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An Easier, More Robust, and Faster Low-flow Quantitative Proteomics Approach

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Introduction

When transitioning from discovery proteomics to targeted protein quantification, the multiple reaction monitoring (MRM)-based LC/MS method plays an essential role. Sample complexity and the low concentration of certain proteins are the major challenges encountered when performing protein analysis using targeted methods. Recent advancements in low flow HPLC and acquisition rates in QQQ instrumentation is enabling MRM-based analytical methods using stable isotope-labeled standard (SIS) peptides for targeted quantitative proteomics to improve sensitivity, linear range, precision, accuracy, robustness, and most importantly throughput in the data acquisition as well as simplified data processing. Sensitivity, linearity, and precision is demonstrated with these fast low flow methods demonstrating inject to inject cycle times under 25 minutes. This study demonstrated the suitability of this new high-performance LC/MS system configuration for high throughput protein quantification, even with very limited sample amount.

Experimental

Plasma (BioIVT) underwent tryptic digestion, was divided into 100 µg aliquots, and dried down. The digest was rehydrated with 10% acetonitrile/ 0.1% formic acid to a concentration of 2 µg/µL. An aliquot of the digest was diluted to 1 µg/µL for a final solution composition of 5% acetonitrile/0.1% formic acid . This was used for preparing the calibration curve. Stable isotope-labeled standard peptides from a biomarker assay kit (MRM Proteomics) were rehydrated and diluted with the digest. MRM assays had already been optimized for the biomarker assay kit, although retention times needed adjustment. Three of the five standard Evosep methods were utilized. Peptides were separated with the 60, 200, and 300 Sample Per Day (SPD) methods at flow rates of 1, 2, and 4 µL/min respectively on the Evosep One™ HPLC system and peptide quantitation was performed with a triple quadrupole mass spectrometer. Data was analyzed with Skyline and Agilent's Quantitative Analysis™ and Qualitative Analysis™ software.

Experimental

Figure 1. The Evosep One system. All methods are standardized for easier and more robust proteomics.

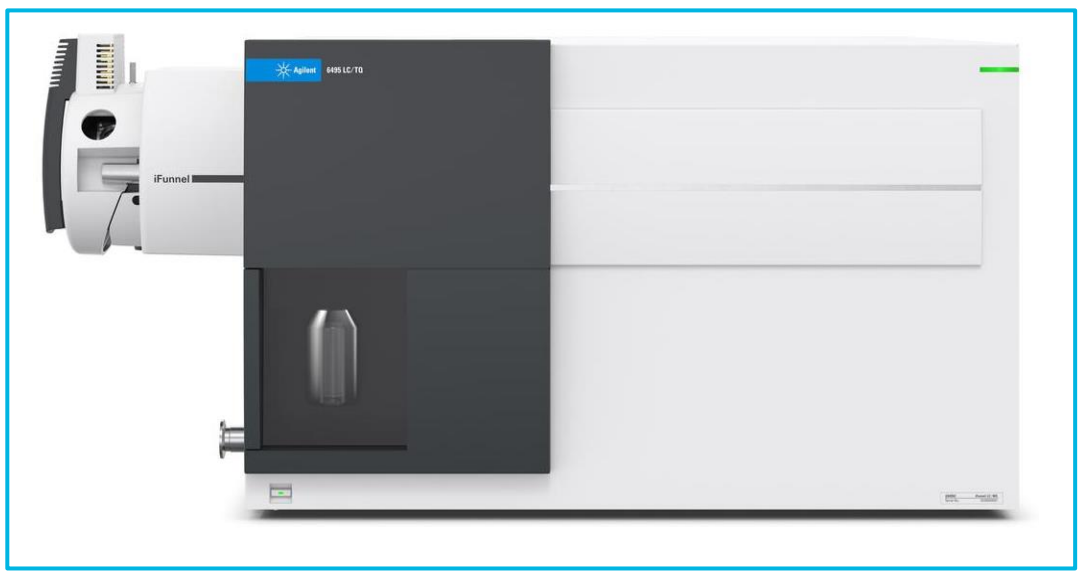


Figure 2. The 6495C LC/TQ was used for this assay. This system has the analytical sensitivity needed for quantifying peptides in a heavy matrix.

