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Development of an integrated electrochemical biosensor for sucrose and its implementation in a continuous flow system for the simultaneous monitoring of sucrose, fructose and glucose

E. Vargas [a], M. Gamella [a], S. Campuzano [a], A. Guzmán-Vázquez de Prada [b], M.A. Ruiz [b], A.J. Reviejo [a], J.M. Pingarrón [a,*]

[a] Departamento de Química Analítica, Facultad de CC. Químicas, Universidad Complutense de Madrid, Avenida Complutense s/n, 28040 Madrid, Spain
[b] InBea Biosensores S.L. Parque Científico de Madrid, C/Santiago Grisolía 2, 28760 Tres Cantos, Madrid, Spain

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ABSTRACT

An integrated amperometric sucrose biosensor involving a 3-mercaptopropionic acid (MPA) self-assembled monolayer (SAM)-modified gold disk electrode (AuE) and coimmobilization of the enzymes invertase (INV) and fructose dehydrogenase (FDH) as well as the redox mediator tetrathiafulvalene (TTF) by means of a dialysis membrane is reported. Amperometry in stirred solutions at a detection potential of +0.10 V provided a linear calibration plot for sucrose over the 1.2×10^{-6}–3.0×10^{-3} mol L^{-1} concentration range, with a limit of detection of 3.6×10^{-7} mol L^{-1}. The practical usefulness of the biosensor was demonstrated by determining sucrose in condensed milk and in an infant food reference material with good results. Additionally, the biosensor was implemented together with commercial fructose and glucose amperometric biosensors in a continuous flow system to perform the multiplexed quantification of sucrose, fructose and glucose in a single experiment. The operational characteristics of the biosensors in this novel flow system were evaluated and their applicability was demonstrated through the simultaneous determination of the three sugars in the above-mentioned reference material.

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1. Introduction

Sucrose is the organic compound commonly known as table sugar and sometimes called saccharose [1]. Since sucrose is a component of foodstuffs and beverages, precise information on the sucrose presence and content is very important for assessment of food quality [2]. The determination of sucrose can be carried out by a wide variety of analytical methods such as polarimetry, isotope dilution, chromatography and optical methods, which exhibit some practical disadvantages including expensive equipment and quite complex sample pretreatment [3]. Other available methods based on determination of density or refractory index, though simpler and faster, are less precise and sensitive to the presence of interfering components. Therefore, there is a demand to develop fast, inexpensive, selective and sensitive methodologies for sucrose determination.

In this context, enzyme-based electrochemical biosensors have been found to constitute versatile analytical devices with high selectivity and meeting the above-mentioned requirements for sucrose determination [4–7]. Most of the enzymatic cascade reactions reported for this purpose are based on the INV/mutarotase/glucose oxidase multienzyme system coupled with electrochemical detection of the produced H_2O_2 or consumed oxygen [8,9]. Alternatively, the INV/mutarotase/glucose dehydrogenase system has been also used with the spectrophotometric or electrochemical detection of generated NADH. Oxygen consumption was also shown to be suitable for the detection of sucrose based on a microbial cell-based biosensor [10].

The knowledge of the qualitative and quantitative distributions of sugars in fruits, vegetables, honey and other different natural foods is essential because these compounds are the major constituents of these products, and are involved in very important characteristics, such as flavor, maturity, quality, authenticity, storage conditions (sugars content diminishes rapidly during storage at ambient temperature), etc. Therefore, the determination of sugars is highly relevant in the food industry [11]. Simultaneous multidetection of sugars is being traditionally approached by means of chromatographic separation coupled to different detection techniques [11]. Among these, the refractive index-based measurement remains the most commonly used due to the lack of more selective physical/chemical properties of

* Corresponding author. Tel.: +34 913944315; fax: +34 913944329.
E-mail address:
pingarro@quim.ucm.es (J.M. Pingarrón).

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carbohydrates [12]. Attempts to increase the sensitivity have been successfully achieved by pulsed amperometry [13,14] and mass spectrometry [15]. Irrespective of the sensitivity of the detection mode, the efficient separation of closely related carbohydrates is compromised by their co-elution, thereby needing sample derivatization [16]. The selective detection of carbohydrates has been mainly achieved through catalytic reactions that produce new species that can be easily measured [17,18].

Attempts for the parallel determination of different sugars using coupled enzymes in specifically adapted flow-injection systems were in principle successful either using enzymatically catalyzed consumption of the monosaccharide prior to the determination of the respective disaccharide or by subtraction of the monosaccharide concentration from the sum of the total sugar signal [19].

In should be noted that most of the sucrose biosensors developed so far were not suitable for the simultaneous determination of different sugars. Due to the enzyme reactions involved in the functioning of these biosensors, samples containing both sucrose and glucose are difficult to be quantified selectively because glucose present in the sample will interfere in the determination of sucrose. Therefore, glucose has either to be removed from the sample solution or the corresponding glucose response has to be subtracted from the total response [6]. To eliminate these problems, a multienzyme electrode system using a time lag arising from reaction and diffusion has been developed [9].

