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Selective application of two rapid, low-cost electrochemical methods to quantify glycerol according to the sample nature

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ABSTRACT

The selection of the method to quantify glycerol largely depends on the amount present in the sample, the matrix nature where glycerol has to be determined, and the potential interferences accompanying the analyte. We compared different electrochemical methods to determine glycerol in terms of convenience, from the point of view of the sample nature, the time spent to accomplish the analysis, the preferably use of green consumables, the limit of detection (LD), and the operational cost. We studied two alternative methodologies based on amperometric measurements to determine glycerol. In aqueous media, we use the versatile gold electrode, being the linear range 2.5×10^{-5} to 2.0×10^{-3} M, and the LD 10 μM ($3 \times SD_b$, standard deviation of the blank). In extremely complex matrixes, where many electroactive species are expected to be present, we propose to use a new approach based on biosensor technology, which takes advantage of an inexpensive, soluble redox mediator, and uses only one enzyme with its cofactor in solution. The substrate was a glassy carbon paste electrode; the system used the enzyme glycerol dehydrogenase, soluble NAD^+ as cofactor, and ferricyanide as charge mediator. The response showed to be linear between 7.0×10^{-5} and 1.8×10^{-3} M, and the LD was 20 μM. The biosensor displayed more than two month stability without the enzyme losing activity when kept dried at 4 °C. The time taken to complete the analysis was 10 min, counting from the moment the sample was taken until the signal was recorded. The operational cost of the whole analysis was less than that derived of using other biosensors or a spectrophotometric assay.

1. Introduction

The determination of glycerol has been gaining importance during the last decades because its use has been growing in several industries such as pharmaceutical, automotive, alimentary, and textile, among others. Certainly, the compound is an important fermentation product of most alcoholic beverages, its dosage being useful to follow up the process [1]. It is also a non desirable component of biodiesel, its concentration being indicative of the fuel condition [2]. Besides, glycerol is an additive of many foodstuff, therefore serving as food quality indicator [3]. In addition, it is used as lubricant, and as a pharmaceutical excipient to prepare medicines in a form suitable for administration [3]. Consequently, glycerol occurring in many variable concentration ranges must be quantified in a number of diverse matrixes, some of which are extremely complex.

Many techniques have been used for glycerol quantitation purposes, such as spectrophotometric/spectrofluorometric ones [4–6], including enzyme-based ones [7–9], which incidentally, have been considered to be expensive [7], electrochemical ones [10–12], and the chromatographic ones, the latter having been officially recommended by the AOAC, as methods 968.09 and 972.10 [13]. However, even though the chromatographic methods permit to effectively determine glycerol in the presence of many interfering compounds, this technique is considered to be ecologically unfavorable because of the use of solvents which may be dangerous for environment, and intrinsically onerous because of the usually high cost of solvents and equipment [11].

Abbreviations: ANOVA, analysis of the variance; AOAC, association of official analytical chemists; ATP, adenosine triphosphate nucleoride; CV, cyclic voltammetry; DMSO, dimethyl sulfoxide; DP, diaphorase; DPV, differential pulse voltammetry; FAD, flavin adenine dinucleotide; FIA, flow injection analysis; GK, glycerol kinase; GlDH, glycerol dehydrogenase; GO, glycerol oxidase; GPO, glycerol phosphate oxidase; HRP, horseradish peroxidase; LD, limit of detection; LDH, lactate deshydrogenase; LSV, linear sweep voltammetry; MWCN, multiwalled carbon nanotube; NAD^+, reduced form of nicotinamide-adenine dinucleotide; N.I., not informed; PBS, phosphate buffer saline solution; PK, piruvate kinase; SD, standard deviation; T, Tween 20; W/W, weigh-to-weigh ratio.

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On the one hand, the enzymatic spectrophotometric proposals to determine glycerol usually rely on the use of relatively expensive either single or multi-enzymatic systems, which are disposed after have being used for a single determination. In these cases, expensive enzymes and/or cofactors such as nicotinamide-adenine dinucleotide (NAD^+) or adenosine 5′-triphosphate (ATP) are often consumed [7–9,12]. As an example, Li's proposal to determine glycerol in complex matrixes is based on the use of three enzymes, namely glycerol kinase (GK), pyruvate kinase (PK), and lactate dehydrogenase (LDH), apart from using ATP and NAD^+ [8]. This method produces good results in terms of sensitivity and specificity, but it consumes 1 U of GK, 1.5 U of PK, 2.2 U of LDH, 2×10^{-6} moles of ATP, and 3.3×10^{-7} moles of NAD^+ per determination. Therefore, the derived cost per analysis is considered to be quite expensive, particularly when a high number of samples must be analyzed.

