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Full Length Research Paper

Chemical composition and volatile compounds in the artisanal fermentation of mezcal in Oaxaca, Mexico

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The differences among the several varieties of mezcal produced in Mexico, besides the *Agave* species, consist essentially in the individuality of the traditional methods used in the elaboration or fermentation process. Therefore, it is necessary to understand the artisanal fermentation processes and to make clear the factors and management practices that have an influence on the chemical composition. Two mezcal artisanal factories of *Agave angustifolia* Haw. under two fermentation seasons were investigated in Oaxaca, Mexico. Volatile compounds were analyzed using a gas chromatograph and a capillary column HP-FFAP. Reference standards were used for the identification and quantification of volatile compounds. Samples were successfully separated, and the main volatile compounds identified corresponded to ethyl acetate, ethanol, methanol, 3-methyl-1-butanol, propanol, 2-methyl-1-propanol, and acetic acid. The chemical composition of the artisanal fermentation process differs significantly from factory to factory, season to season, and the interaction between the factories in which the fermentation process takes place and the seasons. Addition of ammonium sulfate reduces the fermentation time and affects significantly the production of ethanol, propanol and butanol, but decreases the methanol, ethyl acetate, and acetic acid production, and this practice is more convenient in fall than in spring.

Key words: Fermentation, ammonium sulfate, volatile compounds, higher alcohol, gas chromatography, mezcal.

INTRODUCTION

Mezcal is an artisan distilled alcoholic beverage produced from agave (*Agave angustifolia* Haw.) in Oaxaca and other regions of Mexico. The production process involves four stages: cooking the agave stem (leafless flowering axis), mashing the cooked stem, fermenting the mashed stem, and distilling the fermented stem. The process starts when the stems are cooked inside rustic ovens dug in the ground. Then, fructans contained in the stem are hydrolyzed into glucose and fructose (Mancilla-Margalli and López, 2006). After, the cooled cooked stems are mashed in stone mills driven by horses or donkeys, then the mashed product is placed in a wooden fermentation tank, where the producer adds water. This mixture is called "must", and a spontaneous fermentation begins due to the natural microbiota present in the must or in the environment. In general, the fermentation process lasts from five to nine days. Among the factors that have an influence on the artisanal fermentation process of mezcal are the agave species, initial sugar concentration, environmental conditions, and in certain cases the addition of ammonium sulfate (Vera-Guzmán et al., 2009). When the spontaneous fermentation process ends, the fermented product is transferred to copper distillers to produce the distilled mezcal.

During the fermentation process, yeasts produces ethanol and carbon dioxide which promote the synthesis of alcohols, esters, organic acid, and such compounds

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determine the flavor and aroma of the alcoholic beverages (Cedeño, 1995; Santillán and García, 1998; Díaz-Montaño et al., 2008). The pathways of synthesis, type and concentration of these compounds also depend on the microorganisms present during the fermentation process (Escalante-Minakata et al., 2008), the chemical composition of the raw materials, ratio of C/N and the environmental conditions (Pinal et al., 1997; Santillán and García, 1998). Nitrogen concentration and composition in the must determines a higher alcohol formation (Pinal et al., 1997; Arrizon and Gschaedler, 2007). Ough and Bell (1980) mentioned that in wine production, the total nitrogen is correlated negatively with higher alcohol levels. On the other hand, in the cider production, Vidrich and Hribar (1999) found a greater concentration of isobutanol and isoamyl alcohol present in low concentrations of nitrogen in the raw materials. In general, it is accepted that the sugar concentration and the addition of nitrogen in the must are the factors that modify fermentation time, ethanol yield, and volatile compounds production (Pinal et al., 1997; Arrizon and Gschaedler, 2002; Arrizon and Gschaedler, 2007).

The effect of the addition of ammonium sulfate to the natural fermentation process in artisanal mezcal production has not been studied. Normally, the mezcal producers do not have control of the natural fermentation process, and therefore they produce mezcal with a quality control that varies not only from production lot to production lot, but also between factories and communities. Based on this information, we hypothesized that the natural fermentation conditions, season and artisanal factories location have an influence on the mezcal production in relation to the compounds and characteristics of the distilled product. The aim of this study was to evaluate the profiles of must compounds during the spontaneous fermentation processes, taking as references the fermentation procedures at two mezcal artisanal factories and two fermentation seasons, and the effect of the addition of ammonium sulfate on fermentation products, all this in two communities of Oaxaca, Mexico, located in the region of the original source of mezcal, as denominated by the Mexican Ministry of Commerce and Industry Regulation, in 1994.

MATERIALS AND METHODS

The agave plant cores ('piñas' in Spanish) used in this study was harvested at *A. angustifolia* Haw. plantations from the communities of Matatlan (16° 52' 30'' N, 96° 23' 44'' W, 1740 masl) and Tlacolula (16° 58' 42'' N, 96° 32' 11'' W, 166 masl) Oaxaca, Mexico. Both communities present a climate temperate sub-humid, with summer rainfall, and they are inside the region of the original source of mezcal as denominated by the Mexican Ministry of Commerce and Industry Regulation, in 1994. In the same communities, the mezcal artisanal factories ('palenques' in Spanish) are also located, and they were used as study subjects for this work, so the artisanal factory names correspond to community names.

In the artisan factories of Tlacolula and Matatlan, agave plant cores ('piñas') were cooked in rustic ovens (earth ovens) for 72 h. After that, cooked agave was mashed in a horse-driven stone mill, and bagasse juice was transferred to wooden tanks, and then water is added to start the fermentation process. In both artisan factories, the fermentations are carried out without the addition of inoculum just with native microflora disseminated in the environment as a spontaneous process, and also without temperature control (Lappe-Oliveras et al., 2008; Verdugo-Valdez et al., 2011). For this work, all artisanal mezcal fermentations were carried out as commonly as is performed by traditional factories. Later, multiple samples were taken during the fermentation processes where each sample of fermented product (1 L) was filtered using a No. 4 Whatman filter paper and then stored at -75°C until its analysis. In order to obtain an estimation of the microbial populations in the spontaneous fermentation, a yeast count was performed using a Neubauer's counting chamber in the fermentations carried out at Tlacolula and Matatlan factories at spring as well as fall. Viability was determined by staining of the cells with methylene blue reagent, and the results are reported as averages of viable cells per ml to each fermentation process.

