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Review On Recent Analytical Advances In Evaluation Of Impurities And Host Cell Proteins For Biosimilar.

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Abstract

Background: Biologics, derived from living organisms, are pivotal in treating life-threatening conditions such as cancer and autoimmune diseases. This article aims to provide a comprehensive overview of biologics and biosimilars, emphasizing their types, molecular characterization, and recent analytical advancements.

Main Body of the Abstract:

This review addresses:

Introduction to Biologics: Covering their diverse nature and applications in treating various illnesses.

Types of Biologics: Expanding on monoclonal antibodies, recombinant DNA technology products, and biological response modifiers and their therapeutic significance.

Introduction to Biosimilars: Discussing the challenges in replicating these drugs due to inherent variability and complex manufacturing processes.

Molecular Characterization of Biosimilars: Exploring primary and higher-order structures, glycosylation, product-related variants, and process-related variants as key areas of analysis.

Recent Analytical Advances: Highlighting advancements in techniques such as liquid chromatography-mass spectrometry (LC-MS), two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and enzyme-linked immunosorbent assay (ELISA) for host cell protein (HCP) analysis.

Short Conclusion : Recent analytical advancements address limitations in HCP detection and identification, contributing significantly to ensuring the quality, safety, and efficacy of biologics in the pharmaceutical industry.

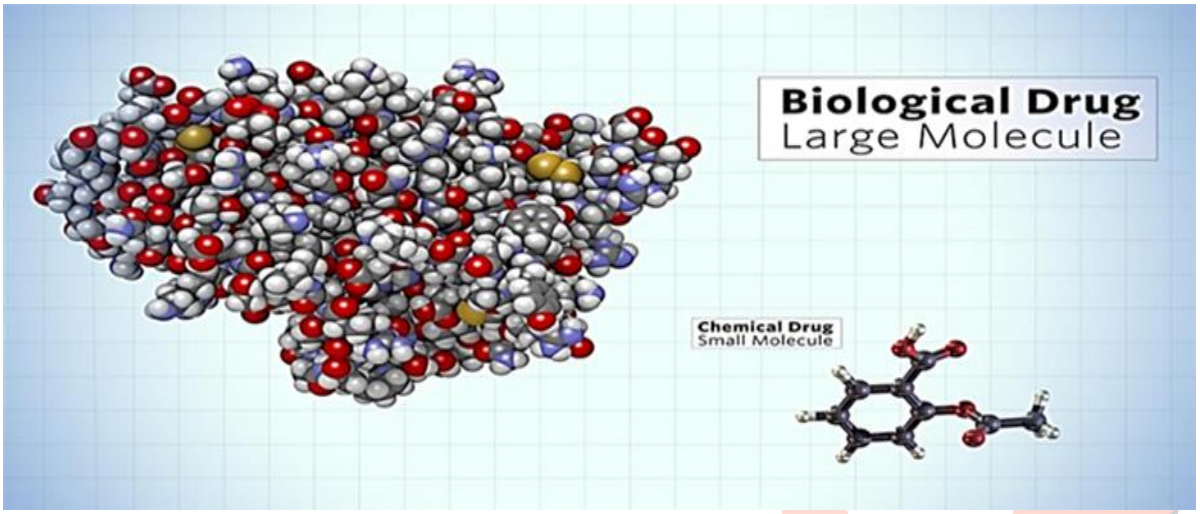
Keywords:

Biosimilars, Molecular Characterization, Host Cell Proteins (HCPs), ELISA, 2D-PAGE, LC-MS, MWCO, AAE, LC-MS/MS, Data-independent Acquisition (DIA)

Background

Introduction to Biologics

Biologics, or biological pharmaceuticals, are medications created using biotechnology from living systems like animals, plants, or microbes. they are larger, more complex molecules used in treatments for life-threatening and rare illnesses like cancer, diabetes, anaemia, rheumatoid arthritis, and multiple sclerosis.[1]



(Fig 1. Small molecules versus large biologic molecule) [2]

Types of Biologics

Monoclonal Antibodies [3]

Monoclonal antibodies (mAb) are monospecific and homogeneous antibodies produced by a single B cell clone, making them useful in development of therapy and diagnostics.

Product obtained by recombinant DNA technology [4]

Recombinant DNA (rDNA) molecules, formed through genetic recombination, are created by combining genetic material from multiple sources, requiring expertise in molecular biology, microbiology, analytical chemistry, enzymology, and immunology.

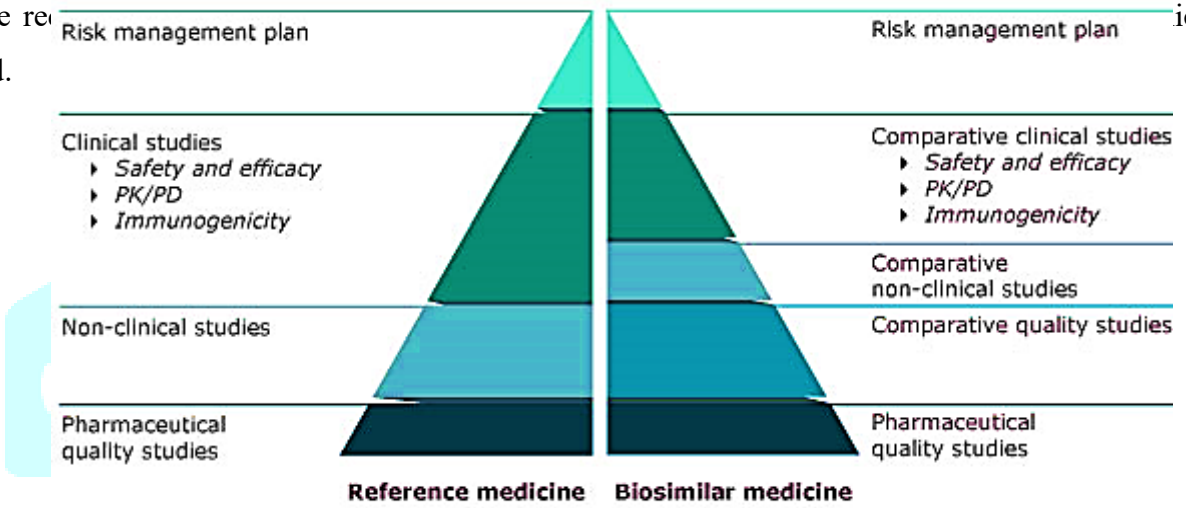
Biological response modifier [5]

Biological response modifiers (BRMs) are substances that modify immune responses, either endogenously or as pharmaceutical drugs. They can enhance or suppress immune responses, serving as immunomodulators in immunotherapy, such as cancer treatment and autoimmune diseases like arthritis and dermatitis. Most BRMs are biopharmaceuticals, including TNF, Lymphotoxin, interleukin 2, interferons, and colony-stimulating factors.