Conditions	Value
Column (60 SPD)	EV1109 (Performance)
Column (200/300 SPD)	EV1107 (Endurance)
Emitter	EV1117 Agilent emitter
Source	Agilent nano ESI source
Gas Temp	200°C
Gas Flow	11 L/min
Capillary Voltage	1700 V (60 SPD)/1850V (200/300 SPD)

Table 1. LC/MS Conditions

60 SPD Method Results

125 peptides in plasma were quantified with a 21-minute method, the 60 SPD method, using a ≤15% CV as a lower limit of quantitation (LLOQ). Converting the 125 Protein PeptiQuant Plus Kit from a 60 minute method to a 21 minute method meant pushing the dwell times of the 6495C LC/TQ as low as 7 msec. Therefore, additional tests were performed to demonstrate precision.

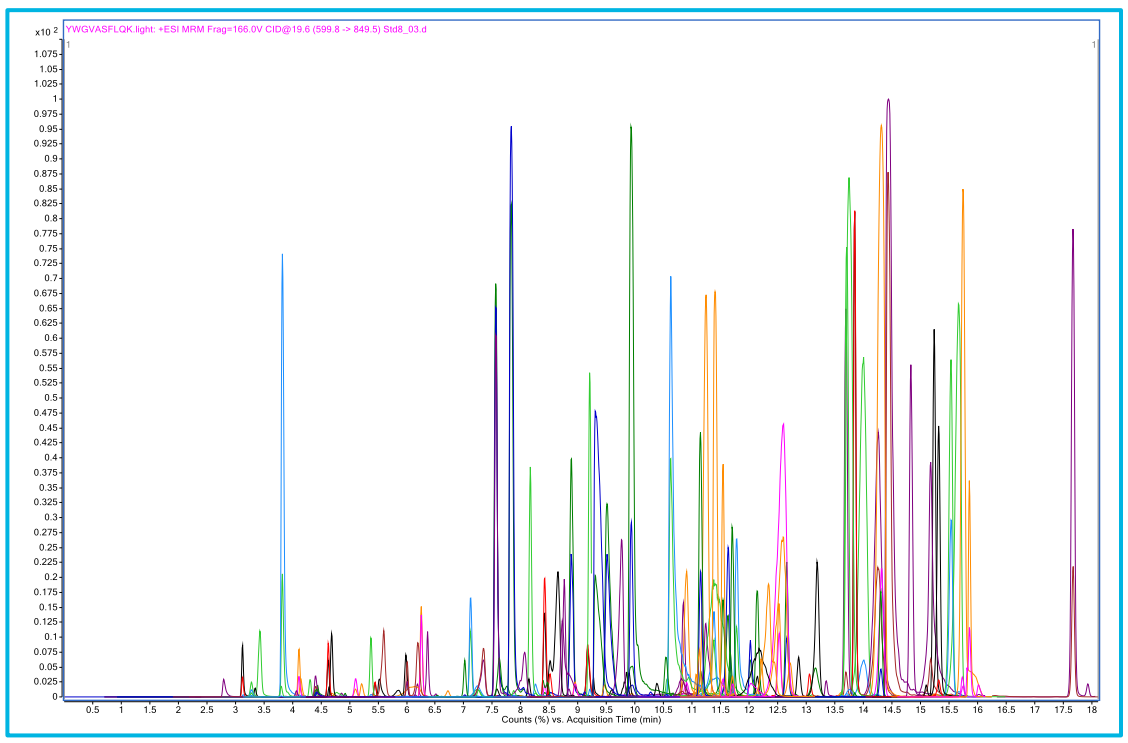


Figure 3. All 250 transitions elute before 18 minutes. Peak width at full width half max for the majority of peptides is at or under 3.6 seconds.

