Analysis of Monoclonal Antibodies and Their Fragments by Size-Exclusion Chromatography Coupled with an Orbitrap Mass Spectrometer

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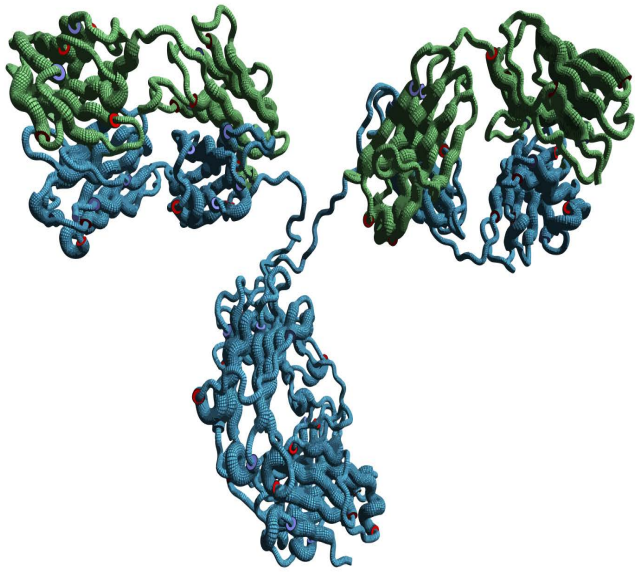
Key Words
MAbPac SEC-1, Exactive Plus EMR Orbitrap, monoclonal antibody, mAb, mAb heavy chain (HC) and light chain (LC), mAb Fab and Fc fragments

Abstract
The Thermo Scientific™ MAbPac™ SEC-1 column uses size-exclusion chromatography (SEC) and is designed for monoclonal antibody (mAb) analysis, including monomers, aggregates, and fragments. Coupling size-exclusion chromatography with a high-resolution Thermo Scientific™ Exactive™ Plus EMR Orbitrap™ mass spectrometer (SEC-MS) enables accurate mass measurement of mAb and its fragments in either native or denatured state. When using a non-denaturing volatile eluent, such as 20 mM ammonium formate, the intact mass of mAb can be measured in its native state. When using denaturing volatile eluents, such as 20% acetonitrile, 0.1% formic acid and 0.05% trifluoroacetic acid, mAb heavy chain (HC), light chain (LC), Fab, and Fc fragments are successfully separated on MAbPac SEC-1 and their masses are determined accurately.



Introduction
The biopharmaceutical industry continues to increase its focus on the development of biotherapeutic monoclonal antibody (mAbs) drugs. Early in the development of recombinant mAbs, a large number of harvest cell culture (HCC) samples must be screened for IgG titer, aggregations, and charge variants.

In a typical workflow, mAbs are first purified by Protein A affinity chromatography and then characterized by ion-exchange chromatography to identify charge variants and size-exclusion chromatography to quantify aggregations. Thermo Fisher Scientific has a complete portfolio of HPLC consumables and systems for the affinity, SEC, and IEX applications, namely the Thermo Scientific™ MAbPac™ Protein A column, Thermo Scientific™ ProPac™ WCX-10 column, Thermo Scientific™ MAbPac™ SCX-10 column, Thermo Scientific CX-1 pH Gradient Buffer Kit, Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system and UltiMate 3000 PCM-3000 pH and Conductivity Monitor. [1]

For final biopharmaceutical product approval and subsequent manufacturing, a comprehensive characterization of mAb purity, aggregate forms, and charge variants is required by regulatory agencies. MAbs

