# Flavonoid Annotation Using a Product Ion-Dependent MS$^n$ Data Acquisition Method on a Tribrid Orbitrap Mass Spectrometer

Reiko Kiyonami[1], Iwao Sakane[2], Seema Sharma[1], Graeme McAlister[1], Caroline Ding[1] and Andreas Huhmer[1]
[1]Thermo Fisher Scientific, San Jose, CA, USA, [2]ITO EN, LTD, Tokyo, Japan

## ABSTRACT

**Purpose:** Develop a product ion-dependent LC-MS$^n$ workflow to collect more structurally relevant fragment ion information for the annotation of flavonoids from natural products with increased coverage and confidence.

**Methods:** All the MS and MS$^n$ data were collected with Thermo Scientific™ Orbitrap ID-X™ Tribrid™ mass spectrometer. We collected MS/MS spectra on precursor ions detected in the survey MS scan within a 1.2 second cycle time. High order MS$^{n\ (3\text{-}5)}$ spectra were only collected when the MS detected the sugar neutral loss fragment ions in MS/MS and/or MS$^{3\text{-}4}$ data. The MS$^n$ spectral tree data were processed using Thermo Scientific™ Mass Frontier ™ 8.0 and Thermo Scientific™ Compound Discoverer™ 3.0 software.

**Results:** Collecting more structurally relevant fragment ion information from the high order of MS$^{n\ (3\text{-}5)}$ enables the identification of more unknown flavonoid compounds from natural products. Two times more flavonoids were annotated using this new LC-MS$^n$ workflow from three fruit and vegetable juice samples.

## INTRODUCTION

Flavonoids are secondary metabolites that play important biological roles in plants. Flavonoids are found in almost all fruits and vegetables, and are powerful antioxidants with anti-inflammatory and immune system benefits. The untargeted profiling of flavonoids provides insights into their biological functions and potential health benefits for humans. However, comprehensive identification of flavonoids remains challenging because of the limited availability of authentic standards and the structural diversity of this class of compounds. Previous studies relied upon extensive expert knowledge about fragmentation rules and structures of flavonoids. Plus, simple MS-MS based analyses are often not sufficient for complete structural characterization. Here we present a new flavonoid profiling workflow that uses comprehensive fragment ion information from HCD/CID MS-MS and higher order CID FTMS$^n$ for rapid flavonoid annotation on an Orbitrap HD-X Tribrid mass spectrometer.

## MATERIALS AND METHODS

### Sample Preparation

Three commercial available fruit and vegetable juice samples (Naked® Kale Blazer, Odwalla® Berries Gomega®, and Odwalla® Red Rhapsody®) were used. Each juice sample was filtered and diluted two times with methanol.

### HPLC Conditions

A Thermo Scientific™ Vanquish™ UHPLC system performed separations. Mobile phase A was water with 0.1% formic acid and mobile phase B was methanol with 0.1% formic acid. The column was a Thermo Scientific™ Hypersil Gold™ column (2.1 x 150mm, 1.9µm) that operated at 45 ℃ and a flow rate of 200 µL/min. Separation of compounds was carried out with gradient elution profile: 0 min, A:B 99.5:0.5; 1 min, A:B 90:10; 10 min, A:B 70:30, 18 min, A:B 50:50, 22 min, A:B 1:99; total 30 min. The injection volume was 2 µL. Each sample was analyzed in duplicate.

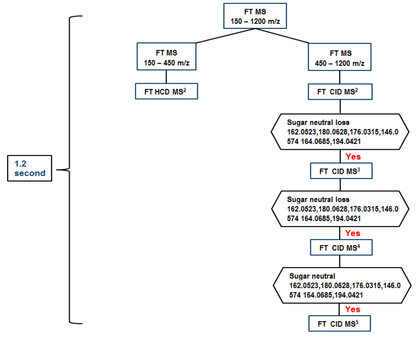
### MS Conditions

All the data was collected on a Thermo Scientific Orbitrap ID-X Tribrid mass spectrometer. The mass spectrometer set up is shown in Table 1. For the precursor ion mass range between 150 – 450 m/z, data dependent HCD MS/MS scans were collected. For the precursor ion mass range between 450 – 1200 m/z, an intelligent product ion-dependent MS$^n$ approach was used, in which an HRAM full MS scan was followed by CID MS/MS scans. The product ions generated from each MS-MS scan are monitored by instrument and an MS$^3$ scan is triggered if one or multiple pre-defined neutral sugar molecular were detected from an MS-MS scan. An MS$^4$ and or $^5$ scan is further followed if pre-defined neutral sugar molecular were detected from the MS$^3$ and or $^4$ scan. Figure 1 shows the flowchart of the developed product ion-dependent MS$^n$ data acquisition instrument method.

