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Automated Enzymatic Assays in a Renewable Fashion Using the Multisyringe Flow Injection Scheme with Soluble Enzymes

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In this paper, a novel flowing stream scheme based upon the multisyringe flow injection (MSFI) technique is presented as a powerful tool to perform automated enzymatic assays. The exploitation of enzymes in homogeneous phase circumvents typical drawbacks associated with the commonly used packed-bead or open tubular permanent columns, namely, malfunctions of the reactor, carryover effects, flow resistance, loss of binding sites, large reagent consumption, and use of harmful organic solvents during immobilization procedures.The proposed MSFI system is able to handle minute volumes of soluble enzymes and accommodate reactions with divergent kinetic and pH demands, as demonstrated via the indirect chemiluminescence determination of trace levels of glucose. The procedure is based on the on-line glucose oxidase-catalyzed oxidation of β-glucose in homogeneous phase to β-glucono-δ-lactone and hydrogen peroxide. Subsequently, the generated oxidant merges downstream with an alkaline slug of 3-aminopthalhydrazide and a metal-catalyst zone (viz., Co(II)) at a total flow rate as high as 72 mL/min aiming to warrant maximum light collection from the fast CL reaction. Under optimum conditions for both sequentially occurring reactions, a glucose concentration as low as 90 μg/L may be easily detected at a 1000-fold photomultiplier gain. A second-order polynomial regression equation of light emission versus substrate concentration is found over the range 90 μg/L−2.7 mg/L glucose, although a maximum concentration of 180 mg/L may be determined by suitable gain selection without requiring manifold reconfiguration. An injection throughput of 20 h^{-1}, a repeatability better than 2.5% at the 1 mg/L level, and a 3σ detection limit of 72 μg/L are the analytical features of the designed analyzer. The proposed approach was applied to the analysis of ultralow glucose content soft drinks as well as fruit juices suitable for diabetic consumers. The accuracy was assessed using the spectrophotometric batch glucose−Trinder method as an external reference methodology for the determination of the target species in parenteral solutions.

Organic analytes are commonly determined in analytical methodologies by chemiluminogenic reactions in the liquid phase. In this context, the recent reviews published by Calokerinos et al.[1,2] and García-Campaña and Baeyens[3] are especially relevant. Although direct determinations have been used in biomedical chemistry for organic compounds of pharmaceutical interest, the most widely extended chemiluminescence (CL) applications are related to indirect determinations. They are based either on the quenching of a certain CL reaction[4] or on the enzymatic conversion of the target compound into an ingredient of the CL reaction.[5] In the latter case, when the indirect CL determination is carried out by flowing stream systems, mainly by flow injection (FI) methods, the most widely employed strategy involves the implementation of either packed-bead reactors with enzymes covalently immobilized onto activated inert supports (e.g., controlled pore glass beads silanized with aminopropyl moieties)[6] or open tubular chemically modified Nylon reactors[6,7] into the flow network. These schemes are commonly used in flowing stream systems, since they warrant both the enzyme stability with time and the low consumption of the often costly catalysts, even with the continuous forward flow of the FI technique.[8] To this aim, a plethora of oxidase-based flow-through electrochemical or optical sensors were devised exploring the covalent immobilization on eggshell membranes,[9] the entrapment into electropolymerized pyrrole films[10] or cellulose acetate membranes,[11] or the encapsulation within sol−gel matrixes[12,13] attached to the surface of the working

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electrode or coupled to oxygen-sensitive optrode membranes. Although retention of enzyme activity at a large extent is asserted for numerous membrane-based procedures, the most severe drawback reported is the lengthy response and regeneration times exhibited as a result of the mass transfer steps of the substrate (or detectable product) into (or out of) the bulk of the modified thin layer.[12]

As a consequence of the expenses of the involved reaction ingredients, few flow applications use the enzyme in the liquid phase. Additional strategies, such as the merging zones approach exploiting either the intermittent pumping or the multiple injection valve scheme, need to be adopted.[14] Nonetheless, to compensate for small flow-rate drifts in the two merging channels owing to the deterioration of the flexible tubing of the liquid driver, the volume of enzyme should be larger than that of the sample, thus making the saving quite marginal. Despite these shortcomings, many analytical procedures demand the application of soluble enzymes, owing to the complexity of the sample matrix, which might in turn reduce drastically the lifetime of immobilized enzyme-containing columns/membranes, to the lack of proper immobilization capabilities of certain enzyme preparations or to the blockage of the active sites during the retention procedure, making them inaccessible for the substrate.[8,14] Recently, the multicommuted flow injection concept has been presented as a powerful tool to handle minute volumes of enzymes in homogeneous phase, as demonstrated by the determination of glucose in soft drinks. However, the high detection limits obtained together with the periodical recalibration of the flow network due to the progressive aging of Tygon tubing hindered its applicability to low-sugar-content samples and restricted its monitoring capabilities.[15] To the best of our knowledge, no automated flow system able to determine trace concentrations of glucose in complex samples using soluble enzymes has been reported so far.

