SCIEX Solutions for Host Cell Protein Analysis

Host cell protein impurities can be difficult to detect and characterize in biological products, but SWATH® Acquisition from SCIEX can overcome these challenges.

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INTRODUCTION

The manufacture of biological-based medical treatments, such as those in cell and gene therapies or other biotherapeutics, carries the added risk of biological component impurities, which can elicit adverse reactions in patients using the therapies. Given the high degree of complexity inherent in biological systems, being able to identify and quantify potentially unforeseen contaminants can pose a challenge. Routine analytical techniques including the use of enzyme-linked immunosorbent assays (ELISA) and Western blots lack the key combination of features (e.g., broad analyte applicability while maintaining sensitivity and providing fast, accurate species identification) that may be required for a quality control method to detect host cell proteins (HCPs) and related impurities, particularly when the target analytes are unknown. In this regard, mass spectrometry is uniquely capable of providing sensitive, selective, and specific analytical data that can alert analysts to these types of contaminants, while doing so on a timescale compatible with the quick pace of manufacturing quality control. This article delves into the analysis of HCPs in bioprocessing and highlights approaches that can detect and quantify them.

PROCESS-RELATED IMPURITIES IN BIOTHERAPEUTICS

The advent of biotherapeutics has been a significant breakthrough in creating more effective, better targeted treatments for incurable or hard-to-treat diseases. An often-overlooked aspect of manufacturing biotherapeutics, however, is the presence of harmful impurities from the HCPs and the need to detect and further purify end products, such as those involving the use of viral vectors, production/packaging cell lines, and growth media and enzyme-based reagents.

- **Patient safety.** HCP contamination can provoke unwanted immune responses or drug interactions in patients, which may result in an adverse event. It can also cause immunogenicity, making the therapeutic ineffective or less effective. In addition, bioimpurities like HCPs may retain biological activity and, in that way, pose risks to anyone receiving the therapies.
- **Product stability.** Accelerated rates of degradation, modification, or activity loss of the intended product may also occur as a result of HCP impurities and other process-related impurities, which can have negative consequences for patients. Impurities may even cause excipients to degrade, which can also negatively impact overall product stability.
- **Manufacturing consistency and product purity**. Not identifying the presence of HCP impurities can negatively affect manufacturing consistency and product purity. This can result from interference in the efficiency of established purification processes if biological impurities are not taken into account.

For all these reasons, it is essential to monitor the removal of HCPs in drug product during bioprocess development. United States and European agencies regulating the manufacture of drugs and associated therapeutics speak to monitoring these types of process-related impurities, but do not set firm directions on how to do so. The US Food and Drug Administration (FDA) recommends that manufacturing processes have a mechanism to detect and remove process-related impurities with associated checks to verify the levels of the species but does not indicate a specific class of analytical technique to use (1). Similarly, the European Medicines Agency (EMA) mentions that manufacturers should set specifications for upper limits of impurities and that the levels should be monitored in products, but also is ambiguous as to the actual mechanism to use (2). Both agencies define this type of process-related impurities in a similar manner as being inclusive of, but not limited to, species such as residual cell components, extraneous nucleic acid sequences, cytokines, growth factors, antibodies, and serum.

MASS SPECTROMETRY AND BIOIMPURITIES

Unlike other commonly used techniques that cannot provide identification of previously unknown species, mass spectrometry (MS) can accurately identify even unexpected contaminants. Mass spectrometric analysis of samples that first undergo separation by liquid chromatography (LC) is the gold standard for detection and identification of biomolecules—especially for peptides and proteins. Peptides and proteins undergo consistent, reproducible fragmentation mechanisms that result in fragment ion patterns that can be readily deciphered even without knowing the identity of species beforehand. Furthermore, with comprehensive SWATH® Acquisition for HCP analysis with the TripleTOF® 6600+ System from SCIEX, the sensitive, robust, and accurate analyses via mass spectrometry include reproducible automated sample preparation and software to identify and quantify HCPs present in each sample.

HCP content analysis leveraging SWATH® Acquisition can be simplified to a three-phase procedure that includes automated sample preparation, separation, and analysis.

- The initial stage of this protocol involves protein digestion so that the protein components of a sample are cleaved into peptides for bottom-up protein identification. This also includes the

Figure 1: Host cell protein amounts in bioprocess samples—comparison of in-process samples.

