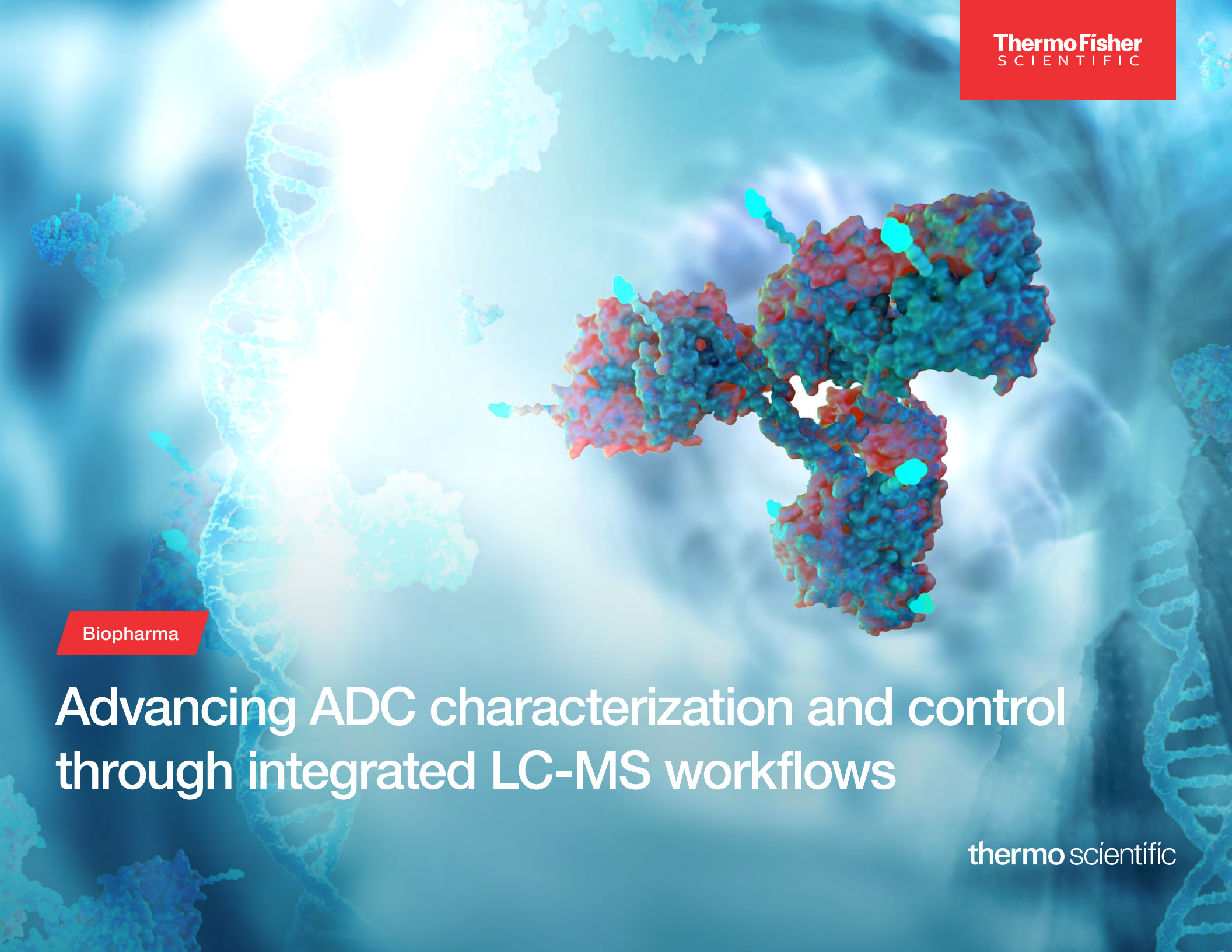


Biopharma

Advancing ADC characterization and control through integrated LC-MS workflows

thermo scientific

Table of contents

Click on titles to jump to page.

Introduction

Antibody-drug conjugates (ADCs) are an emerging class of biotherapeutics that combine the selectivity of monoclonal antibodies with the potency of cytotoxic payloads. However, their structural complexity—arising from heterogeneous drug-to-antibody ratios (DAR), conjugation sites, and post-translational modifications—directly impacts safety, stability, and efficacy. As a result, detailed characterization of ADC heterogeneity, including charge variants and conjugation patterns, is essential for ensuring product quality and regulatory compliance.

Mass spectrometry-based approaches, including bottom-up, top-down, and middle-down workflows, are widely used for ADC characterization. Bottom-up methods provide high sequence coverage but can introduce artifacts and lose information on combinatorial payload distribution. Top-down approaches preserve intact structure but offer limited sequence coverage for large molecules. Middle-down strategies bridge these gaps by enabling higher coverage at the subunit level, yet challenges remain in resolving DAR species and confidently localizing payload conjugation sites across heterogeneous populations.

These limitations highlight the need for integrated, multi-level analytical workflows that can resolve ADC heterogeneity while maintaining structural context. Addressing these challenges requires advanced approaches capable of combining separation, detection, and detailed structural analysis to enable more confident and comprehensive ADC characterization.

The gap between existing analytical capabilities and the demands of ADC characterization underscores a set of interconnected challenges that directly impact data confidence, consistency, and quality assessment. Limitations in resolving heterogeneity, integrating structural insights, and enabling reproducible workflows collectively hinder comprehensive evaluation of these complex modalities. Addressing these constraints requires a clearer understanding of the key analytical bottlenecks shaping ADC development and quality control.

Key analytical challenges in ADC characterization

Key analytical challenges in ADC characterization

1 Achieving comprehensive structural characterization of ADCs

ADCs are highly complex, multi-component molecules requiring simultaneous characterization of the antibody backbone, linker, and cytotoxic payload. Obtaining deep structural insight is hindered by limitations in sequence coverage, fragmentation efficiency, and the ability to capture multiple attributes in a unified analysis. The need to resolve sequence variants, modifications, and conjugation sites across heterogeneous populations adds further complexity. Integrating these diverse data layers into a coherent and reliable structural profile remains a significant analytical challenge.

2 Confident localization of payload conjugation sites

Accurate identification of payload attachment sites remains a major challenge due to the inherent heterogeneity of ADCs, including variable DARs and positional isomers. Complex fragmentation patterns and overlapping signals further complicate the assignment of diagnostic ions to specific conjugation sites. Conventional approaches often fail to provide sufficient resolution or structural context, limiting the ability to precisely map payload distribution across antibody subunits. This lack of clarity directly impacts the understanding of structure–function relationships and consistency across

3 Comprehensive analysis of charge heterogeneity

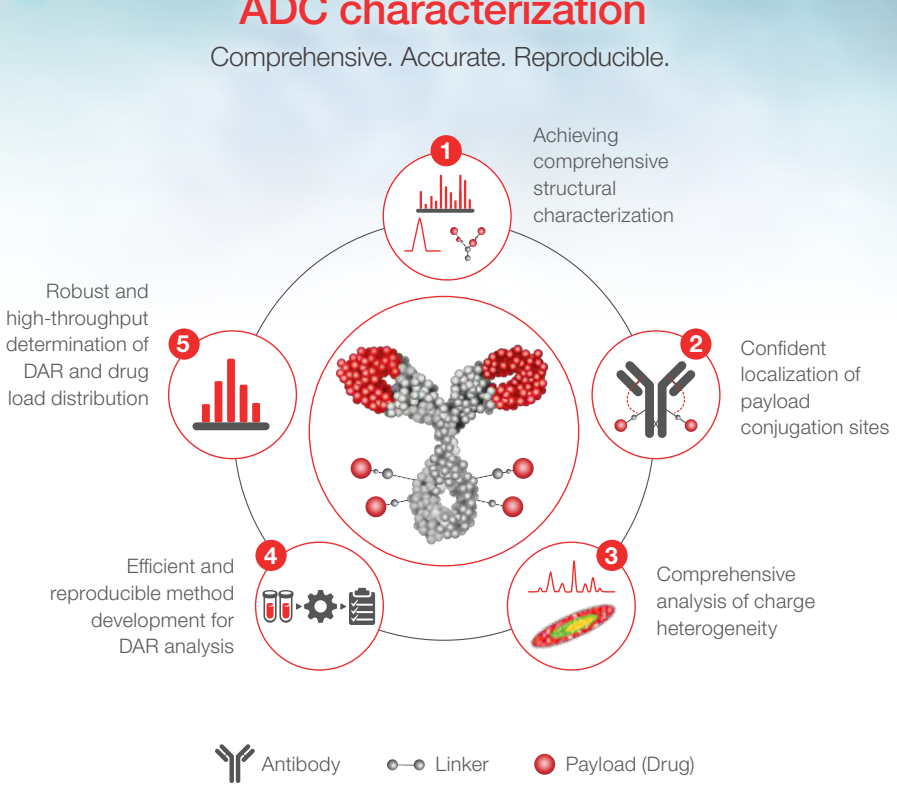
Charge heterogeneity in ADCs arises from conjugation processes, post-translational modifications, and degradation pathways, producing closely related variants with subtle physicochemical differences. Resolving and characterizing these species can be difficult using conventional single-dimension methods. Additional challenges include maintaining native structure during analysis and ensuring compatibility between separation techniques and downstream detection. The presence of low-abundance variants further complicates detection and confident identification, impacting overall quality assessment.

