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Development of a Disposable, Amperometric Glycerol Biosensor Based on a Screen-Printed Carbon Electrode, Modified with the Electrocatalyst Meldolas Blue, Coated with Glycerol Dehydrogenase and NAD$^+$: Application to the Analysis of Wine Quality

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Featured Application: Measurement of glycerol content for wine safety and quality.

Abstract: This paper describes the design and development of a novel electrochemical biosensor for measuring glycerol in wine. Our initial detailed studies were aimed at deducing the optimum conditions for biosensor operation by conducting hydrodynamic voltammetric and amperometric studies. The resulting voltammograms revealed a maximum electrocatalytic current at 0.0 V vs. Ag/AgCl, which we used for all further studies. We also examined the effect of pH (8–10) on the amperometric responses of different glycerol concentrations over a range of 0.04 to 0.20 mM. Based on our findings, we propose that pH 9 would be suitable as the supporting electrolyte for further studies with the amperometric biosensor. The biosensor was constructed by immobilising 10 units of GLDH and 660 µg NAD$^+$ onto the MB-SPCE surface using glutaraldehyde (GLA) as a cross-linking agent. Calibration studies were performed with glycerol over the 1.0–7.5 mM concentration range. Chronoamperometry was the electrochemical technique chosen for this purpose as it is convenient and can be performed with only 100 µL of sample directly deposited onto the biosensor's surface. In the current study, we observed linear calibration plots with the above standard solutions using current measurements at a selection of sampling times along the chronoamperograms (30–340 s). We have evaluated the glycerol biosensor by carrying out an analysis of commercially available red wine. Overall, these findings will form a platform for the development of novel rapid technology for point-of-test evaluation of glycerol in the production and quality control of wine.

Keywords: food safety; food quality; wine; amperometric glycerol biosensor; hydrodynamic voltammetry; chronoamperometry; screen-printed carbon electrode; meldolas blue

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1. Introduction

Glycerol is a biologically important compound that is both a naturally occurring triose sugar and a food additive (E 422) [1]. Therefore, its concentration in foods and beverages needs to be controlled for food quality and safety purposes. Consequently, reliable analytical methods for determining it in different and often challenging matrices are urgently needed.

One of the most powerful analytical techniques used in measuring glycerol in beverages is High-Performance Liquid Chromatography (HPLC); some examples of analytical approaches developed for beverages are discussed below. Some include the possible chemical constituents accompanying glycerol in the beverage matrix to illustrate the important

analytical challenges [2]. An HPLC method involving a cation ion exchange column, preceded by a guard column, in conjunction with a refractive index detector was described by Li et al. [3], which proved successful for the measurement of glycerol in beers. This group reported [3] that controlling glycerol was important, as levels below or above the threshold could be detrimental to the product's flavour. The authors showed complete chromatographic separation of glycerol from ethyl alcohol, as well as earlier eluting unidentified compounds; levels of glycerol were between 0.925 and 1.502 gL^{-1}, and ethanol ranged from 3.0 to 5.0% (v/v) in a variety of commercial beers.

HPLC methods have also been investigated in the context of wine analysis. Lopez and Gomez [4] successfully developed an HPLC method for wine analysis that could measure glycerol, as well as other sugars and carboxylic acids. The authors determined that the glycerol content of white wine was 6.45 gL^{-1}. A similar study was also reported by Calull et al. [5]. Moro et al. [6] reported on an HPLC method that was applied to the determination of glycerol, glucose, and fructose in wines to ascertain whether the drinks were high- or standard-quality red and white wines. This assay was achieved using HPLC in combination with a modified polar-bonded NH_2–silica phase column and a refractive index detector. The authors indicated that the concentration of glycerol in high-standard red wine was 3.30–7.67 gL^{-1} and in white wine, the levels were 1.65–5.87 gL^{-1}.

An interesting method involving HPLC combined with an FTIR detection system was applied to the determination of glycerol in red wine [7]. It was shown that lactic acid co-eluted with glycerol on the column, but the on-line FTIR detection system was able to resolve the responses of the two compounds for quantification. The glycerol concentration was determined for seven samples of red wine, and the range was reported to be 7.88–9.29 gL^{-1}.

Linget et al. [8] have developed a reproducible and high-throughput technique for on-line clean-up, coupled with HPLC, for routine analysis of glycerol and other small molecules such as amino acids. An ion exchange analytical column was used in conjunction with an RI detector, and the concentration found in red wine was found to contain 6.9 gL^{-1}. In addition to glycerol, Linget et al. [8] simultaneously determined a variety of other species by the reported method. In red wine (Cabernet Sauvignon), 0.45 gL^{-1} of citric acid, 1.99 gL^{-1} of tartaric acid, 0.46 gL^{-1} of malic acid, 0.7 gL^{-1} of succinic acid, 1.91 gL^{-1} of lactic acid, and 0.51 gL^{-1} of acetic acid were present and determined. Glucose 0.15 gL^{-1}, fructose, and ethanol 122 gL^{-1} were also present but did not appear to interfere with the determination of glycerol. The HPLC-based methods described above have shown how glycerol may be determined effectively in a range of alcoholic drinks, even in the presence of a variety of other naturally occurring species.

