

An Fe₃O₄-nanoparticles-based amperometric biosensor for creatine determination

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Abstract

An amperometric biosensor for the detection of creatine was designed, based on carbon paste electrode modified with Fe₃O₄ nanoparticles. Electron transfer properties of unmodified and Fe₃O₄-nanoparticles-modified carbon paste electrodes were investigated by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) methods. Fe₃O₄ nanoparticles increased the surface area and electric conductivity of the electrode, thus enhancing the sensitivity of the electrode. Optimum pH, buffer concentration, working potential and enzyme loading were selected as 7.0; 0.05 mol L⁻¹; +0.30 V and 2.0 Unit creatinase (CI), 1.0 Unit sarcosine oxidase (SO), respectively. The purposed biosensor exhibited linear response from 2.0×10^{-7} mol L⁻¹ to 3.8×10^{-6} mol L⁻¹ and from 9.0×10^{-6} mol L⁻¹ to 1.2×10^{-4} mol L⁻¹ with a detection limit of 2.0×10^{-7} mol L⁻¹. Biosensor was used for determination of creatine in commercial creatine powder samples and showed a good sensing performance.

Keywords: Amperometry, carbon paste, creatinase, enzyme electrode, nanoparticle, sarcosine oxidase

Introduction

Creatine is an amino acid derivative synthesized from L-arginine, glycine and S-adenosylmethionine in the kidneys, liver and pancreas (Greenhaff, 1997; Paddon-Jones, Borsheim, & Wolfe, 2004). Creatine is mostly stored in the muscles, and there is a small of creatine found in brain and testes (Balsom, Soderlund, & Ekblom, 1994; Hultman, Soderlund, Timmons, Cederblad, & Greenhaff, 1996). Most of the creatine is converted to phosphocreatine in muscle tissue in a reversible reaction with adenosine triphosphate (ATP). Phosphocreatine is a major energy storage form in the body and plays an important role in muscle energy metabolism. The creatine level in blood serum and urine is clinically used as a parameter of muscle damage. Creatine is also one of the most popular nutritional supplements as ergogenic aid, and many sportsmen use creatine to enhance their performance (Stefan van Staden, Bokretson, Van Standen, & Aboul-Enein,

2003). Therefore, a precise and fast determination of creatine concentration is very important. Most of the methods for creatine and creatinine determination have been based on the Jaffé reaction, in which the active methylene group reacts with alkaline sodium picrate to give a yellow-red complex (Jaffé, 1886). However, Jaffé's method is rather unspecific, since many metabolites normally present in biological fluids cause interference (Weber & van Zanten, 1991). Other various instrumental methods such as HPLC (Persky, Hochhaus, & Brazeau, 2003; Karatzaferi, De Haan, Offringa, & Sargeant, 1999), capillary electrophoresis (Smith-Palmer, 2002), HPLC-mass spectrophotometry (Yasuda et al., 1997; Schwedhelm et al., 2000), IR (Pezzaniti, Jeng, McDowell, & Oosta, 2001), nuclear magnetic resonance (Sewell, Murphy, & Iles, 2002; Feng et al., 2002) were also proposed for the determination of creatine and creatinine. However, these methods are usually laborious, expensive, time-consuming and/or complex to perform. Alternatively, biosensors, allowing direct measurements of creatine in sample, promise economy of time and saving of costs in pharmaceutical and clinical analysis. Several potentiometric and amperometric biosensors for creatine and creatinine have been proposed (Hsiue, Lu, & Jyh-Chern, 2004; Yadav, Kumar, & Pundir, 2011; Ramanavicius, 2007; Stefan van Staden & Bokretson, 2006; Erlenkötter, Fobker, & Chemnitius, 2002; Karakus, Pekyardımcı, & Kılıç, 2006; Erden, Aslan, Pekyardımcı, & Kılıç, 2006). In amperometric creatine biosensors, creatine is converted to electroactive H₂O₂ by two enzymatic reactions catalyzed by CI and SO, respectively (Tsuchida & Yoda, 1983). Amperometric determination of H₂O₂ requires high anodic potential, and this high potential makes biosensors responsive to interfering substances. To avoid this drawback, either the elimination of the interfering substances by the pretreatment of the sample or lowering of the working potential can be used. To obtain a high-performance creatine biosensor use of nanoparticles is also very attractive. Recently, various nanomaterials have found numerous applications in biosensor applications. These interesting materials exhibit desirable properties such as large surface-to-volume ratio, high surface reaction activity, high catalytic efficiency and strong adsorption ability

