

REVIEW ARTICLE



A Review on Bioanalytical Methods for Quantifying and Structural Determination of Therapeutic Monoclonal Antibodies

Phanisai Yeminedi, Murali Karthik Jalla, Bhavya Sri Kakarla, Prabhas Palepogu, Rajesh Guntupalli*

Department of Pharmaceutical Analysis, Malineni Perumallu Pharmacy College, Pulladigunta, Guntur, Andhra Pradesh, India

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Abstract: Therapeutic monoclonal antibodies require robust and validated bioanalytical methods to support pharmacokinetic evaluation, pharmacodynamic modeling, immunogenicity characterization, biosimilar comparability, and commercial quality assurance. The macromolecular nature of these tetrameric glycoproteins characterized by a high molecular mass of approximately 150 kDa, extensive post-translational modifications, variable glycan micro-heterogeneity, and susceptibility to chemical and physical degradation presents significant analytical challenges across all stages of biopharmaceutical development. Bioanalytical workflows rely on selective sample preparation methodologies, including organic solvent and acid precipitation, solid-phase extraction, immobilized Protein A or Protein G affinity chromatography, and targeted ligand or anti-idiotypic immunocapture to extract the analyte from complex physiological fluids while minimizing matrix suppression. Quantitative assessment leverages ligand-binding assays such as enzyme-linked immunosorbent assays for high-throughput screening, whereas liquid chromatography coupled with tandem mass spectrometry offers orthogonal molecular specificity through the multiple reaction monitoring of surrogate peptides generated via site-directed enzymatic proteolysis. Advanced structural characterization incorporates ultra-high-performance liquid chromatography, size-exclusion chromatography, and native high-resolution mass spectrometry across intact, middle-down, and top-down methods to resolve size variants, charge distribution profiles, disulfide linkages, and critical quality attributes. Persistent technical challenges include non-specific matrix effects, circulating anti-drug antibody complexation, and digestion-induced modifications. Multi-attribute methods, automated microfluidics, ion mobility spectrometry, and alternative microsampling can enhance analytical throughput and regulatory concordance.

Keywords: Monoclonal antibodies; LC–MS/MS; Immunocapture; Glycosylation; Critical quality attributes.

1. Introduction

Monoclonal antibodies (mAbs) have emerged as the primary class of biopharmaceuticals across medical oncology, autoimmune disorders, and systemic inflammatory diseases [1, 2]. Therapeutic antibodies can selectively target specific biological epitopes, yielding distinct pharmacological specificity by engineering the complementarity-determining regions (CDRs) of the fragment antigen-binding (Fab) variable domains [3-5]. Following the regulatory approval of early murine and chimeric antibodies, advances in recombinant mammalian cell expression systems predominantly Chinese hamster ovary (CHO) cell cultures have led to humanized and fully human immunoglobulin G (IgG) scaffolds, expanding the therapeutic landscape [6, 7].

The structural framework of an intact IgG molecule comprises two identical heavy chains (~50 kDa each) and two identical light chains (~25 kDa each) linked through a defined network of interchain and intrachain disulfide bridges [8, 9]. Beyond this primary sequence, therapeutic antibodies exhibit structural micro-heterogeneity driven by post-translational modifications (PTMs) occurring during cell culture, downstream purification, and storage [10, 11]. These modifications include core and terminal N-glycosylation at conserved asparagine (Asn-297) residues within the crystallizable fragment (Fc) region, N-terminal glutamine or glutamate cyclization into pyroglutamate, C-terminal lysine cleavage, methionine and tryptophan oxidation, and asparagine deamidation [12, 13]. Because these structural variations can influence target binding affinity, Fc receptor-mediated effector functions (such as antibody-dependent cellular cytotoxicity [ADCC] and complement-dependent cytotoxicity [CDC]), clearance rates, and clinical immunogenicity, rigorous bioanalytical characterization is required [14, 15]. Quantifying monoclonal antibodies within physiological fluids (such as whole blood, serum, plasma, and cerebrospinal fluid) presents distinct bioanalytical hurdles [16, 17]. Unlike small-molecule xenobiotics, monoclonal antibodies must be quantified at nanogram-per-milliliter to microgram-per-milliliter concentrations within a background of endogenous serum proteins totaling 60 to 80 mg/mL, including 7 to 16 mg/mL of

* Corresponding author: Rajesh Guntupalli

endogenous polyclonal human IgG and 35 to 50 mg/mL of human serum albumin [18, 19]. This excess of homologous host proteins creates high non-specific background signals and ion suppression during analytical measurements [20, 21].

The structural diversity of therapeutic antibodies *in vivo* adds further analytical complexity [22, 23]. Circulating proteases, oxidative microenvironments, and endogenous ligands can induce biotransformations, partial proteolysis, or conformational unfolding [24, 25]. Furthermore, the patient's immune response may generate anti-drug antibodies (ADAs), which bind the therapeutic antibody to form circulating immune complexes [26, 27]. These complexes can mask binding epitopes, altering the observable free drug concentration and confounding the interpretation of pharmacokinetic and pharmacodynamic data [28, 29].

Historically, bioanalysis relied heavily on ligand-binding assays (LBAs), primarily enzyme-linked immunosorbent assays (ELISA), because of their high analytical sensitivity and suitability for automated, high-throughput sample processing [30, 31]. However, LBAs depend on paratope accessibility and are susceptible to cross-reactivity, matrix interferences, and reagent variability, while offering limited capacity to resolve structural variants or biotransformation products [32, 33].

To overcome these constraints, hyphenated mass spectrometric methods principally liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and high-resolution mass spectrometry (HRMS) have become essential bioanalytical platforms [34, 35]. Bottom-up workflows utilize enzymatic proteolysis to monitor unique surrogate peptides using multiple reaction monitoring (MRM) on triple-quadrupole instruments, providing high sequence specificity [36, 37]. In parallel, middle-down, top-down, and native mass spectrometry workflows interrogate intact protein domains and native proteoforms, revealing higher-order structural details, non-covalent interactions, and aggregate distributions [38-40].

