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Rapid enzymatic chemiluminescent assay of glucose by means of a hybrid flow-injection/sequential-injection method

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Abstract

This work reports a hybrid flow-injection analysis (FIA)/sequential-injection analysis (SIA) method for the rapid enzymatic assay of glucose with soluble glucose oxidase (GOD). The method relies on the sequential injection of segments of the sample and of a solution of enzyme by means of a multi-port selection valve in a flowing water stream. As the two zones are swept downstream, they overlap and merge so that the glucose in the sample is enzymatically oxidised. The generated hydrogen peroxide is merged with an alkaline luminol solution and the chemiluminescence (CL) intensity is monitored and related to the glucose concentration in the sample. The linear range of the method for glucose determination is 0.01–1 mmol L^{-1}, the relative standard deviation is 3.9% at the 0.08 mmol L^{-1} level ($n = 8$), the limit of detection at the 2σ level is 4 μmol L^{-1} glucose and the injection rate is 80 h^{-1}. The method was applied to the analysis of energy drinks and honey with relative errors in glucose determination in the range $100 \pm 4.3\%$. The advantages of the proposed method are the wide linear range, the simple instrumentation used, the low consumption of sample and reagents, the elimination of catalysts and immobilised enzymes and the high sample throughput.

Keywords: Glucose; Chemiluminescence; Flow-injection analysis; Sequential-injection analysis; Glucose oxidase

1. Introduction

Over the last 30 years, flow-injection analysis (FIA) has been established as the most widely used flow method of analysis [1]. FIA is based on the injection of a discrete segment of sample into a carrier stream which mixes on-line with other flow streams of various reagents followed by the determination of the reaction products in a suitable flow-through detector. The main advantages of FIA compared to batch procedures are its rapidity, precision and scope for automation. Sequential-injection analysis (SIA) has been developed to address some drawbacks of FIA, namely the high consumption of reagents and the use of multi-channel manifolds [2]. SIA relies on the aspiration of zones of sample and reagents into a holding coil of a single-channel manifold and delivery of the stacked zones towards the detector. As the zones move, mutual overlap occurs resulting in the generation of a measurable product.

Chemiluminescence (CL) refers to the phenomenon of production of light in the course of chemical reactions. CL detection relies on the measurement of the intensity of light emitted in a reaction between the analyte of interest and specific chemiluminescent reagents [3]. The advantages of CL as an analytical technique are its high sensitivity and simple instrumentation but, on the other hand, CL suffers from lack of specificity and from strong temporal dependence of the CL signal. FIA is ideally suited to CL detection since the spatial and temporal conditions of the on-line mixing are precisely controlled and its utility in conjunction with CL detection has been well documented [4,5]. On the other hand, SIA has found only limited applications in CL detection [2]. The main reason is that conventional SIA manifolds are not well suited to handle the fast reaction kinetics of most commonly used CL reactions [6].

In order to address the problem of specificity of the CL detection, enzymatic CL reactions have been adapted to FIA

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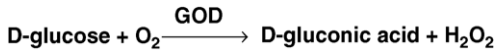
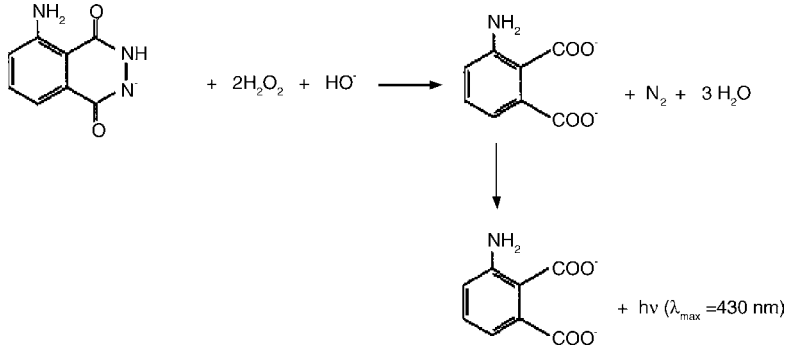


Scheme 1. Enzymatic oxidation of glucose by glucose oxidase (GOD).

