Application Note

Biotherapeutics

Quantification of Host Cell Protein Impurities Using the Agilent 1290 Infinity II LC Coupled with the 6495B Triple Quadrupole LC/MS System

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Introduction

Host Cell Protein (HCP) impurities are low-level product-related and process-related protein impurities in biopharmaceuticals derived from the host organisms during manufacturing. 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, 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 the high background of drug product matrix.

This Application Note demonstrates a workflow for sensitive quantification of host cell proteins including:

- AssayMAP Bravo platform for automated sample preparation
- Agilent 1290 Infinity II LC system for sample separation
- Agilent 6495B Triple Quadrupole for data acquisition
- Agilent automation tool in Skyline software for MRM method development
- A combination of Skyline and Agilent MassHunter Quantitative Analysis software for data analysis

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

Experimental

Instrumentation

- Agilent AssayMAP Bravo system
- Agilent 1290 Infinity II LC system including:
  - Agilent 1290 Infinity II high speed pump
  - Agilent 1290 Infinity II multisampler
  - Agilent 1290 Infinity II thermostatted column compartment
- 6495B triple quadrupole
- Agilent Dual Jet Stream ESI source

Materials

Human IgG1 mAb (an R&D product from a partner) was produced from Chinese Hamster Ovary (CHO) cells, and purified with protein A. Proteomics Dynamic Range Standard Set (UPS2) was purchased from Sigma-Aldrich. 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 analysis and amino acid analysis.

Sample preparation

UPS2 was spiked into the purified mAb at a 1:1,000 ratio, followed by denaturation, reduction, alkylation, and trypsin digestion using the AssayMAP Bravo system. SIL peptides were mixed at equal molar, 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 for each SIL peptide) for standard curve analysis.

LC/MS analysis

Samples were analyzed by the 6495B triple quadrupole LC/MS in dynamic multiple reaction monitoring (dMRM) mode using a nine-minute LC gradient (Tables 1 and 2). The LC-dMRM method was automatically optimized using the Agilent Automation tool integrated with Skyline and Agilent MassHunter workstation software.

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 minutes → 3 %B 1 minute → 3 %B 10 minutes → 21 %B 10.5 minutes → 90 %B 12 minutes → 90 %B 12.5 minutes → 3 %B
Stop time	13 minutes
Post time	1 minute
Column temperature	60 °C

Table 2. 6495B Triple quadrupole dMRM method.

Parameter	Setting
Ion mode	Jet Stream, 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

Data processing

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

Results and discussion

LC-dMRM method development

To evaluate quantitative performance for HCP analysis, UPS2 protein standards were spiked into the purified mAb at a 1:1,000 (w/w) ratio before digestion, resulting in protein levels from 0.0004 to 313 ppm (ng/mg). The sample was then denatured, reduced, alkylated, and digested by trypsin using the AssayMAP Bravo automation system. This digest sample was used as a mAb background matrix in the following experiments. Based on a previous HCP discovery study in the same spiking sample[2], three peptides were selected for targeted quantification, including two peptides matched to two of the UPS2 proteins (SUMO1 and SYHC), and one peptide matched to an endogenous CHO HCP, protein S100-A11 (Table 3)[2]. The SIL peptide standard has a purity greater than 95 % for all the peptides.

A nine-minute LC separation was performed on the reversed-phase C18 column with charged surface[2]. 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 Agilent Automation tool, MRM methods and worklists were automatically created and executed to determine peptide retention time, optimize transition collision energy, analyze data, and export the final LC/MS method[3].

Table 3. Targeted proteins, peptides, and transitions.

Targeted protein	Protein origin	Protein concentration (ng/mg)	Targeted peptide sequence	SIL peptide quality (%)	Monitored transitions (*m/z*)
SUMO1_HUMAN	UPS2 protein standards	18.3	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	2.7	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	N.A.	DPGVLDR	95.1	386.2 → 656.4 386.2 → 559.3 386.2 → 502.3 386.2 → 403.2
					391.2 → 666.4 391.2 → 569.3 391.2 → 512.3 391.2 → 413.2

