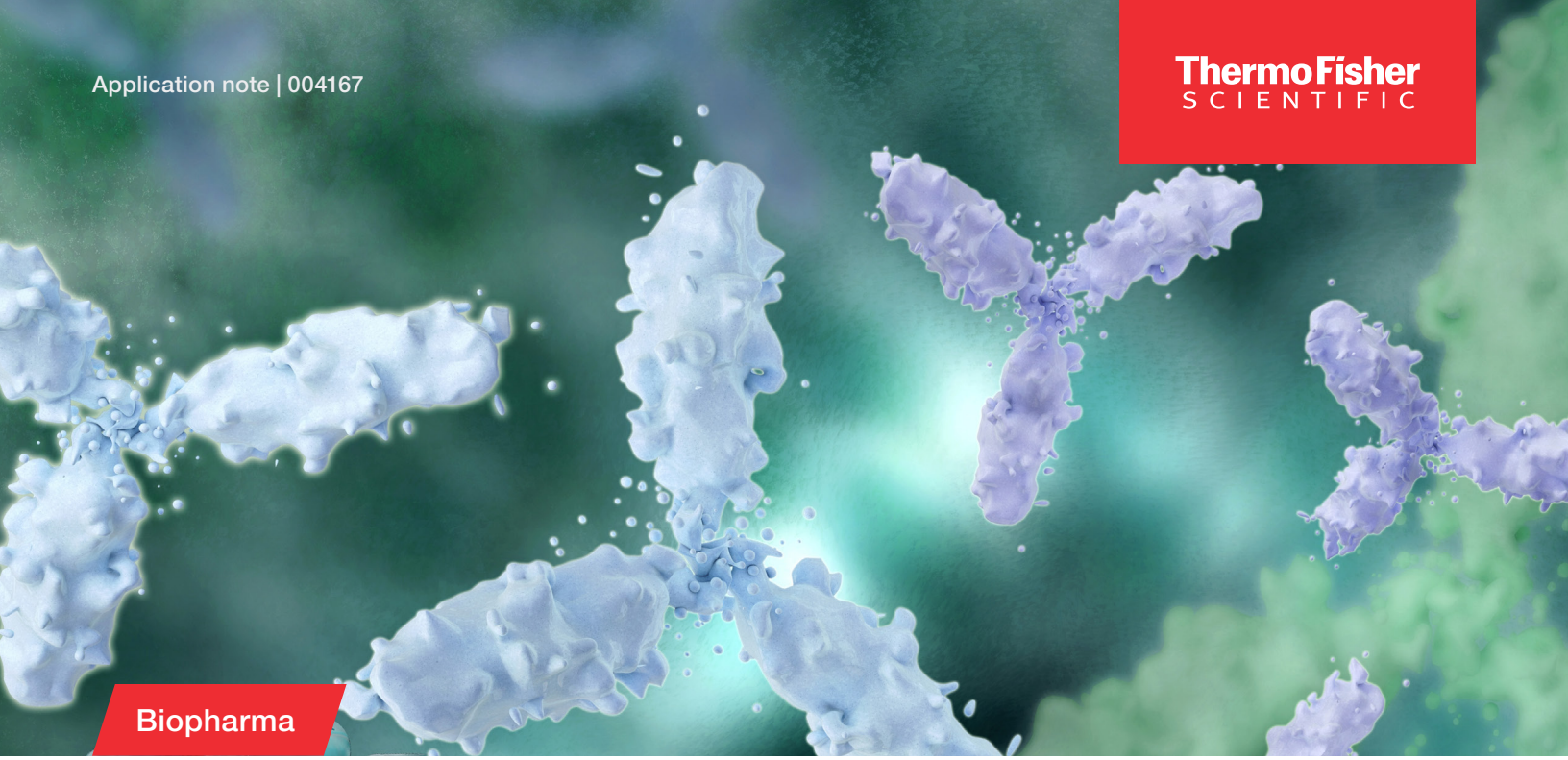
Application note | 004167



Biopharma

# Charge variant analysis of cysteine-linked antibody-drug conjugates using an online multiple heart-cut 2D-SCX-SEC-LC HRAM MS approach


## Authors

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## Keywords

Polatuzumab vedotin, cysteine-linked antibody-drug conjugates (ADCs), charge heterogeneity, multiple heart-cut 2D-LC, strong cation exchange (SCX), size exclusion chromatography (SEC), Orbitrap Exploris 240 mass spectrometer, Chromeleon CDS, BioPharma Finder software


## Application benefits

- The online multiple heart-cut two-dimensional liquid chromatography (2D-LC) system provides an efficient approach for in-depth charge heterogeneity characterization of cysteine-linked antibody-drug conjugates.
- The 250 µL fractionation loops offer adjustable fraction volumes in the first dimension.
- The backflush system sharpens the size exclusion chromatography (SEC) peak in the second dimension and improves sensitivity.
- The high sensitivity of the Thermo Scientific™ Orbitrap Exploris™ 240 Mass Spectrometer allows for the identification of low-abundance charge variants

## Goal

Analyze the charge heterogeneity of polatuzumab vedotin under native conditions using 2D-LC coupled with high-resolution accurate-mass mass spectrometry (HRAM MS).

## Introduction

Antibody-drug conjugates (ADCs) are a rapidly emerging class of biotherapeutics used to treat cancer and autoimmune diseases. ADCs typically comprise a monoclonal antibody (mAb) covalently linked to a cytotoxic drug via a chemical linker. This design leverages the precise selectivity of antibodies to deliver cytotoxic agents directly to targeted cells. Charge heterogeneity is a critical quality attribute of ADCs, impacting

the product quality, safety, stability, and efficacy. Regulatory agencies require detailed characterization of charge variants as part of ADC quality control and assurance. In addition to the heterogeneity of the naked mAb arising from post-translational modifications (PTMs), amino acid substitutions, and other biochemical processes, the process of linker-payload conjugation introduces additional charge heterogeneity to ADC molecules. Consequently, analyzing charge heterogeneity in ADCs presents significant challenges in their characterization.

Cation exchange chromatography is commonly utilized to separate charge variants and can be performed with either salt or pH gradients. The pH gradient method, facilitated by commercially available pH gradient buffers such as Thermo Scientific™ CX-1 pH Gradient Buffers, has emerged as a preferred technique over the conventional salt gradient method. These buffer systems streamline method development by providing generic, rapid, reproducible, linear pH gradients suitable for mAbs and ADCs with different isoelectric points (*pI*).[1] To characterize the ADCs after the liquid chromatography (LC) separation, mass spectrometry is the most widely used technique. However, commercially available buffers are usually not MS-compatible, limiting their use in MS-based characterization. Online 2D-LC addresses this by enabling desalting in the second dimension ($^2$D) and offering multi-attribute analysis in one method.

