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Fluorometric sensors based on chemically modified enzymes Glucose determination in drinks☆

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Abstract

In this paper an enzymatic fluorometric sensor for glucose determination in drinks is presented. The sensor film was obtained by immobilisation of glucose oxidase chemically modified with a fluorescein derivative (GOx-FS) in a polyacrylamide polymer. During the enzymatic reaction the changes in the fluorescence intensity of the GOx-FS are related to the glucose concentration. Working in FIA mode, the optimum conditions found were: 0.7 ml min^{-1} flow rate, 300 μl sample injection and pH 6.5. The sensor responds to glucose concentrations ranging from 400 to 2000 mg l^{-1}, the reproducibility is around 3% and the life-time is at least 3 months (more than 350 measurements). The sensor was applied to direct glucose determination in drinks with good accuracy; interference caused by the filter effect was avoided by the kinetics of the reaction.

Keywords: Fluorometric sensors; Enzymes; Glucose oxidase

1. Introduction

Analytical methods based on the combination of an enzymatic reaction and molecular fluorescence are one of the most interesting and promising analytical alternatives (recently, we have updated [1] the exhaustive compilations carried out by Guilbault [2] and Schenk [3]). In the most cases, the application of these methods for small molecules (lactate, pyruvate, cholesterol, ethanol and many others) in determinations requires the combination of one (or more) enzymatic reaction with a detection reaction (involving a fluorescent reagent). These methodologies are undoubtedly of great use, but have two main drawbacks when used in optical sensors: (1) complexity (many reagents need to be immobilised at the same time in the same film); and (2) irreversibility (due to the nature of the detection reaction).

In recent years, our research group has developed new alternatives in enzymatic-fluorometric methods for analyte determination in batch mode. Firstly, it was seen that the intrinsic fluorescence of some enzymes changes during the enzymatic

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reactions in according to the substrate concentration, thus avoiding the combination of the enzymatic reaction with a reaction in which a fluorophore is involved. Glucose was determined by using the intrinsic fluorescence of glucose oxidase (GOx) [4]. In this method, the addition of glucose to a solution containing GOx did not produce an immediate change in the fluorescence intensity of the enzyme (I_0), but after a certain time (t_a) there was a sudden increase in the intensity until a final value (I_1) was reached. If the reaction was allowed to continue, the intensity decreased again to the initial value (I_0). The analyte concentration did not affect the I_1 value but was related to t_a; after a theoretical study had been carried out, a mathematical model was developed which permitted correlation of t_a with the glucose concentration. Considering that GOx is a FAD-containing enzyme, during the enzymatic reaction FAD is reduced to $FAD \cdot H_2$; it was shown that I_0 and I_1 are the intensity when GOx-FAD and GOx-$FAD \cdot H_2$ are the predominant species, respectively. This method is very simple because the only reagent required is GOx; the main disadvantage is that the excitation and emission wavelengths of the enzyme are in the UV region of the spectrum. Like most of flavin derivatives, FAD display excitation (bands at 360 and 460 nm) and fluorescence (band at 520 nm) spectra (its intensity being dependent on the degree of oxidation) which could be of use for fluorometric determinations; however, the fluorescence decreases sharply when FAD form part of an enzymatic structure, which has impeded the generalized use of these properties, and analytical applications have only occasionally been found [5].

An alternative way of overcoming this problem consisted of covalently bonding the enzyme to a fluorophore whose fluorescence changes during the enzymatic reaction. Firstly, a coumarine derivative (HC) [6] was tested and subsequently a fluorescein derivative (FS) [7]. In both cases the fluorescence intensity of the chemically modified enzyme (GOx-HC: $\lambda_{exc} = 327$, $\lambda_{em} = 452$ nm; GOx-FS: $\lambda_{exc} = 489$, $\lambda_{em} = 520$ nm), changes in a similar way to the changes described for GOx, but at the excitation and emission wavelengths of the modifier. Using FS methods for batch determination of glucose [7], total cholesterol [8] and bilirubin [9] were also studied. In all cases, only the chemically modified enzyme was used as the reagent.

In the present paper, which is the next step in our research line, a sensor for glucose determination based on GOx-FS immobilised in polyacrylamide is reported for the first time. The sensor can work in continuous mode or in FIA mode (only the FIA mode has been employed to date), has a life-time of over 2 months (more than 250 measurements) and has been applied to glucose determination in drinks without sample treatment (only dilution).