that are useful in biosensing applications. Nanomaterials have the unique ability of promoting fast electron transfer between electrode and the active site of the enzyme. Among them, magnetite nanoparticles have gained much attention due to biocompatibility, lower mass transfer resistance, strong superparamagnetic property and low toxicity (Kaushik et al., 2009). In this study, creatinase and sarcosine oxidase was immobilized into the Fe_3O_4 -nanoparticles-modified enzyme electrode to develop a simple, low-cost, high-performance biosensor for creatine determination. Optimum working parameters, analytical characteristics and application of Fe_3O_4 -CPEE to real samples were investigated.

Experimental

Instrumentation and reagents

The electrochemical studies were carried out with IVIUM electrochemical analyzer (Ivium Technologies, Netherlands) and a three-electrode cell stand (Bioanalytical Systems, Inc., USA). The working electrode was a modified carbon paste electrode. The counter and the reference electrodes were a Pt wire (BAS MW 1034) and Ag/AgCl (BAS MF 2052) electrode, respectively (Bioanalytical Systems, Inc., USA). The pH values of the buffer solutions were measured with ORION Model 720A pH/ion meter and ORION combined pH electrode (Thermo Scientific, USA). Double-distilled deionized water was supplied from Human Power I+, Ultra Pure Water System (Produced by ELGA as PURELAB Option-S).

CI (E.C. 3.5.3.3 from *Arthrobacter* sp. with a specific activity of 8.7 Units mg^{-1} solid), SO (E.C. 1.5.3.1 from *Basillus* sp. with a specific activity of 37 Units mg^{-1} solid), Fe_3O_4 nanoparticles, uric acid, ascorbic acid and glutaraldehyde were purchased from Sigma (St. Louis, MO, USA). Sodium monohydrogenphosphate and sodium dihydrogenphosphate were supplied from Riedel-de Haën (Seelze, Germany). Creatine, bovine serum albumin (BSA), graphite powder, paraffin oil and glucose were supplied from Fluka (Buchs, Switzerland). Commercial creatine powder was supplied from GNC. All other chemicals were obtained from Merck (Darmstadt, Germany). Standard solutions of creatine were prepared freshly every day by dissolving creatine in phosphate buffer solution.

Preparation of carbon paste and modified carbon paste electrodes

Carbon paste was prepared in the following proportions for unmodified electrode (UCPE): 77.6% graphite powder and 22.4% paraffin oil. Fe_3O_4 nanoparticles modified carbon paste electrode (Fe_3O_4 -CPE) was composed of 64.66% graphite powder, 12.94% Fe_3O_4 nanoparticles and 22.4% paraffin oil. The modified electrode was prepared by hand-mixing graphite powder with the nanoparticles and then adding paraffin oil and thoroughly mixing for approximately 20 minutes to form homogeneous modified carbon paste electrode. Graphite powder and Fe_3O_4 nanoparticles were mixed and enzyme solution (8 μL CI (250 Unit mL^{-1}), 20 μL SO (50 Unit mL^{-1}), 1.5 mg BSA and 10 μL 1.25% glutaraldehyde) was added to form Fe_3O_4 -CPEE. Paraffin oil was added after the evaporation of water and mixed for approximately 20 minutes until a uniform paste was obtained. Unmodified

enzyme electrode (UCPEE) was also prepared in a similar way. In all cases, the paste was placed into the bottom of the working electrode body and the electrode surface was polished against clean paper to have a smooth surface. The electrodes were washed with distilled water and working buffer between measurements. Electrodes were stored in refrigerator at $+4^\circ\text{C}$ when not in use.

Amperometric measurements

Electron transfer properties of unmodified and modified electrodes were examined in 1 mmol L^{-1} $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$ (1:1) solution containing 0.1 mol L^{-1} KCl by CV and in 5 mmol L^{-1} $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$ (1:1) solution containing 0.1 mol L^{-1} KCl by EIS. CVs of UCPE and Fe_3O_4 -CPE were recorded between (-0.2) V and $(+0.6)$ V. EIS measurements were performed at the frequency range of 10^5 – 5×10^{-2} Hz with 10 mV amplitude under open-circuit potential (E_{OCP}) conditions. All other amperometric measurements were performed in phosphate buffer solution (0.05 mol L^{-1} , pH 7.0). Measurements were carried out at room temperature ($23 \pm 2^\circ\text{C}$).

