

Novel Dual-Pressure Linear Ion Trap Mass Spectrometer Offers Breakthrough Performance in Proteomics Experiments

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Key Words

- LTQ Velos
- Linear Ion Trap
- Proteomics
- Protein Identification
- Peptide Sequencing
- Sensitivity
- Proteome Discoverer

Introduction

Comprehensive definition of a proteome is a necessary foundation for subsequent investigations of proteome dynamics. Proteome characterization should be as efficient, reliable, and exhaustive as possible. Tandem mass spectrometry, particularly using linear ion traps and trap-based hybrid mass spectrometers, has become the technology of choice for peptide and protein identification. The preference for this instrumentation is attributable to robustness, ease of use, superior MS and especially MS/MS performance.

Undiscovered components within a proteome frequently include dynamically modified forms which exist at abundance levels undetectable by most instrumentation. Therefore, the development of more sophisticated instrumentation is essential for a more exhaustive characterization of complex proteomes.

This application note addresses the challenging analysis of a complex, multi-organ peptide digest of *Caenorhabditis elegans* (*C. elegans*) to assess the performance of an innovative new dual-pressure linear ion trap mass spectrometer. Performance of the new instrument is benchmarked against the previous state-of-the-art linear ion trap and also against a well-known quadrupole time-of-flight (Q-TOF) mass spectrometer.

Instrument Innovations

The instrument evaluated in this note is the Thermo Scientific LTQ Velos ion trap mass spectrometer. It includes a novel dual-pressure linear ion trap and a high-pressure stacked ring ion guide (S-lens), as shown in Figure 1.

The radio frequency (RF) S-lens significantly increases transmission of ions into the mass analyzer. This reduces the time required to inject the desired ion population into the linear ion trap.

The first cell of the dual-pressure trap is held at a higher pressure ($\sim 5 \times 10^{-3}$ Torr) than in previous linear ion trap systems to improve efficiency of trapping, isolating, and fragmenting ions of interest. The increased isolation efficiency produces a 4-fold decrease in the time required for precursor ion isolation. The geometry of the LTQ Velos™ ion trap allows better selectivity for low-abundance precursors in the presence of abundant interfering ions, improving dynamic range for MS/MS analysis. The higher pressure also reduces the required CID activation time by 67%, while maintaining the same efficiency of fragmentation. The second cell, held at a lower pressure ($\sim 4 \times 10^{-4}$ Torr), allows for a faster mass analysis scan with increased resolution.

In conjunction with the new dual-pressure linear ion

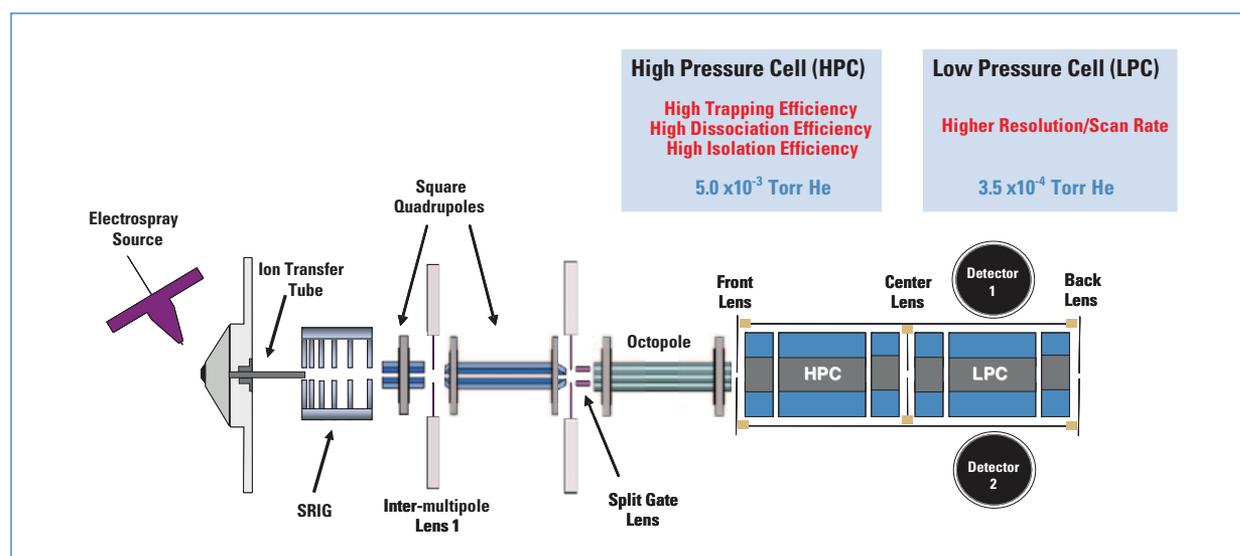


Figure 1: Schematic representation of the LTQ Velos mass spectrometer, containing a stacked-ring ion guide (S-lens) and a dual-pressure ion trap with differential pressure regulation.

