

Mass Spectrometry & Spectroscopy

Ambient Ionisation MS and GC-MS Analysis and Identification of Aroma Compounds in Agave Based Alcoholic Beverages

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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 flavor that distinguishes mezcal from tequila. Reposado ('rested') tequila is aged for between two months and one year in oak barrels.

Volatile aromas from three different varieties of mezcal and a reposado tequila were analysed by using three different mass spectrometers: a time-of-flight mass spectrometer equipped with a Direct Analysis in Real Time (DART) ion source (JEOL AccuTOF-DART 4G), a quadrupole GC-MS system (JEOL Q1500 GC), and a GC- high-resolution time-of-flight MS (GC-HRTOFMS) system (JEOL AccuTOF GCX Plus). The different systems provided different, and often complementary, pieces of information. Ambient ionisation rapidly distinguished between the different samples and identified specific chemical differences but did not distinguish between isomers. Chemometric analysis (SpectralWorks' AnalyzerPro XD) found compound-specific differences between the samples using data from the quadrupole GC-MS system. The GC-HRTOFMS and data analysis software (JEOL msFineAnalysis) gave the most detailed and specific analysis of each sample by integrating data from electron ionisation (EI) and soft ionisation (photoionisation and field desorption).

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.

Ambient Ionisation Mass Spectrometer (AccuTOF-DART 4G)

Data were acquired, calibrated, summed and background subtracted with JEOL *msAxel* software. Oxygen Adduct Formation (OAF) [1] and negative ion measurement was carried out using capillary action to sample each beverage with a narrow-diameter 5 μ L glass micropipette. Liquid was vacuum aspirated into the sampling orifice of the mass spectrometer (Orifice 1) by positioning the tip of the pipette against the orifice. 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 [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 μ L of each liquid in a GC autosampler vial by holding the mouth of the vial against the open end of the tee.

Gas Chromatograph

A PAL SPME Arrow (Restek 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.

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.

Quadrupole Mass Spectrometer (Q1500 GC)

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

Table 2. Q1500 MS Conditions.

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

GC-TOF Mass Spectrometer (AccuTOF-GCX Plus)

GC-MS

Samples were measured with the combination electron ionisation/photoionisation (EI/PI) source. Samples were alternately measured in EI mode and PI mode and some samples were measured 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.

Table 3. AccuTOF-GCX Plus MS Conditions.

Ionisation (EI/PI)	
EI (70 eV, 300 μ A) / PI (Deuterium lamp, 10.8 eV)	
Ionisation (EI/FI)	
EI (70 eV, 300 μ A) / 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

Results

Ambient ionisation: AccuTOF-DART 4G

Positive-ion DART mass spectra were dominated by the proton-bound dimer of ethanol, with minor contributions from other components. Although solid-phase microextraction (SPME) can improve the positive-ion DART sensitivity for minor components, negative-ion DART was chosen because ethanol is not readily deprotonated.

Negative-ion DART is very sensitive to acidic compounds such as carboxylic acids and phenols. Acidic compounds appeared in the negative-ion mass spectra as deprotonated molecules $[M - H]^-$ with odd nominal m/z values and oxygen adducts $[M + O_2]^-$ were detected as even nominal m/z values (Figure 1). Although the ethanol m/z 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 oxygen adducts. Peaks at m/z 60.994 and 70.993 are background peaks for HCO_3^- and HCO_4^- that are incompletely 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 abundant octanoic acid and decanoic acid peaks at m/z 143.105 and 171.138.