However, using this sophisticated instrument has drawbacks. These include the high cost of the instrument, which requires special expertise to operate and maintain, and the latter is often time-consuming. The actual sample preparation and chromatographic analysis may also be time-consuming.

In contrast, electrochemical biosensors have been developed based on screen-printing technology [9,10]. These devices have the advantage that they can be mass-produced in a wide range of geometries at low cost, particularly when carbon materials are employed to construct the electrodes. We explored the possibility of developing a screen-printed biosensor incorporating glycerol dehydrogenase (GLDH) as the biorecognition element. This enzyme requires the cofactor NAD^+ for the oxidation of glycerol, which results in the formation of NADH. Consequently, it was considered possible to measure glycerol indirectly by monitoring the generation of NADH during the oxidation reaction.

Our group was the first to develop an electrochemical strategy for the measurement of NADH based on a Meldolas Blue-Screen-printed Carbon Electrode (MB-SPCE) [11]. Meldolas Blue acts as an electrocatalyst for NADH oxidation, and the resulting anodic current constitutes the analytical response [11,12]. The main advantage of this approach is that the amperometric detection of NADH can be performed at potentials of 0.0 V vs. Ag/AgCl, whereas potentials in excess of +0.5 V vs. Ag/AgCl are required to obtain the

limiting oxidation current at plain carbon electrodes [13]. Consequently, the selectivity is expected to be greatly improved with the former, which is highly beneficial for the analysis of real samples that may contain electroactive interferences.

We have exploited the above approach, employing biosensors based on MB-SPCEs to measure a variety of target analytes in different sample types, and all of the following examples have employed this platform.

A lactate biosensor was fabricated by coating the surface of the screen-printed electrode surface with lactate dehydrogenase and the cofactor NAD^+. This device was successfully applied to the measurement of lactic acid in serum [14]. This analysis could be performed by simply depositing 20 µL aliquots of solution onto the biosensor strip for chronoamperometric measurements. The resulting data demonstrated the possibility of applying the lactate biosensor to biomedical studies. In the area of environmental monitoring, a disposable screen-printed ammonium ion biosensor was constructed by immobilising the enzymes glutamate dehydrogenase, 2-oxoglutarate, and NAD_+ onto the electrode surface and was successfully applied to river water analysis [15]. This biosensor was subsequently incorporated into a commercial hand-held instrument for the measurement of ammonium ions in sewage effluent [16].

Additionally, we explored the possibility of adapting the MB-SPCE platform for the fabrication of amperometric biosensors for applications in the food industry. An amperometric glutamate biosensor was constructed by immobilising glutamate dehydrogenase onto the electrode surface after it had been modified with the biopolymer chitosan [17]. This was successfully applied to the determination of glutamate in a stock cube following a simple dissolution step in buffer solution, to which was added NAD^+. In order to circumvent the need to add NAD^+ to the sample, an improved glutamate biosensor was subsequently developed. This involved incorporating GLDH and NAD^+ into a hydrogel formed by chitosan and carbon multi-walled nanotubes (MWNTs), which were deposited onto the electrode surface using a layer-by-layer approach [18]. This novel biosensor was successfully applied to the determination of glutamate in a stock cube.

In recent years, we have investigated the possibility of developing a biosensor for androstenone, as this compound, together with skatole, is responsible for an off-taint in pig meat known as boar taint [19]. We immobilised the enzyme hydroxysteroid dehydrogenase (HSD) onto the screen-printed electrode by an adsorption procedure. It was demonstrated that androstenone could be measured by directly inserting the biosensor into the adipose tissue layer of the meat sample and that the androstenone concentration may indicate boar taint above a designated threshold level.

The electrocatalytic biosensor platform described above has also been adapted for the development of a biosensor for use in the alcoholic beverage industry. The MB-SPCE surface was modified by the immobilisation of ethanol dehydrogenase, NAD^+, and trehalose, using cellulose acetate to encapsulate the reaction layer. The resulting biosensor was successfully applied to measuring ethanol in gin [14].

The aim of the present study was to investigate the possibility of developing a robust, disposable electrochemical glycerol biosensor based on the MB-SPCE transducer. This biosensor would have important attributes, including mass production at a low cost and the possibility of rapid measurements by simply depositing small aliquots of liquid samples directly onto the flat biosensor surface. The chronoamperometric technique is very suitable for this purpose and is both simple to perform and easy to interpret. This approach would alleviate the requirement for laboratory glassware and help to achieve the portability of an analytical biosensor system. This paper describes the steps involved in fabricating and optimising the biosensor, as well as its application to measuring glycerol in wine. This method provides a foundation for the development of innovative, rapid point-of-test technology and should have a considerable impact on food and beverage quality and safety assessment.

