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Analysis of the Main Components of the Aguamiel Produced by the Maguey-Pulquero (*Agave mapisaga*) throughout the Harvest Period

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The main characteristics of the aguamiel (maguey-pulquero sap) during the harvest period of the *Agave mapisaga* plants were assessed to establish its stability through time and the industrial potential of its components. Only minor differences in aguamiel composition were detected among samples collected at different time points of the harvest period. The aguamiel analyzed contained 11.5 wt % of dry matter, which was composed mainly of sugars (75 wt %). Among these sugars, 10 wt % were fructo-oligosaccharides (FOS), which are known to be important in the food industry for their prebiotic properties. Other components include 0.3 wt % of free amino acids (with most essential amino acids and four neurotransmitters: GABA, GLY, GLX, and ASX), 3 wt % of proteins, and 3 wt % of ashes.

KEYWORDS: Aguamiel (maguey-pulquero sap); *Agave* spp. (*A. mapisaga*); fructans; fructo-oligosaccharides; amino acids

INTRODUCTION

In semidesert areas of Mexico, the growing of agave species such as maguey-pulquero (*Agave salmiana*, *A. mapisaga*, *A. atrovirens*, *A. americana*, *A. ferox*) (*1*) is very important: it limits drift from the land and also prevents soil erosion. These agaves are mainly used to produce pulque, one of the oldest alcoholic beverages on the American continent (*2*), obtained by fermentation of aguamiel (maguey-pulquero sap). Actually, aguamiel production is induced from these plants at the time when the plant's flower stem shoots up (around 8−10 years old) by making a hollow in the center. The collection is done every 12 h after scraping the stem of the agave, and the production lasts from 4 to 6 months. The volume of aguamiel changes throughout the harvesting period. At the beginning one can collect about 0.4 $L \cdot plant^{-1} \cdot day^{-1}$, and this amount increases to 4 or 6 $L \cdot plant^{-1} \cdot day^{-1}$ during the first 2 months and then decreases to 0.4 $L \cdot plant^{-1} \cdot day^{-1}$ toward the end of production time.

Fermentation occurs thanks to microorganisms naturally present in the aguamiel or those incorporated during the collection, transport, etc. It can also be accelerated by the addition of a portion of pulque previously produced. Thus, the beverage is the result of the action of a large bacterial diversity (*1, 3*).

In the first half of the 20th century, pulque production constituted a profitable industry in Mexico, but nowadays it is not economically attractive at all, the pulque price varying from US$0.26 to 0.43 per liter (*4*). Furthermore, pulque consumption has dramatically decreased in favor of beer, which represents >54% of the total ethanol consumption as compared to 11% for pulque (*5*). As a result, the culture of the agave pulquero plant has been discouraged because it did not represent a profitable source of income anymore.

The tradition of the "Tlachique" (aguamiel harvest) as well as the sowing and use of maguey-pulquero should be preserved as part of the Mexican heritage. Thus, finding new alternatives for a wider application and/or industrialization of this raw material should be a priority.

Up to now, aguamiel research has been focused on the microbial and enzyme diversity that participate in the aguamiel fermentation process for the production of pulque (*1, 3, 6, 7*). In addition, its chemical composition has been also partially elucidated, highlighting the importance of the amino acid content as well as the presence of ascorbic acid and iron in aguamiel (*3, 8–10*). In fact, it has been suggested that this beverage may constitute

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a food alternative in places were the water quality is poor and meat consumption is limited (*9*).

Martinez del Campo-Padilla (*11*) reported the presence of fructans in aguamiel from *A. atrivirens*, attracting the attention of other researchers looking for potential industrial applications. Basically, fructans are oligomers or polymers consisting of a chain of β-fructofuranosyl units connected to the fructose residue of a sucrose molecule through β-(2→1) and/or β-(2→6) linkages. The degree of polymerization (DP) can vary from 3 to 60; if the DP is below 10, these polymers are known as fructo-oligosaccharides (FOS), but if it is higher than 10, then they are called inulins (*12*). Fructans are known as food ingredients. In particular, chicory inulin is currently added in yogurts, spreads, and ice cream to replace fat. Besides their use as food additives, fructans are also regarded as prebiotic because they are not digested in the stomach and reach the colon intact, where they promote the proliferation of beneficial flora (*Bifidobacteria*) (*12–14*). The fructans found in aguamiel have been proposed as thermoprotective prebiotics in the spray-drying of *Bifidobacterium bifidum* (*15*).

