High resolution LC-MS/MS solution for accurate quantification of host cell proteins

Featuring the SCIEX TripleTOF® 6600+ LC-MS/MS System

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Host cell proteins (HCPs) are process-related impurities present in the biotherapeutics manufacturing process. It is important to accurately detect and quantify HCPs, because they can adversely affect drug safety and efficiency. Unlike polyclonal ELISA-based methods which only provide the quantitative information of a total HCP level, LC-MS/MS offers improved selectivity to provide the identification and quantification of individual HCPs. As the levels of HCPs vary dramatically during the manufacturing/purification process, their concentrations in the late stage or final drug product are significantly lower than the biotherapeutic protein. Therefore, the traditional data dependent acquisition (DDA) with MS1 level quantification shows limitations when analyzing HCP samples with strong matrix interference and a wide dynamic range of protein concentrations.

The SCIEX TripleTOF® 6600+ System offers two high resolution MS/MS based workflows for HCP quantification, data independent approach using SWATH® Acquisition and targeted approach using MRM^{HR}. SWATH Acquisition measures every detectable analyte in the sample with superior quantification capability. MRM^{HR} allows ultra-sensitive quantification of target proteins with high analysis throughput. Both workflows demonstrate higher selectivity over the MS1 based quantification, thereby improving assay sensitivity to quantify HCPs in single-digit ppm or sub-ppm levels.

Key features of the high-resolution MS/MS solutions for HCP quantification

The data-independent approach using SWATH Acquisition offers:

- Superior quantification capability based on high resolution MS/MS spectra on every detectable analyte in the sample
- Ease of method development with minimum method optimization requirement
- Variable Q1 window acquisition by optimizing window width based on MS/MS spectra *m/z* density of precursors

The targeted approach using MRM^{HR} acquisition offers:

- Ultra-sensitive quantification of target proteins based on high resolution MS/MS spectra
- MRM-like analysis throughput with <10 min total run time
- Comprehensive data processing solution to cover the entire workflow from protein identification to quantification

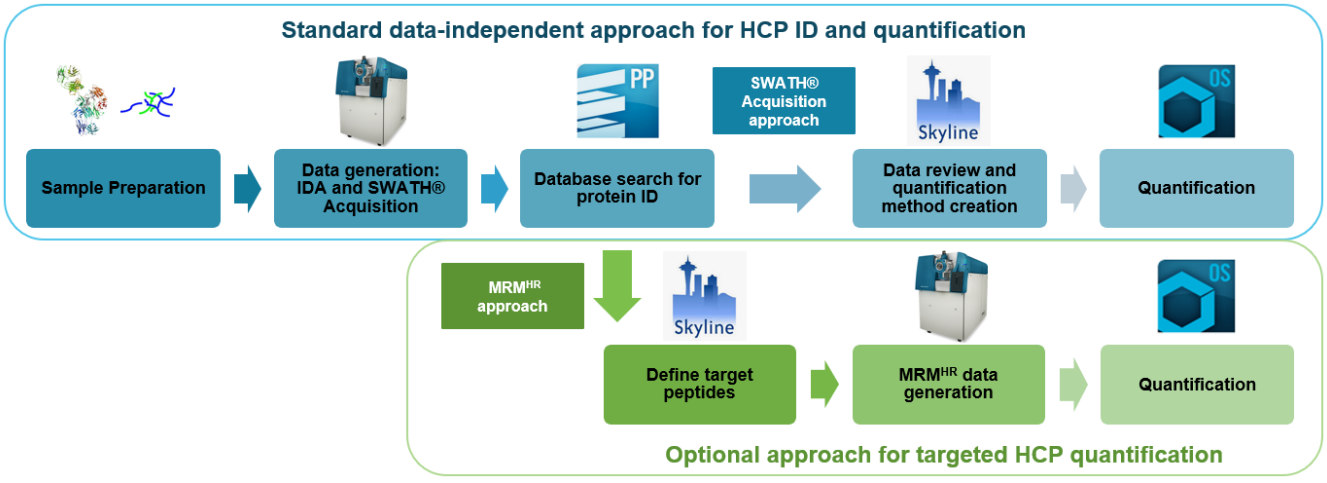

Figure 1. The high-resolution LC-MS/MS workflows for HCP quantification, from sample preparation, data generation to data processing. Both data independent approach using SWATH Acquisition and targeted approach using MRM^{HR} are demonstrated.

