Poster Reprint

ASMS 2020
ThP487

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

Linfeng Wu [1] and Nicolai Bache [2]

[1]Agilent Technologies, Santa Clara, CA;

[2]Evosep Biosystems, Odense, Denmark

Introduction

In clinical research applications, it is commonly necessary to analyze low abundance protein biomarkers from large sample cohorts, which therefore requires the LC/MS platform to be highly sensitive, rapid, and robust for analytical analysis. Nanoflow LC separation has often been used to separate complex biological samples prior to MS analysis owing to the sensitivity requirement. However, nanoflow LC/MS is usually neither fast nor robust. Conversely, conventional flow LC/MS setups are faster and much more robust but requires more sample loading.

This study was performed to evaluate the robustness, reproducibility and analytical sensitivity of a new low flow solution, Evosep One, using a preformed gradient and disposable trap columns when coupled to an Agilent nanospray source and a high-performance 6495 Triple Quadrupole (TQ) LC/MS for high throughput quantitative proteomics.

Experimental

Instrumentation

Evosep One (EV1000) coupled to an Agilent Nanospray source (G1992A) and 6495 triple quadrupole LC/MS (G6495B) (**Figure 1**).

Materials

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

Data processing

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

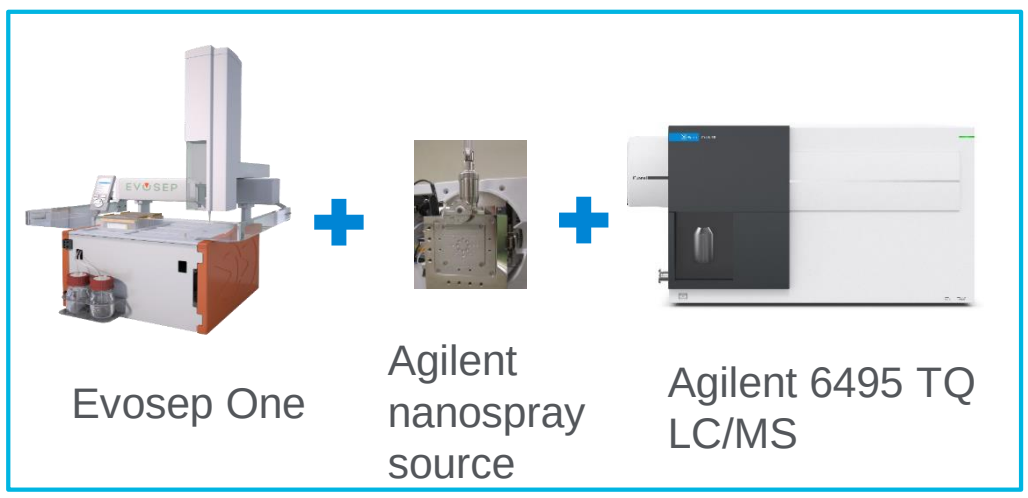


Figure 1. Evosep One coupled to an Agilent 6495 triple quadrupole LC/MS system with Nanospray source.

Experimental

Sample preparation

Human plasma was prepared by denaturation, reduction, alkylation, and trypsin digestion; then lypholized using SpeedVac. The plasma digest was reconstituted and spiked with the balanced Stable Isotope-labeled Standard (SIS) peptide mixture from the Biomarker assessment Kit followed by serial dilution for standard curve analysis. In addition, a large stock of plasma digest sample spiked with 0.7 nmol/mL of the SIS peptide mixture was also prepared for robustness test. All the SIS-spiked plasma digest was directly loaded on the Evotips with ~1µg digest per Evotip without further SPE cleanup.

LC/MS analysis

Peptide samples were separated using a standardized 60 SPD method which is a pre-formed 21-min gradient on a 100µm x 8 cm C18 column from Evosep (Table 1). A stainless-steel emitter was implemented 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 for 33 pairs of heavy and endogenous peptides matching to 31 protein biomarkers.

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 min
Cycle time	24 min
Throughput (samples/day)	60
Agilent 6495 Triple Quadrupole mass spectrometer	
Ion mode	nanoESI, Positive
Gas temperature	200 ºC
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
Min. / Max. dwell Time	5.90 ms/80.59 ms
Total MRMs	198

Robustness of LC Retention Time

To assess system robustness, replicate injections of SIS peptide-spiked human plasma digest were carried out with 10fmol SIS peptides in ~1µg plasma matrix on column per injection. A total of 574 injections was performed consecutively on the same column without any adjustment on spray needle or mass spectrometer.

The retention time of all the targeted peptides show excellent reproducibility across the test (**Figure 2**), with the RSD ranging from 0.43%~2.75%. The back pressure of the analytical column did not change over the entire duration of the experiment.

