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Characterization of fructans from *Agave durangensis*

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Agave **plants are members of the** ***Agavaceae*** **family and utilize crassulacean acid metabolism (CAM) for CO_2 fixation. Fructans are the main photosynthetic products produced by** ***Agave*** **plants, and are their principal source of storage carbohydrates. The aim of this work was to determine the chemical and molecular characterization of fructans from** ***Agave durangensis*****. Fructans were extracted from 10 year old** ***A. durangensis*** **plants. Trimethylsilyl derivatization was employed to determine the monomer composition. The linkage types in these carbohydrates were determined by methylation followed by reduction and O-acetylation, and finally analysis by gas chromatography-mass spectrometry (GC-MS). Samples were shown to contain** ***t*****-β-D-Fru*****f****,** ***t*****-α-D-Glu*****p****,** ***i*****-α-D-6-Glu*****p*** **and 1,6-di-β-D-Fru*****f*** **linkages. The analysis of the degree of polymerization (DP) was confirmed by MALDI-TOF-MS, showing a wide DP ranging from 2 to 29 units. The analyses performed revealed that fructans from** ***A. durangensis*** **are formed of 97.11% fructose and 2.89% glucose, and are a complex mixture of fructooligosaccharides of the neo-fructan type containing principally β(2-1) and β(2-6) linkages, with branch moieties.**

Key words: Degree of polymerization (DP), GC-MS, MALDI-TOF-MS.

INTRODUCTION

Mexico has been considered the center of origin and biodiversity of the *Agave* genus, due to the taxonomic diversity found within its borders. Of the 310 species reported, about 272 can be found in this country. Members of the Agavaceae family are distributed throughout Mexico, and are well adapted to, both arid and semiarid regions (García-Mendoza and Galván, 1995). They have undergone both morphological and

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Abbreviations: DMSO, Dimethyl sulfoxide; **TFA,** trifluoroacetic acid; **EtOH,** ethanol; **HMDS,** hexamethyldisilazane; **NaOH,** sodium hydroxide; **CH_3I,** iodomethane; **$NaBD_4$,** sodium borodeuteride; **NH_4OH,** ammonium hydroxide; **N_2,** nitrogen; **CO_2,** carbon dioxide; **H_2O,** water; **CAM,** crassulacean acid metabolism; **PAAMs,** partially methylated alditol acetates; **WSC,** water soluble carbohydrates; **DP,** degree of polymerization; **GC-MS,** gas chromatography-mass spectrometry; **MALDI-TOF-MS,** matrix-assisted laser desorption time-of-flight mass spectrometry.

physiological adaptations to survive in such adverse conditions (López et al., 2003). One such physiological adaptation of the plant is the use of crassulacean acid metabolism (CAM), which serves to minimize water loss (Santamaría et al., 1995) by opening stomata at night when the temperature is cooler (Nobel and Linton, 1997). The principal photosynthetic products of CAM in *Agave* plants are fructans (Sánchez-Marroquín and Hope, 1953), which are synthesized and stored in the stems, and whose primary function in such plants is storage. Nevertheless, agave plants also are source of saponins and polyphenols, which are compounds considered agents with several activities such as anticancer, antifungal, anti-inflammatory, antidiabetic, anti-inflammatory, among others (Santos-Zea et al., 2012). Fructans may also act as osmo-protectants during drought (Wang and Nobel, 1998), which represents a secondary physiological adaptation. In plants, ~ 15% of higher species contain fructans, and in certain species fructans constitute the plant's sole reserve of carbohydrates. Fructans are polymers or oligomers of fructofuranosyl residues, are commonly water soluble, and are synthesized from sucrose accumulated in the vacuole of the plant (Vijn and Smeekens, 1999). Agave fructans have been used as ingredients of a granola bar, resulting in a product which has a moderate glycemic index (Zamora-Gasga et al., 2014). Indigestible fraction of this granola bar showed potential prebiotic activity, since it affected anaerobic batch cultures inoculated with human gut flora, demonstrating that agave ingredients are good sources of fermentable dietary fiber (Zamora-Gasga et al., 2015). Another work (Crispín-Isidro et al., 2015) reported that agave fructans enhanced sensory attributes of a reduced milk-fat yogurt.

