A Method for Quantifying Carbohydrate Fractions in Forage Plants

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Monosaccharides and polysaccharides of glucose and fructose are extracted from plant material with an ethanol dilution gradient series, hydrolyzed, and quantitized by a modification of the ferrocyanide reduction method. Glucose and fructose are determined simultaneously through differences in oxidation rates at 100 and 55°C. The ferrocyanide arsenomolybdate complex is measured spectrophotometrically at 745 mμ. This method is accurate and sensitive for analyzing fructosan moieties. Ethanol extraction and clarifying with lead acetate does not significantly interfere with color development.

Perennial grasses native to the temperate latitudes appear to accumulate fructosans together with sucrose in their stem bases and rhizomes (DeCugnac, 1931; Weinmann and Reinhold, 1946; Smith, 1968; Ojima and Isawa, 1968). Plants native to semitropical or tropical areas, in contrast, appear to store starch together with sucrose. However, in all plants the major reserve carbohydrates are polysaccharides of the monomers glucose and fructose and previous methods of analyses have not routinely differentiated the structural complex of these polysaccharides. Smith (1967), working with a number of forage species, utilized ethanol dilution gradient extractions to separate carbohydrate fractions. This extraction method combined with acid hydrolysis, thin-layer chromatography, and copper reduction quantitation was utilized to differentiate between fructosans and glucosans. Recent developments in carbohydrate chemistry have made it possible to routinely quantitate individual monosaccharides simultaneously in the same sample (Ting, 1956; Reid *et al.*, 1970).

Most methods of determining total monosaccharides are based on the oxidation of sugars with an alkaline solution of metal salts, such as copper or iron ferrocyanides. The reduced metals are then determined by gravimetric, titrimetric, or colorimetric means (Smith, 1969; Ting, 1956). However, few colorimetric methods allow for differential determination of individual carbohydrates. Selective determination of fructose in the presence of glucose generally depends on a choice of alkalizing agents and temperature of the reaction bath during oxidation. Jackson and Matthews (1950) used carbonate and bicarbonate, whereas Becker and Englis (1941) employed a mixture of sodium carbonate and sodium phosphate buffer for selective oxidation, and determined the reduction product titrimetrically with sodium thiosulfate or ceric sulfate (Englis and Becker, 1939).

The method described in this paper employs a combination of the ethanol dilution gradient extraction and acid hydrolysis (Smith, 1967) and an improved version of the colorimetric quantitation method presented by Ting (1956). The simplicity of this method has an advantage in routine analyses of free and combined glucose and fructose content in forage material, and is relatively inexpensive when analyzing large numbers of samples.

EXPERIMENTAL

Combined samples of tall fescue (*Festuca arundinacea* Schreb.) culm base and root tissue were collected at a 15-cm depth below ground level from an established pasture stand. Samples were immediately placed in an oven at 100°C for 1 hr and drying was completed at 70°C in a forced-air oven. The dried plant material was ground to pass a 40-mesh sieve and stored in sealed bottles. A 500-mg sample of dried plant material was extracted successively with 95, 92.5, 90.0, 85.0, 80.0, 60.0, 30.0, and 0% ethanol solutions made from 95% ethanol. Samples were mechanically shaken for 1 hr with 30 ml of extractant at room temperature, beginning with the most concentrated ethanol dilution. Plant material and eluent were centrifuged at *ca.* 2000 × *g* for 10 min and eluent was removed for hydrolysis and carbohydrate analyses. Plant residue was returned to the flask using next lower ethanol dilution. This procedure was repeated throughout the ethanol dilution series.

The alcohol was replaced with water during evaporation on a hot plate and the solution clarified with 2 ml of 10% lead acetate and centrifuged. Excess lead was precipitated with *ca.* 100 mg of potassium oxalate. The solution was refrigerated for 5 hr and precipitate removed by centrifugation at *ca.* 2000 × *g* for 10 min. The decanted solution was diluted to volume (50 ml) and an aliquot utilized for determination of reducing sugars. A 10-ml aliquot of diluted solution was hydrolyzed with 2 ml of 1 *N* H_2SO_4 in test tubes placed in a boiling water bath for 15 min. The cooled hydrolysate was neutralized with 1 *N* NaOH and diluted to volume with water and aliquots for colorimetric analyses. Residue from the final ethanol extraction was analyzed for starch content using clarase "900" enzyme extraction and titrimetrically quantified according to the method of Smith (1969).