Mezcal fermentation and sampling at artisanal factory of Tlacolula

The mezcal producer at Tlacolula put the juice and bagasse from 508 kg of cooked and mashed agave in a wooden tank and it remained there during 48 h. After, 600 L of water was added, and one homogenization was made, and the mixture remained there for 120 h until the spontaneous fermentation finished. Six samples were taken from the initial stage at every 24 h until the fermentation process had finished. Two spontaneous fermentation processes were performed and sampled, one in spring and another in fall.

Mezcal fermentation and sampling at artisanal factory of Matatlan

At the factory of Matatlan, the mezcal producer added the juice and bagasse from 900 kg of cooked and mashed agave plus 228 L of water in a wooden tank, and the mixture remained there during 36 h. After, 589 L of water was added and one homogenization was made. In this factory, the fermentation finished 192 h later. Nine samples were taken from the homogenized mixture at the initial stage and every 24 h until the fermentation process was finished. Similar fermentation procedures were carried out and sampled in spring, as well as in the fall season.

Spontaneous fermentation with supplementation of ammonium sulfate

In this case, the juice and bagasse from 784 kg of cooked and mashed agave were mixed with 142 L of a solution of ammonium sulfate 1.4% (14 g/L) in a wooden tank, and then the mixture was left there for its spontaneous fermentation during 36 h. Later, 570 L of water was added and again the mixture was homogenized and remained there until the end of the fermentation process (42 h). In this fermentation, five samples were taken from the initial phase and every 12 h, until the fermentation process was finished. Four fermentation procedures were performed to complete the treatments of ammonium sulfate in addition, with and without the supplementation of ammonium sulfate during spring, and other similar treatments were carried out in the fall.

Determination of the sugars and nitrogen in the musts

The method reported by Dubois et al. (1956) was used to measure the total sugars. Reducing sugars were determined by Nelson's method (Nelson, 1944). Ammonium nitrogen was calculated using the phenol salt method (Clesceri, 1992) and total nitrogen by Kjeldahl´s method (955.04) from (AOAC, 1990). Each analysis was performed with three replicates.

Gas chromatography (GC) analysis

100 ml were distilled from the must sample into an ice-cooled flask to avoid loss. 2 μl of sample were injected into a chromatograph (Perkin Elmer, model Autosystem XL) equipped with a flame ionization detector (FID) and a capillary column HP-FFAP (30 m × 0.25 mm d.i., 0.25 μ film thickness, Agilent). Operating conditions were as follows: direct injections were performed in splitless mode at 180°C, helium was used as the carrier gas at 1.3 ml/min and FID was operated at 230°C. The column temperature started at 40°C for 3 min, then increased to 200°C at the rate of 6°C/min and held for 20 min (Vidrih and Hribar, 1999; López and Guevara, 2001). Compounds quantification was based on the external standard method by using diluted solutions ranging from 1 to 1250 mg/L of esters, alcohols and acetic acid quantification. Standard solutions of ethyl acetate, methanol, propanol, 2-methyl-propanol, 3-methyl-butanol, pentanol, butanol, 2-butanol, and acetic acid were obtained from Sigma-Aldrich and Fluka. Calibration curves reported a coefficient of determination (r^2) ≥ 0.99 for each compound. Sample analysis was performed by injecting each sample three times (replicates), so that a minimum of 15 data points were obtained for each fermentation condition and used for statistical analysis.

Statistical analysis

Data of compounds evaluated during the artisanal mezcal fermentations were analyzed by an analysis of variance (ANOVA) using a bifactorial lineal model; as factor A, the fermentation place (Tlacolula and Matatlan artisanal factories); factor B, season of fermentation (spring or fall), and the interaction A*B, combinations of the levels from A and B factors. A second one-way ANOVA was performed to test the effect of the supplementation of ammonium sulfate on compounds synthesis during fermentation. ANOVA mean squares were reported to indicate the high variation of the fermentations evaluated. A Tukey's multiple comparison test ($P \leq 0.05$) was carried out when differences were found in the sources of variation. All data analysis was done using SAS software (SAS, 1999).

RESULTS AND DISCUSSION

Initial characteristics of the agave musts

Initial chemical composition and microbiological population of the musts are indicated in Table 1. The initial composition of the musts was variable in sugars and nitrogen between artisanal factories and seasons of fermentation. The values indicate that agave plantations vary from field to field crop, and also from season to season of each harvest due to sugar concentration in the agave plants which can be affected by dry seasons. Also, the values suggest a high variation in the artisanal fermentation processes. Mancilla-Margalli and López (2006) reported a high variation in carbohydrate production by different agave species, and they proposed that carbohydrate content has high environmental influence. Such variation in sugars content in the agave musts can be influenced by the spontaneous fermentation. In addition, it is important to note that, each mezcal producer has different selection criteria for the age of the plant to be used, since no guidelines exist because of the inherent variability of such a traditional crop. Likewise, agave cooking is not homogenous because the ovens are rustic without temperature control, and probably a caramelization process and/or burning of the strains occurs. In all fermentation processes, the initial pHs varied from 4.2 to 4.5, such values did not differ from that reported (4.7) by Soto-García et al. (2009) for the fermentation of mezcal from Durango.

