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A Dual Pressure Linear Ion Trap Orbitrap Instrument with Very High Sequencing Speed*[S]

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Since its introduction a few years ago, the linear ion trap Orbitrap (LTQ Orbitrap) instrument has become a powerful tool in proteomics research. For high resolution mass spectrometry measurements ions are accumulated in the linear ion trap and passed on to the Orbitrap analyzer. Simultaneously with acquisition of this signal, the major peaks are isolated in turn, fragmented and recorded at high sensitivity in the linear ion trap, combining the strengths of both mass analyzer technologies. Here we describe a next generation LTQ Orbitrap system termed Velos, with significantly increased sensitivity and scan speed. This is achieved by a vacuum interface using a stacked ring radio frequency ion guide with 10-fold higher transfer efficiency in MS/MS mode and 3–5-fold in full scan spectra, by a dual pressure ion trap configuration, and by reduction of overhead times between scans. The first ion trap efficiently captures and fragments ions at relatively high pressure whereas the second ion trap realizes extremely fast scan speeds at reduced pressure. Ion injection times for MS/MS are predicted from full scans instead of performing automatic gain control scans. Together these improvements routinely enable acquisition of up to ten fragmentation spectra per second. Furthermore, an improved higher-energy collisional dissociation cell with increased ion extraction capabilities was implemented. Higher-collision energy dissociation with high mass accuracy Orbitrap readout is as sensitive as ion trap MS/MS scans in the previous generation of the instrument. ***Molecular & Cellular Proteomics 8: 2759–2769, 2009.***

Proteomics experiments typically involve the analysis of peptide mixtures obtained by the enzymatic digestion of proteomes that can be as complex as complete cell lysates (1, 2). Dynamic range of peptide abundances and the sheer number of peptides encountered in these mixtures require extremely sensitive and fast peptide detection and fragmentation (3). Although a first comprehensively identified and quantified proteome has recently been reported (4), further gains in instrumental performance are clearly needed to reduce overall measurement time, improve sequence coverage of identified proteins, and for the in-depth analysis of mammalian proteomes.

Among many different instrumental formats (5), the combination of a linear ion trap (6) with a Fourier transform (FT)[1] mass spectrometer has rapidly become a popular technological platform in proteomics because it combines the sensitivity, speed, and robustness of ion traps with the high resolution capabilities of FT instruments. The first implementation of this principle used an ion cyclotron resonance instrument with a 7T magnet as the high resolution device (7). Later, the Orbitrap™ analyzer developed by Makarov was coupled to the LTQ, combining the linear ion trap with a very small and powerful analyzer (8–11).

Here we describe a next generation linear ion trap-Orbitrap instrument with significant improvements in ion source transmission and with a new ion trap configuration. We show that this instrument, termed the LTQ Orbitrap Velos, is capable of much higher scan speeds compared with the current LTQ Orbitrap. Furthermore, we implemented more efficient ion extraction for the higher-energy collisional dissociation (HCD) cell (12). Due to this improvement and the 10-fold higher transmission of ions from atmosphere, high resolution and high mass accuracy MS/MS can now routinely be obtained at very high sensitivity and at scan speeds of up to 5 Hz acquisition rates. A related instrument, the LTQ-Velos, which does not contain the Orbitrap analyzer for high resolution measurements, has been described very recently (13).

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[1] The abbreviations used are: FT, Fourier transform; S-lens, stacked ring ion guide; CID, collision-induced dissociation; HCD, higher-energy collisional dissociation; BSA, bovine serum albumin; MS/MS, tandem mass spectrometry; LTQ, linear quadrupole ion trap; LC-MS/MS, liquid chromatography tandem mass spectrometry; AGC, automatic gain control; RT, room temperature; RF, radio frequency; SILAC, stable isotope labeling by amino acids in cell culture; HPLC, high pressure liquid chromatography.

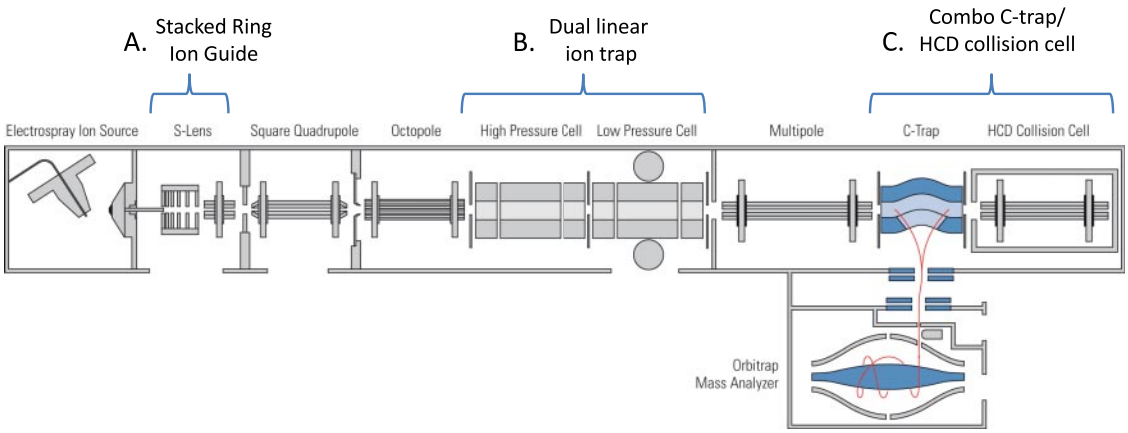

FIG. 1. **Schematic of the LTQ Orbitrap Velos MS instrument with three new hardware implementations.** *A*, the stacked ring ion guide (S-Lens) increases the ion flux from the electrospray ion source into the instrument by a factor 5–10. *B*, the dual linear ion trap design enables efficient trapping and activation in the high-pressure cell (*left*) and fast scanning and detection in the low pressure cell (*right*). *C*, the combo C-trap and HCD collision cell with an applied axial field with improved fragment ion extraction and trapping capabilities.

EXPERIMENTAL PROCEDURES

Construction of an Improved Linear Ion Trap Orbitrap Instrument—The LTQ Orbitrap Velos instrument described in this paper is a further development of the LTQ Orbitrap product (10). The three major novel hardware elements are (i) the introduction of a more efficient ion transfer system (Stacked Ring Ion Guide or "S-lens"), (ii) a dual pressure ion trap, and (iii) a more efficient HCD cell (Fig. 1) .

Introduction of an S-lens—The tube lens/skimmer assembly has been replaced by a set of stainless steel apertures to which an RF voltage is applied to form a so-called S-lens. In addition, the transfer tube has been halved in length to make space for this S-lens and to further improve transmission. The apertures in the S-lens are spaced by a progressively increasing gap and also arranged in two sequential sets: the first set of apertures has a larger ID of 7.5 mm to capture the entire expansion from the transfer tube, and the second has a smaller inner diameter of 5.0 mm, to focus the ion beam through an exit lens. As no direct current gradient appeared to be necessary in such a construction, all odd-numbered apertures have been connected to one phase of a RF voltage and the even-numbered apertures to the other. Thus only the amplitude of this RF voltage is varied to optimize transmission. As demonstrated by Smith and co-workers (14), focusing of ions by RF fields in the low-millibar pressure range drastically reduces ion losses, up to an order of magnitude in favorable cases. Although we employ a considerably simpler construction, we observe a similar gain in signal for all tested compounds. The pressure is increased by about 50% as the transfer tube is halved in length compared with the previous LTQ design. Transport of droplets and solvent clusters from the transfer tube into the ion optics downstream of the S-lens is minimized by slightly curving a quadrupole ion guide after the S-lens, which blocks the line-of-sight.

