White paper | 003007

Mass spectrometry

Transitioning biomarkers from discovery to validation at unprecedented scale with the Stellar mass spectrometer

Expanded target capacity, higher sensitivity with greater specificity with unrivaled quantitative productivity for translational research

Authors

Neloni Wijeratne, Cristina Jacob, Alan Atkins, Philip Remes, Scott Peterman

Keywords

Stellar mass spectrometer, biomarker verification, targeted quantitative solutions, protein, metabolite, lipid, dynamic retention time adjustment, automated method creation, fast polarity switching, translational research

Introduction

The Thermo Scientific™ Stellar™ mass spectrometer establishes a new paradigm to drive biomarker verification by achieving 10X the sensitivity for 5X more compounds at scale compared to existing technologies. By synergistically combining the robust quantitative performance of triple quadrupole technology with the sensitive, rapid full scan MS^n acquisition of dual-pressure linear ion trap technology, the Stellar mass spectrometer extends the unprecedented analytical capabilities to a wider range of compounds. Single-ion detection capabilities expand robust targeted quantitation for single cell or low protein load studies minimizing the potential for missing data. Novel software tools streamline highly multiplexed targeted quantitative method creation, implementation, and data acquisition, bypassing lengthy and costly replicate injections associated with existing technology. The new experimental capabilities of the Stellar mass spectrometer make it ideally suited for transitioning putative biomarker candidates from discovery to validation for translational proteomics, metabolomics, and lipidomics research.

The instrument

Stellar mass spectrometer architecture

The new instrument has been fully optimized to confidently quantitate analyte precursor mass ranges of 30-2,000 *m/z* spanning a dynamic range greater than five orders of magnitude. The front end of the instrument features the Thermo Scientific™ OptaMax™ Plus ion source, increasing ionization efficiency for more diverse compound classes when using analytical LC flow rates. Alternatively, the Thermo Scientific™ EASYSpray™ ion source is used for chromatographic flow rates of 0.1 to 10 µL min^{-1} which covers low micro-, capillary-, or nanoflow chromatography. The high-capacity transfer tube (HCTT), electrodynamic ion funnel and advanced active beam guide enhance ion capture and efficiently transfer them into the Stellar mass spectrometer. This results in a higher ion flux without compromising instrument robustness. The Thermo Scientific™ Auto-Ready™ ion source for calibration is an integrated ion source for performing mass calibration without manual intervention, improving ease-of-use and ruggedness of the Stellar mass spectrometer, making on-demand or scheduled system calibration more convenient. The QR5 Plus quadrupole mass filter delivers increased transmission efficiency and stability across a wide mass range with filtering resolution capabilities down to 0.4 Th with minimal ion loss. The transmission shutter gate reduces ion gating times enabling ion gating times of 1-2 µs extending the upper limit of quantitation (ULOQ) range for highly abundant target analytes. The ion concentrating routing multipole (ICRM) is a curved collision cell performing both beam-type fragmentation (HCD) and precursor/product ion trapping capabilities. Subsequent ion packet concentration from the focused ion beam transmission is managed by dynamic automatic gain control (AGC) and synchronized with ion packet management in the dual-pressure linear ion trap for maximum ion utilization rates. The ICRM can also receive ions from the high-pressure linear ion trap for fast higher order MS^n scan events. The hyper-fast dual-pressure linear ion trap mass analyzer is ideally suited for targeted quantitative analysis. The dual-pressure configuration expands analytical capabilities while maximizing fast and confident data acquisition. The high-pressure linear ion trap maintains a constant helium pressure to maximize ion accumulation and collisional cooling prior to transfer to the low-pressure linear ion trap for detection. In addition,

Figure 1. Stellar mass spectrometer coupled to the Thermo Scientific™ Vanquish™ Neo UHPLC system with the Tandem Direct Injection workflow.

RF excitation can be applied to either eject or excite precursor or product ions for higher order tandem MS scan events. The low-pressure linear ion trap receives ion packets from the high-pressure linear ion trap, traps them in the center of the trap and radially ejects the ions for fast detection. Synchronization of ion packet trapping, transfer, and detection throughout the instrument enables paralleled handling of two separate packets of ions simultaneously for data acquisition rates up to 140 Hz, single ion detection sensitivity, and high dynamic range MS^n acquisition. Due to the extended analytical capabilities of the dual-pressure linear ion trap and high ion utilization rate, the Stellar mass spectrometer can perform many acquisition strategies, including label-free quantitation data-independent acquisition (LFQ DIA), MS^n acquisition using on-resonance CID excitation, targeted multiplexed quantitation with synchronous precursor selection (SPS) MS^3 Tandem Mass Tag™ (TMT™), and multi-notched isolation/activation for enhanced MS^3 acquisition (Figure 2).

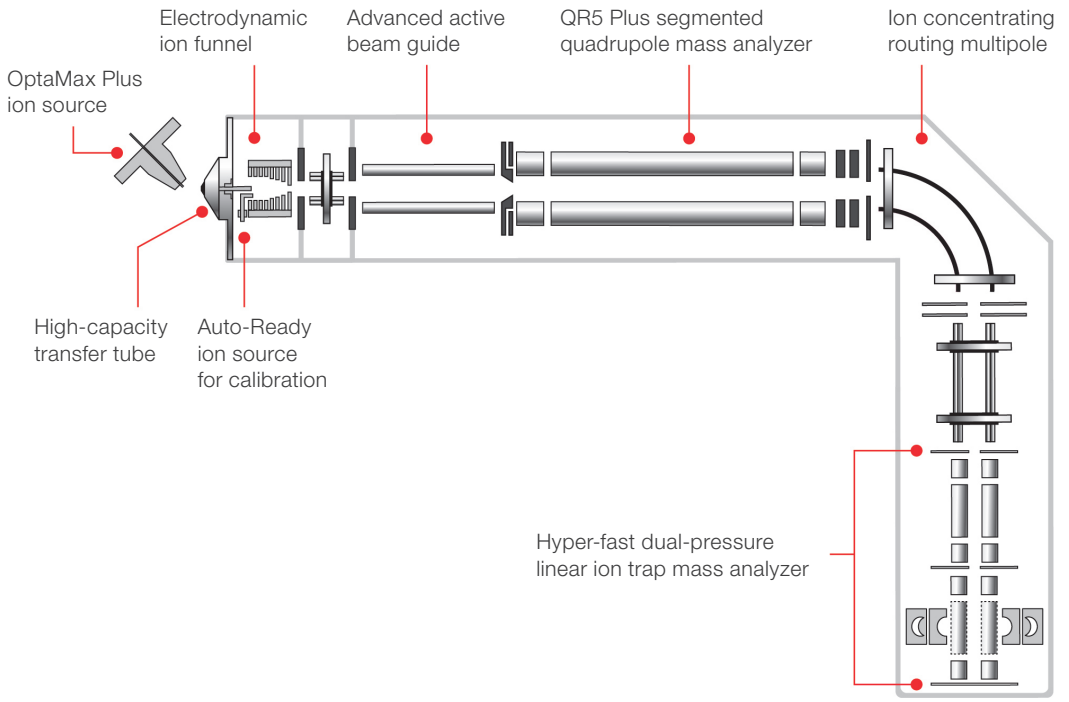

Figure 2. Instrument design of the Stellar mass spectrometer.

Dual-pressure linear ion trap operation

The dual-pressure linear ion trap mass analyzer had been previously implemented in Thermo Scientific™ Orbitrap™ Tribrid™ mass spectrometers, but the hardware and performance has been improved with the dual-purpose ICRM collision cell and synchronized ion management to deliver sensitive, high dynamic range tandem mass spectra at a high acquisition rate. The combined performance characteristics of the QR5 Plus mass filter and ICRM performance with the hyper-fast dual-pressure linear ion trap mass analyzer enables increased quantitative sensitivity with faster acquisition speed and greater throughput for more analytes across a wider compound class without sacrificing quantitation accuracy or precision.