According to the manner that the β-fructofuranosyl units are linked, five main types of fructan can be identified: (i) linear inulins with β(2-1)-fructofuranosyl linkages, (ii) levans with β(2-6) linkages, (iii) graminans, which are mixed fructans containing type (i) and (ii) linkages, (iv) neoseries inulins, which contains a glucose residue between two fructofuranosyl units containing β(2-1) linkages, and (v) neoseries levans, formed by β(2-1) and β(2-6)-fructofuranosyl linkages (Mancilla-Margalli and López, 2006; Sims et al., 2001). Fructans are usually present in plants as a heterogeneous mixture with varying degrees of polymerization (DP) and structure as a result of both environmental conditions and the developmental stage of the plant (Sims, 2003).

The presence of fructans in *Agave* was first reported in 1888 (Suzuki, 1993), with *Agave veracruz* and *Agave americana* being the most studied species (Aspinall and Gupta, 1959; Bathia and Nandra, 1979). More recently, Sims et al. (2001) reported the presence of a similar fructan structure in members of the *Asparagales* order, in which the *Agavaceae* family is included. However, in *Agave* species more than one fructan structure has been reported. Sánchez-Marroquín and Hope (1953) and Bathia and Nandra (1979) reported inulins the principal storage carbohydrate in *Agave tequilana* and *Agave americana*, respectively. Meanwhile, reports (Aspinall and Gupta, 1959; Dorland et al., 1977) on *Agave veracruz* showed the presence of a complex mixture of highly branched fructans with an internal glucose and containing both *β*(2-1) and *β*(2-6) linkages. More recently, Wang and Nobel (1998) reported the presence of a DP5 in *Agave deserti*, primarily in the vascular tissue. Therefore, different agaves contain fructans with a wide variety of structures, so it is necessary to characterize the fructans of each species of agave. Thus, the aim of this work was to determine the chemical and molecular characterization of fructans from *Agave durangensis*.

MATERIALS AND METHODS

Standard material

1-Kestose and Nystose standards (inulin series DP3 and DP4, respectively) were supplied by Sigma; 1, 1, 1-Kestopentaose (inulin DP5) was from Megazyme. Fructans from *Agave durangensis* were extracted and derivatized to trimethylsilyl (TMS) oximes as described below. Derivatization reagents were supplied by Sigma.

Plant materials

Ten year old *Agave durangensis* plants were harvested in the wild, in the zone of Nombre de Dios, Durango, Mexico.

Extraction of *Agave* fructans

The pines of *Agave* were cut off, the cuts were small and uniform (2×2×2 cm). Five kilograms of pine produced from mature *A. durangensis* heads were placed in a container with 10 L of distilled water at 75°C and heated for 3 h to extract the fructans content. The obtained juice was then filtered through a filter paper Whatman No. 4 (non.sterile) and stored at -20°C until further analysis (Waleckx et al., 2007).

Isolation of fructans

Four fructan fractions were obtained from the supernatant and four from the pellet by precipitation of individual samples with different amounts of EtOH (final concentrations: 100% v/v, 80% v/v, 60% v/v and 40% v/v) at 4°C overnight. The fructan fractions were collected by centrifugation (5000 g; 10 min), washed twice with the respective EtOH concentration and freeze-dried to give a white product (Wack and Blaschek, 2006).

TMS Derivatization

A test tube containing 500 µL of extract, 57 µL of 2 M acetic acid and 20 µL of inositol (as internal standard) was placed on a heating block for 45 min at 75°C and the solvent evaporated to dryness with a stream of dry nitrogen. Sugars were initially converted to their oximes by the addition of 500 µL of methoxyamine hydrochloride (25 mg/mL in pyridine) and heated for 30 min at 70°C. Sugars were then trimethylsilylated with a mixture of 900 µL HMDS (hexamethyldisilazane) and 100 µL TFA (trifluoroacetic acid), and

