JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. **2009,** *57,* 3995–4003 **3995**
DOI:10.1021/jf8039389

Structural Analysis of Fructans from *Agave americana* Grown in South Africa for Spirit Production

Neil Ravenscroft,*,† Paola Cescutti,‡ Meredith A. Hearshaw,†
Ronica Ramsout,† Roberto Rizzo,‡ and Elizabeth M. Timme§,⊥

†Bioanalytical and Vaccine Research, Department of Chemistry, University of Cape Town, Rondebosch 7701, South Africa, ‡Department of Life Sciences, University of Trieste, via L. Giorgieri 1, 34127 Trieste, Italy, §CSIR, P.O. Box 395, Pretoria 0001, South Africa, and ⊥Present address: Department of Chemistry, University of Cape Town

Fructans isolated from *Agave americana* grown in South Africa are currently used for spirit production. Structural studies on water-soluble fructans were performed to facilitate the development of other applications including its use as a prebiotic. Acid hydrolysis followed by HPAEC-PAD analysis confirmed that the fructan was composed of glucose and fructose, and size analysis by HPAEC-PAD and size exclusion chromatography indicated that the saccharides have a DP range from 6 to 50. An average DP of 14 was estimated by ^{1}H NMR analysis. Linkage analysis and ESI-MS studies suggest that *A. americana* has a neofructan structure consisting of a central sucrose to which (2→1)- and (2→6)-linked β-D-Fru*f* chains are attached. The (2→1)-linked units extend from C-1 of Fru and C-6 of glucose, whereas the (2→6)-linked β-D-Fru*f* units are attached to C-6 of the central Fru. This structure accounts for the presence of equimolar amounts of 1,6-linked Glu and 1,2,6-linked Fru found in linkage analysis and the multiplicity of the NMR signals observed. Detailed ESI-MS studies were performed on fructan fractions: native, periodate oxidized/reduced, and permethylated oligomers. These derivatizations introduced mass differences between Glc and Fru following oxidation and between 1,2-, 1,6-, 2,6-, and 1,2,6-linked units after methylation. Thus, ESI-MS showed the presence of a single Glc per fructan chain and that it is predominantly internal, rather than terminal as found in inulin. These structural features were confirmed by the use of 1D and 2D NMR experiments.

KEYWORDS: *Agave americana*; fructans; linkage; degree of polymerization; electrospray ionization mass spectrometry; nuclear magnetic resonance spectroscopy

INTRODUCTION

Agave americana, originally a native of Mexico, is one of the few species able to grow in the arid regions of the Eastern Cape Province of South Africa. The plant belongs to the Agavaceae family, which uses crassulacean acid metabolism and produces fructans as the principal reserve carbohydrate (*1*, *2*).

Chemical profiling of "inulin type fructans" shows they contain exclusively or mainly units joined by (2→1) fructosyl-fructose linkage to form linear oligo- or polysaccharides, usually with a terminal glucose unit (*3*). However, within the order Asparagales, of which the *Agave* species are members, a number of different linkage types including 1,6 and 1,6-branched Fru*f* as well as 6-Glc*p* and terminal Glc*p* have been identified (*4*).

Studies on agave species have shown *Agave deserti* to have a DP5 chain containing neokestose, (*5*), whereas *Agave vera cruz* presented a complex mixture that contained 1-kestose and neokestose as well as branched molecules with β-(2→1) and (2→6) linkages (*6*, *7*). Early work on *Agave tequilana* and *A. americana* reported mainly (2→1) linkages for the fructans (*8*, *9*), but recent investigation of *A. tequilana* Weber var. *azul* showed that although the main linkage was (2→1), there was (2→6) branching present (*10*). Further investigations of members of the Asparagales order proposed fructan structural patterns based on linkage type and DP and suggested their classification into three groups (*11*).

These findings suggest that fructans from *A. americana* may also have a branched complex structure, which has given rise to the need to accurately define the chemical structure and molecule size using modern techniques if the potential commercial health and food applications, as opposed to alcohol production (*2*, *12*), are to be realized. To answer these questions, a water extract of locally grown agave has been characterized by use of physicochemical methods including size and anion-exchange chromatography, linkage analysis, NMR, and ESI-MS.

MATERIALS AND METHODS

Preparation of the Fructan Material. Tissues from the heart of *A. americana* plants, around 7 years old and obtained from Graaf Reinet, South Africa, were cut into blocks (~0.5 × 3.0 × 3.0 mm) prior to

*Corresponding author (telephone +27 21 650 4354; fax +27 21 689 7499; e-mail Neil.Ravenscroft@uct.ac.za).

 Published on Web 4/6/2009

comminution by homogenization in distilled water (1:2 w/w plant/water). The fructans were extracted by heating the homogenate, with agitation, to 75 ± 3 °C for 30 min. Insolubles were removed by centrifugation (8000*g* at 20 °C for 30 min), and the supernatant was filtered. The filtrate was passed through an activated charcoal column and then freeze-dried to produce "total extract". Portions were then dialyzed to remove any free sugars and small oligomers to yield the "agave saccharide".

General Procedures. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was performed using a BioLC chromatography system (Dionex) composed of a GS50 gradient pump, an AS50 autosampler, an ED50 electrochemical detector, an EG50 eluent generator, and an LC30 chromatography oven housing the columns and detectors. Sample injections were performed by the autosampler using a 25 μL sample loop. Detection was by pulsed amperometry, with a quadruple waveform with a disposable gold electrode, and data analysis by use of Dionex Chromeleon software.

Ion exclusion HPLC was performed using an Aminex HPX-87C column at 85 °C with deionized water as mobile phase and a pump rate of 0.6 mL/min (*13*). Detection was by refractive index, and conditions allowed for separation of polymer of 4 or more units from trimer, dimer, and free glucose and fructose.

Analytical GLC was performed on a Perkin-Elmer Autosystem XL gas chromatograph equipped with a flame ionization detector and an SP2330 capillary column (Supelco, 30 m), using He as the carrier gas. A temperature program from 150 to 250 at 4 °C/min was used for analysis of the methylated alditol acetates. GLC-MS analyses were carried out on a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 5971 mass selective detector.

ESI mass spectra were recorded on a Bruker Esquire 4000 ion trap mass spectrometer connected to a syringe pump for the injection of the samples. Samples were injected at 180 μL/h and detected in the positive mode. The instrument was calibrated using a tune mixture (provided by Bruker).

Compositional Studies. The composition of the *A. americana* saccharide material was determined by HPAEC-PAD on a CarboPac PA10 column after mild hydrolysis using 0.5 M TFA at 60 °C for 1 h. The column was calibrated using monosaccharide standards (Dionex).

Linkage Analysis. A sample (10 mg) of agave saccharide was methylated according to the method of Harris et al. (*14*). The permethylated product was divided into five aliquots, three of which were used to identify the optimum hydrolysis conditions. For this purpose, hydrolysis with 2 M TFA was conducted at 60, 100, and 125 °C for 40 min. The products were derivatized to alditol acetates (*15*) and analyzed by GLC and GLC-EI-MS. Molar ratio values were corrected by use of effective carbon-response factors (*16*). Another aliquot of permethylated polysaccharide was hydrolyzed at 125 °C, and the products were reduced to alditols with $NaBD_4$ to label the anomeric carbon atoms, acetylated, and analyzed by GLC-MS. The ratio between 2,6- and 2,1-linked fructofuranosyl units was determined by calculating the ratio of peaks *m*/*z* 189/190 and *m*/*z* 162 /161 from six spectra after correction for ^{13}C content (7.77% of *m*/*z* 161 and 8.88% of *m*/*z* 189); the purity of $NaBD_4$ (98%) was also taken into account (*17*).