4 Efficient and reproducible method development for DAR analysis

Developing robust methods for DAR determination, particularly using chromatographic techniques, requires careful optimization of multiple interdependent parameters. Variability in ADC physicochemical properties means that methods are not easily transferable across different molecules, necessitating repeated optimization. This process can be labor-intensive and time-consuming, creating challenges in achieving both analytical rigor and operational efficiency. Ensuring reproducibility while maintaining method performance across conditions remains a key hurdle.

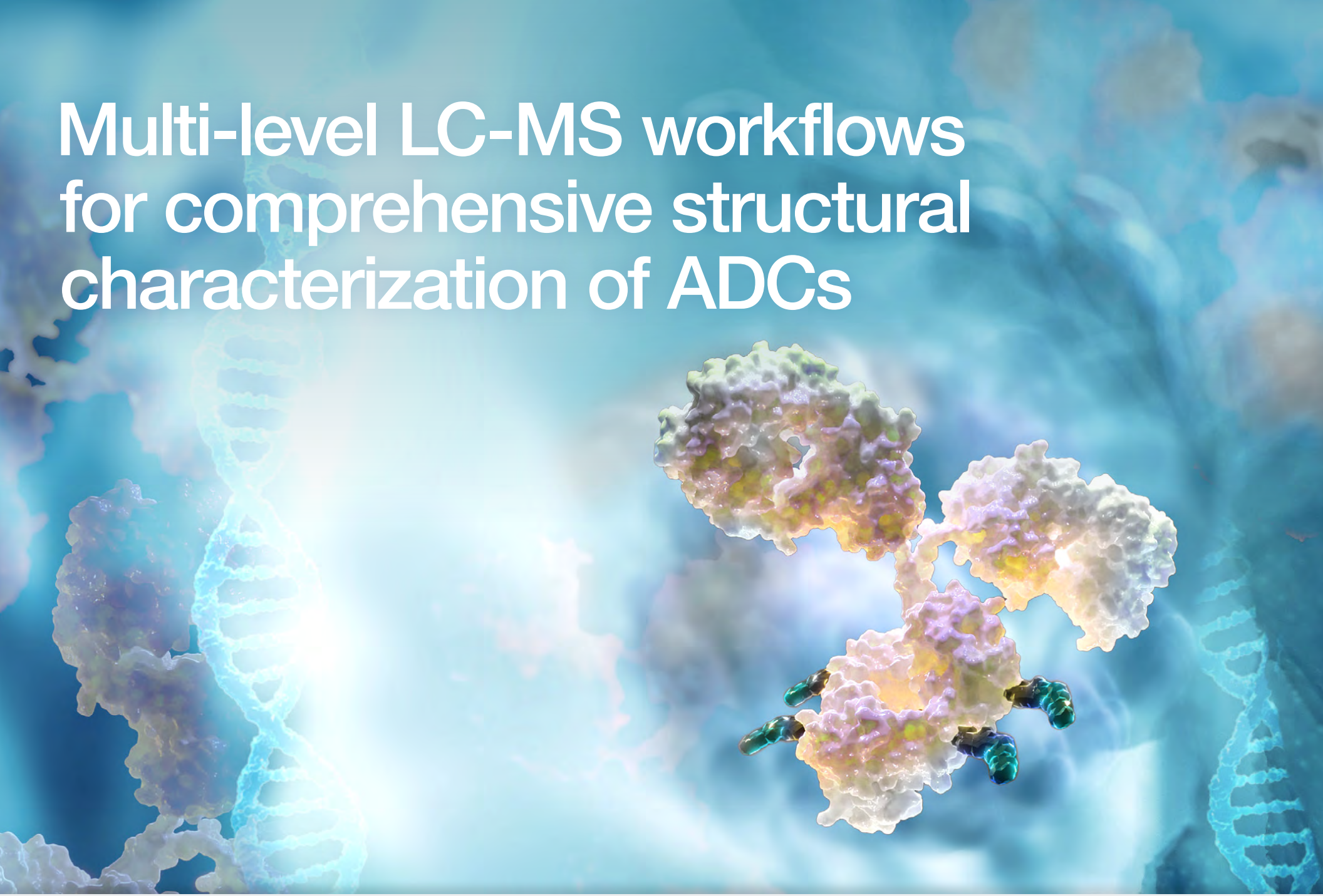
5 Robust and high-throughput determination of DAR and drug load distribution

Accurate determination of DAR and drug load distribution is critical for assessing ADC efficacy, stability, and batch consistency. However, traditional workflows often involve manual processing, multiple analytical steps, and time-intensive data interpretation. These limitations reduce throughput and introduce variability, making routine monitoring challenging. As ADC development scales, there is an increasing need for streamlined approaches that can deliver consistent, reproducible results with minimal complexity.



Addressing these analytical bottlenecks requires more than incremental improvements, it demands integrated workflows designed to resolve ADC complexity with confidence. Thermo Fisher Scientific brings together complementary analytical capabilities to enable sensitive, reproducible, and high-resolution characterization across multiple attributes. The following solutions demonstrate how targeted approaches can overcome these challenges and support reliable ADC development and quality assessment.

Multi-level LC-MS workflows for comprehensive structural characterization of ADCs



Multi-level LC-MS workflows for comprehensive structural characterization of ADCs

Achieving structural characterization of antibody-drug conjugates such as KADCYLA™ (ado-trastuzumab emtansine) remains challenging due to their multi-component architecture, heterogeneous DAR distribution, and the need to simultaneously resolve sequence, modifications, and conjugation sites. Conventional approaches often lack sufficient sequence coverage or fragmentation efficiency to integrate these attributes. A multi-level LC-MS strategy combining intact, subunit, and peptide-level analysis provides a unified, high-resolution structural understanding of complex ADCs.

The workflow integrates complementary analytical levels to capture ADC structural complexity. Native intact mass spectrometry provides molecular weight and DAR distribution while preserving higher-order structure (Figure 1). Subunit analysis characterizes antibody fragments with distinct payload distributions, while peptide mapping delivers sequence coverage, PTM identification, and localization of conjugation sites.

Fragmentation using higher-energy collisional dissociation (HCD) and electron-transfer/higher-energy collision dissociation (EThcD) enhances peptide identification while preserving labile modifications. The fast and efficient EThcD fragmentation improves sequence coverage and enables confident localization of payload conjugation sites, even within structurally complex regions of the ADC.

