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Workflow for differential analysis of whisky using an Orbitrap Exploris GC 240 mass spectrometer

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Keywords

SPME Arrow, spectral deconvolution, Flavor and Fragrance HRAM spectral library, unknown screening, gas chromatography, Orbitrap mass spectrometer

Goal

The goal of this application note is to describe a simple and effective workflow for the differential analysis of whiskies. The Thermo Scientific™ Orbitrap™ Exploris™ GC 240 mass spectrometer in combination with Thermo Scientific™ Compound Discoverer™ software incorporating a high resolution accurate mass flavor and fragrance library provides a fast and comprehensive route to compound identification.

Introduction

Whisky is a premium spirit beverage that is distilled by following long-established methods that involve a complex aging process. It is produced by the mixing of various grains with water to form a mash that is fermented with yeast, distilled to generate an alcoholic distillate, and finally matured in wooden barrels or casks. This is a complex and traditional process that results in a beverage that has both a high value and high degree of variability. The production technology plays a significant role in the chemical composition and hence the flavor characteristics of the final whisky product.

As a result of these distinguishing features and the rising global demand, whisky has become an economically important commodity in many regions of the world. As whisky has a high retail price, counterfeiting and/or adulteration is commonplace and is a threat to the integrity of the industry. Adulteration can take many forms and can occur on both small and large scales. For example, one of the most extensive forms of adulteration is to add the main chemical constituents of whisky to an alternative cheaper spirit to

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create an "artificial" whisky. This is of particular concern as there is no safety control over which chemicals are being added, their quality, or concentration. Another form of adulteration includes the labeling of bottles with more expensive brands and falsely claiming the length of time the whisky was matured in the barrel. The latter type of adulteration can be performed either by the addition of artificial colors or by heating during the aging process to speed up the coloration to mimic the aging process. The bottles are then mis-labeled claiming that the whisky was aged for an extended period in the barrel, which then justifies a higher price. Both processes can appear to achieve within days/months what normally would have taken many years.

Gas chromatography-mass spectrometry (GC-MS) has been widely used to characterize whisky as it provides analytical advantages of chromatographic resolution, reproducibility, peak capacity, and, most importantly, extensive spectral libraries to aid in identification of volatile and semi-volatile chemical constituents. In this study, we take advantage of the performance of the Orbitrap Exploris GC 240 mass spectrometer for the profiling of whiskies of different origins, ages, and types. An additional aim is to evaluate the application of a complete untargeted chemometric workflow using Compound Discoverer software to detect and identify chemical components in whisky. It will also show the process of identifying chemical differences in whiskies of different origins. Samples were analyzed using a full scan non-targeted acquisition and high mass resolving power (120,000 resolution FWHM at m/z 200) to obtain accurate mass measurements. This is important to enable elucidation of the elemental composition and discrimination of co-eluting and isobaric compounds.

These features in combination with unique software algorithms for automated deconvolution and sample comparison create a powerful solution for comprehensive characterization, quality control, and product brand protection.

In this study, the performance of the Orbitrap Exploris GC 240 high resolution accurate mass (HRAM) spectrometer together with the headspace solid phase micro extraction (SPME Arrow) for chemical profiling of whisky is demonstrated.[1] Differences in chemical profiles are easily visualized using the statistical tools incorporated into Compound Discoverer software with streamlined identification using the Thermo Scientific™ Flavour and Fragrance HRAM library. This provides analysts with a means of accurate identification of chemical profiles in whisky to maintain product quality control and differentiate between whiskies suspected of fraudulent activities (additives and mislabeling).

Experimental
Sample preparation
A total of five different whiskies were analyzed in this study, all originating from Scotland (sample ID: 1-5). In addition to the whisky samples, one sample of Brandy (sample ID: 6) was also analyzed to serve as a negative control towards whisky classification and identification.

For identification purposes, 5 μg/mL C_7-C_{40} alkane mixture (Sigma Aldrich, Germany) was prepared in hexane to establish the retention time index. A volume of 20 μL was added to a headspace vial and analyzed in total vaporization mode using the same method conditions for sample analysis outlined in Tables 1–3.

