Rapid Determination of the Glucose Content of Molasses Using a Biosensor

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A knowledge of the sugar content of molasses is of commercial importance to a number of industrial fermentations. Hence the feasibility of using a glucose oxidase biosensor to determine the glucose content of molasses samples was investigated. This method was compared with standard high-performance liquid chromatographic (HPLC) and gas - liquid chromatographic (GLC) procedures and with the use of a commercially available glucose analyser. A good correlation was obtained between the standard acetic anhydride GLC and glucose oxidase biosensor results (correlation coefficient = 0.98). Rapid and accurate measurements could be carried out using the biosensor without the need to employ the sample preparation step required in standard GLC methods. It was concluded that the use of the biosensor technique for the determination of glucose in molasses samples has distinct advantages over conventional methods.

Keywords: *Glucose determination; biosensor; molasses*

The sugars present in molasses (the residue from the crystallisation of sucrose from sugar beet and sugar cane) provide a cheap substrate for a number of industrial fermentations including the production of ethanol, baker's yeast, food and feed yeasts and citric acid. A knowledge of the sugar content of molasses allows the most efficient fermentation feed regime to be determined. Classical procedures for the analysis of molasses are based on the ability of certain sugars to reduce copper(II) sulphate[1] or to rotate a beam of polarised light. Although these methods can be very precise, they suffer interference from other materials present in molasses. The use of commercially available enzyme test kits suffers from interference caused by the opacity and colour of molasses (cane molasses samples in particular show considerable opacity on dilution) as these kits rely on spectrophotometric analysis. Recently, high-performance liquid chromatographic (HPLC)[2] and gas - liquid chromatographic (GLC) methods have been applied to the determination of the sugar composition of molasses, but these methods also suffer from the interference of other sugar and non-sugar components.[3] With GLC, derivatisation of the molasses is also required to render the sugars volatile and stable under the conditions employed and to remove the anomeric forms[4,5]; this has the disadvantage of increased sample preparation time and involves the use of unpleasant reagents.

An amperometric biosensor for glucose, based on glucose oxidase and the mediator ferricinium, has been described previously.[6] This biosensor, which was originally developed for clinical use, has been adapted for use in the determination of the shelf-life of meat,[7] the determination of the glucose concentration in ripening banana fruit,[8] fermentation monitoring of *Escherichia coli* in batch culture[9] and baker's yeast propagation monitoring.[10] A microbial electrode, based on immobilised whole cells of *Pseudomonas fluorescens*, and an oxygen electrode have been developed for the determination of the glucose content of molasses.[11] The Yellow Springs Instrument analyser, which also uses glucose oxidase and is based on the electrochemical detection of hydrogen peroxide,[12] has been used to determine the glucose composition of molasses, "directly and quickly" owing to its "unique enzyme technology which provides measurements that are specific for the sugar being analysed,"[13] but suffers from interferences.[13]

This paper describes an evaluation of the glucose oxidase based biosensor for the determination of glucose in various molasses samples. The results are compared with those given by other methods.

Experimental

Chemicals

All reagents used were of analytical-reagent grade and were purchased from Aldrich (Gillingham, UK), BDH (Atherstone, UK), Strem Chemicals (Newburyport, MA, USA) or Sigma (Poole, UK) unless specified otherwise. The graphite foil (1 mm thick Papyex) was purchased from Le Carbone (Great Britain) (Portslade, UK). Glucose oxidase, Glucox PS grade (E.C. 1.1.3.4, from *Aspergillus niger*, relative molecular mass 186 000), was obtained from Sturge Enzymes (Selby, UK).

High-performance Liquid Chromatographic Analysis of Molasses

The method used was a standard technique for the determination of sugars in molasses and is used by the analytical services section of the Distillers Company (Yeast).

