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ORIGINAL PAPER

Simultaneous Determination of Glucose and Choline Based on the Intrinsic Fluorescence of the Enzymes

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Abstract It has been possible to perform the simultaneous determination of choline and glucose using the intrinsic fluorescence of the corresponding enzyme as an analytical signal. This can be done in two ways. First, for low glucose and choline concentrations (about 0.55 mM and 0.75 μM respectively) two differentiated signals, without mutual interference, are obtained for both analytes in the same measurement. Second, when glucose and choline concentrations are higher, a new model has been designed which permits the concentrations to be accurately determined in samples containing from 0.55 mM to 3.75 mM glucose and from 0.75 μM to 11.0 μM choline; the method has been applied to simultaneous glucose and choline determinations in serum samples with good results. This method gives a better performance than multivariate calibration based on Partial Least Squares Regression. The methodology here shown could be also used for the simultaneous determination of other pairs of analytes.

Keywords Intrinsic fluorescence · Enzymatic analysis · Glucose · Choline · Simultaneous determination

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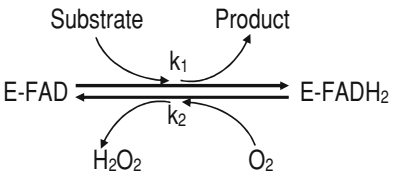
Introduction

Enzymes (and proteins in general) are one of the most useful families of analytical reagents. Their importance has traditionally centred on their virtual selectivity, so a method in which an enzymatic reaction is combined with an indicator (colorimetric or fluorometric) reaction is already a classical methodological scheme for the determination of analytes in many kinds of samples [1, 2]. As new enzymes are continuously discovered and new reagents are synthesized, the applicability of these methods is growing in importance [3]. However, as many of the reagents give irreversible reactions, these methods have a limited application (for example, they are impractical for optical sensors). Enzymes are catalysers (and therefore reversible in essence) and then this problem can be overcome if the analytical signal could be obtained from the enzyme itself, avoiding in this way the indicator reaction.

All proteins containing aromatic aminoacids (phenylalanine, tyrosine and tryptophan) present intrinsic fluorescence due to the presence of these groups [4–6]; besides, some proteins have specific sources of additional UV–vis spectroscopy properties, such as the Green Fluorescence Protein family [7], and some enzymes possess these properties when the cofactor is covalently attached, such as some metalloproteins (containing iron or cupper) or flavoenzymes (containing FAD or FMN). These spectroscopic properties

were traditionally very few used from the analytical point of view, but with the developments in optical sensors they have attracted the attention of researchers in recent decades [2, 8–13]. Unfortunately, they can only be used in few cases, and in such cases a kinetic control of the reaction is needed in order to find the conditions in which they will be evident. However, the use of the intrinsic fluorescence properties of proteins also permits the kinetic aspects to be exploited for analytical use, this being the subject of this paper.

The kinetic model of the enzymatic reactions catalysed by enzymes containing FAD can be simplified to a two step process as follows:



(1)

E-FAD and E-FADH$_2$ being the oxidized and reduced forms of the enzyme respectively, and k_1 and k_2 being the kinetic constants. During the enzymatic reaction FAD is reduced to FADH$_2$ by the substrate and then is regenerated by the oxygen. The variation of the fluorescence is due to a differential energy transfer between tryptophan and FAD or FADH$_2$; as tryptophan groups transfer more energy to FAD than to FADH$_2$, the fluorescence constant for the E-FAD form (K_{ox}) is lower than for E-FADH$_2$ (K_{red}). Since E-FAD is the initial and final form of the enzyme, during the reaction an increase (when E-FAD.H$_2$ is formed) and a later decrease (when E-FAD is regenerated) is observed which can be kinetically related to the initial substrate concentration. As a conclusion of the studies carried out on different reactions following Eq. (1), it is deduced that there are two kinds of behaviour in the FAD-containing enzyme depending on the relative speed of both processes (given by k_1 and $k_2[O_2]_0$ respectively):

