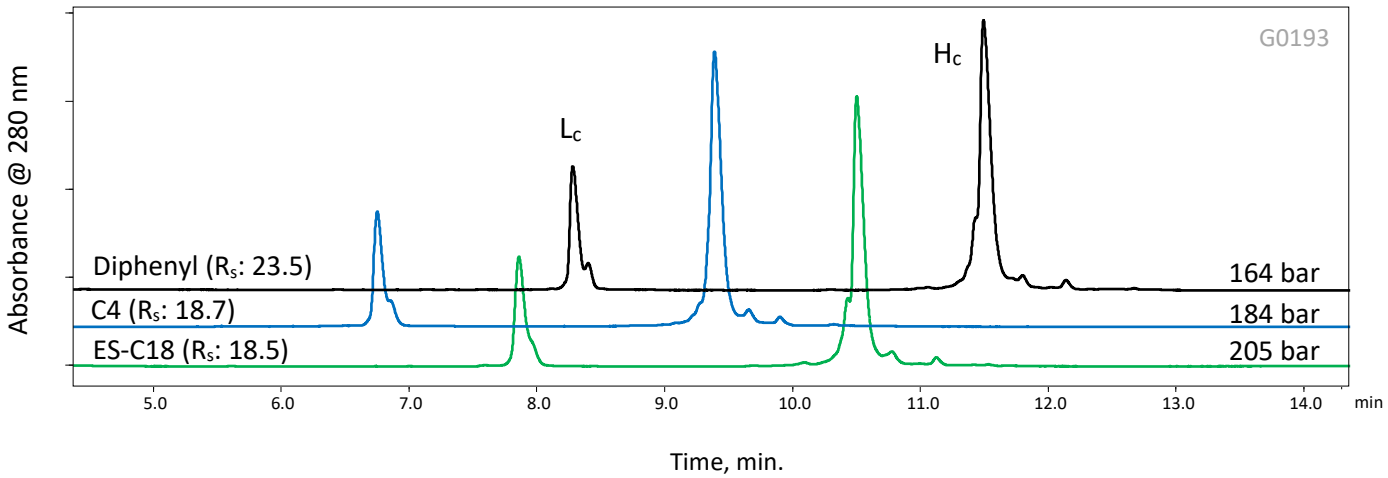
HALO® | Fused-Core® Particle Technology

Application Note: 199-PR

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



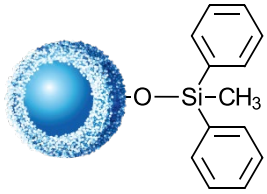
TEST CONDITIONS:

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:

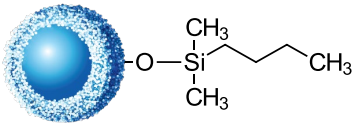
Time (min.)	%B
0.0	30
14.0	40

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

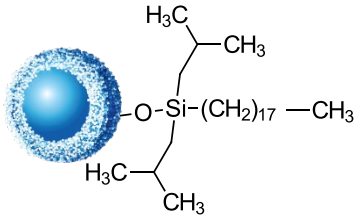
STRUCTURES



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 assess, narrower peaks and superior separations at high temperatures.

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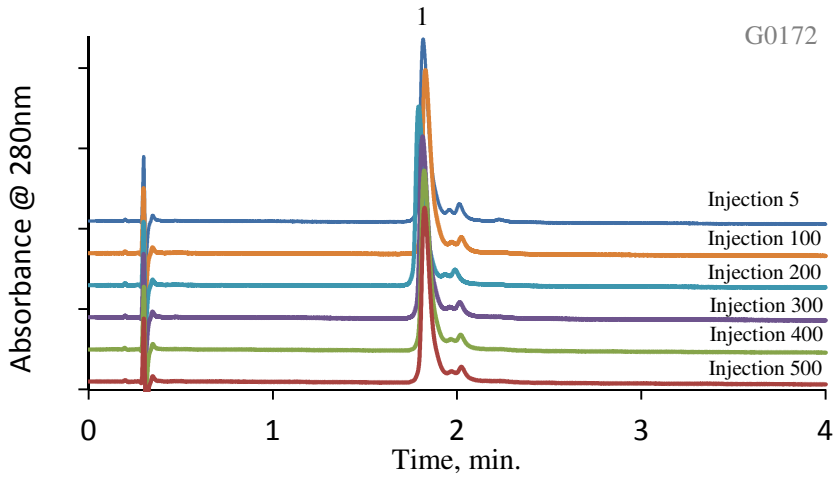
Application Note: 178-PR

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



PEAK IDENTITY:

1. Trastuzumab

TEST CONDITIONS:

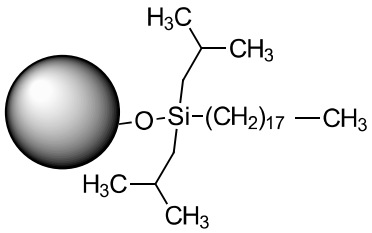
Column: HALO 1000 Å ES-C18, 2.7 µm, 2.1 x 50mm
Part Number: 92712-402
Mobile Phase A: Water/ 0.1% TFA
Mobile Phase B: Acetonitrile/ 0.1% TFA
Gradient:

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

STRUCTURES:

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.

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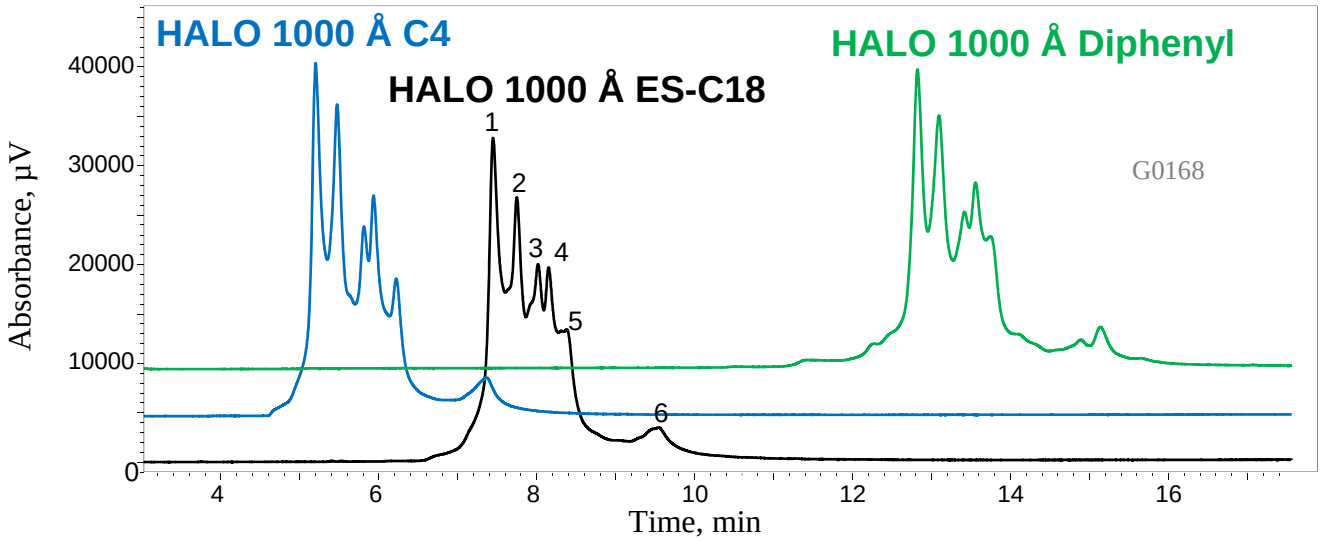
Application Note: 174-PR

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/H_2O
+ 0.1% difluoroacetic acid (DFA)
Mobile Phase B:
70:20:10 n-propanol/ACN/H_2O + 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.

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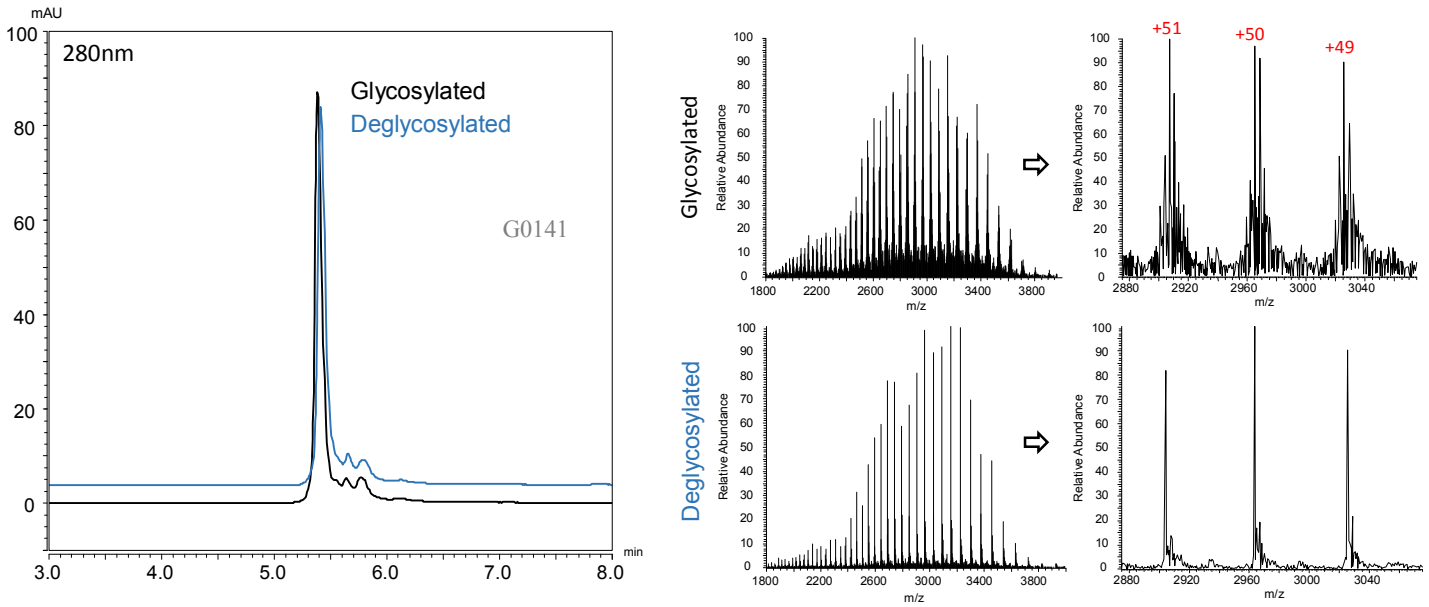
Application Note: 151-PR

LC-MS Analysis of Trastuzumab Using HALO 1000Å C4



LC Test Conditions:

Column: HALO 1000Å C4, 2.7 µm, 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 µL of 2 mg/mL trastuzumab (glycosylated/deglycosylated)
Sample Solvent: 0.1% DFA in 70/30 water/acetonitrile
LC System: Shimadzu Nexera

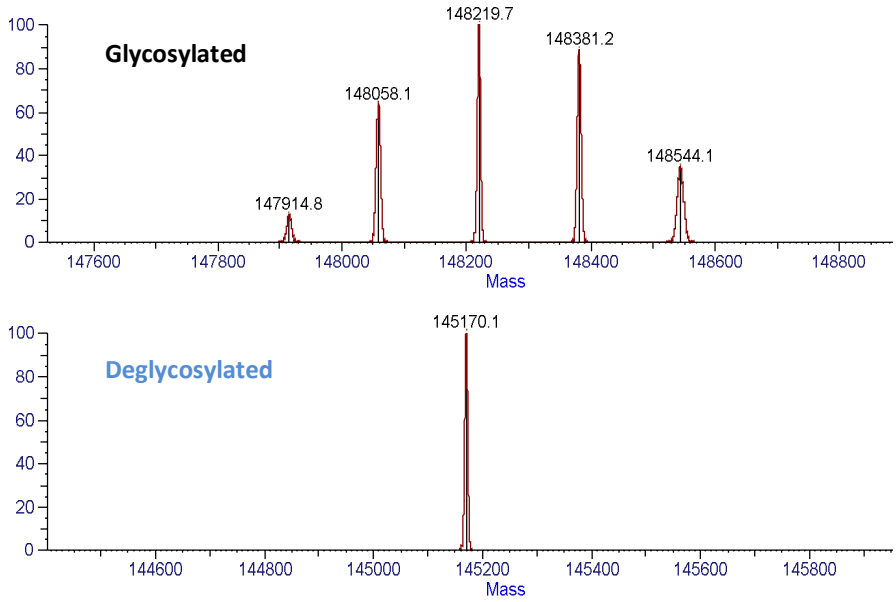
MS Test Conditions:

MS System: Thermo Fisher Orbitrap VelosPro ETD
Scan Time: 6 µscans/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



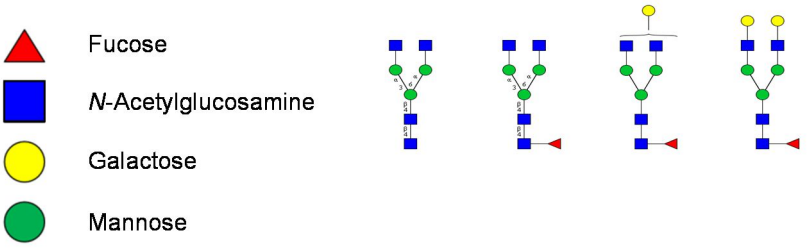
The structure of trastuzumab consists of two heavy chains and two light chains. Glycosylation occurs on the two heavy chains. One or more of the same or different carbohydrate moieties can be present on each heavy chain. Table 1 contains the combinations of sugars that correspond to the masses that were 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
ΔMass (glyc) Trastuzumab	2744	2745	2890	2888	3052	3050	3214	3211	3376	3374	—	3

T = Theoretical mass
M = Measured mass
[1]All masses reported in Daltons

Glycan Descriptions:

