Biopharma Whitepaper

Advanced host cell protein (HCP) analysis with TIMS QTOF MS powers biopharmaceutical development

Introduction

The recent rapid expansion of the biopharmaceutical field is driving the growth of biotherapeutic analysis and bioprocess development. The percentage of large molecules in the pharmaceutical pipeline rose from 30% in 2010 to 42% in 2017 [1] and there are nine Food and Drug Administration (FDA) approved biosimilars on the market, five of which are from 2017. Combined worldwide sales are expected to reach almost US$125 billion by 2020 [2], and such growth will continue to help the industry address the unmet medical needs for the world's most challenging diseases. There is, therefore, an ever increasing requirement for analytical methods with rigorous impurity testing, to monitor contaminants during manufacture and prior to product release.

Impurities in biopharmaceutical products are characterized according to whether they are derived from the manufacturing process, such as during cell culture, from downstream processes or cell substrates (process-related impurities), or are non-active molecular variants arising during expression, manufacture or storage (product-related impurities). A key step in biopharmaceutical development and a requirement by the US Food and Drug Administration (FDA) is the identification and quantification of both process- and product-related impurities. Control strategies must then be established to reduce or remove impurities from the final product.

Key process-related impurities include DNA, leachables, Protein A, cell culture components, and antibiotics. Process-related impurities derived from the host organism can also contaminate drug products. For example, during the expression of a recombinant protein drug, host cell systems can express numerous endogenous proteins, known as host cell proteins (HCPs).

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These contaminants may significantly affect drug efficacy and sometimes cause immunogenicity, making their removal highly important to ensure drug safety and purity. Biotherapeutics, such as monoclonal antibodies (mAbs), are purified using chromatographic techniques designed to remove residual HCPs, DNA and viruses, as well as product-related impurities, such as fragments and aggregates. However, low level HCPs often remain after purification, so the detection and quantification of these residual impurities is critical for biopharmaceutical companies to comply with regulatory guidelines.

The amount of residual HCP in a drug product is generally considered a critical quality attribute (CQA), due to its ability to impact product safety and efficacy. HCPs are measured in parts per million (ppm), often expressed as nanograms per milligram of the intended recombinant protein. The general target for analysis is to ensure identification of HCPs at least down to the 100 ppm level. However, many contaminating enzymes in the HCP mixture can still be active at levels below 100 ppm, so highly sensitive detection technologies are, therefore, desirable for biopharmaceutical manufacturing organizations.

The effect of HCPs

There are several ways in which biotherapeutics contaminated with residual HCPs could compromise patient safety. The drug's efficacy and toxicity can be affected, and the therapeutic window in which the drug acts can be altered. Immunogenicity is the primary concern, where the HCP can invoke an unwanted immune response and cause damage to the patient, even at trace levels. For example, hamster phospholipase B-like 2 (PLBL2) protein, which can be found in some mAbs derived from Chinese hamster ovary (CHO) cell lines, elicited an immune response in patients treated with lebrikizumab at levels as low as 0.2–0.4 ppm. In ongoing clinical trials, lebrikizumab is being investigated for the treatment of severe asthma that cannot be adequately controlled by inhalable glucocorticoids. Data from the clinical studies demonstrated that ~90% of subjects developed a specific and measurable immune response to PLBL2, although the observed response showed no direct link with any clinical adverse effects [3].

The stability of biotherapeutics may also be compromised by the presence of residual HCPs. They can contain enzymes, such as oxidases and lipases, which gradually break down the therapeutic protein or excipient, affecting the stability of the product over time. For example, presence of residual proteases in the drug substance can significantly influence the long-term storage stability [4]. HCPs are also capable of mimicking the action of therapeutic proteins in assays, possibly resulting in the misformulation of the product outside the therapeutic window [5].

Analytical tools for HCP evaluation

HCP-related risks can be controlled either during cell line and upstream process development, or by robust downstream processes, to minimize their impact on product quality, safety and efficacy. The 'gold standard' technique for HCP analysis is the enzyme-linked immunosorbent assay (ELISA), which is a commonly-used immunoassay for measuring antibodies, antigens, proteins and glycoproteins in biological samples.

