

*Full Length Research Paper*

# Influence of the harvest age on fructan content and fructosyltransferase activity in *Agave atrovirens* Karw pine

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The effect of age on the composition and concentration of non-structural carbohydrate was determined in the “pine” or “head” of *Agave atrovirens* Karw. The influence of plant age on the 1-FFT enzymatic activity, the electrophoretic pattern of proteins and the non-structural carbohydrate yield were also determined to establish the optimum age for harvest. The carbohydrate composition of 6 years old pine was 69% sucrose and 4.4% inulin-type fructans. The 1-FFT enzymatic activity was higher at this age, and these results suggested a relationship between the activity of the 1-FFT enzyme and the concentration of fructans. Moreover, the results of the 1-FFT-enzymatic activities that differed among different years after planting of the agave suggest three phases of development in this plant. In addition, the age of the plant corresponded with changes in the conformation of the enzymatic complex, a trimer or dimer were observed at early and late ages, whereas 6 years old pines showed a monomer (MW 70 kDa). This last conformation is responsible for the chain elongation that forms inulin-type fructans with a DP $\geq$ 30. In conclusion, the age of the plant has an influence on the 1-FFT enzymatic activities and the enzymatic complex conformation, therefore on the concentration of carbohydrates in *A. atrovirens*.

**Key words:** Carbohydrates, maguey, 1-FFT, inulin, *Agave atrovirens*.

## INTRODUCTION

Maguey (*Agave atrovirens* Karw) is a semelparous plant with succulent leaves that are arranged in a basal rosette. During its development, a homogeneous structure commonly known as a “pine” forms in which the plant stores reserve carbohydrates, which are then used to develop a traditional Mexican drink called “pulque” (Chellapandian et al., 1998; Escalante et al., 2004). To obtain this drink, Mexicans usually use approximately

nine years old plants. This stage is considered the proper age for harvesting because at this age, the plant has finished growing; and at this stage, it produces an enzymatic complex which is responsible for the hydrolyses of the fructans that are stored in the pine, resulting in a juice (“aguamiel”) with high content of fructose, glucose and sucrose. In this way, it is easier to carry these carbohydrates to higher positions in the flowering stalk (Srinivasan and Bathia, 1954), enabling the development of inflorescence. However, while the agave plant is still growing, fructans are produced by the 1-SST enzyme (sucrose: sucrose 1-fructosyltransferase, EC 2.4.1.99), which catalyzes the production of 1-kestose

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and glucose and then, the chain elongation is performed by the active participation of the 1-FFT enzyme (fructan: fructan 1-fructosyltransferase, EC 2.4.1.100) (Edelman and Jefford, 1968). These compounds are stored in the pine and are used as a reserve source for vegetative growth, sexual reproduction (Watson, 1984) and for protection against conditions that are adverse for development (Vergauwen et al., 2000; Waleckx et al., 2008). On the other hand, fructans are a non-toxic, non-caloric sugar with a high potential for the development of new foods. A promising source of these sugars are the plants of the genus *Agave*, which store high concentrations of inulin-type fructans and its branched structures (Aspinall and Das Gupta, 1959; Waleckx et al., 2008; Montañez et al., 2011; González-Cruz et al., 2011). The degree of fructan polymerization in these plants can vary from 3 in *Agave tequilana* to 30 in *Agave vera cruz*. However, age may also influence the composition and concentration of the fructans, during growth, it increases the concentration of fructans until the plant reach its physiological maturity; later, this concentration can decrease during senescence (Shiomi et al., 1997). The objective of this study was to determine the changes in the concentration and composition of the non-structural carbohydrate that are present in the pine of *A. atrovirens* and to determine the enzymatic activity of 1-FFT to establish the most optimal harvesting period for this plant.

## MATERIALS AND METHODS

### Vegetal material

The pines of *A. atrovirens* Karw were collected in the summer of 2007 in Singuilucan, Hidalgo State, Mexico (altitude 2640 m; 19°59'20" N, 98°27'52" W). To avoid the high concentrations of acid that are caused by the metabolic process, samples were taken at 16:00 ± 1 h. After separation from the plant, the leaves were cut into approximately 3 cm cubes, and 250 g lots were placed in polyvinyl chloride (PVC) bags and stored in a freezer (LG, Model GR-452SH) at -4°C for no more than eight days. Samples were taken from plants within the age of 3, 6 and 9 years old.

### Non-structural carbohydrate extraction

Initially, three batches of 50 g frozen pine with cubes of 1 cm were obtained. Lot 1 was grounded in an industrial blender, after which the juice was extracted using a press and then, filtered through a cotton cloth, producing the raw extract. Lot 2 was placed in water (1/1 w/v), boiled for 20 min (Prosky and Hoebregs, 1999), grounded and filtered to produce the aqueous extract. Lot 3 was placed in 70% ethanol (1/1 w/v) for 20 min, grounded and filtered to produce the alcohol extract (Jaime et al., 2001; Mancilla-Margalli and López, 2006). All extracts were stored at -4°C until analysis.

