0273-2289/95/5303—0215$06.60

A Colorimetric Fructose Assay

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Received December 7, 1993; Accepted August 23, 1994

ABSTRACT

A new spectrophotometric method for measuring fructose is presented. The method uses Tryptamine in HCl acid, is carried out at 60°C, and is complete within 60 min. The assay is read at 518 nm and shows very low interference from other sugars. The method can be used for fructose, fructosans, and inulin.

Index Entries: Fructose; fructosan; inulin; tryptamine; colorimetric assay; sugar analysis.

INTRODUCTION

Fructose is a ketohexose monomer sugar with the same molecular weight as glucose. The sugar is found in plant materials in the free form, in sucrose, in storage carbohydrate fructosans, and inulin. In many plant materials, the fructose sugars can make up a large portion of the readily available carbohydrate fraction. In grasses, for example, many fructans are in oligomers of 2–6 U linked in a β chain terminating in a sucrose unit. The longest fructans are in the stem base tissues. In Brome grass, the degree of polymerization is typically 26, whereas in Timothy grass, this may rise to 260 U. These fructans are usually soluble in cold water and are very acid-labile. In temperature grasses, fructans are the most abundant of the water-soluble carbohydrates, and values as high as 12% of dry matter have been reported for Perennial Ryegrass. Fructose sugars are concentrated in stem tissues, and although the leaf matter of Ryegrass does not exceed 4% of the dry matter, the stems are often over 15%. Fructan quantities increase with plant age and decrease with wilting (*1*).

Colorimetric fructose assays have been reported previously (2,3), but these are complex and require many hours to perform. A simpler method was sought to quantitate fructose. Previous work in this laboratory has indicated that fructose is more susceptible to modification by mineral acids than the aldohexase and pentose sugars. This characteristic made it a source of interference in other colorimetric assays for sugars currently under development. It was thought that this quality could be exploited to assay for fructose itself.

The combination of tryptophan or tryptamine with a sugar in the presence of strong acid gives a chromagen with a red to purple color. This reaction was used to develop the assay.

MATERIALS AND METHODS

Chemicals Used

Concentrated HCl and soluble starch were from Fisher Scientific. Avicel PH-101 was from Fluka, and all other chemicals were from Sigma.

Reagents

1. Concentrated HCl.
2. Tryptamine reagent: Tryptamine hydrochloride [3-(2-Aminoethyl)indole HCl], 10 m*M* in 0.1*M* HCl.

Standard Method

From 0 to 50 μg of fructose in 100 μL of solution were placed in 16 × 150 mm borosilicate test tubes. Tryptamine reagent (100 μL) and HCl (3 mL) were added, and the tubes placed in a 60°C water bath for 15 min. After cooling the tubes to room temperature in a water bath, they were allowed to stand for at least 40 min. The absorbance was read at 518 nm against water.

RESULTS

Absorbance Curve of Chromagen

Figure 1 shows the absorbance curve of the chromagen against a reagent blank containing no fructose. Twenty micrograms of fructose were used to generate this curve. The wavelength of maximum absorbance was determined in several tests to be between 518 and 522 nm. A wavelength of 518 nm was chosen for the assay.