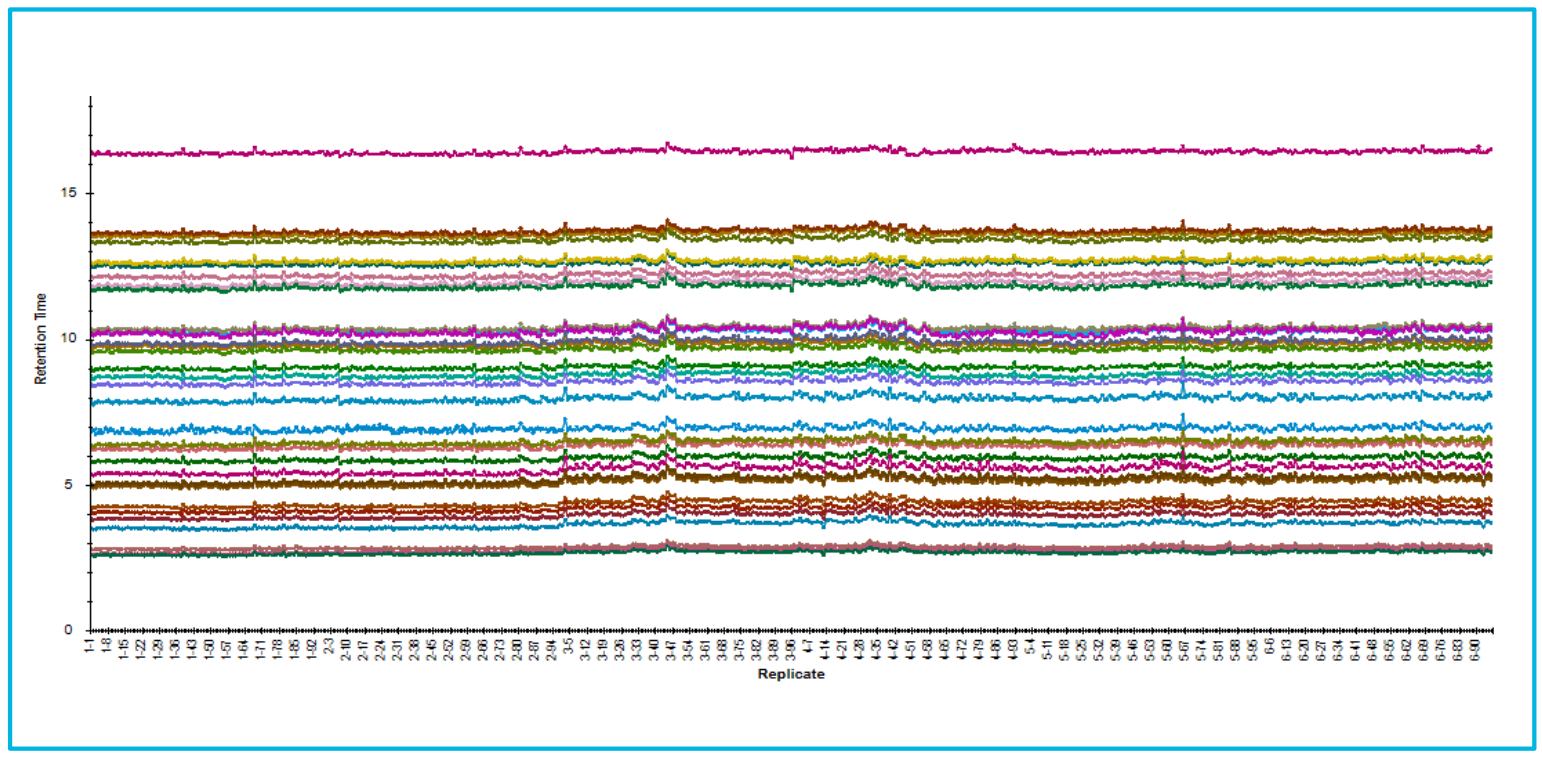


Figure 2. Retention time distribution of all the targeted peptides from 574 replicate injections during robustness test. The different peptides were color-coded.

Reproducibility of MS Signal Response

The relative standard deviation (RSD) of MRM peak area of each targeted peptide is displayed in a histogram plot (**Figure 3**). 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 and unstable in solution. The other pair shows severe matrix interference, causing variation in peak integration. Therefore, the high RSD of these two pairs of peptides was not due to instrument variation