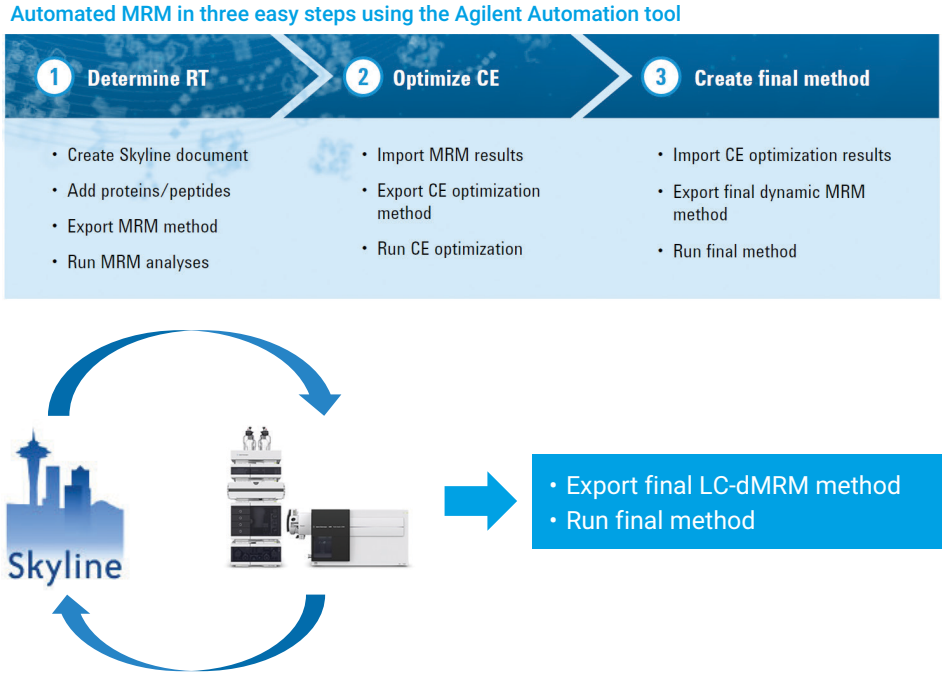

Figure 1. The Agilent automation tool on Skyline.

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 injections (n = 7) were made at all levels, from 6.25 amol/µg to 125 fmol/µg, with an 8-µg sample loading per injection (Tables 4 to 6). The standard curve gives a range from low sub-ppm to over 1,000 ppm for all targeted proteins, which covers a wide range related to HCP analysis. Retention time (RT) reproducibility was determined across all samples (n = 56), and peak area reproducibility and quantification accuracy was determined for each level:

- Excellent linearity for the levels tested with R^2 = 0.9996 for LLLEYLEEK, R^2 = 0.9983 for VFDVIIR, and R^2 = 0.9996 for DPGVLDR (Figures 2B to 4B)
- Excellent precision and accuracy observed at all levels, including the lower limit of quantitation (LLOQ) levels (Tables 4 to 6)
- Low-level sensitivity with sub-ppm LLOQ for all three proteins (Figures 2A to 4A and Tables 4 to 6, 0.24 ppm for SUMO1_HUMAN, 0.7 ppm for SYHC_HUMAN, and 0.13 ppm for CHO Protein S100-A11)
- Note that there is some interference in the background matrix for the SIL peptide DPGVLDR; even so, a limit of detection (LOD) of 0.065 ppm and a LLOQ of 0.13 ppm was achieved for the targeted CHO protein S100-A11 (Figure 4A).
- Excellent RT reproducibility using all 56 injections (RSD = 0.06 % for LLLEYLEEK, 0.07 % for VFDVIIR, and 0.32 % for DPGVLDR)

Note that the column used in this experiment has a higher loading capacity than 8 µg[2,4]. Therefore, a lower LLOQ could potentially be achieved with a higher amount of sample loading on-column, if needed.

Table 4. Precision and accuracy for the SIL peptide LLLEYLEEK in mAb matrix.

Targeted protein	SUMO1_HUMAN		
Protein MW	38,815 Da		
Peptide sequence	LLLEYLEEK		
SIL peptide level (amol/µg)	**%RSD (n = 7)**	**% Accuracy**	**Protein level* (ppm)**
6.25	17.0	110.3	0.24
12.5	17.3	107.1	0.48
25	10.7	96.8	0.95
62.5	10.5	91.6	2.38
125	5.7	93.8	4.77
250	3.3	91.7	9.53
12,500	2.8	100.9	476.45
125,000	2.1	99.9	4,764.54

* Adjusted with SIL peptide purity

Table 5. Precision and accuracy for the SIL peptide VFDVIIR in mAb matrix.