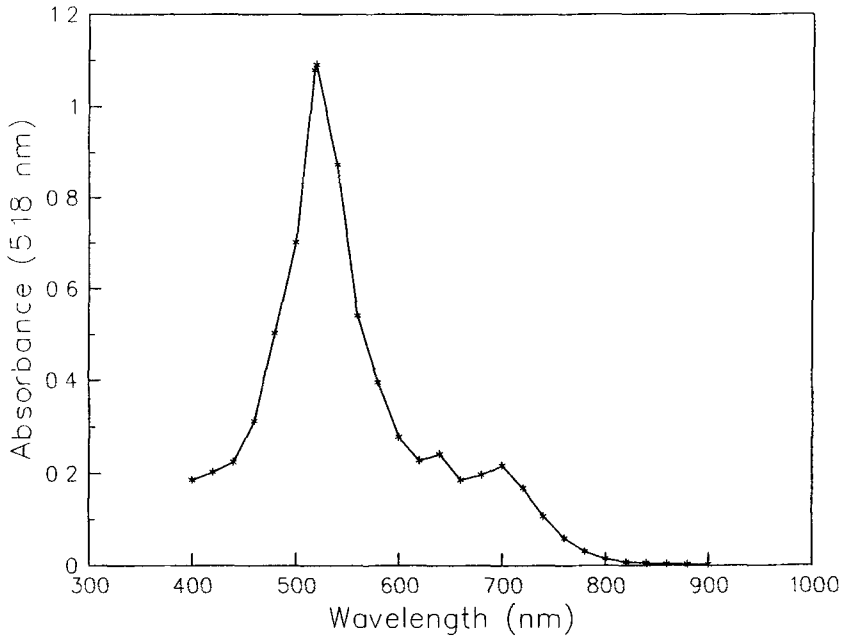

Fig. 1. Absorption spectrum of the chromagen produced using 20 μg of fructose. Maximum absorbance was determined to be at 518 nm.

Amount of Tryptamine Required

Figure 2 shows the absorbance reached as a function of the amount of tryptamine added to the assay; 20 μg fructose were used. When 10 times the amount of fructose was used in the assay, the same curve shape was obtained. The assay was run at room temperature in this case. In both cases, the maximum absorbance was obtained at 100 μL (10 m*M*) tryp t-amine (0.3 m*M* in the assay).

When tryptamine is replaced with tryptophan, there is a drop in response of approx 70%. A volume of 100 μL (of 10 m*M*) Tryptamine was chosen for the assay.

Amount of HCl Required

There is a drop of at least 50% in response when the volume of HCl is reduced from 3 to 2.5 mL, the rest of the 3-mL vol being made up with water. This is true regardless of assay conditions. Three milliliters of concentrated HCl were considered necessary for the assay.

Temperature and Time of the Assay

When the assay was conducted at room temperature, the absorbance was still rising after 3 h. At that time, the glucose response was 0.7% that

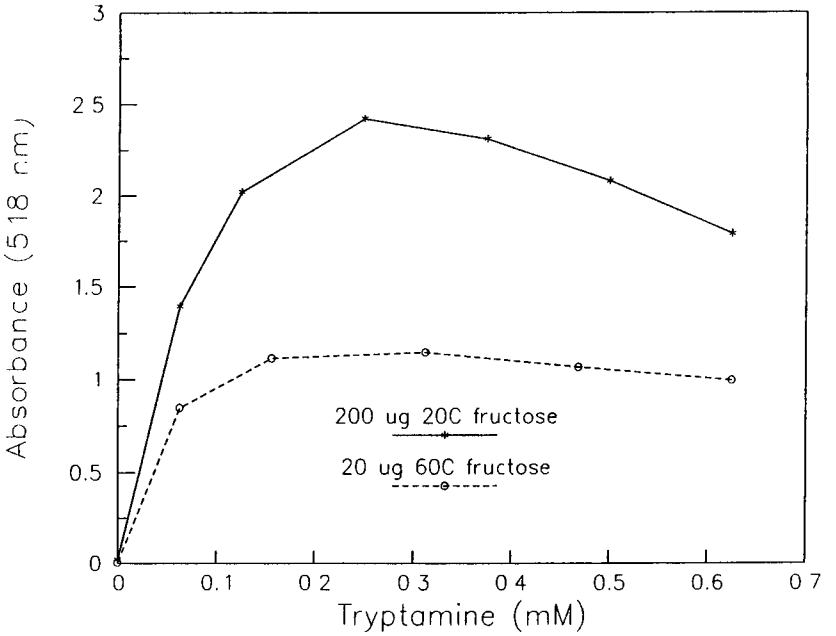

Fig. 2. Absorbance against the amount of tryptamine provided in the assay. The bottom line is for 20 μg fructose incubated for 15 min at 60°C. The top line shows the results for 200 μg fructose incubated at room temperature for 2 h.

of fructose on an equal-weight basis. At 20 h, the room-temperature incubation of fructose reached an absorbance approximately one-half that of the higher-temperature incubations. Glucose interference at this time was about 1.2% on an equal-weight basis. No advantage to an overnight room temperature incubation was seen.

