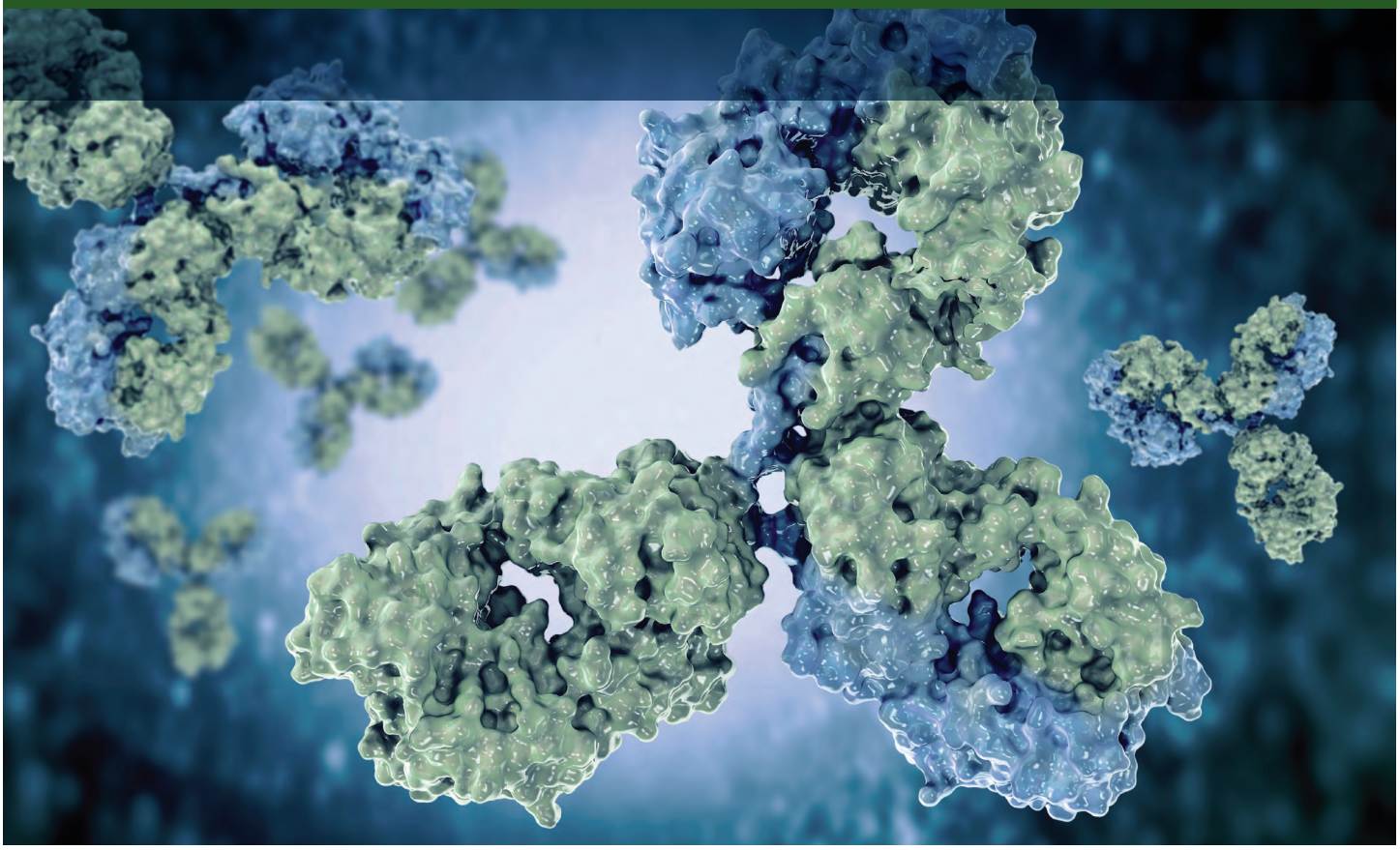
Agilent
Trusted Answers

Agilent-NISTmAb

Intact Analysis Using Hydrophobic Interaction

Agilent BioHPLC Columns
Application Compendium



Contents

Agilent-NISTmAb Standard (P/N 5191-5744; 5191-5745) was aliquoted from NISTmAb RM 8671 batch. Quality control (QC) testing is performed using Agilent LC-MS system. QC batch release test includes aggregate profile, charge variants and intact mass information. A certificate of analysis (CoA) can be found in each product shipment with test results.

Please note that authors used various monoclonal antibodies including Agilent-NISTmAb Standard and NISTmAb RM 8671 to demonstrate critical quality attribute workflows.

4 Intact Native Protein Analysis Hydrophobic Interaction Chromatography 02

4.1 Introduction 02

4.2 Quick Start Guide 07

4.3 Featured Application Notes 10

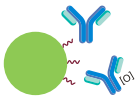
4.4 AdvanceBio HIC: a Hydrophobic HPLC Column for Monoclonal Antibody (mAb) Variant Analysis- 5994-0199EN 10

Intact Protein Analysis using Hydrophobic Interaction Chromatography (HIC)

Introduction

Recombinant proteins are subject to many post translational modifications (PTMs) during processing, delivery, and storage[1]. Some of the most PTMs found in therapeutic monoclonal antibodies (mAbs) include glycosylation, disulfide bond formation, and proteolytic cleavage of the protein backbone (e.g., oxidation, deamidation, glycation, pyroglutamate [pyro-Glu] formation). Hydrophobicity-based HPLC methods, such as reversed-phase liquid chromatography (RPLC) and hydrophobic interaction chromatography (HIC), are often used to characterize these mAb variants. HIC technique is often used for large scale protein purification for removing protein aggregates and contaminants such as host-cell proteins [2, 3]. In more recent years, gained popularity for small scale analytical separations. Due to its unique selectivity, HIC is complementary to techniques like size exclusion, ion exchange, and RP. Of these, HIC is most like RP, in that separation is strongly correlated with protein hydrophobicity. But there are two key differences between HIC and RP: 1) HIC separations are driven by a decreasing gradient of salt, rather than an increasing gradient of organic solvent as in RP. 2) HIC separations maintain the native state of a protein, and thus the protein remains biologically active following the separation. This allows collection of purified peaks for downstream potency assays, for example. Furthermore, the native state of the protein means that features on the surface of a protein tend to drive selectivity in HIC mode. Therefore, HIC is particularly well suited to separating PTMs or degradation products of a protein, which is sometimes very difficult to achieve by other modes of chromatography.

