



Sensitive determination of dihydronicotinamide adenine dinucleotide and ethanol with a nano-porous carbon electrode

XIAOQIN LIU, BUHAI LI and CHUNYA LI*

*Key Laboratory of Analytical Chemistry of the State Ethnic Affairs Commission,
College of Chemistry and Materials Science, South-Central University
for Nationalities, Wuhan 430074, China*

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Abstract: A nano-porous carbon electrode, fabricated in 0.1 mol L⁻¹ NaOH by the electrochemical technique, was used for the electrochemical detection of dihydronicotinamide adenine dinucleotide, NADH, with an overpotential decrease of about 270 mV and a linear range from 1.0×10⁻⁶ to 1.0×10⁻⁴ mol L⁻¹. Amperometric sensing of ethanol cooperating with alcohol dehydrogenase and nicotinamide adenine dinucleotide was successfully demonstrated. A linear response in the range from 5.0×10⁻⁵ mol L⁻¹ to 1.0×10⁻² mol L⁻¹ was obtained, with a detection limit of 1.0×10⁻⁵ mol L⁻¹. The method was successfully employed to determine ethanol in beer with high precision.

Keywords: nano-porous carbon; NADH; ethanol; electrochemical process; amperometry.

INTRODUCTION

The electrochemical oxidation of dihydronicotinamide adenine dinucleotide, NADH, to the enzymatically active NAD⁺ in aqueous solution has attracted considerable interest due to its significance as a cofactor for dehydrogenase enzymes and in the development of biosensors. NAD⁺ as a co-enzyme can catalyze the oxidation of various compounds of great interest in analytical chemistry, such as alcohols, aldehydes and carbohydrates, in cooperation with enzymes, including dehydrogenases and oxidoreductases, to generate NADH. Therefore, the electrochemical detection of NADH and a highly selective and sensitive NADH transducer is desired.¹ However, the direct electrochemical oxidation of NADH at a bare glassy carbon electrode requires a high overpotential. Although the reversible potential of the NADH/NAD⁺ couple is estimated to be -0.56 V (*versus* SCE) in neutral pH, the direct oxidation of NADH at unmodified electrodes often occurs at a potential of about 1.0 V.² The large overpotential always

* Corresponding author. E-mail: lcychem@yahoo.com
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results in interferences from the real samples. In addition, the electrode surface is often fouled by the adsorption of reaction intermediates that are generated during the oxidation of NADH.³ Hence, considerable effort has been made to modify the electrode surface to decrease the overpotential for the oxidation of NADH and to minimize the surface passivation effects.

Many methods have been adopted to immobilize the mediator on the electrode surface to reduce the overpotential for NADH oxidation. Electrodes modified with carbon nanotubes,^{4,5} CdS nanoparticles,⁶ nanostructured TiO₂,⁷ boron-doped diamond⁸ and conducting polymer nanotubes⁹ have been developed for the electrochemical detection of NADH. However, the stability of the mediator limits the reproducibility and the operational lifetime of these modified electrodes. Therefore, sensitively and selectively in the electrochemical detection of NADH are still challenging tasks.

Various kinds of activation methods, such as mechanical polishing,¹⁰ ultrasonification,¹¹ vacuum heating,¹² laser irradiation and electrochemical pretreatment¹³ have been developed to improve the electrochemical performance of glassy carbon electrodes. Among these activation methods, electrochemical pretreatment is one of the most commonly used methods to improve the electrochemical responses to biological compounds, due to its good reproducibility and simple operation. The higher density of functional groups produced on the surface of glassy carbon electrodes by electrochemical activation have been characterized by various techniques, including X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM) and electrochemical impedance spectra (EIS).¹⁴ The results indicate that the existence of carboxyl groups on the electrode surface can accelerate electron transfer between the electrode surface and the solution.^{15,16} These conclusions initiated this investigation on the electrochemical performance of an electrochemically-treated glassy carbon electrode in the determination of NADH.