The HPLC analysis of beet and cane molasses was carried out using a Waters liquid chromatography system (Waters Associates, Milford, MA, USA) fitted with an amine-modified silica column (1 m × 8 mm i.d.) with a 10-μm particle size μSilica-Pak cartridge. The solvent used was acetonitrile - water (77 + 23 *m*/*V*) containing silica amine modifier (SAM) reagent 1 (Waters Associates) and was pumped at a flow-rate of 3 ml min^{-1}. The system was operated at 30 °C. The column was calibrated with glucose solutions of known concentration prior to injection of the beet or cane molasses samples (10% *m*/*V*).

Gas - Liquid Chromatographic Analysis of Molasses

Both methods described below are used routinely for the determination of sugars in molasses by the analytical services section of the Distillers Company (Yeast).

Trimethylsilyl derivatisation

GLC of trimethylsilyl derivatives was carried out using a 5711A chromatograph equipped with a 3380A integrator - recorder (Hewlett-Packard, Avondale, PA, USA) and fitted with a glass column packed with 3% OV-17 on Chromosorb W-HP, 80–100 mesh (2 m × 2 mm i.d.). The column temperature was increased from 120 to 320 °C at a rate of 8 °C min^{-1}. The injector and detector temperatures were 250 and 350 °C, respectively. Nitrogen was used as the carrier gas at a flow-rate of 30 ml min^{-1}.

Samples (1 ml) of beet and cane molasses (10% *m/V*) were dried for 72 h in a vacuum oven at low temperature (40 ± 5 °C) in vials containing small silica balls. A 1-ml volume of STOX oxime [Pierce (UK), Chester, UK] internal standard reagent (consisting of 25 mg ml^{-1} of hydroxylamine hydrochloride and 6 mg ml^{-1} of phenyl-D-glucopyranoside in pyridine) was then added to each vial and the vials were closed and placed in an oven at 75 °C for 30 min. The resulting oximes were allowed to cool, hexamethyldisilazane (0.9 ml) was added and the samples were mixed thoroughly. Trifluoroacetic acid (0.1 ml) was then added and the samples were again mixed to ensure dispersion. The resulting silylates were allowed to stand at room temperature until the white precipitate that formed had settled out. The GLC column was calibrated by injecting an aliquot (1 µl) of the derivatised glucose supernatant prior to injection of the same volume of molasses supernatant.

Acetic anhydride derivatisation

GLC analysis of molasses derivatised with acetic anhydride was carried out using a Series 204 chromatograph equipped with an AR 55 linear recorder (Philips Analytical, Cambridge, UK) and fitted with a matched pair of glass columns (1.5 m × 2 mm i.d.) packed with 1% ethylene glycol succinate (EGS) on Chromosorb W-HP. The column temperature was increased from 140 to 190 °C at a rate of 2 °C min^{-1}. The injector and detector were both held at a temperature of 250 °C. Nitrogen was used as the carrier gas at a flow-rate of 40 ml min^{-1} and hydrogen and air were supplied to the FID at flow-rates of 40 and 250 ml min^{-1}, respectively.

Samples (1 ml) of beet (4.5–9.0% *m/V*) and cane molasses (2.5–5.0% *m/V*) were dried overnight in a vacuum oven at low temperature (40 ± 5 °C). A 1-g amount of the internal standard/oxime-forming reagent (consisting of 2.5 g of hydroxylammonium chloride dissolved in 100 g of 0.2% *m/V* xylitol in pyridine) was added to each hydrolysate and the vials were closed and placed in an oven at 90 °C for 30 min. The vials were shaken at intervals to ensure dispersion of the sample. When the solutions were cool, acetic anhydride (2 ml) was added to each vial, the mixture was shaken and the vials were placed in the oven for a further 30 min. The GLC column was calibrated by injecting an aliquot (1 µl) of the derivatised glucose solution prior to injection of the same volume of the derivatised molasses solution.

