Volatile compounds generation during different stages of the Tequila production process. A preliminary study

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ABSTRACT

Samples obtained during 8 stages of Tequila's production process were analyzed to follow generation and/or disappearance of minor volatile compounds. Volatile compounds were extracted with the liquid-liquid batch extraction method and analyzed by gas chromatography coupled with a flame ionization detector and mass-selective detector. A total of 327 compounds were identified and 316 relatively quantified. Analysis of variance showed that 90 compounds had significant differences ($p \leq 0.05$) between process stages, but only dipropyl disulfide ($p = 0.048$) had significant differences between batches and furfuryl alcohol ($p = 0.022$), myristic acid ($p = 0.039$), 3-methyl-cyclopentanone ($p = 0.044$), and 9-hydroxypyrimido[1,6-a]pyrimidin-4-one ($p = 0.048$) between factories. Principal component analysis (PCA) made it possible to describe two groups including juices and musts (J&M) and distilled samples (S) separated mostly by PC1. Using general discriminant analysis (GDA) of the volatile compounds data set, made it possible to distinguish samples according to 8 sampling 90.3% of the time.

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1. Introduction

Tequila is a Mexican distilled alcoholic beverage produced since the mid-sixteenth century. Production methods have changed since then, becoming more efficient and reproducible (Cedeño & Álvarez-Jacobs, 2003) by standardization of processes, including the use masonry ovens or autoclaves instead of soil ovens, selected yeast instead of spontaneous fermentation, designs of distillation equipment, etc. After the *Agave tequilana* Weber var. azul plants are harvested, they are cooked in masonry ovens (24—48 h) or autoclaves (12—24 h). Next, the agave hearts are milled to extract the juice. If 100% Tequila is to be made from only agave sugars, sugar content is adjusted with water before fermentation according to the distillery's experience (Cedeño & Álvarez-Jacobs, 2003) but if a Tequila is to be produced, not more than 49 g of foreign sugars/100 g total sugars may be added before fermentation (NOM-006-SCFI-2012, 2012). Fermentation step is performed according with each distillery practices: spontaneous fermentation, controlled fermentation using commercial, native *Saccharomyces cerevisiae* yeast, or by request prepared yeasts consortium. Next, the obtained must is distilled twice. From the first distillation, a product called "ordinario" is obtained (40 proof ethanol to 60 proof ethanol). After the second distillation, white Tequila is obtained (76 proof ethanol to 136 proof ethanol) which could be matured to produce aged tequilas (NOM-006-SCFI-2012, 2012).

Tequila flavor is the main characteristic that consumer associate with quality. This characteristic is given by a wide variety of different volatile compounds, some of them, that, present in high amounts, are controlled by law such as methanol, acetaldehyde, ethyl acetate, superior alcohols, furfural, etc. (NOM-006-SCFI-2012, 2012). There are also a large number of volatile compounds present in very low concentrations that have a huge impact on Tequila's flavor (Escalona-Buendia et al., 2004). Each of the Tequila process stages could have a big influence in its final organoleptic

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characteristics. There are several studies that evaluate volatile compounds' profile in Tequila as a final product (Benn & Peppard, 1996; Cardeal & Marriott, 2009) and all those studies showed a complex volatile compounds profile including acetals, aldehydes, ketones, alcohols, esters, terpenes, lactones, etc. Additionally, studies have been carried out to evaluate the volatile compounds production during specific stages (Díaz-Montano, Délia, Estarrón-Espinosa, & Strehaiano, 2008; Flores et al., 2013; Prado-Ramírez et al., 2005). In general, in all these works, only major compounds regulated by Mexican law were evaluated, but minor volatile compounds which have an impact in the aromatic quality of Tequila were not considered in the specific production stages but only after distillation. There is scarce information about minor volatiles throughout Tequila's elaboration process; therefore, the aim of this work was to follow the generation of the minor volatile compounds in selected stages of the Tequila process.

2. Materials and methods

2.1. Samples

Samples were obtained from two Tequila Distilleries in Los Altos region in Jalisco (coded A and B) during regular production steps. In each Distillery, 2 batches of Tequila (51 g agave sugars/100 g total sugars) (coded 1 and 2) were followed at 8 sampling points: Raw agave (MP), cooked agave (C), after milling (M), wort formulation (Fo), fermentation start after yeast addition (FEI) and end (FEF), first distillation (D) and second distillation (R). At each sampling point, 1–1.5 L was obtained for liquid samples, and 1 kg of homogenous solid samples. All the samples were analyzed three times. To assure the samples' integrity, they were frozen at $-18\ ^\circ$C at the Distilleries, transported in a cooler to the laboratory, and kept at $-18\ ^\circ$C until their analysis.

