

Ultra High Resolution Linear Ion Trap Orbitrap Mass Spectrometer (Orbitrap Elite) Facilitates Top Down LC MS/MS and Versatile Peptide Fragmentation Modes*[§]

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Although only a few years old, the combination of a linear ion trap with an Orbitrap analyzer has become one of the standard mass spectrometers to characterize proteins and proteomes. Here we describe a novel version of this instrument family, the Orbitrap Elite, which is improved in three main areas. The ion transfer optics has an ion path that blocks the line of sight to achieve more robust operation. The tandem MS acquisition speed of the dual cell linear ion trap now exceeds 12 Hz. Most importantly, the resolving power of the Orbitrap analyzer has been increased twofold for the same transient length by employing a compact, high-field Orbitrap analyzer that almost doubles the observed frequencies. An enhanced Fourier Transform algorithm—incorporating phase information—further doubles the resolving power to 240,000 at m/z 400 for a 768 ms transient. For top-down experiments, we combine a survey scan with a selected ion monitoring scan of the charge state of the protein to be fragmented and with several HCD microscans. Despite the 120,000 resolving power for SIM and HCD scans, the total cycle time is within several seconds and therefore suitable for liquid chromatography tandem MS. For bottom-up proteomics, we combined survey scans at 240,000 resolving power with data-dependent collision-induced dissociation of the 20 most abundant precursors in a total cycle time of 2.5 s—increasing protein identifications in complex mixtures by about 30%. The speed of the Orbitrap Elite furthermore allows scan modes in which complementary dissociation mechanisms are routinely obtained of all fragmented peptides. *Molecular & Cellular Proteomics* 11: 10.1074/mcp.O111.013698, 1–11, 2012.

In many mass spectrometric applications, the resolving power of the instrument is of pivotal importance. Ultimate resolution has so far been obtained by Fourier Transform Mass Spectrometry (1) and in a recent example, Marshall and co-workers detected more than 26,000 components in a single spectrum of a crude oil mixture (2). In ion cyclotron resonance (ICR)¹ Fourier transform mass spectrometry, resolution is determined by the length of the transient and by the strength of the magnetic field. Increasingly larger magnets have allowed resolution in excess of one million for small molecules. The relatively recently introduced OrbitrapTM analyzer utilizes a different physical principle to obtain high resolution (3–6). The signal is recorded from the image current produced by ion packets which oscillate around and along the spindle-shaped inner electrode of the trap: the higher the electric field, the larger the number of oscillations per unit time and the higher the resolving power. To increase field strength, several design options can be pursued, including increasing the radius of the inner electrode of the device (7). Here we describe an Orbitrap analyzer that achieves higher resolving power through reduced trap dimensions. Resolution is further increased by making use of the phase information during Fourier Transformation (8–11). This ultra high resolution Orbitrap analyzer was combined with other instrumental improvements to construct a novel linear ion trap Orbitrap hybrid mass spectrometer termed the Orbitrap Elite.

We describe principles of this instrument and characterize its operation for both intact protein analysis and for bottom up peptide mixture analysis. Top down protein analysis has previously mainly been performed with Fourier transform (FT) ICR instruments because of their very high resolving power (12–

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¹ The abbreviations used are: AGC, automatic gain control; CID, collision induced dissociation; ETD, electron transfer dissociation; FDR, false discovery rate; FT, Fourier transform; HCD, higher energy collisional dissociation; HPLC, high performance liquid chromatography; ICR, ion cyclotron resonance; IPI, International Protein Index; MS/MS, tandem mass spectrometry; SIM, selected ion monitoring.

14). One of the challenges in using top down approaches in proteomics has been to obtain cycle times commensurate with liquid chromatography tandem MS (LC MS/MS) time scales (15). The linear ion trap Orbitrap has also been employed for top down proteomics (16–19). Here we take advantage of the ultra high resolution of the Orbitrap Elite to enable fast LC MS/MS compatible top-down scan methods.

In bottom-up proteomics typically very complex peptide mixtures are analyzed (20–22). Online LC MS runs contain evidence for tens of thousands of peptides (23, 24) and this places a premium on the resolution of the survey (MS) scans. A popular shotgun proteomics method on the linear ion trap Orbitrap (LTQ Orbitrap or LTQ Orbitrap Velos) is a “1 s” survey scan with 60,000 resolution at m/z 400 (768 ms transient), and ion trap collision-induced dissociation (CID) scans of the ten or twenty most abundant ions (“high resolution” “low resolution” or “high–low” top10 method). Here we explore topN methods with much higher resolution survey scans as well as an increased number of fragmentation events per cycle enabled by “rapid CID” scans. A “high–high” strategy (high resolution MS as well as MS/MS (25)) has been routinely made possible on Orbitrap instruments by higher energy collisional dissociation (HCD) with the advent of the LTQ–Orbitrap Velos (26). We show that this strategy benefits from the shorter transients and higher resolving power possible on the Orbitrap Elite.

It has been demonstrated that a combination of two fragmentation methods can greatly augment sequence related information in peptide MS/MS (27–29) and we explore this dual approach with CID and HCD fragmentation of the same precursor ions.

EXPERIMENTAL PROCEDURES

The Orbitrap Elite is a further development of the LTQ Orbitrap Velos (26). This hybrid instrument combines a Velos PRO dual cell differential pressure linear ion trap mass spectrometer with a high field Orbitrap mass analyzer (Fig. 1A). The Velos PRO builds on the LTQ Velos (30) and its extensions include (1) a new generation of ion optics consisting of a 45° rotated bent quadrupole Q0, a neutral beam blocker, and an octopole ion transfer device, (2) faster ion trap mass analysis scan speed of 66,000 amu/s, and (3) a higher dynamic range detection system for improved quantitation performance, and (4) the addition of beam-type collisional dissociation capabilities for the stand-alone ion trap system.

New Generation Ion Optics—The S-lens consists of a set of stainless steel apertures to which an RF voltage is applied, alternate lenses having opposite (180°) phase. This device is used in a high pressure regime (low millibar) to efficiently focus the ion beam emerging from a transfer tube through a final exit lens (31). Droplets and solvent clusters exiting the transfer tube are kept from passing into the downstream ion optics by a curved quadrupole ion guide. The ion guide has been rotated 45° with respect to the orientation in the LTQ Velos so that noncharged droplets and solvent clusters can pass through the gap between the quadrupole rods, rather than impinge on the rod surface itself. This significantly reduces the potential for contamination of the quadrupole ion guide. The new geometry allows for a stainless steel rod to be positioned in the region of curvature of the quadrupole ion guide where it serves as a neutral beam blocker.

The combination of the 45° rotated quadrupole ion guide and the neutral beam blocker reduces the rate of contamination and improves the longevity of the ion optics system. A short octopole, Q00 (r_0 5.56 mm, rod diameter 2 mm, length 28.58 mm operated at 3 MHz, 800 Vpp), located between the exit lens and curved quadrupole, has also been added and replaces the quadrupole device in this region in the LTQ Velos. This octopole tends to be more robust to contamination and it is also used as a dissociation device in the stand-alone Velos PRO (32).