Size Analysis Using HPAEC-PAD and Direct ESI-MS. The size distribution of the agave extract and saccharide was profiled using HPAEC-PAD on a CarboPac PA200 column (3 × 250 mm) fitted with a guard column (3 × 50 mm) using an eluent gradient of 0−150 mM NaOAc in 100 mM NaOH over 80 min at a flow rate of 0.4 mL/min. The profile for the agave saccharide was compared to that obtained for chicory inulin (Sigma) and monosaccharide standards. Direct infusion ESI-MS analysis was conducted on a 1 mg/mL sample of the agave saccharide in water, diluted 100 times with 50% aqueous acetonitrile/20 mM ammonium acetate.

Fractionation of Saccharides Using Size Exclusion Chromatography Followed by ESI-MS. An aliquot of agave saccharide (15 mg) was fractionated by size exclusion chromatography on a Bio-Gel P10 column (1.6 cm i.d. × 90 cm) equilibrated in water, at a flow rate of 6 mL/h. Detection was achieved by use of a refractive index detector (WGE Dr. Bures, LabService, Analytica). Fractions were collected every 15 min, and single fractions (tubes 48, 59, 70, and 80) were subjected to ESI-MS after appropriate dilution in 50% aqueous acetonitrile/10 mM ammonium acetate.

Periodate Oxidation, Size Fractionation, and Characterization of Some Fractions by ESI-MS. Forty milligrams of agave saccharide was oxidized with $NaIO_4$ (*18*, *19*) followed by reduction with $NaBH_4$ to give the sample designated OR. The excess of reducing agent was destroyed by the addition of 50% acetic acid followed by evaporation at low pressure at 45 °C. The sample was washed three times with 10% acetic acid in methanol and three times with methanol to eliminate borate. After freeze-drying, the product was fractionated on a Bio-Gel P10 column (1.6 cm i.d. × 90 cm) equilibrated in water at a flow rate of 6 mL/h. Fractions were collected every 15 min, and single fractions (60-OR, 70-OR, 77-OR) were chosen and, after appropriate dilution with 50% acetonitrile in water/10 mM ammonium acetate, analyzed by ESI-MS. Fractions 61-OR, 69-OR, and 76-OR were permethylated (*20*) to yield oxidized, reduced, and permethylated fructans (ORM). After purification, they were dissolved in 50% methanol in water/10 mM ammonium acetate and subjected to ESI-MS analysis by direct infusion.

NMR Spectroscopy. NMR studies were carried out on the dialyzed extract (agave saccharide) to avoid spectral interference from free fructose, glucose, and low molecular weight oligomers. Samples (20−30 mg) were prepared for NMR analysis by repeatedly dissolving and drying the sample in D_2O. NMR spectra were acquired in D_2O and recorded at 300K. 1D (^1H and ^{13}C) and 2D (COSY, TOCSY, HSQC and HMQC) NMR spectra were obtained using a Varian Mercury 300, Varian Unity 400, or Varian Inova 600 instrument. The HSQC experiment was optimized for J = 140 Hz (for directly attached $^1H-^{13}C$ correlations), and the HMQC experiment was optimized for a coupling constant of 8 Hz (for long-range $^1H-^{13}C$ correlations). Spectra were referenced by the addition of DMSO with the methyl ^1H signal at 2.71 ppm and the ^{13}C signal at 39.39 ppm (*21*)

RESULTS AND DISCUSSION

Composition. The HPAEC-PAD chromatogram of the hydrolyzed sample showed that the agave saccharide consists of Glc and Fru, which is consistent with the composition expected for inulin. However, the ratio of Glc to Fru could not be estimated by this technique as the fructose is partly destroyed during acid hydrolysis, and it was therefore determined by NMR analysis. The NMR spectra of chicory inulin standard (**Figure 1**) contained characteristic small signals from the α-Glc*p* residue and large resonances due to β-Fru*f*, thus confirming the presence of glucose and fructose. The peaks were assigned on the basis of the comparison of chemical shifts with literature values (*22*) and confirmed by use of 2D NMR experiments (data not shown). The ^1H NMR spectrum of agave saccharide (**Figure 2A**) contained a small broad signal at 5.40 ppm assigned to H1 of α-Glc*p*, whereas the remaining complex signals between 4.40 and 3.50 ppm were ascribed to H2−H6 of Glc*p* and the β-Fru*f* residues. The ratio of integration of H1 of α-Glc*p* to the remaining ring protons of glucose and fructose indicated a ratio of 1Glc*p*:13 Fru*f*. In a linear polymer such as chicory inulin, this would correspond to an average degree of polymerization of 14; however, the structure of agave saccharide is clearly more complex. The multiplicity of Fru*f* ^{13}C NMR signals for agave (**Figure 2B**) compared to that obtained for chicory (**Figure 1B**) suggests the presence of additional linkages and branching, compared to inulin, a linear 1,2-linked β-Fru*f* polymer.

Linkage Analysis. The methylated agave saccharide was hydrolyzed using 2 M TFA for 40 min at 60, 100, and 125 °C, $NaBH_4$ reduced, and acetylated. GLC and GLC-MS analysis showed the presence of t-Fru, t-Glc, 1,2-Fru (or 2,6-Fru), 6-Glc, and 1,2,6-Fru. In contrast, methylation analysis of chicory inulin was shown to contain only t-Fru, t-Glc, and 1,2-Fru

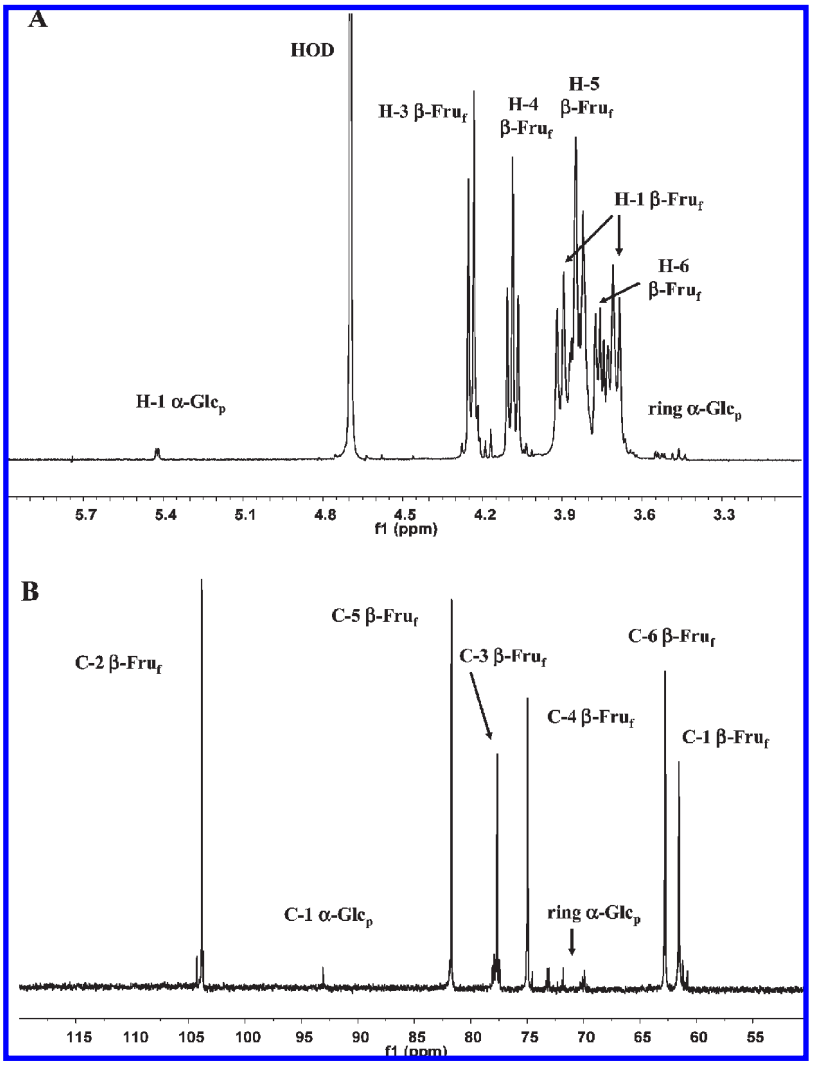


Figure 1. ^{1}H and ^{13}C NMR spectra of chicory inulin (400 MHz). Key assignments have been labeled.