Yellow Springs Instrument (YSI) Model 23AM Glucose Analyser

Beet (10% *m/V*) and cane molasses (2.5% *m/V*) were analysed using a YSI Model 23AM glucose analyser (Yellow Springs Instrument Co., Yellow Springs, OH, USA) according to the manufacturer's instructions.[13] The molasses samples were prepared in the specified phosphate buffer, the calibration of the analyser was checked before each assay and duplicate measurements were carried out for each sample.

Construction of Glucose Oxidase Biosensor

Immobilisation of glucose oxidase

The immobilisation procedure was based on the method described previously by Brooks *et al.*,[9] which was modified as follows. Graphite discs (4.5 mm in diameter, 1 mm thick) were soaked in hexadecylamine (1 mg ml^{-1} solution in toluene) for 15 min and allowed to dry. The discs were then soaked in dimethylferrocene (20 mg ml^{-1} solution in toluene) for 15 min and again allowed to dry, after which the activated discs were placed in periodate-modified glucose oxidase for 90 min. The enzyme coated discs were placed in adipic dihydrazide (2.5 mg ml^{-1} solution in 0.2 M acetate buffer, pH 5.5) for 15 min to immobilise the enzyme and then soaked in periodate-modified dextran for 15 min. Finally, the discs were placed in adipic dihydrazide solution for 15 min to cross-link the enzyme.

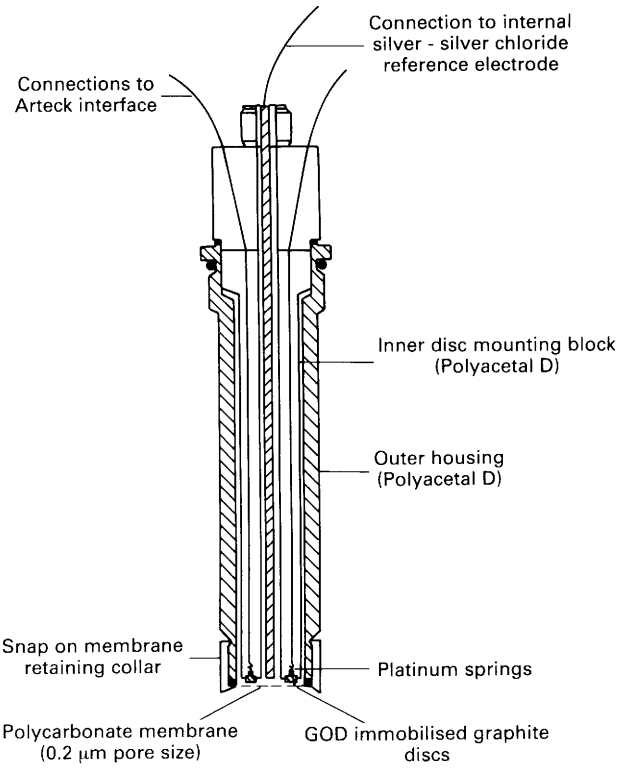

Fig. 1. Schematic diagram of the ferrocene-mediated glucose oxidase based biosensor used for the determination of the glucose content of molasses

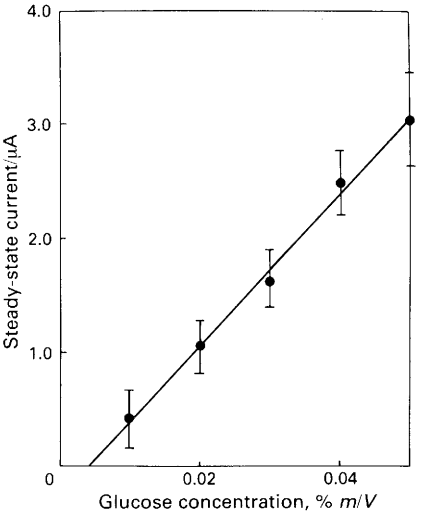

Fig. 2. Calibration of the glucose oxidase biosensor in beet - cane (60 + 40) "spent wash" (pH 7.0), containing potassium chloride (100 mM). Results show linear regression analysis ($n = 6$, $r = 0.99$) and 95% confidence limits

