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Agave americana HONEY FERMENTATION BY *Kluyveromyces marxianus* STRAIN FOR "COMITECO" PRODUCTION, A SPIRIT FROM MEXICAN SOUTHEAST

FERMENTACIÓN DE MIEL DE *Agave americana* UTILIZANDO *Kluyveromyces marxianus* PARA PRODUCIR "COMITECO", UNA BEBIDA ESPIRITUOSA DEL SURESTE DE MÉXICO

C. Lara-Hidalgo[1], A. Grajales-Lagunes[2], M.A. Ruiz-Cabrera[2], C. Ventura-Canseco[1], F.A. Gutiérrez-Miceli[1], V.M. Ruiz-Valdiviezo[1], M. Abud Archila[1]*

[1] *División de Estudios de Posgrado e Investigación. Instituto Tecnológico de Tuxtla Gutiérrez. Tecnológico Nacional de México. Carr. Panamericana km. 1080, C.P. 29050, Tuxtla Gutiérrez, Chiapas, México.*

[2] *Facultad de Ciencias Químicas. Universidad Autónoma de San Luis Potosí. Av. Dr. Manuel Nava No. 6. C.P. 78210 S.L.P. México.*

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Abstract

The aim of this study was to obtain white and aged "comiteco" spirit using the yeast *Kluyveromyces marxianus* strain LEV-03-ITTG during fermentation of *Agave americana* L. and panela honey, as well as to determine volatile compounds in white and aged "comiteco" spirit obtained. Three ratios (60:40, 65:35 and 70:30 v:v) of *Agave americana* L. honey and panela honey respectively to 22° Brix were used for must formulation and three times of fermentation (24, 48 and 72 hours) were used. Volatile compounds of white and aged "comiteco" spirits were identified by Gas Chromatography Mass Spectrometry, and sensory profile was determined using sommeliers and judged semi-trained. The ratio 65:35 v/v (*Agave americana*: panela honey to 22 ºBrix) fermented by 48 hours had the higher ethanol yield 30.85 g/L and allowed to obtain "comiteco" with the best sensorial properties. Ageing process improved the sensory properties of white "comiteco" spirit and decreased alcoholic odor. Therefore, *Kluyveromyces marxianus* strain LEV-03-ITTG could be considered as yeast with potential to industrial production of "comiteco" spirit.

Keywords: "comiteco" spirit, volatile compounds, white and aged spirit.

Resumen

El objetivo de este estudio fue obtener "comiteco" blanco y añejo usando *Kluyveromyces marxianus* LEV-03-ITTG durante la fermentación de miel de panela y de *Agave americana*, así como determinar los compuestos volátiles del "comiteco" blanco y añejo obtenido. Tres proporciones (60:40, 65:35 y 70:30 v:v) de mieles de *Agave americana* y panela a 22º Brix fueron usados para la formulación del mosto de fermentación misma que se llevó a cabo durante 24, 48 o 72 horas. Los compuestos volátiles del "comiteco" blanco y añejo fueron identificados por cromatografía de gases acoplado a espectrometría de masas y el perfil sensorial fue determinado usando "sommeliers" y jueces semientrenados. La proporción de 65:35 v/v (miel de *Agave americana*:panela a 22º Brix ambos) con 48 h de fermentación proporcionó el mayor rendimiento de etanol 30.85 g/L y permitió obtener comiteco blanco con las mejores propiedades sensoriales. El proceso de añejamiento mejoró las propiedades del "comiteco" blanco y desarrolló mejor perfil sensorial. Por lo tanto, con base en los resultados, *Kluyveromyces marxianus* LEV-03-ITTG podría ser utilizada para producir "comiteco" blanco y añejo a nivel industrial.

Palabras clave: "comiteco", compuestos volátiles, bebida espirituosa blanca y añeja.