Introduction to Biosimilar [6-7]

Biosimilars are biological medicines that closely resemble existing EU-approved reference medicines in terms of structure, activity, efficacy, safety, and immunogenicity. The first biosimilar was approved in 2006, but are not considered generics due to their inherent variability and complex manufacturing. Biosimilars, derived from

living systems, are difficult to produce identical copies due to differences in manufacturing processes and molecular differences in clinically inactive components. Examples include glycosylation, which involves the attachment of sugar residues to amino acid chains containing amino or hydroxyl groups, and post-translational protein modifications (changes to the C or N terminals). Comparing biosimilars to the reference biologic, it is possible to find differences in the 3D structure, isoform patterns, and protein aggregation. These variations are not clinically meaningful in terms of efficacy and safety. The FDA requires pharmacodynamic, pharmacokinetic, and immunogenicity studies to demonstrate biosimilarity, but biosimilars must have identical therapeutic amino acid sequences. The ultimate goal is to demonstrate biosimilarity, not reestablish primary efficacy and safety. Analytical studies are conducted to understand the biosimilar molecule's physical characteristics, followed by preclinical and clinical studies. If uncertainty remains, additional clinical studies may be re ical trials is needed.



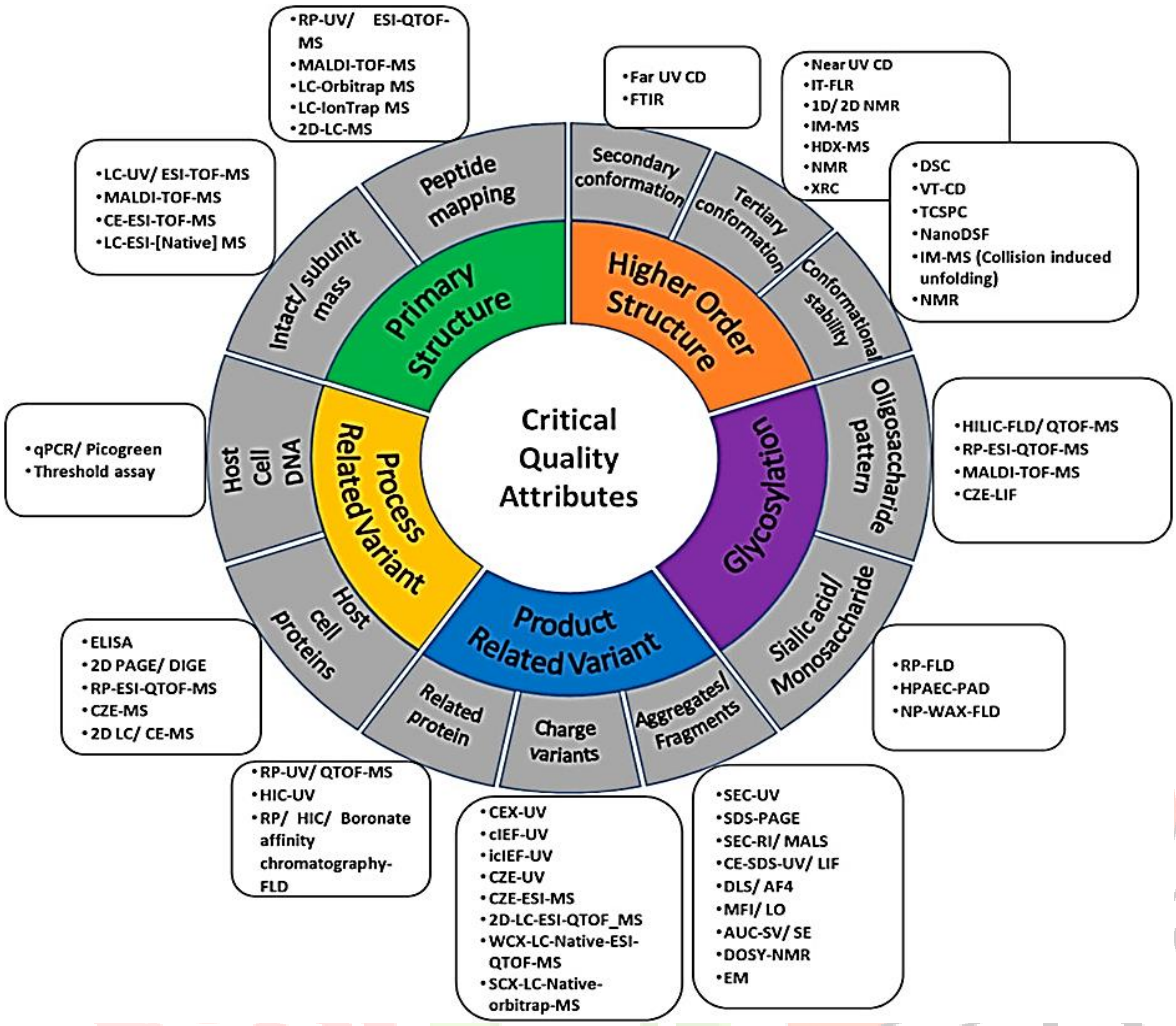
(Fig 2. Comparison of data requirements for approval of a biosimilar versus the reference innovator medicine) [8]