2. Materials and Methods

2.1. Instrumentation

All voltammetric and amperometric measurements were carried out with an Emstat Blue potentiostat (PalmSens, Houten, The Netherlands), which was interfaced to a PC for data acquisition via PS Trace Software version 5.10.

MB-SPCEs were supplied by Gwent Electronic Materials Ltd. (Newport, UK). The working electrode was fabricated using carbon-based ink with CoPC (C2030408P3), and the reference electrode was fabricated using Ag/AgCl ink (C2130809D5). The working electrode area (3 × 3 mm) was defined using electrical insulation tape.

All pH measurements were performed using a Testo 205 (Testo Limited, Alton, Hampshire, UK) pH meter. Solutions were stirred using a colour squid (IKA, Tunbridge Wells, UK) and warmed using a HAAKE P5 water bath (Thermo Scientific, Loughborough, UK).

Optical digital microscopy (OM) was conducted using the Keyence optical microscope (Keyence, Itasca, IL, USA). The High Dynamic Range (HDR) imaging function was utilised to capture multiple images at varying shutter speeds, resulting in an image with enhanced colour gradation and contrast for a more detailed observation. Additionally, the optical shadow effect mode enabled the visualisation of surface details on the sensors. All images were captured at 80× magnification.

The characterisation of the biosensor surface was performed using an attenuated total reflection (ATR) FTIR spectrometer (Nicolet 50, Thermo Fisher Scientific, Loughborough, UK) with built-in ATR. An analysis was performed to confirm the presence of all compounds on the full glycerol biosensor surface and a sample of glutaraldehyde. Scans were performed across a range of 4000–400 cm^{-1}, averaging 128 scans at a 4 cm^{-1} resolution with a 1 cm^{-1} interval. Background absorption was subtracted before analysis.

2.2. Raw Materials, Chemicals, and Reagents

Red wine (Merlot) from California was purchased at Sainsbury's, a UK supermarket chain, and stored at 4 °C. GLDH was obtained from Merck (Darmstadt, Germany). All other chemicals were purchased from Sigma Aldrich (Dorset, UK). Deionised water was obtained from a Purite RO200 Stillplus HP System (Oxon, UK). Stock solutions of monosodium, disodium, and trisodium orthophosphate were prepared at a concentration of 0.2 M by dissolving the appropriate mass in deionised water and were then titrated to achieve the desired pH and diluted in the cell to achieve a working concentration of 0.1 M. Sodium chloride was prepared to a concentration of 1.0 M by dissolving the appropriate mass in deionised water and was diluted in the cell, giving a final concentration of 0.1 M. Ascorbic oxidase (AO; 1000 U mg^{-1}, 189,724) from cucumber (*Cucurbita* sp.) was purchased by Merck (Darmstadt, Germany).

2.3. Glycerol Assessment

The glycerol estimation was obtained using a commercial glycerol assay kit (Megazyme International Ireland, Wicklow, Ireland). The glycerol is phosphorylated in this assay using adenosine-5′-triphosphate (ATP). The formed adenosine-5′-diphosphate (ADP) is used to phosphorylate d-glucose, which is oxidised with nicotinamide adenine dinucleotide (NADH) formation. The amount of NADH was detected by measuring absorbance at 340 nm using a UV-vis spectrophotometer (GENESYS 150, Thermo Scientific, UK).

2.4. Biosensor Fabrication

For the fabrication of the GLDH biosensors, the surface of the MB-SPCE working electrodes was coated with 10 µL of a mixture containing 10 U of GLDH and 660 µg of NAD^+ in a 0.1 M PBS, pH 9, solution. This layer was allowed to dry overnight under a vacuum at −0.6 MPa, at 4 °C. Next, a 10 µL aliquot of glutaraldehyde (0.01% in phosphate buffer) was deposited on top of the enzyme/NAD^+ layer and again left overnight, under vacuum, to allow crosslinking to occur. These biosensors were refrigerated until they were ready for use for calibration purposes and wine analysis.

2.5. Hydrodynamic Voltammetry of NADH in Alkaline Solutions Using an MB-SPCE

To deduce the optimum oxidation potential of NADH required for the operation of the biosensor, hydrodynamic voltammetry was performed with the MB-SPCE from an initial potential of −0.3 V to a final potential of +0.3 V vs. Ag/AgCl, and the current measurements were recorded at 50 mV intervals. Aliquots (10 mL) of either 0.1 M pH 8.0, 9.0, or 10 phosphate buffered saline (PBS) containing 5 mM NADH were transferred to the electrochemical cell, and the solutions were stirred at 250 rpm at a temperature of 30 °C. A fresh MB-SPCE was used to record the hydrodynamic voltammogram of the three buffer solutions.

