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Tequila Volatile Characterization and Ethyl Ester Determination by Solid Phase Microextraction Gas Chromatography/Mass Spectrometry Analysis

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Solid phase microextraction (SPME) and gas chromatography were used for tequila volatile characterization and ethyl ester quantitation. Several factors determined the differences in tequila volatile profiles obtained by the SPME technique, namely, sampling mode, fiber coating, and fiber exposure time. Each of these factors determined the most suitable conditions for the analysis of volatile profiles in tequila. Volatile extraction consisted of placing 40 mL of tequila in a sealed vial kept at 40 °C. A poly(dimethylsiloxane) fiber was immersed in the liquid for 60 min and desorbed for 5 min into the gas chromatograph. The identified volatiles by mass spectrometry were mainly alcohols, esters, and ketones. The calibration curves for ethyl hexanoate, octanoate, and decanoate followed linear relationships with highly significant ($p < 0.001$) determination coefficients ($R^2 = 0.99$). The coefficients of variation of less than 10% for ethyl ester concentrations indicated that the technique was reproducible. The limits of quantitation for ethyl esters were 0.05 parts per million, which were below the concentration range (0.27−15.03 ppm) found for different tequila samples. Quantitative differences in ethyl esters were found for the four most commonly known tequila types: silver, gold, aged, and extra-aged.

KEYWORDS: Tequila volatiles; ethyl esters; solid phase microextraction gas chromatography

INTRODUCTION

Tequila is an internationally known distilled spirit associated with Mexico. This beverage is obtained by the distillation of fermented juice from the agave plant (*Agave tequilana* Weber var. blue). When 100% (w/v) of the sugars come from the agave juice, it is called "tequila 100% agave". However, when up to 49% (w/v) of the sugars come from a source other than the agave, usually sugar cane, the product is called "tequila"(*1*).

Tequila's delicate flavor is a combination of aroma and taste, and both determine the consumer's acceptance of it. Although aroma, which is determined by volatiles, is considered to have a major impact on flavor perception in all distilled spiritious drinks, these compounds together with nonvolatiles constitute a complex mixture in a water−ethanol matrix (*2*). Fermentation is the most important part of the process since it is during this step that sugars are converted to ethanol and other compounds such as esters and organic acids; these compounds, along with other substances derived from the cooked agave, give the characteristic flavor and taste to tequila (*3*).

In 1997, tequila was recognized by the European Union as being an alcoholic drink of Mexican origin, which could only be produced in certain parts of Mexico. The product known as silver or white tequila is clear with no aging, produced from a fermented wort containing not less than 51% sugars from the agave plant (*1*). Gold tequila is the result of a mixture of the white product with aged and/or extra-aged tequila, which may contain caramel color, sugar syrup, natural oak extract, and/or glycerin in no more than 1% total (w/w) to smoothen the flavor (*1*). Aged and extra-aged tequila are white tequila matured in wood containers or oak casks for at least 2 and 12 months, respectively, and may contain the above ingredients to smoothen the flavor (*1*).

Mexican regulations specify chemical differences among tequila types based on the total esters determined by a volumetric method (*1*); however, important ester compositional differences cannot be obtained by this methodology. By determining ester concentrations individually rather than as a group, a clearer distinction between tequila types may be achieved.

Although tequila is a very popular drink that is well-known internationally, there are few studies on its volatile composition. More than 175 components were identified in a tequila dichloromethane extract, from which 60 odorants were detected (*4*). However, efforts at reconstituting the tequila flavor from its component parts were not successful, indicating that further significant contributors to the tequila flavor remain to be identified (*4*). In another study, qualitative and quantitative differences among tequila types were determined by gas

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chromatography (GC)−olfactrometry of dichloromethane extracts obtained by liquid−liquid extraction (*5*).