1 Introduction

Mexico has several native plants of *Agave* genus and some are used for autochthon alcoholic beverages production (Segura-García *et al*., 2015). The most popular alcoholic beverages are tequila, which is produced from *A. tequilana* Weber (blue variety), and mezcal (González-Hernández *et al*., 2012) that can be produced from different Agave genus like *A. salmiana, A. potatorum* or *A. durangensis* and bacanora which is produced from *A. angustifolia* (De León *et al*., 2006). Another less popular alcoholic beverage produced from *A. americana* L. is called "comiteco", this

* *Corresponding author. E-mail*: miaba69@hotmail.com
Tel. 96-16-15-04-61

beverage is an artisanal spirit produced in Chiapas (Mexico). The production process of "comiteco" is different from other spirits because is produced by *Agave americana* L. sugars which are not obtained by cooking of agave pines as tequila. The sap is collected from the apical incision made to the Agave plant and evaporated by boiling until to obtain a syrup with about 60ºBrix. After, this syrup is used to prepare the fermentation must by addition of water until 22ºBrix to produce "comiteco". This difference makes that "comiteco" spirits could have sensorial attributes different of tequila, mezcal and others spirits. However, there are little scientific information about fermentation process production, volatile compounds of white and aged "comiteco" spirits and their sensorial attributes. At present for "comiteco" production, Comiteco Balun Canan S. de R.L. de C.V. is a company dedicated to the artisanal production of "comiteco". They use baker's yeast for production of "comiteco". The company seeks also carry out studies on the influence of the composition of the must and the time of fermentation and how they influence the chemical composition, as well as the sensorial characteristics of the "comiteco" spirit. Also, the "comiteco" industrials are looking for a strain with high ethanol production and which is exclusive to the production of the "comiteco" spirit. Recently, in our laboratory *K. marxianus* strain LEV-03-ITTG wild yeast was isolated of naturally fermented *Agave americana* L. sap, and showed higher ethanol production than other strain isolated. Therefore, the aim of this study was to produce white and aged "comiteco" spirits using *K. marxianus* strain LEV-03-ITTG yeast by fermentation of *A. americana* L. and panela honey, as well as to determine volatile compounds and sensorial properties of white and aged "comiteco" spirit obtained through distillation.

2 Materials and methods

2.1 Raw material

Firstly, an apical incision was made to the Agave plant. After, the sap was collected daily and evaporated by boiling until to obtain a solution with about 60ºBrix. This solution was called honey. After, this honey was used to prepare the Agave honey by addition of deionized water until 22ºBrix to produce "comiteco". On the other hand, "panela" was purchased in a local supermarket. The "panela" was diluted in deionized water also to obtain the panela honey solution to 22ºBrix which was used in the fermentation must. Several proportion of Agave and "panela" honeys (v/v) were used in fermentation must (Table 1). The fermentation must was not sterilized to simulate industrial conditions for "comiteco" production (data not shown).

Glucose, fructose, sucrose and inulin of Agave and "panela" honeys were quantified by HPLC (PerkinElmer, series 200) with a refractive index detector (PerkinElmer, series 200a). The honeys to 22ºBrix were then centrifuged at 30ºC during 10 minutes at 10,000 rpm. The supernatant was diluted ten times and filtered with 0.25 μm Millipore membrane. A column Hi-Plex Ca (Agilent Technologies, Germany) was used and maintained at 30ºC. Ten microliters were injected, and isocratic conditions were used with a flow of 0.4 mL/min, using tridistilled water as mobile phase.

Table 1. Ethanol production after "comiteco" white fermentation.

Treatment	Proportion Agave and panela honeys (v/v) of the must	Fermentation time (h)	Ethanol Production (g/L)
1	60:40	24	28.96^b
2	60:40	48	33.03^a
3	60:40	72	28.67^b
4	65:35	24	27.73^b
5	65:35	48	30.85^a
6	65:35	72	28.71^b
7	70:30	24	22.67^b
8	70:30	48	27.85^b
9	70:30	72	31.49^a

Treatments with different letters in a column are statistically different (p <0.05).