With regards to the microbial population, the initial numbers of yeast were similar in the four musts (1.5 to 5.0×10^7 cell/ml), being slightly higher in the fall season in Tlacolula (Table 1). These yeast averages were similar to reported values for fermentation processes of tequila under controlled conditions (2×10^7 cell/ml) and lower than artisanal tequila processes (6×10^7 to 15×10^7 cell/l by Arrizon and Gschaedler (2002, 2007). So the measured quantities of yeasts in the spontaneous fermentation are within normal range. The kinetic profiles of reducing sugar and ethanol during musts fermentation at Tlacolula and Matatlan is presented in the Figure 1. As usual, in any fermentation process, the increase of ethanol is related to decreasing of the sugar concentration throughout the fermentation time, but the alcohol yield depends on the sugar content in the must at the initial phase. Also, the yield of the final product (mezcal) has a direct relationship with the total nitrogen and C/N ratio (Arrizon and Gschaedler, 2002), a substrate of the yeasts. In this work, the productivity values of the musts varied from 0.32 to 0.48, lower than the values reported by Soto-García et al. (2009) for mezcal from Durango, Mexico. The fermentation time at Matatlan was longer (228 h) than at Tlacolula (168 h), and it was defined by a mezcal producer (Figure 1) and as the main objective was to evaluate the artisanal fermentation, we did not interfere with the process. Nevertheless, these differences can be affected by the chemical composition of the final product and confer additional variations to the fermentation processes evaluated (Arrizon and Gschaedler, 2002).

Variations in the volatile compounds production

Significant differences were determined among the fermentation processes of reducing sugar, total sugar, total nitrogen, ammonium nitrogen, ethanol, ethyl acetate, methanol, propanol, 2-methyl-propanol, 3-methyl-butanol and acetic acid by ANOVA to the sources of variation of place (artisanal factories), fermentation season, and interaction place-season. The exception was the butanol content which only showed significant differences

Table 1. Composition of *A. angustifolia* musts from Tlacolula and Matatlan at initial phase of the fermentations.

Compound	Tlacolula		Matatlan	
	Fall	Spring	Fall	Spring
Reducing sugar (g/L)	217.9±2	230.4±2	174.4±2	294.3±2
Total sugar (g/L)	228.0±2	250.2±2	270.1±2	308.0±2
Total nitrogen (mg/L)	173.8±7	209.2±1	127.2±10	142.2±2
Ammonium nitrogen (mg/L)	0.59±0.02	0.60±0.17	0.25±0.05	0.21±0.02
C/N ratio value	1.2	1.1	1.4	2.0
pH	4.5	4.2	4.2	4.3
Temperature	25.4	25.3	19.0	23.2
Yeast (cell/ml)	5×10^7	1.6×10^7	3×10^7	1.5×10^7

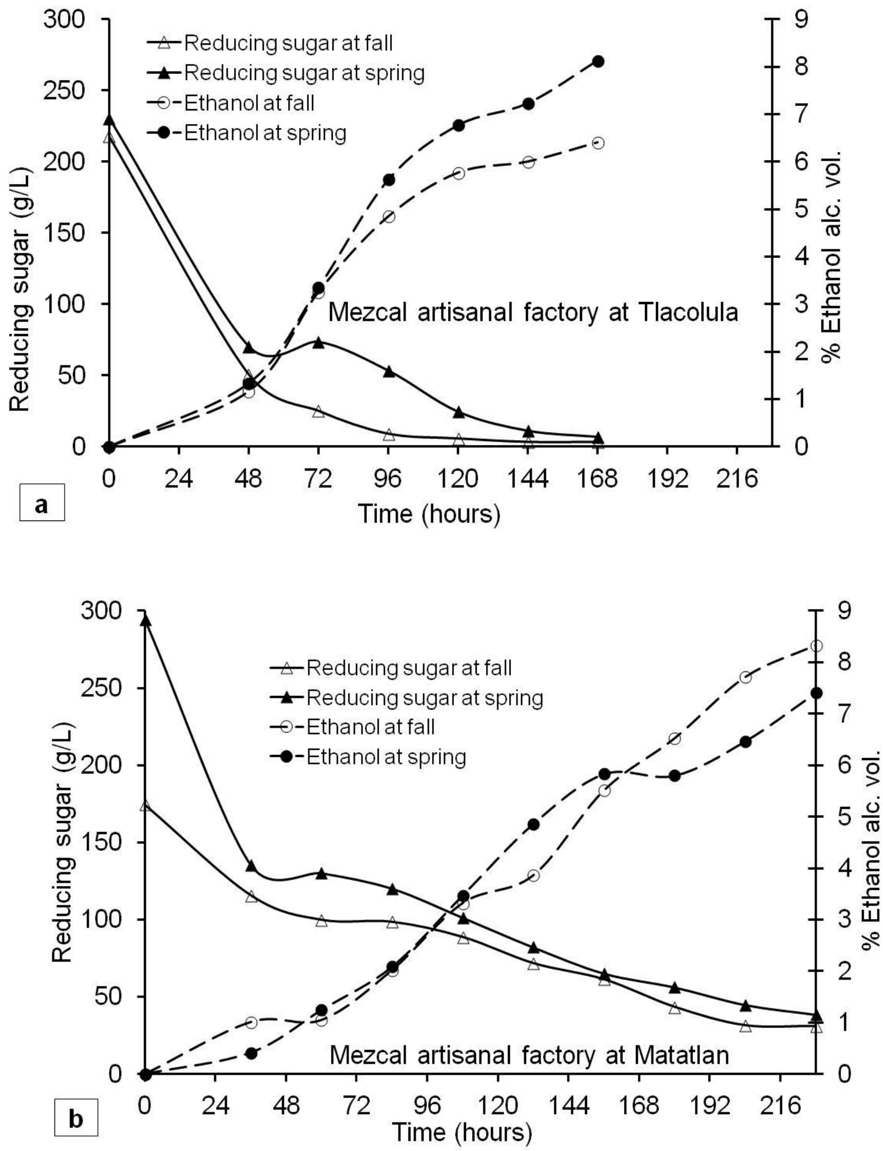

Figure 1. Kinetic profiles of reducing sugar and ethanol during the fermentation of *A. angustifolia* musts at two mezcal artisanal factories and during two fermentation seasons.

