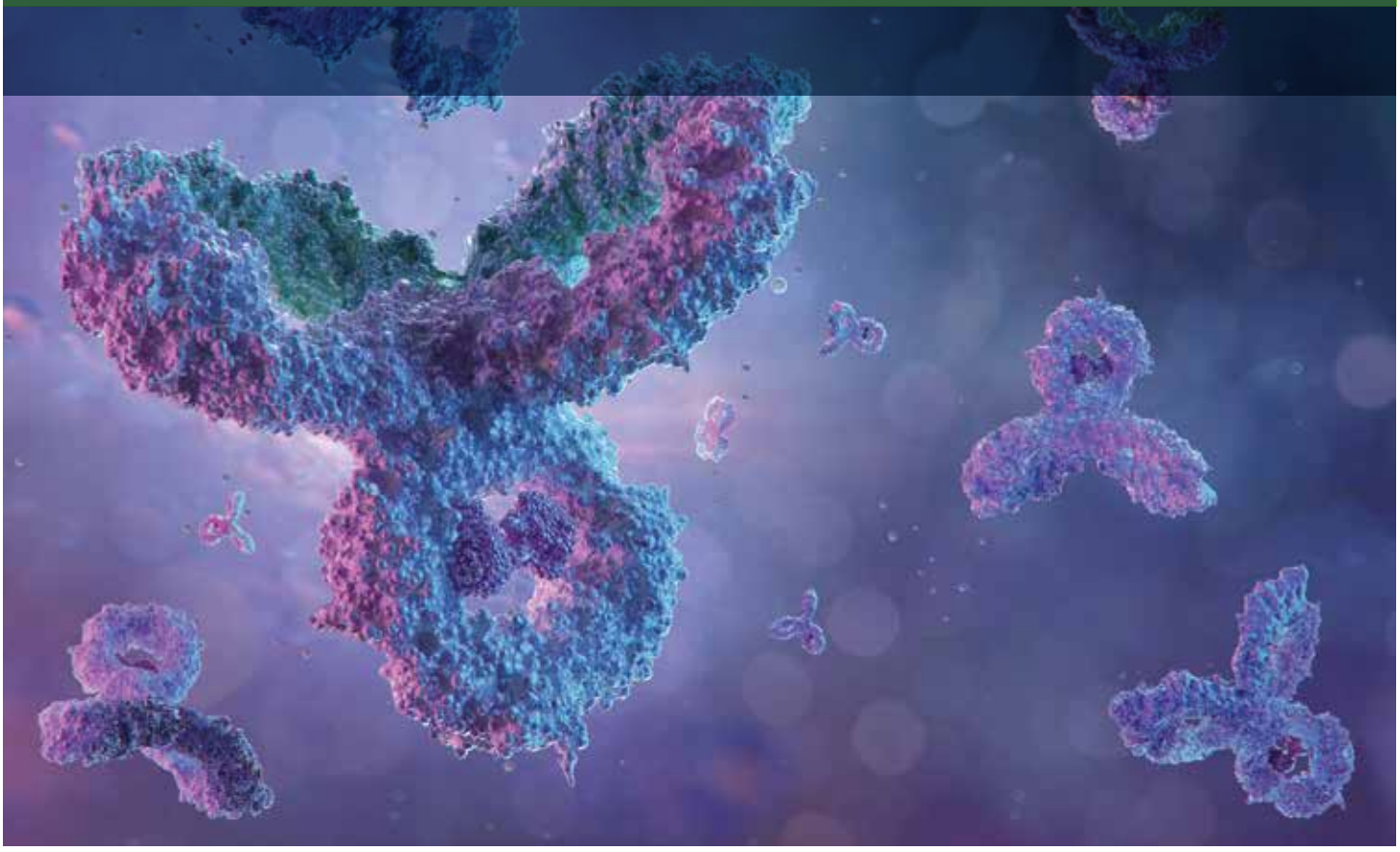
Agilent

Trusted Answers

Agilent Biocolumns

Critical Quality Attributes

Application Compendium



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Critical Quality Attributes Application Compendium

Foreword

The future of biopharmaceuticals looks promising with life changing treatments. The field keeps growing and is powered by innovative, ground-breaking therapies in cancer treatment and auto immune diseases. Advancing these novel biotherapeutics safely in the clinic requires reliable manufacturing and quality control processes. The complex heterogenous nature of biotherapeutics requires accurate and robust analytical testing methodologies with dependable chromatographic separations. Identifying critical quality attributes (CQA) is the most difficult step in implementation of quality by design (QbD) for development and production of biopharmaceuticals. Defining each product attribute is extremely challenging and therefore, consistency of product quality becomes even more important. We at Agilent, designed and manufactured our AdvanceBio columns and consumables to match our customers' needs.
In this compendium, we have therefore selected applications to illustrate the state of-the-art chromatographic separation for each CQA using either HPLC-UV or light scattering detectors. We provide an overview of different chromatographic separation technologies for each CQA using the diverse range of chemistries with in our biocolumns portfolio. There are examples of reversed-phase, size exclusion, ion exchange, and hydrophilic interaction chromatographic analyses of therapeutic proteins, mAbs, and antibody-drug conjugates. Let us help you improve your productivity, method robustness, and reliability of your analytical results. We want to stand by you on this journey in developing safer and effective biotherapeutics.
Your success is our success.

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Introduction

Biotherapeutic proteins are highly complex molecules, which are typically produced by fermentation using recombinant methodologies. This production process however, results in the generation of many different variants of these proteins. Ensuring the quality of such materials is paramount. This means confirming the product is correctly manufactured, any impurities are identified and quantified, and the potency of the protein is determined.

As a result, it is necessary to perform tests on the intact, nondenatured molecule. Something as large as a monoclonal antibody may contain more than 1,300 individual amino acids and have a mass of more than 145,000 daltons. However, identifying a single minor impurity such as deamidation of asparagine resulting in a mass difference of just one dalton, which may occur at any of perhaps twenty or more different asparagine positions throughout the molecule, is challenging. Only by breaking down the molecule into fragments (such as light and heavy chains) and then into smaller polypeptide chains through enzymatic treatment is it possible to begin to pinpoint some of these subtle differences.

Many different types of variant can be created and these are often referred to as post-translational modifications, or PTMs. They arise after the protein has been expressed, and can be a consequence of the manufacturing conditions, or exposure to conditions that cause changes to occur. Fluctuations in temperature, pH, concentration, or exposure to enzymes can all lead to variants developing. Glycosylation in particular is highly variable but is of major importance to the efficacy of many proteins.

Understanding the different types of impurity and the risks each pose forms the basis of Critical Quality Attribute (CQA) monitoring.

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The purpose of this document is to highlight some of the HPLC applications suitable for the different aspects of CQA monitoring. As well as providing guidance for the appropriate liquid chromatography column for the different types of detection that may be required, and to provide a valuable reference for future consideration.

Titer Determination

Affinity chromatography

Ideal for mAb titer determination during process development

Glycan Analysis

Hydrophilic interaction chromatography

Fast, high-resolution, reproducible glycan separation

Charge Variant Analysis

Ion exchange chromatography

Enhances the accuracy and speed of biomolecule characterization

Amino Acid and Cell Culture Analysis

Small molecule chromatography (<150 Å)

Delivers robust, high-resolution separations

Peptide Mapping

Reversed-phase chromatography (<150 Å)

Reliably characterizes primary sequence and detects PTMs

Aggregate/ Fragment Analysis

Size exclusion chromatography

Accurate, precise quantitation for a broad range of biomolecule separations

Intact and Subunit Purity

Large molecule chromatography (>150 Å)

Selectivity options for every separation need

Be Agilent Sure in Your CQA Monitoring

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Titer Determination

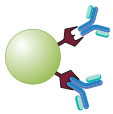
Background

In biotherapeutic manufacture, titer determination is the measurement of the concentration of the target protein in the fermentation broth. There are two notable occasions when accurate titer determination is required. The first is during the clone selection process, selecting only those transfected clones that provide sufficient amounts of the target protein, since not all clones will be equally effective. The second is during scale up of the fermentation process to monitor the concentration of the target protein. Optimization of the cell culture conditions and determining the best harvest time relies on accurate titer determination.

For monoclonal antibodies, one of the most effective ways of titer determination is to use affinity chromatography. By absorbing the IgG molecule onto a Protein A or Protein G affinity chromatography column, the remaining impurities and byproducts from the fermentation broth can be removed. Elution of the purified monoclonal antibody and quantification by comparing the peak area to a calibration curve allows rapid measurement of the protein concentration.

Employing a monolithic column helps to eliminate the risk of clogging from cell culture debris and provides rapid (sub 1 minute) results.

These columns may also be used for purifying sufficient material for subsequent CQA analysis by another complementary technique, such as Aggregate Analysis or Charge Variant Analysis, and can easily be combined into a 2D workflow.



Titer Determination

Affinity chromatography

Ideal for mAb titer determination during process development

BioMonolith Protein A and BioMonolith Protein G

Native Protein A or Protein G

Attribute	Advantage
Fast separation	Shorter method development times
High binding capacity	Greater application flexibility
Minimal clogging	Less system down time

Getting Started

In selecting an affinity column for titer determination, the first point to consider is the target protein to be purified or analyzed. Different immunoglobulins (IgG 1, 2, etc.) from different sources (human, mouse, etc.) have different affinities for Protein A versus Protein G. For example, Human IgG3 binds tightly to Protein G, but not at all to Protein A. Guidelines on selecting a Protein A or a Protein G column, as well as suggested mobile phases and a sample method, can be found in the "How- to-Guide" that follows. Mobile phase B, the eluting buffer in an affinity experiment, is one method parameter that can be optimized.

Affinity Chromatography for Titer Determination: A "How-To" Guide

Introduction

Affinity chromatography is a powerful technique, which takes advantage of highly specific molecular interactions, frequently between specific proteins (e.g. antigen/antibody). Agilent offers several specialty affinity products, including monolithic Protein A and Protein G columns for the isolation and quantitation of monoclonal antibodies (mAbs).

In recent years, mAbs have become one of the major biopharma products in response to the need to treat various diseases. These antibodies have been engineered with a specific genetic make up for better targeting of disease agents. During the development of these antibodies, Protein A and G analytical affinity columns are used to determine antibody titer or concentration from various cell culture supernatants, to select the high-yield clone.

Column Selection

Protein A and G columns have high affinity for antibodies, and so they bind only to antibodies in cell-culture supernatants. However, they have different selectivity. For example, Agilent Bio-Monolith protein A columns have high affinity for human subclasses IgG1, IgG2, and IgG3 and no affinity for IgG3, whereas Agilent Bio-Monolith Protein G columns have high affinity for human subclasses IgG1, IgG2, IgG3, and IgG4. IgG 1 is the most commonly used as biotherapeutic for its stability (long half life), abundance in serum, and less aggregation formation. IgG 3 is often not used because it's prone to aggregate and less stable. Conversely, the Protein G column has no affinity for human subclass monoclonal antibodies such as IgA and IgD, but the Protein A column binds to both these antibodies (Table 1). Together, these columns complement each other, so Protein G has affinity for mAbs that do not bind to Protein A and vice versa (Figure 1). They therefore and enable titer determination of the various mAb subclasses and fragments currently in development as biotherapeutics.

Table 1. Binding affinity of Protein A and G to different human and mouse IgG subclasses [(1), (2)].

Antibody	Antibody	Protein A	Protein G
Human	Human IgG1	++++	++++
	Human IgG2	++++	++++
	Human IgG3	-	++++
	Human IgG4	++++	++++
	Human IgA	++	-
	Human IgD	++	-
	Human IgE	++	-
	Human IgM	++	-
Mouse	Mouse IgG1	+	++
	Mouse IgG2a	++++	++++
	Mouse IgG2b	+++	+++
	Mouse IgG3	++	+++
	Mouse IgM	+/-	-

Antibody Fragments	Protein A	Protein G
Human Fab	+	+
Human F(ab')2	+	+
Human scFv	+	-
Human Fc	++	++
Human K	-	-
Human λ	-	-

Key code for relative affinity of Protein A & G for respective antibodies:
++++ = Strong affinity
+++ = Moderate affinity
++ = Weak affinity
+ = Slight affinity
- = No affinity

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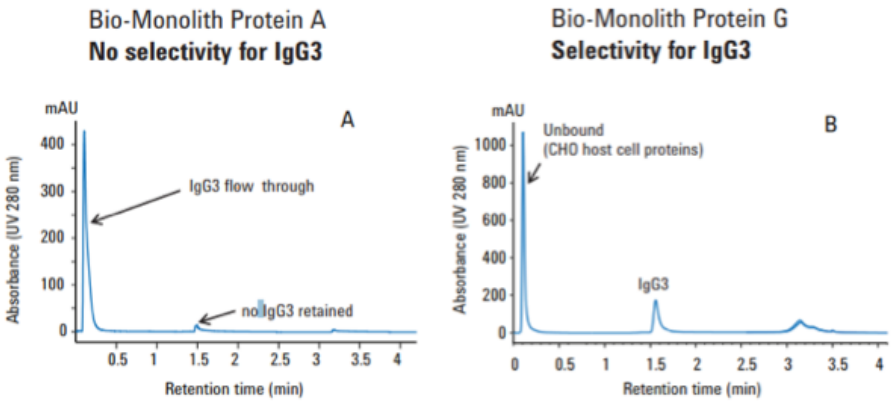


The Bio-Monolith Protein A has no affinity for IgG3 but the Bio-Monolith Protein G does.

Figure 1. From 5991-6087EN or application note 5991-6094EN.

HPLC system considerations - Protect your proteins during analysis

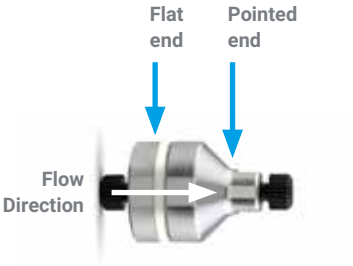
These monolithic columns are compatible with HPLC and UHPLC systems, however an ideal choice for this type of analysis is the Agilent 1260 Infinity II bio-inert LC. It handles challenging solvent conditions with ease, such as extreme pH values of pH 1 to pH 13, and buffers with high salt concentrations. Corrosion resistant titanium in the solvent delivery system and metal-free materials in the sample flowpath create an extremely robust instrument.

Sample Preparation

Sample preparation for affinity chromatography is similar to that for any protein analysis for HPLC. With some minor sample preparation being required before injection to optimize column performance and extend column lifetimes.

- Centrifuge or filter samples to remove host cell debris and particulates from the supernatant or lysate, to prevent blockage of the columns.
- For serum/plasma samples, it is also best to remove lipids from sample. Lipids will strongly bind to the columns and can cause fouling of columns and instruments.

The Bio-Monolith protein A column has a white band and Bio-Monolith Protein G has a yellow band around the column.



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Conditions

Sample injection

For samples containing 1 to 5 mg/mL of mAb injection volumes of 1 to 5 µL are recommended. Samples can be dissolved in H_2O or mobile phase A. Up to 50 µL or up to 400 to 500 mg mAb/injection can be injected on the columns.

Flow rate

Columns can be run at 1.0 to 3.0 mL/min for high speed.

Temperature

For successful separations, 25 °C is a typical temperature. Columns can be operated from 4 to 40 °C.

Detection

Detection by UV at 280 nm being is recommended, at this wavelength absorbance is due to amino acids with aromatic or more conjugated side chains.

Mobile phase

Mobile phase A Binding and washing buffer

Mobile phase A is the binding buffer: 50 mM sodium phosphate buffer, pH 7.4.

Binding/washing buffers should be freshly made. In addition, filtration of buffers through a 0.22 or 0.45 µm membrane is recommended to reduce buffer impurities that build up on the frits inside the column. This filtration will help to prevent column blockage.

Mobile phase B eluting buffer

Bio-Monolith Protein A and G columns are compatible with many low pH buffers that are used for mAb elution, see Table 2 for details. Citric acid, glycine, HCl, and acetate acid, are commonly used. If a low concentration sample is used and baseline noise and artifact peaks are of concern, HCl can be used as an eluent due to its low refractive index.

Note: Commonly, elution buffers for affinity columns have a refractive index (RI) that is very different from binding/washing buffers; therefore, baseline noise and an artifact peak could appear when the eluents start flowing. This peak could interfere with the quantitation of low concentration samples. To minimize this effect, high-quality chemicals are recommended to be used and blank runs should be included to establish the artifact peak. Blank runs can be used for baseline subtraction if desired.

Table 2. Compatible eluting buffers.

Column	Eluting Buffer	Concentration	pH
Bio-Monolith Protein A and rProtein A	Citric acid	0.1 M	2.5 to 3.0
	Glycine	0.1 M	2.5 to 3.0
	Acetic acid	5–20 %	
Bio-Monolith Protein G	Citric acid	0.1 M	2.5 to 3.0
	Glycine	0.1 M	2.5 to 3.0
	Acetic acid	5–20 %	

Fast separation protocols

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 study 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.

High-throughput mAb titer analysis

Experimental

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)

HPLC Method Conditions

Parameter	Value	
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	
Binding Buffer (Eluent B):	100 mM citric acid, pH 2.6	
Gradient Profile:	Time (min) 0.0 to 0.2 0.3 to 0.65 0.66 to 0.90 (0.1 min postrun)	% B 0 (binding) 100 (elution) 0 (reconditioning)
Flow rate:	3 mL/min	
Column Temperature:	25 °C	
Detection:	UV, 280 nm	
Injection volume:	4 µL (10 µg loading)	

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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 was then generated by plotting peak area versus injection quantity (Figure 3). Results indicated excellent linearity response (R2 = 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.

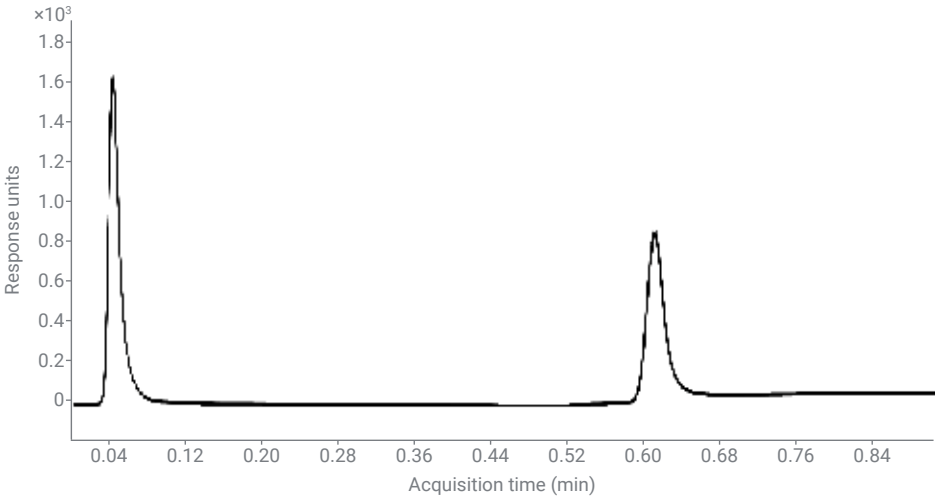

Figure 1. Agilent Bio-Monolith rProtein A column: Overlaid chromatograms of 60 consecutive injections. First peak indicates host cell protein impurities in culture supernatant; second peak is purified mAb.

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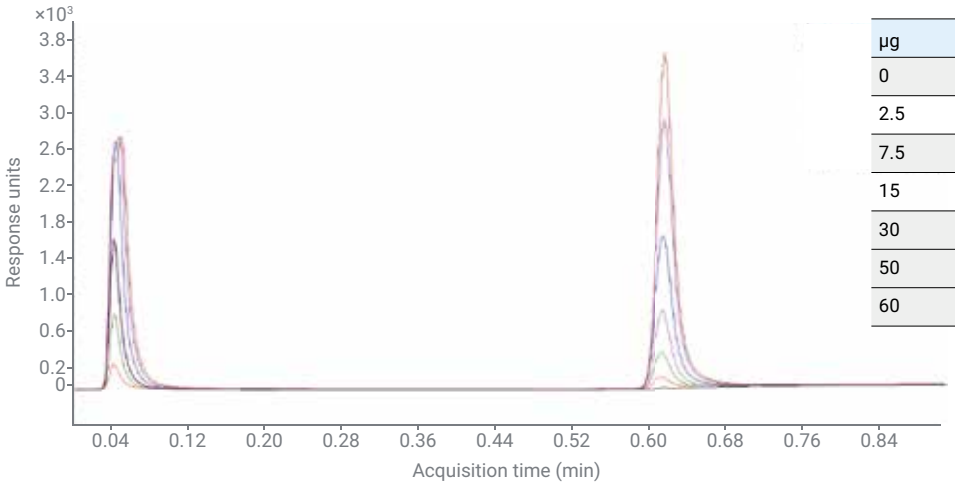
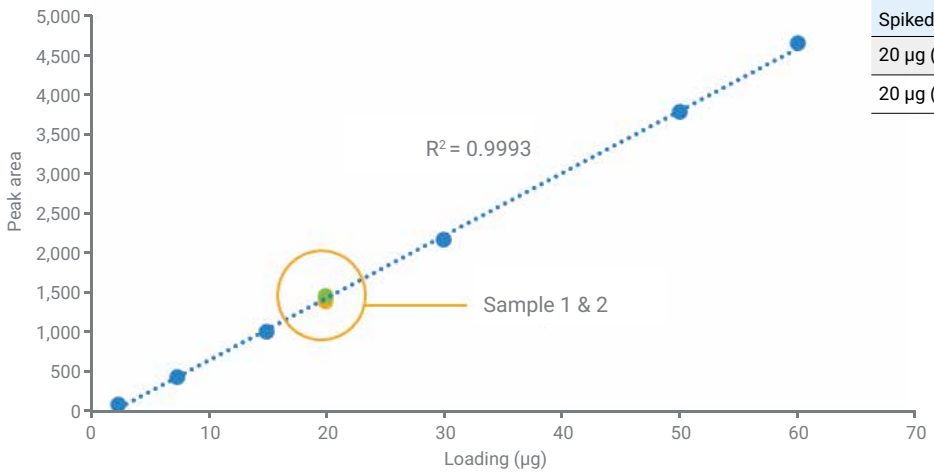


µg	Area	Height
0	0	0
2.5	105.8	97.3
7.5	453.3	365.0
15	1019.1	807.5
30	2186.5	1627.8
50	3811.8	2885.6
60	4696.1	3604.6

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



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

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

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Bridging Study

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

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 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

HPLC Method Conditions

Parameter	Value	
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	
Binding Buffer (Eluent B):	100 mM citric acid, pH 2.6	
Gradient Profile:	Time (min) 0.0 to 0.5 0.6 to 2.6 2.7 to 4.0	% B 0 (binding) 100 (elution) 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)	

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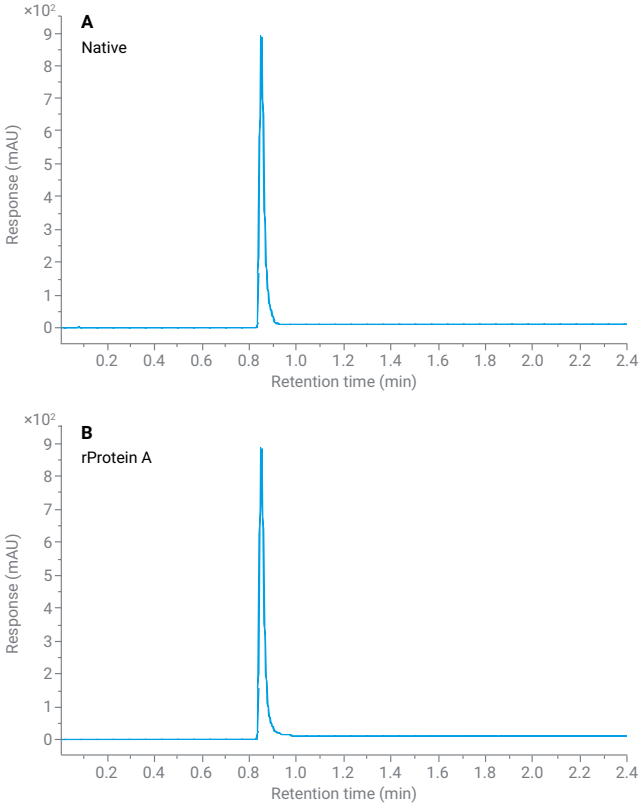
Results and Discussion

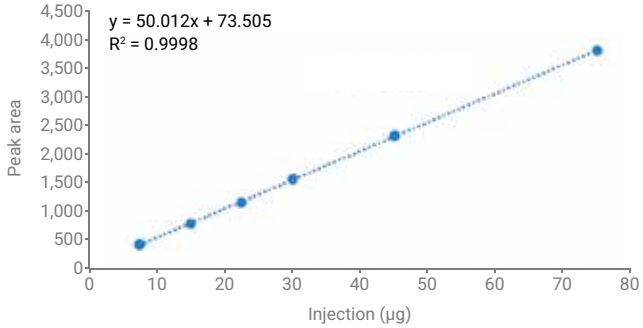
Bridging study

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).



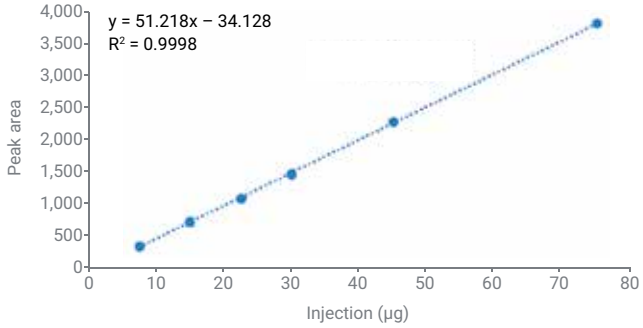
	Native	rProtein A
Retention Time (Min)	0.850 ± 0.001	0.850 ± 0.001
Peak Height	880.9 ± 8.1	881.8 ± 5.3

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



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

Figure 5. rProtein 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 6. Native Protein A column: Linearity response

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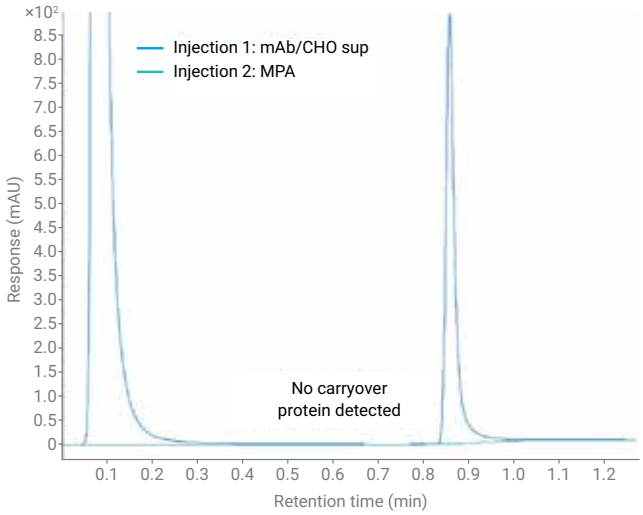

Figure 7. rProtein A column: Carryover Analysis. Subsequent injection of binding buffer (MPA) showed no detectable protein carryover.