Besides application potential, it would be important that aguamiel production be stable during the harvest period to standardize its industrialization. None of studies on aguamiel have looked at the changes in concentrations of its different components throughout its production, and this information may be important, given that the volume of aguamiel is not constant during its production.

The aim of this work was to examine the composition of the aguamiel obtained from *A. mapisaga* throughout the harvest period in order to evaluate its stability through time. This information may help to re-evaluate the economical importance of this plant and encourage its cultivation.

MATERIALS AND METHODS

Samples. The samples of aguamiel were collected in the community of Lomas de Romero, Tecamachalco, Puebla, Mexico (18° 53′ N, 97° 44′ W; altitude 2020 m). Three plants of *A. mapisaga* 'Blanco' were used in this study. All of these plants were at the beginning of their blooming period. The agaves were prepared for the extraction of aguamiel following the traditional procedures (*9*), beginning June 1, 2005.

To study the changes in composition, 500 mL of aguamiel of each plant was collected at 0.5, 1.0, 2.3, 3.5, and 4.5 months of the harvest period. The sampling dates were fixed according to the climatic conditions. No sample was collected when it rained, and we waited 2 days before taking samples after a rain period. Samples were frozen and stored at –18 °C with 0.02 wt % sodium azide to avoid microbial growth. Each collected sample was analyzed in triplicate.

Physicochemical Analysis. Titrable acidity, pH, total soluble solid content (TSS), dry matter, and ashes were determined in accordance with standard methods described by the Association of Official Analytical Chemists (AOAC) (*16*). The protein content was measured according to the Lowry method (*17*). Cation determination was carried out by atomic absorption spectrometry on a "SpectrAA 220 SFC" with the acidic solution obtained by dissolution of ashes in 1 M HCl.

Analysis of Sugars. The concentrations of D-glucose, D-fructose, and sucrose were determined using an enzymatic kit (Boehringer Mannheim UV method 10 716 260 035 R-Biopharm) following the manufacturer's instructions. All of the samples were centrifuged at 17600*g* for 10 min at 10 °C, and the supernatant was used for the analysis.

Verification of the Presence of Fructans. The presence of fructans in the samples was verified indirectly by successive enzymatic degradation with inulinase (from *Aspergillus niger*, Fructozyme L Novozymes) and amyloglucosidase (Fluka-France). Inulinase (10 μL·mL^{-1}) was added to aguamiel diluted 10-fold in 100 mM acetate buffer (pH 4.7). This mixture was left at 50 °C for 12 h under agitation and then heated at 100 °C for 10 min to stop the enzymatic reaction. An aliquot of this hydrolyzed product was withdrawn for chromatographical analysis, whereas the rest was subjected to a second hydrolysis with 10 μL·mL^{-1} of amyloglucosidase. This new mixture was then incubated at 50 °C for another 12 h under agitation. The enzyme reaction was stopped by heating the sample at 100 °C for 10 min, and a new aliquot was taken for analysis. The presence of fructans was determined qualitatively by high-performance anion-exchange chromatography (HPAEC) on a Dionex BioLC chromatography system equipped with a pulsed amperometric detector (PAD), using a CarboPac PA100 column (0.4 × 25 cm; Dionex, USES) with a CarboPac PA100 guard column (0.4 × 5 cm). The columns were first equilibrated at 25 °C with 100 mM NaOH at flow rate of 1 mL·min^{-1} during 10 min before the injection of 30 μL of each aliquot of hydrolyzed or nonhydrolyzed aguamiel separately. The fructans were eluted with a sodium acetate gradient (0–240 mM sodium acetate from 11 to 60 min and 240–600 mM sodium acetate from 60 to 70 min) in 100 mM NaOH. All of the samples were centrifuged at 17600*g* for 10 min at 10 °C, and the supernatant was used for the analysis. 1-Kestose (DP 3) and nystose (DP 4) (Fluka-France) and inulins of Jerusalem artichoke and chicory (Sigma-France) were used as fructan standards.

