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Detailed Host Cell Protein analysis of downstream process samples by Mass Spectrometry

Ejvind Mørtz[1],*, Katrine Pilely[1], Rikke Raaen Lund[1], Thomas Kofoed[1], Thomas K. Jorgensen[2] and Lars Skriver[2]
[1]Alphalyse A/S, Odense, Denmark, [2]Savara Aps, Hørsholm, Denmark

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Introduction

A detailed HCP analysis of in-process samples for manufacturing process development is somewhat difficult by ELISA and 2D-PAGE techniques. The samples contain many HCPs that may not be detected by ELISA or 2D-PAGE and with complex matrices interfering with the analysis.

However, Mass Spectrometry (LC-MS) is well suited for analysing complex in-process samples, identifying and quantifying individual host cell protein, and quantifying the total HCP content. The robust and reproducible method enables optimization of purification parameters and comparability between batch runs. Furthermore, the HCP analysis method is generic and can be applied to other biopharmaceutical processes within weeks.

LC-MS method for HCP analysis

The Alphalyse method is based on:

1. Generic sample preparation and digestion protocol applicable for process samples in different matrices.
2. Fast and robust microflow HPLC method.
3. Data-independent MS acquisition.
4. Data analysis workflow for confident HCP identification and label-free quantification by spike-in of protein standards.

In the following study, we applied the method to in-process samples of a drug substance expressed in *E. coli* inclusion bodies. However, it can also be used for other expression systems, such as CHO cells, *Lactococcus lactis*, human carcinoma cells (A549), etc.

Advantages of mass spectrometry HCP analysis

‘Total and individual HCP quantitation for each process step’
‘Identification incl. physiochemical properties of each HCP’
‘Detailed evaluation of HCP clearance efficiency and risk assesment’
‘Full assay development in 4-8 weeks’

2. The bioprocess case

Downstream process

- A 15 kDa protein drug substance expressed in *E. coli* and recovered in inclusion bodies.
- The downstream process includes renaturation, filtration, and chromatographic purification steps.
- A generic ELISA-HCP assay showed HCP levels of a few ppm.
- It was challenging to develop a process-specific ELISA as the inclusion bodies contained drug substance.

Manufacturing objectives

- Transfer of manufacturing process to new CMO and show comparability of the old and new process.
- Process optimization of purification steps.
- Develop a relevant, sensitive, specific HCP analysis to cover the safety aspects according to ICH guidelines.

HCP analysis workflow

Sample preparation & LC-MS
In-process samples were acetone precipitated, reduced/alkylated, and digested with trypsin. The peptides were cleaned up and concentrated on SPE 96-well plates, followed by chromatography on an Eksigent LC system with a Waters CSH column 150x0.3 mm at 5 ul/min, 80 min gradient.

MS data were acquired on a Sciex 6600 TripleTof LC-MS instrument, first in the information-dependent mode for generation of peptide (HCP) ion-libraries, then in data-independent SWATH™ mode for quantitative analysis.

HCPs were identified by searching the information-dependent mode MS data against the UniProt E. coli protein database using ProteinPilot. Quantification was performed by summarizing the peak areas of each peptide's six most intense fragment ions and all the peptide areas for each protein (SumAll quantification).

The quantitative calculations were performed in PeakView software, SWATH™ micro App, and Excel.

Label-free quantification

Quantification was performed by spike-in of five protein standards in known amounts. The individual response curve for each protein standard shows high linearity.

Figure 1 on page 4 shows that each standard protein has its own unique MS signal response

factor; this is also true for individual HCP. For label-free HCP quantification, the median response factor of the five protein standards.

This SumAll quantification method provides the HCP amount in ppm (ng HCP/mg drug substance) of HCPs >LLOQ (50ppm).

Dilutional linearity of each HCP

Dilutional linearity for individual HCPs was investigated by running five dilutions of the drug sample. The individual HCPs showed quantitative linearity from 6500 ppm to approximately 50 ppm (Figure 2, page 5).

