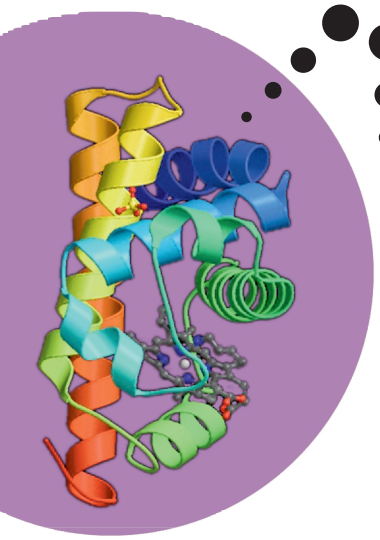

Rapid Protein Characterization Using Microflow LC/MS/MS Techniques

Application Note

Protein Analysis

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Introduction

Automated tandem mass spectrometry (MS/MS), coupled to online microcapillary liquid chromatography is now becoming a commonly used technique for identifying proteins. This technique typically involves digestion of a protein followed by microflow LC separation of the peptide fragments. As each peptide fragment elutes, it is automatically selected for collision induced dissociation (CID) by using powerful mass spectrometric techniques, including the real-time, automatic selection of parent ions using automated data-dependent scan functions. Protein identification is then accomplished by using computer algorithms that correlate uninterpreted protein and peptide tandem mass spectral data with sequences in protein or nucleotide databases. This method not only provides the potential for automated high-throughput data analysis, it also imparts a high degree of specificity to database searches because the tandem mass spectrum representing every amino acid sequence displays a unique fingerprint-type pattern.

Recently, the scope of this method was expanded to increase the number of proteins and peptides that can be analyzed by mass spectrometry. The technique permits the identification of 100 proteins in a single analysis using multidimensional chromatography with MS/MS detection using automated parent ion selection for high-throughput proteomics laboratories.[1] This multidimensional liquid chromatography/MS/MS methodology precludes the isolation of individual proteins using two-dimensional gel electrophoresis and permits the rapid identification of protein mixtures.

We have evaluated the use of microflow LC/MS/MS techniques for identifying proteins from a complex mixture of five tryptic digests using a single reversed-phase capillary LC separation and ion trap MS detection.

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Experimental

All experiments were done using an Agilent 1100 Series LC/MSD Trap mass spectrometer coupled to an Agilent 1100 Series Capillary LC system. The system was operated with the electrospray ionization (ESI) source in the positive ion mode.

Individual tryptic digests were performed on each of the five proteins. The digests were then diluted and combined in one sample tube at a concentration of 1 pmol/µL each. For use as a reference in order to monitor the sensitivity of the system, a standard peptide (Glu-Fib) was prepared and spiked into the digest mixture at a concentration of 1 pmol/µL.

The analysis was carried out using a microelectrospray interface from coupling of an Agilent Capillary LC system to the LC/MSD Trap ion trap mass spectrometer. A 40-min gradient was used for separation of peptides from the digest mixture at a flow rate of 2 µL/min on a 180 µm ID capillary column.

Reagent grade chemicals and HPLC grade solvents were used in preparing mobile phases and standards.

Results and Discussion

Figure 1 shows the chromatographic separation of a mixture of tryptic digests from five different proteins and a reference peptide. The total amount of material injected on-column represents 1 pmol per total tryptic digest.

The top trace in Figure 1a shows the base peak chromatogram from LC/MS analysis of the digest mixture. For use as a reference in monitoring the sensitivity of the system, 1 pmol of a peptide standard was injected. The extracted ion chromatogram corresponding to the peptide standard (Fig. 1b) shows a strong signal response for 1 pmol of total material injected.

