AN ABSTRACT ON THE THESIS OF

Stefanie K. Nguyen for the degree of Master of Science in Food Science and Technology presented on June 6, 2008.

Title: Hydrolytic Methods for the Quantification of Fructose-Equivalents in Herbaceous Biomass

Abstract approved:

Michael H. Penner

A low, but significant, fraction of the carbohydrate portion of herbaceous biomass may be composed of fructose/fructosyl-containing components ("fructose equivalents"); such carbohydrates include sucrose, fructo-oligosaccharides, and fructans. Standard methods used for the quantification of structural-carbohydrate-derived neutral monosaccharide-equivalents in biomass are not particularly well suited for the quantification of fructose equivalents due to the inherent instability of fructose in conditions commonly used for hemicellulose/cellulose hydrolysis (> 80% degradation of fructose standards treated at 4% sulfuric acid, 121^{o}C, 1 hr). Alternative time, temperature and acid concentration combinations for fructan hydrolysis were

considered using model fructans (inulin, β-2,1 and levan, β-2,6) and a grass seed straw (Tall Fescue, *Festuca arundinacea*) as representative feedstocks. The instability of fructose, relative to glucose and xylose, at higher acid/temperature combinations is demonstrated, all rates of fructose degradation being acid and temperature dependent. Fructans are shown to be completely hydrolyzed at acid concentrations well below that used for the structural carbohydrates, as low as 0.2%, at 121^{o}C for 1 hr. Lower temperatures are also shown to be effective, with corresponding adjustments in acid concentration and time. Thus, fructans can be effectively hydrolyzed under conditions where fructose degradation is maintained below 10%. Hydrolysis of the β-2,1 fructans at temperatures $\geq$ 50^{o}C, at all conditions consistent with complete hydrolysis, appear to generate difructose dianhydrides. These same compounds were not detected upon hydrolysis of levan, sucrose, or straw components. It is suggested that fructan hydrolysis conditions be chosen such that hydrolysis goes to completion, fructose degradation is minimized, and difructose dianhydride production is accounted for.

Hydrolytic Methods for the Quantification of Fructose-Equivalents in Herbaceous Biomass

By
Stefanie K. Nguyen

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 6, 2008
Commencement June 2009

Master of Science thesis of Stefanie K. Nguyen presented on June 6, 2008.

APPROVED:

Major Professor, representing Food Science and Technology

Head of the Department of Food Science and Technologoy

Dean of the Graduate School

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.

Stefanie K. Nguyen, Author

ACKNOWLEDGEMENTS

It is difficult to overstate my gratitude to my advisor, Dr. Michael Penner. As a mentor, a teacher, and an advisor, he has helped me make sense of the confusion. Throughout my graduate career, he provided encouragement, sound advice, and provided good teaching. Without his guidance, his persistence, and his support, this would have not been possible.

I would like to thank the faculty and staff in the Food Science department at Oregon State University. They have made the department a welcoming place for education, enjoyment, and have provided numerous opportunities for students to learn and grow.

Finally, but most importantly, I would like to thank my friends and family, for enduring this long process with me, offering emotional support and love. Without their words of encouragement, their humor, and their sense of judgment, I would have not had the strength to better prepare me for my future endeavors.

CONTRIBUTION OF AUTHORS

Supaporn Sophonputtanaphoca. assisted with data collection for the 50°C sample conditions and in the interpretation of the data. Dr. Michael Penner was involved with the writing of the manuscript.

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INTRODUCTION

There is widespread interest in expanding the use of lignocellulosic biomass as a means of enhancing global bio-based economies. This interest has fostered research programs directed at the development of processes for the production of biofuels from a broad spectrum of lignocellulosic starting materials [1]. A necessary component of essentially all such research programs is an ability to monitor the changes in the major chemical components of the starting materials. In many cases the components of primary interest are the carbohydrates, which are present in large amounts and may be processed to generate relatively simple sugars for subsequent fermentation and/or chemical transformation.

In this study we have focused on the quantitative determination of the fructose-equivalents in lignocellulosic materials. “Fructose-equivalents”, in the context of this study, refers to the amount of fructose theoretically available for subsequent processing following complete hydrolysis of the fructosyl-containing polysaccharides to their constituent monosaccharides. The analytical approach considered herein is the same as that used to quantify the analogous glucose or xylose equivalents; hydrolysis of the parent polysaccharides followed by chromatographic separation of the resulting

monosaccharides and quantification using an appropriate detector. The amount of fructose-equivalents in lignocellulosic materials is typically not high relative to glucose and xylose, but it can be significant. Chen et al. [2] recently showed that monomeric sugars (primarily glucose and fructose) account for 30-46% of the total corn stover (4-12% of the dry weight of feedstocks) and herein we show that fructose-equivalents represent a significant fraction of a representative grass seed straw. Carbohydrate equivalents in lignocellulosic biomass are most commonly quantified using the two stage hydrolysis method developed for the quantification of structural carbohydrates [3]. This procedure entails an initial 72% sulfuric acid treatment followed by a 4% sulfuric acid, 121^{o}C treatment for one hour. The drawback of using this method for the determination of fructose equivalents is that fructose is relatively unstable, compared to other monosaccharides typically analyzed, under these hydrolysis conditions. Thus, the correction factors used in calculating the number of fructose equivalents in a feedstock, determined by measuring the extent of degradation of a known amount of pure fructose under equivalent hydrolysis conditions, are excessive. It is generally recognized that these correction factors should be kept to a minimum since they provide only approximations of actual sugar losses [4].

In the present study we have evaluated time, temperature, and acid concentration relationships that are pertinent to the hydrolysis and subsequent quantification of fructose-equivalents in lignocellulosic biomass. The study considered temperatures to 121^{o}C and sulfuric acid concentrations to 4%;

these being the conditions specified for the secondary hydrolysis step of traditional methods [3]. Conditions were thus considered in the context of established hydrolysis parameters that are used for the quantification of neutral sugar equivalents in lignocellulosic biomass. The hypothetical basis of this study is that hydrolyses using relatively low acid concentrations and temperatures are preferable for the quantification of fructose equivalents due to the relative ease of hydrolysis of fructosidic linkages and the relative instability of fructose under low pH, high temperature condition.

LITERATURE REVIEW

BIOFUELS FROM BIOMASS; AN ALTERNATIVE FUEL SOURCE

As the price of gasoline continues to rise the dependence upon fossil fuels is increasingly becoming evident, resulting in a considerable interest in alternative energy resources. Current petroleum prices are at a record breaking $135 a barrel in the United States, resulting in aggressive research in the science and technology of biomass as an alternative fuel source [5]. In 2005, the U.S. Department of Agriculture estimated that farmers will pay $8.2 billion for petroleum [5]. Adding in the cost of supplies for maintaining a farm, such as fertilizers and pesticides both of which are derived from petroleum and natural gas products, farmers are expected to pay more for energy-related items [6].

Production of ethanol from biomass can promote a more environmentally sustainable economy, independence from foreign fuel sources, and stimulate a sustainable domestic biomass industry. Utilization of biofuels can help relieve dependence on oil, decrease greenhouse gas emissions, enhance U.S energy security, and provide economic support in rural areas [7]. Currently, America accounts for 25% of global oil consumption, yet only hold 3% of the world's known oil reserves. Increasing strain on world oil supply is expected as developing countries become more industrialized and require more energy [8].

In 2004, ethanol use reached 3.4 billion gallons per year, and by the end of 2006, fuel ethanol use has reached 4.9 billion gallons annually. The majority of the feedstock used for ethanol production in the United States has been corn, grain, and sorghum (cereal) [9]. Future prospects for ethanol production will come from cellulosic resources such as crop residues (stalks, leaves), forestry residues (dead trees), energy crops (fast growing trees and grasses), and other cellulose containing materials (municipal solid waste) [10].

PRODUCTION OF BIOETHANOL

The production of bioethanol can be generally described as a series of steps. Pretreatment is the initial step and refers to the solubilization and separation of components of biomass as needed to expose the cellulose and hemicellulose structures. This step increases the susceptibility for further chemical or biological treatment. Hydrolysis, or “saccharification,” is the next step in the production process with the use of acids or enzymes. Hydrolysis breaks down the glycosidic bonds in the cellulose and hemicellulose yielding the monomeric moieties. Fermentation to ethanol, or other products, is the last step and generally requires microorganisms, such as yeast, which have the ability to ferment various sugars [11-14].

LIGNOCELLULOSIC MATERIALS

Raw materials for ethanol production can be categorized into three types: simple sugars (e.g. glucose), starch (e.g. corn), and cellulosics (e.g.

wheat straw), often referred to as lignocellulosics. Among lignocellulosic materials, there is wood, municipal solid waste, waste-paper, crop residue, and energy crops (e.g. switchgrass).

