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Capillary LC-MS/MS Identification of Phosphorylated Peptides from Data-Dependent Neutral-Loss Scans on the Finnigan LTQ Linear Ion Trap Mass Spectrometer

Key Words

- Phosphorylation
- Neutral Loss
- Data Dependent™
- MS^3

Chromatography and Mass Spectrometry Application Note

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Introduction

Phosphorylation is recognized as one of the most important post-translational modifications of proteins, and is associated with many proteins that have a regulatory function in cells. Phosphoproteins are generally found in low quantities within cells. This low abundance, coupled with the higher acidity of phosphopeptides, increases the complexity of their analysis by mass spectrometry in positive ESI mode. In tandem mass spectrometry, phosphopeptide precursor ions typically exhibit a prominent neutral loss of a phosphate group (98 Da) during fragmentation. However, identification of the phosphoprotein and determination of the exact site of phosphorylation is often limited by inadequate peptide fragmentation and diagnostic sequence ion information. To overcome the lack of peptide fragmentation information typical in phosphopeptide analysis, a new Data Dependent experiment was created to selectively trigger MS^3 scans on only the MS/MS fragment ions for which a specific, prominent neutral loss ion was detected. This new instrumental method was designed, in part, to take advantage of the superior sensitivity afforded by the design of the new linear ion trap mass spectrometer, the Finnigan™ LTQ.™ The increased ion capacity and improved trapping efficiency of the linear ion trap significantly enhances the quality of the MS^2 data, which, in turn, improves the quality and abundance of ions for the MS^3 data that is key to unambiguous identification of phosphorylated proteins.

Goal

- Demonstrate functionality of the data dependent neutral loss algorithm for detection of phosphorylated peptides
- Demonstrate exceptional sensitivity of the new Finnigan LTQ linear ion trap mass spectrometer

Experimental

Chromatographic Conditions

HPLC system:	Finnigan Surveyor™ MS pump with a flow splitter
Column:	150 µm × 10 cm column packed with 5 µm C18 stationary phase
Injection volume:	2 µL
Flow rate:	800 nL/min post spilt
Mobile phase	A: Water containing 0.1% formic acid B: Acetonitrile containing 0.1 % formic acid
Gradient:	2–60% B in 30 min, 60–80% B in 3 min, and at 80% B for 5 min

Mass Spectrometeric Conditions

Mass spectrometer:	Finnigan LTQ linear ion trap mass spectrometer
Ion source:	NanoSpray Ion Source
Ion transfer tube temperature:	200 °C

Standards: Alpha and beta caseins (1 mg/mL in 6 M guanidine hydrochloride) were reduced (DTT), alkylated (IAA) and buffer exchanged with 100 mM ammonium bicarbonate and subsequently enzymatically digested. Appropriate dilutions of the casein digests were separated by capillary liquid chromatography.

Results and Discussions

The flow chart of scan events for the Neutral Loss Data Dependent experiment is shown in Figure 1. An MS survey scan is initially performed, followed by three MS/MS scan events. If the MS/MS scan event detects a neutral loss ion and this neutral ion intensity is among the top 3 intensities (or top 5, depending on the user's setting), it will trigger the MS/MS/MS scan event. If a neutral loss is not detected, it will repeat with another MS scan and continue the process.

The setup of the acquisition parameters for data dependent scan events is shown in Figure 2. In the data

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dependent settings, the detection of phosphorylated peptides with the neutral loss of 98, 49, or 32.7 (corresponding to +1, +2, and +3 charge states) triggers scan event 3 (MS/MS/MS) to fragment ions exhibiting the neutral loss only if their intensities were within top three.

Figure 3 shows the base peak chromatogram generated by capillary LC/MS/MS analysis of the casein digest using the Finnigan LTQ linear ion trap mass spectrometer. This data was generated using data dependent acquisition parameters to detect neutral loss ions for 10 fmol casein

