Evaluation Of A Novel Disposable Amperometric Glycerol Biosensor Based On A Meldolas Blue-Modified Screen-Printed Carbon Electrode For Juice Beverage Analysis

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ABSTRACT

This study details the design and development of an electrochemical biosensor for measuring glycerol, an important compound in food and beverages, due to its role as a naturally occurring triose sugar and food additive (E 422). Monitoring glycerol concentration is essential for ensuring food quality and safety. The biosensor operates by enzymatically oxidizing glycerol using glycerol dehydrogenase (GLDH) in the presence of oxidized Nicotinamide adenine dinucleotide (NAD^+), resulting in the production of its reduced form (NADH) and dihydroxyacetone. The NADH then undergoes electrocatalytic oxidation at a Meldolas Blue-modified Screen-printed Carbon Electrode (MB-SPCE), with the resulting current serving as the analytical response. This response is directly proportional to the glycerol concentration. Standard addition calibration studies using chronoamperometry were performed with glycerol concentrations between 1.0 to 3.0 mM, utilizing only 100 uL of diluted (0.1 M phosphate buffer) grape juice (GJ) directly on the biosensor surface. Calibration plots were constructed by taking current measurements at 100 s after application of the applied potential; this demonstrated that the glycerol biosensor produced a linear response across the concentration range studied. The amperometric biosensor was successfully applied to the measurement of glycerol in commercially available GJ, representing non-alcoholic beverages. These analyses have the ability to be conducted outside the laboratory using commercially available, portable potentiostats. Overall, this approach shows promise to form a platform for the development of novel rapid technology for point-of-test evaluation of glycerol in the production and quality control of non-alcoholic beverages.

Keywords: *Food safety and quality, amperometric glycerol biosensor, chronoamperometry, screen-printed carbon electrode, Meldolas Blue, grape juice*

INTRODUCTION

It has been reported that the consumption of grape juice (GJ) is increasing worldwide because of its sensory characteristics and nutritional value (Bendaali et al., 2022). It is, therefore, important to be able to ascertain the quality of GJ for commercial purposes. Kupina (1984) suggested that glycerol, a triose sugar, is one of the crucial indicators of GJ quality. Caputi *et al.* (1992) have developed a High-Performance Liquid Chromatographic (HPLC) method for the measurement of glycerol, which involves a strong cation exchange analytical column in conjunction with a guard column, together with a refractive index detector. The method required sample preparation, including a membrane filtration step. The evaluation of the method was performed using 12 GJ samples in a collaborative study, and the authors reported that the procedure had been officially recommended (Caputi et al., 1992). Linget *et al.* (1998) reported an alternative HPLC approach for GJ analysis, which could also reliably measure the glycerol GJ concentration. This method involves a complex HPLC system incorporating an on-line clean-up step using a dialysis procedure, enabling simultaneous measurement of other analytes, including amino acids, sugars and organic acids.

While these chromatographic procedures are very reliable, and effective, they have some important drawbacks. HPLC is a high-cost technique, and the operation and maintenance of

its instruments require highly skilled technical personnel. The overall analysis time can be quite long, owing to the serial nature of the final measurement step with long elution times; this is particularly the case when many analyses are required. Therefore, alternative approaches, which offer more rapid analyses, lower costs, and require simpler instrument operation, are highly desirable. One such approach involves the use of electrochemical biosensors, which are fabricated using screen-printed technology. These devices have been effectively employed to address complex analytical challenges in different fields including agri-food, environmental, and biomedical analyses (Gareth Hughes et al., 2016). Biosensors offer significant advantages such as low cost, particularly as they can be mass-produced using carbon materials and can be fabricated easily in a wide range of planar geometries. The surface of the base screen-printed carbon electrodes is readily tailored with a wide range of enzymes and electrocatalysts to produce biosensors with high selectivity, which is a prerequisite for the analysis of complex matrices (Smart et al., 2020). Consequently, it was considered that this approach provides a platform for developing and applying a glycerol biosensor.

