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3 The nature of fructooligosaccharides in Agave plants

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Abstract

Agaves are plants whose carbohydrates have been used since ancient times on a variety of applications, and actually they are mainly used on the elaboration of ethnic alcoholic beverages like tequila and mezcal. More than 60% of their carbohydrates on dry weight basis are constituted by fructans, which are stored in the stems. Fructans in Agaves are present as a mixture of heterogeneous structures evidenced by HPAEC-PAD and confirmed by ^{13}C-NMR and linkages analysis. Thier pattern distribution and degree of polymerization of fructans are also demonstrated

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using techniques like H-NMR and MSMALDI-TOF. Structures based on β(2-1) and β(2-6)-fructosyl linkages are proposed for Agave fructans, and different Agave species defers on the contribution of branched points and presence of internal and terminal α-D-glucopyranose moieties. These peculiarities have allowed the clustered of Agave species on three main groups. The structural diversity found in Agaves might be related with their ability to successfully grown in drought environments.

1. Introduction

1.1. Importance of the Agave genus

Members of the Agavaceae family, with eight genus, including ancient plants that have support the Mesoamerican civilization since the first inhabitants (more than 9000 years ago) until the present time [1]. Agave is the most exploited genus and very important, from a commercial point of view. There are not many crops like Agaves, which have been utilized in an integral form, and according to unique characteristics from each species such as food, fiber, sweeteners, supplement ingredients and material for house constructions among many other applications.

Metl, was the náhuatl word that prehispanics used to name this "sacred" plant, considered a gift from the goddess. Its domestication was significant as an adaptive strategy for a complementary to annual seed-based agriculture, and also a decisive factor during the conquer to drier highland regions of central and north-central Mexico [2]. On the other hand, Agave (from greek *noble* and latin *admirable*), was the word used by Charles Linneo to describe this genus (1753), referring to the notable ability of these plants to grow within extremely dry environments, where sometimes this plant is the predominant or exclusive flora in a geographic zone. Agave plants, however, can also be found in very diverse ecosystems, such as productive highlands and elevated humidity [3].

The botanic diversity found for this plant is the result of a prehistoric human selection, with the empiric objective to increase specific qualities in a diversified and specialized productive Agave system [2], being Mexico the origin center and endemic region for the majority of the species in this genus.

1.2. Uses of carbohydrates

The most highly appreciated characteristic of Agave plants since ancient times, is their outstanding soluble carbohydrate content, which represent about 80% of its weight on a dry basis [4,5]. Actually, it is known that a great majority of these carbohydrates are constituted by fructans, polydisperse molecules with fructofuranosyl-linkages [6].

Actually, Agaves are, in addition to chicory and artichoke Jerusalem, one of the three important crops whose fructans are utilized industrially [7]. Many

of these plants are the raw material used on the production of alcoholic beverages. Fructans stored in Agave stems are hydrolyzed by heat and fermented, this practice date around 1300 b.C., when the Aztec civilization fermented the sap that was emanated after the Agave stem incision. Pulque, the obtained product of this practice, is consumed even now as a nutritious beverage, especially for diabetic patients, due to its high fructose content [3]. After the Spaniards arrival to the new continent, the distillation process was introduced, giving rise to important distillated beverages like tequila, mezcal, bacanora and sisal.

Tequila is the most remarkable distilled beverage among all, with more than 780 years of tradition [8], whose industry has had an impressive growth worldwide during the last decades. This beverage is protected by the tequila origin denomination which establish that *A. tequilana* Weber in its "azul" (blue) variety and grown in a restricted geographic regions of Mexico, is the only material allowed for tequila production [6], consequently, this variety in the most exploited.

The international recognition of tequila "tequila boom" in the last decades, caused an shorting of plants and a increment of their price [8]. The tequila boom, opened the door for the acceptance of other ethnic alcoholic beverages elaborated from different Agave plants like mezcal, and in lesser way sotol and bacanora.

1.3. Carbohydrate metabolism

The cycle life of Agave plants takes from 7 to 12 years and even 50 years (*A. deserti*) according to species and environmental conditions. During the first years carbohydrates are employed for vegetative development, followed by the accumulation of fructan in the stem, main storage organ in these plants. Upon reaching maturity, fructans are hydrolyzed and soluble carbohydrates are allocated for the preparation of the floral structure emerging [9]. The majority of Agaves are monocarpic plants, since the high energetic demand for their flowering, make plants to die after this event [3].

There are not many reports on fructan metabolism in Agave; however this carbohydrate might be implicated during the crassulacean acid metabolism (CAM); photosynthetic mode used for this plant [10] and considered as determinant physiologically as the adaptation mechanism in inhospitable environments [6]. *A. guadalajarana* was classified, according to Christopher and Holtum [11], as a CAM plant that uses the NAD-malic enzyme during malic acid decarboxilation and extrachloroplastic carbohydrates (fructans) for phosphoenol pyruvate generation. This agrees with the very low amount of starch observed in Agave stems and with the inverse relationship between fructans and malic acid content observed in *A. sisalana*. Those facts suggest that fructans, rather than glucans, provide the substrate (phosphoenol pyruvate) for the synthesis of malic acid during the darkness period of photosynthesis [12].

2. Fructans in Agaves

2.1. Evidence of fructan presence

Although the Agavaceae-like species constitute one of the oldest remains of fructan flora [13], the presence of this kind of carbohydrate in Agave was reported in 1888, more than eight decades later than Rose (1804), who described for the first time fructans in a vegetative species (*Inula helenium*) [14]. Since then, not many studies have been done on fructans in Agave plants.

Agave vera cruz, referred now as *A. lurida* and nowadays almost extinguished species [3], was extensively studied by Satyanarayana group in the 70´s [15,16]. The presence of 1-kestotriose (trisaccharide synthesized from sucrose by a fructosyl-β(2-1) addition) was evident in this species. However, other fructooligosaccharides were also clearly distinguished among inulin, the linear fructan with β(2-1)-linkages, described in the Asteraceae *Helianthus tuberosus* [17].

More recently, Wang and Nobel [18] described the presence of fructooligosaccharides in vascular tissue of *A. deserti*, a species known as the century plant for its longevity. This result was interesting, since in addition to the report in oat [19], it was the first time that extrachloroplastic fructans were reported. The presence of fructooligosaccharides in vascular tissues could be explained as leakage from plasmmalema damaged or by the action of enzymes involved in the fructan metabolism (fructosyl-transferases and fructan-exohydrolases) that should be present in these tissues [18,19].