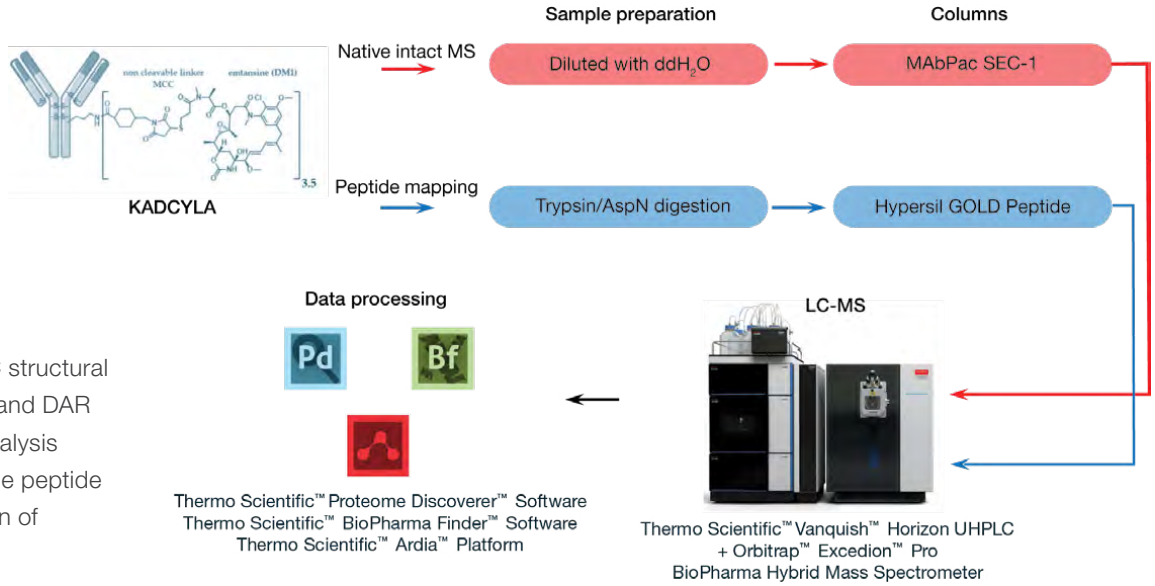
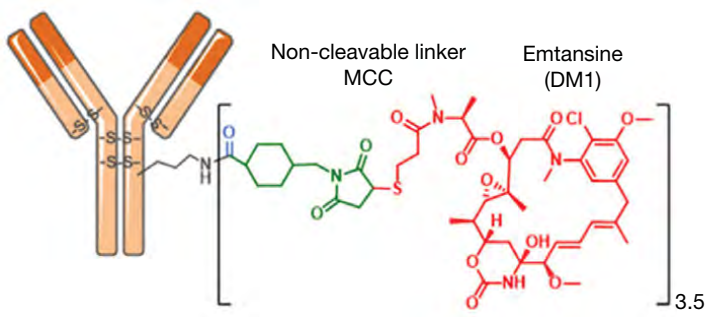


Figure 1. The UHPLC-HRAM MS characterization workflow for KADCYLA.

Multi-level LC-MS workflows for comprehensive structural characterization of ADCs

How was this performed?

In the study, KADCYLA, a lysine-linked ADC with a DAR distribution ranging from 0 to 8, was analyzed across multiple structural levels (Figure 2). Native intact analysis was performed to determine molecular weight and DAR distribution. Subunit analysis involved IdeS digestion followed by reduction to generate antibody fragments for LC-MS characterization.



anti-HER2 Kadcyla (ado-trastuzumab emtansine)

Figure 2. Structural representation of KADCYLA, highlighting the MCC linker and DM1 payload contributing to ADC heterogeneity.

Peptide mapping was conducted using trypsin and AspN digestion, enabling complementary sequence coverage. Chromatographic separation was performed using Thermo Scientific™ Vanquish™ Horizon Binary UHPLC System with Thermo Scientific™MAbPac™ SEC-1 and Hypersil GOLD™ Peptide Columns, coupled to a Thermo Scientific™ Orbitrap™ Excedion™ Pro BioPharma Mass Spectrometer. Data acquisition incorporated both HCD and EThcD fragmentation, while analysis was carried out using Thermo Scientific™ BioPharma Finder™ Software, Thermo Scientific™ Proteome Discoverer™ Software, and Thermo Scientific™ Ardia™ Platform to extract structural and modification information.

Multi-level LC-MS workflows for comprehensive structural characterization of ADCs

What the study shows?

The integrated workflow enables simultaneous characterization of multiple critical quality attributes across ADC structural levels. Native intact MS resolved DAR species from 0 to 8 with accurate quantification, while subunit analysis revealed payload and glycoform distributions across antibody fragments.

Peptide mapping achieved 100% sequence coverage in a single injection (Figure 3), enabling comprehensive identification of sequence variants and modifications. The use of EThcD fragmentation enabled confident localization of 43 out of 46 potential conjugation sites, including peptides containing multiple modification sites (Figure 4A and 4B). High sensitivity further supported detection of low-abundance species, enabling deeper insight into ADC heterogeneity.

The multi-level LC-MS approach delivers deep, high-sensitivity structural insight into complex ADCs such as KADCYLA. It enables accurate DAR determination, complete sequence coverage, and confident identification of PTMs and conjugation sites within a single integrated workflow.

By combining high sensitivity with efficient EThcD fragmentation, this approach significantly improves structural resolution and analytical confidence, supporting comprehensive characterization of conjugation patterns, sequence integrity, and modifications. This enables more reliable assessment of ADC quality, consistency, and function, ultimately supporting robust development and quality control of complex biotherapeutics.

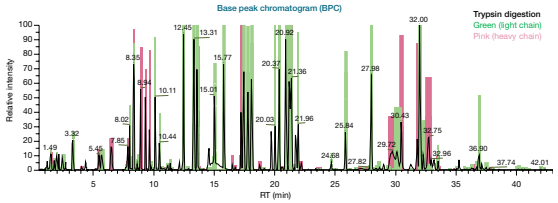
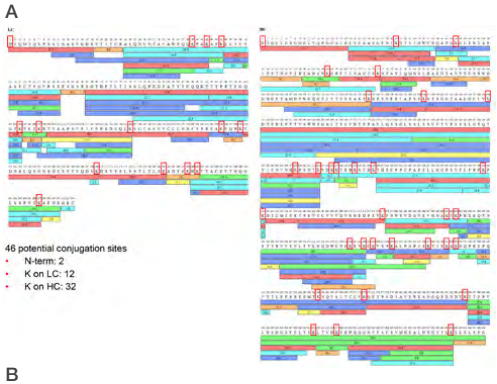


Figure 3. Peptide mapping workflow achieving complete sequence coverage of light and heavy chains, enabling comprehensive structural characterization.



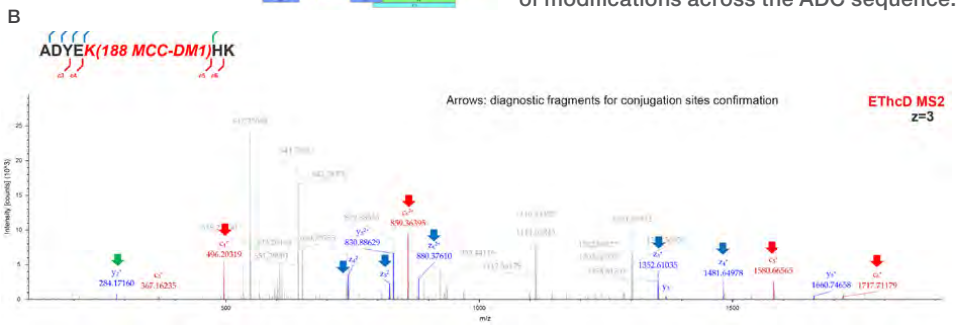


Figure 4A and B. Identification of payload conjugation sites using EThcD fragmentation, enabling confident mapping of modifications across the ADC sequence.

Resolving spectral complexity for high-confidence payload conjugation site localization

Resolving spectral complexity for high-confidence payload conjugation site localization

Accurate localization of payload conjugation sites in heterogeneous ADCs remains a critical challenge due to overlapping fragment ions, variable DAR distributions, and convoluted spectra. Typical intact MS analysis can lack the resolution required to confidently assign diagnostic ions across antibody subunits. A middle-down mass spectrometry workflow integrating advanced fragmentation and spectral decongestion enables precise mapping of payload attachment sites.