Results and discussion

Electrochemical characterization of unmodified and modified electrodes

Electron transfer properties of UCPE and Fe_3O_4 -CPE were investigated in 0.1 mol L^{-1} KCl solution containing 1 mmol L^{-1} $\text{Fe}(\text{CN})_6^{3-/4-}$ at 50 mVs^{-1} (Figure 1A). In case of UCPE surface, well-defined oxidation and reduction peaks of $\text{Fe}(\text{CN})_6^{3-/4-}$ were observed. When Fe_3O_4 nanoparticles are incorporated into the carbon paste matrix, increased peak current is obtained. Fe_3O_4 nanoparticles also decreased peak-to-peak separation ($E_{\text{pa}} - E_{\text{pc}} = \Delta E_{\text{p}}$) for $\text{Fe}(\text{CN})_6^{3-/4-}$ waves. The ΔE_{p} for UCPE and Fe_3O_4 -CPE were found as 270 mV and 240 mV, respectively. These results are attributed to the presence of Fe_3O_4 nanoparticles with increased electron mobility at the surface of the electrode, resulting in enhanced electron transfer (Yang, Ren, Tang, & Zhang, 2009; Yao & Shiu, 2007; Lu & Chen, 2006; Lin et al., 2008). The electrochemical surface area of Fe_3O_4 -CPE was calculated from the voltammetric peak current by the use of the Randles-Sevcik equation (Wang, 2006; Ding, Chang, Wu, Lai, & Chang, 2005). The effective electrochemical surface area of Fe_3O_4 -CPE (0.117 cm^2) is higher than the surface area of the UCPE (0.091 cm^2). It can be concluded that the higher surface area will enhance the sensitivity of electrode. Figure 1B shows electrochemical impedance spectra, Nyquist curves, of UCPE and Fe_3O_4 -CPE. The Nyquist curves include a depressed semicircle portion and a linear portion. The semicircle part corresponds to the electron transfer limited process and its diameter is equal to the electron transfer resistance value (R_{ct}) that controls the electron transfer kinetics of the redox probe at the electrode/solution interface (Wang, 2006; Liu et al., 2006). The R_{ct} values for UCPE and Fe_3O_4 -CPE are approximately 2.24 and 0.72 k Ω , respectively. The results show that the Fe_3O_4 nanoparticles decrease the resistance of the electrode and hold higher electron transfer efficiency (Yang et al., 2009; Lu & Chen, 2006).

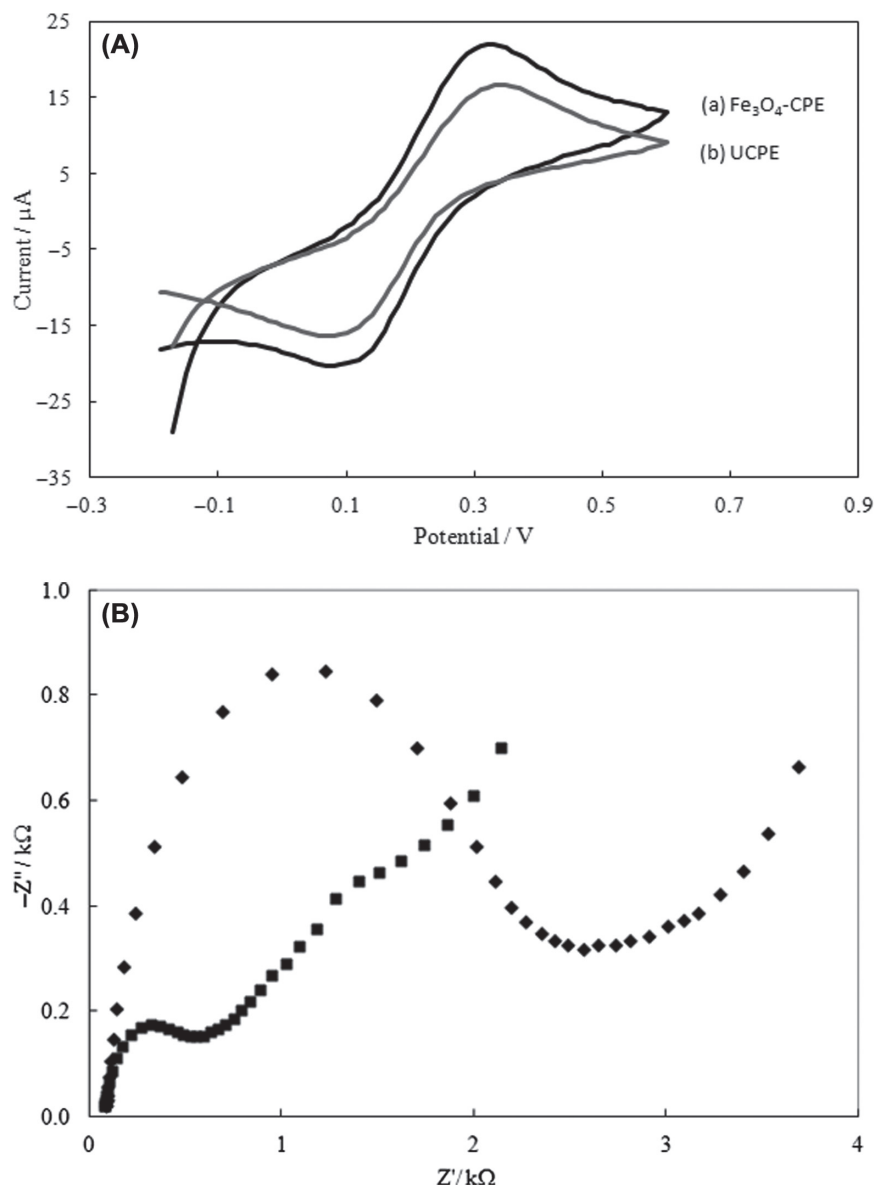
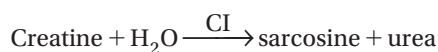