2.2. Fructan content

The stem constitutes the most appraisal vegetative organ of Agave plants, since it is the part where the higher amount of carbohydrates is stored; and in fact, frequently the value of Agaves is esteemed according to fructans and reducing-sugar contents stored in the stems (pines). Figure 1 shows the content of fructans determined in five Agave species commonly used for alcoholic beverages production. Those values ranged from 360 mg/g to 735 mg/g on dry weight basis, being higher than most reported in other fructan-storing plants like dahlia, chicory and perennial ryegrass with values of 350, 240 and 370 mg/g on dry weight, respectively [9,20,21]. Fructans in Agaves represent about 60% or up to 85% of water soluble carbohydrates, other important carbohydrates in Agaves are those related with fructan metabolism: glucose, fructose and sucrose [4,9].

The content of fructans stored in Agave stems has been suggested to be influenced by abiotic factors like climate, rainfall, altitude and soil [9]. Agave plants from the same species but grown in distinct environments presented significant differences on fructan content, such as *A. angustifolia* grown in

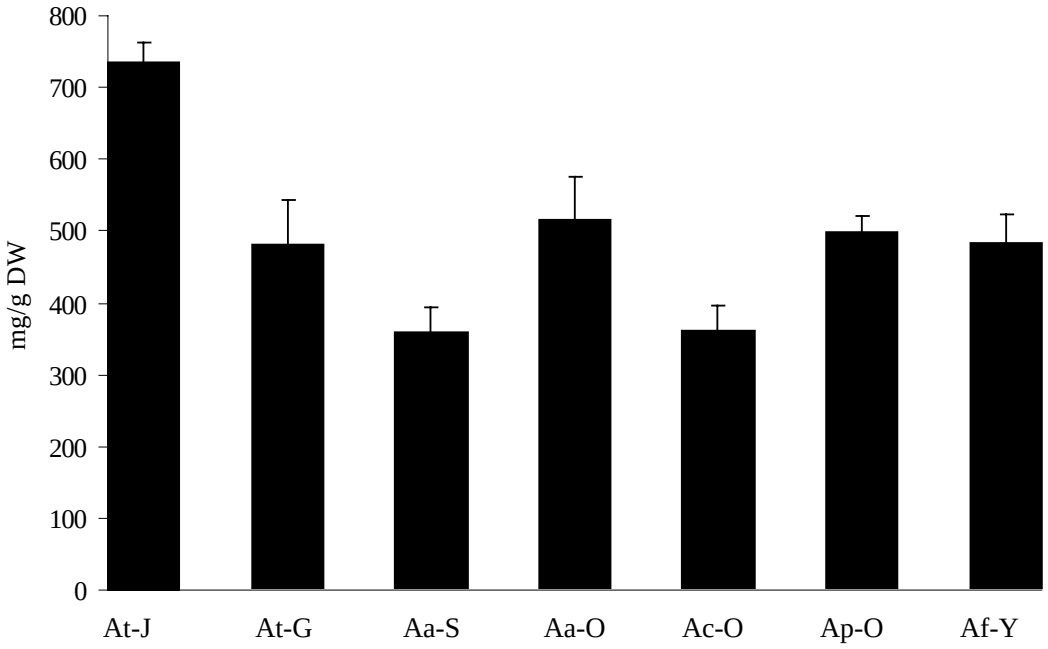

Figure 1. Fructan content determined in the stem of different *Agave* species. All plants were harvested at 6 years old and collected in different geographic zones of the Mexican Republic: At-J, *Agave tequilana* from Jalisco; At-G, *A. tequilana* from Guanajuato; Aa-S, *A. angustifolia* from Sonora; Aa-O, *A. angustifolia* from Oaxaca; Ac-O, *A. cantala* from Oaxaca; Ap-O, *A. potatorum* from Oaxaca; Af-Y, *A. fourcroydes* from Yucatán.

Sonora and Oaxaca with dry-very warm and sub-humid rainy climates, respectively, and contrasting pluvial precipitation and altitude conditions. This fact was more evident in *A. tequilana* from Jalisco and Guanajuato regions. Plants grown in Jalisco presented almost 50% higher fructan content than those from Guanajuato regions, although they are considered genetically identical, since the vegetative propagation way (by rhizomes) of this azul variety [8]. This difference was explained to be influenced by higher altitude and, specially, fresher nocturnal temperature found in Jalisco zone, which favors the uptake of CO_2, consequently, carbohydrate accumulation [9,22].

3. Structural characterization of Agave fructans

3.1. Chromatographic profile

Fructans are generally found in plants as a polydisperse molecules with different degree of polymerization (DP) and/or distinct fructosyl-linkages even when they are extracted from the same tissue. High performance anionic exchange chromatography (HPAEC) is the preferred technique to determine fructan distribution in a sample. This tool allows the separation of different fructan series (inulin, levan, neofructans, etc), but also permits to distinguish

among fructooligosaccharides isomers, such as the DP3 isomers (1-kestotriose, 6-kestotriose and 6G-kestotriose) [23-25].

The HPAEC profiles of fructans from *A. tequilana*, *A. potatorum* and *A. angustifolia* are shown in Figure 2 and compared with those from chicory and onion. Chicory from the Compositae family accumulates inulin in its root.

