Analysis and Identification of Mezcal and Tequila Aromas by Ambient Ionisation MS, GC-MS, and GCxGC-MS

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Introduction

Mezcal and tequila are alcoholic beverages produced from the Agave plant. Tequila is produced exclusively from the blue Weber agave (Agave tequilana) while mezcal can be produced by any of approximately 30 different agave species. Agave hearts are roasted in the process of making mezcal, giving it a characteristic smoky flavour that distinguishes mezcal from tequila. Reposado ('rested') tequila is aged for between two months and one year in oak barrels.

The volatile aromas from three different varieties of mezcal and a reposado tequila were analysed by using three different mass spectrometers: the AccuTOF-DART, the Q1500 GC, and the AccuTOF-GCX Plus. The different systems provide different, and often complementary, pieces of information.

Experimental

Samples

Two single-varietal mezcals were analysed, one from Agave tobala (Agave potatorum) and one from Agave americana var. oaxacensis, sub-varietal arroqueño. The third mezcal ('Ensamble') was a blend containing A. tobala, A. arroqueño, and three other agaves. A reposado tequila was also measured for comparison. Two separate bottles of Ensamble from different batches were analysed by AccuTOF-DART and AccuTOF-GCX.

AccuTOF-DART 4G Ambient Ionisation Mass Spectrometer

Data were acquired, calibrated, summed and background subtracted with JEOL msAxel software. Oxygen Adduct Formation (OAF) [1] and negative ion measurement was carried out by using capillary action to sample each beverage with a narrow-diameter 5 µL glass micropipette. The liquid was aspirated into the sampling orifice of the mass spectrometer (Orifice 1) by positioning the tip of the pipette against the orifice. Liquid was rapidly drawn into Orifice 1 by the mass spectrometer vacuum. Eight or more replicate measurements were made for each sample at a rate of 9-10 seconds per sample. The RF ion guide was set to 500V to pass ions larger than approximately m/z 60. The atmospheric pressure interface potentials were Orifice 1: -15V, Ring Lens: -5V, Orifice 2: -5V and orifice 1 was heated to 120°C. Fomblin Y was measured as an external mass reference standard for exact mass measurements [2]. Mass Mountaineer software (massmountaineer.com) was used for data interpretation and chemometric analysis of the high-resolution mass spectra.

Positive-ion DART mass spectra were measured by inserting a glass tee between the DART ceramic insulator and Orifice 1 and sampling the headspace from approximately 200 µL of each liquid in a GC autosampler vial by holding the mouth of the vial against the open end of the tee.

Gas Chromatography

A Restek PAL SPME Arrow (1.10 mm: DVB/C-WR/PDMS Cat# 27875) was used to sample beverage headspace for all GC-MS analysis. The gas chromatograph conditions are given in Table 1.

Q1500 GC Mass Spectrometer

Data were acquired with JEOL msPrimo software. SpectralWorks' AnalyzerPro XD software was used for analysis. Three replicate injections were made for each sample.

Table 1. GC Conditions.

-Gas chromatograph-	Agilent 7890B
Inlet	Split/splitless
Injector	260°C, 20:1 split
Injector liner	Restek Topaz Liner (SPME) 1.8 mm x 6.5 x 78.5 Cat# 23280
Column	Restek BAC-1 Plus 30m x 0.32mm ID 1.8 µ df
Carrier gas flow	2 mL/min (He, constant flow)
Oven temperature program:	40°C (1 min), 10°C/min to 260°C.

Table 2. Q1500 MS Conditions.

Ionisation:	EI (70 eV, 50 I
Ion source temperature	200°C
GC interface temperature	250°C
Scan	m/z 35-500, step 0.1, cycle 460 ms

Table 3. AccuTOF-GCX Plus MS Conditions.

Ionisation (EI/PI)	EI (70 eV, 300 Ⅰ / PI (Deuterium lamp,10.8 eV)
Ionisation (EI/FI)	EI (70 eV, 300 Ⅰ / FI (JEOL FI Emitter)
Resolving power	>10,000 (FWHM)
Ion source temperature	250°C (EI and PI) or 40°C (FI)
GC interface temperature	250°C
Spectral acquisition	m/z 35-800, 0.4 s cycle

