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Fluorescence anisotropy: application in quantitative enzymatic determinations

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Abstract

In this work a method is presented for the enzymatic determination of glucose using fluorescence anisotropy. During the enzymatic reaction a change in the fluorescence anisotropy of the glucose oxidase (GOx) is produced; the reaction time at which this change appears (t_m) depends on the glucose concentration. A theoretical study has been developed which enables: (a) the correlation of this change in anisotropy with changes in the intensity and the lifetime of the enzyme fluorescence; from this a model which could be generalized to other flavo-enzymes is proposed; (b) the linking of t_m with glucose concentration.

After optimisation, the proposed method allows the determination of glucose over the range 100–1000 mg l^{-1}. The detection limit is 90 mg l^{-1} and the reproducibility is better than 4% (n = 6, [glucose] = 250 mg l^{-1}). Anisotropy is more selective than conventional fluorescence intensity, and this method has therefore been applied to direct glucose determination in fruit juices without the interference caused by the inner filter effect.

Keywords: Enzymatic determination; Polarised fluorescence; Anisotropy; Glucose oxidase

1. Introduction

Methods based on the combination of an enzymatic reaction (because of its inherent selectivity) and molecular fluorescence (because of its intrinsic sensitivity) are among the most interesting and promising of current analytical alternatives. Recently, we have updated [1] the exhaustive compilations of these methods carried out by Guilbault [2] and Schenk [3]. In most cases, the application of these methods in the analysis of substrates involves the combination of one or several enzymatic reactions of the analyte with another detection reaction (involving a fluorescent reagent), consequently the methods are complicated and not reversible (because the detection step is generally irreversible).

Of the various new alternatives being proposed to avoid the detection reaction [1,4], our research group is developing new enzymatic–fluorometric methods for analyte determinations based on changes in the UV intrinsic fluorescence of the enzyme (mainly due to tryptophan) during the enzymatic reaction. Firstly, glucose (β-G) was determined using the UV intrinsic fluorescence of glucose oxidase (GOx) [5]. Fig. 1 shows how the UV fluorescence intensity of GOx changes during the enzymatic reaction when different glucose concentrations were used. After glucose addition, the initial fluorescence intensity decreases to I_1 because of the dilution effect and remains constant; after a certain time (t_m) the fluorescence intensity increases up to a final value (I_2) and later decreases to the I_1 initial value (not shown in Fig. 1). The I_1 and I_2 values do not depend on the glucose concentration but do depend on the enzyme concentration. However, the analyte concentration is related to the t_m and a mathematic model was developed which allowed this correlation to be

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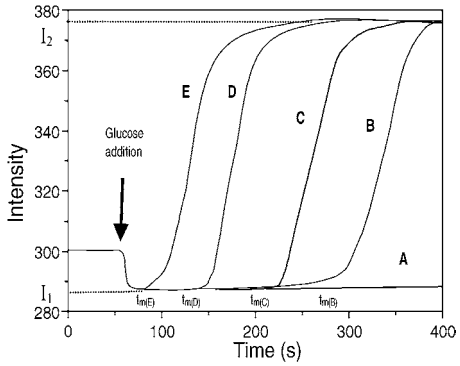

Fig. 1. Variation of fluorescence intensity during enzymatic reactions at different glucose concentrations ($[G]_A = 0$, $[G]_B < [G]_C < [G]_D < [G]_E$). Conditions: pH 6.5, $\lambda_{exc} = 278$ nm, $\lambda_{em} = 340$ nm, $[GOx]_0 = 10$ U ml^{-1}. *Note*: These signals have been measured using a Perkin-Elmer LS 50 luminometer.

established as follows:

$$t_m = \frac{1}{k_1[GOx]_0} \ln\left(\frac{[\beta\text{-}G]_0}{[\beta\text{-}G]_0 - [O_2]_0}\right) \quad (1)$$

k_1 being a velocity constant of the enzymatic reaction; t_m the time from sample addition until the fluorescence intensity increases by 10% of the total increase produced (here called I_m):

$$I_m = I_1 + 0.1\,(I_2 - I_1) \quad (2)$$

This time was selected because it is easier to measure than the time at which the intensity begins to grow (t_a). In order to explain the change of intensity from I_1 to I_2, it should be taken into account that GOx is a FAD-containing enzyme; during the enzymatic reaction FAD is reduced to $FADH_2$ by glucose and is later regenerated by the oxygen. It has been shown [5] that I_1 and I_2 correspond to the moments when GOx–FAD and GOx–$FADH_2$ are the predominant species in solution, respectively. The mechanism responsible for the fluorescence intensity change was thought to be the differential energy transfer between tryptophan and FAD or $FADH_2$. Tryptophan groups transfer more energy to FAD than to $FADH_2$, thus the GOx–FAD UV fluorescence (I_1) is lower than GOx–$FADH_2$ (I_2). This mechanism was later confirmed by the results obtained by Alpert and co-workers [6].

