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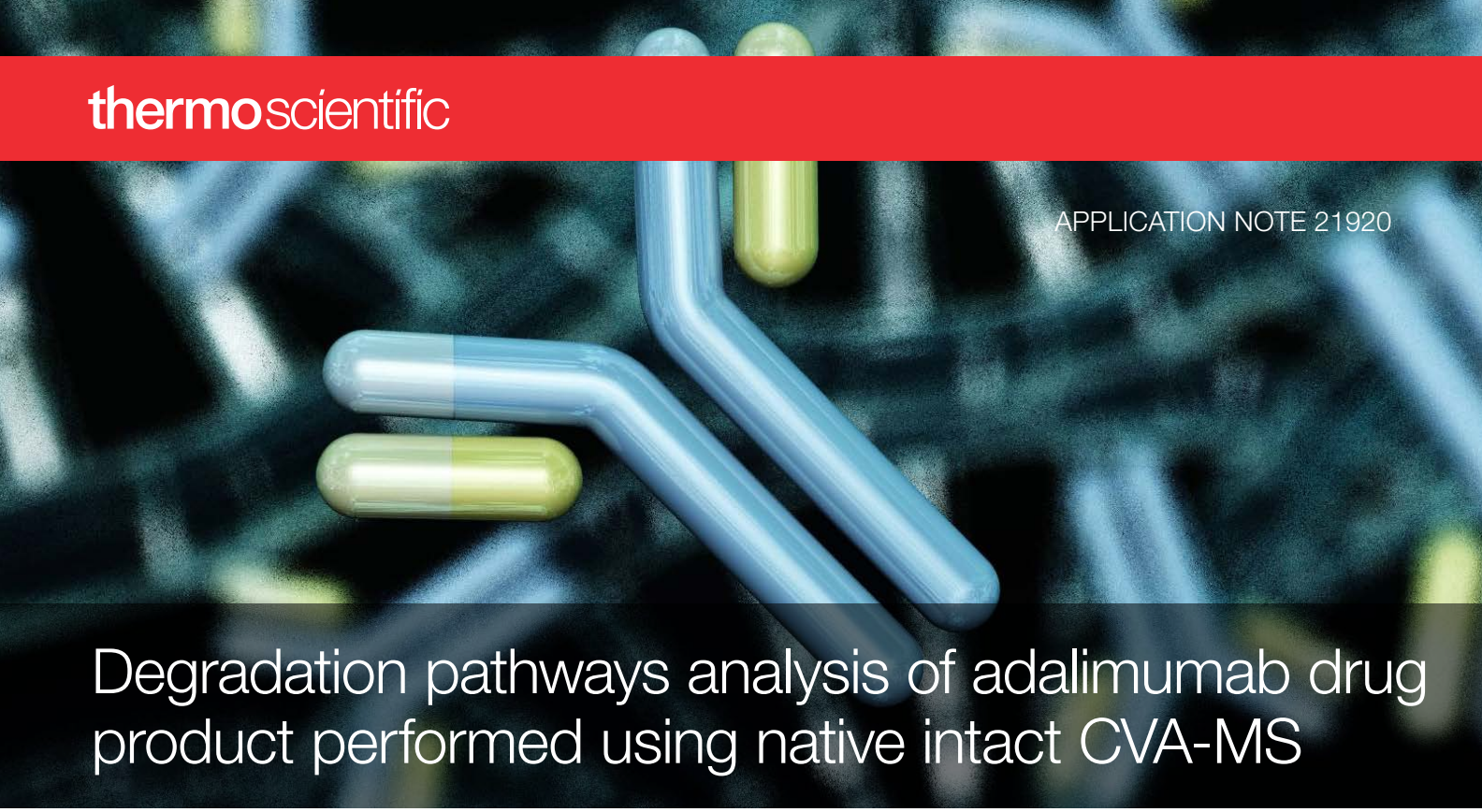

Degradation pathways analysis of adalimumab drug product performed using native intact CVA-MS

