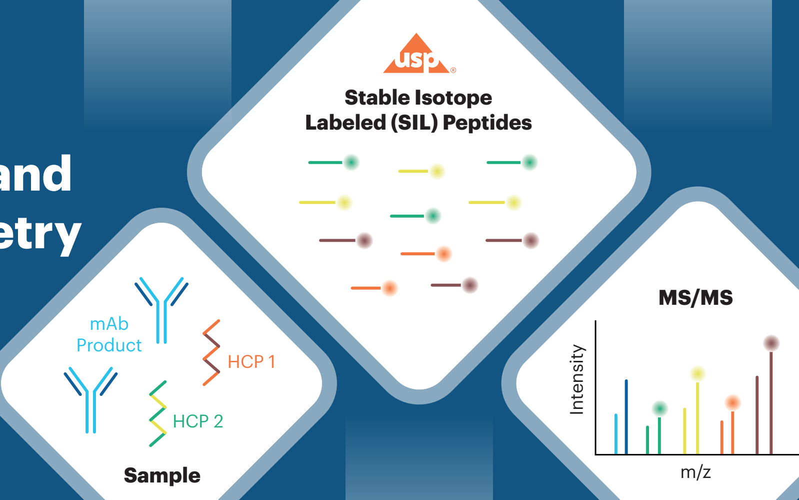
Application Note

Host cell protein identification and quantitation by mass spectrometry enabled by USP analytical reference materials



Residual host cell proteins (HCPs) in biopharmaceuticals are critical considerations for product quality and safety. HCPs can be defined as unwanted proteins that can cause unintended immune responses, alter biologic activity, and/or impact stability of drug products. Traditionally, total HCP content has been assessed using immunoassays, as described in USP General Chapter <1132> Residual Host Cell Protein Measurement in Biopharmaceuticals[1]. Mass spectrometry (MS) has emerged as a powerful orthogonal technique for identification and quantitation of HCPs, because, unlike immunoassays, MS has the advantage of being able to identify and quantify individual HCPs. MS achieves this by examining peptide fragments following the digestion of HCPs. The quantitation of individual problematic HCPs enables a comprehensive risk assessment, supports process optimization, and helps ensure stringent quality control in biotherapeutic production.

In response to stakeholder feedback on the need for best practices for the implementation of MS-based HCP analysis, USP created an Expert Panel of industry representatives and FDA Government Liaisons to develop a General Chapter on HCP analysis by MS, <1132.1> Residual Host Cell Protein Measurements in Biopharmaceuticals by Mass Spectrometry[2].

Quantitation of specific HCPs is a key topic addressed in USP Chapter <1132.1>, with three commonly used approaches discussed in detail:

- relative to product protein – a known amount of product is used to quantitate the amount of HCPs by comparing signals;
- relative to spiked-in proteins – intact protein(s) with known concentration(s) is spiked into samples and used as a standard to quantify the HCP peptides; and
- relative to spiked-in peptides – known peptides with known concentration are spiked into samples and used as calibration standards for HCP measurements.

Quantitation of HCPs is inherently challenging, due to their low levels in in-process samples and final products (typically parts per million (ppm)), as well as the variability in physical properties of each HCP, such as size, post-translational modifications, and ability to bind to other proteins. Quantitative values can routinely vary ±2-fold, which can be attributed to a variety of factors[2]. Furthermore, the lack of standards can further contribute to the variability of HCP quantitation across labs, making comparison of HCP data across organizations difficult. To support more consistent quantitation of HCPs, stakeholders recommended development of a common set of reference materials for problematic HCPs. When using the relative to spiked-in peptides quantification approach, stable isotope labeled (SIL) peptides are often used because they can be added directly to samples without interfering with native peptides. Additionally, they have the same ionization efficiency, fragment behavior, and recovery patterns as native peptides.