Main Text

Molecular characterization of biosimilars

Biosimilars have structures similar to the original drug, but may have minor differences in physiochemical properties due to the complexity of the protein structure and manufacturing process. Comparability studies are necessary to demonstrate similarity between biosimilars and their originator, focusing on variations in critical quality attributes within the innovator's range.[9]

(Fig 3. Different critical quality attributes i.e.., primary structure, higher order structure, glycosylation, product -related and process-related variant used in analytical similarity assessment) [9]



Primary structure [9-16]

Primary structure analysis involves peptide mapping of the amino-acid sequence and measuring the exact mass of a biosimilar compared to the innovator product. Amino acid sequence identity can be established using Edman degradation and mass spectrometry (MS). MS has become the more popular technique for both sequence and mass identity due to its sensitivity, versatility, fast turnaround, and enhanced resolution. Platforms for primary structure analysis include MALDI-TOF-MS, RPESI-QTOF-MS, RP-ESI-Ion Trap/Orbitrap/Q-Exactive-MS, and LC-ESI-QTOF-[Native] MS. Intact mass analysis assesses a protein's total molecular weight without prior digestion or fragmentation. Recent advancements in LC-ESI-[Native] MS-based methods have provided an orthogonal alternative to traditional denaturing RP-ESI-MS. Peptide mapping investigates differences in protein sequences and PTMs between the biosimilar candidate and the innovator product. Data independent acquisition reverse-phase LC-MS with alternate low and elevated collision energy scanning (LC-MSE) offers additional benefits for peptide maps.

Higher order structure [17-21]

Higher-order structure (HOS) refers to three-dimensional (3D) conformations in biosimilars, which can be compared using various orthogonal tools. These tools include CD, FTIR, and fluorescence spectroscopy. The secondary structure of biosimilars can be determined through the overlay of far UV CD spectra, while FTIR provides information on the vibrational states of molecules. Tertiary structures are 3D conformations, and near UV CD and fluorescence spectroscopy are traditionally used for tertiary structure determination. X-ray crystallography (XRC) is limited due to its high cost and time requirements. The evolution of HOS assessment in biosimilarity studies has led to the use of multiple orthogonal techniques, such as single dimension NMR, multi-dimension NMR, HDX-MS, IM-MS, antibody conformational array, aptamer-based enzyme linked apta-sorbent assay, and small angle X-ray scattering (SAXS). Confirmational studies measure conformational stability using thermal denaturation by variable temperature (VT)-CD, differential scanning calorimetry (DSC), and time-correlated single-photon counting (TCSPC).

Glycosylation [22-24]

Glycosylation is an enzymatic PTM in eukaryotic proteins, including antibodies and glycoproteins. Glycan profiling uses a multi-level technique to analyze levels for glycoprotein, glycopeptides, and released glycans. N- or O-linked glycans are common in biopharmaceuticals and account for post-translational modifications. The ability to produce true biogenerics or biosimilars depends on accurately and quantitatively duplicating the nature, location, and levels of various glycans at specific amino acid locations.

Product related variants and impurities [9,25]

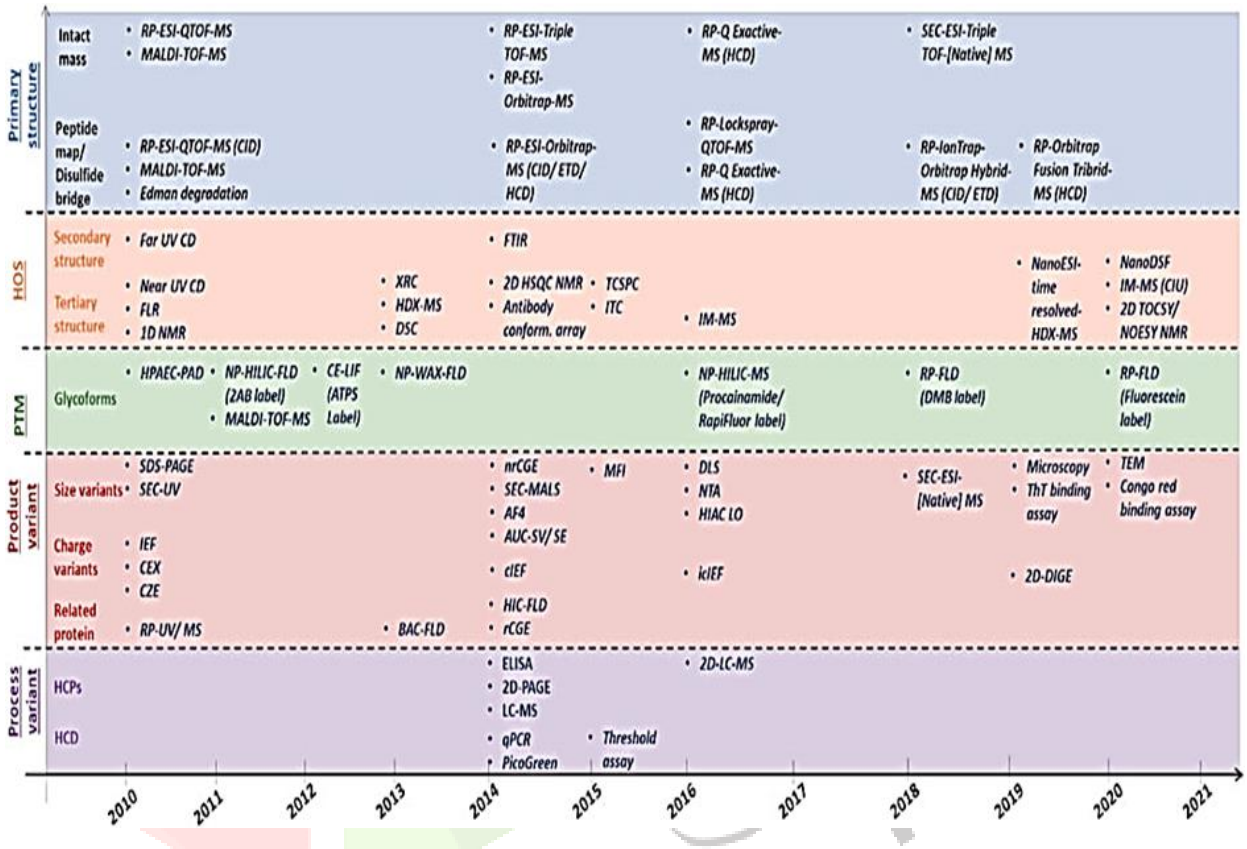
Product Related Variants (PRVs) are formed during the manufacturing, handling, and storage of biosimilars, mainly consisting of size-based heterogeneities like aggregates or fragments, charge variants (acidic and basic variants), and Related protein. Aggregation/fragmentation occurs due to protein unfolding of hydrophobic patches, which can trigger an immune response if present in significant amounts. Size exclusion chromatography (SEC)-UV is the best approach for evaluating these variants, offering quick analysis, resolution, robustness, and repeatability. Charge variants are proteoforms produced at different stages of the production process in distinct colloidal matrices. Non-enzymatic PTMs include modifications such as oxidation, phosphorylation, sulfation, acetylation, methylation, and hydroxylation. RP-HPLC with UV/FLD is the preferred method for quantifying oxidized and reduced species, while hydrophobic interaction chromatography (HIC) and Boronate affinity chromatography (BAC) are less widely used for the separation of product-related variants.

