

LC-MS Analysis of Digested mAbs Using a Reversed-Phase Desalting Cartridge and High-Resolution, Accurate Mass Spectrometry (HRAMS)

Martin Samonig,¹ Shane Bechler,² and Kelly Flook²

¹Thermo Fisher Scientific, Germering, Germany

²Thermo Fisher Scientific, Sunnyvale, CA, USA

Key Words

Desalting, MSPac, monoclonal antibody, mAb, fragment, Fc, Lc, Fd', reversed phase, proteomics, mass spectrometry, biopharmaceutical, biomolecules, glycosylation, carry-over, intact protein

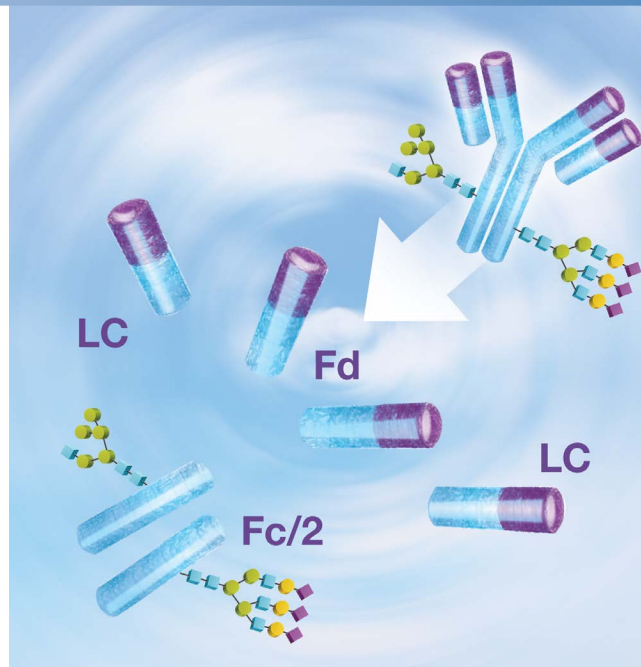
Goal

To describe a proof of concept analysis that demonstrates a simple, straightforward desalting method to optimize chromatographic separations for reduced analysis time, increased resolution, and improved MS detection of monoclonal antibody (mAb) fragments (Lc, Fc/2, and Fd') following digestion and reduction of a pharmaceutical mAb. Desalting and separation were conducted on a 2.1 × 10 mm Thermo Scientific™ MSPac™ DS-10 cartridge using conventional water/acetonitrile based eluents.

Introduction

The development and application of mAbs as biopharmaceutical therapeutics has grown rapidly in the last decade. This class of biomolecular drugs has proven effective for the treatment of a broad spectrum of diseases including cardiovascular, autoimmune disorders, and cancers.¹ While the fidelity of recombinant mAb production is generally very high, a range of biochemical modifications can occur during cellular production, mAb recovery, purification, and storage. These variations in structure can influence the safety and efficacy of the mAb, and it is therefore essential to fully characterize the nature and occurrence of structural variation and the subsequent effects on drug properties.²

Mass spectrometry (MS) is an essential tool in the characterization of mAbs, providing molecular weight determinations and structural information of intact as well as digested mAbs. Direct infusion of the sample into the MS is the simplest approach to characterize the sample. However, salts, detergents, and other matrix components can interfere with accurate MS characterization by decreasing sensitivity and resulting in instrument fouling, ultimately leading to downtime through extended instrument maintenance periods. Additionally, information regarding the types and locations of post-translational protein modifications can be difficult to determine without chromatography.



Reduction and/or digestion of mAbs to generate smaller protein fragments is a common analytical approach. Reducing the disulfide bonds breaks the mAb into smaller fragments. These are easier to characterize by MS than the intact mAb, therefore improving identification of structural changes across the mAb structure. Reducing the size of the proteins in the sample allows the acquisition of isotopically resolved charge states and the possibility of deconvolution to exact monoisotopic masses. Additionally, structural changes to the mAb can be isolated to a specific region (e.g., Fc, Lc, or Fd'). These sample preparation steps often utilize buffers and reagents rich in salts and other compounds that will interfere with MS detection. Liquid chromatography can be used to remove salts and other buffer components prior to MS detection, enabling complete characterization of all species. In some cases, users have applied short reversed-phase columns for the purpose of trapping their analyte while allowing salts to elute to waste.

