



Simultaneous quantitation and discovery (SQUAD) analysis of the fruit juice metabolome

Authors

Mengyuan Zhu¹,
Adrian Sanchez-Woehler¹,
Susan S. Bird², Amanda E. Lee²,
Ralf Tautenhahn², Brandon Bills²

¹Thermo Fisher Scientific,
West Palm Beach, FL

²Thermo Fisher Scientific, San Jose, CA

Application benefits

Enhances productivity by combining accurate quantitation of known polyphenols with discovery and structural elucidation of unknown natural products in a single run.

Goal

This study demonstrates a comprehensive simultaneous quantitation and discovery workflow for commercial fruit juice analysis, enabling structure-based flavonoid profiling using the Thermo Scientific™ Orbitrap™ Ascend MultiOmics Tribrid™ Mass Spectrometer (MS) and Thermo Scientific™ Compound Discoverer™ software.

Keywords

Simultaneous quantitation and discovery, SQUAD, Orbitrap Ascend MultiOmics Tribrid mass spectrometer, Compound Discoverer software, natural product analysis, structure elucidation, structural characterization, molecular networks, differential analysis

Introduction

Fruit juice is a rich source of bioactive compounds, including sugars, organic acids, amino acids, vitamins, and phenolic compounds. Interest in fruit juice metabolomics has grown rapidly because comprehensive characterization of juice composition provides valuable insights into nutritional and health benefits, as well as the impact of food processing and manufacturing practices. Among these constituents, flavonoids—polyphenolic compounds abundant in fruit juices—are well recognized for their antioxidant activity and immune-supporting benefits.¹ Most flavonoids occur as β -glycosides conjugated to one or more sugar moieties, with notable

exceptions such as catechins. We present a simultaneous SQUAD workflow² for the analysis of commercially available fruit juice samples, enabling comprehensive chemical profiling, discovery, and structural elucidation of novel natural products, identification of discriminating compounds across different juice types, and accurate quantitation of targeted polyphenols.

Five commercially available fruit juice samples were analyzed using reversed-phase liquid chromatography on a Thermo Scientific™ Vanquish™ Flex UHPLC System coupled to an Orbitrap Ascend MultiOmics Tribid MS. A metabolomics SQUAD workflow (Figure 1) was employed, integrating targeted and untargeted acquisition strategies within a single analytical run. Comprehensive full-scan and HCD/CID MS²/MS³ data

Experimental details

Sample preparation

Five commercially available fruit juice samples—cranberry, grape, tart cherry, lemonade, and watermelon lemonade—were selected for analysis. Prior to analysis, each sample was filtered and diluted 1:1 (v/v) with methanol. Polyphenol calibration standards, including rutin trihydrate, quercetin, and rosmarinic acid, were prepared in water:methanol (1:1, v/v). Calibration curves were generated over a concentration range of 10–1,000 ppb for quantitative analysis.

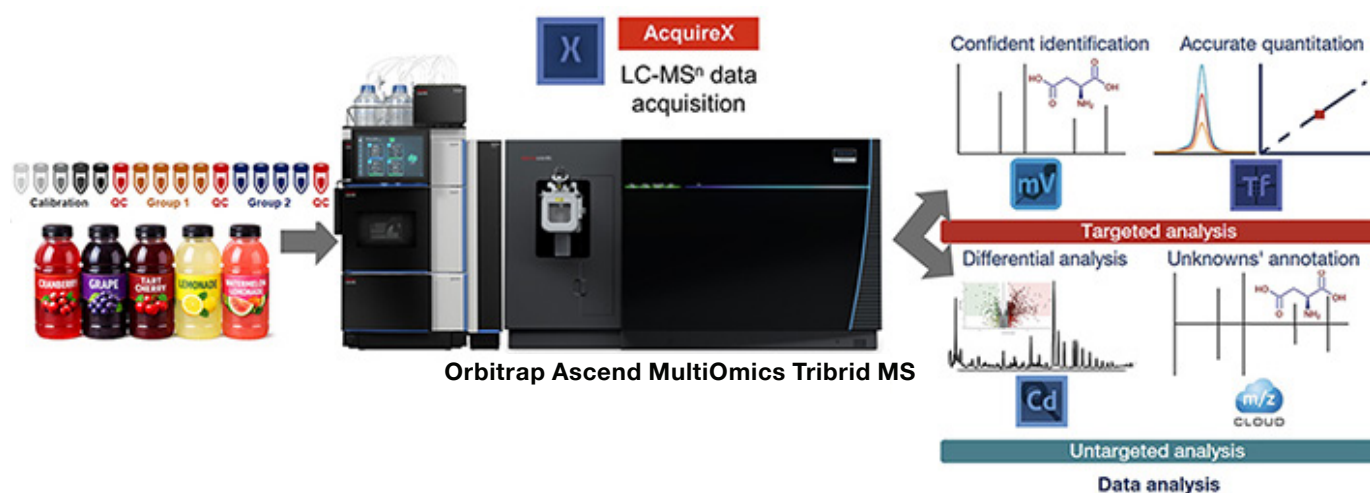


Figure 1. Overview of the SQUAD workflow for comprehensive analysis of fruit juice matrices.

were acquired in both positive and negative ionization modes, and the Thermo Scientific™ AcquireX™ Deep Scan Intelligent Data Acquisition Workflow was used to enhance fragmentation coverage of relevant compounds. In addition to conventional HCD-MS², the Orbitrap Ascend MultiOmics Tribid MS uniquely enables multi-stage (MSⁿ) fragmentation using its dual-pressure linear ion trap, delivering enhanced structural elucidation for compound identification.^{3,4} Targeted quantitation was performed using Thermo Scientific™ TraceFinder™ software version 5.2, while untargeted compound profiling, structural elucidation, and differential analysis were carried out using Compound Discoverer software version 3.5, enabling comprehensive characterization of natural products in complex juice matrices.

Instrumentation

Liquid chromatography

Ultra-high-performance liquid chromatography (UHPLC) was performed using a Vanquish Flex UHPLC system. All chromatographic conditions are listed in Table 1.

Table 1. Chromatographic conditions.

