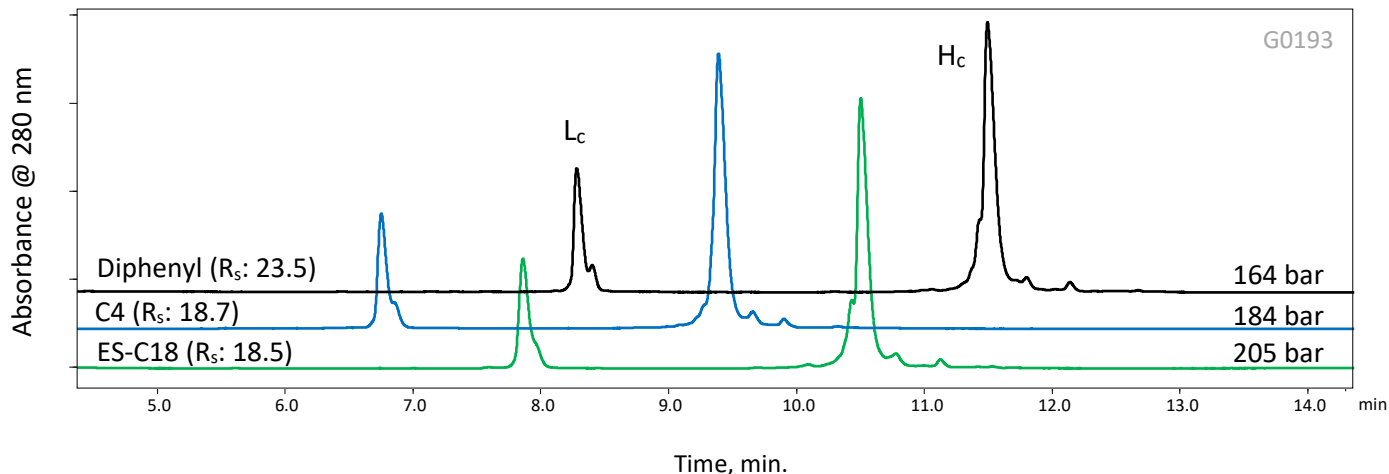


Reduced IgG1 (Trastuzumab) Retention Comparison on Three HALO® 1000 Å Phases



TEST CONDITIONS:

Columns: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150mm

Part Number: 92712-726

HALO 1000 Å C4, 2.7 µm, 2.1 x 150mm

Part Number: 92712-714

HALO 1000 Å ES-C18, 2.7 µm, 2.1 x 150mm

Part Number: 92712-702

Mobile Phase A: Water/ 0.1% TFA

Mobile Phase B: Acetonitrile/ 0.1% TFA

Gradient:

Time (min.)	%B
0.0	30
14.0	40

Flow Rate: 0.4 mL/min

Temperature: 80°C

Detection: 280 nm, PDA

Injection Volume: 2 µL

Sample Solvent: Water

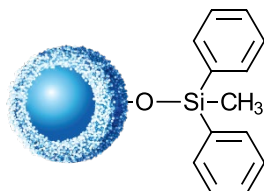
Data Rate: 12.5 Hz

Response Time: 0.25 sec.