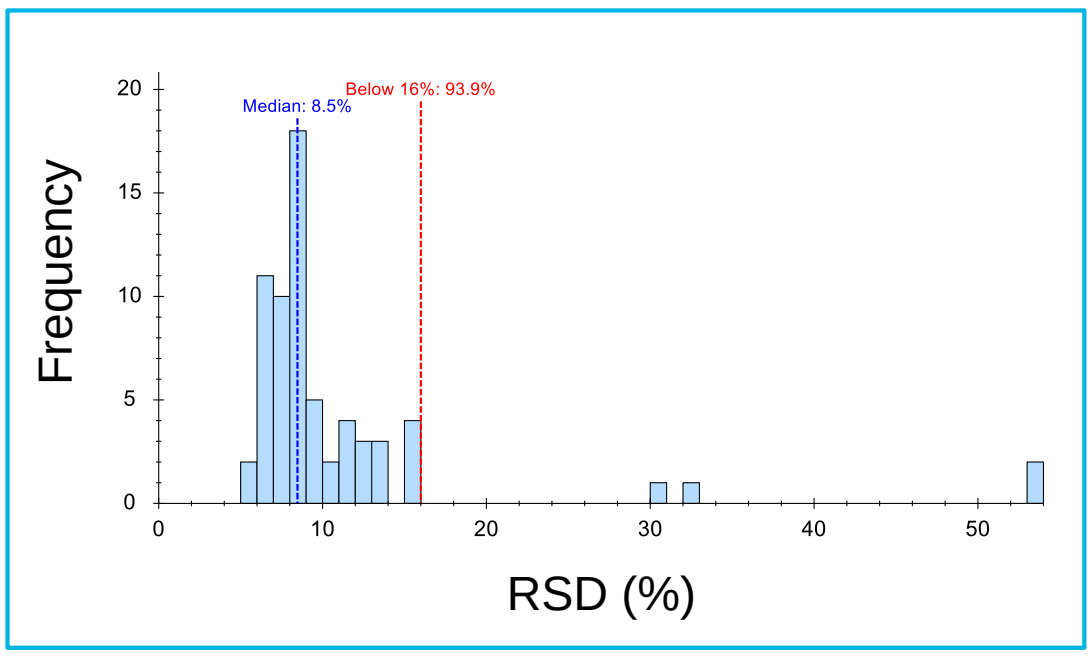
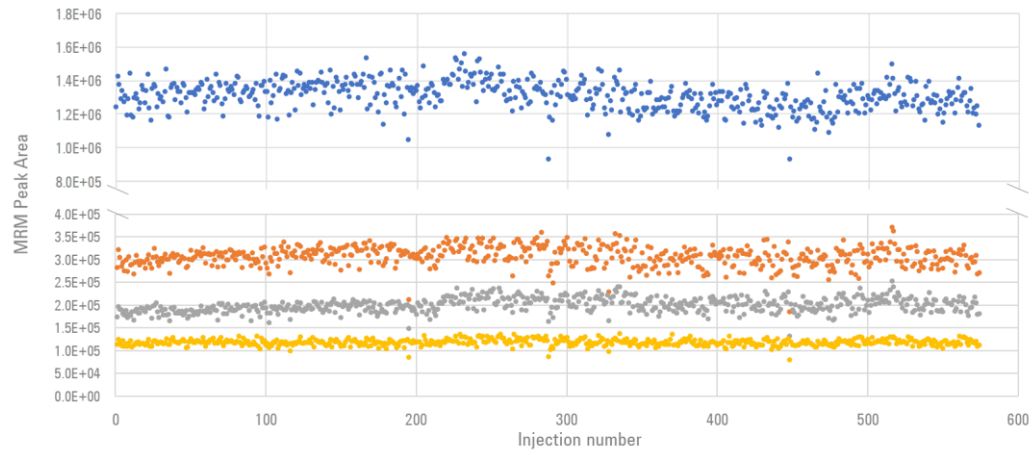


Figure 3. Histogram of peak area RSD for all the targeted peptides during robustness test. The blue dash line labels the median RSD of 8.5%. The red dash line marks the 93.9% of the peptides having an RSD below 16%.

Reproducibility of Four Selected Peptides

The MS signal response of four selected peptides matching to four protein biomarkers shows outstanding stability for the 574 replicate injections (**Figure 4**):

- Very stable MS response (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)



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 for robustness Test.

Results and Discussion

Standard Curve Analysis

To evaluate analytical sensitivity for protein quantification in heavy matrix, the SIS peptide mixture was spiked into human plasma digest at eight different concentrations. Replicates (n=5) of each calibration sample was directly loaded onto Evotip with ~1µg plasma digest on column per injection. Standard curve analysis was carried out both before and after robustness test on the same column for robustness test. The two standard curves for the SIS peptide SGIPIVTSPYQIHFTK from Complement C3 in plasma were very similar with LLOQ of 10 amol on column (**Figure 5**).