On the other hand, biosensor technology, particularly the amperometric-based ones, have been proposed as remarkable versatile tools to quantify glycerol [11,14]. Indeed, amperometric biosensors provide enough sensitivity and selectivity to allow for the analyte determination, together with an appropriate rapidness to complete the analysis. Actually, amperometric biosensors relaying on enzymes as the biological component used as recognizing elements give rise to a significant selectivity, because of their ability to react specifically with their substrate. The latter is often the analyte of interest, so that it can be efficiently discriminated from other structurally similar components occurring in the sample [15]. Devices built up with enzymes can often be reused, and therefore the cost per determination can be significantly reduced, as compared with the spectrophotometric methods mentioned above.

In this work, we present two amperometric methods to quantify glycerol. One of the proposals is based on the use of a solid gold electrode, at pH 13, for very simple matrixes. The second one uses a new low-cost biosensor built up with the enzyme glycerol deshydrogenase (GlDH), able to be used in extremely complex matrixes and to detect slight changes of the analyte concentration in the μM order. Our proposal uses the inexpensive, widely used, soluble redox mediator $Fe(CN)_6^{3-}$, and we set the amounts of the expensive enzyme and its cofactor to the minimum, but enough to achieve suitable sensitivity to detect slight concentration changes, thus importantly reducing the total operative cost of the analysis.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical grade, supplied by Sigma–Aldrich, except otherwise stated. The enzyme was glycerol dehydrogenase (EC 1.1.1.6) from *Cellulomonas* sp., lyophilized powder, 70 units mg^{-1}. The glassy carbon powder was spherical 2–12 μm particles 99.99% metals basis. K_2CO_3, $KHCO_3$, NH_3, dimethyl-sulfoxide (DMSO) and nitric acid were from Merck. A 1 M glycerol stock solution was prepared by diluting the necessary amount in distilled water, and it was fractioned and stored frozen until use. A stock 0.47% w/w solution of Middlebrook 7H9 broth (supplied by Difco) was prepared, fractioned, and stored frozen until use.

2.2. Instrumentation

All electrochemical measurements were performed in a three-electrode cell with either gold tips or the assembled biosensor as the working electrode, a 3 M KCl Ag/AgCl reference electrode, and a glassy carbon rod as the counter electrode. Amperometric measurements and cyclic voltammetries (CV) were recorded with an Autolab Electrochemical analyzer, equipped with a PGSTAT 30 differential electrometer amplifier, and the 4.9 General Purpose Electrochemical System (GPES) software (ECO Chemie, The Netherlands).

2.3. Experiments with metallic electrodes

2.3.1. Gold electrodes conditioning

Polycrystalline gold electrodes embedded in a Teflon® rod (gold tips) were cleaned using the following protocol: (i) degreasing, by rubbing the tip on a polishing cloth moistened with DMSO for 5 min; (ii) thoroughly washing with distilled water; (iii) further polishing on a polishing cloth with 0.3 μm particle diameter wet alumina up to get a mirror surface; (iv) final thoroughly wash with distilled water. The electrodes were then sonicated for 5 min in a Cole-Palmer 8890 sonicator, washed with abundant distilled water, and immersed in 9 M HNO_3 at 60 °C for 1 min. Finally, the electrodes were rinsed thoroughly with distilled water.

2.3.2. Electrochemical measurements with gold electrodes

The area of the electrodes was determined by CV in 0.5 M H_2SO_4 using the oxygen adsorbed monolayer method [16]. The reference charge used for polycrystalline gold was 390,210 pC cm^{-2} [17]. The clean electrodes were immediately immersed in N_2-degassed 0.1 M NaOH, and preconditioned by a 0.50 V pulse potential for 50 s. Then, the amperometric experiments were performed at 0.10 V vs. a 3 M KCl, Ag/AgCl reference electrode, at 25 °C. Once the equilibrium was reached (usually at approximately 50 s) the basal current value (i_b) was recorded. Standard glycerol solution was then added so as to reach the desired concentration. The current i_s was then recorded until stabilization (generally at 400 s). The signal, catalytic current (i_c) was calculated as $i_c = i_s - i_b$. Three independent experiments were carried out to evaluate signals at every concentration.

2.4. Biosensors assembly

The biosensors were built up on the basis of Álvarez-González's proposal, with several modifications [18]. The carbon paste electrode was prepared in an agate mortar by mixing 68% w/w glassy carbon powder, 30% w/w mineral oil, 2% w/w GlDH (firstly dispersed in mineral oil) for 15 min so as to obtain a homogeneous mixture. The paste was then packed tightly into a 3-mm diameter Teflon® frame, and rubbed firmly on a clean paper tensed onto a flat glass to remove paste excess and to smooth the exposed surface.

