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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · JUNE 2006

Impact Factor: 2.91 · DOI: 10.1021/jf060094h · Source: PubMed

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 3911–3915 **3911**

Quantification of Selected Volatile Constituents and Anions in Mexican *Agave* Spirits (Tequila, Mezcal, Sotol, Bacanora)

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A large collection ($n = 95$) of Mexican *Agave* spirits with protected appellations of origin (Tequila, Mezcal, Sotol, and Bacanora) was analyzed using ion and gas chromatography. Because of their production from oxalate-containing plant material, all *Agave* spirits contained significant concentrations of oxalate (0.1−9.7 mg/L). The two Tequila categories ("100% *Agave*" and "mixed") showed differences in the methanol, 2-/3-methyl-1-butanol, and 2-phenylethanol concentrations with lower concentrations in the mixed category. Mezcal showed no significant differences in any of the evaluated parameters that would allow a classification. Sotol showed higher nitrate concentrations and lower 2-/3-methyl-1-butanol concentrations. Bacanora was characterized by exceptionally high acetaldehyde concentrations and a relatively low ethyl lactate content. The methanol content was the most problematic compound regarding the Mexican standards: two Tequilas (4%), five Sotols (31%), and six Bacanoras (46%) had levels above the maximum methanol content of 300 g/hL of alcohol. In conclusion, the composition of Mexican *Agave* spirits was found to vary over a relatively large range.

KEYWORDS: ***Agave*; Agavaceae; Tequila; Mezcal; Sotol; Bacanora; volatile compounds; ion chromatography; gas chromatography**

INTRODUCTION

The *Agave* genus, with more than 200 species, occurs natively in arid and tropical regions from the southern U.S. to northern South America and throughout the Carribean. There are around 150 species of *Agave* plants native to Mexico (*1*, *2*). The most important economic use of *Agaves* is the production of alcoholic beverages such as Mezcal (*Agave angustifolia* Haw., *A. potatorum* Zucc., *A. salmiana* Otto, and other species), Sotol (*Dasylirion ssp.*), and Bacanora (*A. angustifolia* Haw.). All of these spirits are obtained from the fermentation of agavins (fructooligosaccharides) from the different *Agave* species (*3*). However, the most popular contemporary alcoholic beverage made from *Agave* is Tequila, which is recognized worldwide. The production of Tequila is restricted to the blue *Agave* (*Agave tequilana* Weber var. *azul*, Agavaceae) and also to defined geographic areas, primarily to the State of Jalisco in West-Central Mexico (*2*, *4*). Following Tequila's appellation of origin, the other *Agave* distilled beverages from the States of Oaxaca, Guerrero, San Luis Potosi, Chiapas, Guajanuato, Zacatecas (Mezcal), Chihuahua, Coahuila, Durango (Sotol), and Sonora (Bacanora) were granted equal recognition. All of these regional drinks are subject to official standards, and their production is supervised by the Mexican government (Table 1) (*5*−*8*).

Until now, only Tequila and more recently Mezcal have reached international recognition, and especially in the past decade, the consumption of Tequila has increased tremendously worldwide. Tequila and Mezcal are protected under the North American Free Trade Agreement (NAFTA) and an "Agreement between the European Union and the United Mexican States on the mutual recognition and protection of designations for spirit drinks" (*9*).

Because of frequent fraud (e.g., adulteration with grain spirits), the authenticity control of *Agave* spirits is of a high concern. To establish the authenticity of Tequila, different analytical studies have been performed, which include the detection of volatile compounds using gas chromatography (GC) or sensory techniques as well as SPME-GC-MS, FTIR, SIMCA-NEAR, GC-O, and GC-IRMS analyses (*10*−*20*).

In this work, ion chromatography (IC) and GC are used for the first time to assess a large collection of commercial Mexican *Agave* spirits. The use of oxalate as a marker for Tequila authenticity is evaluated. The contents of inorganic anions, as well as methanol and higher alcohols, are discussed regarding quality aspects and conformity to the Mexican standards.

MATERIALS AND METHODS

Samples. Authentic Tequila samples of "100% *Agave*" ($n = 31$) and "mixed" categories ($n = 25$) were available from controlled Tequila

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10.1021/jf060094h CCC: $33.50
Published on Web 04/28/2006

Table 1. Excerpt of Mexican Standards Regarding Spirits with Origin Denomination

	Tequila	Mezcal	Sotol	Bacanora
Mexican norm	NOM-006-SCFI-1994	NOM-070-SCFI-1994	NOM-159-SCFI-2004	NOM-168-SCFI-2004
plant type	*Agave tequilana* Weber var. *azul*	several *Agave* species	*Dasylirion* spp.	*Agave angustifolia* Haw.
alcoholic strength (vol %)	35.0–55.0	36.0–55.0	35–55	38–55
higher alcohols (g/hL of alcohol)	20–400	100–400	20–400	100–400
methanol (g/hL of alcohol)	30–300	100–300	0–300	30–300

production facilities in the Jalisco region. Authentic Mezcal ($n = 10$), Sotol ($n = 16$), and Bacanora ($n = 13$) were purchased in the corresponding Mexican regions of production.