In this work an integrated biosensor for sucrose determination based on the system INV/FDH has been developed for the first time. Both enzymes and the redox mediator TTF were co-immobilized by physical entrapment using a semipermeable dialysis membrane on a gold disk electrode modified with a MPA-SAM. The analytical performance of this biosensor has been evaluated under batch conditions. Additionally, a simple flow analysis system was successfully developed to perform the simultaneous determinations of sucrose, fructose and glucose in a single experiment by the appropriate integration of this sucrose biosensor with commercial fructose and glucose biosensors developed previously by our group. The performance of the new INV/FDH/TTF biosensor for the analysis of real samples was demonstrated by analyzing sucrose in condensed milk and in a reference material by batch amperometry. Moreover, the simultaneous determinations of sucrose, fructose and glucose in a reference material were accomplished by amperometric detection at the corresponding biosensors under continuous flow conditions.

2. Materials and methods

2.1. Apparatus and electrodes

Amperometric measurements were carried with single and bi-channel amperometric detectors purchased from InBea Biosensores S.L. (Madrid, Spain). A P-Selecta ultrasonic bath and a P-Selecta Agimatic magnetic stirrer were also used. Flow experiments were carried out using a Spetec Perimax-12 peristaltic pump.

XBAS-NS-AU gold disk electrodes (Ø~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. Fructose and glucose commercial biosensors developed previously by our research group and commercialized by InBea Biosensores S.L. were employed for the analysis of fructose and glucose, respectively. A 10 mL glass electrochemical cell was used for batch experiments, while a homemade methacrylate wall-jet cell (10 mL) was employed for flow injection measurements.

2.2. Reagents and solutions

Stock 0.1 mol L^{-1} D(+)-sucrose (Fluka) and D(+)-glucose (Panreac) solutions were prepared in 0.05 mol L^{-1} phosphate buffer of pH 6.0, while stock 0.1 mol L^{-1} D(−)-fructose (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. A 40 mmol L^{-1} MPA (Aldrich) solution, prepared in a 75/25% v/v EtOH/H_2O mixture, was employed for the SAMs formation. A 15 U μL^{-1} INV solution (Sigma, EC 3.2.1.26 from *Saccharomyces cerevisae*, 332.8 U mg^{-1}) prepared in phosphate buffer solution of pH 6.0 and a 5.15 U μL^{-1} FDH solution (MP Biomedical EC 1.1.99.11 from *Gluconobacter sp.*, 169 U mg^{-1}) prepared in phosphate buffer solution of pH 4.5 were used for the preparation of the INV–FDH–TTF–MPA–AuE biosensor. Moreover, a 0.5 mol L^{-1} TTF (Aldrich) solution in acetone was prepared. Dialysis membranes (10 K MWCO) were purchased from Cultek®. Muva-KI-1102 infant food reference material containing sucrose (4.01 ± 0.10)%, fructose (1.06 ± 0.03)%, glucose (2.44 ± 0.04)%, starch (24.78 ± 1.59)% and vitamin C (0.08291 ± 0.00935)% was purchased from Muva Kempten®.

Other solutions employed were: 2 mol L^{-1} KOH (Panreac) in water, 0.1 mol L^{-1} stock solutions of lactose, D-fructose, L-arabinose (Sigma), D-glucose (Panreac), D-galactose, lactulose (Fluka), citric acid (Merck), malic acid (Merck) and ascorbic acid (Fluka), prepared in 0.05 mol L^{-1} phosphate buffer of pH 6.0.

All chemicals used were of analytical-reagent grade and water was obtained from a Millipore Milli-Q purification system.

2.3. Procedures

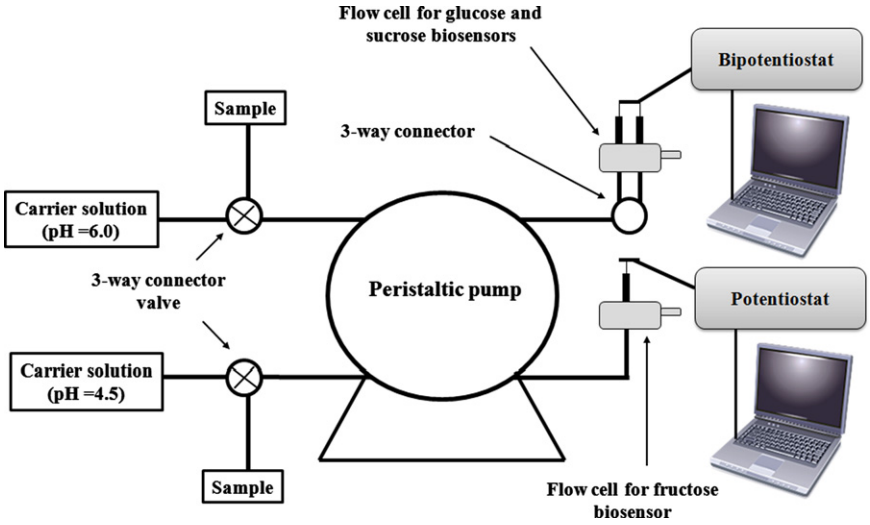
2.3.1. Sucrose biosensor construction

Before the SAM deposition, the gold disk electrode was pretreated as described previously [20]. MPA-SAMs were formed by immersion of the treated AuE in a 40 mmol L^{-1} MPA solution in EtOH/H_2O (75/25, v/v) for at least 15 h. The monolayer modified electrode was rinsed with deionized water to remove physically adsorbed thiols and dried with a nitrogen stream.

