

Automated High-Throughput Data Processing for Targeted Multiplexed Insulin Analog Detection and Quantification

Scott Peterman¹, Kwasi Antwi², Bryan Krastins¹, Eric E. Niederkofler², and Mary Lopez¹

¹Thermo Fisher Scientific BRIMS, Cambridge, MA

²Thermo Fisher Scientific, Tempe, AZ

Key Words

Q Exactive, insulin, insulin variants, Pinpoint, HRAM, high resolution, accurate mass, MSIA, mass spectrometric immunoassay, automation, research

Goal

Present an automated, multiplexed, high-throughput data processing workflow for detection and targeted quantification of insulin and its analogs at concentration ranges of 3.75 to 960 pM in biological matrices.

Introduction

The development of robust biotechnology-based methods to create recombinant peptide hormones with altered peptide sequences has produced hormone variants designed to fit specific needs such as insulin analogs. The development of these variants requires accompanying advances in the analytical technologies used for their detection and quantification in research applications. Routine, global sample preparation, data acquisition, and data processing methods that address expected concentration levels in biological matrices are needed.¹ Traditional global sample preparation and detection methods tend to decrease assay selectivity and sensitivity. Rapid, targeted quantitation of many closely related analytes places significant demands on the software tools used.

To address the analytical requirements of routine detection and quantification of peptide variants in biological matrices, a complete workflow that employs multiplexed Thermo Scientific™ MSIA™ (mass spectrometric immunoassay) technology was created.² MSIA technology couples global affinity capture sample preparation with high-resolution, accurate-mass (HRAM) spectrometric detection.³ The Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap™ mass spectrometer was used to collect full-scan HRAM data.⁴

Thermo Scientific™ Pinpoint™ software provided automated qualitative and quantitative HRAM data processing. The complementary research paper published in *Proteomics* provides a detailed description of the MSIA-HRAM method and results for targeted quantification of intact insulin and its analogs in human serum and plasma.⁵

Experimental

Reagents

Insulin analogs Humulin® S (Lilly, 100 IU/mL), Apidra® (Sanofi Aventis, 100 U/mL), Lantus® (Sanofi Aventis, 100 U/mL), NovoRapid® (Novo Nordisk, 100 U/mL), and Hypurin Porcine (CP Pharmaceuticals, 100 U/mL) were provided by Dr. Stephen Morley (Sheffield Hospital, UK). Bovine serum albumin (BSA, Calbiochem) prepared at 50 g/L in phosphate-buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4) served as the biological matrix. Bovine insulin, TWEEN® 20, and phosphate-buffered saline were obtained from Sigma-Aldrich®. Ultra-pure water, trifluoroacetic acid, and ammonium acetate were obtained from American Bioanalytical. ACTH 1-24 was obtained as a carrier peptide from Bachem®. LC-MS grade water, LC-MS grade acetonitrile, and formic acid were Fisher Chemical brand. Thermo Scientific™ MSIA™ D.A.R.T.'S (Disposable Automation Research Tips) were coupled with anti-human insulin antibody.

Sample Preparation

Two sets of samples were prepared. First, a dilution series of Humulin S, Apidra, Lantus, NovoRapid, and bovine insulin, prepared in the presence of porcine insulin (50 pM) and covering an analytical concentration of 1.5 to 960 pM, were spiked into a phosphate-buffered saline-bovine serum albumin (PBS/BSA) matrix. The second set consisted of Apidra, Lantus, and NovoRapid spiked individually into human plasma at the same concentration range (1.5 to 960 pM). For quantitation curve development, both Apidra and Lantus were spiked into plasma across the same concentration range (1.5 to 960 pM). Porcine insulin was again spiked into each sample at 50 pM as an internal standard.

Mass Spectrometric Immunoassay

The affinity capture of insulin was achieved using insulin-specific MSIA D.A.R.T.'S mounted onto the 96-channel pipetting head of the Thermo Scientific™ Versette™ automated liquid handler. After rinsing the insulin MSIA D.A.R.T.'S with 15 cycles of a single aspiration and dispensing 150 μ L 10 mM PBS, the insulin MSIA D.A.R.T.'S were immersed into the samples and 100 aspiration and dispense cycles of 250 μ L were performed. The multiple cycles allowed simultaneous affinity enrichment of all of the insulin analogues as well as the internal standard. The MSIA D.A.R.T.'S were then rinsed with PBS (15 cycles) from another microplate, followed twice by water (15 cycles) from two additional microplates (150 μ L aspirations and dispenses, from 200 μ L in each well).

The affinity-captured insulin analogs were eluted to a microplate by aspirating and dispensing 80 μ L of 15 μ g/mL ACTH 1-24 in 33% acetonitrile/0.4% (v/v) trifluoroacetic acid (TFA) 100 times from a total of 100 μ L volume in each well. The eluates were dried down in a Thermo Scientific™ Speed Vac™ concentrator until dry and then resuspended in 100 μ L reconstitution buffer (25% acetonitrile/0.2% formic acid (v/v)). The microplate was sealed and vortexed for 30 seconds to ensure proper reconstitution, and then spun-down prior to loading samples onto the LC.

LC/MS Method

Samples were analyzed using a generic LC/MS method. A Thermo Scientific™ UltiMate™ 3000 RSLCnano LC system was used for all LC/MS experiments. To begin, 100 μ L of each sample was separated on a 1 x 250 mm Thermo Scientific™ ProSwift™ RP-4H column using a linear gradient (10–50% in 10 minutes) comprised of A) 0.1% formic acid in water and B) 0.1% formic acid in acetonitrile. The column was heated to 50 °C.