Faster Scan Speed—The dual cell differential pressure linear ion trap allows for substantially accelerated scan rates and higher resolution owing to the lower pressure in the mass analyzing cell of the ion trap (30). The normal scan rate on the LTQ Velos is 33,000 amu/s, which typically achieves peak widths of 0.34 amu at m/z 1822 and is sufficient to separate isotopes of triply charged ions. By optimizing operating conditions such as the resonance ejection amplitude and the phase relationship between the resonance ejection and trapping RF signals, a small sacrifice in resolution can give a large improvement in scan rate. In the Velos PRO, the scan rate has been doubled to 66,000 amu/s while still maintaining better than unit resolution, achieving an average peak width at half height of 0.47 amu at m/z 1822. With this rapid scan rate up to 12.5 MS/MS scans can be performed per second (rapid CID or rCID).

Higher Dynamic Range Detection System—Because of the faster scan rates the ion currents generated when performing mass analysis are also increased. Therefore, a higher dynamic range detection system is required. Discrete dynode electron multipliers have been developed (SGE Analytical Science Pty Ltd, Australia) that replace the continuous channel electron multipliers in the LTQ Velos. These new electron multipliers have linear outputs up to 160 μ A yielding six orders of magnitude dynamic range. A 24 bit analog-to-digital converter is employed in the electrometer circuitry which matches the performance of the discrete dynode multipliers. The wider linear dynamic range of this detection system increases the precision and accuracy for doing quantitative analysis while also offering enhanced limits of detection.

High-field Orbitrap Analyzer—The Orbitrap mass analyzer generally consists of an outer barrel-like electrode of maximum radius R2 and a central spindle-like electrode along the axis of maximum radius R1, with the outer electrode maintained at the virtual ground of the pre-amplifier, while the central electrode is at a voltage $-U_r$ ($U_r > 0$ for positive ions) (3). In a standard Orbitrap analyzer, R1 = 6 mm and R2 = 15 mm (5), whereas the high-field analyzer described here is more compact with R1 = 5 mm and R2 = 10 mm (Fig. 1C), *i.e.* the outer electrode is scaled down by a factor of 1.5. Similarly to an analyzer described recently (7), a decrease of the R2/R1 ratio from 2.5 to 2 allows an increase in the frequency in addition to the above scaling factor, thus bringing the total gain of frequency to \sim 1.8-fold. This is accompanied by an increase of the injection ion energy of \sim 1.4-fold. Despite the increase in space charge density in the analyzer by a factor of $(1.5)^3 \approx 3.4$, the additional shielding provided by the relatively thicker central electrode keeps space-charge induced frequency shifts even slightly below those in the standard analyzer.

As the injection slot was scaled down by the same factor as the outer electrode to avoid compromising the quality of the field inside the analyzer, an additional focusing of the incoming ion beam became necessary. This was achieved by adding a miniature Einzel lens in the form of a 1-mm plate with a 2 mm ID orifice at a voltage of up to 1000 V, which was sandwiched between two similar plates at 0 V and separated by gaps of 1 mm. This assembly was mounted on the same block as the deflector at the entrance to the Orbitrap analyzer.

Scaling down of the outer electrodes appeared also to slightly reduce their capacitance to ground and to each other, which in turn allowed the use of lower-capacitance transistors in the image current

preamplifier. The resulting sensitivity increase of about 30% resulted in the same signal-to-noise ratio for the same number of ions as in the standard analyzer even for twice shorter transients.

We found that reduction of the gap between the outer and the central electrodes requires an almost proportional improvement of machining accuracy of the electrodes. This was achieved by rigorous refinement of the existing manufacturing and measurement techniques.

Transient Processing with eFT—FT of a digitized transient is a fast processing method but it requires relatively long detection times to achieve high resolving powers. It is thus desirable to further increase the resolving power for a given acquisition time. We applied a newly developed enhanced version of the Fourier Transformation (eFT™), which is also employed in another novel instrument, the Q Exactive (11). Details of the technique can be found in ref (10). Briefly, both eFT and conventional FT make use of complex numbers, which can be represented by magnitude and phase. As the initial phase of the ion package typically depends on initial parameters of the ions in a very complicated way (8), FT spectra normally have to be presented in the so-called magnitude mode, which amounts to disregarding the phase information. However, in Orbitrap mass spectrometers the built-in excitation-by-injection mechanism (33) provides an initial phase of ion oscillations that is almost independent of m/z . This synchronization allows converting spectra in such a way that they correspond to zero initial phase for all m/z values (so-called absorption spectra) and exhibit narrower peaks. In practice, eFT uses a combination of magnitude and absorption spectra along with Hanning apodization, triple zero-filling, and additional filtering to improve mass accuracy and peak shape.

Better accuracy of spectra conversion is achieved if detection starts as early as possible after ion injection. Therefore, the following modifications of the preamplifier and Orbitrap analyzer were introduced: (1) High-speed diode bridges have replaced mechanical relays that were previously used for protection of the preamplifier during pulsing of the Orbitrap central electrode, such that the preamplifier is always ready for detection, (2) the capacitance between deflector at the entrance to the Orbitrap analyzer and each of detection electrodes was balanced by modifying the deflector geometry, (3) the capacitance between each of detection electrodes and ground was reduced and also balanced by replacing ceramic isolators with quartz ones as well as by changing the geometry of the Orbitrap holder. (4) The capacitance between Einzel lens elements at the entrance to the Orbitrap analyzer and each of these detection electrodes was minimized by implementing this lens as a miniature ceramic printed-circuit board.

Together, these measures allowed reducing the delay between ion injection and start of transient detection from almost 10 ms to a fraction of a millisecond. In addition to improved eFT, this reduction of delay allows to capture the entire first beat of the transient (see e.g. (34)) even for large proteins like intact antibodies and therefore significantly improves sensitivity of Orbitrap detection for top down analysis.

The practical implementation of the eFT achieves up to twofold increase of resolving power for the same transient. For rapidly decaying signals, for example from proteins, this gain is reduced to about 1.4-fold because of background collisions (8). The dual-spectrum online processing is computationally demanding but still fast enough to be completed in the LC MS time scale. Thus cycle time is still determined by transient acquisition and ion injection times and not by processing of the data. The eFT method is sensitive to precise synchronization of the instrument electronics and remaining shot-to-shot jitter, so that final mass accuracy is comparable to that of traditional magnitude mode FT spectra. Side-lobes in eFT spectra are comparable to those in conventional FT spectra.

Sample Preparation—Intact proteins (all from Sigma Aldrich) were dissolved in buffer A (98% water, 2% acetonitrile (ACN), 0.1% formic acid) prior to LC MS analysis. For direct infusion experiments, protein stock solutions were diluted in 50% ACN, 50% water, 0.1% formic acid.

