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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