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An integrated bienzyme glucose oxidase–fructose dehydrogenase–tetrathiafulvalene-3-mercaptopropionic acid–gold electrode for the simultaneous determination of glucose and fructose

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Abstract

A bienzyme biosensor for the simultaneous determination of glucose and fructose was developed by coimmobilising glucose oxidase (GOD), fructose dehydrogenase (FDH), and the mediator, tetrathiafulvalene (TTF), by cross-linking with glutaraldehyde atop a 3-mercaptopropionic acid (MPA) self-assembled monolayer (SAM) on a gold disk electrode (AuE). The performance of this bienzyme electrode under batch and flow injection (FI) conditions, as well as an amperometric detection in high-performance liquid chromatography (HPLC), are reported. The order of enzyme immobilisation atop the MPA-SAM affected the biosensor amperometric response in terms of sensitivity, with the immobilisation order GOD, FDH, TTF being selected. Similar analytical characteristics to those obtained with single GOD or FDH SAM-based biosensors for glucose and fructose were achieved with the bienzyme electrode, indicating that no noticeable changes in the biosensor responses to the analytes occurred as a consequence of the coimmobilisation of both enzymes on the same MPA–AuE. The suitability of the bienzyme biosensor for the analysis of real samples under flow injection conditions was tested by determining glucose in two certified serum samples. The simultaneous determination of glucose and fructose in the same sample cannot be performed without a separation step because at the detection potential used (+0.10 V), both sugars show amperometric response. Consequently, HPLC with amperometric detection at the TTF–FDH–GOD–MPA–AuE was accomplished.

Glucose and fructose were simultaneously determined in honey, cola softdrink, and commercial apple juice, and the results were compared with those obtained by using other reference methods.

Keywords: Glucose; Fructose; Bienzyme biosensor; Self-assembled monolayers; Amperometric detection

1. Introduction

Nowadays, much attention is devoted to the determination of food components using specific biosensors. Thus, in many food processes, combinations of sugars, especially glucose and fructose, are particularly important, and measurements of these species are necessary for effective process and product control, nutritional purposes, and monitoring of the ripening of fresh fruits [1,2]. An interesting alternative to classical methods for the analysis of different saccharides would be the development of a biosensor for the continuous determination of analytes. This biosensor should allow both the specific determination of each sugar and an easy automation of the process [3]. Concerning the simultaneous determination of glucose and fructose in the same sample, the only amperometric biosensor for this purpose found in the literature was developed by Antiochia and Palleschi [4]. This biosensor involved coimmobilisation of three enzymes, fructose dehydrogenase (FDH), glucose dehydrogenase, and diaphorase, on the surface of platinum electrode, and allowed the batch determination of fructose and glucose in the 5.0×10^{-6}–2.0×10^{-4} M concentration range. The biosensor retained 50% of enzymatic activity after 8 days and was successfully applied for the determination of both analytes in a honey sample.

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The determination of glucose and fructose is not only of interest in the food area. Thus, besides the well-known need of control of the glucose level in the blood, low concentrations of fructose are present in the blood and urine of healthy individuals. However, high fructose concentrations are found in the blood, as well as excretions, of patients affected by essential fructosuria, a relatively rare disease characterised by the inability of accepting fructose from the diet [5].

Recently, we have reported on the construction and performance of robust integrated amperometric biosensors for glucose [6] and fructose [7] in which the corresponding enzymes [glucose oxidase (GOD) and FDH, respectively] were coimmobilised, together with the mediator, tetrathiafulvalene (TTF), by cross-linking with glutaraldehyde atop a 3-mercaptopropionic acid (MPA) self-assembled monolayer (SAM) on a gold disk electrode (AuE). This biosensor design led to a very good analytical performance in terms of sensitivity, time of response, stability, and reproducibility when compared with other biosensors reported in the literature. Moreover, these biosensors are useful in the analysis of real samples. Since, except pH, both amperometric biosensors operate at similar experimental conditions, the development of a bienzyme biosensor for the simultaneous determination of glucose and fructose by coimmobilising GOD, FDH, and TTF on the MPA-modified AuE should be possible. In this article, the performance of this bienzyme electrode under batch and flow injection (FI) conditions, as well as an amperometric detector in high-performance liquid chromatography (HPLC), is reported.

2. Experimental

2.1. Apparatus and electrodes

Voltammetric and amperometric measurements were performed with an ECO Chemie Autolab PSTAT 10 potentiostat using the software package GPES 4.9. A P-Selecta ultrasonic bath and a P-Selecta Agimatic magnetic stirrer were also used. FI experiments were carried out using a Gilson Minipuls-2 peristaltic pump and a Rheodyne Model 5020 injection valve with variable injection volumes. Chromatographic measurements were performed using a Jasco PU-980 HPLC pump and a Rheodyne 7725i valve, connected to a Polymer Labs PL Hi-Plex Pb column (300 × 7.7 mm).

