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Enzymatic Microreactors for the Determination of Ethanol by an Automatic Sequential Injection Analysis System

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Abstract

A sequential injection analysis system with two enzymatic microreactors for the determination of ethanol has been designed. Alcohol oxidase and horseradish peroxidase were separately immobilized on glass aminopropyl beads, and packed in 0.91-mL volume microreactors, working in line with the sequential injection analysis system. A stop flow of 120 s was selected for a linear ethanol range of 0.005–0.04 g/L ± 0.6% relative standard deviation with a throughput of seven analyses per hour. The system was applied to measure ethanol concentrations in samples of distilled and nondistilled alcoholic beverages, and of alcoholic fermentation with good performance and no significant difference compared with other analytical procedures (gas chromatography and high-performance liquid chromatography).

Index Entries: Alcohol oxidase; ethanol; horseradish peroxidase; immobilized enzymes; sequential injection analysis; biosensors.

Introduction

Highly sensitive analytical systems for use in the clinical, forensic, pharmaceutical, food, fuels, bioprocess monitoring, and control industries are being developed to obtain fast and reliable results *(1)*. Several analytical techniques that use enzymatic reactions have been applied to quantify low ethanol concentrations. Of these, the use of flow injection analysis (FIA) promises to be reliable, reproducible, reagent saving, and a readily automated technique *(2)*. FIA has been used for ethanol detection in alcoholic beverages and in gasohol mixtures *(3–5)*. The systems use sequential enzymatic

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microreactors packed with the alcohol oxidase (AOD) and horseradish peroxidase (HRP) enzymes, immobilized on chitosan or glass beads. Sequential injection analysis (SIA), with a time-based aspiration of defined samples and reagent zones, is an alternative to FIA systems that use smaller sample and indicator volumes, reduce the waste volume, and have more versatile sample handling capabilities *(6,7)*. SIA has been applied with free or immobilized enzymes for bioprocess automation and parameters control *(8–11)*. Amperometric detection of hydrogen peroxide or quinohemoprotein alcohol dehydrogenase complexed with a redox polymer and spectrophotometric detection reaction with NAD^+ cofactor dependent enzyme, were reported for ethanol analyses *(9,12,13)*. The aim of this work is to design and develop a sequential injection analysis system with two enzymatic microreactors using AOD and HRP immobilized separately, with a spectrophotometric detection reaction, for the determination of ethanol in distilled and nondistilled beverages and for alcoholic fermentation bioprocess monitoring.

Materials and Methods

Chemicals

All reagents were analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. AOD and HRP (Toyobo of Brazil) were immobilized separately on aminopropyl glass beads treated with 2.5% (v/v) glutaraldehyde as previously described *(4)*. The composition of the indicator solution was: 4-aminophenazone 0.395 g/L and phenol 0.875 g/L prepared in a 0.1 *M* sodium phosphate buffer solution (pH 7.0). The phosphate buffer was also used as carrier. Reactions were carried out at room temperature (20°C).

Enzymatic Reactions

$$\text{Ethanol} + O_2 \rightarrow \text{Acetaldehyde} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} \xrightarrow{\text{AOD}} \text{monoimino-}p\text{-benzoquinone-4-phenazone} + 4H_2O$$

The resulting colored product, monoimino-*p*-benzoquinone-4-phenazone, is detected with a spectrophotometer at 470 nm.

The Sequential Injection Analysis System

The analyzer (Easi Technologies, Cerdanyola del Vallés, Spain) is consisted of four modules, connected by a RS-485/RS-232 interface and powered by a single 12V/2.5A source. The integrated analyzer system includes a five-way eight-roller peristaltic pump (Model 1201/06-5-0), a colorimeter (Model 1203/470/Z10), a module with two three-port rotary valves (Model 1202/3), and a six-port rotary valve (Cheminert Model 4162510, Valco

