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Analytical Methods Section

Removal of ascorbic acid interference in the determination of glucose and sucrose in non-alcoholic beverages

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A flow-injection system with an immobilized-enzyme, single-bead string reactor has been used to study the ascorbic acid interference in the determination of glucose and sucrose. Glucose was enzymatically determined in standard solutions and in samples of non-alcoholic beverages. After the measurement of glucose, the samples were spiked with different amounts of ascorbic acid, which was found to introduce serious errors even in low concentrations. A procedure for treating the samples containing ascorbic acid with ascorbase is proposed. The treatment completely removes the interference effect and ensures the recovery of the true concentration of glucose present in the measured samples. The method was applied to determinations of glucose and sucrose in soft drinks and in natural orange juice. The relative standard deviation of the procedure is ±3%.

INTRODUCTION

Ascorbic acid causes a well-known interference effect in analytical reactions involving peroxidase (Saunders *et al.*, 1964). This interference has been particularly well studied in the enzymatic determination of glucose (Okuda & Miwa, 1973; Pileggi & Szustkiewkz, 1974). Here the H_2O_2 produced in the enzyme reaction oxidizes a dye in the presence of peroxidase to form a colored product. The interference has been postulated to result from several possible mechanisms (White-Stevens, 1987). For example, the ascorbic acid could compete with the dye for an intermediate formed by the interaction of peroxidase with H_2O_2. Furthermore, radicals generated by ascorbic acid, such as superoxide ions, could react with the H_2O_2 produced in the primary reaction before it can oxidize the dye. Alternatively, ascorbic acid could cause reversal of the dye reaction to regenerate the colorless reduced form. In any case, the presence of ascorbic acid can lead to large errors in the determination of glucose, or other sugars that produce glucose, in food samples.

In this work the interference of different levels of ascorbic acid (20–100 mM) on the spectrophotometric determination of glucose and sucrose is reported, and a method for the removal of this interference is described. Flow-injection analysis (FIA) (Ruzicka & Hansen, 1988; Valcarcel & Luque de Castro, 1987) was used to determine glucose (Patton & Crouch, 1986; Ang *et al.*, 1987; Toei, 1987) with immobilized glucose oxidase via reactions (I) and (II).

$$H_2O + O_2 + \beta\text{-D-glucose} \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2 \quad \text{(I)}$$

$$H_2O_2 + \text{AAP} + \text{DCPS} \xrightarrow{\text{peroxidase}} \text{red dye} + H_2SO_4 \quad \text{(II)}$$

AAP = 4-aminoantipyrine, DCPS = 3,5-dichloro-2-hydroxyphenylsulfonic acid

The Trinder reaction (II) gives a colored product absorbing at 510 nm and is utilized as an indicator reaction (Trinder, 1969; Gorton & Ogren, 1981; Worsfold, 1983). Glucose oxidase was immobilized on non-porous glass beads, which were then packed in a single-bead string reactor (SBSR) (Reijn *et al.*, 1981; Stults *et al.*, 1987). This SBSR was combined with the flow-injection system shown in Fig. 1. The system was optimized as previously reported (Stults *et al.*, 1987).

The proposed method has been applied to standard solutions of glucose and sucrose as well as to real samples containing added amounts of ascorbic acid. Seven soft drinks and natural orange juice, preserved in the refrigerator for about one week, were used for this study. The proposed treatment is shown to remove the ascorbic acid interference quickly and effectively for the samples tested.

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MATERIALS AND METHODS

Apparatus

The flow-injection apparatus shown in Fig. 1 consisted of a twelve-channel peristaltic pump (Ismatec) with flow-rated pump tubing (Technicon), a pneumatically activated injection valve with a 30 μl sample loop (Rheodyne), and a miniaturized flow-through filter colorimeter that was designed and constructed in this laboratory (Patton & Crouch, 1986). An IBM-compatible microcomputer equipped with an analog I/O board (RTI-815, Analog Devices) controlled the pump speed, sample injection, and data acquisition. Software was written in QUICK BASIC (Microsoft Corp.). Additional details are given in the thesis of Stults (Stults, 1987).