Host Cell Protein	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Mass	pI	Protein name
sp|P0C058|IBPB_ECOLI	4.274	2.905	2.154	186	229	111	16.093	5,2	Small heat shock protein IbpB
sp|P0A9A9|FUR_ECOLI	158	284	296	142	147	94	16.795	5,7	Ferric uptake regulation protein
sp|P0ABK5|CYSK_ECOLI	597	913	711	618	200	68	34.490	5,8	Cysteine synthase A
sp|P69783|PTGA_ECOLI	33	250	378	256	185	62	18.251	4,7	PTS system glucose-specific EIIA component
sp|P0A8J4|YBED_ECOLI	432	215	253	222	112	21	9.827	5,5	UPF0250 protein YbeD
sp|P02930|TOLC_ECOLI	41	283	187	417	57	11	53.741	5,2	Outer membrane protein TolC
sp|P62623|ISPH_ECOLI	312	1.146	855	231	62	15	34.775	5,2	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
sp|P0ADP9|YIHD_ECOLI	33	28	32	11	10	18	10.273	5,1	Protein YihD
sp|P0A763|NDK_ECOLI	106	240	100	349	113		15.463	5,6	Nucleoside diphosphate kinase
sp|P35340|AHPF_ECOLI	67	291	171	174	48		56.177	5,5	Alkyl hydroperoxide reductase subunit F
sp|P08200|IDH_ECOLI	390	271	166	355	42		45.757	5,2	Isocitrate dehydrogenase
sp|P69797|PTNAB_ECOLI	284	339	240	25	26		35.048	5,7	PTS system mannose-specific EIIAB component
sp|P0A7I7|RIBA_ECOLI	345	297	106	48	45		21.836	5,6	GTP cyclohydrolase-2
sp|P0AEN1|FRE_ECOLI	741	870	849	1.404	33		26.242	5,3	NAD(P)H-flavin reductase
sp|P36683|ACNB_ECOLI	129	87	82	9	8		93.498	5,2	Aconitate hydratase B
sp|P0ADE8|YGFZ_ECOLI	18	113	45	150	26		36.094	5,2	tRNA-modifying protein YgfZ
sp|P0AB91|AROG_ECOLI	56	231	188	42	22		38.010	6,1	Phospho-2-dehydro-3-deoxyheptonate aldolase
sp|P0A825|GLYA_ECOLI	128	332	204	263	13		45.317	6,0	Serine hydroxymethyltransferase
sp|P0A6K3|DEF_ECOLI	129	136	55	61	26		19.328	5,2	Peptide deformylase
sp|P0ABP8|DEOD_ECOLI	33	113	78	19	20		25.950	5,4	Purine nucleoside phosphorylase DeoD-type
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%**			

addition of known proteins in specific quantities that can serve as internal standards to verify that the methods are operating effectively but can also function as quantification standards to generate internal calibration curves for quantifying HCPs present. The protein components are all subject to denaturation, reduction and alkylation of any disulfide bridges, and cleaved into peptides via a LysC digestion. Each portion of this step 1 process can be fully automated and thereby avoid operator variation impacting the analysis.

- The analysis of samples by LC separation of the complex peptide mixture occurs seamlessly in this protocol. Peptides are separated out as they elute for detection and thereby simplifies the mass spectra obtained at each moment in time during analysis.
- Data is processed automatically to obtain a comprehensive overview of the sample components, even down to the identity of the proteins. This is accomplished by comparing mass spectra yielded by the sample to a protein mass spectra database that for identification, which presents a list of proteins that may be in the sample. From there, proteins are identified by a minimum of two different peptides within the sample that match with an identified protein.

EXAMPLE OF BIOPROCESS ANALYSIS AT MULTIPLE STAGES OF PROCEDURE

An examination of HCP content within bioprocess samples taken at six different stages through the manufacturing process serves as a case example of the capability offered by mass spectrometry for monitoring contamination (FIGURE 1).