[7–9] and, to a lesser extent, SIA [10–12]. The main difficulty associated with the use of enzymes in FIA is the handling of the enzyme itself. The great majority of published work on CL flow enzymatic assays rely on re-usable immobilised enzymes [7–12]. The enzyme is immobilised in a reactor and, as the sample is introduced in the manifold, the analyte interacts with the enzyme inside the reactor and the enzymatic conversion takes place on-line. This methodology minimises the consumption of expensive enzymes and simplifies the flow manifold. Yet, there are also severe limitations related to enzyme immobilisation schemes [6,13,14]: (a) some enzymes do not lend themselves to immobilisation; (b) immobilisation is time-consuming and requires trained personnel and harmful organic solvents; (c) there is usually a gradual decrease in the activity of the immobilised enzyme with time as the enzyme is continuously leached from the support or the reactor is inactivated by the matrix components in the sample. Therefore, the use of soluble enzymes in flow analysis is highly desirable but is also difficult to implement bearing in mind that minimal consumption of the enzyme is imperative. Regarding FIA, the obvious choice of using the soluble enzyme as a carrier or in a separate flow line is unacceptable due to high consumption of the enzyme solution. A scheme for improved enzyme economy is intermittent pumping in which enzyme solution is placed in a separate flow line but its flow is activated only as the sample plug reaches the confluence point [13]. As an alternative, the "merging-zones" or "chasing-zones" approach has been suggested: this involves injection of a train of zones of sample and enzyme solution that overlap and merge as they move towards the detector [13]. The drawback of these approaches is the rather complex instrumentation and operational sequence. As far as SIA is concerned, although its potential in the manipulation of minute volumes of enzyme has been realized quite early [15], few applications with soluble enzymes have been reported.

Glucose can be enzymatically determined by taking advantage of its enzymatic oxidation to gluconic acid and hydrogen peroxide in the presence of glucose oxidase (GOD) according to Scheme 1. Based on the detection of hydrogen peroxide, several FIA and SIA methods with electrochemical or optical detection have been developed [14]. CL detection of hydrogen peroxide relies on its well-known CL reaction with luminol (Scheme 2). However, most reported flow procedures for the determination of glucose, including those with CL detection, again rely on immobilised GOD and only a few make use of soluble enzyme [14]. An interesting CL flow method that exploits soluble GOD for the determination of glucose is based on multi-syringe FIA (MSFIA) but the manifold is rather complicated requiring several solenoid valves, it employs an additional reagent (i.e. a Co(II) solution as catalyst), has a narrow linear range and the operational sequence is rather complicated [6]. Besides, a FIA scheme has been reported for the determination of glucose relying on on-line pre-mixing of soluble GOD and sample before the injection point and electrochemiluminescent (ECL) detection [16]. However, this method requires a potentiostat and associated electrodes and is prone to electrode fouling.

In this work, we propose a new simplified procedure for the enzymatic CL assay of glucose by means of a hybrid FIA/SIA method with soluble enzyme. The suggested method is based on the merging-zones principle implemented with a multiport selection valve configured as a multiple injection valve. The zones of sample and enzyme are selected and sequentially injected by simply rotating the selection valve to the required port. The two adjacent segments overlap on-line and the generated hydrogen peroxide merges with an alkaline luminol stream in front of the CL detector. The proposed methodology shares characteristics from both FIA and SIA. From FIA, it borrows the unidirectional and continuous flow in a multi-channel manifold. From SIA, it borrows the multi-port selection valve for solution introduction and the timed-based sequential nature of zone introduction (stacking). In addition to the elimination of the drawbacks associated with the use of immobilised enzyme, further advantages of the proposed method are the simple instrumentation (manifold, injector and detector), the low consumption of sample and enzyme, the elimination of catalysts and the high sample throughput.



Scheme 2. The CL reaction between luminol and hydrogen peroxide in alkaline media.

2. Experimental

2.1. Reagents

Chemicals were purchased from Merck (Darmstadt, Germany). Stock solutions of 0.1 mol L^{-1} glucose, fructose, sucrose, maltose, lactose, galactose and ascorbic acid were prepared in de-ionised water. A 0.01 mol L^{-1} solution of luminol was prepared in 0.01 mol L^{-1} NaOH. Glucose oxidase (GOD) with activity of 24 units mg^{-1} was purchased from Fluka (Buchs, Switzerland). The enzyme solution for the analysis was prepared in 0.1 mol L^{-1} phosphate buffer (pH 7) and contained 24 units mL^{-1}. Solution containing different concentrations of Co(II) and Cu(II) were prepared from atomic standard solutions containing 1000 mg L^{-1} of the respective metal. The samples were commercial energy drinks and honey. Before analysis, the cans containing the energy drinks were opened and left for 2 h to expel carbon dioxide. Then, the samples were diluted 1/500 with de-ionised water. 0.1 g from the honey sample were added to 10 mL de-ionised water, treated for 5 min at 60 °C in an ultrasonic bath and the solution was diluted to 500 mL with de-ionised water.