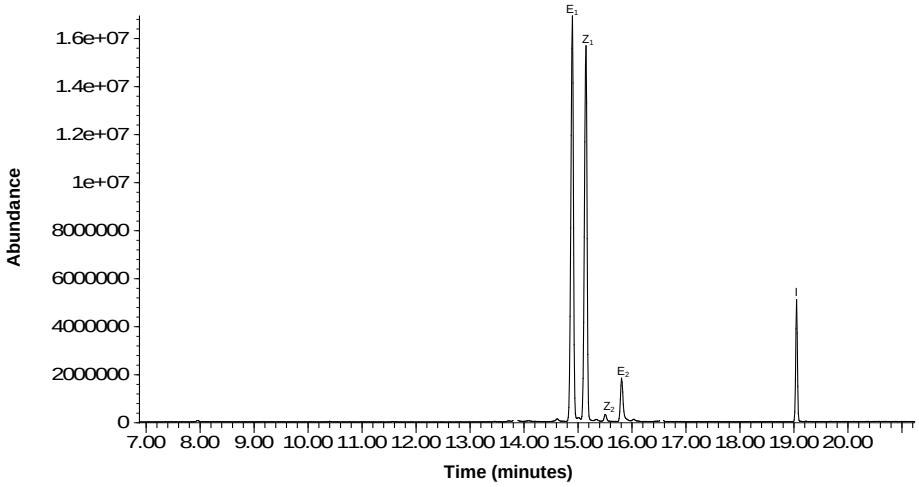

Figure 1. Capillary GC-MS separation of oxime-TMS derivatives of fructans from *Agave durangensis*. Peak identification; E_1: *syn* isomer of fructose; Z_1: *anti*-oxime isomer of fructose; E_2: *syn* isomer of glucose; Z_2: *anti*-oxime isomer of glucose, and I: inositol as internal standard.

the resultant mixture was heated for 1 h at 100°C (5, 8, 12). One microliter was injected on a gas chromatograph and separated on a 15 m x 0.25 mm x 0.25 µm DB-1 column (Hewlett-Packard) with an initial temperature of 150°C for 4 min followed by a temperature program: 4°C/min until 192°C for 0.5 min, 10°C/min until 240°C for 7 min, and then 10 °C/min until 300°C held for 10 min. The injector and detector temperatures were 260 and 310°C, respectively (Total elution time: 42.80 min).

Standard substances (1 mg/mL): fructose, glucose, inositol, sorbitol, mannitol, mannose, xylose and galactose (Sigma). The ionization spectra of all compounds were compared with those from derivatized standards.

Glycosyl linkage analysis by methylation

One milligram of *Agave durangensis* fructans were dissolved in 20 drops of DMSO, stirred on low speed overnight or until complete dissolution. Derivatization to PAAMs was carried out using the method of Ciucanu and Kerek with some modifications (Ciucanu and Kerek, 1984). Methylation was carried out by subsequent additions of NaOH (prepared from 50% aqueous NaOH in DMSO by sonication and washing twice the precipitate with DMSO) and CH_3I (2 M stabilized by copper, Sigma). Permethylated carbohydrates were extracted once with methylene chloride, washed with water, and dried under a stream of nitrogen. Those derivatives were hydrolyzed under acidic conditions with 2 M TFA at 121°C for 2 h. Reduction was carried out with $NaBD_4$ dissolved in 1 M NH_4OH at room temperature for 3 h. Excess borate was neutralized with acetic acid, and the products were taken to complete dryness with repeated addition of 9:1 acetic acid in a methanolic solution. Acetylation was performed at 50°C for 20 min using 250 µL of acetic anhydride and 250 µL of concentrate TFA. The products were extracted with methylene chloride; the organic phases were washed with water and dried under a stream of N_2. The derivatized carbohydrates were separated and identified by GC-MS. Samples were dissolved in 100 µL of methylene chloride, and 1 µL was injected into the GC-MS. Derivatized monosaccharides were separated on a 30 m x 0.25 mm i.d. x 0.25 µm SP-2330 column (Supelco, Bellefonte, PA), using helium as the carrier gas at 2.5 mL/min. The oven temperature was 80°C for 2 min and then ramped at a rate of 30°C/min to 170°C and then at 4°C/min to 240°C and held for 20 min. Injector and detector temperatures were 300°C, and column head pressure was kept at 5 psi.

