Application Note

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Quantification of Host Cell Protein Impurities using the Agilent 6495C Triple Quadrupole LC/MS

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Abstract

This Application Note describes a method for sub-ppm host cell protein quantification in a mAb product. The workflow featured an Agilent AssayMAP Bravo platform, an Agilent 1290 Infinity II LC, and an Agilent 6495C triple quadrupole LC/MS.

Introduction

Host cell protein (HCP) impurities are low-level protein impurities derived from the host organisms during the biopharmaceutical manufacturing process. Due to their potential to affect product safety and efficacy, HCPs must be monitored and controlled in drug products according to regulatory requirements[1]. Traditionally, the enzyme-linked immunosorbent assay (ELISA) is the standard method for quantifying HCPs in protein therapeutics. However, ELISA lacks the specificity and coverage to identify and quantify individual HCPs. Therefore, LC/MS technologies have become an alternative for HCP analysis. The main challenge during LC/MS-based quantitative analysis of HCPs exists in the coelution of low-abundance HCP peptides with the highly abundant peptides from the drug product. This requires sensitive and reproducible quantification of low-abundant peptides in high background of drug product matrix.

This Application Note demonstrated a full workflow solution for sensitive quantification of host cell proteins including:

- Agilent AssayMAP Bravo platform for automated sample preparation
- Agilent 1290 Infinity II LC for sample separation
- Agilent 6495C triple quadrupole LC/MS for data acquisition
- Agilent Automation tool in Skyline software for MRM method development
- Data processing using both Agilent MassHunter Quantitative Analysis software and Skyline software

Using the multiple reaction monitoring (MRM)-based isotope dilution method, we showed that HCPs at low sub-ppm (ng/mg) level could be accurately quantified.

Experimental

Instrumentation

- Agilent AssayMAP Bravo system (G5571AA)
- Agilent 1290 Infinity II LC 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 6495C triple quadrupole LC/MS
- Agilent Jet Stream ESI source (G1958-65138)

Materials

Human IgG1 mAb (an R&D product from a partner) was produced from Chinese hamster ovary (CHO) cells and purified with protein A. Heavy stable isotope-labeled (SIL) peptide standards were custom-synthesized and provided by a third-party vendor (Table 1). All the SIL peptides were HPLC-purified, and their quality was determined by LC/MS and amino acid analysis.

Table 1. Liquid chromatography parameters.

LC Parameters	
Analytical Column	Reversed-phase C18 column with charged surface
Mobile Phase A	H_2O, 0.1% formic acid
Mobile Phase B	90% Acetonitrile in H_2O, 0.1% formic acid
Flow Rate	0.5 mL/min
Injection Volume	20 µL
Gradient	0 min → 3% B 1 min → 3% B 10 min → 21% B 10.5 min → 90% B 12 min → 90% B 12.5 min → 3% B
Stop Time	13 minutes
Post Time	1 minute
Column Temperature	60 °C

Sample preparation

The mAb sample was subjected to denaturation, reduction, alkylation, and trypsin digestion using the AssayMAP Bravo system. SIL peptides were combined at equal molar concentration and spiked into the sample digest at eight different levels (6.25, 12.5, 25, 62.5, 125, 250, 12,500, and 125,000 amol/µg per SIL peptide) for quantitative analysis.

LC/MS analysis

Samples were analyzed by the 6495C triple quadrupole LC/MS in dMRM mode using a nine-minute LC gradient. Tables 1 and 2 list detailed experimental conditions for chromatography and mass spectrometry. The LC-dMRM method was automatically optimized using the Agilent Automation tool, which is integrated with Skyline and Agilent MassHunter workstation software.

Data processing

Data analysis for peptide quantitation was carried out using Agilent MassHunter workstation software and Skyline software.

Table 2. Agilent 6495C Triple Quadrupole dMRM method.