Figure 3A shows the effect of continued incubation at 60°C. Glucose response rises continuously for 1 h, whereas fructose response peaks at 15 min. In this experiment, 20 μg of fructose and 100 μg of glucose were used. At 15 min, glucose response was 1.6% of fructose on an equal-weight basis. Figure 3B shows the effect of incubating in a steam-heated water bath (approx 95°C). Glucose (100 μg) and fructose (20 μg) were measured. The glucose response is one-fifth that of fructose (equal-weight basis) at 4 min. At 1 min, the absorbance of glucose is $<2\%$ that of fructose on an equal-weight basis. This interference from glucose is the result of the generalized reaction of hexose sugars with tryptophan or tryptamine in strong acids.

On this basis the assay was run at 60°C for 15 min or in a boiling water bath for 1 min. In either case, the tubes were cooled rapidly to room temperature using another water bath to limit glucose interference. The rapid cooling was also necessary to limit the reduction of fructose response owing to longer incubation times at elevated temperatures.

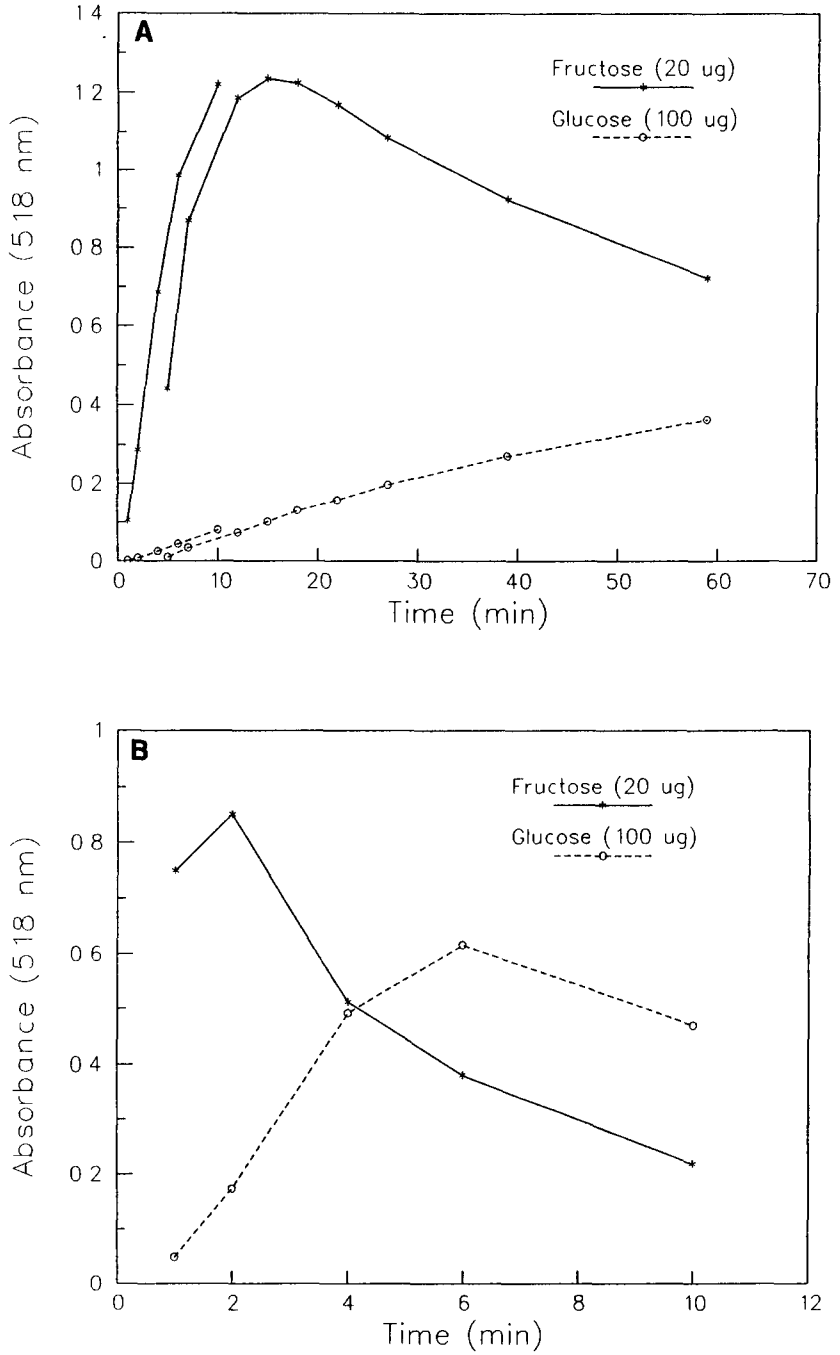

Fig. 3. **(A)** The effect of incubation time at 60°C on the absorbance of the assay. The upper line is for 20 μg fructose, whereas the lower is for 100 μg glucose. **(B)** The effect of incubation time at 100°C on absorbance for fructose at 20 μg and glucose at 100 μg.