1. When $k_1 > k_2[O_2]_0$ the enzyme fluorescence varies during the reaction as indicated in Fig. 1a. This is the case of Choline Oxidase [14] (ChOx; substrate= choline, product=betaine). After choline addition to a ChOx solution the fluorescence intensity reaches a maximum (I_{max}) (due to the reduced form of the enzyme being formed nearly instantaneously) which depends on the choline concentration. Later the reduced form is oxidized by the oxygen and the intensity returns to I_0. The difference between I_1 and I_0 is due to dilution. Considering that the O_2 concentration does not change along the reaction (which is the case because of the choline concentration range tested), the mathematical model describing this process is given in a general case by Eq. (2a) and for low choline concentrations by Eq. (2b):

$$\frac{1}{\frac{I_{max}^{ChOx}-I_0^{ChOx}}{I_0^{ChOx}}} = \left(\frac{K_{ox}}{K_{rd}-K_{ox}}\right) + \left(\frac{K_{ox}}{K_{rd}-K_{ox}}\right)\frac{k_{2,ChOx}[O_2]_0}{k_{1,ChOx}}\frac{1}{[Choline]_0} \quad (2a)$$

$$\frac{I_{max}^{ChOx}}{I_0^{ChOx}} = 1 + \left(\frac{K_{rd}-K_{ox}}{K_{ox}}\right)\frac{k_{1,ChOx}}{k_{2,ChOx}[O_2]_0} \times [Choline]_0 \quad (2b)$$

K_{ox} and K_{rd} being the fluorescence constants for the E-FAD and E-FADH$_2$ forms of the enzyme.

2. If $k_1 < k_2$ the fluorescence during the reaction varies as shown in Fig. 1b. The Glucose Oxidase (GOx; substrate=glucose, product=glucuronic acid) reaction belongs to this group [15]. In this case the E-FADH$_2$ concentration depends on the O_2 concentration in solution (which for its part depends on the analyte concentration), giving a different fluorescence intensity

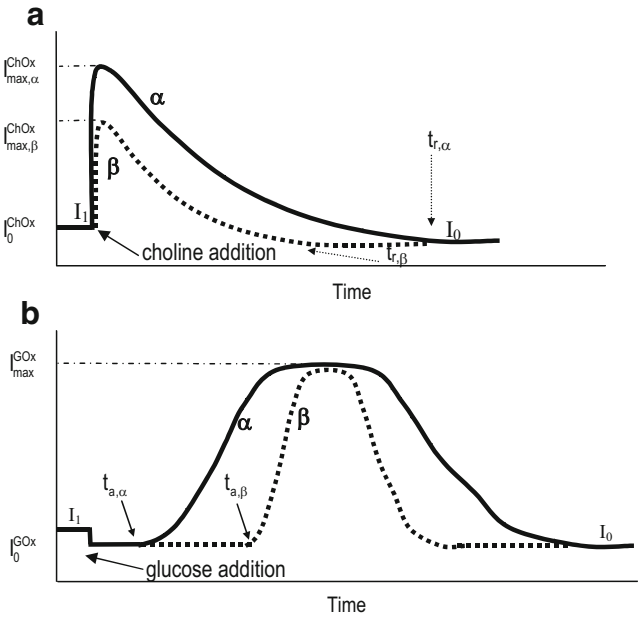


Fig. 1 Variation of the intrinsic fluorescence of oxidase enzymes during the reaction with their corresponding substrates: **a** Choline Oxidase (ChOx) and **b** Glucose Oxidase (GOx). *Dotted lines* (β) correspond to registers obtained with a lower analyte concentration than that of the *continuous lines* (α)

variation. Obviously, it is necessary for the O_2 to be consumed in order to see the fluorescence change. For most of the analyte concentrations, I_{max} does not depend on the glucose concentration but is related to the t_a (time at which the intensity begins to rise). A mathematic model was developed which allowed this correlation to be established for a general case (3a) or for high glucose concentrations (3b):

$$t_a = \frac{1}{k_{1,GOx}[GOx]_0} \ln\left(\frac{[Glucose]_0}{[Glucose]_0 - [O_2]_0}\right) \quad (3a)$$

$$t_a = \frac{[O_2]_0}{k_{1,GOx}[GOx]_0} \times \frac{1}{[Glucose]_0} \quad (3b)$$

As can be seen, there is a delay between the signal of glucose and the signal of choline. If, then, a sample containing glucose and choline was added to a mixture of GOx and ChOx, a combined signal would be obtained, which could be used for the simultaneous determination of choline and glucose in the same experiment. This is a very important difference compared to conventional analytical schemes based on the electrochemical (amperometric sensors) or spectroscopic (using fluorophores or chromophores) H_2O_2 measurement. In this paper the intrinsic fluorescence of two enzymes has been combined for the simultaneous determination of two different analytes such as choline and glucose by using the difference in their kinetic profiles.

