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IMPROVING *Agave duranguensis* MUST FOR ENHANCED FERMENTATION. C/N RATIO EFFECTS ON MEZCAL COMPOSITION AND SENSORY PROPERTIES

MEJORAMIENTO DEL MOSTO DE *Agave duranguensis* PARA LA FERMENTACIÓN MEJORADA. EFECTOS DE LA RAZÓN C/N SOBRE LA COMPOSICIÓN DE MEZCAL Y LAS PROPIEDADES SENSORIALES

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Abstract

Mezcal is a traditional distilled spirit beverage obtained by artisan fermentation of agave sugars. In this work we determined the effects of the C/N ratio and the initial concentration of sugars in *Agave duranguensis* must on the fermentation kinetics, composition and sensory evaluation of the mezcal produced. The fermentation of *A. duranguensis* must was enhanced by adding 50% more nitrogen (respect to the original content) as ammonium sulfate at an initial sugar concentration of 150 g/L. These initial conditions in the must increased the production of ethanol and volatile compounds such as ethyl acetate, 2-butanol and n-propanol, but decreased the amount of acetic acid, during fermentation. The acceptability of the final product was also increased. The commercial application of these initial conditions in the must composition can improve the production of mezcal, since it may increase the proficiency of raw materials, leaving less residual sugar and improve product acceptability.

Keywords: C/N ratio, mezcal composition, volatile metabolites, sensory evaluation.

Resumen

El mezcal es una bebida tradicional destilada que se obtiene por fermentación de los azúcares de agave. En este trabajo se determinaron los efectos de la relación C/N y la concentración inicial de azúcares en el mosto de *Agave duranguensis* sobre la cinética de fermentación, la composición y la evaluación sensorial del mezcal producido. La fermentación del mosto de *A. duranguensis* fue mejorada mediante la adición de un 50% de nitrógeno, respecto al contenido original, como sulfato de amonio, a una concentración inicial de azúcar de 150 g/L. Estas condiciones iniciales en el mosto de fermentación permitieron el aumento de la producción de de etanol y compuestos volátiles, tales como acetato de etilo, 2-butanol y n-propanol, así como la disminución de la cantidad de ácido acético, durante la fermentación. La aceptabilidad del producto final también se incrementó. La aplicación comercial de estas condiciones iniciales en la composición del mosto puede mejorar el proceso de producción de mezcal, ya que puede aumentar el aprovechamiento de las materias primas, dejando menos azúcar residual y mejorar la aceptabilidad del producto.

Palabras clave: relación C/N, composición del mezcal, metabolitos volátiles, evaluación sensorial.

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1 Introduction

Mezcal is a traditional distilled spirit beverage obtained by the fermentation of *Agave duranguensis* sugars and is produced in the state of Durango, Mexico (Soto-García *et al*., 2009). Although Mexican spirits (such as mezcal, tequila and bacanora) have been prepared for hundreds of years, only recently has the production process been studied in detail. Arrizon and Gschaedler (2007) reported that using wine yeasts to ferment agave must with high sugar concentrations (300 g/L) provided low yields, while both agave yeasts and wine yeasts showed similar behavior in grape must. De León-Rodríguez *et al*. (2008) tried to optimize fermentation conditions to produce mezcal from *A. salmiana* and their results showed that it is not possible to obtain a high ethanol concentration and a high production rate simultaneously. Pérez *et al*. (2013) reported that the interaction between temperature and sugars concentration was the most significant factor influencing the ethanol concentration during mezcal production from *Agave cupreata*. Pinal *et al*. (2009) studied the fermentation to produce tequila, and they found that sugar consumption and ethanol production varied as a function of cultivation field and agave age. They also reported that the production of ethyl acetate, 1 propanol, isobutanol, and amyl alcohols were influenced in varying degrees by yeast strain, agave age, and cultivation field. In addition, methanol production was affected only by agave age; and 2 phenylethanol production was influenced only by yeast strain. Soto-García *et al*. (2009) demonstrated that the fermentation of filtered musts at 32°C is a useful means of improving both the quality and throughput of mezcal production.

