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Cereal and commercial grass-seed straws are abundant plant biomass materials that are currently being considered for application in industrial processes aimed at the production of biofuels, biomaterials, and biochemicals. The carbohydrate fraction of these straws is of particular interest in this context due to its chemical functionality and relative ease of fermentation. Thus there is a global effort to develop economical methods through which the carbohydrate fractions of straws may be obtained in a readily utilizable form. The research presented in this thesis is designed to contribute to that effort by determining appropriate conditions for the analysis of the fructose-equivalent content of water extracts of straw. The basis of the method is to hydrolyze the parent fructose-

containing poly/oligosaccharides and then quantitatively measure the resulting fructose. The focus of the study is the hydrolysis, which is to be done using conditions similar to those used for the analysis of the other neutral sugars in biomass materials. Experiments were done using model fructans and water extracts from a representative wheat straw and Tall Fescue straw. The results demonstrate that the standard conditions used for the analysis of raw biomass (minimum acid concentrations for hydrolysis being 4% H_2SO_4) are too severe for the quantification of fructans; under these conditions greater then 50% of the fructose is degraded. It is shown that fructans are effectively hydrolyzed at acid concentrations as low as 0.1%. Application of the method demonstrates that fructose makes up a significant percentage of the total neutral sugars extracted with hot water from either of these straws.

Quantification of Fructose Equivalents in Straw Extracts

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Eugene Kim, Author

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1. INTRODUCTION

There is widespread interest in obtaining alternatives to petroleum-based products, including fuels, chemicals and materials. One potential source of alternative products is plant biomass, such as the cereal and grass seed straws that are evaluated in this study. Straw biomass may be used “as is”, e.g. fibers in composite materials, or converted to industrially relevant “biochemical” products, including fuels. Processes that aim to convert biomass to relevant industrial products are typically dependent on the application of relatively inexpensive unit operations that can economically generate utilizable components. Carbohydrates are among the most utilizable of the biomass components due to their high degree of functionality for chemical processing and their ease of utilization in biological/microbial processes. Thus, the carbohydrate fraction of biomass is typically viewed as being the predominant industrially relevant starting material in biomass (hence the relatively recent term “Carbohydrate Economy”). When considering the carbohydrate fraction of biomass, it is reasonable to focus on

aqueous extraction methods, making use of the relatively high water-solubility of low molecular weight carbohydrates, for obtaining readily utilizable/fermentable sugars.

A number of studies have evaluated the extraction and fractionation of the carbohydrate fractions within cereal and grass straws. Only a limited number of these studies focused on water as the sole solvent for carbohydrate recovery. Within this group of papers, several have considered fructan/sucrose/fructose recovery. This is important because they make up a significant fraction of the total water-soluble carbohydrate in these species and they are of primary concern to the present project. It is generally accepted that the low molecular weight fructans and the sucrose of plants, which are viewed as being a part of the plant's carbohydrate reserves, are water soluble and that the quantities of these components change with the age and the environmental state of the plant (Griffith, 1992; Griffith, 2000; Mancilla-Margalli and Lopez, 2006).

Several methods have been used to assess the total amount of water-soluble carbohydrates in straws. It is clear that the straw should not be oven-dried prior to analyses, as this result in significant decreases in water-soluble yields (Kerepesi and Boross, 1996). The term water-soluble may be viewed,

from a research perspective, in several ways – two of which are most relevant to the current study. In some cases "water-soluble" refers to those components that are extracted into water (typically defined temperature and time) following an organic solvent extraction (Xu et al., 2007). In other cases the water extraction is done on the raw material prior to any pretreatment (other than particle size reduction; Thammasouk et al., 1997). The resulting soluble fructans/sucrose/other poly/oligosaccharides are typically hydrolyzed using hydrochloric (Slominski et al., 1993) or sulfuric (Chen et al., 2007) acids as catalysts. The resulting monosaccharides are then quantified by liquid chromatography (anion exchange, Xu et al., 2007; or ion-mediated, Thammasouk et al., 1997) or gas liquid chromatography (Lawther et al., 1995).

Fermentation-based biomass utilization schemes are most typically based on the utilization of biomass-derived monosaccharides. Thus, low cost operations that result in high yields of fermentable sugars are essential to such schemes. As stated above, aqueous-based processes would appear to be ideal for such applications. When considering the efficiency of such operations it is important to have an estimate of the amount of fermentable sugar that is theoretically available in the starting material (the biomass feedstock) and the

resulting water extract. In the present study we have focused on the development of methods for assessing the amount of fructose that is theoretically available in aqueous extracts of straw feedstocks. Specifically, the objective of the present study was to determine appropriate acid (H_2SO_4)-catalyzed hydrolysis conditions for the quantification of fructose-equivalents in straw extracts; the conditions to be considered in the context of established assays for other neutral sugar equivalents in biomass.

The hypothetical basis of this study is that relatively low acid concentrations are preferable for the quantification of fructose equivalents in straw extracts resulting from the presence of water-soluble fructosyl-containing poly/oligosaccharides.

2. LITERATURE REVIEW

BIOMASS AS AN ALTERNATIVE FUEL

High gasoline prices, global warming, national security, and the limitations of global petroleum resources have regenerated worldwide interest in renewable resources as a feedstock for liquid transportation fuels, particularly those derived from cellulose. Production of fuels and commodity chemicals from low-cost cellulosic would provide unparalleled environmental, economic, and strategic benefits (Liu and Wyman, 2003).

Biomass has been shown to be a considerable good source of raw material for gaseous fuels, and certain petrochemical intermediates (Shah et al., 1984). As an alternative resource, biomass is a promising choice not only because it is abundant, but also because it has the benefit of being renewable and sustainable source of energy. More importantly, this renewable resource is cheap (because it has no competing food values), readily available, hardly increase in atmospheric carbon dioxide, and contribute to solve the problem of waste disposal (White and Plaskett, 1981; Spilda et al., 1991).

Biomass refers to the mass of substance generated by the growth of living

organisms, including micro-organisms, plants and animals (Wayman and Parekh, 1990). Specifically, lignocellulosic or cellulosic biomass refers to agricultural and forestry residues, such as corn stover or wheat straw, herbaceous and woody plants, and even municipal solid waste. It is the most abundant renewable resource on earth (Liu and Wyman, 2003).

World production of biomass is estimated at 146 billion metric tons a year, mostly wild plant growth. Biomass can be used directly (e.g. burning wood for heating and cooking) or indirectly by converting it into a liquid or gaseous fuel through bioconversion process (e.g. alcohol from sugar crops or biogas from animal waste). The net energy available from biomass when it is combusted ranges from about 8MJ/kg for green wood, to 20 MJ/kg for dry plant matter, to 55MJ/kg for methane, as compared with about 27 MJ/kg for coal. One analysis done by the United Nations Conference on Environment and Development (UNCED) has estimated that biomass could potentially accommodate about half of the present world primary energy consumption by the year 2050 (Demirbas, 2001).

The basic process steps in producing ethanol from biomass are (1) Pretreatment to make the cellulose and hemicellulose components more accessible

and to remove any impurities that may hinder any further processing steps (2) Hydrolysis to break down the polymers into their primary sugars by using acids or enzymes (3) Fermentation of the six-carbon and/or five-carbon sugars into ethanol using microorganisms that convert sugars to ethanol (4) Separation and concentration of ethanol that was produced by fermentation (Kerstetter and Lyons, 2001).

Biomass is different from other alternative energy sources in that the resource is varied, and it can be converted to energy through many conversion processes. Biomass resources can be divided into three major categories such as wastes, forest products and energy crops. Energy crops include short rotation woody crops, herbaceous woody crops, grasses, starch crops (corn, wheat and barley), sugar crops(cane and beet), forage crops (grasses, alfalfa and clover)or oilseed crops(soybean, sunflower safflower) (Demirbas, 2001).

AGRICULTURAL RESIDUE AND ENERGY CROP

The Gramineae (Poaceae-more recent name) or grass family is one of the largest and by far the most important family of flowering plants containing about 600 genera and 10,000 species. Major types of this grass family are cereals such

as wheat, rice, maize (corn), barley, and most of the forage and concentrated feeds consumed by domestic animals (Jones, 1985).

Wheat (*Triticum spp.*), one of main grain crop, is very important food sources and many other food products in western countries and food in east Europe and Asia. It is the largest cereal crop, at 462 million tones in 1981 being 28% of the total production of the eight major cereals (Wayman and Parekh, 1990).

After harvesting, the grain is removed from wheat and the leftover such as straw and chaff remain as potential biomass. The annual production of straw and chaff is enormous; 1.5-4 tonnes of straw/tonne of grain and 100-150kg/tonne of chaff. These consist mostly of cellulose and hemicellulose; straw, for example, contains 73% polysaccharides, with about equal quantities of cellulose and hemicellulose. Chaff contains 68% polysaccharides of which 45% is cellulose (Wayman and Parekh, 1990). In addition, wheat straw contains about 37% glucose, 21% xylose, 17% lignin, 10% ash and 12% extractives (Kerstetter and Lyons, 2001).

