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An Integrated Amperometric Biosensor for the Determination of Lactose in Milk and Dairy Products

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An integrated amperometric biosensor for the determination of lactose is reported. The bioelectrode design is based on the use of a 3-mercaptopropionic acid (MPA) self-assembled monolayer (SAM)-modified gold electrode on which the enzymes β-galactosidase (β-Gal), glucose oxidase (GOD), peroxidase (HRP) and the mediator tetrathiafulvalene (TTF) are coimmobilized by a dialysis membrane. β-Gal catalyzes the hydrolysis of lactose, and the produced glucose is catalytically oxidized to gluconic acid and H_2O_2, which is reduced in the presence of HRP. This enzyme reaction is mediated by TTF, and the reduction of TTF^+ at 0.00 V (vs Ag/AgCl) gives rise to an amperometric signal proportional to the lactose concentration. The biosensor exhibits a good repeatability of the measurement carried out with the same biosensor, a good reproducibility of the responses obtained with different biosensors and a useful lifetime of 28 days. A linear calibration plot was obtained for lactose over the 1.5×10^{-6} to 1.2×10^{-4} M concentration range, with a limit of detection of 4.6×10^{-7} M. The effect of potential interferents (sucrose, lactulose, fructose, arabinose, maltose, galactose, glucose and uric and ascorbic acids) on the biosensor response was evaluated. Furthermore, the bioelectrode exhibits a suitable performance in flow-injection systems in connection with amperometric detection. The developed biosensor was applied to the determination of lactose in milk and other foodstuffs (chocolate, butter, margarine, yogurt, cheese and mayonnaise), and the results obtained were validated by comparison with those provided by using a commercial enzyme test kit.

KEYWORDS: Self-assembled monolayers; enzyme electrodes; lactose; milk

INTRODUCTION

Lactose is the characteristic carbohydrate of milk and dairy products, its concentration in the milk of healthy cows being approximately 4.1−5.0%. Lactose concentration is a basic marker for the evaluation of milk quality and the detection of abnormalities. For instance, milk from cows suffering mastitis has low lactose levels. In addition, lactose is a basic parameter in wastewater control and veterinary medicine (*1*). The determination of lactose is also clinically relevant. An excessive amount of lactose in blood indicates gastrointestinal malignancy. As lactose must be hydrolyzed by the enzyme lactase to glucose and galactose in order to be absorbed by the body, it is also important to know the relative amount of lactose in milk and its derivatives to prevent "lactose intolerance" (*2*). Therefore, the quantification of lactose is an important challenge in many areas of economical significance. Several methods appear in the literature, including spectrophotometry (*3*), polarimetry (*4*), gravimetry (*5*), infrared spectroscopy (*6*), titrimetry (*7*), enzymatic methods (*8*) and chromatography (*9*), but most of these methods involve long analysis times and expensive instruments and are difficult to automate (*2*, *10*). For these reasons, direct and rapid online monitoring of lactose in milk and dairy products using simple analytical devices with a high performance is still a challenge.

Enzyme-based amperometric biosensors have been found to constitute versatile analytical devices with high selectivity that can be operated by unskilled personnel. Accordingly, they can be envisaged as serious competitors for conventional techniques, representing an attractive alternative for small industries. **Table 1** summarizes the main characteristics of some lactose electrochemical biosensors reported recently in the literature. The enzyme(s) and the redox mediator used as well as the biomolecules' immobilization strategies and the corresponding analytical characteristics are listed in **Table 1**.

Robust integrated amperometric enzyme biosensors making use of 3-mercaptopropionic acid (MPA) self-assembled monolayer modified gold electrodes have been reported by our group for the determination of lactulose in milk, gluconic acid in musts and wines and glycerol in wines (*17*−*19*). In this work, a related approach has been addressed for the construction of a lactose biosensor. The biosensor functioning is based on three coupled enzyme reactions using β-galactosidase, glucose oxidase and horseradish peroxidase. The enzymes are coimmobilized with the redox mediator tetrathiafulvalene (TTF) on a MPA-SAM-modified

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Table 1. Lactose Electrochemical Biosensors Reported in the Literature[a]