(data not shown), consistent with previous results(*22*) and the structure shown below.

$$\alpha\text{–D–Glc}p\text{–}(1\rightarrow2)\text{–}[\beta\text{ – D – Fru}f]_n\text{–}(1\rightarrow2)\text{–}\beta\text{–D–Fru}f$$

The Fru*f* residues appeared as their glucitol and mannitol PMAA derivatives. Therefore, to calculate the relative molar ratios of t-Fru and the branched 1,2,6-Fru, the areas of the respective mannitol and glucitol derivatives were summed. The in-chain Fru*f* residues also appeared as both derivatives; however, the 1,2-Fru and 2,6-Fru residues are symmetrical and would therefore yield identical mass fragmentation patterns. To establish the type of glycosidic linkages present in agave (1,2- or 2,6- or both), a further aliquot of permethylated sample was hydrolyzed at 125 °C for 40 min, reduced with $NaBD_4$ to label C2 of fructose and C1 of glucose, and acetylated. The derivatives were analyzed by GLC-MS, which showed that the peak eluting at 21.83 min contained both 1,2-Fru and 2,6-Fru, whereas the peak eluting at 22.00 min was attributed only to 1,2-Fru (**Table 1**). The two alditol derivatives arising from the 2,6-Fru did not separate on the column used. From the ratio of the fragments *m*/*z* 189/190 and *m*/*z* 162/16 in the peak eluting at 21.83 min, it was found that 1,2-Fru and 2,6-Fru were present in roughly equal amounts. Therefore, half of the 21.83 min peak area corresponded to the 2,6-Fru, and the other half could be added to the 22.00 min peak area to obtain the value corresponding to 1,2-Fru. The corrected molar ratios relative to t-Fru for the three different hydrolysis conditions are presented in **Table 1**. Fructose, a keto sugar, is known to be more labile to acid hydrolysis than glucose, hence the use of the three different hydrolysis conditions (60, 100, and 125 °C) to obtain quantitative information. The results showed an increased amount of the two Glc derivatives with increasing temperature, which may be attributed to a combination of increased release of the Glc derivatives and increasing decomposition of the Fru residues at higher temperatures. Nevertheless, both t-Glc and 6-Glc were detected in all samples, with 6-Glc present as the major component and t-Glc as a minor component (7−8%). This result proves that the Glc residue in agave must be predominantly internal (6-Glc), not terminal (t-Glc), and therefore the agave saccharide should be classified as a fructan rather than inulin (*23*). The presence of 1,2,6-Fru indicates a branching Fru. The relative molar proportion of this derivative is highest for the 100 °C hydrolysis and decreases when the hydrolysis is performed at higher temperature, probably due to increased decomposition. When averaged over the three hydrolyses, there are approximately equimolar amounts of the 1,2,6-Fru and Glc, suggesting the presence of a structural motif characteristic of agavins or neofructans, in which glucose is attached to a branched fructose (*11*). Further evidence supporting this proposal follows from the presence of both 1,2-Fru and 2,6-Fru in the approximate average molar ratio of 3.3 to 1. According to the agavin model, the 1,2-Fru chain extends from

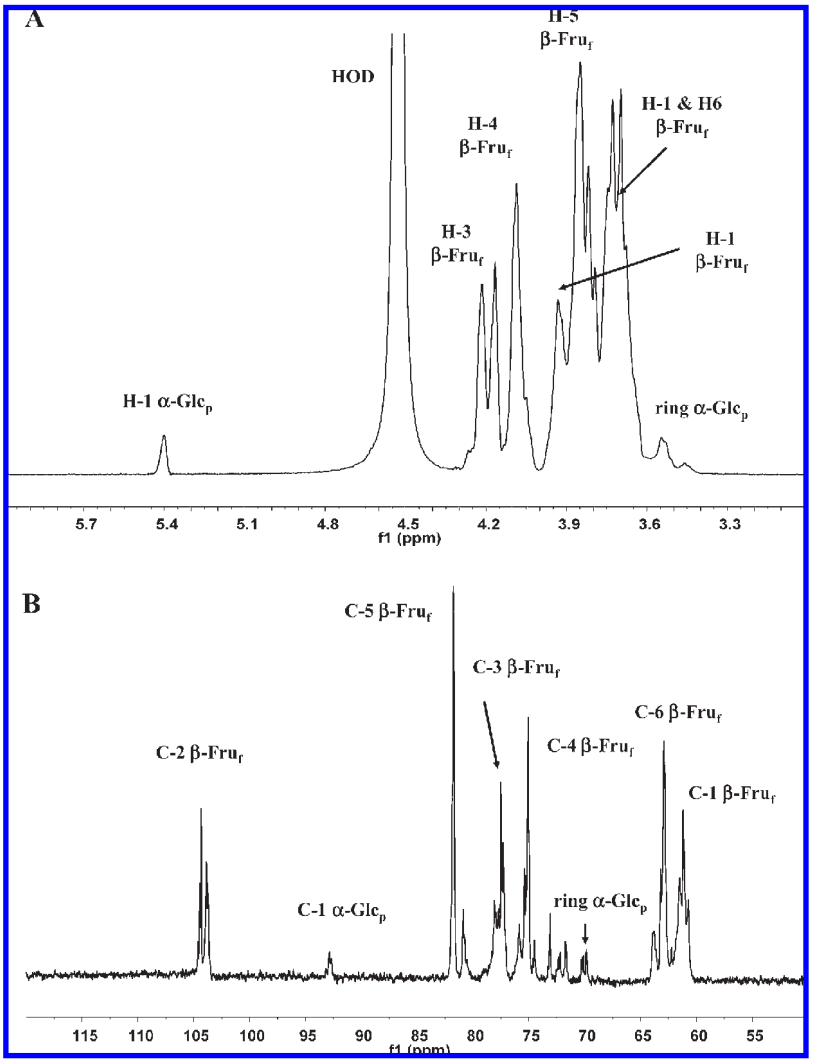
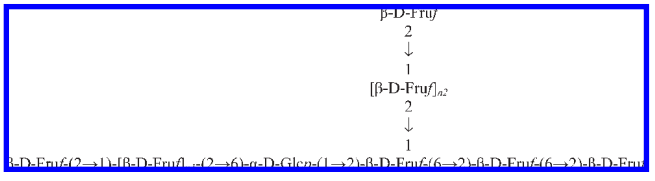


Figure 2. ^{1}H and ^{13}C NMR spectra of agave saccharide (600 MHz). Key assignments have been labeled.

