

# Metabolomic Screening of Human Plasma Using U-HPLC Coupled to a Bench-top Non-hybrid Orbitrap Mass Spectrometer with HCD Fragmentation

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## Abstract

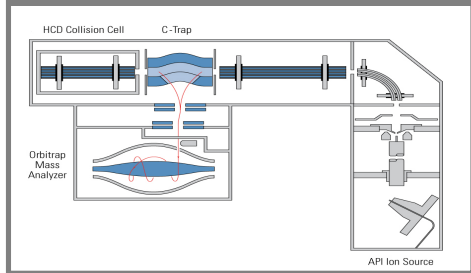
Metabolomics is the comprehensive analysis of wide arrays of endogenous metabolites in biological samples. Metabolomic screening of numerous technical and biological replicates is crucial for results to be statistically and biologically meaningful. Therefore, discovery phase metabolomics relies on rapid full scan analysis to measure as many metabolites as possible. However it is impossible to detect and quantify chemical species which are not adequately mass resolved. One current screening approach utilizes ToF instruments coupled to UHPLC delivering high mass accuracy (~5ppm) at a maximum mass resolution of <15,000. The inability to detect ions with mass resolution of at least 50,000 can lead to inaccurate mass measurements caused by unresolved background matrix interferences.

In this work we highlight a full mass scan screening approach using a novel single stage orbitrap mass spectrometer coupled to U-HPLC, capable of providing high mass accuracy at a range of resolutions: 10,000, 25,000, 50,000 and 100,000. Chromatography was performed using an Accela U-HPLC equipped with a 2.1 mm id Hypersil Gold C18 column packed with 1.9 μm particles at a flow rate of 600 μl/min and 40°C column heating.

The analysis focused on the detection and quantification of low molecular weight components of human plasma. U-HPLC coupled with a small particle column afforded a fast analysis time while maintaining very high chromatographic resolution (peak width <3 seconds at half height). The mass accuracy data (mass difference less than 2 ppm with external mass calibration) was used to confirm elemental composition. Identification of several compounds was facilitated by using HCD fragmentation, while also enabling semi-quantitative determinations.

Because discovery stage metabolomics strives to uncover as broad a range of metabolites as possible, acquisition of data in both positive and negative ionization mode becomes important. The Exactive allows fast positive/negative polarity switching without loss of mass accuracy, enabling ~50% more metabolites to be profiled.

**FIGURE 1 LC-Orbitrap Footprint.** This new instrumentation setup allows direct introduction of ions from ionization source into the C-trap for injection into the Orbitrap mass analyzer with the following specifications: Resolution: 100,000 at 1 scan per second → 10,000 at 10 scans per second; Mass accuracy: <2 ppm; Sensitivity: To be specified; Dynamic Range: >4000 within a spectrum; Scan speed: Up to 10 scans per second; Mass Range: m/z 50 – 4000; Polarity switching: Yes, 1 full cycle <1 sec.



## Materials & Methods

### Protein Precipitation of Human Plasma:

ACN, 0.1% FA (B1)  
 ACN, 0.1% FA, stepwise addition over 12h, kept at 4°C  
 200-fold dilution with 50% ACN, 0.1% FA, kept at 4°C  
 Dry down under N2  
 Add 200 μl of 95:5 H2O:MeOH, vortex, centrifuge  
 Inject 5 μl of supernatant for analysis

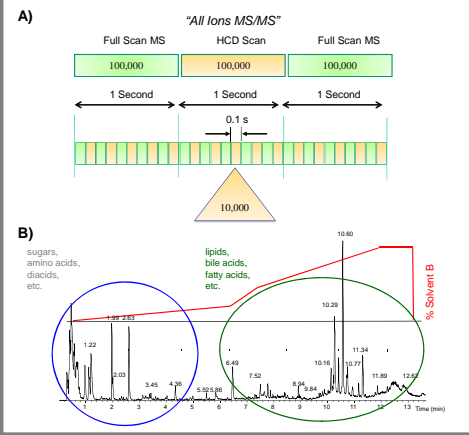
### Chromatography conditions:

Accela HPLC system  
 Column: Hypersil GOLD™ C18 100 x 2.1 mm, 1.9 μm particle size and 40°C column heating  
 Mobile phase: (A) water with 0.1% formic acid  
 (B) acetonitrile with 0.1% formic acid  
 Flow rate: 600 μl/min  
 Injection volume: 10 μl  
 Gradient: Linear gradient Figure 3.