Full scan EI and CI data acquisition → **Accurate mass peak deconvolution** → **Statistical comparison** → **Mass spectral library match** → **Molecular ion and confirmation with CI**
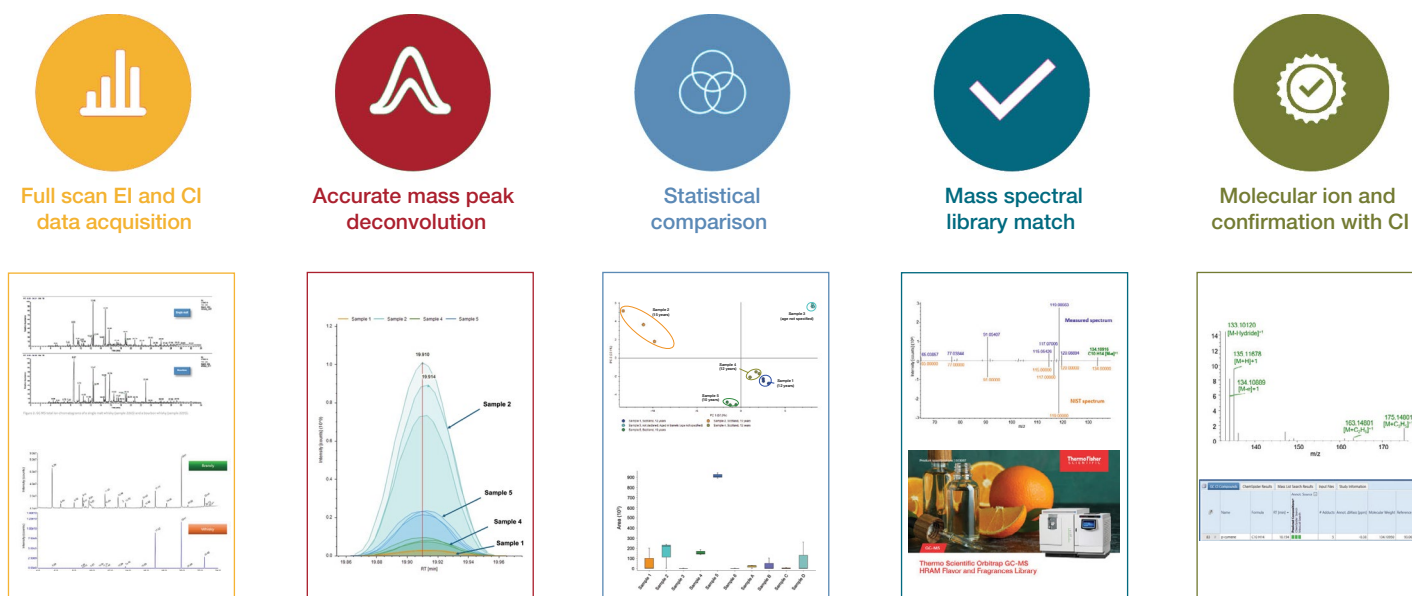
Figure 1. Workflow from left to right showing how from data acquisition in full scan we move from peak detection, to identifying unique components in a sample, to finally making a compound identification

Table 1. TriPlus RSH SMART and GC conditions

Parameter	Setting
TriPlus RSH SMART SMPE Arrow parameters	
Incubation temperature (°C)	40
Incubation time (min)	10
Agitation speed (rpm)	500
Extraction time (min)	10
Needle depth in vial (mm)	30
Needle speed in vial (mm*s^{-1})	20
Fiber injection	
Injection liner depth (mm)	70
Penetration speed (mm*s^{-1})	40
Injection desorption time (min)	3
SPME Arrow fiber conditioning	
Conditioning temperature (°C)	290
Post-conditioning time (min)	10