Stability of Absorbance at Room Temperature

Fructose (20 μg) and glucose (200 μg) were incubated in a 60°C water bath for 10, 15, and 20 min, and in a boiling water bath for 1, 2, and 4 min. The absorbances were recorded over time as the assay mixtures stood at room temperature.

The results for the 60°C incubation are shown in Fig. 4A. Both 10- and 15-min incubations (60°C) reach the same maximum absorbance by about 40 min (at room temperature), whereas the 20-min incubation shows a reduction in the maximum value reached. The maximum absorbances for glucose increase with incubation time. At 44 min (room temperature), the glucose response for the 10-min incubation is 2.2%, for 15 min, 2.7%, and for 20 min, 3.9%. These are all on an equal-weight basis. Although the glucose interference was slightly higher for the 15-min incubation than that of the 10-min incubation, 15-min incubation was chosen for the assay, since it gave a slightly more linear standard curve and a fructose absorbance that was closer to its maximum immediately after incubation.

When the boiling water bath was used to develop the assay, the 1-min time period developed to a maximum value that was comparable to the 60°C assay (Fig. 4B). The 2-min incubation, although showing a higher value immediately after cooling to room temperature, developed a much lower maximum absorbance when allowed to sit at room temperature. This is likely because of destruction of the fructose. In either case, the assay must be left to stand at room temperature for at least 2 h to reach a stable maximum absorbance before being read. When using a 1-min boiling time, the glucose interference is comparable to that in the 15-min 60°C incubation. If the assay is allowed to boil any longer, the glucose interference rises rapidly, whereas the maximum fructose response drops.

Other Sugars and Substances Showing A Response

Table 1 gives the results of tests on other substrates for color and recovery of added fructose. The effect of different levels of glucose on the response of the assay and on the recovery of added fructose is shown in Fig. 5. On a weight-to-weight basis, the average response of glucose in the assay is 2.8%. The fructose recovery ranges from 96% at 20 μg glucose to 84% at 400 μg glucose. This suggests some competitive interference may be occurring at higher glucose levels.

Sugars Dissolved in Feed Extractants

Since the purpose of this assay is to determine the fructose levels in plant materials, some of the sugars commonly found in plants were dissolved in some potential extraction solutions. Absolute ethanol, 0.005,