Solid phase microextraction (SPME) is a sampling technique for introducing analytes into the gas chromatograph that may be useful in this application since it is solvent-free and sample handling is minimized. An additional advantage is that it offers the possibility to directly sample the vapor phase in equilibrium with the matrix (headspace, HS SPME) (*6*). A second sampling mode consists of immersing the fiber into a liquid sample or a sample extract (liquid sampling, LS SPME) (*6*). SPME is based on volatile partitioning from a liquid or gaseous sample onto an immobilized stationary phase and has been recommended for the quantitative analysis of flavor and fragrance compounds (*7*−*9*). The SPME technique was applied to nonalcoholic drinks such as fruit juices (*8*) and coffee beverages (*6*) and to alcoholic drinks such as wine (*10*, *11*), beer (*12*), and vodka (*13*). Thus, the objective of this study was to establish a SPME and GC method that allows tequila volatile characterization and ethyl esters quantitation.

MATERIALS AND METHODS

Reagents. Ethyl esters (C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}, and C_{18}), methyl esters (C_7, C_{10}, and C_{12}), ethanol, and 1-propanol standards were from PolyScience, Co. (Niles, IL).

Samples. Tequila labeled as white, gold, aged, and extra-aged 100% agave was purchased from the local market. The particular tequila brands selected for the study were popular brands in the Mexican market. For quantitative analysis, the samples were analyzed in triplicate.

SPME Procedure. The SPME device was purchased from Supelco Co. (Bellefonte, PA) as were the fused silica fibers coated with poly(dimethylsiloxane) (PDMS, 100 μm), PDMS/divinylbenzene (DVB) (65 μm), carbowax (CW)/DVB (65 μm) and poly(acrylate) (PA, 85 μm). For direct LS, the syringe of the SPME was introduced through a septum into a 60 mL vial where a 40 mL sample was maintained at 40 °C and stirred at 75 rpm in an Orbit shaker bath (Labline, Chicago, IL). The fiber was then drawn into the tequila in such a way that it was just immersed into the liquid. For HS sampling, the fiber was exposed to the space above a 40 mL sample saturated with 28% NaCl, maintained at 40 °C, and stirred at 75 rpm in an Orbit shaker bath (Labline). When the sampling modes were compared, a PDMS fiber was exposed for 60 min. Different sampling times (30, 60, and 90 min) were compared by using a PDMS fiber and direct LS. After sampling, the SPME device was retracted into its housing and removed from the sample vial, immediately inserted into the GC injector, pushed outside its housing, and thermally desorbed for 5 min at 150 °C. When the sampling modes and sampling times were compared, a PDMS fiber was used.

Chromatographic Analysis. The GC used was a Hewlett-Packard 6890 provided with a HP splitless SPME liner (0.75 mm i.d, Supelco Co.). The capillary column used was a HP-5 (30 m × 0.32 mm i.d., 0.25 μm film thickness; Hewlett-Packard). The chromatographic conditions were as follows: 35 °C for 2 min, increased at 5 °C/min to 230 °C, and maintained at this temperature until a 60 min run was completed. Helium was used as a carrier gas at a flow rate of 1.8 mL/min, and the flame ionization detection temperature was maintained at 250 °C.

GC-MS Analysis. GC-MS analyses were carried out on a GC 3400CX/MS Saturn 3 (Varian, Walnut Creek, CA). The ion trap was operated at 180 °C in the electron impact mode with an energy of 70 eV, scanning from *m*/*z* 34 to 400 at 0.6 s/scan. Capillary GC separations were carried out with the same column and under conditions analogous to those reported in the previous paragraph. For GC-MS, fiber exposure and desorption times were limited to 3 min and 30 s, respectively. The compounds were tentatively identified by comparing their mass spectra with those obtained in the NIST92/EPA/NIH Mass Spectral Database.