### Non-structural carbohydrate (quantification and identification)

Inulin-type fructans, 1-kestose, nystose, sucrose, fructose and glucose were measured using high performance anion-exchange chromatography, attached to an evaporative light-scattering

detector (HPAEC-ELSD) with a CarboPac, PA-1 column (Dionex, Sunnyvale, CA, US). The processing temperature was 80°C, and the flow rate was 1 mL min<sup>-1</sup> with a 20 min elution time with water. Samples were diluted with ultrapure water at a 1/10 v/v ratio, and 20 µl of solution was used in the column.

For the quantification and identification of non-structural carbohydrates, standard solutions containing 200, 400, 600, 800 and 1000 µg ml<sup>-1</sup> of dahlia tubers inulin-type fructans (Fluka Biochemika), 1-kestose (Fluka Biochemika), nystose (Fluka Biochemika), sucrose (Sigma-Aldrich), fructose (Sigma-Aldrich) and glucose (Merck Chemical) were used as external standards; the samples were injected into the high-performance liquid chromatography (HPLC), and the carbohydrates were quantified using the calibration curve. Unidentified inulin-type fructans with DP<sub>≥</sub>30 were quantified using the calibration curve of the known inulin-type fructans with DP=29.

## Enzymatic activity of 1-FFT

### Extraction

Protein was extracted by suspending 1000 g of frozen pine from each age group in 1000 ml of extraction buffer (100 mM citric acid, 2 mM DL-Dithiothreitol (DTT), 20 mM ascorbic acid (pH 5.0) and blending it in an industrial blender for 2 min at 4°C. The resulting mixture was filtered through two layers of cotton cloth, and the filtrate was centrifuged at 15,000 × g for 20 min at 4°C. Ammonium sulfate was added to the supernatant until 60% saturation was reached (raw extract). After settling overnight, this solution was then centrifuged at 15,000 × g for 30 min at 4°C, and the precipitate was resuspended in a solubilization buffer (50 mM citric acid, 1 mM MnCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 500 mM NaCl, 0.05% sodium azide (pH 5.5)). The insoluble proteins were separated by centrifuging at 15,000 × g for 25 min, and the supernatant was saved (Lüscher et al., 1996).

### Affinity chromatography

The protein extract from 3, 6 and 9 years old pines (30, 45 and 130 ml, respectively) was concentrated by ultra filtration using a Microcon concentrator (Amicon Bioseparation, Millipore, Bedford, Mass., USA) with a 10 kDa cutoff, to a final volume of 15, 25 and 80 ml, respectively (to obtain a protein concentration of about 2.2 mg ml<sup>-1</sup>). This ultrafiltered extract was applied in 1 ml fractions to an 18 × 1.5 cm Concanavalin A-Sepharose 4B column (GE Healthcare), which was previously equilibrated with solubilization buffer. The column was washed with the same buffer until the wash buffer contained only traces of proteins. After this, 34 ml of 500 mM MES buffer (pH 6.5), (Buffer A) was added and in this way, proteic fractions that do not contain enzyme was removed. Subsequently, a linear gradient from 0 to 500 mM methyl α-D-mannopyranoside (Fluka Biochemika) in 26 ml of buffer A was applied at a flow rate of 0.2 ml min<sup>-1</sup> (Lüscher et al., 1996). Fractions of 2 ml were collected and assayed for protein and enzyme activity. Fractions that contain enzyme (19 to 31) were pooled and concentrated by ultrafiltration with a 10 kDa cutoff (Amicon) to a 4 ml final volume (sample 1<sup>a</sup> Con A). This sample was recirculated using the aforementioned procedure until it reached a 2 ml final volume, and it was then dialyzed using five times its volume of buffer A and reconcentrated to 4 ml (Sample 2<sup>a</sup> Con A).

### Ion-exchange chromatography

The 2<sup>a</sup> Con A sample was applied in 1 ml fractions onto a 5 ml ion-exchange column (High Q, Biorad), which was previously

**Table 1.** Non-structural carbohydrate contained in pines of 3, 6 and 9 years old (mg g<sup>-1</sup> d.b.).

Carbohydrate	Crude extract			Aqueous extract			Alcoholic extract		
	3	6	9	3	6	9	3	6	9
Inulin-type fructans	10.10±0.09	4.40±0.10	23.30±3.20	25.50±3.20	44.30±3.90	18.70±1.30	6.60±0.70	17.50±2.20	3.50±0.20
1-kestose	0.72±0.02	0.44±0.06	0.03±0.01	ND	ND	ND	1.00±0.35	ND	ND
Sucrose	124.60±13.20	102.50±15.20	75.40±4.5	85.40±6.20	692.20±65.50	453.20±55.20	260.90±25.50	624.70±59.00	490.80±62.10
Glucose	10.50±0.12	7.98±0.85	1.10±0.25	1.00±0.07	8.84±0.93	6.50±0.75	20.20±2.50	12.60±1.80	8.10±1.20
Fructose	9.10±0.85	ND	1.00±0.03	1.2±0.08	ND	8.30±0.95	19.70±2.20	ND	10.40±2.20
Total non-structural carbohydrate content	155.02	115.32	100.83	113.10	745.34	486.70	308.40	654.80	512.80

ND: non-detectable.

equilibrated and washed with buffer A until the wash buffer contained only traces of proteins. Subsequently, a linear gradient of 0 to 500 mM NaCl in buffer A was applied at a flow rate of 1 ml min<sup>-1</sup>. Fractions of 1 ml were collected and assayed for protein and enzyme activity. Fractions that had 1-FFT enzymatic activity were pooled and concentrated to obtain a protein concentration of about 0.1 mg ml<sup>-1</sup>.

