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Exploring frontiers of orbitrap performance for long transients

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ABSTRACT

Experiments on a modified Orbitrap Exploris instrument have shown that even in a compact instrument it is possible to achieve a resolving power in excess of 2,000,000 when appropriate tolerance and tuning requirements are met. Such levels are achieved for a 4-s detection time.

A new Orbitrap design with improved pumping allowed, for the first time, isotopic resolution of intact monoclonal antibodies even when using standard nitrogen supply in the Ion Routing Multipole. Longer transients further improve signal-to-noise ratios, approaching levels needed for single-charge resolution in charge detection mass spectrometry. Further steps for improvement of instrumentation are discussed.

1. Introduction

It is a distinct pleasure and honor for the authors to take part in this Special Issue of the International Journal of Mass Spectrometry, and utilize this opportunity to celebrate the decades-long bridge connecting Finnigan MAT of Curt Brunnee's times to the modern Bremen facility of Thermo Fisher Scientific.

Although the authors are working for essentially the same research and development organization led originally by Dr. Curt Brunnee, tectonic shifts in the world of mass spectrometry changed this organization to perhaps even a greater degree than they transformed the famous mass spectrometry archipelago described in Curt's review of 1987 [1] (Figure 183). Coincidentally, it was in fact this review that inspired one of authors (AM) to initiate his own quest for an ideal mass analyzer. This quest eventually enrolled more and more people, first in Manchester (UK) where another author (ED) joined the effort in 2001, and then moving to Bremen (Germany) as a part of the re-birth of the factory based on new instrument technology. There, an ever-growing team finally crystallized and then started steadily growing another island in this archipelago-the Orbitrap™ island [2]. Linked to the "quadrupole" and "ion trap" islands of mass analyzers by expansive bridges of hybrid [3–9] and Tribrid™ [10,11] architectures over the following 15 years, it brought a progressively rising tide of high-resolution accurate-mass (HRAM) analysis to the ocean of analytical science.

Curt Brunnee himself was delighted to witness this transformation as he visited the Bremen site in 2009 after a long absence when he was preparing to award AM with the prize named after him. Following his retirement from the post of R&D director in the factory in early 1990s, he devoted his life to studying philosophy and pursuing different hobbies, as was eloquently described in the literature [12]. Nevertheless, as we toured the lab, he appeared to be surprised to find himself excited to rediscover his past passion for mass spectrometry. While paying tribute to the previously unimaginably high performance of the new instrument breeds, he also was delighted to see that his lifelong work on magnetic sectors has been actively advanced in new models of those instruments. This started a new tradition of AM occasionally giving a tour of new instruments to Curt Brunnee, usually by quietly sneaking out from a yearly Retiree Celebration in December. The last such tour was devoted to the latest Orbitrap generation: the Orbitrap Exploris family [13,14], that became the testbench for results of this publication.

While most of the development efforts for the Orbitrap Exploris platform focused on reducing instrument size and improving ease of use, serviceability, robustness, and intelligent acquisition, there were also some important changes introduced to the seemingly mature Orbitrap technology. This article explores how even such a compact benchtop mass spectrometer could be nevertheless used to reach new levels of Orbitrap performance.

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2. Materials and methods

Experiments were carried out on a standard Orbitrap Exploris 480 mass spectrometer (MS) [13,14] using a high-field Orbitrap assembly specially selected from a batch of serial assemblies. Fig. 1 presents schematics of the instrument and overviews the main units and operating pressures in each of regions, with a detailed description presented in Ref. [14].

As shown in Fig. 2, the horizontally aligned and symmetrically supported Orbitrap assembly is open to pumping on both ends and combines improved mechanical and electrical balancing with better vacuum inside the trap.

