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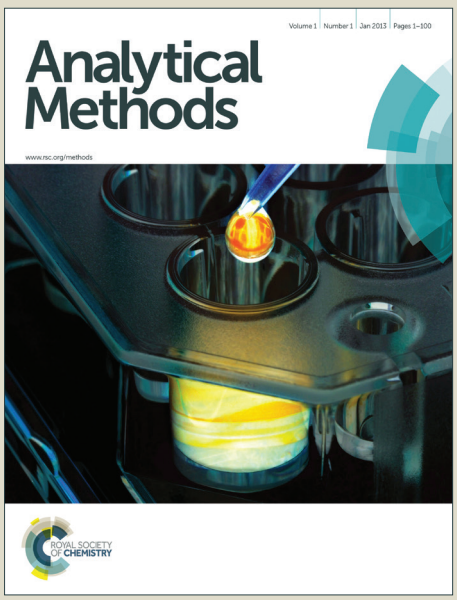

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Precise micromolar-level glucose determination using a glucose test strip for the quick and approximate millimolar-level estimation

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ABSTRACT: Various test strips for quick and rough estimation of analytes are commonly and reasonably available. Although the quickness is one of advantages of test strips, the detection limit could be assumed to be much greater than the actually achievable values because the quickness suggests the possibility of increasing the sensitivity by extending the reaction time. In this study, a test strip product for urinary glucose detection at ordinary homes was used. When the reaction time of 30 seconds indicated in the instruction was extended to be three hours, the sensitivity increased 56-fold. A detection limit of 3.7 μM glucose was achieved while the lowest concentration for a colour shown in the instruction was 2.78 mM. The sensitivity is moderately good among various methods for glucose determination and comparable to widely used high performance liquid chromatography and capillary electrophoresis, and recently emerging non-enzymatic sensors. The application was demonstrated by determining glucose generated by hydrolysis of cellulose in soil-water-cellulose suspensions. The sensitivity-increasing utilization of the convenient, reagentless, safe, cost-effective, and thus highly feasible product by extending the originally-instructed reaction time is reported herein.

KEYWORDS: colour models, Km value, glucose oxidase, optical scanner

1. INTRODUCTION

Test strip is convenient, rapid, reagentless, safe, economical, and thus highly feasible for estimation of analyte concentration. As one of the advantages, the quickness was highly appreciated in rough estimation of concentrations of analytes such as nitrate[1] and phosphate.[2] These test strips show results within some ten seconds. A glucose test strip is a similar product that enables rapid and handy detection/confirmation of urine glucose and rough estimation of glucose concentration within 30 seconds.[3] The extension of the reaction time from 30 seconds could increase the sensitivity beyond the detection limit of 2.78 mM shown in the instruction. In this study, the increase in sensitivity of glucose detection using the commercially available glucose test strip was investigated by extending the reaction time from 30 seconds. The detection limit achieved by the time extension was compared with widely used methods for glucose detection. The method was applied for observation of glucose generation in cellulose-soil-water suspensions incubated for 20 days.

2. MATERIALS AND METHODS

2.1 *Glucose test strip*

In this study, the urine test strip, New Uri-Ace Ga (Terumo, Corporation, Tokyo, Japan) was used. In the manufacturer's instruction, the test strip was described to have been developed to detect glucose in urine for diagnoses of diabetes and other disorders at ordinary homes. On the strip, around the edge, there is a yellow 3 × 4 mm rectangular pad on which glucose oxidase, peroxidase, and *ortho*-tolidine are immobilized.[4]

In the first reaction, glucose oxidase oxidizes glucose to gluconic acid, generating hydrogen peroxide which is subsequently oxidized by peroxidase generating water and oxygen. Then oxygen generated in the previous reaction oxidizes *ortho*-tolidine which becomes a coloured radical cation.[5]

A sample liquid is applied on the yellow rectangular pad. Then, after 30 seconds at room temperature, light green colouration occurs if the sample liquid has glucose at 2.78 mM. The

instruction shows a dark green colour which occurs if the sample has 111 mM glucose as the assumed maximum in the instruction. Thus the intensity of greenness and glucose concentration has a correlation in the range up to 111 mM glucose.