#### Protein concentration and molecular weight estimation

Protein concentration was measured following the Bradford method (Bradford, 1976), using Bovine Serum Albumin (BSA) as a standard for all samples (raw extract, 1<sup>a</sup> Con. A, 2<sup>a</sup> Con A and High Q). All samples were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to measure the degree of separation and the molecular weight (MW) of the enzymatic complex using an 8% polyacrylamide gel (Laemmli, 1970). Due to the low protein concentration of the samples, the gels were stained with silver nitrate (Merril et al., 1981).

#### 1-FFT enzymatic activity

Enzymatic activity was measured assuming sucrose to be a fructosyl group acceptor (Itaya et al., 2007) and inulin-type fructans to be a fructosyl group donor in the 1-kestose formation process. To determine the enzymatic activity, a reaction mixture (100 µl) of 25 mM dahlia tubers inulin-type

fructans (Fluka), 50 mM sucrose and 1 µg of protein (Lüscher et al., 1996) was used. The incubation period was 360 min at 25°C (Saengthongpinit and Sajjaanantakul, 2005), and the reaction was stopped by injection into the HPAEC-ELSD. The enzymatic reaction product (1-kestose) was quantified by HPAEC-ELSD as was described previously for the non-structural carbohydrate measurement. Enzymatic complex activity was reported as nKat mg<sup>-1</sup> protein, where nKat is the amount of enzyme needed to produce 1 nmol 1-kestose min<sup>-1</sup>.

#### Statistical analysis

The quantitative data were expressed as the mean ± standard deviation, and the analysis of variance (ANOVA) was carried out followed by a Tukey's test. SAS software was used for the data analysis, and all experimental determinations were assayed in triplicate.