A Metrohm 6.1204.020 (3 mm ϕ) gold disk electrode, pretreated as described previously [6], was used as the electrode substrate to be modified with the alkanethiol, SAM. A BAS MF-2063 Ag/AgCl/KCl 3 M reference electrode and a Pt wire counter electrode were also used. A 10-ml glass electrochemical cell was employed for batch measurements, while a large-volume (50 ml) home-made glass wall jet cell was used for flow measurements.

2.2. Reagents and solutions

A concentration of 0.5 M glucose (Panreac) or fructose (Sigma) stock solution was prepared in 0.05 M phosphate buffer of pH 5.5 and 4.5, respectively, and let to stand overnight before use to allow equilibration of anomers. More dilute standards were prepared by suitable dilution with 0.05 M phosphate buffer, pH 6.5, which was used as supporting electrolyte both in batch and flow injection measurements.

A 40-mM mercaptopropionic acid (MPA) solution, prepared in a 75/25% vol/vol ethanol/water mixture, was used for the formation of SAMs.

A 5.1-U μl^{-1} FDH (EC 1.1.99.11 from *Gluconobacter* sp., 112 U mg^{-1}; Sigma) solution and a 2.5-U μl^{-1} GOD (EC 1.1.3.4, type X-S from *Aspergillus niger*, 245,900 U g^{-1}; Sigma) solution were prepared in the phosphate buffer solution of pH 4.5 or 5.5, respectively. A 25% glutaraldehyde (Aldrich) solution and a 0.5-M TTF (Aldrich) solution in acetone were also prepared.

Other solutions employed were: a 2-M KOH (Panreac) solution prepared in deionised water; a 0.1-M uric acid (Merck) stock solution prepared in 0.01 M NaOH; 0.25-M stock solution of caffeine, ascorbic acid, citric acid, and urea (Merck); D-galactose (Sigma), L-arabinose (BDH), and D-sucrose (Fluka) prepared in 0.05 M phosphate buffer of pH 6.5. All chemicals used were of analytical reagent grade, and water was obtained from a Millipore Milli-Q purification system.

A D-glucose/D-fructose test combination kit (R-biopharm) was used to compare the results obtained in the analysis of fructose in real samples.

2.3. Procedures

MPA-SAMs were formed by immersion of the pretreated AuE in the 40-mM MPA solution mentioned above for 15 h. Then the modified electrode was rinsed with deionized water.

Immobilisation of enzymes by cross-linking with glutaraldehyde was carried out as follows: 5 μl of the 2.5-U μl^{-1} GOD solution was deposited on the MPA-modified AuE and let to dry at ambient temperature. Then, 4 μl of the 5.1-U μl^{-1} FDH solution was also deposited on the electrode surface. Once the electrode surface had dried, a 3-μl aliquot of the 0.5-M TTF solution was deposited and let to dry again. Then, the electrode was immersed in the 25% glutaraldehyde solution for 1 h at 4 °C.

Amperometric measurements in stirred solutions were carried out at a potential of +150 mV vs. Ag/AgCl and steady-state current was allowed to be reached.

FI and HPLC measurements with amperometric detection were carried out at an applied potential of +100 mV vs. Ag/AgCl. The carrier stream for FI experiments was a 0.05-M phosphate buffer of pH 6.5 with a flow rate of 1.40 ml min^{-1}. According to the column specifications, the mobile

phase used in HPLC experiments was filtered Milli-Q water with a flow rate of 0.6 ml min^{-1}.

2.4. Sample treatment

Precinorm 4® and Precipath 4® (Roche) blood serum samples with certified glucose content were analysed by FI with amperometric detection. The only sample treatment required consisted reconstitution with 5.0 ml of deionized water and appropriate dilution with the carrier solution: 1:10 and 1:25 for Precinorm 4 and Precipath 4, respectively.

Fructose and glucose were analysed in three real samples: honey, cola softdrink, and commercial apple juice, by HPLC with amperometric detection. Honey samples were heated in a water bath at 60 °C for 15–20 min until the sample was melted. Then 0.25 g of honey was accurately weighed and dissolved in 25 ml of Milli-Q water. A 600-μl aliquot of this solution was diluted to 10.0 ml of Milli-Q water, and then 20 μl of this solution was injected into the column.

In the case of the cola sample, this was degassed by sonication for some minutes. Then, 0.5 ml was dissolved in 10.0 ml of Milli-Q water, and the resulting solution was 10-fold diluted with Milli-Q water. A 20-μl aliquot of this solution was then injected into the column. Concerning the analysis of the commercial apple juice, only a 1:1000 sample dilution in Milli-Q water was necessary prior to the injection of a 20-μl aliquot.