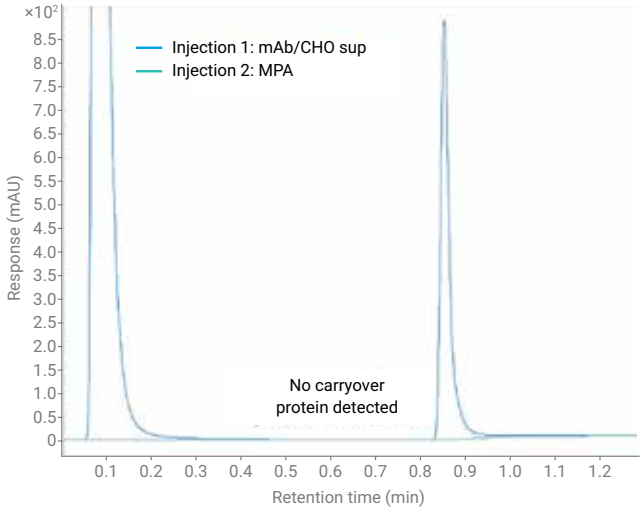

Figure 8. Native Protein A column: Carryover analysis. Subsequent injection of binding buffer (MPA) showed no detectable protein carryover.

Recovery analysis

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

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

HPLC Method Conditions

Parameter	Value	
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	
Binding Buffer (Eluent B):	100 mM citric acid, pH 2.6	
Gradient Profile:	Time (min) 0.0 to 0.4 0.5 to 1.3 1.31 to 4.0	% B 0 (binding) 100 (elution) 0 (reconditioning)
Flow rate:	2 mL/min	
Column Temperature:	25 °C	
Detection:	UV, 280 nm	
Injection volume:	4 µL (10 µg loading)	

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Result 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.

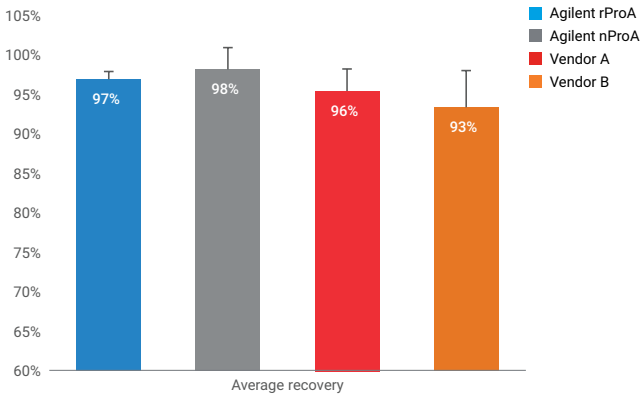

Figure 9. mAb recovery results comparison.

Conclusion

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.

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Maximizing column lifetime

Column regeneration

A major benefit of using a monolithic disk is that the presence of channels instead of pores decreases the likelihood of column clogging when injecting cell-culture samples. This increases robustness and reduces cleaning efforts. Column contamination can be reduced by running a blank gradient injection after every 30–50 samples.

It column deterioration is observed (tailing or broad peaks) the following cleaning procedure is recommended. Column regeneration is the first step. It performance is still suboptimal, the clean in place procedure can be used, which will reduce the amount of Protein A available.

Column regeneration

- Wash with 2 mL (20-column volumes (CV)) of 100 mM phosphate buffer + 1 M NaCl, pH 7-8, at 0.5-1.0 mL/min.
- Wash with 2 mL (20 CV) if low-pH solution (such as elution buffer)
- Re-equilibrate with binding buffer.

Clean-in-place

- Wash with 1 to 2 mL (10–20 CV) of 0.1 M NaOH (reverse flow direction) at 0.2 to 0.5 mL/min.
- Wash with 1 to 2 mL (10–20 CV) of DI water at 0.5–1.0 mL/min.
- Wash with 1 to 2 mL (10–20 CV) of concentrated buffer (0.1 to 0.5 M) to restore normal pH (7.0 to 7.4)
- 4Re-equilibrate with 5 mL (50 CV) of binding buffer.

If the impurities are highly hydrophobic or lipidic, and are not easily removed from the column, 2-propanol (up to 30 %), or guanidine hydrochloride (up to 3 M) can be used to remove these impurities. After using these alternative cleaning solutions, follow steps 1 through 4.

WARNING: When you wash the column with these cleaning solutions, always decrease the flow rate on the column to avoid generation of high pressures that might exceed the maximum allowed pressure over the column.

Short-term storage

For storage, overnight or for a few days, the columns can be flushed with binding buffer, disconnected from the instrument, capped, and stored at 4 to 8 °C. Columns should be equilibrated before the first injection after short-term storage.

Long-term storage

If the column will not be in use for more than two days, it should be washed with at least 1 mL (10 CV) of DI water and afterwards flushed with at least 2 mL (20 CV) of 20 % ethanol with 20 mM Tris buffer, pH 7.4 at a flow rate of 0.2 to 0.5 mL/min. It should then be sealed with column end stops and stored at 4 to 8 °C (39 to 46 °F).

References

1. Richman, D. D., Cleveland, P. H., Oxman, M. N., and Johnson, K. M. 1982. *"The binding of 1. Staphylococci protein A by the sera of different animal species."* J. Immunol. 128: 2300–2305.
2. Frank, M. B. 1997. *"Antibody Binding to Protein A and Protein G beads".* 5. In: Frank, M. B., ed. Molecular Biology Protocols. Oklahoma City.

Ordering information

Part Number	Description
5190-6903	Bio-Monolith Recombinant Protein A, 4.95 x 5.2 mm
5069–3639	Bio-Monolith Protein A, 4.95 x 5.2 mm
5190–6900	Bio-Monolith Protein G, 4.95 x 5.2 mm

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Robust, Reliable, Recombinant Protein A Monolith Column for Antibody Titer Determination

Authors

Te-Wei Chu and
Andrew Coffey

Agilent Technologies, Inc.

Abstract

Rapid screening of crude cell culture supernatant allows decisions to be made on the optimum time for harvest during the manufacture of biotherapeutic antibodies. There are several advantages to using a Bio-Monolith column with recombinant Protein A affinity ligand. First, the Bio-Monolith structure has wide through-pores that minimize the risk of clogging. Second, the use of recombinant Protein A provides the selectivity towards IgG that is associated with native Protein A, but with a higher ligand purity and a more robust structure. Finally, the column can also be used for small-scale purification so that other analytical techniques can be applied, particularly in the determination of critical quality attributes (CQAs).

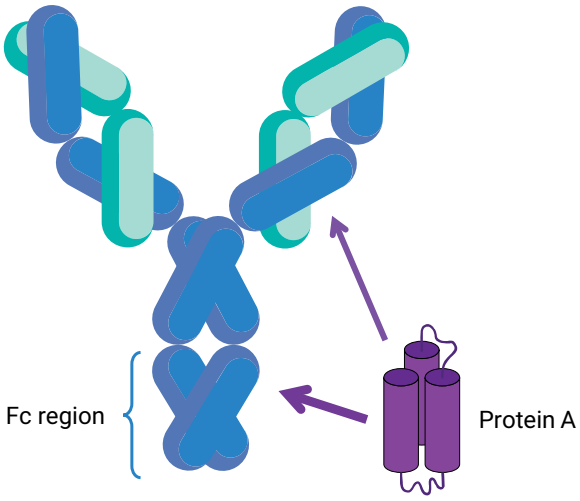

Figure 1. Protein A interaction with immunoglobulin G (IgG).

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Introduction

Native Protein A is a surface protein isolated from Staphylococcus aureus, which has a high binding affinity for the Fc region of many different types of immunoglobulin from different species. Native Protein A affinity chromatography has become the method of choice for the purification of monoclonal antibodies from crude cell culture supernatant. Recombinant Protein A can provide some extra benefits since it can be produced in a purer form and can be engineered to ensure that its immobilization onto a stationary phase creates the ideal orientation for optimum binding.

It is also helpful for improving column lifetime, which can otherwise be compromised due to the crude nature of cell culture supernatant. This is because it can withstand the harsh conditions required for column cleanup better than native Protein A columns.

This application note tests the lifetime of a new Agilent recombinant Protein A Bio-Monolith column.

Experimental

Reagents and chemicals

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 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:

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

Conditions, HPLC

Parameter	Value	
Column:	Agilent Bio-Monolith rProtein A, 4.95 × 5.2 mm (p/n 5190-6903)	
Binding Buffer	Eluent A) 50 mM phosphate, pH 7.4	
Eluting Buffer	Eluent B) 100 mM citric acid, pH 2.6	
Cleanup Buffer	1) 1 M NaCl in 100 mM sodium phosphate, pH 7.4 2) 20% isopropanol in 50 mM sodium phosphate, pH 7.4	
Gradient:	Time (min) 0 to 0.5 0.6 to 1.8 1.9 to 4.0	% B 0 (binding) 100 (elution) 0 (reconditioning)
Flow rate:	1 mL/min	
Injection volume:	As required (1 to 20 µL)	
Column temp:	24 °C	
Detection:	UV at 280 nm	

Results and discussion

A crude cell culture supernatant solution containing much higher levels of host cell proteins than in the previous work[1] was chosen to investigate the robustness of Bio Monolith rProtein A columns.

A repetitive sequence involving step gradients for binding, elution, and column reconditioning was used and the results from every 250th injection are shown in Figures 2, 3, and 4. After 1,500 injections, a column regeneration step (see Method conditions) was introduced using a cleanup buffer, which was performed every 500 injections thereafter. As expected from such a challenging crude sample matrix, a gradual build-up of pressure was observed (Figure 5).

However, with regular cleanup, the column continued to provide consistent, reliable peak area and titer analysis during the 3,000 injections, as shown in Figures 6 and 7.

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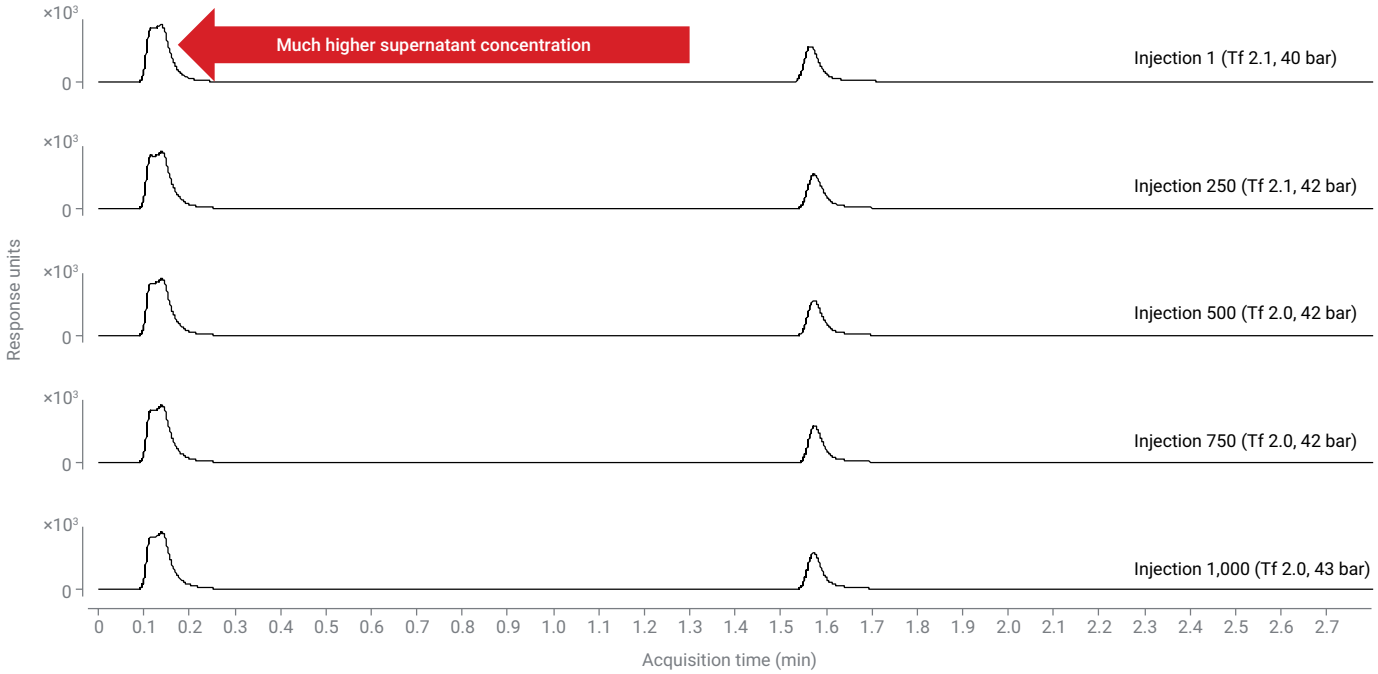

Figure 2. Agilent Bio-Monolith rProtein A column lifetime: Injections 1 to 1,000..

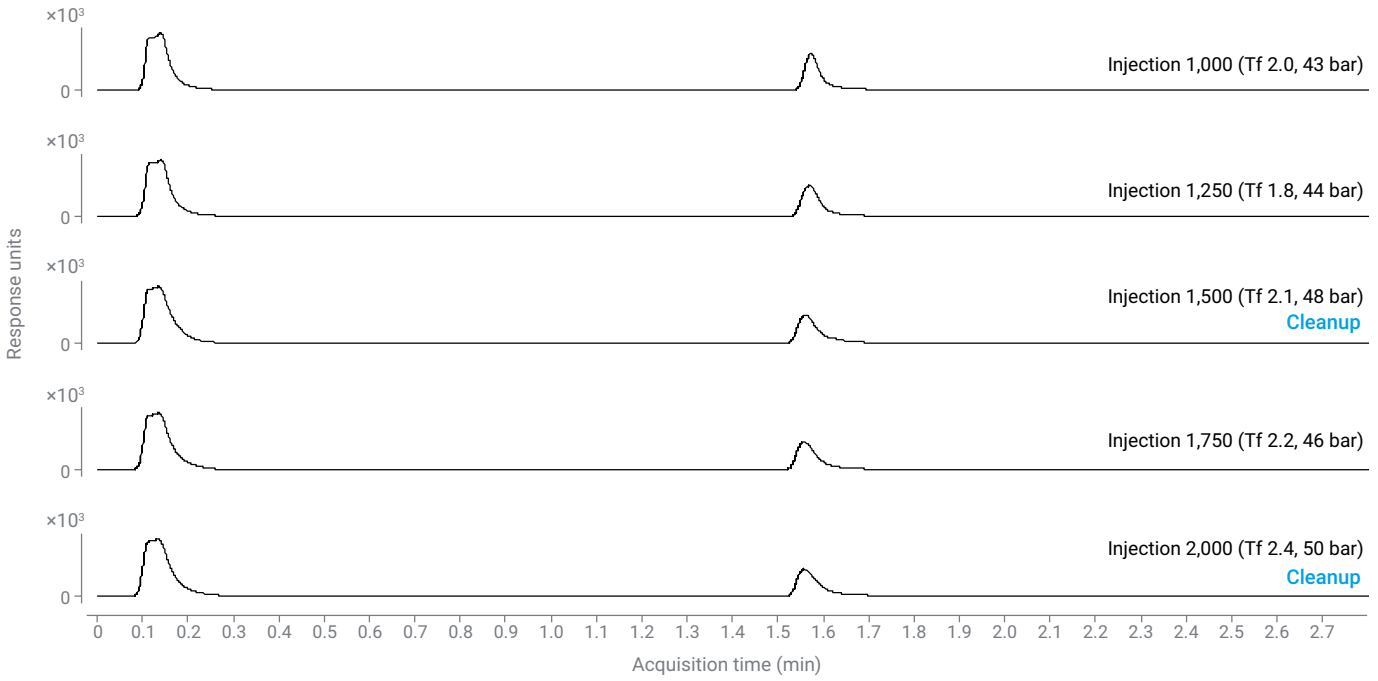

Figure 3. Agilent Bio-Monolith rProtein A column lifetime: Injections 1,000 to 2,000.

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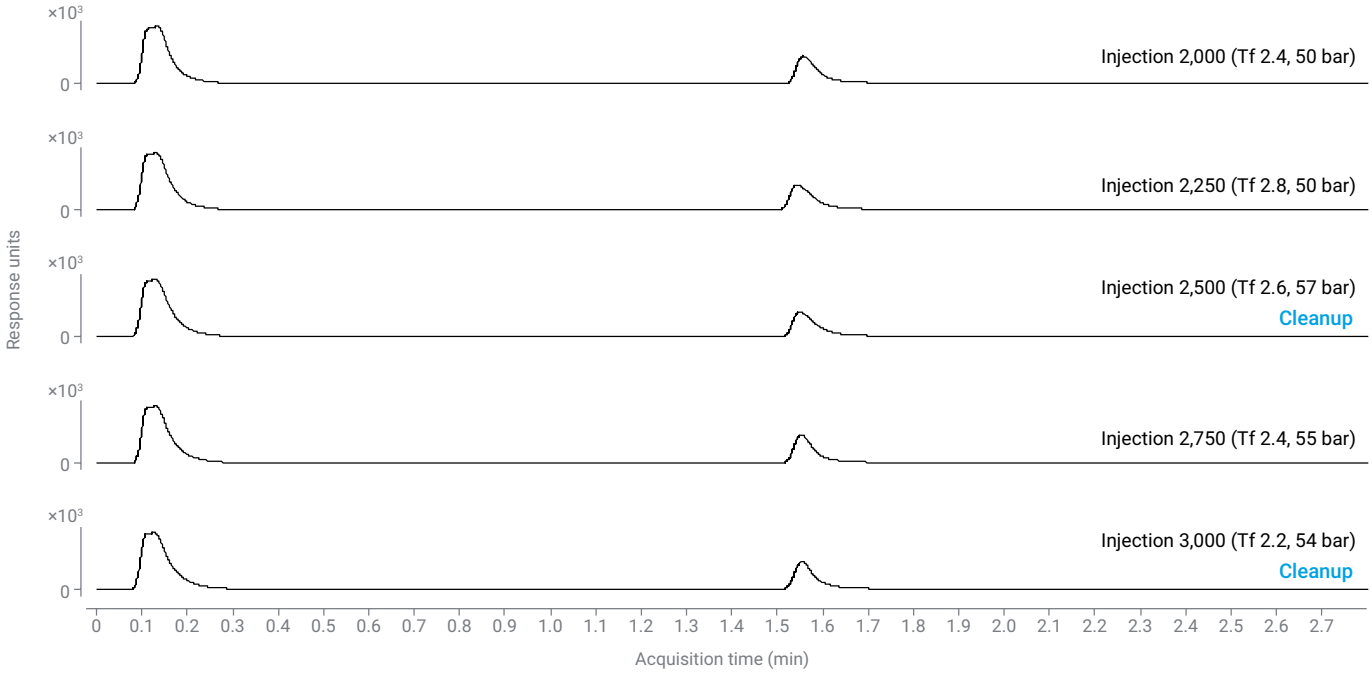

Figure 4. Agilent Bio-Monolith rProtein A column lifetime: Injections 2,000 to 3,000.

Table 1. Column pressure versus injection number during lifetime.

N-glycans	Innovator	Biosimilar
1	38.0	40.0
250	41.0	41.5
500	43.0	42.0
750	48.0	42.0
1,000	-	43.0
1,250	-	44.0
1,500	-	48.0

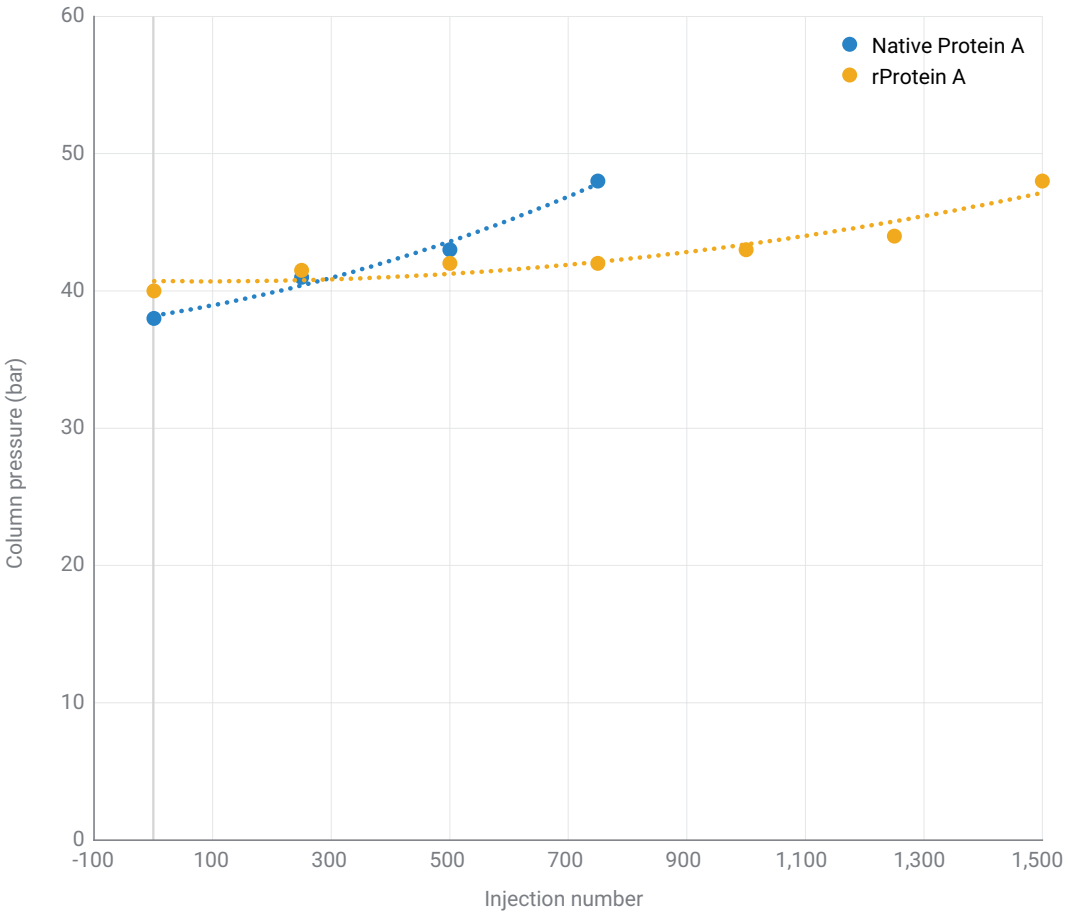

Figure 5. Column pressure versus injection number during lifetime.

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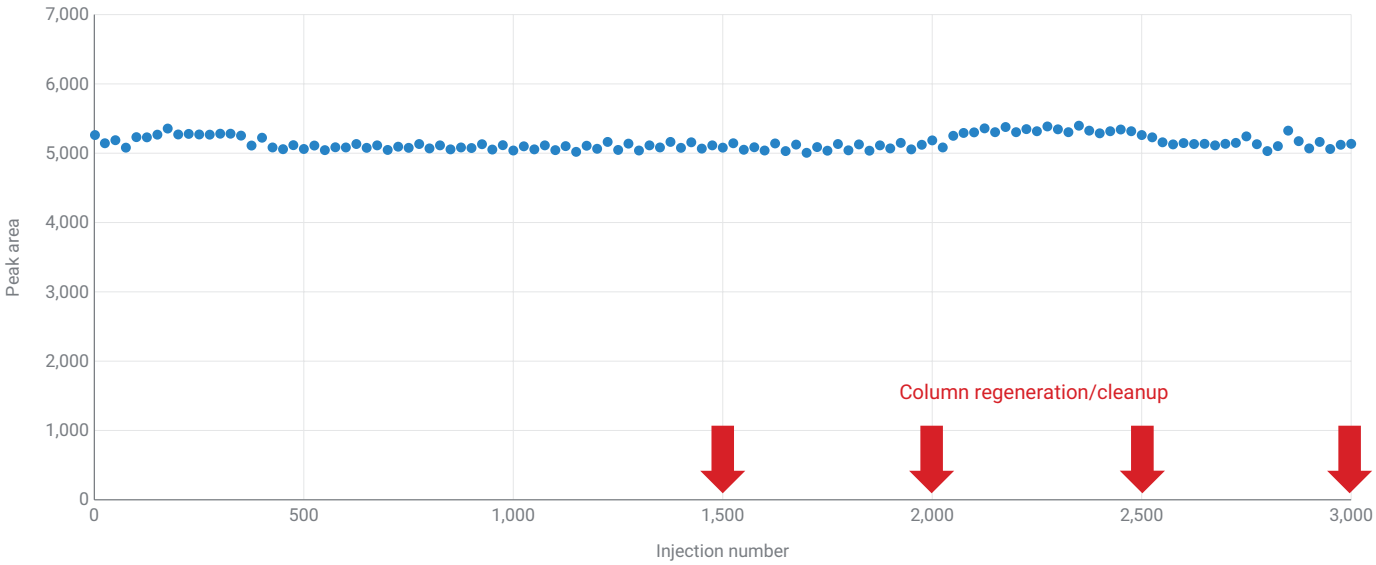

Figure 6. Peak area versus injection number during column lifetime.

Table 2. Peak area versus injection quantity (µg) during column lifetime.

Quantity (µg)	Initial	After 2,000 Injections	After 3,000 Injections
1	654	633	688
2	1,363	1,323	1,308
5	2,766	2,984	2,979
10	5,526	5,699	5,666
15	7,706	7,653	7,699
20	10,541	10,268	10,347

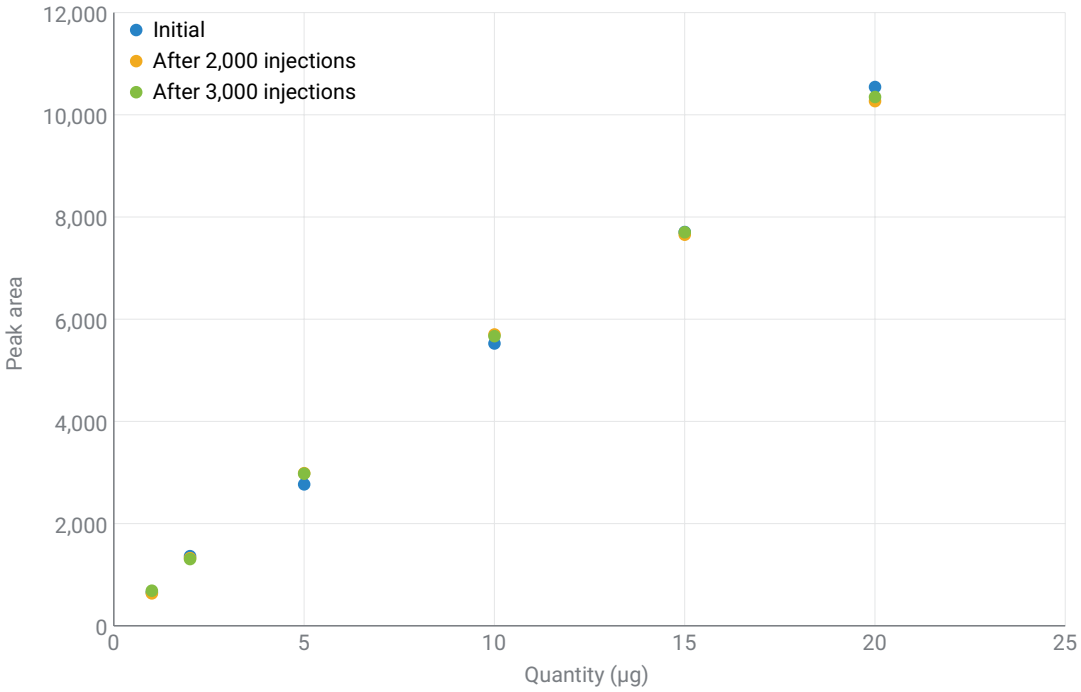

Figure 7. Peak area versus injection number during column lifetime.