Parameter	Setting
TRACE 1610 GC system parameters	
Injector	Thermo Scientific™ iConnect™ SSL
Liner	Thermo Scientific™ SPME Arrow liner ID 1.7 mm, (P/N 453A0415-UI)
Injection mode	Split
Split flow (mL·min^{-1})	60
Injector temperature (°C)	250
Carrier gas (mL·min^{-1})	1.2
Oven temperature program	
Initial temperature (°C)	40
Hold time (min)	3
Rate 1 (°C·min^{-1})	10
Temperature 1 (°C)	270
Final hold time (min)	5
Total analysis time (min)	45

Table 2. EI source and mass spectrometer conditions

Orbitrap Exploris GC MS parameters for EI	
Transfer line (°C)	280
Thermo Scientific™ ExtractaBrite™ ion source temperature (°C)	300
Electron energy (eV)	70
Acquisition mode and scan range (*m/z*)	Full scan, 40–600
Resolving power (at 200 *m/z*)	120,000
Emission current (µA)	50
C-Trap offset (V)	0
Mass accuracy on lock mass	5 ppm
Internal lock mass calibration (column bleed, *m/z*)	207.02235, 281.05114, 355.06993

Table 3. PCI Ion source and mass spectrometer conditions

Orbitrap Exploris GC MS parameters for PCI	
Transfer line (°C)	280
ExtractaBrite ion source temperature (°C)	200
Reagent gas and flow (mL·min^{-1})	Methane; 1.1
Ionization mode	Positive
Acquisition mode and scan range (*m/z*)	Full scan; 50–600
Resolving power (at 200 *m/z*)	120,000
Emission current (µA)	50

For sample preparation, 100 µL of whisky (ETOH content approximately 40%) was diluted with 900 µL of ultrapure water (18.2 Ω) with a final alcohol content of 4%. Three blank samples (1 mL 4% ETOH in water) were prepared in addition to assessing background contamination. A QA/QC sample was prepared by mixing 100 µL of each whisky together to a total volume of 1 mL. This serves as a control for assessing the statistical analysis performance for unknown identification within Compound Discoverer software.

Instrument and method setup

Headspace extraction and injection of whisky samples was performed using the Thermo Scientific™ TriPlus™ RSH SMART autosampler equipped with the Thermo Scientific™ SMART SPME Arrow 1.1 mm DVB/C-WR/PDMS fiber (P/N 36SA11T3-SM). Incubation and extraction were performed online followed by sample injection/desorption. After sample injection, the SPME Arrow fiber was re-conditioned at high temperature under a nitrogen flow using an SPME conditioning station to avoid sample carryover between injections. Further details surrounding the SPME Arrow operating parameters can be found in Table 1.

A Thermo Scientific™ TRACE™ 1610 GC equipped with a Thermo Scientific™ TraceGOLD™ TG-624SilMS (30 m × 0.25 mm I.D. × 1.4 μm film) capillary column (P/N 26085-3320) was used to perform the chromatographic separation. Oven program conditions can be found in Table 1. Data acquisition was carried out in full scan analysis mode using both EI and PCI with the Orbitrap Exploris GC 240 mass spectrometer. Additional MS method parameters are summarized in Tables 2 and 3. External mass calibration was performed prior to analysis, while characteristic ions representing column bleed were used as lock masses when scanning in EI to perform internal mass calibration. Sample acquisition and qualitative processing were performed using the Thermo Scientific™ Chromeleon™ version 7.3.2 Chromatography Data System (CDS) software. Unknown analysis and identification were performed using the Compound Discoverer version 3.3 software.

Results and discussion

The objective of this study was to analyze the whisky samples using a non-target full scan data acquisition and to identify, using statistical tools, if there were differences between the samples and to propose a compound identity to any differences observed.

The workflow used to achieve these objectives is summarized in Figure 1. The samples in the study can vary in their chemical complexity. An example of this can be seen in the total ion chromatogram between Brandy and a whisky sample shown in Figure 2.

