"The BioPharmaSpec Approach": Mass Spectrometry Based Host Cell Protein Identification and Quantitation

1. Introduction

As part of the development of any biopharmaceutical product, the impurities present must be examined, minimized and where possible characterized (1). These impurities fall into two broad categories: product-related impurities (derived specifically from the drug product itself) and process-related impurities (derived from material associated with the production, processing or purification of the sample). Host cell proteins (HCPs) are process-related impurities that require specific analysis due to the multitude of naturally occurring proteins expressed in the production cell line.

The analysis of HCPs has historically been performed using ELISA-based tests. A number of off-the-shelf products (e.g. Cygnus kits) are available and the testing is relatively straightforward. It has been recognized for some time that these kits generally allow detection of a maximum of 60 to 70% of the HCPs present in biopharmaceutical products. The level of coverage can be improved to some extent with the use of in-house developed, product-specific HCP-ELISAs, but a significant number of HCPs are still likely to be missed. This is due to the fact that it is not possible to produce antibodies covering the entire spectrum of HCP species in any anti-HCP antibody pool. Very weakly immunogenic, or non-immunogenic species will not be detected.

In contrast to HCP-ELISA, which relies on the presence of polyclonal antibodies for positive HCP detection, Mass Spectrometric (MS) approaches have the distinct advantage of detecting a wider population of HCPs in a *de novo* manner, without prior knowledge, specialized polyclonal antibody reagents or product/ process specific method development. With this capability and the continued availability of new, more capable MS technologies, and more intuitive, specialized software, groups in the biopharmaceutical industry have been progressively adopting MS as an orthogonal approach to HCP analysis. In turn this trend has swayed regulators worldwide, who welcome HCP assessments using the more decisive analytical technique of MS. It is envisioned that the use of MS as an orthogonal approach to HCP-ELISA is likely to become an expected exercise as part of the normal process and product development workflow.

2. The BioPharmaSpec Approach

BioPharmaSpec has developed a service for MS based HCP detection and quantitation as a support to HCP-ELISA assays. The service is set up as a phased approach with each phase of the analysis designed to provide increasing depth information and ultimately building a detailed qualitative and quantitative understanding of the HCP profile of the sample.

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a. Phase I – Qualitative analysis to identify HCPs present in samples

The initial work focuses on the analysis of samples separated out into their constituent components using 1D or 2D gel electrophoresis. Gels are stained with a MS compatible stain, the spots or bands excised and then subjected to in-gel proteolytic digestion followed by extraction of the peptides. These peptides are analyzed using Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS). So called "MS/MS" analyses are performed within the mass spectrometer on the mixture of peptides from each spot to generate fragment ion data from mass selected peptides, which allows precise amino acid sequence information to be determined (Figure 1).

In parallel with the MALDI-MS analysis, the mixture of peptides is also analyzed by electrospray ionization through an on-line UPLC system coupled to one of BioPharmaSpec's several state-of–the-art Waters Xevo G2 and G2-S Q-TOF mass spectrometers (on-line LC/ES-MS). This coupling of chromatographic and mass spectrometric analyses serves to provide extended and supportive structural data to that obtained by MALDI-MS. Again, MS/MS analyses are performed through data-dependent acquisition (DDA) and/or advantage can be taken of the unique ability of the Q-TOF instrument's so called high energy fragmentation, MS^e, capability (Figure 1).

