A Platform to Identify Endogenous Metabolites Using a Novel High Performance Orbitrap MS and the mzCloud Library

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Overview

Purpose: To demonstrate the capability of the Thermo Scientific™ Orbitrap Fusion™ Tribrid™ Mass Spectrometer in combination with MS^n library for metabolite structural identification.

Methods: UHPLC coupled to the Orbitrap Fusion MS to simultaneously perform global profiling and structural elucidation with MS^n.

Results: Within UHPLC timescale (2-3 second FWHM), >10 cycles can be finished with fast ion trap HCD MS^2 for top 11 precursors, each with ion trees of MS^2-MS^3(top2)-MS^4(top2).

Introduction

The structural identification of metabolites represents a significant challenge in metabolomics study. Multistage mass spectrometry (MS^n) is a powerful tool for compound identification and structural elucidation that goes beyond identifying the exact same compounds, to discovering additional compounds with structural homology to those in the library. Combining with high resolution accurate mass (HRAM) measurement, MS^n is highly effective in identifying the unknown but biologically relevant compounds in metabolomics studies [1]. However, the speed on current platforms is yet to be effectively compatible with UHPLC frontend, which limited such application in biological samples. Presented here is a new platform to identify endogenous metabolites using a novel high performance Orbitrap hybrid instrument in conjunction with UHPLC and an MS^n library.

Method

Sample Preparation

Urine samples from an adult male were analyzed. The urine samples were diluted 1 to 10 with water and processed with a 3kD MWCO filter.

For plasma samples, proteins were removed with cold methanol and centrifugation, followed by centrifugal evaporation at 35°C.The residue was reconstituted in MeOH/Water, 1/9, and also processed with a 3kD MWCO filter.

Liquid Chromatography

UHPLC separation was implemented on a Dionex Ultimate 3000 HPG (high-pressure gradient) pump using Hypersil GOLD RP C18 column at 450 µL/min, column temperature at 55°C. LC solvents were 0.1% FA (A) and 0.1% FA in MeOH (B). A linear gradient was applied from 0.5-50% B for 5.5 min, followed by increasing to 98% at 6 min, held 98% B for 6 min, then decreased to 0.5% at 13 min, and equilibrated for another 2 min.

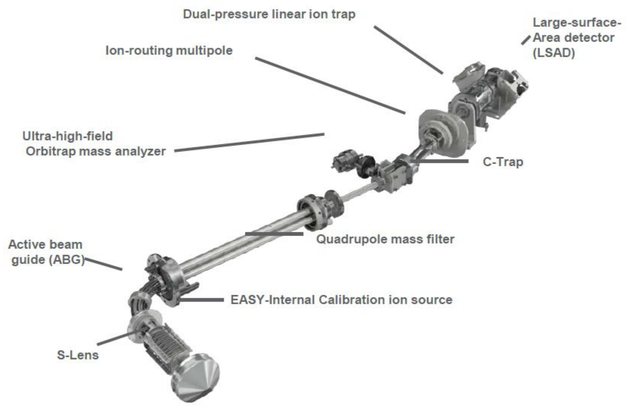
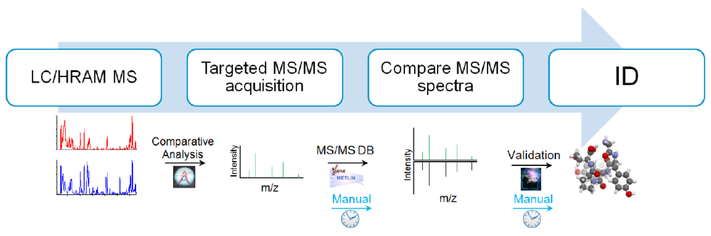


Figure 1. Schematic of Orbitrap Fusion Tribrid Mass Spectrometer

An Improved Metabolomics Workflow

A traditional workflow for metabolite profiling and identification is based on a full scan LC-MS experiment followed by targeted MS/MS confirmation.



The major problems associated with this methodology are:

1. Instrumentation limitations
   - Does not allow simultaneous profiling and MS^n in a single run, the scan speed suffers.
   - Identification is usually based on accurate mass and MS^2, but a large number of LC peaks remain not identifiable.
2. MS/MS library limitations
   - MS/MS spectral search is usually manually performed and time-consuming.
   - Automated scoring for spectral matching is usually not available.
   - The fragmentation needs to be manually interpreted, which is not only time-consuming but also prone to errors.
3. There is no good solution for unknowns.