Table 4. GC x GC Column setup

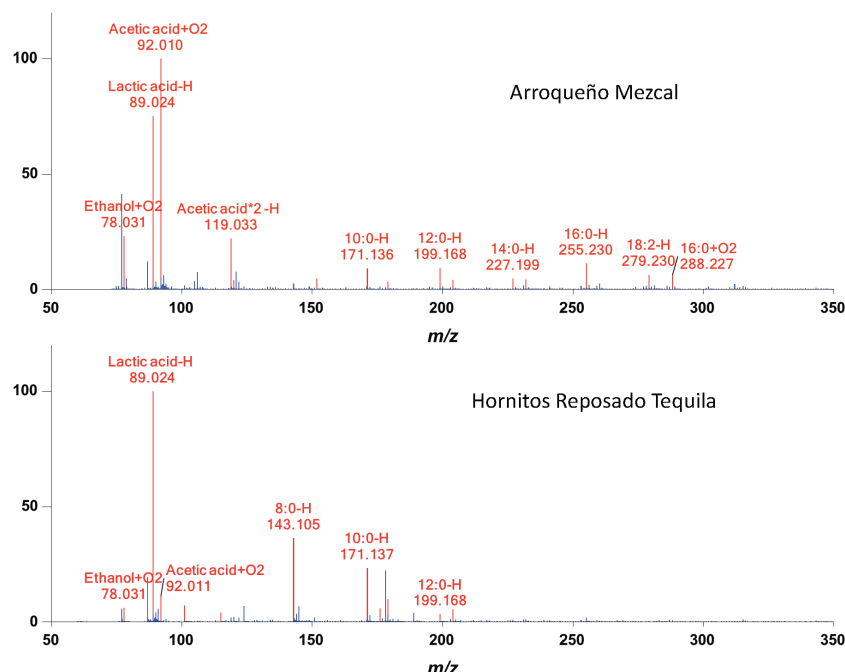|	
Guard column	Blank tube, 1m x 0.25mm
Column 1	ZB-5HT, 30m x 0.25mm x 0.25μm
Modulator loop	Blank tube, 1m x 0.18mm
Column 2	ZB-35HT, 1m x 0.18mm x 0.18μm
Transfer line to ion source	Blank tube, 1.5m x 0.18mm

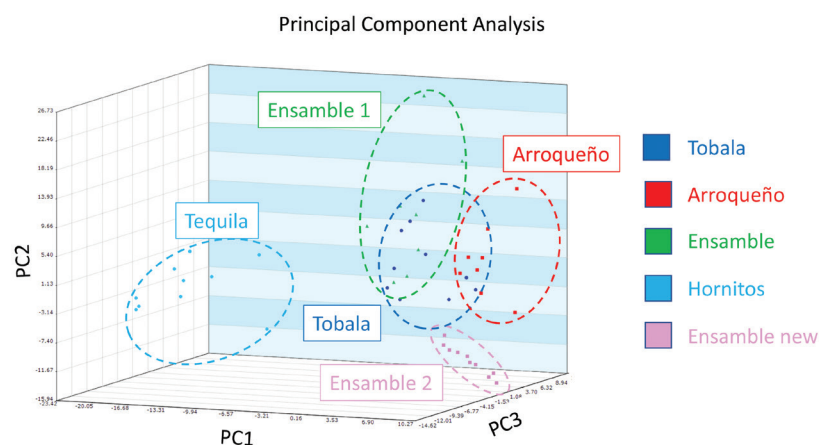*Figure 1. Negative-ion DART mass spectra for Arroqueño mezcal (top) and reposado tequila (bottom).*

Figure 2. Principal Component Analysis of the mezcal and tequila samples.

AccuTOF-GCX Plus GC Mass Spectrometer

GC-MS

The combination electron ionisation (EI) and photoionisation (PI) source was used for all measurements. Each sample was measured once in EI mode and alternately by PI mode using the combination EI/PI ion source and JEOL msAxel software. The samples were measured again using field ionisation (FI) with the combination EI/FI ion source. JEOL msFineAnalysis (version 2) software was used for automatic chromatographic deconvolution and reporting of EI and PI data. The msFineAnalysis report combined exact mass and isotope information with results of searching the NIST 2020 mass spectral database into an integrated report with optional interactive inspection of each assignment.

GC x GC/MS

Comprehensive two-dimensional gas chromatography (GC x GC) was carried out by using a Zoex ZX-2 thermal modulator installed in the Agilent 7890B gas chromatograph with a high-temperature column set. The column configuration is shown in Table 4:
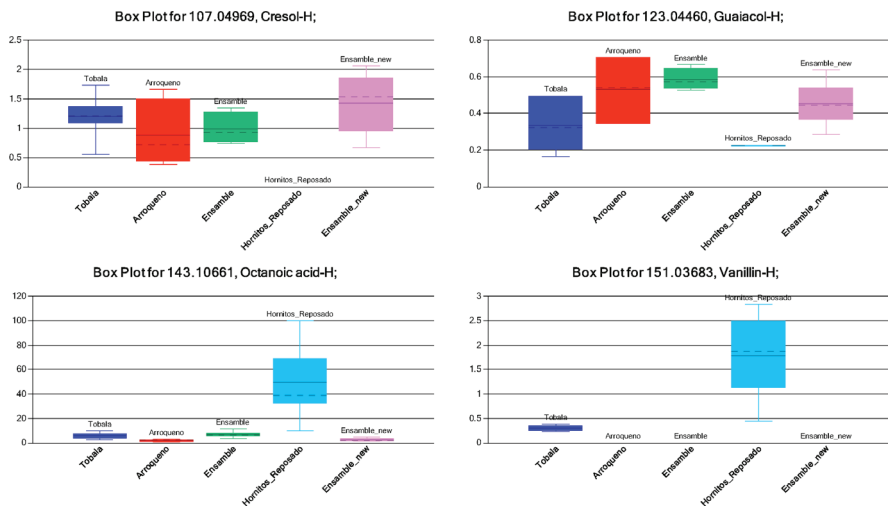
The injector, carrier gas, and GC oven parameters were identical to those used for the one-dimensional GC analysis. The thermal modulation period for the Zoex ZX-2 was set to 6 seconds. The auxiliary hot-gas-jet heater was programmed to start at 150°C and track with the oven temperature program. AnalyzerPro XD software was used to process the GC x GC/MS data exported from the JEOL msAxel program in netCDF format.

