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Determination of glucose using a coupled-enzymatic reaction with new fluoride selective optical sensing polymeric film coated in microtiter plate wells

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Abstract

The determination of glucose in beverages is demonstrated using newly developed fluoride selective optical sensing polymeric film that contains aluminum (III) octaethylporphyrin (Al(OEP)) ionophore and the chromoionophore ETH7075 cast at the bottom of wells of a 96-well polypropylene microtiter plate. The method uses a dual enzymatic reaction involving glucose oxidase enzyme (GOD) and horseradish peroxidase (HRP), along with an organofluoro-substrate (4-fluorophenol) as the source of fluoride ions. The concentration of fluoride ions after enzymatic reaction is directly proportional to the glucose level in the sample. The method has a detection limit of 0.8 mmol L^{-1} , a linear range of $0.9\text{--}40 \text{ mmol L}^{-1}$ and a sensitivity of 0.125 absorbance unit/decade of glucose concentration. Glucose levels in several beverage samples determined using the proposed method correlate well with a reference spectrophotometric enzyme method based on detection of hydrogen peroxide using bromopyrogallol red dye (BPR). The new method can also be used to determine H_2O_2 concentrations in the $0.1\text{--}50 \text{ mmol L}^{-1}$ range using a single enzymatic reaction involving H_2O_2 oxidation of 4-fluorophenol catalyzed by HRP. The methodology could potentially be used to detect a wide range of substrates for which selective oxidase enzymes exist (to generate H_2O_2), with the high throughput of simple microtiter plate detection scheme.

Keywords

Fluoride optical sensor; Al(III)-porphyrin; Glucose determination; Glucose oxidase; (GOD); Horseradish peroxidase (HRP)

The catalytic oxidation of para-halogenated aromatic compounds by peroxidase enzymes in the presence of H_2O_2 was first suggested in the early 1950's [1,2], and subsequently studied extensively by a number of groups [3-8]. Among them, Siddiqi introduced a binary enzymatic reaction scheme for glucose measurements based on fluoride ion release from several para-fluoroaromatic compounds using the enzymes glucose oxidase (GOD) and horseradish peroxidase (HRP), and detecting the liberated fluoride via a solid-state potentiometric fluoride sensor [9]. The pathway suggested involves a two-step reaction:

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According to the proposed mechanism, one mole of fluoride ion is released for every mole of substrate or mole of H_2O_2 . Several others used this bi-enzyme system for the determination of organic substrates, hydrogen peroxide, as well as oxidase and peroxidase activities [10-19]. Herein, we describe the adaptation of this chemistry to determine glucose in soft drinks using a new fluoride selective optical sensing film [20] deposited at the bottom of microtiter plate wells as the transduction element.

Gooding et al. introduced a method for the determination of glucose in sport drinks based on the conversion of ferrocyanide into ferricyanide and H_2O by H_2O_2 produced from glucose oxidase [21]. This group also described a glucose enzyme biosensor based on immobilized GOD on a platinum electrode for determination of glucose in beverages [21]. Others described an enzymatic assay of glucose with soluble enzymes using a multi-syringe flow injection system detected by chemiluminescence, with 3-aminophthalhydrazide as the chemiluminogenic reagent to quantitate liberated peroxide [22]. A flow injection analysis method for glucose assay is also introduced based on glucose inhibition of the electrochemiluminescence of $\text{Ru}(\text{bpy})_3^{2+}$ -triprobylamine [23].

In this paper, we introduce a fast and simple method for determining glucose in beverages based on a recently developed highly selective fluoride sensing optical film based on incorporating aluminum octaethylporphyrin (Al[OEP]) ionophore and the pH-sensitive dye, ETH7075, within a thin polymeric film deposited at the bottom of polypropylene microtiter plate wells [24]. The method uses the bienzymatic reaction system introduced by Siddiqi [9], and is capable of detecting glucose levels in a variety of beverages with the high throughput capabilities of microtiter plate absorbance measurements.