Lignocellulosic materials are comprised of cellulose, hemicellulose, and lignin. On a dry-weight basis, cellulosic materials contain 35-50% cellulose, 20-35% hemicelluloses, and 12-20% lignin, along with a small amount of minerals and various extractives [13]. Cellulose molecules are long chains of glucose moieties (linked β-1,4) and have a structurally different configuration than starch (linked α-1,4). Hemicellulose is a molecule consisting of several sugars, usually a mixture of glucose and xylose, along with varying amounts of arabinose, galactose, mannose, galactose, and acetic acid. Lignin, although intertwined with cellulose and hemicelluloses is unlike either of the molecules and does not have a sugar based structure [11].

HERBACEOUS BIOMASS

Herbaceous biomass are non-woody lignocellulosic plant materials and can be used as a feedstock for ethanol production. Examples of herbaceous crops are grasses (switchgrass) and straws (wheat straw) which are often perennial plants. Herbaceous crops can offer energy and environmental advantages over current biofuels sources (e.g. corn), and require fewer agricultural inputs then annual crops and can be grown on marginal lands [15].

Herbaceous biomass is not commonly used as an energy source in North America. However, these crops are commonly used as energy sources

in other parts of the world, such as Europe and China. Crop residues and dedicated perennial grass crops can potentially displace 30% of our current petroleum consumption [16].

POACEAE

The Poaceae (also known as gramineae or grass family) are a major potential source of herbaceous biomass. It is one of the largest plant families comprising 635 genera and 9,000 species. This family contains some important feed crops; wheat, corn, barley, rice, and oats; all of which are harvested for human and animal consumption.

Forage grasses and straws such as tall fescue (*Festuca arundinacea*), switchgrass (*Panicum virgatum* L.), Kentucky bluegrass (*Poa Pratensis L.*), perennial ryegrass (*Lolium perenne L.*), and wheatgrasses (belonging to the genus *Agropyron)* are some examples of perennial plants being evaluated as cellulosic bioenergy crops. The usage of grasses and straws can relieve the competition of feed and food demands on grain supplies and prices [15].

WATER SOLUBLE CARBOHYDRATES

Water soluble carbohydrates (WSC) consist of glucose, fructose, sucrose, and fructans. Nonstructural carbohydrates (NSC) contain starches and WSC [17]. Many plants store reserve carbohydrates in vegetative tissues. The amount of WSC and NSC will diminish as the plant matures or is exposed to stressful environments, [18].

Chen et. al. [2] demonstrated that water-soluble materials in corn stover account for as much as 27% of the dry weight material. The soluble compounds of corn stover includes alditols, aliphatic acids, inorganic ions, oligomeric sugars, and phenolic glycosides. Monomeric sugars (primarily glucose and fructose) account for 30-40% of the water soluble materials in corn stover. In addition, Tava et.al. [19] reported three tall fescue varieties containing 133 g/kg WSC.

In the study conducted by Mayland et.al. [17], eight tall fescue varieties were used to study their NSC composition. The average NSC concentrations in the tall fescue varieties contained 14 g/kg glucose, 14 g/kg fructose, 5 g/kg sucrose, 40 g/kg fructan, 23 g/kg insoluble starch, and 129 g/kg total NSC. Monosaccharide and disaccharide sugars made up about 45% of the total NSC in the tall fescue varieties.

Typical extraction procedures for the quantitative determination of total water soluble carbohydrates (WSC) are based on the extractions of samples with water, ethanol, or a combination of both followed by hydrolysis and analysis. For example, S. Griffith et.al. [20] discovered the major components of WSC found in the stems of Italian ryegrass were sucrose and fructans. WSC were extracted at 75°C for two hours, centrifuged then hydrolyzed in hydrochloric acid solution and assayed for reducing sugars . Slominski et.al. [21] also developed a method to measure water soluble carbohydrates in feedstuffs. Following extraction and hydrolysis, the quantitative determination of glucose, fructose, and sucrose were determined by a colorimetric method

and gas-liquid chromatography (GLC).

FRUCTANS

In contrast to glucans, which are polymers of glucose, fructans are polymers of fructose with β-D-fructosyl linkages [22]. There are several structural variations that are categorized by the series of linkage patterns. Fructans can be described according to the formula GFn, G = glucose units, F = fructose units, and n = number of fructose moieties. Inulin are β-2,1 linear linkages and can be found in abundance in certain plants. Inulin is usually found in plant species belonging to the order Asterales, such as chicory, and Jerusalem artichoke, which both contain high amounts of inulin and are used today in the industry for food and medicinal purposes [23, 24]. Fructans linked by β-2,6 bonds are termed levan type fructans . Levan can be found in most types of grasses. The fructosyl moieties of levan-type fructans can be associated with either the 1 or 6 position of glucose. Mixed fructans containing both β-2,1 and β-2,6 bonds are also called graminan type fructans and can be branched. This type of fructan is commonly found in most plants species belonging to the Poaceae family [25, 26].

The starting materials for fructan synthesis are a sucrose molecule and fructose [23]. Although most fructan chains contain a glucose moiety, there are fructans that contain only β-2,1 linked fructose molecules, such as in some species of the Asteraceae [27]. Those fructans without a terminal glucose can be the result of internal rearrangements or depolymerization reactions in

fructan metabolism [25].

FRUCTAN OCCURENCE IN POACEAE

Essentially all plants store non-structural carbohydrates as an energy reserve. There are three types of energy reserve used in most plants, which are starch, sucrose, and fructans. Temperate grasses in the Poaceae family store their energy reserve as fructans with starch levels being low or absent [22, 23, 25]. As an energy reserve, fructans are stored in the vegetative tissue of grasses, and are located in the vacuole of the plant as water soluble carbohydrates. Starch, on the other hand, is located in the plastids and is an insoluble polymer.

Fructans in forage grasses are predominantly stored in the leaf base and sheath regions of the grass, and can also accumulate in leaf blades and roots [28]. The amount of fructans in temperate forage grasses is highly influenced by several environmental factors such as the age of the plant [20, 29] . Fructans in the subfamily Pooideae can account for as much as 73-95% of the storage carbohydrates. Fructans can vary depending on the type of grass and which subfamily it belongs to. It can vary by as much as 5-30% of the storage carbohydrates [30].

As an example, approximately 25% of the dry matter is composed of water soluble carbohydrates with over 70% in the form of fructan in perennial ryegrass [31]. The fructans that are accumulated in forage grasses varied in molecular size and linkage type. High molecular weight polymers with a

degree of polymerization (DP approximately 26+)can be found in *Dactylis glomerata* and *Phalaris arundinaceae*. Lower degrees of polymerization are also found in grasses with a DP value between 10-26, and can be found in *Lolium perenne,* and *Festuca arundinaceae,* [30]. Seasonal changes in fructan and other nonstructural carbohydrates in many grasses have been documented [32-34].

MEASUREMENT OF FRUCTANS

There are several procedures for the measurement of fructans in plant material and food products. Following extraction, enzyme- or acid-catalyzed hydrolysis is used to make free fructose. The value of fructose released from fructans is used to calculated total fructans[23, 35]. High-performance liquid chromatography (HPLC), capillary gas chromatography (CGC) , paper chromatography [36], and thin-layer chromatography (TLC) [37] are just some examples of the methods used to analyze and measure fructans after hydrolysis [38].

Acid hydrolysis can be carried out using various acids. However, the most common acid used is hydrochloric acid (HCl) [39, 40]. Szambelan et.al. [41] demonstrated the use of sulfuric, hydrochloric, and phosphoric acid and measured the fructose released in Jerusalem artichoke (H. *tuberosus* L.). Sulfuric acid yielded 6-13% higher values than hydrochloric acid or phosphoric acid for this study. Enzyme hydrolysis relies on the treatment of the sample with a mixture of invertase (hydrolyzes sucrose) and inulinase (hydrolyzes the

ß-2,1 linkages of inulin) [41, 42].

An example of the measurement of fructans is a method developed to measure total fructans in foods and feeds by Hoebregs [43]. The method extracts fructans from the sample with boiling water. The extracts treated with enzymes are measured by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The extract prior to hydrolysis is measured for free fructose and sucrose. An aliquot of the extract is hydrolyzed with amyloglucosidase. Finally, an aliquot of the extract that was treated with amyloglucosidase is treated with inulinase and will yield the total amount of glucose and fructose. The amount of glucose and fructose release from the fructans is calculated by the difference from these determinations.

INSTABILITY OF FRUCTOSE

Fructose, when exposed to procedures involving dilute acid, undergoes rapid degradation to 5-hydroxy methylfurfural which can interfere with the quantification of fructose [44]. When compared to the stability of D-glucose, D-fructose is approximately 40 times more susceptible to degradation in thermal acid conditions [45-49]. Such studies have shown the influence of pH (1-6) on the rates and yields of degradation products from fructose [45-49]. Ninety eight percent of the reaction products of D-fructose in acid media consist of 5-hydroxy-2-methylfurfural (HMF), levulinic acid, and formic acids [45, 46, 49].

There are several reasons as to why fructose yields more HMF than

glucose. Glucose is a stable ring structure and the fraction of open chain forms in solution and enolization rate is relatively slow. Fructose forms an unstable ring structure that frequents the open chain form, becoming highly susceptible to enolization, which is believed to be the rate determining step for HMF formation [50].

