Application Note: 344

Top-Down Analysis of the Low Molecular Weight Human Plasma Proteome Using Hybrid Ion Trap-Fourier Transform Mass Spectrometry

Jennifer N. Sutton[1,5] Leo E. Bonilla[1], Tori Richmond[1], Robert E. Gerszten[2], Emerson Liu[2], Xui Shi[2], Michael Senko[3], Vladimir Zabrouskov[3], Neil L. Kelleher[4], Andrew Forbes[4], Robert G. Harper[5]

[1]Biomarker Research Initiatives in Mass Spectrometry (BRIMS) Center, Thermo Fisher Scientific, Boston, MA; [2]Cardiology Division and Center for Immunology & Inflammatory Diseases, Massachusetts General Hospital and Harvard Medical School, Boston, MA; [3]Thermo Fisher Scientific, San Jose, CA; [4]Center for Top Down Proteomics, Department of Chemistry, University of Illinois, Urbana, IL; [5]Department of Chemistry, West Virginia University, Morgantown, WV

Key Words

- LTQ FT™
- Intact Protein Analysis
- ProSight PTM™
- Protein Identification
- Ultrafiltration

Introduction

Characterization of the plasma proteome is a daunting analytical task due to the large number of proteins thought to be present and the extremely large dynamic range of their concentrations. Historically, several different analytical approaches have been incorporated to identify proteins belonging to the low molecular weight (LMW) plasma proteome, including various enrichment schemes and numerous bottom-up mass spectrometric analyses.

For bottom-up analysis, the protein is digested with an enzyme to give a mixture of peptides which are identified from database analysis of their CID fragmentation spectra to lead to the identity of the source protein. By contrast, top-down analysis enables the study of the intact protein, allowing identification, primary structure determination and localization of post-translational modifications (PTMs) directly at the protein level, without the need for enzymatic digests. In this report, a novel top-down approach is used for characterizing the LMW proteome and plasma peptidome using the Thermo Scientific LTQ FT, a hybrid linear ion trap-Fourier transform mass spectrometer.

Goal

Demonstrate the effectiveness of a specific enrichment methodology, along with top-down analysis on the LTQ FT, to detect and identify both intact LMW plasma proteins and peptides belonging to the plasma peptidome in an enzyme-free protocol.

Sample Preparation & Experimental

Enrichment of the LMW fraction was achieved by ultrafiltration as described in the Villanueva et al protocol[1]. Preparation of the 30 and 50 kDa centrifugal filter membranes was performed according to the manufacturer's specifications. One mL of human plasma was processed by first dividing the sample into 5×200 µL aliquots, then diluting with 120 µL of deionized water and 20% v/v acetonitrile. Proteins were denatured at 40 °C for 15 min then centrifuged at 10,000×*g* for 10 min to remove any precipitated material that could clog the filters. Cleared supernatants were then applied to the prepared centrifugal filters and spun at 1,500×*g* for 90 min at 4 °C. Approximately 25% of the total volume was passed through each filter, pooled and concentrated by lyophilization. Aliquots for MS analysis were dissolved in 100 µL of 5% acetonitrile in 0.1% formic acid. For the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, the filtrates were lyophilized to dryness and resuspended in 30 µL of Laemmli sample buffer, boiled for 10 min, then loaded onto Bio-Rad Criterion Tris-HCl Ready-gels using a 4–15% linear gradient. Silver staining was performed per the Rabilloud protocol[2].

LC Separation and MS Analysis

HPLC

Plasma peptides were captured and concentrated for on-line reversed phase (RP) liquid chromatography using a nonporous silica-based (NPS) polymerically bonded column (3.3 cm×3.0 mm) with 1.5 µm packed particles. Separations were performed at 60 °C at a flow rate of 200 µL/min. Mobile phases A and B each contained 0.1% (v/v) formic acid; acetonitrile was used as the organic modifier (5% v/v in Solvent A and 95% v/v in Solvent B). The linear gradient profile increased the percentage of Solvent B from 0 to 100% over a 30 min period, held the percentage of B at 100% for 5 min, and decreased the percentage of B from 100 to 0% over a 5 min period (total time 50 min).