This method is very simple because the only reagent required is GOx. However, as the fluorescence is measured in the UV, it is prone to inner filter interferences from samples, this being the main disadvantage of the method. One way to avoid this problem could be to translate the detection of the enzymatic reaction to the visible region, for which two main alternatives have hitherto been proposed:

1. Using the flavin fluorescence of the GOx [7]. As is known, FAD shows intrinsic fluorescence ($\lambda_{exc} = 367$ and 445 nm, and $\lambda_{em} = 520$ nm) but $FADH_2$ does not. However, the FAD fluorescence becomes highly quenched by the proteinic environment of the GOx (the FAD quantum yield falls to 0.03 or lower) [8] and a higher enzyme concentration is therefore necessary for accurate FAD fluorescence measurement (according to our experiments about 8 times more than for tryptophan). This increase in the GOx concentration to be used presents two problems: (a) obviously the cost of the determination increases; (b) the rate of the enzymatic reaction sharply increases and, according to the model (1), the linear response range shortens.
2. A chemical modification of the enzyme with a fluorophore whose fluorescence changes during the enzymatic reaction. Different fluorophores (such as a coumarine [9], rhodamine and fluorescein derivatives [10]) have been tested, and the best results were obtained with fluorescein, which has also been used for total cholesterol [11] or bilirubin [12] determination. Recently, pyrene has also been proposed as a modifier [13].

The aim of this paper is to present another alternative based on enzyme fluorescence, which minimizes or avoids the inner filter effect and probably other spectral interferences. As has been commented before, the fluorescence intensity variation during the enzymatic reaction depends on which of the species is predominant in solution, GOx–FAD or GOx–$FADH_2$. That is to say, it is due to a structural change in the GOx molecule, which could also affect the fluorescence polarisation and a change in anisotropy (or polarisation) could be observed at t_m. Based on this, a method for glucose determination in drinks is now presented. The glucose determination should be considered as a model, and the methodology could be further applied to other enzymatic reactions involving flavo-enzymes.

2. Experimental

2.1. Apparatus

(1) All fluorescence measurements were carried out in a Photon Technology International (PTI) Time Master fluorescence lifetime spectrometer working in an L-configuration (model TM-2/2003-PTI). This instrument has two radiation sources and two detectors; depending on the configuration selected, two kinds of measurement can be made:
Configuration A: Lifetime or time-decay measurements. A nanosecond flash lamp (filled with H_2 or He/N_2) is selected as the radiation source and a patented stroboscopic detection system [14] is used as the detector. Data are handled with PTI TimeMaster™ software.
Configuration B: Anisotropy steady-state measurements. An arc-Xe lamp illumination system (lamp power supply LPS-220B, arc lamp housing A-1010B and igniter LPS-221B) is selected as the radiation source and a photomultiplier analog/digital (PTI 814) as the detector. The instrument is equipped with quartz polarizers for both excitation and emission sides. Data are handled with PTI FeliX™ software.

Both configurations use monochromators for excitation and fluorescence wavelength selection (a slit width of 20 nm was used for both) and a sample compartment permitting continuous stirring; a 4 ml Hellma QS 101 quartz cuvette of 1 cm pathlength was used.

(2) UV-visible molecular absorption measurements were performed with a diode array spectrophotometer (Hewlett Packard 8452). A 4 ml Hellma quartz cuvette of 1 cm pathlength was used.

2.2. Reagents

A phosphate buffer of pH 8 was made fresh daily from H_2KPO_4 0.1 M and HNa_2PO_4 0.1 M. Glucose oxidase (GOx) was taken from *Aspargillus niger* EC 1.1.3.4 (Sigma G-7141) of 157500 U g^{-1} of lyophilized solid. Solutions were prepared by dissolving the solid in the above-mentioned buffer solutions.

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

A glucose oxidase chemically modified with a fluorescein derivative (GOx–FS, FS being Fluorescein-5(6)-carboxamido-caproic acid *N*-hydroxy-succinimide) was also used. The preparation of this derivative was made according to a procedure previously described [10].