Introduction of a Dual-pressure Linear Ion Trap—In the LTQ Orbitrap Velos mass spectrometer, a dual-pressure ion trap assembly is used instead of the linear trap of the LTQ, comprising two identical linear quadrupole cells separated by a center lens. The two cells have a common supply of RF (1.2 MHz) and excitation AC voltages but independent DC voltages that allow the transfer of ions from one cell to the other. Compared with the previous LTQ, the first trap is operated at a higher pressure of helium bath gas (5.0×10^{-3} torr) whereas the second trap has a lower pressure (3.5×10^{-4} torr). This allows the dual trap to overcome inherent compromises of the previous single-pressure design by ensuring fast isolation, high capture efficiency into the linear ion trap, which is now higher than 90%, and efficiency of fragmentation of about 70–80% at higher pressure. The dual trap also allows reducing of the activation time used in an LTQ XL to one-third (10 ms) as well as drastically accelerated scanning rates at lower pressure compared with the LTQ XL. Helium is introduced into the high pressure trap via an open-split interface and some fraction leaks into the low-pressure trap via the center lens with its 2.5-mm diameter aperture. This lens and the conductance apertures placed on the back lens of the low pressure trap are used to define its pressure. The design of both traps differs significantly from the previous trap in the LTQ: the stretched rod layout of the latter is superseded by a symmetrical arrangement of four identical quadrupolar rods. In particular, each rod included an ejection slit whereas previously only the two rods used for ejection of ions had these slits. This allows a significant reduction in the misbalance of the RF potential along the axis and thus reduces the dependence of ion capture on the RF amplitude at the moment of ion entry into the device.

Introduction of an Integrated C-trap/HCD Collision Cell Combination—Additional correction electrodes have been installed in the HCD collision cell to create a weak axial field in a way similar to Fig. 2 in ref (15). Equally spaced electrodes have been integrated on a ceramic printed-circuit board that also serves as a carrier for a resistive divider distributing voltages to these electrodes. Penetration of the resulting electric field distribution up to the axis of the device has been ensured by locating the printed circuit board in the center of each gap between four RF-only rods of the cell. This design enables rapid extraction of all ions from the HCD cell and allows increasing speed and sensitivity of the instrument in HCD mode. As a result, the recommended target values for peptide fragmentation in HCD mode are reduced from 50,000–200,000 in the LTQ Orbitrap XL to only 20,000–50,000 for detection in the Velos type Orbitrap analyzer.

To reduce gas load on the Orbitrap vacuum compartment in HCD mode, the nitrogen gas line to the C-trap and the ceramic plates enclosing the C-trap from top and bottom have been removed. By moving the HCD cell closer to the trap electrode of the C-trap a suitable flow of gas from the HCD cell has been established through the C-trap. An aperture of 2.5-mm ID in the trap electrode ensures that gas pressures both in the HCD cell and the C-trap allow uncompromised operation of each of these units. Thus a single gas line and regulator now provide both the collision gas in the HCD cell and the bath gas in the C-trap.

Finally, the integrated C-trap/HCD cell was separated from the linear trap by a conductivity restrictor located at the exit aperture of the linear trap. An additional 70-l/s turbomolecular pump (Pfeiffer, Asslar, Germany) was installed on this newly formed vacuum com-

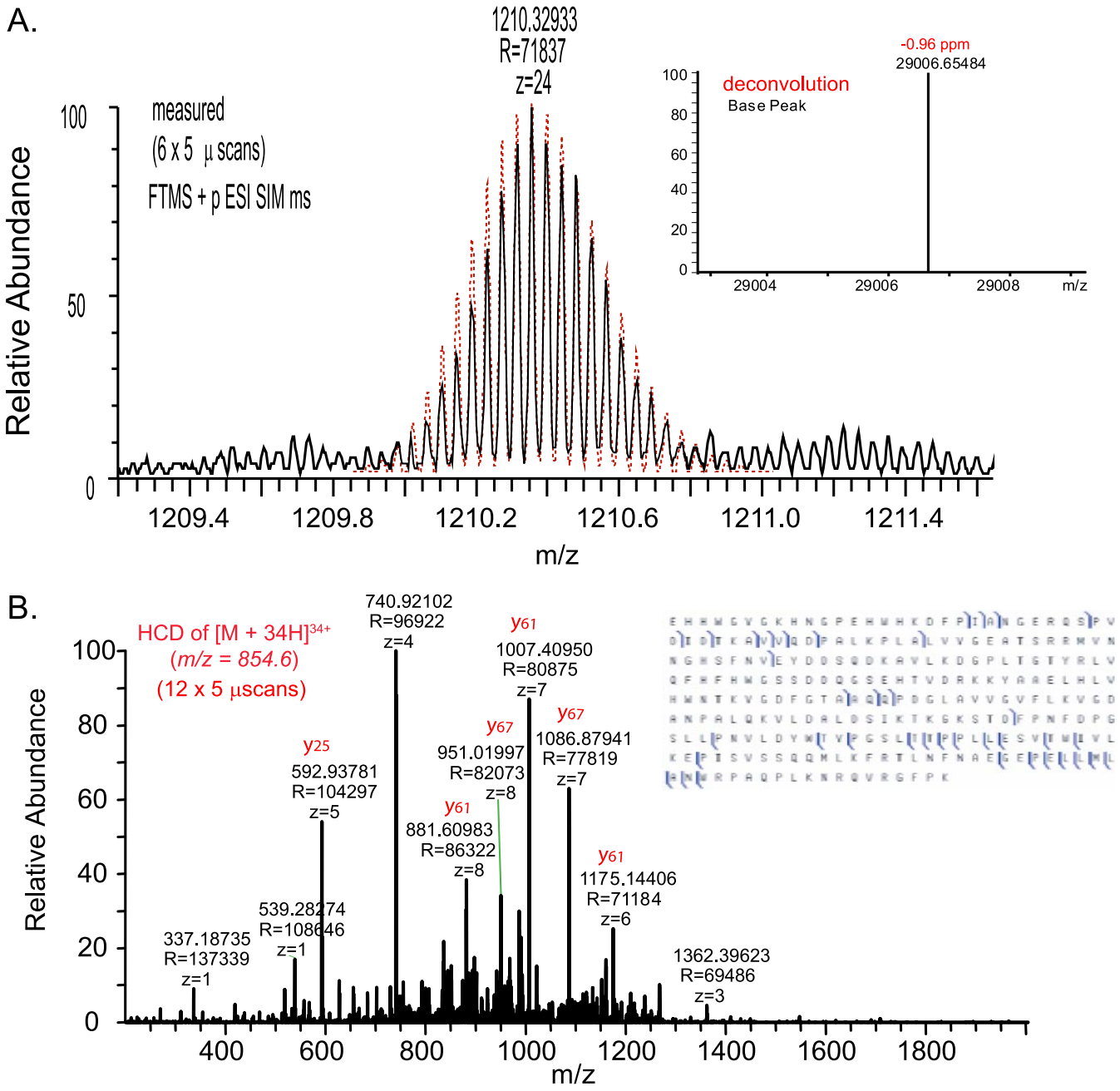

FIG. 2. **Top-down analysis of intact bovine carbonic anhydrase II by LTQ Orbitrap Velos.** *A*, Orbitrap MS full scan of $(M + 24\ H)^{24+}$ acquired at full isotopic resolution. *Inset*, deconvolution of base peak. Note that the mass error is less than one p.p.m. with external calibration. *Red dotted line* indicates the *in silico* simulation of carbonic anhydrase $(M + 24\ H)^{24+}$ at resolving power of 72,000. *B*, HCD of $[M + 34\ H]^{34+}$ of carbonic anhydrase. The *inset* shows the cleavage coverage of the protein sequence by HCD.

partment to make its pumping independent of the helium gas load in the linear trap compartment. This essentially eliminated previously observed carryover of helium into the Orbitrap analyzer chamber.