Table 1. Analysis of molasses using a glucose oxidase biosensor, HPLC, GLC and a YSI glucose analyser

Molasses sample	Mean glucose concentration, % *m/V*				
	GLC (AA)*	HPLC†	GLC (TMS)‡	YSI§	GOD biosensor¶
Egyptian beet	0.47	5.70 ± 1.63‖	0.51 ± 0.07‖	0.22	0.23 ± 0.14‖
Ely beet	1.21	1.91 ± 0.73	0.83 ± 0.07	0.63	0.89 ± 0.13
Peterborough beet	0.56	1.50 ± 0.35	0.42 ± 0.06	0.20	0.54 ± 0.07
Polish beet	0.59	1.91 ± 2.14	0.43 ± 0.07	0.21	0.45 ± 0.09
York beet	0.59	2.29 ± 0.76	0.48 ± 0.06	0.31	0.44 ± 0.07
Brazilian cane	6.87	7.14 ± 3.75	8.57 ± 1.63	8.40	5.21 ± 0.55
Indonesian cane	6.84	8.38 ± 0.46	8.71 ± 0.67	7.86	3.05 ± 0.40
Iranian cane	7.48	15.07 ± 1.17	12.29 ± 1.02	15.60	5.44 ± 0.70
Senegalese cane	6.47	9.37 ± 1.87	9.39 ± 0.60	8.97	4.05 ± 0.46
Thailand cane	5.03	5.89 ± 1.34	8.49 ± 0.06	7.46	2.91 ± 0.27

* GLC (AA) = gas - liquid chromatography of acetic anhydride derivatives (n = 2).
† HPLC = high-performance liquid chromatography (n = 12).
‡ GLC (TMS) = gas - liquid chromatography of trimethylsilyl derivatives (n = 12).
§ YSI = glucose determination performed using the Yellow Springs Instrument Model 23AM glucose analyser (n = 2).
¶ GOD biosensor = glucose oxidase immobilised biosensor (n = 12).
‖ Tolerances are standard deviations calculated about the mean.

Table 2. Linear regression analysis of the $\log_{10}$ data obtained using HPLC, GLC of trimethylsilyl derivatives, the YSI glucose analyser and the glucose oxidase (GOD) biosensor carried out against the results of GLC of acetic anhydride derivatives. Linear regression analysis was performed on the $\log_{10}$ of the glucose concentrations obtained for the ten molasses samples given in Table 1

	Gradient	Correlation coefficient (r)
HPLC	1.53	0.82
GLC (TMS)	1.23	0.99
YSI glucose analyser	1.50	0.99
GOD biosensor	0.94	0.98

The enzyme coated discs were stored in 10 mM sodium phosphate buffer (pH 7.0) at 4 °C until required. When in use, the discs were placed flat side uppermost in the recesses of the probe and were secured firmly in place by a polycarbonate membrane (0.2 μm pore size) (Sterilin, Feltham, UK) (Fig. 1).

Operation of glucose oxidase biosensor

The glucose oxidase biosensor had the configuration of a fermenter probe (Fig. 1), based on the design of Brooks *et al.*,[9] modified to allow rapid exchange of the enzyme-coated discs and made of a hard plastic material [Polyacetal D (Nylonic Engineering, Cumbernauld, UK)] normally used in stainless-steel fermentation vessels.

The biosensor was operated at a potential of −220 mV *versus* a silver - silver chloride reference electrode in sodium phosphate buffer (10 mM, pH 7.0) containing potassium chloride (100 mM) using a four-channel programmable biosensor interface (Artek, Lavendon, UK) connected to an Acorn BBC microcomputer (Acorn, Cambridge, UK). Molasses samples (20% *m/V*) were injected into the electrolyte to give a concentration of 0.5–2.0% *m/V*. The current generated was linearly related to the glucose concentration between 0.01 and 0.05% *m/V* [correlation coefficient (r) = 0.99, n = 6] (Fig. 2). The calibration graph (Fig. 2) does not pass through the origin owing to electrochemical inhibition of the probe response caused by unknown components in the "spent wash."

