Application Note

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# Peptide Quantification in Plasma Using the Agilent 6495 Triple Quadrupole LC/MS Coupled with the Agilent 1290 Infinity II LC System


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## Abstract

This application note showcases the quantitative performance of multiple reaction monitoring (MRM)-based LC/MS analysis of peptides derived from protein biomarkers in human plasma using the Agilent 1290 Infinity II LC system coupled to the Agilent 6495 triple quadrupole LC/MS with Jet Stream ionization source. Results demonstrated outstanding performance of peptide quantification in human plasma using this standard flow-based LC/TQ system.

## Introduction

In translational research, increased throughput as well as increased multiplexing mass spectrometry using MRM-based LC/MS methods have become popular for targeted, bottom-up quantification of protein biomarkers. Researchers often target signature peptides from a limited number of proteins in large cohorts and monitor their expression levels during a specified time period, often leading to hundreds or thousands of biological samples. In such large-volume studies, high-throughput, robustness, and reproducibility are essential when deploying LC/MS methods. For this reason, standard-flow triple quadrupole LC/MS (LC/TQ) is superior to other low-flow LC/MS platforms. In addition to reproducibility and robustness, analytical sensitivity is another important consideration for peptide quantification. The 6495 triple quadrupole LC/MS system coupled with an Agilent Jet Stream (AJS) ionization source is a standard flow-based LC/TQ platform that provides outstanding performance for targeted peptide quantification due to its high sensitivity, reproducibility, and robustness as well as ease of handling and maintenance.[1,2]

This application note demonstrates the quantitative analytical sensitivity of 99 signature peptides from 75 protein biomarkers in plasma using the 6495 triple quadrupole LC/MS system and AJS ionization source. To assess overall quantitative performance for protein biomarker peptides, a commercially available mixture of stable isotope-labeled standard (SIS) peptides was spiked into human plasma digest at nine different concentrations. Standard curves of 99 SIS peptides were generated to determine their lower limit of quantification (LLOQ) in plasma matrix. The results show excellent analytical performance under standard flow conditions with this LC/TQ system for targeted peptide quantification in heavy matrix.

## Experimental

### Instrumentation

- Agilent 1290 Infinity II LC system including:
  - Agilent 1290 Infinity II High Speed Pump (G7120A)
  - Agilent 1290 Infinity II Multisampler (G7167B) with sample cooler option (option 100)
  - Agilent 1290 Infinity II Thermostatted Column Compartment (G7116B)
- Agilent Jet Stream source (G1958-65638)
- Agilent 6495C triple quadrupole LC/MS system (G6495C)

### Materials

Raw human plasma was purchased from Bioreclamation (catalog no. HMPLEDTA2). PeptiQuant Biomarker Assessment Kit (BAK-A6495-76) was purchased from Cambridge Isotope Laboratories.

### Sample preparation

Human plasma was diluted with 25 mM ammonium bicarbonate followed by denaturation with TFE, reduction with DTT, and alkylation with Iodoacetamide. The sample was then trypsin digested overnight at 37 °C. The digested plasma was dried down, then reconstituted at around 1 µg/µL and spiked with the balanced stable-isotope standard (SIS) peptide mixture at nine different concentrations ranging from 5 amol/µL to 100 fmol/µL (which is converted to 350 fmol/mL to 7 nmol/mL in the original raw human plasma).

### LC/MS analysis

All samples were injected with replicates (n = 5) and analyzed using the LC/MS acquisition method provided by the PeptiQuant Biomarker Assessment Kit with minor modifications.[3] As briefly summarized in Table 1, 10 µL of samples were loaded onto an Agilent ZORBAX RRHD Eclipse Plus C18 column (2.1 × 150 mm, 1.8 µm, part number 959759-902) and separated using the same 43-minute LC gradient provided by the kit. On the current system, the peptide retention times were first updated using the *Dynamic MRM Update Options* in Agilent MassHunter software to adjust the minor retention time shift, then AJS source parameters were optimized using the Source Optimizer. The final MS parameters are listed in Table 1. All the other LC/MS parameters, such as MRM transitions and their collision energies, were directly copied from the original method. A total of 99 SIS peptides from 75 proteins with MRM responses in the original SIS peptide mixture were used in the final method for standards curve creation.

