

Tequila Authenticity Assessment by Headspace SPME-HRGC-IRMS Analysis of $^{13}\text{C}/^{12}\text{C}$ and $^{18}\text{O}/^{16}\text{O}$ Ratios of Ethanol

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By use of headspace SPME sampling and a PLOT column, on-line capillary gas chromatography–isotope ratio mass spectrometry was employed in the combustion (C) and the pyrolysis (P) modes (HRGC-C/P-IRMS) to determine the $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^{18}\text{O}_{\text{VSMOW}}$ values of ethanol in authentic ($n = 14$) and commercial tequila samples ($n = 15$) as well as a number of other spirits ($n = 23$). Whereas with $\delta^{13}\text{C}_{\text{VPDB}}$ values ranging from -12.1 to -13.2% and from -12.5 to -14.8% similar variations were found for 100% agave and mixed tequilas, respectively, the $\delta^{18}\text{O}_{\text{VSMOW}}$ data differed slightly within these categories: ranges from $+22.1$ to $+22.8\%$ and $+20.8$ to $+21.7\%$ were determined for both the authentic 100% agave and mixed products, respectively. The data recorded for commercial tequilas were less homogeneous; $\delta^{13}\text{C}_{\text{VPDB}}$ data from -10.6 to -13.9% and $\delta^{18}\text{O}_{\text{VSMOW}}$ values from $+15.5$ to $+22.7\%$ were determined in tequilas of both categories. Owing to overlapping data, attempts to differentiate between white, rested, and aged tequilas within each of the two categories failed. In addition, discrimination of tequila samples from other spirits by means of $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^{18}\text{O}_{\text{VSMOW}}$ data of ethanol was restricted to the products originating from C_3 as well as C_4/CAM raw materials.

KEYWORDS: Agave (*Agave tequilana* Weber var. Azul); tequila; authenticity; spirits; ethanol; isotope ratio analysis; carbon isotope; oxygen isotope; HRGC-C/P-IRMS

INTRODUCTION

There are around 135 species of agave plants native to Mexico (*J*). Some agaves are used to produce alcoholic beverages such as mezcal (*A. angustifolia*; *A. potatorum*; *A. salmiana*), sotol (*A. dasylirion*; *Dasylirion* spp.), bacanora (*A. angustifolia*; *A. potatorum*; *A. pacifica*), and pulque (*A. salmiana*), but the production of tequila is restricted by law to the blue agave (*A. tequilana* Weber var. Azul) as well as to specifically designated geographic areas, primarily the state of Jalisco in west-central Mexico (2).

Blue agave is cultivated for 6–12 years before it reaches sexual maturity. This redirects the sugars into the central stalk, swelling it into a large bulbous shape that contains a sweet juicy pulp. The plant is then cut from its roots and the long sword-shaped leaves are removed. The remaining piña (25–100 lb) delivers the raw material for subsequent ethanol production. At the distillery the piñas are cut and then slowly baked in steam ovens or autoclaves (oversized pressure cookers) to hydrolyze inulin. The next step comprises milling to extract the sweet juice.

The subsequent fermentation stage determines whether the final product will be 100% agave, i.e., use of solely agave juice mixed with some water, or mixed (mixto), i.e., addition up to 49% (w/v) of an adjunct sugar, mainly from cane. By Mexican law all 100% agave (and aged tequila; cf. below) must be bottled in Mexico. This high-quality category is always declared on the bottle label; if it does not say 100% it is a mixed (although that term is seldom used on the label). Distillation is traditionally performed in a two-step process in pot stills.

Beyond the mentioned two basic categories of tequila there are four types: Silver or blanco/white tequilas are clear, with little (no more than 60 days in stainless steel tanks) or no aging. They can be either 100% agave or mixed. Gold tequila is unaged silver tequila that has been colored and flavored with caramel; it is usually a mixed one. Reposado (rested) tequila is aged in wooden tanks or casks for a legal minimum period of 2 months, with the better-quality brands aged 3–9 months in wood. It can be either 100% agave or mixed. Añejo (aged) tequila is aged in wooden barrels (maximum size 600 L) for a minimum of 12 months. The best-quality añejos are aged 18 months to 3 years for mixed and up to 4 years for 100% agaves.

