Short Communication

Effects of Reduced Glutathione, Surface Active Agents, and Ionic Strength on the Detection of Metallothioneins by Using of Brdicka Reaction

Sona Krizkova,[a] *Ivo Fabrik*,[a] *Vojtech Adam*,[a,b] *Jiri Kukacka*,[c] *Richard Prusa*,[c] *Libuse Trnkova*,[d] *Jan Strnadel*,[e] *Vratislav Horak*,[e] *Rene Kizek*[a]*

[a] Department of Chemistry and Biochemistry, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-613 00 Brno, Czech Republic
*e-mail: kizek@sci.muni.cz
[b] Department of Animal Nutrition and Forage Production, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-613 00 Brno, Czech Republic
[c] Department of Clinical Biochemistry and Pathobiochemistry, 2nd Faculty of Medicine, Charles University, V Uvalu 84, CZ-150 06 Prague 5, Czech Republic
[d] Department of Chemistry, Faculty of Science, Masaryk University, Kotlarska 2, CZ-611 37 Brno, Czech Republic
[e] Laboratory of Tumour Biology, Department of Animal Embryology, Cell and Tissue Differentitation, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, v.v.i., CZ-277 21 Libechov, Czech Republic

Received: June 25, 2008
Accepted: September 19, 2008

Abstract
We investigated the effects of two surface active agents (sodium dodecyl sulfate, SDS, and polyoxyethylene sorbitol, Tween-20), ionic strength and reduced glutathione (GSH) on MT signals measured by adsorptive transfer stripping differential pulse voltammetry Brdicka reaction. If we compared the GSH and MT signals at the accumulation time of 120 s, the MT signal was more than two hundred times higher than the GSH one. The influence of Tween-20 was investigated in a broad concentration range (from 10^{-8} to 10^{-11} %, v/v). We found that the catalytic Cat2 signal decreased for more than 60% with increasing Tween-20 content. In the case of using SDS (10^{-3} – 10^{-6} M), the increase of Cat2 peak in the presence of the highest SDS concentration was determined. The height of Cat2 peak enhanced with increasing ionic strength mimicking by NaCl concentration. Based on the enhancement of the Cat2 peak we determined the ionic strength of fibroblasts sample and recalculated the concentration of MT determined there.

Keywords: Differential pulse voltammetry, Adsorptive transfer stripping technique, Proteins, Metallothionein, Brdicka reaction

DOI: 10.1002/elan.200804406

Dedicated to Professor Joseph Wang, on the Occasion of His 60th Birthday

Methods for determination of MT in clinical samples are often based on relatively time-consuming and not enough sensitive immunohistochemistry [8]. Thus proposing new methods and procedures for routine and sensitive detection of MT is needed. More than 70 years ago Brdicka discovered the catalytic evolution of hydrogen in the presence of cobalt(III) solution and proteins [9]. Brdicka used the catalytic signals to determine the changes of the proteins concentration in a blood serum of patients with cancer [10]. Unfortunately, the electrochemical techniques have been replaced by electromigration methods and other robust analytical techniques in the following decades. Ten years ago the catalytic signals were investigated again by means of the new electrochemical instruments and methods to treat the data obtained [11 – 19]. Recently we have studied the electrochemical behavior of metallothionein at the surface of mercury electrode by chronopotentiometric stripping voltammetry and by differential pulse voltammetry both coupled with adsorptive stripping transfer technique [12, 20 – 24].

In spite of the fact that electrochemical techniques have been utilizing for determination of MT in many types of real samples, the effects of fundamental physicochemical properties of samples such as ionic strength, content of active surface agents as well as content of low molecular mass thiols are not clear. Thus we investigated the effects of two surface active agents, ionic strength and reduced glutathione as the most abundant low molecular mass thiol on MT signals measured by adsorptive transfer stripping differential pulse voltammetry Brdicka reaction.