HeLa cells were lysed in urea (6 M) and thiourea (2 M) solution. The protein mixture was reduced with dithiothreitol (1 mM) for 30 min at room temperature and alkylated with iodoacetamide (55 mM) for 20 min. The proteins were first digested with LysC (1 $\mu\text{g}/50 \mu\text{g}$ protein) (Wako, Richmond, VA) for 3 h at room temperature. The sample was diluted (1:4) with water before 12 h incubation with trypsin (1 $\mu\text{g}/50 \mu\text{g}$ protein) (Promega, Charbonnières, France) at room temperature. Formic acid (3%) was added to the mixture to quench enzyme activity. The peptide mixture was desalted on reversed phase C₁₈ StageTips (35) and eluted into 8 well autosampler vials with 60 μl buffer B (80% ACN in 0.5% acetic acid). ACN was removed in a SpeedVac concentrator. The sample volume was adjusted with buffer A (2% ACN in 0.1% TFA) to 12 μl .

LC MS/MS Analysis—Intact proteins were separated on a nano-bore analytical column (75 μm ID \times 10 cm) with an integral fritted nanospray emitter (PicoFrit®, New Objective, Inc., Woburn, MA) containing 5 μm polymeric reversed-phase media (1000 Å pore size) using an EASY-nLC system (Thermo Scientific), which was operated at a flow rate of 300 nL/min. A linear gradient of each 20 min 10–50% buffer B and 50–80% buffer B (80% ACN in 0.1% formic acid) was applied. This setup was extended with a trap column (150 μm ID \times 2 cm) containing identical chromatographic material. MS data were acquired by a relatively low resolution survey scan (10 microscans, resolution 15,000 at m/z 400), followed by a data dependent selected ion monitoring (SIM) scan of the most abundant (5 microscans, resolution 120,000 at m/z 400, isolation width 10 Th) and a data dependent HCD scan of the most abundant ion of the SIM scan (5 microscans, resolution 120,000 at m/z 400, isolation width 10 Th, normalized collision energy 18%). Dynamic exclusion duration of 10 s was enabled. A cycle time of 5.8 s was achieved. A modified method in which the data dependent SIM scan was replaced by a data dependent HCD scan at a different normalized collision energy, was used to increase the number of identified fragment ions.

The peptide mixture from a tryptic HeLa digest was separated with a linear gradient of 5–60% buffer B (80% ACN and 0.5% acetic acid) at a flow rate of 300 nL/min on a C₁₈-reversed phase column (75 μm ID \times 15 cm) packed in-house with ReproSil-Pur C₁₈-AQ 3 μm resin (Dr. Maisch GmbH) in buffer A (0.5% acetic acid). An Easy-nLC chromatography system (Thermo Scientific) was on-line coupled to the Orbitrap Elite instrument (Thermo Scientific) via a Nanospray Flex Ion Source (Thermo Scientific). MS data were acquired in a data-dependent strategy selecting the fragmentation events based on the precursor abundance in the survey scan (300–1650 Th). The resolution of the survey scan was varied between 60,000, 120,000, and 240,000 at m/z 400 Th with a target value of 1e6 ions and 1 or 2 microscans. Low resolution CID MS/MS spectra were acquired with a target value of 5000 ions in normal and rapid CID scan mode. MS/MS acquisition in the linear ion trap was partially carried out in parallel to the survey scan in the Orbitrap analyzer by using the preview mode (first 192 ms of the MS transient). The maximum injection time for MS/MS was varied between 25 ms and 200 ms. HCD MS/MS spectra were acquired with a resolution of 15,000 and a target value of 40,000, setting the first mass to 120 Th. Dynamic exclusion was 60 s and early expiration was disabled. The isolation window for MS/MS fragmentation was set to 2 Th.

Data Analysis—High-resolution mass spectra of the intact proteins were deconvoluted using the Xtract software (Thermo Scientific) and further processed with ProSightPC (36, 37) (Thermo Scientific). The

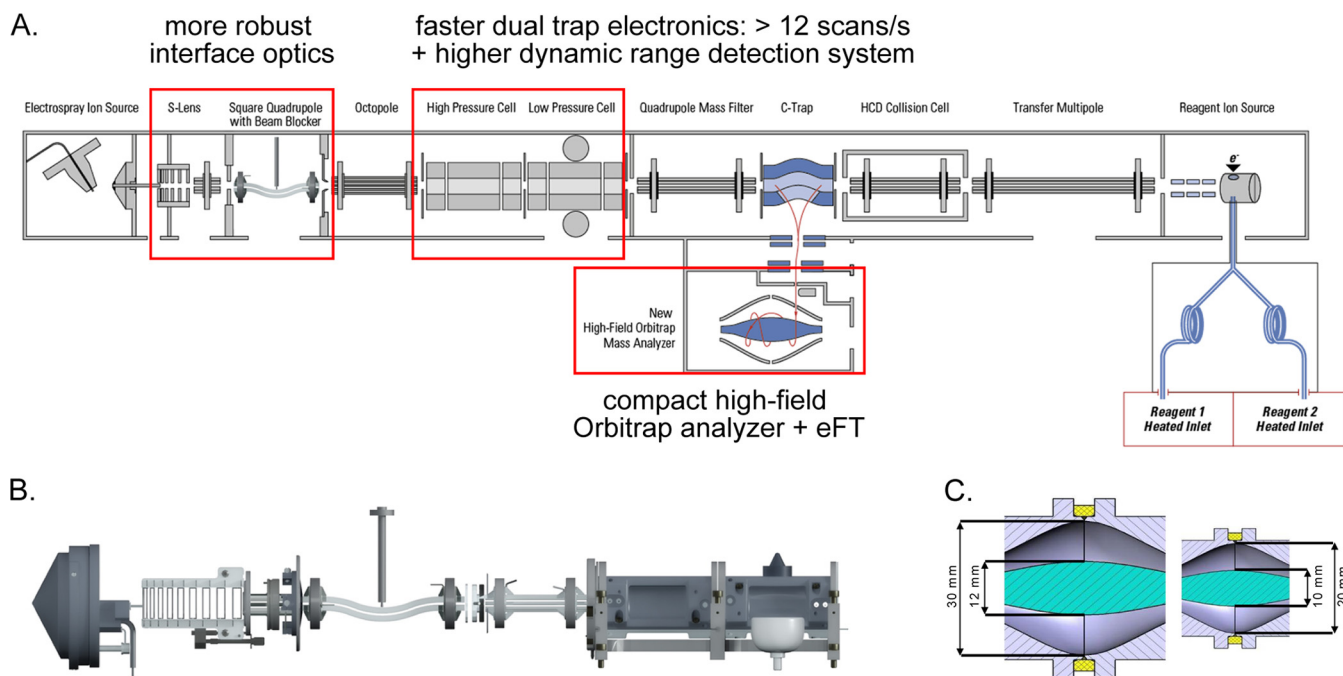


FIG. 1. The Orbitrap Elite mass spectrometer. *A*, Novel elements compared with the LTQ Orbitrap Velos are highlighted and encompass the source region, the dual linear ion trap and the Orbitrap analyzer. ETD fragmentation is optional. *B*, Computer model of the inlet ion optics, showing the S-lens on the left and the dual ion trap on the right. The bent, square transfer quadrupole allows neutrals to leave the ion optics and impinge on the depicted beam blocker. *C*, Comparison of dimensions of the standard (*left*) to the compact, high-field Orbitrap analyzer (*right*).