Data acquisition efficiency was optimized through use of data-dependent techniques. In order to maximize the number of precursor ions that are selected for MS/MS, the threshold for ion current intensity is typically set by the operator at a relatively low level. This ensures that the most intense ion in every MS scan that exceeds the threshold automatically triggers an MS/MS experiment. Since the operator has no knowledge *a priori* about what ions are eluting from the column, there are no preset mass lists. In this manner, a tremendous amount of information is generated in these experiments. However, by using these techniques it is possible that less intense peptide ions can go unanalyzed since they are missed during the selection to obtain CID spectra in the presence of more abundant coeluting peptides. The "number-of-parents" parameter in the LC/MSD Trap software can prevent this by collecting CID spectra from a user-specified number of the most abundant ions from a single MS scan. For instance, the first scan event acquires an MS scan, after which the three to five most abundant ions in the MS scan are acquired. This technique also minimizes redundancy and improves data acquisition efficiency by precluding the reacquisition of tandem mass spectra once a specific *m/z* ion has triggered an MS/MS scan event. In this manner, a maximum number of unique precursor ions, including those of lower abundance, can be included in the analysis. In many cases, however, the second most abundant peak in an MS scan is the ^{13}C isotope of a predominant peptide. The isotopic exclusion feature permits exclusion of the carbon isotopes of the peptide ions triggering MS/MS experiments. A 3 u mass width window is the default. Taken together, these techniques permit automatic acquisition, including "number-of-parents" and isotopic exclusion, which translates into a significant increase in the number of peptide ions selected for CID.