2.5. Biosensors conditioning

The bioactive surface was covered with a layer of poly(*o*-phenylenediamine), obtained by CV electropolymerization. The electrode potential was cycled between −0.51 and +0.69 V (vs Ag/AgCl) at 0.050 V s^{-1} in an oxygen-free 0.1 M $K_2CO_3/KHCO_3$ pH 10 buffer, containing 5×10^{-4} M *o*-phenylenediamine, under N_2 atmosphere. The biosensor thus prepared was used as the working electrode to perform amperometric measurements. When not in use, the biosensor was stored in 0.1 M $K_2CO_3/KHCO_3$ pH 10 buffer at 4 °C.

2.6. Electrochemical experiments with biosensors

Amperometric experiments with biosensors were performed at 25 °C and 0.380 V (vs. 3 M KCl, Ag/AgCl reference electrode) in a cell containing 3 mL of a solution composed of 1.0×10^{-3} M $K_3Fe(CN)_6$ and 5×10^{-4} M NAD^+ in 0.1 M $K_2CO_3/KHCO_3$, pH 10 buffer. The

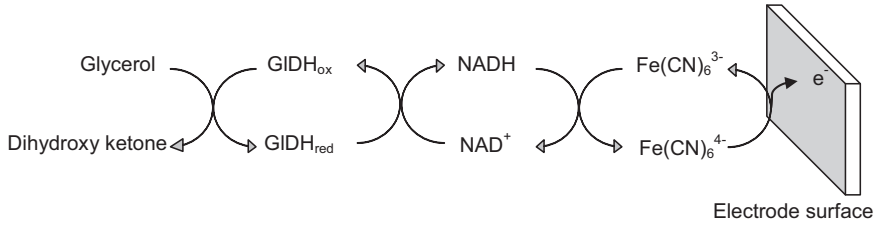

Fig. 1. Schematic representation of the biosensor layout, displaying the catalytic cycle that originates the signal.

system was preconditioned at 0.380 V for 50 s, and once the equilibrium was reached (usually at about 300 s) the current value was recorded as i_b. Standard glycerol solution was then added so as to reach the desired concentration. The current i_s was recorded until stabilization (generally at 500 s). The signal, catalytic current (i_c) was calculated as $i_c = i_s - i_b$. Three independent experiments were performed to evaluate signals at all concentrations.

The catalytic cycle that originates the signal is shown in Fig. 1.

2.7. Ancillary experiments

Several experiments were performed to evaluate the signal obtained using other experimental conditions. In case of aqueous glycerol solutions, we evaluated using Pt and glassy carbon electrodes as alternative working electrodes in several media, such as NaOH solution pH = 13, perchloric acid pH = 1 and neutral medium. In these cases, as well as for Au tip working electrode, we appraised using both amperometry and differential pulse voltammetry (DPV) techniques. For glycerol samples in complex matrixes we assayed to cover the biosensor with other polymers, such as 4-vinylbenzyl thymine copolymerized with 4-vinylbenzyl triethyl ammonium chloride and vinyl-phenyl sulfonate (positive and negative net charge, respectively). Also, we tested PO_4^{3-}/HPO_4^{2-} and NH_3/NH_4^+ as bathing buffers, several NAD^+ final concentrations in the cell, and other soluble mediators, such as ferrocenemethanol and Prussian blue. In addition, we evaluated the incorporation of carboxylic functionalized multi-wall carbon nanotubes as mediator in the carbon paste, according to other authors' previous suggestion [19,20]. To make a decision on which were the final experimental conditions to be used, the criterion was to choose those rendering the higher signal-to-noise relationship, with a total time of analysis not longer than 10 min, i.e. the period of time spent when performing the colorimetric determination when using the commercial kit.

2.8. Validation of the method

The commercial TG Color GPO/PAP AA (liquid line) from Wiener lab (Argentina) was used to compare the new approach with a standardized, accepted method. In these assays different glycerol sample dilutions were processed as serum samples, according to the manufacturer instructions, and performed in triplicate. The results, reported as glycerol concentration recovery, were obtained with a multiparametric autoanalyzer Technicon® RA-XT. When comparing the gold tip method with the spectrophotometric one, we used the same concentration set of glycerol dilutions added to the 0.1 M NaOH solution, while carrying out the electrochemical method. When comparing our biosensor with the spectrophotometric kit, we used the same set of solutions of Middlebrook 7H9 broth, supplemented with glycerol.

2.9. Data analysis

Results were expressed as mean ±1.5 standard deviation (SD), therefore, error bars in graphs represent 3 SD. Linear regression was performed by least squares method, previous verification of normal variance distribution (Shapiro–Wilk Test) and constant variance value. One-way ANOVA followed by the *F*-test were used to check linearity.