electrode	enzyme/s (redox mediator)	immobilization type	E_{app}/V	L.R./M	sensitivity	LOD	stability	reference
Pt	GOD, β-Gal (−)	immobilization with BSA and polyazetidine in cellulose acetate membranes on electrode surface						*11*
field effect transistor	GDH, β-Gal (−)	cross-linking with GA					20 days of continuous measurements	*10*
Ingold potentiometric electrode with membrane permeable to CO_2	β-Gal (−)	physical entrapment with a dialysis membrane		0.0146−0.292			2 months	*12*
GCE	GOD, β-Gal, mutarotase (ferrocene)	cross-linking with GA and a β-cyclodextrin polymer	+0.35 vs Ag/AgCl	50×10^{-6} to 13.5×10^{-3}		10 μM	Maintains 85.7% of initial sensitivity after 2 months' storage	*2*
GCE	HRP, GOD, β-Gal (5-aminosalicylic acid)	cross-linking with GA	0 vs SCE	2.9×10^{-5} to 9.9×10^{-4}			Maintains 40% of initial sensitivity after 140 days of extensive use	*13*
ITO-coated glass plates	GaO, β-Gal (−)	immobilization in LB films of P3HT/SA	+0.40 vs Pt	0.029−0.175			Has a self-life above 120 days	*14*
GCE	GOD, β-Gal (−)	immobilization of the enzymes onto the electrode with Hg thin film	−0.20 vs Ag/AgCl	1.0×10^{-4} to 3.5×10^{-3}	52.1 nA M^{-1}	1.0×10^{-4} M	40 measurements	*1*
solid spectrographic graphite electrode	CDH (−)	physical adsorption	+0.3 vs Ag/AgCl	$(1-100) \times 10^{-6}$[b]	1.3 mA mM^{-1}[b]	1 μM[b]	Maintains 98% of its initial signal after 11 h of continuous use	*15*
gold interdigitated electrodes	GOD, β-Gal (−)	cross-linking with GA		$(30-600) \times 10^{-6}$			28.5% loss of signal after 5 days of measurements	*16*
gold disk electrode modified with a MPA-SAM	β-Gal, GOD, HRP (TTF)	entrapment with a dialysis membrane	0.0 vs Ag/AgCl	$(1.8-120) \times 10^{-6}$; $(5.0-1{,}000) \times 10^{-6}$[b]	(6.04) x10^5 nA M^{-1}; $(1.11) \times 10^4$ nA M^{-1}[b]	0.47 μM; 3.81 μM[b]	28 days of continuous measurements	this work

[a] β-Gal: β-galactosidase. CDH: cellobiose dehydrogenase. GA: glutaraldehyde. GCE: glassy carbon electrode. GDH: glucose dehydrogenase. GOD: glucose oxidase. HRP: horseradish peroxidase. ITO: indium−tin−oxide. LR: linear range. M: molar concentration. MPA: 3-mercaptopropionic acid. P3HT: poly(3-hexylthiophene). SA: stearic acid. SAM: self-assembled monolayer. SCE: saturated calomel electrode. TTF: tetrathiafulvalene. [b] Flow injection analysis mode.

gold disk electrode. The performance of the trienzyme electrode under both batch and flow injection conditions and its usefulness for the determination of lactose in milk and dairy products were critically evaluated by comparing the results with those obtained by a reference commercial enzymatic kit.

EXPERIMENTAL SECTION

Apparatus and Electrodes. Voltammetric and amperometric measurements were carried out with a CHI812B potentiostat controlled by CHI812B software. A Varian Cary-3 Bio UV−visible absorption spectrophotometer, a P-Selecta ultrasonic bath and a P-Selecta Agimatic magnetic stirrer were also used. Flow injection (FI) experiments were carried out using a Spetec Perimax-12 peristaltic pump and a Rheodyne model 5020 injection valve with variable injection volumes.

XBAS-NS-AU gold disk electrodes (∅ ∼ 3 mm) were used as electrode substrates to be modified. A BAS MF-2052 Ag/AgCl/KCl (3 M) reference electrode and a Pt wire counter electrode were also employed. A 10 mL glass electrochemical cell was used in batch experiments, while a 10 mL homemade methacrylate wall-jet cell was employed for flow injection measurements.

Reagents and Solutions. Stock 0.01 M lactose (Sigma) solutions were prepared in 0.05 M phosphate buffer of pH 6.0 containing 1 mM Mg^{2+} (in the form of $MgCl_2 \cdot 6H_2O$, Merck). More dilute standards were prepared by suitable dilution with the same phosphate buffer solution, which was also used as the supporting electrolyte. A 40 mM mercaptopropionic acid (MPA) (Aldrich) solution, prepared in a 75/25% v/v ethanol/water mixture, was employed for the SAMs formation.

A 2.3 U μL^{-1} β-Gal solution (grade VIII from *Escherichia coli*, EC 3.2.1.23, Sigma), a 2.46 U μL^{-1} GOD solution (from *Aspergillus niger*, EC 1.1.3.4, Sigma) and a 12.1 U μL^{-1} HRP solution (Type II from *A. rusticana*, EC 1.11.1.7, Sigma) all prepared in phosphate buffer solution of pH 6.0 were used for the preparation of the β-Gal-GOD-HRP-TTF-MPA-AuE biosensor. Moreover, a 0.5 M TTF (Aldrich) solution in acetone was prepared. Dialysis membranes (10K MWCO) were purchased from Cultek.

Other solutions employed were 2 M KOH (Panreac) in water; 0.01 M stock solutions, prepared in 0.05 M phosphate buffer of pH 6.0 containing 1 mM Mg^{2+}, of ascorbic acid (Fluka), uric acid (Merck), D-glucose (Panreac), fructose (Sigma), galactose (Sigma), lactulose (Fluka), sucrose (Fluka), maltose (Fluka) and L-arabinose (Sigma).

All chemicals used were of analytical-reagent grade, and water was obtained from a Millipore Milli-Q purification system. Moreover, the galactose and lactose spectrophotometric enzymatic kit (Enzyplus, EZS784+) was used to compare the results obtained with the biosensor for the determination of lactose.

