Adeno-Associated Virus Host Cell Protein Profiling Using Micro-flow Separation on a UHPLC-HRAM MS Platform

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ABSTRACT

Purpose: Develop a robust micro-flow LC-MS/MS method for AAV residual host cell protein (HCP) impurities identification and relative quantification.

Methods: Crude harvest AAV6 sample and purified AAV6 sample were enzymatically digested using trypsin. The digested samples were analyzed using a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer coupled with Thermo Scientific™ Vanquish™ UHPLC system. The collected MS and MS/MS data were searched against the Human-uniprot-proteome database for HCP identification using Thermo Scientific™ Biopharma Finder™ software.

Results: Close to 1000 HCPs were identified from the crude harvest AAV6 sample with at least three unique peptides. The identified number of HCPs from the purified AAV6 sample was significantly decreased to 30, demonstrating that the purification method was very efficient to remove the HCPs from the crude harvest AAV6 sample.

INTRODUCTION

Recombinant Adeno-associated viral (rAAV) vectors have emerged as the leading gene delivery vehicles for gene therapy due to their high-efficiency transduction and safety. HCP is one of the process-related impurities that needs to be well characterized and controlled throughout biomanufacturing processes in order to assure the safety of the AAV products. Unlike therapeutic proteins manufactured in CHO cells, most AAVs are manufactured in human cell lines with a more complex proteome background, presenting more challenges for HCP analysis. Micro flow LC-MS/MS method offers higher sensitivity compared to high flow LC MS/MS, while maintaining comparable method robustness and provides a great analytical solution to address the AAV HCP analysis challenges. Taking advantages offered by micro flow separation, we developed a micro flow UHPLC MS/MS approach using the Orbitrap Exploris 480 mass spectrometer coupled with the Vanquish UHPLC system. Combining the increased sensitivity benefits offered by the micro flow separation and the brighter ion source design of the Orbitrap Exploris 480 mass spectrometer, close to 1000 HCPs were identified from the crude harvest AAV6 sample and 30 from the purified AAV6 sample with high confidence. The analytical results are reported here.

MATERIALS AND METHODS

Sample Preparation

Crude harvest AAV6 sample generated from transient transfection in HEK293 cell was collected. Partial crude harvest AAV6 sample was further purified using POROS AAVX affinity resin.

250uL of the crude harvest and purified AAV6 samples containing spiked-in 200ng intact protein (Streptococcus Protein AG chimeric) were buffer exchanged to 7M Guanidine HCl 100 mM Tris, respectively. The buffer exchanged AAV samples were reduced with DDT (Dithiothreitol) and alkylated with IAC (iodoacetic acid). The reduced and alkylated samples were further buffer changed to 50 mM Tris and enzymatically digested in-solution using trypsin at 37℃ over 2.0 h. The digestion was terminated by the addition of 10% formic acid. The digested sample was used for LC-MS/MS analysis directly.

HPLC conditions

The Vanquish Horizon UHPLC system performed separations. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The column was a Thermo Scientific™ PepMap™ 100 C18 Column (1.0x150mm, 3μm) that operated at 50 ℃ and a flow rate of 80 μL/min. The gradient condition used was listed in Table 1. The injection volume was 60 μL. Each sample was analyzed in triplicate.

Table 1. HPLC gradient condition

Time	Flow (ml/min)	%A	%B
0	0.08	98	2
5	0.08	98	2
6	0.08	95	5
106	0.08	95	35
113	0.08	10	90
117	0.08	10	90
117.1	0.08	98	2
125	0.08	98	2

MS Conditions

All the data was collected on a Thermo Scientific Orbitrap Exploris 480 mass spectrometer. MS/MS data acquisition was carried out using a data dependent MS/MS set up. The ESI and mass spectrometer set ups are shown in Table 2 and 3, respectively.