Table 2. Mean squares of the ANOVAs of 12 compounds evaluated during the fermentation process of mezcal at Matatlan and Tlacolula, Mexico.

Compound	Mean square			CV (%)
	Place (P)	Season (S)	Interaction P-S	
Reducing sugars	55701.8**	7608.0**	458.8**	7.6
Total sugars	57907.4**	260.3**	3460.2**	24.7
Total nitrogen	81658.5**	33839.5**	31518.1**	3.8
Ammonium nitrogen	2.7**	2.0**	1.4**	17.8
Ethanol	10.8**	2.1**	5.6**	12.1
Ethyl acetate	8349.0*	9682.7**	9793.4**	12.8
Methanol	524.7**	4803.9**	8626.6**	3.5
Propanol	178.8**	25.1**	6.7**	9.8
2-Methyl-propanol	59.7**	64.1**	554.1**	3.1
3-Methyl-butanol	467.9**	245.6**	4383.5**	3.6
Butanol	0.05[NS]	0.20*	0.04[NS]	16.3
Acetic acid	1162005.3**	964113.1**	1692363.5**	3.8

[NS]Not significant at P > 0.05; *Significant at P ≤ 0.05; **significant at P ≤ 0.01; CV, coefficient of variation.

Table 3. Means comparison of the evaluated compounds in the spontaneous fermentations at mezcal artisanal factories from Matatlan and Tlacolula, Mexico.

Compound	Artisanal factory		Fermentation season		Interactions factories-season			
					Matatlan		Tlacolula	
	Matatlan	Tlacolula	Spring	Fall	Spring	Fall	Spring	Fall
Direct reducing sugars (g/L)	79.0[a†]	28.2[b]	67.6[a]	49.7[b]	86.1[a]	71.9[b]	39.9[c]	16.5[d]
Total reducing sugars (g/L)	94.1[a]	42.3[b]	74.0[a]	72.7[a]	89.6[a]	98.5[a]	50.5[b]	34.1[c]
Total nitrogen (mg/L)	115.2[b]	181.0[a]	159.2[a]	122.8[b]	116.6[c]	113.7[c]	220.8[a]	136.3[b]
Ammonium nitrogen (mg/L)	0.44[b]	0.81[a]	0.45[b]	0.72[a]	0.41[c]	0.47c[b]	0.51[b]	1.11[a]
Ethanol (% v/v)	4.2[b]	5.0[a]	4.6[a]	4.4[a]	4.1[c]	4.4[c]	5.4[a]	4.6b[c]
Ethyl acetate (mg/L)	14.8[b]	34.5[a]	14.0[b]	31.3[a]	14.6[b]	15.0[b]	13.0[b]	55.9[a]
Methanol (mg/L)	100.1[a]	94.9[b]	107.1[a]	88.7[b]	117.1[a]	82.4[d]	92.1[c]	97.7[b]
Propanol (mg/L)	5.9[b]	8.8[a]	6.5[b]	7.7[a]	5.1[c]	6.7[b]	8.6[a]	9.1[a]
2-Methyl-propanol (mg/L)	27.7[a]	26.2[b]	25.9[b]	28.4[a]	24.5[c]	31.1[a]	27.9[b]	24.4[c]
3-Methyl-butanol (mg/L)	81.8[b]	86.4[a]	84.1[a]	83.2[a]	76.5[c]	87.1[b]	95.4[a]	77.5[c]
Butanol (mg/L)	0.07[a]	0.12[a]	0.14[a]	0.04[b]	0.14[a]	ND	0.14[a]	0.09[a]
Acetic acid (mg/L)	404.6[b]	650.5[a]	420.7[b]	580.1[a]	436.4[b]	372.7[c]	395.7[c]	891.1[a]

[†]In row, means within the same letter are not differ significantly (Tukey, P ≤ 0.05). ND, Not detected.

between seasons (Table 2). Total sugar values presented the highest coefficient of variation (24.7%), indicating a high variability among samples evaluated. The comparisons of volatile compounds between artisanal factories indicate that the Tlacolula factory presented the higher productivity in total nitrogen, ammonium nitrogen, ethanol, ethyl acetate, propanol, 3-methyl-butanol and acetic acid, but reducing sugars, methanol and 2-methyl-propanol were significantly higher in Matatlan (Table 3). Both factories satisfy the levels permitted by the Mexican Ministry of Commerce and Industry Regulation (1994) for ethyl acetate, propanol, 2-methyl-propanol, 3-methyl-butanol, butanol, and acetic acid. The difference between Tlacolula and Matatlan artisanal factories are related to the differences in their fermentation processes. A similar differential pattern was observed between spring and fall seasons, the higher concentrations of ammonium nitrogen, ethyl acetate, propanol, 2-methyl-propanol, and acetic acid content was determined in fall fermentations, while reducing sugars, total nitrogen, methanol and butanol values were higher in the spring. For combined effect of artisanal factory and seasons of fermentation, the results showed that the fermentation carried out in the spring, in general, presented higher values of volatile