Biosensor Construction. Gold disk electrodes (AuE) were treated as described previously (*20*). Briefly, the AuE was polished with 3 μm diamond powder (BAS MF-2059) for 1 min. Then, it was sonicated in deionized water for 1 min and immersed for 1 h in a hot 2 M KOH solution. Next, the electrode was rinsed with water, immersed in concentrated H_2SO_4 and concentrated HNO_3 for 10 min each, and rinsed again with deionized water. Finally the electrode was dried thoroughly under a N_2 flow. Then, MPA-SAMs were formed by immersion of the treated AuE in a 40 mM MPA solution in EtOH/H_2O (75/25, v/v) for at least 15 h. The modified electrodes were rinsed with deionized water and dried with a nitrogen stream. The enzymes and the mediator were immobilized onto the MPA-modified AuE as follows: a 2 μL aliquot of the 0.5 M TTF solution was deposited on the modified electrode surface. Once the surface dried at room temperature, a 2 μL aliquot of the 12.1 U μL^{-1} HRP solution (or Cat solution) was deposited and allowed to dry again. Then, a 5 μL aliquot of the 2.46 U μL^{-1} GOD and 4 μL of the 2.3 U μL^{-1} β-Gal solution was dropped on the modified electrode surface (waiting between drops for drying at room temperature). Finally, a 1.5 cm^2 piece of the dialysis membrane was fixed on top of the electrode surface and secured with an appropriate O-ring.

Amperometric measurements were performed by applying a potential of 0.00 V (vs Ag/AgCl). FI experiments were performed with a 0.85 mL min^{-1} flow rate and a 500 μL injection volume.

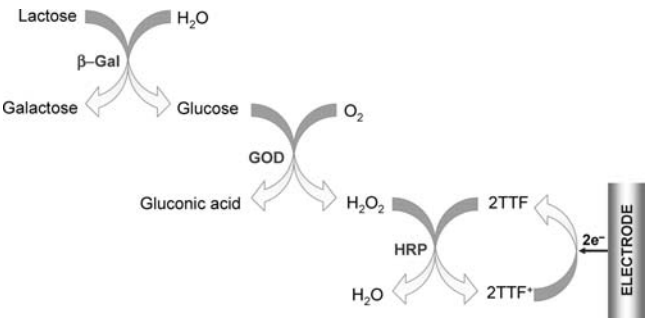
Determination of Lactose in Milk and Other Foodstuffs. As it will be commented below, no matrix effect was observed and, therefore, lactose concentrations were calculated by interpolation of the corresponding amperometric signals from the sample solutions into a calibration graph constructed with standard solutions. The determination of lactose was carried out in 25 samples. Liquid samples were analyzed directly after homogenizing by stirring and adequately diluting with the buffer solution to fit the sample lactose concentration into the linear range of the calibration plot. Solid or semisolid samples were treated as follows.

- *Condensed milk, yogurt, heavy cream and mayonnaise*: Approximately 1 g of sample was accurately weighed and 15 mL of water were added and stirring for 10 min. The resulting suspension was transferred to a 25 mL volumetric flask and diluted to the mark with distilled water.
- *Cheese, chocolate*: About 2 g of sample was accurately weighed and 70 mL of distilled water was added and heated for 15 min at 70 °C while stirring occasionally. The resulting suspension was transferred to a 100 mL flask and diluted to the mark with distilled water.
- *Butter, margarine*: About 5 g of sample was accurately weighed and 40 mL of distilled water was added with mechanical stirring and heated to melt fat. Then, it was allowed to cool in an ice bath and the aqueous phase collected and transferred to a 100 mL flask. The procedure was repeated with the solid phase, and the resulting aqueous phase was added to the same 100 mL flask. Finally, distilled water was added to the mark.
- *Milk, cream*: only 10 times diluted with the corresponding buffer solution.
- *Powdered milk*: About 1 g of sample was accurately weighed and dissolved in approximately 80 mL of distilled water. The resulting solution was added to a 100 mL flask and diluted to the mark with distilled water.

Next, an appropriate volume of the resulting sample solution (10 μL for condensed milk and chocolates, 500 μL for cheese, butter, margarine and mayonnaise and 30 μL for the other samples) was added to the electrochemical cell containing 10.0 mL of 0.05 M phosphate buffer solution of pH 6.0 containing 1 mM Mg^{2+} which was used as supporting electrolyte. The amperometric measurements were carried out by applying a potential of 0.00 V (vs Ag/AgCl) and allowing the steady-state current to be reached.

The analysis of samples with the commercial enzyme kit implied following the sample treatment and procedures described in the corresponding leaflet.

Scheme 1. Schematic Diagram Displaying the Enzyme and Electrode Reactions Involved with the β-Gal-GOD-HRP-TTF-MPA-AuE Biosensor for Lactose Determination



RESULTS AND DISCUSSION

The biocatalytic reactions on which the functioning of the lactose biosensor is based are schematically depicted in **Scheme 1**. β-Gal catalyzes the hydrolysis of the disaccharide lactose to D-galactose and D-glucose, this being oxidized by oxygen in the presence of GOD to produce gluconic acid and hydrogen peroxide. The generated hydrogen peroxide is subsequently reduced in the presence of HRP, the regeneration of the HRP reduced form being mediated by TTF. The oxidized form of the mediator, TTF^+, is then reduced at the electrode at an applied potential