Back To Contents Page



Intact Native Protein Analysis

Hydrophobic interaction chromatography

Resolves various protein variants (PTMs) including oxidation in mAbs and drug-antibody species observed in ADcs

AdvanceBio HIC

Designed to Address the Separation of Challenging Biomolecules applications

Attribute	Advantage
Single Chemistry	Multiple applications with better peak shape
Faster method development and higher throughput for greater productivity	Meet vital deadlines
Enhanced robustness, batch-tobatch consistency	Reduce rework

HIC separates analytes in order of increasing hydrophobicity; in other words, less hydrophobic species typically elute first. Both enthalpic (i.e., hydrophobic/hydrophilic interactions) and entropic (i.e., hydration shell) interactions are responsible for retention[4,5]. A weakly hydrophobic stationary phase is used to bind the analyte in the presence of high concentrations of polar salts. A mobile phase gradient of decreasing salt concentration is applied. As the concentration of salts decreases, analytes desorb into the mobile phase. This phenomenon relates to the "salting out" often observed with proteins. Salts can screen the electrostatic and dipole–dipole interactions as well as disrupt the solvation shell of the molecules. Figure 1 presents the effects of various salts on the stability and solubility of proteins in solution. Chaotropic salts appear on the right (e.g., $NaClO_4$) and will improve the protein's solubility, but tend to disrupt protein folding where they unravel from their tertiary structure. Salts on the left, however, often stabilize the protein's structure, but increase the likelihood of adsorption and precipitation. Ammonium sulfate is the most commonly used salt for HIC because of its ability to induce adsorption onto the column. There is also an entropy-driven component to the separation. Water tends to form an ordered hydration layer around the protein as well as the stationary phase. These layers become disrupted and disordered when the protein approaches the stationary phase. The resultant increase in entropy makes retention favorable. Furthermore, selection of a hydrophobic stationary phase, salt type, temperature, pH and use of organic modifier mobile phase, can be used to adjust the selectivity of the separation for the particular analyte or analytes of interest.

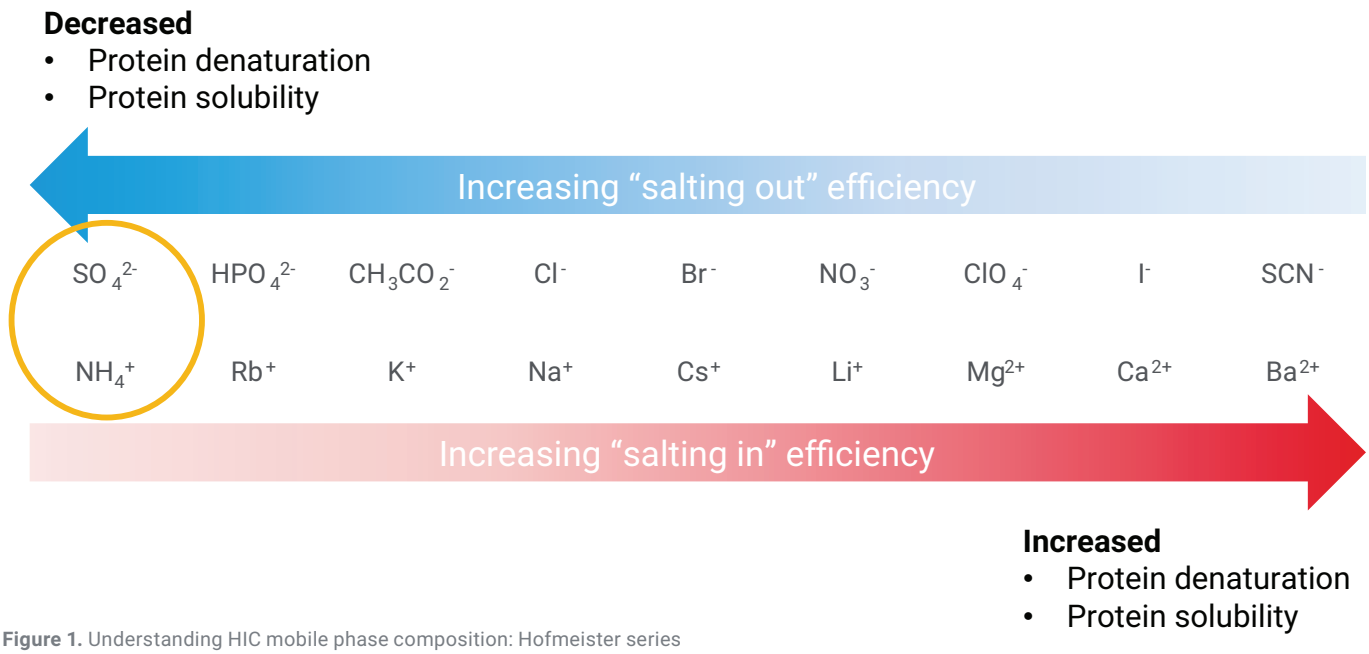


Figure 1. Understanding HIC mobile phase composition: Hofmeister series

With careful column and experimental design, HIC can be characterized by very good repeatability, both injection to injection, and column to column. Like reversed phase (or any chromatography), good mass transfer is essential to having high efficiency and narrow peaks. To accommodate the large molecular size of the simple species, HIC columns tend to have much larger pore sizes, up to 450 angstroms or more. The column capacity is kept high by using fully porous particles. The viscosity of the mobile phase presents some unique challenges. The slow mass transfer associated with large molecules in a viscous environment would make smaller particles the preferred choice, but the high viscosity puts pressure limits on how small the particles can become. Most columns use between 3 µm-5 µm particle size as the optimal compromise. Because of the drastic changes in viscosity that result from the variation in salt concentrations, it is not recommended to rapidly switch back to the initial mobile phase at the end of the gradient, as is typically done in reversed phase chromatography. Such a sharp change could damage the column. Rather, a relatively slow reverse gradient over several minutes should be used, followed by a 2–3 column volumes to complete the re-equilibration.