Stable-isotope labeled peptides support consistent HCP analysis

To support the consistency of quantitation of problematic HCPs, USP has developed a set of well-characterized analytical reference materials (ARMs) consisting of tryptic SIL peptides corresponding to specific Chinese Hamster Ovary (CHO) HCPs of concern. These peptide reference materials were selected based on detectability in in-process samples, solubility, and stability assessments, and have been tested for purity, concentration, and identity. For specifications on each SIL peptide, please review their respective Product Information Sheet, which can be found on the USP website. By creating a common source of well characterized ARMs, USP is providing the industry an opportunity to standardize HCP quantitation, which can lead to more precise results and easier comparisons across different laboratories.

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Table 1. USP SIL HCP peptides

Peptide1	Description	Catalog number	Concentration	Suggested use
ITGLDPAGPNFEYAEAPSR	LPL SIL peptide 1	1130763	0.59 mg/mL	Confirmation of identity and trending[3]
GLGDVDQLVK	LPL SIL peptide 2	1130774	0.86 mg/mL	Quantitation and identification[2]
LVGNDVAR	LPL SIL peptide 3	1130785	0.75 mg/mL	Confirmation of identity and trending[3]
SLLNSLEEAK	Clusterin SIL peptide 1	1130730	0.84 mg/mL	Confirmation of identity and trending[3]
LTQQYNELLHSLQTK	Clusterin SIL peptide 2	1130741	0.80 mg/mL	Quantitation and identification[2]
LFDSDPITVVLPEEVSK	Clusterin SIL peptide 3	1130752	0.84 mg/mL	Confirmation of identity and trending[3]

[1] SIL (^{13}C and ^{15}N) labeled on R and K
[2] These peptides exhibited the highest recovery and/or lowest level of modifications. Therefore, these peptides are recommended for quantitation and can also be used for identification and trending purposes.
[3] These peptides exhibited lower recovery and/or higher levels of modifications (e.g. deamidation, oxidation, non-specific cleavage), which can complicate quantitation. Therefore, they are recommended for confirmatory purposes to confirm identification and trending.

USP's initial launch of HCP peptides targets two problematic HCPs (**Table 1**), Clusterin and Lipoprotein Lipase (LPL), that have been identified as high-risk CHO HCPs. Clusterin can interact with the Fc and Fab domains, while LPL can degrade key excipients commonly found in drug product formulation buffers[3]. There are three SIL peptides available for each HCP (Clusterin and LPL) that can be used for the identification, quantitation, and trending of their respective protein in in-process samples and final products (**Table 1**). SIL peptides allow for more accurate quantitation by serving as an internal standard that can be directly spiked into samples, thus forgoing the need for external standard curves, which would otherwise be needed if performing quantification with unlabeled peptides. Herein, we will demonstrate how USP SIL peptides can be used in an MS-based procedure to identify and quantify Clusterin and LPL. Future SIL peptide sets will be made available for other problematic CHO HCPs and will be characterized in a similar manner.

Use of SIL peptides in an MRM-based HCP assay

To demonstrate the use of these SIL peptides in the assessment of these target HCPs, we developed an LC-MRM (multiple reaction monitoring) method for each target peptide and used in-process samples and spiked samples to illustrate the use of the SIL peptides. Specificity, linearity (including upper and lower limits of quantitation, ULOQ and LLOQ), range, and accuracy were also assessed. The general principles described in General Chapter <1132.1> were followed in this study. More information on the MRM method development (including MS conditions), sample preparation, and method feasibility testing can be seen in the **Supplemental information**.

All protein samples were digested using a standard trypsin digestion method, which consisted of denaturation, reduction, alkylation, and enzymatic digestion. LC separation was performed on a SCIEX ExionLC AD UHPLC instrument connected to a SCIEX Triple Quad 7500 MS. SCIEX OS software was used for MS data acquisition and processing.

For each SIL peptide and associated unlabeled native peptide, an MRM MS method was developed. To accomplish this, SIL peptides were directly injected into the instrument using syringe infusion. The method was subsequently optimized to find the most abundant fragment ions by determining the optimal parameters, such as voltage, collision energy, dwell times, etc. Since native peptides have the same fragmentation behavior as SIL peptides, the transitions of the native peptides were calculated using software with a fragment ion calculator and confirmed using upstream in-process samples. The MRM transition parameters are shown in **Table 2**.

