Application Note

Proteomics

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Robust and Reproducible Protein Quantification in Plasma using the Evosep One and the Agilent 6495 Triple Quadrupole LC/MS

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Abstract

This application note showcases the reproducibility, robustness, and analytical sensitivity of MRM-based LC/MS analysis of human plasma proteins using the Agilent 6495 triple quadrupole LC/MS with the Evosep One LC system. Results demonstrated excellent retention time reproducibility over a 12-day analysis period, and equivalent sensitivity before and after robustness testing, underlining the suitability of the system for high-throughput protein quantification.

Introduction

As targeted workflows for protein biomarker verification using multiple reaction monitoring (MRM)-based methods are getting more popular, sample complexity and the low concentration of certain proteins are still the major challenges. Consequently, the development of MRM-based analytical methods using stable isotope-labeled standard (SIS) peptides for targeted quantitative proteomics in biological fluids is focused on improving method reproducibility, sensitivity, and robustness. Nanoflow liquid chromatography/mass spectrometry (LC/MS) uses the sample concentration effect of electrospray ionization (ESI) by producing the highest ionization efficiency using a small internal diameter column. However, nanoflow LC/MS typically requires a high degree of maintenance, and the limited nanocolumn capacity results in less robust chromatography for complex samples. Agilent Jet Stream technology coupled to the 6495 triple quadrupole LC/MS incorporating iFunnel technology has demonstrated outstanding analytical sensitivity and robustness for targeted protein quantification in complex matrices using standard flow LC/MS.[1,2] Nonetheless, there is still a need to address the prevalent challenges associated with throughput and robustness of nanoflow LC/MS for applications with limited amounts of samples. A conceptually novel chromatography system, called Evosep One, uses four low-pressure pumps in parallel to elute samples from a disposable and single-use trap column, the Evotip, while also using a pre-formed gradient, specifically designed to deliver the robustness and throughput required for clinical research applications.[3] This application note demonstrates the robustness, reproducibility, and analytical sensitivity performance of an Evosep One instrument coupled to a 6495 triple quadrupole LC/MS with an Agilent nanospray source (Figure 1).

To illustrate instrument robustness under challenging conditions, a balanced SIS peptide mix was spiked into human plasma digest and loaded directly onto Evotips. 574 replicate injections were analyzed on the LC/MS platform in a consecutive manner. To evaluate the precision and accuracy of the protein quantification, standard curve analyses were carried out both before and after the robustness test. A pre-formed gradient was used for separation, allowing 60 sample injections per day for both experiments. The instruments were not cleaned, adjusted, or tuned during the 12 days of continuous operation. The results show excellent robustness, reproducibility, and analytical sensitivity performance for targeted quantitative protein analysis in plasma.