2. Pre-Analytical Sample Preparation Techniques

Sample preparation is a critical phase in the bioanalytical workflow for biotherapeutics, directly dictating assay selectivity, analyte recovery, lower limits of quantification, and reproducibility [41, 42]. The primary goal is to isolate and concentrate the intact antibody or its constituent peptides from endogenous serum macromolecules and non-protein contaminants while preserving the analyte's chemical integrity [43, 44].

2.1. Protein Precipitation and Depletion

Protein precipitation (PP) involves destabilizing the solvation layer of dissolved proteins using organic solvents (such as methanol, acetonitrile, or ethanol), concentrated salts (ammonium sulfate), or strong acids (trichloroacetic acid or perchloric acid) [45]. In small-molecule bioanalysis, precipitation removes macromolecular proteins to leave analytes in the supernatant [46]. In protein bioanalysis, organic solvent precipitation can be used either to precipitate the therapeutic antibody alongside host proteins (followed by pellet re-solubilization in a denaturing digest buffer) or to selectively precipitate high-abundance proteins [47, 48].

Acidic precipitation using ammonium sulfate or trichloroacetic acid operates via salting-out and hydrophobic association mechanisms [49, 50]. Although protein precipitation provides high throughput, low per-sample costs, and complete workflow automation, it possesses low analytical selectivity [51, 52]. Endogenous immunoglobulins co-precipitate with the target therapeutic antibody, leading to high background complexity that requires subsequent chromatographic resolution [53, 54]. Consequently, protein precipitation is typically reserved for early discovery screening, preclinical species lacking human IgG cross-reactivity, or as an initial cleanup step prior to solid-phase extraction [55, 56].

2.2. Solid-Phase Extraction (SPE)

Solid-phase extraction isolates target analytes through differential partitioning between a liquid sample matrix and a functionalized solid sorbent [57, 58]. In therapeutic antibody workflows, SPE is applied either at the intact protein level using wide-pore sorbents or at the post-digestion peptide level using reversed-phase (C₁₈), ion-exchange, or mixed-mode chemistries [59, 60].

Mixed-mode SPE sorbents, which combine hydrophobic retention with cation- or anion-exchange interactions, provide orthogonal separation of tryptic peptides [61, 62]. Peptides containing basic residues are retained via strong cation exchange (SCX) at acidic pH, allowing neutral and negatively charged matrix interferents to be washed away prior to step-elution using elevated ionic strength or organic modifiers [63, 64]. Sorbent bed conditioning, loading dynamics, wash protocols, and elution solvents must be optimized to prevent non-specific secondary adsorption and maximize analyte recovery [65, 66]. The integration of 96-well microtiter SPE plates into automated robotic liquid-handling systems supports high-throughput quantitative pipelines [67, 68].

2.3. Affinity Purification via Protein A and Protein G

Bacterial affinity ligands, predominantly Protein A (derived from *Staphylococcus aureus*) and Protein G (derived from Group C and G *Streptococci*), demonstrate high binding affinity for the constant heavy-chain Fc region (C_H2–C_H3 interface) of immunoglobulins [69, 70]. Immobilized onto agarose, cross-linked dextran, or functionalized magnetic beads, these ligands capture IgG antibodies from serum or plasma matrices under neutral physiological pH conditions [71, 72].

Elution is achieved using acidic buffers (typically 0.1 M glycine-HCl or 1% formic acid, pH 2.5–3.0), which protonate critical histidine residues at the binding interface and disrupt hydrophobic interactions [73, 74]. While Protein A and G purification provides high analyte recovery and removes non-immunoglobulin proteins (such as serum albumin), it cannot differentiate human therapeutic IgG from endogenous polyclonal human IgG because both share conserved Fc domains [75, 76]. As a result, Protein A/G capture is primarily utilized in non-human preclinical pharmacokinetic studies, biomanufacturing downstream processing, and biosimilar batch-release testing where endogenous human IgG background is absent [77, 78]. Table 1 summarizes the operational principles, matrix compatibilities, recovery profiles, and primary bioanalytical trade-offs across common sample preparation workflows utilized for therapeutic monoclonal antibodies.

Table 1. Comparison of Pre-Analytical Sample Preparation Strategies for Monoclonal Antibodies in Biological Matrices

Sample Preparation Method	Principle	Matrix Compatibility	Advantages	Limitations	Optimal Downstream Instrumentation
Organic Solvent Precipitation	Solvation layer displacement causing protein denaturation and bulk co-aggregation	Preclinical serum, plasma, cell culture media	Rapid processing, low cost, minimal consumable requirements, automation-compatible	Lacks target specificity, co-precipitates endogenous IgG, causes severe ESI ion suppression	Microflow LC–MS/MS, peptide mapping
Solid-Phase Extraction (Mixed-Mode SCX/RP)	Dual retention via hydrophobic partition and secondary electrostatic cation exchange	Serum, plasma, urine, digested peptide lysates	High extract cleanliness, reproducible recovery, effective phospholipid and salt depletion	Requires extensive conditioning and pH tuning; secondary irreversible adsorption losses	UPLC–MS/MS (MRM/PRM), nano-LC–MS
Protein A / Protein G Affinity Purification	Specific high-affinity binding to constant Fc domain (C _H 2 – C _H 3 interface)	Non-human preclinical serum, bioprocess harvest fluids	High extraction recovery, robust removal of host cell proteins and serum albumin	Co-purifies endogenous human polyclonal IgG; requires low pH (2.5–3.0) elution stress	Intact HRMS, SEC–HPLC, middle-down LC–MS
Target Antigen Immunocapture	Functional binding between antibody paratope and immobilized recombinant receptor/ligand	Human serum, plasma, synovial fluid	Isolates free pharmacologically active drug; low non-specific background	Under-quantifies drug in the presence of neutralizing anti-drug antibodies (ADAs)	IA-LC–MS/MS, sandwich ELISA, SPR
Anti-Idiotypic Magnetic Bead Immunocapture	Paratope-directed or framework-directed affinity capture using anti-CDR antibodies	Whole blood, serum, plasma, CSF	High sensitivity (low ng/mL limits), customizable for total vs. free drug profiling	High critical reagent development cost; batch-to-batch antibody reagent variability	Nano-LC–MS/MS, high-resolution MRM