Agave is cooked to hydrolyze fructans into fermentable sugars (fructose and glucose), which causes nitrogen loss by Maillard reactions, leading to low levels of nitrogen in the agave musts (Pinal *et al*., 2009; Mancilla-Margalli and López, 2002). Since nitrogen content in grape musts affects some aspects of wine elaboration, such as fermentation kinetics and the production of aromatic compounds, ethanol, and glycerol (Albers *et al*., 1996), nitrogen supplementation is a common practice in wine production (Hernandez-Orte *et al*., 2005), but nitrogen supplementation has been also used to produce ethanol for energy (Morales-Martínez *et al*., 2014). Yeasts have a tendency to use ammonium ions as the nitrogen source, so ammonium sulfate is the most suitable nitrogen source due to its easy availability and low price. Arrizon and Gschaedler (2007) determined that fermentation efficiency was increased by almost 30% by increasing the initial concentration of sugar to 170 g/L, when a mixture of amino acids and ammonium sulfate was added during the exponential phase growth. Several studies have analyzed the effect of nitrogen addition to wine fermentation; however, only a few studies have determined the effect of nitrogen addition to agave must to produce Mexican spirits.

Therefore, the aim of this work was to determine the effect of the C/N ratio and the initial concentration of sugars in *A. duranguensis* must on the fermentation kinetics, composition, and sensory evaluation of the mezcal produced.

2 Materials and methods

2.1 Biological material

Saccharomyces cerevisiae strain ITD-00185, which was isolated from the fermentation musts in an artisan mezcal factory in Durango, Mexico, was used to ferment the musts in this work. Identification of the strain was performed by restriction fragment length polymorphisms (Páez-Lerma *et al*., 2013). This strain was selected for its good fermentative capacity (high sugar consumption, high ethanol production and low acetic acid production) and because it showed a neutral killer phenotype. *A. duranguensis* juice was kindly provided by "Productora Mexicana de Mezcal S. de R.L.M.I.", a local company.

2.2 Fermentations

Fermentations were performed in 50 mL tubes containing 40 mL of must during the first experimental stage, in order to determine the best initial must conditions (C/N ratio and the initial concentration of sugars). These fermentations were inoculated with 2×10^7 cells/mL and incubated at 28°C for 72 h. Independent variables were the C/N ratio (44, 55, and 73 g C/g N) and the initial concentration of sugars in the must (90, 120, and 150 g/L), according to a 3^2 factorial experimental design. Experiments were performed in triplicate. Samples were taken every 12 h, filtered, and frozen at -20°C, until analysis. The biomass concentration was measured by direct counting in a Neubauer chamber; while concentrations of glucose, fructose, acetic acid, and ethanol were measured by high performance liquid chromatography (HPLC).

For mezcal production, fermentations were performed in stainless steel cubes using 15 L of must. These fermentations were carried out using artisan must and enhanced must. Artisan must had an initial concentration of sugars of 120 g/L, without nitrogen addition (C/N ratio of 110). The C/N ratio and the initial concentration of sugars in the enhanced must were adjusted to the values obtained during the first experimental stage. These fermentations were inoculated with 2×10^7 cells/mL and incubated at 28°C, in duplicate. Samples were taken every 8 h, filtered, and frozen at -20°C, until analysis. The biomass concentration was measured by direct counting in a Neubauer chamber; while concentrations of glucose, fructose, acetic acid, and ethanol were measured by HPLC. Fermented musts were distilled twice into a stainless steel steel distiller. First, distillation was conducted until an alcoholic concentration of 20% (v/v) was reached, in order to obtain the so-called "agua-vino". This "agua-vino" was distilled again, discarding the initial 1% distillate, because it is rich in methanol. The final distillate reached an ethanol concentration of 45% (v/v). It was analyzed by gas chromatography with flame ionization detector to determine the concentration of major compounds (ethanol, methanol, acetic acid, ethyl acetate, and high alcohols), which are the substances with concentrations greater than 10 mg/L (De León-Rodríguez *et al.*, 2006). The presence of minor compounds in the headspace was detected by solid-phase microextraction combined with gas chromatography mass spectroscopy (SPME-GC/MS).