Discovery workflow

High-resolution accurate mass data in full scan mode is collected on the Orbitrap Exploris GC 240 mass spectrometer with Chromeleon CDS software. This full scan EI data is then processed in Compound Discoverer software to reveal differences and compound identity. Compound Discoverer software provides a complete solution of accurate mass deconvolution of the data to identify all features across all samples. Compound identification is carried out using matching with spectral libraries or, in the case of true unknowns, proposed elemental compositions through accurate mass and isotope pattern matching. The statistical tools within the software are used to compare the samples and identify differences, if any. These tools can help to isolate peaks or features in the data that are unique to a particular sample. All of these are functionality held within Compound Discoverer software making it a powerful for research and discovery applications.
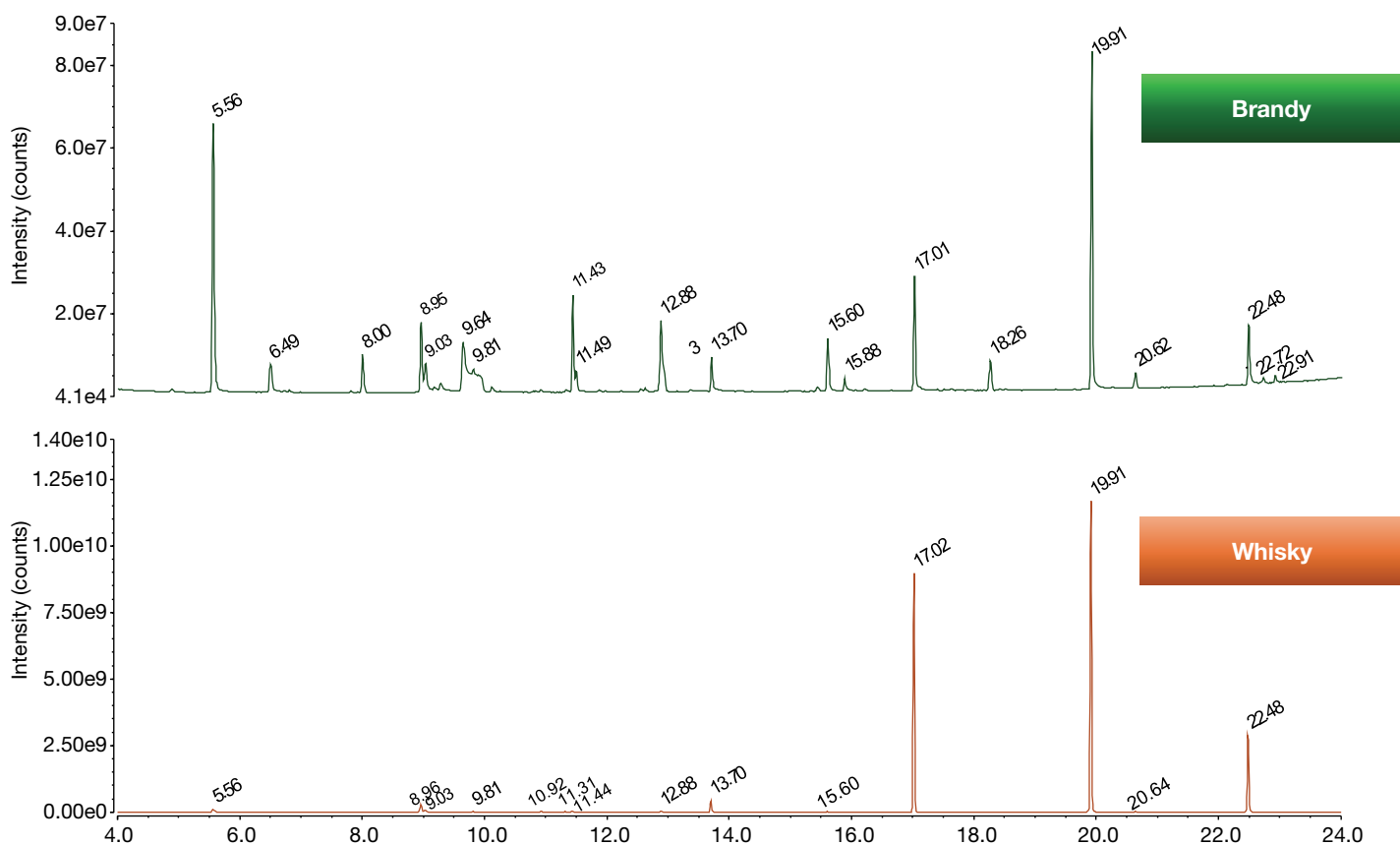

Figure 2. Total ion chromatogram of SPME Arrow extracted Brandy (upper) and whisky (lower) samples. Deconvolution is necessary to reveal the peaks that exist beneath the full scan base line.