On a worldwide basis, straw and chaff as well as stover have the greatest potential for motor fuel supply among present crops. If half of all straw and

chaff were collected, hydrolysis and fermented alcohol production would amount to 480000 million 1/year, equivalent to 3000 million barrels of oil, about 15% of all crude oil production (Wayman and Parekh, 1990).

Tall fescue (*Festuca arundinacea*), a perennial, cool-season bunchgrass, is grown as forage crops for pasture, hay, and silage. In the United States, there are about 20 species, all perennial (excluding Vulpia). Tall fescue is adapted to a wide range of climatic conditions and is heavily used in the central and southeastern United States and is the only cool-season grass that can persist in many parts of the south. Large quantities of tall fescue are produced for seed in Oregon, in addition to its use as a pasture grass throughout Washington and Oregon. It is one of the most drought-tolerant forage grasses, is also tolerant of poor drainage, alkalinity and salinity (Hannaway et al., 1999).

A paper investigated on chemical composition of Tall fescue of different maturity stages resulted as following: protein, 10.42-11.51%; ether extract, 2.92-3.54%; soluble ash,5.98-6.09%; Lignin,6.08- 6.47%; cellulose,28.34-29.36%; crude fiber,29.29-31.12%; reducing sugar,3.74-4.17%; sucrose,4.51-7.36%; total sugars, 8.25-11.53%; fructosan , trace amount to 1.35%, depending on the degree of maturity attained by the grass at the time of cutting (Phillips et al, 1954).

Switchgrass (*Panicum virgatum* L.) is a sod forming, warm season grass, which is an important component of the native, highly productive North American Tallgrass Prairie. It is one of several warm-season forage grasses that have been identified as potential biomass crops in North America including eastern Canada. It has high yields, nutrient usage efficiency, and wide geographic distribution as well as its positive environmental attributes (Madakadeze et al., 1999; McLaughlin and Walsh, 1998).

COMPOSITION OF LIGNOCELLULOSIC BIOMASS

Lignocellulosic biomass composed mainly of cellulose, hemicellulose, lignin and extraneous substances, often referred to as extracts (Madan et al., 1983). The relative amount of each of three major structural components varies greatly depending on the biomass source (Wayman and Parekh, 1990). The typical composition of lignified cell walls is about 35-40% cellulose, 20-25% hemicellulose, and 20-25% lignin (Lewin and Goldstein, 1991). Monosaccharides commonly present in structural polysacchrides are aldohexoses (D-glucose, D-galactose, D-mannose), aldopentose (D-arabinose, D-xylose), and uronic acids.

Approximately, 5% of the material in cell wall consists of minor, non-structural components that are categorized as extracts and mineral substances. Examples of the extracts are waxes, fats, gums, tannins, and soluble sugars. Important mineral elements in cell walls are Ca, P, and Si. Protein in plants presents in small amount as glycoproteins rich in hydroxyproline, arabinose and galactose (Jung et al., 1993).

Nonstructural carbohydrate content and type depends on the plant species, plant part, stage of development, and environmental conditions such as root and shoot temperatures during growth as well as light intensity and duration, plant nutrient availability, and water status (Chatterton et al., 2006). Herbaceous crops contain much more minerals, proteins, soluble sugars and other water soluble extracts than do hardwoods (Torget et al., 1990). Also, a low content of pectic substances is in grasses. This group of amorphous polysaccharide consists of polygalacturonic acids, rhamnose, arabinose and galactose.

Cellulose

Cellulose is a linear polymer composed of anhydro-D-glucopyranose units linked by ß-1, 4 glycosidic bonds. The degree of polymerization is

between 5,000 to 10,000 (Brett and Waldron, 1990). These long molecules are hydrogen bonded to their neighboring linear polymers and form a crystalline lattice which results in a structure with very high tensile strength. However, cellulose is paracrystalline, having both crystalline and amorphous regions (Sjostrom, 1993). Because of the high degree of H-bonding and crystalline structure, cellulose is quite resistant to the chemical reaction and is insoluble in water or in common solvents. It dissolves in strong acids (72% H_2SO_4, 85% H_3PO_4, and 41% HCl) or in alkaline copper solutions (Wayman and Parekh, 1990).

The glucose subunits obtained from cellulose saccharification (enzyme or acid hydrolysis of the cellulose) can be converted to ethanol by yeast fermentation. Saccharification of cellulose to glucose can be accomplished by using cellulose enzyme systems, such as those produced by the fungus *Trichoderma reesi* and *Aspergillus niger*. Then, glucose is converted to ethanol by fermentation with *Saccharomyces cerevisiae*, a common brewer's yeast. The saccharification and fermentation can take place separately in a separate hydrolysis and fermentation process (SHF) or simultaneously in a process called simultaneous saccharification and fermentation (SSF) (Wayman and Parekh, 1990).

Hemicellulose

Like cellulose, most hemicelluloses function as supporting material in the cell walls. In contrast to cellulose, hemicellulose is branched and heterogeneous polysaccharide with a lower degree of polymerization than cellulose, approximately 150 to 200 molecules. It is branched with glucose, arabinose, mannose, galactose, and uronic acid side chains and does not form crystalline regions. The composition of hemicellulose varies greatly among cell types and species. Gramineae hemicellulosic structures in grasses vary considerably, depending on the species and tissues (Coughlan and Hazlewood, 1993). Xylan as present in cell walls of monocotyledon (grasses and cereals) consist of linear chains of β-D-(1,4)-linked D-xylopyranosyl residues, which can generally be substituted with α-L-arabinofuranosyl at the 2-*O* and/or 3- *O*-position(s) and α-D-glucopyranosyl uronic acid or its 4- *O*-methyl derivative at the 2- *O*-position (Kabel et al., 2005).

Some hemicelluloses are readily hydrolyzed to their monomeric sugars, acetic acid, and other compounds at 185˚C under steam or at lower temperature under mild acidic conditions (Wayman and Parekh, 1990). Saccharification and subsequent fermentation of the xylan backbone can be performed using xylanse

enzymes and the xylose fermenting yeast, *Pichia stiptis* (Wayman et al., 1987).

Extracts

Extracts or extraneous components are those substances which are removed from lignocellulosic cell walls by extraction with neutral solvents. These materials are deposited after cell wall formation and are not considered as essential structural components of the cell wall (Soltes, 1983). Extracts can be classified as follows (Lewin and Goldstein, 1991):

1. Volatile materials, e.g., terpenoids and related compounds
2. Non-volatile resinous extracts, e.g., resin acid, fatty acids, and unsaponifiable substances
3. Phenolic extracts, e.g., stilbenes, lignans, tannins, and flavonoids
4. Soluble carbohydrates and other polar extract, e.g., monosaccharides, sucrose, arabinogalactans, pectins, cyclitols, and low molecular weight carboxylic acids

RESERVE CARBOHYDRATE

When sugar production exceeds the energy requirement of the plant for

growth and development, they are converted into storage, or reserve carbohydrates in vegetative (non - reproductive) tissue of grasses (Longland and Byrd, 2006). Reserves of attainable carbohydrates are crucial to survival and to the production of plant tissues during periods when carbohydrate utilization exceeds photosynthetic activity. Cool-season grasses (C3 plants), those of temperate origin, grown under cool temperature, accumulate soluble sugar, starch, and fructan (oligo and polyfructosyl sucrose), whereas warm-season grasses (C4 plants) accumulate soluble sugars and starch but no fructan as their reserve carbohydrate (Chatterton et al., 2006).

The sum of the simple sugars and fructans comprise the water-soluble carbohydrate (WSC) fraction of the plant, and non-structural carbohydrate (NSC) or reserve carbohydrate consists of WSC and starch. Typically, warm season grasses have lower NSC contents than cool season species. Generalizations about NSC concentrations in forages are often difficult because of the many environmental and plant growth related factors that influence carbohydrate metabolism and accumulation in forage. It is often assumed that mature plants are higher in fiber and lower in NSC content than immature plants (Longland and Byrd, 2006).

Fructan

Fructans are oligomers or polymers with ß-fructofuranosyl residues, commonly water soluble and synthesized from sucrose accumulation in the vacuole (Margalli and Lopez, 2006). It is known to be used as short term carbohydrate storage, for osmoregulation of cellular activity, adaptation to low temperature photosynthesis, and indirect protection from freezing stress of the plants (Livingston III, 1990).