Other Differences to the LTQ Orbitrap—In addition to these major changes several minor ones were introduced. The bake-out procedure of the Orbitrap analyzer chamber has been changed to increase the temperature of the Orbitrap analyzer during bake-out from 110 °C to 180 °C. To protect the image current preamplifier from overheating under these conditions, a water-cooling line has been attached to its base. Along with the new gas supply of the C-trap this routinely achieves pressures in the 10^{-11}m bar range in the Orbitrap analyzer compartment. Such pressure levels facilitate very long transients and thus measurement with isotopic resolution of not only small proteins, but also of proteins beyond 30 kDa. More importantly, this can now be done with the HCD cell in full operation. As a result, on-line top-down and middle-down analysis using the HCD cell is now compatible with the mass analysis of intact proteins (Fig. 2).

The software controlling the instrument now includes a new mode of predictive automatic gain control (pAGC). In this mode the injection

times of precursor ions for MS/MS are no longer determined by a dedicated pre-scan in the LTQ, but from a full-MS spectrum acquired from either the linear trap or the Orbitrap mass analyzer. Mass calibration has also been modified to include spline functions. This has recently been shown to result in a better fit of the mass calibration curve (16).

BSA Standard Digests—350 μg of bovine serum albumin fraction V (Sigma, BSA No. A7638) was resolubilized in an 8 M urea buffer (6 M urea + 2 M thiourea in 10 mM HEPES, pH 8). Cysteine disulfide bonds were reduced by addition of 7 μg dithiothreitol for 30 min, and alkylated with 35 μg iodoacetamide for 20 min. The protein was digested with 5 μg LysC for 3 h at room temperature (RT) and further digested overnight using 5 μg of a protease (sequencing grade from Promega) that cleaves C-terminal to arginine and lysine residues. The resulting peptide mixture was diluted 10-fold with 5% acetonitrile in 1% trifluoroacetic acid, and the equivalent of 5 pmol BSA digest was loaded onto a C_{18}-Empore disc StageTip (17), washed once with 20-μl 0.5% acetic acid, and eluted with 200-μl 50% MeOH in 0.5% formic acid.

HeLa SILAC Cytoplasmic Extract 1:3 Standard—Two populations of HeLa S3 cervix carcinoma cells were grown in suspension and SILAC-labeled (18) in RPMI 1640 medium supplemented with dialyzed bovine serum and two distinct isotopic variants of arginine ($^{13}C_6$,$^{15}N_4$-L-Arg) and lysine ($^{13}C_6$,$^{15}N_2$-L-Lys). The two cell populations were harvested in 50-ml tubes by spinning for 5 min at 400 *g* (Hereaus multifuge, 4 °C), washed once with phosphate-buffered saline, and combined (heavy-to-light 3:1), and lysed by douncing (30–40 strokes) in 5 volumes of a 10 mM Hepes KOH, pH 7.9, 1.5 mM $MgCl_2$, and 10 mM KCl in a homogenizer on ice. The nuclear and cellular debris were pelleted by centrifugation for 15 min at 3900 rpm. The crude cytoplasmic supernatant was transferred to a new tube and ultra-centrifuged for 1 h at 60,000 × *g*. The supernatant was diluted with glycerol (10% final concentration) and NaCl (150 mM final concentration), and the obtained cytoplasmic extract was snap-frozen in liquid nitrogen and stored at −80 °C. The frozen SILAC cytoplasmic extract was resolubilized in an 8 M urea buffer (6 M urea + 2 M thiourea in 10 mM HEPES, pH 8). The soluble proteins were reduced for 30 min at RT with 1 mM dithiothreitol and then alkylated for 15 min by 5.5 mM iodoacetamide. Endoproteinase Lys-C (Wako) was added 1:100 w/w, and the lysates were digested for 4 h at RT. The resulting peptide mixtures were diluted 4-fold with de-ionized water to achieve a final urea concentration below 2 M. A modified protease that cleaves C-terminally to arginine and lysine residues was added 1:100 w/w, and the sample was digested overnight. Protease activity was quenched by acidification of the reaction mixtures with a 10% trifluoroacetic acid solution to pH ~2. The peptide mixture was aliquoted, desalted, and concentrated on a C_{18}-StageTip, and eluted with 20 μl of 80% acetonitrile in 0.5% acetic acid.

Nanoflow HPLC-MS/MS —The peptides mixtures were analyzed by online nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) on an EASY-nLC™ system (Proxeon Biosystems, Odense, Denmark) connected to the LTQ Orbitrap Velos instrument (Thermo Fisher Scientific, Bremen, Germany) through a Proxeon nanoelectrospray ion source. 5 μl of the peptide mixtures were autosampled directly onto the analytical HPLC column. They were separated in a 15-cm analytical column (75-μm inner diameter) in-house packed with 3-μm C_{18} beads (Reprosil-AQ Pur, Dr. Maisch) with a 120-min gradient from 5% to 50% acetonitrile in 0.5% acetic acid. The effluent from the HPLC column was directly electrosprayed into the mass spectrometer. The LTQ Orbitrap Velos instrument was operated in data-dependent mode to automatically switch between full scan MS and MS/MS acquisition. Instrument control was through Tune 2.6.0 and Xcalibur 2.1.

For the low-resolution CID-MS/MS top20 method, full scan MS spectra (from *m/z* 300–1700) were acquired in the Orbitrap analyzer after accumulation to a target value of 1e6 in the linear ion trap. Resolution in the Orbitrap system was set to $r = 60{,}000$ (all Orbitrap system resolution values are given at *m/z* 400). The 20 most intense peptide ions with charge states ≥2 were sequentially isolated to a target value of 5,000 and fragmented in the high-pressure linear ion trap by low-energy CID with normalized collision energy of 35% and wideband-activation enabled. The resulting fragment ions were scanned out in the low-pressure ion trap at the "normal scan rate" (33,333 amu/s) and recorded with the secondary electron multipliers. Ion selection threshold was 500 counts for MS/MS, and the maximum allowed ion accumulation times were 500 ms for full scans and 25 ms for CID-MS/MS measurements in the LTQ. An activation $q = 0.25$ and activation time of 10 ms were used.

For the HCD top10 method, survey full scan MS spectra (from *m/z* 300–1700) were acquired in the Orbitrap system with resolution $r = 30{,}000$ (after accumulation to a target value of 1e6 in the linear ion trap). The ten most intense peptide ions with charge states ≥2 were sequentially isolated to a target value of 3e4 or 5e4 and fragmented in the HCD collision cell with normalized collision energy of 40%. The resulting fragments were detected in the Orbitrap system with resolution $r = 7{,}500$. The ion selection threshold was 5,000 counts for HCD, and the maximum allowed ion accumulation times were 500 ms for full scans and 250 ms for HCD.