trap, a novel method for controlling the ion population in the trap produces a dramatic increase in the practical scan rate achievable in a typical data-dependent tandem MS experiment. This function, termed “predictive AGC”, eliminates pre-scans conducted prior to each tandem MS scan. It predicts the injection time required for the specified ion population based on the ion flux and the relative intensity of parent ions measured in the preceding full MS scan.

Experimental

Sample Preparation

The soluble fraction of mixed-stage *C. elegans* homogenates were diluted in a buffer of ammonium bicarbonate (pH ~7.8) and 0.1% concentration of RapiGest surfactant, followed by reduction of disulfide bonds with dithiothreitol (DTT) at 100 °C, and alkylation of cysteines with iodoacetamide. The sample was then enzymatically digested for 4 hours with a K/R specific protease. The digest was acidified and stored at -80 °C. Samples were diluted in 0.1% formic acid prior to analysis by reverse-phase HPLC-MS/MS.

LC-MS/MS Analysis

For comparative purposes and to benchmark performance, analyses were run on a Thermo Scientific LTQ XL linear ion trap mass spectrometer and on an Agilent 6520 Quadrupole Time-of-Flight mass spectrometer (Q-TOF), as well as on the new LTQ Velos dual-pressure linear ion trap mass spectrometer. A proteolytic digest of *C. elegans* was separated by reverse phase chromatography for each LC-MS/MS run. Details of the chromatographic conditions are listed in Table 1. Dilutions of the injection amount were also investigated to assess the effects of decreasing sample quantity.

Data-dependent tandem MS acquisition methods were used for all experiments. A minimum of three replicate runs were acquired at each gradient on each instrument.

For data-dependent acquisition on the linear ion traps, the method was set to analyze the top ten most intense ions. Exclusion conditions were optimized according to expected chromatographic peak width, which varied as a function of the reverse-phase gradient length. Due to the greater ion transmission of the ion source on the LTQ

Velos mass spectrometer, a lower maximum injection time (IT) was selected for full MS scans (10 msec). The same maximum IT was allowed for MS/MS scans to capture more ions and potentially identify more low-abundance peptides (where precursor ion abundance is low and AGC injection times reach their maximum). A correspondingly higher full MS signal threshold was used for data-dependent selection of precursors on the LTQ Velos because of the expected stronger signal intensities. The ion transfer tube temperature on the LTQ Velos was set to 250 °C, higher than that used on the LTQ XL to compensate for a shorter capillary path length. The activation time for resonance CID fragmentation was reduced from 30 msec to 10 msec to take advantage of the increased fragmentation efficiency in the higher-pressure cell. The LTQ Velos dual-pressure ion trap offers both increased resolution and increased scan rate for normal scan acquisition, making rejection of singly-charged precursors possible on the fly. This feature was utilized during data acquisition. A detailed summary of the acquisition parameters is given in Table 2.

The acquisition parameters used for the 6520 Q-TOF are detailed in Table 3. Both 3 and 6 Hertz (Hz) scan rates were utilized to maximize peptide identification, producing effective scan rates of 2.5 Hz or 5.1 Hz, respectively. Conditions used were recommended by Agilent Technical Support and were similar to those recommended in literature¹.

Database Search

Data were searched using Thermo Scientific Proteome Discoverer 1.0 software with the MASCOT[™] v2.1 search engine (Matrix Sciences Ltd., London, UK) to allow a comparison between ion trap and Q-TOF data. The conditions used for the database search are listed in Table 4. The reverse database search option was enabled in MASCOT, and all data were filtered to satisfy a false discovery rate (FDR) of 1% or better. Data from the 6520 Q-TOF were converted into mzdata format using Agilent MassHunter[™] software prior to submission to Proteome Discoverer. Proteome Discoverer software automatically calculates the expectation score (or peptide score, if chosen) required to filter the data to achieve any specified FDR, and applies this filter to the dataset.