The ability of HIC to separate even closely related proteins makes it useful application in the analysis of the purity of protein samples. This method could be used to analyze stressed samples (e.g., heat, light) that might cause an increase in hydrophobic variants. Because this method is performed under conditions that do not irreversibly impact the structure of the antibody, collection of variants and testing by potency- or binding-related biological assays could be conducted to further understand the impact and significance of these species. One of the main success stories relates to ADC characterization [4]. In this case, HIC can be used to separate ADCs based on the number of conjugated small molecule drugs, and is thus useful for calculating the drug to antibody ratio or DAR [5]. HIC has also been shown to separate Asp/IsoAsp isomers as well as succinimide variants [6] and oxidation variants of methionine and tryptophan [7,8]. Bispecific mAbs, a modality of increasing importance, can be analyzed using HIC, in particular for assessing the population of parental mAbs vs the intended bispecific product [9]. Relative hydrophobicity of proteins can also be assessed, as a potential indicator of aggregation [4].

The Application Note (5994-0199EN) describes the separation of oxidized NISTmAb variants from their native form using the Agilent AdvanceBio HIC column.

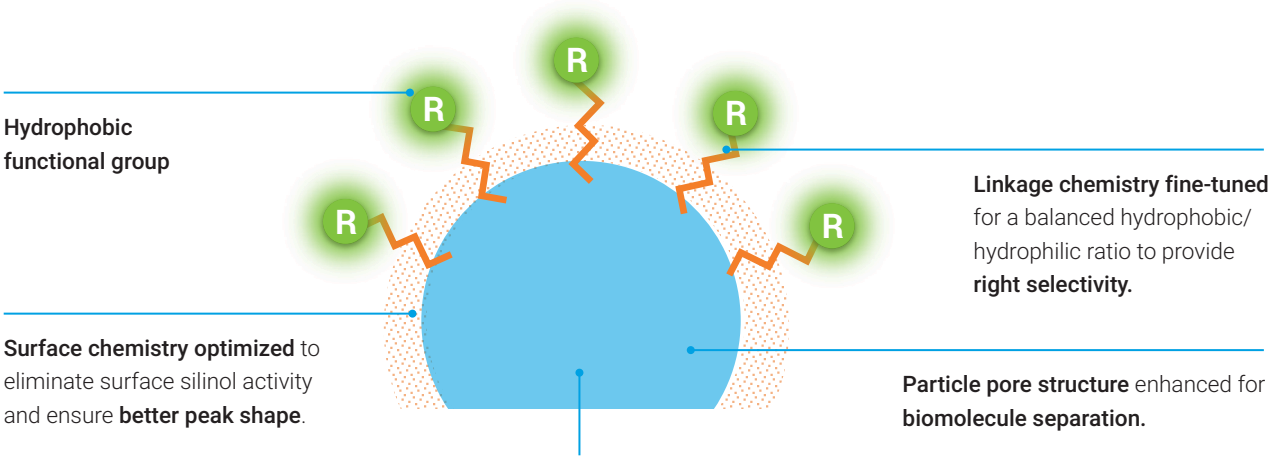
Back To Contents Page

Column Specifications

Pore Size	Particle Size	Temperature Limit	pH Range	Pressure Limit	Flow rate*
450Å	3.5 µm	60 C (at pH 7)	2.0 - 8.0 (at 35 C)	400 bar (Typical operating pressure <200 bar)	0.5 - 1.0 mL/min (4.6 mm id)

* In some cases, lowering the flow rate to 0.3 ml/min and extending gradient time may further improve resolution.

Novel hydrophobic interaction chemistry optimized to ensure best selectivity and robustness



AdvanceBio HIC columns deliver high resolution, robust, and reproducible separations of native proteins at the intact level. Built using the capabilities of the ZORBAX fully porous particles and proprietary bonding technology, these columns provide new levels of hydrophobicity and versatile single chemistry to address particularly challenging molecules such as monoclonal antibodies (mAbs), antibody drug conjugates (ADCs), and other recombinant proteins.

Together with the 1260 Infinity II Bio-inert LC system, the AdvanceBio HIC provides uncompromised performance and data consistency during characterization and validation.

- Optimized selectivity: ideal for mAb oxidation and ADC DAR ratios.
- Single chemistry: reduces the need for multiple-column screening for different CQAs.
- Enhanced robustness: improved column lifetime for ultimate confidence in your data.
- Proven performance: every batch of media is tested with NISTmAb.
- High quality: each column is individually tested to ensure packing efficiency.
- Greater productivity: shorter columns reduce analysis time while maintaining separation performance

Back To Contents Page

References

1. Zhang, Y.; Martinez, L.; Woodruff, B.; Goetze, A.; Bailey, R.; Pettit, D.; Balland, A. Hydrophobic interaction chromatography of soluble Interleukin I receptor type II to reveal chemical degradations resulting in loss of potency, ***Anal. Chem.*** **2008**, ***80(18)***, 7022-8.
2. Jiang, C., et al., Defining process design space for a hydrophobic interaction chromatography (HIC) purification step: application of quality by design (QbD) principles. ***Biotechnol Bioeng***, **2010**. ***107(6)***: p. 985-97.
3. McCue, J.T., et al., Modeling of protein monomer/aggregate purification and separation using hydrophobic interaction chromatography. ***Bioprocess Biosyst Eng***, **2008**. ***31(3)***: p. 261-75.
4. Lienqueo, M.E. and A. Mahn, Predicting Protein Retention Time in Hydrophobic Interaction Chromatography. ***Chemical Engineering & Technology***, **2005**. ***28(11)***: p. 1326-1334.
5. Fekete, S., et al., Hydrophobic interaction chromatography for the characterization of monoclonal antibodies and related products. ***J Pharm Biomed Anal***, **2016**. ***130***: p. 3-18.
6. Wakankar, A., et al., Analytical methods for physicochemical characterization of antibody drug conjugates. ***mAbs***, **2014**. ***3(2***): p. 161-172.
7. Valliere-Douglass, J., A. Wallace, and A. Balland, Separation of populations of antibody variants by fine tuning of hydrophobic-interaction chromatography operating conditions. ***J Chromatogr A***, **2008**. ***1214(1-2)***: p. 81-9.
8. Boyd, D., T. Kaschak, and B. Yan, HIC resolution of an IgG1 with an oxidized Trp in a complementarity determining region. ***J Chromatogr B Analyt Technol Biomed Life Sci***, **2011**. ***879(13-14)***: p. 955-60.
9. Zwolak, A., et al., Rapid Purification of Human Bispecific Antibodies via Selective Modulation of Protein A Binding. ***Sci Rep***, **2017**. ***7(1)***: p. 15521.