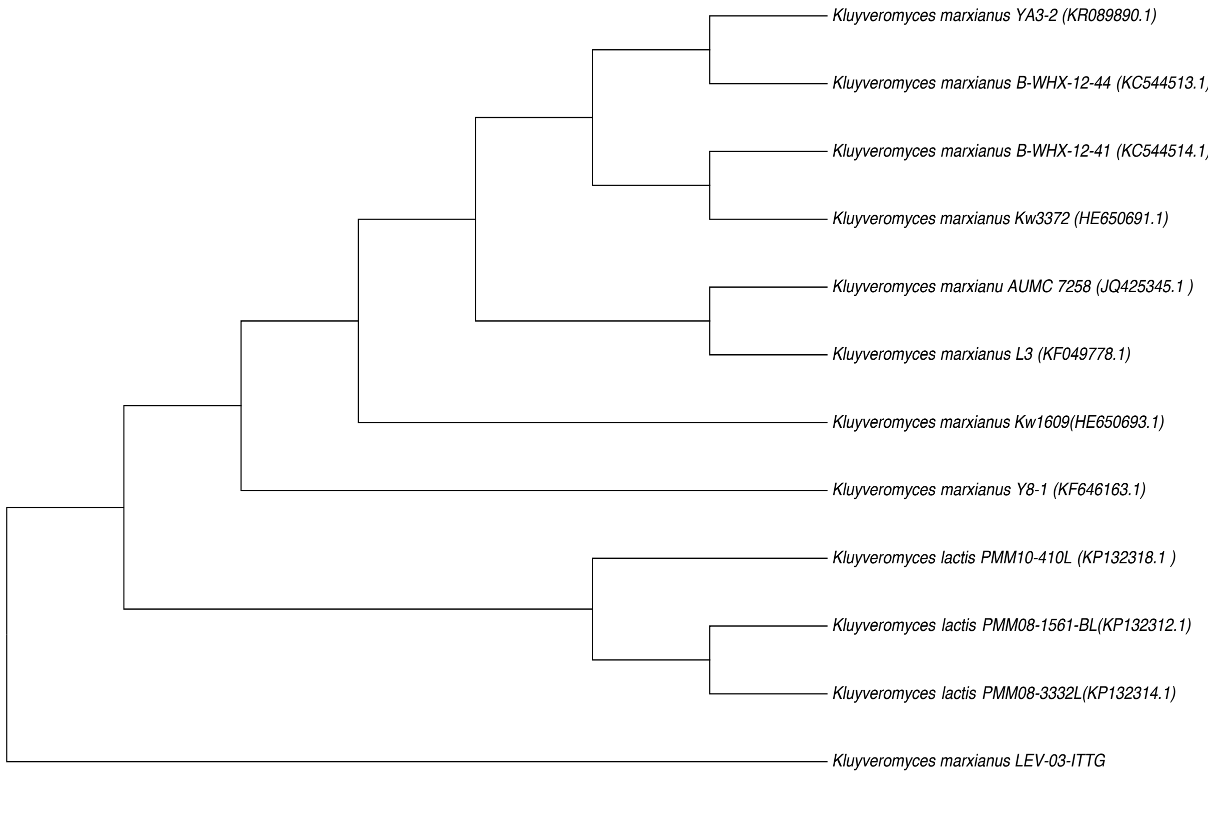

Fig. 1. Phylogenetic analysis based on 18S rRNA gen of strain *Kluyveromyces marxianus* isolated from Agave honey naturally fermenting. Using method Neihbor-Joining with 1000 bootstrap.

2.2 *Molecular identification of Kluyveromyces marxianus strain LEV-03-ITTG*

Kluyveromyces marxianus strain LEV-03-ITTG was isolated from *Agave americana* L. sap naturally fermented in Comitán, Chiapas, México. Thus, Genomic DNA was isolated using the method reported by Stringini *et al*. (2009). Molecular identification of the strain LEV-03-ITTG was performed by phylogenetic analysis based on the 18S rRNA gene (Figure 1). The accession number in GenBank NCBI for *Kluyveromyces marxianus* strain LEV-03-ITTG was KT971341.

2.3 *Inoculum preparation*

The strain LEV-03-ITTG was grown on PDA agar slants tube for 72 h. A suspension of five tubes was inoculated into 120 mL of sterilized YM broth, which was incubated at 30±3°C under stirring at 100 rpm for 8 hours. The Erlenmeyer flasks were covered only with a thin cloth. After, the culture media was allowed to settle for 10 minutes, then 25 mL of media were taken to start inoculum stage containing about 1×10^8 cel/mL. Finally, 25 mL of mixture were added to 25 mL of the must fermentation as a fed batch system until to reach 1.5 L of inoculum must.

