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Research paper

SP3-based host cell protein monitoring in AAV-based gene therapy products using LC-MS/MS

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A B S T R A C T

Residual host cell proteins (HCPs) represent a critical quality attribute of biotherapeutic drug products. Workflows enabling reliable HCP detection in monoclonal antibodies and recombinant proteins have been developed, which facilitated process optimization to improve product stability and safety, and allowed setting of acceptance limits for HCP content. However, the detection of HCPs in gene therapy products such as adeno-associated viral (AAV) vectors has been limited. Here, the use of SP3 sample preparation followed by liquid chromatography-mass spectrometry (LC-MS) analysis for HCP profiling in various AAV samples is reported. Suitability of the workflow is demonstrated and provided data constitutes an important reference for future work aiming towards a knowledge-driven improvement of manufacturing conditions and characterization of AAV vector products.

1. Introduction

Residual host cell proteins (HCPs) constitute process impurities that require reliable methods for detection, identification and quantitation if present in biopharmaceutical products. HCPs impact product safety and stability and require monitoring throughout the development of a manufacturing process and during final product release. Numerous studies investigating HCP content in monoclonal antibodies (mAbs) have been reported and commonly observed HCPs have become well characterised, which has enabled more targeted observation, removal strategies, and the establishment of acceptance thresholds (<100 ppm) [1,2]. However, methods are limited to determine the content and identity repertoire of HCPs in next generation biotherapeutics.

Gene therapy is gaining momentum as an advanced therapeutic modality with numerous viral-based products in clinical trials, (https://www.clinicaltrials.gov). Due to their low immunogenicity and high efficiency of delivery, a commonly used vector for gene therapy is based on recombinant adeno-associated virus (rAAV). AAV vectors are comprised of a protein capsid that harbours a therapeutic genome and are commonly produced in HEK293 mammalian cells or Sf9 insect cells [3]. While mammalian cells are most frequently used for AAV vector production, insect cells are more easily scaled-up, allowing an uninterrupted scale up for commercial manufacturing [4]. Additionally, different methods of transfection or purification can influence product yield and purity [5]. In either case, HCPs that co-purify with AAV products are poorly investigated with only few studies reported for HEK293-derived HCPs in rAAV2 vector preparations [6,7].

Multiple techniques are available for HCP detection with ELISA traditionally used to assess overall HCP content. However, ELISA has limited specificity and has been shown to underestimate the overall HCP content of AAV produced in mammalian cells, due to limitations of the antibodies in the ELISA kit raised against mammalian host cells [8]. Therefore, orthogonal methods such as liquid chromatography coupled to mass spectrometry (LC-MS) are often utilized for in-depth analysis of HCP impurities. The use of nano flow-LC enables the achievement of high sensitivity while only requiring minute sample amounts, an important feature when analysing AAV samples so as to minimize the amount of vector used in laboratory testing. HCP analysis represents a challenge for LC-MS due to the high dynamic range required. Various strategies for sample pre-treatment and preparation have been presented in recent years trying to address this challenge [8].

Here, we demonstrate the use of a semi-automated version of the SP3

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protocol for HCP analysis [9]. High reproducibility has been reported for the SP3 protocol even when working with sample amounts in the low µg-range, which is especially beneficial when working with samples with low protein content, such as rAAV. Additionally, we exploit the reduced efficiency of trypsin to digest AAV capsid proteins under the conditions used [10]. Using trypsin, full digestion of HCPs is facilitated while AAV capsid protein digestion is limited, lowering their contribution to the overall MS signal and aiding detection of low abundance HCP peptides.

2. Materials and methods

2.1. Chemicals and reagents:

All reagents and solvents used were ACS reagent grade or better. Research-grade HEK293-derived AAV2 was provided by Patheon Viral Vector Services. Research-grade Sf9-derived AAV8 was purchased from Virovek (Hayward, CA, USA).

