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Determination of Total Nonstructural Carbohydrates in Forage Tissue by *p*-Hydroxybenzoic Acid Hydrazide Flow-Injection Analysis

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An extraction procedure for total nonstructural carbohydrates (TNC) in grasses was developed for *p*-hydroxybenzoic acid hydrazide (PAHBAH) flow-injection analysis. The extraction requires a minimum of 40 min of hydrolysis in 10 mL of boiling 2.5 mM H_2SO_4 to hydrolyze sucrose and fructans. Recoveries of added glucose and fructose ranged from 100 to 107%. The boiling treatment also served to gelatinize starch, allowing rapid starch hydrolysis using amyloglucosidase. Gelatinized preparations of corn, potato, and wheat starch were hydrolyzed to about 80–89% reducing sugars in less than 1 h after enzyme addition. Shoots and stem bases of wheat (*Triticum aestivum*) and Caucasian bluestem (*Bothriochloa caucasica*) and rhizomes of johnsongrass (*Sorghum halepense*) were analyzed for completion of hydrolysis by 0-, 24-, 48-, or 72-h incubations treated with a 0.5% solution of amyloglucosidase at 50 °C. Nearly complete TNC extraction of wheat and Caucasian bluestem occurred at 0 h while a 24-h incubation was necessary for johnsongrass rhizomes to allow effective tissue penetration by amyloglucosidase. Samples stored in the refrigerator for up to 50 days gave similar values when reanalyzed for TNC.

Plants allocate carbon into structural and nonstructural forms (White, 1973). Structural forms include cellulose and hemicellulose and nonstructural carbohydrates include starch (amylose, amylopectin), fructans (inulins, levans), disaccharides (sucrose, maltose), and monosaccharides (glucose, fructose) (Smith, 1981; White, 1973). Glucose and fructose are reducing sugars that react with a variety of compounds to produce colors that are measured spectrophotometrically. The major poly- or disaccharides present in most forage plants can be hydrolyzed to glucose or fructose chemically or enzymatically (Weir et al., 1977; Smith, 1981); hence, the total nonstructural carbohydrates (TNC) of a tissue sample may be estimated spectrophotometrically.

Many methods have been used for measuring TNC as reducing sugars in plant tissues. Common procedures include potassium ferricyanide (FCN) adapted for use with an autoanalyzer (Gaines, 1973; Wolf, 1975; Weir et al., 1977) and the Shaffer–Somogyi titrimetric method using copper reduction (Smith, 1981). Several authors (Powell and Lever, 1972; Lever, 1972, 1973; Lever et al., 1973; Fingerhut, 1973; Davis, 1976) have suggested a colorimetric method based on the reaction of *p*-hydroxybenzoic acid hydrazide (PAHBAH) with reducing sugars. Fingerhut (1973) suggested that PAHBAH was more specific for glucose than is the FCN procedure. In addition, Davis (1976) found that PAHBAH was satisfactory for measuring reducing sugars in tobacco. He suggested that the PAHBAH method was superior to the FCN method because substances other than glucose and fructose contributed to the reducing power of tobacco samples analyzed with FCN.

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Table I. Integrator Input Parameters (s) Required for the *p*-Hydroxybenzoic Acid Hydrazide (PAHBAH) and Ferricyanide (FCN) Flow-Injection Schemes

	PAHBAH	FCN		PAHBAH	FCN
cycle time	92	83	load time	45	45
sample time	8	19	window delay	182	214
load delay	17	29	peak window	90	81

Little information is currently available regarding TNC measurement with flow-injection analysis. Because the CUI and FCN methods are used routinely for measuring TNC in plants, our intention was to develop an automated PAHBAH and FCN procedure using flow-injection that is comparable to the Shaffer–Somogyi CUI method and to document the hydrolysis, incubation, and storage time for TNC extraction from forage tissue.