MALDI-TOF-MS Analysis

Five hundred microliters of fructans from agave were dissolved in 200 µL of DMSO, purged with dry nitrogen, and sonicated for 10 min. After this time, 300 µL of NaOH and 150 µL of CH_3I were added to the sample, stirred and sonicated again for 15 min. The products were extracted with methylene chloride; the organic phases were washed with water and dried under a stream of N_2. Permethylated glycans were dissolved in 25 µL of 100% methanol, and the matrix was 2,5-dihydroxybenzoic acid; sample mixtures from 0.5 to 1 µL were applied onto the plate and quickly dried under N_2. The sample solution was serially dried with matrix to obtain optimal sensitivity. A mixture of oligosaccharides was used as the calibration standard. MALDI-TOF-MS measurement was performed using a Hewlett-Packard (Cupertino, CA) LDI AOOXP MS in the positive ion mode. The instrument was operated at an accelerating voltage of 30 kV and an extractor voltage of 9 kV. The pressure was ~2.1 x 10^{-6} Torr (Stahl et al., 1997).

RESULTS AND DISCUSSION

Composition of Fructans from *Agave durangensis*

The content of fructose and glucose was determined by gas chromatography coupled to mass (GC-MSof their TMS derivatives Carbohydrates are commonly analyzed by gas chromatography (GC) as their trimethylsilyl (TMS) derivatives. Because tautomeric forms of reducing sugars can produce multiple peaks, approaches have been taken in order to suppress the anomeric center before silylation, the most popular being the formation of oximes from the carbonyl group. The major peak, which always eluted first (Figure 1) was assigned to *syn* (*E*) isomer and

Table 1. Quantification of glucose and fructose presents in the different fractions from fructans of *A. durangensis*.

Sample	% of Fructose	% of Glucose
Fructan raw	98.63 ± 0.30	1.37 ± 0.31
Fructan in 80% of EtOH-H_2O (supernatant)	95.79 ± 1.20	4.86 ± 3.22
Fructan in 60% of EtOH-H_2O (supernatant)	97.05 ± 1.91	2.94 ± 1.68
Fructan in 80% of EtOH-H_2O (pellet)	96.96 ± 1.32	3.03 ± 1.08

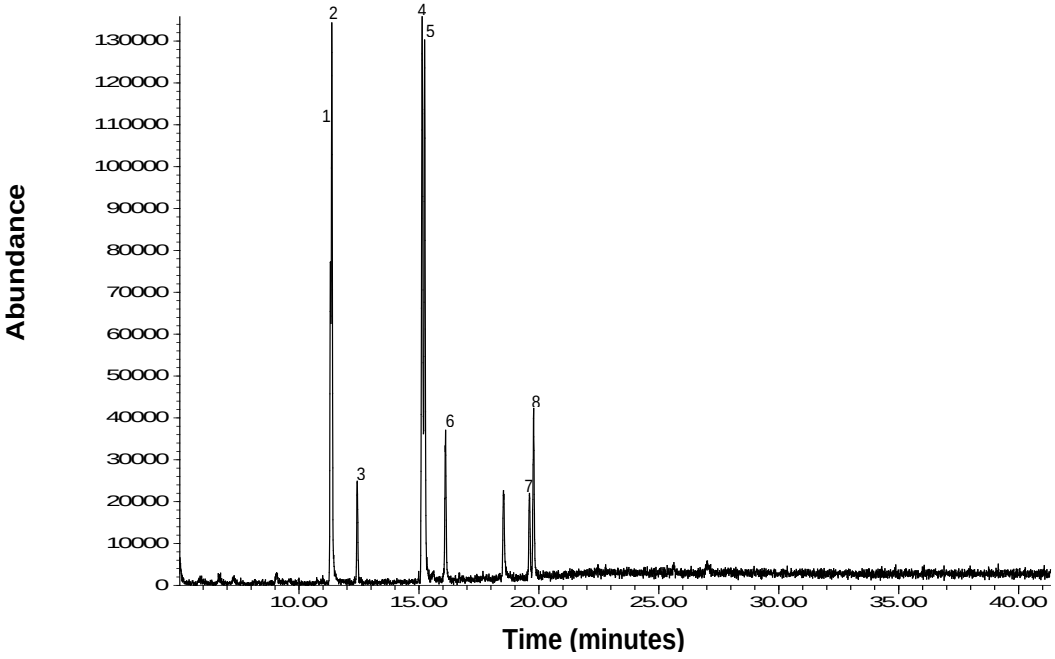

Figure 2. Chromatographic profile of derivatization products of fructans from *Agave durangensis*. Numbered peaks correspond to elution order, and they were identified as indicated in Table 2.

the minor to the *anti*-oxime (*Z*) isomer, according to the results found by Sanz et al. (2003). The *syn* (*E*) and *anti*-oxime (*Z*) isomers produced in the reaction can be separated by GC.