Coimmobilization of the enzymes and the mediator was carried out as follows: a 3-μL aliquot of the 0.5 mol L^{-1} TTF solution was deposited on the MPA-modified electrode surface. Once the surface dried at room temperature, a 4-μL aliquot of the 5.15 U μL^{-1} FDH solution was deposited and allowed to dry again. Then, a 3-μL aliquot of the 15 U μL^{-1} INV solution was dropped on the modified electrode waiting to dry 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. The use of the dialysis membrane gave rise to a more stable coimmobilization of the enzymes and the mediator on the modified AuE than that obtained using other immobilization approaches such as crosslinking with glutaraldehyde. In this latter case, a significant loss of enzymes was observed after immersion of the biosensor in the solutions.

2.3.2. Electrochemical detection

Amperometry in stirred solutions with the sucrose biosensor was performed by applying a potential of +0.10 V (vs Ag/AgCl). Flow measurements were made using the system depicted in Scheme 1. The flow system was designed to allow the simultaneous detection of glucose, sucrose and fructose and consists of two channels, one for monitoring glucose and sucrose and a second channel for monitoring fructose. The corresponding flow cells were connected to peristaltic pumps and three-way connector valves were placed at the beginning of each flow channel



Scheme 1. Schematic diagram of the flow system developed for the multiplexed detection of glucose, fructose and sucrose using enzyme bioelectrodes as amperometric biosensors.

to control the passage of carrier or sample solution. The carrier streams used were a 0.05 mol L^{-1} phosphate buffer of pH 6.0 for sucrose and glucose monitoring, and of pH 4.5 for the measurement of fructose. Sucrose, glucose and fructose biosensors were polarized at +0.10, 0.00 and +0.15 V vs Ag/AgCl, respectively.

2.3.3. Analysis in real samples

2.3.3.1. Determination of sucrose in condensed milk and in a reference material sample. As it will be commented below, no matrix effect was observed and, therefore, sucrose concentrations were calculated by interpolation of the corresponding amperometric signals recorded from the sample solutions into a calibration graph constructed with standard solutions. The samples were treated as follows:

- *Condensed milk*: approximately 0.5 g of sample were accurately weighed and dissolved by stirring in 10 mL of 0.05 mol L^{-1} phosphate buffer solution (pH 6.0). This sample was also analyzed after spiking with a known sucrose amount; approximately 0.5 g of sample and 0.058 g of sucrose were accurately weighed and dissolved by stirring in 10 mL of 0.05 mol L^{-1} phosphate buffer solution (pH 6.0).
- *Reference material*: about 0.25 g of sample were accurately weighed and dissolved by stirring in 5 mL of distilled water for 10 min. 2.5 mL of the resulting suspension were transferred to a 5-mL volumetric flask and diluted to the mark with 0.05 mol L^{-1} phosphate buffer solution of pH 6.0.

Subsequently, 20-μL aliquots of condensed milk samples (non-spiked or spiked samples) or 100 μL of the reference material solutions were added to the electrochemical cell containing 10 mL of 0.05 mol L^{-1} phosphate buffer solution, pH 6.0, which was used as the supporting electrolyte. The amperometric measurements in stirred solutions were carried out by applying a potential of +0.10 V (vs Ag/AgCl) and allowing the steady-state current to be reached.

2.3.3.2. Simultaneous determinations of sucrose, fructose and glucose in a reference material by amperometric detection under continuous flow conditions. The developed INV–FDH–TTF–MPA–AuE biosensor together with commercial fructose and glucose biosensors purchased from InBea Biosensores S.L. were employed for the multiplexed determination of the target analytes in the reference material. The analysis was implemented in a single experiment with the flow analysis system described in Section 2.3.2. The sample solution was prepared by weighing accurately 0.125 g approximately of the reference material and dissolving by stirring in 10 mL of distilled water for 10 min. Then, 2 mL of the resulting suspension were transferred to a 10-mL volumetric flask and diluted to the mark with 0.05 mol L^{-1} phosphate buffer solution of suitable pH for the analysis of each sugar (pH 6.0 for sucrose and glucose and pH 4.5 for fructose). The determination of each sugar was carried out by passing the samples through the appropriate flow channel, and interpolating the amperometric signal obtained into the calibrations plots constructed previously for each sugar under flow conditions.

Scheme 2. Scheme showing the enzyme and electrochemical reactions involved in the sucrose determination with a INV/FDH biosensor.

3. Results and discussion

3.1. Bienzyme biosensor for sucrose detection

The construction of a bienzyme biosensor for sucrose based on the system INV/FDH was addressed for the first time in this work. The biocatalytic reactions on which the functioning of the sucrose biosensor relied, are depicted in Scheme 2. INV catalyzes the hydrolysis of the disaccharide sucrose to α-D-glucose and β-D-fructose. Then fructose oxidation to 5-keto-D-fructose is specifically catalyzed by FDH, giving rise to the reduction of the enzyme PQQ (pyrroloquinoline quinone) group to H_2PQQ. Reoxidation of H_2PQQ is achieved by means of the mediator oxidized form, TTF^+, and the electrochemical oxidation of the TTF generated at the bioelectrode is used for monitoring the overall reaction [21]. The immobilization of the enzymes was carried out on an MPA-SAM-modified gold electrode due to the demonstrated SAM role in the improvement of the stability and sensitivity of the resulting enzyme biosensors [20,21].