2.2. SP3 sample digestion

The protein concentration present in the AAV samples was determined using a NanoOrange™ Protein Quantitation Kit (Fisher Scientific, Dublin, Ireland) following the manufacturer's protocol. 10 µg of protein were reduced and alkylated with 0.2 M 1,4-dithiothreitol and 0.4 M iodoacetamide (Merck, Wicklow, Ireland) respectively before undergoing tryptic digestion using a Thermo Scientific KingFisher Duo Prime purification system using a modified version of the single-pot solid-phase-enhanced sample preparation (SP3) protocol [9]. In short, sample volumes were adjusted to 95 µL using 50 mM ammonium bicarbonate prior to reduction and alkylation. Five microliters each of hydrophobic and hydrophilic beads per sample were combined, washed three times, and resuspended in water before being used for the semi-automated trypsin digestion on magnetic beads in a ratio of 1:50. After 2 h, the reaction was quenched with 0.1 % (v/v) TFA, and samples were evaporated to dryness using vacuum centrifugation. For the Sf9-derived AAV8, triplicate digestions were prepared while for the HEK293 derived AAV2, a single digestion was performed due to limited sample availability. Prior to analysis, samples were dissolved in 0.1 % FA at a concentration of 0.2 µg.µL^{-1} and Hi3 *E. coli* (Waters, Milford, MA, USA) standard was added to reach a final concentration of 50 fmol.µg^{-1} of protein injected.

2.3. LC-MS analysis

HCP analysis was performed using a Thermo Scientific UltiMate 3000 RSLCnano system (Thermo Scientific, Germering, Germany) coupled to a Thermo Scientific Orbitrap Exploris 240 mass spectrometer equipped with an EASY-Spray source (Thermo Scientific, Bremen, Germany) and a Thermo Scientific Easy-Spray PepMap RSLC C18 column, 2 µm, 75 µm × 50 cm (Thermo Scientific, Sunnyvale, CA, USA). Using the loading pump at 20 µL.min^{-1}, 1 µg was injected onto a PepMap C18, 5 µm, 5 mm × 30 µm trap column (Thermo Scientific, Sunnyvale, CA, USA). Injections were performed in triplicate for AAV2 and duplicate for each digestion of Sf9-AAV8. Separation was performed using a linear gradient of 2–40 % B (ACN with 0.1 % FA, Optima, Fisher Scientific, Dublin, Ireland) over 105 min, followed by two wash steps at 80 % B. The column was then re-equilibrated for 20 min at 2 % B. The flow rate was 250 nL.min^{-1} and the column temperature 45 °C.

MS parameters were as follows: full scans were performed in positive ion mode with a scan range between 200 and 2000 *m/z* and a resolution of 120,000 (at *m/z* 200), RF lens was set to 60 %, normalized AGC target was 100 % with a maximum injection time of 100 ms and the number of microscans was 1. Data-dependent fragment scans (ddMS2) were acquired using a resolution setting of 15,000 with an AGC target of 100 %, a maximum injection time of 200 ms, an isolation window of *m/z* 2.0 with no isolation offset selected and the number of microscans set to 1.

Table 1
Sequence databases searched for HCP analysis using Sequest HT in Proteome Discoverer 2.5.

Database	Download Date	Source	HEK293 AAV2	Sf9 AAV8
AAV2 (Reviewed)	May 8th 2021	Uniprot	✓	
AAV Associated Proteins (Reviewed)	May 8th 2021	Uniprot	✓	✓
Human Adenovirus C Serotype 5 (Reviewed)	November 2nd 2021	Uniprot	✓	
Human (Reviewed)	May 8th 2021	Uniprot	✓	
AAV8 (Reviewed)	May 27th 2022	Uniprot		✓
Autographa californica nuclear polyhedrosis virus (Reviewed)[2]	June 3rd 2022	Uniprot		✓
Sf9 (All)[3]	March 15th 2022	Uniprot		✓
Hi3 *E. coli* standard (for LFQ)	Provided by supplier	Waters	✓	✓

HEK293 cells were modified with human adenovirus C serotype 5.
[1] Baculovirus used for production of AAVs in Sf9 cell line.
[2] Due to the poorly characterized nature of the Sf9 genome, the full Sf9 sequence database available on Uniprot was searched instead of just the reviewed entries.