This method has been used to analyze different monosaccharides (Scanlon and Willis, 1985; Willis, 1983). Although several disaccharides have been analyzed as their TMS oximes, GC retention data for these derivatives are relatively scarce. This methodology was used to analyze the four fractions of fructans (100, 80, 60 and 40% in EtOH-H_2O), obtained from the supernatant, pellet and fructan raw extract. The analysis revealed only the presence of glucose and fructose.

The chromatograms of certain fractions showed only the presence of fructose and were discarded from the analysis. These included the 40 and 100% EtOH fractions of the supernatant, and the 40, 60, and 100% fractions of the pellet. The percentages of glucose and fructose in the remaining fractions are shown in Table 1, these results show that fructans from *A. durangensis* contain fructose in a proportion higher than 90%. This table also shows that the fraction of raw fructan, showed the highest content of fructose (98.63 ± 0.30%), because this fraction did not undergo any modification, unlike the rest, which were precipitated with EtOH at different concentrations. Figure 1 shows the different sugars present in the samples, and the fructose and glucose each yielded two peaks, possibly α and β isomers (Chapman and Horvat, 1989), in a 1:1 ratio for fructose and 6:1 for glucose.

Glycosyl linkage types

The results of the methylation analysis (Ciucano and Kerek, 1989) of agave fructans provided highly valuable information on the linkage types presents in *A. durangensis*. Figure 2 shows a typical chromatogram of the reductive cleavage of agave fructans. A good separation of all methylated compounds can be

Table 2. Glycosyl linkage types identified in fructans from *Agave durangensis.*

Peak[a]	t_R[b]	Derivative compound	Fragmentation pattern[c]	Linkage type[d]
1	11.31	2,5-di-*O*-acetyl-(2-deuterio)-1,3,4,6-tetra-*O*-methyl-*D*-mannitol	129-**162**-**161**-87-101-102-75-145-72-146-205	*t*-β-D-Fru*f*
2	11.36	2,5-di-*O*-acetyl-(2-deuterio)-1,3,4,6-tetra-*O*-methyl-*D*-glucitol	129-**162**-**161**-87-101-102-75-72-145-146	*t*-β-D-Fru*f*
3	12.42	1,5-di-*O*-acetyl-(1-deuterio)-2,3,4,6-tetra-*O*-methylglucitol	102-129-118-101-145-71-72-87-162-161-**205**	*t*-α-D-Glu*p*
4	15.12	2,5,6-tri-*O*-acetyl-(2-deuterio)-1,3,4-tri-*O*-methylglucitol 1,2,5-tri-*O*-acetyl-(2-deuterio)-3,4,6-tri-*O*-methylglucitol	129-87-**161**-190-162-101-100-71-72-75- 118-**189**	*(2-1)/(2-6)*-β-D-Fru*f*
5	15.24	1,2,5-tri-*O*-acetyl-(2-deuterio)-3,4,6-tri-*O*-methylmannitol	129-87-161-**190**-101-100-71-72-75-145	*(2-1)*-β-D-Fru*f*
6	16.10	1,5,6-tri-*O*-acetyl-(1-deuterio)-2,3,4-tri-*O*-methylglucitol	102-118-129-87-101-**162**-71-189-145-233	*i*-α-D-6-Glu*p*
7 y 8	19.60 19.78	1,2,5,6-tetra-*O*-acetyl-(2-deuterio)-3,4-di-*O*-methylhexitol	129-87-**190**-**189**-100-99-60-71-72	1,6-di-β-D-Fru*f*

[a]Peak numbers correspond to the elution order shown in Figure 3. [b]Retention time (minutes) in the SP-2330 column. [c]Values in black color are the fragmentation primary patterns. [d]*t*, terminal; *i*, internal.

observed.