Experimental

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 *Aspergillus niger* EC 1.1.3.4 (Sigma G-7141) of 157,500 U g^{-1} of lyophilized solid. Solutions of 40 U mL^{-1} were prepared by dissolving the solid in the above-mentioned buffer solutions.
* Choline oxidase (ChOx) was taken from *Alcaligenes species* EC 1.1.3.17. (Sigma C-5896). One hundred units were dissolved in 2 mL of doubly distilled water and divided into fractions of 330 μL which were immediately frozen. One fraction was thawed prior to use and diluted to 900 μL with buffer solution.
* Glucose stock solutions were prepared by dissolving the appropriate amount of β-D(+)-glucose (Sigma G-5250) in the phosphate buffer solution.
* Choline stock solutions were prepared by dissolving the appropriate amount of Choline (Sigma. C-1879) in the phosphate buffer solution.

Apparatus

All measurements were carried out in a Photon Technology International (PTI) Time Master fluorescence spectrometer working in a L-configuration (model TM-2/2003-PTI). Slit widths of 2 nm (excitation) and 5 nm (emission) were selected. A 4 mL Hellma QS 101quartz cuvette of 1 cm pathlength was used. 286 nm and 340 nm were chosen as the excitation and emission wavelength respectively.

Measurement procedure

The cuvette was filled with 100 μL of GOx of 40 IU mL^{-1}, 100 μL of ChOx of 20 IU mL^{-1} and 1,700 μL of buffer solution. The mechanical stirrer was then started and the fluorescence intensity was measured at 340 nm (with excitation at 286 nm). After 90 s 100 μL of the sample (containing glucose and choline) were added to the cuvette and the variation of fluorescence during the reaction was followed during 1,200 s. For the simultaneous glucose and choline determination in a sample, the buffer solution can be fully or partially replaced by sample solution (if previously has been buffered).

For calibration and validation purposes standards solutions containing different glucose and choline concentrations were prepared (see Table 1). The Intensity *versus* time representation (I_t=$f(t)$) was obtained and before applying any method the representation was divided by the initial fluorescence intensity (corresponding to the GOx plus ChOx initial intensity):

$$I_{t,n} = \frac{I_t}{I_0^{GOx} + I_0^{ChOx}} = \frac{I_t}{I_0} \quad (4)$$

In section "Simultaneous determination from separated signals" these solutions were just using for calibration purposes.

Partial least squares regression (PLS) multivariate methods

For multivariate methods a part of the experimental results obtained with solutions indicated in Table 1 were used for calibration purposes (indicated with C) and another part for

Table 1 Glucose/choline standard solutions used for calibration and validation purposes

β-Glucose, mM → / Choline, μM ↓	0.55	1.00	1.50	2.25	3.00	3.75
0.75	1 C	2 V	3 C	4 V	5 C	6 C
2.00	7 V	8 C	9 V	10_1 C 10_2 C	11 V	12_1 V 12_2 V
3.75	13 C	14 V	15 C	16 V	17 C	18 C
5.50	19 V	20 C	21 V	22_1C 22_2C	23 V	24 V
7.50	25 C	26 V	27 C	28 V	29 C	30 C
11.0	31 C	32 V	33_1C 33_2C	34 V	35 C	36 C

Each solution is represented by a number (from 1 to 36) and a letter (*C* calibration; *V* validation); replicates are indicated as a subindex

validation purposes (indicated with V). The statistic relative error (RE) was used as:

$$\mathrm{RE} = 100\sqrt{\frac{\sum_{i=1}^{n}(\hat{c}_i - c_i)^2}{\sum_{i=1}^{n} c_i^2}} \qquad (5)$$

$\hat{c}_i$ and c_i being the estimated and added analyte concentration respectively, for sample *i*. RE can be applied to the calibration ($\mathrm{RE_{cal}}$) and prediction ($\mathrm{RE_{val}}$) sets. The $I_t=f(t)$ was transformed into a MS-Excel format to be treated with the UNSCRAMBLER version 7.5 software package (Camo A/S, Trondheim, Norway) for PLS application. All data were centred before applying any chemometric treatment. The PLS calibration models were first constructed by applying the cross-validation procedure to the calibration set (one sample was left out each time), the model was then applied to the prediction set and the $\mathrm{RE_{val}}$ was calculated for different number of latent variables (LVs). The number of LVs chosen was the lowest number whose $\mathrm{RE_{val}}$ did not differ significantly from the minimum value of $\mathrm{RE_{val}}$ according to an *F*-test with a probability $P=0.25$ [16].