Instrument	Thermo Scientific Ion Traps	Q-TOF
HPLC	Thermo Scientific Surveyor MS Pump	Agilent 1200 Series HPLC
Ionization source	Nanospray I	Agilent HPLC-Chip/MS System
Analytical Column	Michrom Magic C18AQ packed tip 150 mm x 75 µm I.D. x 15 µm tip 5 µm particle, 250 Å pore size	Agilent Protein ID Chip #3 (up to 4 µg capacity) SB-ZORBAX C18 150 mm x 75 µm I.D. 5 µm particle, 300 Å pore size
Flow Rate	~ 300 nL/min at 50% organic	300 nL/min
Gradient	2-25% acetonitrile in 0.1% formic acid (either 60 minutes or 180 minutes)	5-30% acetonitrile in 0.1% formic acid (either 60 minutes or 180 minutes)

Table 1. Conditions used for the one dimensional reverse phase separation of peptide digests.

Mass Spectrometer	LTQ Velos	(LTQ XL)
Capillary Temperature	250 °C	(200 °C)
Source Parameters	S-Lens 40%	(Tube Lens 100 V)
AGC Targets Full	MS: 3e4 MSn: 1e4	
Maximum Injection Times	Full MS: 10 msec (Full MS: 50 msec) MSn: 100 msec	
Full MS Mass Range	400-1200 <i>m/z</i>	
Isolation Window	2 <i>m/z</i>	
Activation Type	CID	
Normalized Collision Energy	30%	
Activation Time for CID	10 msec (30 msec)	
Default Charge State	3	
Threshold for DDA Selection	1000 (10,000)	
Method	Top 10 most intense DDA	
Number of Microscans	1	
Scan Rate	Normal	
Rejection of Singly Charged Precursors	Yes (No)	
Dynamic Exclusion Enabled	Exclusion duration: 15 sec (60 min), 30 sec (180 min) List size: 500 Repeat count: 1 Mass width: low-1.0, high 1.5	

Table 2: Acquisition parameters used for LTQ Velos and LTQ XL mass spectrometers.

As such, the high-confidence peptide filter was selected to achieve a desired FDR of 1%. The number of unique peptides and corresponding proteins identified in the database search was compared.

Mass Spectrometer	Agilent 6520 Q-TOF
ESI Condition	HPLC-Chip NSI Interface
Chip Gas Temperature	300 °C
Drying Gas Temperature	4 L/min
MS Acquisition Rate	3, 6, or 8 Hz
MS/MS Acquisition Rate	3 or 6 Hz
Full MS Mass Range	200-2000 <i>m/z</i>
Collision Energy	Slope: 3 V Offset: 2 V
Method	Top 6 DDA Precursors sorted by abundance
Charge State Selection	Rejection of singly-charged
Dynamic Exclusion Enabled	Duration: 30 sec (60 min), 60 sec (180 min) Repeat count: 1

Table 3: Acquisition parameters used for the Q-TOF mass spectrometer¹.

Results and Discussion

LTQ Velos identifies a greater number of unique peptides than both LTQ XL and Q-TOF

Compared to the LTQ XL ion trap and the Q-TOF, the LTQ Velos dual-pressure ion trap mass spectrometer identified a significantly larger number of unique peptides from the proteolytic digest of *C. elegans*. An increased number of proteins were also identified. Figure 2 depicts

Software Platform	Proteome Discoverer
Search Engine	MASCOT v 2.1
Database	NCBI, April 2008
Maximum Number of Missed Cleavages	1
Precursor Mass Tolerance	Ion trap data: 1.4 Da Quadrupole-TOF data: 40 ppm
Fragment Mass Tolerance	Ion trap data: 0.8 Da Quadrupole TOF data: 100 mmu
Protein Mass	Unrestricted
Reverse Database Search	Enabled
Instrument Type	Ion trap data: ESI-TRAP Q-TOF data: ESI-Q-TOF
Modifications	Fixed: carbamidomethyl cysteines (C)
Scoring	MUDDPIT scoring Protein cut-off: 20 Peptide cut-off: 10
DTA Generation Conditions	Total intensity threshold: 100 Minimum ion count: 10 S/N threshold: 2 Precursor mass range: 700-5000 <i>m/z</i> (ion traps), 700-7000 <i>m/z</i> (Q-TOF)

Table 4: Database search conditions used for analysis of data.