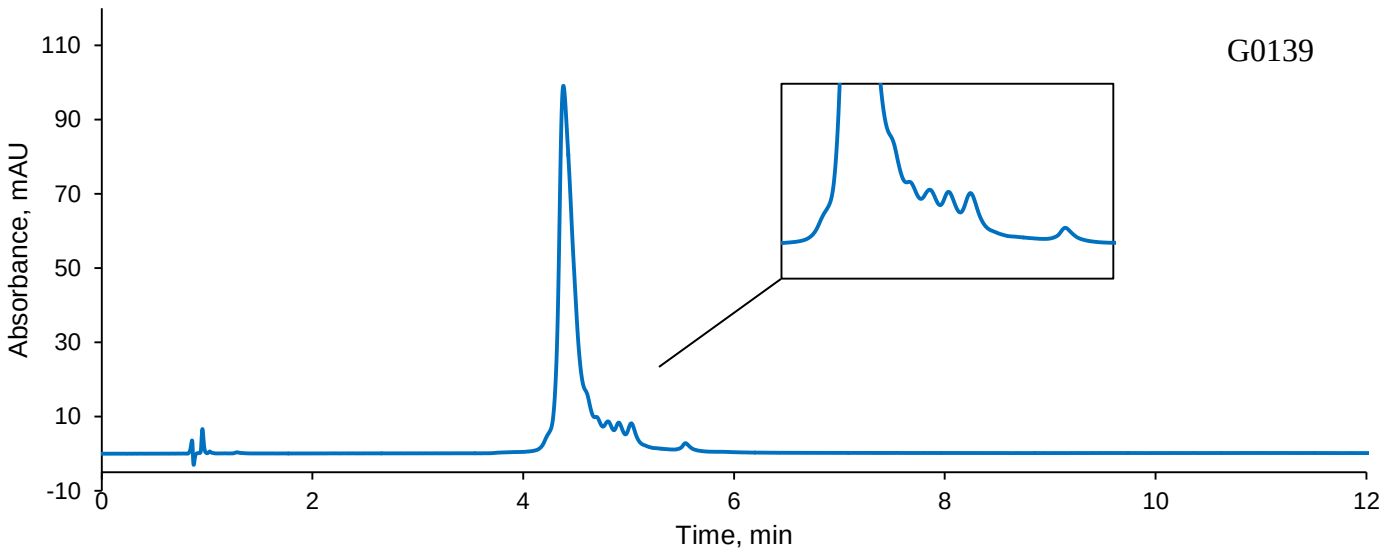


Deconvolution Parameters:

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:

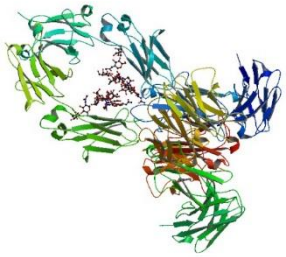


Image from the RCSB PDB (www.rcsb.org) of PDB ID 1HZH (E.O. Saphire, P.W. Parren, R. Pantophlet, M.B. Zwick, G.M. Morris, P.M. Rudd, R.A. Dwek, R.L. Stanfield, D.R. Burton, I.A. Wilson) (2001) Crystal structure of a neutralizing human IGG against HIV-1: a template for vaccine design Science 293: 1155-1159)

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.

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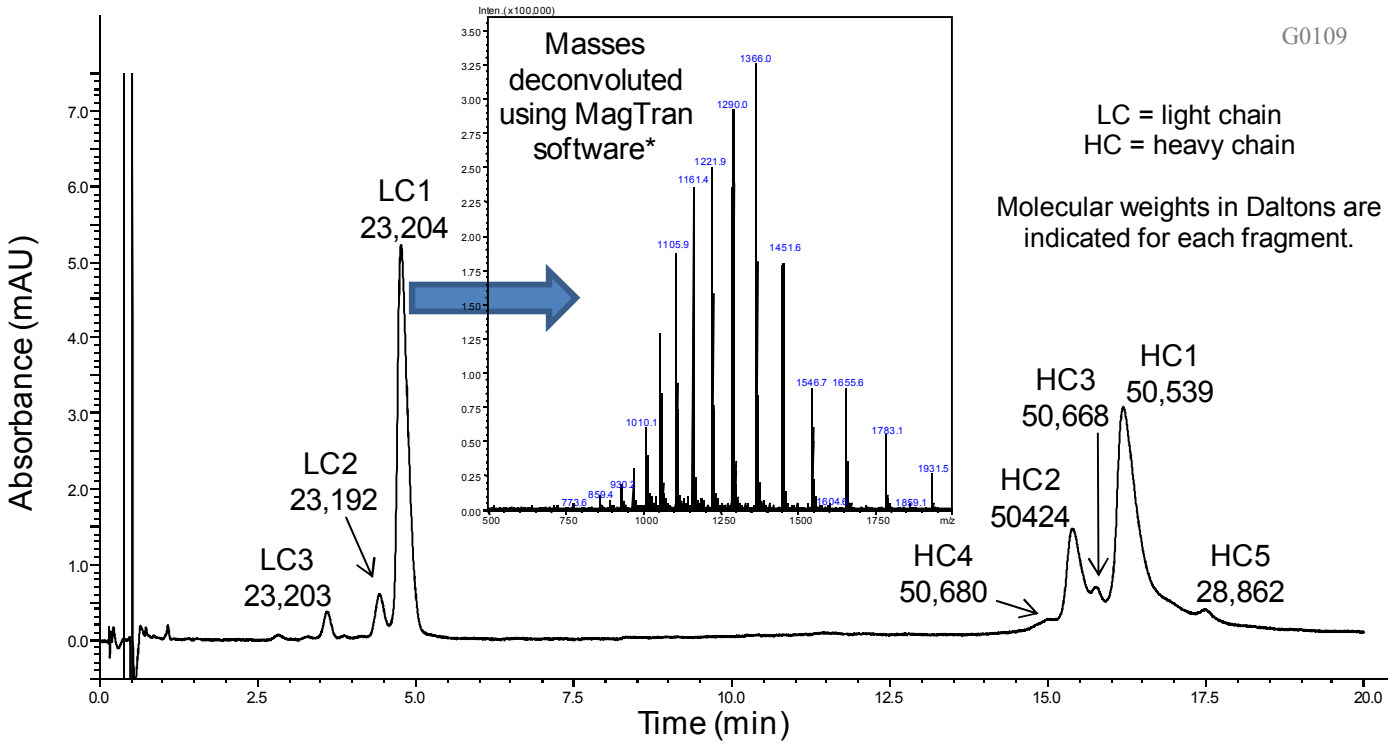
HALO® | Fused-Core® Particle Technology

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.

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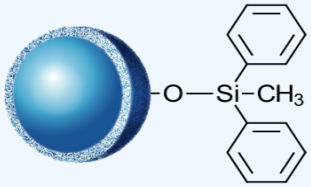
The HALO® BioClass Diphenyl Phase: Discussion and Best Uses

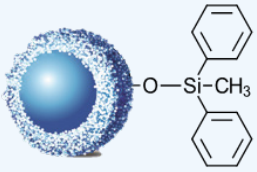
Advanced Materials Technology offers two diphenyl bondings on the well-known superficially porous particle. The new HALO 400 Å Diphenyl, 3.4 µm column and 1000 Å Diphenyl, 2.7 µm columns are primarily used for protein and monoclonal antibody analysis due to its larger pore size. The columns provide narrow peak shapes and better sample recoveries for large biomolecules that range from 2kDa and higher when compared to smaller pore sizes and fully porous particles. The new 3.4 µm superficially porous particle consists of a 3 µm core and a 0.2 µm shell with 400 Å pores while the 2.7 µm superficially porous particle consists of a 1.7 µm core and a 0.5 µm shell with 1000 Å pores. Figure 1 below shows a comparison of the two particles.

Figure 1. HALO® BioClass Diphenyl Options

HALO 400 Å Diphenyl, 3.4 µm Particle

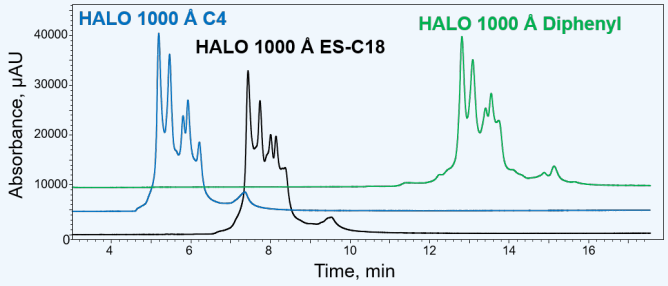


HALO 1000 Å Diphenyl, 2.7 µm Particle



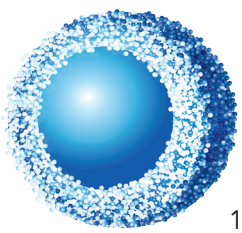
The diphenyl phase offers a unique selectivity to help separate complex samples such as IgG1 and IgG2 monoclonal antibodies. Figure 2 shows a comparison resolving denosumab isoforms (mAb used to help treat bone cancer) on three different 1000 Å bonded phases. The diphenyl phase is retained the longest. While there are minor differences for this IgG2 in this particular comparison, since biopharmaceutical production involves designing custom mAbs with particular characteristics, screening multiple bonded phases could reveal important differences. This is especially true when looking at protein variants.

Figure 2. IgG2 Comparison On HALO 1000 Å C4, ES-C18, And Diphenyl



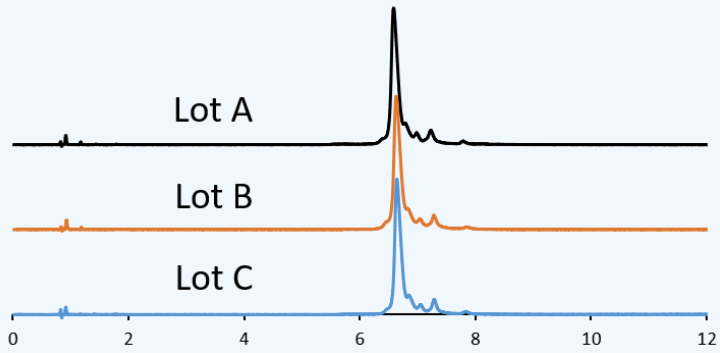
TEST CONDITIONS

Columns: HALO 1000 Å C4, 2.7 µm, 2.1 x 150 mm
HALO 1000 Å ES-C18, 2.7 µm, 2.1 x 150 mm
HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm
Flow Rate: 0.2 mL/min
Temperature: 80°C
Injection Volume: 2 µL of 2 mg/mL denosumab in water/0.1% TFA
Instrument: Shimadzu Nexera
Detection: PDA at 280 nm
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



Both 400 and 1000 Å Diphenyl phases show excellent lot to lot reproducibility in order to maintain reliable and repeatable results for the user. Tight manufacturing processes used by Advanced Materials Technology ensure that the highest quality column performance is achieved. For example, Figure 3 shows trastuzumab (mAb used to treat breast cancer) on three different lots of HALO 1000 Å Diphenyl. Resolution of minor components are repeatable along with the retention time of the IgG1 monoclonal antibody.

Figure 3. Lot-To-Lot Reproducibility Of HALO 1000 Å Diphenyl

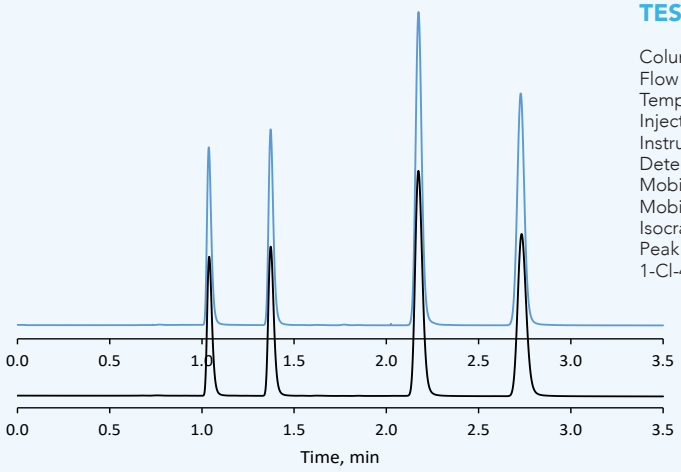


TEST CONDITIONS

Column: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm
Flow Rate: 0.4 mL/min
Temperature: 80 °C
Injection Volume: 2 µL of 2 mg/mL trastuzumab in water/0.1% TFA
Instrument: Shimadzu Nexera
Detection: PDA at 280 nm
Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/ Water/0.085% TFA
Gradient: 40-47.5% B in 12 min

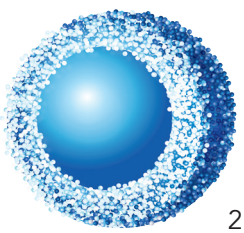
Both 400 Å and 1000 Å Diphenyl columns are also very stable allowing for long column lifetimes. Figure 4 shows the results of a high-pressure stability test using neutral compounds on a HALO 400 Å Diphenyl column. The column has experienced 10,000 column volumes at 600 bar and maintained its peak shape, retention, and held a consistent back pressure.

Figure 4. HALO 400 Å Diphenyl Stability



TEST CONDITIONS

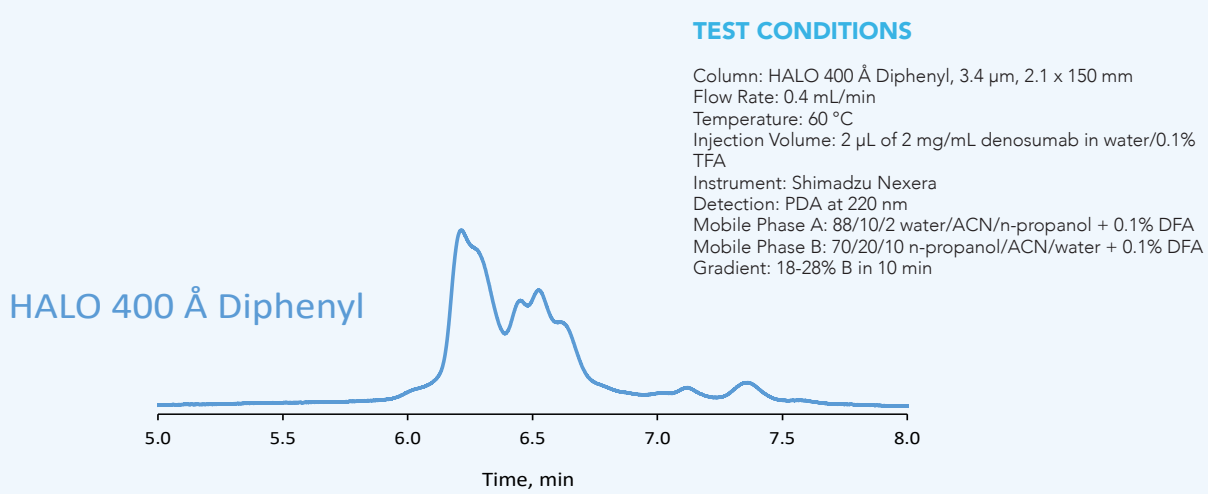
Column: HALO 400 Å Diphenyl, 3.4 µm, 2.1 x 150 mm
Flow Rate: 0.3 mL/min
Temperature: 30 °C
Injection Volume: 0.5 µL
Instrument: Shimadzu Nexera
Detection: PDA at 254 nm
Mobile Phase A: water
Mobile Phase B: ACN
Isocratic: 75/25 A/B
Peak Identities: uracil, phenol, propiophenone, 1-Cl-4-nitrobenzene (in elution order)



HALO 400 Å DIPHENYL: BEST USES

For analysts whose goal is high throughput and/or less emphasis on ultimate resolution yet looking for the critical quality attributes of their biopharmaceutical in the shortest amount of time, the larger particle size and thinner shell of the HALO 400 Å Diphenyl may be used to their advantage while developing release assay methods. The HALO 400 Å Diphenyl with 3.4 µm particle size will have lower overall back pressures when compared to the 1000 Å, 2.7 µm particle or a sub-2 µm fully porous particle. Figure 5 shows a fast analysis of denosumab in under 8 minutes on the HALO 400 Å Diphenyl column. Shorter run times allow reduced mobile phase consumption and higher throughput.