Standard mass spectrometric conditions for all experiments were: spray voltage, 2.2 kV; no sheath and auxiliary gas flow; heated capillary temperature, 200 °C; predictive automatic gain control (AGC) enabled, and an S-lens RF level of 50–60%. For all full scan measurements with the Orbitrap detector a lock-mass ion from ambient air (*m/z* 445.120024) was used as an internal calibrant as described (19). We also chose to deactivate selected ion monitoring injection of the lock mass, in order to save time. However, when present the lock mass was still used for real time correction of the mass scale.

Raw LC-MS Data Analysis—Fragmented peptides were identified and quantified by MASCOT and MaxQuant; Raw Orbitrap full-scan MS and ion trap CID-MS/MS and Orbitrap HCD spectra from the SILAC-labeled HeLa cytoplasmic extracts were processed by MaxQuant as described (20, 21). In brief, all identified SILAC doublets were grouped, accurate precursor masses determined using the entire LC elution profiles and MS/MS spectra were merged into peak-list files (*.msm). Peptides were matched to tandem mass spectra by Mascot version 2.2 (Matrix Science, London, UK) by searching an in-house curated concatenated target/decoy database. This was a forward and reversed version of the human International Protein Index (IPI) sequence database (version 3.37; 138,632 forward and reversed protein sequences from EBI Database) supplemented with common contaminants such as human keratins, bovine serum proteins, and proteases. Scoring was performed in MaxQuant as described previously. Tandem mass spectra were initially matched with a mass tolerance of 7 ppm for precursor masses and 0.5 Da for CID fragment ions and 0.02 Da for HCD fragments. We required strict enzyme specificity and allowed for up to two missed cleavage sites. Cysteine carbamidomethylation (Cys +57.021464 Da) was searched as a fixed modification, whereas *N*-acetylation of proteins (N-term +42.010565 Da), and oxidized methionine (+15.994915 Da) were searched as variable modifications.

Peptide Filtering and Protein Identification —The resulting Mascot result files (*.dat) were loaded into the MaxQuant software suite for further processing. In MaxQuant we fixed the estimated false discovery rate of all peptide and protein identifications at 1%, by automatically filtering on peptide length, mass error precision estimates, and peptide score of all forward and reversed peptide identifications (20). All reported protein groups have been identified by at least two peptides, one of which has to be unique to the protein. All identifications can be found in supplemental Table 1.

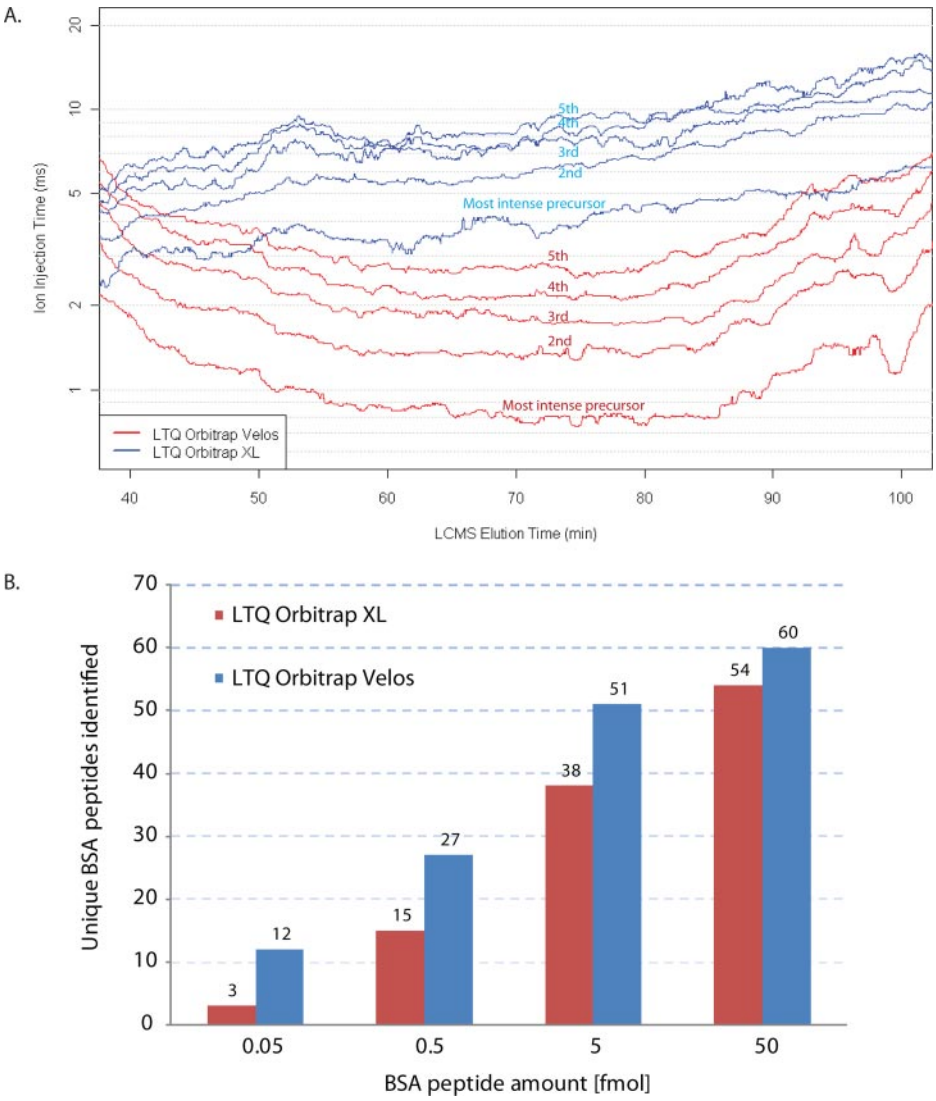

FIG. 3. *A*, S-lens ion injection times for CID-MS/MS compared with the previous ion source configuration. The same SILAC-labeled HeLa extract separated with a 2 h LC gradient were analyzed on an LTQ Orbitrap XL instrument (*blue lines*) and an LTQ Orbitrap Velos instrument (*red lines*) with ion trap-CID MS/MS. Injection times of the top5 most intense peaks from each scan cycle are shown as running medians of 1000 scans. *B*, LC-MS/MS of serial dilutions of a simple BSA digest on both the XL and Velos type LTQ Orbitraps under identical conditions: 0.05, 0.5, 5, and 50 fmol of the digest loaded on column and analyzed by top5 CID in the ion trap. Unique peptides identified by the XL at the different concentrations are indicated in the *red bars*, whereas peptide identifications from the Velos are displayed in the *blue bars*.

RESULTS AND DISCUSSION

In "shotgun" proteomics experiments mass spectrometers have to analyze extremely complex peptide mixtures at very high sensitivity and scan speeds while providing excellent data quality for MS and MS/MS scans. Even the most advanced instruments still do not completely analyze all observed peptides, leading to apparent irreproducibility between experiments. Here we describe the LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Fisher Scientific), which directly addresses several of the key challenges in the analysis of complex peptide mixtures.

