AN ULTRASENSITIVE CHEMICAL TEST FOR QUANTITATIVE CHROMATOGRAPHY OF SUGARS*

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INTRODUCTION

Various sugars often occur in widely differing amounts in biological materials. In order to obtain enough of a minor sugar to measure accurately, it may be necessary to overload the chromatogram with the major sugars, especially when the sugars are separated by thin-layer chromatography. Overloading results in poor separation. The need for overloading can be minimized by using a more sensitive sugar test.

Two general methods are used for quantitation of non-radioactive sugars separated by paper and thin-layer chromatography. By the first method, sugars are sprayed with a suitable reagent, while still on the chromatogram, and the quantity of sugar present is then estimated by size of the spot, absorbance, reflectance, or by eluting the colored material and determining its absorbance in a photometer. By the second method, marker strips are sprayed and then used to locate sugars on the unsprayed portion of the chromatogram[1,2]. The unsprayed areas which contain the sugars are removed and the sugars are eluted and estimated by appropriate chemical tests. The second method is probably more precise because color development is more easily controlled in a test tube than on a chromatogram.

A large number of sugar tests are available. Most of them depend either upon the reducing properties of sugars or upon reaction with a strong acid and a suitable color developer. The anthrone test of DREYWOOD[3] has been used for quantitation of sugars separated by paper[1,4] and thin-layer[2] chromatography. A similar test using phenol[5] was developed for quantitative paper chromatography. Both of these tests require the use of concentrated sulfuric acid. Therefore, it is necessary to remove all traces of cellulose from the sugar extracts, because sulfuric acid would hydrolyze cellulose and the glucose liberated would result in erroneously high readings. The anthrone test[3] and the phenol–sulfuric acid test[5] are both positive for cellulose. Filtration through glass wool[5] and washed filter papers[1] have been used for removing cellulose. Filtration can introduce errors through dilution if the filter is wet, or through cross contamination if the same filter is used for more than one sample. The smaller the sample volume, the larger these errors are likely to be.

Ideally, the sugar test used for quantitation should be sufficiently sensitive to

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measure very small amounts of sugars, but it should be insensitive to the presence of cellulose. WHISTLER AND HICKSON[6] tested four methods for quantitation of sugars separated by paper chromatography, and then mentioned that they preferred the ferricyanide test[7] over the four other methods because the results were more reproducible and because cellulose did not react. The ferricyanide test has been modified a number of times since its introduction by HAGEDORN AND JENSEN[7] in 1923. The modification of PARK AND JOHNSON[8] is the most sensitive. Although the ferricyanide method was developed for glucose, there is no reason to suppose that other reducing sugars would not give a positive test.

This paper reports the results of applying the ferricyanide submicromethod[8] to 17 sugars in addition to glucose, and also reports conditions which permit acid hydrolysis of sucrose in the presence of cellulose fibers without subsequent interference by cellulose in the ferricyanide sugar test.

MATERIALS AND METHODS

Acetaldehyde, arabinose, 2-deoxy-D-glucose, 2-deoxy-D-ribose, dihydroxyacetone, erythrose, fructose, fructose-1,6-diphosphate, galactose, glucose, glucose-1-phosphate, glucose-6-phosphate, glyceraldehyde, hydrolyzed lactose, hydrolyzed sucrose, inositol, lactose, mannose, ribose, sorbose, sucrose, and xylose were tested in the ferricyanide submicromethod of PARK AND JOHNSON[8]. Duplicate 1-ml samples, containing 0.03 μmoles, were used. In addition, standard curves were prepared for 10 sugars over the range from 0 to 0.06 μmoles per ml.

