Chemicals and material

$FeSO_4$ and other chemicals were purchased from Sigma Aldrich (St. Louis, USA). Stock standard solutions were prepared with ACS grade water (Sigma-Aldrich, USA) and stored in the dark at –20 °C. Working standard solutions were prepared daily by dilution of the stock solutions. To pipette volumes down to micro and nanolitres, pipettes used were purchased from Eppendorf Research (Eppendorf, Germany) with the highest certified deviation (±12 %). 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).

2.2 Automated voltammetric analyser

The 797 VA Computrace is voltammetric measuring stand that is connected to a PC via a USB port. PC software controls the measurement, records the measuring data and evaluates it. Operation is straightforward due to the well-laid-out structure of the program. The analyser (797 VA Computrace from Metrohm, Switzerland) employs a conventional three-electrode configuration with a hanging mercury drop electrode (HMDE; ACS mercury from Fluka, USA) working electrode: 0.4 mm2, Ag/AgCl/3M KCl as reference electrode, and a platinum auxiliary electrode (Fig. 1).

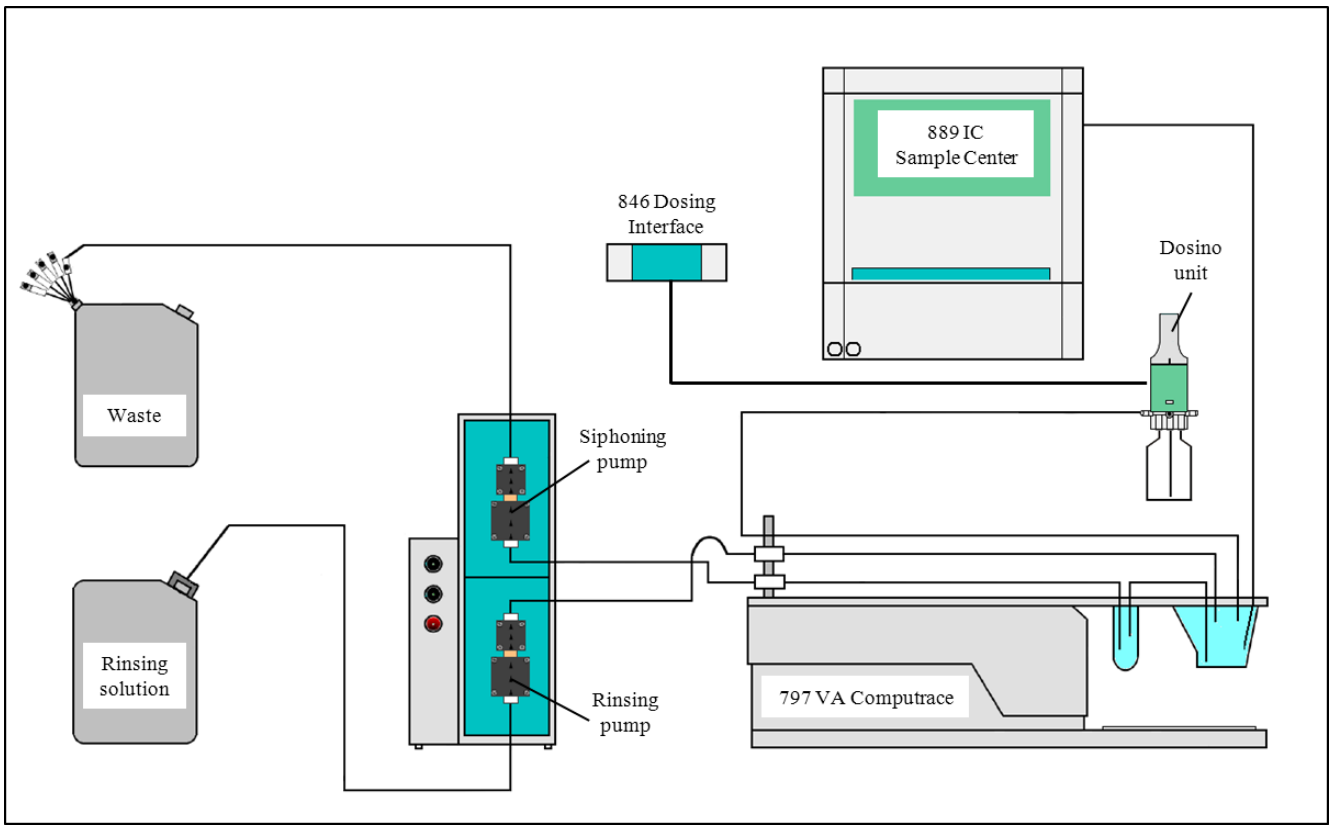

Figure 1. Scheme of voltammetric trace analyser. The main part is 797 VA Computrace, which is connected to Dosino unit and 889 IC Sample Center. System of two pumps control washing the electrochemical cell and dosing the samples.