In this study, a glassy carbon electrode was treated in 0.1 mol L⁻¹ NaOH by electrochemical oxidization at 1.80 V for 3 min followed by reduction at -1.00 V for 1 min. This electrochemical process resulted in the formation of nano-porous cavities on the electrode surface. This nano-porous carbon electrode exhibited excellent electro-catalytic activity towards the oxidation of NADH at a low potential in phosphate buffer at pH 7.0. Furthermore, an electrochemical method for ethanol determination at the nano-porous carbon electrode in presence of alcohol dehydrogenase, ADH, and NAD⁺ using amperometry was developed.

EXPERIMENTAL

Apparatus and reagents

The electrochemical measurements were performed with an EC 550 electrochemical workstation (Gaoss Union Technology Co., Ltd, Wuhan, China) in an electrochemical cell containing 10.0 mL of 1/15 mol L⁻¹ phosphate buffer solution at a pH of 7.0. A conventional three-

-electrode system was used with a pretreated glassy carbon electrode, GCE (diameter 3 mm), as the working electrode, a Pt wire as the auxiliary electrode and a saturated calomel electrode (SCE) as the reference electrode (all potentials are expressed vs. SCE). All experiments were realized at room temperature. AVG Multilab 2000F X-ray photoelectron spectroscope (Thermo Electron Corp., USA) and a JSM-6700F field emission scanning electron microscope (JEOL Ltd., Japan) were applied for surface analysis of the nano-porous carbon electrode. The electrochemical impedance spectra (EIS) measurements were performed in a 0.5 mol L⁻¹ KCl solution containing 2.0 mmol L⁻¹ K₄[Fe(CN)₆]/K₃[Fe(CN)₆] at a measuring potential of 0.19 V.

Nicotinamide adenine dinucleotide (NAD⁺), nicotinamide adenine dinucleotide reduced form (NADH) and alcohol dehydrogenase ADH (EC 1.1.1.1) from baker's yeast in the form of a lyophilized powder were obtained from Sigma. All other chemicals were of analytical grade and all of the solutions were prepared with ultrapure water obtained from a molecular water purification system.

Preparation of the nano-porous carbon electrode

A glassy carbon electrode was polished with fine emery paper and alumina slurries followed by rinsing thoroughly with distilled water. The electrodes were successively sonicated in nitric acid, ethanol, and distilled water, and then allowed to dry at room temperature. A freshly polished electrode was electrochemically treated in 0.1 mol L⁻¹ NaOH at 1.80 V for 3 min, and then reduced at -1.0 V for 1 min, whereby a nano-porous carbon electrode was obtained. After successive cyclic voltammetric scanning for 20 cycles in phosphate buffer solution in the potential range from 0.0 to 0.80 V, the nano-porous carbon electrode was taken out and washed thoroughly with ultrapure water. The same procedure was employed for the electrochemical treatment of a glassy carbon electrode in acid, only 0.1 mol L⁻¹ H₂SO₄ solution was used instead of NaOH solution. The density and diameter of the formed nano-porous cavities could be modulated easily by the adjustment of the potential and the time of electrochemical treatment.

Electrochemical measurements

NADH detection was performed under stirring using amperometric batch analysis in an electrochemical cell containing 10 mL of 1/15 mol L⁻¹ phosphate buffer solution (PBS) at pH 7.0 with an applied potential of 350 mV. The nano-porous carbon electrode was dipped in the buffer solution, then, after a stable background current was observed, NADH was successively added and a fast current response was obtained.

RESULTS AND DISCUSSION

Characterization of the nano-porous carbon electrode

Scanning electron microscopy was used to characterize the morphology of the obtained nano-porous carbon electrode surface. The SEM images of the glassy carbon electrode before (a) and after (b) electrochemical treatment in 0.1 mol L⁻¹ NaOH are presented in Fig. 1. A slide surface was observed for the glassy carbon electrode before treatment. However, many cavities of nano-meter size were found on the surface of the electrochemically treated electrode. The rough and lacunose construction of the nano-porous carbon electrode offers more electroactive sites and a larger surface area, which is responsible for easier electron-transfer processes and the enhancement of the current response.