Mass Spectrometry

Mass spectrometer:	LTQ FT
Ionization mode:	Positive µ-ESI ionization
Spray voltage:	4.00 kV
Sheath gas:	25 units
Capillary temperature:	275 °C
Capillary voltage:	100.0 V
Tube lens voltage:	130 V

Cytochrome-C from rabbit was used to optimize the instrument for intact protein analysis. Full MS measurements were made using the FT-ICR mass analyzer with an AGC target setting of 7e5. Two microscans were averaged for each full MS scan and allowed to reach the maximum AGC target value in 500 ms. Fragmentation occurred in the linear ion trap and detection in the FT with an AGC target value of 4e5 achieved in a maximum of 3 s with one microscan.

Thermo SCIENTIFIC

Spectral Processing and Database Searching

Intact protein spectra were analyzed using a deconvolution program[3] with manual validation of Mr values reported with isotopic resolution. MS/MS spectral analysis was performed using the THRASH algorithm[4], and the resulting peak lists, together with the intact protein Mr values, were sent to ProSight PTM software for identification and characterization of the intact proteins[5]. Precursor masses were searched against the human proteome database. Peptides matching the precursor mass within a specified tolerance were checked against the listed fragment masses to see if any b/y ions matched, according to the appropriate fragmentation method. Those peptides with more than three fragment matches were returned in the output file. ProMass Deconvolution software was used for deconvolution of intact protein spectra to confirm the identification of two overlapping proteins.

Results and Discussion

Analysis of the human plasma proteome is considered one of the most challenging tasks for proteomics researchers interested in identifying new disease biomarkers. The analytical complexity of the plasma proteome is due to its extremely wide dynamic range reported to extend over 12 orders of magnitude. High-abundance proteins such as albumin and IgG comprise approximately 80% of the human plasma proteome, leaving only 20% of the total protein content for low abundance proteins that may have the most impact in regulating signaling pathways crucial to disease. For this reason, reduction of sample complexity is a critical step for the detection and identification of the LMW plasma proteome, along with a mass spectrometry technique that provides the highest sensitivity and resolving power. In this experiment, the LTQ FT was chosen for its unique ability to provide accurate mass and high resolution data on a fast chromatographic timescale –without the need for proteolytic digestion.

Figure 1 shows a schematic of the LTQ FT mass spectrometer – a hybrid instrument that combines the high sensitivity and superior fragmentation capability of the LTQ linear ion trap, with the accurate mass and high resolution of FTICR-MS. The analysis conditions listed are specific for intact protein analysis. Tuning the instrument in this way ensured maximum transmission of the multiply-charged ions for the best MS/MS sensitivity and subsequent protein identification. Note that only one microscan was used for every MS/MS scan, preventing the need to average several spectra.

Ultrafiltration was used to enrich the LMW fraction of the human plasma sample. Figure 2 shows a schematic of this process, in which the sample was first divided into five aliquots prior to centrifugation. This ultimately reduced

