LC GC's CHROMacademy
powered by element

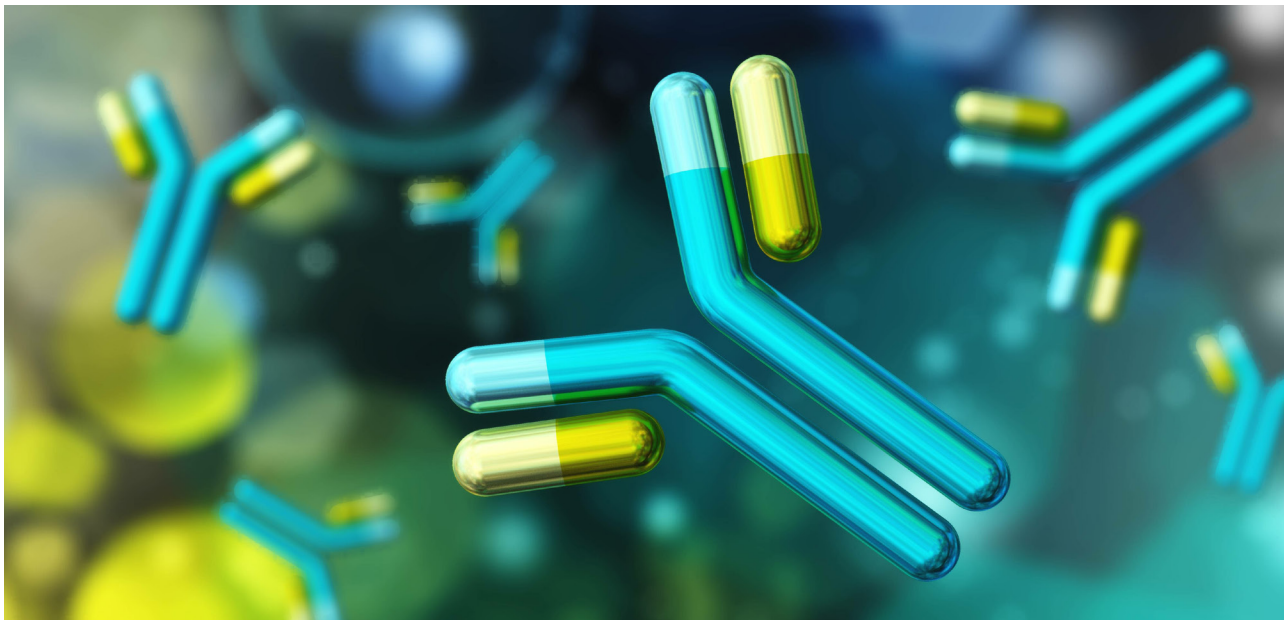
DECEMBER 2022

Simplify Your Lab Operations by Implementing Platform Methods

- mAb Titer Determination in 60 Seconds
- High-throughput and Sensitive Size Exclusion Chromatography
- Separation of Deamidated Peptides
- Improved Workflow for Sialic Acid Profiling and Quantitation

This custom ebook is sponsored by Agilent and presented in partnership with *LCGC's CHROMAcademy*.

Agilent



mAb Titer Determination in 60 Seconds Using the Agilent Bio-Monolith rProtein A Column

By Dr. Te-Wei Chu and Ivan Huang

High-throughput clonal selection for process development and process optimization

INTRODUCTION

The Agilent Bio-Monolith rProtein A (recombinant protein A) analytical column is the latest addition to the Bio-Monolith and affinity chromatography family. The column enables high-speed analysis of monoclonal antibody (mAb) titer and small-scale purification, and can easily be integrated into other analytical workflows, such as 2D-LC. This application note tests the recombinant protein A column at the maximum flow rate and performs the bridging study against the native protein A column. A chromatography bind/elute method for mAb titering is demonstrated, which delivers an ultrafast run time

(1 minute) suitable for high-throughput applications such as clonal selection, process development, and optimization. In the bridging study, performance characteristics such as retention time, linearity and deviation of the standard curve, sample carryover, and recovery showed no detectable difference between the native and the recombinant columns. This work serves to give confidence to those who are transitioning from the native protein A column to the rProtein A column.

EXPERIMENTAL

1. High-throughput mAb titer analysis

Chemicals and reagents

All chemicals and reagents were HPLC grade or higher and were obtained from Sigma-Aldrich (now Merck) or VWR Scientific. Water was purified using a Milli-Q A10 (Millipore).

Sample

The sample was a crude Chinese Hamster Ovary (CHO) cell culture supernatant collected from a bioreactor that contained 1 mg/mL of recombinant IgG monoclonal antibody.

Instrumentation

Agilent 1260 Infinity II Bio-Inert LC comprising:

- 1260 Infinity II Bio-Inert pump (G5654A)
- 1260 Infinity II Bio-Inert multisampler (G5668A) with sample cooler (option 100)
- 1260 Infinity II multicolumn thermostat (G7116A) with Bio-Inert heat exchanger (option 019)
- 1260 Infinity II variable wavelength detector (G7114A)

Method conditions

HPLC Conditions	
Column	Agilent Bio-Monolith rProtein A, 4.95 × 5.2 mm (p/n 5190-6903)
Binding Buffer (Eluent A)	50 mM sodium phosphate, pH 7.4
Eluting Buffer (Eluent B)	100 mM citric acid, pH 2.6
Gradient Profile	Time (min) %B 0.0 to 0.2 0 (binding) 0.3 to 0.65 100 (elution) 0.66 to 0.90 0 (reconditioning) (0.1 min postrun)
Flow Rate	3 mL/min
Column Temperature	25 °C
Detection	UV, 280 nm
Injection Volume	4 µL (10 µg loading)

2. Bridging study

Chemicals and reagents

The same chemicals and reagents as used in the high-throughput mAb titer analysis experiment.

Sample

The samples were crude Chinese Hamster Ovary (CHO) cell culture supernatant collected from a bioreactor that contained 1.5 mg/mL of recombinant IgG monoclonal antibody and purified recombinant IgG monoclonal antibody at the same concentration.

Instrumentation

The Agilent 1290 Infinity II Bio LC system consists of the following components:

- 1290 Infinity II Bio high-speed pump (G7132A)
- 1290 Infinity II Bio multisampler (G7137A)
- 1290 Infinity II multicolumn thermostat with bio heat exchanger (G7116B)
- 1290 Infinity II diode array detector (G7117B) and variable wavelength detector with respective bio flow cell

Method conditions

HPLC Conditions	
Column	Agilent Bio-Monolith rProtein A, 4.95 × 5.2 mm (p/n 5190-6903)
	Agilent Bio-Monolith Protein A, 4.95 × 5.2 mm (p/n 5069-3639)
Binding Buffer (Eluent A)	50 mM sodium phosphate, pH 7.4
Eluting Buffer (Eluent B)	100 mM citric acid, pH 2.6
Gradient Profile	Time (min) %B 0.0 to 0.5 0 (binding) 0.6 to 2.6 100 (elution) 2.7 to 4.0 0 (reconditioning)
Flow Rate	1.5 mL/min
Column Temperature	25 °C
Detection	UV, 280 nm
Injection Volume	5 to 50 µL (25 µg loading)

RESULTS AND DISCUSSION

High-throughput mAb titer analysis

With the high-throughput method, high-speed mAb titering with a 1-minute chromatography run time was demonstrated (FIGURE 1). The retention time of the purified (bound/eluted) mAb was approximately 0.61 minutes, well separated from the impurities peak at ~0.05 minutes containing host cell proteins from the CHO cell culture supernatant. In FIGURE 1, repeated injection of crude supernatant spiked with mAb showed consistent and robust performance of 60 samples/hour throughput with backpressure leveling at 125 bar. Throughout the study, there was no noticeable change in peak shape, retention time, and backpressure. FIGURE 2 showed the chromatograms of different sample loading amounts. A calibration curve

FIGURE 1: Agilent Bio-Monolith rProtein A column: Overlaid chromatograms of 60 consecutive injections. First peak indicates host cell protein impurities in culture supernatant; second peak is purified mAb.

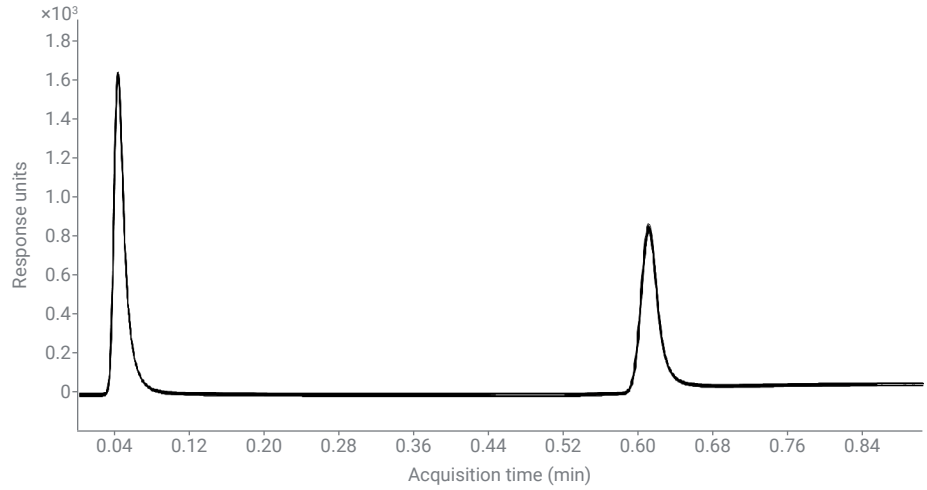
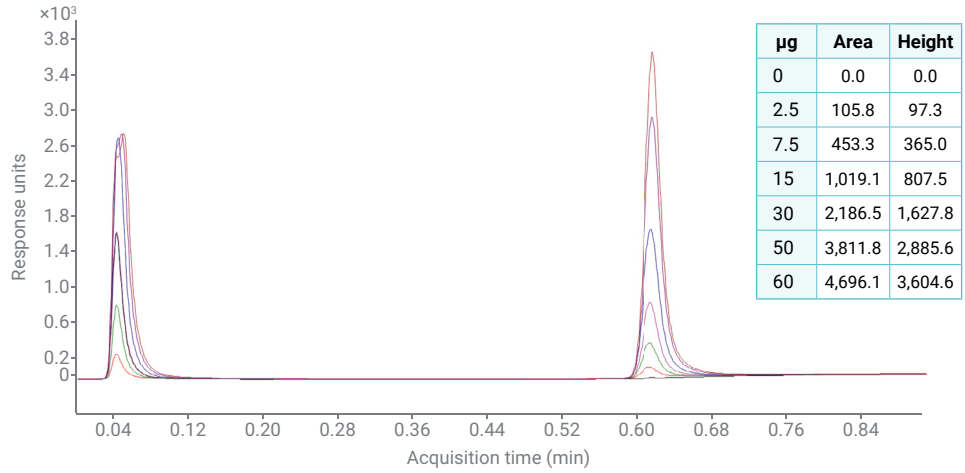
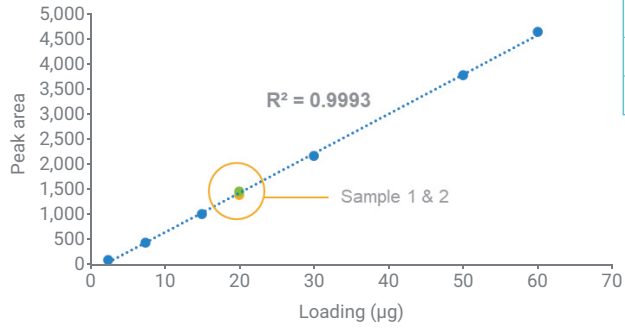


FIGURE 2: Agilent Bio-Monolith rProtein A column: Calibration curve. Overlay chromatograms of increasing sample loading amount for calibration curve generation.



µg	Area	Height
0	0.0	0.0
2.5	105.8	97.3
7.5	453.3	365.0
15	1,019.1	807.5
30	2,186.5	1,627.8
50	3,811.8	2,885.6
60	4,696.1	3,604.6

FIGURE 3: Agilent Bio-Monolith rProtein A column: Standard curve linearity response and % deviation.



Spiked Sample	Measured	% Deviation
20 µg (Sample 1)	19.53 µg	-2.35%
20 µg (Sample 2)	20.51 µg	+2.53%

was then generated by plotting peak area versus injection quantity (FIGURE 3). Results indicated excellent linearity response (R^2 = 0.9993), as shown in the calibration curve, and accurate measurement of mAb quantity from two separate sets of samples. These data demonstrated the feasibility of accurate mAb titer measurement using this fast analysis method.

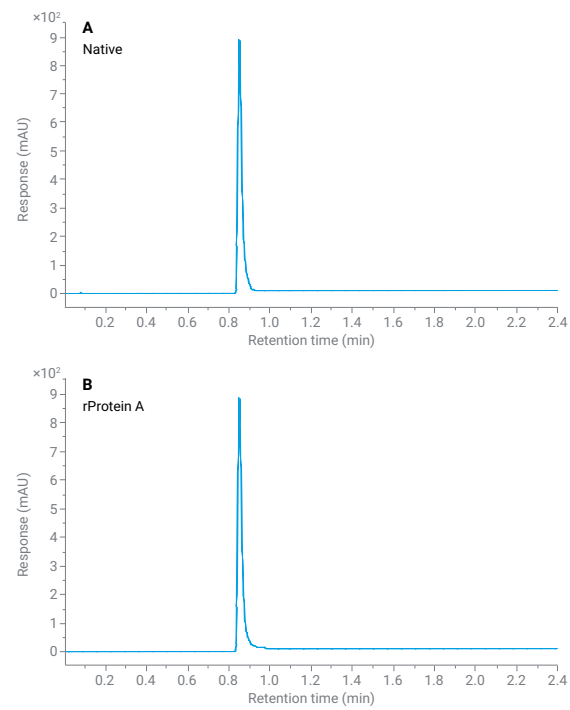
Bridging study

Performance of the two Bio-Monolith protein A columns were tested under the same conditions. All characteristics showed little or no difference between the native and the rProtein A columns, including retention time and peak shape of the purified mAb (FIGURE 4), linearity response of the standard curve and spiked sample recovery (FIGURES 5 AND 6), and sample carryover (FIGURE 7).

Recovery analysis

In addition to comparing the recovery between native and the rProtein A columns,

FIGURE 4: Chromatogram and mAb peak result comparison between Native and rProtein A columns.

