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*Research Article*

# Determination of Different Saccharides Concentration by Means of a Multienzymes Amperometric Biosensor

**Marianna Portaccio and Maria Lepore**

*Dipartimento di Medicina Sperimentale, Università della Campania "Luigi Vanvitelli", Via S.M. di Costantinopoli, 16-80134 Napoli, Italy*

Correspondence should be addressed to Maria Lepore; maria.lepore@unicampania.it

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A three-electrode amperometric biosensor for the detection of two different saccharides (lactose and glucose) in aqueous solutions is described. On the graphite working electrode, the glucose oxidase (GOD) and $\beta$-galactosidase ($\beta$-gal) were coimmobilized by means of covalent bonding. The response of the biosensor as a function of the relative concentration of the two immobilized enzymes was investigated and the best working conditions were identified by measuring the sensitivity and the linear range response. In particular, our best lactose biosensor shows a linear range up to 0.010 mM and a limit of detection (LOD) and a sensitivity equal to 0.001 mM and 850 ± 81 $\mu$A/mM, respectively. For glucose, the values of the above-mentioned parameters are equal to 0.015 mM for the linear range, 0.001 mM for LOD, and 505 ± 55 $\mu$A/mM for the sensitivity. The working parameters of our biosensors are significant in comparison with other biosensors developed for concentration determination of the two saccharides investigated in the present work. In particular, low (LOD) and high sensitivities are obtained for lactose and glucose. To challenge our biosensor with real samples, it was tested using fruit juices, skim milk, and whey.

## 1. Introduction

Saccharides play a relevant role in the food industry and the research for the development of reliable and low-cost biosensors is always very active [1]. Lactose and glucose are two of the most important saccharides and the determination of their concentration is very important for different reasons. In particular, lactose is a disaccharide that consists of one molecule of galactose and one of glucose bonded through a $\beta$-1→4 glycosidic linkage. It is present in milk in a percentage varying from 2 to 8% (by weight) depending on different species and individuals [2]. In industry, the quantitative determination of lactose in milk and in dairy products is mandatory because lactose content is a fundamental parameter for evaluating milk quality [3, 4]. Moreover, in the last years, the determination of this parameter in foods has acquired a significant role since many individuals manifest lactose intolerance and therefore cannot consume milk and dairy products without suffering and gastrointestinal disorders [5]. This intolerance is related to lack of the required enzyme lactase ($\beta$-galactosidase) in the digestive system that inhibits the metabolization of lactose into galactose and glucose [6]. Then the determination of lactose concentration is important for industry and for medical applications.

As far as glucose is concerned, it is a monosaccharide and it is the simplest form of sugar that can be absorbed into the bloodstream. It is naturally present in foods such as grains, fruits, and vegetables and it is also one of the major ingredients in many sweeteners and processed foods. The control of glucose level especially in soft drinks is nowadays mandatory due to their role in caloric overconsumption and body weight gain [7].

Various methods have been used for determining the concentration of these two saccharides such as spectrophotometry [8, 9], polarimetry [10], high performance liquid chromatography [11], and infrared spectroscopy [12]. All the above-mentioned techniques are time-consuming, expensive, and they require sample pretreatment and considerable technical skills. Therefore, enzyme-based biosensors can offer a rapid, simple, and robust method to quantify lactose

and glucose in food. These devices are particularly useful since they combine the high specificity of the enzymes with the versatility of the transducer. In the last years, different types of enzymatic biosensors for lactose [13–19] and glucose [4, 20–24] determination have been developed for application in food industry, but few of them have been reported for the determination of both sugars. One of the first similar devices used different enzymes (invertase, $\beta$-galactosidase, amyloglucosidase, mutarotase, and glucose oxidase) covalently immobilized on a cellulose membrane [25]. Lactose and glucose concentrations were also simultaneously determined by using a measuring cell containing lactose and glucose electrodes made by mixing galactosidase/glucose oxidase and glucose oxidase, respectively, with carbon paste [26]. Another biosensor sensitive to glucose and lactose was developed using coimmobilized ferrocene, glucose oxidase, $\beta$-galactosidase, and mutarotase on $\beta$-polymer. The ferrocene was entrapped in the $\beta$-cyclodextrin polymer and glucose oxidase, $\beta$-galactosidase, and mutarotase were cross-linked with the $\beta$-cyclodextrin polymer. In this case, cyclic voltammetry and amperometric measurements were employed in order to show the efficacy of electron transfer between immobilized glucose oxidase and a glassy carbon electrode via ferrocene included in the cavities of the $\beta$-cyclodextrin polymer [27]. More recently, Soldatkin et al. [28] reported the development of an array of biosensors for the simultaneous determination of four carbohydrates in solution. Several enzyme systems selective to lactose, maltose, sucrose, and glucose were immobilized on the surface of four conductometric transducers and served as biorecognition elements of the biosensor array. The main working characteristics of these devices are reported in Table 1.