Column	Thermo Scientific™ Accucore™ aQ column (100 x 2.1 mm, 2.6 µm particle size)		
Mobile phase	A: 0.1% formic acid in water B: 0.1% formic acid in methanol		
Gradient	Time (min)	% A	% B
	0.0	97	3
	0.1	97	3
	12.0	50	50
	16.0	5	95
	18.0	5	95
	18.1	97	3
	20/0	97	3
Flow rate	0.4 mL/min		
Column temperature	35 °C		
Autosampler temperature	10 °C		
Injection volume	2 µL		

Mass spectrometry

Mass spectrometry data were acquired using the Orbitrap Ascend MultiOmics Tribrid MS equipped with a Thermo Scientific™ OptaMax™ NG heated electrospray ionization (H-ESI) source. The Orbitrap Ascend MultiOmics Tribrid MS incorporates softer ion funnel optics that minimize in-source fragmentation of labile compounds while maintaining high ion transmission efficiency. Reduced MS¹ fragmentation significantly lowers false-positive identifications during natural product characterization. Dual ion routing multipoles (IRMs) enable true parallelized operation, allowing ions to be accumulated, stored, and fragmented using higher-energy collisional dissociation

(HCD) in the front IRM while simultaneous analyses occur in the linear ion trap or back IRM. This architecture frees additional ion accumulation time and enhances scan rates, enabling deeper coverage for both discovery and quantitative workflows. Multiple orthogonal fragmentation techniques—including collision-induced dissociation (CID), HCD, and ultraviolet photodissociation (UVPD)—are available at any stage of MSⁿ, with detection in either the Thermo Scientific™ Orbitrap™ or linear ion trap analyzer. The ion path of the Orbitrap Ascend MultiOmics Tribrid MS⁵ is shown in Figure 2.

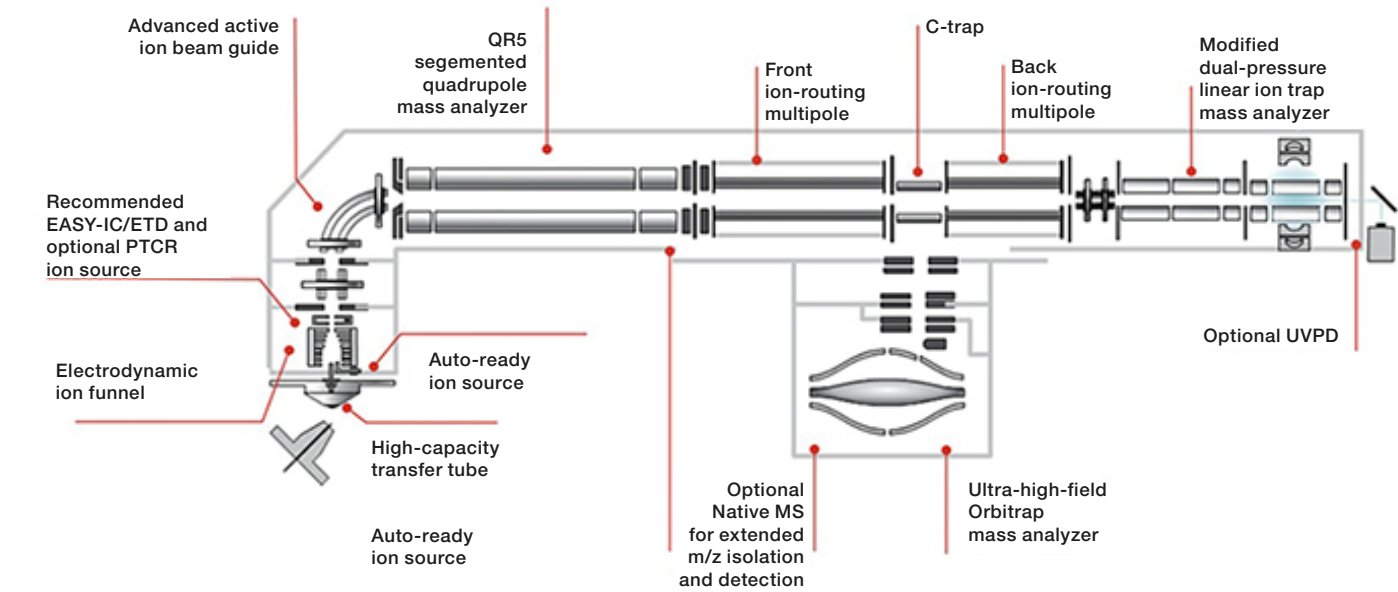


Figure 2. Ion path of the Orbitrap Ascend MultiOmics Tribrid MS.

Data were acquired in full-scan polarity-switching mode for polyphenol calibration standards and individual juice samples. For comprehensive untargeted analysis, the AcquireX Deep Scan intelligent data acquisition workflow was applied to a pooled juice sample to generate full-scan ddMSⁿ data in both positive and negative ionization modes. A Thermo Scientific™ EASY-IC™ internal calibration ion source was employed for all data acquisitions to ensure high mass accuracy throughout data collection. MS source conditions and key MS experiment parameters are detailed in Tables 2–4.

Table 2. MS H-ESI source conditions overview.

Ion source properties	Value
Source voltage	+3,500 V, –2,500 V
Sheath gas (Arb)	50
Aux gas (Arb)	10
Sweep gas (Arb)	0
Vaporizer temperature	350 °C
Ion transfer tube temperature	325 °C
Internal mass calibration	RunStart EASY-IC

Table 3. Full-scan MS method parameter overview.

Scan parameters	Value
Orbitrap resolution	120,000 (FWHM at <i>m/z</i> 200)
Scan range	<i>m/z</i> 150–1,200
RF lens (%)	50
AGC target	Standard
Maximum injection time mode	Auto
Microscans	1
Polarity	Both positive and negative
Source fragmentation	False

Table 4. Full-scan data-dependent MS²/MS³ method parameter overview.