Results

Ambient ionisation: AccuTOF-DART 4G

Positive-ion DART mass spectra were dominated by the proton-bound dimer of ethanol, with only minor contributions from other components. For that reason, this analysis focuses on the negative-ion DART mass spectra.

Negative-ion DART is very sensitive to organic acids. Acidic compounds were detected in the negative-ion mass spectra with odd nominal m/z values as deprotonated molecules [M – H]⁻ and oxygen adducts [M + O$_2$]⁻ were detected as even nominal m/z values (Figure 1). Although the ethanol *m/z*

Figure 3. Box plots showing the distribution of selected compounds in the mezcals and tequila.

Single quadrupole GC-MS: Q1500 GC

The AccuTOF-DART provides rapid and convenient analysis and is sensitive to more polar compounds that may or may not be convenient for analysis by gas chromatography. It is a useful approach for differentiating samples and quickly identifying differences. However, assignments for compounds in a complex mixture are based solely upon accurate mass and isotope data. GC-MS is the classical approach when more information is needed about the specific isomers that make up a mixture. The JEOL Q1500 with JEOL msPrimo and Escrime software provides a simple and inexpensive way to combine GC separations with mass spectral database searching to identify components.

Chromatographic deconvolution and statistical analysis were carried out by using the AnalyzerPro XD software package (SpectralWorks Ltd) which provides powerful and easy-to-use capabilities for chemometric analysis of GC-MS data from the JEOL Q1500GC GC-MS system. The AnalyzerPro XD analysis identified differences in the aroma composition between each of the beverages.

For this example, three replicate measurements (the minimum number required for statistical analysis) were made for each of three mezcal samples (Arroqueño, Tobala, and Ensamble) and the reposado tequila.

is below the detected range, its oxygen adduct is present at *m/z* 78.031. The majority of peaks have elemental compositions assigned to deprotonated organic acids or their oxygen adducts. The peaks at *m/z* 60.994 and 70.993 are background peaks for HCO_3— and HCO_4— that are not completely subtracted. Lactic acid is present in all samples and acetic acid is present in the mezcal samples, but not in the tequila. The tequila sample showed relatively abundant octanoic acid and decanoic acid peaks at *m/z* 143.105 and 171.138.

Principal Component Analysis (Figure 2) shows some clustering of the mezcals visible in three dimensions, but the tequila sample is clearly distinguished from the mezcals. The two Ensamble samples are clearly

different. Leave-One-Out Cross Validation (LOOCV) for Support Vector Machine (SVM) classification shows 100% accuracy for identifying the mezcals and tequila.