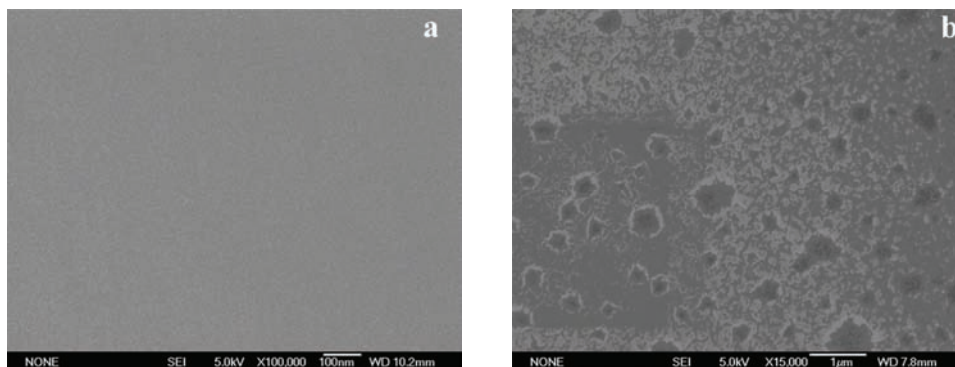


Fig. 1. Morphology of the glassy carbon electrode a) before and b) after electrochemical treatment in 0.1 mol L^{-1} NaOH solution.

It was found that electrodes that had been freshly polished with emery paper and alumina showed relatively poor activity toward the oxidation of NADH, compared to electrodes that had been electrochemically pretreated. It was suggested that pretreatment introduces or alters the nature of functional groups on the electrode surface and that such groups might serve as mediators of electrons between the electrode and the electroactive species. After electrochemical oxidation, phenolic, quinoidal, and carboxyl functional group were generated on the electrode surface. According to an early report,¹⁴ carboxyl groups on the surface of a GCE treated by an electrochemical process can mediate charge transfer reactions. Thus, in the present, XPS was used to determine the carbon and oxygen content of the electrochemically treated electrode surface, and the results are shown in Fig. 2, from which it was found that the oxygen-to-carbon (O/C) ratios

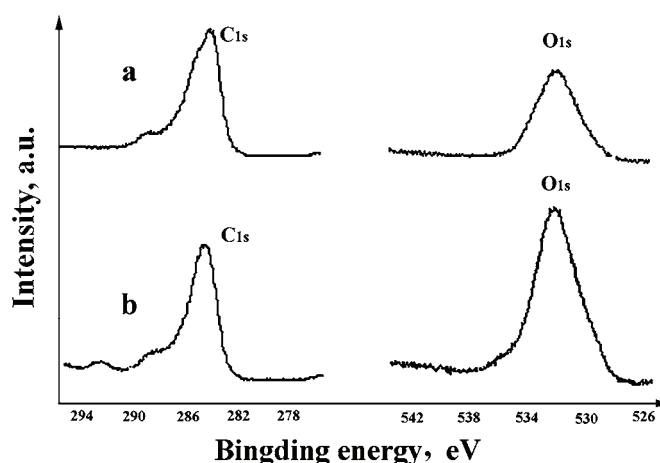


Fig. 2. XPS Spectra of the O (1s) and C (1s) regions: a) bare GCE; b) nano-porous carbon electrode.

of the surface of the bare GCE and the nano-porous carbon electrode were 0.27 and 0.46, respectively. The increase in the O/C ratio indicated that the electrochemical process could increase the number of oxygen-containing functional groups on the GCE surface, especially those groups which were negatively charged, which would experience an electrostatic repulsion towards NADH, which is negatively charged in PBS at pH 7.0.