2.6. Effect of pH on Amperometric Responses of Glycerol Solutions Containing Free GLDH and NAD^+

Next, we examined the effect of pH (8–10) on the amperometric responses of different glycerol concentrations. All the solutions were stirred at 250 rpm and contained 10 units of GLDH and 5 mM NAD^+, and the solution temperature was controlled at 30 °C. A fresh MB-SPCE was used to record the amperograms for each pH over the 0.04–0.2 mM concentration range.

2.7. Calibration Procedure and Analysis of Wine

Calibration studies were performed with glycerol solutions, prepared in 0.1 M PBS pH 9, over the 1.0–7.5 mM concentration range. The electrochemical technique employed was chronoamperometry, using an applied potential of 0.0 V vs. Ag/AgCl, preceded by an incubation time of 3 min. Aliquots of 100 µL of standards and samples were directly deposited onto the glycerol biosensor surface, which was warmed to 30 °C by placement onto a thermostated surface. A fresh biosensor was used to measure each concentration of glycerol. In the current study, we constructed calibration plots with the above standard solutions by taking current measurements at a selection of sampling times along the chronoamperograms at 30, 100, 200, 300, and 340 s.

For the analysis of red wine, an initial dilution step was carried out in order to adjust the concentration of glycerol to be in the linear range. Wine dilutions were carried out using 0.1 M PBS, pH 9. In addition, a final treatment of the sample was carried out with the addition of AO to enzymatically remove any ascorbic acid present in the sample.

2.8. Statistical Analysis

All data acquired were expressed as mean ± standard deviation (SD), and the Excel Microsoft® Office 365 (ver. 16.48) was used to plot graphs. Data were analysed using the paired two sample means *t*-test with IBM® SPSS® statistics 26 software for macOS (SPSS Inc., Chicago, IL, USA) at a 5% level of significance.

3. Results

3.1. Fabrication and Operation of the Glycerol Biosensor

Figure 1 presents the steps involved in fabricating the glycerol biosensor. Initially, a mixture containing GLDH and NAD^+ in phosphate buffer was deposited on a base of MB-SPCE. This layer was allowed to dry overnight under vacuum at 4 °C. Next, a 10 µL aliquot of a solution containing GLDH in PBS was deposited onto the enzyme/NAD^+ layer and again left overnight under vacuum to allow crosslinking to occur. These biosensors were refrigerated until they were ready for use.

Figure 2 shows the sequence of reactions that occur during the operation of the amperometric glycerol biosensor. The biosensor's operation principle is that glycerol is enzymatically oxidised by GLDH in the presence of NAD^+, which results in the production of NADH and dihydroxyacetone. NADH then undergoes electrocatalytic oxidation at an MB-SPCE, and the resulting electrocatalytic oxidation current constitutes the analytical response. This response is directly proportional to the glycerol concentration. It should be emphasised that the advantage of exploiting the electrocatalytic oxidation reaction of

NADH is that the operating potential is much lower (0.0 V vs. Ag/AgCl) than is possible by direct oxidation at bare carbon electrodes. This aspect is discussed later.

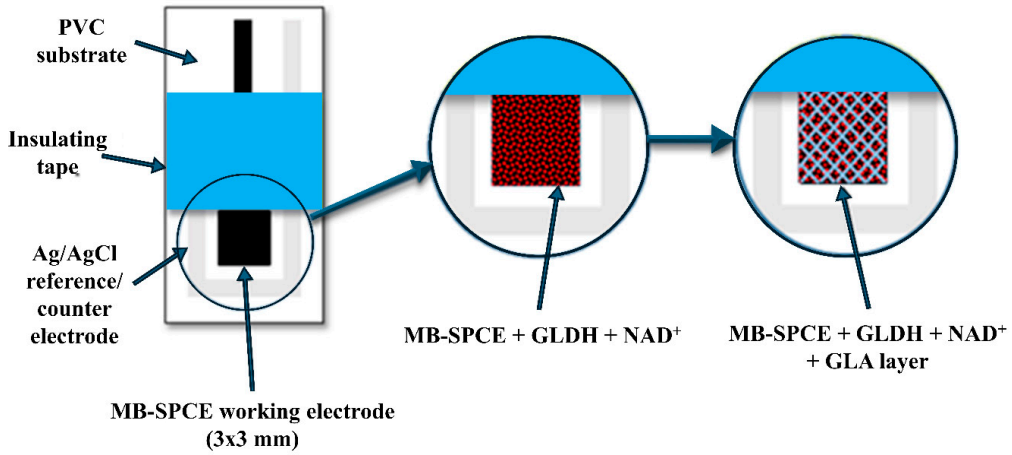

Figure 1. Schematic diagram of the biosensor fabrication involving the deposition of GLDH and NAD^+ followed by the application of the GLA cross-linking layer.

