Application Note

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Instrument: Pegasus® BT 4D

Statistical Differentiation of Tequila Spirits Using SPME-GCxGC-TOFMS and ChromaTOF® Tile Software

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Introduction

Tequila, one of Mexico's most iconic distilled spirits, is produced by fermenting and distilling sugars obtained from Agave tequilana Weber var. azul. Current regulations permit producers to use a minimum of 51% agave-derived sugars and up to 49% from other sources, while "100% agave" tequila must be made exclusively from agave sugars. Its designation of origin is legally protected, restricting production to specific regions of Mexico, including Jalisco, Nayarit, Tamaulipas, Michoacán, and Guanajuato.

The production process consists of several stages. The first is harvesting the agave stem and removing its leaves, followed by cooking to hydrolyze inulin into fermentable sugars. Fermentation is then carried out, typically by Saccharomyces cerevisiae, converting these sugars into alcohols. A double distillation and dilution step produces a spirit with an alcohol content of approximately 40–50%. Tequilas are classified according to their maturation stage: *blanco* (silver or white) are bottled immediately after distillation; *joven,* or gold, produced by blending tequila *blanco* with aged tequilas or adding caramel to color them golden amber; *reposado* (less aged) is aged in oak barrels for at least two months; *añejo* (aged) has matured for a minimum of one year; and *extra-añejo* (extra aged) has been aged for more than three years. These production differences play a key role in the wide variability of flavors and aromas that define tequila. Other factors, including geographical region of production, can also contribute to the variability of different tequila products.

This application note presents the analytical workflow for evaluating ten different commercial tequila samples using headspace solid-phase microextraction (HS-SPME) combined with a comprehensive two-dimensional gas chromatography (GCxGC) system coupled to time-of-flight mass spectrometry (TOFMS). The LECO *Pegasus* BT 4D GCxGC-TOFMS system delivers excellent separation capacity, wide dynamic range, and high sensitivity, enabling the detection of aroma-relevant compounds. Data processing was carried out with *ChromaTOF* Tile Software, which supports mass spectral (MS) and retention index (RI) library searches and generates a composite feature table for the entire sample set, facilitating the identification of trends, patterns, and significant differences among the tequilas.

Experimental

Tequila samples (100% agave) with different brands, from different geographical regions, and with various aging classifications were acquired in liquor stores (see Table 1). Triplicates for each tequila sample were prepared by placing 250 µL of each into 20 mL headspace vials for further analysis. Table 2 summarizes the parameters applied for the HS-SPME incubation, extraction, and desorption, as well as the GCxGC-TOFMS instrumental parameters. An n-alkane standard (C7-C30) was analyzed for calculation of linear retention indices (RIs).

Table 1: Different tequila samples classified by aging process and region of production.

Sample Code	Aging Classification	Region
3G_B	*Blanco*	Jalisco
3G_R	*Reposado*	Jalisco
3G_A	*Añejo*	Jalisco
Co_B	*Blanco*	Guanajuato
Co_R	*Reposado*	Guanajuato
Co_A	*Añejo*	Guanajuato
JC_B	*Blanco*	Jalisco
D.Ra_B	*Blanco*	Jalisco
LA_R	*Reposado*	Guanajuato
H_R	*Reposado*	Jalisco

Table 2: Aroma sampling and GCxGC analysis parameters.

	HS-SPME
SPME Arrow Fiber (1.8 cm)	120 µm coated DVB/CAR/PDMS
Extraction Conditions	50 °C for 2 min (500 rpm)
Desorption Time	2 min
Split Ratio	1:20
GCxGC	**Agilent 8890 with LECO Cryogen Free QuadJet™ Thermal Modulator**
Injector	260 °C
Carrier Gas	He 1.0 mL/min
Columns	^{1}D: HP-5ms 30 m x 0.25 mm i.d. x 0.25 µm coating ^{2}D: RXI-17Sil MS 1,2 m x 0.25 mm i.d. x 0.25 µm coating
Modulation Period	2.5 s
Oven Program	40 °C (2 min); ramp: 6 °C/min to 200 °C; 25 °C/min to 235 °C (5 min)
Secondary Oven Temp	+5 °C (relative to the GC oven temperature)
Modulator Temp	+15 °C (relative to the secondary oven temperature)
Transfer Line	260 °C
TOFMS	**LECO *Pegasus* BT**
Ion Source Temp	260 °C
Mass Range	30-400 m/z
Acquisition Rate	200 spectra/s

Results and Discussion

GCxGC-TOFMS provides significant analytical advantages for the characterization of these complex samples. In Figure 1, it is possible to see the complexity of a representative blanco tequila sample, where over 170 different compounds were successfully annotated with a similarity score of at least 800, S/N higher than 10, and a maximum ΔRI of 10.