C-6 of Glc and C-1 of the branched Fru, whereas the 2,6-Fru units are attached to C-6 of the branched Fru. The presence of relatively low amounts of t-Fru is consistent with the high DP of the agave saccharide (see HPAEC-PAD Profiling).



Size Analysis by Use of Ion Exclusion Chromatography and HPAEC-PAD. Ion exclusion chromatography did not permit profiling of the agave saccharides; however, it did demonstrate that the agave water extract contained small amounts of low DP material, whereas the dialyzed fraction (agave saccharide) contained only oligomers of DP4 and greater (confirmed by the corresponding HPAEC-PAD chromatograms, data not shown). Detailed analysis of the standard (chicory inulin) profile (**Figure 3A**) showed that the DP values of the polymers range from 2 to 38. The slightly broader peaks and the lack of peak resolution observed in the agave saccharide chromatogram (**Figure 3B**) compared to the chicory profile recorded under the same conditions indicated differences in the saccharide structure of agave compared to inulin, thus supporting the proposal of a branched structure for agave. The lower DP of agave was estimated to be 6, but poor resolution at high DP prevented accurate determination of the DP range; instead, the upper limit was determined by use of size exclusion chromatography with calibration using ESI-MS.

Table 1. Relative Molar Ratio of PMAA from Permethylated Agave at Three Different Temperatures of Hydrolysis

	relative molar ratios				
sugar	125 °C (D-labeled)	60 °C	100 °C	125 °C	average[a]
t-Fru	1.00	1.00	1.00	1.00	1.00
t-Glc	0.11	0.03	0.05	0.11	0.06
2,6-Fru	0.52	0.58	0.76	0.60	0.65
(1,2-Fru)	(+0.52)[b]				
1,2-Fru	0.68	2.11	2.76	1.59	2.15
6-Glc	1.03	0.38	0.59	1.35	0.77
1,2,6-Fru	0.41	0.81	1.14	0.51	0.82

[a] The average relative molar ratio was calculated from the three hydrolyses. [b] Detailed analysis of the deuterated derivatives showed that 50% of the 2,6-Fru peak area is due to 1,2-Fru; the relative areas of the three hydrolyses were corrected accordingly.

Size Analysis of Agave Saccharides by Use of ESI-MS Directly and after Fractionation Using Size Exclusion Chromatography. Direct ESI-MS of agave saccharide (**Figure 4**) showed mainly doubly charged mixed ions (NH_4^+ and Na^+) ranging from DP 3 to 26. Less abundant ions were attributed to single Na^+

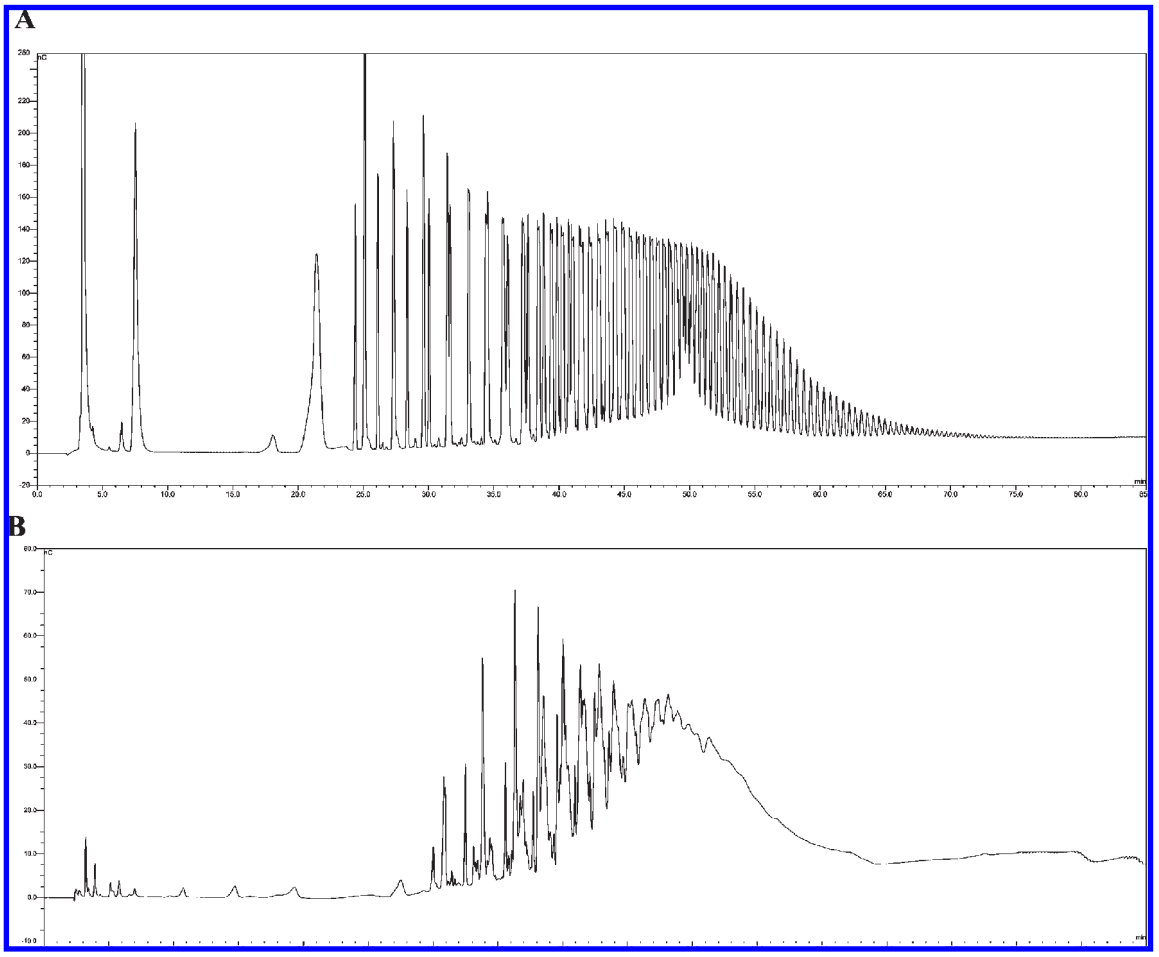


Figure 3. HPAEC-PAD profiles of chicory inulin (**A**) and agave saccharide (**B**).

adducts ranging from DP 2 to 13. Because it is known that polysaccharides give better MS spectra when their polydispersity is decreased (*24*), agave saccharide was fractionated on a Bio-Gel P10 column (S1). Single fractions of high, intermediate, and low molecular weight (48, 59, 70, and 80) were collected and analyzed by ESI-MS; the pseudomolecular ions detected together with their assignments are reported in **Table 2**. Oligosaccharides in fraction 48 were revealed as triply charged sodium species ranging from DP 26 to 31. Oligomers of DP 7 and 8 were also present in this fraction, revealing that oligosaccharides of higher DP coeluted with oligomers of lower DP due to aggregation as water was used as the chromatographic eluent (*25*). Although better separation is usually obtained using eluents of higher ionic strength (e.g., $NaNO_3$), the salts introduced would have interfered with the MS analysis. Fraction 59 showed doubly charged sodium ions from DP 17 to 21, the most abundant being DP 19. Fraction 70 showed doubly charged sodium ions corresponding to oligosaccharides from DP 7 to 13, the most abundant being DP 12. Finally, fraction 80 showed $[M + Na]^+$ ions due to oligomers from DP 4 to 8, the most abundant being DP 7. Therefore, after fractionation of the sample, it was evident that the DP of the fructans ranged from 3 to at least 31. These results broadly agree with the large DP range indicated by the HPAEC-PAD analysis, although lower DP values were detected by the sensitive ESI-MS. However, the Glc/Fru ratio in the oligomers could not be determined as both hexoses have the same molecular mass. Additionally, identification of the major saccharides present in the four fractions permitted calibration of the Bio-Gel P10 column and estimation of the highest molecular weight saccharide as DP 50.