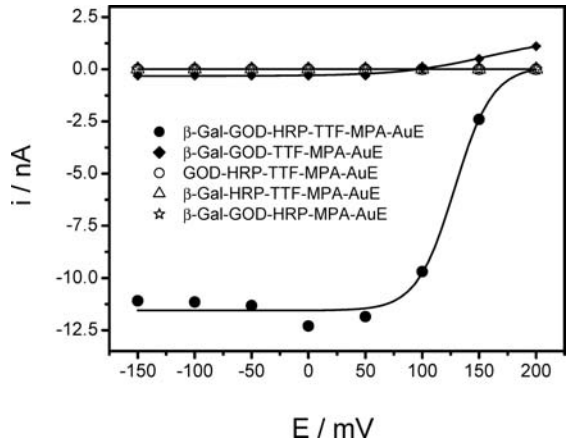


Figure 1. Effect of the applied potential on the amperometric signal for 2.0×10^{-5} M lactose obtained at β-Gal-GOD-HRP-TTF-MPA-AuE, GOD-HRP-TTF-MPA-AuE, β-Gal-GOD-TTF-MPA-AuE, β-Gal-HRP-TTF-MPA-AuE and β-Gal-GOD-HRP-MPA-AuE biosensors. Supporting electrolyte: 0.05 M phosphate buffer (pH 6.0) containing 1 mM $MgCl_2$.

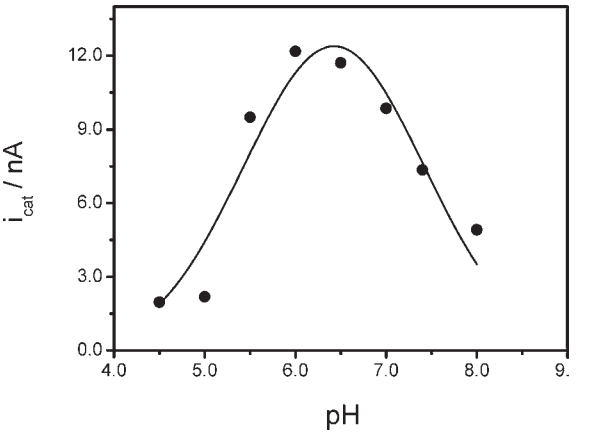


Figure 2. Influence of pH on the steady-state current measured with the β-Gal-GOD-HRP-TTF-MPA-AuE biosensor for 2.0×10^{-5} M lactose. E_{app} = 0.00 V (vs Ag/AgCl).

more negative than the formal potential of the TTF/TTF^+ redox couple, resulting in an amperometric signal proportional to the lactose concentration (*21*).

Optimization of Working Variables. The optimization of variables for the functioning of the trienzyme biosensor was accomplished by amperometry in stirred solutions taking the highest amperometric signal for 2.0×10^{-5} M lactose as the criterion of selection. Regarding the biosensor composition, the GOD and HRP loadings as well as that of the mediator TTF were the same as those used previously for the development of glucose and hydrogen peroxide biosensors (*20*, *21*). Therefore, only the effect of the β-Gal loading was evaluated. The current measured at 0.00 V increased with β-Gal loading up to a value of 9.2 U and then decreased for higher loadings. This was attributed to the fact that a high amount of β-Gal could block the HRP and GOD active centers and then decrease the rate of the second and third enzyme reactions. Consequently, the composition of the trienzyme electrode for further work was 9.2 U β-Gal/12.3 U GOD/24.2 U HRP/1.0 μmol TTF.

The influence of the applied potential on the biosensor response to lactose was examined in the +0.20 to −0.15 V range. **Figure 1** shows that the cathodic current reached a practically constant value for potentials more negative than 50 mV. Moreover, as expected considering the involved enzyme reactions, no significant cathodic amperometric signals were found in the whole potential range for bioelectrodes constructed without one of the three enzymes or without mediator. Also, **Figure 1** displays that an anodic current was observed with a β-Gal-GOD-TTF-MPA-AuE above +0.15 V, in agreement with the mechanism proposed for the oxidation of glucose in the presence of GOD and TTF (*20*). An applied potential value of 0.00 V was chosen in order to accomplish a sensitive detection and also to minimize the number of potential interferents able to be reduced at the electrode surface.

Regarding selection of the working pH, optimum pH ranges are reported for each of the three employed enzymes: 6 to 8 for β-Gal, a broad range between 4 and 7 (with best performance at 5.5) for GOD, and pH 7.0 for HRP (*13*). Therefore the effect of pH on the three-enzyme electrode response to lactose was evaluated in the 4.5−8.0 range. **Figure 2** shows that the biosensor provided the highest current values at pHs comprised between 5.5 and 6.5. Accordingly, a 0.05 M phosphate buffer solution of pH 6.0 (containing 1.0 mM Mg^{2+}) was chosen for further work.

Since it is well-known that divalent magnesium ions (Mg^{2+}) are required for the *E. coli* β-Gal optimal activity (*13*, *22*), their concentration in the electrochemical cell was also optimized. The amperometric response increased significantly with Mg^{2+} concentration up to a concentration of 1 mM and then decreased for higher concentrations. Therefore, 1 mM Mg^{2+} was added to the electrolyte solution in further experiments.

