

High Resolution, High Accuracy Measurement and Fragmentation Analysis for Metabolite Identification in Broccoli Samples – Meta-Phor Research Initiative Results

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Overview

Purpose: To establish methodology for metabolome-wide analysis in plant extracts

Methods: Metabolite profiling and fragmentation were both performed using three high-resolution, high mass accuracy platforms. The mass spectrometers were mass calibrated prior to starting the sequence of injections. All data were acquired using external calibration.

Results: Masses were measured with mass accuracies of < 2 ppm, leading to identifications based on elemental composition analysis. Unambiguous identification of 4 analytes targeted in the META-PHOR study (citric acid, chlorogenic acid, phenylalanine and UDP-D-glucose) were used to validate the performance of the different MS/MS fragmentation regimes. Identifications were carried out either via resonance excitation CID or via higher energy collisional activation (HCD) experiments, and were validated by IRMPD fragmentation of standards. Results obtained using both hybrid and non-hybrid systems for metabolite profiling and identification experiments provide evidence that the strategies selected can be successfully applied to future LC-MS based projects in plant metabolomic studies.

Introduction

Food quality, nutritional value, flavor and resistance to pathogens are among the traits monitored by governments and the food industry alike, in an attempt to promote the creation of robust, healthy, nutrition-rich cultivars that contribute to sustained agro development. Metabolomics has been identified as a key mass spectrometry-based approach in the analysis of such traits. As a partner to the Metabolomics for Plants, Health and OutReach project (META-PHOR) which is financed primarily by the European Union (www.meta-phor.eu) we provide an overview of the use of high resolution, accurate mass measurement systems and the benefits they provide for the identification of small molecule compounds. The work presented here describes a workflow, and a general strategy for metabolome analysis and tools supporting these applications, which may be used for further plant metabolomics projects.

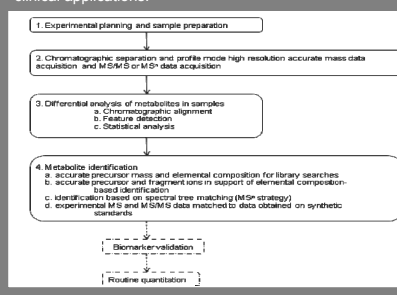
Methods

Sample preparation: Frozen broccoli samples (genotypes Monaco, Chevalier and Iron Man) were extracted as 1500 mg frozen vegetable weight in 4.5 ml of extraction solution (0.1% formic acid in methanol), vortexed, sonicated for 15min and filtered through PTFE filters into a clean 2 ml polypropylene tube (Eppendorf, Germany) prior to aliquoting (500µl) and freezing at -80 °C. Samples were shipped on dry ice and kept at -80 °C prior to analysis. Before injection into the mass spectrometer, samples were vortexed, centrifuged and re-filtered (0.22µm, PVDF Hydrophilic, Milipore; 1ml syringe from BD Plastipak, Becton Dickinson, Spain).

Chromatography: Separations were performed on either a 100 x 2.1 mm Hypersil Gold™ or Hypersil Gold PFP™ 1.9µ column (Thermo Fisher Scientific, Runcorn, UK) using an Accela U-HPLC (Thermo Fisher Scientific, San Jose, CA) operated in high pressure mode together with mass spectrometric analyses performed on the Exactive™ LC-MS and the LTQ FT Ultra™ hybrid mass spectrometer. For LTQ Orbitrap XL™ mass analysis a Surveyor MS Pump Plus™ (Thermo Fisher Scientific, San Jose, CA) was used for liquid chromatographic separations. The column was maintained at 30 °C.

Either a long or a short gradient was employed, where for the long, 30 min gradient (flow rate of 300mL/min for the LTQ FT Ultra runs and 150mL/min for the LTQ Orbitrap XL runs) the mobile phase separation ran from 95-60% A over 25 min then 60-5% A for 1 min and maintained at isocratic flow for another 2 min followed by 5-95% A in 1 min where it was kept for 1 min prior to a new separation. A short, 15 min gradient was used (flow rate of 500mL/min) together with mass spectrometric analysis on the Exactive, where mobile phase separation ran from 95-67% A over 11 min, 67-55 % A in 0.2 min, 55-5% A in 0.3 min where it was

FIGURE 1. Metabolomics workflow representing the sequence of events involved in metabolite biomarker discovery (1-4) and progressing towards development of clinical applications.



was kept for 0.9 min, and returned to 95% A in 0.6 min and where it was kept for 2 min prior to a new separation. 5 µL of sample was injected on the column and column eluent was directed into the ion source of the mass spectrometer.

Blank injection : 0.1% formic acid in methanol
Quality control sample: French red wine Les Charmes de Kirwan, Margaux (cuvee, Boreaux, years 2003 and 2005, Thermo Scientific Application Note 30173), (1).