Many scientists have been paying attention to electrochemical determination of metallothionein as stress marker in environmental research. However it seems that these proteins can play somewhat key role in the processes of tumor development and this role remains still unclear. Therefore, the increased interest in metallothionein deter-

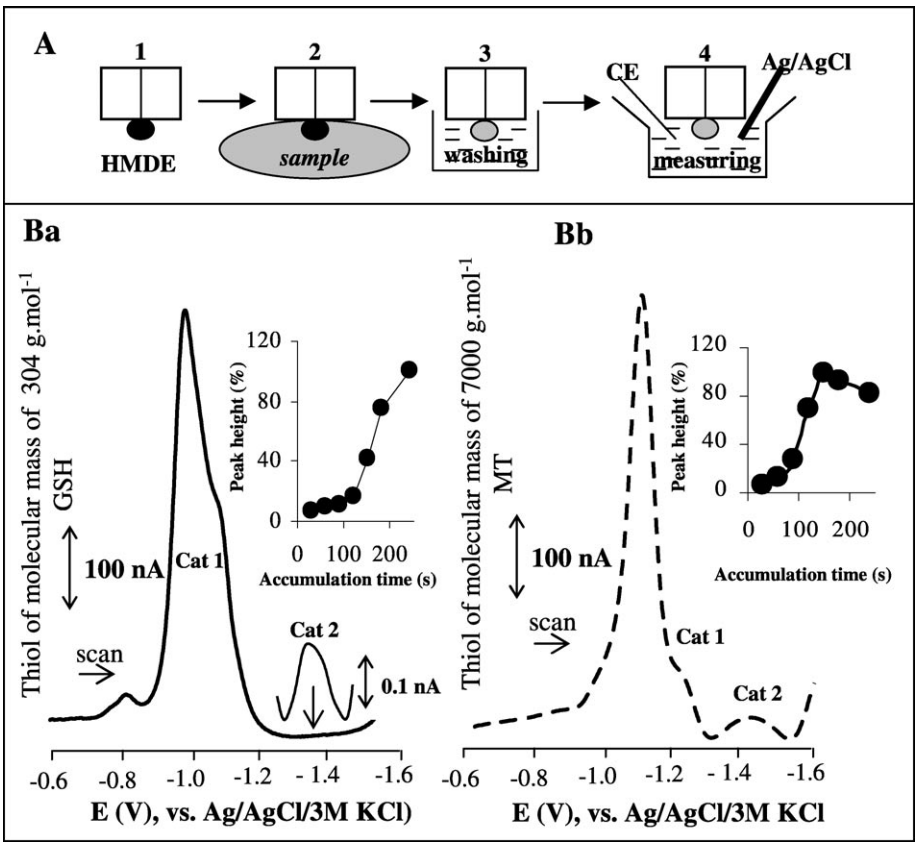

Fig. 1. Scheme of adsorptive transfer technique: 1) renewing of the hanging mercury drop electrode surface of 0.4 mm^2; 2) adsorbing of MT in a drop solution onto the HMDE surface at open circuit; 3) washing electrode in a MiliQ water electrolyte; 4) analysis of MT by DPV Brdicka reaction (A). Typical DP voltammograms of GSH and MT both at 100 nM (Ba and Bb, respectively), in insets: dependence of the Cat2 peak height on accumulation time.

mination in biological fluids like human blood and blood serum can be expected.

Adsorptive transfer technique using HMDE represents a unique tool for a detection of peptides and proteins rich in thiols moieties and nucleic acids (Fig. 1A). In the very first experiment with AdTS it was supposed that small molecules such as peptides can not be adsorbed on the HMDE surface [25]. Nowadays, when the more sophisticated software and instruments are using, it has been shown that the adsorption of such small molecules (even down to 1 kDa) on HMDE surface can be accomplished, but it is still several folds less intensive compared to adsorption and analysis of large proteins and nucleic acids. Recently we demonstrated that even small molecules like glutathione (GSH) interact with HMDE surface and are detectable by adsorptive transfer technique [26, 27]. Protein as metallothionein with molecular mass about 7 kDa can be also detected by AdTS [12].