Targeted protein	SYHC_HUMAN		
Protein MW	58,233 Da		
Peptide sequence	VFDVIIR		
SIL peptide level (amol/µg)	**%RSD (n = 7)**	**% Accuracy**	**Protein level* (ppm)**
12.5	14.1	98.2	0.70
25	9.9	100.3	1.40
62.5	7.5	83.4	3.50
125	3.3	85.4	7.00
250	4.5	82.4	14.01
12,500	2.8	93.7	700.25
125,000	3.8	100.7	7,002.52

* Adjusted with SIL peptide purity

Table 6. Precision and accuracy for the SIL peptide DPGVLDR in mAb matrix.

Targeted protein	Protein S100-A11 (G3HUU6)		
Protein MW	11,241Da		
Peptide sequence	DPGVLDR		
SIL peptide level (amol/µg)	**%RSD (n = 7)**	**% Accuracy**	**Protein level* (ppm)**
12.5	8.3	106.9	0.13
25	10.8	112.3	0.27
62.5	10.3	102.9	0.67
125	8.7	89.0	1.34
250	7.4	93.2	2.67
12,500	1.2	95.3	133.63
125,000	0.9	100.5	1,336.27

* Adjusted with SIL peptide purity

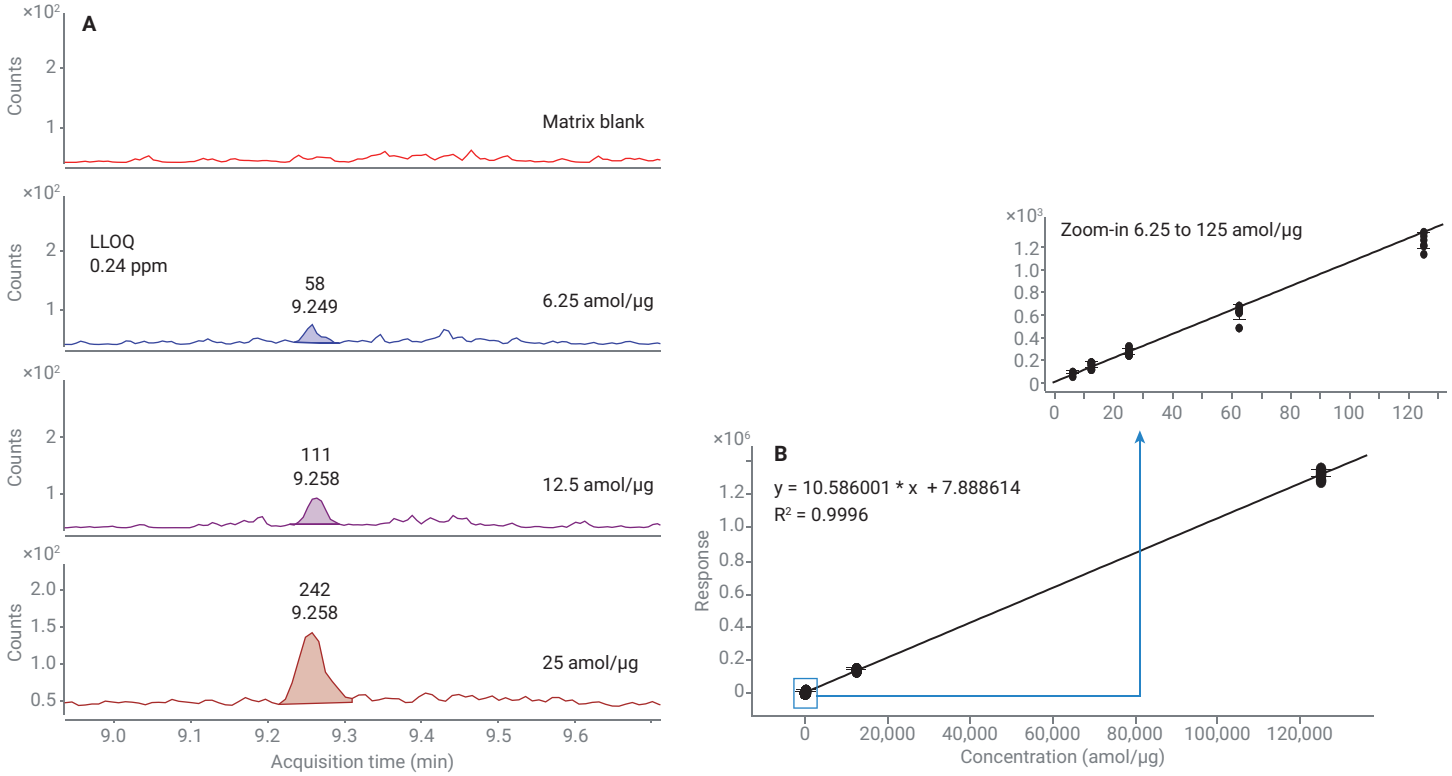

Figure 2. Quantitative results for the heavy peptide standard LLLEYLEEK in trypsinized mAb matrix. A) Stacked extracted ion chromatograms showing the LLOQ. B) Standard curve with inset showing detail for the curve from 6.25 to 125 amol/µg.

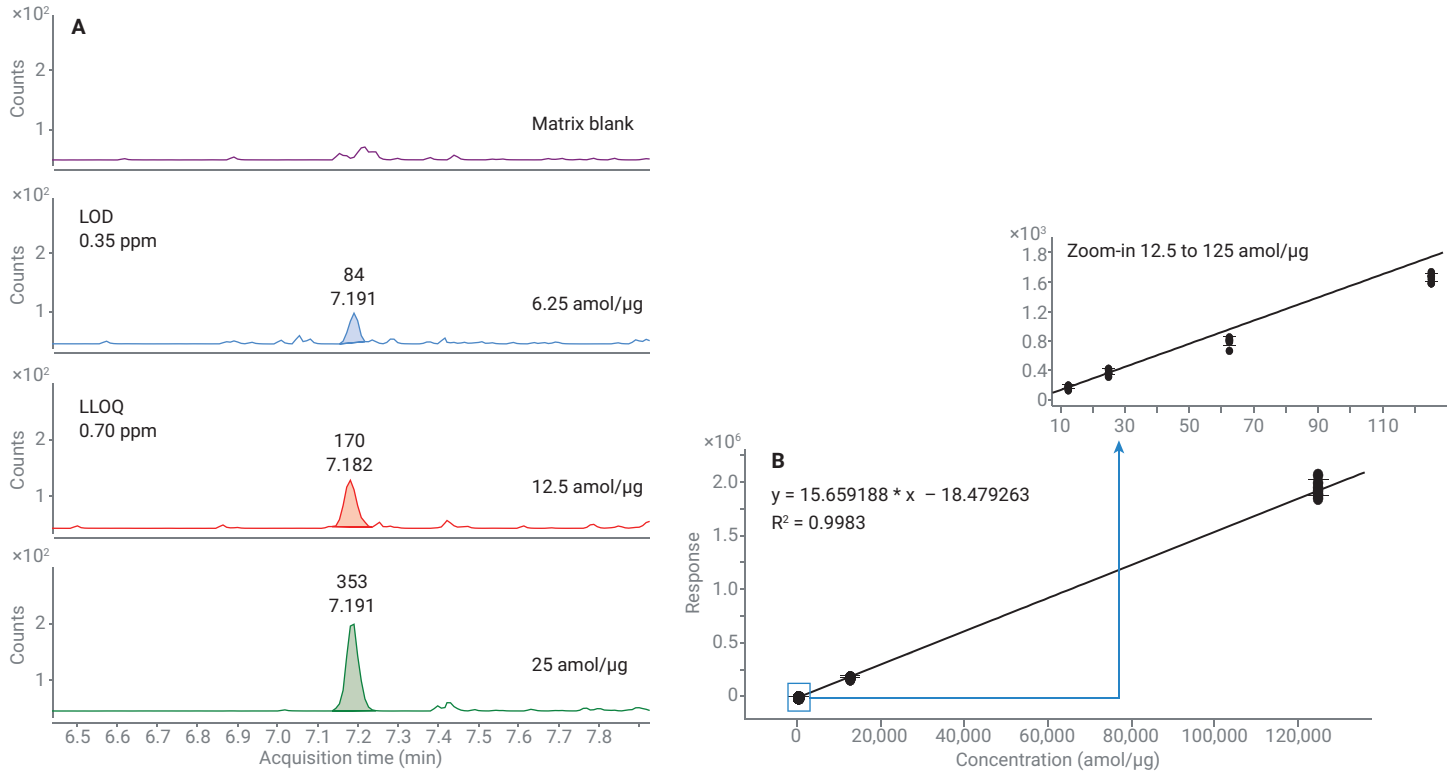

Figure 3. Quantitative results for the heavy peptide standard VFDVIIR in trypsinized mAb matrix. A) Stacked extracted ion chromatograms showing the LOD and LLOQ. B) Standard curve with inset showing detail for the curve from 12.5 to 125 amol/µg.