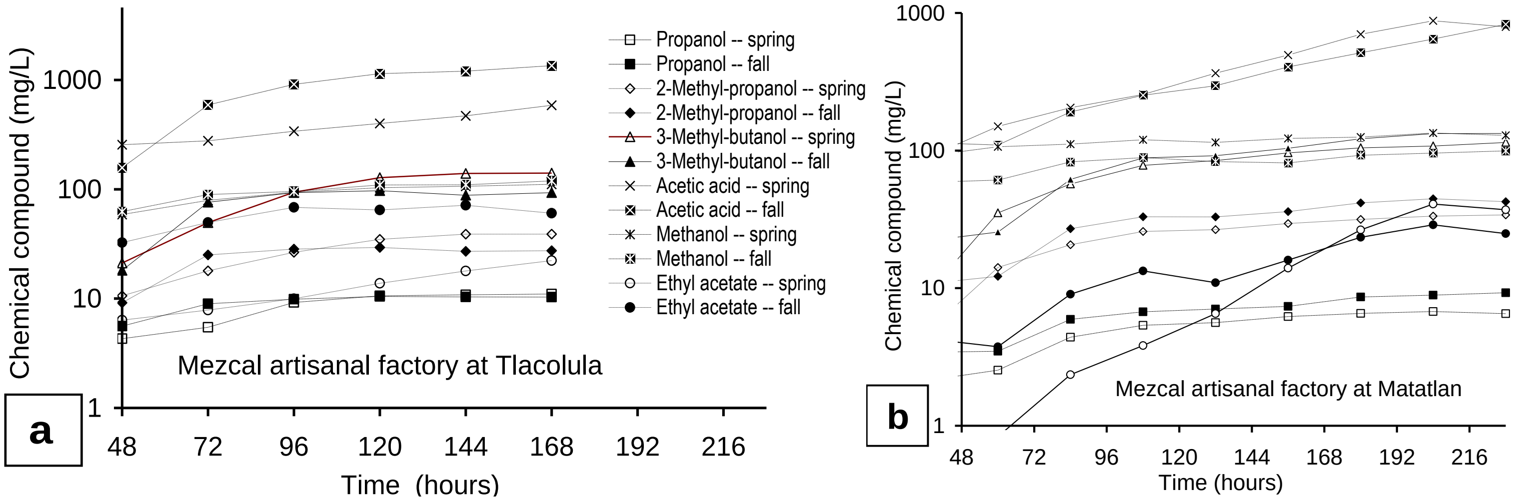

Figure 2. Kinetic profiles of chemical compounds on fermentation of *A. angustifolia* musts at two mezcal artisanal factories.

compounds than in the fall. Such behavior was determined, in part, by the higher temperatures (around 30°C) in the spring than in the fall (less than 20°C), Table 3. For example, in ethanol, methanol, total nitrogen, reducing sugars, propanol and 3-methyl-butanol, the results confirm a high variability in the composition of musts during mezcal fermentation due to the high variability of the fermentation conditions.

Ethyl acetate production is commonly regulated by the type and the density of microorganisms present during fermentation as well as the nitrogen content. Díaz-Montaño et al. (2008) found that fermentation of *Agave tequilana* by *Kloeckera africana* and *Kloeckera apiculata* produced ethyl acetate more abundantly than *Saccharomyces cerevisiae*. However, a direct relation was not found between the initial content of nitrogen and yeasts with average of acetyl acetate. Methanol differences depend on many factors. For example, non-uniform cooking induces differential de-methylation of agave pectin because there was no temperature or pH control (Téllez, 1998). Also, the yeast species and abundance influence the methanol content in the must fermentations. Tequila musts fermentation, pectinesterases are released by *Saccharomyces* (Gainvors et al., 1994; Arrizon and Gschaedler, 2007). Pectinesterases catalyze hydrolysis of methyl ester bonds in pectin, producing pectic acid and methanol (Singh et al., 2005). The observed differences in higher alcohols such as propanol, 2-methyl-propanol, 3-methyl-butanol, and n-butanol, are difficult to separate because there are no studies on microorganism present during fermentation of *A. angustifolia* musts. Mezcal producers perform a spontaneous fermentation with just yeasts present around factory and wooden tanks of fermentation. Nevertheless, this type of mezcal production can lead to health hazards because during the fermentation process, undesirable microorganisms could be present and also these conditions are adequate for the growth of wild yeast that favor the fermentation of agave musts (Díaz-Montaño et al., 2008; Escalante-Minakata et al., 2008). Concentrations of n-butanol lower than 1 mg/L were found probably because musts can be contaminated with *Clostridium* bacteria or *Kloeckera* spp. yeast (Díaz-Montaño et al., 2008).

The differences in acetic acid concentration showed that agave cooking and fermentation at each traditional factory influences the synthesis of this compound. The artisanal process of the fermentation favors the inoculation of acetic acid bacteria (Delfini and Formica, 2001). Aside from the artisan process, age, environment, and factories, the characteristic and conditions of growth of the agave plant also determine the chemical composition of the musts and the final product, mezcal. Figure 2 shows the evolution of the average values of propanol, 2 methyl-propanol, 3-methyl-butanol, acetic acid, methanol

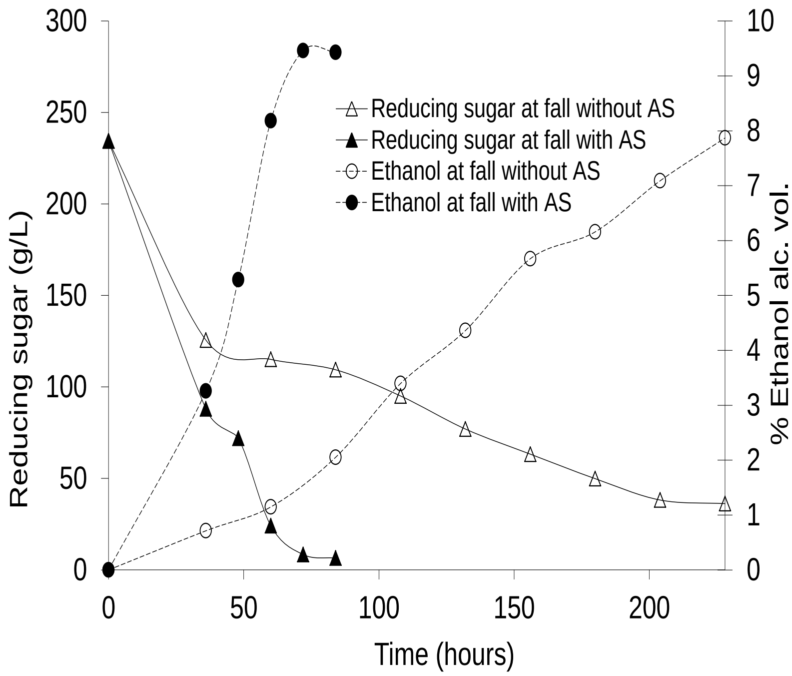

Figure 3. Kinetic profiles of the *A. angustifolia* musts fermentation on the base of reducing sugar and ethanol production with and without ammonium sulfate (AS) addition.

