From spectra to certainty: end-to-end workflows for confident metabolite identification

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Abstract

Purpose: To demonstrate an end-to-end workflow for increasing confidence in untargeted metabolite annotation by combining acquisition on Thermo Scientific™ Orbitrap™ Excedion™ Mass Spectrometer, Thermo Scientific™ AcquireX™ Deep Scan workflow, Thermo Scientific™ Compound Discoverer™ 3.5 software, Thermo Scientific™ mzCloud™ Mass Spectral Library, and AI/ML confidence scoring, and automated confidence-level reporting.

Methods: NIST SRM 1950 plasma extract was analyzed by reversed-phase LC-MS/MS on an Orbitrap Excedion Mass Spectrometer.

Results: The workflow moved untargeted metabolomics analysis beyond feature detection toward metabolite annotation. Accurate mass alone produced 920 caffeine candidate structures, but combined MS1, adduct, MS/MS, and structure-consistency evidence reduced the candidate space to one defensible annotation. AI/ML confidence improved separation of methylxanthine positional isomers, molecular networking added chemical-family context, and automated confidence-level assignment converted complex evidence into transparent, reportable metabolite-confidence levels.

Introduction

Metabolite identification confidence is not a single score; it is an evidence chain spanning acquisition, annotation, and reporting. Untargeted LC-HRMS generates many molecular features, but isotopes, adducts, multimers, contaminants, and in-source fragments can obscure which signals represent true molecular entities. We therefore combine hardware designed to preserve intact precursors with software designed to prioritize, interpret, and report evidence.

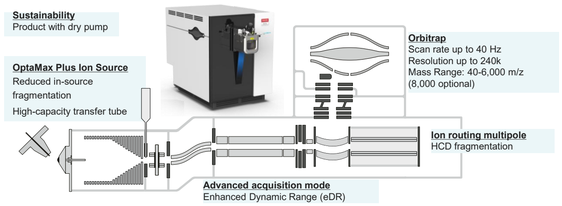

Figure 1. Orbitrap Excedion and its schematics.

Orbitrap Excedion Mass Spectrometer supports gentler ion transfer through a labile-compound-optimized ion funnel, helping reduce unintended MS1 fragmentation and preserve precursor integrity for MS/MS. The AcquireX Deep Scan workflow uses blank exclusion, sample-specific inclusion lists, and iterative list updating to target new sample-related precursors across replicate injections. Compound Discoverer Software integrates isotope/adduct grouping, in-source fragment recognition, spectral searching, molecular networking, AI/ML confidence scoring, and automated confidence-level assignment to produce transparent, evidence-ranked metabolite annotations. Together, these co-optimized hardware and software components convert complex data into transparent, evidence-ranked metabolite annotations.

Materials and methods

Sample Preparation

NIST SRM 1950 plasma extract was prepared by methanol protein precipitation followed by centrifugation, evaporation, and reconstitution prior to LC-MS analysis. The final injected plasma equivalent was 4.583 µL plasma per injection.

Liquid chromatography, mass spectrometry and data analysis

Analysis was performed on a Thermo Scientific™ Vanquish™ Horizon UHPLC System with a Thermo Scientific™ Hypersil GOLD™ VANQUISH™ C18 UPLC Column, 150 × 2.1 mm, 1.9 µm, coupled to a Orbitrap Excedion Mass Spectrometer operated in positive ion mode. Separation used water and methanol, both with 0.1% formic acid, at 0.30 mL/min with an 18 min gradient. Full-scan MS1 data were acquired over m/z 67-1000 at 120,000 resolution with enhanced Dynamic Range enabled using 16 auto-defined windows. Fragmentation data were acquired using AcquireX iterative Deep Scan workflow, stepped HCD collision energies of 10, 35, and 60%, and 30k MS2 resolution. Raw data were processed using Compound Discoverer 3.5 software and Thermo Scientific™ FreeStyle™ 1.8 SP2 Software.

Results

Exact mass is not identification - even caffeine gives 920 candidates

Even for a small molecule such as caffeine, accurate mass alone is insufficient for confident identification. A ChemSpider monoisotopic mass search for m/z 195.08775 $[M+H]^+$ within approximately 5 ppm returned 920 possible structures, illustrating that exact mass is a high-recall but low-decision filter. Confidence increased only after combining orthogonal evidence: the isotope pattern supported the expected elemental composition, the presence of a sodiated adduct supported the same neutral molecular entity, and the experimental MS/MS spectrum showed an excellent match to caffeine reference fragmentation.

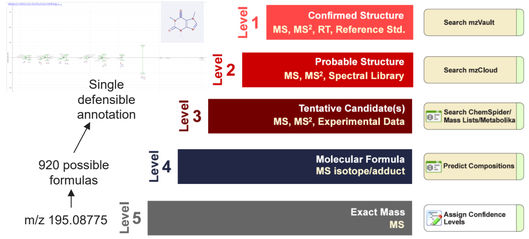

Figure 2. From accurate mass to confident annotation: integrated MS1, MS/MS, adduct, and structure-consistency evidence resolves caffeine from 920 candidate structures. Assigned annotation confidence levels based on the framework proposed by Schymanski et al. [1, 2]

AI/ML confidence resolves methylxanthine positional isomers

Positional isomers are a difficult case for library-based metabolite annotation because they share the same formula, precursor mass, and often partially overlapping fragment ions. In this example, 1-, 3-, and 7-methylxanthine all match the observed precursor m/z 167.05655 [M+H]+ and return plausible conventional similarity scores. However, the traditional scores do not create a clear decision boundary: some legacy metrics remain close across candidates or even score the wrong positional isomers higher.