The sample taken from the first phase of the process was found via LC-MS analysis to contain 562 distinct HCPs, which were identified by comparing mass spectra to

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databases of known proteins. The analysis returned a total HCP content by weight of 19.32%. After Step 4, the HCP content found to be below 1%, but still with 67 different proteins found to have been sourced from host cells during manufacturing. Only in the sample taken during Step 6 was the HCP content reduced to 0.04%, wherein the total number of HCPs identified was a mere eight proteins. Mass spectrometry as an analytical technique is unrivaled in its capability of providing accurate identification of hundreds of proteins within a sample, even at trace ppm levels. Furthermore, the information-rich data provided about which HCPs are present at a given production stage can help in tailoring purification and manufacturing methods throughout a given process.

USING MASS SPECTROMETRY TO ASSESS ELISA COVERAGE OF HCPS

Having mass spectrometry on hand can also permit the evaluation of ELISA methods regarding how well they can capture and represent the actual HCP content of a sample. An outline of how to verify ELISA methods using mass spectrometry has been published by Pilely et al. (3). The procedure begins much like traditional ELISA protocols with antibody immobilization, addition of antigen, and a wash to remove unbound species. Trypsin is then added to cleave the proteins in preparation for mass spectral analysis, which can then provide a list of the specific proteins detected by the ELISA and compare with the sample as analyzed before the immunosorption stage.

A primary advantage of HCP coverage analysis for ELISA methods using mass spectrometry is that the antibody binding conditions mirror those used in traditional ELISA under native sample conditions, rather than under the denaturing conditions required by SDS-PAGE and Western blots. Additionally, data obtained from mass spectrometry is information-rich with complete identification of each protein present in the sample, rather than simply appearing as dots on a gel that indicate the presence of general protein content with a rough molecular weight approximation. The amount of antibody necessary for these ELISA coverage assessments is approximately 0.5 mg, which is a large savings of costly antibody relative to immunoaffinity columns that require 10–15 mg. Finally, the mass spectra can also reveal the presence of impurities within the drug substance, thereby informing about the presence of unforeseen contaminants.

In a case example of evaluating HCPs, an immunotherapy manufacturer wanted to see how applying a SWATH®-based LC-MS system could inform their quality assessment practices. Their objectives included comparing the HCP profiles of three product batches obtained via a SWATH®-based LC-MS/MS method and also investigating the HCP coverage by a generic commercial ELISA kit the manufacturer had been using for detecting HCPs that could result from adenovirus expressed in A549 human cell line within their purified drug product. The HCP ELISA results had been varying from 10 ng/ml to 100 ng/ml when batches were measured by a commercial generic ELISA kit.

Figure 2: Using the SWATH® Acquisition LC-MS method, 13 distinct adenovirus HCPs were identified and quantified within all samples.

Adenovirus protein	Mass (Da)	No of peptides	Batch A	Batch A	Batch B	Batch B	Batch C	Batch C
Hexon protein	107,949	70	23,694	22,516	21,671	16,466	16,662	19,295
Hexon-associated protein IX	14,458	13	4,623	4,361	4,290	7,139	4,275	4,555
L2 pVII	6,724	9	4,390	3,725	3,376	3,312	2,848	4,226
L2 pV	41,546	26	3,872	3,860	3,618	3,372	2,965	3,365
L1 pIIIa	65,253	42	3,779	3,779	3,577	3,457	3,024	3,024
L3 pVI	26,996	10	1,252	1,269	1,211	1,664	1,180	1,163
L4 pVIII	24,677	8	747	895	639	1,007	624	673
Fiber protein (L5 pVI)	61,585	21	623	527	529	399	418	382
52 kDa protein (L1 52, 55K)	47,034	16	258	248	275	174	189	183
Fiber protein (L5 protein IV)	34,815	8	232	203	231	164	198	196
Protease (Adenain)	23,068	11	140	143	119	70	80	92
E2B IVa2	50,878	19	78	77	85	75	69	66
E2B pTP 75K	74,689	23	64	68	69	54	40	39
Total amount ng/ml			**43,752**	**41,671**	**39,690**	**37,353**	**32,572**	**37,259**

