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Intelligence Driven Metabolomics Workflows: Hardware and Software Innovations for Improved Quantification and Annotation

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ORBITRAP-BASED MASS SPECTROMETRY FOR METABOLOMICS

High-Quality Data for High-Quality Results

Metabolomics is the comprehensive, qualitative, and quantitative study of all endogenous small molecules in each biological system.[1] The collection of these small molecules reflects the biochemical phenotype, which in turn aids in understanding the physiological function and associated pathologies. Yet, the physical-chemical properties of these endogenous compounds are vastly diverse including a range of molecular weight, polarity, structural possibilities, and concentration creating analytical challenges in any metabolomics analysis. The detection of metabolites by electrospray ionization mass spectrometry is further challenged with spectra containing molecular features derived from external sources that are experimentally unrelated or multiple ion species reflecting the same molecule such as adduct formation. **Metabolomics analysis requires (1) high resolution to distinguish closely related masses in complex matrices, (2) accurate mass measurements for confident spectral peak assignments, and (3) consistent results from scan-to-scan and run-to-run over extended periods.**

Leading Orbitrap-based mass spectrometers (Figure 1) provide high-resolution accurate mass (HRAM) measurements and sensitivity required to measure metabolites in complex matrices. High resolution distinguishes spectral features of similar mass, which is required to differentiate isobaric species and determine fine-isotopic patterns (Figure 2). Accurate mass measurements are paramount for confident spectral assignment (Figure 3).

When combined with advanced separations, high throughput and quantitative capabilities expand the scope of what we know about metabolites and their role in several different areas of study (Figure 1).

Figure 1. Thermo Scientific™ Orbitrap™ based Mass Spectrometers provide HRAM measurements suitable for metabolomics analysis of sample types including animal, plant and cellular components. The Thermo Scientific™ Orbitrap Exploris™ 240 MS instrument is equipped with a quadrupole mass filter and Orbitrap mass analyzer for HRAM MS and MS/MS while the Thermo Scientific™ Orbitrap Tribrid™ Series instruments include a dual pressure linear ion trap in addition to a quadrupole mass filter and Orbitrap mass analyzer for HRAM MS, MS/MS (HCD), CID, UVPD and MS^n spectra. Optional 1M resolution available.

Thermo Scientific™ Vanquish™ Duo UHPLC System

Thermo Scientific™ Orbitrap Exploris™ 240 MS

Thermo Scientific™ Orbitrap IQ-X ™ Tribrid™ MS

Figure 2. Fine isotopic distribution for the A1 ion cluster of biotin. Data collection was on an organic extraction of human plasma reference material, NIST SRM 1950, using a Orbitrap Exploris™ 240 MS with a resolution setting of 120K FWHM @ 200 *m/z*.

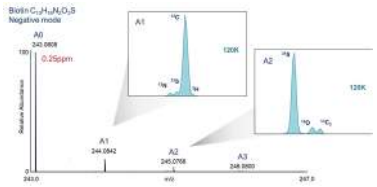
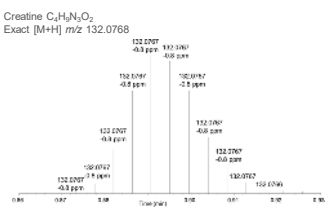


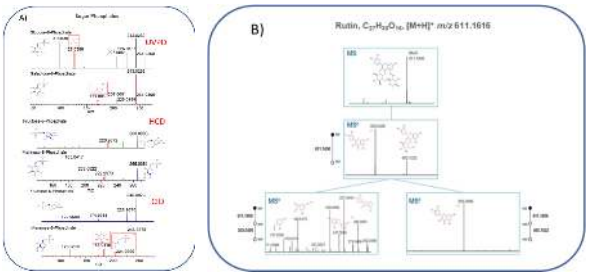
Figure 3. Excellent scan-to-scan mass measurement accuracy was obtained with the Orbitrap Tribrid MS. Sub-ppm mass measurement accuracy for creatine detected over the LC-MS elution profile.



Multiple Dissociation for More Spectral Information

Unique to the Tribrid MS platform are multiple fragmentation techniques: (1) higher-energy collision dissociation (HCD) in a high-pressure collision cell (2) collisional-induced dissociation (CID) in an ion trap (3) multi-stage fragmentation or stepwise MS^n and (3) Ultraviolet Photodissociation (UVPD) for annotating unknown metabolite structures in untargeted metabolomics and lipidomics experiments.