Table 2 shows all derivatized compounds found. 2,5-di-*O*-acetyl-(2-deuterio)-1,3,4,6-tetra-*O*-methyl-D-mannitol (Peak 1) and 2,5-di-*O*-acetyl-(2-deuterio)-1,3,4,6-tetra-*O*-methyl-D-glucitol (Peak 2) were the products of a terminal fructose (*t*-β-D-Fru*f*). 1,5-di-*O*-acetyl-(1-deuterio)-2,3,4,6-tetra-*O*-methylglucitol (Peak 3) resulted from the presence of a terminal glucose (*t*-α-D-Glu*p*). The compound 2,5,6-tri-*O*-acetyl-(2-deuterio)-1,3,4-tri-*O*-methylglucitol and 1,2,5-tri-*O*-acetyl-(2-deuterio)-3,4,6-tri-*O*-methylglucitol (Peak 4) indicated the existence of branches in the fructans, while, the presence of internal glucose was confirmed by the compound 1,5,6-tri-*O*-acetyl-(1-deuterio)-2,3,4-tri-*O*-methylglucitol (Peak 6). Finally the compound 1,2,5,6-tetra-*O*-acetyl-(2-deuterio)-3,4-di-*O*-methylhexitol (Peaks 7 and 8) was due to the presence of a 1,6-di-β-D-fructofuranose. The derivatization products of *Agave durangensis* were compared with those from well-studied *Agave tequilana* Weber var. *azul* (López et al., 2003; Mancilla-Margalli and López, 2006). The fructan structural characteristics determined for *A. durangensis* coincided with those reported for other *Agave* species; the linkages, the fragmentation patterns and degree of polymerization were similar. The identity of each compound derivative was determined by comparison with standards and fragmentation patterns of spectra generated by gas chromatography coupled to mass spectrometry, and can be seen in Table 2.

In this study the fractions of fructans (80, 60 and 40% from the supernatant, and 80% from the pellet) analyzed that contained some glucose all had the same number of peaks (eight peaks), and each had the same retention time compared with other fractions, the differences were only in the abundance of each peak.

Reduced fructose produces mannitol and glucitol epimers, in the case of the terminal β-D-fructofuranose (*t*-β-D-Fru*f*), both epimers were resolved well in the column used (SP-2330) and correspond to peaks 1 and 2 (Figure 2), indicating the presence of short chain fructans [~DP3-10 (14)]. These molecules are characterized by the presence of a doublet at m/z 161 and 162 as primary fragments and doublets at m/z 145 and 146, and m/z 101 and 102 as secondary fragments, which can be observed in the spectra of Figure 4.

The elution of peak 3 corresponds to the terminal α-D-glucopyranoside (*t*-α-D-Glc*p*) with a primary fragment at m/z 205. (2-1)-β-D-fructofuranosyl and (2-6)-β-D-fructofuranosyl linkages were found in peak 4, with primary fragments at m/z 161 and 189 respectively, these linkages indicate the presence in *A. durangensis* of the Neo-fructans class.

The fragmentation pattern of an aditional peak (Peak 6) indicates the presence of internal α-D-6-glucopyranose (*i*-