2.2. Apparatus

The 10-port selection valve with microelectric actuator was obtained from (Vici Valco, Schenton, Switzerland). The peristaltic pump was a Gilson Miniplus 3 (Villiers-le-Bel, France) featuring 0.5 mm i.d. Tygon tubing. The CL detector was a miniature Hamamatsu H6780 photomultiplier tube (Hamamatsu Photonics, Japan) operating at 600 V. The optical flow-cell was a spiral homemade glass unit and was placed in front of the detector in a light-tight box. The tubing was PTFE 0.75 mm i.d. (Jour Research, Sweden). A 6025 E PCI multi-function interface card (National Instruments, Austin, TX) was used to interface the valve, pump, and detector to a personal computer. The experimental set-up is illustrated in Fig. 1. The control and acquisition programme was developed in LabVIEW 5.1 (National Instruments, Austin, TX) and allowed complete automation of the experimental sequence.

2.3. Experimental procedure

The experimental sequence is illustrated in Table 1. Initially, the valve was maintained in port 1 (C, carrier) allowing water to flow through the manifold. A zone of sample or standard was injected by switching the selection valve to port 2 (S, sample) and maintaining it in this position for a precisely controlled time (step 1). Then, a zone of GOD solution was injected by switching the selection valve to port 3 (E, enzyme) and maintaining it in this position for a precisely controlled time (step 2). Then, the selection valve was switched back to port 1 (C, carrier) that directed the two zones towards the detector through a reaction coil (step 3). During their travel in the reaction coil, the two zones overlapped and the enzymatic reaction took place. The hydrogen peroxide generated by the reaction merged with a stream of alkaline luminol (L) and a second stream of water (C) at the confluence point M where the CL reaction was initiated. The CL solution was directed into the flow cell where the CL intensity was monitored by the detector, recorded, displayed on the screen of the PC and saved as an ASCII file. Note that the second stream of water was introduced in order to increase the total flow rate of the final CL solution and to minimise the light losses before the CL solution reached the flow cell. The same effect could be achieved by increasing the flow rates of the luminol and carrier streams at the expense of higher luminol consumption. Thus, if manifold simplicity is critical, the current three-channel manifold could be further simplified to a two-channel manifold by proper adjustments of the experimental parameters.

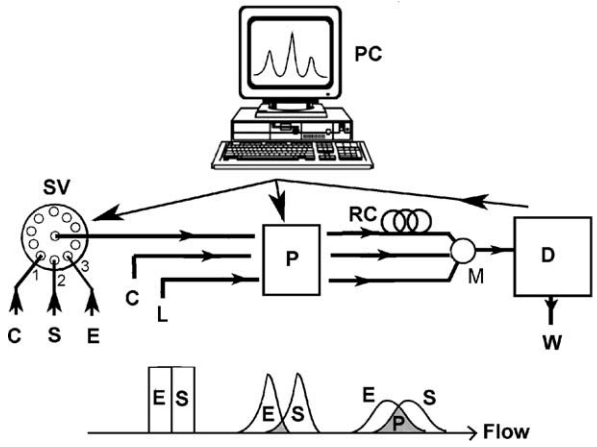


Fig. 1. The experimental set-up of the proposed method (SV, selection valve; P, pump; D; detector; RC, reaction coil; M, confluence junction; PC, personal computer; C, carrier (de-ionised water); S, glucose standard or sample; E, enzyme solution (24 units mL^{-1} of GOD in phosphate buffer pH 7); L, luminol solution (0.2 mmol L^{-1} luminol in 0.15 mol L^{-1} NaOH); W, waste. The lower part gives a schematic representation of the zone profiles of the sample, S, enzyme, E, and product, P, during the analysis.