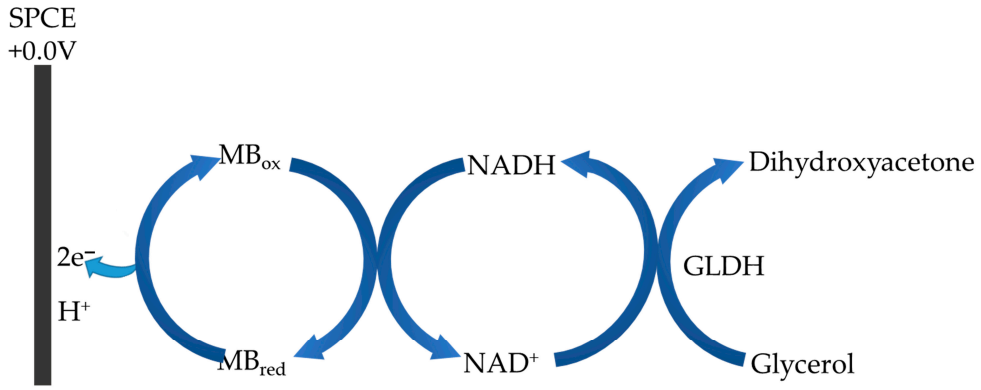

Figure 2. The sequence of reactions involved in the operation of the amperometric glycerol biosensor.

3.2. Surface Characterisation of the Biosensor

High-resolution digital microscopy was used to investigate the surface morphology of the glycerol biosensor during the fabrication process. Figure 3A–C displays three images captured during the process. Figure 3A shows the surface of the initial MB-SPCE, with clearly visible striations due to the screen design used in the screen printing process. Figure 3B depicts the image obtained after the deposition of the GLDH/NAD^+ mixture, showing an evenly dispersed layer over the surface, although the striations are still partially visible. Finally, Figure 3C represents the image obtained after the deposition of a GLA layer over the enzyme/cofactor layer, which had been allowed to dry and crosslink. This cross-linked layer encapsulates the underlying reaction layer, which results in strong immobilisation on the surface of MB-SPCE. This is evident from the latter image, where the upper layer is opaque and no striations are visible.

It was of interest to deduce the extent of cross-linking of glutaraldehyde with GLDH, so we next performed FTIR spectroscopic analysis on the biosensor surface. The FTIR spectrum obtained for glutaraldehyde alone (Figure 4) clearly shows a strong, sharp band at 1725 cm^{-1}, resulting from the C=O stretching mode. However, the spectrum obtained for the biosensor surface (Figure 4) does not exhibit this band, which indicates that a reaction of the C=O with the amino moiety of the enzyme has occurred. Therefore, the FTIR study indicates that complete cross-linking of glutaraldehyde with the enzyme has occurred, resulting in the formation of a Schiff base. Similar observations and conclusions have been reported previously by Lović et al. [20].

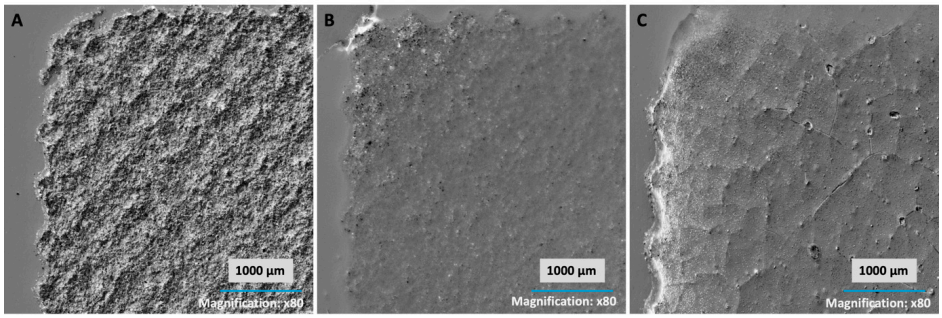

Figure 3. High-resolution (digital) microscopy images at 80× magnification; (**A**) the surface of the MB-SPCE; (**B**) the MB-SPCE with a deposited layer of GLDH/NAD^+; (**C**) the GLDH/NAD^+ layer cross-linked to the MB-SPCE surface with glutaraldehyde.