Ions are detected with 4 kV applied between the central and the outer electrodes, which is somewhat lower than the 5 kV used in previous instruments to facilitate a more compact and robust design. Unlike previous Orbitrap instrument families, pulsing of the central electrode has an adjustable starting point in time prior to the pull-out pulse on the C-trap. With an adjustable starting voltage and a fixed ramp time constant determined by the Orbitrap capacitance and an input resistor, this enables electrodynamic squeezing in the trap [3] of a very broad mass range: m/z 40 to 8000. This feature additionally allows the use of a single calibration voltage set for the entire mass range.

Being integrated with the C-trap and suspended on a vibration-damping plate, the Orbitrap assembly is enclosed in an aluminum block directly bolted on a six-stage SplitFlow XL turbomolecular pump (Pfeiffer Vacuum, Asslar, Germany). The use of a single, purpose-developed, turbomolecular pump to evacuate the entire analyzer enabled drastic reduction of instrument footprint and volume. At the same time, it created the need to explore whether any limitations on Orbitrap performance could result from such an unprecedented attempt to cover ten orders of magnitude from ultra-high vacuum (UHV) to forevacuum in a single pump. The authors addressed this need by going beyond the already increased resolving power settings of a standard instrument.

For the most pressure-sensitive analysis of intact proteins [15], experiments took advantage of a wide range of pressure control in the instrument in the intact protein mode, with the pressure of the ultra-high purity nitrogen bath gas directly measured in the Ion Routing Multiple (IRM) and controlled by an open-split flow controller in the gas line. Nitrogen flows from the IRM into the C-trap, where it is used to thermalize ions prior to injection into the Orbitrap analyzer. As gas also affects UHV pressure in the analyzer via both leakage through slits in ion optics and back-streaming through the turbopump, the design of the instrument was optimized to ensure that pressure rise in the UHV region remains less than 5 parts per billion of IRM pressure.

While a standard Orbitrap Exploris 480 MS features resolving powers from 7500 to 480,000 at m/z 200 (16–1024 ms transients, respectively), an internally used developer's license enables higher-resolution analysis with length of transients limited only by the available electronics. In this work, 2048 and 4096 ms transients were employed (corresponding to resolving powers around 1 and 2 million at m/z 200, respectively), and 8192 ms transient duration was also probed.

As already mentioned in the article devoted to the previous endeavor to conquer the one-million resolution barrier [16], such performance requires that the mechanical accuracy of electrodes lies well in the nanometer range, thus formally (though a little bit superficially) placing the Orbitrap analyzer into the realm of nanotechnology. Since the article [16] got published, the one-million resolution option became commercially available on the Orbitrap Fusion Lumos and Orbitrap Eclipse instruments (as used e.g. in Ref. [17]), which provided new data useful for developing improved manufacturing and testing processes for the Orbitrap Exploris analyzer. Other publications also demonstrated similar or even higher resolutions on instruments of Orbitrap Elite™ [18,19] and Q Exactive type [20].

Due to operation outside of the realm of standard calibration settings, the instrument has to be manually tuned and mass calibrated. The higher the required resolving power, the more accurate the tuning of the deflector voltage must become, as it strongly and directly affects the quality of the electrostatic field inside the analyzer. Tuning of other voltages appear to affect the quality of