Scan parameters	Value
Cycle time	0.9 s
MS ¹ resolution	60,000 (FWHM at <i>m/z</i> 200)
MS ¹ mass range	<i>m/z</i> 150–1200
MS ² intensity threshold	50,000
Dynamic exclusion	3 s
MS ² /MS ³ Orbitrap resolution	30,000 (FWHM at <i>m/z</i> 200)
MS ² targeted mass exclusion list	Populated using AcquireX Deep Scan workflow and matrix blank sample
MS ² targeted mass list	Populated using AcquireX Deep Scan workflow and pooled juice reference sample
MS ⁿ decision tree	<420 Da: MS ² -HCD, >420 Da: MS ² -CID + MS ³ -CID, MS ³ criteria: saccharide neutral loss, >20,000 minimum intensity, precursor ion exclusion (up to 2) (Figure 4)

Scan parameters	Value
MS ² /MS ³ -CID collision energy	35%
MS ² -HCD collision energy (normalized, stepped)	20, 40, 70%
MS ² maximum injection time	100 ms
MS ³ maximum injection time	200 ms

Software

Data acquisition was performed using Thermo Scientific™ Xcalibur™ software version 4.7 with the AcquireX Deep Scan workflow for intelligent fragmentation. Targeted quantitative analysis was performed using TraceFinder software version 5.2, while untargeted compound profiling, structural elucidation, and differential analysis were conducted using Compound Discoverer software version 3.5. This integrated workflow enabled simultaneous quantitative analysis and untargeted discovery of the fruit juice metabolome.

Results and discussion

SQUAD approach

An example batch order implemented using the SQUAD approach is illustrated in Figure 3. By incorporating preselected polyphenol calibration standards directly into the acquisition sequence, the SQUAD workflow enables accurate targeted quantitation while simultaneously supporting in-depth discovery of unknown compounds.

MSⁿ data acquisition approach

The Orbitrap Ascend MultiOmics Tribrid MS full-scan ddMS³ HCD/CID method employs an intelligent, decision-tree-based acquisition strategy that dynamically adapts fragmentation in real time based on precursor mass and acquired spectral quality (Figure 4). This adaptive approach maximizes the collection of relevant multi-stage MSⁿ data and enhances confidence in compound identification. Within a cycle time of 0.9 s, data-dependent HCD MS² scans were acquired for precursor ions in the *m/z* range of 150–420. For precursor ions between *m/z* 420–1,200, an intelligent product-ion-dependent MSⁿ strategy was applied, in which a high-resolution accurate-mass (HRAM) full MS scan was followed by CID MS² acquisition. When one or more predefined sugar neutral losses were detected, up to two additional CID MS³ scans were triggered, enabling detailed structural elucidation of flavonoid glycosides. Figure 5 illustrates representative data acquired using this decision-tree-based method.

SQUAD study in fruit juice

Order of samples in analytical batches

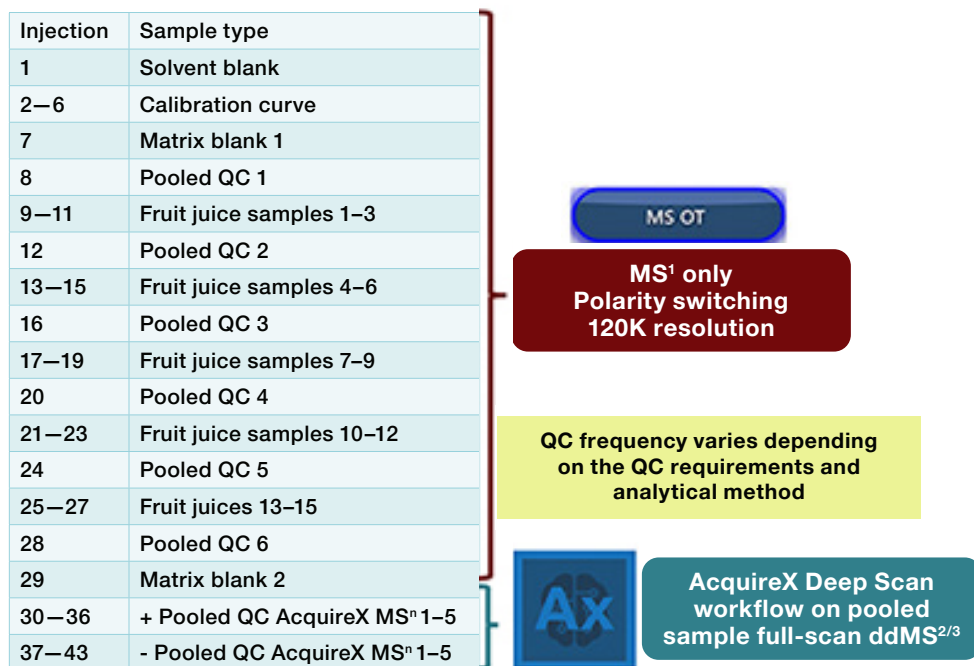


Figure 3. Example batch order implemented using the SQUAD approach.

Orbitrap Ascend MultiOmics MS ddMS^{2/3} method using a CID/HCD decision tree and AcquireX Deep Scan workflow

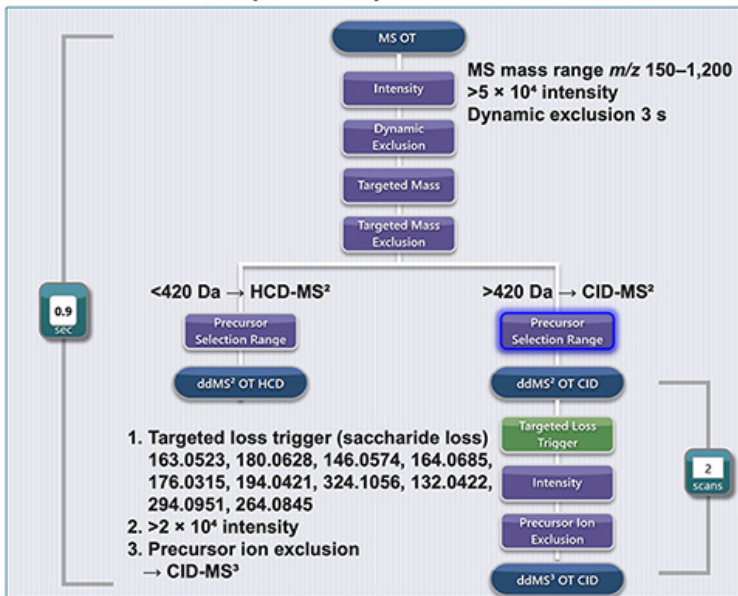


Figure 4. Instrument decision tree for versatile acquisition of multi-stage MSⁿ data.