2.3. Measurement procedure

2.3.1. Anisotropy measurements during the reaction

The instrument was set at configuration B, and the excitation and emission monochromators set at the desired wavelengths. The cuvette was excited with vertically polarized light and the fluorescence intensity components I_{vv} and I_{vh} monitored alternately (due to the L configuration of the instrument), I_{vv} being the fluorescence intensity when both the excitation and emission polarizers are parallel and I_{vh} being the fluorescence intensity when the excitation and emission polarizers are perpendicular. The anisotropy (r) was calculated simultaneously by the instrument using equation:

$$r = \frac{I_{vv} - G \times I_{vh}}{I_{vv} + 2 \times G \times I_{vh}} \quad (3)$$

The G factor (which corrects anisotropy introduced by the optical components of the detection system) was obtained by the instrument before each anisotropy measurement.

To perform the enzymatic determination with GOx, the excitation and emission monochromators were set at 280 and 340 nm, respectively. The luminometer cuvette was filled with 2.8 ml of a standard solution of glucose or the sample. The mechanical stirrer was then started. 0.2 ml of a GOx solution of 157 U $ml^{-1}$was then added to the cuvette and the anisotropy measurement started. The time t_m was used as the analytical parameter.

The same procedure was used in the experiments with GOx–FS; in this case the excitation and emission monochromators were set at 490 and 520 nm, respectively.

For the determination of glucose in drinks, the sample only needs dilution before being submitted to this procedure.

2.3.2. Lifetime measurements

In order to obtain the lifetime of the GOx and GOx–FS (with flavin in FAD or $FADH_2$ form), the instrument was set at configuration A.

After selection of the excitation and emission wavelengths (the same as those given in the previous paragraph), the cuvette was filled with a colloidal solution in order to perform the instrumental response function (IRF). To obtain the GOx–FAD lifetime, the cuvette was then filled with the enzyme solution, the decay curve obtained and subsequently fitted using an iterative fitting procedure. The best fit was determined when χ^2 was minimized to a value of $0.9 < \chi^2 < 1.2$. To obtain the GOx–$FADH_2$ lifetime, a large excess of glucose was added to a solution containing with GOx–FAD. After 5 min (time enough for all the enzyme to become reduced) the decay curve was made and fitted. The same procedure was employed for obtaining the lifetimes of the GOx–FS(FAD) and GOx–FS ($FADH_2$).

As is known, the fluorescence curve decay is $f(t) = \sum \alpha_i \exp(t/\tau_i)$, α_i being the pre-exponential factor and τ_i the decay time for the ith emitting component. The average fluorescence decay time used in the fluorescence quenching treatment was $\langle\tau\rangle = \sum \alpha_i \tau_i / \sum \alpha_i$.

The fluorescence lifetime of quinine sulphate in H_2SO_4 0.05 M was obtained to evaluate the instrument performance, yielding a τ = 19.30 ± 0.15 ns (n = 5, χ^2 = 1.1), in good agreement with the value found in the bibliography [15].

3. Results and discussion

3.1. Analytical signal and its prediction

Fig. 2 shows the I_{vv} (A) and I_{vh} (B) component variations of the GOx fluorescence during the enzymatic reaction for different glucose concentrations; in this figure t = 0 represents the point when GOx is added. As can be seen, both components change in the same way as the total fluorescence intensity shown in Fig. 1. The t_m as previously defined also appears in this figure. When anisotropy is calculated from this value, Fig. 2C is obtained. At the beginning of the reaction, the anisotropy presents an initial value r_1 and after a reaction time (which depends on the glucose concentration), a decrease in anisotropy is observed to a r_2 final value; if the monitoring continues, the anisotropy increases again up to the initial r_1 value. The negative peak observed is in fact an instrumental artefact: as the instrument only has one channel, it measures I_{vv} and I_{vh} alternately during the reaction so that these curves are out of phase. The time at which the negative peak appears is called t_p (peak time) and is, in fact,