Fragmentation of the ten most abundant precursor ions was performed using a normalized collision energy of 28 %, a signal intensity threshold of 1.0×10^4, a dynamic exclusion for 45 s with a ±5 ppm mass tolerance and included charge states were +2–8.

2.4. Data analysis

Spectra were searched against appropriate sequence databases (Table 1) via Sequest HT using Thermo Scientific Proteome Discoverer, version 2.5 (Thermo Scientific, San Jose, CA, USA). For protein identification a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da were allowed. False discovery rate targets were set to 0.01 (strict) and 0.05 (relaxed). Trypsin was set as the digestion enzyme with a maximum of 2 missed cleavages. Search criteria included carbamidomethylation of cysteines as static modification and oxidation, N-terminal acetylation, and N-terminal methionine loss as dynamic modifications.

Protein identification was considered valid if a minimum of two unique peptides were identified. The common Repository of Adventitious Proteins (cRAP) and MaxQuant contaminant databases were used to identify and filter potential contaminants. Label Free quantification (LFQ) was performed by Hi3 quantitation. LFQ abundances were normalized to the concentration of the Hi3 peptides and protein abundances were then calculated using the average LFQ-values of the 3 most abundant peptides of each identified protein. Identified HCPs were classified based on a manual search carried out using the BioPhorum host cell protein database (https://www.biophorum.com/host-cell-proteins) and relevant references [1,2].

3. Results and discussion

Throughput and reproducibility are preferential during characterization of biotherapeutic products, especially in QC environments. The use of magnetic bead-based sample preparation allowing for automation is ideal in addressing those needs [9]. In combination with nano flow-LC, which offers increased sensitivity, reliable in-depth analysis of HCPs is enabled while requiring minute sample amounts. In the present study, a semi-automated version of the SP3 protocol followed by nano LC-MS/MS was used to detect and quantify HCP protein impurities in research-grade AAV samples.

As a first step, reproducibility of the workflow was evaluated based on retention time (RT) stability and peak areas of HCP-unique peptides