2.4. Target-Specific and Anti-Idiotypic Immunocapture

To achieve high analytical sensitivity and selectivity in clinical human matrices, target-directed immunocapture is widely employed [79, 80]. This technique utilizes biotinylated or covalently coupled capture reagents immobilized onto streptavidin-coated magnetic beads or microtiter plates [81, 82]. Capture reagents generally fall into two categories:

2.4.1. Recombinant Target Antigens

The specific extracellular domain of the pharmacological target is functionalized as the capture reagent, isolating only those antibody molecules that maintain functional, accessible paratopes [83, 84]. This approach selectively quantifies the pharmacologically active, unbound drug fraction [85, 86].

2.4.2. Anti-Idiotypic Antibodies

Monoclonal or polyclonal antibodies raised against the hypervariable CDRs of the therapeutic antibody bind exclusively to the unique idiotypic determinants of the drug [87, 88]. Depending on whether the anti-idiotypic antibody is blocking or non-blocking, it can be configured to capture free, partially bound, or total therapeutic antibody concentrations [89, 90].

Paramagnetic bead-based immunocapture workflows provide high surface-area-to-volume ratios, favorable binding kinetics in solution, and seamless compatibility with automated multi-channel magnetic particle processors [91, 92]. Following automated magnetic isolation and rigorous washing to eliminate unbound endogenous proteins, the enriched therapeutic antibody can be eluted via acidic disruption or processed directly on-bead through sequential denaturation, reduction, alkylation, and enzymatic digestion [93, 94].

3. Quantitative and Structural Analytical Techniques

3.1. Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assays represent an established quantitative methodology for therapeutic protein monitoring in clinical development [95, 96]. Assay configurations rely on immobilizing capture reagents within polystyrene microplates [97, 98]. In a standard sandwich ELISA, the therapeutic mAb is bound between an immobilized anti-idiotypic capture antibody and an enzyme-conjugated detection antibody (such as horseradish peroxidase [HRP] or alkaline phosphatase [AP]) [99, 100]. Addition of a chromogenic substrate (e.g., 3,3',5,5'-tetramethylbenzidine [TMB]) produces a colorimetric signal proportional to the analyte concentration [101, 102].

The primary advantages of ELISA are operational simplicity, high sample throughput, and low picomolar limits of quantification without requiring extensive instrumental hardware [103, 104]. However, traditional ELISA formats have several limitations:

3.1.1. Narrow Dynamic Range

The sigmoidal response curve typically provides a quantifiable range of only two to three orders of magnitude, requiring sample dilutions to match the linear range [105, 106].

3.1.2. Matrix and Soluble Target Interference

Soluble target receptors, circulating endogenous ligands, and high concentrations of rheumatoid factors can competitively block the capture paratope, leading to under- or over-estimation of the circulating antibody concentration [107, 108].

3.1.3. Anti-Drug Antibody Interference

Neutralizing ADAs compete with the capture and detection reagents for paratope binding, masking drug detection unless acid-dissociation pre-treatment protocols are implemented [109, 110].

3.1.4. Lack of Structural Specificity

Standard ELISA cannot differentiate fully intact, bioactive molecules from partially degraded forms, deamidated species, or C-terminal truncated variants [111, 112].

Recent developments have introduced nanomaterial-based signal amplification and alternative detection modes to enhance sensitivity for trace targets [113, 114]. Nanopore arrays and robotic fluidic systems further enable multiplexed bioanalysis of trace target antibodies and clinical biomarkers [115, 116]. Nevertheless, structural mutations and non-canonical glycosylation, such as atypical O-linked Fab glycans identified on IgG4 constructs like dupilumab, require mass spectrometric verification to elucidate their specific molecular architecture [117, 118]. Table 2 provides the fundamental performance metrics, dynamic working ranges, selectivity attributes, and susceptibility to matrix interferences distinguishing ELISA from hybrid IA-LC-MS/MS platforms. Figure 1 shows the parallel operational workflows of targeted immunoaffinity LC-MS/MS surrogate peptide quantification and intact/subunit high-resolution mass spectrometry (HRMS) structural profiling