Polydispersity of Fructans. The chain length of fructans was determined qualitatively by HPAEC with the same Dionex BioLC chromatography system described before. Once the columns were equilibrated, 30 μL of a 10-fold water-diluted aguamiel sample was injected, and fructans were eluted in the same sodium acetate gradient as before.

Determination of the Degree of Polymerization and Quantification of Fructans. To determine the degree of fructan polymerization, 50 μL of a commercial preparation of inulinase from *A. niger* (Fructozyme L, Novozymes) was added to a 5 mL of aguamiel diluted 10-fold in 100 mM acetate buffer (pH 4.7). This mixture was incubated at 50 °C for 12 h. After that time, the samples were placed for 10 min into boiling water to inactivate the enzyme and were submitted to total glucose and fructose analysis using the enzymatic kit described above. In parallel, a second assay was carried out in the same conditions except that samples were immediately placed at 100 °C for 10 min after enzyme addition to inactivate this biocatalyst. This allowed us to determine free glucose, fructose, and sucrose in the aguamiel.

The average degree of polymerization (DP) was calculated using eq 1 (*18*).

$$\mathrm{DP} = \frac{F}{G} + 1 \qquad (1)$$

where F = total fructose − free fructose − 0.52 free sucrose and G = total glucose − free glucose − 0.52 free sucrose.

The sum of glucose and fructose produced by hydrolysis ($F + G$) was considered here as the fructan concentration.

Determination of Endogenous Enzymatic Activity. Endogenous enzymatic activity was determined by dissolving 10 mg·mL^{-1} of chicory inulin (Sigma-France) in aguamiel that already contained 0.02% of NaN_3. One milliliter of this solution was either incubated directly at 50 °C for 110 h (enzyme test) or heated at 100 °C for 10 min to inactivate endogenous enzymes and then incubated at 50 °C for 110 h (blank test).

An aliquot of each test solution was taken before and after the incubation and diluted 20-fold before being analyzed by HPAEC as above. The columns were equilibrated at 25 °C with 120 mM sodium acetate in 100 mM NaOH at flow rate of 1 mL·min^{-1} during 10 min, and then 30 μL of the diluted test sample was injected. The fructans were first eluted with 120 mM sodium acetate in 100 mM NaOH from 11 to 15 min and then in a linear gradient up to 460 mM sodium acetate from 15 to 70 min.

Identification and Quantification of Amino Acids. The identification and quantification of amino acids were achieved using a high-performance liquid chromatograph (HPLC) with a Dionex chromatography system equipped with a DAD UVD 340U detector and with both a Waters Symmetry column (Uptisphere UP5ODB-25QS, 250 × 4.6 mm, 5 μm) and a Waters Symmetry 20 mm guard column (Uptisphere UP5OBD). Dabsylated amino acids were injected in a mixture (78:22 v/v) of two solvents [solvent A, 9 mM sodium dihydrogenophosphate,