In this application note, we introduce an online multiple heart-cut 2D strong cation exchange (SCX)-SEC-HRAM MS system for characterizing the charge heterogeneity of polatuzumab vedotin, a cysteine-linked ADC marketed as Polivy™, developed by Genentech. This system integrates a Thermo Scientific™ Vanquish™ Online 2D-LC System with an Orbitrap Exploris 240 mass spectrometer. Figure 1 provides an overview of the experimental study design. The CX-1 pH gradient buffer and Thermo Scientific™ ProPac™ 3R SCX Column were used in the first dimension ($^1$D) to effectively separate charge variants. Then, the $^1$D peaks were cut into the sample loops based on VWD and were eluted to $^2$D SEC separation for the HRAM-MS characterization. To remove the salts introduced from the $^1$D and maintain the ADCs in their native state, SEC with the MS-compatible salt ammonium acetate was used in the $^2$D. This approach prevents the disruption of non-covalent interactions in cysteine-linked ADCs, which can occur when reversed-phase (RP) chromatography is applied for desalting in the $^2$D, ensuring the analysis of intact ADCs. The fluidic setup of the system (Figure 2A) enables the backflush of fractions to minimize sample dispersion in the fractionation loops and enhance peak shape and sensitivity in the $^2$D, as there is no refocusing at the column head for SEC separation. The Orbitrap Exploris 240 mass spectrometer offers high sensitivity, high resolution, and high mass accuracy, facilitating the identification of all the charge variants, even those present at very low abundance. By integrating multiple separation techniques into a single workflow, this system enhances analytical efficiency and provides comprehensive insights into charge heterogeneity-related PTMs of polatuzumab vedotin for quality assessment.

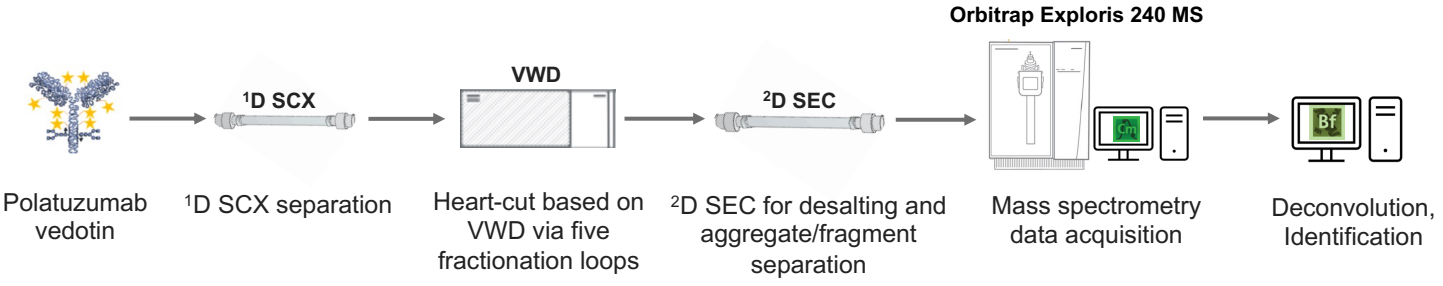



**Figure 1. Overview of the experimental study design for polatuzumab vedotin charge variant analysis using the online multiple heart-cutb2D SCX-SEC-LC HRAM MS approach.**

## Experimental

### Chemicals

| Chemical name | Cat. No. |
|---|---|
| Deionized water with 18.2 MΩ·cm resistivity or higher, purified by a Thermo Scientific™ Barnstead™ GenPure™ Pro Water Purification System | 50131948 |
| Thermo Scientific™ CX-1 pH Gradient Buffer A, 10X concentrate | 302779 |
| Thermo Scientific™ CX-1 pH Gradient Buffer B, 10X concentrate | 302780 |
| Fisher Chemical™, Ammonium acetate, Optima™ LC-MS grade | A11450 |

### Sample handling

| Item name | Cat. No. |
|---|---|
| Thermo Scientific™ SureSTART™ 2 mL Polypropylene Screw Top Microvials | 6ESV9-04PP |
| Thermo Scientific™ SureSTART™ 9 mm Vial Caps with Septum | 6ASC9ST1 |
| Thermo Scientific™ Sorvall™ Legend™ Micro 21R Microcentrifuge | 75002545 |
| Thermo Scientific™ Finnpipette™ F3 Variable Volume Single Channel Pipettes | 4640030, 4640040, 4640050, 4640060 |

### Sample preparation

Polatuzumab vedotin solution with a protein concentration of 4.2 mg/mL in water was provided by one of our customers and used directly in the experiment.

### Instrumentation

A Vanquish Flex Simple Switch 2D-LC system for multiple heart-cut was used, enabling the collection of up to five fractions from a $^{1}$D separation and the transfer to $^{2}$D separation. The fluidic setup is depicted in Figure 2.

### Liquid chromatography

| Module | Cat. No. |
|---|---|
| **Thermo Scientific™ Vanquish™ 2D UHPLC System consisting of:** | |
| Thermo Scientific™ Vanquish™ System Base | VF-S01-A-02 |
| Thermo Scientific™ Vanquish™ Binary Pump F ($^{1}$D pump) | VF-P10-A-01 |
| Thermo Scientific™ Vanquish™ Quaternary Pump F ($^{2}$D pump) | VF-P20-A |
| Thermo Scientific™ Vanquish™ Dual Split Sampler F | VF-A40-A-02 |
| Thermo Scientific™ Vanquish™ Column Compartment H ×2 | VH-C10-A-03 |
| Thermo Scientific™ Vanquish™ Variable Wavelength Detector ×2 | VF-D40-A |
| Thermo Scientific™ Vanquish™ Switching Valves, 2-position/6-port 150 MPa bio ×2 | 6036.2520 |
| Thermo Scientific™ Vanquish™ Switching Valves, 6-position/7-port 150 MPa bio ×2 | 6036.2530 |
| Thermo Scientific™ Vanquish™ Viper Fractionation Loop, MP35N, 250 µL ×6 | 6823.0030 |

### Chromatographic conditions

The chromatographic conditions are listed in Tables 1-3.

**Table 1. $^{1}$D chromatographic conditions.**

| | |
|---|---|
| Column | ProPac 3R SCX column, 100 × 4 mm, 3 µm (Cat. No. 43103-104068) |
| Mobile phase | A: CX-1 pH gradient buffer A (10-fold diluted)<br>B: CX-1 pH gradient buffer B (10-fold diluted) |
| Column temperature | 40°C |
| Autosampler temperature | 4°C |
| Autosampler wash solvent | 10% MeOH in water |
| Injection volume | 10 µL for method optimization and system setup<br>20 µL for charge heterogeneity characterization |
| Detector | 280 nm, 0.2 Hz |

**Table 2. $^{2}$D chromatographic conditions.**

| | |
|---|---|
| Column | Thermo Scientific™ MAbPac™ SEC-1 Column, 300 × 4 mm, 5 µm (Cat. No. 074696) |
| Mobile phase | 100 mM ammonium acetate |
| Column temperature | 30°C |
| Autosampler temperature | 4°C |
| Autosampler wash solvent | 10% MeOH in water |
| Injection volume | $^{1}$D fraction volume |
| Detector | 280 nm, 0.2 Hz |