Process related variants [9,26]

Process Related Variants, including Host cell proteins (HCPs) and Host cell DNA (HCD), are crucial for product safety and efficacy. Enzyme-linked immunosorbent assay (ELISA) and real-time or quantitative PCR are used for HCP and HCD detection and quantitation. Low levels of HCPs can cause product fragmentation, immunogenic responses, and formulation changes, affecting product shelf life. Proteomic techniques like 2D-gel electrophoresis and LC-MS/MS-based platforms are replacing qualitative approaches.

Evaluation of host cell proteins (HCPs) [27-28]

Residual host cell proteins (HCPs) are impurities derived from cell substances used to produce target proteins, which must be monitored for clinical safety due to potential immunogenicity or proteolytic activity. Evaluation includes visualisation, identification, and quantification. HCPs can be monitored in both upstream and downstream processes, revealing potential protein abundance and potential interactions with APIs. Monitoring HCPs helps determine purification strategy adjustments and alternative methods. USFDA guidelines recommend orthogonal analytical platforms for comparative analytical assessment of biosimilars, with the development of methods and techniques being a burgeoning field.



(Fig 4. Evolutionary timeline of analytical platforms used for host cell proteins with respect to analytical bio similarity assessment) [9]

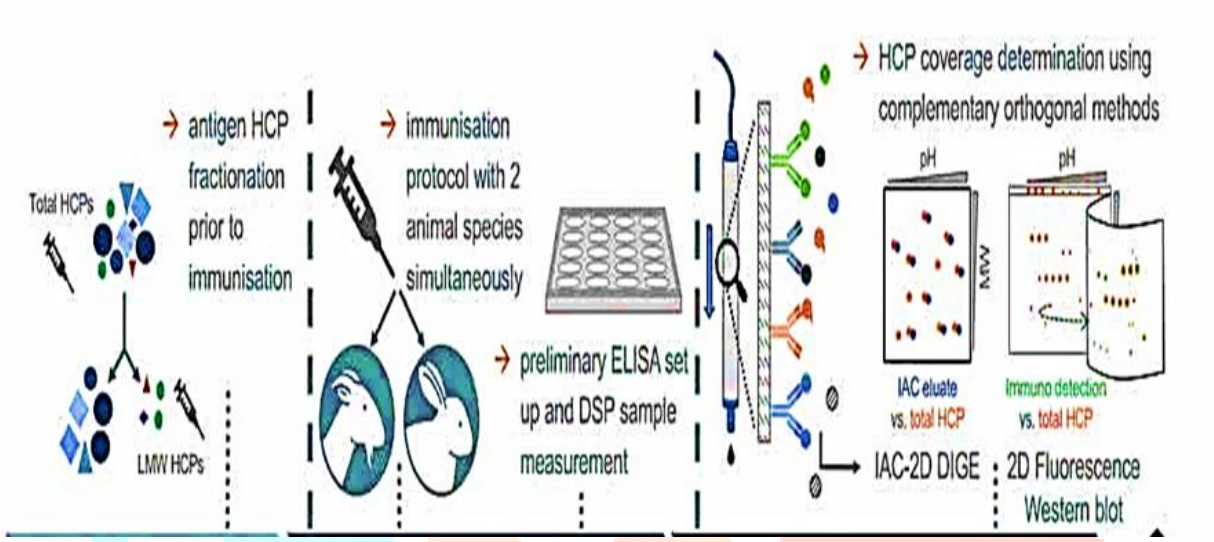
Enzyme-linked immunosorbent assay (ELISA) [29-31]

ELISA is a widely used method for analyzing Host Cell Proteins (HCP) in biopharmaceutical products, ensuring safety and efficacy. It can detect HCPs at very low concentrations using antigen-antibody interactions between the ELISA reactive components and the actual HCPs. ELISA tests have become a powerful method due to its versatility, high throughputs, and relatively simple experimental setup.

The procedure involves injecting HCP mixture into animals, which generate anti-HCP antibodies against foreign proteins. These antibodies can recognize most, if not all, of the proteins contained in the HCP mixture. A multi-analyte sandwich ELISA is developed, which enriches the HCPs from the sample and immobilizes them to a 96-well plate. Detection antibodies, conjugated directly with an enzyme or through a biotin-avidin

magnification, bind to the captured HCPs, generating a colorimetric, chemiluminescent, or fluorescent signal that correlates with the number of HCPs in the test sample.