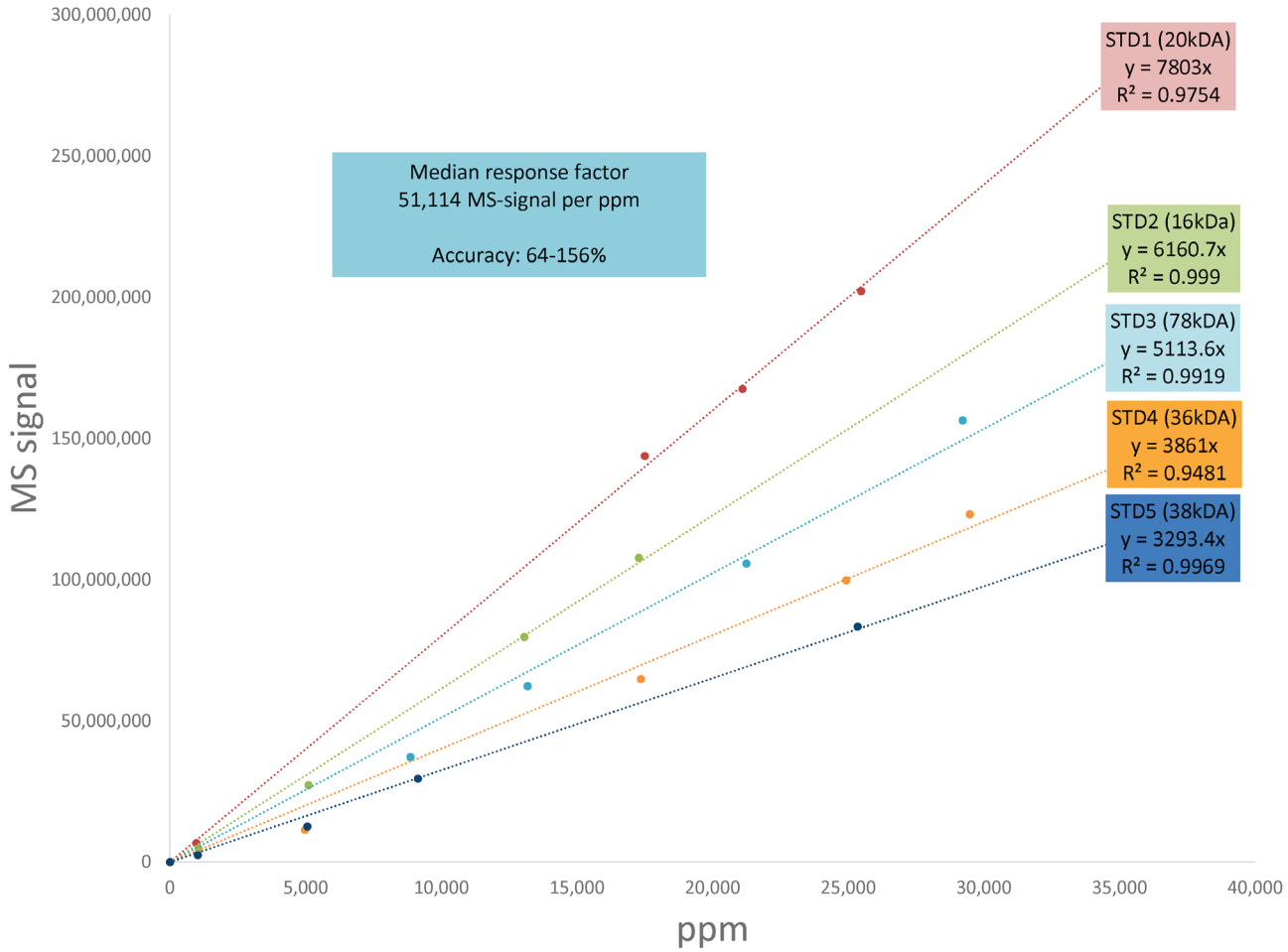

Figure 1: Five standard proteins each spiked in at four different concentrations. SumAll MS signal versus ppm of five spiked-in protein standards. Linearity of the five proteins of different molecular weights is observed. The median response factor is used for SumAll label free quantification of HCPs.