**Table 3. Gradient and column switching valve position.**

| Fraction collection from SCX | | | |
|---|---|---|---|
| | Time (min) | A | B |
| $^{1}$D pump gradient | 0 | 100 | 0 |
| | 2 | 100 | 0 |
| | 2.1 | 95 | 5 |
| | 32 | 50 | 50 |
| | 33 | 0 | 100 |
| | 35 | 0 | 100 |
| | 36 | 100 | 0 |
| | 41 | 100 | 0 |
| $^{1}$D pump flow rate | 0.3 mL/min | | |
| $^{2}$D pump gradient | Isocratic elution, 41 min | | |
| $^{2}$D pump flow rate | 0.10 mL/min | | |
| Column switching valve position | CutValve: 1–2<br>DivertValve: 1–6<br>ArrayValves: Triggered by the valve switching time set in the $^{1}$D | | |
| **SEC separation and MS data acquisition** | | | |
| $^{1}$D pump gradient | Isocratic elution with 100% A, 30 min | | |
| $^{1}$D pump flow rate | 0.10 mL/min | | |
| $^{2}$D pump gradient | Isocratic elution, 30 min | | |
| $^{2}$D pump flow rate | 0.12 mL/min | | |
| Column switching valve position | CutValve: 1–6<br>DivertValve: 0–10 min (1–6); 10–25.5 min (1–2); 25.5–30 min (1–6)<br>ArrayValves: Corresponding to the fractionation loop that was eluted into the $^{2}$D | | |

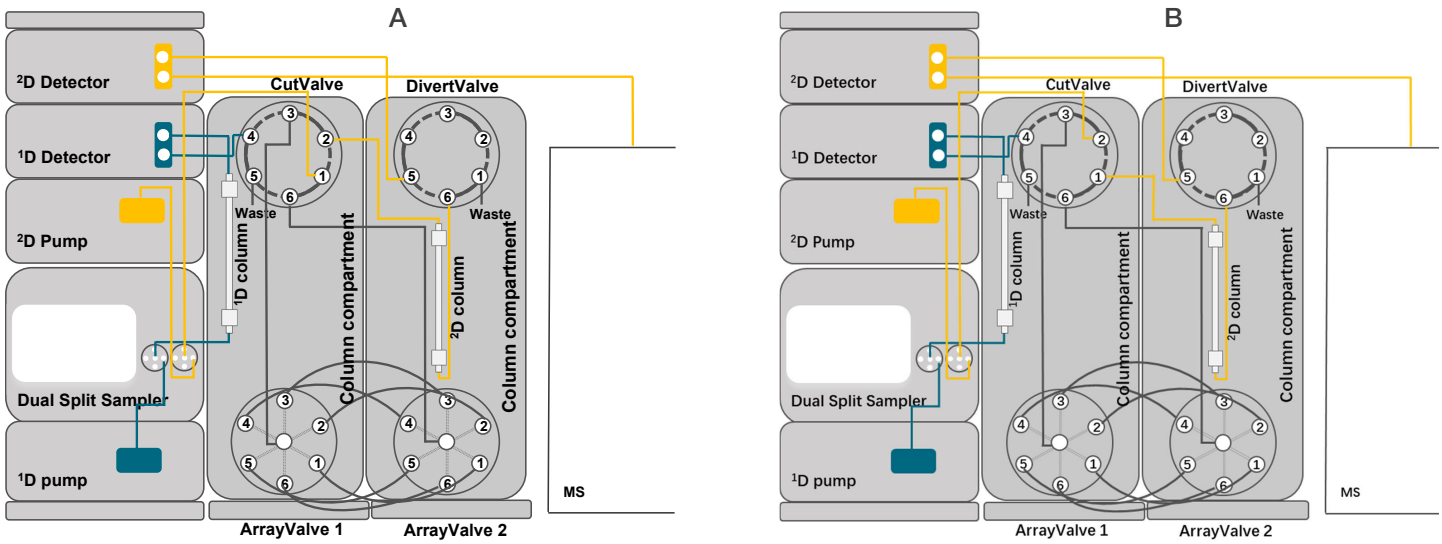



**Figure 2. Fluidic scheme of multiple heart-cut 2D-LC-HRAM MS setup for (A) backflush and (B) forward flush.** The 250 µL fractionation loops combined with the backflush system offer adjustable fraction volumes in the $^{1}$D and sharpen the SEC peaks in the $^{2}$D.

## Mass spectrometry

| System | Cat. No. |
|---|---|
| Orbitrap Exploris 240 mass spectrometer | BRE725535 |
| BioPharma Option (factory installed) | BRE725539 |

## Software

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) version 7.3.2 was used for instrument control, data acquisition, and UHPLC data analysis. Thermo Scientific™ BioPharma Finder™ Software version 5.3 was used for HRAM MS data deconvolution and analysis (Table 5).

**Table 4. Instrument and scan settings for the mass spectrometer.**

| Parameters | Value/settings |
|---|---|
| Spray voltage: Positive ion (V) | 3,800 |
| Sheath gas (Arb) | 35 |
| Aux gas (Arb) | 10 |
| Ion transfer tube temp. (°C) | 250 |
| Vaporizer temp. (°C) | 175 |
| Orbitrap resolution | 60,000 |
| Microscans | 10 |
| AGC target | Custom |
| Normalized AGC target (%) | 300 |
| RF lens (%) | 200 |
| Maximum injection time mode | Custom |
| Data type | Profile |
| Source fragmentation (eV) | 120 |
| Intact protein mode | High pressure |

**Table 5. Deconvolution parameter settings used in the BioPharma Finder software.**

| Parameter | Value |
|---|---|
| Algorithms | Respect™ (for isotopically unresolved spectra) with Sliding Windows algorithm.[2] |
| *m/z* range | 5,000–7,000 |
| RT range | 17.8–25 min |
| Target avg. spectrum width | 0.2 min |
| Target avg. spectrum offset | 1 |
| Merge tolerance | 30 ppm |
| Max. RT gap | 1 min |
| Min. number of detected intervals | 3 |
| Output mass range | 145,000–160,000 |
| Model mass range | 145,000–160,000 |
| Charge state range | 5–100 |
| Minimum adjacent charges | 4–4 |
| Deconvolution mass tolerance | 15 ppm |
| Target mass | 160,000 |

## Results and discussion

### Multiple heart-cut 2D-LC-MS system setup

The multiple heart-cut 2D-LC-MS system was configured based on our previously published application note for infliximab charge variants characterization, which provides detailed information regarding the fluidic connections, hardware configuration, and instrument control.[3] In this study, the RP method in the $^{2}$D was replaced by SEC to benefit from the native conditions and to remove salts introduced from the $^{1}$D and provide orthogonal separation for the ADCs under the native state. For cysteine-linked ADCs, the interchain disulfide bonds in the mAb are reduced for conjugation. The heavy and light chains are held together by non-covalent interactions, which may separate under the high organic solvent conditions in RPLC. Figure 2A depicts the fluidic scheme. The $^{1}$D SCX peaks were collected and stored in up to five 250 µL fractionation loops with the aid of one 2-position/6-port (2-p/6-p) valve (CutValve) and two 6-position/7-port (6-p/7-p) valves (ArrayValves). After the $^{1}$D separation was completed, the fractions were successively collected and stored in the fractionation loops. The fractions were then transferred to the SEC stationary phase under aqueous conditions one by one. The detection in the $^{2}$D was toggled between UV-MS and waste by the second 2-p/6-p valve (DivertValve) to avoid nonvolatile salts being eluted to the MS.

The fractionation loops employed in this 2D-LC system offer flexible $^{1}$D fraction volumes up to 250 µL but can also contribute to the dispersion of fractions in the loops when the fraction volume is less than 250 µL. To minimize this dispersion effect, the fluidic connections at the CutValve were set up in a way to enable reverse flow directions in the loops for fraction loading by the $^{1}$D and fraction transfer to the $^{2}$D (backflush, Figure 2A). This can significantly sharpen the SEC peaks in the $^{2}$D, as the peak shape in SEC is highly affected by the injection volume.[4] The benefits of the backflush fraction transfer, compared to forward flush, are illustrated in Figure 3 for three $^{1}$D SCX peaks (main, acidic, and basic peak). The UHPLC conditions for this test are listed in Tables 1 to 3. After implementing the backflush, sharper SEC peaks were detected, and the sensitivity increased. This improvement was particularly noticeable for small-volume SEC columns (data not shown here).