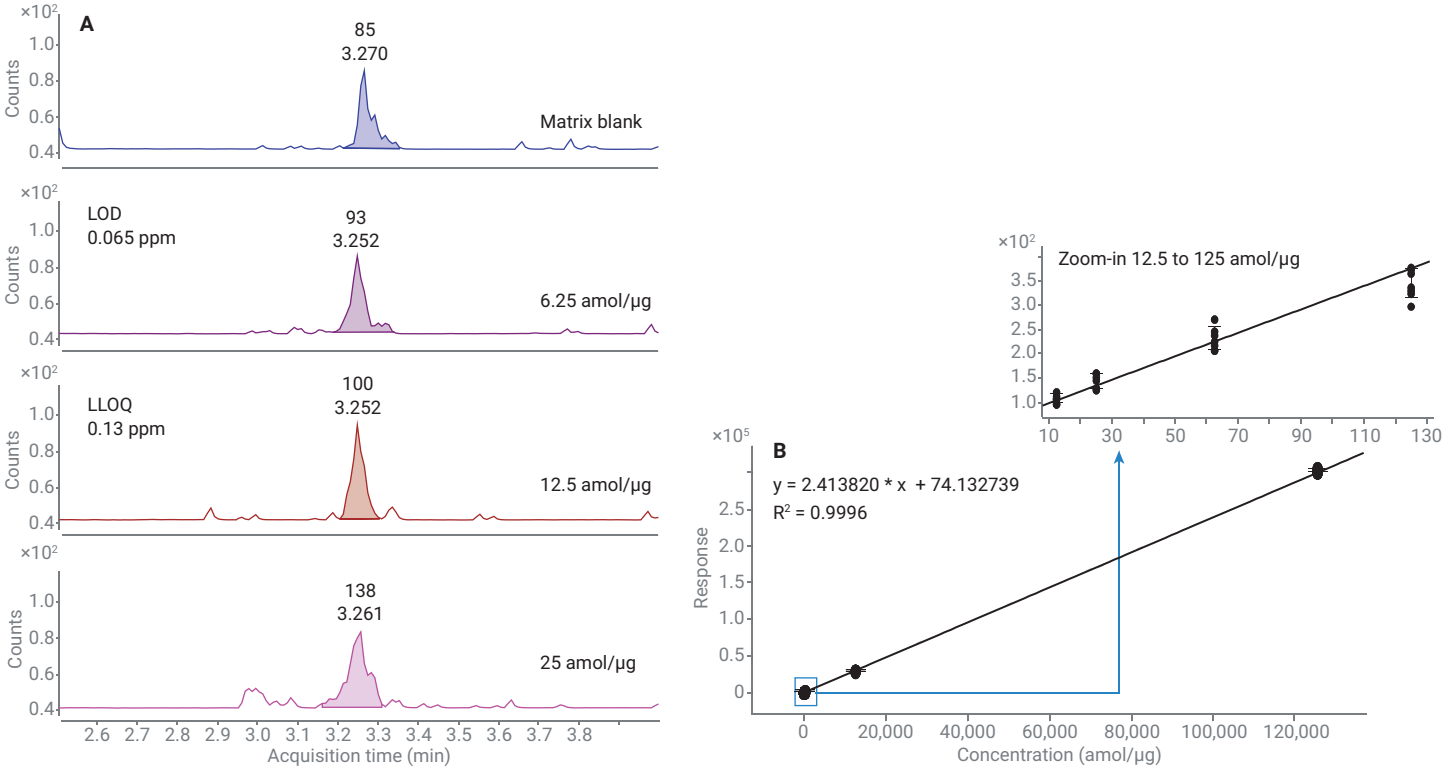

Figure 4. Quantitative results for the heavy peptide standard DPGVLDR in trypsinized mAb matrix. A) Stacked extracted ion chromatograms showing the LOD and LLOQ. B) Standard curve with inset showing detail for the curve from 12.5 to 125 amol/µg.