Under the selected conditions, the amperometric responses of the biosensor upon additions of lactose or glucose at the same concentration level 2.0×10^{-5} M to the supporting electrolyte were compared. A response 1.88 times higher was obtained when no previous hydrolysis reaction occurred, which is likely related to the ratio of the enzyme loadings and the hydrolyzed lactose percentage under the experimental conditions employed.

Stability of the β-Gal-GOD-HRP-TTF-MPA-AuE Biosensor. The repeatability of the measurements was evaluated by constructing ten different calibration plots with the same biosensor in the 1.0×10^{-5} to 5.0×10^{-5} M lactose concentration range. A relative standard deviation (RSD) value of 3.6% was obtained for the slope values of these calibration plots, indicating a good repeatability of the measurements carried out with the biosensor with no need of cleaning or pretreatment for the bioelectrode surface. Moreover, a RSD value of 3.0% was obtained for the steady-state current values corresponding to 10 repetitive measurements of 2.0×10^{-5} M lactose.

The reproducibility of the responses obtained with eight different biosensors was evaluated by measuring the amperometric signals obtained for 2.0×10^{-5} M lactose, and also by measuring the slope values corresponding to the calibration plots for lactose in the 2.0×10^{-5} to 8.0×10^{-5} M range with the eight biosensors. RSD values of 6.5 and 6.6% were obtained, respectively, thus demonstrating that the fabrication procedure of the biosensors was reliable, allowing reproducible amperometric responses to be obtained with different biosensors constructed in the same manner following the described methodology.

Also, the useful lifetime of one single biosensor was checked by performing daily six amperometric measurements of 2.0×10^{-5} M lactose. Once all the experiments of the daily working were completed, the biosensor was stored in the buffer solution at 4 °C. After 28 days of continuous use, the biosensor response remained within the limits set $\pm 3s$ of the mean value for 12 successive measurements carried out on the first day, where s is the standard deviation calculated from these 12 measurements. The response of the biosensor decreased to 65% after 34 days of use, which is likely due to the denaturation of the immobilized enzymes.

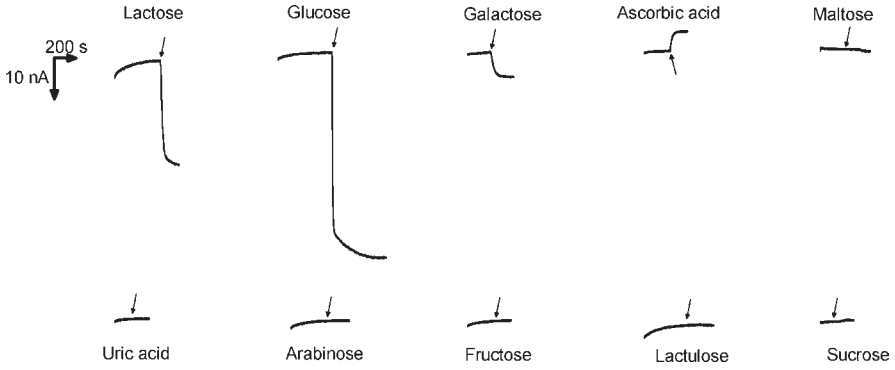


Figure 3. Amperometric responses obtained at the β-Gal-GOD-HRP-TTF-MPA-AuE upon 50 μL additions of 0.01 M solutions of different potential interferents to 0.05 M phosphate buffer (pH 6.0) containing 1 mM $MgCl_2$; E_{app} = 0.00 V (vs Ag/AgCl).

Kinetic Constants and Analytical Characteristics. The overall reaction involves four different reactions, three of them enzyme-catalyzed and the electrode reaction involving TTF. Assuming that this electrode reaction is rapid, the limiting step would be one of the three enzyme reactions.

The reaction rate constants were calculated from the slope values of the corresponding ln *i* vs time plots, constructed from the current−time recordings obtained for 5.0×10^{-5} M lactose, glucose and H_2O_2. The mean values from three measurements were (0.43 ± 0.03) s^{-1} for H_2O_2, (1.23 ± 0.03) × 10^{-2} s^{-1} for glucose and (4.02 ± 0.07) × 10^{-3} s^{-1} for lactose, thus demonstrating that, as expected, the rate limiting step is the enzymatic hydrolysis of lactose. After the addition of lactose, the steady-state current was reached in approximately 5 min, which means a remarkably improved response time in comparison with other reported biosensors for lactose determination (*2*, *12*, *16*).

A linear log[(i_{max}/i) − 1] versus log [lactose] plot with a slope value close to 1 (1.13 ± 0.02) proved that the immobilization procedure did not alter the Michaelis−Menten type kinetics. The apparent Michaelis−Menten constant and the maximum rate of the reaction were calculated from the Lineweaver−Burk plot. Values of K_M^{app} (0.96 ± 0.02) mM and i_{max} (3.33 ± 0.05) × 10^{-7} A were obtained, with the confidence intervals calculated for a significance level of 0.05 (n = 3). The K_M^{app} value for the β-Gal-GOD-HRP-TTF-MPA-AuE was even smaller than that reported for the *E. coli* β-Gal reaction in solution (with Mg^{2+} as cofactor at 30 °C, 1.35 mM), which indicates that the affinity of the enzyme for the substrate is practically nonaffected by the immobilization procedure used.