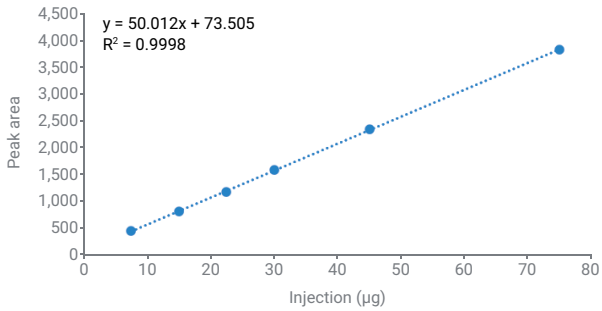


	Native	rProtein A
Retention Time (Min)	0.850 ±0.001	0.850 ±0.001
Peak Height	880.9 ±8.1	881.8 ±5.3

two non-Agilent rProtein A columns were included in this study. Flow rate was adjusted to 2 mL/min to accommodate a non-Aglient column's operating flow rate. Additional mAb samples were included:

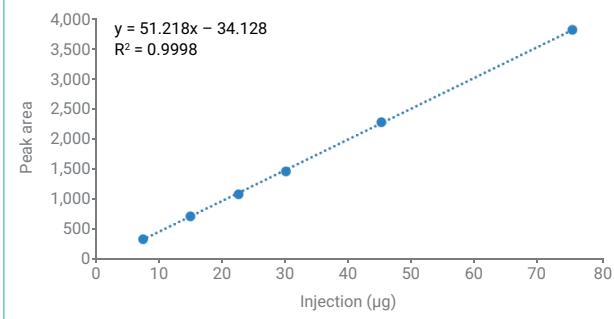
- Agilent-NISTmAb (part number 5191-5744)
- Sigma SiLu mAb from Sigma-Aldrich (SiLu Lite, part number MSQC4)

FIGURE 5: rProtein A column: Linearity response.



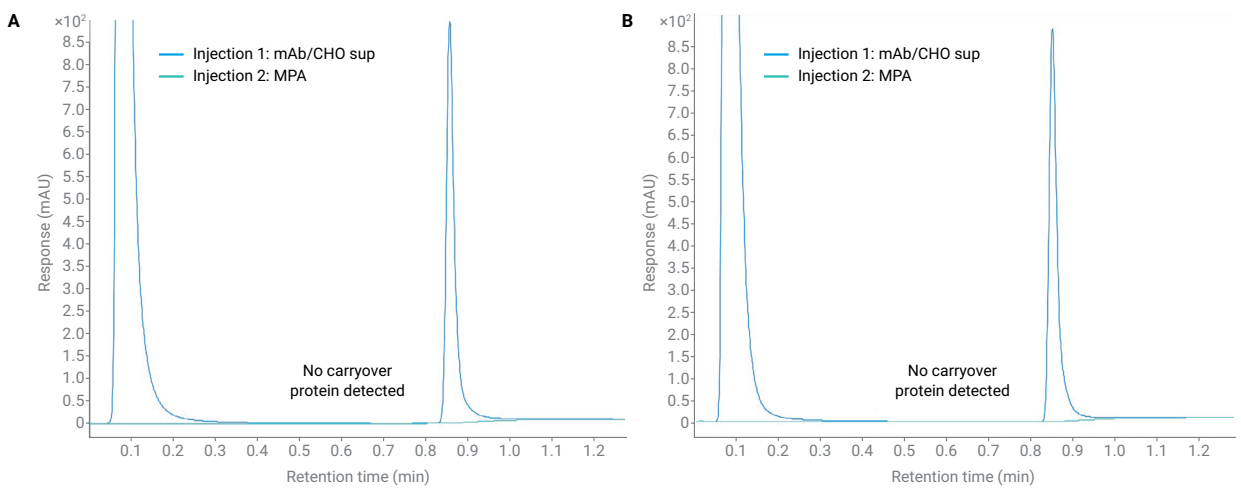
Spiked Sample	Measured	% Deviation
25 µg (pure)	25.25 µg	0.99%
25 µg (in sup.)	26.09 µg	4.35%

FIGURE 5: Native Protein A column: Linearity response.



Spiked Sample	Measured	% Deviation
25 µg (pure)	24.94 µg	−0.23%
25 µg (in sup.)	25.78 µg	3.12%

FIGURE 7: Carryover analysis. Subsequent injection of binding buffer (MPA) showed no detectable protein carryover. (A) rProtein A column. (B) Native Protein A column.



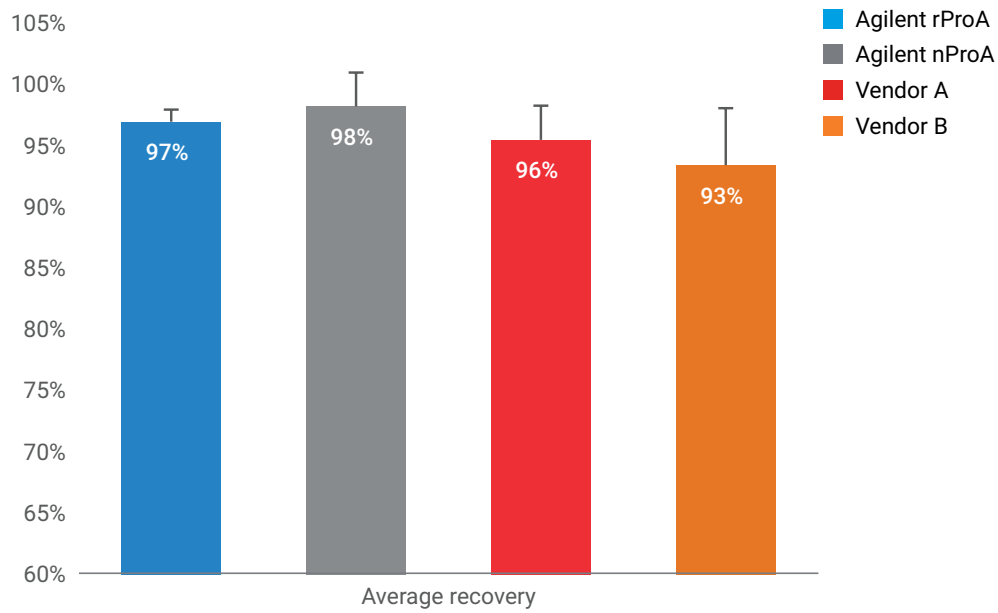
Baseline area under the curve (AUC) of the mAb peak was obtained by injecting purified mAb sample, which was diluted with mobile phase B, without a column (with a union).

The column was applied and AUC of eluted mAb was obtained. The same amount of mAb sample as baseline AUC was used.

Recovery % = (AUC of eluted mAb/Baseline AUC) × 100

FIGURE 8: mAb recovery results comparison.

HPLC Conditions	
Column	Agilent Bio-Monolith rProtein A, 4.95 × 5.2 mm (p/n 5190-6903)
	Agilent Bio-Monolith Protein A, 4.95 × 5.2 mm (p/n 5069-3639) Vendor A & B rProtein A column
Binding Buffer (Eluent A)	50 mM sodium phosphate, pH 7.4
Eluting Buffer (Eluent B)	100 mM citric acid, pH 2.6
Gradient Profile	Time (min) %B 0.0 to 0.4 0 (binding) 0.5 to 1.3 100 (elution) 1.31 to 4.0 0 (reconditioning)
Flow Rate	2 mL/min
Column Temperature	25 °C
Detection	UV, 280 nm
Injection Volume	4 µL (10 µg loading)



Result and analysis

The average recovery of the rProtein A column was 1% lower than the native protein A column but still showed better recovery than the two non-Agilent columns. While the nProtein A column took the slight lead in recovery, it was the rProtein A column that demonstrated the most robust recovery across the three mAb samples.

CONCLUSION

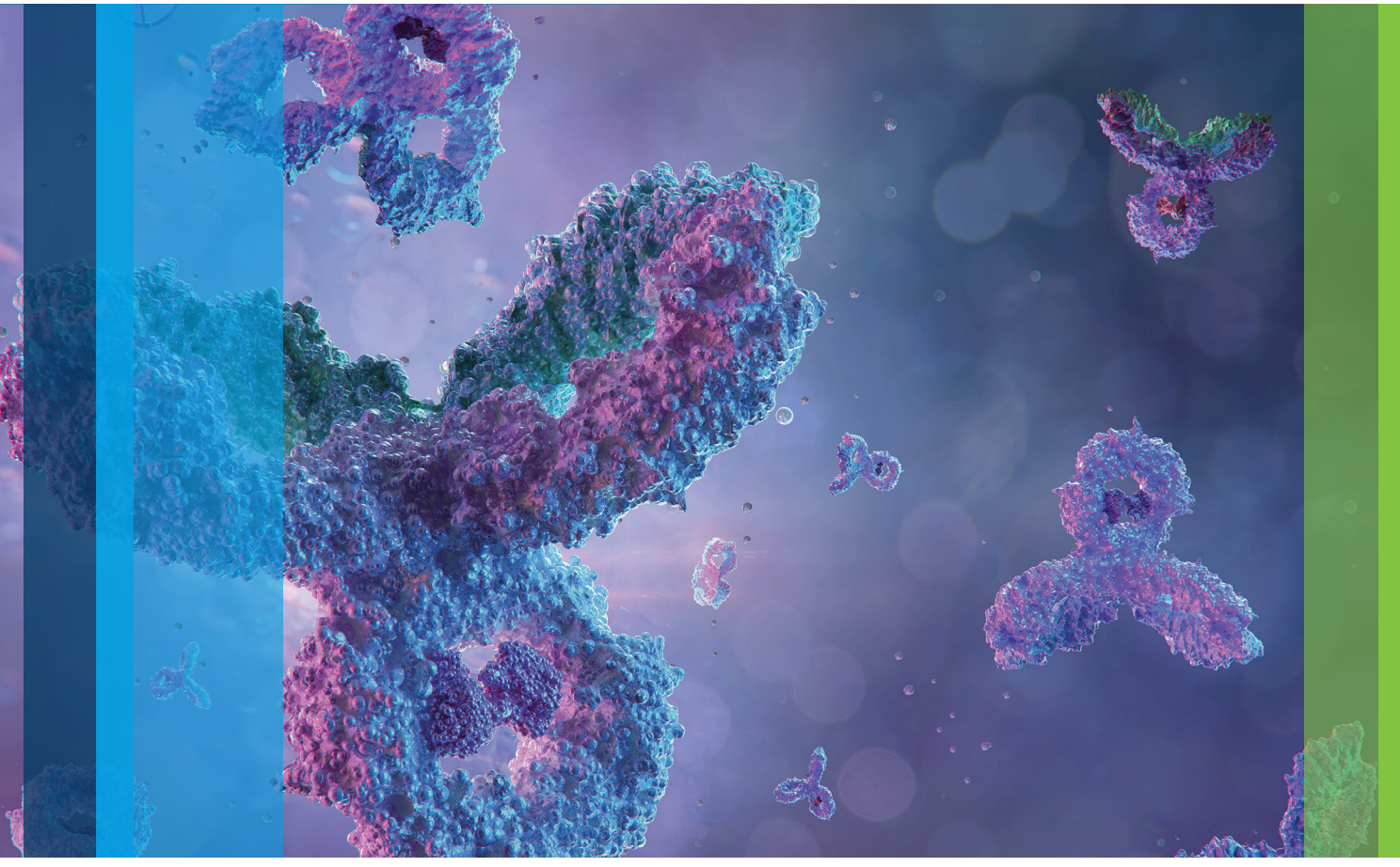
This application note has shown that, at the maximum flow rate of 3 mL/min, the Agilent Bio-Monolith rProtein A column delivers a robust performance. In the second part of this application note, the bridging study between native column and the rProtein A column has demonstrated that rProtein A delivers a similar or equivalent performance to the native protein A column.

Dr. Te-Wei Chu and Ivan Huang

Agilent Technologies Inc.

Agilent

Trusted Answers



Be Agilent Sure in Your CQA Monitoring

Measure what matters

Understanding the attributes of a biologic drug, and the processes used it create it, is critical to ensuring safety, efficacy, and pharmacokinetics.

Agilent AdvanceBio columns deliver results you can count on when analyzing complex biotherapeutic molecules. They can help you confidently monitor CQAs.

www.agilent.com/chem/advancebio

Agilent offers BioHPLC columns for the analysis of:

- Amino acids and cell culture
- Intact and subunits
- Intact using HIC
- Aggregates and fragments
- Charge variants
- Peptide mapping
- Glycans
- Protein titer

High-throughput and Sensitive Size Exclusion Chromatography (SEC) of Biologics Using Agilent AdvanceBio SEC Columns

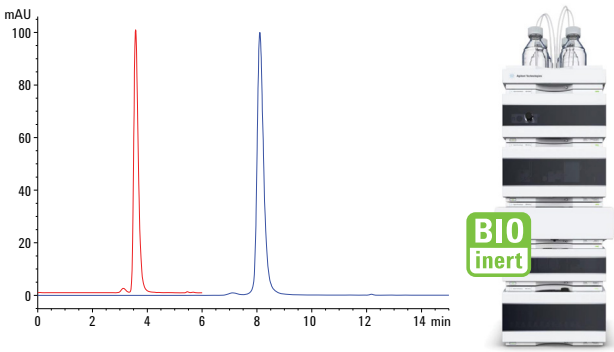
By M. Sundaram Palaniswamy

Agilent AdvanceBio SEC 300 Å, 2.7 µm columns

ABSTRACT

Monoclonal antibody (mAb) aggregation can arise due to multiple mechanisms during cell culture, harvest, purification, storage, and filling. Size Exclusion Chromatography (SEC) is a standard method for size-based separation of mAbs. It is considered as a reference and powerful technique both for qualitative and quantitative assessment of aggregates. SEC analysis of multiple in-process samples not only takes several hours to complete but can also critically influence aggregation status as the samples are held in an unfavorable environment. This study demonstrates the use of shorter and narrower Agilent AdvanceBio SEC columns for fast,

high-resolution, sensitive, and reproducible separation and quantitation of mAbs and Antibody Drug Conjugate (ADC). Separation and quantitation was achieved in less than 4 minutes, and more importantly, this method was able to monitor and detect aggregates created due to stress.



INTRODUCTION

Proteins frequently aggregate when exposed to stress conditions such as changes in pH, temperature, or concentration. Aggregation can occur at many different stages of the production process: upstream, downstream, or simply during storage. Size Exclusion Chromatography (SEC) is a method for monitoring and characterizing aggregates of monoclonal antibodies (mAbs) and antibody drug conjugates (ADCs). However, SEC separations are usually carried out with large columns operated at comparatively low flow rates, and analysis times are often prolonged. More recently, Ultra High Performance Liquid Chromatography (UHPLC) using sub-2 µm columns has been employed to overcome these challenges, demonstrating much shorter analysis times. However, when applying very fine particles and high flow rates, thermal and shearing forces can become critical for temperature or pressure-sensitive proteins [1]. Furthermore, SEC analysis of ADCs using aqueous mobile phase provides poor peak shape and unacceptable resolution between aggregates and monomers. These problems may be explained by nonspecific interactions between hydrophobic cytotoxic drugs and the stationary phase. To solve this problem and improve peak shape, various organic modifiers are added to the SEC mobile phase. However, these organic modifiers could potentially damage the protein and compromise column lifetime.

Agilent has made several advancements in SEC to improve the quality of information that can be obtained. Among them, is the development of shorter (150 mm) and narrower (4.6 mm) columns having an optimum pore volume and pore size, combined with a unique hydrophilic polymer coating. These developments ensure that peaks are well resolved and sharp without the need to add any organic modifiers to the aqueous mobile phase. This application note demonstrates the application of shorter and narrow Agilent AdvanceBio SEC columns for fast high-throughput SEC analysis of mAbs and ADCs. This study also shows the usage of these columns for the quantitation of these molecules.