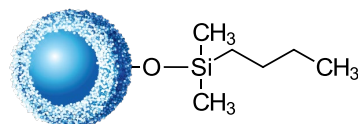
Flow Cell: 1 µL

LC System: Shimadzu Nexera X2

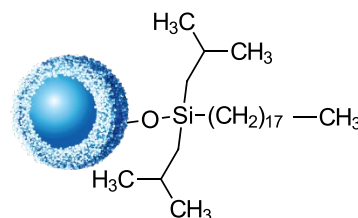
STRUCTURES



HALO 1000 Å Diphenyl



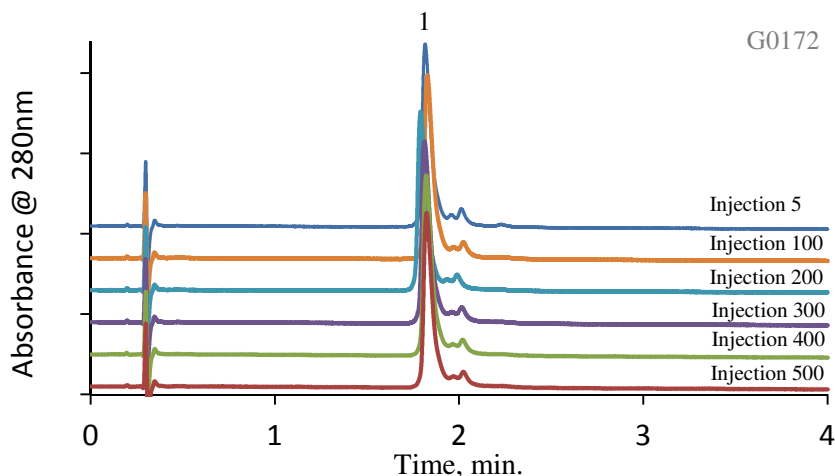
HALO 1000 Å C4



HALO 1000 Å ES-C18

Trastuzumab is a monoclonal antibody used to treat breast cancer. Enhanced resolution of trastuzumab's heavy and light chains is demonstrated in the chromatograms above using three different HALO® bonded phases. The 1000 Å pores of the HALO® Protein columns readily accommodate large biomolecules, and allow unrestricted pore access, narrower peaks and superior separations at high temperatures.

High Temperature/ Low pH Stability of HALO 1000 Å ES-C18, 2.7 μm



PEAK IDENTITY:

1. Trastuzumab

STRUCTURES:

TEST CONDITIONS:

Column: HALO 1000 Å ES-C18, 2.7 μm, 2.1 x 50mm

Part Number: 92712-402

Mobile Phase A: Water/ 0.1% TFA

Mobile Phase B: Acetonitrile/ 0.1% TFA

Gradient:	Time	%B
	0.0	32
	4.0	38

Flow Rate: 0.4 mL/min

Initial Pressure: 81 bar

Temperature: 80 °C

Detection: UV 280 nm, PDA

Injection Volume: 1.2 μL

Sample Solvent: Water

Data Rate: 40 Hz

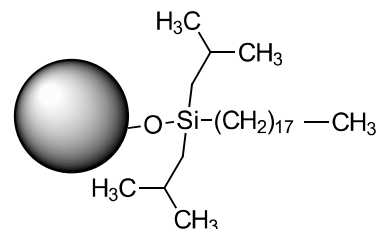
Response Time: 0.025 sec

Flow Cell: 1 μL

LC System: Shimadzu Nexera X2



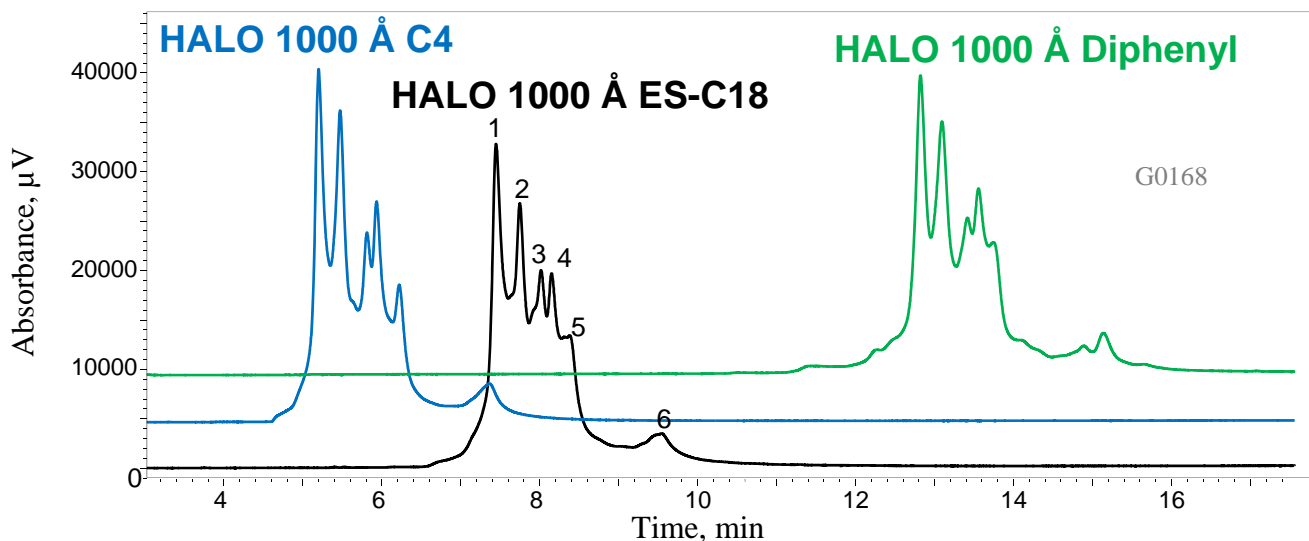
1000 Å 2.7μm particle



ES-C18 bonded phase

Trastuzumab (MW ~148 kDa) is a monoclonal antibody used to treat breast cancer. A stability experiment using a HALO 1000 Å ES-C18 column shows excellent reproducibility for 500 injections of trastuzumab. The sterically protected C18 bonded phase enables rugged stability at the elevated temperature and low pH conditions that are typically used for protein analysis.

IgG2 Comparison on HALO 1000 Å C4, ES-C18, and Diphenyl



TEST CONDITIONS:

Columns:

HALO 1000 Å C4, 2.7 µm, 2.1 x 150 mm

Part Number: 92712-714

HALO 1000 Å ES-C18, 2.7 µm, 2.1 x 150 mm

Part Number: 92712-702

HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm

Part Number: 92712-726

Mobile Phase A:

2:10:88 n-propanol/ACN/H₂O

+ 0.1% difluoroacetic acid (DFA)

Mobile Phase B:

70:20:10 n-propanol/ACN/H₂O + 0.1% DFA

Gradient: 16–26% B in 20 min

Flow Rate: 0.2 mL/min

Temperature: 80°C

Instrument: Shimadzu Nexera

Detection: PDA 280 nm; 350 nm reference

Injection Volume: 2 µL of 2 mg/mL denosumab

Sample Solvent: water (0.1% TFA)

PEAK IDENTITIES:

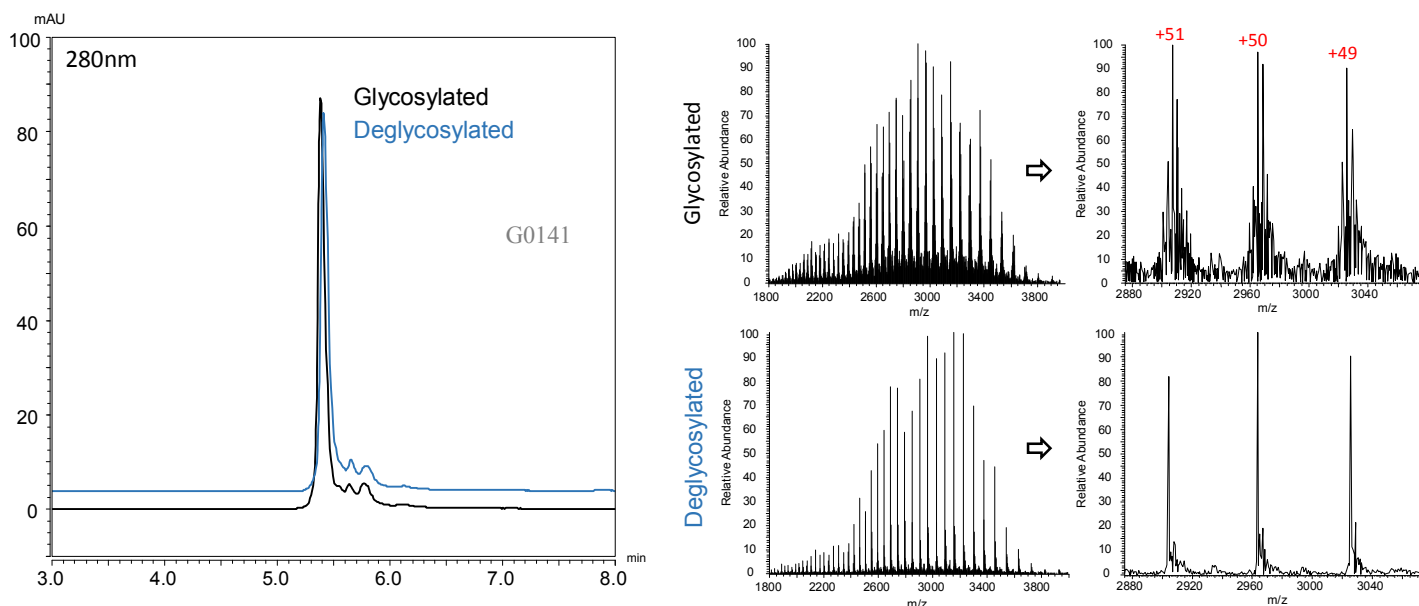
1. IgG2-B
 2. IgG2-B
 3. IgG2-A/B
 4. IgG2-A/B
 5. IgG2-A
 6. IgG2-A*
- } disulfide bridge isoforms of IgG2