Parameter	Setting
Ion Mode	JetStream, Positive
Gas Temperature	150 °C
Drying Gas Flow	19 L/min
Nebulizer Gas	35 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	4,000 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	500 ms
Minimum/Maximum Dwell Time	28.85 ms/60.39 ms

Results and discussion

LC-dMRM method development

To evaluate quantitative performance for HCP analysis, the purified mAb was subjected to denaturation, reduction, alkylation, and trypsin digestion using an AssayMAP Bravo automation system. This sample digest was used as a mAb background matrix in the following experiments. Three SIL standard peptides were spiked into the sample digest for standard curve analysis, including two peptides matched to two extraneous proteins (SUMO1 and SYHC) and one peptide matched to an endogenous HCP, CHO protein S100-A11 (Table 3)[2]. All the SIL peptide standards have a purity greater than 95%.

The LC-dMRM method was optimized using a MassHunter and Skyline Automation workflow (Figure 1). In this workflow, targeted peptides and transition ions were first created in Skyline software. Using the Automation tool, MRM methods and worklists were automatically created and executed to determine peptide retention time, optimize collision energy, analyze data, then export the final LC/MS method[3].

Quantification of SIL peptide standards in mAb matrix

Sensitivity performance for quantification of the three SIL peptide standards was evaluated in the mAb background matrix. Following blank injections to establish system cleanliness, replicate (n = 5) injections were made at all the levels from 6.25 amol/µg to 125 fmol/µg with 8 µg sample loading per injection (Table 4). The standard curve gives a range from low sub-ppm to over 1,000 ppm for all the targeted proteins, and covers a wide range related to HCP analysis. Retention time (RT) reproducibility was determined across all samples (n = 40), and peak area reproducibility and quantification accuracy was determined for each level:

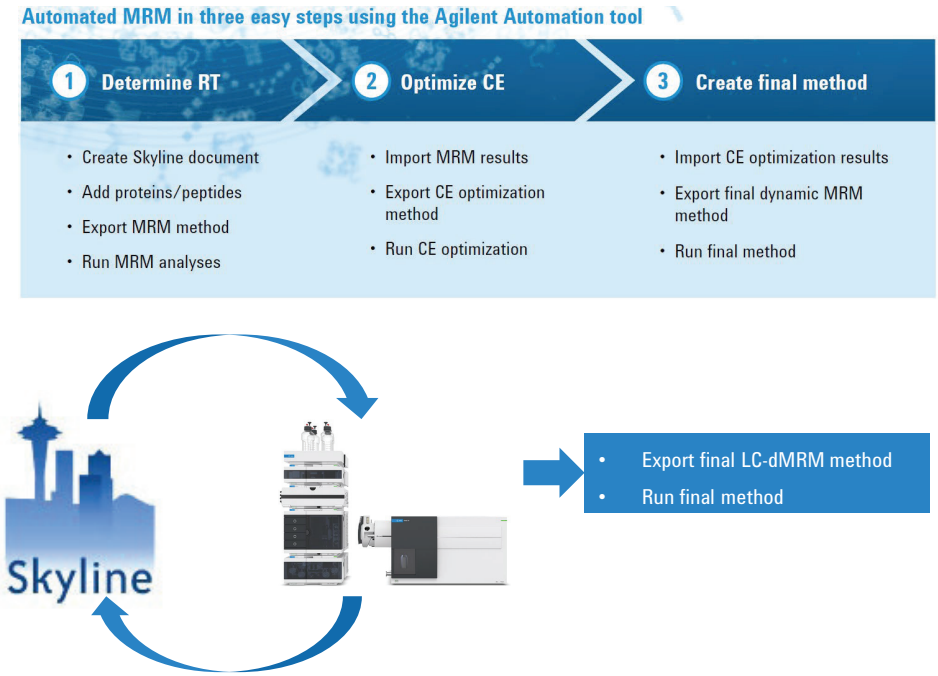

Figure 1. Agilent automation tool on Skyline.