3. Results and discussion

The selection of the values of the geometrical and chemical parameters was carried out univariately and were

Table 1
Experimental sequence of the proposed method (valve positions refer to Fig. 1)

Step	Valve position	Operation	Time (s)	Data acquisition	Flow rate (mL min^{-1})
1	2	Sample injection	6.25	No	1.2
2	3	Enzyme injection	6.25	No	1.2
3	1	Delivery to detector and recording of signal	32.5	Yes	1.2

studied at two concentration levels of glucose, $c_{glucose}$, i.e. 10^{-3} and 10^{-4} mol L^{-1}. The selection process was guided by a compromise between sensitivity, sample throughput, sample and reagent consumption and linearity. The initial conditions were: amount concentration of NaOH, c_{NaOH}, 0.1 mol L^{-1}; flow rate, Q_v, 1.5 mL min^{-1}; volume of glucose, $V_{glucose}$, 125 μL; volume of enzyme, V_{enzyme}, 125 μL; length of reaction coil, $l_{reaction\ coil}$, 30 cm. The flow rate refers to the flow rate of each flow channel so that the total flow rate was three times the quoted flow rate.

Fig. 2a shows the effect of the luminol concentration on the CL intensity in the range of 0.01–1 mmol L^{-1}. For a concentration of glucose of 10^{-3} mol L^{-1}, increasing the concentration of luminol caused an increase in the CL intensity up to 0.1 mmol L^{-1} luminol while at higher luminol concentrations the CL intensity decreased. On the contrary, for a glucose concentration of 10^{-4} mol L^{-1}, the CL intensity was almost constant up to 0.2 mmol L^{-1} of luminol and decreased at higher concentrations. Therefore, there was a marked difference at low concentration of luminol between the 10^{-3} and the 10^{-4} mol L^{-1} glucose solutions. For the glucose concentration of 10^{-3} mol L^{-1}, a significant amount of hydrogen peroxide was produced so that this compound was in excess with respect the dilute luminol solution. Therefore, the CL intensity was controlled by the concentration of the luminol solution. As the luminol concentration increased, so did the CL intensity. For the glucose concentration of 10^{-4} mol L^{-1}, a lower amount of hydrogen peroxide was produced and, even at low luminol concentrations, there was enough luminol to react with the hydrogen peroxide generated. Therefore, the CL intensity was mostly independent of the luminol concentration. The decrease at higher luminol concentrations was attributed to quenching of the light produced by the luminol solution. Consequently, the selection of the concentration of the luminol solution should be made in such a way as to always provide an excess of luminol but to avoid excessively high luminol concentrations. A luminol concentration of 0.2 mmol L^{-1} was selected for subsequent experiments.

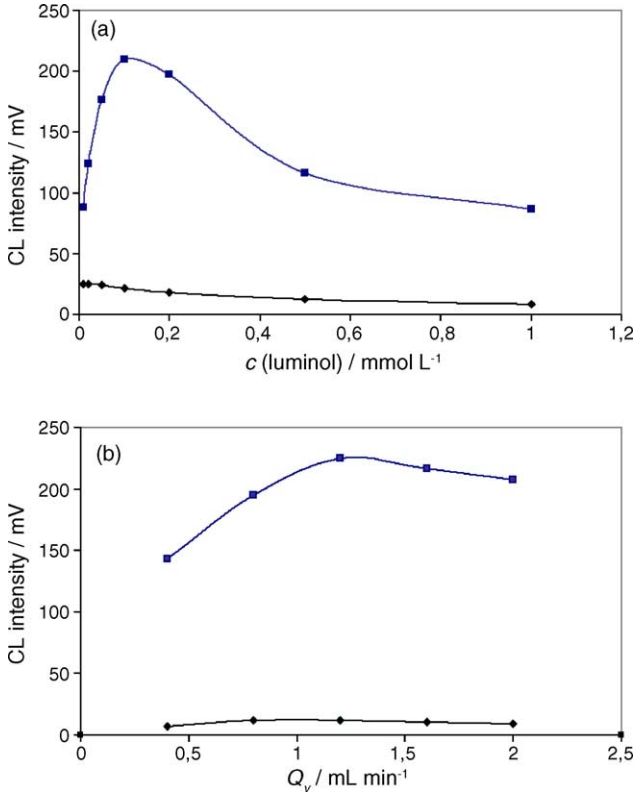


Fig. 2. The effect of different parameters on the CL intensity of a solution containing 10^{-3} mol L^{-1} (■) and 10^{-4} mol L^{-1} (♦) glucose: (a) the luminol amount concentration and; (b) the flow rate.