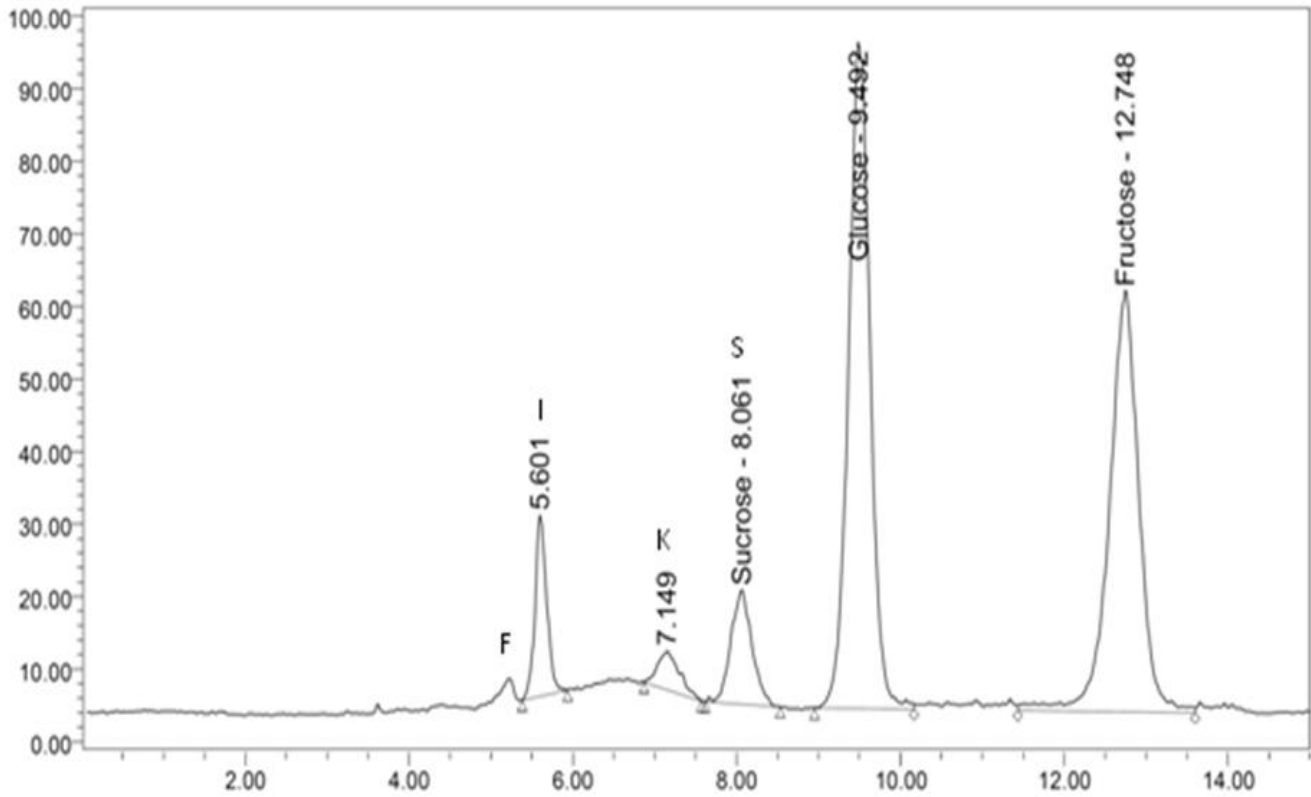
## RESULTS AND DISCUSSION

#### Non-structural carbohydrate content

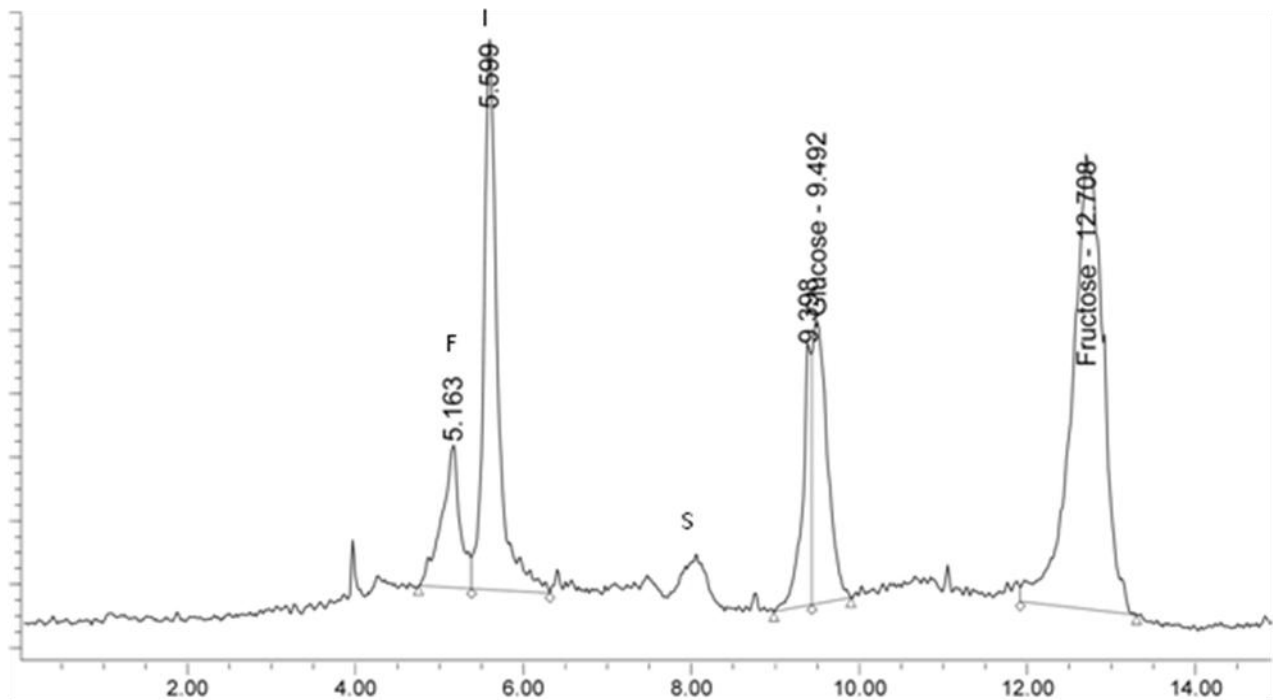
The carbohydrates of *A. atrovirens* are composed of monosaccharides (fructose and glucose), disaccharides (sucrose), inulin-type fructans and trisaccharides (1-kestose, retention time, 7.14

min) (Table 1). Inulin-type fructans for both dahlia tubers (standard) and *A. atrovirens* (pine) had the same retention times (5.60 min). Because the retention times were the same, the inulin-type fructans in the pine have a DP = 29. However, also present in the pine were fractions with retention time of 5.20 min (Figure 1), which indicate the presence of inulin-type fructans with DP > 30.

The results obtained using different extraction methods showed that the aqueous extract of the 6 years old pine contained the highest concentration of carbohydrate (740 mg g<sup>-1</sup> d.b.), of which the majority were inulin-type fructans and sucrose (Figure 2), the aqueous extract had the highest concentration of carbohydrate in each one of the 3 developmental stages, although the compound concentration was influenced by age (Saengthongpinit and Sajjaanantakul, 2005; González-Cruz et al., 2011). The concentration of inulin-type fructans was 42 and 58% lower in the 3 and 9 years old pines, respectively, when compared with the 6 years old pine, whereas the sucrose concentration in the 3 and 9 years old pines was 88 and 35% lower, respectively, when compared to the 6 years old pine. On the other



**Figure 1.** Chromatogram of carbohydrates present in the *Agave atrovirens* pine at 6 years old (crude extract). I, inulin-type fructans (DP = 29); S, sucrose; K, 1-kestose; F, inulin-type fructans (DP  $\geq$  30).



**Figure 2.** Chromatogram of carbohydrates present in the *Agave atrovirens* pine at 6 years old (aqueous extract). I, Inulin-type fructans (DP = 29); S, Sucrose; F, Inulin-type fructans (DP  $\geq$  30).

**Table 2.** Purification of fructan-fructan 1-fructosyltransferase (1-FFT) from 3 years old pine of *Agave atrovirens* (1000 g wet weight).

Purification step	Total volume (ml)	Total activity (nKat)	Total protein (mg)	Specific activity (nKat mg <sup>-1</sup> protein)	Purificationfold	Yield (%)
Crude extract	1520	790.04	307.41±4.02	2.57	1.00	100
Precipitate	15	156.18	34.40±0.21	4.54	1.77	19.77
1a Con A	4	4.18	0.76±0.02	5.50	2.14	0.53
2a Con A	4	2.30	0.34±0.01	6.75	2.63	0.29
High Q	0.5	0.45	0.05±0.01	8.86	3.45	0.06

**Table 3.** Purification of fructan-fructan 1-fructosyltransferase (1-FFT) from 6-year old pine of *Agave atrovirens* (1000 g wet weight).

