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Development and Application of an Integrated System for Monitoring Ethanol Content of Fuels

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Abstract

An automated flow injection analysis (FIA) system for quantifying ethanol was developed using alcohol oxidase, horseradish peroxidase, 4-aminophenazone, and phenol. A colorimetric detection method was developed using two different methods of analysis, with free and immobilized enzymes. The system with free enzymes permitted analysis of standard ethanol solution in a range of 0.05–1.0 g of ethanol/L without external dilution, a sampling frequency of 15 analyses/h, and relative SD of 3.5%. A new system was designed consisting of a microreactor with a 0.91-mL internal volume filled with alcohol oxidase immobilized on glass beads and an addition of free peroxidase, adapted in an FIA line, for continued reuse. This integrated biosensor-FIA system is being used for quality control of biofuels, gasohol, and hydrated ethanol. The FIA system integrated with the microreactor showed a calibration curve in the range of 0.05–1.5 g of ethanol/L, and good results were obtained compared with the ethanol content measured by high-performance liquid chromatography and gas chromatography standard methods.

Index Entries: Biosensors; gasohol; immobilized enzymes; alcohol oxidase; horseradish peroxidase.

Introduction

Since 1975, Brazil has supported a governmental program to design a new car engine technology using (95%) hydrated ethanol as biofuel.

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Table 1
Enzymatic Ethanol Biosensors

Enzyme	Support	Transductor	Detection range
Alcohol dehydrogenase *(6)*	Flat glass	Amperometric	0.4–3.9 *M* ethanol in air
AOD and HRP *(8)*	poli(carbamoil)sulfonate-poliethilenimine hydrogel	Amperometric	0.02–3.75 m*M*
AODand HRP *(4)*	Not immobilized	Spectrophotometric	1.1–21.7 m*M*
AOD and HRP *(9)*	Chitosan	Spectrofluorometric	0.01–0.04 m*M*
Alcohol dehydrogenases and NAD^+/ NADH *(5)*	Not immobilized	Spectrophotometric	0.01–2.5 m*M*
AOD and catalase *(10)*	Controlled pore glass	Thermometric	1.9–3.9 *M*
Alcohol dehydrogenase and diaphorase *(11)*	Semiconductor chip	X-ray photoelectron spectroscopic	0.005–1.0 m*M*

Nowadays a worldwide focus is on renewable fuels, and ethanol has been considered an interesting option to replace petroleum derivate gasoline. It has been reported that the United States, France, Switzerland, Australia, Canada, China, Russia, India, South Africa, and the European Community are considering gasoline ethanol blends as fuel options. Recently, Brazilian government funds have been awarded to laboratories in universities and research centers in different regions of the country, and a strict control of the physicochemical characteristics of the gasohol blend and hydrated fuel alcohol for combustion machines is necessary to prevent adulteration *(1,2)*.

Among the physicochemical methodologies developed to identify chemicals, biosensors have been studied in the last 10 yr as analytical instruments that can be applied in clinical, food, and environmental analyses. Biosensors to be used as analytical instruments should present some important technical characteristics, such as low response time, high selectivity, relatively long lifetime, stability under the analytical conditions, and reproducibility of the measurements *(3)*. Biosensors, which have many advantages, can be miniaturized and/or introduced in on-line proceedings as analytical instruments to detect chemical concentrations with a very rapid response. Recently, increased applications of integrated biosensors and flow injection analysis (FIA) systems in monitoring and controlling biochemical processes have been reported *(4–6)*. Table 1 lists various enzymatic ethanol biosensors published in recent literature. Amperometric, spectrophotometric, thermometric, and X-ray photoelectron spectroscopic methods were used to detect ethanol samples using free and immobilized enzyme systems. Alcohol dehydrogenase and NAD^+/NADH, alcohol dehydrogenase and diaphorase, alcohol oxidase (AOD) and catalase, or horseradish peroxidase (HRP), have usually been applied in ethanol biosensors, as shown in Table 1.

To improve the quality control process of gasohol and hydrated ethanol, an automated FIA system was developed using AOD and HRP enzymes, and addition of 4-aminophenazone and phenol. A colorimetric detection method was used in two different methods of analysis, with free *(4)* and immobilized enzymes. Both systems have shown good results when compared with established methods such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) *(4,7)*.