analysis of the mass spectrometric RAW data was carried out using the MaxQuant software environment (developmental version 1.2.0.23) applying standard settings unless otherwise noted. Peptide scoring for identification is based on a search with an initial allowed mass deviation of the precursor ion of up to 7 ppm. To further improve the precursor mass accuracy a time-dependent recalibration algorithm was applied. Fragment mass tolerance was 0.5 Da for low resolution and 20 ppm for high resolution spectra. Enzyme specificity was defined as C-terminal to Arg and Lys including proline bond cleavage and a maximum of two missed cleavages. Carbamidomethylation of cysteine was set as fixed modification and N-terminal protein acetylation and methionine oxidation as variable modifications. MS/MS spectra were searched against the IPI human data base (version 3.68, 87,061 entries) combined with 262 common contaminants by the Andromeda search engine, with 2nd peptide identification enabled (38). The FDR for proteins and peptides (which had to have at least 6 amino acids) was set to 0.01. Results provided by MaxQuant were further analyzed using the R scripting and statistical environment. The data sets are provided at Tranche (www.proteomecommons.org) using the following hash: ZtXtC5NwSlxEwDzZKID3b14XYnUCo7nKb-WAnlGRZXORy3eoXWSodhh/w7SZBxTAZbqoDPDs8FGkVcBB-pe8N1fC1+M1EAAAAAAAAANYw==.

RESULTS AND DISCUSSION

Overview of the Orbitrap Elite—Like its predecessors, the LTQ Orbitrap and LTQ Orbitrap Velos, the instrument is a combination of two mass analyzers, a linear ion trap and an Orbitrap mass analyzer (Fig. 1). It offers up to three fragmentation modes, CID or ETD in the dual linear ion trap, or HCD in its dedicated collision cell. The fragment ions produced by CID or ETD are normally analyzed in the ion trap at low

resolution and in parallel with the acquisition of the MS transient. However, they can also be transferred to the C-Trap and recorded in the Orbitrap analyzer. HCD fragments are always analyzed in the Orbitrap analyzer.

In the Orbitrap Elite instrument, as discussed in detail in EXPERIMENTAL PROCEDURES, the interface optics in the front part of the instrument now incorporates a rotated square transfer quadrupole with a beam blocker preventing neutral or low charged material from passing. The dual ion trap was equipped with new electron multipliers that tolerate up to 10-fold higher ion currents, which improves the dynamic range of the device. This in turn allowed further speed-up of the low resolution CID fragmentation mode. This scan mode was named rCID for rapid CID and it allows acquisition of up to 12.5 MS/MS spectra per second (EXPERIMENTAL PROCEDURES). The changes in interface optics together with the improved dual ion trap constitute the Orbitrap Velos Pro instrument, whereas the central feature of the Orbitrap Elite instrument described in this publication is a novel Orbitrap analyzer with drastically improved resolving power.

A detailed description of the principles and construction of the novel Orbitrap analyzer is given in EXPERIMENTAL PROCEDURES. Briefly, the major hardware change was a reduction in the inner diameter of the outer electrode from 30 to 20 mm whereas the size of the spindle-shaped inner electrode was only reduced from 12 to 10 mm (Fig. 1C). These smaller dimensions, with an increased ratio of inner to outer electrode

diameters, lead to a higher field strength, resulting in almost doubling of the resolving power at the same scan time. An improved signal processing algorithm (eFT), which takes phase information into account (EXPERIMENTAL PROCEDURES), provides a further boost by a factor up to 2. Together, the Orbitrap Elite instrument achieves about fourfold higher resolution at the same transient length. This translates into 240,000 resolution at m/z 400 with the standard 768 ms transient—a radical improvement over the 60,000 resolving

power achieved by the predecessor instrument. This very high resolution per unit time can alternatively be used to shorten cycle times at the same resolving power. An overview of the different resolution settings as a function of transient length can be found in Table I. Even the shortest transient (48 ms), results in a resolving power of 15,000, twice that of the 96 ms scan typically used for MS/MS on the Orbitrap Velos. In principle, even shorter transients would produce sufficient resolution for MS/MS. However, we decided against such scan modes because the ratio of useful transient time compared with overhead times and ion filling times would become unfavorable.

Top Down at an LC Time Scale—High resolution is an important requirement for resolving the different charge states of intact proteins investigated in top-down proteomics. We selected carbonic anhydrase II, a frequently used standard in top-down proteomics, to investigate the advantages of the increased resolving power and sequencing speed of the instrument. We devised a method that alternates between a survey scan with 15,000 resolution followed by a high resolution SIM scan of a particular charge state acquired in a data dependent manner. This same precursor m/z is subsequently

TABLE I

Resolution and transient length of Orbitrap hybrid instruments. The highest resolution on Orbitrap Elite (indicated by an asterisk) can only be activated when using the developer's kit

Resolution		Transient
LQ Orbitrap	Orbitrap Elite	
–	15,000	48 ms
7500	30,000	96 ms
15,000	60,000	192 ms
30,000	120,000	384 ms
60,000	240,000	768 ms
120,000	480,000*	1536 ms