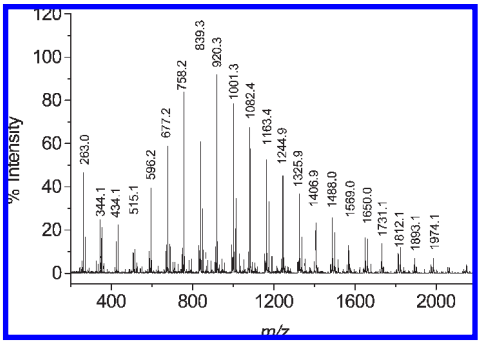


Figure 4. ESI-MS spectrum of agave saccharide.

Periodate Oxidation, Size Fractionation, and Characterization of Some Fractions by ESI-MS before and after Permethylation. To gain information on the Glc/Fru ratio in the oligomers, a sample of agave saccharide was oxidized with $NaIO_4$ and reduced with $NaBH_4$ to generate sample OR. The OR sample was fractionated on a Bio-Gel P10 column (S1), and three fractions (60-OR, 70-OR, 77-OR), similar to those collected for the native material, were collected and analyzed by ESI-MS. The oxidation and reduction reactions conserved the polymeric form of the saccharide but gave different molecular weights for the two residues. In fact, all of the fructose residues could only be cleaved at C3−C4, resulting in a Δ mass of 164.07 amu (monoisotopic mass), whereas all of the glucose residues cleaved at C2−C3 and C3−C4, resulting in a Δ mass of 134.06 amu (monoisotopic mass), regardless of the number of carbon atoms involved in glycosidic linkages. The MS spectra

Table 2. Assignment of Pseudomolecular Ions in Fractions 48, 59, 70, and 80 Obtained from Size Exclusion Chromatography of Agave Saccharide

fraction	observed *m/z*	charge	DP
48	1175.5	$[M + Na]^+$	7
	1337.6	$[M + Na]^+$	8
	1434.2	$[M + 3Na]^+/3$	26
	1488.2	$[M + 3Na]^+/3$	27
	1542.3	$[M + 3Na]^+/3$	28
	1596.4	$[M + 3Na]^+/3$	29
	1650.3	$[M + 3Na]^+/3$	30
	1704.3	$[M + 3Na]^+/3$	31
59	1410.1	$[M + 2Na]^+/2$	17
	1491.1	$[M + 2Na]^+/2$	18
	1572.1	$[M + 2Na]^+/2$	19
	1653.1	$[M + 2Na]^+/2$	20
	1734.1	$[M + 2Na]^+/2$	21
70	923.4	$[M + 2Na]^+/2$	11
	1004.4	$[M + 2Na]^+/2$	12
	1085.4	$[M + 2Na]^+/2$	13
	1175.4	$[M + Na]^+$	7
	1337.5	$[M + Na]^+$	8
	1499.6	$[M + Na]^+$	9
	1661.6	$[M + Na]^+$	10
	1823.7	$[M + Na]^+$	11
	1985.8	$[M + Na]^+$	12
	2147.8	$[M + Na]^+$	13
80	663.5	$[M + Na]^+$	4
	851.3	$[M + Na]^+$	5
	1013.4	$[M + Na]^+$	6
	1175.4	$[M + Na]^+$	7
	1337.5	$[M + Na]^+$	8

of the three fractions are shown in **Figure 5**, and the corresponding assignments are given in **Table 3**. The *m/z* values are in agreement with a complete oxidation of vicinal diols followed by complete reduction. Importantly, the pseudomolecular ions indicated that only one glucose residue is present in each molecule. Although the data in this experiment did not permit elucidation of the specific Glc linkage (t-Glc and 6-Glc have the same Δ mass value), linkage analysis has already shown that the majority of oligomers contain 6-Glc and only a small proportion of t-Glc.

Additional derivatization was performed with the aim of distinguishing among the different residues (Fru/Glc) and linkage positions by ESI-MS. OR fractions (61-OR, 69-OR, 76-OR) adjacent to those subjected to ESI-MS were permethylated and purified to give ORM fractions and analyzed by ESI-MS. In this way all of the residues that compose the polysaccharide (apart from 2,6-Fru and 1,2-Fru) had a different Δ mass value: t-Fru = 220.13; 1,2-Fru = 2,6-Fru = 206.11; 1,2,6-Fru = 192.10; t-Glc = 176.10; 6-Glc = 162.09. The ESI mass spectrum of fraction 76 ORM is reported in **Figure 6**, together with the M^2 and M^3 fragmentations. Fraction 76 ORM is mainly constituted of oligosaccharides DP 7 and 8. Pseudomolecular ions for $[M + NH_4]^+$, $[M + Na]^+$, and $[M + K]^+$ at *m/z* 1462.7, 1467.9, and 1483.8, respectively, were assigned to a completely oxidized, reduced, and permethylated heptasaccharide (DP7, one glucose and six fructose). Ions at *m/z* 1668.7, 1673.9, and 1689.8 were attributed to $[M + NH_4]^+$, $[M + Na]^+$, and $[M + K]^+$ pseudomolecular ions derived from an octasaccharide (DP 8, one glucose and seven fructose). Peaks

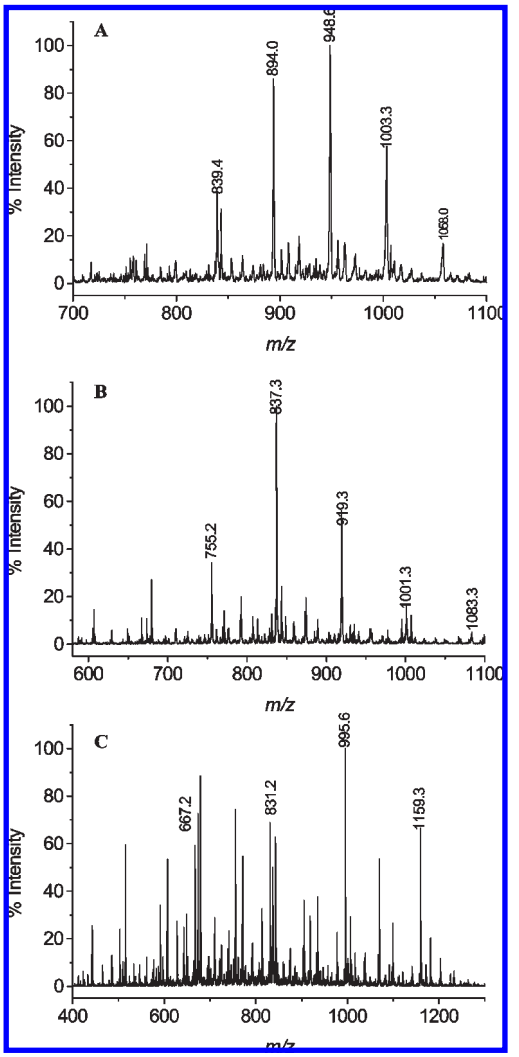


Figure 5. ESI-MS spectra of fractions 60-OR (**A**), 70-OR (**B**), and 77-OR (**C**).