We can view how individual components vary between samples by using box plots or volcano plots. The top image in Figure 3 shows the box plot for *m/z* 107.049 corresponding to deprotonated cresol and guaiacol, two of the components that gives mezcal a smoky aroma and taste that differentiate it from tequila. As expected, the cresol and guaiacol are present in all mezcal samples, but is absent in the reposado tequila. The bottom image shows the box plot for deprotonated octanoic acid and vanillin which are an indication of tequila maturation [3].
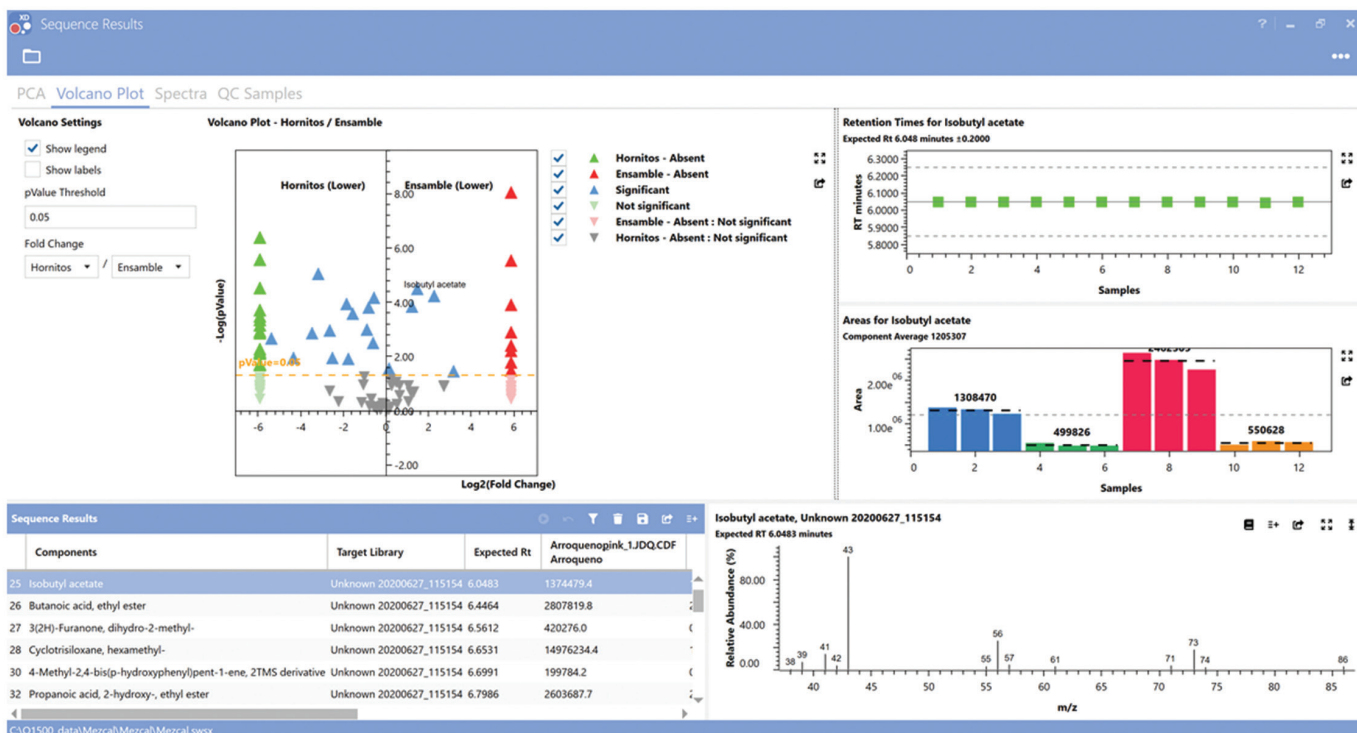

Figure 4. Volcano plot comparing tequila and Ensamble mezcal and bar graph showing the distribution if isobutyl acetate in 3 mezcals and one tequila.
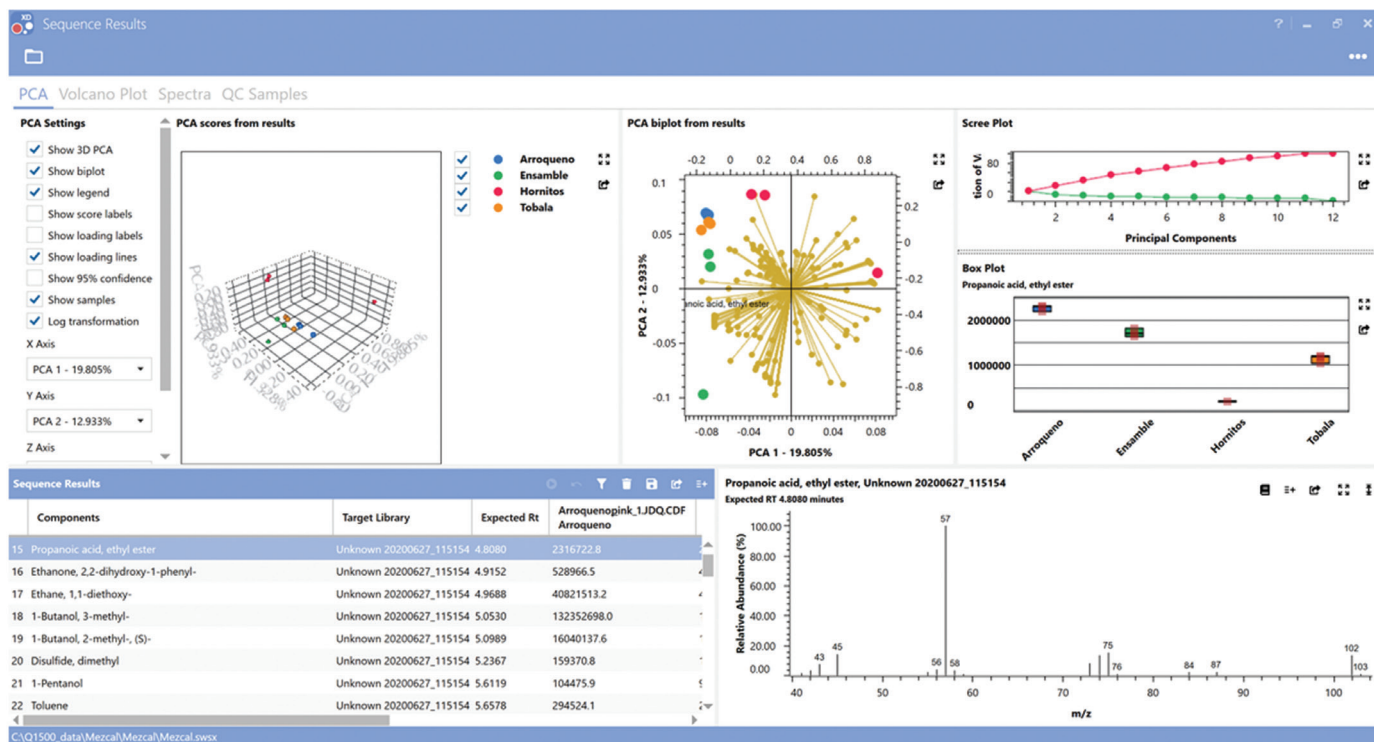
Figure 5. Principal Component Analysis and distribution of ethyl propanoate in the mezcals and tequila.