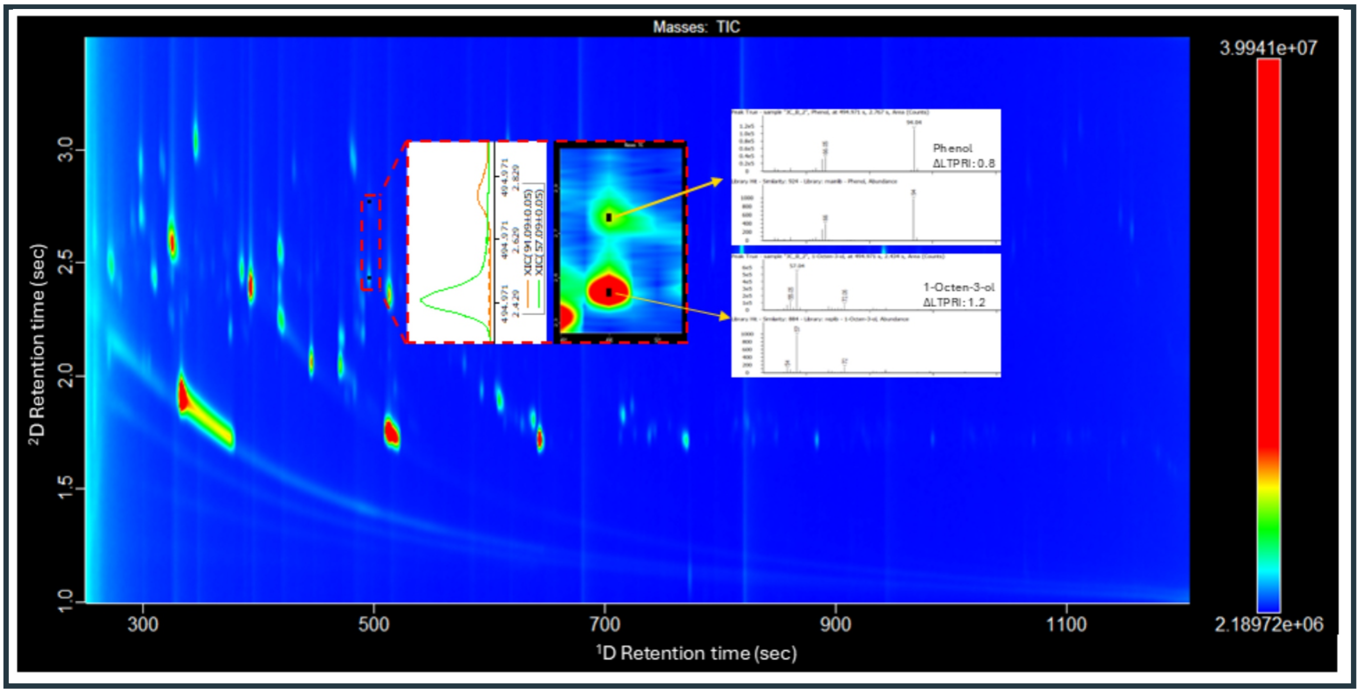

Figure 1: GCxGC-TOFMS Total ion current (TIC) contour plot of a blanco tequila aroma. The highlighted region shows the separation of 1-octen-3-ol and phenol, which would have coeluted in a traditional one-dimensional GC analysis.

The GCxGC contour plot demonstrates the superior separation power of the technique relative to a single GC separation, which can be seen in the highlighted separation of the 1-octen-3-ol and phenol. These two compounds have the same first dimension retention times, but thanks to the second orthogonal separation, the overlap in conventional one-dimensional GC were successfully resolved. A spectral comparison performed using the NIST MS library revealed a similarity score of 924/1000 for phenol and 884/1000 for 1-octen-3-ol as well as excellent ΔRI. The enhanced peak capacity and identification allow an accurate qualitative and quantitative analysis of individual components, even in highly complex matrices. Both analytes are likely to contribute to the aroma profile. Phenol has phenolic aroma descriptors and 1-octen-3-ol has earthy descriptors.[3] Another important feature in GCxGC contour plots is the structural and class separation throughout the chromatographic space (Figure 2).