According to the instruction, the reaction period of 30 seconds is important because the green colour development further proceeds as time goes by. This description indicates that lower concentrations of glucose than 2.78 mM may cause visible colour development on the strip if the incubation period is prolonged. To observe colour development on the pad at lower glucose concentrations than 2.78 mM, standard glucose solutions of 0, 0.00556, 0.0556, 0.556, and 5.56 mM were prepared. Ten µL of the solution were applied on the rectangular pad of the test strip. In this study, the test strips were incubated for three hours at 26 °C in the dark. In the incubation, the test strips were put on Parafilm (Pechiney Plastic Packing, Chicago) in a plastic box including a cup of water. The plastic box was covered with plastic film to avoid desiccation of the test strips.

2.2 *Glucose generation from cellulose in soil-water-cellulose suspension*

In this study, glucose generation in soil suspension mixed with cellulose was observed. Three soils were used. A field soil was sampled from a pepper field in Tochigi prefecture, Japan, passed through 2 mm sieve, and kept moist in a plastic bag at room temperature.[6] This soil will be referred as the original soil hereafter. From the original soil, a nutrient-exploited soil was prepared by planting various vegetables on the soil, harvesting the vegetables, putting various plant materials in a plastic box, and incubating for 1 year with occasional mixing and aeration at room temperature in the dark. Hereafter, this soil will be referred as the exploited soil because the soil nutrients were exploited.[6] The third soil was prepared by air-drying the exploited soil. Hereafter, this soil will be referred as the exploited and air-dried soil. As previously described elsewhere, [6] physicochemical characteristics[7] of the original and the exploited soils were determined.

A soil suspension was prepared by suspending 25 g (105 °C dry basis) of soil in 125 mL of water and reciprocal shaking at 120 rpm for 30 minutes at room temperature. Twenty mL of this suspension and two g of cellulose (Nacalai Tesque Inc., Kyoto, Japan) were vigorously mixed to

prepare a soil-water-cellulose suspension. This suspension was incubated at 26 °C in the dark for 20 days by mixing the suspension every day. In the incubation period, 200 μL of the suspension was sampled after 0, 23, 46, 72, 144, 312, and 480 hours of incubation. The sampled suspension was kept at -25 °C for up to 20 days before the suspension was used for glucose determination. After melting the frozen suspension at room temperature, the suspension was centrifuged at 10,000 *g* for 10 minutes. The supernatant was used for glucose determination. Ten μL of the supernatant was applied on the glucose test strip. Standard glucose solutions at 0 to 3.33 mM were also prepared to confirm the reliability of a formula derived from readings of colour development on the rectangular pad. Two replications were used for 0, 1.11, and 3.33 mM standard solutions, and three replications for 0.022, 0.111, and 0.556 mM standard solutions. To confirm significant contribution of colour of the supernatant, changes in colour of filter paper was observed by loading 10 μL of the supernatant on quantitative filter paper No. 390 (Munktell Filter, Falun, Sweden) cut into a 3 × 4 mm rectangle, put on Parafilm.[8] The filter paper was incubated for 3 hours at 26 °C in the dark as in the case of the test strip. As the reference, ten μL of water were applied to the filter paper rectangle to obtain the reference colour.

2.3 *Determination of colour development*

The rectangular pad of the test strip was optically scanned[9] using the Epson ES-2000 optical scanner[10],[11] Each test strip was scanned at 300 dots per inch in the colour mode. The images of the test strips were saved as JPEG files, and spectral profiles of the rectangular pads were obtained by reading the intensity values of red, green, blue, cyan, magenta, yellow, black, and *L** and values of *a** and *b** with Adobe Photoshop™ 7.0 computer software.[12] The values were provided as digital numbers between 0 and 255.[13]

2.4 *Data analysis*

The statistical software SPSS10.0.1 (SPSS Inc.) was used to examine relationships between changes in glucose concentration and those in (the intensity) values of the colour components.