Fig. 1 presents a schematic of the LTQ Orbitrap Velos instrument. As in the original instrument, ions produced by the electrospray ion source are sampled from atmosphere by a metal ion transfer tube and transmitted into a differentially pumped atmosphere-to-vacuum interface. Then they reach the linear trap mass analyzer via RF-only multipoles. In the linear trap ions can either be radially ejected to a pair of secondary electron multipliers using an RF amplitude/resonance ejection mass analysis scan or released axially toward the C-trap, where they are stored prior to the injection into the Orbitrap mass analyzer. The Orbitrap analyzer then utilizes image current detection and Fourier transformation for mass analysis. Multiple steps of isolation and fragmentation can precede each of these detection steps.

The three main areas of improvement of the LTQ Orbitrap Velos are indicated in Fig. 1: (*A*) The introduction of a much more efficient atmosphere/vacuum ion transfer system; (*B*) a novel dual-pressure linear ion trap that allows separation of the capture and fragmentation processes from the mass scanning and detection process, and (*C*) a highly efficient integrated C-trap/HCD collision cell combination. The hardware implementation of these three elements is described above in "Experimental Procedures".

Performance of the S-lens—To increase the ion current transmitted from the electrospray region of the source, which

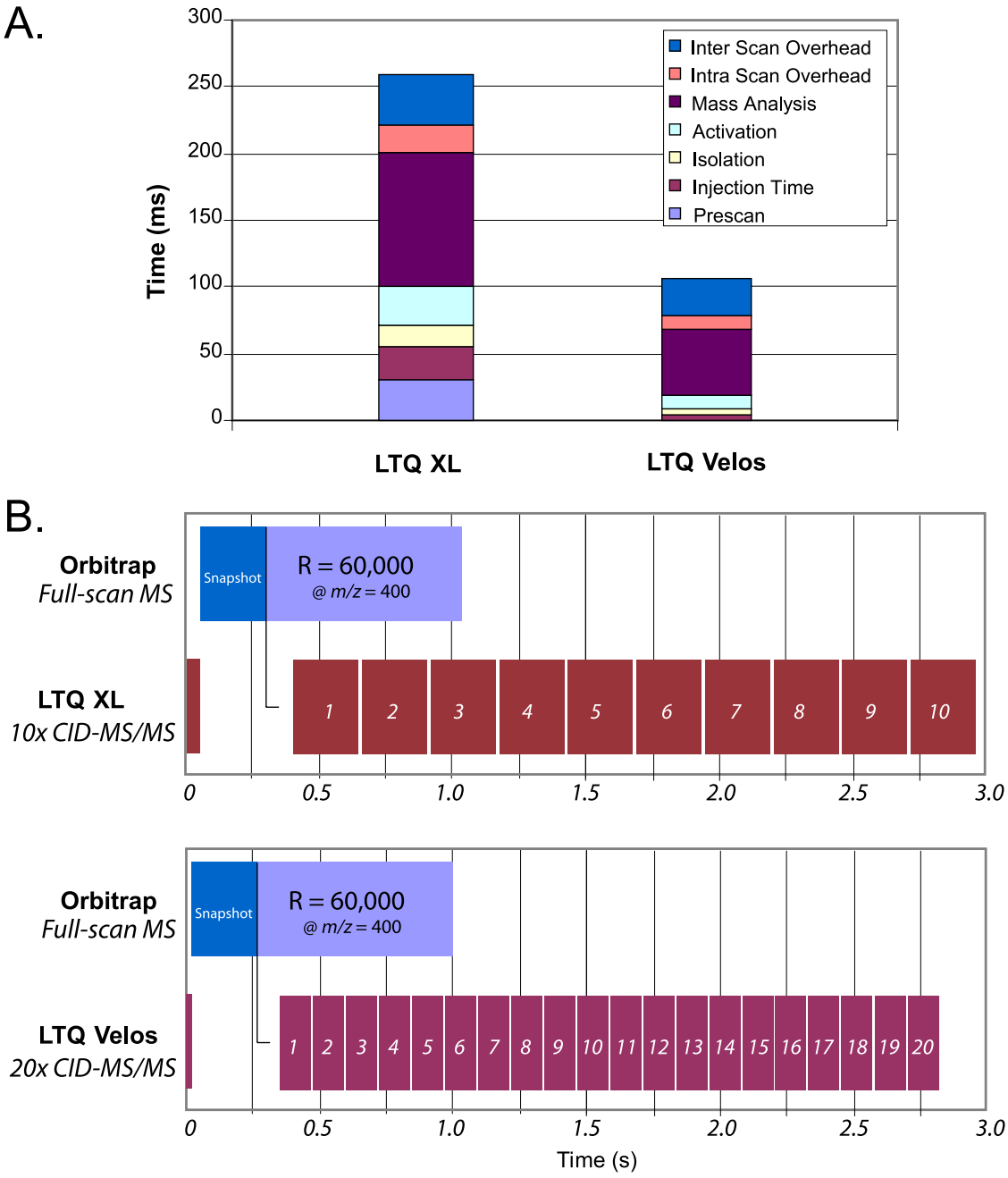

FIG. 4. **CID-MS/MS in the dual-pressure linear ion trap top20 cycle times for shotgun proteomics.** *A*, full scan data from a complex SILAC-labeled HeLa cell cytoplasmic fraction using a top10 CID method on the LTQ Orbitrap XL instrument. *B*, full scan data from the same HeLa sample analyzed with a top20 method on the LTQ Orbitrap Velos instrument.

is at atmospheric pressure, to the vacuum of the mass spectrometer, we implemented a simplified and robust variant of a stacked ring RF ion guide, the most well known implementation being an ion funnel (14). This focusing element operates at an intermediate pressure of a few millibar and keeps charged particles on the center line using an RF field, while the gas admitted through the source orifice expands out. According to its construction, the lens is termed a "Stacked Ring Ion Guide" or S-lens. Since the optimal RF voltage depends on the *m/z* to be passed through the S-lens, this value is automatically adjusted for each precursor mass. In evaluation on model compounds, ion current through the S-lens was increased more than 10-fold at low *m/z* and about 2-fold at *m/z* 1000. For full range mass measurement, the RF is adjusted in three steps for low, medium, and high mass range. In this mode, ion current increased about 3-fold.

To test the performance of the S-lens for MS/MS with proteomic samples, we determined the injection time nec-