The method discrimination ability between two different concentrations was assessed as the concentration error, S_c, calculated as:

$$S_c = \frac{S_r}{m}\sqrt{\frac{1}{N}}$$

where S_r is the standard error of the estimate, m is the regression line slope of the calibration plot, and N is the number of replicates.

The limit of detection (LD) was assessed as the glycerol concentration rendering a signal 3 times the SD of the current registered when adding to the cell the matrix studied, either water or Middlebrook 7H9 broth without glycerol, both used as models of simple and complex matrixes, for ten independent experiments.

Elliptical joint confidence region (EJCR) test for the slope and intercept of predicted versus nominal concentrations plot was used for method comparisons.

The level of significance was set at $P < 0.05$ in all statistical tests.

3. Results and discussion

We determined glycerol concentration by amperometry using two different working electrodes, namely (i) gold tip and (ii) a new glassy carbon-GlDH biosensor. In an attempt to evaluate the convenience of choosing one or the other according to the sample nature, we used two quite different kinds of matrixes, a very simple one, aqueous solution, and an extremely complex, Middlebrook 7H9 broth. This latter medium is useful for the preparation of inocula for antimicrobial assays, biochemical tests, maintenance of stock bacteria strains and, when supplemented with other nutrients such as glycerol, supports the growth of a number of mycobacteria. Indeed, Middlebrook 7H9 broth contains, among others, ammonium sulfate, glutamic acid, sodium citrate, pyridoxine, biotin, ferric ammonium citrate, zinc and copper sulfate, all of these being potential electrochemical interferences because of their redox properties.

3.1. Calibration curve and figures of merit obtained with the gold electrode

A typical chronoamperometric response obtained with a gold tip working electrode, and conditions detailed in Section 2.3.2 is displayed in Fig. 2(a). A well-defined stationary oxidation current was registered after addition of the analyte. This indicates that an oxidation process takes place at the applied potential of the electrode, followed by a time-dependent decay due to diffusion of glycerol to the electrode surface. Fig. 2(b) shows the calibration line obtained with the gold electrode in aqueous alkaline solutions of glycerol

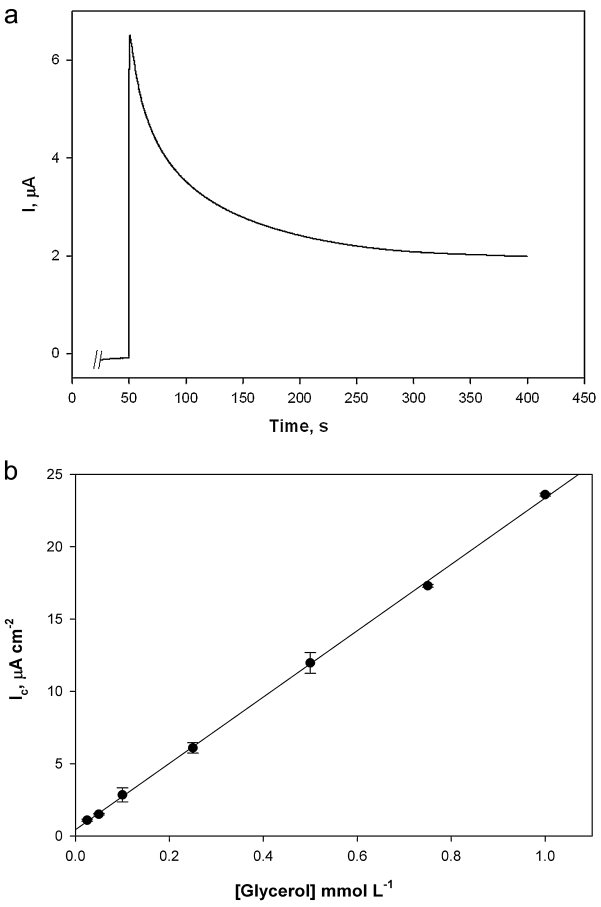

Fig. 2. (a) Chronoamperometric response registered in a cell with a Ag/AgCl reference electrode and a gold tip working electrode, at 0.100 V, in 1×10^{-4} M glycerol solution, pH 13. (b) Calibration plot depicting the signals recorded using gold electrodes as a function of glycerol concentration. Current/area ratio, measured at 0.100 V constant voltage vs. Ag/AgCl, in 0.1 M NaOH, for 3 replicate experiment sets at each concentration, with error bars showing 3 SD.

