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Development of Potentiometric Lactate Biosensor Based on Composite pH Sensor

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Abstract: *In this study, a micro-sized lactate sensitive biosensor based on polyvinylchloride, quinhydrone and graphite composite pH sensing platform was developed. Lactate oxidase was immobilized on the composite layer as the biorecognition element. Transformation reaction of lactate to pyruvate and hydrogen peroxide was the basis of this biosensor system. In the reaction, hydrogen peroxide undergoes to give hydronium ions into solution, and the pH sensitive membrane detects the adjunct hydronium ions potentiometrically. The surface of lactate biosensor based composite pH sensing matrice was first examined for electrochemical elucidation by using cyclic voltammetry and electrochemical impedance spectroscopy. A linear response in concentration range from $5x10^{-5}$ to $1x10^{-1}$ mol/L was obtained with a detection limit of $2x10^{-5}$ mol/L. The lactate biosensor developed was successfully applied for highly precise and efficient determination of lactate in food preparations. The biosensor exhibited a fast response time (10 s), had good stability, and had an extended lifetime.*

Keywords: Lactate, lactate oxidase, pH sensitive biosensor, impedance, quinhydrone.

1. INTRODUCTION

Biosensors, which are combination of a biorecognition receptor and a transducer, are widely used in health and environmental applications [1-6]. This technology provides selective and sensitive analysis of target analyte by using biomolecules. This selectivity and sensitivity can be improved by selecting proper immobilization materials, which located on transducer for proper biorecognition receptor. Combination of proper immobilization materials and biological materials is capable of measuring especially the food ingredients [7-9]. As it is known, foods have additives against microbial or chemical disruption. Therefore, checking our supplies is very crucial for our health.

Among the food ingredients lactate has a very important place in food industry and it is used as food additive [10] or food quality marker [11]. Accordingly lactate amount in food must be kept under control. However a number of analyzing methods need a professional staff and a complicated device and these methods must be precise, easy and economical. Lactate can be analyzed by an effective method such as biosensors [12]. As it was mentioned above the compatibility and specificity of biomolecules provide sensitive detections in biosensor technology. Regarding other methods, electrochemical methods are very suitable for this kind of measurements, especially in blur and non-homogenous samples. In our system, potentiometry was used as the measurement method. This method provides determination of ion concentration in sample solutions; however it is needed to be prevent from ion interferences in selectivity. Therefore, a lactate oxidase (LOx) immobilized pH selective conductive membrane was constructed. As it is known, LOx enzyme catalyzes the transformation of lactate into pyruvate and hydrogen peroxide on the surface of the membrane. Hydrogen peroxide component dissolves and becames decomposed by generating hydronium ions into solution. The constructed biosensor membrane was selective for hydronium ions, and therefore this reaction is the fundamental of our measurement system. The composite pH membrane constructed by using quinhydrone that was the combination of hydroquinone and quinone in 1:1 mole ratio in the membrane matrice. The composite pH matrice can allow miniaturization [13] and could not be affected by atmospheric pressure [14] consequently needed for measurements. The application of a robust and miniaturized matrice appears to be advantageous, especially when monitoring over a long period of time and measurement over low sample volumes are required [15].