2.3 Sensory analysis

The members of the tasting panel were chosen through a screening test (selective-discriminative), which determined the sensitivity to low concentrations of the four basic tastes (sweet, sour, salty, and acidic). Samples were evaluated in triplicate by 15 non trained panelists in three different consecutive sessions. Panelists were provided with a set of three mezcal samples (mezcal from an artisan must, mezcal from a nitrogen-enhanced must and a commercial mezcal). Samples were served at room temperature (~25°C) under normal white fluorescent lighting. A nine-point hedonic scale was used in which '1' indicated extreme dislike, '5' indicated neither like nor dislike and '9' indicated like extremely (Stone, 1993; Sancho *et al.*, 2002, Corona-González *et al.*, 2013). The panelists evaluated their preferences on product overall acceptability.

2.4 Nitrogen content quantification

A sample of agave must (10 mL) was dehydrated at 60°C for 6 days to measure the nitrogen content by the micro-Kjeldahl method (AOAC, 1984).

2.5 Chromatographic analysis

High performance liquid chromatography to measure fructose and glucose concentrations was performed as follows. Five microliters were injected into the HPLC. An ionic exchange column (Organic acid H^+[8%], 300 x 7.8 mm, PHENOMENEX Rezex ROA) was employed with a pre-column (organic acid [8%], 50 x 7.8 mm, PHENOMENEX ROA). H_2SO_4 0.005 N at 0.5 mL/min was used as mobile phase. The column and refractive index detector temperatures were 65°C and 35°C, respectively.

Concentrations of the so-called major compounds of the mezcal (ethanol, methanol, ethyl acetate, 2-butanol, n-propanol, 2-methyl-1-propanol, isoamyl alcohol and acetic acid) were quantified by gas chromatography using direct injection of the mezcal samples. An Agilent 6890 N gas chromatograph was used with an HP-Innowax column (30 m length × 0.25 mm id × 0.25 μm thick). Nitrogen (4.8, Praxair) was used as the carrier gas at 1.5 mL/min. Temperatures of the injector and the detector were fixed at 220°C and 250°C, respectively. A split of 10:1 was used in the injector. The oven temperature was programmed as follows: 35°C for 2 min, a gradient of 10°C/min to 210°C, and hold for 1 min (De-León-Rodríguez *et al.*, 2006). Methanol, ethanol, acetic acid, 1-butanol, 2-butanol, n-propanol, 2-methyl-propanol, and isoamyl alcohol from Sigma^{TM} were used as standards.