A typical calibration curve for an enzyme system was obtained for lactose. A linear plot was obtained over the 1.5×10^{-6} to 1.2×10^{-4} M concentration range (r = 0.999, slope = (6.04 ± 0.05) × 10^2 μA M^{-1}, intercept = −0.6 ± 0.3 nA). The limits of detection and determination were calculated according to the $3s_b/m$ and $10s$ criteria, respectively, where m is the slope of the linear calibration plot and s_b was estimated as the standard deviation (n = 10) of the amperometric signals from 2.0×10^{-6} M lactose. These values were 4.6×10^{-7} and 1.5×10^{-6} M, respectively.

Selectivity. Sucrose, lactulose, fructose, arabinose, uric acid, ascorbic acid, maltose, galactose and glucose were checked as potential intereferents due to their possible presence in the samples to be analyzed. The influence of these compounds, at a concentration level of 5.0×10^{-5} M on the quantification of lactose was investigated. As can be seen in **Figure 3**, only ascorbic acid, galactose and glucose gave a significant amperometric response under the working conditions. The interference of glucose was not unexpected due to the presence of GOD in the biosensor. This problem can be overcome by removing glucose prior to lactose measurement or, as it will be demonstrated below, by subtracting the glucose content (quantified with a biosensor prepared in the absence of β-Gal, GOD-HRP-TTF-MPA-AuE) from the total content of glucose and lactose provided by the β-Gal-GOD-HRP-TTF-MPA-AuE biosensor. The ascorbic acid interference was due to the electrochemical oxidation of this compound at the applied potential to the bioelectrode, and to the reported catalytic oxidation of ascorbic acid by TTF (*23*). Nevertheless, taking into account that the expected content of lactose in the samples to be analyzed (see below) is much higher than the possible content of ascorbic acid (between 4.4×10^{-5} and 1.0×10^{-4} M in UHT milk samples (*24*)), no practical drawbacks could be envisaged from the presence of ascorbic acid. Similarly, the low response observed for galactose did not produce significant interference in the lactose determination because galactose is only present in some of the foodstuffs but in a very low concentration level (*2*). These results clearly demonstrate the high selectivity of the developed biosensor for the determination of lactose in dairy products containing other saccharides or organic acids.

Flow-Injection with Amperometric Detection at the β-Gal-GOD-HRP-TTF-MPA-AuE. The behavior of the developed biosensor in flow-injection systems with amperometric detection was evaluated. Characteristic FI parameters such as flow rate and injection volume were optimized using a detection potential of 0.00 V. The largest $i_p/W_{1/2}$ ratio, where i_p is the FI peak height and $W_{1/2}$ is the peak width at half-height, was obtained for a flow rate of 0.85 mL min^{-1} and an injection volume of 500 μL. This large sample volume was needed to obtain a sufficiently high analytical signal (for instance, a 100 μL injection volume gave rise to a 3 times lower i_p value). Under these conditions, the sampling frequency was 2.3×10^{-3} s^{-1} (one sample every 435 s). The effect of these hydrodynamic variables on the hydrolysis percentage produced in the FI system was analyzed by comparing the i_p values obtained after injection of 1.0×10^{-4} M solutions of glucose and lactose. As expected, the hydrolysis percentage decreased slightly as the carrier flow rate was higher and increased with the injected volume up to 500 μL. Under the hydrodynamic conditions selected, a hydrolysis percentage of 16% was obtained in comparison with the 53.2% obtained for the batch mode.

The repeatability of the measurements was evaluated by performing a set of 15 repetitive injections of 1.0×10^{-4} M lactose. The RSD value obtained for i_p, 7.2%, suggested a good stability of the enzymes and the mediator atop the SAM-modified electrode in spite of the hydrodynamic conditions. A linear calibration plot for lactose was obtained over the 1.3×10^{-5} to 1.0×10^{-3} M concentration range (r = 0.999), slope (1.113 ± 0.007) × 10^4 nA M^{-1} and intercept (0.14 ± 0.02) nA. The limits of detection and determination, calculated according to the same criteria mentioned above, were 3.8×10^{-6} and 1.3×10^{-5} M, respectively.

Moreover, an interference study for the same compounds tested in the batch mode was carried out by following a similar

Table 2. Determination of Lactose in Different Foodstuffs Using the β-Gal-GOD-HRP-TTF-MPA-AuE Biosensor and the Commercial Enzyme Kit