The volcano plot and bar graph in Figure 4 shows that isobutyl acetate is present at significant amounts only in Arroqueño mezcal and reposado tequila.

The Principal Component Analysis (PCA) plot and biplot in Figure 5 show that the four different beverages have distinctive aroma profiles and the same figure shows that ethyl propionate (pineapple aroma) is present in all three mezcals, but absent in the reposado tequila.

The box plots in Figure 6 show the differences in the distribution of the two isomers 2-methyl-1-butanol with an odour described as 'roasted winey onion fruity fusel alcoholic whiskey' [4], and 3-methyl-1-butanol with an odour described as 'fusel alcoholic pungent ethereal cognac fruity banana molasses' [5].

GC-High-Resolution Time-of-Flight MS: AccuTOF-GCX Plus

GC-MS

Despite a relatively fast GC oven program, over 300 compounds in each sample were detected by the JEOL msFineAnalysis software chromatographic deconvolution function. Electron ionisation (EI) mass spectra were searched against the NIST 2020 Mass Spectral Database. The exact masses and isotope data for the molecular ions in the photoionisation (PI) mass spectra and Field ionisation (FI) mass spectra provided elemental composition information to complement the database search results. Including the elemental composition

for fragment ions in the EI mass spectra provided a comprehensive report with detailed information about each compound.

Ethyl hexanoate (aroma described as 'sweet fruity pineapple waxy green banana' [6]. elutes at 9.14 minutes. The EI mass spectrum for ethyl hexanoate does not show a molecular ion, but a molecular ion is present in the FI mass spectrum that is used to confirm the molecular weight and elemental composition.

The detailed view for ethyl hexanoate identification (Figure 7) shows a summary of the database search results from the EI mass spectrum, elemental composition determination from exact mass and isotope data in the FI mass spectrum, and fragment ion coverage from accurate mass information in the EI mass spectrum. Similar results were obtained by using the combination EI/PI ion source.

In comparing PI and FI soft ionisation methods, both show molecular ions that are weak or absent in the EI mass spectra, but field ionisation generally shows a higher abundance for the molecular ion and less fragmentation than the photoionisation. FI is operated with a cold combination EI/FI ion source, and EI is operated with a heated ion source. A benefit of the combination EI/PI source is that photoionisation can be carried out with the ion source heater on, so switching between EI and PI does not require heating or cooling the source.