MATERIALS & METHODS

Materials

Sugar standards: inulin, levan, sucrose, glucose, and fructose were obtained from Sigma (USA). Levan was also purchased from Montana Polysaccharides Corporation (USA). Tall fescue grass seed straw was obtained from commercial Pacific Northwest grass seed farms. Straws were 6-8% moisture when taken from the field. Straws were milled to pass a 20-mesh per inch screen. The milled and sieved straw was stored under dry conditions at room temperature prior to use.

Monosaccharide decomposition studies

Sugars were dried at 45°C for 12 hours and stored with desiccant prior to use. Solutions containing 1 mg/mL of the test sugars at specified acid concentrations were prepared just prior to testing. Acid concentrations ranged from 0.02 to 4.00 percent (wt/wt, 0.002 to 0.418 molar). Molar concentrations of the acid solutions were determined by titration with standardized base (0.1N NaOH) to a phenolphthalein endpoint.

Autoclave and water-bath hydrolysis method

For the autoclave hydrolysis method, sugar solutions (25mL of 1 mg/mL in appropriate acid concentration) were transferred to 100mL autoclavable, Teflon-lined, screw-cap bottles. The samples were then autoclaved at 121°C

for 1 hour – the one hour time period started once the autoclave had reached 121°C. For the water bath hydrolysis method, sugar solutions (25mL of 1 mg/mL in appropriate acid concentration) were transferred to 100mL autoclavable, Teflon-lined, screw-cap bottles. The samples were then placed in an appropriate temperature water bath and allowed to react for the given time periods– the timed periods started once the bottles were immersed in the water bath. Following the hydrolysis period, the samples were transferred to an ice bath for rapid cooling. Once the samples reached room temperature, they were transferred to 50mL flasks and solid $CaCO_3$ was added to raise the pH to between 6 and 7 (determined by pH meter). Samples were then set aside for 60 minutes at room temperature to allow precipitate development and settling of solids.

Hydrolysis of inulin, and levan

Inulin and levan solutions containing 1 mg/mL of inulin or levan at specified acid concentrations were prepared and hydrolyzed in accordance with the methods described above.

Tall Fescue extraction and hydrolysis

Approximately 6g of sample, weighed to the nearest 0.1mg, was placed in a 250mL screw-top Erlenmeyer flask containing 94mL of double distilled pre-heated water. The flask was maintained at 60˚C for 2 hours in a thermo-regulated water bath with orbital mixing at 200 rpm. At the completion of the

extraction, the contents were cooled and filtered using a No.1 Whatman filter paper. Approximately 15-20 g of filtrate, weighed to the nearest 0.1mg, was transferred to a pre-dried, weighed porcelain crucible and dried in a convection oven at 105˚C for 12 hours for “solids” determination. The filtered Tall Fescue extract was hydrolyzed using the autoclave and water bath methods as described above. Hydrolysis was done on 10mL of filtrate diluted to 25mL with aqueous sulfuric acid to give the appropriate acid concentration.

HPLC

Prior to HPLC analyses, portions of the sample hydrolysates were filtered through 0.22µm Acrodisc© syringe filters (Pall, USA) into auto-sampler vials. Analyses were done using a Waters HPLC system equipped with an Aminex HPX-87P column (300 x 7.8 mm, BIO-RAD,USA), and a refractive index detector. Analysis conditions were as follows: injection volume: 20µl, mobile phase: Milli-Q grade H_2O, flow rate: 0.6ml/min, column temperature: 85˚C , running time: 50 minutes.

Levan Purification

Crude levan (from Montana Polysaccharide Corp.), 5g, was washed three times by suspending in 25mL of 100% ethanol for 15 minutes. The washed levan was then suspended in 50mL of dionized water, stirred for 20 minutes, filtered (Whatman No.1 filter paper), and then re-precipitated by the addition of 500mL 100% ethanol. The suspension was centrifuged for 10min

at 10,000 RPM and the supernatant decanted and discarded. This process was repeated a second time. The sample was then washed with absolute ethanol and dried for 16 hours in a convection oven at 37°C. The dried levan was stored in a desiccator until ready for use.

RESULTS & DISCUSSION

Figure 1 illustrates the relative lability of fructose at high temperature/low pH conditions (121^{o}C, acid concentrations to 4%) and, thus, the motive for this study. Treatment of fructose solutions using traditional secondary hydrolysis conditions, 4 % acid/121^{o}C/1 hr, results in the degradation of nearly 90% of the initial fructose. For reference, the degradation of glucose and xylose under these same conditions is typically less than 10% and 20%, respectively. Hence, the correction factor used in calculating the amount of the monosaccharide theoretically available in a feedstock, using these hydrolysis conditions, is inordinately large for fructose (for discussion of use of correction factors based on degradation of standard monosaccharide solutions see Sluiter et al., 2008). Figure 2 illustrates that there is significant loss of fructose from aqueous solutions at 121^{o}C even in the absence of acid. These results prompted our consideration of hydrolysis at lower temperatures; representative data from such experiments at 100^{o}C are included in Figure1. The data show the extent of fructose degradation was 10% or less when solutions were incubated at 100^{o}C for 1 hr at acid concentrations up to 1% (the stability at higher acid concentrations was not tested because 1 % acid was sufficient for hydrolysis of the tested fructan linkages, as discussed below). Yet lower temperatures were tested (50^{o}C for 1 hr at acid concentrations up to 4%); under these conditions fructose degradation was not observed, neither by a significant decrease in measured

fructose nor the generation of hydroxy methyl furfural (Figure 6).

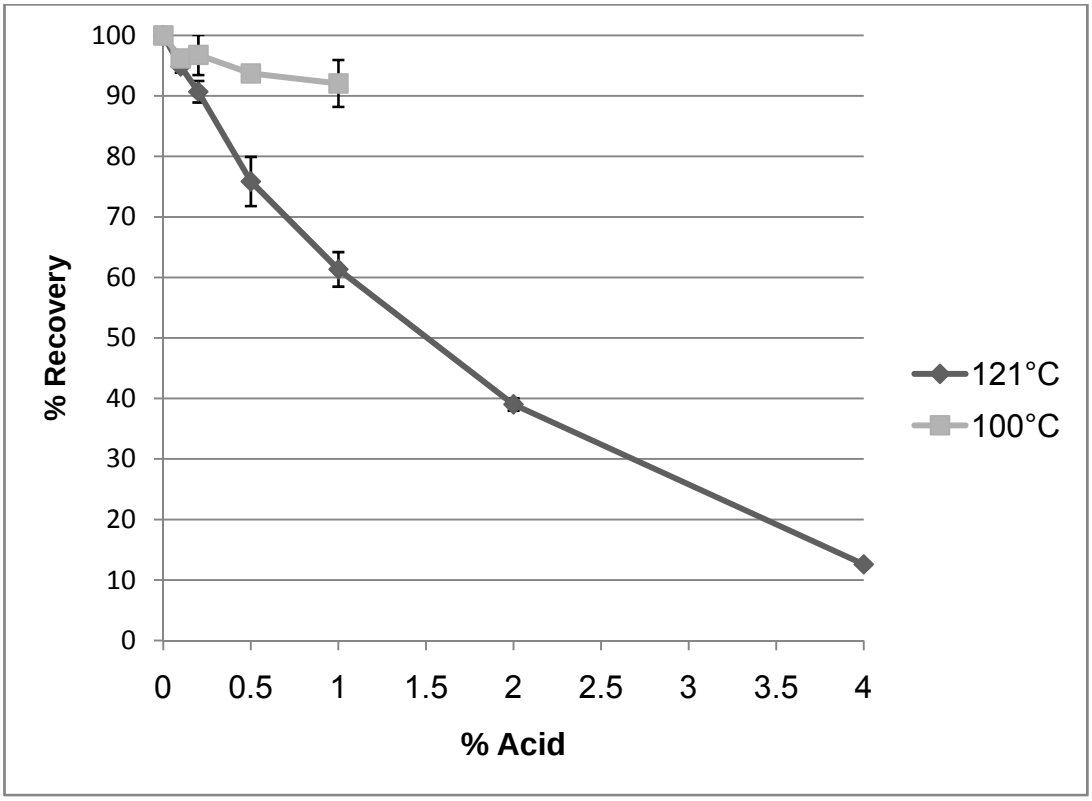

Figure 1. Fructose degradation after 1 hour at different acid and temperature combinations. Error bars +/- 1 standard deviation.