Table 3. Targeted proteins, peptides and transitions

Targeted Protein	Protein Origin	Targeted Peptide Sequence	SIL Peptide Quality (%)	Monitored Transitions (*m/z*)
SUMO1_HUMAN	UPS2 protein standards	LLLEYLEEK	98.2	575.3 → 1036.6 575.3 → 923.5 575.3 → 810.4 575.3 → 681.3 579.3 → 1044.6 579.3 → 931.5 579.3 → 818.4 579.3 → 689.4
SYHC_HUMAN	UPS2 protein standards	VFDVIIR	96.2	431.3 → 762.5 431.3 → 615.4 431.3 → 500.4 431.3 → 401.3 436.3 → 772.5 436.3 → 625.4 436.3 → 510.4 436.3 → 411.3
Protein S100-A11 (G3HUU6)	CHO cell	DPGVLDR	95.1	386.2 → 559.3 386.2 → 502.3 386.2 → 403.2 391.2 → 569.3 391.2 → 512.3 391.2 → 413.2

- Excellent linearity for the levels tested with R^2 = 0.9993 for LLLEYLEEK, R^2 = 0.9990 for VFDVIIR, R^2 = 0.9995 for DPGVLDR (Figure 2A to 4A)
- Excellent precision and accuracy observed at all levels, including the lower limit of quantitation (LLOQ) levels (Table 4)
- Low-level sensitivity with a LLOQ of sub-ppm for all the three proteins (Figure 2B to 4B and Table 4, 0.48 ppm for SUMO1_HUMAN, 0.7 ppm for SYHC_HUMAN, and 0.13 ppm for CHO Protein S100-A11)
- There is some interference in the background matrix for the SIL peptide VFDVIIR and DPGVLDR. Even so, sub-ppm LLOQ was achieved for the targeted proteins (Figure 3B and 4B).
- Excellent RT reproducibility using all 40 injections (RSD = 0.06% for LLLEYLEEK, 0.08% for VFDVIIR, and 0.47% for DPGVLDR)

The column used in this experiment has a higher loading capacity than 8 µg[2]. Therefore, a lower level of the LLOQ could potentially be achieved with a higher amount of sample loading on-column, if needed.

Table 4. Precision and accuracy for the SIL peptides in mAb matrix. The LLOQ level is highlighted in red.

Protein Name / Molecular Weight	SUMO1_HUMAN / 38,815 Da			SYHC_HUMAN / 58,233 Da			Protein S100-A11 (G3HUU6) / 11,241 Da		
Peptide Sequences	LLLEYLEEK			VFDVIIR			DPGVLDR		
Sil Peptide Spiked-in Level (amol/µg)	%RSD (n=5)	%Accuracy	Protein Level* (ppm)	%RSD (n=5)	%Accuracy	Protein Level* (ppm)	%RSD (n=5)	%Accuracy	Protein Level* (ppm)
6.25	11.3	144.0	0.24	18.1	160.1	0.35	15.8	137.5	0.07
12.5	14.5	110.2	0.48	3.3	114.8	0.70	8.2	106.2	0.13
25	3.4	98.2	0.95	8.7	92.9	1.40	8.3	103.2	0.27
62.5	1.5	84.2	2.38	3.7	80.5	3.50	5.3	82.9	0.67
125	4.0	80.3	4.77	2.7	80.1	7.00	9.0	87.8	1.34
250	2.2	81.5	9.53	3.1	80.1	14.01	4.9	88.1	2.67
12,500	2.3	94.0	476.45	1.2	90.9	700.25	1.2	93.8	133.63
125,000	1.9	100.7	4,764.54	0.6	101.0	7,002.52	0.6	100.7	1,336.27