SPONSORED CONTENT

Agilent AdvanceBio SEC for Peptides, Proteins & ADC Analysis

MATERIALS AND METHODS

Instruments, columns, and standards

A biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC system with a maximum pressure of 600 bar consisting of the following modules was used:

- Agilent 1260 Infinity Bio-Inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-Inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment containing bio-inert click-in heating elements (G1316C, option 19)
- Agilent 1260 Infinity Diode Array Detector with 60 mm Max-Light high sensitivity flow cell (G4212B option 33)
- Agilent AdvanceBio SEC, 300 Å, 7.8 × 150 mm packed with 2.7 µm particles (p/n PL1180-3301)
- Agilent AdvanceBio SEC, 300 Å, 4.6 × 150 mm packed with 2.7 µm particles (p/n PL1580-3301)
- AdvanceBio SEC 300 Å Protein Standard, lyophilized. 1.5 mL (p/n 5190–9417)

Software

Agilent ChemStation B.04.03 (or higher)

SEC parameters

TABLE 1 shows the chromatographic parameters for SEC using an Agilent 1260 Bio-inert LC System.

Reagents, samples, and materials

Innovator and biosimilar rituximab, Herceptin, and ADC were purchased from local pharmacy and stored according to the manufacturer's instruction. Monobasic and dibasic sodium hydrogen phosphate, hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich. All chemicals and solvents used were HPLC grade, and highly purified water from a Milli-Q water purification system (Millipore Elix 10 model, USA) was used.

TABLE 1: Chromatographic parameters used for SEC HPLC.

Parameter	Conditions
Mobile phase	150 mM sodium phosphate, pH 7.0 (mobile phase A)
TCC Temperature	Ambient
Isocratic run	Mobile phase A
Injection volume	5 µL for 7.8 × 150 mm column and 2 µL for 4.6 × 150 mm column
Flow rate	1 mL/min for 7.8 × 150 mm column 0.35 mL/min for 4.6 × 150 mm column
UV detection	220 and 280 nm

Calibration of Agilent AdvanceBio SEC columns

AdvanceBio SEC columns were calibrated by measuring the elution volumes of Agilent 300 Å Protein standards (thyroglobulin (670 KDa), γ-globulin (158 KDa), ovalbumin (44 KDa), myoglobin (17 KDa), and angiotensin II (1,000 Da). The log molecular weight (logMW) values of the AdvanceBio SEC protein standards were

plotted against the elution volumes to determine the exclusion limit.

Limit of quantitation (LOQ) and limit of detection (LOD)

As an example, Herceptin and ADC were used for LOD and LOQ measurements. The protein concentration that provided a signal-to-noise ratio (S/N) > 3 was considered the LOD, and S/N > 10 was considered the LOQ.

Procedure

To calculate area and retention time (RT) deviation, 5 µL and 2 µL of mobile phase was injected as blank, followed by six replicates of intact and stressed mAbs.

Preparation of rituximab aggregates

Innovator rituximab and ADC were used for aggregate analysis as a representative example of mAbs and ADC. Aggregates were induced as described earlier with slight modification [2].

1. 1 M HCl was slowly added drop-wise to the sample solution (2 mg/mL) to change the pH from 6.0 to 1.0.
2. 1 M NaOH was added to adjust the pH to 10.0.
3. 1 M HCl was added again to adjust the pH back to 6.0.

There was a waiting time of approximately 1 minute between the pH shifts, while stirring constantly at 500 rpm. The resulting solution was incubated at 60 °C for 60 minutes.

SPONSORED CONTENT

Analyzing Monoclonal Antibody (mAb) Fragments and Dimers Using Size Exclusion Chromatography (SEC)

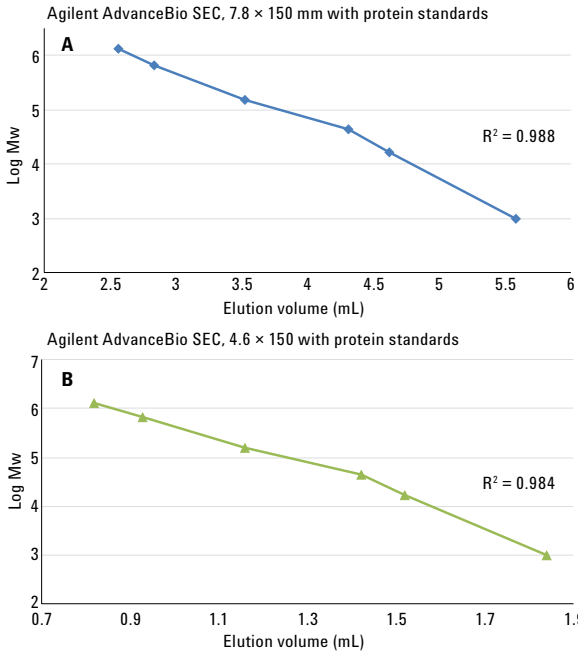
Results and Discussion

Separation and detection

The AdvanceBio SEC columns were calibrated using a series of Agilent 300Å protein standards with known molecular weight. Thyroglobulin aggregates (void peak) in the protein marker were used to calculate the void volume. These aggregates elute at 2.56 minutes on an AdvanceBio SEC, 7.8 × 150 mm column, with a void volume (V_0) = 2.56 mL, and 2.35 minutes on 4.6 × 150 mm column, corresponding to a V_0 = 0.805 mL, respectively. The calibration curve for proteins separated on the AdvanceBio SEC column shows a linear relationship, and defines the exclusion limit (670 kDa) and total permeation limit (1,000 Da) for the protein range analyzed. The molecular weight of an unknown protein can then be determined from its elution volume using this plot (FIGURE 1).

The objective was to improve the analysis throughput by reducing the run time. The flow rate determines run time in SEC for a given column dimension, however, at higher flow rates, resolution could be compromised. To achieve faster run time, the ratio of column void volume to the flow rate needs to be

FIGURE 1: Calibration curve for protein standards on 7.8 × 300 mm (A) and 4.6 × 150 mm (B) Agilent AdvanceBio SEC 300Å columns.



decreased. Reducing the column length and increasing the flow rate is the straightforward approach for faster SEC [2,3]. FIGURE 2 shows the SEC chromatographic profiles of rituximab biosimilar and innovator, Herceptin, and ADC on an AdvanceBio SEC, 7.8 × 150 mm column. These chromatograms demonstrate excellent separation of the monomer in less than 4 minutes under chromatographic conditions.

To achieve better sensitivity, the separation was carried out on an AdvanceBio SEC, 4.6 × 150 mm column. FIGURE 3 shows that this resulted in superior separation performance.

In both the cases, the absence of an early or late eluting peak suggests that the marketed mAb preparation is homogenous without any indication of aggregation or degradation. Analysis of hydrophobic ADC with aqueous mobile phase using both these columns resulted in a symmetrical peak, indicating no secondary interactions of the hydrophobic payload with the stationary support. The shorter AdvanceBio SEC columns were able to separate and resolve ADC aggregates. This separation indicates its suitability to characterize ADC with sufficient information to support development, lot release, and stability studies.

Precision of RT and area

To establish the method precision, relative standard deviation (RSD) values for RT and area of all four biologics were calculated at a 10 µg on-column concentration for 7.8 × 150 mm, and 4 µg for 4.6 × 150 mm columns. TABLE 2 displays the average RTs and area RSDs from six replicates of samples. The highest observed area RSD value was 0.21%, and RT RSD was 0.02%. The area and retention time RSDs demonstrate excellent reproducibility of the method, and thus the precision of the system.

Quantification of Herceptin and ADC using shorter and narrow Agilent AdvanceBio SEC columns

LOD and LOQ

The on-column LOD and LOQ using 7.8 × 150 mm and 4.6 × 150 mm columns for Herceptin and ADC are summarized in

FIGURE 2: SEC chromatographic profiles of native rituximab innovator and biosimilar, Herceptin, and ADC on an Agilent AdvanceBio SEC, 300Å, 7.8 × 150 mm, 2.7 µm column.

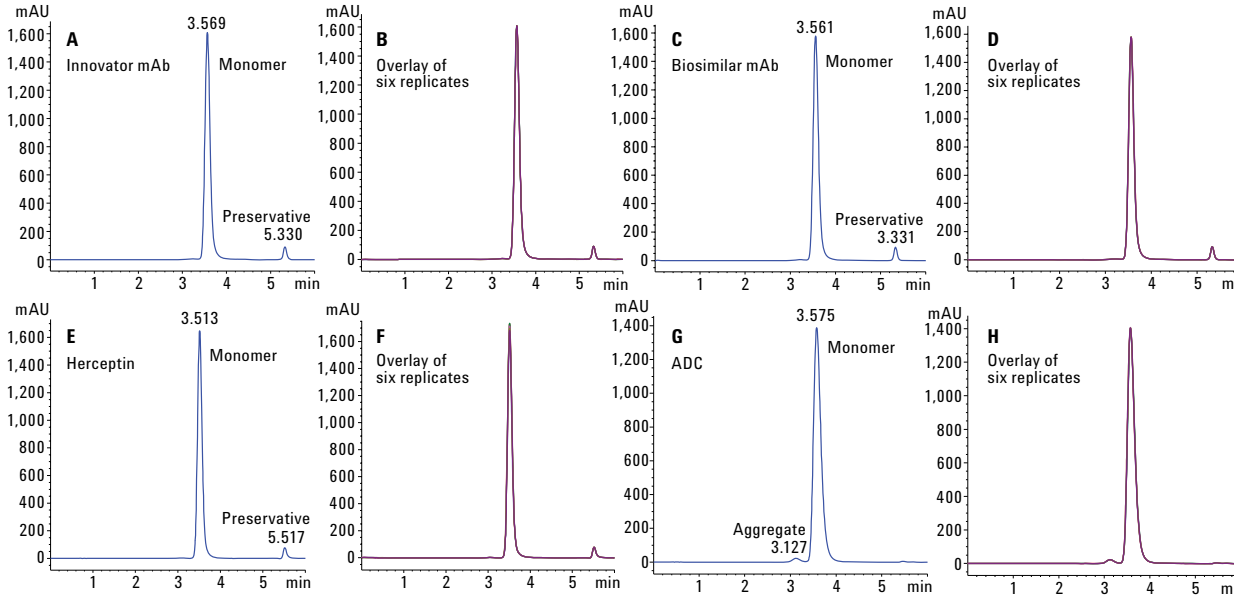


FIGURE 3: SEC chromatographic profiles of native rituximab innovator and biosimilar, Herceptin, and ADC on an Agilent AdvanceBio SEC, 300Å, 4.6 × 150 mm, 2.7 µm column.

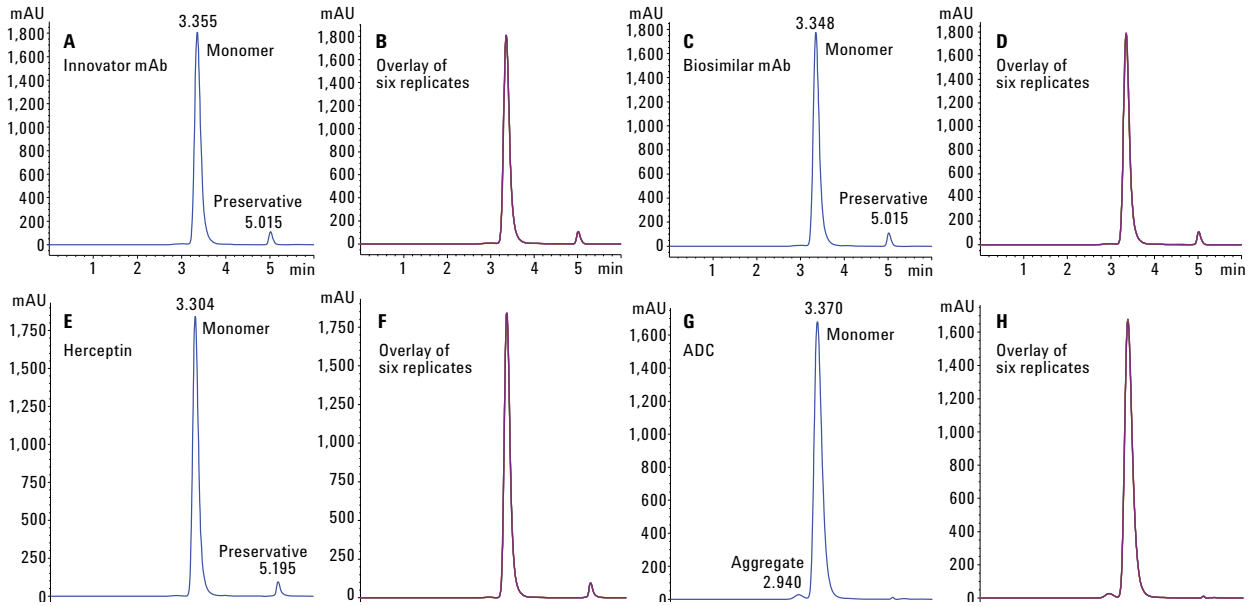


TABLE 3. FIGURE 4 shows an overlay of LOD and LOQ chromatograms with the blank for the Herceptin and ADC samples. Note that the narrow 4.6 × 150 mm column provided a sensitive analysis of the biologics.

TABLE 2: RT and peak area precision (n = 6) of samples.

	Agilent AdvanceBio SEC, 300Å, 7.8 × 150 mm, 2.7 µm		Agilent AdvanceBio SEC, 300Å, 4.6 × 150 mm, 2.7 µm	
Sample	RT RSD	Peak area RSD	RT RSD	Peak area RSD
Rituximab innovator	0	0.15	0.02	0.02
Rituximab biosimilar	0	0.04	0.01	0.01
Herceptin	0	0.21	0.01	0.02
ADC	0	0.01	0	0.02

TABLE 3: LOD and LOQ values for samples.

	Agilent AdvanceBio SEC, 300Å, 7.8 × 150 mm, 2.7 µm		Agilent AdvanceBio SEC, 300Å, 4.6 × 150 mm, 2.7 µm	
Sample	LOD	LOQ	LOD	LOQ
Herceptin	78 ng	156 ng	31.2 ng	62.4 ng
ADC	78 ng	156 ng	31.2 ng	62.4 ng

Linearity

The observed linearity curves for Herceptin and ADC were constructed from the LOQ level to the highest concentration level in the study using area response and concentration of Herceptin/ADC. FIGURE 5 shows the linearity curve for Herceptin and ADC in the concentration range of 15.6 to 2,000 µg/mL on both columns. The observed regression coefficient (R^2) for the mAbs and ADC indicate that the method is quantitative in the range analyzed.