Materials and Methods

Chemicals

AOD, 4-aminophenazone, phenol, glutaraldehyde, dialysis sacs, and aminopropyl glass beads were from Sigma (St. Louis, MO). Toyobo of Brazil donated the HRP. All other chemicals were of analytical reagent grade.

Immobilization

AOD was immobilized on aminopropyl glass beads that were first treated with 2.5% glutaraldehyde, staying in contact with the enzyme for 24 h at 30°C in a rotatory shaker incubator at 50 rpm. The protein concentration in buffer solution was quantified before (PCBI) and after (PCAI) the immobilization proceeding, using the Lowry method. The retention efficiency was calculated using Eq. 1.

$$\text{Retention Efficiency} = \left[\frac{\text{PCBI (g/L)} - \text{PCAI (g/L)}}{\text{PCBI (g/L)}}\right] \cdot 100 \quad (1)$$

Enzymatic Reactions

Ethanol determination is based on the enzymatic reactions of AOD and HRP:

$$\text{Ethanol} + O_2 \xrightarrow{\text{AOD}} \text{Acetaldehyde} + H_2O_2$$

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

The second reaction gives a colored product that can be detected in a spectrophotometer at 555 nm. The free-enzyme FIA system used an enzyme-reagent solution as described in a previous work *(4)*. The immobilized AOD (0.1 mL of AOD with 4.5 mL of phosphate buffer, pH 7.0) integrating the biosensor-FIA system worked with phosphate buffer (pH 7.0), HRP (0.5 mg/mL), and reagent solution consisting of 0.875 g/L of phenol and 0.305 g/L of 4-aminophenazone was added.

Integrated FIA System

The FIA system consisted of TMI modules (Técniques Mesura Instrumentació), a five-channel peristaltic pump, an eight-channel injection valve, an eight-channel distributed valve, and a colorimeter connected to an interface and to an IBM-PC microcomputer. A specific application software, Qcontroll®, was used for data acquisition, with a continuous historical trend, and a scheduled control program for sequential analysis. In a previous scheme as in Fig. 1, free enzymes were used and the whole system was calibrated for a linear range of 0.05–1.0 g of ethanol/L of standard ethanol solutions, and the product of the reaction was passed through the colorimeter.

As indicated in the schematic diagram in Fig. 1, AOD was mixed with HRP, 4-aminophenazone, and phenol in phosphate buffer (pH 7.0) solution, and the colored reaction occurred with the mixture flowing through a 100-cm-long coil. All tubes used had a 0.8-mm id and the 25-cm injection valve loop permitted injection of 0.185 mL of enzymes-reagents solution per analysis. After each analysis, the solution produced containing the enzymes was directed to waste.

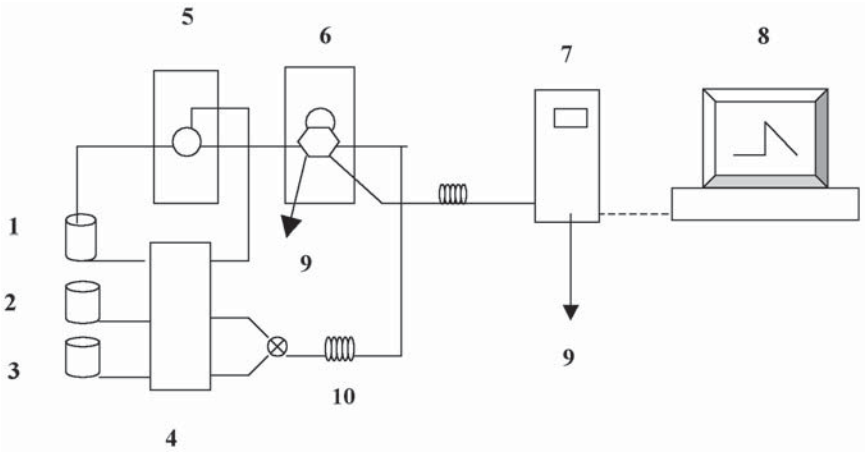

Fig. 1. Free enzymes FIA system: 1, enzymes and reagents solution; 2, sample solution; 3, buffer solution; 4, peristaltic pump; 5, three-channel valve; 6, six-channel injection valve; 7, colorimeter; 8, computer; 9, waste; 10, coil.