Figure 5. Fast Separation Of Denosumab On HALO 400 Å Diphenyl



If resolution is more important than speed, then the method may be adjusted accordingly. The 400 Å Diphenyl phase shows excellent resolution for monoclonal antibodies when compared to similar columns on the market. For example, denosumab was analyzed again on a HALO 400 Å Diphenyl column compared to a 300 Å fully porous diphenyl column. The HALO 400 Å column outperformed the 300 Å fully porous diphenyl column by providing much better resolution at 2.5x lower back pressure along with a shorter analysis time. See Figure 6.

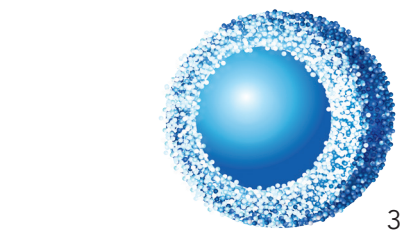
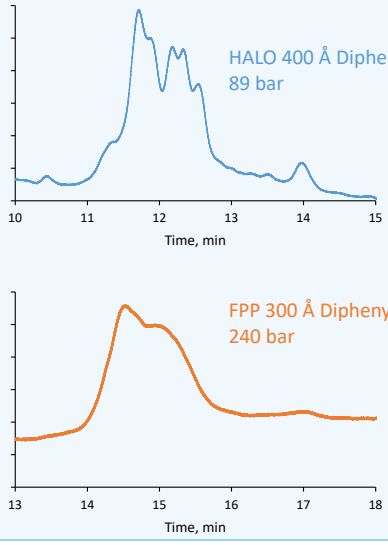


Figure 6. Increased Resolution And Lower Back Pressure with HALO 400 Å Diphenyl Compared To An FPP 300 Å Diphenyl



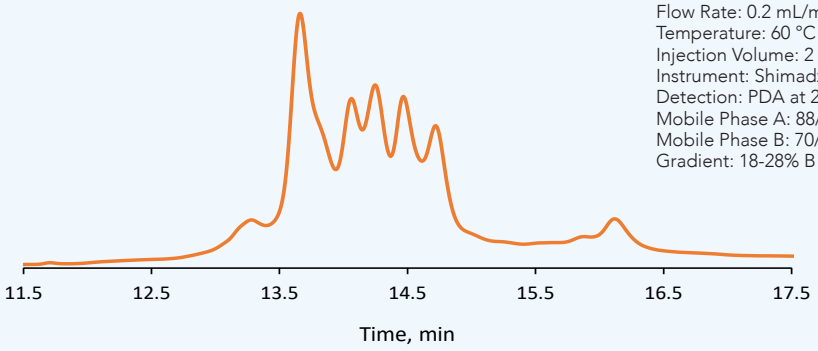
TEST CONDITIONS

Columns: HALO 400 Å Diphenyl, 3.4 µm, 2.1 x 150 mm
FPP 300 Å Diphenyl, 1.8 µm, 2.1 x 150 mm
Mobile Phase A: 88/10/2 water/ACN/n-propanol + 0.1% DFA
Mobile Phase B: 70/20/10 n-propanol/ACN/water + 0.1% DFA
Gradient: 18-28% B in 20 min
Flow Rate: 0.2 mL/min
Temperature: 60 °C
Injection Volume: 2 µL of 2 mg/mL denosumab in water/0.1% TFA
Instrument: Shimadzu Nexera
Detection: PDA at 220 nm

HALO 1000 Å DIPHENYL: BEST USES

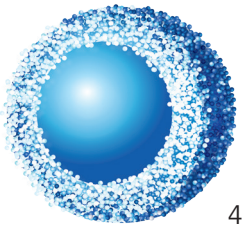
For the ultimate resolution of monoclonal antibodies or other large proteins, HALO 1000 Å Diphenyl phase is recommended. The large pores allow unrestricted access of mAbs to the bonded phase, while the higher surface area/thicker shell enables high resolution separations of various mAb isoforms. Figure 7 shows a high resolution separation of denosumab.

Figure 7. High Resolution Separation Of Denosumab On HALO 1000 Å Diphenyl Column



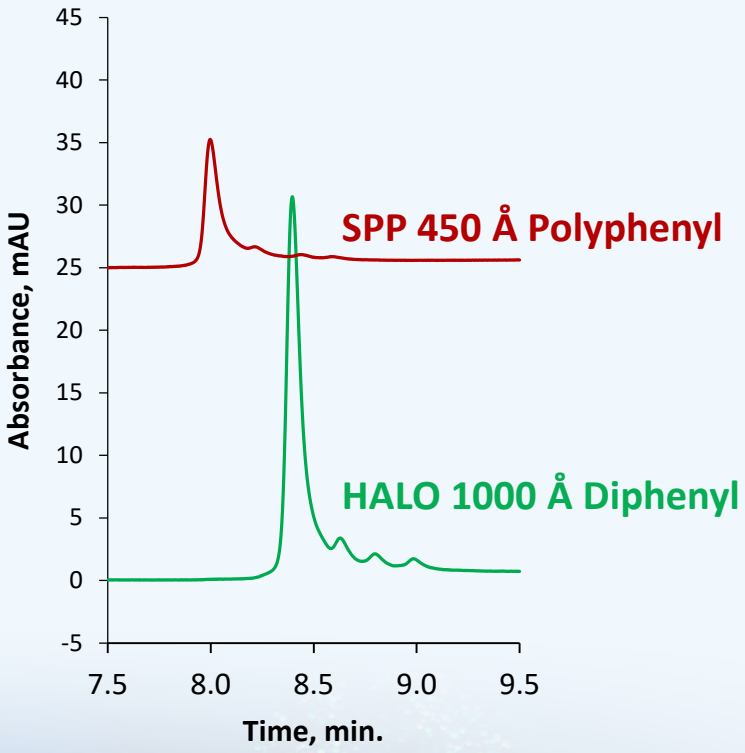
TEST CONDITIONS

Column: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm
Flow Rate: 0.2 mL/min
Temperature: 60 °C
Injection Volume: 2 µL of 2 mg/mL denosumab in water/0.1% TFA
Instrument: Shimadzu Nexera
Detection: PDA at 220 nm
Mobile Phase A: 88/10/2 water/ACN/n-propanol + 0.1% DFA
Mobile Phase B: 70/20/10 n-propanol/ACN/water + 0.1% DFA
Gradient: 18-28% B in 20 min



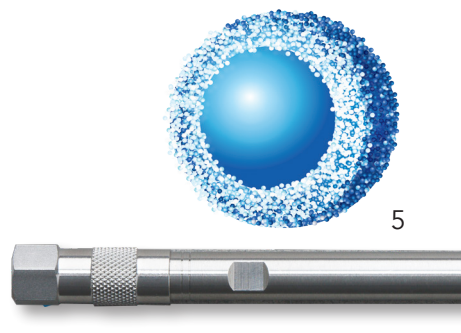
Both HALO 400 Å and 1000 Å Diphenyl columns are stable up to 90 °C for high temperature separations. It is generally accepted that sample recovery of proteins and mAbs improves as temperature increases, therefore temperatures of 60 °C or greater are often used to maximize sample recovery. However, temperature related artifacts have been observed when operating at these higher temperatures. Because of this, a temperature gradient is recommended and operating at lower temperature may prove desirable. A comparison of trastuzumab (Figure 8) at 40 °C on both a HALO 1000 Å Diphenyl column and a 450 Å SPP Polyphenyl column shows impressive protein recovery with the HALO® column. The HALO® Diphenyl column also demonstrates improved resolution, retention, and peak area compared to the competitor SPP column. This increased retention and resolution clearly demonstrate the benefit of unrestricted large pore access to the bonded phase and that high recoveries are possible with lower operating temperatures.

Figure 8. Comparison of HALO 1000 Å Diphenyl to Competitor Polyphenyl at 40 °C



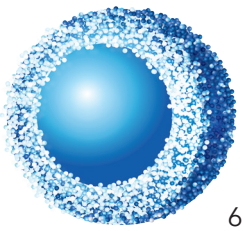
TEST CONDITIONS

Columns: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm
SPP 450 Å PolyPhenyl, 2.7 µm, 2.1 x 150 mm
Mobile Phase A: water/0.1% TFA
Mobile Phase B: ACN/0.1% TFA
Gradient: 30-45% B in 15 min
Flow Rate: 0.4 mL/min
Temperature: 40 °C
Injection Volume: 2 µL of 2 mg/mL trastuzumab in water/0.1% TFA
Instrument: Shimadzu Nexera
Detection: PDA at 280 nm



CONCLUSIONS

For biopharmaceutical separation scientists, the HALO 400 Å and 1000 Å Diphenyl columns are two beneficial additions to the protein chemist's separation toolbox for mAb release assays and characterization methods. The HALO® Diphenyl offers a unique selectivity compared to C4 and C18 and demonstrates excellent stability with good sample recovery while outperforming the competitors to deliver quality and performance – every time.



Analyses of Large Proteins, Antibodies, and Modified Proteins Using A New Series of Advanced Large Pore HPLC Materials

Introduction

The list of FDA-registered and commercialized therapeutic proteins continues to expand rapidly, including more than 400 biotherapeutics, such as enzymes, monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), and hybrid antigen binding structures. Various human and veterinary diseases, such as cancers, autoimmune diseases, neurodegenerative diseases, and many others can be treated using these new molecules, employing a more effective targeted approach [1].

Protein biopharmaceuticals have higher molecular weights (MWs), are large in aqueous solution, with shapes that can be very complex having tertiary and quaternary structural elements. The polypeptide chains fold in specific ways, with highly hydrated charged amino acid side chains oriented towards the surface containing domain structures that are stabilized by specific and cumulative ion pairs and hydrophobic interactions. Many of these proteins are composed of more than one polypeptide chain, associated by both covalent and non-covalent bonds. In many cases, the proteins are based on molecules from the human or animal immune systems, and often their MWs usually exceed 100 kilodaltons (kDa). For example, the MWs of antibodies, including therapeutic mAbs, range between 140 and 150 kDa (Figure 1). The volumes of these molecules in solution are very large, compared to small molecule pharmaceuticals, peptides, or even small to medium MW polypeptides and proteins.

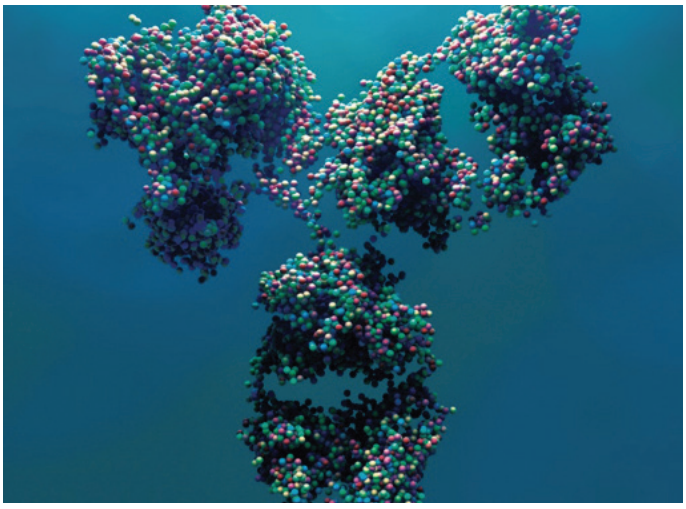


Figure 1. Representative Therapeutic Monoclonal Antibody Structure

In addition to their high MW and hydrated volumes, large biotherapeutic proteins have additional structural complexities due to inherent or induced heterogeneity. For example, many biotherapeutics are glycoproteins, often with a mixture of neutral and acidic glycan structures at one or more specific sites of the protein structures. As mentioned above, proteins may be composed of multiple protein chains, and some are covalently linked by specific interchain or intrachain disulfide linkages, or may have a mixture of fully bridged disulfide and free sulfhydryl groups. Moreover, the polypeptide chains themselves may have heterogeneous lengths, with terminus variations of one or two amino acids (ragged ends). In many therapeutic proteins these various sources of heterogeneity may coexist to a varying degree, as the biological and production process variables that lead to these heterogeneities are not all under complete control during the preparation of a useful therapeutic biopharmaceutical.

Characterization of Biopharmaceutical Proteins

The complexity of these biopharmaceuticals, and the regulatory requirements for thorough characterization of novel entities, or of biosimilars, to ensure identity, purity and quality, are substantial compared to those of small molecule drugs. Among the important analytical techniques that are applied for characterization of biotherapeutics such as mAbs, are various modes of liquid chromatography (LC) for separations of intact mAbs, their fragments, their enzymatic digests to yield smaller peptide fragments, and their associated glycans. A key analytical approach for protein characterization remains reversed-phase HPLC (RPLC), due to the unique and informative capabilities of this method.

Reversed-Phase HPLC as a Preferred Method for Protein Analysis

For several decades, RPLC has been the dominant method for pharmaceutical and small molecule analyses, and it is employed at every stage of drug discovery and development, manufacturing and quality assurance processes. Since the early 1990s, a central role for RPLC for the analysis of proteins has emerged—initially with protein fragments (chemical and enzymatic digests), protein subunits, and, more recently, for intact protein analyses.

This growing importance and application of RPLC for protein analyses coincides with the development of increasingly useful and appropriately designed chromatographic materials. Methods for their use include the analysis of protein enzymatic digests, intact polypeptides and intact proteins, and the various derivatives of all of the above.

The increased role of RPLC has also been accelerated by the development of improved LC instrumentation (hardware and software), and the advancements in high speed detectors, most notably, high speed and high resolution mass spectrometers (MS). Most advanced MS platforms are capable of being efficiently interfaced with HPLC and UHPLC instruments via electrospray ionization (ESI). Unlike some HPLC modes, RPLC is easily accommodated by MS detection, and RPLC-MS can be used at scaled flow rates ranging from nanoliter/min to mL/min accommodating nano (< 300 µm ID), capillary (300 µm –1 mm ID) and analytical (1 – 4.6 mm ID) columns.