For the fastest MS^2 acquisition in the linear ion trap mass analyzer, the incoming ion beam is filtered in the QR5 Plus quadrupole mass filter to a user-defined precursor *m/z* value with isolation resolution down to 0.4 Th, dissociated and trapped in the ICRM for a duration dynamically controlled by the AGC routine. The ion packet is then transferred into the high-pressure linear ion trap for collisional cooling in the presence of a static helium pressure and ultimately transferred into the low-pressure linear ion trap for detection. Maintaining static neutral gas pressures in each linear ion trap maximizes performance and greatly improves the overall acquisition speed while reducing ion loss during transfer between linear ion traps and radial ejection into the dual conversion dynode/PTM detector assembly. Ejection of MS^2 product ions occur from low-to-high *m/z* values by applying a resonant excitation RF. The overall detection time is therefore dictated by the user-defined scan speed and the product ion mass range. The scan rate options are 33, 67, 125, and 200 kDa/s. For most highly multiplexed targeted acquisition methods, a scan rate 125 kDa/s is used to balance acquisition speed and quantitative data quality and enables a 140 Hz acquisition rate for most small-molecule methods and 75 Hz for targeted peptide quantitation based on the average product ion mass range associated with each compound class. Table 1 lists the approximate time needed to cover an average product ion mass range associated with each compound class. For bottom-up proteomics applications, 1,000 Da covers the average product ions associated with the y_3 to last y-type ion (y_n). The overall sensitivity and selectivity of resulting full scan MS^2 spectra has been demonstrated to be greater than full scan MS^2 spectra providing an ideal solution for the verification class mass spectrometer. (Heil L. R., 2021)

Table 1. List of the scan speeds from the low-pressure linear ion trap for detection. The selected product ion mass ranges represent typical experimental parameters for small and large molecules.

Scan speed (kDa/s)	Scan time for each product ion mass range (ms)	
	500 Da	1,000 Da
33	15	30.3
67	7.5	15
125	4	8
200	2.5	5

The resulting product ions are excited radially from the low-pressure linear ion trap analyzer and detected by the extended scale detector. The extended scale detector is comprised of a dual conversion dynode assembly located on opposite sides of the low-pressure linear ion trap with aligned focal points that lead into a photomultiplier tube for single ion sensitivity. The use of low neutral gas pressure and the dual dynode configuration results in *ca.* 84% ion detection to improve detection levels while requiring minimal ion accumulation times for faster overall data acquisition.

The fastest MS^3 acquisition leverages multiple components of the Stellar mass spectrometer for synchronized acquisition of sensitive data. Precursor ion filtering in the QR5 Plus mass analyzer enables the ICRM to perform continual fragmentation and concentration as governed by dynamic AGC. As described above, the MS^2 ion packet is transferred to the high-pressure linear ion trap once the previous MS^3 ion packet has been ejected from the low-pressure linear ion trap and measured. Once in the high-pressure linear ion trap, selected MS^2 product ions are isolated. Then, for HCD fragmentation, those ions are then accelerated back to the ICRM where MS^3 fragmentation is performed. The resulting MS^3 product ions are transferred back to the high-pressure linear ion trap, cooled to the center of the trap, transferred to the low-pressure linear ion trap and subsequently ejected out of the low-pressure linear ion trap at 125 kDa/s scan rates up to 40 Hz acquisition rates. For CID fragmentation, which has slightly slower MS^3 acquisition rates, the isolated MS^2 product ions can also be fragmented in the high-pressure linear ion trap, using on-resonance RF (CID) colliding with helium. The benefit of using CID is to reduce secondary fragmentation thereby concentrating MS^2 abundance in fewer product ions. This occurs because fragment ions (with different *m/z* values than the precursor *m/z* value) are no longer resonant with the drive RF and cool back to the center of the high-pressure linear ion trap. CID fragmentation generally takes about 10 ms as opposed to performing HCD fragmentation which takes ~3 ms.

The PRM Conductor software tool

The greater challenge to performing accurate and precise quantitation for highly multiplexed PRM methods is creating and refining instrument methods. The PRM Conductor software streamlines the translational research workflow through Skyline software to expedite method development, implementation, and data processing. (Heil, 2022) The user inputs the targeted set of proteins or analytes into Skyline software based on the research objective. Next, the user imports the non-targeted data into Skyline to create the spectral library from which LC and MS^n information can be read into the subsequent PRM acquisition table. Once the protein list and non-targeted search results are loaded into Skyline, the user sets the refinement thresholds to identify analytes based on data-dependent acquisition (DDA) or DIA data. For proteomics, the most common non-targeted discovery approach is to use DIA as the resulting product ion XIC-based data, similar to PRM data acquisition, and allows for more advanced transition filtering.

The PRM Conductor software interface is shown in Figure 3 and is broken down into three steps as shown along the left-hand side of the GUI. The first step is to refine the list of plausible analytes considered for targeted quantitation using a set of user-defined thresholds including area values, signal-to-noise ratio, interference, retention time and peak width, and minimum number of product ions surpassing the stated thresholds. The second step is to define the experimental methods that will be used in the instrument method editor. Settings include the scan rates, minimum ion accumulation/concentration time, acquisition type, cycle time (or points per peak), scheduled time window used for targeted data acquisition, and number of required data points, which establish the cycle time. Lastly, the targeted window duration sets the maximum concurrent PRM events per time. Step 3 builds the targeted method based on the final two user-defined settings, balanced target selection, and one precursor *m/z* value per analyte.

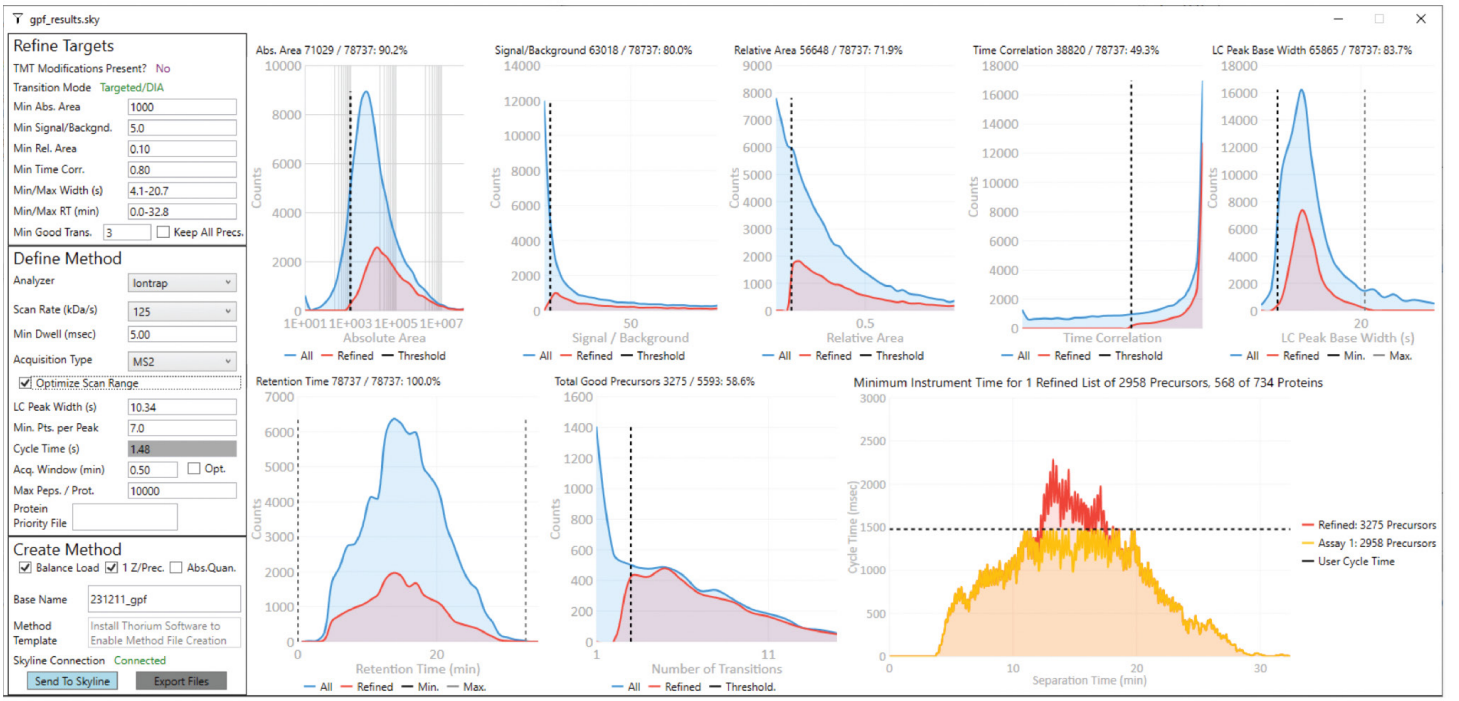

Figure 3. Screen capture showing the PRM Conductor software used to filter non-depleted plasma for a highly multiplexed targeted method.