A sample changer (Metrohm 889 IC Sample Center) performs the sequential analysis of 96 samples in plastic test tubes (Fig. 2A). Dosing needle, bottle for the needle washing and multi-way valve are the accessories of equipment. For the addition of standard solutions and reagents, automatic dispenser (Metrohm 800 Dosimat) is used, while peristaltic pump station (Metrohm 843 Pump Station, Fig. 2B) is employed for transferring the rinsing solution in the cell and for removing solutions from the voltammetric cell (Fig. 2C). Automatic dispenser and peristaltic pump station are controlled by central unit (Metrohm 846 Dosing Interface). Electrolyte is transferred into electrochemical bottle by the dosing unit via multi-way valve. The whole instrument is shown in Fig. 2D.

2.3 Electrochemical procedure for iron ion analysis

Differential pulse voltammetric measurements for determination of iron ions were carried out under the following parameters: deoxygenating with argon 120 s; start potential -0.6 V; end potential -1.3 V; deposition potential 0 V; accumulation time 0 s; pulse amplitude 0.05 V; pulse time 0.06 s; step potential 5.951 mV; time of step potential 0.1 s; volume of injected sample 20 µl; cell was filled with 2400 µl of electrolyte: 0.1M $KBrO_3$, 0.3M NaOH, 0.01M triethanolamin (TEA).

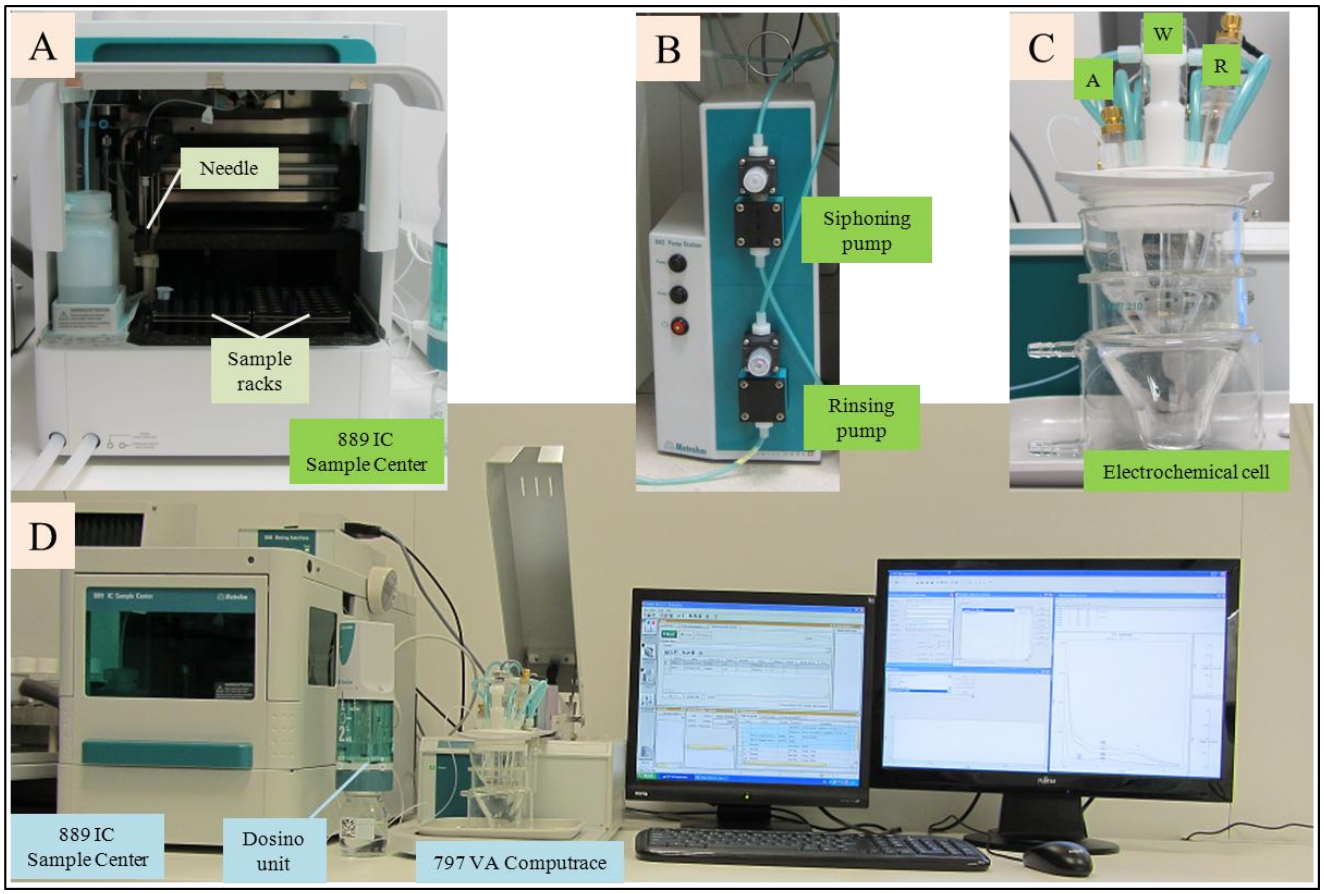

Figure 2. Photography of voltammetric trace analyser. The analyser consists from (A) 889 IC Sample Center, (B) pumps, (C) electrochemical cell containing A – auxiliary electrode, W – working electrode, R – reference electrode, (D) the whole instrument.