Results and discussion

Simultaneous determination from separated signals

Parameter optimization

Although the ChOx and GOx reactions give different fluorescence profiles, they are not always separated. This depends on the concentrations, firstly of both enzymes and secondly of both analytes; in addition the kinetics of both reactions are also affected by the pH.

As the glucose signal appears after that of choline, it is necessary to use conditions which permit the t_a to be delayed as much as possible. According to (3a), this can be achieved using low GOx concentrations (see Fig. 2). Furthermore, low GOx concentrations are necessary in order to fulfil one of the conditions applied for the deduction of Eq. (2a) which is that the O_2 concentration does not appreciably change at the beginning of the reaction (i.e. the glucose is not quickly oxidised). On the other hand, as the GOx concentration diminishes the speed of the enzymatic reaction decreases and the linear response range is shortened due to the mechanism (a balance between enzyme regeneration by O_2 and glucose consumption). Regarding choline, it can be deduced from Eq. (2a) that the higher the analyte concentration, the higher the maximum intensity. However, this model does not show

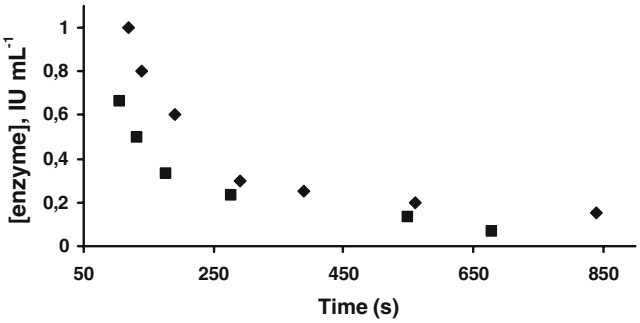


Fig. 2 Effect of enzyme concentrations on the t_a of the GOx reaction (■) and the relaxing time (t_r) for the ChOx reaction (♦). For the sake of clarity GOx concentrations have been divided by 30

that the lower the ChOx concentration, the longer the relaxing time (the time necessary for the fluorescence intensity to decrease to the initial value; t_r in Fig. 1). Bearing in mind all these considerations, concentrations of 2.0 and 1.0 IU mL^{-1} GOx and ChOx were finally selected as optimal. In these conditions $I_{t,n}=f(t)$ representations as that indicated in Fig. 3 are obtained in which two zones can be differentiated: "choline zone" and "glucose zone".

The activity of GOx and ChOx depends on the pH and the kind of buffer solution used, because both of them affect the kinetic of the enzymatic reaction. For GOx the optimum pH zone for using the intrinsic fluorescence ranges from 4 to 8 because the t_a is minimum and independent of the pH [15]; outside this range the time is longer because the reaction is slower; above pH 8.5 and below pH 3 the reaction does not take place within a reasonable time. Regarding ChOx [14], for pH values below 7 no reaction occurs while for pH values higher than 11 enzyme denaturation quickly begins. The optimum pH value is 9.5 but it is possible to work in a range between pH 8 and 10. For these reasons and after testing that the reaction of both enzymes occurs satisfactorily, pH 8 was selected as a compromise.

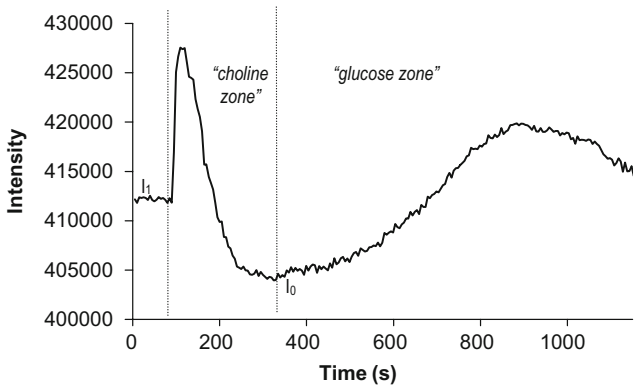


Fig. 3 Fluorescence intensity variation with a sample containing 0.55 mM glucose and 5.50 μM choline; [GOx]=2.0 U mL^{-1}, [ChOx]=1.0 U mL^{-1} in a buffer solution phosphate pH 8. Instrumental conditions as indicated in "Experimental" section

Figures of merit and analytical determination

The $I_{t,n}=f(t)$ representations for each solution indicated in Table 1 are compiled in Figure 1S (see Electronic supplementary material). Different calibration studies were performed for choline (using the parameters indicated in Eq. (2a)) with the results obtained at fixed glucose concentrations; similar calibration studies were performed for glucose (using the t_a parameter, Eq. (3b) using fixed choline concentrations. The results obtained indicated that the linear response range for each analyte depends on the fixed concentration of the other.