The example shown in Figure 3 is for a non-depleted plasma study reading in DIA discovery data from the Stellar mass spectrometer. The entire search results were considered in building the PRM method for targeted MS^2 acquisition which consisted of 734 proteins, 5,593 peptide precursors and 78,737 product ions. Each graph shown in Figure 3 shows the distribution of product ions relative to the user-defined thresholds as described above and used to identify only the product ions, precursor *m/z* values that create the greatest potential for successful targeted quantitation.

A refined list of 3,275 peptides representing 524 proteins were considered to create the final method. However, the library retention times associated with all 3,275 peptides resulted in an excess target capacity at the middle of the gradient. LC peak capacity and maintaining the number of desired MS data points per peak truncated the final list to 2,958 peptides representing the 524 proteins. The final set of peptides were selected based on expected retention times to balance the overall elution profile more evenly across the entire gradient profile maximizing utilization rates for increased data quality.

To further enhance tMSn acquisition speed for peptides, the PRM Conductor software can further leverage the imported discovery LFQ DIA data to customize the product ion *m/z* range scanned in the low-pressure linear ion trap mass analyzer for each target. Based on previous studies, detection of four product ions can generate peptide-specific data that cover on average 440 Da as opposed to a static mass range covering the y_3 to the y_n product ion mass range. This approach reduces the overall scan times by *ca.* 4 milliseconds per peptide, which equates to 400 milliseconds for each timed acquisition window with 100 concurrently eluting peptides, enabling more peptides eluting in the middle of the LC gradient to be verified.

Adaptive RT routine to manage dynamic retention time adjustments

Expanding the experimental scale by either increasing the number of analytes quantified, shortening the gradient for higher throughput or a combination of both, requires more efficient use of cycle time to maintain improved quantitation. Incorporating scheduled/timed acquisition windows reduces concurrent scan events per unit time and improves duty cycle for co-eluting analytes. The narrower the time windows used either expands the analyte target capacity for the entire method or reduces the concurrent scan events per cycle time thus improving duty cycle. The risk of using narrow RT windows without a dynamic alignment strategy, however, is that any changes to the gradient profile can shift target analyte elution profiles out of their timed acquisition windows resulting in missing values.

Current solutions involve spiking RT calibrator standards to be used as landmarks enabling on-the-fly RT alignment. The analytes comprising the RT calibrator kits generally have high ionization potential, cover a wider range of hydrophobicity factors for reversed-phase (RP) chromatography, elute across

most gradients. (Kiyonmai, 2011) Researchers must determine the optimal spiking amount relative to the biological sample: spiking too much of the RT calibration kit can suppress ionization potential for co-eluting endogenous targets and spiking too little can result in missed detection which reduces the effectiveness of dynamic retention time adjustments. Once the optimal spike in amount is determined, the number of analytes used in the kits can also create challenges. Too few targets leave long retention time windows between markers that may miss key shifts in the LC performance and too many markers take up valuable instrument time. Lastly, targeted peptide quantitation has been the primary application to create commercially available sets. Moving to metabolites and lipids requires utilization of stable isotope labeled (SILs) analytes to be used which may or may not meet all the requirements for robust retention time adjustments.

The Stellar mass spectrometer utilizes the Adaptive RT routine to perform real-time RT adjustments. (Remes, 2020) The PRM Conductor tool creates a reference dataset that is embedded in the instrument method editor to routinely analyze the background for alignment without using spiked standards. Configuring and utilizing Adaptive RT is fully automated within the software and operates within that instrument control software.

The user-defined targeted quantitative method establishes product ion features per precursor *m/z* values at estimated retention times. Figure 4 shows the steps performed to enable the Adaptive RT routine. Prior to the start of any study, a representative sample is analyzed using the LC gradient in a discovery DIA experiment on the Stellar mass spectrometer or on a Thermo Scientific™ Orbitrap™-based mass spectrometer and processed using the PRM Conductor tool to associate the correct retention time values with the targeted analytes. Next, 12 x 50 Th wide DIA windows that are repetitively acquired at a frequency equivalent to the user-defined peak width divided by 3.5 or about every two cycles to create the Alignment Map file. (Figure 6A) The 12 x 50 Th DIA scan events covers enough of the background to establish retention time landmarks throughout the gradient based on known targets as well as background ions. The instrument method used for routine sample analysis also incorporates the 12 x 50 DIA scan events at the start of every other cycle prior to switching over for the tMSn scan events. The results from each DIA spectrum are matched to the Alignment Map in real time and the resulting retention time windows per target analyte are adjusted accordingly. Cycling back to the DIA scan events at an increased frequency ensures accurate dynamic retention time alignment is performed improving data confidence.

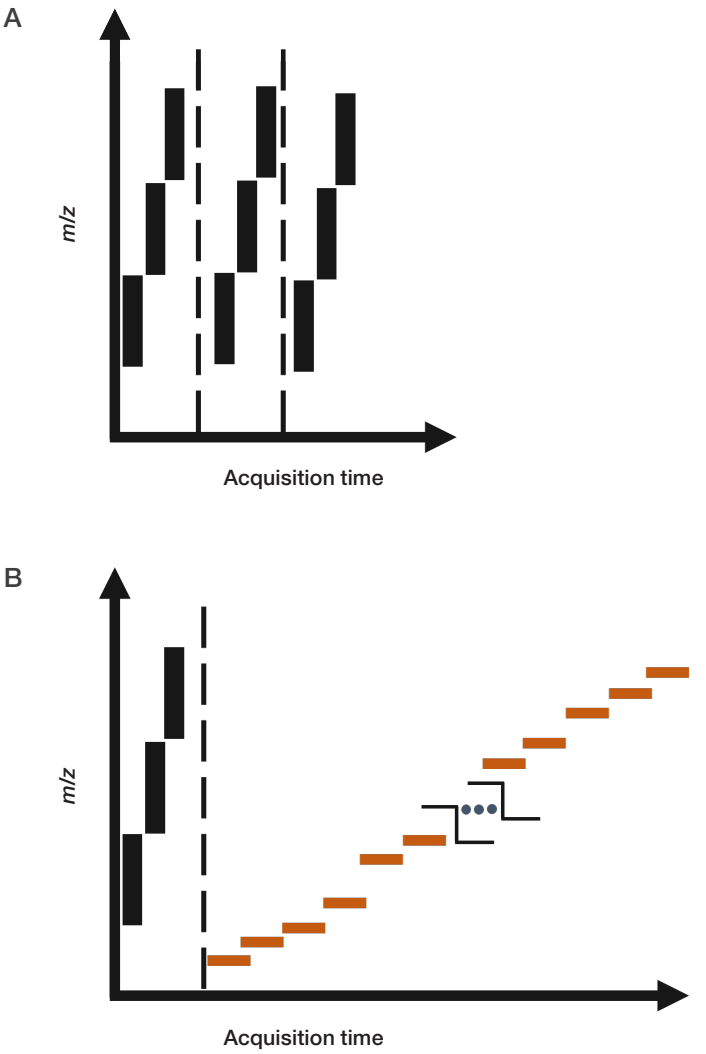

Figure 4. Two-step process for implementation of Adaptive RT. The Alignment Map file is created by repetitively stepping across 12x 50 Th DIA scan events acquired at a scan rate of 200 kDa/s. (Figure 4A). For the sample analysis method, two experiments are performed in a cycle, starting with the three 50 Th DIA scan events followed by the tMS2 scan events which uses a scan rate of 125 kDa/s. (Figure 4B).