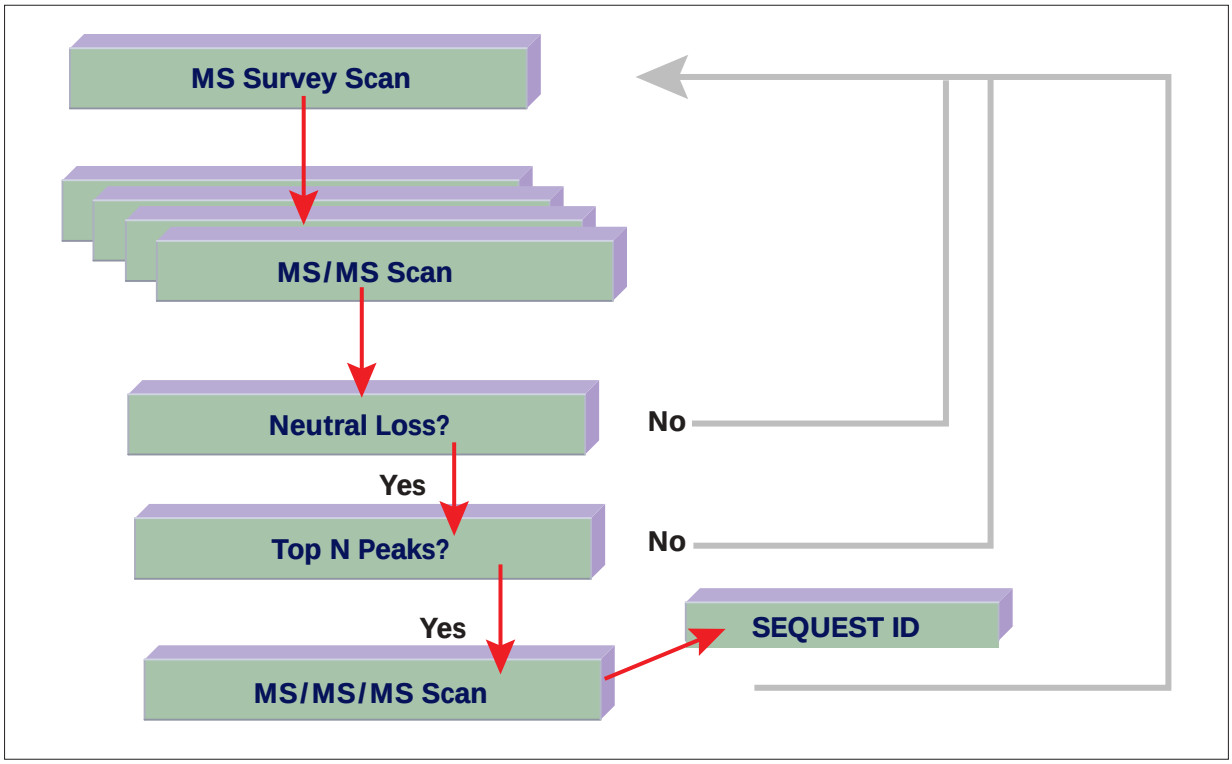

Figure 1. Automated phosphorylation site analysis using Data Dependent MS^3 on the Finnigan LTQ.

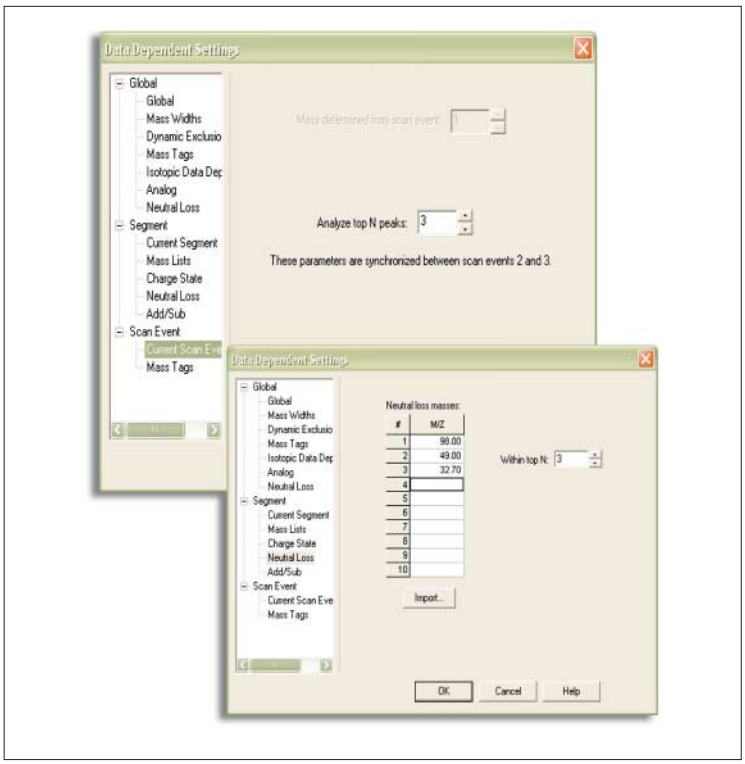

Figure 2. Data Dependent acquisition parameters for phosphorylated peptides.

injected on-column. The full-scan survey detects a peptide at m/z of 831.6 which upon MS/MS shows a neutral loss of 49 resulting in a peak at m/z of 782.1. The Data Dependent neutral loss algorithm then triggers an MS/MS/MS event on the ion at m/z 782.1 in the subsequent scan. Figure 4 shows MS/MS and MS^3 spectra of three phosphorylated peptides found in the casein digest. Figure 5 shows the TurboSEQUEST™ search parameters set up in BioWorks 3.1 and the results for the phosphorylated peptide at m/z of 831.6. The neutral loss ion