2. Experimental

2.1. Apparatus

All fluorescence measurements were carried out on a Perkin–Elmer LS-50B luminometer fitted with an optical fiber Plate Reader accessory (the flow cell containing the sensor film is located inside). Spectral bandwidths of 15 (excitation) and 20 nm (emission) were selected. The FIA system consisting of a Wilson peristaltic pump and a six-way manual injection valve; 0.5 mm inner-diameter PTFE tubes were used. An UV Atom 75-Fotomatic lamp was also used.

Occasionally, the separation of the chemically modified enzyme was carried out with a low pressure chromatography system consisting of a glass column (13 cm long, 1 cm diameter) filled with Sephadex G-50 (Sigma G-50-150). The eluent was supplied with a peristaltic pump (M312 Miniplus 3, Gilson), and the end of the column was connected to an UV-M filter photometric detector (Pharmacia) (filter 254 nm) followed by a recorder (Unicam AR-25).

2.2. Reagents

0.1 M phosphate solution of pH 6.5 (from solid KH_2PO_4 and solid Na_2HPO_4) and 0.1 M hydrogencarbonate solution of pH 8.5 (from $NaHCO_3 \cdot H_2O$).

GOx was taken from *Aspergillius niger*, EC 1.1.3.4 (Sigma G-7141) was of 245 900 U g^{-1} of lyophilised solid. Solutions were prepared by dissolving the solid in the above-mentioned buffer solutions.

The FS solution was prepared by dissolving 1 mg of fluorescein-5(6)-caproxamido-caproic acid *N*-hydroxy-succinimide ester, a solid reagent obtained from Sigma (F-1756), in 1 ml dimethyl sulfoxide (DMSO). This solution was used for the chemical modification of GOx.

Glucose stock solutions were prepared by dissolving the appropriate amount of β-D(+)-glucose (Sigma G-5250) in the phosphate buffer solution.

2.3. GOx-FS preparation procedure

Depending on if it is necessary or not to discard the excess of FS, two different methods were applied for GOx-FS preparation.

- Without FS excess elimination. Two hundred microlitres of FS solution in DMSO were mixed with 300 μl hydrogencarbonate solution containing 0.02 g GOx. The mixture was allowed to react in darkness at room temperature with continuous stirring for 60 min. This was the procedure generally applied.
- With FS excess elimination. One hundred microlitres of FS solution in DMSO were mixed with 300 μl hydrogencarbonate solution containing 0.02 g GOx. The mixture was allowed to react in darkness at room temperature with continuous stirring for 60 min. The excess FS was then separated from the GOx-FS using the chromatographic system previously described. The carbonate solution was used as the eluent at a flow-rate of 1.5 ml min^{-1}. The first 4.5 ml were discarded and the next 3.5 ml recovered; the eluent was passed through the column for another 15 min to remove any remaining FS.

2.4. GOx-FS sensor film preparation

The preparation of the polyacrylamide GOx-FS sensor was based on a general enzyme immobilisation procedure described elsewhere [10]. To this end, 0.1 ml of a phosphate buffer solution containing 20 mg of acrylamide, 4 mg of bis-acrylamide and 8 mg of ammonium persulphate (as a reaction precursor) were mixed with 0.1 ml of the GOx-FS solution. Dissolved oxygen was eliminated by bubbling nitrogen through the solution. The cocktail was spread on a 0.5 mm hollow made in a glass film ($20 \times 9 \times 0.1$ mm), covered with a Mylar film and a glass film, and irradiated with the UV-lamp (254 nm) for 50 min. The film was then stored in the phosphate buffer solution at 4 °C.

2.5. Flow cell for the film

The flow cell was designed in our laboratory (Fig. 1A). The main part of the cell (a) was a stainless steel piece ($2 \times 2.5 \times 2$ cm) with a hollow ($0.5 \times 1.5 \times 0.3$ cm) in which the sensor film (b) is fixed with a holed Mylar film (c). The flow cell was covered with a methacrylate piece (d) containing two stainless steel tubes (2 mm, outer diameter) for circulation of the fluid in the cell; the space between tubes was just wide enough for the optical fiber termination (Fig. 1B). Pieces (a) and (d) were joined by means of four screws and a silicon washer (e) to avoid fluid loss. The sensor had a 225 μl capacity.