3. RESULTS and DISCUSSION

Incubation of the test strips loaded with 10 μL glucose solutions at up to 5.56 mM for three hours generated light to dark greenish colours on the pad (Fig. 1). The colour for the greatest glucose concentration (5.56 mM) looked to be somewhat bluish. The visual appearance of the incubated pad loaded with the 0.556 mM glucose solution looks to be close to that of the standard colour for 27.8 mM glucose in the manufacturer's instruction. Therefore, the conditions adopted in this study could give 50-fold higher sensitivity than the conditions recommended in the instruction for detecting urinary glucose at ordinary homes.

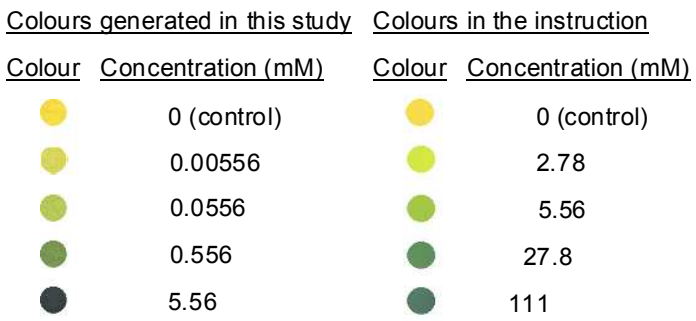

Fig. 1. Colour development of the test strip pad loaded with standard glucose solutions at different concentrations and incubated for three hours at 26 °C in the dark (left) and the relationship between urinary glucose concentration and colour development shown in the manufacturer's instruction (right). For each glucose concentration, 417 pixels were copied from the original JPEG file obtained using the scanner.

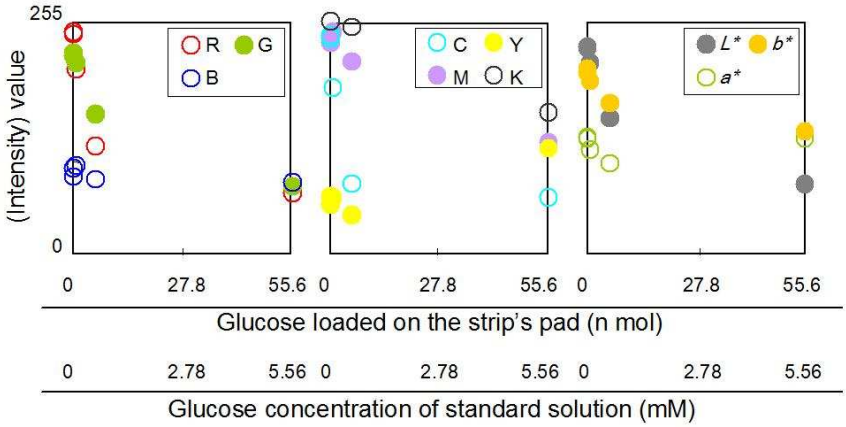

Fig. 2. Changes in (the intensity) values of red-green-blue (RGB), cyan-magenta-yellow-key black (CMYK), and *L*a*b** color components in relation to glucose amount loaded on the strip pad.
The reaction took three hours at 26 °C in the dark.

As great amounts of glucose were loaded, the intensity values of R, G, cyan, magenta, key black, and *L** and value of b* dropped, indicating dark colours appeared on the pad. Fitting hyperbolic curves,[14] the following relationship was found for . R, G, M, K, *L**, and *b** ($R^2 > 0.90$).

Glucose concentration (mM) = [numerator/(the intensity) value of the colour component] + constant [1]

By performing non-linear multiple regression analysis, four colour components (G, M, K, and *L**) were chosen as those most significantly describe glucose concentration.