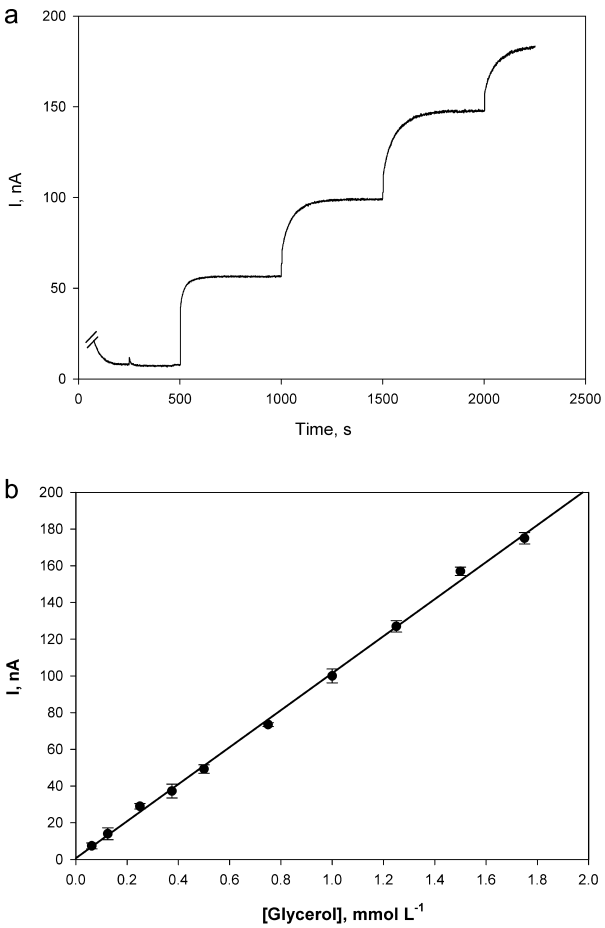

Fig. 3. (a) Chronoamperometric response registered in a cell with a Ag/AgCl reference electrode and the glycerol biosensor, at 0.380 V, after consecutive additions of 30 µL of Middlebrook 7H9 broth, glycerol 67 mM in 4 mL 0.1 M K_2CO_3/$KHCO_3$, pH 10 buffer. (b) Calibration plot depicting the signals recorded with biosensors built up with GlDH as a function of glycerol concentration. Catalytic current at 0.380 V constant voltage vs. Ag/AgCl, in 1.0×10^{-3} M $K_3Fe(CN)_6$, 5.0×10^{-4} M NAD^+ in 0.1 M K_2CO_3/$KHCO_3$ pH 10 buffer, for 3 measurement sets at each concentration, with error bars showing 3 SD.

in the 2.5×10^{-5} to 2.0×10^{-3} M range. It is possible to see that the response of the electrode is linear within the whole range of concentrations analyzed.

3.2. Calibration curve and figures of merit obtained with the biosensor

The amperometric response obtained with our biosensor operating as working electrode, and the conditions stated in Section 2.6 is shown in Fig. 3(a). Similarly to the behavior of the gold-tip electrode, an oxidation current was registered after each addition of Middlebrook 7H9 broth supplemented with glycerol. However, the process taking place is the catalytic cycle illustrated in Fig. 1 and, in this case, the current observed corresponds to the oxidation of the reduced redox mediator, $Fe(CN)_6^{4-}$, at the applied potential. Fig. 3(b) shows the calibration plot obtained with our GlDH biosensor, as described in Section 2.6. In this figure it can be observed linearity of the signal produced by the biosensor with glycerol concentration in the range 7.0×10^{-5} to 1.8×10^{-3} M.

It is worth highlighting that the main differences between our biosensor and others using GlDH are, firstly that we use a single-enzyme system which is mixed in the glassy carbon paste at significantly less proportion than others previously reported and secondly, that we changed the mediator formerly proposed by Álvarez-González's method [18] by an inexpensive soluble mediator. Regarding the enzyme cofactor, we used NAD^+ in solution instead of adding it mixed in the carbon paste at high proportions, as accounted by Álvarez-González et al. because our experiments showed that reproducibility enhanced when the cofactor was in solution. A possible explanation for this is that when NAD^+ is mixed in the paste, there might be a leaking of the cofactor during consecutive analysis, therefore, diminishing its activity and augmenting variability between independent consecutive assays.

3.3. Stability and reusability of the biosensor

Once the glassy carbon paste containing the enzyme was prepared, it was stable for 10 weeks with no enzymatic activity loss, provided it was kept dry at 4 °C. After the paste was mounted on the tip and the polymer was electrodeposited, the GlDH-biosensors were used for 8 h with negligible sensitivity loss. Indeed, changes in signals consecutively acquired were within the method intrinsic error given by the instrument noise. This feature allowed us to envisage the device potentiality to be used in automatic equipment. Although our biosensors could be used in the following days, the signal-to-noise decreased with time, about 15% and 50% of the original value during the third and fifth day, respectively. We therefore recommend using the biosensor during one working day, and then replace the paste by the leftover that, provided it is kept dried at 4 °C, it is stable for 10 weeks.