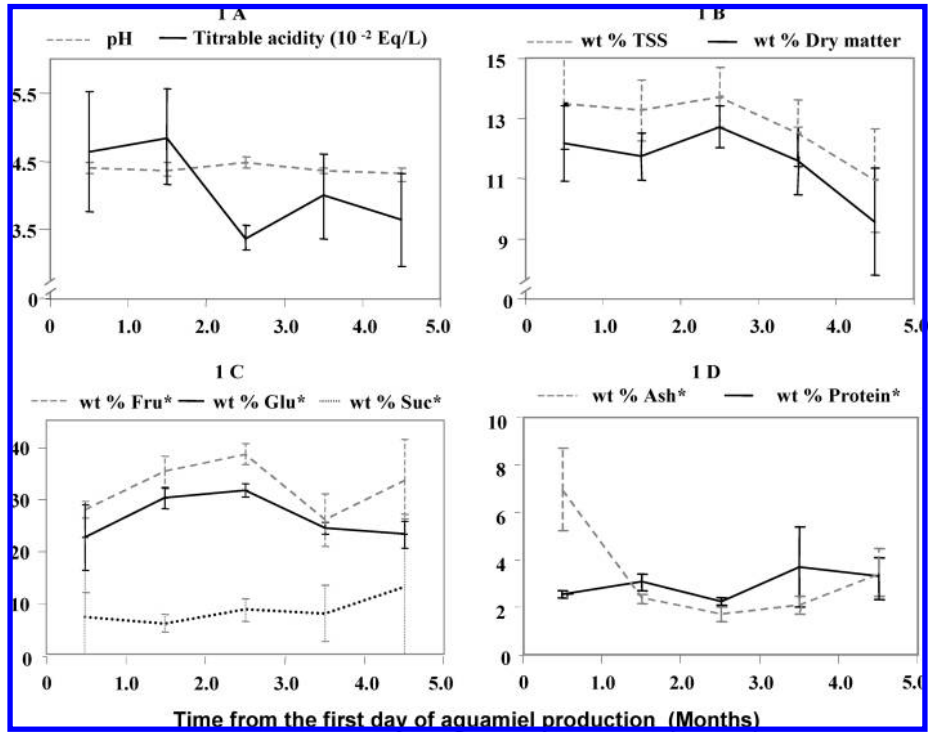

Figure 1. Behavior of some physicochemical characteristics of aguamiel during its production in *A. mapisaga*: **A**, pH and acidity; **B**, wt % TSS and dry matter; **C**, wt % sucrose (Suc), glucose (Glue), fructose (Fru); **D**, ash and protein. *, wt % in dry matter.

4% dimethylformamide and 0.1% triethylamine (TEA), pH 6.55; solvent B, 80% (v/v) aqueous acetonitrile] and then eluted at a flow rate of 1 $mL \cdot min^{-1}$ that increased linearly the percentage of solvent B. Detection was performed at 436 nm. AA-S18 standard solutions and GABA (Sigma-France) were used for calibration.

Statistical Analysis. A single classification analysis of variance was performed to determine significant differences ($P < 0.05$) of aguamiel components among plants and throughout its harvest period with the statistical software Statgraphics 5.1 (Stsc, Rockville, MD).

RESULTS AND DISCUSSION

Physicochemical Properties and Main Components of the Aguamiel. The evolution of mean values of the analyzed physicochemical properties of the aguamiel produced by the three plants at different production times is shown in **Figure 1**. No significant effect of time on titrable acidity was observed ($p < 0.0846$). However, this factor slightly decreased in the samples collected after 3 months of harvest. The pH values remain at around 4.5 in all aguamiel samples throughout the study (**Figure 1A**). The dry matter content did not change significantly ($p < 0.0699$) and represented on average 11.5% of the total weight of the aguamiel. In addition, the dry matter content showed a good correlation with the TSS measurements (**Figure 1B**), indicating that most of the dry matter is related to the mono- and disaccharide contents. In fact, the sum of glucose, fructose, and sucrose content represented >65 wt % of the dry matter, but the concentration of sucrose was always significantly lower than that of the two monosacharides (**Figure 1C**). However, whereas no major differences in the concentration of each of these three sugars were observed through time, the fructose concentration decreased significantly ($p < 0.0314$) in the samples collected at 3.5 months after the beginning of the harvest. The protein concentration was about 3 wt % of the dry matter and remains almost the same during the harvest period ($p < 0.3368$). In contrast, the ash concentration presented a significant decrease in the first 2 months of harvest ($p < 0.003$) (**Figure 1D**), mainly due to the decrease in potassium concentration ($p < 0.0011$). Indeed, this cation varied from 1614 ± 22 to 960 ± 8 $mg \cdot L^{-1}$ during this period. After 3 months of harvesting, there was a leveling out in the ash concentration associated with a slight increase in Ca content (from 35 ± 1 to 126 ± 36 $mg \cdot L^{-1}$). Rendiles et al. (*19*) reported a very similar K behavior in *Psidium guajava* L. and other fruit-bearing trees. They attributed the initial K foliar decrease to the formation and filling of fruit and, later, the increase in K and Ca to the beginning of a new vegetative phase of growth in the plant. Actually, potassium plays a vital role in a wide range of biochemical and biophysical processes in plants. It is a highly mobile charge carrier, which neutralizes the effects of anions and plays an important role in enzyme activation and membrane transport. No significant variation was observed in sodium and magnesium contents, which were maintained at 116 ± 25 and 72 ± 9 $mg \cdot L^{-1}$, respectively. Ashes also contained traces of iron (1.1 $mg \cdot L^{-1}$), zinc (0.8 $mg \cdot L^{-1}$), copper (0.3 $mg \cdot .L^{-1}$), and manganese (0.3 $mg \cdot L^{-1}$). Although it has been reported that aguamiel contains ascorbic acid (*8, 9*), this compound could not be detected in our samples.

