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Quantitative Characterization of Nonstructural Carbohydrates of Mezcal Agave (*Agave salmiana* Otto ex Salm-Dick)

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Fructans are the reserve carbohydrates in *Agave* spp. plants. In mezcal factories, fructans undergoes thermal hydrolysis to release fructose and glucose, which are the basis to produce this spirit. Carbohydrate content determines the yield of the final product, which depends on plant organ, ripeness stage, and thermal hydrolysis. Thus, a qualitative and quantitative characterization of nonstructural carbohydrates was conducted in raw and hydrolyzed juices extracted from *Agave salmiana* stems and leaves under three ripeness stages. By high-performance liquid chromatography (HPLC), fructose, glucose, sucrose, xylose, and maltose were identified in agave juice. Only the plant fraction with hydrolysis interaction was found to be significant in the glucose concentration plant. Interactions of the fraction with hydrolysis and ripeness with hydrolysis were statistically significant in fructose concentration. Fructose concentration rose considerably with hydrolysis, but only in juice extracted from ripe agave stems (early mature and castrated). This increase was statistically significant only with acid hydrolysis.

KEYWORDS: Agave salmiana; thermal hydrolysis; fructose; glucose; sucrose; xylose; maltose; high-performance liquid chromatography (HPLC)

INTRODUCTION

Mezcal agave (*Agave salmiana*) displays crassulacean acid metabolism (CAM), which is a biochemical and physiological adaptation to low CO_2 levels resulting from an efficient water economy (*1, 2*). During photosynthesis, glucose is produced directly in the leaves, generally by the action of a phosphatase on glucose-1-phosphate (G1P) (*3*). Afterward, as a result of hexokinase, glucose is converted into glucose-6-phosphate (G6P); then, the enzyme hexose phosphate isomerase transforms G6P into fructose-6-phosphate (F6P) (*3*). Finally, F6P loses the phosphate molecule and releases fructose (*4–6*). Glucose and fructose are the most important hexoses as the energy source for the plant (*3*). These, along with other less abundant sugars, are found in plants in a dynamic state, since the energy they contain is required in many cell reactions, including protein and lipid syntheses, among others (*7*). The main substance involved in carbohydrate transport through the plant is sucrose, the synthesis of which is regulated by sucrose-phosphate synthase, an enzyme that catalyzes the bond of one glucose and one fructose molecule (*8*). When the plant requires energy, sucrose is hydrolyzed by sucrase (*9*), releasing fructose and glucose. Sucrose is also the substrate for the synthesis of inulin, the reserve polysaccharide in *Agave* spp. plants, formed in this case by the polymerization of 35 fructose and one glucose unit as an average per inulin molecule. In agave plants, inulin and other fructans are synthesized and stored in the stalk during the plant's long vegetative period (*10, 11*). At the onset of the reproductive period, the formation and maintenance of the big reproductive organs, which means a great energy demand, is met from fructose and glucose after fructans are hydrolyzed by exoinulinase (*12, 13*).

Because of its high reserve carbohydrate content, ripe agave is used as a raw material in mezcal production. Mezcal factories use only agave pineapples or heads, which are formed by the stem and leaf bases; the remaining parts of the leaves are discarded in the field. Rather than plant size, mezcal agave processing requires consideration of the plant ripeness stage, since it determines the extent of reserve carbohydrate accumulation, and in wild agave populations, ripeness is poorly related to plant size (*14*). When agave shows signs of having entered the reproductive phase (shoot apex thinned at its base and

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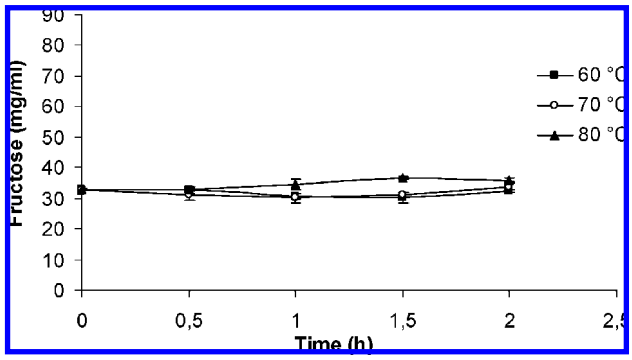

Figure 1. Temperature effect on fructose content of raw head agave juices in a bain-marie heater.

youngest leaves shorter and displaying black and shiny spines), it is called "quiotillo", and then, the apical meristem may be removed (castration); from that moment on and up to 1−2 years following castration, the agave is regarded as under optimal ripeness for use (*14*). Through cooking in mezcal factories, fructans in agave heads are hydrolyzed, thereby releasing fructose and glucose, along with other mono- and disaccharides probably present in juice, which afterward undergo fermentation to produce alcohol.