Instruments, Houston, TX). The sampling lines of the SIA system were made up of PTFE tubing (0.8 mm i.d.) joined by polyvinylchloride (PVC) fittings. A 1 m length of polytetrafluoroethylene (PTFE) tube was used as a holding coil. Samples and reagent solutions were aspirated and delivered to the six-port rotary valve by two automatic microburettes (Crison MicroBU Model 2031, Alella, Spain) with two syringes of 1-mL and 0.5-mL volume each (Hamilton Model 1002 Teflon Luer Lock, Hamilton Bonaduz AG, Bonaduz, Switzerland). A personal computer with a RS-485/RS-232 interface was used to control and for data acquisition using software developed in C^{++}. Figure 1A shows the SIA system schematic. The system has two acrylic microreactors, each with 0.91 mL void volume packed with AOD and HRP immobilized enzymes. The sequence zone structure and the time schedule implemented in the SIA system are shown in Fig. 1B. A 1.2-mL diluted sample was used with 0.14 mL of reagent solution in each run with the proposed time schedule: coil cleaning, 30 s; colorimeter cleaning, 100 s; sequential aspiration of sample and reagent solutions, 89 s; impulsion 1, 9 s; stop-flow at AOD immobilized microreactor, 120 s; impulsion 2, 150 s.

Microorganism, Medium, and Culture Conditions

A batch fermentation with baker's yeast *Saccharomyces cerevisiae* (AB Mauri División, Córdoba, Spain) was monitored in 2-L bioreactor (Braun Biostat ED, Braun Biotech International, Melsungen, Germany), with 1 L of sterilized medium, conducted at 30°C and 500 rpm. The fermentation medium consisted of 100 g/L glucose, 1 g/L KH_2PO_4, 1 g/L $MgSO_4{\cdot}7H_2O$, 2 g/L $(NH_4)_2SO_4$, 1 g/L NaCl, and pH 4.5 sterilized at 121°C for 30 min. The samples were centrifuged at 5000*g* and filtered before being diluted for the SIA analyses.

Biomass Analysis

Biomass was measured by withdrawing 3-mL samples from the bioreactor. After filtration and washing (Whatman GF/F, Maidstone, UK) the samples were dried at 105°C to a constant weight. Alternatively, biomass was measured by optical density at 570 nm, using a dry cell weight linear relation (Absorbance = $3.027.C_e + 0.094$) with correlation coefficient 0.9957. Biomass concentration (C_e) was expressed as dry cell weight/mL. Both determinations were performed by duplicate and the RSD was about 5%.

Off-Line Ethanol Analysis

A Hewlett Packard (Paolo Alto, CA) 5890 gas chromatograph with a HP-INNOWAX Agilent column (30 m × 0.53 mm × 1 μm) was also used to analyze standard diluted ethanol samples for comparison. Operating conditions were 220°C and 280°C for injection and detector temperatures, respectively; oven temperature profile 40°C for 2 min, followed by 20°C /min up to 180°C , maintained for 2 min. Helium was used as a carrier gas with a flow rate of 8 mL/min. Injection volumes were 1 μL with splitless injection.