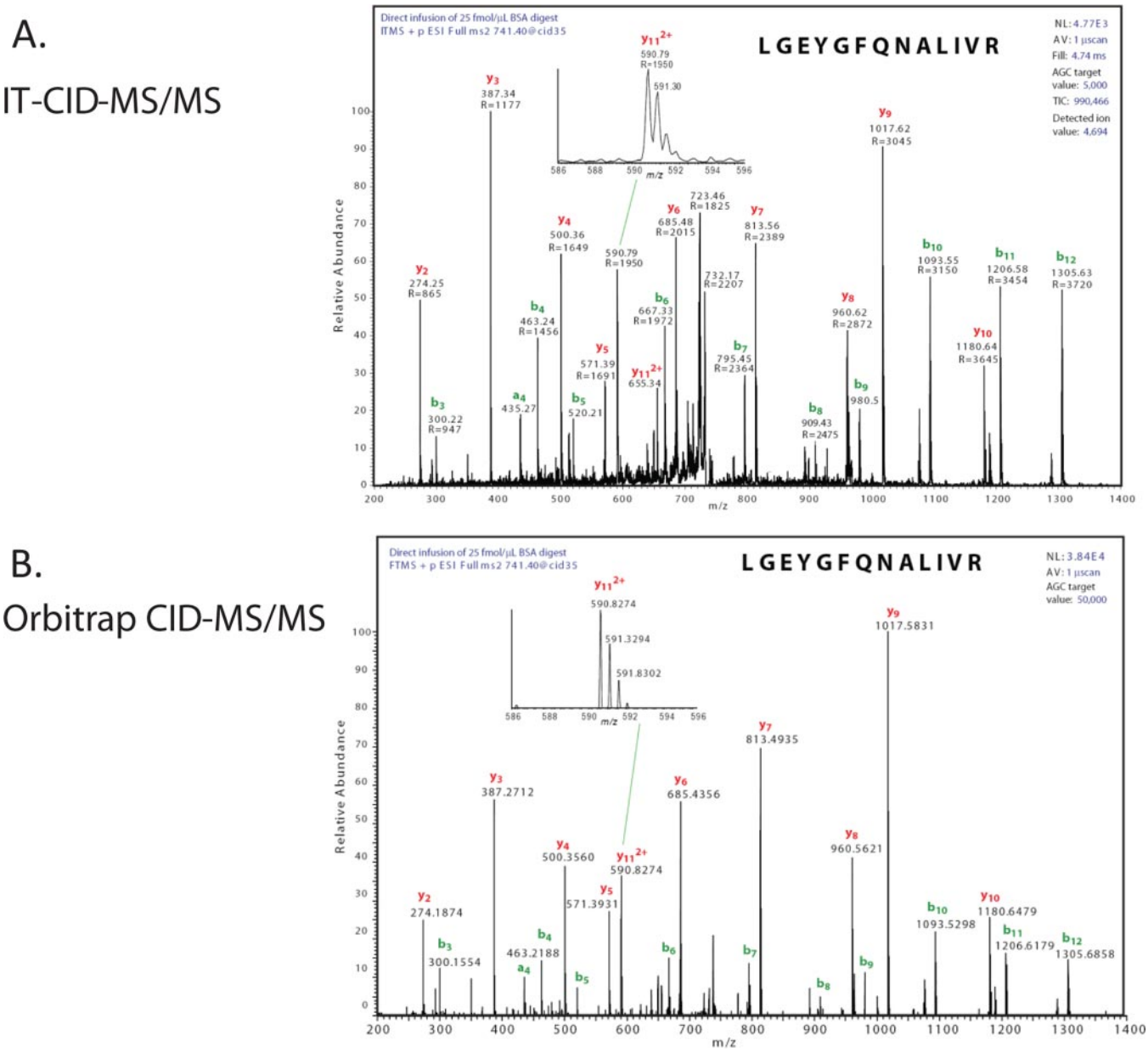

FIG. 5. **Profile data of MS/MS detected in the linear ion trap and in the Orbitrap instrument.** *A*, CID-MS/MS spectrum acquired in the linear ion trap. 5000 ions of a doubly charged BSA peptide at *m/z* 740.40 from a 25 fmol/µl BSA standard mixture infusion are isolated in 4 ms and fragmented by 10 ms resonant excitation in the high-pressure trap using normalized collision energy of 35%. Fragment ions are moved to the low-pressure trap and scanned out to the two electron multiplier detectors with a scan speed of 33,300 amu/sec. The spectrum is recorded from *m/z* 200 to 2000, which results in a scan time of 50 ms. Note that the natural isotope cluster of doubly charged fragment ions are resolved (*inset*). *B*, low-energy CID spectrum of the same peptide acquired in the Orbitrap analyzer. 50,000 ions are isolated in the low pressure linear ion trap by 50 ms injection time and activated by 35% normalized collision energy. The fragment ions are detected in the Orbitrap analyzer with a resolution of 7500, which is achieved by 100 ms transient detection time. All fragment ion isotope clusters are baseline resolved (*inset*).

essary for ion trap fragmentation. We chose a target value of 5,000, which is commonly used for ion trap MS/MS. Fig. 3*A* shows a plot of these injection times across the major elution window of a complex HeLa cell lysate. Because injection times in topN experiments depend on *N*, we separately plotted the first to fifth peptides picked from the full scan. Clearly, median injection times are very low and typically in the range of a few milliseconds at most. For the first picked ion they are generally below one millisecond. Thus they do not appreciably contribute to cycle time. In fact, most of the peptides could have been accumulated at least 10-fold longer without being limited by the maximum

TABLE I
Comparison of the Velos to previous instrument versions
2 h gradient analysis of a HeLa SILAC cytoplasmic extract. All identifications can be found in supplemental Table I.

Instrument	MS	MS/MS	Peptide IDs	Protein IDs	Protein IDs (> = 2 peptides)
7T-LTQ-FT ultra	4761	17091	8036	1177	854
LTQ-Orbitrap classic	4872	17568	8387	1261	910
LTQ-Orbitrap XL	4329	17609	8494	1301	954
LTQ-Orbitrap Velos IT-CID	5416	33138	11306	1581	1171
LTQ-Orbitrap Velos HCD	4434	22460	9686	1355	988

injection times for CID MS/MS, set at 25 ms in our experiment.

To directly compare the performance of the S-lens to the vacuum interface of the LTQ Orbitrap XL, we superimposed the injection time distribution for the two instrument types. Over the entire graph, injection times using the S-lens was at least 5-fold lower than that of the LTQ Orbitrap.

The high ion current provided by the S-lens should be a pronounced advantage when analyzing very low amounts of sample, which typically require somewhat longer injection times. To assess this aspect of proteomic sensitivity we performed LC-MS/MS analyses of serial dilutions of a simple BSA digest on both the XL and Velos type LTQ Orbitraps under identical conditions. We injected 0.05, 0.5, 5, and 50 fmol of the digest on column and analyzed it with a standard top5 CID-MS/MS method with Orbitrap full scan acquisition at a resolution of 60,000 with parallel MS/MS detection with the ion trap detection system. The XL and the Velos have similar coverage of BSA peptides in the 50 fmol analysis. Both identify more than 50 unique peptides that cover more than 70% of the BSA protein sequence (Fig. 3*B*). However, when analyzing sub-fmol amounts of BSA the Velos was superior in its sensitivity and was capable of identifying up to four times more BSA peptides compared with the XL.

We next inspected injection times for the entire mass range, when loading the C-trap with 1,000,000 ions for Orbitrap analysis. On the LTQ Orbitrap Velos, these were extremely short, with a median of less than one millisecond for most of the gradient. As expected they were several-fold longer on the LTQ Orbitrap XL. However, in neither case would they add substantially to the overall analysis time in these experiments. Finally, we noticed that the time to accumulated ions in the LTQ for full scan analysis was extremely short and frequently in the microsecond range (see supplemental Fig. 1 for example).

Top20 Method with Fast Cycle Time—To achieve very fast MS/MS scan rates, all associated overhead functions were minimized. As established above, injection times do not appreciably contribute to total cycle duration. The time required for isolation of the precursor, voltage stabilization, and the like was also kept in the millisecond range. Standard fragmentation time was reduced from 30 ms to 10 ms, which did not lead to a major decrease in fragmentation efficiency in the higher pressure linear ion trap of the LTQ Orbitrap Velos.