The obtained results with these food samples were compared with those obtained with a commercial enzyme kit from R-biopharm using spectrophotometric detection. This method is based on the phosphorylation of D-glucose and D-fructose by hexokinase and adenosine-5′ -triphosphate (ATP) to D-glucose-6-phosphate and D-fructose-6-phosphate with the formation of adenosine-5′ -diphosphate (ADP). D-fructose-6-phosphate is converted by phosphoglucose isomerase to D-glucose-6-phosphate, and this later is oxidised to D-gluconate-6-phosphate by nicotinamide–adenine–dinucleotide phosphate (NADP) in the presence of the enzyme, glucose-6-phosphate dehydrogenase. The reduced form of NADP, NADPH, formed in the D-glucose-6-phosphate oxidation is measured spectrophotometrically at 340 nm.

Glucose in the cola sample was also analysed by an FI method proposed by Mottola [8], in which a spectrophotometric detection of the reaction product formed in the reaction between H_2O_2 and *o*-dianisidine was employed.

Fructose in the apple juice was analysed by applying Taylor's method [9]. Thus, 2.8 ml of 75% (vol/vol) sulfuric acid and 0.1 ml of 2.5% (wt/vol) cysteine (Sigma) solution were added to 0.1 ml of diluted sample solution (7.5 μl of sample aliquot diluted to 10.0 ml with deionised water). The mixture was heated and kept at 45 °C for approximately 10 min in a water bath, and then mixed with 1.0 ml of 0.1 M HCl containing 100 μg of tryptophan (Sigma). After standing for 20 min at room temperature, the absorbance was read at 518 nm. As no matrix effect was observed, the content of fructose was calculated by interpolation of the absorbance in a calibration graph constructed with standard fructose solutions in the 0.1–0.6 mM concentration range subjected to the same procedure.

3. Results and discussion

The biosensor design, using TTF as mediator, implies that both the enzyme reactions with GOD and FDH were monitored by the electrochemical oxidation of TTF. Fig. 1 shows cyclic voltammograms obtained at the TTF–FDH–GOD–MPA–AuE from a phosphate buffer background solution and from a 5-mM glucose (a) or fructose (b) solution, respectively.

3.1. Optimization of variables

The optimisation of the working variables affecting the behaviour of the bienzyme biosensor was carried out taking

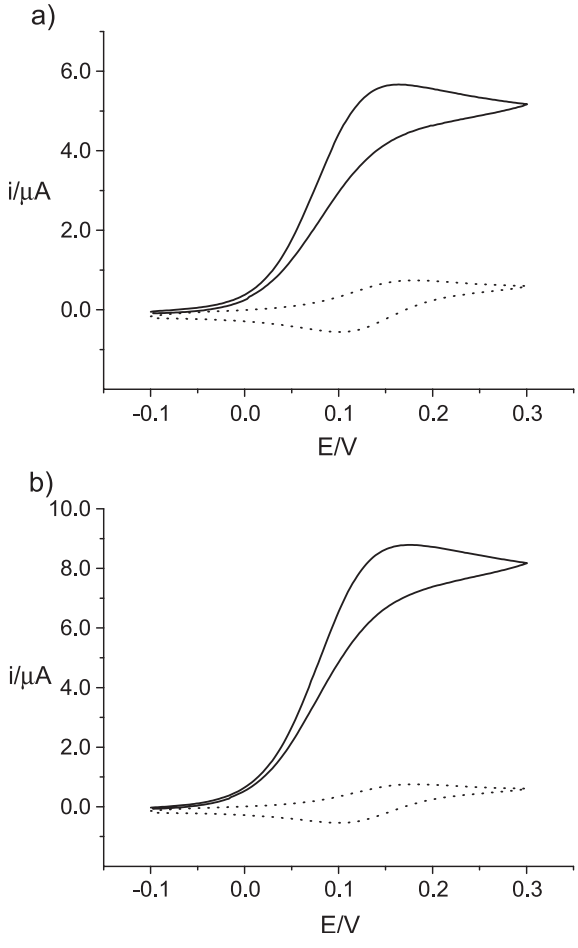

Fig. 1. Cyclic voltammograms obtained at 25 mV s^{-1} for the background solution (phosphate buffer of pH 6.5) (. . .) and for 5.0 mM glucose (a) and fructose (b) solutions at TTF–FDH–GOD–MPA–AuE.

as criterion of selection of the highest slope value obtained both for the glucose and fructose calibration graphs in the 2.0×10^{-4}–1.0×10^{-3} M concentration range.

It was verified that the order of enzyme immobilisation atop the MPA-SAM affected the biosensor amperometric response in terms of sensitivity. According to the results obtained, the immobilisation order GOD, FDH, TTF was that selected for the fabrication of the biosensors. The enzymes loadings used, as well as that of TTF, were the same as those optimised in previous works [6,7].

The effect of the pH on the bienzyme amperometric response was evaluated over the 4.5–7.5 range. As expected, higher slope values were obtained for fructose in the 5.0–5.5 pH range, while for glucose, they were in the 7.0–7.5 pH range. As a compromise to achieve good sensitivity for both analytes with the same biosensor, a phosphate buffer of pH 6.5 was chosen for further work.