Conclusions

This application note has shown that the Bio-Monolith rProtein A column is capable of consistent and reliable performance in titer analysis beyond what we have observed with equivalent native Protein A columns.

References

1. Coffey, A.; Kondaveeti. Improved Lifetime of Bio-Monolith Protein A Columns for Titer Determination. *Agilent Technologies Application Note*, publication number 5994-2168EN, **2020**.

Application Note

Titer Determination

Agilent

Trusted Answers

Cell Clone Selection Using the Agilent Bio-Monolith Protein A Column and LC/MS.

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Abstract

This application note describes how the Agilent Bio-Monolith Protein A column was applied to determine recombinant monoclonal antibody titer in Chinese hamster ovary cell-culture supernatants, and how the column was used to enrich µg amounts of antibody for further structural characterization by mass spectrometry. The workflow provides guidance for the clone selection process in biopharmaceutical and biosimilar development.

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Introduction

Monoclonal antibodies (mAbs) are currently in widespread use for the treatment of life-threatening diseases, including cancer and autoimmune diseases. Over 30 monoclonal antibodies are marketed, nine displayed blockbuster status in 2010, and five of the 10 top-selling biopharmaceuticals in 2009 were mAbs [1]. mAbs are currently considered the fastest growing class of therapeutics. The knowledge that the topselling mAbs are, or will become, open to the market in the coming years has resulted in an explosion of biosimilar activity. The first two monoclonal antibody biosimilars were approved in 2013, and both contain the same active substance, infliximab [2].

Whether developing innovator or biosimilar mAbs, well thought out clone selection is critical early on in the development process. This application note describes how the Agilent Bio-Monolith Protein A column can guide this process. This HPLC column is composed of a poly(glycidyl methacrylate-co-ethylene dimethacrylate) highly porous monolithic support coated with Protein A from Staphylococcus aureus. It combines the advantages of monoliths, that is, fast and efficient separations with limited carry-over, with the selectivity of the Protein A receptor for the Fc region of immunoglobulin G (IgG). As such, it represents an ideal tool for the high-throughput determination of mAb titer and yield directly from cell-culture supernatants, and for purifying mAbs at analytical scale for further measurements, for example by mass spectrometry (MS), ion exchange (IEX), size-exclusion chromatography (SEC), or hydrophobic interaction chromatography (HIC).

We have illustrated the selection of trastuzumab- biosimilar-producing Chinese hamster ovary (CHO) clones, based on titer and structural characteristics, using the Bio-Monolith Protein A column. Trastuzumab has been marketed as Herceptin since 1998, and is still in widespread use in the treatment of HER2 positive breast cancer [3]. This major biotherapeutic becomes open to the market in 2014 in Europe and 2018 in the US. To select clones based on biosimilar mAb titer, absolute concentrations were determined making use of a calibration curve generated with the Herceptin originator. To assess the structural characteristics and to compare with the originator molecule, the Protein A column was used to enrich analytical-scale quantities of the mAbs prior to mass spectrometric analysis.

Experimental

Materials

Acetonitrile, water, and isopropanol were obtained from Biosolve (Valkenswaard, The Netherlands). Citric acid, formic acid, NaH_2PO_4. Na_2HPO_4, and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich, Corp. (St. Louis, MO, USA). Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland). Trastuzumab biosimilar CHO cell-culture supernatants were obtained from a local biotechnology company.

Sample preparation

Herceptin stock solution present at 21 mg/mL was diluted in mobile phase A for construction of the calibration curves. Cell supernatants were diluted 1:1 in 50 mM Na_2HPO_4. Supernatants were centrifuged at 5.000 g for 5 minutes prior to injection. Collected fractions were reduced at room temperature for 1 hour by adding 10 mM TCEP.

Instrumentation

Bio-Monolith Protein A measurements were performed on:

- Agilent 1100 Series Quaternary Pump (G1311A)
- Agilent 1100 Series Autosampler (G1313A)
- Agilent 1100 Series Diode Array Detector (G1315A)
- Agilent 1200 Infinity Series Analytical-scale Fraction Collector (G1364C)

LC/MS measurements were performed on:

Agilent 1290 Infinity Binary LC equipped with:

- Agilent 1290 Infinity Binary Pump (G4220B)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Q-TOF with Agilent Jet Stream LC/MS (G6540A)

Software

- Agilent Technologies OpenLAB CDS ChemStation revision C01.05 (35)
- Agilent Technologies MassHunter for instrument control (B05.01)
- Agilent Technologies MassHunter for data analysis (B06.00)
- Agilent Technologies BioConfirm software for MassHunter (B06.00)

Conditions, Bio-Monolith column

Parameter	Value	
Column:	Agilent Bio-Monolith Protein A (p/n5069-3639)	
Mobile phase:	A) 50 mM phosphate, pH 7.4 B) 100 mM citric acid, pH 2.8	
Gradient:	Time (min) 0 to 0.5 0.6 to 1.7 1.8 to 3.5	% B 0 (binding) 100 (elution) 0 (regeneration)
Flow rate:	1 mL/min	
Injection volume:	50 µL	
Detection:	UV at 280 nm	
Fraction collection:	Time-based	

Conditions, LC/MS

Parameter	Value	
Cartridge:	Online desalting cartridge, 2.1 × 10 mm	
Mobile phase:	A) 0.1% formic acid in water (v:v) B) 0.1% formic acid in acetonitrile (v:v)	
Flow rate:	400 µL/min	
Injection volume:	Variable (corresponding to a protein amount of 1 µg)	
Needle wash solvent:	60 % acetonitrile, 35 % water, 5 % isopropanol	
Autosampler temperature:	7 °C	
Gradient:	Time (min) 0 0.5 2 3 3.10 5	% B 5 5 80.0 80.0 5 5
Q-TOF source:	Agilent Jet Stream, positive ionization mode	
Drying gas temperature:	300 °C	
Drying gas flow rate:	8 L/min	
Drying gas flow rate:	35 psig	
Nebulizer pressure:	350 °C	
Sheath gas temperature:	11 L/min	
Nozzle voltage:	1,000 V	
Capillary voltage:	3,500 V	
Fragmentor voltage:	200 V	
Q-TOF detection:	Mass range 3,200 amu	
Data acquisition range:	500 to 3,200 m/z	
High-resolution mode	(4 GHz)	
Data acquisition rate: mode	1 spectrum per s	
Profile acquisition		
Diverter valve:	Time (min) 0 1 3.5	Flow to waste MS waste

Results and Discussion

Clone selection through determination of trastuzumab titer

Figure 1 shows an overlay of the Protein A chromatograms of the supernatant of a specific trastuzumab-producing clone and a Herceptin originator. The unbound material eluted in the flow-through while the mAb was only released after lowering the pH. In the case of the originator, no material was observed in the flow-through, which is not surprising since this represents the marketed product. In the case of the supernatant, a substantial signal resulting from the unbound material was seen.

Figure 2 shows an overlay of the Protein A chromatograms of 12 trastuzumab-producing clones, generated in the framework of a biosimilar development program. From these chromatograms, a distinction can already be made between low and high producing clones. Absolute mAb concentrations can be determined by linking the peak areas to an external calibration curve constructed by diluting Herceptin originators.

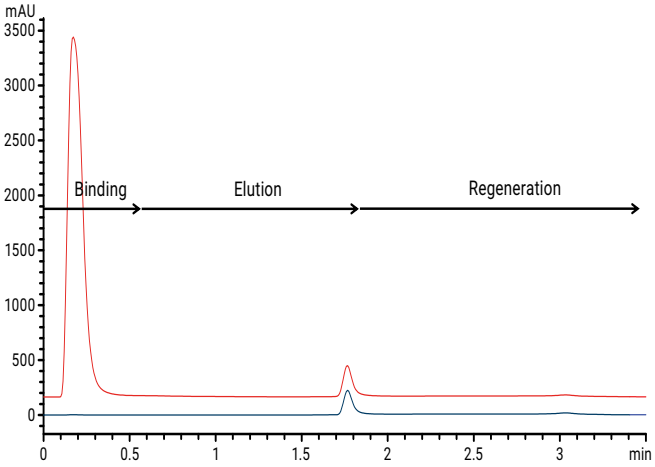

Figure 1. UV 280 nm Agilent Bio-Monolith Protein A chromatogram of a trastuzumab-producing CHO clone, clone 9 (red), and of a Herceptin originator diluted in 50 mM Na-phosphate pH 7.4 to 0.2 mg/mL (blue). Note that the supernatant was diluted 1:1 in phosphate buffer.

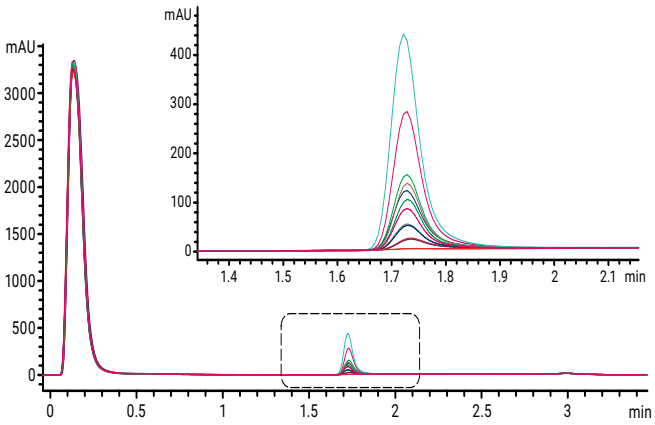

Figure 2. Overlaid UV 280 nm Agilent Bio-Monolith Protein A chromatograms of 12 trastuzumab-producing CHO clones.

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The calibration curve and corresponding chromatograms of the Herceptin calibration points are shown in Figures 3 and 4. Good linearity was obtained between 0.02 and 2 mg/mL, which is the typical mAb titer range in CHO cells. Obtained mAb titers are reported in Table 1 and are pictured graphically in Figure 5. From these findings, clear decisions could be made for further biosimilar development, that is, high-producing clones 9 and 10 could readily be selected and sub cloned. Table 1 also shows the titers obtained when growing the CHO clones in two different cell-culture media, and clearly shows the benefit of one over the other, linking the peak areas to an external calibration curve constructed by diluting Herceptin originators.

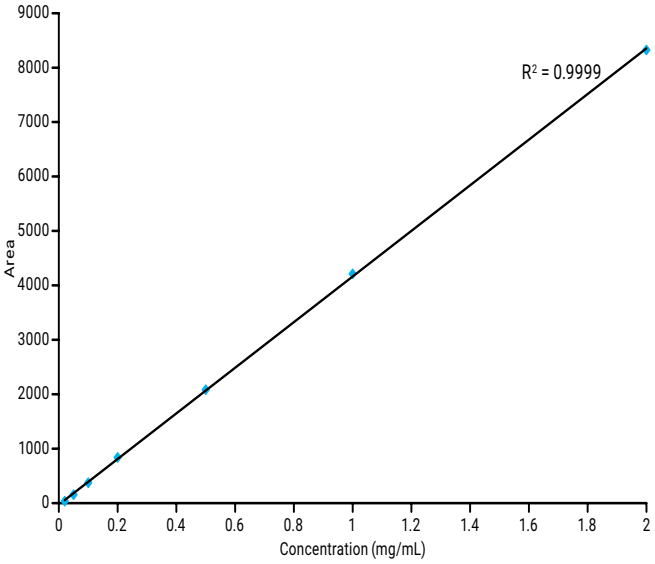

Figure 3. Herceptin Agilent Bio-Monolith Protein A calibration curve, 0.02 to 2 mg/mL.

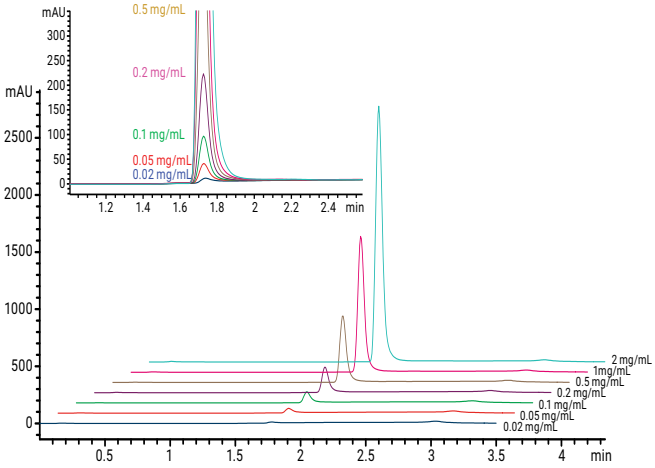

Figure 4. Overlaid UV 280 nm Agilent Bio-Monolith Protein A chromatograms of Herceptin calibration points.

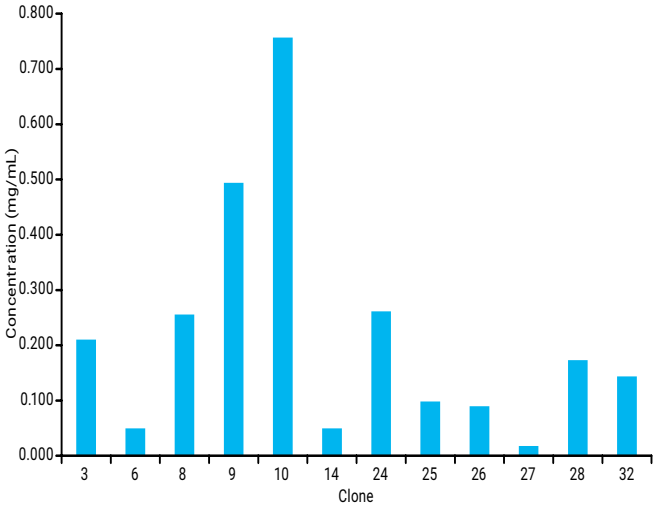

Figure 5. Graphical representation of the biosimilar mAb titer, expressed in mg/mL, in the different trastuzumab CHO clones.

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Table 1. Absolute trastuzumab biosimilar concentrations determined in the different CHO clones grown on two different media.

CHO Clone Medium A	Concentration (mg/mL)	CHO Clone Medium B	Concentration (mg/mL)
3	0.156	3	0.210
6	0.048	6	0.050
8	0.155	8	0.256
9	0.215	9	0.494
10	0.311	10	0.757
14	0.038	14	0.050
24	0.082	24	0.262
25	0.049	25	0.098
26	0.037	26	0.090
27	-	27	0.018
28	0.117	28	0.173
32	0.156	32	0.144

Clone selection by assessing structural characteristics

Next to the mAb titer, the second important criterion in clone selection is based on the structural aspects. In the case of biosimilar development, the structure should be highly similar to the originator product, within the originator batch-to-batch variations. Therefore, Protein A fractions were collected and measured on high-resolution mass spectrometry following disulfide-bond reduction giving rise to the light and heavy chain. This strategy allowed verification of the amino acid sequence and revealed the glycosylation pattern.

To be able to reduce the mAb directly in the collection vial containing acidic buffer, TCEP was chosen instead of the more common reductant dithiothreitol (DTT). The former allows reduction over a broad pH range including low pH values, while the latter's reducing capacities are limited to pH values above seven. Reduced fractions were delivered to the MS system following online desalting. Figures 6 and 7 show the deconvoluted light and heavy chain spectra of one Herceptin originator and two high yield trastuzumab biosimilar-producing clones.

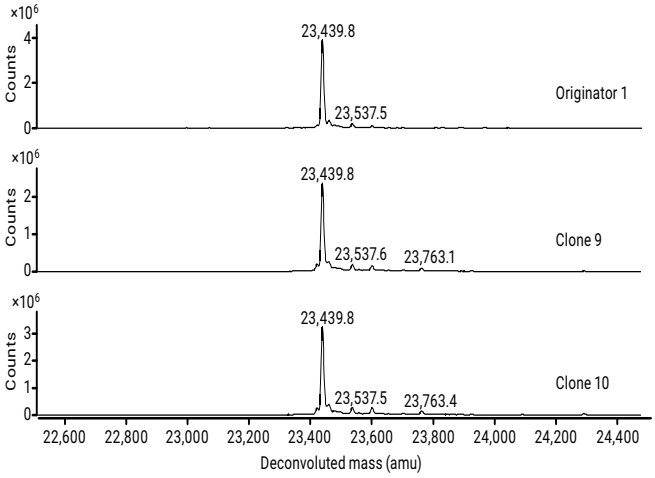

Figure 6. Deconvoluted light chain spectra of a Herceptin originator and two trastuzumab-producing clones.

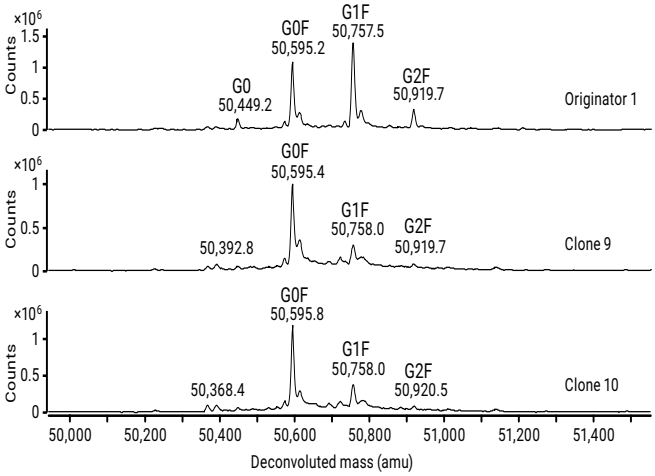

Figure 7. Deconvoluted heavy chain spectra of a Herceptin originator and two trastuzumab producing clones. The abbreviations G0, G0F, G1, and G2F refer to the N-glycans attached to the mAb backbone..

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Tables 2 and 3 display the measured MW values and relative intensity of the main glycoforms in four originator production batches and 12 trastuzumab clones. From this, it can be concluded that the Herceptin originators and clone derived trastuzumab displayed the same light and heavy chain molecular weight values.

In addition, the same N-glycans, which are of the complex type, were observed on the heavy chain of the originators and clone derived mAbs.

These are considered the most important attributes of biosimilarity according to US and European regulatory authorities (the primary sequence should be identical and glycosylation should be preserved). While glycosylation is similar from a qualitative perspective, quantitative differences were seen. A separate application note describes how the Protein A Bio-Monolith was used in the tuning of the growth medium to fit the glycosylation to the originator specifications [4].

Table 2. Measured light and heavy chain MW values in the originators and trastuzumab clones.

MW (Da)	Originator 1	Originator 2	Originator 3	Originator 4	Clone 3	Clone 6
Light chain	23,439.8	23,439.8	23,439.8	23,439.8	23,439.8	23,440.2
Heavy chain *	49,149.9	49,150.2	49,150.1	49,150.1	49,150.5	49,151.0

MW (Da)	Originator 8	Originator 9	Originator 10	Originator 14	Clone 24	Clone 25
Light chain	23,439.8	23,439.8	23,439.8	23,439.9	23,439.8	23,439.9
Heavy chain *	49,150.6	49,150.1	49,150.5	49,150.2	49,150.6	49,151.1

MW (Da)	Originator 26	Originator 27	Originator 28	Originator 32
Light chain	23,440.0	23,441.4	23,439.8	23,439.9
Heavy chain *	49,150.9	49,151.9	49,150.7	49,150.9

*Theoretical deglycosylated MW values.

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Table 3. Relative intensity of the main glycoforms in four originator production batches and trastuzumab clones.

Glycoform	Originator 1	Originator 2	Originator 3	Originator 4	Clone 3	Clone 6
% Man 5	1.6	1.6	1.3	1.1	2.7	1.6
% G0F-GlcNAc	1.5	2.7	3.3	2.4	3.2	3.2
% G0	5.7	5.9	5.0	4.9	2.8	3.3
% G0F	35.2	44.8	50.5	48.2	66.1	56.2
% G1F	45.2	38.4	34.0	36.8	20.6	27.7
% G2F	10.7	6.6	5.9	6.7	4.7	8.1

Glycoform	Clone 8	Clone 9	Clone 10	Clone 14	Clone 24	Clone 25
% Man 5	2.6	3.3	5.0	1.2	1.9	5.1
% G0F-GlcNAc	3.8	4.8	4.6	2.1	3.6	4.2
% G0	1.7	2.9	2.9	3.9	2.2	2.3
% G0F	69.9	66.1	64.1	64.6	68.6	60.7
% G1F	18.4	18.5	19.5	22.9	19.4	20.9
% G2F	3.6	4.3	3.8	5.3	4.3	6.7

Glycoform	Clone 26	Clone 27	Clone 28	Clone 32
% Man 5	5.4	0.0	1.5	3.1
% G0F-GlcNAc	5.8	0.0	2.9	4.3
% G0	1.8	0.0	1.2	2.7
% G0F	61.6	67.2	61.6	64.3
% G1F	19.5	32.8	26.3	20.3
% G2F	5.8	0.0	6.5	5.3

Conclusions

The Agilent Bio-Monolith Protein A column was successfully applied in the selection of trastuzumab-biosimilar-producing clones based on both titer and structural similarity to the originator. This clone selection process is of utmost importance early in the development of innovator and biosimilar mAbs.

References

1. 1. K. Sandra, I. Vandenheede, P. Sandra. *J. Chromatogr. A.,* **1335**, 81 (2014).
2. www.ema.europa.eu
3. www.gene.com
4. 4. E. Dumont, *et al.,* Cell Culture Optimization Using an Agilent Bio-Monolith Protein A Column and LC/MS, *Agilent Technologies Application Note,* publication number 5991-5124EN (**2014**).

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Application Note

Titer Determination

Agilent

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mAb Titer Analysis with the Agilent Bio-Monolith Protein A Column

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Abstract

Monoclonal antibodies (mAbs) are becoming increasingly important in thetreatment of various diseases. During development of recombinant mAbs, proteintiter and yield from various cell culture supernatants must be monitored. Thisapplication note describes how the Agilent Bio-Monolith Protein A column was successfully applied in the determination of mAb concentrations.

Introduction

Protein A from Staphylococcus aureus has a very strong affinity for the Fc domain of immunoglobulins (IgG), allowing its capture from complex matrixes such as cell-culture supernatants. Affinity chromatography making use of Protein A is the gold standard in therapeutic monoclonal antibody (mAb) purification, and typically represents the first chromatographic step in downstream processing. Protein A chromatography finds applications beyond this large-scale purification. At the analytical scale, it is used early in the development of mAbs for the high-throughput determination of mAb titer and yield directly from cell culture supernatants, and to purify µg amounts of material for further measurements, for example, by mass spectrometry (MS), ion-exchange (IEX), size exclusion chromatography (SEC), or hydrophobic interaction chromatography (HIC).

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This application note describes the use of the Agilent Bio-Monolith Protein A column in mAb titer analysis. This HPLC column (Figure 1) has a 5.2 mm id, a column length of 4.95 mm, and is composed of a highly cross-linked poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolithic disk coated with native Protein A from S. aureus. Its monolithic nature, characterized by well-defined channels of 1,200 to 1,500 nm, and by the absence of pores and voids, delivers fast and efficient separations with negligible carryover and excellent robustness. These are features typically expected from a column for mAb titer analysis, to successfully guide clone selection and cell-culture optimization. We present the best practice for use of the column in the determination of absolute mAb concentrations in Chinese hamster ovary (CHO) cell-culture supernatants. Data from a trastuzumab biosimilar project are used for illustration purposes. Trastuzumab, marketed as Herceptin since 1998, is used in the treatment of HER2 positive breast cancer, and comes out of patent in 2014 in Europe, and 2018 in the United States.

Experimental

Materials

Water was obtained from Biosolve (Valkenswaard, The Netherlands). Citric acid, acetic acid, NaH_2PO_4 and Na_2HPO_4 were purchased from Sigma-Aldrich, Corp. (St. Louis, MO, USA). Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland). Trastuzumab biosimilar CHO cell culture supernatants were obtained from a local biotechnology company.

Sample preparation

Herceptin stock solution present at 21 mg/mL was diluted in mobile phase A prior to injection. Cell supernatants were diluted 1:1 in 50 mM Na_2HPO_4. Supernatants were centrifuged at 5,000 g for 5 minutes prior to injection.

Instrumentation

Agilent Bio-Monolith Protein A measurements were performed on:

- Agilent 1100 Series Quaternary Pump (G1311A)
- Agilent 1100 Series Autosampler (G1313A)
- Agilent 1100 Series Diode Array Detector (G1315A)

Software

- Agilent Technologies OpenLAB CDS ChemStation revision C01.05 (35)

Conditions, Bio-Monolith column

Parameter	Value	
Column:	Agilent Bio-Monolith Protein A (p/n5069-3639)	
Mobile phase:	A) 50 mM phosphate, pH 7.4 B) 100 mM citric acid, pH 2.8 mM acetic acid, pH 2.6	
Gradient:	Time (min) 0 to 0.5 0.6 to 1.7 1.8 to 3.5	% B 0 (binding) 100 (elution) 0 (regeneration)
Flow rate:	1 mL/min	
Injection volume:	Variable (50 µL, optimized for CHO cell culture supernatants)	
Detection:	UV at 280 nm	

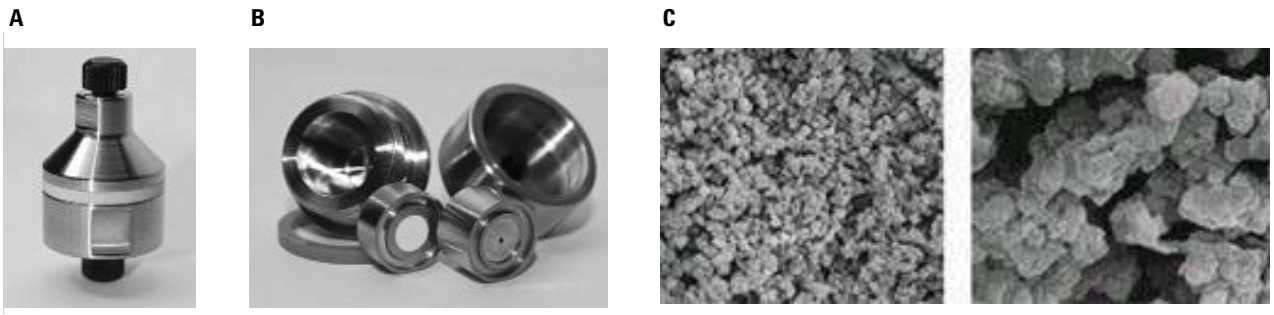

Figure 1. UV 280 nm Agilent Bio-Monolith Protein A chromatogram of a trastuzumab-producing CHO clone, clone 9 (red), and of a Herceptin originator diluted in 50 mM Na-phosphate pH 7.4 to 0.2 mg/mL (blue). Note that the supernatant was diluted 1:1 in phosphate buffer.