The different protocols for preparation of the sample for MT determination are based on removal of high molecular proteins out of the sample using chemical or heat denaturating steps [12, 28]. The precipitated proteins are further separated by centrifugation. In the supernatant, low-molecular mass and thermo-stable proteins and peptides including MT are present. The influence of other low molecular mass compounds in a sample on MT determination has not been investigated. To evaluate the possible interferences, reduced glutathione (GSH) as one of the most abundant non-protein thiols with molecular mass of 304 g mol^{-1} was used. The typical DP voltammogram of GSH (100 nM) is shown in Figure 1Ba. We detected two catalytic signals called Cat1 and Cat2. These signals correspond to the reduction of hydrogen at the mercury electrode [29]. Petrlova et al. reported on that Cat2 signal only is proportional to concentration of MT [12]. Therefore, we further aimed our attention on Cat2 signal. The experimental confirmation of GSH accumulation on HMDE surface is clearly shown, when the dependence of signal height on the time of accumulation (30, 60, 90, 120, 150, 180, and 240 s) is investigated (inset in Fig. 1Ba). The same experiment was done with MT, which has more than twenty times higher molecular mass compared to GSH (Fig. 1Bb). The DP voltammograms of GSH and MT differed substantially. In the case of GSH analysis, Cat2 signal was low with increasing time of accumulation, but from time of accumulation higher than 120 s the more apparent increase of the peak height was observed (inset in Fig. 1Ba). When we detected MT using AdTS DPV Brdicka reaction, the height of Cat2 signal increased up to 150 s long accumulation time and then the signal slightly decreased (inset in Fig. 1Bb). This phenomenon is in good agreement with previously published results