Methods

Sample preparation: NISTmAb monoclonal antibody (NISTmAb) and the Universal Proteomics Standard (UPS) are purchased from Sigma-Aldrich. In this experiment model, NISTmAb is serving as the biotherapeutic molecule, while the human UPS proteins are mimicking the targeted HCPs for quantification. The UPS proteins are spiked into NISTmAb solution and serial diluted. Bovine serum albumin (BSA) is used as the internal standard. The UPS proteins and BSA are spiked into NISTmAb solution and serial diluted. The level of UPS proteins ranges from 4.9 to 5000 fmol per 100 µg NISTmAb. The ppm level range for each individual protein varies based on the protein molecular weight (MW).

Samples are denatured by incubating with N-octyl-glucoside (OGS), reduced by dithiothreitol (DTT) and alkylated by iodoacetamide (IAM). A trypsin/Lys-C digestion was performed at 37 °C for overnight, with an enzyme-protein ratio at 1:25. Formic acid was spiked into the samples to abort digestion. The samples are centrifuged at the speed of 12000 g and the supernatants are injected into LC-MS analysis.

LC-MS conditions: A TripleTOF 6600+ LC-MS/MS System coupled with an ExionLC™ system are utilized for high resolution data generation. The MRM^{HR} analysis is performed for targeted quantification of selective HCPs. The information dependent acquisition (IDA) analysis followed by the SWATH Acquisition are performed for general HCP identification and quantification. The experimental details are summarized in Tables 1-5.

Table 1. HPLC condition for IDA analysis and SWATH Acquisition.

Parameter	Value
Stationary phase	*C18 column, 2.1 X 150 mm, 1.7 µm*
Mobile phase A	*0.1% formic acid in water*
Mobile phase B	*0.1% formic acid in acetonitrile*
Gradient	*B% ramping from 5% to 40% in 42 min*
Total run time	*60 min including equilibration*
Flow rate	*0.2 mL/min*
Column temperature	*40 °C*

Table 2. Mass spectrometer parameters for IDA analysis.

Parameter	Value	Parameter	Value
MS range:	*350-1500*	*MS/MS range:*	150-1500
MS accumulation time:	*150 ms*	*MS/MS accumulation time:*	*30* ms
Curtain gas:	*30 psi*	*Source temperature:*	450 *°C*
Ion source gas 1:	*50 psi*	*Ion source gas 2:*	50 *psi*
Number of MS/MS triggered per circle:	*25*	*Ion spray voltage:*	5500 V

Table 3. Mass spectrometer parameters for SWATH Acquisition.

Parameter	Value	Parameter	Value
MS range:	*350-1500*	*MS/MS range:*	150-1500
MS accumulation time:	*50 ms*	*MS/MS accumulation time:*	*30* ms
Curtain gas:	*30 psi*	*Source temperature:*	450 *°C*
Ion source gas 1:	*50 psi*	*Ion source gas 2:*	50 *psi*
Number of SWATH windows:	*30*	*Ion spray voltage:*	5500 V

Table 4. HPLC condition for MRM^{HR} analysis.

Parameter	Value
Stationary phase	*Phenomenex Kinetex C18 column, 3 X 50 mm, 2.6 µm*
Mobile phase A	*0.1% formic acid in water*
Mobile phase B	*0.1% formic acid in acetonitrile*
Gradient	*B% ramping from 12% to 32% in 5 min*
Total run time	*8 min including equilibration*
Flow rate	*0.5 mL/min*
Column temperature	*40 °C*
Divert valve set-up	*1-6.2 min to MS*

Table 5. Mass spectrometer parameters for MRM^{HR} analysis.