Electrochemical impedance spectroscopy (EIS) is a powerful tool for studying the interface properties of nano-porous carbon electrodes and can provide information on the impedance changes between the interface between the electrode surface and electrolyte solution. Nyquist plots are presented in Fig. 3 for the bare GCE (a) and the nano-porous carbon electrode (b) in 0.5 mol L^{-1} KCl solution containing 2.0 mmol L^{-1} $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ using an amplitude of 0.005 V and a frequency range of $1 \text{ mHz} - 100 \text{ kHz}$. The Randles circuit (inset of Fig. 3) was chosen to fit the obtained impedance data. The resistance to charge transfer (R_{ct}) and the diffusion impedance (W) were both in parallel to the interfacial capacitance (C_{dl}). The diameter of the semicircle corresponded to the interfacial electron-transfer resistance (R_{ct}). By fitting the data, R_{ct} was estimated to be 1081Ω at the bare GCE, the R_{ct} decreased to 103.8Ω at the nano-porous carbon electrode. This result demonstrates that the nano-porous surface of the carbon electrode could function as an efficient electron conducting tunnel between the electrode surface and electrolyte solution due to the enormous numbers of nano-cavities and electroactive sites.

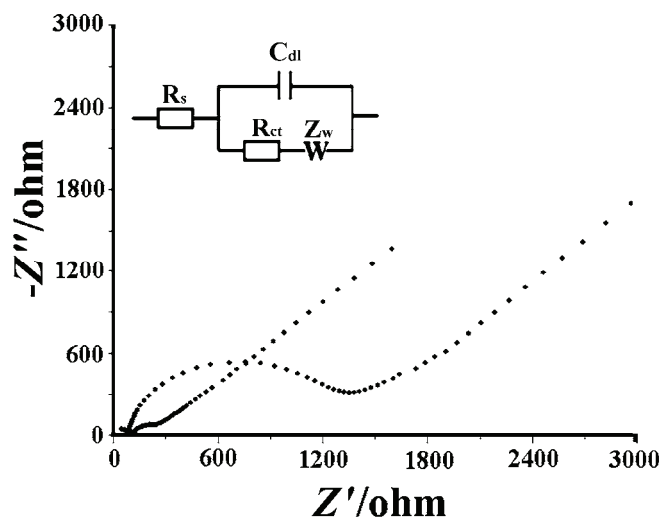


Fig. 3. Nyquist plots for the bare GCE (a) and nano-porous (b) carbon electrode in the presence of 2.0 mmol L^{-1} $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ in 0.5 mol L^{-1} KCl solution. The inset is the Randle equivalent circuit used to fit the EIS measurements.

Electrochemical behavior of NADH

Considering the higher surface density of functional groups on the nano-porous carbon electrode, the electrochemical oxidation of NADH could be reasonably catalyzed.^{15,16} Accordingly, the electrochemical behavior of 1.0 mmol L⁻¹ NADH at the nano-porous carbon electrode was investigated in 1/15 mol L⁻¹ PBS (pH 7.0) by differential pulse voltammetry (DPV). The obtained voltammograms are shown in Fig. 4. When the GCE was electrochemically treated in different supporting electrolyte solutions, the change in the structure of the electrode surface was different, which resulted in different decreases in the overpotential for electrochemical oxidation. For the bare GCE, the recorded difference pulse voltammogram shows an oxidation peak at 0.62 V (curve a). After electrochemical treatment in 0.1 mol L⁻¹ H₂SO₄ solution (curve b), the oxidation peak was negatively shifted to 0.42 V. However, after electrochemical treatment in 0.1 mol L⁻¹ NaOH solution, the oxidation peak appeared at 0.35 V (curve c). No peak at 0.35V was observed at the nano-porous carbon electrode in the absence of NADH (curve d). Therefore, the oxidation peak at 0.35 V can be assigned to the catalytic oxidation of NADH by functional groups on the nano-porous carbon electrode. It can be seen that the response of the carbon electrodes to NADH depended on the nature of the pretreatment. The main difference in the electrochemical pretreatment process was the composition of the electrolyte solution, which includes both the pH of the solution and the nature and concentration of the electrolyte ions besides H⁺. Thus, it can be concluded that the functional groups obtained on the electrode surface by pretreatment in a 0.1 mol L⁻¹ NaOH solution were different from that in 0.1 mol L⁻¹ H₂SO₄ solution. It can also be seen from Fig. 4 that the oxidation overpotential of NADH was decreased by 270 mV and the oxidation peak current was improved significantly after the glass carbon electrode had been electrochemically treated.