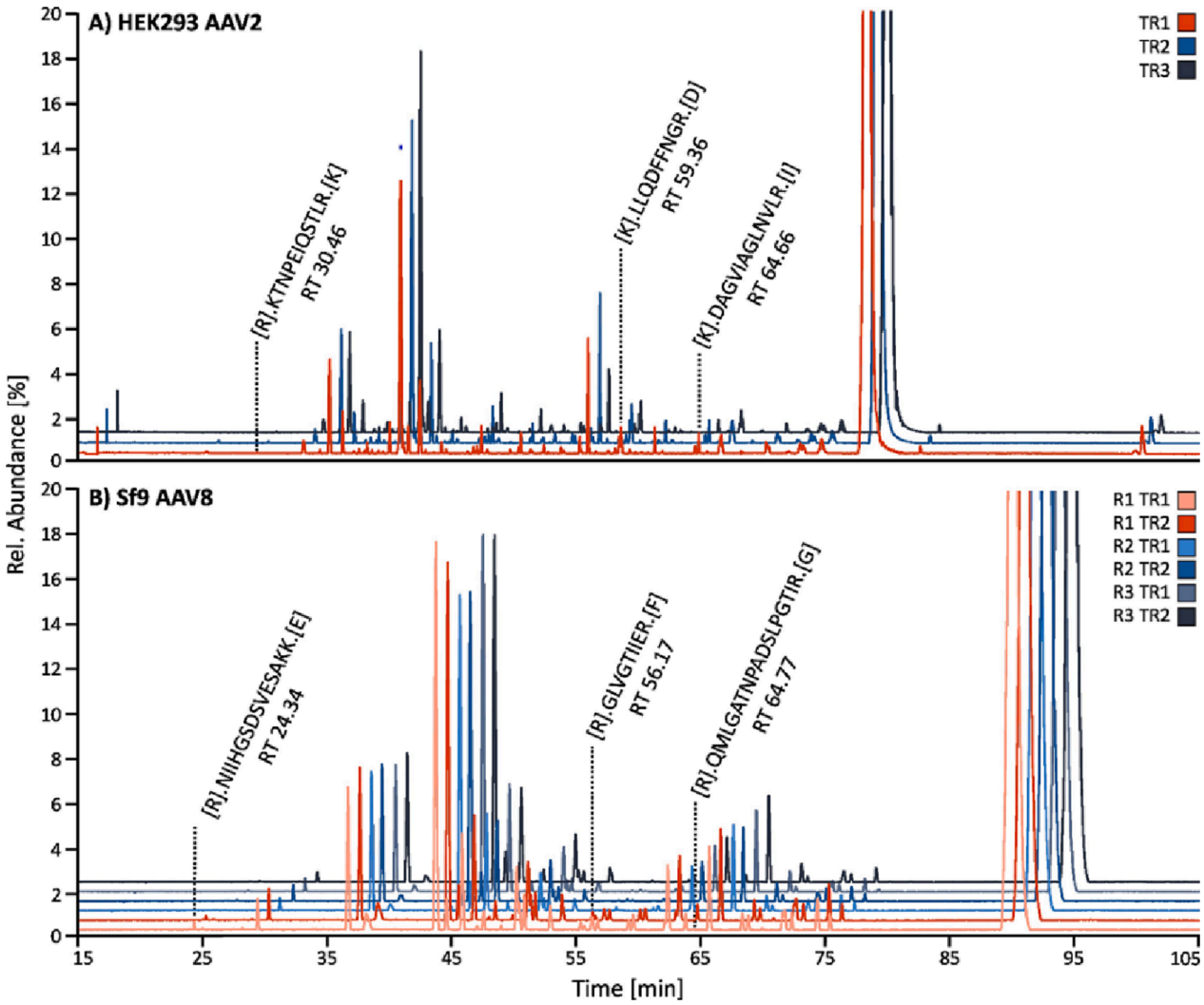

Fig. 1. Overlayed base peak chromatograms (BPCs). (A) Replicate injections (TRs) of HEK293 AAV2. (B) Replicate digests (R1-3) and injections (TRs) of Sf9 AAV8. Highlighted are HCP-unique peptides and corresponding retention time (RT) used to assess reproducibility.

Table 2
HEK293 and Sf9 HCP-derived peptides and corresponding average (avg.) retention time (RT) and peak areas as well as coefficient of variation in % (HEK293 n = 3; Sf9 n = 6). Results of AAV8 samples were normalized on total ion count (TIC) to account for variation during sample preparation.

Sample	HCP Peptide	Avg. RT	% CV RT	Avg. Peak Area	% CV Area
HEK293	[R].KTNPEIQSTLR.[K]	30.46	0.44	24301.33	4.94
AAV2	[K].LLQDFFNGR.[D]	59.36	0.16	229361.33	10.38
	[K].DAGVIAGLNVLR.[I]	64.66	0.18	493671.00	10.40
Sf9 AAV8	[R].NIIHGSDSVESAKK.[E]	24.34	0.38	1877617.54	8.03
normalized	[R].GLVGTIIER.[F]	56.18	0.19	1192040.62	17.17
	[R].QMLGATNPADSLPGTIR.[G]	64.77	0.16	336287.23	7.47

highlighted in Fig. 1. Peptides used for evaluation were chosen based on differences in retention time (24–65 min) and LFQ protein abundances (2–1000 ppm) to cover a wide range of conditions.