Authors

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Keywords

NIBRT, biopharmaceutical, biotherapeutic, monoclonal antibody (mAb), IgG, intact mass, fragmentation, multiple critical attributes, MAbPac columns, Vanquish Flex Binary UHPLC system, Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer, BioPharma Finder, sliding windows

Application benefits

- High information content with no sample preparation using native mass analytical methods for the quantification of multiple mAb critical quality attributes and the identification of mAb degradation pathways that may have an impact on the protein activity and stability
- Global applicability of the workflow using a Thermo Scientific™ MAbPac™ SCX-10 column for mAb charge variant analysis with volatile buffer-aided pH gradients to allow direct coupling to high-resolution mass spectrometry
- High data quality obtained using a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer with BioPharma Option for intact native high-resolution accurate-mass analysis with on-line charge variant separation of monoclonal antibodies
- Ease of use of Thermo Scientific™ BioPharma Finder™ 3.0 software for intact mass analysis

Goal

To highlight intact protein mass analysis under native conditions for the comprehensive characterization of biotherapeutics. To show the unique direct coupling of high-resolution accurate-mass MS to ion exchange separations of proteins. To demonstrate how the method can be used as a global method for mAb characterization and their degradation products.

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Introduction

Monoclonal antibodies, which offer high specificity and low side effects, are used to treat many types of cancer, autoimmune, and inflammatory diseases. These attributes drive the exponential growth of the biopharmaceutical market and their success as human medicines.[1] The intrinsic heterogeneity of these drugs requires specific analytical technologies developed to allow monitoring of critical quality attributes that may affect product potency, stability, and biological activity. Between these features, special relevance is given to the N-glycan profile, C-terminal lysine truncation, oxidation of tryptophan and methionine residues or asparagine and glutamine deamidations. Moreover, the steps going from protein expression to product fill finish may have an influence on other aspects of the drug product, such as the presence of aggregates or fragments derived from incorrect handling or storage of the drug. As they are unavoidable, these drug by-products need to be monitored and efforts are made to design the bioprocess in such a way that their formation is kept to a minimum and consistent from batch to batch. Forced degradation studies are also used to investigate the potential modifications and degradation pathways under extreme conditions. This may include high temperature, freeze-thaw cycles, agitation, high pH, low pH, light exposure, oxidation, and glycation. These stress factors usually reflect the conditions the products are potentially exposed to during processing, packaging, shipping, and handling.[2,3] Information-rich analytical methods, in particular liquid phase separations like size exclusion, reversed-phase or ion-exchange chromatography, coupled with mass spectrometry can address these challenges and identify the many possible variants and degradation products.

Cation-exchange chromatography is normally considered incompatible with mass spectrometry due to the high concentrations of salt used in the eluent system.[4] The current trend to use pH gradients for protein elution from the ion exchange column reduces the salt requirement of the buffer system.[5] Going one step further to employ volatile buffers in the eluent system for pH gradient elution has allowed charged variant analysis (CVA) to be directly coupled to high-resolution mass spectrometry (HRMS)[6,7] resulting in a hyphenated method called CVA-MS. This technique delivers the chromatographic resolution of ion-exchange chromatography and allows identification of the separated variants by HRMS.

In this study, CVA-MS was applied for the identification of degradation products derived from long term storage of adalimumab drug product.[7] Degradation of adalimumab drug product was generated with accelerated aging conditions estimated from the Arrhenius equation to simulate theoretical storage durations of 2, 6, 12, 18, 24, 30, and 36 months at 4 °C using an aging factor of 2.0. The charge variant profile of the original drug was compared to the forced degradation product to highlight variants originating from the simulated study. A Thermo Scientific™ MAbPac™ SCX-10 column was used with a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system. The LC system was coupled to a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer equipped with the BioPharma Option allowing mass detection up to *m/z* 8,000.

The buffer system used in our setup keeps proteins in their native form and preserves the three dimensional structure, resulting in a smaller exposed surface to accept charges compared to the analysis under denaturing conditions involving organic solvents and acids. Thus the charge distribution detected by MS under native conditions consists of a smaller number and lower charge states typically detected between *m/z* 4800 and 6800. The benefit of the shift to lower charge states detected at higher *m/z* values is the increased spatial resolution. Detection of species beyond the standard mass range up to *m/z* 6000 is enabled by the BioPharma Option enabling mass detection up to *m/z* 8000, which is essential for the intact mAb analysis under native conditions.