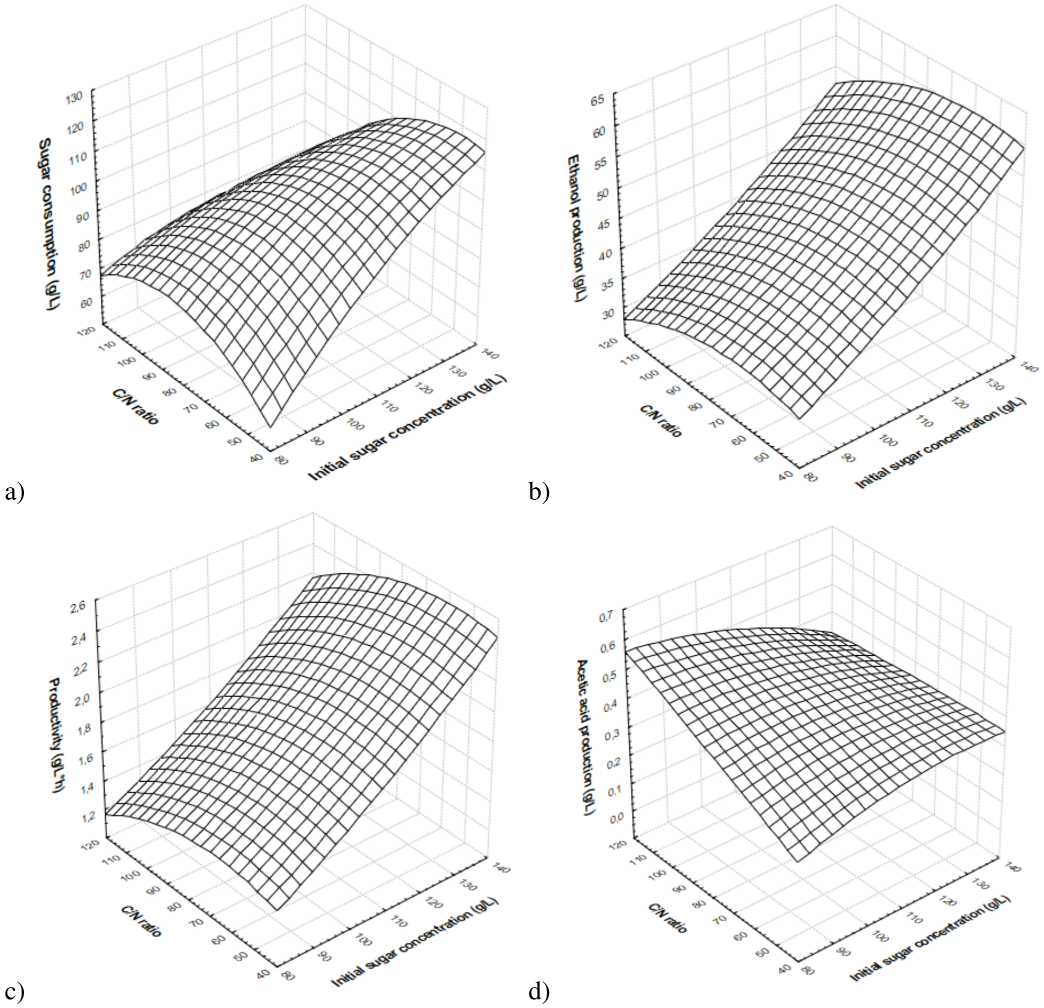
The presence of minor compounds in the mezcal was determined by SPME-GC/MS as follows. The volatile compounds were extracted by headspace SPME (HS SPME) with a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Bellefonte, PA, USA). Analyses were performed on a Hewlett Packard 5890 gas chromatograph in splitless mode with an SPME (0.75 mm id, Supelco) inlet liner. A Hewlett Packard 5972 mass selective detector was used. For compound separation, an HP FFAP column (30 m length × 0.32 mm id × 0.25 μm thick; Agilent Technologies) was used. High purity helium (5.0, Praxair) at a flow rate of 2 mL/min was used as the carrier gas. The injector and detector temperatures were 180°C and 230°C, respectively. The oven temperature was increased from 40ºC to 200ºC using the following program: the initial temperature was maintained for 3 min, a gradient of 3°C/min to 120ºC, hold for 1 min, a gradient of 3°C/min to 200°C, and hold for 5 min. The ionization voltage was 70 eV. The volatile compounds were identified by comparing the mass spectra obtained with those in the NIST database as made previously (Calvo-Gómez *et al*., 2004, Palmerin-Carreño *et al*., 2014).

Data analysis was carried out using *Statistica*® 5.5 by a comparison of averages for the least significant difference (LSD), and $p < 0.05$ was considered to be statistically significant.

3 Results and discussion

3.1 Effect of C/N ratio and initial sugar concentration on the fermentation kinetics

The nitrogen content of the artisan *A. duranguensis* must was 0.49 g N/L, which corresponds to a C/N ratio of 110. Thus, 50, 100, and 150% were added to the original nitrogen content to obtained C/N ratios of 73, 55 and 44 g C/g N, respectively. Figure 1 shows the effect of the C/N ratio and the initial sugar concentration on sugar consumption (1A), ethanol production (1B), productivity (1C) and acetic acid production (1D) on the fermentation of *A. duranguensis* must after 24 h of incubation.



a)

b)

c)

d)

Fig. 1. Effects of the C/N ratio and the initial sugar concentration on the sugar consumption (A), ethanol production (B), productivity (C) and acetic acid production (D) after 24 h of incubation.