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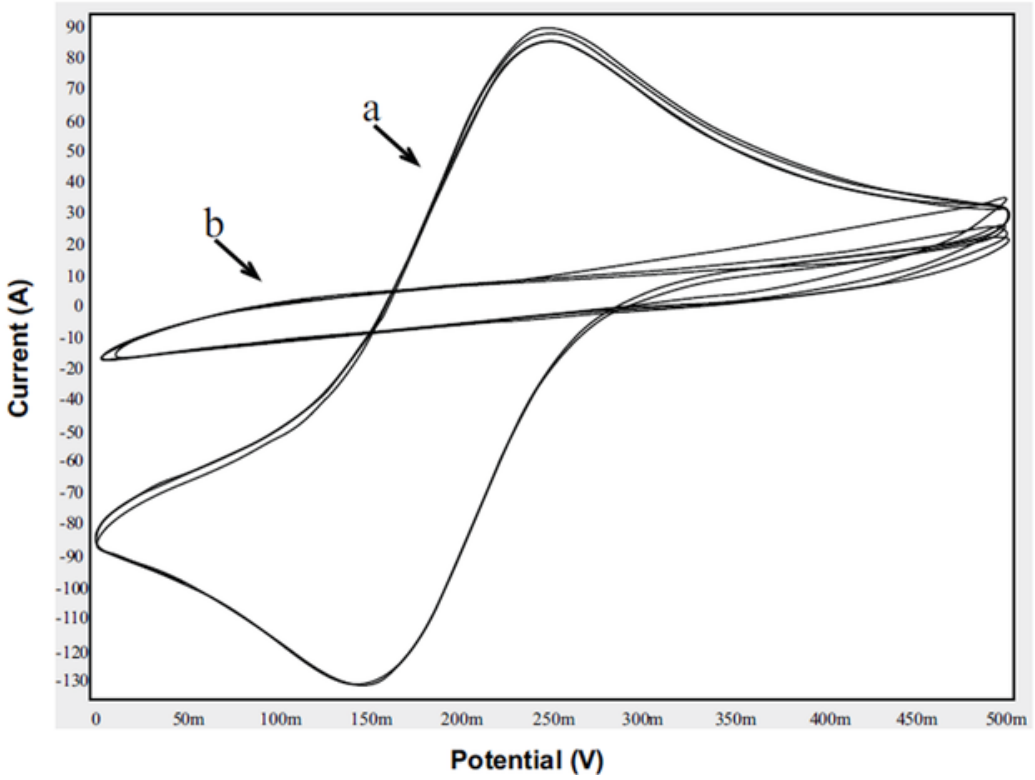

Figure 1. CV results of PVC-NH_2-Quinhydrone-Graphite composite electrode (a) LOx immobilization on PVC-NH_2-Quinhydrone-Graphite composite electrode (b).

In continuation of our previous works on the development of potentiometric biosensors for some of the biologically important compounds such as urea, glucos, creatinine, and creatin [13,14,16,17]. This work was concerned with the development of a potentiometric pH dependent biosensor system for lactate determination. Preliminary studies for elucidation of the electrochemical behavior of graphite-quinhydrone-PVC-NH_2 composite matrice were carried out by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The constructed lactate biosensor was then used in an assay for the potentiometric lactate determination in industrial food products.

2. MATERIALS AND METHOD

2.1. Materials

Polyvinylchloride (PVC), o-nitrophenyloctylether (o-NPOE), methanol, tetrahydrofuran (THF) and graphite were obtained from Fluka (Milwaukee, USA). Lactate oxidase, glutaraldehyde, potassium chloride, sodium lactate, quinhydrone and 1,4-diamino butane were obtained from Merck (Darmstadt, Germany). Epoxy resin was received from Araldite. Ppolymethylmetacrilate (PMMA), potassium dihydrogenphosphate and acrylic liquid were obtained from Sigma Aldrich (St. Louis, USA). Doubly distilled, deionized water was used for preparing all of the solutions and throughout the experiments. The supporting electrolyte solutions were 0.05 mol/L phosphate buffers (pH: 5.0-7.4) and were prepared from 0.1 mol/L solutions of phosphoric acid, potassium dihydrogen phosphate, potassium hydrogen phosphate, and potassium hydroxide, using a pH meter. Solutions of lactate were freshly prepared as required, in phosphate buffer at the desired pH and protected from light during investigation. Voltammetric experiments were carried out in phosphate buffer solutions, deoxygenated by pure nitrogen. In the study, the electrochemical lactate biosensor system was characterized electrochemically by VersaSTAT3 potentiostate/galvanostate (Princeton Applied Research, Oak Ridge, USA), and impedimetric measurements were carried out by high-impedance input galvanostate (Princeton Applied Research, Oak Ridge, USA). A conventional three-electrode cell was used with a lactate biosensor, a saturated Ag/AgCl reference electrode (BASi), and a Pt wire as the counter electrode. Potentiometric measurements were made by a multichannel potentiometric measurement system laboratory-made. It was controlled by a computer that has a home-made software program.