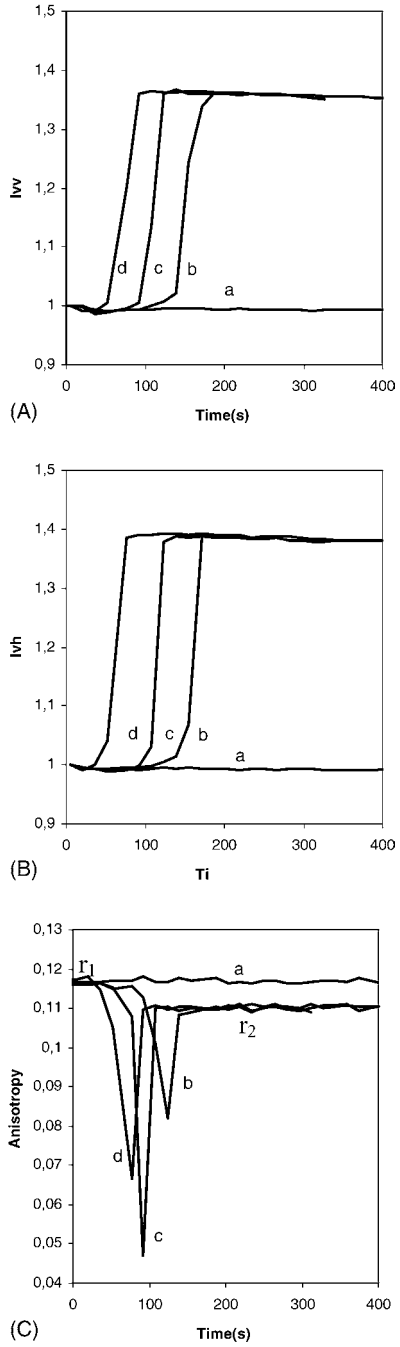

Fig. 2. Variation of the I_{vv} (A), the I_{vh} (B) and the anisotropy (C) of the GOx during the enzymatic reaction. Conditions: λ_{exc} = 280 nm, λ_{em} = 340 nm, $[GOx]_0$ = 10 U ml^{-1}, $[G]_d$ = 160 mg l^{-1}, $[G]_c$ = 130 mg l^{-1}, $[G]_b$ = 120 mg l^{-1}, $[G]_a$ = 0 mg l^{-1}. *Note*: I_{vv}, I_{vh} and r return to the baseline (not shown). I_{vv} and I_{vh} are divided by I_0.

very close to t_m so that the mathematical model (1) is also followed as will be demonstrated later. In this work, t_p is used instead of t_m because it has the advantage that calculations are unnecessary and it is more precise than t_m.

According to the mechanism described above, the r_1 value corresponds to the GOx–FAD anisotropy and the r_2 to that of GOx–$FADH_2$. A hypothesis has been elaborated explaining this change in anisotropy. As is known, the energy transfer efficiency (E) can be alternatively calculated by:

$$E = 1 - \frac{I_{AD}}{I_D} \tag{4a}$$

or

$$E = 1 - \frac{\tau_{AD}}{\tau_D} \tag{4b}$$

I_{AD} and τ_{AD} being the fluorescence intensity and the lifetime of the donor in the presence of the acceptor, and I_D and τ_D being the same parameters for the donor in the absence of the acceptor. In this case, I_D and τ_D correspond to the apo-enzyme (apo-GOx) and two different values for I_{AD} and τ_{AD} can be observed depending on whether the enzyme is in the oxidised (GOx–FAD) or the reduced (GOx–$FADH_2$) form. Eq. (4a) can accordingly be expressed as:

$$E_{GOx\text{–}FAD} = 1 - \frac{I_{GOx\text{–}FAD}}{I_{apo\text{-}GOx}},$$

$$E_{GOx\text{–}FADH_2} = 1 - \frac{I_{GOx\text{–}FADH_2}}{I_{apo\text{-}GOx}} \tag{5}$$

A combination of both equations for the same enzyme concentration yields gives:

$$\frac{E_{GOx\text{–}FAD} - 1}{E_{GOx\text{–}FADH_2} - 1} = \frac{I_{GOx\text{–}FAD}}{I_{GOx\text{–}FADH_2}} \quad \text{or}$$

$$\frac{E_{GOx\text{–}FAD} - 1}{E_{GOx\text{–}FADH_2} - 1} = \frac{K_{GOx\text{–}FAD}}{K_{GOx\text{–}FADH_2}} \tag{6}$$

$K_{GOx\text{–}FADH_2}$ and $K_{GOx\text{–}FADH_2}$ being the proportionality constants between fluorescence intensity and concentration for both forms of the enzyme. A similar treatment of the lifetimes gives the following equation:

$$\frac{E_{GOx\text{–}FAD} - 1}{E_{GOx\text{–}FADH_2} - 1} = \frac{\tau_{GOx\text{–}FAD}}{\tau_{GOx\text{–}FADH_2}} \tag{7}$$

Finally, the combination of Eqs. (6) and (7) yields:

$$\frac{\tau_{GOx\text{–}FAD}}{\tau_{GOx\text{–}FADH_2}} = \frac{I_{GOx\text{–}FAD}}{I_{GOx\text{–}FADH_2}} = \frac{K_{GOx\text{–}FAD}}{K_{GOx\text{–}FADH_2}} \tag{8}$$

According to this equation, the quotient of the fluorescence constants for both enzyme species (or the intensities when the both enzyme concentrations are the same) is equal to the quotient of the lifetime of both species.