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Results and Discussion

Buffer selection

Figure 2 shows a typical chromatogram from the Protein A column. The example chromatogram is one injection of the supernatant of a specific trastuzumab-producing CHO clone. The unbound material eluted in the flow-through while the mAb was retained at neutral pH (binding) and was only released (elution) after lowering the pH upon applying a step gradient. In this case, 50 mM Na-phosphate at pH 7.4 was used for binding/loading, and 100 mM citric acid at pH 2.8 for elution. This represents a good starting condition for any application.

When developing a new method for a Protein A column, both binding and elution buffers should be optimized. For binding buffers, 50 mM Na phosphate, pH 7.4, is a good starting point, and can be optimized between pH 7 and 8. For elution buffers, the 100 mM citric acid used here is a good starting point. Other possible elution buffers are 500 mM acetic acid, pH 2.6, 100 mM glycine HCl, pH 2.8, and 12 mM HCl, pH 1.9.

Figure 3 compares the elution of a Herceptin originator with acetic acid and citric acid. Very similar peak shape and area were observed, although peaks were slightly sharper using citric acid. In the case of this Herceptin originator, no material was seen in the flow-through, which was not surprising since this represented the marketed product and was devoid of host-cell proteins. In the chromatograms shown, the flow rate was set at 1 mL/min. The monolithic nature of the support, characterized by convective instead of diffusive mass transfer, allowed for near flow-rate independence and, hence, high-throughput separations. This is highly desirable in mAb titer determination, which typically requires the processing of a wide range of samples. The maximum flow rate that can be applied on the column is 2 mL/min, which allows fast, sub-2-minute separations.

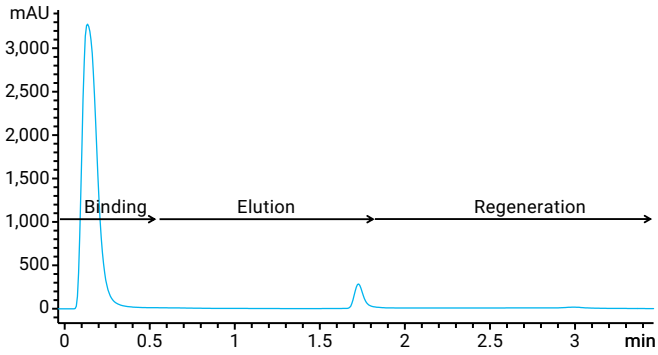

Figure 2. UV 280 nm Protein A chromatogram showing the supernatant of a trastuzumab-producing CHO clone. Injection volume was 50 µL. Peak width at half height was 0.10 minutes for the unbound material and 0.06 minutes for the retained mAb.

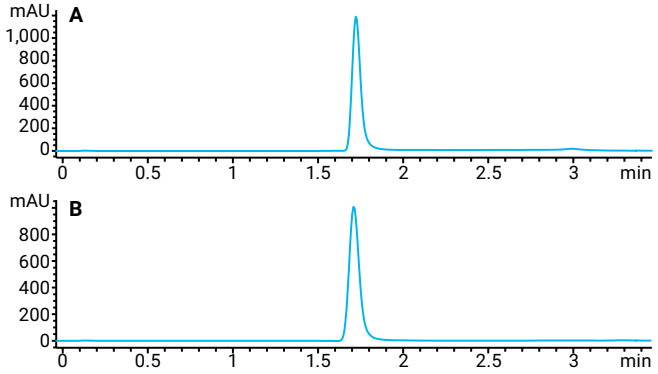

Figure 3. UV 280 nm Protein A chromatogram of Herceptin originator diluted in 50 mM Na-phosphate, pH 7.4, to 0.5 mg/mL (50-µL injection, 25-µg column load). Elution was achieved using citric acid (A) and acetic acid (B). Peak width at half height is 0.057 and 0.067 minutes for citric acid and acetic acid, respectively.

Precision, linearity, carryover, and injection size

Precision is critically important in the determination of the mAb titer. Table 1 shows the peak area and retention time repeatability that can typically be expected upon injecting a Herceptin originator 10 times. Chromatograms are shown in Figure 4. More than acceptable relative standard deviation (RSD) values were obtained for both citric acid and acetic acid as elution buffers. Carryover was simultaneously assessed by injecting a buffer blank after the mAb injection sequence (Figure 5). At a 10-fold column load of 5 µg, carryover appeared to be nonexistent, which can again be attributed to the use of a monolithic support. Carryover at 1% levels became apparent upon a single load of 500 µg of mAb onto the column. This represents the maximum column load and is one typically not encountered in real-life experiments. It is worth noting that carryover was eliminated after the injection of a second buffer blank.

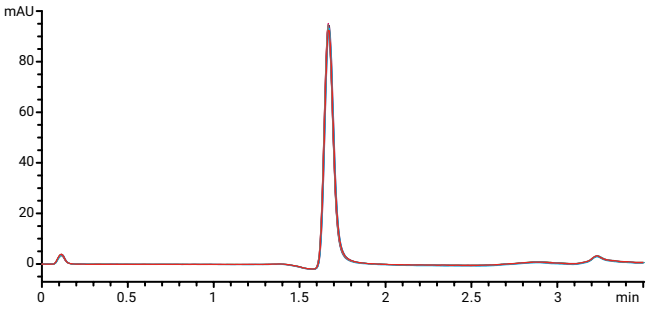

Figure 4. Replicate (n = 10) UV 280 nm Protein A chromatograms of a Herceptin originator diluted in 50 mM Na phosphate, pH 7.4, to 0.5 mg/mL (injection volume 5 µL). Elution was achieved using acetic acid.

Table 1. Retention time and peak area RSD values obtained for the 10-fold analysis of a Herceptin originator at 0.5 mg/mL (5 µL injection volume).

	Acetic acid		Citric acid	
	Peak area	RT (min)	Peak area	RT (min)
1	361	1.669	383	1.666
2	362	1.668	372	1.666
3	373	1.668	365	1.665
4	365	1.669	389	1.667
5	370	1.669	383	1.666
6	373	1.669	378	1.666
7	367	1.671	379	1.678
8	365	1.668	377	1.666
9	366	1.670	376	1.667
10	360	1.670	377	1.667
Mean	366	1.669	378	1.667
S	4.64	0.001	6.52	0.001
% RSD	1.27	0.06	1.73	0.06

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The limit of detection (LOD) was around column loads of 0.5 µg. This put some demands on injection volume. If samples have low mAb levels, high volume injections are required. Figure 6 shows the linearity obtained when increasing the injection volume from 5 to 50 µL for a 1 mg/mL Herceptin originator. With the knowledge that 50 µL injections are perfectly feasible and that the lowest detectable amount on-column is 0.5 µg, samples with mAb concentrations at 10 µg/mL are within reach.

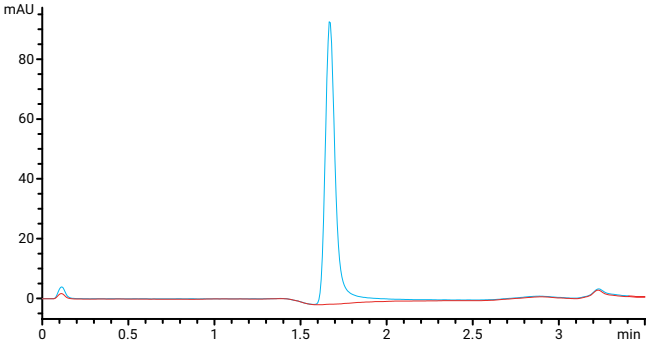

Figure 5. UV 280 nm Protein A chromatograms of a Herceptin originator diluted in 50 mM Na phosphate, pH 7.4, to 0.5 mg/mL, and a blank buffer analyzed after a sequence of 10 Herceptin injections. Elution was achieved using acetic acid, and injection volumes were 5 µL.

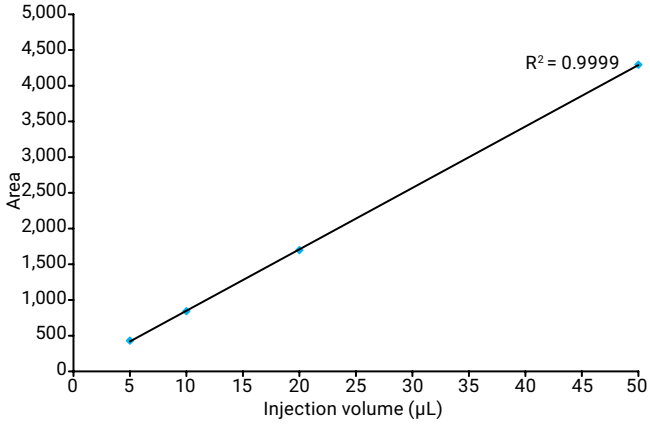

Figure 6. Linearity obtained by increasing the injection volume of a Herceptin originator (0.5 mg/mL) from 5 to 50 µL.

In mAb titer determination, it is important to be able to assess absolute mAb concentrations. These can be found by linking the peak areas measured in cell-culture supernatants to an external calibration curve constructed by diluting a mAb standard. For the Herceptin biosimilar project, this standard was found in the originator product, which was accurately formulated at 21 mg/mL. The calibration curves of a dilution series of Herceptin originators using citric acid and acetic acid as elution buffers are shown in Figure 7. The corresponding chromatograms are shown in Figure 8. In both cases, linearity was excellent, between 0.02 mg/mL and 2 mg/mL, which is the typical mAb titer range in CHO cells.

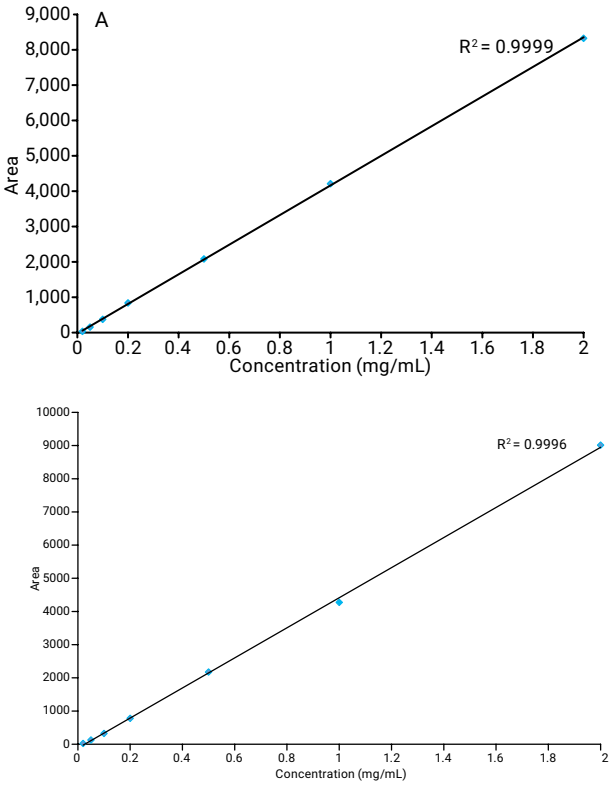

Figure 7. Herceptin Protein A calibration curve (0.02 to 2 mg/mL) using citric acid (A) and acetic acid (B) as elution buffer.

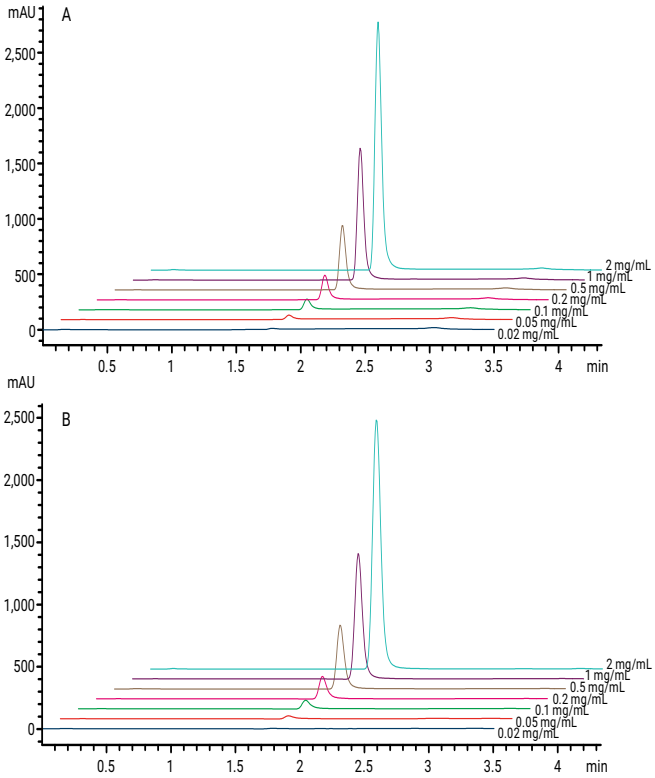

Figure 8. Overlaid UV 280 nm Protein A chromatograms of Herceptin calibration points using citric acid (A) and acetic acid (B) as elution buffer.

Application in mAb titer determination

The method possesses all the characteristics for the determination of mAb titer in cell-culture supernatants. It is fast, precise, and linear in the expected mAb concentration range and does not suffer from carryover. To illustrate this, nine trastuzumab-producing clones, generated in the framework of a Herceptin biosimilar development program, were analyzed using the Bio-Monolith Protein A column to determine absolute mAb concentrations. Chromatograms are displayed in Figure 9, and Table 2 reports the obtained mAb titers using both citric acid and acetic acid as elution buffer. Very consistent data were generated using both elution buffers. These results allow clear decisions to be made early in the development of mAbs. High-producing clones can be readily selected and subjected to further development.

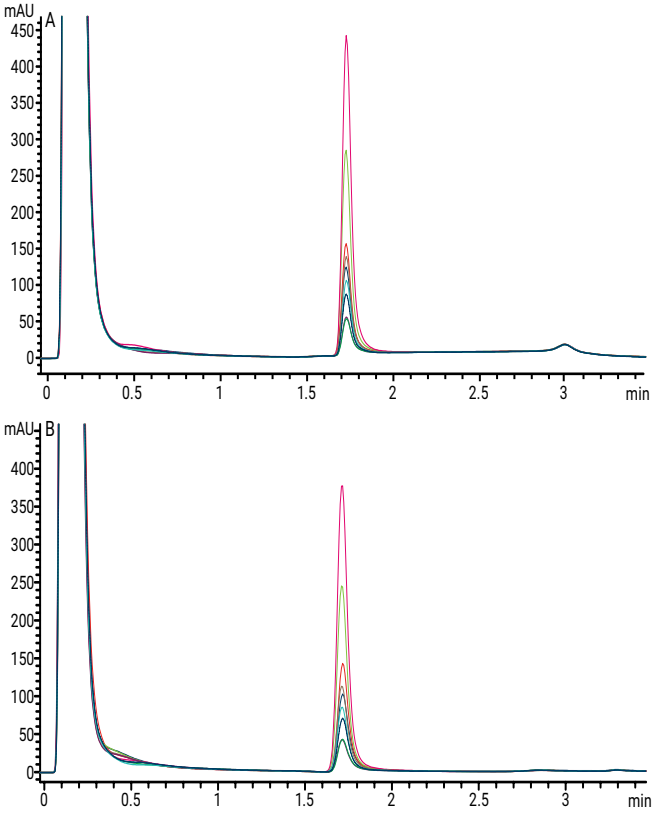

Figure 9. Overlaid UV 280 nm Protein A chromatograms of nine trastuzumab-producing CHO clones using citric acid (A) and acetic acid (B) as elution buffer.

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Maximizing column lifetime

Column regeneration

A major benefit of using a monolithic disk is that the presence of channels instead of pores decreases the likelihood of column clogging when injecting cell-culture samples. This increases robustness and reduces cleaning efforts. Column contamination can be reduced by running a blank gradient injection after every 30 to 50 samples. If column deterioration is observed (tailing or broad peaks), the following cleaning procedure is recommended. Column regeneration is the first step. If performance is still suboptimal, the clean-in-place procedure can be used, which will reduce the amount of Protein A available.

Column regeneration

1. Wash with 2 mL (20 column volumes (CV)) of 100 mM phosphate buffer + 1 M NaCl, pH 7 to 8, at 0.5 to 1.0 mL/min.
2. Wash with 2 mL (20 CV) of low-pH solution (such as elution buffer).
3. Re-equilibrate with binding buffer.

Clean-in-place

1. 1. Wash with 1 to 2 mL (10 to 20 CV) of 0.1 M NaOH (reverse flow direction) at 0.2 to 0.5 mL/min.
2. Wash with 1 to 2 mL (10 to 20 CV) of DI water at 0.5 to 1.0 mL/min.
3. Wash with 1 to 2 mL (10 to 20 CV) of concentrated buffer (0.1 to 0.5 M) to restore normal pH (7.0 to 7.4).
4. Re-equilibrate with 5 mL (50 CV) of binding buffer.

Conclusions

The Agilent Bio-Monolith Protein A column was successfully applied in the selection of trastuzumab-biosimilar-producing clones based on both titer and structural similarity to the originator. This clone selection process is of utmost importance early in the development of innovator and biosimilar mAbs

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Additional Application Notes

Publication Number	Title
5991-2990EN	Agilent Bio-Monolith Protein A Monitors Monoclonal Antibody Titer from Cell Cultures
5991-4723EN	Reducing Cycle Time for Quantification of Human IgG Using the Agilent Bio-Monolith Protein A HPLC Column
5991-5125EN	Cell Culture Optimization Using an Agilent Bio-Monolith Protein A Column and LC/MS
5991-6094EN	Bio-Monolith Protein G Column - More Options for mAb Titer Determination

Additional Information

Agilent Bio-Monolith columns are also available in ion exchange formats.

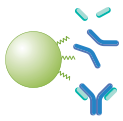
Intact & Subunit Purity

Background

Reversed-phase chromatography remains one of the most valuable tools in the chromatographer's armory. It is a well understood technique relying on hydrophobic interactions between the analyte and the stationary phase. For intact proteins, the technique uses gradients of organic solvents as mobile phase, typically with an ion pair reagent. Under these conditions, the molecule is likely to become denatured. It is a sensitive technique as the sample is concentrated as it is retained by the column and it is useful with mass spectrometry. It is therefore suitable for determining the accurate mass of an intact protein.

For large proteins, such as monoclonal antibodies, and even for smaller fragments such as heavy and light chains or Fab and Fc regions of an IgG molecule, wide pore columns are recommended. Agilent offers 300 and 450 Å products in many configurations including fully porous, Poroshell technology featuring superficially porous particles, and a selection of alkyl bonded phases. The bonded phase is typically shorter chain length (C8 or C4/C3) or more unique ligands such as diphenyl that may offer different selectivity.

With the accuracy of reversed-phase chromatography it is possible to use intact and fragment analysis to compare biosimilars with originator biotherapeutics. However, it is always necessary to perform extra tests to identify the specific location of the different variants that may be detected.



Intact and Subunit Purity

Large molecule chromatography (>150 Å)

Selectivity options for every separation need

AdvanceBio RP-mAb

Ideal for monoclonal antibodies

Attribute	Advantage
450 Å pore, superficially porous particles	Optimum design for high-resolution mAb separations
Extended column lifetime	Lower operating costs

ZORBAX RRHD 300 Å 1.8 µm

UHPLC separations

Attribute	Advantage
1200 bar maximum pressure	UHPLC-compatible
1.8 µm particles	Maximum resolution

PLRP-S*

Ideal formic acid performance for MS detection

Attribute	Advantage
Polymeric particle with no silanol interactions	Better peak shape, better recovery, and lower carryover
Durable, resilient particles	Reproducible results over longer lifetimes

*** Available in PEEK lined stainless hardware**

Getting Started

Selecting a reversed-phase column for intact protein analysis requires consideration of several interrelated factors: Sample molecular weight and the best suited particle pore size, column chemistry, the instrumentation to be used particularly the type of detector, mobile phase conditions, and speed or throughput requirements to name a few.

Larger analytes require larger pore sizes. With some exceptions, pore sizes for intact protein analysis are typically 300–500 Å. As a rule of thumb, the pore size should be at least three times the hydrodynamic radius of the protein. The AdvanceBio RP-mAb column has 450 Å pores ZORBAX RRHD 300 Å, ZORBAX 300SB, and Poroshell 300 all have 300 Å pores, and PLRP-S is available in many pore sizes. While substantially larger than what is commonly used for intact proteins, the 1000 Å, 5 µm PLRP-S columns give excellent results for intact protein and protein fragment analysis.

For reversed-phase columns, a general guideline for choosing a column chemistry is, the higher the molecular weight, the shorter the alkyl chain should be. Hence, C18 columns are commonly used for peptides while C8, C4, and C3 columns are commonly used for intact protein separations. In addition to linear alkyl chains, diphenyl phases are available for the AdvanceBio RP-mAb, ZORBAX RRHD 300, and ZORBAX 300SB columns. Sometimes, the alternate selectivity of the diphenyl phase can provide the separation needed. PLRP-S is a polymeric particle rather than a silica-based particle. It gives a typical reversed-phase separation, with somewhat different selectivity and the advantage of wide pH tolerance.

The instrumentation available determines what maximum pressure can be achieved. One can certainly use a column with a 600 bar pressure maximum on a UHPLC capable of 1200 bar. But care should be taken not to over pressure the column, which can lead to premature column failure. Within Agilent's reversed-phase portfolio, the ZORBAX RRHD column has a maximum backpressure of 1200 bar, and can thus be used for high speed, high-pressure separations.

When considering instrumentation and backpressure capabilities, it is worth considering whether the method under development will ever need to be transferred to another LC system with a different maximum backpressure. If so, it would be cost- and time-effective to develop a method that can be run on all platforms.

Detector selection and mobile phase conditions are often related. For protein separations, this is commonly a decision between using UV detection or mass spectrometry (MS). Traditionally trifluoroacetic acid (TFA) has been used as an ion pairing agent for separations with UV detection, while formic acid is preferred for MS detection. TFA is typically used for UV detection as it gives excellent peak shape on silica-based columns, however it leads to ion suppression in mass spectrometry. Formic acid preserves MS sensitivity, but gives less than ideal peak shape on silica-based columns, therefore polymeric PLRP-S column is recommended for formic acid mobile phases. With an understanding of the trade-offs, one can use formic acid mobile phase with silica-based columns, or TFA with mass spectrometry. There's also no disadvantage to using formic acid or PLRP-S with UV detection.

Water/acetonitrile gradients are commonly used for reversed-phase separations of intact proteins and monoclonal antibody fragments and are generally suitable for Agilent reverse-phase columns. A different organic solvent, such as methanol or isopropanol may produce a helpful change in selectivity in the case of some separations. The AdvanceBio RP-mAb columns give their best results with an organic mobile phase containing isopropanol, acetonitrile, and water. Application note number 5991-6274EN gives more detail on this column and mobile phase pairing.

The AdvanceBio Desalting-RP product offers a cartridge-format, PLRP-S based approach to online desalting. This cartridge is typically used before mass spectrometry analysis, but could be used with UV detection and fraction collection to desalt samples before subsequent analysis.

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Monitoring Product-Related mAb Fragments

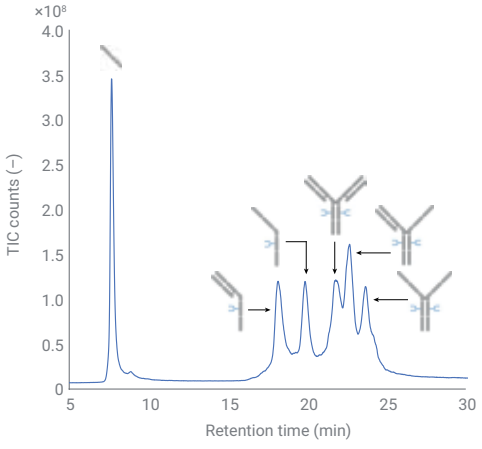
Intact protein analysis with the Agilent 1290 Infinity II Bio LC System enables UV and MS detection of low molecular weight species

Author

André Feith
Agilent Technologies, Inc

Abstract

Product-related impurities such as low molecular weight (LMW) or high molecular weight (HMW) species are considered critical quality attributes (CQAs) in therapeutic monoclonal antibody (mAb) products and need to be monitored across the drug production process. This application note developed an RPLC method based on the excellent performance of the Agilent 1290 Infinity II Bio LC System combined with the PEEK-lined Agilent PLRP-S column. By analyzing the reduced heavy and light chains of the NISTmAb, excellent relative retention time and area deviations were observed, even with extremely shallow gradient slopes. After method development, all relevant LMW fragments, such as two heavy chains (H2) or two heavy chains and one light chain (H2L), could be separated and detected. Due to the sequential coupling of the UV and MS detector, this method can be used in several areas of the biopharmaceutical production chain. The method also stands as an alternative to SDS-PAGE/CE-SDS with the possibility to analyze two CQAs – LMW species and post-translational modifications (PTMs) – in one run.



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Introduction

mAbs are a major product class of biopharmaceuticals and have been used successfully to tackle various diseases.[1] These biomolecules possess a conserved heterotetrameric structure, consisting of two heavy chains and two light chains connected by disulfide bonds. During manufacturing or improper storage, product-related impurities such as LMW species (see Figure 1) or HMW species (e.g., antibody dimers) can be formed. Those impurities can be present even after extensive purification steps, making it essential to monitor them as a CQA for a drug product. HMW species such as antibody dimers, trimers, or higher aggregates can routinely be analyzed and separated by size exclusion chromatography (SEC) with UV detection.[2] Coupling of SEC with MS detection can be performed to further characterize impurities regarding molecular weight and PTMs.[3] The analysis of LMW species such as heavy chain (H), light chain (L), or H2L fragments can be carried out by capillary electrophoresis-sodium dodecyl sulfate (CE-SDS).[4] Unfortunately, CE-SDS cannot be coupled to MS detection due to high ion suppression caused by SDS, and therefore proposed identities of LMW species are often based on empirical knowledge. This application note shows an alternative analysis of LMW species of mAbs based on the excellent performance of the 1290 Infinity II Bio LC and the PEEK-lined PLRP-S column. Due to the reversed-phase liquid chromatography (RPLC) mode, all relevant reduction-induced LMW fragments of the NISTmAb can be detected with UV and MS for routine or in-depth analysis as needed.

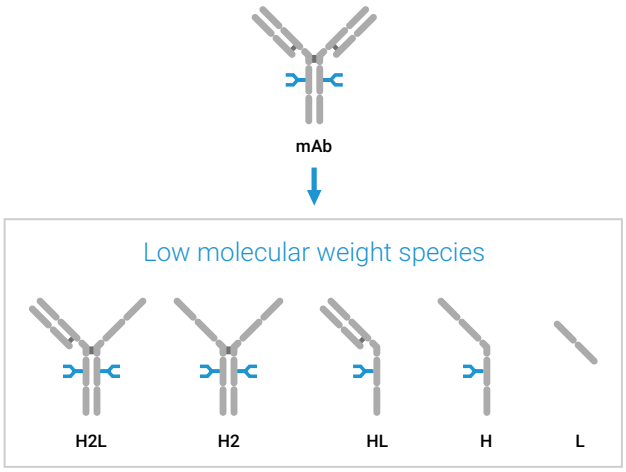

Figure 1. Schematic overview of reduction-induced LMW species of monoclonal antibodies (mAb). Abbreviations: H2L (two heavy chains and one light chain), H2 (two heavy chains), HL (one heavy chain and one light chain), H (heavy chain), and L (light chain).

