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SHORT COMMUNICATION

Analysis of free glycerol in biodiesel using an electrochemical assay based on a two-enzyme platinum microelectrode system

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Abstract An electroenzymatic method based on two coupled enzymatic activities (glycerokinase and glycerol-3-phosphate oxidase) was developed using a platinum microelectrode for the determination of free glycerol in biodiesel samples. The electroenzymatic method proposed showed a good linear correlation coefficient (R = 0.9894), with a linear response in the concentration range of 8.2×10^{-5} to 5.7×10^{-4}% (w/w) and a limit of detection of 6.0×10^{-5}% (w/w). The results obtained for the free glycerol content of select biodiesel samples were compared with their gas chromatography (GC) analyses. The relative errors for glycerol determination using this enzymatic-amperometric method were in the range of −8.0 to 3.0%. The proposed method was shown to be promising for the analysis of glycerol in biodiesel samples and a simple and inexpensive method in comparison to gas chromatography.

Keywords Microelectrodes · Free glycerol · Hydrogen peroxide · Biodiesel amperometric detection

1 Introduction

Due to the massive development of renewable biodiesel fuel, which is composed of a mixture of fatty acid methyl esters and intended to be an alternative to diesel fuel, it is absolutely necessary to establish standards for the description of the quality of this product [1]. Free glycerol content, which mainly depends on the technical process of transesterification, is an important parameter for defining the quality of biodiesel.

The transesterification of oils and fats results in the production of glycerol as a byproduct in biodiesel. During this process, free glycerol can easily be removed with washing steps [1], but if the purification process is not effective, high free glycerol content could cause problems during storage or in fuel systems due to the separation of glycerol. It could also lead to injector fouling or the formation of higher aldehyde emissions. Accordingly, international regulations typically specify a maximum free glycerol content of 0.020% (w/w) [2].

Immobilized or dissolved enzymes, such as glycerokinase (GK) and glycerol-3-phosphate oxidase (G3PO), have been extensively applied to develop prospective methods for the measurement of glycerol in clinical analysis, food products and biotechnological processes [3–9]. In these methods, glycerol is converted into dihydroxyacetone phosphate by the sequential action of the GK and G3PO enzymes, while consuming oxygen and producing hydrogen peroxide in proportion to the initial content of glycerol in the sample, as described in Fig. 1. Several amperometric sensors have been described in the literature that take advantage of this oxygen consumption [7, 9, 10] and hydrogen peroxide production presented in reactions 1 and 2 [3–5].

Microelectrodes have received increasing attention for both kinetic studies of electrode processes and quantitative analyses. Some advantages of microelectrodes include the low current necessary to achieve a negligible ohmic drop and a fast response due to a small electrode capacitance. Another advantage is that the electrodes reach steady-state conditions in a short time. In principle, these properties allow microelectrodes to be employed directly in resistive media without the addition of supporting electrolytes [11–15].

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$$\text{glycerol} + \text{adenosine triphosphate} \xrightarrow{GK} \text{adenosine diphosphate} + \text{glycerol 3-phosphate} \quad (1)$$

$$\text{glycerol 3-phosphate} + O_2 \xrightarrow{G3PO} \text{dihydroxyacetone phosphate} + H_2O_2 \quad (2)$$

Fig. 1 Enzymatic reaction scheme involving glycerol conversion to dihydroxyacetone phosphate by the enzymes glycerokinase and glycerol-3-phosphate oxidase

In the present work, an amperometric detection method based on a GK + G3PO enzyme-platinum microelectrode system was developed to analyze free glycerol in biodiesel samples.

2 Experimental section

All biodiesel samples used in this work were previously analyzed by GC at Petrobras in order to obtain reference values. Density values were also provided by Petrobras.

First, free glycerol was extracted by adding 400 μL of a biodiesel sample to a centrifuge tube containing a mixture of 800 μL of distilled water, 800 μL of absolute ethanol and 1600 μL of heptane. This mixture was then shaken in a vortex mixer. The phase separation was assisted by placing this tube in a centrifuge for about 2 min. The bottom phase, containing the free glycerol of the biodiesel sample, was analyzed in the described electrochemical cell.

A 50 μL aliquot of the aqueous phase was analyzed in a two-electrode cell placed in a Faraday cage at 25 °C that contained 3 mL of the commercial enzymatic kit. Prior to amperometric detection, this mixture was stirred in the electrochemical cell for about 10 min in order to convert all of its glycerol content into dihydroxyacetone phosphate and H_2O_2 by the enzymes glycerokinase and glycerol-3-phosphate oxidase. The amperometric detection of the hydrogen peroxide produced was performed at +0.65 $V_{Ag/AgCl}$ for 200 s. The reference/counter electrode was Ag/AgCl/KCl (3 mol L^{-1}) and the working electrode was a platinum ultramicroelectrode. In order to prepare this electrode, a platinum wire with a diameter of 100 μm (purchased from Heraeus Vectra do Brasil) was sealed directly into soft glass. The resulting platinum electrode was polished mechanically with 1500 and 2000 emery paper.