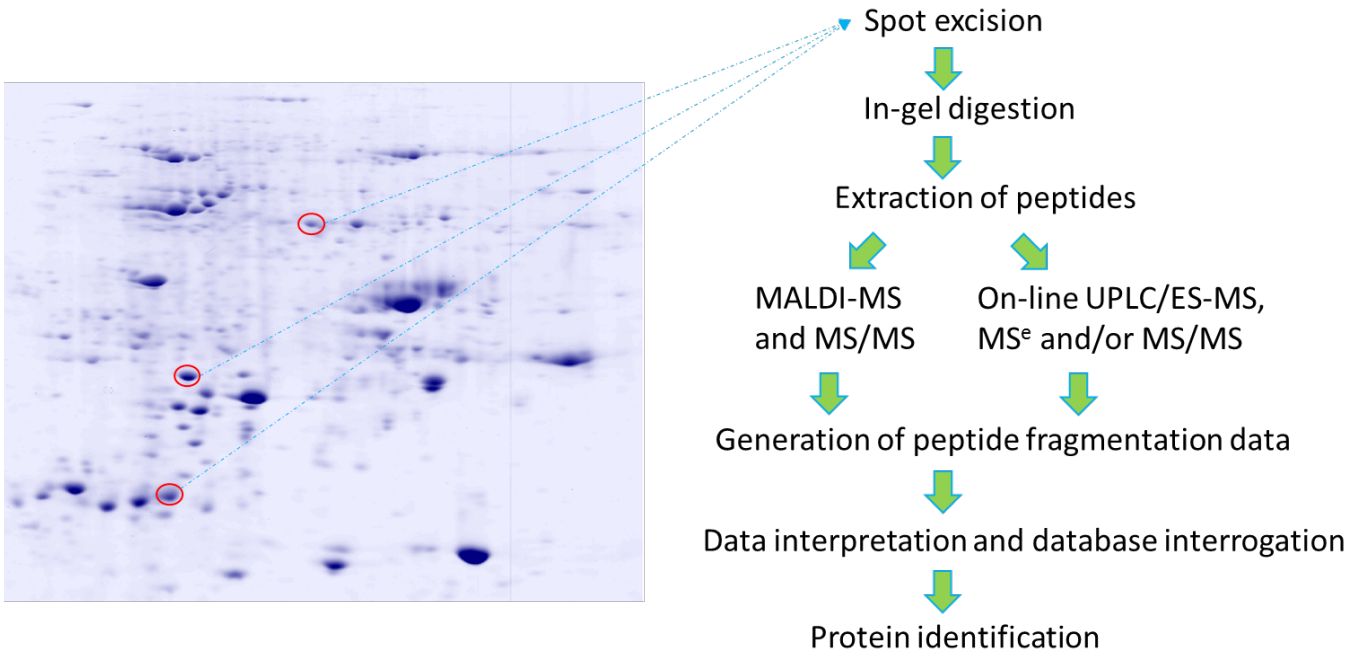

Figure 1: Phase I workflow: from 2D gel separation of a protein mixture through to protein identification

The fragment ions generated from either or both approaches are then used for database interrogation and identification of each protein (Figure 2).

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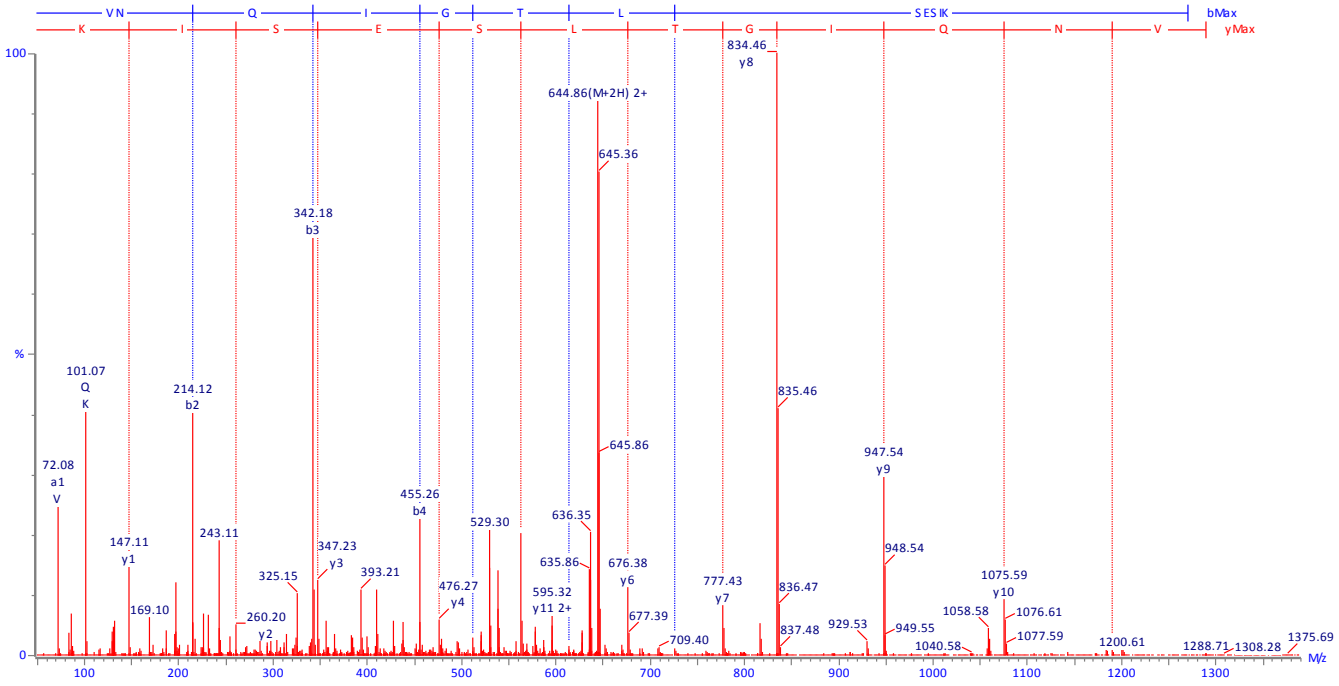

Figure 2: Automated sequence analysis of MS/MS generated fragment ions from a peptide during on-line UPLC/ES-MS analysis

