Full Paper

A Lactulose Bienzyme Biosensor Based on Self-Assembled Monolayer Modified Electrodes

*Susana Campuzano, María Pedrero, F. Javier Manuel de Villena, José M. Pingarrón**

Dpto. Química Analítica, Facultad de CC. Químicas, Universidad Complutense de Madrid, E-28040 Madrid, Spain
*e-mail: pingarron@quim.ucm.es

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Abstract
A bienzyme biosensor in which the enzymes β-galactosidase (β-Gal), fructose dehydrogenase (FDH), and the mediator tetrathiafulvalene (TTF) were coimmobilized by cross-linking with glutaraldehyde atop a 3-mercaptopropionic acid (MPA) self-assembled monolayer on a gold disk electrode, is reported. The working conditions selected were $E_{app} = +0.10$ V and $(25 \pm 1)\,^{\circ}C$. The useful lifetime of one single TTF-β-Gal-FDH-MPA-AuE was surprisingly long, 81 days. A linear calibration plot was obtained for lactulose over the $3.0 \times 10^{-5} - 1.0 \times 10^{-3}$ mol L^{-1} concentration range, with a limit of detection of 9.6×10^{-6} mol L^{-1}. The effect of potential interferents (lactose, glucose, galactose, sucrose, and ascorbic acid) on the biosensor response was evaluated. The behavior of the SAM-based biosensor in flow-injection systems in connection with amperometric detection was tested. The analytical usefulness of the biosensor was evaluated by determining lactulose in a pharmaceutical preparation containing a high lactulose concentration, and in different types of milk. Finally, the analytical characteristics of the TTF-β-Gal-FDH-MPA-AuE are critically compared with those reported for other recent enzymatic determinations of lactulose.

Keywords: Self-assembled monolayers, Enzyme electrodes, Lactulose, Milk

1. Introduction

Lactulose (4-*O*-β-Galactopyranosyl-D-fructofuranose) is a synthetic disaccharide consisting of galactose and fructose. It is formed from lactose in milk by heat treatment [1], and it has been proposed by the International Dairy Federation and by the European Commission [2] as an analytical index to distinguish ultra high temperature (UHT) milk from in-container sterilized milk. Moreover, lactulose is used in infant formula, due to its ability to promote the proliferation of *lactobacillus bifidus*, as well as for prevention and treatment of chronic constipation, portal systemic encephalopathy and other intestinal or hepatic disorders [3].

Different analytical methods have been used for the determination of lactulose in milk, including gas chromatography [4], liquid chromatography with an ion exchange column [5–6], and enzymatic methods based on spectrophotometric detection [7]. These methods exhibit some drawbacks, such as the need of using six kinds of enzymes and a long time (about 15 h) to perform the analysis, in the case of the enzymatic photometry, and the unsuitability of the liquid chromatographic method for the determination of low concentrations of lactulose in milk [8]. More recently, different methodologies have been developed to improve the determination of lactulose in milk. Thus, an enzymatic spectrophotometric assay entailing the use of β-galactosidase (β-Gal), which hydrolyses lactulose giving fructose and galactose, and fructose dehydrogenase (FDH) which reacts with fructose in the presence of a tetrazolium salt giving a colored compound which can be detected at 570 nm, has been reported [9]. A flow system was developed by immobilizing β-Gal in a reactor, and the amount of fructose produced was measured with an electrochemical biosensor based on FDH, $K_3[Fe(CN)_6]$ as mediator and a Pt based electrochemical transducer [10]. Moreover, the automated determination of lactulose in milk using an enzyme reactor and flow analysis with integrated dialysis has been reported [3]. However, the only real bienzyme biosensor found in the literature for the determination of lactulose is the one reported by Sekine and Hall [8], in which FDH and β-Gal were immobilized by covering the electrode surface with a dialysis membrane, and a ring electrode onto which TTF^+-$TCNQ^-$ salt was physically packed, was employed. This biosensor exhibited a good sensitivity and selectivity, but it could be used only for 2 days with recalibration, and different sensors prepared with the procedure proposed by the authors could not be made with identical current response due to the variability in the precise electrode area achieved.

Recently, we have reported on the construction of a fructose biosensor based on a 3-mercaptopropionic (MPA) self-assembled monolayer (SAM)-modified gold disk electrode (AuE). The enzyme FDH and the redox mediator tetrathiafulvalene (TTF) were coimmobilized by cross-linking with glutaraldehyde [11]. This biosensor exhibited a very good sensitivity, time of response, stability and reproducibility when compared with other fructose biosensors reported in the literature. Moreover, the capability of the biosensor to be used in a flow system was also demonstrated. Considering the excellent analytical per-

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formance of the TTF-FDH-MPA-AuE, we decided to construct a bienzyme biosensor in which β-Gal, FDH and the mediator TTF were coimmobilized by cross-linking atop a MPA-SAM on a AuE. In similar coupled enzyme reactions to that commented above, lactulose was hydrolysed to D-fructose and D-galactose by β-Gal, and the hydrolyzed D-fructose was oxidized by FDH which was simultaneously reduced to the reduced form FDH-$PQQH_2$. The FDH-$PQQH_2$ is reoxidized by TTF^+, and the generated TTF is amperometrically reoxidized at the modified electrode, with the resulting current being dependent on the lactulose concentration. The construction and performance of the bienzyme electrode, both under batch and flow injection conditions, and its applicability to the analysis of real samples is discussed in this paper.