To support this type of experiment, Compound Discoverer software contains template workflows for GC EI, as well as GC PCI data. In this study, the EI data were used for statistical analysis and compound identification, whereas the PCI data were used for confirmation purposes. PCI is an alternative and complimentary form of ionization, which is considered a softer ionization that can give molecular ion information through mass adduct patterns and lower fragmentation. Although not mandatory, the combination of EI and PCI data is advisable as it increases the confidence of identification due to molecular ion confirmation. The main features of the EI workflows in Compound Discoverer software are spectral deconvolution, compound identification, and statistical analysis. Compound identification is based on the library search using both high-resolution and nominal mass spectral libraries such as NIST 2023.

For various statistical analyses, zero values within the sample set can lead to erroneous results. To avoid this type of error, Compound Discoverer software provides methods for inputting missing chromatographic peak areas for detected compounds across the set of input files. This is the role of the "Missing

Value Imputation" node. Additionally, two extra nodes—"Apply QC Correction" and "Mark Background Compounds"—were aggregated to the template nodes present in the workflow. The "Apply QC Correction" node is useful when a long sequence of samples is acquired and compensates for time-dependent batch effects. To use this node, a QC sample is required. To create the QC sample, a small aliquot from each sample must be pooled in one vial. The pooled sample is injected at regular intervals along the sequence. The "Mark Background Compounds" node is applied to flag compounds that are found not only in the sample but also in the instrumental or matrix blanks.

Discovering differences between samples

The first objective was to identify if there was any significant difference between the whisky samples. This was achieved through a PCA plot of the replicate injections of each sample. Figure 3 shows the PCA plot that demonstrates that there are clear differences in the identified chemical profiles between the samples and good agreement of the replicate injections, for example between samples 2 and 3.
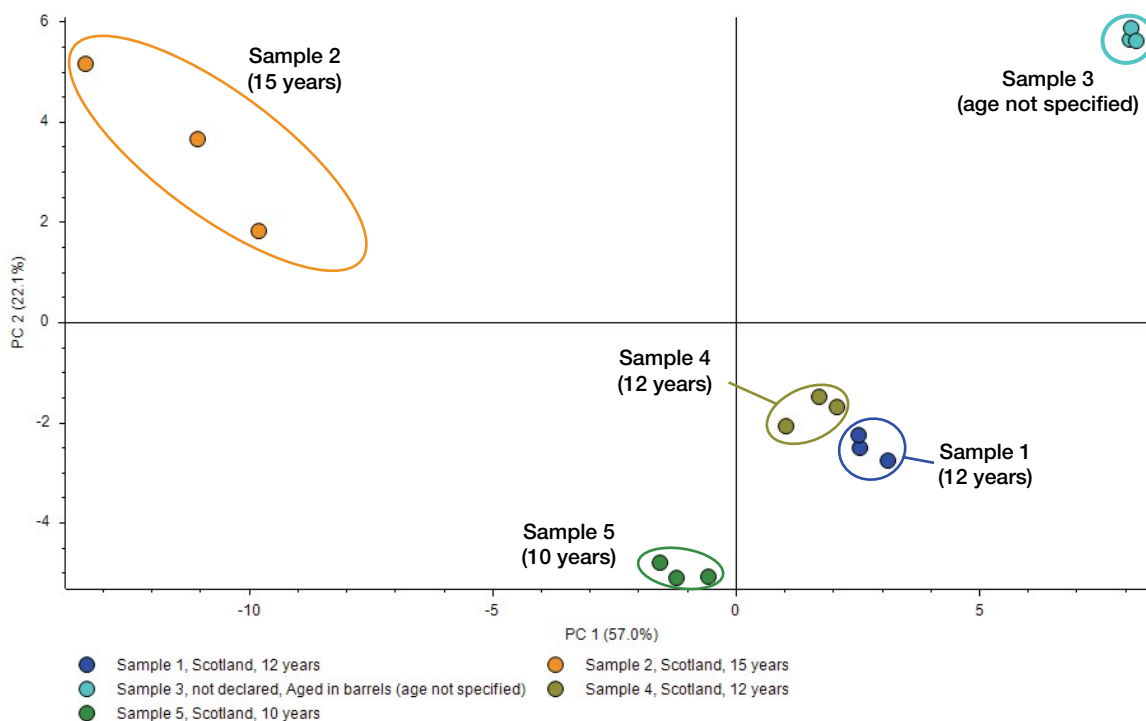