Table 1. Average Composition of Aguamiel from Three *A. mapisaga* Plants throughout Their Harvest Period

	av wt % (in dry matter)
fructose	32.4 ± 3.9
glucose	26.5 ± 2.7
sucrose	8.8 ± 6.5
fructo-oligosaccharides	10.2 ± 5.7
AA free	0.26 ± 0.05
protein	3.0 ± 0.6
ash	3.3 ± 0.8

Despite small variations observed, the composition of aguamiel was mostly stable during the harvest period, and its average composition is shown in **Table 1**.

Fructan Analyses. The ionic chromatographic profiles of the aguamiel samples obtained before and after hydrolysis with

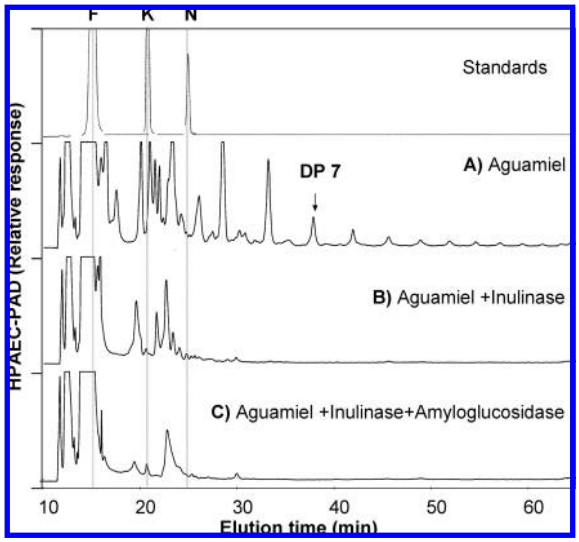

Figure 2. HPAEC-PAD profiles of aguamiel of *A. mapisaga* before and after enzymatic hydrolysis compared to elution time of some standards (F, fructose; K, kestose; N, nystose).

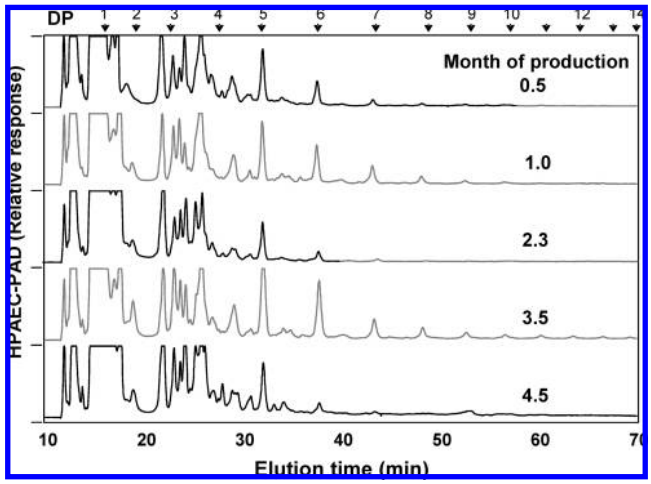

Figure 3. Chromatographic profiles of aguamiel from *A. mapisaga* at different harvest times obtained by HPAEC-PAD.

inulinase and amyloglucosidase were compared to determine the presence of fructans (**Figure 2**).

The HPAEC profile of aguamiel before hydrolysis (**Figure 2A**) showed many peaks that disappeared after the addition of inulinase (**Figure 2B**). The presence of inulin-type fructans in our samples was thus confirmed. Furthermore, we can assume that almost all of the peaks eluted after 25 min were fructans. In addition, after the hydrolysis with amyloglucosidase (**Figure 2C**), a new degradation of peaks was observed, indicating the presence of gluco-oligosacharides. The peaks that are still present after hydrolysis (**Figure 2C**) may correspond to a few β-fructofuranosyl units linked through both β-(1→2) and β-(2→6) linkages. Thus, they cannot be hydrolyzed by the inulinase used. The presence of these highly branched fructans involving different types of linkage has been already reported in several agave species (*18–20*). Because their structures are more complex than that of inulin, they are referred to as agavines (*20*).