Calibration Curves for Ester Quantitation. Standard solutions of ethyl esters, hexanoate, octanoate, and decanoate were used for constructing calibration curves for the quantitation of major esters present in tequila. Sampling conditions for constructing the calibration curves or for analyzing tequila samples were as follows: samples (40 mL) at 40 °C; direct liquid as sampling mode; a PDMS fiber; and a 60 min sampling time. After sampling, the fiber was thermally desorbed for 5 min at 150 °C in the GC injection port. The chromatographic conditions were those described in the section entitled Chromatographic Analysis. A standard stock solution (20 000 ppm) was prepared by dissolving the ethyl esters in 96% ethanol and storing it at 4 °C. To prepare the standard mixture solutions, 50 mL of deionized water:ethanol (60:40) was mixed to simulate tequila composition and aliquots of the stock solution were used to prepare 0.5, 2.5, 5.0, 10, and 20 ppm final concentrations of ethyl esters. The standard mixture solutions and tequila samples containing methyl octanoate (10 ppm) as an internal standard were analyzed in triplicate. The concentrations of ethyl dodecanoate, tetradecanoate, hexadecanoate, and octadeanoate were calculated based on the calibration curves constructed for ethyl decanoate. Attempts to construct their corresponding calibration curves failed since replicate analyses were highly irreproducible possibly due to problems with the solubility of the standards. The calibration curves for individual ethyl esters were constructed by applying linear regression analysis using Systat/Sygraph (Systat, Inc., Evanston, IL) on concentrations (ppm) vs ethyl ester peak area/internal standard area. The precision of the method was determined by performing five consecutive analyses. The limit of quantitation for each ester was calculated to be the concentration that produced a signal-to-noise ratio of 5.

Table 1. Effect of Sampling Mode on Extraction of Ethyl Esters from Tequila[a]

	sampling mode			
	direct liquid[a]		HS[b]	
ethyl ester	mean peak area	CV[c] (%)	mean peak area	CV[c] (%)
octanoate	173.8	1.6	80.4	25.2
decanoate	1030.1	1.8	251.6	13.6
dodecanoate	930.5	2.5	41.9	3.5
tetradecanoate	320.3	5.2	ND[d]	
hexadecanoate	2120.1	6.3	ND[d]	
octadecanoate	2534.8	8.6	ND[d]	

[a] Sample, gold tequila (40 mL) at 40 °C; fiber, PDMS; fiber exposure time, 60 min. [b] Sample contained 28% NaCl. [c] CV, coefficient of variation, $n = 5$. [d] ND = not detectable.

Table 2. Effect of Sampling Time on Extraction of Ethyl Esters from Tequila[a]

	sampling time (min)					
	30		60		90	
ethyl ester	mean peak area	CV[b] (%)	mean peak area	CV[b] (%)	mean peak area	CV[b] (%)
octanoate	138.1	3.2	173.8	1.6	185.51	0.96
decanoate	463.6	9.2	1030.1	1.8	1067.44	6.4
dodecanoate	322.5	11.9	930.5	2.5	940.60	11.2
tetradecanoate	186.4	13.3	320.3	5.2	322.17	2.6
hexadecanoate	325.7	9.6	2120.1	6.3	1980.23	14.6
octadecanoate	456.9	17.8	2534.8	8.6	2402.0	12.5

[a] Sample, gold tequila (40 mL) at 40 °C; sampling mode, direct liquid; fiber, PDMS. [b] CV, coefficient of variation, $n = 5$.

RESULTS AND DISCUSSION

SPME Conditions. Several factors were shown to determine qualitative and quantitative differences in tequila volatile profiles obtained by the SPME technique, namely, sampling mode, fiber coating, and fiber exposure time. Each of these factors was investigated to determine the most suitable conditions for the analysis of volatile profiles in tequila. Particular interest was given to ethyl esters since preliminary experiments showed

