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Application of a long shelf-life biosensor for the analysis of L-lactate in dairy products and serum samples

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Abstract

An L-lactate biosensor using an enzyme-immobilised eggshell membrane and an oxygen electrode for L-lactate determination has been developed. An ionotropic gelatinous solution of L-lactate oxidase and chitosan was deposited on an eggshell membrane and subsequently covered the surface of the oxygen electrode. The detection scheme was based on the depletion of dissolved oxygen content upon exposure to L-lactate solution. The decrease in oxygen concentration was monitored by the oxygen electrode which was connected to a datalogging system for data acquisition and processing. The effects of pH, dissolved oxygen content, salt concentration, temperature and potential interferants have been studied in detail. The relative standard deviation ($n = 10$) of the response was 5% for a 0.10 mM L-lactate standard. The t_{95} response time is ≈60 s and the recovery time is ≈90 s. A linear calibration curve, decrease in dissolved oxygen concentration = 11.67[L-lactate] + 0.039; $r^2 = 0.9998$, where dissolved oxygen concentration is in mg L^{-1} and [L-lactate] is in mM, was obtained from the biosensor. The limit of detection was calculated to be 8.6 μM. The response of the membrane to L-lactate was reversible and fast. The L-lactate biosensor demonstrated long shelf-life of at least 1 year and it maintained its activity above 80% after being kept at 4 °C for a year. The biosensor has been successfully applied to the determination of L-lactate content in dairy products and serum samples.

Keywords: L-Lactate; Biosensing; Eggshell membranes; Dairy products

1. Introduction

The determination of various substances in food samples is important in the control of food processing and maintenance of their quality. Some success applications have already been reported on the analysis of different food samples using enzyme-based biosensors (Bilitewski, 1994; Kuswandi, Andres, & Narayanaswamy, 2001). Biosensors are useful analytical tools that combine high selective biocomponents with physical transducers. Usually, it is the biocomponent that limits the shelf-life and stability of a biosensor. Thus, there is an increasing need for temperate immobilising methods and biocompatible enzyme-immobilised platforms. Conventional methods of enzyme immobilisation including physical adsorption, covalent binding, and entrapment or micro-encapsulation in polymeric or sol–gel matrices have been attempted. Although entrapment of purified enzymes in these matrices has been done, enzymes often fail to retain their native stability and activity upon immobilisation. One of the possible effective ways of improving the stability and activity of an immobilised enzyme is to incorporate the enzyme into a biomaterial as they are more biocompatible with each other. There have been numerous reports that the lifetimes of enzymes were much extended when they were immobilised onto eggshell membranes (Choi, Pang, Xiao, & Wu,

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2001; Choi & Yiu, 2004; Deng, Yuan, Xu, Xiao, & Wang, 1998; Hu, Fang, Zhou, & Zhu, 1995; Xiao & Choi, 2002). The backbone of an eggshell membrane is protein fibres, a natural polymer to be biocompatible and highly permeable and flexible in water. Enzymes can be physically and chemically entrapped in the three-dimensional interpenetrating network and are insoluble in aqueous solution. Borrowing this novel enzyme immobilisation idea, we endeavour to develop several biosensors (Choi et al., 2001; Choi & Yiu, 2004; Xiao & Choi, 2002) consisting of enzyme-entrapped eggshell membranes and an oxygen transducer.

Typical fields of application for lactate biosensor are in food industry for the control of dairy products and in sports medicine for exercise control. Determination of lactate is also essential in clinical analysis for the diagnosis of lactate acidosis as a result of metabolic, respiratory, or haemodynamic disturbance. Lactate oxidase (LOx) is widely employed in biosensors to determine L-lactate concentration. One of the problems with such biosensors is that LOx has limited stability. Although to date many lactate biosensors have been developed (Anzai, Takeshita, Kobayashi, Osa, & Hoshi, 1998; Collier, Lovejoy, & Hart, 1998; Hart, Matthews, & Collier, 1999; Mizutani, Yabuki, & Hirata, 1995; Palmisano, Quinto, Rizzi, & Zambonin, 2001; Rohm, Genrich, Collier, & Bilitewski, 1996; Yao, Yano, & Nishino, 2004), some of them still involve lengthy and cumbersome procedures and, the long-term stability of these biosensors is not satisfactory.