For choline, Fig. 4 shows the $I_{t,n}=f(t)$ representations obtained using a 0.55 mM glucose concentration. As can be seen the fluorescence in the "choline zone" varies with the choline concentration but is not affected by glucose; in addition, the fluorescence in the "glucose zone" is not affected by choline. The GOx contribution to the fluorescence intensity (as I_0^{GOx}) was subtracted and after the application of Eq. (2a) and the least squares method the following equation was given:

$$\frac{1}{\frac{I_m-I_0}{I_0}} = 5.2 + 1.09 * 10^{-5} \frac{1}{[\text{Choline}]_0} \qquad r = 0.9997 \qquad (6a)$$

Which is in reasonable agreement with the calibration results previously obtained for choline alone [14]. As the glucose concentration increases, the linear response range quickly decreases. Table 2 shows the linear response range

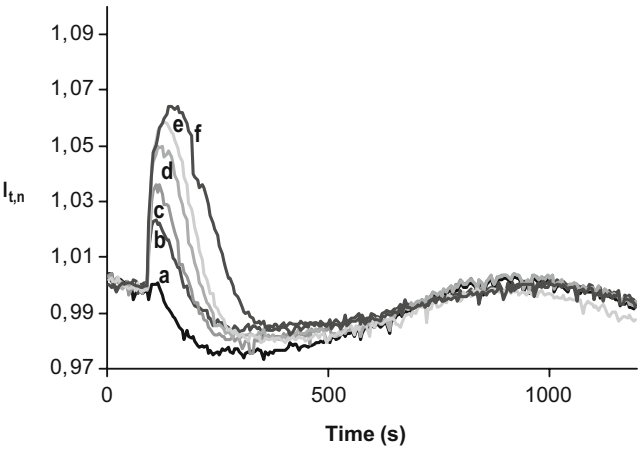


Fig. 4 $I_{t,n}$ representations obtained for a choline calibration study using a fixed glucose concentration (0.55 mM). Experimental conditions: [GOx]=2.0 IU mL^{-1}, [ChOx]=1.0 IU mL^{-1}in a buffer solution phosphate pH 8. Choline concentrations: **a** 0.75 μM; **b** 2.00 μM; **c** 3.75 μM; **d** 5.50 μM; **e** 7.50 μM; **f** 11.0 μM

Table 2 Effect of the glucose concentration on the choline linear response range, calibration line and correlation coefficient

Glucose (mM)	Choline linear range (μM)	Slope (μM)	Intercept	*r*
1.00	2.00–11.0	19	3.4	0.9990
1.50	3.75–11.0	17	2.8	0.9961
2.25	3.75–11.0	14	2.4	0.9982
3.00	No linear range			
3.75	No linear range			

and the corresponding fits when using the $I_{r,n}$ obtained with the solutions indicated in Table 1.

For glucose determination, Fig. 5 shows the representations obtained for this analyte when using a fixed choline concentration (0.75 μM). This figure shows how the choline signals appear clearly differentiated for low glucose concentrations (0.55 and 1.00 mM) but for higher glucose concentrations, the choline signal overlaps the "glucose zone". A new calibration set including concentrations between 0.50 and 1.00 mM of glucose was prepared (six points). The application of Eq. (3b) and the least squares method to glucose concentrations ranging from 0.50 to 1.00 mM gives a linear response range according to the following equation:

$$t_m = 2 + \frac{0.278}{[\text{Glucose}]_0} \qquad r = 0.998 \tag{6b}$$

In this case also, the calibration line agrees with that obtained without choline. As would be expected, the linear response range decreases as the choline concentration increases (see Table IS in Electronic supplementary material).

The above results show that for the simultaneous determination of glucose and choline Eqs. (6a) and (6b) have to be fulfilled simultaneously and the concentration of both analytes should be low enough in the sample solution (see Table 1). This permits the determination of both analytes in samples such as dietary supplements or infant milk formulations but not for simultaneous determination in serum and other kinds of samples.

Simultaneous determination from overlapping signals

Two different approaches were studied: one based on multivariate calibration and another based on a theoretical approximation performed from the kinetic models summarized in Eqs. (2a) and (3a). To do these studies, the choline and glucose standard solutions indicated in Table 1 were considered.