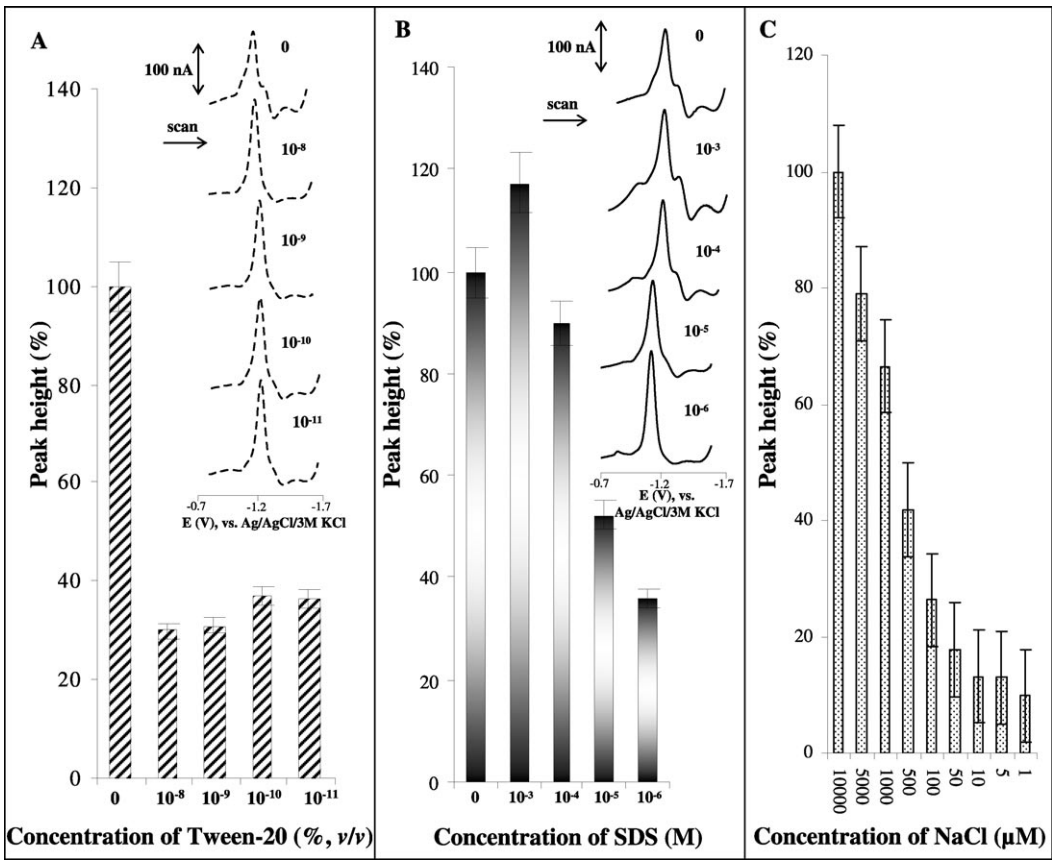

Fig. 2. Influence of Tween-20, SDS and NaCl on height of the Cat2 peak of MT (100 nM, t_A 120 s). The concentration of Tween-20 varied from 10^{-8} to 10^{-11} % (A), sodium dodecyl sulfate from 10^{-3} to 10^{-6} M (B), NaCl from 10^{-2} to 10^{-8} M (C). Peak of height of 12 nA corresponds to 100%.

[12]. If we compared the GSH and MT signals measured at the accumulation time of 120 s, the MT signal was more than two hundred times higher than GSH one. Based on the results obtained it can be concluded that GSH have more less affinity to HMDE than MT, thus the low molecular mass thiols are disadvantaged in binding to electrode surface compared to metallothioneins. Their influence on an analysis of real samples can be considered as negligible.

In biological samples there are present many compounds, which can influence a measurement. Furthermore, it is obvious to add compounds for preventing degradation of the molecule of interest into a sample. We were interested in the issue how the common compounds routinely used for sample preparation influence the catalytic signals of MT. Particularly we focused on the investigation of the effect of the surface-active agents (sodium dodecyl sulfate, SDS, and polyoxyethylene sorbitol, Tween-20) and change of ionic strength.

More than 35 years ago Palecek and Pechan reported on the study of the influence of surfactant Triton X on Brdicka catalytic signals [30]. In the present paper we were interested whether it is possible to observe the similar changes as they observed after addition of the surfactants to MT sample. The changes were investigated in the broad concentration range (from 10^{-8} to 10^{-11}%, v/v or from 8.2×10^{-11} to 8.2×10^{-14} M) of Tween-20. We found that the catalytic Cat2 signal decreased for more than 60% with increasing Tween-20 content (Fig. 2A). The changes of MT Cat2 signals are well observable also in the DP voltammograms shown in inset in Figure 2A. In the case of using SDS ($10^{-3} - 10^{-6}$ M) as a surfactant with negative charge, the increase in the Cat2 peak height in the presence of the highest concentration of SDS (10^{-3} M) was determined (Fig. 2B). The lower concentrations of SDS resulted in the lowering of the Cat2 peak height. MT signals (100 nM) were well separated and developed in the presence of 10^{-3} M SDS (inset in Fig. 2B). It clearly follows from the results obtained that interfering surfactants can have considerable influence on a determination of proteins content using AdTS DPV Brdicka reaction. Further the affecting of Cat2 peak height by different ionic strength (NaCl from 10^{-2} to 10^{-8} M) was investigated (Fig. 2C). The height of Cat2 peak enhanced with increasing NaCl concentration.

To compare the influence of the surface active agents and NaCl on MT signal, their concentrations, where the largest effect was determined (Tween-20 10^{-8}%, SDS 10^{-6} M, NaCl 10^{-2} M), were chosen (Fig. 2). The Cat2 peak heights decreased for more than 60% measured in the presence of the surfactants used in comparison to MT Cat2 signal measured without addition of these compounds. The

calibration curves obtained were strictly linear with the correlation coefficients $R^2 = 0.995$ (Tween-20) and $R^2 = 0.998$ (SDS), with relative standard deviation below 5%. In the case of NaCl the catalytic signal enhances for more than 70%. The calibration curve obtained was linear with $R^2 = 0.990$. It clearly follows from the results obtained that the quantification of MT content in biological samples is very difficult. If we consider that NaCl concentration inside a cell is in units of mM level, the enhancement of MT signal could be 60%.