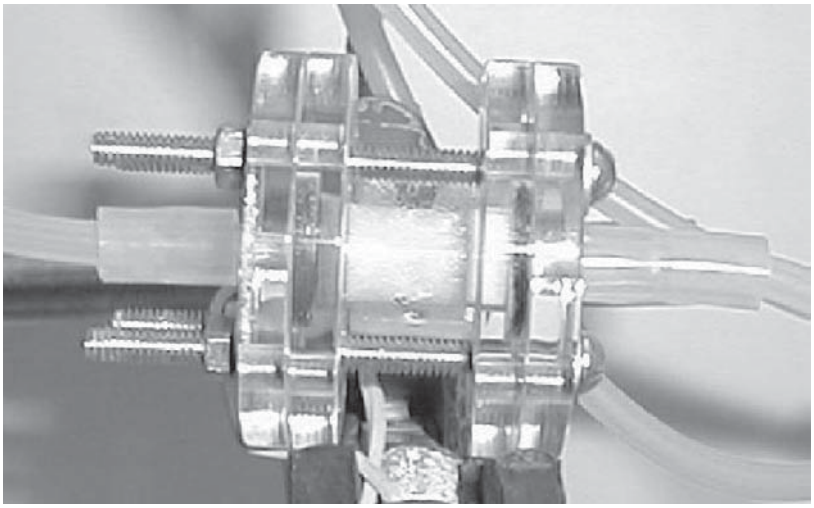

Fig. 2. Microreactor, made of acrylic packed with AOD immobilized on glass beads.

For periodic reuse of the enzymes a new project was developed including a microreactor incorporated into the FIA system. The micro-reactor shown in Fig. 2, made of acrylic acid and with a 0.91-mL void volume, and length-to-diameter ratio of 3:1, was packed with AOD immobilized on glass beads. The beads were retained in the microreactor with a 110-mesh nylon screen and two rubber O-rings with an 11.4-mm external diameter. The lids were attached to the microreactor with four stainless steel screws.

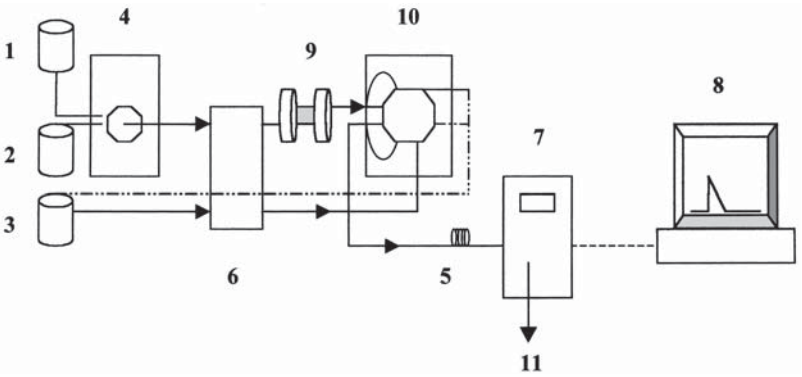

Fig. 3. Proposed integrated FIA system, with microreactor packed with immobilized AOD: 1, buffer solution; 2, sample solution; 3, horseradish peroxidase and reagents solution; 4, eight-channel distribution valve; 5, coil; 6, peristaltic pump; 7, colorimeter; 8, computer; 9, micro reactor; 10, eight-channel injection valve; 11, waste.

Flexible tubes with a 2.4-mm id were connected to each side of the microreactor as sampling lines of the integrated FIA system. In this new biosensor integrated FIA system, HRP was still used in solution with the other two reagents and injected into the FIA line after the sample reacted with the immobilized AOD. Figure 3 shows the new integrated system.

Actuator and Monitoring Equipment

In both systems studied, with free and immobilized enzymes, an automatic analysis system was coupled to the integrated biosensor-FIA system, and analysis was carried out with a specific programmed software for data acquisition and pump and valve control. The computer program controlled the analysis timing sequence, scheduling the signals to the distribution and injection valves to introduce the sample and enzymes-reagents solutions into the system. The difference between the peak heights formed and the baseline were referenced to the absorbance values detected.

Results and Discussion

Free Enzymes System

The free enzymes FIA system permitted analysis in a linear range of 0.05–1.0 g of ethanol/L, a sampling frequency of 15 analyses/h, and a relative SD of 3.5%. The total volumetric flow was 5 mL/min, and the six-channel injection valve permitted the introduction of 0.185 mL (loop volume) of enzymes-reagents solution per analysis *(4)*. Ethanol solutions of different concentrations were used to determine the linear working range and for the calibration curve construction (Fig. 4), presenting a linear relation up to 1 g of ethanol/L with a correlation factor of 0.9899 for six samples.