The workflow analyzes antibody subunits (~25 kDa) generated through controlled proteolysis and reduction, preserving structural context while improving sequence coverage. Fragmentation using electron transfer/higher-energy collision dissociation (EThcD) generates complementary fragment ions, while proton transfer charge reduction (PTCR) at the MS^3 level disperses ions across a wider *m/z* range, simplifying spectral interpretation (Figure 5). This combined approach improves fragment ion clarity and supports confident localization of conjugation sites in heterogeneous ADC subunits.

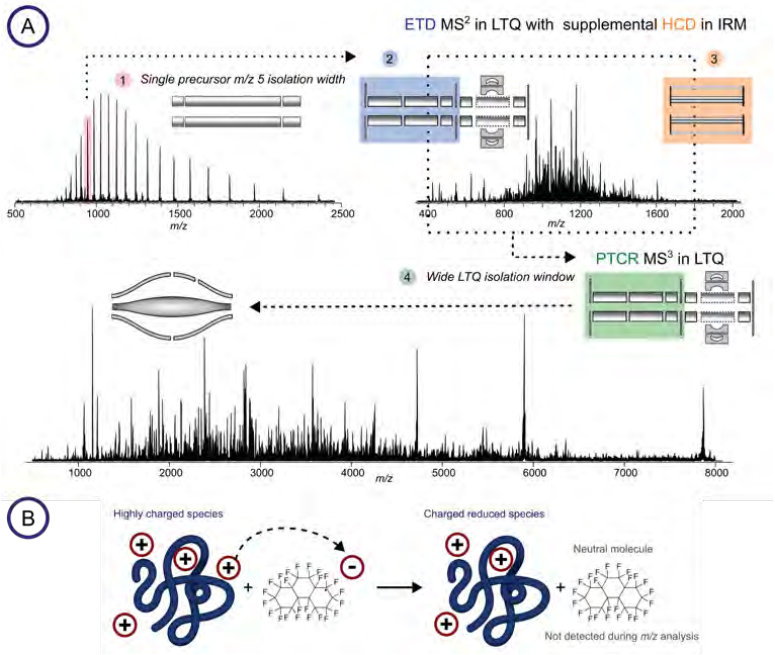


Figure 5. Middle-down LC-MS workflow incorporating EThcD fragmentation and PTCR-based spectral decongestion out using an Thermo Scientific™ Orbitrap Ascend BioPharma MS. (A) Main steps of data acquisition, namely: (1) quadrupole isolation of an individual precursor charge state, (2) ETD reaction performed in the high-pressure cell of the dual-pressure LTQ followed by (3) the HCD-based supplemental activation of ETD products in the back ion-routing multipole (IRM), and—if experiments are performed at the MS3 level (4) isolation of EThcD products, that are subjected to PTCR prior to detection in the Orbitrap mass analyzer. (B) Reaction scheme of the PTCR event: a single proton is transferred from a multiply charged cation to the PTCR reagent anion, yielding a charge-reduced cation and a neutral molecule.

The workflow also increases the number of identifiable fragment ions and complementary fragment ion pairs, strengthening sequence confirmation and enabling more accurate mapping of conjugation sites. Enhanced fragmentation coverage further supports confident structural assignment across heterogeneous ADC populations (Figure 6).

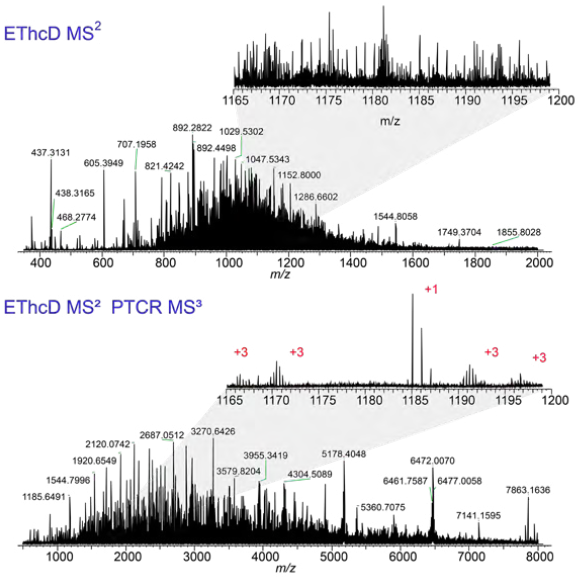


Figure 6. PTCR before vs after spectrum: PTCR reduces spectral complexity, enabling clearer identification of fragment ions and improved confidence in product ion matching.

Resolving spectral complexity for high-confidence payload conjugation site localization

How was this performed?

In the study, a SigmaMAb ADC mimic was subjected to IdeS digestion followed by reduction to generate ~25 kDa subunits (Fc/2, Lc, and Fd) with varying payload attachments. The samples were analyzed using reversed-phase LC on a Thermo Scientific™ Vanquish™ Flex UHPLC Systems coupled to an Thermo Scientific™ Orbitrap™ Ascend BioPharma Tribrid™ Mass Spectrometer equipped with ETD, EThcD, UVPD, and PTCR capabilities.

A targeted LC-MS workflow was employed, where precursor ions corresponding to specific subunits were isolated and fragmented using EThcD (MS^2), followed by PTCR (MS^3) for spectral simplification. Data analysis was performed using BioPharma Finder software to enable fragment matching and sequence interpretation.

What the study shows

The integration of PTCR with EThcD significantly reduces spectral congestion, enabling the detection of previously obscured diagnostic fragment ions and improving confidence in fragment ion assignment (Figure 7). This allows clear differentiation of positional isomers and precise localization of payload conjugation sites across antibody subunits. fragment matching and sequence interpretation.