### Mass spectrometry settings:

Exactive Mass Spectrometer  
 Positive and Negative Electrospray Ion Source  
 All methods: Full scan MS and All Ions MS/MS in Orbitrap with a mass resolutions of 10,000, 25,000, 50,000, and 100,000 resolutions.

**FIGURE 2 LC-MS Method.** A) MS scan mode schematic showing duty cycle at 10,000 and 100,000 resolutions. Similarly, at 10,000 resolution, positive and negative mode ionization can be alternated to obtain a more comprehensive metabolite profile. B) LC gradient with Solvent A: 0.1% FA in water; Solvent B: ACN, 0.1% FA, Column temp: 40°C, Sample temp: 4°C.



## Results

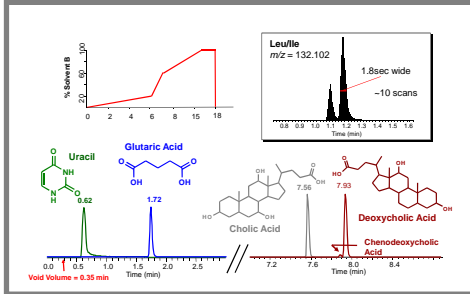
**FIGURE 3.** Number of Endogenous Metabolite Components Detected in Plasma on LTQ-Orbitrap XL. This work was done on the LTQ-Orbitrap XL in collaboration with Mark Sanders to determine optimal sample prep methodology so that the greatest number of components would be detected by the LC-Orbitrap prototype instrument. Components were detected using Genedata Expressionist™ Software.

Protein Precipitation						Solid Phase Extraction					
ACN	ACN	ACN	MeOH	MeOH	MeOH	C2	C8	C18	CN	Oasis	NH <sub>2</sub>
FA	FA	step	FA	FA	-20°C						
# 568	695	702	572	672	696	489	476	464	489	391	238
# 512	629	699	566	614	688	521	604	421	690	390	176
# 74	83	68	70	73	77	73	82	73	66	65	18
% 7.4	6.7	5.5	6.6	6.0	6.4	7.8	9.1	9.0	6.5	9.1	4.6
# 922	1168	1165	998	1140	1130	873	815	739	947	651	378

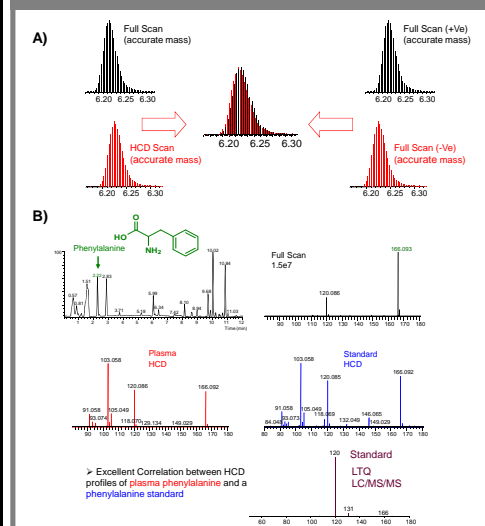
Although MeOH looked good here it didn't get rid of the protein/peptides as well as ACN

- # of components pos ion
- # of components neg ion
- # of overlapping components
- % of overlapping components
- Total # of components (pos + neg - overlap)

**FIGURE 4.** Tentative Identifications and Extracted Ion Chromatograms. Shown below are tentative IDs for compounds found in human plasma samples. Identification was accomplished via accurate mass database searching as well as structural analysis of HCD fragmentation spectra. There were a sufficient number of scans across the ~2 sec UHPLC peaks for better than 2 ppm mass accuracies as well as reproducible fragmentation spectra.



**FIGURE 5.** A) Strategies to maximize throughput and information. Due to the increased scan speed of the Exactive, data acquisition can either provide structural information by alternating full scan and HCD MS/MS scans, or provide increased metabolite profiling by acquiring full scans in alternating positive and negative ionization modes. B) Structural identification of phenylalanine is accomplished with the all ions MS/MS approach. There is a high correlation between the HCD spectra of the phenylalanine standard and the HCD spectra of the eluting phenylalanine species in human plasma.



## Conclusions

Metabolomic profiling of human plasma was accomplished in a high throughput, comprehensive fashion. Acquisition in both positive and negative ionization modes allowed over 1000 unique components to be measured with only 5-10% overlap between the two modes. The increased scan speed of the Exactive also enabled structural information to be acquired in the same method with enough scans across the chromatographic peaks to be UPLC compatible. The increased scan speed, high mass accuracy and resolution, and positive and negative polarity switching make the Exactive an ideal screening instrument for applications such as metabolomics.