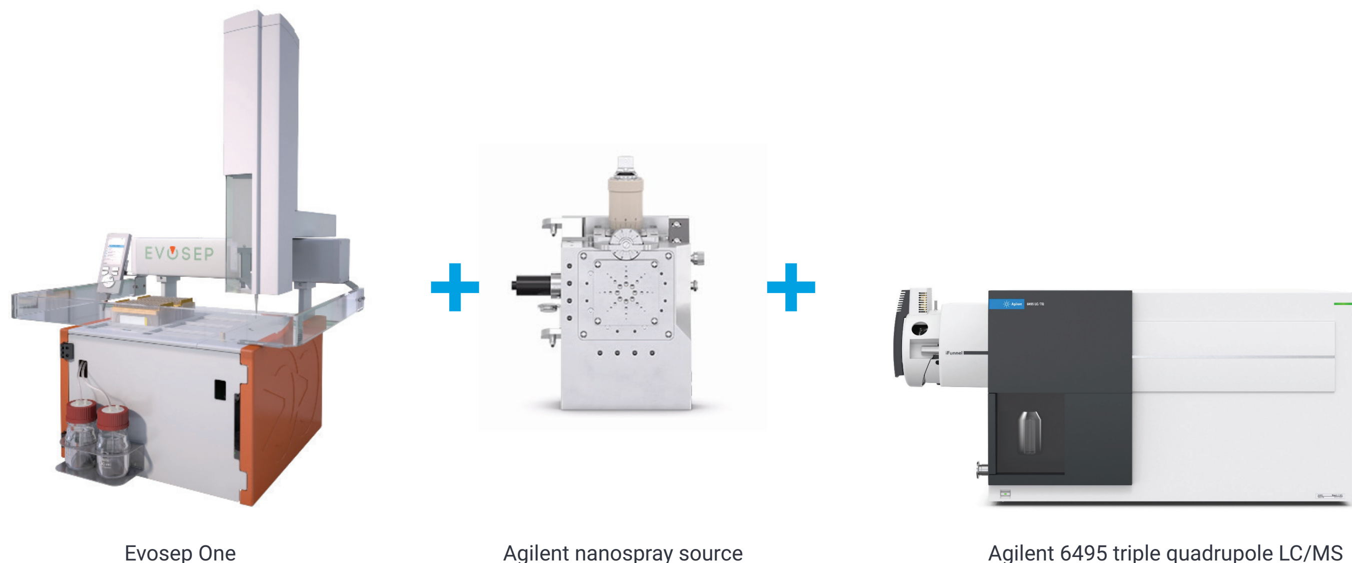


Figure 1. Evosep One coupled to an Agilent 6495 triple quadrupole LC/MS system with nanospray source.

Experimental

Instrumentation

- Evosep One
- Agilent nanospray source (G1992A)
- Agilent 6495 triple quadrupole LC/MS (G6495B)

Materials

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 prepared by denaturation, reduction, alkylation, and trypsin digestion followed by lyophilization using a Speedvac. The plasma digest was reconstituted and spiked with the balanced SIS peptide mixture followed by a serial dilution for standard curve analysis. The final SIS peptide concentration ranged from 280 fmol/mL to 7 nmol/mL relative to the original plasma sample. A large stock of plasma sample spiked with 0.7 nmol/mL of the same SIS peptide mixture was also prepared for robustness testing. All samples for the 12-day continuous measurement were loaded on Evotips (~1 µg load) before starting the measurements and kept cold until analysis.

LC/MS analysis

All samples were separated by the Evosep One using a standardized pre-formed 21-minute gradient with a Pepsep column (100 µm × 8 cm) packed with 3 µM C18 beads, allowing 60 sample injections per day (Table 1). A stainless-steel emitter was incorporated into the needle holder (clamshell) for the Agilent nanospray source. LC/MS data was acquired using the Agilent 6495 triple quadrupole LC/MS in dMRM mode. A transition list for 33 pairs of heavy and endogenous peptides (198 transitions, 66 peptides matching to 31 protein biomarkers) was selected for the final dMRM method.

Table 1. LC/MS acquisition parameters.

Evosep One LC System	
Analytical Column (Length/ID/C18 Bead Size)	8 cm/100 µm/3 µm
Flow Rate	1 µL/min
Gradient Length	21 minutes
Cycle Time	24 minutes
Throughput	60 (samples/day)
Agilent 6495 Triple Quadrupole Mass Spectrometer	
Ion Mode	nanoESI, Positive
Gas Temperature	200 °C
Drying Gas Flow	11 L/min
Capillary Voltage	1750 V
High/Low Pressure RF Voltage	200/110 V
Delta EMV	200 V
Q1 And Q3 Resolution	Unit/Unit
Cycle Time	500 ms
Minimum/Maximum Dwell Time	5.90 ms/80.589 ms
Total MRMs	198

Data processing

Data analysis for targeted peptide quantification was carried out using the Agilent MassHunter workstation software (v10.0) and Skyline software (v19.1.0.193).

Results and discussion

Robustness test: Complex sample analysis

To assess the system robustness, 1 µg of human plasma digest spiked with the balanced SIS peptide mixture was injected 574 times in a consecutive manner, leading to 10 fmol of SIS peptide mixture on column per injection. The Evotip was used as a disposable trap column and the plasma sample was directly loaded onto this without further SPE cleanup. The sample was separated using a 21-minute LC-dMRM method. The retention time (RT) alignment of the targeted 33 pairs of heavy and endogenous peptides from 574 consecutive injections provided good and consistent reproducibility across all injections for the targeted peptides (Figure 2). This observation demonstrated the excellent reproducibility and robustness of the Evosep One for high-throughput studies.