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System coupled to the Agilent 6545XT AdvanceBio LC/Q-TOF comprised the following modules:

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) equipped with a Standard Flow Quick
- Connect Bio Heat Exchanger (G7116-60071) and two Agilent Thermal Equilibration Devices (G7116-60013)
- Agilent 1290 Infinity II Variable Wavelength Detector (VWD) (G7114B), equipped with a Bio Micro Flow Cell VWD, 3 mm, 2 µL, RFID.
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6545XT)

Software

- Agilent MassHunter workstation data acquisition (B.09.00 or later)
- Agilent MassHunter Qualitative Analysis (10.0 or later)
- Agilent MassHunter BioConfirm (10.0 or later)

Columns

Agilent PLRP-S 5 µm 1000 Å, 2.1 × 100 mm PEEK-lined

(part number PL1912-2502PK)

Chemicals

Agilent InfinityLab Ultrapure LC/MS acetonitrile (part number 5191-4496) and the Agilent-NISTmAb (part number 5191-5744) were used. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). DL-dithiothreitol (DTT) was purchased from Merck (Darmstadt, Germany).

Sample preparation

To partially reduce the NISTmAb, 40 µg were incubated with 1 mM DTT in an amber glass vial directly in the 1290 Infinity II Bio Multisampler at 4 °C. Full reduction into heavy chain (H) and light chain (L) was achieved by incubating 40 µg of NISTmAb with 10 mM DTT at 60 °C for 30 minutes. Injection concentration was 1 mg/mL NISTmAb or reduced NISTmAb.

Method

Table 1. LC method for analyzing the intact NISTmAb and corresponding LMW species with the Agilent 1290 Infinity II Bio LC.

Parameter	Value
Column	Agilent PLRP-S 5 µm 1,000 Å, 2.1 × 100 mm PEEK-lined
Solvent	A) Water + 0.1% formic acid B) Acetonitrile + 0.1% formic acid
Gradient	0.00 min – 25% B 9.00 min – 30% B 34.00 min – 38% B 34.01 min – 100% B 36.00 min – 100% B 36.01 min – 25% B 40.00 min – 25% B
Flow rate	0.400 mL/min
Temperature	60 °C with thermal equilibration devices installed
UV Detection	VWD: 280 nm, 10 Hz/MS: see Table 2
Injection	Injection volume: 0.3 µL Sample temperature: 4 °C Wash: 3 s with water (flush port)

Table 2. Source and MS parameters for the analysis of the intact NISTmAb and corresponding LMW species.

Parameter	Value
Instrument	Agilent 6545XT AdvanceBio LC/Q-TOFn
Gas Temperature	350 °C
Drying Gas Flow	12 L/min
Nebulizer	35 psig
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
VCap	4,000 V
Nozzle Voltage	2,000 V
Fragmentor	180 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Acquisition Mode	Positive, extended (m/z 10,000) mass range
Mass Range	*m/z* 100 to 10,000
Acquisition Rate	1 spectrum/sec
Reference Mass	*m/z* 922.0098

Results and discussion

The analysis of biopharmaceuticals throughout the production process from manufacturing to quality control demands the best performance possible from LC systems. To evaluate the 1290 Infinity II Bio LC's performance regarding the analysis of mAb fragments, the NISTmAb was entirely reduced with DTT, resulting in H and L fragments. Figure 2 shows the relative retention time and area standard deviations (RSD) based on seven consecutive injections. It shows that the retention time and area precision of the 1290 Infinity II Bio LC coupled to the 6545XT AdvanceBio LC/Q-TOF is excellent and perfectly suited for analyzing mAb fragments with shallow gradients. Even though the LC method consists of two linear gradient steps with slopes of 0.32 and 0.55 %B/min, the RSD values remain low with 0.190% (L) and 0.056% (H) for the retention time and 0.530% (L) and 0.744% (H) for the area precision.

One of the major challenges when analyzing LMW species with RPLC is the insufficient resolving power to separate antibody fragments such as H2 or H2L due to their similarity in hydrophobicity compared to the actual mAb. These fragments can occur in the fermentation process or by partial reduction in the final product. However, these fragments can also be generated artificially by partial reduction over time with a low amount of DTT and decreased temperature. With this technique, an RPLC method based on the PEEK-lined PLRP-S and the 1290 Infinity II Bio LC was developed.

The dynamic reduction of the NISTmAb in the 1290 Infinity II Bio Multisampler can be seen in the chromatogram of Figure 3. All of the relevant mAb fragments depicted in Figure 1 can be nicely resolved and change over time due to the addition of DTT. In particular, the separation of the H2, H2L fragments, and the NISTmAb is exceptionally good for RPLC, rendering the combination of the PEEK-lined PLRP-S column and the 1290 Infinity II Bio LC the method of choice for the analysis of LMW. Thanks to the RPLC mode, the 1290 Infinity II Bio LC System can easily be coupled to the 6545XT AdvanceBio LC/Q-TOF, and MS data can be analyzed in Agilent MassHunter BioConfirm. After deconvolution, the spectra in Figure 3 depict the main glycoforms of the respective fragments. The characteristic glycosylation of the NISTmAb shows that it is possible to analyze PTMs of the different fragments easily with this method. Additionally, Figure 4B shows the extracted ion chromatograms (EIC) of representative ions for the fragments clustering around the mAb peak. These EICs also offer good peak shape owing to the resolving power of the PEEK-lined PLRP-S column.

Since the instrumentation setup comprises the 1290 Infinity II Variable Wavelength Detector and the 6545XT AdvanceBio LC/Q-TOF in sequence, UV and MS detection is possible in one run with little to no band broadening and convenient method transfer from process development to quality control (Figure 4A).

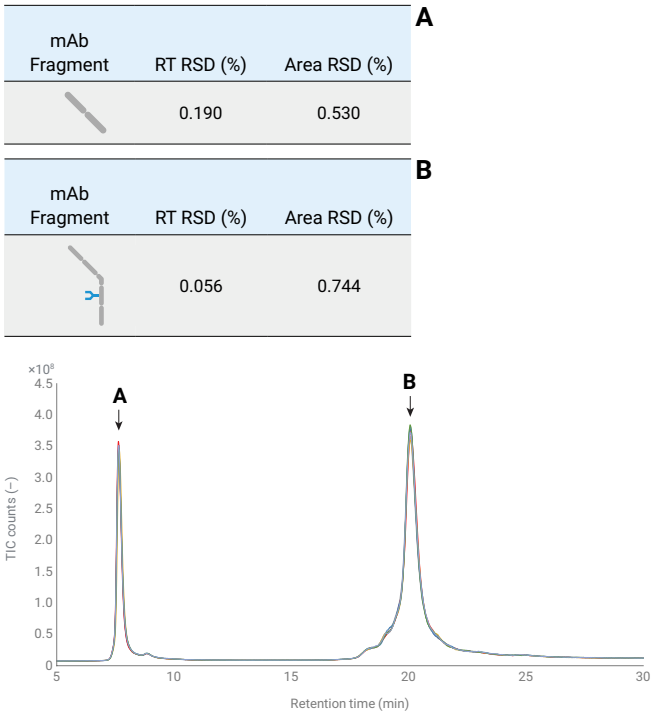

Figure 2. Relative retention time and area precision (RSD, n = 7) values for the Agilent 1290 Infinity II Bio LC analyzing heavy and light chain fragments derived by reduction of the NISTmAb.

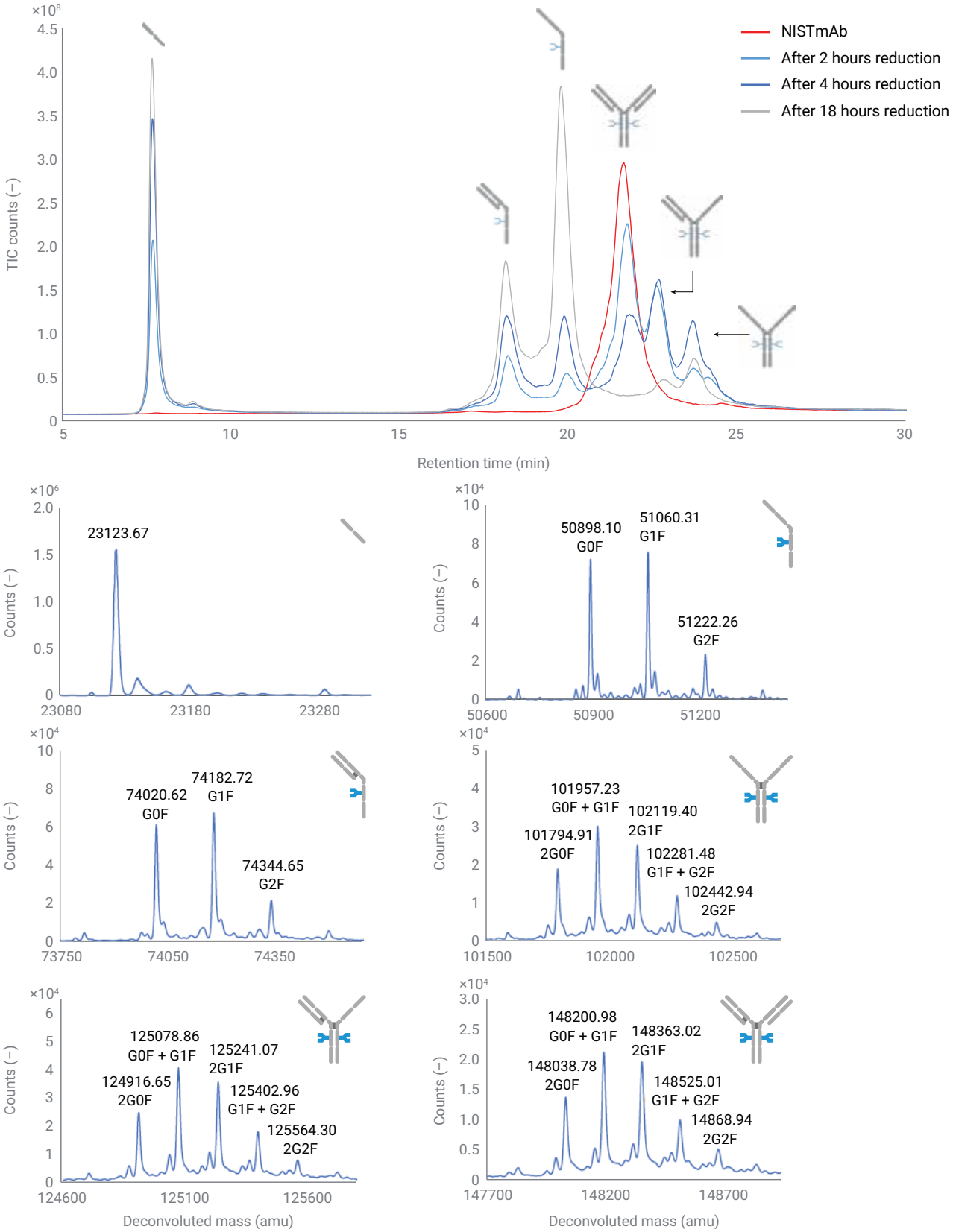

Figure 3. Chromatograms of the dynamic partial reduction of the NISTmAb separated by the Agilent 1290 Infinity II Bio LC and detected with the Agilent 6545XT AdvanceBio LC/Q-TOF. Corresponding extracted spectra of the respective fragments show the characteristic glycosylation of the NISTmAb.

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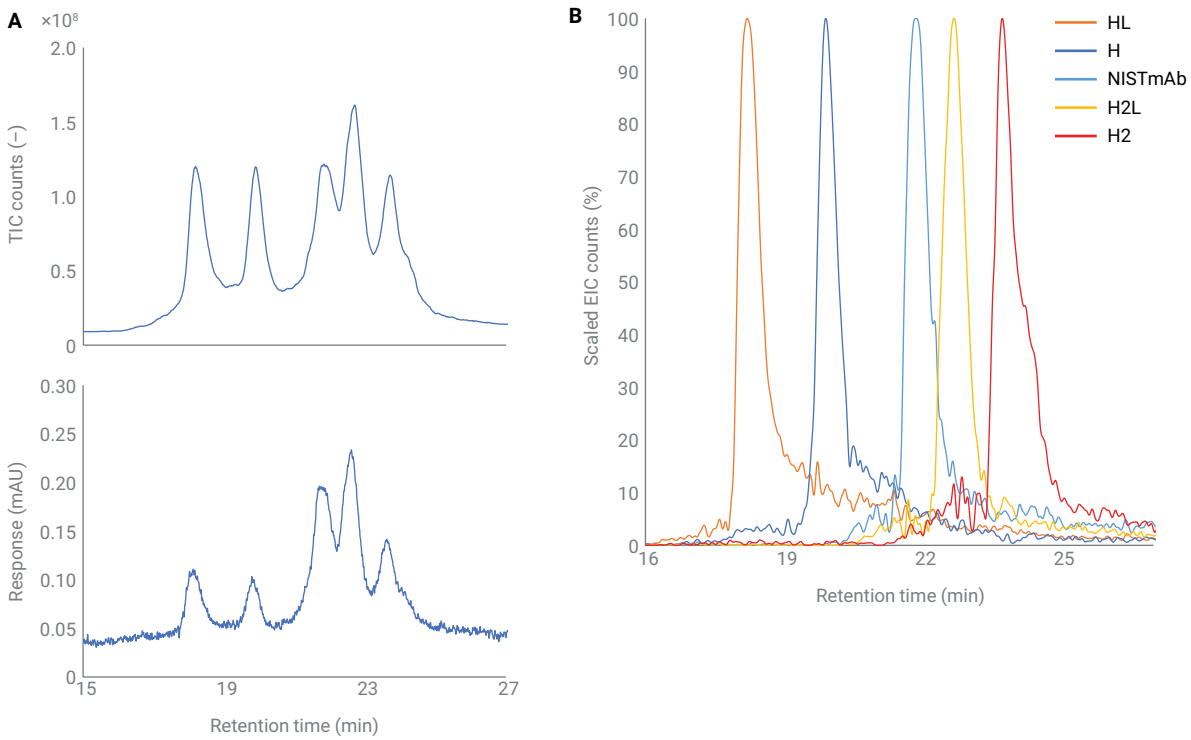

Figure 4. MS and UV chromatogram of the fragments clustering around the NISTmAb acquired in one run (A). Extracted ion chromatograms of NISTmAb fragments showing excellent peak shape (B).

Conclusion

Traditional SDS-PAGE and the modern equivalent CE-SDS are widely used to analyze product-related impurities like LMW and HMW species. However, structural identification of LMW species with these methods has been challenging and primarily based on empirical knowledge. This application note presents an RPLC method capable of separating all relevant reduction-induced LMW species of the NISTmAb. The 1290 Infinity II Bio LC showed excellent retention time and area precision values based on the heavy chain and light chain fragment analysis. Dynamic reduction of the NISTmAb in the 1290 Infinity II Bio Multisampler and subsequent detection with the 6545XT AdvanceBio LC/Q-TOF showed the potential of the method to analyze post-translational modifications. When combined with fragment analysis, this capability can accelerate biopharmaceutical development. That is why the PEEK-lined PLRP-S column and the 1290 Infinity II Bio LC are a future-proof combination for the analysis of biopharmaceuticals across the production process up to final quality control.

References

1. Walsh, G. Biopharmaceutical Benchmarks 2014. Nat. *Biotechnol*. **2014**, *32*, 992–1000.
2. Nägele, E. Elevate Your mAb Aggregate Analysis: High-resolution SEC with the Agilent 1290 Infinity II Bio LC System. *Agilent Technologies* application note, publication number 5994-2709EN, **2020**.
3. Vandenheede, I. et al. SEC Coupled to High-Resolution Mass Spectrometry for Detailed Characterization of mAbs and ADCs. *Agilent Technologies* application note, publication number 5994-0303EN, **2018**.
4. Rustandi, R. R.; Washabaugh, M. W.; Wang, Y. Applications of CE SDS Gel in Development of Biopharmaceutical Antibody-Based Products. *Electrophoresis* **2008**, *29*, 3612–3620

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Determination of Drug-to-Antibody Distribution in Cysteine-Linked ADCs.

An Analysis of ADCs of IgG1 and IgG2 Subclasses

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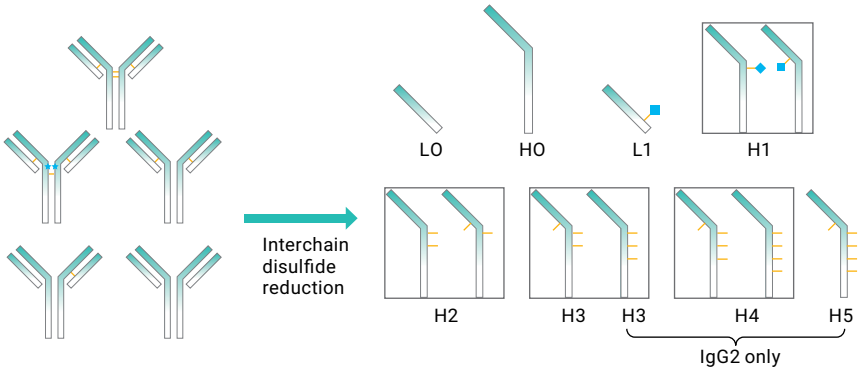
Abstract

This Application Note shows the advantages of using sub-2 µm reversed-phase columns to gain extra resolution and accuracy in the determination of drug-to-antibody ratios. These ratios were determined in antibody-drug conjugates derived from antibody intermediates of the IgG1 and IgG2 subclasses in this study.



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Introduction

Antibody-drug conjugates (ADCs) represent a growing class of anticancer therapeutics that combine the specificity of an antibody with the potency of chemotherapeutic agents using covalent and chemically stable linkages. The ADC field is expanding with an increasing number of conjugation technologies being developed. One dominant class of ADCs includes conjugation to cysteine residues that are involved in the formation of interchain disulfide bonds through maleimide linkages. One of the principal critical quality attributes for ADCs that directly correlates with potency is the drug-to-antibody ratio (DAR). Up to eight or 12 drug-linkers may be conjugated per antibody, depending on the IgG antibody subclass1.

Hydrophobic interaction chromatography (HIC) is a common approach for determining conjugate distribution, and calculating DAR for ADCs manufactured from IgG1 mAbs. However, monitoring conjugate distribution and DAR for ADCs manufactured from IgG2 mAbs by HIC is challenging, due to incomplete resolution between positional isomers and variably conjugated species. Reversed-phase (RP) chromatography can be used as an alternative or orthogonal technique for determining the DAR of ADCs following reduction of interchain disulfide bonds. Using this technique, the DAR may be calculated experimentally from the distribution of unconjugated and conjugated light and heavy chains. For ADCs manufactured from IgG2 antibodies, RP is a more suitable method. This is because elution between unconjugated and variably conjugated light and heavy chains is dictated by the number of conjugated drug-linkers, regardless of the site of conjugation.

A limited variety of suitable HIC and RP columns are available for these applications. This Application Note describes the use of the Agilent ZORBAX RRHD SB300-C8 column for characterizing the distribution of unconjugated and variably conjugated light and heavy chains, and for determining the average DAR. Here, we describe RP UHPLC methods suitable for ADCs manufactured from both IgG1 and IgG2 antibodies. Compared to methods using common HPLC columns, the ZORBAX RRHD SB300-C8 column offers improved peak resolution, and yields similar distributions of unconjugated and conjugated light and heavy chains and DARs.

Materials and Methods

Reagents, samples, and materials

ADCs manufactured from fully human IgG1 and IgG2 antibody intermediates are proprietary. DL-dithiothreitol (DTT) was purchased from Thermo Scientific (Pierce NoWeigh DTT). All solvents used were HPLC grade, and were purchased from either VWR or Fisher Scientific.

Samples in their respective formulation buffers (pH 5–6) were diluted to 5 mg/mL, and the pH was adjusted to approximately pH 8 with 1 M tris pH 9. Partial reduction of the interchain disulfide bonds was achieved by incubation in 40 mM DTT at 37 °C for 15 minutes. After cooling to room temperature, reduced samples were diluted 1:1 with 2 % formic acid in 50 % acetonitrile to quench the reduction reaction.

UHPLC method

Parameter	Value
Column	Agilent ZORBAX RRHD SB300-C8, 50 mm × 2.1, 1.8 µm
Other columns	Vydac 214MS, C4, 2.1 × 50 mm, 5 µm, 300 Å Agilent PLRP-S, 2.1 × 50 mm, 5 µm, 1,000 Å
Mobile phases	A) 0.1 % TFA in H2O B) 0.08 % TFA in 90 % ACN
Column temperature	80 °C (IgG1) 70 °C (IgG2)
Post-column cooler	35 °C
Injection volume	2 µL (IgG1) 3 µL (IgG2)
Flow rate	1 mL/min (IgG1) 0.8 mL/min (IgG2)
Detection	UV at 214 and 280 nm
Autosampler temperature	10 °C
IgG1 Gradient	Time (min) %B 0 34.5 3 38.0 5.5 38.5 25 55.0 25.1 75.0 26 75.0 26.1 34.5 Post time: 4 minutes
IgG2 Gradient	Time (min) %B 0 30.0 3 30.0 21 45.0 21.1 75.0 22 75.0 22.1 30.0 Post time: 2 minutes

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Instruments

UHPLC with DAD detection system from an external vendor.

The equivalent Agilent instrument for UHPLC analysis is the Agilent 1290 Infinity II LC system, which is expected to delivery comparable, or better performance

Peak assignments

Peak identities were confirmed by coupling the UHPLC with in-line mass spectrometry (data not shown). The major peaks corresponded to unconjugated and variably conjugated light and heavy chains. Peaks eluting as trailing shoulders from the major peaks were identified as having one or more intrachain disulfide bonds reduced. Multiple peaks corresponding to conjugated heavy chains were observed having the same mass, and were identified as being positional isomers, where the drug-linker was conjugated at different cysteine residues.

DAR calculation

The DAR value was calculated from the analysis of the UV chromatogram, using Equation 1.

$$DAR = 2\left(\sum_{n=0}^{1}\frac{\text{LC peak area}\times n_{drug}}{\text{Total LC peak area}} + \sum_{n=0}^{j}\frac{\text{HC peak area}\times n_{drug}}{\text{Total HC peak area}}\right)$$

Figure 1.

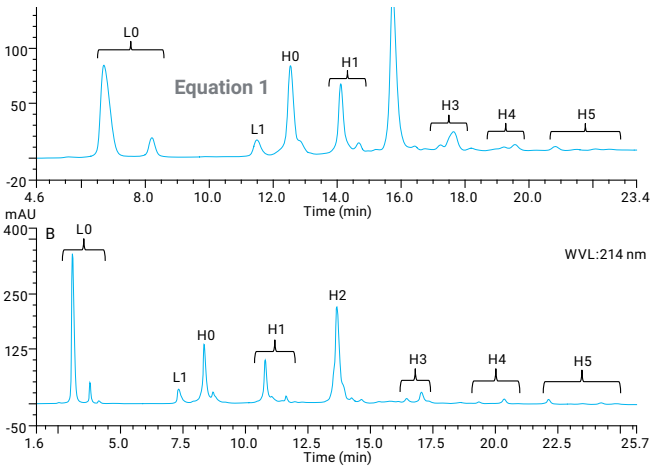

Figure 1. Comparison of reduced RP profiles obtained using a C4 HPLC column (A) and an Agilent ZORBAX RRHD SB300-C8 (B) for a cysteine-conjugated IgG2 ADC.

Results and Discussion

Figure 1 compares the reversed-phase chromatographic profiles of an ADC manufactured from an IgG2 mAb intermediate using a conventional C4 HPLC column and the ZORBAX RRHD SB300-C8 column. The peaks observed in the chromatogram corresponding to the UHPLC method using the ZORBAX RRHD SB300-C8 column are sharper, and show better resolution compared to the C4 HPLC column. Peak separation and resolution achieved using the ZORBAX RRHD SB300-C8 column enabled improved peak integration accuracy, and the DAR value was calculated to be 0.1 higher as a result. resolution achieved using the ZORBAX RRHD SB300-C8 column enabled improved peak integration accuracy, and the DAR value was calculated to be 0.1 higher as a result.

Figure 2 shows RP chromatograms of a reduced ADC manufactured from an IgG1 mAb resulting from analyses using both the Agilent PLRP-S column and the ZORBAX RRHD SB300-C8 column. Separation of the unconjugated and conjugated light and heavy chains on the ZORBAX RRHD 300SB-C8 column resulted in sharper peaks and improved resolution of the minor species in comparison to the PLRP-S HPLC column. The DAR value calculated from the results using the ZORBAX RRHD column was 0.1 higher than the result from the PLRP-S column. This difference can be attributed to more accurate peak integration.

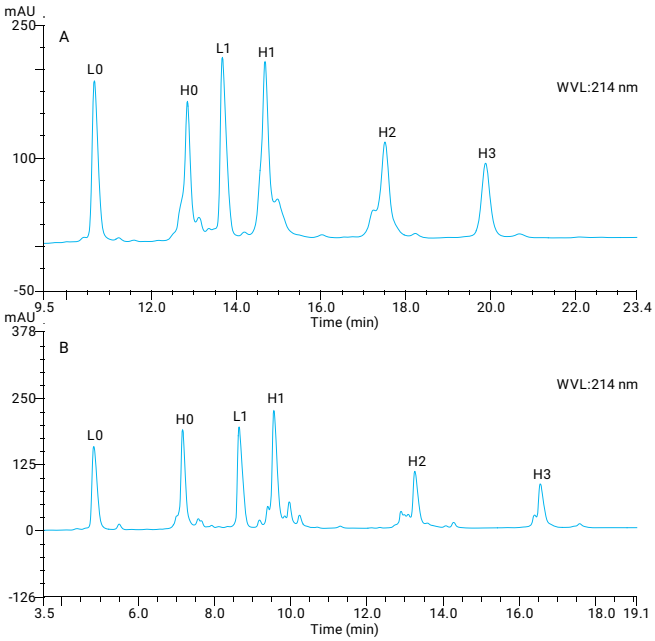

Figure 2. Comparison of PLRP-S HPLC (A) and Agilent ZORBAX RRHD SB300-C8. 1.8 µm (bottom) profiles for a cysteine-conjugated IgG2 ADC.

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Distributions and DAR value

Table 2 shows the distribution of unconjugated and conjugated light and heavy chains, and the calculated average DAR for the ZORBAX RRHD column and the two different HPLC columns. Relative peak areas and concomitant DAR values calculated using results obtained with the ZORBAX RRHD column are similar to those determined using the HPLC columns. The major advantage of using a sub-2 µm column is the improved peak resolution. This improved resolution results in better separation of minor species that were coeluting with the main peaks using the HPLC columns. The gradients presented in this Application Note can also be used to detect minor changes in sample stability and characterization of minor peaks by LC/MS. Also, the increased peak resolution and the higher pressure compatibility of the ZORBAX RRHD columns allow for development of shorter gradients for high-throughput testing.

Table 2. Relative peak areas of each species and average DAR value using an Agilent ZORBAX RRHD SB300-C8 compared with HPLC columns.