Concerning the applied potential for amperometry under batch conditions, the bienzyme electrode exhibited a similar behaviour to that reported previously for each single GOD or FDH–TTF–MPA–AuE [6,7], with a maximum limiting current in the +0.15–+0.20 V range.

3.2. Operational characteristics of the TTF–FDH–GOD–MPA–AuE amperometric biosensor under batch conditions

The repeatability of the amperometric responses at +0.15 V was checked by performing 10 successive measurements from solutions formed with 10 ml of 0.05 M phosphate buffer of pH 6.5, to which 10 or 50 μl of 0.1 M fructose or glucose solution was added, respectively. Relative standard deviations (RSD) of 2.8% and 1.7% for the limiting current were obtained for fructose and glucose, respectively. Moreover, 10 successive calibration plots were constructed both for glucose and fructose in the 0.1–1.0 mM concentration range with the same biosensor. RSD values of 3.8% and 5.1% were obtained, respectively, for the slopes of such calibration graphs, indicating good reproducibility of the measurements with no cleaning or pretreatment of the modified electrode.

Linear calibration graphs were obtained for glucose and fructose over the concentration ranges 1.0×10^{-5}–1.0×10^{-2} M and 1.0×10^{-5}–2.0×10^{-3} M, with slope values of $(9.7 \pm 0.3) \times 10^{-4}$ and $(1.9 \pm 0.1) \times 10^{-3}$ A M^{-1}, respectively. The limits of detection were calculated according to the $3s_b/m$ criteria, where m is the slope of the calibration plots and s_b is estimated as the standard deviation ($n = 10$) of the amperometric signals from 1.0×10^{-5} M glucose or fructose. These values were 2.6×10^{-6} and 2.7×10^{-6} M, respectively. These analytical characteristics were similar to those obtained with the single-enzyme SAM-based biosensors for glucose and fructose, in spite of the somewhat different working conditions (mainly pH). This indicates that no noticeable changes in the biosensor responses to the analytes occurred as a consequence of the coimmobilisation of the both enzymes on the same MPA–AuE.

3.3. Flow injection with amperometric detection at the TTF–FDH–GOD–MPA–AuE

The main objective of this work was to evaluate the performance of the bienzyme biosensor under flowing conditions in order to use it for a simultaneous measurement of glucose and fructose in real samples as it will be discussed below. Therefore, the TTF–FDH–GOD–MPA–AuE was characterised under these conditions using a FI system.

The influence of the detection potential on the FI response was evaluated from 150-μl injections of 5.0×10^{-4} M solutions of glucose or fructose into a carrier solution consisting of 0.05 M phosphate buffer solution of pH 6.5 (Fig. 2). The shape of this figure is rather similar to that obtained for batch amperometry with a maximum peak current at +0.10 V, which was selected for further work.

Characteristic flow injection parameters, such as flow rate and sample volume injected, were optimised. Concerning flow rate, the usual behaviour for enzyme-based FIA was observed, with a decrease of the FI peak height with relatively high flow rates [10]. Moreover, the peak width increased as the flow rate decreased. As a compromise between sensitivity and sample frequency, a flow rate of 1.40 ml min^{-1} was selected. On the other hand, taking into account the $i_p/W_{1/2}$ ratio, where $W_{1/2}$ is the peak width at half height, a sample volume of 150 μl was chosen as the injection volume.

Different aspects regarding the stability of the TTF–FDH–GOD–MPA–AuE under flow conditions were evaluated. First, the repeatability of the measurements was tested by constructing 10 successive calibration plots for glucose and fructose in the 0.1–1.0 mM concentration range. RSD values of 5.1% and 4.6% were obtained for the slopes of such calibration graphs, respectively.

Fig. 3 shows successive injections of 5.0×10^{-4} M glucose and fructose solutions. As can be seen, the order

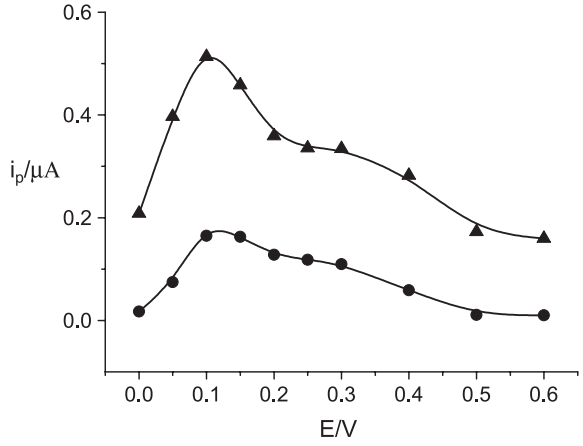

Fig. 2. Peak current vs. potential plots obtained under flow injection conditions at a TTF–FDH–GOD–MPA–AuE from 150-μl injections of 5.0×10^{-4} M solutions of glucose (●) and fructose (▲) in 0.05 M phosphate buffer solution of pH 6.5; flow rate, 1.40 ml min^{-1}.