**Table 1. Orbitrap ID-X instrument set up**

| ESI source | Orbitrap-ID-X |
|---|---|
| Sheath gas 35 | Pos ion (150-1200 amu) |
| Aux gas 5 | MS: R=60K (FWHM at m/z 200) |
| Spray volt. 3.4 kV | MSn: R=15K (FWHM at m/z 200) |
| RF-Lens 40 | Cycle time: 1.2 second |
| Cap. temp. 300°C | MS$^2$ Isolation width: 1.6 Da |
| Heater temp. 300°C | MS$^n$ Isolation width: 1.6 Da (MS2) → 2.0 Da (MSn) |

**Figure 1. Flowchart of sugar neutral loss triggered high order MS$^n$ data acquisition on the Orbitrap ID-X instrument. The method is delivered as an instrument method template on the ID-X to allow easy set up of the MS$^n$ method**




### Data Processing

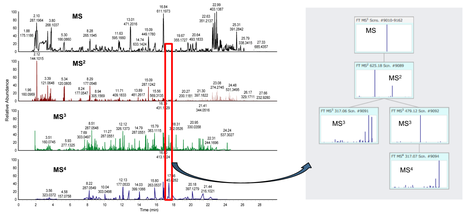
Mass Frontier 8.0 and Compound Discoverer 3.0 software were used for flavonoid identification, structure annotation, and statistical analysis. Multiple databases were employed in the processing workflow including mzCloud, ChemSpider, Arita lab flavonoid structure database[1] and a custom flavonoid mass list for unknown flavonoid structure annotation.

## RESULTS

### MS$^n$ spectral tree data generation using the developed instrument method template

Three fruit and vegetable juice samples were analyzed using the instrument method. Up to MS4 tree data were collected from all three juice samples. Figure 2 shows the base peak chromatograms from the Kale Blazer juice and one example of the MS$^n$ spectra tree data.

**Figure 2. Left: Extracted base peak chromatograms for MS, MS2, MS3, and MS4 of the Kale Blazer juice sample. Right: MS$^n$ spectral tree of precursor ion 625.1772**




### Flavonoid annotation using a new database informative data processing approach

Currently, more than 9000 flavonoid species have been reported from plant sources. Because only hundreds of flavonoid standards are available for now, the exact MS/MS (MS$^n$) spectral matches against a mass spectral library are very limited. Traditionally, flavonoid annotation has required extensive expert knowledge about the fragmentation rules of flavonoid and extensive time to do a manual interpretation. In order to address this bottleneck, we have developed a new flavonoid annotation workflow to facilitate the flavonoid annotation process without the need to have expert knowledge about fragmentation rules and diversified chemical structure of flavonoids (Figure 3).

**Figure 3. Class informative flavonoid structure annotation approach. This facilitates flavonoid structure annotation significantly.**

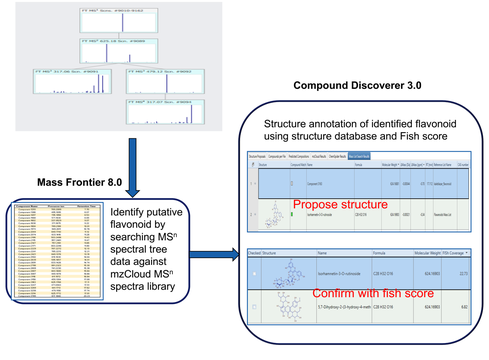



**Figure 4. Example of identifying unknown compound (m/z 581.1861) from the Kale Blazer juice sample as belonging to a specific flavonoid class using subtree search against and mzCloud library**