	biosensor				enzymatic kit	
sample no./description	[glucose] (n = 2)	[lactose] (n = 5)	$RSD_{n=5}$/%	time/min	[lactose] (n = 1)	time/min
1/homogenized pasteurized semiskimmed milk	$(6.12 \pm 0.06) \times 10^{-2}$ g L^{-1}	(44 ± 3) g L^{-1}	4.5	6	42 g L^{-1}	95
2/UHT semiskimmed milk with calcium	(0.21 ± 0.02) g L^{-1}	(53 ± 1) g L^{-1}	1.7	6	51 g L^{-1}	95
3/skimmed milk with calcium	(0.14 ± 0.06) g L^{-1}	(49 ± 4) g L^{-1}	4.8	6	53 g L^{-1}	95
4/skimmed milk	(0.11 ± 0.02) g L^{-1}	(47 ± 4) g L^{-1}	6.4	6	44 g L^{-1}	95
5/UHT skimmed milk with calcium	(0.08 ± 0.03) g L^{-1}	(64 ± 6) g L^{-1}	6.0	6	57 g L^{-1}	95
6/UHT semiskimmed milk	(0.14 ± 0.03) g L^{-1}	(47 ± 3) g L^{-1}	3.7	6	46 g L^{-1}	95
7/UHT semiskimmed milk with calcium	(0.10 ± 0.02) g L^{-1}	(58 ± 6) g L^{-1}	7.0	6	55 g L^{-1}	95
8/calcium plus milk powder	(0.23 ± 0.13)%	(40 ± 4)%	2.2	6	35%	110
9/condensed whole milk	(0.91 ± 0.06)%	(11.1 ± 0.3)%	0.8	23	11%	110
10/condensed whole milk	(0.81 ± 0.06)%	(5 ± 1)%	10.5	23	8%	110
11/condensed skimmed milk	(0.54 ± 0.13)%	(18 ± 3)%	5.8	23	17%	110
12/milk chocolate	(0.73 ± 0.06)%	(8.0 ± 0.8)%	5.3	28	9%	130
13/white chocolate	(0.6 ± 0.3)%	(21 ± 5)%	8.9	28	13%	130
14/cheese slices	0%	(5.0 ± 0.5)%	6.3	27	3.5%	130
15/cream cheese	0%	(2.7 ± 0.2)%	6.5	27	2.7%	130
16/hard cheese	0%	0%		27	0.00%	130
17/Camembert cheese	0%	0%		27	0.00%	130
18/grated cheese	0%	(0.73 ± 0.06)%	2.7	27	0.52%	130
19/butter	0%	(0.65 ± 0.02)%	2.2	37	0.72%	135
20/margarine	0%	0%		37	0.00%	135
21/cream	0 g L^{-1}	(24 ± 2) g L^{-1}	4.1	6	26 g L^{-1}	95
22/heavy cream	0%	(1.6 ± 0.2)%	3.9	20	0.80%	110
23/skimmed natural yogurt	(0.24 ± 0.13)%	(3.7 ± 0.1)%	1.7	22	3.7%	110
24/mayonnaise	0%	0%		22	0.00%	110
25/mayonnaise	0%	0%		22	0.06%	110

procedure. Galactose, uric acid, arabinose and fructose behaved similarly to that observed in the batch mode. However, glucose and ascorbic acid produced an interference approximately ten times higher than that found under batch conditions. Also, maltose, lactulose and sucrose gave rise to small FI peaks, with i_p values at least ten times lower than that measured for lactose. This behavior can be attributed to the commented much lower lactose hydrolysis percentage achieved under flow conditions. This makes the lactose analytical signal lower and the presence of interfering compounds becomes more important than that observed for the batch mode.

Determination of Lactose in Real Samples. The developed biosensor was applied to the analysis of lactose in milk from several brands as well as in other foodstuffs: powder and condensed milk, chocolate, butter, margarine, yogurt, cheese and mayonnaise. Both batch amperometry and flow-injection with amperometric detection modes were employed. No matrix effect was observed for any of these samples. This was stated by comparing statistically, using Student's t test, the slope values of the calibration graphs obtained with the standard addition method for all the tested samples with that of the calibration graph prepared with lactose standards in the 2.0×10^{-5} to 8.0×10^{-5} M concentration range. Therefore, analyses were accomplished in all cases by interpolation of the corresponding amperometric signals into the calibration plot.

Samples without or with very low glucose content were analyzed as described in the Experimental Section. However, some samples contain this monosaccharide in an important amount. Therefore, in order to consider this interference and after applying the described sample treatment, we proceeded to determine also glucose with a GOD-HRP-TTF-MPA-AuE biosensor. The lactose content was then calculated by subtracting the number of moles obtained of glucose from the number of moles obtained from the signal measured with the biosensor for lactose. Both measurements were made separately.

The batch amperometric method in stirred solutions was applied to the determination of lactose in 25 different samples (8 milks, 3 condensed milks, 2 chocolates, 5 cheeses, 1 butter, 1 margarine, 2 creams, 1 yogurt and 2 mayonnaises, see **Table 2**). Five replicates were made for each sample. As an example, **Figure 4a** shows the amperograms obtained for the calibration graph constructed with lactose standard solutions and after addition of 30 μL (samples 1 and 2) and 500 μL (samples 18 and 19) of 4 different sample solutions prepared as described in Determination of Lactose in Milk and Other Foodstuffs. **Table 2** summarizes the obtained results. The confidence intervals were calculated for a significance level of 0.05, and RSD values were in all cases $< 10\%$.

The results obtained with the biosensor were compared with those provided by means of a commercial enzyme kit for lactose. **Figure 4b** shows the correlation plot of the results obtained by using both methodologies. The confidence intervals, calculated for a significance level of 0.05, for the slope and intercept values of the correlation plot, 1.04 ± 0.06 and 0.3 ± 1.7, respectively (r = 0.991) include the unit and the zero values, respectively, indicating that the developed method has no systematic errors (*25*), and demonstrating that the biosensor can be successfully used for the determination of lactose in milk and other foodstuffs.