Back To Contents Page

AdvanceBio HIC Columns

Agilent
Trusted Answers

Quick Reference Guide

In this document, Agilent applications chemists share their recommendations for an optimum LC system and its configuration for performing hydrophobic interaction chromatography (HIC).

They offer guidance on generic methods for protein and monoclonal antibody separations, and for separation of antibody drug conjugates (ADCs). These methods can then be modified to meet your exact requirements.

Additional application information is available at **www.agilent.com/chem/advancebio-hic**

Introduction

Hydrophobic interaction chromatography (HIC) relies on the effect of "salting out" to cause proteins to absorb onto the weakly hydrophobic HIC column. The most common salt used in this technique is ammonium sulfate. This is readily soluble at a concentration of 2 M, which is required in the analysis of many proteins. It is necessary to use a regular buffer to maintain protein solubility and to stabilize pH. For this purpose, sodium phosphate at neutral pH in concentrations from 20 to 100 mM is recommended. Proteins are eluted from the column in order of increasing hydrophobicity using a gradient from high to low ammonium sulfate concentration.

Back To Contents Page

Operating Guidelines

An Agilent 1260 Infinity II Bio-inert LC is recommended due to the high salt concentrations used. Gradients should typically last from 10 to 20 column volumes for good resolution. Flushing and re-equilibration should last at least five column volumes. High salt concentrations can cause issues with some LC systems. Use of a fully bio-inert LC is recommended, ideally with a quaternary pump so that additional channels can be used for flushing. It is not advisable to leave 2 M ammonium sulfate solution in either the HIC column or the LC; flush the column with sodium phosphate buffer after use. Additional precautions, such as pump seal washing and needle washing, should be taken where possible.

Mobile phase solutions should be UV transparent and have little background absorption, allowing detection at low wavelengths for maximum sensitivity. However, it is advisable to use the highest solvent grade available. The Agilent AdvanceBio HIC column is available in two formats: 4.6 × 30 mm for fast separations, and 4.6 ×100 mm for higher resolution separations. For proteins, the optimum flow rate is typically around 0.4 to 0.5 mL/min. It is important to also take the viscosity of the mobile phase into consideration.

Maintaining temperature control is also vital: Samples should be kept in a refrigerated autosampler to avoid deterioration. Temperature control is also recommended during HIC separation: Many proteins are temperature sensitive, and changes in retention time and peak shape may be observed at different temperatures.

Storage

AdvanceBio HIC columns are shipped containing 100 % acetonitrile. **It is important that the columns are returned to 100% acetonitrile for storage after use.** Care must be taken not to mix 100 % acetonitrile with high salt mobile phase in case of precipitation.

Please refer to the User Guide at **www.agilent.com/chem/advancebio-hic-userguide** for column conditioning, use, and storage.

Reagents and chemicals

All reagents should be HPLC grade or higher.

Sample preparation

Dissolve samples in high concentration ammonium sulfate solution. A final concentration of 1 mg/mL should be sufficient for most needs.

Mobile phase preparation

Care should be taken to ensure that all salts are fully dissolved and the pH has been adjusted to its target value. It is necessary to filter all mobile phase solutions through a 0.22 µm membrane filter before use. Do not leave mobile phase on the instrument longer than necessary, and replace regularly.

Instrumentation

A 1260 Infinity II Bio-inert LC is recommended.

Gradients should typically last from 10 to 20 column volumes for good resolution. Flushing and re-equilibration should last at least five column volumes.

Agilent AdvanceBio HIC Columns

Description	Part No.
AdvanceBio HIC, 4.6 x 100 mm, 450 Å, 3.5 µm	685975-908
AdvanceBio HIC, 4.6 x 30 mm, 450 Å, 3.5 µm	681975-908

Back To Contents Page

Suggested starting conditions

HPLC Conditions		
Column	Agilent AdvanceBio HIC, 4.6 × 100 mm, 450 Å, 3.5 µm (p/n 685975-908)	
Mobile phase	Eluent A:	50 mM sodium phosphate, pH 7.0
	Eluent B:	2 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0
	Eluent C:	propan-2-ol
	Eluent D:	water (for flushing)
Flow rate	0.5 mL/min	
Temperature	25 °C	
Injection volume	1 to 10 µL	

Gradient profile: Proteins

Time	%A	%B	%C
0	0	100	0
20	100	0	0
25	100	0	0
30	0	100	0
40	0	100	0

After all samples have been completed, flush the column with eluent A

Gradient profile: Monoclonal antibodies (mAbs)