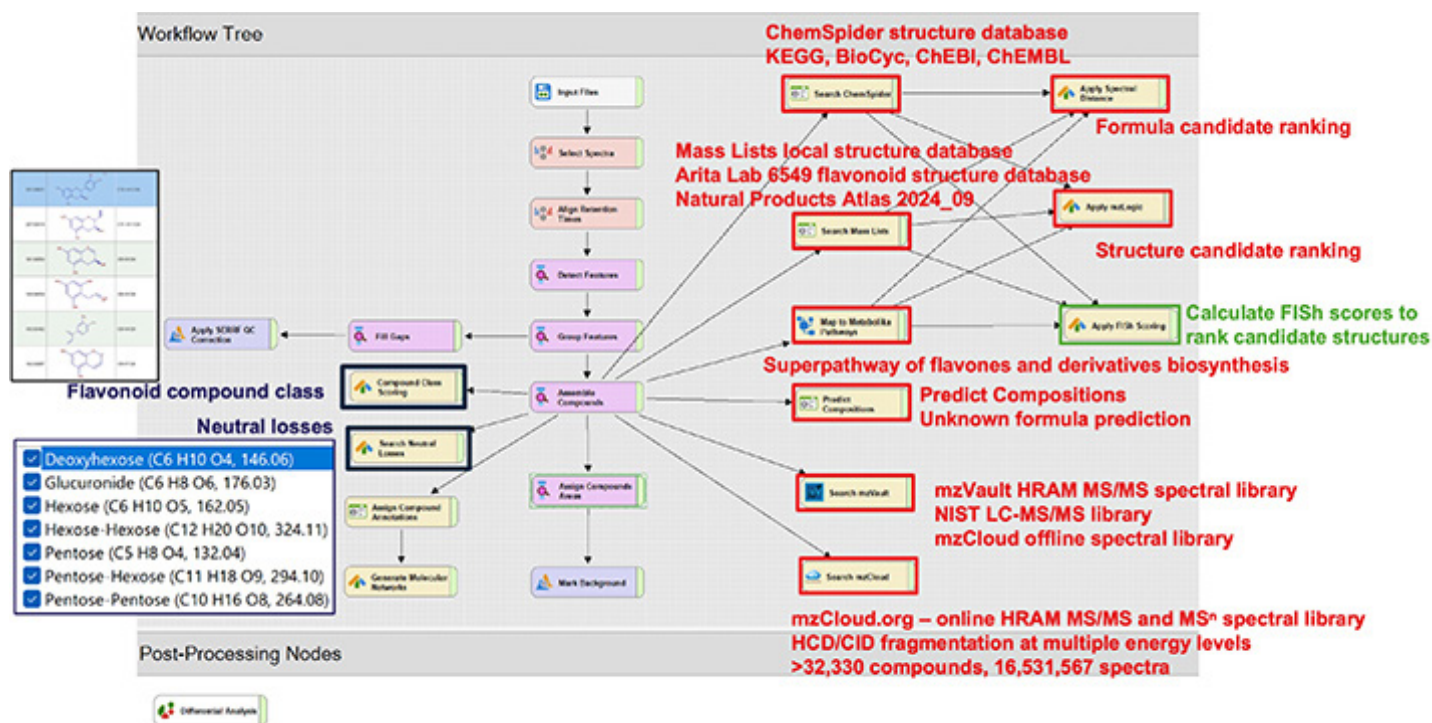


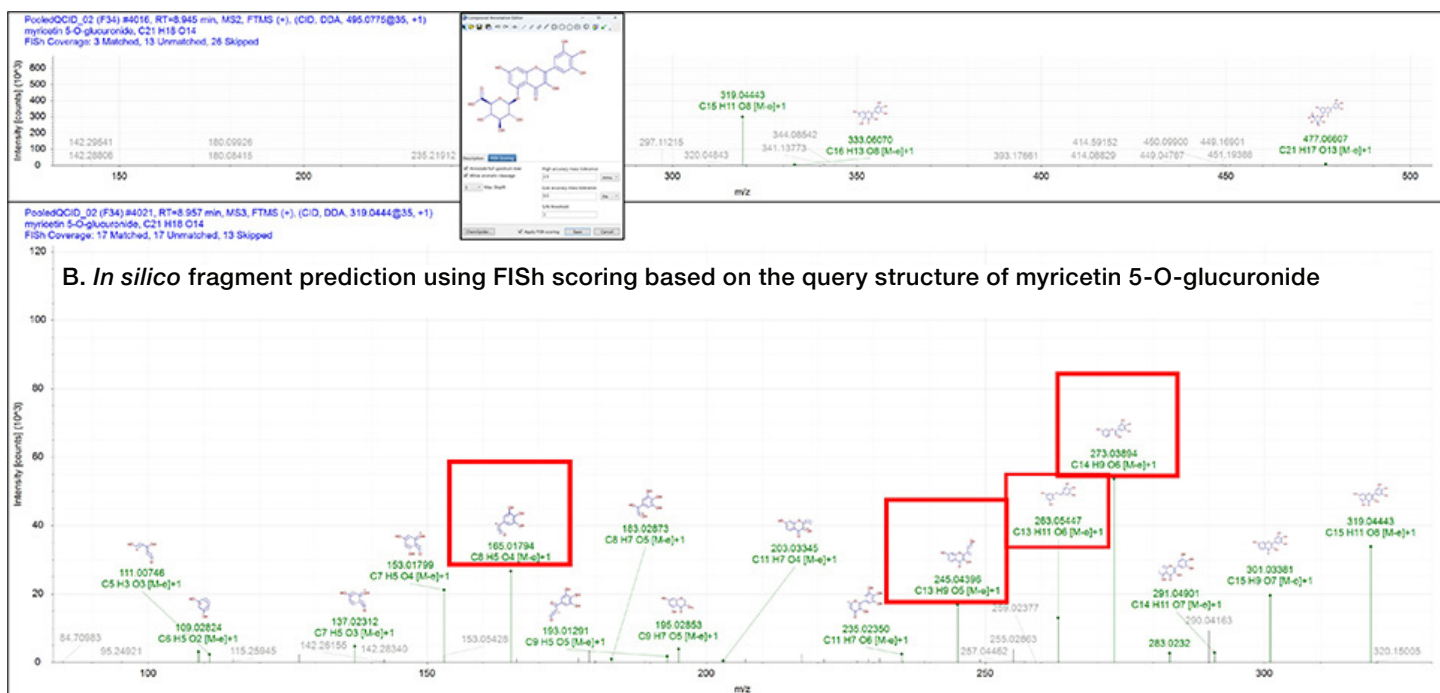
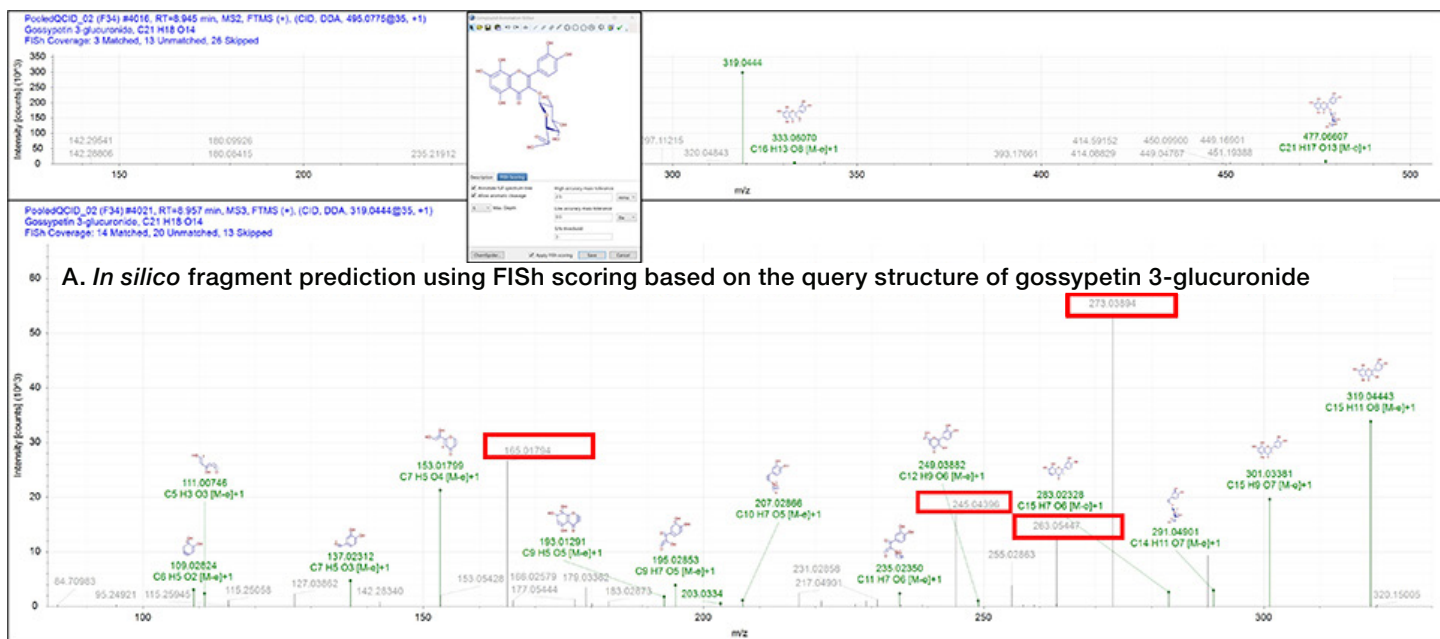
Figure 6. Compound Discoverer workflow tree used in this study, modified from the built-in Natural Product Unknown ID w Stats Online and Local Database Searches template.

Compound Discoverer results view

Compound Discoverer software provides an integrated approach to reveal the chemical composition of different fruit juice types while enabling robust statistical analysis. All unknown compounds detected through the workflow are compiled in the Compounds table (Figure 7), which delivers comprehensive information on extracted features and multiple annotation results, including Annotation Source, Group Areas, Class Coverage, and Neutral Losses. The results view table is fully interactive with the corresponding Chromatogram and Mass Spectrum views. The related tables reveal detailed information for a selected compound in the main table, including matching results from multiple Thermo Scientific™ and third-party party annotation sources (Thermo Scientific™ mzCloud™ and Thermo Scientific™ mzVault™ spectral libraries, ChemSpider [The Royal Society of Chemistry], Mass Lists, Thermo Scientific™ Mass Frontier™ software, Metabolika Pathways, predicted elemental composition, etc.), search results for class coverage and neutral loss matches, as well as detected adduct ions and in-source fragments associated with the selected feature.

Unknown compounds identification by spectral library matching