To handle enzymes in homogeneous phase, the use of software-controlled piston pumps as enzyme drivers is especially suited as a guarantee of minimum consumption. The automatic burets, typically associated with sequential injection (SI) manifolds, are able to aspirate/dispense minute, well-controlled microvolumes of solutions at the required time for the analysis, yet the interfacing of SI methods with CL detection is mainly limited to reactions with moderately slow kinetics, owing to the requirement of stacking reagent and sample slugs in a holding coil before delivering to the flow-through cell in a reversed-flow mode.[16] To extend its applicability to enzyme-based approaches relying on the fast oxidation of organic species by the oxidant on-line generated, modification of a classical SI system by merging the reaction coil with an exterior reagent line has been reported.[7,17]

The recently developed multisyringe flow injection (MSFI) technique[18,19] assembles some features that makes it better adapted than SI to implement enzymatic-based CL methodologies in a homogeneous (renewable) fashion. It involves a maximum of four glass syringes (as used in SI systems) connected in block to the same step-by-step motor and coupled to three-way solenoid valves. This novel configuration, similar to FI methods, enables the forward movement of solutions but additionally ensures the precise injection of minute volumes of costly reagents, such as enzymes, by proper switching of the commutators. Furthermore, it allows the automatic selection of the flow rate for each operation of the analytical protocol as well as the application of stopped-flow strategies.

In this paper, a novel multisyringe flow injection system interfaced with CL detection is proposed for the ultratrace determination of glucose, as a model of sugars, using soluble enzymes contained in one of the syringes of the module. The methodology is based on the oxidation of the β-anomeric form of the substrate by the glucose oxidase(GOD) flavoprotein to β-glucono-δ-lactone and hydrogen peroxide using molecular oxygen as the electron acceptor. Subsequently, the generated oxidant merges downstream with an alkaline slug of 3-aminophthalhydrazide (luminol) and a metal catalyst zone (viz., Co(II)) aiming to develop the CL reaction into a laboratory-made spirally shaped flow-through cell placed in front of a metal case-type photomultiplier. The outstanding feature of the designed assembly is its versatile applicability for accommodating and executing under individual optimum conditions, two sequentially occurring reactions (namely, enzymatic conversion and Co-catalyzed light emission) with divergent kinetic and pH requirements. The potentials of the developed MSFI methodology were assessed through the determination of ultralow glucose concentrations in dietetic drinks and beverages suitable for diabetic consumers.

EXPERIMENTAL SECTION

Reagents and Solutions. All chemicals were of analytical reagent grade, and Millipore Milli-Q ultrapure water was used throughout.

A stock liquid solution of glucose oxidase (GOD) obtained from mycelia of *Aspergillus niger* fungus (5.7 mg prot/mL; 340 unit/mg protein) was purchased from Sigma, St. Louis, MO (ref. G 9010). The low catalase content in the enzyme preparation prevents hydrogen peroxide decomposition prior to the CL reaction. Standard solutions were prepared by diluting 1.08 mL of the stock to 25 mL with Milli-Q water. Although this enzyme solution is stable for more than 2 weeks at room temperature,[20] storage at 4 °C is recommended when not used for prolonged periods of time.

The chemiluminogenic reagent consisted of 1.4 mg of 3-aminophthalhydrazide dissolved in 1.0 mL of 3 M NaOH solution and made up to 100 mL with water. The reagent was prepared daily to avoid high CL background signals.

A stock standard solution of cobalt(II) was prepared by dissolving 4.93 g of $Co(NO_3)_2 \cdot 6H_2O$ in water and diluting to 1000 mL. A 1 mg/L working solution of the metal catalyst was obtained by serial dilution of the stock.

A 0.1 mol/L standard solution of glucose was prepared weekly from the stable crystalline α-D-glucose form (Sigma). It has been proved that this solution should be prepared 4 h before use to achieve the mutarotation equilibrium between anomeric forms.

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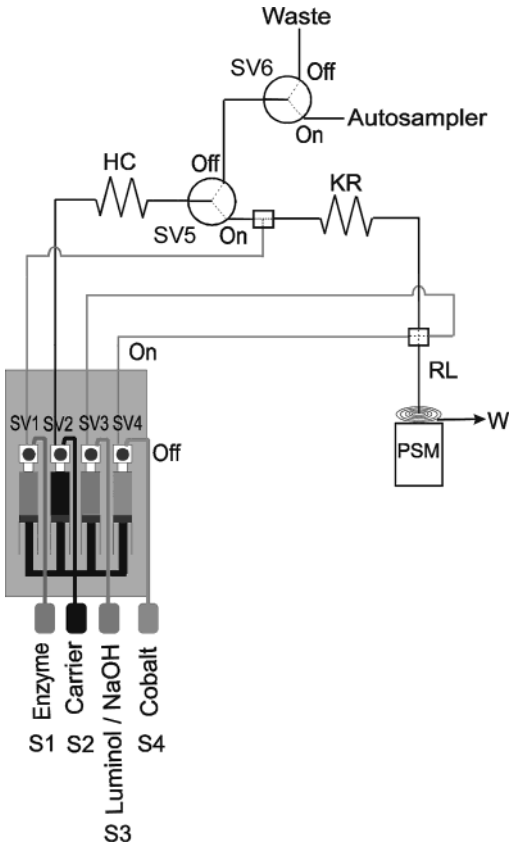

Figure 1. Schematic representation of the multisyringe flow injection setup assembled for the chemiluminescence determination of glucose at ultratrace levels using soluble enzymes. S1−S4, Syringe pumps; SV1−SV6, three-way solenoid valves; HC, holding coil; KR, knotted reactor; RL, reactor line; PSM, photosensor module; and W, waste.

The working glucose solutions were prepared daily by proper dilution of the stock in a 2 mM acetic acid/sodium acetate buffer medium (pH = 5.2), so that the suitable chemical conditions to guarantee maximum enzymatic conversion were attained. Sample pH was properly adjusted after dilution using the same buffer concentration.