Table 3. Assignment of Pseudomolecular Ions in Fractions 60-OR, 70-OR, and 77-OR Obtained from Size Exclusion Chromatography of Oxidized and Reduced Agave Saccharide

fraction	observed *m/z*	charge	DP/composition
60-OR	839.4	$[M + 3Na]^+/3$	15/1Glc + 14Fru
	894.0	$[M + 3Na]^+/3$	16/1Glc + 15Fru
	948.6	$[M + 3Na]^+/3$	17/1Glc + 16Fru
	1003.3	$[M + 3Na]^+/3$	18/1Glc + 17Fru
	1058.0	$[M + 3Na]^+/3$	19/1Glc + 18Fru
70-OR	755.2	$[M + 2Na]^+/2$	9/1Glc + 8Fru
	837.3	$[M + 2Na]^+/2$	10/1Glc + 9Fru
	919.3	$[M + 2Na]^+/2$	11/1Glc + 10Fru
	1001.3	$[M + 2Na]^+/2$	12/1Glc + 11Fru
	1083.3	$[M + 2Na]^+/2$	13/1Glc + 12Fru
77-OR	667.2	$[M + Na]^+$	4/1Glc + 3Fru
	831.2	$[M + Na]^+$	5/1Glc + 4Fru
	995.3	$[M + Na]^+$	6/1Glc + 5Fru
	1159.3	$[M + Na]^+$	7/1Glc + 6Fru

due to undermethylation (−14 amu) were also present. Isolation and fragmentation of the ion at *m/z* 1467.9 gave useful structural information. At least two series of ions could be discerned: one

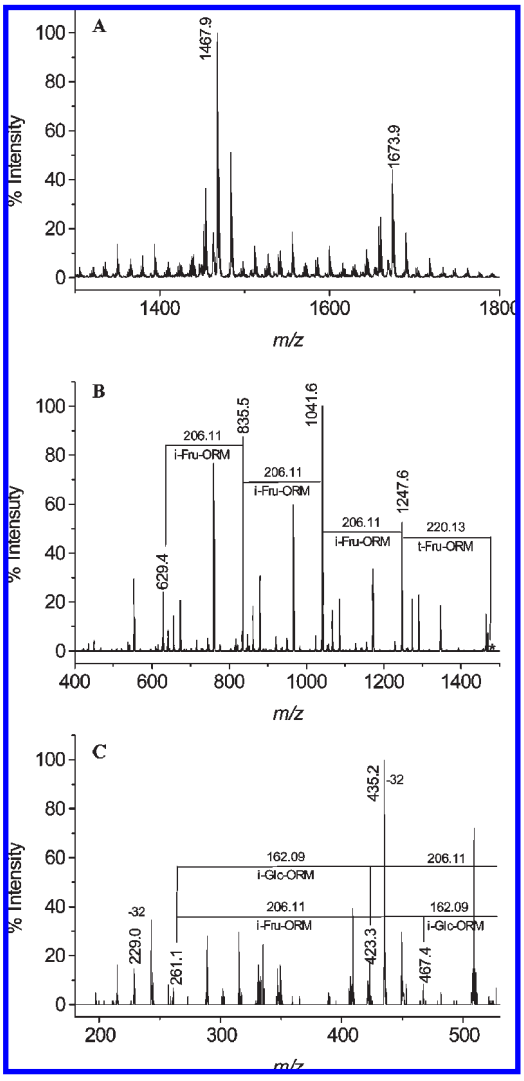


Figure 6. ESI-MS spectra of fraction 76-ORM (**A**) and MS^2 fragmentation (**B**) and MS^3 fragmentation (**C**) of *m/z* 629.5 ion, corresponding to a trimer. Peaks involved in establishing the location of Glc are shown together with those derived from loss of methanol (−32 amu).

series started with the loss of 176.10 amu, which corresponds to a t-Glc residue, and the second with the loss of 220.13 amu, which corresponds to t-Fru. The second series must therefore contain an internal Glc, and they were subjected to isolation and MS^3 fragmentation to investigate the position of the Glc. The fragments at *m/z* 629.4, 835.5, 1041.6, 1247.6, all deriving from loss of one t-Fru and three, two, and one internal fructose units, respectively, were isolated and subjected to MS^3 fragmentation. They all showed a fragment ion due to the loss of an internal Glc residue (−162.09 amu), usually accompanied by a −32 ion, attributable to the loss of methanol, thus indicating that the 6-Glc might occupy any position inside the DP 7 oligomer. The shorter the fragment subjected to MS^3, the more intense the M − 162.09 ion detected. This can be summarized, regardless of the branching points, with the following formula: Fru-$(Fru)_{n1}$-Glc-$(Fru)_{n2}$-Fru, where both *n*1 and *n*2 must be comprised between 0 and 4, to give a final DP of 7. Fraction 69-ORM was analyzed in the same way. It contained fructans of DP 10, 11, and 12, detected as doubly charged sodium adducts at *m/z* 1055.7, 1158.7, and 1261.7, respectively. The ion at *m/z* 1157.7 was isolated and fragmented. The fragments were present as singly or doubly charged sodium adducts, increasing the complexity of the spectrum. For fraction 69-ORM, only the fragments deriving from loss of t-Fru and subsequent losses of internal Fru were isolated again and subjected to MS^3. These ions were at *m/z* 1047.6 and 944.4 (both as doubly charged sodium adducts) and *m/z* 1660.4, 1453.7, 1247.7, 1041.7, 835.7, and 629.4 (all as singly charged sodium adducts). Their isolation and fragmentation gave rise to ions at −81 amu for the doubly charged species and at *m/z* −162., always accompanied by a 32 amu loss attributable to methanol. In two cases, ions at *m/z* 629.4 and 1247.7, only the peak corresponding to loss of both 6-Glc and methanol was present. Therefore, analysis of fraction 69-ORM showed that the 6-Glc can occupy any position inside the fructan chain, and the formula Fru-$(Fru)_{n1}$-Glc-$(Fru)_{n2}$-Fru, where both *n*1 and *n*2 must be comprised between 0 and 6 to give a final DP of 11 applies. Analysis on fractions ORM of higher DP was not feasible due to the instrumental limits (maximum *m/z* 2200), which resulted in very complex spectra containing singly, doubly, and triply charged ions.

NMR Spectroscopy. 1D and 2D NMR spectra of agave saccharide were recorded to investigate structural features indicated by the composition, linkage analysis, and mass spectrometric studies. Assignments were made by comparison with literature values for inulin (**Figure 1**) and related fructans (*7*, *22*, *26*−*28*) and supported by use of proton−proton and proton−carbon correlation experiments. The 1D spectra are shown in **Figure 2**, and the HSQC spectrum is given in **Figure 7**. The TOCSY and HMQC spectra are available as Supporting Information (S2 and S3).

The NMR spectra of agave saccharide (**Figure 2**) contain large signals from the β-Fru*f* residues and small resonances attributed to α-Glc*p*. Although many peaks were overlapping, two Fru spin systems were identified. Use of the COSY and TOCSY experiments permitted assignment of the H-3 (4.20 ppm), H-4 (4.09 ppm), and H-5 (3.85 ppm) resonances of the major Fru*f* spin system (S2). The corresponding carbon resonances were assigned at 77.46, 75.06, and 81.71 ppm, respectively, using the HSQC experiment (**Figure 7**). These assignments were confirmed by the HMQC experiment (S3), which showed intraresidue correlations from H-3 (to C-4), H-4 (to C-3 and C-5), and H-5 (to C-4). In addition, this experiment established correlations to the rest of the Fru*f* spin system. Key crosspeaks were observed between H-3 and C-1 (at 61.15 ppm) and between H-4, H-5 and C-6 (at 62.90 ppm). The corresponding proton assignments for H-1 (at ∼3.72 and ∼3.88 ppm) and H-6 (at ∼3.70 and ∼3.82 ppm) followed from the HSQC experiment. Finally, the C-2 resonances near 104 ppm showed a strong long-range correlation to the H-1 resonances (S3); this was attributed to both intraresidue correlations (between H-1 and C-2) and inter-residue correlations between H-1 and C-2 of the adjacent residues. The major Fru*f* spin system identified was assigned to 1,2-linked β-Fru*f* on the basis of comparison with assignments for chicory inulin (**Figure 1**) and literature values for inulin (*22*, *27*, *28*).