* Adjusted with SIL peptide purity

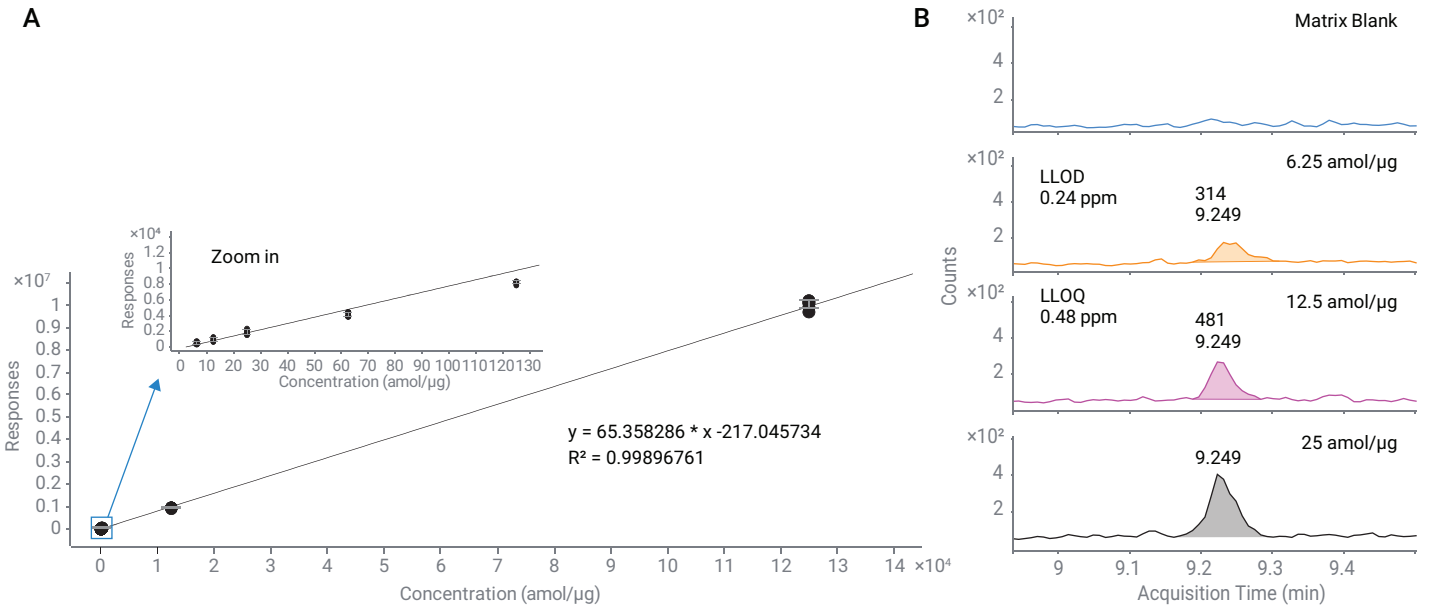

Figure 2. Quantitative results for the heavy peptide standard LLLEYLEEK in trypsinized mAb matrix. A) Standard curve with inset showing detail for the curve at low levels. B) Stacked extracted ion chromatograms showing the LOD and LLOQ.