2.4 *"Comiteco" production*

The fermentations for "comiteco" production were carried out under anaerobic conditions at 28-32°C in plastic bottles of 3 L, containing 1.5 L of must culture during 24, 48 and 72 h. Briefly, 750 mL of the must was inoculated with 750 mL of inoculum must (developed in 2.3) containing a *Kluyveromyces marxianus* strain of about 1×10^8 cells/mL. This inoculum resulted in a proportion of at least 1×10^6 cell/mL in the fermentation must. The must was prepared using Agave and panela honeys mixed in three different ratios (60:40, 65:35 and 70:30 v/v) and were supplemented with ammonium sulfate (3.3 g/L).

After fermentation, must fermentation was distilled at 96ºC until depletion using a Corning equipment, the distillate obtained was diluted with deionized water to reach 35° G.L. to obtain white "comiteco" spirit. For production of aged "comiteco" spirit, the white spirit was stored in an Erlenmeyer flask hermetically sealed containing 2 g of toasted white oak per 100 mL of distillate during 3 months at 30ºC. Ethanol concentration in distillates was determined using an electronic density meter Anton Paar (DMA-500, Graz, Austria), their content was calculated with the relation between ethanol content and sample density.

2.5 Determination of volatiles compounds content in "comiteco"

Volatile compounds analysis in distillated after Erlenmeyer flask fermentation and white and aged "comiteco" spirits were determined by Gas Chromatograph (GC) using an Agilent Technologies 5975C interfaced with Agilent 7890a mass spectrometry and a DB-WAX column (30 m x 0.32 mm, 0.5 μm). For major volatile compound determination, the samples were dehydrated with sodium sulfate until saturation, after were centrifuged at 4,000 rpm, and filtered using Millipore filter of 0.2 μm and then were injected to GC. To determine the minor volatile compounds, 5 mL of distillate were extracted with 10 mL of dichloromethane, the extract was washed and dried over anhydrous sodium sulfate. Sample was reduced to 2 mL by evaporation of solvent and 1 μL of sample was injected to the CG-MS. In both test, chromatographic conditions were 40°C for 3 min, increased at 3°C/min to 120°C and 6°C /min to 200 °C, the last temperature was maintained for 60 min (De León *et al*., 2006). Helium was used as carrier gas at a flow of 1.0 mL/min and the injector and detector temperatures were 180 and 230°C respectively. The MS ionization potential was 70 eV and transfer line temperature was 230°C. Compounds were identified with retention index using HP CHEMSTATION-NIST MS A.00.00 library.

2.6 Sensory analysis

The white "comiteco" (treatments 2, 5 and 9) at 35 °G.L was firstly evaluated by sommeliers panel. Visual, olfactory and taste test were carried out to determine their higher sensorial attributes. Finally, sommeliers panel selected the better treatment for higher sensorial attributes. The white "comiteco" spirit was aged for sensorial evaluation. After that, seven judges were trained to identify 30 different flavors, which were identified previously by sommeliers. Then, sensory descriptive analysis was carried out by semi-trained judge panel (Meilgaard *et al*., 1999). Each sample of white and aged spirits was judged for intensity of color, odor and taste using a 5-point scale where 1 was 'of low intensity' and 5 was 'of high intensity'.

2.7 Statistical analysis

ANOVA tests (p <0.05) was performed using the Statgraphics software XV (StatPoint, Inc. Rockville, MD software) to evaluate the effect of factors on the response variables in "comiteco" spirit. Least Significant Difference was calculated with Tukey test, all treatments were carried out by triplicate.

3 Results and discussions

3.1 Fermentation kinetics in Erlenmeyer flasks

The values of μmax (h^{-1}) during fermentation kinetics of *K. marxianus* were found in the range of 0.094 to 0.186 h^{-1}. The μmax values was increased according to the proportion of Agave honey. These results were similar to those reported by Graciano-Fonseca *et al*. (2007) 0.1 h^{-1} for *K. marxianus* ATCC 26548, however these values were higher (0.0189 h^{-1}) than those reported by Siqueira *et al*. (2008). Duplication time (td) decreased for *K. marxianus* when the proportion of Agave honey increased because their growth was quickly. These results indicated that *K. marxianus* use the nutrients of Agave honey easily.