2.4 Animal blood samples

The miniature pigs are housed in the Institute of Animal Physiology and Genetics of the Academy of Sciences of the Czech Republic, Libechov. The experimental herd of laboratory pigs was founded by importation of 5 animals of the Hormel strain from the USA in 1967. These animals were crossbred for porcine blood group studies with several other breeds or strains: Landrace, Large White, Cornwall, Vietnamese pigs and miniature pigs of the Göttingen origin. Different cross-breeding produced more than 2000 descendants without any signs of melanoma. Nevertheless, a few black

piglets with melanoma had occurred in this genetically heterogeneous population by 1989. They originated from mating two male brothers with four related sows. These parents had no visible skin tumours. The MeLiM strain with hereditary melanoma was established using selective breeding [48]. The MeLiM minipigs with melanoma progression (n = 8) and melanoma-free controls (n = 8) were used in our study. Blood samples (4 ml of peripheral blood) were collected in test-tubes coated by ethylenediaminetetraacetic acid (EDTA), centrifuged to obtain plasma and erythrocytes. The obtained erythrocytes were twice times washed with physiological solution and were immediately deep frozen prior to use. All experiments were authorized by Ethic Committee.

2.5 Preparation of samples

Samples were centrifuged (Eppendorf, Germany) at 2000 rpm for 10 min. Blood plasma was subsequently removed. For analysis itself, only erythrocytes were used. For the first, 0.1 g of erythrocytes was transferred into Eppendorf tubes. After that, the sample was frozen with liquid nitrogen for 5 min to disrupt the cells. Then we added 500 µl of 1mM HCl and sample was ultrasonicated for 2 minutes at 40 W by the using the needle (Bandelin, Germany). Homogenates were then vortexed for 10 minutes at 400 rpm (Genie, USA) and then centrifuged for 30 minutes at 16000 rpm (Eppendorf, Germany) prior to next analysis. The obtained supernatant (100 µl) was pipetted into Eppendorf tubes with 100 µl TFA solution (20 % trifluoroacetic acid). Diluted sample was then centrifuged for 10 minutes at 16000 rpm (Eppendorf, Germany) and supernatant (20 µl) was used for electrochemical determination.

2.6 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®, Microsoft, USA). Accuracy, precision and recovery of iron ions were evaluated with homogenates (erythrocytes extracts) spiked with standard of the ions. Before extraction, 100 µl iron ions standard and 100 µl water were added to erythrocytes. Homogenates were assayed blindly and iron ions concentrations were derived from the calibration curves. The spiking of iron ions was determined as a standard measured without presence of real sample. Accuracy was evaluated by comparing estimated concentrations with known concentrations of thiol compounds. Calculation of recovery was carried out as indicated by Causon [49] and Bugianesi et al. [50].

3. RESULTS AND DISCUSSION

Analytical instrumentation for trace and ultra-trace analysis is aimed not only at sensitivity but also possibility of high throughput measurements. The aim of this progress is to establish the complex system covering sample preparation and analysis with minimal operator actions [51,52]. Such

commercial systems are very often limited in the point of view of number of detected compounds. Composition of sample matrix is in this case limited too. Determination of biological samples brings another complication to this issue and brings other demands for such systems. Biological instability is a great problem at detection of wide range of samples or at long time monitoring. Solving of this issue is very often hidden in cooling of apparatus (sample-rack area or whole measuring system).

3.1 Automated electrochemical system

Electrochemical analysis of biological samples is often connected with need of cooling samples. Presented system consists from four basic parts: electrochemical measuring stand, autosampler (889 IC Sample Centre), dosing unit (800 Dosino Dosing Unit, 846 Dosing Interface) and pumps (843 Pump Station), which is shown in Fig. 2. Uniqueness of presented system is in connection of cooling sample area in autosampler and possibility of samples injection in units of µl. Samples way through the measuring system begin in Eppendorf tube, which is positioned in autosampler rack. Then sample is taken up with needle and after that it is transported through injection valve and loop to measuring vessel. Electrolyte is in glass bottle and is injected by dosing unit into electrochemical cell. Communication between software and hardware is provided by 846 Dosing Interface. All procedures are controlled by two software programmes as MagIC Net 2.3 and Computrace Software. Both programmes are synchronized by the programming their applications.