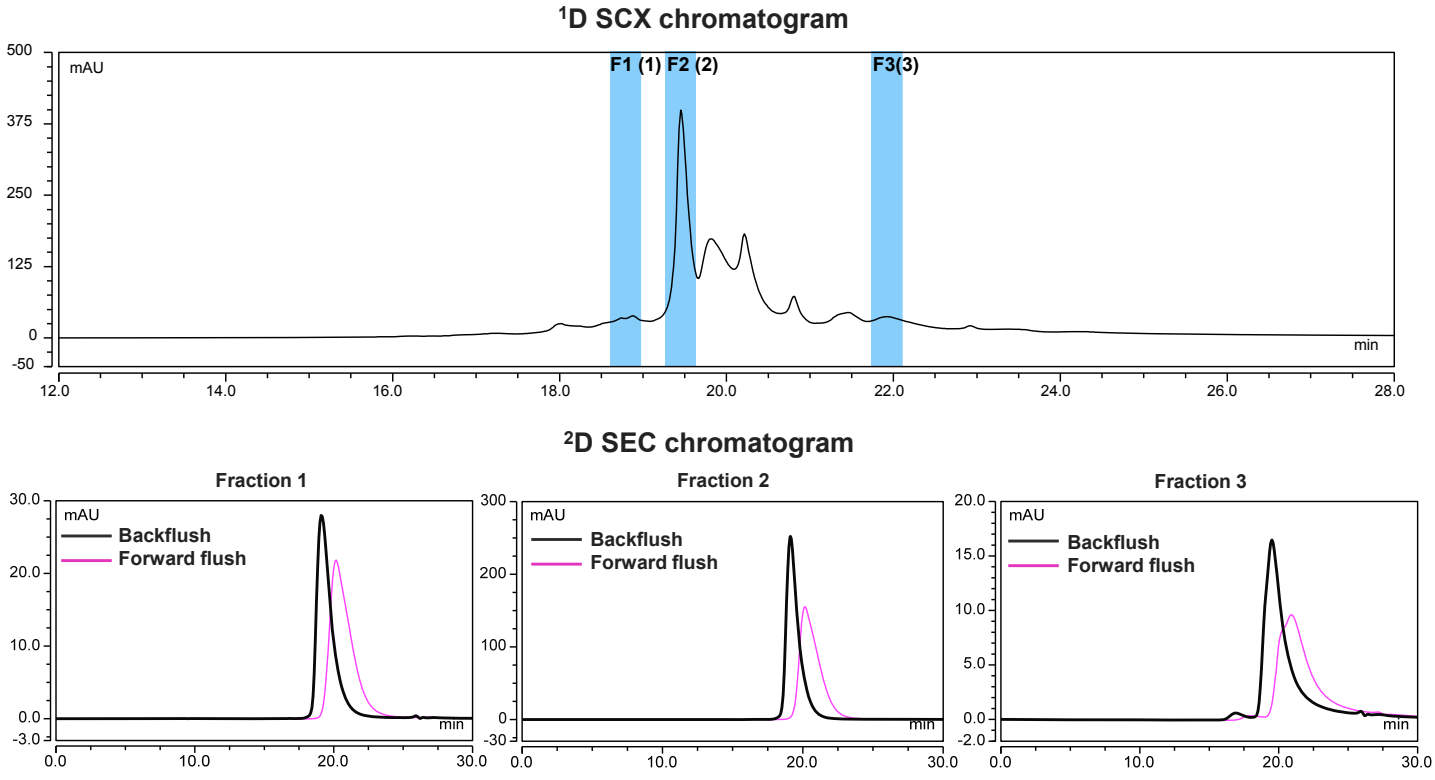



**Figure 3. The $^{1}$D SCX (top) and $^{2}$D SEC UV chromatograms (bottom) for three fractions, with a fraction volume of 105 µL from $^{1}$D, demonstrating significant improvements in peak shape and sensitivity when fractions were transferred in backflush mode compared to forward flush.**

## Method optimization

The objective of the 2D-LC-MS application was to characterize the charge heterogeneity of polatuzumab vedotin using the CX-1 pH gradient buffer. For the $^{1}$D analysis, a ProPac 3R SCX column with a dimension of 100 × 4 mm, 3 µm was used. This size allows for the loading of a substantial sample amount, which is particularly advantageous for analyzing charge variants with very low abundance. The $^{1}$D gradient was optimized under LC-UV conditions; the final parameters are detailed in Table 3. The $^{2}$D analysis was primarily aimed at the desalting of $^{1}$D fractions. Several SEC column dimensions were evaluated for the $^{2}$D analysis, including 50 × 2.1 mm (3 µm), 150 × 2.1 mm (5 µm), and 300 × 4.0 mm (5 µm). The results indicated that the 300 × 4.0 mm column provides the best peak shapes and separation for the salts and ADCs.

The influence of fraction volume from the $^{1}$D analysis was also studied. While larger fraction volumes can enhance signal intensity in the $^{2}$D and ensure comprehensive $^{1}$D peak coverage, thereby preventing the loss of early or late coeluted peak information, this may also cause peak broadening in the $^{2}$D SEC, potentially resulting in inadequate separation from salts. Fraction volumes of 45 µL, 105 µL, and 210 µL for three peaks were investigated, and the results are shown in Figure 4. Although the SEC peaks broadened with increasing fraction volume, the separation remained adequate with a fraction volume of 210 µL when the 300 × 4.0 mm SEC column was used. Utilizing a 210 µL fraction volume, the fraction collection time translated into 0.70 min, ensuring the transfer of the entire $^{1}$D peak to the $^{2}$D.

## $^{2}$D charge heterogeneity characterization of polatuzumab vedotin

In the $^{1}$D chromatogram, 18 peaks were separated and subsequently transferred to the $^{2}$D system for the native intact mass analysis. Four injection cycles were conducted. For the first three injections, five fractions were collected and transferred for each cycle. For the fourth injection, three fractions were collected. Fraction volumes were determined based on the peak width, ranging from 48 µL (0.16 min width) to 150 µL (0.5 min width), as listed in Figure 5. Relative abundances of the separated charge variants were calculated by their $^{1}$D peak areas.