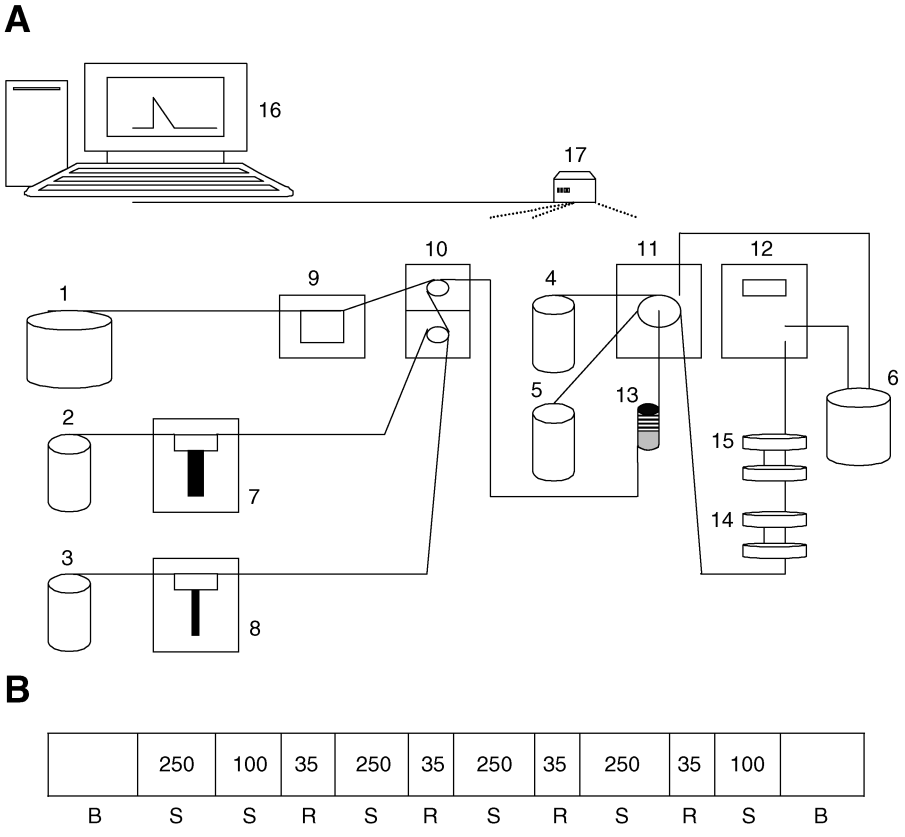

Fig. 1. The SIA **(A)** 1–3—pH 7.0 phosphate buffer solutions, 4—diluted ethanol sample, 5—phenol and 4-aminophenazone solution, 6—waste, 7—1000 µL microburette, 8—500 µL microburette, 9—peristaltic pump, 10—two three-way valves, 11—six channels distribution valve, 12—colorimeter at 470 nm, 13—coil, 14—AOD immobilized microreactor, 15—HRP immobilized microreactor, 16—computer and software control, 17—Interface RS-232/RS-485 and **(B)** Schematic diagram of the sequenced zone structure implemented in the SIA system. B, phosphate buffer; S, sample; and R, reagent solution. Quantities are expressed in microliter.

Ethanol was also determined using high-performance liquid chromatography (HPLC) (Hewlett Packard 1050) fpor samples of beverages and for monitoring batch fermentation using an Aminex HPX-87H column, at 25°C, and a refraction index detector. The mobile phase was 15 m*M* sulphuric acid in MilliQ water at 0.6 mL/min, and injection volume was 20 µL.

Off-Line Glucose Analysis

Glucose present in the fermentation medium was measured by the HPLC and confirmed with a YSI (Yellow Springs, OH) 2700 SELECT biochemistry analyzer.

Results and Discussion

Operational Conditions of the SIA System

The retention efficiencies obtained in the immobilization of AOD and HRP were 95.07 ± 2.33% and 55.60 ± 5.37%, respectively. The retention efficiency was calculated as reported in previous work *(4)*. Preliminary experiments were conducted in the SIA system to select between two different approaches: continuous- and stop-flow strategies, in order to obtain maximum signal response. Three continuous flows of 1.7, 3.3, and 7.4 mL/min were studied in order to analyze the performance of the system in terms of linear range of measurements and sample frequency. Initial results obtained with experiments using a standard solution of 0.05 g/L ethanol rejected the intermediate flow rate. In Table 1 the main parameters obtained for the two flows using a set of ethanol standard solutions are presented.

As can be seen, the selection of the optimum flow is dependent on the detection limit or sample frequency. Trying to amplify the output signal the stop-flow method was used for the same linear range of ethanol solutions. Evaluating different stop-flow times at the first AOD immobilized microreactor with a standard solution sample of 0.05 g/L ethanol, 120 s was selected as an intermediate value between sample frequency and linear range. With this configuration a linear range up to 0.04 g/L ethanol was attained with a coefficient correlation of 0.9922, as shown in Fig. 2. The repeatability of the response signal for different ethanol concentrations is shown in Fig. 3. The sample frequency was seven analyses per hour and the calculated RSD was lower than 0.6% with a detection limit of 2.1×10^{-3} g/L, calculated as three times the standard deviation of the background noise. After 60 analyses the enzymatic activity decreased by 50% at which point the immobilized enzymes had to be replaced with new lots.

In Fig. 4, a comparison of results between the proposed SIA and gas chromatography analysis shows with a good correlation ($r^2 = 0.9923$). The performance of the proposed SIA system was also evaluated for the analysis of ethanol concentration of nine diluted samples of distilled and nondistilled beverages. The results obtained are presented in Table 2. A maximum relative error of 7% was observed in samples of white wine, tequila, and vodka. Nevertheless the relative error for the other beverages was lower than 3% compared with HPLC analysis results.