1. Materials and methods

1.1 Chemicals

4',5'-Dibromofluorescein octadecyl ester (ETH-7075), high molecular weight poly(vinyl chloride) (PVC), *o*-nitrophenyloctyl ether (*o*-NPOE) and dioctyl sebacate (DOS) plasticizers, tetrahydrofuran (THF), and cyclohexanone were obtained from Fluka (St. Louis, MO). (*R,R*)-*N,N'*-Bis(3,5-di-*tert*-butyl-salicylidene)-1,2-cyclohexane diamino-aluminium(III) chloride ionophore (Al-Sal), sodium fluoride (NaF), disodium hydrogen phosphate, phosphoric acid, *p*-fluorophenol, glycine, glucose, horseradish peroxidase (HRP), glucose oxidase (GOD), and Bromopyrogallol red (BPR) were obtained from Sigma-Aldrich (St. Louis, MO). Chloro-aluminum(III)-octaethylporphyrin (Al[OEP]) was obtained from Frontier Scientific (Logan, UT). Polyurethane (PU) (Tecoflex SG-80A) was obtained from Thermedics Polymer Products (Wilmington, MA). All reagents were prepared using $18\text{-M}\Omega\text{cm}^{-1}$ deionized water produced by a Milli-Q water purification system (Millipore, Bedford, MA).

1.2 Reagents

A glycine-phosphate buffer solution, pH 3.0, was used as the final assay solution for fluoride detection. This buffer was prepared by dissolving L-glycine (6.066 g) in 950 mL water. The solution pH was adjusted to pH 3.0 using concentrated phosphoric acid and the volume was then diluted to the 1 L with water. A 10 mmol L^{-1} phosphate buffer, pH 5.5, was used for the initial enzyme reaction step of the assay. This solution was prepared by dissolving 2.6807 g of disodium hydrogen phosphate in 950 mL water. The solution pH was then adjusted to pH 5.5 using concentrated phosphoric acid, and then the solution volume was brought up to 1 liter with water. A 0.1 mol L^{-1} NaF stock standard solution was by dissolving 0.42 g of NaF in glycine-phosphate buffer, pH 3.0, and complete to the volume.

All glucose standards were prepared in the pH 5.5 phosphate buffer, covering the range of 0.01 mmol L⁻¹ to 70 mmol L⁻¹. The glucose assay reagent was prepared by dissolving 67.26 mg 4-fluorophenol, 2.2 mg HRP, and 13.7 mg GOD in 100 mL phosphate buffer, pH 5.5.

All of the above buffer solutions, standard solutions, and reagent solutions were stored at 4° C.

1.3 Optical membrane composition and preparation

The Al[OEP] and Al[SAL]/ETH7075-based optical fluoride sensing films were prepared according to the method reported by Badr and Meyerhoff [24]. Twenty-five mg of film components (65 w% plasticizer, 25 mmol Al[OEP] or Al[SAL], 100 mole% ETH 7075 (relative to aluminum(III) porphyrin or salophen), 15 mg PVC, and 15 mg PU) were dissolved in 1 mL THF/cyclohexanone solvent (1:1) to prepare a 2.5 wt.% membrane cocktail solution. Ten µL aliquots of this cocktail were delivered into the U-bottomed micro-wells of polypropylene plates (model M4404, Sigma Chemical Co.) by means of a Hamilton microsyringe (Reno, NV). The plates were then fixed on a 600 rpm shaker (Labnet Int. Inc., Berkshire, UK) for 8 h in a dark, well ventilated atmosphere for solvent evaporation and uniform film casting. Measurements of the background absorbance values of the dry films in the plate wells after casting at the wavelength that corresponds to the deprotonated form of the ETH 7075 dye (540 nm) was used to estimate the variation in film thickness. The manual procedure used here to coat the wells was found to be fairly reproducible with background absorbances exhibiting a coefficient of variation typically in the 5-10% range. This variation limits the overall precision that can be achieved in the assays of peroxide and glucose, using the current manual method of film deposition.