In the present work, we discuss the functioning of an amperometric biosensor based on the use of $\beta$-galactosidase ($\beta$-gal) and glucose oxidase (GOD) that has been prepared for lactose and glucose concentration determination in aqueous solution. The immobilization of enzymes on graphite electrodes has been carried out by means of covalent bonding [29]. The response of the biosensors as a function of the relative concentration of the two immobilized enzymes has been investigated and the best working conditions have been identified by measuring the linear range response and sensitivity of each prepared biosensor. The working parameters of the prepared devices have been compared with the performances of other similar biosensors reported in the literature. This comparison has confirmed the effectiveness of multienzymes biosensors for lactose and glucose concentration determination. The proposed biosensor has been tested with real samples using fruit juices, skim milk, and whey.

## 2. Experimental

*2.1. Materials.* In this work, glucose oxidase (GOD) (E.C. 1.1.3.4) from *Aspergillus niger* (145 $Umg^{-1}$) and $\beta$-galactosidase ($\beta$-gal) (E.C. 3.2.1.23) from *Aspergillus oryzae* (>8 $Umg^{-1}$) were used.

Nitric acid, N,N′-dicyclohexylcarbodiimide, hexamethylenediamine (HMDA), and glutaraldehyde (GA) were employed for the process of enzyme immobilization. In particular, HMDA and GA were used as the spacer and coupling agents, respectively, while the carbodiimide was used as the activator of the carboxylic groups produced on the graphite electrode surface.

Graphite rods (4 mm in diameter) were purchased from Agar Scientific (Agar Scientific Limited, 66a, Cambridge Road Stansted, Essex CM24 8DA, England). The platinum and the Ag/AgCl electrodes were purchased from Radiometer Analytical (Radiometer Analytical SAS, Villeurbanne Cedex, Lyon, France).

In order to test the real functioning of our biosensor, we used four real samples of the food chain to compare the saccharides concentrations measured with our biosensor with those obtained by using Sigma colorimetric assay kits (Catalogue Number MAK011 for lactose determination and Product Code GAHK-20 for glucose determination). The used real samples were pineapple juice, orange juice, skim milk, and whey. The tests were performed in triplicate on opportunely diluted samples, and the results were presented as the mean and standard deviation.

All chemicals, including the enzyme, were purchased from Sigma (Sigma, Milan, Italy) and used without further purification.

*2.2. Apparatus.* The electrochemical cell (Figure 1(a)) was a three-electrode cell where the enzyme modified graphite electrode acted as a working electrode and the platinum electrode (type M241Pt) as a counter electrode. All measurements were carried out versus an Ag/AgCl reference electrode (type REF321). A +700 mV potential drift between the electrodes was ensured by means of a low current potentiostat/galvanostat (model 2059 from Amel, Milan, Italy) interfaced to a PC through a board (PCI-6221) purchased from National Instruments Corporation (National Instruments, Austin, TX, USA). Electric current measurements were performed by means of a flow injection analysis (FIA) system, also shown in Figure 1(a). A continuous flow of carriers (the washing buffer solution: 0.1 M sodium acetate, pH 5.0; $T = 25^{\circ}$C) or of the mixture containing the substrate was injected through the electroanalytic cell under the control of an electrovalve (RS Components s.p.a., Cinisello Balsamo, Milan, Italy). The injected volume was 200 $\mu$L and the electrical response, which constituted the output signal from the biosensor, was acquired using the Labview software package (National Instruments, Austin, TX, USA). The software accounted for the values of the background current, which was continuously subtracted from the subsequent value of the measurement. The electrical current was produced by the oxidation of the substrate by the immobilized enzyme according to the following reaction scheme:

$$\text{lactose} + H_2O \xrightarrow{\beta\text{-gal}} \beta\text{-D-glucose} + \beta\text{-D-galactose} \quad (1)$$

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{GOD}} \text{D-glucone-1,5-lactone} + H_2O_2 \quad (2)$$