Aggregation/degradation analysis

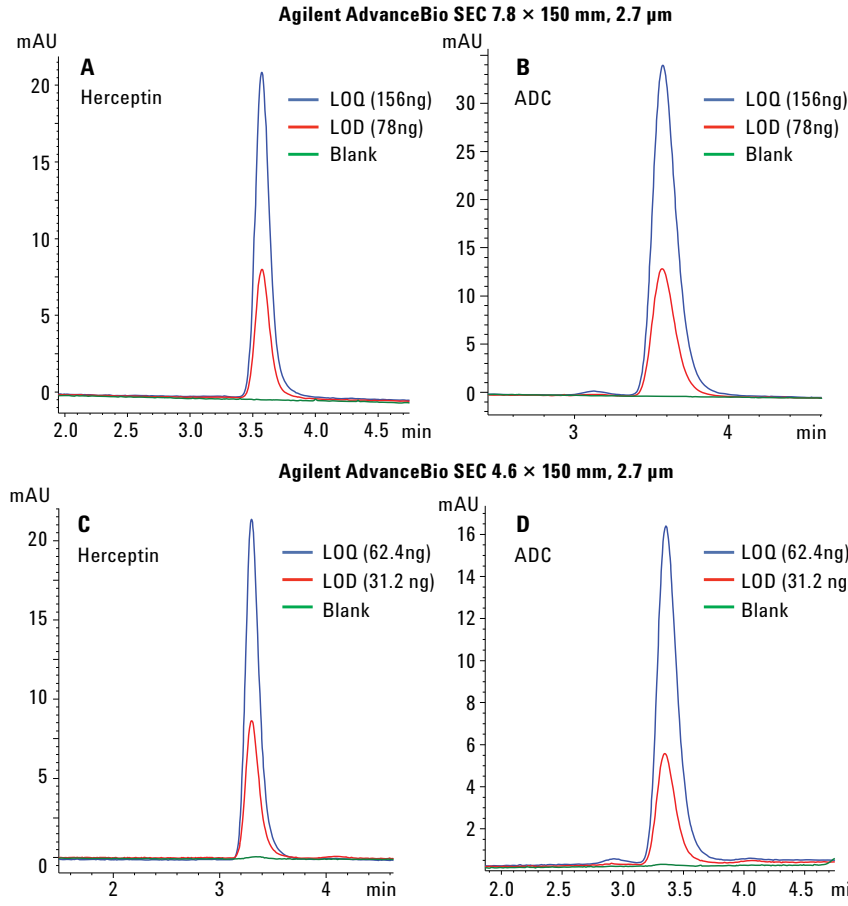
The control of protein aggregation is always a concern during the purification, formulation, and manufacture of protein products. We compared the native and forced-stress Herceptin and ADC between AdvanceBio SEC columns for monitoring aggregates and degradants. Any peaks from the chromatographic run eluting before the monomeric form were considered as aggregates, and any eluting later were considered as fragments/degradants.

The chromatograms of pH/heat-induced aggregates show that both of these AdvanceBio SEC columns were able to separate and detect aggregates and fragments. Monomer, aggregates, and degradants were distinctly and well separated from each other, as shown in FIGURES 6 AND 7. There was also a significant decrease in the peak height of monomer as a result of stress (data not shown).

CONCLUSION

SEC is widely used for the characterization of protein aggregates. Due to their potential immunogenicity, the aggregate levels have to be thoroughly controlled during production of recombinant proteins. This study demonstrates the use of shorter and narrower Agilent AdvanceBio SEC columns with 2.7 µm particles for the

FIGURE 4: SEC chromatographic profiles for LOD and LOQ of Herceptin and ADC overlaid with the blank for the 7.8 × 150 mm and 4.6 × 150 mm Agilent AdvanceBio SEC 300Å columns.



high-throughput separation of monomers, aggregates, and fragments in less than 4 minutes. AdvanceBio SEC columns were able to provide superior peak shapes of hydrophobic ADC without using organic modifiers in the mobile phase. Area and RT precision was excellent, and demonstrate the reliability of the method for routine use. The method was also quantitative and accurate, as determined by the coefficient of linearity values for the concentration range evaluated. Finally, the AdvanceBio SEC shorter and narrower columns provided certainty of monitoring aggregates and fragments based on forced-stress studies. These results indicate that these columns are suitable for applications where high-throughput and sensitivity are required.

FIGURE 5: Linearity curve with eight standard concentrations of Herceptin and ADC on Agilent AdvanceBio SEC columns ranging from 15.6 to 2,000 µg/mL, showing excellent coefficient values.

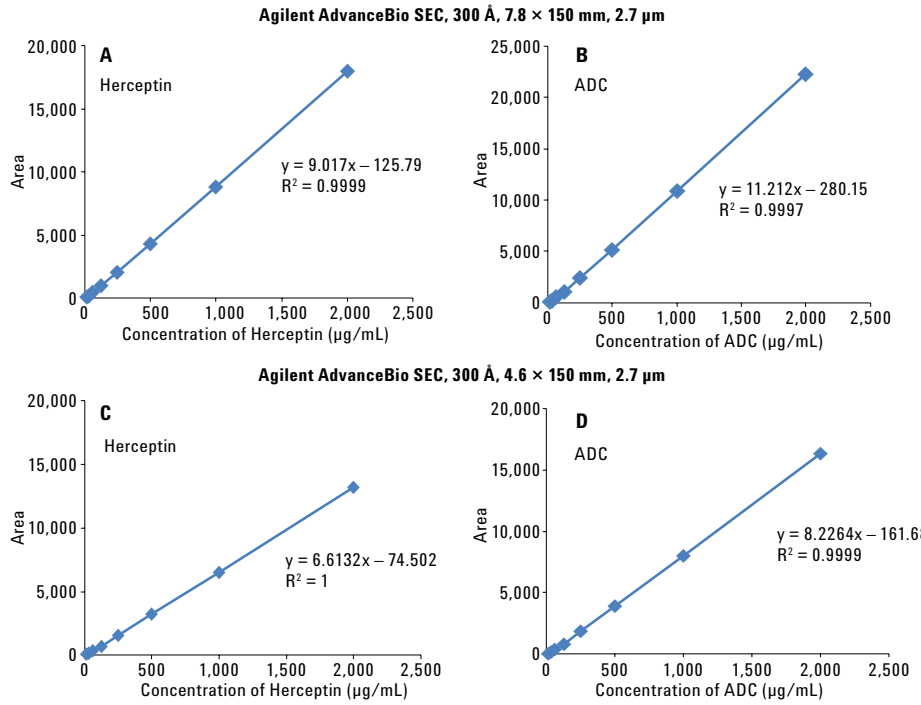


FIGURE 6: Chromatogram of native (control, red trace) innovator mAb, Herceptin, and ADC overlaid with heat/pH stressed (blue trace) using an Agilent AdvanceBio SEC 300 Å, 7.8 × 150 mm, 2.7 µm column.

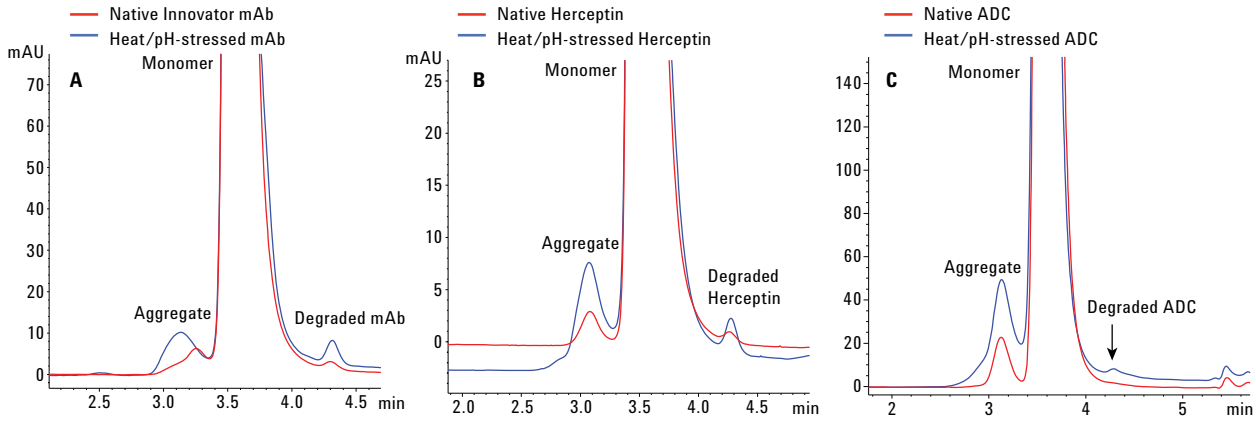
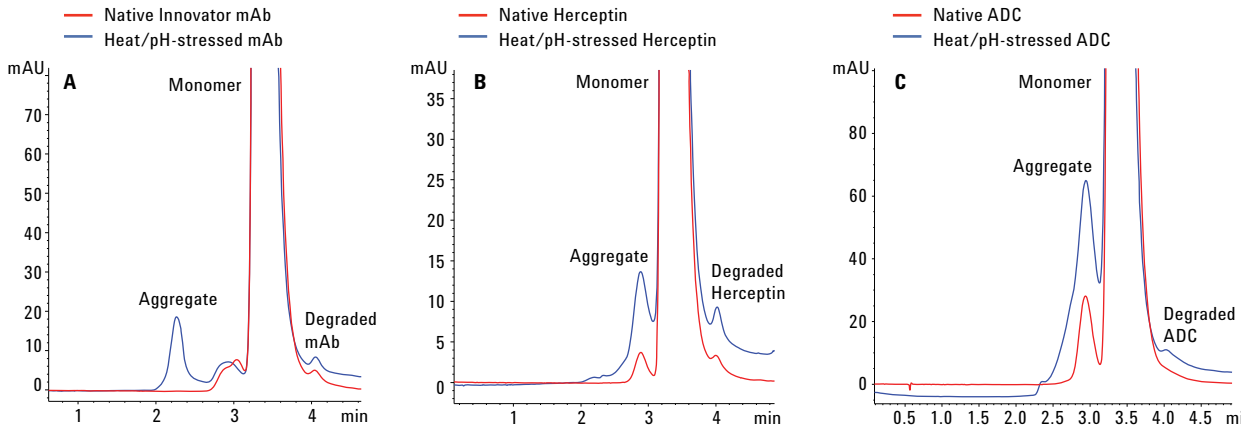


FIGURE 7: Chromatogram of native (control, red trace) innovator mAb, Herceptin, and ADC overlaid with heat/pH stressed (blue trace) using an Agilent AdvanceBio SEC 300 Å, 4.6 × 150 mm, 2.7 µm column.



REFERENCES

1. Fekete, S.; Beck, A.; Veuthey, J-L.; Guillarme, D. Theory and practice of size exclusion chromatography for the analysis of protein aggregates. *J. Pharm. and Biomed. Anal.* **2014**, *101*, 161–173.
2. Kükrer, B.; Filipe, V.; van Duijn, E.; Kasper, P. T.; Vreeken, R. J.; Heck, A. J. R.; Jiskoot, W. Mass Spectrometric Analysis of Intact Human Monoclonal Antibody Aggregates Fractionated by Size-Exclusion Chromatography. *Pharmaceutical Research* **2010**, *27*, 2197–2204.
3. Coffey, A. *Fast, High-Resolution Size Exclusion Chromatography of Aggregates in Biotherapeutics*; Application note, Agilent Technologies, Inc. Publication number 5991-6458EN, **2015**.

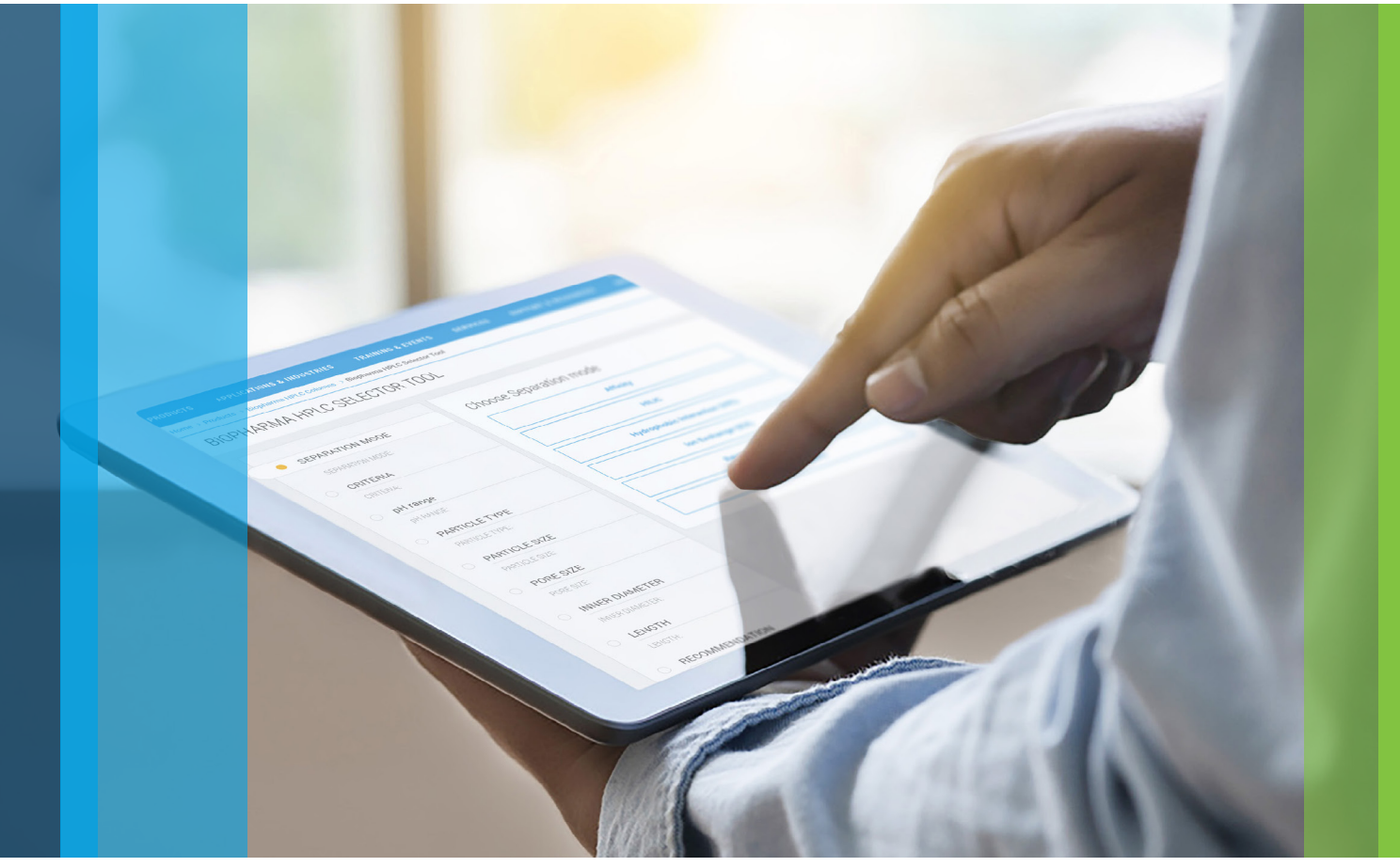
For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.