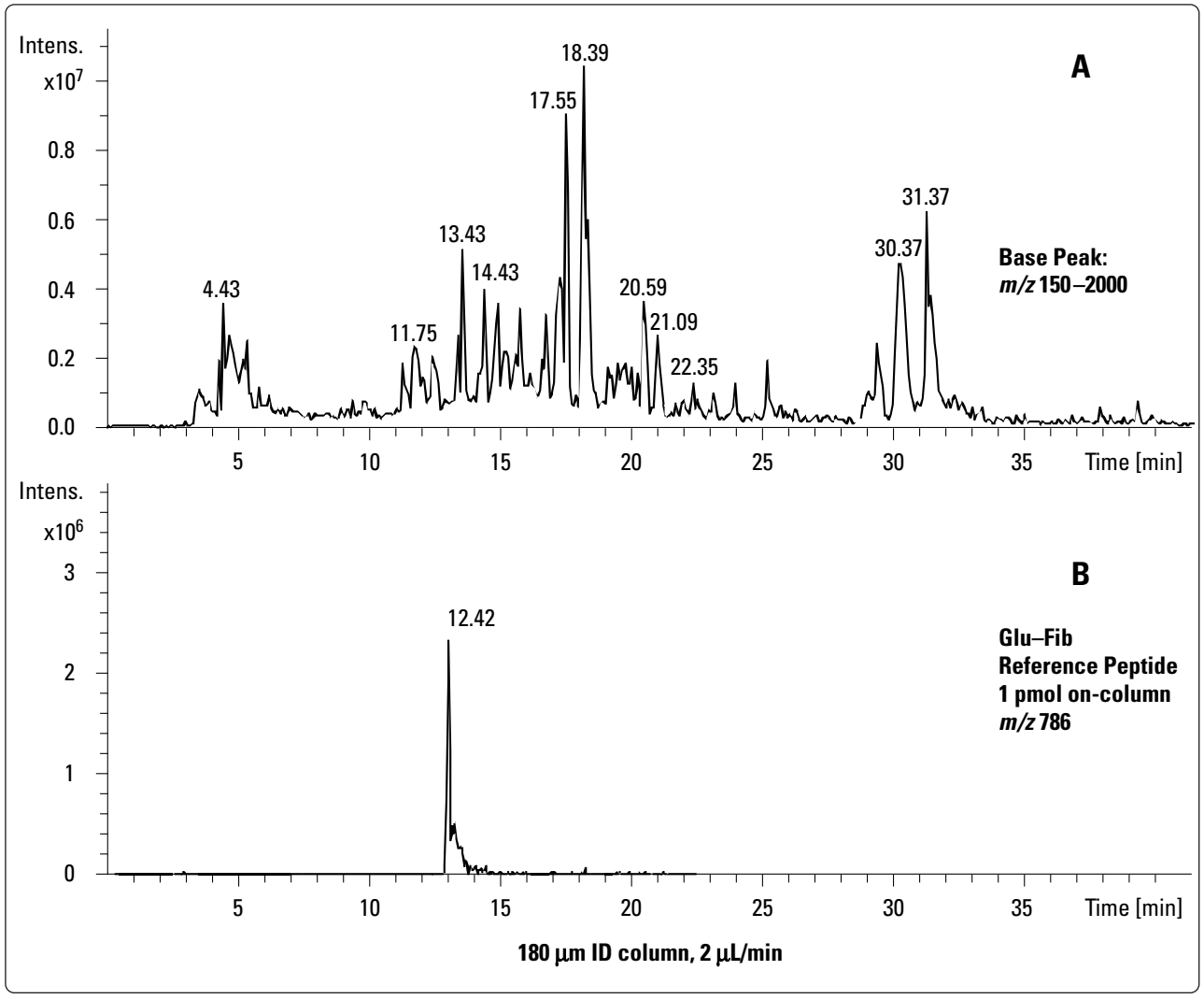


Figure 1. Base peak (A) and extracted ion chromatogram (B) generated from analysis of a complex mixture of protein digests

ANALYSIS METHOD:

Capillary LC/MS^n

Flow rate:	2 µL/min
Injection volume:	1 µL
Mobile phase:	A = 0.1% formic acid in H_2O
Mobile phase:	B = acetonitrile

MS Conditions

Source:	ESI
Drying gas flow:	10 L/min
Nebulizer:	10 psig
Drying gas temperature:	200°C
Skim 1:	15.0 V
Cap exit offset:	60.0 V
Averages:	2
ICC:	On
Max accu time:	100 ms
Target:	50000
Ion mode:	Positive
Isolation width:	4 *m/z*
Fragmentation amp:	1.1 V

Figure 2 shows the total ion chromatogram (TIC) which includes all of the MS and MS/MS experiments that were triggered during the entire chromatographic run. The spiky appearance of the chromatographic trace is a result of the instrument switching between MS and MS/MS experiments—the total ion current decreases in the MS/MS mode.

Whenever an ion that is present in a scan exceeds the pre-set threshold during the automated run, a product ion spectrum is acquired. The instrument continues to alternate between MS and MS/MS scans until the ion intensity drops below the ion current threshold.

Figure 3 shows the full scan MS spectrum corresponding to the chromatographic peak at 17.55 min. The doubly charged molecular ion at *m/z* 807 exceeds the threshold and triggers a tandem MS experiment. The observed MS/MS spectrum of *m/z* 807 along with the output from a database search using the MASCOT (Matrix Science, Ltd.) program is shown in Figure 4. The y-ion series for the peptide, whose sequence is also shown, matches that of the observed CID spectrum. This represents one of the peptides that was automatically found by the MASCOT program and that matched that of the human apolipoprotein, one of the proteins digested and combined in the original protein mixture.

The MASCOT database search algorithm correctly identified three of the five proteins present in the complex tryptic digest mixture. Although two of the proteins were not identified by the algorithm, these results represent only a single chromatographic analysis. Further optimization of the capillary LC–ion trap system is being studied in an effort to increase the number of correctly identified proteins. Even though this was not a coverage experiment, approximately 30 percent sequence coverage was obtained for each of the three identified proteins, as shown in Figure 5.