and ethyl acetate, which increased throughout the time of fermentation. Based on all of the results presented, we confirm that each traditional mezcal factory produces unique liquors with different tastes for a wide variety of consumers. While the Mexican Ministry of Commerce and Industry Regulations (1994) tries to regulate mezcal production, consumers seek a wider variety of tastes and favors instead of uniformity. The quality of the final product is influenced during the fermentation process by strain, origin and composition of the agave plants, agave cooking, musts pH, ratio carbon/nitrogen, and species and the abundance of microorganisms.

Effect of the ammonium sulfate supplementation on fermentation

At initial phase of the experiment related with ammonium sulfate (AS) addition, the treatment with AS presented 1.7 $\times 10^7$ cell/ml of native yeasts, and it exhibited higher reducing sugar consumption than treatment without AS with 1.5 $\times 10^7$ cell/ml of yeasts. It means that ammonium sulfate addition acts on sugars during fermentation; the ammonium ion activates sugar transport as well as protein synthesis, such as an alosteric activator to phosphofructokinase in glycolysis (Albers et al., 1996; Alexandre and Charpentier, 1998). This event increases sugar consumption and decreases the fermentation time, in our experiment the reduction was drastic from 228 to 78 h (Figure 3). More ethanol was produced with ammonium sulfate addition (9.74% alcohol v/v) than without ammonium sulfate addition (7.41% alcohol v/v) (Figure 3). The productivity values of the agave musts were of 1.12 and 0.35 with and without ammonium sulfate, respectively. These values are explained in part, by the differences in the initial content of reducing sugar and the total nitrogen in the musts. Also, the addition of ammonium sulfate promotes the growth of a yeast population as a consequence of the availability of carbon and nitrogen in the fermentation process (Thomas and Ingledew, 1990; Arrizon and Gschaedler, 2002).

In the ANOVAs, significant differences were determined ($P \leq 0.01$) between the treatments of AS, fermentation seasons and interaction seasons AS addition to all compounds evaluated (Table 4). The coefficients of variation (CV) of ammonium nitrogen and reducing sugars determined were 23.0 and 24.2%, respectively. The concentration of volatile compounds differ from season to season of fermentation but without a clear pattern; for example, in spring fermentations, the sugar content of total nitrogen, methanol and acetic acid were higher than in fall, but the other compounds presented major values in this season; again it seems that non-controlled fermentation influence the musts composition (Table 5). Such a pattern was also observed with AS supplementation but in this case the AS enhanced the

Table 4. Mean squares of the ANOVAs of 12 compounds evaluated during the fermentation processes with treatments of ammonium sulfate addition.

Compound	Mean square			CV (%)
	Season (S)	Ammonium sulfate addition (AS)	Interaction S-AS	
Direct reducing sugars	15020.9**	28691.9**	3881.7**	7.8
Total reducing sugars	7807.7**	16165.7**	17308.9**	24.2
Total nitrogen	27199.5*	656887.5**	25445.4**	3.2
Ammonium nitrogen	11743.5**	1531476.3**	12379.2**	23.0
Ethanol	23.3**	153.7**	16.7**	10.5
Ethyl acetate	738.4**	139.4**	717.1**	11.3
Methanol	6345.9**	112.9**	5244.0**	3.1
Propanol	828.9**	10824.78**	499.8**	4.8
2-Methyl-propanol	477.8**	780.0**	55.6**	3.9
3-Methyl-butanol	3353.0**	0.68**	148.4**	4.6
n-Butanol	0.84**	11.30**	2.49**	68.9
Acetic acid	1392.7**	1687750.9**	61409.3**	5.8

**Significant differences between treatments at P ≤ 0.1; CV, Coefficient of variation.

Table 5. Means comparison of the compounds evaluated during the fermentation of *A. angustifolia* musts with ammonium sulfate (AS) supplementation at two seasons.

Compound	Season		Treatment		Interaction season-AS			
					Spring		Fall	
	Spring	Fall	No AS	With AS	No AS	With AS	No AS	With AS
Direct reducing sugars (g/L)	75.8[a†]	52.7[b]	79.0[a]	36.5[b]	86.1[a]	56.0[c]	71.9[b]	18.2[d]
Total reducing sugars (g/L)	90.0[a]	77.5[b]	94.1[a]	65.1[b]	89.6[a]	90.7[a]	98.5[a]	39.6[b]
Total nitrogen (mg/L)	199.0[a]	172.1[b]	115.2[b]	303.6[a]	116.6[c]	341.8[a]	113.7[c]	265.4[b]
Ammonium nitrogen (mg/L)	92.0[b]	110.2[a]	0.44[b]	282.2[a]	0.41[c]	256.9[b]	0.47[c]	307.6[a]
Ethanol (% v/v)	4.8[b]	5.7[a]	4.2[b]	7.1[a]	4.1[c]	6.1[b]	4.4[c]	8.1[a]
Ethyl acetate (mg/L)	11.8[b]	16.1[a]	14.8[a]	12.3[b]	14.6[b]	5.3[c]	15.0[b]	18.3[a]
Methanol (mg/L)	110.2[a]	87.4[b]	100.1[a]	97.3[b]	117.1[a]	97.8[b]	82.4[c]	96.8[b]
Propanol (mg/L)	11.9[b]	16.9[a]	5.9[b]	29.8[a]	5.1[d]	24.1[b]	6.7[c]	35.9[a]
2-Methyl-propanol (mg/L)	22.8[b]	28.2[a]	27.1[a]	21.2[b]	24.5[b]	19.7[d]	31.1[a]	22.8[c]
3-Methyl-butanol (mg/L)	75.6[b]	87.8[a]	81.8[a]	81.3[a]	76.5[b]	73.9[b]	87.1[a]	89.2[a]
Butanol (mg/L)	0.28[b]	0.40[a]	0.07[b]	0.83[a]	0.14[c]	0.54[b]	ND	1.12[a]
Acetic acid (mg/L)	308.5[a]	288.5[b]	404.6[a]	101.4[b]	436.4[a]	78.3[d]	372.7[b]	126.1[c]