Table 1: Working parameters of biosensors for concentration determination of lactose and glucose. The asterisk means that the numerical values of sensitivity were evaluated from published linear calibration. n.a. stands for not available.

| Immobilization support & method | Substrate | Linear range (mM) | LOD (mM) | Sensitivity | Stability | References |
|---|---|---|---|---|---|---|
| Triacetyl cellulose membranes | Lactose<br>Glucose | Up to 180<br>Up to 5 | n.a.<br>n.a. | $\sim 4\ 10^{-3}$ $\mu$A/mM*<br>n.a. | More than 10 days | [25] |
| Carbon paste | Lactose<br>Glucose | Up to 2.5<br>Up to 2.5 | 0.1<br>0.05 | $5.6\ 10^{-2}$ $\mu$A/mM<br>n.a. | More than 10 days | [26] |
| $\beta$-Cyclodextrin polymer | Lactose<br>Glucose | Up to 4<br>n.a. | n.a.<br>n.a. | $\sim 4\ 10^{-1}$ $\mu$A/mM*<br>n.a | Two months | [27] |
| Conductometric transducer using gold electrodes | Lactose<br>Glucose | Up to 1–3<br>Up to 1–3 | 0.001<br>0.001 | $\sim 12$ $\mu$S/mM*<br>n.a. | Four months | [28] |
| Graphite electrode | Lactose<br>Glucose | Up to $1.0\ 10^{-2}$<br>Up to $1.5\ 10^{-2}$ | 0.001<br>0.001 | $850 \pm 81$ $\mu$A/mM<br>$505 \pm 55$ $\mu$A/mM | Two months | Present work |

The hydrogen peroxide from reaction (2) was oxidized at the anode and generated a current proportional to the lactose concentration:

$$H_2O_2 \xrightarrow{700\,\text{mV}} O_2 + 2H^+ + 2e^- \quad (3)$$

The electric pathway in the production of the biosensor response is reported in Figure 1(b). A typical electrical response is illustrated in Figure 1(c), where it is possible to note the reproducibility of the measurements and the small "response time" of the biosensor. The $I_{\text{peak}}$ value was used as current response signal for different substrate concentrations.

*2.3. Graphite Electrode Preparation.* The enzyme immobilization was carried out on the electrode using a covalent bond [29]. A spacer (HMDA) was used to bind the enzyme to the functional carboxylic groups induced on the graphite electrode with nitric acid. The enzyme immobilization phase for all electrode types was preceded by a cleaning phase of the electrode surface using gamma alumina powder, after which the electrode was washed and sonicated in 5% (v/v) ethanol aqueous solution. The carboxylic groups on the electrode were obtained by treating the graphite electrode for 24 h with an aqueous solution of nitric acid (20% v/v). At the end of the treatment, the graphite electrode was washed in deionized water. In particular, the treated electrodes were dipped for 2 h in a 0.1 M sodium acetate buffer solution, pH 4.8 and $T = 60^\circ$C, containing 14 mg/mL of carbodiimide. At the end of this step, the graphite electrodes were dipped for 30 min at room temperature in an aqueous solution of HMDA (5% v/v) and then washed in deionized water for 1 min. After this treatment, the electrode was dipped for 30 min at room temperature in a 10% (v/v) GA aqueous solution containing different quantities of two enzymes. A large series of electrodes were prepared and tested in order to establish the relative quantities of GOD and $\beta$-gal enzymes able to give the best results in terms of sensitivity and stability. The whole process of enzyme immobilization is shown in Figure 1(d).

*2.4. Electrode Stability.* Electrode stability was tested daily by measuring the electrical response under standard conditions: 0.08 mM lactose, pH 6.5, and $T = 25^\circ$C. After a 2-day period, during which the response decreased by 20% with respect to the first set of measurements, fairly stable conditions were obtained. All the experiments reported in the following section were carried out under similarly stable conditions. The electrodes were discarded when the daily measure values differed by 10% from the standard reference measure. When not in use, the biosensors were stored in 0.1 M sodium phosphate buffer, pH 6.5 at 4°C.