Absolute quantification of protein

HCP impurity concentrations are monitored and regulated in the manufacture of biologic drugs. During this process, it is important to measure the absolute concentration of the targeted HCP. MS-based analyses are extremely specific and accurate for this application, provided that suitable reference standards are available. The gold standard for absolute protein quantification by MS is the addition of a SIL version of the targeted protein to the samples at an early stage of sample processing. However, SIL proteins are often not commercially available, and custom synthesis could be very expensive. As an alternative, SIL peptides containing the amino acid sequence of the tryptic peptides have been used for absolute quantification of proteins[5]. In a SIL peptide approach, surrogate peptides were selected for protein quantitation. SIL peptides with matched sequences were generally added to the samples after protein digestion. They do not allow concentration correction for protein losses that may occur during sample preparation, nor do they take into account the yield of the protease digestion step. Therefore, the calculated protein concentration by the SIL peptide method often underestimates the real protein concentration.

This study performed the absolute quantification of the three targeted proteins using SIL peptides. The accuracy of absolute quantification for two of the spiked proteins, SUMO1 and SYHC, was evaluated by comparing to the gold standard, which is the addition of UPS2 protein standards in mAb before sample digestion. Figure 5 shows the chromatograms of three pairs of light and heavy peptides in the mAb matrix, including LLLEYLEEK, VFDVIIR, and DPGVLDR. Figure 6 shows a peak area comparison of light and heavy peptides for the three pairs of peptides with the heavy SIL peptides spiked at 250 amol/µg.

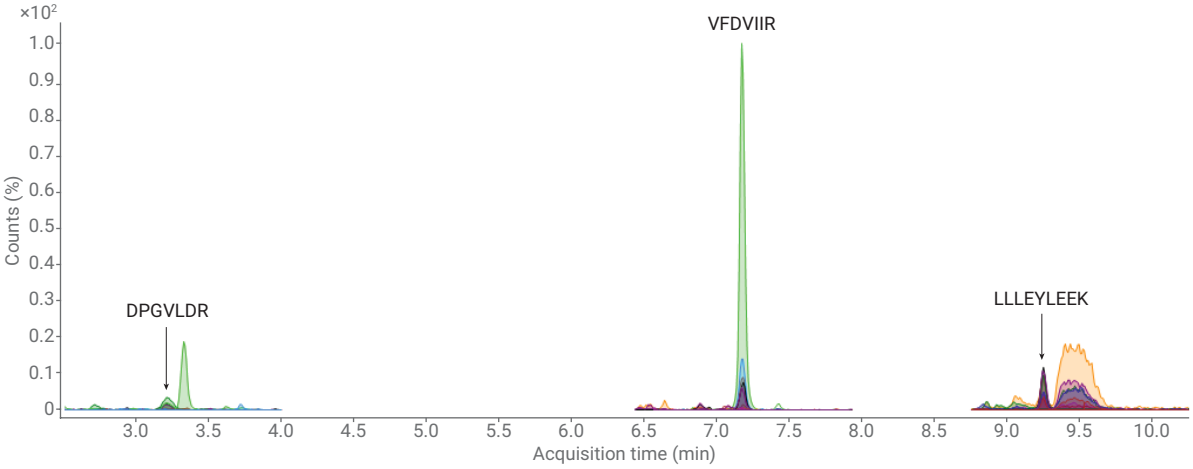

Figure 5. Absolute quantification of proteins using the heavy SIL peptides as internal standards. Chromatograms of three pairs of monitored light and heavy peptides in trypsinized mAb matrix include LLLEYLEEK, VFDVIIR, and DPGVLDR peptides.