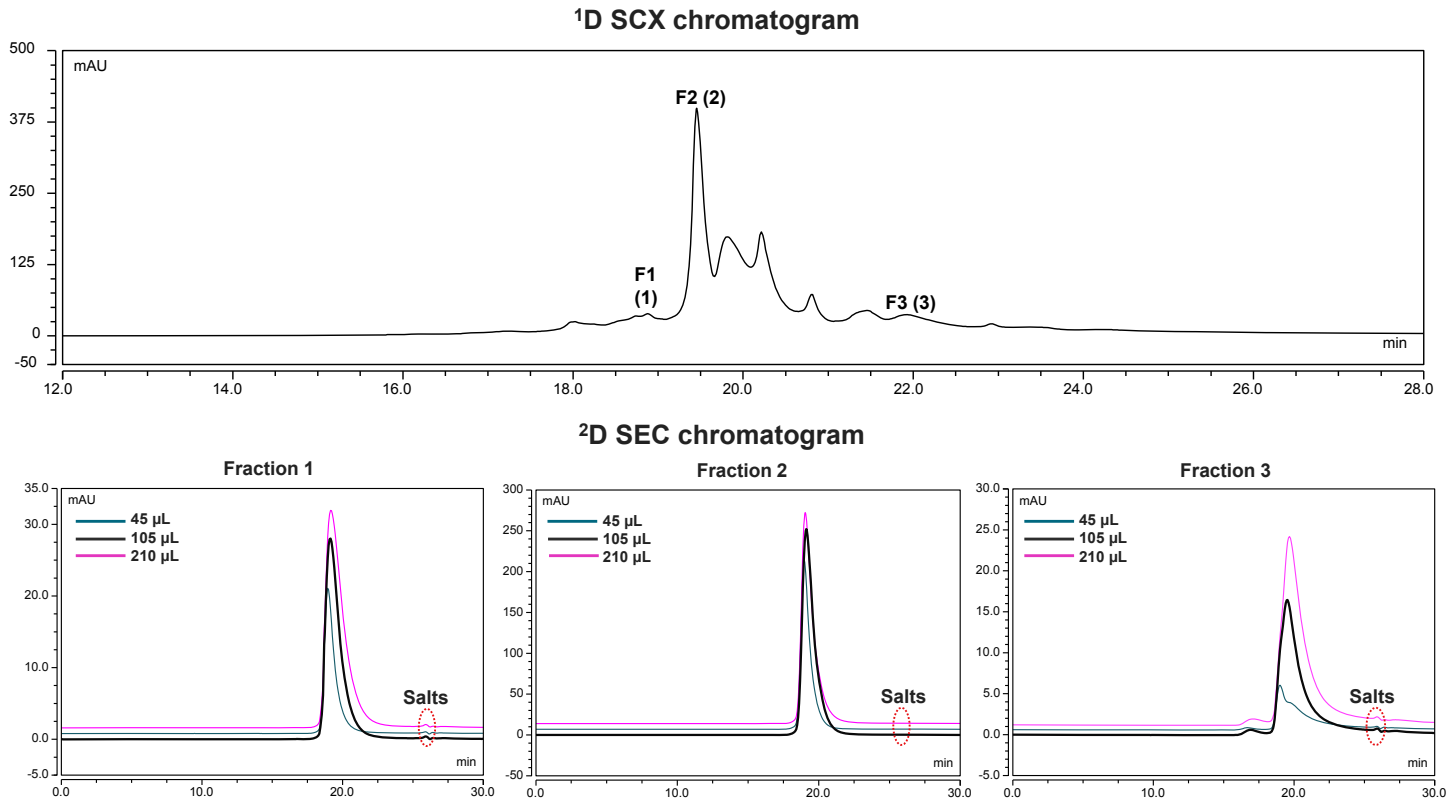
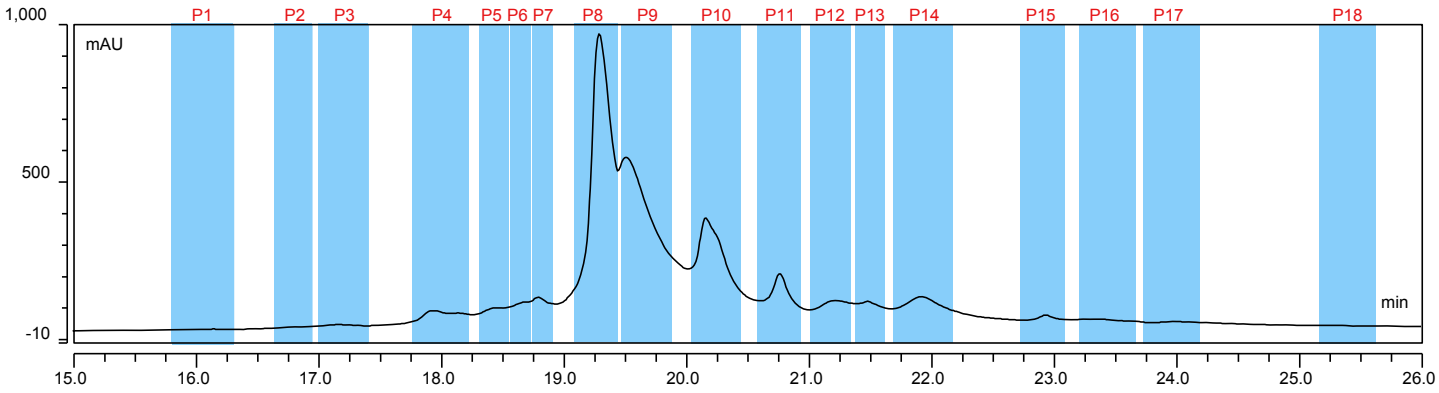



**Figure 4. The $^{1}$D SCX (top) and $^{2}$D SEC UV chromatograms (bottom) of three different fractions, with fraction volumes of 45 µL, 105 µL, and 210 µL, collected from $^{1}$D.** Despite SEC peaks being broadened with increasing fraction volume, separation of protein and salts remained adequate with a fraction volume of 210 µL when the 300 × 4.0 mm SEC column was used.

After acquiring the MS data using Chromeleon CDS, raw data were exported and spectra deconvolution was performed using the ReSpect™ algorithm for isotopically unresolved spectra within BioPharma Finder software, applying deconvolution parameters as detailed in Table 5. The results indicate that payload conjugation altered the surface charge of the mAbs, allowing ADCs with different drug-to-antibody ratio (DAR) to be separated in the SCX chromatogram. For example, the main components in peaks 8 and 9 in $^{1}$D were identified as DAR2 and DAR4, respectively, as shown in Figure 6. And the predominant glycosylation pattern for DAR 2 and DAR 4 was identified as A2G0F+A2G0F, along with some low-abundance forms such as A2G0F+A2G0 and A2G0F+A2G1F, demonstrating the high sensitivity of the Orbitrap Exploris 240 mass spectrometer.

Besides the payload conjugation, other modifications such as deamidation, oxidation, and lysine (Lys) truncation affect the *pI* of ADCs and were separated and identified in this method. C-terminal Lys truncation variants are commonly observed in mAbs and increase the *pI* value of the molecule, causing a later elution in SCX chromatography. Peaks 11 and 12 in Figure 7 were used to show this modification. Compared with the DAR2 isoform detected in peak 8 and the DAR4 isoform detected in peak 9, the average mass of the main components detected in peaks 11 and 12 showed a mass shift of 128 Da in Figure 7C and Figure 7F, indicative of a Lys truncation. Additionally, the Lys truncation variants were separated and identified with a $DAR_n$ + 1 Lys and $DAR_{n+2}$ + 0 Lys pattern in polatuzumab vedotin. DAR2+1 Lys and DAR4, DAR4+1 Lys with a minor amount of DAR6 and DAR2 were identified in peak 11 and 12, respectively. This lysine truncation modification was also identified for the subsequent SCX peaks 13, 15, 16, 17, and 18, and the $DAR_n$ + 1 Lys and $DAR_{n+2}$ + 0 Lys pattern was found in peaks 13 and 17.