*2.5. Determination of Biosensor Working Parameters.* As well known, the electrical response of an amperometric biosensor is well described by Michaelis–Menten equation:

$$I_{\text{peak},C} = \frac{I_{\text{peak,max}} C}{K_{m,\text{el}}^{\text{app}} + C}, \quad (4)$$

where $I_{\text{peak},C}$ is the peak current at the substrate concentration $C$, $I_{\text{peak,max}}$ is the peak current at saturating substrate conditions, and $K_{m,\text{el}}^{\text{app}}$ is a constant with the same meaning as the Michaelis–Menten constant $K_m^{\text{app}}$. The apex "app" was used to differentiate this value from that of the free enzyme. Indeed, when an enzyme is immobilized, the kinetic constant $K_m$ changes owing to the diffusional limitations introduced by the graft carrier. $K_{m,\text{el}}^{\text{app}}$ and $I_{\text{peak,max}}$ are known as electrokinetic parameters and can be used for quantifying the biosensor's performances. Moreover from Michaelis–Menten curve, it is possible to evaluate the linear calibration range and the sensitivity can be estimated as the slope of the calibration curve.

*2.6. Treatment of the Experimental Data.* Each experimental point in the figures is the average of six independent measurements performed under the same experimental conditions. The experimental error never exceeded 5%.

## 3. Results and Discussion

The first part of the experimental work concerned the quantitative determination of the relative quantities of GOD and $\beta$-gal enzyme that enabled us to obtain the best sensitivity