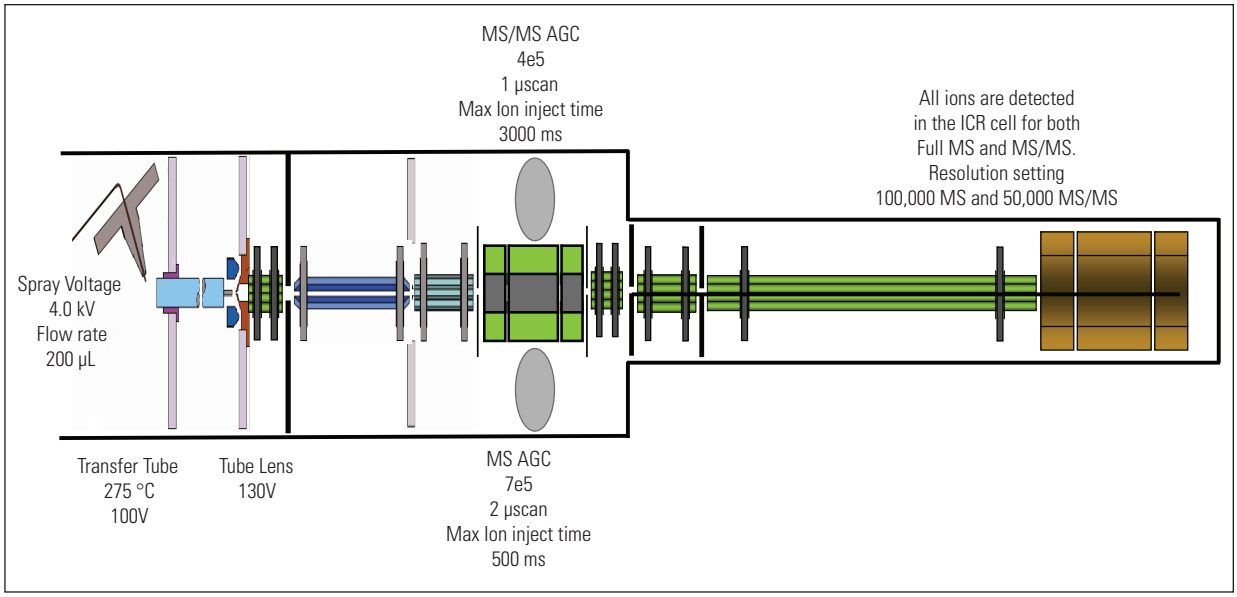

Figure 1: LTQ FT schematic describing conditions ideal for Top Down analysis