Chemically, fructan molecules can be described in a simple way by the formula GF_n, in which G = glucosyl unit, F = fructosyl unit, and n-number of fructosyl units linked ($n \geq 2$). Fructans also contain minor amounts of F_n fructans, in which the glucosyl end-unit is not present, and branched molecules. According to the way that ß-fructofuranosyl units are linked, five major types of fructans can be identified: (1) linear inulin with ß(2-1)-fructofuranosyl linkages, widely described in *Asteraceae*, (2) levan (or phlein) with ß(2-6) linkages found in grasses like *Phleum pratense*, (3) graminans, which are mixed fructans containing type (1) and (2) linkages, (4) inulin neoserie, which contains a glucose moiety between two fructofuranosyl units extended by ß(2-1) linkages, characterized in onion and asparagus , and (5) levan neoserie, formed by ß(2-1)-

and ß (2-6)-linked fructofuranosyl units on either end of a central sucrose molecule, which has been reported in oat. The fructans that occur in most cool-season grasses, including the small grains wheat, barley, and oat, are phleins. Those found in dicotyledonous plants are known as inulins. Graminan type fructans of mixed β (2-1) and β (2-6) structure are found in low concentration in grains of several grasses and cereals, particularly in rye and wheat (Chatterton et al., 2006; Paul et al., 2004).

Fructans are usually present in plants as a heterogeneous mixture with different degrees of polymerization (DP) and structures. The type of fructans found in plants and the presence of a particular type of fructan have been found to be species specific and highly influenced by the environmental condition as well as the developmental stage of the plant (Margalli and Lopez, 2006). Bonnett et al. (1997) has separated the supertribes of the Poaceae on the basis of the structure of the fructans that they accumulate.

Extraction of total available carbohydrate has been accomplished by acid hydrolysis or with water since fructan is soluble in water (Smith, 1964). The analytical methods measuring fructans quantitatively can be divided into two main groups: direct methods which measure the individual chain length of the fructans,

and indirect methods which measure the total amount of fructans by hydrolysis of fructans to glucose and fructose. GC and HPLC are commonly used to separate shorter fructans, such as kestose, but the lack of commercially available standards is a serious limitation on the quantification of the individual fructans (Livingstonelll, 1990).

EXTRACTION

Different kinds of solid-liquid extraction methods can be used for the early purification of natural products or extracts from plant materials. No one method removes all the extracts from the cell wall structure. As a result, extracts can be classified on the basis of their extraction or solubilization with cold, hot water, or neutral solvents such as alcohol, benzene, acetone, or ether. It should be noted that no single solvent is capable of solubilizing all extracts, and selection of the solvent must be based on the aim of the work.

Water extraction can remove compounds such as inorganic salt, sugars, polysaccharides, cyclones, cylitols, and some phenolic substances (NREL LAP 010). Water extraction is used widely in silage fermentation studies of both grasses and legumes, assuming that little or no starch exists in most herbage tissue

or that the starches are not hydrolyzed to sugars during the fermentation process. While amlyose is water-soluble, especially in hot water, amylopectin is not. Therefore, water extraction does not accurately estimate the total non-structural carbohydrate concentration when amylopectin starch is present in the sample (Smith, 1971).

Organic solvents can be use to extract resins, fatty acids, waxes, unsaponifiable substance, pigmets, etc. Soxhlet extraction utilizing 95% ethanol has been found to be effective as well as being non-toxic in comparison to benzene (NREL LAP 010). One difficult with organic solvents such as benzene and ethanol is that a significant amount of the solvent is absorbed by the biomass residue. The absorbed solvent is difficult to remove (TAPPI, 1988). Subsequent washing with hot water can remove the absorbed solvent but the small advantage gained can be offset by the loss of water soluble materials (Browning, 1967).

Temperature and duration of extraction are crucial factors which affect the amount and type of extract removed by a given solvent. It is found that the amount of substances soluble in water tends to increase with time, which may be a result of the solution becoming acidic from the hydrolysis and release of the

acetyl groups from the biomass (Browning, 1967).

Supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) and pressurized solvent extraction (PSE) are fast and efficient methods developed for extracting analytes from solid matrixes. MAE and PSE are emerging as attractive alternatives to conventional extraction methods such as Soxhlet extraction, maceration, percolation, digestion, sonication and some cases steam distillation.

The development of microwave assisted extraction was firs reported by Ganzler (1986) and co-workers. Unlike classical conductive heating methods, microwaves found to be heating the whole sample simultaneously. In the case of extraction, the advantage of microwave heating was the disruption of weak hydrogen bounds promoted by the dipole rotation of the molecules (Kaufmann and Christen, 2002). The applicability of microwave irradiation to the extraction of various types of compounds showed that microwave extraction method was more effective than the conventional methods (Ganzler et al., 1986).

Pressurized solvent extraction (PSE) has been developed as one of an alternative SLE technique to current extraction methods, offering benefits with respect to extraction time, solvent consumption, extraction yields and

reproducibility. PSE uses organic solvents at elevated pressure and temperature to increase the efficiency of the extraction process. Kaufman and Christen (2002) reported that “Increased temperature accelerates the extraction kinetics and elevated pressure keeps the solvent in the liquid state, thus enabling safe and rapid extraction.”

3. MATERIALS AND METHODS

MATERIALS

Tall fescue sample was provided by United States Department of Agriculture (USDA). Wheat straw was gathered from a local farm in Willamette valley. All samples were milled to pass a 20 mesh screen and stored at room temperature prior to analyses using a Tecator Cycltec 1093 sample mill (Eden Prairie, MN). Sugar standards: inulin, levan, sucrose, glucose, fructose, xylose, and arabinose were obtained from Sigma (USA). *Exo–Inulinase* (EC 3.2.1.80; fructan β-fructosidase) from *Aspergillus niger* was purchased from Megazyme (Ireland).

TOTAL SOLIDS/ MOISTURE

All analyses are calculated on a dry weight basis. Moisture content of the samples was thus determined using NREL Standard Biomass Analytical Procedure LAP 001. Approximately 1 g of sample was weighed to the nearest 0.1 mg into a tared, dried aluminum weighing dish and dried to constant weight which usually took 16 hours in a convection oven at 105±3˚C. The sample was

placed in a desiccator to cool to room temperature and then weighed again.

Calculation:

$$\text{Solid (\%) at } 105^{\circ}C = \frac{(\text{wt. sample plus dish} - \text{wt. dish})_{\text{after drying}}}{(\text{wt.sample plus dish-wt.dish})_{\text{prior to drying}}} \times 100$$

$$\text{Moisture (\%) at } 105^{\circ}C = \left[1 - \frac{(\text{wt. sample plus dish} - \text{wt. dish})_{\text{after drying}}}{(\text{wt. sample plus dish-wt.dish})_{\text{prior to drying}}}\right] \times 100$$

WATER EXTRACTION

Approximately 2 g of sample, weighed to the nearest 0.1mg, was placed in a 250ml screw-top Erlenmeyer flask containing 98g of double distilled pre-heated water. The flask was heated at 90˚C for 2 hours in thermo-regulated water bath with orbital mixing at 200 rpm. At the completion of the extraction, the contents were cooled and filtered using a Whatman filter paper No.1 (VWR, USA). Approximately 15-20 g of filtrate, weighed nearest to 0.1mg, was transferred to a pre-dried, weighed porcelain crucible and dried in convection oven at 105˚C for 12 hours for “solids” determination.

Calculation:

$$\text{Water Extractive (\%)} = \frac{\text{wt.crucible plus residue-wt.crucible}}{\text{total dried solid in raw sample}} \times \frac{\text{filtrate}_{\text{total}}}{\text{filtrate}_{\text{used}}} \times 100$$

HYDROLYSIS OF GLUCOSE/FRUCTOSE/SUCROSE

200mg of each glucose or fructose or sucrose were suspended in 200ml of distilled water and mixed for 5-10 minutes at room temperature. To 25ml of each solution was added; 21.3, 42.5, 107.1, 215.1, 433.5 and 885.8µl of 72% H_2SO_4 resulting in hydrolysis solutions that are 0.1, 0.2, 0.5, 1.0, 2.0, and 4.0% H_2SO_4 (wt H_2SO_4/wt total), respectively. The resulting solutions were mixed and autoclaved at 121˚C for 1 hour (American Sterilizer Company, PAT. 2470776).

HYDROLYSIS OF INULIN/LEVAN

Inulin or levan, 200 mg, was suspended in 200ml distilled water and mixed for 5-10 minutes at room temperature. The resulting suspension was filtered using Whatman 0.22µm GFC filter paper and the filtrate used as the inulin preparation. A qualitative test for soluble inulin was done by adding 4ml of 100% ethanol to 1ml of filtrate (final 80% ethanol, v/v). Upon mixing there was a precipitate that indicated the presence of polysaccharide. To 25ml of each filtrate was added; 21.3, 42.5, 107.1, 215.1, 433.5 and 885.8µl of 72% H_2SO_4, resulting in hydrolysis solutions that were 0.1, 0.2, 0.5, 1.0, 2.0, and 4.0% H_2SO_4 (wt H_2SO_4/wt total), respectively. The resulting solutions were mixed and autoclave at 121˚C for 1hour (same as above).