A second β-Fru*f* spin system was also identified. The TOCSY experiment showed correlations between H-3 (4.20 ppm), H-4 (4.06 ppm), and H-5 (3.92 ppm), and the corresponding carbon resonances were assigned (77.46, 75.06, and 80.85 ppm, respectively) using the HSQC experiment. Although the entire spin system could not be elucidated, the H-4 resonance showed long-range correlations to C-5 and a carbon signal at 63.77 ppm in the HMQC experiment (S3). The latter resonance was attributed to C-6 of this residue, and the H6 signals at 3.71 and 3.96 ppm were assigned using the HSQC experiment. The relative deshielding of C-6 (63.77 versus 62.75 ppm) and shielding of C-5 (80.85 versus 81.71 ppm) compared to 1,2-linked β-Fru*f* in chicory inulin confirmed that this residue must be 1,2,6-linked.

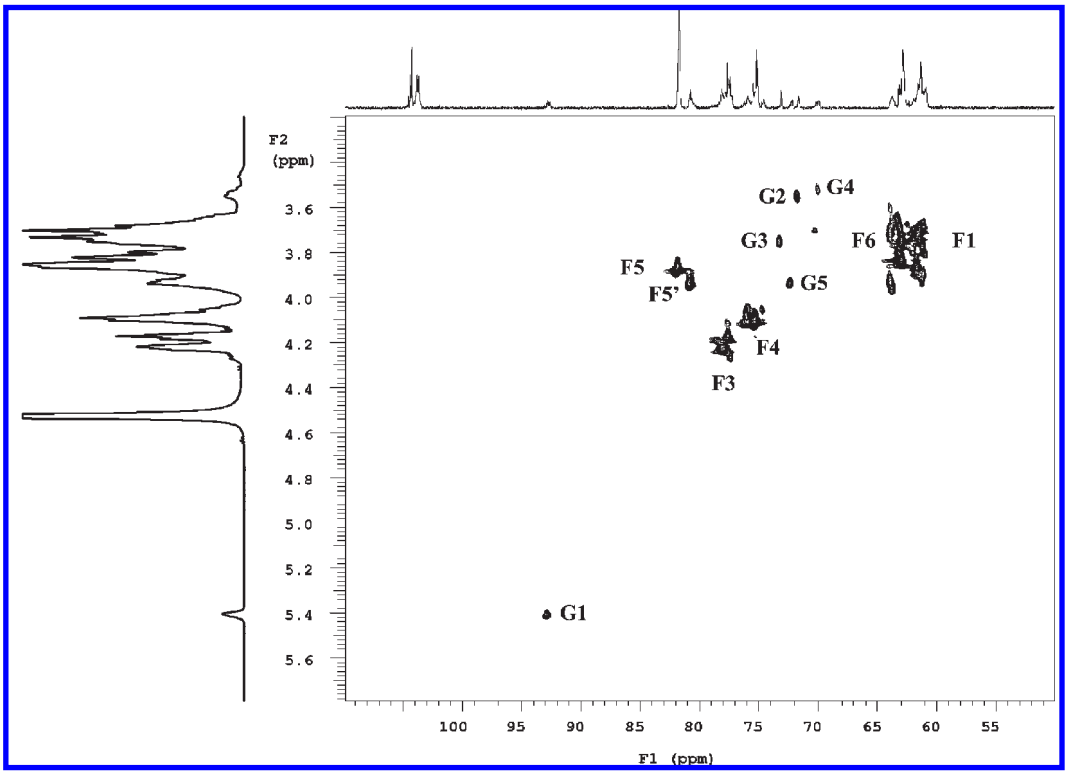


Figure 7. HSQC NMR spectrum of agave saccharide (600 MHz). Major crosspeaks have been labeled (e.g., G1 = H-1,C-1 crosspeak for α-Glc*p*; F5 = H-5,C-5 crosspeak for 1,2-linked β-Fru*f*, F5′ = H-5,C-5 crosspeak for 1,2,6-linked β-Fru*f*).

This assignment is in agreement with the interpretation of ^{13}C chemical shift data reported for fructans from *A. tequilana* Weber that contain 6-linked Fru*f* (*10*). Thus, the NMR data show that Fru is present predominantly as 1,2-linked β-Fru*f* with a smaller proportion present as 1,2,6-linked β-Fru*f*, in agreement with linkage analysis. Signals emanating from the minor 2,6-linked Fru*f* component could not be unambiguously identified.

The small signals were attributed to Glc; the small broad signal at 5.40 ppm assigned to H-1 of α-Glc*p* served as the starting point for scalar correlations established using the COSY and TOCSY (S2) experiments. This identified H-2−H-5 of the Glc spin system at 3.55, 3.75, 3.52, and 3.93 ppm, respectively. The corresponding carbon assignments for C-1−C-5 (at 92.82, 71.71, 73.08, 69.80, and 72.15 ppm) were made by use of the HSQC experiment (**Figure 7**). Corroboration of these assignments followed from the HMQC experiment (S3), which showed crosspeaks for correlations from H-1 (to C-2, C-3, and C-5), H-2 (to C-1 and C-3), and H-3 (to C-2). The crosspeaks from H-4 to C-3 and C-5 confirmed these assignments, and the crosspeak to the carbon signal at 61.05 ppm was assigned to the H-4, C-6 correlation. Thus, the ^{1}H and ^{13}C resonances of Glc were identified as labeled on the 2D NMR plots. The linkage of α-Glc*p*-(1→2)-β-D-Fru*f* was confirmed by the HMQC crosspeaks between H-1 of Glc and C-2 of Fru (near 104 ppm), whereas the β-D-Fru*f* -(1→6)-α-Glc*p* linkage was evidenced by the ^{13}C chemical shifts obtained for C-5 and C-6 of Glc compared to reference saccharides. Chicory inulin containing terminal Glc has C-5 and C-6 at 73.07 and 60.80 ppm, respectively, whereas the agave resonances are at 72.15 and 61.01 ppm. The small deshielding of the linkage position C-6 and slight shielding of the adjacent carbon (C-5) are characteristic of the effect of β-D-Fru*f* attached to C-6 of α-Glc (*28*). Thus, the presence of 1,6-linked α-Glc*p* shown by linkage analysis was confirmed by NMR analysis.

In summary, 1D and 2D NMR experiments identified the key spin systems of 1,2-linked β-Fru*f*, 1,2,6-linked β-Fru*f*, and 1,6-linked α-Glc*p*; these data together with the results from linkage analysis and ESI studies support the agavin structure proposed for the fructan produced by *A. americana* grown in South Africa.