Despite its high throughput, sensitivity and selectivity capabilities, the ELISA can overlook weak and non-immunoreactive HCPs, which still carry the potential to impact drug safety and efficacy. It is also process-specific, meaning a new assay is required after each process change, which may take several months.

Recent developments in mass spectrometry (MS)-based techniques have enhanced downstream process development activities to remove high-risk HCPs [6]. MS can rapidly monitor and identify multiple protein analytes in the same sample, and detect very low amounts of HCP in a non-targeted manner. This is crucial, considering that even very low level HCPs can cause immunogenicity and impact drug quality. Whereas an ELISA can only measure the total HCP, MS provides detailed information on the level of each individual HCP. Advanced MS techniques not only monitor, but characterize several impurities using a single method, bringing the high discriminatory power needed to separate impurities, while providing high sensitivity to detect and quantify low level HCPs.

In-depth analysis with MS

MS-based HCP analysis provides a high confidence identification, enabled by accurate mass, tandem MS sequencing (MS/MS), and retention time. Although MS is gaining popularity as a powerful technique, there are still some limitations to overcome. Basic MS-based methods are well-suited to detect qualitative differences in HCP populations derived from early purification stages, but their identification in highly purified biotherapeutics is far more challenging, as HCPs are present in trace amounts. Additionally, if HCPs co-elute with the biopharmaceutical, a mass spectrometer with up to six orders of magnitude dynamic range is required to directly detect 1 to 100 ppm of HCPs in the sample, which is out of the range of many current instruments [7]. One way of overcoming the issue of dynamic range for HCP characterization is to add another dimension of separation, such as two-dimensional liquid chromatography (2D-LC), which has shown to increase sensitivity and resolution, but results in longer analysis times compared with one-dimensional LC (1D-LC) [8].

Alternatively, additional dimensions of separation can be achieved using ion mobility MS. Some modern instruments use trapped ion mobility spectrometry (TIMS) quadrupole time-of-flight (QTOF) MS to achieve high confidence in identification and deep coverage, without compromising speed. TIMS is a separation technique in gas phase, which resolves sample complexity with an added dimension of separation, in addition to high performance liquid chromatography (HPLC) and MS. This method increases peak capacity and confidence in compound characterization and, combined with parallel accumulation–serial fragmentation (PASEF) technology, enables high sequencing speeds while generating high quality spectra. TIMS separates ions based on collisional cross section (CCS) using two TIMS cells in series, making it possible to focus precursor ions in a small time window with a specific mass-to-charge ratio (m/z) and CCS value using PASEF scan mode. The high resolving power of TIMS MS makes it useful for the analysis of complex samples, such as those of biological origin.

PASEF scans implemented on TIMS QTOF MS/MS can combine fast acquisition speeds (>100 Hz MS/MS) with high resolution detectors, for high resolution quantifiable data from a complex sample, in a single run. When coupled with an advanced HPLC system, such as the Evosep One (Evosep Biosystems, Denmark), TIMS QTOF MS/MS with PASEF can detect HCPs using a gradient as short as 21 minutes. PASEF enables almost 100% duty cycle, for maximum use of all ions and therefore superior sensitivity.

There are two approaches for HCP analysis by MS:

1. HCP discovery

This method is useful in early process development, where critical problems arise in production or stability testing. HCP discovery uses longer runs for increased depth of analysis, typically two hours or more. Quantitation is not always necessary, as the primary concern is identifying all the proteins in the mixture. Maximum depth of coverage can be achieved, to identify low level proteases/lipases that can affect stability, as well as other problematic proteins. Hundreds of peptides can be identified in a single run (Figure 1).

For a 1.5 µg NISTmAb tryptic digest, nanoLC provides a 210 minute gradient for deep coverage of HCPs. Sample preparation can either be a standard tryptic digest of the entire mixture (220 HCPs identified), or a native tryptic digest (using protocol from [7]), where the mAb is left in its native structure, which provides a better dynamic range (280 HCPs identified).