In our previous work (Choi et al., 2001; Choi & Yiu, 2004; Xiao & Choi, 2002), glutaraldehyde was employed to cross-link different enzymes onto eggshell membranes for fabrication of glucose, aspartame and H_2O_2 biosensors, respectively. Unfortunately adopting this similar enzyme immobilisation approach, the LOx-immobilised eggshell membrane had very low sensitivity to the L-lactate solutions. It was believed that glutaraldehyde could deactivate the immobilised-LOx (Sirkar, Revzin, & Pishko, 2000). Recently, it has been found that chitosan is an attractive biomaterial for immobilising LOx through the formation of polyelectrolyte complexes between the molecules of LOx and polysaccharide chains of the biopolymer chitosan (Wei, Zhang, & Gorski, 2003). Chitosan possesses favourable properties of excellent film-forming ability, high permeability towards water, good adhesion and biocompatibility. In addition, it has antifungal properties and can be used as a semipermeable coating material for fresh fruits and vegetables to help extend shelf-life (Park & Zhao, 2004). In the present work, chitosan was chosen to immobilise LOx onto eggshell membrane for the fabrication of L-lactate biosensor. The main objective of this work is to design a long-lived L-lactate biosensor that can be applied to the analysis of real samples. The proposed work is simple, safe and convenient to use. To our knowledge, this is the first report on a L-lactate biosensor constructed from an eggshell membrane with LOx–chitosan polyelectrolyte complexes. The developed biosensor has been demonstrated to be almost interference-free and is adequate for L-lactate determination in untreated or diluted dairy products and human serum samples.

2. Materials and methods

2.1. Materials

Lactate oxidase (EC 1.1.3.2 from *Pediococcus species*) with a specific activity of 38 U mg^{-1} of solid was obtained from Sigma (St. Louis, MO, USA). L-Ascorbic acid, 4-acetamidophenol, bovine serum albumin, chitosan (high molecular weight), disodium hydrogen phosphate, monosodium dihydrogen phosphate, sodium L-lactate, trisodium phosphate and uric acid were from Aldrich (Milwaukee, WI, USA). Milk and yoghurt samples were purchased from a local supermarket. Human sera were obtained from Chiron Diagnostics Corporation (East Walpole, MA, USA). All the other chemicals were of analytical-reagent grade or above and used as received. The buffer solution for preparing L-lactate standards was 0.10 M sodium phosphate solution at pH 7.50. All solutions were prepared with deionised (DI) water.

2.2. Immobilisation of lactate oxidase on eggshell membrane

A 0.30% (w/v) chitosan solution was prepared by dissolving 0.06 g chitosan flakes in 10 mL hot 0.05 M HCl (80–90 °C). After the solution had been cooled to room temperature, the pH was adjusted to 5.4 by about 5 mL 0.1 M NaOH, and then diluted to 20 mL with DI water. The solution was filtered through a Whatman #1 35 mm diameter filter paper (Whatman International Ltd., Maidstone, Kent, UK) to obtain a clear chitosan solution. A 1.3% (w/v) LOx solution was prepared by dissolving 13 mg LOx in 1.0 mL pH 7.50 phosphate buffer. An eggshell membrane was carefully peeled off from a broken fresh eggshell after the albumen and yolk had been removed. It was cleaned with a copious amount of DI water. The membrane was placed in a clean petri dish and it was cut into a circle of diameter about 2 cm. An aliquot of 10 μL 1.3% (w/v) LOx solution was added. Then, 100 μL 0.3% (w/v) chitosan solution as a gelation agent was dropped onto the surface of the membrane. A small spatula was gently used to mix the chitsoan solution with the LOx solution and spread it evenly on the membrane surface. The enyzme-immobilised membrane was dried at ambient conditions for 6 h. Before use, the membrane was immersed in and washed with a pH 7.50 phosphate buffer for 10 min to

remove any loosely bound enzyme. The LOx-immobilised eggshell membrane was kept at 4 °C until further use.

2.3. Assembly of L-lactate biosensor

The LOx-immobilised eggshell membrane was positioned on the surface of a Pasco CI-6542 oxygen sensor (Pasco Scientific, Roseville, CA, USA) and kept in a steady position by an O-ring. The electrode was then immersed into a stirred phosphate buffer solution, pH 7.50 at 22 ± 1 °C. Various volumes (4.0–20 μL) of standard or sample L-lactate solutions were injected into the phosphate buffer by a 100-μL syringe. The dissolved oxygen signal was captured at a sampling rate of 10 s^{-1} and processed by a datalogger system consisting of a ScienceWorkshop 500 interface, serial cables, a power supply, and control software (Pasco Scientific, Roseville, CA, USA). The data were logged in a personal computer for real-time display and processing. Unless otherwise stated, all test solutions were air-saturated by a stream of compressed air at flow rate of 20 mL min^{-1} for 20 min before measurement. When the L-lactate biosensor was not in use, the LOx-immobilised eggshell membrane was removed from the oxygen electrode and kept at 4 °C. The pH measurements of all solutions were taken on a combined pH glass electrode (Orion, Chicago, IL, USA).