Experimental

Recommended consumables

- Water, Optima™ LC/MS grade (Fisher Chemical) (P/N 10505904)
- Acetic acid (ACS reagent grade, ≥99.7%), (P/N 32209-M)
- Ammonium bicarbonate (BioUltra, reagent grade, ≥99.7%), (P/N 09830)
- Ammonium hydroxide solution (BioUltra, 1 M) (P/N 09859)
- MAbPac SCX-10 RS column, 5 µm, 2.1 × 50 mm (P/N 082675)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)

- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)

Sample handling equipment

Vanquish Flex Binary UHPLC system including:

- Binary Pump F (P/N VF-P10-A)
- Column Compartment H (P/N VH-C10-A)
- Split Sampler FT (P/N VF-A10-A)
- System Base Vanquish Horizon VH-S01-A
- VWD-3400RS Rapid Separation Variable Wavelength Detector (P/N 5074.0010)
- Thermo Scientific™ UltiMate™ 3000 PCM-3000 pH and conductivity monitor (P/N 6082.2005)
- Thermo Scientific™ UltiMate™ VWD semi-micro flow cell (P/N 6074.0300)

- Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer with BioPharma Option (P/N IQLAAEGAAPFALGMBDK)

Sample preparation

Adalimumab was provided by St. Vincent's University Hospital in Dublin, Ireland. 50 to 100 µg of antibody were injected directly from formulation buffer.

210 µg of adalimumab in formulation buffer were incubated for 2, 5, 11, 16, 21, 27, and 32 days at 55 °C in an incubating mini shaker. According to the Arrhenius equation, time points were chosen to resemble theoretical storage durations of 2, 6, 12, 18, 24, 30, and 36 months at 4 °C using an aging factor of 2.0. Sample condensation during elevated temperature incubation was counteracted by daily centrifugation of the samples at 1000 rounds per minute for 1 minute. After heat exposure, samples were stored at -20 °C.

LC conditions

Mobile phase A:	25 mM ammonium bicarbonate [1.975 g in 200 mL for 5X concentrate] and 30 mM acetic acid in water [1.8 mL in 200 mL for 5X concentrate] final unadjusted pH 5.3
Mobile phase B:	10 mM ammonium hydroxide in water [10 mL of 1 M solution in 200 mL for 5X concentrate] final unadjusted pH 10.9 [results in pH 10.2 from the column due to column buffering effects]
Flow rate:	0.4 mL/min
Column:	MAbPac SCX-10 RS, 5 µm, 2.1 × 50 mm
Column temperature:	30 °C
Pre-column heater:	30 °C
Autosampler temp.:	5 °C
Injection:	50 to 100 µg
Injection wash solvent:	Methanol/water, 10:90
Needle wash:	Enabled pre-injection
Gradient:	See Table 1 for details

Table 1. Mobile phase gradient for CVA analysis. Re-equilibration starts with a 100% buffer A flush for 2 minutes, then equilibration to the starting conditions for a further 8 minutes.

mAb	Time [min]	%B	Curve
Adalimumab [pI 8.9][8]	0	40	
	10	100	5

MS conditions

Table 2. Summary of tune and method parameters

Tune Parameters	Spray Voltage	3.6 kV
	Capillary Temperature	275 °C
	Sheath Gas	20 arbitrary units
	Aux Gas	5 arbitrary units
	Probe Heater Temperature	275 °C
	S-Lens RF Level	200
	HMR Mode	On
	Trapping Gas Pressure Setting	1
Method Parameters	Polarity	Positive
	In-Source CID	150 eV
	Microscans	10
	Resolution (@ *m/z* 200)	17,500; 35,000; 70,000
	AGC Target	3e6
	Maximum IT	200 ms
	Scan Range	2500–8000 *m/z*
	Spectrum Data Type	Profile