1.4 Fluoride and glucose calibrations

Optical fluoride membrane sensors at the bottom of the micro-wells were conditioned prior to use with 200 µL aliquots of 50 mmol L⁻¹ glycine-phosphate buffer, pH 3.0 for 20 min. Two hundred µL of fluoride standard solutions were then delivered to the wells after removing the blank buffer, each in triplicate, and absorbance was measured at 540 and 690 nm after 10 min and the difference value plotted against the logarithm of fluoride concentration.

For glucose measurements, to the preconditioned films in the micro-wells, 196 µL aliquots of the glucose assay reagent (see above) and 4 µL of glucose standards were delivered and incubated at 30° C for 10 min. The pH of the solutions was then lowered to pH 3.0 by adding 50 µL of 2 mol L⁻¹ glycine-phosphate buffer, pH 3.0. The membranes were incubated for another 20 minutes with the liberated fluoride ions to achieve equilibrium. Absorbance values were then measured at 540 and 690 nm and a glucose calibration curve was prepared by plotting absorbance change vs. log glucose concentration.

1.5 Beverage sample analysis

Several beverage samples with low and high glucose content were analyzed for glucose using the proposed optical fluoride sensor based method and a standard spectrophotometric reference method using bromopyrogallol red (BPR) dye to detect the hydrogen peroxide liberated from the GOD/HRP reaction mixture [25]. The low sugar samples were diet 7up, diet Coke, diet Pepsi, low-sugar cranberry-grape juice (Old Orchard), and low-sugar apple juice (Old Orchard). The high sugar samples were regular 7up, Coke, and Pepsi. For the optical film-based method, the microtiter plate films were preconditioned with phosphate buffer, pH 5.5, for 20 min. Then 196 µL aliquots of the glucose assay reagent were delivered to each well, followed by 4 µL of the test samples. The plates were then incubated for 10 min at 30°C followed by 50 µL of 2 mol L⁻¹ glycine-phosphate buffer, pH 3.0 and the absorbance was then measured at 540 and 690 nm

2. Results and discussion

The proposed glucose assay is based on using a mixed enzyme solution containing both GOD and HRP in the presence of the suitable HRP substrate, 4-fluorophenol, and quantitating the liberated fluoride ions using optical fluoride sensing films adhered to the bottom of microtiter plate wells. As shown in Fig. 1, the oxidation of glucose in aqueous solutions is catalyzed by the GOD producing equivalent amount of H_2O_2 [26]. The produced H_2O_2 is immediately consumed in the catalytic oxidation of 4-fluorophenol by HRP yielding an equivalent amount of fluoride ions [27]. The fluoride ions are co-extracted with hydrogen ions into a polymeric membrane containing fluoride ionophore (Al[OEP] or Al[SAL]), L^+ , and lipophilic acidic dye (ETH 7075), C^- , resulted in a decrease in the dye absorbance at 540 nm [20]. It should be noted that the polymer films containing Al[OEP] as ionophore to detect fluoride (and indirectly peroxide and glucose, see below) are fully reversible in their optical response; however, when used in the microtiter plates, the adhesion of the films to the bottom of the wells may not be fully secure (and excessive washing, etc. may cause some delamination) and hence it is simply easier and quite inexpensive to use a new well for every assay.