By direct comparison with spectral library search results, known natural products were readily identified through MS¹ accurate-mass measurements and MS² fragment ion matching. For example, kaempferol 3-robinobioside, highlighted in the Compounds table in Figure 7, is visualized across multiple sample groups in the Chromatograms view, which is populated with the extracted ion chromatogram (XIC) of the selected compound (formula C₂₇H₃₀O₁₅; retention time 10.84 min). The Mass Spectrum view displays the corresponding MS¹ scans and data-dependent MS²/MS³ scans of the preferred ion. The MS¹ spectrum illustrates the isotopic pattern of the selected compound, with green rectangles highlighting peaks that match the theoretical isotopic distribution. The mzCloud library search results pane presents mirror plots comparing experimental raw-file spectra with library reference spectra across MSⁿ levels, supporting the putative identification of kaempferol 3-robinobioside (Figure 8).



Name	Formula	Annot. Source # Annots.	Annot. ΔMass [Da] Annot. ΔMass [ppm] Calc. MW m/z Reference Ion RT [min] Polarity
myricetin 5-O-glucuronide	C21 H18 O14	Predicted Compositions • Metabolia Search mcCloud Search ChemSpider Search MassList Search Predicted Compositions • Metabolia Search mcCloud Search ChemSpider Search MassList Search	0.00108 2.18 494.07073 493.06352 [M-H]⁻1 8.970 +/-

Figure 9. Example of FISH scoring applied to structural proposals for gossypetin 3-glucuronide and myricetin 5-O-glucuronide retrieved from Mass Lists and ChemSpider search results, respectively, for a compound detected at m/z 493.065 (negative ion mode) with a retention time of 8.97 min. Comparison of MS^2/MS^3 fragmentation spectra explained by each structural proposal indicates that myricetin 5-O-glucuronide is the more plausible candidate.