Figure 3. Principal component analysis loading plot of whisky samples based on identified chemical profile from Compound Discoverer software. Whisky age and country of origin (if provided) have been displayed as additional explanatory variables.

The following steps then help identify which peaks contribute to the differences so that a compound identification can be proposed. As an example, Figure 4 shows a volcano plot for sample 3 versus 2. The volcano plot is a type of scatter plot for replicate data where the x axis represents the log2 of the fold change between two sample groups (generated ratio), and the y axis represents the negative log10 of the p-value (test of significance) of the fold change. In other words, when a point (compound) is more on the left (positive values on x axis), the peak area of that compound is much higher in sample 2 than in sample 3, while points that are higher on the graph are statistically more significant. For example, higher levels of 2/3 methyl-1-butanol are typically associated with Scotch malt whiskey, as these are reduced in the grain whiskey due to the distillation processes within column stills. This is further supported by the higher Furfural content also found in sample 2, which is present in higher amounts in malted grains. High ester content is also associated with age and cask maturation (i.e., bourbon casks), indicating sample 2 has undergone a much longer and different maturation process compared to sample 3.).
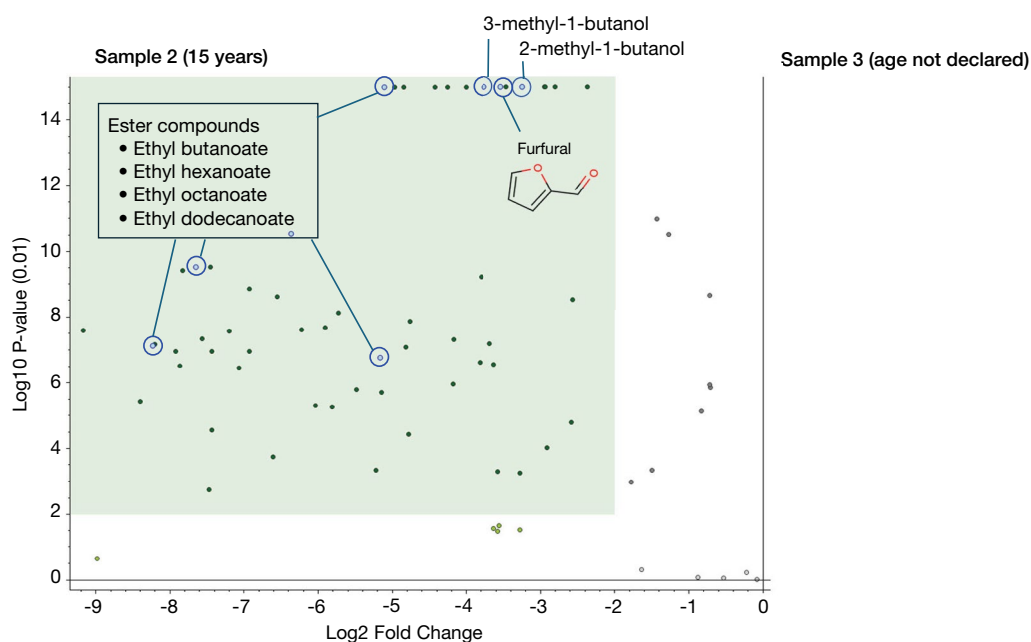