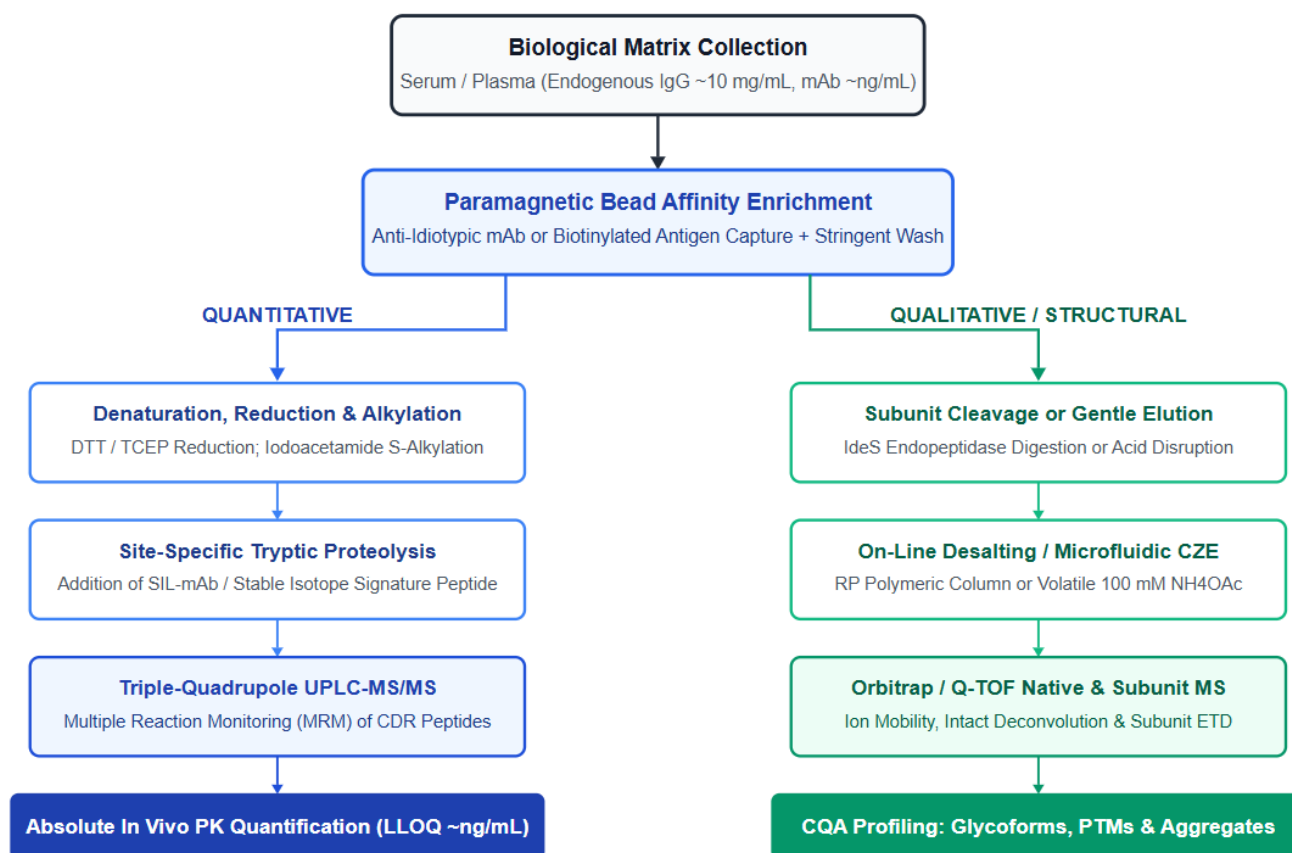


Figure 1. End-to-End Analytical Process Comparing Immunoaffinity-LC-MS/MS and Intact HRMS Characterization

3.2. Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS)

Triple-quadrupole LC-MS/MS provides a selective, multiplexed alternative to ligand-binding assays, separating analytes by chromatographic retention and detecting them via precursor-to-product ion transitions in multiple reaction monitoring (MRM) mode [119, 120]. The primary quantitative methodology is the bottom-up or surrogate peptide approach [121, 122]. The therapeutic antibody is denatured (using surfactants like sodium deoxycholate or urea), reduced (with dithiothreitol [DTT] or tris(2-carboxyethyl)phosphine [TCEP]), alkylated with iodoacetamide (IAM) to block free thiols, and digested using sequence-specific endoproteases most commonly trypsin, which cleaves peptide bonds C-terminal to lysine and arginine residues [123, 124].

Surrogate peptide selection requires careful theoretical and experimental optimization:

- Peptides must originate from the unique hypervariable CDRs of the heavy or light chains to avoid sequence homology with endogenous human immunoglobulins [125, 126].
- Peptides should contain between 7 and 20 amino acid residues to ensure suitable chromatographic retention and electrospray ionization efficiency [127, 128].
- Sequences prone to chemical instability such as methionine or tryptophan residues susceptible to oxidation, asparagine-glycine motifs prone to deamidation, and terminal glutamine residues prone to cyclization should be avoided to ensure reproducible signal response [129, 130].

- Cleavage sites adjacent to proline or dibasic residues (Arg-Arg, Lys-Lys) that exhibit variable digestion kinetics must be excluded [131, 132].

Table 2. Comparison of Ligand-Binding Assays (ELISA) versus Hybrid Immunoaffinity LC–MS/MS

Bioanalytical Parameter	Enzyme-Linked Immunosorbent Assay (ELISA)	Hybrid Immunoaffinity LC–MS/MS (IA-LC–MS/MS)
Analytical Principle	Epitope-paratope recognition with enzymatically amplified colorimetric/chemiluminescent readout	Selective immunocapture coupled with tryptic surrogate peptide separation and mass-to-charge transition detection (m/z)
Linear Dynamic Range	Narrow (typically 2 to 3 orders of magnitude; sigmoidal curve)	Broad (typically 3 to 5 orders of magnitude; linear regression)
Lower Limit of Quantification (LLOQ)	0.01 – 1.0 ng/mL (highly sensitive for compliant binding pairs)	0.05 – 5.0 ng/mL (dependent on peptide ionization efficiency and capture recovery)
Molecular Selectivity	Low to Moderate; cannot differentiate intact drug from deamidated, oxidized, or cleaved fragments	Absolute structural specificity; resolves primary sequence variations, PTMs, and catabolites
Susceptibility to ADA Interference	High; circulating anti-drug antibodies block paratopes, causing false under-quantification	Low; acid-dissociation pre-treatment and denaturing digestion liberate surrogate peptides from immune complexes
Soluble Target / Receptor Interference	High; soluble circulating ligands mask antibody paratopes	Negligible when using generic framework capture or post-digestion chromatographic separation
Multiplexing Capability	Very low; single-analyte per assay well (unless employing complex planar arrays)	High; simultaneous monitoring of multiple signature peptides, biotherapeutics, and biomarkers
Internal Standardization	None available; susceptible to uncorrected pipetting and matrix variation	Excellent; uniform $^{13}\text{C}/^{15}\text{N}$ full-length SIL-mAb or stable isotope-labeled signature peptides
Sample Throughput & Equipment Cost	High sample throughput; low initial capital investment and operational cost	Moderate throughput; high capital expenditure, instrumentation maintenance, and analyst expertise

To ensure high quantitative accuracy, stable isotope-labeled internal standards (SIL-IS) are added [133, 134]. SIL-IS formats include:

Stable-Isotope Peptides: Synthetic surrogate peptides synthesized with ^{13}C - and ^{15}N -labeled arginine or lysine residues; these correct for electrospray ionization variability and matrix suppression but do not compensate for extraction losses or incomplete enzymatic cleavage [135, 136].