All data were acquired using a Q Exactive mass spectrometer operated in data-dependent/dynamic exclusion mode. A resolution setting of 70,000 (FWHM) at m/z 200 was used for full-scan MS and 17,500 for MS/MS events. Full-scan MS data were acquired using a mass range of 800–2000 Da. A targeted inclusion list was used to trigger all data-dependent events.

Data Analysis

All data were processed using Pinpoint software (revision 1.4). HRAM MS data extraction was used for quantification. To provide additional levels of qualitative analysis, the three most abundant precursor charge states per insulin variant were used, as well as the six most abundant isotopes per charge state. A mass tolerance of ± 7 ppm was used to extract all data. Qualitative scoring was based on mass error, precursor charge state distribution, isotopic overlap, and corresponding LC elution peak profiles measured for each sample. Product ion data were used for sequence verification. The measured area-under-curve (AUC) values for porcine insulin were used as the internal standard for all samples.

Results and Discussion

To assess the workflow, the insulin variants were spiked into two different matrices and processed. The effects of the matrix, competitive binding/extraction of all insulin variants, and automated data extraction, verification, and quantitation were evaluated. The HRAM data acquisition capability of the Q Exactive mass spectrometer enabled downstream automated qualitative and quantitative data processing using Pinpoint software. By acquiring data in a nontargeted manner, post-acquisition methods can be used to process the data for any insulin variant sequence or modification. To increase the qualitative information obtained, multiple target-specific attributes per insulin variant were chosen for analysis by the software.

Qualitative Data Processing Strategy

Figure 1 shows the base peak chromatogram for the human plasma sample spiked with 960 pM of Lantus and Apidra insulin analogs and 50 pM of porcine insulin extracted using MSIA. The data acquisition time range used was 3.5 to 5.5 minutes. The chromatographic trace shows two peaks eluting, with the peak at 4.52 minutes attributed to the Lantus insulin analog and the peak at 4.72 minutes attributed to porcine, human, and Apidra insulin analogs. The inset shows the averaged HRAM mass spectrum around the +5 precursor charge states for the insulin analogs. The observed relative abundance of Apidra to porcine (ca. 6%) was in close agreement with the spiked amounts of 960 to 50 pM, respectively. The observed relative abundance of endogenous human insulin was equivalent to that of porcine. The remaining peaks in the mass spectrum were attributed to adduct formation during ionization. Despite the large difference in the amounts present in the plasma samples, there was little interference observed when detecting all insulin variants. The resolution of the Q Exactive mass spectrometer was more than sufficient to baseline resolve the isotopic profiles for the +5 charge state across the dynamic range.