Results

GLC of acetic anhydride derivatives was taken as a standard measure of the glucose concentration in molasses as the technique separated glucose from other monosaccharides known to be present such as galactose, whereas the use of HPLC, GLC of trimethylsilyl derivatives and the YSI glucose analyser suffered interference from other sugars.[4,5,14] Table 1 shows the glucose concentration in real molasses samples as determined by the various techniques. The glucose concentrations fall into two distinct groups corresponding to beet and cane molasses samples, with the former containing significantly less glucose. Correlation coefficients for the various methods, measured against GLC of acetic anhydride derivatives, indicated that the glucose oxidase biosensor gave an accurate measurement; HPLC, the YSI glucose analyser and the glucose oxidase biosensor gave correlation coefficients of 0.82, 0.99 and 0.98, respectively (n = 10). GLC of trimethylsilyl derivatives gave a correlation coefficient of 0.99 when measured against GLC of acetic anhydride derivatives; however, although this technique is more accurate than the glucose oxidase biosensor it is limited by the sample preparation time of approximately 3 d compared with a few minutes for the biosensor.

Table 2 and Fig. 3 show the correlation coefficients and linear relationships of the methods examined measured against GLC of acetic anhydride derivatives, calculated on logarithmically transformed data to compensate for the dependence of variances on the means.

Stability and Reproducibility of Response of the Glucose Oxidase Biosensor

The glucose oxidase biosensor was used to test 30 molasses samples before the immobilised discs were replaced; the results obtained fell within the 95% confidence limits of the original response. The biosensor can be used continually for at least 12 h to within the 95% confidence limits of the original response. The glucose oxidase immobilised discs gave a response that was within the 95% confidence limits of the original response after storage for more than 1 month at 4 °C (for glucose concentrations in the range 0.01–0.05% *m/V*).

Interferences

The complex composition of molasses[3,15,16] makes the source of the interference on the results difficult to define; however, the interference from sugars known to be present in molasses was determined using the YSI and glucose oxidase biosensor systems. It was found that all the sugars tested produced a larger interference on the YSI system; the results are shown in Table 3. A typical molasses sample causes a negative interference of approximately 10.2% (of the response given by a 0.05% *m/V* glucose solution at an equivalent molasses concentration) on the glucose oxidase biosensor owing to electrochemical inhibition; the higher operating potential of