HYDROLYSIS OF EXTRACTS

Acid - catalyzed Hydrolysis

To 25ml of each Tall Fescue and Wheat Straw extracts were added; 21.3, 42.5, 107.1, 215.1, 433.5 and 885.8µl of 72% H_2SO_4 resulting in hydrolysis solutions that are 0.1, 0.2, 0.5, 1.0, 2.0, and 4.0% H_2SO_4 (wt H_2SO_4/wt total), respectively. The resulting solutions were transferred to Pyrex™ screw cap bottles (VWR) and autoclaved at 121˚C for 1 hour (same as above). After sample was cooled to room temperature, it was poured into 50ml Erlenmeyer flask and neutralized with calcium carbonate to pH 5-6, measured by pH meter (Oracle, USA). Precipitate was allowed to settle at 4˚C (refrigerator) for 1-2 hours. Calcium sulfate may remain in supersaturated state or finely dispersed state (Englis and Fiess, 1942) so would like to avoid precipitation following filtration. A small portion of the neutralized sample(following precipitation at 4˚C) was filtered through 0.22µm Acrodisc® syringe filter (Pall, USA) into an autosampler vial for HPLC analysis. A series of standards were prepared by mixing together pure carbohydrates in double distilled water to mimic the carbohydrate profiles of feedstock. Samples were all analyzed using Waters HPLC system with Aminex HPX-87P lead form, 300 x 7.8 mm (BIO-RAD, USA) column and refractive index detector. De-ashing catridges (BIO-RAD) containing a cationic H^+ catridge and an anionic CO^{3-} catridge were use as guard columns to remove salts from the samples. The conditions used for the analysis were:

Injection Volume: 20µl

Mobile Phase: Milli-Q grade H_2O

Flow Rate: 0.6ml/min

Column Temperature: 85˚C

Running Time: 50 minutes

Calculation:

$$\text{Sugar recovered (\%)} = \frac{\text{sample sugar peak area}}{\text{known conc. of sugar standard peak area}} \times 100\%$$

$$\text{Corrected Sugar concentration (mg/ml)} = \text{known conc. of sugar standard (mg/ml)} \times \frac{\text{sugar recovered (\%)}}{100(\%)}$$

Acid Solutions:

Table1 is provided as a reference for the interconversion of solutions expressed as percent H_2SO_4 and normality of H_2SO_4. Analogous values for HCl solutions are included because HCl, instead of H_2SO_4, is sometimes used as the acid-catalyst for fructan hydrolysis. The U.S. Department of Energy (NREL) standard procedure for determining the neutral sugar content of plant biomass is specified in terms of % H_2SO_4; so that convention was used in this study. Included in the table are the measured pH values for extracts that have been adjusted to the specified percent H_2SO_4. These values, when compared to the same values for water, demonstrate the buffering capacity that is inherent in the extracts.

Enzyme - catalyzed Hydrolysis

Exo–Inulinase (EC 3.2.1.80; fructan β-fructosidase) from *Aspergillus niger* (Megazyme) was used for the enzymatic hydrolysis experiments. The

activity of the stock enzyme preparation was determined by quantifying the amount of fructose generated in a reaction mixture containing 1% inulin or levan in a 0.1M sodium acetate (Sigma) buffer, pH 4.5 at 40˚C for 10 minutes (assay conducted as prescribed by enzyme manufacturer, Megazyme; AZFR5 11/99). Enzyme solution was diluted in 0.1M sodium acetate buffer and further diluted, as required to obtain the correct range of activity for assay. One unit (U) of enzyme activity is defined as the amount of enzyme required for hydrolyzing substrate (inulin or levan) to produce 1 μmol of fructose reducing-sugar/min under the standard assay conditions. In experiments testing for the presence of enzyme-susceptible fructans, 50 μl (18U, Megazyme; 15U, Laboratory assay) of enzyme solution was added to the reaction mixture (5ml of sodium acetate buffer + 5ml of extractive) and mixed using vortex and incubated for 30 minutes at pH 4.5 in 50˚C water bath. The reaction was terminated by incubating sample at 80-90˚C for 2-3 minutes and the sample was prepared for the analysis by HPLC following the same procedure described above in acid hydrolysis section.

STATISTICAL ANALYSIS

SPSS 14.0.2 for Windows was used to analyze for the statistically significant differences between treatments using a general linear model. The sources of observed difference were identified by Tukey's post hoc test. There were one control and three treatment groups: 0.2% acid treated, 2% treated, and enzyme treated group.

4. RESULTS

The quantification approach considered in this study is based on the hydrolysis of the glycosidic linkages of the existing poly/oligosaccharides and the subsequent quantification of the resulting monosaccharides by chromatographic methods. The quantification of fructose-equivalents using this approach is somewhat complicated due to the relative instability of the fructose monosaccharide. Figure 1 demonstrates the high temperature/high acid-instability of fructose relative to glucose. This instability is particularly relevant to biomass analyses in that the standard conditions used for the hydrolysis of the carbohydrate components of straw feedstocks, prior to quantification of the resulting monosaccharides, is 4% sulfuric acid at 121˚C for 1 hour (NREL LAP, 2002). The data of figure 1 shows that > 85% of free fructose is degraded under such hydrolysis conditions. The figure shows that, in contrast to fructose, glucose is relatively stable under these same conditions. The different degradation rates for each of the monosaccharides are taken into account in the final calculations of theoretically available monosaccharide-equivalents by including monosaccharide “controls” in all hydrolysis experiments. The extents

of degradation of the monosaccharide controls, over the course of hydrolysis, are assumed to equal the extents of degradation of the monosaccharides in the biomass feedstock. The control degradation values thus provide "correction factors" that are used to estimate total available monosaccharides. The data showing the extensive degradation for fructose under standard hydrolysis conditions suggests that alternative hydrolysis conditions should be considered.

Alternative hydrolysis conditions were considered in this study by following the hydrolysis of three model fructose-containing oligo/polysaccharides: sucrose, inulin and levan. Sucrose is a fructose-glucose polysaccharide (α-D-glucopyranosyl-(1→2)-β-D-fructofuranose), inulin is a fructan polymer with β-(2-1)-fructofuranosyl linkages , and levan is a fructan polymer comprised of β-(2-1)-fructofuranosyl linkages. The data depicted in figure 2 shows that the lowest acid concentration tested (0.1% H_2SO_4) was sufficient for complete hydrolysis of sucrose (based on lack of sucrose peak in subsequent HPLC). The data also demonstrates that the highest fructose yields were obtained at the lowest acid concentrations. This result is expected when the data of figure 1 is consider with reference to the observation that the sucrose/fructans appear to be completely hydrolyzed under the least severe

(lowest acid) hydrolysis conditions. It is worth emphasizing that the lower fructose yields at the higher acid concentrations are not due to incomplete sucrose hydrolysis, but due to increased conversion of the resulting fructose to degradation products. The data for inulin (Figure 3) and levan (figure 4) also demonstrate the ease with which the glycosidic linkages of the fructans may be hydrolyzed. Neither inulin nor levan poly/oligosaccharides could be detected following hydrolysis at the lowest acid concentrations tested. The fructose: glucose ratios in the hydrolysates obtained from inulin and levan are high due to their monomeric composition, provided the hydrolysis is done at the lower acid concentrations. The fructose:glucose ratios in the hydrolysates resulting from the more severe hydrolyses (higher acid concentrations) are lower due to the high rate of degradation of fructose relative to glucose.

The acid concentrations used in the experiments described above with model compounds were also applied to the analysis of hot-water (90^{o}C) extracts obtained from Tall Fescue straw and wheat straw. The data for both Tall Fescue straw (Figure 5) and wheat straw (Figure 6) demonstrate the same pattern as observed for the model compounds. Maximum fructose yields were obtained from samples hydrolyzed at the lowest acid concentrations. Comparison of the

sugar yields from the two straws also demonstrates that aqueous extractions of Tall Fescue straw yield significantly higher amounts of fructan than analogous extractions of wheat straw.

The analytical approach of quantifying total (theoretically) available monosasccharides by acid-catalyzed hydrolysis of the parent polysaccharides/oligosaccharides and subsequent quantification of the resulting monosaccharides may be considered with respect to the following reaction scheme that depicts the reactions that occur under hydrolysis conditions.