Figure 1. (A) Cyclic voltammograms of (a) UCPE, (b) $\text{Fe}_3\text{O}_4\text{-CPE}$ at 50 mVs^{-1} . (B) The Nyquist curves of (◆) UCPE and (■) $\text{Fe}_3\text{O}_4\text{-CPE}$ in 0.1 mol L^{-1} KCl solution containing $5 \text{ mmol L}^{-1} \text{Fe}(\text{CN})_6^{3-/4-}$.

CI catalyzes the conversion of creatine to sarcosine and urea, and sarcosine oxidase (SO) catalyzes the conversion of sarcosine to glycine, formaldehyde and hydrogen peroxide, as depicted in the following reaction sequence:



The quantification of creatine is based on the electrochemical detection of enzymatically generated H_2O_2 , thus the sensor sensitivity is dependent on the electrochemical response of the electrode to H_2O_2 . Electrodes with high catalytic efficiency to H_2O_2 would achieve high sensitivity to creatine. Figure 2A shows the current difference values versus H_2O_2 concentration obtained with UCPE and $\text{Fe}_3\text{O}_4\text{-CPE}$. The sensitivity of $\text{Fe}_3\text{O}_4\text{-CPE}$ is higher than that of unmodified carbon paste electrode to H_2O_2 . It can be concluded

that $\text{Fe}_3\text{O}_4\text{-CPE}$ shows good catalytic activity towards H_2O_2 determination.

Oxygen is the natural electron acceptor for SO; however, we also investigated the mediating capability of Fe_3O_4 nanoparticles. We determined the creatine response of the modified enzyme electrode ($\text{Fe}_3\text{O}_4\text{-CPEE}$) in nitrogen-saturated buffer solution and compared this result with that obtained in oxygen saturated solution to clarify the detection mechanism of creatine. $\text{Fe}_3\text{O}_4\text{-CPEE}$ showed very small catalytic activity towards creatine in nitrogen-saturated solution in contrast to the response in oxygen saturated solution (Figure 2B). This indicates that Fe_3O_4 nanoparticles cannot catalyze the electron transfer between the reduced enzyme and electrode surface efficiently and do not show a significant mediating capability. Figure 2B also shows that the sensitivity of $\text{Fe}_3\text{O}_4\text{-CPEE}$ is higher than that of UCPEE which may be attributed to the fact that Fe_3O_4 nanoparticles increase the surface area and electric conductivity of the electrodes, thus enhancing the sensitivity of the electrodes.