### Data processing

Agilent MassHunter workstation Quantitative Analysis software (v10.1) was used for peptide quantification analysis.

## Results and discussion

### Chromatography of standard-flow LC/MS

The 1290 Infinity II LC system, the next generation in UHPLC, gives excellent chromatography resolution and higher retention precision. The human plasma digest spiked with SIS peptides at 10 fmol/µL was injected onto a ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm column using a 43-minute LC/MS analysis performed in dynamic MRM mode. Excellent peak shapes and separation were achieved (Figure 1). All targeted peptides eluted between 2.4 to 36.5 minutes, showing a median full width at half maximum (FWHM) of 3.5 seconds. Figure 1B shows the excellent peak shapes of four selected SIS peptides

**Table 1.** LC/MS parameters.

| Agilent 1290 Infinity II LC Parameters | |
|---|---|
| Analytical Column | Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm (p/n 959759-902) |
| Mobile Phase A | $H_2O$, 0.1% formic acid |
| Mobile Phase B | Acetonitrile, 0.1% formic acid |
| Flow Rate | 0.4 mL/min |
| Injection Volume | 10 µL |
| LC Gradient Time | 43 min |
| Column Temperature | 50 °C |

| Agilent 6495 LC/TQ Parameters | |
|---|---|
| Ion Mode | Agilent Jet Stream, positive |
| Gas Temperature | 150 °C |
| Drying Gas Flow | 17 L/min |
| Nebulizer Gas | 30 psi |
| Sheath Gas Temperature | 250 °C |
| Sheath Gas Flow | 12 L/min |
| Capillary Voltage | 3500 V |
| Nozzle Voltage | 0 V |
| High-/Low-Pressure RF Voltage | 200/110 V |
| Delta EMV | 200 V |
| Q1 and Q3 Resolution | Unit/Unit |
| Cycle Time | 600 ms |
| Minimum Dwell Time | 7.02 ms |
| Total MRM Transitions | 606 |
| HED in Tune File | –20 kV |