2.1 Fluoride calibration at different pH's

Initial fluoride ion calibration was carried out at three different pH's: 3.0, 5.5, and 6.5. Solutions with pH 3.0 yield the most sensitive fluoride measurements because at this low pH level, hydroxide anion concentration will be minimum ($\sim 10^{-11} \text{ mol L}^{-1}$). Hydroxide is one of the strongly interfering anions in fluoride measurements using this optical sensing system [24, 28]. However, the pH range of 5.5 – 6.0 is most suitable for enzymatic reactions involving GOD and HRP [9]. Figure 2 indicates that fluoride measurements using Al[OEP]-based fluoride optical sensors at pH 5.5 and 6.0 have poor detection limits ($10^{-3} \text{ mol L}^{-1}$ and $3 \times 10^{-2} \text{ mol L}^{-1}$, respectively), while fluoride calibration at pH 3.0 has a very good detection limit of $5 \times 10^{-6} \text{ mol L}^{-1}$. These results suggest that the final fluoride measurements must be performed at pH 3.0, not pH 5.5 or 6.0.

Table 1 lists the different analytical performance characteristics of the fluoride calibration curve at pH 3.0 using both Al[OEP]- and Al[SAL]-based sensors. It is clear that use of the Al[OEP] as the fluoride selective ionophore yields a more sensitive assay for fluoride using this sensing configuration.

2.2 Effect of H_2O_2 on the performance characteristics of fluoride calibration curve

The effect of hydrogen peroxide, a strong oxidant, on the response of the optical sensing films to fluoride was examined in detail. Fluoride calibrations using the Al[OEP] and Al[SAL] ionophore systems were carried out in the presence of a $50 \text{ mmol L}^{-1} \text{ H}_2\text{O}_2$ standard solution. The response characteristics of the two configurations in the presence and absence of peroxide are reported in Table 2. The presence of H_2O_2 did not have any effect on the detection limit and linear response range of the Al[OEP]-based optical sensor. It had only a minor effect on the other performance characteristics. However, fluoride calibration obtained with the Al[SAL]-based sensor suffered from a very slight increase in the limit of detection (see Table 2), accompanied with a noticeable decrease in the linear response range. Therefore, the Al[OEP]-based optical sensors were selected for all further experiments to determine hydrogen peroxide and glucose.

2.3 Determination of H_2O_2

Prior to glucose determinations, the feasibility of detecting H_2O_2 concentrations in samples was assessed using the proposed fluoride sensing polymer film modified microtiter plates. After soaking the optical films in 50 mmol L^{-1} glycine-phosphate buffer, pH 3.0, for 20 min, the soaking solution was replaced with $196 \mu\text{L}$ of H_2O_2 assay solution followed by $4 \mu\text{L}$ of

H₂O₂ standard solutions in the range 0.1 – 50 mmol L⁻¹. The plates were incubated at 30°C for 20 min and absorbance was measured at 540 and 690 nm. A H₂O₂ calibration curve (Fig. 3) was created by subtracting the background absorbance at 690 nm from the absorbance measured at 540 nm. A detection limit of 0.1 mmol L⁻¹ H₂O₂ (initial concentration in 4 µL of sample), a linear range of 0.1–50 mmol L⁻¹, and a sensitivity of 0.103 absorbance unit/decade was found (see Fig. 3).

2.4 Glucose calibration with external and internal incubation

Calibration curves for glucose were determined using two different approaches: a) one with all the components (glucose assay reagent and glucose standards) pre-incubated outside the fluoride selective microtiter plate wells; and b) with the components incubated inside the same well that contained the Al[OEP]-based polymer film at the bottom. For the external incubation experiment, a calibration curve (Fig. 4) yielded a glucose detection limit of 0.5 mmol L⁻¹ glucose (initial concentration in sample), a linear range of 0.5 – 20 mmol L⁻¹, and a sensitivity of 0.173 absorbance/decade. When the experiment was conducted with internal incubation within the same wells that had the fluoride selective polymeric film, the detection limit increased to 0.8 mmol L⁻¹ glucose, with a narrower linear range of 0.9 – 40 mmol L⁻¹ and sensitivity decreased to 0.125 absorbance/decade. Although differences exist between the values of the external and internal incubation methods, they are not significant in terms of the accuracy and precision observed in determining glucose levels in real samples. Given the simplicity (i.e., all in one well) of the internal incubation method, this approach was selected for the determination of glucose levels in various beverage samples.