	IgG2 ADC		IgG1 ADC	
Peak ID	**% by HPLC (C4)**	**% by UHPLC (Agilent ZORBAX RRHD)**	**% by HPLC (PLRP-S)**	**% by UHPLC (Agilent ZORBAX RRHD)**
L0	22.6	23.7	14.3	13.3
L1	2.3	3.4	15.6	17.7
H0	17.3	15.6	16.8	15.4
H1	12.5	13.1	26.2	27.8
H2	32.4	30.9	16.8	15.8
H3	7.4	8.1	10.5	10.0
H4	2.1	3.2	–	–
H5	3.3	2.2	–	–
DAR	3.5	3.6	3.6	3.7

Conclusion

Reversed-phase methods can determine the distribution of unconjugated and conjugated light and heavy chains and for calculation of the DAR for ADCs.
The Agilent ZORBAX RRHD SB300-C8 column has been shown to be suitable for these purposes to support characterization of ADCs derived from both IgG1 and IgG2 antibody intermediates. Peaks eluting from the ZORBAX RRHD SB300-C8 column were observed to be sharper and better resolved in comparison to the HPLC columns included in the comparison. This improvement enabled more accurate peak integrations and concomitant DAR values. The improvement in peak resolution from the ZORBAX RRHD SB300-C8 column also allowed detection of conjugation site positional isomers. RP methods using the ZORBAX RRHD SB300-C8 column have been shown to be suitable for characterizing the conjugate distribution of partially reduced ADCs in place of, or orthogonal to, hydrophobic interaction chromatography.

Reference

1. Wiggins, B.; *et al.* Characterization of Cysteine-Linked Conjugation Profiles of Immunoglobulin G1 and Immunoglobulin G2 Antibody–Drug Conjugates. *J. Pharm. Sci.* **2015**, 104(4), 1362-1372.

Application Note

Intact & Subunit Purity

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Fast and High Resolution Analysis of Intact and Reduced Therapeutic Monoclonal Antibodies (mAbs)

The Agilent Bio-inert LC and AdvanceBio RP-mAb Columns

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Abstract

Therapeutic monoclonal antibodies (mAbs) have become the most rapidly growing class of therapeutics in development for many diseases. Novel mAbs are entering clinical trials at a rate of 40 per year. There is also an urgent need for an analytical method that can be used for high-throughput analysis of large number of samples to support the growing biopharma development. This Application Note describes a fast and high-resolution method for the analysis of intact and reduced therapeutic Innovator and Biosimilar mAbs by reverse phase HPLC. Separation was achieved using an Agilent 1260 Infinity Bio-inert LC system with Agilent AdvanceBio RP-mAb C4 and Diphenyl columns. RP-mAb columns give the advantage of superficially porous 3.5 µm particles with 450 Å wide pores for improved accuracy and short analysis time compared to fully porous particles of the same size. The bio-inertness of the system, together with high resolution, and short and reproducible methods makes it highly suitable for biopharma QA/QC analysis.

Introduction

Evaluating the molecular similarity of a biosimilar to the reference or the innovator molecule is crucial during biosimilar development. A number of physicochemical methods are required by regulatory agencies involving a wide range of comparability programs. The authorities want to see comparability data on platforms that the previous company or the innovator submitted, primarily high-performance liquid chromatography (HPLC), TOF, Q-TOF mass spectrometry, and capillary electrophoresis. HPLC is a well-established technique for the determination of intact protein by size exclusion or ion exchange. However, technological developments in the fi eld of reverse phase (RP) chromatographic stationary phases (a large pore size of 300 Å or fused core particles with short alkyl chains) have made them promising tools for analyzing intact proteins1.

Historically, mAbs and their fragments are analyzed with limited success using widepore, totally porous particle RP HPLC. Due to their large size and limited diversity, analysis times are often unacceptably long, and mAb peaks can elute as broad bands, compromising resolution. In contrast, high effi ciency superfi cially porous columns easily separate mAbs and their fragments in minutes with high effi ciency.

In this work, we have demonstrated the suitability of the Agilent 1260 Bio-inert Quaternary LC system and Agilent AdvanceBio RP-mAb columns to achieve high resolution and rapid separation of intact and fragmented therapeutic innovator and biosimilar rituximab. The unique design of the AdvanceBio RP-mAb column offers unique selectivity due to its superfi cially porous particles (3.5 µm) with wide pores (450 Å). The column delivers a significant speed and resolution advantage while maintaining compatibility with all instruments..

Experimental

Equipment

A completely 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 RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm (p/n 799775-944)
- Agilent AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm (p/n 799775-904)

The complete sample flow path is free of any metal components, therefore, the sample never contacts metal surfaces. Solvent delivery is free of any stainless steel or iron components.

Software

Agilent ChemStation B.04.03 (or higher)

Reversed-phase HPLC parameters

Chromatographic parameters for intact and reduced mAb analysis using AdvanceBio RP-mAb columns are shown in Table 1.

Table 1. Chromatographic parameters used for intact and reduced analysis.

Parameter	HPLC (intact and reduced mAbs)
Mobile phase	A) Water + 0.1 % TFA B) IPA:ACN:Water (70:20:10) + 0.09 % TFA
Columns	Agilent AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm Agilent AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm
Gradient	Time (min) % B 0 15 0.5 25 1.5 35 1.51 35 3.0 60 4.0 60
Post time	2 minutes
Injection volume	1 µL
Flow rate	1.0 mL/min
TCC	80 °C
UV detection	220 and 280 nm

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Reagents, samples, and materials

AInnovator and biosimilar rituximab were purchased from a local pharmacy and stored according to the manufacturer's instruction. PBS and tris(2-carboxyethyl) phosphine (TCEP) were purchased from Sigma-Aldrich. All chemicals and solvents were HPLC grade, and highly purified water from a Milli-Q water purification system (Millipore Elix 10 model, USA) was used. Acetonitrile and 2-propanol were purchased from Lab-Scan (Bangkok, Thailand). For intact and reduced analysis, rituximab samples were diluted to 2 mg/mL using PBS.

Sample preparation

Reduction of mAbs

For the separation of the light and heavy chains, an aliquot of 0.5 MTCEP stock was added to the mAb samples to obtain a final concentration of 10 mM. The mixture was held at 60 °C for 30 minutes.

Results and Discussion

The AdvanceBio RP-mAb column with superficially porous particles and wide pores delivers higher resolution and faster run times to provide accurate, reproducible results when analyzing monoclonal antibodies for biopharma discovery, development, and QA/QC applications. Combined with the Agilent 1260 Infinity Bio-inert Quaternary LC System with a power range up to 600 bar, it can be used for mAb separation. The mobile phase was a combination of isopropanol (IPA), acetonitrile (ACN), water, and trifluoroacetic acid (TFA). Figures 1 and 2 depict the optimized RP HPLC elution profile of intact innovator and biosimilar rituximab on an AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm and AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm column, respectively, demonstrating excellent peak shape and fast separation in 4 minutes. Comparing Figures 1 and 2 demonstrates that different selectivity can be obtained through the use of different bonded phases using the same chromatographic conditions, with the diphenyl phase resolving in finer detail.

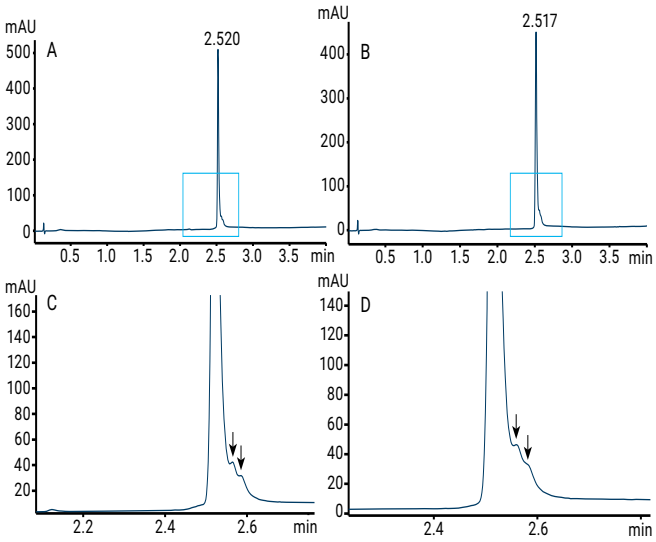

Figure 1. RP-HPLC analysis of innovator rituximab (A) and biosimilar rituximab (B) separated on an Agilent AdvanceBio RP-mAb Diphenyl 2.1 × 50 mm, 3.5 µm column. C and D show zooms.

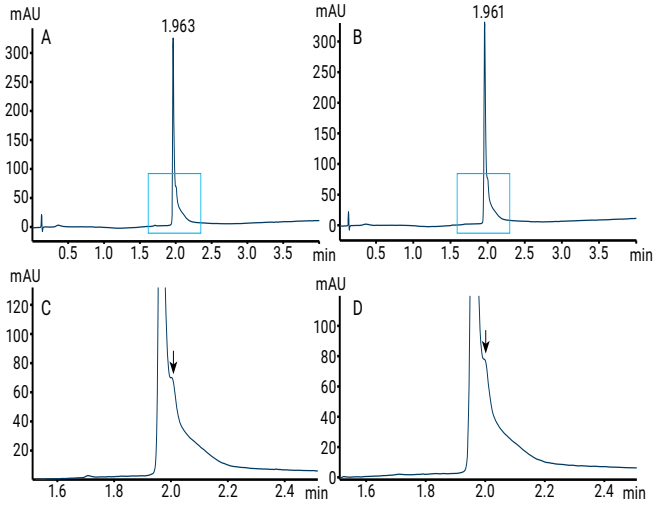

Figure 2. RP-HPLC profile of intact innovator rituximab (A) and biosimilar rituximab (B) on an Agilent AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm column. C and D show zooms.

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Reduced mAb analysis

TCEP was used to separate free antibody light and heavy chains. AdvanceBio RP-mAb columns are very effective in providing fast and high-resolution separations of antibody fragments. The profiles in Figures 3 and 4 show a rapid reversed-phase analysis optimized for the separation of antibody fragments in approximately 4 minutes using C4 and diphenyl phases, respectively.

In both cases, due to reduction of the disulfide bonds, mAbs eluted as distinct light chain (LC) and heavy chain (HC) separations with high effi ciency. The same gradient used for the intact analysis was able to resolve the LC and HC for the reduced samples. As we have seen with intact mAb analysis, the LC and HC show different selectivities with diphenyl and C4 columns. RP HPLC analysis of intact and reduced innovator and biosimilar using AdvanceBio RP-mAb diphenyl and C4 columns indicates that the mAb pair are highly similar.

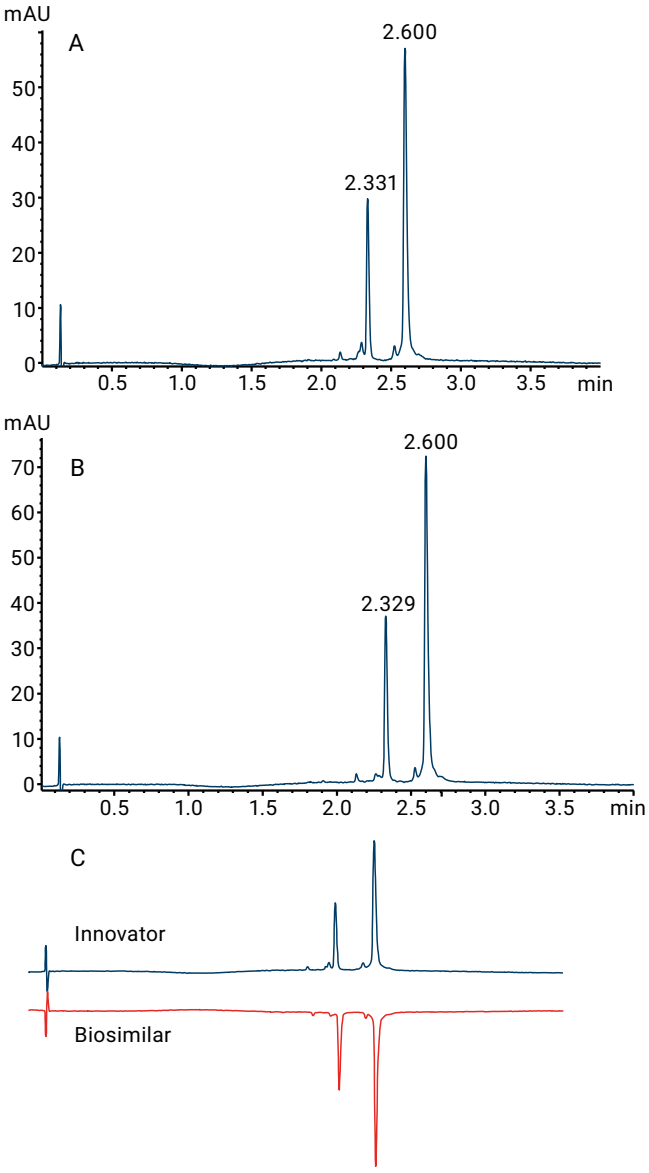

Figure 3. RP-HPLC profiles of innovator rituximab (A) and biosimilar rituximab (B) separated on an Agilent AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm column. Mirror plot image overlays (C).

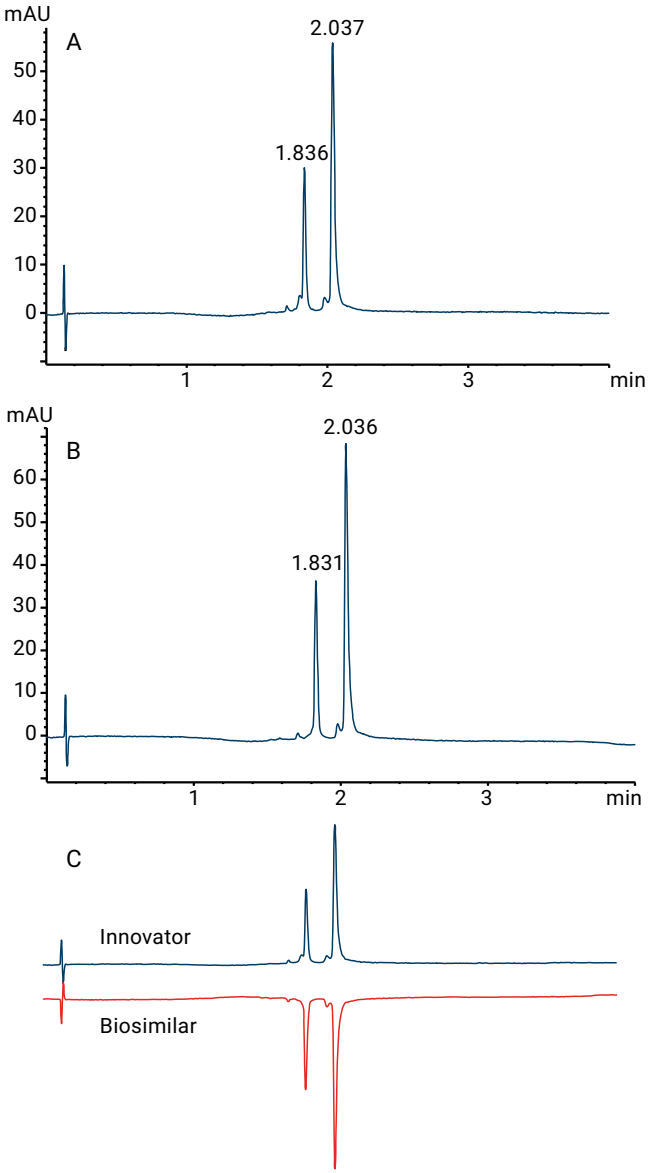

Figure 4. RP-HPLC profiles of innovator rituximab (A) and biosimilar rituximab (B) separated on an Agilent AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm column. Mirror plot image overlays (C).

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Precision of retention time and area

Tables 2 and 3 present the average retention times and area RSDs from six replicates of intact and TCEP reduced innovator, and biosimilar rituximab for the diphenyl and C4 phases. The results show that both columns provide precision of RT and area within the acceptable limit of ±3 % and ±5 %, respectively.

Conclusion

In this application note, we have demonstrated a simple LC-UV-based approach to define the molecular similarity between a biosimilar and its innovator reference. We first used the Agilent 1260 Bio-inert Quaternary LC system with Agilent AdvanceBio RP-mAb Diphenyl and C4 columns to develop a high-resolution and rapid separation of intact mAbs. Using the same method, we were also able to show the separation of light chain and heavy chain after TCEP reduction. Area and RT precision of intact and reduced analysis using AdvanceBio RP-mAb columns were excellent, and show the reliability of the method. Such fast, simple, and reproducible methods for intact and reduced analysis of mAbs, coupled with bio-inertness of the system makes this solution suitable for the comparability analysis of mAbs for the biopharma industry.

Reference

1. Navas, N; *et al.,* Anal. Bioanal. Chem. 2013, 405, pp 9351-9363.

Table 2. Retention time and peak area RSD (%), n = 6 for intact analysis

	Retention time		Peak area	
Samples	**Mean (min)**	**RSD**	**Mean (mAU/min)**	**RSD**
Agilent AdvanceBio RP-mAb, C4, 2.1 × 50 mm, 3.5 µm				
Innovator rituximab	1.96	0	71.61	1.98
Biosimilar rituximab	1.95	0.26	77.3	0.47
Agilent AdvanceBio RP-mAb, Diphenyl, 2.1 × 50 mm, 3.5 µm				
Innovator rituximab	2.51	0.20	66.7	0.458
Biosimilar rituximab	2.51	0	73.3	1.86

Table 3. Retention time and peak area RSD (%), n = 6 for reduced analysis

	Retention time		Peak area	
Samples	**Mean (min)**	**RSD**	**Mean (mAU/min)**	**RSD**
Agilent AdvanceBio RP-mAb, Diphenyl, 2.1 × 50 mm, 3.5 µm				
Innovator rituximab LC	2.32	0.60	19.71	4.24
Innovator rituximab HC	2.58	1.52	57.33	1.57
Biosimilar rituximab LC	2.32	0.07	23.56	3.25
Biosimilar rituximab HC	2.60	0.05	58.40	5.61
Agilent AdvanceBio RP-mAb, C4, 2.1 × 50 mm, 3.5 µm				
Innovator rituximab LC	1.83	0	21.5	1.4
Innovator rituximab HC	2.03	0.04	51.2	2.25
Biosimilar rituximab LC	1.83	0.03	24.47	3.84
Biosimilar rituximab HC	2.03	0.06	52.66	0.84

Application Note

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Disulfide Linkage Analysis of IgG1 using an Agilent 1260 Infinity Bio-inert LC System with an Agilent ZORBAX RRHD Diphenyl sub-2 µm Column

Authors

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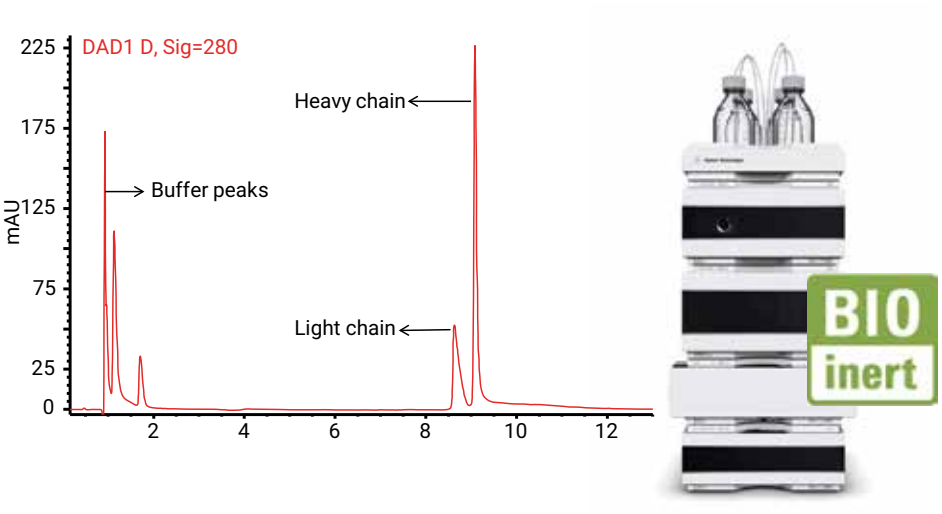
Abstract

This Application Note describes a simple method for the analysis of disulfide linkages in monoclonal antibodies (mAbs) by reversed-phase HPLC. Separation was achieved using an Agilent 1260 Infinity Bio-inert LC System and an Agilent ZORBAX RRHD 300 Diphenyl sub-2 µm particle column. Diphenyl 1.8 µm columns deliver UHPLC performance for reversed-phase separations of intact proteins and peptide digests. Together with UHPLC instruments, these versatile columns enable higher order characterization with shorter analysis times. The 1260 Infinity Bio-inert LC System has a power range up to 600 bar and is capable of handling the higher pressures demanded by emerging column technologies with smaller particles down to 1.7 µm.



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Introduction

Although recombinant mAb therapeutics have advanced enormously in recent years, little is known about their disulfide bond patterns. Complete disulfide bond assignment of IgG1 antibodies can be challenging due to their large size and substantial number of disulfide linkages. Disulphide bonding is critical to maintaining immunoglobulin (IgG) tertiary and quaternary structure for therapeutic monoclonal antibodies (mAb). Both inter- and intra-chain disulphide bonds are formed intracellularly in the expression host prior to secretion and purification during mAb production processes. Disulphide bond shuffling has previously been reported for IgG2 and disulphide-mediated arm-exchange for IgG4, reflecting innate behaviour of these IgG classes1, 2. However, a typical and significant reduction in the number of disulphide bonds has been observed in IgG13 that present significant issues for manufacturing of therapeutic mAbs. This Application Note demonstrates the suitability of the 1260 Bio-inert Quaternary LC System for separating and analyzing the disulfide linkages of IgG1 by reversed-phase HPLC on ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm column. Ultrahigh performance liquid chromatography (UHPLC) separation using sub-2 µm particles improves resolution per time and sensitivity, shortens run times, and thus enables the analysis of IgG1, reduced IgG1, and the peptides resulting from digestion of IgG1.

Equipment

Instrumentation

A completely 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 ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm column (p/n858750-944).

The complete sample flow path is free of any metal components such that the sample never gets in contact with metal surfaces. Solvent delivery is free of any stainless steel or iron components.

Software

Agilent OpenLAB CDS ChemStation Edition for LC & LC MS Systems, Rev. C.01.04

HPLC analysis

Table 1 Chromatographic parameters used for RP HPLC.

Parameter	Conditions	
Mobile phase A	Water + 0.1% TFA	
Mobile phase B	Acetonitrile + 0.09% TFA	
Gradient	Time (min)	Mobile phase B (%)
	0 minutes	25
	3 minutes	35
	4 minutes	40
	5 minutes	40
	15 minutes	90
	16 minutes	25
	Post time	5 minutes
Injection volume	3 µL (Needle with wash, flush port active for 7 seconds)	
Flow rate	0.3 mL/min	
Data acquisition	214 and 280 nm	
Acquisition rate	20 Hz	
Flow cell	60 mm path	
Column oven	50 °C	
Sample thermostat	5 °C	

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Reagents, Samples and Materials

The human monoclonal antibody IgG1 was a proprietary pharmaceutical molecule. DL-Dithiothreitol (DTT), iodoacetamide, trizma base, and Endoproteinase Lys C were purchased from Sigma Aldrich. All chemicals and solvents used were HPLC grade and highly purifi ed water from Milli Q water purification system (Millipore Elix 10 model, USA) was used. Acetonitrile was of gradient grade and purchased from Lab-Scan (Bangkok, Thailand).

Reduction and alkylation of Intact IgG1

IgG1 was diluted to 2 mg/mL using 100 mM Tris HCl and 4 M Gu HCl, pH 8.0. An aliquot of 10 µL of 0.5 M DTT stock was added to obtain a final concentration of 5 mM. The mixture was held at 37 °C for 30 minutes. The reaction mixture was cooled briefly to room temperature (RT). An aliquot of 26 µL of 0.5 M Iodoacetamide stock was added for a fi nal concentration of 13 mM. It was allowed to stand for 45 minutes. Once removed, the solution was quenched with 20 µL of DTT for a final concentration of 10 mM.

Lys C digestion of IgG1 and reduction

IgG1 was diluted to 1 mg/mL using 100 mM Tris HCl, pH 8.0. Endoproteinase Lys C in 100 mM Tris HCl, pH 8.0 was added at an enzyme protein ratio of 1:100 (w/w). The mixture was incubated overnight at 37 °C. The reaction was stopped by lowering the pH to 6.0 by adding 10 % TFA. Later, the reduction of Lys C digested IgG1 was carried out as described earlier in this Application Note.

Results and Discussion

Separation and Detection

A ZORBAX RRHD 300 Diphenyl 1.8 µm column has the advantage of low pH and temperature stability, and, combined with the 1260 Infinity Bio-inert Quaternary LC System with a power range up to 600 bar and capabilities of handling the higher pressures, can be used for protein separation. Figure 1 **A** depicts the optimized RP HPLC elution profile of intact IgG1 on a ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm column demonstrating excellent retention of IgG1 in 15 minutes. The reproducibility of analysis was tested with six replicates. Figure 1 **B** shows the overlay of six replicates.

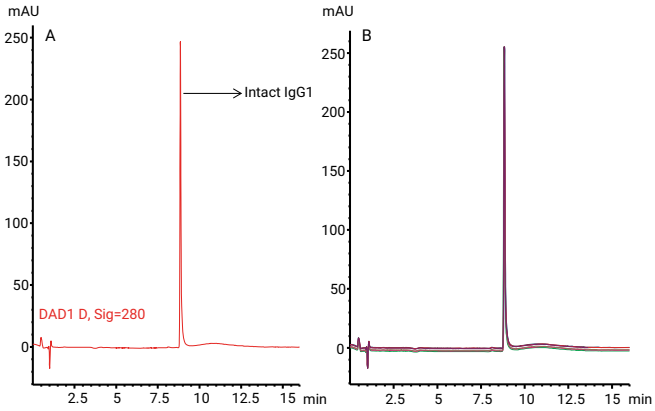

Figure 1. RP HPLC profile of intact IgG1 on an Agilent ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm column (A), and an overlay of six replicates (B).

Table 2 Retention time and area RSD (%), n = 6 for intact IgG1

Retention time		Peak area	
Mean (min)	RSD (limit: ±3.0 %)	Mean (mAU/min)	RSD (limit: ±5.0 %)
8.838	0.086	1,170	0.461

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The effect of reduction and alkylation of the disulfide bonds in intact IgG1 was tested. Figure 2 shows the reversed-phase chromatogram of A reduced and alkylated IgG1 B overlay with reduction/alkylation buffer blank and C overlay of six replicates showing separation reproducibility. Due to the reduction of the disulfide bonds, the IgG1 is separated into its light and heavy chains. The IgG1 eluted as distinct light chain (LC) and heavy chain (HC) as indicated in Figure 2; however, this was not confi rmed by mass spectral analysis.

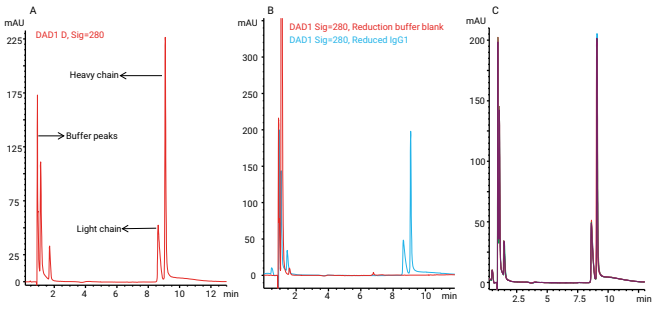

Figure 2. RP HPLC profile of (A) Reduced and alkylated IgG1, (B) overlaid with buffer blank, (C) overlay of six replicates.

