Biopharma

Intact mass analysis of monoclonal antibody charge variants by multi heart-cut 2D-LC/MS coupling ion-exchange and reversed-phase chromatography

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Abstract

Purpose: Identification of Infliximab charge variants by two-dimensional liquid chromatography (2D-LC) and intact mass analysis.

Methods: A Thermo Scientific™ Vanquish™ Horizon Simple Switch™ 2D-LC system for multiple loop heart-cutting was used to collect fractions from an ion-exchange chromatography (IEC) first dimension (^{1}D). Fractions were transferred to a reversed-phase (RP) second dimension (^{2}D) for focusing, desalting, and subsequent intact mass analysis with a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer (MS).

Results: Multi heart-cut 2D-LC facilitated the overcoming of the inherent incompatibility of non-volatile IEC mobile phases with MS. Three major IEC peaks could be identified as Infliximab variants with none, one, and two C-terminal lysine residues, each with a consistent baseline resolved glycosylation pattern of the major glycoforms. Secondly, five minor ^{1}D peaks were targeted. An automated workflow enabled the fivefold online enrichment of one peak to attain sufficient MS signal intensity. Deconvoluted mass data suggested the identification of minor peaks as variants with one deamidation, or with sialylation of glycan side chains, combined with different numbers of C-terminal lysine residues. Therefore, the capabilities of multi heart-cut 2D-LC for the direct transfer of IEC fractions to mass analysis were demonstrated for the identification and monitoring of major and minor intact charge variants of Infliximab.

Introduction

IEC is the method of choice for the profiling of charged variants of biopharmaceutical products. Application of pH gradients or buffered salt gradients for the separation usually hinder a direct hyphenation with MS for deeper characterization. However, 2D-LC provides a straightforward way to facilitate the hyphenation and circumvent the need for manual fraction collection and LC-MS re-analysis.[1] Online multiple heart-cutting 2D-LC is showcased here for the intact mass analysis of charged variants of Infliximab, a mAb for the treatment of several autoimmune diseases.

Materials and methods

Sample Preparation

Infliximab CRS (Y0002047)[2] reference material was prepared in water at a concentration of 3.5 mg/mL.

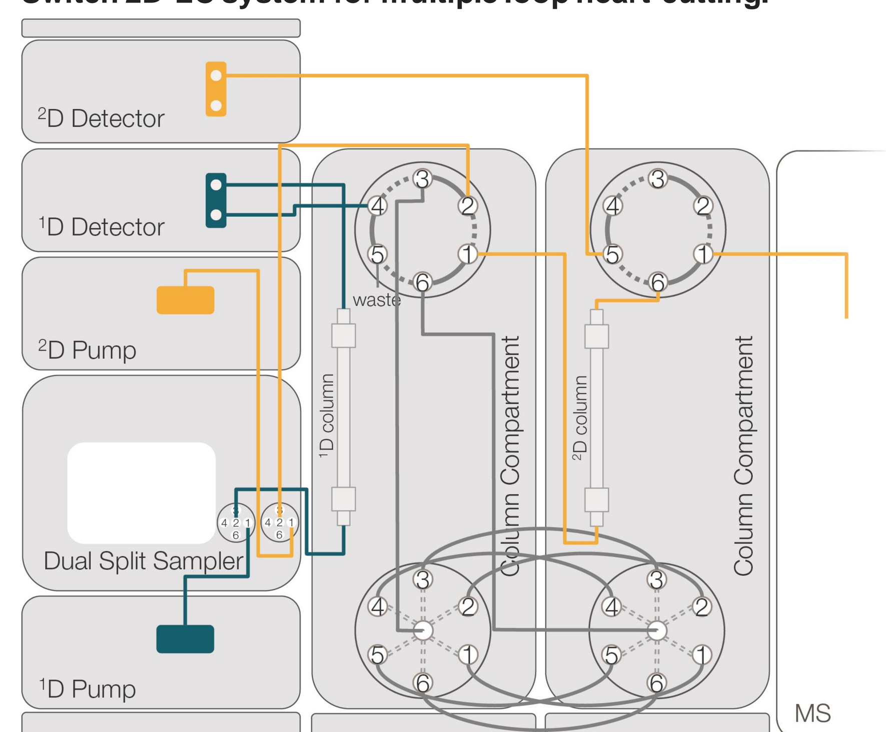
Instrumentation

The Vanquish Horizon Simple Switch 2D-LC system for multiple loop heart-cutting consisted of:

Module	Part Number
Vanquish System Base	VF-S01-A-02
Vanquish Stack Stabilizer	6036.1710
Vanquish Binary Pump H (^{2}D pump)	VH-P10-A-02
Vanquish Quaternary Pump F (^{1}D pump)	VF-P20-A
Vanquish Dual Split Sampler HT	VH-A40-A-02
2x Vanquish Column Compartment H	VH-C10-A-03
2x Valve 2-position/6-port 150 MPa bio	6036.2520
2x Valve 6-position/7-port 150 MPa bio	6036.2530
5x Sample loop, MP35N, 250 µL	6823.0030
Vanquish Diode Array Detector FG (^{1}D detector)	VF-D11-A-01
Flow Cell, semi-micro biocompatible, 7 mm, 2.5 µL	6083.0550
Vanquish Variable Wavelength Detector (^{2}D detector)	VF-D40-A
Flow Cell, semi-micro biocompatible, 7mm, 2.5 µL	6077.0300
Orbitrap Exploris™ 240 Mass Spectrometer	BRE725535

The fluidic configuration, depicted in Figure 1, enabled for 2D-LC-MS with up to five ^{1}D fractions in 250 µL loops, or for the operation of standard 1D-LC or 1D-LC-MS separations. Independent injection units in both flow paths make method development simple and straightforward. The ^{2}D is accessible independently from the ^{1}D, which is convenient for ^{2}D LC and MS parameter optimization or 1D-LC/MS applications without replumbing.

Figure 1. Fluidic scheme of the Vanquish Horizon Simple Switch 2D-LC system for multiple loop heart-cutting.



Test Methods

The multiple heart-cutting 2D-LC method allowed for flexible selection of up to five fractions from the ^{1}D IEC eluent with consequent fraction storage in loops and transfer of each fraction onto the RP ^{2}D. Here, the protein material was trapped under highly aqueous conditions for desalting. The flow was directed to UV/waste for 3 min to flush off non-volatile salts. Afterwards it was eluted in a fast gradient under denaturing conditions into the MS for intact mass analysis. Details are given in Tables 1 and 2.

Data Analysis

Thermo Scientific™ Chromeleon™ 7.3.1 CDS was used for data acquisition and data analysis. Thermo Scientific™ BioPharma Finder™ 5.1 software (BPF) was used for MS data deconvolution and evaluation.

Table 1. Chromatographic conditions.