It is well known that the CL reaction of luminol oxidation by hydrogen peroxide is facilitated at alkaline pH. In this work, an alkaline environment was ensured by adding NaOH in the luminol solution. The effect of the NaOH concentration on the CL intensity was studied in the range of 0.05–0.4 mol L^{-1}. Increase in the NaOH concentration up to 0.1 mol L^{-1} led to an increase in the CL intensity, whereas at higher NaOH concentrations a decrease of the CL intensity was observed. Therefore, a NaOH concentration of 0.15 mol L^{-1} was selected.

Then, the order of injection was investigated. It was found that the order of injection of the sample and the enzyme solution did not practically affect the CL intensity, an observation that corroborated the efficient merging of the sample and enzymes zones in the manifold.

The effect of the volume of glucose solution injected on the CL intensity was investigated in the range of 25–250 μL. It was observed that upon increasing the volume of the glucose solution, the CL intensity also increased. As the zone overlap was significant, a larger volume of glucose solution caused the production of a larger amount of hydrogen peroxide and, therefore, led to an increase in the CL intensity. However, at higher glucose injection volumes, the peaks became wider due to increased dispersion which also dictated a decrease in the sampling rate The selected volume was 125 μL which allowed a satisfactory sampling rate and low sample consumption as well as adequate sensitivity.

It was found that the CL intensity was not significantly affected by the volume of the GOD solution injected in the range of 25–250 μL. This was attributed to the fact that using the solution of enzyme with activity 24 units mL^{-1}, GOD was always in great excess over glucose. It must be noted that the enzyme activity is highest in the pH range of 5–7 and the enzyme solution (prepared in phosphate buffer pH 7) also served as a buffer for the sample solution. In order to ensure that adequate buffering capacity was provided, a volume of 125 μL of enzyme (i.e. at least equal to the volume of the sample) was selected.

The effect of the reaction coil on the CL intensity was studied in the range of 30–300 cm. The CL intensity increased as the length of the reaction coil increased up to 230 cm and remained constant for longer reaction coils. This was due to the more efficient zone overlap taking place as the length of the reaction coil increased. However, increasing the length

of the reaction coil also caused widening of peaks due to increased dispersion and also resulted in a narrower linear range. A length of 110 cm was selected as a compromise between sensitivity, sampling rate and linearity.

The effect of the flow rate was studied in the range of 0.4–2 mL min^{-1} as shown in Fig. 2b. It must be emphasized that the flow rate refers to the flow rate of each single channel and that the three channels were set at the same flow rate. Thus, the total flow rate in the flow cell was three times the quoted flow rate. It was observed that the CL intensity increased up to a flow rate of 1.2 mL min^{-1} and decreased at higher flow rates. The initial increase was due to the elimination of light losses after the solutions were mixed at the confluence point, as the final CL solution was transported faster to the flow cell. It must be noted that the auxiliary water flow line was incorporated in the manifold for this very purpose. Increase in the flow rate beyond 1.2 mL min^{-1} did not allow sufficient time for the sample and enzyme zones to mix and the CL intensity decreased. The value of 1.2 mL min^{-1} was finally selected. For quantitative analysis, the conditions shown in Table 2 were employed.