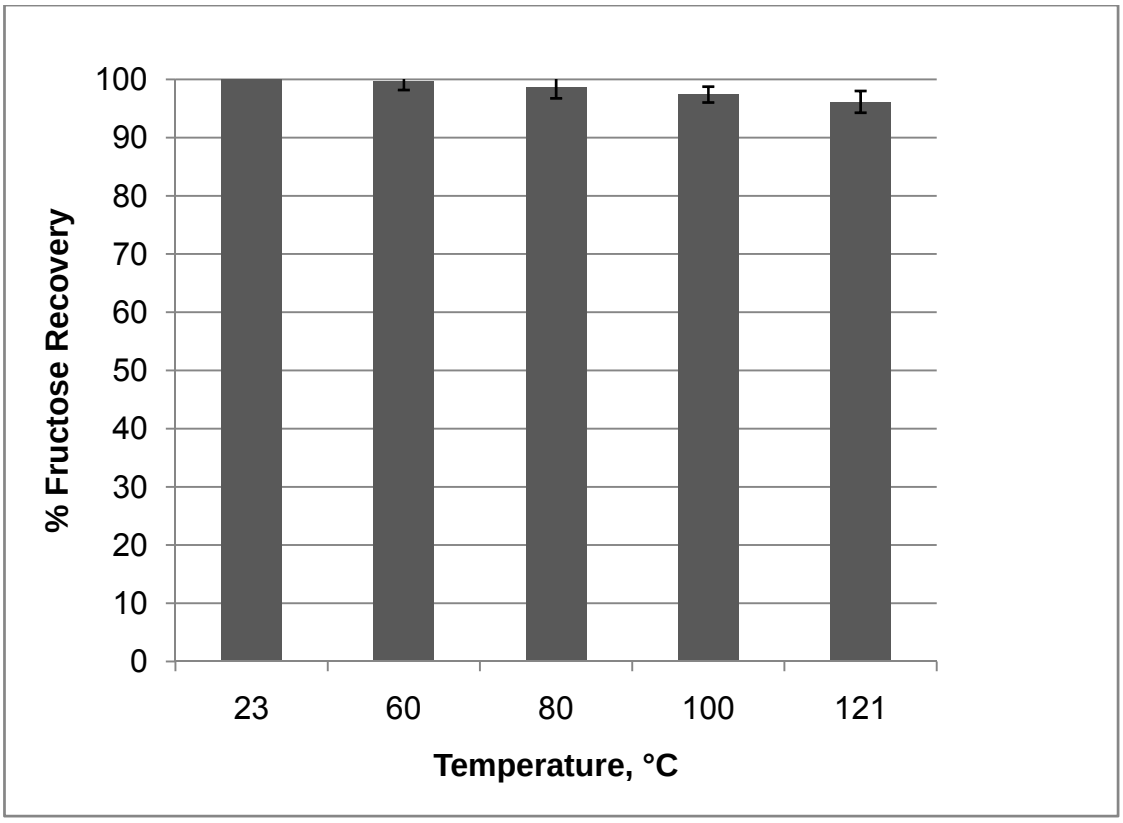

Figure 2. Fructose degradation in water after 1 hour at different temperatures. Error bars +/- 1 standard deviation.

The primary linkages in fructans from grass and cereal straws are expected to be β-2,1 and β-2,6 [26]. Hence, our hydrolysis studies used inulin as a model β-2,1 fructan and levan as a model β-2,6 fructan. Initial studies with inulin, summarized in Figures 3-8 demonstrate that it is readily hydrolyzed, compared to the structural polysaccharides, under rather modest conditions. At 121^{o}C for 1 hr, inulin was effectively hydrolyzed at acid concentrations down to 0.1 % (Figure 3). Shorter reaction times, at 121^{o}C, were not considered due to the common use of the one hour treatment for the hydrolysis of structural polysaccharides (typically 4% acid/121^{o}C/1 hr). Time and acid combinations at 100^{o}C showed the β-2,1 fructan was hydrolyzed in as short as 10 minutes

at 0.5% acid (Figure 4) or at acid concentrations as low as 0.1 % if treated for 1 hour (Figure 5). Experiments done at yet lower temperatures, 50°C, with incubation times of 1 hr and acid concentrations of 0.2, 0.5 and 1.0% acid resulted in extents of inulin hydrolysis of 33, 72 and 96% respectively (Figure 6); treatments at 50°C for 1 hr at the higher acid concentrations (≥ 2%) resulted in complete hydrolysis (Figure 6). Hydrolysis at the lower acid concentrations, at 50°C, also revealed sucrose as an intermediate hydrolysis product, as expected based on the ease of hydrolysis of inulobiose relative to sucrose [51], there being one potential sucrose moiety per molecule inulin.

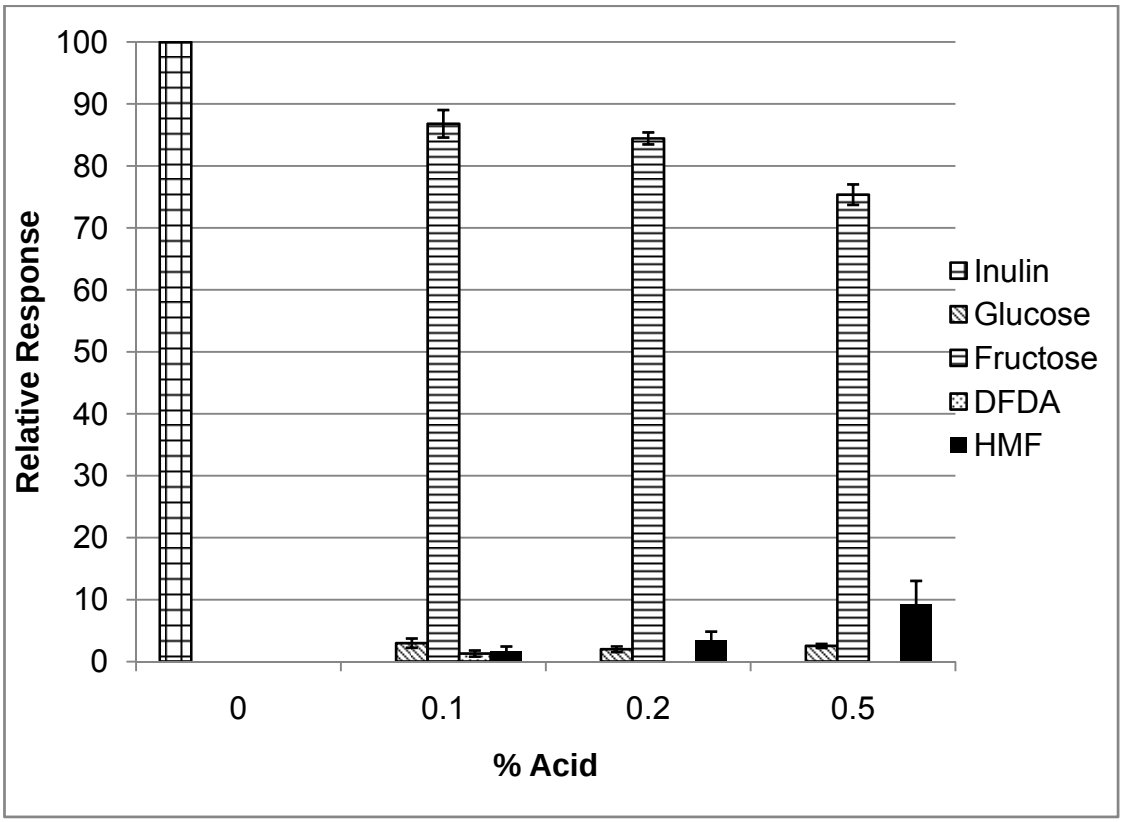

Figure 3. Hydrolysis of inulin at 121°C, 1 hour, at different concentrations of sulfuric acid. DFDA, difructose dianhydride; HMF, hydroxy methylfurfural. "Relative response" is relative to 1mg/mL

fructose or glucose solution. Error bars +/- 1 standard deviation.