2.6. Procedure

The phosphate buffer solution flowed across the flow cell at 0.7 ml min^{-1} and the fluorescence intensity began to be monitored at $\lambda_{exc} = 490$ and $\lambda_{em} = 520$ nm (I_0 being the initial fluorescence intensity of the film). Three hundred microlitres of the sample (or glucose standard solution) were injected and a transient signal obtained, I_{max} being the fluorescence intensity at the maximum. Different parameters of the signal are related to the glucose concentration: I_{max}, recovery time (t_c, time between the maximum and the end of the signal), signal area (A) and I_r defined as:

$$I_r = 100\frac{I_{max} - I_0}{I_0}.$$

Any of these parameters can be used for glucose determination. During the optimisation study I_r and A were used as a measurement of the

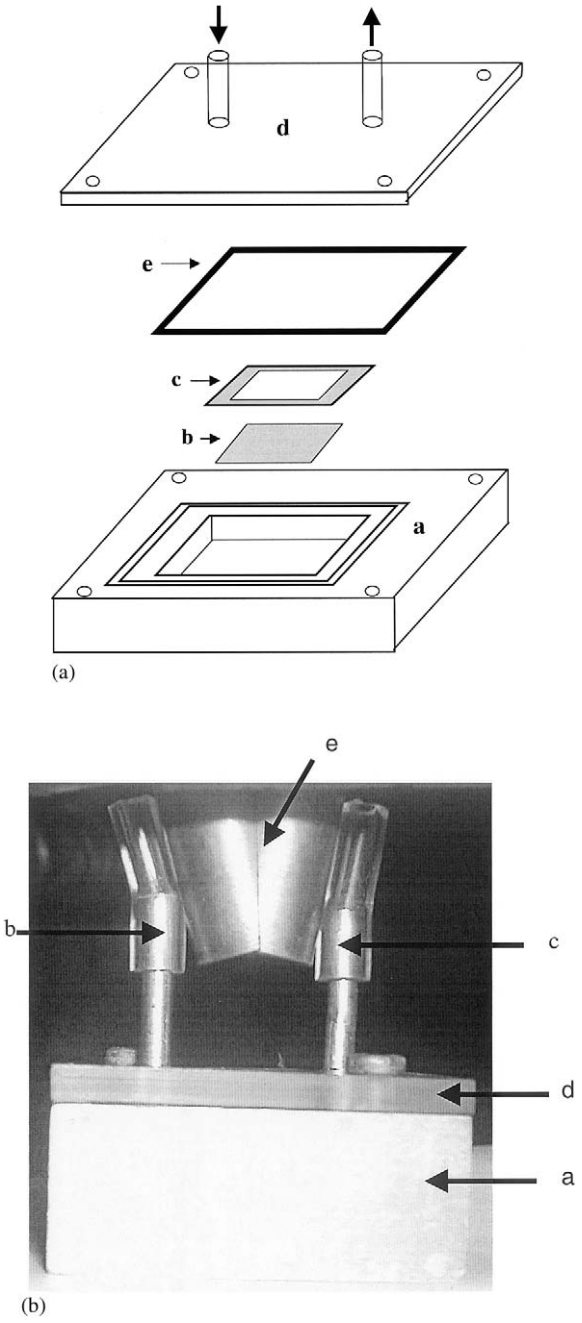

Fig. 1. (A) Scheme of the flow cell used in the measurements (see test for description); (B) Picture of the flow cell placed into the sample compartment of the luminometer. (a) Stainless steel piece; (b) y; (c) inlet and outlet; (d) methacrylate piece; (e) optical fiber.

sensitivity and t_c was used as an estimation of the sampling rate (as the t_c increases, the sampling rate decreases).