Poly/Oligosaccharide → Monosacchardie → Degradation Products

In view of this scheme, it can be seen that "perfect" hydrolysis conditions would be those for which there was complete conversion of the poly/oligosaccharides to monosaccharides with no degradation of the resulting monosaccharides. If this were the case, then all that remains would be the quantification of the resulting monosaccharides by any convenient method (HPLC, GLC, enzyme assay, etc.). Since "perfect" conditions for acid-catalyzed poly/oligosasccharide hydrolysis are heretofore unattainable, it is common to account for the extent of degradation by simultaneously "hydrolyzing" monosaccharide standards (and the use of "correction factors" as described above). This approach assumes that the extent

of degradation of the monosaccharide standards (which are monosaccharides throughout the hydrolysis period) is roughly equivalent to the extent of degradation of the monosaccharides resulting from poly/oligosaccharides hydrolysis (these monosaccharides being generated over the course of the hydrolysis period). The validity of this assumption is dependent on the relative rates of hydrolysis and degradation – but complete hydrolysis of the poly/oligosaccharides is assumed when using this approach. We previously discussed the data of figures 5 & 6 with respect to the maximum amount of fructose recovered. Considering the data in this way provides a first approximation of appropriate hydrolysis conditions. However, the reaction scheme depicted just above suggests that maximum fructose yields are not likely to coincide with the point of complete hydrolysis of the fructan poly/oligosaccharides. This is particularly important when considering that the analytical approach most commonly used, i.e. hydrolysis combined with the application of "correction factors" for monosaccharide degradation, assume complete poly/oligosaccharide hydrolysis. The importance of this issue led us to do a set of experiments to determine whether or not the low acid hydrolysis conditions, those corresponding to maximum fructose yields, actually resulted in

complete hydrolysis of the fructans.

The experiments testing the extent of hydrolysis of the fructan poly/oligosaccharides are based on the use of an enzyme preparation known to hydrolyze plant fructans (fructan β-fructosidase). A hot-water extract obtained from Tall Fescue straw was divided into three fractions, one fraction was not hydrolyzed, a second fraction was hydrolyzed at 0.1 % sulfuric acid and the third fraction was hydrolyzed using 0.2% sulfuric acid. Half of each of these fractions was subsequently treated with enzyme; the other half was not. Figure 7 summarizes glucose and fructose yields for these six treatments. The data shows that the enzyme treatment significantly increased the free glucose and fructose levels in the extracts that had not been previously acid-hydrolyzed. In contrast, the enzyme treatments had no discernable effect on extracts that had previously undergone acid-catalyzed hydrolysis, be it at either the 0.1% or 0.2% acid levels. The implication from this experiment is that there is essentially complete hydrolysis of the fructans present in the extracts when low acid hydrolysis conditions are used. Included in figure 7 are results obtained by monitoring what we believe to be the combined oligomeric “fructan” peaks. Comparison of the fructan peaks from the non-hydrolyzed samples with and without subsequent

enzyme treatments shows the expected decrease in fructans due to enzyme activity; although the data also indicates that not all of the assumed "fructans" are susceptible to enzyme-catalyzed hydrolysis under the conditions employed in this experiment. These same "fructan" peaks are absent from chromatograms of analogous extracts that have undergone acid hydrolysis. Further study is required to determine whether the peaks in question are actually fructans and, if so, why they are resistant to enzyme hydrolysis.

The results presented thus far may be used as a guide for selecting the most appropriate conditions for the hydrolysis of straw extracts when wanting to assess the total fructose-equivalents available in that extract. The most appropriate conditions are assumed to be those that result in complete hydrolysis of the fructose containing poly/oligosaccharides with minimal fructose degradation. The data herein suggests that hydrolysis at final concentrations of 0.2% sulfuric acid (121^{o}C, 1 hour) is appropriate for this purpose. It must be kept in mind that the optimum hydrolysis conditions for measuring fructose-equivalents may not coincide with the optimum hydrolysis conditions for assessing other monosasccharides, e.g. xylose-equivalents. It is generally recognized that furanosides (*e.g.* fructans) are hydrolyzed faster than pyranosides (*e.g.* xylans)

(BeMiller, 1967). This suggests that optimum conditions for xylose quantification are likely to be more severe than those for fructose quantification (keeping in mind the requirement for complete hydrolysis of the monosaccharide-containing parent poly/oligosaccharides). The reality of this is illustrated in the data presented in figure 6; the figure summarizes a series of analyses of wheat straw extract using conditions analogous to those discussed for extracts from Tall Fescue straw (data of figure 5). As with Tall Fescue straw, the fructose yields from wheat straw are at a maximum when hydrolyses are done using low acid concentrations – again suggesting that these lower acid concentrations are most appropriate for the measurement of total available fructose. However, as the severity of the hydrolysis increased, up to approximately 2.0% acid, there was a corresponding increase in free xylose. This suggests that the soluble xylans in the wheat straw extracts were not completely hydrolyzed under the relatively low acid conditions. (This was not observed with extracts from Tall Fescue straw due to the apparent lack of extractable xylans in that straw.) Thus, if one is interested in the quantification of the theoretically available xylose, or total neutral sugar content, of an extract, then the more severe hydrolysis conditions (2.0 % acid) are to be employed(Adams and Castagne,1951; Lawther et al.,1996).

The drawback of using these more severe conditions when quantifying fructose is that a relatively large correction factor will be required to account for fructose degradation (data of Figure 1 suggests that > 50% of the fructose is degraded under these conditions).

A comparison of the theoretically available (total equivalents) fructose and glucose in Tall Fescue straw and Wheat straw extracts, as determined by HPLC quantification of the monosaccharides resulting from different methods of hydrolysis, is presented in Table 2. The methods included the direct analysis of the extract (no hydrolysis), analysis of the extract following hydrolysis at 0.2 % acid, analysis of the extract following hydrolysis at 2.0 % acid, and analysis of the extract following enzyme-catalyzed hydrolysis. The results presented in this table have taken into account the appropriate "correction factors" for the different acid concentrations. The fructose values for the extract from Tall Fescue straw increase as a result of the application of any of the methods of hydrolysis, indicating the polymeric nature of the fructose-containing component. The acid-catalyzed hydrolysates gave higher values than the corresponding enzyme-catalyzed hydrolysates. The values for the fructose content of the hydrolysates resulting from the 0.2 % and the 2.0 % acid-treatments differ appreciably,

according to the statistical analysis presented in Table 3. However, we have greater confidence in the value obtained from the extract hydrolyzed at the lower acid concentration due to the greater response (higher sensitivity) of the detection system and the application of a smaller correction factor (both due to less fructose degradation during hydrolysis at the lower acid concentration). The glucose values for the hydrolyzed Tall Fescue extracts were all higher than for the non-hydrolyzed samples, and they appear to differ among the different hydrolysis treatments according to the statistical analysis shown in Table 3. The fructose and glucose values for the wheat straw were quite constant for all hydrolysis conditions as shown in Table 3; this is thought to be attributable to the relatively low amounts of these monosaccharides that can be extracted from wheat straw – thus pushing the limits of our quantification system.

5. DISCUSSION

Traditional quantitative determinations of the theoretically available carbohydrates in biomass preparations are based on the hydrolysis of the polysaccharides and oligosaccharides inherent in the preparations, to their constituent monosaccharides, and then quantification of the resulting monosaccharides. A complicating factor in such analyses is that sugar degradation is occurring as hydrolysis proceeds. Optimum conditions for hydrolysis are, thus, those that rapidly result in complete hydrolysis of the poly/oligosaccharides with minimum sugar degradation. The extent of degradation in such analyses is taken into account by a correction factor determined using the free monosaccharide. Extents of degradation of free monosaccharides may not directly correlate to extents of degradation for those monosaccharides that are being generated over the course of the hydrolysis treatment (as is the case during poly/oligosaccharide analysis). Thus, it is beneficial to run the acid hydrolysis step using as mild of conditions as possible to minimize monosaccharide degradation (Puls, 1993).

The most common catalyst for hydrolyses of this type is sulfuric acid. In the traditional method, soluble poly/oligosaccharides are hydrolyzed in 4% sulfuric acid at 121^{o}C for 1 hour (NREL LAP 002, 2005; Puls, 1993). As shown in this study, these conditions result in extensive degradation of fructose. The objective of the present study was thus to determine an appropriate acid (H_2SO_4) concentration for hydrolysis of soluble fructosyl-containing poly/oligosaccharides, such that the other parameters for hydrolysis (time, temperature) match those of the traditional assay. It was expected that lower acid concentrations could be used for the hydrolysis of fructosyl-containing poly/oligosaccharides due to the relative ease of hydrolysis of oligofructoses (Blecker et al., 2002) and previously used analytical methods based on the use of hydrochloric acid (Slominski et al., 1993; Negro et al., 2006). In the present study we identified an acid concentration of 0.5% sulfuric acid as appropriate for measurement of fructose-equivalents. This acid concentration is recommended because it was the minimum acid concentration necessary for complete hydrolysis of the fructosyl-containing poly/oligosaccharides in all samples analyzed. The presented data also indicates that 2% sulfuric acid is sufficient for hydrolysis of the other neutral monosaccharide-equivalents in the soluble phase. This acid concentration is to

be considered as an attractive alternative to the traditional 4% sulfuric acid due to the lower extents of sugar degradation under these conditions.