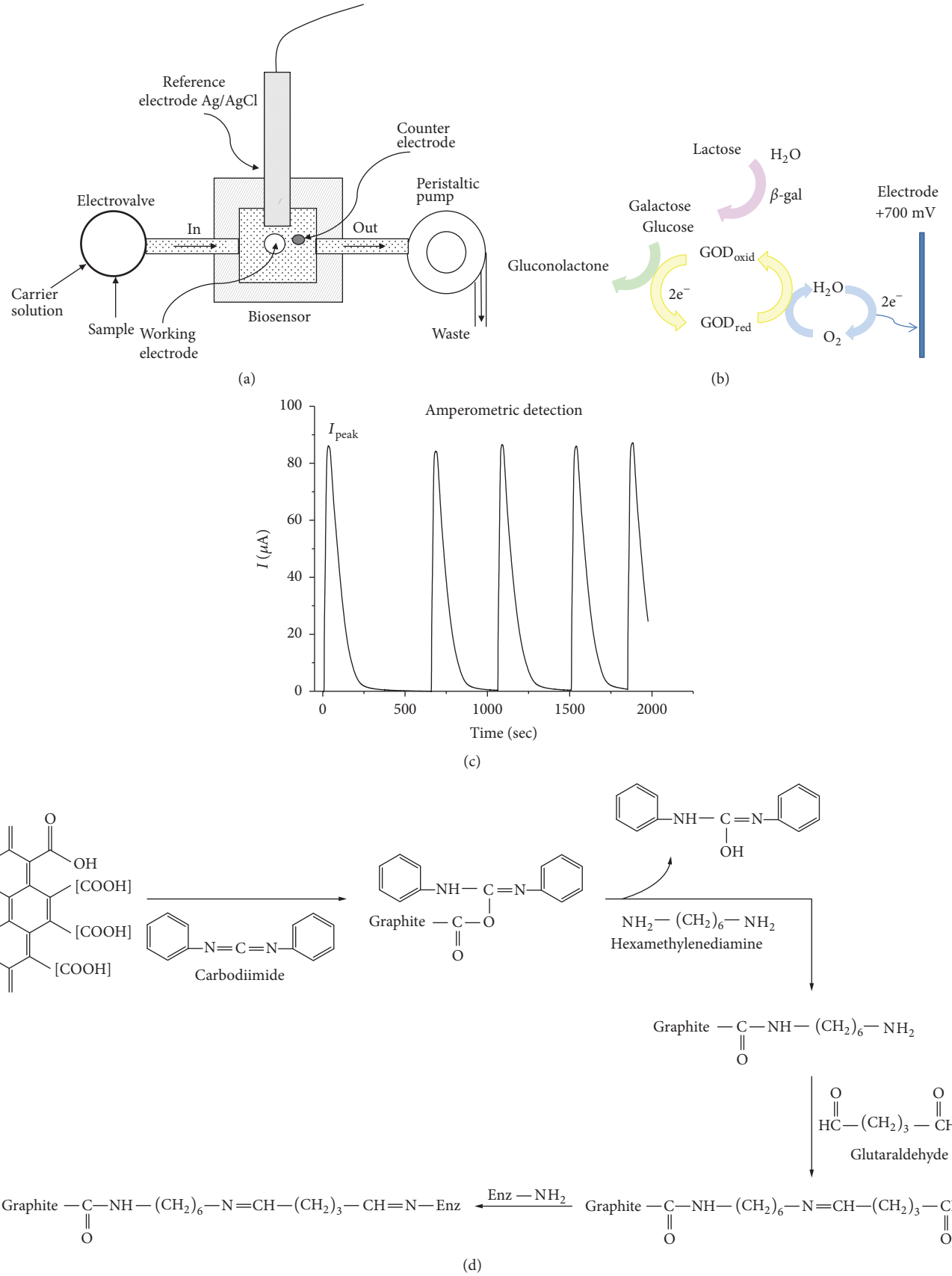


FIGURE 1: (a) Representation of the electrolytic cell and FIA system; (b) electric pathway for the production of biosensor response; (c) a typical signal obtained with a 0.5 mM solution of lactose, at pH 6.5 and $T = 25^{\circ}$C, with an electrode prepared with $\beta$-gal = 6 mg/ml and GOD = 2 mg/ml; (d) schema of the enzyme immobilization process on graphite electrodes.

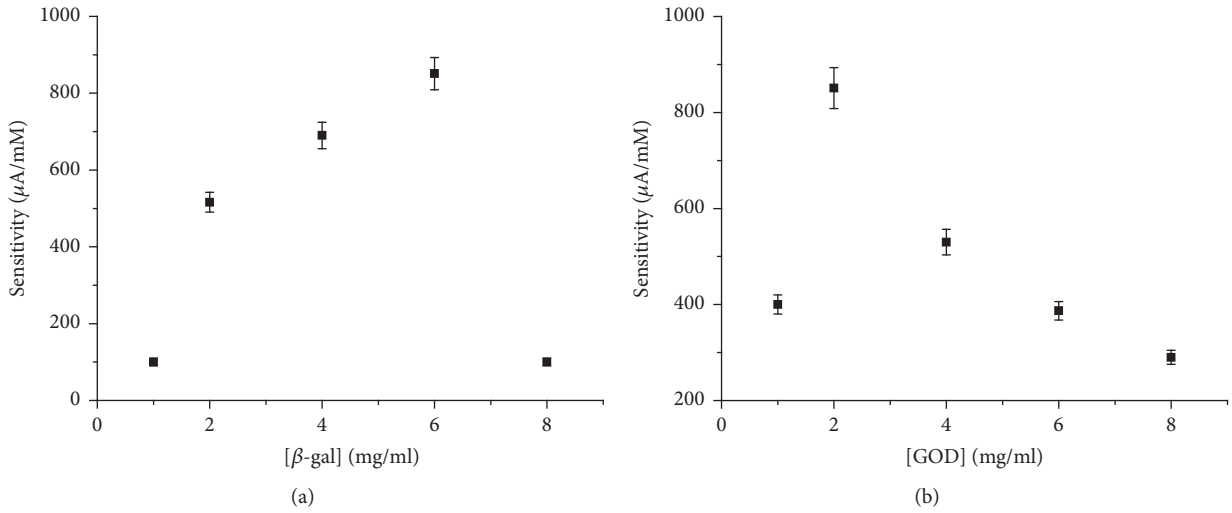


Figure 2: Sensitivity of different electrodes using lactose as substrate; (a) the electrodes were prepared with fixed GOD concentration (2 mg/ml) and varying $\beta$-gal concentrations; (b) the electrodes were prepared with fixed $\beta$-gal concentration (6 mg/ml) and varying GOD concentrations, [lactose] = 0.5 mM and pH = 6.5.