2. Experimental

2.1. Apparatus and Electrodes

Voltammetric and amperometric measurements were carried out with an ECO Chemie Autolab PSTAT 10 potentiostat using the software package GPES 4.9 (General Purpose Electrochemical System). A P-Selecta ultrasonic bath, and a P-Selecta Agimatic magnetic stirrer were also used. Flow-injection (FI) experiments were carried out using a Gilson Minipuls-2 peristaltic pump, and a Rheodyne Model 5020 injection valve with variable injection volumes.

A Metrohm 6.1204.020 gold disk electrode (3 mm ∅) was used as electrode substrate to be coated with the modified MPA-SAM. A BAS MF-2063 Ag | AgCl | KCl 3 mol L^{-1} reference electrode and a Pt wire counter electrode, were also employed. A 10-mL glass electrochemical cell was used in batch experiments while a large volume (50 mL) home-made glass wall-jet cell was employed for flow injection measurements.

2.2. Reagents and Solutions

Stock 0.5 mol L^{-1} lactulose (Sigma) solutions were prepared in 0.05 mol L^{-1} phosphate buffer of pH 4.5. More dilute standards were prepared by suitable dilution with the same phosphate buffer solution, which was also used as the supporting electrolyte both in batch and flow injection measurements.

A 40 mmol L^{-1} mercaptopropionic acid (MPA) (Research Chemicals Ltd.) solution, prepared in a 75/25% v/v ethanol/water mixture, was employed for the formation of the monolayers. The solutions used for the enzyme immobilization were a 5.1 U μL^{-1} solution of FDH (Sigma, EC 1.1.99.11 from *Gluconobacter sp.*, 112 U mg^{-1}), a 0.45 U μL^{-1} solution of β-Gal (Sigma, EC 3.2.1.23 from *Aspergillus oryzae*, 9.1 U mg^{-1}) prepared in the above mentioned phosphate buffer solution, and a 25% glutaraldehyde (Aldrich) solution. Moreover, a 0.5 mol L^{-1} tetrathiafulvalene (TTF, Aldrich) solution in acetone was prepared.

Other solutions employed were: a 2 mol L^{-1} KOH (Panreac) solution prepared in deionized water; and stock 0.5 mol L^{-1} fructose (Sigma), 0.25 mol L^{-1} ascorbic acid (Merck), 0.5 mol L^{-1} lactose (Sigma), glucose (Panreac), galactose (Sigma) and sucrose (Fluka) solutions prepared in 0.05 mol L^{-1} phosphate buffer of pH 4.5, for the interferences study.

Solutions used for the deproteinization of milk samples were: citric phosphate buffer prepared from a mixture of 0.1 mol L^{-1} citric acid (Merck), dibasic potassium phosphate (Scharlau) and 0.1% Tween 20 (Scharlau) in deionized water; 7.2% w/v $K_4Fe(CN)_6.3H_2O$ (Sigma) solution prepared in deionized water (Carrez I), and 14.4% (w/v) $ZnSO_4.7H_2O$ solution prepared in deionized water (Carrez II). All chemicals used were of analytical-reagent grade, and water was obtained from a Millipore Milli-Q purification system.

2.3. Procedures

MPA-SAMs were formed as described previously [12]. Immobilization of enzymes by cross-linking with glutaraldehyde was carried out as follows: 4 μL of the 5.1 U μL^{-1} FDH solution were deposited on the SAM-modified AuE and let to dry at ambient temperature. Then 3 μL of the 0.45 U μL^{-1} β-Gal solution were also deposited on the electrode surface. Once the electrode surface had dried out at ambient temperature, a 3-μL aliquot of the 0.5 mol L^{-1} TTF solution was deposited on and let to dry again. Then, the electrode was immersed in the 25% glutaraldehyde solution for 1 h at 4 °C.

Amperometric measurements were performed by applying in all cases a potential of +100 mV (vs. Ag/AgCl). The carrier stream for FI experiments was a 0.05 mol L^{-1} phosphate buffer of pH 4.5, with a flow rate of 1.40 mL min^{-1}.

2.4. Sample Treatment

Only an appropriate dilution with the supporting electrolyte solution was needed for the analysis of lactulose in the pharmaceutical preparation Duphalac. Thus, 515 μL of the syrup were diluted to 10 mL with the phosphate buffer of pH 4.5. Then 50 μL of this solution were added to the electrochemical cell for batch experiments, and 150 μL of this diluted solution were injected in the carrier solution for FI measurements.