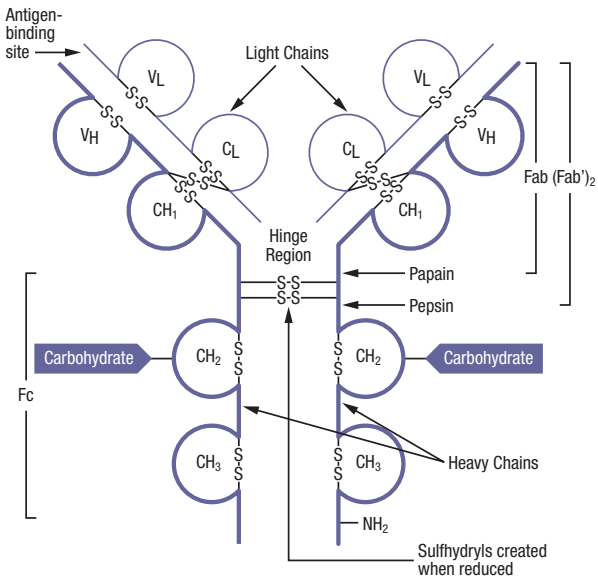


Figure 1: IgG structure

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produced from mammalian cell culture may contain significant amounts of dimers and higher-order aggregates. Studies show that these aggregates present in drug products may cause severe immunogenic and anaphylactic reactions. Size-exclusion chromatography (SEC) is a well-accepted technique for the detection and accurate quantification of protein aggregates in biological drug products. It is also routinely used for the characterization and quality control of mAb products.

There is a growing requirement to determine intact mass information, as well as the glycan profile in the QC of mAbs, using high resolution mass spectrometry. Most commonly employed LC/MS methods require the mAb to be desalted prior to MS analysis, and this is typically done by reversed-phase chromatography. However, the low pH and high concentration of organic solvent condition used in reversed-phase chromatography often denatures the mAb. This problem is further exacerbated in the particular case of antibody-drug conjugate (ADC) with interchain cysteine-linked drugs. The harsh solvent condition dissociates the heavy and light chains of the ADC and prevents the measurement of intact mass.

SEC chromatography using ammonium formate buffers, in front of mass spectrometry detection, enables mass measurement of intact mAb in its native state without the need for desalting. Since ammonium formate is a volatile buffer, it is compatible with MS and preserves intact protein structure. Full characterization of a mAb includes mass determination of the fragments, such as the heavy chain and light chains generated by reduction of the disulfide bonds. SEC-MS enables intact mass detection of mAb under non-denaturing condition and fragments (including heavy chain, light chain, Fab, and Fc) under denaturing condition (Figure 1).

The MAbPac SEC-1 column is a size-exclusion chromatography (SEC) column designed for monoclonal antibody (mAb) analysis, including monomers, aggregates, and fragments. Its proprietary column chemistry ensures low column bleed and and compatibility with MS detection. The compatibility of the MAbPac SEC-1 column with the Exactive Plus EMR Orbitrap mass spectrometer is demonstrated here. The Exactive Plus EMR MS combines high-resolution accurate-mass with the extended mass range (EMR). It has an *m/z* range up to 20,000 and improved transmission of higher-mass ions for stronger signals. All these features make the Exactive Plus EMR mass spectrometer a superb tool for accurate intact mass measurement of mAb and high-performance screening of mAb glycosylation profile.

This application note compares SEC-MS analysis of heavy chain and light chain mAb fragments under denaturing conditions. Additionally, the Fc and Fab fragments were analyzed after papain digestion (Figure 1) under denaturing conditions.

Experimental Details

Consumables		**Part Number**
All reagents were purchased from reputable suppliers. Monoclonal antibodies were kindly supplied by a biotechnology company.		
Column	MAbPac SEC-1, 5 µm, 4 × 300 mm	074696
Non-denaturing SEC mobile phase	20 mM ammonium formate. Solution pH was measured at 6.3.	
Denaturing SEC mobile phase	20% acetonitrile, 0.1% formic acid, and 0.05% trifluoroacetic acid (TFA)	

Chromatographic Conditions	
HPLC experiments were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano system equipped with an SRD-3400 Membrane Degasser, NCS-3500RS Dual-Gradient Pump and Column Compartment, and WPS-3000TPL Rapid Separation Thermostatted Autosampler.	
Gradient:	Isocratic
Flow rate:	0.2 mL/min
Total run time:	20 min
Temperature:	30 °C

MS Conditions

The Exactive Plus EMR Orbitrap mass spectrometer was used for this study. Intact mAb or mAb fragments were analyzed by ESI-MS for intact molecular mass.