Prior to each experiment, the electrode surface was electrochemically activated by cycling the potential 50 times over the range −0.4 to 1.75 V at 500 mV s^{-1} in 0.5 mol L^{-1} sulfuric acid solution and then characterized by cyclic voltammetry at 100 mV s^{-1} over the range −0.25 to 1.45 V in 0.5 mol L^{-1} sulfuric acid solution. All measurements were performed using an Autolab potentiostat, model PGSTAT 100, from Ecochemie with a current amplifier module controlled by GPES 4.8 software.

Calibration curves were generated by adding glycerol standards (87%, purchased from Merck) in place of the sample. The kit for enzymatic conversion of glycerol to dihydroxyacetone phosphate was obtained from Bioclin. This kit included 0.250 mmol L^{-1} ATP, 15 mmol L^{-1} magnesium chloride, glycerokinase > 1,500 U/L and glycerol-3-phosphate oxidase > 4,000 U/L, and PIPES buffer pH 7.0 (100 mmol/L), among other reagents.

3 Results and discussion

The quantification of glycerol was followed by the sequential action of glycerokinase and glycerol-3-phosphate oxidase in the presence of ATP and O_2, which metabolized the analyte and produced hydrogen peroxide proportional to the initial content of glycerol in the sample, and was amperometrically detected at +0.65 $V_{Ag/AgCl}$ due to its oxidation to O_2.

Figure 2 presents the calibration curve obtained by fitting the data to the linear regression model. For this, all of the data for the standard samples were obtained in triplicate and submitted to Grubb's test to verify possible outlier values. All measurements were found to be within the 95% confidence interval. The Cochran value calculated (0.340) was lower than the tabulated value (0.561), which characterized a homoscedastic behavior for the data. Good

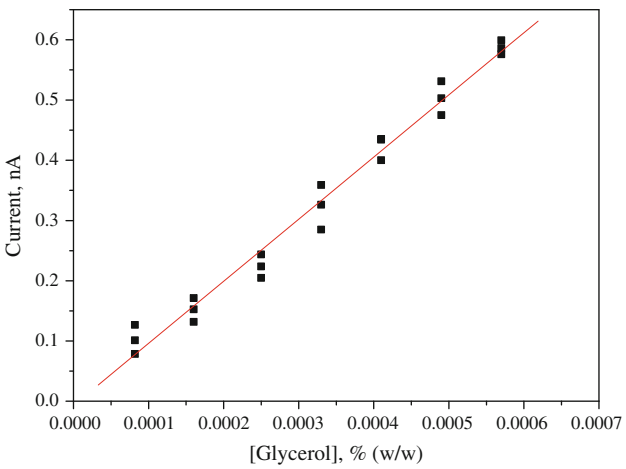

Fig. 2 Calibration curve obtained for glycerol standards in 3 mL of the commercial enzymatic kit ranging from 8.2×10^{-5} to 5.7×10^{-4}% (w/w)

Table 1 Comparison of free glycerol content in biodiesel samples measured by gas chromatography (GC) and enzymatic-amperometric (EA) methods

Samples	Free glycerol content (%, w/w)			
	GC	EA	Recovery (%)	δ (%)
1	0.21	0.212 ± 0.002	101.0	1.0
2	0.20	0.184 ± 0.002	92.0	−8.0
3	0.22	0.221 ± 0.004	100.5	0.5
4	0.20	0.206 ± 0.003	103.0	3.0
5	<0.01	<0.013	–	–
6	<0.01	<0.013	–	–
7	<0.01	<0.013	–	–
8	<0.01	<0.013	–	–

The EA analyses were performed in triplicate and mean values are reported. *GC* gas chromatography, *EA* enzymatic-amperometric method, δ (%) relative errors

linearity was observed, with a 0.9889 correlation coefficient (R), and the response was linear in the concentration range of 8.2×10^{-5} to 5.7×10^{-4}% (w/w). A detection limit of 6.0×10^{-5}% (w/w) was obtained from the experimental criteria, which corresponded to 0.013% (w/w) of free glycerol in the biodiesel sample. This value was lower than the maximum allowable free glycerol content of 0.020% (w/w) specified by international regulations.

The results obtained for the free glycerol content of select biodiesel samples were compared with GC analyses, as shown in Table 1. The relative errors for glycerol determination using the enzymatic-electrochemical method were in range of −8.0 to 3.0%.

The accuracy of the reported method was evaluated using the results of the recovery study with one biodiesel sample having a glycerol content of <0.01% (w/w) by GC. Fortifying this sample with 0.05% (w/w) glycerol, the results obtained intra- and interday for the free glycerol content of this sample was 0.049 ± 0.002% (w/w) and 0.048 ± 0.002% (w/w), respectively. These results were performed in triplicate and mean values were reported corresponding to 98 and 96% recovery, respectively.

4 Conclusions

An electrochemical assay based on a two-enzyme platinum microelectrode system was developed for the analysis of free glycerol in biodiesel samples. The method presented in this work provided good signal levels, good linearity, short response times (approximately 10 min), a low detection limit, good sensitivity, a suitable working range and excellent accuracy. In comparison with gas chromatography results used as reference values, the proposed method presented low relative errors for the analysis of free glycerol in different biodiesel samples. The method was thus shown to be promising for the analysis of glycerol in biodiesel samples using a simpler and less expensive procedure compared with the gas chromatography technique.

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