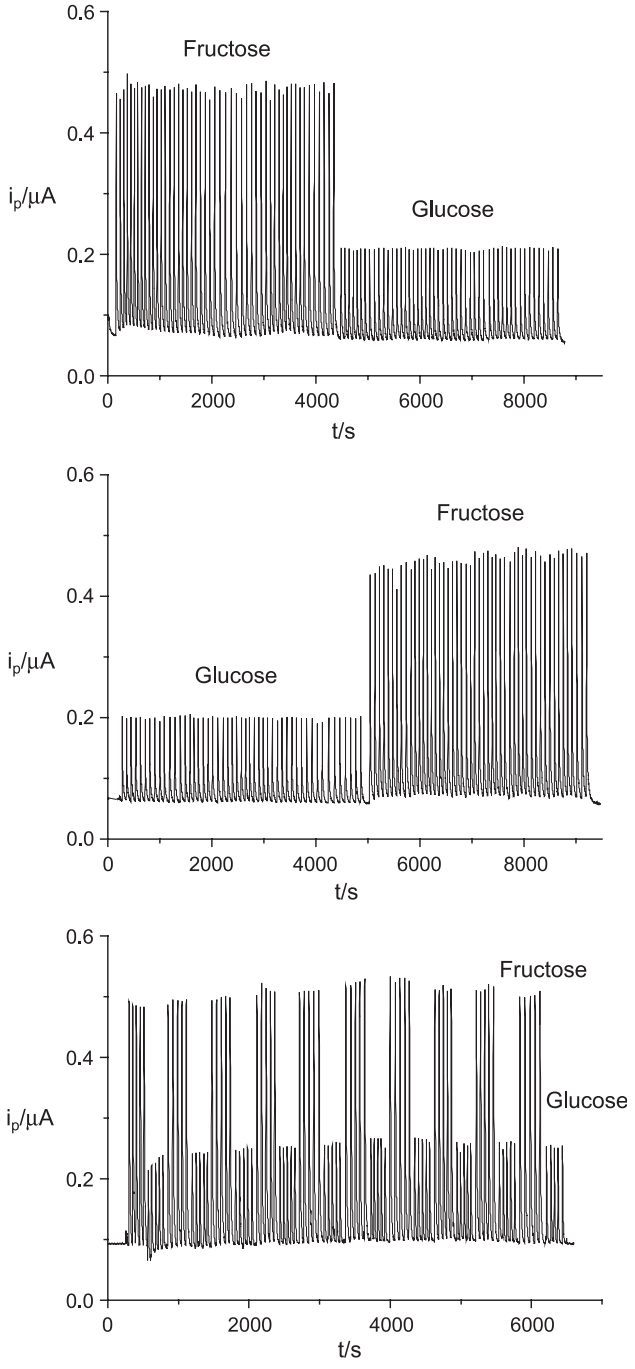

Fig. 3. Flow injection responses of a TTF–FDH–GOD–MPA–AuE for successive injections of glucose and fructose, 5.0×10^{-4} M. $E_{app} = +0.10$ V. Other conditions as in Fig. 2.

of injection of the analytes as well as the alternation of these injections did not affect the good repeatability of the FI responses. Thus, RSD values between 2.5% and 5.3% for i_p ($n = 50$) were obtained. This demonstrated the stability of the enzymes and the mediator atop the SAM-modified electrode in spite of the hydrodynamic conditions.

The reproducibility of the responses obtained with different TTF–FDH–GOD–MPA–AuEs was also evaluated. The RSDs for the slope values of the corresponding calibration plots for glucose and fructose in the 0.1–1.0 mM concentration range, obtained with five different biosensors, were 9.1% and 9.2%, respectively.

Finally, the useful lifetime of one single TTF–FDH–GOD–MPA–AuE was checked by constructing calibrations graphs for glucose and fructose in the 0.1–1.0 mM concentration range after storing the biosensor in phosphate buffer of pH 6.5 at 4 °C. The control chart constructed, taking the mean value of 10 successive calibration plots for each analyte (obtained the same day of the biosensor fabrication) as the central value and ± 3S.D. of this value as the upper and lower limits of control, showed that the slope values (taken as the mean value of three successive calibration plots for each analyte) remained within the control limits for around 388 h (approximately 16 days) for fructose, and for around 96 h (4 days) for glucose.

Under hydrodynamic conditions, the enzyme reactions for glucose and fructose at the TTF–FDH–GOD–MPA–AuE also obeyed a Michaelis–Menten kinetics, as demonstrated by the slope values (1.05 ± 0.01 for glucose and 1.01 ± 0.02 for fructose) of the Hill's plots. Calculation of the apparent Michaelis–Menten constants and the maximum rate of the reactions was carried out from Lineweaver–Burk plots. Values of $K_M^{app} = 25 \pm 6$ and 5.0 ± 0.8 mM, and $i_{max} = 3.5 \pm 0.6$ and 3.4 ± 0.5 μA were obtained for glucose and fructose, respectively.