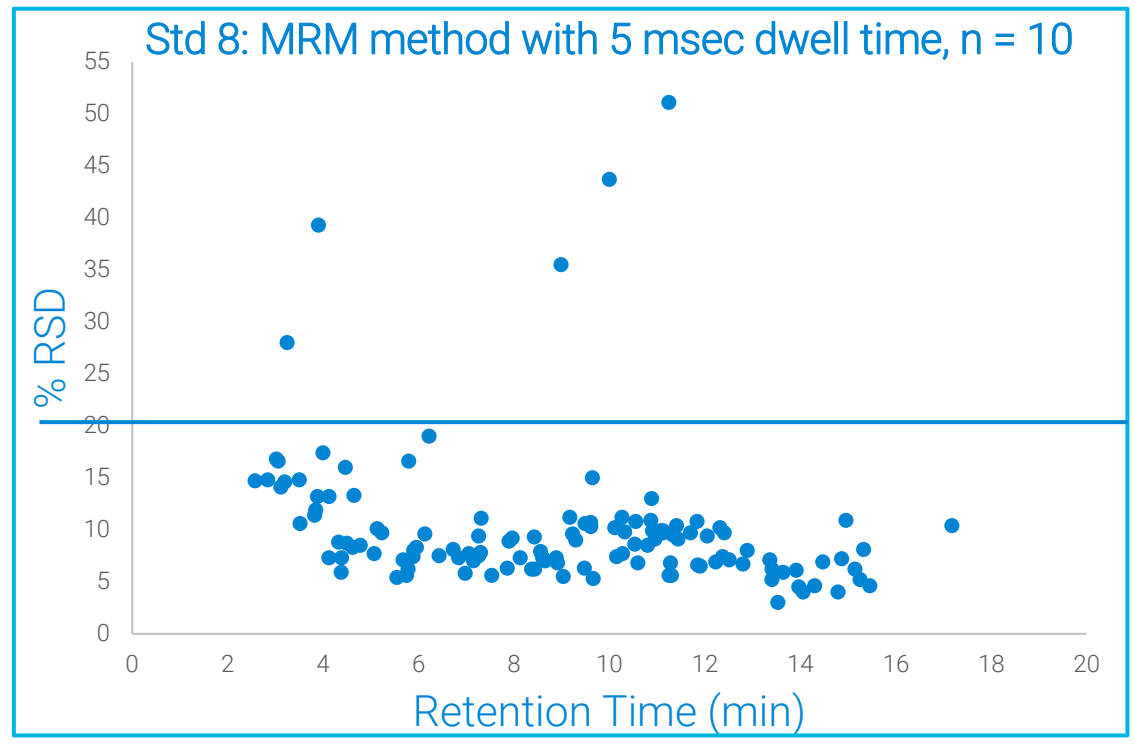


Figure 5. Reducing the dwell time can greatly affect the reproducibility. Here we are looking at the %RSD of 10 replicates of all peptides where the dwell time is set to 5 msec. 91% of the peptides are <15% RSD and 96% are < 20% RSD.