Purification step	Total volume (ml)	Total activity (nKat)	Total protein (mg)	Specific activity (nKat mg <sup>-1</sup> protein)	Purification fold	Yield (%)
Crude extract	1531	1340.70	311.79±5.06	4.3	1.00	100
Precipitate	25	534.93	55.78±0.34	9.59	2.23	39.90
1a Con A	4	50.20	3.52±0.03	14.26	3.32	3.74
2a Con A	4	45.63	1.21±0.02	37.71	8.77	3.40
High Q	4.7	52.58	0.46±0.03	114.31	26.58	3.92

hand, the results showed that both the aqueous and alcohol extracts were unsuitable for the extraction of 1-kestose.

The agave pine has similar compositions of carbohydrates as other vegetables that store fructans as reserve carbohydrates, such as the Burdock root (Imahori et al., 2010) and the onion (Jaime et al., 2001). On the other hand, the agave's non-structural carbohydrate composition depends on the species, the developmental stage and the part of the plant that is analyzed. Although some authors suggest that fructans are the most common carbohydrates in agave (*Agave angustifolia*, *Agave cantala*, *Agave potatorum* and *Agave tequilana*) (Mancilla-Margalli and López, 2006; Waleckx et al., 2008), others affirm sucrose as the most common soluble carbohydrate (Wang and Nobel, 1998), which is congruent with the results obtained in this study.

Nevertheless, if only the vascular tissue is considered, the fructan concentration is greater. In the pine (which is considered to be a reserve structure), short chain fructans were not detected in high concentrations (1-kestose and nystose), confirming the results previously reported by Wang and Nobel (1998), that fructans do not exist in the chlorenchyma or water storage parenchyma. As previously thought, if the purpose is to obtain sucrose or fructans inulin-type with DP> 30, 6 years old agave crops were most suitable for this purpose; at 3 years old, the pine in the agave is not yet differentiated, and the carbohydrate content is less because the plant is mostly composed of leaves.

#### Determination of 1-FFT enzyme activity

The activity of 1-FFT was lower in the 3 years old

agave pine (Table 2) and it was higher in the 6 years old agave pine (Table 3), while enzyme activity in the 9 years old agave pine was slightly lower than in the 6 years old (Table 4). 1-FFT was markedly active in the last developmental stage of *A. atrovirens*, however in the 3 and 9 years old pine, the purification invariably resulted in uncontrollable losses of activity and yield.

The agave pine has 3 phases of enzyme production. The 3 year old (phase I) plant is in a growth period, showing lower 1-FFT enzyme activity (Table 2). The carbohydrate synthesis in this phase is carried out to satisfy the structural requirements during plant growth, generating the leaves that form the basal rosette that during the growth period will widen to form the pine, which will serve as a reservoir of carbohydrates and water. This pattern is congruent with the results obtained in other plants such as *Cichorium*

**Table 4.** Specific activity in Agave pine.

Age (years)	Specific activity (nKat mg <sup>-1</sup> protein)
3	8.9 <sup>c</sup>
6	114.3 <sup>a</sup>
9	97.8 <sup>b</sup>

Different letters in each column indicate significant statistical differences ( $P \leq 0.05$ ).

**Table 5.** Purification of fructan:fructan 1-fructosyltransferase (1-FFT) from 9 years old pines of *Agave atrovirens* (1000 g wet weight).

Purification step	Total volume (ml)	Total activity (nKat)	Total protein (mg)	Specific activity (nKat mg <sup>-1</sup> protein)	Purification fold	Yield (%)
Crude extract	1250	1275.00	307.23±4.13	4.15	1.00	100
Precipitate	80	841.63	177.56±0.47	4.74	1.14	66.00
1a Con A	4	149.73	7.34±0.40	20.40	4.92	11.74
2a Con A	4	49.86	2.43±0.20	20.52	4.94	3.91
High Q	8	7.42	0.76±0.02	97.75	23.55	0.58

*intybus* L. where, during the early stages, there is less fructosyltransferase activity, resulting in a high yield of the hexoses that are used for cell division processes and for basal metabolism (Druart et al., 2001). Similarly, the 3 year old agave pine contains low concentrations of sugar and zero inulin-type fructans with DP  $\geq 30$ .

The 6 year old pines (Table 3) are in phase II (storage and maintenance), in which 1-FFT activity is increased, 12 times (114.31 nKat mg<sup>-1</sup>) compared to the 3 year old pine and only 0.16 times compared to the 9 year old pine (Table 4). Therefore, given that the highest activity of 1-FFT, as well as the highest concentration of carbohydrates is present in pines of 6 years old, we consider this age of the plant as the ideal time to carry out the harvest of the culture.