MATERIALS AND METHODS

Apparatus. The flow-injector was a Lachat Quickchem System IV, Model 170 analyzer [Lachat Chemicals Inc., Mequon, WI; mention of a trade name does not constitute endorsement by the USDA]. Schematic drawings of the flow-injection systems for PAHBAH and FCN are shown in Figures 1 and 2, respectively. Flow-injection and sample loop tubing were made of Teflon and had internal diameters of 0.813 and 0.559 mm, respectively (Lachat Chemicals, Inc.). Other tubing within the system had an internal diameter of 0.813 mm. The heating coil was made of Teflon tubing (i.d. = 1.6 mm). The proportioning pump speed was set on 80% maximum. Injected sample volumes were calculated from internal tubing dimensions. Approximate sample volume was 44.9 μL with a sample loop

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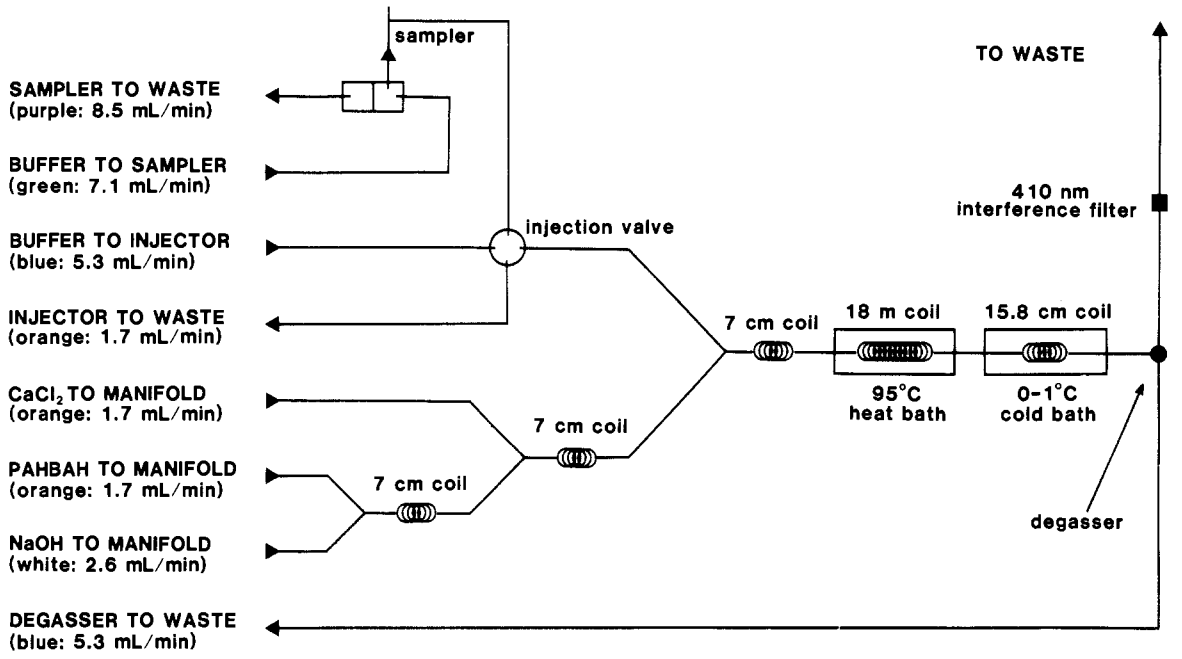

Figure 1. Flow-injection system for *p*-hydroxybenozic acid hydrazide (PAHBAH) methodology. Tubing color codes and flow rates are shown in parentheses.