The high sensitivity of the Orbitrap Exploris 240 mass spectrometer allows for the identification of charge variants even when they are present at very low abundance levels. As illustrated in Figure 8, peak 18, which has an abundance of merely 0.43% as detected by the UV, still exhibits a high signal-to-noise ratio (S/N) in the raw mass spectra. This high S/N of raw data facilitated the generation of a deconvoluted mass spectrum that allows for precise identification of peak 18. The analysis determined that peak 18 is a composite of DAR4, DAR6, DAR8, DAR6 + 2 Lys, and DAR8 + 2 Lys.




| Peak no. | Relative abundance (%) | Fraction volume (µL) | Peak no. | Relative abundance (%) | Fraction volume (µL) |
|---|---|---|---|---|---|
| 1 | 0.24 | 150 | 10 | 11.26 | 120 |
| 2 | 0.47 | 90 | 11 | 5.10 | 105 |
| 3 | 0.91 | 120 | 12 | 4.41 | 99 |
| 4 | 4.15 | 135 | 13 | 2.76 | 69 |
| 5 | 2.45 | 72 | 14 | 6.54 | 135 |
| 6 | 1.53 | 48 | 15 | 1.80 | 105 |
| 7 | 2.53 | 51 | 16 | 1.67 | 135 |
| 8 | 27.90 | 105 | 17 | 1.72 | 135 |
| 9 | 24.14 | 120 | 18 | 0.43 | 135 |

**Figure 5. A total of 18 peaks were separated in the $^{1}$D SCX chromatogram.** The relative abundance of the charge variants peaks and fraction volume that transferred to the $^{2}$D SEC separation are shown in the table.

The summary of the identified principal species for each fraction is listed in Figure 9 and Table 5. The sequential elution of the DAR0, DAR2, DAR4, and DAR8 species suggests that the payload conjugation alters the surface charge of the mAbs, thereby increasing their retention on the SCX column. Additionally, deamidation and oxidation also affect the *pI* of ADCs, resulting in the formation of acidic variants, whereas Lys truncation at the C-terminus of the heavy chain leads to the formation of basic variants. Identifying charge variants from intact mass analysis presents substantial challenges due to the multitude of potential PTMs and their combinations. Further in-depth characterization can be achieved through peptide mapping experiments; however, once these variants are identified and confirmed, intact mass analysis serves as a convenient tool for monitoring the identified and confirmed variants.

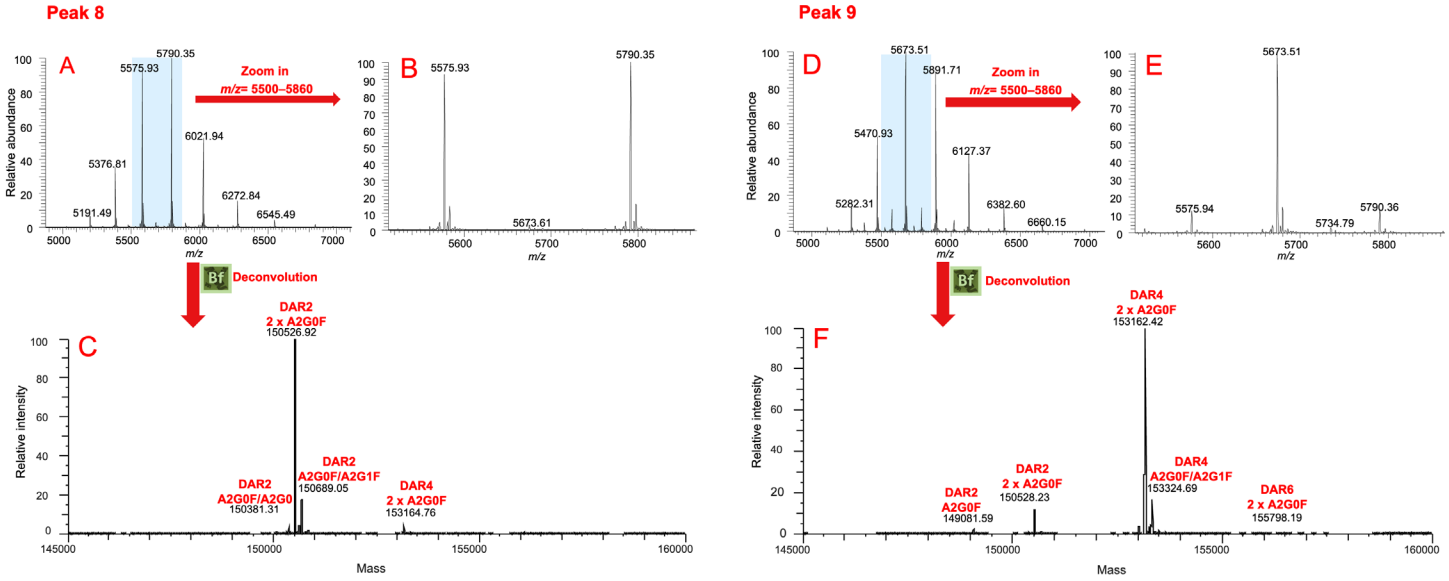



**Figure 6. The full mass spectra (A and D) and deconvoluted spectra (C and F) of peak 8 and peak 9 in Figure 5.** The blue shaded area in the full mass spectra indicates the location of the zoomed-in spectra to the right. The major payloads/glycoforms are labeled in the deconvolution spectra C and F. Peak 8 was identified as a mixture of DAR 2 with a minor amount of DAR 4, while peak 9 was identified as a mixture of DAR 4 with a minor amount of DAR 2 and DAR 6.