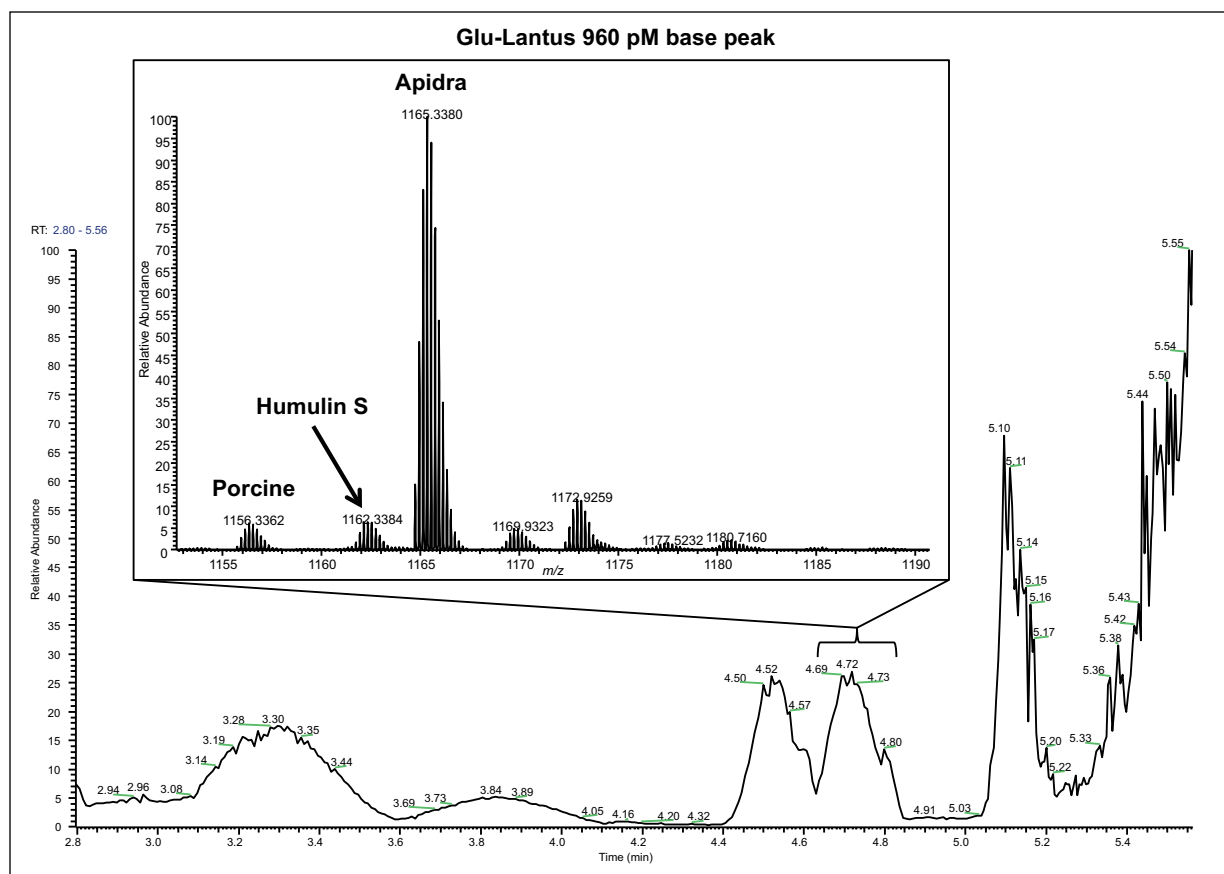


Figure 1. Base peak chromatogram for the MSIA-extracted human plasma sample spiked with 960 pM of both Apidra and Lantus insulin variants and 50 pM of porcine insulin (internal standard). The inset shows the summed mass range covering three of the four insulin variants.

Figure 2a shows the initial data extract using multiple isotopes per charge state. Pinpoint software determined the isotopic distribution and m/z list and created the theoretical profile based on user-defined sequence and possible modifications. Each isotopic m/z value was used to create an extracted ion chromatogram (XIC) with a ± 7 ppm window, providing first-level qualitative analysis. The resulting XICs were overlaid to determine the retention time (Figure 2b) and AUCs were calculated for all isotopes. The overlaid peak profiles enabled scoring the LC component based on common peak shapes (peak state/end, apex, and symmetry) for the collection of isotopes of one or more precursor charge states. The color-coding capability of Pinpoint software facilitated data review.

The AUC values for each isotope were calculated and then used to determine background interference. Figure 2c shows the Pinpoint software-generated bar chart used to evaluate the isotopic distribution profile of human insulin.

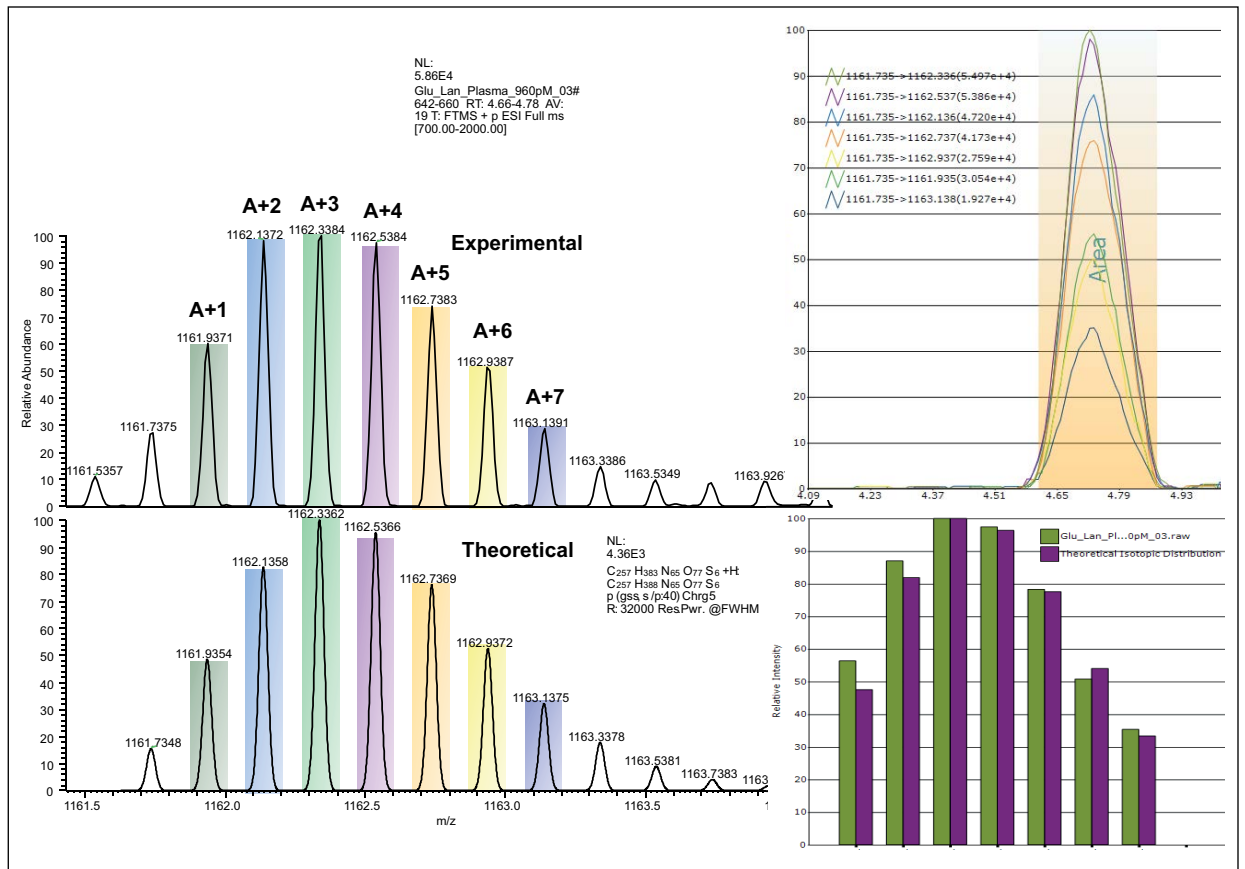


Figure 2. Data processing using Pinpoint software. Figure 2a shows the targeted data extraction based on isotopic m/z values for the seven most abundant isotopes, and a ± 7 ppm extraction tolerance based on the theoretical isotopic distribution. Figure 2b shows the overlaid XICs for each of the targeted isotopes. The AUC values for each isotope were used to evaluate the scoring shown in Figure 2c, where the relative AUC values for the collective isotopic distribution were compared to the theoretical value.