HPLC analysis showed differences in glucose, fructose, sucrose and inulin contents between Agave (19, 26.2, 45.1 and 12.8 g/L, respectively) and panela (71.8, 23.5, 10.6 and 1.3 g/L, respectively) honeys. The *Agave americana* honey content was 12.8 g of inulin/L; this value is higher than reported in *Agave tequilana* juice used for tequila. That can be due to obtain syrup for "comiteco" and tequila production. In this case, Agave honey to 60ºBrix is obtained by boiling of sap juice to atmospheric pressure, where the inulin could be partially hydrolyzed, at difference to process to obtain syrup for tequila production. López *et al*. (2003) reported that inulin has the higher content of carbohydrates in *Agave tequila* Weber, but Arrizon and Gschaedler (2002) reported the composition of *A.*

tequilana Weber Var. Azul juice with 94% of fructose, 5% of glucose and 1% of sucrose.

In relation to ethanol production (g ethanol/L of the must), values were between 22.67 and 33.03 g/L for all proportion of Agave:panela honey and fermentation time (Table 1). The results of this study were three times larger than the value (10.1 g/L) reported by Kumar *et al*. (2011) and Ortíz-Méndez *et al*. (2017) which reported 25.4 g/L of ethanol. However, our values were lower than 58.78 g ethanol/L as was reported by López-Alvarez *et al*. (2012) and 7.2%V reported by González-Hernández *et al*. (2012). These differences could be due that fermentation must was realized with Agave tequilana honey for tequila production and for the use of different strains.

3.2 Volatile composition for "comiteco"

Table 2 shows the volatile composition results for the distilled of treatments with higher ethanol production (2, 5 and 9). According of analysis in GC-MS, it was observed the presence of ethanol, higher alcohols as 1-propanol, 2-methylpropanol, 3-methylbutanol and 1-pentanol, as well as acetaldehyde and ethyl acetate. These compounds have been reported in mezcal spirit also (Rios-Deras and Rutiaga-Quiñonez, 2015). The acetaldehyde is commonly found in distillates beverages from agave, such tequila or mezcal (Molina-Guerrero *et al*., 2007, Arellano *et al*., 2008) and is synthesized from pyruvate by pyruvate decarboxylase. Ethyl acetate, which imparts fruity flavor in mezcal (De león *et al*., 2006; Rios-Deras and Rutiaga-Quiñonez, 2015), is the main ester that occurs in distilled beverages (Arellano *et al*., 2008). According of ANOVA test, no significant differences (p >0.05) were observed in volatile compounds of these treatments, due to similarity of ethanol production and volatile compounds, for that, the treatment 5 (Agave and panela honey ratio of 65:35 and 48 hours of fermentation time) was chosen for the white and aged spirit production.

The major and minor volatile compounds of white and aged "comiteco" spirits were summarized in Table 3 and 4 respectively. Significant differences ($p \leq 0.05$) were observed in ethyl acetate and pentanal, between white and aged spirits. 2-propenic acid-1-methylundecylester, 3-methyl-oxiran-2-methane, 3-methylbutanamine, 2-hydroxyhexadecylbutanoate were only found in aged spirit, which could be used as markers in "comiteco" aged. With respect of minor compounds (Table 4), 19 compounds were detected which belong to ketones, furans, higher alcohols, esters, amine and acetal, of these compounds, 41 and 59% were detected in white and aged spirit respectively. 2-tertiary-butyl-4-methylfuran and 2-butanol are only present in white spirit i.e during aged these components were probably volatilized. Centi *et al*. (2011) reported that the occurrence of furan derivatives is due to degradation of lignin. The furan derivatives have been extracted from toasted wood, and can participate in many reactions that take place in the wine during this process. The compounds detected (Table 4) to our knowledge (Web of Science, 2017), these have not been reported in "comiteco" spirit. Therefore, they may be unique compounds of "comiteco" and could be used as markers for the authenticity of "comiteco" spirit. In "comiteco" spirit, these components may be correlated with sensory profile, but it is difficult to make a direct correlation between volatile compounds and sensory profile (Lopes *et al*., 2007).