Using the combination of 50 Th DIA windows and the fastest scan rate of 200 kDa/s, the Alignment Map routine takes only ~100 milliseconds every other tMS2 cycle of acquisition time for the stated cycle times. For the example presented in Figure 3, this is only 3% of the overall cycle time, ensuring maximum target capacity and/or quantitative data quality. The stability of the Adaptive RT has been tested over four weeks of continual acquisition maximizing instrument productivity. Figure 5 shows a screen capture of the Adaptive RT taken 108 hours following the original Alignment Map acquisition. The measured time shift for the example shown was around 12 seconds for most of the gradient which is well within the user-defined timed duration of 30 seconds. The increased retention time stability is attributed to the Thermo Scientific™ Vanquish™ Neo UHPLC system delivering robust performance. There is one retention time period at about 8 minutes where the time shift jumped to 24 seconds, yet the fast background sampling enabled dynamic RT adjustment resulting in continual high-quality data acquisition.

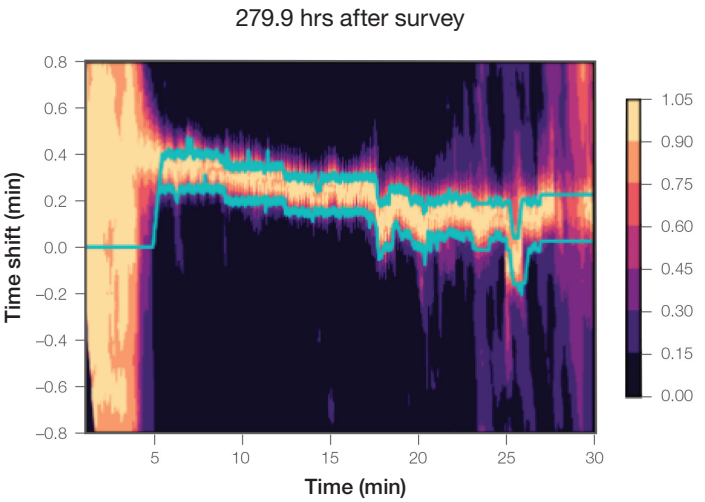

Figure 5. Example of the Adaptive RT for routine targeted data acquisition. The screen capture was taken 108 hours following the Alignment Map acquisition. The left-hand y-axis shows the measured time shift while the right-hand y-axis shows the heat map for background feature correlation as a function of retention time. The teal lines bracket the correlation apex and show the algorithm-determined spread in retention time drift throughout the gradient.

The Stellar mass spectrometer for proteomics applications

Verification of candidate biomarkers becomes critical to ensure effective translation to validation minimizing false positives. Unbiased discovery studies using LFQ DIA analyzes well-defined samples originating from different biological conditions to identify peptides and consequently proteins representing biomarker candidates. While LFQ DIA experiments have become extremely effective at profiling putative candidates, they can have challenges maintaining reliable quantitation at lower intensity levels and, depending on the number of samples, may be statistically underpowered and thus unable to accurately define small changes presenting the risk of false negatives. Thus, the verification stage looks to leverage the improved sensitivity and quantitative accuracy targeted data acquisition affords to mitigate for risks.

The challenge, however, is to maintain quantitative accuracy for experiments with expanded scale. The Stellar mass spectrometer enables this by targeting a greater number of peptides across a wider dynamic range with faster throughput. The gold standard for targeted quantitation is utilizing triple quadrupole mass spectrometers (QQQ MS) performing selected reaction monitoring (SRM) data acquisition. SRM acquisition measures the ion flux of the user-defined precursor and product ion *m/z* value for the quantitative response. To ensure specificity, three SRM transitions are determined per peptide requiring the user to set the collision energy value and dwell time values for all SRM transitions and then test the resulting method in a matrix-matched curve.

When increasing the experimental scale for SRM applications on QQQ MS, it becomes more difficult to maintain high performance for quantitative analysis at lower expression levels. Each SRM transition is an individual scan event and proteomics experiments use a minimum of three SRM transitions per peptide for specificity but up to five in the initial method to overcome the probability of one or more SRM transitions having background interference. Thus, researchers are faced with lengthy method creation and refinement steps to determine the optimal peptides per protein, precursor and product ion charge state and *m/z* values, collision energy, and the final dwell time based on cycle times and duty cycles needed per peptide. Either direct infusion analysis or replicate LC analysis of stock synthetic peptides are performed to ensure confident determination of the resulting SRM parameters setting which can take weeks or months depending on the scale of the experiment.

The Stellar mass spectrometer introduces unprecedented productivity for building, refining, and implementing highly multiplexed targeted peptide quantitation routines. The instrument acquires a parallel reaction monitoring (PRM) scan event, also known as full scan targeted MS2 (tMS2) per peptide enabling simultaneous measurement of all product ions per scan event, enabling post-acquisition data processing to select the best set of MS^2 product ions for maximum quantitative data and minimizing the need for method refinement. The fast and sensitive data acquisition of the Stellar mass spectrometer enables high-quality data acquisition requiring only 5-30 milliseconds acquisition time per peptide. The Stellar mass spectrometer also utilizes normalized collision energy (NCE) settings and implements similar tandem MS mechanisms as Orbitrap-based mass spectrometers ensuring direct method transition from discovery to targeted analysis. Finally, the Stellar mass spectrometer incorporates dynamic AGC to intelligently determine the ion accumulation times on-the-fly to ensure confident data acquisition without setting manual AGC values that compromise on speed or sensitivity. Post-acquisition software strategies designed to intelligently determine which set of MS^n product ions combine to measure the greatest S/N value per peptide reduce the barriers to productivity in both building methods and processing.

To demonstrate the improved limits of detection and quantitation (LOD and LOQ, respectively), a comparative set of experiments were performed on a premier QQQ mass spectrometer and the Stellar mass spectrometer, quantifying 786 plasma peptides in 30 minutes. To ensure similar qualitative and quantitative analysis is maintained, up to 5 SRM transitions were monitored per peptide for a total of 3,950 SRM transitions in 30 minutes whereas the tMS2 method on the Stellar mass spectrometer used 786 scan events monitoring over 5,500 product ions. The same timed window parameters were used for each experiment. To evaluate the quantitative performance, the reference human plasma digest was diluted into digested chicken serum. The dilution series utilized 3-fold dilution steps ranging from samples containing 50% human plasma down to 0.2% analyzed in triplicate. LODs were determined by the bilinear turning point method and LOQs by ≥ 20% relative standard deviation (%RSD), externally calibrated.

Figure 6 shows the relationship between concurrent scan events and available dwell times as used for SRM acquisition and accumulation times for tMS2 methods. Increased SRM concurrency costs QQQ experiments more sensitivity as the greater number of scan events reduces the available dwell time down to 1-3 milliseconds per SRM transition. Shorter dwell times at lower intensity levels reduce the quantitative accuracy for any premier triple quadrupole mass spectrometer. On the other hand, acquiring full scan MS^n spectra requires only one scan event to measure most of the product ions per peptide, which at the middle of the gradient profile is still 10 milliseconds per peptide, enabling 10X lower LOQs on average.