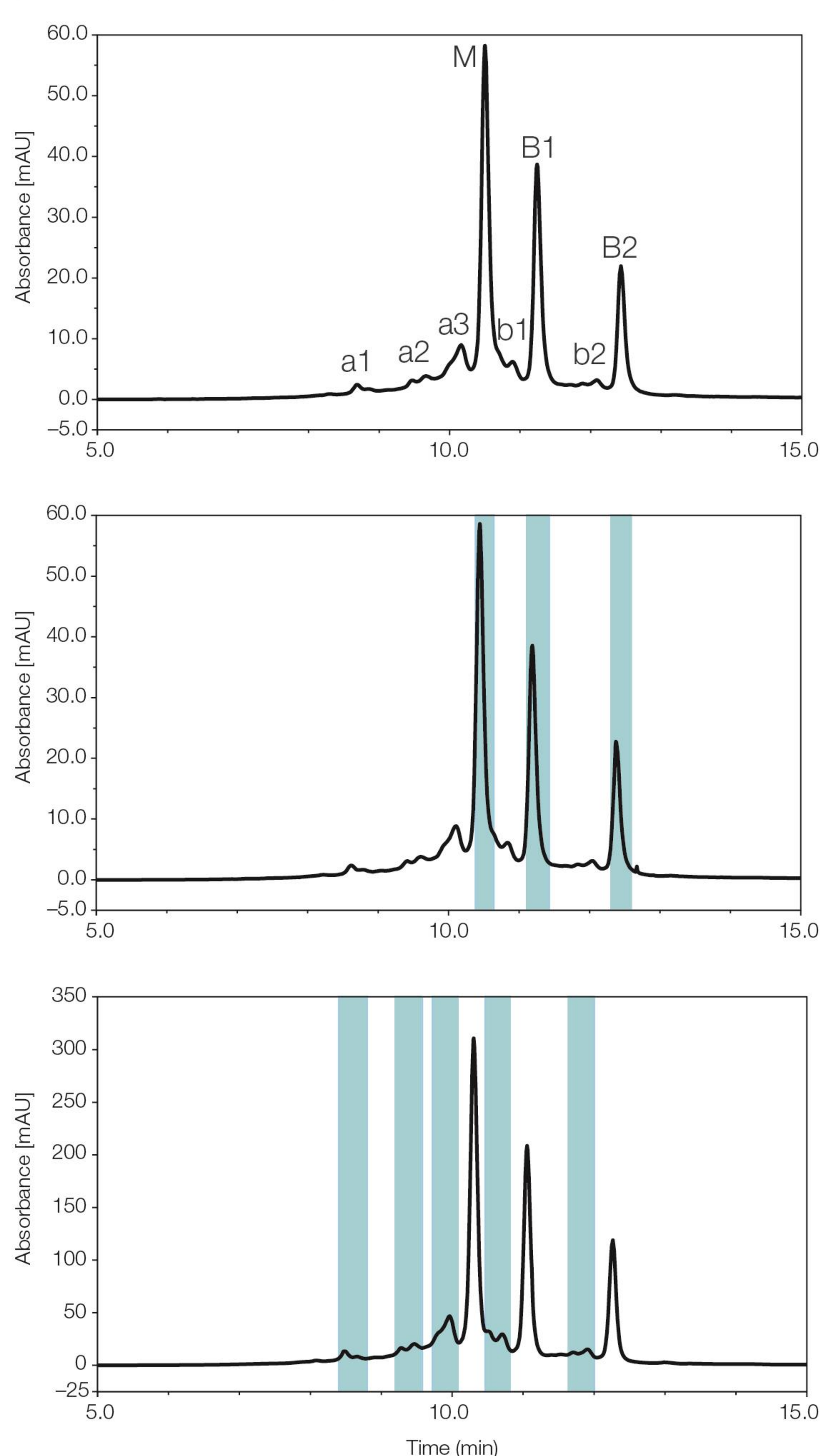
Parameter	^{1}D		^{2}D	
Column	Thermo Scientific™ MAbPac™ SCX-10, 5µm, 4x250mm (PN 078655)		Thermo Scientific™ MAbPac™ RP, 2.1x100mm, 4µm (PN 088647)	
Mobile phase	A: 20 mM MES + 20 mM NaCl pH6.5 B: 20 mM MES + 500mM NaCl pH6.5		A: 0.1 % formic acid in water B: 0.1 % formic acid in acetonitrile/water (90/10, v/v)	
Flow rate	0.6 mL/min		0.3 mL/min	
Injection volume	5-25 µL		5 µL or ^{1}D fraction volume	
Gradient				
	Time [min]	**%B**	**Time [min]**	**%B**
	0.0	0	0.0	10
	1.0	0	3.0	10
	13.0	13	4.0	60
	14.0	100	6.0	60
	17.0	100	6.1	10
	17.3	0	10.0	10
	22.0*	0		
Column temperature	40˚C (forced air incl. active eluent preheating)		80˚C (still air incl. active eluent preheating)	
Autosampler temperature	5˚C		5˚C	
UV data collection	280 nm; 10 Hz; 0.5 s response time		210 nm, 280 nm; 2 Hz; 2 s response time	

Table 2. MS parameter settings.