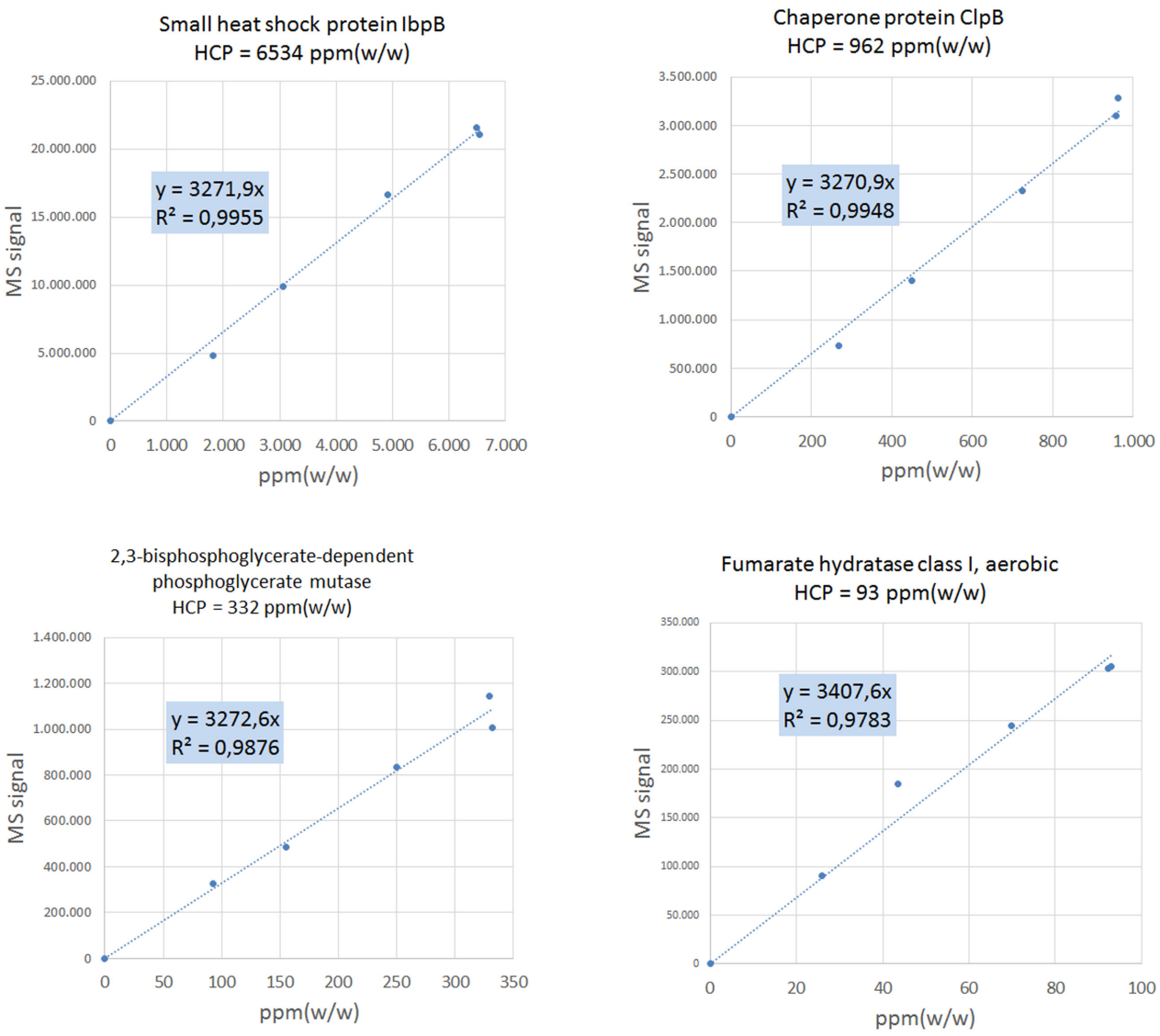

Figure 2: Dilutional linearity on four HCPs between 6500 ppm and 90 ppm

High reproducibility

A high method reproducibility of technical replicates of an early process sample was observed (Figure 3, page 6).

HCPs in bioprocess samples

HCPs were analyzed after each of the six purification steps in downstream processing. The analysis shows clearance of the majority of the HCP species. Of 560 HCPs identified in the harvest sample, only 8 HCPs remain after step 6 (Table 1, page 6).