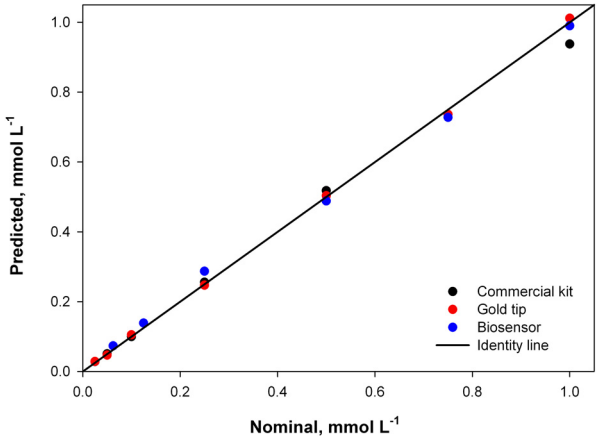

Fig. 4. Comparison of the results obtained for the determination of glycerol between both of our proposed methods and one spectrophotometric, commercial kit. Regression line displaying predicted versus nominal glycerol concentration. Points represent the media of three replicates for every concentration.

3.4. Validation of the proposals

In order to validate our methodologies we carried out a parallel assay with an alternative method, namely one current, extensively used, commercial spectrophotometric kit (Wiener lab). Fig. 4 shows the regression line obtained for predicted versus nominal glycerol concentration, obtained when testing the different sample dilutions for the three methods, plotted as the mean of 3 replicates.

It is evident that both of our proposed methods appropriate correlate with the identity line. This is clearly confirmed observing Fig. 5 where the three elliptical joint confidence regions (EJCR) contain the (0; 1) point quite centered, indicating the good correlation between both of our methods and the spectrophotometric, commercially available one.

3.5. Comparison with several methods that quantify glycerol

We examined different methods already published to determine glycerol, and studied which were the more convenient to quantify this compound, in terms of achieving the best balance between sensitivity to detect tiny differences in glycerol concentrations, fast performance and low cost; the two factors latter mentioned are critical when a large number of samples has to be processed. We have omitted chromatographic and mass spectroscopic methods since, from the green chemistry and economical points of view,

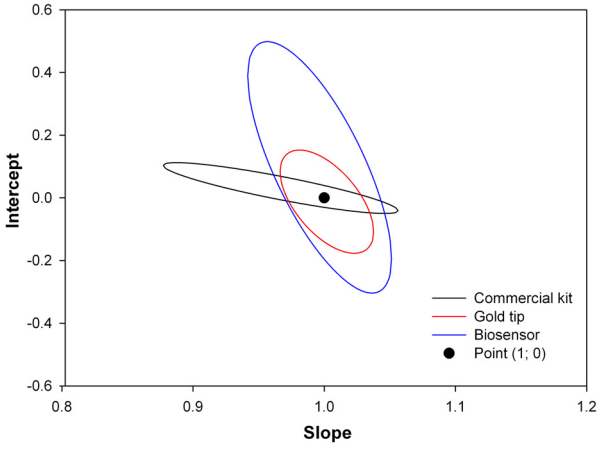

Fig. 5. Elliptical joint confidence regions (EJCR) for the three mentioned methods.

Table 1
Analytical features of several recent reported methods to determine glycerol.