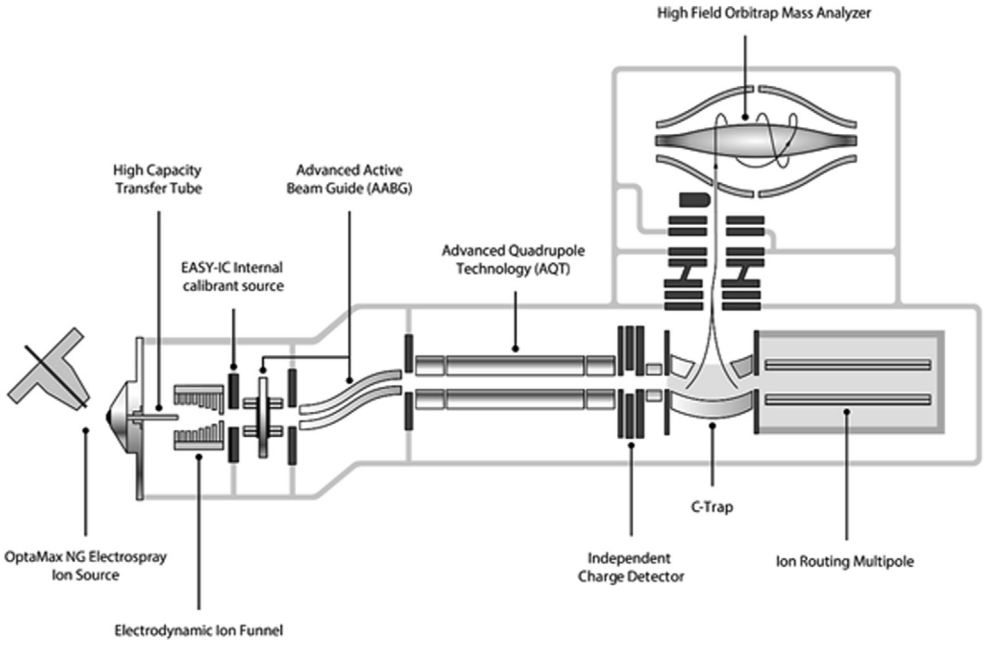

Fig. 1. Schematics of Orbitrap Exploris 480 mass spectrometer with main units indicated by their names. Typical operational pressures in mbar are shown, with measured values shown in bold and settings used in this work underlined.

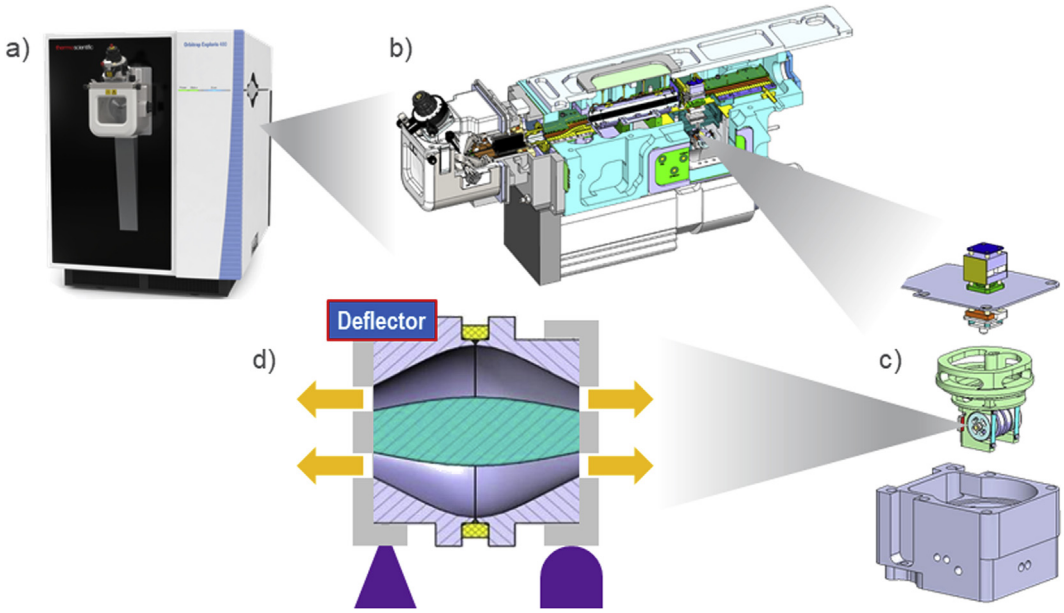

Fig. 2. Layout of the Orbitrap Exploris 480 quadrupole-Orbitrap mass spectrometer.
a) An exterior view,
b) A cut-out view of the mass spectrometer showing the entire ion-optical path,
c) An explosion view of the Orbitrap block, with C-trap and lens assembly at the top and Orbitrap assembly below in a holder
d) A schematic view of the Orbitrap assembly with deflector and fixed support on the left side and sliding support on the right side. The outer electrodes are separated by a quartz ring and used for detection and therefore are maintained at the virtual ground of the preamplifier, while the central electrode is used for trapping at a voltage −Ur (Ur > 0 for positive ions). Quartz end-pieces are shown in gray and pumping - by arrows.

spectra only indirectly, via changes in the initial parameters of the ion packets.