Tequila is protected under the North American Free Trade Agreement (NAFTA), but Mexico has to defend tequila's exclusivity in bilateral negotiations with the European Union

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and, most recently, with China (3). Considering this situation together with the increase of the international demand for tequila, which has skyrocketed over the past few years (3), it will be obvious that there is a fundamental interest to evaluate parameters to assess tequila's authenticity.

As to the raw material, *A. tequilana* Weber var. Azul, first attempts have been made to assay the levels of genetic variation (4). Tequila has been characterized by analyzing the composition of its flavor constituents by instrumental and sensory techniques (5, 6), as well as by coupling gas chromatography and electronic nose (7). The successful assignment of raw materials and authentication of spirits obtained by MS and ^2H NMR isotope ratio measurements (8–10) including $^{13}\text{C}/^{12}\text{C}$ and $^{18}\text{O}/^{16}\text{O}$ determinations of ethanol (11–14) encouraged us to apply the on-line capillary gas chromatography–isotope ratio mass spectrometry in the combustion (C) and the pyrolysis (P) modes (HRGC-C/P-IRMS) in combination with the headspace SPME technique (15) for the authenticity assessment of various types of tequila. The results obtained in this study, in which authentic white, rested, and aged tequilas, as well as commercial samples were included together with a number of spirits of other sources, are reported in this paper.

MATERIALS AND METHODS

Samples. Authentic tequila samples both of 100% agave ($n = 7$) and mixed categories ($n = 7$) were available each as white, rested, and aged from controlled tequila production in the Jalisco region. In addition, commercial tequila samples (100% agave, $n = 6$; mixed, $n = 9$), as well as spirits from other sources (rum, $n = 8$; vodka, $n = 6$; wine distillates, $n = 9$), were investigated. Reference ethanol was purchased from Riedel de Haën (Sigma-Aldrich, Seelze, Germany, no. 32205, batch no. 10530) and dried over molecular sieve (3 Å, Fluka, Switzerland).

Headspace SPME. Headspace SPME sampling was performed after 12 h equilibration (25 °C) of 500- μL samples in 20-mL vials (closed with Teflon septa and aluminum caps) using 65- μm fiber coated with poly(dimethylsiloxane)/divinylbenzene (Supelco). Adsorption and desorption times of 10 and 60 s, respectively, were used. The injector temperature was kept at 180 °C.

Gas Chromatography–Isotope Ratio Mass Spectrometry (HRGC-IRMS). A Finnigan Delta plus XL isotope ratio mass spectrometer coupled by an open-split via a combustion/pyrolysis (C/P) interface to an HP 6890 gas chromatograph (GC) was used. The GC was equipped with an HP PLOT-Q column (30 m \times 0.32 mm i.d.; $d_f = 0.32 \mu\text{m}$). The following conditions were used: temperature program, raised from 100 to 110 °C at 5.5 °C/min, then with 10 °C/min at 230 °C; flow, helium at constant pressure of 25 psi; blackflush time, 600 s, to cut off the water peak of the samples.

Interfaces. $^{13}\text{C}/^{12}\text{C}$: combustion by oxidative reactor (Al_2O_3 , 0.5 mm i.d., 1.5 mm o.d., 320 mm) with Cu, Ni, Pt (each 240 mm \times 0.125 mm) to CO_2 at 960 °C; water separation by Nafion membrane.

Pyrolysis. $^{18}\text{O}/^{16}\text{O}$: pyrolysis in the reactor (Al_2O_3 ; 1.5 mm o.d., 320 mm; Pt, Ni) to CO at 1250 °C, using auxiliary ("magic-mix") gas, 1% hydrogen in helium, flow 0.7 mL/min.