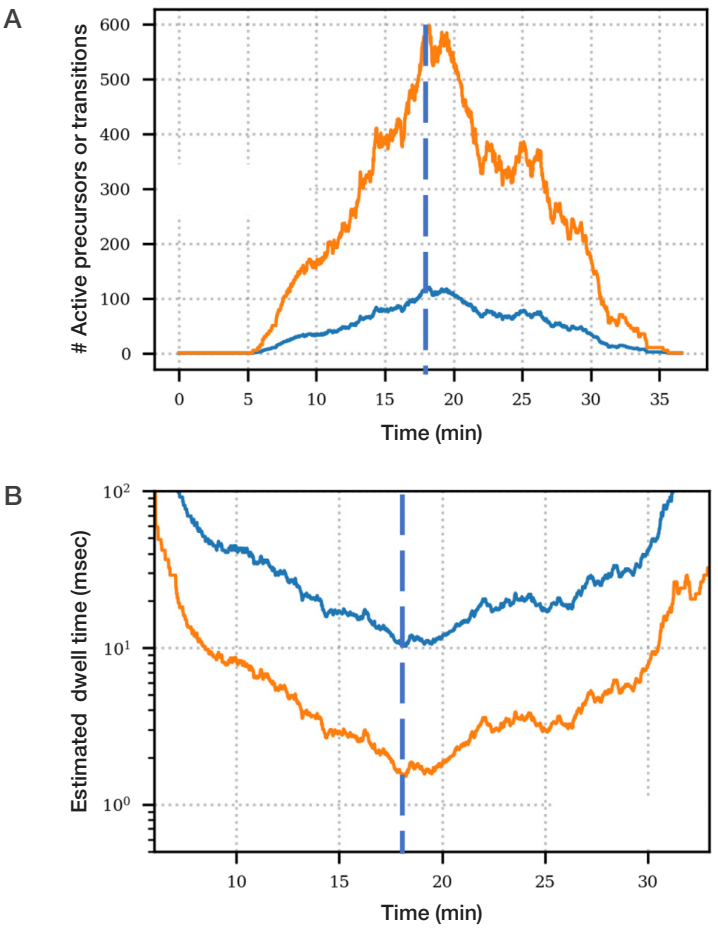

Figure 6. Correlation between concurrent scan events and dwell/accumulation time for the two different experiments. Figure 6A shows the number of concurrent scan events as a function of retention time and Figure 6B shows the corresponding dwell/accumulation times for the 823 targeted peptides for the triple quadrupole mass spectrometer method using SRM acquisition (orange line) and the Stellar mass spectrometer using PRM (blue line). The dashed line provides alignment between Figures 6A and 6B.

An example of the comparative performance is shown in Figure 9 for the peptide ATWSGAVLAGR from the A2-glycoprotein. The A2 glycoprotein is a medium-level protein in human plasma and further dilution into chicken serum presents greater ion suppression. Additionally, the retention time of 18.8 minutes is at the maximum concurrency reducing the available dwell time to *ca.* 2 milliseconds per SRM transition. Collectively, this enables signal measurement for only the most intensive product ion at 5.5% dilution level which is not sufficient for quantitation.

Timed-MS2 acquisition using the Stellar mass spectrometer shows sensitive detection and about 10X better LOQ as compared to SRM acquisition. The targeted peptide underwent similar matrix effects in both the tMS2 and SRM experiments, along with the same level of dilution. The only difference was the use of a 10-millisecond product ion accumulation time in the tMS2 experiments. Figure 7D shows that the Stellar mass spectrometer measured all five productions at the 5.5% spiking level and that the measured ion ratios resulted in a dot product correlation coefficient of 0.85 which adds increased specificity to the target peptide. The %CV and RSD were less than 20% with a S/N *ca.* 10 meeting the stated metrics used to establish reliable quantitation.

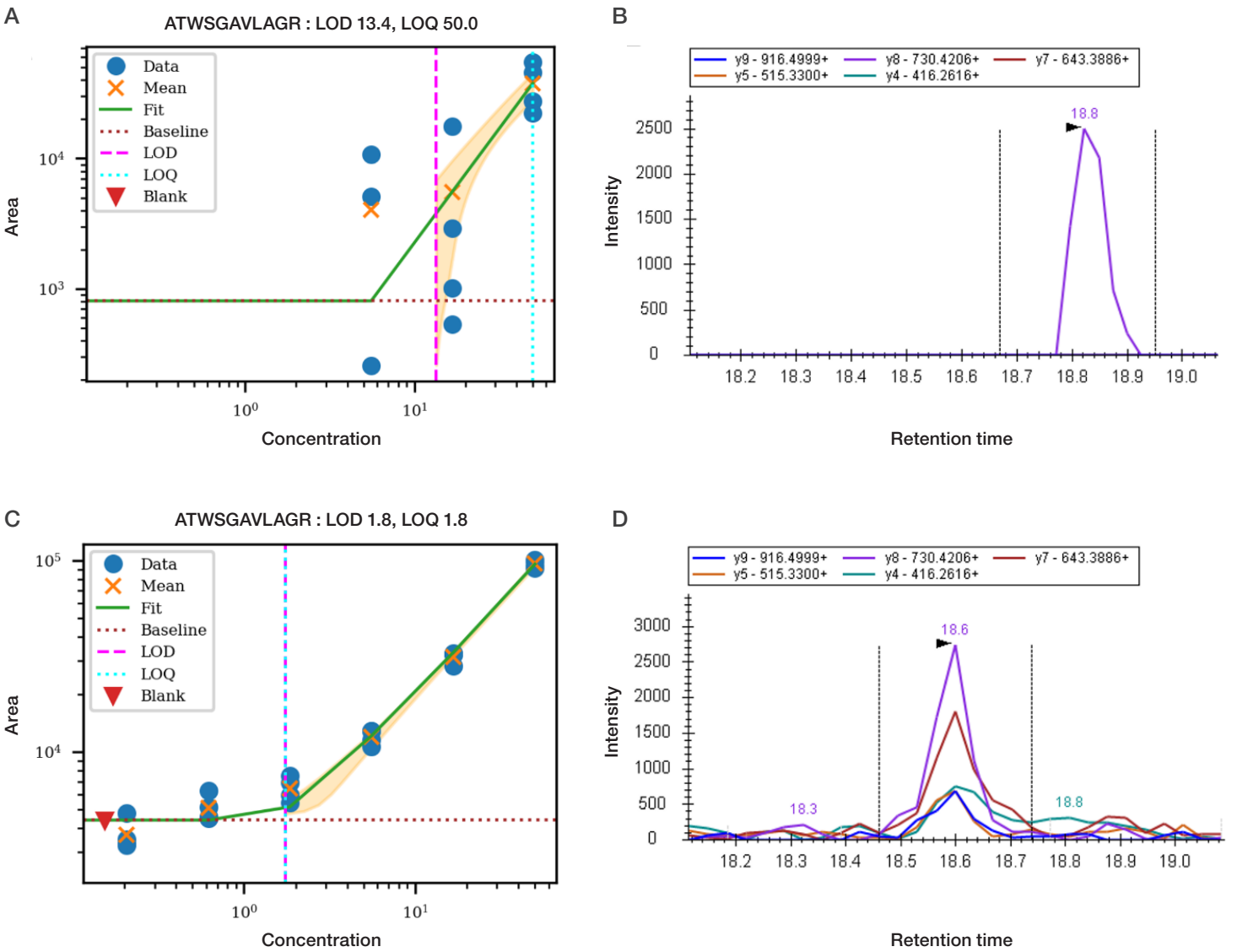

Figure 7. Linear quantitative response comparison for the peptide ATWSGAVLAGR at each level of the matrix-matched curve analysis. Figure 7A shows the measured response at the dilution levels of 50, 16.6, and 5.5% as spiked into chicken serum and Figure 7B shows the resulting XIC for the set of SRM transitions at 5.5%. Figure 7C shows the quantitative response for the same peptide using tMS2 acquisition on the Stellar mass spectrometer with consistent measurements at all levels. Figure 7D shows the resulting overlaid XIC plot for the targeted peptide at the 5.5% dilution level.