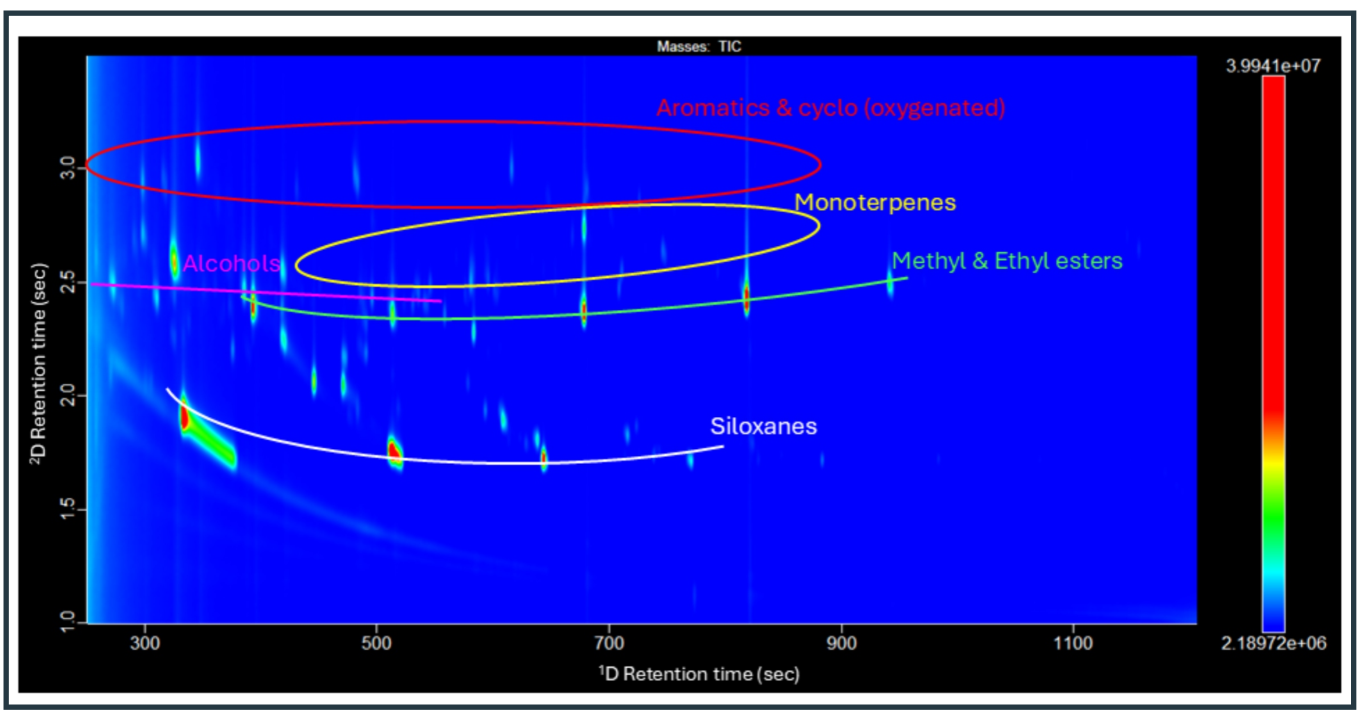

Figure 2: Different compound classes and elution patterns found in the GCxGC-TOFMS analysis of a blanco tequila aroma help with sample characterizations.

Figure 2 shows how GCxGC delivers an easily interpretable chromatogram with clear class-based separation. Therefore, the main compound classes found in the tequila aroma samples were aromatics and cyclo-oxygenated compounds, monoterpenes, alcohols, and esters. These classes can be clearly differentiated based on their structured chromatographic distribution patterns and corroborate with existing data in the literature.[1,2] This organized separation facilitates the interpretation of compositional trends and the recognition of characteristic chemical fingerprints associated with different aroma profiles. LECO's TOFMS detection adds another layer of analytical strength for evaluating the samples by offering high acquisition speed and non-skewed spectra optimized for deconvolution, enabling reliable compound identification even in cases of partial coelution.

Historically, the alignment and comparison of multiple chromatograms has presented a major challenge in GCxGC-MS data processing. However, new software tools have streamlined these analysis tasks making them more reliable and efficient. Here, it was possible to find differences among the analyzed tequila samples in a straightforward manner utilizing *ChromaTOF* Tile. This processing software platform uses a tile-based approach to rapidly compare multiple GCxGC-TOFMS data sets in a fast and user-friendly way. Statistically significant differences are highlighted, reducing the time required to locate chromatographic differences between the sample sets. In this work, the F-ratio comparison mode was used to highlight chemical features that differentiated the tequila samples listed in Table 1. For example, Figure 3 shows an example of an identified difference in the samples using *ChromaTOF* Tile. The tentative identification, 2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethenyl)-, trans, was supported with mass spectral and RI matching.