The relative simplicity of RPLC and the widespread availability of automated, computer-controlled instrumentation and qualified software have made the technique indispensable for protein identification and quantitation. It was shown in the 1980s that RPLC of even moderate size proteins (ca. 15 – 20 kDa), because of their molecular size, required larger-pore-size column packing materials [2], and careful selection of mobile phases and analysis conditions that maintain protein solubility and enhance recovery. Most analytical RPLC protein separations use acidic mobile phase additives (e.g., formic or trifluoroacetic acid), elevated column temperatures (40 – 90 °C), and an organic modifier-water gradient using acetonitrile or acetonitrile mixed with a short chain aliphatic alcohol (propanol, butanol or isopropanol) [3]. An example RPLC separation of a mixture of intact proteins, separated using a typical acetonitrile/ water gradient, with UV absorbance detection at 215 nm, is shown in Figure 2.

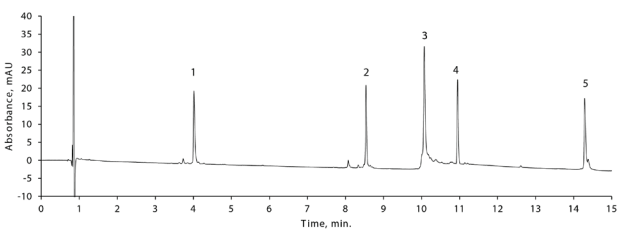


Figure 2. Gradient RPLC Separation of a Mixture of Intact Proteins

TESTING CONDITIONS:

Column: HALO 1000 Å ES-C18, 2.7 µm, 2.1 x 150 mm
Mobile phase A: Water (0.1% TFA)
Mobile phase B: 80/20 ACN/Water/0.085% TFA
Gradient: 27–60 %B in 15 min
Flow rate: 0.4 mL/min
Temperature: 60 °C
Injection volume: 2 µL
Instrument: Shimadzu Nexera XR
Detection: UV at 280 nm
Sample: (1) ribonuclease A; (2) lysozyme; (3) SigmaMAb; (4) α-lactalbumin; (5) enolase

Until very recently, researchers have used column technology for RPLC of intact mAbs, ADCs and their resulting fragments which has been fundamentally unchanged since the mid-1990s. Typically, such packing materials have consisted of 200 – 300 Å pore size, silica-based, fully porous particle (FPP) bonded phases. However, new, very wide, 1000 Å pore size superficially porous packings for RPLC of very large molecules have been developed to improve the resolution for, and the information gained from, higher resolution chromatographic separations of protein mixtures.

HALO® 1000 Å Protein Column Series

The HALO 1000 Å Protein columns are innovative products that are built upon the success and performance of the family of small particle diameter

COLUMN NAME	PORE SIZE (Å)	PARTICLE SIZE(S) (um)	SURFACE AREA (m^2/g)	STATIONARY PHASES	TARGET ANALYTES
HALO Glycan	90	2.7	135	Proprietary	Glycans, glycopeptides, glycoproteins < 20 kDa
HALO Peptide	160	2,2.7,5	65, 90, 60	ES-C18, ES-CN, Phenyl-Hexyl	Peptides and polypeptides 100 Da < MW < 15 kDa
HALO Protein	400	3.4	15	C4, ES-C18	Peptides, polypeptides, and proteins 2 kDa < MW < 500 kDa
HALO Protein	1000	2.7	22	C4, ES-C18, Diphenyl	Large proteins, mAbs, mAb fragments, and ADCs > 50 kDa

Table 1. HALO Fused-Core Column Family: Pore Size Designated for Target Analytes

Fused-Core® superficially porous particle (SPP) HALO columns (Table 1), a particle type pioneered by Advanced Materials Technology. The objective in the design of the various HALO columns has always been to develop particles whose pore size had been chosen carefully to balance retention and pore access for the targeted analyte sizes.

The performance improvement afforded by HALO 1000 Å, 2.7 µm particles (Figure 3) for RPLC analyses of large biotherapeutics, compared to commercially available, smaller pore size columns, is attributable to these main advantages:

- Larger pore size allows completely unrestricted access of biomolecules to the interior domain of particles, and movement within the volume element adjacent to the particle surface and associated bonded phase.

- Superficially porous layer provides a shorter diffusion path (even vs. fully porous sub-2-µm particles) for larger biomolecules having much lower diffusion coefficients, while maintaining sufficient surface area for necessary loading capacity, resulting in reduced peak broadening and high resolution of minor components (impurities and structural variants).

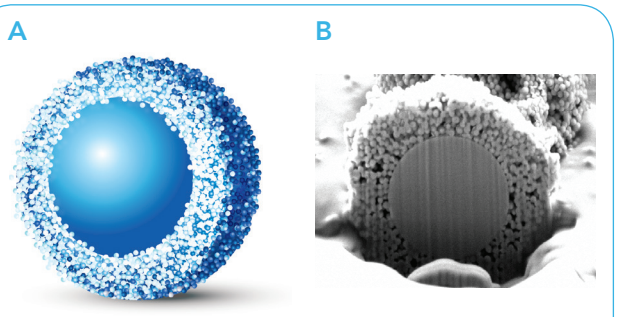


Figure 3. (A) Schematic drawing of a 1000 Å superficially porous 2.7 µm particle; (B) Focused-ion-beam-sliced SEM image of a 1000 Å SPP showing the 1.7 µm core with 0.5µm shell

Unrestricted Pore Access

Unrestricted pore access by large biomolecules to the large 1000 Å pores produces narrow peaks, which allows high resolution separations of protein and mAb variants, in addition to improved sample load tolerance before band broadening. For existing columns with smaller pore sizes, molecular exclusion and restricted diffusion of large molecules occurs and produces broader peaks, poorer loading behavior, and less resolution. While the minimum pore size required to fully accommodate very large biomolecules is determined by many factors, consideration of the effects of diffusion into an open cylindrical channel suggests [4, 5] that particle pore size should be on the order of 10 times the effective hydrodynamic diameter of an analyte for optimal chromatographic performance.

The HALO 1000 Å pore size distribution permits free access for mAbs and larger proteins to the stationary phase available within the porous shell structure.

Short Diffusion Path

Another contributing factor to the superior performance of the HALO 1000 Å particle is its porous shell morphology, which it shares with the other Fused-Core® product series (see Table 1 and Brief History Section). The unique particle design of SPPs includes a solid silica core surrounded by a porous shell of sub-micron thickness. This thin shell does not require that the slowly-diffusing biomolecules traverse the entire radius of the particle as it does for FPPs. This reduced diffusion path confers improved mass transfer and sharper peaks, and permits faster separations. For a review on SPPs, see Hayes, et al. [6], and for descriptions of superficially porous particles and their advantages for larger protein separations, see Kirkland, et al. [7].

Sample Loading

The very wide pores and short diffusion distances not only provide narrower peaks and improved resolution for large biotherapeutic molecules, but they also enable greater sample load capacity and tolerance. What this means is that a larger amount (mass) of sample can be injected, with less peak broadening, so that minor impurities such as subtle variants of mAbs and ADCs can be detected and quantified.

The ability of the HALO 1000 Å Protein SPP columns to tolerate increasing load for protein separations have been investigated using various preparations. The effects of sample load for a highly purified IgG1 mAb peak width are shown in Figure 4 both for a HALO 1000 Å C4 column and a 300 Å FPP C4 column (2.1 x 150 mm sizes) using trastuzumab as a model. These results show that, for all load levels, the HALO 1000 Å C4 column afforded smaller peak widths than those for the 300 Å FPP C4 column.

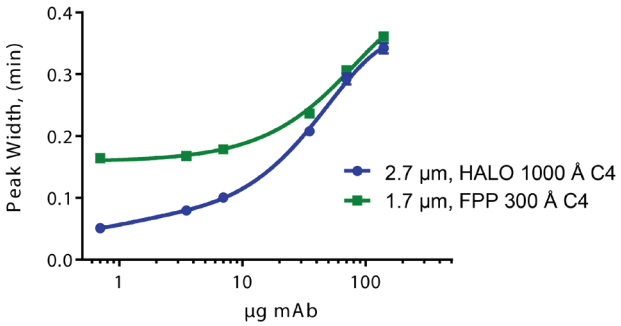


Figure 4. Effect of Sample Mass on Peak Width

TESTING CONDITIONS:

Column: 2.1 x 150 mm
Mobile phase A: Water (0.1% DFA)
Mobile phase B: ACN (0.1% DFA)
Gradient: 27–37% B in 10 min
Flow rate: 0.5 mL/min
Temperature: 80 °C
Sample: trastuzumab
Injection volume: 0.1, 0.5, 1, 5, 10, and 20 µL of 7 mg/mL mAb in Water
Instrument: Shimadzu Nexera
Detection: UV at 280 nm with 350 nm reference wavelength

Note: Peak widths measured at 50% height

These results are counterintuitive, when one considers the actual surface areas of these two silica column packing materials. The surface area (nitrogen adsorption, surface BET analysis) of the 300 Å FPPs is about 90 m2/g compared to about 20 m2/g for the 1000 Å SPPs. The surface area of the latter is about 4.5-fold smaller, yet the sample loading capacity (inversely correlated with peak width) is much better for the 1000 Å SPPs at lower sample loads, and comparable to the FPPs at high loads. These findings suggest that in the example of trastuzumab, it has much greater access to the bonded phase surface of the 1000 Å SPPs, compared to the 300 Å FPPs, which have higher absolute surface area.

The HALO® 1000 Å Protein Column Bonded Phase Series

The traditional bonded phase for RPLC separations of proteins has been the short chain alkyl bonded phase, typically based on a form of butyl-silane (C4). This material works well, and has repeatedly shown high performance separations of proteins using default gradient RP conditions. To enhance the utility of 1000 Å wide pore bonded phases for protein development, Advanced Materials Technology has recently introduced specially-selected versions of the octyldecyl-silane (ES-C18) and diphenyl-silane (Diphenyl) surface modified bonded phases (surface structures shown in Figure 5).

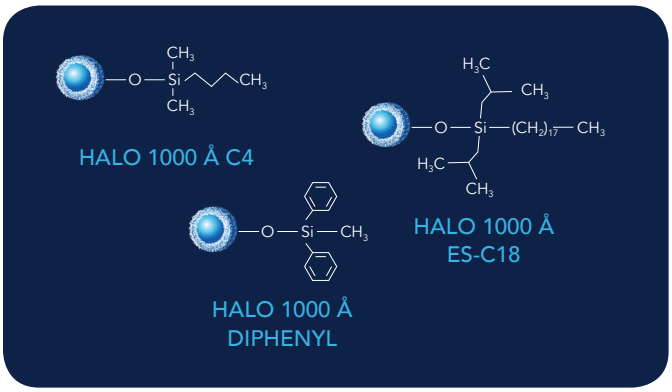


Figure 5. HALO 1000 Å Bonded Phase Structures

The three bonded phase RPLC materials selected for the HALO 1000 Å Protein series was derived from the comparison of many materials, with specific reference to stability for applications of low pH and elevated temperature that are often required to obtain high recovery of proteins in RPLC separations. All three materials exhibit acceptable column lifetimes when operated under such conditions, and an example of the HALO 1000 Å Diphenyl stability, when challenged with highly aggressive low pH (<2), elevated temperature conditions (90°C) is shown in Figure 6.

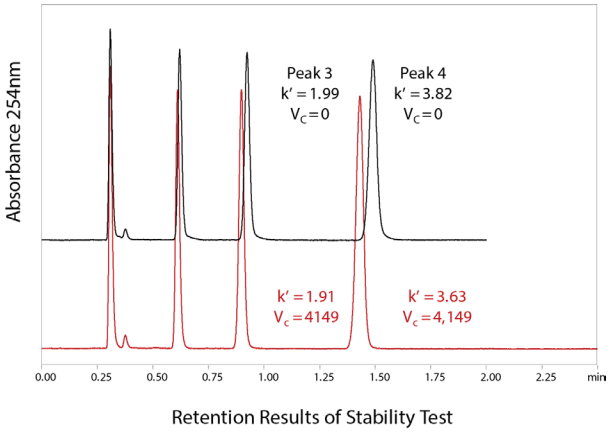


Figure 6. High Temperature and Low pH Stability

TESTING CONDITIONS:

Column: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 50 mm
Mobile phase A: Water
Mobile phase B: ACN
Isocratic: 55/45 A/B
Flow rate: 0.4 mL/min
Temperature: 25 °C
Injection volume: 0.2 µL
Instrument: Shimadzu Nexera
Detection: UV at 254 nm
Sample: (1) uracil; (2) hexanophenone; (3) octanophenone; (4) decanophenone

Black trace shows the initial results and red trace shows the column performance after 4000 column volumes.

Examples of HALO® 1000 Å Protein Column RPLC Performance

HALO® 1000 Å SPP C4 vs. 300 Å FPP C4

RPLC separations of intact denosumab (IgG2 type) are compared for HALO 1000 Å SPP and 300 Å FPP columns in Figure 7. For the HALO 1000 Å C4 column separation, the denosumab peak widths are not only narrower compared to that for the 300 Å C4 column, but there is also much better resolution of the IgG2 disulfide bridge isoform variants. The narrower peak widths using HALO 1000 Å have also been observed and previously described using IgG1 type mAbs [8].

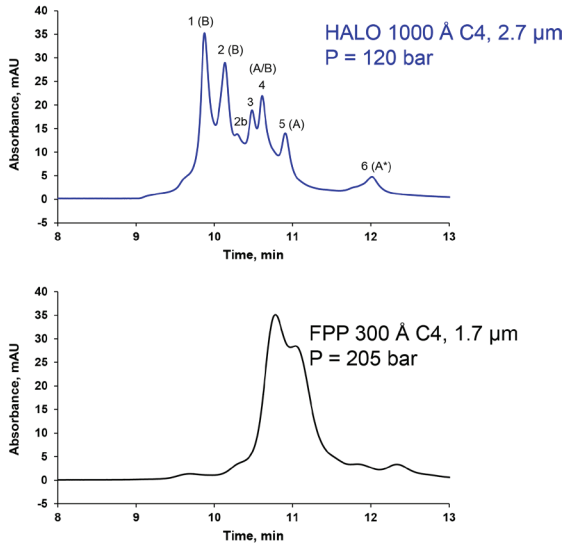


Figure 7. Intact Denosumab Separation Using 1000 Å SPPs and 300 Å FPPs

TESTING CONDITIONS:

Columns: 2.1 x 150 mm
Flow rate: 0.2 mL/min
Mobile Phase A: 88/10/2 H2O/ACN/n-Propanol + 0.1% DFA
Mobile Phase B: 70/20/10 n-Propanol/ACN/H2O + 0.1% DFA
Gradient: 14–24% B in 20 min
Injection Volume: 2 µL of 2 mg/mL denosumab in Water + 0.1% DFA
Temperature: 80 °C
Detection: PDA at 280 nm

These observations, along with the sample loading results shown in Figure 4 demonstrate that large molecules have much greater pore access for bonded phase interactions using the HALO 1000 Å SPP column, compared to the 300 Å FPP column.