Then, different sources of interferences were investigated. Provided that the enzyme preparation does not contain other enzymes (other oxidases, catalases, invertases), the main potential interferences are saccharides, metal cations and reducing agents. Both monosaccharides and disaccharides are commonly found in samples containing glucose and can interfere in many ways. GOD lacks absolute specificity and glucose stereoisomers (i.e. galactose) may also undergo catalysis, causing a positive error [17]. In addition, most saccharides are reducing agents and can reduce the hydrogen peroxide generated in the enzymatic reaction between glucose and hydrogen peroxide, leading to a negative error [6]. Finally, disaccharides containing a glucose molecule (i.e. sucrose) may hydrolyse in the course of the analysis or monosaccharides (i.e. fructose) may undergo isomerisation [18]; both these transformations produce glucose, resulting in a positive error. In this work, sucrose, fructose, lactose, maltose and galactose were investigated and the tolerance levels are shown in Table 3. All the saccharides caused a decrease in the CL intensity of a solution of 1×10^{-4} mol L^{-1} glucose, suggesting that the reduction of hydrogen peroxide by these compounds was the prevailing interference factor. Metal cations can have an enhancing or inhibiting effect on the CL intensity. For instance, it was interesting to find that Cu(II) has a strong inhibiting effect on the CL intensity, presumably due to poisoning and inactivation of the enzyme. On the other hand, Co(II) had a strong enhancing effect upon the CL intensity due to its well-known catalytic activity on the luminol-hydrogen peroxide reaction. Thus, the tolerance levels for Co(II), Cu(II) and Fe(II), shown in Table 3, were rather low. Other metals, more abundant in many samples, such as K^+, Na^+, Mg^{2+}, Ca^{2+} caused no interference (Table 3). Ascorbic acid was selected as a model of a strongly reducing agent and it was found to interfere severely causing a dramatic drop of the CL intensity. This was attributed to the scavenging of the produced hydrogen peroxide by ascorbic acid and suggested that the utility of the proposed method might be compromised in media containing high concentrations of strongly reducing compounds. One of the features of the proposed method is that it allows extensive dilution of the sample so that interferences due to the presence of metal cations and reducing compounds are alleviated.

Table 2
Selected conditions for the determination of glucose

Parameter	Range studied	Selected value
$c_{luminol}$ (mmol L^{-1})	0.01–1	0.2
c_{NaOH} (mol L^{-1})	0.04–0.4	0.15
$V_{glucose}$ (μL)	25–250	125
V_{enzyme} (μL)	25–250	125
$l_{reaction\ coil}$ (cm)	60–330	110
Q_v (mL min^{-1})	0.4–2	1.2

Table 3
Interference study

Interfering species	Tolerance level[a]
Fructose, galactose	0.5 mmol L^{-1}
Lactose, sucrose	1 mmol L^{-1}
Maltose	2 mmol L^{-1}
K^+, Na^+, Mg^{2+}, Ca^{2+}	100 mg L^{-1}
Co^{2+}, Fe^{2+}, Cu^{2+}	0.05 mg L^{-1}
Ascorbic acid	0.01 mmol L^{-1}

[a] Tolerance level is the concentration of interferent that caused a ±10% error in the determination of 0.1 mmol L^{-1} glucose.

Fig. 3 illustrates a calibration curve for glucose in the range of 0.01–5 mmol L^{-1}. The calibration curve had the shape predicted by the Michaelis–Menten theory and obeyed a second order equation:

$$I = -21.741\,(c_{glucose})^2 + 213.14\,(c_{glucose}) + 9.0859$$

where I is the CL intensity (mV), $c_{glucose}$ the amount concentration of glucose (mmol L^{-1}).The regression coefficient was $R^2 = 0.998$.

The calibration curve was linear in the range 0.01–1 mmol L^{-1} which is particularly useful for practical appli-

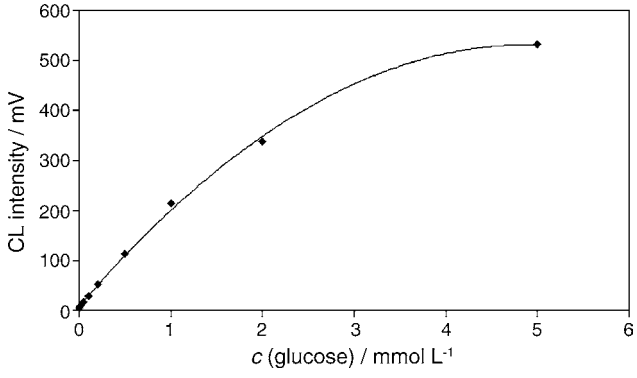


Fig. 3. Calibration curve for glucose with the proposed method in the range of 0.01–5 mmol L^{-1}.