LC-MS methods, particularly those employing HRAM detection, provide significant advantages over enzyme-linked immunosorbent assay (ELISA), capillary electrophoresis (CE), and ultraviolet (UV) methods because the selectivity of MS allows detection of co-eluting analogs. Three co-eluting insulin analogs shown in Figure 1 were easily separated based on the accurate m/z values of each precursor charge state and corresponding isotopes. Comparative analyses for the three insulin variants (porcine, human, and Apidra), including XICs and the total isotopic distribution, are shown in Figure 3. Pinpoint software automatically calculated the dot product correlation coefficient for the charge states that was used to evaluate isotopic distribution overlap and filter results. Here the dot product scores for each charge state and analog were greater than 0.9, an excellent match.

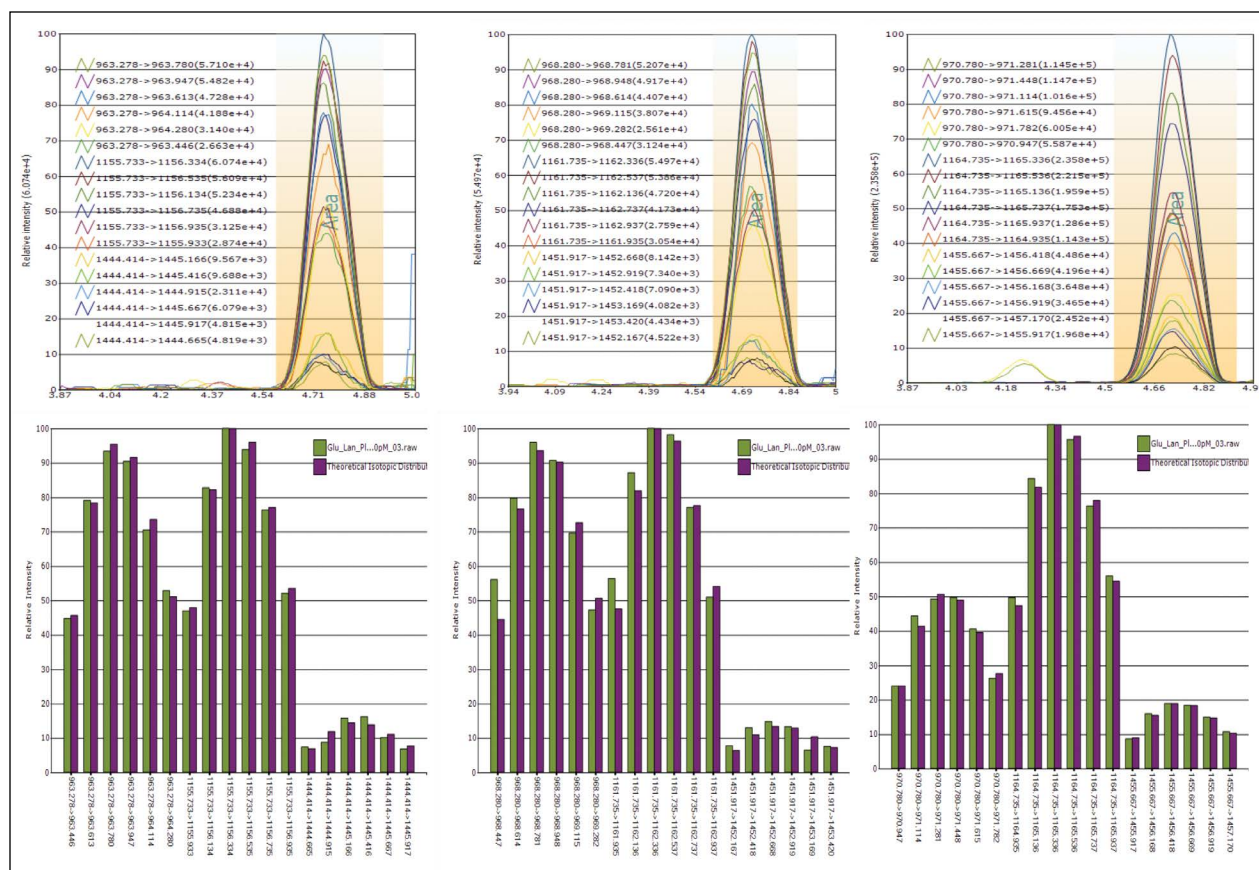


Figure 3. Comparative analysis of the three co-eluting insulin variants. Overlaid XICs for the six isotopes and three precursor charge states per insulin variant for (3a) porcine, (3b) human, and (3c) Apidra. The isotopic distribution analysis for the collection of precursor charge states and Pinpoint software calculated dot product correlation coefficients per charge state are shown.

The data analysis method provided an additional dimension of qualitative scoring beyond the isotopic distribution of a single charge state: the relative distribution of precursor charge states. For example, the measured abundances of the +5 and +6 charge states of porcine and human insulin were nearly equivalent, while the measured abundance of the +5 charge state of Apidra was twice that of the +6 charge state. The +4 precursor for all three showed relatively poor response and would likely be excluded from the final quantitation method.