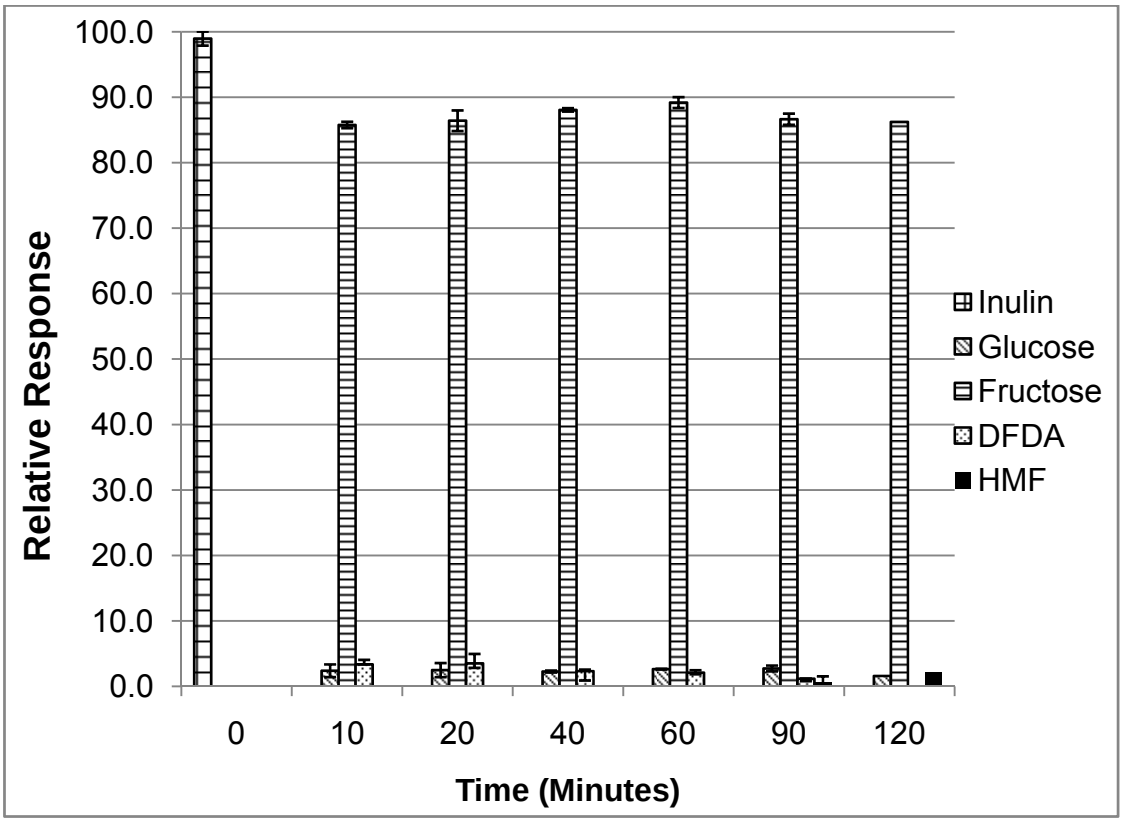

Figure 4. Time course of inulin hydrolysis at 100°C, 0.5% sulfuric acid. DFDA, difructose dianhydride; HMF, hydroxy methylfurfural. "Relative response" is relative to 1mg/mL fructose or glucose solution. Error bars 1 +/- standard deviation.

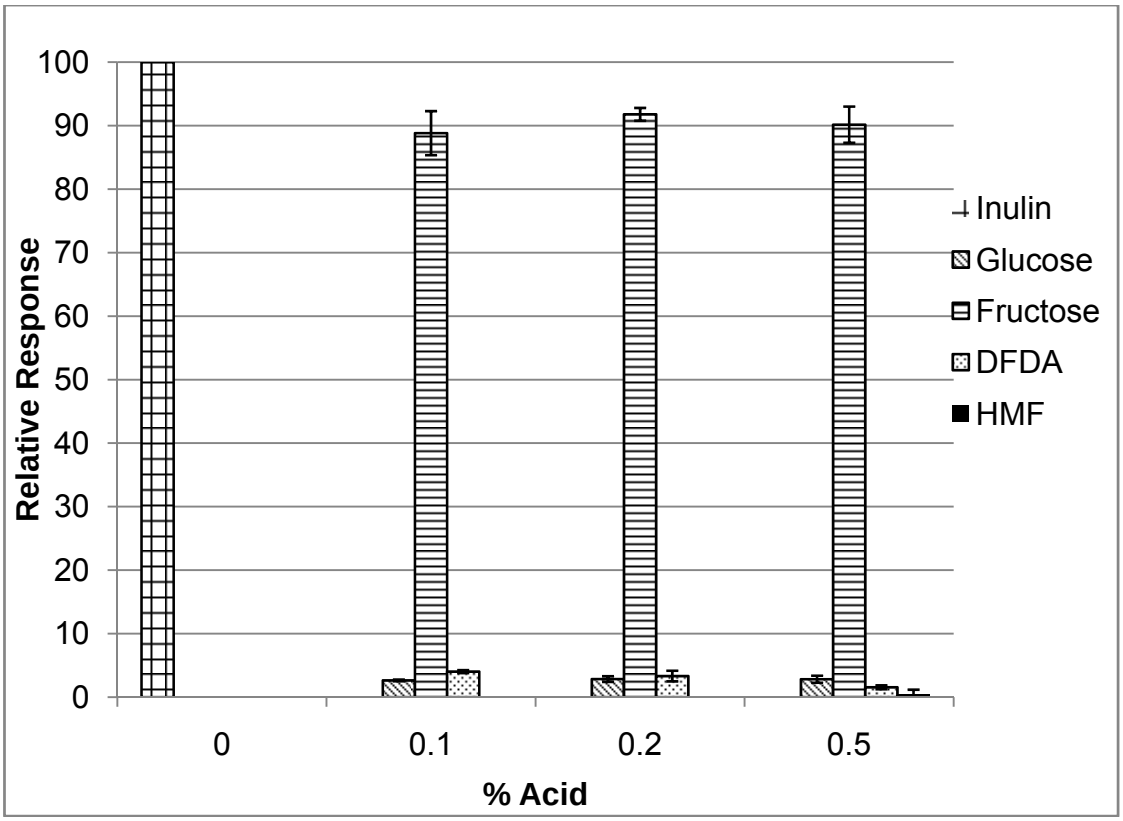

Figure 5. Hydrolysis of inulin at 100°C, 1 hour, at different concentrations of sulfuric acid. DFDA, difructose dianhydride; HMF, hydroxy methylfurfural. "Relative response" is relative to 1mg/mL fructose or glucose solution. Error bars +/- 1 standard deviation.

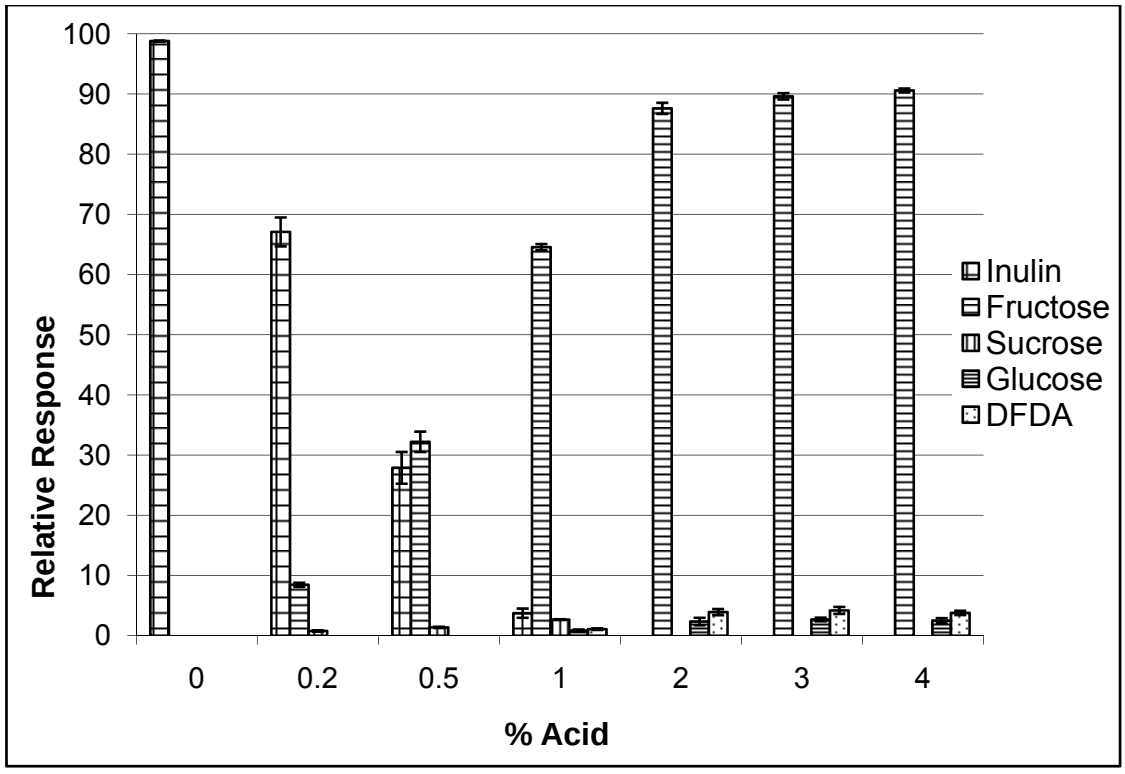

Figure 6. Hydrolysis of inulin at 50°C, 1 hour, at different concentrations of sulfuric acid. DFDA, difructose dianhydride; HMF, hydroxy methylfurfural. "Relative response" is relative to 1mg/mL fructose or glucose solution. Error bars +/- 1 standard deviation.

The data of Figures 3-8 illustrate a potential complication in using acid as the catalyst for the hydrolysis of inulin-type fructans; the complication being the production of a hydrolysis byproduct which we have tentatively identified as difructose dianhydrides (DFDA). The tentative identification is based on the observation that, a) the compound is relatively resistant to acid-catalyzed hydrolysis, b) the only detectable product resulting from hydrolysis is fructose, it, c) is resistant to an enzyme preparation for fructan hydrolysis ("Fructozyme", Sigma Chemical Co.), d) its retention time is consistent with that of disaccharides, and, e) DFDA are known to be generated as a result of acid-catalyzed hydrolysis of 2,1-linked fructans [52]. The presence of DFDA

presents a complication in quantifying fructose equivalents for inulin-type fructans because they are not accounted for by traditional correction factors. The correction factors used when quantifying structural carbohydrate-derived neutral sugars are based on the degradation of the monosaccharides of interest (under the chosen hydrolysis conditions). This approach will not work for the generation of DFDA since they are apparently not formed from the monosaccharide under the conditions used in this study, but are derived directly from inulin (we see no evidence for the formation of DFDA when fructose alone is incubated at the hydrolysis conditions evaluated in this study).