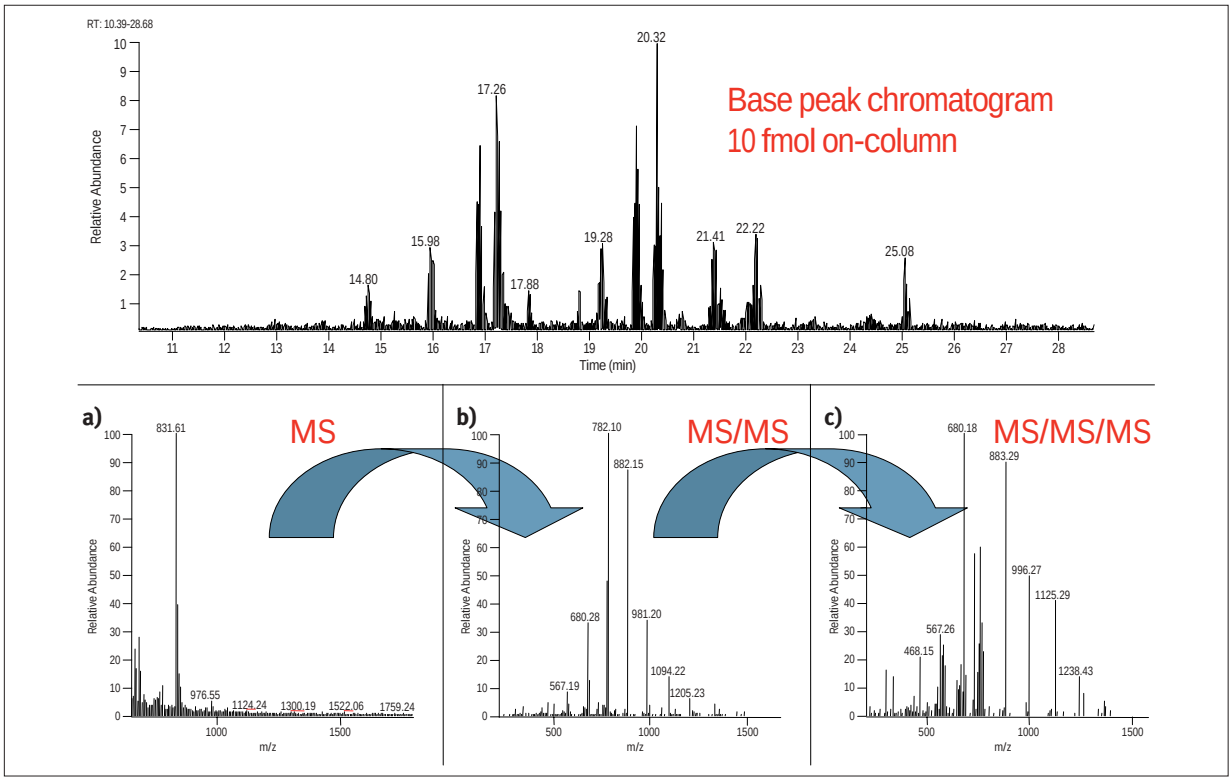

Figure 3. LC-MS/MS analysis with Data Dependent neutral loss MS^3 scanning of casein digest using the Finnigan LTQ. a) T: + c ESI Full ms [500.00-1800.00]; b) T: + c ESI Full ms2 831.61@22.00 [215.00-1675.00]; c) T: + c ESI Full ms3 831.61@22.00 782.10@22.00 [205.00-1575.00].