Non-denaturing, MAb Monomer:	
EMR mode:	On
Mass range:	*m/z* 400–20,000
Spray voltage:	4.3 kV
Sheath gas:	30 arb. units
Auxiliary gas:	10 arb. units
Capillary temperature:	275 °C
S-lens level:	200
In-source CID:	100 eV
HCD CE:	10
Microscans:	5
AGC target:	1×10^6
Maximum IT:	300 ms
Resolving power:	35,000
Probe temperature:	400 °C
Denaturing:	
EMR mode:	On
Mass range:	*m/z* 400–6,000
Spray voltage:	4.3 kV
Sheath gas:	30 arb. units
Auxiliary gas:	10 arb. units
Capillary temperature:	275 °C
S-lens level:	200
In-source CID:	100 eV
HCD CE:	n/a
Microscans:	1
AGC target:	1×10^6
Maximum IT:	200 ms
Resolving power:	17,500
Probe temperature:	200 °C

Data Processing

MS spectra of intact mAbs and HC, LC, Fab, and Fc fragments were analyzed using Thermo Scientific™ Protein Deconvolution 2.0 software, which utilizes the ReSpect™ algorithm for molecular mass determination. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the mAb or their fragments. The averaged spectra were subsequently deconvoluted using an input range of *m/z* 4000 to 6000 for spectra acquired under non-denaturing conditions (or *m/z* 2000 to 4000 for spectra acquired under denaturing conditions). An output mass range of 140,000 to 160,000 Da with a target mass of 150,000 Da for mAb, or an output mass range of 45,000 to 55,000 Da with a target mass of 50,000 Da for mAb fragments, and a minimum of at least eight consecutive charge states from the input *m/z* spectrum was used to generate the deconvolution results.

Reduction of mAb to Heavy Chain (HC) and Light Chain (LC) Subunits

Reduction of inter-chain disulfides in a mAb (1 mg/mL) was achieved by incubation of mAb with 20 mM dithiothreitol (DTT) at 50 °C for 30 min. The reduced sample was acidified with formic acid to a final concentration of 0.1%.

Papain Digestion of mAb to Generate Fab and Fc Subunits

The digestion was carried out by incubating mAb (1 mg/mL) with papain (0.04 mg/mL) in 100 mM Tris-HCl, pH 7.6, 4 mM EDTA, and 5 mM cysteine buffer at 37 °C. After 4 hours, the digestion was stopped by addition of formic acid to a final concentration of 0.1%.

Results

Analysis of mAb by Non-denaturing SEC-MS

The analysis of mAbs by SEC is typically performed under non-denaturing conditions at physiological pH (6.8) with phosphate buffer and 300 mM sodium chloride. However, the non-volatile nature of phosphate buffer and high salt content makes this buffer incompatible with online mass spectrometry detection.

In this application we used a volatile buffer, 20 mM ammonium formate, for SEC separation and directly coupled the SEC column to the Exactive Plus EMR instrument. Figure 2 shows the SEC-MS analysis of an mAb, where (a) is the extracted ion chromatogram of *m/z* at 5483.08–5483.31 and (b) is the charge envelope of +24 to +29 in the *m/z* range of 5100–6200.

Normally under acidic conditions, the charge envelope of mAb is in the *m/z* range of 2000–4000 but because the 20 mM ammonium formate eluent has a pH of 6.3, the charge envelope of mAb shifts to higher mass range. The detection of such high *m/z* charge envelopes (greater than 6000) is made possible with the extended mass range of the Orbitrap instrument. Figure 2c shows the deconvoluted mass spectra of the mAb, with a main peak at *m/z* 148,033 and adjacent peaks at *m/z* 148,198, and 148,359, corresponding to different glycoforms with one and two additional hexoses. An adjacent peak at *m/z* 148,163 is 130 amu above the main peak, corresponding to a lysine variant.

MAb average mass can be measured by SEC-MS under denaturing condition as long as the mAb structure remains intact using eluent such as 20% acetonitrile, 0.1% formic acid, and 0.05% TFA. When comparing the charge distribution of mAb under acidic and near neutral conditions, mAb is carrying more charge in acidic conditions with charge envelope in the range of +33 to +60 (data not shown).