In addition, coupling the elemental analyzers (EA) ($^{13}\text{C}/^{12}\text{C}$, Euro Vector EA 3000, Milano, Italy; temperature, 1000 °C; $^{18}\text{O}/^{16}\text{O}$, HT Sauerstoff-Analysator, HEKATech, Wegberg, Germany; temperature, 1460 °C) to the IRMS was realized for off-line control determination of ethanol reference.

Daily system stability checks were carried out by measuring a reference ethanol sample with known $^{13}\text{C}/^{12}\text{C}$ and $^{18}\text{O}/^{16}\text{O}$ ratios. Stability check of the used reference gases was continuously performed by measuring International Atomic Energy Agency (IAEA) standards (NBS 22, IAEA-CH-7, VSMOW, and IAEA-CO-9).

The isotope ratios are expressed in per mil (‰) deviation relative to the VPDB and VSMOW international standards. For $^{13}\text{C}/^{12}\text{C}$ determinations the mass spectrometer was calibrated against reference CO_2 gas (Messer Griesheim, Frankfurt, Germany) with a defined

$\delta^{13}\text{C}_{\text{VPDB}} = -24.9\text{‰}$. Results are expressed in $\delta^{13}\text{C}_{\text{VPDB}}$ values as

$$\delta^{13}\text{C}_{\text{VPDB}} [\text{‰}] = \left(\frac{R_{\text{sample}} - R_{\text{VPDB}}}{R_{\text{VPDB}}} \right) \times 1000 \quad (\text{a})$$

where R is the isotope ratio $^{13}\text{C}/^{12}\text{C}$.

Noncertified CO gas (Linde AG, Oberschleißheim, Germany) was used as working standard. Owing to the parallel connection of a pyrolysis EA to the IRMS, the $\delta^{18}\text{O}_{\text{VSMOW}}$ value of the CO gas could be determined by measuring the interantional IAEA standard VSMOW (with known $\delta^{18}\text{O}_{\text{VSMOW}}$ value of 0). The calculation of the $\delta^{18}\text{O}(\text{CO})_{\text{VSMOW}}$ was then performed according to

$$\delta^{18}\text{O}(\text{CO})_{\text{VSMOW}} [\text{‰}] = \left[\frac{\left(\frac{\delta^{18}\text{O}(\text{VSMOW})_{\text{VSMOW}} + 1}{1000} \right)}{\left(\frac{\delta^{18}\text{O}(\text{VSMOW})_{\text{CO}} + 1}{1000} \right)} - 1 \right] \times 1000 \quad (\text{b})$$

thus, indirect calculation of $\delta^{18}\text{O}_{\text{VSMOW}}$ values of substances to be determined could be realized. As mean value for the CO reference gas, $\delta^{18}\text{O}_{\text{VSMOW}} = +7.8 \pm 0.2\text{‰}$ was calculated. Daily performed control analyses using the IAEA standard VSMOW or water samples with known $^{18}\text{O}/^{16}\text{O}$ ratios revealed high stability of the CO gas within the given standard deviation.

In general, 5-fold determinations were carried out, and standard deviations were calculated.

RESULTS AND DISCUSSION

As already learned from previous HRGC-P-IRMS studies carried out with several volatile compounds, pyrolysis shows, in contrast to the C-IRMS mode, structurally dependent kinetics (16–18) which requires determination of the range of sample amounts in which not only reproducibility but also linearity will be obtained. Thus, first, the $\delta^{18}\text{O}_{\text{VSMOW}}$ values of various amounts of reference ethanol were reproducibly determined off-line via the equipped elemental analyzer (EA). In subsequent HRGC-P-IRMS analyses the kinetics of ethanol pyrolysis was also checked. The $\delta^{18}\text{O}_{\text{VSMOW}}$ values for the reference ethanol were $+16.5 \pm 0.2\text{‰}$ and $+16.4 \pm 0.5\text{‰}$ determined by EA-P-IRMS and on-line HRGC-P-IRMS analyses, respectively. Thus, the on-line-determined $\delta^{18}\text{O}_{\text{VSMOW}}$ data agreed well with those measured by EA analysis. In EA measurements, reproducible linearity was observed from <0.5 to $2.5 \mu\text{L}$. In on-line HRGC determinations, linearity was found at broad ethanol sample volumes (ethanol concentrations in the gas phase not evaluated), but at volumes $>500 \mu\text{L}$ the dynamic of IRMS detection reached its limit.

