

Bottom-up Label-Free HT Differential Analysis of Human Plasma Using an Integrated Fractionation/nanoESI Device Coupled with LC-FTMS Detection

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Overview

Purpose: To develop a methodology for extensive, in-depth analysis of human plasma proteins using a bottom-up, label-free integrated fractionation/nanoESI device coupled with LC-FTMS Detection.

Methods: Following protein partition, the simplified plasma proteins were analyzed by a semi-preparative split workflow that coupled the TriVersa™ NanoMate™ (Advion Biosciences, Ithaca, NY) with a Finnigan™ LTQ FT™ (Thermo Electron, San Jose, CA) and simultaneously collected LC fractions for gas phase fractionation MS and MS/MS analyses. All peptide identifications were made using BioWorks™ 3.2 software (Thermo Electron, San Jose, CA).

Results: Demonstrates the effectiveness and reproducibility of split workflow and gas phase fractionation to provide comprehensive analyses of complex biological samples.

Introduction

Identification of protein/peptide biomarkers in plasma is a daunting analytical task due to the extremely large concentration, dynamic range and complexity of this biological fluid. Intrinsic to traditional bottom-up strategies, proteolytic digestion further increases the complexity of the original protein sample, and multidimensional separations have been employed in an effort to ameliorate this problem. In this work we present an alternative strategy for high-throughput differential analysis of human plasma based on a fully automated, robust post-column split experiment in combination with gas-phase fractionation. This allows for a more thorough, real-time characterization of the eluting peptides, as well as rapid, targeted evaluation of those fractions that are indicative of differential expression.

Methods

Sample Collection

Plasma samples were collected from healthy volunteers and stored at -80°C.

Plasma Partition and Enzymatic Digestion

The twelve most abundant plasma proteins were depleted from the plasma using affinity-based partitioning spin columns (Genway Sepro mixed 6) according to the manufacturer's protocol. Enzymatic digestion was performed on the flow-through fractions using the procedure described below. Proteins were then dissolved in 25 µl of 100mM NH₃CO₃ pH 7.8, reduced with 10 µl of 100mM dithiothreitol for 1 hour at 56°C, then alkylated using 10 µl of 300mM iodoacetamide for 1 hour at room temperature in the dark. Proteolytic enzyme was added at ~ 1:20 w/w enzyme-to-protein ratio and the solution was incubated for 15 hours at 37°C. The reaction was quenched with 5 µl of acetic acid and stored at -80°C.

Sequential LC-MS/MS Analysis

Plasma digests were analyzed by LC MS/MS using a Finnigan LTQ FT mass spectrometer (San Jose, CA) equipped with a TriVersa NanoMate (Advion BioSciences, Ithaca, NY). Peptides were captured and concentrated for on-line with RP-HPLC using a C18 capillary column (2.1mm i.d. x 50cm), with 5.0 µm packed particles (Higgins Analytical Inc., Mountain View CA). The flow was split 1:1000 (NanoMate LTQ FT to sample plate) using a MicroTee (Upchurch Scientific, Oak Harbor, WA). The low-flow branch of the split was directed into the mass spectrometer and full scan (MS) data was collected in the FT-ICR MS in profile mode between m/z 400-1800 with simultaneous Data Dependent MS/MS scanning of the top five most abundant ions performed in the ITMS. The high-flow branch was collected into a 96-well plate using a time-based fractionation of 36 sec/fraction.

Gas Phase Fractionation Analysis (Infusion)

The collected samples were lyophilized to dryness and then reconstituted in 25 µl of 50% Methanol with 0.1% acetic acid. Sample fractions were infused using the TriVersa NanoMate. A ten minute analysis was performed using less than 5 µl of the samples, while performing the MS scans described in Figure 4.

Results

FIGURE 2. Does fraction collection and LC-MS align correctly? Peptide at m/z 523.7740 elutes at 22.63 min during the LC-MS analysis; therefore, according to the ChipSoft software, this peptide should be in sample position C07. Results below show the presence of this peptide in C07 but not present in the adjacent fractions.