Table 3. BioPharma Finder 3.0 software parameter settings for MS data processing

Parameter	Intact mAb	Fragments
m/z Range	5000–7000	2500–5000
Deconvolution Algorithm	ReSpect™	ReSpect
Model Mass Range	145,000–152,000	40,000–60,000 (LMWF) 90,000–110,000 (HMWF)
Mass Tolerance	10 ppm	10 ppm
Charge State Range	20–30	10–20 (LMWF) 20–30 (HMWF)
Minimum Adjacent Charges	3 - 3	3 - 3

Results and discussion

Product development in the biopharmaceutical industry is focused on understanding the relationship between drug structure and its function, so there is an increasing need for detailed characterization with faster and more efficient assays. The possibility of implementing quick and reliable analytical methods to characterize monoclonal antibody variants is important to evaluate lot-to-lot consistency, investigate potential problems emerging along the production pipeline, and biosimilar product characterization to establish comparability with their innovator.

In this study we used a recently developed method that involves charge variant analysis hyphenated with high-resolution accurate mass analysis for the characterization of adalimumab drug product (Figure 1).[7] Several charge variants could be distinguished using this method, with a single analysis and no sample preparation required.

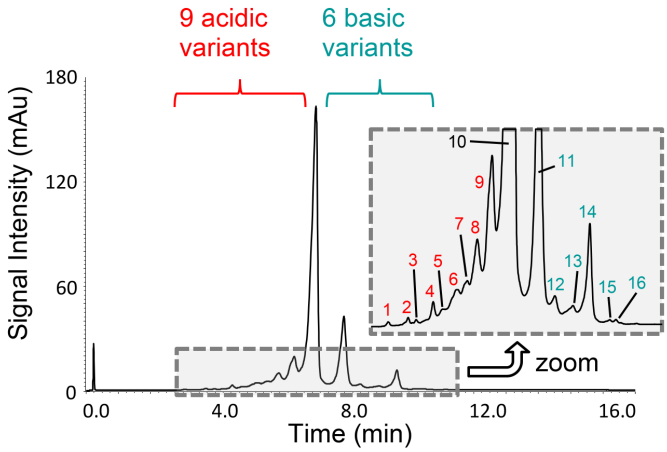

Figure 1. UV chromatogram highlighting the charge variant separation of adalimumab. Sixteen different species were separated as detailed in the zoom window. The data were acquired using the optimized gradient for adalimumab (Table 1).

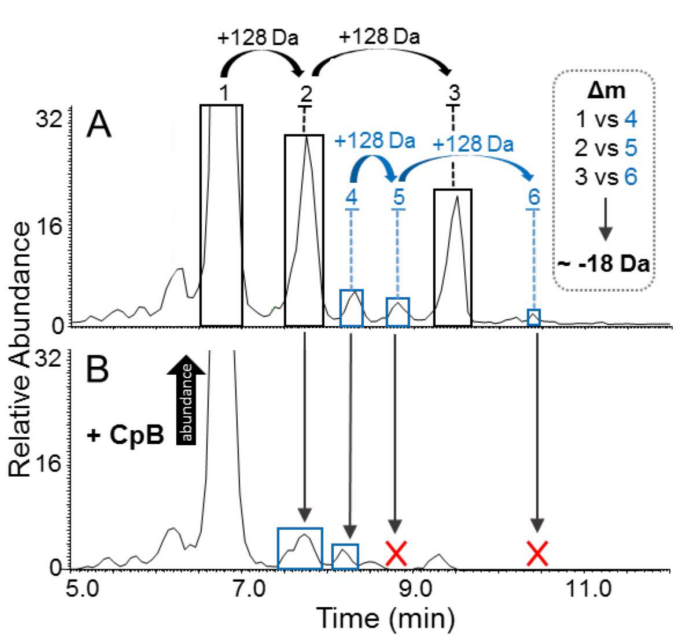

Figure 2. A) Magnification of the base peak chromatogram (BPC) of an undigested and unstressed adalimumab sample, acquired at a resolution setting of 35,000. The most abundant peaks labeled 1 to 3 are the main lysine variants. Peaks 4 to 6 labeled in blue correspond to the succinimide Asp containing versions. **B) BPC of a carboxypeptidase B (CpB) digested sample to remove lysine truncation variants.** Former positions of peaks that have completely disappeared following CpB digestion are indicated by a red "X". The main species is increasing in abundance upon digestion as indicated by the arrow.