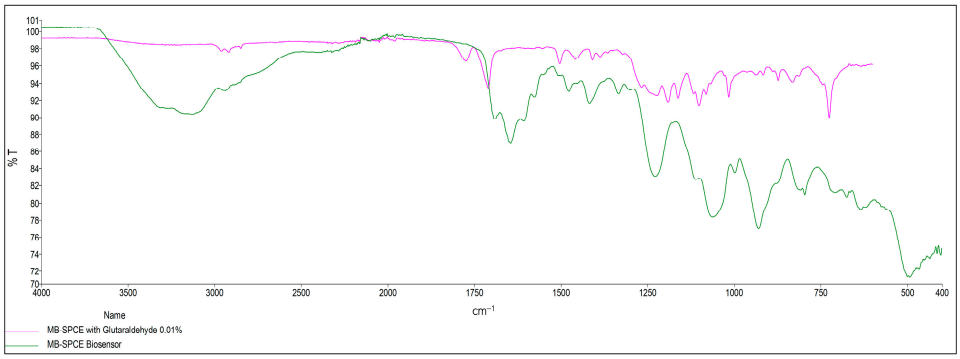

Figure 4. FTIR analysis of the glycerol MB-SPCE biosensor surface (green line) and glutaraldehyde alone (pink line).

3.3. Optimisation of the Biosensor Conditions

3.3.1. Hydrodynamic Voltammetry

Hydrodynamic voltammetric studies were performed with NADH under alkaline buffer conditions (pH 8–10) using the MB-SPCEs only. The resulting voltammograms exhibited a maximum electrocatalytic current at 0.0 V vs. Ag/AgCl (Figure 5). Therefore, this potential was used for all further studies. The electrocatalytic oxidation reaction is illustrated in Figure 2. This shows that 2 electrons per NADH molecule are transferred to the oxidised form of Meldolas Blue (MB_{ox}) to produce the reduced form (MB_{red}), which is subsequently transferred to the SPCE, generating the response current.

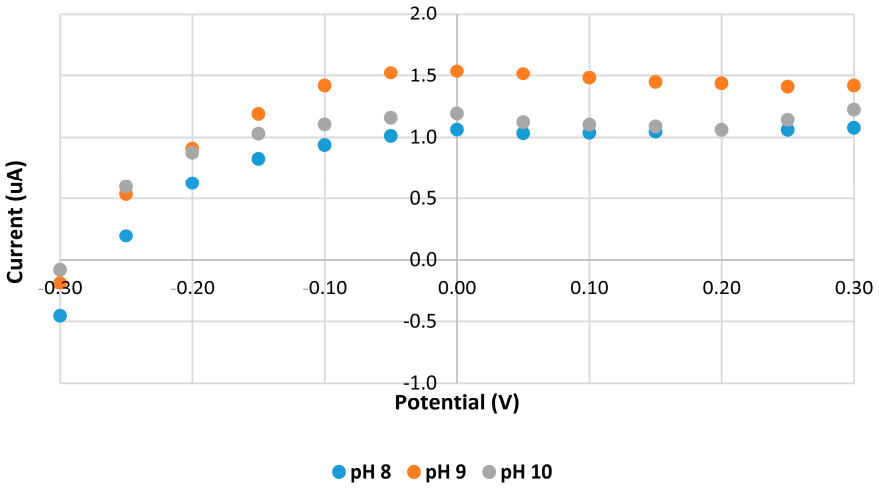

Figure 5. Hydrodynamic voltammograms for NADH were obtained using an MB-SPCE in buffer solutions of pH 8, 9, and 10.

3.3.2. Effect of pH on Amperometric Responses of Glycerol Solutions Containing Free GLDH and NAD^+

Next, we examined the effect of pH (8–10) on the amperometric responses of different glycerol concentrations over a range of 0.04 to 0.20 mM (Figure 6). All the solutions were stirred at a fixed speed and contained 10 units of GLDH and 5 mM NAD^+, and the solution temperature was controlled at 30 °C. Figure 5 shows the current response–concentration profiles obtained with these solutions, which indicates that pH 10 produces a slightly higher current than pH 9, with pH 8 producing the lowest response. It was shown that the response–concentration profiles obtained with these solutions follow Michaelis Menten's behaviour. We also analysed the data using Hanes plots, which confirmed Michaelis Menten's behaviour (Figure S1). Enzymes generally have bell-shaped pH profiles with an optimal pH in the middle, showing increased activity as pH rises initially, followed by decreased activity with further pH increase. The results obtained by Ali & El-Ghonemy [21] indicated an optimum pH for NAD^+ at pH 8–9. From these results, we concluded that pH 9 would be appropriate as the supporting electrolyte for further studies with our amperometric biosensor.