2.5 Beverage sample analysis

Eight beverage samples were analyzed and the glucose levels determined are provided in Table 3. On average, the proposed method showed somewhat better precision than the reference BPR-based method in terms of a relative standard deviation of 4.95 % vs. 6.98% for the reference method (n=3 determinations for each of the 8 samples assayed). Results from both methods are in agreement, without significant differences. The two methods assayed samples as colored as Coke and Pepsi without the need for sample dilution. This was possible because of the high dilution of the sample in the final assay mixture (~ 2% of final volume was the sample).

3. Conclusion

The present work introduces a new method for the determination of glucose in beverage samples. The glucose analysis involved a double enzymatic reaction including glucose, GOD, HRP, and 4-fluorophenol. Samples with high glucose levels such as non-diet 7up, Coke, and Pepsi, and samples with low glucose levels, diet 7up, diet Coke, diet Pepsi, low-sugar natural cranberry-grape and apple juices were all analyzed successfully using the proposed fluoride optical sensing film based method. Data compared favorably between the new method and a previous redox dye-based method for detecting the peroxide generated from the dual enzymatic reaction. The method is fast, simple and allows the analysis of a large number of samples, as many as 96 samples, using the 96-well microtiter plates and reader. Further, it is likely the same chemistry and transduction method described herein could be used to devise a new type of glucose strip-test for monitoring glucose levels in undiluted blood. Such a strip would have dry reagents within upper layers (enzymes, fluorophenol, etc.), with a bottom polymeric layer containing the fluoride selective optical sensing film. Measurement of liberated fluoride can provide some added selectivity over redox species that may interfere with the typical optical measurement of enzymatically generated hydrogen peroxide.

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References

1. Hughes GMK, Sanders BS. J Chem SOC 1954:4630.
2. Hughes GMK, Sanders BS. Chem Ind 1954:1265.
3. Yamazaki I, Mason HS, Piette L. J Biol Chem 1960;235:2444. [PubMed: 13846434]
4. Critchlow JE, Dunford HB. J Biol Chem 1972;247:3703. [PubMed: 5033385]
5. Dunford HB, Stillman JS. Coord Chem Rev 1976;19:187.
6. Osman AM, Boeren S, Veeger C, Rietjens IMCM. Chem Biol Int 1997;104:147.
7. Deyhimi F, Salamat-Ahangari R. Talanta 2003;61:493.
8. Fenoll LG, García-Molina F, Gilabert MA, Varón R, García-Ruiz PA, Tudela J, García-Cánovas F, Rodríguez-López JN. Biol Chem 2005;386:351. [PubMed: 15899697]
9. Siddiqi IW. Clin Chem 1982;28:1962. [PubMed: 7127811]
10. Pilosof D, Nieman TA. Anal Chem 1982;54:1698. [PubMed: 7149258]
11. Pilosof D, Malavolti N, Nieman TA. Anal Chim Acta 1985;170:199.
12. Kobos RK, Abbott SD, Levin HW, Kilkson H, Peterson DR, Dickinson JW. Clin Chem 1987;33:153. [PubMed: 3802466]
13. Jameison F, Sanchez RI, Dong L, Leland JK, Yost D, Martin MT. Anal Chem 1996;68:1298.
14. Karyakin AA. Electroanalysis 2001;13:813.
15. Kiba N, Miwa T, Tachibana M, Tani K, Hoizumi H. Anal Chem 2002;74:1269. [PubMed: 11922293]
16. Xu Y, Peng W, Liu X, Li G. Biosens Bioelect 2004;20:533.
17. Vojinović V, Azevedo AM, Martins VCB, Cabral JMS, Gibson TD, Fonseca LP. J Mol Cat B, Enzym 2004;28:129.
18. Takagi K, Nakao M, Ogura Y, Nabeshima T, Kunii A. Clin Chim Acta 1994;226:67. [PubMed: 8070135]
19. Edwards R, Townshend A, Stoddart B. Analyst 1995;120:117.
20. Badr IHA, Meyerhoff ME. J Am Chem Soc 2005;127:5318. [PubMed: 15826159]
21. Gooding JJ, yang W, Situmorang M. J Chem Edu 2001;78:788.
22. Pizà N, Miró M, Estela JM, Cerdà V. Anal Chem 2004;76:773. [PubMed: 14750875]
23. Wang C, Huang H. Anal Chim Acta 2003;498:61.
24. Badr IHA, Meyerhoff ME. Anal Chim Acta 2005;553:169.
25. Guo Z, Li L, Shen H. Anal Chim Acta 1999;379:63.
26. Weibel MK, Bright HJ. J Biol Chem 1971;246:2734. [PubMed: 4324339]
27. Pirzad R, Newman JD, Dowman AA, Cowell DC. Analyst 1994;119:213.
28. Badr IHA, Meyerhoff ME. Anal Chem 2005;77:6719. [PubMed: 16223262]