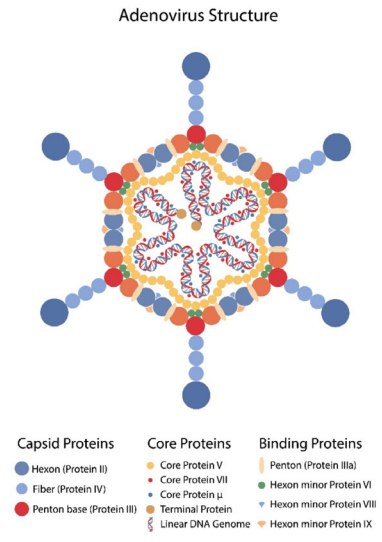
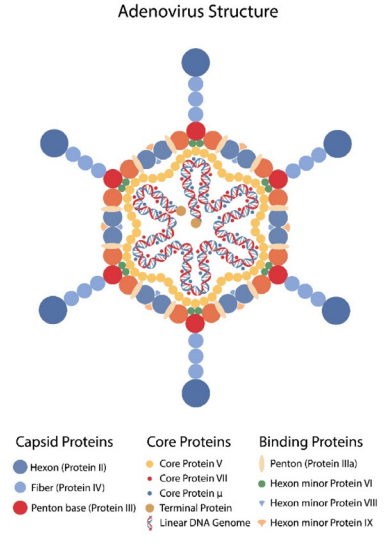


Figure 3: Smaller total quantities of HCP were detected in the batches A, B, and C.

Human host cell proteins	Mass (kDa)	No of peptides	Batch A	Batch A	Batch B	Batch B	Batch C	Batch C
HCP 1	34	5	19	19	16	24	15	14
HCP 2	14	4	7	7	9	6	8	8
HCP 3	19	4	7	7	8	6	8	7
HCP 4	24	6	8	7	7	6	6	6
HCP 5	22	5	5	5	6	4	4	5
HCP 6	18	5	3	3	4	3	3	3
HCP 7	23	5	3	3	3	3	3	2
Total amount of HCP ng/ml			**52**	**51**	**53**	**52**	**47**	**45**



Examination of the HCP profile for two replicates of each of the three batches (FIGURE 2) shows that the 13 distinct adenovirus HCPs could be identified and quantified within all samples, with an average total HCP content of about 39,000 ng/mL and good agreement among the individual batches. The total adenovirus HCP concentration variation within batch replicates differed by no more than 15%, and the difference in total adenovirus HCP among the three batches did not exceed 34%.

For the proteins originating from the human cell line, far smaller total quantities of HCP were detected in the three batches (FIGURE 3) and with similar agreement in the measurements as had been seen with the adenovirus HCPs. The batches each had a total human HCP content in the range of 45–53 ng/mL, which translated into no more than 18% difference between the highest and lowest human HCP content measurements. For coverage evaluation of their ELISA kit, the company compared the HCP profiles obtained through traditional sample workup to the HCP profile resulting from examining the captured HCPs. Of the 682 distinct HCPs identified with traditional sample preparation and mass spectrometric analysis, their ELISA kit had been able to capture only 246 HCPs. The resulting HCP coverage of the ELISA kit used by the manufacturer was a mere 36% of the total HCPs present in the samples, indicating a large gap of impurities that is missed entirely when relying on ELISA alone for quality assurance checks.