Table 2. ESI source parameter set ups

MS source setting	Value
Sheath gas	25
Aux gas	8
Sweep gas	0
Spray voltage (+V)	3500
Capillary temp. (℃)	250
Vaporizer temp. (℃)	100

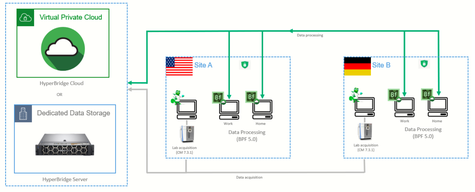
Table 3. MS parameter set ups

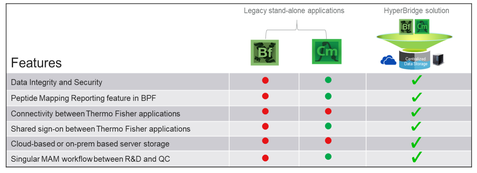
MS full MS/dd MS2 (top10) setting	Value
General	
Application mode	Peptide
Pressure mode	Standard
RF lens (%)	50
Full MS	
Scan range (m/z)	300 - 1800
Resolution	60,000 at m/z 200
AGC target value (%)	300
Max inject time (ms)	100
dd-MS/MS (top10)	
Resolution	17500 at m/z 200
Isolation window (m/z)	2
AGC target value (%)	100
Max inject time (ms)	200
Fixed first mass (m/z)	75
Targeted backgound exclusion	on
HCD collision energy (V)	28

Data Analysis

All data were processed using Biopharma Finder 5.0 software.

Figure 1. Data processing flow chart using Thermo Scientific™ HyperBridge™ . Collaboration with BioPharma Finder software is enabled by HyperBridge connectivity providing data processing capabilities to any computer connected to the virtual private cloud or using the dedicated data storage. Data acquisition and processing can be performed by multiple users across multiple locations.



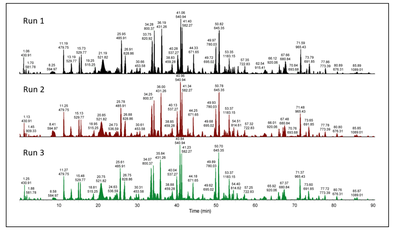


RESULTS

Retention time reproducibility

The trypsin digests of a crude AAV6 sample and the purified AAV6 product were each analyzed in triplicate. Figure 2 shows the base peak chromatograms from the triplicate runs of purified AAV6 sample. Excellent retention time reproducibility and separation efficiency were achieved using the micro flow separation.

Figure 2. Base peak chromatograms of purified AAV6 sample

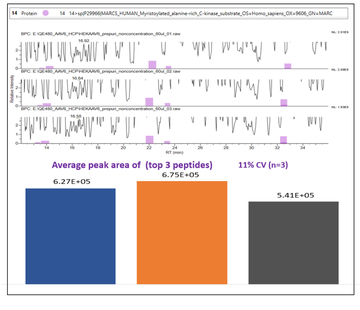


HCP identification

The 6 raw files were processed using the peptide mapping workflow via the host cell protein analysis feature in the Biopharma Finder 5.0 software. The Human-uniprot-proteome database appended with the sequence for Streptococcus protein was used for HCP identification.

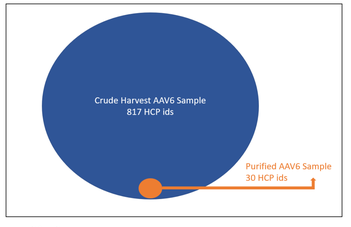
817 HCPs were identified with at least three unique peptides in the crude harvest AAV6 sample. With the high separation efficiency and great sensitivity offered by this micro UHPLC-MS/MS approach, the MS/MS data were obtained over a wide dynamic range. As shown in Figure 3, although the peaks of detected peptides from the HCP - myristoylated alanine-rich C-kinase substrate (highlighted as purple color) was buried in the background because of their low intensity signals, the Orbitrap Exploris 480 MS still triggered MS/MS acquisition and generated high quality MS/MS spectral data for confident peptide identification. Using the spiked-in 200ng intact Streptococcus Protein AG_chimeric as reference, the estimated concentration of this HCP was calculated to be 3.6pmol/mL in the crude harvest AAV6 sample using the average area of top 3 peptides. In addition, the integrated peak areas for these low abundant peptides demonstrated good reproducibility. The percentage coefficient of variance of the average peak area (top 3 peptides) across the triplicate runs was 11%.