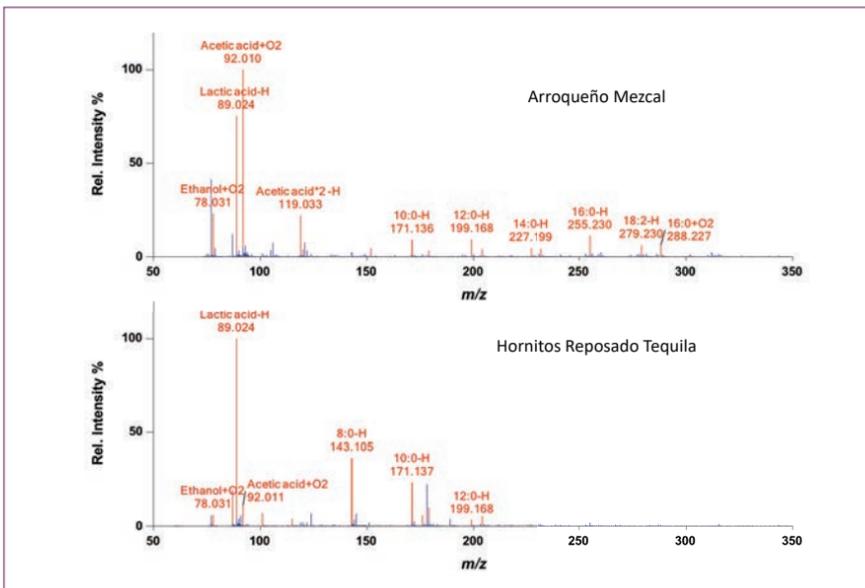


Figure 1. Negative-ion DART mass spectra for Arroqueño mezcal (top) and reposado tequila (bottom).

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

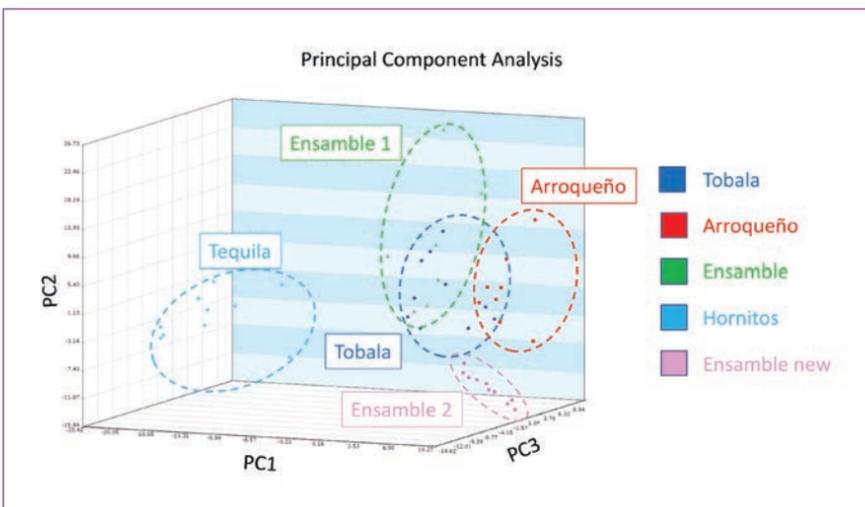


Figure 2. Principal Component Analysis of the mezcal and tequila samples.

We can view sample-dependent variations for individual components 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. Cresol and guaiacol are present in all mezcal samples, but absent in the reposado tequila. The bottom image shows the box plot for deprotonated octanoic acid and vanillin which are indicators of tequila maturation [3].

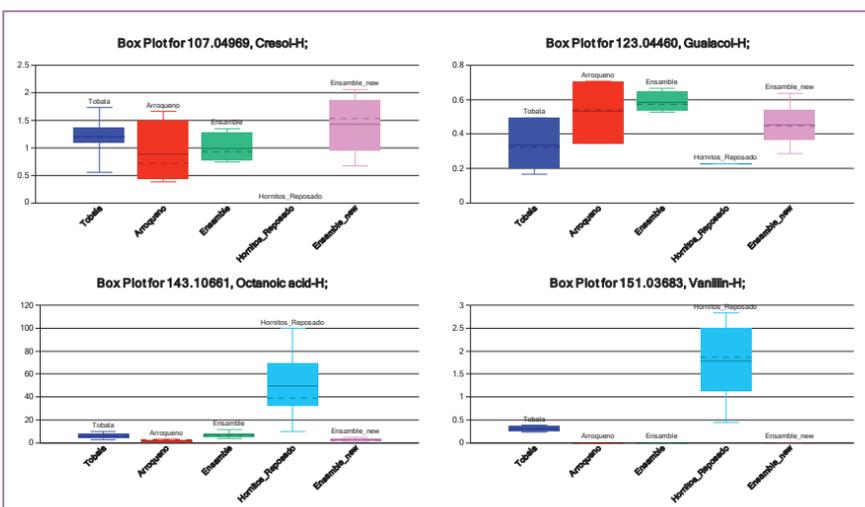


Figure 3. Box plots showing the distribution of selected compounds in the mezcals and tequila.