The SIL peptides were used to quantify Clusterin and LPL in a variety of in-process samples from a standard mAb production, including a host cell culture fluid (HCCF) pool, Protein A pool, and ion exchange (IEX) Pool. All in-process samples were prepared using sample preparation techniques described in USP Chapter <1132.1> and quantified against the spiked-in SIL peptides (**Supplemental information**). Results for this experiment, which successfully showed clearance of HCPs in downstream mAb processes, can be seen in **Figure 1**. MRM based quantitation of in-process samples demonstrated clearance of Clusterin and LPL throughout the purification process. The Protein A purification step yielded more than a 23-fold clearance for Clusterin and a 100-fold clearance for LPL, while the IEX purification step yielded an additional 31-fold and 7-fold clearance as compared to Protein A for LPL and Clusterin, respectively. Based on these data, as well as accuracy assessment during method feasibility, we suggest using LPL Peptide 2 and Clusterin Peptide 2 for quantification. The additional SIL peptides did

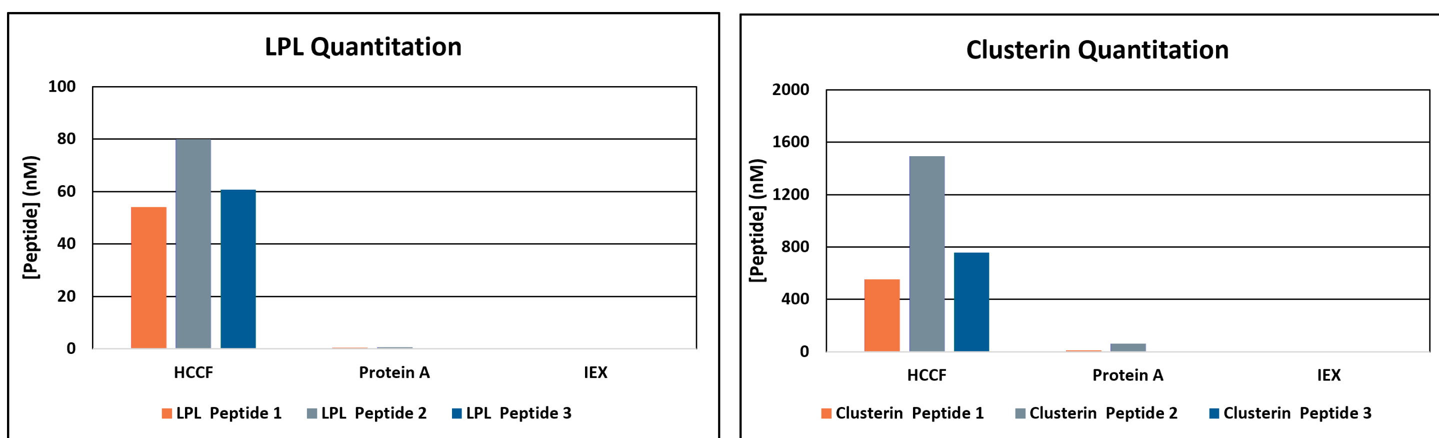

Figure 1. Quantitation of LPL (left) and Clusterin (right) in various in-process samples.

Table 2. MRM transitions

Description	Peptide	Precursor Ion (m/z)	Product Ion (m/z)	Retention time (min)
LPL Peptide 1	ITGLDPAGPNFEYAEAPSR[1]	1008.0	1290.6[2]	19.15
	ITGLDPAGPNFEYAEAPSR	1003.0	1280.6[2]	19.15
LPL Peptide 2	GLGDVDQLVK[1]	526.3	881.5[2]	17.45
	GLGDVDQLVK	522.3	873.5[2]	17.45
LPL Peptide 3	LVGNDVAR[1]	427.2	641.3[2]	7.30
	LVGNDVAR	422.2	631.3[2]	7.30
Clusterin Peptide 1	SLLNSLEEAK [1]	556.3	911.5[2]	18.68
	SLLNSLEEAK	552.3	903.5[2]	18.68
Clusterin Peptide 2	LTQQYNELLHSLQTK[1]	608.7	677.4[2]	19.29
	LTQQYNELLHSLQTK	606.0	673.4[2]	19.29
Clusterin Peptide 3	LFDSDPITVVLPEEVSK[1]	948.5	1318.8[2]	24.94
	LFDSDPITVVLPEEVSK	944.5	1310.8[2]	24.94