The high reproducibility and robustness of the MS signal is illustrated by the distribution of the relative standard deviation (RSD) of peak area for all targeted peptides during the robustness test. The median RSD is 8.5%, with 62 out of the 66 peptides (93.9%) showing an RSD below 16%. Only two pairs of heavy and endogenous peptides show an RSD greater than 16%. One pair is hydrophilic peptides (RT = 2.6 minutes) and unstable in solution, as a signal degradation (>50%) was observed

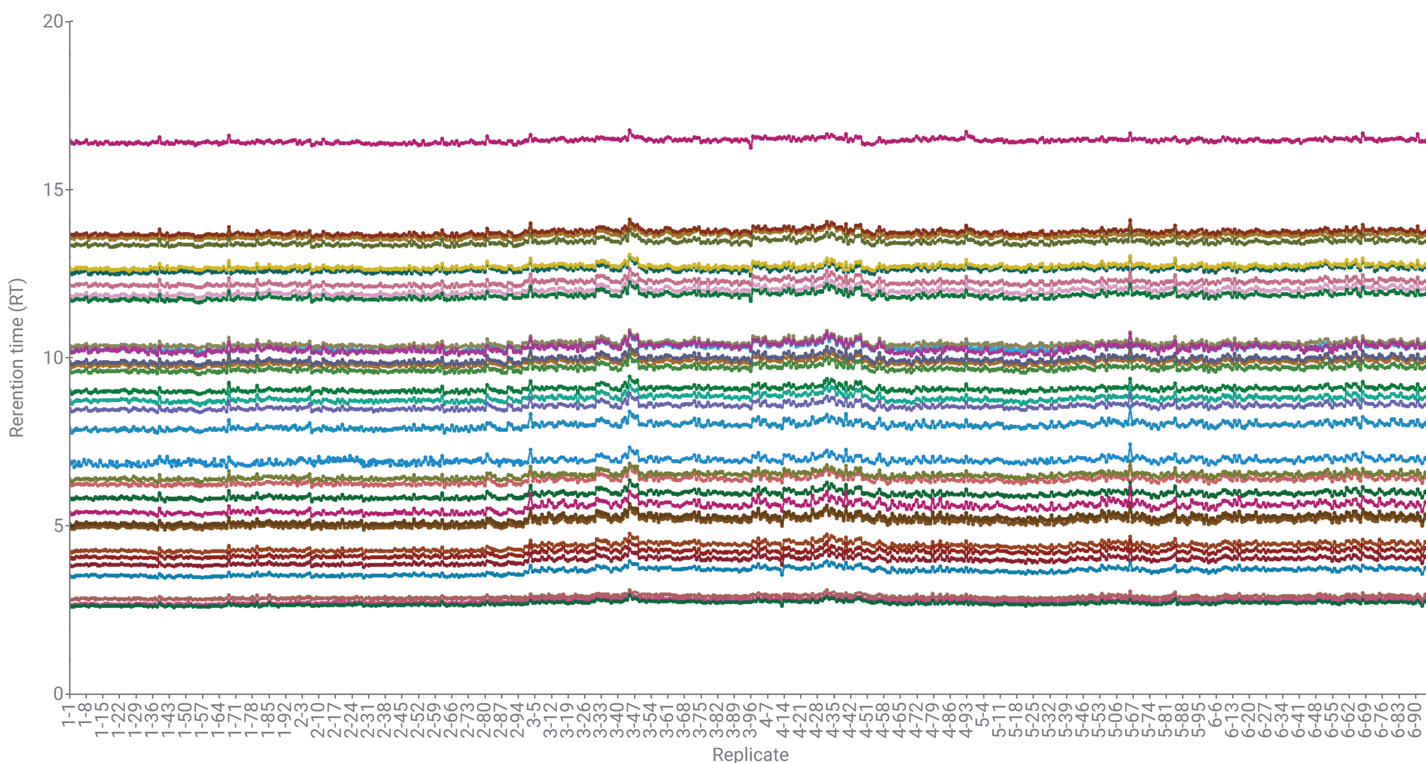


Figure 2. Retention time distribution of all targeted 33 pairs of peptides from 574 replicate injections during robustness test. The different peptides were color-coded.

after overnight storage in a refrigerator. The other pair suffered severe matrix interference, causing variation in peak integration. Therefore, the high RSD of these two pairs of peptides was not due to instrument variation (Figure 3).