Table 3. Retention time and area RSD (%), n = 6 for Light chain

Retention time		Peak area	
Mean (min)	RSD (limit: ±3.0 %)	Mean (mAU/min)	RSD (limit: ±5.0 %)
8.638	0.091	504.33	2.780

Table 4. Retention time and area RSD (%), n = 6 for Heavy chain

Retention time		Peak area	
Mean (min)	RSD (limit: ±3.0 %)	Mean (mAU/min)	RSD (limit: ±5.0 %)
9.084	0.152	1,520	0.390

Peptide maps resulting from Lys C digestion of intact IgG1 under nonreducing conditions resulted in a less intricate RP HPLC profile. A representative chromatogram of the IgG1 digest (Figure 3 A) displays the two (baseline separated) peaks that were selected for area and RT precision. The overlay results in sharp peaks with good resolution and excellent separation reproducibility (Figure 3 B).

Further, we wanted to compare the reversed-phase profile of IgG1 under nonreduced and reducing conditions to determine the peptides bound through disulfide linkages. The overlay of Lys C peptide maps of nonreduced IgG1 (red trace) and reduced IgG1 (blue trace) is depicted in Figure 4. The appearance of additional peaks (indicated by an asterix) after reduction of Lys C digested IgG1 confirms they are bound through disulfide linkages.

Precision of retention time and area

The precision of the retention time and area for intact IgG1, reduced IgG1 and endoproteinase Lys C digested IgG1 under nonreduced conditions are given in Tables 2, 3, and 4. The results show that the ZORBAX diphenyl sub-2 µm column shows precision of RT and area to be within 3% and 5 % respectively.

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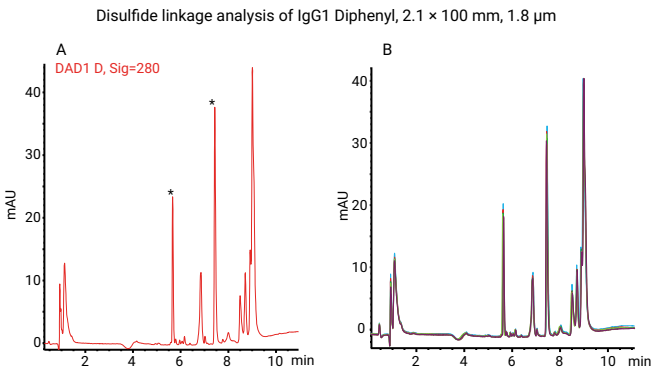

Figure 3. RP HPLC profile of (A) Lys C digested IgG1 and (B) overlay of six replicates. Peaks selected for RT and area RSDs are indicated by an asterix.

Table 5 Retention time and area RSD (%), n=6 for Lys C digested IgG1

	Retention time		Peak area	
	Mean (min)	RSD (limit: ±3.0 %)	Mean (mAU/min)	RSD (limit: ±5.0 %)
Peak 1	5.525	0.307	60	0.544
Peak 2	7.444	0.140	132.45	1.113

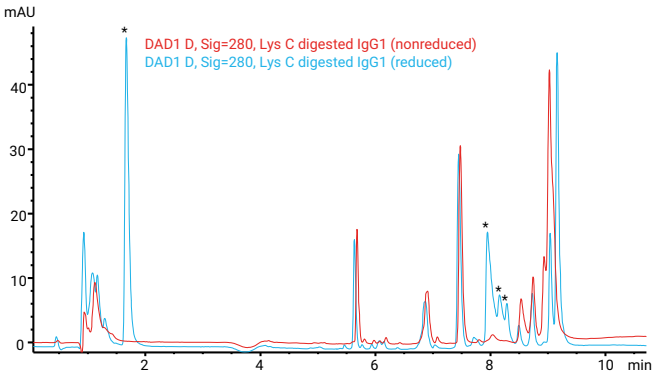

Figure 4. Comparison of peptide maps of Lys C digested IgG1 under nonreducing condition (red trace) followed by reduction (blue trace). Peptides bound through disulfide linkages are indicated by an asterix.

Conclusion

Disulfide linkage analysis is important to study some of the post-transitional modifications of proteins for biopharma process development and monitoring. We have shown the combination of an Agilent 1260 Infinity Bio-inert Quarternary LC System and an Agilent ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm column to perform reproducible and high resolution analysis of disulfide linkage analysis of monoclonal antibodies for biopharma process development and monitoring. Area and RT precision of the method were excellent and proved reliability. Further, the 1260 Infinity Bio-inert LC has a power range up to 600 bar and is capable of handling the higher pressures demanded by emerging column technologies with smaller particles down to 1.7 µm. The bio-inertness and corrosion resistance of the instrument coupled with a simple and reproducible method make this solution particularly suitable for the QA/QC analysis of monoclonal antibody for the biopharmaceutical industry.

References

1. R. Mhatre, J. Woodard, C. Zeng, Strategies for locating disulfide bonds in a monoclonal antibody via mass spectrometry spectrometry, Rapid Commun. Mass Spectrom. 13 (**1999**) 2503–2510.
2. T.-Y. Yen, H. Yan, B.A. Macher, Characterizing closely spaced, complex disulfide bond patterns in peptides and proteins by liquid chromatography/electrospray ionization tandem mass spectrometry, *J. Mass. Spectrom.* 37 (**2002**) 15–30.
3. Mullan et al. BMC Proceedings **2011**, 5 (Suppl 8):P110

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Application Note

Charge Variant Analysis

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Convenient and Reliable Analysis of Antibody Drug Conjugates

Drug-to-antibody determination with ternary gradients on the Agilent 1260 Infinity II Prime Bio LC

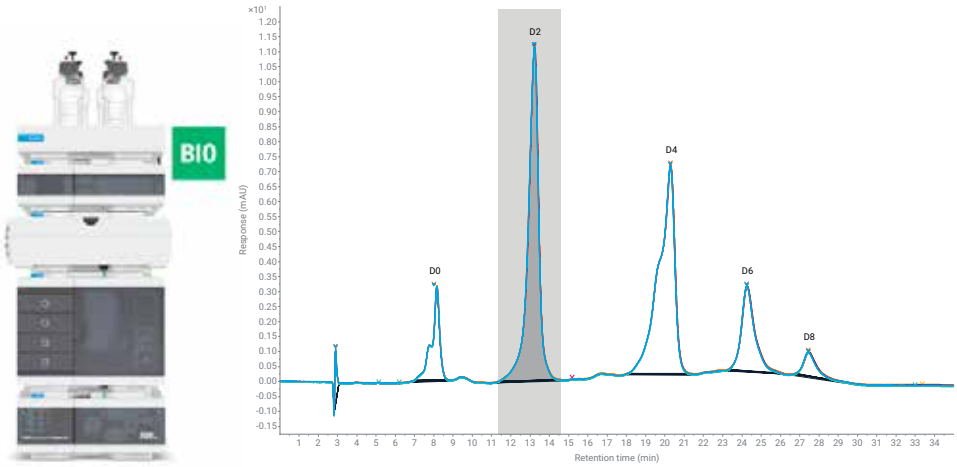
Author

Sonja Schneider
Agilent Technologies, Inc

Abstract

The addition of organic modifiers like isopropanol in hydrophobic interaction chromatography (HIC) can be an important parameter to decrease the retention of hydrophobic antibody drug conjugates (ADCs) as well as to adjust selectivity.

This application note demonstrates the drug-to-antibody (DAR) determination of brentuximab vedotin using a ternary gradient with isopropanol as organic modifier in the third channel. Excellent reproducibility was found for this challenging combination of high-salt-containing buffer and organic solvent, making the Agilent 1260 Infinity II Prime Bio LC the next generation of Agilent high-end liquid chromatography systems for the highest confidence in generated data.



Introduction

ADCs are monoclonal antibodies (mAbs) to which a cytotoxic small molecule drug is chemically linked.[1] Compared to their corresponding antibodies, the structure is more complex and heterogeneous.

Cysteine-linked ADCs such as brentuximab vedotin are generated after the reduction of the interchain disulfides, resulting in free sulfhydryl groups that can be conjugated to specific maleimide linkers. The number of free sulfhydryl groups limits the number of defined positions for the drug to be conjugated, resulting in a mixture of zero (D0), two (D2), four (D4), six (D6), and eight (D8) drugs per antibody.

Due to the hydrophobicity of the high DAR species in particular, the addition of an organic modifier such as isopropanol is helpful to enable full elution from the HIC column. Typically, in binary gradients, the modifier is added to the mobile phase used for elution (usually a buffer containing little or no salt). The Agilent 1260 Infinity II Bio Flexible Pump, as a quaternary pump, enables the use of a third channel to add the organic modifier solvent. The combination of high-salt-containing buffers with organic mobile phases can be critical due to potential formation of salt crystals when the two solvents mix in the pump.

The 1260 Infinity II Prime Bio LC is the next generation of Agilent high-end liquid chromatography systems, specially designed for conditions used in bio chromatography: The sample flow path is completely free of stainless steel (SST) or iron; all capillaries and fittings throughout the multisampler, multicolumn thermostat, and detectors are made of MP35N, a nickel-cobalt alloy. For this reason, the 1260 Infinity II Prime Bio LC is optimally suited to the conditions used in bio chromatography, with the high concentrations of corrosive salts typically used in HIC, to avoid potential corrosive damage to the system.

Experimental

Equipment

The Agilent 1260 Infinity II Prime Bio LC System comprised the following modules:

- Agilent 1260 Infinity II Bio Flexible Pump (G7131C)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with standard flow biocompatible heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2 µL

Software

Agilent OpenLab CDS version 2.5 or later versions

Columns

Agilent AdvanceBio HIC column 3.5 µm, 4.6 × 100 mm (part number 685975-908)

Chemicals

All solvents were LC grade. Isopropanol was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, and ammonium sulfate were obtained from Sigma-Aldrich (Steinheim, Germany).

Samples

Brentuximab vedotin (trade name Adcetris by Takeda, Tokyo, Japan) dissolved in 50% water: 50% solvent A (see below) at 100 mg/mL.

Note: As Adcetris contains many adjuvants, the concentration mentioned here is not the protein concentration only, but the total concentration of all components of the drug.

Buffer preparation

A) 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7

B) 50 mM phosphate buffer at pH 7

C) Isopropanol

For 2 L of 50 mM phosphate buffer, pH 7, 5.84 g of sodium phosphate monobasic monohydrate and 15.47 g of sodium phosphate dibasic heptahydrate were added to an amber-colored 2 L bottle and filled up to 2 L using ultrapure water. The pH value was checked and adjusted, if necessary, to pH 7 (buffer B). 198.21 g of ammonium sulfate for a total of 1.5 M was added to an empty amber-colored 1 L bottle and filled up to 1 L using the prepared phosphate buffer (& buffer A). The pH value was checked and adjusted, if necessary, to pH 7 (the addition of high amounts of salt can change the pH). Both prepared buffers were filtered using a 0.2 µm membrane filter.

Method

Parameter	Value
Solvent	A) 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7 B) 50 mM phosphate buffer at pH 7 C) Isopropanol
Gradient	Gradient: 0 min 55% A, 40% B, 5% C 25 min 0% A, 75% B, 25%C Stop time: 35 min Post time: 10 min
Flow rate	0.400 mL/min
Temperature	25 °C
Detection	280 nm, 10 Hz
Injection	Injection volume: 15 µL Sample temperature: 10 °C Needle wash: 3 s in water

Note: WThe high concentrations of salt used in HIC require a robust LC system, and the completely stainless steel (SST)/ iron-free flow path of the 1260 Infinity II Prime Bio LC prevents potential corrosion from high-salt-containing buffers. In addition, washing features like seal wash and needle wash help to avoid issues with salt precipitation. However, it is still important to avoid leaving either the LC system or the column in a concentrated salt solution for any length of time.

Note: When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method. For example, for solvent A, which features 1.5 M ammonium sulfate, use Ammonium Sulfate 1.5 M rather than Generic Aqueous or Water in the solvent selection field in the pump method. High amounts of salts change the compressibility of the solvent, and so using the preconfigured solvent tables enables best pump performance.

Results and discussion

Figure 1 shows the analysis of brentuximab vedotin with five main peaks that correspond to the mAb containing zero, two, four, six, and eight small molecule drugs, respectively. Each peak in Figure 1 corresponds to an intact mAb species with an increasing number of attached drugs molecules (zero to eight bound molecules, D0 to D8). The peaks were identified by comparing the HIC chromatogram to chromatograms found in literature for brentuximab vedotin.[2]

The analysis was evaluated for the precision of retention time (RT) and area. With seven subsequent runs, an excellent RT precision of lower than 0.055% relative standard deviation (RSD) was found. This proves the excellence of the quaternary pump to run ternary gradients even with very challenging combinations of high-salt-containing buffers (such as the 1.5 M ammonium sulfate buffer used here) and isopropanol as organic modifier. The area precision was also excellent, with RSDs lower than 0.46%, except for the last peak (see the table in Figure 1).

The HIC analysis allowed both the characterization of the distribution of drug-linked species, as well as the determination of the DAR. By integrating the peak areas of each peak and its respective drug load, it is possible to calculate the overall DAR (Equation 1).

Equation 1.

$$DAR = \sum_{n=0}^{8} \frac{\text{LC peak area} \times n_{drug}}{\text{Total LC peak area}}$$

The integration of the five observed peaks and the area percentage calculation revealed a DAR of ~3.3 (see Table 1).

Table 1. DAR species results.

DAR Species	RT (min)	Area	Area %	DAR Calculated
D0	8.00	89.18	8.11	0
D2	13.22	427.04	38.83	0.78
D4	20.29	405.58	36.88	1.48
D6	24.27	140.51	12.78	0.77
D8	27.48	37.38	3.4	0.27
			DAR	**3.3**

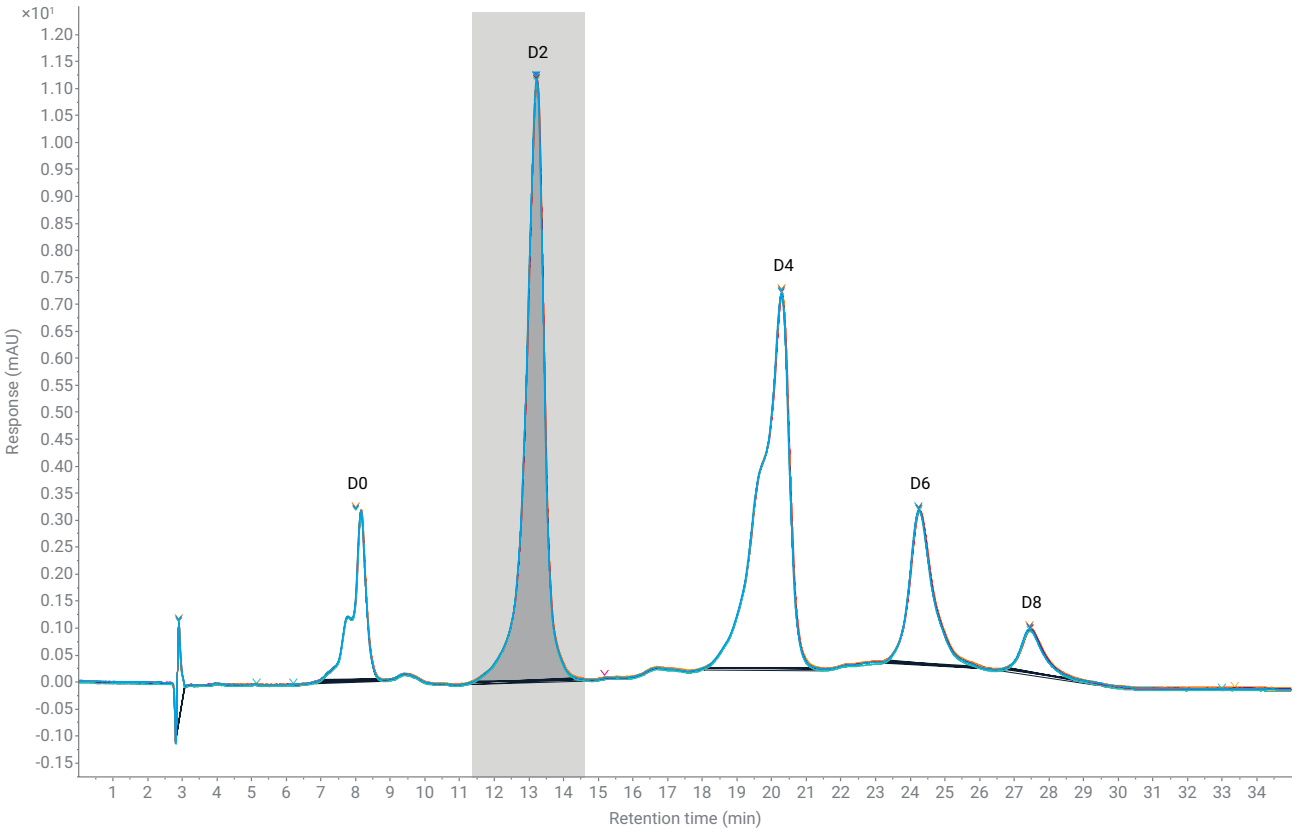


Figure 1. Separation of brentuximab vedotin on the Agilent 1260 Infinity II Prime Bio LC. D0–D8 refers to different DAR species. An overlay of seven subsequent runs is displayed. Blank Subtraction was applied to filter out the baseline drift caused by the ammonium sulfate salt in buffer A using blank injections run in the same sequence.

Conclusion

Brentuximab vedotin was analyzed using HIC in a ternary gradient with isopropanol in a third channel as organic modifier. All five expected ADC species were well separated, corresponding to the mAb containing zero, two, four, six, and eight small molecule drugs, respectively. The HIC analysis allowed both the characterization of the distribution of drug-linked species as well as the determination of the DAR, calculated to 3.3 drug molecules per antibody. The challenging solvent combination was managed outstandingly by the Agilent 1260 Infinity II Prime Bio LC including the Agilent 1260 Infinity II Bio Flexible Pump. The reproducibility of retention times was excellent, with relative standard deviations below 0.055%, allowing binary like performance for highest confidence in generated data.

References

1. McCombs, J. R.; Owen, S. C. Antibody Drug Conjugates: Design and Selection of Linker, Payload and Conjugation Chemistry. *J. Amer. Assoc. Pharm. Sci.* **2015**, 17(2), 339–51.
2. Rodriguez-Aller, M. *et al*. Practical Method Development for the Separation of Monoclonal Antibodies and Antibody-Drug-Conjugate Species in Hydrophobic Interaction Chromatography, Part 1: Optimization of the Mobile Phase. *J. Pharm. Biomed. Anal.* **2016**, 118, 393–403.

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High Salt—High Reproducibility

Analysis of antibody drug conjugates using hydrophobic interaction chromatography with the Agilent 1290 Infinity II Bio LC System

Author

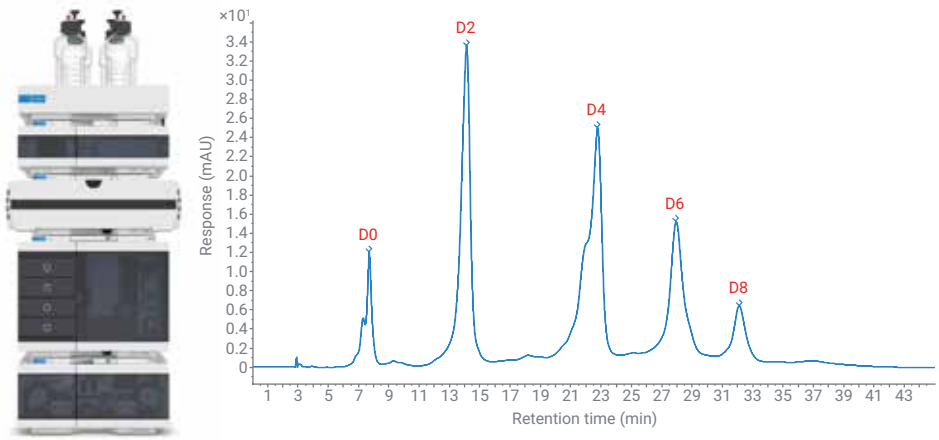
Sonja Schneider

Agilent Technologies, Inc.

Abstract

The determination of the drug-to-antibody ratio (DAR) is typically performed using hydrophobic interaction chromatography (HIC). The eluents for this mild, nondenaturing analysis method contain high concentrations of corrosive salts, which challenge the liquid chromatography (LC) system.

The Agilent 1290 Infinity II Bio LC System including High-Speed Pump, with its completely iron-free flow path, is optimally suited for the conditions used in biochromatography—avoiding potential corrosive damage to the system. This application note demonstrates the DAR determination of brentuximab vedotin using HIC. The DAR was calculated to 3.7 drug molecules per antibody. Excellent reproducibility was found, demonstrating that the 1290 Infinity II Bio LC belongs to the next generation of Agilent high-end liquid chromatography systems for the highest confidence in generated data. “Blank subtraction”, as a software feature of Agilent OpenLab CDS, removes drifting baselines due to less pure ammonium sulfate, enabling smooth integration.



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Introduction

Antibody-drug Antibody drug conjugates (ADCs) are monoclonal antibodies (mAbs) to which a cytotoxic small molecule drug is chemically linked.[1] Compared to their corresponding antibodies, the structure is more complex and heterogeneous.

Cysteine-linked ADCs[2] (such as brentuximab vedotin, Adcetris by Takeda) has the small molecule attached to the free thiol groups of the partially reduced mAb.[3,4] The number of free sulfhydryl groups limits the number of defined positions for the drug to be conjugated, resulting in a mixture of zero, two, four, six, and eight drugs per antibody. The average number of drugs conjugated to the mAb is one of the most important quality attributes of an ADC because it can directly affect safety and efficacy. The DAR determines the amount of payload that can be delivered to the desired tissue.[5]

HIC is the reference technique to separate cysteine-linked ADC molecules loaded with different numbers of drugs per antibody.[6] The relative hydrophobicity increases with the drug load of the ADC because the small molecules attached to the mAb are often relatively hydrophobic. Therefore, HIC is perfectly suited to monitor the DAR.

HIC is a nondenaturing analysis technique maintaining the native protein structure. It is typically performed at neutral pH, separating the proteins with a gradient from high to low salt concentration. The separation principle is the same as found in protein salting-out experiments.[6] In the high-concentration salt buffer used in mobile phase A, the proteins lose their hydration shell and are retained on the hydrophobic surface of the stationary phase. Mobile phase B is usually the same buffer (mostly phosphate) without added salt. With an increasing amount of mobile phase B in the gradient, the proteins re-assemble the water shell and are eluted from the column. The addition of a small amount of organic solvent such as isopropyl alcohol can also help to elute the proteins from the column.

The 1290 Infinity II Bio LC is the next generation of Agilent high-end liquid chromatography systems, specially designed for conditions used in biochromatography: high salt concentrations such as 2 M NaCl,[7] up to 8 M urea, and high- and low-pH solvents such as 0.5 M NaOH or 0.5 M HCl. The sample flow path is completely free of stainless steel (SST) or iron; all capillaries and fittings throughout the multisampler, multicolumn thermostat, and detectors are built of MP35N, a nickel-cobalt alloy. With this material, potential corrosion from high salt-containing buffers is reduced and protein modifications caused by the presence of iron ions (e.g., oxidation, protein complex formation) can be avoided.

This application note presents the analysis of brentuximab vedotin with HIC for the determination of DAR, evaluating the precision of retention time and area. In addition, the advantages of the software feature "Blank Subtraction" in the processing method of OpenLab 2 are demonstrated to filter drifting baselines.

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System comprised the following modules:

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with standard flow biocompatible heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2 µL

Software

Agilent OpenLab CDS Version 2.5

Columns

Agilent AdvanceBio HIC column, 3.5 µm, 4.6 × 100 mm (p/n 685975-908)

Chemicals

All solvents were LC grade. Isopropanol was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, and ammonium sulfate were obtained from Sigma-Aldrich (Steinheim, Germany).

Samples

Brentuximab vedotin (Trade name Adcetris by Takeda, Tokyo, Japan) dissolved in half water, half solvent A (see below) at 100 mg/mL.

Note: As Adcetris contains many adjuvants, the concentration mentioned here is not the protein concentration only, but the total concentration of all components of the drug.

Buffer preparation

A: 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7

B: 50 mM phosphate buffer at pH 7 + 20% isopropanol

For 2 L of 50 mM phosphate buffer, pH 7, 5.84 g of sodium phosphate monobasic monohydrate and 15.47 g of sodium phosphate dibasic heptahydrate were added to an amber-colored 2 L bottle and filled up to 2 L using ultrapure water. The pH value was checked and adjusted,

Buffer preparation

A: 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7

B: 50 mM phosphate buffer at pH 7 + 20% isopropanol

For 2 L of 50 mM phosphate buffer, pH 7, 5.84 g of sodium phosphate monobasic monohydrate and 15.47 g of sodium phosphate dibasic heptahydrate were added to an amber-colored 2 L bottle and filled up to 2 L using ultrapure water. The pH value was checked and adjusted, if necessary, to pH 7. Then, 198.21 g of ammonium sulfate for a total of 1.5 M was added to an empty, amber-colored 1 L bottle and filled up to 1 L using the prepared phosphate buffer (&buffer A). The pH value was checked and adjusted, if necessary, back to pH 7 (the addition of high amounts of salt can change the pH). 200 mL of isopropanol and 800 mL of the prepared 50 mM phosphate buffer, pH 7 was mixed and added to an empty, amber-colored 1 L bottle (&buffer B). Both prepared buffers were filtered using a 0.2-µm membrane filter.

Note: The presence of small hydrophobic drug molecules conjugated to the mAb increases the overall hydrophobicity considerably. Consequently, it is necessary to include some organic modifier in the mobile phase (here: 20% isopropanol).

Method

Table 1. Salt gradient chromatographic conditions.

Parameter	HPLC (intact and reduced mAbs)
Solvent	A) 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7 B) 50 mM phosphate buffer at pH 7 + 20% isopropanol
Gradient	0 minutes 30% B, 30 minutes 100% B Stop time: 45 minutes Post-time: 10 minutes
Flow rate	0.400 mL/min
Temperature	25 °C
Detection	280 nm, 10 Hz
Injection	Injection volume: 15 µL Sample temperature: 10 °C Needle wash: 3 s in water

Note: The high concentrations of salt used in HIC require a robust LC system, and the completely stainless steel (SST)/iron-free flow path of the 1290 Infinity II Bio LC prevents potential corrosion from high salt-containing buffers. In addition, washing features like seal wash and needle wash help to avoid issues with salt precipitation. However, it is still important to avoid leaving either the LC system or the column in a concentrated salt solution for any length of time.

Note: When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method. For example, for solvent A, including 1.5 M ammonium sulfate, use "Ammonium Sulfate 1.5 M" rather than *Generic Aqueous or Water* in the solvent selection field in the pump method. High amounts of salts change the compressibility of the solvent, and so using the preconfigured solvent tables enables best pump performance.

Results and discussion

Figure 1 shows the analysis of brentuximab vedotin, revealing five main peaks that correspond to the mAb containing zero, two, four, six, and eight small molecule drugs, respectively. The HIC analysis allowed both the characterization of the distribution of drug-linked species, as well as the determination of the DAR.