We attempted to confirm this presumption by experiment with fibroblasts. Based on the previously published results [31], we processed sample containing 100 000 cells to obtain the highest response with the best correlation to number of the cells. Typical DV voltammogram of fibroblasts' sample is shown in inset in Figure 3. MT level was 1.2 ± 0.1 μM ($n = 10$), which correlated with the results published by Krizkova et al. [31]. Moreover we measured conductivity of cell samples, because conductivity very well corresponded to ionic strength of a sample (Fig. 3). The conductivity of fibroblasts' sample containing app. 100 000 cells was 600 ± 12 μS. According to equation shown in Figure 3 we calculated 'ionic strength' (ca. concentration of NaCl) of this sample as 5.3 ± 0.1 μM. MT standard samples used for calibration was prepared with water of ACS purity and gave app. same signal as samples prepared with 1 μM NaCl (Fig. 2). The height of MT Cat2 peak measured in this sample was 10%. The height of Cat2 peak of MT standard prepared with 5 mM NaCl (similar 'ionic strength' to those determined in fibroblasts) was 79%. The enhancement was 69%. If we considered this enhancement, we recalculated concentration of MT in fibroblasts according to following equation: $[c_{\text{recalculated}} = c_{\text{determined}} - c_{\text{determined}} \times 0.69]$. Recalculated concentration of MT in fibroblasts was 0.4 μM. It follows from the results that we confirmed our previous presumption with enhancing of signal. However consideration of influence of other components of such complex matrix as cells needs to be done.

A biological sample contains many surface active agents in unspecified amounts, which negatively influence the adsorption of MT on the electrode surface. From the experimental point of view it is very complicated to determine the degree of their positive and negative effects. In the present paper we attempted to mimic influence of ionic strength and surface active agents on MT determination. It can be concluded that the effect of both is considerable.

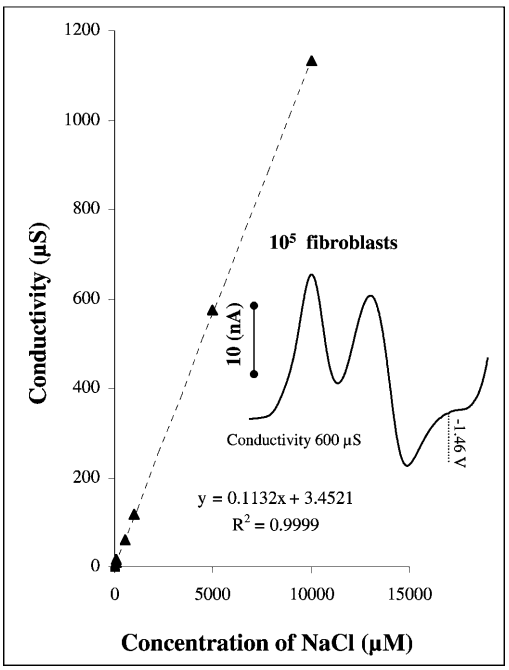

Fig. 3. Dependence of conductivity on ionic strength of NaCl; inset: typical DP voltammogram of fibroblasts.

Experimental

Rabbit liver MT (MW 7143 g mol^{-1}), containing 5.9% Cd and 0.5% Zn, was purchased from Sigma Aldrich (St. Louis, USA). $Co(NH_3)_6Cl_3$ and other chemicals used were purchased from Sigma Aldrich (Sigma-Aldrich, USA) unless noted otherwise. The stock standard solutions of MT (10 μg mL^{-1}) was prepared with ACS water (Sigma-Aldrich, USA) and stored in the dark at $-20\,^{\circ}$C. Working standard solutions were prepared daily by dilution of the stock solutions with ACS water. The pH value and conductivity was measured using WTW inoLab Level 3 with terminal Level 3 (MultiLab Pilot; Weilheim, Germany).