As summarized in Table 2, using a combination of triplicates prepared during sample preparation and technical replicates revealed excellent RT stability (CV < 0.5 %) as well as only minor variation of peak areas (CV = 5–17 %) highlighting consistency of obtained results.

One substantial challenge during untargeted data-dependent acquisition LC-MS-based HCP analysis is the dynamic range that is required to allow for detection of low abundant HCPs while high amounts of AAV drug product is present. There are numerous approaches described in literature to address this problem [8]. Here, we exploit the previously reported limited efficiency of trypsin to digest AAV capsid proteins (VPs) [10]. Incomplete digestion of AAV Capsid VPs results in fewer peptides that potentially could co-elute with HCP-derived peptides. Indeed, sequence coverage ranged from 69.4–72.3 % and 60.1–65.9 % for AAV8 and AAV2, respectively (data not shown).

Overall, 113 HEK293-derived HCP proteins were detected in AAV2 and 102 Sf9 HCP proteins were found in AAV8. Based on Hi3 quantitation, 69 proteins were quantified in AAV2, and 72 proteins quantified in AAV8. A complete list of identified and quantified proteins can be found in the supplementary information. The 10 most abundant proteins found in the 2 AAV samples analysed, are highlighted in Table 3.

For other biotherapeutics, such as e.g., mAbs, which are commonly

Table 3
Top 10 most abundant HCPs quantified in HEK293- and Sf9-derived AAV samples. Shown are the average MS abundance (HEK293 n = 3; Sf9 n = 6) as well as calculated quantities in fmol and ppm (ng/mg). Additionally, HCP quantities are shown as ng per 1e14 AAV capsids, based on the assumption that 1.6e14 particles equal approximately 1 mg of protein.

Sample	Uniprot ID	Description	Average Abundance	CV	fmol	ppm (ng/mg)	ng/1e14 capsids
	P83916	Chromobox protein homolog	40203418.1	0.17	37.91	811.27	507.04
	P35968	Vascular endothelial growth factor receptor 2	2448698.795	0.15	2.31	349.58	218.49
	P17948	Vascular endothelial growth factor receptor 1	2347939.91	0.01	2.21	333.65	208.53
	Q13185	Chromobox protein homolog 3	15183184.42	0.10	14.32	297.79	186.12
	P01857	Immunoglobulin heavy constant gamma 1	6191654.044	0.20	5.84	210.77	131.73
HEK 293 AAV2	P0DMV9	Heat shock 70 kDa protein 1B	2061926.441	0.05	1.94	136.10	85.06
	P62424	60S ribosomal protein L7a	966972.4792	0.03	0.91	27.35	17.10
	P07900	Heat shock protein HSP 90-alpha	341087.6343	0.03	0.32	27.21	17.01
	P15924	Desmoplakin	68658.62224	0.30	0.06	21.47	13.42
	P45973	Chromobox protein homolog 5	955454.4865	0.06	0.90	20.00	12.50
	A0A2H1VK95	Nucleoside diphosphate kinase	16579701.28	0.30	59.06	1009.91	631.19
	A0A2H1VTD3	SFRICE_000994	2218000.578	0.30	7.90	471.68	294.80
	A0A2H1VL96	Inositol-3-phosphate synthase	3119838.185	0.31	11.11	431.19	269.50
	A0A2H1VCY7	Adenosylhomocysteinase	1379482.623	0.36	4.91	232.43	145.27
SF9	Q06691	Telokin	2865366.504	0.21	10.21	202.09	126.31
AAV8	B1P868	Beta-hexosaminidase	779457.321	0.15	2.78	195.74	122.34
	A0A2H1WCS9	SFRICE_006789 (Fragment)	2442599.59	0.25	8.70	184.46	115.29
	A0A2H1WB52	Proteasome subunit alpha type	799900.7693	0.47	2.85	155.86	97.41
	L8B8V1	Glucosidase II alpha-subunit	414169.5523	0.28	1.48	155.06	96.91
	A0A2H1W7W9	Proteasome subunit alpha type	1513141.967	0.39	5.39	146.61	91.63