Thermo Scientific™ Pierce™ LTQ ESI Positive Ion Calibration Solution mixture, Thermo Scientific™ Pierce™ Intact Protein Standard Mix, Sigma- 2522 Sigma Carbonic Anhydrase Isozyme II from bovine erythrocytes (Millipore Sigma) and Herceptin® (trastuzumab) obtained in manufacturer's formulation buffer (Genentech), were directly infused using a HESI II ion source.

3. Results and discussion

Resolving power in excess of 2,000,000 could be demonstrated for *m/z* below 200 (Fig. 3) and fine structure of isotopes could be resolved and used for unequivocal determination of molecular composition, as shown for caffeine (*m/z* 195.0877 for A0). Minor isotopes could be also measured with high mass accuracy at low intensity levels as shown in the inset. SIM scans were used to improve signal-to-noise for such minor isotopes.

For larger molecules, isotopic fine structure at the A+2 peak in the peptide MRFA can easily be resolved (Fig. 4), with a signal-to-noise ratio that allows isotope abundance determination with accuracies that match spectral fit and pattern coverage requirements of such software packages as e.g. Thermo Scientific Compound Discoverer version 2.0 or higher. In particular, the ^{18}O peak and the $^{33}S^{13}C$ peak can be separated at the baseline (1.4 mDa). As seven detected peaks at *m/z* 526.63 in Fig. 3 reflect the abundance of ^{34}S, $^{15}N^{13}C$, ^{18}O, $2{\cdot}^{13}C$ isotopes in (A+2) of doubly charged MRFA peptide, they could be employed for direct deduction of composition stoichiometry of the molecule.

The new geometry of the Orbitrap mass analyzer appeared to drastically decrease transient decay even for larger proteins that are known to be the most sensitive to collisions with gas at the high energies typical for the Orbitrap mass analyzer.

Measurements of intact carbonic anhydrase under native conditions were performed at reduced pressure conditions in the IRM and demonstrated for the 10+ ion resolving power in excess of 500,000 at m/z 2,900, with 17 beats visible in the 4-s transient (see Fig. 5). When compared for the same transient duration, the instrument achieves higher resolving powers than theoretically calculated for Fourier Transform Ion Cyclotron Resonance instruments equipped with the strongest available super-conducting magnets (21 T) even in the absorption mode.

Furthermore, for the first time it became possible to achieve baseline isotopic resolution on intact 150 kDa monoclonal antibodies both under denaturing and native conditions in infusion mode while employing a conventional nitrogen supply (in contrast to helium as employed in a similar experiment on a much better pumped Orbitrap Elite MS with six turbopumps in Ref. [21]). Fig. 6 shows a 3-s transient of a SIM scan recorded at reduced pressure conditions for the denatured intact trastuzumab monoclonal antibody [22]. A resolving power >300,000 at m/z 2800 was required to baseline isotopically resolve 53+ ions of the four most abundant trastuzumab glycoforms. For the Full MS analysis of the native intact trastuzumab antibody, a 4 -second transient was required to obtain baseline isotopic resolution as shown in Fig. 7. Although only achieved on a single well-baked and hand-tuned instrument, these results demonstrate feasibility of using multi-stage turbopumps under full gas load from the electrospray source to achieve the demanding UHV conditions needed for such performance.