Quantitative Data Processing Strategy

Robust data processing and reporting incorporate both qualitative and quantitative strategies. After data were collected using the HRAM global acquisition method, a Pinpoint software Main Workbook for the targeted insulin sequences was created, the processing parameters set, and the RAW files loaded for automated data processing. This was performed in three steps. Figure 4 shows the list of insulin variant sequences imported from a FASTA file containing all of the insulin variant sequences. A FASTA file is a text-based format for representing either nucleotide sequences or peptide sequences, in which nucleotides or amino acids are represented using single-letter codes.

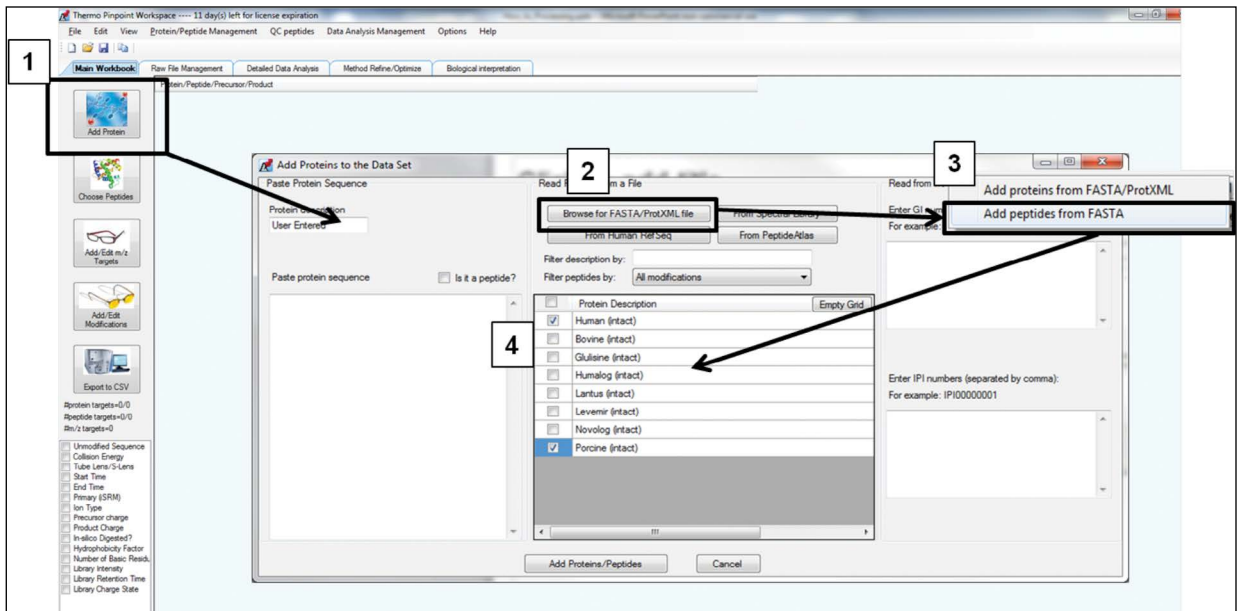


Figure 4. The targeted insulin variant list was created by opening the “Add Protein” window (1), selecting means of importing variant sequences (2), defining each entry as a peptide, and then selecting analytes to be included (4).

Modifications were then added to the sequences. Because insulin is comprised of multiple disulfide bonds linking the alpha and beta chain, mass shifts were added to determine the correct chemical formula (Figure 5).

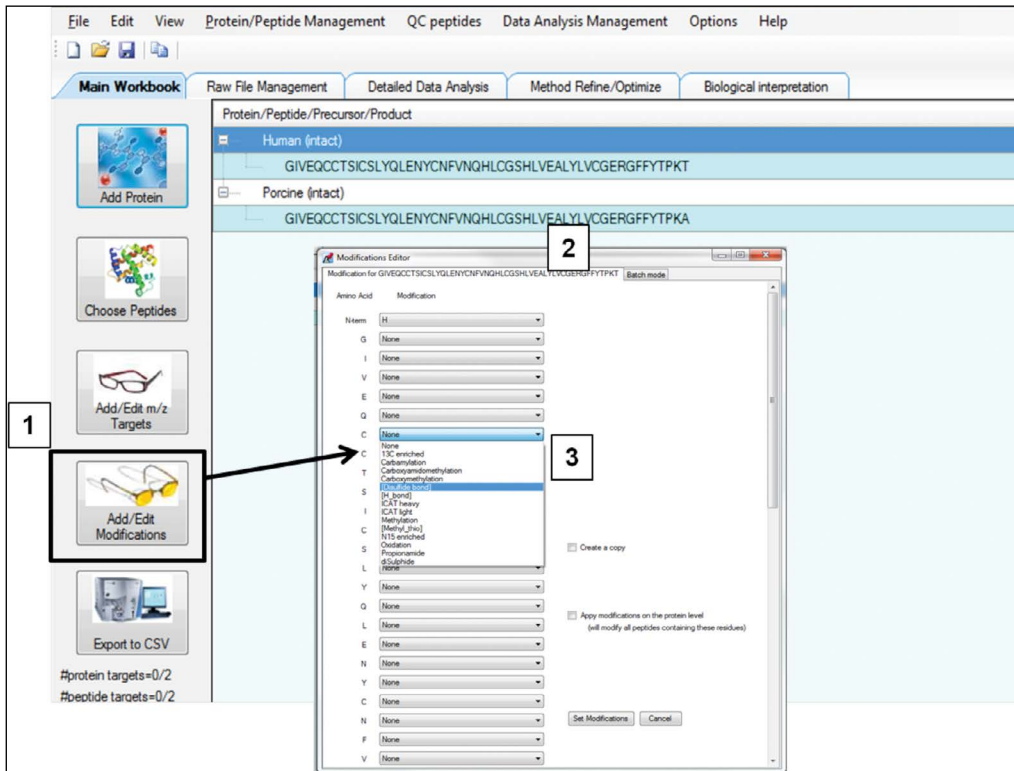


Figure 5. Mass shifts can be applied globally or locally by choosing Add/Edit Modifications (1), which opens the working window (2). Here the specific amino acid residue to which the modification was applied was selected and desired modification chosen (3).