Flow Manifold and Instrumentation. The automated MSFI flow system designed for the indirect CL determination of glucose is depicted in Figure 1. It comprises a multisyringe piston pump with programmable speed (MicroBu 2030, Crison, Alella, Barcelona) controlled by a Pentium 166 MHz PC. Each syringe has a three-way commutation valve (N-Research, Caldwell, NJ) at the head. The flow assembly also includes two additional solenoid valves used as a sampling device, one of them being connected to a 40-position autosampler (Crison, Alella, Barcelona). The other valve connected to waste enables the rinsing of the sampling tube without propelling solutions through the manifold, thus reducing risks of carryover between consecutive samples. Standard and buffer solutions placed in containers of the autosampler are injected at preset intervals of time to assess the performance of the system and control the background CL signals. In the MSFI system, similar to FI manifolds, once the flow rate of a certain channel is selected, the flow velocity of the remaining lines is automatically fixed, which in the case of the multisyringe module is directly related to the syringe volume.

All MSFI tubing was made from PTFE of 0.8-mm i.d., except for the holding coil (HC), which was of 1.5-mm i.d. The lengths of the HC and the knotted reactor (KR) for the enzymatic reaction were 65 and 105 cm, respectively. The reaction line for the fast CL luminol oxidation was merely 4 cm long. The three- and four-way connectors assembled in the setup were built from poly(methyl methacrylate) (PMMA). The control of the experimental variables, CL data acquisition, and treatment of the recorded transient signals were performed using the software package Autoanalysis written in Delphi (version 5.0) and Visual C++ (version 6.0) (Sciware, sciware-sl@teleline.com).

The chemiluminescence detector involved a Hamamatsu HS5784 photosensor module (PSM) incorporating a high-voltage power supply circuit, a low-noise amplifier circuit, and a metal package photomultiplier tube.[21] A purpose-made PMMA spirally shaped flow-through cell with an inner volume of 40 mL was mounted over the PSM window. A particular feature of the devised CL detector was the adjustment of the methodology sensitivity through the selection of the suitable voltage of the implemented feedback circuit (over the range from 0.3 to 0.8 V), which enabled the detector gain to be straightforwardly varied from 1 to 1000.

Analytical Procedure of the Automated MSFI Glucose Analyzer. The experimental MSFI procedure (detailed in Table 1) for the determination of ultratrace concentrations of glucose ensuing the GOD-based enzymatic reaction and the CL Co(II)-catalyzed luminol oxidation required the use of the four syringes of the module (S1, S2, S3, and S4) together with their three-way solenoid valves (SV1, SV2, SV3, and SV4). In addition, two independent commutation valves (SV5 and SV6) were assembled in the manifold to allow system cleanup as well as to inject minute and well-defined volumes of sample (or buffer) at the appropriate time, which were delivered downstream using the carrier (viz., 2 mM acetic acid/sodium acetate, pH = 5.2) contained in S2.

S1, S2, S3, and S4 with a capacity of 5, 5, 2.5, and 2.5 mL, respectively, contained the enzyme solution, buffer, 3-aminophthalhydrazide/sodium hydroxide reagent, and metal catalyst, respectively. To aspirate (or dispense) accurate microliter volumes of solutions, it is strongly recommended that one should avoid directional changes of the piston bar during consecutive operations of the analytical protocol, since otherwise, the loss of steps of the liquid-driver motor has been proved to upset the handled volumes (step 2 in Table 1). The first commands of the timing schedule involved the proper rinsing of the manifold lines and sample aspiration tubing with buffer and following sample, respectively, so that carryover risks were minimized (steps 3, 4 and 5). Thereafter, S2 was set to aspirate 200 μL from the sample reservoir once SV5 and SV6 were switched off and on, respectively. A minute volume of the sample stored in the HC (viz., 60 μL) was next directed forward to the PMMA three-way connector following SV5 activation (stage 8). Then, S1 and S2 were programmed to dispense simultaneously and at the same flow rate the soluble enzyme and the sample plug into the KR, where the enzymatic substrate oxidation took place, with the subsequent generation of hydrogen peroxide (step 9). An incubation time of 150 s was forthwith applied with a goal of attaining the maximum reaction yield. Afterward, the PSM was activated, and measurement of light intensity within the wavelength interval ranging from 185 to 850 nm was initiated. The fast reading rate of the PSM (viz., 14 Hz)

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Table 1. Automated Procedure for Glucose Determination Exploiting CL Detection Following Enzymatic Transformation

step	instrumentation	protocol	commentary
1	multisyringe piston pump	dispense 625 μL at 17.6 mL min^{-1} for syringe S1 with heads SV1 off, SV2 off, SV3 off, SV4 off, SV5 off, and SV6 off	initialization
2	multisyringe piston pump	pick up 125 μL at 1.7 mL min^{-1} for syringe S1 with heads SV1 off, SV2 off, SV3 off, SV4 off, SV5 off, and SV6 off	piston bar positioning
3	multisyringe piston pump	pick up 500 μL at 5.0 mL min^{-1} with heads SV1 off, SV2 on, SV3 off, SV4 off, SV5 off, and SV6 on	cleanup sample tubing
4	multisyringe piston pump	dispense 1000 μL at 17.6 mL min^{-1} with heads SV1 off, SV2 on, SV3 off, SV4 off, SV5 off, and SV6 off	
5	multisyringe piston pump	dispense 1200 μL at 17.6 mL min^{-1} with heads SV1 off, SV2 on, SV3 off, SV4 off, SV5 on, and SV6 off	cleanup manifold lines
6		start loop	
7	multisyringe piston pump	pick up 200 μL at 3.5 mL min^{-1} with heads SV1 off, SV2 on, SV3 off, SV4 off, SV5 off, and SV6 on	wample loading into HC
8	multisyringe piston pump	dispense 60 μL at 1.8 mL min^{-1} with heads SV1 off, SV2 on, SV3 off, SV4 off, SV5 on, and SV6 off	driving sample volume to the T-connector
9	multisyringe piston pump	dispense 200 μL at 3.5 mL min^{-1} for syringe S1 with heads SV1 on, SV2 on, SV3 off, SV4 off, SV5 on, and SV6 off	merging sample plug with enzyme solution
10	stopped-flow	wait 150 s	enzymatic conversion
11	CL detector	light collection with a 500-fold (or 1000-fold) photomultiplier gain	14 readings per second
12	multisyringe piston pump	dispense 1000 μL (syringe S2) at a total flow rate of 72 mL min^{-1} with heads SV1 off, SV2 on, SV3 on, SV4 on, SV5 on, and SV6 off	merging generated oxidant with CL reagents
13	CL detector	stop chemiluminescence measurement	
14	multisyringe piston pump	pick up 1060 μL at 17.6 mL min^{-1} for syringe S1 with heads SV1 off, SV2 off, SV3 off, SV4 off, SV5 off, and SV6 off	adjusting piston bar position
15		repeat *n* times from loop	n = number of injections

