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Black Pepper Authenticity Analysis Workflow with LC-QTOF and Novel Software Solution

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Introduction

Food authenticity as a global issue

Complex supply chains characterize the global market of herbs and spices. Black pepper is one of the most popular spices; it is used globally as a flavoring or seasoning in a wide variety of different cuisines and accordingly is produced in several countries globally. Pepper, as with most other herbs and spices, is typically grown on smaller farms, reaching the consumer after passing through a series of complex and opaque intermediaries, the ideal scenario for various frauds to be perpetrated. Geographic origin fraud is increasingly problematic in a market where tariffs and tax evasion pose significant financial risks to both businesses and governments.

Untargeted analysis using HRMS followed by statistical analysis has been demonstrated as powerful methodology to address these challenges.[1] However, such analytical workflow can be complex with multiple acquisition modes, requiring expert statistical and analytical knowledge and therefore time consuming. Herein is a novel software MassHunter Explorer 2.0 that integrates complex analyses into a simplified workflow, making it easier to interpret various data file types and providing users with clear and confident insights.

Figure 1. Revident LC/Q-TOF with 1290 Infinity III LC

Authentic black pepper samples from 5 geographical origins (Vietnam, Cambodia, India, Indonesia and Brazil) were used for this study.

Experimental

Sample preparation

Grinded black pepper samples (20 mg) were extracted with 1.5 mL of a methanolic solution (MeOH:H_2O; 4:1 v/v). Resulting mixture were vortexed and shaken for 20 minutes at 30 C then sonicated for 30 minutes at 30 C. Samples were centrifuged and supernatant collected, then filtered before analysis on the LC/MS system. Quality Control (QC) samples were generated by aliquoting each sample into a single vial and injecting at the start of the sequence to condition the column and every 5 samples during analysis.

All data were acquired on a 1290 LC combined with Revident LC/Q-TOF (Figure 1) with MS mode and iterative AutoMSMS to increase MS/MS coverage for identification.

Table 1. LC method and Revident LC/Q-TOF source parameters.

LC Conditions	
Column	Agilent Zorbax Eclipse Plus C18, 2.1 x 150 mm, 1.8 µm
Column temperature	45 °C
Autosampler temp	4 °C
Injection volume	1 µL
Mobile phase	**A:** Water, 4.5 mM NH_4HCO_2, 0.5 mM NH_4F, 0.1% HCO_2H **B:** Methanol, 4.5 mM NH_4HCO_2, 0.5 mM NH_4F, 0.1% HCO_2H
Flow rate	0.4 mL/min
Gradient program	**Time** **%B** 0.0 2 1.5 2 15.0 100 19.0 100 19.1 2 20.0 2
Post time	4 min
Total run time	24 min
Revident LC/Q-TOF MS Conditions	
Acquisition rate	MS: 3 spectra/s AutoMS/MS: 3 spectra/s
Gas temperature, Flow	325 °C, 10 L/min
Nebulizer pressure	35 psi
Sheath gas temp, Flow	375 °C, 12 L/min
Nozzle voltage	200 V
Capillary voltage	2500 V

Results and Discussion

Novel Software providing streamline data analysis workflow

The analysis of black pepper is complicated by the nature of the matrix, which is considered to be one of the harsher on both the UHPLC system and the MS itself. It is known to be feature rich and therefore challenging both for the instrument and the analyst to interpret results.