Additionally, targeted acquisition using tMS2 enables post-acquisition data refinement to increase performance. Measuring multiple product ions per peptide offers the opportunity to determine which product ions are used for quantitative analysis as some that are measured with low intensity have background interference that reduces the S/N. An additional software tool has been created to evaluate the resulting tMS2 data to automatically determine which sets of product ions are used to determine the area-under-the-curve (AUC) for each of the 786 peptides. Figure 8 shows the LOQ distribution plots considering all measured product ions from the two sets of data. The results for the SRM experiment show almost all targeted peptides had LOQ values at 50% dilution as compared to the tMS2 results showing most had LOQ values between 1 and 10%. When applying the post-acquisition data processing to increase S/N, the resulting LOQ distribution has shifted to lower spiking levels where the majority of LOQ values now range between 0.2 and 2% dilution in chicken serum.

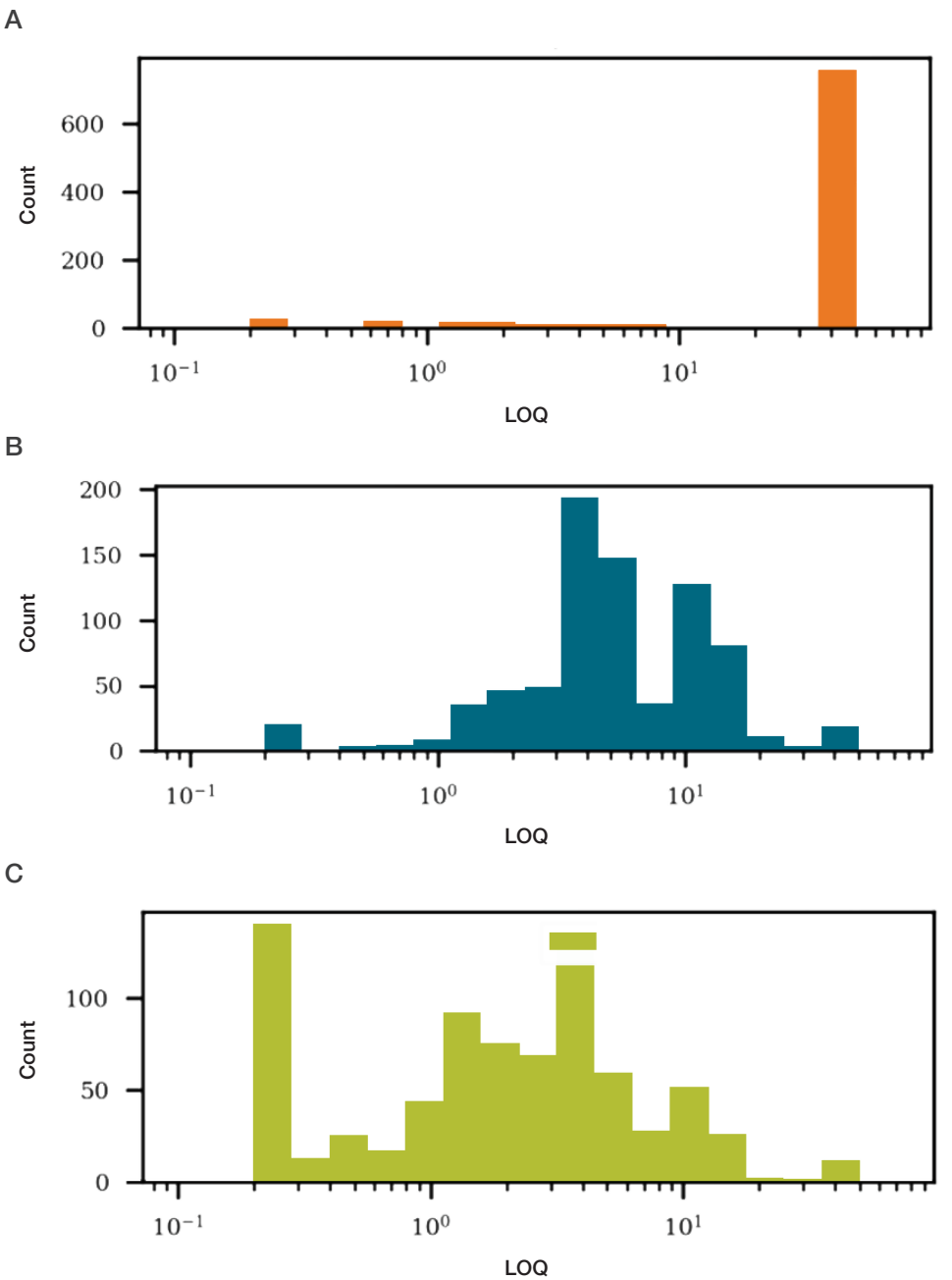

Figure 8. Measured LOQ distribution plot for the targeted plasma peptides across the dilution percentage in chicken serum digest. The distribution plots were measured for the SRM (Figure 8A) and PRM (Figure 8B) experiments when considering all product ions. Figure 8C shows the refined LOQ distribution plot following post-acquisition data processing to identify the best set of product ions to create the greatest S/N and lowest LOQ.

The Stellar mass spectrometer for small-molecule quantitation

Targeted quantitation for small-molecule panels introduces additional challenges compared to peptide quantitation, requiring additional instrumental capabilities to increase experimental productivity. Small-molecule panels involving metabolites and lipids contain analytes with a wider range of physicochemical properties that may result in better ionization response in negative and positive mode electrospray ionization (-ESI than +ESI) as well as alternative collision dissociation mechanisms to ensure formation of diagnostic product ions that contain isotopic enrichment sites. The combination of the QR5 Plus quadrupole mass filter, ICRM, and hyper-fast dual-pressure linear ion trap mass analyzer provide differentiated capabilities unique to the Stellar mass spectrometer.

While the PRM Conductor software can read in discovery data from high-resolution, accurate mass (HRAM) mass spectrometers, laboratories may not have access to additional instruments, therefore relying on one instrument for all experimental aspects of the study. The Stellar mass spectrometer leverages the fast acquisition speeds and industry-leading polarity switching speeds to fully characterize the targeted analytes in an LC-timescale expediting method building.

Figure 9 shows the acquisition scheme that was employed to characterize amino acids in one sample injection. The Stellar mass spectrometer surpasses triple quadrupole mass spectrometers for characterization due to fast full scan MS^n acquisition (almost 6X faster) to evaluate the most abundant precursor formed in either +ESI or -ESI or identify putative background matrix interference. Corresponding full scan MS^n spectra can also be acquired to determine optimal product ions used for tMS2 or tMS3 quantitation. As described above, the Stellar mass spectrometer also utilizes NCE settings as opposed to absolute CE values simplifying method building. Finally, the Stellar mass spectrometer offers HCD and CID fragmentation options expanding compound characterization steps. While the options are much greater than that using a QQQ mass spectrometer, the instrument method editor simplifies experimental set up with drag-and-drop methods and a few user-defined settings with drop-down options.

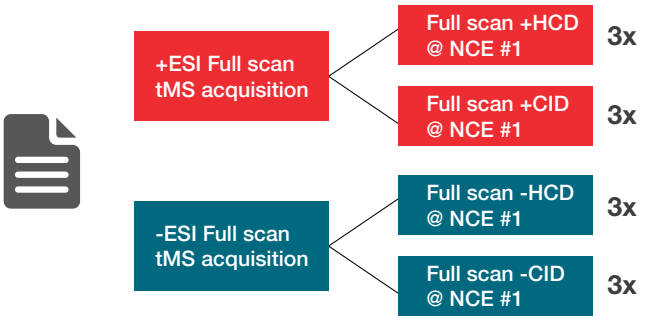

Figure 9. Full analyte characterization relying on the user-defined import compound list. Compound characterization can utilize polarity switching for full scan MS acquisition, HCD and/or CID fragmentation, and normalized collision energy at either multiple settings or using stepwise fragmentation.

The exhaustive acquisition strategy outlined above was tested for a mixture of 15 amino acid standards and corresponding SILs. A 5-minute LC gradient was used for separation for the entire run and the precursor *m/z* values for all target compounds were imported into the targeted mass list. Figure 10 shows that the average peak width for the amino acids is between 3 and 4.5 seconds and for each elution profile there are numerous scan events acquired in each ESI polarity and MS^n level.