Mass spectrometry: was performed on three high-resolution, high mass accuracy platforms. The mass spectrometers were mass calibrated prior to starting the sequence of injections. All data were acquired using external calibration.

The **Exactive** (Thermo Scientific, Bremen, Germany) mass spectrometer used a heated electrospray ionization (HESI) source and was operated in negative ion mode. The sheath gas was set to 30 (arbitrary units) at a temperature of 300 °C, the aux gas set to 20 (arbitrary units) and the capillary temperature set to 250 °C. The capillary voltage and spray voltage were set to 50 V and 3.5 kV respectively. The instrument was operated in full scan negative mode, from *m/z* 120-1000 at 100,000 resolving power. The data acquisition rate was 1 Hz, with 250 ms maximal injection time and the AGC (Automatic Gain Control) target set at 1 million charges. Each full scan was followed by a same-polarity, "all-ion-fragmentation" HCD scan with data acquisition rate of 4 Hz and resolution set at 25000, *m/z* 60-1000, 250 ms maximal injection times and the AGC target value of 3 million. The HCD was set at 35 eV. The **LTQ Orbitrap XL** hybrid mass spectrometer was operated in full scan negative mode, from *m/z* 100-1400 at 100,000 resolving power. Fragmentation data was acquired in the linear ion trap with a normalised collision energy of 70, in Data Dependant™ fashion, whereby the 3 most intense analytes detected in the full scan were selected for linear ion trap resonance excitation fragmentation, with resolving power set at 7500 for mass analysis in the Orbitrap analyzer.

The **LTQ FT Ultra** hybrid mass spectrometer was operated in negative ion mode, from *m/z* 100-1400 at 100,000 resolving power, without Data Dependent subsequent fragmentation events. Standards provided were analyzed by direct infusion with either CID or IRMPD as dissociation techniques.

FIGURE 2. Schematic of the Thermo Scientific Exactive orbitrap mass spectrometer.

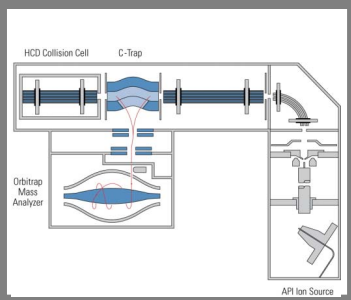
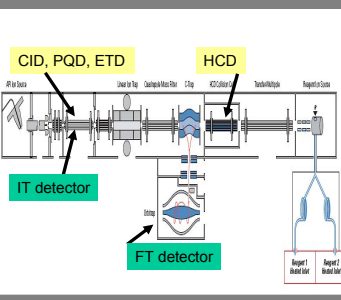


FIGURE 3. Schematic of the LTQ Orbitrap XL hybrid mass spectrometer (equipped with EDT).



Results

For all samples and instruments (LTQ FT Ultra instrument with 5 biological replicates of 3 broccoli genotypes and 5 technical replicates of pooled broccoli samples; biological triplicates of 2 cultivars analyzed in full scan mode followed by HCD fragmentation on the Exactive system or trap-based CID fragmentation on the LTQ Orbitrap XL) the 4 standards provided (citric acid, chlorogenic acid, phenylalanine and UDP-D-glucose) were mass measured at high resolution using external mass calibration. During a set of LC-MS analyses performed using the Exactive mass spectrometer covering approximately 12 hours of acquisition time the mass measurement error for UDP-D-Glucose ranged from 0.30 to 2.03ppm (data not shown).

Data acquisition was in profile mode at high resolution on all systems and compatible with HPLC and fast UPLC chromatographic separations. Peak shapes were well defined and chromatographic widths varied from 4 s (U-HPLC) to under 30 s (HPLC). As seen from table 1, RMS values for all three systems tested displayed mass errors well within instrument specifications, with data acquired on the LTQ Orbitrap XL and the LTQ FT Ultra instruments showing results across multiple acquisitions with mass accuracy better than 1ppm.

FIGURE 4. (A). Calculation of RMS error for UDP-D-Glucose measured in three non-consecutive LCMS analyses (analysis 3, 11, 23). (B). Theoretical isotopic distribution is close to a perfect match to the observed distribution.