Colorimetric analyses were carried out essentially as described by Ting (1956) with the following modifications. A Beckman Model DB-G spectrophotometer was used to measure absorbance at 745 mμ wavelength using 1.00-cm silica cells and 2-ml sample aliquots. Absorbance measurements were made 30 to 60 min after the addition of the arsenomolybdate solution using a blank treated the same as samples. Concentrations were calculated from a standard curve obtained from subjecting known concentrations of glucose and fructose to the same test. Since Beer's law was valid for the concentration range of interest, total reducing sugar values were determined from the value A_{100}, measured absorbance from the reaction at 100°C and according to the formula $Ct = Cg + Cf = (A_{100}/a_{100})\ D$, where Ct was total concentration of glucose (Cg) and fructose (Cf) in mg per g of plant material. The dilution factor (D) in this study was 20 for nonhydrolyzed samples and 40 for hydrolyzed samples. Glucose concentration was found from the formula $Cg = (A_{100} - A_{55})/(a_{100} - a_{55})$. Values of a_{100} and a_{55} are the slopes of the lines of absorbance (A_{100}) *vs.* concentration of

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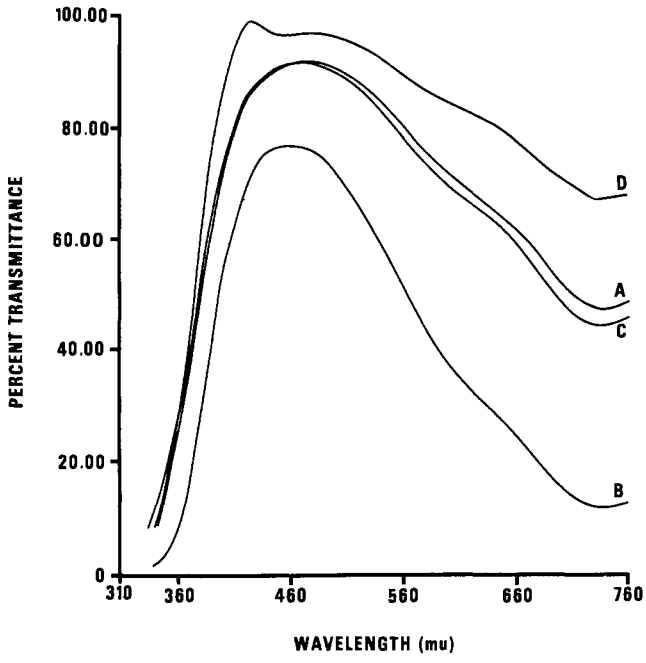

Figure 1. Transmittance spectra of ferrocyanide arsenomolybdate complex from: (A) 90% ethanol plant extract (reaction temp 55°C); (B) 90% ethanol plant extract (reaction temp 100°C); (C) 200 μg of fructose (reaction temp 55°C); and (D) 100 μg of glucose (reaction temp 100°C)

standard glucose and fructose solutions and of absorbance (A_{55}) *vs.* the concentration of glucose standard, respectively. Fructose concentration is given by Cf = Ct − Cg.

Prior to analyses of reducing sugars in plant samples, paper and gas–liquid chromatographic separation of hydrolyzed sample extracts were made to identify the major reducing sugars present. The separation was effected by paper chromatography according to the method of Sunderwirth *et al.* (1964) using ethyl acetate, acetic acid, and water (6:3:2) solvent system, and by gas–liquid chromatography of the sugar trimethylsilyl ethers according to a method that will be reported elsewhere (Phillips and Smith, 1971).