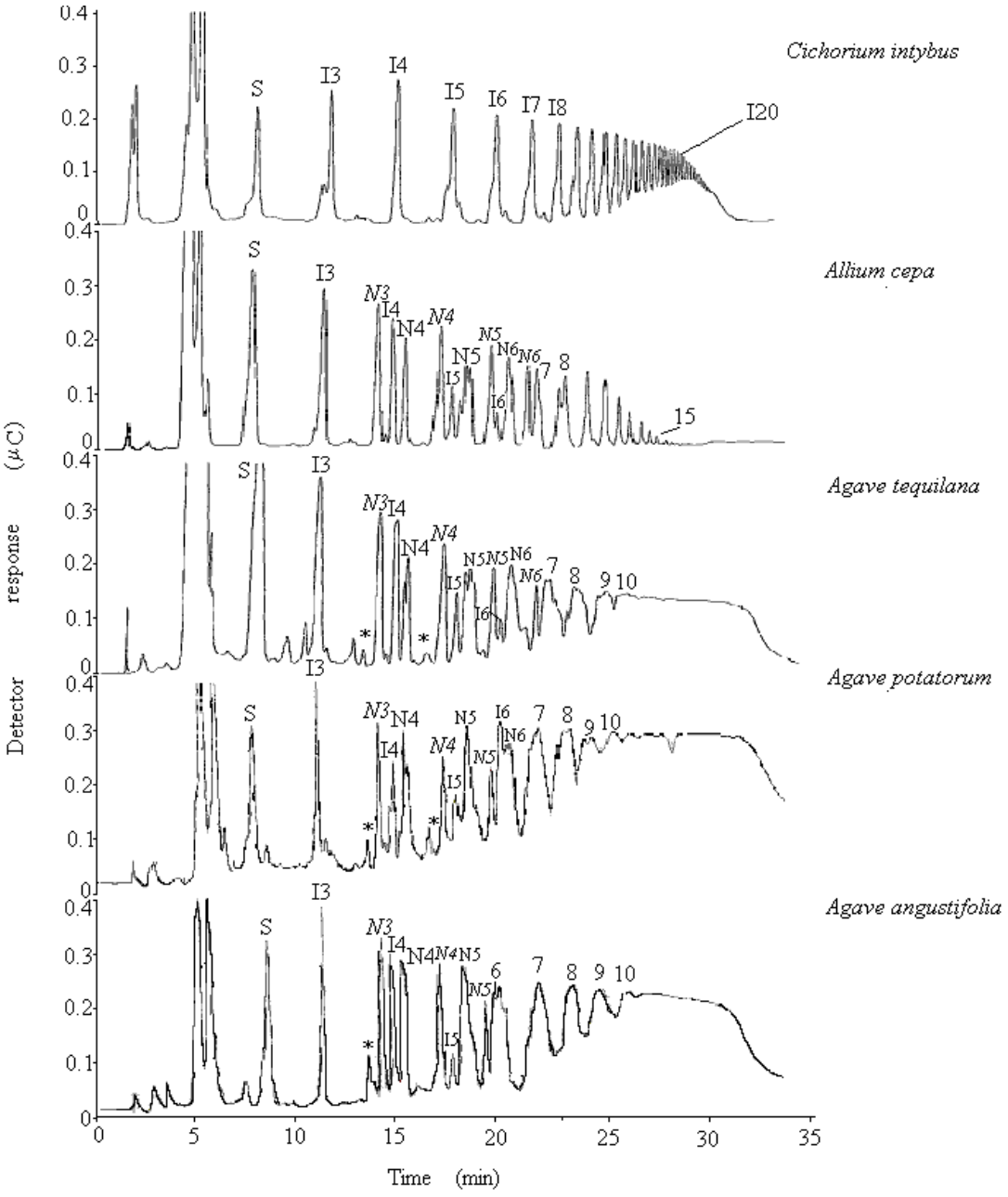

Figure 2. HPAEC profile of Agave fructans compared with fructans from chicory (inulin) and onion (neofructans). Ix, indicates inulin series with DPx; meanwhile, Nx and Nx indicates if the molecule is elongated on both ends or only at the glucose side, respectively. Asterisks marks unidentified extrapeaks observed in Agave fructans.

Although, there are evidences of branched residues in these species [26,27], they are not abundant and only a series of linear fructans with β(2-1)-fructofuranosyl units (Ix) and progressive increase in its DP is observed in Figure 2. On the other hand, the chromatographic profile from onion fructans has been reported [28,29]; they are characterized by the presence of inulin molecules and predominant neofructans, compounds with internal-α-D-Glucopyranose (*i*-α-D-Glc*p*) moieties. The 6G-kestotetraose (or neokestose) is the most abundant of this series and neofructans that by notation are distinguished according to Ernst et al. [28] like either Nx when the molecule is elongated by fructosyl-moieties on both ends or *Nx* when this is prolonged only at the glucose side.

By the retention time showed on eluted peaks, it is evident that in Agave fructans the presence of inulin series is evident in Agave fructans, in addition to both neofructan molecules, Nx and *Nx* series, such those in onion, are also present. However, unidentified smaller peaks as well as wide signals from higher DP´s, might indicate the presence of other isomer mixtures. Probably these peaks are due to molecules linked by β(2-6)-fructosyl-units or branch moieties, as those reported for Asparagales members taxonomic more related to Agaves such as *Cordyline australis*, *Urginea maritima* and *Phormium* spp [30-33]. Profile patterns for the Agave fructans shown in Figure 2 are very similar among them; however differences in the abundance of peaks and isomers are clearly observed.

3.2. ^{13}C-NMR

Nuclear magnetic resonance of ^{13}C is a method widely assisted for fructan structural determination, since it gives useful information without sample processing [27,30,34,35]. The observed complex fructans in Agave species by HPAEC was confirmed by ^{13}C-NMR [6]. Figure 3 shows the carbon spectra of *Agave tequilana* fructans precipitated by ethanol to 80% as final concentration; meanwhile, Table 1 lists the chemical shifts of these different signals and they are compared with assignments reported for other fructan species. The anomeric region corresponding to C2 fructose (δ 103-106 ppm) gives useful information about the nature of fructosyl-linkage in *A. tequilana*. Four signals

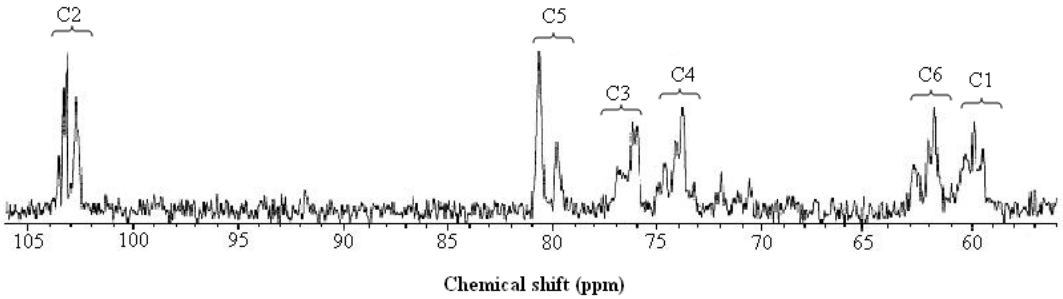

Figure 3. Carbon nuclear magnetic resonance of fructans from *Agave tequilana*. *Cx*, indicates the resonance region of carbon *x* in fructosyl-moieties.

Table 1. Chemical shifts (δ in ppm) from fructosyl-residues of ^{13}C-fructans from *A. tequilana* compared with other species.