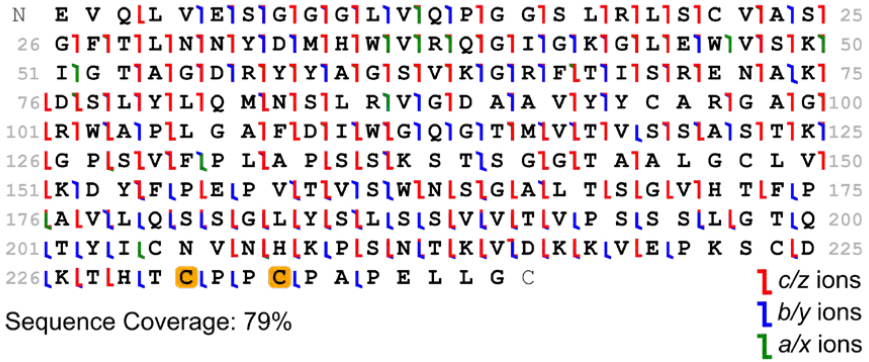
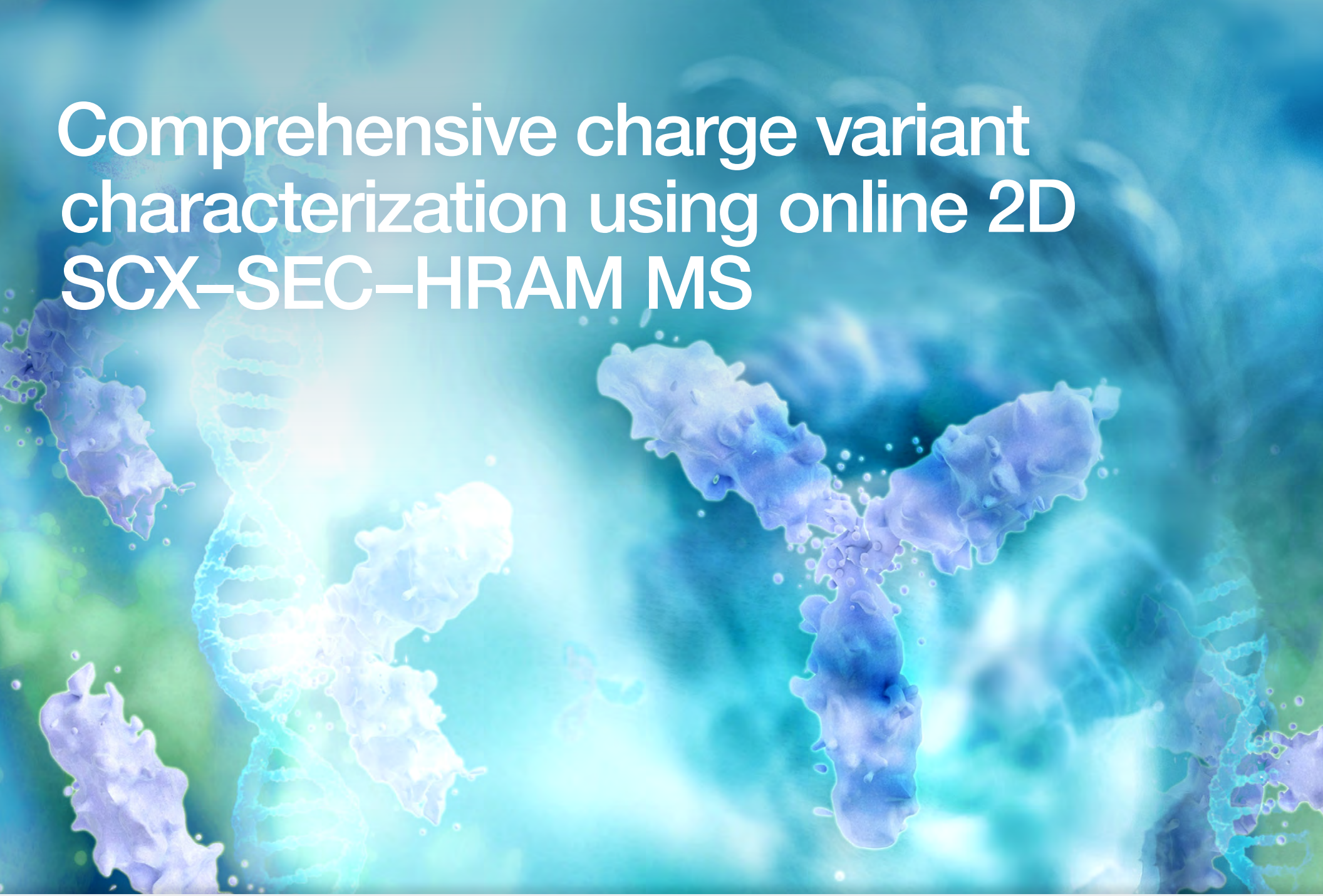


Figure 7. Enhanced sequence coverage enables precise mapping of conjugation sites across antibody subunits. Cysteine residues identified as drug conjugation site are highlighted in yellow.

The combined EThcD–PTCR workflow enables unambiguous localization of payload conjugation sites in variable-DAR ADCs. Spectral deconvolution results in a 16–20% increase in sequence coverage, with overall coverage exceeding 75% when multiple activation methods are combined.

By bridging the gap between intact and peptide-level analysis, this approach delivers site-specific structural insight with reduced analytical complexity, significantly improving confidence in ADC characterization and supporting more informed decision-making during development.

Comprehensive charge variant characterization using online 2D SCX–SEC–HRAM MS



Comprehensive charge variant characterization using online 2D SCX–SEC–HRAM MS

Charge heterogeneity in ADCs introduces closely related variants arising from conjugation, post-translational modifications, and degradation, making them difficult to resolve using single-dimension methods. An integrated hybrid 2D SCX–SEC–LC–HRAM MS workflow enables detailed characterization of charge variants while preserving native structure.

An online multiple heart-cut two-dimensional LC workflow combines strong cation exchange (SCX) separation with SEC desalting and high-resolution accurate-mass detection (Figure 8). SCX separates variants based on isoelectric differences, while targeted fraction transfer enables MS-compatible desalting and preserves native structure for downstream analysis. SEC removes non-volatile salts and maintains non-covalent interactions for HRAM MS. This setup, implemented on a Thermo Scientific™ Vanquish™ Simple Switch 2D-LC System with a Thermo Scientific™ ProPac™ 3R SCX Column coupled to a Thermo Scientific™ Orbitrap Exploris™ 240 Mass Spectrometer, with Thermo Scientific™ Chromeleon™ CDS Software control and BioPharma Finder software, supports seamless acquisition, processing, and identification of charge variants.

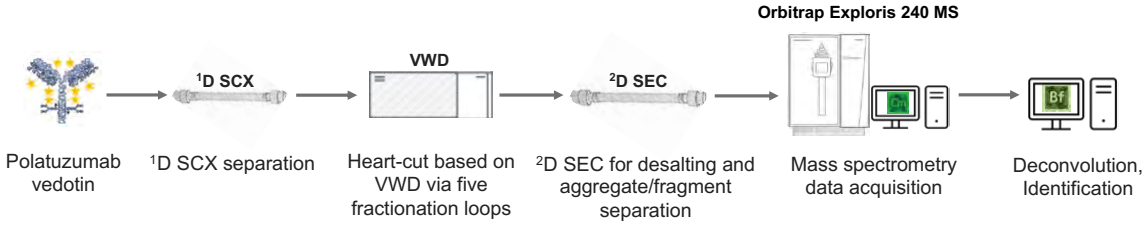


Figure 8. Overview of the experimental study design for polatuzumab vedotin charge variant analysis using the online multiple heart-cutb2D SCX-SEC-LC HRAM MS approach.

How was this performed?

In the study, polatuzumab vedotin was analyzed under native conditions without additional sample preparation. SCX separation resolved multiple charge variants, which were selectively captured using fractionation loops and transferred to the SEC dimension for desalting and intact mass analysis. The 2D workflow enabled direct coupling to Orbitrap Exploris 240 system, with data processed using the ReSpect™ deconvolution algorithm in BioPharma Finder software to identify charge variants associated with DAR species, glycoforms, and PTMs.

Comprehensive charge variant characterization using online 2D SCX–SEC–HRAM MS

What the study shows?

The study demonstrates that SCX separation resolves distinct charge variants across ADC populations, enabling detailed profiling of heterogeneity (Figure 9). A total of up to 18 charge variant peaks were resolved, reflecting the complexity of ADC charge heterogeneity. The workflow enables identification of variants associated with DAR distribution and PTMs, while high sensitivity allows detection of low-abundance species down to <0.5% (Figure 10). Online 2D-LC enables targeted fraction transfer and MS-compatible desalting, ensuring native-state analysis and accurate characterization of structurally similar variants.

This integrated workflow provides deep insight into ADC charge heterogeneity and associated modifications, enabling accurate and confident characterization across a wide abundance range. By combining orthogonal separation, native-state analysis, and high-resolution detection within a single platform, it reduces reliance on multiple orthogonal methods while improving analytical efficiency and throughput for ADC quality assessment.

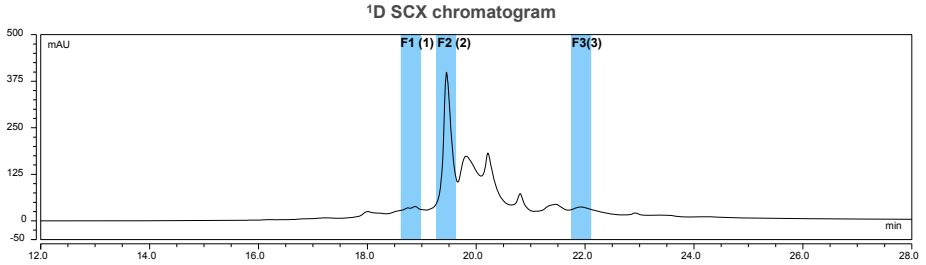


Figure 9. SCX separation resolves distinct charge variants, enabling detailed profiling of ADC heterogeneity.