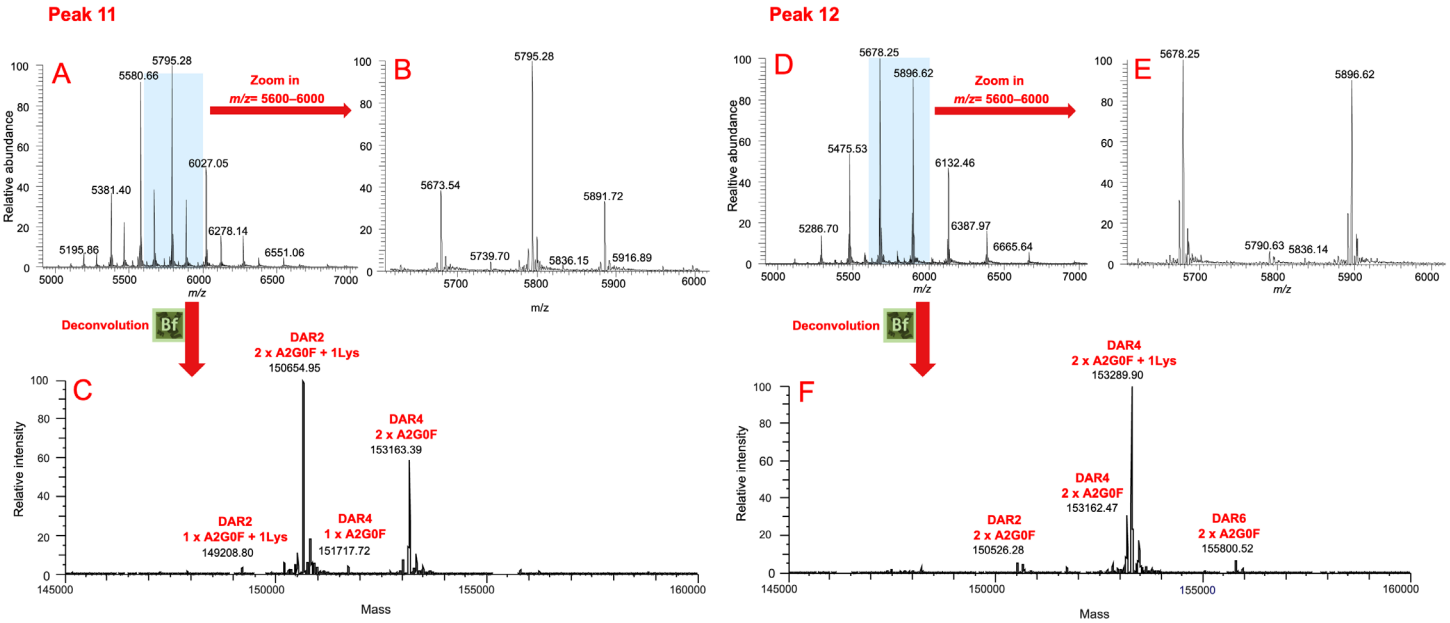



**Figure 7. The full mass spectra (A and D) and deconvoluted spectra (C and F) of peak 11 and peak 12 in Figure 5.** The blue shaded area in the full mass spectra indicates the location of the zoomed-in spectra to the right. The major payloads/glycoforms/PTMs were labeled in the deconvolution spectra C and F. It shows the Lys truncation variants were separated and identified with a $DAR_n$ + 1 Lys and $DAR_{n+2}$ + 0 Lys pattern.

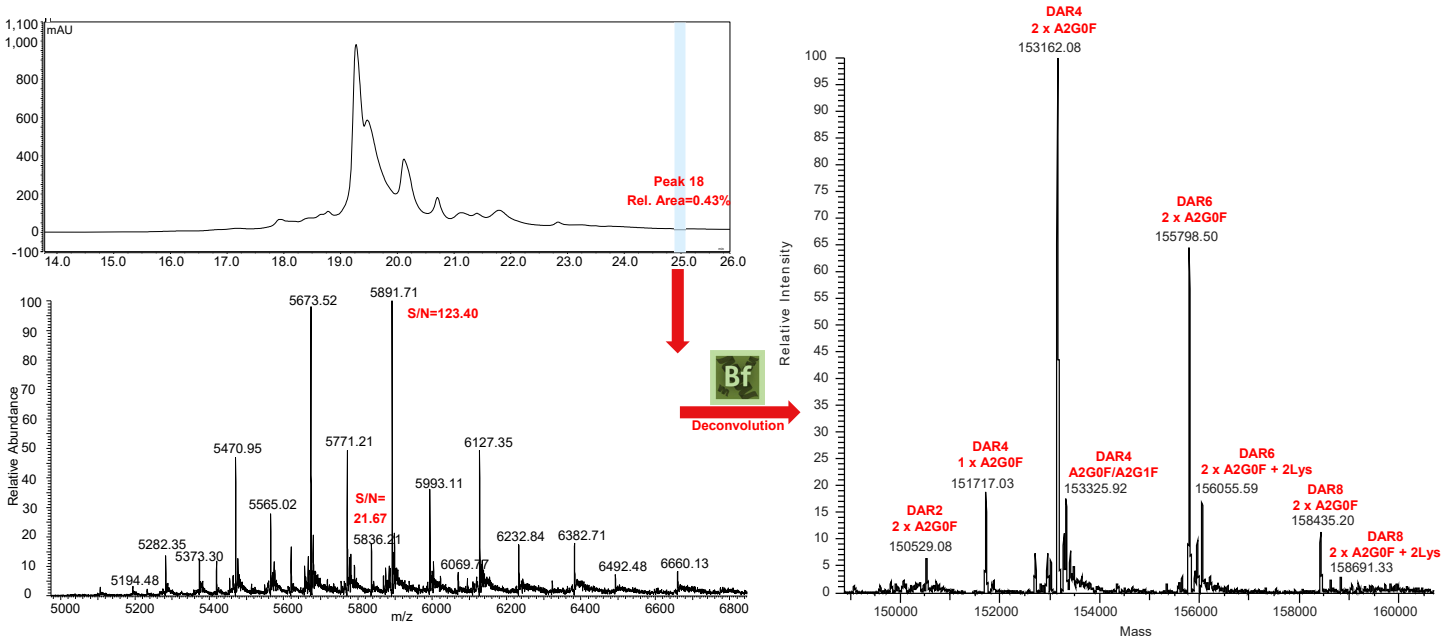



**Figure 8. The [1]D UV chromatogram and [2]D mass spectrum before and after deconvolution for peak 18.** The identification results were labeled on the deconvolution mass spectrum.

| Peak | Main species |
|---|---|
| Peak 1 | DAR0 related compounds |
| Peak 2 | DAR2 +98 Da |
| Peak 3 | DAR2 + Oxidation, DAR2 +6 Da, DAR2+98 Da |
| Peak 4 | DAR2 +6 Da, DAR2 +101 Da, DAR2 + Oxidation |
| Peak 5 | DAR4 + Oxidation, DAR2+Deamidation, DAR0+Deamidation |
| Peak 6 | DAR2+Deamidation, DAR4 + Oxidation, DAR3 |
| Peak 7 | DAR2+Deamidation, DAR4 + Deamidation, DAR0 |
| Peak 8 | DAR2 |
| Peak 9 | DAR4,DAR2 |
| Peak 10 | DAR4, DAR0 |
| Peak 11 | DAR2 + 1Lys, DAR4 |
| Peak 12 | DAR4 + 1Lys, DAR6 |
| Peak 13 | DAR4 + 1Lys, DAR0 + 1Lys, DAR2 |
| Peak 14 | DAR6, DAR4, DAR2 |
| Peak 15 | DAR4, DAR2 + 2Lys, DAR6 |
| Peak 16 | DAR4, DAR4 + 2Lys, DAR6 + 1Lys, DAR6, DAR0 + 2Lys |
| Peak 17 | DAR4, DAR6, DAR6 + 1Lys, DAR8 |
| Peak 18 | DAR4, DAR6, DAR6 + 2Lys, DAR8, DAR8 + 2Lys |

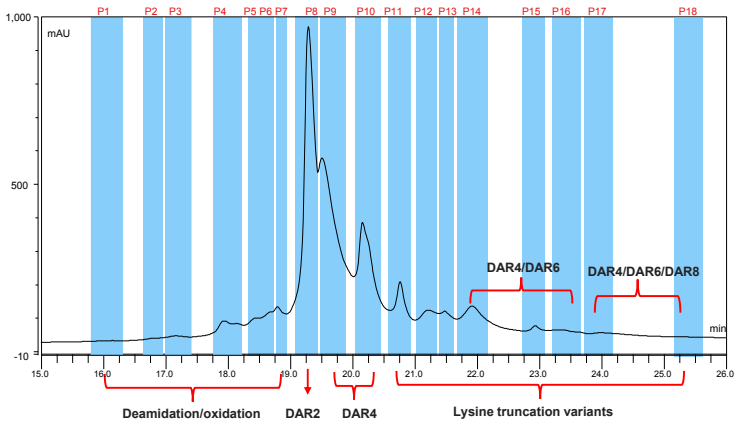



**Figure 9. The left table summarizes the main species identified for all charge variant peaks, and the SCX chromatogram shows how the DAR value and modifications affected the SCX separation.** Deamidation and oxidation result in acidic variants, while Lys truncation at the C-terminus leads to basic variants. Increased payload conjugation increases retention on the SCX column.