HCP ANALYSIS AND SWATH WORKFLOWS OVERVIEW

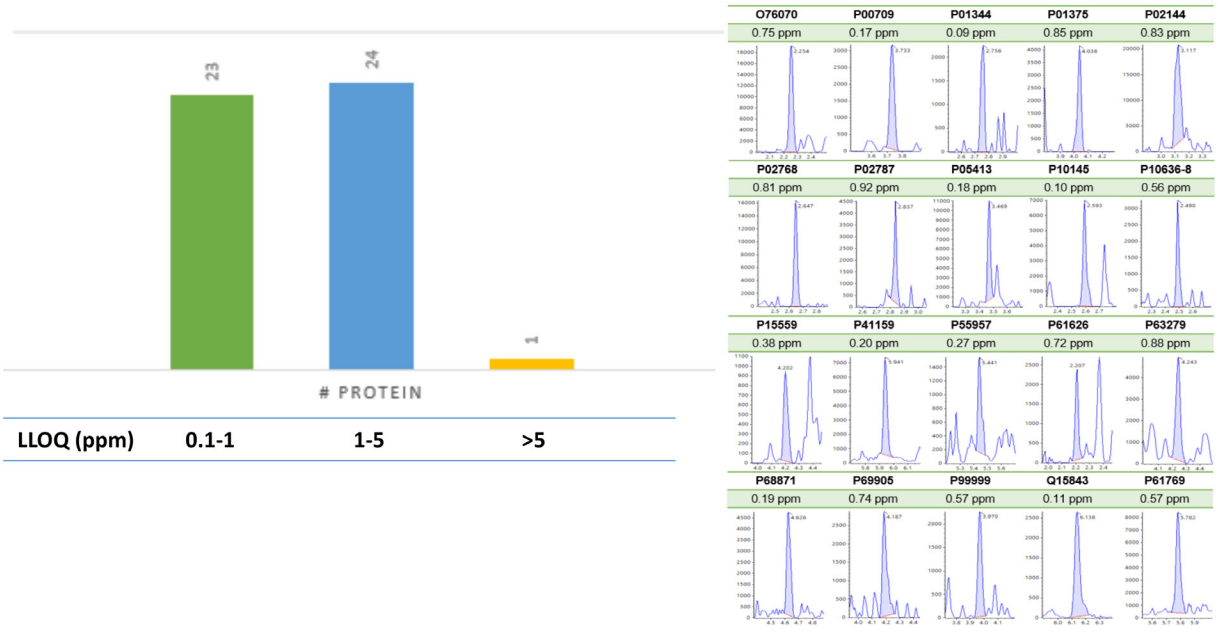
After establishing the efficacy of mass spectrometry methods for characterizing HCP impurities applied across a large dynamic range and in highly complex matrices in biotherapeutics production, the discussion can expand into greater detail pertaining to the method workflows. The two major classes of analytical workflows can be grouped into targeted approaches for better-understood systems that require accurate HCP quantification, and into non-targeted data-independent approaches for global HCP identification and quantitation throughout the full complement of sample components. The targeted approach can be performed using either traditional multi-reaction monitoring (MRM) or MRM with high-resolution mass spectrometry (MRMHR). For the data independent approach, the SWATH® Acquisition method is required to probe the full breadth of analytes across the entirety of mass spectra.

TARGETED APPROACH DETAILS

The targeted approach has the important role of offering great analyte sensitivity and analytical throughput for HCP detection. With the targeted approach, thousands of individual proteins can be profiled and quantified to ppm levels, which can allow manufacturers to build catalogs of HCPs that need to be detected during bioprocesses. Following the methods put forth by SCIEX, the mass spectrometry system used was the SCIEX QTRAP® 6500+ LC-MS system, which can operate with microflow and high flow LC sample intake as well as with multistage functionality including MRM operating modes. The overall workflow involved with information-dependent analysis (IDA) includes construction of the peptide library, development of the MRM methods, acquisition of the sample data, and data processing and species concentration calculation.

Peptide library build-up. Libraries can be generated through multiple routes. Mass spectra can be imported if high-resolution data is available, thereby permitting the creation of custom libraries provided that the user has access to high resolution mass spectrometry. In a laboratory without the ability to generate high-

Figure 4: MRM quantification summary.



resolution mass spectra, the peptides can be cataloged via an *in-silico* digestion through the Skyline software program. With a peptide library in hand, the assignment of fragment ions can be made for each peptide, followed by the creation of protein sequence coverage maps.

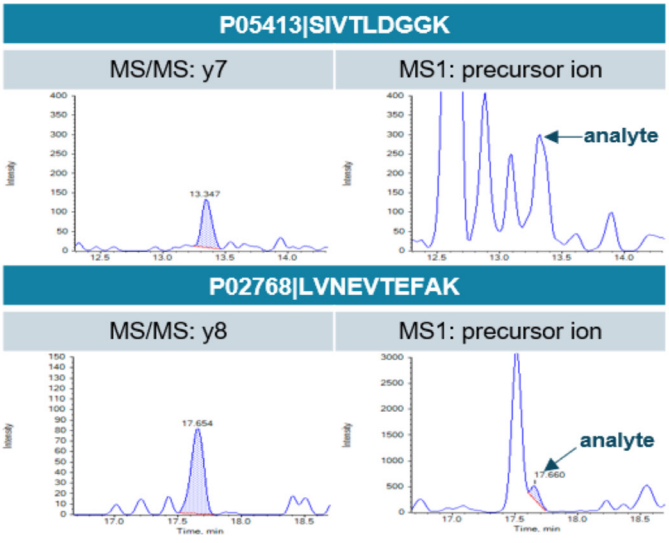
MRM development. Once the peptide library has been established, MRM method development can begin. This starts with importing the peptide library into Skyline, which will generate predictions of which MRM transitions should be monitored with the method. Once MRM transitions are selected, the MRM approach can be optimized for sensitivity and selectivity. Major fragment ions will be compiled according to signal intensity and the ions to monitor will be selected along with associated collision energy (CE) and declustering potential (DP) values, with the software automatically selecting optimal values for each peptide ion. Furthermore, the retention times can be optimized at this point for the targeted analytes. The completed method can then be exported from Skyline to the SCIEX interface for data acquisition.

