Utilizing Ultra High Resolution for Selective Component Separation in Complex Matrices

Tim Stratton, Seema Sharma, Jesse Canterbury
Thermo Fisher Scientific, San Jose, CA, USA

ABSTRACT

Purpose: To demonstrate the use of ultra high resolution mass spectral data to selectively separate components of interest in complex samples based on 'atom counting' of the fine isotopic patterns.

Methods: Ultra-high resolution (1,000,000 @ *m/z* 200) full MS1 data were acquired for several fruit extracts. To identify potential compounds of interest (flavonoid conjugates), two complementary algorithms were applied to create extracted ion traces for the fine isotopic pattern of high oxygen containing compounds or the direct scoring of unknown peak detection results based on high oxygen count.

Results: Access to the fine isotopic information and direct observation of the 18O signal allowed both approaches to selectively separate potential compounds in matrices containing 4,000-6,000 compounds, aiding in selective analysis to determine structures.

INTRODUCTION

The complexity of most biological samples is a fundamental cause for the difficulty in their analysis. Many methods of attempting to resolve or separate their many components to aid in analysis have been developed over the long history of analytical development, including separations in time (LC and GC) and separation by mass. Increased capability beyond unit mass resolution and into high resolution allows for the separation of more components in a complex sample by separating their otherwise overlapping *m/z* values. Moving beyond high resolution into even higher mass resolving power enables the detection of fine isotopic patterns – direct observation of the presence and abundance of individual elements. This kind of resolution enables a new possibility of separation based on specific isotopic fingerprints for groups of molecules. Flavonoids have long been an area of study for multiple reasons including their potential health benefits. Flavonoids, and their multiple conjugates, can be characterized as having very high C/O ratios in their composition which, combined with resolving powers high enough to separate the ^{18}O and $2^{*13}C$ signal, can be used to selectively separate potential compounds of interest from a complex sample.

MATERIALS AND METHODS

Sample Preparation

Samples of juice extracted from orange and grapefruit were centrifuged to remove particulates. Aliquots of the supernatant were diluted with 4 volumes of RO water and used for injection. No further sample preparation was performed.

Mass Spectrometer Acquisition Conditions

Samples were separated on a 100 X 5mm, 3um C18 column maintained at 35ºC (Thermo Scientific™ Hypersil GOLD™ columns); ionization was performed by electrospray ionization in positive ionization mode. The initial ultra high resolution acquisition obtained full MS^1 data at a resolution of 1,000,000 (FWHM @ *m/z* 200). For subsequent putative flavonoid conjugate triggering injections, the compounds found through the fine isotopic data from the MS^1 injections were targeted for Orbitrap HCD MS^2 (20% normalized collision energy, NCE) fragmentation with subsequent HCD MS^2 data (50% NCE) acquired on peaks observed to give common neutral loses for known conjugating species.

Mass spectrometer: Thermo Scientific™ Orbitrap Fusion™ Lumos™ MS with 1M option

LC: Thermo Scientific™ Dionex™ UltiMate™ U3000 LPG pump and wellplate autosampler.

Table 1. LC Gradient for Sample Analysis

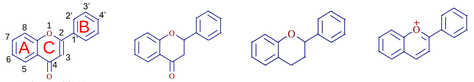
Time (min)	% A (Water + 0.1% Formic)	% B (ACN + 0.1% Formic)
0	98	2
0.5	98	2
8	65	35
11.5	5	95
12	5	95
12.5	98	2
16	98	2

RESULTS

Flavonoid Conjugates Detection

Fruit extracts are complex matrices which contain several thousand detectable components under the LC-MS conditions used; for example, orange provided an average of 4,500 compounds while grapefruit contained an average of 4,250. In this experiment, we focused on detecting flavonoids and their conjugates and selectively separating them from the complexity of the sample for subsequent targeted analysis. Flavonoids are a broad category of plant secondary metabolites used in a variety of metabolic processes (Figure 1). They are a common component of the average diet, coming from many sources, and have been studied significantly for many years for their potential health benefits and influence on crop health. Broadly, flavonoids break down into 5 major categories which can undergo single or multiple conjugations with diverse chemistries.

Figure 1. Major Flavonoid Classes



Anthoxanthines (Flavone shown, 3-OH → Flavonol) | Flavanones and Flavanonols (3-OH) | Flavans (Including 3-ol, 4-ol, and 3,4-diol) | Anthocyanidins

These core flavonoids typically have multiple hydroxy or methoxy substitutes at the 6, 7, 8, 2′, 3′, and 4′ positions, which gives rise to the broad diversity of this chemical class, especially when combined with the multiple conjugation patterns with multiple species. Our focus was on glycoside or gallate conjugates (Table 2), which each add multiple additional oxygens to the resulting conjugate molecule.