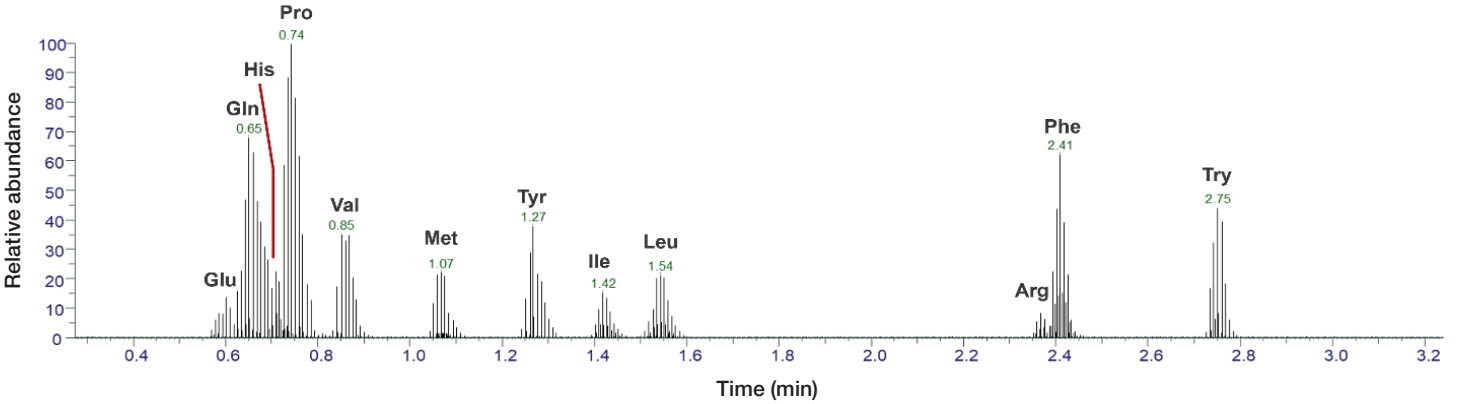

Figure 10. Shows the acquisition time for the set of scan events used to profile the set of amino acids only.

To demonstrate the acquisition speed for full scan MS and MS^2 spectra, methionine was profiled. Figure 11 shows the various scan events acquired during the methionine elution profile. Data was collected in both ESI polarity modes for full scan MS and MS^2 for the SIL and unlabeled methionine standard. Additional precursors were analyzed by full scan MS^2 events in between the set of full scan MS. A total of 160 scan events were acquired in 4.2 seconds that included nine MS in each polarity, 142 full scan MS^2, and 33 polarity switching events which took only 165 milliseconds (4%) of the total instrument run time.

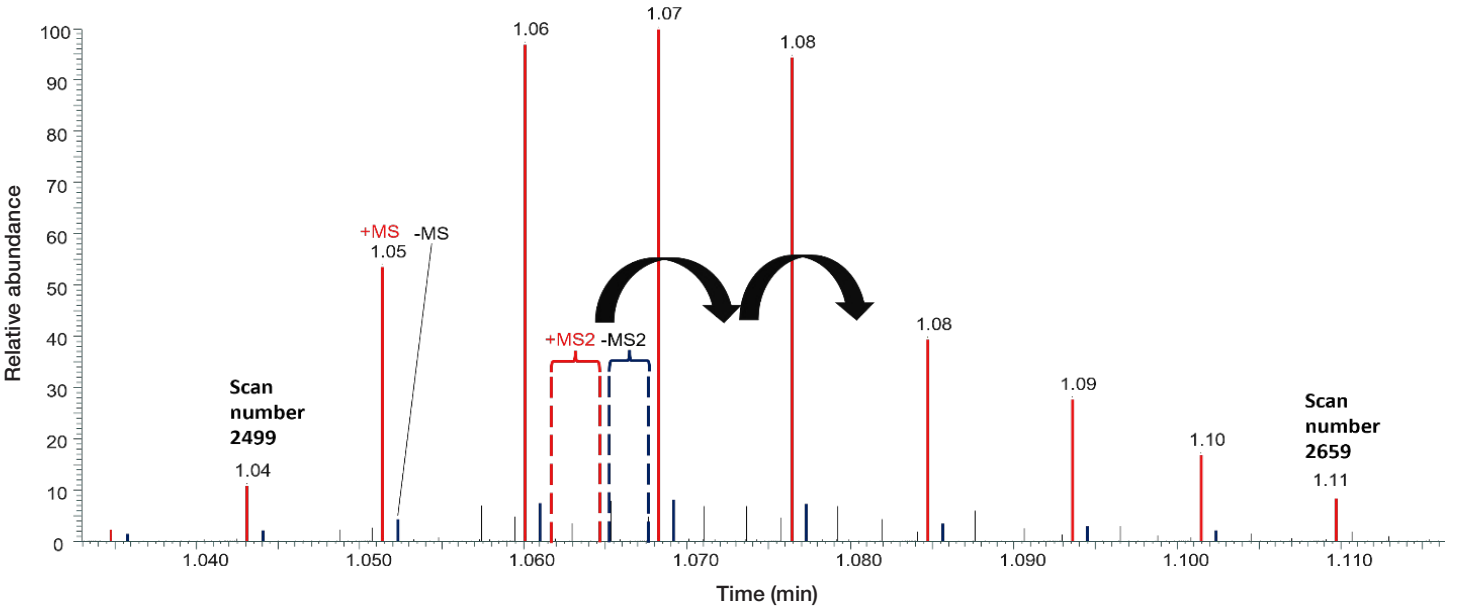

Figure 11. Stick plot representing the set of MS and MS^2 scan events in +ESI (shown in red) and -ESI (shown in blue) polarity. Solid lines represent full scan MS and dashed lines represent the time range used to acquire nine full scan MS^2 scan events for both +ESI and -ESI modes. The acquisition series is repeated across the elution profile.

Figure 12 shows the full scan MS analysis for methionine and the corresponding internal standard (IS) with precursors measured at *m/z* 150 and 154 in +ESI mode, and *m/z* 148 and 152 in -ESI mode, respectively. The full scan analysis also enables researchers to evaluate the potential for alternative forms of ionization, especially in +ESI mode for protonation ($[M+H]^+$ or alternative adduct formation including ammonia, sodium, and potassium that could present better options for targeted analysis. In addition, the full scan MS analysis enables inspection of co-eluting isobaric interference reducing tMS2 performance.