The sample passed first through an SBSR composed of chemically modified 0·6-mm-diameter non-porous glass beads (Proper Mfg, New York, NY) in 0·86-mm-i.d. Teflon tubing (Benton-Dickinson). This SBSR contained glucose oxidase immobilized onto the glass beads (Stults *et al.*, 1987). The sample was then mixed with the peroxidase-reagent solution in a tee-mixer and, after reaction, flowed through a plain SBSR to the detector (colorimeter).

The appropriate choice of pump tubing made the flow rate of the carrier ten times that of the reagent in order to minimize both the consumption of peroxidase and the dilution of the sample. The lengths of the immobilized-enzyme SBSR and of the plain SBSR were 10 cm and 40 cm, respectively. These were chosen so that a minimal amount of glucose oxidase was required, and a measurable absorbance value at the maximum flow rate could be obtained.

Reagents

All chemicals (reagent grade) were used without further purification. The stock solutions were prepared in distilled water and diluted with 0·05M phosphate buffer. Anhydrous α-D(+)glucose (grade III), sucrose (grade II), and L-ascorbic acid (all from Sigma Chemical Co.) were used to compare the standard solutions. The phosphate buffer was prepared by dissolving KH_2PO_4 (3·39 g) and Na_2HPO_4 (3·53 g) in distilled water and diluting to 1 litre.

The reagent for the Trinder reaction contains: peroxidase (0·8 mg ml^{-1}), 4-aminoantipyrine (AAP) (1 mM) and 3,5-dichloro-2-hydroxyphenyl sulfonic acid (DCPS) (1 mM). The enzymes used were: horseradish peroxidase (Sigma, Type II, activity 150–200 units mg^{-1}); glucose oxidase (Sigma, Type II, from *Aspergillus niger*, activity *c.* 17 800 units g^{-1}, working concentration, 5 mg ml^{-1}); invertase (Sigma, grade VII, from Bakers Yeast, activity approximately 400 units mg^{-1}, working concentration, 0·6 mg ml^{-1}); and ascorbase (Sigma, from Cucurbita species, activity approximately 1700 units mg^{-1}, working concentration, 0·3 mg ml^{-1}).

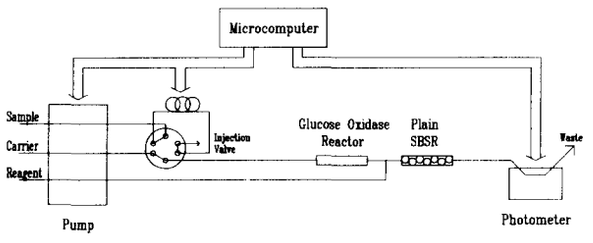

Fig. 1. FIA system for determination of glucose.

Procedures

Study of the interference of ascorbic acid

An appropriate volume of an aqueous stock solution of 0·1110M glucose was diluted with 0·05M phosphate buffer to make a working standard with a glucose concentration of 3·3mM. The standard solution was measured with the flow-injection system, and its absorbance maximum (A_{max}) at 510 nm was recorded.

An appropriate volume of a freshly prepared 4mM ascorbic acid solution was spiked in a second working standard solution of the same glucose concentration to reach the desired concentration of ascorbic acid. Glucose was measured in the spiked solution at different time intervals up to 2 hours, and the A_{max} values were recorded against time.

Treatment of the samples to remove the ascorbic acid interference

Four 250-μl aliquots of soft-drink or standard sugar solution (0·1110M glucose or 0·1170M sucrose) were transferred into four 25-ml volumetric flasks. One aliquot was diluted to volume with 0·05M phosphate buffer, and glucose was measured immediately with the FIA system. The second aliquot was treated with invertase, and, after 2 min, the treated samples were diluted to the mark with the buffer. The A_{max} values were recorded and converted to glucose concentration in order to determine the extent of sucrose inversion. The third aliquot was first treated with invertase and then spiked with ascorbic acid to give a final concentration of 40 μM in the 25-ml flask. Measurements were taken immediately to estimate the ascorbic acid interference. The fourth aliquot was prepared as for the third but was also treated with ascorbase in order to remove the ascorbic acid interference.