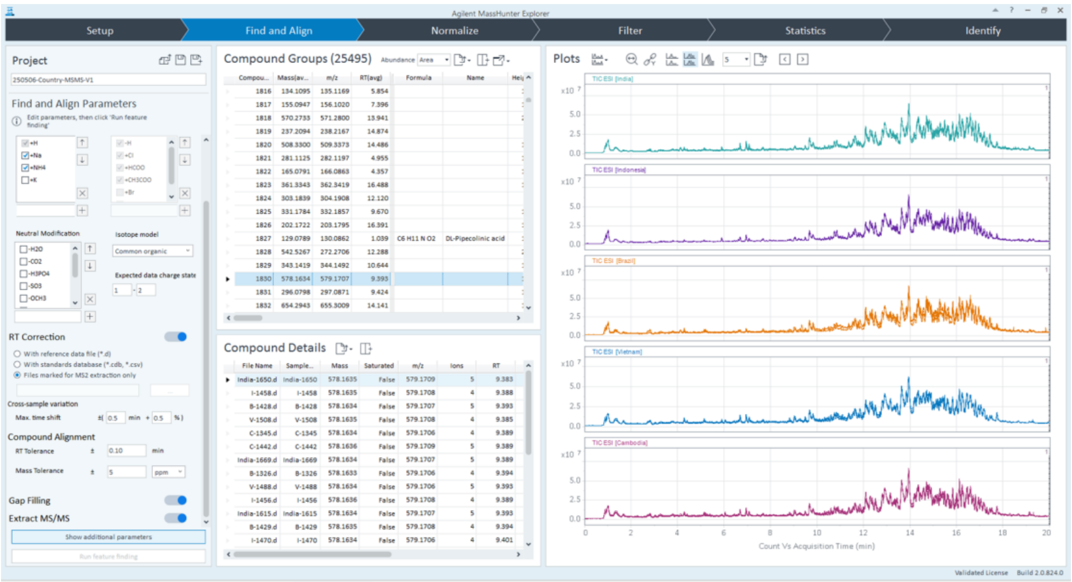


Figure 2. MH Explorer 2.0 Find and Align settings with TIC of various black pepper extracts

MassHunter Explorer 2.0 simplifies the analysis process into six key steps: setup, find and align, normalization, filtering, statistics, and identification (Figure 2). Data files are directly imported from acquisition. Subsequently, files are batched and sorted using custom groupings and easily selectable types. Feature extraction settings, such as mass extraction limits, adduct possibilities, and height filters, are chosen. Compound groups are then normalized and filtered based on abundance, variability, and frequency. Various statistical analyses, including HCA, volcano plots, PCA, ANOVA, unique features, and fold change analysis, can be performed. Finally, identification is completed using mass accuracy and isotope analysis, available retention times if present, and library fragmentation spectra.

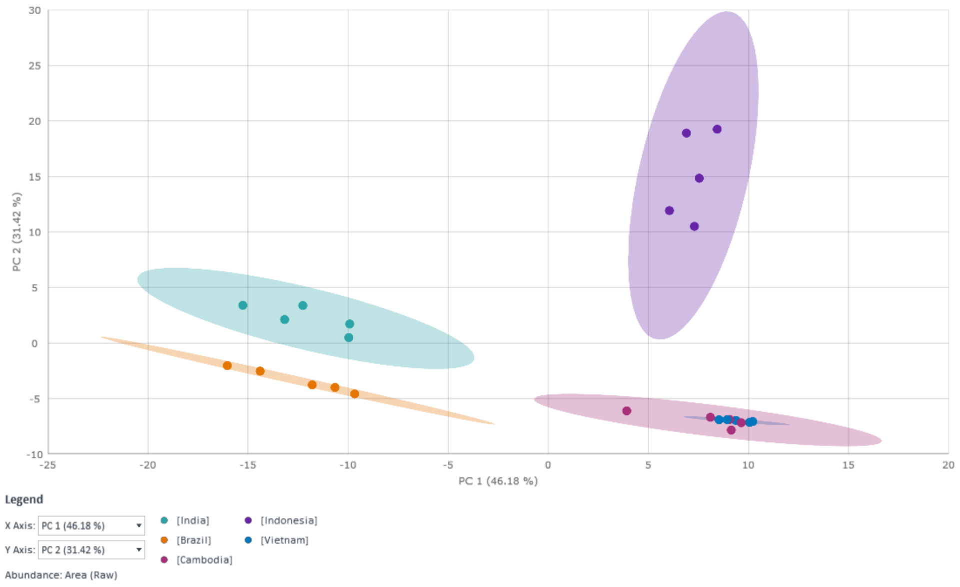


Figure 3. PCA plot of black pepper extracts after data filtration

Integrated data filtering and statistical analysis

More than 25,000 compounds were detected using a height filter of 2,000 including Na^+ and NH_4^+ adducts. Dimensionality of the data was reduced by applying a relative filter on abundance (25 to 100%), variability (less than 25% variation in the 5 group) and frequency (100% in each group).