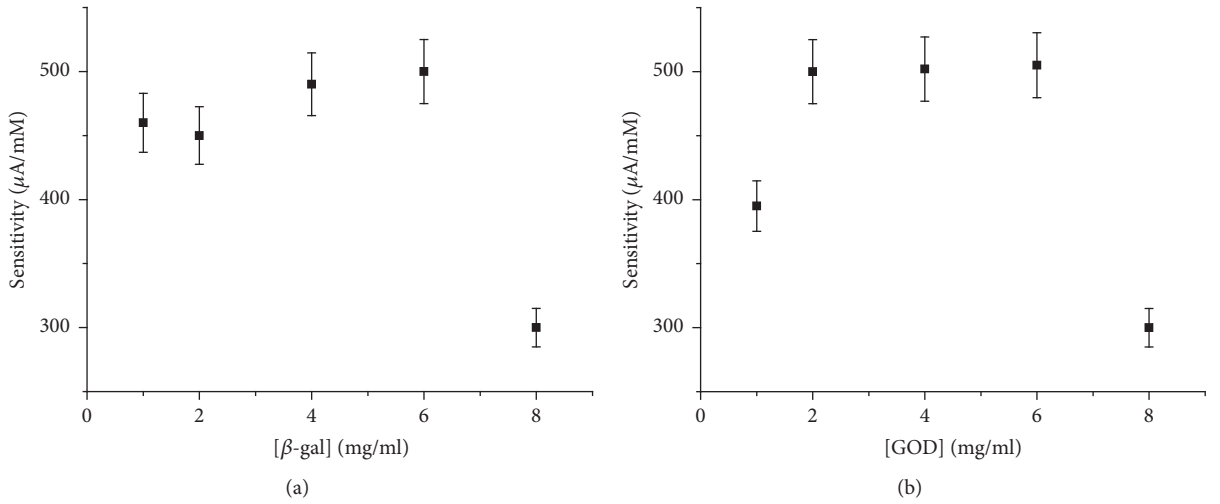


Figure 3: Sensitivity of different electrodes using glucose as substrate; (a) the electrodes were prepared with fixed GOD concentration (2 mg/ml) and varying $\beta$-gal concentrations; (b) the electrodes were prepared with fixed $\beta$-gal concentration (6 mg/ml) and varying GOD concentrations, [glucose] = 0.5 mM and pH = 5.5.

and stability. For this purpose, different electrodes were prepared using different amounts of GOD and $\beta$-gal. In particular, the amounts of two enzymes varied in the range 2–8 mg/ml. For each of them, the linear range calibration curves were measured and the different sensitivities were determined from their slopes. In Figures 2(a) and 2(b), the results of this series of measurements are reported using lactose as substrate. In particular, in Figure 2(a), the sensitivities for electrodes prepared with a fixed amount of GOD (2 mg/ml) and varying amounts of $\beta$-gal are reported. The values of sensitivity range in the 150–850 $\mu$A/mM interval and the highest sensitivity is obtained for the electrodes prepared using 2 mg/ml of GOD and 6 mg/ml of $\beta$-gal. In Figure 2(b), the sensitivities for electrodes prepared with a fixed amount of $\beta$-gal (6 mg/ml) and varying amounts of GOD are reported. In this case, the sensitivities range between 380 and 850 $\mu$A/mM. These results show that the highest sensitivity is still obtained for the electrode prepared with 2 mg/ml of GOD and 6 mg/ml of $\beta$-gal. In Figures 3(a) and 3(b), the results of similar measurements are reported using glucose as substrate. In this series of experiments, when electrodes were prepared using a fixed amount of GOD (2 mg/ml) and varying amounts of $\beta$-gal (Figure 3(a)), the sensitivities range in the 300–500 $\mu$A/mM interval and the highest sensitivity (460) is still obtained for the electrode prepared with 2 mg/ml of GOD and 6 mg/ml of $\beta$-gal. In Figure 3(b), sensitivities for electrodes prepared with a fixed amount of $\beta$-gal (6 mg/ml) and varying amounts of GOD are reported. In this case, the sensitivities range between 300 and 500 $\mu$A/mM. Sensitivity values around 500 $\mu$A/mM are