ELISA is used in clinical diagnosis, biomedical research, and immunology research. It is used for detecting specific antibodies or antigens in blood or body fluids, detecting antibodies in biological samples, and identifying potential drug candidates for further development. However, ELISA has limitations, such as its preference for proteins with high abundance or immunogenicity, and its inability to detect 100% of HCPs coproduced with the recombinant protein.



(Fig5. Schematic representation of ELISA for host cell proteins) [32]

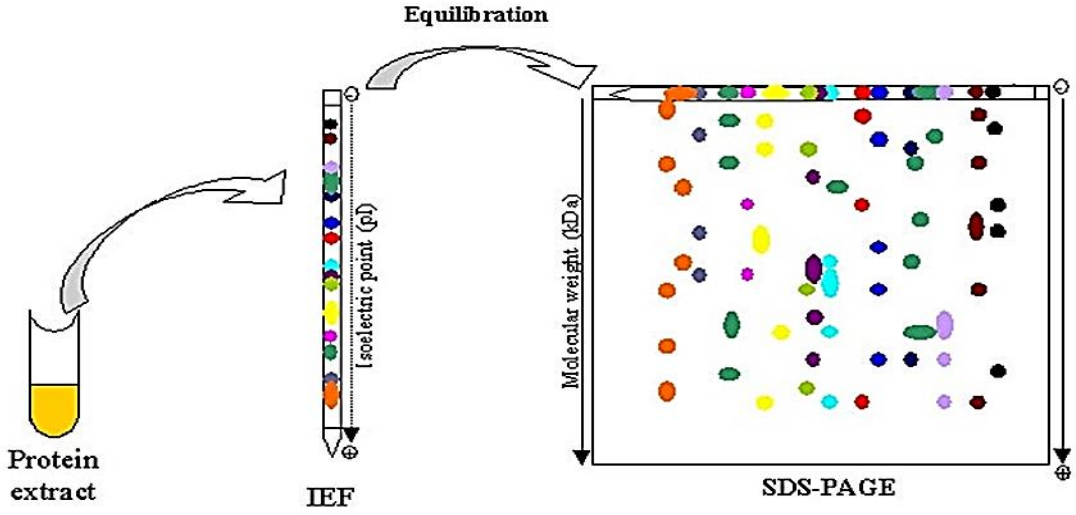
Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [33-36]

2D PAGE is a powerful orthogonal tool for classifying Human Cytoplasmic Protein (HCP) and visually characterizing HCP impurities in biologics purification processes. It is used for separation and fractionation of complex protein mixtures from biological samples. The process involves two steps: isoelectric focusing (IEF) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The first-dimension separates proteins based on isoelectric points (PI) and molecular weights (Mr), allowing thousands of proteins to be separated.

The second dimension is often performed using SDS-PAGE, an electrophoretic method for separating polypeptides according to their molecular weights. This method can make proteins denaturing and bind to the backbone at a constant molar ratio. Staining methods include Coomassie Blue staining and silver staining.

2D PAGE can be applied to characterize HCP contamination during the production of biologics and verify that the ELISA's key reagent, HCP antibody, covers the broad spectrum of HCP present in the drug product. Advantages of 2D PAGE include high-throughput, high resolution, computer-based tools, cost-efficiency, and high flexibility. However, reproducibility has been an issue with 2D-PAGE, especially when profiling two protein mixtures. The development of IPGs has improved resolution by allowing ultrazoom gels with a limited pH gradient range to customize the pH gradient for maximum resolution.

Despite its limitations, 2D PAGE continues to be a valuable tool for protein analysis, providing additional supporting information for the production of biological products.



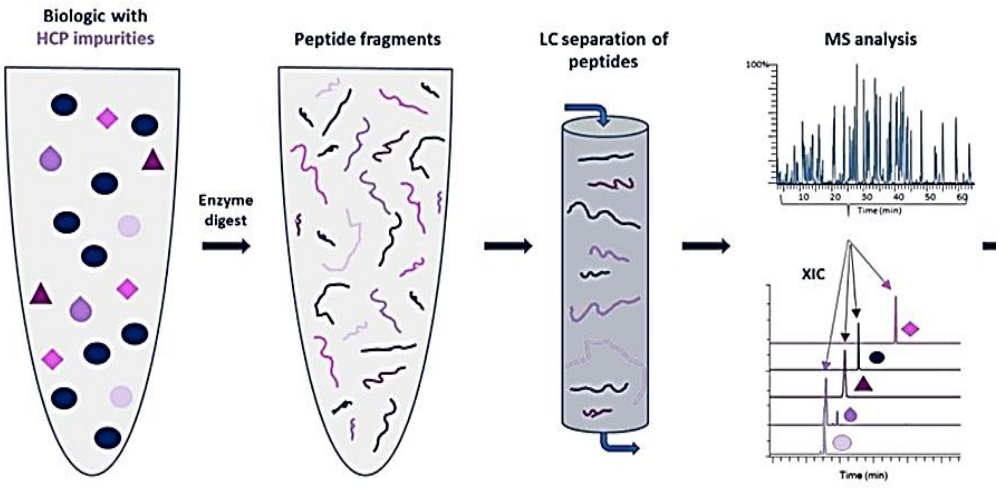
(Fig 6. 2D PAGE for Host cell protein Analysis) [34]

Liquid chromatography-Mass spectrometry (LC-MS) [37-41]

LC-MS analysis is a two-step process that involves liquid chromatography (LC) and mass spectrometry (MS). The LC phase divides the sample based on chemical characteristics, while the MS phase provides detailed information about the chemical makeup and structure of each component. LC-MS is a crucial technique in analytical chemistry, enabling the separation and analysis of complex mixtures of substances.

Tryptic digestion was performed using Thermo Scientific's SMART Digest™ trypsin kit and the KingFisher Duo Prime system. LC-MS has advantages such as high sensitivity, enabling the detection of even the smallest compounds at concentrations that would otherwise be undetected by other methods. It covers a wide range of compounds, including small molecules, peptides, proteins, and nucleic acids, providing accuracy in results and insights into molecular structure.