RPLC analyses of four additional mAbs were carried out using both columns. The peak widths of these four mAbs (in addition to trastuzumab) were, on average, 69% narrower using the HALO 1000 Å C4 column compared to those obtained using the 300 Å FPP C4 column (Figure 8).

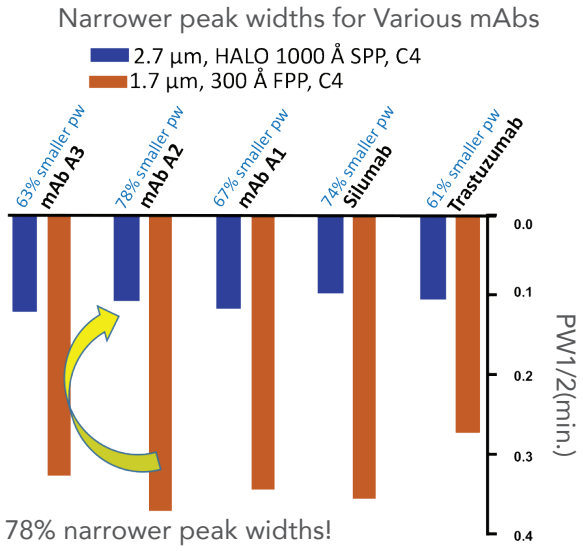


Figure 8. Narrower Peak Widths for Various mAbs. This bar graph compares the average peak widths for five different mAbs using a HALO 1000 Å C4 column and a 300 Å FPP C4 column.

TESTING CONDITIONS:

Columns: 2.1 x 150 mm
Mobile phase A: Water (0.1% DFA)
Mobile phase B: ACN (0.1% DFA)
Gradient: 27–37% B in 20 min
Flow rate: 0.4 mL/min
Temperature: 80 °C
Injection volume: 2 µL (1 µg each in 0.1% TFA)
Instrument: Shimadzu Nexera
Detection: UV at 280 nm with 350 nm reference wavelength
Samples: As Indicated

Note: Peak widths measured at 50% height

HALO® 1000 Å C4 SPP vs. 1500 Å Polymeric RP FPP

RPLC separations of intact trastuzumab were also compared using the HALO 1000 Å, 2.7 µm C4 column and a 1500 Å, 4 µm, polymeric FPP column (Figure 9). The trastuzumab peak was about 24% narrower using the 1000 Å C4 column, compared to the larger pore size polymeric column. The trastuzumab peak may be sharper due to the combination of smaller particle size and the thin shell of the 1000 Å SPP column (2.7 µm with 0.5 µm shell) compared to the 4 µm particle size of the 1500 Å fully porous polymeric particles. Resolution of the minor trastuzumab variants was much better using the 1000 Å C4 SPP column, which is expected, as generally the case with silica particles, to exhibit high pressure and mechanical shock resilience, in comparison to polymeric particles.

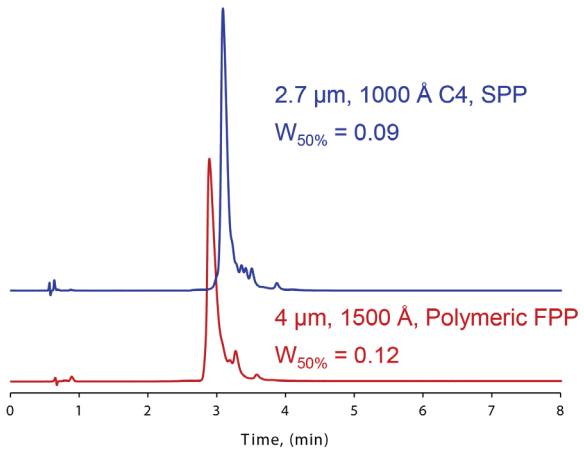


Figure 9. Intact Trastuzumab RPLC Separations Using 1000 Å C4 SPP and 1500 Å FPP Columns

TESTING CONDITIONS:

Columns: 1000 Å SPP and 1500 Å FPP columns 2.1 x 100mm
Mobile phase A: Water (0.1% TFA)
Mobile phase B: 80/20 ACN/Water (0.085% TFA)
Gradient: 40–47.5% B in 8 min
Flow rate: 0.4 mL/min
Temperature: 80 °C
Sample: trastuzumab
Injection volume: 2 µL of 2 mg/mL in Water
Instrument: Shimadzu Nexera
Detection: UV at 280 nm with 350 nm reference wavelength

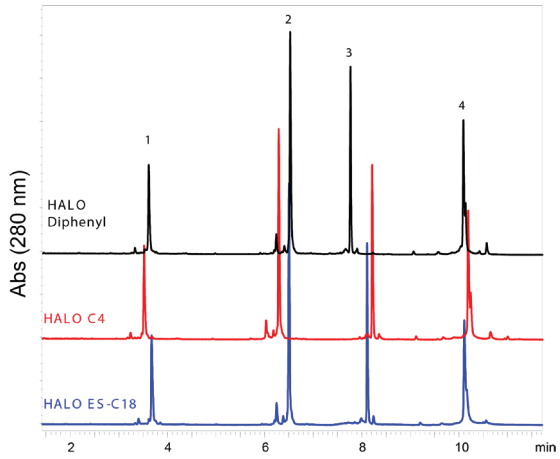


Figure 10. Comparison of Three HALO 1000 Å Bonded Phases Using a Protein Mix

TESTING CONDITIONS:

Columns: HALO 1000 Å, phase as indicated, 2.1 x 150 mm, 2.7 µm
Mobile phase A: Water/0.1% TFA
Mobile phase B: ACN/0.1% TFA
Gradient: 20–60 %B in 15 min
Flow rate: 0.4 mL/min
Temperature: 80 °C
Injection volume: 2 µL
Instrument: Shimadzu Nexera
Detection: UV at 280 nm
Sample: (1) Ribonuclease A; (2) lysozyme; (3) α-lactalbumin; (4) enolase

Use of HALO® 1000 Å Protein Bonded Phase Options for Protein Separations

The HALO 1000 Å Protein series is the only commercially available RPLC material that is a very wide pore SPP particle, and has available three highly stable and reproducible bonded-phases specific for resolving protein mixtures. The separation of four standard proteins is shown in Figure 10, using typical gradient RPLC conditions. Although the Diphenyl bonded phase is a weak RPLC retention material for small molecules, these results show that this bonded phase is comparable in retention to C4 and C18 for protein mixtures (for example comparing retention of peaks 1 and 4 for each column). In addition, this figure also shows that the three bonded phases exhibit subtle selectivity differences, appreciated when examining the relative elution positions for peaks 2 and 3, across the different bonded phase surfaces. Each bonded phase exhibits a unique pattern of separation for this mixture. Such selectivity differences, although appearing modest, become highly relevant when employing gradient elution conditions that are less aggressive, for example as is employed for protein variant analysis for a highly purified protein.

In Figure 11, a high resolution separation of the biotherapeutic mAb trastuzumab is shown. In this example, the materials of interest are the small peaks that elute after the main, correctly formed and fully disulfide bridged intact mAb structure. Following this main peak, a collection of smaller peaks is variably resolved by each of the specific bonded phases. The selectivity differences between these columns show up as a complex collection peaks for the many small protein variant structures. Subsequent studies have revealed that these later eluting peaks are subtle structural variants of trastuzumab, predominantly variants with a single reduced disulfide bridge, leading to pairs of free thiol variants. Similar disulfide bridge variants have been shown in previous studies of other IgG1 and IgG2 mAbs [10,11].

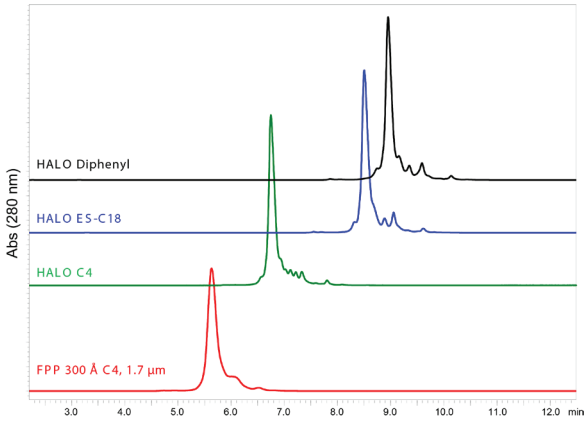


Figure 11. Comparison of Three HALO 1000 Å 2.7 µm Bonded Phases to a 300 Å 1.7 µm FPP C4 Column Using Trastuzumab

TESTING CONDITIONS:

Columns: as indicated, 2.1 x 150 mm
Mobile phase A: Water/0.1% TFA
Mobile phase B: ACN/0.1% TFA
Gradient: 32–40 %B in 16 min
Flow rate: 0.4 mL/min
Temperature: 80 °C
Injection volume: 2 µL
Instrument: Shimadzu Nexera
Detection: UV at 280 nm

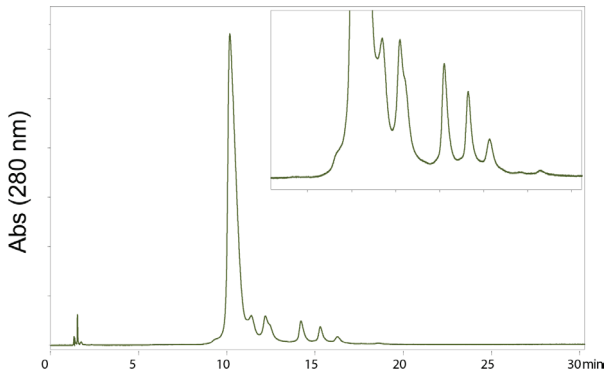


Figure 12. Example of Optimized Trastuzumab Method Development Using a HALO 1000 Å Diphenyl Column

TESTING CONDITIONS:

Mobile phase A: Water (0.1% DFA)
Mobile phase B: 50/50 ACN/n-propanol/0.1% DFA
Gradient: 29–33 %B in 29 min
Flow rate: 0.25 mL/min
Temperature: 60 °C
Injection volume: 2 µL of 2mg/mL trastuzumab in water/0.1% TFA
Instrument: Shimadzu Nexera
Detection: UV at 280 nm

The subtle selectivity difference in the HALO 1000 Å Protein bonded phases can be employed with gradient selectivity optimization and mobile phase modification as independent variables. As an example, Figure 12 shows the high resolution results that can be obtained with bonded phase, temperature and mobile phase optimization to yield a highly informative separation of the main peak and at least 7 variant protein structures present in this biotherapeutic mAb. The separation using this highly efficient HALO 1000 Å Protein Diphenyl column is completed in less than 30 minutes, while being compatible with both UV detection, as well as online high resolution MS detection.

List of Abbreviations:

ADC: antibody-drug conjugate **BET:** Brunauer, Emmett and Teller (method for calculating surface area) **DFA:** difluoroacetic acid **FPP:** fully porous particle **IgG:** immunoglobulin **kDa:** kilodaltons **mAb:** monoclonal antibody **SEM:** scanning electron microscope **SPP:** superficially porous particle **TFA:** trifluoroacetic acid

Conclusions

As pharmaceutical companies have shifted their development focus to large-molecule biotherapeutics, the ability to separate intact monoclonal antibodies and antibody-drug conjugates for characterization purposes has become extremely important. To enable this characterization work, new HPLC particle technology has been required. The large 1000 Å pore size of the superficially porous particles used for RPLC described herein enables full access to the bonded phase surface for these larger biomolecules. This improved access to the bonded surface produces narrower peak widths and enhanced resolution of minor mAb variants, and can lead to increased retention under most analysis conditions. Together with new mass spectrometric instrumentation and software, wide-pore superficially porous particle HPLC columns will greatly aid in the advancement of large-molecule biopharmaceutical characterization and development. The recent expansion of the very large pore superficially porous particle family to include several bonded phases (C4, C18, Diphenyl) permits very high resolution separations of lower abundance protein variants, permitting structure analysis and quantification of these variants.

Authors

Dr. Barry Boyes and Dr. Stephanie Schuster of Advanced Materials Technology, Inc. For more information, please contact info@advanced-materials-technology.com

A Brief History of Superficially Porous Particles for HPLC and UHPLC

- Superficially porous particles (SPPs) were originally developed during the 1960s, but enjoyed a new renaissance in the mid-2000s with the commercialization of HALO® Fused-Core® technology by Advanced Materials Technology. This revolutionary HALO 90 Å, 2.7 µm SPP column, first introduced in 2006, rivals the speed, resolution and performance of sub-2-µm columns, which had been introduced in 2003-2004, for small molecule analyses. This new SPP technology has rapidly changed the HPLC materials landscape, and has become very popular in the last ten years.

- These particles consist of a non-porous silica core, surrounded by a porous shell (Figure 3A). The interest in and popularity of these particles is due to the very high column efficiencies at modest back pressures [9]. This superficially porous particle morphology has been so successful that it has been adopted and commercialized by a number of other column manufacturers.

- It is well known that selection of the correct pore size is important to allow unhindered analyte access to the silica surface. Pore access is not a consideration for all but the largest pharmaceuticals (e.g., macrocyclic antibiotics such as tylosin, etc.). However, for peptides, proteins and larger biopharmaceuticals, larger pore size is a critical factor in achieving high efficiencies and narrow peak widths.

- Building upon the success of the 90 Å HALO particles, 160 Å pore size HALO Peptide particles, designed for fast and high resolution separations of peptides and small polypeptides, were commercialized in 2011 [12]. HALO Peptide particles are bonded with sterically-protected silanes to promote excellent stability at the low pH, high temperature conditions often used for peptide mapping and other peptide analyses.

- Subsequently, in 2013 a new 400 Å, 3.4 µm HALO particle was introduced with C4 and sterically-protected C18 bonded phases to provide high efficiency gradient separations of larger polypeptides and proteins.

- The HALO 1000 Å, 2.7 µm particle is the newest addition to the HALO BioClass series of columns, and was designed to deliver superior performance for monoclonal antibodies, their fragments, and antibody-drug conjugates.

- Advanced Materials Technology is the innovator in this area of superficially porous packing materials, being first to offer very wide pore SPP materials, and is the only company that offers the choice of 400 Å and 1000 Å pore particle materials.

- The HALO 1000 Å Protein series has been extended to include the C4, ES-C18 and Diphenyl bonded phases, allowing manipulation of separation selectivity, while assuring highly stable and reproducible columns for high resolution separations.