Table 2 compares also the time elapsed since sample uptake until the result delivering for the two compared methodologies. As can be seen, the analysis time with the biosensor, defined as the time elapsed from sample uptaking until the measurement readout, is much shorter (approximately 90 min less) than that required when the spectrophotometric enzymatic kit was used. It should be noted that filtration and deproteinization steps are not necessary with the biosensor.

Furthermore, the FI methodology was applied to the analysis of 4 milk samples. **Table 3** compares the obtained results with those found by applying the enzymatic kit. As it can be seen, there is a high degree of agreement between the results obtained with both methodologies, the calculated Student's t value = 0.774 being much lower than the tabulated t value = 3.182 for a 95% confidence. Thus, there is not evidence for systematic differences

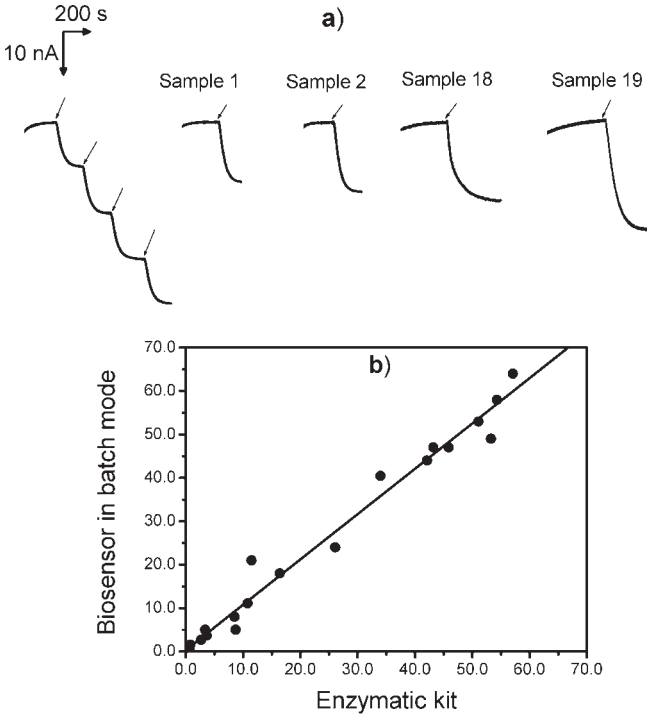


Figure 4. (**a**) Amperograms in stirred solutions obtained with the β-Gal-GOD-HRP-TTF-MPA-AuE biosensor for the calibration graph constructed with lactose standard solutions in the 2.0×10^{-5} to 8.0×10^{-5} M concentration range and upon 30 μL additions (samples 1 and 2) and 500 μL additions (samples 18 and 19) of 4 different sample solutions. (**b**) Correlation plot of the results for the determination of lactose in several foodstuffs, obtained with the β-Gal-GOD-HRP-TTF-MPA-AuE biosensor and the commercial enzymatic kit (values from **Table 2**).

Table 3. Determination of Lactose in Milk Samples by FIA Using the β-Gal-GOD-HRP-TTF-MPA-AuE Biosensor as Amperometric Detector

	FIA		
sample	[lactose]/g L^{-1}	$RSD_{n=5}$/%	enzymatic kit (n = 1)/g L^{-1}
skimmed milk	(54 ± 2)	3.3	54
semiskimmed milk	(56 ± 1)	1.8	54
whole milk with calcium	(53 ± 2)	2.4	55
semiskimmed milk	(48 ± 1)	1.7	52

between the three sets of results obtained by using the biosensor in both modes and the enzymatic kit. It is important to remark that the methods derived from the use of the integrated multienzyme biosensor exhibit important operational advantages, such as simplicity (only an appropriate dilution of liquid samples is needed), rapidity and lower cost in comparison with the enzymatic kit.

Comparison with Other Lactose Biosensors. The analytical performance of the β-Gal-GOD-HRP-TTF-MPA-AuE biosensor was compared with that reported for other electrochemical lactose biosensors (**Table 1**). In general, it can be claimed that the biosensor presented here has relevant advantages. The first one relies on the simple enzymes and mediator immobilization procedure. Moreover, the biosensor shows a wide linear dynamic concentration range. The detection limit achieved by batch amperometry in stirred solutions is the lowest reported until now, and allows quantification in samples with low lactose content, provided that the sample does not contain any major interference (glucose) or its content is determined previously and subtracted from the total content given by the biosensor. In this context, it should be remarked that the detection is accomplished at 0.00 V, and therefore the interference from electroactive substances can be minimized. The sensitivity of the biosensor using cellobiose dehydrogenase is reported to be higher than that obtained in this work. However, its applicability in the dairy industry is limited because it is not a commercial enzyme and possesses broad substrate specificity: cellobiose (main substrate of the enzyme), higher cellodextrins, or lactose (*15*).

CONCLUSIONS

The lactose biosensor constructed by coimmobilization of β-Gal, GOD, HRP and the mediator TTF atop a MPA-SAM on a gold disk electrode exhibits a good analytical performance, involving simple and rapid analytical methodologies by batch amperometry in stirred solutions and flow injection with amperometric detection. The described results demonstrate fairly well that the integrated biosensor accomplishes the requirements of precision, rapidity, sensitivity, simplicity, and low cost required to be considered as a useful analytical tool for the dairy industries.

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