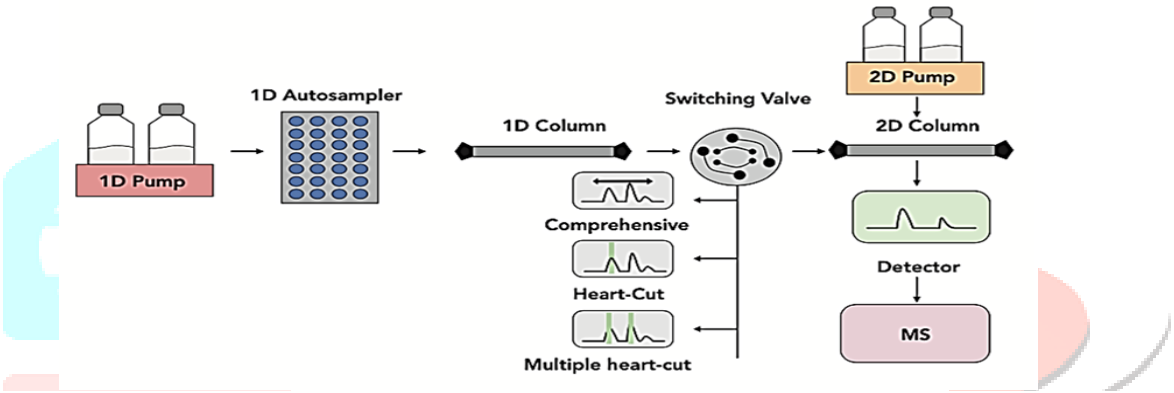
However, LC-MS has limitations, such as its high price, narrow dynamic range, and the need for experienced operators. It also depends on protein fragments' ability to ionize, making relative quantification using established spike standards less reliable.



(Fig 7. Schematic diagram of hcp analysis using LC-MS) [39]

Two-dimensional liquid chromatography- mass spectrometry (2D LC-MS) [42-43]

The 2D-LC-MS method is a method for analyzing host cell proteins using a set of pumps and loop-valves between two chromatographic chambers. The first dimension involves a 7-step fractionation process using different mobile phases, with a 0.1% formic acid solution added to dilute the eluent. The second dimension uses a gradient of 3%–50% mobile phase B, with the nanoACQUITY UPLC HSS T3 1.8 µm column serving as the second dimension. The MS data is gathered for each fraction using a XEVO G2 tandem mass spectrometer and MassLynx software. The LC/MSE data is processed using PLGS 2.5 software for HCP identification and protein quantification. The system offers advantages such as robust and fully automated separation, easy setup, high resolution, user-defined fraction width and number, easy optimization, chemical manipulation, and a reanalysis option. However, the SCX column operates far from its optimum under these conditions, limiting resolution and the number of salt steps.



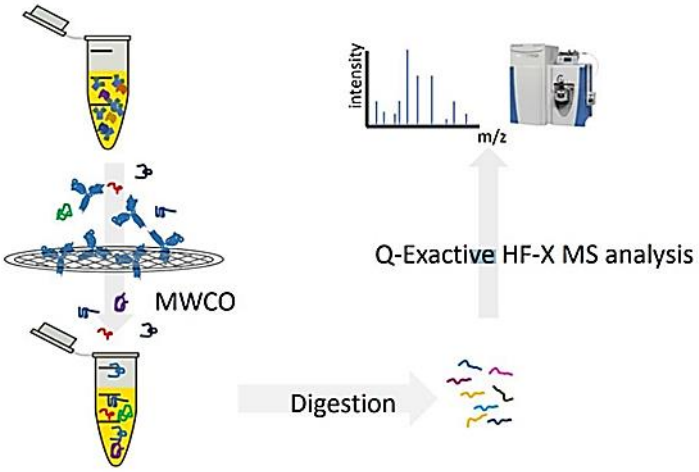
(Fig 8. Schematic representation of 2D LC-MS) [42]

Recent analytical advances in analysis of Host cell proteins.

Host cell proteins (HCPs) are impurities in biopharmaceutical products like therapeutic antibodies. ELISA monitors HCP levels, but LC-MS offers qualitative and quantitative information. Accurate detection of low abundance HCPs is challenging due to potential differences in concentration. Recent analytical methods can overcome these limitations for HCP identification and detection. Some of them are described below;

Molecular weight cut-off (MWCO) [44]

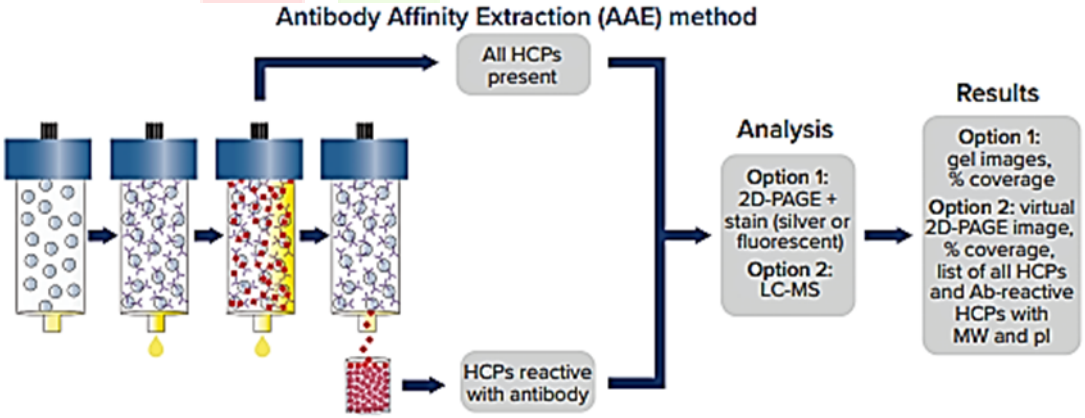
The enzyme-linked immunosorbent assay (ELISA) is a commonly used method for detecting high-affinity peptides (HCPs) in therapeutic protein synthesis. However, it struggles to quantify specific HCP fractions and may not identify less immunogenic or non-immunogenic HCPs. To monitor HCPs, complementary assays like 1D/2D-PAGE and MS-based methods have been developed. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is an orthogonal supplemental analysis approach to ELISA. However, the concentration differential between HCPs and target proteins is a major issue with MS-based approaches. Two methods are available to address this problem: enriching HCPs through affinity purification or molecular weight cut-off (MWCO) ultrafiltration, or adding separation methods like 2D-LC and/or ion mobility. The MWCO-based HCP characterization technique involves sample preparation, protein digestion, LC-MS/MS analysis, and parallel reaction monitoring (PRM) proteomic analysis. Data analysis involves searching against the UniprotKB murine protein database and manually curated using targeted proteomics software for HCP identification.