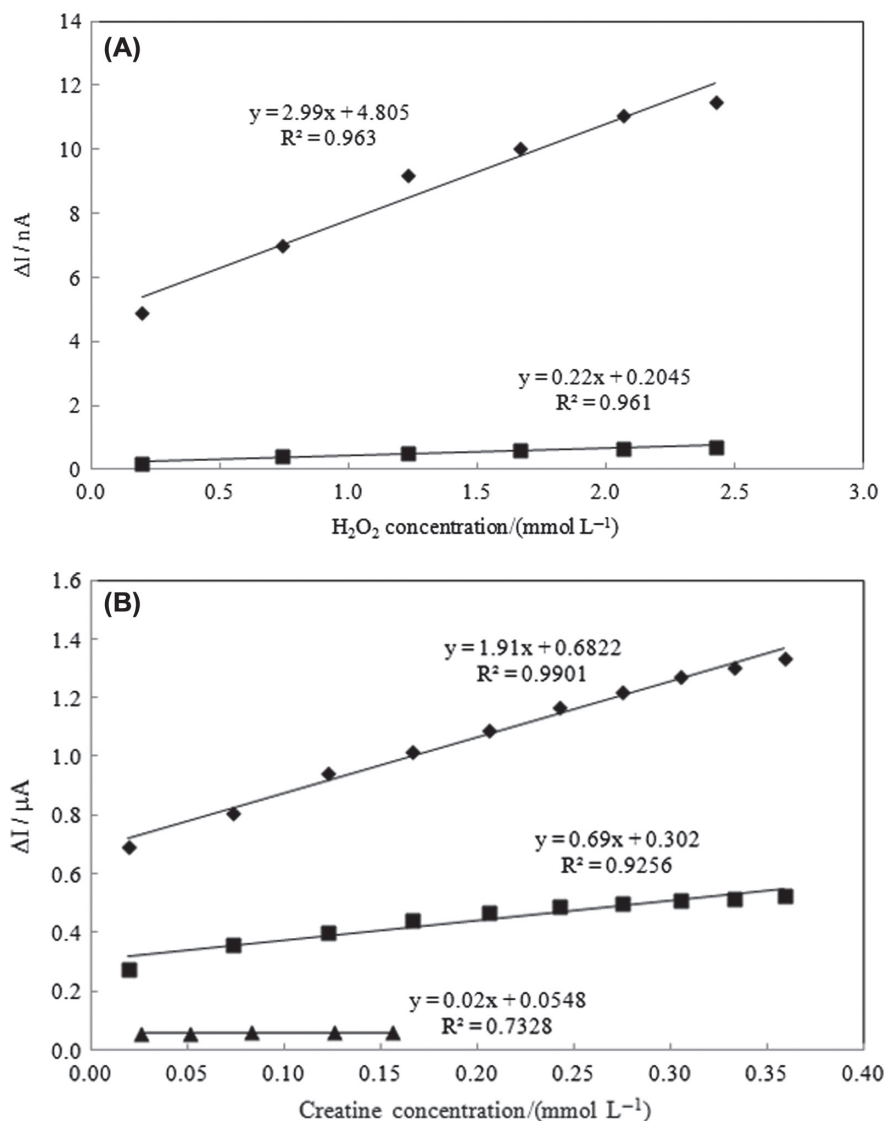


Figure 2. (A) Calibration curves for H_2O_2 : (■) UCPE, (◆) $\text{Fe}_3\text{O}_4\text{-CPE}$. (B) Calibration curves for creatine: (■) UCPEE, (◆) $\text{Fe}_3\text{O}_4\text{-CPEE}$ (oxygen saturated solution), (▲) $\text{Fe}_3\text{O}_4\text{-CPEE}$ (nitrogen saturated solution) (0.05 mol L^{-1} pH 7.0, phosphate buffer, +0.30 V).

Creatine is converted to electroactive H_2O_2 by two consecutive enzymatic reactions catalyzed by CI and SO. The ratio and amount of these enzymes have an important effect on the performance of the creatine biosensors. In this study, different compositions of SO and CI were immobilized into the carbon paste matrix by cross-linking with glutaraldehyde and BSA to investigate the optimum composition. Maximum response current and sensitivity were observed with 1.0 U SO and 2.0 U CI, and this composition was used for further experiments. The pH of the buffer solution is a crucial factor affecting the amperometric response of the biosensor. The dependence of enzymatic activity of the immobilized SO and CI on pH was investigated at 0.09 mmol L^{-1} creatine concentration over the pH range from 6.0 to 9.0 (Figure 3). The response current increases with increasing pH from 6.0 to 7.0 and then decreases. The highest response was obtained at pH 7.0, which was selected as optimum pH. The amperometric response of $\text{Fe}_3\text{O}_4\text{-CPEE}$ was determined at different phosphate concentrations of 0.05 mol L^{-1} , 0.10 mol L^{-1} , 0.15 mol L^{-1} and 0.20 mol L^{-1} , and the best response for $\text{Fe}_3\text{O}_4\text{-CPEE}$