ABBREVIATIONS USED

DP, degree of polymerization; GLC, gas−liquid chromatography; GLC-MS, gas−liquid chromatography−mass spectrometry; HPLC, high-performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; HSQC, heteronuclear single quantum coherence; HMQC, heteronuclear multiple quantum coherence; HPAEC-PAD, high-performance anion exchange chromatography−pulsed amperometric detection; TOCSY, total correlation spectroscopy.

ACKNOWLEDGMENT

Dr. F. Zanetti (Eurand International SpA, Trieste, Italy) is gratefully acknowledged for the use of the Hewlett-Packard 5971 mass selective detector. ESI mass spectra were run at the Centro Servizi Polivalenti di Ateneo (University of Trieste), and NMR spectra were recorded by Dr. J. McKenzie on a Varian Inova 600 spectrometer (University of Stellenbosch).

Supporting Information Available: Size exclusion chromatography profiles of agave saccharide and oxidized and reduced agave saccharide together with the TOCSY and HMQC spectra of agave saccharide. This information is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Nobel, P. S.; Castañeda, M.; North, G.; Pimienta-Barrios, E.; Ruiz, A. Temperature influences on leaf CO_2 exchange, cell viability and cultivation range for *Agave tequilana*. *J. Arid Environ.* **1998**, *39*, 1–9.

(2) Iniguez-Covarrubias, G.; Diaz-Teres, R.; Sanjuan-Duenas, R.; Anzaldo-Hernandez, J.; Rowell, R. Utilisation of by-products from the tequila industry. Part 2: potential value of *Agave tequilana* Weber azul leaves. *Bioresour. Technol.* **2001**, *77*, 101–108.
(3) Van Laere, A.; Van den Ende, W. Inulin metabolism in dicots: chicory as a model system. *Plant Cell Environ.* **2002**, *25*, 803–813.
(4) Sims, I. M. Structural diversity of fructans from members of the order Asparagales in New Zealand. *Phytochemistry* **2003**, *63*, 351–359.
(5) Wang, N.; Nobel, P. S. Phloem transport of fructans in the crassulacean acid metabolism species *Agave deserti*. *Plant Physiol.* **1998**, *116*, 709–714.
(6) Dorland, L.; Kamerling, J. P.; Vliegenthart, J. F. G.; Satyanarayana, M. N. Oligosaccharides isolated from *Agave vera cruz*. *Carbohydr. Res.* **1977**, *54*, 275–284.
(7) Sims, I. M.; Cairns, A. J.; Furneaux, R. H. Structure of fructans from excised leaves of New Zealand flax. *Phytochemistry* **2001**, *57*, 661–668.
(8) Sánchez-Marroquin, A.; Hope, P. Fermentation and chemical composition studies of some species of agave juice. *J. Agric. Food Chem.* **1953**, *1*, 246–249.
(9) Bhatia, I. S.; Nandra, K. S. Studies on fructosyl transferase from *Agave americana*. *Phytochemistry* **1979**, *18*, 923–927.
(10) Lopez, M. G.; Mancilla-Margalli, N. A.; Mendoza-Diaz, G. Molecular structures of fructans from *Agave tequilana* Weber var. azul *J. Agric. Food Chem.* **2003**, *51*, 7835–7840.
(11) Mancilla-Margalli, N. A.; Lopez, M. G. Water-soluble carbohydrates and fructan structure patterns from *Agave* and *Dasylirion* species. *J. Agric. Food Chem.* **2006**, *54*, 7832–7839.
(12) Casas, R. Between traditions and modernity: technological strategies at three tequila firms. *Technol. Soc.* **2006**, *28*, 407–419.
(13) Zuleta, A.; Sambucetti, M. Inulin determination for food labelling. *J. Agric. Food Chem.* **2001**, *49*, 4570–4572.
(14) Harris, P. J.; Henry, R. J.; Blakeney, A. B.; Stone, B. A. An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydr. Res.* **1984**, *127*, 59–73.
(15) Albersheim, P.; Nevins, D. J.; English, P. D.; Karr, A. A method for the analysis of sugars in plant cell-wall polysaccharides by gas−liquid chromatography. *Carbohydr. Res.* **1967**, *5*, 340–345.
(16) Sweet, D. P.; Shapiro, R. H.; Albersheim, P. Quantitative analysis by various GLC response-factor theories for partially methylated and partially ethylated aliditol acetates. *Carbohydr. Res.* **1975**, *40*, 217–225.
(17) Carpita, N. C.; Shea, E. M. Linkage structure of carbohydrates by gas chromatography−mass spectrometry (GC-MS) of partially methylated alditol acetates. In *Analysis of Carbohydrates by GLC and MS*; Biermann, C. J., McGinnis, G. D., Eds.; CRC Press: Boca Raton, FL, 1989; pp 157−216.
(18) Hay, G. W.; Lewis, B. A.; Smith, F. Periodate oxidation of polysaccharides: general procedures. *Methods Carbohydr. Chem.* **1965**, *5*, 357–361.
(19) Goldstein, I. J.; Hay, G. W.; Lewis, B. A.; Smith, F. Controlled degradation of polysaccharides by periodate oxidation, reduction, and hydrolysis. *Methods Carbohydr. Chem.* **1965**, *5*, 361–370.
(20) Dell, A. Preparation and desorption mass spectrometry of permethyl and peracetyl derivatives of oligosaccharides. *Methods Enzymol.* **1990**, *193*, 647–660.
(21) Gottlieb, H. E.; Kotlyar, V.; Nudelman, A. NMR Shifts of common laboratory solvents as trace impurities. *J. Org. Chem.* **1997**, *62*, 7512–7515.
(22) Wack, M.; Blaschek, W. Determination of the structure and degree of polymerisation of fructans from *Echinacea purpurea* roots *Carbohydr. Res.* **2006**, *341*, 1147–1153.
(23) Bruggink, C.; Wuhrer, M.; Koeleman, C. A.; Barreto, V.; Liu, Y.; Pohl, C.; Ingendoh, A.; Hokke, C. H.; Deelder, A. M. Oligosaccharide analysis by capillary-scale high-pH anion-exchange chromatography with on-line ion-trap mass spectrometry. *J. Chromatogr., B* **2005**, *829*, 136–143.
(24) Garrozzo, G.; Impallomeni, G.; Spina, E.; Sturiale, L.; Zanetti, F. Matrix-assisted laser desorption/ionization mass spectrometry of polysaccharides. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 937–941.
(25) Cescutti, P.; Rizzo, R. Divalent cation interactions with oligogalacturonides. *J. Agric. Food Chem.* **2001**, *49*, 3262–3267.
(26) Chen, X. M.; Tian, G. Y. Structural elucidation and antitumor activity of a fructan from *Cyathula officinalis* Kuan. *Carbohydr. Res.* **2003**, *338*, 1235–1241.
(27) Cérantola, S.; Kervarec, N.; Pichon, R; Magné, C.; Bessieres, M. A.; Deslandes, E. NMR characterisation of inulin-type fructooligosaccharides ast the major water-soluble carbohydrates from *Matricaria maritima*. *Carbohydr. Res.* **2004**, *339*, 2445–2449.
(28) Shiomi, N. Structure of fructopolysccharide(asparagosin) from roots of asparagus (*Asparagus officinalis* L.). *New Phytol.* **1993**, *123*, 263–270.

Received for review December 19, 2008. Revised manuscript received March 12, 2009. Accepted March 24, 2009. The University of Cape Town and the National Research Foundation are thanked for funding. The Italian Ministry of Foreign Affair and the National Research Foundation are acknowledged for funding the joint Italian - South African projects for the years 2005-2007 and 2008-2010. Ms Ramsout was funded by a CSIR bursary.