Table 4
List of potentially problematic HCPs highlighted on the BioPhorum website (https://www.biophorum.com/host-cell-proteins) or identified by relevant references[1,2]. Quantified proteins in HEK293- and Sf9-derived AAV samples are shown, the average MS abundance (HEK293 n = 3; Sf9 n = 6) as well as calculated quantities in fmol and ppm (ng/mg). Additionally, HCP quantities are shown as ng per 1e14 AAV capsids, based on the assumption that 1.6e14 particles equal approximately 1 mg of protein.

Sample	Uniprot ID	Description	Average Abundance	CV	fmol	ppm (ng/mg)	ng/1e14 capsids
	P0DMV9	Heat shock 70 kDa protein	2061926.441	0.05	1.94	136.10	85.06
	P07900	Heat shock protein HSP 90-alpha	341087.6343	0.03	0.32	27.21	17.01
	Q5QNW6	Histone H2B type 2-F	969057.0272	0.08	0.91	12.70	7.94
	P11142	Heat shock cognate 71 kDa protein	143036.202	0.17	0.13	9.56	5.98
	P62805	Histone H4	704579.1685	0.08	0.66	7.57	4.73
	Q16777	Histone H2A type 2-C	447256.0418	0.11	0.42	5.90	3.69
	P10809	60 kDa heat shock protein	82344.53663	0.02	0.08	4.74	2.96
HEK 293 AAV2	P08238	Heat shock protein HSP 90-beta	58462.26269	0.15	0.06	4.59	2.87
	P10412	Histone H1.4	182385.8103	0.11	0.17	3.77	2.35
	P07237	Protein disulfide-isomerase	56881.51963	0.08	0.05	3.06	1.91
	P14618	Pyruvate kinase PKM	55307.76613	0.31	0.05	3.02	1.89
	P62937	Peptidyl-prolyl cis–trans isomerase A	160163.8254	0.19	0.15	2.72	1.70
	Q16778	Histone H2B type 2-E	95807.14853	0.54	0.09	1.26	0.78
	P32119	Peroxiredoxin-2	60519.66632	0.15	0.06	1.25	0.78
	P07355	Annexin A2	25554.35036	0.07	0.02	0.93	0.58
	A0A2H1WB52	Proteasome subunit alpha	799900.77	0.47	2.85	155.86	97.41
	A0A2H1W7W9	Proteasome subunit alpha	1513141.967	0.39	5.39	146.61	91.63
	A0A2H1X1L9	Proteasome subunit alpha	2327030.133	0.34	8.29	145.06	90.66
	A0A2H1WH41	Proteasome subunit alpha	1227471.043	0.42	4.37	121.55	75.97
	A0A2H1WHA1	Proteasome subunit alpha	1216594.065	0.43	4.33	120.91	75.57
	A0A2H1VS21	Proteasome subunit alpha	1160132.35	0.35	4.13	110.75	69.22
SF9 AAV8	A0A2H1VM92	Proteasome subunit alpha	588023.6631	0.47	2.09	54.25	33.91
	A0A2H1WFA2	Heat shock 70 kDa protein*	179332.929	0.32	0.64	45.67	28.55
	A0A7G9U6I8	Heat shock protein*	112928.953	0.22	0.40	30.41	19.01
	A0A7S6RMB6	Heat shock cognate 70 protein*	103012.4468	0.23	0.37	26.82	16.76
	P25783	Viral cathepsin*	100410.442	0.53	0.36	13.20	8.25
	A0A2H1V328	Peptidyl-prolyl cis–trans isomerase*	29245.09747	0.52	0.10	1.84	1.15

produced in CHO cells, much is now known about HCP impurities. Therefore, problematic proteins potentially affecting product stability or patient safety can be targeted for separation during purification and monitored in a targeted way [1]. However, information on residual HCPs in gene therapy products is limited. Additionally, insect cell protein databases are poorly annotated making an appropriate risk assessment even more difficult. Nevertheless, amongst the identified proteins, a few potentially problematic HCPs were found in both samples (Table 4).