The MRM peak area of four selected peptides matching four protein biomarkers demonstrated the outstanding robustness of the overall analytical platform (Figure 4):

- Very stable MS response without any adjustment on the LC/MS system (MRM peak area RSD = 6.5, 7.0, 7.9, and 6.0%, respectively, for n = 574)
- Good RT reproducibility (RSD = 0.69, 0.80, 1.04, and 0.59%, respectively, for n = 574)

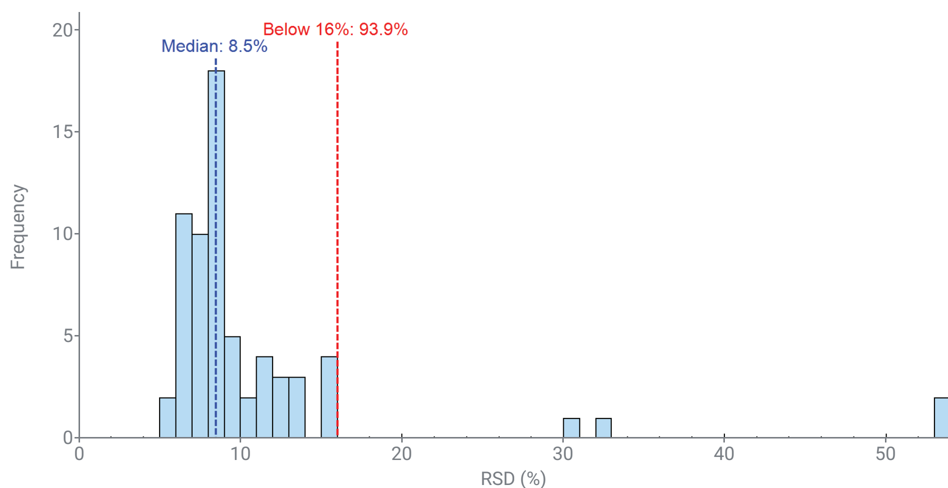
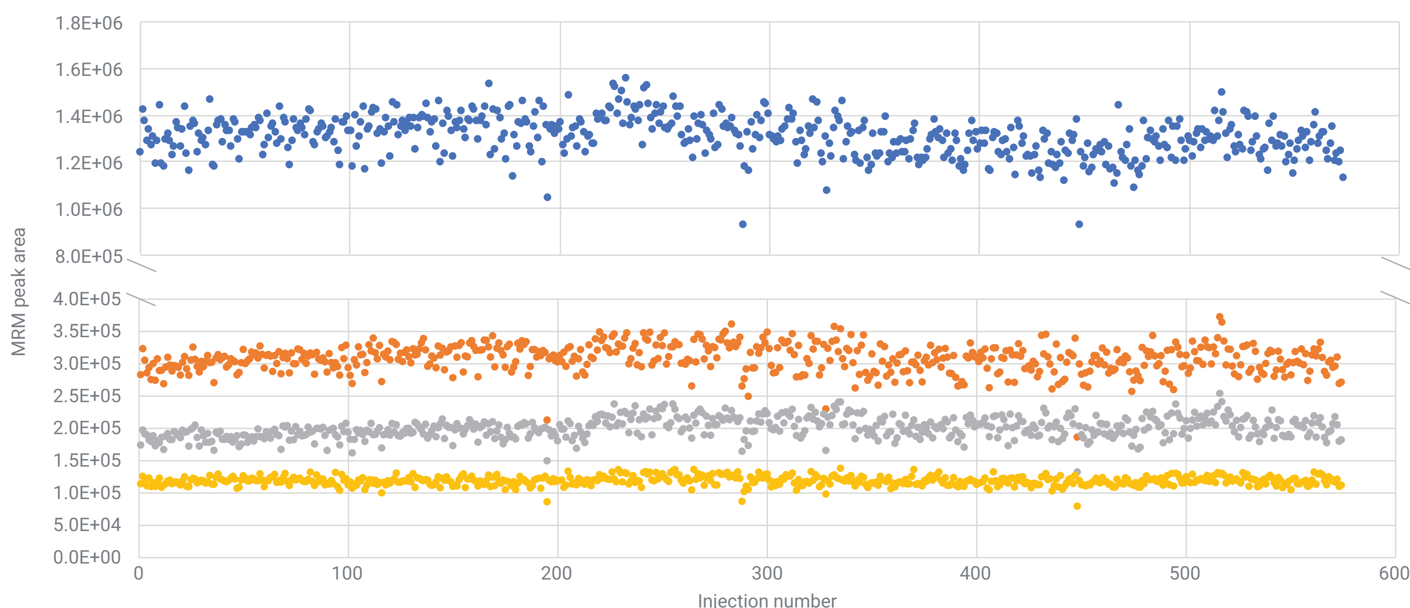


Figure 3. Distribution of peak area RSD for all targeted peptides from 574 replicate injections. The blue dash line represents the median RSD of 8.5%. The red dash line marks the 93.9% of the peptides having an RSD below 16%.