3.1.1. Optimization of working variables

The optimization of all the experimental variables implied in the bienzyme biosensor performance was accomplished by amperometry in stirred solutions. Regarding biosensor preparation, only the influence of the INV loading was checked, whereas both FDH and TTF loadings were the same than those optimized previously for a fructose biosensor [21]. The response of the biosensor for 1.0×10^{-4} mol L^{-1} sucrose using a detection potential of +0.10 V increased with INV loading up to a value of 45 U and showed a decrease for larger loadings. Considering that the protocol used for the biosensor preparation implied a former immobilization of FDH, a high amount of INV could block the FDH active centers and decrease the rate of the coupled enzyme reaction. Consequently, the selected composition of the bienzyme electrode for further work was: 45 U INV/ 20.6 U FDH/1.5 micromol TTF.

The influence of the applied potential on the biosensor response to 1.0×10^{-4} mol L^{-1} sucrose was examined in the 0.00–0.30 V range (Fig. 1). A catalytic effect could be observed at TTF–INV–FDH–MPA–AuE between 0.00 and +0.30 V, with a maximum steady-state current measured at +0.10 V. The decrease of the amperometric response above +0.10 V is similar to that reported previously [20] and can be attributed to the non reversible oxidation of TTF^{+} to TTF^{2+} that decomposes and leaks from the electrode surface. Accordingly, a working potential of +0.10 V was chosen for further work to accomplish a sensitive detection, to ensure the stability of the integrated biosensor and also to minimize the effect of potential interferents able to be oxidized at the electrode surface. It should be noted than no amperometric signal was observed for sucrose in the whole potential range at TTF–FDH–MPA–AuE and TTF–MPA–AuE modified electrodes.

The influence of pH on the biosensor amperometric response for a sucrose concentration of 1.0×10^{-4} mol L^{-1} was evaluated over the 4.0–8.0 pH range (data not shown). Although the maximum activity was reported to occur at pH 4.5 for free INV [22] and between pH 4.5 and 5.0 for free FDH [23], immobilized INV showed activity over a broader pH range (4.5–6.0) [22,24]. In good agreement with these previous observations, the integrated biosensor provided the highest value for the steady-state current at pH 6.0. According to this, a 0.05 mol L^{-1} phosphate buffer solution of pH 6.0 was chosen as the working medium.

Under the selected conditions, the amperometric responses of the biosensor upon additions of sucrose or fructose at the same concentration level 1.0×10^{-4} mol L^{-1} were compared. A response 3.4 times higher was obtained when no previous hydrolysis reaction occurred, which is likely related to the ratio of the enzyme loadings and the hydrolyzed sucrose percentage under the experimental conditions employed.

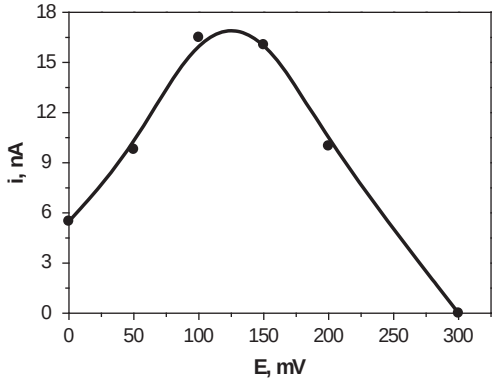


Fig. 1. Effect of applied potential on the amperometric signal measured for 1.0×10^{-4} mol L^{-1} sucrose in a 0.05 mol L^{-1} phosphate buffer solution (pH 6.0) at the INV/FDH biosensor.

3.1.2. Stability of the sucrose amperometric biosensor

Taking into account that the stability of the biosensor response is one of the most critical factors for assessing the possibilities of a biosensor to be applied in control processes and routine monitoring, different aspects concerning this aspect were considered.

The repeatability of the measurements was evaluated from the steady-state current values corresponding to repetitive measurements ($n=10$) of 1.0×10^{-3} mol L^{-1} sucrose, a RSD value of 4.2% being obtained. Moreover, the reproducibility of the responses obtained with five different INV–FDH–TTF–MPA–AuEs prepared in the same manner was also checked. A RSD value of 6.9% was calculated from the slope values of the corresponding calibration plots for sucrose in the 1.0×10^{-4}–2.0×10^{-4} mol L^{-1} concentration range. This result demonstrated that the fabrication procedure of the bienzymatic biosensor was reliable, allowing reproducible amperometric responses to be obtained with different biosensors constructed in the same manner following the described methodology. Finally, the lifetime of one single INV–FDH–TTF–MPA–AuE was evaluated by performing daily calibration graphs for sucrose in the above-mentioned concentration range. After use, the biosensor was stored in phosphate buffer of pH 6.0 at 4 °C. No statistically different slope values for the calibration plots were observed for the first 8 days.

3.1.3. Analytical characteristics of the bienzyme biosensor

A linear calibration plot for sucrose was obtained over the 1.2×10^{-6}–3.0×10^{-3} mol L^{-1} concentration range ($r=0.9991$) with a slope value of $(1.38\pm0.02)\times10^{5}$ nA mol^{-1} L, and an intercept of 17 ± 3 nA. The limits of quantification and detection were estimated according the IUPAC criteria, $10\times s_b/m$ and $3\times s_b/m$, respectively, where m is the slope of the calibration graph and s_b is the standard deviation of the blank current, which was measured each second for a period of time of 100 s before recording the analytical signal. The values obtained were 3.6×10^{-7} and 1.2×10^{-6} mol L^{-1}, respectively.