the base peak chromatograms from analysis of a 60-minute gradient of a reverse-phase separation on the LTQ Velos mass spectrometer with split flow from a Surveyor MS pump versus that obtained from the Q-TOF with a 1200 HPLC-Chip. For a typical 60-minute separation, the LTQ Velos mass spectrometer identified 67% \pm 3.3 more unique peptides at 1% FDR than did the LTQ XL mass spectrometer and 165.7% \pm 3.1 more unique peptides than did the Q-TOF operated at 3 Hz. As a result, the number of proteins identified by the LTQ Velos ion trap was 240% \pm 8 more than the number of proteins identified by the Q-TOF for equivalent experiments (Figure 3). All numbers are an average of three replicate runs with a relative standard deviation of less than 10% in the number of proteins or unique peptides identified for each equivalent experiment. The average percent increase in ID is achieved in comparison for each run, and is not an increase considering the consensus of all replicates.

A significant overlap existed in the sets of proteins identified between instruments, as illustrated in Figure 4. For a separation of 60 minutes, the LTQ Velos dual-pressure ion trap identified >88% of the proteins identified by the LTQ XL ion trap and >90% of the proteins identified by the Q-TOF, plus 570 proteins (53%) not detected by the other instruments. Therefore, both the LTQ XL and the Q-TOF identified a subset of the proteins identified by the LTQ Velos, rather than any significant augmentation to the complement of proteins. Within replicate runs on the LTQ Velos, overlap of the identified proteins was greater than 70%, and 85% of proteins were identified in two out of three runs on average, indicating high reproducibility.

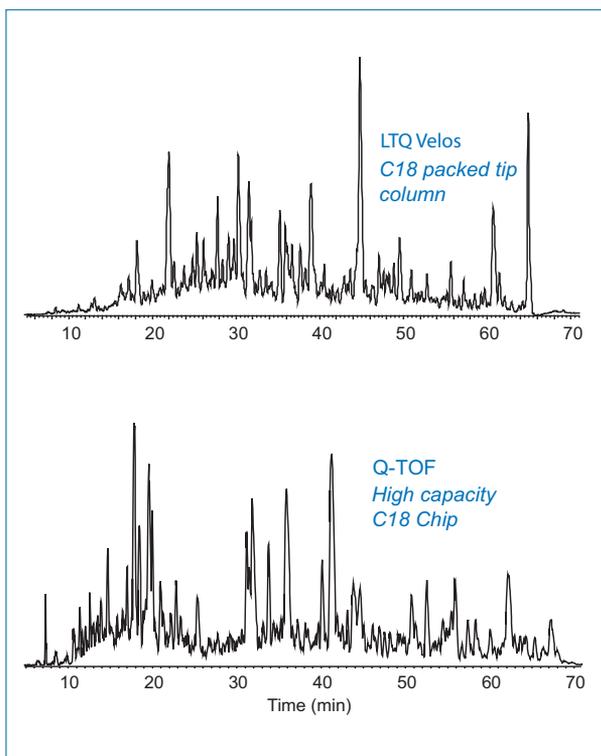


Figure 2: Base peak chromatogram for a 60-minute reverse-phase separation of one microgram of a tryptic digest of *C. elegans*.

LTQ Velos offers greater sensitivity

Sensitivity in a complex mixture requires not only raw sensitivity (greater signal), but also selectivity, efficiency of isolation, and speed of acquisition to adequately interrogate complex systems, as precursors of lower abundance exist within an environment of high dynamic range. The performance enhancements of the LTQ Velos mass spectrometer offer increased sensitivity of detection for crucial low-abundance proteins *within* a highly complex environment.

The protein identification results were annotated by

Proteome Discoverer software, using the included Inforsense Virtual Machine to retrieve relevant biological information from publicly available protein databases (such as Swissprot or NCBI), including gene ontology references for function or subcellular location. The identified proteins from a single analytical run with a 180-minute gradient were classified. The LTQ Velos dual-pressure ion trap demonstrated a 133% increase in the detection of signal transduction proteins compared to the LTQ XL ion trap, and also detected more than twice the number of kinases and phosphatases (Table 5). Additionally, only the LTQ Velos dual-pressure ion trap identified the low-abundance transcription factors ntl-3, involved in growth regulation, and F4369, involved in locomotion.