Multivariate calibration

Several studies were carried out using multivariable calibration (PLS) in order to solve mixtures, because this method gave successfully results (compared to PCR and neural networks) for bilirubin [17] and alcohol speciation using the intrinsic fluorescence of the corresponding enzymes. For glucose determination the range of variables were (in all cases, t=0 time corresponds to the moment of the substrate addition):

(a) the complete range of time (because glucose needs more time than choline for full signal decay);
(b) 0–350 s, time which includes the increment in the glucose signal in all samples;
(c) a further two ranges, one being the same as (b) but excluding the time interval at which the choline signal is maximum (50–350 s) and other one being from 50 to 1,000 s.

For choline determination the range of variables is the zone due to choline alone (0–50 and 0–110 s). In the best conditions the RE (Eq. (5)) for both calibration and validation were higher than 20% (see Table IIS, in Electronic supplementary material).

In general few LV are necessary to minimize RE_{cal} and RE_{val}, using the rule of Haaland [16], but the value of RE_{cal} and RE_{val} are high. In any case, when a PCA model was made and a plot of PC2 versus PC1 represented, the score matrix was much distorted with respect to the calibration

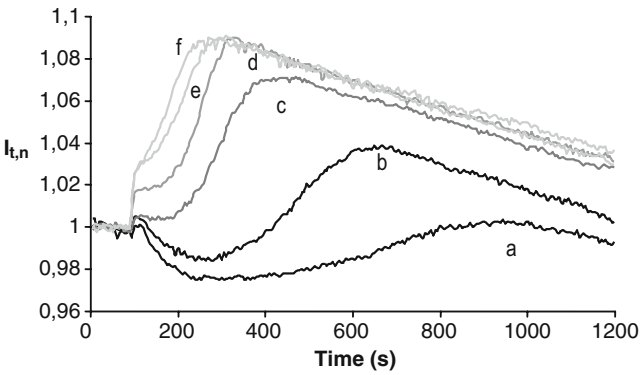


Fig. 5 $I_{t,n}$ representations obtained for a glucose calibration study using a fixed choline concentration (0.75 μM). Experimental conditions: [GOx]=2.0 IU mL^{-1}, [ChOx]=1.0 IU mL^{-1}in a buffer solution phosphate pH 8. Glucose concentrations: **a** 0.55 mM; **b** 1.00 mM; **c** 1.50 mM M; **d** 2.25 mM; **e** 3.00 mM; **f** 3.75 mM

matrix, indicating the presence of non-linearities. In the case of glucose this could be due to the fact that both the intensity and the t_{max} of the signals vary with the glucose concentration, but the latter parameter does not depend linearly on the glucose concentration.

As it is demonstrated in Figure 2S (Electronic supplementary material), when the glucose signal appears separated from the choline signal, the kinetic of both enzymes do not affect each other. However when the signals overlap and the analyte concentrations are high (Figure 3S in Electronic supplementary material), choline reaction affects the fluorescence intensity of the other so the intensity are not additive.

Univariate method: a fit for purpose mathematical model

A) Model for glucose

The main difference between the $I_{t,n}$ representations obtained using GOx and GOx plus ChOx is that the maximum intensity due to GOx depends on the analyte concentration (Fig. 5).

This could be explained in terms of the GOx concentration used in this study (2 U mL^{-1}), which is much lower than that previously used. In these conditions some oxygen remains in solution when all the glucose has been consumed; consequently a part of the enzyme is not in the reduced form and the intensity of the maximum is lower. To test this hypothesis, Figure 4S (Electronic supplementary material), shows a calibration study done using 2 U mL^{-1} GOx. As can be seen the same type of register as previously indicated is obtained. For high glucose concentrations the oxygen is fully consumed and the maximum intensity becomes independent of the glucose concentration.

From an analytical point of view this result is very interesting because it allows the maximum intensity (I_{max}) and the time of the maximum (t_{max}) to be used as the analytical parameters for glucose determination. However, the experimental data indicated that the I_{max} gave poor results and consequently the t_{max} is finally applied. From the results obtained with the calibration solutions indicated in Table 1, the following equation was obtained by least squares:

$$t_{max} = 70.358 + 0.4099\frac{1}{[\beta - \text{Glucose}]_0} \qquad r = 0.9995 \quad (7)$$

For method validation, the solution in the validation set (Table 1) containing the same glucose concentration were considered replicates (assuming that choline does not interferes), the results obtained were interpolated in the previous equation and the corresponding glucose concentration were obtained. Table 3 compiles the results obtained. For all glucose concentrations, the application of the corresponding statistical significant test did not revel significant different between added and obtained concentrations [18].