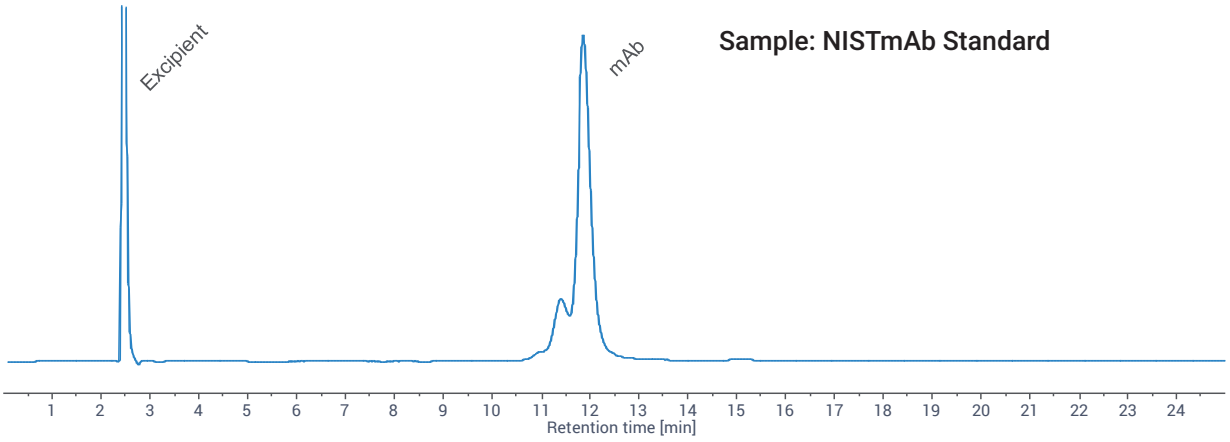
Time	%A	%B	%C
0	50	50	0
20	100	0	0
25	100	0	0
30	50	50	0
40	50	50	0

After all samples have been completed, flush the column with eluent A

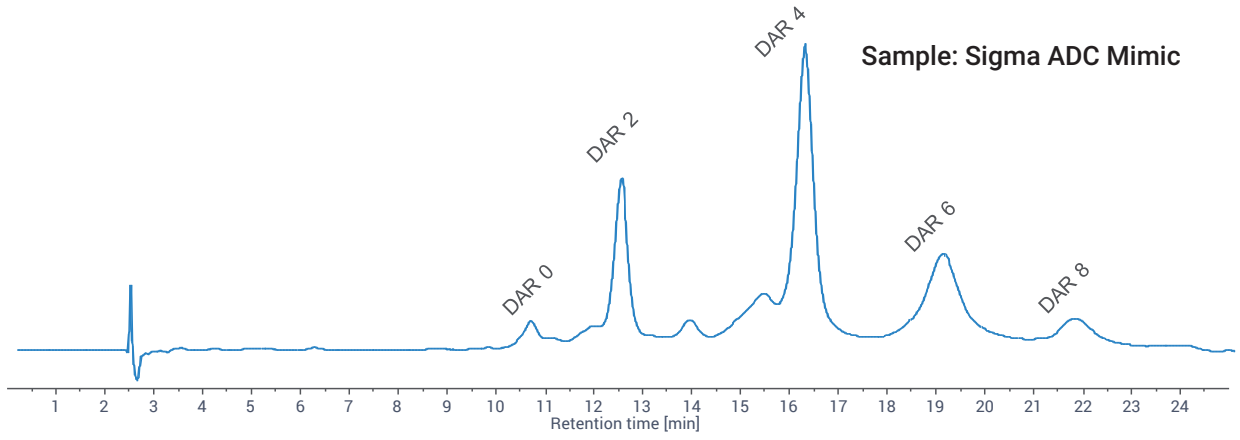
Gradient profile: Antibody drug conjugates (ADCs)

Time	%A	%B	%C
0	45	50	5
20	75	0	25
25	75	0	25
30	45	50	5
40	45	50	5

After all samples have been completed, flush the column with eluent A



Sample: NISTmAb Standard



Sample: Sigma ADC Mimic

Back To Contents Page

Application Note

Biologics Research

Agilent

Trusted Answers

AdvanceBio HIC: a Hydrophobic HPLC Column for Monoclonal Antibody (mAb) Variant Analysis

Using the Agilent 1260 Infinity II Bio-Inert LC

Authors

Andrew Coffey and
Sandeep Kondaveeti
Agilent Technologies, Inc.

Introduction

This Application Note describes the separation of oxidized monoclonal antibody (mAb) variants from their native form using the Agilent AdvanceBio HIC column. Oxidation of exposed amino acid side chain residues such as methionine, cysteine, and tryptophan is a common degradation pathway for monoclonal antibodies, and presents a major analytical challenge in biotechnology. Often, oxidized mAbs have decreased potency compared to their native form1. Therefore, to ensure the therapeutic efficacy of the mAb products, analysis of such degradation is critical. Oxidation of amino acid residues on an mAb can alter the hydrophobic nature of the mAb by the increase in polarity of the oxidized form, or also due to resulting conformational changes2. HPLC methods for separating biomolecules based on differences in hydrophobicity include reversed-phase and hydrophobic interaction chromatography (HIC). HIC can be applied to characterize mAb variants resulting from post-translational modifications (PTMs). The AdvanceBio HIC column provides excellent resolution of oxidized mAb variants from unmodified forms, and can resolve oxidized species without mAb digestion into subunits or other sample preparation methods.

Back To Contents Page

Introduction

mAbs and related products such as antibody drug conjugates (ADCs) and bispecific antibodies (bsAbs) are the fastest growing classes of biotherapeutics. Recombinant mAbs are subject to many PTMs during processing, delivery, and storage. Among these modifications, oxidation of exposed amino acid side chains such as methionine (Met) and tryptophan (Trp) is a common occurrence. Various researchers have reported that oxidation of mAbs has an adverse effect on product shelf life and bio-activity[1,2]. Therefore, developing analytical methods to detect oxidized mAb variants has gained interest. The sulfoxide and sulfone side chains of methionine-oxidized mAb products are larger and more polar compared to the native form, which may alter protein structure, stability, and biological function. Hydrophobicity-based HPLC methods, such as reversed-phase liquid chromatography (RPLC) and HIC, are often used to characterize mAb variants. Recently, several studies have indicated that HIC can be applied to monitor oxidation of recombinant mAbs with reasonable selectivity and ease, as an excellent alternative to RPLC[3].

HIC is similar to RPLC in that separation of analytes is based on hydrophobic interactions with the stationary phase. The elution order in HIC enables proteins to be ranked based on their relative hydrophobicity. Unlike RPLC, HIC employs nondenaturing conditions, does not require the use of organic solvents or high temperatures, and separations are carried out at physiological pH, allowing for the preservation of protein structure. Thus, conformational changes in the native form of the protein may be analyzed using HIC[4].