Many time-temperature-acid concentration combinations were tested in an attempt to avoid DFDA production. In general, the lower temperature treatments appeared most promising. However, the data of Figure 7, which summarizes the time course of hydrolysis of inulin at a relatively low temperature, 50^{o}C, 0.5% H_2SO_4, suggests that DFDA are generated prior to the completion of inulin hydrolysis. Experiments at yet a lower temperature 20^{o}C, 5% acid, showed no sign of DFDA production after 4 hours incubation (data not shown) - but the calculated pseudo half-life for total inulin hydrolysis under these conditions was greater than 4 hours. Therefore these conditions were deemed unreasonable for routine analyses based on the extensive time it would require for “complete” hydrolysis. DFDA production at 20^{o}C, 5% acid, was thus not tested beyond four hours. Note that DFDA production is estimated to be 5% or less under all conditions tested in this study.

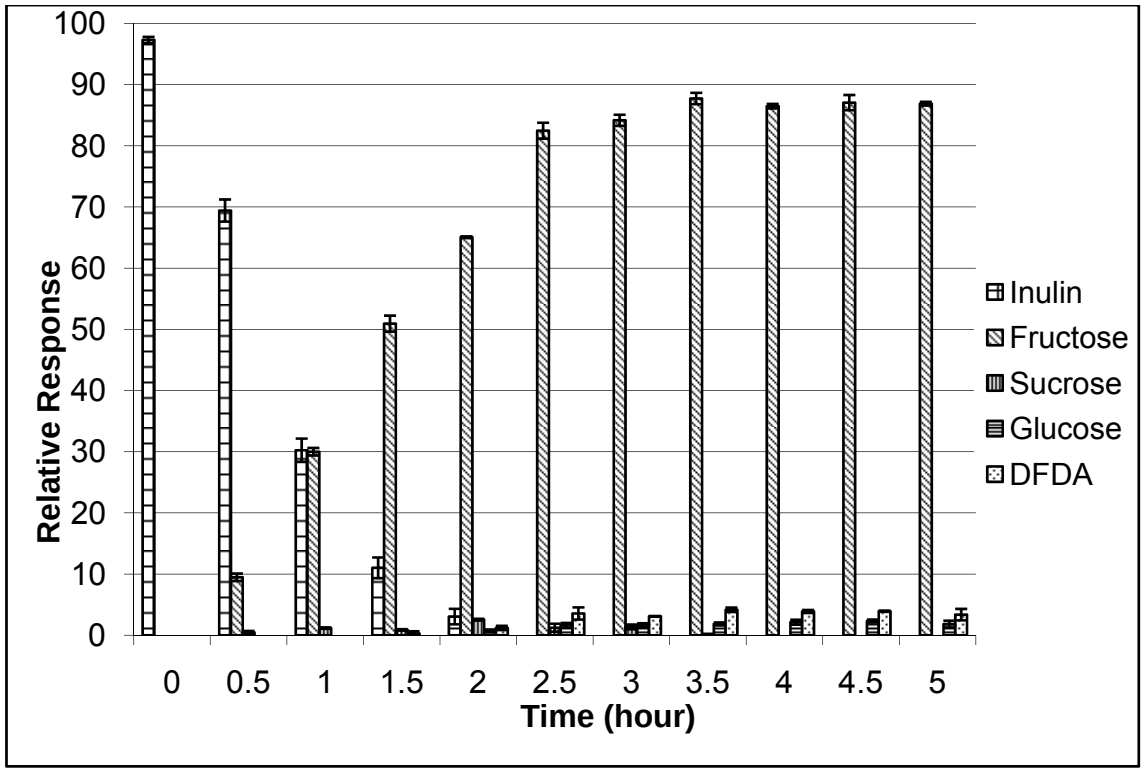

Figure 7. Time course of inulin hydrolysis at 50°C sulfuric acid. DFDA, difructose dianhydride. "Relative response" is relative to 1mg/mL fructose or glucose solution. Error bars +/- 1 standard deviation.

DFDA are relatively resistant to hydrolysis, but upon hydrolysis are expected to yield fructose [52, 53]. Thus, one approach to accounting for their presence is to use hydrolysis conditions sufficiently harsh to hydrolyze the DFDA (essentially treating the DFDA as an uncommon intermediate in the hydrolysis scheme). This approach is summarized in Figure 8, which depicts the time course of inulin hydrolysis at 100^{o}C and 1% acid. It can be noted that complete hydrolysis of the DFDA occurred after 90 minutes. Similarly, the data of Figure 3 shows that no DFDA was present following hydrolysis at 121^{o}C, for 1 hour, at acid concentrations $\geq$0.2%. It is to be kept in mind that fructose degradation (principally to hydroxy methylfurfural) increases as the

severity of the hydrolysis conditions increase. Fructose degradation of this nature, i.e. degradation via hydroxy methylfurfural, may be accounted for by using the traditional correction factors (standard fructose solutions incubated under equivalent conditions) – with the objective of keeping the extent of degradation as low as possible [4].

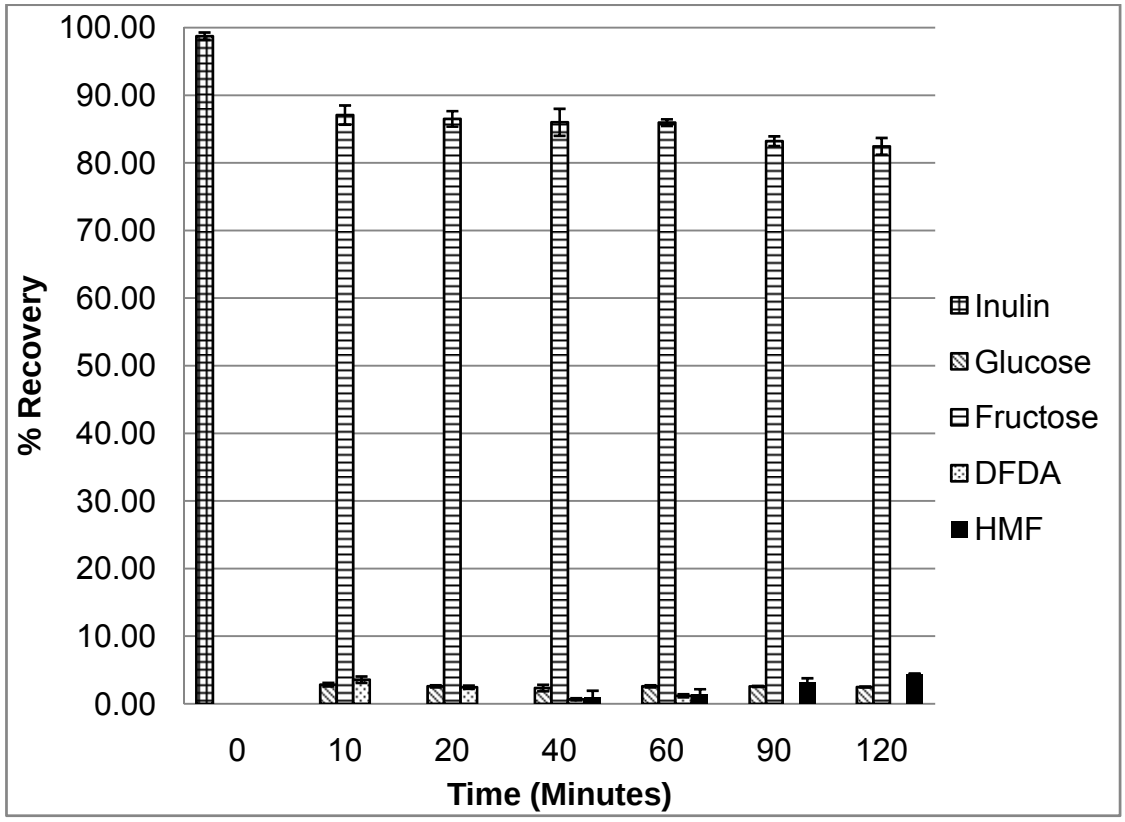

Figure 8. Time course of inulin hydrolysis at 100°C, 1% sulfuric acid. DFDA, difructose dianhydride; HMF, hydroxy methylfurfural. "Relative response" is relative to 1mg/mL fructose or glucose solution. Error bars +/- 1 standard deviation.

The levan-type fructans (2,6- fructans) were found to be readily hydrolyzed under the same conditions found effective for hydrolysis of the 2,1-type fructans. Thus, effectively all of the levan was hydrolyzed following

treatment at 100°C with 0.1 % acid for 1 hour. The hydrolysis of levan produced no detectable DFDA, allowing the choice of conditions for hydrolysis of 2,6-fructans to be dictated simply by time of hydrolysis and extent of fructose degradation.