2.2. Method

A copper wire used as the electrical conductor that was coated by hydrogen ion selective membranes. Firstly polyvinylchloride-aminated (PVC-NH_2) polymer was synthesized [14]. Briefly; 1.43 g PVC, 11.5 mL 1,4-diaminobutane and 3.5 mL triethylamine were mixed and refluxed under atmospheric pressure for 3.5 hours. Then obtained polymer was washed with HCl and methanol. Subsequently, the impurities were removed by THF and the solution was filtrated and dried. The solid state pH layers were prepared by the addition of quinhydrone- PVC-NH_2 polymer-graphite composite

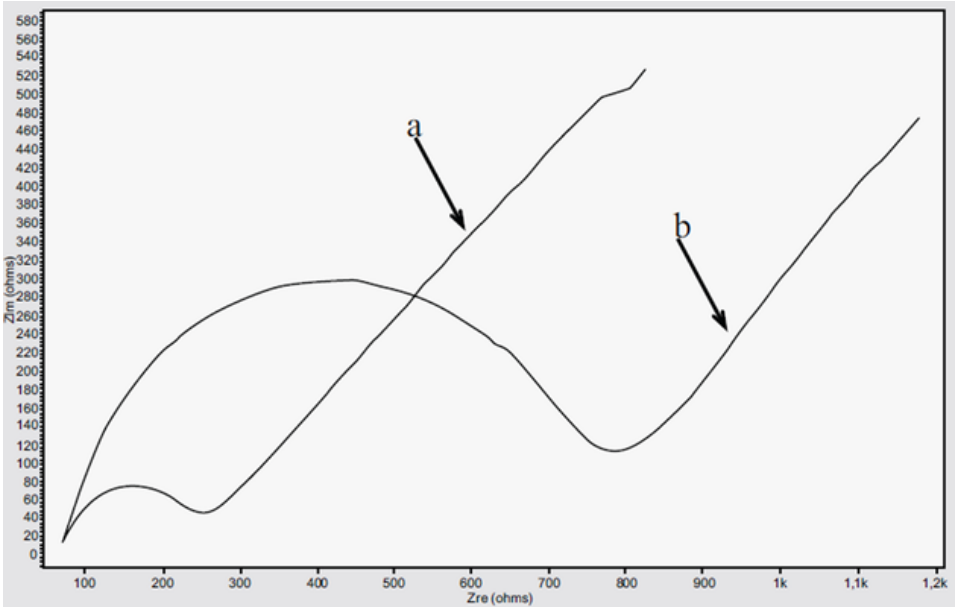

Figure 2. EIS results of PVC-NH_2-Quinhydrone-Graphite composite electrode (a) LOx immobilization on PVC-NH_2-Quinhydrone-Graphite composite electrode (b).

[14]. The graphite composite layer as transducer was formed by using 50% graphite (w/w), 35% epoxy (w/w) and 15% hardener (w/w) after grinding with a hand-mill. The pH sensitive composite electrodes were dried in room temperature for one week. Afterwards LOx was immobilized onto the pH layer via glutaraldehyde, a bifunctional linker. This biosensor layer was characterized with three electrode combination in phosphate buffer solution containing 0.1 mol/L KCl at pH 7.2 including $Fe(CN)_6^{3-/4-}$ redox couple by using CV and EIS data. CV method was performed under the experimental conditions: scan rate of 50 mV/s, 0 V to 0.5 V potential range. EIS spectrums were obtained by applying 0.180 mV bias potential between 10000 and 0.05 Hz. LOx modified composite solid state biosensor experiments on lactate measurements were carried out potentiometrically by two electrode system, and real food samples were also tested and compared to standard product ingredients.

3. RESULTS AND DISCUSSION

3.1. Preparation and CV characterization of composite pH electrode and lactate biosensor

Preliminary experiments to elucidate the catalytic activity of the lactate biosensor based on composite pH sensor membranes toward lactate were performed using CV and EIS. Cyclic voltammograms were recorded using composite pH sensor and the lactate biosensor in nitrogen saturated solutions. The processes associated with the electrochemical oxidation of lactate are illustrated in Fig. 1, by cyclic voltammograms of 0.01 mol/L lactate at pH 7.2. Lactate solutions recorded after several preliminary scans at the surface of the lactate biosensor in the potential range of 0 to 0.5 V vs Ag/AgCl reference electrode. As can be seen, the direct oxidation of lactate at the surface of the composite pH sensor showed no measurable wave in plain supporting electrolyte icluding in the potential range studied (Fig. 1b).

The biosensor exhibited a remarkable increasement in the anodic and cathodic peak currents when the LOx immobilized onto the composite pH sensor membrane (Fig. 1a). When the lactate biosensor is thoroughly rinsed with water and a new voltammogram is recorded in electrolyte solution, the response is retained and two pairs of anodic and cathodic peaks were observed as reported in literature [18-20].

This behavior, which was observed at different concentrations of lactate and several potential scan rates, clearly demonstrates that the electrode functions electrocatalytically toward lactate.