3.1.1 889 IC Sample Centre

Sample Center is controlled with software MagIC Net 2.3 (Figs. 1 and 2). This autosampler was selected for its opportunity to cool samples and to choose low volumes to be injected. The whole system consists from five main parts: 2 sample racks (each for 48 vials), needle arm (with air needle and drives for horizontal and vertical positioning), injection valve (with six connectors and two switching positions), syringe module (for aspirating and transferring the samples), and cooling module (for cooling the samples). Sample capacity of system is 2 microtiter plates according to the SBS standard, with 96 cavities (high/low shape) and 384 cavities (low shape), 48 vial- or 12 vial-racks. In our system, we use two 48-vial racks with adapters to Eppendorf tubes for each position (Fig. 2A). For vial recognition in a rack, a sensor tracking missing vials is used. Injection valve has six connectors and is capable to work in three modes: *Full-Loop*, *Partial Loopfill* and *Pickup mode*. Individual modes differ in purpose of injection. Full-Loop injection is the best for full precision (100 µl), Partial Loopfill for full flexibility (1 to 50 µl) and Pickup mode for smallest loss of sample (1 to 27 µl). The following range of sample volumes can be covered with the various injection modes using the 500 µl syringe, in combination with the standard buffer loop (1000 µl) and the standard sample loop (100 µl). Analytical characteristic are different for the individual injection modes. For the Full-Loop injection relative standard deviation is $\leq$ 0.3%. For the Partial Loopfill injection relative standard deviation is $\leq$ 0.5%, when injection volume is > 10 µl. For the Pickup injection relative standard deviation is $\leq$ 1.0%, when injection volume is > 10 µl. A sample cooling is performed by built-in Peltier element. It can be used

within the range from 24 °C to 25 °C. Air temperature in the sample vessel: 4 ±2 °C (at the temperature sensor).

3.1.2 797 VA Computrace

797 VA Computrace is standard electrochemical measuring platform, in our case, standard three electrode configuration with a hanging mercury drop electrode (HMDE) working electrode with surface of 0.4 mm^2 (Multi-Mode Electrode), Ag/AgCl/3MKCl as reference electrode, and a platinum auxiliary electrode was used (Fig. 2C).

3.1.3 Dosing instruments

Dosing unit 800 Dosino Dosing Unit (Metrohm) is used for electrolyte dosing into electrochemical cell. In our case, dosino unit has maximal injection volume of 2 ml. Dosino has resolution of 10000 steps per cylinder volume. Maximal systematic dosing deviation is ±0.5 %. This part of system takes care of rinsing and siphoning with rinsing solution (MiliQ water) in electrochemical cell. Pump station has two membrane pumps. One is for siphoning and the second is for rinsing. Membrane pumps are made from PTFE, which ensure high chemical resistance. Pumps has delivery rate > 450 ml/min.

3.1.4 Measuring software

MagIC Net 2.3 software is used for the control of operation with samples by 889 IC Sample Center (Methrom). Two basic modes of measurement are available – single determination and determination series. 797 VA Computrace Software electrochemical determinations are controlled by this software. All electrochemical methods and parameters are available to choose. Measurement as determination series or single step is also possible. Evaluation of determination curves is automatic after assignment of characteristic peaks potential. Coordination of programming steps as autosampler and measuring stand is needed due to the time synchronization. Both programmes allow defining the steps by time and other parameters. Time as a parameter is the primary key, which creates the connection between software and instruments.

MagIC Net 2.3 is the first software to be used from the point of view of programming. There is possible to define parameters, which allow communicating with 797 VA Computrace software. The programme consists from two parts; one is for the first sample in measurement series and the second for other samples in the rack (Fig. 3A). First sample programme begins by initializing the communication with 797 VA Computrace. Second step consists in dosing the sample. This procedure consists from three steps as injecting of sample, electrolyte dosing and electrolyte filling. Measurement using 797 VA Computrace software is the last step of this programme sequence. After this sampler needle is moved to wash position and is washed. Subsequently, electrochemical cell is rinsed with miliQ water. For second and other samples in the sample rack, second programme is chosen (Fig. 2A).

In this case, the programme begins with dosage of sample. This procedure has the same structure as in the first programme.