Table 2. Abundance of major volatile compounds found in distilled with higher ethanol productions during fermentation.

Compound name	Abundance (%)		
	Treatment 2 (60:40, 48 h)	Treatment 5 (65:35, 48 h)	Treatment 9 (70:30, 72 h)
Acetaldehyde	0.59	0.11	0.36
Ethyl acetate	0.34	0.079	0.20
Ethanol	96.37	98.91	96.35
1-propanol	0.86	0.13	0.53
2-methylpropanol	0.25	0.01	0
3-methylbutanol	0.98	0.16	1.48
1-pentanol	0.15	0	0

No statistical difference (p <0.05) were found between treatments for each compound.
Values are the average of three injections.

Table 3. Abundance of major volatile compounds found in "comiteco" white and aged spirits.

Compound name	Abundance (%)		
	Spirits		
	White	Aged	Aroma
Acetaldehyde	0.020[A]	0.015[A]	Walnut, sherry and green leaves[a]
Ethyl acetate	0.013[A]	0.024[B]	Fruity, pineapple[a]
Pentanal	0.002[A]	0[B]	Sweet[a]
Ethanol	98.132[A]	99.028[A]	Alcoholic[a]
3-methylbutanol	1.370[A]	0.467[A]	Alcoholic, fruity and sweet[a]
3-hydroxybutanal	0.005[A]	0.005[A]	-
5-methylheptanoamine	0.003[A]	0[A]	-
1-pentanol	0.451[A]	0.411[A]	Marzipan and sweet[b]
Butanol	0.004[A]	0[A]	Banana[a]
2-propenic acid-1-methylundecylester	0[A]	0.015[A]	-
3-methyl-oxiran-2-methanol	0[A]	0.023[A]	-
3-methylbutanamine	0[A]	0.008[A]	-
2-hydroxyhexadecylbutanoate	0[A]	0.004[A]	-

The data were mean values of triplicate samples; compound with same capital letters in a row are not statistically different (p <0.05). [a]Bénes *et al.* (2015) and [b]Lambrechts and Pretorius (2000).

Table 4. Minor volatile compounds abundance percentage in "comiteco" white and aged spirits.

Compound name	Abundance (%)		
	Spirits		
	White	Aged	Aroma
Diacethyl	0.67[A]	0[A]	Butter[a]
6-amine-2-methylheptanol	0.33[A]	0[A]	-
Etanimidic acid ethyl ester	0.42[A]	0[A]	-
Isobutanol	9.05[A]	0[A]	Alcoholic[b]
Butanol	0.50[A]	0[A]	Alcoholic[b]
1-pentanol	41.41[A]	43.11[A]	Marzipan and sweet[b]
Tributilfosfate	45.04[A]	45.91[A]	-
1,5-buthyl-4-methyldihydroxy furanone	2.33[A]	0[A]	-
Isopentylamine	0[A]	0.40[A]	-
Tetrahydro-3,4-furandiol	0.22[B]	0[A]	Smoked[c]
Ciclopropanecarboxyl-4-methylpentil ester	0[A]	0.13[A]	Fruity[c]
2-propanamine	0[B]	0.25[A]	-
1,6-methyl-2-pirimidinamine	0[A]	0.24[A]	-
5-amine valeric acid	0[B]	2.74[A]	-
4-fluorohistamine	0[A]	0.12[A]	-
Methacryilic acid methyl ester	0[A]	0.25[A]	-
Ethyl acetimidate	0[B]	0.78[A]	-
2-methyl-1-propanol	0[B]	5.30[A]	-
1-methylundecylester	0[A]	0.40[A]	-

The data were mean values of triplicate samples; compound with same capital letters in a row are not statistically different (p <0.05). [a]Bénes *et al.* (2015), [b]Lambrechts and Pretorius (2000) and [c]Lehtonen (1983).