Note: Labels on ES-C18 chromatogram also apply to C4 and Diphenyl chromatograms.

There are currently three bonded phases available on HALO 1000 Å Fused-Core® particles – C4, ES-C18, and Diphenyl. Each shows unique selectivity for the separation of monoclonal antibodies. In this example, denosumab isoforms are resolved using a shallow gradient with the addition of n-propanol. Diphenyl phase is the most retentive phase, followed by ES-C18, and then C4. All three phases are recommended to be screened to determine which one yields the optimum separation for mAbs under investigation.

Application Note: 151-PR

LC-MS Analysis of Trastuzumab Using HALO 1000Å C4



LC Test Conditions:

Column: HALO 1000Å C4, 2.7 μ m, 2.1 x 150 mm
 Part Number: 92712-714
 Mobile Phase A: 10 mM difluoroacetic acid (DFA) in water
 Mobile Phase B: 10 mM difluoroacetic acid in 10/90 water/acetonitrile
 Gradient: 32-42% B in 10 min
 Flow Rate: 0.35 mL/min.
 Pressure: 184 bar
 Temperature: 80 °C
 Detection: 280 nm
 Injection Volume: 1 μ L of 2 mg/mL trastuzumab (glycosylated/deglycosylated)
 Sample Solvent: 0.1% DFA in 70/30 water/acetonitrile
 LC System: Shimadzu Nexera

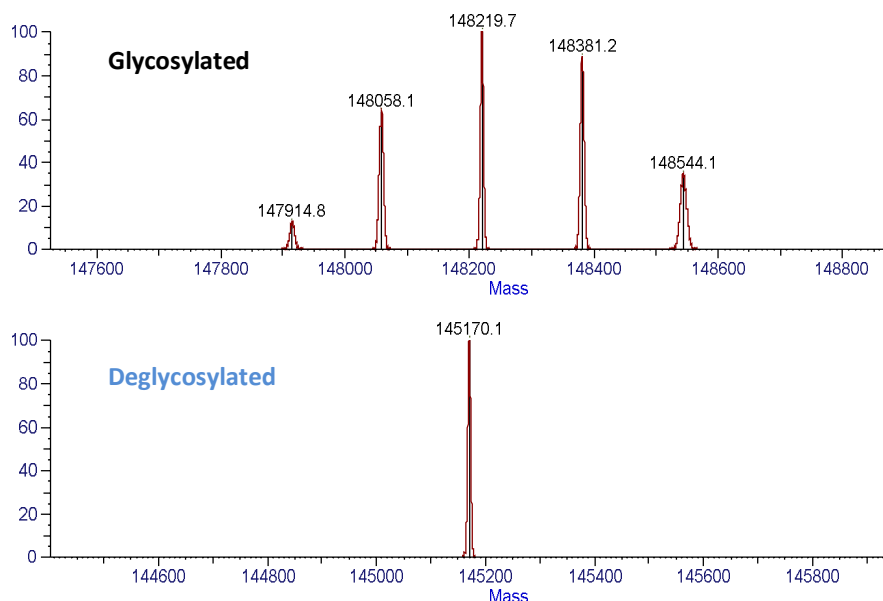
MS Test Conditions:

MS System: Thermo Fisher Orbitrap VelosPro ETD
 Scan Time: 6 μ scans/250 ms max inject time
 Scan range: 1800 to 4000 m/z
 MS parameters: Positive ion mode, ESI at +4.0 kV, 225°C capillary

LC-MS analysis using a HALO 1000Å C4 Protein column has been used to analyze two samples of the monoclonal antibody, trastuzumab: glycosylated and enzymatically deglycosylated. Minor variant structures are observed in both the glycosylated and deglycosylated monoclonal IgG (small peaks after main peak), indicating that the polypeptides are structure variants.

The glycosylation profile of therapeutic mAbs is an important characteristic, which must be monitored throughout the manufacturing process. Determination of the mass of the deglycosylated IgG confirms the identity and integrity of the protein.

Deconvoluted Spectra and Peak Information



The structure of trastuzumab consists of two heavy chains and two light chains. Glycosylation occurs on the two heavy chains. One or more of the same or different carbohydrate moieties can be present on each heavy chain. Table 1 contains the combinations of sugars that correspond to the masses that were observed upon deconvolution of the mass spectrum on the previous page. The last column is the mass of the deglycosylated trastuzumab, which results from enzymatic cleavage of the glycans by PNGase F.

Table 1:

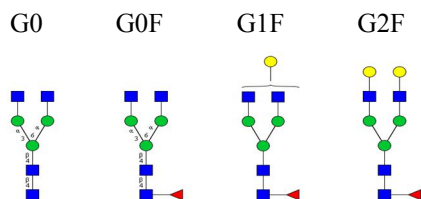
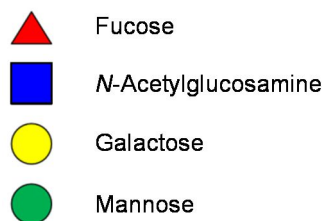
GLYCANS:	G0/G0F		G0F/G0F		G1F/G0F		G1F/G1F, G2F/G0F		G1F/G2F		Deglycosylated Trastuzumab	
	T ¹	M ¹	T	M	T	M	T	M	T	M	T	M
Trastuzumab	147911	147915	148057	148058	148219	148220	148381	148381	148543	148544	145167	145170
Δ Mass (glyc) Trastuzumab	2744	2745	2890	2888	3052	3050	3214	3211	3376	3374	—	3

T = Theoretical mass

M = Measured mass

¹All masses reported in Daltons

Glycan Descriptions:

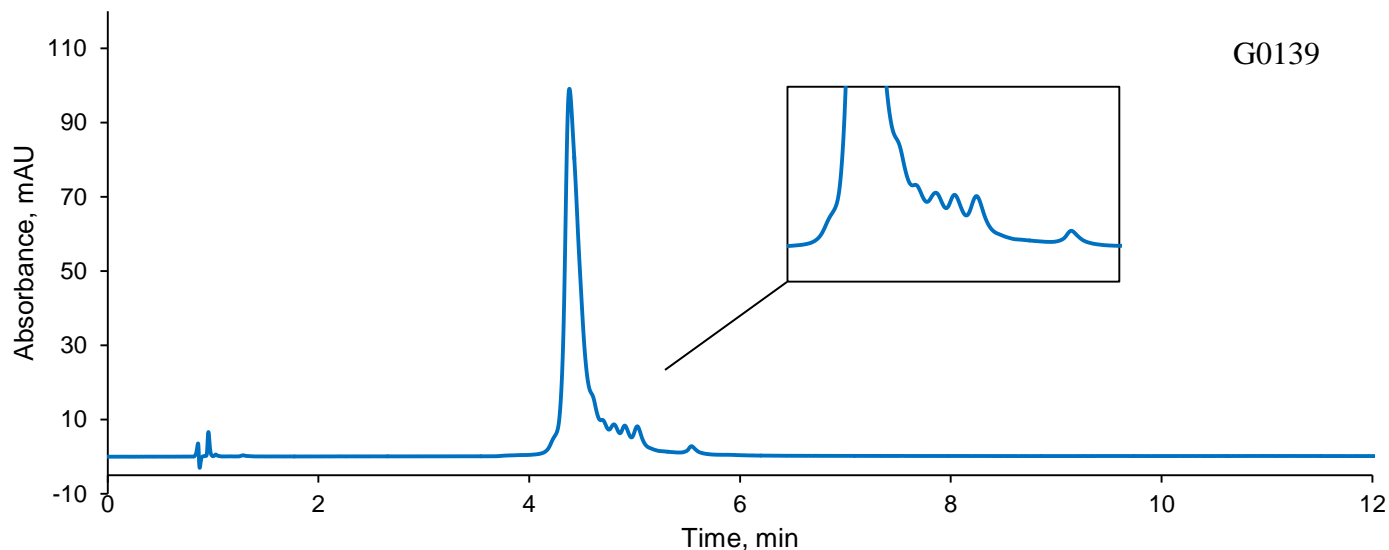


Deconvolution Parameters:

Minimum Adjacent Charges 3 - 6
 Noise Rejection 95% Confidence
 m/z Range 1800 - 4000
 Mass Tolerance 20 ppm
 Charge State Range 40 - 120
 Choice of Peak Model Intact Protein

Application Note: 149-PR

HALO 1000Å C4 Protein Column for High Resolution Separation of a Monoclonal Antibody



TEST CONDITIONS:

Column: HALO 1000Å C4, 2.7µm, 2.1 x 100 mm

Part Number: 92712-614

A= Water, 0.1% TFA

B= 80/20 ACN/Water, 0.085% TFA

Gradient:

Time (min.)	%B
0.00	40
12.00	47.5

Flow Rate: 0.4 mL/min

Pressure: 210 bar

Temperature: 80 °C

Injection Volume: 2 µL

Sample Solvent: 70/30 Water/ACN

Detection: UV 280 nm, PDA

Data Rate: 12.5 Hz

Response Time: 0.05 sec.

Flow Cell: 1 µL

LC System: Shimadzu Nexera X2

Monoclonal Antibody Structure:

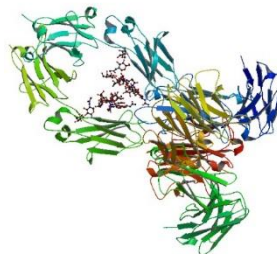
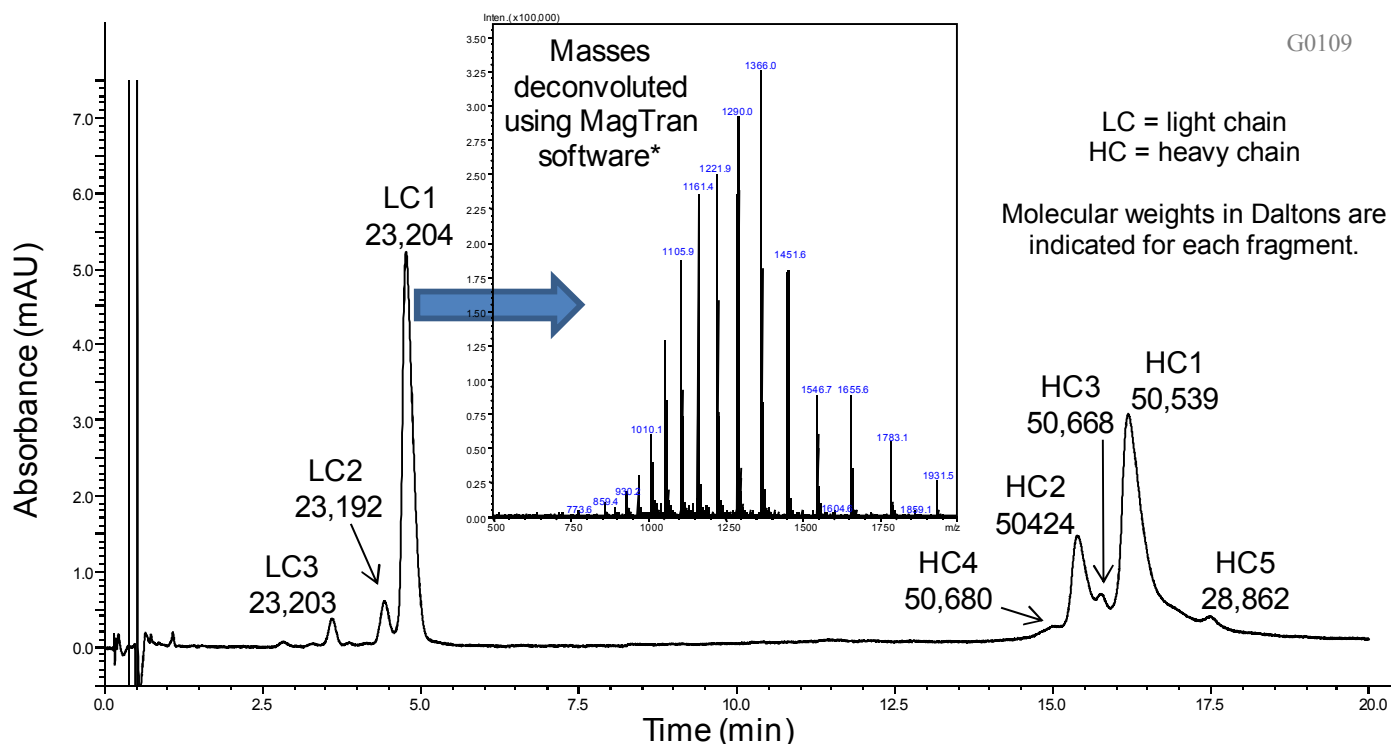