M. Sundaram Palaniswamy
Agilent Technologies Inc.

Agilent

Trusted Answers



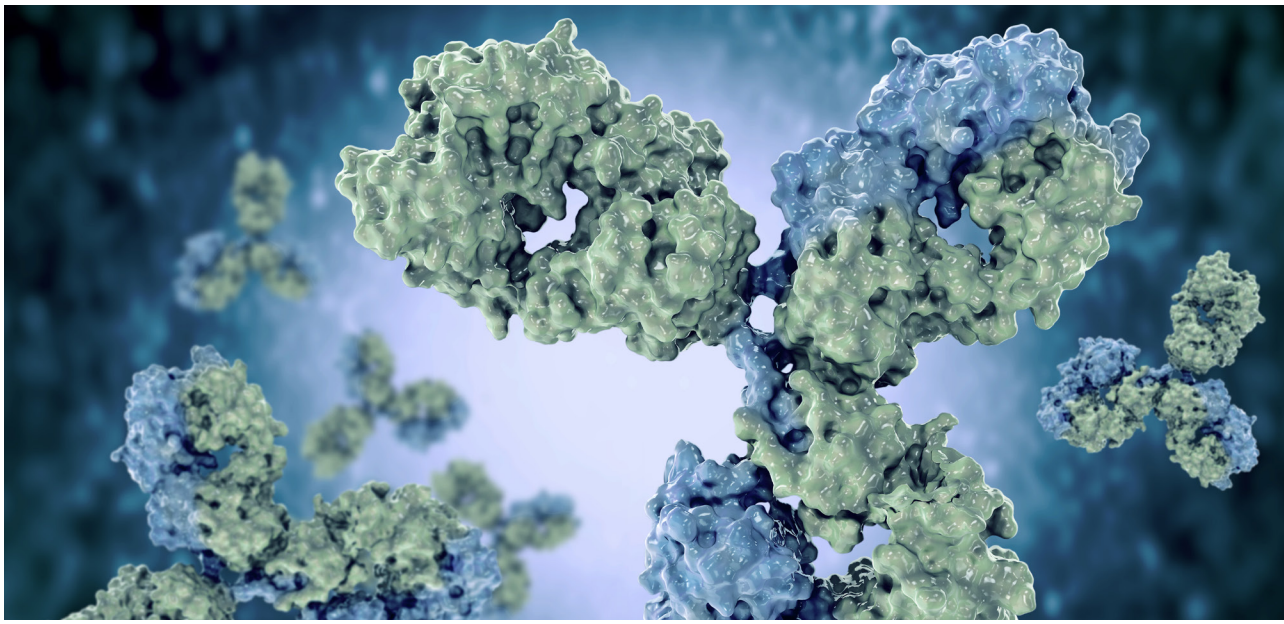
Easily and Quickly Find the Right Biopharma HPLC Column for Your Needs

Agilent AdvanceBio HPLC columns are designed and produced to provide results you can be sure of when analyzing highly complex biotherapeutic molecules and monitoring their purity, potency, and other critical quality attributes.

Our interactive eSelector tool helps easily identify an appropriate column by desired phase chemistry, application, or other manufacturer's part number.

Try the biopharma HPLC column selector tool:

agilent.com/chem/eselectortools



Separation of Deamidated Peptides with an Agilent AdvanceBio Peptide Plus Column

By Oscar Potter and Veronica Qin

ABSTRACT

Deamidations of glutamine and asparagine are amongst the most common degradations affecting proteins. However, analysis of deamidation by mass spectrometry is challenging due to the small mass shift of less than 1 Da versus the unmodified form. Site-specific deamidation is often determined by protease digestion followed by LC/MS analysis, but even this approach can fail when the unmodified and deamidated forms are not chromatographically resolved. Fortunately, a charged surface C18 column dramatically improves the resolution of deamidated peptides from their unmodified variants. Furthermore, mobile phase optimization can provide additional control over the resolution of these analytes.

INTRODUCTION

Analysis of protein deamidation is important for multiple stages of biopharmaceutical drug development and production. From an analytical perspective, deamidation eliminates an amide group and introduces a new carboxylic acidic group, potentially enabling analysis by charge-based methods such as IEX and isoelectric focusing.[1] However, these techniques do not always resolve deamidated products and cannot confirm deamidation at a given site within the protein. Therefore, many analysts perform a protease digest of the sample and analyze the resultant peptides by reversed-phase LC/MS. Deamidation of asparagines or glutamines is identified by a mass increment of 0.9840 Da versus the unmodified form of the peptide. In some cases, peptides containing these degraded sites are well-separated, and relative quantification can be performed based on the relative signal intensity of the different forms.[2] However, deamidated peptides sometimes coelute with their nondeamidated forms since conversion of asparagine/glutamine to their corresponding carboxylic acids does not result in a large change in hydrophobicity at low pH.[3] Such coelutions result in an overlap of the deamidated peptide signal with the highly abundant ^{13}C isotopes of the unmodified form, as illustrated in FIGURE 1. This can impact the quantitation of deamidation, and in some cases, may even prevent detection of the deamidated variant.

This application note demonstrates that a charged surface C18 column greatly enhances selectivity for deamidated variants of peptides versus their unmodified forms when compared to a traditional C18 column. This increases confidence in the ability to detect and quantify deamidation at the peptide level.

Based on Agilent superficially porous Poroshell technology, Agilent AdvanceBio Peptide Plus columns feature a hybrid, endcapped C18 stationary phase on a 120 Å pore size, 2.7 µm particle modified to have a positively charged surface. This provides alternative selectivity compared to traditional C18 columns

SPONSORED CONTENT

Agilent AdvanceBio Peptide Plus 2.7 µm Column for Peptide Characterization

EXPERIMENTAL

Materials

The mAb sample was expressed and purified from chinese hamster ovary cells. The sample was digested by trypsin, adjusted to a pH of approximately 11 using reagent-grade ammonium hydroxide (Sigma-Aldrich), and incubated for 4 hours at 60 °C to accelerate deamidation. LC/MS-grade formic acid (part number 533002) and acetonitrile (part number 900667) were also purchased from Sigma-Aldrich.

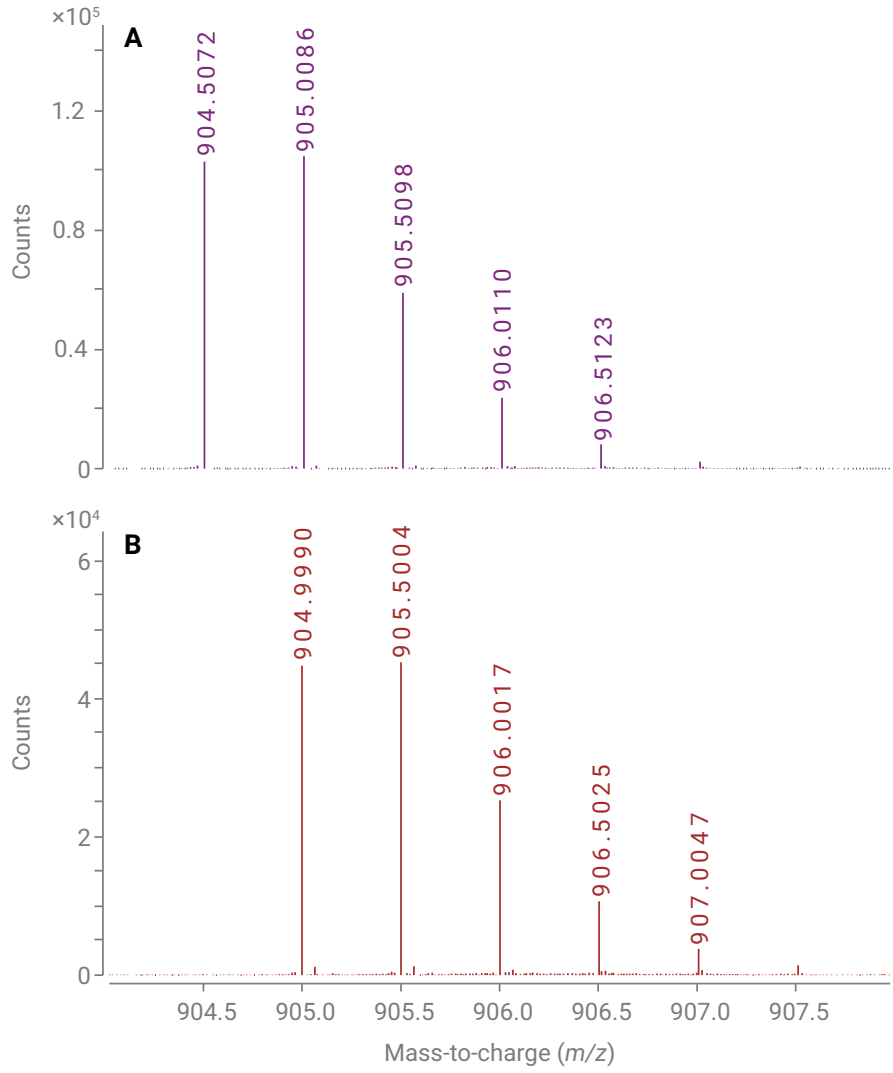
Instrumentation

LC system

An Agilent 1290 Infinity II LC system with the following configuration was used:

- Agilent 1290 Infinity II binary pump (G4220A)
- Agilent 1290 Infinity II autosampler (G4226A)
- Agilent 1290 Infinity II thermostatted column compartment (G1316C)

FIGURE 1: Mass spectrum of VVSVLTVLHQDWLNGK (A) and a deamidated variant of that peptide (B), showing the overlap between mass spectra.



MS system
Agilent 6546 LC/Q-TOF

Data processing
LC/MS data were processed by Agilent MassHunter BioConfirm software (version 10.0 SP1) and MassHunter Qualitative Analysis software (version 10.0).

Results and discussion
Five peptides in the mAb digest showing partial deamidation were identified, shown in TABLE 1. These peptides were used to investigate how the choice of column and mobile phase affects the separation of the unmodified peptide from its deamidated variants.

TABLE 1.

Peptide	Sequence (Nondeamidated Form)	m/z of $[M+2H]^{2+}$
A	NQVSLTCLVK	581.8103
B	FNWYVDGVEVHNAK	839.4047
C	VVSVLTVLHQDWLNGK	904.5071
D	NTAYLQMNSLR	655.8300
E	GLEWVGYIDPSNGETTYNQK	1136.0323

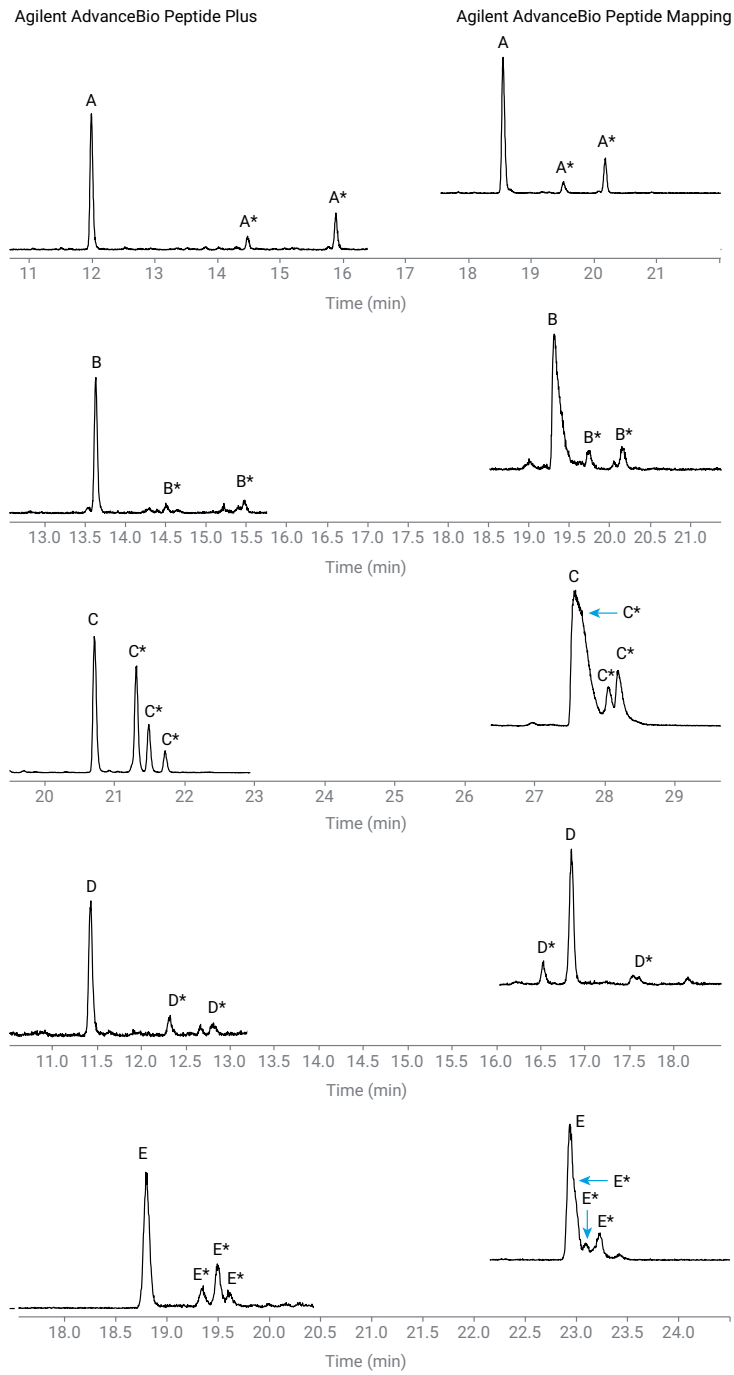
Column type
FIGURE 2 shows the separation of peptides and their deamidated variants on two different C18 columns in a typical LC/MS analysis method using a formic acid-modified water/acetonitrile gradient.

LC/MS conditions

Parameter	Agilent 1290 Infinity II LC
Column	Agilent AdvanceBio Peptide Plus, 2.1 × 150 mm, (p/n 695775-949) Agilent AdvanceBio Peptide Mapping column, 2.1 × 150 mm, (p/n 653750-902)
Column Temperature	60 °C
Mobile Phase	A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile
Flow Rate	0.4 mL/min
Gradient	Time (min) % B 0 3 2 3 40 40 50.5 100 53 3
Post Time	7 minutes
Injection Volume	3 µL

Parameter	Agilent 6546 Q-TOF
Source	Agilent Jet Stream
Gas Temperature	323 °C
Drying Gas Flow	13 L/min
Nebulizer Gas	35 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Fragmentor	125 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	*m/z* 300 to 1,700
MS Scan Rate (spectra/s)	5
Acquisition Mode	Positive mode, extended dynamic range (2 GHz) Centroid data format

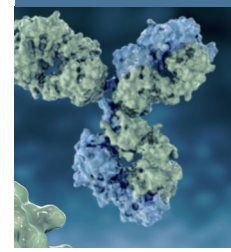
FIGURE 2: Separation of peptides and their deamidated variants (indicated by *) on the Agilent AdvanceBio Peptide Mapping column (elevated and eluting later) and the Agilent AdvanceBio Peptide Plus column (eluting earlier) under the same conditions with 0.1% formic acid mobile phase modifier.



The AdvanceBio Peptide Mapping column uses an endcapped C18 silica based on 2.7 µm superficially porous particles with a 120 Å pore size. On this column, deamidated variants typically eluted slightly later than the unmodified form. At least two deamidated variants are detected in each case, likely representing conversion of asparagine into aspartate and isoaspartate. However, in the case of peptide D, one deamidated variant elutes before the unmodified form, while for peptides C and E, a deamidated variant coelutes with the unmodified form. These findings demonstrate that a standard C18 column will not resolve deamidated variants from their unmodified forms in a significant minority of cases.

Meanwhile, the AdvanceBio Peptide Plus column incorporates a positively charged surface on the same type of particle with similar C18 functionalization and endcapping. On this column, all deamidated variants were well resolved from the unmodified form. Furthermore, all deamidated variants of all five peptides eluted later than their modified forms.

A likely explanation for the difference in behavior between the two columns starts with the observation that the positively charged C18 phase is less retentive for peptides in general versus the standard C18 phase. This reduced retention may result from ionic interactions with peptides since they generally carry a positive charge in the presence of 0.1% formic acid.[4] This retention-reducing effect is stronger for highly basic peptides, and becomes less significant on peptides with greater numbers of acidic amino acids. Since deamidation introduces an additional acidic group, deamidated peptide variants are less basic than their unmodified forms and therefore show greater retention on the charged column.