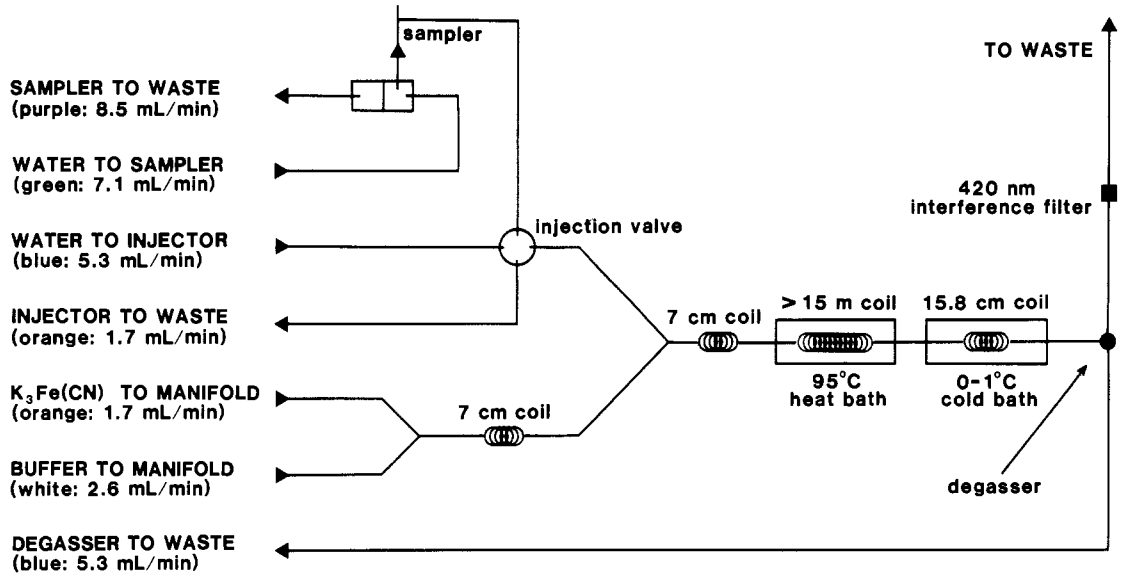

Figure 2. Flow-injection system for potassium ferricyanide (FCN) methodology. Tubing color codes are shown in parentheses.

length of 18.3 cm for PAHBAH (i.d. = 0.559 mm) and 311 μL for FCN with a sample loop of 60 cm (i.d. = 0.813 mm). Watson–Marlow silicone tubing (Lachat Chemicals, Inc.) was used in conjunction with the proportioning pump. Tubing length and heating times at 95 °C were 18.0 m and 190 s for PAHBAH and 15.0 and 160 s for FCN. Time was calculated beginning with entry of the sample into the heating coil and ending with maximum detector response. System controller operating parameters are given in Table I.

Reagents. A solution containing 2.5% PAHBAH and 2.1% citric acid monohydrate was prepared in 0.5 M HCl. A 5.0 mM solution of calcium chloride monohydrate was prepared by adding 735 mg of $CaCl_2 \cdot H_2O$ and 0.5 mL of Brij 35 surfactant to 1000 mL of distilled water. Reagent concentrations for the FCN procedure were those of Wolf and Ellmore (1975) and consisted of 0.2 M phosphate buffer ($Na_3PO_4 \cdot 12H_2O$) and 4.7 mM $K_3Fe(CN)_6$. Absorbance was measured by a colorimeter interfaced to a programmer/printer and recorded with a strip chart recorder. Reagents for the CUI titration method were mixed according to the Shaffer–Somogyi CUI procedure as described by Smith (1981). For the above procedures standard stock solutions of D-glucose and D-fructose were mixed at 10 g/L in 2.5 mM H_2SO_4. Glucose and fructose standard solutions were made separately by diluting stock solutions to 200, 400, 600, 800, 1000, 1200, and 1400 mg/L with 2.5 mM H_2SO_4.

TNC Extraction. Shoots of Caucasian bluestem [*Bothriochloa caucasica* (Trin.) C. E. Hubbard], rhizomes of johnsongrass [*Sorghum halepense* L. (Pers.)], and shoots and stem bases of winter wheat (*Triticum aestivum* L.) cv. TAM 101 were taken from the field, washed, microwaved for 60 s, and dried in a forced-air oven at 65 °C for 24 h. Samples were ground through a 1.0-mm screen using a cyclone mill (Udy Inc., Fort Collins, CO). Six replicates of each tissue (35 mg of johnsongrass and 100 mg for wheat and Caucasian bluestem) were added to flasks containing 10 mL of 2.5 mM H_2SO_4. In addition, 10 mL of standards was added to 25-mL Erlenmeyer flasks.

Flasks were stoppered with a cap that was fitted with a syringe needle to eliminate pressure buildup within the flask while minimizing evaporation. Flasks were immersed in boiling water for 1 h and then cooled. Nine milliliters of 0.2 M acetate buffer (Smith, 1981) was added to each flask. The flasks were agitated to mix buffer and acid, and 1 mL of 0.5% amyloglucosidase was added to raise the total volume of each flask to 20 mL. The final sample solution had a pH of 4.4 and enzyme concentration of 0.025%. The enzyme solution was made by diluting 500 mg of amyloglucosidase [glucoamylase; 1,4-α-D-glucan glucohydrolase; 11 800 units/g (from *Rhizopus*)] to 100 mL of acetate buffer. The final enzyme activity was 59 units/mL, and the resulting glucose and fructose standard concentrations (50% diluted) were 100, 200, 300, 400, 500, 600, and 700 mg/L.