Finally, the proposed SIA system was applied for monitoring a batch of alcoholic fermentation. Culture broth samples were collected every 30 min, filtered to obtain a biomass-free sample, and diluted manually before analysis in the SIA system. Figure 5 shows the production of ethanol measured using the SIA system and fermentation parameters as biomass-glucose, obtained by other measurement techniques. A good agreement between SIA and HPLC results were obtained and no problems of interference with other culture components were detected with a maximum relative error of 4.9%.

Table 1
Continuous Flow Effects on the SIA System

Flow (mL/min)	Ethanol linear range (g/L)	Correlation factor (r^2)	Ethanol detection limit (g/L)	Sample frequency (1/h)	RSD (%)
1.7	0.005–0.02	0.9917	2.1×10^{-4}	2	0.70
7.4	0.005–0.08	0.9717	1.5×10^{-3}	9	0.74

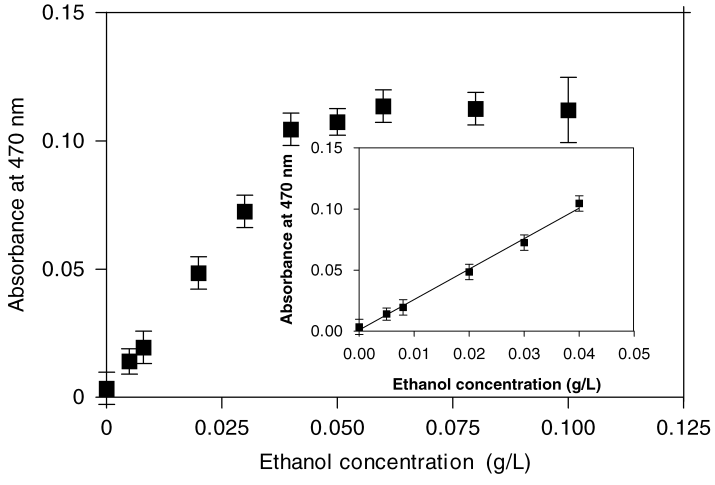

Fig. 2. Calibration curve for the 120 s stop-flow at the AOD immobilized microreactor.

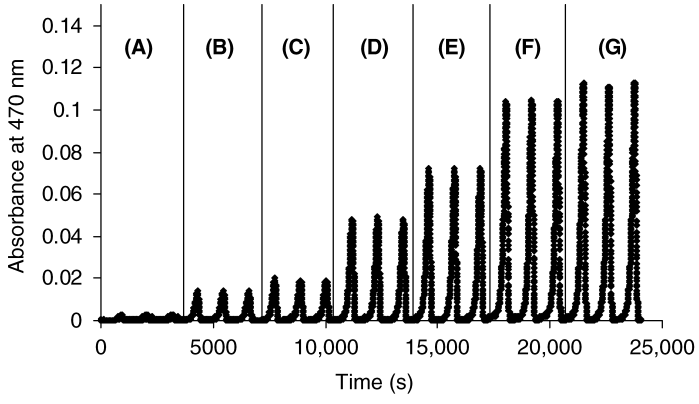

Fig. 3. Response signal repeatability obtained in SIA: **(A)** Phosphate buffer pH 7.0, **(B)** 0.005 g/L ethanol, **(C)** 0.008 g/L ethanol, **(D)** 0.020 g/L ethanol, **(E)** 0.030 g/L ethanol, **(F)** 0.040 g/L ethanol, and **(G)** 0.060 g/L ethanol.

Hence, the reliability of the method proposed was corroborated. A relative error of 4.4% was also reported in the literature for ethanol analysis in fermentation samples, working with an amperometric detection device and immobilized AOD in a SIA system *(9)*. The advantage of the SIA system