3. Results and discussion

3.1. Origin of the analytical signal

As stated in the Section 1, the fluorescence change in GOx-FS during the enzymatic reaction is due to a reduction of FAD to FAD·H_2. Fig. 2A shows how the fluorescence intensity of the sensor changes when the flow-cell is fed in a continuous mode with solutions of different glucose concen-

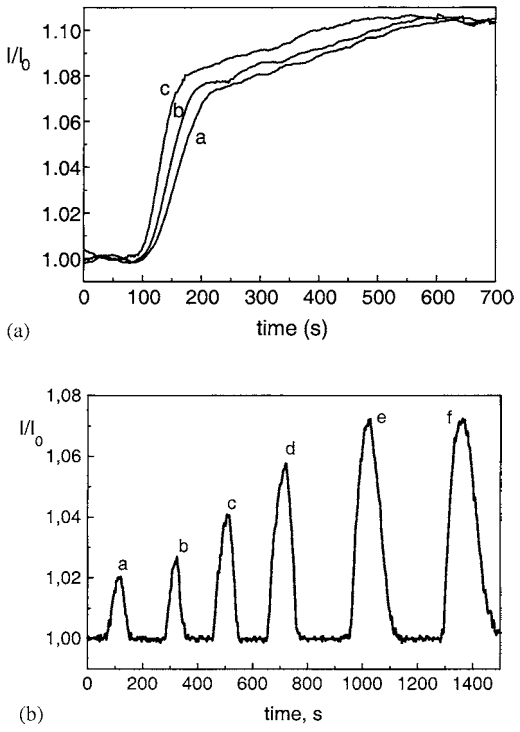

Fig. 2. (A)Variation of relative fluorescence intensity (I/I_0) of GOx-FS with glucose concentration working in continuous mode. Working conditions as indicated in Section 2.6. (a) 500; (b) 800; (c) 1000 mg l^{-1} glucose. (B) Variation of fluorescence intensity of GOx-FS with glucose concentration working in FIA mode. Working conditions as indicated in Section 2.6. Glucose concentrations, in mg l^{-1}, are: (a) 500; (b) 600; (c) 750; (d) 900; (e) 1200; (f) 1500.

tration; as the glucose concentration increases the rate of the intensity variation with time increases but the maximum intensity changes very slightly; if a random time t is considered after the beginning of the glucose feed and before the maximum is reached, the intensity at this time (I_t) changes with the glucose concentration. When glucose solutions are injected in FIA mode (discrete sample volume) two opposite effects take place: (1) the increase in fluorescence intensity due to the enzymatic reaction; and (2) the decrease in intensity due to dilution of glucose and regeneration of the enzyme. These opposite effects give a FIA-like peak in which the analytical parameters described in Section 2.6, change with glucose concentration (Fig. 2B).

3.2. Optimisation of the flow cell

Prior to the system shown in Fig. 1, two other flow cell systems were designed. Firstly, a system (Fig. 3A) consisting of a methacrylate piece (a; $7.7 \times 5 \times 0.4$ cm) with a central hollow (b; $1.1 \times 0.5 \times 0.5$ cm; 275 μl volume) and two lateral orifices (c and d; 0.5 mm diameter) for sample inlet and outlet. Two Mylar films (e, f) fixed with silicone closed the central hollow; the rear film (e) contained the polyacrylamide-GOx-FS sensor film (g). Finally, a reflecting mirror (h) was fixed to the back part of the system to increase the optical path-length. With this system similar signals to those with the above-described system were obtained and so the system can be used as an alternative. However, the system has two main drawbacks: (1) operative problems arise due to the frequent formation of bubbles on the sensor film which perturb the signals (this can be avoided if a second pump is connected to the outlet tube); (2) as the sensor is composed of a large number of films, the system has to be tested frequently for liquid leakage.

The second system designed is shown in Fig. 3B. A plastic cuvette ($4 \times 1 \times 1$ cm) is divided into two sections; the upper section is discarded. In the diagonal of the lower part (which is showed in the figure, $1.5 \times 1 \times 1$ cm), a polyester film (a), in which the GOx-FS sensor (b) has been fixed, is located. The back part (c) is filled with silicone and