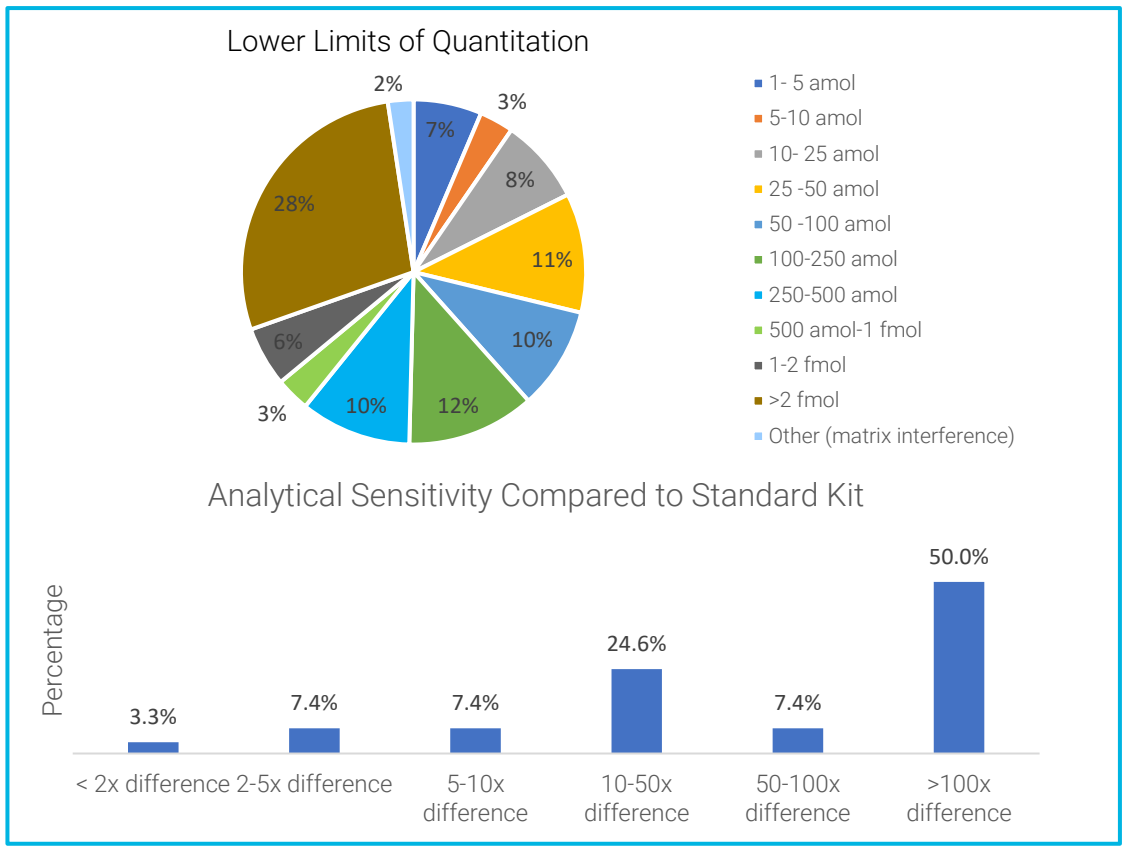


Figure 4. A summary of the LLOQs of the 125 quantified peptides with concentration factors accounted for. %RSD for LLOQs was capped at 15%. A combination of diluting the calibration curve and using the Evosep for chromatography contributed to a significant jump in sensitivity when compared to the published values of the kit.

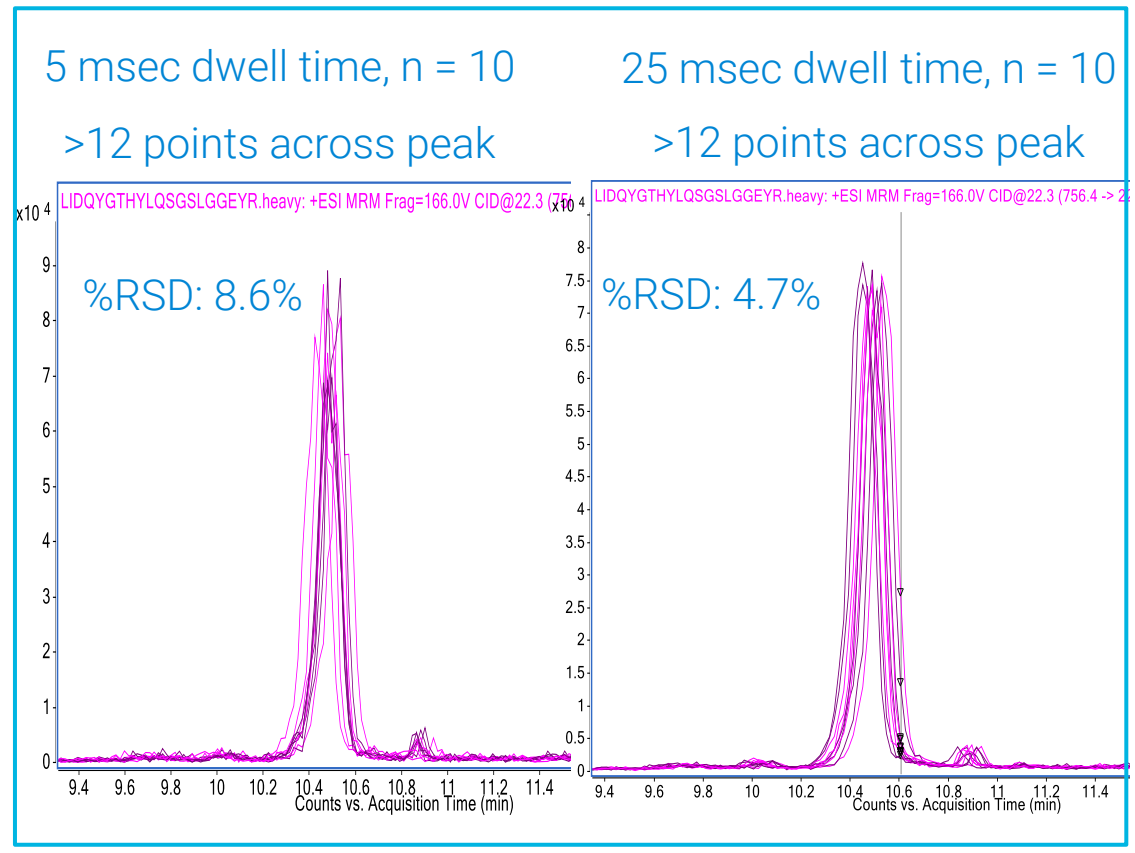


Figure 6. Reproducibility with short dwell times at the low end of the calibration curve holds very well. Here is an example of a peptide close to its LLOQ at 208.8 amol on column. When all 250 transitions are set to 5 ms, the %RSD of this peptide is still well within the limit at 8.6% for an LLOQ.