Parameter	Value	Parameter	Value
Curtain gas:	*30 psi*	*Source temperature:*	550 *°C*
Ion source gas 1:	*65 psi*	*Ion source gas 2:*	65 *psi*
CAD gas:	*12 psi*	*Ion spray voltage:*	5500 V
MS accumulation time:	*40 ms*	*MS/MS accumulation time:*	*40* ms

Data processing: Figure 1 describes the general workflows for SWATH Acquisition based (data independent approach) and MRM^{HR} based (targeted approach) HCP quantification.

For protein ID purposes, the IDA data are generated and submitted to ProteinPilot™ Software 5.0 for database search. A .fasta file including sequences of NISTmAb and USP proteins is used as the database. The search result file is generated and imported into Skyline software for peptide library creation.

Skyline software is used for data review and quantification method generation. The most abundant fragment ions observed in the MS/MS spectra of the peptide library are selected to create the quantification method, which can be imported into SCIEX OS Software 1.7. The quantification data are then processed by using the Analytics function in SCIEX OS Software 1.7.

Protein identification with IDA analysis

To identify the HCPs in the sample and select appropriate peptides for quantification, an IDA peptide mapping analysis is performed on a TripleTOF 6600+ LC-MS/MS System to analyze the digested NISTmAb-UPS protein mix. The IDA data are processed by ProteinPilot Software 5.0, searched against the protein sequences of potential HCPs and biotherapeutics. In this experiment, they are NISTmAb and UPS proteins. It is worth noting that multiple IDA data can be co-submitted to ProteinPilot Software 5.0 for combined database search to achieve comprehensive protein ID coverage. The information of peptide ID, sequence coverage and MS/MS fragment ion assignment is covered in the search result file (Figure 2). This result file is imported into Skyline, served as the peptide library.

Figure 2. Example database search result shown in ProteinPilot 5.0 Software. The result file provides information including (from top to bottom panel): the list of identified protein, the summary of peptides identified per protein, the MS/MS fragment ion assignment for each peptide, the sequence coverage map.

Data generation: data independent approach using SWATH Acquisition

For HCP quantification, two approaches are developed with SCIEX high-resolution MS platforms. In the data independent approach, SWATH Acquisition, combined with IDA, was utilized for general HCP identification and quantification to look into global HCP levels during process development. It generates high-resolution MS/MS spectra for every detectable analyte in the sample (Figure 3). There are multiple benefits using SWATH Acquisition. Compared to DDA, SWATH Acquisition constantly measures all fragment ions and thereby allowing XIC generation for MS/MS level quantification of every analyte in the sample. Compared to MS1 level quantification, it offers enhanced selectivity when monitoring the high-resolution fragment ions. The method is also straightforward to set up, by using generic method parameters with minimum method optimization requirement.

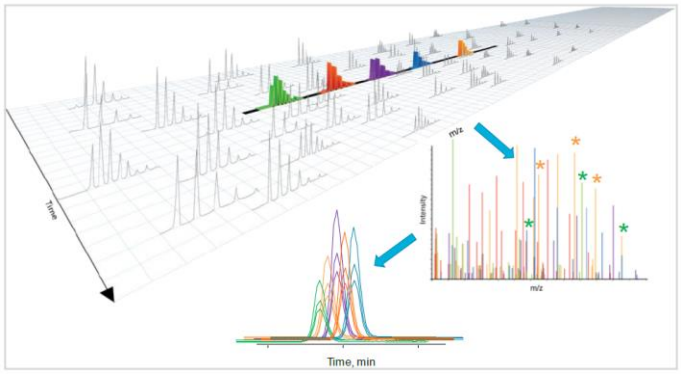

Figure 3. SWATH Acquisition. In this workflow, instead of the Q1 quadrupole transmitting a narrow mass range through to the collision cell, a wider window containing more analytes is passed. Because the fragment ions are high resolution, high quality XICs can be generated post-acquisition to produce the MRM-like data. This Q1 window can be stepped across the mass range, collecting full scan composite MS/MS spectra at each step, with an LC compatible cycle time.