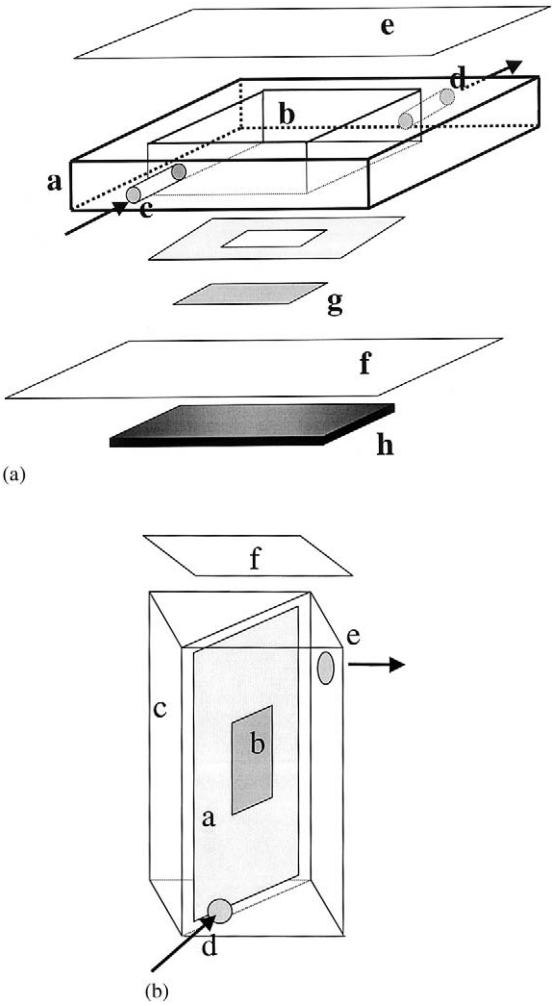

Fig. 3. (A) Scheme of an alternative flow cell for 180° illumination. (B) Scheme of a flow cell for 90° illumination.

the front part is provided with two orifices for sample inlet (d) and outlet (e). Finally, the system is closed with a methacrylate cover (f). This flow cell is located in the sample compartment of the luminometer. This system has a 750 μl capacity and is recommended when conventional illumination (90%, horizontal) has to be used. However, this system presents low sensitivity; only concentrations higher than 1000 mg l^{-1} glucose can be determined.

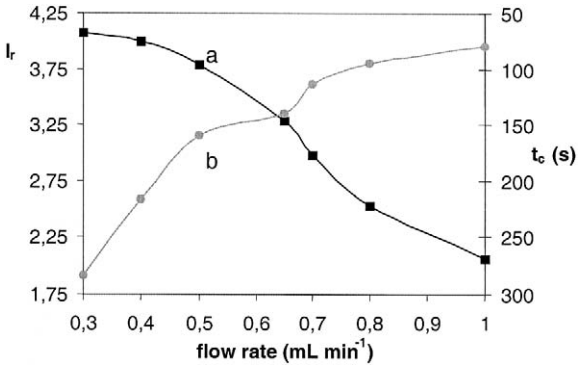

Fig. 4. Effect of the flow rate in: (a) I_r; and (b) t_c. Working conditions as indicated in Section 2.6 (700 mg l^{-1} glucose).

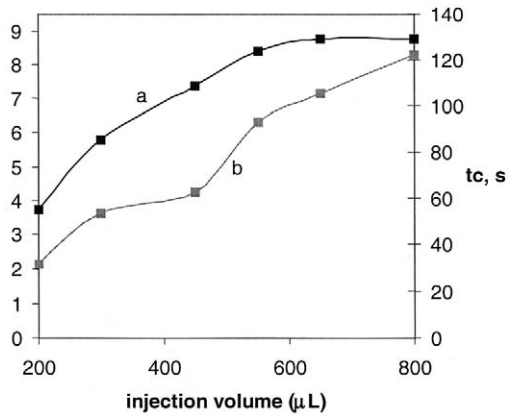

Fig. 5. Effect of the injection volume on: (a) I_r; and (b) t_c. Working conditions as indicated in Section 2.6 (700 mg l^{-1} glucose).

3.3. Optimisation of the flow conditions

3.3.1. Flow rate

Fig. 4 shows the effect of the flow-rate on the sensor response. As the flow increases the I_r decreases whereas the t_c increases. The optimum flow rate chosen will depend on the particular problems of the application. If a high sampling rate is needed, lower sensitivity will be obtained and, conversely, if high sensitivity is required, then the sampling-rate will be limited. Another important parameter, which may be affected by the flow rate, is the precision; it has been stated that precision is better as the volume increases [11]. In our system this effect was not very important (Table 1). 0.7 ml min^{-1} would seem to be a good compromise flow-rate.

Table 1
Effect of the flow rate in the precision (as RSD, $n = 5$) of the determination

Flow rate (ml min^{-1})	I_r (%)	A (%)
0.3	4	6
0.4	5	8
0.5	4	5
0.65	4	4
0.7	3	4
0.8	5	5
1.0	4	5

Working conditions as established in Section 2.6.