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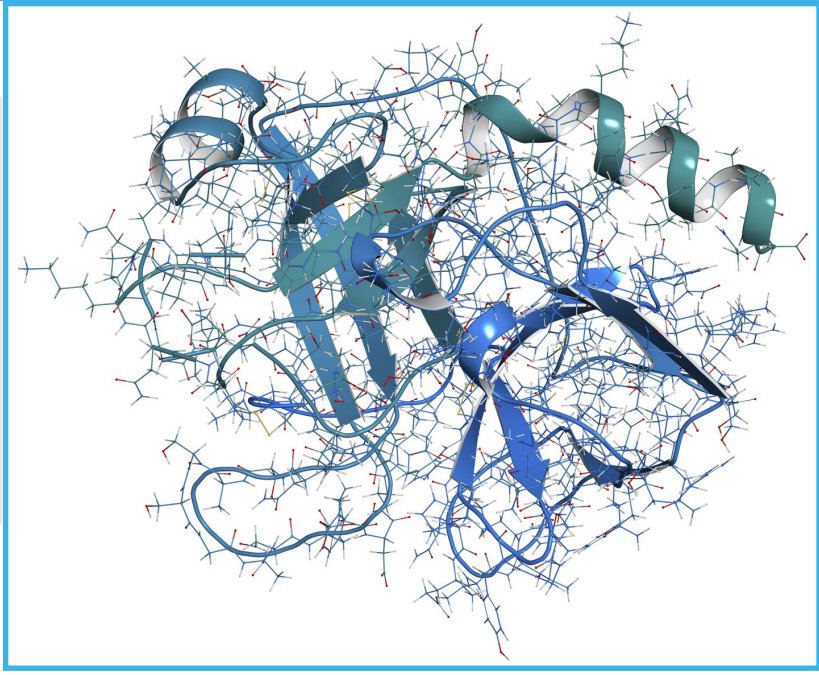
HALO®

TECHNICAL REPORT: AMT_TR_BIO_21

TITLE: **CAPILLARY HILIC LC/MS SEPARATIONS OF MONOCLONAL ANTIBODY POLAR MODIFICATION DEAMIDATION PRODUCTS**

MARKET SEGMENT: BIOCLASS



AUTHOR:

Arianne Soliven Ph.D., Application Scientist
Ben Libert AMT R&D Staff Scientist
Andrew Harron Ph.D., Application Scientist

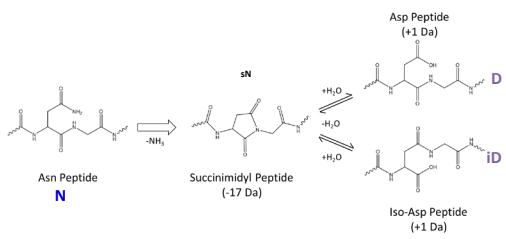
ABSTRACT

This study highlights a capillary HALO® Penta-HILIC column incorporated in an LC hyphenated high resolution MS workflow for the purpose of separating deamidation polar modification products of a monoclonal antibody, represented by a trastuzumab tryptic digest. The capillary HALO® Penta-HILIC column is an attractive alternative to the commonly used reversed-phase approaches for hyphenation with electrospray ionization MS. In addition to exploiting a low volumetric flowrate of 12 µL/min, this HILIC separation also utilizes a higher organic composition HILIC gradient.

INTRODUCTION

Deamidation is a reaction in which an amide functional group in the side chain of the amino acids asparagine or glutamine is removed or converted to another functional group. Deamidation products are of increasing importance in proteomics because they can alter a protein's structure, or possibly its function and stability, resulting in degradation. The rate of deamidation is dependent on multiple factors, including pH, the higher order structure of the protein, solution components, and temperature.

In the case of asparagine, the reaction proceeds with the loss of ammonium, then a succinimide intermediate is formed. This intermediate can then be interconverted to succinimidyl-, aspartyl- and iso-aspartyl peptides, provided it is in an aqueous solution. The analysis of protein deamidation products can be challenging for a number of reasons. The mass shifts associated with these interconversions are very well known, and can enable identification of the peptides from mass spectrometry, however, for many practical purposes, mass analysis alone is insufficient to resolve aspartate from iso-aspartate sequences, and chromatographic separation is needed. Protein deamidation has been commonly analyzed by reversed-phase liquid chromatography (RPLC) through peptide mapping, however RP separations often result in limited chromatographic selectivity for resolution of deamidated peptides from their unmodified counterparts. Recent advancements, however, have shown that hydrophilic interaction chromatography (HILIC) has advantages to RP separations [1–6]. Peptide deamidation products exhibit increased hydrophobicity, which affects peptide selectivity differences between HILIC and RP separations. Furthermore, operating in HILIC mode offers improved chromatographic separations for peptide deamidation products that are not resolved in RP [1–6]. Here we present the HALO® Penta-HILIC column for the analysis of deamidation products of trastuzumab in a high-resolution workflow and to enable identification of the polar modifications.



KEY WORDS:

HILIC, capillary, tryptic digest, peptide mapping, polar modifications, deamidation monoclonal antibody, trastuzumab

MATERIALS AND METHODS:

All solvents used were HPLC grade. Methanol, acetonitrile, mobile phase additives, and individual standards were obtained from MilliporeSigma (St. Louis, MO), unless specified otherwise. Trypsin digestion of the mAb - trastuzumab (as obtained for pharmaceutical purposes). Reduced and alkylated proteins were digested at 1:20 enzyme to protein overnight in 50 mM Tris-HCl (pH 7.8)/1.5M Guanidine-HCl, followed by 0.5% formic acid acidification and direct injection on to capillary LC/MS using the Orbitrap/IT.

Columns of HALO® Penta-HILIC were produced at Advanced Materials Technology Inc. (Wilmington, DE). These materials employ superficially porous Fused-Core® silica particles of 2.7 µm diameter, shell thicknesses of 0.5 µm, and pore sizes of 90 Å. Analyses of trastuzumab tryptic fragments employed a 0.5 mm ID x 150mm Penta-HILIC capillary column, operated at 12.0 µL/min, and 60°C. A trap column of the same material, of 2.6 µL size (0.5 mm x 12.5 mm) was obtained from Optimize, Inc. Analytical gradient conditions were from 80%B for 4min, then 80-48%B in 64 min using a flow rate of 12 µL/min. Mobile phase modifiers were obtained from Pierce (TFA, FA), Sigma/Millipore (TFA, formic acid, ammonium formate). Acetonitrile was MS grade from JT Baker. Mobile phase A: 50 mM ammonium formate pH 4.4 and mobile phase B: 0.1% formic acid in acetonitrile.

Capillary column separations used the Dionex RSLC 3000 connected to the Orbitrap VelosPro MS (ThermoScientific, Inc.), with the low flow IonMax ESI capillary needle operated at 3.8 kV potential. MS spectra were obtained, using 30,000 resolution, with CID MS/MS obtained using a Top N=4 data dependent regime. MS data were analyzed using Chromeleon v. 7.2 for integration. Chromatographic peak widths are reported as half height (PW1/2).

Results and Discussion

We highlight the HALO® Penta-HILIC capillary column to facilitate online LC/MS to determine deamidation and isomerization of protein asparagine and aspartate residues. The HALO® Penta-HILIC separation is an attractive front-end LC separation technique for use with LC/MS, due to a low volumetric flow-rate of 12 µL/min and a HILIC gradient employing a highly organic mobile phase environment. Clearly, the ESI is amenable to these conditions, as evidenced by the large number of charged species shown in Figure 1, and no evidence of ion suppression. The sufficient ion current dictates that the HILIC phase can be used for these types of analysis.

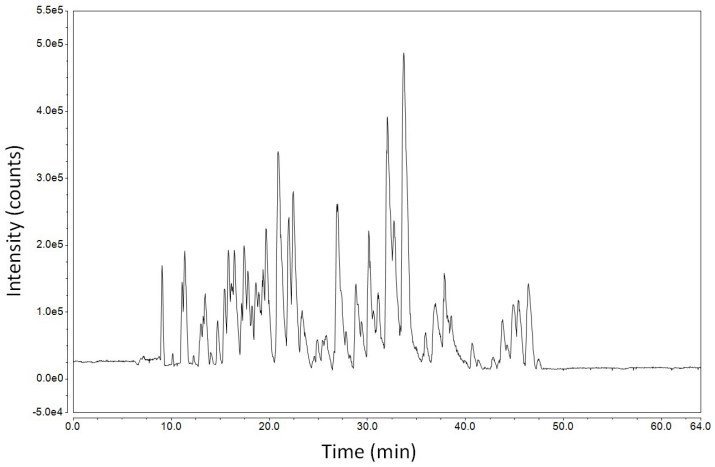


Figure 1. Total ion chromatogram of a tryptic digest between 0-64 min.

The deamidation products from the tryptic digest of the monoclonal antibody are shown in the peptide fragment GFYPSDIAVEWESNGQPENNYK and is one of many in a long list of peptide fragments identified by their mass to charge ratios (m/z) with the use of Biopharma Finder. Figure 2 illustrated the presence of the asparagine peptide (N) and the aspartyl- (D) and iso-aspartyl (iD) peptides in the peptide fragment. Both D and iD have equivalent m/z values that were separated by the capillary HALO® Penta-HILIC column. The retention pattern was supported by the analysis of standard synthetic peptides containing N, D and iD outlined previously [7].

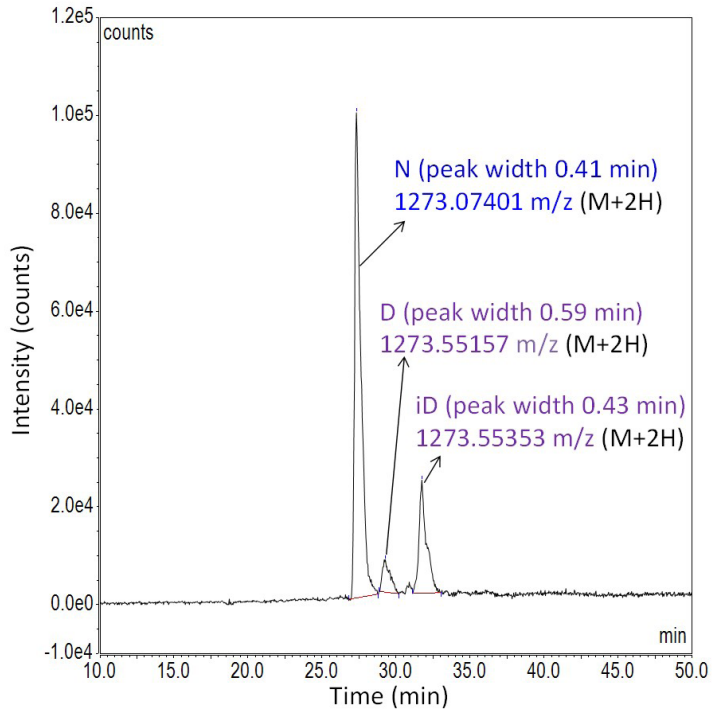


FIGURE 2. Extracted ion chromatogram 1272.5-1273.7 m/z of the peptide fragment GFYPSDIAVEWESNGQPENNYK.

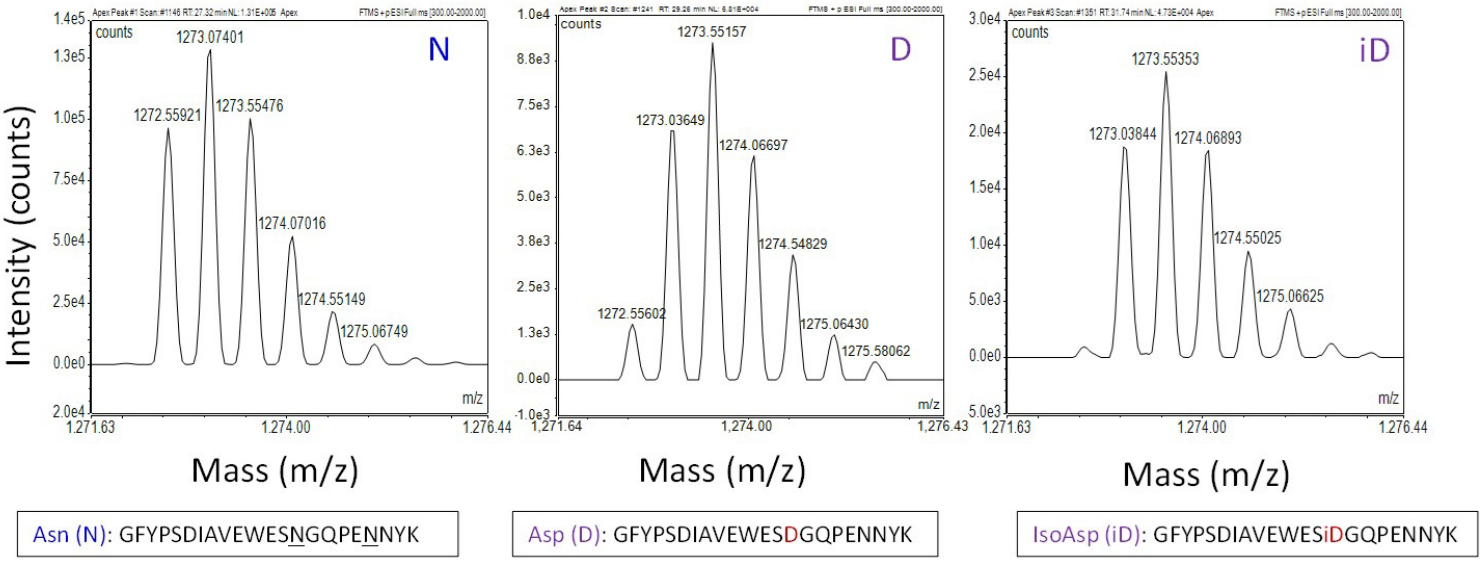


FIGURE 3. Mass spectra of the three peaks shown in Fig 2 in the order of elution.

Furthermore, the spectra for each of the asparagine peptide (N) and the aspartyl- (D) and iso-aspartyl (iD) peptide peaks represented in Figure 2 are shown in the order of elution in Figure 3. The spectra and EIC of the peptide fragment of GFYPSDIAVEWESNGQPENNYK evidenced the deamidation polar modification products of the trastuzumab tryptic digest experiments. This exercise may be repeated by searching other peptide fragments and their respective m/z that have a similar EIC pattern and spectra associated with the deamidation and isomerization of asparagine peptide and their retention pattern using standard peptides for N, D and iD [7].

CONCLUSION:

The capillary HALO® Penta-HILIC column facilitated coupling of microflow LC conditions that are ideal for use with ESI, with a low volumetric flowrate of 12 µL/min and a higher organic composition HILIC gradient. This report highlighted an LC/MS workflow for examining peptide deamidation and isomerization products of Asn, Asp, and isoAsp forms via HILIC [7].

ACKNOWLEDGEMENTS:

This technical note was based on a conference proceeding at ASMS 2017 'HILIC LC/MS Analytical Approach for Identification of Protein Deamidation and Isomerization Modifications' by Barry E Boyes, Majors Badgett, Benjamin Libert, and Ronald Orlando. Freely available to download online at fused-core.com

AUTHOR CONTRIBUTIONS:

AS figures and writing; BP experimental, editing; AH writing.