Sucrose, another potential source of fructose-equivalents, can be a significant component of herbaceous feedstocks, as demonstrated by the data of Figure 9 for Tall Fescue straw (discussed below) and as recently shown for corn stover [2]. Studies evaluating the hydrolysis of sucrose at relatively low acid concentrations, 0.2% acid, and 100°C demonstrate that it is effectively hydrolyzed at 10 minutes under these conditions (Figure 10). The data contained in Figure 9 demonstrate that the sucrose component of Tall Fescue is effectively hydrolyzed within 10 minutes in 1% acid at 100°C. Similarly, the inulin-derived sucrose, noticeable during the time-course of inulin hydrolysis at 50°C, 0.5% acid, was found to be completely hydrolyzed in 130 minutes (Figure 7).

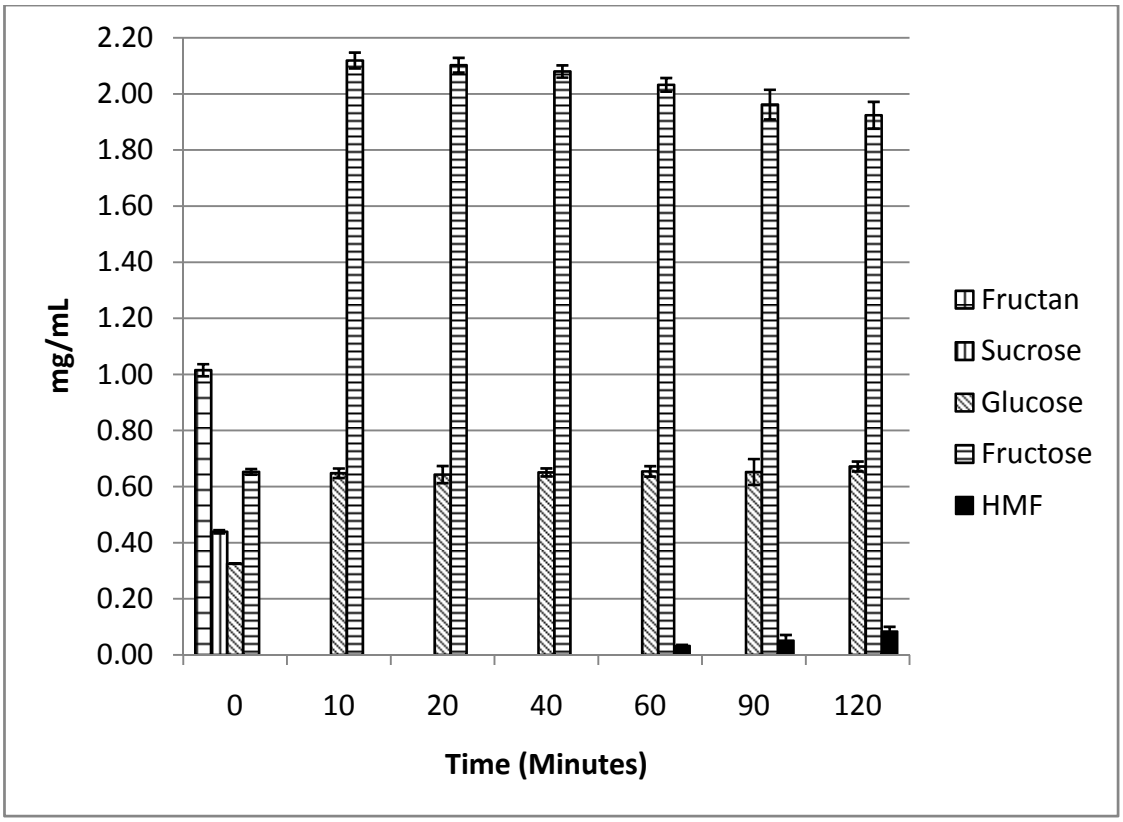

Figure 9. Time course of Tall Fescue hydrolysis at 100°C, 1% sulfuric acid. DFDA, difructose dianhydride; HMF, hydroxy methylfurfural. Error bars +/- 1 standard deviation.

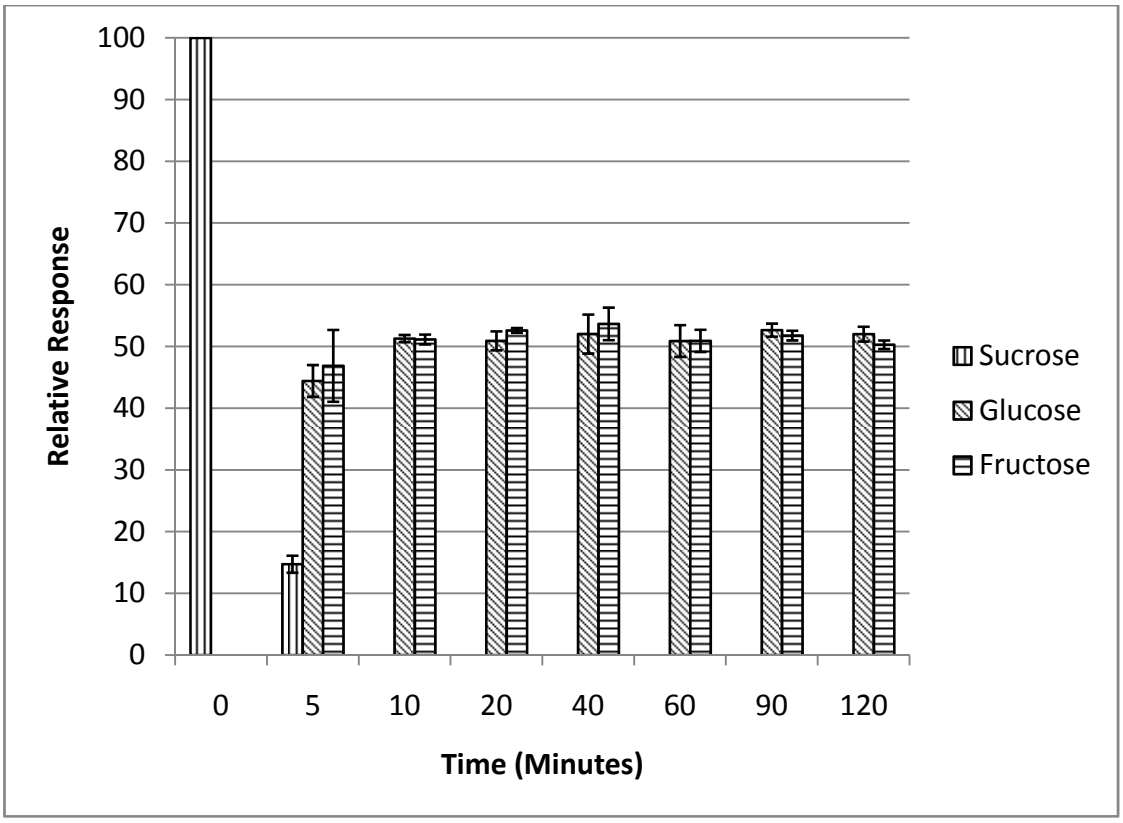

Figure 10. Time course of sucrose hydrolysis at 100°C, 0.2% sulfuric acid. "Relative response" is relative to 1mg/mL fructose or glucose solution. Error bars +/- 1 standard deviation.

All of the data summarized to this point suggest that the major fructose-containing compounds are readily hydrolyzed in 1% acid at 100^{o}C, and that the rate of fructose degradation under these conditions is low, relative to rates of hydrolysis. The fructose equivalents of a representative grass seed straw (Tall Fescue) were thus determined using these conditions, with a one hour hydrolysis period. In this case, i.e. the analyses of fructose-equivalent in Tall Fescue straw, there was no evidence for the production of DFDA during hydrolysis and, hence, the fructose-equivalents could be determined simply by the application of the appropriate correction factor (~10% of the standard

fructose solution was lost under these hydrolysis conditions). Using this approach, the analysis indicated that the straw contained ~76g fructose equivalents per Kg straw (dry weight basis).

The above value for grams of fructose equivalents per Kg straw is based on analysis of the fructose-equivalents in a 60°C water extract of the straw (see methods). A separate analysis was done by directly hydrolyzing the straw, without fractionation by water extraction. The results from the two approaches were not significantly different; the value obtained by hydrolysis of a water extract of the straw was 76.5 ±1.3 and the value obtained by directly hydrolyzing the unextracted straw was 75.9 ±2.9. It is generally assumed that the fructans are water soluble, thus it is common to see an extraction prior to hydrolysis [32]. If extractions are to be done, then it is important to again consider the temperature. As noted above, there was detectable degradation of fructose in water at 121°C after 1 hour. Further experiments at 100°C showed a 10% decrease in the fructose content of a standard fructose solution after six hours at this temperature (data not shown). This suggests, where possible, extended extraction times at high temperatures are to be avoided due to the potential fructose degradation under such conditions. This caution is pertinent to the use of soxhlet-type apparati for extended periods.

CONCLUSION

The data summarized above provide information that may be used in deciding the appropriate conditions for hydrolysis when attempting to quantify fructose-equivalents. It is generally recognized that degradation correction factors are to be kept to a minimum when determining monosaccharide equivalents [4]. This suggest that the hydrolysis conditions typically employed for hydrolysis of structural carbohydrates are not appropriate for the hydrolysis of fructans and sucrose. We have shown a number of milder conditions that may be employed for such hydrolyses. The analysis of the fescue straw was done using 1% acid, 100^{o}C, 1 hour incubation, because this combination resulted in relatively low fructose degradation (<10%) and this acid/temperature combination is sufficient for the timely hydrolysis of DFDA should it be present in the hydrolysate.