One ml of sample was mixed with 1 ml of ferricyanide (0.5 g of potassium ferricyanide per liter of water stored in a brown bottle) and 1 ml of carbonate–cyanide (5.3 g of sodium carbonate and 0.65 g of potassium cyanide per liter of water). The tubes were capped with clean marbles and heated 15 min in boiling water. The contents were then cooled by placing the tubes in tap water for 5 min. Five ml of ferric iron solution (1.5 g of ferric ammonium sulfate plus 1 g of sodium lauryl sulfate per liter of 0.05 N sulfuric acid) were added to each tube and the contents mixed. With reducing sugars, a blue color developed. Absorbancies were determined at 680 mμ in a spectrophotometer, using 1-cm square cells, 15 min after ferric iron was added.

Lactose and sucrose were tested both before and after hydrolysis. Solutions which contained 0.3 μmole per ml were hydrolyzed by mixing 1 ml of sugar solution with 1 ml of N sulfuric acid and heating in boiling water for 30 min in capped test tubes. The solutions were neutralized with 1 ml of N sodium hydroxide and the volume made to 10 ml with water. These hydrolyzed samples were then carried through the ferricyanide test.

A test was conducted to determine if cellulose would be partially hydrolyzed by the above acid hydrolysis of lactose and sucrose. A 5.5-cm disk of Whatman No. 1 filter paper was vigorously agitated in 10 ml of water until many fibers were in suspension. Three ml of this suspension were mixed with 3 ml of N sulfuric acid, heated in boiling water for 30 min, and then neutralized by adding 3 ml of N sodium hydroxide. One-ml portions were subjected to the ferricyanide test, along with 1-ml samples of fiber suspension which was not heated with acid. This test was repeated twice. Another test was conducted in which a whole disk of filter paper was subjected to acid hydrolysis.

Absorbancies of all samples were determined at 680 mμ against a reagent blank, which remained pale yellow.

RESULTS AND DISCUSSION

Although sensitivity differed, all free reducing sugars tested gave a positive test (Table I). In general, the hexose sugars gave higher readings on a molar basis than the lower molecular weight sugars. Sucrose and glucose-1-phosphate failed to react, probably because the conditions of the test did not cause hydrolysis to yield a reducing group. The presence of phosphate groups in other positions decreased sensitivity, but did not prevent a positive test. Glucose-6-phosphate and fructose-1,6-diphosphate gave lower readings than free glucose and fructose.

TABLE I

A COMPARISON OF SENSITIVITIES OF THE MODIFIED FERRICYANIDE SUGAR TEST WITH 0.03 μMOLES OF ACETALDEHYDE, INOSITOL, AND 18 SUGARS

Free sugars	*O.D. at 680 mμ**	*Phosphorylated sugars, acetaldehyde, and inositol*	*O.D. at 680 mμ**
Hydrolyzed sucrose	0.832	Glucose-6-phosphate	0.180
Hydrolyzed lactose	0.634	Fructose-1,6-diphosphate	0.051
Lactose	0.555	Glucose-1-phosphate	0
Fructose	0.442	Acetaldehyde	0
Glucose	0.403	Inositol	0
Sorbose	0.394		
Mannose	0.379		
Xylose	0.353		
Galactose	0.329		
Arabinose	0.310		
Dihydroxyacetone	0.288		
Ribose	0.271		
Glyceraldehyde	0.258		
Erythrose	0.155		
2-Deoxy-D-ribose	0.118		
2-Deoxy-D-glucose	0.091		
Sucrose	0		

* The data are averages of duplicate determinations.

The results suggest that the presence of a hydroxy group is necessary for a positive test; glyceraldehyde gave a positive test but acetaldehyde did not. Further, the fact that deoxyribose and deoxyglucose gave lower readings than ribose and glucose suggests that the hydroxy group should be on a carbon atom adjacent to the carbonyl carbon for maximum color development.

The ferricyanide test followed Beer's law reasonably well over the range tested (Fig. 1).