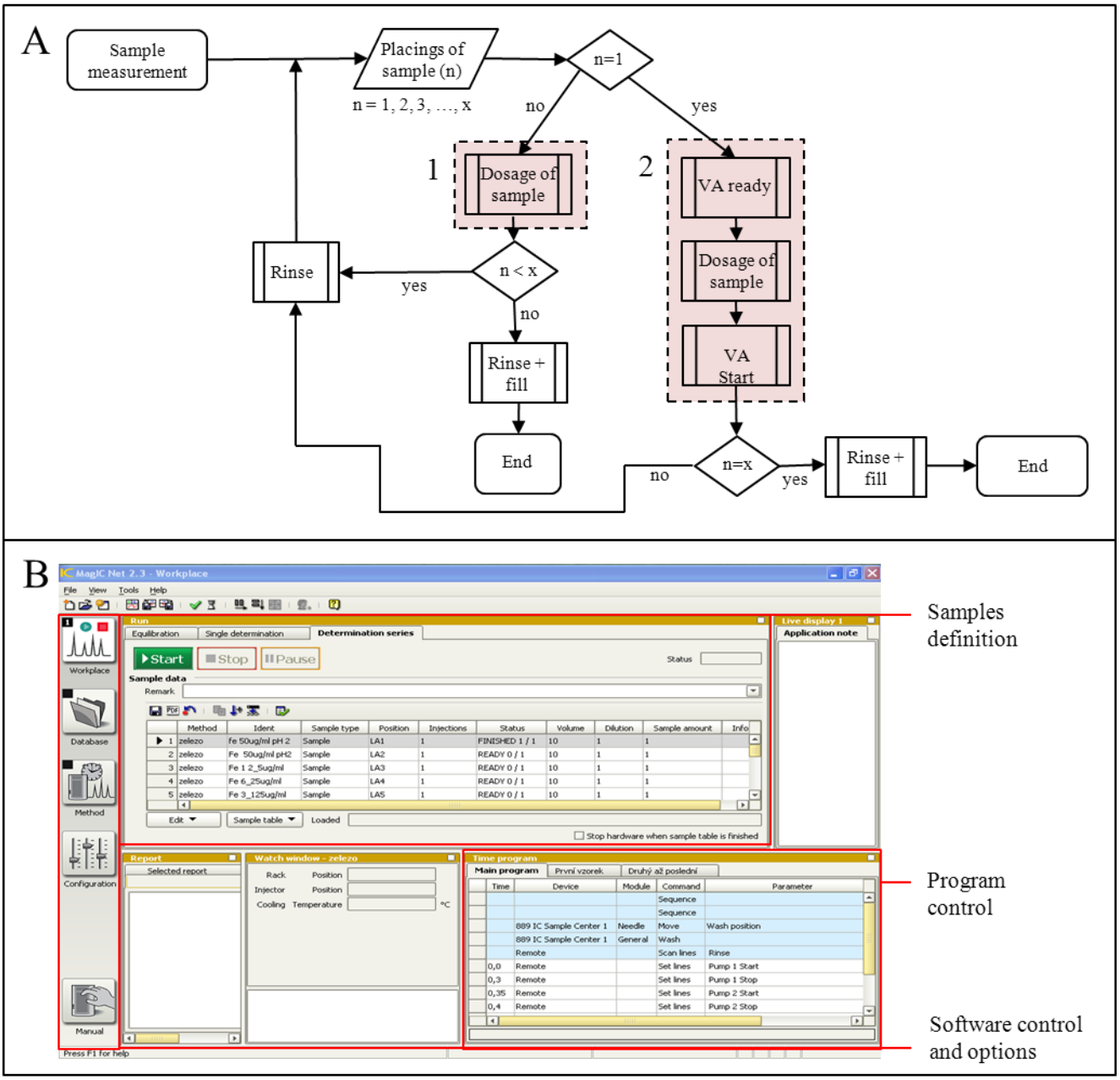

Figure 3. (A) Sample measuring flow chart: automated electrochemical system, where n is number of sample in the rack. Program of measurement is divided into two basic parts (1 and 2), which differs in placing of a sample in a rack. First measured sample in the rack requires software connection between MagIc Net and VA 797 Computrace software. After that both programmes run parallel. (B) Software window for autosampler control.

Connection with 797 VA Computrace software is taken up already in first programme and next coordination is controlled only with time. After sample measuring the same steps follow to the first

programme. Rinsing of electrochemical cell is defined by MagIC Net 2.3 command. There are commands in 797 VA Computrace software for rinsing too. These commands reflect only the time of rinsing, because 797 VA Computrace has no connection with 846 Dosing interface. After last analysis, electrochemical cell is twice rinsed with miliQ water and the last step is filling with miliQ water. The print screen from the window with the programme is shown in Fig. 3B.

Programme (first or second); Needle move and wash; Rinsing of measuring vessel (Pump 1 – rinse; Pump 2 – fill); Pump 1 Start (0 min); Pump 1 Stop (0.30 min); Pump 2 Start (0.35 min); Pump 2 Stop (0.40 min); Pump 1 Start (0.45 min); Pump 1 Stop (1.45 min); Pump 2 Start (1.50 min); Pump 2 Stop (1.55 min). First programme: Scan lines - VA Ready (1 min.); Pump 1 start (0 min); Pump 2 stop (1 min); Pickup injection (Injection volume: From Sample Data; Transport Volume: 1× Syringe Volume; Needle Height: 6mm; Syringe speed: normal; Air segment: off; Head Space Pressure: off; Wash After Injection: on); Dosing electrolyte (Port 1; Volume: 2 ml; Dosing Rate: max; Filling Rate: max); Fill electrolyte (Port 2; Rate: maximum); Set lines - VA Start (Time 0 min; Output Signal: VA Start).

Second programme: Pump 1 start (0 min); Pump 2 stop (1 min); Pickup injection (Injection volume: From Sample Data; Transport Volume: 1× Syringe Volume; Needle Height: 6 mm; Syringe speed: normal; Air segment: off; Head Space Pressure: off; Wash After Injection: on); Dosing electrolyte (Port 1; Volume: 2 ml; Dosing Rate: max; Filling Rate: max); Fill electrolyte (Port 2; Rate: maximum).