For each insulin variant, three different disulfide bonds were selected and assigned to three different Cys residues as well as the addition of H₂O to account for two chains. Since the targeted quantitation was performed on the MS signal, the specific Cys residues were not important.

After the sequences had the correct modifications (chemical formulas), the m/z values were assigned (Figure 6). Determination of precursor charge states and isotopes linked the m/z values used for targeted data extraction to the appropriate insulin variant sequence.

Figure 6. Method to assign m/z values to each targeted insulin variant. “Add/Edit m/z Targets” (1) was selected to display sequence-specific information for the highlighted sequence. The Batch Mode Tab was used to apply the settings globally. To determine the m/z for a specific sequence, the precursor charge state was selected (2), and the isotopes checked (3).

Figure 6. Method to assign m/z values to each targeted insulin variant. “Add/Edit m/z Targets” (1) was selected to display sequence-specific information for the highlighted sequence. The Batch Mode Tab was used to apply the settings globally. To determine the m/z for a specific sequence, the precursor charge state was selected (2), and the isotopes checked (3).

For larger targets, at least six isotopes per charge state were selected to increase the qualitative information used for quantitation. In addition, multiple charge states were incorporated into the automated data extraction.

After the Main Workbook was created, the RAW files were batch processed as shown in Figure 7.

Figure 7. RAW data for processing was imported by clicking on the top bar (1) and selecting RAW files. The extraction parameters—including precursor mass tolerance (2) and retention time window for data extraction (3)—were set. After the extraction values were selected and data processing had begun, the group names (4) used for data organization were assigned.

Figure 7. RAW data for processing was imported by clicking on the top bar (1) and selecting RAW files. The extraction parameters—including precursor mass tolerance (2) and retention time window for data extraction (3)—were set. After the extraction values were selected and data processing had begun, the group names (4) used for data organization were assigned.

For quantitative experiments, the expected values per group were entered (Figure 8).

After all values were set, automated data processing was completed in 30 minutes. Pinpoint software consolidated qualitative and quantitative results in an interactive display that facilitated review and customized reporting (Figure 9). The top left table displays the AUC values for

the levels of the spiked analytes (e.g. isotopes, precursor charge states, and the summed values). The method of reporting AUC values enabled display of specific values for each level, which could be expanded or reduced as desired. Each entry was scored based on the calculated dot product for isotopic distribution, making it easy to determine which rows failed the filter.

Figure 8 shows the 'Absolute amount description' dialog box in Thermo Pinpoint software. The dialog is open over a table of raw files. The 'Reference Group' is set to '1_5'. The 'Absolute Amounts Definition' is selected. The 'Quantification strategy' is 'Standard Normal Curve'. The 'Absolute amount units' are 'fmol' and the 'Weighting scheme' is '1/x'.

Figure 8. Quantitative parameters were defined by clicking on “Absolute Amounts Determination” (1) to open the dialog box containing user-defined group names (2). Values were assigned or defined as unknown. Finally the units to display and the weighting scheme to apply to the calibration curve (3) were selected.