In order to confirm this find, the lifetime for both glucose oxidase species were obtained following the procedure indicated in Section 2.3.2. The results obtained are shown in Table 1 where the fluorescence intensity is also included. From these data it can be deduced that Eq. (8) is fulfilled:

$$\frac{I_{GOx\text{–}FAD}}{I_{GOx\text{–}FADH_2}} = \frac{K_{GOx\text{–}FAD}}{K_{GOx\text{–}FADH_2}} = \frac{I_1}{I_2} = 0.77 \quad \text{and}$$

$$\frac{\tau_{GOx\text{–}FAD}}{\tau_{GOx\text{–}FADH_2}} = 0.75$$

Table 1
Lifetime, intensity and anisotropy measurements for GOx and GOx–FS in their oxidised and reduced forms

	$<\tau>$ (ns)	χ^2	I (10^6)	r
GOx(FAD)	2.7 ± 0.1[a]	1.14	1.58[c]	0.113[c]
GOx ($FADH_2$)	3.6 ± 0.1[a]	1.18	2.06[c]	0.085[e]
GOx(FAD)–FS	3.3 ± 0.1[b]	1.02	0.65[d]	0.135[d]
GOx($FADH_2$)–FS	3.4 ± 0.1[b]	1.07	0.70[d]	0.133[f]

[a] λ_{exc} = 280 nm, λ_{em} = 340 nm; pH = 8; [GOx] = 30 U ml^{-1}.
[b] λ_{exc} = 490 nm, λ_{em} = 520 nm; pH = 8; [GOx–FS] = 40 U ml^{-1}.
[c] As indicated in[a] except [GOx] = 10 U ml^{-1}.
[d] As indicated in[b] except [GOx–FS] = 20 U ml^{-1}.
[e] As indicated in[c] and with glucose addition.
[f] As indicated in[d] and with glucose addition.

As is known, the Perrin equation describes depolarisation in the excited state due to rotation:

$$r = \frac{r_0}{1 + \tau/\phi} \tag{9}$$

r_0 and ϕ being the intrinsic anisotropy and the rotational correlation time respectively. Assuming that r_0 and ϕ are equal for GOx–FAD and GOx–$FADH_2$ it can be stated that:

$$r_1 = \frac{r_0}{1 + \tau_{GOx–FAD}/\phi} \quad \text{and} \quad r_2 = \frac{r_0}{1 + \tau_{GOx–FADH_2}/\phi} \tag{10}$$

Combining both expressions:

$$\frac{r_2}{r_1} = \frac{\phi + \tau_{GOx–FAD}}{\phi + \tau_{GOx–FADH_2}} \tag{11}$$

This equation confirms that the anisotropy variation between both forms of the enzyme is due to the changes in the lifetime. The substitution of anisotropy and lifetimes obtained in Eq. (11) permits a ϕ calculation for GOx yielding 15 ns. To obtain the real ϕ of GOx it would be necessary to make measurements of polarised fluorescence in the time domain, which is not possible with the instrumentation at hand. Furthermore, no bibliographic data relating to this parameter for GOx has been found.

It is known that ϕ can be theoretically calculated by using the following equation [16]:

$$\phi = \frac{\eta M}{RT}(\bar{v} + h) \tag{12}$$

M being the molecular weight of GOx (155000 g mol^{-1}), $\bar{v}$ being the GOx specific volume (0.75 g ml^{-1}) and h being the hydration shell of the protein (about 0.23 g H_2O per gram of protein). This equation yields a value of about 100 ns for GOx, much higher than the experimental value. However, Lakowicz et al. [17,18] have studied the rotational motions of tryptophan residues in different proteins such as carbonic anhydrase, carboxypeptidase A and bovine and human serum albumin, finding ϕ ranging from 1 to 12 ns, also much lower than the theoretical values (obtained after applying (12)). They concluded that the tryptophan residues in these proteins display remarkable freedom of motion within the protein matrix, so the "effective rotating volume" is much lower than the total volume of the protein. On this basis, the ϕ found for GOx can be explained and, in conclusion, the anisotropy changes can be ascribable to the different lifetimes of GOx–FAD and GOx–$FADH_2$.