While long transients enable probing the limits of resolving power, they also open opportunities to improve signal-to-noise ratio of image current detection proportionally to the square root of transient duration. Ultimately, a noise band substantially smaller than one elementary charge $\bar{e}$ enables high-fidelity charge detection mass spectrometry (CDMS) as already experimentally proven for a linear electrostatic trap [23,24]. As harmonic motion of ions in the Orbitrap analyzer allows detection of hundreds and thousands of species in parallel, there was substantial progress made towards

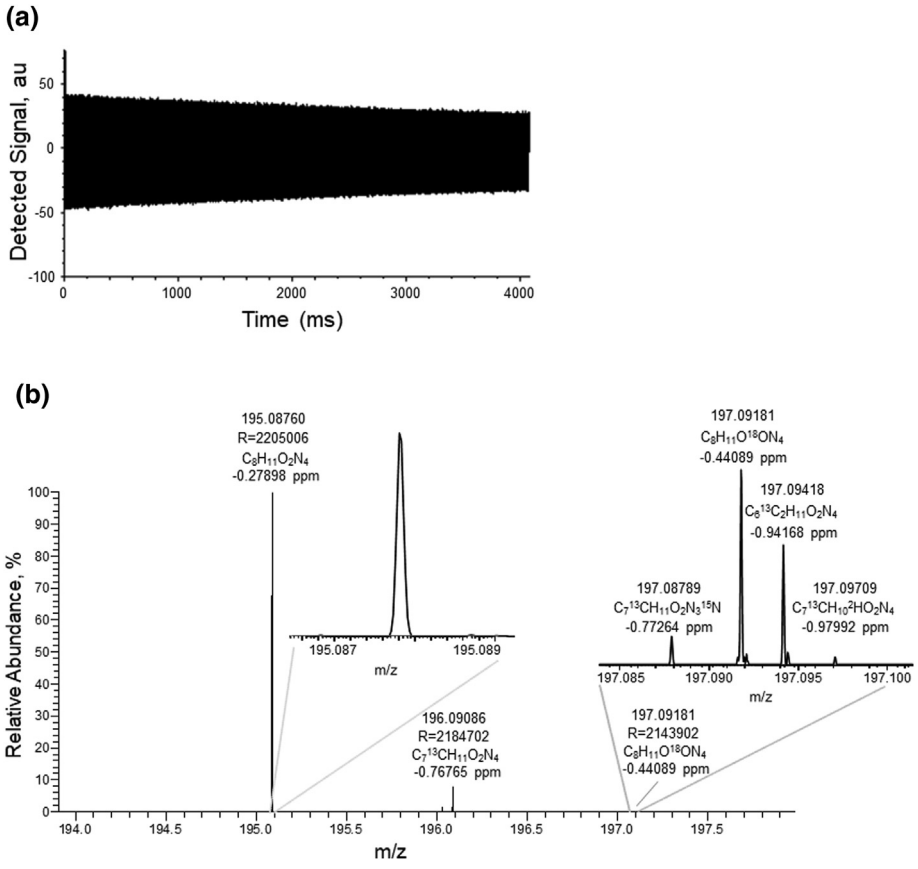

Fig. 3. A single selected ion monitoring (SIM) scan of caffeine utilizing 4 s transient (a), demonstrating experimental resolving power > 2,000,000 and isotopic fidelity also for low abundance isotopologues (b). Isolation width 10 Th and AGC target 5e5 were used.