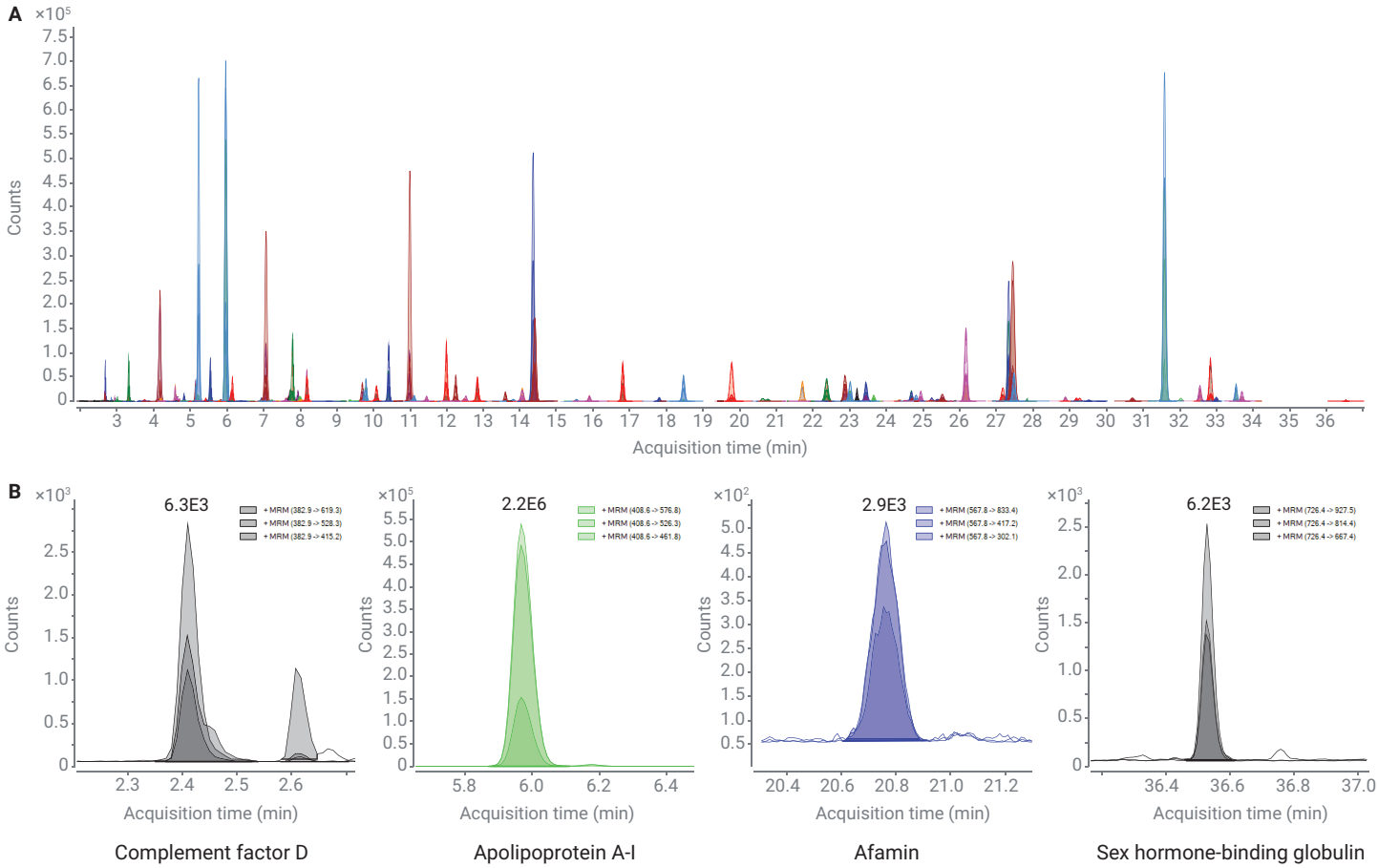


**Figure 1.** MRM chromatograms of heavy and endogenous peptides in human plasma digest spiked with SIS peptide mixture at 10 fmol/µL. (A) total dynamic MRM chromatograms of all targeted heavy and endogenous peptides. (B) MRM peak chromatograms of four selected SIS peptides spanning wide ranges of retention time (2.4 ~ 36.5 minutes) and signal response (peak area 2.9E3 ~ 2.2E6).

spanning a wide range of retention time (2.4 ~ 36.5 minutes) and signal response (peak area 2.9E3 ~ 2.2E6). The targeted SIS peptides differ widely in terms of signal response when loaded equally on column. This indicates broad differences in the analytical sensitivity of various peptides.

To assess the precision of peptide retention time (RT), the relative standard deviation (RSD) of all targeted endogenous peptides were calculated from all the 45 injections (9 levels × 5 replicates) since they have good and equal signal response across all the spiking samples. Distribution of peptide retention time versus its corresponding RSD shows the RSD ranges from 0.01 to 0.42%, with a median RSD of 0.13% (Figure 2). This result shows much better RT reproducibility compared to a low microflow LC system.[4] It demonstrated the excellent retention time precision of the standard flow 1290 Infinity II LC system.

## Quantification of peptide standards in human plasma

To evaluate quantitative performance of the 6495 triple quadrupole LC/MS with AJS ionization source for peptide quantification, the balanced SIS peptide mixture was spiked at nine different concentrations ranging from 5 amol/µL to 100 fmol/µL in 1 µg/µL plasma digest. Replicate (n = 5) injections were made for all samples to make linear standard curves. Eighty-one percent (80 out of 99) of targeted SIS peptides show $R^2$ equal to or greater than 0.99. The LLOQ of each SIS peptide was determined as the lowest spiking level with MRM response RSD <20% and linear quantification accuracy between 80 to 120%. As expected, the LLOQs of all 99 SIS peptides show a wide range of difference (Figure 3). For well-responding peptides, LLOQs as low as 5 amol/µL in complex plasma matrix could be achieved. This indicates the importance of peptide and MRM transition selection to achieve the best quantitative sensitivity.