To study the effect of dissolved oxygen (O_2) content on the L-lactate biosensor response, various dissolved O_2 concentrations of L-lactate solutions were prepared. Pure nitrogen (N_2), air and oxygen (O_2) cylinder gases of high purity (>99.9%) were purchased from Chun Wang Industrial Gases (Shenzhen, China). N_2–O_2 gas mixture of various specified compositions, spanning the range 6.0–35% v/v O_2, was generated with two mass flow controllers (Brooks Instrument BV, Veenendaal, The Netherlands). These N_2–O_2 gas mixtures were employed to prepare various dissolved O_2 concentrations of L-lactate solutions. The dissolved O_2 levels of L-lactate standards were controlled by passing through various O_2 gas standards (6.0–35% v/v) for 20 min and their dissolved O_2 concentrations were then measured by a commercial oxygen electrode (model 58, Yellow Springs Instrument, Yellow Springs, OH, USA).

3. Results and discussion

3.1. Response behaviour of L-lactate biosensor

The oxygen electrode acting as an oxygen transducer was employed to measure the oxygen consumption in the enzymatic oxidation of L-lactate (Anzai et al., 1998; Rohm et al., 1996; Wei et al., 2003):

$$CH_3CH(OH)CO_2^- + O_2 \xrightarrow{LOx} CH_3COCO_2^- + H_2O_2$$

The analytical signal of the L-lactate biosensor is the decrease in the dissolved oxygen content upon exposure to L-lactate solution. A typical response curve of the L-lactate biosensor is shown in Fig. 1. The decrease in the oxygen level was proportional to the L-lactate concentration. A linear calibration curve plotting the decrease in the dissolved oxygen concentration against the concentration of L-lactate is displayed in the inset in Fig. 1. The calibration curve displays excellent linearity, decrease in dissolved oxygen concentration = 11.67[L-lactate] + 0.039; $r^2 = 0.9998$; $n = 5$, where [L-lactate] is in

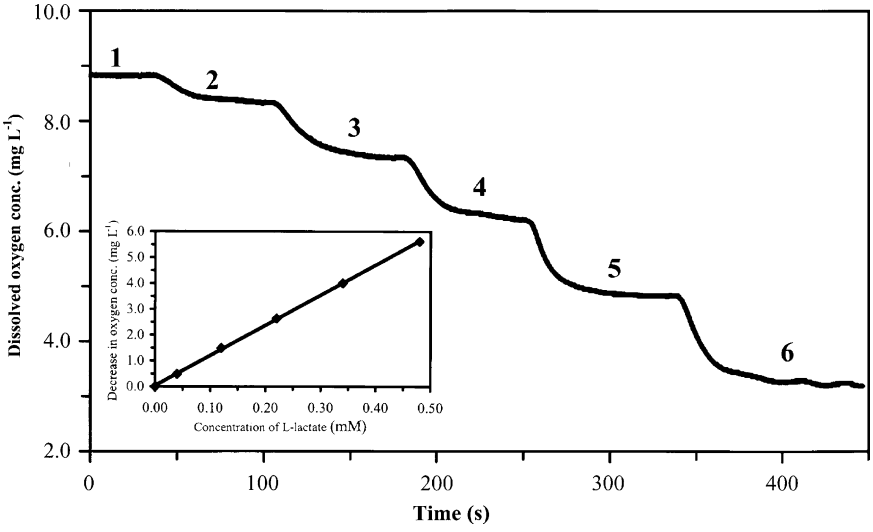

Fig. 1. Typical response curves of the L-lactate biosensor when subjected to various concentrations of L-lactate at pH 7.50 phosphate buffers (0.10 M). (1) 0.00; (2) 0.040; (3) 0.080; (4) 0.10; (5) 0.12; and (6) 0.14 mM. The inset displays the calibration curve of the L-lactate biosensor by plotting the decrease in the dissolved oxygen concentration against the concentration of L-lactate.

mM and dissolved oxygen concentration is in mg L^{-1}. The slope of the calibration curve represents the response sensitivity of the biosensor. The linear range covers from 0.00 to 0.48 mM. The limit of detection was calculated to be 8.6 μM from the calibration plot as that L-lactate concentration which produced an analytical signal equal to three times the standard deviation of the signal at zero value (Miller & Miller, 1993).