Detection method (previous steps)	LD μM	Linear range/M	Samples assayed	Total analysis time/min	Expensive reagents or apparatus	Reference
Spectrophotometric (analyte extraction)	22	4×10^{-5} to 2×10^{-4}	Biodiesel	20	–	Bondioli and Bella [4]
Spectrophotometric (analyte extraction)	10	5×10^{-5} to 5×10^{-4}	Biodiesel	5–30	–	Silva and Rocha [5]
Spectrofluorometric (analyte extraction)	0.4	1×10^{-6} to 5×10^{-4}	Biodiesel	4	–	Lima et al. [6]
Spectrophotometric, multienzymatic	N.I.	2×10^{-2} to 1×10^{-1}	Sugar cane juice	5	GK, GPO, ATP	Kronka et al. [7]
Spectrophotometric, multienzymatic	8	8×10^{-6} to 2.5×10^{-4}	Human sera	10	GK, PK, LDH, ATP, NAD^+	Keymeulen et al. [8]
Spectrophotometric, enzymatic	65	2×10^{-2} to 1×10^{-1}	Wine	5	GlDH, NAD^+	Fernandes and Reis [9]
Electrochemical, DPV (analyte extraction)	33	5×10^{-4} to 1.2×10^{-2}	Biodiesel	15	–	Tehrani and Ghani [10]
Electrochemical, CV (analyte extraction)	3	3.3×10^{-5} to 1.7×10^{-3}	Biodiesel	30	FIA	Maruta and Paixao [21]
Electrochemical, CV (analyte extraction)	25	3.3×10^{-5} to 1.7×10^{-3}	Biodiesel	20	C18 cartridge	Lourenço and Stradiotto [22]
Electrochemical, amperometry (analyte extraction)	N.I.	1.6×10^{-4} to 1.6×10^{-3}	Biodiesel	60	–	Stradiotto et al. [23]
Electrochemical LSV, CV, and amperometry	10	2.3×10^{-5} to 2.3×10^{-4}	Aqueous pH = 13	5	Silver functionalized MWCNT	Manea et al. [24]
Electrochemical, amperometry	10	2.5×10^{-5} to 2.0×10^{-3}	Aqueous pH = 13	8	–	This work
Amperometric biosensor	50	5×10^{-5} to 2.3×10^{-2}	Phosphate buffer	5 estimated	GO	Goriushnika et al. [1]
Amperometric biosensor	0.4	1.0×10^{-6} to 1.0×10^{-4}	Plant extract syrup	15 estimated	GlDH[a], NAD^+	Álvarez et al. [18]
Amperometric	0.4	1×10^{-6} to 2×10^{-5}	Wine	3	GlDH, DP, NAD^+	Gamella et al. [11]
biosensors	0.3	1×10^{-6} to 1×10^{-5}	Wine	3	GK, GPO, HRP, ATP	
Amperometric biosensor	4	5×10^{-6} to 1×10^{-4}	Wine	5	GK, GPO, ATP	Ghica and Brett [14]
Amperometric biosensor	20	7.0×10^{-5} to 1.8×10^{-3}	Middlebrook 7H9 broth	10	GlDH[b], NAD^+	This work

ATP, adenosine triphosphate; CV, cyclic voltammetry; DP, diaphorase; DPV, differential pulse voltammetry; FIA, flow injection analysis; GlDH, glycerol dehydrogenase; GK, glycerol kinase; GPO, glycerol phosphate oxidase; HRP, horseradish peroxidase; LDH, lactate deshydrogenase; LSV, linear sweep voltammetry; MWCN, multiwalled carbon nanotubes; NAD^+, nicotinamide-adenine dinucleotide; N.I., not informed; PK, piruvate kinase.

[a] High GlDH consumption (7% w/w) per biosensor.

[b] Low GlDH consumption (2% w/w) per biosensor.

Table 2
Comparative reagents consumption between the method displaying the best figures of merit [11] and ours.

	Glycerol dehydrogenase/per biosensor	Diaphorase/per biosensor	Tetratiofulvalene/per biosensor	NAD^+/per analysis
Gamella et al.	7.5 U	1.62 U	1.5×10^{-6} M	5×10^{-3} M
This work	2.3 U	–	–	5×10^{-4} M

these methodologies are not recommended due to the use of solvents potentially risky for the environment, and the expensive equipment necessary to carry out the determinations.

Table 1 summarizes the reviewed methods to determine glycerol together with those performed in this work, tabulated regarding their LD (μM), the linear concentration range (M), the type of sample for which the method has been used, the estimated total time of analysis, and the use of relatively expensive reagents (chemicals or enzymes). We focus in these several methods because they met our requirements, in terms of the time spent to perform the analysis and the operational cost.