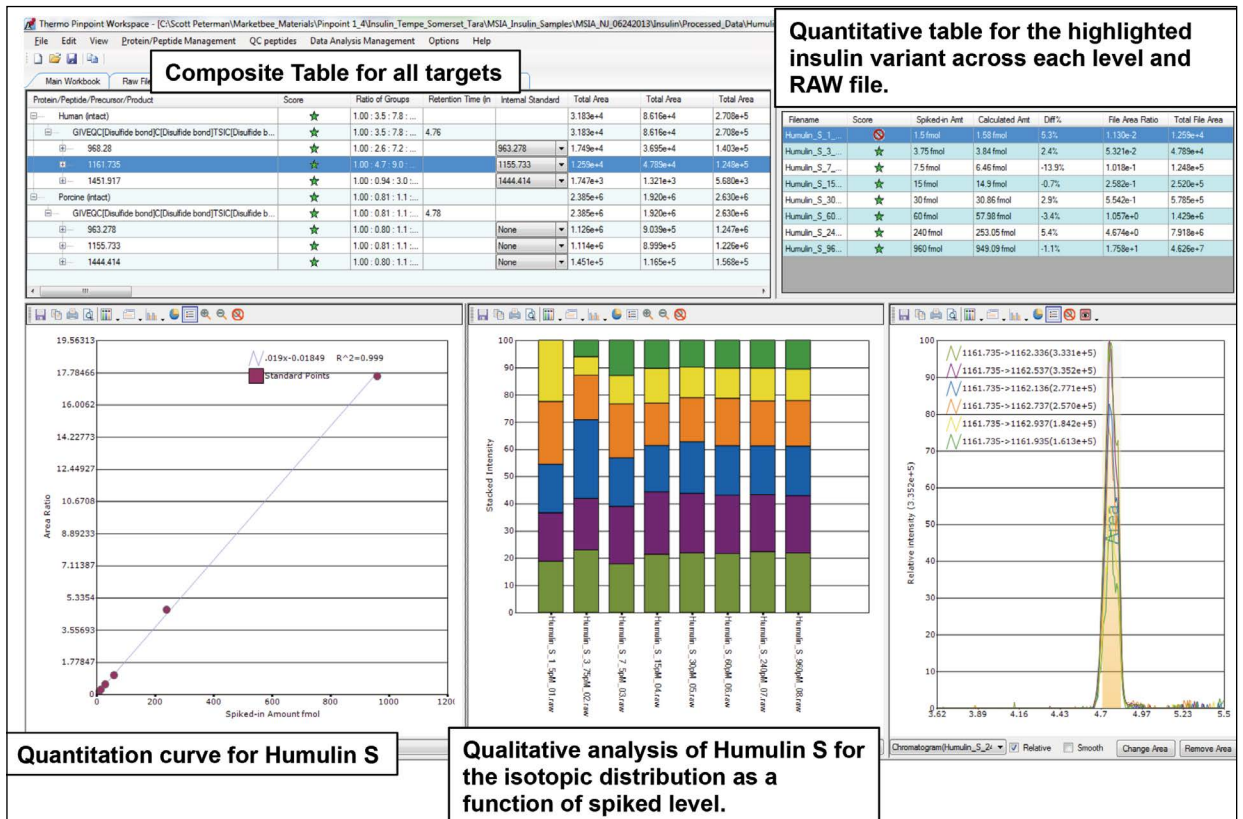


Figure 9. Automated data processing using Pinpoint software. The Detailed Data Analysis tab provided the composite quantification results for Humulin S from 1.5 to 960 pM spiked into PBS/BSA matrix.

Quantitation curves were generated based on user-defined levels. To best match the relative AUC values, each charge state was used to normalize the corresponding charge state for human insulin. This provided a significant benefit as relative abundance values are generally not equivalent, and simply summing and normalizing AUC response per target will bias towards the most abundant charge state. Normalizing at each charge state and showing the summed response for each individual charge state, takes advantage of HRAM data to increase the discriminatory power of the method.

As shown in Figure 9, the top right table shows the cumulative quantitation results for the row highlighted and contains all of the values used to evaluate the quantitation, including the %RSD and calculated amounts. Specific values can be added or subtracted. When a row in the table is selected (highlighted in blue), the accompanying results are graphically displayed in the bottom half of the window. The bottom left of Figure 9 shows the normalized quantitation curve for human insulin. The curve is weighted by $1/x$ and the equation is displayed. The bar chart (bottom middle) displays the relative AUC values for the six isotopes across the spiked levels. As reported in the upper right table, only the lowest spiked amount (1.5 pM) could not be used to identify all six isotopes. The graph on the lower right displays the XIC data for any single RAW file selected and can be changed by clicking on any point in the quantitation curve or bar chart.

The cumulative results from individual quantitative curves for the first sample set were overlaid to demonstrate that the workflow was global in its ability to quantify the different insulin variants (Figure 10). Each curve, when normalized to porcine insulin, had a linear regression of 0.98 or greater for all precursor charge states, isotopes, and reported summed AUC values. The difference in slopes was attributed to the relative ionization efficiency and antibody binding coefficients of the different insulin analogs. The workbook used to process the insulin quantitation curves spiked into PBS/BSA was also able to process the insulin quantitation curves spiked into human plasma.

Figure 11 summarizes the qualitative aspects of the quantitation as a function of the levels spiked and the confidence in the isotopic overlap between experimental and theoretical values. The response at each level represents over 30 measurements where all charge states across each quantitation curve were considered. As expected, the lower levels resulted in a lower average dot product correlation coefficient (0.58 for all insulin variants spiked at 1.5 pM). However with six isotopes considered, the coefficient was discriminatory and accurately defined potential background interference that would disrupt the true isotopic distribution in the four most abundant isotopes. Even at very low analyte levels, the quality of the quantitation is quite high. All other levels generated average dot product correlation coefficients greater than 0.8. The average dot product coefficients for porcine measurements were consistently above 0.97 for the 50 pM level.

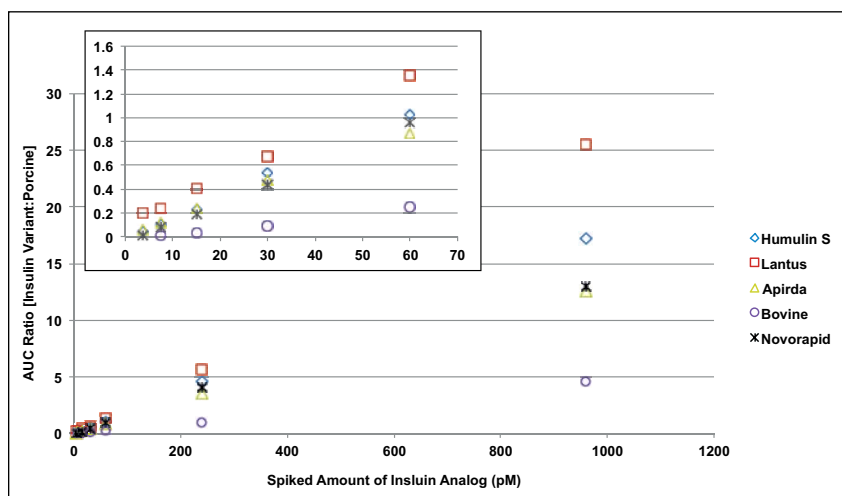


Figure 10. Comparative quantitation curves for all insulin analogs spiked into PBS/BSA matrix. The AUC values were normalized against the measured AUC value for porcine.