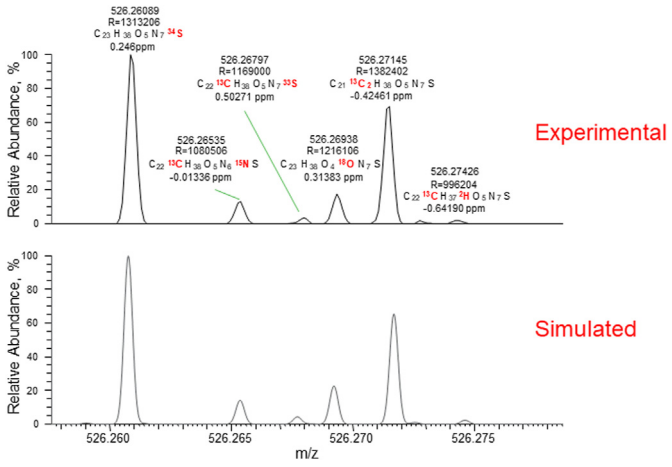

Fig. 4. A single SIM scan of singly charged MRFA peptide utilizing 4 s transient demonstrating resolving powers above one million and high isotopic fidelity for A2 isotopologue cluster, comparing theoretical and experimental spectra. Isolation width 50 Th and fixed ion time of 8 ms were used.

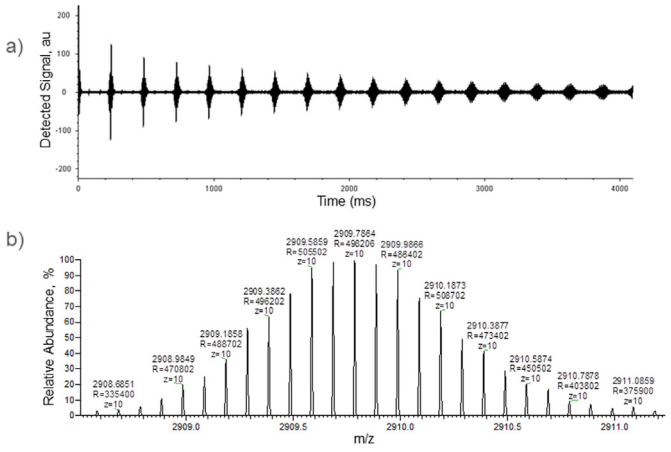

Fig. 5. SIM scan of the native intact carbonic anhydrase ($C_{1312}H_{1996}N_{358}O_{384}S_3Zn$) 10+ ion, using 4 s transient. Isolation width 50 Th, S-lens RF level 100%, in-source CID 35V, fixed ion time of 3 s and 9 microscans were used.

applying this analyzer for CDMS [25–27]. However, sub-elementary charge precision remained elusive, presumably because of the higher noise associated with the higher capacitance of the Orbitrap assembly. Encouraged by the ability to experiment with longer transients, the authors explored how far the error of charge measurement could be reduced.

Experiments were carried out using individual ions to build histograms of signal-to-noise ratios S/N as described in Refs. [15,28]. An example of such a histogram is presented in Fig. 8 for 4+ ions of Angiotensin 1, with the first maximum corresponding to S/N from a single ion, the second maximum from two ions detected in the same peak, the third maximum from three ions in the same peak, etc. Noise N is determined automatically by Xcalibur software in the phase-independent magnitude mode, while signal S is determined by eFT and takes advantage of its known phase [29].