Figure 4. Multiple dissociation techniques provide more fragment ion information for structure characterization and elucidation of unknowns using the Orbitrap IQ-X Tribrid MS. A) CID, HCD, and UVPD dissociation provide unique spectral information to distinguish sugar phosphates. B) Higher order fragmentation generates MS^n spectral trees enabling the systematic breakdown of the flavonoid rutin.

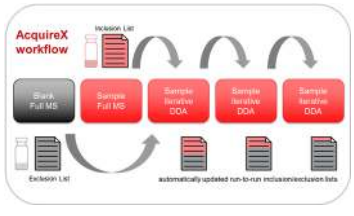


INTELLIGENCE DRIVEN MASS SPECTROMETRY

AcquireX Data Acquisition to Collect More Biologically Meaningful Data

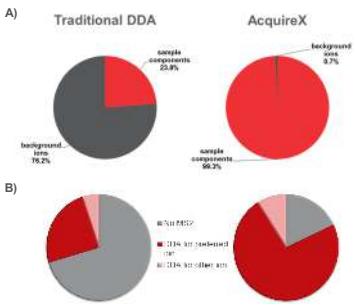
Insufficient metabolome annotation has limited the biological interpretation of untargeted metabolomics studies. Fragmentation spectra provide more spectral information to increase the confidence of unknown annotations. **The AcquireX intelligent acquisition generates more fragmentation spectra for sample relevant compounds, avoids unrelated background ions, and removes redundancies by using fully automated iterative inclusion and exclusion lists**. Available on the Orbitrap Exploris™ 240 MS and Tribrid MS systems, AcquireX takes advantage of knowledge to drive acquisition where the blank sample generates a list of ions to exclude for subsequent fragmentation and a matrix sample generates a list of true sample components to prioritize for data dependent MS^2 and MS^n acquisition (Figure 5). AcquireX leads to information-rich fragmentation of more experimentally relevant compounds (Figure 6).

Figure 5. The AcquireX Deep Scan acquisition workflow for improved compound annotation.



- First, the AcquireX process obtains the LC-MS data for the blank and a pooled sample
- The AcquireX process creates an exclusion list from the blank and an inclusion list from the sample data
- The first data dependent MS^2 run is acquired and the inclusion/exclusion lists are updated after the run
- On the second injection, MS^2 spectra are acquired for compounds remaining on the inclusion list
- This process is repeated for a user-specified number of injections

Figure 6. A) Obtaining MS^2 information on compounds vs. background with traditional data dependent acquisition (DDA) compared to AcquireX. B) Sample components with no MS^2 or MS^2 selected for the preferred ion (M + H) or associated ion (M+ Adduct) for traditional DDA compared to AcquireX, indicating increased MS^2 primarily avoid compound dereplication, during characterization.



Human plasma (NIST SRM1950), C18, 15 min gradient, data dependent LC-MS^2

SOFTWARE BUILT TO ANNOTATE UNKNOWNS

Thermo Fisher™ Compound Discoverer™ 3.3 Software

Compound Discoverer is a qualitative data-processing application that uses accurate mass data, isotope pattern matching, fragment matching, and mass spectral library searches for the structural identification of small molecules. It can process the accurate-mass spectra from the entire product line of Thermo Scientific high-resolution mass spectrometers. The Compound Discoverer 3.3 application includes the following new features:

1. New Peak Detection
2. Improvements to mzCloud library search including MS^n search
3. Optimization for large datasets
4. Enhancement for GC/MS workflow and molecular networking.

Figure 7. The new peak detection feature can detect peaks at very low intensities which would have been missed earlier.

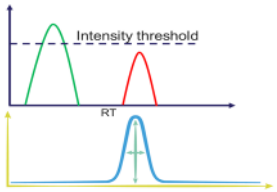
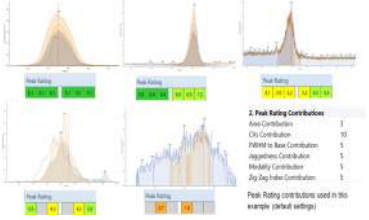


Figure 8. New peak rating filter nodes provides peak thresholding based on the *quality* of the chromatographic peaks. Improving detection and relative quantification.



Mass Spectral Library and library match

The mzCloud mass spectral library is a highly curated, a public library of endogenous and exogenous small molecules containing over **5 million fragmentation spectra**. Each compound entry in the library generally includes two fragmentation techniques: HCD and CID. This highly diverse spectral library (Table 1) contains true mass spectra generated from purified reference standards. Each spectrum is recalibrated for exact mass and noise removed and is further structurally annotated making this an ultra high-quality spectral library. The mzCloud library is fully integrated into the Compound Discoverer and the Thermo Scientific™ Mass Frontier™ spectrum interpretation software. In addition to HRAM measurements of the precursor ion, fragmentation spectra provide an additional layer of knowledge about the molecular makeup of a compound and subsequently increase unknown annotation confidence. Fragmentation spectra generated from the experimental results are compared against reference spectra within the library (Figure 9). Associations based on structural relationships to the parent structure in the reference library enable unknown annotations.