Table 2. Common Flavonoid Conjugates (Monosaccharides and Gallic Acid)

Conjugate	Formula	# Added Oxygen	Δ MW
Arabinose	$C_5H_{10}O_5$	4	132.1146
Glucose	$C_6H_{12}O_6$	5	162.1406
Fructose	$C_6H_{12}O_6$	5	162.1406
Xylose	$C_5H_{10}O_5$	4	132.1146
Rhamnose	$C_6H_{12}O_5$	4	146.1412
Gallic acid (gallates)	$C_7H_6O_5$	4	152.1043

Fine Isotope Pattern Methods

Given the high oxygen content of our analytes of interest, and the capability to acquire ultra high resolution data, we first utilized the fine isotopes of ^{16}O as a fingerprint to single out potential compounds of interest. Full MS^1 data at a 1,000,000 resolving power were acquired, and two different orthogonal approaches were applied to select potential compounds of interest. In the first, the data were passed through a fine isotope pattern algorithm which detects the presence of one or more specified patterns in every spectra and reconstructs a summed extracted ion chromatogram trace (XIC) based on observed matching signals in each MS^1 spectra. The second approach combined untargeted peak detection and fine isotopic patterns, scoring the peaks and flagging those of interest for further study.

Both approaches were possible using Thermo Scientific™ Compound Discoverer™ software which is a workflow-based software application. We utilized the Pattern Tracer and Pattern Scoring combined with Detect Unknown Compounds in a workflow to process the data and provide results for both approaches. The pattern entry field was the same for either approach with the output being a set pattern-based XIC's or a untargeted peak table with flags for each detected components (outlined in Figure 2). The patterns were entered into the algorithm based on a general formula with a ratio of C/O between 1.5 and 3 (unconjugated, mono-, di-, or trisaccharide conjugation). Alternatively, one could consider the pattern scoring was selectively flagging peaks with greater than a set number of oxygen atoms.

Each of several thousand detected components in the result table received a flag for the patterns searched (match or no match) and an overall score for the closeness of the pattern match (Figure 3.). The untargeted peak detection table was then filtered for peaks with a molecular weight greater than 350 (average flavonoid + monosaccharide) combined with a positive score for at least one of the requested isotope patterns.

In effect, the access to the fine isotopic fingerprint for ^{18}O allowed compounds to be "binned" by atom counting with attention paid to compounds with greater than 6 oxygen atoms. This filtering resulted in a total of 128 putative flavonoid conjugates for orange, and 84 putative conjugates for grapefruit. A comparison of the raw data for orange and grapefruit versus the selected putative flavonoid conjugates is shown in Figures 4, and 5.

Figure 2. Fine Isotopic Pattern Search and Score Results in Table

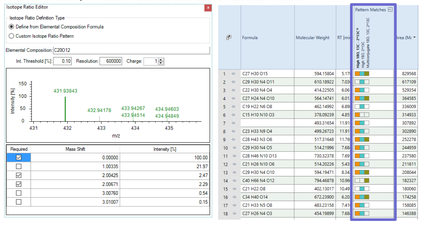
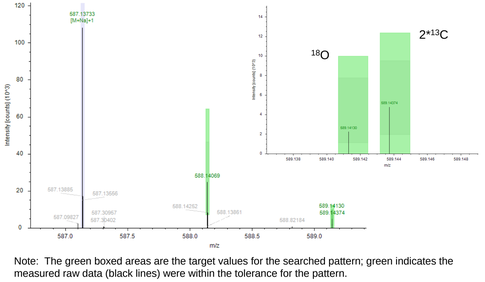


Figure 3. Pattern Score for the [M+Na]+ Adduct of Potential Compound of Interest in Orange



Note: The green boxed areas are the target values for the searched pattern; green indicates the measured raw data (black lines) were within the tolerance for the pattern.