Method type	Full MS
Scan range (m/z)	1800-4000
Resolution	30000
RF lens (%)	100
AGC target value	300
Max inject time	200
Microscans	10
Source fragmentation (V)	60

Source settings	
Spray voltage (+)	3800
Ion transfer tube temperature (˚C)	320
Sheath gas	25
Aux gas	5
Sweep gas	0
Vaporizer temperature (˚C)	150

Figure 2. ^{1}D UV chromatograms of Infliximab; top - without heartcutting; middle - heart-cutting of three major peaks (M, B1, B2); bottom - heart-cutting of five minor peaks (a1, a2, a3, b1, b2).



Results

Method development

For the separation of charged Infliximab variants, a strong cation-exchange ^{1}D method was developed under LC-UV conditions. After the main peak (M) at around 10.5 min, two more major peaks are visible in the chromatogram depicted in Figure 2 (top) at around 11.2 min and 12.4 min, thus labeled as basic variants B1 and B2. In addition, there were five regions of minor peaks, three eluting before M, thus labeled as acidic variants a1-a3, and two eluting later than M and between the major peaks, labeled as b1 and b2.

Figure 3. Deconvoluted spectra of the three major charge variant peaks M (top), B1 (middle), and B2 (bottom).

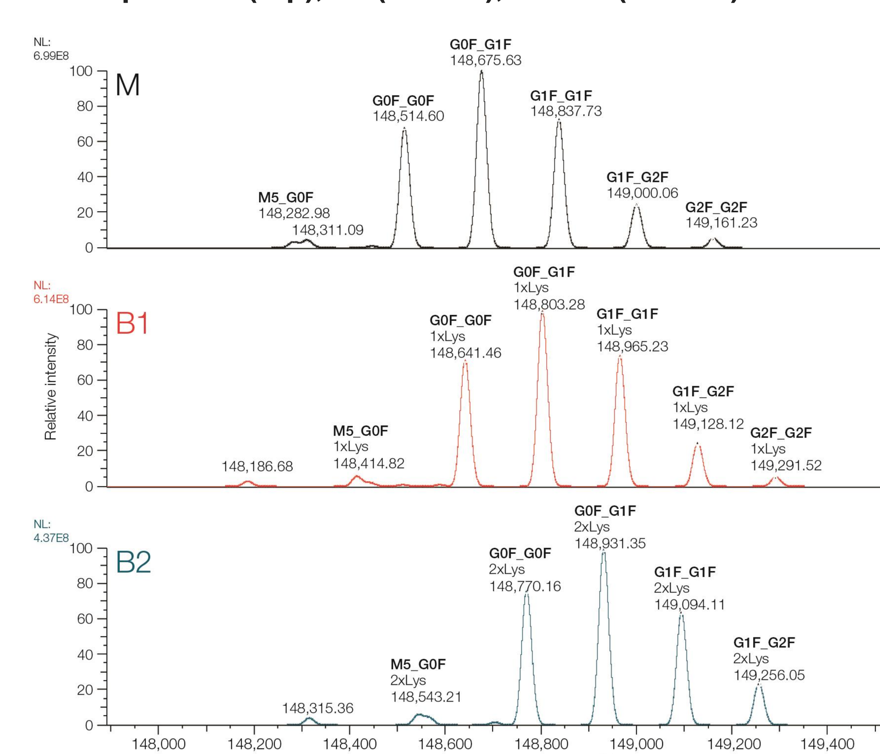
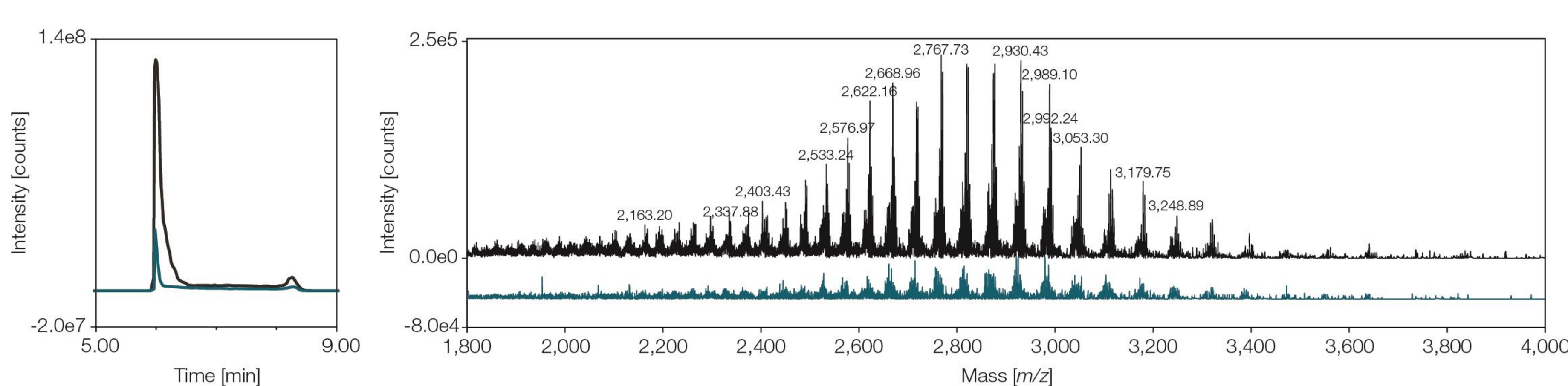