Table 1. Concentrations of ethanol (%, v/v), methanol (mg/100 mL of anhydrous ethanol), and other major compounds (mg/L) quantified in the mezcals produced from artisan must (initial sugar concentration of 120 g/L and a C/N ratio of 110) and nitrogen-enhanced must (initial sugar concentration of 150 g/L and a C/N ratio of 73).

RETENTION TIME (MIN)	COMPOUND	MEZCAL FROM ARTISAN MUST	MEZCAL FROM ENHANCED MUST
4.560	Ethanol	$39^a \pm 4$	$41^a \pm 2$
3.697	Methanol	$213^b \pm 27$	$283^a \pm 13$
3.605	Ethyl acetate	$238^b \pm 35$	$857^a \pm 29$
5.409	2-butanol	$81^b \pm 17$	$119^a \pm 1.06$
5.619	n-propanol	$263^b \pm 55$	$359^a \pm 6.84$
6.493	2-methyl-1-propanol	$304^a \pm 36$	$260^a \pm 13.4$
7.372	1-butanol	$9^a \pm 3$	$12^a \pm 2$
8.189	Isoamyl alcohol	$0.97^a \pm 0.10$	$1.15^a \pm 0.06$
11.491	Acetic acid	$452^b \pm 36$	$560^a \pm 50$
	High alcohols**	$657^a \pm 102$	$750^a \pm 21$

**sum of the alcohols with three or more carbons. Means sharing the same letter within a row are not significantly different at confidence level of 95%.

These graphics were done at 24 h because complete or almost complete sugar consumption was observed at this timepoint in all of the fermentation musts with nitrogen addition, while artisan must (without nitrogen addition) showed total sugar consumption at 36 h of incubation (data not shown).

Figure 1 (A) shows that sugar consumption was enhanced at a low C/N ratio and a high initial sugar concentration, while simply increasing the initial sugar concentration did not increase the consumption appreciably. Figures 1 (B) and (C) show that initial sugar concentration is the main factor which enhances ethanol production and productivity, while the C/N ratio had less effect on these parameters. According to Figure 1 (D), acetic acid production was diminished at a low C/N ratio and a high initial sugar concentration, while the C/N ratio had an appreciable effect only at a low initial sugar concentration. Therefore, a C/N ratio of 73 was considered as optimal, since the addition of more nitrogen did not improve the fermentation substantially. Arrizon and Gschaedler (2007) attributed the positive effect of adding ammonium sulfate to the activation of protein synthesis. Our results show that *A. duranguensis* must fermentation, which is done by an artisan method without nitrogen addition (Soto-García *et al*., 2009), should be carried out at an initial sugar concentration of 150 g/L with the addition of ammonium sulfate to reach a C/N ratio of 73. These conditions could lead to an enhanced and more efficient fermentation.

3.2 Effect of C/N ratio and initial sugar concentration on mezcal composition

Mezcals were produced using the artisan must (initial sugar concentration of 120 g/L and a C/N ratio of 110) and a nitrogen-enhanced must (initial sugar concentration of 150 g/L and a C/N ratio of 73). Musts were fermented using a native strain of *S. cerevisiae* in both cases. Nine compounds, which have been reported in mezcal produced from Agave salmiana (De León-Rodríguez *et al*., 2006), were quantified. As shown in Table 1, all the compounds met the specifications of the Mexican Official Norm (Secretaría de Economía, 1978). This norm considers particularly important that the methanol concentration is within the limits prescribed, since larger concentrations of methanol and higher alcohols are considered indicators that mezcal has not been adulterated. The methanol concentrations presented here (Table 1) are slightly higher than those previously reported (De León-Rodríguez *et al*., 2006) for mezcal made from *A. salmiana* (204 ± 18 mg/100 mL of anhydrous alcohol). It is very important that the methanol concentration is not outside the limits permitted by the standard norm, due to toxic effects such as neurological damage and blindness (Cedeño, 1995).