3.2. Effect of enzyme loading

Since the response sensitivity of the L-lactate biosensor strongly depends on the enzymatic activity of the oxidation of L-lactate, any change in enzyme concentration on immobilisation would affect the sensitivity of the L-lactate biosensor. Enzyme-immobilised eggshell membranes were prepared by adding various concentrations of LOx (0.1–1.5% w/v) on the eggshell membranes. The response sensitivity of each membrane was determined by exposing it to various concentrations of L-lactate solutions (0.04–0.14 mM). It was found that each of these membranes exhibited similar response behaviour towards L-lactate. The sensitivity of the biosensor increased with increasing enzyme loading. However, there would be no further increase in the sensitivity of the biosensor when the LOx immobilisation concentration reached 1.3% w/v. As a result, 1.3% w/v LOx was chosen as the optimum enzyme immobilisation solution for our biosensor.

3.3. Effect of pH

The pH effect was investigated over the range pH 4.57–10.0. Fig. 2 shows the normalised response sensitivity of the biosensor against pH when the biosensor was subject to 0.04–0.14 mM L-lactate standards at various pH phosphate buffer solutions. The results show that the optimal pH values are about 6.50–9.00 and the sensitivity can be maintained at or above 90% for this pH range. The L-lactate biosensor is irreversibly deactivated at pH < 4.5. Since the p*I* of LOx is 4.6 (Mizutani et al., 1995), LOx will mainly exist as cations at pH < 4.5, it will no longer electrostatically bind with the cationic chitosan. As a result, LOx will be dissolved and washed away by solution with pH < 4.5. In brief, the biosensor can function satisfactorily over the pH range from 6.50 to 9.00 and has a relatively broad pH working range.

3.4. Effect of KCl concentration

The effect of KCl concentration on the response sensitivity of the biosensor was investigated by subjecting the biosensor to 0.04–0.14 mM L-lactate standards containing 0.0–0.50 M KCl at pH 7.5. The sensitivity at 0.10 M KCl was 4% higher than without any KCl. Although the sensitivity was slightly higher at low salt concentration, the biosensor was virtually insensitive to salt concentrations up to 0.50 M. Increasing the salt concentration from 0.10 to 0.50 M decreased the sensitivity by 4%. The response sensitivity of the biosensor was relatively constant throughout the range of 0.0–0.50 mM and its performance was not affected much by the salt concentration.

3.5. Effect of dissolved oxygen concentration

In this study, the response sensitivity of the biosensor was investigated by the variation of the partial pressure of oxygen on L-lactate solutions. L-Lactate standard solutions (0.04–0.14 mM) were subject to various oxygen gases (6.0–35.0% v/v) and the response sensitivity were then determined as displayed in Fig. 3. When the dissolved oxygen concentration was lower than 0.2 mM, the sensitivity decreased substantially with the decrease in dissolved oxygen concentration. This can be explained by the fact that the enzymatic reaction requires oxygen to quantitatively convert L-lactate to H_2O_2 and pyruvate. On decreasing the dissolved oxygen

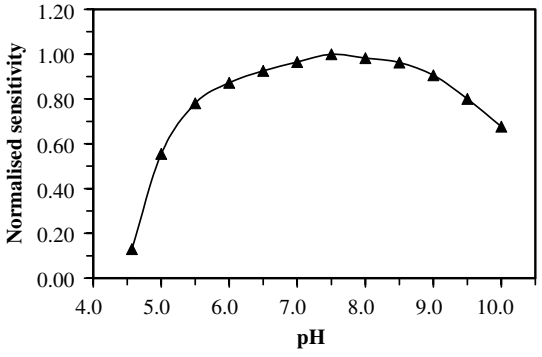

Fig. 2. Effect of pH on the normalised response sensitivity of the L-lactate biosensor upon exposure to 0.04–0.14 mM L-lactate at various pH phosphate buffers.