Results and Discussion

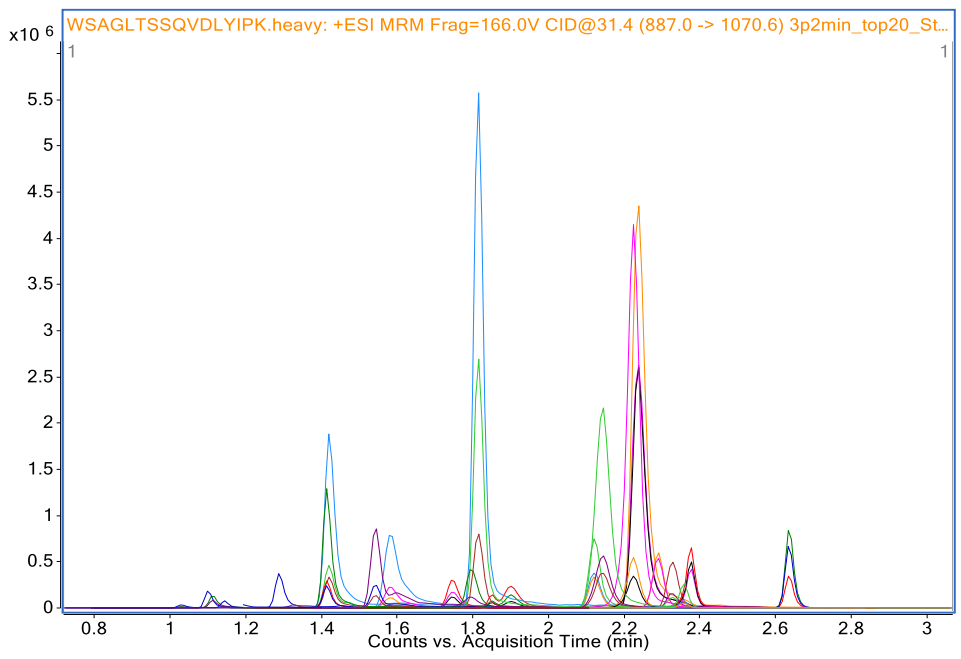
300 and 200 SPD Methods

If the desired target list contains fewer peptides, a shorter method can save time without sacrificing significant sensitivity. Twenty peptides from the 125 were selected across the retention time range. Two qualifier ions were selected and optimized using the automated Skyline workflow.

Robust Performance

- Requires only finger-tight fittings.
- Disposable trap columns contribute to a very robust system with very low carryover and cross-contamination.
- To date, the columns and emitter have undergone over 1500 injections with no decrease in performance.