AdvanceBio HIC is a silica-based HPLC column designed for the separation of mAbs and related products. Its unique proprietary bonded phase chemistry provides high resolution and desired selectivity for the analysis of mAbs and mAb variants. This Application Note describes the separation of oxidized NISTmAb variants using an AdvanceBio HIC column.

Experimental

Equipment and Materials

All chemicals and reagents were HPLC grade or higher, and were obtained from Sigma-Aldrich (now Merck) or VWR Scientific. Humanized IgG1k mAb sample (product item no. 8671) was obtained from NIST SRM Standards. Water was purified using a Milli-Q A10 water purification system (Millipore).

Instrumentation

Agilent 1260 Infinity II bio-inert LC comprising:

- Agilent 1260 Infinity II bio-inert pump (G5654A)
- Agilent 1260 Infinity II bio-inert multisampler (G5668A) with sample cooler (option no. 100)
- Agilent 1260 Infinity II multicolumn thermostat (G7116A) with bio-inert heat exchanger (option no. 019)
- Agilent 1260 Infinity II diode array detector WR (G7115A) with bio-inert flow cell (option no. 028)

Software

Agilent OpenLab 2.2 CDS

mAb Oxidation with t-BHP treatment

A solution of 1 mL of NISTmAb (1 mg/mL) in formulation buffer was incubated with 0.2 % (v/v) of 70 % tert-butyl hydroperoxide (t-BHP) solution at room temperature for 24 hours. Residual oxidant was removed using Amicon Ultra-10 centrifugal filters with a molecular weight cut-off of 10 kDa. Samples were then buffer exchanged with 50 % mobile phase B.

Reaction conditions used to obtain Figure 6 data: 2 % (v/v) of 70 % t-BHP solution was added to a 1-mL sample of NISTmAb (1 mg/mL), and the reaction mixture was injected onto the column. The sample vial was held at 7 °C, and multiple injections from the same vial were carried out.

mAb Oxidation with H_2O_2 treatment

A solution of 1 mL of NISTmAb (1 mg/mL) in formulation buffer was incubated with 0.2 % (v/v) of 50 % hydrogen peroxide (H_2O_2) solution at room temperature for 24 hours. Residual oxidant was removed using Amicon Ultra-10 centrifugal filters with a molecular weight cut-off of 10 kDa. Samples were then buffer exchanged with 50 % mobile phase B

Back To Contents Page

Results and discussion

Protein oxidation is frequently monitored in stability studies or stressed samples during formulation development (for example, samples exposed to a chemical oxidant such as hydrogen peroxide (H_2O_2), UV light, or metal ions). In this study, t-BHP and H_2O_2 were used as chemical oxidants to promote oxidation of NISTmAb samples. It was previously reported that both of these reagents tend to specifically oxidize Met side chain residues of the mAb. H_2O_2 more readily oxidizes less accessible, buried residues, whereas t-BHP is known to target more surface-exposed Met residues7. Figure 1 illustrates the reaction scheme for Met oxidation induced by chemical oxidants.

The NISTmAb (humanized IgG1k) amino acid sequence in Figure 2 shows that there are six possible surface-accessible Met residues located on both heavy chains of the mAb. Based on prior studies for most human IgG1-subclass antibodies, Met residues localized to the CH2 and CH3 domains of the antigen binding, or Fc, region are known to be highly susceptible to oxidation[5]. In the case of NISTmAb, Met 255 and Met 431 correspond to the amino acid residues prone to oxidation. This is depicted by the illustration in Figure 3.

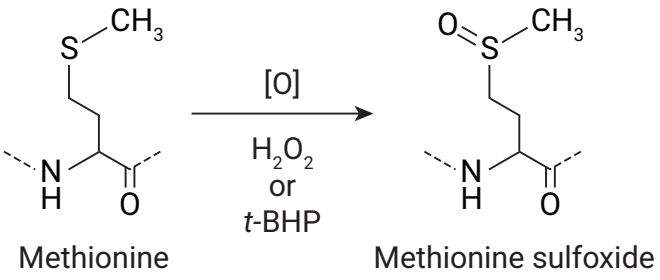


Figure 1. Methionine oxidation induced by chemical oxidant.

Method conditions

	HPLC Conditions
Column	AdvanceBio HIC, 4.6 × 100 mm (p/n 685975-908)
Mobile phase	Eluent A) 50 mM sodium phosphate, pH 7.0 Eluent B) 2 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0
Flow rate	0.3 to 0.5 mL/min
Column temperature	25 °C
Injection volume	5 µL
Final sample concentration	1 mg/mL
Detection	UV, 220 nm
Gradient profile	Flow rate: 0.5 mL/min Time %A %B 0 50 50 20 100 0 25 100 0 30 50 50 40 50 50

Back To Contents Page

Heavy chain

QVTLRESGPA LVKPTQTLTL TCTFSGFSLS TAGMSVGWIR QPPGKALEWL ADIWWDDKKH YNPSLKDRLT
ISKDTSKNQV VLKVTNMDPA DTATYYCARD MIFNFYFDVW GQGTTVTVSS ASTKGPSVFP LAPSSKSTSG
GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS
NTKVDKRVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW
YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ
VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW
QQGNVFSCSV MHEALHNHYT QKSLSLSPGK

Light chain

DIQMTQSPST LSASVGDRVT ITCSASSRVG YMHWYQQKPG KAPKLLIYDT SKLASGVPSR FSGSGSGTEF
TLTISSLQPD DFATYYCFQG SGYPFTFGGG TKVEIKRTVA APSVFIFPPS DEQLKSGTAS VVCLLNNFYP
REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSSTLTL SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC

Figure 2. NISTmAb amino acid sequence.