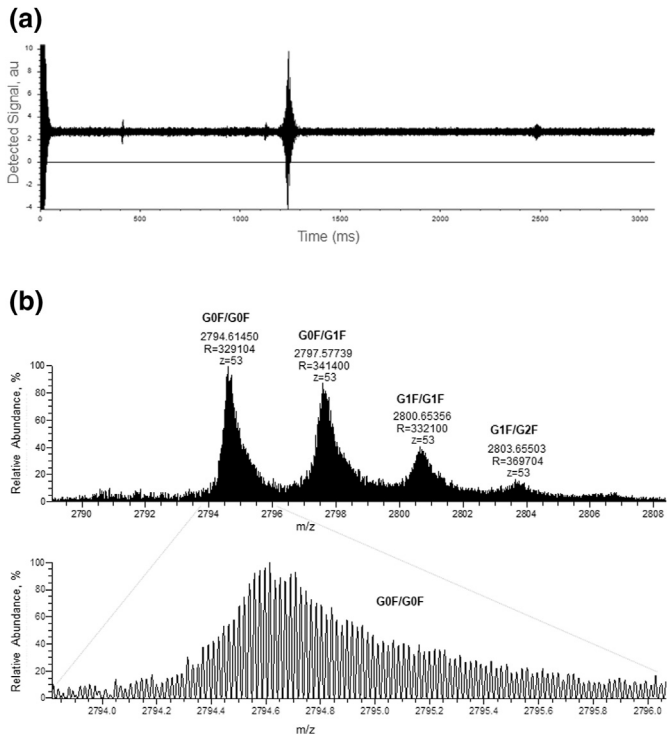

Fig. 6. SIM scan of 53+ ion of the denatured intact trastuzumab antibody with 16 S–S bonds and 2 Lys clippings [22]. Using 3 s transient (a), glycoform profile is obtained as shown in b), with zoom-in confirming isotopic resolution for G0F/G0F glycoform (C_{6560} H_{10132} N_{1728} O_{2090} S_{44} with methionine oxidation associated with infusion). Isolation width 50 Th, S-lens RF level 80%, in-source CID 80V, fixed ion time of 20 ms and 1430 microscans were used.

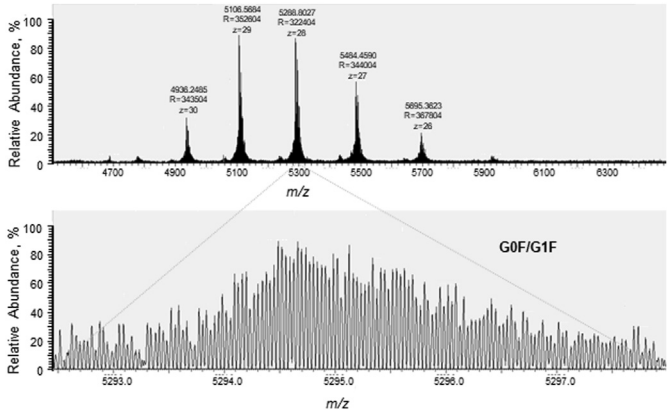

Fig. 7. Full MS scan of the native intact trastuzumab antibody with 16 S–S bonds and 2 Lys clippings. Using 4 s transient and standard pressure conditions, isotopic resolution was obtained as shown for G0F/G1F glycoform (C_{6566} H_{10142} N_{1728} O_{2095} S_{44} with methionine oxidation associated with infusion) on zoom-in of panoramic spectrum. In-source CID 150V, S-lens RF level 200%, fixed ion time of 100 ms and 140 microscans were used.

While the width of maxima is defined directly by noise of image current detection, tailing of peaks towards lower values reflects decay of signal due to collisions or dephasing and becomes especially pronounced for two or more ions co-detected in a single detection event. Although such tailing could be corrected using, e.g., the STORI algorithm from Ref. [25], in this work we use full width half-maximum (FWHM) $\boldsymbol{\Delta_{FWHM}}$ of just the first peak as an upper-limit estimation of charge measurement error. Based on the