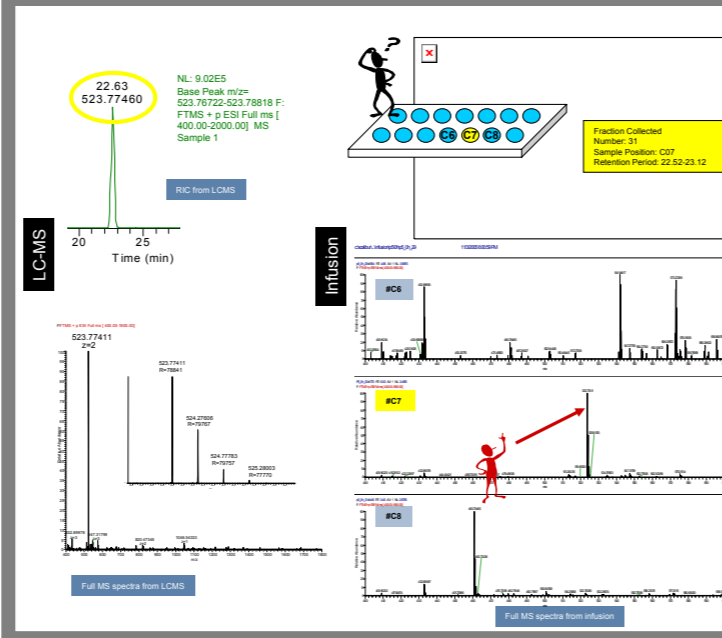


FIGURE 3. Is it reproducible? Three samples were analyzed using the workflow described in Figure 1. Shown below are RIC for m/z 523.77460 (LC-MS) and full MS spectra (infusion). Note the reproducibility of the retention time (LC-MS) and the intensity of the signal (infusion).

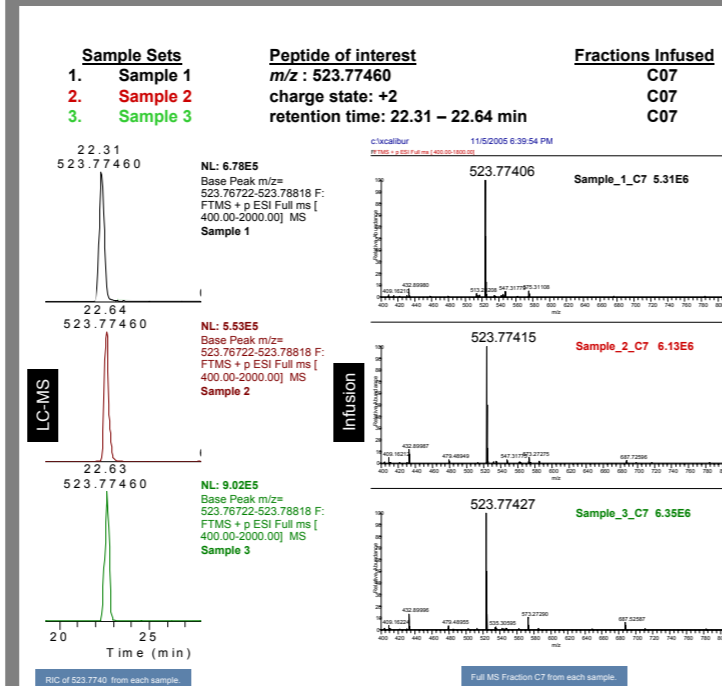


FIGURE 4. Gas phase fractionation MS and MS/MS analysis. Ninety-six fractions are infused into the LTQ FT for ten minutes using the NanoMate. The MS is programmed to follow the scan events shown below.

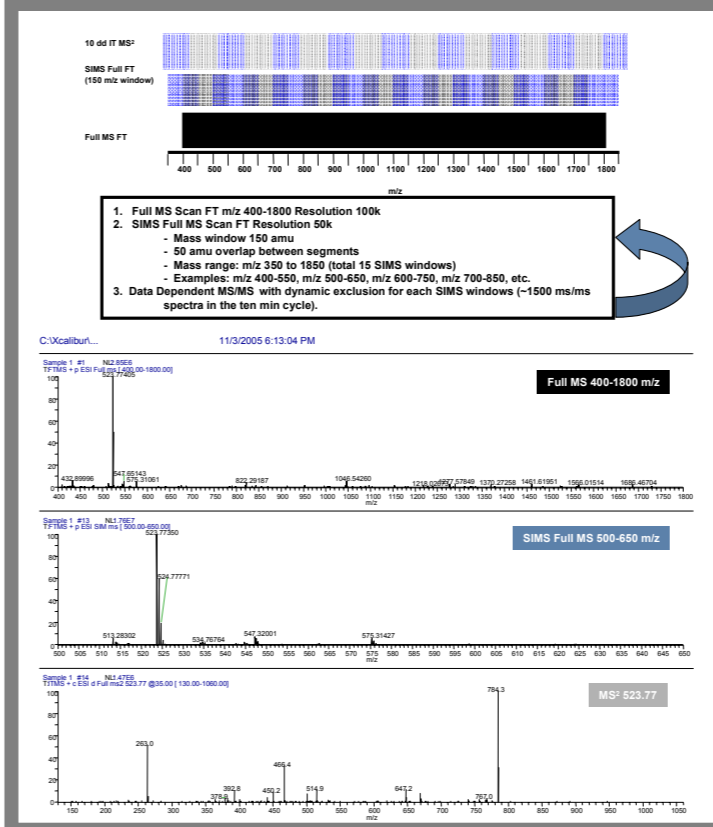


FIGURE 5: Comparison of the protein and peptides search results from Sequest for sample 2 from both the LC-MS and the gas phase infusion analysis.