Ion Chromatography. A previously developed and validated method for the determination of anions in spirits was applied (*20*, *21*). The sample preparation was modified to include an inline-dialysis step to prevent contamination of the column, e.g., by caramel or polyphenols contained in gold, reposado, or añejo spirits. The chromatographic conditions were modified using a column with higher capacity (4 × 250 mm i.d. instead of 4 × 100 mm i.d.) for the sensitive detection of oxalate.

The chromatographic analyses were performed on a Compact IC 761 system (Deutsche Metrohm, Filderstadt, Germany) equipped with an IC Dialysis Sample Processor 788 and a conductometric detector including a temperature-compensated conductivity cell and an MSM packed-bed suppressor. Substances were separated on an anion-exchange column (Metrosep A Supp 5, 4 × 250 mm i.d.) fitted with a guard column (RP-Guard, 4 × 25 mm i.d.). The eluent consisted of 3.2 mM sodium carbonate and 1.0 mM sodium bicarbonate. The samples were diluted 1:10. The dialysis step was executed automatically prior to analysis according to the standard procedure of the manufacturer (10-min dialysis time using a cellulose acetate membrane with a pore size of 0.15 μm). After that, the dialyzed solution was transferred into an injection loop of 50-μL volume and then analyzed. Separations were carried out at a flow rate of 0.7 mL/min. The volume of the conductivity flow cell was 0.5 μL. The IC Net chromatography software was used for instrument control, data acquisition, and data processing.

Quantification was done based on the validated procedure ISO 10304-2 (*22*). The calculation was carried out automatically using the standard software supplied by the manufacturer against a previously prepared calibration. References for all anions were purchased in "pro analysis" quality from Merck (Darmstadt, Germany). The linear measurement range was 0.1−10 mg/L for oxalate and 0.5−100 mg/L for chloride, nitrate, and sulfate. Analytical results of samples below the lowest calibration point are reported as "not detected" (ND).

Gas Chromatography. The quantitative determination of the volatile compounds methanol, acetaldehyde, 1-propanol, 1-butanol, 2-butanol, isobutanol, 2-/3-methyl-1-butanol, 2-phenylethanol, 1-hexanol, benzyl alcohol, methyl acetate, ethyl acetate, benzyl acetate, ethyl lactate, ethyl caprylate, ethyl benzoate, and benzaldehyde using gas chromatography with flame ionization detection was based on the Community reference methods for the analysis of spirits drinks (*23*). References for all volatile substances were purchased in pro analysis quality from Sigma-Aldrich (Taufkirchen, Germany).

The GC system used for analysis was a Trace 2000 gas chromatograph (Thermo Electron Scientific Instrument Division, Dreieich, Germany). Data acquisition and analysis were performed using the Chromeleon Chromatography Information Management System (Dionex, Idstein, Germany). Substances were simultaneously separated on two fused silica capillary columns, which were attached to the inlet using an inlet splitter (Graphpack 2M dual-column injector adapter, Gerstel Analytical Equipment, Mülheim/Ruhr, Germany). This dual-column injector adapter permits the simultaneous connection of two fused silica capillary columns with different polarities to one injector and thus the parallel production of two chromatograms. Because of the design of the adapter, no split distortions due to contact of the capillary column with the inside wall of the inlet liner can occur.

Column 1: CP-WAX 52CB, 60 m × 0.32 mm i.d., film thickness 0.5 μm, Varian Deutschland GmbH, Darmstadt, Germany. Column 2: CP-SIL 5CB, 60 m × 0.32 mm i.d., film thickness 5 μm, Varian Deutschland GmbH, Darmstadt, Germany. Temperature program: 40 °C hold for 15 min, 4 °C/min to 200 °C, hold for 10 min, 15 °C/min to 230 °C, hold for 10 min. The temperature for the injection port was set at 260 °C. After addition of an internal standard (*n*-amyl alcohol), the spirits were directly injected using split injection mode (2 μL, 1:5) with helium at a constant flow rate of 6.5 mL/min as the carrier gas. For quantification, peak area ratios of the analytes to the internal standard were calculated as a function of the concentrations of the substances. As result, the average of the two columns is calculated.