warranted proper collection of emitted light during CL reaction. Once the stopped-flow scheme was concluded, the commutation valves of S2, S3, and S4 were turned on, and the multisyringe pump was set to the dispense mode. Thus, the generated oxidant species in the KR merged downstream with the chemiluminogenic reagent and metal catalyst delivered by S3 and S4, respectively, the interdispersed zone being driven by the buffer solution to the flow-through spirally shaped cell for CL measurements (stage 12). A total flow rate as high as 72 mL/min is available in the devised configuration, so that the kinetic requirements of the fast Co(II)-mediated luminol oxidation were readily fulfilled without deterioration of the ruggedness of the flow assembly. Finally, the cleanup protocols were again started, rendering the system ready for the next analytical cycle.

RESULTS AND DISCUSSION

Features of the Automated Flowing Stream CL System Handling Soluble Enzymes. To explore the fast CL reactions based upon luminol oxidation, continuous forward-flow systems have been demonstrated to be well-suited.[8] Nevertheless, the opposed kinetic requirements of both reactions involved in the present application (viz., catalytic conversion and chemiluminescence reaction) made the use of conventional flow injection analyzers troublesome, since the narrow flow-rate range available for the different setup channels impeded the simultaneous optimization of both reactions.

One advantage of the proposed MSFI approach is the capability to accommodate both discontinuous and continuous operation modes in a single protocol due to the use of syringe pumps as liquid drivers, thus allowing whatever sequence of desired procedures to be performed automatically. In addition, it offers the likelihood of selection among 150 different flow rates, which makes the flow technique especially adaptable to the demands of the assays.

The implementation of small-size syringes with a capacity of 1.0 or 2.5 mL that are able to handle reagent volumes at the microliter level with high precision and the forward movement of the injected slugs, which is a basic difference with respect to automated SI methods, have opened new perspectives in the field of enzymatic assays coupled to CL detection, as will be demonstrated in this paper. The exploitation of soluble enzymes in flow systems offers unrivaled analytical features in comparison with the conventional enzyme-containing permanent reactors. The immediate consequence is that the immobilization procedures, which are time-consuming[22] (typically last more than 8 h) and may involve the use of harmful organic solvents,[6,7,17] are completely circumvented. Moreover, as a result of the renewable fashion used, deactivation or contamination of the packed-bead or open tubular reactors, which is described as one of the most serious drawbacks for the monitoring of substrates (mainly sugars) in complex matrixes,[14] is overcome. Furthermore, risks of carryover effects between consecutive sample runs are completely eliminated. The buildup of backpressure in unidirectional flow injection assemblies furnished with packed enzyme columns is often the cause of the impaired performance of the analyzer, which detracts from its long-term applicability.[6,17] It should also be stressed that the reproducibility of the analytical results depends drastically on the column packing and active-surface preparation. It should be noted as an additional advantage of the performance of enzymatic reactions in homogeneous phase that typical immobilization yields are ~50−60%, thus causing the loss of an important fraction of active species during sorbent preparation. With regard to enzymes entrapped in optrode membranes or layers attached to electrochemical or optical sensors, high sampling throughput is ensured with the proposed scheme, as mass transfer steps involved in the former systems may result in regeneration/response times higher

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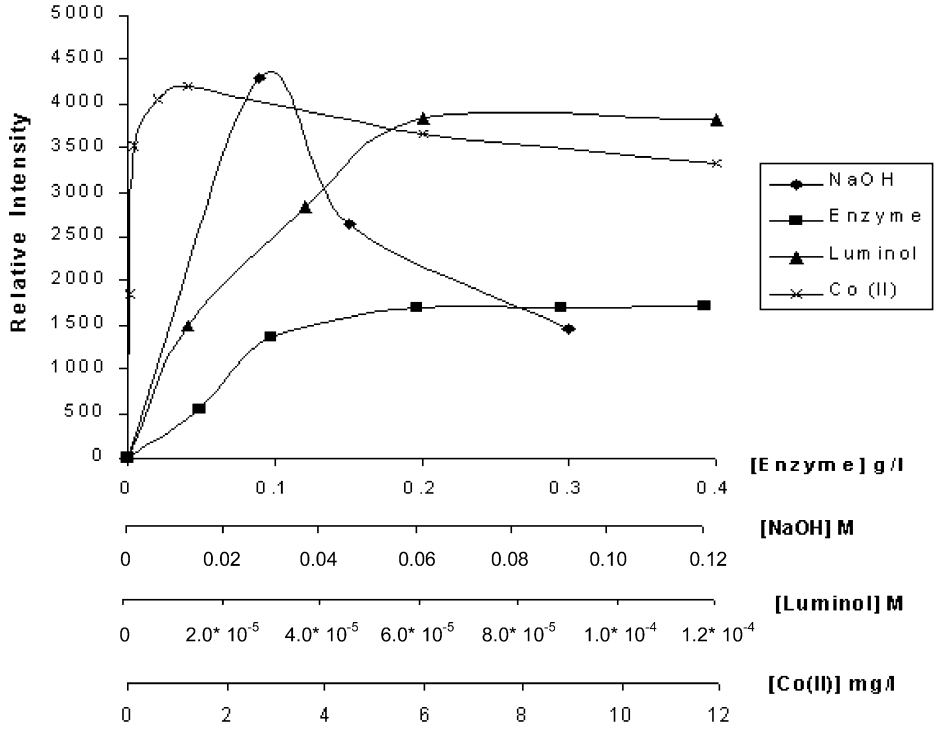