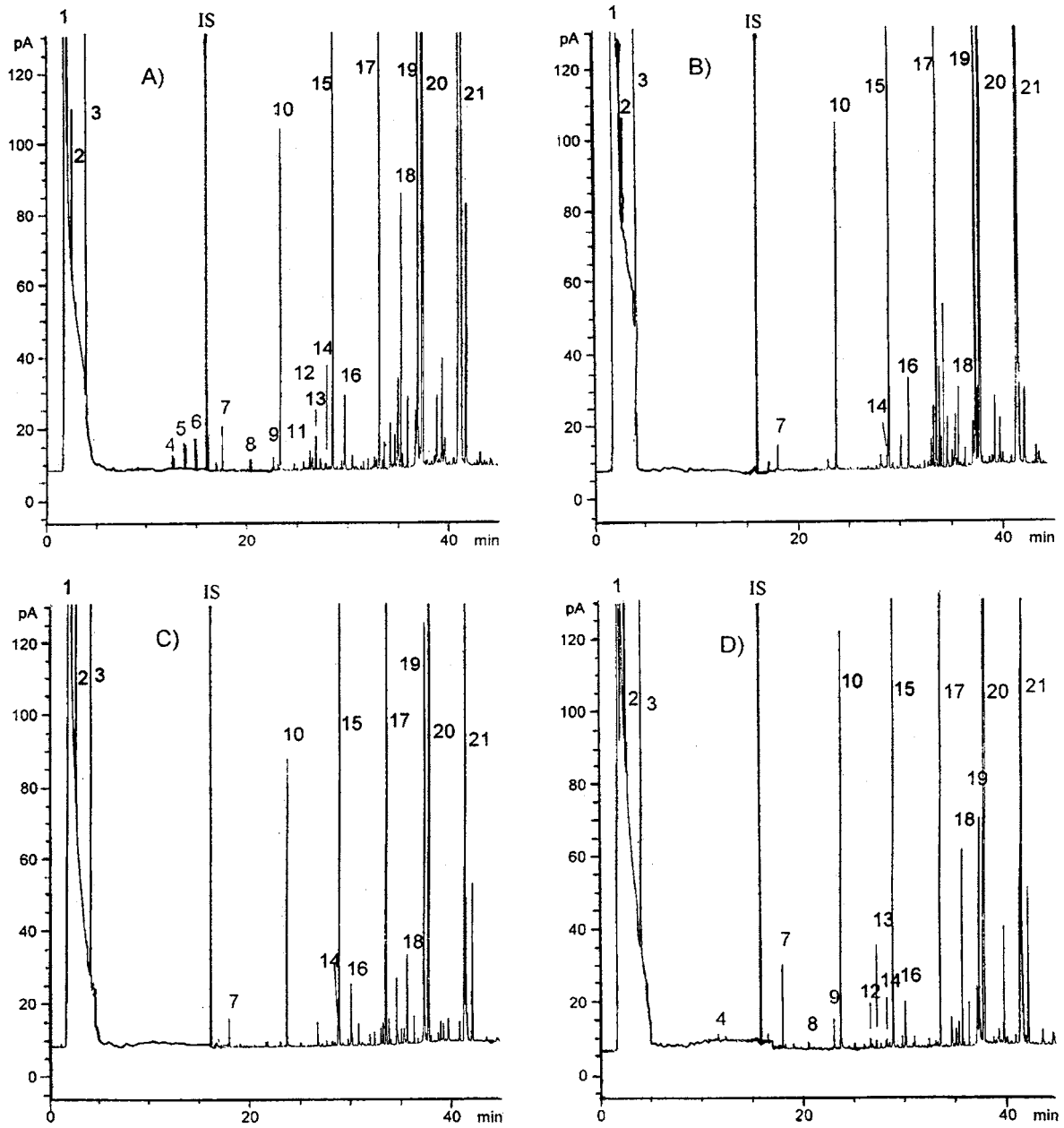

Figure 1. Typical volatile profiles of white (**A**), gold (**B**), aged (**C**), and extra-aged (**D**) tequila. Peaks: 1, ethanol;[a] 2, 1-propanol;[a] 3, 3-methylbutanol;[b] 4, ethyl hexanoate;[a] 5, 4-methylheptanol;[b] 6, methyl heptanoate;[a] IS, internal standard, methyl octanoate; 7, ethyl octanoate;[a] 8, methyl decanoate;[a] 9, 2-buten-1-one;[b] 10, ethyl decanoate;[a] 11, 3-methyl butyl octanoate;[b] 12, propyl decanoate;[b] 13, methyl dodecanoate;[a] 14, butyl decanoate;[b] 15, ethyl dodecanoate;[a] 16, 3-methyl ethyl decanoate;[b] 17, ethyl tetradecanoate;[a] 18, 2-phenyl ethyl octanoate;[b] 19, 3-hexanone;[b] 20, ethyl hexadecanoate;[a] and 21, ethyl octadecanoate.[a] [a]Positively identified by SPME GC-MS and retention times of authentic analytical standards. [b]Tentatively identified by SPME GC-MS.

marked profile differences among tequila types. Other factors such as sampling temperature and sampling volume were kept constant at 40 °C and 40 mL. For sampling mode selection, a PDMS fiber was used since it is the most popular coating.