[1] SIL (^{13}C and ^{15}N) labeled on R and K;
[2] transition used as quantifier

also show similar behavioral trends across in-process steps, further confirming clearance trends. These data suggest that USP SIL peptides can be used for accurate identification and quantification of troublesome HCPs.

Conclusion

Based on the need for qualified standards to streamline HCP MS measurements, USP is expanding its portfolio of tools to support HCP quantitation. In addition to the General Chapters <1132> and <1132.1>, as well as these six SIL peptides, USP offers the CHO PLBL2 ARM (USP Catalog # 1582716), which can be used for the identification of CHO PLBL2, which is another problematic HCP. USP now offers a CHO Null Cell Harvest Cell Culture Fluid (HCCF) ARM (USP Catalog # 1544913) that can aid in mAb titer determination, HCP LC-MS analysis setup, and assess immunoassay coverage. Furthermore, USP is developing additional sets of SIL peptides for other high risk CHO HCPs. For each subsequent set of SIL peptides, USP will provide additional resources to aid users with incorporating each SIL peptides into their LC-MS/MS workflow. Future USP work will continue to focus on developing physical standards that can help improve the accuracy and consistency of HCP quantitation. These products can be used to identify and monitor HCPs in various workflows, as has been demonstrated in this study.

References

1. USP General Chapter *<1132> Residual Host Cell Protein Measurement in Biopharmaceuticals*
2. USP General Chapter *<1132.1> Residual Host Cell Protein Measurement in Biopharmaceuticals by Mass Spectrometry*
3. M. Jones et.al. "High-risk" host cell proteins (HCPs): A multi-company collaborative view, Biotechnol. Bioeng. 2021;118:2870–2885

More information: https://www.usp.org/biologics/host-cell-proteins
Questions: uspbiologics@usp.org
Ordering information: store.usp.org

Supplemental information

HCP quantitation method

Samples were prepared by combining 20 µL of protein (~100 µg) with 141 µL of triethylammonium bicarbonate (100 mM). Samples were then digested using a standard trypsin digestion method, which involved denaturation, reduction, alkylation, and enzymatic digestion, described below:

- Denaturation and reduction were achieved by adding 5 µL of TCEP-HCl (100 mM) and incubating for 1 hour at 60°C. After 1 hour incubation, 10 µL of iodoacetamide (150 mM) was added and the mixture was stored in the dark for 30 minutes for alkylation.
- Digestion was performed by adding 10 µL of trypsin (0.2 µg/µL) and incubating at 37°C overnight, and subsequently quenched using 4 µL of 20% formic acid.
- A Sep-Pak C18 cartridge was used for desalting before injection into the LC-MS. The final reconstitution buffer was 250 µL of 5% acetonitrile/2% formic acid.
- LC separation was performed on a SCIEX ExionLC AD UHPLC instrument connected to a SCIEX Triple Quad 7500 MS. SCIEX OS software was used for MS data acquisition and processing. A summary of the LC and MS method parameters can be seen in **Table S1**.

Ten (10) µL of SIL peptides (20 nM) was added following digestion. For each SIL peptide and associated unlabeled peptide, an MRM MS method was developed. The MRM parameters can be seen in **Table S2**. A sample chromatogram for each SIL peptide can be seen in **Figure S1**.

To properly assess the suitability of the USP peptides for targeted HCP quantitation, we first examined the feasibility of using the LC-MRM method for peptide quantitation and evaluated the specificity, precision, linearity, range, and accuracy of the method with the peptides. The results of these experiments revealed that this method is indeed suitable for its intended use of quantify Clusterin and LPL by the relative to spiked-in peptides approach using USP SIL peptides.