Figure 3. Example of low abundant HCP (estimated concentration: 3.6pmol/mL) identification and quantification from triplicate runs of the crude harvest AAV6 sample



The number of HCP decreased significantly after the purification and only 30 HCPs which have at least 3 unique peptides were identified from the purified AAV6 sample (Figure 4).

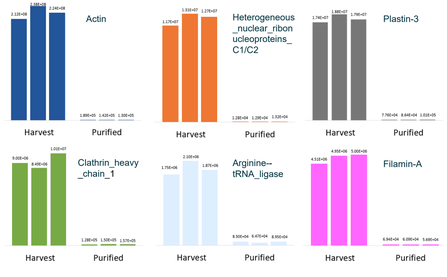
Figure 4. The numbers of identified HCPs with at least three unique peptides from the harvest vs purified AAV6 samples using the Human-uniprot-proteome database



HCP relative quantification

The developed micro flow UHPLC-MS-MS/MS method showed great retention time reproducibility and integrated peak area reproducibility, enabling precise relative quantification between the culture harvest AAV6 sample and its purified AAV6 sample. Figure 5 shows the relative quantification results for several HCPs identified in both crude harvest and purified AAV6 samples. Overall, all HCPs showed lower concentrations in the purified AAV sample, demonstrating that the POROS AAVX affinity resin was able to remove the HCPs efficiently from the AAV6 harvest.

Figure 5. Quantitative trend of representative HCPs between the crude harvest and purified AAV6 samples using the average peak area of top 3 peptides



Data reporting with BioPharma Finder and HyperBridge

Data visualization tools and reporting provide a critical component for HCP analysis and review that enable trend identification and insight into the effectiveness of the purification process. When connected to HyperBridge, BioPharma Finder provides customizable reporting tools designed to enhance data review and streamline collaboration.

These customizable reporting tools provide comprehensive reporting features, including dynamic data tables, column filters, custom calculations, charts and graphs, and plot images (Figure 6 & Figure 7). Individual report layouts can be managed across multiple page tabs within the report display and saved and stored as templates for future implementation. Additionally, all generated reports can be exported in excel and PDF format for additional evaluation or data archiving.

Figure 6. New peptide mapping reporting feature in BioPharma Finder provides visualization of the identified HCPs in a stacked bar chart.

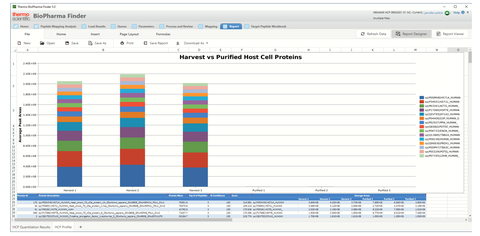
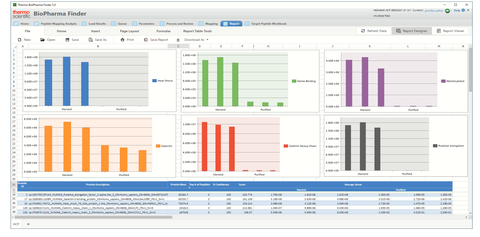


Figure 7. Quantitative trend of representative HCPs visualized in BioPharma Finders new report designer. Reports can be downloaded as PDF and XLs greatly expanding the customization of results.



CONCLUSIONS

- A robust micro flow UHPLC MS-MS/MS method was developed and applied to AAV6 HCP analysis successfully.
- The increased sensitivity benefits offered by both micro flow separation and the Orbitrap Exploris 480 mass spectrometer allowed low abundant HCP identification from complex human cell matrix.
- The micro flow UHPLC MS-MS/MS method provided excellent retention time and integrated peak area reproducibility, yielding precise relative quantification of HCPs between the crude harvest and purified AAV6 samples.
- New data processing solution for HCP analysis in BioPharma Finder that provides new reporting capabilities, increased data integrity and security, using a cloud-base or on-prem software solution.
- Expanding the collaboration of data processing and reporting by enabling access to BioPharma Finder from multiple sites around the world.

TRADEMARKS/LICENSING

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