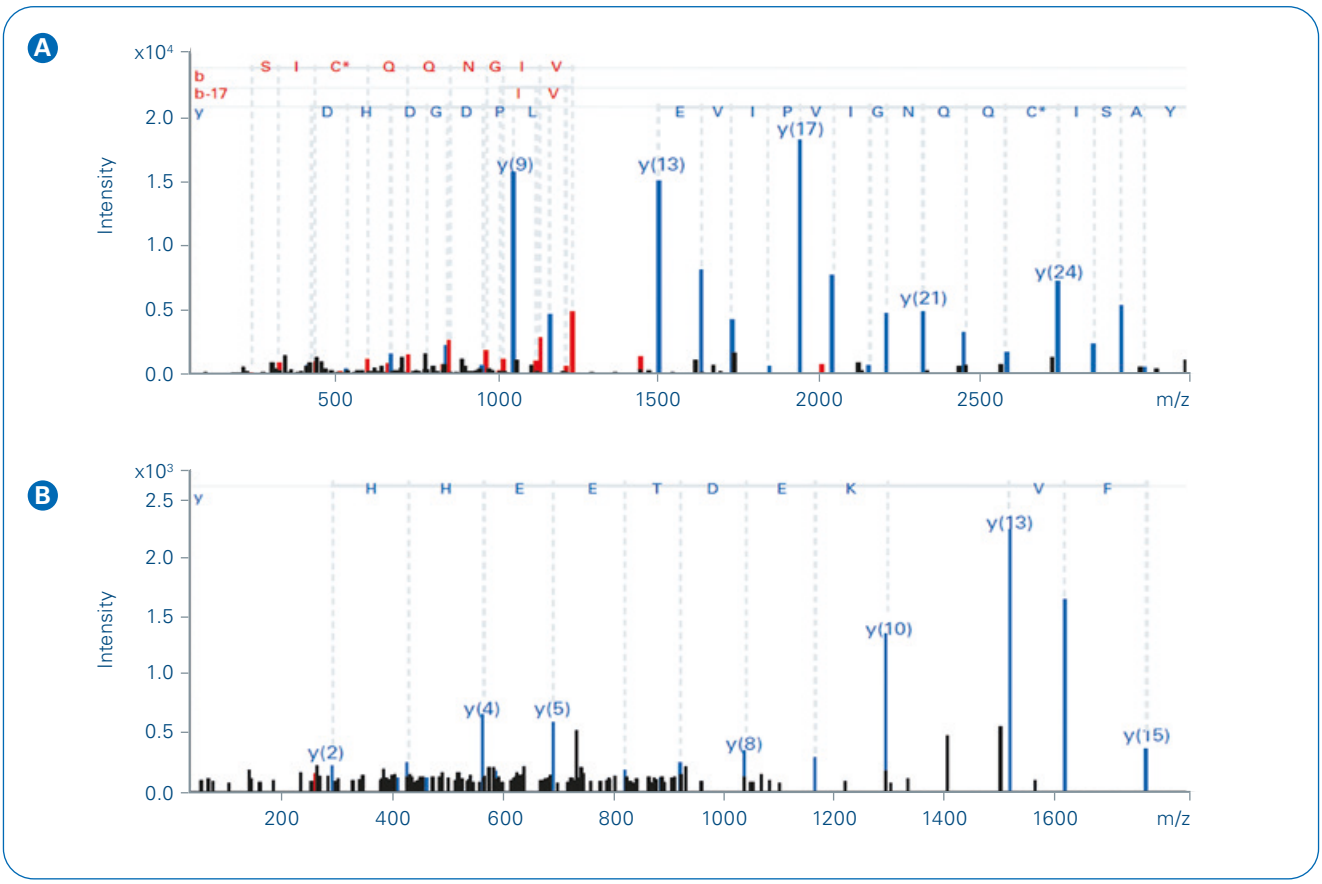

Figure 1.A,B: High quality TIMS QTOF MS/MS with PASEF spectra (timsTOF Pro, Bruker Daltonics), featuring high sequencing speeds of >100 Hz and sensitive detection of low abundant proteins A *Fructose-biphospahte adolase A and* B *Hetergeneous nuclear ribonucleoprotein A1.*