3.2 Determination of iron in erythrocytes

Suggested electroanalytical system was used for determination of iron ions in erythrocytes. Optimization experiment was focused on the testing of three potentially suitable electrolytes for analysis (Electrolyte No. 1: ammoniumoxalate (0.25M) + NH_3 (1M) + NaOH (10M); Electrolyte No. 2: ammoniumoxalate (0.25M) + triethanolamin (TEA 0.01M); Electrolyte No. 3: $KBrO_3$ (0.1M) + NaOH (0.3M) + TEA (0.01M)). Fig. 4A shows that the most suitable electrolyte was electrolyte No. 3, which was mixture of 0.1M $KBrO_3$ + 0.3M NaOH + 0.01M TEA, where significant increase in signal (for more than 80 %) was detected compared to electrolytes Nos. 1 and 2. The observed signal of iron ions was highly dependent on the pH of supporting electrolyte. Promising increase of signal height for more than 40 % was detected under pH values between 10 and 11. pH values from 11 to 12 led to decreasing of signal,(in inset Fig. 4A). Based on the results obtained it can be concluded that the optimal pH of the supporting electrolyte No. 3 was 11. We also tested the effect of deposition potentials (0, -0.3, -0.6, -0.9 and -1.2 V) on the electrochemical response. Signal of iron ions gradually decreased with the applied negative potential (y = -8x). Deposition potential of 0 V was the most suitable for determination of iron ions. There are no changes in the observed electrochemical signal of iron ions in the case of pre-concentration test (Fig. 4B). Our results indicate that electrode process of complexes formation between constituents of supporting electrolyte and iron ions takes place on the HMDE working electrode surface.

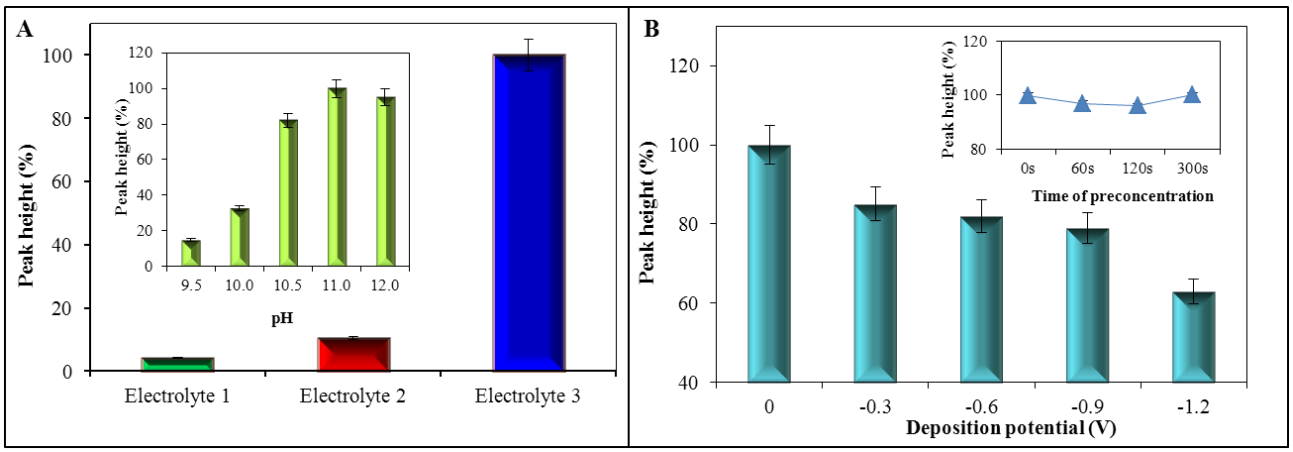

Figure 4. (A) Influence of electrolyte composition on iron signal height. Electrolyte 1: ammoniumoxalate (0.25M) + NH_3 (1M) + NaOH (10M); Electrolyte 2: ammoniumoxalate (0.25M) + triethanolamin (TEA 0.01M); Electrolyte 3: $KBrO_3$ (0.1 M) + NaOH (0.3M) + TEA (0.01M); in inset: the influence of pH values of electrolyte No. 3 on iron signal height. (B) Dependence of signal intensity on deposition potential; in inset: influence of time of accumulation on iron signal height. Both characteristics were measured in the presence of electrolyte No. 3 ($KBrO_3$ + NaOH + TEA).