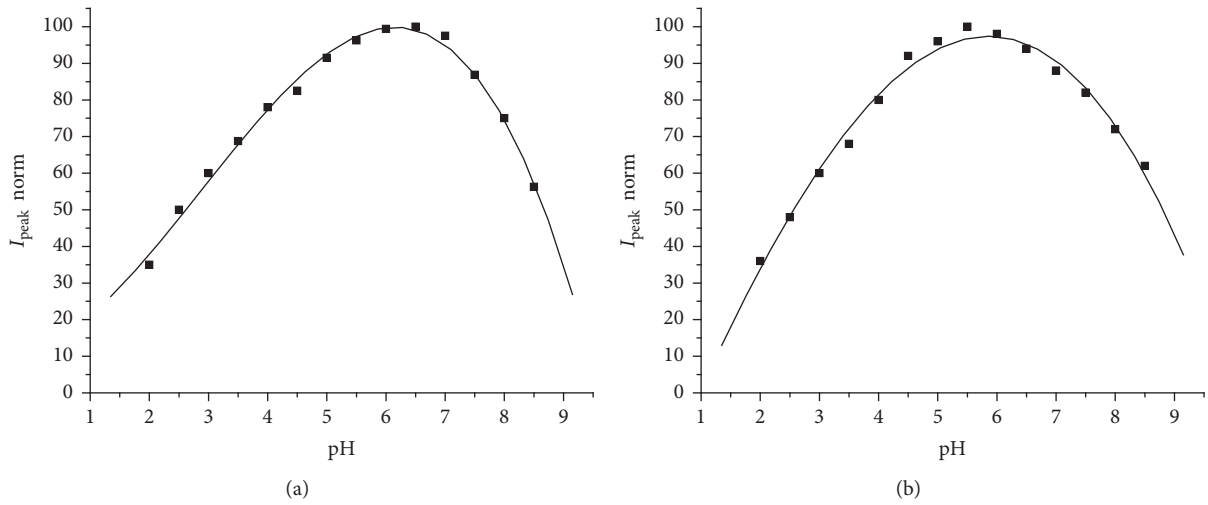


FIGURE 4: Relative $I_{peak,norm}$ values as a function of pH for best working electrode; (a) [lactose] = 0.5 mM and $T = 25^{\circ}$; (b) [glucose] = 0.5 mM and $T = 25^{\circ}$.

obtained for electrodes prepared with 2, 4, and 6 mg/ml of GOD and assuming a fixed 6 mg/ml of $\beta$-gal. These results confirm that the highest sensitivity for glucose is obtained for an electrode prepared with 2 mg/ml of GOD and 6 mg/ml of $\beta$-gal. Hence, the same kind of electrode that ensures the highest sensitivity for lactose, it is able to work well using glucose as substrate, even though a lower sensitivity is obtained.

After this step, the pH solution dependence of enzyme reaction was investigated. Since an enzyme reaction is strongly dependent on the pH of the solution, especially when the catalyst is immobilized [30–34], the biosensor response was studied at different values of the pH in the range from 3.5 to 6.5. In Figures 4(a) and 4(b), the results of this investigation are reported as the relative values of $I_{peak}$ as a function of pH for lactose and glucose, respectively. The glucose and lactose concentration was 0.5 mM and the temperature 25°C. The results in the figure clearly show that the best electrical response occurs at pH 6.5 for lactose and at pH 5.5 for glucose. The width of the bell-shaped response curves depends on the analyte.

Once it is established that the optimum value of the peak current for our biosensor occurs at pH 6.5 for lactose, experiments were conducted under these conditions in order to obtain calibration curves for this saccharide. The results of the experiment are shown in Figure 5, where the peak currents are reported as a function of lactose concentration. In the (a) inset, the signal obtained for a 0.5 mM lactose concentration is shown. The experimental data clearly indicate that the electrical response of biosensor resembles Michaelis–Menten behavior. The electrokinetic parameters $K^{app}_{m,el}$ and $I_{peak,max}$ for this biosensor are equal to 0.108 ± 0.004 mM and to 96.12 ± 1.28 $\mu$A, respectively. The (b) inset represents the linear range of the electrical response, that is, the calibration curve. A linear range up to 0.010 mM is evidenced. In addition, the developed biosensor shows a sensitivity equal to 850 ± 81 $\mu$A/mM and a limit of detection (LOD) equal to 0.001 mM. LOD is evaluated as signal-to-noise ratio equal to 3.

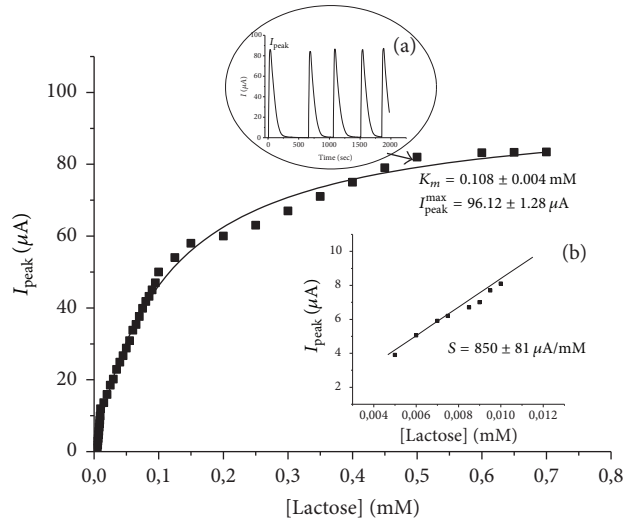


FIGURE 5: Trend of peak current value as a function of the concentration of lactose; (a) inset: biosensor response signal for a lactose concentration equal to 0.45 mM; (b) inset: linear range of the biosensor response.