Single quadrupole GC-MS: Q1500 GC

Ambient ionisation with 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 to separate and identify isomers in a mixture. Quadrupole mass spectrometers like the Q1500 GC are widely used for GC-MS analysis because of their low cost and ease of operation and maintenance. Compounds are identified by searching EI mass spectra against the mass spectral databases and using retention index matching to distinguish isomers having similar database search scores.

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

Three replicate measurements (the minimum number required for statistical analysis) were made for each of three mezcal samples (*Arroqueño*, *Tobala*, and *Ensemble*) and the *reposado* 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.

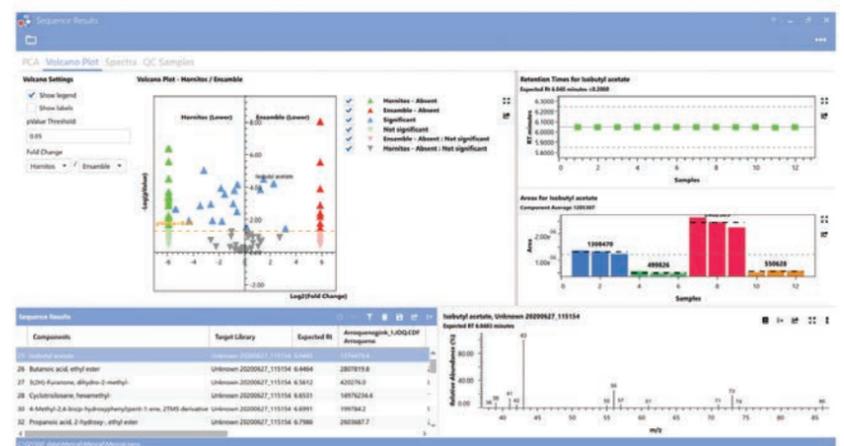


Figure 4. Volcano plot comparing tequila and Ensemble mezcal and bar graph showing the distribution of isobutyl acetate in 3 mezcals and one tequila.

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

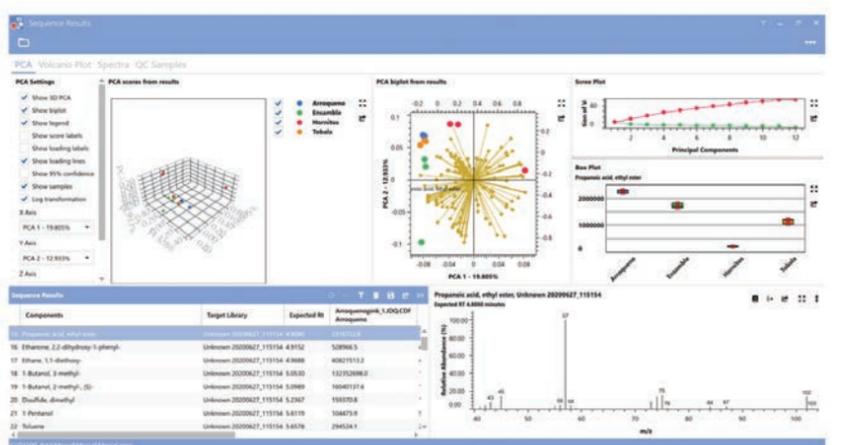


Figure 5. Principal Component Analysis and distribution of ethyl propionate in the mezcals and tequila.

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].