Concerning the analysis of milk samples, only a 0.5 mL-addition of the sample to the 10 mL-electrochemical cell was necessary. Then, the standard additions method involving successive additions of the 0.1 mol L^{-1} lactulose stock solution, was used for the lactulose determination by amperometry in stirred solution at +100 mV.

Recovery tests of lactulose in milk samples were carried out by adding lactulose to pasteurized, UHT and sterilized milk at a concentration level of 685 mg L^{-1}. These recovery

studies were carried out both in deproteinized milk and non-deproteinized milk samples. Deproteinization was performed as follows: 10 mL of milk were transferred into a 50-mL conical flask. Then, 1.75 mL of each Carrez I and II were added and the resulting solution stirred for 2 – 3 min. Then 6.5 mL of citric phosphate buffer were added. The solution was well mixed for 2 – 3 min, left to rest for 30 min, and then filtered through a filter paper, eliminating the first 2 – 3 mL of filtrate [9]. The filtrate solution was spiked at the concentration level mentioned above.

After stabilization of the lactulose sensor (1 – 1.5 min) in the phosphate buffer solution, a 0.5 mL-aliquot of the spiked sample was added to the electrochemical cell. Amperometry in stirred solutions was performed and the analysis was carried out by applying the standard additions method as mentioned above.

3. Results and Discussion

The optimization of experimental variables concerning the behavior of the bienzyme biosensor was accomplished by amperometry in stirred solutions. Regarding biosensor preparation, only the influence of the β-Gal loading was checked, because both FDH and TTF loadings were optimized previously for the fructose biosensor [11]. Moreover, the same working medium used for fructose, i.e., a 0.05 mol L^{-1} phosphate buffer solution of pH 4.5, was employed with the bienzyme biosensor. The highest slope value obtained for lactulose calibration plots in the $2.0 \times 10^{-4} - 1.0 \times 10^{-3}$ mol L^{-1} concentration range, was taken as the criterion of selection for this variable. The sensitivity of the biosensor for lactulose at +0.20 V (potential at which the detection of fructose is ensured) increased with β-Gal loading up to a value of 1.4 U, then decreasing for higher loadings. As FDH was inmmobilized first in the used procedure, a high amount of β-Gal could block the FDH active centers, then decreasing the rate of the second enzyme reaction. Consequently, the composition of the bienzyme electrode for further work was: 20.6 U FDH/1.4 U β-Gal/1.5 μmol TTF.

The influence of the applied potential on the biosensor response to 2.0×10^{-4} mol L^{-1} lactulose was examined in the 0.00 to +0.60 V range. The amperometric responses of the TTF-FDH-MPA-Au, TTF-β-Gal-MPA-Au, and TTF-MPA-Au electrodes were also tested. Figure 1 shows that no amperometric signal was observed in the whole potential range at TTF-β-Gal-MPA-AuE and TTF-MPA-AuEs. Similarly to that reported for glucose [10], lactulose gave a very small response (100-fold smaller than at the bienzyme electrode) with the single enzyme fructose biosensor. A catalytic effect was observed at the TTF-β-Gal-FDH-MPA-AuE between 0.00 and +0.50 V, with a maximum limiting current at +0.10 V. The shape of the current vs. E plot for this biosensor is similar to that obtained with the single TTF-FDH-MPA-AuE for fructose [11], the decrease of the response above +0.10 V being attributed to a leakage of TTF from the electrode surface at more positive potentials [13]. An applied potential of +0.10 V was chosen for further work in order to obtain the highest sensitivity. Cyclic voltammograms obtained at the TTF-β-Gal-FDH-MPA-AuE from a 5.0×10^{-3} mol L^{-1} lactulose solution and from the supporting electrolyte solution are displayed in the inset of Figure 1.

Finally, although it was observed that the bienzyme biosensor showed a higher response for lactulose in the 35 – 40 °C temperature range, the thermal stability of β-Gal is lower than at room temperature [3, 14], and therefore it was decided to keep temperature at (25 ± 1) °C.

Under the selected conditions, the amperometric responses of the biosensor upon additions of the same amount of lactulose or fructose to the electrochemical cell were compared. A response 57% higher was obtained when no previous hydrolysis reaction occurred, which should be related with the ratio of the enzyme loadings and the lactulose hydrolysis percentage.

3.1. Stability of the TTF-β-Gal-FDH-MPA-AuE Biosensor

Different aspects concerning the stability of the biosensor were considered.