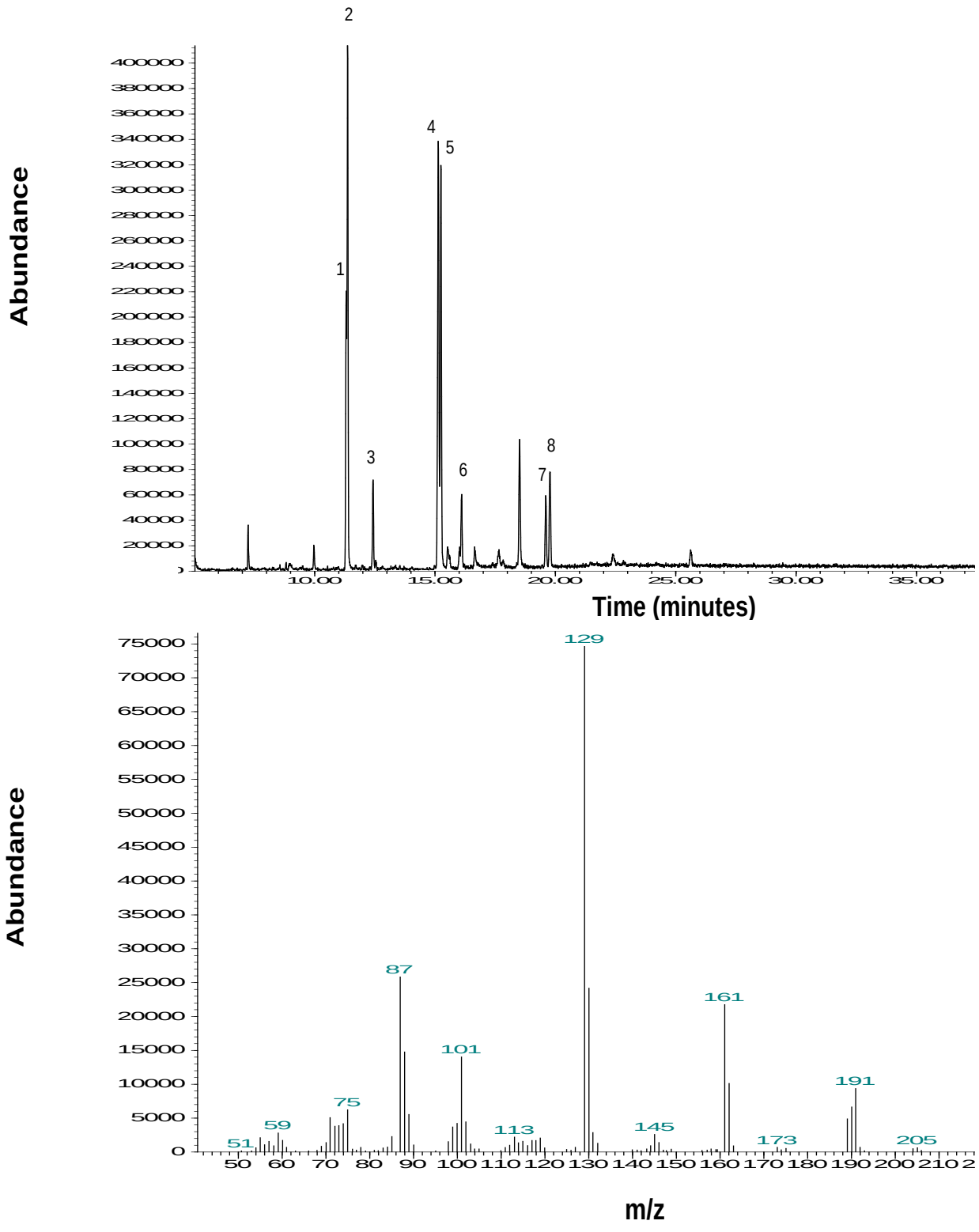

Figure 3. Separation by GC-MS of derivatives of fructans from *Agave durangensis* (a); fragmentation primary patterns (b).

α-D-6-Glu*p*), with a fragment at m/z 162, indicative of an additional acetyl group in the C_6 position (Figure 3). Finally, 1-6-di-β-D-fructofuranosyl linkage was identified in peak 7 and 8, meaning the presence of branched fructans. The six different linkages above were found in all fructan fractions differing only in the abundance of