Structure-based flavonoid profiling

Flavonoids share a common core structure consisting of a 15-carbon skeleton with two phenyl rings (A and B) and a heterocyclic ring (C).¹ Structural modifications at different positions of this core give rise to diverse flavonoid classes with a wide range of chemical structures. As a result, flavonoids exhibit characteristic diagnostic fragment ions in mass spectrometry. Many flavonoids occur naturally as β-glycosides conjugated to one or more sugar moieties; therefore, common neutral losses corresponding to sugar residues are frequently observed in the MSⁿ fragmentation spectra of flavonoid glycoconjugates. Within the Compound Discoverer software workflow, the Compound Class Scoring node annotates compounds that share MSⁿ fragment ions characteristic of flavonoids. The Search Neutral Losses node screens MSⁿ spectra and flags compounds

exhibiting sugar-related neutral losses in the Compounds table. In addition, the Molecular Network node generates clusters of structurally related flavonoids and reveals potential relationships based on fragmentation similarity and chemical transformations. Together, these tools provide a comprehensive workflow that leverages intrinsic flavonoid attributes, such as diagnostic product ions and sugar neutral losses, to effectively discriminate flavonoid natural products in complex matrices and facilitate the discovery of new structurally related flavonoid species. Figure 10 displays an example of a compound annotated by mzCloud library matching as rhamnetin 3-galactoside, whose MSⁿ spectra are annotated by matching fragments from a user-curated flavonoid compound class library and a hexose neutral loss. The comprehensive Molecular Network diagram created using the Flavonoid compound class as seed is shown in Figure 11.

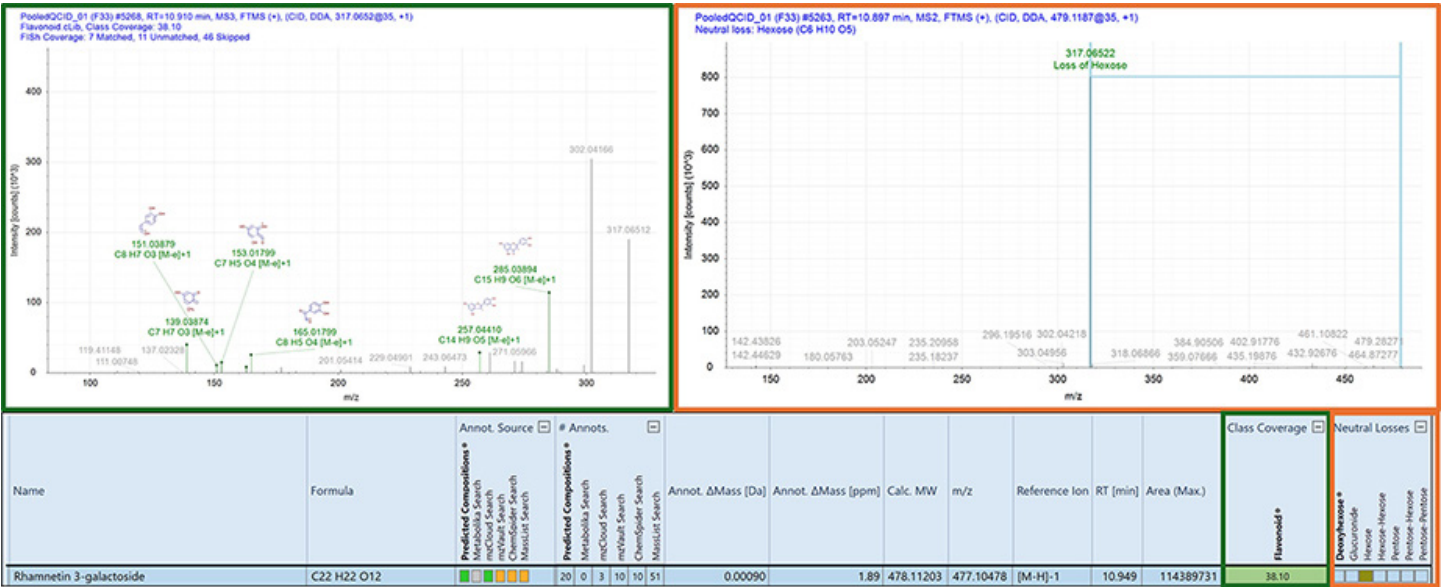


Figure 10. The Class Coverage column shows the percentage of MSⁿ fragments matched to the user-curated flavonoid compound class library, with the matched fragment structures annotated in the MSⁿ spectra. The Neutral Losses column highlights compounds exhibiting characteristic neutral losses, which are annotated directly within the fragmentation spectra.