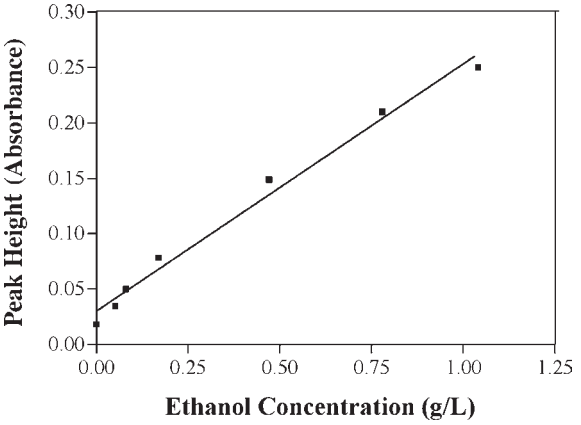

Fig. 4. Free enzymes FIA system calibration curve.

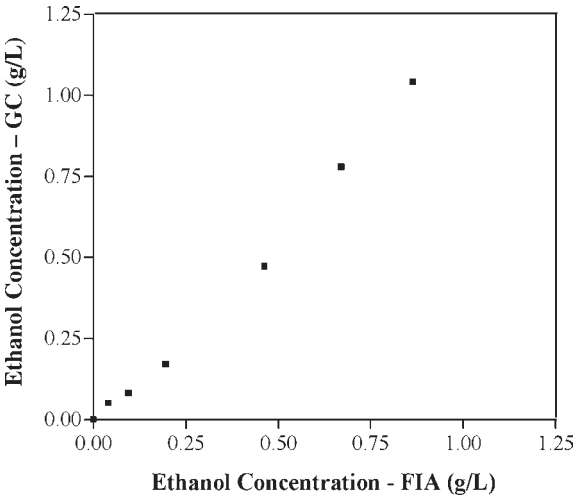

Fig. 5. Ethanol analysis by GC compared to proposed free enzymes FIA system.

The results obtained using GC for the same samples are shown in the comparative plot of Fig. 5. The curve presented a correlation factor of 0.9928, which shows the reliability of the biosensor system proposed.

Immobilization of Enzymes

AOD was successfully immobilized on aminopropyl-functionalized glass beads by covalent bonding through glutaraldehyde with an average retention efficiency of 95.14% (*see* Table 2). The method used for enzyme immobilization showed good reproducibility with a relative SD of 2.85%.

Table 2
Retention Efficiency of AOD
on Aminopropyl Glass Beads

Experiment	Retention efficiency (%)
1	92.6
2	91.9
3	96.3
4	98.8
5	96.1

Table 3
Extracted Ethanol Concentrations
from Gasohol Blends

Gasohol blend (% [v/v])	Extracted ethanol (% [v/v])
4	3.7
6	5.5
8	7.5
10	9.6
15	15.1
20	19.3

Immobilized AOD in Integrated Biosensor-FIA System

The new system was applied for standard ethanol solutions prepared in phosphate buffer solutions (pH 7.0). Extracted ethanol solutions were also used with 10% (w/v) NaCl by NBR 13992:1997 from gasohol blends *(12)* (Brazilian Association Technical Standard). Table 3 shows the concentrations of the extracted ethanol solutions measured by HPLC and respective gasohol blends. This new integrated system biosensor-FIA was used for the range of 0.05–1.5 g of ethanol/L, and good results were obtained compared with the ethanol content measured by the HPLC standard method.

Flow Adjustment of Integrated Biosensor-FIA System

Volumetric flow rate was adjusted working with three different flow rates: 0.47, 0.9, and 3.6 mL/min. Figure 6 shows the profiles obtained for the lower (0.47 mL/min, Fig. 6A) and the higher (3.6 mL/min, Fig. 6B) flow applied to the integrated biosensor-FIA system. Concerning the 0.5 g of ethanol/L sample, output signal with a short response time was achieved when the system worked with 3.6 mL/min.