One of the analytical techniques used for the identification and quantification of chemicals in complex mixtures is high-performance liquid chromatography (HPLC) (*15*). This technique has been extensively used in carbohydrate identification in plants (*10*), food (*16*), beverages (*17, 18*), and sweeteners (*19*).

As there are not previous studies about carbohydrate characterization in *A. salmiana*, the qualitative and quantitative characterization of nonstructural carbohydrates in mezcal agave, depending on plant organ, ripeness, and hydrolysis, is essential to assess, and ultimately improve, the current mezcal process efficiency. Thus, the purpose of this study was to identify and quantify the nonstructural carbohydrates present in raw, thermal, and acid hydrolyzed juice from the stems and the four transversal identifiable leaf sections (base, neck, wing, and tip) of immature, early mature or "quiotillo", and one-year castrated mezcal agave plants, through HPLC.

MATERIALS AND METHODS

Plant Material. Nine whole mescal agave plants were collected at El Llano, Ipiña ejido, Ahualulco, San Luis Potosí, Mexico, in July 2006; three of them corresponded to each of the three ripeness stages: young or unripe (less than one year to start its reproductive period), early mature or with an incipient reproduction, and one-year after being castrated. One random chosen quarter of the stem of each plant was used as subsample, resulting from two crossed longitudinal cuts, and four ripe leaves (with a 45° inclination from the central axis), each oriented toward one of the four cardinal points.

Juice Extraction and Filtration. The juice from the stem was obtained using an International centrifuge juice extractor (Mexico City, Mexico). Leaves were sectioned into the base, neck, wing, and tip. For the leaf sections juice extraction, a stainless-steel Gerrey sugar cane mill (Bogota, Colombia) was used. Fiber debris was removed using a 5 μm pore-diameter Pentek cellulose filter (Sheboygan, WI). The juice was stored in tightly closed containers at 4 °C.

Hydrolysis. *Thermal Hydrolysis.* In a previous assay, juice samples from each plant fraction were placed in 5 mL test tubes and then immersed in a Precision Scientific water bath (Chicago, IL) at three different temperatures (60, 70, and 80 °C) for 2 h. Fructose concentration in the juices processed at 80 °C was statistically higher. However, the increase in fructose over 2 h was minimum (**Figure 1**), so that the process was prolonged at 80 °C, with sample analysis at 3 h intervals.

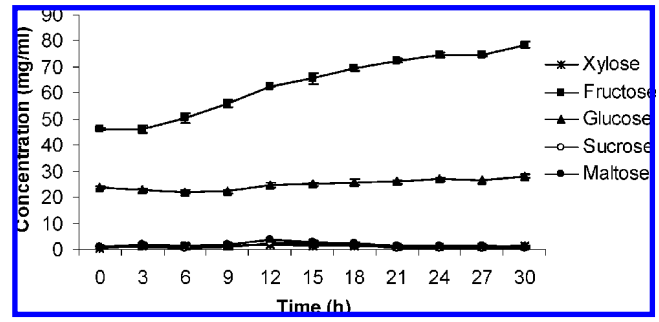

Figure 2. Effect of 80 °C bain-marie heating on concentration of carbohydrates in raw head agave juices. Standard deviations are ±0.1 or less.