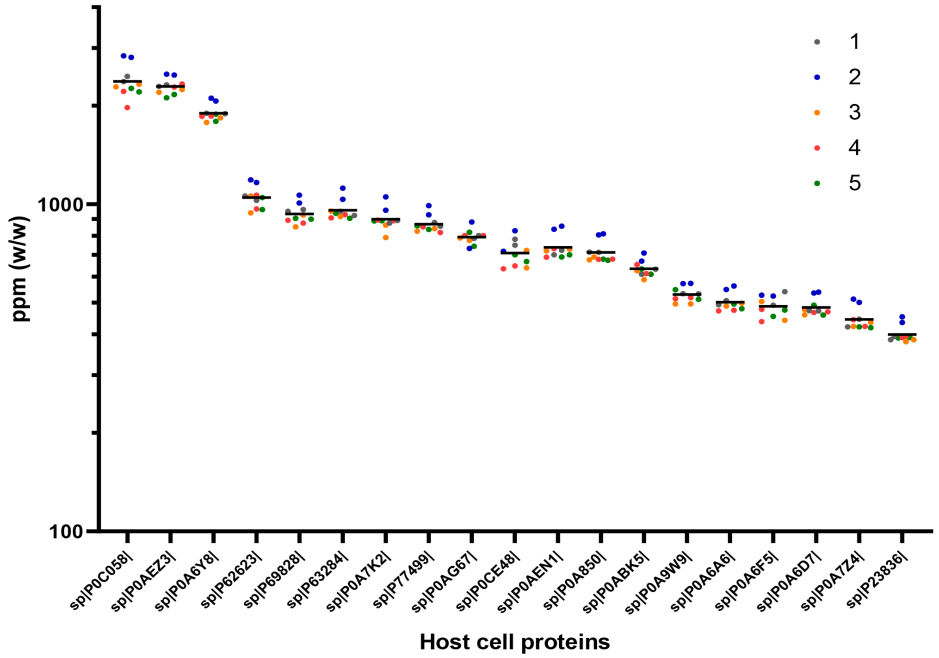

Figure 3: Reproducibility of absolute quantification of HCPs. An early process sample was spiked with five different standard protein mixtures, prepared in parallel and analyzed in duplicate runs. Calculated ppm values of the most abundant 20 HCPs are shown.

Protein Purification Step	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6
LC-MS Number of HCPs	562	245	206	67	25	8
Number of HCPs > 50ppm	380	142	86	34	8	4
Amount of HCP in % (w/w)	18.8%	4.6%	3.1%	0.9%	0.1%	0.03%
Amount of HCP in ppm (w/w)	188,441	45,813	30,573	8,819	1,105	335
ELISA generic kit Amount of HCP in ppm (w/w)	4,979	920	not analyzed	33	4	16

Table 1 HCP content after each of the six purification steps. Measured by LC-MS and by generic ELISA method.

Comparison of LC-MS and ELISA

HCP amounts were analyzed by both a generic ELISA and by LC-MS. The HCP removal was followed in six purification steps. The two orthogonal methods show comparable removal efficiency for total HCP content, approximately 310 fold for ELISA and 560 fold for MS data (Table 1 on page 6). The reduction factors for the individual eight proteins in the final DS are shown in Figure 4 and Table 2 below.