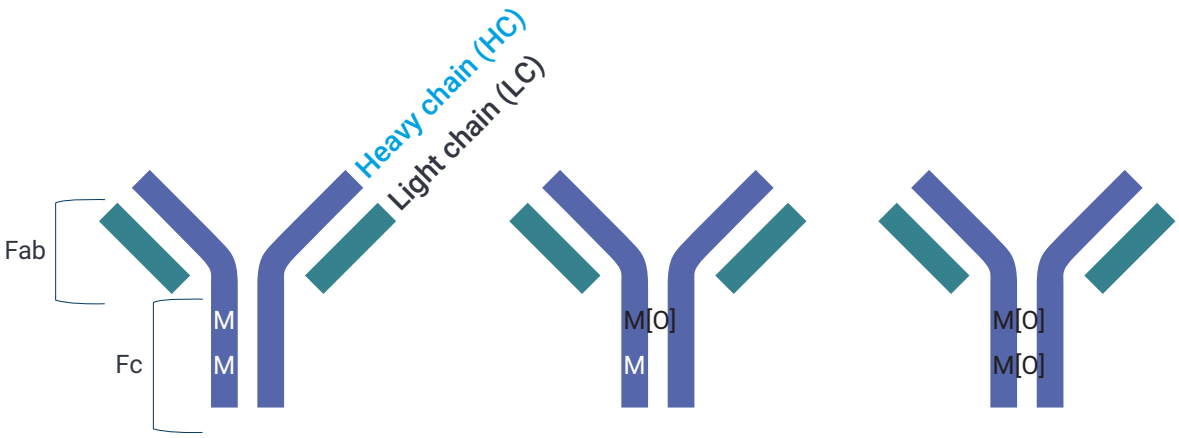
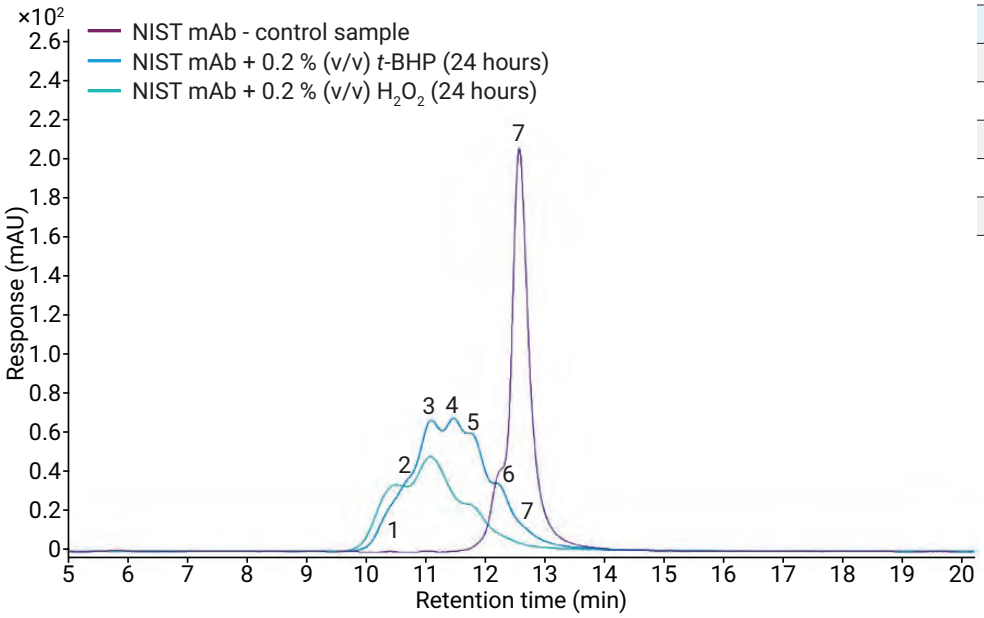


Figure 3. Methionine residues located in Fc region are most susceptible to oxidation in human IgG1 mAbs.

Back To Contents Page

An AdvanceBio HIC column was able to differentiate oxidized mAb variants from the untreated mAb sample under low salt starting conditions. Oxidation of the NISTmAb with t-BHP under reported experimental conditions resulted in multiple peaks with shorter retention times, presumably due to conformational change. The HIC chromatogram (Figure 4) showing earlier retained peaks labeled 1 to 6 likely indicates the result of oxidized Met residues on the mAb, and peak 7 with a retention time of approximately 12.6 minutes, corresponds to nonoxidized mAb. For the H_2O_2-treated mAb sample, complete oxidation occurred, with three peaks eluting in a shorter retention time, indicating more aggressive oxidation of Met residues. These differences in the chromatograms of the IgG1k mAb sample incubated with two different oxidation reagents suggest that reactivity is governed by solvent accessibility of the Met residues and steric limitations of the oxidizing agent, as previously reported[6].

To further improve the resolution, a slower and shallower gradient was used. Using a flow rate of 0.3 mL/min and a starting ammonium sulfate concentration of 1.2 M with a lower gradient rate of 25 mM/min, better resolution was achieved with a relatively short analysis time (Figure 5). In this chromatogram, multiple mAb-oxidized species are clearly observed from the untreated mAb sample.



Time	%A	%B
0	50	50
20	100	0
25	100	0
30	50	50
40	50	50

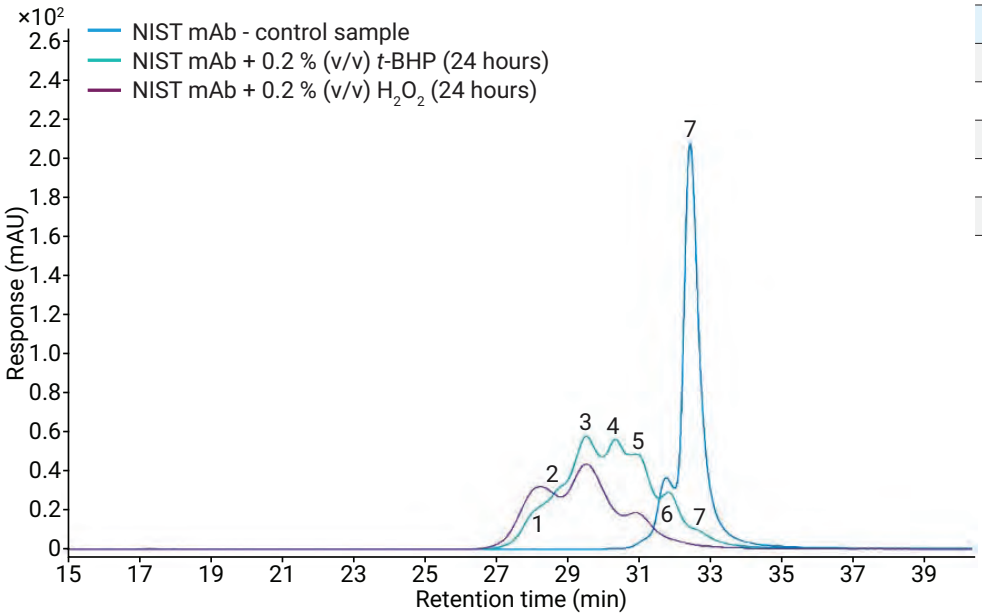
50 mM/min

Figure 4. Separation of oxidized NISTmAb variants using lower starting salt concentration.