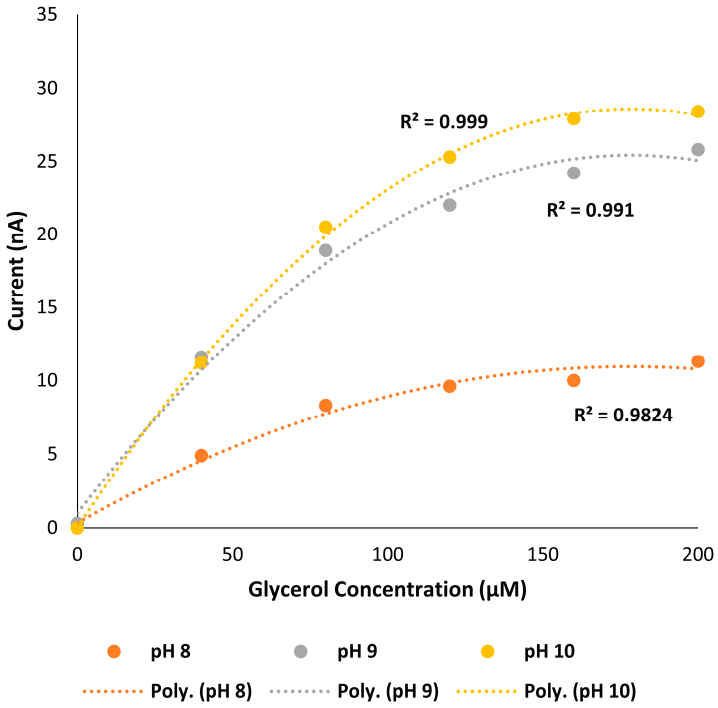

Figure 6. Plot of amperometric responses versus glycerol concentration, obtained with an MB-SPCE in solutions containing glycerol, GLDH (10 U), and NAD^+ (5 mM) at an applied potential of 0.0 V vs. Ag/AgCl.

3.3.3. Calibration Study

A calibration study was performed with the glycerol biosensor in conjunction with chronoamperometry (Figure 7). For this purpose, a 100 µL aliquot of the standard glycerol solutions prepared in 0.1 M PBS pH 9.0 was deposited on top of the working/reference electrodes, and the concentration range of glycerol was 1 to 7.5 mM. Chronoamperometry was performed using an applied potential of 0.0 V vs. Ag/AgCl, preceded by an incubation time of 3 min. Figure 7A,B show the chronoamperomgrams obtained for the standard solutions.

The current was measured at 30 s, 100 s, 200 s, 300 s, and 340 s for each concentration and plotted against the corresponding concentrations. The limits of detection (LOD) were calculated based on the standard error of the regression line (STE_{YX}) of the current response and the slope of the calibration curve (S) according to the formula: $LOD = 3.3 \times (STE_{YX}/S)$.

Table 1 shows the linear regression equations, R^2 values, and the LOD for every current sampling time. Based on this data, we considered that a measurement time of 100 s would provide a reliable response for the analytical application described in the next section.

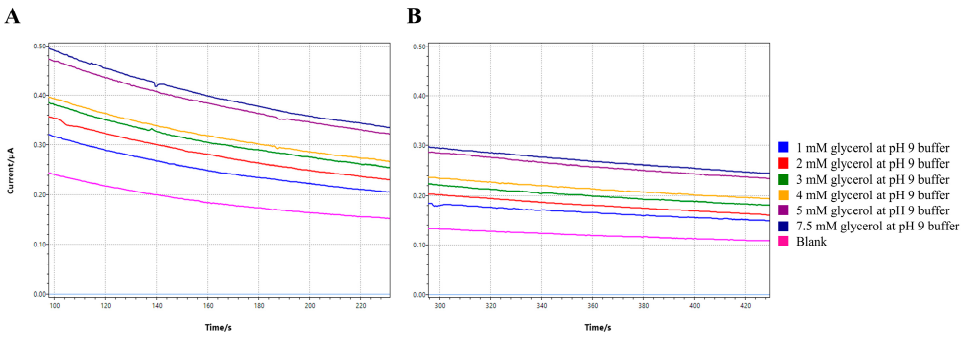

Figure 7. Chronoamperometric responses obtained with the glycerol biosensor for concentrations between 1 and 7.5 mM at (**A**) 100–200 s and (**B**) 300–400 s.

Table 1. The calibration equation, R^2 values, and LOD were obtained using chronoamperometry with the glycerol biosensor at different current sampling times.

Time (s)	Equation	R^2	LOD (mM)
30	y = 0.0317x + 0.4676	0.8916	1.76
100	y = 0.0263x + 0.2643	0.9597	0.87
200	y = 0.0200x + 0.1879	0.9321	1.06
300	y = 0.0181x + 0.143	0.9673	1.25
340	y = 0.0156x + 0.11392	0.9557	1.73

3.4. Analytical Application

The influence of different levels of glycerol on the overall quality of wine continues to be a contentious subject. Previous reports have relied on expensive and time-consuming methods such as phosphorus Nuclear Magnetic Resonance (p-NMR), Gas Chromatography-Mass Spectrometry (GC-MS), and HPLC to detect and quantify glycerol in red wine [22,23].

Table 2 presents the glycerol content recovered from commercial red wine compared to a standard spectrophotometric assay kit for glycerol determination. The mean glycerol concentration detected in red wine (Merlot) using the MB-SPCE glycerol biosensors (11.88 gL^{-1}) was hypothesised not to be different than the glycerol concentration detected using the standard spectrophotometric technique (11.91 gL^{-1}). This hypothesis was confirmed since there was no significant difference between the mean values obtained by the two different methods ($p > 0.05$). The above results demonstrate that our developed MB-SPCE glycerol biosensors can be used as a rapid and efficient analytical method for the determination of glycerol in wines, using only a 100 µL aliquot, which makes this method suitable also for the large-scale control of alcoholic beverages.