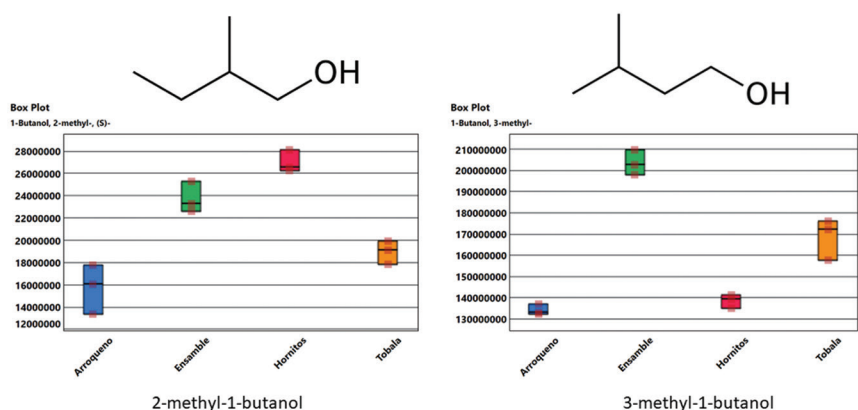2-methyl-1-butanol

3-methyl-1-butanol

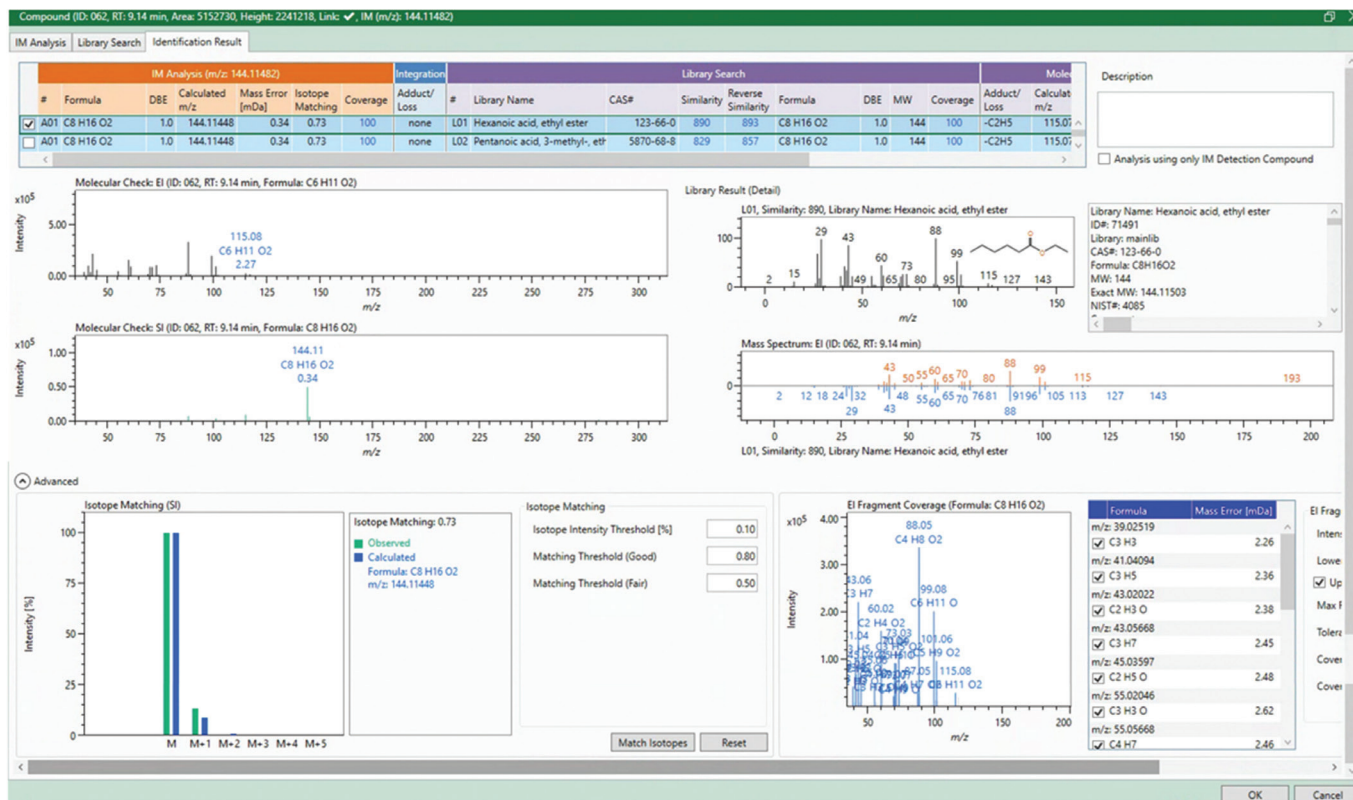Figure 6. Distribution of two isomers in the mezcals and tequila.

Figure 7. Detailed summary of ethyl hexanoate data from both EI and FI mass spectra.

GC x GC-MS

Comprehensive gas chromatography (GC x GC) combined with the AccuTOF GCX combination ion sources provides powerful separation for very complex mixtures such as petroleum products. However, the choice of hardware configuration for the thermal modulator is an important factor for the analysis of volatile compounds. This analysis used the ZX-2 system which uses a closed cycle refrigerator/heat exchanger unit to cool the nitrogen jet. This eliminates the need for a liquid nitrogen supply, but the cold gas jet temperature is only effective for compounds with boiling points equivalent to roughly seven carbons or more. Many of the small organic compounds in these

samples are not efficiently trapped by the -90°C nitrogen jet in the ZX-2 as can be seen from the 2D GC chromatogram in Figure 8. The x-axis represents the retention time on Column 1 and the y-axis represents the retention time on Column 2. The figure shows contour lines and the small orange dots represent compounds detected by the AnalyzerPro XD software. Note that small compounds with retention times below about 5.1 minutes show poor separation. These compounds include ethanol, acetic acid, propanol, etc. Better separation of the small organic compounds could be achieved by using the ZX-1 thermal modulator. The ZX-1 system requires liquid nitrogen, but is effective for compounds with boiling points

corresponding to approximately 3 carbons or more. Beyond 5.1 minutes, compounds are well separated in both dimensions in regions that roughly correlate to compound classes such as silicones from column bleed, organic esters and acids, aromatics, and terpenes.

Compounds that overlap in the one-dimensional GC separation are separated in the two-dimensional separation. Figure 9 shows the region around 10 minutes with labels for selected compounds. Naphthalene, anethole (p-methoxyphenylpropene, anise aroma), α-terpineol and ethyl octanoate are separated in two dimensions, providing mass spectra that are identified by database searching.