20 Peptides (60 Transitions) in 3.2 minutes



20 Peptides (60 Transitions) in 5.6 minutes

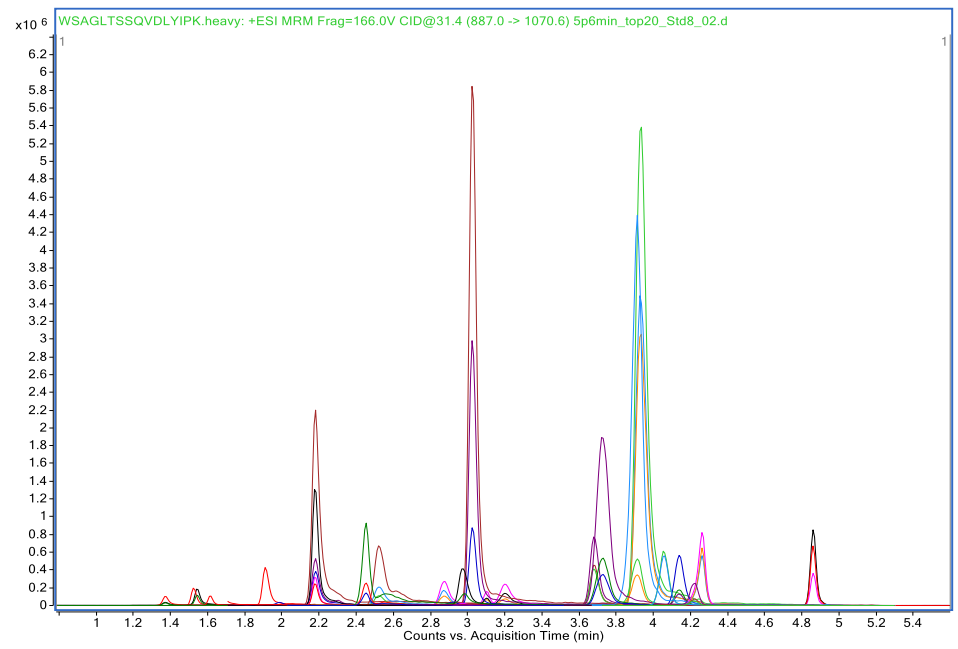
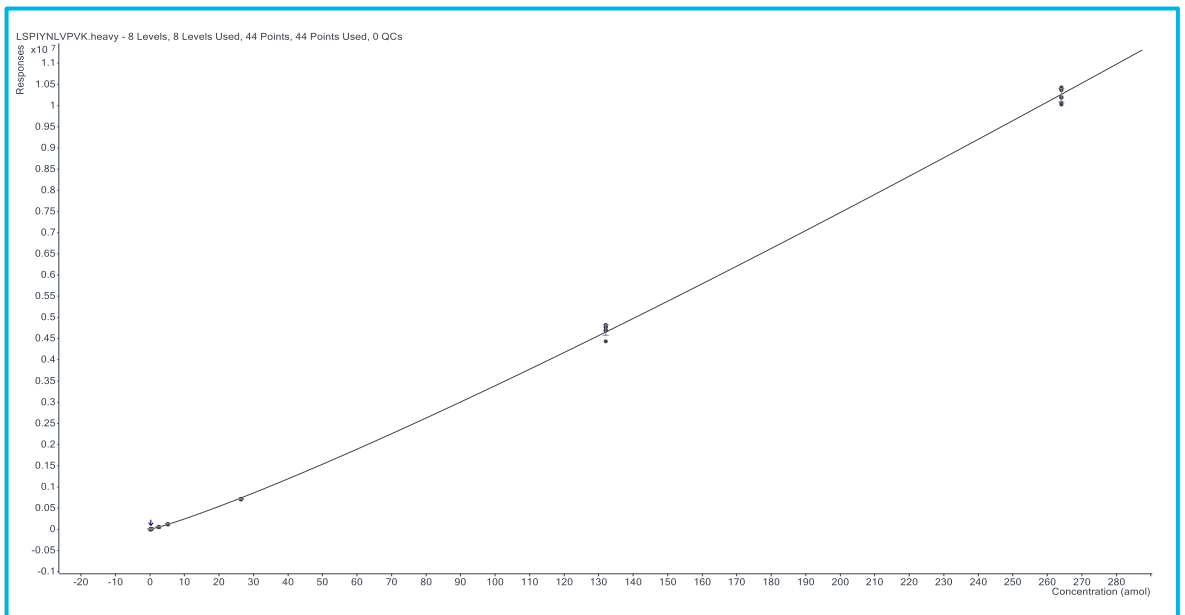


Figure 7. Peptides eluting across the full retention range were selected to represent the higher-throughput methods. Correcting for concentration multipliers with the PeptiQuant Kit, both the 300 SPD and 200 SPD methods had 30% of peptides with an LLOQ under 50 amol and 55% had an LLOQ <500 amol.



Level	1	2	3	4	5	6	7	8
amol	0.76	1.8	3.7	17.9	36.6	182.2	910.8	1821.6
%CV	28	11.8	12.8	6.1	8.8	3.6	8.2	1.9
Acc.	119.8	116.5	106.4	98.7	101.4	96.5	100.8	99.8

Figure 8. An example of a peptide quantified with the 300 SPD method, where n = 5. Excellent analytical sensitivity, precision, and accuracy is achieved with a high throughput method.

Conclusions

- Coupling the Evosep One to the 6495C LC/TQ for peptide quantitation in conjunction with the PeptiQuant kits allows for
  - High Analytical Sensitivity
  - Accuracy
  - Linearity
  - Throughput
  - Robustness
  - Ease of Use
- 6495C LC/TQ provides short dwell times with high precision enabling higher throughput.

References

[1]Bache, N et al. A Novel LC System Embeds Analytes in Pre-formed Gradients for Rapid, Ultra-robust Proteomics. Mol. Cell Proteomics 2018, 17(11), 2284–2296

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