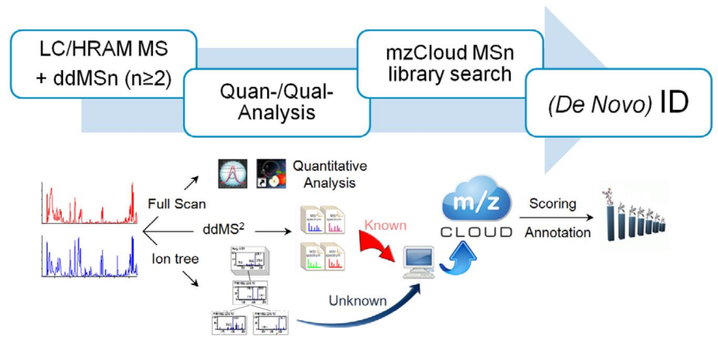
The Orbitrap Fusion MS enables fast parallel data acquisition on a UHPLC timescale with a variety of dissociation choices

The Orbitrap Fusion MS offers ultimate flexibility in fragmentation methods at all stages of MS^n analysis, with fragment ions detected by either mass analyzer. CID-HCD, HCD-CID, and HCD-HCD can all be set up as simple as the conventional CID-CID. This complete flexibility facilitates the examination of fragmentation pathways that leads to the most comprehensive structural information.

mzCloud MS^n library enables MS^n search for both high and low resolution

mzCloud is a multistage MS library. Based on the spectral tree similarity, an algorithm called precursor ion finger printing (PIF) enables the identification of compound seven if they are not in the library, and the fragmental peaks can be annotated [2].

Powered by the novel MS and the mzCloud library, an improved, more connected workflow is shown below:



Major benefits of the improved workflow:

1. HCD MS/MS can be performed simultaneously with full scan MS on the UHPLC timescale with ion trap detection at >22 Hz.
2. Simultaneous ddMS^n ion tree with flexible combinations of CID and/or HCD fragmentation with ion trap and/or Orbitrap™ detection.
3. Both the MS^2 and MS^n will be automatically searched in mzCloud, the matching entry will be scored based on PIF, and the fragments will be annotated.
4. *De novo* structural identification is enabled by using this workflow.

Results

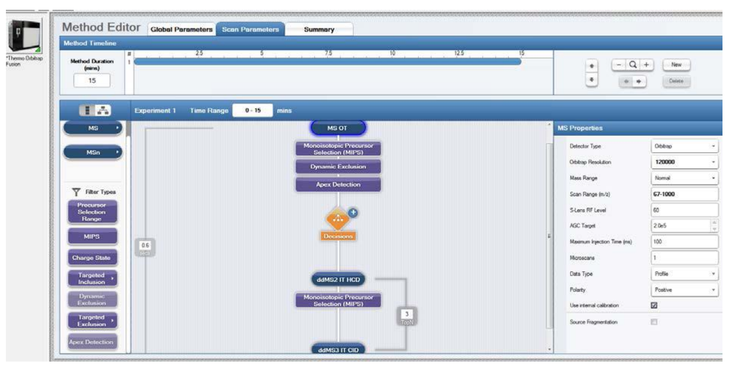


FIGURE 1. The Orbitrap Fusion MassSpectrometer and the Drag and Drop Instrument Method Editor.

Metabolite Identification Requires High Resolution and Isotope Ratio Measurements

Structure elucidation of unknown small molecules by mass spectrometry is a great challenge. The first crucial step is to obtain correct elemental compositions.

Natural occurring elements can be monoisotopic (F, Na, P, I) or polyisotopic (H, C, N, O, S, Cl, Br) [1]. The molecule L-Methionine $C_5H_{11}NO_2S$ contains multiple polyisotopic elements, especially the S, which result in multiple isotopic peaks needing resolving power above 240,000 at m/z 200 (FWHM). Figure 2A shows the better isotope pattern of molecular ion $[M+1+H]^+$. With increasing resolution up to 450,000 (FWHM), all five isotope peaks are clearly resolved. Figure 2B shows the simulated isotope pattern.