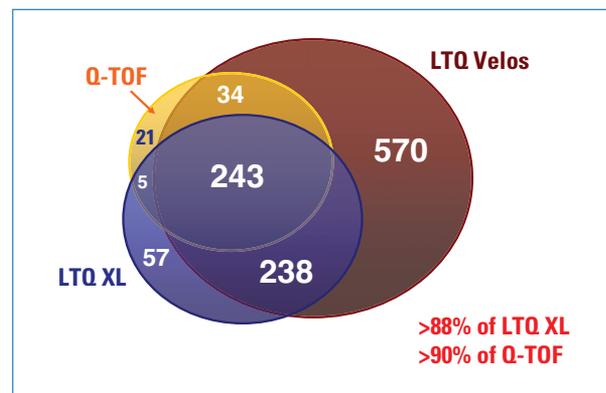


Figure 4: Venn diagram illustrating the overlap in proteins identified between instruments for a 60-minute reverse-phase separation of 1 microgram of *C. elegans* (Data reported at 1% FDR).

	LTQ XL	LTQ Velos	% Increase
Signal Transduction Proteins	21	49	133%
Kinases	11	24	118%
Phosphatases	15	31	107%

Table 5. Increase in identification of signal transduction proteins achieved with the LTQ Velos ion trap versus the LTQ XL ion trap.

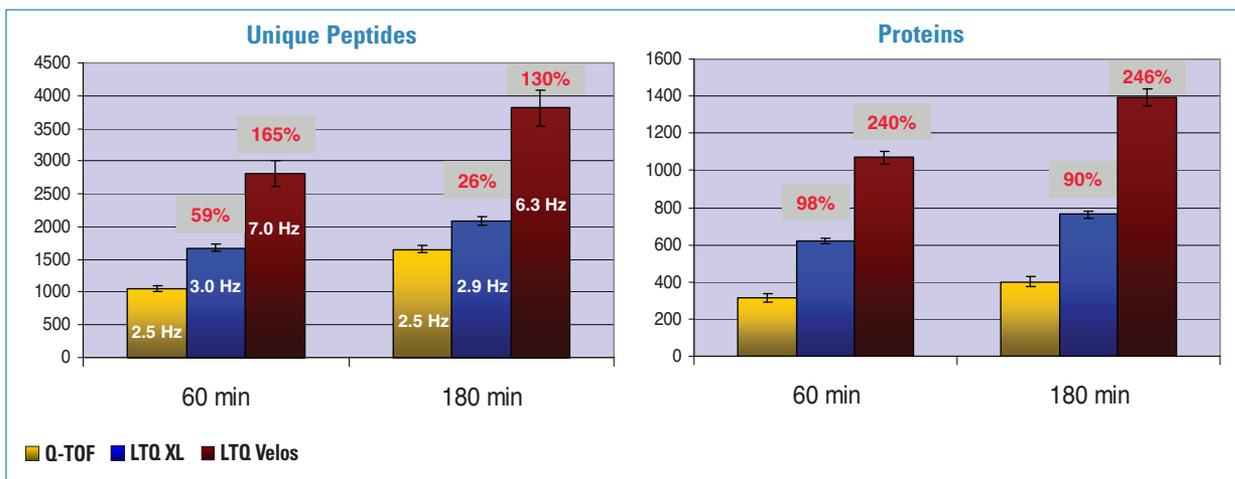


Figure 3: Increase in identification of unique peptides and proteins comparing LTQ Velos dual-pressure ion trap to the LTQ XL ion trap and the Q-TOF operated at 3 Hz for a reverse-phase separation of a 1-µg tryptic digest of *C. elegans*. Results from two gradients are shown. Practical acquisition rates are displayed for each instrument.

To further assess the ability of the LTQ Velos dual-pressure ion trap to analyze compounds of lower intensity, such as proteins or peptides that exist naturally at lower abundance, the sample was diluted 50-fold and analyzed on both linear ion trap instruments. The number of unique peptides identified by the LTQ Velos was 152% greater than that achieved by the LTQ XL, the former gold standard for sensitive MS/MS identification (Figure 5). The difference can be directly attributed to the greater ion transmission of the LTQ Velos ion source. For weak precursors, a “brighter” ion beam delivers more ions to the ion trap during the limited maximum injection time in the same amount of time, thus increasing MS/MS sensitivity. The greater scan rate of the LTQ Velos boosts this benefit, as it allows more frequent sampling of weak precursors.