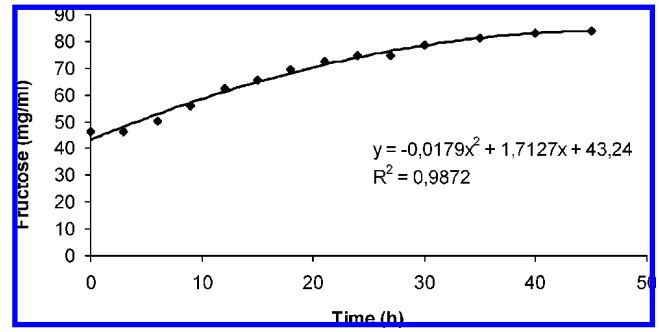

Figure 3. Relationship between time of thermal hydrolysis and fructose concentration in agave head juice.

At 30 h, fructose concentration tended to stabilize (**Figure 2**). The regression model best fitted to these empirical data was the polynomial one (**Figure 3**). According to the corresponding regression equation, thermal hydrolysis during 35 and 40 h could only increase fructose content by 3.49 and 5.82%, respectively. Thus, 80 °C and 30 h were set as the thermal hydrolysis conditions, similar to those used in mezcal (30 h at ± 110 °C) (*20*) and tequila factories (32 h ± 100 °C) (*21*).

Acid Hydrolysis. Raw juice samples similar to the ones above were poured into 5 mL test tubes. The pH of these samples was adjusted to 1 with concentrated sulfuric acid; then, tubes were immersed in a Precision Scientific water bath at 80 °C for 2 h (*22*). Finally, the pH of the samples was adjusted to 5 (the normal value in raw juices) using 10% sodium hydroxide; both reagents used were from Merck (Darmstand, Germany). Raw juice and acid hydrolyzed juice were considered as reference points for thermal hydrolyzed juice.

HPLC Analysis. Acetonitrile and HPLC-grade water from JT-Baker (Deventer, Holland), plus the CAR-11 carbohydrate kit (arabinose, fructose, galactose, glucose, lactose, maltose, mannose, ribose, sucrose, and xylose) from Sigma-Aldrich (Steinheim, Germany), were used. Juice samples were diluted to 20% with a 50:50 (v/v) acetonitrile/water solution, filtered through nylon-membrane filters (0.45 μm) coupled to 5 mL polypropylene syringes, both from Waters (Milford, CT), and analyzed immediately.

Agilent HP series 1100 chromatography equipment (Waldbronn, Germany) was used, consisting of a degassing device, quaternary pump, column compartment, and refractive-index detector. Samples were injected into the system with an Agilent 50 μL Lc syringe (Sydney, Australia), and the manual injector was Rheodyne, 20 μL (Cotati, CA). A carbohydrate-specific Zorbax column (4.6 mm i.d. × 250 mm, 5 μm particle size) from Agilent (Palo Alto, CA) was used as the stationary phase.

Chromatographic Conditions. Chromatographic carbohydrate separation was achieved in a 75:25 (v/v) acetonitrile/water mobile phase. The diluent flow was 1.4 mm/min. The injected sample volume was 20 μL. The chromatographic separation time was 15 min. The temperatures of the column compartment and the detector were kept at 30 °C. Environmental temperature was kept constant at 20 °C. The HP Chem Station for LC Rev A. 09.03 software from Agilent (Palo Alto, CA) was used for system control and data analysis.

Table 1. Retention Time (min) of Standards Used in the Zorbax Column for Carbohydrates and Correlation Coefficients between Samples and Standards Refraction Values

carbohydrate	t_R (min)	correlation coefficients (r)
ribose	4.32	
xylose	4.71	0.9999[a]
arabinose	5.30	
fructose	5.66	0.9996[a]
mannose	6.03	
glucose	6.30	0.9997[a]
galactose	6.78	
sucrose	8.48	0.9980[a]
maltose	10.04	0.9994[a]
lactose	11.51	

[a] $p < 0.05$.

Identification and Quantification. Sugars present in agave juice were identified by comparing the retention times with those of the CAR-11 carbohydrate kit standards. All the carbohydrate standards could be separated in a single operation. **Table 1** includes the retention times for the standards, which were relatively constant, with slight variations when the laboratory temperature exceeded 20 °C. Only xylose, fructose, glucose, sucrose, and maltose were identified in mezcal agave juice, as previously observed in other *Agave* species (*10, 11, 21, 23*). The other carbohydrates were either not found or could be present at a trace concentration below the detection limit. Carbohydrate affinity toward the stationary phase displayed the following order: monosaccharides (pentoses < hexoses) < disaccharides. This agrees with observations in HPLC chromatograms to identify carbohydrates in the common mushroom (*Agaricus bisporus*) (*24*).