Figure 4. Base Peak and XIC of Potential Flavonoid Conjugates in Grapefruit

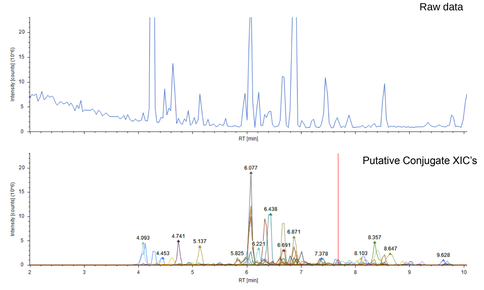
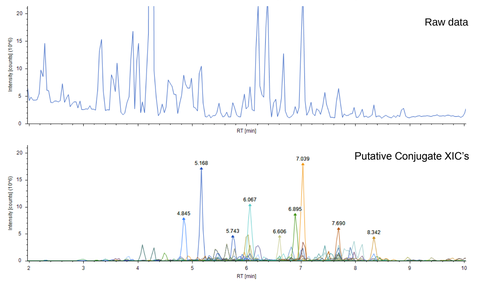


Figure 5. Base Peak and XIC of Potential Flavonoid Conjugates in Orange



Ultra-High Resolution Directed Conjugate Confirmation

The putative flavonoid conjugate lists generated using the very fine isotopic search enabled a selective follow up experiment to confirm both their identification as a flavonoid conjugate, but also to obtain information on the specific conjugates and the flavonoid species. The detected peak lists which met the criteria for fine isotopic pattern for high ^{18}O content were exported as a target inclusion list for subsequent MS^n analysis. Peaks from this target list were subjected first to low energy HCD MS^2 fragmentation (NCE 20%) to trigger loss of the conjugating species (Figure 6 for two examples). Those confirmed to display a neutral loss consistent with one of the known conjugation species (or combinations of them in the case of di- or trisaccharides) triggered further MS^3 scans which gave fragmentation of the flavonoid core. Identification of the compounds confirmed as flavonoid conjugates was achieved by performing a fragmentation spectral library search against the mzCloud™ database for both the MS^2 (Figure 7A, to provide putative conjugate candidates) and for the MS^3 (Figure 7B, to identify the flavonoid core structure). In a single injection, the putative identification of a flavonoid conjugate was confirmed which also obtaining identification of the flavonoid.

The example spectra searches displayed here are also included as SpectraQR, which can be scanned and searched against the mzCloud library using the mzCloud smartphone app (available in iPhone® or Android® versions)

Figure 6. Confirmation and Identification of Two Flavonoid Conjugates

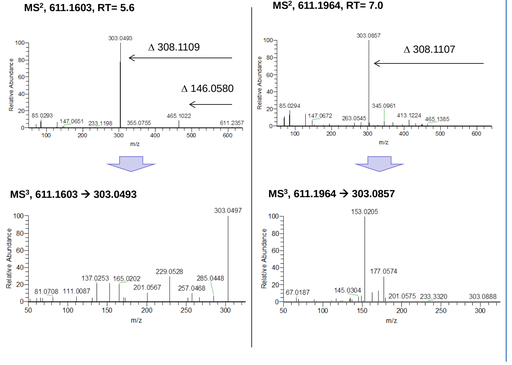
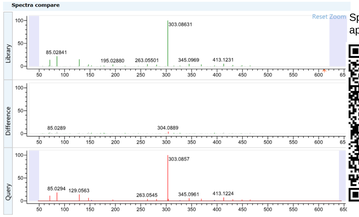


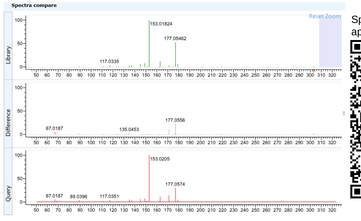
Figure 7. Identification Example – Hesperidin MS^2 and MS^3 Spectral Searches

Part A: MS^2, Hesperidin Query (RT = 7.0 min) Spectral Library Search Result



SpectraQR, Download mzCloud app and scan to see search.

Part B: MS^3, Hesperidin (RT = 7.0 min) → 303.0493 (Hesperitin) Spectral Library Search



SpectraQR, Download mzCloud app and scan to see search.

Note: The top spectra is the matched library spectra, bottom spectra is the query spectra from the raw data. Middle window is a difference spectra by subtracting query and library.

CONCLUSIONS

- Access to hyperfine isotopic signature from very high resolution data allows for selective separation of compounds of interest in a complex sample through direct observation of elements.
- "Atom counting" of fine isotopic pattern enables effective binning of compounds based on observed elemental fingerprints.
- Intelligently guided fragmentation allowed the identification of a set of putative conjugates which were easily targeted in subsequent MS^n analysis.

TRADEMARKS/LICENSING