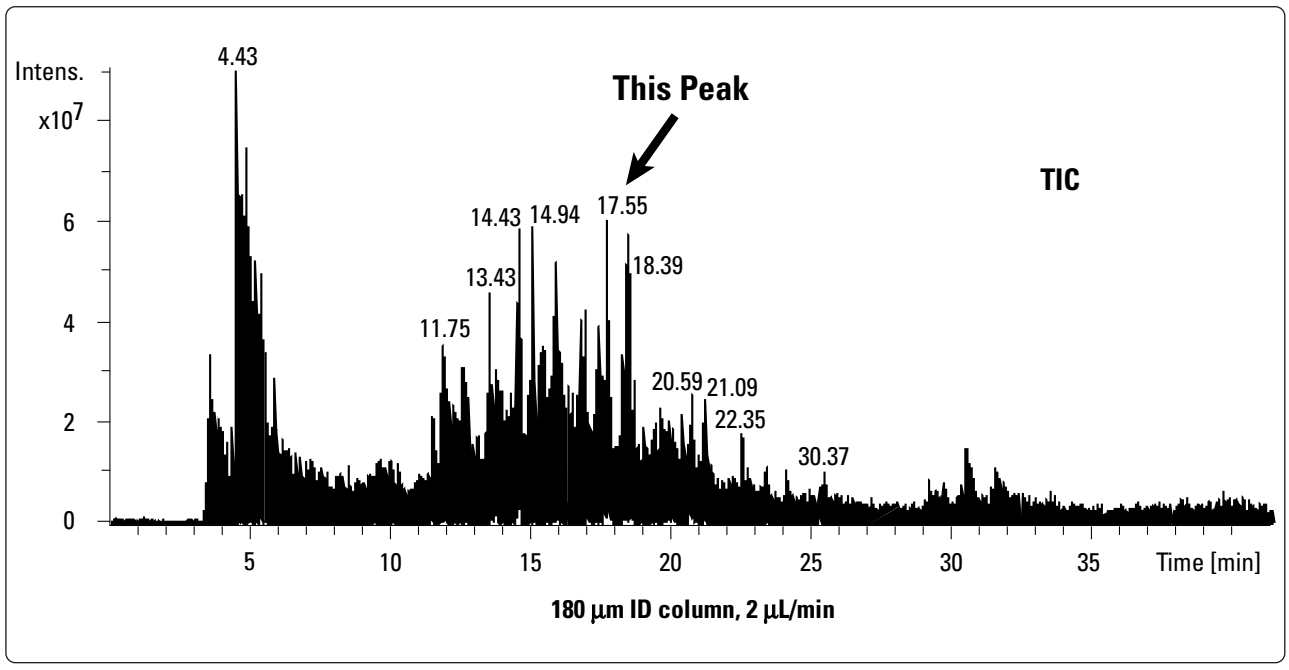


Figure 2. Total ion current (TIC) chromatogram generated from data-dependent analysis of a mixture of protein digests