The repeatability of the measurements was evaluated by constructing 10 successive calibration plots for lactulose in the $2.0 \times 10^{-4} - 1.0 \times 10^{-3}$ mol L^{-1} concentration range with the same biosensor. A relative standard deviation (RSD) value of 8.4% was obtained for the slope values of such

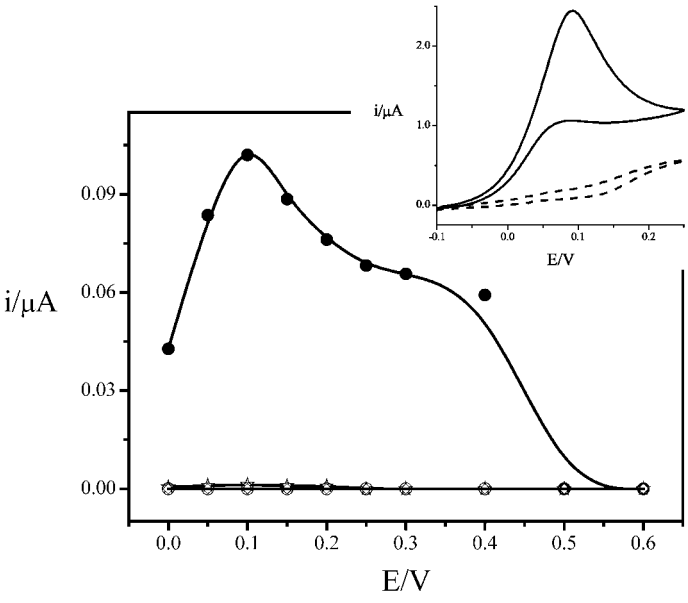

Fig. 1. Effect of the applied potential on the amperometric signal for 2.0×10^{-4} mol L^{-1} lactulose in a 0.05 mol L^{-1} phosphate buffer solution (pH 4.5) at: a TTF-β-Gal-FDH-MPA-AuE (●), a TTF-FDH-MPA-AuE (⋆), a TTF-β-Gal-MPA-AuE (△), and a TTF-MPA-AuE (◇). Inset: cyclic voltammograms at the TTF-β-Gal-FDH-MPA-AuE from a 5.0×10^{-3} mol L^{-1} lactulose solution (–); background voltammogram (– – –); $v = 1$ mV s^{-1}.

calibration plots, indicating an acceptable repeatability of the measurements with no need to apply a cleaning or pretreatment procedure to the biosensor. Moreover, a RSD of 6.6% was obtained for the steady-state current corresponding to 10 repetitive measurements of 3.0×10^{-4} mol L^{-1} lactulose.

The useful lifetime of one single TTF-β-Gal-FDH-MPA-AuE was checked by constructing calibration plots for lactulose after storing the biosensor in 0.05 mol L^{-1} phosphate buffer of pH 4.5 at 4 °C. Figure 2 shows the control chart constructed taking the mean value of 10 successive calibration plots obtained the first day of this study as the central value (dotted line). The upper and lower limits of control were set at $\pm 3 \times$ S.D. of this initial value. The values shown after the first day correspond to the mean of three successive calibration plots. The slope value remained within the control limits for the surprisingly high period of time of approximately 81 days. It is important to remark that this period is considerably longer than that found for a TTF-FDH-MPA-AuE (30 days). This may be due to two facts: a) the enzyme FDH is more protected because β-Gal is placed above it; b) as the hydrolysis is not a 100% completed reaction, the amount of fructose detected is lower with the bienzyme electrode, and then the enzyme is less damaged with time.

3.2. Kinetic Constants and Analytical Characteristics

The overall reaction comprises three different reactions, two of them are catalyzed by enzymes and the other one is the electrode reaction involving TTF. Assuming that the electrode reaction is rapid, the limiting step would be one of the two enzyme reactions. The kinetics of these reactions obeyed a Michaelis-Menten behavior, with Hill's parameter of 0.98 and 0.97 for fructose and lactulose, respectively. 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 after a 100 μL addition of a 0.1 mol L^{-1} solution of fructose or lactulose to 10 mL of the phosphate buffer solution.

The mean values from three determinations were (0.6 ± 0.1) s^{-1} for fructose and (0.26 ± 0.03) s^{-1} for lactulose, demonstrating that, as expected, the rate limiting step is the enzymatic hydrolysis of lactulose.

A linear calibration plot was obtained for lactulose over the $3.0 \times 10^{-5} - 1.0 \times 10^{-3}$ mol L^{-1} concentration range ($r = 0.999$), with a slope value of $(4.73 \pm 0.06) \times 10^{-4}$ A mol^{-1} L and an intercept of $(1.2 \pm 0.3) \times 10^{-8}$ A. The limits of detection and determination were calculated according to the 3 s_b/m and 10 s criteria, respectively, where m is the slope of the calibration plot and s_b was estimated as the standard deviation ($n = 10$) of the amperometric signals from 3.0×10^{-5} mol L^{-1} lactulose. The values obtained were 9.6×10^{-6} and 3.2×10^{-5} mol L^{-1}, respectively.

The effect of potential interferents on the biosensor response was evaluated. The substances tested were lactose, glucose, galactose, sucrose and ascorbic acid. The addition of 50 μL of 0.1 mol L^{-1} solution of galactose to the lactulose solution gave rise to a decrease in the biosensor response to lactulose (Fig. 3) suggesting inhibition of some of the enzyme reactions involved. Galactose did not produce this type of negative responses upon its addition in the absence of lactulose, which demonstrated that the inhibited reaction was the hydrolysis one. The fact that β-Gal is inhibited by one of the enzyme reaction products justifies the difficulty to achieve a complete hydrolysis of the substrate.