The results presented here suggest that the total neutral sugar-equivalent content of a straw extract is best determined by hydrolyzing, under traditional time/temperature conditions, a representative sample of the extract at 0.5% sulfuric acid (for quantification of fructose-equivalents) while simultaneously hydrolyzing a second representative sample at either 2 or 4% sulfuric acid (for the remaining neutral sugar equivalents).

6. CONCLUSION

In summary, it is recommended that the quantification of fructose-equivalents in straw extracts be done using lower acid concentrations than are currently used for the analysis of neutral sugars in biomass feedstocks. An acid concentration of 0.2 %, at 121^{o}C for 1 hour, appeared to effectively hydrolyze the hot water-extracted fructans in Tall Fescue straw and wheat straw and the degradation of fructose under these conditions appears to be less than 5 percent.

Figures and Tables

Figure 1. Stability of fructose and glucose while undergoing simulated hydrolysis at different acid concentrations. To pure fructose and glucose solutions (1 mg per ml water) was added sulfuric acid (as the independent variable in this experiment) and the solutions incubated at standard autoclave conditions (121^{o}C) for 1 hour. Solutions ranged in acid concentrations from 0 to 4.0 %. Fructose and glucose concentrations were determined at the end of the simulated hydrolysis periods; values are reported as percent of sugar recovered relative to non-hydrolyzed sample.

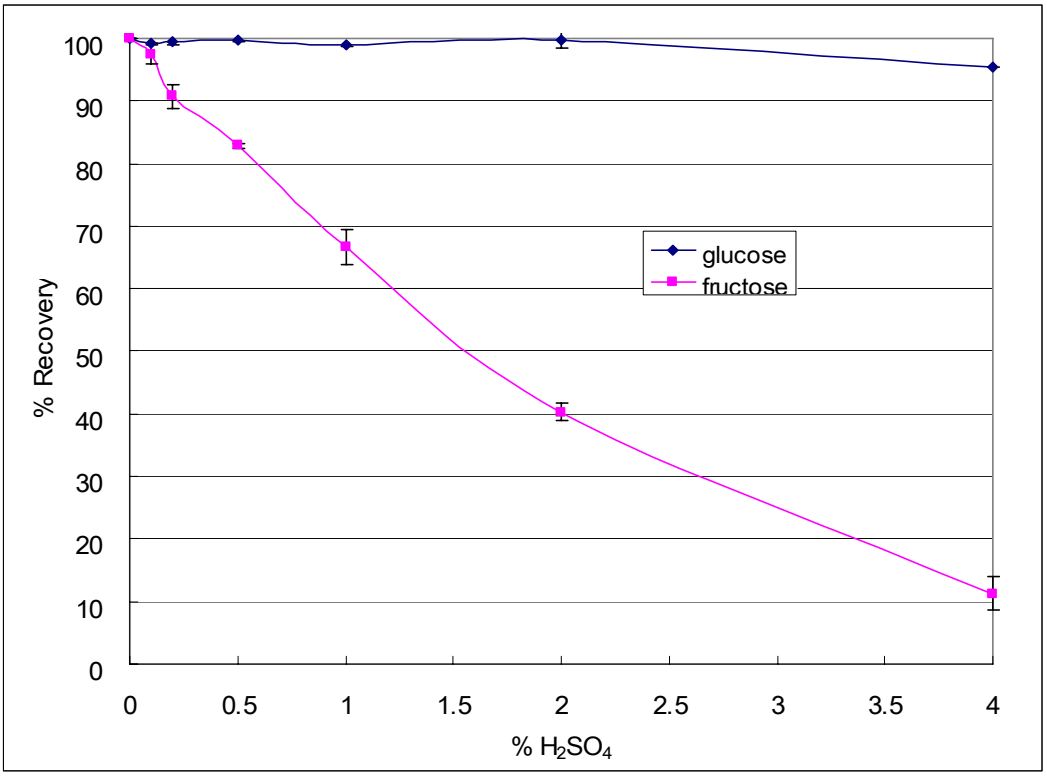

Figure 2. Hydrolysis of sucrose and concomitant recovery of glucose and fructose under hydrolysis conditions differing with respect to concentration of the acid-catalyst. To a pure sucrose solution (1 mg per ml water, 2.92 mM) was added sulfuric acid (as the independent variable in this experiment) and the solutions incubated at standard autoclave conditions (121°C) for 1 hour. Solutions ranged in acid concentrations from 0 to 4.0 %. Sucrose, fructose and glucose concentrations were determined at the end of the hydrolysis periods; values are reported as mM concentrations recovered following hydrolysis.

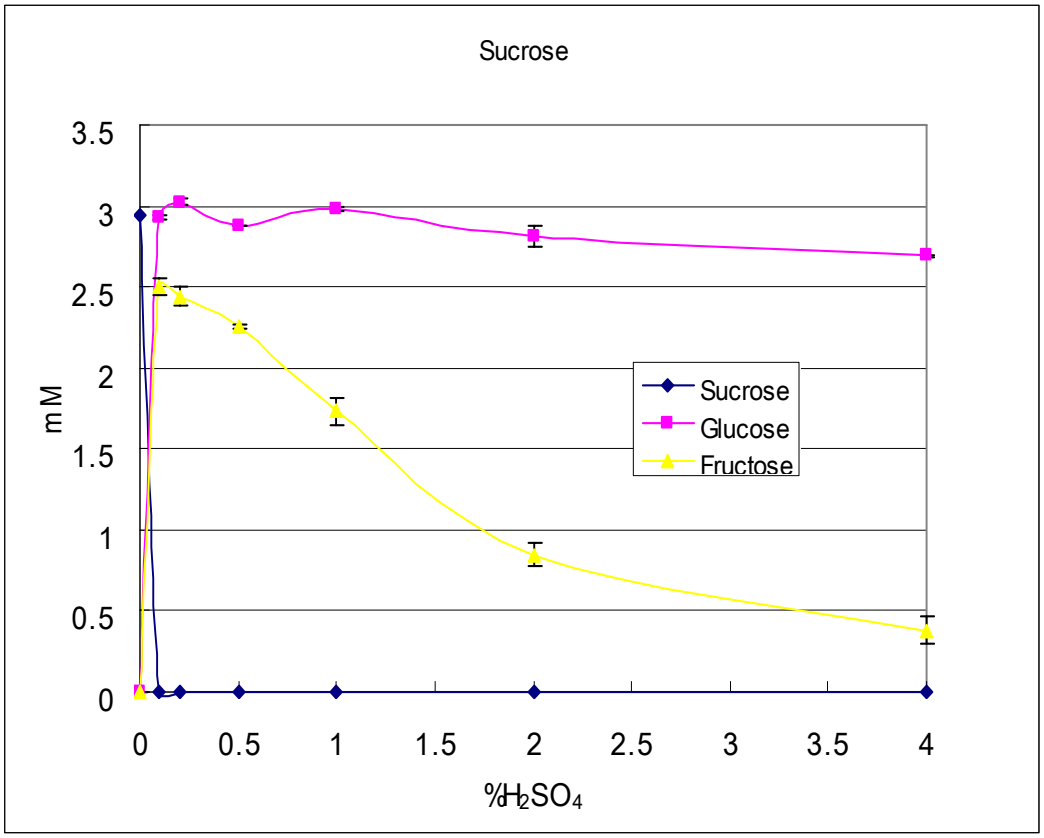

Figure 3. Hydrolysis of inulin and concomitant recovery of glucose and fructose under hydrolysis conditions differing with respect to concentration of the acid-catalyst. To a pure inulin solution (~1 mg per ml water) was added sulfuric acid (as the independent variable in this experiment) and the solutions incubated at standard autoclave conditions (121°C) for 1 hour. Solutions ranged in acid concentrations from 0 to 4.0 %. Inulin, fructose and glucose concentrations were determined at the end of the hydrolysis periods; values are reported as mg saccharide per ml recovered following hydrolysis.