3.3.2. Injection volume

As can be seen in Fig. 5, volumes of 550 µl or more do not change the sensitivity of the method but the sampling flow rate decreases as the injection volume increases. In addition the injection volume also affects the linear response range. Working with 550 µl linear response ranges from 400 to 700 mg l^{-1} (for I_r) and from 500 to 1200 mg l^{-1} (for A) were obtained, which are shorter than those observed with 300 µl (see Section 3.4). We recommend to use 300 µl, but higher can be use to increase the sensitivity. This volume is slightly higher than the volume of the flow-cell. It can therefore be deduced that if a lower sample volume is required another flow-cell with a similar design but with a lower inner volume should be designed.

3.4. Optimisation of the reaction conditions

3.4.1. pH

The pH and type of the buffer solution used could affect the fluorescence of the GOx-FS and enzyme activity. With regard to GOx-FS fluorescence at pH values lower than 4.5, the fluorescence is very weak (due to fluorescein protonization), which makes it difficult to follow the reaction satisfactorily. As far as the pH is concerned, different values were tested (Fig. 6). As occurs with the flow-rate, the higher the sensitivity the higher the regeneration time and the lower the

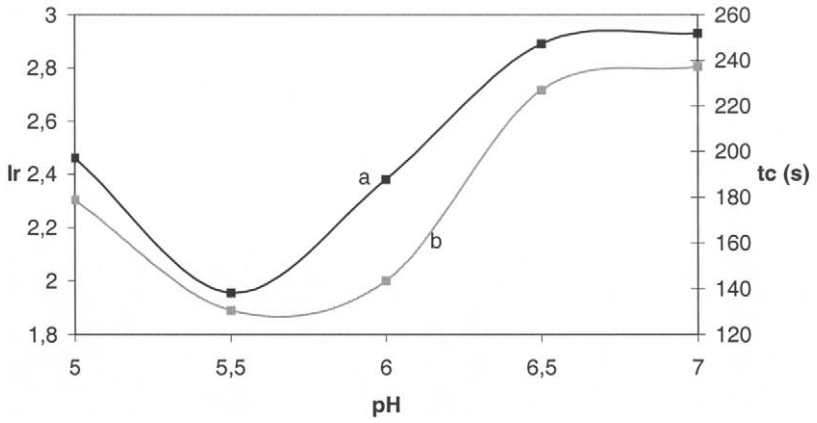

Fig. 6. Effect of the pH on: (a) I_r; and (b) t_c. Working conditions as indicated in Section 2.6 (700 mg l^{-1} glucose).

sampling rate. We propose 6.5 as the optimum pH but another pH can be selected, depending on the sample characteristics. It should be pointed out that the optimum pH obtained was 1 unit higher than that obtained when working with GOx-FS in dissolution [7], indicating the effect of the polyacrylamide on the kinetics of the enzymatic reaction.

3.4.2. GOx-FS amount in films

Using the procedure described, different sensor films were prepared in which the amount of the enzyme added to sensor was modified. The results show that as the enzyme amount increases, the regeneration time decreases due to the increase in the rate of the enzymatic process. Two other effects can be attributed to the enzyme amount: the sensitivity of the method and the response range. Table 2 shows the linear response range and the slope of the calibration graph, working with I_r and A. As can be seen, the higher the enzyme amount the higher the slope of the calibration graph. However, when the highest enzyme amount was used, the sensor films formed displayed high inhomogeneity and poor mechanical resistance, so it was impossible to carry out reproducible measurements.

The effect of the enzyme amount on the linear response range, working with the I_r, can be explained by the kinetics of the process: as the enzyme amount increases, the kinetics of the process speeds up, so the difference between the time between sample injection and the beginning of the signal and the time needed to reach the maximum in Fig. 2A is shorter. In these conditions the glucose concentration required to reach the

Table 2
Effect of enzyme amount on slope of the calibration graph and linear response range

GOx-FS (mg)	I_r		A	
	Slope (mg^{-1} l)	Linear response (mg l^{-1})	Slope (mg^{-1} l)	Linear response (mg l^{-1})
7	0.0120	400–600	0.0100	400–800
13	0.0130	400–750	0.0120	400–1800
20	0.0135	400–1200	0.0130	400–2000
40	0.0135	600–1200	0.0140	600–1800

Working conditions as established in Section 2.6.

maximum is lower. On the other hand, the regeneration time is longer, so the A of the signal continues to increase.