Complement C3
Hemopexin
Serotransferrin
Alpha-1B-glycoprotein

Protein	Peptide	Average MRM Peak Area	MRM Peak Area %RSD (n = 574)	Average RT (min)	RT %RSD (n = 574)
Complement C3	SGIPIVTSPYQIHFTK	1.31E+06	6.5	12.2	0.69
Hemopexin	NFPSPVDAAFR	3.08E+05	7.0	10.4	0.80
Serotransferrin	EGYYGYTGAFR	2.00E+05	7.9	8.8	1.04
Alpha-1B-glycoprotein	LETPDFQLFK	1.18E+05	6.0	13.4	0.59

Figure 4. MRM peak area of four selected SIS peptides from 574 replicate injections during the robustness test.

Quantitation of peptide standards in human plasma

To evaluate the analytical sensitivity for quantification of proteins in plasma, the SIS peptide mixture was spiked into human plasma digest at eight different concentrations ranging from 280 fmol/mL to 7 nmol/mL relative to the original plasma sample, then loaded directly on Evotips (~1 µg plasma digest). A standard curve was measured before and after the robustness test, with each injection being from Evotips, which were loaded in parallel before initiating the robustness test and stored cold until analysis. Five replicate injections of each dilution were measured before and after the robustness test to evaluate the precision and accuracy of the quantification up-front and with the impact of the robustness test.

The results from the two standard curves, measured before and after the robustness test, for the SIS peptide SGIPIVTSPYQIHFTK from Complement C3 are summarized below:

- Low amol-level sensitivity with a limit of detection (LOD) of 4 amol on column and a lower limit of quantification (LLOQ) of 10 amol on column in heavy matrices for both standard curves using a quantitative criterion of RSD <15% and accuracy of 80 to 120% (Figure 5 and Table 2)
- Excellent standard curve fitting for a dynamic range over 4 orders of magnitude (4 amol to 100 fmol on column) in heavy matrices with R^2 = 0.9987 and 0.9998 for the two standard curves, respectively (Figures 5A and 5B)
- Excellent precision and accuracy observed at all the tested levels including the LLOQ levels (Table 2)
- Good RT reproducibility (RSD = 0.43 and 0.32%, respectively, for n = 40)

The highly reproducible results between the two standard curves demonstrate the excellent instrument robustness and sample storage stability on Evotips even for the low-amol peptides. The samples for the standard curve measured after the robustness test were stored cold at 4 °C for about 10 days before being subjected to LC/MS. Despite this, the LOD of 4 amol and LLOQ of 10 amol remained the same as for the freshly prepared Evotips loaded just prior to analysis before the robustness test.