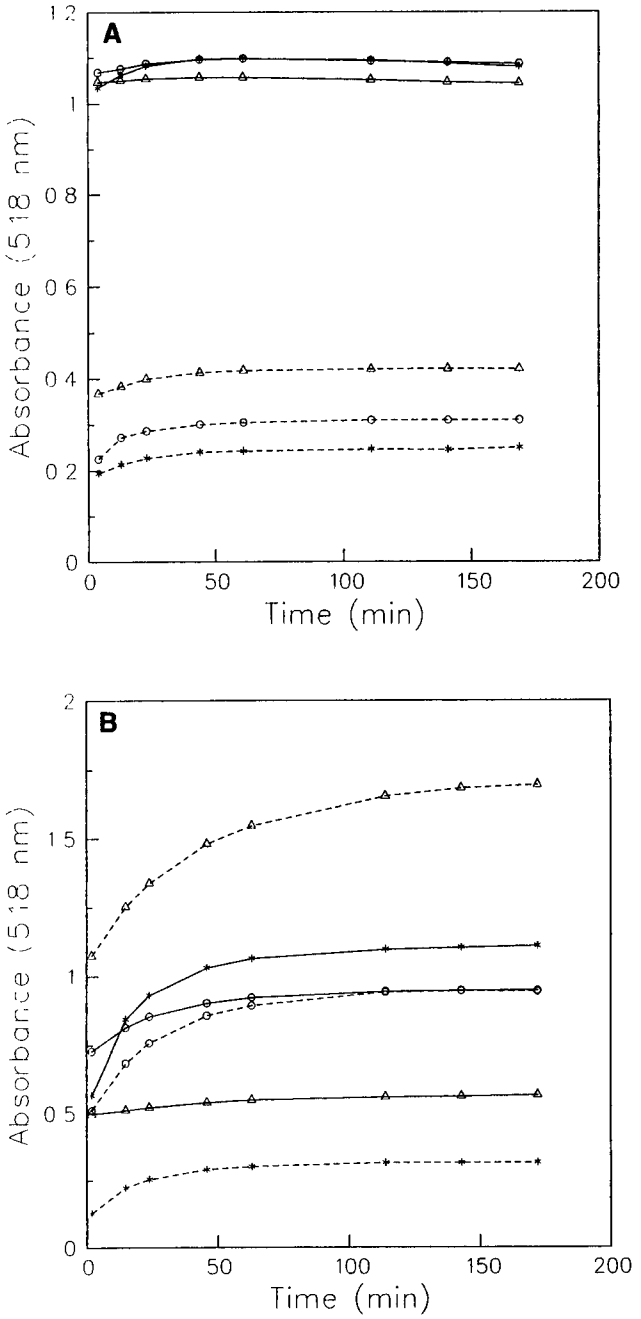

Fig. 4. (**A**) The effect of the time of incubation at room temperature for 20 μg of fructose (solid lines) or 200 μg glucose (dashed lines) after incubating at 60°C for 10 (*), 15 (○), 15, or 20 (△) min. (**B**) The effect of the time of incubation at room temperature for 20 μg of fructose (solid lines) or 200 μg glucose (dashed lines) after incubating at 100°C for 1 (*), 2 (○), or 4 (△) min.

Table 1
Reactivity of Various Substances
and Recovery of Added Fructose

Sugar	μg	Apparent fructose%, w/w
Amygdalin	50	nd[1]
D-Arabinose	50	nd
	100	nd
D-Cellobiose	50	3
L-Fucose	50	nd
	200	nd
D-Galactose	100	nd[2]
D-Glucose	50	nd
Heparin	100	nd[2]
Lactose	100	20
Maltose	100	3
Mannan	50	nd
D-Mannose	50	6
	200	.2
Melibiose	50	nd
L-Rhamnose	50	5
	200	.1
Rutin	50	nd
D-xylose	50	nd
	100	nd
D-Ribose	20	nd
D-Xylulose	50	32
D-Ribulose	50	12
D-Arabitol	100	nd[2]
Dulcitol	100	nd[2]
D-Mannitol	100	nd[2]
Myoinositol	100	nd[2]
	200	nd
D-Sorbitol	100	nd[2]
D-Galacturonic acid	100	nd[2]
	50	nd
D-Glucuronic acid	100	nd[2]
Pectin	100	nd[2]
6-Phosphogluconate	50	nd
Poly-galacturonate	100	nd[2]
Acetaldehyde	78.8	nd
Benzaldehyde	105	10
Formaldehyde	37	9
Glutaraldehdye	100	3
Glyceraldehyde	100	20
Hexanal	83	nd
Octanal	82	nd
Propionaldehyde	81	nd
2-Heptanone	82	nd

(*continued*)

Table 1 (*continued*)

Sugar	μg	Apparent fructose%, w/w
D-Saccharic acid	100	nd[2]
D-Gluconic acid	100	nd[2]
D-Galactonic acid	100	nd[2]
Phytic acid	50	nd
Lactic acid	25	nd
D-Glucosamine	100	nd[2]
D-Mannosamine	100	nd[2]
D-Galactosamine	100	nd[2]
Inulin (artichoke)	20	95
Inulin (dahlia)	20	98
Sucrose	20	69
D-Raffinose	50	31
Stachyose	20	26
L-Sorbose	20	66
Brome grass		2
Alfalfa		2.4
Broccoli leaf midrib		20.2
leaf minus midrib		3.9

[1]nd = not detected.
[2]Recovery > 95% with other sugars at 5X excess.