The observed anodic and cathodic catalytic currents were proportional to lactate concentration. In order to determine whether the current due to the oxidation process of lactate was diffusion controlled, the effect of scan rate on the cyclic voltammograms for oxidation of 0.01 mol/L lactate at the electrode was investigated. A linear dependence of ip vs square root of the scan rate, n1/2, obtained suggests that the oxidation of lactate at the lactate biosensor is a diffusion controlled process. The oxidative current of the lactic acid on the lactate biosensor are pH dependent. The anodic and cathodic peak potentials shift to more negative and positive values with increasing pH. The experiment was performed by adding concentrated phosphoric acid dropwise to 10.0 mL of 0.1 mol/L unbuffered lactic acid solution while the peak currents and pH were monitored simultaneously. Such a behavior, together with the observed increase in curent values with pH, suggests that dissociation of lactate occurs at or before the rate-determining step [21]. The direct oxidation of lactate by LOx leading to pyruvate and hydrogen peroxide, has been the object of many kinetic investigations, due to its basic biochemical and potential significance. The same conditions were also available in impedance measurements. Electron transfer resistance of the electrode surface increased because of the insulating layer that is the LOx layer (Fig. 2). As a result of

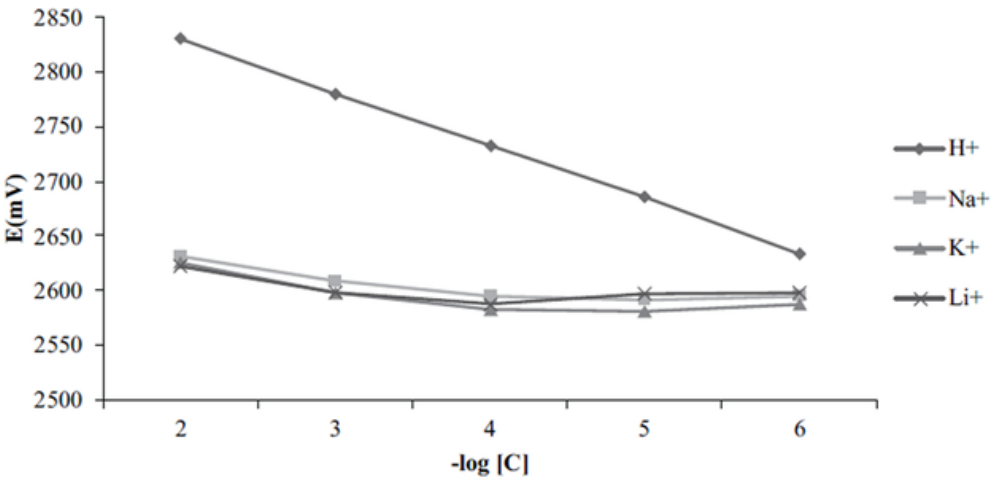

Figure 3. Selectivity of pH sensitive lactate biosensor for different ion concentrations (H^+: proton ions, Na^+: sodium ions, K^+: potassium ions, Li^+: lithium ions).