Linear calibration graphs were obtained over the 1.0×10^{-5}–1.0×10^{-2} M concentration range for glucose [$r = 0.998$, slope=$(1.74 \pm 0.03) \times 10^{-4}$ A M^{-1}, intercept=$(4 \pm 1) \times 10^{-8}$ A], and 1.0×10^{-5}–1.0×10^{-3} M for fructose [$r = 0.998$, slope=$(9.0 \pm 0.2) \times 10^{-4}$ A M^{-1}, intercept=$(3.7 \pm 0.9) \times 10^{-8}$ A]. The limits of detection, according to the same criteria mentioned above, and estimation of s_b as the standard deviation ($n = 10$) of the FI signals from 10 injections of 1.0×10^{-5} M glucose or fructose were 5.7×10^{-6} and 2.5×10^{-6} M for glucose and fructose, respectively. Again, these analytical characteristics are rather similar to those obtained with the single-enzyme MPA–AuEs for glucose and fructose, which demonstrates that no significant worsening in the analytical performance towards the analytes is produced by the coimmobilisation of GOD and FDH on the same MPA–AuE.

Potential interferents for the FI responses of glucose and fructose were checked. The substances tested were caffeine, other sugars such as arabinose, galactose, and sucrose, as well as citric acid and ascorbic acid. Fig. 4 shows the amperometric FI responses for injections of 5-mM solutions of each of the above mentioned substances in 0.05 M phosphate buffer of pH 6.5. Table 1 summarises the analyte-to-interferent molar ratio for which a relative error lower than 10% was obtained in the slopes of the calibration plots for glucose and fructose in the 0.1–1.0 mM concentration range. The interference from ascorbic acid is due to the electrochemical oxidation of this compound at the applied potential. Although the presence of the SAM inhibits this oxidation process in a high extent, it has been

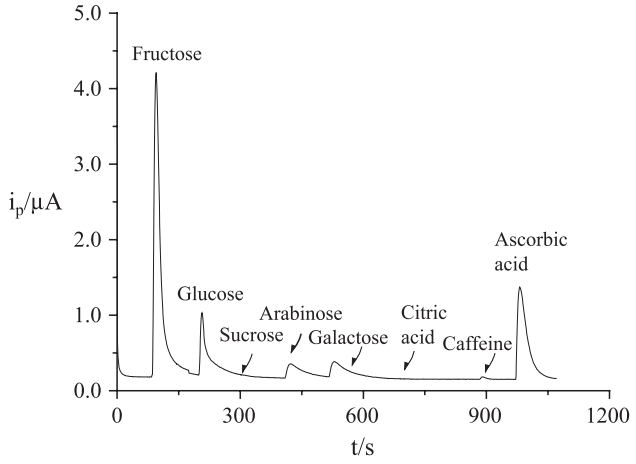

Fig. 4. Flow injection responses of a TTF–FDH–GOD–MPA–AuE for injections of 5.0×10^{-3} M solutions of different potential interferents in 0.05 M phosphate buffer solution of pH 6.5. Other conditions as in Fig. 3.

described that the presence of TTF catalyses the ascorbic acid oxidation [11], and therefore an important interference was produced. However, the interference from citric acid and caffeine should be related with the enzyme reactions because no significant amperometric responses were found for these compounds at both the bienzyme biosensor and TTF-MPA–AuE in the absence of the substrates. As expected, taking into account the sensitivity of the bienzyme biosensor for the analytes, the selectivity was considerably better for fructose, which, in fact, is again similar to that achieved with the single-enzyme TTF–FDH–MPA–AuE [7]. The ratios shown in Table 1 are sufficient to ensure that no significant interference should be produced in the analysis of real food samples.

The suitability of the bienzyme biosensor for the analysis of real samples under flow injection conditions was tested by determining glucose in two certified serum samples. One of the serum samples (Precinorm 4) had a glucose content (96 ± 14 mg dl^{-1}) corresponding to a healthy individual, whereas another sample (Precipath 4) contained glucose (243 ± 36 mg dl^{-1}) within the established interval for a diabetes sick person.

The possibility of interference from urea and uric acid present in the samples was evaluated by comparing the slopes of the calibration graph for glucose in the presence and absence of these compounds. No interference was found even for an analyte-to-interferent molar ratio of 1:20 for urea and 5:1 from uric acid. These ratios are lower than those usually found in blood serum [12].

No matrix effect was found with these samples, and the very simple treatment described in Experimental was applied before interpolation of the amperometric signal in a previously constructed glucose calibration plot in the 2.0×10^{-4}–1.0×10^{-3} M concentration range. The results obtained from seven replicates were 88 ± 3 mg dl^{-1} for Precinorm 4 and 244 ± 9 mg dl^{-1} for Precipath 4. No significant differences were found with respect to the certified values by applying the Student's t test, thus demonstrating the suitability of the TTF–FDH–GOD–MPA–AuE for the rapid, easy, and automated determination of glucose in human serum samples.