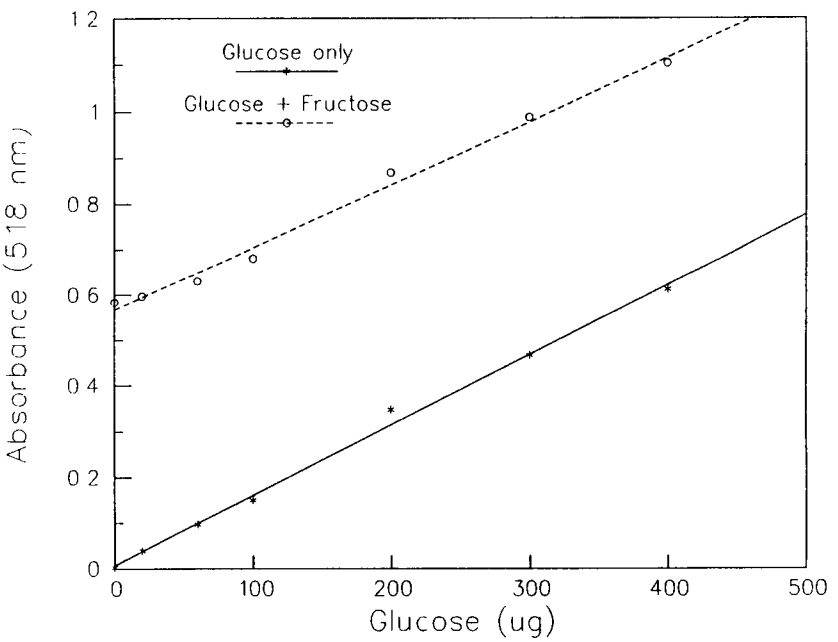

Fig. 5. The absorbance of glucose at different levels is shown here represented by the solid line. The absorbance of different levels of glucose plus 10 μg fructose is also shown (dashed line). The incubation was carried out for 15 min at 60°C and then held at room temperature for 45 min.

Table 2
Percent (w/w) Fructose Found for Some Sugars
Dissolved in Various Potential Extractants

Sugar	Extractant			
	EtOH	0.005*N*	0.02*N*	1*N*
Fructose	107.8	107.3	105	98.6
Glucose	3.8	0	0	0.2
Sucrose	26.2[a]	57.7	56.0	52.7
Sol. starch	0	0	0	0
Oat spelt xylan	0	0	0	0

[a]Sucrose is not completely dissolved in absolute ethanol.

0.02, and 1*N* sulfuric acid solutions were used to boil the sugars. A ten-milliliter extractant was added to 20 mg of sugar in a screw-cap tube. The tubes were boiled for 1.5 h and cooled to room temperature. Twenty microliters of the supernate, representing a potential 40 μg of sugar, were tested under standard assay conditions. The results as "percent fructose" using a standard curve of 0–50 μg fructose are shown in Table 2.

Fructose Recovery

A variety of sugars and feeds were sequentially extracted with absolute ethanol (boil 1 h), 0.5% EDTA in water (pectin extractant, boil 1.5 h) and then 1*N* sulfuric acid (boil 2 h). Forty microliters of the supernatants were assayed. This represented a potential sugar quantity of 80 μg, since approx 20 mg of sample were extracted with 10 mL of solution. The extractant solutions were tested for fructose levels and to each, 10 μg of fructose were also added to check recovery. The results are presented as percent fructose and percent fructose recovered in Table 3.

DISCUSSION

This article presents a simple and specific method of quantitatively determining fructose. The method is quick and requires only two reagents, HCl and Tryptamine, as well as a sugar standard. Very few sugars show any reaction at all. If temperature control is maintained within reasonable limits, the interference owing to glucose is $<3\%$.

Two approaches commonly used to estimate total soluble sugars in feeds are the phenol sulfuric acid method of Dubois et al. (*4*) or a reducing sugar assay (e.g., *5*).