The calibration curve for each carbohydrate was derived using 2, 4, 6, 8, and 10% standard solutions. Correlation coefficients (r) were calculated between refraction values in samples and standards for each sugar to estimate the detector's consistency in terms of concentration amplitude; r values ranged between 0.9980 and 0.9999 (**Table 1**). All the determinations were carried out in triplicate, and a mixture of standards was injected daily in order to identify any potential calibration variations.

Experimental Design and Statistical Analyses. The experiment was conducted according to a completely randomized design, with factorial treatment arrangement ($3 \times 5 \times 3$). Factors and levels were as follows: (a) ripeness stage— young or unripe plants, early mature plants or under incipient reproduction, and one-year after being castrated plants; (b) plant organ or fraction— stem, and leaf base, neck, wing, and tip; (c) hydrolysis— raw juice, heat-hydrolyzed juice, and acid-hydrolyzed juice. Carbohydrate concentrations in raw and hydrolyzed juice were subjected to an analysis of variance (SAS, ver. 8, SAS Inc., Cary, NC). Regression analyses were made using Sigma Plot software for PC (Jandel Scientific, version 9.0.1, San Jose, CA).

RESULTS AND DISCUSSIONS

Sugars in Agave Juice. Only noticeable amounts of xylose, fructose, glucose, sucrose, and maltose were recorded (**Table 2**). Sucrose is the first disaccharide to be hydrolyzed by the plant when energy is required for several metabolic processes (*25, 26*). Xylose has only been found in plants at low concentrations, associated with structural polysaccharides as hemicellulose, or in the primary cell wall associated with xyloglycan (*27*). Maltose is formed only from the hydrolysis of transitory starch, which takes place at night, and is immediately used as an energy source (*28, 29*). The concentrations of these sugars in agave juice samples (**Table 2**) showed specific variations according to plant part, ripeness stage, and hydrolysis. Thus, higher contents of xylose were in leaf raw juices, and fructose was only lower or similar to glucose in raw juices and showed higher levels in hydrolyzed stem juices.

Fructans seem to be the reserve carbohydrates in *Agave salmiana* because heat and acid hydrolysis increased fructose concentration compared to raw juice, as has also been observed in *Agave tequilana* Weber (*21*). The highest fructose concentration was observed in hydrolyzed juice from the stem, confirming that this portion of the plant accumulates the highest amount of fructans as energy reserve (*10*). Hydrolysis led only to a slight rise in glucose concentration, since fructans contains one glucose unit per molecule in addition to fructose (*11*) and the juice probably lacks other polysaccharides with glucose. The absence of all other standard carbohydrates in the juice is explained by their nature and origin (*30, 31*). Since glucose and fructose were found at the highest concentrations, analyses of variance were conducted only for these two sugars; calculated F values and test are presented in **Table 3**.

Table 2. Average Concentration of Carbohydrates (mg/mL) in Agave Head Juices ($n = 3$)[a]