3.4. HPLC with amperometric detection at the TTF–FDH–GOD–MPA–AuE

From the results discussed above, it is obvious that a simultaneous determination of glucose and fructose in the same sample cannot be performed without a separation step because, at the detection potential used under FI conditions, both sugars are amperometrically detected and then an overall response would be obtained. Consequently, HPLC with amperometric detection at the TTF–FDH–GOD–MPA–AuE was accomplished.

Both the mobile phase flow rate and the applied potential were optimised by working with Milli-Q deionised water as mobile phase, according to the specifications of the manufacturer for the chromatographic column used (see Experimental). The results obtained from injections in the chromatographic system of 1:1 mixtures of 5.0×10^{-3} M fructose and glucose led us to select a flow rate of 0.6 ml min^{-1} as a compromise between good resolution and sensitivity and low retention times and peak widths. According to the column specifications, the retention times for glucose and fructose, using water as mobile phase at a flow rate of 0.6 ml min^{-1} and with the column thermostated at 80 °C are 13.9 and 19.3 min, respectively. Under our experimental conditions (i.e., with Milli-Q water at 0.6 ml min^{-1} and at 25 °C), the retention times for glucose and fructose were 862 s (~ 14.4 min) and 1501 s (~ 25.0 min), respectively (Fig. 5).

Concerning the detection potential, again a +0.10 V value gave rise to the largest peak areas for both sugars. Ten successive chromatograms from 1:1 glucose and fructose mixtures at a concentration level of 7.5×10^{-4} M yielded RSD values for peak area of 3.1% and 9.3% for glucose and fructose, respectively, thus demonstrating a good stability of the biosensor in the chromatographic system.

Linear relationships between peak area and concentration were found between 2.5×10^{-4} and 3.0×10^{-3} M ($r = 0.995$) for glucose, and 2.0×10^{-4} and 3.0×10^{-3} M

Table 1
Analyte-to-interferent molar ratio for which a relative error lower than 10% was obtained for the slopes of the calibration plots for glucose and fructose in the 0.1–1.0 mM concentration range obtained by FI with amperometric detection at a TTF–FDH–GOD–MPA–AuE

Interferent	Glucose	Fructose
Ascorbic acid	40	3
Citric acid	1	0.25
Caffeine	0.25	0.25
Galactose	0.33	0.05
Arabinose	1	0.05
Sucrose	0.05	0.05

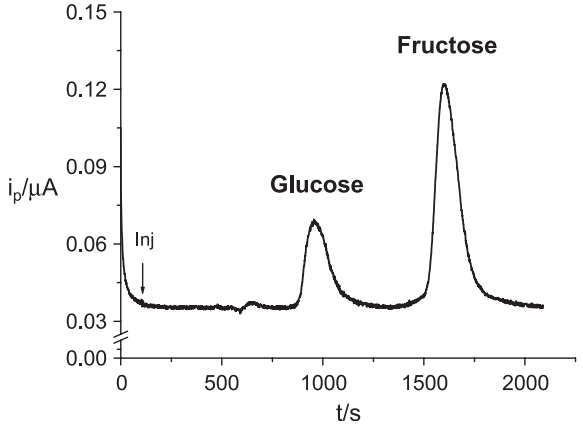

Fig. 5. Chromatographic peaks for glucose and fructose obtained with amperometric detection at a TTF–FDH–GOD–MPA–AuE. Mobile phase, Milli-Q water; flow rate, 0.6 ml min^{-1}; $E_{app} = +0.10$ V.

($r = 0.999$) for fructose. The slopes of these calibration plots were $(6.3 \pm 0.3) \times 10^{-4}$ and $(1.08 \pm 0.02) \times 10^{-3}$ A s M^{-1}, respectively, with intercepts of $(1.7 \pm 0.5) \times 10^{-8}$ and $(2.8 \pm 0.3) \times 10^{-7}$ A s, respectively.

Detection limits, calculated according the abovementioned criterion, and estimation of s_b as the standard deviation ($n = 10$) of the peak area values for mixtures of glucose and fructose at concentrations of 2.5×10^{-4} and 2.0×10^{-4} M, respectively, were 7.3×10^{-5} M for glucose and 6.0×10^{-5} M for fructose.

3.5. Simultaneous determination of glucose and fructose in real samples by HPLC with amperometric detection at the TTF–FDH–GOD–MPA–AuE

Fructose and glucose were determined in honey, cola softdrink, and commercial apple juice.

Concerning honey sample, no matrix effect was observed and then interpolation in external calibration plots could be used to determine both analytes. Following the procedure described in the Experimental and assuming the usual contents of these sugars in honey, their concentration in the analytical solution injected is around 1×10^{-3} M.

Regarding cola samples and given that a matrix effect was observed, the standard additions method was used to minimise this effect. Thus, the degassed and diluted cola sample (the dilution factor was 1:200) was injected into the chromatographic system, and then solutions prepared with the same amount of sample and growing amounts of glucose and fructose always in a 1:1 molar ratio were injected.