In order to reduce the amount of FS to be used, different text were performed using a mix of GOx and GOx-FS, maintainned the same GOx total amount. In general the I_r decreases as the proportion of GOx-FS in the mix decreases. For example, for a 0.16 GOx-FS/GOx the I_r decreases about 50% respect to use GOx-FS.

3.5. Analytical figures of merit

In the best conditions considered (0.7 ml min^{-1} flow rate, 300 μl samples, pH 6.5 and 20 mg GOx-FS), the linear response range goes from 400 to 1000 for I_r, and from 400 to 2000 for A. The reproducibilities (as RSD) obtained were 3 and 4% for I_r and A, respectively ($n = 5$). The sampling rate depends on the glucose concentration tested (because of the regeneration time); for 1000 mg l^{-1} glucose, the sampling rate was about 30 h^{-1}. Fig. 7 shows the variation of the I_r for 1000 mg l^{-1} on 90 days; on these days the sensor was used for conventional measurements. As can be seen, the sensor life-time is at least 3 months (about 350 measurements). From day-to-day the sensors was stored in phosphate buffer at 4 °C (in a refrigerator).

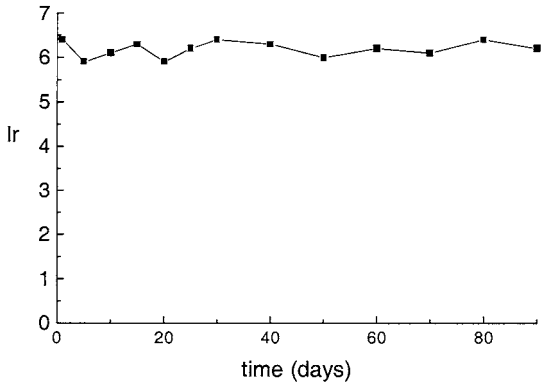

Fig. 7. Values of I_r for 1000 mg l^{-1} glucose at different days. Working conditions as indicated in Section 2.6.

4. Application

4.1. Filter interferences

The method was applied to the determination of glucose in drinks. The selectivity of the enzymatic reaction virtually impedes chemical interferences. Physical interferences affecting the kinetics of the reaction (viscosity or density of the sample) can be avoided by using the standard addition method. For this application the effect was not observed, so determination was carried out by interpolation on the calibration graph.

The most important interferences are caused by species that absorb at excitation or emission wavelengths of the GOx-FS (filter interferences). However, the system itself avoid this interference because of the kinetics of the reaction is a phase-out between the passage of the sample through the flow-cell and the signal appearance, so the filter interference is negligible.

4.2. Determination

The sensor was applied to glucose determination in three commercial fruit juices; the only sample treatment was dilution. The results obtained (Table 3) were compared with those obtained by the GOx-FS batch method [7], which had been previously validated. The results obtained by both

Table 3
Application of the method to glucose determination in fruit juice

Fruit juice	GOx-FS in solution method	Sensor method (peak height)	Sensor method (peak area)
Orange	18.5±0.7	18.9±1.4	18.8±1.8
Pineapple	40.4±0.9	41.1±1.3	41.3±1.7
Peach	44.4±1.0	46.3±1.5	55.2±2.1

Glucose concentration in g l^{-1}, $n = 5$.

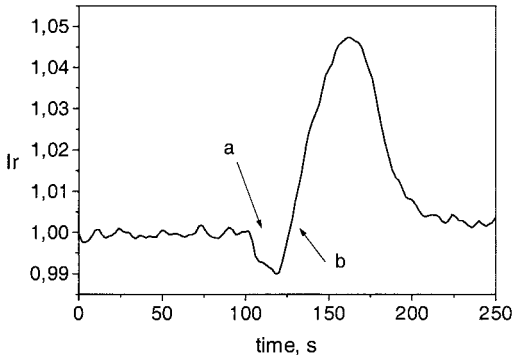

Fig. 8. Recorder of a determination of glucose in fruit zume. Point (a) refers to inner filter effect caused by zume; point (b) refers at the moment in which sample has just finished to pass across the cell. Working conditions as indicated in Section 2.6.

methods were statistically compared (f-test) and no significant differences were observed. Fig. 8 shows a recorder of $I=f(t)$ for a sample juice; as can be seen the absorption of the juice matrix does not interfere in the glucose determination.

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