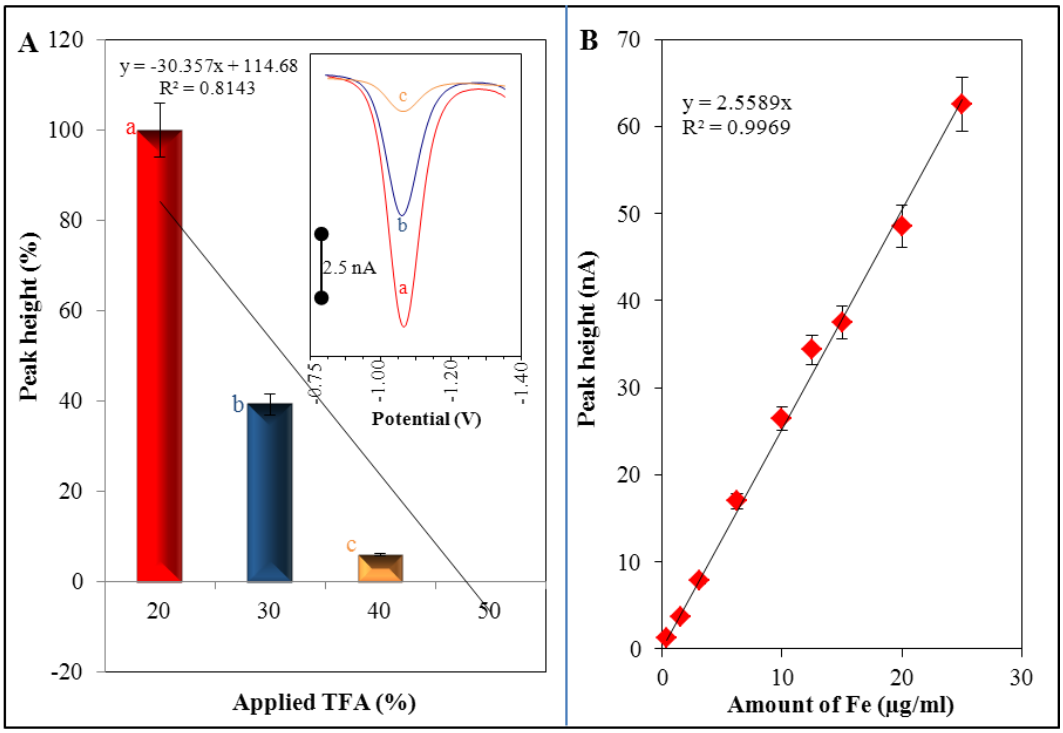

Figure 5. (A) Influence of concentrations of TFA (20 "a", 30 "b", 40 "c" and 50 "d"%, *v*/*v*) on iron signal from erythrocytes of MeLiM minipigs; in inset, typical voltammograms of samples prepared using various concentration of TFA. (B) Dependence of signal height of iron ions added into thousand fold diluted real sample of minipigs erythrocytes on electrochemical response. 100 µg of sample (erythrocytes) was placed into Eppendorf tube with 100 µl of TFA solution with subsequent centrifugation. 20 µl of supernatant was taken away and mixed with 2400 µl of the electrolyte No. 3 ($KBrO_3$ (0.1M) + NaOH (0.3M) + TEA (0.01M)).

Analysis of real sample is almost in all cases very complicated and complex procedure that often needs robust sample preparation. Three different ways of releasing of iron ions from erythrocytes of MeLiM minipigs were thus tested. Erythrocytes were weighted into micro test-tubes. Subsequently, TFA in the concentrations of 20, 30, 40 and 50% (*v*/*v*, with water) was added. Sample was subsequently homogenized using the ultrasound pulses for 2 min at 100 W. After it, mixture was vortexed (500 rpm for 30 min). Homogenized sample was immediately centrifuged to remove erythrocytes (16,000 g for 30 min). Removed supernatant was analysed using the optimised and fully automated method. Maximal response of iron ions was observed under the use of 20 % of TFA. Increasing concentration of TFA led to decreasing of the observed signal according to the following equation as y = -30.4x (Fig. 5A). The obtained voltammograms demonstrated well distinguishable and reproducible signals at -1.1 V (inset in Fig. 5A). Typical dependence of iron ions signal height on their concentration in thousand fold diluted real sample is shown in Fig. 5B. The obtained dependence was strictly linear (R^2 = 0.9969). Relative standard deviations of determination in interday analysis was 2.1 % (n=5) and in intraday analysis was 3.5 % (n=5).

Reproducibility and recovery were tested in the following experiments (Fig. 6). In the case of repeated analysis of the same samples, the signal change was 1.60 % (n=20, Fig. 6A). Variability between different samples was from 1 to 6 %. Strictly defined amount of iron ions was added into prepared sample in the case of recovery testing. Average recovery of iron ions detected in the sample of minipig erythrocytes was 91 % (Fig. 6B).