By analyzing Table 1, it is apparent that when quantifying glycerol in aqueous alkaline media which do not contain interferences, relatively inexpensive methodologies can be used. It should be considered that, when determining glycerol in biodiesel samples, its quantitation requires withdrawal of the analyte from the matrix, as can be seen in the lately proposed methods [4–6,10,21–23]. These either spectroscopic or electrochemical methods use preceding steps, such as the extraction, centrifugation or purification, previously to the analyte determination. Along with this, even Lima's proposal, which is the only method that makes a noticeable favorable difference when comparing the figures of merit (lowest LD, 0.4 μM, with one of the shortest analysis time per sample, 4 min) needs sample pretreatment [6]. Despite its advantages, Lima's methodology relies on the use of a relative complicated multifaceted flow-batch system. Indeed, it is assisted by six three-way solenoid valves, a peristaltic pump, a stirrer, and a spectrofluorometer as detection system, the whole system being automatically controlled by a computer. Similarly, Tehrani's approach, using a nanonickel modified graphite electrode working at pH = 13, is carried out with prior extraction of glycerol from biodiesel samples [10]. In this case, the LD was 33 μM, whereas by using gold electrodes to perform amperometry in the same medium we reached 10 μM. Maruta and Paixao have recently proposed an amperometric method using a copper electrode working at 0.65 V vs. Ag/AgCl, adapted in a FIA system [21], whereas Lourenço et al. reported on one electrochemical method that requires aqueous extraction of glycerol from the sample, and further purification by elution through a C18 cartridge, subsequent concentration by evaporation, and finally, CV analysis [22]. Also, other proposals report on the determination of glycerol by amperometry, relaying on the use of modified electrodes to electrocatalyze its oxidation, such as a nickel nanoparticle modified boron doped diamond electrode [23], and a silver-functionalized multi-wall carbon nanotube composite electrode [24]. Although both methods proved to be useful, different operational steps are required to eliminate potential interferences such as other alcohols usually accompanying the analyte that could be oxidized as well at the working electrodes potentials, 0.65 V and up to 1.30 V, respectively. In this regard, our results obtained by amperometry using gold electrodes in aqueous alkaline medium, pH 13, are in the same order than those obtained by Pop et al. by LSV [24], using a silver-functionalized MWCNT composite electrode. Indeed, this latter and our methodologies display the best figures of merit, among the inexpensive electrochemical methods which do not consume enzymes. Moreover, our experiments with the versatile Au electrode show the best results regarding linear range (2.5×10^{-5}–2.0×10^{-3} M vs. 2.3×10^{-5}–2.3×10^{-4} M), and also our method perceives as the most inexpensive one because of reusability of the electrode, which can be renewed simply by polishing it.

Table 1 also shows that, as the number of interfering compounds accompanying glycerol in the sample increases, so does the complexity of the methodology used to quantify it. In this regard, there are a number of advantages when using methodologies that rely on the use of enzymes that selectively react with the analyte to render products that are to be detected. The use of enzymes aims avoiding sample pretreatment steps, provided the sample matrix where glycerol is to be quantified does not denaturalize the enzyme. The spectrophotometric, enzymatic methods though are not laborious, have some disadvantages, such as their relatively high consumption of enzyme; every sample needs the addition of the costly biological reagent because it cannot be reused. Also the LDs of spectrophotometric, enzymatic methods are generally higher than those displayed by enzymatic biosensors. Actually, in Table 1, it is shown that biosensors display the lowest LDs and the wider linear range, as compared with other approaches, either electrochemical or spectrophotometric ones. Only Lima's spectrofluorometric flow-batch methodology [6] displays a sensitivity in the same order than the lowest of biosensors being, however, a more complex and expensive methodology than the latter ones.

Analyzing the whole range of biosensors displayed in Table 1, it is noteworthy that Gamella's biosensors [11] displays the lowest LD and total analysis time. In our method, as a trade of between the mentioned attributes and the operational cost, we changed the platform on which the biosensor is assembled from gold to glassy carbon powder, diminished the amount of enzyme and cofactor, and used a very cheap soluble redox mediator, such as $Fe(CN)_6^{3-}$. A summary of the amount and concentration of consumables spent per biosensor or per analysis to evaluate operational costs of Gamella's, the best featured biosensor, and ours is depicted in Table 2.

It is readily apparent from Table 2 that our proposal is less expensive, a fact that it is important and should be evaluated when a large number of samples is to be analyzed. On the other hand, by lowering the amount of GlDH, the LD was raised from 0.4 to 20 μM, though it is still suitable to appropriately detect changes in the 1×10^{-5} M glycerol concentration order. Concurrently, by avoiding the use of DP, the total time of analysis was extended from 3 to 10 min. Therefore, Gamella's biosensors should be used in circumstances in which these factors become critical. Nonetheless, even in these cases it could be considered that it is still possible to augment both the GlDH proportion in the glassy carbon paste and the cofactor concentration to achieve better quality figures of merit. As a whole, our studies confirmed that the changes introduced to previously reported biosensors, such as using only one enzyme at low proportion, replacing the redox mediator by soluble ferricyanide, and working with soluble NAD^+ cofactor, provided an inexpensive reliable system to quantify glycerol.

4. Conclusions

A study to appraise glycerol determination with low LD was performed, considering several proposals that include benefits, such as being green, low cost, and friendly methodologies. Among the alternatives to quantify the analyte in alkaline media, which may include aqueous extracts of biodiesel samples, we evaluated that

amperometry using the versatile gold electrode is a suitable, economical proposal. A low cost amperometric biosensor that uses GlDH dispersed on glassy carbon powder together with soluble NAD^+ cofactor, and ferricyanide as redox mediator proved to be useful for sensitive and specific detection of glycerol, when the analyte is in a complex matrix, such as Middlebrook 7H9 broth. The new biosensor could be reused in consecutive assays during a whole working day, additionally demonstrating its potential utility for automated analysis.

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