Like other plants (Ameziane et al., 1997), when agave reach physiological maturity, they store large amounts of carbohydrates such as inulin-type fructans with DP  $\geq 30$  (Figures 1 and 2), and at this time, the base of the leaves of the agave reach their maximum diameter and the pine is completely formed. On the other hand, with the accumulation of carbohydrates, the plant initiates the formation of new basal or rhizomatic shoots (Arizaga and Ezcurra, 2002) and the generation of the flower stalk. However, with the absence of leaves in this part of the plant, photosynthesis cannot occur. Therefore, during the bud of the scape, it is necessary to take the carbohydrates that are stored in the pine through a translocation process; a similar process occurs in the stems of the Jerusalem artichoke, where fructans are stored before their translocation to the tuber (Monti et al., 2005).

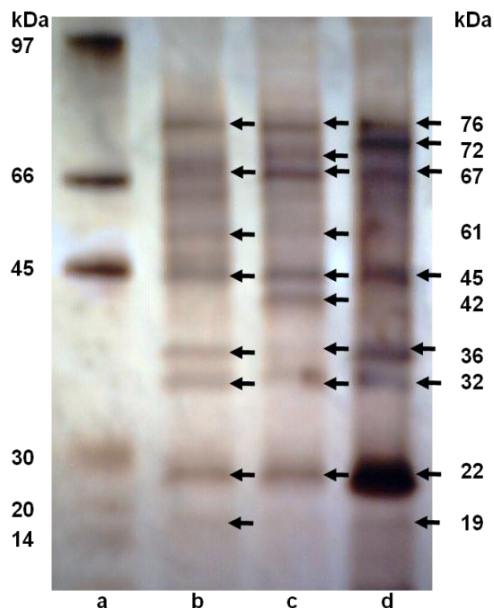
The 1-FFT enzyme activity in 9 years old pines decreased by approximately 15% (Table 5) when compared with the 6 years old pines; these results indicate that the plant has started phase III (senescence),

in which the enzymatic machinery is more complex, reducing the fructosyltransferase concentration. The senescence process ends with the formation of the inflorescence, which requires a process of translocation upward. To facilitate this translocation, the fructans are hydrolyzed by the 1-FEH (Fructan 1-Exohydrolase, EC 3.2.1.153) and acid invertase enzymes, generating hexoses that enable the carbohydrates to transfer to the scape (Srinivasan and Bhatia, 1954). This process, which occurs naturally in agave, has been observed in response to an induced defoliation of chicory plants (De Roover et al., 1999) or damage to the photosynthetic apparatus of the plant, resulting in a decrease of fructans and an increase of sucrose, during which it is necessary to cease the synthesis of fructans and diminish the 1-FFT enzymatic activity.

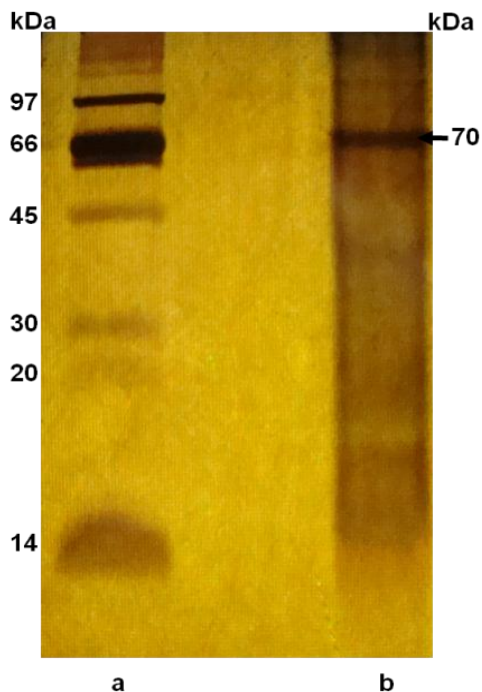
The quantification of enzymatic activity is a good parameter for determining the best age for agave harvest. Although the plant has a lower concentration of carbohydrates at nine years, the levels are adequate for use in the food industry. However, it is necessary to harvest the plant before the initiation of the flower stalk formation because during that period, the agave pine is exposed to dehydration processes and carbohydrate loss (Wang and Nobel, 1998).

#### Effect of harvest age on the electrophoretic pattern of 1-FFT

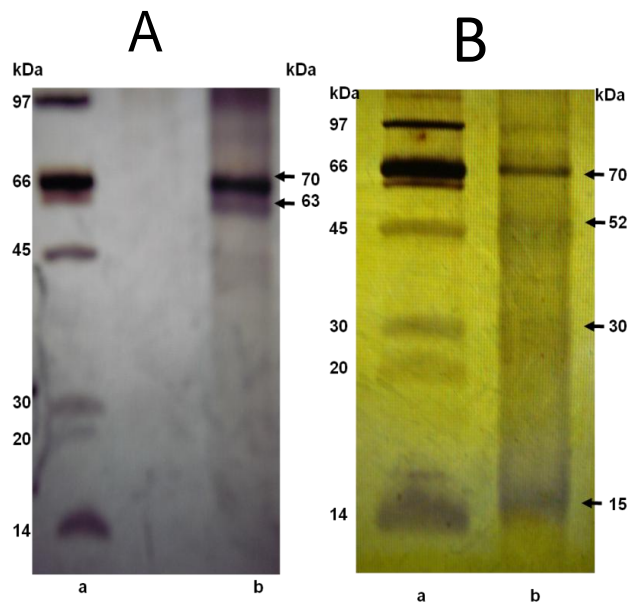
The 1-FFT enzyme, which is responsible for the synthesis of inulin-type fructans in *A. atrovirens*, was partially purified in five purification steps; the overall activity was purified 3.45, 26.58 and 23.55-fold from the 3, 6 and 9 years old pines, respectively. The proteins from the peaks with 1-FFT enzymatic activity that were



**Figure 3.** SDS-PAGE analysis of *Agave atrovirens* pine. Samples obtained from Con A pH 6.5 were separated on an 8% (w/v) acrylamide gel and stained with silver nitrate. Lane a: molecular weight marker, lane b: 3 years old pine, lane c: 6 years old pine, lane d: 9 years old pine.