Statistical analysis was then applied to the remaining 6000 Compound Groups to focus on analytes that were significantly different between the groups. A 1-way ANOVA (pair group) test was followed by:

a Fold Change (pair group) to obtain a list of 239 features exacerbating significant difference between the different country of origin for the black pepper extract and

PCA plot (Figure 3) showed clear difference between 4 groups with samples originating from Cambodia and Vietnam having high similarities likely due to geographical reasons, making them difficult to differentiate.

Integrated MS and MS/MS identification workflow from curated spectral libraries and databases

The identification procedure has been enhanced to evaluate fragmentation information from MS/MS data in complement of accurate mass and isotopologue matching (Figure 4).

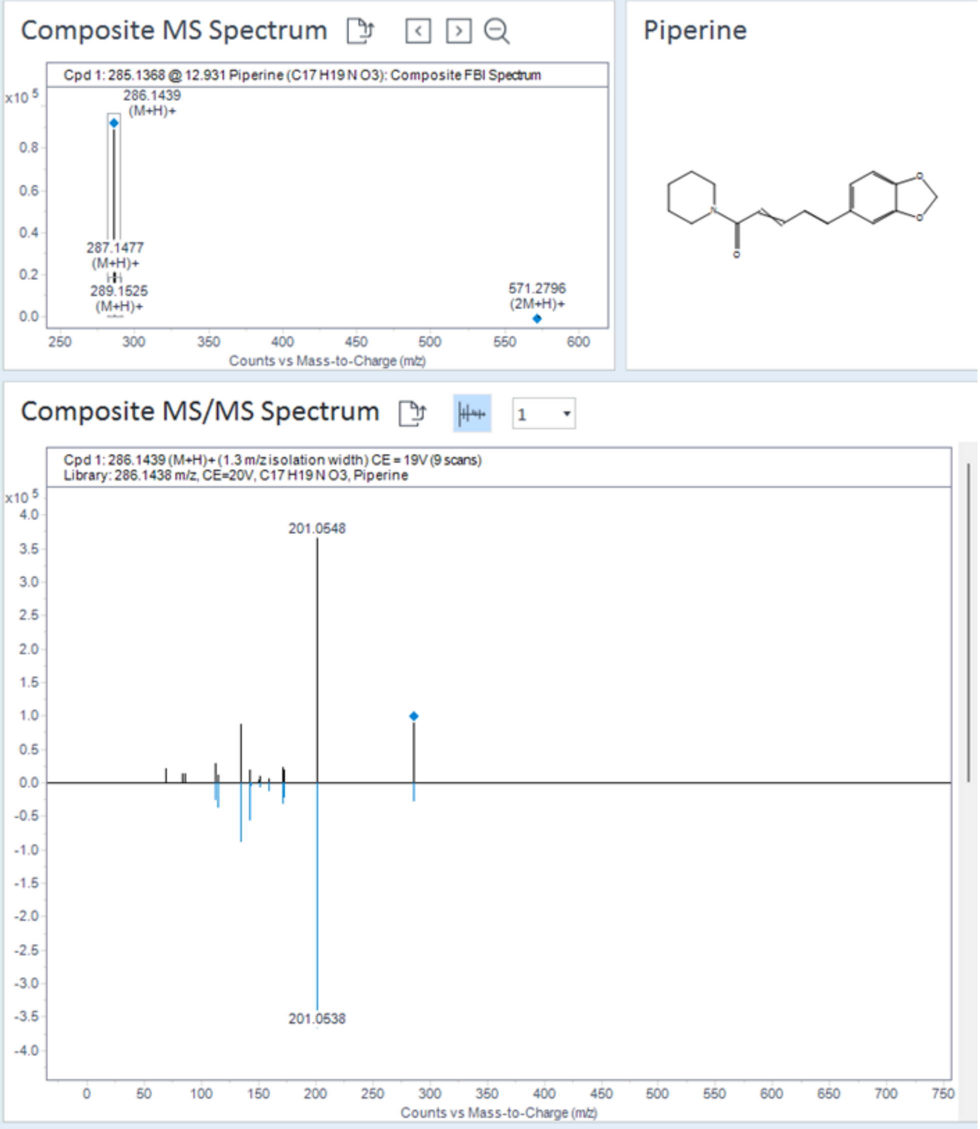


Figure 4. Identification step displaying isotopologue matching (top) and fragment spectra mirror plot (bottom).