Image from the RCSB PDB (www.rcsb.org) of PDB ID 1HZH (E.O. Saphire, P.W. Parren, R. Pantophlet, M.B. Zwick, G.M. Morris, P.M. Rudd, R.A. Dwek, R.L. Stanfield, D.R. Burton, I.A. Wilson) (2001) Crystal structure of a neutralizing human IGG against HIV-1: a template for vaccine design Science 293: 1155-1159)

Trastuzumab (MW ~148 kDa) is a monoclonal antibody used to treat breast cancer. Enhanced resolution of trastuzumab and its variants is demonstrated in the chromatogram above. The pores of the HALO 1000Å C4 Protein particles accommodate larger biomolecules enabling superior separations at high temperatures.

Application Note: 125-PR

LC-MS Analysis of Reduced IgG1 Monoclonal Antibody Fragments Using HALO 400 Å C4



TEST CONDITIONS:

Column: HALO 400 Å C4, 3.4 µm, 2.1 x 100 mm
 Part Number: 93412-614
 Mobile Phase A: 0.5% (v/v) formic acid with 20 mM ammonium formate
 Mobile Phase B: 45% acetonitrile/45% isopropanol/0.5% (v/v) formic acid/9.5% water with 20 mM ammonium formate
 Gradient: 29-32% B in 20 min
 Flow Rate: 0.4 mL/min.
 Pressure: 20 bar
 Temperature: 80°C
 Detection: 280 nm and MS using 2 pps scan rate from 500 to 2000 m/z
 Injection Volume: 2 µL of 2 µg/µL reduced and alkylated IgG1
 Sample Solvent: 0.25% (v/v) formic acid in water
 MS parameters: Positive ion mode, ESI at +4.5 kV, 400°C heat block, 225°C capillary
 LC-MS System: Shimadzu Nexera and LCMS-2020 (single quadrupole MS)

HALO 400 Å C4 has the low pH and high temperature stability that is required to analyze reduced and alkylated IgG1 using MS-compatible mobile phase. The use of 80 °C enables improved peak shape while the high resolution MS allows complete analysis of the IgG1 fragments that are present.

Adapted from *J. Chromatogr. A* 1315 (2013) 118-126.

*Z. Zhang, A.G. Marshall, *J. Am. Soc. Mass Spectrom.* 9 (1998) 225.