**Figure 4.** SDS-PAGE analysis of 6 years old pine of *Agave atrovirens*. The High Q purification at pH 6.5 (pool of 6 fractions) was separated on an 8% (w/v) acrylamide gel and stained with silver nitrate. Lane a: molecular weight marker, lane b: 6 years old pine.



**Figure 5.** SDS-PAGE analysis of 3 years old pine of *Agave atrovirens*. The High Q purification at pH 6.5 was separated on an 8% (w/v) acrylamide gel and stained with silver nitrate. Lane a: molecular weight marker, lane b left: pool of 2 peaks with 1-FFT.

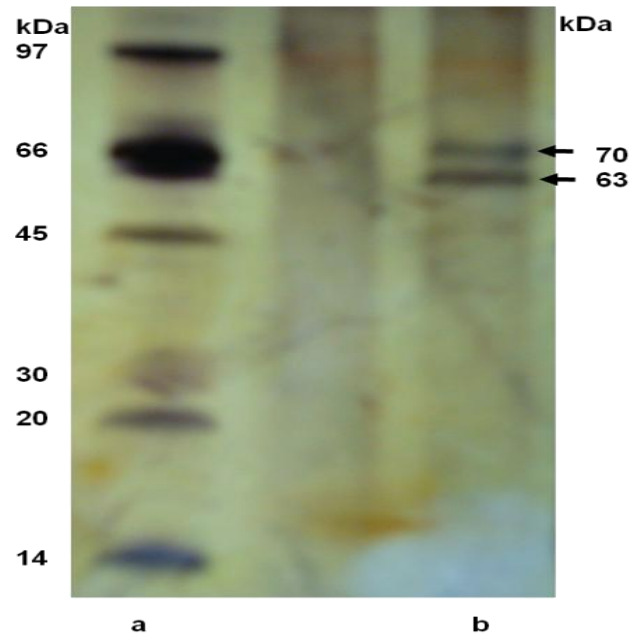
eluted with Con A chromatography were run on SDS-36, 32 and 22 kDa (Figure 3).

PAGE, resulting in 6 similar bands from the agave

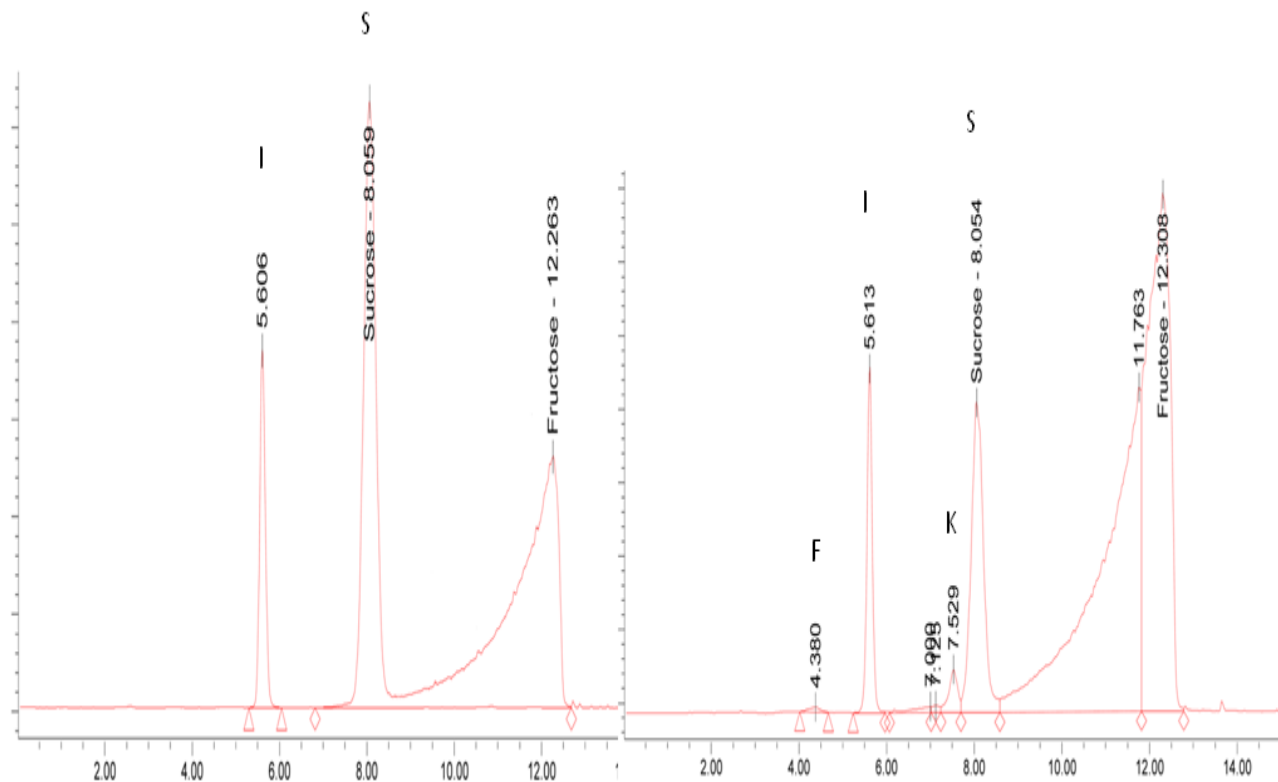
The elution of the High Q column at pH 6.5 yielded 12, 7 and 8 protein peaks from the 3, 6 and 9 years old pines, respectively. In the 3 years old pines, 3 peaks showed 1-FFT activity; while in the 6 and 9 years old pines, 6 and 4 peaks, respectively, showed 1-FFT activity. The separation by SDS-PAGE of the peaks from the 6 years old pine yielded a single band with the apparent MW of 70 kDa (Figure 4), which is similar to that which was reported for *Helianthus tuberosus* (Lüscher et al., 1993; Koops and Jonker, 1994; Van der Meer et al., 1998). The separation by SDS-PAGE of 2 of the peaks from the 3 years old pines yielded 2 bands with the apparent MWs of 70 and 63 kDa (Figure 5A), and 1 peak yielded 4 bands with MWs of 70, 52, 30 and 15 kDa (Figure 5B).