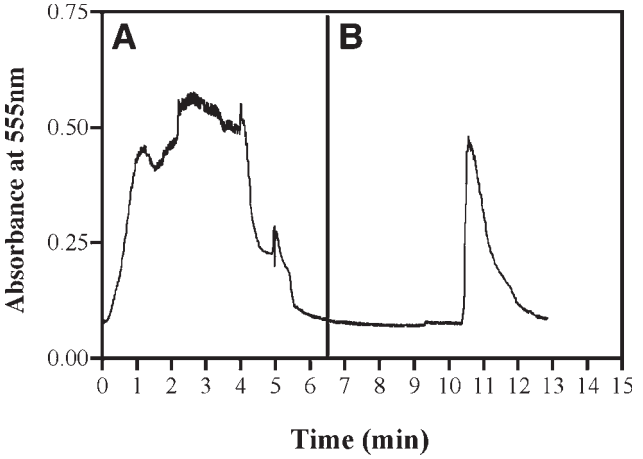

Fig. 6. Profiles registered for 0.5 g of ethanol/L: **(A)** 0.47 mL/min; **(B)** 3.6 mL/min.

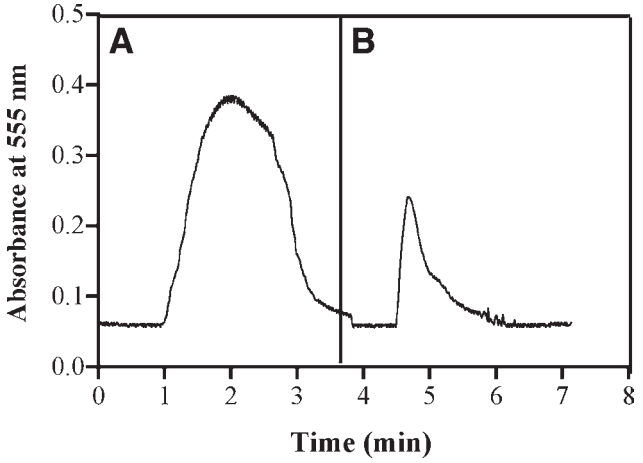

Fig. 7. Profiles registered for 0.1 g of ethanol/L: **(A)** 0.9 mL/min; **(B)** 3.6 mL/min.

Figure 7 shows the response profiles registered when 0.9 and 3.6 mL/min were applied with a 0.1 g of ethanol/L working sample. In Fig. 7A the profile was wider than that obtained when 3.6 mL/min (Fig. 7B) was used, and a maximum height of the peak (0.385) for 0.9 mL/min was observed when compared with 0.242 for 3.6 mL/min.

The measurements of extracted ethanol from gasohol and hydrated fuel alcohol, properly diluted, determined with the integrated biosensor-FIA system showed the registered signals in Fig. 8.

Good reproducibility of the registered signals was obtained with the proposed automatic analysis system. The working features used in the experiments were constant loop volume, scheduled time control program,

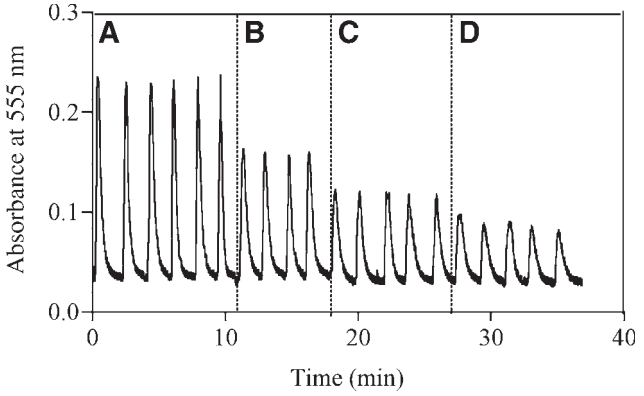

Fig. 8. Biosensor output signals for range of 0.05–1.5 g of extracted ethanol/L samples: **(A)** 1.5 g/L; **(B)** 0.3 g/L; **(C)** 0.1 g/L; **(D)** 0.05 g/L.