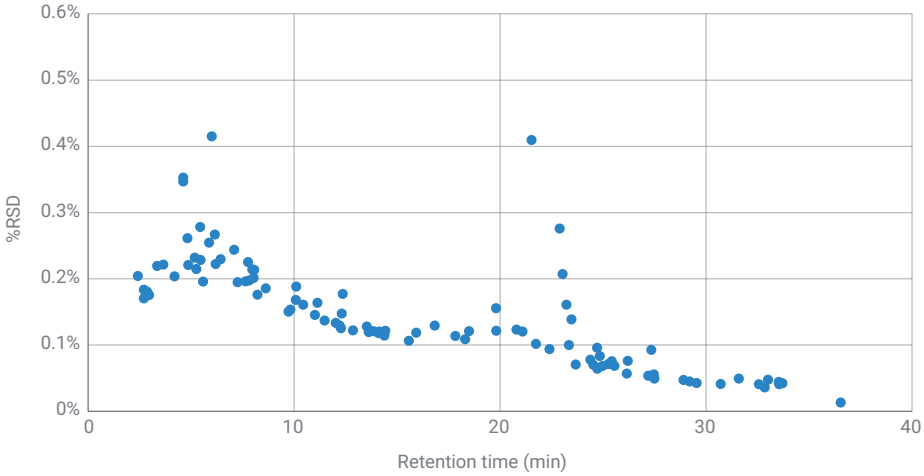


**Figure 2.** Distribution of retention time versus its corresponding RSD for 99 targeted endogenous peptides in human plasma digest (n = 45).

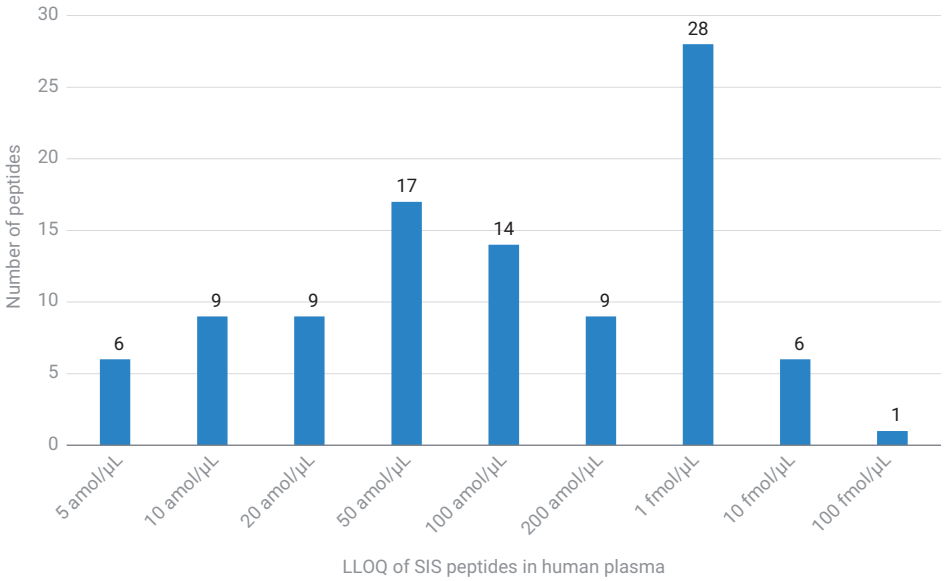


**Figure 3.** Distribution of LLOQ of SIS peptides spiked into human plasma. 10 µL of each standard sample was injected leading to 10 µg plasma digest matrix on column. The LLOQ of SIS peptide was determined as the lowest spiking level, with MRM response RSD<20% and linear quantification accuracy between 80 to 120%.