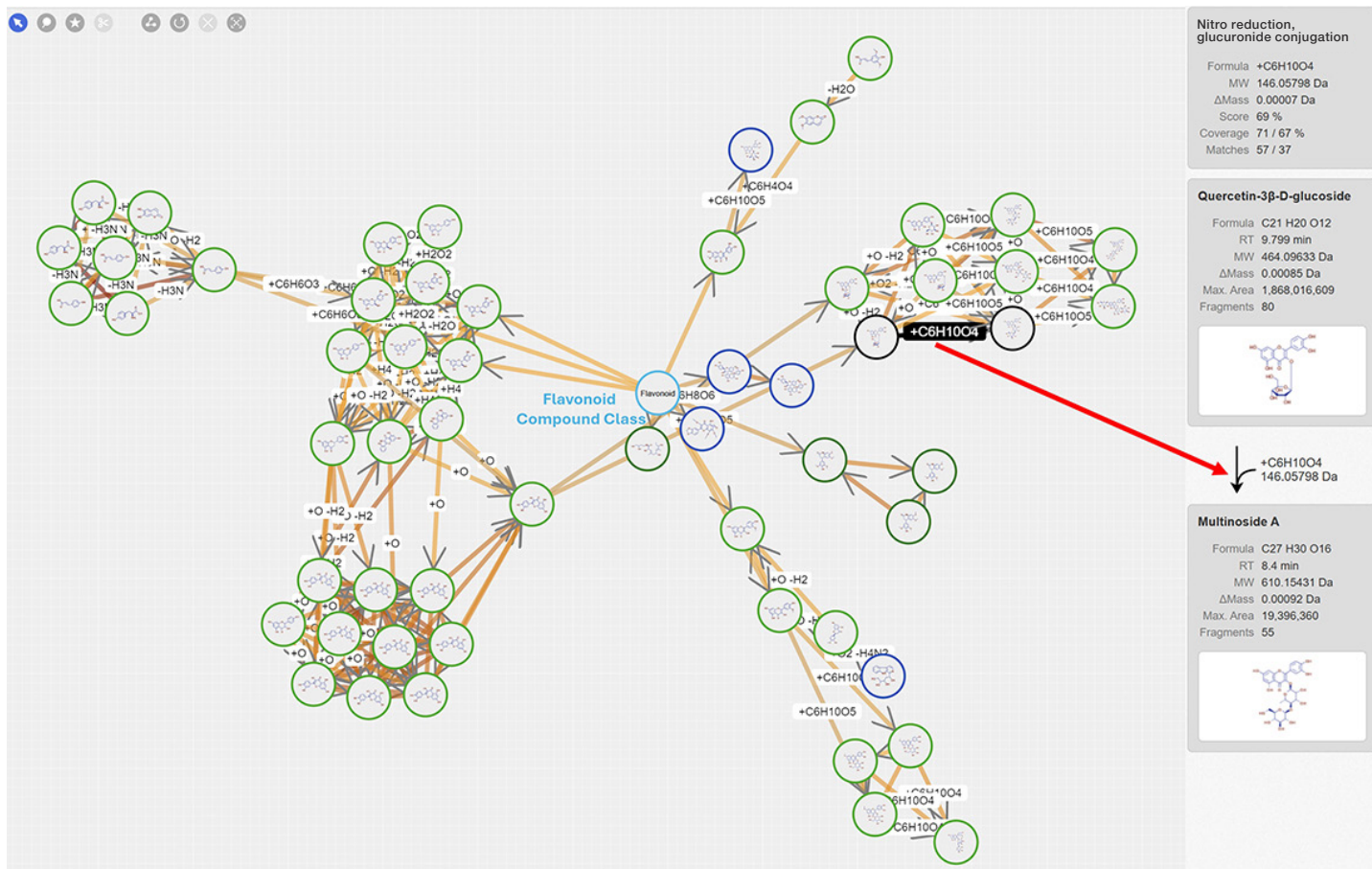


Figure 11. The molecular network was generated using the flavonoid compound class as seed compound to form clusters of structurally related features. Transformations between highlighted compounds are shown on the right, providing insight into structural modifications and facilitating the discovery of new natural products.

Differential analysis

Compound Discoverer software supports both univariate and multivariate statistical analyses and provides multiple visualization tools to explore differences between samples and identify compounds that discriminate among fruit juice types. As shown in Figure 12, principal component analysis (PCA) reveals relationships and similarities among sample groups, while hierarchical cluster analysis visualizes correlations between detected compounds and selected samples as a two-dimensional, color-coded heat map. Differential analysis, displayed as a volcano plot, highlights compounds that differ significantly between two juice groups, while trending charts compare chromatographic peak areas of selected compounds across multiple sample groups.

Targeted quantitative analysis using TraceFinder software

Absolute quantitation was performed using TraceFinder software. Calibration curves for rosmarinic acid (negative ion mode) and rutin (positive ion mode) are shown in Figure 13. Fast polarity switching combined with detection at a resolution of 120,000 (FWHM at m/z 200) enabled the use of both positive and negative ionization modes within a single analytical run while maintaining sufficient data points across chromatographic peaks, thereby ensuring accurate and precise quantitative performance.