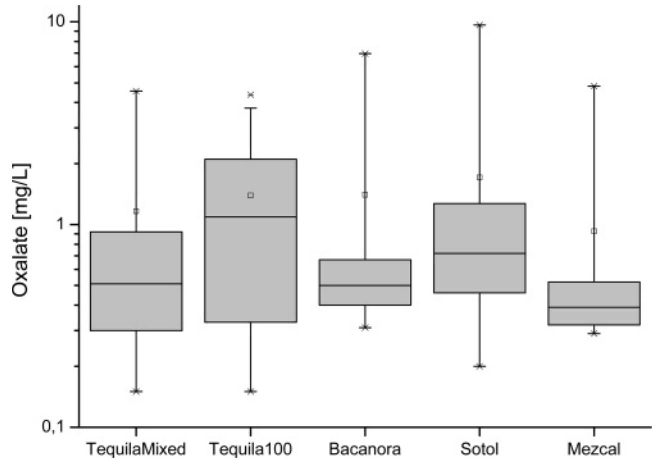

Figure 1. Box chart of the oxalate contents of Mexican *Agave* spirits (no significant difference between Tequila, Mezcal, Sotol, or Bacanora).

Statistics. All data were evaluated using Excel V9.0 (Microsoft, Unterschleissheim, Germany) and Origin V7.5 (Originlab, Northampton, MA). Statistical significance was assumed at below the 0.05 probability level. One-way analysis of variance (ANOVA) was used to test whether three or more cases have the same mean including the Bonferroni post hoc means comparison. Box and whisker plots were used for visualization of data [box 25th−75th percentile; line in the box, median; whiskers, minimum and maximum (maximum 1.5 times the length of the inner quartiles), data points outside are outliers].

RESULTS

The analytes 1-hexanol, benzyl alcohol, methyl acetate, benzyl acetate, ethyl caprylate, ethyl benzoate, and benzaldehyde were not detectable in the majority of cases. Using ANOVA, no significant differences between the spirit types could be determined for the parameters chloride, oxalate, 1-propanol, 2-butanol, and ethyl acetate. As an example, the concentrations of oxalate are shown in Figure 1. In contrast, sulfate, methanol, acetaldehyde, 1-butanol, isobutanol, 2-/3-methyl-1-butanol, 2-phenylethanol, and ethyl lactate showed significant differences in the subgroups of Mexican *Agave* spirits. These significant parameters are depicted in Figure 2.

The two Tequila categories showed differences in the methanol, 2-/3-methyl-1-butanol, and 2-phenylethanol concentrations, with lower concentrations in the mixed category. Mezcal showed no significant differences in any of the evaluated parameters that would allow a classification. Sotol showed higher nitrate concentrations and lower 2-/3-methyl-1-butanol concentrations than any other class of spirits under investigation.