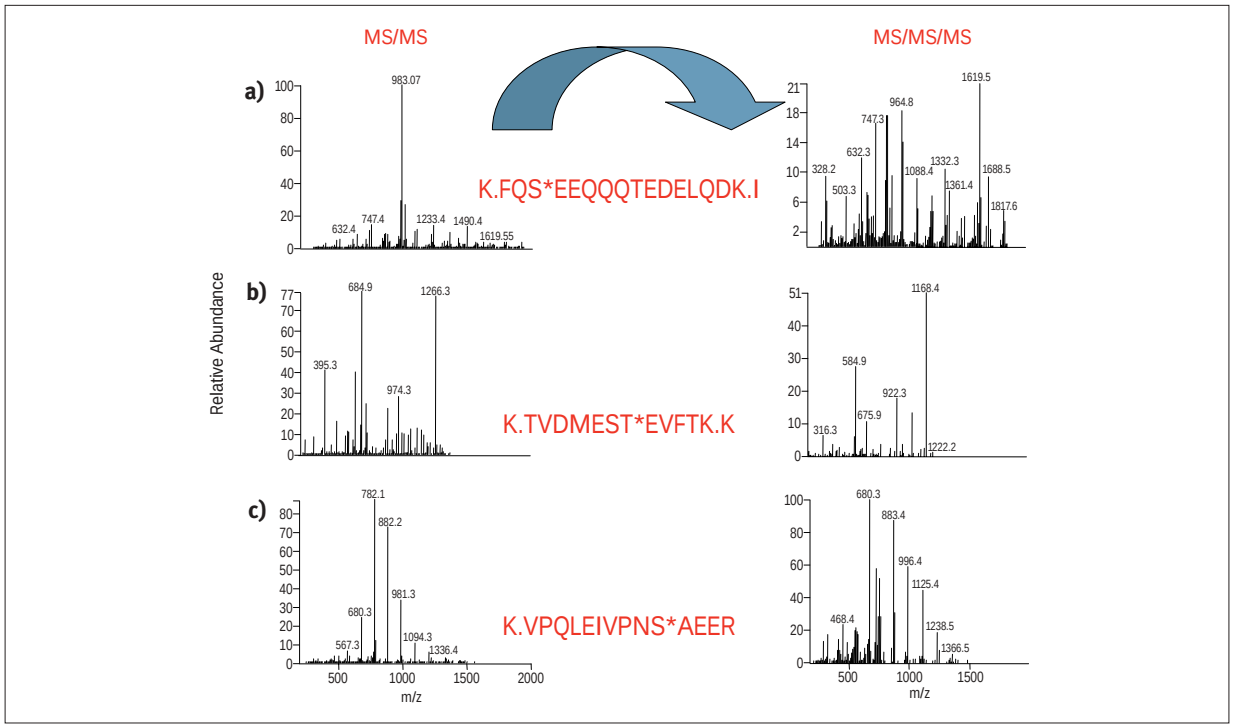

Figure 4. MS/MS and MS^3 spectra of three phosphorylated peptides found in the casein digest.
a) F: + c ESI Full ms2 1032.24@22.00 [270.00-2000.00], F: + c ESI Full ms3 1032.24@22.00 983.07@22.00 [260.00-1980.00];
b) F: + c ESI Full ms2 734.70@22.00 [190.00-1480.00], F: + c ESI Full ms3 734.47@22.00 684.9@22.00 [175.00-1380.00];
c) F: + c ESI Full ms2 831.46@22.00 [215.00-1675.00], F: + c ESI Full ms3 831.42@22.00 782.1@22.00 [205.00-1575.00].

(at m/z 782.1) has lost an H_3PO_4 moiety, but is still an intact peptide. Further fragmentation of this ion (in MS^3) yields a classical peptide fragmentation pattern, with a y- and b-ion series searchable by TurboSEQUEST within BioWorks. As shown in Figure 5, the match of y- and b-ions are labeled in blue and red, respectively. The peptide sequence and the position of phosphorylation (e.g. at Ser) is automatically identified as shown in the left column.

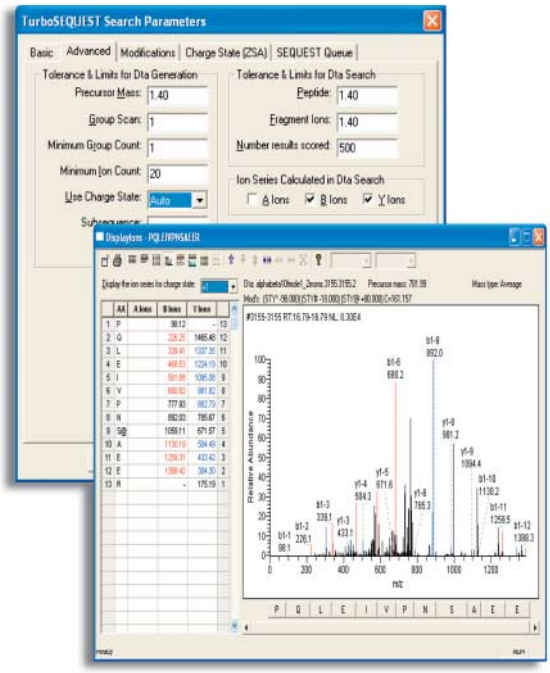

Figure 5. TurboSEQUEST search on MS^3 spectra for peptide at m/z 831.6 using BioWorks.

Conclusions

The Finnigan LTQ linear ion trap mass spectrometer shows unparalleled sensitivity for the analysis of a digest of casein, which is evident by the detection of peptides at levels as low as 10 fmol of digest injected on-column. The automated Data Dependent neutral loss scanning algorithm outlined here is of great utility in identifying sites of phosphorylation on peptides whose fragmentation using only MS/MS would not normally be sufficient for identification. The MS^3 data was searched using TurboSEQUEST within BioWorks and was successful in identifying the exact site of phosphorylation on the peptide.

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