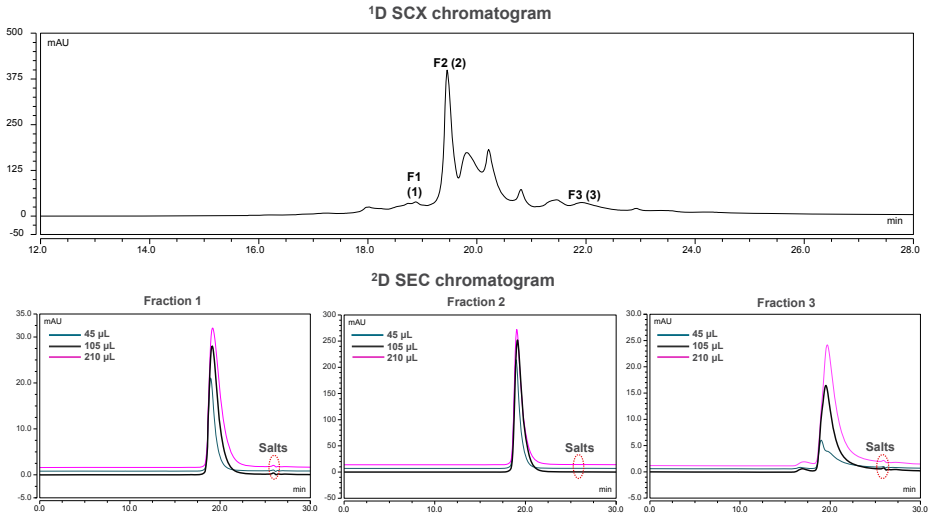


Figure 10. The ¹D SCX (top) and ²D SEC UV chromatograms (bottom) of three different fractions, with fraction volumes of 45 µL, 105 µL, and 210 µL, collected from ¹D. Despite SEC peaks being broadened with increasing fraction volume, separation of protein and salts remained adequate with a fraction volume of 210 µL when the 300 × 4.0 mm SEC column was used.

Accelerating robust DAR method development with AQbD-driven HIC optimization

Accelerating robust DAR method development with AQbD-Driven HIC optimization

Developing robust and reproducible hydrophobic interaction chromatography (HIC) methods for DAR determination is challenging due to the need to optimize multiple interdependent parameters across different ADCs. To address this, an automated method optimization workflow enables rapid, systematic evaluation of chromatographic conditions, significantly reducing development effort while ensuring consistent performance.

An analytical quality by design (AQbD) driven HIC workflow integrates automated design-of-experiment (DoE) optimization with chromatographic method execution. Using Fusion QbD™ software with the Vanquish Flex UHPLC system and Chromeleon CDS software, multiple parameters—including gradient profile, salt concentration, column temperature, and flow rate—are systematically evaluated within a structured experimental design. The software generates optimized experimental sequences, models parameter interactions, and defines a method operable design region (MODR), accelerating method development while ensuring robustness and reproducibility.

How was this performed?

In the study, three cysteine-linked (Figure 11) ADCs—brentuximab vedotin, disitamab vedotin, and polatuzumab vedotin—were analyzed using a HIC-based workflow. Samples were prepared in a mixture of eluent A and water and separated on a Thermo Scientific™ MAbPac™ HIC-Butyl HPLC Columns using a decreasing salt gradient with ammonium sulfate and phosphate buffer, combined with isopropanol as an organic modifier. A 30-run DoE generated by Fusion QbD software enabled automated evaluation of key chromatographic parameters, with sequences executed directly through Chromeleon CDS software on the Vanquish Flex UHPLC system. The resulting data were modeled to predict optimal conditions, which were experimentally verified, with predicted and observed retention times showing strong agreement.

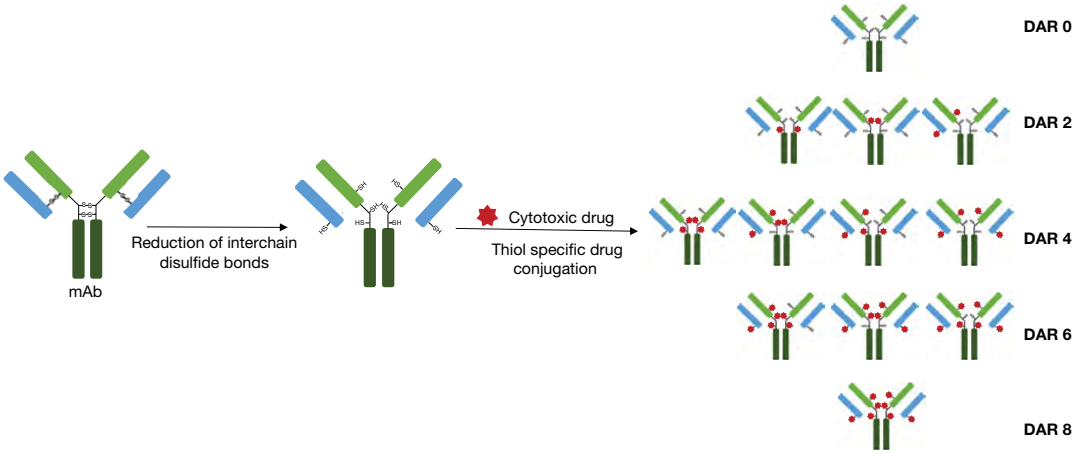


Figure 11. Schematic of the synthesis of cysteine-linked ADCs.

Accelerating robust DAR method development with AQbD-Driven HIC optimization

What the study shows?

The study demonstrates rapid optimization of HIC conditions for DAR separation across multiple ADC samples through automated evaluation of chromatographic parameters (Figure 12). The workflow enables systematic screening of gradients, salt concentration, column temperature, and flow rate, significantly reducing method development time compared to manual approaches. High agreement between predicted and experimental retention times (average error ±0.22 min; relative retention difference <6 s) confirms model accuracy, while robust method performance meets resolution and peak quality criteria. The defined MODR ensures consistent operation across parameter ranges, and reproducibility is demonstrated with retention time variability <0.9% RSD and peak area variability <2.0% RSD.

The automated workflow accelerates the development of robust HIC methods for DAR determination, reducing time to results while ensuring reliable and reproducible separation of ADC variants. By enabling rapid identification of optimal conditions and consistent performance across operating ranges, it enhances analytical confidence and operational efficiency in ADC characterization.