In a previous study (Sprules et al., 1994), we developed a sensor based on a screen-printed carbon electrode modified with the electrocatalyst Meldolas Blue (MB-SPCE) and reduced nicotinamide adenine dinucleotide (NADH). This device has the advantage of operating at 0 V (*vs* Ag/AgCl), which results in highly selective measurements. We showed that this device could be converted to a lactate biosensor by immobilizing the enzyme lactate dehydrogenase onto the surface of the MB-SPCE (Sprules, Hartley, et al., 1996). Additionally, it was found that the MB-SPCE could be readily converted into various biosensors by immobilizing a suitable dehydrogenase enzyme, together with the cofactor of oxidized Nicotinamide adenine dinucleotide (NAD^+), for the analyte of interest. For example, an ethanol biosensor was developed to assess the quality of a beverage by immobilizing alcohol dehydrogenase (Sprules, Hart, et al., 1996). In a separate study, a glutamate biosensor was developed by immobilizing glutamate dehydrogenase onto the MB-SPCE surface and was applied to assess food quality (G. Hughes et al., 2016; Gareth Hughes et al., 2014). Bearing the above discussion in mind, a glycerol biosensor was developed by immobilizing glycerol dehydrogenase and NAD^+ onto an MB-SPCE. The intention was to apply the prototype device to determine glycerol in GJ and deduce its performance in this complex matrix. This paper describes the procedure for the fabrication and operation of a glycerol biosensor; its potential for applications in the area of juice beverage analysis is demonstrated using a commercial GJ product. The described approach could have broad applications in the areas of food quality and safety.

MATERIAL AND METHODS

Raw materials, chemicals, and reagents

Purple commercially available GJ was purchased at Tesco'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). Deionized 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 deionized water and were then mixed to achieve the desired pH and subsequently diluted in the cell to a working concentration of 0.1 M. Sodium chloride was prepared to a concentration of 1.0 M by dissolving the appropriate mass in deionized water and was diluted in the cell, giving a final concentration of 0.1 M.

Instrumentation

An Emstat Blue potentiostat (PalmSens, The Netherlands) was used for the voltammetric and amperometric measurements. The potentiostat was connected to a PC for data acquisition *via* PS Trace Software.

Gwent Electronic Materials Ltd (Pontypool, UK) supplied all disposable MB-SPCEs. The working electrode (containing MB-SPCE) was fabricated using carbon graphite-based ink with Meldolas Blue (C2030519P5), and the reference electrode was fabricated using Ag/AgCl ink (C2130809D5). The working electrode's area (3 × 3 mm) was defined using electrical insulation tape.

pH measurement

The pH of all samples was recorded using a Testo 205 pH meter (Testo Limited, UK). All solutions were stirred using a colour squid (IKA, UK) and warmed in a HAAKE P5 water bath (Thermo Scientific, UK).

Assessment of glycerol concentration

A commercial glycerol assay kit (Megazyme International Ireland, Ireland) was used to estimate the glycerol concentration in the GJ samples. The amount of NADH was detected by measuring absorbance at 340 nm using a UV-vis spectrophotometer (Thermo Scientific, UK) as described in our previous study (Ekonomou et al., 2024). Initially, the absorbance difference (A1-A2) for both blank and sample was determined. Next, the concentration of glycerol was calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{glycerol} \; [g/L]$$

Where:
V = final volume [mL]
MW = molecular weight of glycerol [g/mol]
ε = extinction coefficient of NADH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for glycerol:

$$c = \frac{2.34 \times 92.1}{6300 \times 1.0 \times 0.10} \times \Delta A_{glycerol} \; [g/L]$$

The sample was diluted during preparation and multiplied by the dilution factor, F.

Fabrication of glycerol biosensor

A mixture of 10 uL containing 10 U of GLDH and 660 ug of NAD^+ in a 0.1 M PBS, pH 9 solution was drop-coated on the surface of the MB-SPCE working electrodes. The layer was allowed to dry overnight under a vacuum at –0.6 MPa, at 4°C. Next, a 10 uL aliquot of glutaraldehyde (GLA; 0.01% in phosphate buffer) was placed on top of the enzyme/NAD^+ layer and left overnight under vacuum to allow crosslinking to occur. The fabricated biosensors were then refrigerated until they were ready for calibration and GJ analysis.

Standard addition calibration procedure and analysis of GJ

A standard addition calibration study was performed with solutions prepared in 0.1 M PBS pH 9 spiked with 1, 2, and 3 mM glycerol concentrations. 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 uL of GJ and GJ samples spiked with glycerol were directly deposited onto the glycerol biosensor surface, which was warmed to 30°C on a thermostated surface and current measurements were taken at 100 s. A new biosensor was used for each glycerol concentration measurement.

The analysis of GJ was performed in a similar manner to that described above, including an initial dilution step to adjust the concentration of glycerol to be in the linear range. All GJ dilutions were carried out using 0.1 M PBS, pH 9.