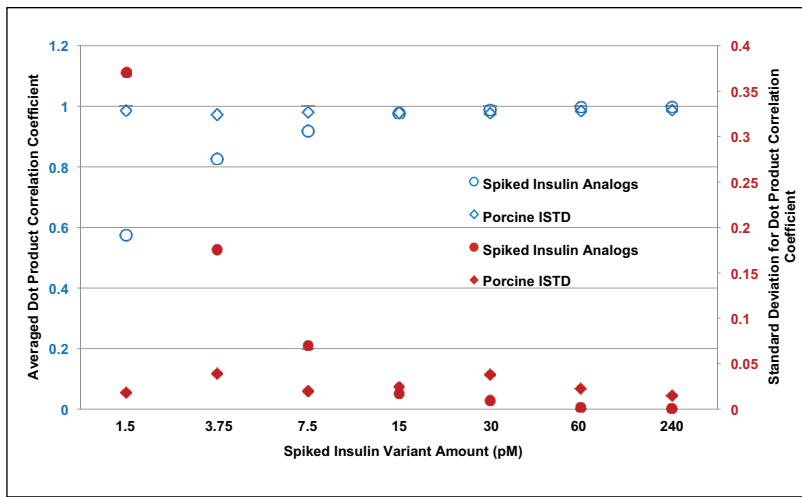


Figure 11. Dot product correlation coefficients for each insulin analog spiked into both matrices, and for porcine insulin. The standard deviation was also plotted per spiked level.

In combination with the average dot product coefficient, the standard deviation for the measurements demonstrated the workflow's effectiveness. The deviation in the isotopic distribution showed that each insulin variant was routinely detected with four or more isotopes. Predicted relative abundance values were maintained at the 1.5 pM level.

Figure 12 shows the RSD values for all insulin analogs as a function of amounts spiked in the PBS/BSA and human plasma matrix. The greatest spread in measured %RSD was at the 1.5 pM level where three of the 11 measurements exceeded 20% spread. Only one measurement at the 3.75 and 30 pM level exceeded 20% spread in %RSD. The errors were attributed to using only one measurement per level, per quantitation curve. The overall groupings per level were well within acceptable error. It should also be noted that the lowest four levels used in this study were 50–100 times lower than the previously published lower limits of quantitation.

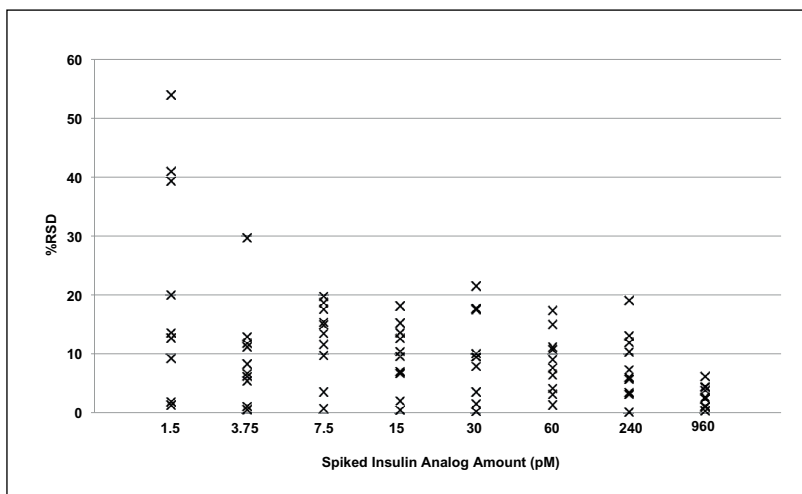


Figure 12. Spread in %RSD values for all insulin analogs across the spiked levels. The %RSD values are based on the individual quantitation curves.

Conclusion

An automated, high-throughput data processing workflow for detection and targeted quantification of insulin and its analogs at low levels in biological matrices was presented for use in research applications.

Full-scan MS data could be analyzed due to the low noise and selectivity of the MSIA extraction technology. Co-eluting insulin analogs were easily separated and identified based on the accurate m/z values of each precursor charge state and corresponding isotopes. Detection and quantification ranges were 1.5 to 960 pM in a plasma matrix. Even at low analyte levels, the quality of the quantitation was high. Linear regression values for the method were all better than 0.98 using a $1/x$ -weighting scheme. The average dot product coefficients for the porcine insulin ISTD were consistently above 0.97 at the 50 pM level. Though the lowest four levels used in the study were 50–100 times lower than previously published, %RSD values were acceptably low.

The HRAM data acquisition capabilities of the Q Exactive mass spectrometer enabled streamlined qualitative and quantitative data processing and reporting using Pinpoint software. This approach enabled quantification of HRAM MS data using the precursor charge state distribution as well as the isotopic distribution for evaluation of potential background interference. By acquiring data in a nontargeted manner, post-acquisition methods can be used to process the data later for any insulin variant sequence or modification. Data processing was performed in 30 minutes using a single Pinpoint software workbook containing all of the targeted insulin variants. To achieve robust quantitation, the Pinpoint software data processing method used the precursor charge state distribution and isotopic distribution analysis for evaluation of potential background interference. Pinpoint software capabilities, such as color-coding capability and the Main Workbook, which consolidates qualitative and quantitative tables and graphs in an interactive display, facilitated data review.

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