SPONSORED CONTENT

Analysis of a Synthetic Peptide and Its Impurities

The enhanced selectivity for deamidated peptide variants versus their unmodified forms greatly reduces the chance of a coelution between these analytes on the charged column, thereby avoiding any challenges that would arise from their overlapping mass spectra.

Mobile phase

While all five peptides were well-resolved from their deamidated variants on the AdvanceBio Peptide Plus column in 0.1% formic acid, these separations are also greatly affected by mobile phase choice.

FIGURE 3 shows the separation of peptide C from its variants when the aqueous and

FIGURE 3: Separation of peptides on Agilent AdvanceBio Peptide Plus with increasing concentrations of formic acid mobile phase modifier. Selectivity for deamidated peptide variants over their native forms is maximized at lower concentrations.

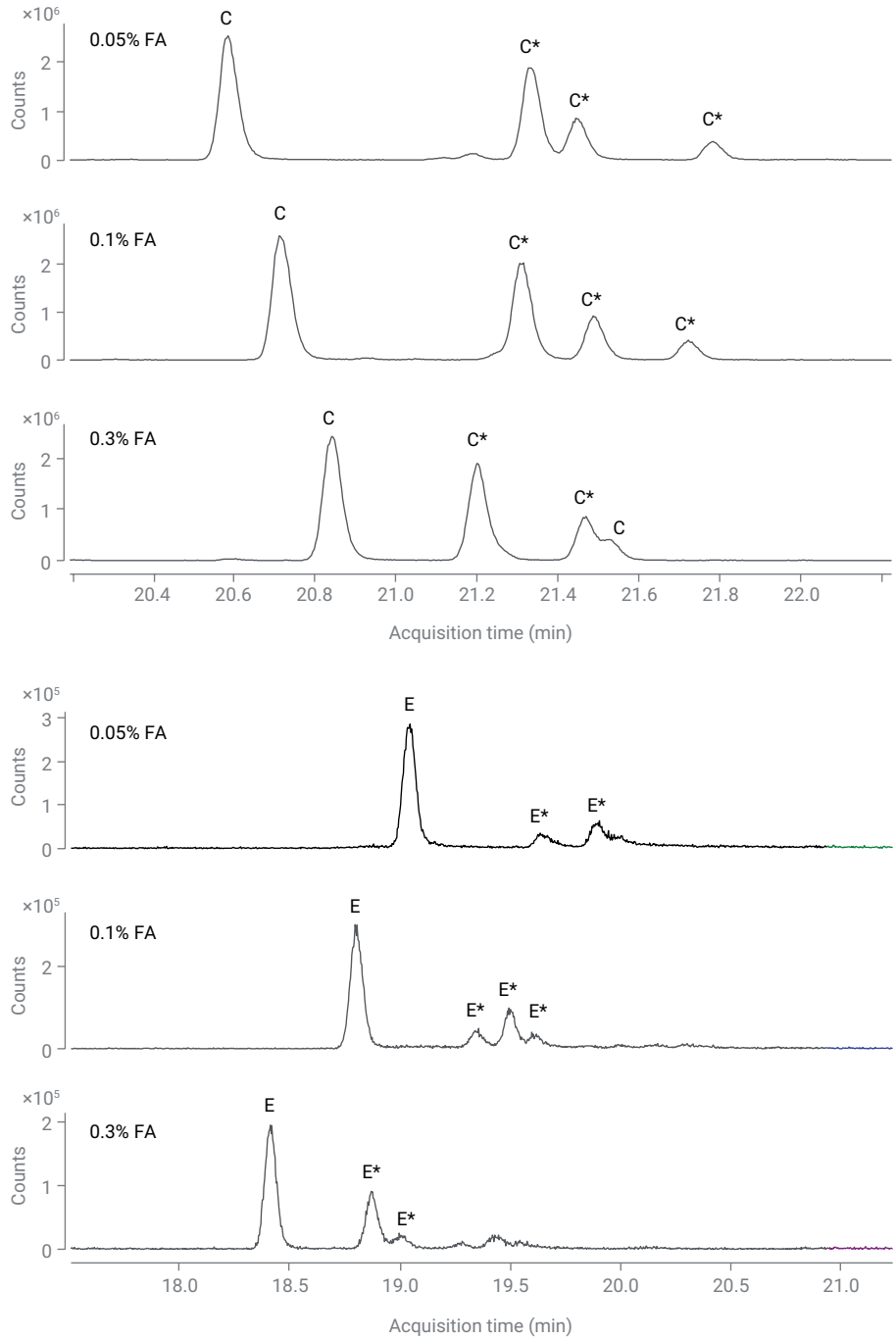
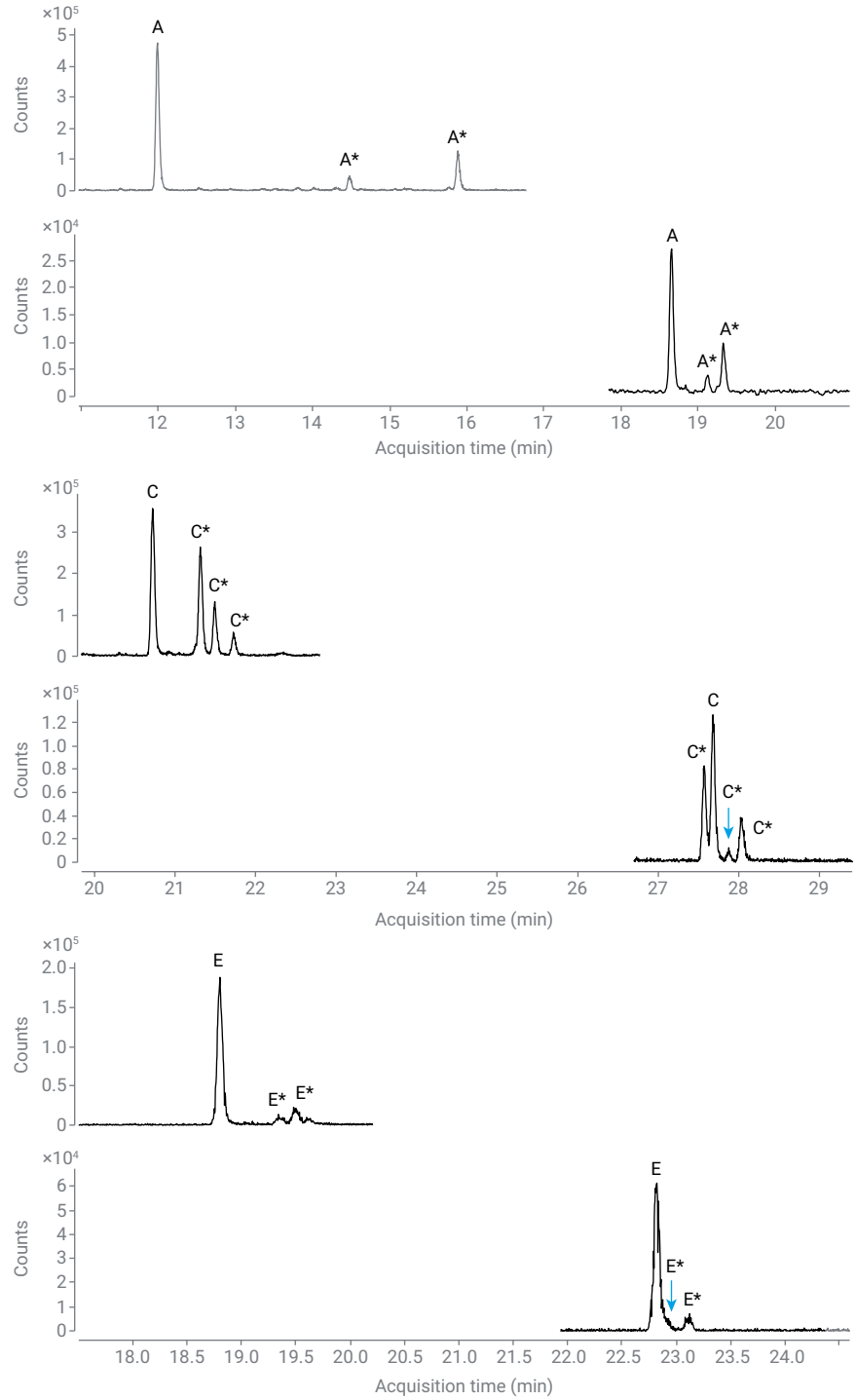


FIGURE 4: Separation of peptides and their deamidated variants on Agilent AdvanceBio Peptide Plus with 0.1% formic acid mobile phase modifier compared to 0.1% TFA.



organic mobile phases are modified with 0.05%, 0.1 or 0.3% formic acid. Dropping the concentration to 0.05% increased selectivity and resolution, while at 0.3% formic acid, resolution was somewhat reduced. A similar pattern is observed for peptide E.

While formic acid is often the favored mobile phase modifier for LC/MS peptide separations, trifluoroacetic acid (TFA) is sometimes used to improve peak shape, even though it has a detrimental effect on ESI-MS sensitivity.[5] TFA lowers mobile phase pH more than formic acid, and therefore suppresses the ionization of the carboxylic acids formed by deamidation. Furthermore, TFA is reported to reduce the impact of ionic interactions by acting as a stronger ion pair reagent than formic acid. These effects may impact the ability of the AdvanceBio Peptide Plus column to separate deamidated variants of peptides from their unmodified form.

FIGURE 4 shows the effect of substituting 0.1% formic acid for 0.1% TFA. Under this condition, selectivity for deamidated variants over the unmodified form was significantly reduced. For peptide A, this simply resulted in less baseline between the unmodified form and the variants. However, in the case of peptide C, one of the deamidated variants eluted before the unmodified form. Meanwhile, resolution was lost between the unmodified form of peptide E and one of its deamidated variants.

Overall, the general pattern of deamidated peptides eluting later than the unmodified variant was preserved in the 0.1% TFA condition on AdvanceBio Peptide Plus. However, analysts should be aware that the chances of coeluetion when using TFA are much higher than when using formic acid.

CONCLUSION

The AdvanceBio Peptide Plus column shows greater selectivity for deamidated peptide variants versus their unmodified form when compared to a standard C18 column. This dramatically improves confidence that deamidated forms of peptides can be detected and quantified either manually or by automated analysis software by preventing issues with overlapping mass spectra. Selectivity can be altered by increasing or decreasing the concentration of formic acid mobile phase modifier. These findings may be useful to anyone analyzing deamidation of proteins using LC/MS, as described in the application note *Quantitation of Chemical-Induced Deamidation and Oxidation on Monoclonal Antibodies*.[2]

References

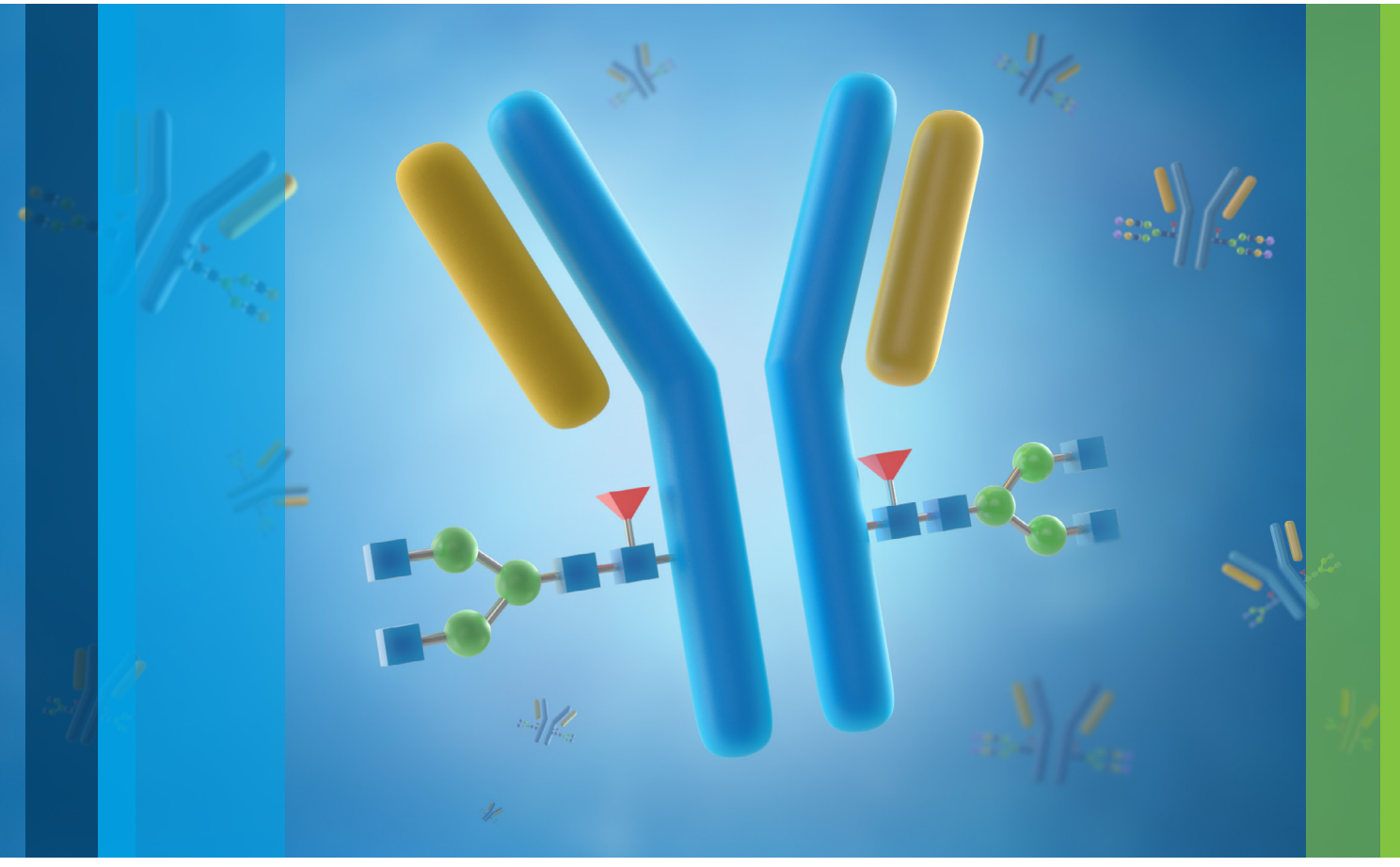
1. Gervais, D. Protein Deamidation in Biopharmaceutical Manufacture: Understanding, Control and Impact. *Journal of Chemical Technology and Biotechnology* **2015**, *91*, 569–575.
2. Linfeng, Wu. Quantitation of Chemical Induced Deamidation and Oxidation on Monoclonal Antibodies. Agilent Technologies, **2018**.

3. Wang, W. et al. Quantification and Characterization of Antibody Deamidation by Peptide Mapping with Mass Spectrometry. *International Journal of Mass Spectrometry* **2011**, *312*, 107–113.
4. Nogueira, R.; Lämmerhofer, M.; Lindner, W. Alternative High-Performance Liquid Chromatographic Peptide Separation and Purification Concept Using a New Mixed-Mode Reversed-Phase/Weak Anion-Exchange Type Stationary Phase. *Journal of Chromatography A* **2005**, *1089*, 158–169.
5. Apffel, A. et al. Enhanced Sensitivity for Peptide Mapping with Electrospray Liquid Chromatography-Mass Spectrometry in the Presence of Signal Suppression Due to Trifluoroacetic Acid-Containing Mobile Phase. *Journal of Chromatography A* **1995**, *712*, 177–1190.