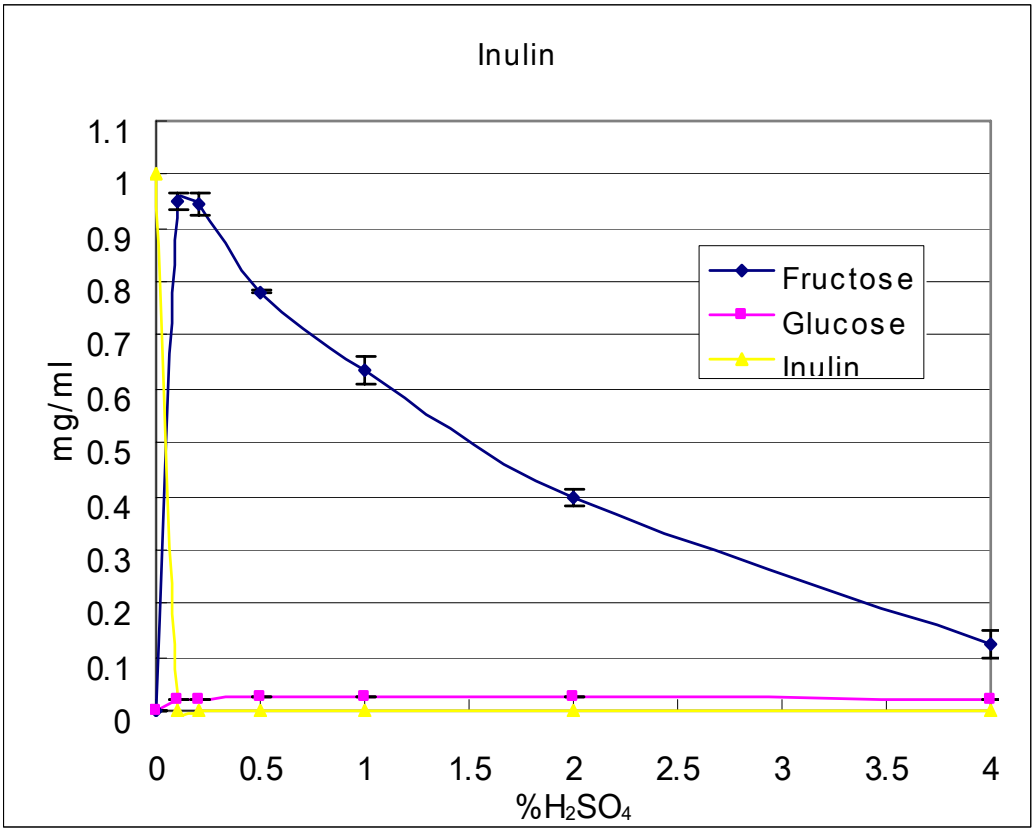

Figure 4. Hydrolysis of levan and concomitant recovery of glucose and fructose under hydrolysis conditions differing with respect to concentration of the acid-catalyst. To a pure levan solution (~1 mg per ml water) was added sulfuric acid (as the independent variable in this experiment) and the solutions incubated at standard autoclave conditions (121°C) for 1 hour. Solutions ranged in acid concentrations from 0 to 4.0 %. Fructose and glucose concentrations were determined at the end of the hydrolysis periods; values are reported as mg monosaccharide per ml recovered following hydrolysis.

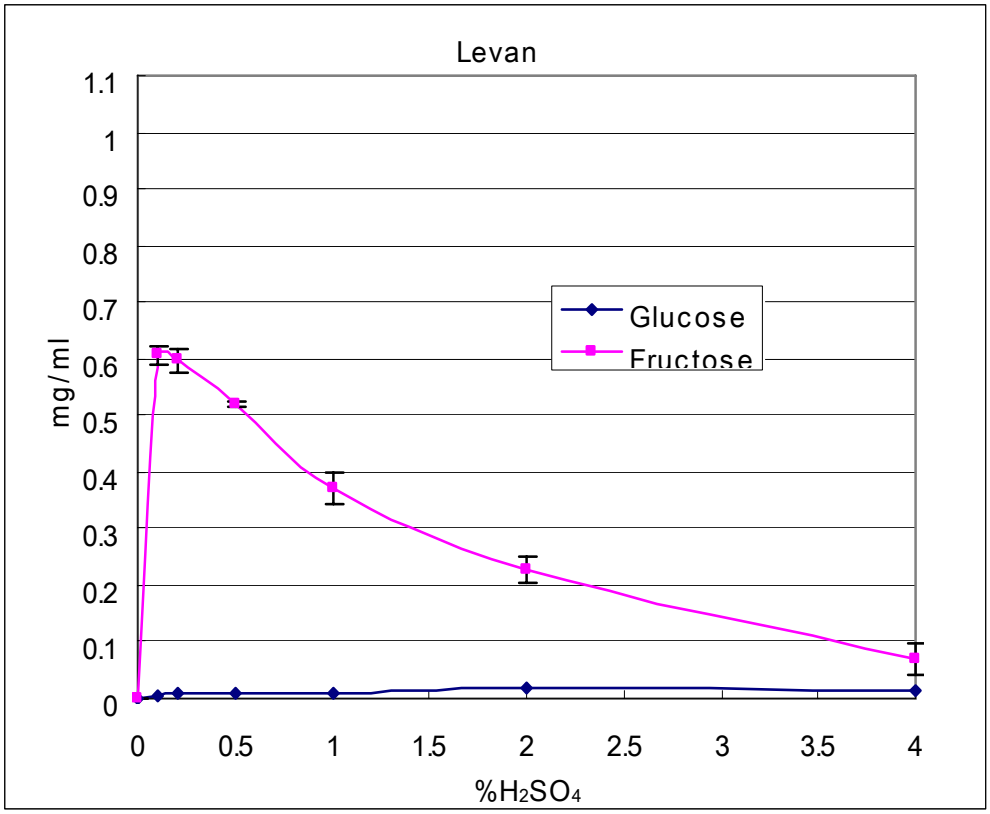

Figure 5. Hydrolysis of Tall Fescue straw extract (obtained by extracting 2 g straw for 2 hours with 98 ml 90°C water) and concomitant recovery of neutral sugars under hydrolysis conditions differing with respect to concentration of the acid-catalyst. To twenty five ml of extract was added sulfuric acid (as the independent variable in this experiment) and the solutions incubated at standard autoclave conditions (121°C) for 1 hour. Solutions ranged in acid concentrations from 0 to 4.0 %. Neutral sugars were determined at the end of the hydrolysis periods; values are reported as total mg monosaccharides per ml extract for each hydrolysis condition (total bar height) and specific monosaccharides are indicated by differences in tone within bars.

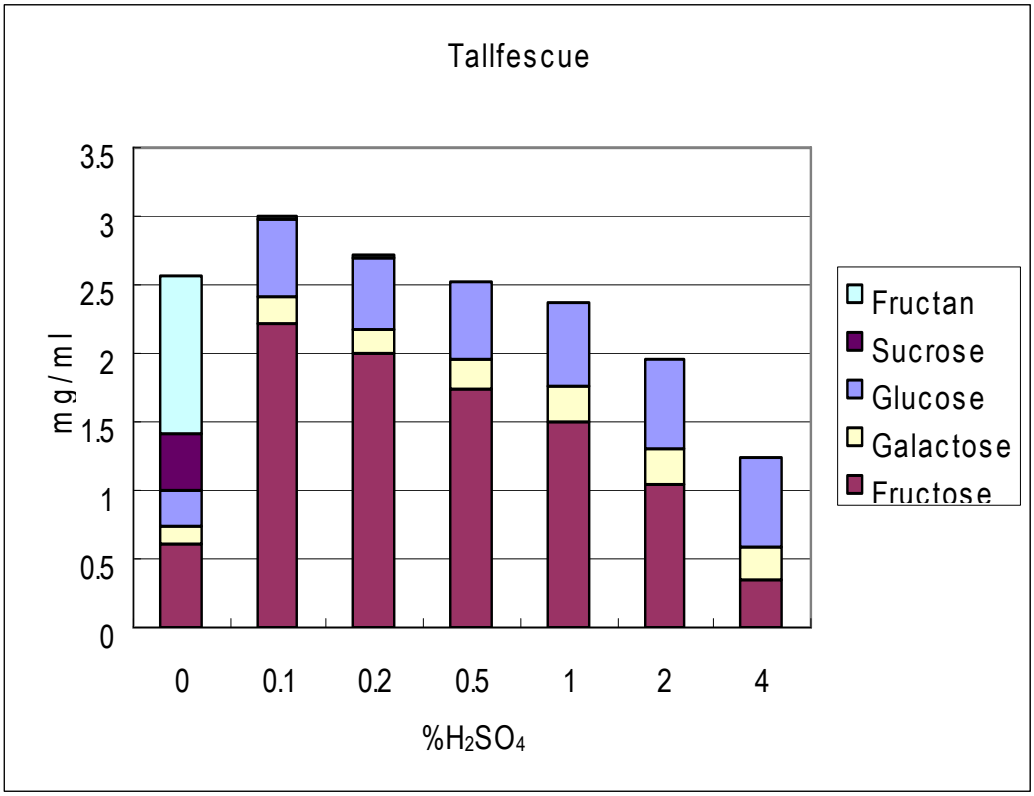

Figure 6. Hydrolysis of wheat straw extract (obtained by extracting 2 g straw for 2 hours with 98 ml 90°C water) and concomitant recovery of neutral sugars under hydrolysis conditions differing with respect to concentration of the acid-catalyst. To twenty five ml of extract was added sulfuric acid (as the independent variable in this experiment) and the solutions incubated at standard autoclave conditions (121°C) for 1 hour. Solutions ranged in acid concentrations from 0 to 4.0 %. Neutral sugars were determined at the end of the hydrolysis periods; values are reported as total mg monosaccharides per ml extract for each hydrolysis condition (total bar height) and specific monosaccharides are indicated by differences in tone within bars.