In the case of GOx–FS, the following quotient can be calculated from data shown in Table 1:

$$\frac{I_{GOx–FAD}}{I_{GOx–FADH_2}} = \frac{K_{GOx(FS)–FAD}}{K_{GOx(FS)–FADH_2}} = \frac{I_1}{I_2} = 0.93 \quad \text{and}$$

$$\frac{\tau_{GOx(FS)–FAD}}{\tau_{GOx(FS)–FADH_2}} = 0.97$$

Assuming the same ϕ value for GOx–FS as for GOx, the anisotropy quotient obtained is:

$$\frac{r_2}{r_1} = \frac{\phi + \tau_{GOx(FS)–FAD}}{\phi + \tau_{GOx(FS)–FADH_2}} = 0.995 \quad \Rightarrow r_1 \approx r_2$$

As can be seen, the anisotropy values for both forms of the chemically modified GOx are similar, thus no changes in anisotropy would be observed during the enzymatic reaction. Experimentally, we have not observed changes in the anisotropy when working with GOx–FS (see Table 1). This result is in accordance with the hypothesis and prevents to use this methodology for chemically modified enzymes, at least with FS.

3.2. Mathematical model and its confirmation

Using anisotropy as a measurement parameter, Eq. (1) can be applied; in this case t_m is defined as the time for r anisotropy to reach r_m:

$$r_m = r_1 + 0.1(r_2 - r_1) \tag{13}$$

A drawback of the mathematical model (1) is that the O_2 concentration needs to be known. In this work a simplification of the previous model is proposed, useful in cases when slightly higher glucose concentrations are to be measured. According to this, Eq. (1) can be rearranged as:

$$e^{-(t_m \times k_1 \times [GOx]_0)} = \frac{[\beta\text{-}G]_0 - [O_2]_0}{[\beta\text{-}G]_0} \tag{14}$$

The order of magnitude of $[GOx]_0$ and k_1 are about 10^{-7} and 10^4, respectively. If t_m is low (moderately high glucose concentration), the following approximation is possible:

$$1 - (t_m \times k_1 \times [GOx]_0) = \frac{[\beta\text{-}G]_0 - [O_2]_0}{[\beta\text{-}G]_0} \tag{15}$$

which can be rearranged to obtain the following expression:

$$t_m = \frac{[O_2]_0}{k_1 \times [GOx]_0 \times [\beta\text{-}G]_0} \tag{16}$$

This mathematical model linearly relates t_m with $1/[\beta\text{-G}]_0$. It is not necessary to know $[O_2]_0$, although it must be constant.

Fig. 3 shows the calibration lines using the new mathematical model and with t_p as the analytical parameter for different

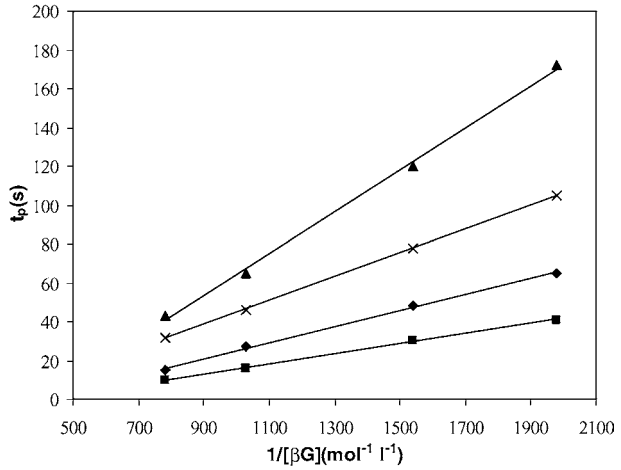

Fig. 3. Variation of t_p = f $(1/[\beta G]_0)$ at different GOx concentrations. (▲) 5 U ml^{-1} (×) 7.5 U ml^{-1} (♦) 10 U ml^{-1} (■) 15 U ml^{-1} GOx. Conditions: pH 8, λ_{exc} = 280 nm, λ_{em} = 340 nm.

concentrations of enzyme. Table 2 shows the slope, the standard deviation of the slope and the correlation coefficients. The good agreements of the results can be seen. If the slopes of the calibration graphs are represented against 1/[GOx], a straight line is obtained as the model predicts:

$$\text{slope} = -0.002 + 1.8 \times 10^{-8}\left(\frac{1}{[\text{GOx}]_0}\right) \quad r = 0.998$$

The slope in this line corresponds to $[O_2]/k_1$. From these data and the O_2 concentration measurement, k_1 was calculated to be 12841 mol^{-1} l s^{-1}. This value is comparable to those found in the literature [19]. These results validate the model.