Visual inspection of the mirror plots supports 1-methylxanthine as the best spectral explanation, with more diagnostic fragment agreement than the 3- or 7-methylxanthine alternatives. The AI/ML confidence score converts this expert-like spectral judgement into a better quantitative separation, assigning 86.1 to 1-methylxanthine but only 3.5-3.6 to the near-miss isomers. This wider score gap makes the top annotation easier to defend and helps prevent overinterpretation of chemically plausible but weakly supported isomeric candidates. This is illustrated in Figures 3 and 4.

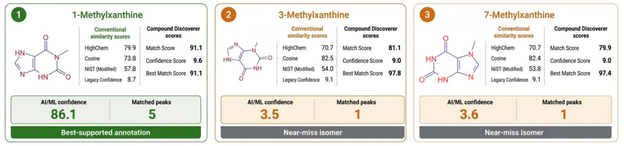

Figure 3. AI/ML confidence improves decision-making in separation for methylxanthine positional isomers. Conventional similarity scores returned multiple plausible candidates, whereas AI/ML confidence strongly prioritized 1-methylxanthine over near-miss 3- and 7-methylxanthine annotations.

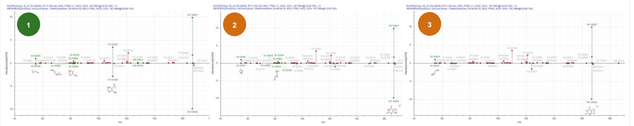

Figure 4. MS/MS mirror plots for methylxanthine positional isomers show the strongest diagnostic fragment agreement for 1-methylxanthine (1), while 3-methylxanthine (2) and 7-methylxanthine (3) produce weaker near-miss matches.

Molecular networking adds chemical-context evidence

Molecular networking added an orthogonal confidence layer by testing whether proposed annotations formed chemically coherent families rather than isolated database hits. The caffeine-centered subnetwork grouped caffeine with expected xanthine metabolites and positional isomers, supporting the plausibility of the caffeine-related annotations through consistent transformation and MS/MS fragmentation relationships.

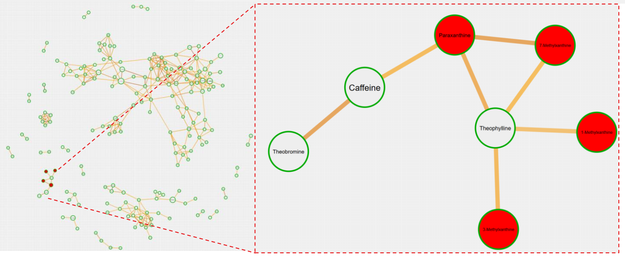

Figure 5. Molecular networking contextualizes individual annotations.

Automatic confidence scoring converts evidence into reportable annotation levels

After compound detection, annotation, and spectral matching, the final step was to convert each assigned compound entry into a standardized confidence level. In this example, 1-, 3-, and 7-methylxanthine were detected as separate chromatographic features at different retention times, each with its own MS/MS evidence and library match. The confidence-scoring node evaluated the available evidence and assigned the resulting confidence level. It utilizes evidence such as predicted composition, Thermo Scientific™ mzVault™ Library, mzCloud Mass Spectral Library, and ChemSpider results, as described by Krakko et al. [2]

Name	Formula	ConfLevel
1-Methylxanthine	C6 H6 N4 O2	2
3-Methylxanthine	C6 H6 N4 O2	3a
7-Methylxanthine	C6 H6 N4 O2	3a

Figure 6. Confidence is reported for each detected methylxanthine entry separately.

Conclusions

From feature detection to confidence-ranked annotation. The Orbitrap Excedion MS-based workflow integrates intelligent acquisition, MS1/MS2 evidence, molecular networking, AI/ML scoring, and automated confidence-level reporting. Accurate mass alone produced 920 caffeine candidates, but orthogonal evidence resolved one defensible annotation; AI/ML confidence similarly separated methylxanthine isomers for clearer, reproducible reporting.

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References:
[1] Schymanski, E. L.; Jeon, J.; Gulde, R.; Fenner, K.; Ruff, M.; Singer, H. P.; Hollender, J. **Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence.** *Environ. Sci. Technol.* **2014**, *48* (4), 2097–2098. https://doi.org/10.1021/es5002105
[2] Krakko, D.; Tautenhahn, R.; Stutts, W. L. **Implementing Annotation Confidence Scoring in Untargeted Mass Spectrometry Workflows for Small Molecule Analysis.** *Anal. Chem.* **2026**, *98*, 10352–10359. https://doi.org/10.1021/acs.analchem.5c03229