The biosynthesis of higher alcohols is a result of deamination and decarboxylation reactions (Mallouchos and Kanellaki, 2002; Ter Schure *et al*., 1998). In alcoholic beverages, it is common to find n-propanol, 2-butanol, phenylethanol, 2-methyl-1-propanol, and amyl alcohols (a mixture of 2-methyl-1-butanol, 3-methyl-1-butanol, and 1-pentanol), called fusel oil (Ter Schure *et al.*, 1998). In general, higher alcohols enhance the aroma and taste of drinks (Ter Schure *et al.*, 1998; Benn and Peppard, 1996). However, n-propanol is an exception and does not present a desirable aroma (Ter Schure *et al.*, 1998; Legin *et al.*, 2005).

Thirty-two volatile compounds were detected by SPME-GC/MS in the analyzed mezcals (Table 2), most of which have been reported in tequila (Benn and Peppard, 1996; Vallejo-Córdoba *et al.*, 2004) and mescal (De León-Rodríguez *et al.*, 2006).

Table 2. Minor compounds detected by GC/MS in the mezcals produced from artisan must (initial sugar concentration of 120 g /L and a C/N ratio of 110) and enhanced must (initial sugar concentration of 150 g /L and a C/N ratio of 73).

FAMILY	INDEX OF KOVATS*	COMPOUND	FROM ARTISAN MUST	MEZCAL FROM ENHANCED MUST
Esters	600	Ethyl butanoate	1.0	1.74
	680	Ethyl 3-methylbutanoate	7.07 ± 4.14	6.84
	800	Ethyl hexanoate	6.26	8.78
	921	2-hidroxyethyl propionate	28.00 ± 24.74	14.02 ± 5.33
	1000	Ethyl octanoate	103.63 ± 33.63	58.39 ± 39.41
	1100	Ethyl nonanoate	2.80	ND
	1200	Ethyl decanoate	125.41 ± 64.28	48.66 ± 4.36
	1210	3-methylbutyl octanoate	4.65 ± 0.71	ND
	1210	3-methylbutyl dodecanoate	15.16	ND
	1290	2-Phenylethyl acetate	6.72 ± 0.66	2.96 ± 1.08
	1409	3-methylbutyl pentanoate	7.22 ± 3.13	ND
	1600	Ethyl tetradecanoate	2.37	ND
	1693	1-methylethyl hexadecanoate	2.23	ND
Acids	1029	Acetic acid	20.97 ± 10.34	40.99 ± 1.00
	1119	Propanoic acid	ND	1.77 ± 0.37
	1141	Propanoic acid, 2,2-dimethyl	1.25	ND
	1406	Hexanoic acid	1.80 ± 1.03	ND
	1610	Octanoic acid	4.60 ± 078	9.71 ± 5.18
	43.38	Decanoic acid	3.13	ND
Alcohols	601	Propanol	8.11 ± 3.11	10.37 ± 0.39
	668	2-Methyl-1-propanol	41.09 ± 4.93	24.60 ± 4.06
	789	3-Methyl-1-butanol	351.93 ± 28.73	149.04 ± 59.34
	867	2-hydroxi-2-butanol	ND	5.60
	1442	Phenylethyl alcohol	27.25 ± 2.99	7.37 ± 2.34
	1485	2-methyl-phenol	2.54 ± 0.59	3.55 ± 1.11
	1623	2-ethyl-phenol	1.08	ND
	1627	4-methyl-phenol	ND	2.14
Terpenes	747	Limonene	1.59	1.00
	1233	α-Terpineol	4.12	6.04
	1165	4-Terpineol	ND	1.13 ± 0.18
Ketones	754	Cyclopentanone	1.79 ± 0.03	2.76 ± 0.24
	782	Cyclopentanone, 3-methyl	1.26	1.34 ± 0.38

*Kovats index based on short and long ethyl esters

Table 3. Comparison of acceptability of the mezcals produced from artisan must (initial sugar concentration of 120 g/L and a C/N ratio of 110), nitrogen enhance must (initial sugar concentration of 150 g/L and a C/N ratio of 73), and a commercial mezcal.