The flasks were agitated to mix enzyme with sample solution, and flask trays were placed in a shaker bath at 50 °C. After the incubation period, samples were centrifuged at 5650 RCF (relative centrifuge force) for 15 min. An aliquot of extract was taken for the CUI method and the remainder used for flow-injection analysis of TNC using PAHBAH and FCN.

Hydrolysis and Storage. The effect of hydrolysis on glucose, fructose, sucrose, inulin, and corn starch was tested in 20 mL of boiling 2.5 mM H_2SO_4. Each carbohydrate was analyzed at two concentrations: 200 and 500 mg/L. Samples were boiled in a water bath for 20, 40, and 60 min. The experiment was repeated three times for replication, and reducing sugars were measured by PAHBAH flow-injection analysis.

Hydrolysis of 10 mL (500 mg/L) of corn, potato, and wheat starch (three replications) was compared with and without a 1-h gelatinization in boiling 2.5 mM H_2SO_4. After gelatinization, 9 mL of 0.2 M acetate buffer was added to each flask, followed by 1 mL of 0.5% amyloglucosidase (resulting in starch concentrations of 250 mg/L). Samples were incubated at 50 °C for 0, 24, and 48 h. The 48-h incubation was started first, the 24-h incubation began 1 day later, and the 0-h treatment was synchronized to finish its 1-h gelatinization step at the completion of the 48-h incubation. Reducing sugars were determined by PAHBAH flow-injection analysis. Percent recovery values of hydrolyzed polysaccharides and disaccharides were corrected for water addition in the following manner: 1.0 mg/L starch equals 1.1 mg/L glucose; 1.0 mg/L inulin would be hydrolyzed to 1.1 mg/L fructose and glucose; and 1.0 mg/L sucrose would be hydrolyzed to 1.05 mg/L of 1:1 glucose and fructose. Maximum removal of TNC in shoots and stem bases from Caucasian bluestem and winter wheat and rhizomes from johnsongrass was determined by PAHBAH flow-injection analysis after 0–72-h incubations. A single gelatinization was used for all samples, and a subset of samples was measured for TNC at 0, 24, 48, and 72 h following gelatinization. Three replications of each tissue type were used, and the experiment was conducted twice. All samples were reanalyzed 7 and 50 days after the 72-h reading to assess whether samples could be stored in the refrigerator without a change in the TNC measurement.

The data for each experiment were analyzed by the statistical analysis system (SAS, 1982) analysis of variance procedure (ANOVA), and treatment means were compared by least significant differences (LSD).

RESULTS AND DISCUSSION

PAHBAH, FCN, and CUI Procedures. Reagents for the PAHBAH procedure were mixed initially according to the method of Davis (1976), who used 5.0% PAHBAH and 0.5 M NaOH to measure tobacco reducing sugars on a Technicon autoanalyzer (Tarrytown, NY). In our procedure, precipitation resulted and caused in-line clogging; consequently we found 2.0 M NaOH more suitable with 2.5% PAHBAH.