RESULTS AND DISCUSSION

Chromatographic data indicated glucose and fructose to be the major monosaccharides in the hydrolyzed plant extracts. Transmittance spectra of the ferrocyanide arsenomolybdate complexes, following reaction of ferrocyanide with plant extracts and with standard solutions of glucose and fructose (Figure 1), indicate transmittance minima in the 740 to 750 mμ region. By measuring absorbance at 745 mμ, where absorptivity is maximum, the sensitivity and accuracy of the method were greatly improved over the method presented by Ting (1956). Absorbance *vs.* concentration curves of fructose and glucose oxidized at 100°C and fructose oxidized at 55°C gave the same slope. However, equivalent glucose samples oxidized at 55°C presented lower absorptivities. Both curves followed Beer's law over the concentration range.

The influence of ethanol extraction and extract clarification on color development were studied further. Absorbance readings of sample blanks oxidized at 55°C were not significantly influenced by clarifying with lead acetate and potassium oxalate (Table I). However, interference equivalent to 1.03 μg of glucose was received at the 100°C oxidizing temperature. Similar effects were noted when testing 1 ml of 95% ethanol (extractant) plus 1 ml of clarified sample blank. The influence was considered well below experimental error when analyzing plant extracts containing more than 100 μg of glucose equivalents. The colorimetric method was checked for efficacy in starch analyses using the clarase "900" extraction procedure (Smith, 1969). Results indicated that the enzyme preparation drastically interfered with the color development (Table I), eliminating the possible use of this method for quantitizing enzymatically hydrolyzed starch samples. Method precision and accuracy were determined using tall fescue foliage, including culm and leaf material samples (referred to as plant extract) separately and spiked with known quantities of glucose and fructose (Table II). Data indicated a high degree of both precision and accuracy, with standard deviations (SD) of less than 5%.

Data obtained from plant samples extracted with the ethanol dilution series and quantified by the arsenomolybdate colorimetric method are presented in Table III. Tall fescue,

Table I. Influence of Enzyme and Ethanol Extraction on Ferrocyanide Arsenomolybdate Complex

Sample description	Glucose equivalents, μg
Cleared enzyme extracted starch blank	356.6[a]
Carbohydrate blank cleared by lead acetate–potassium oxalate (oxidation temp: 55°C)	0
Carbohydrate blank cleared by lead acetate–potassium oxalate (oxidation temp: 100°C)	1.03
Carbohydrate blank cleared by lead acetate–potassium oxalate + 1 ml ethanol (oxidation temp: 55°C)	0
Carbohydrate blank cleared by lead acetate–potassium oxalate + 1 ml ethanol (oxidation temp: 100°C)	1.78

[a] Average of six replicates.

Table II. Precision and Accuracy of Ferrocyanide Arsenomolybdate Method of Differential Carbohydrate Analyses [Total Reducing (Ct), Glucose (Cg), and Fructose (Cf)]

Description	μg reducing sugars Ct	SD[b]	Cg	SD	Cf	SD
Plant extract	138.4[a]	± 3.2	71.2	± 1.4	67.2	± 1.3
Plant extract + 200 μg of glucose	340.0	± 5.1	272.0	± 4.1	68.4	± 1.3
Plant extract + 200 μg of fructose	348.3	± 6.2	73.1	± 2.2	275.2	± 4.6

[a] Average of six replicates. [b] SD, standard deviation.

Table III. Ethanol Gradient Extracted Sugars from Tall Fescue Differentially Analyzed by Ferrocyanide Arsenomolybdate Colorimetric Method

Percent ethanol gradient extraction	Sugar concentration in plant material (mg/g) Ct	Cg	Cf	Cf/Cg
95.0 unhydrolyzed	4.24[a]	0.82	3.42	4.2
95.0 hydrolyzed	5.27	1.37	3.90	2.9
92.5 hydrolyzed	4.95	2.42	2.53	1.1
90.0 hydrolyzed	3.77	1.50	2.27	1.5
85.0 hydrolyzed	4.34	1.92	2.42	1.3
80.0 hydrolyzed	6.25	1.52	4.73	3.1
60.0 hydrolyzed	11.56	1.18	10.38	8.8
30.0 hydrolyzed	4.90	0.30	4.60	15.3
0 hydrolyzed	4.31	0.20	4.11	20.6