Table S1. Method parameters

LC Parameter	LC condition
Column	Waters ACQUITY UPLC HSS T3 column (150 x 2.1 mm, 1.8 µm)
Column temperature	40°C
Flow rate	0.2 mL/min
Mobile phase A	0.1% Formic acid in water
Mobile phase B	0.1% Formic acid in acetonitrile
Gradient	0 min: 92% A 3 min: 92% A 23 min: 70% A 30 min: 20% A 35 min: 20% A 36 min: 92% A 40 min: 92% A
Injection volume	20 µL
Autosampler temperature	4°C

MS parameter	MS condition
Ionization mode	Electrospray ionization, positive mode
Spray voltage	5500 V
Source temperature	450°C
Curtain gas	40 psi
Ion source gas 1	60 psi
Ion source gas 2	55 psi
CAD gas	9 units
Detector	Triple quadrupole mass spectrometer

Specificity

To assess the specificity of the method with the SIL peptides, the following samples were analyzed: Diluent (8% acetonitrile in water), 1 nM SIL peptides in diluent, and samples (USP mAb003, CHO HCCF, CHO Protein A Pool, CHO IEX Pool) without spiked-in SIL peptides. In specificity samples lacking spiked-in SIL peptides, no SIL peptide peaks were observed at the expected retention time using the MRM method, while each SIL peptide peak was observed in samples containing spiked-in peptides. An example of overlaid chromatograms (for LPL SIL Peptide 2) can be seen in **Figure S2**. Furthermore, unlabeled peptides were not detected in the 1 nM SIL peptides solution, suggesting that no detectable contamination of unlabeled peptides is present in USP's SIL peptides.

Range and linearity

The range and linearity were analyzed for each SIL peptide by preparing 11 levels of peptide solution at different concentrations ranging from 0.003 nM – 150 nM per peptide. The LLOQ was defined as the lowest concentration with

Table S2. MRM Transition Table

Description	Peptide	Precursor Ion (m/z)[4]	Collision energy (V)	Product Ion (m/z)	Retention time (min)	Dwell time (ms)
LPL peptide 1	ITGLDPAGPNFEYAEAPSR[1]	1008.0	54	1290.6[2]	19.15	47
			54	1347.6[3]		
			54	1418.6[3]		
	ITGLDPAGPNFEYAEAPSR	1003.0	54	1280.6[2]	19.15	47
			54	1337.6[3]		
			54	1408.6[3]		
LPL peptide 2	GLGDVDQLVK[1]	526.3	23	881.5[2]	17.45	103
			24	709.4[3]		
			23	610.4[3]		
	GLGDVDQLVK	522.3	23	873.5[2]	17.45	103
			24	701.4[3]		
			23	602.4[3]		
LPL peptide 3	LVGNDVAR[1]	427.2	22	641.3[2]	7.30	135
			19	740.4[3]		
			34	355.2[3]		
	LVGNDVAR	422.2	22	631.3[2]	7.30	135
			19	730.4[3]		
			34	345.2[3]		
Clusterin peptide 1	SLLNSLEEAK [1]	556.3	24	911.5[2]	18.68	47
			25	798.4[3]		
			26	684.4[3]		
	SLLNSLEEAK	552.3	24	903.5[2]	18.68	47
			25	790.4[3]		
			26	676.4[3]		
Clusterin peptide 2	LTQQYNELLHSLQTK[1]	608.7	29	677.4[2]	19.29	53
			39	584.4[3]		
			35	721.4[3]		
	LTQQYNELLHSLQTK	606.0	29	673.4[2]	19.29	53
			39	576.4[3]		
			35	713.4[3]		
Clusterin peptide 3	LFDSDPITVVLPEEVSK[1]	948.5	46	1318.8[2]	24.94	135
			41	809.5[3]		
			43	696.4[3]		
	LFDSDPITVVLPEEVSK	944.5	46	1310.8[2]	24.94	135
			41	801.4[3]		
			43	688.4[3]		

[1] SIL (^{13}C and ^{15}N) labeled on R and K;
[2] transition used as quantifier;
[3] transitions used as qualifiers for additional confirmation.
[4] An Entrance Potential of 10 V was applied for each peptide.