A similar standard additions method was used in the analysis of the commercial apple juice. As stated in Experimental, only a 1:1000 sample dilution was needed as a sample treatment in this case.

The results obtained from replicates of each sample are summarised in Table 2, where the confidence intervals were calculated for a significance level of 0.05. These results were compared with those obtained by using other reference methods. When the commercial R-biopharm enzyme kit was employed, only a good agreement of the results could be found for the honey sample and for the determination of glucose in the apple juice. The concentrations of glucose and fructose in the cola sample, and of fructose in apple juice, were much higher. This type of divergence has also been observed by other authors [13], and it was attributed to the fact that the multienzyme reaction in the kit method may allow interferences from other sugars. However, using the HPLC method, the different components of the samples are separated before the amperometric detection. Nevertheless, in order to confirm the validity of the results obtained with the bienzyme SAM-based biosensor, glucose was also analysed in the cola sample by a FI reference method proposed by Mottola [8] (see Experimental). Five determinations gave a mean value of 0.099 ± 0.004 M, which is in good agreement with the result obtained by HPLC and the TTF–FDH–GOD–MPA–AuE.

Fructose in apple juice was analysed by applying Taylor's method [9] (see Experimental). As can be observed in Table 2, the concentration obtained agrees fairly well with that found with the HPLC-biosensing detection method. A recovery study for fructose in the cola sample was also carried out. Thus, a degassed and 200-fold diluted cola sample was spiked with 0.1 M fructose. A 20-μl aliquot of the diluted noncontaminated sample solution was injected in the chromatographic system, followed by a 20-μl aliquot of the contaminated diluted sample solution. Then, solutions of the contaminated diluted sample containing also growing fructose additions were injected to construct the standard additions calibration graph by plotting the peak area (to which the peak area of the nonspiked sample was subtracted) vs. fructose concentration. A mean recovery ($n = 5$) of $98 \pm 4\%$ (RSD = 3.4%) was obtained, thus demonstrating good accuracy of the fructose determination in this kind of sample.

Table 2
Determination of glucose and fructose in real samples by HPLC with amperometric detection at the TTF–FDH–GOD–MPA–AuE, and comparison with the results obtained by other methods

Sample	TTF–FDH–GOD–MPA–AuE		Enzyme kit		Mottola method	Taylor method
	Glucose	Fructose	Glucose	Fructose	Glucose	Fructose
Honey (%)	36 ± 2 ($n = 5$)	30 ± 2 ($n = 5$)	39 ± 4 ($n = 3$)	28 ± 3 ($n = 3$)		
Cola (M)	0.105 ± 0.005 ($n = 3$)	0.119 ± 0.004 ($n = 3$)	0.35 ± 0.05 ($n = 3$)	1.08 ± 0.01 ($n = 3$)	0.099 ± 0.004 ($n = 5$)	
Apple juice (M)	0.32 ± 0.03 ($n = 5$)	0.40 ± 0.04 ($n = 5$)	0.30 ± 0.05 ($n = 3$)	1.0 ± 0.4 ($n = 3$)		0.40 ± 0.03 ($n = 5$)

Finally, the analytical performance of the TTF–FDH–GOD–MPA–AuE was also checked by carrying out recovery studies of fructose in blood serum, in order to evaluate its usefulness for the detection of fructosuria. These studies were performed using the Precinorm 4 serum sample. Firstly, the absence of endogenous fructose in this serum was verified by injecting in the chromatographic system an aliquot of the reconstituted undiluted sample and an aliquot of the same serum contaminated with 1.0 mM fructose. Once this was verified, the serum was spiked with fructose at a concentration level of 1.0×10^{-3} M (18.0 mg dl^{-1}), which is close to the highest concentration reached in blood after the intravenous administration of 0.2 g kg^{-1} fructose to an individual with fructosuria. The peak areas obtained by the injection of the spiked serum samples in the chromatographic system were interpolated in a calibration graph previously constructed in the 5.0×10^{-4}–2.0×10^{-3} M concentration range. Four determinations yielded a fructose concentration of $(9.8 \pm 0.7) \times 10^{-4}$ M, with a mean recovery of 98 ± 7%, which demonstrates that the HPLC method using biosensing detection at the TTF–FDH–GOD–MPA–AuE is also useful for the determination of the fructose level in blood serum for patients with intolerance to this substance.

4. Conclusions

The integrated bienzyme glucose oxidase–fructose dehydrogenase–tetrathiafulvalene electrode, constructed by coimmobilisation of the enzymes and the mediator by cross-linking with glutaraldehyde atop an MPA-SAM on a gold electrode, exhibits good analytical performance, both under batch and flow injection conditions in terms of sensitivity, stability, and reproducibility for the analysis of glucose and fructose. The simultaneous determination of these analytes in the same sample can be accomplished by using the biosensor as amperometric detector in HPLC, with the usefulness of the methodology having been demonstrated with several real samples.

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