In this experiment, a variable window acquisition strategy is applied in SWATH Acquisition, by setting up different sizes of the Q1 isolation window based on the density of analyte precursor masses.[1,2] A total of 30 variable isolation windows are built in this method for optimal mass range coverage and high quantification performance.

Data generation: targeted approach with MRM^{HR}

In the targeted approach, the selected HCP targets are monitored for ultra-sensitive quantifications, commonly applied in manufacturing and QC stages. The MRM^{HR} acquisition is performed on a defined list of signature peptides for

quantification. The signature peptides for each protein are selected based on the mass spectrometer signal abundances and sequence uniqueness. The mechanism of a MRM^{HR} workflow for quantification is to acquire full scan TOF MS/MS spectra for each species of interest. The high resolution extracted ion chromatograms (XICs) of specific product ions or a sum of ions can then be used post-acquisition for quantification to achieve optimum sensitivity and selectivity (Figure 4). The selection of signature fragment ions for quantification is not required prior to data acquisition, offering the flexibility to choose different fragment ions based on matrix effects. The MRM^{HR} workflow also offers significantly higher selectivity over a full scan TOF MS approach.

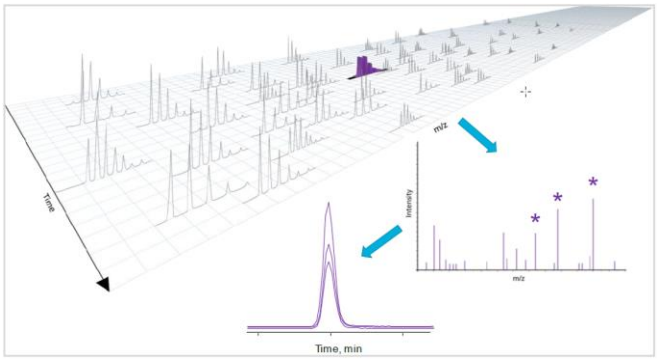

Figure 4. MRM^{HR} workflow using the TripleTOF systems. In the MRM^{HR} workflow, a fixed number of analytes are targeted and high-resolution MS/MS spectra are collected across an LC run. Precursor masses are selected at narrow resolution such that mainly the target compound is passed into the collision cell (top). This produces a full scan MS/MS spectrum enriched for the analyte of interest (right). Then, any number of fragment ions can be extracted at high resolution post-acquisition to generate MRM-like data (bottom).

In this project, a two-period MRM^{HR} acquisition method is used to quantify selective HCPs, in which multiple MS/MS experiments are set up per period for improved multiplexing capability. The total run time for MRM^{HR} is only 8 min, as short as a regular MRM based method, for high throughput analysis.

Fragment ion selection for quantification

To identify the appropriate fragment ions for quantification, the database search result file is imported into Skyline software, served as the peptide library. HCP protein sequences are imported as well, the list of peptides and fragment ions are generated based on peptide/transition settings and peptide library matching. The precursor ion can also be included for MS1 based data processing. Optionally, representative MRM^{HR} or SWATH Acquisition data files can be imported into Skyline to generate XICs for peak assignment verifications and modifications. A quantification method or transition list is generated and ready to be imported into SCIEX OS Software for quantification.

Protein quantification

Both SWATH Acquisition and MRM^{HR} data are processed by using the Analytics function in SCIEX OS Software 1.7, with the following information provided: XIC extractions, peak integrations, quantification result summaries, calibration curves, statistical summaries, etc. (Figure 6). Several example XICs and calibration curves are shown in Figure 7 and 8, demonstrating outstanding quantification capabilities for both workflows. Please note: additional semi-quantitative SWATH Acquisition data processing workflow is available for HCP level estimation, demonstrated in the previously published application note.[3]