Experimental

Consumables

- MSPac DS-10 Desalting Cartridge, 2.1 × 10 mm, 2/pack (P/N 089170)
- Thermo Scientific™ Acclaim™ Guard Cartridge Holder (P/N 069580)
- Thermo Scientific™ National Mass Spec Certified 2 mL clear vial with blue bonded PTFE silicone cap (P/N MSCERT5000-34W)
- Fisher Scientific™ LC-MS grade water (P/N W/011217)
- Fisher Scientific LC-MS grade acetonitrile (P/N A/0638/17)
- Thermo Scientific™ Pierce™ LC-MS grade formic acid (P/N 28905)
- Molecular Probes™ Tris-(2-Carboxyethyl)phosphine, Hydrochloride (TCEP) (P/N T-2556)
- FabRICATOR® (IdeS protease), purchased from Genovis

Note: Unless stated otherwise, consumables are from Thermo Fisher Scientific.

Sample Pretreatment and Sample Preparation

Rituximab (10 mg/mL, 147 kDa) from Hoffmann-La Roche Ltd (Basel, Switzerland) in the formulation buffer:

- 0.7 mg/mL polysorbate 80
- 7.35 mg/mL sodium citrate dehydrate
- 9 mg/mL sodium chloride
- Sterile water adjusted to pH 6.5 using sodium hydroxide

IdeS protease was used to digest the rituximab mAb according to the manufacturer's protocol. Following sample digestion, the resulting protein fragments were reduced in 5 mM TCEP for 30 min at 60 °C.

Separation Conditions

Instrumentation	Thermo Scientific™ Vanquish™ System Base (P/N VH-S01-A)
	Vanquish Binary Pump H (P/N VH-P10-A)
	Vanquish Split Sampler HT (P/N VH-A10-A)
	Vanquish Column Compartment H (P/N VH-C10-A)
	Vanquish Active Pre-heater (P/N 6732.0110)
	Vanquish Diode Array Detector (P/N VH-D10-A)
	LightPipe Flow Cell, 10 mm (P/N 6083.0100)
	Vanquish MS Connection Kit Vanquish (P/N 6720.0405)
	Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer

Gradient Conditions

Mobile phase A	Water + 0.1% formic acid
Mobile phase B	20:80 (v/v) water/acetonitrile + 0.1% formic acid
Flow rate	0.2 mL/min
Temperature	50 °C

Table 1. LC gradient conditions.

Time (min)	A	B
0	75	25
1	75	25
11	30	70
11.1	0	100
13	0	100
14	75	25
18	75	25

MS Conditions

Source	HESI-II
Sheath gas pressure	40 psi
Auxiliary gas flow	10 arbitrary units
Capillary temperature	260 °C
S-lens RF voltage	50
Source voltage	3.5 kV
Full MS mass range	550–3000 <i>m/z</i>

Full MS parameters

Resolution settings	240.000/15.000
Target value	3e6
Max injection time	200 ms
Microscans	3/10
SID	10 eV

Data Processing

The Thermo Scientific™ Dionex™ Chromeleon™ 7.2 SR2 Chromatography Data System and Thermo Scientific™ Protein Deconvolution™ 3.0 software were used for data acquisition and analysis.

Results and Discussion

Effectiveness of Desalting

Figure 2 shows the separation and MS detection of the IdeS digested and TCEP reduced monoclonal antibody rituximab on a 2.1×10 mm MSPac DS-10 cartridge. This illustrates the benefits of the desalting protocol with multiple species being detected during the gradient separation.

The peak at 0.24 minutes shows the elution of salts and other sample matrix components that do not bind to the polymeric stationary phase and would normally interfere with MS detection. Typically, the MS diverter valve is used to send salts to waste during loading; however, here we show detection of the eluted sample salts to demonstrate their removal from the protein prior to MS detection.