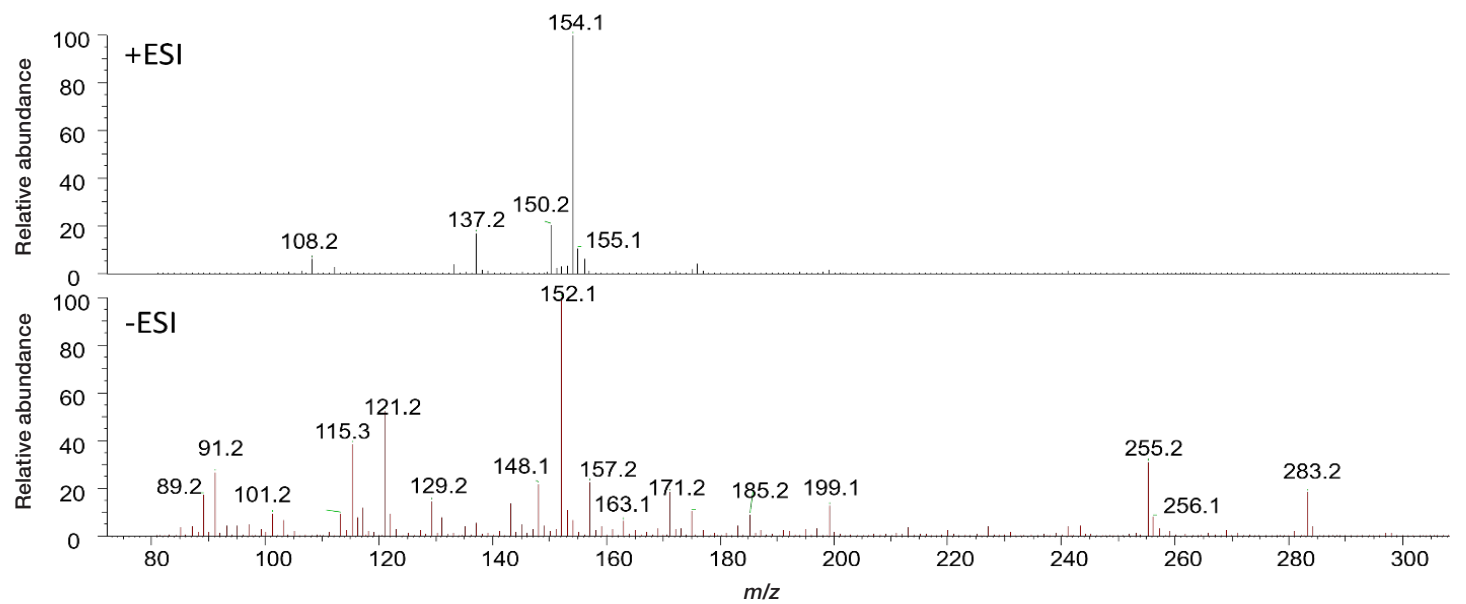

Figure 12. Full scan MS analysis in +ESI (top) and -ESI (bottom) for the methionine and methionine IS elution.

Figures 13 and 14 show the full scan MS^2 spectra acquired in both ESI polarity modes for methionine and its internal standard. Acquiring the full scan MS^2 enables evaluation of comparative product ion ratios and the localized site of isotopic enrichment that can be critical in establishing confirmation metrics. Comparison of product ion spectra acquired in both positive and negative polarity can enable decisions to be made that may sacrifice signal intensity for specificity and improved signal-to-noise. For methionine, the HCD spectrum acquired in -ESI mode resulted in a single product ion at a low *m/z* value that can be more susceptible for background interference.

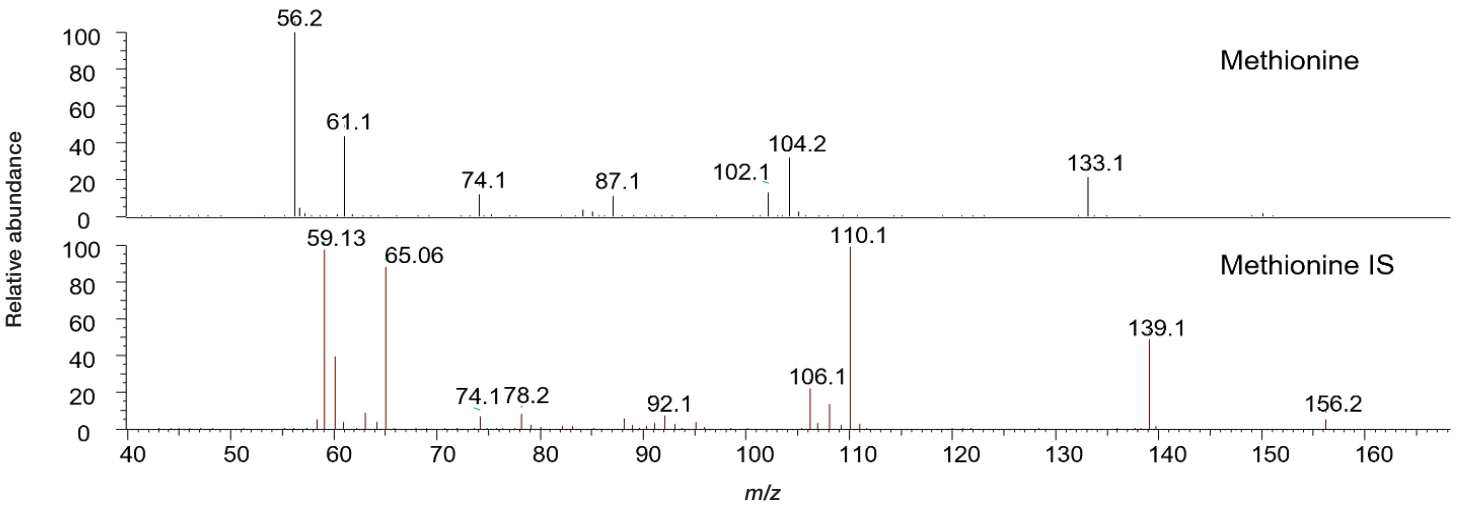

Figure 13. Comparative +ESI full scan MS^2 for methionine (top) and the internal standard (bottom) acquired using HCD with a NCE setting of 30.

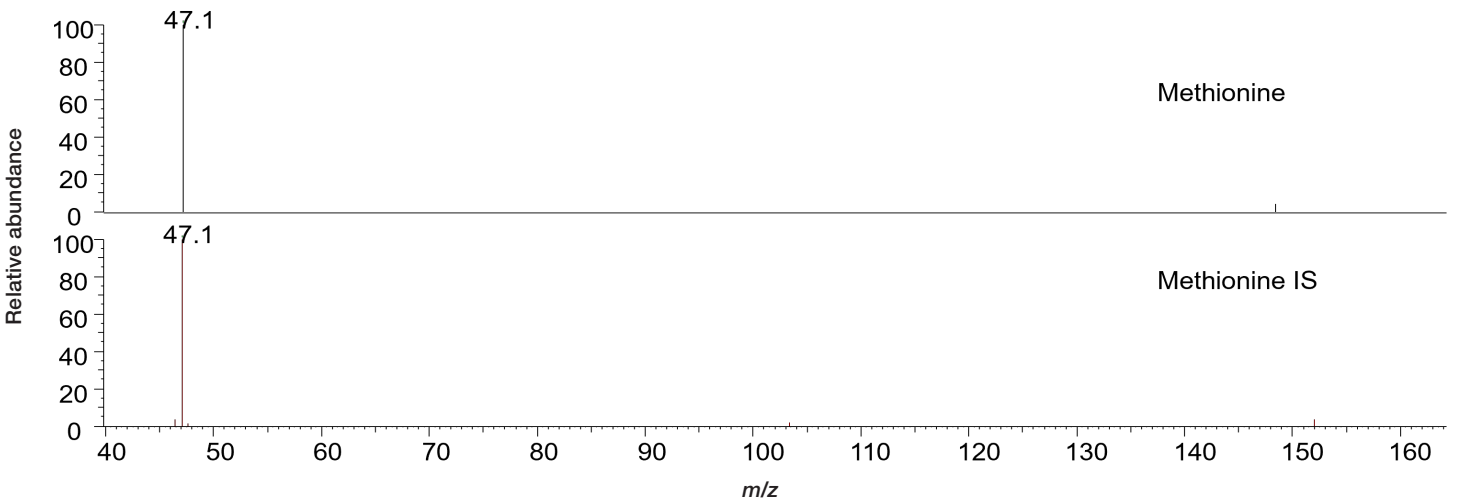

Figure 14. Comparative -ESI mode full scan MS^2 for methionine (top) and the internal standard (bottom) acquired using HCD with a NCE setting of 30.

Improved environmental impact

In addition to the improved targeted quantitative performance the Stellar mass spectrometer delivers, it has been designed to lower the environmental impact in operation. The footprint of the instrument is 710 × 760 × 840 mm (h, w, d – 28 × 30 × 33 in) freeing up more benchtop space. The mass spectrometer has one Edwards nXR120i dry-pump requiring no maintenance or oil, as well as an advanced triple-inlet turbomolecular pump integrated with the vacuum manifold. The use of one mechanical and turbo pump generates a maximum heat output of 1,002 W (3.416 Btu/h) and total system heat output of 1,550 W (5,300 Btu/h). The system noise output is 69 dB recorded at a 1-meter distance that collectively reduces its laboratory impact.

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