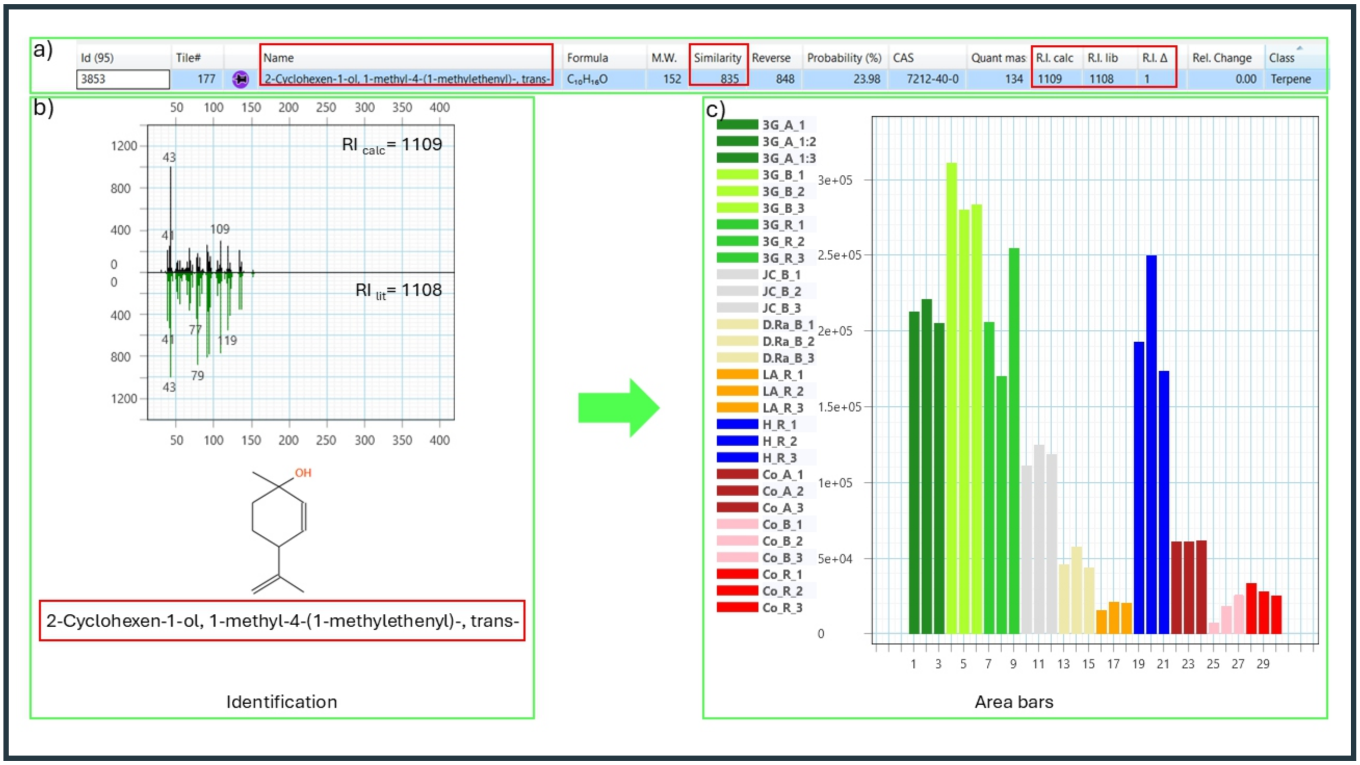

Figure 3: ChromaTOF Tile compound identification visualization: a) feature table (highlighted: name, similarity and RI columns); b) mass spectral comparison (highlighted: compound name); and c) area bars plot for 2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethenyl)-, trans, analyzed in tequila aroma.

Another important capability of *ChromaTOF* Tile is Principal Component Analysis (PCA), which is useful to reveal overall trends between the ten different tequila samples. For this work, compounds with F-ratio lower than 75, similarity score lower than 750/1000, or features determined to be column and fiber bleed were not considered for PCA. By using this filtering strategy, 40 different chemical features were identified and selected for PCA. These compounds are likely to contribute to the tequila aroma differentiation, and Figure 4 shows the PCA results.