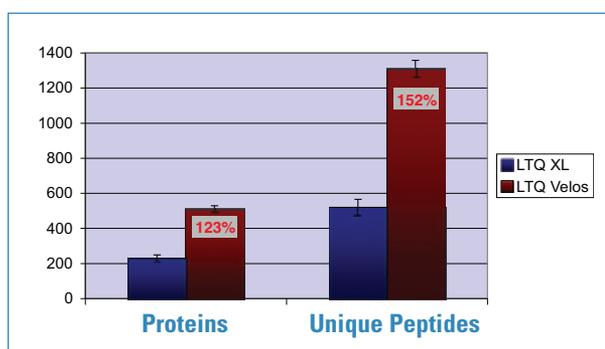


Figure 5: Increase in identification achieved for samples of lower abundance (here a 20-ng injection) for LTQ Velos dual-pressure ion trap versus the LTQ XL ion trap (60 min gradient)

LTQ Velos dual-pressure ion trap improves experimental throughput

A faster scan rate not only improves duty cycle but also allows for the use of shorter chromatographic gradients to increase experimental throughput. The LTQ Velos dual-pressure ion trap identified more unique peptides and proteins in a 60-minute gradient than the LTQ XL ion trap identified in a 180-minute gradient of the same complex mixture (Figure 3). A significant increase in throughput for analysis of simple mixtures with high dynamic range (i.e. gel bands) can, therefore, be anticipated. Experiments requiring multiple runs/replicates, such as method development experiments or experiments involving biological and/or technical replicates, would benefit from increased throughput. Quantitative experiments would also benefit from the increased scan rate as more scans are acquired across narrow chromatographic peaks. This makes the LTQ Velos ideally suited for ultra-high pressure chromatographic separations and short run times.

Why did the Q-TOF instrument identify fewer proteins?

Detailed analysis of the data acquired from the Q-TOF instrument indicated that the full MS scan is of comparable quality to that obtained with the LTQ Velos

dual-pressure ion trap (Figure 6). The signal-to-noise (S/N) ratio and number of features in the spectra were similar, though the Q-TOF showed better mass accuracy (9 ppm RMS for identified peptides at 1% FDR). Consequently, neither full-scan sensitivity and dynamic range nor mass accuracy was the limiting factor in the outcome of the peptide identification experiments. Scan rate is possibly a limiting factor in a typical length analysis (i.e. 60 min); indeed with longer separations where scan rate is less influential, an increase in peptide and protein IDs was observed (Figure 3). For a detailed discussion of the contribution of scan rate, see “Effect of Q-TOF scan rate on sensitivity” on the next page.

The average Mascot score (1113.4) of the top 20 proteins identified by the LTQ Velos ion trap was 119% higher than the average Mascot score (506.7) of the top 20 proteins identified by the Q-TOF operated at 3 Hz

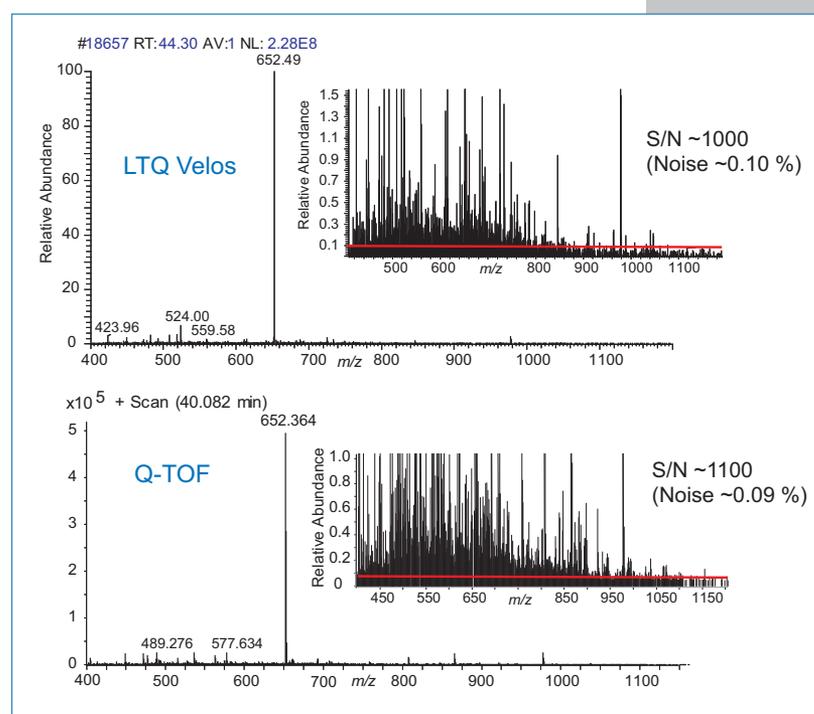


Figure 6: Comparison of the full-scan dynamic range for the Q-TOF and the LTQ Velos ion trap from the base peak MS scan of a reverse-phase separation of 1 microgram of a *C. elegans* tryptic digest.

scan rate. The lower quality of Q-TOF MS/MS scans appeared to contribute to a significantly lower number of peptides identified. Better mass accuracy in the Q-TOF MS and MS/MS scans did not compensate for lower-quality MS/MS scans, i.e. those with fewer sequence ions than equivalent spectra obtained by the linear ion trap. The quality of ion trap MS/MS scans was high enough that, even when data was searched with nominal mass accuracy (1.4 Da for MS and 0.8 Da for MS/MS), results from either linear ion trap system were superior to those of the Q-TOF instrument.