Quail fibroblasts were used in our experiments. The harvested cells (approximately 100 000 cells) were transferred to a test tube and then deep frozen by liquid nitrogen to disrupt cells. The frozen cells were mixed with extraction buffer (100 mM potassium phosphate, pH 8.7) to a final volume of 1 ml and homogenized using hand-operated homogenizer ULTRA-TURRAX T8 (IKA, Germany) placed in an ice bath for 3 min at 25 000 rpm [32]. The homogenate was centrifuged at 10 000 g for 15 min and at 4 °C (Eppendorf 5402, USA).

The processed cells were prepared by heat treatment. Briefly, the sample was kept at 99 °C in a thermomixer (Eppendorf 5430, USA) for 15 min. with occasional stirring, and then cooled to 4 °C. The denatured homogenates were centrifuged at 4 °C, 15 000 g for 30 min. (Eppendorf 5402, USA). Heat treatment effectively denatures and removes high molecular weight proteins out from samples [33].

Counting of BY-2 suspension cells was carried out using a Fuchs – Rosenthal haemocytometer (Germany). Aliquots of suspension were diluted with distilled water and loaded into the heamocytometer according to the instructions of the manufacturer. The counting of cells was performed manually using a microscope (Olympus, Japan).

Electrochemical measurements were performed with AUTOLAB Analyzer (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was employed as the working electrode. An Ag/AgCl/3 M KCl

electrode served as the reference electrode. Glassy carbon electrode was used as the auxiliary electrode. For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed.

Principle of the adsorptive transfer stripping technique (AdTS) is based on the strong adsorbing of the target molecule on the electrode surface at an open electrode circuit [34]. The electrode is washed in a rinsing buffer. The electrode is further transferred to the supporting electrolyte and measured. The 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; surface-active agent was not added. The samples of the MT were reduced before each measurement by 1 mM tris(2-carboxyethyl)phosphine addition [16, 20]. AdTS DPV Brdicka reaction parameters were as follows: an initial potential of -0.35 V, an end potential -1.8 V, a modulation time 0.057 s, a time interval 0.2 s, a step potential of 1.05 mV, a modulation amplitude of 250 mV, $E_{ads} = 0$ V. All experiments were carried out at 4 °C (Julabo F12, Germany).

Acknowledgement

Financial support from Grant GAAV IAA401990701 is highly acknowledged.