In a series of additional fundamental studies the conditions for the headspace SPME sampling were elaborated. In accordance with previous experiences made by others in the course of headspace IRMS analyses (11), a rather extended equilibration time of 12 h at room temperature was required to obtain reproducible data, in particular, regarding $^{13}\text{C}/^{12}\text{C}$ determination. As another critical parameter the ethanol concentration of the samples was found. As shown from **Figure 1**, reproducible $^{18}\text{O}/^{16}\text{O}$ measurements were only possible at ethanol concentrations $>30 \text{ vol } \%$.

Finally, in a series of optimizing the HRGC conditions, the most suitable temperature program was evaluated, including the elaboration of a blackflash time of 600 s in order to cut off the water peak in the samples and to provide an ideal peak form for the ethanol to be analyzed.

After having evaluated reproducible optimal conditions for the headspace SPME HRGC-IRMS determinations, three series of analyses were performed, comprising (i) authentic 100%

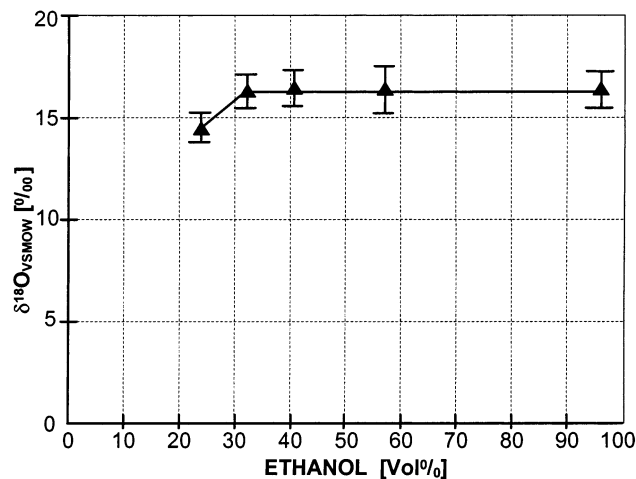


Figure 1. $\delta^{18}\text{O}_{\text{VSMOW}}$ data of reference ethanol determined by headspace SPME HRGC-P-IRMS in water/ethanol mixtures.

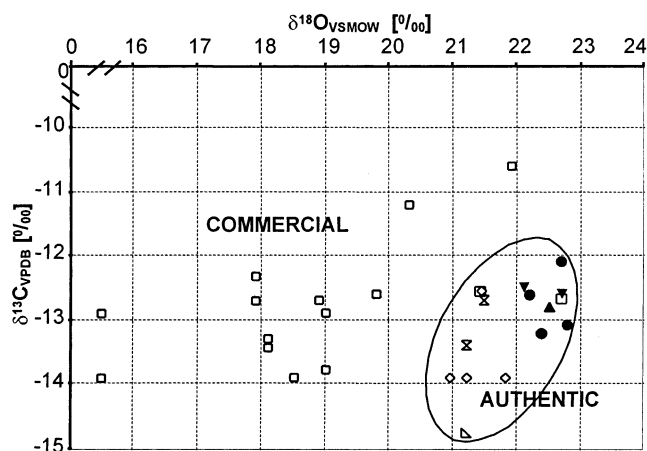


Figure 2. Correlation of $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^{18}\text{O}_{\text{VSMOW}}$ data of ethanol determined by headspace SPME HRGC-C/P-IRMS in authentic and commercial tequila samples (mean values from five determinations with standard deviations of 0.1–0.2 and 0.2–0.5 for $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^{18}\text{O}_{\text{VSMOW}}$, respectively). 100% agave: white, \blacktriangle ; rested, \bullet ; aged, \blacktriangledown . Mixed: white, (open right triangle); rested, \diamond ; aged, \bar{X} ; commercial tequila, \square .