Figure 1. Bienzymatic pathway, fluoride optical-membrane response mechanism, and a picture of the U-shaped 96-well microtiter plate with the optical membranes.

Figure 2.

Optical film responses to fluoride at different pH's: pH 3 (■), pH 5.5 (▲), and pH 6 (●). Data points represent average $\pm$ s.d. of $n=3$ wells for each concentration tested.

Figure 3. Optical H_2O_2 calibration curve. Data points represent average $\pm$ s.d. for $n=3$ wells containing the same concentration of hydrogen peroxide.

Figure 4. Glucose calibration curves performed with external (■) and internal (▲) sample/enzymes/substrate incubation. Data points represent average $\pm$ s.d. of $n=3$ wells for each concentration tested.

Table 1

Performance characteristics of fluoride optical sensors

Characteristic	Value	
	Al(OEP)-based sensor	Al(SAL)-based sensor
LOD, mol L ⁻¹	5.0×10^{-6}	5.5×10^{-6}
Linear range, mol L ⁻¹	$5.0 \times 10^{-6} - 1.0 \times 10^{-3}$	$5.6 \times 10^{-6} - 5 \times 10^{-4}$
Sensitivity, Abs./decade	0.229	0.152
Correlation coefficient, <i>r</i>	0.9988	0.9929

Table 2

Effect of H_2O_2 (50 mmol L^{-1}) on the performance characteristics of the fluoride optical sensor response towards fluoride.

Characteristic	Value	
	Al[OEP]-based sensor	Al[SAL]-based sensor
LOD, mol L^{-1}	5.0×10^{-6}	5.6×10^{-6}
Linear range, mol L^{-1}	$5.0 \times 10^{-6} - 1.0 \times 10^{-3}$	$5.5 \times 10^{-6} - 1.0 \times 10^{-4}$
Sensitivity, Abs./decade	0.206	0.218
Correlation coefficient, r	0.998	0.997

Table 3

Analysis of beverage samples

Sample	[Glucose], g/l (n = 3)	
	Al[OEP] ⁺ -based F ⁻ sensor	BPR method
Diet 7up	0.137 ± 0.003	0.143 ± 0.005
Diet Coke	0.137 ± 0.002	0.142 ± 0.007
Diet Pepsi	0.139 ± 0.005	0.147 ± 0.008
Regular 7up	8.905 ± 0.843	8.923 ± 0.975
Regular Coke	11.704 ± 1.981	11.771 ± 2.325
Regular Pepsi	13.531 ± 1.152	13.557 ± 1.576
Low-sugar Cranberry-grape juice	3.035 ± 0.341	3.096 ± 0.442
Low-sugar Apple juice	2.951 ± 0.223	2.984 ± 0.383