The degree of interference was estimated by calculating the relative error in the slope of the lactulose calibration plot in the presence and absence of the above mentioned compounds at different concentration levels. Table 1 shows the lactulose-to-interferent molar ratio for which a relative error lower than 10% was found. No interference was observed for glucose and sucrose even at a 1-to-20 molar ratio. However, besides galactose, ascorbic acid and lactose did affect the slope value of the lactulose calibration plot for

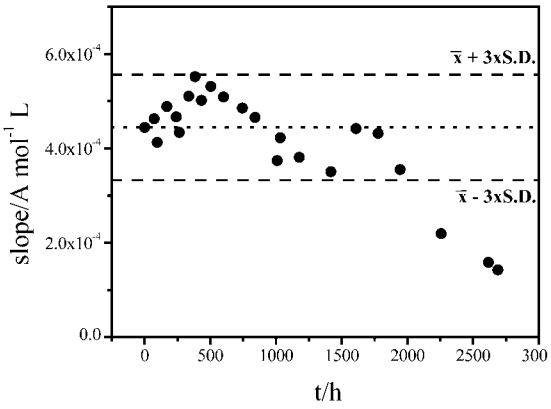

Fig. 2. Control chart constructed for one single TTF-β-Gal-FDH-MPA-AuE. Measurements correspond to the mean slope values of calibration plots for lactulose in the $2.0 \times 10^{-4} - 1.0 \times 10^{-3}$ mol L^{-1} concentration range; 0.05 mol L^{-1} phosphate buffer of pH 4.5; $E_{app} = +0.10$ V.

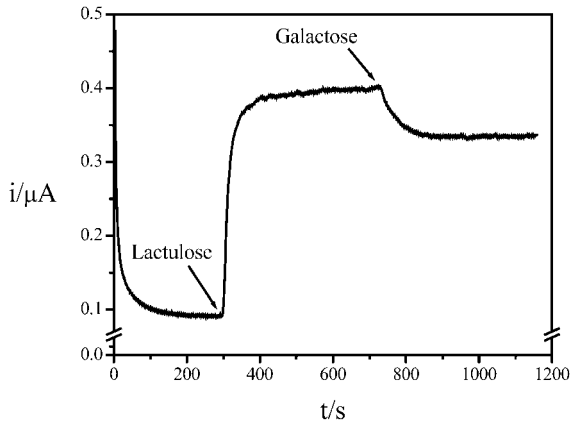

Fig. 3. Amperogram obtained after additions of 50 μL of a 0.1 mol L^{-1} lactulose solution, and 50 μL of a 0.1 mol L^{-1} galactose solution to the 10 mL-electrochemical cell. Other variables as in Figure 2.

Table 1. Lactulose-to-interferent molar ratio for which a relative error lower than 10% was obtained for the slope of the lactulose calibration plot in the 0.2 – 1.0 mol L^{-1} concentration range.

Interferent	Lactulose-to-interferent molar ratio	
	BATCH	FIA
Ascorbic acid	4	20
Lactose	0.2	0.08
D-Glucose	0.05	0.05
L-Galactose	2	0.05
D-Sucrose	0.05	0.05

the ratios shown in Table 1. In the case of ascorbic acid, the degree of interference is similar to that reported for fructose using a TTF-FDH-MPA-AuE [11], and is due to both direct oxidation at the electrode surface (although the presence of the SAM inhibits to a high extent this oxidation process), and to the electrocatalytic oxidation involving the mediator [15]. The interference from lactose is because it is also a substrate of β-Gal [10]. Although β-Gal from *Aspergillus Oryzae* has an activity of 210% for lactulose compared with lactose, this compound can interfere when present in high concentrations.

3.3. Flow-Injection with Amperometric Detection at the TTF-β-Gal-FDH-MPA-AuE

The behavior of the SAM-based biosensor in flow-injection systems in connection with amperometric detection was evaluated. Characteristic FI parameters, such as flow rate and injected volume were firstly optimized using a detection potential of +0.15 V. A higher $i_p/W_{1/2}$ 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 1.40 mL min^{-1} and an injection volume of 150 μL. The effect of these hydrodynamic variables on the hydrolysis percentage produced in the FI system was also analyzed by comparing i_p values obtained after injection of 5.0×10^{-4} mol L^{-1} solutions of fructose or lactulose. As expected for a hydrolysis reaction, the hydrolysis percentage decreased slightly as the carrier flow rate was higher, and increased with the injected volume up to 650 μL. Taking into account that these effects are not very significant, and that $W_{1/2}$ was 3-times larger when passing from an injection volume of 150 μL to 650 μL, it was decided to use 1.40 mL min^{-1} and 150 μL as flow rate and injected sample volume for further work.

Selection of the detection potential was carried out checking the i_p values obtained over the 0.00 to +0.60 V range. A similar behavior to that depicted in Figure 1 for batch conditions was obtained, and therefore, we decided to use the same detection potential, +0.10 V, for further work. Under these conditions, the biosensor response for fructose was a 80% higher than for lactulose when identical concentrations of both sugars were injected.