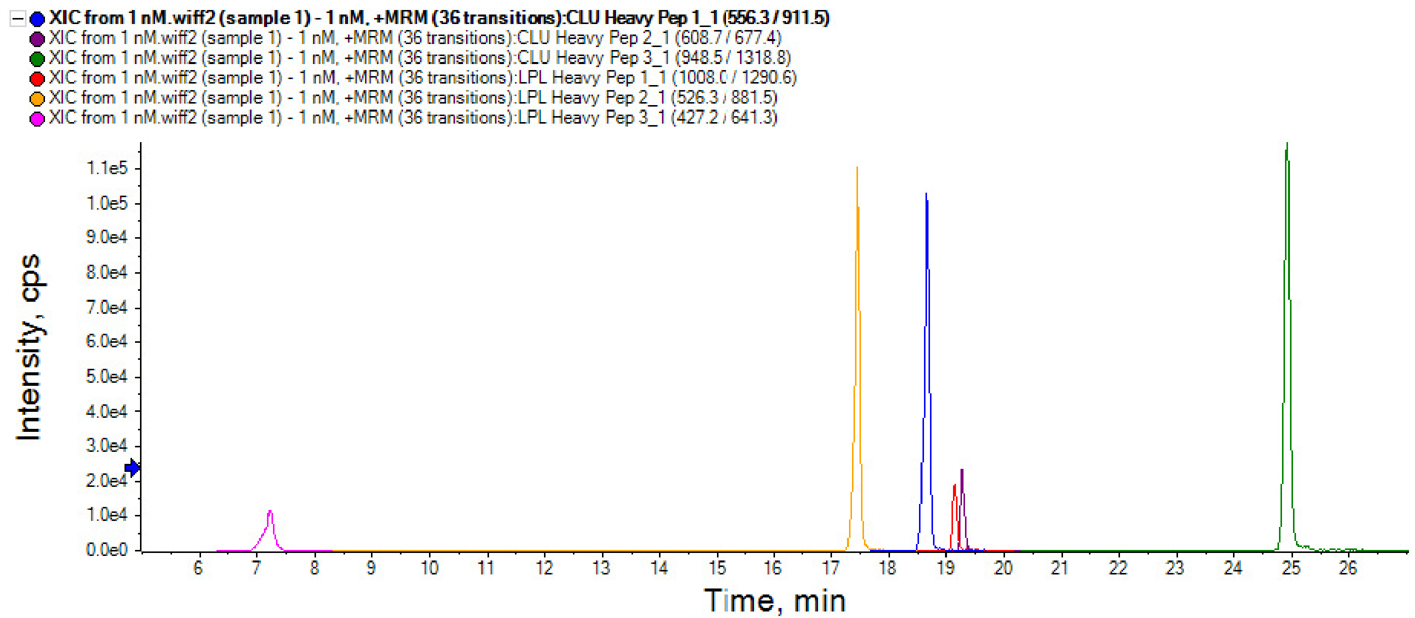

Figure S1. Representative chromatogram with the following SIL peptides (1 nM): Clusterin SIL peptide 1 (blue), Clusterin SIL peptide 2 (purple), Clusterin SIL peptide 3 (green), LPL SIL peptide 1 (red), LPL SIL peptide 2 (yellow), and LPL SIL peptide 3 (pink).