It should be mentioned that there have been other reports describing the successful construction of electrochemical biosensors for the measurement of glycerol (Table 3). The fabrication methods vary, involving different combinations of enzymes, transducers, and immobilisation procedures. While these devices have been shown to be reliable in a laboratory setting, they do not have the attributes required for commercialisation, which, amongst other requirements, are the capability of high-speed mass production at low cost to be attractive to sensor manufacturers and end users. Although biosensors constructed with pencil graphite [24], gold [25], carbon paste-packed tubes [26], and graphite rod substrates [27] have demonstrated innovative approaches, the materials and designs do not lend themselves to an economical fabrication approach. In the approach by Mahadevan & Fernando [25] involving grafting FeS to the cofactor NAD^+ with the enzyme GLDH, the sulphur moiety facilitates the attachment of the complex to the surface of a gold electrode.

However, the complexity of the manufacturing procedure would preclude this approach from being used for commercial exploitation.

Table 2. Recovery of glycerol content (in gL^{-1}) from red wine using a glycerol biosensor and Spectrophotometry.

Sample	Recovery (gL^{-1})	
	Biosensor	Spectrophotometry
1	12.58	11.70
2	12.46	11.94
3	11.74	11.94
4	10.06	12.01
5	12.58	11.94
Mean	11.88 [a]	11.91 [a]
SD	0.964	0.097
CV (%)	8.08	0.81

[a] The lowercase letters indicate no significant differences between methods.

Table 3. Brief fabrication details, operating technique, and performance of glycerol biosensors.

Biosensor Fabrication	Technique	LOD	Linear Range	Potential for Commercialisation	References
GLDH/NAD^+ on a meldolas blue-screen-printed carbon electrode	Chronoamperometry Applied potential 0.0 V vs. AgCl/AgCl	0.87 mM	1.0 to 7.5 mM	Yes	Our approach Appl. Sci. 2024
GLDH/NAD^+/Fe-S on a gold electrode	Amperometry in a stirred solution Applied potential +0.6 V vs. Ag/AgCl	0.43 µM	1 µM to 0.1 mM	No	[25]
Alcohol dehydrogenase from Pseudomonas putida immobilised on graphite rod electrode modified with carbon nanotubes and a redox mediator tetrathiafulvalene.	Amperometry in a stirred solution Applied potential +0.05 V vs. SCE	18 µM	0.05 to 10 mM	No	[27]
GLDH/NAD^+/poly(o-phenylenediamine/carbon paste tube electrode	Amperometry in a stirred solution Applied potential 0.0 V vs. Ag/AgCl	0.43 µM	1 µM to 100 mM	No	[26]
Glycerol kinase/glycerol-3-phosphate nanoparticles (NPs) onto graphene oxide nanoparticles (GrONPs) modified pencil graphite (PG) electrode.	Amperometry Applied potential +0.6 V vs. Ag/AgCl	0.002 µM	1µM to 60 mM	No	[24]

4. Conclusions

This paper has shown proof-of-principle studies to demonstrate the feasibility of fabricating a functioning amperometric glycerol biosensor based on an MB-SPCE with immobilised GLDH and NAD^+. This device is convenient to use due to its planar geometry, which permits a microlitre volume of liquid sample or standard solution to be deposited on the biosensor's surface and interrogated using a chronoamperometric procedure. Such chronoamperometric methods can nowadays be performed with small, commercial hand-held potentiostats, which allow samples to be analysed outside of the laboratory.

Our approach utilises the most popular industrial method for fabricating biosensors, screen printing with low-cost carbon materials. It should be noted that the one-shot glucose biosensors for diabetes monitoring are fabricated using screen-printing technology,

and nowadays, these biosensors have the greatest market share. A simple, single-step enzyme deposition method readily converts the resulting planar transducers into biosensors. In conclusion, our developed MB-SPCE glycerol biosensors can be used as a rapid and efficient analytical method for determining glycerol in wines. This makes the current method suitable for commercialisation and applicable for the large-scale control of alcoholic beverages.

In the future, we will investigate approaches to extend the linear range beyond the limits (1–7.5 mM) described in this paper. This should be readily feasible by adjusting the enzyme and co-factor loadings as well as the biosensor operation. This can help form a platform for developing a novel rapid technology for point-of-test evaluation of glycerol and to assess the practical applicability of the MB-SPCE glycerol biosensors in real-world wine production and quality.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app14146118/s1, Figure S1: Hanes plots obtained for glycerol solutions under alkaline conditions, with GLDH and NAD^+ in free solution.

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