Figure 4. Volcano plot of chemical profile detected in sample 2 and sample 3 after background correction. Results are filtered to compounds with search index (SI) values greater than 700. Compounds highlighted in blue are unique to sample 2 and labeled where identification is known. The X axis represents the difference in response observed by a factor of 2-fold change. The Y axis represents the p-value based on the statistical analysis between the two sample groups. Shaded regions in green represent compounds whose response differs greater than 2-fold and are significantly different at the 95% confidence interval ($p \leq 0.05$).
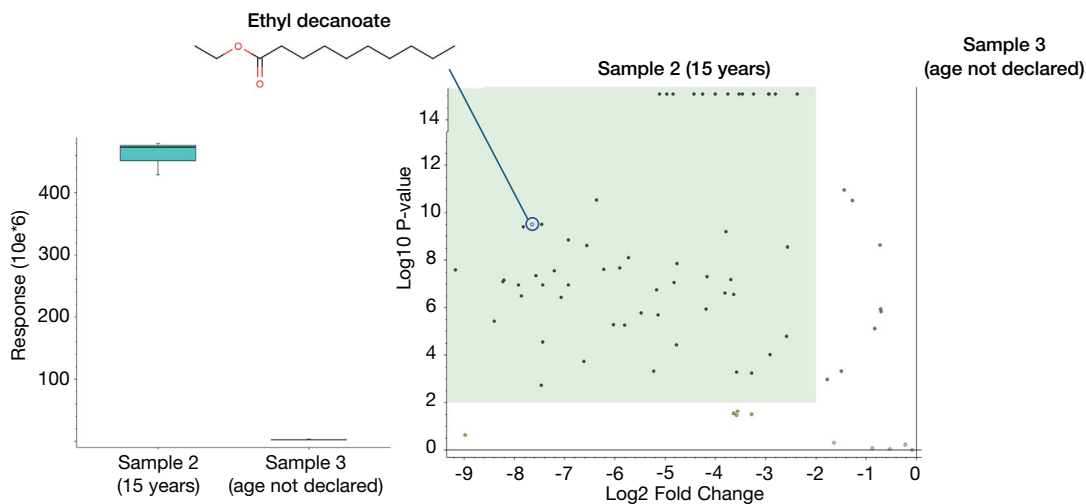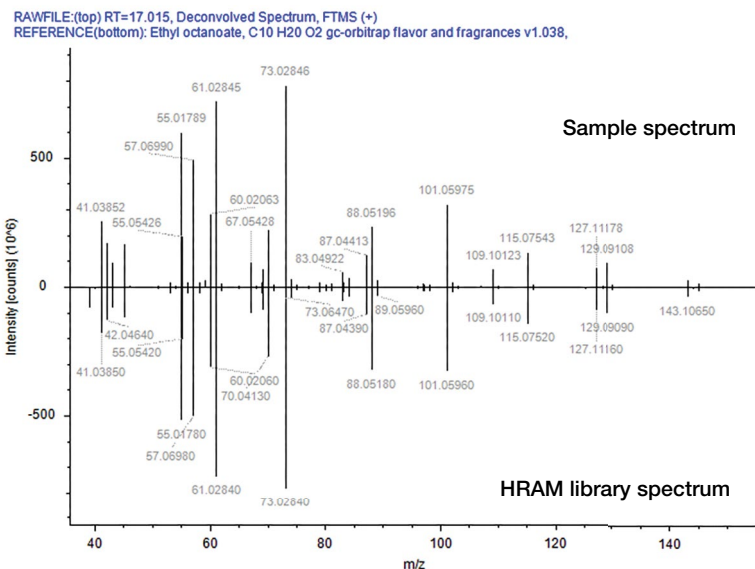

Figure 5. Volcano plot of chemical profile detected in sample 2 and sample 3. Example peak identified as ethyl decanoate is shown as being elevated in sample 2. By clicking on the feature in the volcano plot (right) a separate box plot (left) is displayed showing the difference intensity between the samples and variability within the replicate injections.