Although these compounds are not fully separated by one-dimensional chromatography, they were separated by the msFineAnalysis program. Figure 10 shows the msFineAnalysis chromatographic deconvolution of these compounds in the GC-EIHRMS data (top chromatogram) and the GC-FIHRMS data (bottom chromatogram). The table at the bottom of the figure shows compounds highlighted in light blue that are identified with a high degree of confidence. Compounds with multiple isomers are marked in yellow and compounds with uncertain compositions in white. The deconvolved peaks for
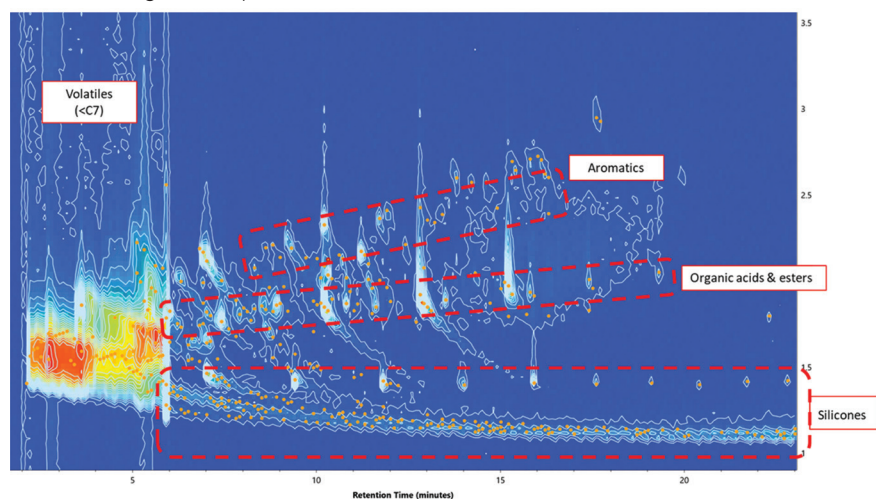

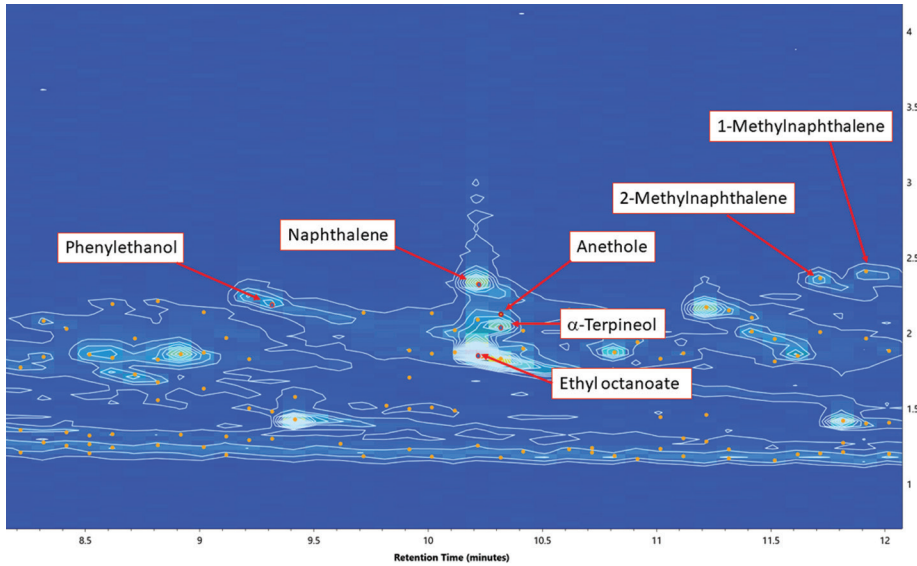Figure 8. GC x GC chromatogram for volatile compounds in Ensamble mezcal.

Figure 9. Enlarged view of the retention time region around 10 minutes with labels for selected compounds identified by AnalyzerPro XD database searching.

phenol are highlighted in both the EI and FI chromatograms. Of course, we could increase the separation in either the GC-MS or GC x GC-MS methods by using a slower oven temperature program, but that was not necessary for this analysis.

Conclusion

The AccuTOF-DART permits extremely rapid analysis with 8-12 replicates of each beverage measured within a total analysis time of 2 minutes. Differences in the chemical composition of the headspace vapor are identified by accurate mass and isotope data combined with chemometric analysis using Mass Mountaineer software. If the goal is to identify the source of a specific sample or to simply to distinguish the different beverages, the AccuTOF-DART provides the fastest and simplest solution. A 'fingerprint' spectrum of volatile aroma compounds can be used to identify the origin of an unknown sample. In addition, the AccuTOF-DART can be somewhat more sensitive than GC-MS for certain compound classes, such as the substituted phenols that contribute to the smoky flavour of mezcal. Although not shown here, it is also possible to use the AccuTOF-DART to rapidly determine the alcohol-by-volume composition in beverages [7].

If the goal is to determine the identity and/or relative abundance of specific compounds including isomers, the Q1500 GC system provides a powerful and cost-effective solution. The JMS-Q1500GC single quadrupole mass spectrometer with SpectralWorks' AnalyzerPro XD software easily identifies differences between aroma profiles with chromatographic separation and mass spectral identification of isomeric compounds such as 2-methyl-1-butanol and 3-methyl-1-butanol. Compounds are identified by database search and GC retention time information and statistical analysis reveals compound-dependent differences between classes.