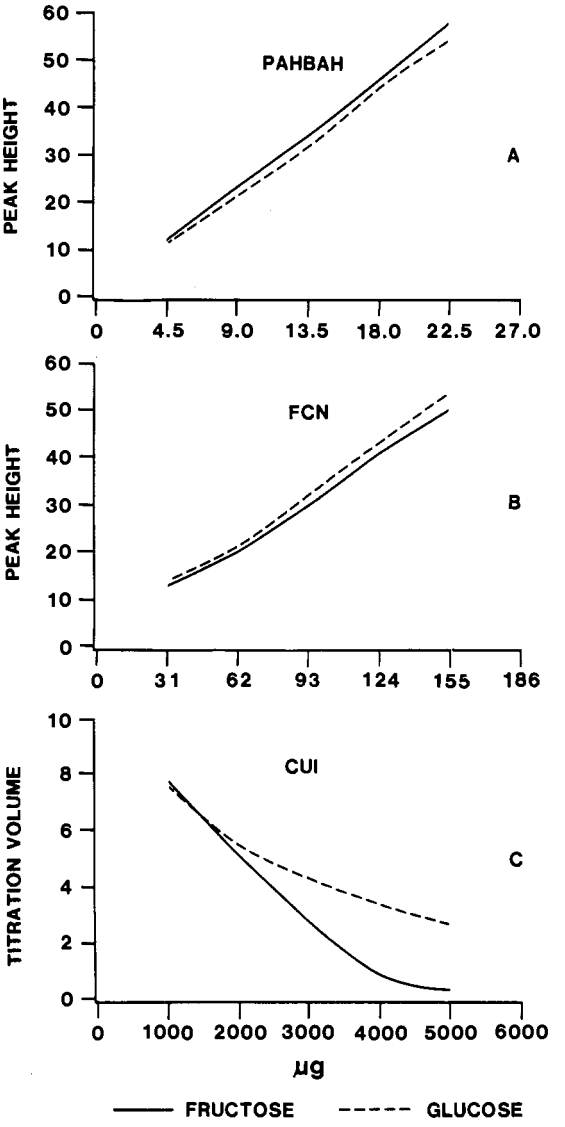

Figure 3. Glucose and fructose standard curves for *p*-hydroxybenzoic acid hydrazide (PAHBAH), potassium ferricyanide (FCN), and the copper iodometric (CUI) methods. Analyzed volumes of standards were 45 µL for PAHBAH, 300 µL for FCN, and 10 mL for the CUI procedures.

Lever (1972) measured low levels (<1 µg) of glucose using PAHBAH–glucose colorimetry. Our procedure resulted in automatic injection of 45-µL solutions that ranged from 100 to 700 mg/L of glucose and fructose and amounted to 4–32 µg of reducing sugar. Glucose measured about 6% lower than fructose (Figure 3A). According to the flow rates indicated in Figure 1, PAHBAH and sodium hydroxide concentrations would be 0.37% and 0.46 M, respectively, after mixing, compared to 0.5% and 0.5 M used by Lever (1972).

In the FCN procedure reagents were similar to those used by Wolf and Ellmore (1975) who used a FCN method with a Technicon autoanalyzer. The final concentrations of reagents after mixing according to the flow rates in Figure 2 were 0.92 mM and 0.039 M for potassium ferricyanide and the acetate buffer, respectively. We measured reducing sugars (sample loop volume was 300 µL) in the range of 30–220 µg. In Figure 3B, glucose response is about

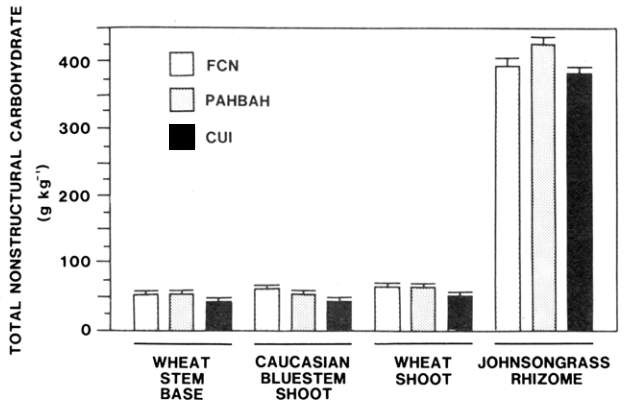

Figure 4. Total nonstructural carbohydrate comparison of dried, ground herbage using potassium ferricyanide (FCN), *p*-hydroxybenzoic acid hydrazide (PAHBAH), and copper iodometric (CUI) procedures. Values shown are the means of six replicates. Error bars indicate 95% confidence interval.

6% higher than fructose and divergence of glucose from fructose is not evident.

Smith (1981) suggested that fructose standards be used for fructan accumulators and glucose standards be used for starch or sucrose accumulators in the range of 1000–3000 μg of reducing sugars. Figure 3C supports Smith's (1981) implication that glucose and fructose do not have equal reducing power in the standard range for the CUI procedure.

For all tissues analyzed, FCN and PAHBAH tended to yield higher TNC values than the CUI method (Figure 4). Repeatability of results [measured as standard deviation (SD)] for wheat shoots, wheat stem bases, and Caucasian bluestem shoots was greater for FCN (SD = 0.23–0.31) and PAHBAH (SD = 0.08–0.12) than for CUI (SD = 0.38–0.61) (Figure 4). For johnsongrass rhizomes, the precision was slightly higher for the CUI method with a SD of 1.10 compared to 1.20 and 1.31 for the FCN and PAHBAH procedures, respectively (Figure 4). When pooled across tissues the assays had coefficients of variation (% CV) of 4.65 (FCN), 4.60 (PAHBAH), and 6.14% (CUI).