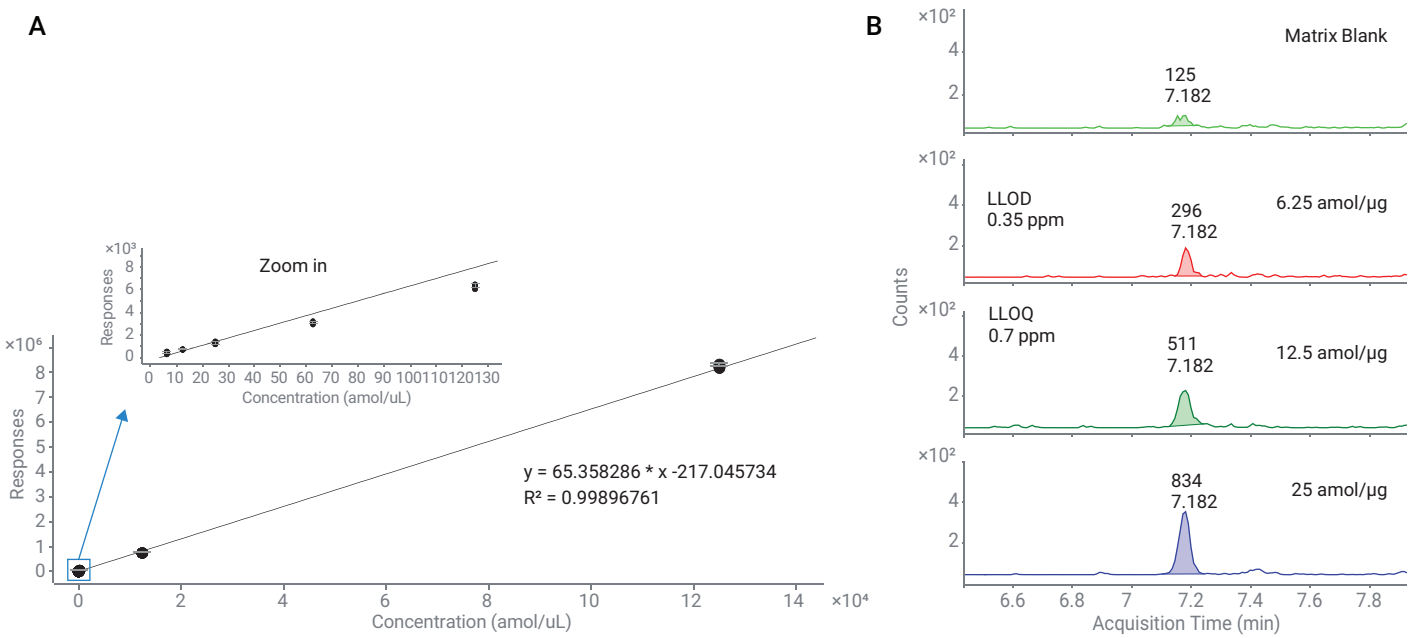

Figure 3. Quantitative results for the heavy peptide standard VFDVIIR in trypsinized mAb matrix. A) Standard curve with inset showing detail for the curve at low levels. B) Stacked extracted ion chromatograms showing the LOD and LLOQ.