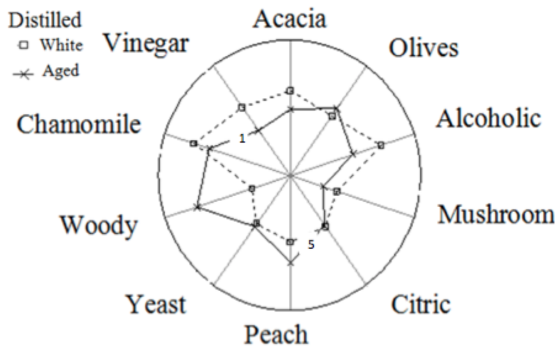

Fig. 2 Flavour of "comiteco" white and aged spirit determined by semi-trained judges panel.

The fruity aromas (fruity, peach, lime, and apple) described in the sensory profile are attributed mainly to the production of esters in mezcal (De León *et al.*, 2006) and tequila (Vallejo-Cordoba *et al.*, 2004). Therefore, flavor profile of alcoholic beverages cannot be attributed to only one component; it is the result of the combination between the different compounds, their content and interactions between compounds (Lambrechts and Pretorius, 2000).

3.3 Sensory analysis

The white "comiteco" spirits obtained with distilled of treatments 2, 5, and 9 were also analyzed by four sommelier judges according to the visual, olfactory and gustative test, as well as sensory taste profile. With respect to visual phase results, no significance differences (p <0.05) were observed between three samples. However, in olfactory phase, the samples 5 and 9 (65:35-48 hours and 70:30-48 hours respectively) achieved the highest scores; and in gustative phase, the white spirit with higher acceptation was the treatment 5. After, the white "comiteco" obtained with treatment 5 was aged and determined their sensory profile. Figure 2 shows the sensory profile of white and aged spirits determined by semi-trained judges. These results indicated that the white spirit had low woody and mushroom aroma with values of 1.5±0.5. Values between 3±0.5 and 3.5±0.5 were obtained for peach, yeast, vinegar, olives, acacia and citric aroma. The higher value 4±0.5 was obtained for alcoholic and chamomile aroma. However, after aged woody and peach aroma were increased to 4 and 3.5 values respectively; while vinegar, alcohols and mushroom aroma were decreased to 2, 3 and 1.5±0.5 respectively. These kind of aromas have been reported in others alcoholic beverages such as tequila, a sensory evaluation of different commercial tequilas was reported by Benn and Peppard (1996). They found woody, chocolate, vanilla, cream, beans, green grass, sweet, whiskey, rum, dried fruit and alcohol aromas, many of these were found in our spirits. The appearance and disappearance of compounds after ageing caused changes in the taste and aroma of spirits (Mosedale and Puech, 1998). These changes may be due by direct extraction of compounds from wood, reactions between the wood components and the constituents of the spirit or the evaporation of volatile compounds (Nishimura and Matsuyama, 1989). According to Mosedale and Puech (1998), the extraction during ageing is the most important for sensory characteristics of the final product. Madrera *et al.* (2011) suggests that in ageing process occur chemical reactions of esterification, acetylation and hydrolysis, which generate new compounds, reducing ethyl ester content and increasing fatty acid esters in distillate cider. In our study, the concentration of ethyl acetate in aged spirit increased significantly and 2-butanol decreased. These results are in agreement with sensory profile reported by judges, who detected lower alcoholic aroma in aged spirit than in white spirit. Disappearance of 2-butanol could be caused by evaporation as suggest Mosedale and Puech (1998) for some alcohol.

Conclusions

A proportion of must fermentation (v/v) of 65:35 (*Agave americana* L.:panela honey to 22ºBrix both) can be used to produce an white and aged "comiteco" spirit beverage during 48 hours of fermentation using *Kluyveromyces marxianus* strain KT971341. Thus, ageing process in white oak causes the appearance or disappearance of the several volatile compounds in aged spirit. Thirteen major volatile compounds and twenty minor volatile compounds were found in white and aged "comiteco" spirits. This synergy of volatile compounds is responsible of sensorial properties of "comiteco" spirits and could represent the difference of "comiteco" and others spirits like tequila and mezcal. In addition, *Kluyveromyces marxianus* LEV-03-ITTG (accession number in GenBank NCBI KT971341) can be considered as yeast with potential to industrial production of "comiteco" spirit.

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