[a] Average of six replicates.

a fructosan-accumulating species (Smith and Grotelueschen, 1966), was used to evaluate this method for characterizing and quantitizing carbohydrate fractions in forage plants. Data indicated that roots of this species have a relatively high free fructose content. The increasing fructose/glucose (Cf/Cg) ratio corresponding with decreasing ethanol extractions beginning with the 92.5% hydrolyzed extraction indicated the presence of fructosans of varying chain lengths and the greatest quantity of fructose was extracted with 60% ethanol. These data corroborate the findings of Smith and Grotelueschen (1966). Fructosans in grasses are reported to be similar to the inulin type found in *Compositae*, but are thought to occur in chain lengths shorter than 35 fructofuranose residues linked 2:6 and terminated by a sucrose residue (Bacon, 1960; Edleman, 1960). In this experiment, fructose/glucose ratios increased with decreasing ethanol concentration indicating the presence of fructosans with average chain lengths of 1.05 (sucrose) at 92.5% ethanol extraction to 20.6 (inulin) at 0% ethanol extraction, in tall fescue roots.

Colorimetric determination of fructose in the presence of glucose can be used advantageously for differentiating these two sugars in plant extracts where they are present in greatest quantities. This method, in combination with the ethanol dilution extractions and acid hydrolyses, allows for routine quantitative description of carbohydrates in plant material, with the exception of starch. Because of the simplicity of this method, it can be used for routine determination of sugar components in forage samples, in particular the fructosan accumulating species.

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Identification and Estimation of Tocopherols and Tocotrienols in Vegetable Oils Using Gas Chromatography–Mass Spectrometry

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A one-step method to estimate and identify TMS derivatives of tocopherols and tocotrienols using gas chromatography and mass spectrometry is described. Thin-layer chromatography is used as a pretreatment of unsaponifiable material when the critical isomers β- and γ-tocopherols are present together. The contents of individual tocopherols and trienols in oats, wheat germ, barley, soybean, and coconut oils are recorded. Wheat germ oil has a total tocopherol content of 212 mg/100 g of oil; oats, barley, and coconut oils have less than 3 mg/100 g of oil. Oats and barley oils are found to contain major amounts of α-tocotrienol, whereas coconut oil contains α-tocotrienol. The bulk of the tocopherols are distributed between α- and β-tocopherols in wheat germ oil and γ- and δ-tocopherols in soybean oil. Barley oil seems to possess almost all the known tocopherols and trienols. However, the presence or absence of δ-tocotrienol has not been determined due to nonavailability of the standard compound.

Separation of individual tocopherols from the nonsaponifiable material of oils and fats has been one of the major activities in this field. Of the eight tocopherols and trienols (α-, β-, γ-, and δ-, and their unsaturated counterparts, Figure 1), the positional isomers β- and γ-tocopherols are the most difficult to separate. Earlier, Quaife (1948) adopted a method to differentiate tocopherols under two main classes: α-tocopherol and non-α-tocopherols. This classification was made on the basis that β-, γ-, and δ-tocopherols reacted with nitrous acid to give a yellow nitroso derivative which could be determined by the colorimetric procedure of Emmerie and Engel (1939). Since the three nitroso derivatives have different extinction coefficients, the only disadvantage of this method was that the most important α-tocopherol was measured by difference.

The second method most popular during this period was the Dianisidine coupling reaction (Weisler *et al.*, 1947). The γ- and δ-tocopherols couple with diazotized *o*-dianisidine in alkali solution. The dye can be extracted and determined colorimetrically. However, the method is valid only when β-tocopherol is absent, but when present it is measured with α-tocopherol. With the discoveries of tocotrienols, the method became more complicated since 5,8-dimethyl tocotrienol (ε- or β-T-3) and 7,8-dimethyl tocotrienol (η- or γ-T-3) both gave the nitroso reaction, in addition 7,8-dimethyl tocotrienol (γ-T-3) coupled with dianisidine; therefore, its presence complicates the validity of an α- and γ-tocopherol determination.

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