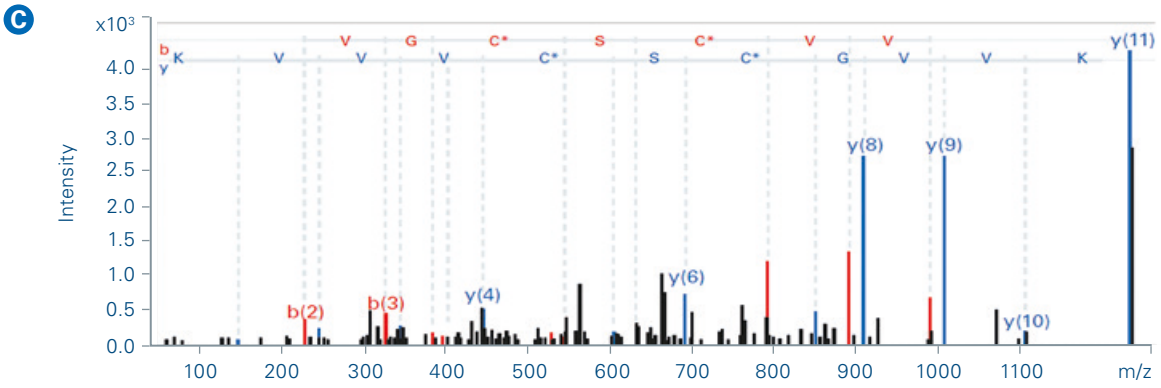

Figure 1.C: High quality TIMS QTOF MS/MS with PASEF spectra (timsTOF Pro, Bruker Daltonics), featuring high sequencing speeds of >100 Hz and sensitive detection of low abundant proteins **C** *40S ribosomal protein S12.*

2. Fast HCP screening

This method provides rapid analysis of downstream processes, with fast runs and high throughput, enabling batch-to-batch fingerprinting. Quantitation is often necessary. Faster analysis, enabled by the Evosep One chromatographic system [9] combined with Bruker's timsTOF Pro, is advantageous for more routine applications, but coverage of 50+ HCPs is still attainable at 1% false discovery rate (FDR) using a 21 minute gradient (Figure 2).

Description	Coverage	#Peptides	#Unique	Avg. Mass
Fructose-bisphosphate aldolase A OS=Mus m...	74%	35	30	39356
Glucose-6-phosphate isomerase OS=Mus mus...	46%	26	26	62767
Fructose-bisphosphate aldolase C OS=Mus m...	53%	18	13	39395
Semaphorin-4B OS=Mus musculus GN=Sema...	10%	7	7	91392
Ig gamma-3 chain C region OS=Mus musculu...	7%	7	6	43929
Protein ABHD11 OS=Mus musculus GN=Abhd1...	35%	6	6	33561
Protein disulfide-isomerase A6 OS=Mus musc...	13%	4	4	48100
Low affinity immunoglobulin gamma Fc region...	11%	4	4	36695
Polypeptide N-acetylgalactosaminyltransferase...	7%	3	3	71537
Syntaxin-12 OS=Mus musculus GN=Stx12 PE=...	18%	3	3	31195
NSFL1 cofactor p47 OS=Mus musculus GN=Ns...	16%	3	3	40710
Fumarate hydratase, mitochondrial OS=Mus ...	9%	3	3	54357
Methionine--tRNA ligase, cytoplasmic OS=Mus...	5%	3	3	101431
Nucleoside diphosphate kinase B OS=Mus mu...	26%	3	3	17363
Adenylate kinase 2, mitochondrial OS=Mus m...	21%	3	3	26469

Figure 2: Host cell proteins (HCPs) identified in 21 minutes with 3 or more peptides. Data were acquired on the timsTOF Pro (Bruker Daltonics) applying a 0.5 sec cycle consisting of 1 TIMS MS scan and 4 PASEF MS/MS scans.