The AccuTOF-GCX Plus with msFineAnalysis version 2 software provides the most complete analysis of samples. Electron ionisation (EI) mass spectra and photoionisation (PI) mass spectra or field ionisation (FI) mass spectra can be easily measured for each beverage by using the combination EI/PI or combination EI/FI ion sources. The integrated report from msFineAnalysis combines information about molecular ion composition from the PI data with database search results from the EI
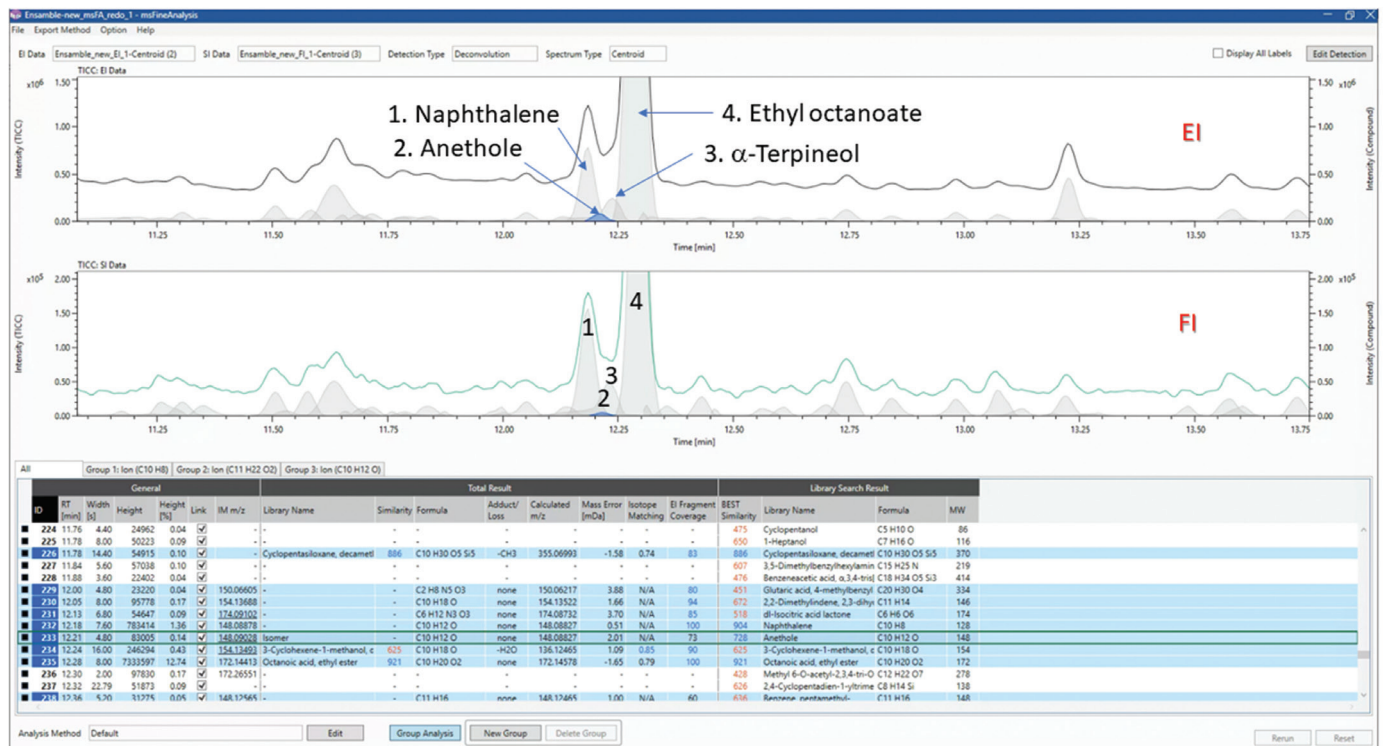

Figure 10. Chromatographic deconvolution and comprehensive identification of trace components in the Ensamble mezcal. The anethole peak (0.14% relative height) is selected and highlighted in blue.

data with accurate mass and isotope data obtained for molecular ions and fragments. Field ionisation (FI) mass spectra measured with the combination EI/FI ion source generally show more abundant molecular ions and less fragmentation than the PI mass spectra. However, the combination EI/PI source permits alternating EI and PI analysis for each sample without changing the ion source temperature. Although it was not used in this particular example, the SpectralWorks AnalyzerPro XD software can also be used for statistical analysis of AccuTOF GCX data.

GC x GC-MS analysis with the AccuTOF-GCX separated components with at least 7 carbons but the ZX-2 thermal modulator is not suited for analysis of smaller compounds. Changing to the ZX-1 modulator would give better separation of those compounds. Although GC x GC-MS is often the best method for separating complex mixtures, one-dimensional GC-MS with the msFineAnalysis software was the preferred method for the AccuTOF-GCX analysis, giving smaller data files with excellent separation and identification of chromatographically overlapping trace components with integrated reporting that combines all of the data from EI and soft ionisation analyses.

References

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