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Identification of marker compounds

Once unique features are isolated, the subsequent compound identification was aided through library matching with NIST 2023 and the Thermo Scientific™ Flavor and Fragrance HRAM library (P/N 834-009400). An example is shown in Figure 6 with the compound ethyl octanoate match in Compound Discoverer software. An excellent SI score of 990 is made with this library entry. An additional identification point is the high resolution filtering score (HRF). The HRF score is the percentage of the measured spectrum that is explained by the chemical formula proposed from the best library hit elements. Additionally, for the compounds that had an RI value in the library, a criterion of ΔRI < 50 was applied. This is displayed in the results table in Compound Discoverer software for a quick review.

With EI spectra, the molecular ion frequently does not appear in the spectrum. Therefore, to confirm the compound's identity or in the case of a true unknown, to propose an elemental composition, it is essential to review the chemical ionization spectrum. This will lead to identification of the molecular ion based on the adduct pattern formation. Compound Discoverer software automatically searches for these ions and labels them as shown in Figure 7. The ethyl octanoate mass adducts of $[M+H]^+$, $[M+C_2H_5]^+$, and $[M+C_3H_5]^+$ are automatically labeled in Compound Discoverer software to support compound confirmation. The final step for unequivocal identification is to obtain a certified standard and analyze under the same conditions to match retention time and spectra with the sample data.

Figure 6. Sample mass spectrum (upper) and matching HRAM spectrum (lower) with Flavor and Fragrance Library hit of ethyl octanoate including search results table. SI score of 990 indicates a strong match. Confirmation of the molecular ion in PCI is required to support this identification.
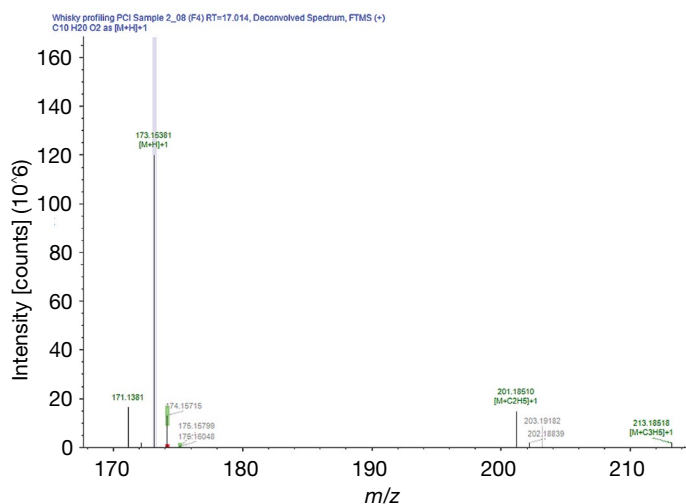
Figure 7. Sample positive chemical ionization (PCI) zoomed mass spectrum for ethyl octanoate to confirm molecular ion. Mass adducts of [M+H], [M+C$_2$H$_5$], and [M+C$_3$H$_5$] are automatically labeled in Compound Discoverer software.

Conclusion

The complex and versatile chemical profile existing among the various whisky types causes challenges in sample profiling and finding marker compounds. A combination of the HS-SPME Arrow, Orbitrap Exploris GC 240 mass spectrometer, and Compound Discoverer software provides an efficient workflow for the analysis of compounds giving analytical advantages including:

- Time savings with minimal sample preparation and online extraction using the TriPlus RSH SMART robotic autosampler

- Full scan acquisition at high mass resolution (i.e., 120,000 at *m/z* 200) providing targeted quantitative analysis together with non-target analysis for chemical profile determination

- Mass spectral deconvolution combined with statistical tools for sample differentiation, all combined within the Compound Discover software

- Dedicated Flavor and Fragrance HRAM library for accurate identification at sub ppm mass accuracy

The workflow on food profiling in this application note can be applied to other areas that involve sample group comparison, screening, and compound identification.[2-4]

Reference

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