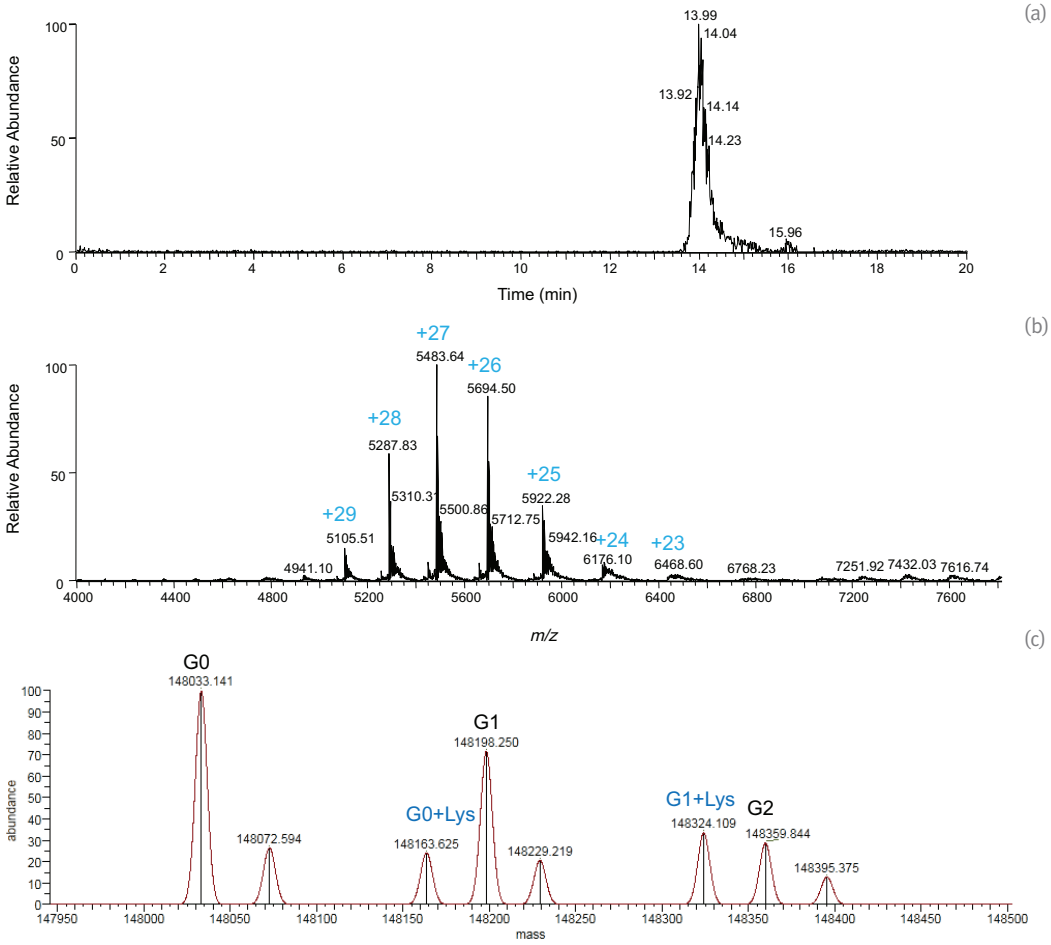

Figure 2: SEC-MS analysis of mAb under non-denaturing condition using 20 mM NH4Fc. (a) Extracted ion chromatogram of mAb (b) Mass spectrum of mAb (c) Deconvoluted spectrum of mAb

Analysis of mAb Fragments by Denaturing SEC-MS

Comprehensive analysis of post-translational modifications, such as deamidation, C-terminal lysine truncation, N-terminal pyroglutamation, methionine oxidation, and glycosylation requires complete digestion of the mAbs and subsequent sequencing of all the peptides; however, "peptide mapping" is time consuming. A simpler and faster way to analyze the mAb variants and locate the modifications is to measure the mass of heavy and light chains, or Fab and Fc fragments. Heavy chain and light chain are generated by the reduction of mAb. Fab fragments are generated by papain digestion. For example, the glycan modification is located in the Fc region of the heavy chain, glycan variants can be detected in the heavy chain and Fc fragment mass profiles, while light chain and Fab fragment mass profiles should only show a single polypeptide chain.

Figure 3 shows the SEC-MS analysis of HC and LC using denaturing conditions. Figure 3a shows the extracted ion chromatogram of HC (*m/z* at 3163.70–3164.89) and LC (*m/z* at 2600.78–2601.88). Using these denaturing eluents HC elutes at 10.15 min and mAb LC elutes at 12.71 min. Different mAbs have been tested and their HC and LC fragments have similar retention times. Therefore, denaturing SEC can be used as a platform method for the separation of HC and LC.

Figure 3b shows the charge envelope of mAb HC (*m/z* 1900-3600). Figure 3c shows the deconvoluted mass spectra of the mAb HC (*m/z* 50614.5) and adjacent peaks corresponding to a C-terminal lysine variant (*m/z* 50742.3) and a different glycoform with one additional hexose (*m/z* 50,776.4). Figure 3d shows the charge envelope of the LC (*m/z* 1500–3500), and Figure 3e shows the deconvoluted mass spectra of the LC (a single peak at *m/z* 23,403.7). The mAb light chain is not glycosylated and does not have C-terminal lysine variants. The intact mass of mAb was determined at *m/z* 148,029 from the equation 2 × (HC+LC)-8. This calculated mass was in good agreement with the measured mass at *m/z* 148,035.