Among the modifications that have been identified using this method are: relative glycoform abundance levels within the lysine truncation variants, glycation and succinimide formation of different lysine truncated forms, deamidation, isomerization, as well as double deamidation, N-terminal Asp loss or C-terminal proline amidation and fragmentation.[7]

The excellent chromatographic resolution obtained from the pH gradient performed allowed the separation and identification of adalimumab variants with 2, 1, or no C-terminal lysines with very high mass accuracy. During lysine variant annotation three other low abundant, basic species that showed mass differences of ~128 Da were noticed as well. These species are shown in blue in Figure 2.

To confirm the identity of these less abundant peaks as lysine variants, a digestion with CpB enzyme was performed, which led to a complete disappearance of the more basic peaks, verifying our assumptions. In comparison with the main lysine variants, we found a constant mass shift of ~18 Da for all three additional basic species. Taking retention time into consideration, we concluded these species likely to be three C-terminal lysine variants of an antibody that had also undergone succinimide formation of an Asp residue. Two main glycoforms for each lysine variant were identified based on triplicate injections and are listed in Table 4.

Table 4. Annotation of Asp succinimide containing adalimumab forms based on the two most abundant glycoforms per peak.
Deconvolution was performed after manual integration using the BioPharma Finder 3.0 software. Mass deviations >20 ppm are likely attributable to the low signal abundance or possibly near isobaric co-eluting substances.

Run #	Variant	Theoretical Mass (Da)	Average Mass (Da)	Δm (ppm)	RT Range (min)
1	0K_G0F/G0F	148,062.0	148,063.2	8.1	8.187–8.386
2	0K_G0F/G0F	148,062.0	148,063.7	11.5	8.218–8.398
3	0K_G0F/G0F	148,062.0	148,063.3	8.8	8.212–8.424
1	0K_G0F/G1F	148,224.2	148,224.6	2.7	8.187–8.386
2	0K_G0F/G1F	148,224.2	148,227.1	19.6	8.218–8.398
3	0K_G0F/G1F	148,224.2	148,224.9	4.7	8.212–8.424
1	1K_G0F/G0F	148,190.2	148,191.6	9.4	8.674–8.933
2	1K_G0F/G0F	148,190.2	148,190.1	-0.7	8.717–8.945
3	1K_G0F/G0F	148,190.2	148,191.7	10.1	8.689–9.000
1	1K_G0F/G1F	148,352.4	148,352.0	-2.7	8.674–8.933
2	1K_G0F/G1F	148,352.4	148,352.4	0.0	8.717–8.945
3	1K_G0F/G1F	148,352.4	148,355.6	21.6	8.689–9.000
1	2K_G0F/G0F	148,318.4	148,322.3	26.3	10.276–10.410
2	2K_G0F/G0F	148,318.4	148,318.8	2.7	10.326–10.478
3	2K_G0F/G0F	148,318.4	148,320.6	14.8	10.330–10.519
1	2K_G0F/G1F	148,480.5	148,479.1	-9.4	10.276–10.410
2	2K_G0F/G1F	148,480.5	148,479.5	-6.7	10.326–10.478
3	2K_G0F/G1F	148,480.5	148,480.5	0.0	10.330–10.519

Succinimide formation of Asp would explain an increase in retention on the SCX stationary phase since the loss of one acidic residue generates a more basic proteoform. Succinimide formation in mAbs has previously been described as correlated to the exposure of the molecule to certain environmental conditions, such as acidic pH and elevated temperature, leading to the potential conversion into aspartic acid and a consecutive possible loss in biological activity or rise of undesirable immunologic responses.[9-11] To verify this hypothesis, forced degradation experiments were performed to simulate long-term storage at 4 °C as described in the Experimental section. After accelerated aging we observed an increase in the respective succinimide Asp species at the intact level with increasing incubation time (Figure 3). Peptide mapping data[7] of the drug product and samples derived from forced degradation revealed the presence of succinimide formation on multiple Asp residues and an increasing level of this modification over incubation time. This is illustrated in Figure 3B with the example of Asp284. This residue undergoes succinimide formation faster than other Asp residues when increasing storage duration. After simulated storage for 24 months at 4 °C, almost 20% of the residue is modified. This provides an additional confirmation of correct peak annotation as the succinimide forms on the intact level, but also gives evidence of Asp succinimide formation as a dominant degradation pathway of adalimumab under non-ideal storage conditions.