Data acquisition. The optimized, targeted method can then be applied to acquiring data from prepared samples using the selected method. As a model system to examine, a trypsin digest was performed using NISTmAb, a monoclonal antibody standard, with added spikes of UPS protein standard. Following serial dilution, the sample protein concentrations fell within the range of 0.1 to 1000 ppm. A total of 48 proteins were targeted for monitoring within the sample; at two peptides to monitor per protein and two transitions per peptide, roughly 200 MRM transitions total were observed over the 8-minute total run time.

Data processing. Once data has been acquired, the SCIEX software can provide the results pertaining to sample characterization. The quantitative values for each analyte, including corresponding calibration curves and peak integration parameters, are user accessible within the interface. In the context of the NISTmAb example, the lower limits of quantitation (LLOQs) that were obtained for the 48 proteins are presented in FIGURE 4. Out

Figure 5: Comparison between MS/MS and MS1 based quantification.

- Significantly better S/Ns are observed when using fragment ion XICs for quantification than using precursor ion XICs, because of the additional level of selectivity provided by HR MS/MS.



of the 48 proteins analyzed, 23 had LLOQs in the 0.1-1 ppm range and 24 fell within 1-5 ppm—only one of the 48 proteins had an LLOQ exceeding 5 ppm. A few selected extracted ion chromatograms (XICs) for the peptides at LLOQs below 1 ppm are shown to demonstrate the high signal to noise ratios that can be achieved with this approach.

For laboratories with access to a high-resolution MS system, such as the SCIEX TripleTOF® 6600+ system, the general MRM targeted approach can be easily adapted to an MRMHR workflow. For the MRMHR adaptation, Skyline can be used to define the targeted peptides, which then defines the masses to target for MRMHR in data generation. The greater scan speeds, extended mass ranges, and shorter accumulation times work in concert with the high mass resolution to produce enhanced signal to noise ratios, even in complex matrices. Furthermore, the systems are capable of performing multi-period MS/MS, wherein different peptides can be monitored in the case of species coeluting.