Carbon	*P. sochifolia* β(2-1)-linkage[1] [37]	*P. peisonis* β(2-6)-linkage [31]	*U. maritima* Complex mixture [31]	*A. tequilana* Complex mixture [6]
C1	61.61-*t*[2]	60.8-*t*	60.80-*t*	61.67-*i,2-1*
	61.84-*i,2-1*[3]	60.8-*i, 2-6*[5]	61.20-*i, 2-1*	61.24-*n*
	62.08-*n*[4]		60.70-*i, 2-6*	60.77-*i, 2-6* and/or -*b*
			60.80-*b*[6]	62.27-*i, 2-6*
C2	104.53-*t*	104.8-*t*	104.50-*t*	104.87-*i, 2-6*
	104-*i*	105.1-*i*	104.00-*i, 2-1*	104.68-*b*
	104.14-*n*		104.90-*i, 2-6*	104.54-*t* and/or -*n*
			104.60-*b*	104.06-*i,2-1*
C3	77.82-*t*	77.5-*t*	77.50-*t*	77.54-*t*
	78.20-*i*	77.2-*i*	77.50-*i, 2-1*	77.32-*i, 2-6*
	77.89-*n*		77.30-*i, 2-6*	77.32
			77.50-*b*	
C4	75.46-*t*	75.4-*t*	75.40-*t*	75.98-*i, 2-6*
	75.38-*i*		75.20-*i, 2-1*	75.46
	74.93-*n*		76.00-*i, 2-6*	75.17
			75.90-*b*	74.60
C5	81.99-*t*	76.1-*i*	81.90-*t*	81.95
	82.00-*i*		81.90-*i, 2-1*	81.12
	82.18-*n*		81.00-*i, 2-6* and/or -*b*	
C6	63.02-*t*	82.0-*t*	63.40-*t*	64.11
	63.10-*i*	81.2-*t*	63.10-*i, 2-1*	63.37
	63.15-*n*		64.00-*i, 2-6* and/or -*t*	63.10

[1], indicates the fructosyl-linkage predominant in these species; [2], terminal fructosyl-residues; [3], internal β2-1-linkages; [4], fructosyl-residue due to neofructan series; [5], β2-6-moities; [6], branched residues.

on this region indicate the presence of fructose moieties bonded at least in four different ways. The signal at δ 104.06 ppm is assigned to the internal-β-(2-1)-D-fructofuranose (β2-1-D-Fru*f*), which is the most intense signal and it evidences the predominance of this kind of linkage. On the other hand, resonance at δ 104.54 ppm corresponds to terminal-β-fructofuranose (*t*-β-D-Fru*f*) and from its considerable intensity it suggests molecules highly branched in *A. tequilana* fructans. The nature of branched residues (1,6-di-β-D-fructofuranose, 1,6-di-β-D-Fru*f*) is confirmed with the signal at δ 104.68 ppm, whose value is similar to the assignment reported for branched residues in *Urginea maritima* [31]. The internal-β-(2-6)-D-fructofuranose-linkages (β2-6-D-Fru*f*) is also demonstrated by the δ 104.87 ppm resonance and confirmed by signals at δ 81.12 and 64.11 ppm due to C5 and C6, respectively, holding a

substitution on O6 [6,32]. The presence of internal-α-D-glucopyranose (*i*-α-D-Glc*p*) shifted the C2-fructosyl-signal around to δ 104.5 ppm [36]; therefore it is possible that shifted at δ 104.54 ppm correspond to an overlaping signal of both *t*-β-D-Fru*f* and *i*-α-D-Glc*p* moieties.

3.3. Glycosyl-linkages

Generally, glycosyl-linkage determination by methyl-derivatives has been used as complementary technique to NMR analysis during elucidation of fructan structures [27,30,32]. The mass spectrometry analysis of fructan derivates of partially methylated alditol acetates (PMAA´s) reported for some Agave species (Table 2), have demonstrated the heterogeneity of this fructan mixture stored in Agave stems [9].

The presence of *t*-β-D-Fru*f* and both β2-1- and β2-6-fructosyl-linkages is evident, due to the glucitol isomers chromatographically unresolved. Derivatives corresponding to branched units, 1,6-di-β-D-Fru*f*, have been identified by fragments of *m/z* 189 and 190 with similar relative intensities [24]. On the other hand, the α-D-Glc*p* residues are shown by a fragmentation pattern with a *m/z* signal at 102 as base fragment and *m/z* 118 as a prominent fragment. The i-α-D-Glc*p* residue due to neofructan series is recognized from t-α-D-Glc*p* residue by the *m/z* 233 fragment, due to an additional acetyl group in the C6 [38].

3.3.1. Differences on glycosyl-constituents in *Agave* species

Although the methylation patterns of fructans from different *Agave* species seem very similar, important differences are observed in the molar contribution of each residue type. In addition, since it has been proposed that fructan structures might be specific depending on the species [32,35], the PMAA´s compounds from *Dasylirion* spp. fructans, a tightly species taxonomically related to Agaves, has been also analyzed [9]. Figure 4 shows the molar contribution of derivative compounds from many species, and it is evidenced that the main difference among them is quantitative more than qualitative.

It is difficult to establish the significance of these quantitative differences; however, according to these data fructans from *Agave* and *Dasylirion* species were clustered into three groups, considering the summatory of *t*- and *i*-α-D-Glc*p* as the unity (Table 3) [9]. Species associated in group I presented more contribution of neofructan series than fructans with *t*-α-D-Glc*p* structure (about 4:1 ratio); this relationship is 2:1 for species clustered in group II, and equal proportions of these fructan structures has been found in *A. tequilana* from Guanajuato region, that according to its more dissimilar distribution was the only species clustered in group III. A major contribution of β(2-1)-linkages is common for all species, with a ratio values of 2, 4 and 3 for groups I, II and III,

Table 2. Partially methylated alditol acetates identified for fructans from *Agave* species.

Rt [1]	Derivative Compound	Linkage type [2]	Fragmentation pattern [3]
50.21	2,5-Di-O-acetyl-(2-deuterio)-1,3,4,6-tetra-O-methyl-D-mannitol	*t*-β-D-Fru*f*	129 (100), 162 (46.6), 161 (30.0), 87 (25.0), 101 (15.8), 102 (15.0), 75 (11.7), 145 (8.3), 72 (8.3)
50.81	2,5-Di-O-acetyl-(2-deuterio)-1,3,4,6-tetra-O-methyl-D-glucitol	*t*-β-D-Fru*f*	129 (100), 162 (38.9), 161 (34.7), 87 (24.5), 101 (15.2), 102 (14.4), 75 (10.1), 72 (10.1), 146 (5.8)
55.07	1,5-Di-O-acetyl-(1-deuterio)-2,3,4,6-tetra-O-methyl-glucitol	*t*-α-D-Glc*p*	102 (100), 129 (62.0), 118 (55.7), 101 (52.4), 145 (40.0), 71, 72 (36.6), 87 (36.0), 162 (27.8), 161 (26.2), 205 (11.4)
63.71	2,5,6-Tri-O-acetyl-(2-deuterio)-1,3,4-tri-O-methyl-mannitol	(2→6)-β-D-Fru*f*	129 (100), 162 (45.2), 87 (35.8), 99 (16.9), 189 (15.0), 71, 72, 102 (13.2), 75 (12.2), 60 (10.3)
64.36	1,2,5-Tri-O-acetyl-(2-deuterio)-3,4,6-tri-O-methyl-mannitol	(2→1)-β-D-Fru*f*	129 (100), 87 (33.8), 161 (25.4), 190 (23.7), 101 (14.4), 100 (13.5), 71, 72 (10.1), 75 (8.47), 145 (6.7)
64.94	2,5,6-Tri-O-acetyl-(2-deuterio)-1,3,4-tri-O-methyl-glucitol + 1,2,5-Tri-O-acetyl-(2-deuterio)-3,4,6-tri-O-methyl-glucitol	(2→1)/ (2→6)-β-D-Fru*f* [4]	129 (100), 87 (30.3), 161 (29.4), 190 (14.1), 162 (11.6), 101 (10.7), 100 (8.9), 71, 72, 75, 118 (7.1)
68.67	1,5,6-Tri-O-acetyl-(1-deuterio)-2,3,4-tri-O-methyl-glucitol	*i*-α-D-Glc*p*	102 (100), 118 (75.8), 129 (55.1), 87 (51.7), 101 (27.5), 162 (22.4), 71 (22), 189 (13.7), 145 (6.8)
78.14	1,2,5,6-Tetra-O-acetyl-(2-deuterio)-3,4-di-O-methyl-hexitol	1,6-di-β-D-Fru*f*	129 (100), 87 (42.5), 190 (20.3), 189 (17.5), 100 (16.2), 99 (14.8), 60 (11.1)