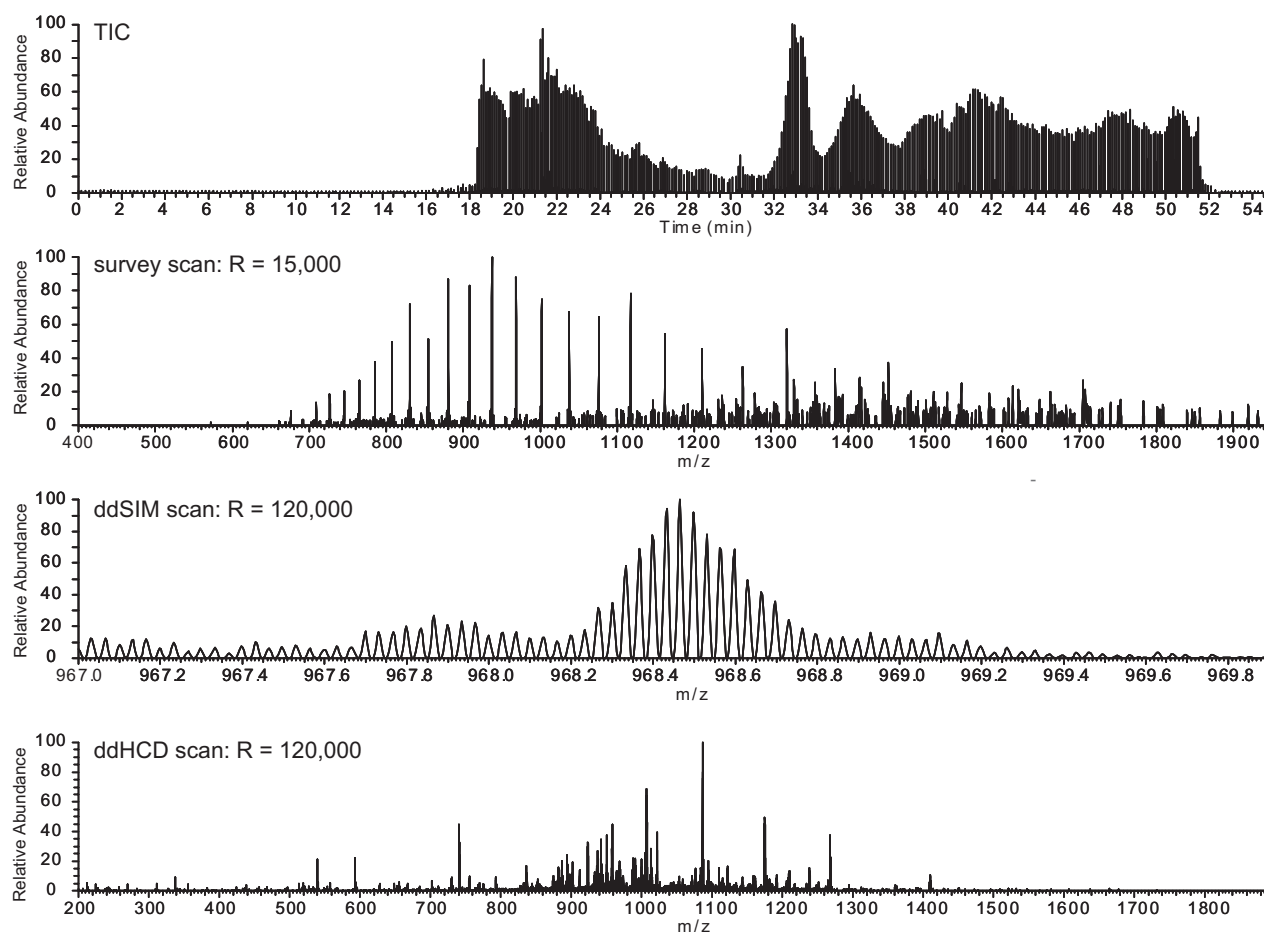


FIG. 2. **Top down method at an LC time scale.** Total ion chromatogram of an LC separation of carbonic anhydrase II (29 kDa). Fast survey scans reveal the charge envelope and are followed by high resolution SIM and HCD scans. Overlapping fragment isotope distributions are clearly resolved from each other.

fragmented by HCD and analyzed at a high resolving power of 120,000 (Fig. 2). We found that the Orbitrap Elite is capable of isotopically resolving and measuring this 29 kDa protein with a root mean square mass accuracy below 2 ppm under these conditions. Averaging times for five to six microscans of the HCD fragmentation spectra were much shorter than in the LTQ Orbitrap Velos, without reducing signal-to-noise. Deconvolution with Xtract of a single, six microscan HCD spectrum, in which charge state 34+ carbonic anhydrase was fragmented, revealed 30 b-type and 31 y-type ions (supplemental Fig. S1). For enolase (46.6 kDa), 15 b-type and 20 y-type ions were identified following fragmentation of the 58+ precursor charge state by HCD. For both of these model proteins optimizing the MS/MS parameters such as increasing the collision energy but mainly averaging of up to 24 microscans increased the number of identified fragment ions to 49 b-type and 48 y-type ions for carbonic anhydrase II and 30 b-type and 42 y-type ions for enolase (supplemental Fig. S1).

Ultrahigh Orbitrap Resolution for Top Down Experiments— The implementation of the compact Orbitrap analyzer and eFT signal processing allows the Orbitrap Elite to reach a comparably high resolution as a 17 Tesla FT ICR instruments at m/z 400 Th and with standard signal processing methods. In contrast to the inversely linear dependence of FT ICR resolving power on m/z , however, the Orbitrap resolving power is inversely proportional to the square root of m/z (1). Therefore, for subsecond acquisition on an LC-MS time scale its resolving power at m/z 1000 already corresponds to a 25 Tesla FT-ICR instrument, and it should be particularly suitable for top down experiments of larger proteins such as BSA (66.4 kDa) and enolase (46.64 kDa). Using static electrospray conditions we found that charge state 47+ of intact yeast enolase could readily be baseline resolved at a resolution setting of 240,000 corresponding to 768 ms transients (Fig. 3A). To reach this resolution, a vacuum better than 10^{-10} Torr was necessary. On the predecessor instrument, partial resolution of intact yeast enolase could occasionally be observed (Fig. 3B).

Parallel topN CID Method for Bottom Up Proteomics— For the analysis of complex peptide mixtures, a standard mode of operation is the acquisition of a survey spectrum in the Orbitrap analyzer while the linear ion trap isolates, dissociates, and scans the fragments. For this “high-low” mode, we evaluated the influence of the higher resolution in the survey spectra and of the faster MS/MS scans. LTQ Orbitrap instruments select precursors for fragmentation on the basis of a snapshot or preview spectrum—the first 192 ms of the MS transient—after which time the CID scans are initiated in the LTQ. However the resolution is increased fourfold, allowing isotopic resolution for all charge states and the entire m/z range (300–1650) at higher signal-to-noise. We found that this increased the quality of precursor ion selection.

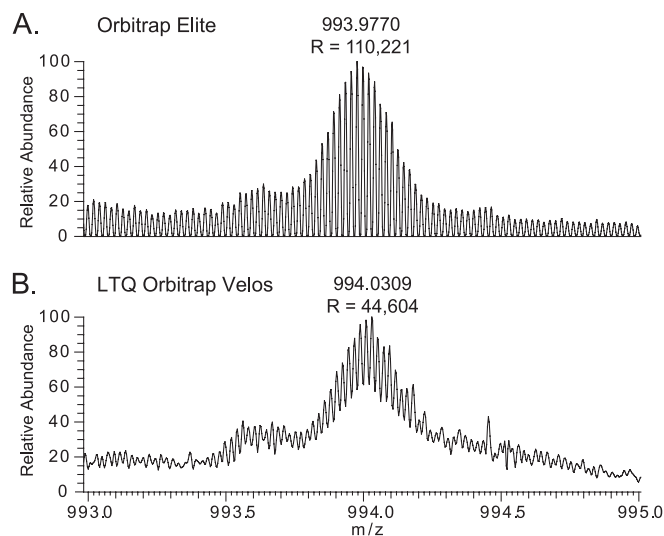


FIG. 3. Isotope resolved spectrum of enolase with Orbitrap Elite and LTQ Orbitrap Velos instruments. Spectra were acquired with transients of *A*, 768 ms on the Orbitrap Elite and *B*, 1536 ms on the LTQ Orbitrap Velos. In these conditions, the superior resolution of the Orbitrap Elite instrument ($>2 \times$ higher in $2 \times$ shorter transient time) helps to baseline resolve the 47+ charge state of the intact yeast enolase.