Winged Extended Peptides: Synthetic peptides extended with flanking amino acid sequences that match the native cleavage boundary, helping to correct for enzymatic digestion rate variations [137, 138].

Full-Length Uniformly Labeled mAbs: Full-length recombinant antibodies uniformly labeled with ^{13}C and ^{15}N ; these are introduced at the start of sample preparation and provide comprehensive normalization across affinity capture, reduction, alkylation, proteolysis, and MS detection [139, 140].

Chromatographic separation of peptide digests typically utilizes reversed-phase octadecylsilane (C_{18}) columns with acidified water-acetonitrile gradients [141, 142]. For polar and hydrophilic analytes, hydrophilic interaction liquid chromatography (HILIC) and chiral chromatography serve as complementary retention modes [143, 144]. Combining immunoaffinity enrichment with LC–MS/MS (IA-LC–MS/MS) unifies the high extraction recovery of affinity capture with the physicochemical mass specificity of tandem mass spectrometry, reaching sub-nanogram per milliliter quantification limits across complex biological matrices [145, 146].

3.3. Ultra-Performance Liquid Chromatography–Tandem Mass Spectrometry (UPLC–MS/MS)

Ultra-performance liquid chromatography (UPLC) platforms utilize sub-2- μm porous or core-shell particle packings within high-pressure fluidic systems operating up to 15,000 psi [147, 148]. Compared with conventional HPLC, UPLC offers several analytical improvements:

High Peak Capacity: Narrow chromatographic band widths (often below 3 to 5 seconds at the baseline) yield high peak capacity, resolving complex peptide digests containing hundreds of closely related fragments [149, 150].

Reduced Matrix Co-Elution: The enhanced chromatographic resolution separates surrogate peptides from co-extracted endogenous phospholipids and non-specific serum proteins, substantially reducing electrospray ionization matrix suppression [151, 152].

Increased Throughput: High linear mobile phase velocities shorten runtime cycles to under 5 to 10 minutes per sample, facilitating the high-throughput demands of clinical pharmacokinetic pipelines [153, 154].

When hyphenated to triple-quadrupole mass spectrometers with fast dwell times and high-speed collision cells, UPLC–MS/MS enables simultaneous, multi-analyte quantification of the primary surrogate peptide alongside confirmatory monitoring peptides to verify structural integrity [155, 156].

3.4. Size-Exclusion Chromatography (SEC–HPLC)

Size-exclusion chromatography (SEC) is the standard analytical technique for profiling and quantifying size variants in biopharmaceutical formulations [157, 158]. The chromatographic mechanism is based on the differential steric exclusion of macromolecules from the defined pore volume of a hydrophilic stationary phase under isocratic, non-denaturing mobile-phase conditions (e.g., 50–200 mM sodium phosphate, pH 6.8–7.0, supplemented with potassium chloride or sodium perchlorate to suppress secondary ionic interactions) [159, 160].

Macromolecules larger than the average stationary phase pore diameter are excluded from the internal pore volume and elute in the column void volume (V_0) [161, 162]. Progressively smaller molecules penetrate deeper into the intra-particle pore network and elute in order of decreasing hydrodynamic volume [163, 164]:

- **High-Molecular-Weight Species (HMWS):** Covalent and non-covalent aggregates, including dimers, trimers, and higher-order oligomers, elute first [165, 166]. Quantifying HMWS is a critical safety parameter because protein aggregates can trigger immunogenic responses in patients [167, 168].
- **Monomeric IgG:** Fully assembled, native 150 kDa immunoglobulin molecules elute as the primary monomer peak [169, 170].
- **Low-Molecular-Weight Species (LMWS):** Proteolytic degradation products, hinge-cleaved Fab and Fc fragments, and unassociated light chains elute after the monomer peak [171, 172].

Mixed-mode size-exclusion chromatography incorporates controlled hydrophobic or electrostatic properties into the column matrix, enabling the separation of conformational variants such as correctly assembled bispecific antibodies versus disulfide-scrambled homodimeric mispairings that have identical molecular weights [173, 174]. Downstream purification monitoring applies SEC–HPLC to identify non-binding aggregate fractions during Protein A capture and downstream chromatographic polishing [175, 176]. Furthermore, SEC-based fractionation methodologies provide non-destructive isolation and characterization across diverse macromolecular systems, including intact virus particles and extracellular vesicle fractions [177, 178]. Table 3 compares the digestion requirements, spectral complexity, structural resolution, and primary analytical applications of bottom-up, middle-down, top-down, and native mass spectrometry workflows.

3.5. High-Resolution and Native Mass Spectrometry Methods

High-resolution mass spectrometry (HRMS) platforms including Quadrupole–Time-of-Flight (Q–TOF) and Orbitrap systems provide high mass resolving power (>50,000 to 200,000 FWHM) and sub-5 ppm mass accuracy, enabling in-depth structural characterization of therapeutic proteins [179, 180]. Characterization strategies generally follow three primary workflows:

3.5.1. Intact Protein Analysis

The intact, fully assembled glycoprotein is desalted and analyzed under denaturing electrospray conditions [181, 182]. Deconvolution of the resulting multi-charge envelope reveals the global mass distribution, highlighting major glycoform assemblies (such as G0F/G0F, G0F/G1F, and G1F/G1F combinations) and verifying bulk drug substance mass [183, 184].