Apart from HCPs commonly detected in mAbs, such as GAPDH, VIM, and S100A9, several heat shock proteins, reported to be potentially immunogenic, were found in HEK293-derived AAV2 [2]. Of special interest is HSP90A, which is amongst the most abundant HCPs detected [11]. Additionally, multiple histones were identified which were also reported to pose a risk to patients if present at higher concentrations [2,12]. Based on gene ontology (GO term) annotation, several identified HCPs are related to cellular mechanisms such as stress response (HSP8, UBA52, or CBX1), cell cycle and proliferation (H4C1, HSPD1), but also apoptosis (H1-2, H1-4) and virus induced apoptosis (SLC25A6). Those proteins not only indicate a cellular stress response to the recombinant

viral production but also suggest an activation of cellular defence mechanisms as reported previously [13]. Therefore, identified HCPs represent potential targets for further optimization of purification strategies to ensure their removal.

Interestingly, based on GO-term annotation, no proteases were found in AAV2 samples. In Sf9-derived AAV8 on the other hand, multiple potential product-degrading HCPs were identified (Table 4). However, interpretation of those results is limited due to the lack of database and proteome annotation available. Along with Sf9 HCPs, proteins associated with the baculovirus infection required to generate AAVs in Sf9 cells were also identified. These included proteins involved in the formation of both the occlusion derived (SLP(GP37), p95, GP41, P79 and ORF142) and budding forms (vUBI and GP41) of the virus, as well as host liquification for horizontal virus transmission (VCATH and CHIA). Such findings indicate that for Sf9-derived AAV vectors, baculovirus proteins need to be monitored along with Sf9 proteins as potential HCP impurities. The complete list of identified proteins is included in the supplementary material.

As mentioned previously, in-depth characterization of other biotherapeutics enabled the establishment of guidelines regarding acceptable HCP thresholds. Due to the limited information available about protein impurities in gene therapy products [7], appropriate thresholds are yet to be determined and need to be interpretated with caution. While some proteins were found to be present at >100 ppm it is currently unknown how this would impact patient treatment and if those concentrations pose a risk to safety but also product stability. Further investigations are required.

4. Conclusion

With an increasing number of AAV-vector based gene therapies being evaluated in the clinic and being approved for market, data concerning residual HCPs present in the AAV products is required to facilitate knowledge-driven process optimization to minimize risk for patients and to increase product stability. The SP3 protocol described here in combination with data-dependent acquisition LC-MS/MS was used to study residual HCPs in AAV samples derived from different production cells. The analytical workflow presented allows for reproducible and sensitive identification of HCP proteins in AAV8 and AAV2 vector preparations produced in mammalian and insect cells, respectively. Furthermore, Hi3 quantitation enabled estimation of HCP abundances in femtomoles and ppm. Interestingly, HEK293 derived AAV2 contained potentially immunogenic HCPs while in Sf9-derived AAV8 various proteasomal proteins were detected. Nevertheless, the aim of the presented work was to demonstrate suitability of the established workflow and to provide a reference for HCPs detected in AAV products. The presented data will be useful to further drive optimization of gene therapy product manufacturing and better characterization of AAV product lots.

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Declaration of Competing Interest

S.G.M. and R.O.S. are employees of Patheon Viral Vector Services. J. B. received funding from Patheon Viral Vector Services to undertake this research. J.S. was employed under the collaborative research engagement between Patheon Viral Vector Services and NIBRT.

Data availability

Raw data was deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identified PXD041536.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2023.06.019.

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