no.	treatment	xylose	fructose	glucose	sucrose	maltose	total
1	I−TT−C	7.958	8.392	8.304	0.825	1.762	27.241
2	I−TT−HT	4.549	52.806	14.421	7.792	2.933	82.501
3	I−TT−HA	3.483	81.338	20.225	2.151	2.775	109.973
4	I−PB−C	6.121	4.858	12.222	2.136	2.202	27.539
5	I−PB−HT	0.922	13.900	9.306	2.038	0.406	26.571
6	I−PB−HA	2.047	29.058	13.947	0.525	4.222	49.798
7	I−PC−C	4.486	9.904	9.528	0.855	1.549	26.321
8	I−PC−HT	2.100	13.350	6.811	0.846	0.494	23.601
9	I−PC−HA	2.872	32.097	12.033	0.892	2.623	50.517
10	I−PA−C	9.073	8.671	8.019	0.793	1.302	27.858
11	I−PA−HT	3.970	15.171	7.269	1.421	0.438	28.268
12	I−PA−HA	2.157	22.012	9.742	0.000	1.598	35.508
13	I−PP−C	12.946	5.262	9.898	4.334	4.857	37.297
14	I−PP−HT	6.017	13.328	7.872	0.934	4.735	32.886
15	I−PP−HA	3.825	29.959	8.963	0.000	0.868	43.615
16	Q−TT−C	2.916	2.946	4.448	2.180	1.397	13.886
17	Q−TT−HT	1.998	68.687	10.456	8.903	4.178	94.221
18	Q−TT−HA	2.310	155.910	26.927	0.843	2.770	188.760
19	Q−PB−C	0.891	4.017	5.978	4.187	9.543	24.616
20	Q−PB−HT	0.633	36.226	13.608	3.904	1.552	55.924
21	Q−PB−HA	3.033	82.605	18.745	1.870	1.968	108.222
22	Q−PC−C	2.428	2.487	7.966	2.370	5.560	20.811
23	Q−PC−HT	3.348	44.274	16.250	3.674	2.646	70.193
24	Q−PC−HA	2.412	84.622	14.703	1.367	1.543	104.647
25	Q−PA−C	13.867	3.668	8.899	0.848	0.677	27.958
26	Q−PA−HT	2.649	26.499	12.901	3.437	2.328	47.813
27	Q−PA−HA	3.410	48.338	14.487	3.702	1.008	70.945
28	Q−PP−C	12.009	7.035	9.272	2.550	3.270	34.136
29	Q−PP−HT	4.841	24.372	12.028	4.624	3.197	49.063
30	Q−PP−HA	1.120	49.718	12.073	5.005	0.000	67.916
31	C−TT−C	2.723	2.487	4.506	2.933	5.599	18.249
32	C−TT−HT	4.259	43.257	11.073	4.787	3.541	66.917
33	C−TT−HA	0.378	145.310	20.773	2.822	0.240	169.523
34	C−PB−C	0.779	3.159	10.845	3.601	11.361	29.744
35	C−PB−HT	2.150	30.413	8.672	3.897	0.828	45.960
36	C−PB−HA	0.670	48.475	15.930	1.987	0.785	67.846
37	C−PC−C	4.209	5.789	10.844	5.330	3.777	29.949
38	C−PC−HT	2.671	25.093	10.033	2.176	2.085	42.058
39	C−PC−HA	0.365	70.420	13.730	1.472	1.415	87.401
40	C−PA−C	3.500	6.504	12.056	2.016	1.062	25.139
41	C−PA−HT	5.958	32.169	14.679	4.290	2.063	59.159
42	C−PA−HA	2.377	67.783	16.036	2.453	0.000	88.649
43	C−PP−C	4.891	4.803	11.280	2.681	1.816	25.472
44	C−PP−HT	4.997	34.713	13.056	5.115	1.932	59.812
45	C−PP−HA	1.688	61.350	15.039	1.028	0.000	79.106

[a] Ripeness: I = immature; Q = early mature; C = castrated. Organ: TT = stem; PB = leaf base; PC = leaf neck; PA = leaf wing; PP = leaf tip. Hydrolysis: C = raw or without hydrolysis; HT = thermal hydrolysis; HA = acid hydrolysis.

Variation in Glucose and Fructose Concentration. According to the F-test, there was no statistical evidence that ripeness modifies glucose concentration in agave juice (**Figure 4**). This is likely due to the fact that, after being produced from photosynthesis, glucose is immediately used as an energy source and any surplus amounts are isomerized into fructose, or along

Table 3. *F* Calculated Values and Test for Fructose and Glucose Concentration (mg/mL) in Agave Juices

	df[a]	glucose	fructose
ripeness	2	0.6033	50.9014[b]
fraction	4	0.2465	66.1515[b]
hydrolysis	2	5.0879[b]	434.8382[b]
ripeness × hydrolysis	4	0.5349	27.0683[b]
fraction × hydrolysis	8	0.8464[b]	27.6592[b]
fraction × ripeness	8	0.2589	3.0578
fraction × ripeness × hydrolysis	16	0.1190	2.7603
error	90	0.2513	2.9231
V.C. (%)		42.101	49.2154

[a] Degress of freedom. [b] $p < 0.05$.