Table 1. The reference library within the mzCloud spectral library consist of 16 categories of small molecules including endogenous metabolites. Sourced from mzCloud.org on 5/18/2022.

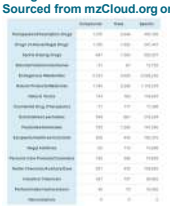
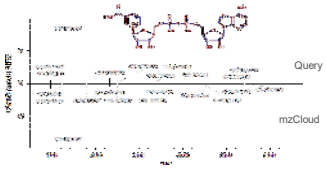


Figure 9. Identity match for the endogenous compound nicotinamide adenine dinucleotide (NAD) with MS^2 experimental data collected from the SRM 1950 plasma extract against the mzCloud spectral library.



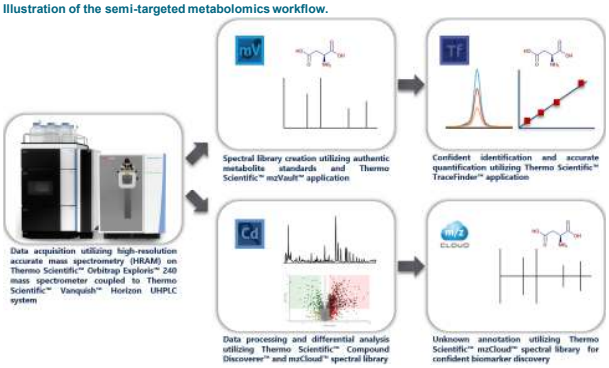
SEMI-TARGETED METABOLOMICS

In order to make meaningful biological interpretations of metabolomic data, researchers need two main insights: first, specific identification and quantification of metabolite changes (targeted analysis), and second, an overview of general changes to the metabolome (untargeted analysis). Both approaches have pros and cons when using LC-MS, which means researchers are often faced with a choice: a low accuracy overview of total molecular changes, or a detailed yet restricted snapshot of a select group of metabolites.

However, a new semi-targeted workflow combining both untargeted and targeted workflows has recently emerged as a middle ground, addressing the limitations of both approaches. The primary focus of the semi-targeted approach is the confident annotation and optionally the accurate quantitation of a targeted set of metabolites, and the secondary focus is to find new molecular connections in a system by performing untargeted analysis **on a single injection** (i.e., by reanalyzing (or retro-mining the data).

Figure 10 illustrates the semi-targeted metabolomics workflow utilizing high-resolution accurate mass spectrometry on Orbitrap technology and sophisticated data processing and analysis application solutions for targeted confident identification and quantification of metabolites, and untargeted differential analysis and unknown annotation for biomarker discovery.

Figure 10, Illustration of the semi-targeted metabolomics workflow.



By enabling simultaneous acquisition of hypothesis-led and discovery-led datasets, semi-targeted workflows allow scientists to gain more knowledge about biological systems in a single experiment. Weighing the pros and cons of untargeted and targeted metabolomics has often held back the tremendous potential of metabolomics in life sciences research. Now, the semi-targeted analysis looks set to help to unlock this potential.

One of the biggest strengths of semi-targeted metabolomics is the ability to perform targeted and untargeted analysis in a single sample injection. In traditional metabolomics experiments, a sample is injected (analyzed) twice; once for untargeted metabolomics analysis and a second time for targeted analysis. A single injection workflow is particularly advantageous for laboratories that have limited access to samples, time, and resources, and offers a powerful and efficient way to gain more knowledge from valuable biological samples.

CONCLUSIONS

- Confidence in compound annotation increases when combining HRAM full scan measurements with multiple dissociations (CID/HCD, MS^2 and MS^n)
- Intelligence-driven data acquisition generates more experimentally relevant spectral information and ignores background and redundancies to increase annotation of unknown compounds
- Semi-targeted workflow combining both untargeted and targeted workflows to address the limitations of both approaches. The semi-targeted approach ensures the confident annotation and optionally the accurate quantitation of targeted metabolites, in addition, it enables performing untargeted analysis on a single injection.

REFERENCES

1. Oliver, S.G., Winson, M.K., Kell, D.B., and Baganz, F. (1998). Systematic functional analysis of the yeast genome. Trends in Biotechnology, 16(9):373-8.

TRADEMARKS/LICENSING

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