A reducing sugar assay will not accurately reflect the amount of available sugars if they are still in polymer or dimer form as fructosans,

Table 3
Percent (w/w) Fructose Detected and Fructose Recovered from a Variety of Sample Matrices

	Extractant					
	EtOH		EDTA		1*N* Sulfuric	
Sample	Fruc.	recovered	Fruc.	recovered	Fruc	recovered
Oat spelt xylan	0	103	0.51	102	0	100
Pectin	0	100	0.83	100	—	—
Avicel	0	98	0	99	0	97
Filter paper	0	98	0	97	—	—
Soluble starch	0	97	2.1	102	—	—
Corn	0.25	102	0.28	102	0.06	104
Soybean meal	0.9	101	3.8	102	0	104
Brome hay	6.0	106	7.1	104	0	104
Alfalfa cubes	0.2	102	0.31	100	0	97
Grass hay	2.9	102	2.8	102	0	98
Corn gluten blood meal	0	101	0	96	0	97
Fish meal	0	100	0	102	0	98

sucrose, or short-chain oligomers of glucose or xylose. A prior digestion with acid is needed to estimate these accurately. The method presented here uses 12*N* HCl, which should easily break down most fructosans to monomer sugars giving an accurate value for fructose.

The phenol sulfuric acid method of Dubois et al. (4), which uses concentrated sulfuric acid, will digest any oligomers and give an estimate of total sugars. The problem with this method, however, is that it does not respond to every sugar in the same way. Fructose gives an absorbance that is only 60% that of glucose in this assay (4), although we have modified the assay to give equivalent values (Taylor, this issue). Since the soluble carbohydrates of many plants include a high percentage of fructose, an additional simple fructose method would be of value in obtaining a more refined value.

The method described here is suitable for measuring fructose and the fructose contents of polymers containing fructose. Table 1 shows that two types of inulin give fructose values of 95 and 98%. Sucrose, raffinose, and stachyose are sugars of 2, 3, and 4 U, each with one fructose unit. The assay gives values of 69, 31, and 26% for these sugars, respectively. Another ketohexose, sorbose, reacts at 66% the value of fructose. Of the other substances tested, only some aldehydes, lactose, xylulose, and ribulose gave values of $>10\%$ on a weight-to-weight basis.

Some wilted and dried forage materials were analyzed using a cold-water extraction. Brome grass showed a fructose content of 2%, whereas alfalfa gave 2.4%. Broccoli leaves were separated into midrib and leaf minus midrib fractions to give values of 20.2 and 3.9% dry matter, respectively.

A method of estimating fructose using 30% HCl and Scatole (3-methyl indole) was reported by Pogell (*2*). The chromagen had a major peak at 510 nm and a minor one of 50% height at 400 nm. This method suffered from an absorbance that continued to rise as the tubes were read requiring a correction factor and careful timing of the assay. The method was later modified with a chloroform extraction step to give a stable color (*6*). The method still required a 6-h incubation at 38°C. The assay was used to estimate inulin and sucrose with no prior acid hydrolysis. Good recovery from animal tissue, sera, and urine was obtained. Glucose, galactose, mannose, maltose, xylose, and arabinose at amounts similar to fructose reacted at 1% or less on an equimolar basis. At a 10 times excess, however, glucose gave a 9% error.

A somewhat similar method is described by Messineo and Mussara (*3*). This assay involves first reacting fructose with cysteine in sulfuric acid, and then with tryptophan to give a chromaphore with a maximum absorbance at 518 nm. At a similar concentration, the assay gives a comparable absorbance to the fructose tryptamine assay presented here. The fructose-cysteine-tryptophan assay is said to be specific for ketohexose sugars with sorbose reacting at about one-half the value of fructose. Inulin, sucrose, and fructose phosphates all react in proportion to their fructose contents. Aldopentose, aldohexose, ketopentose, and triose sugars all give no reaction, or react nonspecifically with the high concentration acid used in the assay. It is likely that the chromagens in these assays are similar to the tryptamine-fructose chromagen described in this article.

CONCLUSIONS

The assay described here will detect as little as 1 μg fructose and gives a linear absorbance response. The assay is not affected by ethanol, EDTA, or 1*N* H_2SO_4 extractants. The chromagen is stable at room temperature after a period of color development. No interference by various feed substrates, e.g., blood meal, corn, hay, fish meal, soybean meal, or alfalfa, is seen. In addition, there is low interference with good recovery of fructose at very high levels of glucose, and 84% recovery when glucose is 40× in excess of fructose. Very low reactivity with other sugars is noted. The assay will quantitate fructose and fructosan oligomers without the need for prior digestion, and has been used to localize fructose contents in broccoli leaves.

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