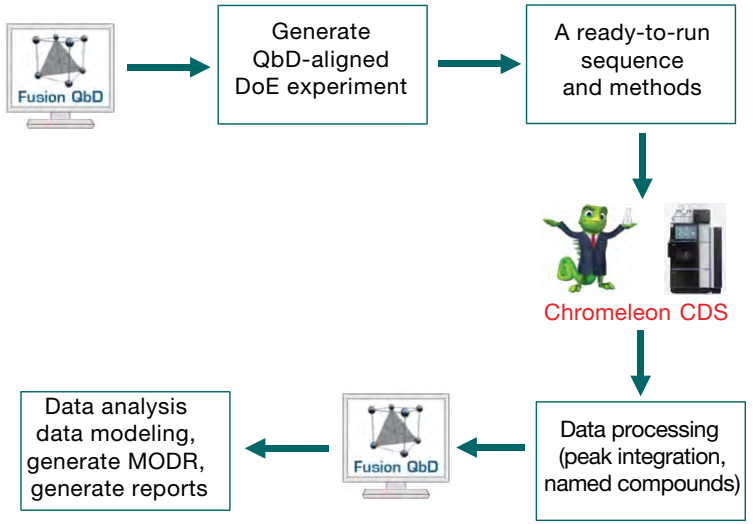
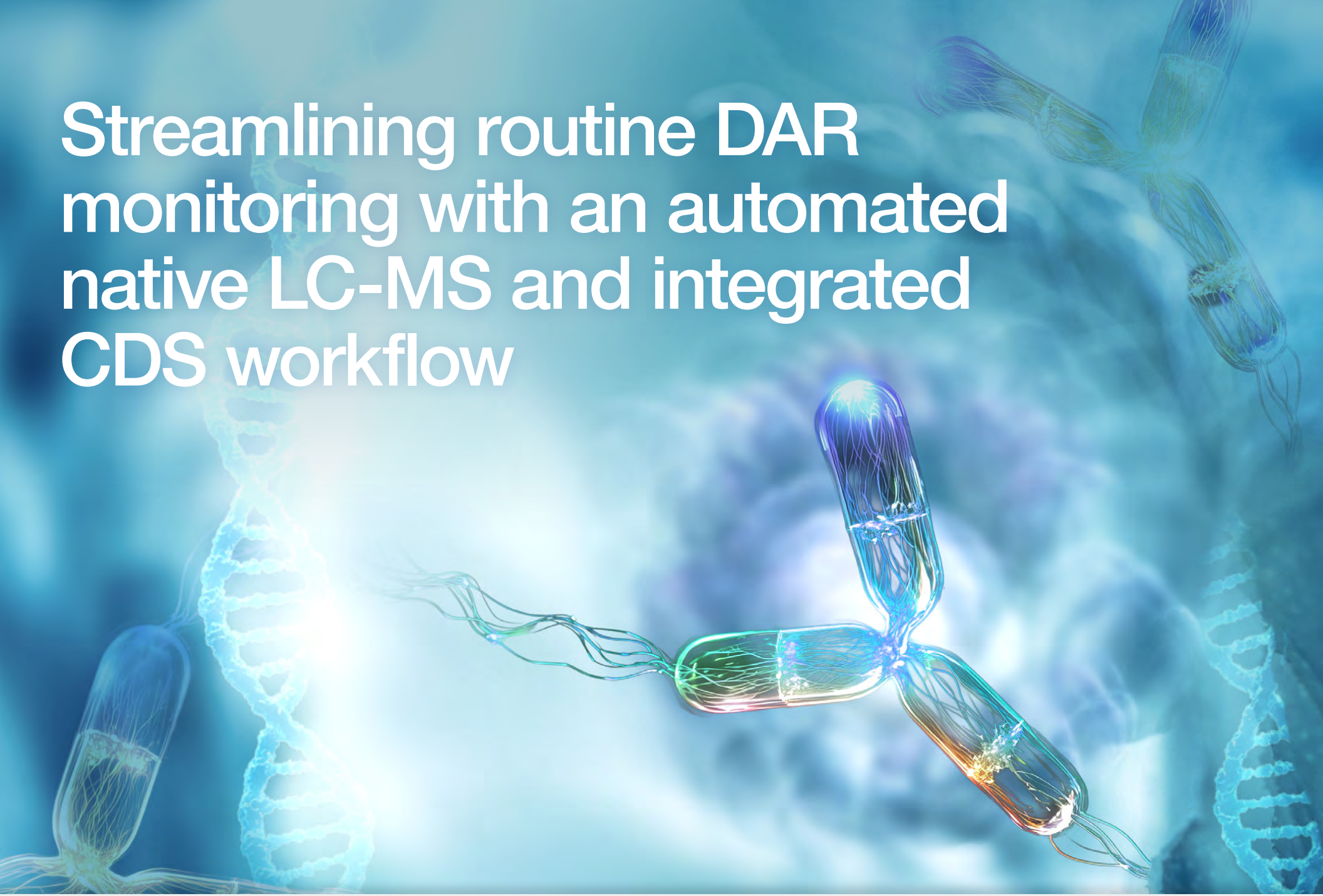


Figure 12. A QbD-based method optimization workflow using Fusion QbD software and Chromeleon CDS software.

Streamlining routine DAR monitoring with an automated native LC-MS and integrated CDS workflow



Streamlining routine DAR monitoring with an automated native LC-MS and integrated CDS workflow

Accurate determination of DAR is essential for monitoring ADC quality, consistency, and batch-to-batch variability. While detailed structural workflows provide deep insight, routine analysis requires simplified, high-throughput approaches that minimize manual intervention and ensure reproducible results. An automated native intact LC-MS workflow integrated within a Chromatography Data System (CDS) enables rapid, reliable DAR determination with end-to-end data processing and reporting (Figure 13).

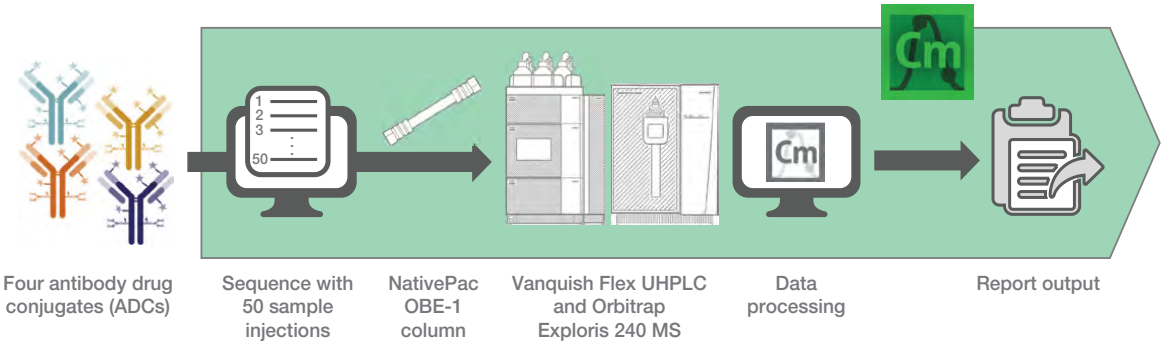


Figure 13. Automated workflow built on a tailored report template for ADC analysis and DAR calculation, fully supported by Chromeleon CDS software.

Native intact mass spectrometry enables direct analysis of ADCs while preserving higher-order structure and non-covalent interactions. UHPLC separation under native conditions, coupled with high-resolution accurate mass detection and spectral deconvolution, transforms complex *m/z* spectra into mass-domain profiles, enabling clear identification of drug load distribution and glycoform variants. Integrated data acquisition, processing, and reporting support automated component identification and accurate DAR determination from deconvoluted mass spectra.

How was this performed?

In the study, multiple therapeutic ADCs, including lysine- and cysteine-linked constructs, were analyzed using a Vanquish Flex Binary UHPLC system with a Thermo Scientific™ NativePac OBE-1 SEC Column coupled to a Orbitrap Exploris 240 mass spectrometer under native conditions. Samples were prepared at 1.0 mg/mL and 2-3 µl injections were analyzed using short 3-minute LC-MS runs, enabling rapid throughput.

Data acquisition, intact deconvolution, glycoform assignment, and DAR calculation were fully automated using the ReSpect deconvolution algorithm within Chromeleon CDS software. Batch sequences of up to 50 injections were processed without manual intervention, generating standardized report outputs for each sample.

Streamlining routine DAR monitoring with an automated native LC-MS and integrated CDS workflow

What the study shows?

The study demonstrates accurate determination of DAR distribution directly from intact ADC analysis through automated deconvolution of high-resolution mass spectra (Figure 14). Complex spectral data are simplified upon spectral deconvolution into easily interpretable mass profiles, enabling clear identification of DAR species (D0–D8) and associated glycoforms.