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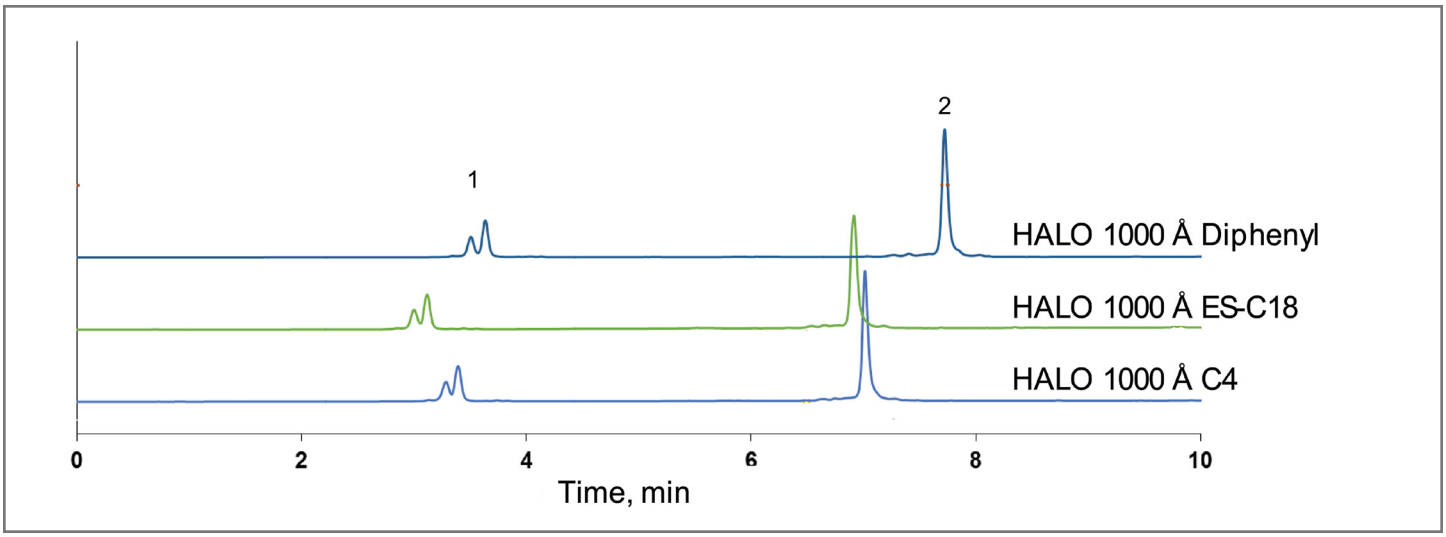
HALO®

BIOPHARMACEUTICALS

Comparison of an IdeS Digested mAb on Different HALO 1000 Å Phases

271-PR



PEAK IDENTITIES:

1. Fc/2
2. $F(ab')_2$

TEST CONDITIONS:

Columns:
HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm
Part Number: 92712-726
HALO 1000 Å ES-C18, 2.7 µm, 2.1 x 150 mm
Part Number: 92712-702
HALO 1000 Å C4, 2.7 µm, 2.1 x 150 mm
Part Number: 92712-714

Mobile Phase A: water/0.1% TFA
Mobile Phase B: ACN/0.1% TFA
Gradient: 30-45% B in 10 min
Flow Rate: 0.4 mL/min
Temperature: 80 °C
Detection: Fluorescence (280 nm ex, 350 nm em)
Injection Volume: 0.5 µL
LC System: UPLC, I-Class

The characterization of mAbs is critically important for protein biotherapeutic drug development. Although the analysis of the heavy and light chain can provide important information, often times site specific information is more critical, and allows for a more thorough characterization of the mAb. IdeS, a cysteine protease, is often used to do a partial digestion of the mAb, and by site specific cleavage, provide heterogeneity information about the structure. Two Fc fragments (Fc/2) and one $(Fab')_2$ fragment are produced, which allows for a thorough characterization of the Fc fragment. The separation of IdeS digested Cetuximab was run on the three stationary phases that are available on the 1000 Å HALO® particle. Slightly different selectivity and retention were observed for the Diphenyl, ES-C18, and C4 with all of them providing excellent resolution and peak shape for the fragments of Cetuximab.

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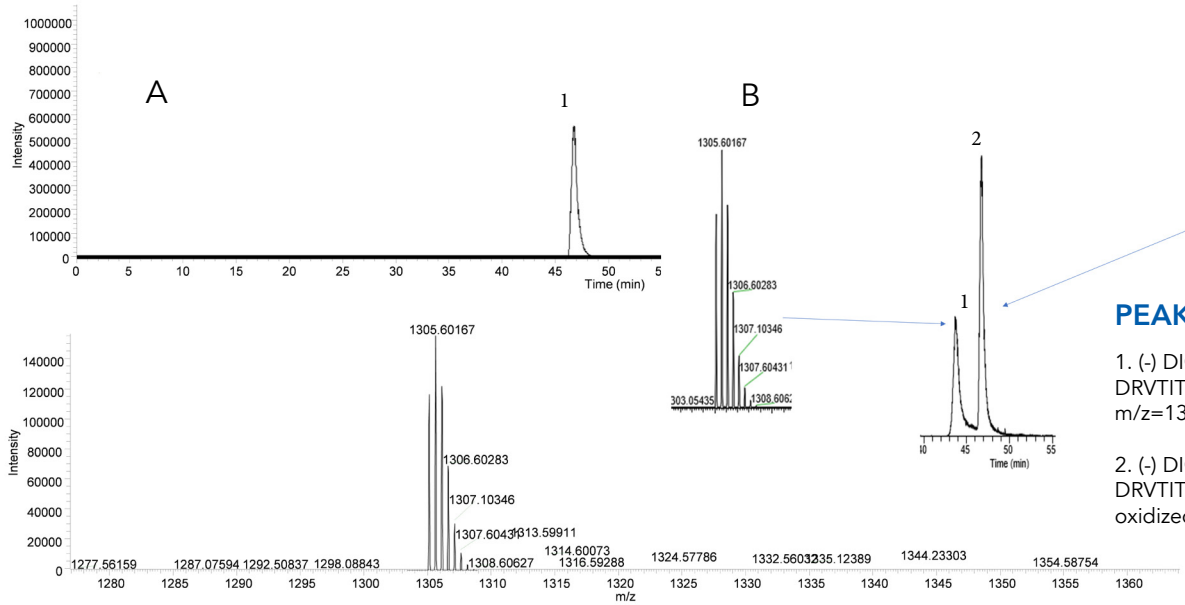
BIOPHARMACEUTICALS

Oxidation of NIST mAb Fragment

274-PE



PEAK IDENTITIES

1. (-) DIQMTQSPSSLSASVG-DRVTITC(Carbamidomethyl)R(A) m/z=1305.60167

2. (-) DIQMTQSPSSLSASVG-DRVTITC(Carbamidomethyl)R(A) oxidized m/z= 1313.59911

(A) Extracted ion chromatogram of (-) DIQMTQSPSSLSASVGDRVTITC(Carbamidomethyl)R(A)
(B) Extracted ion chromatogram oxidized (-) DIQMTQSPSSLSASVGDRVTITC(Carbamidomethyl)R(A) showing a mass shift of 8, as expected for an oxidized doubly charged peptide

TEST CONDITIONS:

Column: HALO® 90 Å Penta-HILIC, 2.7 µm, 0.5 x 150 mm
Part Number: 98215-705
Mobile Phase A: 50 mM Ammonium formate, pH 4.4
Mobile Phase B: 0.1% formic acid in acetonitrile
Gradient:

Time	%B
0.0	80
4.0	80
55	48
59	48
63	80
70	end

Flow Rate: 50 µL/min
Pressure: 158 bar
Temperature: 60 °C (standard)
80 °C (oxidized)
Detection: +ESI
Injection Volume: 5.0 µL
Sample Solvent: 70% ACN, 30% Water
LC System: Shimadzu Nexera X2
MS System: Thermo LTQ VELOS PRO

Post-translational modifications (PTMs), such as oxidation, are a critical variable that must be accounted for during protein analysis. Often times the minor mass shifts associated with these modifications are too small to be resolved during intact protein analysis, due to the charge envelope produced by large proteins, such as monoclonal antibodies (mAbs). However, chromatographically, these compounds will have a difference in retention time relative to the native, and can be separated before getting to the detector. Peptide analysis is an important method of characterization for mAbs because, in addition to revealing modifications such as oxidation, it can provide valuable insight into additional post-translational modifications, which may not be evident during intact mass analysis. In this experiment, the digested NIST mAb was exposed to high temperature in order to induce oxidation, and then analyzed using the HALO® Penta-HILIC capillary column, demonstrating it is an ideal choice for peptide oxidation analysis of mAbs.

MS CONDITIONS:

Ion mode: Positive
Aux gas: 2 arbitrary units
Sheath gas: 4 arbitrary units
Sweep gas: 0 arbitrary units
Rf lens: 55 V
Heater temp: 225°C
Ion transfer tube: 275°C
Capillary Voltage: 3.5 kV

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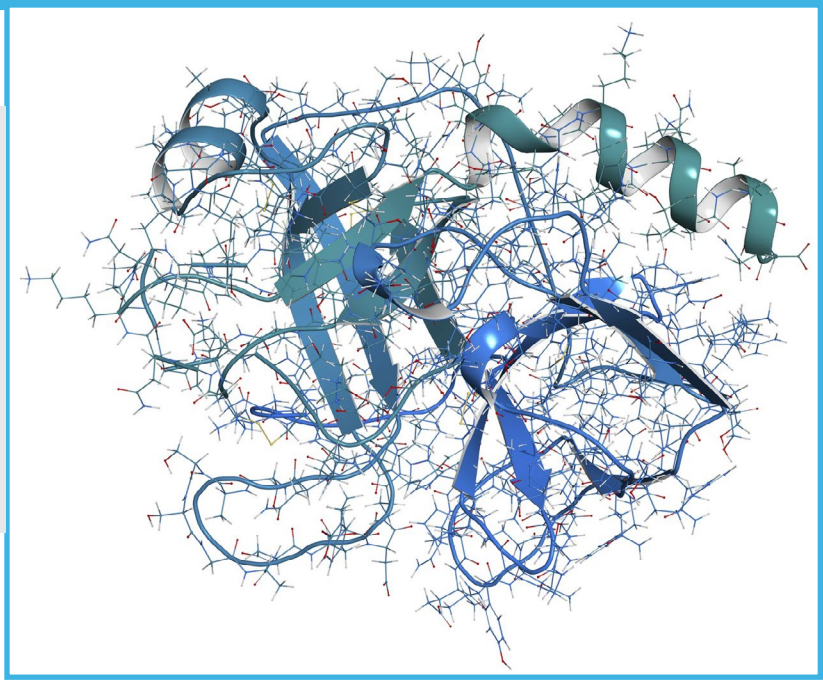
TECHNICAL REPORT: AMT_TR_BIO_21_2

TITLE: **ANALYSIS OF THE OXIDATION OF NIST MAB FRAGMENT USING THE HALO® PENTA-HILIC CAPILLARY COLUMN AND HRAM MS**

MARKET SEGMENT: BIOPHARMACEUTICAL



AUTHOR:

Andrew Harron Ph.D., Application Scientist

ABSTRACT

Post-translational modifications (PTMs), such as oxidation, are a critical variable that must be accounted for during protein analysis. Often times the minor mass shifts associated with these modifications are too small to be resolved during intact protein analysis, due to the charge envelope produced by large proteins, such as monoclonal antibodies (mAbs). However, chromatographically, these compounds will have a difference in retention time relative to the native, and can be separated before getting to the detector. Peptide analysis is an important method of characterization for mAbs because, in addition to revealing modifications such as oxidation, it can provide valuable insight into additional post-translational modifications, which may not be evident during intact mass analysis. In this experiment the digested NIST mAb was exposed to high temperature in order to induce oxidation, and then analyzed by the HALO® Penta-HILIC capillary column, demonstrating it is an ideal choice for peptide oxidation analysis of mAbs.

INTRODUCTION

Monoclonal antibody (mAb) based drugs, are biotherapeutic proteins, which have shown bioactivity against many types of diseases, including cardiovascular and cancer.[1-2] These drugs, like most proteins, can be subject to a multitude of biochemical modifications during their lifetime, which can change the efficacy of the biotherapeutic, and lead to variant forms of the drug.[2] These variants, often do not have the intended effect on the targeted disease, and therefore need to be studied and evaluated. One such variant causing modification, oxidation, is of major concern because it has a negative effect on the shelf life and bioactivity of the drug.[1-4]

The amino acids methionine (Met), cysteine (Cys), tryptophan (Trp), and histidine (His) are susceptible to oxidation, and if this occurs, the hydrophobic nature of the protein can change. In particular, oxidation of Met is of major concern as it can result in a product with altered binding.[4] Similar to other biotherapeutic proteins, mAbs can undergo oxidation at various stages such as during production, the formulation process, or during post formulation storage.[3] Therefore, it is critical to analyze potential oxidation changes during these processes.

The analysis of peptide oxidation is relatively straightforward. The mass shifts associated with these interconversions are very well known, and can enable identification of the oxidized peptides from mass spectrometry. In general, peptide-based mass spectrometry methods are used for analysis of oxidation of biotherapeutics. Although sample prep can be labor intensive and the methods can have longer run times, they can provide better chromatographic resolution of modified and unmodified features for accurate analysis.

KEY WORDS:

NIST mAb, Oxidation, HRAM, HALO® Penta-HILIC capillary

Traditionally, reversed-phase liquid chromatography (RPLC) methods are often used, as it is a hydrophobic based method and well suited to the analysis. However, Hydrophilic interaction chromatography (HILIC), a complementary technique to traditional RPLC, is also an ideal choice for this analysis because polar compounds can be retained while using MS friendly mobile phases and buffers. HILIC can have an advantage over RPLC due to the higher organic content of the mobile phase.[5-7] Whereas RPLC elutes from low to high organic, HILIC elutes from high to low organic. This will allow a higher percentage of organic into the ion source during the elution than RPLC, and will enhance the desolvation of the analyte and contribute to better spray stability, which will therefore lead to an increase in sensitivity.[5] There are also selectivity differences between HILIC and RPLC, which can have definitive advantages depending on the analyte of interest.[6,7]

Here we present the HALO® Penta-HILIC capillary column as an alternative to RPLC for analysis of oxidative stress of peptides.

EXPERIMENTAL DATA

All solvents used were MS grade. Methanol, acetonitrile, mobile phase additives, and individual standards were obtained from MilliporeSigma (St. Louis, MO), unless specified otherwise.

Trypsin digestion of the NIST mAb

The NIST mAb sample was denatured and alkylated using 50 mM Tris-HCl (pH 7.8)/1.5M Guanidine-HCl, and 2-iodoacetamide (Sigma Aldrich). Trypsin (Promega) was added in a ratio of 1:30 (w:w; Trypsin:mAb) followed by an incubation at 37 °C overnight. The reaction was quenched by 0.5% formic acid and analyzed by LCMS.