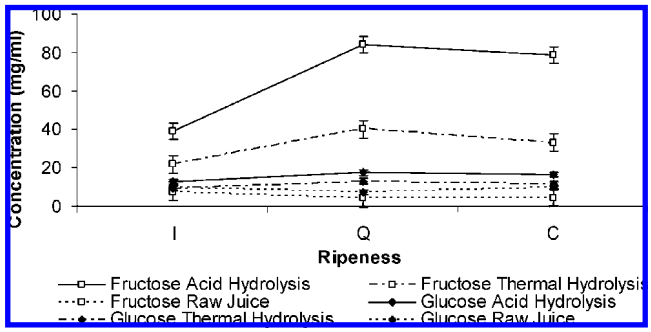

Figure 4. Maturity and hydrolysis effects on fructose and glucose concentration ($\bar{X} \pm$ EE) in raw agave juices (I = immature; Q = incipient reproduction; C = castrated).

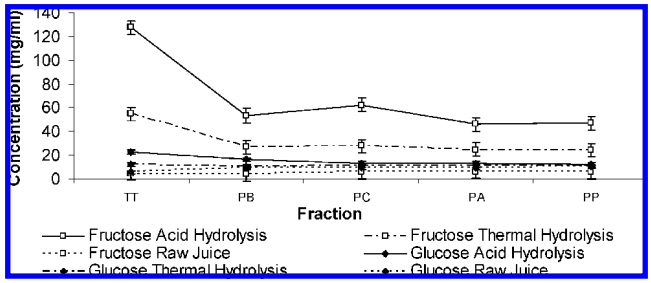

Figure 5. Plant fraction and hydrolysis effects on fructose and glucose concentration ($\bar{X} \pm$ EE) in raw agave juices (TT = stem; PB = leaf base; PC = leaf neck; PA = leaf wings; PP = leaf apex).

with the latter into sucrose, which is then used as a transport sugar (*8*). The significance of the plant fraction with hydrolysis interaction (**Table 3**, **Figure 5**) shows that only the acid hydrolysis, the most aggressive one, increased the amount of glucose in juice, but this increase displayed a gradient from a peak in the stem to nil at the leaf tip. This higher glucose concentration seems to derive mostly from the acid hydrolysis of fructans (*11*), which tends to accumulate in the stem but may also derive from sucrose and maltose hydrolysis, particularly in juice extracted from leaf portions (**Figure 5**).

In regards to fructose, the statistically significant interaction of ripeness with hydrolysis (**Table 3**) demonstrates the direct relation of ripeness with fructans accumulation, which releases fructose after hydrolysis (*21*). **Figure 4** shows that fructose concentration in hydrolyzed juice peaks in the early mature ripeness stage, which may be related to the onset of reproduction, since these reserves are directed for the development and maintenance of reproductive organs if the plant is left intact (*14*). The onset of reproduction determines the start of senescence, and castration delays it and prolongs the plant's period of use with minimum effects on the useful reserves (*14*). This trend of fructose polymer increment according to ripeness in general terms agrees with previous studies (*21*).

The significance of the plant fraction with the hydrolysis interaction (**Table 3**) indicates that the effect of hydrolysis on fructose concentration also depends on the portion of the plant from which the juice was extracted (**Figure 5**). Thus, while fructose concentration in raw juice was minimum and nearly identical in all fractions, it considerably rises in hydrolyzed stem juice, whereas a far less increase is noted in the leaf portions, which drops with distance from the stem. These trends confirm the role of the stem as a reserve carbohydrate (fructans) storage organ (*10, 11, 14*), suggesting that leaf fructose probably rises as a result of sucrose hydrolysis but above all from fructose chains in the process of polymerization to form fructans or from fructans hydrolysis.

It is worthy to point out the considerable difference (above 100%) between the fructose concentration obtained by both hydrolysis types, despite the fact that it only reached statistical significance (Tukey $\alpha = 0.05$) in juice extracted from early mature and castrated agave plants. It is likely that the thermal hydrolysis of fructans in whole agave heads, as currently practiced in mezcal factories, is even more incomplete, and this may be one of the reasons for the inefficiency of mezcal production processes (*14*).

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