On the other hand, the separation by SDS-PAGE of all of the peaks from the 9 years old pine yielded 2 bands with MWs of 70 and 63 kDa (Figure 6). These results indicate that the enzymatic complex from *A. atrovirens* changes with the age of the plant; however, the MWs are similar to results that were obtained from other species (Lüscher et al., 1993; Koops and Jonker, 1994; Van der Meer et al., 1998; Vergauwen et al., 2003; Van den Ende et al., 2005).

The native 1-FFT enzyme in *A. atrovirens* exists as a monomer, dimer or trimer, with inulin-type fructans and sucrose as substrates. However, the monomer (70 kDa)



**Figure 6.** SDS-PAGE analysis of 9 years old pine of *Agave atrovirens*. The High Q purification at pH 6.5 (pool of 4 fractions) was separated on an 8% (w/v) acrylamide gel and stained with silver nitrate. Lane a: molecular weight marker, lane b: 9 year old pine.



**Figure 7.** *In vitro* production of high DP inulin-type fructans. 1-FFT enzymatic complex reaction products with 25 mM dahlia tubers inulin and 50 mM sucrose at 25°C. Left) 0 min reaction time; right) 360 min. I = inulin-type fructan DP<sub>29</sub>; S = sucrose; K = 1-kestose; F= inulin-type fructans DP>30.



that is obtained from 6 years old pines is the most efficient in the formation process ( $114 \text{ nKat mg}^{-1}$ ), generating inulin-type fructans with a  $DP \geq 30$  in addition to the inulin-type fructans with a  $DP \approx 29$ . In this reaction, the generation of glucose was not observed, which indicates that the enzymatic activity is specifically from the 1-FFT enzyme (Figure 7). The dimer that was obtained from the 9 years old pines showed a specific enzymatic activity, about 15% lower than the monomer obtained from the 6 years old pines. On the other hand, when the enzyme is part of a trimer and is associated with other subunits as in the 3 years old pines, the specific enzymatic activity is dramatically decreased by about 90%.

The principal factor that influences the changes to the composition of the 1-FFT enzymatic complex is the age of the plant. In the early stages of development (3 years old), the accumulation of fructans as reserve carbohydrates is not required. Although, the 1-FFT enzyme complex from this age has 3 subunits in addition to the 70 kDa fraction, the specific enzymatic activity is lower than in the 6 years old pines for two possible reasons: the trimer that is observed (MWs 52, 30 and 15 kDa) is less efficient than the monomer (MW 70 kDa) from the 6 years old pines, likely because of the formation of a heterodimer (MWs 52 and 15 kDa); and/or the 30 kDa MW protein does not have 1-FFT enzymatic activity. More work is necessary at this point to obtain specific dates that permit the corroboration of this hypothesis. The behavior observed in the 3 and 9 years old pines could be due to the fact that at these ages, the plant needs to generate new structures; new plant leaves in the 3 years old plant and the flowering scape in the 9 years old plant.

## Conclusions

Here, we demonstrated that the harvest age is associated with the carbohydrate composition and concentration. The optimum harvest age in *A. atrovirens* is 6 years old, considering the higher sucrose and inulin-type fructans concentrations at this age. The carbohydrate content is correlated with the 1-FFT enzymatic activity variations in which are categorized by three developmental phases (growth, maintenance and senescence). The 1-FFT enzyme in *A. atrovirens* may have 3 forms (monomer, dimer and trimer), depending on the age. The monomer (MW 70 kDa) is responsible for the higher 1-FFT enzymatic activity and the formation of inulin-type fructans with a  $DP \geq 30$ . The changes in the carbohydrates and 1-FFT enzyme activity can be considered markers for determining the appropriate harvest periods for *A. atrovirens* Karw.

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