Since plants may have large amounts of starch, fructan, or sucrose, accuracy of TNC measurements would be increased by procedures having spectrophotometric equivalence between glucose and fructose. The FCN and PAHBAH procedures do not require prior knowledge of the predominant storage polysaccharide as is suggested for the Shaffer–Somogyi CUI technique (Smith, 1981). Since a starch-storing plant may have a high proportion of sucrose in its tissue during some phase of its annual growth, the accuracy of TNC measurement would be enhanced with glucose and fructose reducing power equality, as sucrose is a disaccharide composed of glucose and fructose.

Carbohydrate Extraction and Hydrolysis. We chose 10 mL of boiling 2.5 mM H_2SO_4 to hydrolyze sucrose and fructans because Groteleuschen and Smith (1967) showed that acid concentrations of 0.4 M H_2SO_4 resulted in fructose degradation and partial structural carbohydrate hydrolysis, while 2.5 mM H_2SO_4 hydrolyzed fructans and sucrose but left structural carbohydrates intact. They measured reducing sugars in timothy (*Phleum pratense* L.) and alfalfa (*Medicago sativa* L.), a fructan accumulator and starch accumulator, respectively. We measured recovery of reducing sugars upon hydrolysis of sucrose, inulin, and corn starch by 2.5 mM H_2SO_4 (Table II).

There were no statistical differences between 40- and 60-min hydrolysis times for the inulin, sucrose, and starch. After 60 min of hydrolysis in 10 mL of boiling 2.5 mM H_2SO_4, the recovery of 200 and 500 mg/L inulin was 88 and 86%, respectively, while sucrose was 102 and 104% (Table II). Only a small fraction (2–7%) of starch was hydrolyzed during the 60-min period. Recoveries of reagent grade glucose from the 200 and 500 mg/L concentrations were 100 and 101%, while fructose values were 106 and 107%, respectively (Table II).

Table II. Effect of Boiling 2.5 mM H_2SO_4 on Hydrolysis of Sucrose, Inulin, and Starch and on the Stability of Glucose and Fructose at Two Concentrations

	reducing power, mg/L				
time, min	sucrose	inulin	starch	glucose	fructose
	Carbohydrate Source (200 mg/L)				
0	15	10	8	201	208
20	188	186	13	200	194
40	211	193	8	203	205
60	214	193	16	200	211
LSD(0.05)	33	17	10	13	13
	Carbohydrate Source (500 mg/L)				
0	12	25	12	515	517
20	452	455	13	510	516
40	522	461	14	515	519
60	548	474	13	506	534
LSD(0.5)	31	17	9	26	28

Table III. Time Required for Amyloglucosidase To Hydrolyze Corn, Potato, and Wheat Starch Treated with and without Gelatinization

	reducing power starch source (250 mg/L)					
enzyme incubn	no gelatinization			gelatinization		
time, h	corn	potato	wheat	corn	potato	wheat
0	8	2	6	237	221	228
24	179	50	146	245	227	222
48	216	56	167	244	221	238
LSD(0.05)	9	5	8	12	9	15

Table IV. Concentration of Total Nonstructural Carbohydrate (TNC) in Wheat (WH), Johnsongrass (JG), and Caucasian Bluestem (CBS) after Four Periods of Incubation with Amyloglucosidase [Tissue Types Used: Shoots (SH), Stem Bases (SB), Rhizomes (RH)]

	plant and tissue, g TNC kg^{-1} dry matter				
incubn, h	WH SB	WH SH	JG RH	CBS SB	CBS SH
0	308	206	309	77	62
24	309	212	341	84	68
48	310	205	339	86	70
72	296	203	336	82	68
LSD(0.05)	13	19	13	7	6

Subsequent to gelatinization by 2.5 mM H_2SO_4, 80–89% of the corn, potato, and wheat starch was hydrolyzed by amyloglucosidase with no differences among 0-, 24-, or 48-h incubations (Table III). Conversely, when starches were not gelatinized, hydrolysis was incomplete following enzyme addition. Corn starch was 78% hydrolyzed after 48-h incubation while wheat and potato starch hydrolysis were 61% and 20%, respectively.

We analyze more than 300 TNC samples weekly and refrigerate samples for several days prior to reducing sugar analysis. Hence, an experiment was conducted to test storage effects on several different forage extracts. Refrigerated storage of extracts in capped plastic beaker cups at 1 °C did not alter TNC measurements up to 50 days after extraction (P = 0.14). Mean values across plant extracts were 191, 194, and 194 g kg^{-1} TNC after 0, 7, and 50 days of storage, respectively (LSD (0.05) = 5).