[†]In row, means with the same letter, between seasons, sulfate treatments and within interactions seasons-AS treatment, are not statistically different (Tukey, P≤ 0.05). ND, Not detected.

values of nitrogen, ethanol, propanol and butanol in musts during the fermentation process. However it is important to emphasize that in the fall fermentation, there was an increase in the ammonium nitrogen, ethanol, propanol, 3-methyl-butanol, butanol and ethyl acetate content detected with AS supplement-ation. Then when a mezcal producer decides to add AS, the best responses are obtained in the fall fermentations.

A kinetic profile of volatile compounds with and without ammonium sulfate supplementation is presented in the Figure 4. The effect of adding of nitrogen results in the increase in propanol concentration and a reduction in the concentration of acetic acid can be observed. All of the results indicate that the addition of ammonium sulfate to fermentation tanks in fall can produce a unique kind of mezcal, significantly different from the produced one in spring with and without the addition of ammonium sulfate. Our results coincide with several reports on the addition of ammonium sulfate to the fermentation of *A. tequilana* musts. Arrizon and Gschaedler (2007) reported high

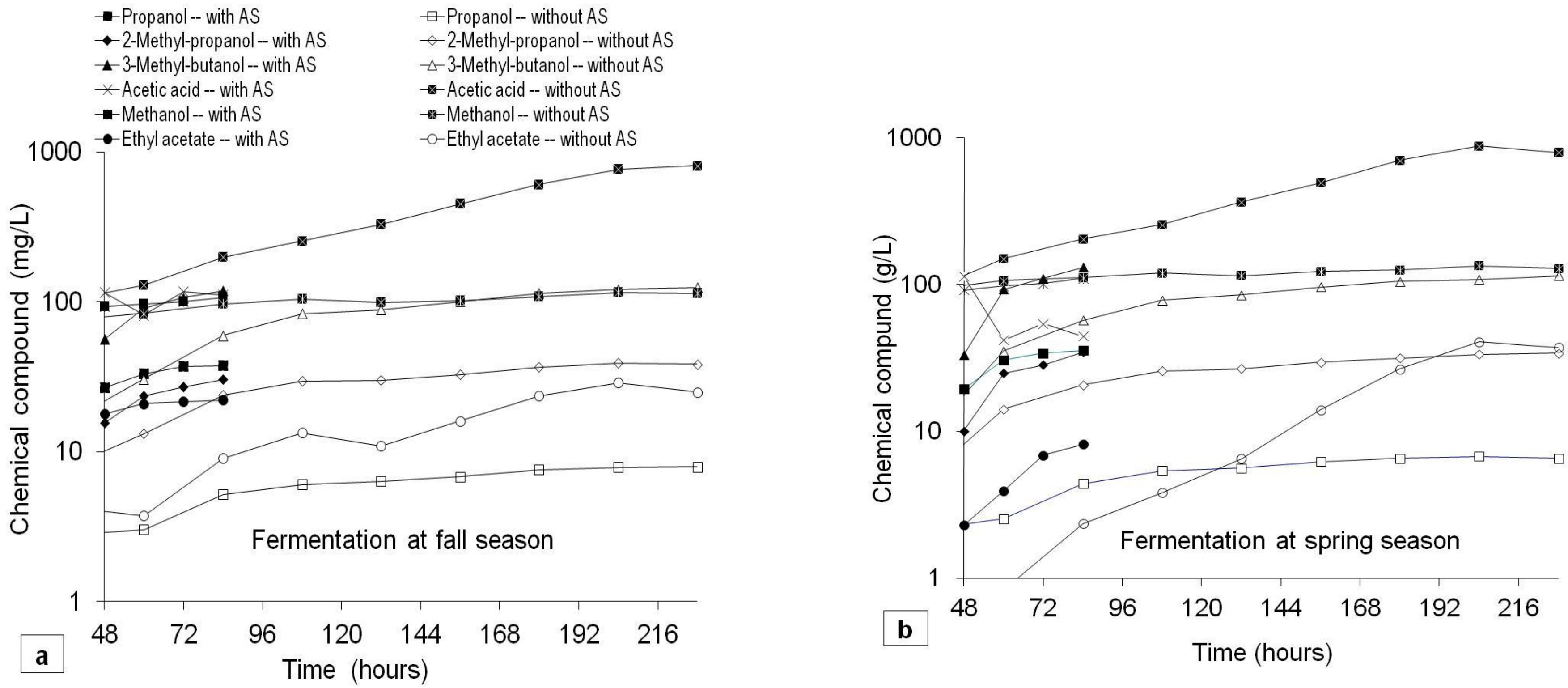

Figure 4. Evolution of chemical compounds during fermentation of *A. angustifolia* musts with and without addition of ammonium sulfate (AS).

propanol production by adding ammonium sulfate, while Berry and Watson (1987) reported a similar tendency on the production of this compound accompanied by higher total nitrogen concentrations.

Conclusion

The traditional and spontaneous fermentation processes evaluated of mezcal showed that the chemical composition varied significantly from artisanal factory to factory due to the particular characteristics of agave plants core, musts used, natural yeasts and without temperature control. It was proved that spontaneous fermentations differ significantly between two fermentation seasons (spring and fall), factories and interactions between the artisanal factories-fermentation seasons. Every evaluated factor presented a significant effect on reducing sugar content, nitrogen, acetic acid, and higher alcohols and thus, on the final product, which is reflected in differential quality, flavor, and smell of the final product (mezcal). Supplementation of ammonium sulfate reduces the fermentation time and affects significantly the production of ethanol, propanol and butanol, but decreases the methanol, ethyl acetate, and acetic acid production. The supplementation of ammonium sulfate is more convenient in the fall than in the spring.