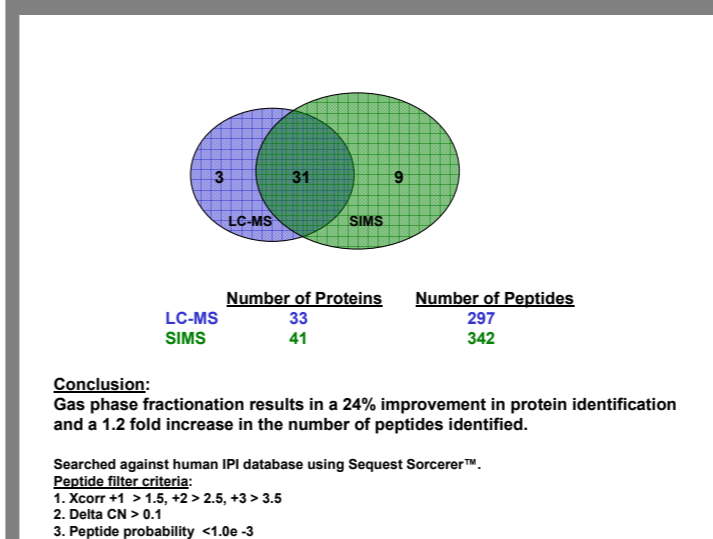
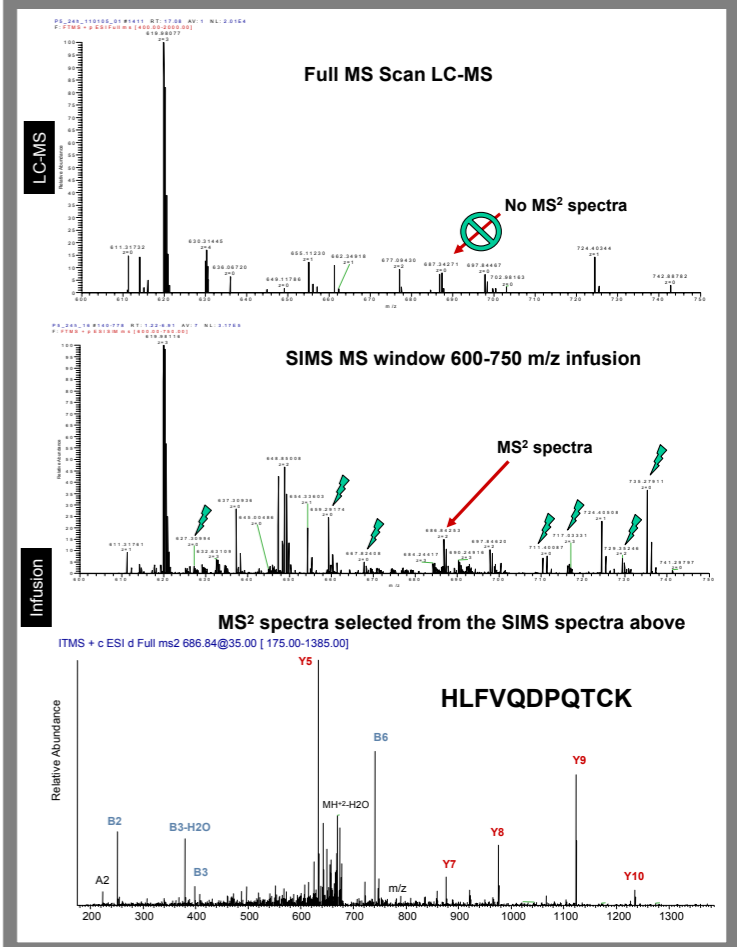


FIGURE 6. Advantages of gas phase fractionation analysis. Ions not selected in data dependent MS/MS experiments can now be identified. Shown below in the LC-MS spectra is a low abundant peak observed at m/z 686.82 - detectable but not selected for MS/MS. SIMS full MS window (m/z 600-750) produces a much stronger signal for this peptide and MS/MS spectra are generated resulting in successful peptide identification. Note: other ions are detected in the SIMS scan that were not detected in the full MS scans.



Conclusions

The strengths of combining LC-MS and infusion MS analysis is presented.

The split experiment allows for ultimate peak parking. In this case, sample fractions were concentrated and multiple MS experiments were performed resulting in the generation of more data than LC-MS alone. In addition, the extra time allowed for improved data quality through signal averaging.

The technique of coupling an integrated fractionation/nanoESI device with an LC-FTMS detector resulted in extremely reproducible data generation. The TriVersa NanoMate provided an automated and versatile setup for high-throughput analysis. Infusion produced an extremely stable, robust, and prolonged nanoESI spray (in- and off-line).

Gas phase fractionation analysis provided increased sensitivity, exact mass measurements and lower detection limits. The smaller mass windows also resulted in trapping more low abundant ions, because the target values are not reached by a more abundant ion, resulting in the generation of higher quality data.

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