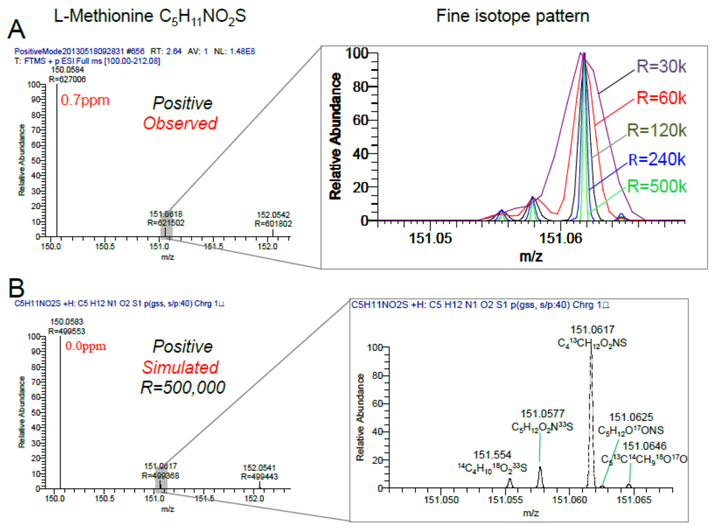


FIGURE 2. Ultrahigh resolution resolves the isotopes

Metabolite Identification and Quantitation in Complex Samples Requires High Resolution

D-Glucose $C_6H_{12}O_6$ and Paraxanthine $C_7H_8N_4O_2$ co-exist in human blood at distinct concentration levels: 5300 µM (D-Glucose) vs. 10 µM (Paraxanthine) [3]. Although D-Glucose is hundreds of fold more concentrated, it is less readily detectable because of its low ionization efficiency. Their masses differentiate by 0.0013 amu (179.0561 vs. 179.0574), making the detection of these individual components very challenging. Figure 3 shows that, with >300,000 resolving power, the 200 µM (D-Glucose) and 1 µM (Paraxanthine) can be readily separated and quantified.

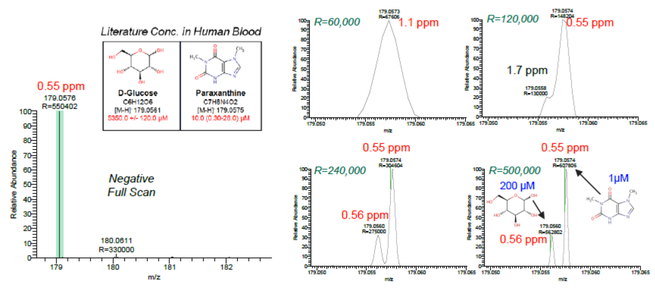


FIGURE 3. Ultrahigh resolution resolves isobaric metabolites.

Metabolite Identification Requires High Accuracy of Mass Measurement in Both MS Full Scan Level and MS/MS Level

For an unknown metabolite, the number of calculated chemically possible formulae could be hundreds (20 ppm tolerance window), but the possibilities significantly reduce to tens (3 ppm) and several if the tolerance window is narrowed to sub-ppm.

However, due to the large amount of known isomeric structures, one single formula may correspond to hundreds of structural isomers, thus structure identification of metabolite isomer using only MS full scan is not feasible. MS/MS and MS^n techniques can help solve this analytical problem.

The Orbitrap Fusion MS has a built-in internal calibration functionality (Easy IC). Figure 4 shows LC-MS/MS data of L-Tryptophan from an urine sample with and without internal calibration. Sub-ppm mass accuracy was observed for MS and MS^2 fragments with IC activated.

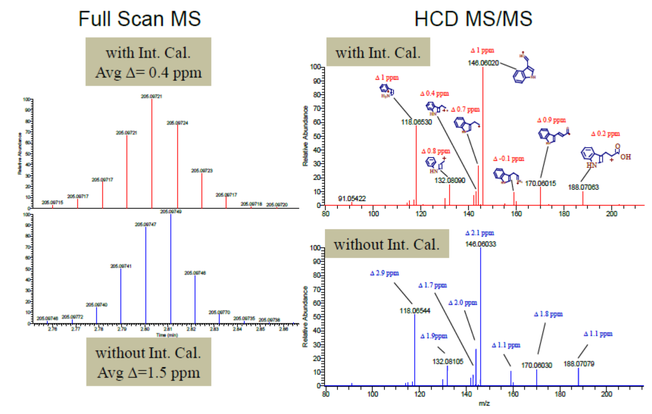


Figure 4. Easy Internal Calibration.