All of the drink samples were simply degassed prior to analysis except the natural orange juice, which also required centrifugation at 5000 r/min for 15 min.

Each FIA measurement made at room temperature (22°C) required 2 min to complete. This time is needed for the injection of the sample and the monitoring of the FIA-detector signal with the digital data-acquisition system. The signal was converted to absorbance and stored in computer memory with a program developed in this laboratory.

Six replicate measurements were taken for each sample. The conditions for all measurements were: flow rate 0·5 ml min^{-1}, temperature 22°C, peroxidase concentration 0·8 mg ml^{-1}, AAP concentration 1mM, DCPS concentration 1 mM, pH of the carrier and the reagent 6·86.

RESULTS AND DISCUSSION

The interference of ascorbic acid on the photometrically obtained values of glucose when glucose oxidase and peroxidase are used is dependent on time and the ascorbic acid concentration, as can be seen in Fig. 2. By the first measurement, taken 2 min after the addition of the ascorbic acid, the peak absorbance (A_{max}) obtained for glucose is very low compared with that obtained for a plain glucose solution having the same concentration (1·1 mM). (The horizontal line in Fig. 2 indicates this A_{max}.) This effect is more noticeable at higher concentrations of ascorbic acid, where the lowest A_{max} values for glucose were obtained.

The A_{max} values increase with time and reach their highest level after 70–80 min. The curves show a maximum, which lies higher than the expected value for the glucose concentration in the sample. At longer times, the curves level off at the expected A_{max} value. This maximum in the curves was highly reproducible. At first, it was hypothesized that the maximum was due to dehydroascorbic acid causing a positive interference on the Trinder reaction. However, experiments with dehydroascorbic added to the glucose solutions showed no increase or any other effect on the A_{max} value. The formation of some other intermediate may be responsible for the increased product concentration. The mechanism of this effect is currently under study.

Because of the relatively high concentration (*c.* 6%) of citric acid in some of the samples, its possible interference was also studied. Experiments similar to those made with ascorbic acid showed no interference of any kind due to citric acid.

Removal of the ascorbic acid interference

As shown in Fig. 2, the ascorbic acid interference on the glucose sample is completely removed after approximately 2 h at pH 6·86. This is undoubtedly due to destruction of the ascorbic acid in the sample. This destruction can be completed within 30 min by diluting the sample containing ascorbic acid with a more

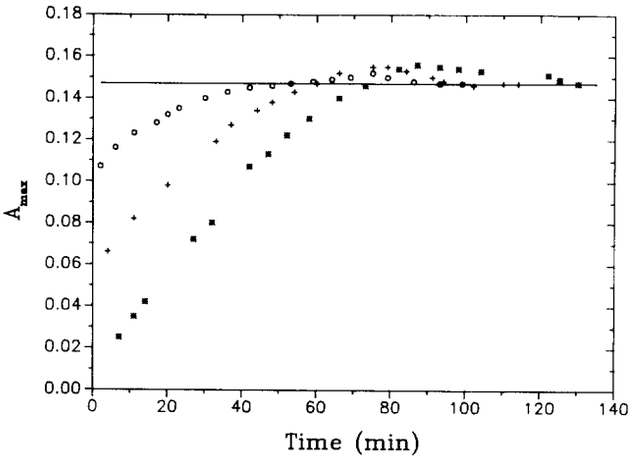

Fig. 2. Interference of ascorbic acid on glucose determinations. Ascorbic acid concentrations: *100 μm, +60 μm, ○ 20 μm.

alkaline buffer (pH ≈ 10). However, this chemical treatment for the removal of the ascorbic acid interference causes lower measurement precision and is not recommended. The instability noted is probably due to deactivation of the immobilized-enzyme reactor in the basic buffer solution.