2.2. Samples conditioning

Agave samples were thawed and milled to extract agave juices by using a semi industrial juice extractor. Musts and juices were thawed and homogenized by manual agitation. Tequila samples were adjusted to 30 mL of ethanol/100 mL by adding distilled water and were verified by the Gay-Lussac scale at 15 $^\circ$C with calibrated alcoholmeters (Dujardin-Salleron, Paris). First distillation samples were analyzed directly without any adjustment since its alcohol content was in the adjustment value (30 mL of ethanol/100 mL).

2.3. Volatile compounds extraction

Volatile compounds were extracted with the liquid–liquid batch method (Martín del Campo et al., 2011) with some modifications for agave juices and musts as shown in previous works (Pinal, 2001). This method was selected since it permits rapid high extraction yields possible with lower amounts of sample and solvents.

For juices and musts, 70 mL of the sample were placed in a centrifuge tube with 12 mL of a mixture of pentane/dichloromethane 3:1 mL/mL (Fisher, Leicester, U.K.). After 5 min agitation, they were centrifuged for 5 min at 7000 $\times$ g and 10 $^\circ$C, the solvent layer was separated, dried with anhydrous Na_2SO_4 (Mallinckrodt, Paris, USA), and preserved in amber flasks at $-18\ ^\circ$C for subsequent concentration. For distillates, 325 mL sample added with 0.2 g of NaCl (J.T. Baker, Phillipsburg, NJ) were extracted with 45 mL of a mixture of Pentane/Dichloromethane 3:1 mL/mL. After 5 min agitation, sample was allowed to stand until solvent layer separation. This was separated, dried with anhydrous Na_2SO_4 (Mallinckrodt) and preserved in amber flasks at $-18\ ^\circ$C for subsequent

concentration. All the extracts were concentrated using a Kuderna-Danish device. The extract was placed in the device in a water bath at 40 $^\circ$C. After solvent evaporation, the final volume of 0.4 mL was adjusted with a nitrogen gas flow. Concentrated extracts were placed in suitable vials and preserved at $-40\ ^\circ$C until their chromatographic analysis.

2.4. Chromatographic analysis

Concentrated extracts were analyzed in a gas chromatography system (Hewlett Packard 6890, Palo Alto, USA) with flame ionization detection (FID) and an auto sampler. Compounds were separated with a capillary DB-Wax column (30 m $\times$ 250 µm ID $\times$ 0.25 µm), using helium as carrier gas at 1.2 mL/min. Injector temperature was 220 $^\circ$C and detector 260 $^\circ$C. Programmed oven temperature was: after 5 min at 40 $^\circ$C, temperature was raised at 2.5 $^\circ$C/min to 220 $^\circ$C and kept for 35 min. Under these conditions, 0.5 µL of sample were injected and a 60:1 split ratio was used. All the extracts were injected twice. Quantification was done by using the % area report of each chromatogram without solvents in order to obtain a relative concentration in the obtained extracts.

Simultaneously, one concentrated extract was chosen from each process step to be analyzed by gas chromatography-mass spectrometry (GC−MS) as well as a model solutions prepared with reference standards with a minimum purity of 90 g/100 g provided by Sigma−Aldrich (St. Louis, MO, USA). The reference compounds were selected among those previously identified in Tequila (Benn & Peppard, 1996; Martín del Campo et al., 2011) that belonged to different chemical families. Model solution contained 10 mg/mL of each one of the compounds in a 30 mL ethanol/100 mL water solution (Lichrosolv, Merck, Darmstadt, Germany). Reference compounds selected are identified as ST in Table 1. For this, we used a gas chromatograph (Hewlett Packard 5890 Series II) coupled with a mass selective detector (HP 5972). Column, injector temperature, oven program, sample volume, and split ratio were the same used for GC-FID. The total ion chromatograms (TIC), as well as the mass spectra, were acquired in the electron impact (EI) mode at 70 eV and traced at 1.6 scans/s. Compounds were tentatively identified by comparing the spectrum of each compound with the Wiley 138 spectra library. Compounds' identity was confirmed by comparing with reference standards and/or by comparison with the Kovats index reported in literature (Kovats.org, 2014).

2.5. Statistical analysis

Chromatograms obtained in the CG-FID analysis were integrated and the peak areas were recorded for each compound. All the statistical analyses were performed with STATISTICA software (StatSoft, Tulsa, USA). First, we performed an analysis of variance (ANOVA) as well as the Fisher's multiple range tests of the minimal significant differences (LSD) to evidence the compounds showing significant differences among process stages. Then, principal component analysis (PCA) was used to extract the information of the whole data set in order to identify the compounds that change the most between process stages. PCA made it possible to evaluate the whole data set instead of individual compounds. Finally, general discriminant analysis (GDA) was applied to the data set in order to evaluate the possibility to discriminate among the 8 process stages. This tool generates discriminant functions that made it possible to classify experimental units in two or more populations defined in a unique way, reducing the amount of initial variables by selecting those having more impact in the discrimination. A backward stepwise method (p inclusion 0.05, p exclusion 0.05) was applied in order to