was obtained at 0.05 mol L^{-1} . Above this concentration, the current difference was found to show a significant decrease. The response of $\text{Fe}_3\text{O}_4\text{-CPEE}$ to constant creatine concentration (0.09 mmol L^{-1}) was determined at different working potentials between (0) V and (+0.70) V. Amperometric current was increased gradually for $\text{Fe}_3\text{O}_4\text{-CPEE}$ in the range from (0) to (+0.70) V. Although the maximum current difference was obtained at +0.70 V, +0.30 V was selected for the amperometric determination of creatine since we obtained sufficient response and calibration curves with good linearity. This working potential also could minimize the risk for interfering reactions of other oxidizable species in real samples.

Figure 4 shows the amperometric response curve of the $\text{Fe}_3\text{O}_4\text{-CPEE}$ recorded as a function of creatine concentration under the optimized experimental conditions. The response current is linear to the concentration of creatine over the range from $2.0 \times 10^{-7} \text{ mol L}^{-1}$ to $3.8 \times 10^{-6} \text{ mol L}^{-1}$ and from 9.0×10^{-6} to $1.2 \times 10^{-4} \text{ mol L}^{-1}$. The regression equation of the first linear part of the curve is $\Delta I = 2.22c_{\text{creatin}} + 0.05$ ($R^2 = 0.9988$) and the second is

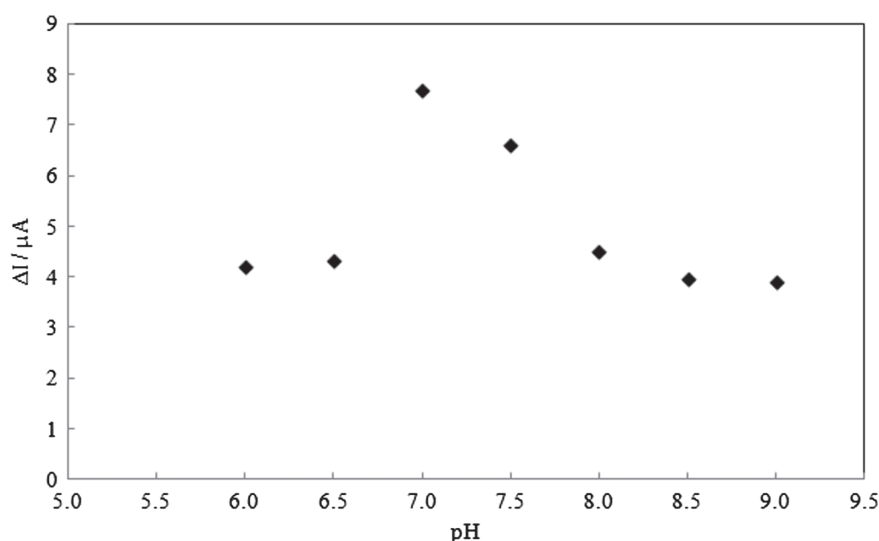


Figure 3. The effect of buffer pH on the response of Fe_3O_4 -CPEE.

$\Delta I = 0.08c_{\text{creatinine}} + 0.07$ ($R^2 = 0.9799$). The limit of detection is $2.0 \times 10^{-7} \text{ mol L}^{-1}$. There is a remarkable improvement in detection limit and linear working range of the Fe_3O_4 -CPEE compared with other creatine biosensors recently reported (Stefan van Staden & Bokretson, 2006; Karakus et al., 2006; Erden et al., 2006). The repeatability of Fe_3O_4 -CPEE was investigated. Five calibration curves were plotted by the use of the same electrode sequentially. The relative standard deviation of the sensitivities was found as 8.7%. We also checked the long-term stability of Fe_3O_4 -CPEE prepared under optimum conditions. The electrode was stored in dry atmosphere at $+4^\circ\text{C}$ when not in use. The current response maintained more than 70% of its original value after 30 days. The loss may result from the decrease of creatinase and sarcosine oxidase activities during storage. The relatively good storage stability implies that the Fe_3O_4 nanoparticles are quite efficient for retaining the activity of creatinase and sarcosine oxidase. The presented biosensor achieved 95% of the steady-state current within about