Chronocoulometry is useful for measuring electrode surface areas, diffusion coefficients, the time window of an electrochemical cell, adsorption of electroactive species, and the mechanisms and rate constants for chemical reactions coupled to electron transfer reactions. The chronocoulometry for the bare GCE and the nano-porous carbon electrode in 0.5 mmol L⁻¹ K₃[Fe(CN)₆] solution containing 0.1 mol L⁻¹ KCl were investigated with a potential step from 0.0 to 0.40 V, a pulse width of 0.25 s and sample interval of 0.00025 s. The results showed that the effective surface areas of the nano-porous carbon electrode and the bare GCE were 0.023 cm² and 0.018 cm², respectively. The increase in the effective surface area of electrode should be partly responsible for the current increase in the oxidation of NADH,¹⁷ but the large number of functional groups on the electrode surface is the most important factor leading to the decrease of the overvoltage for the oxidation of NADH. This result indicated that the electrochemical treatment in 0.1 mol L⁻¹ NaOH solution can improve the electroche-

mical activity of the electrode, which facilitates electron transfer between the electrode surface and the NADH solution.

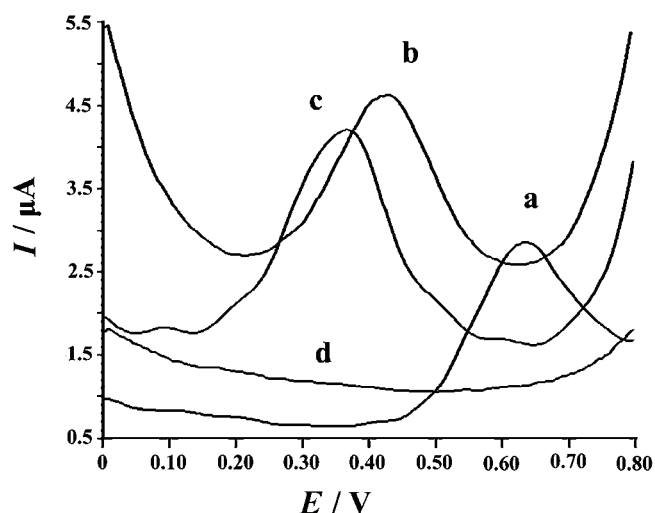


Fig. 4. Differential pulse voltammograms of 1.0 mmol L^{-1} NADH at a) the bare GCG, the nano-porous carbon electrode obtained in b) 0.1 mol L^{-1} H_2SO_4 solution and c) 0.1 mol L^{-1} NaOH solution, and d) the nano-porous carbon electrode obtained in 0.1 mol L^{-1} NaOH solution in the absence of NADH.

NADH determination

The advantageous effect of the functional groups leading to a decrease in the overvoltage for the oxidation of NADH on the surface of the pretreated electrode was successfully used for NADH detection. The amperometric responses of NADH recorded with an initial potential at 0.35 V (vs. SCE) in a stirred buffer solution were investigated. It was found that the increment of the current response at the nano-porous carbon electrode was almost the same for successive injections of $10 \text{ }\mu\text{L}$ of 1.0 mmol L^{-1} NADH into 10 mL PBS at pH 7.0, indicating a good reproducibility. The mechanism of NADH oxidation reaction, which has already been extensively investigated, is that NADH undergoes a two-electron and one proton process to yield the corresponding enzymatic active NAD^+ . Direct electrochemical oxidation of NADH only occurs at high overpotentials and is accompanied by rapid poisoning of the reaction because the electrode surface is fouled by polymeric products of side reactions which occur because of the one-electron oxidation intermediates formed in the reaction. Then, the oxidation current of NADH decreases and the reaction becomes unstable. After a glass carbon electrode was pretreated by electrochemical polarization, many functional groups were generated on the electrode surface that could act as transfer electron oxidants, which were used to lower the overpotential of NADH