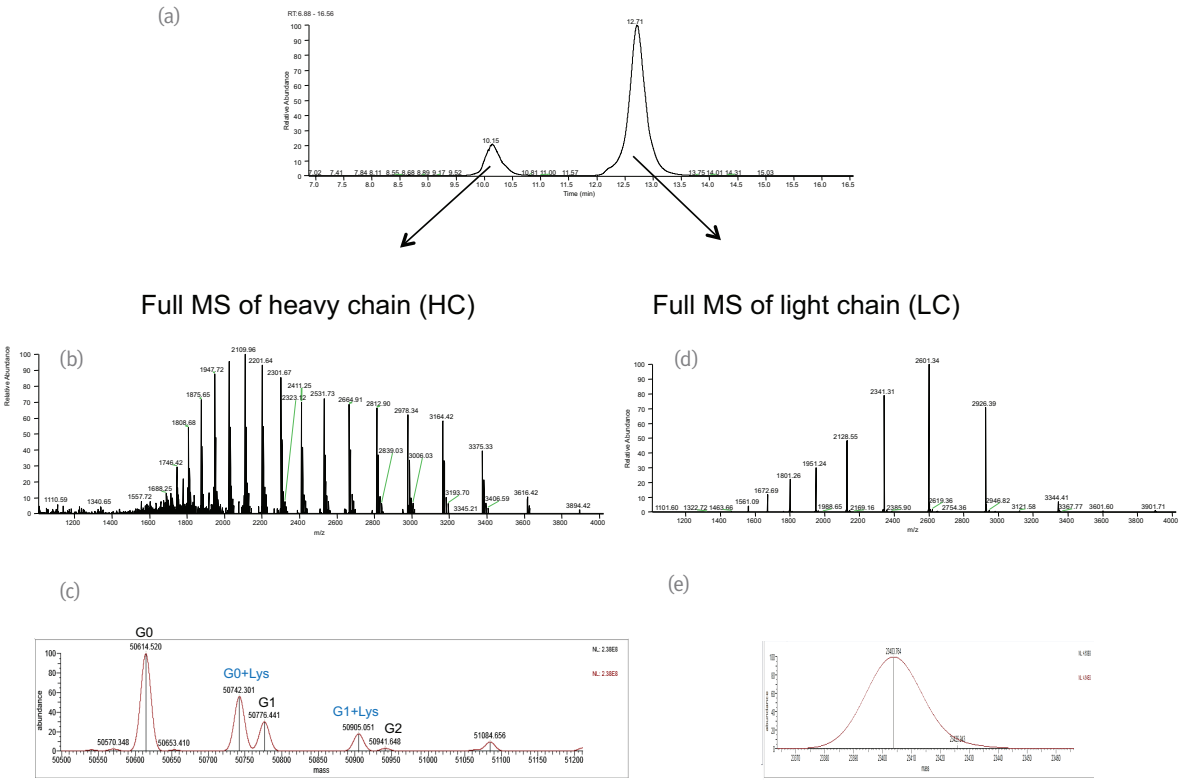

Figure 3: SEC-MS analysis of mAb heavy chain and light chain under denaturing condition using 20% acetonitrile, 0.1% formic acid, and 0.05% trifluoroacetic acid. (a) Extracted ion chromatogram of heavy chain (HC) and light chain (LC) (b) Mass spectrum of heavy chain (HC) (c) Deconvoluted spectrum of heavy chain (HC) (d) Mass spectrum of light chain (LC) (e) Deconvoluted spectrum of light chain (LC)

Using the same chromatographic method, Fc and Fab fragments were eluted off the SEC column at 9.94 and 10.79 min, respectively (Figure 4a), although the separation is not as good as that for HC and LC due to the fact that Fab and Fc fragments are very similar in size. Figure 4b shows the charge envelope of Fc in the *m/z* range of 1500–3500. Figure 4c shows the deconvoluted mass spectra of the Fc, with a main peak at *m/z* 52,752.9 and adjacent peaks at *m/z* 52,880.5 and 52,916.1, corresponding to a lysine variant and a different glycoform with one additional hexose. Figure 4d shows the charge envelope of Fab in the *m/z* range of 1600–3700, and Figure 4e shows the deconvoluted mass spectra of the Fab, with a single peak at *m/z* 47,317.6. The intact mass of mAb is determined at *m/z* 147,387 using the equation 2xFab+Fc. The calculated mass is more than 700 Da away from the measured mass at *m/z* 148,035, indicating an additional fragment generated from the papain digestion.