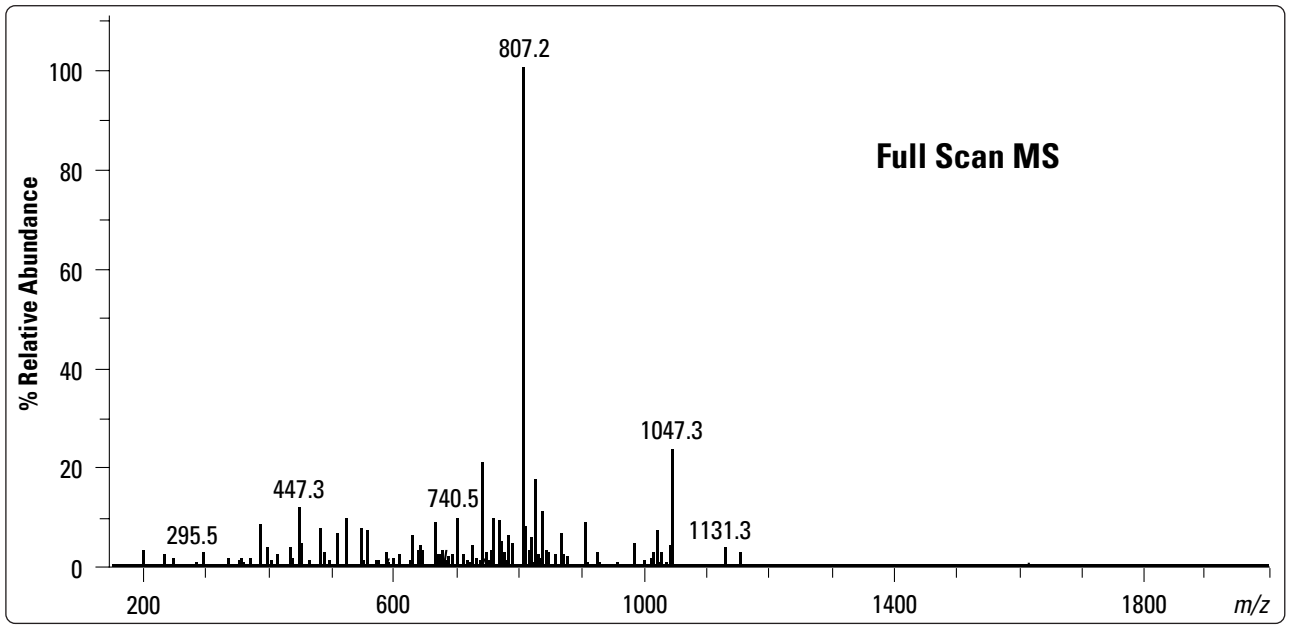


Figure 3. Full scan mass spectrum of peak at 17.55 min acquired from data-dependent analysis of a mixture of protein digests