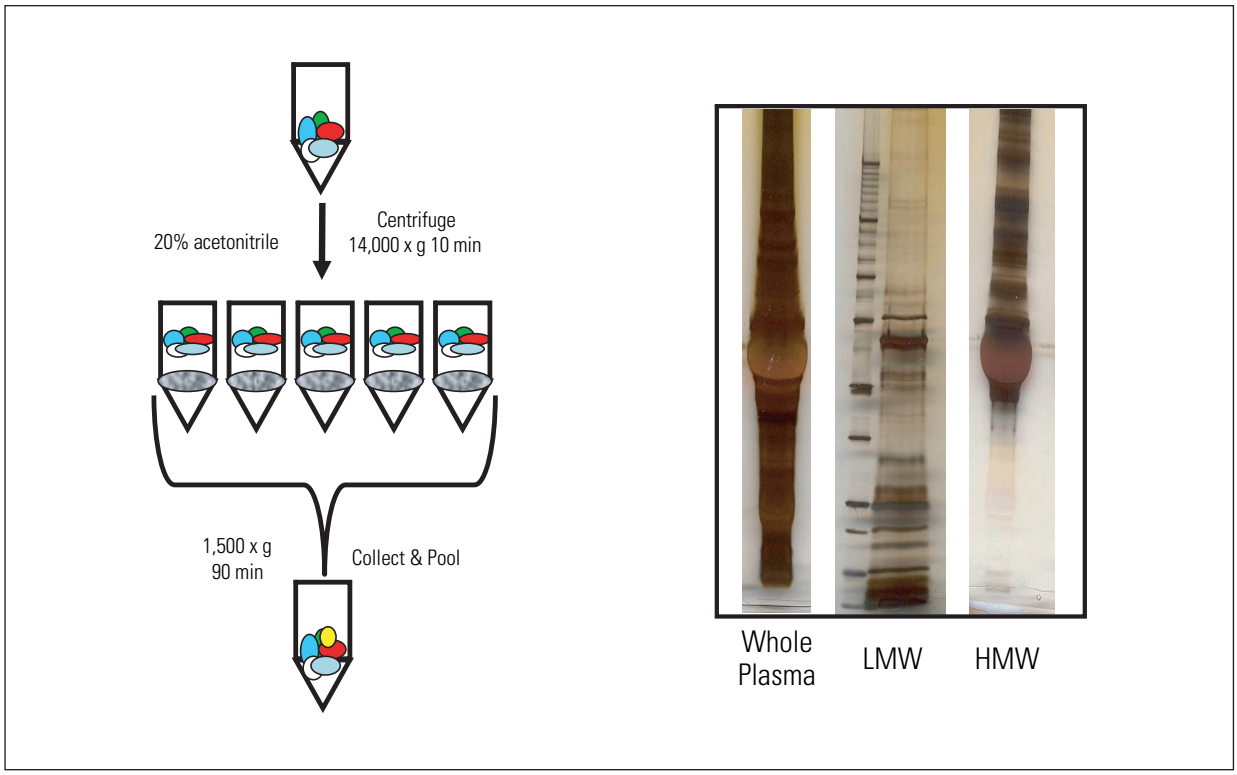

Figure 2: Ultrafiltration purification of human plasma with gel images showing the final fractions