3.1.4. Selectivity

The effect of potential interfering compounds on the biosensor response was investigated under the experimental conditions specified above. The substances tested were glucose, fructose, arabinose, galactose, lactose, lactulose and ascorbic, citric and malic acids. The influence of these compounds, at a concentration level of 5.0×10^{-4} mol L^{-1}, on the quantification of sucrose was tested. As can be observed in Fig. 2, among all of these compounds, only ascorbic acid and fructose gave rise to a significant amperometric response under working conditions. The interference of fructose was not unexpected due to the presence of FDH in the biosensor. This problem can be overcome by removing fructose prior to sucrose measurement or, as it will be demonstrated in the following Section, by performing mathematical calculations. The ascorbic acid interference was due to the electrochemical oxidation of this compound at the applied

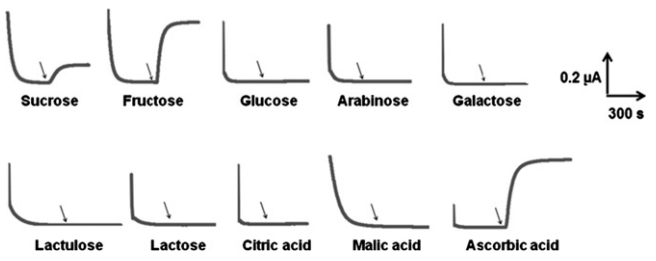


Fig. 2. Amperometric responses obtained at the INV–TTF–FDH–AuE biosensor upon 50 µL additions of 0.1 mol L^{-1} solutions of different interfering compounds to 0.05 mol L^{-1} phosphate buffer (pH 6.0). $E_{app}=+0.10$ V (vs Ag/AgCl).

potential to the bioelectrode, and to the reported catalytic oxidation of ascorbic acid by TTF [25]. Nevertheless, it is important to remark that the content of sucrose in the samples that we have analyzed is high enough with respect to the possible content of ascorbic acid to ensure that no significant interference should be produced. These results clearly demonstrate the appropriate selectivity of the developed biosensor for the determination of sucrose in dairy products containing other saccharides or organic acids.

3.1.5. Determination of sucrose in real samples

Sucrose was determined in a commercial sample of condensed milk and in a reference material by using the developed biosensor. As it was commented in Section 2.3.3., no matrix effect was observed in any of these two samples. Indeed, the slope values of the calibration graphs constructed by applying the standard additions method for both samples were not statistically different, using the Student's test, to that of the calibration graph prepared with sucrose standards in the 1.0×10^{-4}–2.0×10^{-4} mol L^{-1} concentration range. Therefore, analyses could be accomplished in all cases by interpolation of the corresponding amperometric signal into the calibration plot obtained with standards.

The condensed milk sample was analyzed according to the protocol described in the Section 2.3.3.1. The results obtained from three replicates yielded a mean sucrose concentration of $41.2\pm0.9\%$ (RSD=1.7%), the confidence interval being calculated for $\alpha=0.05$. The content value obtained is included between the sucrose ranges reported for condensed milk samples [26]. The accuracy of the determination was evaluated also by carrying out recovery studies by adding a known amount (0.058 g) of sucrose to this sample. A mean recovery of $97\pm1\%$ (RSD=1.9%) was obtained from five replicates of these fortified samples. Taking into account the straightforwardness of the experimental protocol, this can be considered as a very good result, demonstrating the usefulness of the developed biosensor to perform the analysis just upon a simple dilution of the sample.

Sucrose was also determined in a reference material which contained a significant amount of fructose (see Section 2.2.). Therefore, the significant interference from this compound on the biosensor response commented above was addressed by means of the following mathematical correction. A "*K*" factor, defined as the ratio between the current values measured with the same INV–FDH–TTF–MPA–AuE biosensor for sucrose and fructose standards at the same molar concentration within the range of linearity of the biosensors, was defined:

$$K=\frac{i_{\text{sucrose}}}{i_{\text{fructose}}} \tag{1}$$

As the biosensor response is affected by free fructose in the sample, the following equation was employed to calculate sucrose concentration:

$$[\text{sucrose}]=\frac{(i_s/m_s)-(i_f/m_f)}{K} \tag{2}$$

where i_s is the current measured with the INV–FDH–TTF–MPA–AuE biosensor for the sample (corresponding to sucrose and fructose); i_f the signal for fructose in the sample measured with a fructose biosensor; m_s is the slope of the single-pointed calibration obtained with fructose standard (at a concentration of 3.0×10^{-5} mol L^{-1}) with the INV–FDH–TTF–MPA–AuE biosensor; m_f is the slope of the single-pointed calibration obtained with fructose standard with the fructose biosensor and K the factor given by Eq. (1).

By applying this protocol, a sucrose concentration value of $4.0\pm0.8\%$ with a RSD value of 7.6% ($n=4$) was obtained in the analysis of the reference material. Considering that the certified sucrose content was $4.01\pm0.10\%$, the Student's t test ($t_{CAL}=1.73$ vs $t_{TAB}=4.303$, for a significance level of 0.05; $n=3$) confirmed that there were no significant differences between the result obtained with the developed methodology and the concentration value provided by the reference material, demonstrating also the possibility of quantifying the sucrose content in samples containing fructose in a significant amount with the developed biosensor.