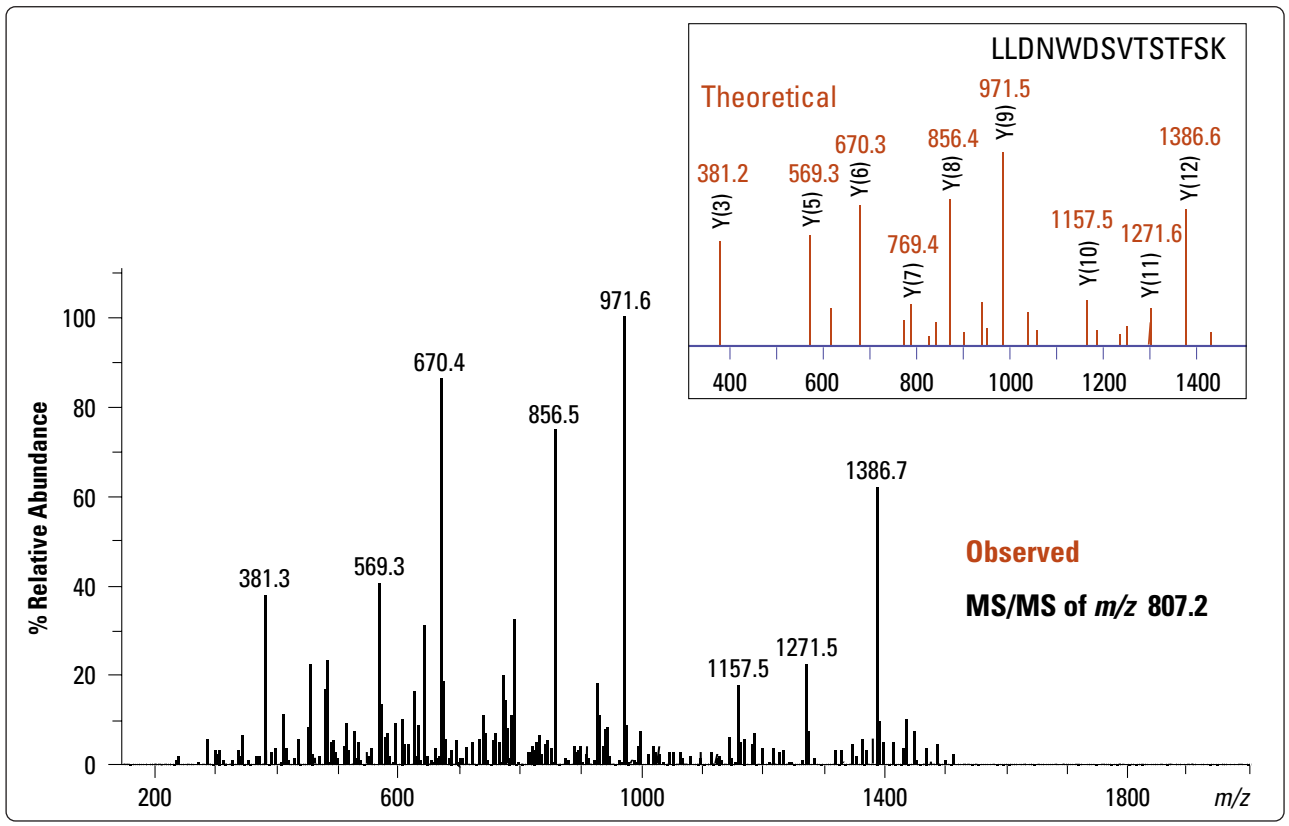
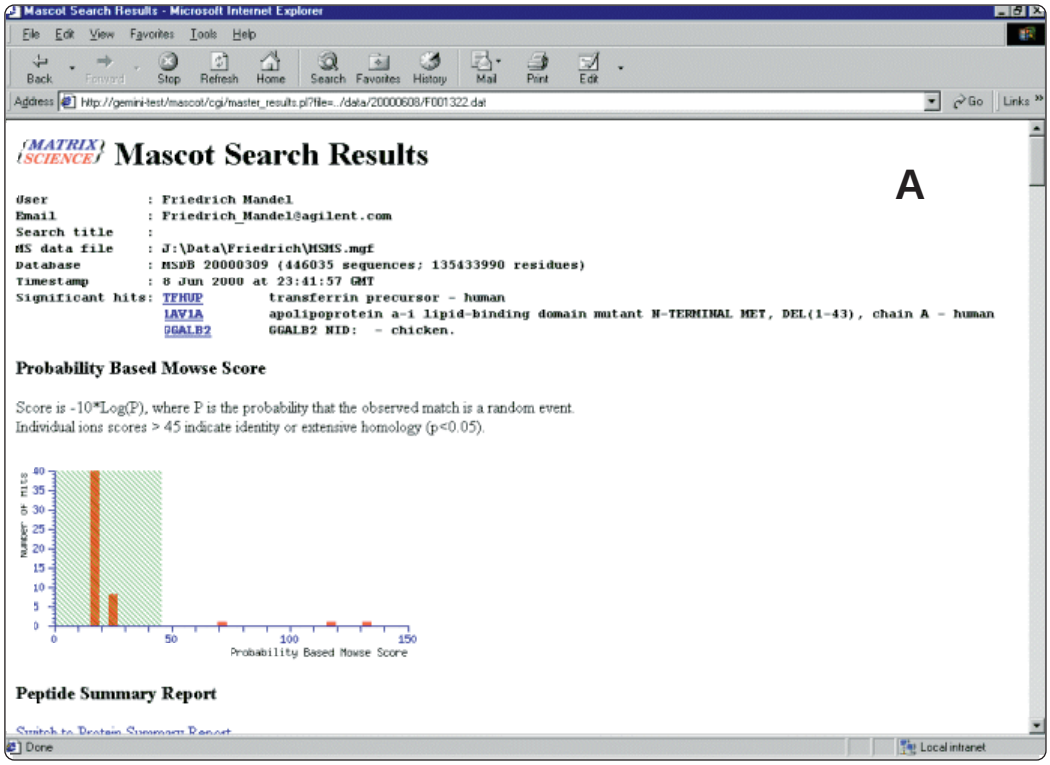