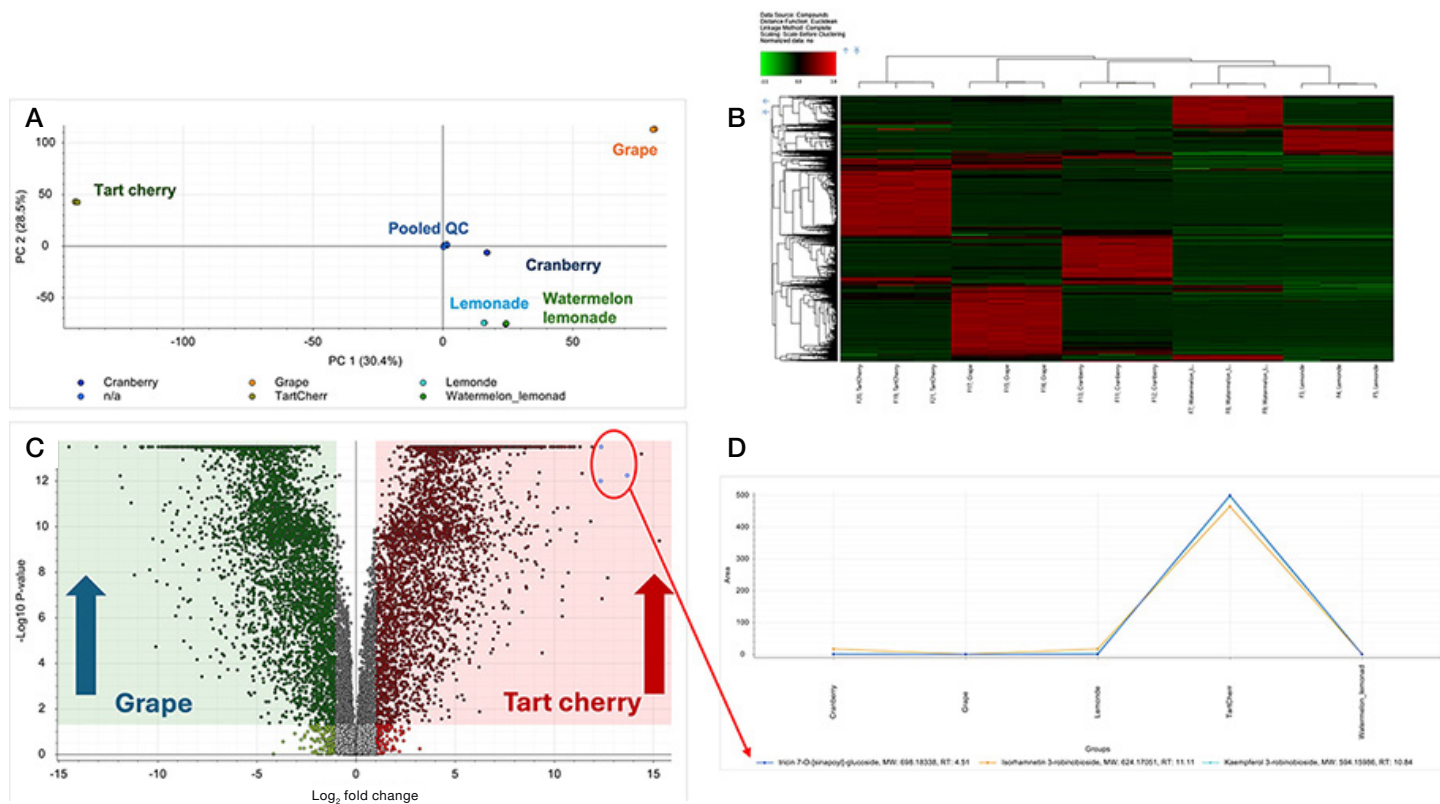


Figure 12. (A) Principal component analysis (B) hierarchical clustering analysis (C) volcano plot comparing tart cherry juice vs. grape juice and (D) trending chart of the selected compounds from the volcano plot.

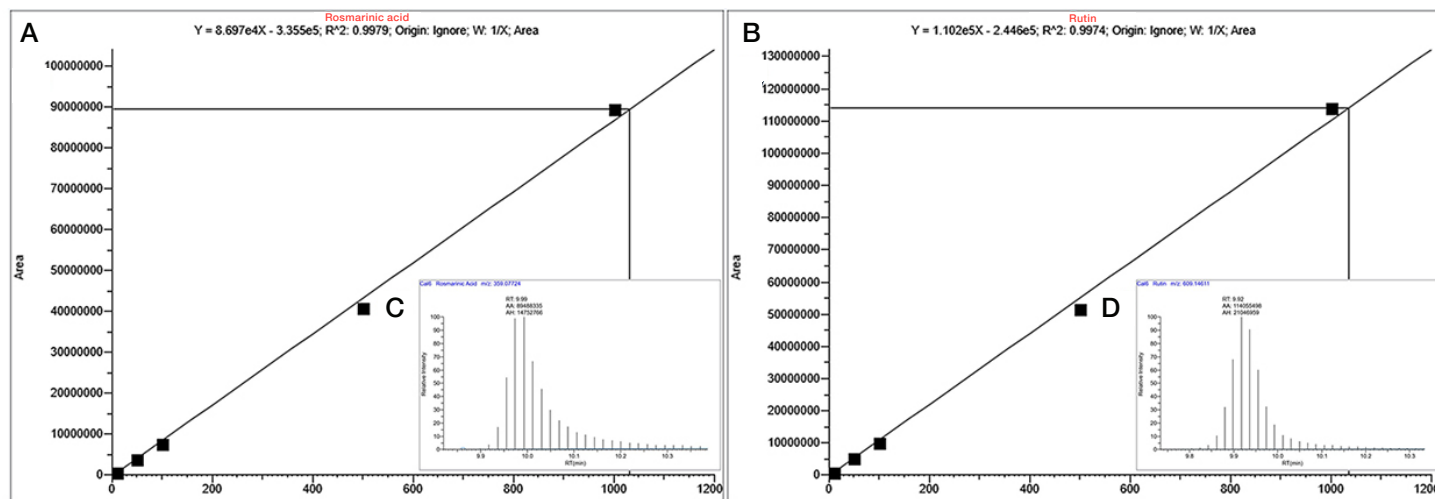


Figure 13. Calibration curves for (A) rosmarinic acid and (B) rutin demonstrated linearity over the range of 10–1,000 ppb. Extracted ion chromatograms of (C) rosmarinic acid and (D) rutin standards are presented.

Conclusion

This study demonstrates a comprehensive SQUAD workflow for the analysis of commercially available fruit juice samples within a single LC-MS run using the Orbitrap Ascend MultiOmics MS together with Compound Discoverer software and TraceFinder software.

High-quality MS and MSⁿ data acquired with high resolution and mass accuracy on the Orbitrap Ascend MultiOmics Tribrid MS enable high-confidence compound identification and annotation. Multi-stage fragmentation further supports structure-based flavonoid profiling and provides sufficient fragment information for structural elucidation.

Compound Discoverer software version 3.5 delivers an integrated and powerful solution for comprehensive characterization of the chemical composition of diverse fruit juice types, facilitating the discovery of novel natural products while providing robust and intuitive statistical analysis.

TraceFinder software enables streamlined and confident targeted quantitation.

The described workflow can be readily applied to the analysis of other compound classes in natural product research and food and beverage applications.

References

1. Thermo Fisher Scientific Application Note 003277: Novel structure-based profiling and annotation workflow-high-throughput analysis of flavonoids using the Thermo Scientific Orbitrap ID-X Tribrid MS.
2. Thermo Fisher Scientific Technical Note 004327: Simultaneous Quantitation and Discovery analysis: Combining targeted and untargeted metabolomics on Orbitrap mass spectrometers.
3. Thermo Fisher Scientific Application Note 003385: Confident transformation site localization of PROTAC drug metabolites facilitated by multi-stage fragmentation LC-MS.
4. Thermo Fisher Scientific Application Note 002154: Increased confidence in drug metabolite identification through intelligent data acquisition strategies and multiple fragmentation techniques on the Orbitrap Tribrid MS platform.
5. Thermo Fisher Scientific Product Specification 002859: Scale up your multiomics research Orbitrap Ascend MultiOmics Tribrid mass spectrometer.

 Learn more at thermofisher.com/squad

General Laboratory Equipment – Not For Diagnostic Procedures. © 2026 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified. This information is presented as an example of the capabilities of Thermo Fisher Scientific products. It is not intended to encourage use of these products in any manner that might infringe the intellectual property rights of others. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details. **AN-004519-EN 0526**

thermo scientific