Table 3. Strategic Mass Spectrometry Methods for Structural Characterization of Therapeutic Monoclonal Antibodies

Characterization Method	Enzymatic / Chemical Cleavage Method	Molecular Mass Observed	Critical Quality Attributes (CQAs) Resolved	Advantages	Analytical Bottlenecks
Bottom-Up Peptide Mapping	Complete enzymatic digestion (trypsin, Lys-C, Asp-N) following reduction and alkylation	0.5 – 3.5 kDa (peptides)	Primary sequence confirmation, site-specific deamidation, methionine oxidation, N-/O-glycosylation localization	High chromatographic resolution; comprehensive sequence coverage (> 98%)	Artificial PTM artifacts induced during alkaline digestion; loss of global proteoform context
Middle-Down / Subunit Analysis	IdeS digestion at hinge region followed by DTT/TCEP reduction	25 kDa (Fd', LC, Fc/2)	Domain-specific glycosylation profiling, hinge cleavage, CDR-localized PTMs, Fc oxidation	Rapid digestion (< 30 min); resolves localized modifications without peptide-level complexity	Incomplete fragmentation across dense disulfide-bonded loops; requires high-resolution Orbitrap/Q-TOF
Top-Down Mass Spectrometry	None (intact denatured protein desalted online)	~150 kDa (intact monomer)	Whole-molecule proteoforms, terminal truncation variants, total payload distribution (ADCs)	Zero sample preparation artifacts; reveals intact combinatorial proteoform landscape	Dense charge envelopes; low sequence fragmentation efficiency across the full 150 kDa backbone
Native MS & Native IM-MS	None (sprayed from physiological volatile ammonium acetate, pH 6.8–7.2)	~150 kDa (monomer) to >300 kDa (aggregates)	Non-covalent oligomers, higher-order structure, receptor-ligand stoichiometries, drug-to-antibody ratio (DAR)	Preserves native tertiary/quaternary assembly; reduces charge-state spectral crowding	Stringent desolvation control required; lower ionization efficiency; requires non-standard ESI parameters

3.5.2. Middle-Down and Subunit Analysis

To localize modifications without the sample-preparation artifacts common in bottom-up tryptic digests, the antibody is enzymatically cleaved into 25–50 kDa subunits [185, 186]. Digestion with the endopeptidase IdeS (*Streptococcus pyogenes*) cleaves below the hinge region at a single site (Gly-Gly), yielding an intact F(ab')₂ fragment and a dimeric Fc fragment [187, 188].

Subsequent reduction with DTT or TCEP yields three distinct fragments: the light chain (LC, ~25 kDa), the cleaved heavy-chain variable domain (Fd', ~25 kDa), and the single-chain Fc/2 domain (~25 kDa) [189, 190]. High-resolution LC–MS of these subunits allows site-specific localization of PTMs:

- Fc/2 characterization reveals conserved N-glycan distribution patterns, C-terminal lysine truncation, and core oxidation [191, 192].
- Fd' and LC subunits isolate modifications within the antigen-binding CDR loops, including deamidation, atypical Fab glycosylation, and leader-sequence clipping [193, 194].
- Electron-transfer dissociation (ETD) and higher-energy collisional dissociation (HCD) fragmentation of these subunits preserves labile PTMs, pinpointing exact residue modifications without inducing chemical degradation [195, 196].

Figure 2 shows a comparative quantitative distribution of critical quality attributes (CQAs) including glycosylation variants, charge modifications, and size heterogeneity typically monitored during biopharmaceutical batch release and biosimilar comparability assessments

