Using LC-MS for Efficient HCP Clearance

Mass spectrometry for host cell protein removal.

Host cell proteins (HCPs) are the most critical process-related impurities in biologics. Due to their potential risk on product safety, efficacy, and quality, HCPs "should be reduced to low ppm levels" (1). HCP reduction is achieved through various purification steps designed to generate the highest possible yield of target protein with the lowest possible levels of HCPs.

Thus, reproducible and reliable HCP information is essential in the development of efficient protein purification processes (2).

Limitations of HCP-ELISA assays

Classical HCP-ELISA provides an estimation of total HCP amount; however, complex purification buffers and the suitability of the antibody reagents are critical parameters in the estimation. Since ELISA only provides limited information about the HCP content after each process step (a simple number), it provides poor guidance for how to improve the process. This results in time-consuming purification optimization and posible delays in clinical development. Therefore, a better tool could reduce development cost and time as well as ensure manufacturing consistency.

TOOL FOR EFFICIENT HCP CLEARANCE

Health authorities increasingly request mass spectrometry (MS) for HCP analysis as an orthogonal method to ELISA. For the past five years, Alphalyse has developed and executed a growing number of HCP analysis projects based on SWATH® LC-MS. These analyses provide information for protein purification and process development of biologics.

SWATH® LC-MS is a data-independent acquisition method that is especially suited for HCP analysis of in-process samples:

- Its large dynamic range **detects low-abundance HCPs** in large amounts of drug substance.
- It is **highly reproducible** and provides robust identification and quantification over time.
- It is **suitable for complex samples in different buffers** to compare process steps and batches.
- It is **generic** and rapidly adjusted to new bioprocesses.

EXAMPLE OF DATA FROM SWATH® LC-MS BASED HCP ASSAY

The SWATH® LC-MS method was used for the analysis of in-process samples of a drug substance expressed in *E. coli* inclusion bodies. The method is also useful for other expression systems such as CHO, yeast, and insect cells as well as human cell lines. The analysis was used for purification optimization and batch comparison, and for PPQ validation of the process's robustness and reproducibility.

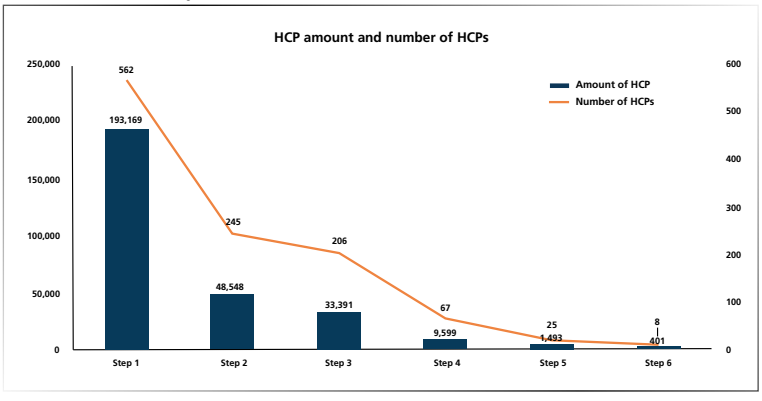
Analysis of purification process samples

The drug substance was purified through a six-step process. By SWATH® LC-MS, the HCPs were identified and quantified in each process sample. Thus, the efficiency of each step and total clearance of HCPs were evaluated through the process (**Figure 1**).

LC–MS for efficient and targeted purification

Examples of the MW and pI of the 10 most abundant individual HCPs are listed in **Table I.** This information makes it easier to optimize a separation step, targeted at specific HCPs. Interestingly, all remaining HCPs after step six were small (<40 kDa) and acidic. The HCP details can be used to adjust individual process steps such as changing buffer pH or using

Figure 1. Host cell protein (HCP) amount (ppm) and number of identified HCPs measured by SWATH® LC-MS.



a different cutoff during size exclusion.

List of identified HCPs with name, mass, and pI

The most abundant HCPs are shown in **Table I** and the change in quantity of individual HCPs through the six steps is shown. The data includes the unique protein name, mass, and pI. This enables risk assessment of individual HCPs and targeting of specific physiochemical properties of the remaining HCPs.

The SWATH® LC-MS method is highly robust to different sample matrices and can be set up for a new biologic within weeks. Subsequent process samples can be analyzed with the same method on an ongoing basis for further process development, process upscaling, or process transfer to a new CMO.

METHOD CHARACTERISTICS

The analysis is divided into three steps: sample preparation, MS analysis, and data analysis. Samples are prepared by adding internal protein standards, acetone precipitation, and trypsin digestion. Peptides are cleaned in SPE 96-well plates.

The MS analysis is performed on a SCIEX TripleTOF® 6600 system. Samples are analyzed in technical triplicates in data-dependent mode to generate peptide ion libraries and in data-independent mode for quantification. Finally, the obtained data is interpreted using ProteinPilot® and the SWATH microapp® for identification and label-free quantification of individual HCPs.

Table 1. Top 10 identified host cell protein (HCPs) in six purification steps, including name, amount, mass and pI of the individual HCP (Protein Accession numbers are also available).

	Purification steps							
Protein name	**Step 1**	**Step 2**	**Step 3**	**Step 4**	**Step 5**	**Step 6**	**Mass**	**pI**
Small heat shock protein IbpB	4.274	2.905	2.154	186	229	111	16.093	5,2
Ferric uptake regulation protein	158	284	296	142	147	94	16.795	5,7
Cysteine synthase A	597	913	711	618	200	68	34.490	5,8
PTS system glucose-specific EIIA component	33	250	378	256	185	62	18.251	4,7
UPF0250 protein YbeD	432	215	253	222	112	21	9.827	5,5
Outer membrane protein TolC	41	283	187	417	57	11	53.741	5,2
4-hydroxy-3-methylbut-2-enyl diphosphate reductase	312	1.146	855	231	62	15	34.775	5,2
Protein YihD	33	28	32	11	10	18	10.273	5,1
Nucleoside diphosphate kinase	106	240	100	349	113		15.463	5,6
Alkyl hydroperoxide reductase subunit F	67	291	171	174	48		56.177	5,5
Number of HCPs	562	245	206	67	25	8		
Total HCP content ppm (w/w)	193.169	48.548	33.391	9.599	1.493	401		
HCP cont % (w/w)	**19,32%**	**4,85%**	**3,34%**	**0,96%**	**0,15%**	**0,04%**		

Data-independent acquisition

The MS-based HCP analysis is favorable in several ways: The high sensitivity enables detection of HCP at low ppms. Further, the method is robust with no carry-over problem and the data independent acquisition method (SWATH®) ensures reproducibility. Finally, the use of multiple, intact internal standard proteins and label-free quantification leads to absolute quantification of individual HCPs.

Label-free quantification

For HCP quantification, intact standard proteins are added prior to precipitation and digestion (i.e., undergoing the same processing as the HCPs). Dilution series of all standard proteins display a linear quantification.

The label free quantification by MS/MS signals are less likely to promote signal saturation compared with their MS precursors. Through generation of an ion library, peptides are assigned to their corresponding proteins.

Quantification of each identified protein is obtained by summing the MS/MS intensity of all peptides in the specific protein (SumAll quantification). ■

REFERENCES

1. *USP* General Chapter <1132>, "Residual Host Cell Protein Measurement in Biopharmaceuticals," *USP 39-NF 34* (Rockville, MD, 2016), 1416–1436.
2. D.G. Bracewell, et al., *Biotechnol. Bioeng.*, 112, (9), 1727–1737 (2015).

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