Figure 4. ^{2}D TIC and m/z charge envelop of fraction b2 with (black) and without (teal) enrichment by 5-fold accumulation.



The ^{2}D RP method was primarily meant as a desalting step, rather than providing an orthogonal separation to the ^{1}D. IEC-RP is an ideal combination for 2D-LC as the aqueous IEC mobile phases induce strong peak focusing on the RP column even when large fraction volumes are transferred.

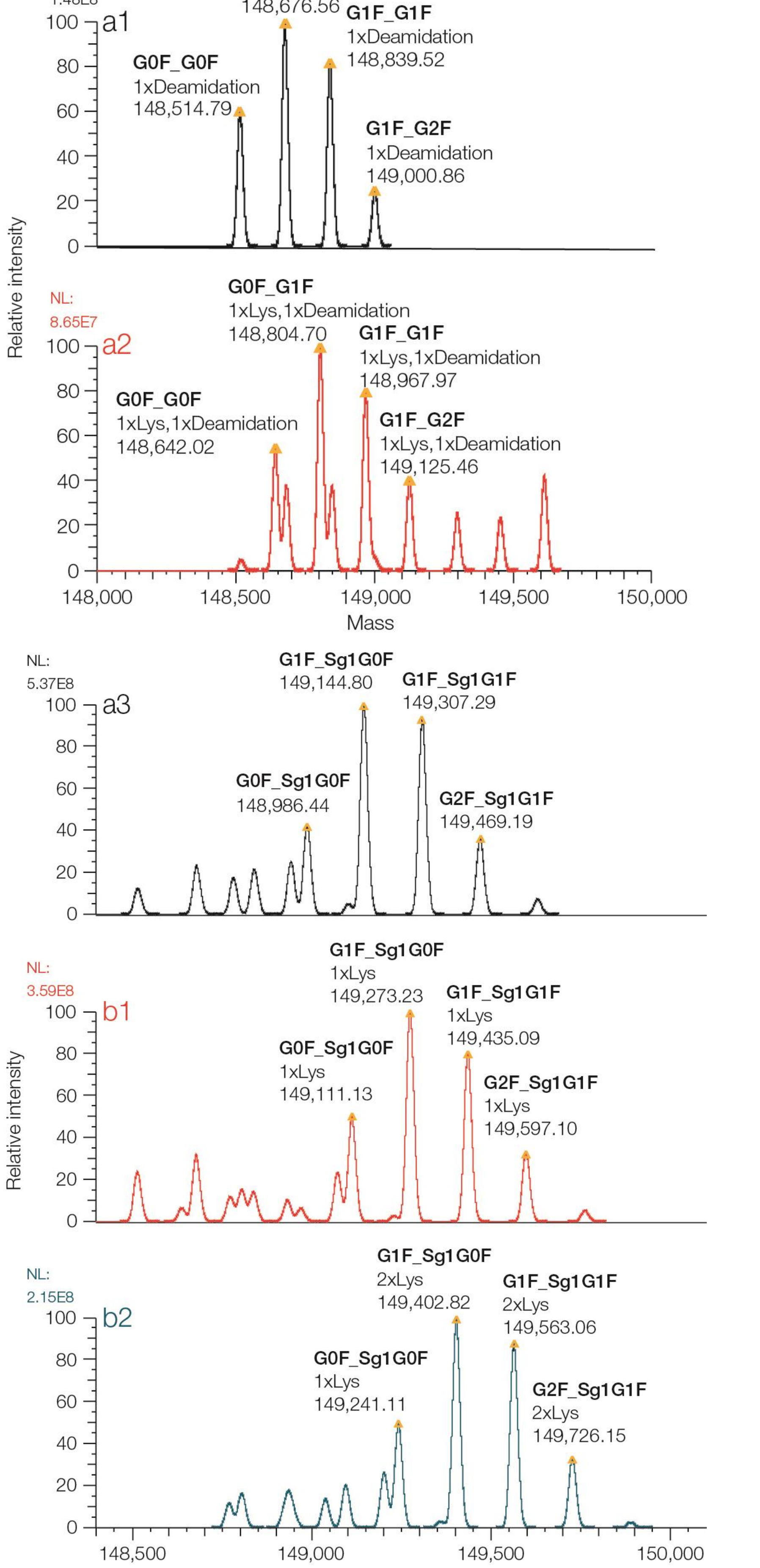
Identification of major peaks

5 µL of sample were injected into the 2D-LC system for the identification of the three major peaks. Three heart-cuts of the ^{1}D, of up to 200 µL volume, were collected and transferred (Figure 2 – middle). For the deconvolution of mass spectra, the ReSpect™ algorithm provided in the BFP software for isotopically unresolved masses was applied. The deconvoluted spectra of the three major peaks M, B1, and B2 reveal baseline-resolved and consistent glycosylation patterns (Figure 3).
Peaks M, B1, and B2 were identified as Infliximab variants with none, one, and two C-terminal Lys residues, which is in accordance with published work.[3] The major protein glycoforms are G0F/G0F, G0F/G1F, G1F/G1F, G1F/G2F, and G2F/G2F. G0F/M5 forms were detected with low intensity. Mass accuracies between 1 and 17 ppm were achieved.

Identification of minor peaks

The sample injection volume was increased to 25 µL for the five heart-cuts of minor peaks a1, a2, a3, b1, and b2 (Figure 2 bottom). Good MS signal intensity was attained, except for fraction b2. To generate adequate MS data, the fractionation of b2 was repeated five times in a row with only the region of b2 being cut. For each ^{1}D run, another loop was used to eventually fill all five loops with fraction b2 and flush one after the other to the ^{2}D column for sample enrichment. This procedure substantially improved the MS signal (Figure 4) and facilitated proper deconvolution. The deconvoluted spectra of the five minor charge variant peaks are displayed in Figure 5.
The masses of fraction a1 present the same glycosylation pattern as the three major peaks M, B1, and B2, with average mass differences of 162 Dalton due to the difference in terminal galactose residues.

Figure 5. Deconvoluted spectra of the five minor charge variant peaks a1, a2, a3, b1, and b2.