The evolution of polydispersity of fructans in the samples throughout the study was evidenced by the analysis of HPAEC-PAD profile shown in **Figure 3**. The aguamiel analyzed contained mostly FOS with low DP (3−6) and small amounts of FOS with DP 7−10 the whole time. But no peaks of

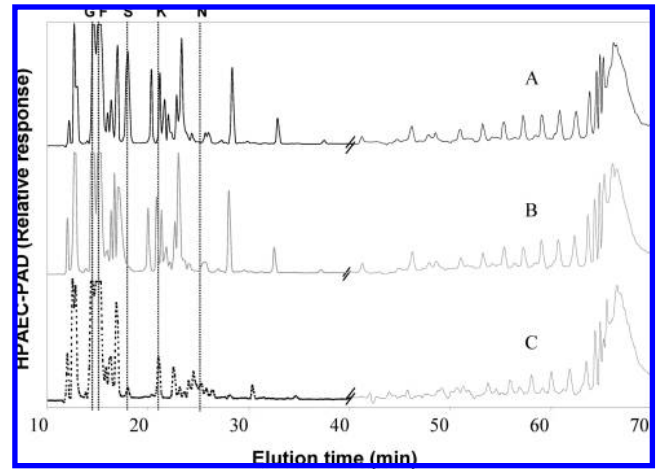

Figure 4. HPAEC-PAD profiles of a 10 $g \cdot L^{-1}$ chicory inulin solution prepared in aguamiel of *A. mapisaga*: **A**, blank and enzyme test before incubation; **B**, blank test after incubation (50 °C for 110 h); **C**, enzyme test after incubation (50 °C for 110 h). The relative response after 40 min of elution is amplified 10 times. G, glucose; F, fructose; S, sucrose; K, kestose; N, nystose.

significant importance in the HPAEC-PAD profile were detected with DP >10 corresponding to inulins, except for samples taken at 3.5 months of production. The average DP of fructan calculated using eq 1 was 4.5. This value is characteristic of mixtures consisting mainly of FOS (*12*). These results strongly suggest that inulins were absent from our samples. However, previous works have reported the presence of inulins in the aguamiel of *A. atrovirens* (*11*). This evidence, together with fact the samples at 3.5 months showed DP >10 peaks correlated with a decrease in fructose concentration (**Figure 1C**), implied that high DP fructans were hydrolyzed in the other samples during the collection or before their storage at –18 °C. In fact, the presence of an inulinase hyperproducing strain of *Kluyveromyces* in the aguamiel has been previously reported by Cruz-Guerrero (*7*). To confirm this hypothesis, the endogenous enzymatic activity was determined by comparing the HPAEC profiles of inulin-enriched aguamiel samples before (enzyme test) and after (blank test) enzyme inactivation and after 110 h of incubation. At the beginning of the incubation the enzyme and the blank tests showed profiles similar to the one shown in **Figure 4A**. This profile was maintained constant in the blank test, even after incubation (**Figure 4B**). However, the enzyme test presented important changes due to the reduction in the number of peaks of FOS and a saturation of the peaks corresponding to monosaccharides (**Figure 4C**). These results clearly show that peaks corresponding to fructan with high DP, or inulins, were hydrolyzed by endogenous enzymes. This fact was confirmed by the monosaccharide analyses performed with the Boehringer enzymatic kit before and after 110 h of incubation. This analysis showed that although the blank test remained unchanged, the fructose and glucose concentrations of the enzyme test increased by 33 and 20%, respectively, and the sucrose concentration decreased by 4.5%. These results confirm the presence of invertase and inulinase in our sample of aguamiel. These enzymes, which were still active after 5 months of storage at –18 °C, may have come from the plant itself or were secreted by microorganisms present in the aguamiel before the addition of NaN_3.