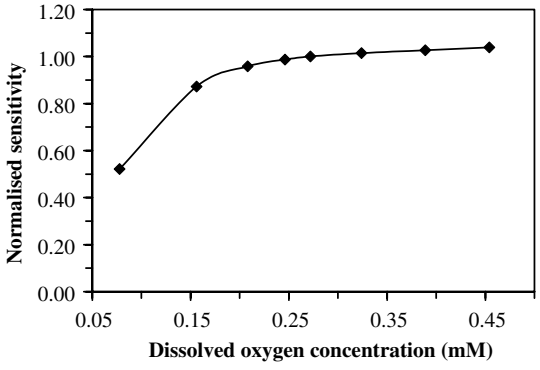

Fig. 3. Effect of dissolved oxygen content on the normalised response sensitivity of the L-lactate biosensor upon exposure to 0.04–0.14 mM L-lactate at pH 7.50 pre-saturated with various oxygen gases.

content, the oxygen supply required for enzyme regeneration will be increasingly important and become the rate-determining step for the enzymatic reaction under low dissolved oxygen environment. As a result, the sensitivity of the biosensor is limited by an oxygen deficit. When the dissolved oxygen concentration was higher than 0.20 mM, the sensitivity of the biosensor only slightly increased with the increase in dissolved oxygen concentration. Since the dissolved oxygen concentration for an air-saturated aqueous solution is 0.272 mM at 22 °C (Pasco, 1997), a variation of 27% of the dissolved oxygen content for an air-saturated aqueous sample solution would not introduce a significant effect on the sensitivity of detection.

3.6. Effect of temperature

The analytical performance of enzyme-immobilised eggshell membrane is anticipated to be temperature dependent. Higher temperature would result in a drop in lifetime of the L-lactate biosensor. On the other hand, increasing working temperatures have a counterbalance effect on the biosensor. The activity of an immobilised-enzyme is governed by the kinetics of the enzymatic reaction. The reaction rate is faster with the increase in working temperature. The response sensitivity for the L-lactate biosensor on exposure to 0.04–0.14 mM L-lactate at various temperatures was thus studied. There was a significant increase in the rate of the reaction from 10 °C to room temperature. The sensitivity increased with the increase in temperature and reached the highest value at 35 °C. Even though the dissolved oxygen concentrations in solutions are lower at higher temperatures, the immobilised-enzyme can still acquire higher activities at higher temperatures and subsequently consume oxygen at a faster rate in the enzymatic reaction of L-lactate. The sensitivity decreased at temperature above 35 °C due to the possible denature of the enzyme. Although the rate of most enzymatic reactions increases with temperature, if the temperature gets too high the reaction will not proceed. At elevated temperatures, the tertiary structure of the protein is disrupted and the enzyme essentially unravels and is not active (Johnson, 2002). Thus, for practical reasons room temperature is recommended to prolong the lifetime of the biosensor.

3.7. Shelf-life of L-lactate biosensor

The operation stability of the L-lactate biosensor has been tested by measuring the response sensitivity to 0.04–0.14 mM L-lactate solutions at room temperature. The sensitivity did not change appreciably during an 8-h period of continuous use. The shelf-lives of the biosensors were tested over 50- and 380-day periods at 22 and 4 °C, respectively. When the LOx-immobilised eggshell membrane was stored in a refrigerator at 4 °C and measured intermittently, the response sensitivity dropped to 85% of its initial value over the first 15 days and remained above 80% over a year. By contrast, when the LOx-immobilised eggshell membrane was kept at 22 °C, the sensitivity dropped dramatically over the first 10 days and finally the biosensor lost completely its sensitivity to L-lactate after 25-day of storage. Thus, the shelf-life of the biosensor can be prolonged if it is kept at 4 °C. The excellent shelf-life of the L-lactate biosensor consisting of LOx–chitosan immobilised on eggshell membrane kept at low temperature is possibly related to the biological compatibility of chitosan and eggshell membrane with the enzyme. Eggshell membrane and chitosan are mainly composed of biopolymers which can supply polycations to stabilise the enzyme (Heller & Heller, 1998; Wei et al., 2003).

3.8. Response time, repeatability and reversibility of L-lactate biosensor

The L-lactate biosensor can continuously monitor the L-lactate contents in aqueous solutions. The precision of the biosensor was evaluated at 0.10 mM L-lactate and the relative standard deviation ($n = 10$) was 5%. The t_{95} response time of the biosensor was 40–60 s on going from air-saturated buffer to 0.10 mM L-lactate and the recovery time was 80–90 s on going from 0.10 mM L-lactate to air-saturated buffer. The response of the biosensor to L-lactate is reversible and fast.