Figure 2. Optimization of the chemical variables using the automated CL-MSFI approach. (▲) Luminol concentration. Experimental conditions: 3.0×10^{-2} M NaOH, 2 mg/L Co(II), 300 μL GOD, 20 UE/injection, 5.0×10^{-5} M glucose, 90-s stopped-flow, and 800-fold PSM gain. (×) Metal catalyst concentration. Working conditions: 8.0×10^{-5} M luminol, 3.0×10^{-2} M NaOH, 300 μL GOD, 20 UE/injection, 5.0×10^{-5} M glucose, 90-s stopped-flow, and 800-fold PSM gain. (◆) Sodium hydroxide concentration. Experimental conditions: 8.0×10^{-5} M luminol, 1 mg/L Co(II), 300 μL GOD; 20 UE/injection; 5.0×10^{-5} M glucose, 90-s stopped flow, and 800-fold PSM gain. (■) GOD concentration. Working conditions: 3.0×10^{-2} M NaOH, 8.0×10^{-5} M luminol, 1 mg/L Co(II), 300 μL GOD, 5.0×10^{-5} M glucose, 90-s stopped flow, and 500-fold PSM gain.

than 5 min.[12] It has been reported in several cases that probe-type flow-through sensors should be frequently recalibrated, mainly in fermentation analyzers and in monitoring schemes, as a consequence of the leaching or contamination of the entrapped (or immobilized) enzyme.[23]

Finally, it should be stressed that the MSFI concept with soluble enzymes, as opposed to permanent reactors, may be considered as a universal flow method for enzymatic assays, since many catalysts, which exhibit lack of proper retention capabilities or the blockage of the active sites during immobilization, may be thus exploited. In the present paper, the determination of ultra-trace levels of glucose in complex matrixes was chosen as an example to assess the potentials of the automated system which may be extended to a large number of other stable enzymatic preparations.

Investigation of Chemical Parameters. The best feature of the performance of enzymatic assays in homogeneous phase is the feasibility of operating at the optimum pH for the catalyzed reaction; thus, the maximum conversion yield for the particular substrate is warranted. For GOD, the maximum activity is attained within the pH range 5.0−5.5, which is not applicable in common immobilization procedures involving enzyme binding with glutaraldehyde owing to the hydrolysis of the generated Schiff base. Hence, proper adjustment of sample pH to neutral values (namely, pH 6.3−7.0) is demanded in these applications.[24]

In the designed MSFI system, the pH of both samples and standards can be adjusted at the desired value, thus overcoming the pH restrictions. According to the above considerations, a 2 mM acetic acid/sodium acetate buffer solution (pH 5.2), also implemented as a carrier stream, was used throughout. The buffer concentration should be carefully selected considering the different requirements of the both reactions involved in the glucose analyzer. Lower concentrations proved ineffective for buffering the reaction medium, leading to multiplicative matrix interferences detected by the method of standard additions. In addition, higher concentrations are also not recommended, since the alkaline medium for the catalyzed luminol oxidation imposes the increase of the sodium hydroxide concentration in the CL reagent that results in incompatibility with the cobalt concentrations (namely, 1 mg/L, see Figure 2) required to provide the best analytical sensitivity. Thus, a 3-fold CL intensity diminution was observed when the sodium hydroxide concentration was increased from 3×10^{-2} to 0.1 M, as a consequence of the precipitation of the metal catalyst as hydroxyoxide species within the spirally shaped flow-through cell. Therefore, the lowest concentration of NaOH able to ensure maximum light emission as well as the solubility of the organic reagent (viz., 3×10^{-2} M) was adopted for subsequent experiments. The use of the common catalyst in flow systems with immobilized GOD, that is, hexacyanoferrate(III),[7,22] was discarded due to the extremely high background signals recorded, which impeded the detection limits to be lowered.