Different aspects concerning the stability of the TTF-β-Gal-FDH-MPA-AuE under these flow conditions were also tested. The repeatability of the measurements was evaluated by constructing 10 successive calibration plots for lactulose in the $2.0 \times 10^{-4} - 1.0 \times 10^{-3}$ mol L^{-1} concentration range, with the same electrode. A RSD value of 2.7% was obtained for the slopes of these calibration plots.

Figure 4 shows a series of 50 repetitive injections of 3.0×10^{-4} mol L^{-1} lactulose. The RSD value obtained for i_p, 3.7%, demonstrated the good stability of the enzymes and the mediator atop the SAM-modified electrode in spite of the hydrodynamic conditions. Furthermore, the reproducibility of the responses obtained with different TTF-β-Gal-FDH-MPA-AuEs was also checked. The RSD for the slope values of the calibration plots for lactulose in the $2.0 \times 10^{-4} - 1.0 \times 10^{-3}$ mol L^{-1} range, obtained with five different bioelectrodes was 6.8%.

A linear calibration plot was obtained over the $5.0 \times 10^{-5} - 1.0 \times 10^{-3}$ mol L^{-1} concentration range ($r = 0.998$), slope $(9.5 \pm 0.2) \times 10^{-5}$ A mol^{-1} L, intercept $(1.0 \pm 0.1) \times 10^{-8}$ A. The limits of detection and determination, calculated according to the same criteria mentioned above, were 1.6×10^{-5} and 5.3×10^{-5} mol L^{-1}, respectively. Moreover, an interference study for the same compounds that in the batch mode was carried out in a similar way. Table 1 also summarizes the analyte-to-interferent molar ratios for which a relative error lower than 10% was obtained in the slope of the calibration plot for lactulose in the $2.0 \times 10^{-4} - 1.0 \times 10^{-3}$ mol L^{-1} concentration range. As can be seen, the interference from ascorbic acid is much higher under flow conditions than in the batch mode, which can be attributed to the fact than FI measurements are carried out under no steady-state conditions. Moreover, although the behavior observed for glucose and sucrose was similar in both working modes, the interference from lactose and galactose is higher in the batch mode. This may be attributed, either to the higher extent of the hydrolysis reaction under this mode in comparison with FI conditions, and/or to the fact that the lower residence time of the substrates close to the enzyme

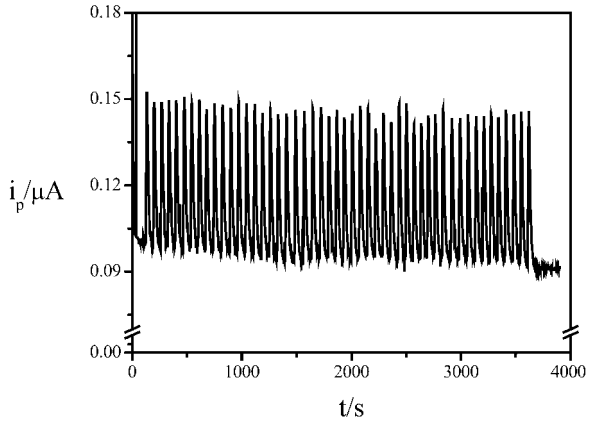

Fig. 4. Amperometric responses at the TTF-β-Gal-FDH-MPA-AuE for 50 repetitive injections of 3.0×10^{-4} mol L^{-1} lactulose under flow-injection conditions. Carrier solution: 0.05 mol L^{-1} phosphate buffer of pH 4.5; flow rate: 1.40 mL min^{-1}; V_i = 150 μL; E_{app} = +0.10 V.

layer in the FI mode caused a decrease of the interference impact.

3.4. Determination of Lactulose in Real Samples

The analytical usefulness of the biosensor was evaluated by determining lactulose in two different samples: a pharmaceutical preparation containing a high lactulose concentration, and, of course, in different types of milks.

The pharmaceutical preparation analyzed was a laxative syrup (Duphalac), with the composition for each 15 mL of: 10 g lactulose, <1.65 g galactose, <0.90 g lactose, <0.70 g epilactose and <0.30 g tagatose. Lactulose was determined using both the batch and the FI methods. No matrix effect was observed employing either methodology and, therefore, interpolation in an external calibration plot could be used. The results obtained from five replicates yielded mean lactulose concentrations (g mL^{-1}) of (0.66 ± 0.07) using the batch method, and (0.69 ± 0.03) by FI with amperometric detection, the confidence interval being calculated for a significance level of 0.05. As expected, no significant differences between the results obtained with both methods were found, which also agreed fairly well with the labeled value (0.67 g mL^{-1}).

Concerning milk samples, semi-skimmed pasteurized, ultra-heat treated (UHT) and sterilized milk purchased in a local supermarket were analyzed. Amperometry in stirred solutions was now used because of the higher sensitivity of this methodology. Milk is a very complex matrix containing electroactive compounds. Therefore, a relative high blank signals can be suspected [3].