Samples were analyzed on a Shimadzu Nexera X2 (Shimadzu Scientific Instruments, USA). Mass spectra were acquired using a Thermo Scientific Velos Pro LTQ Orbitrap mass spectrometer (Bremen, Germany) using a heated electrospray (HESI-II) probe on the Ion Max source.
A 90 Å 2.7 µm, (0.5 mm ID x 150mm)
HALO® Penta-HILIC column was used (Advanced Materials Technology, Inc. Wilmington, DE). Flow rate was 50 µL/min, and 60 °C for initial analysis. In the oxidation experiments the temperature was raised to 80 °C.

LC Gradient

Time	%B
0	80
4	80
55	48
59	48
63	80
70	end

Column: HALO® 90 Å Penta-HILIC, 2.7 µm, 0.5 x 150 mm
Part Number: 98215-705
Flow Rate: 50 µL/min
Pressure: 158 bar
Temperature: 60 °C characterization run, 80°C for oxidation run.
Detection: +ESI
Injection Volume: 5.0 µL
Sample Solvent: 70% ACN 30% water
LC System: Shimadzu Nexera X2
MS System: Thermo Scientific Velos Pro LTQ Orbitrap
Mobile phase A: 50 mM ammonium formate pH 4.4 and
Mobile phase B: 0.1% formic acid in acetonitrile.

MS Conditions

Voltage	3.5 kV
Aux gas	4 arbitrary units
Sheath gas	2 arbitrary units
Sweep gas	0 arbitrary units
Rf lens	45 v
Heater temp	225 °C

Oxidation experiment:

After digestion, the oxidation was carried out by injecting the sample onto the column at 80 °C, then the flow was stopped for 30 minutes. After the 30-minute hold had been completed, the mobile phase gradient was started and the analysis was begun.

RESULTS AND DISCUSSION

In order to determine the validity of the HALO® Penta-HILIC column for oxidative analysis, a rudimentary oxidation experiment was performed. The digested peptide was injected onto the column at an elevated temperature of 80 °C, and left on the column for 30 minutes. After 30 minutes the mobile phase gradient was started and the digest was eluted from the column. Figure 1 shows the extracted ion chromatogram of (-) DIQMTQSPSSLSASVGDRVTITC(Carbamidomethyl)R(A), m/z=1305.60167, before the oxidation experiment.

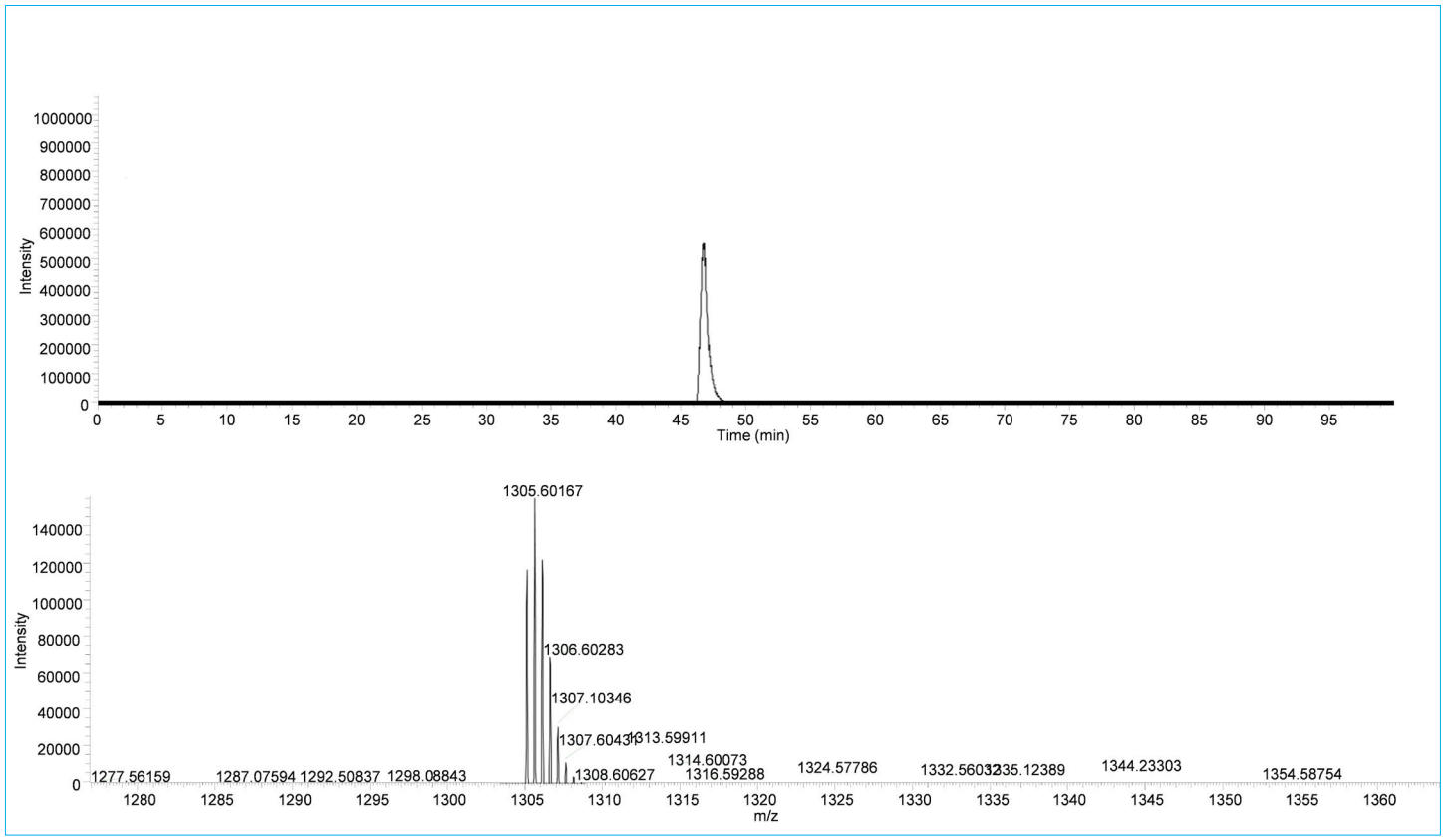


Figure 1. extracted ion chromatogram of (-) DIQMTQSPSSLSASVGDRVTITC(Carbamidomethyl)R(A)

Figure 2 shows the same peptide after the oxidation and shows the characteristic mass shift of 8 Da associated with oxidation for a doubly charged peptide species.

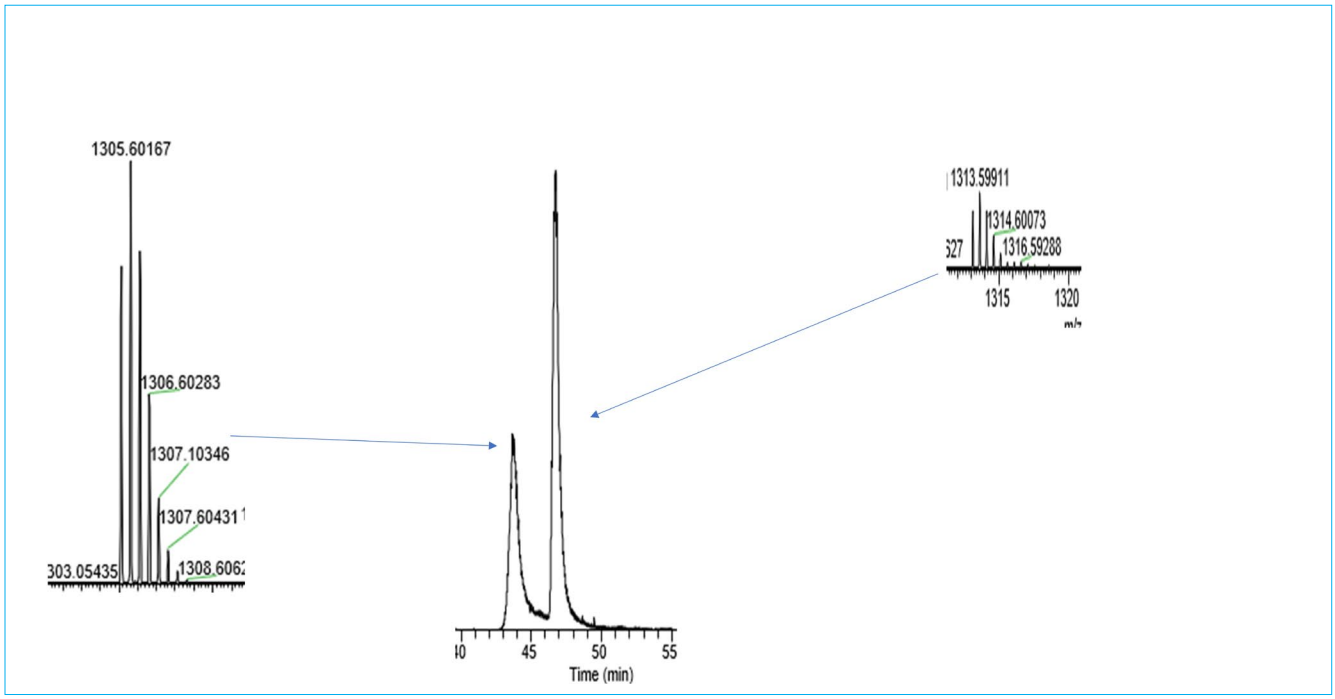


Figure 2. Extracted ion chromatogram oxidized (-) DIQMTQSPSSLSASVGDRVTITC(Carbamidomethyl)R(A) showing a mass shift of 8.

The oxidized species is detected at m/z= 1313.59911, and is 8 Da away from the native as would be expected for a doubly charged peptide. The shift in retention time relative to the native is evident and indicative of a modification having occurred on this peptide.

This pattern was also observed in Figure 3, which shows m/z=959.96868, the extracted ion chromatogram of the doubly charged peptide fragment (R)EPQVYTLPPSREEMTK(N). The oxidized species is detected at m/z=967.46419, and is 8 Da away from the native as would be expected for a doubly charged peptide.

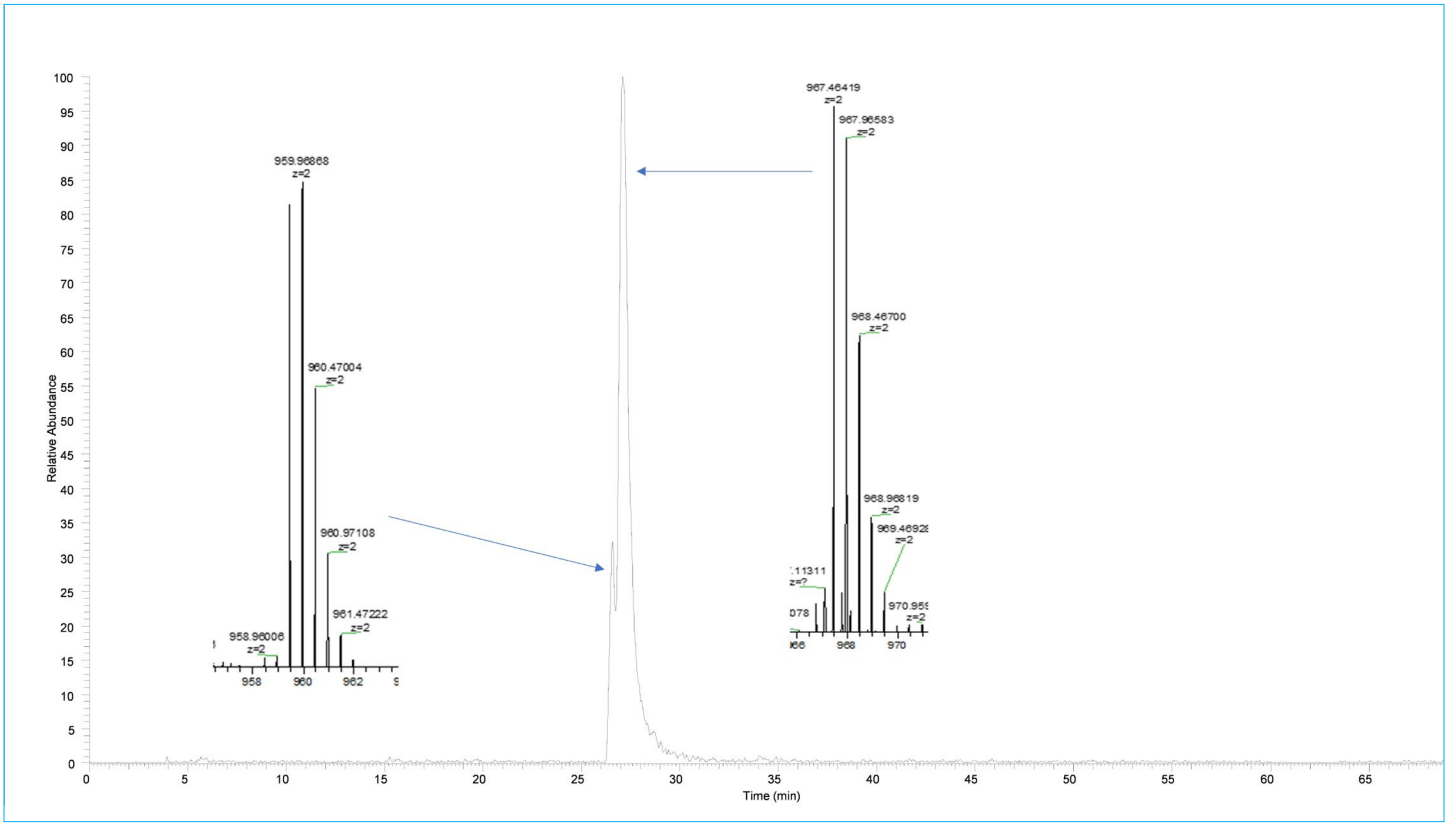


Figure 3. The extracted ion chromatogram of the doubly charged peptide fragment (R)EPQVYTLPPSREEMTK(N) showing a mass shift of 8.

The mass shift that was observed with both doubly charged peptide fragments was indicative of a successful oxidation. Despite the rudimentary aspects of the experiment, the HALO® Penta-HILIC capillary column displayed the selectivity and sensitivity to resolve the structural modification that occurred with oxidation.

CONCLUSION:

Polar modifications, such as oxidation, can have a deleterious effect on the bioactivity of a mAb biotherapeutic, and must be monitored. Peptide analysis is critical to test for oxidization, due to the negative effect that can result from the process, and a loss of integrity of the mAb. The HALO® Penta-HILIC capillary column coupled with HRMS delivers the sensitivity and resolution needed to analyze oxidation of mAbs.

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