Table 3 Validation of the improved method for glucose determination

Glucose, mM (added)	Glucose, mM (found)	*n*	Solutions used[a]
0.55	0.55±0.06	2	7, 19
1.00	1.02±0.07	4	2, 14, 26, 32
1.50	1.53±0.09	2	9, 21
2.25	2.24±0.10	4	4, 16, 28, 34
3.00	3.10	1	11
3.75	3.83±0.34	3	12_1, 12_2, 24

[a] The number corresponds with that used in Table 1

B) Model for choline

When the choline and the glucose signals overlap, the maximum intensity in the "choline zone" is not only due to choline but also to the fluorescence of GOx that has to be considered. To do this an improved mathematical model based on Eq. (2a) has been developed. The intensity in the choline maximum can be now written as:

$$I_{max,r} = \frac{I_{max}^{ChOx} + I^{GOx}}{I_0^{ChOx} + I_0^{GOx}} \quad (8)$$

I_{max}^{ChOx} being the intensity at the maximum due to ChOx and I^{GOx} being the intensity at the choline maximum due to GOx. Considering the I^{GOx} definition:

$$\begin{aligned} I^{GOx} &= K_{ox}^{GOx}[\text{GOx}] + K_{red}^{GOx}[\text{GOx.H}_2] \\ &= I_0^{GOx} + \left(K_{red}^{GOx} - K_{ox}^{GOx}\right)[\text{GOx.H}_2] \end{aligned} \quad (9)$$

In light of the $I_t=f(t)$ representations for glucose we can consider that at the beginning of the reaction (which is when the choline maximum appears) the $[GOx.H_2]$ linearly increases with time:

$$[\text{GOx.H}_2] = \alpha t = \delta[\text{Glucose}]t \quad (10)$$

δ being the proportionality constant which does not depend on the glucose concentration. Substitution of Eqs. (9), (10) and (3a) in (8) and rearranging gives:

$$\begin{aligned} \frac{I_{max}}{I_0} &= 1 + \left(\frac{K_{red}^{GOx} - K_{ox}^{GOx}}{I_0 K_{ox}^{GOx}}\right)\left(\frac{[\text{Choline}]_0}{[\text{Choline}]_0 + B}\right) \\ &\quad + \left(\frac{K_{red}^{GOx} - K_{ox}^{GOx}}{I_0}\right)\alpha t \\ &= 1 + C\left(\frac{[\text{Choline}]_0}{[\text{Choline}]_0 + B}\right) + D \end{aligned} \quad (11)$$

This equation relates the $I_{max,r}$ with the choline concentration. The B, C and D values were obtained by using the experimental values and fitted using Origin 6.0 software. All the solutions indicated in Table 1 were used again as a standard calibration set. The results obtained for each glucose concentration (each column) were then fitted to equation (11) and the C, B and D values were obtained (Fig. 6). As can be seen, the representation of C and B versus glucose is a straight line with a zero slope, so these parameters are independent of $[\beta\text{-Glucose}]_0$ with average values of:

$$C = 0.14(\pm 0.02) \quad B = 4.8(\pm 1.0)*10^{-6} \tag{12}$$

As was expected, a D dependence of $[\beta\text{-Glucose}]_0$ was observed which can be fitted to a straight line. The slope (δ) shows how D varies with $[\beta\text{-Glucose}]_0$.

The next equation can be written:

$$I_{max,r} = 1 + C\left(\frac{[col]_0}{[col]_0 + B}\right) + \delta[\beta - G]_0 \tag{13}$$

where C, D and δ are now known. $[col]_0$ can be written as:

$$[col]_0 = B\left(\frac{I_{max}^{read} - 1 - \delta \times [\beta G]_0}{\delta[\beta G]_0 + C + 1 - I_{max}^{read}}\right) \tag{14}$$

so the choline concentration can be calculated from the I_{max} of the "choline zone". This model was validated using a similar procedure as that described for glucose: the solutions in the validation set (Table 1) containing the same choline concentration were considered replicates (assuming that choline does not interferes) and after interpolating the corresponding choline concentration were obtained (Table 4). In this case, the standard deviation values were higher than those for glucose indicating that the choline determination is less precise than glucose; also in