and decrease the polymeric products of the one-electron oxidation intermediates.¹⁸ Figure 5 shows the amperometric response of the nano-porous carbon electrode to successive additions of different concentrations of NADH into a stirred buffer solution. After each injection of NADH, the current response (I) increased immediately and reached a steady state quickly. It exhibited a linear range from 1.0×10^{-6} to 1.0×10^{-4} mol L⁻¹ according to the equation:

$$I (\mu\text{A}) = 0.00591c (\mu\text{mol L}^{-1}) + 0.00271, r = 0.993 \quad (1)$$

where c is the NADH concentration.

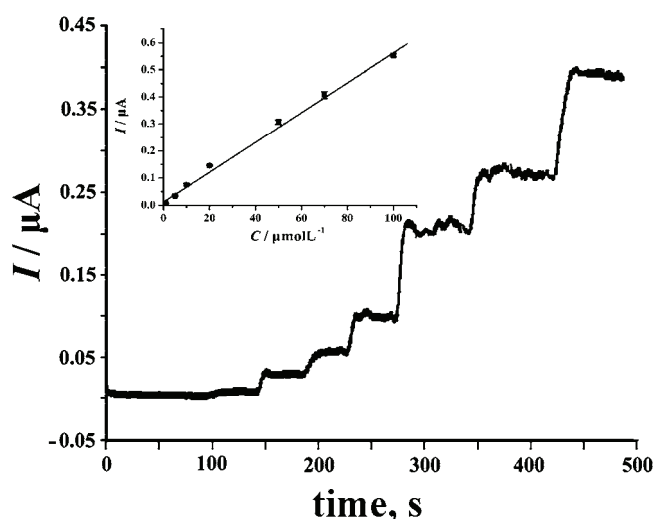


Fig. 5. Amperometric response of NADH at various concentrations in the range from 1.0×10^{-6} mol L⁻¹ to 1.0×10^{-4} mol L⁻¹ in phosphate buffer solution (pH 7.0) at the nano-porous carbon electrode. The inset is the calibration curve for NADH determination.

It is also important to emphasize that an NADH concentration as low as 1.0×10^{-6} mol L⁻¹ was detected with high accuracy, indicating that the nano-porous carbon electrode could be used as a sensitive sensor for NADH.

Application to ethanol determination

Electrochemical sensing of ethanol was performed in a phosphate buffer solution using the nano-porous carbon electrode in the presence of ADH and NAD⁺. The enzymes, together with their principal cofactor NAD⁺, were initially used free in solution at their optimum pH. The reaction that occurs in the solution was consistent with an earlier report,¹⁹ and can be described as follows:



After the background current had reached a steady state, 10 μL of 0.05 mol L⁻¹ ethanol were successively added and the subsequent formation of NADH was

measured as described above. The amperometric response of the nano-porous carbon electrode towards different concentrations of ethanol is shown in Fig. 6. The insert curves are the calibration curves for ethanol determination. It was found that amperometric current (I) was linearly related to ethanol concentration (c) in the concentration range from 1.0×10^{-5} mol L $^{-1}$ to 1.0×10^{-3} mol L $^{-1}$ and 1.0×10^{-3} mol L $^{-1}$ to 1.0×10^{-2} mol L $^{-1}$ according to the equations:

$$I_1 \text{ (nA)} = 56.510c_1 \text{ (mmol L}^{-1}\text{)} + 3.580, r = 0.995 \quad (3)$$

and

$$I_2 \text{ (nA)} = 8.941c_2 \text{ (mmol L}^{-1}\text{)} + 49.26, r = 0.996 \quad (4)$$

At higher concentrations, the slope of the curve decreases, most likely because of control by the enzymatic reaction. A similar behavior was reported previously.⁸