3.2. Amperometric multiplexed detection of sucrose, glucose and fructose using a continuous flow system

To perform the simultaneous detection of sucrose, glucose and fructose, we developed a continuous flow system using as amperometric biosensors the novel sucrose biosensor developed and two commercial biosensors for glucose and fructose. The experimental variables affecting the responses of the biosensors in the continuous system were first optimized. As can be seen in Scheme 1, the determinations of glucose and sucrose were performed at the same flow cell and 0.05 mol L^{-1} phosphate buffer of pH 6.0 was used as the carrier solution in order to achieve a good sensitivity with both biosensors. Fructose detection was accomplished at a different flow cell and using 0.05 mol L^{-1} phosphate buffer of pH 4.5 as the carrier solution because the biosensor lifetime was demonstrated to be much shorter at pH 6.0 [27].

Characteristic flow parameters such as the flow rate and the sample volume were optimized. Experiments regarding the effect of flow conditions on the detection potential showed that the same potential value used under batch conditions provided high current responses and, therefore, we decided to keep the same value, +0.10 V, for the detection of sucrose. Regarding fructose and glucose biosensors, the detection potential values applied (+0.15 V and 0.00 V, respectively) were those recommended by the supplier (InBea Biosensores S.L.). The effect of flow rate was checked at a 30 s fixed time of sample passing through the flow system. The peak current increased with the flow rate up to approximately 25 μL s^{-1}, with a significant decrease for higher flow rates. As a compromise between sensitivity and sampling frequency, flow rates of 4.9, 14.3 and 10 μL s^{-1} were chosen for glucose, fructose and sucrose, respectively. Regarding optimization of the sample volume introduced into the flow system, it was varied between 49 μL and 342 μL for glucose, 143 μL and 1000 μL for fructose and 100 μL and 700 μL for sucrose. These volume ranges are fixed by the different lengths to be covered by the flowing solution for a fixed initial flow value. Using the flow rates mentioned above for each flow channel, these sample volumes implied time range between 10 and 70 s. An increase in the peak current with the sample volume introduced was observed and, therefore, in order to achieve the highest sensitivity for the three biosensors, samples were introduced into the system during 70 s, corresponding to sample volumes of 342 μL (glucose), 1000 μL (fructose) and 700 μL (sucrose). Larger sample volumes (i.e., longer introduction times) produced flow disturbances with generation of bubbles.

The repeatability of the flow amperometric responses was evaluated from series of 10 repetitive measurements from 4.0×10^{-4} mol L^{-1} glucose, 3.0×10^{-5} mol L^{-1} fructose and 1.0×10^{-3} mol L^{-1} sucrose (Fig. 3). RSD values for i_p of 1.8%, 3.2% and 5.4% were obtained for the measurements carried out with the fructose, glucose and sucrose biosensors, respectively, thus demonstrating the good repeatability of their flow amperometric responses in spite of the hydrodynamic conditions. The direction of recorded peaks reflects the oxidation or reduction currents measured.

Under the optimized flow conditions, typical calibration curves for enzyme systems were obtained with three biosensors. Table 1 summarizes the analytical characteristics of the corresponding

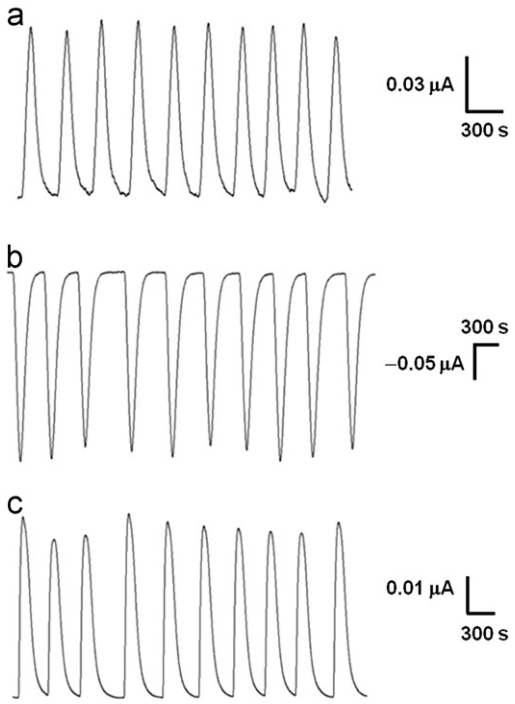


Fig. 3. Flow amperometric responses obtained for 10 repetitive measurements at: (a) the INV/FDH biosensor for 1.0×10^{-3} mol L^{-1} sucrose; (b) the glucose biosensor for 4.0×10^{-4} mol L^{-1} glucose and (c) the fructose biosensor for 3.0×10^{-5} mol L^{-1} fructose. Other experimental conditions as described in Section 3.2.