Due to these uncontrolled hydrolyses, the polysaccharide content of our aguamiel samples could have been changed. Therefore, we suggest that in order to produce maguey-pulquero

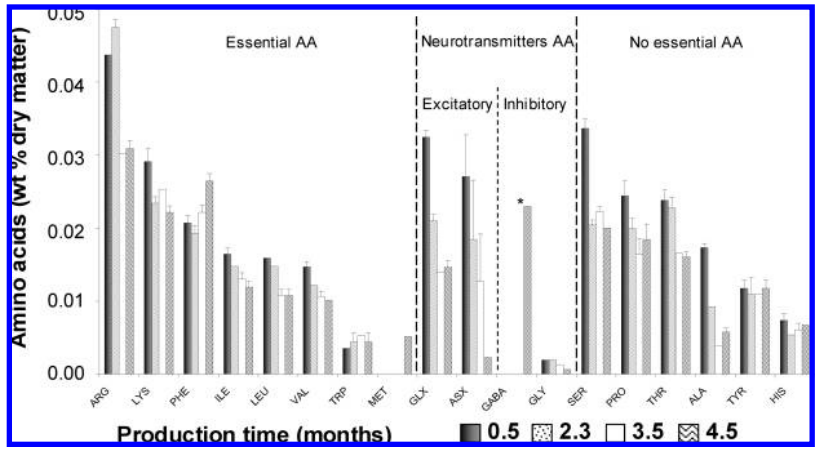

Figure 5. Amino acids in the aguamiel during the period of production of the *A. mapisaga*. *, GABA was analyzed only in the last month of aguamiel production.

sap that contains fructans with higher DP, inactivation treatment is required to avoid any enzymatic degradation after aguamiel harvest.

Free Amino Acid (AA) Content. The concentrations in free AA in the different samples of aguamiel are reported in **Figure 5**. From these results the total amount of free AAs was estimated as 0.26% of dry matter, corresponding to 0.3 $g \cdot L^{-1}$. It is worth noting that almost all of the essential AAs are present in all aguamiel samples except for methionine, which was present at only a very low level (<0.01% of dry basis) in the sample collected after 4.5 months of production.

No significant differences were observed among samples during the harvest period apart from arginine (ARG), aspartic acid/asparagine (ASX), and alanine (ALA) contents, which continuously decreased during the harvest period. Their disappearance can be easily explained by their involvement in metabolic pathways. Actually, alanine can be transformed by a simple transamination reaction into α-keto pyruvate, which is a key intersection in the network of metabolic pathways. If sufficient oxygen is available, pyruvic acid is converted into acetyl-coenzyme A, which is the main input for a series of reactions known as the Krebs cycle, whereas if the oxygen is missing, pyruvate is converted by anaerobic respiration to lactate. In the same way, aspartic acid is the analogue of the oxaloacetate, which is also one of the intermediates of the Krebs cycle. With regard to L-arginine (Arg), this AA is a precursor in the formation of polyamines, which are small polycationic growth regulators implicated in a large range of plant growth and developmental processes (*21*).

Besides these well-known AAs, an unknown peak has been identified in our chromatograms as γ-aminobutyric acid (GABA). This is an AA never found in protein structure, but it is widely distributed throughout the biological world even if its level in plant tissues is low (ranging from 0.03 to 2.00 $\mu mol \cdot g^{-1}$ of fresh weight) (*22*). Here, GABA concentration is estimated to be 0.25 $\mu mol \cdot mL^{-1}$, corresponding to 26 $mg \cdot L^{-1}$ of aguamiel. GABA synthesis is induced by diverse environmental stresses including cold, heat, salt, and mild or transient environmental factors, such as wind and rain; thus, its presence in aguamiel is not surprising.

GABA is known to be the main inhibitory neurotransmitter in the sympathetic nervous system. It also plays an important role in cardiovascular function. Inoue (*23*) has reported that the consumption of only 10 mg of GABA daily for 12 weeks has a positive effect on the reduction of blood pressure (BP) of patients with mild hypertension, whereas normotensive subjects do not exhibit change in their BP. However, even if aguamiel with 26 $mg \cdot L^{-1}$ can be considered as a functional drink, it cannot represent a potential raw material for GABA purification because its concentration is really too low to permit the development of an economically viable process.

In summary, the general composition of the aguamiel was stable throughout the harvest period even though the total amount of this product changed over time. This result suggests that the aguamiel produced by *A. mapisaga* represents a stable raw material, allowing for the standardization of its industrial processing. The aguamiel composition and the low-budget maintenance of maguey-pulquero plants should be good reasons to encourage the cultivation of this species in the arid regions of Mexico.

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