Oscar Potter and Veronica Qin
Agilent Technologies, Inc.

Agilent

Trusted Answers



Advance Your Glycoprotein Analysis

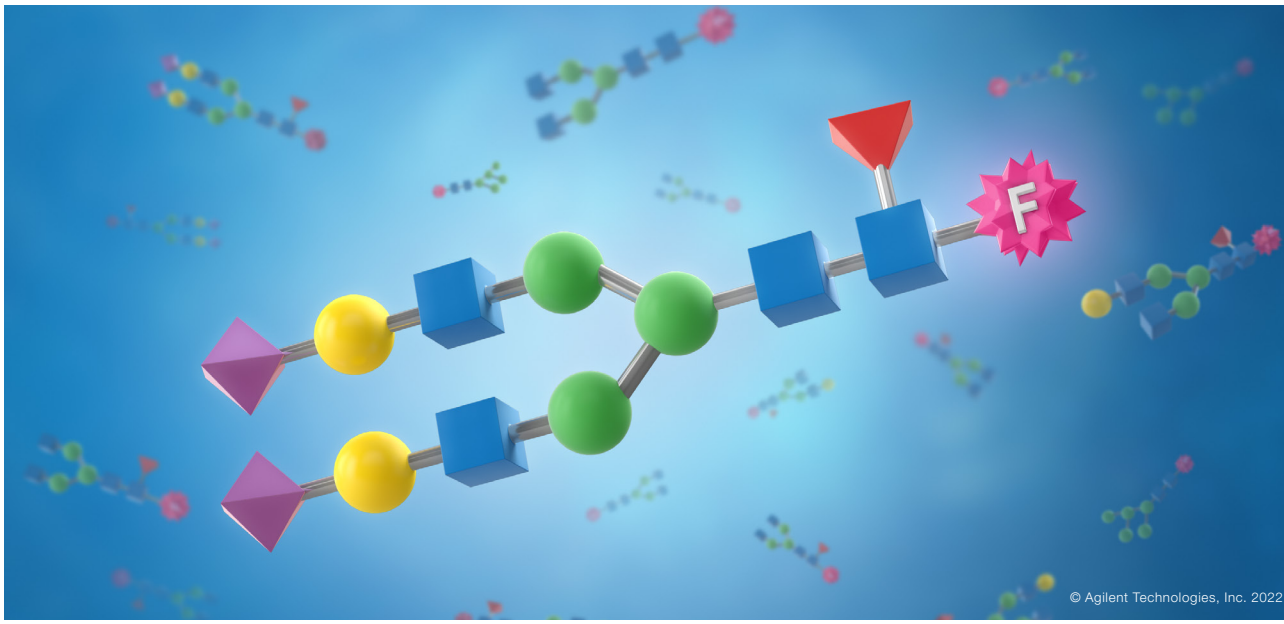
Glycan characterization is essential to the biotherapeutic development process as N-glycans can affect immunogenicity, pharmacokinetics, and pharmacodynamics.

Agilent is your single source for N-glycan sample preparation and analysis tools. With rapid (1 hour) sample preparation, N-glycan standards, and glycoenzymes, Agilent offers improvements to a broad range of released N-glycan sample preparation and analysis workflows.

Together with AdvanceBio Glycan Mapping HILIC columns and instrumentation for liquid chromatography and mass spectrometry, Agilent offers and supports your entire released N-glycan workflow, as well as kit options for total sialic acid quantitation.

Let us help you push your glycan analysis to new levels of performance.

agilent.com/chem/glycoscience



An Improved Workflow for Profiling and Quantitation of Sialic Acids in Biotherapeutics

By Anna Fong, John Yan, Ace G. Galermo, Tom Rice, Aled Jones, and Ted Haxo

ABSTRACT

This application note describes use of the Agilent AdvanceBio Sialic Acid profiling and quantitation kit (p/n GS24-SAP) to profile and perform absolute quantitation of sialic acids present in biotherapeutic glycoproteins as well as the NISTmAb. This kit uses a new and improved high-throughput workflow for the preparation, separation, and detection of sialic acids labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB). Sialic acid capping at the non-reducing terminal of N- or O-glycans can serve a key role in mediating the effectiveness of biotherapeutic glycoproteins.[1]

The workflow described here demonstrates the AdvanceBio Sialic Acid profiling and quantitation kit for release of terminal sialic acid by acid hydrolysis, followed by DMB labeling and both qualitative and quantitative analysis. DMB-labeled sialic acids from samples and standards are separated by reversed-phase liquid chromatography (LC) and quantitated using fluorescence detection (FLD) and structurally confirmed by mass spectrometry (MS).

INTRODUCTION

The composition of glycans present on biotherapeutic glycoproteins can affect immunogenicity, pharmacokinetics, and pharmacodynamics.[2] Glycans are carbohydrates composed of monosaccharides arranged into many different possible oligosaccharide structures based on composition and linkage position. Depending on the molecule and the application, terminal sialic acid may reduce the rate of clearance, reduce antibody-dependent cellular cytotoxicity (ADCC) activity, or can be anti-inflammatory.[3–5] Two forms commonly found in biotherapeutics are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Neu5Ac is usually the predominant species, while Neu5Gc is not synthesized by humans and its presence on biotherapeutics can be immunogenic. Therefore, it is essential to monitor the absolute quantity of sialic acid, as well as the levels of different sialic acid species present in therapeutic glycoproteins.

Presented here is a new high-throughput workflow based on a 96-well plate format for the release, labeling, and analysis of sialic acids from therapeutic glycoproteins using rituximab, etanercept, NISTmAb, and cetuximab as examples. Sialic acid residues are released then labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB) in a two-step procedure. DMB-labeled sialic acids are then separated and analyzed using a rapid 10-minute method based on reversed-phase ultrahigh-performance liquid chromatography (UHPLC) with FLD detection for quantitation and optional MS detection for mass analysis. The workflow offers both qualitative characterization of Neu5Ac, Neu5Gc, and other sialic acid species using a sialic acid reference panel (SARP), as well as absolute quantitation with picomole level sensitivity using included Neu5Ac and Neu5Gc quantitative standards. The workflow enables reliable and reproducible high-throughput sample preparation for the profiling and quantitation of sialic acids. This kit provides a broad detection range and improved sensitivity for molecules with low levels of sialylation.

EXPERIMENTAL

Sample preparation

Samples were prepared using a using a 96-well plate format. Sialic acids were released from rituximab (Rituxan, lot number M190170), etanercept (Enbrel, lot number M190088), NISTmAb (lot number 14HB-D-002), and Erbitux (cetuximab, lot number MI60886) through an acid hydrolysis reaction. The method eliminates the need for a dry-down step, thereby decreasing overall sample preparation time by 1 to 2 hours. The sample amount is typically 200 µg of glycoprotein with low-level sialylation and 5 µg of highly sialylated glycoprotein. Serial dilutions of sialic acid reference standards Neu5Ac and Neu5Gc were used to prepare a standard curve and to determine the limit of quantitation (LOQ) and limit of detection (LOD) for the assay. Released sialic acids, SARP, and standards were then derivatized with DMB following the workflow illustrated in FIGURE 1, release and labeling steps were carried out in a thermocycler.

LC/FLD/MS analysis of DMB-labeled sialic acids

DMB-labeled sialic acids from Rituxan, Enbrel, NISTmAb, and Erbitux were analyzed using reversed-phase (RP) separation with an Agilent 1290 Infinity II LC system with fluorescence detection (FLD) for quantitation. All RP-UHPLC separations were conducted under the conditions described in TABLE 1. Additional inline analysis using an Agilent 6545XT AdvanceBio LC/Q-TOF mass spectrometer (TABLE 2) was also performed to confirm elution order of the DMB-labeled

FIGURE 1: Sialic acid release and DMB labeling workflow (A) overview (B) DMB labeling mechanism of sialic acid Neu5Gc.

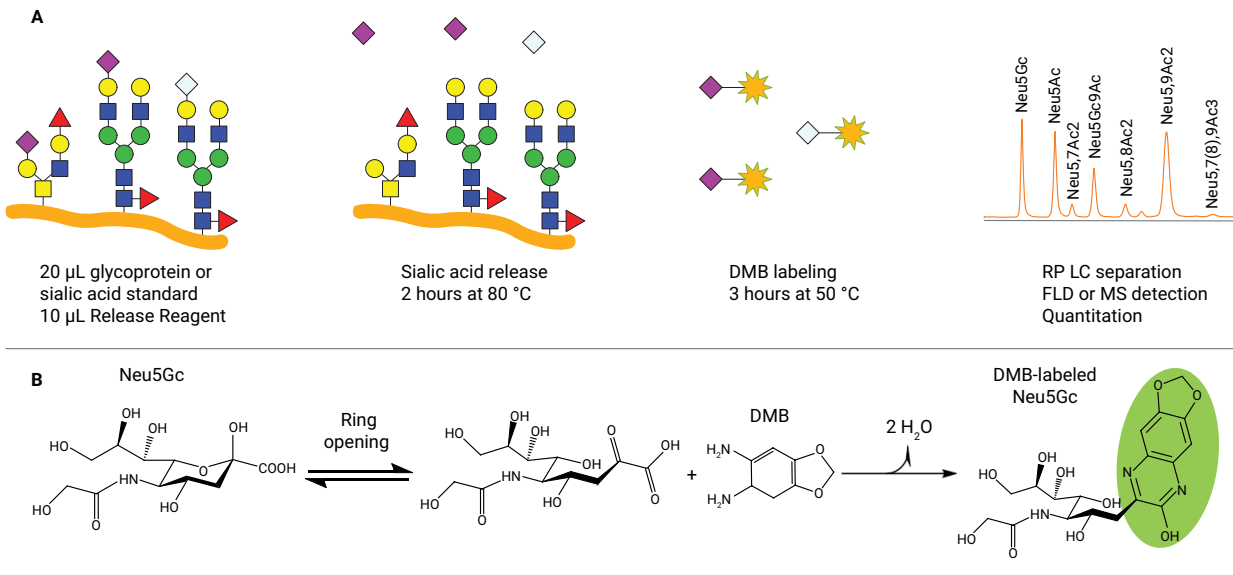


TABLE 1: Reversed-phase UHPLC conditions.

Parameter	Value
Instrument	Agilent 1290 Infinity II LC System
Column	Agilent InfinityLab PoroShell 120 EC-C18, 2.1 × 75 mm, 2.7 µm (p/n 697775-902)
Column Temperature	30 °C
Mobile Phase	A) methanol:acetonitrile:water (4:8:88) B) acetonitrile
Gradient Program	Time (min) %A %B Flow rate (mL/min) 0.00 100 0 0.4 Isocratic 6.00 100 0 0.4 elution 6.25 20 80 0.4 7.30 20 80 0.4 Wash 7.50 100 0 0.4 Re-equilibration 10.00 100 0 0.4
Injection Volume	10 µL
Detection	Agilent 1260 Infinity II FLD λ_{Ex} 373 nm, λ_{Em} 448 nm

sialic acid species present in the SARP. A fixed flow splitter was used post-FLD, diverting approximately 50% of the flow to waste and 50% to the MS. The data was analyzed with Agilent OpenLab CDS 2.3 and MassHunter Qualitative Analysis 10.0 software. Neu5Gc and Neu5Ac were quantified using the calibration curves.

Materials

Acetonitrile (LC/MS grade, Honeywell Burdick & Jackson) was purchased from VWR. Methanol (Optima LC/MS grade) was purchased from Fisher Scientific. Nanopure water generated in-house was used for all experiments.

TABLE 2: Agilent 6545XT AdvanceBio LC/Q-TOF parameters.

6545XT AdvanceBio LC/Q-TOF	
Source	Dual AJS ESI
Gas Temperature	350 °C
Drying Gas Flow	11 L/min
Nebulizer	15 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Vcap	1,400 V
Nozzle Voltage	1,800 V
Fragmentor	120 V
Skimmer	65 V
Mass Range (MS)	*m/z* 400 to 1,000
Mass Range (MS/MS)	*m/z* 100 to 550
Acquisition Mode	High resolution (4 GHz)

Instrumentation

DMB-labeled sialic acid samples were separated using an Agilent InfinityLab Poroshell 120 EC-C18 column (2.1 × 75 mm, 2.7 µm; p/n 697775-902) using the method details in TABLE 1, on an Agilent LC/MS setup composed of:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent Infinity multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1260 Infinity fluorescence detector (G1321B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (parameters in TABLE 2)

Software

- Agilent OpenLab CDS 2.3
- Agilent MassHunter Qualitative Analysis 10.0

RESULTS AND DISCUSSION

LC/FLD/MS analysis of DMB-labeled sialic acids

RP-UHPLC analysis of DMB-labeled SARP results in the separation and detection of seven sialic acid derivatives: Neu5Gc, Neu5Ac, Neu5,7Ac2, Neu5Gc,9Ac, Neu5,8Ac2, Neu5,9Ac2, and Neu5,7(8),9Ac3. While differences in retention times may be observed with different columns, flow rate, solvents, or laboratory conditions, the elution order of DMB-derivatized sialic acids remains consistent. The reference panel is used to evaluate the resolution and accuracy of the chromatographic system at the beginning of the sample sequence. A typical FLD chromatogram of DMB-labeled SARP is shown in FIGURE 2A. Identification of the DMB-sialic acid derivatives was confirmed by mass spectrometry (FIGURE 2B).

Analysis of sialic acid content of biotherapeutics and NISTmAb

DMB-labeled sialic acids identified by applying the workflow to Rituxan, Enbrel, NISTmAb, and Erbitux are shown in FIGURE 3. Both Rituxan (FIGURE 3A) and Enbrel (FIGURE 3B) contain primarily Neu5Ac while NISTmAb (FIGURE 3C) and Erbitux (FIGURE 3D) contain primarily Neu5Gc. Mass spectra of major peaks in DMB-labeled samples from Enbrel and Erbitux confirm their identities as Neu5Ac and Neu5Gc, respectively (FIGURE 4).

Quantitative analysis of sialic acid content

Based on the chromatographic separation and fluorescence response of DMB-labeled Neu5Ac and Neu5Gc standards, a quantitative calibration curve was generated (FIGURE 5). The LOD and LOQ were calculated using the noise determined by OpenLab CDS 2.3 using P2P noise calculation. The detectable mole quantities of Neu5Gc and Neu5Ac from Rituxan, Enbrel, NISTmAb, and Erbitux

FIGURE 2: UHPLC chromatogram of DMB-labeled SARP. (A) fluorescence; (B) extracted ion chromatogram of DMB-labeled sialic acid species, $[M+H]^{1+}$.