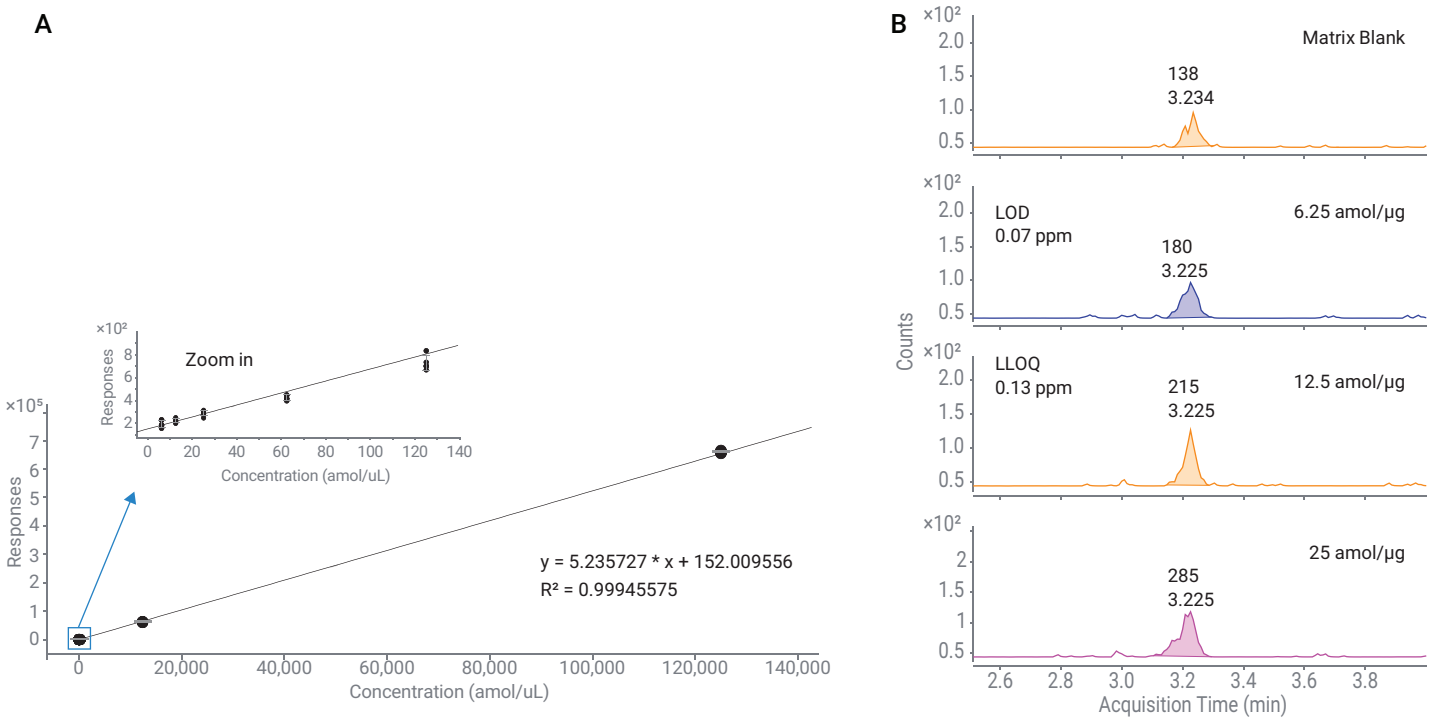

Figure 4. Quantitative results for the heavy peptide standard DPGVLDR in trypsinized mAb matrix. A) Standard curve with inset showing detail for the curve at low levels. B) Stacked extracted ion chromatograms showing the LOD and LLOQ.

Absolute quantification of protein

Host cell protein impurity concentrations are monitored and regulated in the manufacture of biologic drugs. An important request during this process is to measure the absolute concentration of the targeted HCP. MS-based analyses are extremely specific and accurate for this application, as long as suitable reference standards are available. Due to the availability and cost, SIL standard peptides that match to the sequences of targeted proteins were widely used for absolute quantification.

We performed absolute quantification for the endogenous HCP, CHO Protein S100-A11, which was identified in the previous HCP discovery work[2]. Two approaches were performed to calculate absolute protein concentration. In the first approach, the average response from the endogenous peptide DPGVLDR and the standard curve equation in Figure 4A were used to determine the absolute peptide concentration. In the second approach, the response ratio between the light and heavy peptide in the same LC/MS run was used to determine endogenous peptide concentration (Figure 5). As expected, these two approaches yield comparable protein concentrations for CHO Protein S100-A11 (1.05 versus 1.47 ppm). These results further confirm that:

- The Agilent HCP discovery workflow can identify HCP at a low-single digit ppm level[2]
- The 6495C triple quadrupole LC/MS system is reliable for peptide quantification at low levels.