Statistical analysis

All data acquired were expressed as mean ± standard deviation (SD). Data were analyzed using the paired two-sample for means t-test with IBM® SPSS® statistics 26 software for macOS (SPSS Inc.) at a 5% level of significance.

RESULTS AND DISCUSSION

Principal of operation of the amperometric glycerol biosensor

The overall sequence of reactions involved during the operation of the amperometric glycerol biosensor is shown in Fig. 1. The biosensor operates based on the enzymatic oxidation of glycerol by GLDH in the presence of NAD^+. This process leads to the production of NADH and dihydroxyacetone. The NADH then undergoes electrocatalytic oxidation at an MB-SPCE, and the resulting electrocatalytic oxidation current is the analytical response. This response is directly proportional to the glycerol concentration. It's important to note that the advantage of using the electrocatalytic oxidation reaction of NADH is that the operating potential is much lower compared to the direct oxidation at bare carbon electrodes. This aspect will be discussed later.

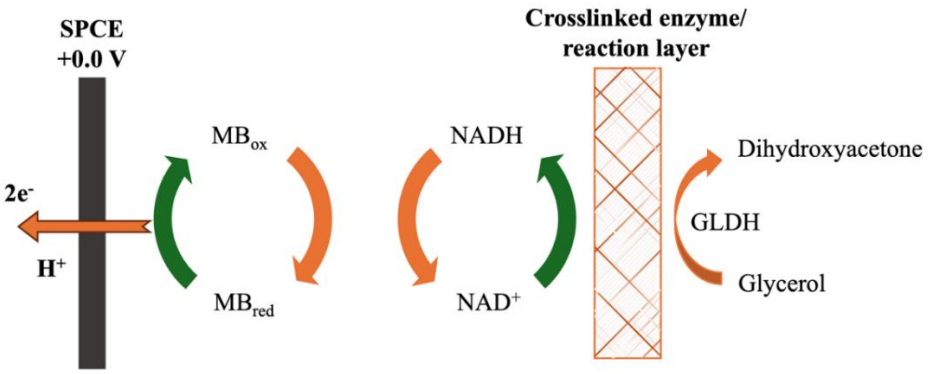

Figure 1. *The sequence of reactions involved in the operation of the amperometric glycerol biosensor.*

Standard addition calibration study and Grape juice analysis

A standard addition calibration study was conducted using the glycerol biosensor along with chronoamperometry (Fig. 2 A-C). To do this, 100 uL aliquots of GJ to which was added glycerol (1 – 3 mM), were deposited on the working/reference electrodes. Chronoamperometry was carried out with an applied potential of 0.0 V *vs* Ag/AgCl, following a 3 min incubation period. Fig. 2 (A) shows the chronoamperograms obtained for the solutions containing only GJ. Fig. 2 (B) shows the chronoamperograms of GJ samples containing additions 1 mM (blue and green lines), 2 mM (orange and purple lines), and 3 mM (haki and red lines) of the appropriate glycerol additions.

The chronoamperometric currents were collected for all GJ samples and standards at 100s. Fig. 2 (C) shows the resulting calibration plot and the regression equation was calculated to be i (uA) = 0.043C (mM) + 0.0017, with an R^2 value equal to 0.9971. Using the standard addition plot above (Fig. 2C), we calculated the unknown GJ concentrations from the Chronoamperometric responses shown in Fig. 2 (A). This data is further discussed below.

In the present work, two methods, the first using a novel glycerol biosensor with amperometric detection and the second using a commercial glycerol assay kit with colorimetric detection, were compared for the determination of glycerol concentration in GJ.

Commercial kits (such as the one used in the current study) have been developed for the determination of glycerol content in GJ. Even though it is a straightforward and convenient enzymatic-colorimetric method, it involves multiple steps, which may lead to spurious data.