The primary phase of the work provides important information on the identity of HCPs present in the sample. Ideally analysis should also be performed in parallel with the Drug Product on a full HCP extract and a HCP extract that has been through the full downstream process. This allows an assessment of any HCPs that may be masked by the significantly higher level of the protein product in the sample.

b. Phase II – Quantitation against the response from a known protein spiked into the sample

During HCP analysis, it is not only important to identify proteins in a qualitative sense but also assess their quantities. This can be achieved through a tiered analytical approach using either a relatively simple comparison to a marker protein(s) or a more detailed and accurate analysis against a selection of isotopically labeled peptide markers. The lower level of quantitative analysis, i.e. relative to a marker protein(s), is considered here as Phase II.

Following assessment of the results from Phase I, digestion of the protein mixture followed by on-line UPLC/ES-MS/MS and/or MS^e analysis of the products is repeated with the addition of a suitable protein (or mix of proteins), to estimate the levels of protein impurities in the sample.

It must be noted that quantitation against a protein standard will provide a quantitation estimate relative to the protein standard or mixture. This estimate will be compromised to an extent by the differences in ionization efficiency between the various peptides in the sample and reference protein mixture, something that cannot be taken into account in the quantitation calculation. As

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such, this form of quantitative analysis will only provide a basic level of quantitation. This may be sufficient for, for example, comparative analysis of different sample preparations to assess the relative merit of different processing regimes or stages in the sample purification. However, the data generated would not be sufficient for provision of accurate quantitative information on the amounts of HCPs present. For this reason BioPharmaSpec also recommends using a more accurate quantitation method to quantify the HCPs of concern or of particular interest. This higher level of quantitation is covered in Phase III.

c. Phase III – Quantitation against heavy labeled marker peptides

The data obtained from Phases I and II are used to identify marker peptides for the HCPs of interest. These marker peptides are those that fulfill certain criteria such as an absence of chemically reactive sidechains, which would serve to alter the concentration of the peptide should they undergo any modification, such as oxidation. These chemically stable peptides are then synthesized as heavy labeled analogs (i.e. having exactly the same chemical properties, including ionization potential, as the identified marker peptides). Known amounts of these isotopically labeled peptides are then spiked into samples of interest. Triple Quadrupole mass spectrometry is then used to develop Multiple Reaction Monitoring (MRM) transitions for the native and heavy labeled peptides. These transitions represent a parent to fragment (daughter) ion transition. The sensitivity of HCP detection and quantitation is based on these MRM transitions. Figure 3 shows an example of a Q-TOF generated MS^e spectrum with the unique transition from the parent ion at m/z 957.5 to a daughter ion and m/z 715.4, which was subsequently used for identification and quantitation of the peptide of interest.