Nanoflow LC-MS (nanoLC-MS) is gaining ground in proteomics applications, including biomarker discovery and validation. Its advantages over traditional analytical flows, including enhanced sensitivity, make nanoLC-MS an ideal tool for detecting low abundant HCPs. Some challenges still remain, however. Samples are usually injected into an LC system to separate the peptides using nanoflow chromatography, but alternative methods may be more suitable for biopharmaceutical applications because nanoflow can pose some practical challenges. By partnering with chromatography experts Evosep, the robustness and speed of the timsTOF Pro can be maximized. Evosep has developed a robust, high-throughput LC system, the Evosep One, with moderately low flow rates that are ideal for HCP analysis.

Both HCP discovery and fast HCP screening methods provide unbiased analysis of proteins, accurate mass measurement and, because of the high resolution of both MS and MS/MS, isotopic fidelity. They also provide CCS values for a new dimension of confidence. These are physical parameters of peptides, often more reproducible than LC retention times. Novel MS instrumentation, such as the timsTOF Pro with PASEF, is now enabling users to combine HCP discovery and screening in one workflow, and accurately and reproducibly measure CCS values. Highly reproducible CCS values eliminate the need to include retention times (RT) in confidence measurements, enabling easy changing of LC methods, columns, flows, and other instrument parameters.

In instances that require deeper coverage of HCPs, samples can be analyzed using a standard proteomics set-up using nano ultra-high performance liquid chromatography (UHPLC).

Optimizing acquisition methods

Historically, data-dependent acquisition (DDA) MS methods have been extensively used in proteomics and biopharmaceutical workflows. In DDA methods, the peptides to be identified are selected by algorithms that locate the peaks in the MS spectrum and then use criteria such as peak intensity, charge state, and m/z to automatically calculate which peptides to target for MS/MS identification. Such algorithms generally also use dynamic exclusion lists so that once a peptide is targeted and fragmented for MS/MS, time will not be wasted in targeting it again.

An advantage of DDA methods is that the mass of the peptide targeted for identification is known and may be used in subsequent identification using the MS/MS data. However, the algorithms that perform the DDA selection rely on the MS data to select what is targeted, and biological variation in the samples as well as technical variations between runs may cause a peptide selected in one run to be missed in a subsequent run.

To overcome these issues, a data-independent acquisition (DIA) approach was proposed, in which, rather than selecting a single ion, all of the ions in a mass window are selected for fragmentation. If the windows are adjacent and scanned, then the DIA approach is deterministic – in principle every peptide eluting from the chromatographic separation will be fragmented.

The drawback of DIA experiments is that many peptides will be fragmented at once and it can be complex to determine which of the fragments belong together and came from a single precursor. However, software packages capable of deconvolution are improving rapidly, and as a result DIA experiments are becoming more common.

The introduction of fast, sensitive QTOF instruments with TIMS has enabled the development of MS acquisition methods to optimize HCP analysis. DDA experiments on instruments such as the timsTOF Pro are performed with unprecedented MS/MS speed and sensitivity, and provide an additional dimension of separation that assists in uncovering isobaric species with the same elution profile, using the PASEF method. The same speed and sensitivity advantages apply to DIA experiments, termed diaPASEF [10], and in this case the additional mobility dimension results in more efficient usage of the ion current, and also provides an additional chromatographic dimension that can be used in MS/MS data alignment to connect fragments to precursors with high confidence.

Continuing innovation

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The low abundance of HCPs continues to present a challenge to the biopharmaceutical industry, and work on the removal of impurities has traditionally required specialized set-ups. PASEF scans implemented on advanced TIMS TOF MS instrumentation can be applied to HCP analysis using both routine analytical or nanoflow configurations, to achieve sensitive detection with enhanced speed and data quality.

The depth of HCP identification provided by PASEF technology allows fingerprinting of biomanufacturing processes and the ability to easily identify the effects of changes in these procedures. Sensitive, rapid, in-depth techniques for HCP analysis are driving the field of biotherapeutics, which is now transitioning into a new phase, thanks to decreased run times conferred by PASEF implemented on the timsTOF Pro. Such technologies are enabling biopharmaceutical manufacturers to accurately identify and quantify HCPs as CQAs and, therefore, comply with increasingly stringent regulatory guidelines.

For more information on the power of PASEF for deep HCP coverage, please visit www.bruker.com/timstofpro

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