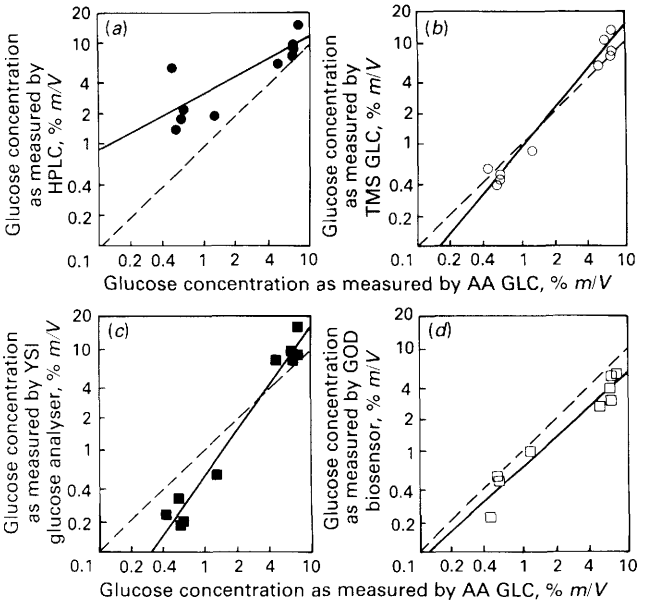

Fig. 3. Comparison of results obtained with (*a*) HPLC; (*b*) GLC of trimethylsilyl (TMS) derivatives; (*c*) the YSI glucose analyser; and (*d*) the glucose oxidase biosensor with those given by GLC of acetic anhydride (AA) derivatives for the determination of glucose in various molasses samples (Table 1). (●) HPLC; (○) TMS GLC; (■) YSI glucose analyser; and (□) glucose oxidase biosensor. (– – – –) Direct proportionality line; and (———) $\log_{10}$ regression analysis line of best fit. Points show the mean glucose concentrations as given in Table 1

the YSI sensor increases the electrochemical inhibition of the signal. Although the analysis of standard sugar profiles by HPLC and GLC shows very good resolution, the analysis of molasses samples was subject to interference from other substances which decreased the accuracy of the analysis and made the interference effects difficult to quantify.

The difference between the results obtained with the glucose oxidase biosensor and with GLC of acetic anhydride derivatives is probably due to the over-all negative interference on the probe response, caused by electrochemical inhibition (the application of correction factors to the results obtained for beet samples to account for the interference due to sugars and electrochemical species eliminates this discrepancy). The much higher operating potential of the YSI sensor compared with the glucose oxidase biosensor probably causes more oxidation or reduction of any species present in the molasses which in turn would increase the electrochemical interference on the signal. The much higher glucose concentrations measured by HPLC and GLC of trimethylsilyl derivatives could be due to the fact that neither of these techniques is able to separate mannose or galactose (possible constituents of molasses) from any glucose present. Further optimisation of HPLC procedures could overcome the problem of resolution.

Discussion

The glucose oxidase biosensor system was found to be as accurate as the complex and time-consuming gas - liquid chromatographic procedures for the determination of the glucose concentration in beet and cane molasses. In terms of the reproducibility of response the biosensor is comparable to GLC procedures and has a higher reproducibility than the HPLC procedure. The over-all additive interference observed with the YSI glucose analyser compared with the biosensor is partially due to the interference of known sugar constituents on the YSI system.

An alternative whole-cell glucose electrode system has been reported to be suitable for the analysis of molasses.[11]

Table 3. Interference effect of some known molasses constituent sugars on the glucose oxidase (GOD) biosensor and the YSI glucose analyser. Results are given as a percentage of the response obtained for glucose at an equivalent concentration, *viz.*, 0.5% *m/V*

	Interference, %	
	GOD biosensor	YSI glucose analyser
L(+)-Arabinose	0	0
D(−)-Fructose	0	0
D(+)-Galactose	3.5	4.5
D(+)-Mannose	8.0	10.3
α-L-Rhamnose	0	0
Sucrose	0.5	3.0
D(+)-Xylose	2.0	2.6

However, the interference from other sugars was reported to be greater than when the immobilised glucose oxidase biosensor was used.[11] In addition, results were given only for high test cane molasses, which is a sugar-enriched molasses containing far fewer metallic and unknown constituents than the traditionally produced beet and cane molasses. High test cane molasses would be expected to have far fewer interference problems.

Conclusions

It has been shown that a ferrocene-mediated glucose oxidase biosensor is not only as accurate as other available analytical techniques, but also it does not require pre-treatment of the sample and does not suffer significantly from interferences from other compounds present in real samples. An additional advantage is that the electrode may be used directly in a flow stream as it has the facility for *in situ* calibration, *i.e.*, if the response to a standard glucose concentration is found to have decreased beyond permissible limits, then the immobilised discs could be replaced rapidly. Hence the glucose oxidase biosensor system could be used for routine quality assurance testing of molasses prior to its use in commercial situations where accurate knowledge of the sugar concentration is of considerable financial importance.

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