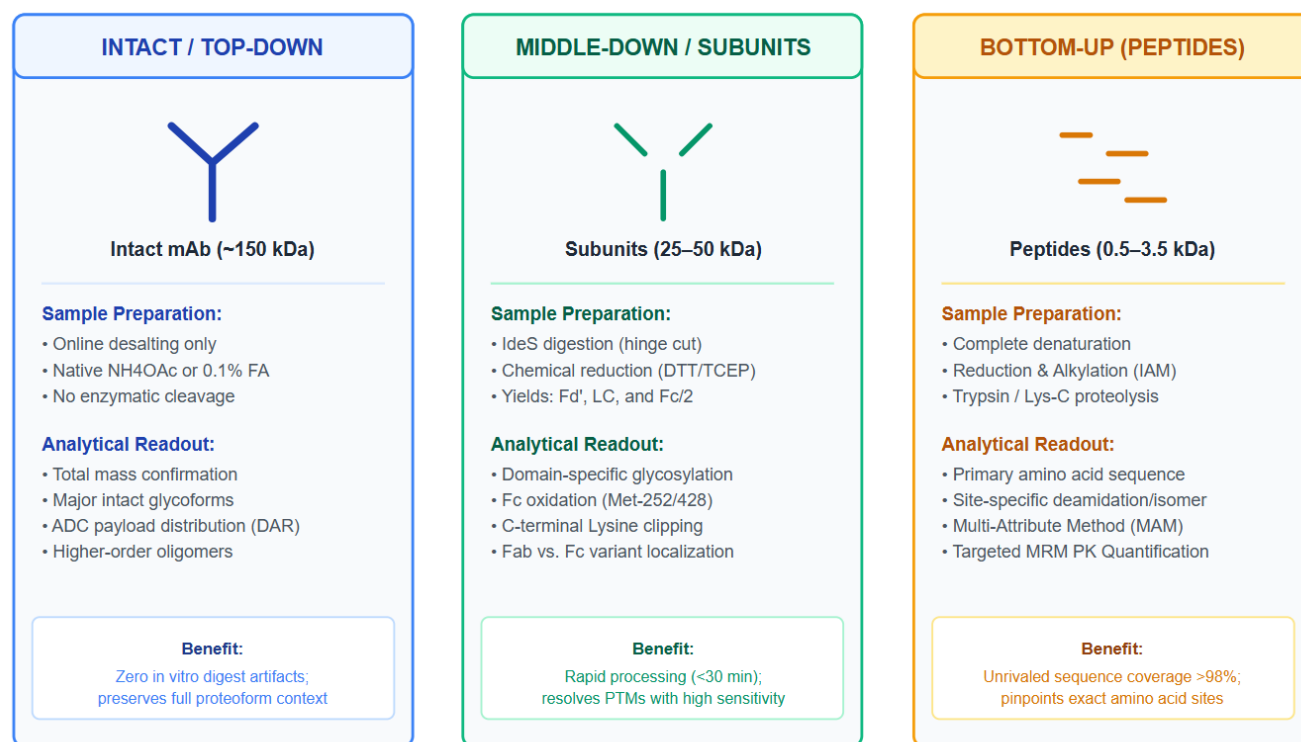


Figure 2. Top-Down, Middle-Down, and Bottom-Up Mass Spectrometry Methods

3.5.3. Native Mass Spectrometry

Native MS introduces the intact antibody or biotherapeutic complex into the vacuum phase directly from volatile, aqueous physiological buffers (typically 50–200 mM ammonium acetate, pH 6.8–7.2) [197, 198]. By preserving non-covalent interactions and the folded tertiary and quaternary structure, surface protonation is reduced, which shifts the multi-charge ion envelope to higher mass-to-charge ranges (m/z 5,000–9,000) [199, 200]. This spectral expansion reduces charge-state overlap, allowing the direct interrogation of native proteoforms, non-covalent multimeric aggregates, and antibody–receptor binding stoichiometries [201, 202].

Combining native MS with ion mobility spectrometry (IM-MS) introduces gas-phase electrophoretic separation based on the collision cross section (CCS) of the ions [203, 204]. IM-MS distinguishes conformational isomers, monitors unfolding pathways, and determines the drug-to-antibody ratio (DAR) distribution of conjugated modalities, such as antibody–drug conjugates (ADCs) [205, 206].

3.6. Recent Trends in Bioanalytical Methods

Emerging bioanalytical workflows are transitioning from isolated single-attribute assays toward integrated, high-density analytical systems [207, 208]:

3.6.1. Multi-Attribute Methods (MAM)

MAM workflows utilize high-resolution peptide mapping coupled with dedicated deconvolution software to simultaneously detect, identify, and quantify multiple critical quality attributes (CQAs) including site-specific deamidation, oxidation, isomerization, and glycan distributions within a single validated analytical run [209, 210]. Designed with automated new-peak detection algorithms, MAM can replace multiple conventional release assays (such as cation-exchange chromatography and capillary isoelectric focusing) during commercial quality control [211, 212].

3.6.2. Sustainable Sample Preparation Materials

Sorbent architectures are incorporating advanced materials, including metal-organic frameworks (MOFs), molecularly imprinted polymers (MIPs), and deep eutectic solvents (DES) [213, 214]. These materials offer tunable pore dimensions, high surface areas,

and selective molecular recognition while reducing the volume of hazardous organic solvents needed for bioanalytical sample preparation [215, 216].

3.6.3. Microsampling in Clinical Studies

Volumetric absorptive microsampling (VAMS) and dried blood spot (DBS) devices enable precise collection of fixed, capillary blood volumes (10–30 μL) from fingerstick sampling [217, 218]. Following desiccation and stabilization, dried matrices are eluted and processed via automated immunoaffinity-LC–MS/MS pipelines, supporting pediatric trials and remote therapeutic drug monitoring [219, 220].

3.6.4. Bioorthogonal Sensors and Microfluidics

Integrated microfluidic chips ("lab-on-a-chip") couple automated sample extraction, chemical reduction, and electrophoretic separation directly with mass spectrometers [221, 222]. These microfluidic platforms limit handling volumes, lower reagent costs, and provide rapid turnaround times for real-time biomanufacturing process monitoring [223, 224]. Table 4 highlights emerging bioanalytical platforms, summarizing their operational advancements over conventional techniques, primary analytical applications, and current regulatory or technical hurdles.

Table 4. Modern Bioanalytical Technologies for Therapeutic Monoclonal Antibodies

Modern Analytical Technology	Technical Innovation & Operational Paradigm	Primary Bioanalytical Application	Distinct Advantage Over Legacy Platforms	Remaining Implementation Hurdle
Multi-Attribute Method (MAM)	Automated, high-resolution LC–MS peptide mapping paired with new-peak detection software	Simultaneous identification and quantification of multiple CQAs in QC release testing	Replaces multiple single-attribute legacy assays (CEX, cIEF, SDS gels) with a single validated workflow	High data processing burden; software validation for automated GMP release compliance
Ion Mobility–Mass Spectrometry (IM-MS)	Gas-phase electrophoretic separation based on ion collision cross section (CCS)	Resolving isobaric conformers, disulfide mispairings, and ADC drug-to-antibody ratio (DAR) distributions	Separates structural and conformational isomers that completely co-elute in traditional liquid chromatography	Requires specialized hardware, calibrants, and standardized collision cross section libraries
Volumetric Absorptive Microsampling (VAMS)	Precision absorption of fixed micro-volumes (10–30 μL) of capillary blood via porous polymeric tips	Decentralized clinical trials, pediatric PK studies, and remote therapeutic drug monitoring (TDM)	Eliminates the volumetric hematocrit bias associated with traditional dried blood spot (DBS) cards	Extraction recoveries vary with desiccation duration; requires validated high-sensitivity IA-LC–MS/MS
Microfluidic Lab-on-a-Chip Electrospray (e.g., ZipChip)	Integration of capillary zone electrophoresis (CZE) and nano-ESI directly within a microfluidic glass chip	Ultrafast charge-variant separation and intact mass profiling of biotherapeutics	Reduces chromatographic runtimes from 45 minutes down to 2–3 minutes with sub-microliter sample consumption	Chip lifetime and surface fouling during continuous loading of complex clinical matrices
Green Sorbent Microextraction (MOFs, MIPs, DES)	Highly porous, customizable sorbents including metal-organic frameworks and deep eutectic solvents	Environmentally sustainable, selective solid-phase microextraction of biotherapeutic digests	Tunable pore geometries provide superior loading capacities while drastically reducing hazardous solvent consumption	Sorbent stability in extreme pH ranges; batch-to-batch synthetic reproducibility across manufacturers

4. Challenges in Bioanalytical Method Development and Validation

Despite advances in high-resolution instrumentation and selective affinity matrices, monoclonal antibody bioanalysis remains subject to specific chemical and instrumental interferences [225, 226]:

Ion Suppression from Endogenous Immunoglobulins: Co-extracted endogenous polyclonal human antibodies generate dense peptide backgrounds that compete for electrospray ionization charge states, causing non-uniform ion suppression and elevating baseline noise during surrogate peptide detection [227, 228].