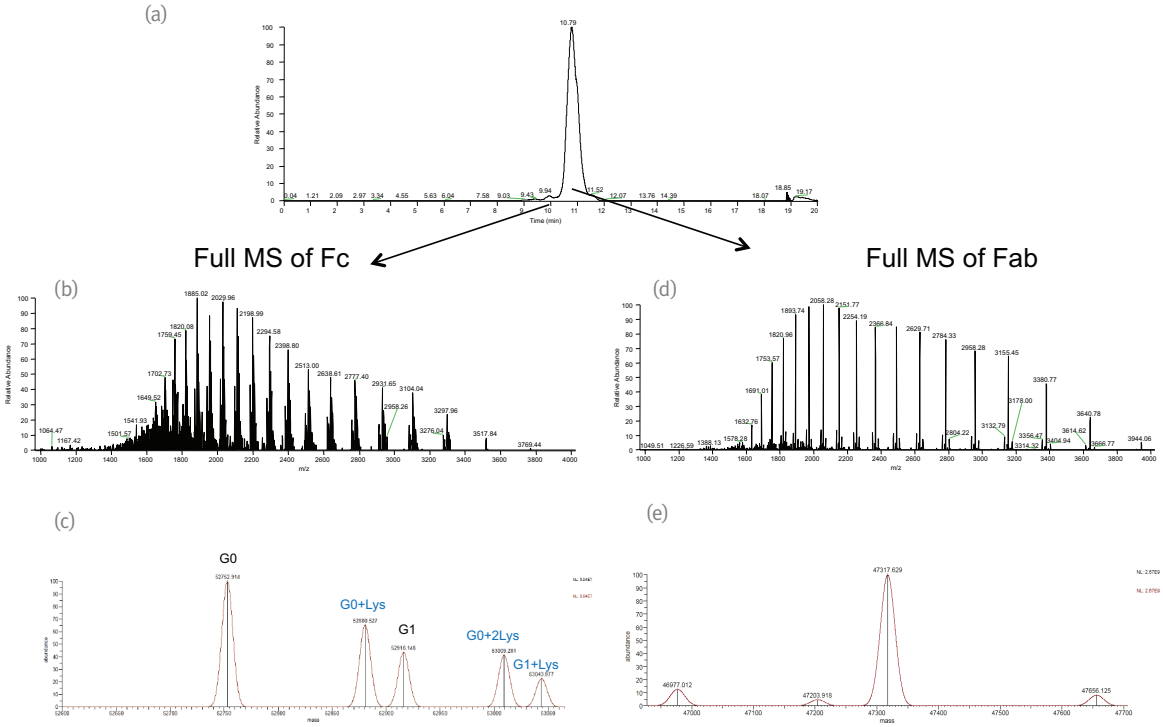

Figure 4: SEC-MS analysis of mAb Fc and Fab under denaturing condition using 20% acetonitrile, 0.1% formic acid, and 0.05% trifluoroacetic acid. (a) Extracted ion chromatogram of Fc and Fab (b) Mass spectrum of Fc (c) Deconvoluted spectrum of Fc (d) Mass spectrum of Fab (e) Deconvoluted spectrum of Fab

Conclusion

- SEC serves as a platform method for mAb fragment analysis capable of resolving intact mAb and its fragments.
- mAb intact mass can be measured by SEC-MS under non-denaturing condition using MS compatible eluents such as 20 mM ammonium formate.
- The Exactive Plus EMR mass spectrometer enables accurate detection of mAbs in the range of *m/z* 350–20,000
- Additionally, the MAbPac SEC-1 column successfully separated the HC and LC, and partially separated the Fab and Fc fragments using denaturing eluents.

Reference

[1] Lin, S.; Rao, S.; Thayer, J.; Agroskin, Y.; and Pohl, C. Automated Monoclonal Antibody 2-Dimensional Workflow: from Harvest Cell Culture to Variant Analysis. Presented at The WCBP Conference, San Francisco, CA, January 23–25, 2012.

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