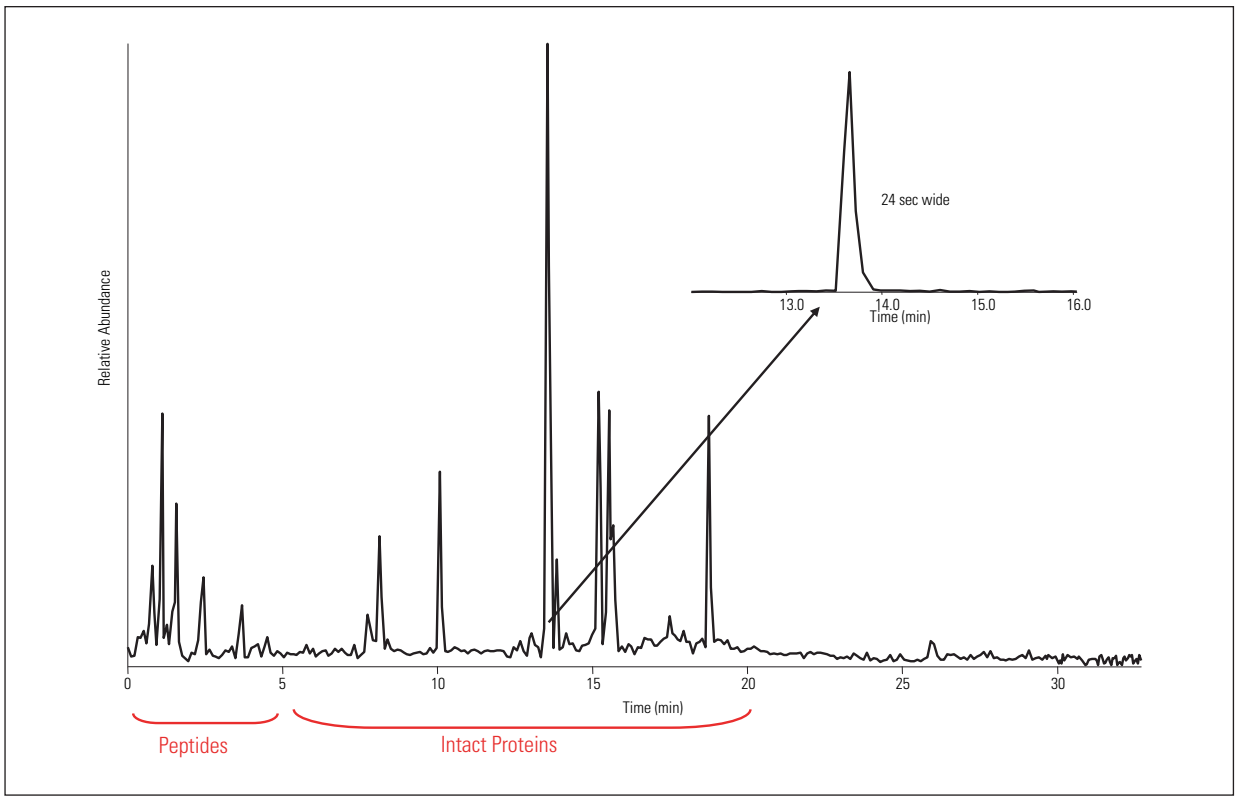

Figure 3: Base peak chromatogram RPLC-MS using NPS column

the concentration of albumin and other abundant plasma proteins by approximately 90%, enriching the lower abundant protein fraction (LMW), in which most biomarkers are believed to exist. Gel images show the total protein content of the entire sample, as well as the HMW and LMW fractions. The LMW proteins were then separated by reversed-phase chromatography and analyzed on the LTQ FT, as shown in the base peak chromatogram in Figure 3. In this data, the first five minutes of the chromatogram represent separated peptides; from 5–20 minutes, intact proteins, both analyzed on a single NPS column. An expanded view of one of the peaks in the intact protein region shows that the peak widths are only 24 seconds wide – ideal for high chromatographic resolution. The NPS column helped contribute to the resolution quality of the peaks, as well as the high resolving power of the LTQ FT.

Figure 4 shows a Full MS spectrum of one of the peaks in the intact protein region, and is representative of the quality of the highly-resolved peaks throughout the run. In this example, each peak is labeled with its *m/z* value, resolution (R) and charge state (z). Closer inspection of the expanded region around the peak at *m/z* 1178 revealed the isotopic envelope of the more abundant of two overlapping multiply-charged proteins. This was confirmed by using ProMass Deconvolution, which successfully deconvoluted two overlapping proteins from the full MS spectrum to expose major proteins with molecular weights of 9420.9 and 9712.2 Da, as shown in Figure 5. The MS/MS spectrum of the larger protein at *m/z* 1178 is shown in Figure 6.

Using ProSight PTM software a putative truncated isoform of Apolipoprotein A-II was identified, as shown in Figure 7. A C-terminal sequence tag was clearly identified from eleven b-ions with an 8.7 ppm average mass error. A crude PScore for this protein identification yielded a value of 6.71e-11, indicating a high probability of a correct identification. A mass difference of 2363.43 Da was observed from the theoretical mass of the predicted protein sequence, indicating a truncated isoform of the apolipoprotein A-II, which may also contain a post-translational modification on the N-terminus.