Luminol concentration should be also accurately selected within the concentration range from 5×10^{-5} to 1.2×10^{-4} M,

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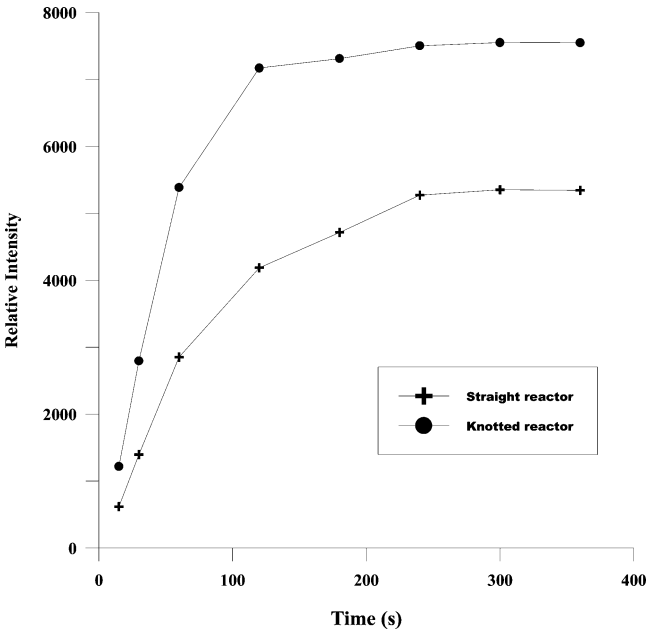

Figure 3. Dependence of the CL-MSFI enzymatic determination of glucose on the reactor configuration and incubation time. Experimental conditions: 8.0×10^{-5} M luminol, 1 mg/L Co(II), 3.0×10^{-2} M NaOH, 200 μL GOD, 14 UE/injection, 5.0×10^{-5} M glucose, and 800-fold PSM gain.

since concentrations above this interval led to a sharp CL peak drop attributed to the contribution of self-absorption phenomena[21] and also caused high blank values.

Because one of the basic requirements of the designed automated system is to guarantee the minimum consumption of the enzyme preparation without deterioration of the analytical performance, according to Figure 2, an optimum concentration of 0.2 mg/mL GOD was selected for further investigations.

Investigation of Physical Parameters. The distinguishing characteristic of the glucose analyzer was the integration of both the enzymatic and CL reactions with opposed kinetic demands. Thus, while a stopped-flow method was adopted for the substrate transformation aiming to obtain the maximum concentration of the oxidizing reagent, the subsequent Co(II)-catalyzed luminol oxidation was performed at extremely high flow rates (viz., 72 mL/min), which are not accessible with conventional flow or sequential injection procedures. In fact, the dependence of the flow rate on light collection by the photomultiplier tube was investigated from 10 to 72 mL/min. A 10-fold increase of the recorded signal was attained at the maximum flow rate available, which reveals the drastic influence of this hydrodynamic parameter on the fast CL reaction selected as detection scheme.

Various configurations of mixing reactors (namely, straight and knotted arrangements) were also assessed for the purpose of ensuring the best performance of the devised system in terms of sensitivity and adaptation to monitoring approaches. As shown in Figure 3, lower incubation times are needed with the KR to obtain the steady-state conditions as a consequence of the higher rate of the enzymatic reaction. In addition, the KR configuration strongly minimizes the axial dispersion of the generated hydrogen peroxide plug, as demonstrated by the 38% CL signal enhancement with regard to the straight coil. These results are a consequence of the existence of toroidal secondary flows in the radial plane, which enhance radial mixing and decrease the axial dispersion. Hence, the implementation of a three-dimensionally disoriented reactor with knots of ~5 mm diameter and selection of an incubation time of 150 s (which provides a sample throughput of 20 h^{-1}) was adopted for further investigations. Higher stopped-flow times are not recommended owing to the possible decomposition of the generated oxidizing agent by interfering matrix constituents prior to reaction with luminol, as described in the following section.

A critical parameter to take into consideration in the present assembly is the injection modality for the soluble enzyme. Different volumes ranging from 100 to 300 μL were delivered into the reaction coil exploring simultaneous injection, binary sampling and tandem flow (typically from multicommutation methods). The injection of GOD volumes below 200 μL resulted inappropriate in the last two approaches as a consequence of the limitation of the axial dispersion between stacked slugs in the KR, which results in a sharp drop (more than 30%) of the analytical signal. Therefore, the simultaneous injection of sample and enzyme (both 200 μL), alike flow injection approaches, was preferred in order to exploit the radial dispersion promoted by the mixing reactor. Under these optimum conditions, the consumption of flavoprotein in the renewable fashion was fixed to 14 enzyme units per injection, which represents a considerable oxidase saving with regard to immobilized reactors when applied to real sample analysis.[6]

Analytical Figures of Merit. Under the optimized conditions, a second-order polynomial equation of light emission versus glucose concentration, commonly described in chemiluminescence methodologies,[25,26] was established over the range 90 μg/L−2.7 mg/L glucose at a 1000-fold photomultiplier tube gain (I_{CL} = 454.4[glucose, mg/L]^2 + 1780.7[glucose, mg/L] − 89.7; n = 6, r = 0.9995). A linear response interval was found within the concentration range of 0.9−2.7 mg/L glucose (I_{CL} = 3530.6[glucose, mg/L] − 1644; n = 4, r = 0.9999), which is especially useful for practical assays. Typical transient redouts recorded during a calibration cycle are depicted in Figure 4. It should be also emphasized that the dynamic working range may be enlarged 2 orders of magnitude, namely, up to a maximum concentration of 180 mg/L glucose, by suitable gain selection without requiring manifold reconfiguration. An injection throughput of 20 h^{-1}, a repeatability better than 2.5% (n = 8) at the 1 mg/L level, and a detection limit of 72 μg/L at the 3σ level were the analytical features of the developed analyzer for the determination of ultratraces of glucose. Thus, an extremely low detection limit, which is 2−3 orders of magnitude better than those recently reported in amperometric or optical flow-through glucose biosensors[27−30] and more than 12-fold improved with respect to those of reactors with immobilized enzymes in CL flow systems,[6,17] was achieved without requiring surfactants to enhance the quantum yield of the luminescence process. The sensitivity