(Fig 9. Experimental work flow of HCP identification using molecular weight cutoff filtration) [44]

Antibody Affinity Extraction (AAE) [45-47]

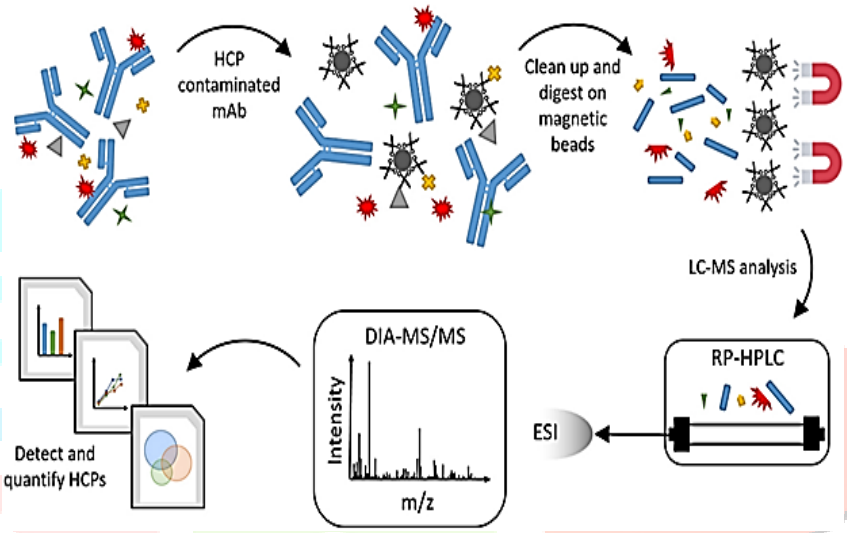
Antibody Affinity Extraction (AAE) is a new technique used in immunoaffinity chromatography to evaluate a polyclonal antibody's coverage of host cell proteins (HCPs) and its reactivity to those HCPs that may co-purify with a drug substance. AAE chromatography is a powerful method for identifying HCPs that persist through downstream purification processes, which are crucial for patient safety, drug efficacy, and stability. It can be used as a sample preparation method to enrich HCPs and eliminate most of the DS in a sample. When combined with AAE, Mass Spectrometry can also assess antibody coverage to HCPs in a given process and identify those HCPs that are reactive with the antibody. AAE-MS is a powerful method for assessing antibody coverage to HCPs in a given process, identifying HCPs in the harvest material and HCPs reactive with the antibody, and yielding protein MW and pI information.



(Fig.10 Antibody coverage by AAE chromatography with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) or mass spectrometric (MS) analysis options) [45]

Data-independent Acquisition (DIA)LC-MS/MS [48-49]

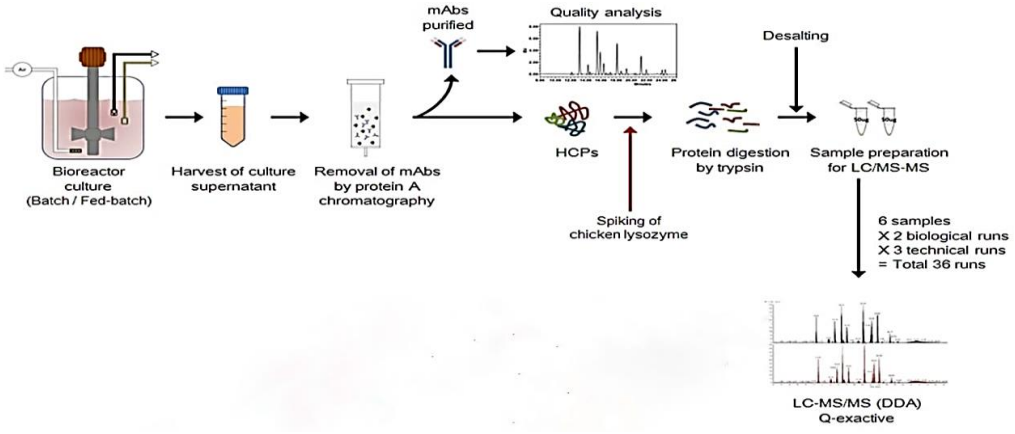
A DIA-based workflow for sensitive HCP analysis has been developed, which can be easily implemented using the same instrumental setup as standard peptide mapping analysis of mAbs. This method combines automation, reproducibility, robustness, and deep HCP coverage in an unprecedented manner, making it ideal for implementation in the biopharmaceutical industry. Data were collected for HCP analysis using variable isolation windows in DIA mode, with a resolution setting of 70,000 and an AGC target of 1e6. The DIA-MS-based HCP detection showed excellent sensitivity, allowing for unbiased quantitation of proteins over a broad abundance range. The method also identified several problematic HCPs, including immunogenic HCPs, proteases, and proteases that affect product stability.