Figure 5 shows amperograms recorded after addition of 500 μL of each type of milk to the electrochemical cell. Although the biosensor was able to distinguish between pasteurized, UHT and sterilized milk, a high blank signal was obtained for pasteurized milk, which has a non-detectable lactulose content [1, 16]. One of the most important interfering compound is, as it has been discussed above, ascorbate, which is present in UHT milk samples in concentration between 7.5 and 18.0 mg kg^{-1} (4.4×10^{-5} and 1.0×10^{-4} mol L^{-1}, respectively) [3].

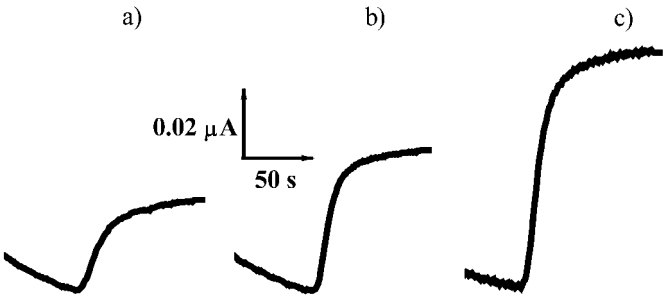

Fig. 5. Amperograms in stirred solutions obtained after addition of 500 μL for: pasteurised milk (a), UHT milk (b) and sterilised milk (c) to the electrochemical cell containing 10 mL of 0.05 mol L^{-1} phosphate buffer of pH 4.5; $E_{app} = +0.10$ V.

Another interference in milk samples is the lactose presence in high concentration since, as commented above, it is also a substrate of β-Gal. The usual concentration of lactose in milk is about 49 g L^{-1} (140 mmol L^{-1}) [9]. Finally, although the biosensor response is based on lactulose which is the only source of fructose, it has been reported that a small amount of free fructose is present in milk [3].

We verified that two completely different pasteurized milk samples (whole milk Pergola and semi-skimmed Lauki) gave the same unspecific amperometric signal. Therefore, we proceeded in a similar way to that reported by other authors [3, 10], and this blank signal was subtracted from the responses obtained with UHT and sterilized milks. The evident matrix effect observed was solved by using the standard additions method. The results obtained from five replicates yielded mean lactulose concentrations of (230 ± 23) mg L^{-1} (RSD = 8.2%) and (661 ± 71) mg L^{-1} (8.7%) for UHT and sterilized milk, respectively. These values are included between the lactulose ranges reported for UHT (100 – 510 mg L^{-1}) and sterilized (600 – 2000 mg L^{-1}) milks [9], and show an acceptable precision taking into account the need of an unspecific blank signal subtraction to carry out the analysis.

The accuracy of the determination was evaluated by carrying out recovery studies by adding a known amount of lactulose (685 mg L^{-1}) to both deproteinized (see Section 2.) and nondeproteinized pasteurized, UHT and sterilized milk samples. Good recoveries were obtained in all cases, ranging between 98.4 and 105.3%, with RSD values ranging between 3.4 and 7.5% ($n = 5$). Although the signals from the standard additions are better defined for deproteinized samples, no significant decrease of the blank signal was observed for these pretreated samples, and therefore, one can conclude that deproteinization prior the analysis does not imply an important advantage.

Finally, we have compared the analytical characteristics of the TTF-β-Gal-FDH-MPA-AuE with those reported for other recent enzymatic determinations of lactulose (Table 2). As can be seen, the sensitivity of the biosensor is lower than that achieved with other methodologies using electrochemical detection, although, indeed, these methodologies are based on the use of enzyme reactors instead of an integrated biosensor like the SAM-based biosensor is. Furthermore, the detection potential is lower with the TTF-β-Gal-FDH-MPA-AuE, which implies an improvement in selectivity. Compared with the only integrated bienzyme sensor reported [8], this has a surprisingly better selectivity against lactose, considering that the same enzyme reactions are involved. However, the useful lifetime of the SAM-based biosensor is very much longer (81 against 2 days), and also reproducible responses were obtained with different biosensors constructed in the same manner. On the contrary, Sekine and Hall [8] reported that different sensors could not be made with identical current response due to the variability in the electrode area achieved. Finally, the methodology involved with the SAM-based biosensor is simpler (no deproteinization and hydrolysis of milk samples is needed), faster (10 min against 1 h) and cheaper (less

Table 2. Comparison of the analytical characteristics of the TTF-β-Gal-FDH-MPA-AuE with those reported for other recent enzymatic determinations of lactulose. β-Gal: β-galactosidase; CPG: controlled-pore glass; FDH: fructose dehydrogenase; L.R.: linear range; D.L.: detection limit.