Table 4
Comparison of the analytical features of the proposed method with the MSFIA/CL and FIA/ECL methods employing soluble GOD, a spectrophotometric multi-commutation (MC)FIA employing soluble GOD, and two CL methods employing immobilised GOD

Method	Reaction	Catalyst	Enzyme	Linear range (mmol L^{-1})	c_L (μmol L^{-1})	Injection rate (h^{-1})	s_r (%) (mmol L^{-1})	Enzyme/ injection (units)	Luminol/ injection (μmol)	Sample/ injection (μL)	Reference
SIA-FIA/CL	Luminol-H_2O_2	NA	Soluble GOD	0.01–1	4	80	3.9 (0.08)	3	0.16	125	Present
MSFIA/CL	Luminol-H_2O_2	Co(II)	Soluble GOD	0.005–0.015	0.4	20	2.5 (0.005)	14	0.08	200	[6]
FIA/ECL	Luminol-H_2O_2	+1.9 V	Soluble GOD	0.027–0.27	4.3	NR	3.3–3.6 (0.11)	NR	NR	NR	[16]
MCFIA/Vis	4-aminophenazone-phenol	NA	Soluble GOD–soluble POD	2.8–11.1	NR	30	0.3 (5.6)	5	NA	112	[19]
MSFIA/CL	Luminol-H_2O_2	Co(II) or HRP	Immobilised GOD	0.0025–0.02 (log-log)	0.8	12	2.5–4 (0.01)	NA	0.064	200	[12]
SIA/CL	Luminol-H_2O_2	$K_3[Fe(CN)_6]$	Immobilised GOD	0.03–0.6	15	54	2.7 (0.05)	NA	0.00375	900	[20]

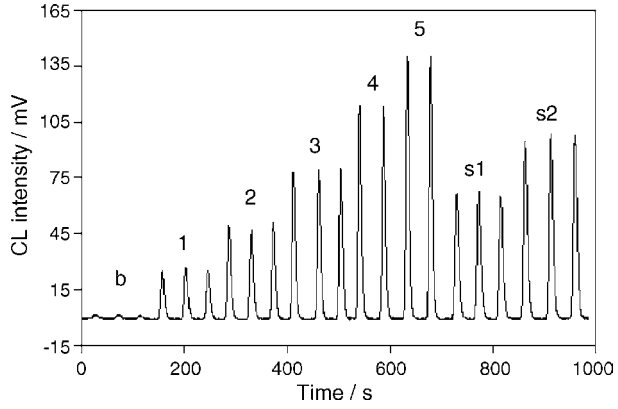


Fig. 4. FIA peaks for standards and samples using the proposed method (b, blank; 1–5, solutions of glucose 0.1, 0.2, 0.3, 0.4 and 0.5 mmol L^{-1}; S1, S2, samples of energy drinks diluted 1/500).

cations and was expressed by the first-order equation:

$$I = (207.9 \pm 2.4)(c_{\text{glucose}}) + (7.8 \pm 1.0)$$

The regression coefficient was $R^2 = 0.9993$.

The coefficient of variation at 8×10^{-5} mol L^{-1} of glucose was $s_r = 3.9\%$ ($n = 8$). The limit of detection of glucose determination at the 2σ level was $c_L = 4 \times 10^{-6}$ mol L^{-1}. The limit of detection was not defined by the sensitivity of detection but by the blank value (which was presumably due to traces of oxidising agents in the enzyme solution) and could be further reduced by employing purer enzyme preparations. The increased costs of such pure reagents can be compensated by the minimal enzyme consumption of the method. The sampling rate was 80 injections h^{-1}.

The analytical features of the proposed hybrid SIA/FIA method are summarised in Table 4 and compared to other flow methods utilising soluble GOD and a two methods employing immobilised GOD and CL detection. Table 4 reveals that the proposed method offers the widest linear range, the highest sample throughput and the lowest consumption of enzyme among the methods employing soluble GOD while the consumption of sample is comparable to the other methods.

The proposed method was applied to the analysis of commercial energy drinks and honey. Fig. 4 shows characteristic peaks of standards and samples used for the determination of glucose in energy drinks. The results of the analysis by the proposed method and by HPLC with refractive index (RI) detector are presented in Table 5. The samples were extensively

Table 5
Results for the determination of glucose by the proposed method and by HPLC with refractive index detector

Sample	Glucose (FIA/SIA)[a]	Glucose (HPLC)	e_r[b]
Energy drink 1 (g L^{-1})	24.3	25.4	−4.3
Energy drink 2 (g L^{-1})	31.0	31.8	−2.5
Honey (g/100 g)	31.6	31.0	1.9

[a] Average of three determinations.
[b] Relative error.

diluted before the analysis, thus minimising interferences from co-existing metal cations and reducing agents. Indeed, the accuracy of the method was in the range of $100 \pm 4.3\%$ which is considered satisfactory for rapid screening of the glucose content in such samples.

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