agave and mixed tequilas, in both categories white, rested, and aged types were available, (ii) commercial tequilas, and (iii) other commercial spirits, i.e., rum, vodka, and wine distillates. The results of the first two series of analyses are summarized in **Figure 2**, in which the $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^{18}\text{O}_{\text{VSMOW}}$ data of ethanol were correlated in a graph. At first glance it is obvious that the isotope data obtained from the authentic samples are much more homogeneous than those of the commercial tequilas. A more detailed check reveals the following information about the authentic products: Whereas with $\delta^{13}\text{C}_{\text{VPDB}}$ values ranging from -12.1 to -13.2‰ and from -12.5 to -14.8‰ similar variations were found for 100% agave and mixed tequilas, respectively, the $\delta^{18}\text{O}_{\text{VSMOW}}$ data differed within these categories: ranges from $+22.1$ to $+22.8\text{‰}$ and $+20.8$ to $+21.7\text{‰}$ were determined for the 100% agave and mixed tequilas, respectively. In the analytically heterogeneous group of commercial products of both categories, the values ranged from -10.6 to -13.9‰ and $+15.5$ to $+22.7\text{‰}$ for $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^{18}\text{O}_{\text{VSMOW}}$, respectively. As also can be seen from **Figure 2**, the data did not allow differentiation between white, rested, and aged types within each of the 100% agave or mixed tequilas.

In **Figure 3**, again as correlation between $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^{18}\text{O}_{\text{VSMOW}}$ values, the data obtained with other commercial

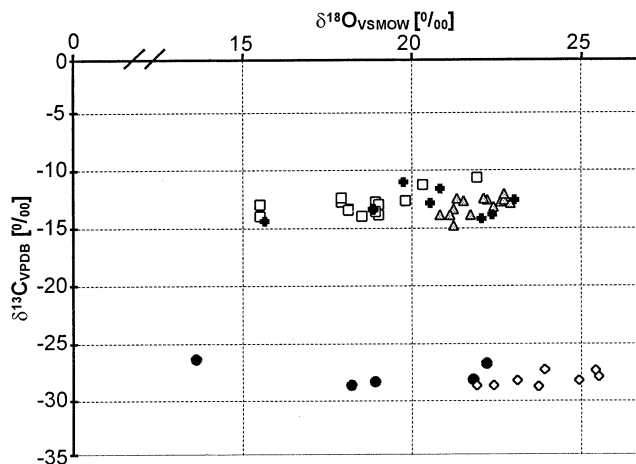


Figure 3. Correlation of $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^{18}\text{O}_{\text{VSMOW}}$ data of ethanol determined by headspace SPME HRGC-C/P-IRMS in authentic and commercial tequilas as well as in other spirits (mean values from five determinations with standard deviations of 0.1–0.2 and 0.2–0.5 for $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$, respectively). Commercial tequila, \square ; authentic tequila, (gray triangle); rum, (black cross); vodka, \bullet ; wine distillates, \diamond .

spirits were compared with those of the tequilas under study. Clear-cut discrimination was obtained between the group of rums (with obviously one adulterated sample) with tequilas vs that of vodkas (with probably three adulterated samples) and wine distillates. Thus, differentiation was restricted to the products originating from C_3 and C_4/CAM raw materials.

In conclusion, this is the first time that defined authentic tequila samples from the Jalisco region were subjected to stable isotope analysis. Despite the limited number of samples, the restriction to one production line, and the lack of control of fermentation conditions (19), the information provided especially by the $^{18}\text{O}/^{16}\text{O}$ ratio analysis of ethanol can be helpful to the industry and the control laboratories for the authenticity assessment of tequila. In addition, with these data the rather scarce knowledge about the $\delta^{18}\text{O}_{\text{VSMOW}}$ values of ethanol from spirits was extended. Whereas discrepancies with few reported values exist (13), our data fit well with earlier information provided by Kornexl (20).

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