To investigate the instrument capability for the analysis of very complex peptide mixtures such as HeLa cell lysate, we started with a digested standard of 400 ng that was analyzed using different methods. Comparison of the normal CID and rapid CID scan modes revealed that rCID produced significantly more fragmentation events and therefore this mode was chosen for all subsequent experiments. The scheme in Fig. 4A shows the timing sequence of MS and MS/MS scans at different MS transient lengths. For a 192 ms survey scan, resolution is 60,000 and there is no parallel operation between MS and MS/MS scans. Due to rCID an entire top20 method takes 2.7 s, easily compatible with peptide LC elution profiles. At 384 ms resolution is 120,000 and a few MS/MS scans are performed in parallel with the survey scan. At the full 768 ms transient (240,000 resolution), about six CID spectra are performed in parallel, while total cycle time is still unchanged (Fig. 4A). Therefore, the longest transients appear to be advantageous because the increased resolution comes “for free” as it does not cost extra measurement time.

We make use of the fixed cycle time to explore the benefits of high resolving power on complex peptide mixture analysis. Three top20 methods were established as outlined above using 60,000, 120,000, and 240,000 resolution for the survey scan (Fig. 4A). When analyzing the HeLa peptide mixture with these methods, we found that the number of potential peptide features (isotope clusters) detected by MaxQuant, nearly doubled from 93,000 at 60,000 resolution to 148,000 at 240,000 resolution under otherwise identical conditions (Table II). Fig. 4B shows a zoom into the LC MS map of the 240,000 reso-

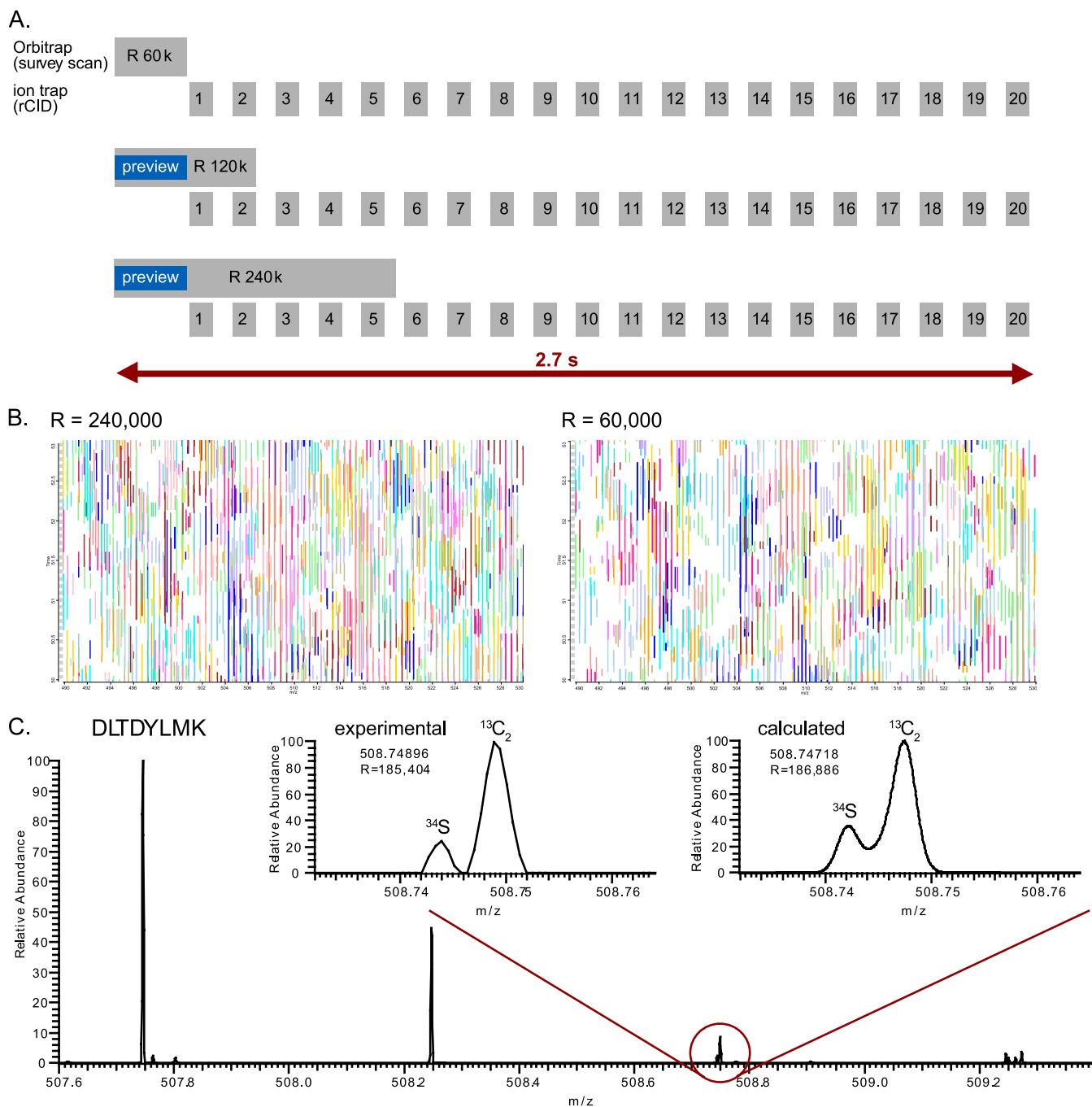


FIG. 4. Parallel CID top20 method and ultra high resolution survey scans. *A*, High resolution MS scan at three different transient lengths followed by 20 CID MS/MS scans in the linear ion trap. Note that cycle time is unaffected by the resolution of the full scan. Preview refers to the portion of the survey scan that is used to select precursor ions for fragmentation. *B*, LC MS heat map of peptides eluting over a 3 min elution time interval in a 40 Th range. More detail is visible in the ultra high resolution setting (*left* panel) compared with the normal resolution setting (*right* panel). *C*, Separation of isobaric species in standard LC MS/MS analysis. The ^{34}S isotope containing peak is clearly resolved from the $^{13}\text{C}_2$ isotope.

lution versus the 60,000 resolution run and demonstrates the rich feature set in complex peptide mixtures with 540 versus 320 isotope clusters, respectively.

ICR Fourier transform mass spectrometry measurements of small molecules can resolve the fine structure in the isotope

patterns of small molecules (isobaric species with the same nominal mass) (39), although to our knowledge this has not yet been reported in proteomics LC MS/MS experiments. We inspected the methionine and cysteine containing peptides and found that the ^{34}S isotope and $^{13}\text{C}_2$ peaks ($\Delta M = 0.011$

Da) were clearly resolved from each other (Fig. 4C). Thus, the high resolution immediately indicates the presence of a sulfur atom in the peptide.