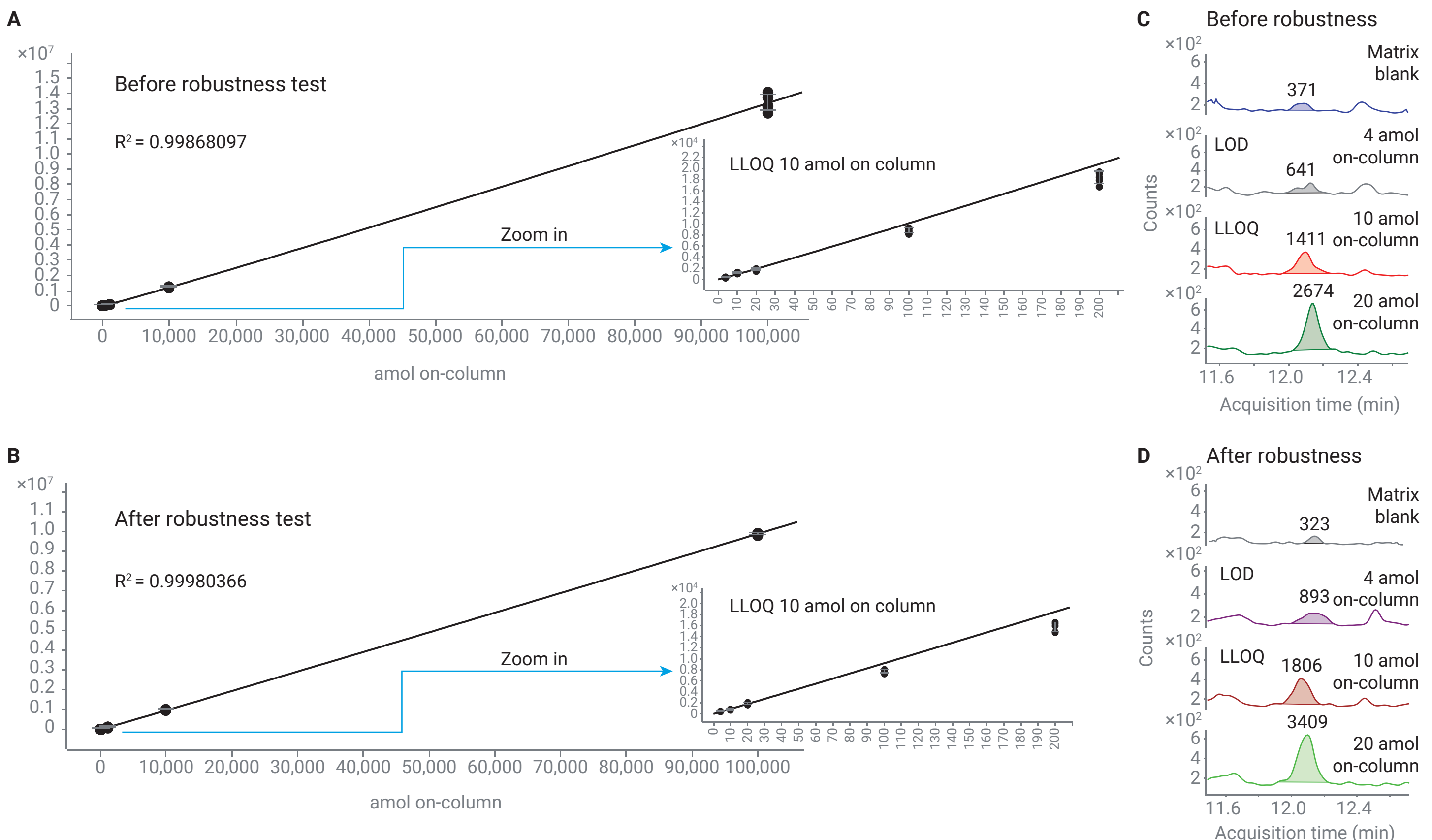


Figure 5. Standard curve analyses of SIS peptide SGIPIVTSPYQIHFTK from Complement C3 in plasma both before and after robustness test. A,B) standard curves before and after robustness test. C,D) Stacked extracted ion chromatograms showing the LOD and LLOQ.

All LC/MS analyses for the robustness test and standard curve evaluation were carried out on a single analytical column and was not replaced. The column was conjugated with the stainless-steel emitter inside the clamshell, which shields the column temperature from room-temperature fluctuation. Throughout the 12-day testing, the backpressure of the analytical column did not increase; the MS signals show excellent stability without spray needle changing, spray voltage adjustment, or mass spectrometer tuning. In summary, all these results demonstrated the outstanding analytical sensitivity, robustness, and reproducibility of this LC/MS platform for high-throughput studies under challenging conditions.

Table 2. Precision and accuracy for the standard curve analysis of the Complement C3 heavy peptide standard SGIPIVTSPYQIHFTK in trypsinized human plasma.

Amount On-column (amol)	Before Robustness Test		After Robustness Test	
	%RSD (n = 5)	%Accuracy	%RSD (n = 5)	%Accuracy
4	17.7	96.4	11.5	126.0
10	10.5	119.5	10.3	87.2
20	11.2	91.0	11.4	97.9
100	5.6	87.3	4.4	83.2
200	5.8	88.5	5.5	84.9
1,000	4.7	97.3	6.2	94.7
10,000	3.3	101.2	1.4	102.2
100,000	4.0	99.9	0.6	99.9

Conclusion

The reproducibility, robustness, and analytical sensitivity of MRM-based LC/MS methods for peptide quantification in biological fluids are important considerations for high-throughput protein biomarker quantification studies. This application note discusses the performance evaluation of the Evosep One LC system when coupled to the nanospray source and 6495 triple quadrupole LC/MS for the quantification of proteins in human plasma. For the analysis of a complex plasma digest matrix over an extended period of 12 days, the LC/MS system maintained excellent response stability and RT reproducibility without any cleaning, adjustment, or tuning. The analytical sensitivity was evaluated both before and after the robustness test and resulted in the same LOD and LLOQ. All these results demonstrate the suitability of this high-performance LC/MS system for high-throughput protein quantification in complex matrices.

References

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