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REFERENCES

Albers E, Larsson C, Lidén G, Niklasson C, Gustafsson L (1996). Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. Appl. Environ. Microbiol. 62(9):187-3195.

Alexandre H, Charpentier C (1998). Biochemical aspects stuck and sluggish fermentation in grape must. J. Ind. Microbiol. Biotechnol. 20(1):20-27.

AOAC (1990). Official Methods of Analysis of the Association of Official Analytical Chemists. Association of Official Analytical Chemist, Washington DC, USA, 2(15):735, 915.

Arrizon J, Gschaedler A (2002). Increasing fermentation efficiency at high sugar concentrations by supplementing an additional source of nitrogen during the exponential phase of the tequila fermentation process. Can. J. Microbiol. 48(11):965-970.

Arrizon J, Gschaedler A (2007). Effects of the addition of different nitrogen sources in the tequila fermentation process at high sugar concentration. J. Appl. Microbiol. 102(4):1123-1131.

Berry D, Watson D (1987). Production of organoleptic compounds. In: Berry D, Russell I and Stewart G (eds), Yeast Biotechnology. London: Allen and Unwen, pp. 345-364.

Cedeño C (1995). Tequila production. Crit. Rev. Biotechnol. 15(1):1-11.

Clesceri L (1992). Método de la sal de fenol para la determinación de amoniaco. In Santos D (ed.), Métodos normalizados de análisis de aguas potables y residuales. Madrid España, pp. 4126-4138.

Delfini C, Formica J (2001). Wine Microbiology. Science and Technology. In Dekker M (ed), Inc. Italy, pp. 339-350.

Díaz-Montaño DM, Délia ML, Estarrón EM, Strehaiano P (2008). Fermentative capability and aroma compound production by yeast strains isolated from *Agave tequilana* Weber juice. Enzyme Microb. Technol. 42(7):608-616.

Dubois M, Gilles K, Hamilton J, Reber P, Smith F (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem. 28(3):350-356.

Escalante-Minakata P, Blaschek HP, Barba de la Rosa AP, Santos L, De León-Rodríguez A (2008). Identification of yeast and bacteria involved in the mezcal fermentation of *Agave salmiana*. Lett. Appl. Microbiol. 46(6):626-630.

Gainvors A, Karam N, Lequart C, Belarbi A (1994). Use of *Saccharomyces cerevisiae* for the clarification of fruit juices. Biotechnol. Lett. 16(12):1329-1334.

Lappe-Oliveras P, Moreno-Terrazas R, Arrizon-Gaviño J, Herrera-Suarez T, Garcia-Mendoza A, Gchaedler-Mathis A (2008). Yeasts associated with the production of Mexican alcoholic nondistilled and distilled *Agave* beverages. FEMS Yeast Res. 8:1037-1052.

López M, Guevara Y (2001). Authenticity of three Mexican alcoholic beverages by SPME-GCMS. Annual Meeting of Institute of Food Technologists, New Orleans LA, pp. 10-3.

Mancilla-Margalli NA, López MG (2006). Water-soluble carbohydrates and fructan structure patterns from *Agave* and *Dasylirion* species. J. Agric. Food Chem. 54(20):7832-7839.

Mexican Ministry of Commerce and Industry Regulations (1994). NOM-070-SCFI-1994. Alcoholic Drinks-Mezcal Specifications. Diario Oficial de la Federación, México.

Nelson N (1944). Photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375-380.

Ough C, Bell A (1980). Effects of nitrogen fertilization of grape wines on amino acid metabolism and higher-alcohol formation during grape juice fermentation. Am. J. Enol. Vitic. 31(2):122-123.

Téllez P (1998). El cocimiento, una etapa importante en la producción del tequila. Bebidas Mex. 7:19-20.

Pinal L, Cedeño M, Gutiérrez H, Álvarez J (1997). Fermentation parameters influencing higher alcohol production in the tequila process. Biotechnol. Lett. 19(1):45-47.

Santillán V, García G (1998). Congeners biosynthesis during alcoholic fermentations. Rev. Lat. Am. Microbiol. 40:109-119.

SAS (1999). STAT Guide for personal computers. 8th ed. SAS Institute, Cary, North Carolina, USA. p. 1643.

Singh RJ, Saxena S, Gupta R (2005). Microbial pectinolytic enzymes: A review. Process. Biochem. 40(9):2931-2944.

Soto-García L, Rutiaga-Quiñones M, López-Miranda J, Montoya Ayón L, Soto-Cruz O (2009). Effect of fermentation temperature and must processing on process productivity and product quality in mezcal fermentation. Food Control 20:307-309.

Thomas K, Ingledew W (1990). Fuel alcohol production: effects of free amino nitrogen on fermentation of very-high-gravity wheat mashes. Appl. Environ. Microbiol. 56(7):2046-2050.

Vera-Guzmán AM, Santiago-García PA, López MG (2009). Compuestos volátiles aromáticos generados durante la elaboración de mezcal de *Agave angustifolia y Agave potatorum*. Rev. Fitotec. Mex. 32(4):273-279.

Verdugo-Valdez A, Segura-Garcia L, Kirchmary M, Ramirez-Rodriguez P, Gonzalez-Esquinca A, Coria A, Gschaedler-Mathis A (2011). Yeast communities associated with artisanal mezcal fermentation from *Agave salmiana*. Antonie van Leeuwenhoek 100:497-506.

Vidrih R, Hribar J (1999). Synthesis of higher alcohols during cider processing. Food Chem. 67(3):287-294.