References

[1] J. H. R. Kagi, A. Schaffer, *Biochemistry* **1988**, *27*, 8509.
[2] S. E. Theocharis, A. P. Margeli, J. T. Klijanienko, G. P. Kouraklis, *Histopathology* **2004**, *45*, 103.
[3] M. Dutsch-Wicherek, T. J. Popiela, M. Klimek, L. Rudnicka-Sosin, L. Wicherek, J. P. Oudinet, J. Skladzien, R. Tomaszewska, *Neuroendocrinol. Lett.* **2005**, *26*, 567.
[4] R. Klimek, *Neuroendocrinol. Lett.* **2001**, *22*, 413.
[5] R. Prusa, M. Svoboda, O. Blastik, V. Adam, O. Zitka, M. Beklova, T. Eckschlager, R. Kizek, *Clin. Chem.* **2006**, *52*, A174.
[6] P. Dziegiel, E. Suder, P. Surowiak, J. Kornafel, M. Zabel, *Appl. Immunohistochem.* **2002**, *10*, 357.
[7] T. Goldmann, A. Moorkamp, K. H. Wiedorn, L. Suter, F. Otto, *Arch. Dermatol. Res.* **2001**, *293*, 115.
[8] B. Zelger, A. Hittmair, M. Schir, C. Ofner, D. Ofner, P. O. Fritsch, W. Bocker, B. Jasani, K. W. Schmid, *Histopathology* **1993**, *23*, 257.
[9] R. Brdicka, *Coll. Czech. Chem. Commun.* **1933**, *5*, 112.
[10] R. Brdicka, *Nature* **1937**, *139*, 1020.
[11] M. Tomschik, L. Havran, E. Palecek, M. Heyrovsky, *Electroanalysis* **2000**, *12*, 274.
[12] J. Petrlova, D. Potesil, R. Mikelova, O. Blastik, V. Adam, L. Trnkova, F. Jelen, R. Prusa, J. Kukacka, R. Kizek, *Electrochim. Acta* **2006**, *51*, 5112.
[13] V. Dorcak, I. Sestakova, *Bioelectrochemistry* **2006**, *68*, 14.
[14] D. B. Luo, J. G. Lan, C. Zhou, C. X. Luo, *Anal. Chem.* **2003**, *75*, 6346.
[15] I. Bontidean, C. Berggren, G. Johansson, E. Csoregi, B. Mattiasson, J. A. Lloyd, K. J. Jakeman, N. L. Brown, *Anal. Chem.* **1998**, *70*, 4162.
[16] V. Adam, J. Petrlova, D. Potesil, J. Zehnalek, B. Sures, L. Trnkova, F. Jelen, R. Kizek, *Electroanalysis* **2005**, *17*, 1649.
[17] M. Dabrio, A. R. Rodriguez, *Electroanalysis* **2000**, *12*, 1026.
[18] I. Sestakova, D. Miholova, H. Vodickova, P. Mader, *Electroanalysis* **1995**, *7*, 237.
[19] N. Serrano, I. Sestakova, J. M. Diaz-Cruz, *Electroanalysis* **2006**, *18*, 169.
[20] R. Kizek, J. Vacek, L. Trnkova, B. Klejdus, L. Havel, *Chem. Listy* **2004**, *98*, 166.
[21] R. Kizek, L. Trnkova, E. Palecek, *Anal. Chem.* **2001**, *73*, 4801.
[22] L. Trnkova, R. Kizek, J. Vacek, *Bioelectrochemistry* **2002**, *56*, 57.
[23] M. Strouhal, R. Kizek, J. Vacek, L. Trnkova, M. Nemec, *Bioelectrochemistry* **2003**, *60*, 29.
[24] R. Prusa, R. Kizek, J. Vacek, L. Trnkova, J. Zehnalek, *Clin. Chem.* **2004**, *50*, A28.
[25] E. Palecek, I. Postbieglova, *J. Electroanal. Chem.* **1986**, *214*, 359.
[26] R. Kizek, J. Vacek, L. Trnkova, F. Jelen, *Bioelectrochemistry* **2004**, *63*, 19.
[27] J. Vacek, J. Petrek, R. Kizek, L. Havel, B. Klejdus, L. Trnkova, F. Jelen, *Bioelectrochemistry* **2004**, *63*, 347.
[28] B. Raspor, M. Paic, M. Erk, *Talanta* **2001**, *55*, 109.
[29] B. Raspor, *J. Electroanal. Chem.* **2001**, *503*, 159.
[30] E. Palecek, Z. Pechan, *Anal. Biochem.* **1971**, *42*, 59.
[31] S. Krizkova, I. Fabrik, V. Adam, J. Kukacka, R. Prusa, G. J. Chavis, L. Trnkova, J. Strnadel, V. Horak, R. Kizek, *Sensors* **2008**, *8*, 3106.
[32] V. Supalkova, J. Petrek, J. Baloun, V. Adam, K. Bartusek, L. Trnkova, M. Beklova, V. Diopan, L. Havel, R. Kizek, *Sensors* **2007**, *7*, 743.
[33] M. Erk, D. Ivanković, B. Raspor, J. Pavičić, *Talanta* **2002**, *57*, 1211.
[34] J. Petrlova, D. Potesil, J. Zehnalek, B. Sures, V. Adam, L. Trnkova, R. Kizek, *Electrochim. Acta* **2006**, *51*, 5169.