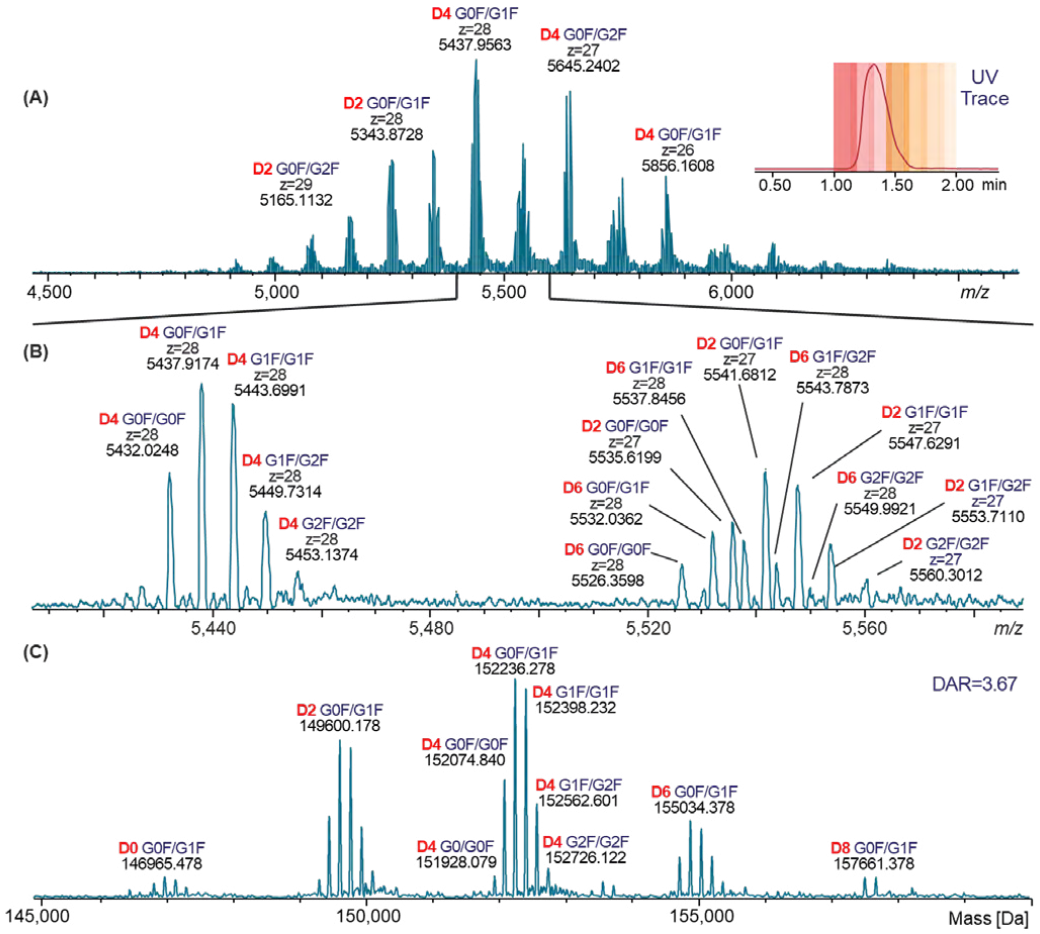


Figure 14. Native intact MS of Aidixi with peak assignments. (A) Full MS spectrum of native intact Aidixi with the insert representing the UV trace. The shaded area of the UV chromatogram indicates the range used for averaging the mass spectrum. (B) Zoom-in of *m/z* range that illustrates sufficient resolution and spectral quality to assign drug load, glycan profile, and charge state, even for crowded areas as seen at *m/z* 5,520–5,560, with overlapping peaks for D2 and D6 at different charge states. (C) Deconvoluted mass spectrum, which shows D0 to D8 identified at different glycoforms as illustrated for D4 components, and the calculated DAR.

Streamlining routine DAR monitoring with an automated native LC-MS and integrated CDS workflow

The workflow supports rapid assessment of batch-to-batch consistency through automated processing of large injection sequences, while reduced sample preparation eliminates the need for subunit or peptide-level workflows. Clear visualization of DAR distributions further enables confident routine monitoring of ADC quality attributes (Figure 15).

The integrated LC-MS workflow enables high-confidence, high-throughput characterization of ADCs within a single analytical platform. Accurate DAR determination is achieved with mass accuracy below 10 ppm for major components, while supporting the analysis of up to 50 injections in under four hours. By combining native MS with automated deconvolution and unified data processing, the approach enhances analytical efficiency, reproducibility, and confidence in assessing ADC heterogeneity.

This is enabled by a customizable Chromeleon CDS software report template that integrates instrument control, data processing, and DAR reporting within a single environment. End-to-end automation—from acquisition through deconvolution, identification, and DAR calculation—eliminates manual intervention, while short 3-minute LC-MS runs support high-throughput operation. The resulting high-resolution data further ensures reliable identification of drug load distribution and ADC variants, supporting consistent and data-driven decision-making.

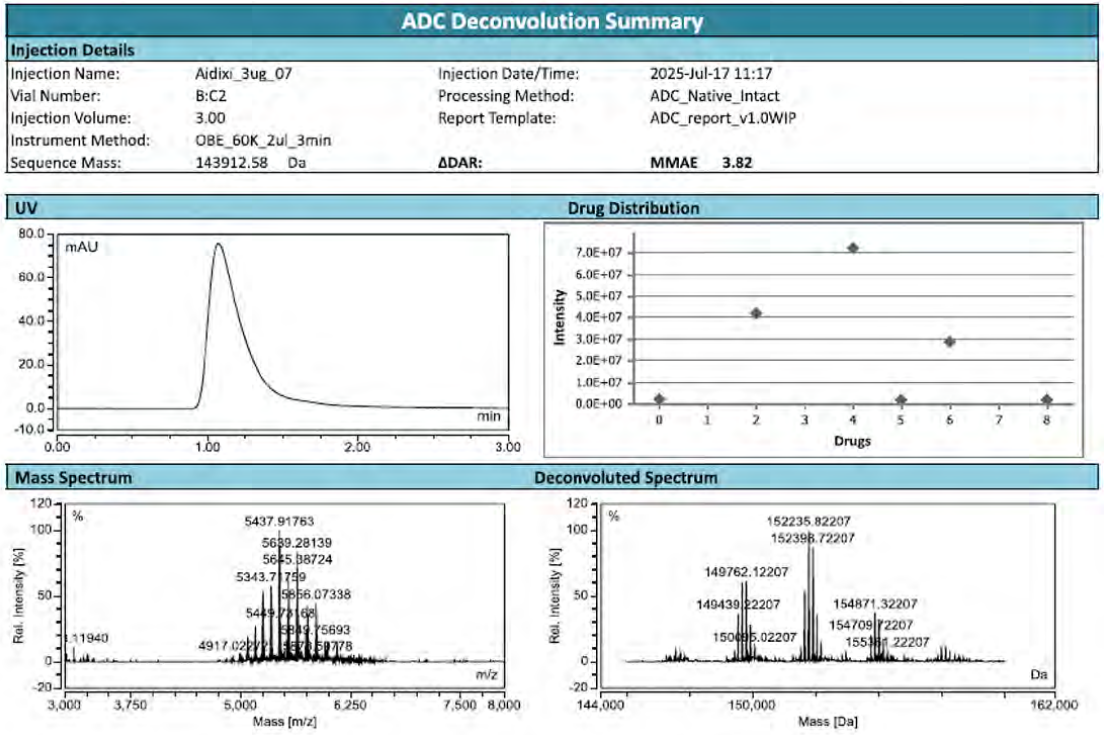


Figure 15. Report example for a single injection of Aidixi ADC. The report includes a header with information about the injection, sequence, processing, report method, and calculated average DAR. The UV trace, mass spectrum, and deconvoluted spectrum are also shown in the report with a figure illustrating the drug load distribution.

Conclusion

Thermo Fisher Scientific enables a unified approach to ADC characterization by integrating complementary analytical strategies within a single platform. Workflows spanning intact mass analysis, middle- and top-down fragmentation, and peptide mapping address molecular complexity across multiple structural levels, enabling comprehensive evaluation of critical quality attributes—from DAR distribution and glycoforms to sequence-level modifications—while maintaining analytical continuity.

By combining advanced separations, high-resolution mass spectrometry, and sophisticated data analysis within streamlined workflows, this approach delivers both depth and efficiency. Seamless progression from intact—and peptide-level characterization enhances confidence in data interpretation, reduces workflow complexity, and streamlines decision-making across ADC development and quality control.

This multi-level characterization strategy supports the characterization of structurally complex, heterogeneous ADCs by linking separation, detection, and data interpretation into a unified analytical framework, aligning structural insight with operational efficiency.

Back to contents

Learn more at **thermofisher.com/bys**