Effect of Q-TOF scan rate on sensitivity

Increasing the scan rate on the Q-TOF from 3 Hz to 6 Hz resulted in a decrease in the number of identified peptides (Figure 7). This is because the quality of both MS and especially MS/MS spectra decreases with increased scan rate on a beam-type mass spectrometer. Increasing the scan rate indiscriminately shortens the ion beam time, and decreases the signal-to-noise of the spectrum, as fewer ions are detected and averaged. Figure 8 demonstrates that increasing the Q-TOF MS scan rate from 3 Hz to 8 Hz leads to a 66% loss of Q-TOF signal and a noticeable decrease in the number of detectable features in the MS spectrum.

Increasing the scan rate for MS/MS acquisition produced a corresponding decrease in the signal-to-noise quality of MS/MS scans and also in the Mascot expectation scores for identified peptides. For the spectra seen in Figure 9, an MS/MS scan was triggered near the apex of the chromatographic peak. The Mascot ion score (49) for the higher quality 3-Hz spectrum was still 53% lower than the ion score (103) obtained from the LTQ Velos ion trap for an injection amount of 80% less material than that analyzed on the Q-TOF. When the Q-TOF was operated with a 6-Hz scan rate, the number of identified peptides decreased by 40% for a gradient of 180 minutes for a one microgram injection (Figure 7). This is why, despite having available scan rates of up to 10 Hz, the manufacturer does not recommend running at rates higher than 3 Hz MS/MS for peptide identification¹.

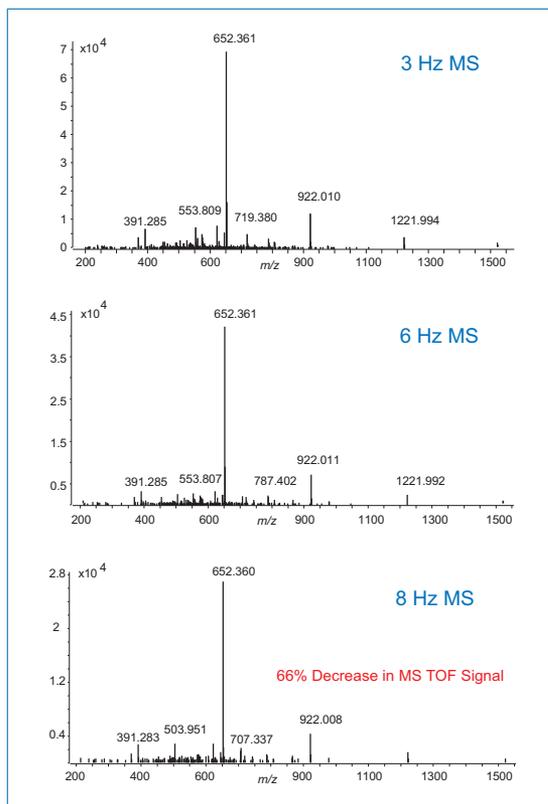


Figure 8: Comparison of the signal intensity for various MS scan rates on the Q-TOF instrument.

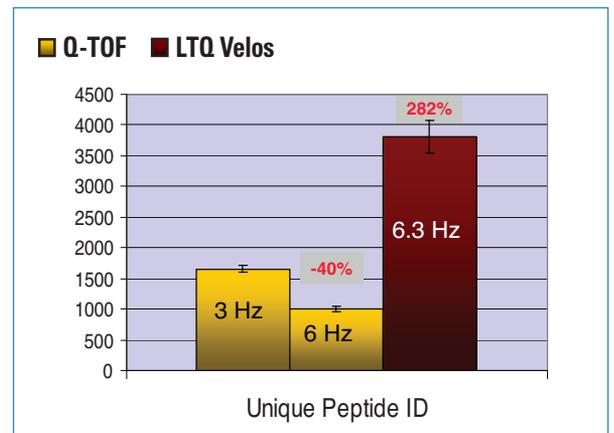


Figure 7: Comparison of the number of identified unique peptides at alternate scan rates (3 Hz and 6 Hz) on the Q-TOF versus the LTQ Velos ion trap for a 180-minute separation of one microgram of a tryptic digest of *C. elegans*.