Results and Discussion

Direct access to SIRIUS and NIST MS Search for features not present in user libraries

Even with access to extensive and well-curated reference libraries, there will be instances where a specific analyte is absent, restricting identification to its chemical formula. However, integrating direct links to SIRIUS CSI:FingerID or NIST MS Search offers the opportunity for a more in-depth exploration of potential identifications.

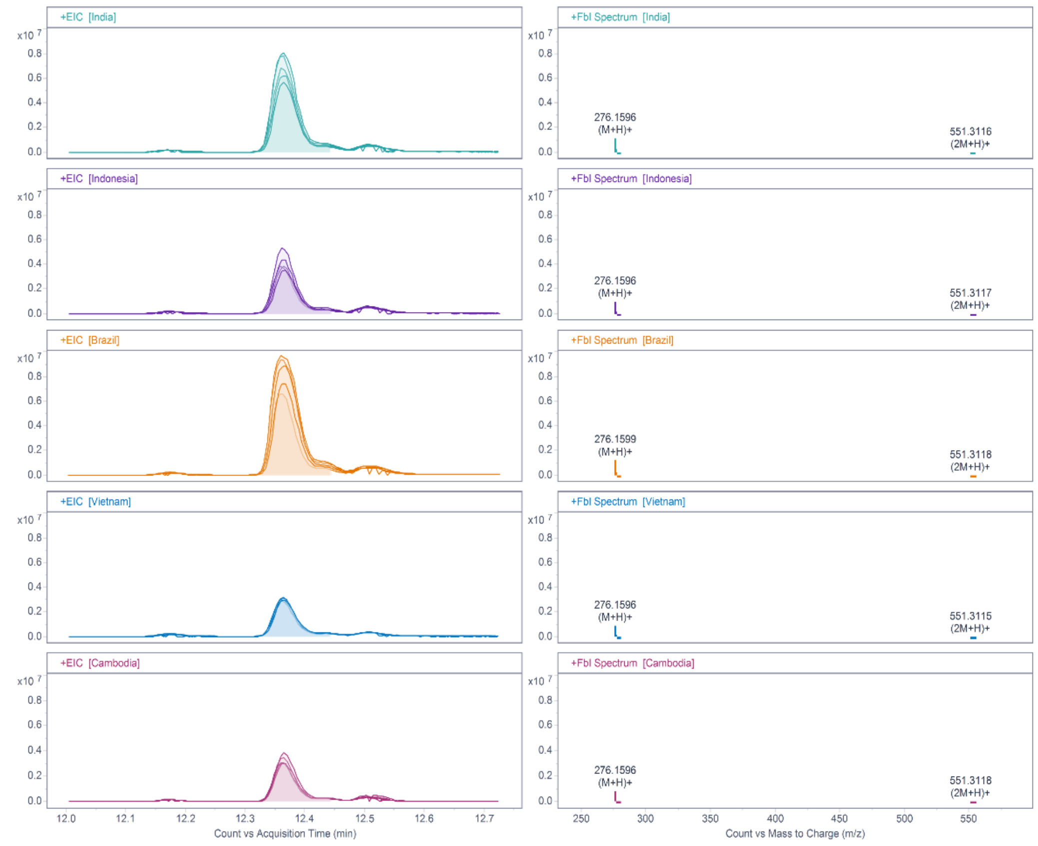
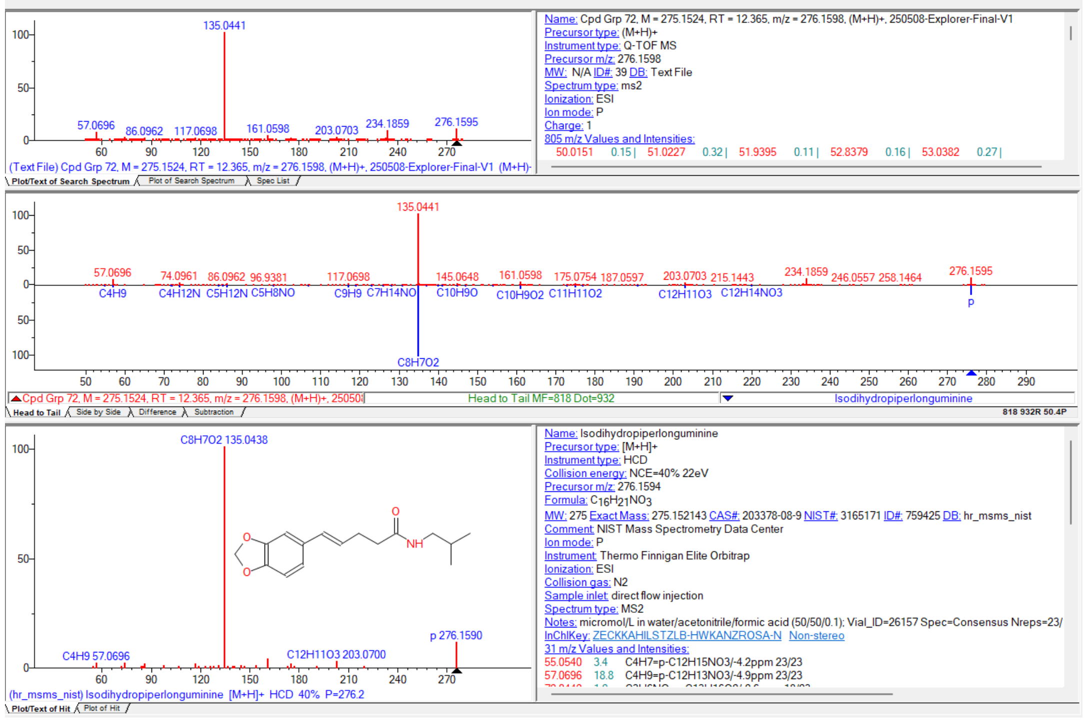