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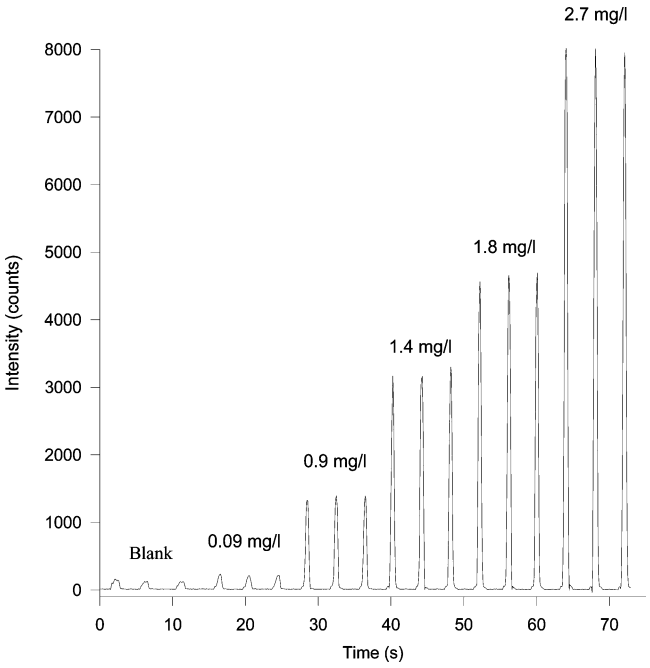

Figure 4. Response of the CL-MSFI system to ultratrace levels of glucose. Concentrations of the target species are depicted above the transient signals.

improvement is due to the accommodation of fast CL reactions within the MSFI manifold, and hence, mineral catalysts such as Co(II), which provides detection limits lower to those of the hexacyanoferrate(III)−luminol system, can be exploited. The glucose conversion yield in the KR, calculated as the peak area ratio between the injection of a certain amount of substrate and the injection of the maximum amount of hydrogen peroxide which might be generated (in the latter case, replacing the enzyme with the buffer solution) was 77%. This value is slightly higher than the β-anomer ratio at the mutarotation equilibrium as a result of the application of the stopped-flow mode for 150 s.

Interference Studies. In this section, potential interfering agents for both reactions accommodated in the automated glucose analyzer are taken into account. It is well-known that the cobalt(II)-catalyzed CL oxidation of luminol is severely affected by the presence of a large number of heavy metals, which behave as competitive species.[31,32] Yet, it has been very recently proved[21] that one of the assets of the exploitation of the Co-catalyzed reaction under dynamic regime at extremely high flow rates (viz., 25 mL/min) is the high metal/cobalt ratio tolerated without masking agents (ratios as high as 50 are reported) as a consequence of the low yield of competitive catalyzed reactions. According to the above paper, copper and lead, which are reported to interfere at trace levels in the CL reaction, were examined as potential interfering agents. The maximum concentration of both heavy metals tolerable at the 10% interference level was 0.1 mg/L so that typical levels found in beverages, juices, and pharmaceutical formulations under investigation do not interfere in the present application.

The outstanding feature of whatever enzymatic conversion is the high selectivity for a particular substrate. Yet, in some instances, a lack of specificity is encountered, since stereoisomeric molecules may also contribute to the analytical measurement.[33] Moreover, the binding of interfering species to the functional groups of the catalyst may dramatically hinder the free access of the target compound to the active sites. Thus, the effect of a sort of monosaccharides, including aldohexoses, ketohexoses, and glucose-containing disaccharides in the GOD-catalyzed transformation of a 5×10^{-5} M glucose standard solution, was thoroughly assessed. As examples of diastereoisomeric glucose molecules, galactose and mannose were considered. The similar environment of galactose and the target sugar at the anomeric carbon (C_1) resulted in the positive interference at the 10% level for a 2×10^{-4} M galactose concentration, while mannose, which is discernible from glucose due to the axial hydroxyl moiety at C_2, may be tolerated at a interfering agent/analyte ratio higher than 40. The possible existence of impurities of galactose oxidase in the GOD preparation[24] may also contribute to the observed interference. Different tolerable concentrations were also found for the disaccharides lactose and maltose (namely, 8×10^{-5} and 8×10^{-4} M, respectively), notwithstanding both sugars present one glucose unit with an unprotected hemiacetal group. This result can be explained considering the different conformations of both disaccharides. The α-glycosidic linkage of maltose (as opposed to the β-linking of lactose) yields a remarkable steric repulsion, thus making this species nonrecognizable by the enzymatic site.

A singular result was obtained when the concentration of galactose was made more than 1 order of magnitude higher than glucose. Instead of recording an increase of the CL peak, as occurred for lower concentrations of the interfering agent, a dramatic drop in the signal intensity (namely, 42%) was observed. It was then decided to evaluate the likelihood of decomposition of the generated oxidizing species by a great excess of reducing sugar. The flow setup was slightly modified, in order to compare the transient signals recorded by direct injection of a zone of 5×10^{-5} M hydrogen peroxide (without flavoprotein) in the presence and absence of aldohexoses (namely, glucose and galactose). A 5−10% lower CL signal was detected under static conditions for aldohexose concentrations 10-fold higher than that of the oxidizing reagent. Thus, the incubation time for the enzymatic reaction in the MSFI manifold is recommended to be as short as possible in order to avoid decomposition of the on-line-generated reagent by oxidation of either the nonconverted glucose or the remainder of aldohexoses present in the sample matrix. In addition, β-glucose is expected to react with hydrogen peroxide faster than the α-anomeric form due to the equatorial disposition of the hydroxyl group at C_1.