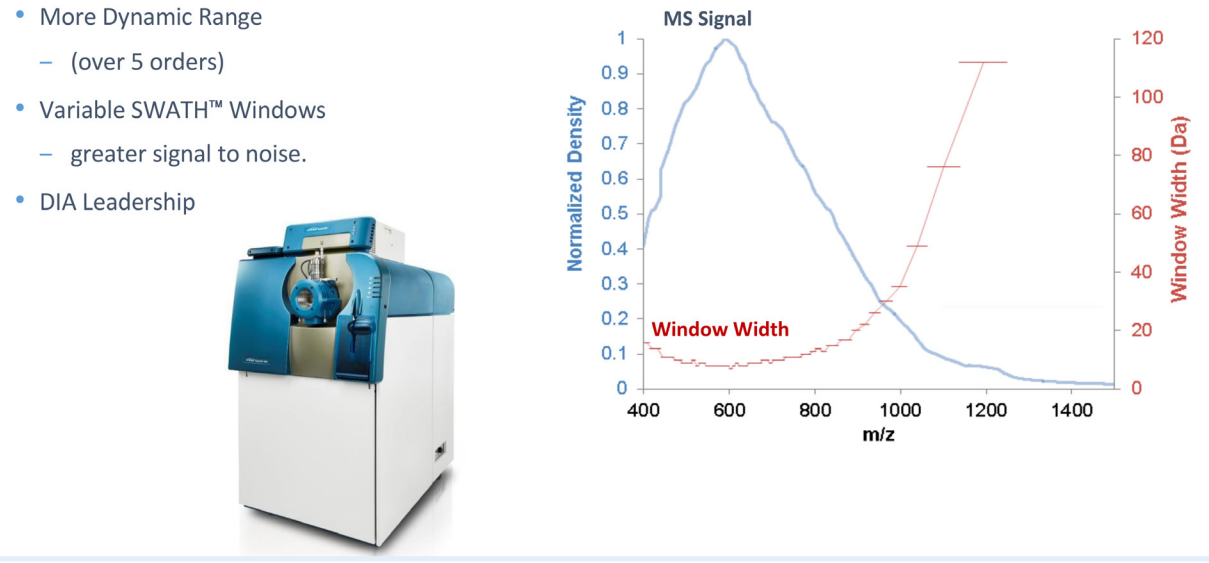
In the case of analytical methods accomplished using mass spectrometry, many systems offer the option to perform either single-stage analyses or tandem mass spectrometry. Although the absolute signal intensities obtained with single-stage MS are often orders of magnitude greater than resulting fragment ion signals in MS/MS spectra, the value of MS/MS lies in enhancement of signal to noise ratios and analyte selectivity. As shown in the two righthand traces in FIGURE 5, the XICs yielded by monitoring MS1 alone can suffer greatly from multiple species overlapping with the target analytes, thereby substantially reducing signal to noise ratios for the species of interest.

In contrast, the same analytes monitored with MS/MS in the lefthand XICs of FIGURE 5 can be readily identified with markedly improved signal to noise ratios and correspondingly enhanced LLOQs. Similarly, summing multiple fragment ion XICs for one peptide can bring tangible signal enhancement versus using a single fragment ion XIC for quantitation.

FUNDAMENTALS BEHIND SWATH® ACQUISITION

For samples where a data-independent approach is preferable, such as those

Figure 6: Variable windows with the SWATH® Acquisition LC-MS system.



with potentially unknown HCPs, SWATH® Acquisition provides an efficient mechanism to comprehensively report on sample contents with minimized front-end preparation. The fundamental feature underlying SWATH® Acquisition is the use of wide isolation windows for obtaining MS/MS spectra. Precursor ions are isolated in small segments that are marched across large mass windows; all ions within the isolated segments, typically fixed around 10-20 Da or set dynamically based on ion density, are subject to fragmentation within the collision cell. As a result, tandem mass spectra are obtained for the full mass range for all species throughout the chromatographic separation in an approach known as MS/MSALL. This permits complete characterization of a sample with tandem MS without needing to have full knowledge of what peptides may be present, thereby functioning as a truly data independent analytical method.

When creating SWATH® Acquisition protocols, the mass windows for segmentation into smaller isolation segments should be tuned as a part of the method development stage. This allows for signal optimization according to the unique sample components and can enhance analyte signal-to-noise ratios and dynamic range. The MS signals can be balanced with the isolation window size to ensure optimal results (FIGURE 6).

DATA-INDEPENDENT WORKFLOW WITH SWATH® ACQUISITION

Within a data independent workflow, the approach is similar on the front end to prior protocols, but with slight modifications to the processing after collection of SWATH® Acquisition data. The approach to take for processing will depend upon the intended end result for the collected data. For accurate quantitative results, the suggested processing approach is to take a brief review of the data in Skyline. This stage is intended to confirm the selected transitions and retention times with the peptide library. Any necessary corrections to peak assignments can be made at this point, including manual peak adjustments. The finalized data can be transferred back into the SCIEX OS for quantification of HCPs and other analytes, assessment of internal standards, and scrutiny of grouped files such as multiple ion transitions for an individual peptide. For large-scale quantitative profiling of peptides,