Figure 4 and Table 2 show the excellent quantitative performance of the SIS peptide AFLLTPR from apolipoprotein M in plasma including:

- Excellent linear dynamic range (5 amol/µL to 100 fmol/µL) with $R^2$ = 0.9998 (Figure 4A)
- Excellent analytical sensitivity with an LLOQ of 5 amol/µL in 1 µg/µL trypsinized plasma (Figure 4)
- Superior precision and accuracy observed at all levels, including the LLOQ level (Table 2)

**Table 2.** Precision and accuracy for the Agilent 6495 triple quadrupole LC/MS analysis of the SIS peptide standard AFLLTPR in 1 µg/µL plasma digest.

| Level (amol/µL) | Average Response | RSD (%) (n = 5) | Accuracy (%) (n = 5) |
|---|---|---|---|
| Matrix blank | 3 | NA | NA |
| 5 | 117 | 9.3 | 114.2 |
| 10 | 251 | 9.7 | 102.4 |
| 20 | 562 | 4.6 | 104.0 |
| 50 | 1,295 | 1.6 | 91.3 |
| 100 | 2,925 | 4.1 | 100.9 |
| 200 | 5,174 | 3.3 | 88.6 |
| 1,000 | 28,396 | 1.3 | 96.4 |
| 10,000 | 302,313 | 2.5 | 102. 5 |
| 100,000 | 2,944,790 | 1.1 | 99.8 |