Glucose concentration (mM) = - 15.7 - 4363/G + 681/M + 4179/K + 3651/*L** ($R^2 = 1.000$) [2]

When equation 2 was used to estimate glucose concentrations represented by the standard colours in the manufacturer's instruction (Fig. 1), they were revealed to represent up to 2.00 mM glucose (Fig. 3). According to Fig. 3, the conditions adopted in this study resulted in 56-fold greater sensitivity than that described in the instruction. In the quantitative use, the minimum concentration which the test strip can indicate would be determined using the following equation:

$$\text{Delta E} = [(L^*_1 - L^*_2)^2 + (a^*_1 - a^*_2)^2 + (b^*_1 - b^*_2)^2]^{0.5} \quad [4]$$

The Delta E is the difference between colours 1 and 2. Humans can discriminate colours that have a Delta E value of around 1.[15] Also, a Delta E value of 1 is the minimum valued that is detectable by the Adobe Photoshop software. Therefore, according to Table 1, 0.0037 mM (0.022 mM/5.89, within 0 to 0.022 mM glucose) to 0.022 mM ([1.11-0.556]/24.9, within 0.556 to 1.11 mM glucose) are the detection limit that human eyes and the current scanner-software combination can discriminate. This sensitivity is comparable to widely used high performance liquid chromatography (e.g., 1.5 μM),[16] capitally electrophoresis (e.g, 7 μM),[17] and one of the most sensitive non-enzymatic nano-composite sensors (4 μM).[18] The current method has greater sensitivity than sophisticated biosensors (some ten μM)[19], [20] while is less sensitive when compared with some gas chromatography (0.42 μM)[21], liquid chromatography-mass spectrometry (0.44 μM)[22], and the most sensitive colorimetiric methods (0.03 μM).[23]

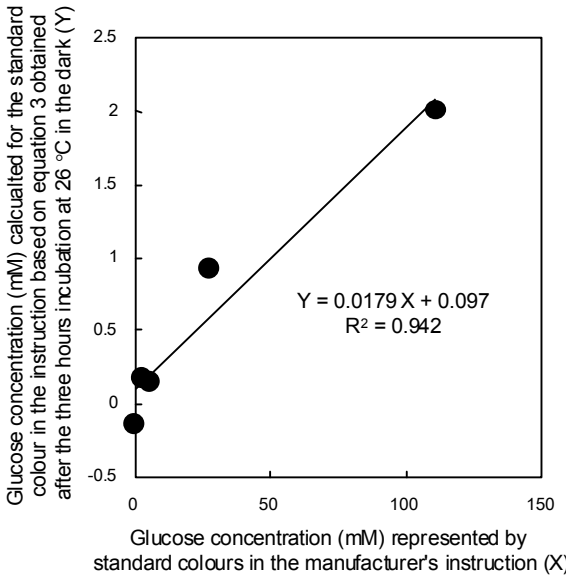

Fig. 3. Relationship between glucose concentration represented by the standard colour in the manufacturer's instruction (horizontal axis) and that calcualted for the standard colour based on equation 3 obtained after the three hours incubation at 26 °C in the dark (vertical axis)

Glucose oxidases are known to have their Km values of up to 100 mM.[24], [25] The Km is the substrate concentration at which a half of the maximum initial velocity of enzymatic reaction is achieved.[26] The glucose concentrations for which the colours are shown in the manufacturer's instruction are close to or smaller than the Km value for glucose oxidases. In the experimental conditions described in Middleton,[4] the reaction mixture contained glucose at 0.139 mM or smaller, glucose oxidase at 3750 units/L, peroxidase at 10 mg/L, and *ortho*-tolidine at 100 mg/L. On the other hand, in this study, the pad absorbed 12.7 ± 1.2 µL (n = 3) liquid sample, and had 53.3 units of glucose oxidase, 0.63 mg of peroxidase, and 24.8 mg of *ortho*-tolidine. When all the chemicals on the pad are assumed to be dissolved in the 12.7 µL liquid, the concentrations of the chemicals are 1119- (glucose oxidase) to 19528-fold (ortho-tolidine) greater than those in the reaction mixture in Middleton[4] while the greatest glucose concentration assumed in the manufacture's instruction (111 mM) is 799-fold greater than the greatest glucose concentration (0.139 mM) tested in Middleton[4] in which a correlation between colour development of the reaction mixture and glucose concentration was observed at the colour convergence stage at 14 minutes

reaction time. The correlation indicates that applying glucose solutions at 0.139 mM and lower may result in simple patterns of changes in (the intensity) values of colour components against glucose concentration as indicated in Fig. 2 in which linearity is suggested for some colour components up to 0.556 mM glucose. In other words, the glucose concentrations (up to 111 mM) assumed in the instruction were quite high. Thus the colour observation exactly 30 seconds after a contact to the sample urine is reasonable for proper diagnoses due to the rapid colour development that continues after 30 seconds. On the other hands, the glucose concentrations applied in the current study were low enough to result in the clearly perceivable differences among the colors/concentrations and equation 2 after the three hours' incubation.