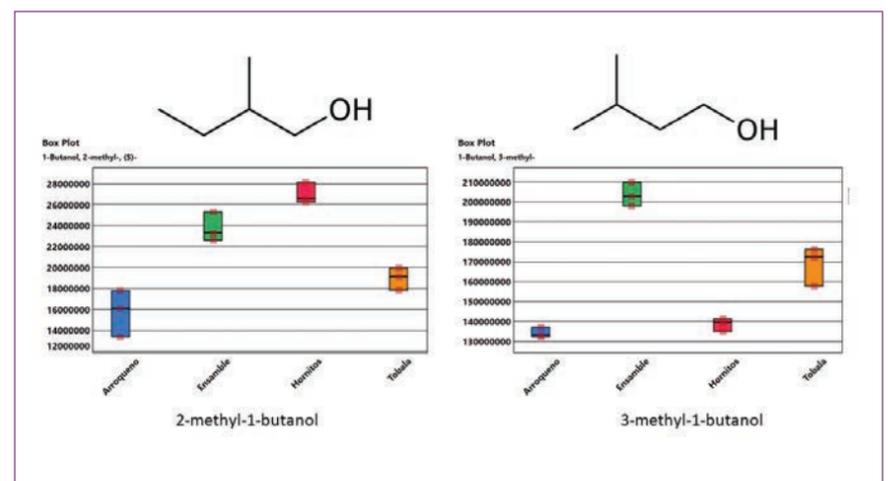


Figure 6. Distribution of two isomers in the mezcals and tequila.

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

GC-MS

The high-resolution TOF mass spectrometer provided the greatest amount of information. High-resolution accurate mass data were acquired for electron ionisation (EI) and soft ionisation (PI and FI) measurements. Chromatographic deconvolution using the accurate mass information finds even low-level components that are not chromatographically resolved. The EI data provide fragmentation that can be searched against the mass spectral databases, and the soft ionisation data provide information to determine the elemental composition of the intact molecule.

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) summarises 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.



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

Both PI and FI soft ionisation methods 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.

Although some compounds are not fully separated by one-dimensional chromatography, they were separated by the *msFineAnalysis* program. Figure 8 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 phenol are highlighted in both the EI and FI chromatograms.

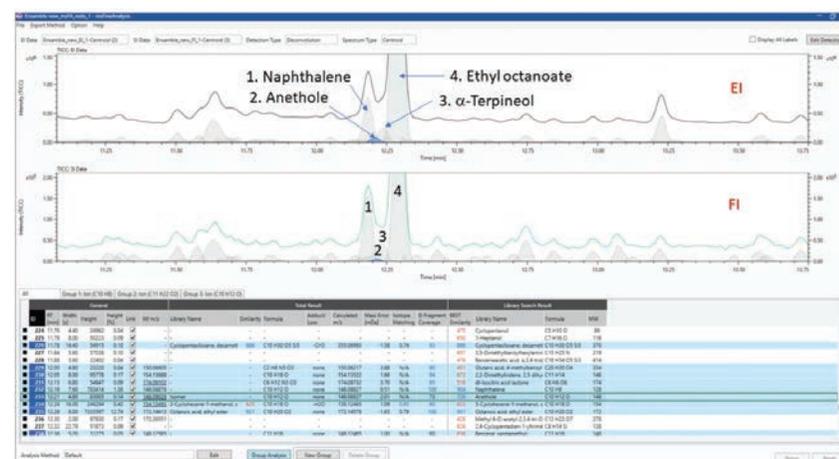


Figure 8 Chromatographic deconvolution and comprehensive identification of trace components in the Ensemble mezcal. The anethole peak (0.14% relative height) is selected and highlighted in blue.

Conclusion

The AccuTOF-DART permitted 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 vapour were 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 provided the fastest and simplest solution. A 'fingerprint' spectrum of volatile aroma compounds could be used to identify the origin of an unknown sample. In addition, the AccuTOF-DART was 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 provided a powerful and cost-effective solution. The JMS-Q1500GC single quadrupole mass spectrometer with SpectralWorks' *AnalyzerPro XD* software easily identified 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 were identified by database search and GC retention indices, and statistical analysis revealed compound-dependent differences between classes.

The AccuTOF-GCX Plus with *msFineAnalysis* version 2 software provided the most complete analysis of samples. Electron ionisation (EI) mass spectra and photoionisation (PI) mass spectra or field ionisation (FI) mass spectra were easily measured for each beverage by using the combination EI/PI or combination EI/FI ion sources. The integrated report from *msFineAnalysis* combined information about molecular ion composition from the PI data with database search results from the EI 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 showed more abundant molecular ions and less fragmentation than the PI mass spectra. However, the combination EI/PI source permitted alternating EI and PI analysis for each sample without changing the ion source temperature.

References

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