Qualitative and quantitative differences were observed for the two types of sampling, HS and direct liquid extraction, when volatiles were adsorbed on a PDMS fiber for 1 h (**Table 1**). Less volatiles, both in number and amount, were recovered by HS than by direct LS, as it was shown by peak areas for ethyl esters (**Table 1**). Thus, direct LS presented higher recoveries and lower detection limits than HS sampling for the compounds tested. Higher coefficients of variation (CV) for HS sampling indicated that this sampling mode was less precise than direct LS (**Table 1**). Semivolatile compounds such as the longer chain ethyl esters (C_{14}, C_{16}, and C_{18}) were not detected in the HS despite sample saturation with NaCl (28%). Saturating the sample with salt affects partitioning of organic analytes out of an aqueous phase; thus, salt addition is frequently used to drive polar compounds into the HS to increase the amount of volatiles extracted (*13*), but it has a relatively insignificant effect on nonpolar compounds (*13*). It appears that longer chain esters, ethyl tetradecanoate, hexadecanoate, or octadecanoate present in tequila were not released into the HS since they are relatively nonpolar.

Practically, direct LS is necessary for compounds of very low volatility. Semivolatile compounds diffuse relatively slow into the HS above the liquid phase and into the fiber; therefore, longer adsorption times are required to achieve a satisfactory response with HS sampling (*14*). Thus, for sample matrices such as tequila, which do not contain contaminants such as fat or protein that may interfere with extraction, direct LS was preferred. Both types of sampling have been used for the

analysis of volatiles in alcoholic beverages. HS SPME was used for determining dimethyl sulfide in beer after saturation with salt in order to increase sensitivity (*15*). Others reported the use of direct LS SPME for characterizing volatiles in commercial vodkas (*12*). Thus, selection of the sampling mode is made by considering the sample matrix, analyte volatility, and its affinity to the matrix (*13*).

An evaluation of different fiber coatings showed that the most suitable fiber was PDMS, since CV of less than 10% were obtained for all peak ethyl ester areas. Similarly, CV of less than 10% were reported when esters and alcohols were added to beer (*11*) or when ethyl esters were analyzed in vodka (*12*). When PA or PDMS/DVB was tested, higher CV (>17%) were obtained. Similarly, volatile profiles obtained with CW/DVB were highly irreproducible since CVs as high as 63% were calculated. Thus, the PDMS fiber coating was used for further work. PDMS was recommended by the manufacturer for the analysis of nonpolar compounds and was the most suitable fiber for the analysis of tequila volatiles. Ethyl esters are relatively nonpolar compounds, and these were after ethanol, the most abundant volatiles in tequila. Although the four different types of fibers tested produced similar volatile profiles, the reproducibility of the PDMS profiles was superior. The optimum coating type for a broad range of compound characteristics requires experimentation with different fibers; however, for different groups of analytes, primary consideration should be given to the most difficult analytes (*13*).

In general, volatiles increased with fiber exposure time during the first 60 min (**Table 2**). Area counts increased dramatically from 30 to 60 min and then reached a plateu from 60 to 90 min, indicating that equilibrium was almost attained. A 60 min extraction time was optimal since recoveries were maximized as shown by higher area counts and variation was minimized as indicated by lower CV (**Table 2**). Thus, a 60 min extraction time was used for further studies. SPME, unlike other sampling techniques, is not based on an exhaustive extraction of the sample but on an equilibrium between the analyte concentration in the sample and that in the solid phase fiber coating. Although the time required to reach the equilibrium is the optimal sampling time, a shorter time can be used as long as the extraction conditions are kept constant (*13*).