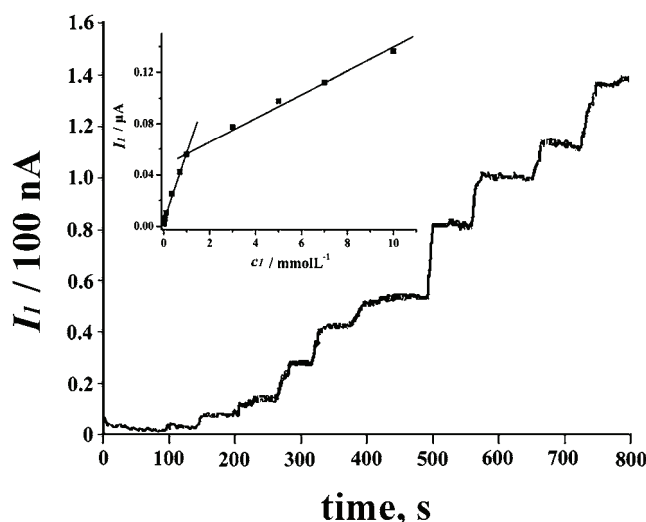


Fig. 6. Amperometric response of ethanol at various concentrations in the range from 1.0×10^{-5} mol L $^{-1}$ to 1.0×10^{-2} mol L $^{-1}$ in phosphate buffer solution containing 1 mg mL $^{-1}$ ADH and 3.0 mmol L $^{-1}$ NAD $^{+}$ at the nano-porous carbon electrode. The inset shows the calibration curves for ethanol determination.

The concentration of ethanol in Budweiser beer was measured using the nano-porous carbon electrode. 10 mL of phosphate buffer solution at pH 7.0 was spiked with 2.0 μ L of beer. Five parallel determinations were performed to determine the ethanol content. The average ethanol content was determined to be 3.7 ± 0.3 (vol. %), which is close to the ethanol nominal of 3.7 (vol. %), showing a good accuracy and high precision as the obtained relative standard deviation was 0.24 %.

CONCLUSIONS

In this present work, a nano-porous carbon electrode was fabricated by electrochemically treatment in NaOH solution with oxidation at 1.80 V following by reduction at -1.00 V. The electrochemical performances of the obtained nano-porous carbon electrode towards NADH were investigated thoroughly by voltammetry and amperometry. The overpotential for the oxidation of NADH was decreased substantially and the current response was improved significantly. In cooperation with NAD^+ and ADH, the nano-porous carbon electrode was successfully utilized for ethanol determination, which indicates that the obtained electrode can potentially be applied in the analysis of ethanol in real samples.

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ИЗВОД

ОСЕТЉИВА МЕТОДА ОДРЕЂИВАЊА NADH И ЕТАНОЛА КОРИШЋЕЊЕМ
НАНО-ПОРОЗНЕ УГЉЕНИЧНЕ ЕЛЕКТРОДЕ

XIAOQIN LIU, BUHAI LI и CHUNYA LI

Key Laboratory of Analytical Chemistry of the State Ethnic Affairs Commission, College of Chemistry and Materials Science, South-Central University for Nationalities, Wuhan 430074, China

Нано-порозна угљенична електрода је формирана електрохемијским поступком у раствору $0,1 \text{ mol L}^{-1}$ NaOH, а затим је коришћена за електрохемијску детекцију NADH уз смањење пренапетости од 270 mV и линеарни опсег од $1,0 \times 10^{-6}$ до $1,0 \times 10^{-4} \text{ mol L}^{-1}$. Такође је демонстрирана могућност амперометријског одређивања етанола уз алкохол дехидрогеназу и никотинамид аденин динуклеотид. Добијен је линеарни одговор у опсегу од $5,0 \times 10^{-5}$ до $1,0 \times 10^{-2} \text{ mol L}^{-1}$. Метода је успешно примењена за одређивање етанола у пиву и показала је велику прецизност.

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