Figure 5. Feature discriminating between some of black pepper country of origin.

For instance, the feature presented in Figure 5 did not yield any identification from the curated database but direct import of the spectral information to NIST MS Search (Figure 6) and to SIRIUS (Figure 7) provided valuable information to help for structure annotation and scoring.


Name: Cpd Grp 72, M = 275.1524, RT = 12.365, m/z = 276.1598, (M+H)+, 250508-Explorer-Final-V1
Precursor type: (M+H)+
Instrument type: Q-TOF MS
Precursor m/z: 276.1598
MW: N/A ID#: 39 DB: Text File
Spectrum type: ms2
Ionization: ESI
Ion mode: P
Charge: 1
805 m/z Values and Intensities:
50.0151 0.15 | 51.0227 0.32 | 51.9395 0.11 | 52.8379 0.16 | 53.0382 0.27 |

Head to Tail MF=818 Dot=932 Isodihydropiperlonguminine 818 932R 50.4P

Name: Isodihydropiperlonguminine
Precursor type: [M+H]+
Instrument type: HCD
Collision energy: NCE=40% 22eV
Precursor m/z: 276.1594
Formula: $C_{16}H_{21}NO_3$
MW: 275 Exact Mass: 275.152143 CAS#: 203378-08-9 NIST#: 3165171 ID#: 759425 DB: hr_msms_nist
Comment: NIST Mass Spectrometry Data Center
Ion mode: P
Instrument: Thermo Finnigan Elite Orbitrap
Ionization: ESI
Collision gas: N2
Sample inlet: direct flow injection
Spectrum type: MS2
Notes: micromol/L in water/acetonitrile/formic acid (50/50/0.1); Vial_ID=26157 Spec=Consensus Nreps=23/
InChIKey: ZECKKAHILSTZLB-HWKANZROSA-N Non-stereo
31 m/z Values and Intensities:
55.0540 3.4 C4H7=p-C12H15NO3/-4.2ppm 23/23
57.0696 18.8 C4H9=p-C12H13NO3/-4.9ppm 23/23

Figure 6. NIST MS Search output from direct feature search from MH Explorer 2.0

https://explore.agilent.com/AOAC2025-Download-Posters

Complementary information from NIST MS Search and SIRIUS.