Table 1
Effect of potential interferants on the L-lactate biosensor

Interferant	Concentration (mM)	Signal change
D-Galactose	0.100	No
D-Glucose	0.100	No
D-Fructose	0.100	No
D-Lactose	0.100	No
Sucrose	0.100	No
Sodium benzoate	0.100	No
Sodium citrate	0.100	No
Sodium salicylate	0.100	No
Potassium sorbate	0.100	No
Aspartame	0.100	No
Saccharin sodium salt	0.100	No
Sodium cyclamate	0.100	No
4-Acetamidophenol	0.100	No
Uric acid	0.100	No
L-Ascorbic acid	0.100	Slight signal drift
L-Cysteine hydrochloride	0.100	Slight signal drift
Bovine serum albumin	0.500[a]	No
Casein	0.050[a]	No
Amarath	0.100	No
Brillant green	0.100	No
Orange II	0.100	No
Tartrazine	0.100	No

[a] In % w/w.

Table 2
Results of L-lactate analysis and the recovery test on various real samples

Sample	L-lactate content[a] (mM)	RSD (%)	L-lactate added (mM)	Recovery[a] (%)	RSD (%)
Pasteruised whole milk	0.111	1.04	0.100	103	5.31
Pasteruised low fat milk	0.119	6.19	0.100	107	1.62
UHT whole milk	0.545	2.75	0.500	105	2.03
Evaporated full cream milk	0.872	1.43	0.500	106	1.08
Yakult (live *lactobacillus* drink)	56.59	2.40	50.00	95.7	2.10
Natural yoghurt	4.679[b]	1.77	5.000[b]	101	5.20
Strawberry fruit yoghurt	5.924[b]	0.84	5.000[b]	103	2.95
Strawberry low fat fruit yoghurt	7.051[b]	1.11	5.000[b]	103	5.12
Serum 1	1.464	6.68	1.500	102	1.66
Serum 2	1.502	5.05	1.500	95.9	3.98
Serum 3	1.615	1.92	1.500	100	2.50

[a] Average values are calculated on three replicates.
[b] In mg g^{-1}.

3.9. Interference study

The effect of potential interferants on the signal change of the L-lactate biosensor was evaluated by exposing the biosensor to interferants in 0.10 M phosphate buffer at pH 7.50. The results are given in Table 1. It is found that most potential interferants did not give any significant interference on the response of the L-lactate biosensor. It is noteworthy to mention that bovine serum albumin and casein, which often cause interference on real food sample analysis (Wei et al., 2003), did not have any effect on our L-lactate biosensor. Common interferants such as 4-acetamidophenol and uric acid for amperometric L-lactate biosensors did not produce any effect either on our biosensor. However, L-ascorbic acid and L-cysteine could produce slight interference as they slowly consume the dissolved oxygen in the solution. Fortunately, this oxidation process is very slow since the drift of the baseline signal is only about 5% h^{-1}. As the response time of the biosensor is 40–60 s, measurement can be taken within a minute to minimise the effect from L-ascorbic acid or L-cysteine. Alternatively, this interference could be reduced to nearly zero if the test solutions are air-saturated for 2.5 h before measurement. In conclusion, most substances often found in dairy products and serum samples do not exhibit significant interference on the determination of L-lactate using our proposed biosensor.

3.10. Sample analysis

The L-lactate concentrations of some commercial milk, yoghurt and human serum samples were also determined by the L-lactate biosensor. The samples were either undiluted or directly diluted by a phosphate buffer at pH 7.50 to yield testing sample solutions for the biosensor. The signal change of the sample solution was then measured and compared with that of a set of L-lactate standard solutions. The results are displayed in Table 2. All the samples contained various concentrations of L-lactate. Among these samples, yoghurt and Yakult had the highest contents of L-lactate. The L-lactate concentrations in human sera determined by the L-lactate biosensor were in good agreement with the findings in the literature (Mizutani et al., 1995; Perdomo et al., 2000). The results demonstrate that the L-lactate biosensor offers a convenient and simple method for determination of L-lactate in real samples. The recovery tests for L-lactate were performed by adding various known amounts of L-lactate in the sample solutions. The amounts of added L-lactate were then evaluated by using the proposed L-lactate biosensor. All sample solutions were air-saturated before testing. The results of the recovery of the samples are summarised in Table 2. The recovery tests demonstrate that the L-lactate biosensor offers an excellent, accurate and precise method for determination of L-lactate in dairy products and serum samples.

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