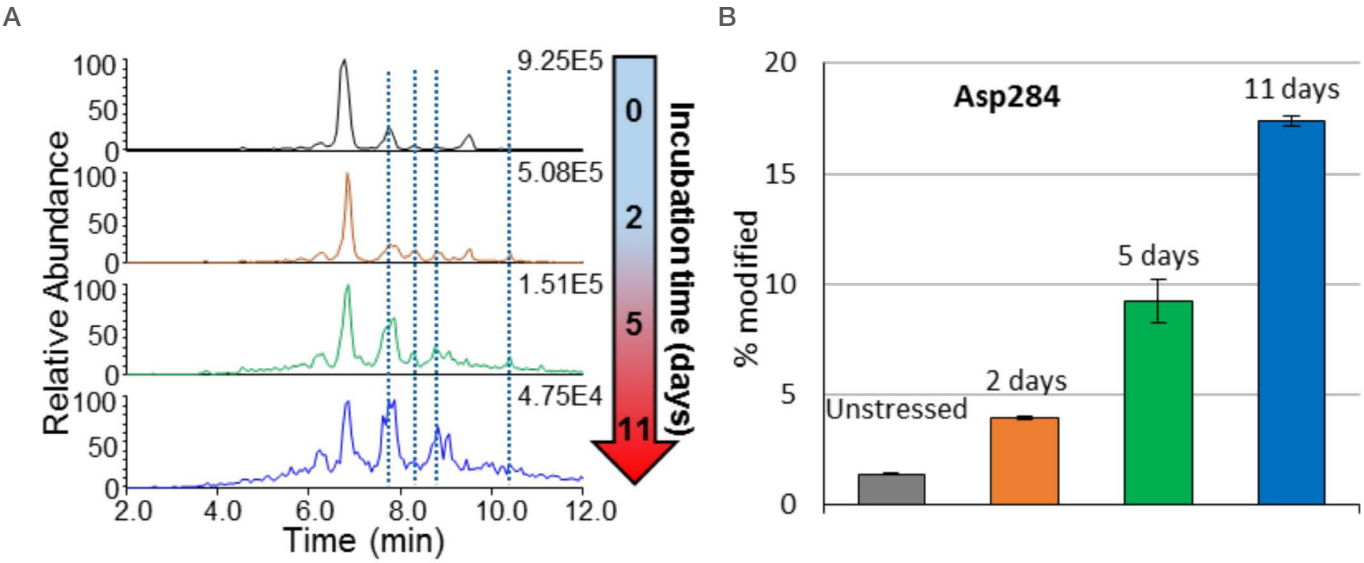

Figure 3. A) BPCs of adalimumab samples incubated at elevated temperature for up to 11 days; B) Increase of succinimide formation on Asp284 with increasing incubation time at elevated temperature revealed by peptide mapping

Conclusions

- CVA-MS proved to be an excellent analytical technique for in-depth analysis of monoclonal antibody charge variants.
- Multiple levels of information are obtained with a single workflow that requires no sample preparation prior to analysis: intact mass, CVA pattern, glycan distribution, levels of lysine truncation, deamidation, succinimide-Asp formation and fragmentation.
- The performance of the Thermo Scientific Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer with BioPharma Option provided high mass accurate identification of detected species in their native form. The confident identification of low abundant species and possible degradation pathways have been highlighted
- BioPharma Finder 3.0 software using the deconvolution options allowed fast data analysis and confident identification of the separated charge variants along with all their glycoforms distribution.

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