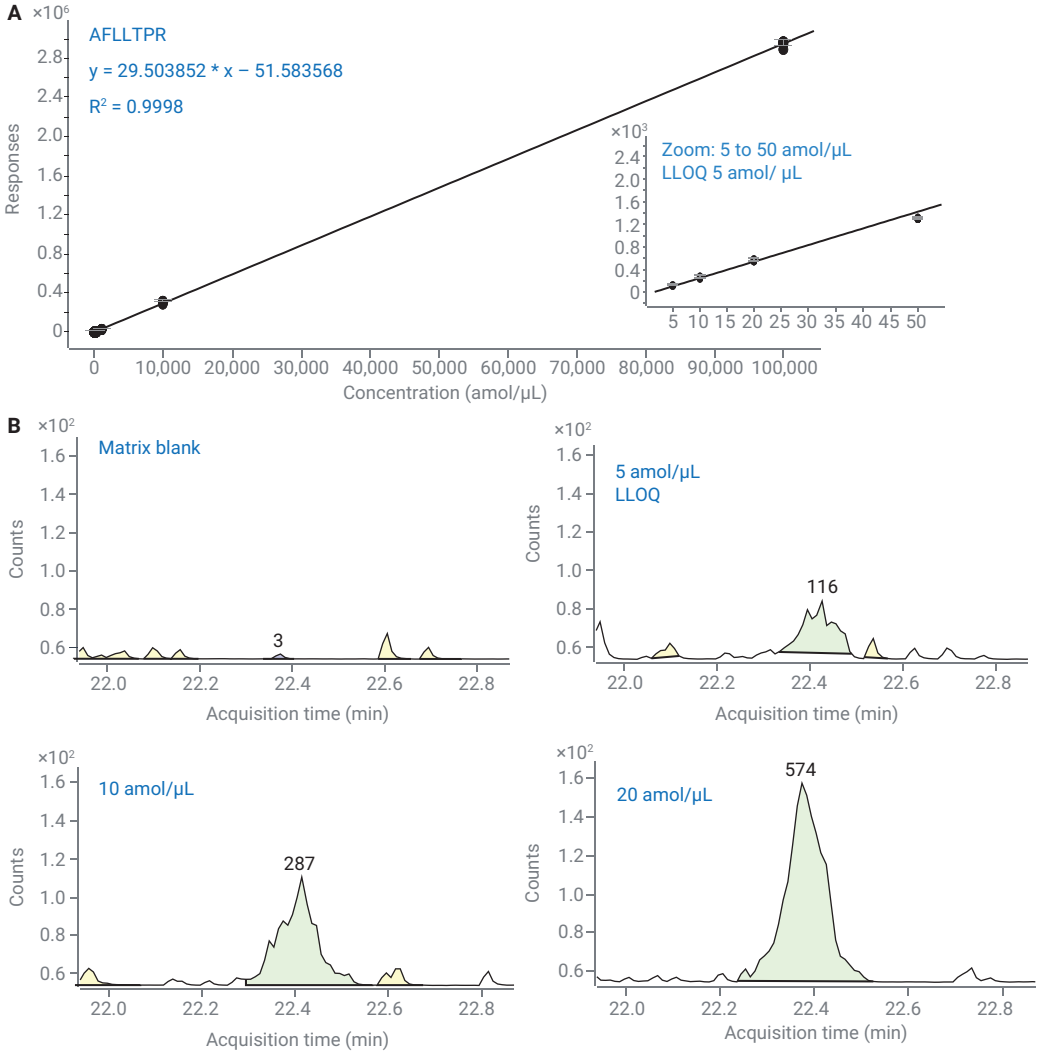


**Figure 4.** Quantitative performance of the SIS peptide AFLLTPR from apolipoprotein M in plasma. (A) standard curve of SIS peptide AFLLTPR for the range from 5 amol/µL to 100 fmol/µL in 1 µg/µL plasma digest with inset showing detail for the range of the curve from 5 to 50 amol/µL. (B) extracted ion chromatograms for peptide AFLLTPR showing the LLOQ.

The LLOQ results show that analytical sensitivity not only depends on the strength of monitored transition signal response, but may also be affected by matrix interference, response reproducibility, linearity, etc. For example, Figure 5 shows extracted ion chromatograms of SIS peptide SGIPIVTSPYQIHFTK from complement C3 at low levels labeled with the response ratio of qualifier ion transition relative to quantifier ion transition. The matrix blank contains interferences, showing the ion response ratios deviated from the expected ratios. These interferences affected the linear quantification accuracy at the low concentration of 5 amol/µL (RSD = 2.7%, accuracy = 157.5% for n = 5). Therefore, the LLOQ of peptide SGIPIVTSPYQIHFTK was determined to be 10 amol/µL (RSD = 2.4% and accuracy = 112.8% for n = 5), even though the linear standard curve $R^2$ of 0.9995 was achieved. Note that the quantitative sensitivity of SIS peptide SGIPIVTSPYQIHFTK is comparable to a previous result using a microflow LC system coupled to 6495 LC/TQ, which has a lower sample loading capacity.[4] This demonstrated that the 6495 triple quadrupole LC/MS, coupled with the 1290 Infinity II LC system and Jet Stream ionization source, can achieve similar quantitative sensitivity to the microflow LC/TQ system if not sample restricted.