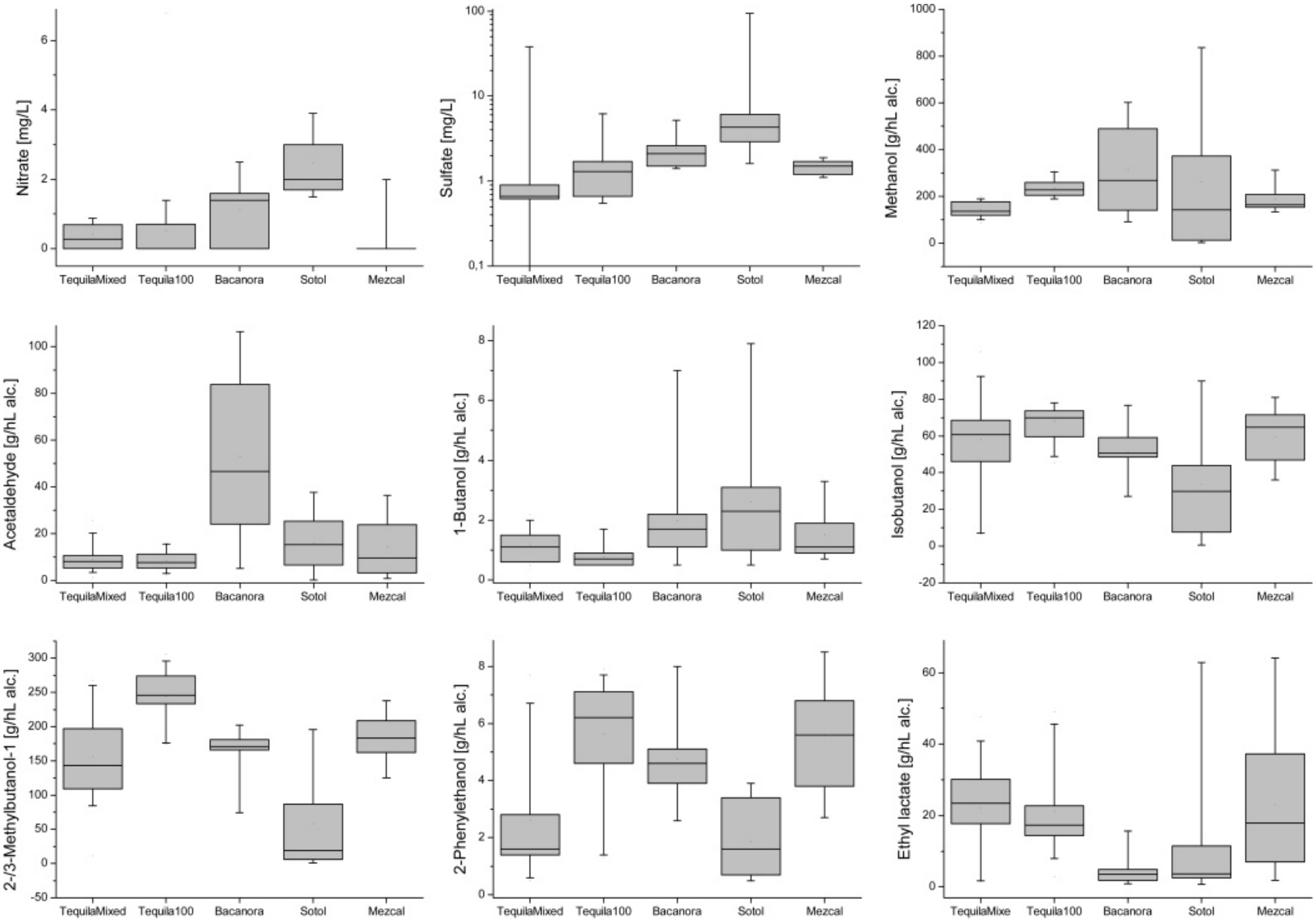

Figure 2. Box charts of analysis results with significant differences between the different types of Mexican *Agave* spirits.

Bacanora was characterized by exceptionally high acetaldehyde concentrations and a relatively low ethyl lactate content.

DISCUSSION

Oxalate as a Possible Authenticity Marker. In a previous study by our working group, there was evidence that oxalate might be a marker substance to determine the Tequila category because oxalate was found predominantly in the 100% *Agave* Tequilas. However, the sample concentrations were in the range or below the limit of quantitation (0.8 mg/L) of our standard method for the determination of anions in spirit drinks (*20*, *21*). Therefore, a chromatographic column with a higher capacity was chosen for this work so that oxalate could be detected in all samples (limit of quantitation 0.1 mg/L). It is obvious from the results that all *Agave* spirits contain significant concentrations of oxalate, but they did not allow a classification between the Tequila types or the other spirits (**Figure 1**).

The origin of oxalate is calcium oxalate crystals (raphides), which are found abundantly in all tissues of *Agave* plants (*24*–*26*). The acidic pH of the *Agave* matrix (*4*, *27*, *28*) apparently liberates the free oxalic acid in part, which is then transferred into the alcoholic distillate. Distillation experiments of oxalate model solutions (100 mg/L at 9 vol % alcohol) showed that only below pH 4 can relatively small concentrations of oxalate be found in the distillate (0.2–1.9% recovery). Oxalate was predominantly found in the last fractions of the distillation (tailings), which are normally discarded. These points clearly demonstrate that oxalate can be used as a marker for spirits distilled from oxalate-containing plant material. Because of the different influences during spirit manufacture (unknown natural content of raphides, pH, distillation conditions), however, oxalate allows no differentiation between subgroups of Mexican *Agave* spirits.

Anionic Profile of Mexican Spirits. An often-neglected aspect of the quality control of spirit drinks is the ionic composition of the water used in their production. As a result of different processing conditions, the anionic profile of Tequilas bottled in Mexico showed major differences from those bottled in the importing countries (*20*). In this study, significant differences were likewise found between Tequila (lower content of anions) and the other Mexican spirits (higher content, especially Sotol). This finding can be explained by the fact that most Tequila distilleries employ technological advances, e.g., water purification by reverse osmosis (*29*), whereas the other products are produced by rudimentary production methods (*30*).