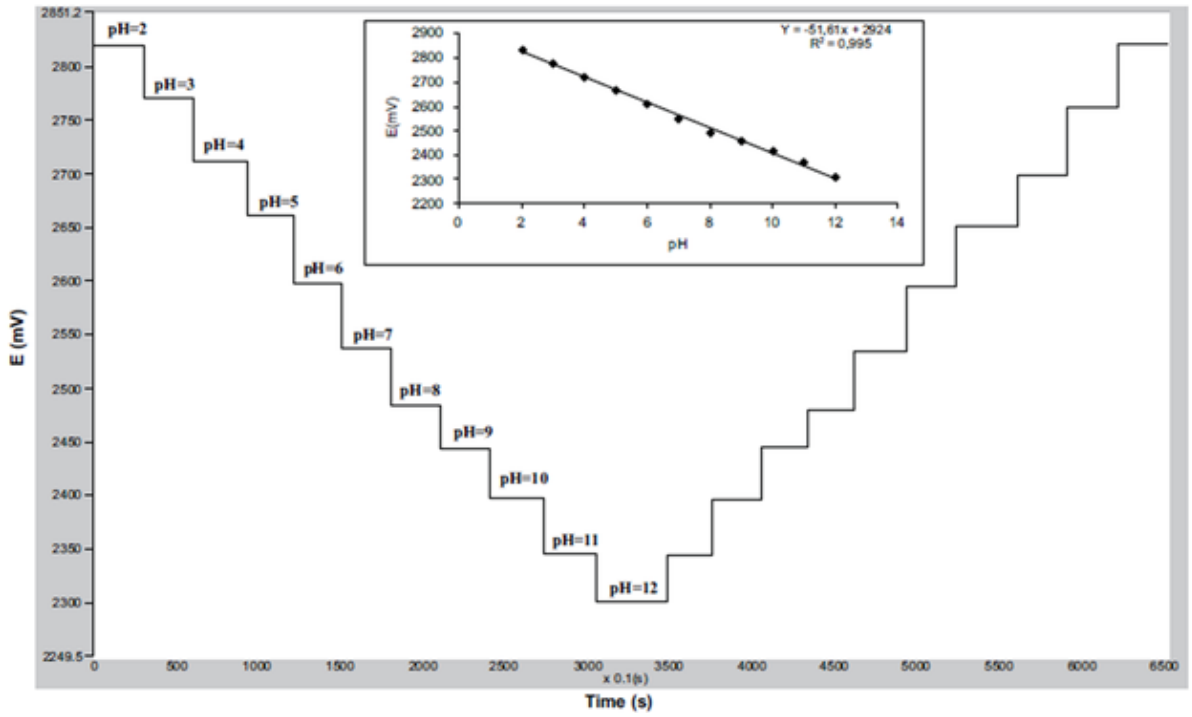

Figure 4. Repeatability of pH sensitive solid-state composite electrode.

enzyme immobilization on pH sensitive membrane, the surface resistance was increased. Peak current of CV was decreased while electron transfer resistance of EIS was increased. These measurements showed that LOx layer was successfully formed on pH sensitive layer.

3.2. Potentiometric behavior of the composite pH electrode and lactate biosensor

The pH sensitive layer was based on the quinhydrone characteristic, which complex molecule formed on the redox probe in the presence of hydrogen ions [22]. For the selectivity coefficient measurements of the composite pH sensor, $1x10^{-6}$-$1x10^{-1}$ mol/L solutions of interfering ions were used (Fig. 3). It can be seen from the results that the composite pH sensor was highly selective for hydronium ions. The repeatability of the composite pH sensor response was tested with measurements taken randomly in the pH 2 and pH 12 buffer solutions as shown in Fig. 4. The composite pH sensor exhibited in some degree good results for hydronium ions by

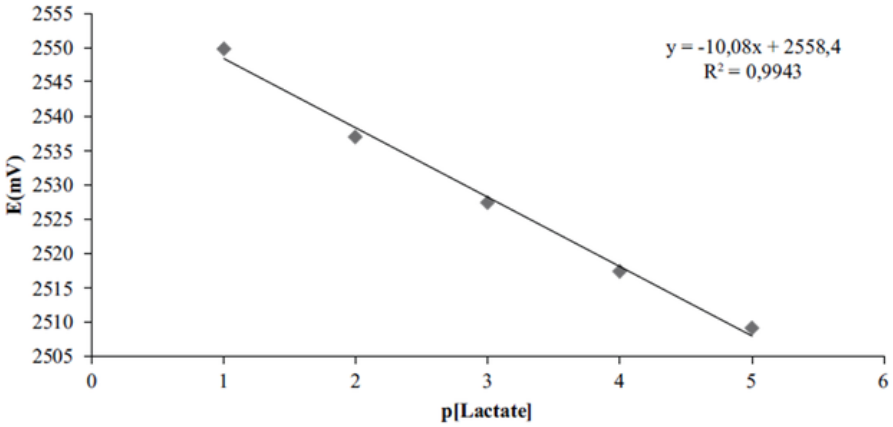

Figure 5. Calibration curve of pH sensitive lactate biosensor.

the comparison of Aquino-Binag *et al.* (1994) and had a good repeatability when compared with the results of Tao *et al.* (2004) [22,23].