Basis	Detection	Analytical characteristics	Stability	Ref.
β-Gal and mediator, $K_3[Fe(CN)_6]$, in solution, FDH immobilized in a CPG reactor. Detection on Pt electrode	Electrochemical (+0.385 V vs. Ag/AgCl)	L.R.: 0.073 – 0.73 mmol L^{-1} [a]; Sensitivity: 468 nA $mmol^{-1}$ [a]	The FDH reactor losses half of the initial activity in two days	[3]
Both enzymes coimmobilized with a dialysis membrane on a TTF^{+}-$TCNQ^{-}$ modified ring carbon paste electrode	Electrochemical (+0.200 V vs. Ag/AgCl)	L.R.: up to 0.030 mmol L^{-1}; D.L.: 0.001 mmol L^{-1}; poor reproducibility with different sensors	The biosensor retains 80% sensitivity after two days	[8]
β-Gal immobilised in CPG reactor. Fructose generated in hydrolysis process is detected on a FDH-Pt membrane electrode. Mediator, $K_3[Fe(CN)_6]$, in solution	Electrochemical (+0.380 V vs. Ag/AgCl)	L.R.: 0.001 – 5 mmol L^{-1} [a]; Sensitivity: 324 nA $mmol^{-1}L$ [a]; D.L.: 0.001 mmol L^{-1} [a]	The enzyme in the membrane retains 50% activity after 3 months	[10]
β-Gal hydrolyses lactulose; generated fructose reacts with FDH in the presence of a tetrazolium salt giving a coloured compound	Spectrophotometric ($\lambda = 570$ nm)	L.R.: 0.058 – 0.233 mmol L^{-1}; D.L.: 0.03 mmol L^{-1}	–	[9]
Immobilization of β-Gal, FDH, and TTF by cross-linking onto a MPA-AuE	Electrochemical (+0.10 V vs. Ag/AgCl)	L.R.: 0.03 – 1.0 mmol L^{-1}; Sensitivity: 4.73×10^{-4} A $mol^{-1}L$; D.L. 9.6×10^{-6} mmol L^{-1}; L.R.: 0.05 – 1.0 mmol L^{-1} [a]; Sensitivity: 9.5×10^{-5} A $mol^{-1}L$ [a]; D.L. 1.6×10^{-5} mmol L^{-1} [a]	81 days	This work

[a] Flow system

enzyme loadings per assay) than the enzymatic spectrophotometric assay [9].

4. Conclusions

The lactulose biosensor constructed by coimmobilization of β-Gal, FDH and the mediator TTF atop a MPA-SAM on a gold disk electrode exhibits a good analytical performance, especially concerning its stability with time, and the simplicity and rapidity of the methodology involved, both by batch amperometry in stirred solutions and flow injection with amperometric detection. Its applicability for the analysis of UHT and sterilized milk samples implies, similarly to that reported previously for other enzymatic determinations of lactulose, the need of a blank signal subtraction from pasteurized milk which has a non-detectable lactulose content.

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6. References

[1] G. R. Andrews, *J. Dairy Res.* **1986**, *53*, 665.
[2] European Commission (EC) (**1992**) Dairy Chemists' Group-Doc VI/5726/92, Project de Decision de la Commission Fixant les Limites et les Methodes Permettant de Distinguer les Differents Types de Laits de Consommations Traites Thermiquement.
[3] M. Mayer, M. Genrich, W. Künnecke, U. Bilitewski, *Anal. Chim. Acta* **1996**, *324*, 37.
[4] A. Olano, M. M. Calvo, V. Corzo, *Food Chem.* **1989**, *31*, 259.
[5] L. A. Th. Verhaar, M. J. M. Van Der Aalst, J. A. W. M. Beenackers, B. F. M. Kuster, *J. Chromatogr.* **1979**, *170*, 363.

[6] J. Von Riel, C. Olieman, *Carbohydr. Res.* **1991**, *215*, 39.
[7] H. Geier, H. Klostermeyer, *Z. Lebensm. Unters Forsch.* **1980**, *171*, 443.
[8] Y. Sekine, E. A. H. Hall, *Biosens. Bioelectron.* **1998**, *13*, 995.
[9] A. Amine, D. Moscone, R. A. Bernardo, E. Marconi, G. Palleschi, *Anal. Chim. Acta* **2000**, *406*, 217.
[10] D. Moscone, R. A. Bernardo, E. Marconi, A. Amine, G. Palleschi, *Analyst* **1999**, *124*, 325.
[11] S. Campuzano, R. Gálvez, M. Pedrero, F. J. Manuel de Villena, J. M. Pingarrón, *Anal. Bioanal. Chem* **2003**, *377*, 600.
[12] S. Campuzano, R. Gálvez, M. Pedrero, F. . Manuel de Villena, J. M. Pingarrón, *J. Electroanal. Chem.* **2002**, *526*, 92.
[13] A. Begum, E. Kobatake, T. Suzawa, Y. Ikariyama, M. Aizawa, *Anal. Chim. Acta* **1993**, *280*, 31.
[14] *β-Galactosidase from Aspergillus sp*, Information bulletin, Toboyo Co., Ltd.
[15] A. S. N. Murthy, Anita, *Biosens. Bioelectronics* **1996**, *11*, 191.
[16] D. De Rafael, M. M. Calvo, A. Olano, *Milchwissenschaft* **1996**, *51*, 552.