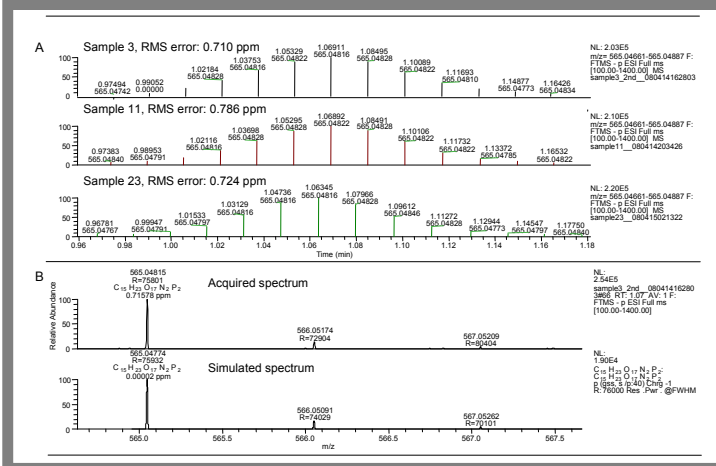


Figure 4 shows the RMS error value as measured for UDP-D-Glucose in 3 non-consecutive analyses. Isotopic distribution of the analyte is extremely reproducible from analysis to analysis as is intensity. High mass accuracy combined with high mass resolution provides powerful data for compound identification: correct isotopic distribution corroborates the evidence provided by elemental composition calculation. Differential analysis was performed with SIEVE™ software, while metabolite identification employed a two-pronged approach based on accurate mass determination for confirmatory elemental composition matching with a secondary strategy of pairing MS/MS product ion data against theoretical fragmentation patterns derived with Mass Frontier™ software.

FIGURE 5. Fragmentation of chlorogenic acid. (A) HCD fragmentation in the Exactive; (B) CID fragmentation on the LTQ Orbitrap XL; (C) MPD fragmentation on the LTQ FT Ultra

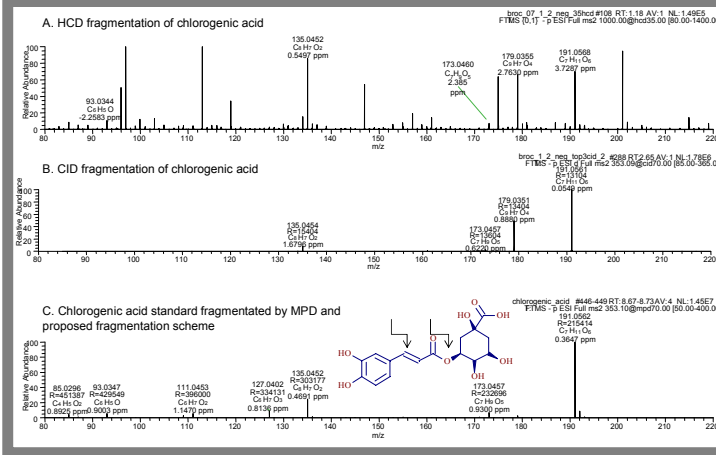


Figure 5 shows a typical fragmentation spectrum for one of the standards provided, chlorogenic acid. While all three types of fragmentation employed here provided adequate means to identify the compound with good confidence, conclusive evidence is provided by a comparison of accurate precursor mass and isotopic pattern and high quality MS/MS data of a standard to the actual mass spectral data acquired for the sample under analysis (C).

Table 1. List of mass measurements, elemental compositions and RMS mass error values obtained for UDP-D-Glucose on the LTQ FT Ultra, LTQ Orbitrap XL and the Exactive mass spectrometers. (*) Total number of data points from 3 LCMS runs.

Instrument	Elemental Composition (Neg. Ion)	Theoretical Mass	N° of data points*	RMS error (ppm)
LTQ FT ULTRA	C15H23O17N2P2	565.04774	30	0.741
LTQ OT XL	C15H23O17N2P2	565.04774	60	0.548
EXACTIVE	C15H23O17N2P2	565.04774	42	1.212

Conclusions

• **Sample preparation for global metabolite discovery can be performed efficiently, with hundreds of features profiled with SIEVE™ software**

• **Chromatographic conditions employed 15 or 30 min gradients, resulting in peak widths of 4 s (when U-HPLC was used), to peakwidths of under 30 s for standard HPLC of abundant components; such conditions were optimal for both metabolite profiling and for metabolite identification experiments**

• **Resolution of 100,000 was used for metabolite fingerprinting, enhancing discrimination of components as well as metabolite identification based on accurate mass measurements, for components identified in publicly available databases and compound libraries.**

• **Metabolite identification, a crucial component in metabolomics experiments, was performed using a two-pronged approach:**

1. accurate mass determination generating elemental composition within a narrow mass tolerance window for identification based on accurate precursor masses
2. MS/MS product ion data matching against theoretical fragmentation patterns derived with Mass Frontier™ software, where standards were available, or supporting *de novo* interpretation

• **Both fragmentations regimes utilized, resonance excitation CID on the LTQ Orbitrap XL as well as higher energy collisional activation (HCD) experiments performed using the Exactive generated high-quality spectra, and MS/MS based compound identification, validated by IRMPD fragmentation of directly infused standards**

References

- (1) Damoc, E., Scigelova, M., Giannakopoulos, A. E., Moehring, T., Pehal, F., and M., H. (2008) Direct analysis of red wine using ultra-fast chromatography and high resolution mass spectrometry Thermo Scientific Application Note 30173.

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