The pattern was also found for the most abundant masses in fractions a2 to b2. The deconvoluted masses of a1 show an average mass difference of +1 Dalton compared to the corresponding ones of M, strongly suggesting their identity as variants with one deamidation. In fraction a2, a combination of one deamidation and one terminal Lys is suggested by the mass differences compared to B1.
For the fractions a3, b1, and b2, an interesting pattern is observed with a common average mass difference of 469.6 Dalton, which is found for the peak pairs M/a3, B1/b1, and B2/b2. Sialylation with one N-glycolylneuraminic acid (Neu5Gc) causes a mass shift of 469 Dalton (see Figure 6). Sialylation is well known for antibody expression in murine cell lines and has been reported for Infliximab.[3] Thus, fractions a3, b1, and b2 are assumed to be sialylated Neu5Gc variants with none, one, and two terminal Lys residues (Figure 5).

Figure 7 summarizes the suggested identifications based on intact mass analysis of all fractions cut by 2D-LC. For a full characterization, peptide mapping experiments may be required, but the 2D-LC/MS approach facilitated a deep insight into the charge variant profile of Infliximab with high sensitivity.

Figure 6. Main and sialylated glycan structures detected in Infliximab sample with mass differences indicated.

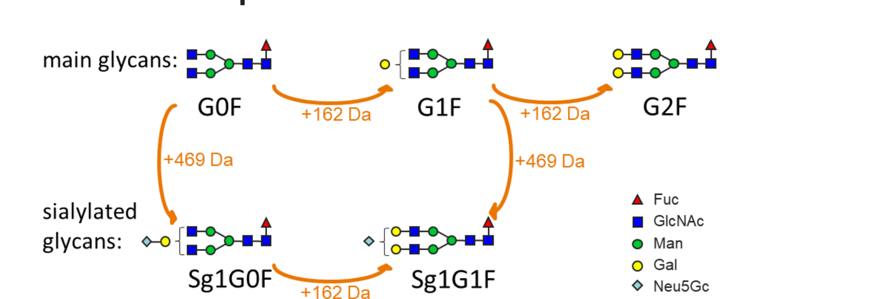
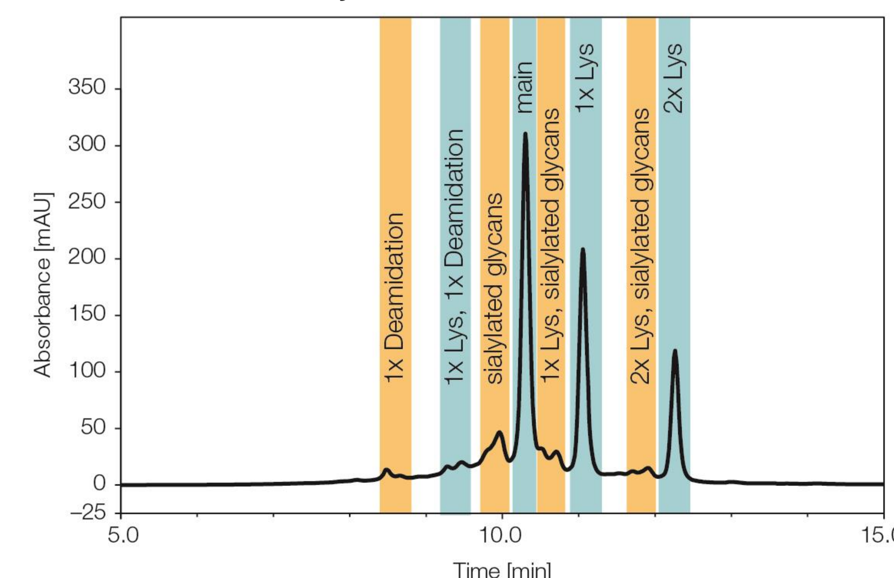


Figure 7. Summary of proposed Infliximab charge variants from intact mass analysis.



Conclusions

- Multi heart-cut 2D-LC enabled the direct transfer of ion-exchange fractions to MS analysis by using RP as a desalting step to overcome incompatibility of IEC mobile phases with MS.
- Intact mass analysis facilitated identification and monitoring of mAb charge variant patterns. Annotations of major and minor charge variant peaks was proposed, including lysine conjugations, deamidations, and sialylations.
- An automated workflow facilitated the enrichment of low abundant components for improved MS signal intensity and data processing.

References

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