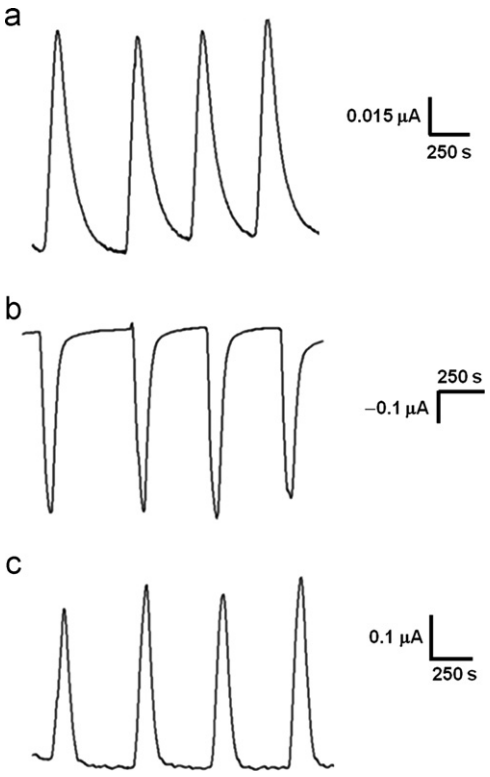


Fig. 4. Amperometric flow responses obtained at a sucrose (a), glucose (b) and fructose (c) biosensor for the multiplexed determination of these three sugars in a reference material. Other experimental conditions are as in Fig. 3.

Table 1
Analytical characteristics obtained from the corresponding calibration plots for fructose, glucose and sucrose using amperometric detection under continuous flow conditions.

Biosensor	Linear range (mol L^{-1})	Slope (nA mol^{-1} L)	r	LOD (mol L^{-1})
Fructose	$(0.0021–1.2)\times10^{-3}$	$(3.30\pm0.02)\times10^{6}$	0.999	6.4×10^{-7}
Glucose	$(0.023–5.0)\times10^{-4}$	$(9.81\pm0.16)\times10^{5}$	0.999	7.0×10^{-7}
Sucrose	$(0.0034–1.0)\times10^{-3}$	$(1.50\pm0.04)\times10^{5}$	0.999	1.0×10^{-6}

Table 2
Determination of fructose, glucose and sucrose in a reference material, using glucose, fructose and sucrose bioelectrodes as amperometric biosensors using the developed continuous flow system.

	Certified content	Biosensors			
Sugar	**Sugar** (%)	**Sugar** (%)	**RSD**$_{n=4}$ (%)	t_{CAL}	t_{TAB}
Fructose	(1.06 ± 0.03)	(1.2 ± 0.2)	9.2	1.175	3.182
Glucose	(2.44 ± 0.04)	(2.6 ± 0.2)	5.0	1.003	3.182
Sucrose	(4.01 ± 0.10)	(4.3 ± 0.6)	9.1	1.073	3.182

calibration graphs. The limits of detection were calculated according to the same criterion commented in Section 3.1.3. It is worth mentioning that these analytical characteristics are slightly better than those reported when working with individual glucose and fructose biosensors in a conventional FIA system [20,21], which demonstrated that no significant worsening in the analytical performance towards the analytes was produced when the bioelectrodes are used as amperometric biosensors in the developed flow system. These results demonstrated that the biosensors could be used in the flow system exhibiting good analytical performance and sampling frequencies of around 10 samples per hour which constitute relevant characteristics for applications in the food industry.

3.2.1. *Multiplexed electrochemical detection of sugars in a certified material under flow conditions*

In order to address and minimize the interference of fructose under flow conditions, we performed calibrations plots by introducing, through the flow channel of glucose and sucrose, mixed standard solutions containing sucrose in the range 2.9×10^{-4}–8.8×10^{-4} mol L^{-1}, glucose in the range 1.7×10^{-4}–5.0×10^{-4} mol L^{-1} and a constant fructose concentration of 1.7×10^{-4} mol L^{-1}. As the presence of free fructose affected only the sucrose biosensor response, the calibration graph for this analyte was constructed by subtracting, from the total measured current provided by the INV–FHD–TTF–MPA–AuE biosensor, the signal corresponding to the 1.7×10^{-4} mol L^{-1} fructose standard measured with the same biosensor:

$$i_{s(s)} = i_{T(s)} - i_{f(s)} \quad (3)$$

where $i_{s(s)}$ is the amperometric signal of sucrose standard; $i_{T(s)}$ is the amperometric signal of mixed standards of sucrose and fructose and $i_{f(s)}$ is the fructose standard amperometric signal.

In order to measure the amperometric response corresponding to sucrose in the sample to be analyzed and thus quantify sucrose by simple interpolation into the above calibration plot, the current corresponding to the free fructose measured the sucrose biosensor was subtracted from the total response:

$$i_s = i_T - i_f \quad (4)$$

where i_s is the amperometric signal due to sucrose only; i_T is the amperometric response obtained for the sample (sucrose and fructose) and i_f is the free fructose amperometric signal calculated

Table 3
Comparison of the analytical characteristics reported for different electrochemical biosensors for the determination of sucrose.