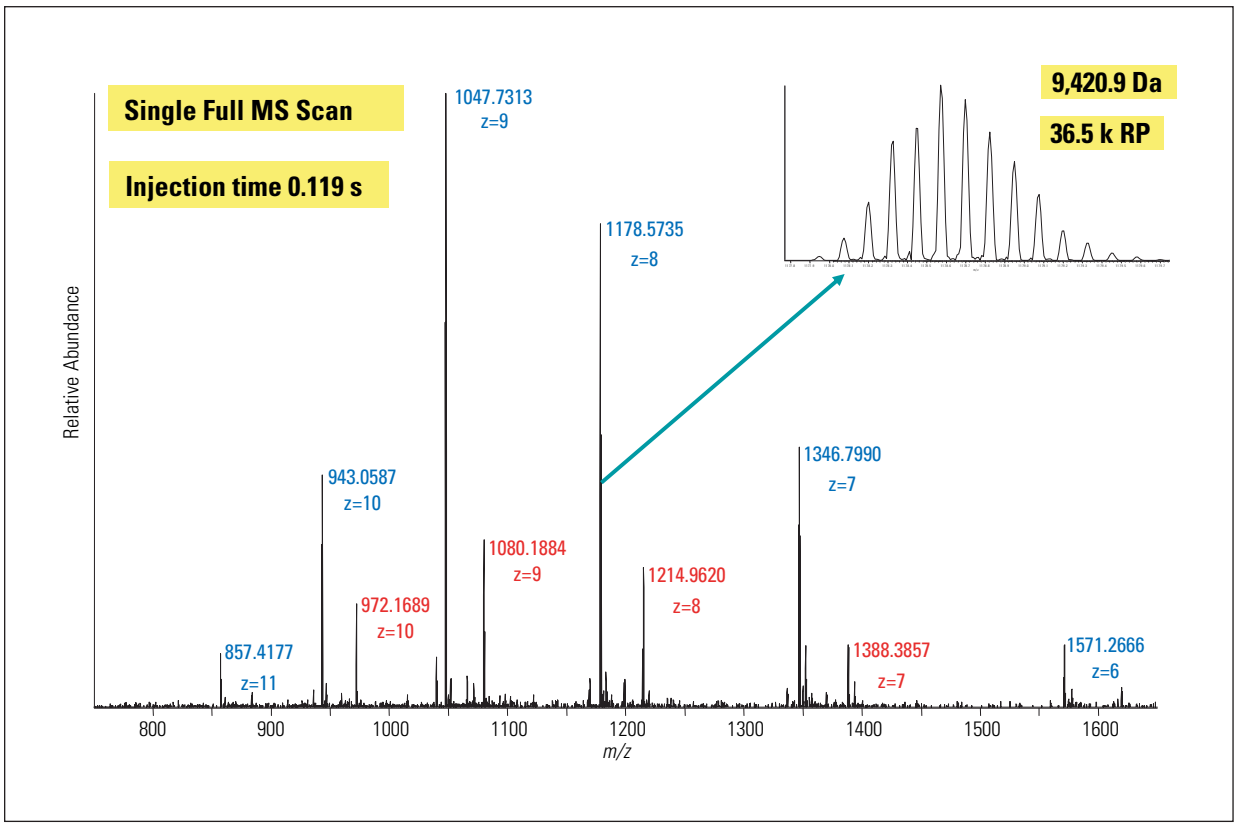

Figure 4: Full MS spectra detected in the LTQ FT

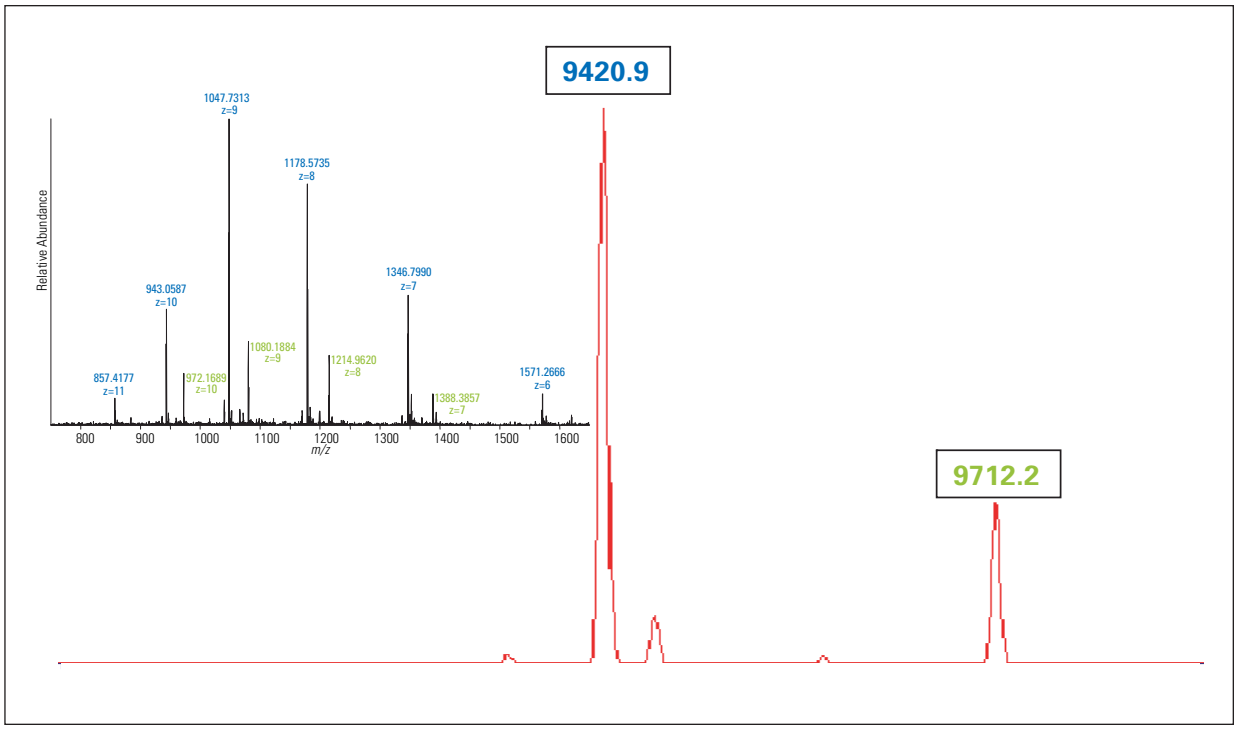

Figure 5: Deconvoluted spectra from ProMass software showing two major proteins

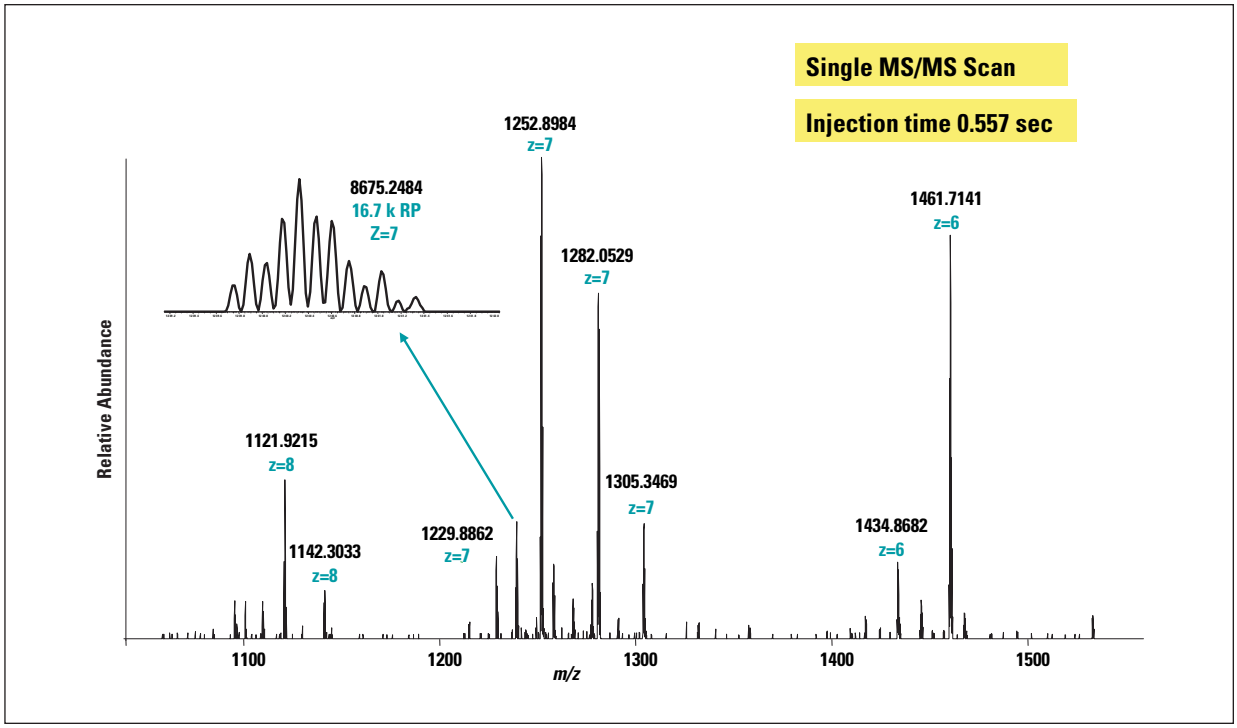

Figure 6: MS/MS spectrum of 1178.6 *m/z* charge +8

Conclusion

The Low Molecular Weight portion of the human plasma proteome is thought to contain a wealth of putative biomarkers for human disease. This study was performed to demonstrate an effective methodology for isolating the LMW protein fraction of plasma and analyzing it directly with Top-Down protein sequence analysis.

To be effective, Top-Down analysis requires high mass accuracy and resolution in order to determine the molecular weight of the intact protein and its fragments. To be practical, Top-Down analysis should be performed during routine LC/MS of LMW plasma protein fractions.