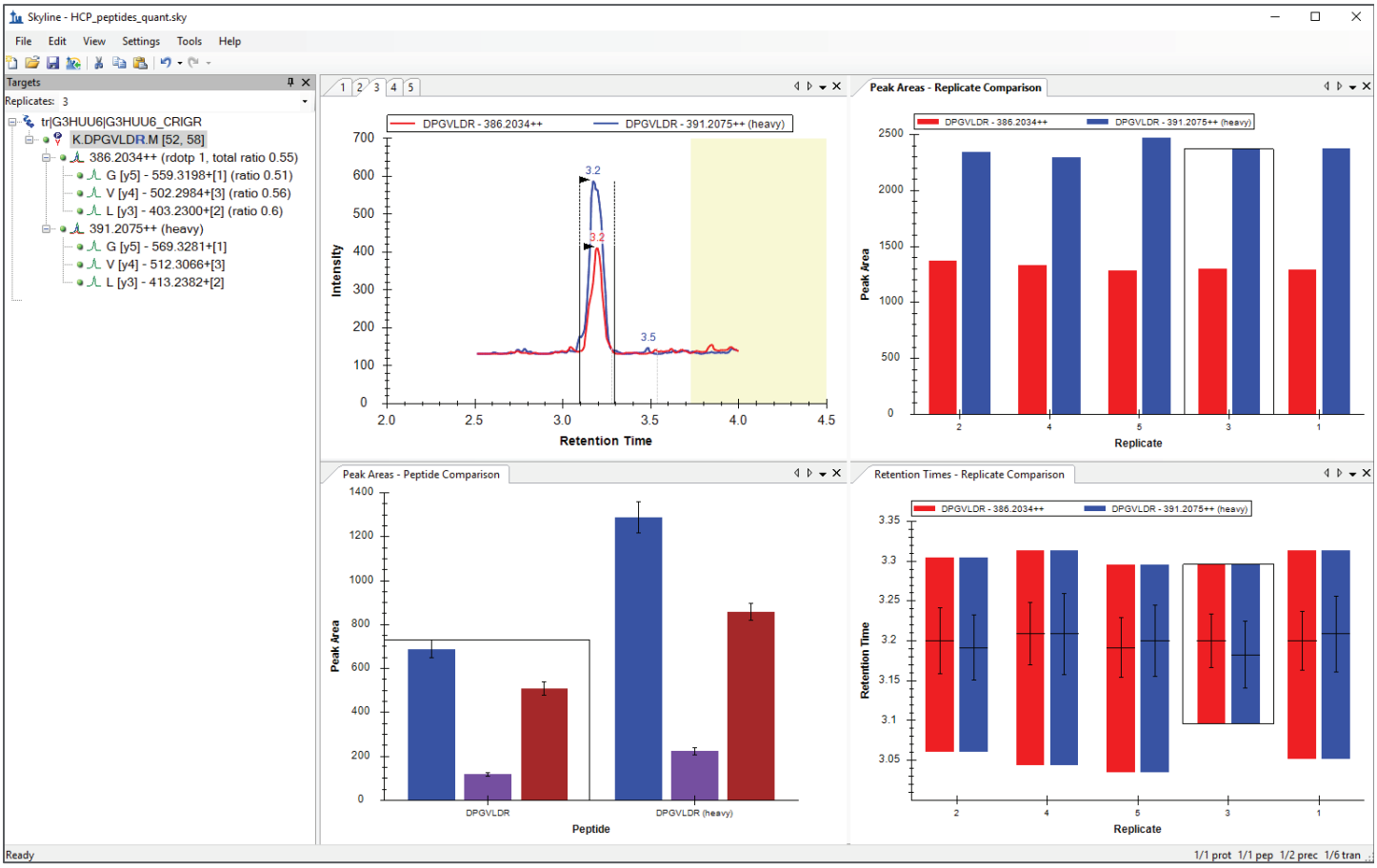

Figure 5. Peak area comparison of light and heavy peptides for the CHO Protein S100-A11 in mAb matrix with the SIL peptide DPGVLDR spiked at 250 amol/µg.

Conclusion

The performance of the Agilent workflow solution for HCP quantification has been demonstrated. The AssayMAP Bravo platform, using task-centric automation protocols, has brought unprecedented reproducibility, scalability, flexibility, and ease-of-use to sample preparation automation. The Agilent 1290 Infinity II LC, the next generation in UHPLC, gives more chromatography resolution and higher retention time precision. The seamless integration between Skyline software and Agilent MassHunter software using the Automation tool provides a straightforward solution for optimizing LC-dMRM method. The accurate quantification of HCP at sub-ppm levels has been demonstrated using the Agilent 6495C triple quadrupole LC/MS. A combination of Skyline and MassHunter software has provided powerful tools for targeted data analysis.

References

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2. Host Cell Protein Analysis Using Agilent AssayMAP Bravo and 6545XT AdvanceBio LC/Q-TOF. *Agilent Technologies Application Note,* publication number 5991-9300EN, **2018**.
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