The enzymatic treatment of the samples by the addition of ascorbase completely removes the ascorbic acid interference within 2 min. The effect on the A_{max} of glucose that remains after the standards and samples have been treated with invertase, ascorbic acid, and ascorbase is shown in Fig. 3. It can be seen that the addition of invertase had no effect on glucose standards and on the six soft-drink samples because of the absence of sucrose. The sucrose standard was completely inverted, as expected. Natural orange juice seems to contain a considerable amount of sucrose. The addition of ascorbic acid had a similar effect on the standards and samples, decreasing the values of A_{max}. Ascorbase increased the A_{max} values to the initial levels in the standards in all but two samples. In ginger ale and Sprite, the A_{max} values increased after the addition of ascorbase but did not reach the levels found by measuring the standards and samples diluted 1 : 100 with

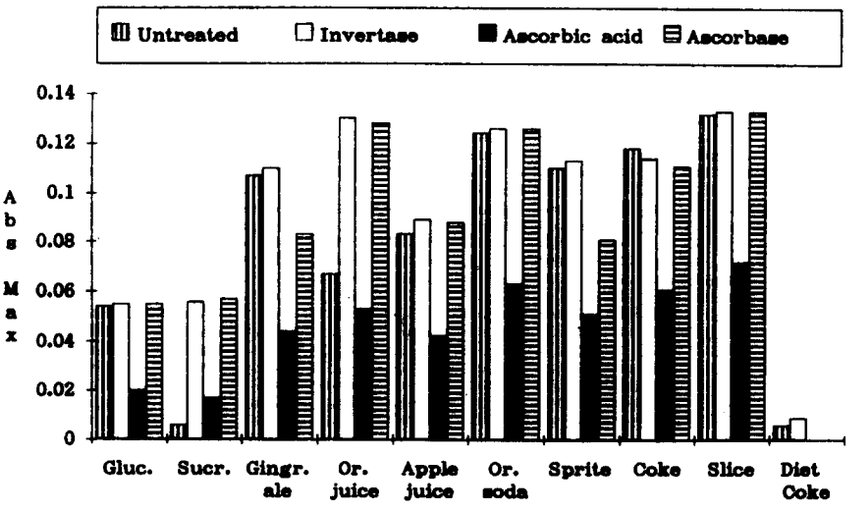

Fig. 3. Determination of glucose in various samples.

Table 1. Results for glucose and sucrose in beverages

Sample	Percent glucose found*				Percent sucrose found
	Untreated	Invertase	Ascorbic acid	Ascorbase	
Standard glucose	2·00	2·03	0·72	2·02	0·05
Standard sucrose	0·12	2·13	0·57	2·09	4·00
Ginger ale	3·92	4·13	1·62	3·08	0·39
Natural orange juice	2·47	4·93	1·98	4·79	4·60
Apple juice	3·01	3·31	1·53	3·26	0·56
Orange soda	4·59	4·68	2·29	4·69	0·15
Sprite	4·03	4·24	1·88	3·00	0·39
Coca Cola	4·34	4·23	2·25	4·13	−0·18
Slice	4·82	4·90	2·67	4·95	0·13
Diet Coke	0·14	0·25			0·11

* The relative standard deviation for six measurements was 2–3%, depending on the sample.

phosphate buffer (0·05M). The reasons for this discrepancy are as yet unknown, but the A_{max} values reached are still >70% of those expected.

Table 1 shows the percentages obtained for glucose and sucrose by this method for the untreated and treated standards and the samples treated with invertase, ascorbic acid, and ascorbase. Glucose was found in all samples except Diet Coke. Only the natural orange juice was found to contain sucrose in significant amounts.

In order to examine the matrix effect in the samples, a standard addition method for glucose was applied. The recoveries were in the range of 99·1–102·7%. The standard deviation of six measurements for the 1·1mM standard solution of glucose was found to be $s = \pm 0{\cdot}03$ or 3% rsd.

This method is fast, simple, and effective; it is therefore recommended for the quick removal of the ascorbic acid interference during enzymatic determination of glucose and sucrose in non-alcoholic beverages.

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