Based on the above observations, we selected a 240,000 resolution survey scan and top20 CID scan as the standard high-low method. Analysis of the 2h gradient of the 400 ng HeLa peptide sample identified 11,543 unique peptides and 2268 proteins (supplemental Tables. S1–S4), an increase of 25–30% over the analysis of the same sample on the LTQ Orbitrap Velos. Although not demonstrated here, quantification accuracy was also observed to increase due to the several-fold higher resolution.

TABLE II
Number of isotope clusters depending on survey scan resolution at equal cycle time

Resolution	Cycle time	MS scans	Isotope clusters
60,000	2.76	1902	93,624
120,000	2.87	1821	124,808
240,000	2.85	1843	148,085

High Resolution MS/MS Methods—The Orbitrap Elite offers CID, HCD and optional ETD fragmentation modes. Analysis of the fragments is either in parallel mode in low resolution in the linear ion trap (for CID and ETD) or in sequential mode in the Orbitrap analyzer (for CID, ETD, and HCD). In principle, all these modes can be mixed and matched according to the analytical question under investigation. Fig. 5 depicts three prototypical combinations of scan modes. In a pure HCD mode, a survey scan is acquired and is followed immediately by N HCD spectra that are also recorded in the Orbitrap analyzer. A 384 ms survey scan already provides resolution of 120,000, which is adequate for most applications and which helps to limit the overall cycle time in this mode. HCD spectra are acquired at the lowest possible resolution (48 ms transients; 15,000 resolution at m/z 400). A complete top15 sequence takes 3.3 s, about the same time required for a top10 method with half the resolution in MS and in MS/MS mode in the predecessor instrument. We measured the 400 ng HeLa cell lysate sample with this method and obtained significantly improved numbers of peptide identifications compared with

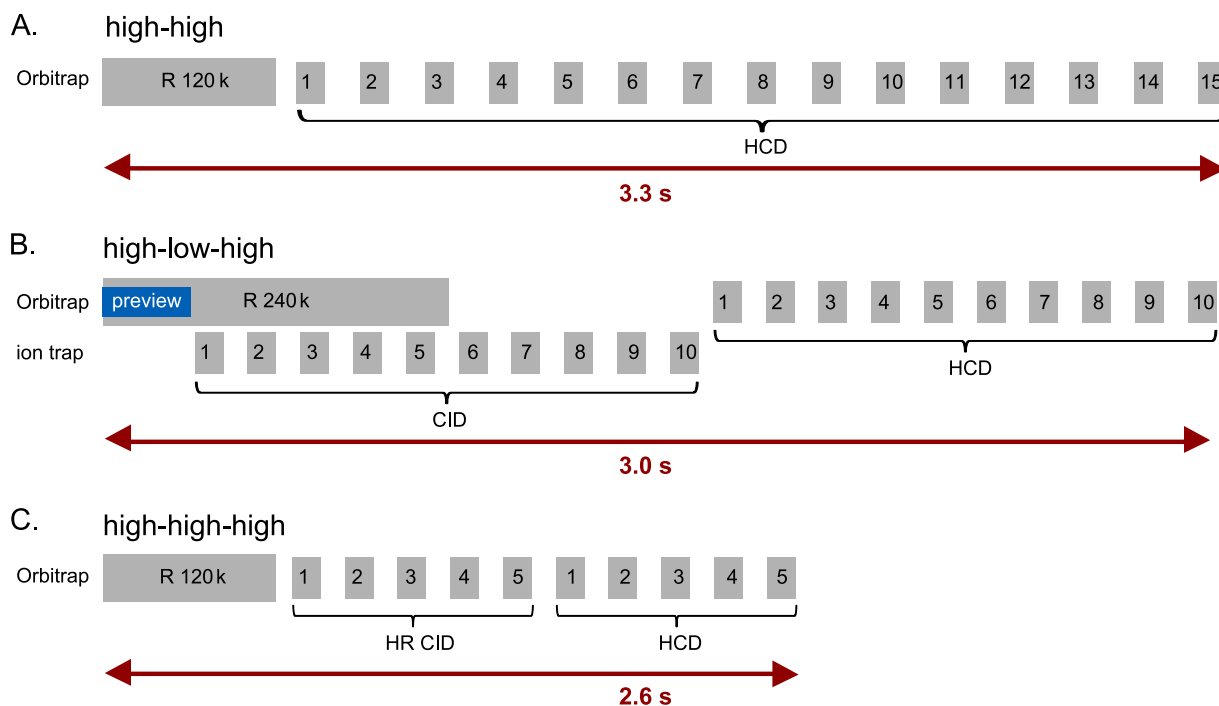


FIG. 5. **Combinations of fragmentation modes.** A, Sequential mode in which a high resolution survey scan with 120,000 resolution (384 ms) is followed by 15 HCD scans at 15,000 resolution (48 ms transients). B, Parallel and sequential mode in which an ultra high survey scan with 240,000 resolution is acquired in parallel with 10 rCID spectra in the linear ion trap and in sequence with 10 HCD scans that are analyzed in the Orbitrap analyzer. The same precursors are analyzed by rCID and HCD. C, Double sequential mode in which a 120,000 resolution survey scan is followed by 5 high resolution CID and 5 HCD spectra of the same precursors.

TABLE III
Protein and peptide identification from HeLa duplicate analysis of a top15 HCD method

	MS spectra	MSMS spectra	Identifications [%]	Unique peptides	Proteins	Isotope clusters
HCDtop15(1)	1556	23,211	45.77	10,847	2082	116,632
HCDtop15(2)	1538	22,930	45.82	10,633	2064	115,370