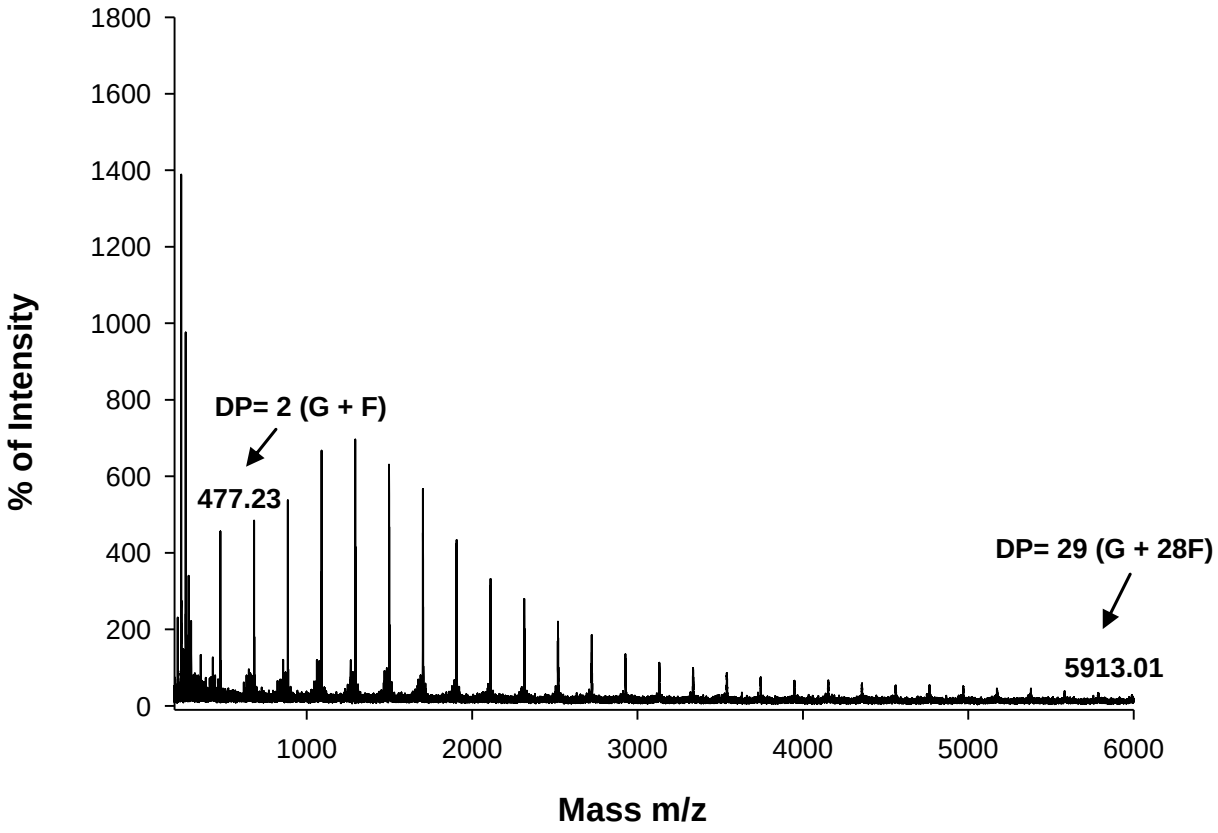

Figure 4. MALDI-TOF-MS mass spectrum of fructans from *Agave durangensis* recorded with 2,5-dihydroxybenzoic acid as the matrix. Numbers in parentheses are the number of fructose units in each fructooligosaccharide.

each peak. These linkages types are characteristic of species included in the *Asparageles* order and *A. tequilana* (López et al., 2003).

Degree of polymerization (DP) of fructans

Among all of the different analytical measurements performed with agave fructans, MALDI-TOF-MS proved to be the best option for establishing the DP distribution of these types of carbohydrates. Since 1991, MALDI has also been successfully applied to glycan analysis and is a superior technique if complex mixtures of oligo- or polysaccharides are to be analyzed as such (Stahl et al., 1997). The spectrum of masses of *A. durangensis* fructans is shown in Figure 4. It can clearly be seen that the extract displayed a complex mixture of fructans molecules; this mixture presented a molecular weight distribution of 273-5936 Da, which corresponds to a range of degree of polymerization (DP) from 2 to 29 units.

Finally, it is important to mention that the physiological functions of fructan metabolism in agave plants need to be studied carefully, because they could point to many other relevant functions such as resistance under the adverse conditions where most agave plants grow.

Conclusions

The fructans from *A. durangensis* are constituted by: 82% of water soluble carbohydrates (WSC), only fructose (97.11 ± 1.17%) and glucose (2.89 ± 1.31%) sugars; the presence of the β(2-1) and β(2-1/2-6) linkages and a molecular weight distribution of 273-5936 Da, which corresponds to a range of degree of polymerization (DP) from 2 to 29 units. These fructans are a complex mixture of oligosaccharides from Neo-fructans type. The presence of different linkages, including β(2-1) and β(2-1/2-6), the former being the most abundant, as well as GC-MS data allowed the establishment of the fructan type present in *A. durangensis*.

Conflict of interest

The authors have not declared any conflict of interest.

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