[1], retention time (min) in HP5 column (30 m μ 0.25 mm i.d.); [2], *t*, terminal and *i*, internal; [3], values in parenthesis indicates relative intensity of the fragments; [4], glucitol isomers of β(2-1)- and β(2-6)-D-Fru*f* after reduction with $NaBD_4$ were unresolved on the chromatographic column used.

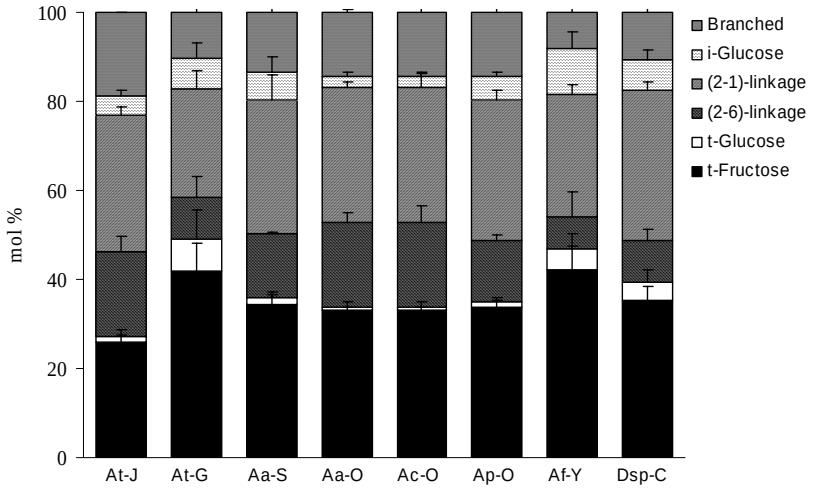

Figure 4. Glycosyl-linkage composition in mol %, of fructans from *Agave* and *Dasylirion* spp. Abbreviations as in Figure 1; Dsp-C, *Dasylirion* spp. from Chihuahua. Bars represent SD of three determinations.

Table 3. Clustering of *Agave* and *Dasylirion* species according to number of fructan glycosyl-components, considering α-D-Glc*p* as the unit.

Group	*t*-α-D-Glc*p*	*i*-α-D-Glc*p*	*t*-β-D-Fru*f*	(2-6)-β-D-Fru*f*	(2-1)-β-D-Fru*f*	1,6-di-β-D-Fru*f*	DP Estimation
I							
At-J	0.20	0.79	4.70	3.46	5.53	3.42	18.12
Aa-S	0.18	0.82	4.51	1.90	3.92	1.74	13.07
Aa-O	0.21	0.79	10.51	6.01	9.64	4.59	31.75
Ap-O	0.17	0.83	5.19	2.12	4.84	2.19	15.34
II							
Ac-O	0.33	0.67	4.27	0.95	3.71	1.24	11.17
Af-Y	0.31	0.69	2.81	0.49	1.82	0.55	6.66
Dsp-C	0.38	0.62	3.21	0.84	3.08	0.96	9.09
III							
At-G	0.52	0.48	2.99	0.65	1.75	0.74	7.13

respectively. Interestingly, a ratio near to 1 was observed among β(2-6)-linkage and branched moieties for group I, a not significantly different value (0.8) was found for the other two groups. Another important data is the ratio found between terminal fructose and branched moieties, which is correlated with the length chain and branching points, since molecules with high DP or highly branched, this ratio should be near to one. In this context, group I presented the lowest value, with a ratio of 2 for all members except for *A. tequilana* (Jalisco) that had a ratio of 1, indicating the presence of fructans highly branched. *A. cantala* and *Dasylirion* spp. presented a ratio of 3, meanwhile lesser branched structures were reported for *A. tequilana* (Guanajuato) and *A. fourcroydes* with ratio values of 4 and 5, respectively.

4. Distribution of degree of polymerization

Although fructans are polydisperse mixtures, present in vegetative tissues like series of different DPs, the kinetic properties of fructosyltransferases, enzymes involved in their metabolism, are reported to be depending on the species [39,40]. Therefore, the distribution pattern on the length of fructan polymers might depend not only on environmental and developmental conditions, but also it is dependent on species [41].

A few reports on the length of fructans from *Agave* species are available. The fructose-glucose ratio was used to determine a DP up to 32 for *A. deserti* [42]; meanwhile, H-NMR and MALDI-TOF-MS are techniques applied to determine the distribution of DP in *A. tequilana* [6].

4.1. H-NMR

Like ^{13}C-NMR, reports of fructans H-NMR are less frequently [34,36]. Figure 5 shows the H-NMR spectra of *A. tequilana* fructans. The resonance at

δ 5.23 ppm due to H1 from the glucose moiety is easily resolved and identified; however, the remaining chemical shifts are hard to assignate due to an extensive overlapping in a ranging from δ 3.40 to 4.18 ppm. The complexity of fructan structure in *A. tequilana* evidenced by ^{13}C-NMR (Figure 3) and the narrow zone where protons are shifted, make difficult the signal resolution and therefore the structural analysis by these methods. However, the overlapped signals integration permitted to calculate a value of 124 protons in this area. This value suggests a DP of 21 glycosyl-moieties [6].