With patented predictive automatic gain control (pAGC) of the ion population in the ion trap, the LTQ Velos instrument maintains an optimal number of ions available for MS/MS, irrespective of the intensity of the precursor. As a result, the LTQ Velos produced 282% more unique peptide IDs at an actual scan rate of ~6.3 Hz than did the Q-TOF operated at 6 Hz over a 180 min gradient (Figure 7).

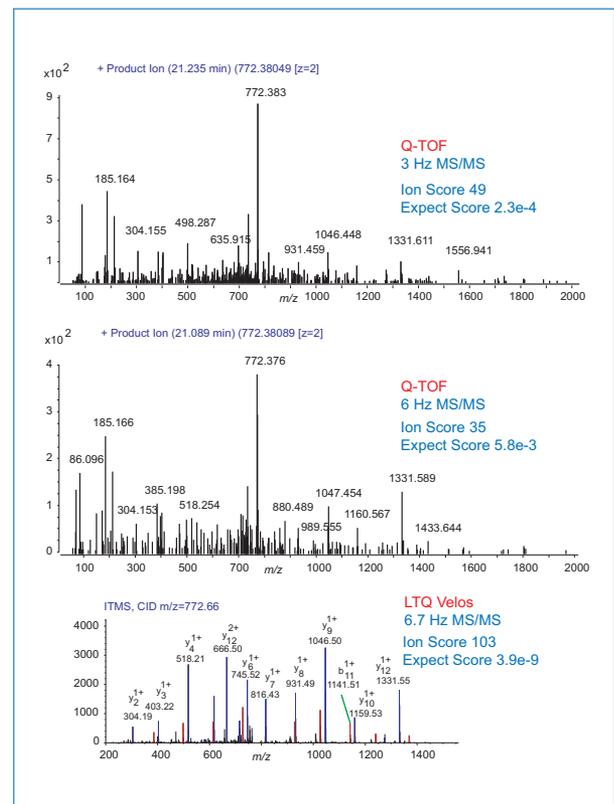


Figure 9: Comparison of MS/MS spectra of precursor, LVGLDDAQQVDVER, 2+, from the Q-TOF at two different MS/MS scan rates versus the spectrum for the same peptide precursor acquired with the LTQ Velos ion trap using 80% less sample.

Conclusions

The sensitive detection and identification of components within a complex proteomic sample is crucial for the characterization and understanding of proteome dynamics. The technological advancements of the LTQ Velos dual-pressure linear ion trap mass spectrometer, including increased speed of acquisition and sensitivity, have resulted in significant improvement in the identification of peptides and proteins, including an increase in identification of lower intensity precursors, when compared to existing state-of-the-art technologies.

- The LTQ Velos ion trap identified ~240% more proteins and ~130% more unique peptides from a highly complex sample than did a Q-TOF, where >90% of the proteins identified by the Q-TOF were also identified by the LTQ Velos.
- The LTQ Velos dual-pressure ion trap demonstrated higher sensitivity for samples at lower levels, with more than a 150% increase in the number of identified unique peptides for a low load of 20 ng, versus the LTQ XL ion trap.
- With the increase in the number of identified proteins, the LTQ Velos offered greater access to low-abundance proteins as shown by a 133% increase in the number of identified signal transduction proteins.
- The LTQ Velos dual-pressure ion trap offered an increase in experimental throughput, identifying more proteins and peptides than the LTQ XL ion trap in 1/3 the separation time for a complex sample.
- The better quality of the MS/MS scans in the linear ion traps produced higher Mascot scores for equivalent peptides compared to those of the Q-TOF instrument. Higher mass accuracy of analytical scans acquired with the Q-TOF was beneficial but not sufficient to produce superior peptide identification.
- Increasing the scan rate on the Q-TOF resulted in a decrease in signal and spectral quality for MS and MS/MS scans and a corresponding decrease in the number of identified peptides.

Literature References:

1. Horn, D.M., Miller, C.A., Miller, B.D. The Effect of MS/MS Fragment Ion Mass Accuracy on Peptide Identification in Shotgun Proteomics. *Application Note 5989-7820 EN*. www.agilent/chem/proteomics

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