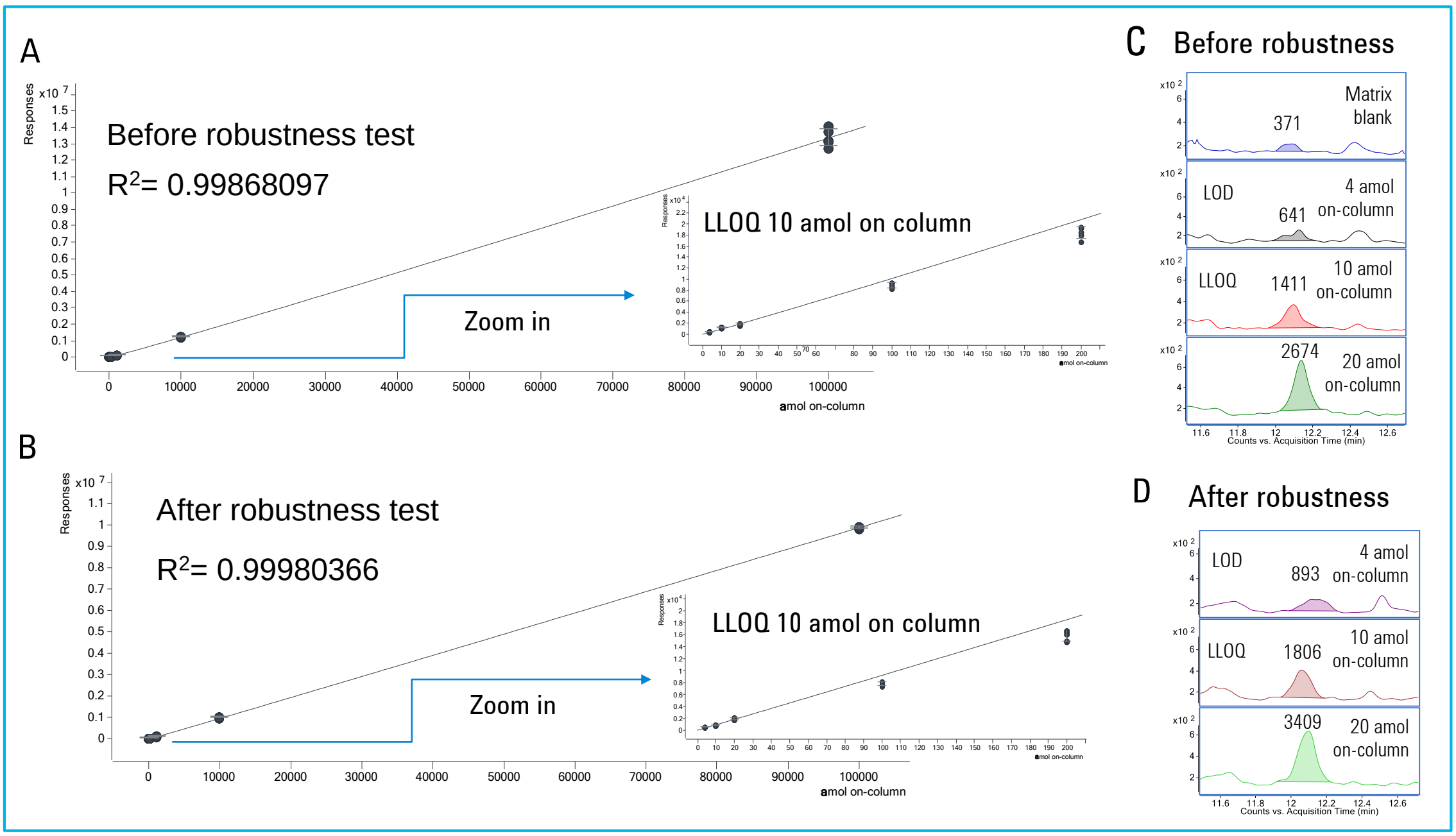


Figure 5: Standard curve analyses of SIS peptide SGIPIVTSPYQIHFTK from Complement C3 in plasma both before and after robustness test. A,B) standard curves before and after robustness test. C,D) Stacked extracted ion chromatograms showing the LOD of 4 amol and LLOQ of 10 amol on-column.

Conclusions

The excellent reproducibility, robustness and analytical sensitivity of a low flow LC/TQ system, including Evosep One LC system, Agilent Nanospray source and Agilent 6495 TQ LC/MS were demonstrated for high throughput protein quantification:

- Reproducible LC retention time for all targeted peptides across over 600 injections on the same analytical column
- MRM signal response shows outstanding stability during consecutive analysis of complex plasma samples over twelve days without any adjustment on spray needle or mass spectrometer
- Similar standard curves were achieved for the example SIS peptide both before and after robustness test
- Robust and reproducible storage of samples loaded on the Evotips

For Research Use Only. Not for use in diagnostic procedures.

This information is subject to change without notice.

© Agilent Technologies, Inc. 2020
Published in USA, June 1, 2020

EVOSEP

Agilent
Trusted Answers