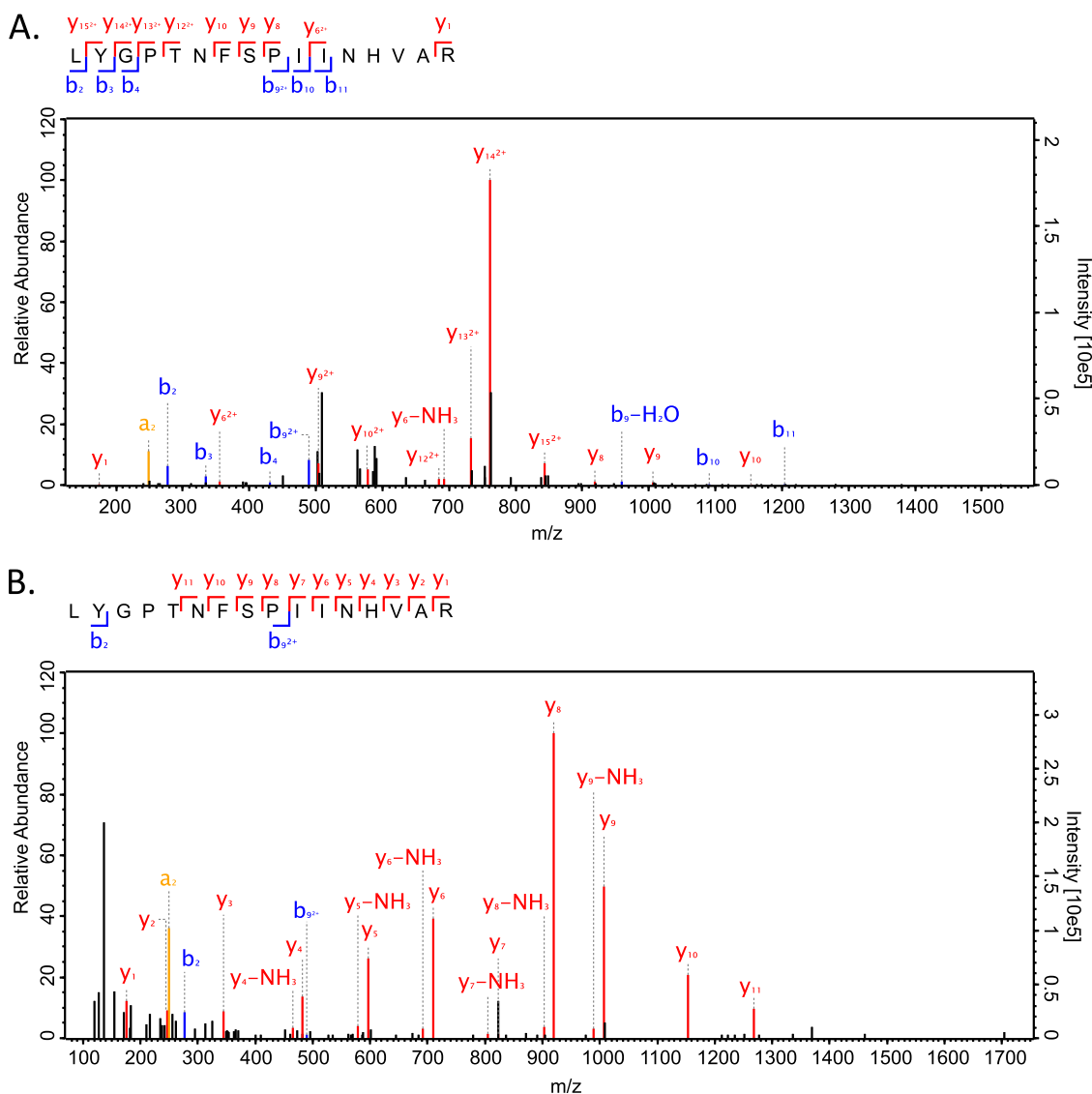


FIG. 6. Complementary CID + HCD MS/MS spectra. Example spectra using the parallel and sequential fragmentation mode depicted in Fig. 5B. *A*, The rCID spectrum features more b-ions than the HCD spectrum. *B*, HCD spectrum with nearly complete y-ion series. Mass accuracy in *B* but not in *A* is in the ppm range (absolute average deviation of 3.67 ppm. *versus* 0.06 Th).

LTQ Orbitrap Velos measurements (Table III). This high-high HCD mode offers high sequencing speed and high mass accuracy MS/MS spectra and is therefore well suited to the analysis of complex mixtures. In comparison to the CID method described above, it achieves similar numbers of peptide fragmentation events because it is slightly faster than CID in the linear ion trap but does not have parallel operation. Target values for fragmentation are somewhat higher in HCD (40,000 *versus* 5,000 ions).

The high scan speeds of this instrument also makes more complex scan modes possible. For instance, the parallel low resolution scan mode can be followed by high resolution, sequential HCD Orbitrap analyzer scans (Fig. 5B). To test this mode, we selected up to 10 precursors for fragmentation by CID and repeated fragmentation of the same candi-

dates by HCD. As can be seen in the schematic, this mode makes particularly good use of the hybrid instrument's capabilities. The initial parallel operation with CID fragmentation allows ample time to perform a survey scan with 240,000 resolution without affecting total cycle time. In principle, it results in two fragmentation spectra for each of the peptides. The advantages are illustrated in Fig. 6, the *upper* panel of which shows the CID spectrum of the peptide LYGPITNFSPIIINHVAR, while the *lower* panel shows the corresponding HCD spectrum acquired subsequently in the same cycle. The CID spectrum has the typical mixture of b- and y-ions, whereas the HCD spectrum contains a nearly complete series of y-ions but few b-ions. Both spectra together account for all possible y-ions as well as a large proportion of all b-ions of this peptide. Importantly, this dual

fragmentation information comes at an acceptable cost in cycle time and sequencing speed. This parallel top10 CID + HCD method had a total cycle time of 3.0 s, very similar to the top15 HCD method. When targeting the same precursors, it sacrifices some sequencing speed but gains in complementary fragmentation information. Thus it may be particularly interesting in applications where relatively high sequencing speed is important but where peptide identification is challenging, for instance in the analysis of post-translational modifications at a large scale.

The final method depicted in Fig. 5C is a further step in the same direction. Here, two fragmentation modes are applied but all MS/MS scans are performed in the Orbitrap analyzer. Therefore this mode is completely sequential and does not use the linear ion trap as a scanning mass spectrometer. A 120,000 resolution survey scan followed by sequential top5 CID + HCD scans takes about 2.6 s. When targeting the same precursors, it sacrifices sequencing events compared with the above methods. However, high resolution MS/MS spectra are obtained by two different fragmentation methods, yielding maximum information of the primary structure. Therefore the sequential top5 CID + HCD scan mode would be very attractive for applications with limited peptide complexity but high demands on peptide characterization. This could be the case in traditional, single protein applications, in proteomics when no complete database is available, or generally when unusual modifications are expected.

CONCLUSIONS AND OUTLOOK

Here we have described the Orbitrap Elite, a mass spectrometer that achieves fourfold improved resolving power by increasing the electric field strength in the Orbitrap analyzer and by enhanced Fourier Transformation. The high resolving power enables ready isotopic resolution of proteins in the BSA mass range as well as characterization of their fragments in a chromatographic time scale. In bottom-up proteomics, the instrument allows high-low topN CID methods featuring ultra high resolution survey scans and a large number of parallel MS/MS experiments in the linear ion trap. For instance, we demonstrated the combination of a survey scan of 240,000 resolution with 20 CID scans all within a 2.7 s cycle time. Remarkably, this high resolution routinely enabled resolving isobars of sulfur-containing peptides. We also explored the acquisition of CID and HCD spectra of the same precursor ions, with either parallel or sequential analysis of the fragmentation spectra. Although not shown here, CID and HCD fragmentation events could also be distributed to different peptide classes. Further fragmentation modes, such as ETD, can also be incorporated. Moreover, the product ions of one fragmentation method could be dissociated again by the same or other fragmentation methods—for example, CID could be followed by HCD and resulting fragments could be recorded in the Orbitrap analyzer—all at a rapid time scale. All these methods are possible in principle and it will be interest-

ing to develop them for a wide range of proteomic and other applications.

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§ This article contains [supplemental Tables S1 to S4 and Fig. S1](#).

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Data availability: Supplementary data is available with this publication at the MCP web site. Raw MS files are uploaded to Tranche (www.proteomecommons.org) as “Michalski et al. Elite”.

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