A 5 min desorption time was determined to be sufficient for transferring all analytes to the injection port, since a second desorption of the same fiber did not present carryover of the analytes (data not shown). Thus, sampling SPME conditions used for further work were direct LS for 60 min with a PDMS fiber.

Major volatiles identified in the different tequila types were alcohols, esters, and ketones (**Figure 1**). The most numerous volatiles detected were esters, and the most abundant volatiles after the alcohols were ethyl esters that showed qualitative and quantitative differences among the different tequila types. These results are in agreement with previous work (*4*), since ethyl esters also represented the largest group isolated from a dicholoromethane tequila extract. Although aging may contribute to ester production, the fermentation step is the major contributor since white tequila, which is not aged, presents a relatively high ester concentration. Ethyl esters were present in other beverages such as whiskey, cognac, and rum as the result of yeast metabolism during fermentation and were associated with pleasant fruity flavors (*2*).

Quantitative Determination of Ethyl Esters in Tequila. Accurate quantitation of individual esters in tequila was achieved since calibration curves constructed for ethyl esters followed linear relationships with highly significant ($p < 0.001$) determination coefficients ($R^2 = 0.99$). The relationships between ester concentrations (y) and ethyl ester GC peak area/GC internal standard area (x) were given by the following equations: $y = 0.0201 + 0.037x$ for ethyl hexanoate, $y = 0.064 + 0.154x$ for ethyl octanoate, and $y = -0.117 + 0.46x$ for ethyl decanoate. CV of less than 10% indicated that the technique was reproducible. The limits of quantitation calculated were 0.05 ppm, which were below the concentration range found in tequila samples (0.27−15.03 ppm) (**Table 3**). Ethyl dodecanoate, tetradecanoate, hexadecanoate, and octadecanoate concentrations were estimated based on the calibration curve constructed for ethyl decanoate assuming that they have comparable responses.

Table 3. Quantitative Determination of Ethyl Esters Present in Tequila

	concentrations (ppm)							
compounds	gold[a]		white[a]		aged[a]		extra-aged[a]	
ethyl esters	mean	CV[b] (%)	mean	CV[b] (%)	mean	CV[b] (%)	mean	CV[b] (%)
hexanoate	0.27		ND[c]		ND[c]		ND[c]	2.0
octanoate	0.65	1.4	0.62	1.2	0.70	1.0	1.98	1.6
decanoate	3.54	2.0	4.25	1.5	4.00	1.5	4.33	0.90
dodecanoate	3.25	2.3	3.18	2.2	3.73	2.4	5.97	1.7
tetradecanoate	1.09	5.3	0.73	4.8	0.87	5.3	2.61	4.5
hexadecanoate	7.42	6.4	8.73	6.8	9.95	7.0	13.08	7.6
octadecanoate	9.85	8.1	10.36	8.7	11.90	8.5	15.03	9.8

[a] Extraction conditions for ethyl esters, tequila (40 mL) at 40 °C; sampling mode, direct liquid; fiber, PDMS; sampling time, 60 min. [b] CV, coefficient of variation, $n = 3$. [c] ND = not detectable.

Extra-aged tequila presented the highest concentration of ethyl esters of all tequila types (**Table 3**). These results were expected since ethyl esters may be formed not only during fermentation but also during aging. It has been reported that the majority of esters may be the result of yeast metabolism or may be formed subsequently during the aging process by esterification of fatty acids in the presence of ethanol at high concentrations (*4*). Ethyl hexadecanoate and octadecanoate were the most abundant ethyl esters of all tequila types. It has been reported that other spirits such as vodka have C_8-C_{18} ethyl esters, with C_{16} and C_{18} being predominant (*12*). Ethyl ester quantitation in tequila by the SPME-GC methodology presented in this work may be a good alternative for the classification of tequila types. However, the analyses of multiple samples are required to be able to establish concentration ranges that allow correct classification.

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