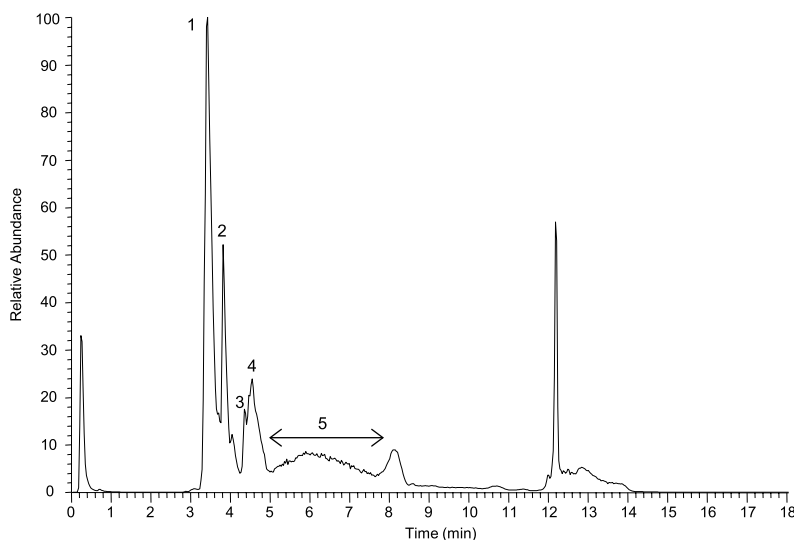


Figure 2. Analysis of IdeS digested and reduced rituximab ($0.8 \mu\text{g}$ total protein) on the MSPac DS-10 cartridge showing the separation of fragments, intact mAb, and partially digested and/or reduced mAb fragments.

Protein Loading

The effective MS characterization of product-related impurities and low-abundant modifications often requires higher sample loading for sufficient signal strength to obtain clean protein mass spectra. For this reason, it is important to be able to load a sufficient amount of protein onto the cartridge. Figure 3 shows the spectra for increasing mass loading of the digested and reduced mAb ranging from 0.1 – $10 \mu\text{g}$ of total protein and the blank run after the $10 \mu\text{g}$ injection.

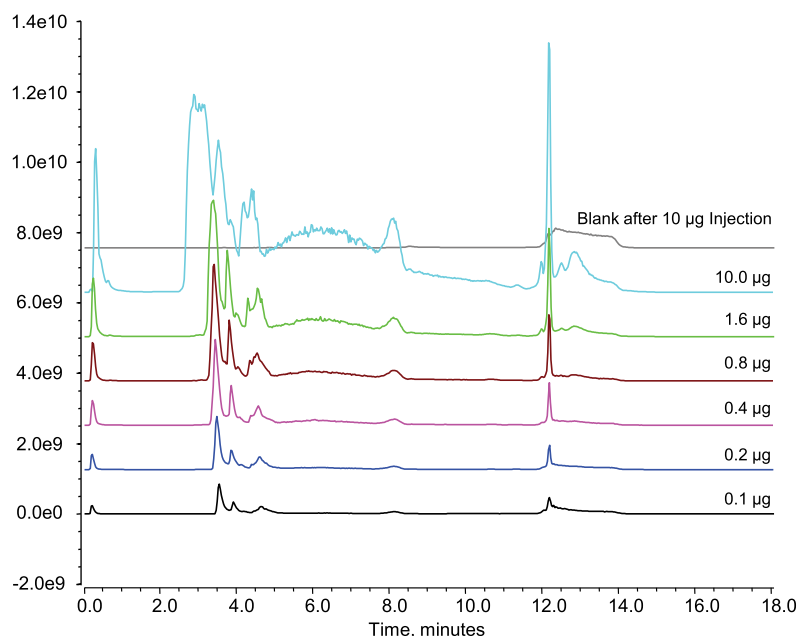
On increased loading, the signal strength increased for all components including salts and other matrix components observed to elute at ~ 0.25 minutes. This indicated effective desalting even at high mass loading amounts. Above $1.6 \mu\text{g}$ total protein loaded, the peak width at half height is observed to increase to the extent that sample component peaks overlap.

Peaks 1, 2, and 3 (retention times of 3.49, 3.86, and 4.35 minutes, respectively) correspond to the Fc/2, Lc, and Fd' species. Peak 4 is the intact mAb, and the broad range of peaks in region 5 are the result of partially digested and/or reduced species (e.g., Hc, Hc+Lc, and $2 \times \text{Lc} + 2 \times \text{Fd}'$). These results illustrate the desalting and separation capabilities of this short cartridge when handling samples composed of a range of ionic and organic matrix components and mAb fragments with similar molecular weights. In order to achieve resolution, the use of an analytical column is recommended. The Thermo Scientific™ MAbPac™ RP column is designed for this purpose and baseline resolution has been shown using the 3×50 mm format.³

Cartridge:	MSPac DS-10 Desalting Cartridge		
Format:	2.1 × 10 mm		
Mobile Phase A:	Water + 0.1% formic acid		
Mobile Phase B:	20/80 (v/v) water/acetonitrile = 0.1% formic acid		
Flow Rate:	0.2 mL/min		
Inj. Volume:	0.8 μL		
Temp.:	50 °C		
Detection:	MS; See methods for details		
Gradient:	Time (min)	A	B
	0.0	75	25
	1.0	75	25
	11.0	30	70
	11.1	0	100
	13.0	0	100
	14.0	75	25
	18.0	75	25
Sample:	Rituximab digested with IdeS and reduced with TCEP; 1 mg/mL		
Peaks:	<ol style="list-style-type: none"> 1. Fc/2 – C-terminal fragment of heavy chain with glycosated N301 2. Lc – Light chain 3. Fd' – N-terminal fragment of heavy chain 4. Intact mAb 5. Undigested and partially reduced species 		