As such, the LTQ FT is the only analytical system capable of performing such an analysis. Data is shown for just one of many proteins identified in this analysis.

Further studies will demonstrate online multi-dimensional chromatography techniques to enhance the separation of the intact proteins for future differential expression work.

References

1. Villanueva, J., Philip, J., Entenberg, D., Chaparro, C., Tanwar, M., Holland, E., Tempst, P. Serum peptide profiling by magnetic particle-assisted, automated sample processing and MALDI-TOF mass spectrometry. *Anal. Chem.* **2004,** 76, 1560-1570.
2. Rabilloud, T., A comparison between low background silver diammine and silver nitrate protein stains. *Electrophoresis* **1992,** 13, 429–439.
3. Johnson, J.R.; Meng, F.; Forbes, A.J.; Cargile, B.J.; Kelleher, N.L. *Electrophoresis* **2002,** 23, 3217-3223.
4. Horn, D.M.; Zubarev, R.A.; McLafferty, F.W. *J. Am. Soc. Mass Spectrometry.* **2000,** 11, 320-332.
5. Taylor, G.K.; Kim, Y.B.; Forbes, A.J.; Meng, F.; McCarthy, R.; Kelleher, N.L. *Anal. Chem.* **2003,** 75, 4081-4086.
6. Meng, F.; Cargile, B.J.; Miller, L.M.; Forbes, A.J.; Johnson, J.R.; Kelleher, N.L. *Nat. Biotechnol.* **2001,** 19, 952-957.

***View additional Thermo Scientific LC/MS application notes at:* www.thermo.com/appnotes**

In addition to these offices, Thermo Fisher Scientific maintains a network of representative organizations throughout the world.

Africa
+43 1 333 5034 127
Australia
+61 2 8844 9500
Austria
+43 1 333 50340
Belgium
+32 2 482 30 30
Canada
+1 800 530 8447
China
+86 10 5850 3588
Denmark
+45 70 23 62 60
Europe-Other
+43 1 333 5034 127
France
+33 1 60 92 48 00
Germany
+49 6103 408 1014
India
+91 22 6742 9434
Italy
+39 02 950 591
Japan
+81 45 453 9100
Latin America
+1 608 276 5659
Middle East
+43 1 333 5034 127
Netherlands
+31 76 587 98 88
South Africa
+27 11 570 1840
Spain
+34 914 845 965
Sweden/Norway/ Finland
+46 8 556 468 00
Switzerland
+41 61 48784 00
UK
+44 1442 233555
USA
+1 800 532 4752

www.thermo.com

AN62498_E 08/16S