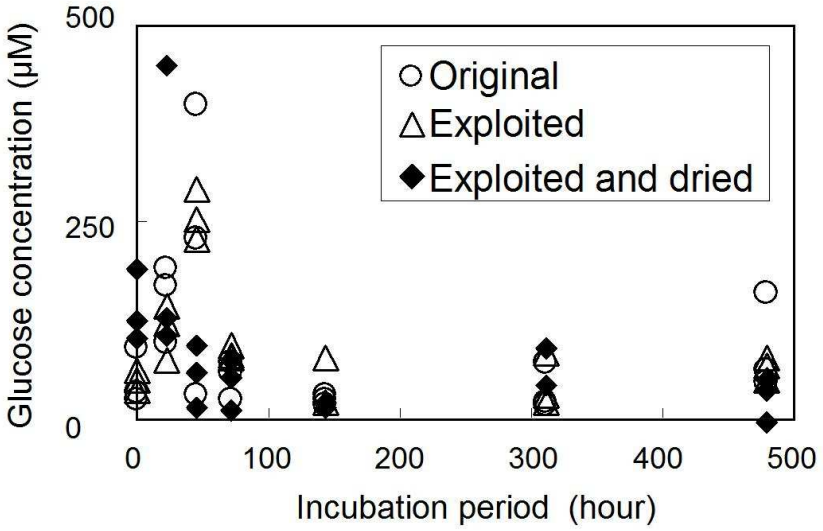

Fig. 4. Changes in glucose concentration in the soil-water-cellulose suspensions prepared from the soils

Glucose was detected in the soil-water-cellulose suspensions. The standard solutions and equation 2 again provided a reliable standard measure (R^2 = 0.997, p < 0.001). Colouration of soil suspension on the filter paper was negligible; 6.28 μM (original soil), -2.57 μM (exploited soil), and 6.33 μM (exploited and air-dried soil) glucose were determined using the filter paper and equation 2. These values were subtracted from raw values. Up to 46 hours, glucose concentrations

increased in the original and the exploited soil suspensions (Fig. 4). Later, the values dropped to close to nil indicating that glucose generated through cellulose hydrolysis was rapidly taken into microbial cells. As for the exploited and dried soil the response to cellulose dose did not last as long as in the case of the other soils. The exploited and dried soil had more free glucose than the other soils as previously reported by Murayama [27] In this study, air-drying took 2 weeks because the air was humid. In the drying processes, fungi, being more tolerant to dryness than bacteria,[28] were likely to have decomposed cellulose originally contained in the exploited soil.[29] On the other hand, in the less aerobic suspension, bacteria could contributed to cellulose decomposition than fungi.[30]

4. CONCLUSIONS

By extending the reaction time from 30 seconds to three hours, the glucose detection sensitivity of the current method increased 56-fold. A detection limit of 3.7 μM glucose was achieved. In these days, various test strips for quick and approximate estimation of analytes are very commonly and reasonably available in the market. Therefore, the extension of reaction time for increasing sensitivity of other test strips, especially those depend on (bio-)chemical reactions, is worth being considered and examined for precise determination of various analytes, taking advantages of the convenience, no waste generation other than the test strips, safety, cost-effectiveness, and thus high feasibility.

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Table 1. Colour differences among the test strips loaded with 10 μL of glucose solutions at different concentrations

Glucose concentration (mM)	Glucose concentration (mM)			
	0.022	0.111	0.556	1.11
0	5.89	17.1	50.0	72.0
0.022		14.2	46.0	67.2
0.111			33.3	56.2
0.556				24.9

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