**Table 5. Summary of all deconvoluted masses obtained for the main species and identification assignment for all the charge variant peaks.**

| $^1$D peak (fractional area)* | Payload | Glycoform | Modification | Average mass (Da) | Mass accuracy (ppm) | Mass accuracy (Da) | $^1$D peak (fractional area) | Payload | Glycoform | Modification | Average mass (Da) | Mass accuracy (ppm) | Mass accuracy (Da) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P1 (0.24%) | 0 | 2*A2G0F | + 446Da | 148336.12 | - | - | P12 (4.41%) | 4 | 2*A2G0F | + 1 Lys | 153290.84 | -12.53 | -1.92 |
| P2 (0.47%) | 2 | 2*A2G0F | +98Da | 150624.32 | - | - | | 4 | 2*A2G0F | - | 153161.47 | -20.37 | -3.12 |
| P3 (0.91%) | 2 | 2*A2G0F | +6Da | 150532.31 | - | - | | 2 | 2*A2G0F | - | 150526.90 | -2.72 | -0.41 |
| | 2 | 2*A2G0F | +101Da | 150627.35 | - | - | | 6 | 2*A2G0F | - | 155799.17 | 10.14 | 1.58 |
| | 2 | 2*A2G0F | + Oxidation | 150542.67 | -4.18 | -0.63 | P13 (2.76%) | 4 | 2*A2G0F | + 1 Lys | 153290.87 | -12.33 | -1.89 |
| P4 (4.15%) | 2 | 2*A2G0F | + Oxidation | 150541.46 | | | | 0 | 2*A2G0F | + 1 Lys | 148019.68 | 9.75 | 1.48 |
| | 2 | 2*A2G0F | +98Da | 150624.32 | - | - | | 2 | 2*A2G0F | - | 150526.59 | -4.78 | -0.72 |
| | 3 | 2*A2G0F | +604Da | 152444.08 | - | - | P14 (6.54%) | 6 | 2*A2G0F | - | 155797.59 | 0.00 | 0.00 |
| | 1 | 2*A2G0F | +470Da | 149677.04 | - | - | | 4 | 2*A2G0F | - | 153161.32 | -21.35 | -3.27 |
| P5 (2.45%) | 4 | 2*A2G0F | + Oxidation | 153179.15 | -9.34 | -1.43 | | 2 | 2*A2G0F | - | 150527.10 | -1.40 | -0.21 |
| | 2 | 2*A2G0F | + Deamidation | 150530.54 | 1.37 | 0.21 | P15 (1.80%) | 4 | 2*A2G0F | - | 153161.90 | -17.56 | -2.69 |
| | 0 | 2*A2G0F | + Deamidation | 147892.70 | 4.49 | 0.66 | | 2 | 2*A2G0F | + 2 Lys | 150783.51 | -0.93 | -0.14 |
| P6 (1.53%) | 2 | 2*A2G0F | + Deamidation | 150528.61 | 1.94 | 0.29 | | 6 | 2*A2G0F | - | 155798.74 | 7.38 | 1.15 |
| | 4 | 2*A2G0F | + Oxidation | 153177.84 | -17.89 | -2.74 | P16 (1.67%) | 4 | 2*A2G0F | - | 153162.41 | -14.23 | -2.18 |
| | 3 | 2*A2G0F | | 151844.41 | -16.79 | -2.55 | | 4 | 2*A2G0F | + 2 Lys | 153418.96 | -12.91 | -1.98 |
| P7 (2.53%) | 2 | 2*A2G0F | + Deamidation | 150528.03 | -1.91 | -0.29 | | 6 | 2*A2G0F | - | 155798.46 | 5.58 | 0.87 |
| | 4 | 2*A2G0F | + Deamidation | 153164.95 | -4.30 | -0.65 | | 6 | 2*A2G0F | + 1 Lys | 155925.68 | -0.51 | -0.08 |
| | 0 | 2*A2G0F | - | 147890.63 | 4.12 | 0.61 | | 4 | 1*A2G0F | - | 151717.72 | 3.23 | 0.49 |
| P8 (27.90%) | 2 | 2*A2G0F | - | 150526.92 | -2.59 | -0.39 | | 0 | 2*A2G0F | + 2 Lys | 148149.03 | 17.96 | 2.66 |
| | 4 | 2*A2G0F | - | 153164.76 | 1.11 | 0.17 | P17 (1.72%) | 4 | 2*A2G0F | - | 153162.58 | -13.12 | -2.01 |
| P9 (24.14%) | 4 | 2*A2G0F | - | 153162.42 | -14.17 | -2.17 | | 6 | 2*A2G0F | - | 155797.40 | -1.22 | -0.19 |
| | 2 | 2*A2G0F | - | 150528.23 | 6.11 | 0.92 | | 6 | 2*A2G0F | + 1 Lys | 155924.15 | -10.33 | -1.61 |
| | 2 | 1*A2G0F | - | 149081.59 | -2.55 | -0.38 | | 4 | 1*A2G0F | - | 151718.13 | 5.93 | 0.90 |
| P10 (11.26%) | 4 | 2*A2G0F | - | 153162.56 | -13.25 | -2.03 | | 8 | 2*A2G0F | - | 158433.62 | 16.22 | 2.57 |
| | 0 | 2*A2G0F | - | 147891.13 | 7.51 | 1.11 | P18 (0.43%) | 4 | 2*A2G0F | - | 153162.08 | -16.39 | -2.51 |
| | 4 | 1*A2G0F | - | 151717.72 | 3.23 | 0.49 | | 6 | 2*A2G0F | - | 155798.50 | 5.84 | 0.91 |
| P11 (5.10%) | 2 | 2*A2G0F | + 1 Lys | 150654.95 | -3.52 | -0.53 | | 6 | 2*A2G0F | + 2 Lys | 156055.59 | 10.64 | 1.66 |
| | 4 | 2*A2G0F | - | 153163.39 | -7.83 | -1.20 | | 4 | 1*A2G0F | - | 151717.03 | -1.32 | -0.20 |
| | 4 | 1*A2G0F | - | 151717.72 | 3.23 | 0.49 | | 8 | 2*A2G0F | - | 158435.20 | 26.19 | 4.15 |
| | 2 | 1*A2G0F | + 1 Lys | 149208.80 | -8.83 | -1.34 | | 8 | 2*A2G0F | + 2 Lys | 158691.33 | 24.83 | 3.94 |

## Conclusions

In this study, an online multiple heart-cut SCX-SEC-HRAM MS system was configured and optimized for the characterization of charge heterogeneity in polatuzumab vedotin. This approach provides detailed and comprehensive insights into charge heterogeneity-related PTMs of polatuzumab vedotin under native conditions.

- The online multiple heart-cut 2D-LC system enables the direct transfer of SCX fractions to mass spectrometric analysis by utilizing SEC as a desalting step, thereby overcoming the incompatibility of CX-1 buffer with MS.
- The backflush transfer of fractions was employed to enhance peak shape and sensitivity in the $^{2}$D SEC separation.
- The $^{2}$D SEC-MS analysis enables the identification of charge heterogeneity of ADCs under native conditions.
- The high sensitivity of the Orbitrap Exploris 240 MS and the straightforward workflow in BioPharma Finder software allow for the accurate identification of all fractions even at very low abundance levels.

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