In Figure 6, the peak currents are reported as a function of glucose concentration at pH equal to 5.5. Also in this case, the electrical response of biosensor resembles Michaelis–Menten behavior. The (a) inset shows the signal obtained for a 0.5 mM glucose concentration. The electrokinetic parameters $K^{app}_{m,el}$ and $I_{peak,max}$ for this biosensor are equal to 0.123 ± 0.009 mM and to 55.66 ± 1.37 $\mu$A, respectively. A linear range up to 0.015 mM is evidenced in the (b) inset. In addition, the developed biosensor has a sensitivity equal to 505 ± 55 $\mu$A/mM and a LOD still equal to 0.001 mM.

The small $K^{app}_{m,el}$ values obtained for the two substrates indicate that there is a good affinity between the immobilized enzymes and the investigated saccharides and there are no significant limitations for substrate diffusion due to the immobilization process.

Table 2: Comparison ofglucose or lactose concentrations in real samples as obtained using our biosensor and commercial kits.

| Beverage | Glucose (mM) | | Lactose (mM) | |
|---|---|---|---|---|
| | Results with our biosensor | Results with the commercial kit | Results with our biosensor | Results with the commercial kit |
| Pineapple juice | 118 ± 19 | 123 ± 7 | | |
| Orange juice | 106 ± 12 | 110 ± 8 | | |
| Skim milk | | | 132 ± 15 | 130 ± 10 |
| Whey | | | 123 ± 13 | 125 ± 8 |

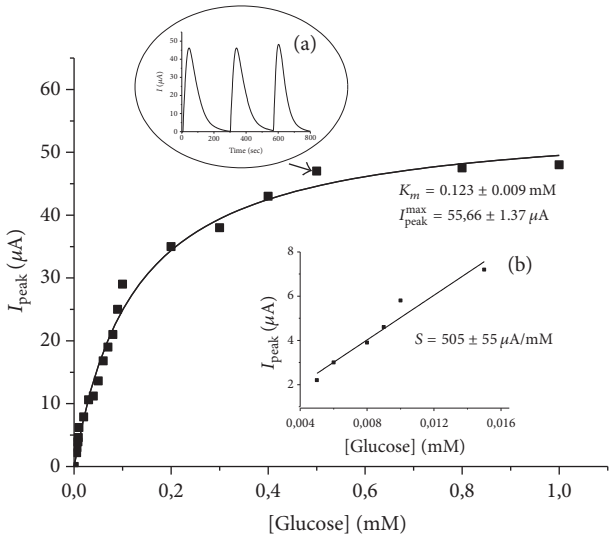


Figure 6: Trend of peak current value as a function of the concentration of glucose; (a) inset: biosensor response signal for a glucose concentration equal to 0.5 mM; (b) inset: linear range of the biosensor response.

All the parameters characteristic of our biosensor are reported in Table 1 together with the ones of other biosensors reported in the literature for the measurements of lactose and glucose. As can be seen, other biosensors [25–28] are characterized by a linear range larger than the one obtained by our biosensor, but its sensitivity is much higher than the others reported in the literature [25–28]. As far as LOD is concerned, it reaches very significant values for both the investigated saccharides. Also the time stability is similar to the one reported for other biosensors.

As far as real samples are concerned, the results of their measurements are reported in Table 2. As is evident, the concentrations of saccharides measured with our biosensor are in good agreement with those obtained using commercial kits. These results demonstrate that the proposed biosensor may be used in measuring glucose or lactose contents in real food samples.

## 4. Conclusions

In the present paper, the working parameters of a three-electrode amperometric biosensor for the detection of two different saccharides (lactose and glucose) have been investigated. Two different enzymes (GOD and $\beta$-gal) were coimmobilized on the working electrode. The best working conditions have been identified by measuring the sensitivity and the linear range response. The working parameters of our biosensors are significant in comparison with the ones of other biosensors developed for concentration determination of the two saccharides investigated in the present work. In particular, low limit of detection and high sensitivities are obtained for lactose and glucose. Our biosensor has been also successfully tested with real samples using different food samples.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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