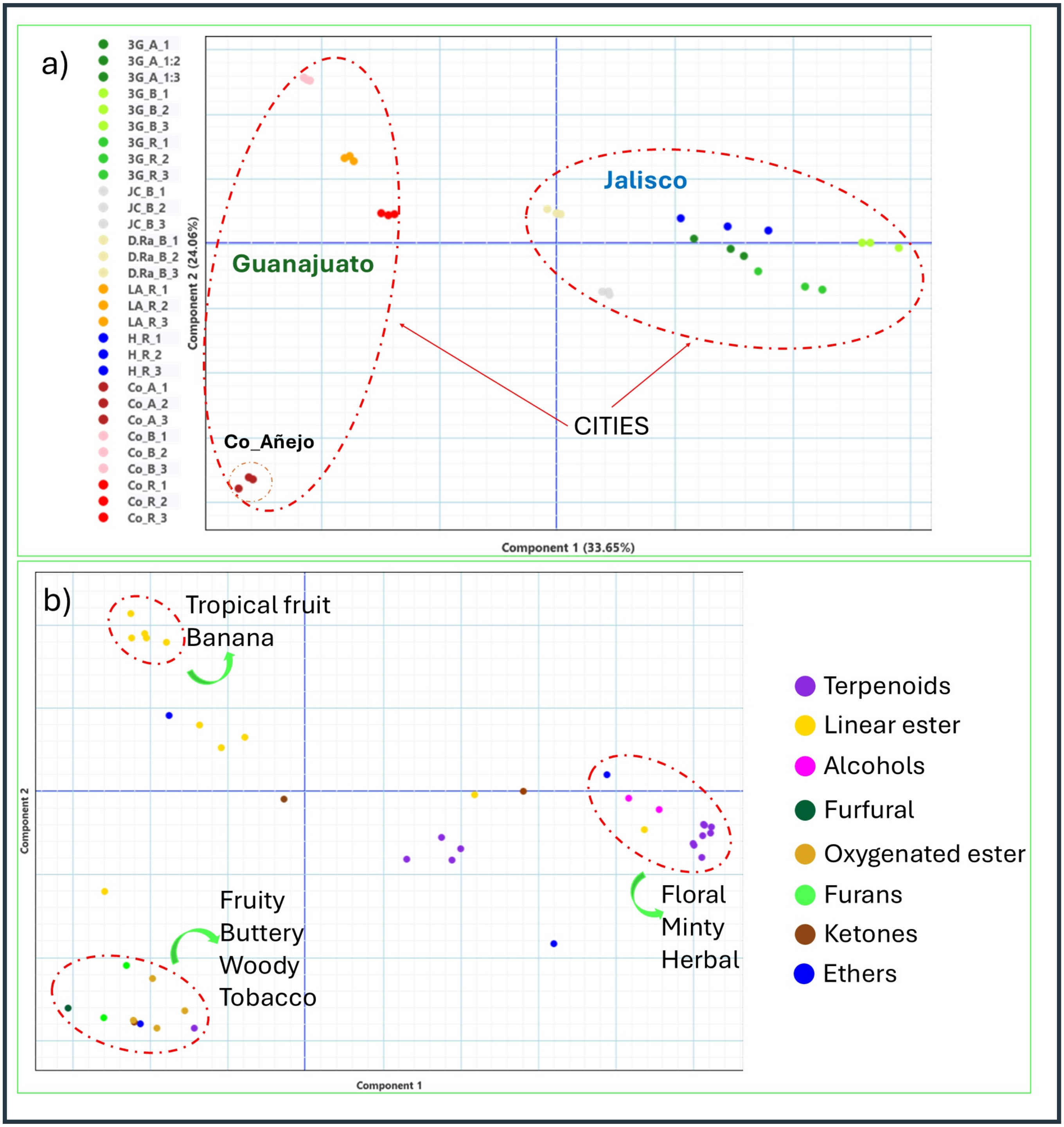

Figure 4: PCA a) scores plot and b) loadings plot displaying clustering of tequila aroma samples and associated variables. For the PCA it was using the mean center function and variance as a scaling method.