(Fig 11. HCP analysis by DIA LC-MS/MS) [49]

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) [50-52]

High-performance liquid chromatography-mass spectrometry (LC-MS/MS) is a method used to analyze the molecular structure and information of substances. It involves separating peptide mixtures from proteolytic digestion, fragmentation using a tandem mass spectrometer, and acquiring MS/MS spectra for each fragmented peptide. The protein is identified by searching the protein database for matching peptides using each MS/MS spectrum. LC-MS/MS provides identification and quantitation of individual hydrogen peroxide (HCPs) with high sensitivity (>10 ng/mg). Samples are broken down by enzymes like trypsin or Lys-C, and peptides are separated using RPLC. An ultrahigh-resolution mass spectrometer detects peptides, which are then used to search protein databases for HCP- and product-related peptides. LC-MS/MS allows direct measurements of individual HCPs in the final DS and throughout the purification process, which is crucial for biotherapeutic manufacturers to ensure quality, safety, and efficacy.



(Fig 12.HCP Analysis by LC-MS/MS) [52]

Recent Regulatory challenges in Host cell proteins (HCPs) [53-59]

Chinese Hamster Ovary (CHO) cell lines are used in manufacturing over two-thirds of recombinant biopharmaceuticals, including over 85% of monoclonal antibody drugs. Residual Host Cell Protein (HCP) impurities derived from these cells can compromise the stability, efficacy, and safety of the therapeutic product. Regulatory authorities like the FDA require manufacturers to monitor and demonstrate HCP clearance and report the amount of HCP impurities in the finished product. HCP detection methods are well-established, but many labs struggle with assay development and qualification. Guidance documents from the US Pharmacopeia, European Pharmacopeia, International Council for Harmonization, FDA, European Medicines Agency, and Chinese Pharmacopeia have been issued on strategies for monitoring HCP impurities. The risk associated with HCP exposure depends on the clinical setting, including dose, route of administration, frequency of exposure, indication, patient population, and the particular impurity. To qualify for use in bioprocess development and manufacturing, HCP immunoassays must be well-characterized and satisfy stringent performance criteria. These include coverage, sensitivity, matrix effect, linearity, and dynamic range.

Conclusion

Biologics are complex and heterogeneous molecules derived from living sources that have a wide range of therapeutic applications. Biosimilars are highly similar but not identical versions of biologics that aim to provide comparable safety and efficacy at a lower cost. Molecular characterization of biosimilars is essential to demonstrate their similarity to the reference biologic in terms of structure, function, and quality. One of the major challenges in biosimilar development is the evaluation of host cell proteins (HCPs), which are impurities that originate from the production cell line and can affect the safety, efficacy, and stability of the product. Recent analytical methods for HCP detection and quantification include ELISA, LC-MS, DIA-LC-MS/MS, MWCO and AAE method. Regulatory agencies have issued guidelines and recommendations for the assessment and control of HCPs in biosimilars, but there are still some gaps and uncertainties that need to be addressed. In conclusion, biosimilars offer a promising alternative to biologics, but they require rigorous molecular characterization and evaluation of HCPs to ensure their quality, safety, and efficacy.

List of abbreviations

- **mAb:** Monoclonal antibodies
- **BRMs:** Biological response modifier
- **TNF:** Tumor necrosis factor
- **CSF:** Colony stimulating factor
- **GM-CSF:** Granulocyte macrophage-colony stimulating factor
- **G-CSF:** Granulocyte-Colony stimulating factor
- **EU:** European union
- **CQAs:** Critical quality attributes
- **MS:** Mass spectrometry
- **PTMs:** Post translational modifications
- **MALDI-TOF-MS:** Matrix-assisted laser desorption ionization-time of flight mass spectrometry
- **RPESI-QTOF-MS:** Reverse phase electrospray ionization quadrupole time-of-flight mass spectrometry
- **LC-ESI-QTOF-[Native] MS:** Liquid chromatography-electrospray ionization-mass spectrometry
- **SEC-ESI-QTOF-MS:** Size exclusion chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry
- **LC-MS:** Liquid chromatography -mass spectrometry
- **LC-MS/MS:** Liquid chromatography-tandem mass spectrometry
- **ADH:** Alcohol dehydrogenase
- **MSE:** Mean square error
- **HOS:** Higher order structure
- **CD:** Circular dichromism
- **FTIR:** Fourier transform infrared spectrometry
- **UV:** Ultra-violet
- **IR:** Infra-red
- **XRC:** X-ray crystallography
- **NMR:** Nuclear magnetic resonance
- **HDX-MS:** Hydrogen deuterium exchange mass spectrometry
- **SAXS:** Small angle x-ray scattering
- **VT:** Variable temperature
- **DSC:** Differential scanning calorimetry
- **TM:** Transition temperature
- **TCSPC:** Time-correlated single-photon counting

- **HILIC:** Hydrophilic interaction chromatography
- **MALS**: Multi-angle light scattering
- **RMS:** Root mean square
- **CIEF**: Capillary isoelectric focusing
- **icIEF:** Image capillary isoelectric focusing
- **WCID:** Whole-column imaging detection
- **BAC:** Boronate affinity chromatography
- **HCPs:** Host cell proteins
- **HCD:** Host cell DNA
- **ELISA**: Enzyme-linked immunosorbent assay
- **PCR:** Polymerase chain reaction
- **qPCR:** quantitative polymerase chain reaction
- **2D-PAGE/DIGE**: Two dimensional-poly acrylamide gel electrophoresis/difference gel electrophoresis
- **HRP:** Horse radish peroxidase
- **HIV:** Human immunodeficiency virus
- **AIDS:** Acquired immunodeficiency syndrome
- **Mr:** Relative molecular weight
- **PI:** Isoelectric point
- **IEF:** Isoelectric focusing
- **IPG:** Immobilized pH gradient
- **DTT**: Dithiothreitol
- **IAA**: Indole acetic acid
- **FA:** Formic acid
- **ACN:** Acetonitrile
- **UPLC**: Ultra performance-liquid chromatography
- **MWCO:** Molecular weight cut-off
- **PRM:** Parallel reaction monitoring
- **AAE:** Antibody affinity extraction
- **DIA:** Data independent acquisition
- **DDA:** Data dependent acquisition
- **2D-DIBE:** Two-dimensional differential in blot electrophoresis
- **CHO**: Chinese hamster ovary

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