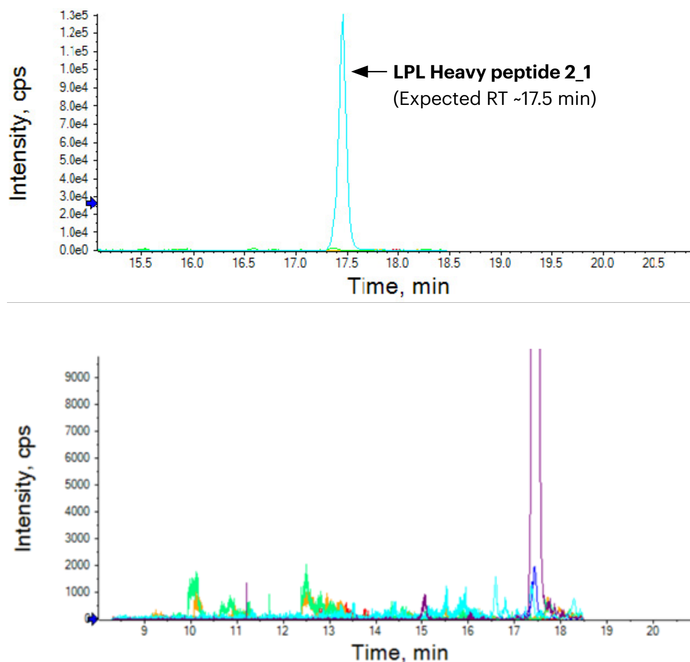

Figure S2. MRM chromatogram for the following samples: 1 nM SIL peptides (blue), USP mAb 003 (pink), and HCCF (green). Full chromatogram shown on top, while bottom displays zoomed in baseline.

signal-to-noise ratio (s/n) ≥10. The linearity was determined as the concentration range of each peptide with a linear fit having an $R^2 \geq 0.99$; the range was determined as a minimum of 5 consecutive concentration levels with a recovery of 80 – 120% of the nominal concentration within a linear range. The LLOQ and ULOQ were defined as the lowest and highest concentrations, respectively, that met both range and linearity acceptance criteria, starting from the LLOQ level. The range of each SIL peptide with the tested method and a representative linearity plot for select peptides can be seen in **Table S3** and **Figure S3**. Note that for the peptides with ULOQ at 150 nm, the actual ULOQ may be higher but was not tested in this work.

The linearity of each SIL peptide with this method was determined experimentally by preparing solutions ranging from 0.003 nM – 150 nM and calculating the linear range of the method for each individual SIL peptide. All concentration levels were prepared from a 5 µM SIL peptide stock mix, which was prepared by mixing the appropriate amount of each peptide into a solution with 8% acetonitrile in water. Eleven levels were assessed for linearity at concentrations from 0.003 nM – 150 nM SIL peptides

To assess the accuracy of the method in relation to known amount of peptide, the recovery at each linearity level was calculated for its associated SIL peptide. For analysis of these data, calibration curves were generated based on the linear regression of peak areas against the nominal SIL peptide concentration.

Table S3. Range and linearity data

SIL peptide	LLOQ* (nM)	ULOQ* (nM)	R^2
ITGLDPAGPNFEYAEAPSR	0.01	50	1.00
GLGDVDQLVK	0.03	50	1.00
LVGNDVAR	0.01	150	1.00
SLLNSLEEAK	0.01	50	1.00
LTQQYNELLHSLQTK	0.03	150	1.00
LFDSDPITVVLPEEVSK	0.003	150	1.00