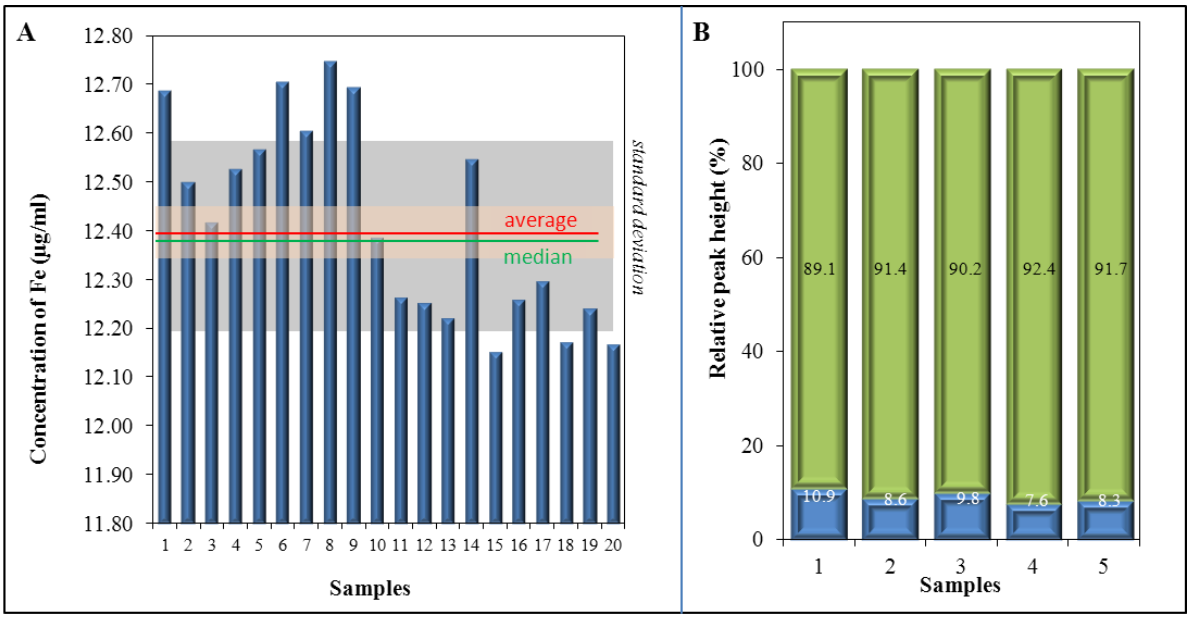

Figure 6. (A) Measurement of the repeatability (sample was twenty times measured). Dependence of iron ions concentration on the number of samples. Concentration of iron standard was 12.5 µg/ml. Average was 12.42 µg/ml, median 12.40 µg/ml, standard deviation 1.64 % and variance 0.32 % (both values were related to an average). (B) Iron recovery - relative parts of total iron signal after iron standard addition to prepared sample of erythrocytes. Blue parts represent iron from erythrocytes; green parts represent iron from standard addition. Presented results are determined for five different samples of erythrocytes with 1.25 µg standard iron addition.

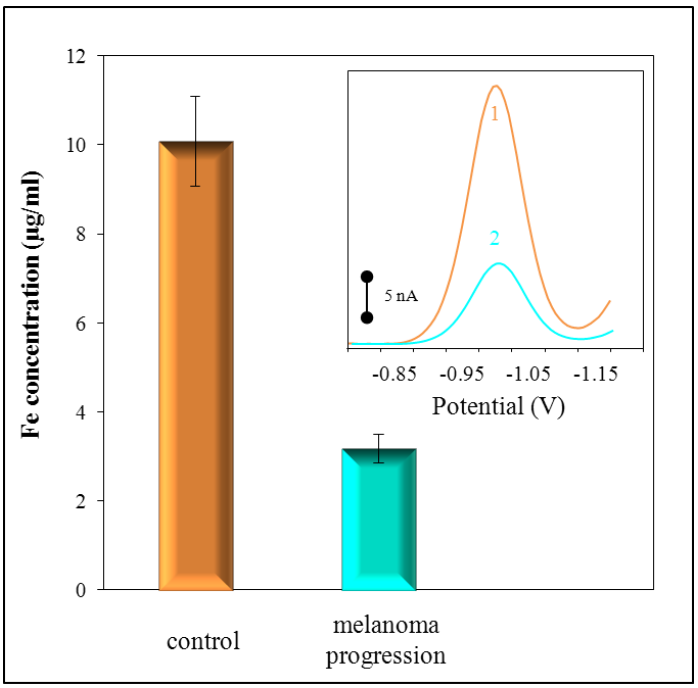

Figure 7. Influence of melanoma on amount of iron ions in erythrocytes in blood of MeLiM minipigs. First was control group of healthy minipigs, second group was minipigs with melanoma; in inset: typical DP voltammograms of the extracts. Parameters of electrochemical determination were as follows: start potential -0.6 V; end potential -1.3 V; deposition potential 0 V; accumulation time 0 s; equilibration time 10 s; purge time 120 s; pulse amplitude 0.05005 V; pulse time 0.06 s; voltage step 0.005951 V; voltage step time 0.5 s; sweep rate 0.0119 V/s. Samples were measured in solutions consist from 20 µl of sample and 2400 µl of electrolyte ($KBrO_3$ + NaOH + TEA).

It has been shown in the previous works that haematological markers connected with iron metabolism were in close relation to melanomas in the MeLiM strain of miniature pigs [53] Due to this fact, monitoring of iron ions represents next supplementing information for understanding the malignant disease. In our work obtained voltammograms were well repeatable, signals were symmetric and well developed. Slight shift of signal potential toward the positive values (signal potential at -1.02 V) was observed in the case of real samples (inset in Fig. 7). Changes in iron content of control group (10 µg Fe ions/ml) and group of animals suffering from melanoma progression (3.5 µg Fe ions/ml) were determined (Fig. 7). More detailed study will be published elsewhere.

4. CONCLUSIONS

In the case of metal determination, voltammetric methods represent the most used analytical techniques [54,55] with detection limits lower than 10^{-10} M [56]. It clearly follows from the results obtained that the electrochemical detection is suitable for rapid and inexpensive determination of iron ions. In addition, it is possible to use the suggested and verified method for analysis of porcine erythrocytes.

ACKNOWLEDGEMENTS
Financial support from NANIMEL 102/08/1546 and CEITEC CZ.1.05/1.1.00/02.0068 is highly acknowledged. The authors wish to express their thanks to Martina Stankova for perfect laboratory assistance.

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