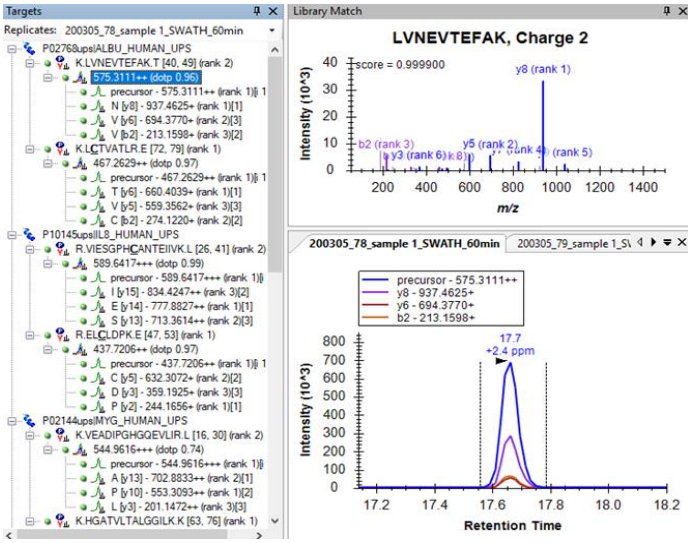

Figure 5. A screenshot of Skyline Software for fragment ion selection. Left: the list of selected peptides and fragment ions to create quantification method. Right top: the representative MS/MS spectrum from the imported peptide library. The most abundant fragment ions (ranked and color labeled) are listed as the signature fragment ions. Right bottom: the XICs of the selected precursor ion and fragment ions for peak assignment verification.

Comparison between MS/MS and MS1 based quantification

For both SWATH Acquisition and MRM^{HR} analysis, there are three ways to calculated XIC peak areas for quantification using: 1) the peak area of a single fragment ion XIC, 2) the sum of peak areas from multiple fragment ion XICs, or 3) the XIC peak area from the precursor ion associated with the target peptide. To identify the ideal data processing method to achieve the lowest LLOQ, the data are processed against all three strategies. Because of the additional level of selectivity provided by HR

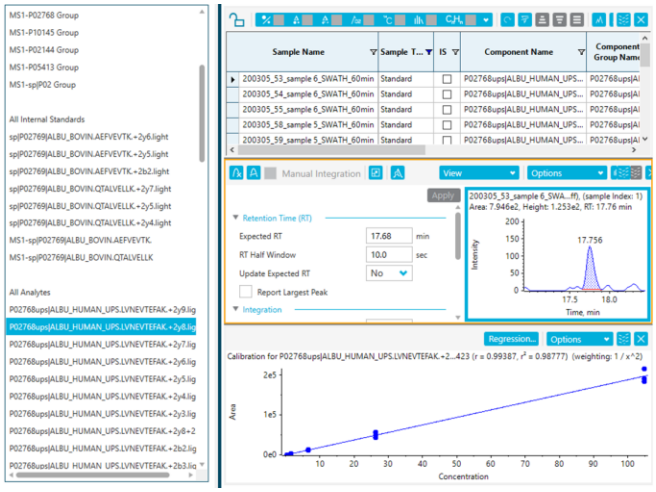

Figure 6. A screenshot of SCIEX OS Software 1.7 for HCP quantification. The following information is shown: analyte list, quantification result table, example XIC, integration parameters and calibration curve.