To regulate the number of ions analyzed by the LTQ FT and LTQ Orbitrap instruments and avoid space charge effects, a "pre-scan" is normally performed previous to each MS or MS/MS scan, in which the number of ions under the current conditions is estimated using a very fast low resolution (less than unit) scan in the linear ion trap (AGC). In the LTQ Orbitrap Velos instrument, this pre-scan is eliminated, and injection times for MS/MS spectra are instead determined "*in silico*" from already acquired MS data (predictive AGC). This step eliminates about 30 ms from each scan event. Together with the faster scan speed enabled by the low pressure linear ion trap, all operations necessary for obtaining an MS/MS spectrum were compressed to about 100 ms thus allowing MS/MS rates of up to 10 Hz.

On the previous instruments, "top5 or top10" methods are commonly employed, in which a 1 s MS scan in the Orbitrap analyzer (60,000 resolution) is coupled to linear ion trap detection of the fragmentation products of the five or ten most intense ions. The total cycle time for a top10 method is about 3 s on an LTQ Orbitrap XL (Fig. 4*A*). Given the very fast MS/MS scan rates on the new instrument we decided to use a "top20 fragmentation" method. This consisted of an Orbitrap precursor scan with 60,000 resolution and 20 product ion scans in the dual pressure linear ion trap. As can be seen in Fig. 4*B*, total cycle times from one MS scan to the next was less than 3 s. Thus the top20 method in the LTQ Orbitrap Velos instrument is about twice as fast and produces twice the number of MS/MS spectra as the top10 method did previously. The figure also shows that the actual MS/MS scan times in this complex mixture analysis were indeed about 100 ms, leading to 10 Hz repetition rates.

The fast scan speed was not achieved at the expense of ultimate resolution – already at "normal scan speed" (33,300 amu/s) the low pressure linear ion trap provides better than unit resolution. This is shown in Fig. 5*A*, where doubly charged fragment ions are easily distinguishable from singly charged fragments when visualized in profile mode.

Next we evaluated MS/MS rates in the complex peptide mixtures typically used in proteomics analysis. A SILAC-labeled cytoplasmic cell extract of HeLa cells was separated and analyzed with a standard 2 h gradient as described in "Experimental Procedures". We compared the LTQ-FT Ultra, LTQ Orbitrap Classic, and LTQ Orbitrap XL to the LTQ Orbitrap Velos. Five micrograms of protein digest were loaded on

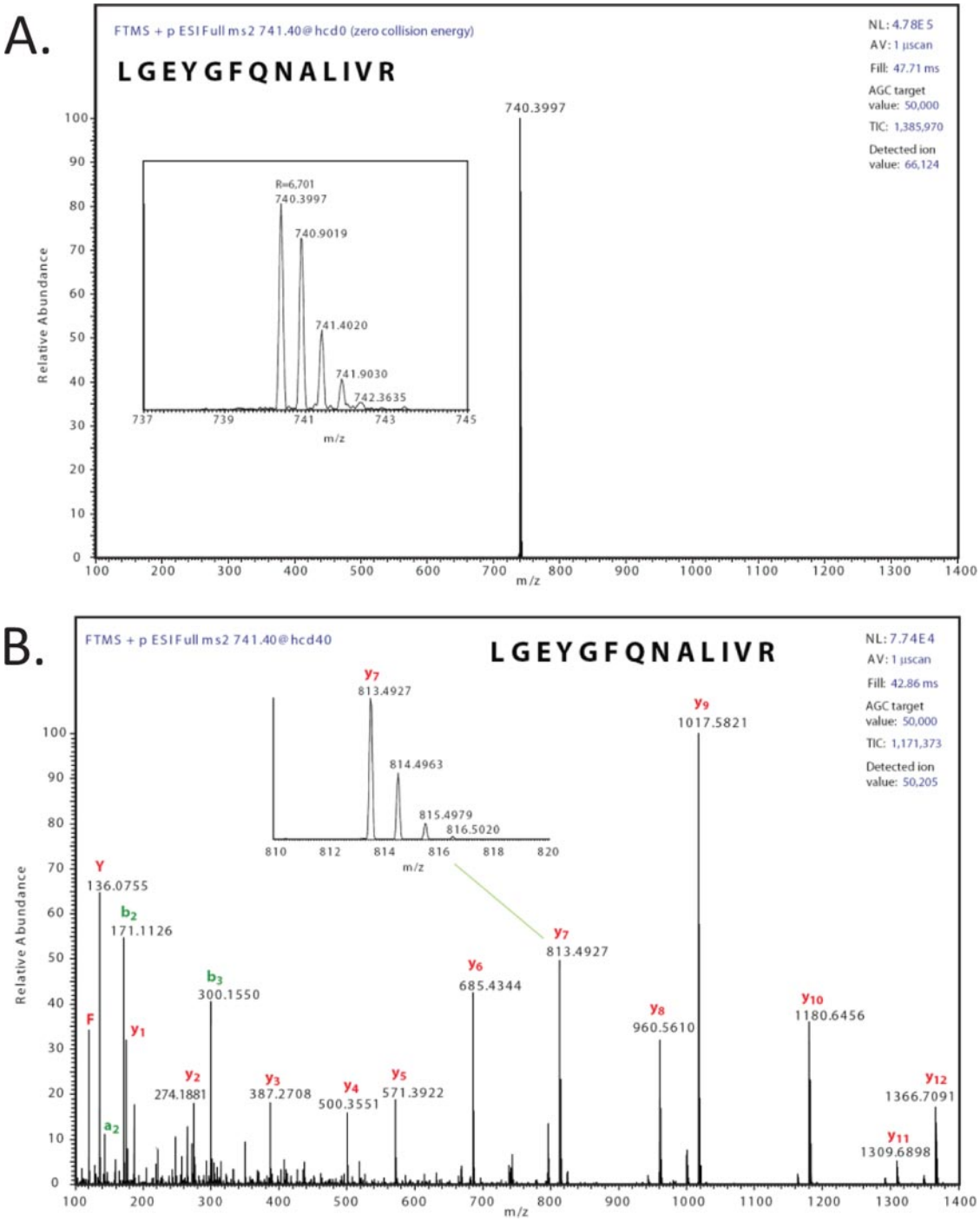

FIG. 6. **HCD fragmentation and extraction efficiency.** *A*, HCD scan at zero collision energy. 50,000 ions of a doubly charged BSA peptide at *m/z* 740.40 from a 25 fmol BSA digest/μl direct infusion experiment are isolated in 48 ms and transferred to the C-trap before detection in the Orbitrap analyzer at a resolution of 7500 at *m/z* 400. *B*, HCD of the same peptide. 50,000 ions of the doubly charged BSA peptide at *m/z* 740.40 are isolated in 43 ms and fragmented in the HCD collision cell by 37 eV acceleration before detection in the Orbitrap analyzer at a resolution of 7500 at *m/z* 400. Natural isotope clusters of fragment ions (*inset*) are baseline resolved.

column in each experiment. As shown in Table I, the number of MS scans was around 5,000 for all instruments. The number of MS/MS scans was also very similar among the previous instruments (about 17,000) but about 2-fold higher on the LTQ Orbitrap Velos. Among the previous instruments the Orbitrap systems identified slightly more peptides than the FT Ultra. In contrast, the LTQ Orbitrap Velos CID experiment resulted in a third more peptide identifications. At the protein level we observed the same trend, with up to one third more proteins resulting from the LTQ Orbitrap Velos run compared with the