Figure 4. Full scan MS/MS spectrum (MS/MS of *m/z* 807), a tryptic peptide from human apolipoprotein acquired from data-dependent analysis, and theoretically predicted MS/MS spectrum (inset) obtained from the MASCOT database search



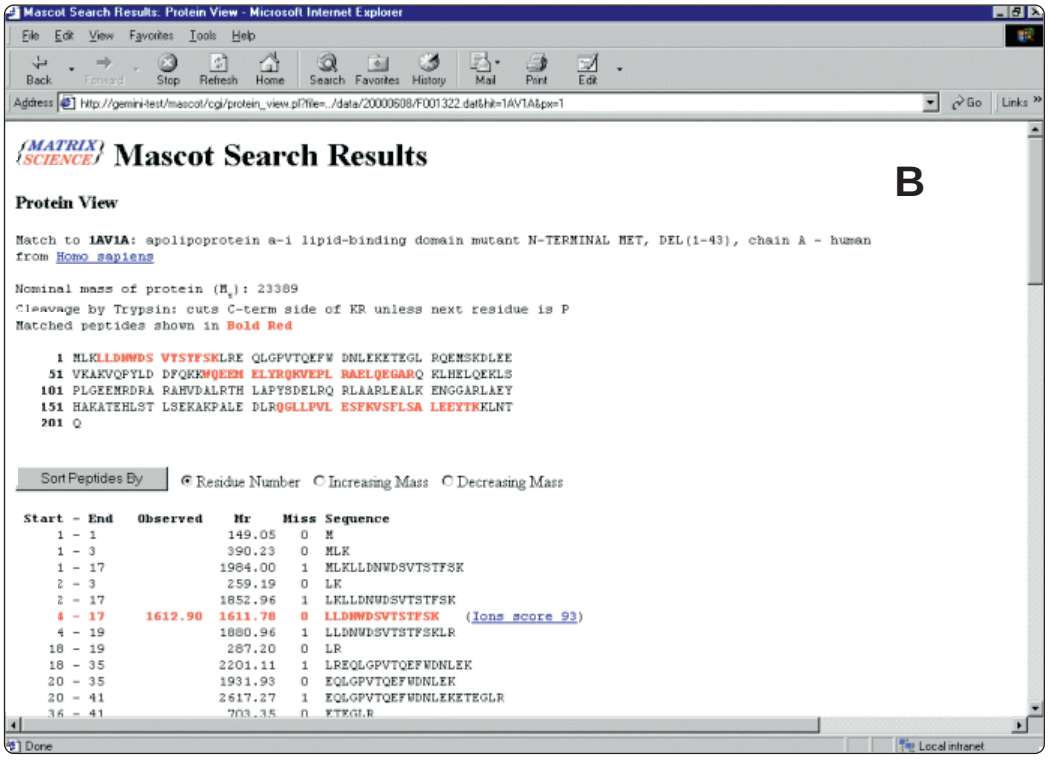


Figure 5. Hit list from results of the MASCOT database search (A) and sequence coverage map (B) showing peptides from human apolipoprotein that were found by the MASCOT search program

Conclusions

By employing data-dependent acquisition of tandem mass spectra, the duty cycle of the instrument is improved and the sampling rate efficiency is increased, thereby providing greater sequence coverage than can be obtained by manual selection of ions for MS/MS. The software on the LC/MSD Trap provides the ability to trigger on a user-specified number of ions in a single MS scan with simultaneous isotopic exclusion, which minimizes redundancy and allows a maximum number of unique peptide ions to be selected for CID. Employing these techniques permits the generation of multiple CID spectra on multiple coeluting peptides within a single chromatographic peak and helps further the goal of more comprehensive protein identification.

Reference

1. Tong, W.; Link, A.; Eng, J. K.; Yates, J. R. *Anal Chem*, **1999**, *71*, p. 2270–8.

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