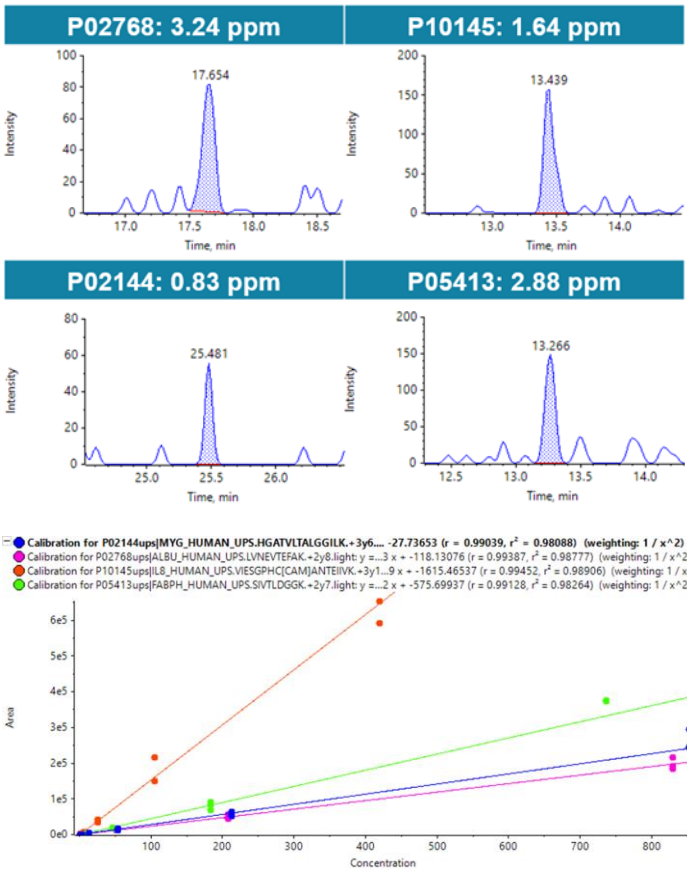

Figure 7. Representative SWATH Acquisition data. Top: XICs of HCPs at their LLOQ level. Bottom: calibration curves

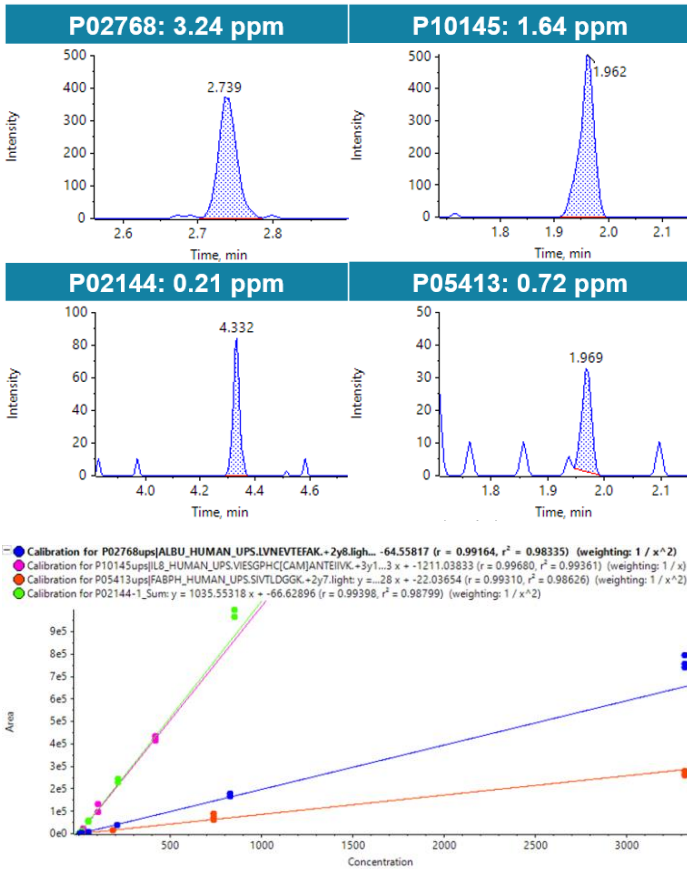

Figure 8. Representative MRMHR data. Top: XICs of HCPs at their LLOQ level. Bottom: calibration curves.

MS/MS, notably better S/N is observed when using fragment ion XICs for quantification than using precursor ion XICs (Figure 9).

A comparison between the single fragment ion XIC and the sum of multiple XICs is also performed. When one single high abundant fragment ion is generated in MS/MS, the single XIC approach typically provides the best S/N. When multiple high abundant fragment ions are generated from the target peptide, the sum of XICs can further enhance the assay sensitivity (Figure 10). Therefore, the selection between these two approaches need to be evaluated based on the CID fragmentation profile of the target peptide.