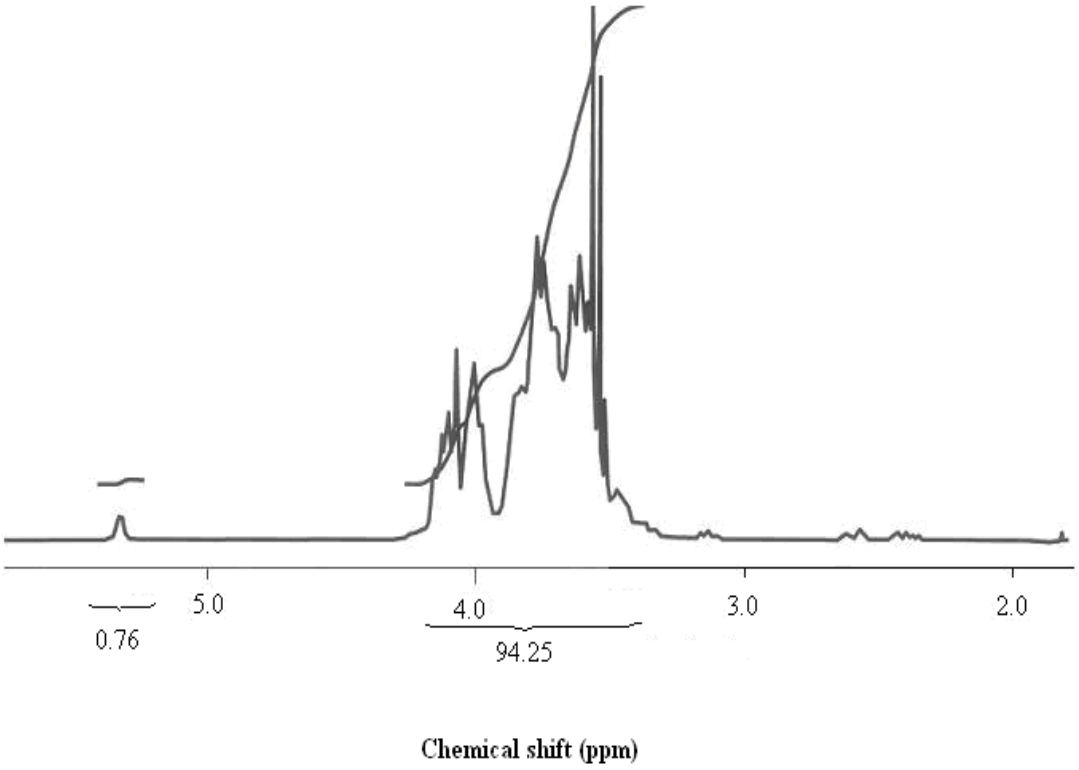

Figure 5. Proton nuclear magnetic resonance of fructans from *Agave tequilana*. The integration of signal was considered according to unique value assigned to 1H belong to glucose moiety (0.76).

4.2. MALDI-TOF-MS

Figure 6 shows the mass spectrum obtained by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) for fructans from *A. tequilana*. This spectrum confirms, with a major resolution and precision, the presence of a heterogeneous mixture in Agave fructans. The mass range observed fall between m/z 527 and 4739 Da and indicates for this species, fructans with a DP among 3 to 29 units forming adducts with Na^+ ($G\text{-}F_n^+ + Na^+$).

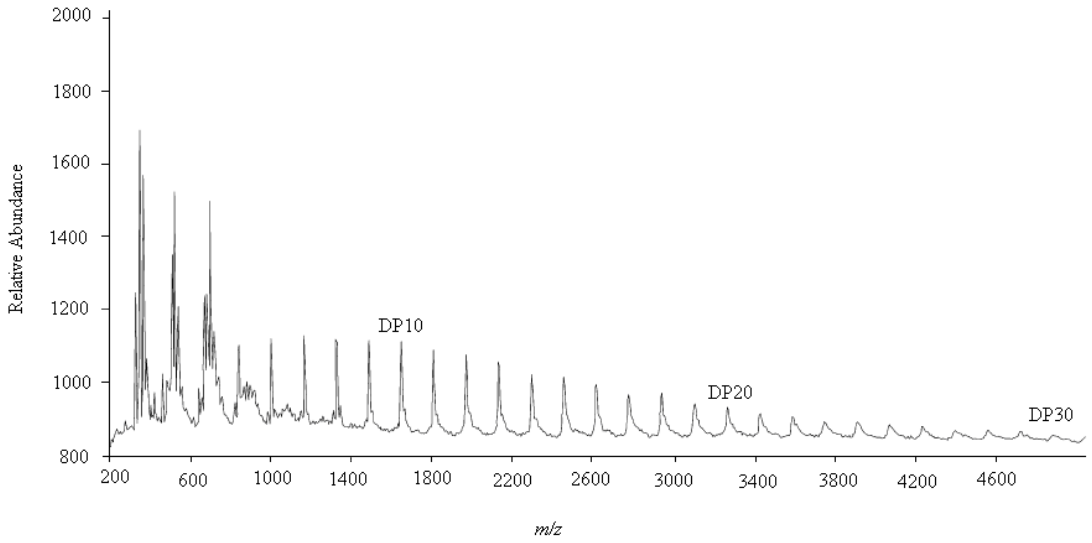

Figure 6. MALDI-TOF-MS of *A. tequilana* fructans. The spectrum was registered in the positive ion form; the matrix was constituted by 2,5-dihydroxybenzoic acid.

4.3. From PMAAs

The estimation of DP in other *Agave* species has been determined based on the number of fructan glycosyl-components from PMAAs derivatives (Table 3). Although this method is less precise than techniques such as MALDI-TOF-MS and size exclusion chromatography, it has been validated since the DP calculation of fructans from onion and dahlia have been done in this manner [43,44].

Based on these data, Agave fructans were clustered again in three groups. Species in group I presented the larger DP (13 to 32); meanwhile the DP range for group II was from 7 to 11 [9].

5. Fructan structure

5.1. Structure determination

The first fructan structure for Agave plants was proposed for *A. vera cruz* [42]. According to that, fructans in this Agave are constituted by a backbone of β(2-1)-fructosyl-linkages from which, multiples branched points are emerged and elongated with *n* residues linked by β(2-6)-fructosyl-residues. On the other hand, for *A. tequilana*, a similar structure has been proposed [6]. Also it is a structure where both β(2-1)- and β(2-6)-linkages are present in a highly branched molecule; however, *i*-α-D-Glc*p* moiety is present in this structure, stressing the dominance of neofructan series in *A. tequilana* species.

In conciliation with those previous structures and from the evident presence of both *i*- and *t*-α-D-Glc*p* residues in the rest of Agaves analyzed (Table 3), Mancilla-Margalli and López [9] concluded that the existence of at

least to types of fructans in Agaves: neoseries and those with terminal glucose moiety. The general structure for these species constitutes a graminan structure, majority branched and, since the ratio of β-(2-6)-D-Fru*f* and 1,6-di-β-D-Fru*f* near to 1, it can be suggested the presence of one β-(2-6)-D-Fru*f*-residue by each branched point in the backbone of a linear β-(2-1)-D-Fru*f*. This means that in each branched point the chain could be elongated by two fructosyl- units. Graminan structures from Agaves could be characterized by either *t*- or *i*-α-D-Glc*p* moieties, and being called agavins. Figure 7 shows these two structures proposed for *Agave* species, where from n_1 up to $n_4 \geq 0$; and n could vary according to plant species and environmental conditions.