*These values may vary depending on method and instrumentation used for testing

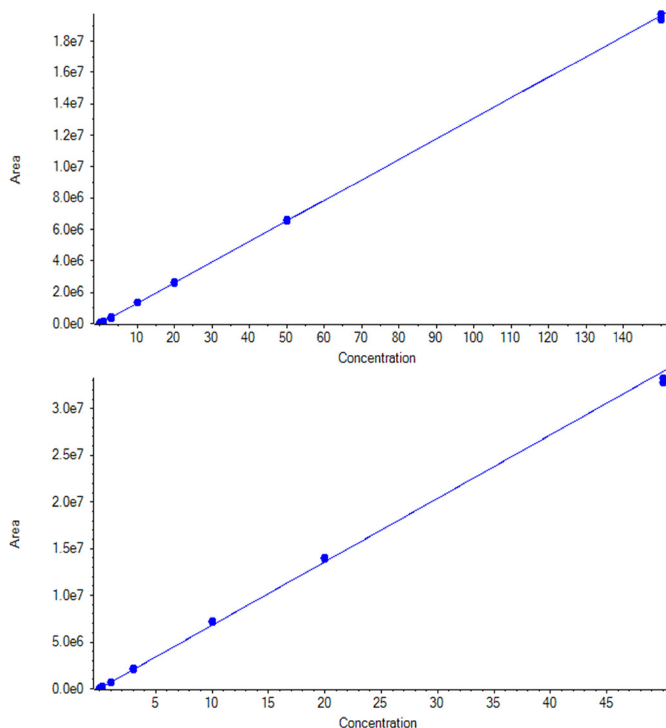

Figure S3. Linearity plot for Clusterin SIL peptide 2 (top) and LPL SIL peptide 2 (bottom).

Peptide recovery

After the calculated concentration for each SIL peptide was determined from the linearity equation, the accuracy was determined using Equation 1 and the results can be seen in **Table S4**. Note that the N/A in the table represents concentrations that were shown to be outside of the linear range, thus not used in the calculation.

Equation 1.

$$\%\ Accuracy = \frac{Calculated\ Concentration}{Nominal\ Concentration} \times 100$$

Accuracy of method

The accuracy was assessed in relation to a known amount of HCP (Clusterin). This was accomplished by using the SIL peptides to quantify known amount of spiked-in recombinant Clusterin. To prepare these samples, known amounts of Clusterin (4.0 nM and 40.0 nM) and SIL peptides (1 nM/peptide) were added to USP mAb 003, which was previously shown to contain no detectable Clusterin. A single point calibration was used to analyze these data (Equation 2). Note, this equation was also used to calculate concentration of in-process samples in main text.

Although the recovery of Clusterin is within the acceptable range (±2-fold of nominal), it is evident that the recoveries of Clusterin Peptides 1 and 3 are lower than Peptide 2 in both samples (**Table S5**). Interestingly, this aligns with internal DIA (data independent acquisition) data that revealed high levels of post-translational modifications (PTMs) on Peptides 1 and 3 of Clusterin expressed in CHO (data not shown). As a result of these PTMs, a portion of peptides would not be identified nor quantifiable using the MRM method, which is highly specific to unmodified peptides. Our data suggest that Peptides 1 and 3 are best suited for use in identification and Peptide 2 for quantitation. Note, this may vary depending on

Table S4. Peptide accuracy

Concentration (nM)	Clusterin SIL peptide 1	Clusterin SIL peptide 2	Clusterin SIL peptide 3	LPL SIL peptide 1	LPL SIL peptide 2	LPL SIL peptide 3
0.003	N/A	N/A	106.3	N/A	N/A	N/A
0.01	88.6	N/A	101.3	106.4	N/A	109.0
0.03	95.4	115.3	97.4	115.6	90.1	96.6
0.1	99.9	97.2	94.6	99.7	94.3	98.7
0.3	105.5	90.3	97.7	91.9	99.7	96.3
1	101.9	96.2	99.8	96.7	104.6	98.2
3	102.9	95.5	99.0	93.4	105.1	100.0

Equation 2. $$[HCP](nM) = \frac{Peak\ Area^{Target\ Peptide}}{Peak\ Area^{SIL\ Peptide}} \times [SIL\ Peptide](nM) \times Dilution\ Factor$$

Table S5. Clusterin recovery accuracy data

Clusterin SIL peptide	Nominal Clusterin concentration	Experimental concentration	% Recovery
SLLNSLEEAK	4 nM	2.2 nM	54.4
LTQQYNELLHSLQTK		4.7 nM	118.1
LFDSDPITVVLPEEVSK		3.0 nM	74.0
SLLNSLEEAK	40 nM	20.7 nM	51.7
LTQQYNELLHSLQTK		41.3 nM	103.2
LFDSDPITVVLPEEVSK		30.8 nM	77.0

manufacturing conditions and a thorough understanding of each HCP is crucial to increase accuracy.

Based on these experiments, we were able to demonstrate that these peptides are fit for their intended use when using the method described above. However, since the performance of these peptides is dependent on the method and instrumentation being used, the end user should perform their own assessment.