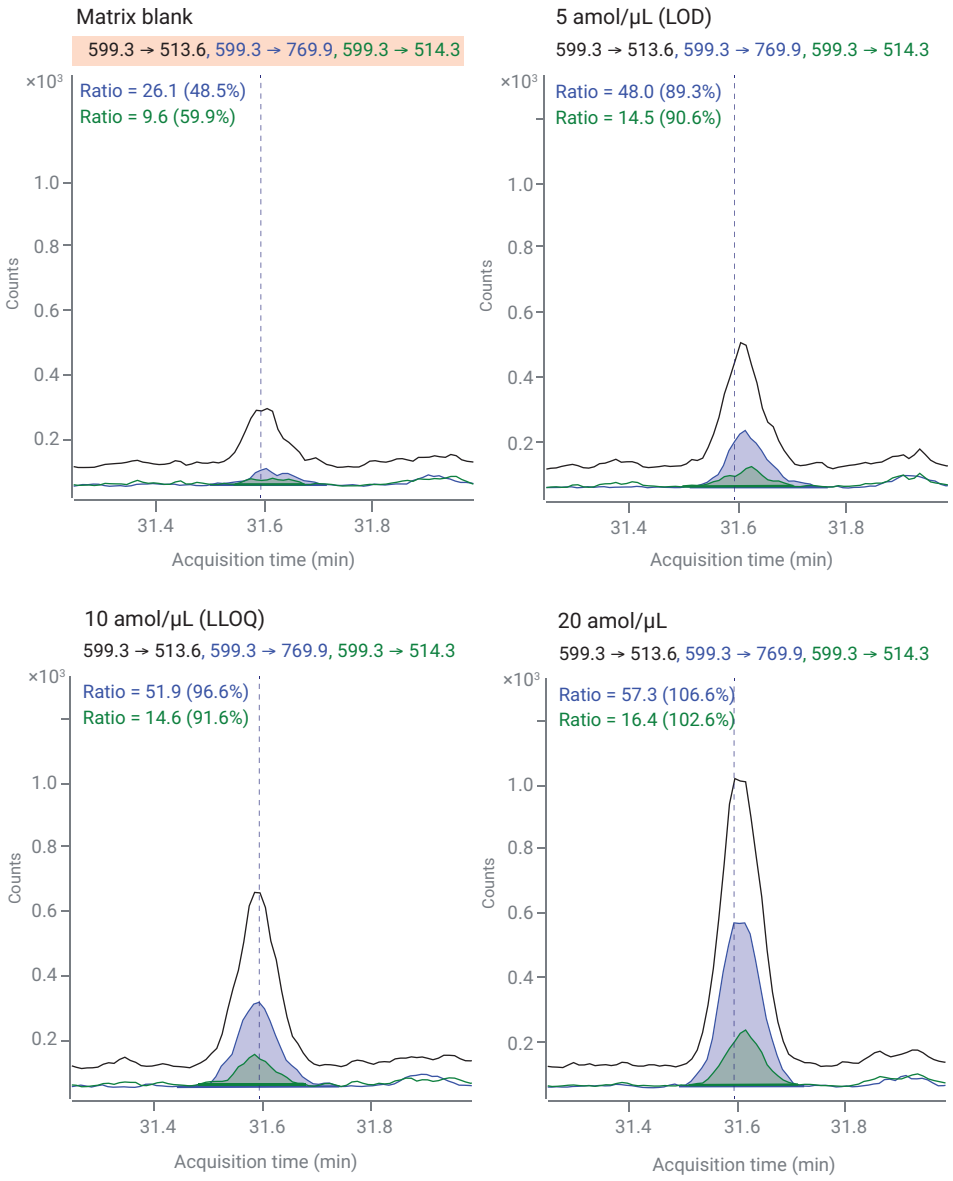


**Figure 5.** Extracted ion chromatograms of SIS peptide SGIPIVTSPYQIHFTK from complement C3 at low levels. The measured response ratios of qualifier relative to quantifier were labeled with percent of expected ratio in brackets. The title of chromatogram was highlighted in color when the percent of expected ratio deviated above 30% uncertainty in this case. The dashed line indicates the reference retention time of the targeted peptide.

## Conclusion

The analytical sensitivity, precision, accuracy, and robustness of MRM-based LC/MS methods for peptide quantification in biological fluids are important considerations for high-throughput biomarker verification. This application note describes the overall analytical sensitivity of the 6495 triple quadrupole LC/MS for peptide quantification in human plasma. Using a commercially available biomarker assessment kit from a third-party vendor, the outstanding performance of the 6495 LC/TQ coupled with the 1290 Infinity II LC system and Jet Stream ionization source was demonstrated for peptide quantification. Based on the physicochemical property of the peptides at hand, various peptides have wide differences in MRM signal response, which leads to huge variation (>four orders of magnitude) on quantitative sensitivity. In this experiment, an LLOQ of 5 amol/µL was achieved in heavy plasma matrix for some peptides, which is comparable to the results using a low-microflow LC/TQ platform.[4] This result demonstrated the excellent analytical sensitivity of the 6495 triple quadrupole LC/MS coupled with the 1290 Infinity II LC system and Jet Stream ionization source.

## References


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