Back To Contents Page

In Figure 6, the NISTmAb sample was incubated with 2 % (v/v) t-BHP, and the oxidation reaction was monitored at various time points using shallower gradient conditions. As represented by an overlay of chromatograms, the mAb oxidation progressed with t-BHP incubation time. Multiple mAb oxidation species were observed within a few hours of the oxidation reaction. This suggested that surface-accessible Met residues in both heavy chains of the mAb sample might be oxidized randomly, which was previously reported[5].

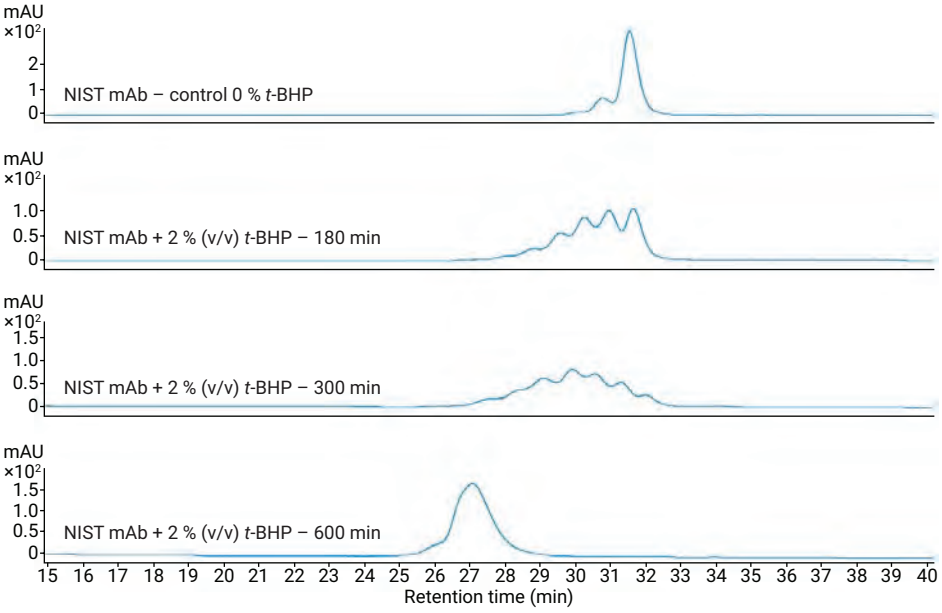
Further oxidation of the mAb sample after 10 hours of reaction led to a broad peak, indicating forced oxidation. It has previously been speculated that oxidation of deeply buried Met residues can lead to a more dramatic structural change, which may cause the mAb to partially unfold[7]. Partially unfolded mAb is likely to have more conformational variation, resulting in a broader peak with a large retention time shift.



Time	%A	%B
0	40	60
40	90	10
45	90	10
50	40	60
60	40	60

25 mM/min

Figure 5. Separation of oxidized NISTmAb variants using a shallow gradient.



Time	%A	%B
0	40	60
40	90	10
45	90	10
50	40	60
60	40	60

25 mM/min

Figure 6. Monitoring the t-BHP oxidized mAb reaction.

Back To Contents Page

Conclusion

The AdvanceBio HIC column demonstrated the separation of oxidized mAb variants from its native form. Using the AdvanceBio HIC column, optimal separation of oxidized mAb variants can be achieved using slower flow rates and shallower gradient conditions, while maintaining relatively short analysis times.

References

1. Zhang, Y.; et al. Hydrophobic interaction chromatography of soluble Interleukin I receptor type II to reveal chemical degradations resulting in loss of potency, *Anal. Chem*. **2008**, *80(18)*, 7022-8.
2. Gaza-Bulseco, G.; et al. Effect of methionine oxidation of a recombinant monoclonal antibody on the binding affinity to protein A and protein G. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2008**, *870(1)*, 56–62.
3. Boyd, D.; Kaschak, T.; Yan, B. HIC resolution of an IgG1 with an oxidized Trp in a complementarity determining region, *J. Chromatogr. B.* **2011**, *879(13–14)*, 955-60.
4. Fekete, S.; et al. Hydrophobic interaction chromatography for the characterization of monoclonal antibodies and related products, *J. Pharm. Biomed. Anal.* **2016**, *130*, 3–18.
5. Chumsae, C.; et al. Comparison of methionine oxidation in thermal stability and chemically stressed samples of a fully human monoclonal antibody. *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci.* **2007**, *850(1–2)*, 285–294.
6. Shen, F. J.; et al. The application of tert-butylhydroperoxide oxidation to study sites of potential methionine oxidation in a recombinant antibody. In *Techniques in Protein Chemistry*; Marshak, D. R., Ed.; Academic Press, Inc.: San Diego, CA, **1996**; 7, 275–284.
7. Liu, D.; et al. Structure and stability changes of human IgG1 Fc as a consequence of methionine oxidation. *Biochemistry* **2008**, *47(18)*, 5088–5100.

Back To Contents Page

Learn more:
www.agilent.com/chem/advancebio

Buy online:
www.agilent.com/chem/store

U.S. and Canada
1-800-227-9770
agilent_inquiries@agilent.com

Europe
info_agilent@agilent.com

Asia Pacific
inquiry_lsca@agilent.com

RA.1719675926

Published in the USA, June 01, 2020
5994-2072EN

Agilent
Trusted Answers