Interference studies were also performed using fructose as a model of typical ketohexose found in fruit juice samples. Although the furanosic conformer of the fructose is rather different from the piranosic structure of the glucose, a surprisingly low tolerance level (identical to that of galactose) was attained. This observation can be explained according to the Lowry de Bruin−Alberde van Eckerstein transformation,[34] that is, the isomerization of the interfering agent at the pH of the enzymatic reaction (possibly

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accelerated in the presence of the flavoprotein) into mannose and glucose.

Finally, the effect of sucrose in the enzymatic assay of glucose was investigated. A maximum tolerance of 3×10^{-4} M was attained at the 10% level. Because sucrose is a nonreducing sugar, the consumption of the oxidizing agent prior to luminol oxidation cannot support this result. The interference caused by sucrose reveals the presence of invertase impurities in the enzyme preparation, as described by Benthin et al.,[24] which in turn makes the disaccharide constituent units (viz., fructose and glucose) available for the enzymatic transformation.

Applications of the Automated Glucose Analyzer. To demonstrate the applicability of the multisyringe flow system under the optimum experimental conditions detailed in the foregoing sections, soft drinks (viz., Coca-Cola light, Pepsi-Cola light, tonic water light, 7-Up light, and Schweppes lemon light) containing ultratrace levels of glucose and fruit juices (namely, orange and peach) destined to diabetic consumers were analyzed. Glucose analysis in juice samples is known to cause severe complications to optical and amperometric biosensors relying upon redox reactions or optical detection due to the color of the sample itself and the presence of reducing agents, such as ascorbic acid (natural and added as conservant). To overcome or at least minimize these effects, additional barrier copolymeric layers or negatively charged membranes have been commonly used to limit the interfering species from reaching the electrode surface or the active microzone.[35−38]

As opposed to the above electrochemical or optical transducer systems, the MSFI methodology proposed is readily adaptable to complex samples because of both the different detection principle exploited, that is, CL detection, and the low detection limit attained. As a result, high sample dilution factors may be applied, which in turn ensure proper adjustment of sample pH with the 2 mM acetic acid/sodium acetate buffer (pH 5.2). The ability of the MSFI approach for implementing on-line dilution procedures via a mixing chamber and splitting flow techniques has been very recently demonstrated.[39] Dilution factors as high as 1000 (which, in fact, are very difficult to achieve with typical flow procedures) may be obtained with great repeatability, being specially suited in our configuration for the determination of glucose in juice beverages. The method of standard additions was applied to the entire set of soft drinks and juices to evaluate the existence of matrix effects on the obtained results. According to Table 2, maximum deviations of 9.0% were found, which revealed the absence of multiplicative matrix effects occasioned by concomitant species in the analyzed samples.

The accuracy of the enzymatic CL-MSFI methodology was assessed using the batch Trinder spectrophotometric method[40] commercialized as a kit for glucose diagnostics. It involves the oxidative coupling of 4-aminoantipyrine with a phenolic derivative (similarly to the phenolic index determination) to provide a quinoneimine dye monitored at 500 nm. This procedure with a dynamic range within the millimolar level of glucose has been proven a suitable analytical method to determine glucose in serum or plasma samples wherein low levels of other carbohydrates are present. Yet, the application to raw fruit juice samples without dilution, as a consequence of its working interval covering the millimolar range, yielded unsatisfactory results, and a 1.5−2.0-fold higher glucose concentration than expected was obtained. This was attributed to the severe contribution of matrix interferences on this particular assay. Thus, a pharmaceutical formulation (viz., Buprex) containing glucose as the main carbohydrate constituent was selected for validation purposes. The glucose content obtained via the automated flow system with soluble enzymes (namely, 4.3 ± 0.1%) was in good agreement with that of the Trinder method (namely, 4.7 ± 0.1%) and the labeled value (viz., 4.5%).

Table 2. Determination of Ultratraces of Glucose in Beverages Using the Designed MSFI Assembly

sample	dilution factor	added (mg/L)	found (mg/L)[a]	recovery (%)
Pepsi-Cola light	1:20		2.42 ± 0.04	
		1.80	4.30 ± 0.07	101.9
		3.60	6.3 ± 0.1	104.6
		5.40	8.2 ± 0.1	104.8
Coca-Cola light	1:20		3.18 ± 0.05	
		0.90	4.01 ± 0.01	98.3
		1.80	4.83 ± 0.02	97.0
		5.40	7.8 ± 0.2	90.9
7-Up light	1:20		4.00 ± 0.02	
		3.60	8.11 ± 0.07	106.7
		5.40	9.3 ± 0.1	98.9
		7.20	11.8 ± 0.1	105.4
lemon light (Schweppes)	1:20		8.7 ± 0.1	
		3.60	12.5 ± 0.2	101.6
		5.40	14.3 ± 0.2	101.4
		7.20	15.8 ± 0.1	99.4
tonic water (Schweppes)	1:50		1.57 ± 0.02	
		2.70	4.21 ± 0.05	98.5
		3.60	5.1 ± 0.1	98.6
		5.40	6.6 ± 0.2	94.7
peach juice (for diabetic consumers)	1:1000		5.4 ± 0.1	
		0.90	6.3 ± 0.1	100.0
		1.80	7.1 ± 0.1	98.6
		3.60	8.6 ± 0.2	95.5
orange juice (for diabetic consumers)	1:1000		7.5 ± 0.2	
		1.80	9.1 ± 0.2	97.8
		3.60	11.4 ± 0.1	102.7
		7.20	13.6 ± 0.2	92.5

[a] The results are expressed as the mean of four determinations ± standard deviation

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