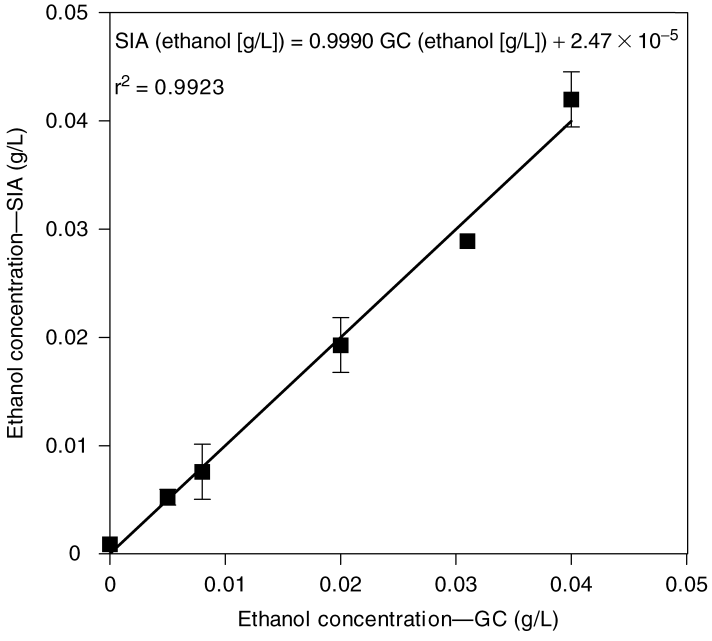

Fig. 4. Comparison of SIA and gas chromatography results.

Table 2
SIA and HPLC Analyses of Distilled and Nondistilled Beverage Samples

Sample	HPLC[a] (g/L ethanol)	SIA[a] (g/L ethanol)	Relative error (%)
Gin	365.2 ± 0.2	378.8 ± 9.0	−3.7
Tequila	359.8 ± 1.1	336.5 ± 6.6	6.5
Vodka	427.8 ± 1.5	455.6 ± 4.5	−6.5
White wine	101.9 ± 0.1	94.5 ± 3.3	7.3
Sugar-cane spirit	320.6 ± 2.5	312.8 ± 5.5	2.4
Whisky	322.8 ± 7.1	324.8 ± 5.7	−0.6
Cognac	375.6 ± 2.0	365.8 ± 9.0	2.6
Wine spirit	254.3 ± 5.1	252.9 ± 10.2	0.6
Peach liqueur	181.2 ± 0.3	181.4 ± 2.3	−0.1

[a]Mean of two analyses.

proposed in this work is that it works with both AOD and HRP enzymes and a colorimetric detection system for ethanol samples with only a simple dilution for the alcoholic beverages, appropriate for the sensible linear measurement range of 0.005–0.04 g/L ethanol. Other authors *(12,13)* reported similar relative errors working with alcohol dehydrogenase, but needed the cofactor NAD^+/NADH, which is not necessary in the present proposed system.

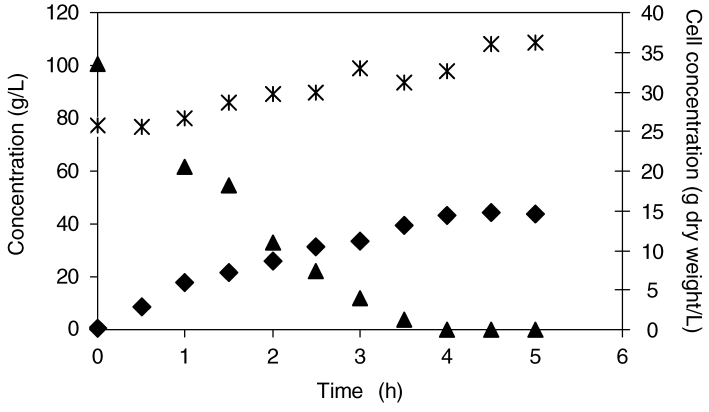

Fig. 5. Ethanol monitoring of alcoholic batch fermentation samples by SIA and HPLC. SIA: ethanol (◆), HPLC and biochemistry analyzer: glucose (▲), and dry cell weight (✳).

Conclusions

A reproducible and reliable SIA analyzer system for ethanol samples was developed. The use of two microreactors with immobilized AOD and HRP permitted an important saving of enzymes reagents in a spectrophotometric detection system. The system was applied to the analysis of distilled and nondistilled beverage samples with similar performances, as reported by other authors working with a more expensive system than the one proposed in this work *(12,13)*. The SIA system performance was also demonstrated to be useful in the monitoring of an alcoholic fermentation showing flexibility and robustness of the analytical equipment. No pretreatment of the samples was needed, simple dilution adequate for the linear range of 0.005–0.04 g/L ethanol was sufficient to analyze the alcoholic beverages and bioprocess medium samples with a detection limit of 2.1×10^{-3} g/L ethanol.

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