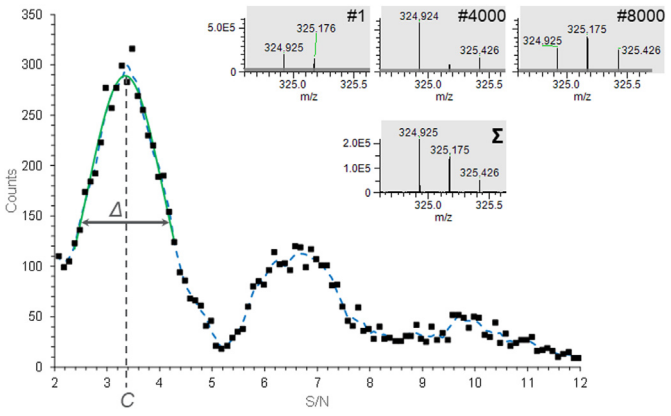

Fig. 8. An example of histogram of ion intensities acquired in a set of 8266 of 1 s transients for 4+ ions of Angiotensin 1 peptide. Data points of the histogram with a step 0.1 are represented by black squares and the dashed line of smoothed histogram reveals multiple maxima corresponding to single-, double- and triple-ions co-detected in a single detection event. Gaussian fit is represented by the solid line (R^2>0.98) and FWHM Δ and centroid C of the first maximum are used then for E_{FWHM} calculation. Insets show typical individual scans (numbers 1, 4000 and 8000, with dark gray band indicating noise level N) as well as a summed scan over the entire data set (denoted as Σ). Isolation width 20 Th and fixed ion time of 0.2 ms were used.

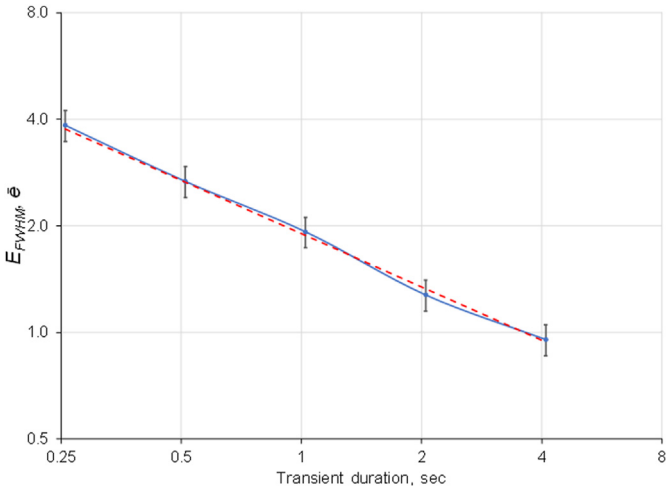

Fig. 9. Measured dependence of E_{FWHM} on the transient duration in log-log scale. The dashed line represents the theoretical square root dependency with a fitted scaling coefficient (R^2>0.99).

known charge state $\boldsymbol{Z}$ corresponding to the corresponding centroid $\boldsymbol{C}$ of a peak in the histogram (Fig. 8), the FWHM $\boldsymbol{E_{FWHM}}$ of error distribution could be estimated as $\boldsymbol{E_{FWHM} = \Delta_{FWHM}Z/C}$. This value corresponds to the band within which >75% of charge measurements would fall and equates to 2.35 standard deviations under the assumption of Gaussian peak shape.

Fig. 9 presents the dependence of $\boldsymbol{E_{FWHM}}$ on the transient duration in log-log scale measured with 10+ Ubiquitin ions for 0.25–1 s transients and 4+ ions of Angiotensin 1 peptide for 1–4 s transients. The fitting line represents the theoretical square root dependency on duration with R^2>0.99 and gives a value of 1.9·ē for 1 s transient. This fits well with an independent statistical measurement of detection limit for a high-field Orbitrap instrument [30]. Intriguingly, the FWHM of error distribution falls to just one ē FWHM (or around 0.4·ē standard deviation) for 4 s transient, coming tantalizingly close to high-fidelity charge determination. Nevertheless, sub-ē charge precision is still not achieved-following

the line of Fig. 9, it could be expected that only 8 or 16 s transients would allow baseline separation of charge states differing by one ē. At the same time, this work shows that several serious technical challenges need to be overcome in order to reliably acquire and process transients of such extended durations.

4. Conclusion

This work enabled exploration of new realms of Orbitrap transient durations that promise even higher resolving powers than ever achieved before in serial instruments. It was shown that even in a benchtop instrument with a new streamlined pumping design, Orbitrap technology is capable of ultra-high resolving power in excess of 2,000,000 when appropriate tolerance and tuning requirements are met.

This capability could find its use for resolution of fine isotopic structure for metabolomics, proteomics and petroleomics, but also for charge detection and ultra-sensitive trace analysis.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: All authors are employees of Thermo Fisher Scientific, manufacturer of Orbitrap mass spectrometers.

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