Carryover and Column Fouling

Following the $10 \mu\text{g}$ protein injection, no detectable carryover was observed illustrating the low fouling properties of the MSPac DS-10 solid phase. As is common in reversed-phase chromatography, hydrophobic sample artifacts are observed to elute as several peaks from 12 to 13 minutes during the step wash at 100% mobile phase B, resulting in a cleaned stationary phase. Together, these results demonstrate the ability to load high levels of mAb fragments onto the cartridge with effective desalting for improved MS signal and characterization with no detectable carryover between subsequent runs.



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18.0	75	25																							

Figure 3. Loading analysis (0.1–10 µg protein) of mAb fragments from IdeS digested and reduced rituximab on a 2.1 × 10 mm MSPac DS-10 cartridge and the blank run following the 10 µg injection.

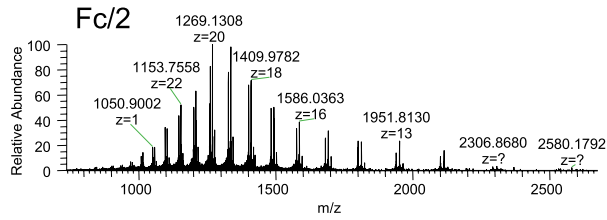
Structural Identification

Figure 4 shows the mass spectra and deconvoluted mass spectra for the Fc/2, Lc, and Fd' fragments of the digested and reduced rituximab mAb for the chromatogram shown in Figure 2. The clean mass spectrum obtained for each component demonstrates the excellent desalting power of the MSPac DS-10 cartridge. The quality of the spectra allowed easy deconvolution to provide the measured molecular weight for each fragment including the glycosylated variants of the Fc/2 fragments. These results are summarized in Table 2 including the deviation from the theoretical monoisotopic molecular weight. The mass deviation of only -4.6 to -1.2 ppm for the molecular weight fragments demonstrates the detection power of the Q Exactive HF mass spectrometer.

In addition to these species, MS analysis and deconvolution of the MS spectra in region 5 (as shown in Figure 2) yielded the measured molecular weight of additional fragment variants and partially digested and/or reduced

fragments. Effective desalting resulted in suitable mass spectra even in this region, which contains multiple protein species; this allowed deconvolution and determination of structural features for these species. These measurements are summarized in Table 3. Generally, observed mass deviations are directly related to the poorer spectrum quality for some of the fragments eluted in this complex region of the chromatogram. Inspection of the data in Table 3 shows that the measured molecular weight of the Hc (Fc/2 + Fd'), Hc + Lc, and 2 × Lc + 2 × Fd' fragments correspond to the calculated theoretical molecular weight of the individual fragments.

Mass Spectrum



Deconvoluted Mass Spectrum

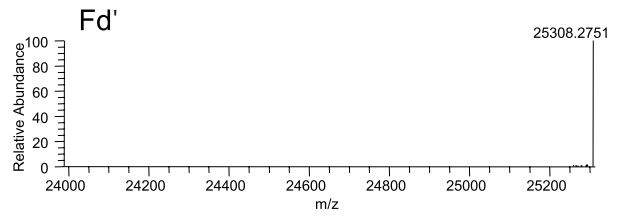
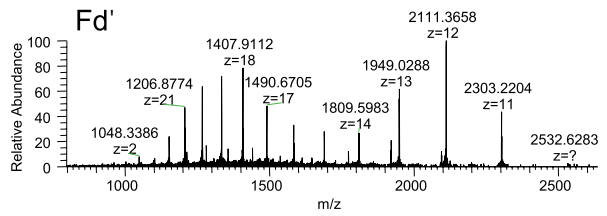
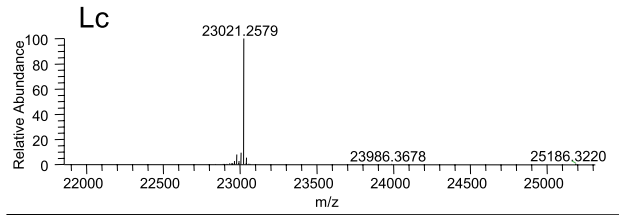
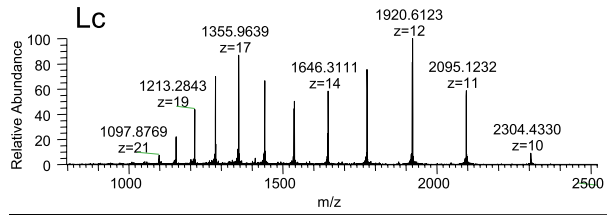
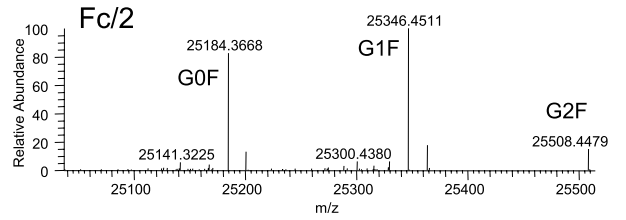