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APPENDICES

Appendix A

Tall Fescue fructose equivalents (g/kg feedstock)		
Day	**Extraction & Hydrolysis**	**Hydrolysis without Extraction**
1	77.91, σ = 1.39	73.80, σ = 0.32
2	75.62, σ = 0.42	74.75 , σ = 2.67
3	75.85, σ = 1.44	79.16 , σ = 1.86
Average	76.46 , σ = 1.26	75.90, σ = 2.86
*Each day represents analysis of three separate experiments		

Appendix B

SULFURIC ACID CONCENTRATIONS

Table of % Concentration, molarity, density, and amount of the stock solution needed to make 25mL of solution using 2.20M H_2SO_4 stock solution

%wt/wt Concentration	Molarity	Density	Amount of stock solution (mL) added to 25mL
*20.00	2.324	1.1398	-
**19.046	2.200	1.1325	-
*4.00	0.418	1.0250	4.750
*2.00	0.206	1.0116	2.341
***1.00	0.102	1.000	1.159
***0.50	0.051	1.000	0.580
***0.20	0.020	1.000	0.232
***0.10	0.010	1.000	0.116
***0.05	0.005	1.000	0.058
***0.02	0.002	1.000	0.023

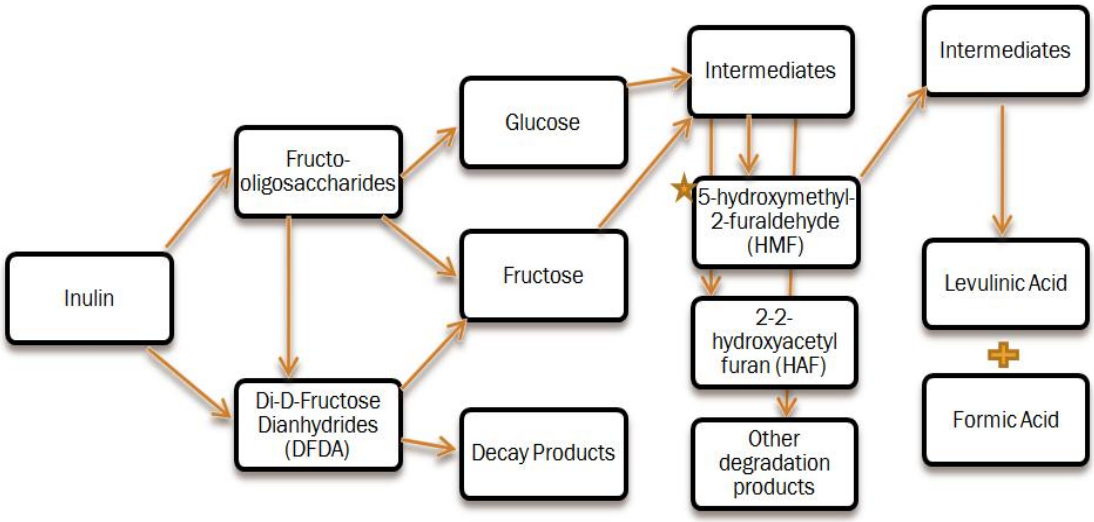
*Theoretical values from CRC Handbook of Chemistry and Physics, 85th Edition

**Calculated values

***Assumed a density of 1.00 to calculate values

Appendix C

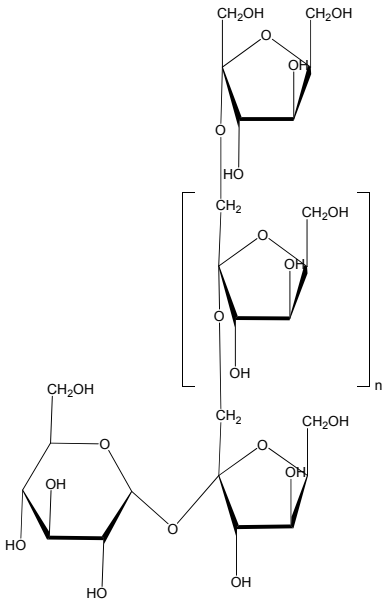
PRESUMED REACTION PATHWAYS FOR THE HYDROLYSIS OF INULIN UNDER HEAD AND ACID CONDITIONS TESTED IN THIS STUDY



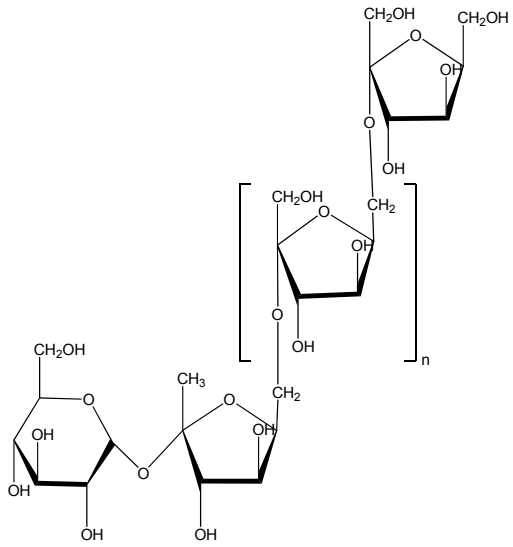
* Hydroxy methylfurfural is the primary degradation product

Appendix D

STRUCTURES OF INULIN AND LEVAN



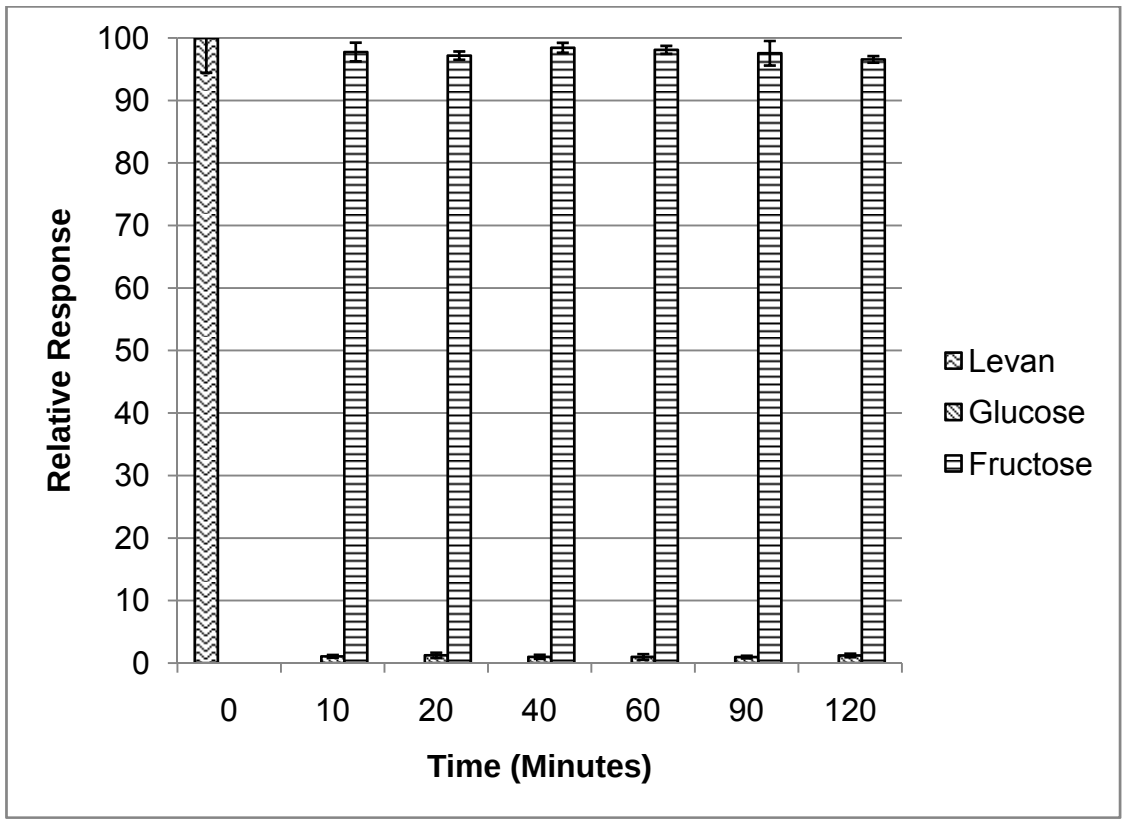
Inulin, ß-(2-1)-fructofuranosyl



Levan, ß-(2-6)-fructofuranosyl

Appendix E

HYDROLYSIS OF LEVAN AT 100°C WITH 0.2% H_2SO_4



Time-course of levan hydrolysis at 100°C, 0.2% sulfuric acid. Error bars +/- 1 standard deviation. "Relative response" is relative to 1mg/mL fructose or glucose solution.