3.3. Optimisation of the determination procedure

3.3.1. pH study

The GOx activity depends on the pH, which affects the kinetics of the process although, as has been pointed out in a previous works [5], the pH has no influence in the range between 5 and 8.5. Fig. 4 shows the influence of the pH on the anisotropy of GOx–FAD (r_1), GOx–$FADH_2$ (r_2) and its difference. It can be observed that there is an effect in the latter case but not in the former. As a result, the energy transfer depends on the pH and the maximum anisotropy variation is obtained at pH 8, which has been selected as the optimum.

This effect of the pH on the energy transfer could be due to two main reasons:

Table 2
Slopes and intercepts of the calibration graphs of glucose obtained for different GOx concentration. Experimental conditions: λ_{exc} = 280 nm, λ_{em} = 340 nm; pH = 8

[GOx] (U ml^{-1})	5	7.5	10	15
Slope (mol l^{-1} s)	0.107	0.062	0.040	0.026
Standard deviation of the slope	0.004	0.001	0.001	0.001
R	0.9994	0.9999	0.9995	0.9998

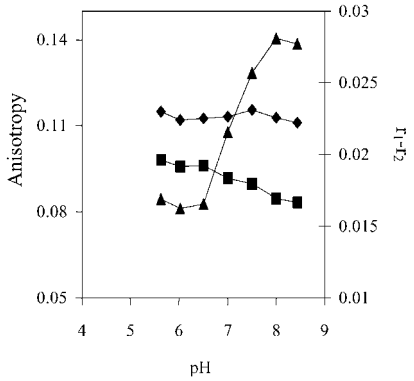

Fig. 4. Variation of the anisotropy of GOx–FAD (r_1) (♦), GOx–$FADH_2$ (r_2) (■) and r_1–r_2 (▲) as a function of pH. Conditions: λ_{exc} = 280 nm, λ_{em} = 340 nm, $[\text{GOx}]_0$ = 10 U ml^{-1}.

Table 3
Molar absorptivity coefficients (M^{-1} cm^{-1}) at 340 nm for GOx–FAD and GOx–$FADH_2$ at different pH values

pH	$\varepsilon_{\text{GOx–FAD}}$ (10^3)	$\varepsilon_{\text{GOx–FADH}_2}$ (10^3)
5	3.8	2.4
6	4.0	2.5
6.5	4.2	2.8
7	4.1	4.0
8	4.2	4.2

- The pH affects the apo-enzyme (tryptophan) quantum yield. However, Alpert and co-workers [6] has shown that this does not occur for apo-GOx in the range mentioned.
- The pH affects the molar absorptivity of the acceptor. The p*K* acid–base of FAD and $FADH_2$ are 10 and 6.7, respectively. Table 3 shows the molar absorptivity at 340 nm obtained for FAD and $FADH_2$ inside GOx at different pH values. As can be seen, in the range studied the GOx–$FADH_2$ changes its molar absorptivity but the GOx–FAD does not, explaining the observed changes in anisotropy.

3.3.2. Integration time

The integration time can affect the accuracy and the precision of the measurement of t_p. A study of the influence of the integration time on the standard deviation was made (n = 7) and the results are shown in Table 4. In order to check if the difference is significant or not, an ANOVA was applied confirming that there is no significant difference and that it is therefore possible to use any time value. About 5 s of integration time was chosen because the noise is lower and the precision for measuring the t_p is greater.

Table 4
Influence of integration time on t_p. Experimental conditions: λ_{exc} = 280 nm, λ_{em} = 340 nm; pH = 8; [GOx] = 10 U ml^{-1}; [G] = 130 mg l^{-1}

Integration time(s)	1	2	5
Average t_p (s)	91.4	93.0	86.9
Standard deviation	6.0	5.0	3.8

Table 5
Results obtained for glucose determination in juice samples. Experimental conditions: λ_{exc} = 280 nm, λ_{em} = 340 nm; pH = 8; [GOx] = 10 U ml^{-1} (5 determinations for each sample)

Sample	Orange juice (g l^{-1})	Pineapple juice (g l^{-1})	Peach juice (g l^{-1})
Proposed method	19.0 ± 0.7	42.8 ± 0.5	44.7 ± 0.5
Validated method	18.4 ± 0.7	40.4 ± 0.5	44.5 ± 0.4

3.4. Analytical characteristics

The linear response range for the method, the sensitivity and the detection limit depend on the GOx concentration used. A concentration of 10 U ml^{-1}at pH 8 provided a linear response range of 100–1000 mg l^{-1}of glucose, and the detection limit was 90 mg l^{-1}of glucose. The reproducibility obtained for a glucose concentration of 250 mg l^{-1} was 4% (n = 6) using t_p as an analytical parameter.