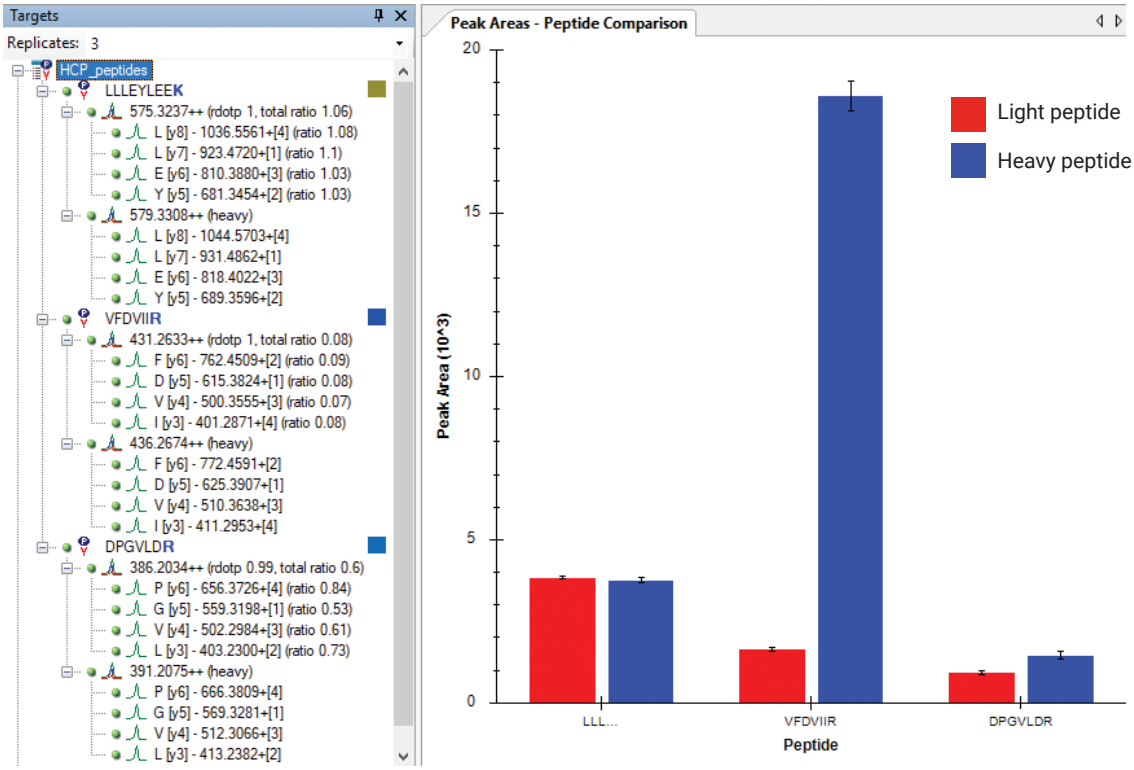

Figure 6. Peak area comparison of light and heavy peptides for the three targeted peptides in mAb matrix with SIL peptides spiked at 250 amol/µg.

Table 7. Comparison of the spiked and measured protein absolute levels for the three targeted proteins in mAb matrix.

Targeted protein	SUMO1_HUMAN	SYHC_HUMAN	Protein S100-A11 (G3HUU6)
Protein MW (Da)	38,815	58,233	11,241
Peptide sequence	LLLEYLEEK	VFDVIIR	DPGVLDR
Spiked protein level (ppm)	18.3	2.7	NA
Measured protein level (ppm)	10.1	1.2	1.6

Table 7 shows a comparison of the spiked and measured protein absolute levels for the three targeted proteins in mAb matrix. The endogenous CHO HCP, Protein S100-A1,1 was calculated as 1.6 ppm in this experiment; the spiked levels of SUMO1 and SYHC protein standards were 18.3 and 2.7 ppm, respectively, and their measured protein levels were 10.1 and 1.2 ppm respectively. As expected, an underestimation (~50 %) of protein concentration with the SIL peptide approach was observed, which is consistent with other reports[6,7]. These results suggest that when using a SIL peptide approach for absolute protein quantification, the recovery of the targeted peptides from protein digestion need to be evaluated for accurate absolute quantification. In addition, maintaining reproducible sample preparation steps in the lab is important for cross-sample or cross-experiment comparisons of absolute protein concentration. The AssayMAP Bravo and its suite of tools for LC/MS sample preparation could help achieve this goal[8,9].

Conclusion

The performance of the Agilent workflow solution for HCP quantification has been demonstrated. The solution included:

- AssayMAP Bravo platform for automated sample preparation
- 1290 Infinity II LC system for peptide separation
- 6495B Triple quadrupole for data acquisition
- The automation tool in Skyline software for MRM method development
- A combination of Skyline and MassHunter software for data analysis

The following was observed:

- 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 system, the next generation in ultra high performance liquid chromatography, gives more chromatography resolution and higher retention time precision.
- The seamless integration between Skyline software and MassHunter software using the Agilent automation tool allows a straightforward solution for optimizing LC-dMRM methods.
- The accurate quantification of HCP at sub-ppm levels has been demonstrated using the 6495B triple quadrupole LC/MS.
- A combination of Skyline and MassHunter software has provided powerful tools for targeted data analysis.

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