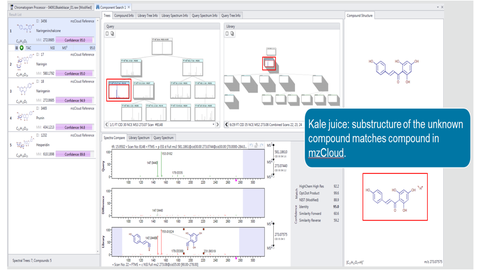



**Figure 5. Compound Discoverer workflow to annotate structures of putative flavonoids that are identified based on the sub-structure match with flavonoid references in the mzCloud library and carrying out the statistical analysis**

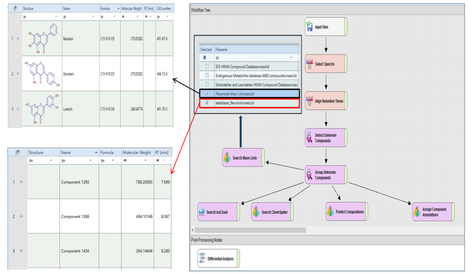
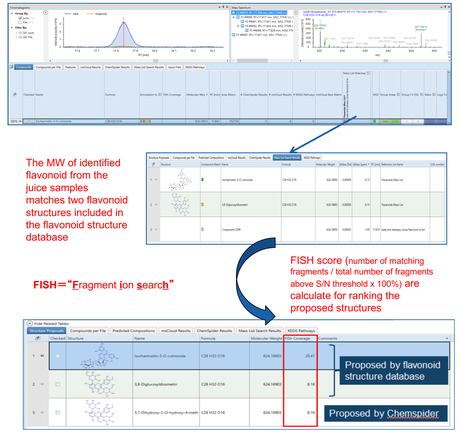


**Figure 6. Example of structural annotation for one unknown compound having a substructure matching a flavonoid reference in mzCloud. The structure candidates which match the molecular weight of the unknown compound are identified from the flavonoid structure database and ranked by matching the observed MS$^n$ fragment ions against the theoretically predicted fragment ions per each structure candidate (Fish score)**

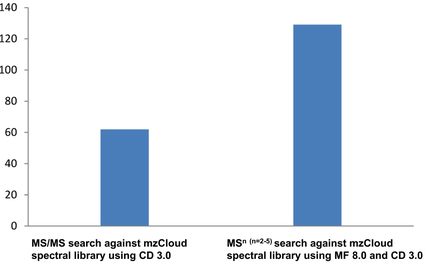



## DISCUSSION

Although it has been known that MS$^n$ spectral tree data provide deeper and more detailed fragmentation pathways, thus enabling more structural information for flavonoid annotation, the use of the MS$^n$ approach for flavonoid annotation is hindered mainly by two factors: (i) MS$^n$ instrument method setup has been difficult for non-mass-spectrometer-expert users, and (ii) MS$^n$ spectral tree data processing has been a bottleneck because it required extensive expert knowledge about flavonoid chemical structure and fragmentation rules and requires excessive time for manual fragment ion assignments. Our new flavonoid workflow addresses both challenges and can be used for the annotation of flavonoids from any type of samples, such as plants, drinks, foods and biological matrix samples.

This new approach takes full advantage of deeper and more structurally relevant fragment ion information offered by MS$^n$, enabling the annotation of many more flavonoid compounds compared to the MS/MS only approach. Figure 7 shows a comparison of annotated flavonoid numbers from the three fruit and vegetable juice samples using MS/MS only data and MS$^n$ spectral tree data; the MSn approach shows a two-fold increase relative to the MS/MS only approach.

**Figure 7. Comparison of identified flavonoids with full structure annotation obtained using MS/MS only and MS$^n$ tree spectral data**




## CONCLUSIONS

A new product ion-dependent MS$^n$ workflow has been developed for rapid flavonoid annotation with increased coverage and confidence.

- The dedicated instrument template on Orbitrap ID-X platform allows easy MS$^n$ spectral tree data acquisition.
- The newly developed class informative MS$^n$ spectral tree data processing workflow allows rapid flavonoid class compound identification and structure annotation without the need to have expertise in flavonoid chemical structure and fragmentation rules.
- 200% more flavonoids are annotated from the three fruits and vegetable juice samples by using MS$^n$ approach compared to MS/MS only approach.

## REFERENCES

1. Masanori Arita and Kazuhiro Suwa. Search extension transforms Wiki into a relational system: A case for flavonoid metabolite database. BioData Mining 2008 **1**:7.

## TRADEMARKS/LICENSING



ThermoFisher SCIENTIFIC

PO65301-EN 0518S