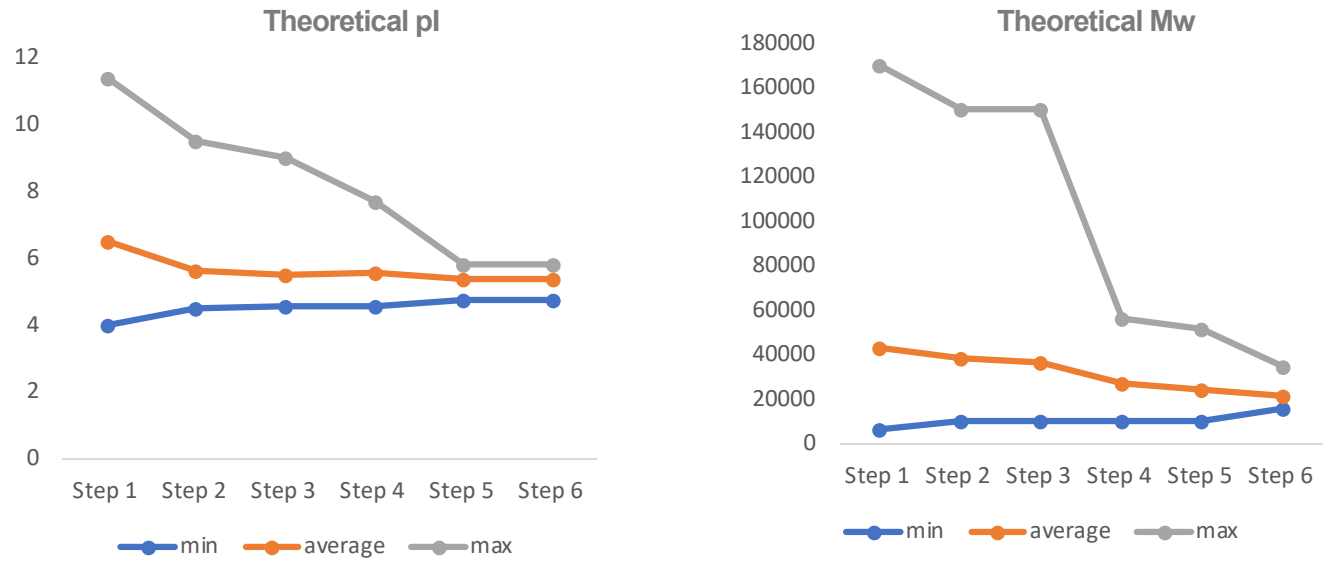

Figure 4: theoretical pI and mw of the HCPs present at EACH step

Uniprot ID	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	pI	Mass (Da)	Protein name
P0C058	4,274	2,905	2,154	186	229	111	5.19	16,093	Small heat shock protein IbpB
P0A9A9	158	284	296	142	147	94	5.68	16,795	Ferric uptake regulation protein
P0ABK5	597	913	711	618	200	68	5.83	34,490	Cysteine synthase A
P69783	33	250	378	256	185	62	4.73	18,251	PTS system glucose-specific EIIA component
P0A8J4	432	215	253	222	112	21	5.50	9,827	UPF0250 protein YbeD
P0ADP9	33	28	32	11	10	18	5.12	10,273	Protein YihD
P62623	312	1,146	855	231	62	15	5.18	34,775	Diphosphate reductase
P02930	41	283	187	417	57	11	5.23	53,741	Outer membrane protein TolC
P0A763	106	240	100	349	113		5.55	15,463	Nucleoside diphosphate kinase
P35340	67	291	171	174	48		5.47	56,177	Alkyl hydroperoxide reductase subunit F
P0A7I7	345	297	106	48	45		5.60	21,836	GTP cyclohydrolase-2
P08200	390	271	166	355	42		5.15	45,757	Isocitrate dehydrogenase [NADP]
P0AEN1	741	870	849	1,404	33		5.29	26,242	NAD(P)H-flavin reductase
P69797	284	339	240	25	26		5.74	35,048	PTS system EIIAB component
P0A6K3	129	136	55	61	26		5.23	19,328	Peptide deformylase
P0AB91	56	231	188	42	22		6.14	38,010	Deoxyheptonate aldolase, Phe-sensitive
P0A825	128	332	204	263	13		6.03	45,317	Serine hydroxymethyltransferase
P0AEZ3	1,217	2,146	809	244	12		5.25	29,614	Septum site-determining protein MinD
P0A9W9	195	664	882	17	10		5.26	20,245	Protein YrdA
P36683	129	87	82	9	8		5.24	93,498	Aconitate hydratase B
	188,441	45,813	30,573	8,819	1,105	335	Total HCP content ppm(w/w)		
	18.8%	4.6%	3.1%	0.9%	0.1%	0.03%	Total HCP content % (w/w)		
	562	245	206	67	25	8	Number of HCPs		
	380	142	86	34	8	4	Number of HCPs >50ppm		

Table 2: HCP removal during purification process of drug substance. Most abundant HCPs, after step 5 and 6, are shown.

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3. Conclusion

The Alphalyse LC-MS method provides robust and reproducible HCP analysis with these characteristics:

- Generic sample preparation protocol for in-process samples of varying complexity.
- Fast and reproducible 1-dimensional microflow LC for high throughput of in-process samples.
- Reproducible label-free absolute quantification method based on SumAll peak areas.

This Alphalyse LC-MS analysis provides:

- Total HCP quantitation for each process step in ppm (ng HCP/mg drug substance).
- Identification and quantitation of each HCP.
- Detailed evaluation of each process step for HCP clearance efficiency.
- HCP clearance efficiency allows for process optimization and risk assessment of well-defined HCP in drug substance batches.

More info available on our website
alphalyse.com/downstreamHCP