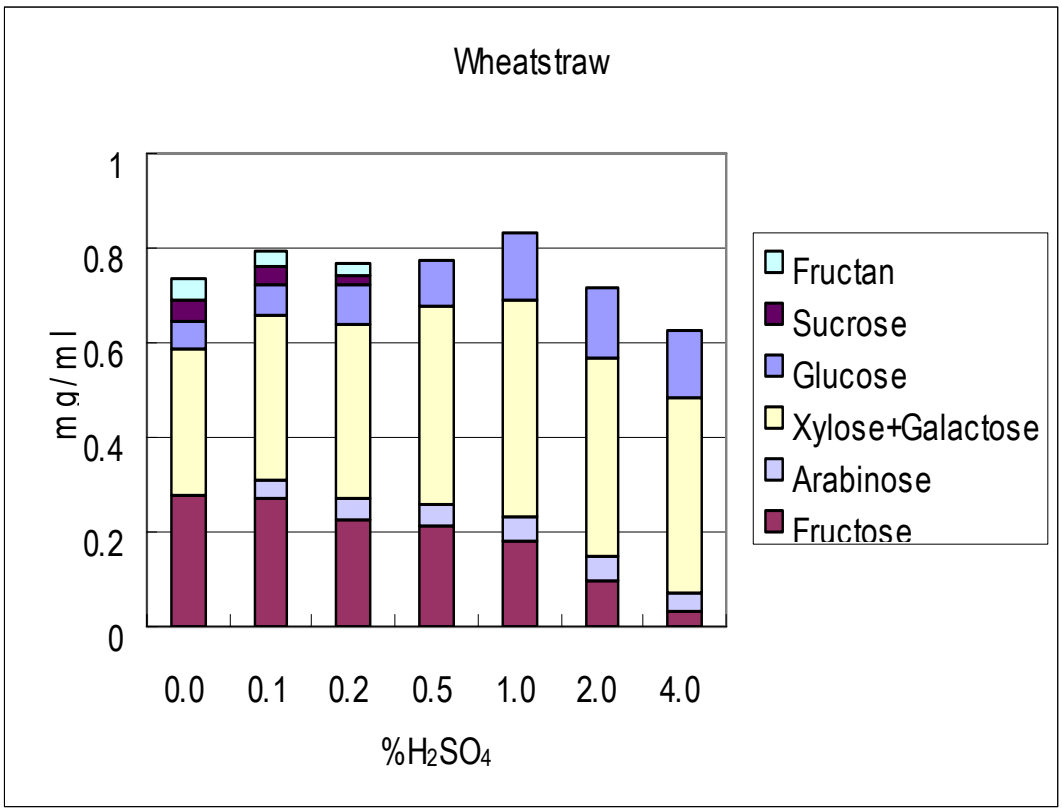

Figure 7. Fructose and glucose amounts in Tall Fescue straw extracts hydrolyzed under different acid-catalyst concentrations with and without subsequent treatment with fructan β-fructosidase. Acid concentrations were 0, 0.1 and 0.2 percent sulfuric acid. Enzyme treatments were done with 15 U fructan β-fructosidase per ml extract at 50°C for 30 minutes. Fructose and glucose concentrations were determined at the end of the hydrolysis periods; values are reported as mg monosaccharide per ml extract recovered following hydrolysis. Included in the figure are apparent fructan values obtained from HPLC peaks with appropriate retention times.

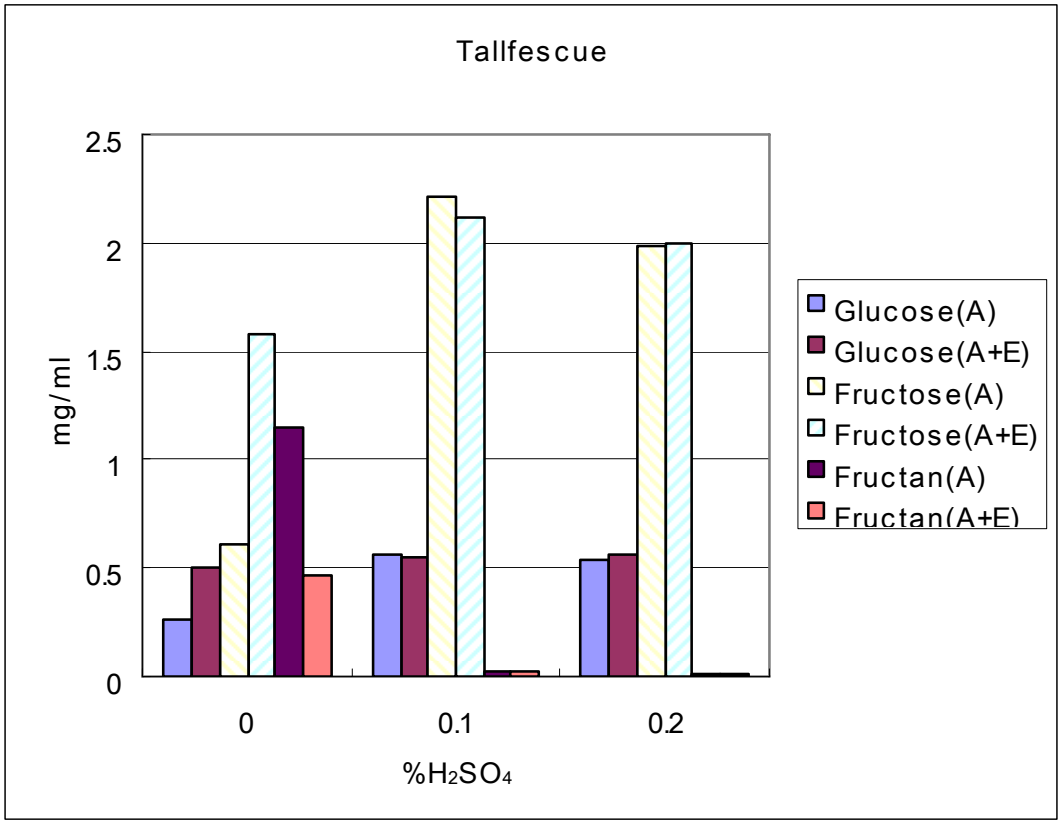

Table1. The interconversion of solutions expressed as percent H_2SO_4 and normality of H_2SO_4 and HCl.

% H_2SO_4	N	pH $_{\text{Tall fescue}}$	pH $_{\text{Wheat}}$	%HCl	N
0.1	0.024	1.99	1.89	0.1	0.027
0.2	0.048	1.72	1.65	0.2	0.055
0.5	0.121	1.32	1.31	0.5	0.138
1.0	0.244	1.05	1.05	1	0.277
2.0	0.487	0.8	0.77	2	0.548
4.0	0.976	0.49	0.49	4	1.096

Table2. Fructose and glucose amounts after calculating with degradation factor in Tall Fescue straw and wheat straw extracts hydrolyzed under different acid-catalyst concentrations with and without subsequent treatment with fructan β-fructosidase. Acid concentrations were 0, 0.1 and 0.2 percent sulfuric acid.

	No treatment		0.2% H_2SO_4		2% H_2S0_4		Enzyme	
sample	fruc	glu	fruc	glu	Fruc	glu	fruc	glu
TFS	0.26	0.60	0.54	2.19	0.66	2.48	0.50	1.58
WS	0.06	0.28	0.08	0.25	0.15	0.23	0.05	0.25

TFS : Tall Fescue Straw
WS : Wheat Straw

Table3. Statistical analysis of fructose and glucose amounts in Tall fescue straw and Wheat straw extracts hydrolyzed under different acid-catalyst concentrations with and without subsequent treatment with fructan β-fructosidase. Acid concentrations were 0, 0.1 and 0.2 percent sulfuric acid.

Sample		No treatment	0.2% H_2SO_4	2% H_2S0_4	Enzyme
Tall fescue_Fruc*	Mean	0.60^{a}	2.19^{b}	2.48^{c}	1.58^{d}
	SD	(0.05)	(0.03)	(0.04)	(0.03)
Tall fescue_Glu*	Mean	0.26^{a}	0.54^{b}	0.66^{c}	0.50^{d}
	SD	(0.01)	(0.01)	(0.00)	(0.01)
Wheat_Fruc ^{ns}	Mean	0.28^{a}	0.25^{a}	0.23^{a}	0.25^{a}
	SD	(0.05)	(0.03)	(0.02)	(0.03)
Wheat_Glu*	Mean	$0.06^{a,b}$	0.08^{b}	0.15^{c}	0.05^{a}
	SD	(0.02)	(0.00)	(0.01)	(0.00)

* Attributes are significant at $p<0.001$

$^{A, b, c, d}$ Treatments means with different superscripts within a row are significantly different from one another

^{ns} Attributes are NOT significant ($p>0.05$)

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