10 seconds which is quite satisfactory for biosensor response. In order to study the selectivity of the creatine biosensor, response of the biosensor to ascorbic acid, uric acid and glucose was investigated at a constant creatine concentration of 0.1 mmol L^{-1} . Addition of 0.1 mmol L^{-1} ascorbic acid, 0.1 mmol L^{-1} uric acid and 0.1 mmol L^{-1} glucose to 0.1 mmol L^{-1} creatine increased the response current by about 74%, 22.2% and 6.5%, respectively. However, when the concentration of ascorbic acid and uric acid decreased to 0.01 mmol L^{-1} , the interference effect also decreased to 8.2% and 1.3%, respectively. Therefore, it can be concluded that dilution reduces the effect of interferences like it was reported (Martinez-Peréz, Ferrer, & Mateo, 2003) and our biosensor presents an acceptable anti-interferant ability.

The proposed creatine biosensor was applied to the direct determination of creatine in commercial creatine powder. Creatine powder was weighed and dissolved in a 100 mL calibrated flask with phosphate buffer solution. The contents of the flask were sonicated for 10 minutes to achieve complete

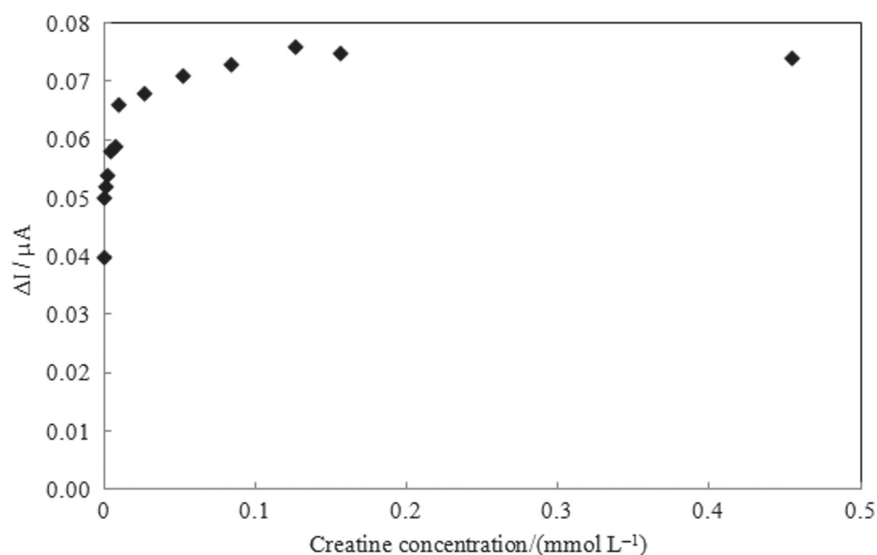


Figure 4. Effect of creatine concentration on the response of Fe_3O_4 -CPEE (0.05 mol L^{-1} pH 7.0, phosphate buffer, $+0.30 \text{ V}$).

dissolution of creatine. Aliquots of this solution were transferred to electrochemical cell containing 5 mL of phosphate buffer and response of the biosensor was measured. Determination of creatine was performed by using direct calibration curve method. Creatine concentration was calculated from the related calibration curve by using the current differences measured by the biosensor. The average recovery value was found as 103.8 ± 3.6 for four different creatine concentrations. This result indicates that the content of creatine in the commercial creatine powder can be safely determined by using proposed biosensor. As a result, Fe_3O_4 -CPEE gives good analytical characteristics of creatine detection and can be used for reliable creatine analysis in commercial creatine powder.

Conclusion

We have successfully demonstrated an Fe_3O_4 -modified carbon paste enzyme electrode as a feasible sensor for creatine determination. Fe_3O_4 -CPEE exhibited a good analytical performance for the amperometric detection of creatine. With low detection limit, rapid response, satisfactory linear range, good stability and selectivity, the presented biosensor was successfully used to determine creatine in commercial creatine powder which is promising in food industry. The method is simple and easy to perform. Furthermore, the cost of the presented biosensor is lower than that of the currently used methods. The purposed strategy can be extended for the development of other biosensors.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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