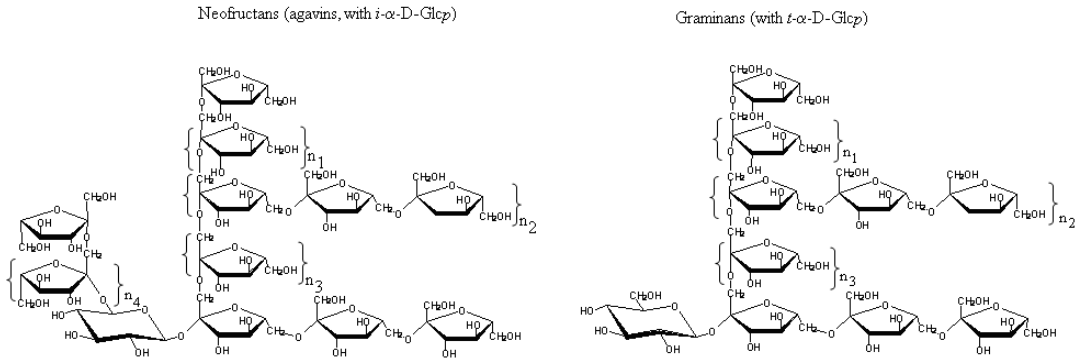

Figure 7. Fructan structures from *Agave* species.

5.2. Structural variation

In monocots, fructans structural variation seems to follow a taxonomic pattern [45]. In this way, strengthening this asseveration, Bonnet et al. [35] after the analysis of fructans from several Poaceae members, proposed that fructan structural determination could be an additional taxonomic criterion in the classification of supertribes Triticodae and Poodae from the Poales order. On the other hand, Sims [33] has found a structural diversity in fructans from Asparagales members; those species more tightly related contains fructan structures more similar than those more taxonomically distant.

In this way, *Phormium tenax* and *P. cookianum* (Phormiaceae family) and *Cordyline australis* (Asteliaceae family) present fructans based on 1-kestotriose (13), 6G-kestotriose (neofructan DP3) and linear- and/or branched graminan; meanwhile, more distant genus like *Allium cepa* (Alliaceae family) and *Asparagus officinalis* (Asparagales family) contain fructans with lineal structures and predominantly with *i*-α-D-Glc*p* unit [32,33].

According to HPAEC-PAD profile, NMR spectrum and PMAAs derivatives from Agave fructans, the presence of a heterogeneous mixture and a structural complexity similar to those reported from *Phormium* spp. and *C. australis* is evident. These species were previously classified into the

Agavaceae family [32]. There, there are not any molecular characterizations that confirm the kind of fructan structures proposed in Figure 7 for *Agave* and *Dasylirion* species, the however fructan type called agavin is very similar to sinistrin, a fructan molecule isolated from the bulbs of red squill (*Urginea maritima*) [31].

In a general way, it could be concluded that Asparagales members could be characterized by the presence of more than one fructan type, with low representation of inulin compared with Asterales members (although Sims [33] reported the exclusive presence of inulin type fructans in Maori onion, *Bulbinella hookeri*). In addition, the presence of β-(2-6)-D-Fru*f* moieties indicates the synthesis of levans and/or their inclusion into the fructans molecules; however, 6-kestotriose (levan DP3) has not been clearly evidenced in Asparagales; therefore levans, if they are, could be present only in small amounts. On the other hand, the presence of branched residues or predominance of either *i*- or *t*-α-D-Glc*p* moieties are more variable characteristics among genera and species [32,33], and they could be useful during subclassification of Asparagales members.

5.3. Significance

The analysis of fructan structures and the study of their physiological roles on developmental and adaptability events in higher plants, have lead to speculate that its structure could be related with its function [46]. In this way, for example, inulin is present as the only fructan type in dicots, stored in reserve vegetative organs, and that is depolymerized and mobilized to cover the energy demanding activities of plants, such sprouting [47] and inflorescence [48]. On the other hand, the more variable structures of fructans found in monocots could play other physiological roles in those plants. This kind of fructans might be related to the adaptability phenomena, since the cryoprotective role of graminans has been demonstrated in cereals such as oat and wheat [19]. In addition, it has been experimentally shown that present major tolerance to drought conditions in transgenic plants accumulating levans [49]. More research is necessary on those aspects, since Vereyken et al. [50] have demonstrated the capability of fructans to interact with lipid headgroups from membranes in both mono- and bi-layer systems; however this interaction is different in levans and inulins, since a DP ≈ 125 levan is necessary to present the same effect than inulin with only a DP ≈ 15 [51].

On the other hand, Chatterton and Harrison [52] suggested that the structural variation found in *Agropyron cristatum* is important for the tolerance of this grass under dryness conditions. Similar situation could be found in *Agave* and *Dasylirion* species: the different fructan types stored in their stems might be a relevant adaptability factor. The significance of the quantitative

differences on glycosyl-constitutes in Agave fructans (Table 3), among the different plant is difficult to establish, probably the predominance of a specific residue over another reflects the peculiar adaptability process of each species growing in different environmental conditions. However, from data in Table 3, it is difficult to establish a relationship among fructan structure and the environmental conditions. For example, *A. angustifolia* plants collected from different regions (Sonora with extremous climate and pluvial precipitation lesser than 400 mm and Oaxaca with average temperature about 27°C and precipitation up to 2000 mm) are in the same cluster (Group I); meanwhile, different species such as *A. potatorum* and *A. canatala*, grown in the same zone (Oaxaca) were classified in different groups (group I and II, respectively). On the other hand, changes in fructan concentration and DP also have been demonstrated to be related not only with environmental factors [53], but also according to developmental conditions [54,55]. With all these evidences, it could be suggested that fructan concentration and DP could be closely related to ontogenic and environmental factors. This last asseveration, might be carefully studied and confirmed, since *Dasylirion* spp. belonging to Nolinaceae family and taxonomically less related to *Agave* species (from Agavaceae family), was clustered together with *A. fourcroydes* and *A. cantala* (Table 3). Meanwhile, *A. tequilana* from Guanajuato region was clustered in different group, although these plants belong to the same species of *A. tequilana* from Jalisco region (Group I).

Finally, in arid regions, where Agave species successfully grow, water availability is very limited. These conditions were, according to Hendry [13], the climatic factors that may have given origin to fructan-storing plants. Therefore, it is possible that fructan metabolism in Agaves, plays a more direct role in the drought tolerance and constitutes an advantage in such conditions.