Although information concerning commercial starch hydrolysis by enzyme was provided (Table III), we felt different tissues should be extracted for TNC and optimum incubation times determined. Concentration of TNC in Caucasian bluestem and wheat shoots and stem bases

showed no significant differences among incubation times; however, johnsongrass rhizomes were significantly lower in percent TNC at 0 h when compared to 24, 48, and 72 h (Table IV). A 24-h incubation was necessary to allow effective penetration by the enzyme to sites of starch localization within the rhizome tissue.

Abbreviations: TNC, total nonstructural carbohydrates; PAHBAH, *p*-hydroxybenzoic acid hydrazide; FCN, potassium ferricyanide; CUI, copper iodometric.

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Registry No. PAHBAH, 5351-23-5; amyloglucosidase, 9032-08-0; sucrose, 57-50-1; D-glucose, 50-99-7; D-fructose, 57-48-7; starch, 9005-25-8.

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Effect of Hydrolysis on Sapogenin Release in Soya

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The effect of various hydrolysis procedures on the sapogenin yield and profile of soya saponins was investigated. Hydrolysis for 3 h with 3% sulfuric acid in an anhydrous methanolic environment gave the highest yield of total sapogenins and also only liberated soyasapogenols A and B. The results show that soyasapogenols B_1, C, D and E are artifacts of the hydrolysis procedure employed.

Saponins are triterpene or spirostan glycosides that hydrolyze to liberate an aglycon (generally referred to as a sapogenin) and the composite sugar residues. Five sapogenins have been isolated from soya: soyasapogenols A, B, C, D, and E. Smith et al. (1958) proposed structures for soyasapogenols A, B, and C, Cainelli et al. (1958) proposed a structure for soyasapogenol D, and Willner et al. (1964) isolated and characterized a further sapogenin, soyasapogenol E. The originally proposed structures for soyasapogenols A, B, and E have recently been revised by Kitagawa et al. (1982). These sapogenins together with soyasapogenol C are shown in Figure 1.

We have recently reported a method for the analysis of soybean sapogenins by normal-phase, high-performance liquid chromatography (HPLC) and mass detection (Ireland and Dziedzic, 1985). Our work identified a previously unknown sapogenin, probably similar in structure to B, which we tentatively named soyasapogenol B_1. Of the five soya saponins that have been isolated and their structures elucidated, three contain soyasapogenol B and two soyasapogenol A as the aglycon (Kitagawa et al., 1976; Kitagawa et al., 1985 a,b). Saponins containing soyasapogenols C, D or E as the aglycon have not yet been isolated, and it has been suggested that some or all of these sapogenins may be artifacts (Dziedzic and Ireland, 1985; Heftmann et al., 1979).

Since sapogenin concentration is often used as a measure of the saponin concentration, it, therefore, seemed pertinent to examine how hydrolysis conditions affect sapogenin concentration and profile.

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EXPERIMENTAL SECTION

Extraction of Saponin Fraction. A single, defatted soya flour (Arkasoy 50; British Arkaday Co. Ltd., Manchester, U.K.) was used throughout this study. A sample of milled, defatted soybeans (USDA Grade II) was also used to monitor the effects of the hydrolysis procedures using aqueous sulfuric acid/1,4-dioxane and sulfuric acid in methanol. The samples were subjected to a 36-h methanol Soxhlet extraction to obtain an extract containing the saponin fraction. The saponin extracts were then hydrolyzed by the different hydrolysis methods outlined below.

Isolation of Standard Soyasapogenols A and B. Standard samples of soyasapogenols A and B were isolated and identified as previously described (Ireland and Dziedzic, 1985).

Effect of Time of Hydrolysis on Sapogenin Yield and Profile. Duplicate samples (15 g) of the flour were extracted, the methanol was evaporated under reduced pressure, and the extract obtained was dissolved in 133 mL of 3 N H_2SO_4 in 1,4-dioxane/water (1:3, v/v) and hydrolyzed for either 30 min or 1, 2, 4, 6, 9 or 12 h under reflux. After hydrolysis the samples were cooled, diluted with water (100 mL), and extracted with diethyl ether (200 mL). The hydrolysis solution was extracted with two further portions of diethyl ether (2 × 100 mL), and the combined ether extracts were washed with 2% aqueous KOH (m/v, 2 × 100 mL) and water (2 × 100 mL). The sapogenin extract was evaporated under reduced pressure and dis-

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