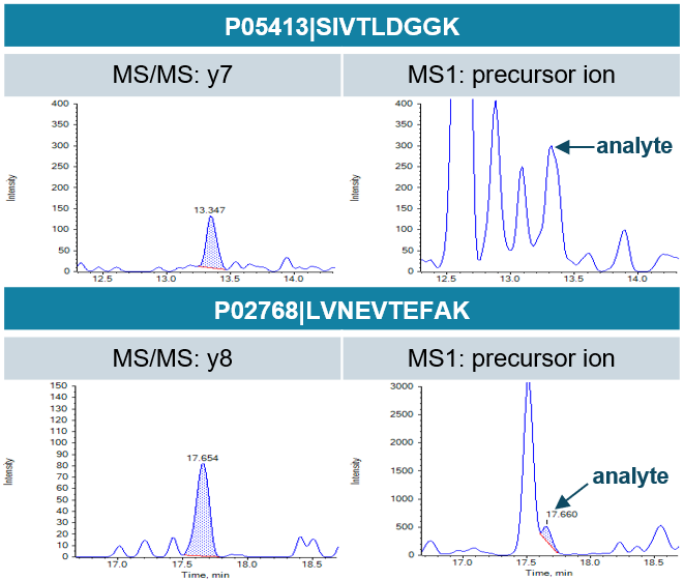

Figure 9. An example comparison between quantification using fragment ion XIC and precursor ion XIC (SWATH ® Acquisition data). Significantly better S/Ns are observed using fragment ion XICs (left).

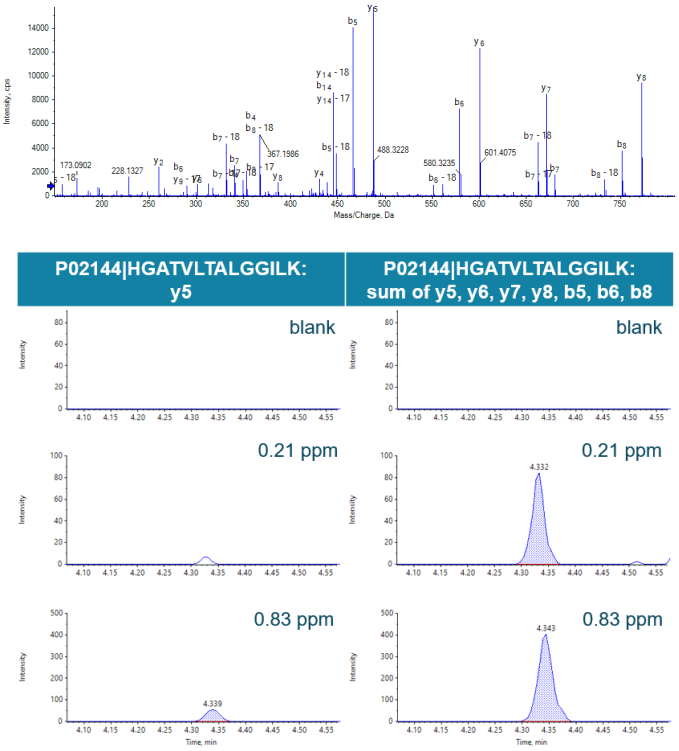

Figure 10. An example comparison between quantification using single fragment ion XIC and sum of multiple fragment ion XICs (MRM^{HR} data). Top: the MS/MS of peptide HGATVLTALGGILK showing multiple fragment ions with high intensity. Bottom: the XICs comparison between y5 only and the sum of y5, y6, y7, y8, b5, b6, b8. Significantly better signal intensity and S/N is observed using the XIC summation. The data is generated with MRM^{HR}.

Conclusions

Two high resolution MS/MS based workflows using the SCIEX TripleTOF 6600+ System are presented to quantify HCPs in single-digit ppm or sub-ppm levels.

- The data-independent approach using SWATH Acquisition offers superior quantification capability based on high resolution MS/MS spectra on every detectable analyte in the sample
- The targeted approach using MRM^{HR} acquisition offers ultra-sensitive quantification of target proteins based on high resolution MS/MS spectra with high analysis throughput
- Comprehensive data processing solution is demonstrated to cover the entire workflow from protein identification to quantification
- Combining with the previous published MRM workflow[4], the most comprehensive LC-MS/MS solution is offered for accurate HCP quantification

References

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