6. Enzymatic activities in Agave fructan biosynthesis

The diverse structures found in Agave fructans may suppose the presence of a more complex enzymatic system, than that of only two enzymes such is the case in Asteraceae. The classic model for the inulin synthesis in artichoke Jerusalem proposed by Edelman and Jeford [17], has been widely characterized and validated in dictos inulin-storing plants like chicory, artichoke, dandelion and dahlia [39,56,57]. Inulin synthesis is started from sucrose by 1-sucrose:sucrose-fructosyl-transferase enzyme (1-SST, E.C.2.4.1.99), transferring a fructosyl-moiety from one donor sucrose to another acceptor sucrose, forming in this way the intermediate 1-kestotriose. The elongation of inulin, is carried out by the 1-fructan:fructan-fructosyl-transferase (1-FFT. E.C.2.4.1.100); this enzyme transfers reversibly fructosyl

units from fructans DP*m* to fructans with DP*n*, where *m* should be ≥3 and $n \geq 2$. However, from a more diverse structures found in monocots, such Agave, the participation of other fructosyltransferases enzymes (FTs) seems evident, as well as enzymes involved in branched linkages.

Figure 8 shows a model proposed for the fructan biosynthesis in *Agave* species. As is reported in another storing fructan species, the biosynthesis of these carbohydrates in Agaves might be induced by sucrose accumulation in the vacuole, where this molecule is substrate for 1-SST and 6-SFT in the formation of 1- and 6-kestotriose, respectively. Satyanarayana [15] reported an 1-SST activity in a chromatographic fraction of proteinic extract from *A. vera cruz*, and the presence of 1-FFT also was suggested in order to explain the *in vitro* synthesis of 1,1-kestotetraose (nystose or 14) and higher inulo oligosaccharides during long incubation periods [58]. However, during enzymatic assays there were not production of fructans based on 6-kestotriose, 6G-kestotriose or branched structures such it had been demonstrated *in vivo* [59]. Dorland et al. [16] discussed the necessity of another activity for the fructosyltransference to C6 of glucose moiety from either 1-kestotriose or higher fructans. This activity was later identified in asparagus [60,61], onion and garlic [62] as fructan:fructan-6-glucose-fructosyltransferase (6G-FFT). The characteristic activity of this enzyme is the fructan synthesis with *i*-α-D-Glc*p* units, by the fructosyltransference to C6 of glucose moiety from 1-kestotriose or higher inulins [63,64]. Branching molecules in Agaves could be synthesized by the sucrose:fructan-6-fructosyltransferase (6-SFT, E.C.2.4.1.10), enzyme that has been identified as responsible for β(2-6)-fructosyl-linkages synthesis during levan formation in grasses like *Phleum pratense* and *Lolium* spp. [25,65,66], or in the branched points formation during the bifurcose and higher branched graminans synthesis [67,68].

The specific fructan patterns of species seems to be regulated by the different catalytic properties of FTs [33,45]; therefore from peculiar kinetics, the diversity of structures in Agave fructans could be explained. The enzyme 1-SST in Agaves should have more affinity for sucrose like substrate than 6-SFT, due to the observed major concentration of 1-kestotriose. However, 6-SFT should be more active in the synthesis of branched fructans, using either inulins or neofructans as substrates (Figure 8). 1-kestotriose may be the central molecule which is used during the synthesis of neofructans by the action of 6G-FFT, elongated to higher inulins by the 1-FFT activity or in the branched fructan bifurcose. The preferential metabolic pathways, probably are substrate threshold and enzyme concentration dependent, and also highly influenced by developmental and environmental conditions as determinants on the expression of genes that codify the involved FT´s enzymes.

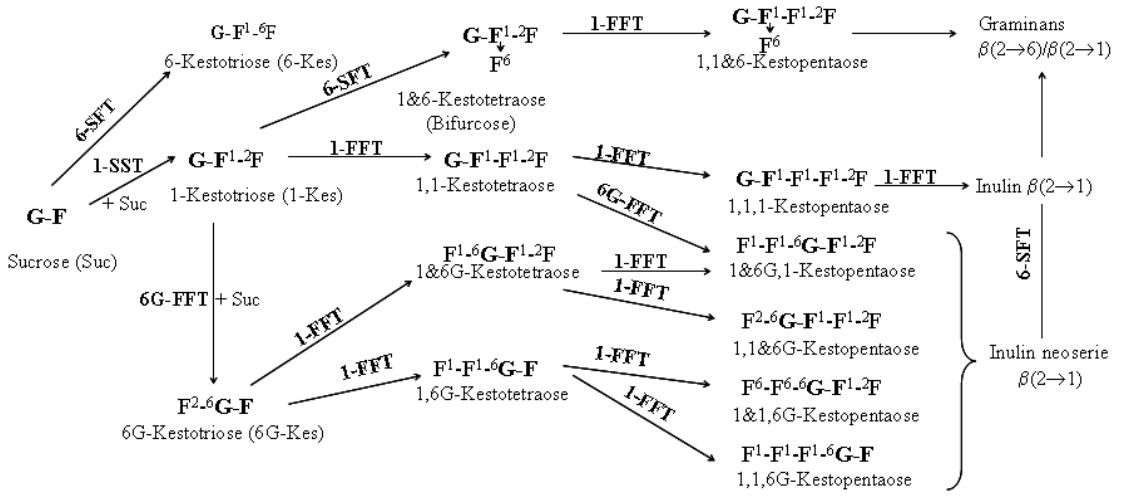

Figure 8. Fructan biosynthesis model proposed by *Agave* species.

7. Perspectives

7.1. Perspective for Agave fructans

Worldwide alcoholic beverages produced from Agaves have become very attractive, especially *A. tequilana* Weber var. azul. However, some specialists have predicted an overproduction of *A. tequilana* with a maximum production around 1.8 million ton for 2008. Only half of this production is being used to make alcoholic beverages [69].

Fortunately, scientists working on Agave plants have focused their research in new exciting areas such fructans. For instance, the elaboration of high fructose syrup elaborated from the hydrolysis of Agave fructans using bacterial inulinase action. This syrup is already commercialized in many different Agave syrup products with different nutraceutical properties [71]. This presents an alternative sweetener, instead of sucrose, Agave syrup is sweeter, high water soluble, less viscous, low energy and its metabolism in humans does not depend on insulin, therefore it can be consumed by diabetic people.

Many other Agave fructan applications are being currently studied in the Centro de Investigación y de Estudios Avanzados in the fructan group of Prof. López [71]. Preliminary data on the impact of Agave fructans in obesity and diabetes areas present new niches for agavin's research as well as health and food applications.

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