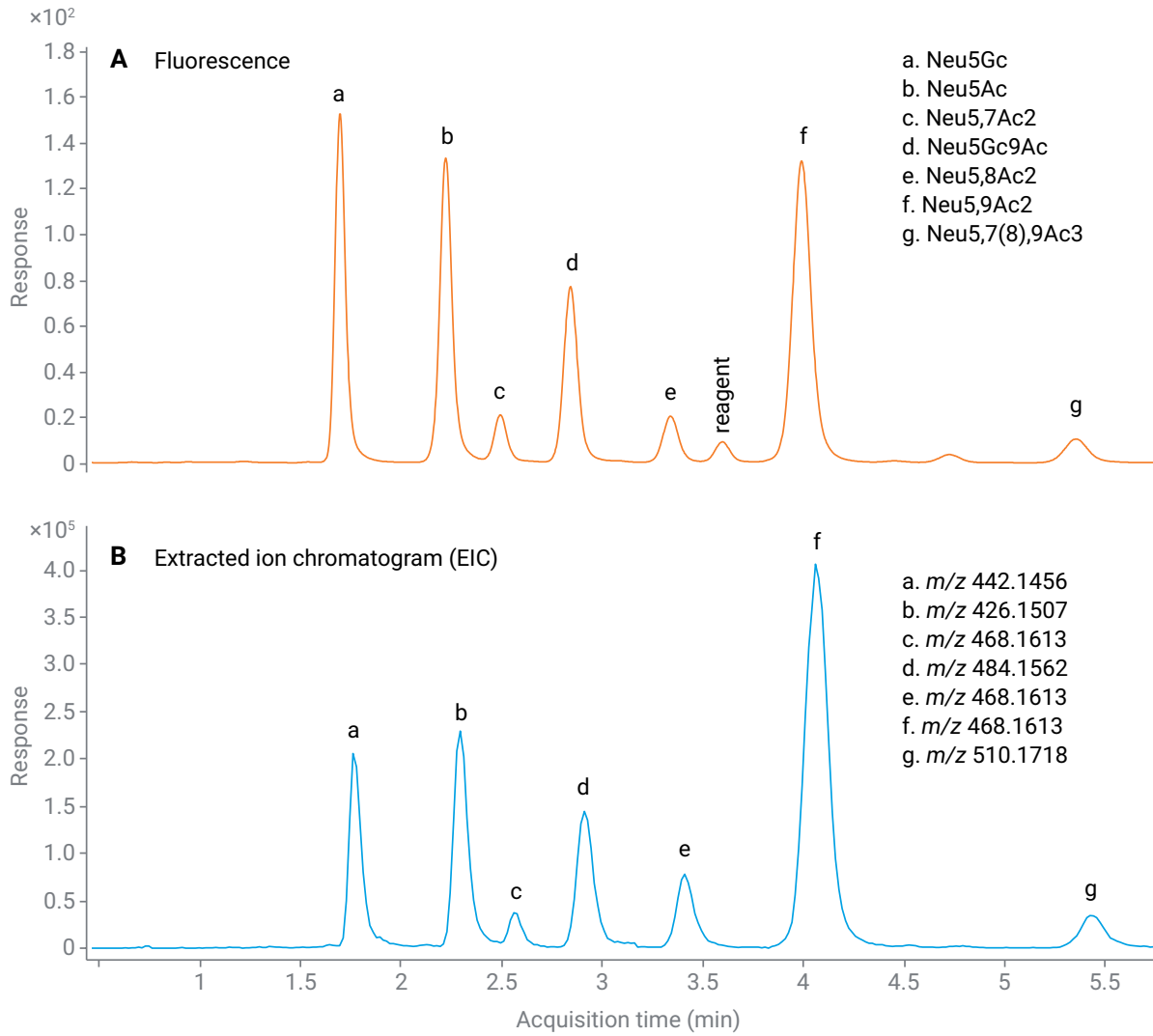
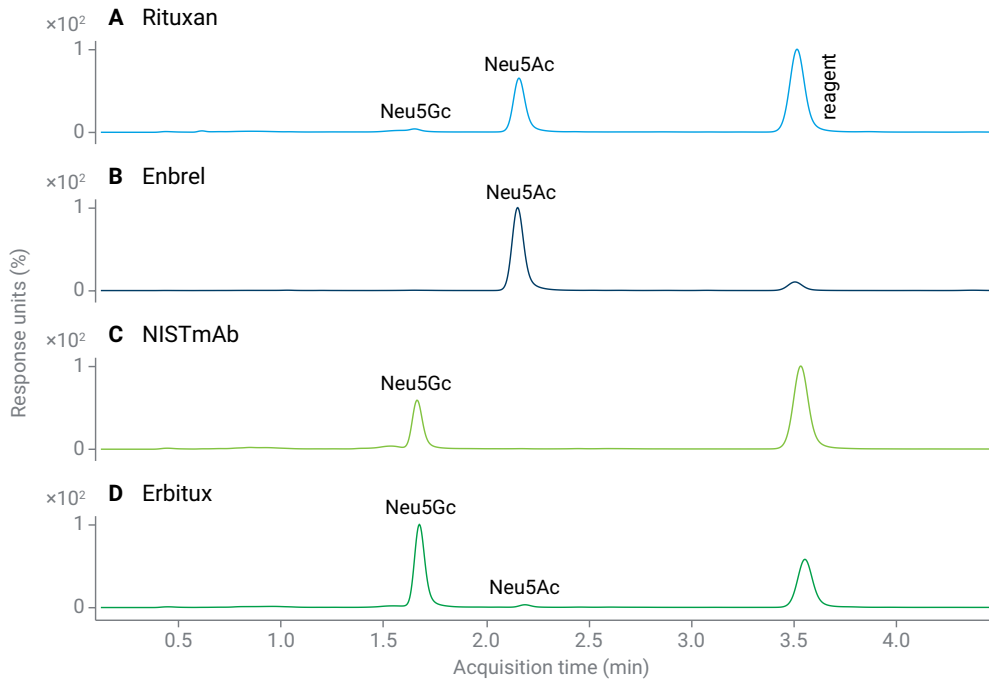


FIGURE 3: UHPLC fluorescence profiles of DMB-labeled sialic acids from different glycoproteins (A) Rituxan; (B) Enbrel; (C) NISTmAb; and (D) Erbitux.



was determined based on integrated peak areas and listed in TABLE 3. Total sialic acid quantitation results are consistent with those obtained from the AdvanceBio Total Sialic Acid quantitation kit (p/n GS48-SAQ) (TABLE 4). The kit also shows improved performance compared to an older DMB labeling workflow (p/n GKK-407) (TABLE 5) by allowing an increased concentration of glycoprotein per sample well as a decrease in sample dilution prior to analysis, resulting in an increase in fluorescence signal for DMB labeled sialic acids.

CONCLUSION

The AdvanceBio Sialic Acid profiling and quantitation kit offers improved sensitivity for proteins with low levels of sialylation such as monoclonal antibodies with a single N-glycosylation site in the Fc region. The updated DMB labeling workflow eliminates the dry down step of samples, decreasing sample preparation time.

This workflow provides a method to determine both absolute and relative quantities of Neu5Ac and Neu5Gc present

FIGURE 4: Mass spectrum of DMB-labeled sialic acid (A) Neu5Ac from Enbrel; and (B) Neu5Gc from Erbitux.

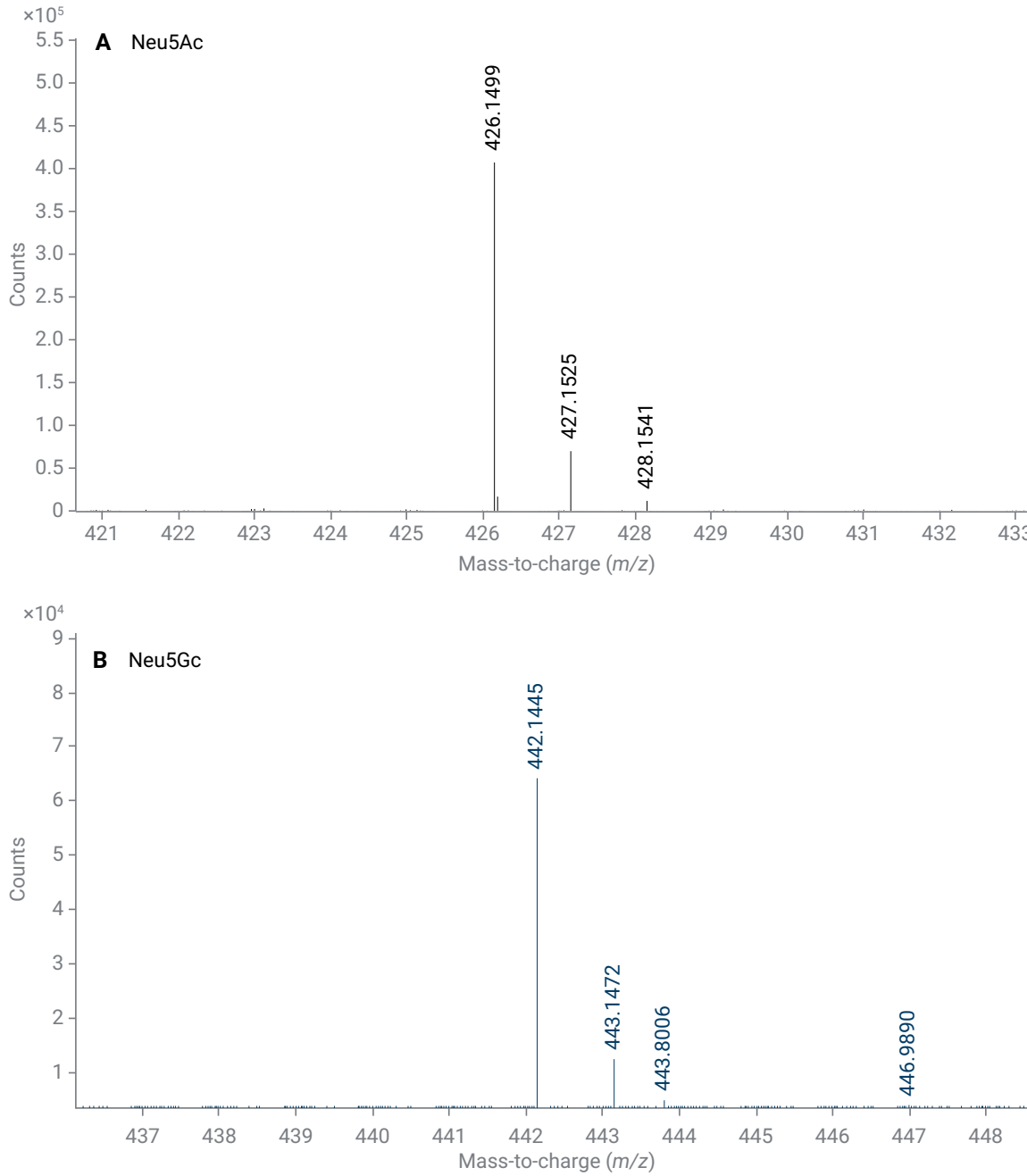


TABLE 3: Average pmol/µg of Neu5Ac and Neu5Gc for each glycoprotein is shown in the table, n = 3. ND = not detected.

	Concentration (mg/ml)	Sample Mass (µg)	Neu5Ac (pmol/µg)	%CV	Neu5Gc (pmol/µg)	%CV
Rituxan	10	200	0.60	4.2%	0.02	1.8%
Enbrel	0.25	5	228	6.9%	ND	-
NIST mAb	10	200	ND	-	0.36	1.8%
Erbitux	2	40	0.12	10.9%	3.72	7.1%

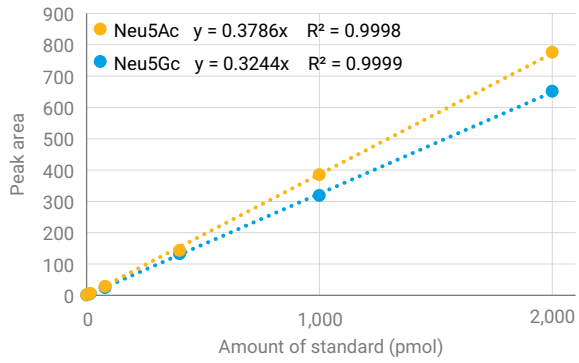
TABLE 4: Total sialic acid (Neu5Ac and Neu5Gc) with the Agilent AdvanceBio Sialic Acid profiling and quantitation kit (p/n GS24-SAP) in comparison to the values obtained with the Agilent AdvanceBio Total Sialic Acid quantitation kit (p/n GS48-SAQ), n = 3.

	Agilent AdvanceBio Sialic Acid Profiling and Quantitation Kit		Agilent AdvanceBio Total Sialic Acid Quantitation Kit	
	pmol/µg	%CV	pmol/µg	%CV
Rituxan	0.62	4.17%	0.47	5.04%
Enbrel	220	1.65%	210	12.34%
Erbitux	3.80	7.26%	3.49	0.69%
Fetuin	226	4.45%	232	7.39%

TABLE 5: Quantitation of Neu5Ac and Neu5Gc (pmol/µg) using the Agilent AdvanceBio Sialic Acid profiling and quantitation kit (p/n GS24-SAP) in comparison to the values obtained with the Signal DMB Sialic Acid labeling kit (p/n GKK-407), n = 3. ND = not detected.

		GKK-407		Agilent AdvanceBio Sialic Acid Profiling and Quantitation Kit	
Glycoprotein	Sialic acid	pmol/µg	%CV	pmol/µg	%CV
Rituxan	Neu5Gc	ND	-	0.02	1.76%
	Neu5Ac	0.58	1.12%	0.60	4.25%
Enbrel	Neu5Ac	226	3.57%	223	2.92%
Erbitux	Neu5Gc	ND	-	3.68	1.02%
	Neu5Ac	ND	-	0.12	4.46%
Fetuin	Neu5Gc	ND	-	4.78	4.90%
	Neu5Ac	201	1.47%	222	4.44%

FIGURE 5: Neu5Ac and Neu5Gc calibration curves, n = 2. LOD and LOQ for Neu5Ac and Neu5Gc are shown in the table.



Sialic Acid	LOD (pmol)	LOQ (pmol)
Neu5Ac	0.016	0.053
Neu5Gc	0.012	0.040

in biotherapeutics. Sample preparation uses a 96-well plate format for high-throughput sample preparation and is highly reproducible. Quantitative data is comparable to older DMB labeling workflows (GKK-407) and AdvanceBio Total Sialic Acid quantitation kit (GS48-SAQ) results.

REFERENCES

1. Varki, A. Sialic Acids in Human Health and Disease. *Trends Mol. Med.* **2008**, *14(8)*, 351–360.
2. Liu, L. Antibody Glycosylation and its Impact on the Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies and Fc-Fusion Proteins. *J. Pharm. Sci.* **2015**, *104(6)*, 1866–1884.
3. Li, Y. et al. Sialylation on O-glycans Protects Platelets from Clearance by Liver Kupffer Cells. *Proc. Natl. Acad. Sci.* USA. **2017**, *114(31)*, 8360–8365.
4. Scallon, B. J. et al. Higher Levels of Sialylated Fc Glycans in Immunoglobulin G Molecules can Adversely Impact Functionality. *Mol. Immunol.* **2007**, *44(7)*, 1524–1534.
5. Kaneko, Y. et al. Anti-inflammatory Activity of Immunoglobulin G Resulting from Fc Sialylation. *Science* **2006**, *313*, 670–673.

Anna Fong, John Yan, Ace G. Galermo, Tom Rice, Aled Jones, and Ted Haxo
Agilent Technologies, Inc.

RA44915.610474537
5994-7771EN