3.5. Application

This method was applied to glucose determination in three commercial fruit juices; the only sample treatment was dilution. The results (Table 5) were compared with those obtained by a validated method [10]. The results obtained by both methods were statistically compared (F-test) and no significant differences were observed.

The most important interferences could be caused by primary or secondary inner filter effects on the GOx fluorescence. Fig. 5A shows the variation of intensity during the enzymatic reaction for an orange juice sample (a) and for a glucose standard (b). In this case the measurement procedure was different in order to show the effect (the cuvette was filled with 2.6 ml of phosphate buffer, and 0.2 ml of a GOx solution of 157 U ml^{-1} was added; after the mechanical stirrer was started and the measurement begun, 0.2 ml of either a standard solution of glucose or the sample was added). As can be seen, the addition of the sample produces a decrease in the signal due to the dilution and the inner filter effect. During the enzymatic reaction the intensity increases, but this value remains under the effect produced by the inner filter. The addition of the standard produces a minor decrease due only to the dilution. Fig. 5B shows a record of $r = f(t)$ for a sample and a standard. In this case, the sample addition does not modify the baseline, and the anisotropy variation is the same as with the standard. This result shows the high possibilities of anisotropy in order to avoid the inner filter effect.

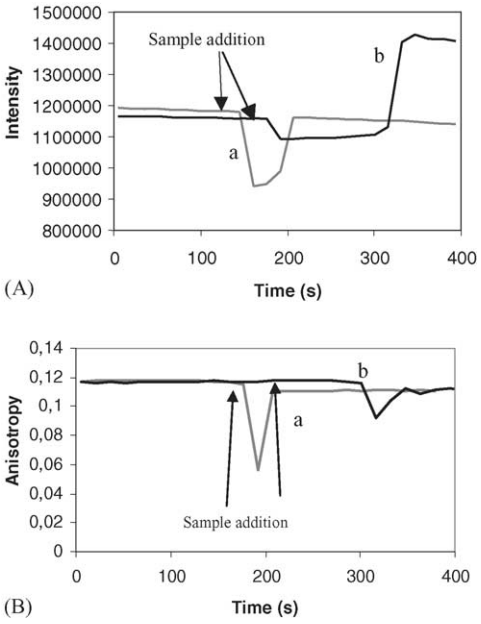

Fig. 5. GOx anisotropy variation (A) and intensity variation (B) during the enzymatic reaction: orange juice sample (a) and standard glucose (b). Conditions: pH 8, λ_{exc} = 280 nm, λ_{em} = 340 nm and $[GOx]_0$ = 10 U ml^{-1}.

4. Future possibilities

As has been commented before, fluorescence decay time is different for the reduced form of the enzyme than the oxidised form. On this basis it is possible to measure the changes in the GOx fluorescence intensity at a given decay time. Tests have been made at different decay times; here we show the result at 45 ns (one of the best). The experimental conditions used were similar to those described in 2.3.1 (only the enzyme concentration needs to be increased). Configuration A of the fluorimeter was obviously chosen (45 ns delay to the excitation pulse). The results are shown in Fig. 6. As can be seen, the intensity changes during the enzymatic reaction as in Fig. 1. However, the noise of the signal is very high (due to the radiation source available), and in our opinion the S/N ratio could be greatly improved with the use of a laser. The

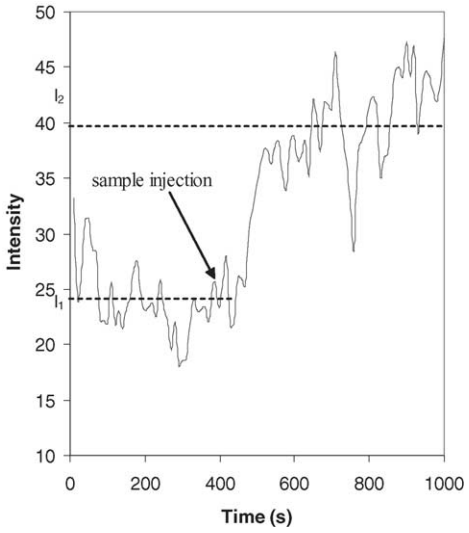

Fig. 6. Variation of intensity during the enzymatic reaction. Conditions: λ_{exc} = 278 nm, λ_{em} = 340 nm, time delay: 50 ns, $[GOx]_0$ = 25 U ml^{-1}, [G] = 120 mg l^{-1}.

use of the decay time could also improve selectivity (avoiding fluorescence interferences).

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