Another advantage of the ferricyanide test is that cellulose does not react; even sucrose did not react unless it was hydrolyzed prior to the test. This freedom from interference by contaminating cellulose is an important advantage because cellulose appears to be the preferred adsorbent for thin-layer[9,10,11] as well as paper chromatography of sugars. The conditions used to hydrolyze sucrose and lactose apparently

caused no significant hydrolysis of cellulose fibers except when the entire disk was heated with acid. When a suspension of fibers was heated in 0.5N sulfuric acid, neutralized with sodium hydroxide, and subjected to the ferricyanide test, no blue color developed, and absorbance at 680 mμ was no higher than that of the reagent blank. However, when the entire disk was heated with acid, neutralized, and 1 ml of the extract subjected to the ferricyanide test, an intense blue color did develop Therefore, sucrose should be extracted from the paper before it is hydrolyzed.

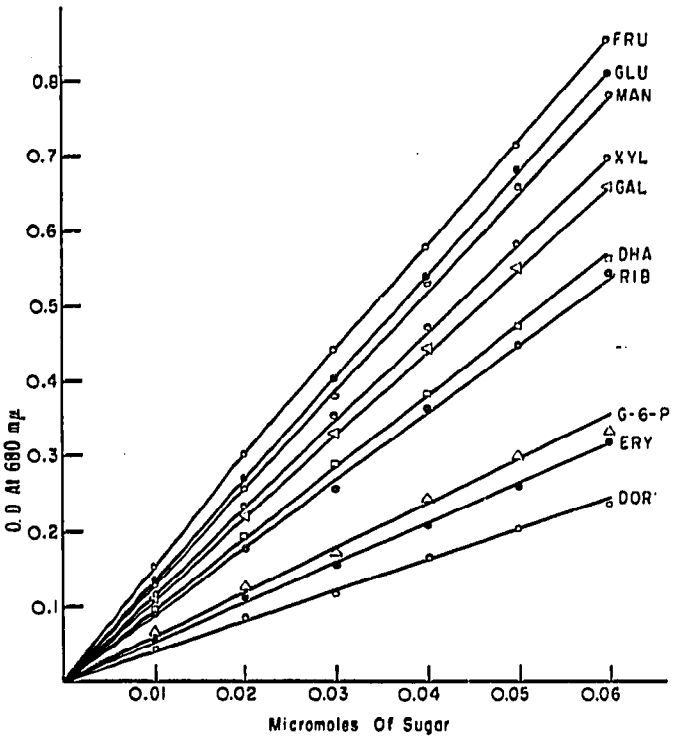

Fig. 1. Standard curves for fructose, glucose, mannose, xylose, galactose, dihydroxyacetone, ribose, glucose-6-phosphate, erythrose, and 2-deoxy-D-ribose by the modified ferricyanide sugar test. All points are averages of duplicate determinations.

A third advantage of the ferricyanide submicromethod is its extreme sensitivity; as little as 0.01 μmole is enough for a reasonably accurate determination of most free reducing sugars (Fig. 1). This high sensitivity permits quantitation of minor components without overloading the chromatogram. It also permits elution of major components in test tubes rather than requiring that a small amount of solvent flow through the adsorbent.

SUMMARY

The ferricyanide test, which was originally developed for the determination of blood sugar, was applied to 17 other sugars in addition to glucose. Although sensitivity differed, all free reducing sugars gave a positive test. Substances without a free reducing group (inositol, glucose-1-phosphate, and sucrose) did not react. Acetaldehyde also failed to give a positive test. Deoxy sugars and phosphorylated sugars gave less color than free sugars with a full complement of hydroxy groups.

Standard curves, prepared for 10 sugars, indicated that Beer's law is followed reasonably well.

The presence of cellulose fibers in suspension did not interfere with the ferricyanide test. It was also possible to hydrolyze sucrose in 0.5N sulfuric acid without significant hydrolysis of cellulose fibers washed from filter paper.

Adherence to Beer's law, freedom from interference by cellulose, and extreme sensitivity (0.01 μmole of sugar) are advantages which should make the modified ferricyanide test well suited for quantitative chromatography of many sugars.

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