The PCA results presented in Figure 4 summarize the variance within the GCxGC-TOFMS dataset obtained from tequila aroma profiles. The first two principal components (PC1 and PC2) explain the major sources of variation among the analyzed samples (57.71%). The combination of scores and loadings plots reveals a strong geographical discrimination between the tequila samples, generating two main clusters. Therefore, it is possible to notice that Jalisco group shows the prevalence of terpenoid compounds (PC1>0). Some of the identified compounds that are likely responsible for floral and herbal notes of the Jalisco samples are 1-methyl-4-(1-methylethenyl) cyclohexanol, α-terpineol 7-octen-2-ol, and 2-methyl-6-methylene.

The Guanajuato group (PC1<0) can be subdivided into two small groups. The samples within the first group have positive values in PC2, presenting a higher number of linear esters species, such as propanoic acid, propyl ester; 2-methyl-propanoic acid, 3-methylbutyl ester; heptanoic acid, 3-methylbutyl ester. Due to the presence of these compounds, these samples have tropical fruit and banana-like notes. The other secondary group (PC2<0) is composed of one single añejo sample, presenting some possible markers of aging process. In this case, the compound classes present in this sample are furans, furfural, and oxygenated esters. Amongst the identified compounds are furfural; 2-furancarboxylic acid, ethyl ester; acetic acid-ethoxy, ethyl ester. These compounds provide woody, buttery, tobacco and fruity notes to this sample.

As can be seen in Figure 5, *ChromaTOF* Tile enabled this fast, intuitive, and highly efficient workflow that took us from raw data to important analytical conclusions in just 60–90 minutes. Expected processing times will vary depending on number of samples, method parameters, complexity of data, and specifications of the data processing computer.

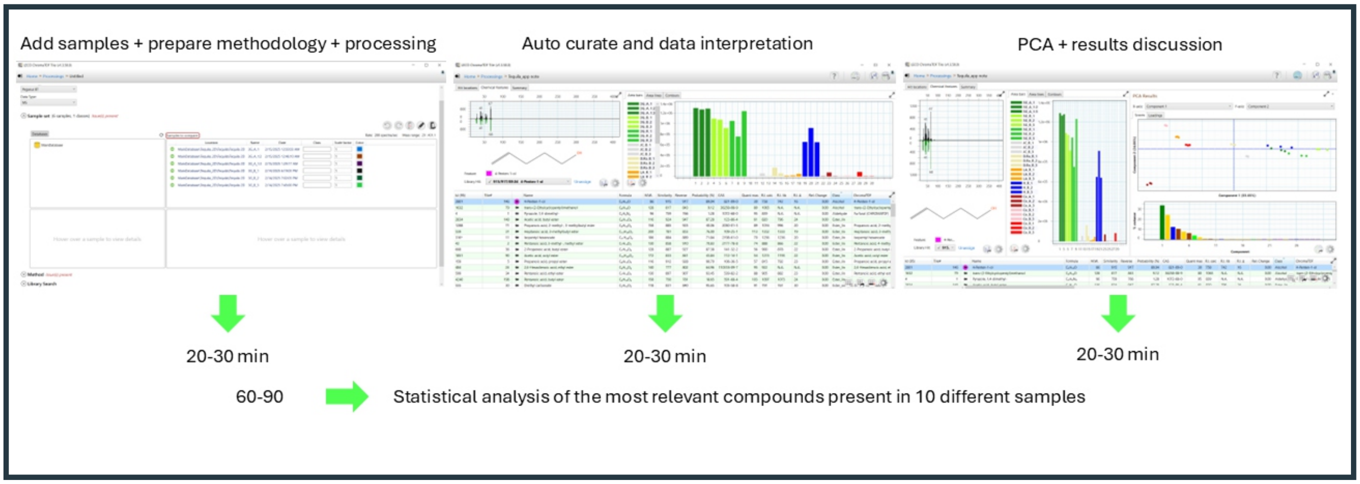

Figure 5: ChromaTOF Tile workflow and respective time consumed in each step of data processing and data handling.

Conclusion

GCxGC-TOFMS combined with *ChromaTOF* Tile was used to analyze the aroma profile of different commercial tequila in a time-effective manner. The analytical workflow, based on LECO's *Pegasus* BT 4D GCxGC-TOFMS technology, generates rich, high-quality data. Compounds of interest were identified based on the comparison of mass spectral information within *ChromaTOF* Tile, using NIST MS libraries and RI calculations. Through the PCA analysis, it was possible to find geographical discrimination between the set of samples as well as compounds that can be used as aging markers in these beverages. This study shows important implications for beverage production, quality monitoring, and reformulation, since better knowledge of the volatile compounds present in tequila aroma were obtained.

References

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