Volatile Composition of Mexican Spirits. Relatively high genetic differences of yeasts isolated from Mexican *Agave* spirits were reported (*30*). Tequila is normally manufactured using selected yeast strains (*29*). The yeast strain is the most important factor influencing the amount of higher alcohols produced along with ethanol. Native strains isolated from Tequila were reported to produce higher amounts of such compounds as isoamyl alcohol and isobutanol compared to yeast strains employed in bakeries. Other factors with an impact on the production of higher alcohols were carbon/nitrogen ratio and temperature of fermentation (*29*, *31*). The subsequent distillation (e.g., type of pot still or rectification time) no longer alters the composition of regulated compounds and the most abundant congeners (*32*). The characteristic compound methanol is generated through hydrolysis of methylated pectins present in the *Agave* plant (*29*).

Other volatile substances (often undesirable ones) might be produced as a result of bacterial contamination of the *Agave* wort (*4*).

Because of these different influences, it was not unexpected that the volatile profile of *Agave* spirits varies over a large range. The contents of methanol and higher alcohols completely comprise the whole range defined in the Mexican Official Standards. The lowest variability was found for Tequila, which is regulated strictly to one type of *Agave* and which is made by production processes that became technologically advanced after its worldwide success. Especially large variances are obvious for the spirits with rudimentary production methods, e.g., the methanol content of Sotol was in the range between 3 and 837 g/hL of alcohol. The higher variability of Mezcal, Sotol, and Bacanora can also be explained beginning from the raw material used. A large variety of *Agaves* are allowed, and the specifications are not as clear as those for Tequila.

In this regard, our study confirms the results of Bindler et al. (*11*) who also found a large variance in the volatile composition and no significant differences in the profile of higher alcohols during the analysis of 21 Tequilas and 11 Mezcals. Our study is likewise confirmed by recent analysis results of Mezcals by De León-Rodríguez et al. (*33*), which found most of the compounds of Mezcal to be very similar to those present in Tequila. Only by use of preconcentration with solid-phase microextraction was it possible to determine minor compounds (e.g., limonene) with differences between the alcoholic beverages.

Our study and the study of De León-Rodríguez et al. (*33*), however, do not confirm Bindler's results (*11*) that Mezcal contains significantly higher contents of acetaldehyde and ethyl acetate than Tequila. Presumably, the production methods of Mezcal have been improved since 1992, when Bindler's study was conducted. The production of acetaldehyde and ethyl acetate as result of spoilage microorganisms (e.g., *Acetobacter*) is often correlated with production hygiene.

The methanol content was the most problematic compound regarding the Mexican standard. Two Tequilas (4%), five Sotols (31%), and six Bacanoras (46%) had levels above the maximum methanol content of 300 g/hL of alcohol. For Tequila, the exceeding (methanol: 304 and 308 g/hL of alcohol) of two 100% Tequilas was not significant. In contrast, Sotol (methanol: 371, 493, 653, 782, and 837 g/hL of alcohol) and Bacanora (methanol: 318, 483, 489, 502, 581, and 601 g/hl of alcohol) showed higher deviations than Tequila. It should be noted that these methanol levels are not yet of toxicological relevance (*34*). Some other spirit types have significantly higher methanol levels than Mexican *Agave* spirits, e.g., German fruit spirits have legally allowed methanol levels up to 1000 g/hl of alcohol (*35*).

These findings verify a previous study of Tellez et al. (*36*) that described methanol as one of the most important problems for the producers of *Agave* beverages. The standards for higher alcohols were observed by all spirits except for Sotol samples (25%), which had concentrations below the minimum of 20 g/hL of alcohol.

As a permanent problem in food control, the basic categories of Tequila (100% *Agave* and mixed Tequila) must be distinguished. For the high-quality category 100% *Agave*, only pure *Agave* juice is allowed to be fermented and distilled. A mixed Tequila is manufactured by adding up to 49% (w/v) of sugar, mainly from sugar cane (*5*). This lower-end Tequila is usually shipped out in bulk containers for bottling in the importing countries, and there is, of course, a high economic incentive for labeling fraud. Our results confirm the recent study of Bauer-Christoph et al. (*18*) that the concentrations of methanol and 2-/3-methyl-1-butanol are the most suitable analytical approach to differentiate 100% *Agave* and mixed Tequila.

ACKNOWLEDGMENT

The skillful technical assistance of S. Gonzalez, H. Heger, M. Jaworski, and K. Müller is gratefully acknowledged. We thank Casa Herradura S.A. de C.V. for donation of tequila samples.

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Received for review January 12, 2006. Revised manuscript received March 27, 2006. Accepted April 2, 2006.

JF060094H