1. **Anti-Drug Antibody Complexation:** Circulating anti-drug antibodies bind therapeutic mAbs to form stable immune complexes [229, 230]. These complexes block target paratopes, which interferes with antigen-based capture techniques and leads to under-quantification in pharmacokinetic studies unless acid-dissociation protocols are validated [231, 232].
2. **Artifacts from Proteolytic Digestion:** Prolonged trypsin digestion under alkaline conditions (pH 7.8–8.5) at 37°C can induce artificial asparagine deamidation, methionine oxidation, and disulfide scrambling [233, 234]. These *in vitro* artifacts complicate the quantification of true critical quality attributes [235, 236].
3. **Physical Adsorption:** Large, amphiphilic proteins readily adhere to standard glass and polypropylene collection tubes, auto-sampler vials, and chromatographic capillaries [237, 238]. Without surface passivation using non-ionic surfactants (e.g., polysorbate-20) or protein carriers, non-specific adsorption causes analyte loss at low nanogram-per-milliliter concentrations [239, 240].
4. **Charge-State Distribution Instability:** Minor variations in electrospray desolvation temperatures, mobile-phase additives, or counter-ion concentrations can alter the charge-state distribution of intact proteins, leading to shifting mass spectral responses and complicating absolute deconvolution [241, 242].

Table 5. Critical Quality Attributes (CQAs) of Therapeutic Monoclonal Antibodies: Analytical Profiling Techniques and Clinical Impact

Critical Quality Attribute (CQA)	Modification Mechanism	Biological & Clinical Impact	Analytical Method	Confirmatory Method
High-Molecular-Weight Species (HMWS)	Physical/covalent aggregation (dimers, trimers, sub-visible particles)	High clinical risk of anti-drug antibody (ADA) induction, altered clearance, severe infusion reactions	Non-denaturing SEC–HPLC (UV / MALS)	Analytical Ultracentrifugation (AUC), Native MS
Low-Molecular-Weight Species (LMWS)	Enzymatic or radical-induced hinge hydrolysis, light-chain dissociation	Reduced serum half-life, loss of bivalent binding avidity, compromised therapeutic efficacy	Microchip CE-SDS (non-reducing)	Reversed-Phase Subunit LC–MS
Core N-Glycan Afucosylation	Absence of core α -1,6-fucose on conserved Asn-297 heptasaccharide	Dramatically increases binding affinity to Fc γ RIIIa, elevating ADCC potency 10- to 50-fold	Hydrophilic Interaction Liquid Chromatography (HILIC–FLR/MS)	Intact Fc/2 Subunit High-Resolution MS
Asparagine Deamidation	Conversion of Asn to Asp/isoAsp via succinimide intermediate (common in CDR loops)	Alters local charge; can cause significant reduction or complete loss of target binding affinity	Cation-Exchange Chromatography (CEX–HPLC/UHPLC)	Bottom-up Multi-Attribute Method (MAM LC–MS/MS)
Methionine / Tryptophan Oxidation	Chemical oxidation (Met-252, Met-428 in Fc; Trp in CDR loops)	Met oxidation impairs binding to neonatal Fc receptor (FcRn), drastically shortening <i>in vivo</i> circulating half-life	Reversed-Phase LC–MS Peptide Mapping	Middle-Down Fc/2 Subunit LC–MS
Charge Heterogeneity (C-terminal Lysine)	Incomplete enzymatic cleavage of heavy-chain C-terminal Lys residues	Clinically benign; rapidly cleaved by endogenous carboxypeptidases <i>in vivo</i> upon systemic circulation	Capillary Isoelectric Focusing (cIEF / imaged cIEF)	Native Mass Spectrometry

Overcoming these bioanalytical challenges require improvements in automation, sample preparation, and computational data processing [243, 244]. Standardized, automated magnetic bead workstations minimize manual pipetting steps, reducing coefficient-of-variation values across high-volume pharmacokinetic studies [245, 246]. Rapid digestion protocols utilizing immobilized trypsin cartridges or pressure-assisted digestion can shorten enzymatic incubation times from hours to minutes, suppressing artificial

chemical deamidation [247, 248]. In data processing, deep-learning deconvolution algorithms are improving the processing of complex, multi-charge native mass spectra, enabling automated annotation of low-abundance glycoforms and post-translational variants in the presence of overlapping background signals [249, 250]. Combining automated microfluidic extraction, native ion mobility-mass spectrometry, and multi-attribute methods establishes a cohesive bioanalytical framework capable of supporting both clinical pharmacokinetic trials and strict commercial quality control for next-generation biotherapeutics [251, 252]. Table 5 lists the major critical quality attributes of therapeutic monoclonal antibodies, specifying their root modification mechanisms, clinical implications, and the orthogonal bioanalytical methodologies used for their evaluation

5. Conclusion

Monoclonal antibodies constitute an essential class of modern biotherapeutics, requiring reliable, selective, and validated bioanalytical methods across preclinical evaluation, clinical trials, and manufacturing quality control. Traditional enzyme-linked immunosorbent assays provide high-throughput capacity, while liquid chromatography coupled with tandem mass spectrometry provides chemical specificity to overcome endogenous matrix interferences. Complementary size-exclusion chromatography and native high-resolution mass spectrometry enable in-depth characterization of size variants, higher-order structures, and critical quality attributes across intact, middle-down, and top-down workflows. The combination of automated sample-handling systems, multi-attribute methods, and data-deconvolution algorithms provide an analytical pipeline capable of accelerating biotherapeutic discovery and maintaining regulatory standards.

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