Enzymes/ mediator	Electrode	Immobilization	*E* (V)	Linear range (mol L^{-1})	LD (mol L^{-1})	Sensitivity (nA mol^{-1} L)	Sample	Ref.
GOD, INV	Pt	Electrostatic interaction with the oxidized polymer ($PVF^{+}ClO^{4-}$) film	+0.70 vs SCE	$(0–6)\times10^{-2}$	–	–	–	[4]
GOD, INV, MUT/ TCNQ	Graphite paste	MUT covalently coupled to CPG. Entrapment of GOD, INV, and TCNQ in graphite paste	+0.20 vs Ag/AgCl	2.5×10^{-5}–2.0×10^{-1}[a]	–	3.5×10^{9}[a]	–	[28]
GOD, INV, MUT	Glassy carbon	Crosslinking with PVA–SbQ polymer	1.0 vs Ag/ AgCl	1.0×10^{-5}–6.0×10^{-3}	1.0×10^{-5}	–	Jam and fruit juice	[29]
GOD, INV, MUT	Pt	GOD entrapped in a poly-1,3-DAB film. MUT/INV crosslinking with glutaraldehyde	+0.70 vs Ag/AgCl	1.0×10^{-3}–4.0×10^{-2}[a]	–	–	Soft drinks	[8]
SP, PGM, G-6PDH/ Osphendione	CPE	Entrapment in the CPE	+0.15 vs Ag/AgCl	$(1.0–5.0)\times10^{-3}$[a]	1×10^{-3}[a]	1.0×10^{3}[a]	Fruit juices	[6]
GOD, INV, MUT/ PB	Glassy carbon	Crosslinking with glutaraldehyde and BSA on the PB modified electrode	−0.05 vs Ag/AgCl	$(4.0–800.0)\times10^{-6}$[a]	4.5×10^{-6}[a]	2.82[a]	–	[24]
INV, MUT, GDH/ Osphendione	CPE	Entrapment in the CPE	+0.15 vs Ag/AgCl	$(1.0–12.0)\times10^{-3}$[a]	1.0×10^{-4}[a]	–	Fruit juices and milk	[18]
GOD, INV, MUT	Thin layer enzyme cell	Enzymes immobilized in a BSA membrane with glutaraldehyde	+0.60 vs Ag/AgCl	1.0×10^{-4}–2.5×10^{-3}	–	–	Fruit juices	[9]
INV, FDH/TTF	Gold disk electrode modified with a MPA-SAM	Entrapment with a dialysis membrane	+0.10 vs Ag/AgCl	1.2×10^{-6}–3.0×10^{-3} 3.4×10^{-6}–1.0×10^{-3}[a]	3.6×10^{-7} 1.0×10^{-6}[a]	$(1.38\pm0.02)\times10^{5}$ $(1.50\pm0.04)\times10^{5}$[a]	Condensed milk and infant food reference material	This work

CPE: carbon paste electrode; DAB: 1,3-diaminobenzene; FDH: fructose dehydrogenase; GDH: glucose dehydrogenase; GOD: glucose oxidase; G-6PDH: glucose-6-phosphate 1-dehydrogenase; INV: invertase; MPA: 3-mercaptopropionic acid; PB: Prusian blue; PGM: phosphoglucomutase; SAM: self-assembled monolayer; SP: sucrose phosphorylase; TCNQ: tetracyanoquinodimethane; TTF: tetrathiafulvalene.

[a] Measurements in continuous mode.

employing the following equation:

$$i_f = [\text{fructose}] \times m_f \quad (5)$$

where i_f is the free fructose signal; [fructose] is the fructose concentration determined with the commercial fructose biosensor and m_f is the single-pointed calibration slope obtained for a fructose standard current measured with the INV–FDH–TTF–MPA–AuE biosensor in the flow system.

Glucose and fructose contents were determined simply by interpolating the signals obtained from the samples into their respective calibration plots.

Fig. 4 shows the amperometric flow responses obtained at a sucrose (a), glucose (b) and fructose (c) biosensor for the multiplexed determination of these three sugars in the reference material. The results are summarized in Table 2. The confidence intervals were calculated for a significance level of 0.05 and RSD values were in all cases $<10\%$. Validation of the obtained results was accomplished by applying a Students' t test. As it can be deduced from Table 2, t_{CAL} values were in all cases lower than t_{TAB} values (for a significance level of 0.05; $n=4$). Therefore, it could be concluded that no significant differences existed between the result obtained by applying the developed methodology and the certified content value for the three sugars.

3.3. Comparison with other sucrose biosensors reported in literature

We have compared the analytical performance of the TTF–FDH–INV–MPA–AuE biosensor with those reported in literature for other sucrose electrochemical biosensors. It is important to emphasize here that this is the first time that a biosensor for sucrose is developed using the system INV/FDH. Characteristics such as the type of used enzyme immobilization and mediator, measurement potential, linearity range of the corresponding calibration graph, sensitivity (slope value of this calibration), limit of detection achieved, and stability of the biosensors are listed in Table 3. In general, it can be claimed that the biosensor presented here possess relevant advantages such as the simple enzymes and mediator immobilization procedure, the wide linear dynamic concentration range and the detection limits achieved both under batch and flow conditions which are among the lowest reported until now. In this context, it should be remarked that the detection is accomplished at a detection potential of $+0.10$ V (vs Ag/AgCl) and, therefore, the interference from electroactive substances can be minimized.

4. Conclusions

The results described above demonstrate fairly well that the use of a novel integrated INV–FDH–TTF–MPA–AuE amperometric biosensor for sucrose accomplishes the requirements of precision, rapidity, sensitivity, simplicity, and low cost required to be considered as a useful analytical tool for the food industry. The developed sucrose biosensor has been successfully implemented together with two other commercialized biosensors in a novel continuous flow system allowing the multiplexed quantification of sucrose, fructose and glucose in a single experiment. The demonstrated good performance, stability of the three biosensors and the usefulness of the developed continuous system in the analysis of a reference material show great promise as an affordable and useful analytical tool for the quality control food industry.

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