The lactate biosensor was used as the indicator electrode in conjunction with a saturated Ag/AgCl reference electrode in a galvanic cell containing phosphate buffer, saturated with air or oxygen, for detection of lactate in non stirred solution. The fundamental of lactate determination was based on the hydronium ions, which were formed by enzymatic transformation of lactate into the pyruvate and hydrogen peroxide. Hydrogen peroxide dissolves in water and decomposes generating hydronium ions into measurement medium which give rise a potential between reference electrode and LOx modified composite pH sensor. An increase in hydrogen ion concentration in the solution alters potential of the lactate biosensor. A calibration curve was prepared by using these signals in a wide concentration range of lactate between $1x10^{-5}$ to $1x10^{-1}$ mol/L using phosphate buffer at pH 7.2. Figure 5 shows the calibration plot for the lactate determination obtained from the potential responses with LOx modified solid-state composite pH electrode. Under the optimized conditions, steady-state potentials showed an inverse relationship with the lactate concentration in the range from $5x10^{-5}$ to $1x10^{-1}$ mol/L of the lactate bioesensor to composite pH sensor. The potential increased steeply to a stable value while lactate concentration decreased. The slope of the potentiometric lactate biosensor was dependent on the solution pH and calculated to be between 10-14 mV/decade of lactate concentration indicating an under-Nernstian response of the lactate biosensor. Maximum sensitivity was observed at pH 7.2 with a calibration slope of 14 mV/decade. The linear dependence of the lactate concentration has a correlation coefficient of 0.9943. The calibration curve for lactate, using LOx modified solid-state composite pH electrode, has a detection limit of $2x10^{-5}$ mol/L, based on three times measurement for the standard deviation of the blank noise (95% confidence level, k:3, n:5). This detection limit is parallel with the detection limits obtained in previously reported potentiometric lactate biosensor [24]. The reproducibility of the potential response of the lactate biosensor was obtained by determining the slope of the calibration graph in a period of one month (n: 5). The biosensor retained its full activity during this period and the slopes of the calibration graphs were reproducible to within 1.5 mV/decade of lactate concentration. In order to investigate the applicability and selectivity of the proposed potentiometric method, the lactate biosensor was applied to the determination of lactate in food preparations using the standard addition method.

3.3. Real sample tests and characteristics of the biosensor

Randomly chosen different buttermilk products and a pickle juice were quantified by the lactate biosensor. Quantification was achieved by extrapolating the calculated best linear fit of potential vs logarithm of ascorbic acid concentration using the method of least squares and the signals for the lactate biosensor were comprised by standard values of product ingredients (Table 1). The precision of the method was assessed by repeating the analyses of the samples several times. These results were tested statistically. Unpaired t-test was used to assess the lactate determination. By using the equation of t test ($t = \bar{x}_d\sqrt{n}/S_d$) , t value was calculated as 0.43 and degree of freedom (n-1) was compared by t-test table and critical t values 2.26 (p: 0.05). The correlation coefficient (r) for the

Table 1. Analysis results of commercial products by pH sensitive lactate biosensor.

Product	Standard Concentration (%) (Product Ingredients)	Lactate Biosensor (%)	Recovery (%)
Buttermilk 1	0.58	0.62	108
Buttermilk 2	0.56	0.59	105
Buttermilk 3	0.57	0.61	107
Buttermilk 4	0.62	0.60	97
Buttermilk 5	0.56	0.58	104
Buttermilk 6	0.59	0.62	105
Buttermilk 7	0.61	0.62	102
Buttermilk 8	0.58	0.61	105
Buttermilk 9	0.59	0.60	102
Pickle Juice	1.00	1.02	102

two sets of results using the potentiometric and the standard values of product ingredients was 0.99 (n: 5). This demonstrates excellent agreement and illustrates that the proposed potentiometric method is reliable for the determination of lactate in the food samples as those described. The obtained results show that the measurement system is acknowledgeable for lactate determination (p: 0.067>0.05).

As a result a new lactate biosensor based on pH sensitive layer employed by quinhydrone, PVC-NH_2 polymer and modified graphite was constructed. The sensor was characterized by EIS and CV measurements, and it showed excellent potentiometric response with high selectivity. The quinhydrone-graphite-PVC-NH_2 composite allowed extensive active sites onto the biosensing layer, resulting in a sensitive and stable biosensor system that was very selective and effective for lactate determination. The real beneficial feature of the biosensor was real sample experiments acknowledged that the sensor system is very useful for lactate determination in food sample analysis. Without using commercialized electrode systems, handmade pH electrode and follow-up lactate biosensor unit used here has low cost, repeatability and reproducibility. The performance of the constructed biosensor is good in terms of sensitivity, selectivity, response time, and reproducibility. The developed biosensor was applied to detect lactate concentration in several beverages such as buttermilk and pickle juice, and satisfactory results were obtained.

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