MEZCAL	ACCEPTABILITY
From enhanced must	$6.250^a \pm 0.42$
From artisan must	$5.455^b \pm 0.13$
Commercial	$5.227^b \pm 0.39$

Means sharing the same letter within a column are not significantly different at confidence level of 95%.

The Kovats index for the detected compounds were very similar to those reported previously in the same column (Martell-Nevárez *et al*., 2011). Esters, acids, alcohols, terpenes and ketones were among the most abundant minor compounds in both mezcals. The presence of these compounds is important because they provide flavor and aroma to the beverage (De León-Rodríguez *et al*., 2006). Terpenes are widespread in natural products that share the common precursor isopentyl pyrophosphate. They are found in extracts of wood resin and as metabolic products and substrates in yeasts (Sjöström, 1993). The odors produced by terpenes vary widely and include floral, citrus, fruity, menthol, spicy, chemical, and pine (Sjöström, 1993). The presence of esters is very favorable, providing a fruity aroma (Benn and Peppard, 1996; Ledauphin *et al*., 2003; Aznar *et al*., 2001). Some of the compounds detected in this work have been reported in the literature as important aroma descriptors. For example, ethyl butanoate (butanoic acid, ethylester) provides a fruity aroma with notes of banana, pineapple, and strawberry; while ethyl hexanoate (Hexanoic acid, ethylester) smells like apple, banana, and strawberry. In addition, ethyl decanoate (decanoic acid, ethylester) gives a sweet aroma (Benn and Peppard, 1996; Ledauphin *et al*., 2003).

Some differences between mezcals shown in Table 2 are the following. The relative abundances of two compounds (acetic acid and octanoic acid) were increased almost twice in mezcal produced from the enhanced must. On the other hand, the relative abundances of six compounds (2-hidroxyethyl propionate, ethyl octanoate, ethyl decanoate, 2-phenylethyl acetate, 2-methyl-1-propanol and 3-methyl-1-butanol) decreased by half in the mezcal produced from the enhanced must. Also, some compounds are present in the mezcal from enhanced must, but not in the mezcal from artisan mus, and vice versa. The specific impact of each of these differences over the sensory properties of the product is difficult to predict. However, sensory evaluation allows a qualitative assessment to determine if changes were favorable to the acceptability of the beverage.

3.3 Sensory evaluation

It was conducted to compare the mezcal produced from the artisan must, mezcal produced from the enhanced must, and a commercial mezcal supplied by a local company. Preference analysis showed differences between the mezcal produced in the laboratory and the commercial mezcal. As shown in Table 3, the mezcal produced from nitrogen-enhanced must showed the highest value of acceptability ($p > 0.95$) with an average of 6.250, while mezcal made from the control must had a mean of 5.455 and the commercial mezcal had a mean of 5.227. This means that sensory properties of the mescal were enhanced by improving the initial C/N ratio of the agave juice.

Conclusions

Collectively, our results demonstrate the importance of the initial must composition on the fermentation process, volatiles compounds (aroma) and sensory quality. The fermentation of *A. duranguensis* must was enhanced by the addition of nitrogen (as ammonium sulfate) to reach a C/N ratio of 73 at an initial sugar concentration of 150 g/L. These initial conditions in the must allowed an increased production of ethanol and other volatile compounds, as well as better acceptability of the final product. A potential commercial application of these initial conditions of the must composition may help improving the production of mezcal in the state of Durango as well as other mezcal producing states, since it would increase the proficiency of raw materials, leaving less residual sugar, and improve product acceptability.

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