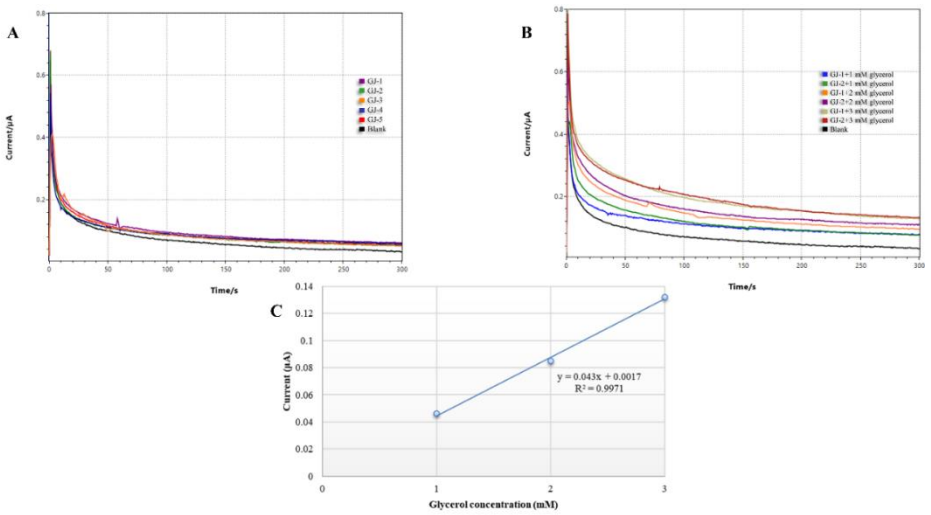

Figure 2. *Chronoamperometric responses obtained with the glycerol biosensor for A) GJ (n=5) and B) GJ spiked with 1, 2, and 3 mM of glycerol at 100 – 300 s. C) Presents the standard addition plot for GJ over the concentration range of glycerol added (1 – 3 mM).*

Next, a comparison was made with our newly developed glycerol biosensor and a commercially available glycerol kit. Table 1 summarises the glycerol recoveries for the individual GJ samples as well as the mean recovery (1.09 gL^{-1}) and precision data with a relative standard deviation (RSD) equal to 11.52% by the biosensor. This Table also compares the results obtained with the glycerol kit, which resulted in a mean recovery of 0.28 gL^{-1} and an RSD equal to 9.59%. Our results, obtained by the biosensor agrees with the results reported by Csutorás *et al.* (2014) in a similar type of GJ product containing 1.21 gL^{-1}. A similar difference in recovery data in GJ has been reported by de Souza *et al.* (2013) when using two different analytical techniques (enzymatic-amperometric and spectrophotometric). de Souza *et al.* (2013) noted that for all the beverage samples they tested, including various wines and sodas, the glycerol content observed was similar for both methods except for the GJ sample, where half the glycerol content was observed when using the enzymatic-spectrophotometric method (de Souza et al., 2013).

Our results reveal that our novel biosensor can successfully detect glycerol concentration in GJ and can be easily and rapidly used in the food industry to test quality. One explanation for the different results obtained with the enzymatic-spectrophotometric kit compared with our biosensor is related to a high lactate concentration (0.025 – 5 gL^{-1}) naturally occurring in the GJ as reported by Coehlo et al. (2018) testing two commercial GJ samples; this only affects the former analytical approach, which has a different enzyme sequence which may be inhibited by lactate in GJ. In previous work performed by our group, it was ascertained that when working at an applied potential of 0.0 V, no significant interferences were detected in various liquid samples (Sprules, Hart, *et al.*, 1996; Hughes *et al.*, 2014; G. Hughes *et al.*, 2016).

Table 1. *Recovery of glycerol content (in gL^{-1}) from GJ using a glycerol biosensor and enzymatic-colorimetric method.*

Sample	**Recovery (gL^{-1})**	
	Biosensor	**Enzymatic-colorimetry**
1	0.91	0.29
2	1.16	0.30
3	1.04	0.30
4	1.04	0.29
5	1.28	0.23
Mean	1.09[a]	0.28[b]
SD (n=5)	0.13	0.03
RSD (%)	11.52	9.59

[a] Different lowercase letters indicate significant differences between methods ($p < 0.05$).

CONCLUSIONS

This paper has demonstrated the possibility of applying a rapid, convenient electrochemical glycerol biosensor for the measurement of glycerol in GJ. It is convenient to use due to its simple geometry, which permits a microlitre volume of liquid sample to be deposited on the biosensor's surface and determined using a chronoamperometric procedure. This offers another advantage for biosensors against spectrophotometric methods, as cloudy and colored solutions can be measured accurately. Such chronoamperometric methods can be performed with small, commercial handheld potentiostats, which allow samples to be analyzed outside of the laboratory. This has the potential to be commercialized and used directly in the food industry setting at the point of production. This approach has the potential to lead to a future platform for developing novel rapid technology for point-of-test evaluation of glycerol in numerous food products and beverages.

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