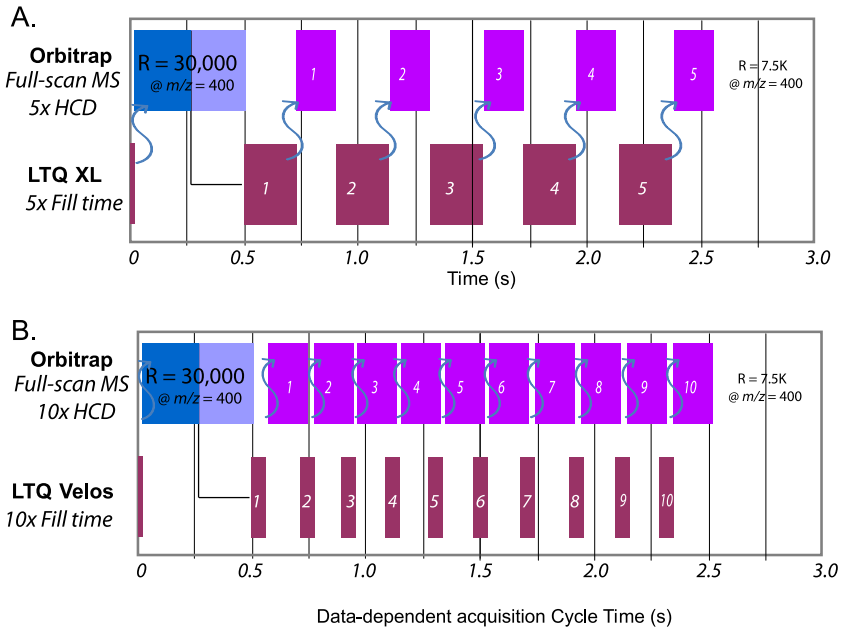

FIG. 7. **HCD scan cycle times.** HeLa cytoplasmic extract was analyzed with 2 h gradient using a top10 HCD method. Orbitrap analyzer full scans had a resolution of 30,000 (0.5 s), and HCD spectra 7500.

earlier Orbitrap system runs. This result clearly indicates the practical utility of the increased MS/MS scan speed in complex mixture analysis. However, more detailed tests will have to be done for different proteomics scenarios. For example, we expect that the sensitivity of the LTQ Orbitrap Velos will be a marked advantage when analyzing very small number of cells. Furthermore, it is probable that optimized acquisition software can employ the sequencing speed of the LTQ Orbitrap Velos mass spectrometer to further advantage.

High Resolution MS/MS Analysis with Low Injection Times—The linear ion trap Orbitrap hybrid instrument allows fragmentation either in the linear ion trap or the HCD cell. Resulting fragments can be measured either with the linear ion trap or with the Orbitrap detectors. Analysis in the linear ion trap is very sensitive, only requiring a few thousand ions. It is also very fast due to high ion trap scan speeds combined with the ability of the LTQ Orbitrap to measure precursors and fragments simultaneously in the two constituent parts of the hybrid mass spectrometer. Conversely, the resolution and mass accuracy of the linear ion trap are moderate, whereas fragments measured in the Orbitrap analyzer achieve low p.p.m. range mass accuracy. Good fragment ion spectra in the Orbitrap analyzer require about an order of magnitude more ions than in the LTQ and this fact has so far limited the routine applicability of high resolution MS/MS for shotgun proteomics experiments on this instrument. In principle, the improvement in ion current of about an order of magnitude reported here should make Orbitrap FT analysis of fragments as sensitive as ion trap MS/MS was before. Thus, the LTQ Orbitrap Velos should routinely be able to acquire data in a "high-high" mode, where both MS and MS/MS spectra are obtained with high mass accuracy instead of the currently used "high-low" mode.

For all experiments in high-high mode we chose very fast transients of 100 ms for the MS/MS spectra, which should be compatible with chromatographic time scales and which still provide ample resolution and mass accuracy for fragments. In Fig. 5, a BSA peptide was fragmented by CID in the ion trap and analyzed in either the ion trap or the Orbitrap analyzer. Besides somewhat less abundant b-ions, which are less stable than y-ions, the spectra look very similar. Injection time in this infusion experiment was 4.7 ms for the ion trap spectrum and 58 ms for the Orbitrap FT spectrum. Without the S-lens, injection time would have been ten times longer, dwarfing the detection times. Fragment ions are baseline resolved and mass deviations from calculated values are in the low p.p.m. range. It is also noteworthy that MS/MS spectra acquired in the Orbitrap analyzer have a much larger dynamic range than ion trap MS/MS spectra (Fig. 5).

The alternative peptide fragmentation method to CID is HCD, which occurs in a separate RF multipole at the far side of the C-trap (12). Analysis of fragments again happens at high resolution and mass accuracy in the Orbitrap analyzer but in contrast to CID fragmentation, HCD has "triple quadrupole-like" behavior, including the preponderance of y-ions and the absence of a low mass cut-off for fragments. In the LTQ Orbitrap Velos, efficiency of the HCD cell has been improved by an additional extraction field and the HCD cell has been directly combined with the C-trap (as described under "Experimental Procedures"). To demonstrate extraction efficiency of the new HCD device, we isolated a precursor ion and either transferred it to the C-trap and measured it in the Orbitrap analyzer or isolated it, fragmented it in the HCD cell, and analyzed the fragment spectrum in the Orbitrap analyzer. Total ion current was similar with and without HCD fragmentation, consistent with very high extraction efficiency from the HCD cell (Fig. 6).

Next we tested if injection times for HCD fragmentation are compatible with routine proteomics experiments. The same HeLa cytosolic lysate as described above was analyzed in a top10 method, consisting of an MS scan with 30,000 resolution followed by 10 HCD fragmentation events, recorded at 7,500 resolution in the Orbitrap analyzer. Fig. 7 shows that this leads to a cycle time of 2.6 s, which is well suited to complex mixture analysis. In fact, the total cycle is comparable with what was possible with ion trap fragmentation before, even though the fact that the Orbitrap analyzer is used for both MS and MS/MS precludes parallel operation. Peptide identifications were also similar or better than those by standard methods in the previous LTQ Orbitrap instruments (Table I). In supplemental Fig. 2 we have superimposed the injection times necessary for the HCD spectra on those previously necessary for the CID spectra. The distributions largely overlap, showing that HCD can indeed be used routinely in shotgun proteomics.

CONCLUSION AND OUTLOOK

Here we have described the design and performance of a next generation linear ion trap Orbitrap instrument. Due to its higher transmission ion source, dual ion trap, and improved HCD cell it opens up several exciting perspectives in proteomic research. Scan speed for MS/MS is doubled, and this leads to significantly more identifications in the analysis of complex peptide mixtures. As we have demonstrated, the injection times necessary to obtain high resolution, high mass accuracy fragmentation spectra are the same as those previously necessary for ion trap fragmentation. This makes it possible to routinely analyze the proteome in a high-high mode, where not only MS spectra but also MS/MS spectra have high mass accuracy. For example, we have analyzed the yeast proteome by HCD alone and found that such a high-high strategy is already capable of large scale mixture characterization. This capability will be an important next step in the continuing quest for "precision proteomics" (3). In particular, it provides new perspectives for modification analysis where identification of single peptides and localization of modifying groups is essential.

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S The on-line version of this article (available at http://www.mcponline.org) contains supplemental Figs. 1 and 2 and Table 1.

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