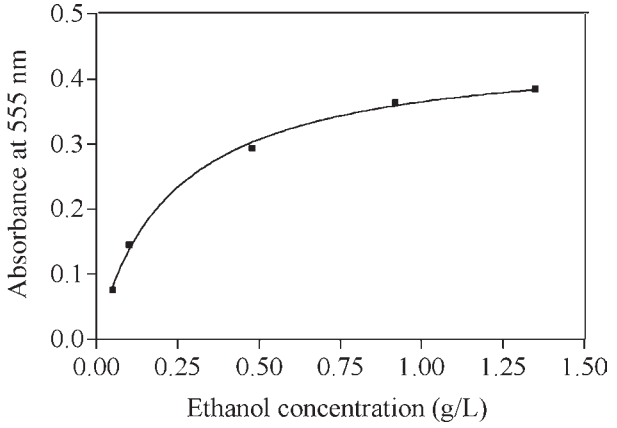

Fig. 9. Calibration curve for phosphate buffer standard ethanol solutions with integrated biosensor-FIA system.

and sample volume. The volume of free HRP mixed with 4-aminophenazone and phenol was 69.4 µL. The schedule time program controlled the timing sequence of the module's operation (cycles of 101 s), always injecting the same volume of the ethanol sample (1.0 mL) and free enzyme with reagent solution. Data were collected in real time, and the registered signal could be watched on the monitor screen as the colorimetric detector showed the absorbance signal during the measurement. The analysis response time spent only 2.0 min, between each operation cycle of the sampling line.

The calibration curves obtained with the output signals are shown in Figs. 9 and 10. Both curves were adjusted by a hyperbolic correlation with correlation coefficients of 0.9972 and 0.9909, respectively, for buffer

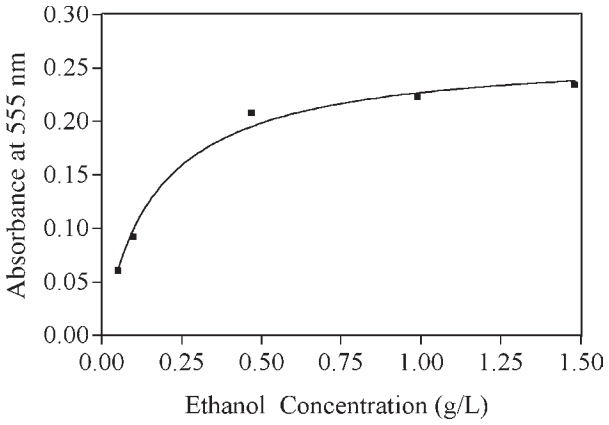

Fig. 10. Calibration curve for extracted ethanol solutions with integrated biosensor-FIA system.

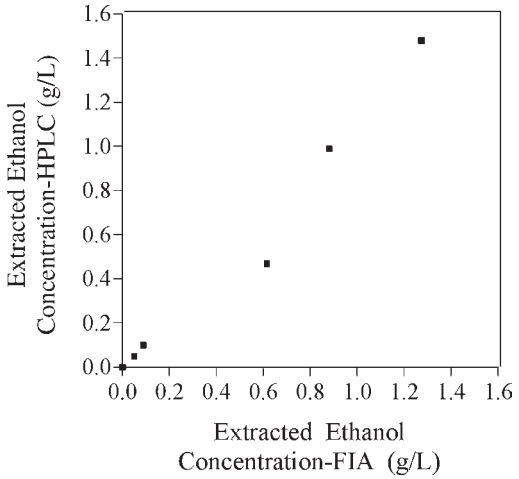

Fig. 11. Results of ethanol concentration solutions analysis by HPLC compared to proposed integrated biosensor-FIA system.

and extracted ethanol solutions. Additionally, comparison of the results obtained with the integrated biosensor-FIA system and the ethanol concentration solutions measured using HPLC is illustrated in Fig. 11.

Conclusion

The free enzyme–FIA system applied to ethanol analysis presented good results, with high reproducibility and reliability in the range of 0.05–1.0 g of ethanol/L with a relative SD of 3.5%. The methodology developed to immobilize AOD on functionalized glass beads presented high retention efficiency of the protein, about 95.14 ± 2.85%. The new, proposed,

integrated immobilized AOD biosensor-FIA system, for the reuse of this enzyme, showed excellent reproducibility and reliability for a range of 0.05–1.5 g of ethanol/L, for ethanol analysis either in buffer standard solutions or in NaCl-extracted ethanol solutions. The proposed integrated biosensor-FIA system presented a simple method, inexpensive and robust for ethanol determination in buffer or extracted ethanol solutions, with good sample frequency analysis and no significant differences compared to other analytical procedures (GC or HPLC). This system is extremely practical, giving the results of the analysis shortly after measurement, with a response time of 2.0 min. This is an advantage for a routine procedure in a quality control program, as proposed in a fuel quality control program. It was possible to perform 70 analyses in 7 d, maintaining the activity of the immobilized AOD.

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