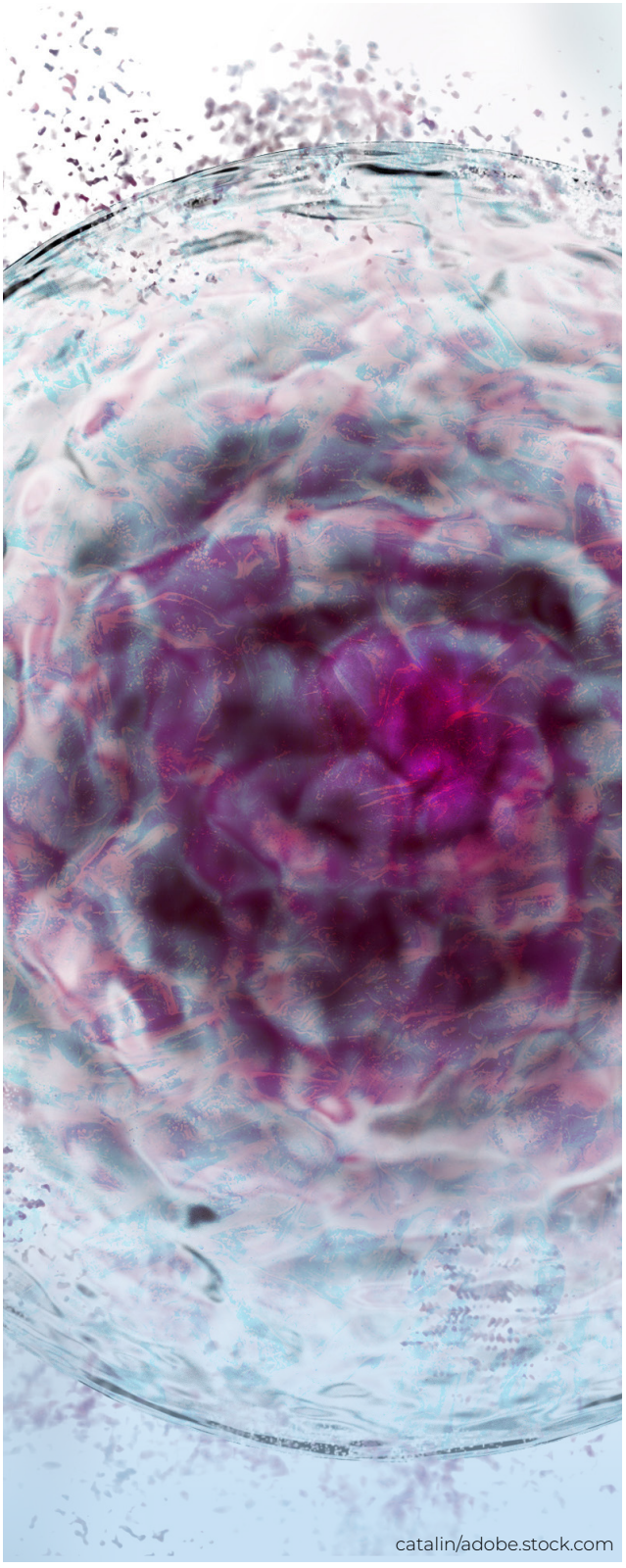
the data review and quantitative profiling can be performed in the PeakView® software, which allows for enhanced visualization within a single viewpoint of XICs from each peptide fragment, total ion current traces from SWATH® Acquisition, and MS/MS spectra including comparison with library matches. Furthermore, the use of MarkerView® allows for observation of quantitative trends for each species across different analyses, which can facilitate quality assessment across multiple sample batches and throughout the manufacturing process.

CONCLUSIONS

Quality assurance of biotherapeutics can be hindered by the difficulty of detecting HCPs and related contaminants using traditional analytical approaches. However, the introduction of methods such as SWATH® Acquisition into impurity monitoring has the potential to both streamline the process while also providing a greater depth of detail into sample components than can be afforded by other methods. The specter of lingering HCP impurities can be minimized with the proactive comprehensive sample characterization that is met with sensitivity, reproducibility, and yet also broad sample applicability through mass spectrometry.

REFERENCES

1. FDA, Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs), January 2020.
2. European Medicines Agency, “Guidelines on the quality, non-clinical, and clinical aspects of gene therapy medicinal products,” March 2018.
3. K. Pilely, et al., “A Novel Approach to Evaluate ELISA Antibody Coverage of Host Cell Proteins - Combining ELISA-based Immunocapture and Mass Spectrometry,” Biotechnol. Prog. (2020) 36(4), 2983. DOI: 10.1002/btpr.2983



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