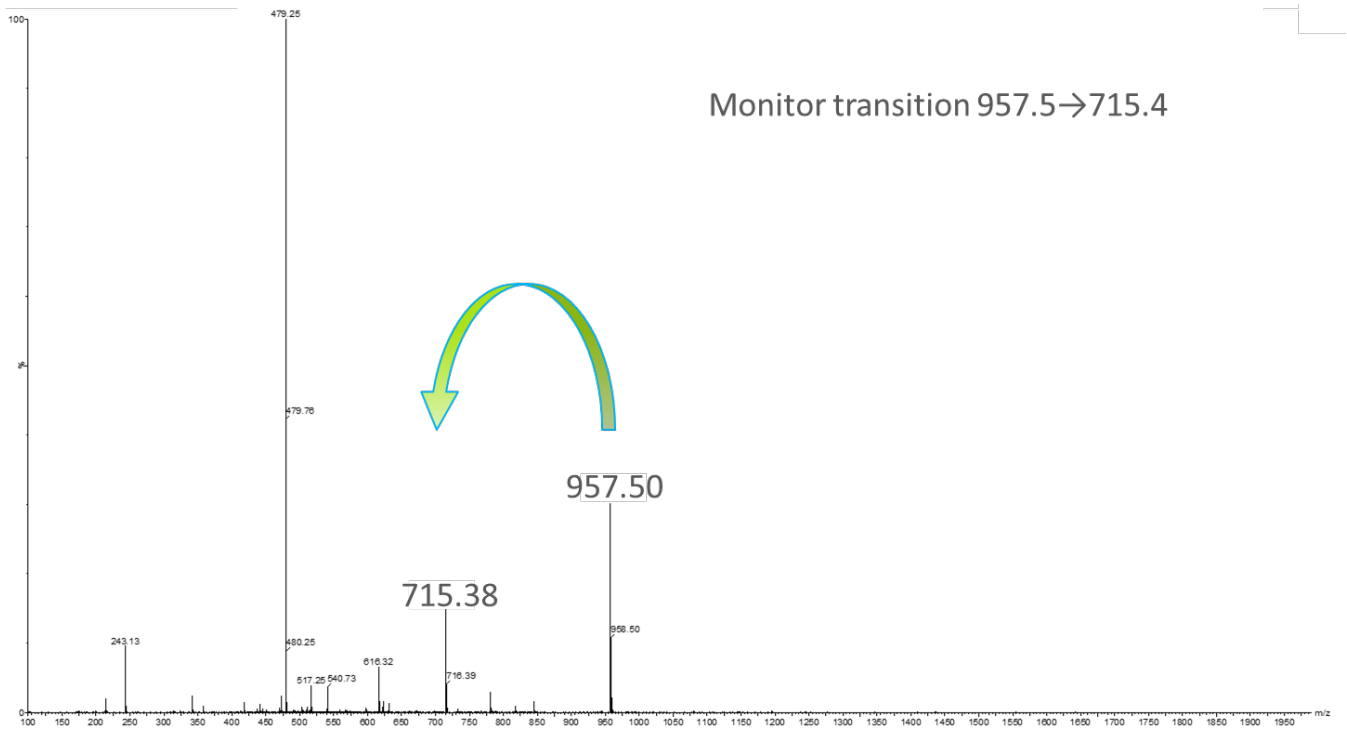

Figure 3: Waters Xevo G2 Q-TOF fragmentation data showing intact peptide and fragment ions signals selected for MRM transition monitoring

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More than one peptide should be monitored from each HCP of interest, to provide statistically meaningful data. At BioPharmaSpec we use a Waters Xevo TQ-S tandem quadrupole mass spectrometers to perform these quantitative analyses. This allows for high-speed mass switching and monitoring of multiple peptides at any one time, giving high detection coverage and a large dynamic range for quantitation. Amounts of the protein of interest are then calculated based on a comparison of the response from the native and heavy labeled peptide analogues. This technique is very selective and sensitive due to the mass filtration of extraneous signal noise and high sensitivity of the MRM monitoring. The analysis provides the ultimate mass spectrometric approach for HCP analysis.

3. Summary

The regulators are increasingly requesting an orthogonal approach to HCP analysis. BioPharmaSpec offers a detailed and thorough mass spectrometry based analytical package to fulfill this requirement. MS based HCP analysis should not be seen as a replacement to HCP-ELISA tests, rather it is an additional set of studies to extend the range of HCPs that can be detected and analyzed, providing the fullest picture possible of the product's HCP profile.

BioPharmaSpec can provide a full service for MS analysis of HCPs including accurate detection of HCPs of interest i.e. those present in amounts >100ng per mg of product or those that are immunogenic.

HCPs should be assessed from early in the product development. It can then be shown that improvements in, for example, the downstream purification through process development are having a positive impact on the levels of HCPs present.

BioPharmaSpec scientists are highly experienced in all aspects of mass spectrometry-based HCP analyses, from qualitative identification of HCP proteins to detailed isotope-based quantitative HCP determinations using multiple marker peptides.

About BioPharmaSpec

BioPharmaSpec is a Contract Research Organisation (CRO) specializing in structural characterization and physicochemical analysis of recombinant biopharmaceuticals including monoclonal antibodies and associated products, Antibody Drug Conjugates (ADCs), blood circulating hormones and Biosimilars with a focus on *Discovery*, *Early* and *Pre-clinical* phases.

BioPharmaSpec was formed by Prof Howard Morris (with 45 years of experience in biopolymer characterization and founder of the M-Scan group of companies) and Dr. Andrew Reason (with 25 years of experience in commercialization of analytical methods for characterizing biopolymers and previously Group Manager of the European M-Scan laboratories and SGS M-Scan Europe).
With over 45 years of experience in the fields of Mass Spectrometry and Liquid Chromatography our teams of expert scientists, from both academic and industrial backgrounds, are skilled in the analysis of Biopharmaceutical products and the interpretation of novel and unusual structures,

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including post-translational modifications (PTMs). BioPharmaSpec can therefore play an important enabling role in your own research and product development.

Our scientists pioneered the now well-accepted strategies of mass spectrometric Peptide Mapping [2,3,4], the use of mass spectrometry in Disulfide Bridge Assignment [5,6] and the discovery and characterization of Glycosylation in natural and recombinant proteins [7,8,9,10,11]. Our scientists continue to be involved in cutting edge discovery research and development as illustrated by (i) the characterization of 24 sites of N-linked Glycosylation in virion-derived gp120 from HIV-1 [12], (ii) the discovery of novel glycoprotein epitopes in hypervirulent strains of *C. difficile* and their characterization as sulfonated peptidylamidoglycans [13] and (iii) the conceptualization and development of the Q-TOF mass spectrometer, now a cornerstone of bioanalytical research [14].

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