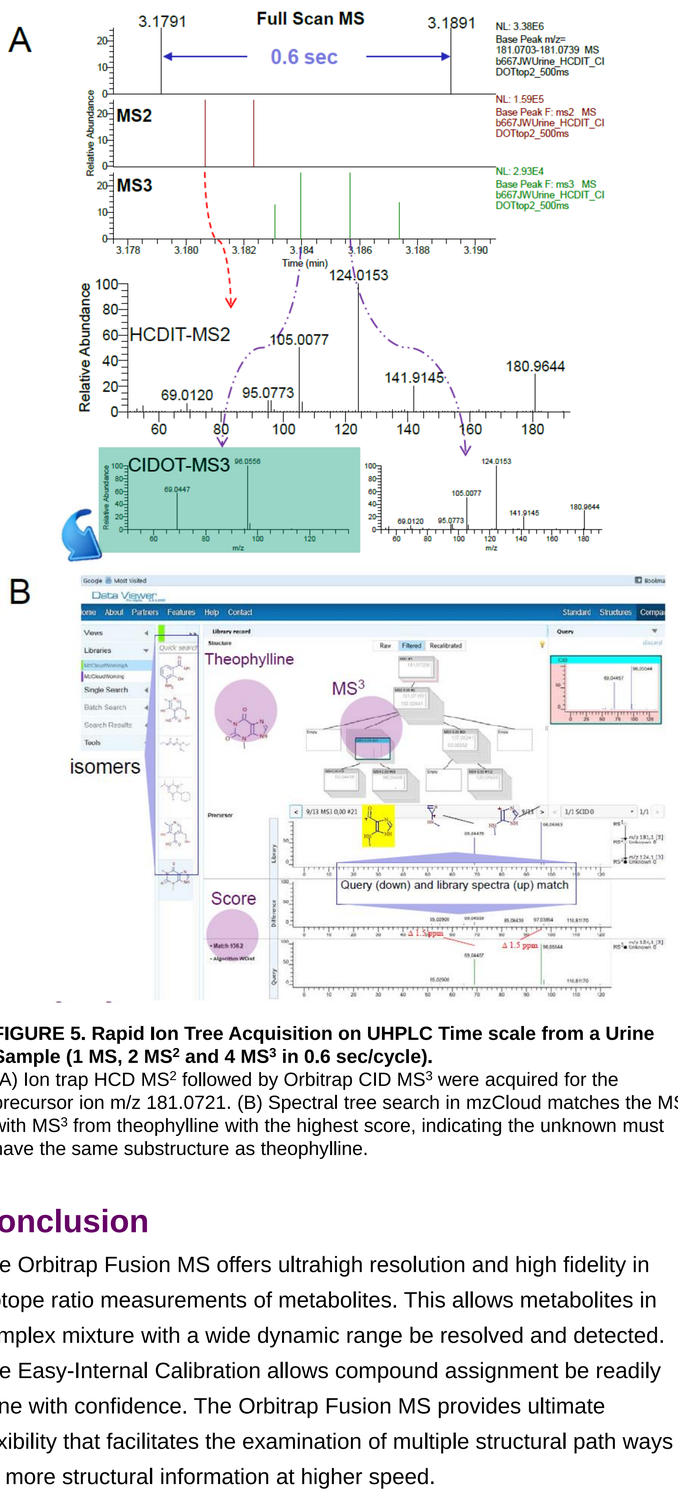


FIGURE 5. Rapid Ion Tree Acquisition on UHPLC Time scale from a Urine Sample (1 MS, 2 MS^2 and 4 MS^3 in 0.6 sec/cycle).
(A) Ion trap HCD MS^2 followed by Orbitrap CID MS^3 were acquired for the precursor ion m/z 181.0721. (B) Spectral tree search in mzCloud matches the MS^3 with MS^3 from theophylline with the highest score, indicating the unknown must have the same substructure as theophylline.

Conclusion

The Orbitrap Fusion MS offers ultrahigh resolution and high fidelity in isotope ratio measurements of metabolites. This allows metabolites in complex mixture with a wide dynamic range be resolved and detected. The Easy-Internal Calibration allows compound assignment be readily done with confidence. The Orbitrap Fusion MS provides ultimate flexibility that facilitates the examination of multiple structural path ways for more structural information at higher speed.

The mzCloud library allows metabolite identification with MS^2 and/or MS^n data in an automated fashion. The identification based on multistage MS spectral trees and PIF algorithm opens the possibility to identify unknown metabolites and isomers. An improved metabolomics workflow is enabled.

References

1. Budzikiewicz, H.; Grigsby, R.D. *Mass Spectrom. Rev.* **2006**, 25(1):146-157.
2. Sheldon, M.T.; Mistrik, R.; Croley, T.R. *J. Am. Soc. Mass Spectrom.* **2009**, 20(3):370-376
3. http://www.hmdb.ca/metabolites/HMDB00122 & HMDB01860

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