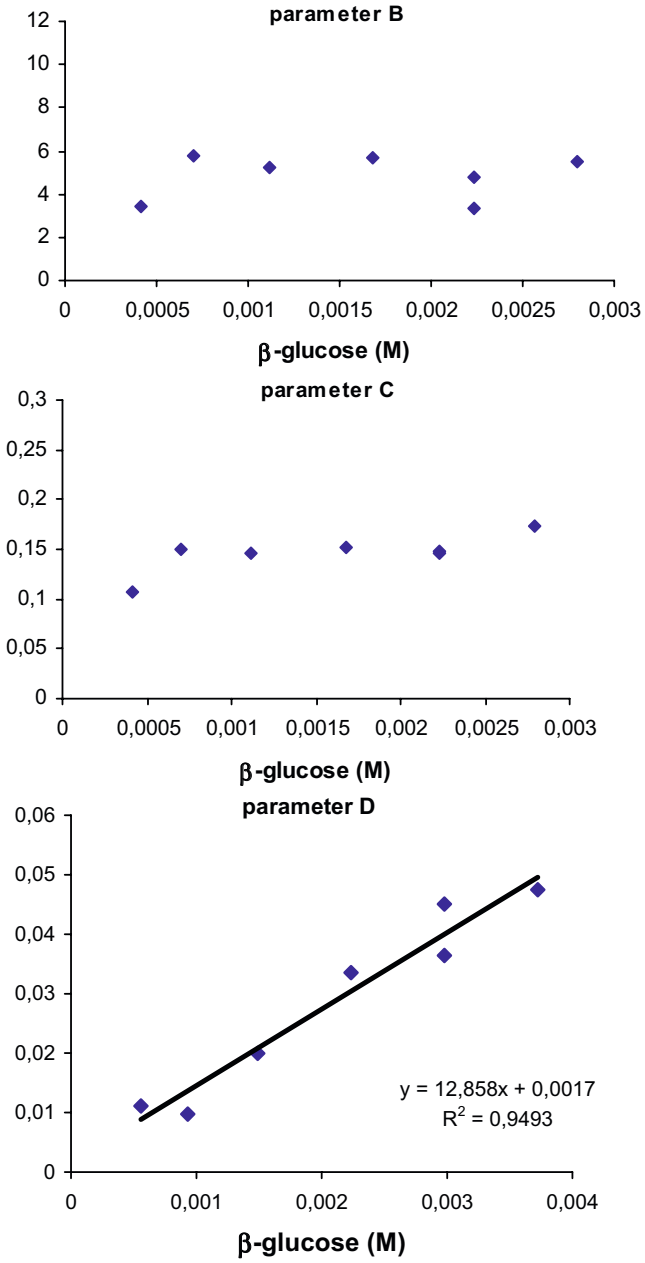


Fig. 6 Variation of B, C and D versus [β-glucose]. Parameter B must be multiplied by 10^{-6}

Table 4 Validation of the improved method for choline determination

Choline, μM (added)	Choline, μM (found)	n	Solutions used[a]
0.75	0.76±0.08	2	2, 4
2.00	2.09±0.17	5	7, 9, 11, 12_1, 12_2
3.75	3.77±0.20	2	14, 16
5.50	5.73±0.40	4	19, 21, 23, 24
7.50	7.20±0.60	2	26, 28
11.0	10.7±1.0	2	32, 34

[a] The number corresponds with that used in Table 1

Table 5 Choline and glucose determination in serum samples (pH 8, ChOx=1 U mL^{-1}, GOx=2.0 U mL^{-1})

Glucose certified (mM)	Glucose found (mM)	Choline added (μM)	Choline found (μM)
4.8±0.7	4.4±0.3	13	11.5±0.1

this case the application of the corresponding statistical significant test [18] did not revel significant different between added and obtained concentrations.

C) Glucose and choline determination

Using this method, glucose and choline were determined in a synthetic serum sample. A commercial synthetic serum (Normal Control Serum, Thermo, TR40001/1902-050) for which the glucose concentration is certified was used. This sample does not contain choline so once the sample was dissolved in water, choline was added. After ultrafiltration the choline and glucose were determined. The results obtained are shown in Table 5.

Conclusions

The determination of choline and glucose contained in a sample can be carried out by measuring the variation in fluorescence intensity of a mixture of choline and glucose oxidase. Spectral discrimination is not necessary, but only the fluorescence intensity measurement over time at one wavelength. The glucose and choline determination using absolute calibrations can be considered as satisfactory, with a lower precision for choline. Simultaneous determination using multivariable methods does not improve the results obtained using absolute calibration. This methodology could be also used for the simultaneous determination of other pairs of analytes.

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