Figure 4. (Left) Mass spectra for Fc/2, Lc, and Fd' rituximab fragments and (right) the deconvoluted mass spectra showing the measured molecular weight of each fragment including glycosylated variants for the Fc/2 fragment.

Table 2. Theoretical monoisotopic MW, measured MW, and mass deviation for rituximab fragments and associated variants.

Fragment	Modifications	# C	# H	# N	# O	# S	MW (monoisotopic) [Da]	MW measured [Da]	Mass deviation [ppm]
Lc	N-terminal pyro Glutamic acid, 2 internal S-S bonds	1016	1570	272	328	6	23021.28593	23021.2579	-1.22
Fd'	N-terminal pyro Glutamic acid, 2 internal S-S bonds	1125	1724	292	354	10	25308.30854	25308.2751	-1.32
Fc/2	G0F glycan 2 internal S-S bonds	1122	1736	286	361	6	25184.46011	25184.3668	-3.71
Fc/2	G1F glycan 2 internal S-S bonds	1128	1746	286	366	6	25346.51294	25346.4511	-2.44
Fc/2	G2F glycan 2 internal S-S bonds	1134	1756	286	371	6	25508.56576	25508.4479	-4.62

Table 3. Measured MW for assumed fragment variants and partially digested and/or reduced rituximab fragments.

Fragment	MW measured [Da]	Theoretical MW [Da]
Lc	23036.352	23035.353
Fc/2+ G1F (2 internal S-S bonds)	25361.822	25362.019
Hc + G1F (4 internal S-S bonds)	50602.141	50668.124
Hc+ Lc + G1F (6 internal S-S bonds)	73680.188	73699.445
2 x Lc + 2 x Fd' (7 internal S-S bonds) ^a	96713.414	96712.9

^a Partially reduced F(ab')₂, with 7 unreduced S-S bonds

Conclusion

The analyses shown here for rituximab demonstrate a broadly applicable desalting method that can be used with a range of intact biomolecules and their associated fragments for the purposes of determining variations in structure and for proper clone selection for production in biopharmaceutical processes. Gradient conditions can be further optimized to develop a high-throughput platform for analyzing hundreds of samples in a short time frame.

- The MSPac DS-10 cartridge provides excellent separation of digested and reduced mAbs resulting in a clean protein spectrum for each fragment with minimum signal overlay.
- Using a reversed phase gradient and a high flow rate salts, adducts, and other sample matrix components are effectively removed from the mAb fragments resulting in clean MS spectra.
- The MSPac DS-10 cartridge provides these benefits without carryover enabling the user to reliably do successive injections of high protein loading amounts without requiring additional blank runs.
- The resolving power of the Q Exactive HF mass spectrometer is able to isotopically resolve mass spectra for all subunits enabling the calculation of the exact monoisotopic masses of each mAb fragment.

References

1. Leavy, O. Therapeutic Antibodies: Past, Present, and Future, *Nature Reviews Immunology*, 2010, 10, 297.
2. Liu, H.; Gaza-Bulsecu, G.; Faldu, D.; Chumsae, C.; Sun, J. Heterogeneity of Monoclonal Antibodies, *J. of Pharmaceutical Sciences*, 2008, 97, 2426.
3. Lin, S., Zhang, T., Wang., H., Josephs, J., and Liu, X., fast Analysis of Therapeutic Monoclonal Antibodies, Fragments, and Oxidation Variants Using a Super-Macro Porous Reversed-Phase Column Coupled with an Orbitrap Mass Spectrometer, ASMS 2015, Thermo Scientific Poster Note PN64398.

Useful Links

AppsLab Library

The eWorkflow and the Chromeleon Backup (cmbx) file can be downloaded at AppsLab Library:

<https://applab.thermoscientific.com/>

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