In this particular case, the feature was identified as a piperlonguminine derivative with only some uncertainty on the double bond position. However, fragmentation tree information provided by SIRIUS can help annotation.

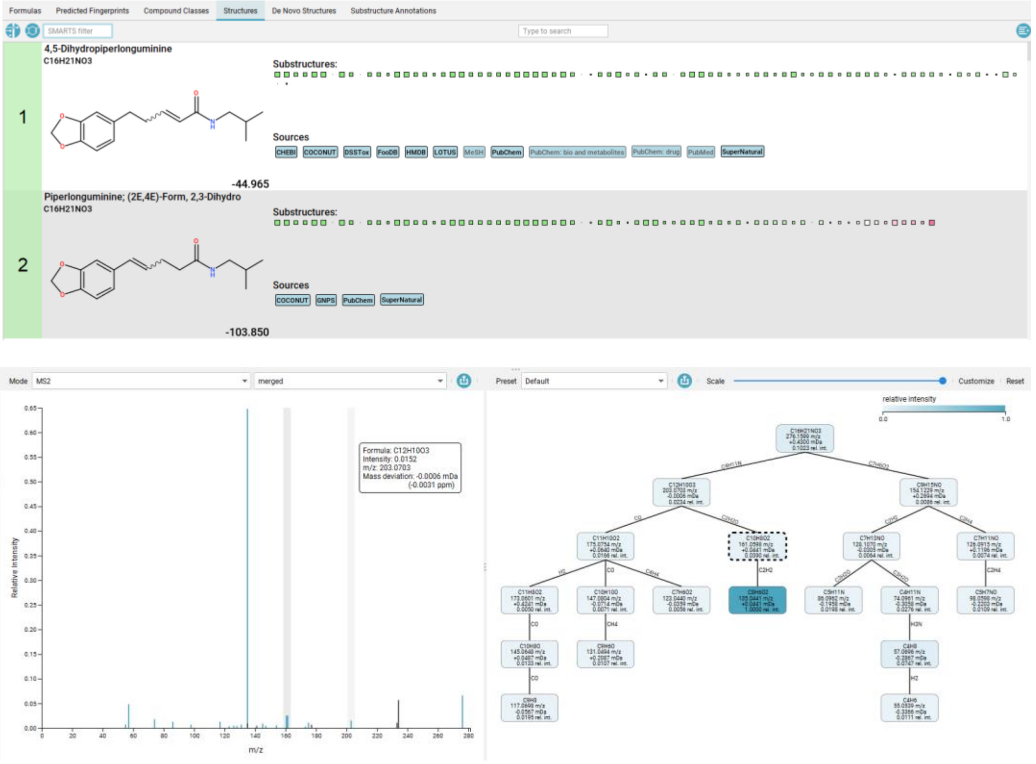

1 4,5-Dihydropiperlonguminine C16H21NO3 -44.965
2 Piperlonguminine; (2E,4E)-Form, 2,3-Dihydro C16H21NO3 -103.850
Formula: C12H10O3
Intensity: 0.0152
m/z: 203.0703
Mass deviation: -0.0006 mDa (-0.0031 ppm)

Figure 7. SIRIUS CSI:FingerID output and fragmentation tree from direct feature export from MH Explorer 2.0.

Conclusions

Novel Software Simplifies Food Authenticity Data Analysis

Black pepper extracts from five different countries can be differentiated

- Molecular features displaying significant differences can be easily highlighted with straightforward guided workflow
- Integrated MS and MS/MS identification workflow
- Direct export to third party software such as SIRIUS and NIST to enhance annotation capabilities

References

[1]Mialon, N et *al*, Untargeted metabolomic approaches in food authenticity: A review that showcases biomarkers. Food Chemistry 2022, 398, 133856.

The authors declare no competing financial interest.

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