Although the interchain disulfide bridges are disrupted and occupied by the conjugated drugs, the combination of covalent linkages and noncovalent forces between the antibody chains is sufficient to maintain the mAb in an intact form during the analysis. This is due to the mild, nondenaturing conditions of HIC, making it ideal for the analysis of cysteine-linked ADCs. Each peak in Figure 1 corresponds to an intact mAb species with an increasing number of attached drugs molecules (zero to eight bound molecules, D0 to D8). The peaks were identified by comparing the HIC chromatogram to chromatograms found in literature for brentuximab vedotin.[6]

By integrating the peak areas of each peak and its respective drug load, it is possible to calculate the overall DAR (Equation 1).

Equation 1.

$$DAR = \sum_{n=0}^{8} \frac{\text{LC peak area} \times n_{drug}}{\text{Total LC peak area}}$$

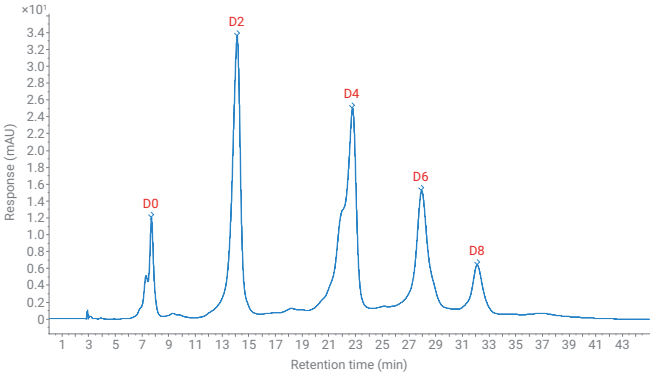

Figure 1. Analysis of brentuximab vedotin on an Agilent 1290 Infinity II Bio LC. D0 to D8 refers to different DAR species.

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The integration of the five observed peaks and the area percentage calculation revealed a DAR of ~3.7 (see Table 2). This value is consistent with the literature.[8]

The analysis was also evaluated for the precision of retention time (RT) and area (Figure 2). After seven subsequent runs, an excellent RT precision of lower than 0.081% relative standard deviation (RSD) was found. The area precision was also excellent, with RSDs lower than 0.282% (see table in Figure 2).

Ammonium sulfate is a very commonly used chaotropic salt in HIC analysis. The concentrations used typically range from 1 to 2 M salt, which is a considerable quantity. If a less pure salt is used in the analysis (which is sometimes even visible in the color of the salt crystals), the baseline of the chromatogram can drift significantly, resulting in potential integration errors. To approach this issue, a software feature called "Blank Subtraction" can be applied to filter out the baseline drift using the blank injection. This feature is found in the processing method of OpenLab 2 (see Figure 3). Figure 4 displays the chromatogram with different baseline behavior before and after the feature was applied.

Table 2. DAR species results.

DAR Species	RT (min)	Area	Area%	DAR Calculated
D0	7.68	378.116	7.59	0
D2	14.12	1537.829	30.84	0.6196
D4	22.78	1756.026	35.22	1.415
D6	27.98	951.983	19.13	1.1506
D8	32.15	340.176	6.79	0.5482
			DAR	3.733

Peak ID.

Precision in RSD	RT (%)	Area (%)
D0	0.074	0.064
D2	0.080	0.043
D4	0.072	0.071
D6	0.065	0.262
D8	0.066	0.281

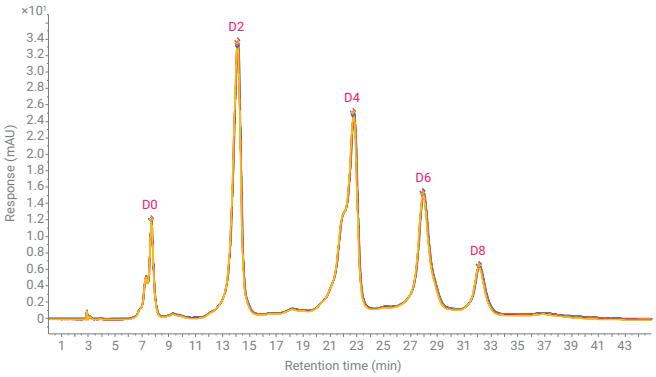

Figure 2. Separation of brentuximab vedotin on an Agilent 1290 Infinity II Bio LC (overlay of seven subsequent runs).

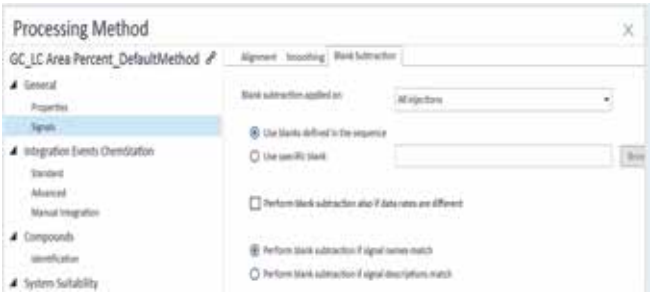

Figure 3. Screenshot of the "Blank Subtraction" feature in the processing method of Agilent OpenLab CDS 2.

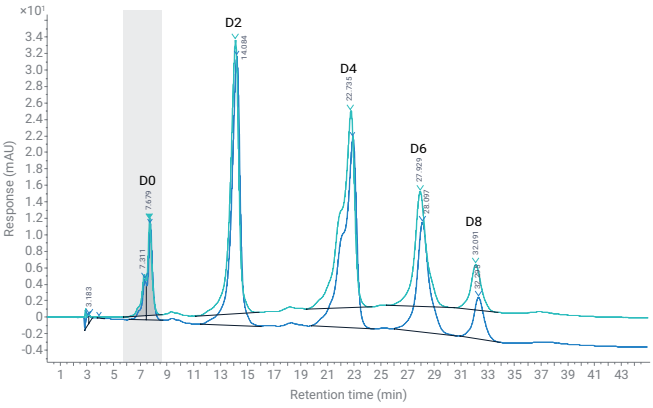

Figure 4. Comparison of HIC chromatogram before (blue) and after (green) blank subtraction.

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Conclusion

Brentuximab vedotin was analyzed using HIC on the Infinity II 1290 Bio LC. All five expected DAR species were well separated corresponding to the mAb containing zero, two, four, six, and eight small molecule drugs, respectively. The HIC analysis allowed both the characterization of the distribution of drug-linked species as well as the determination of the DAR, calculated to 3.7 drug molecules per antibody. The precision analysis of seven subsequent runs revealed excellent reproducibility for RT and area. The eluents used in HIC contain high concentrations of corrosive salts challenging the LC system. Due to its completely iron-free sample flow path, the 1290 Infinity II Bio LC is optimally suited for the conditions used in biochromatography, avoiding potential corrosive damage to the system.

Blank subtraction as a software feature of OpenLab 2 enables users to employ even less pure ammonium sulfate in their analysis without negatively affecting their results. Just by filtering out the blank runs, the baseline can be corrected to enable smooth integration calculation.

The combination of the biocompatible hardware of the 1290 Infinity II Bio LC with new software features of OpenLab 2 results in the highest confidence in generated data.

References

1. McCombs, J. R.; Owen, S. C. Antibody Drug Conjugates: Design and Selection of Linker, Payload and Conjugation Chemistry. *J. Amer. Assoc. Pharm. Sci.* **2015**, *17(2)*, 339–51.
2. Marcoux, J. *et al*. Native Mass Spectrometry and Ion Mobility Characterization of Trastuzumab Emtansine, a Lysine-Linked Antibody Drug Conjugate. *Protein Sci*. **2015**, *24*, 1210–1223.
3. Younes, A. *et al*. Brentuximab Vedotin (SGN-35) for Relapsed CD30-Positive Lymphomas. *N. Eng. J. Med*. **2010**, *363(19)*, 1812–1821.
4. Schneider, S. Analysis of Cysteine-Linked Antibody Drug Conjugates, *Agilent Technologies application note, publication number 5991-8493EN*, **2017**.
5. Wakankar, A. et al. Analytical Methods for Physiochemical Characterization of Antibody Drug Conjugates. *mAbs* **2011**, *3(2)*, 161–172.
6. Rodriguez-Aller, M. et al. Practical Method Development for the Separation of Monoclonal Antibodies and Antibody-Drug-Conjugate Species in Hydrophobic Interaction Chromatography, Part 1: *Optimization of the Mobile Phase. J. Pharm. Biomed. Anal*. **2016**, *118*, 393–403.
7. High Stakes. High Performance. High Confidence: Agilent 1290 Infinity II Bio LC. *Agilent Technologies brochure, publication number 5994-2376DE*, **2020**.
8. van de Donk, N. W. C. J.; Dhimolea, E. Brentuximab Vedotin. *mAbs* **2012**, *4(4)*, 458–465.

Application Note

Intact & Subunit Purity

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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.

Abstract

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 form[1]. 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 changes[2]. 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.

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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 NIST mAb 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 NIST mAb (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 NIST mAb (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 NIST mAb (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.

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 NIST mAb 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 residues[7]. Figure 1 illustrates the reaction scheme for Met oxidation induced by chemical oxidants.

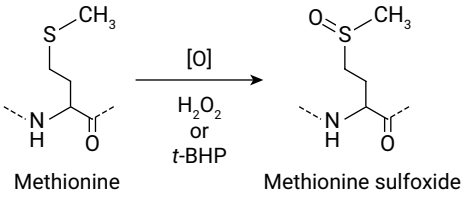

Figure 1. Methionine oxidation induced by chemical oxidant.

The NIST mAb (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 CH_2 and CH_3 domains of the antigen binding, or Fc, region are known to be highly susceptible to oxidation[5]. In the case of NIST mAb, Met 255 and Met 431 correspond to the amino acid residues prone to oxidation. This is depicted by the illustration in Figure 3.

Figure 2. NIST mAb amino acid sequence.

Method conditions

Parameter	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 20
Shallower Gradient	Flow rate: 0.3 mL/min Time %A %B 0 40 60 40 90 10 45 90 10 50 40 60 60 40 60

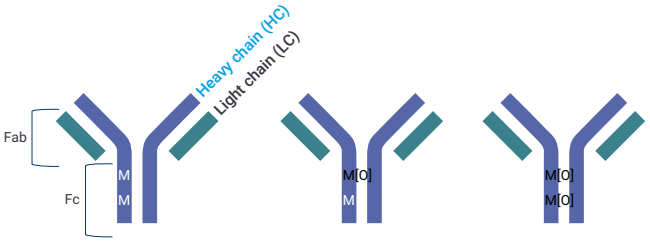

Figure 3. Methionine residues located in Fc region are most susceptible to oxidation in human IgG1 mAbs.

An AdvanceBio HIC column was able to differentiate oxidized mAb variants from the untreated mAb sample under low salt starting conditions. Oxidation of the NIST mAb 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	RT (%)	Area (%)
0	50	50
20	100	0
25	100	0
30	50	50
40	50	50

50 mM/min

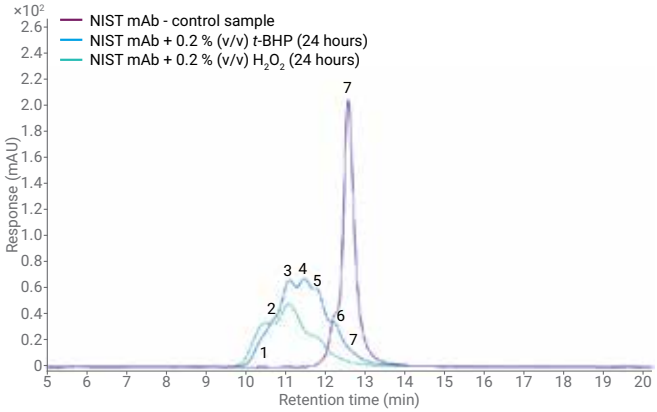

Figure 4. Separation of oxidized NIST mAb variants using lower starting salt concentration.

Time	RT (%)	Area (%)
0	40	60
40	90	10
45	90	10
50	40	60
60	40	60

25 mM/min

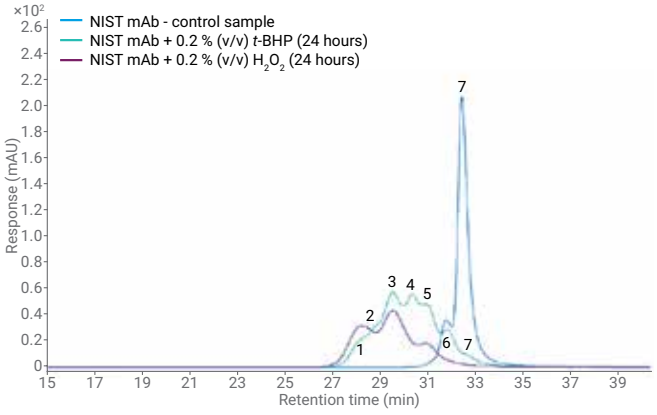

Figure 5. Separation of oxidized NIST mAb variants using a shallow gradient.

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In Figure 6, the NIST mAb 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

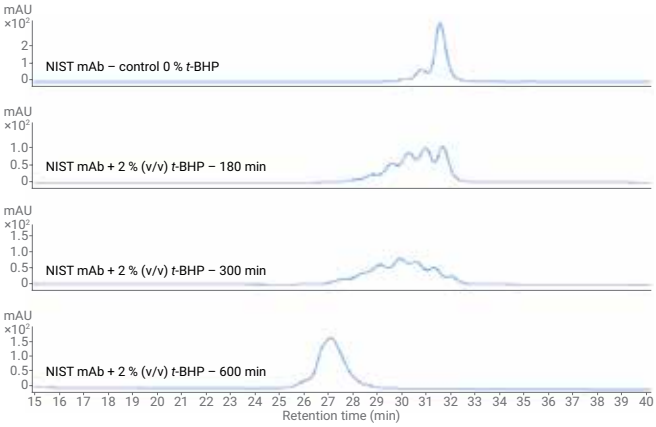


Figure 6. Monitoring the *t*-BHP oxidized mAb reaction.

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.

Additional Application Notes

AdvanceBio RP mAb

Publication Number	Title
5991-6296EN	LC/MS Analysis of Intact Therapeutic Monoclonal Antibodies Using AdvanceBio RP-mAb
5991-4723EN	Reducing Cycle Time for Quantification of Human IgG Using the Agilent Bio-Monolith Protein A HPLC Column
5991-5125EN	Cell Culture Optimization Using an Agilent Bio-Monolith Protein A Column and LC/MS
5991-6094EN	Bio-Monolith Protein G Column - More Options for mAb Titer Determination

PLRP-S

Publication Number	Title
5991-6263EN	Drug-to-Antibody Ratio (DAR) Calculation of Antibody-Drug Conjugates (ADCs) Using Automated Sample Preparation and Novel DAR Calculator Software
5991-6559EN	Measuring Drug-to-Antibody Ratio (DAR) for Antibody-Drug Conjugates (ADCs) with UHPLC/Q-TOF

AdvanceBio HIC

Publication Number	Title
5994-0149EN	An AdvanceBio HIC Column for Drug-to-Antibody Ratio (DAR) Analysis of Antibody Drug Conjugates (ADCs)
5994-1869EN	Analysis of Camelid Single-Domain Antibodies Using Agilent AdvanceBio SEC 120 Å 1.9 µm and AdvanceBio HIC Columns

ZORBAX RRHD 300SB

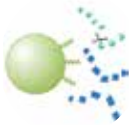
Publication Number	Title
5990-9668EN	Ultra High Speed and High Resolution Separations of Reduced and Intact Monoclonal Antibodies with Agilent ZORBAX RRHD Sub-2 µm 300 Diphenyl UHPLC Column
5990-7988EN	Analysis of Oxidized Insulin Chains using Reversed-Phase Agilent ZORBAX RRHD 300SB-C18
5990-7989EN	Agilent ZORBAX 300SB-C18 1.8 µm Rapid Resolution High Definition Columns for Proteins
5990-9248EN	Fast Separation of Recombinant Human Erythropoietin Using Reversed-Phased Agilent ZORBAX RRHD 300SB-C18, 1.8 µm
5990-9631EN	Rapid UHPLC Analysis of Reduced Monoclonal Antibodies using an Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C8 Column
5990-9016EN	Reversed-phase Separation of Intact Monoclonal Antibodies Using Agilent ZORBAX Rapid Resolution High Definition 300SB-C8 1.8 µm Column
5990-9667EN	Reversed-phased Optimization for Ultra Fast Profiling of Intact and Reduced Monoclonal Antibodies using Agilent ZORBAX Rapid Resolution High Definition 300SB-C3 Columnn

Peptide Mapping

Background

Peptide mapping is a powerful technique that can be used to comprehensively identify the primary structure of a protein. It is also possible to distinguish the exact position of a variant within the protein. Since the primary structure, or amino acid sequence, of a recombinant protein is already known, it is possible to predict the fragments that will be generated when the protein is digested using an enzyme such as trypsin. Trypsin will cleave a protein into fragments by hydrolyzing the bond between lysine or arginine and any other amino acid except proline. Using this approach, trastuzumab will be broken into sixty-two separate fragments and a high resolution reversed-phase separation should be able to separate these out into a classic "fingerprint" chromatogram. Combining the separation with mass spectrometry detection should make it possible to correlate the actual peaks observed in the peptide mapping chromatogram with the expected fragments predicted by the analysis software.

Different proteins will give different peptide "fingerprints" and these will include a wide range of sizes (from individual amino acids and dipeptides up to much larger polypeptides), with varying degrees of hydrophobicity. The recommended column for this type of separation is therefore a C18 reversed-phase in either superficially porous or totally porous particles.



Peptide Mapping

Reversed-phase chromatography (<150 Å)

Reliably characterizes primary sequence and detects PTMs

AdvanceBio Peptide Mapping

Protein identification and PTM analysis

Attribute	Advantage
Endcapped C18 bonded phase	Good retention of hydrophilic peptides
Superficially porous particles	UHPLC-like efficiency at modest back pressure

AdvanceBio Peptide Plus

Ideal formic acid performance for MS detection

Attribute	Advantage
Sharp peaks with formic acid	Good MS sensitivity
High sensitivity	Identify critical low-level modifications
Charge surface chemistry	Preserve high performance with large sample loads
Unique selectivity	Resolve important PTMs such as deamidation

AdvanceBio EC-C18

Ideal for samples susceptible to stainless steel on-column artifacts

Attribute	Advantage
PEEK hardware	Eliminates on-column metal interactions

Getting Started

Sample preparation is key to successful peptide mapping. It can be a time consuming process, with several steps that may need optimization for each protein to be digested. Step-by-step instructions for sample preparation can be found in the "How-To" Guide on the following pages. Users that have a high volume of peptide mapping samples may want to consider automation to improve speed and reproducibility. More information on the AssayMAP Bravo, a sample preparation automation system, may be found at the end of this chapter.

Optimum peak shape is obtained using trifluoroacetic acid as ion pair reagent and for this separation the AdvanceBio Peptide Mapping column is the preferred choice. This column contains a 120 Å pore size Poroshell particle and provides excellent resolution and peak capacity without the need for UHPLC instrumentation. For applications where MS detection will be used, it is often preferable to use formic acid as ion pairing reagent. In such cases, the AdvanceBio Peptide Plus column will provide a better separation profile. The AdvanceBio Peptide Plus is also recommended for cases where large sample loads are necessary to detection minor impurities. For extremely hydrophilic, small peptides AdvanceBio Peptide Mapping is recommended for best retention.

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Peptide Mapping: A "How-To" Guide

Introduction

Peptide mapping – an invaluable tool for biopharmaceuticals – is a very powerful method and the most widely used identity test for proteins, particularly those produced by recombinant means. It most commonly involves enzymatic digestion (usually using trypsin) of a protein to produce peptide fragments, followed by separation and identification of the fragments in a reproducible manner, allowing the detection and monitoring of single amino acid changes, oxidation, deamidation, and other degradation products. It also enables the direct detection of common monoclonal antibody variants such as N-terminal cyclization, C-terminal lysine processing, and N-glycosylation, as well as other post-translational modifications.

A peptide map is a fingerprint of a protein and the end product of several processes that provide a comprehensive understanding of the protein being analyzed. It involves four major steps: isolation and purification of the protein; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and validated analysis of the peptides.

Peptide mapping is considered a comparative procedure that confirms the primary structure of the protein and detects alterations in structure. Additionally, it demonstrates process consistency and genetic stability. A peptide map should include positive identification of the protein, maximize coverage of the complete peptide sequence, and provide additional information and sequence identification beyond that obtained at the non-digested protein level.

The selection of a chromatographic technique to separate peptides and generate peptide maps depends on the protein, experimental objectives, and anticipated outcome. However, the excellent resolving power of reversed-phase chromatography (RPC) makes this technique the predominant HPLC technique for peptide mapping separations. It is also ideal for both analytical and preparative separations because of the availability of volatile mobile phase eluents. It is important to note that the preferred columns for peptide mapping separations are similar to those used for small molecules, but because most peptide mapping separations are performed at low pH and elevated temperature, columns with excellent pH stability and minimal silanol effects are routinely used.

Careful inspection of the complete characterization strategy is required to generate successful peptide maps. A profile may consist of over 100 peaks representing individual peptides and their derivatives, so it requires knowledge of sample preparation methods, powerful separation techniques and validated protocols. Having the skill and information to develop a successful peptide map will help you achieve the best possible separation of your proteolytic digests and deliver a successful and reliable peptide characterization outcome.

The objective of this peptide mapping “how-to” guide is to highlight the areas which are important to generating peptide maps by reversed-phase chromatography, share some of the fundamental techniques used for peptide mapping procedures and emphasize considerations for optimizing your peptide mapping separations to achieve the best possible results.

Protein Digestion:

Preparing Your Protein to Enhance the Peptide Mapping Separation

A good understanding of the steps for digesting a protein prior to analysis will help to ensure a complete, successful digestion and provide a high degree of confidence in your chosen strategy. Often the digestion method requires its own set of development protocols to provide an adequate and stable sample for LC injection. Although there are many options to consider for optimizing the digestion, a number of common approaches should be followed. The five steps used for protein digestion, summarized in Table 1, are (1) sample preparation (2) selection of cleavage agents (3) reduction/alkylation (4) digestion process (5) reduction/alkylation.

Table 1. Five steps for protein digestion

Procedure	Intended Effect	General Experiment
1. Sample Preparation	Preparing sample for digestion	Depletion, enrichment, dialysis, desalting
2. Selection of Cleavage Agent	Specific cleavage requirement	None
3. Reduction and Alkylation	Reduction reduces disulphide bonds Alkylation caps SH groups	Reduction: DTT, 45 min, 60 °C Alkylation: IAM, 1 hr, in the dark
4. Digestion Process	Cleavage of proteins	Digestion: pH 8, 37 °C, overnight Quenching: TFA addition
5. Enrichment/Cleanup	Preparing sample for LC or LC/MS analysis	C18 tips, concentrating, dialysis, affinity columns

Step 1:

Sample preparation

Depending on the size or the configuration of the protein, there are different approaches for pretreatment of your sample. Under certain conditions, it might be necessary to enrich the sample or to separate the protein from added substances and stabilizers used in formulation of the product, especially if these interfere with the mapping procedure. There are many methods for performing these procedures and each protein has its own set of cleanup measures or processes. However, some of the more common approaches used for sample cleanup prior to digestion include depletion/enrichment dialysis and desalting by gel filtration.

Depletion and enrichment strategies have been developed to remove high abundance proteins or isolate target proteins in the sample, respectively. Depletion is more often used in proteomics applications to reduce the complexity of biological samples such as serum, which contain high concentrations of albumin and immunoglobulins. The Agilent Multiple Affinity Removal System (MARS) HPLC columns and spin cartridges enable the identification and characterization of high-value, low abundance proteins and biomarkers found in serum, plasma, and other biological fluids. Through depletion of the 14 high-abundance proteins with MARS, ~94% of the total protein mass is removed. The depletion process is robust, easily automated, and highly efficient.

MARS is available in a variety of LC column dimensions and in spin cartridge formats. Proteins depleted include Albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha2-macroglobulin, alpha1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3, and transthyretin.

Depletion strategies utilize immunoaffinity techniques (e.g., immunoprecipitation, co-immunoprecipitation and immunoaffinity chromatography). Alternatively, enrichment techniques isolate subclasses of cellular proteins based on unique biochemical activity, post-translational modifications (PTMs) or spatial localization within a cell. Post-translational modifications – such as phosphorylation and glycosylation – can be enriched using affinity ligands such as ion-metal affinity chromatography (IMAC) or immobilized lectins, respectively. To introduce unique protein chemistries, other techniques entail metabolic or enzymatic incorporation of modified amino acids or PTMs.

Whether simple or complex, samples often need dialysis or desalting to ensure they are compatible and optimized for digestion. For example, because mass spectrometry (MS) measures charged ions, salts – especially sodium and phosphate salts – should be removed prior to MS to minimize their interference with detection. Dialysis and desalting products allow buffer exchange, desalting, or small molecule removal to prevent interference with downstream processes.

Dialysis is an established procedure for reducing the salt concentration in samples. It requires filling a dialysis bag (membrane casing of defined porosity), tying the bag off, and placing the bag in a bath of water or buffer where the concentration of salt will equilibrate through diffusion. Large molecules that can't diffuse through the bag remain in the bag. If the bath is water, the concentration of the small molecules in the bag will decrease slowly until the concentration inside and outside is the same. Once equilibration is complete, the bag is ruptured and the solution poured off into a collection vessel. Dialysis can be used for volumes up to a few liters, but it is not practical for large sample volumes because it can take several days for complete salt removal.

To desalt samples prior to digestion, Gel Filtration (GF) is the most practical laboratory procedure. This method is a non-adsorptive chromatography technique that separates molecules on the basis of molecular size. Desalting is used to completely remove or lower the concentration of salt or other low molecular weight components in the sample, while buffer exchange replaces the sample buffer with a new buffer.

Gel filtration is one of the easiest chromatography methods to perform because samples are processed using an isocratic elution. In its analytical form, gel filtration (also known as size exclusion chromatography) can distinguish between molecules (e.g. proteins) with a molecular weight difference of less than a factor of 2 times. In these applications, the size difference between the substances being separated is very large (i.e. proteins vs. salts). A gel filtration media is chosen that completely excludes the larger molecules while allowing the smaller molecules to freely diffuse into all of the pore spaces. The column is equilibrated with a buffer, which may be the same or different from that of the sample. Following application of the sample to the column, more of the column buffer (eluting buffer) is added to carry the sample molecules down the column. The larger molecules – which can't enter the pores of the media – elute first from the column, followed by the smaller molecules that diffuse into the pores, slowing them down relative to the larger molecules. If the eluting buffer is different from the sample that was applied, the larger molecules will be displaced from the original salts and elute in this new buffer, completely separated from the original sample buffer.

AdvanceBio SEC columns can effectively classify (by size) and desalt protein mixtures prior to peptide mapping applications.

Captiva Low Protein Binding Filters

Regardless of what sample prep you are performing, it's a good idea to filter your sample with a low protein binding filter.

Agilent PES filters provide superior and consistent low protein binding for protein-related filtration. The PES filter membranes are a better option than PVDF membranes for most LC analyses. Agilent PES has similar compatibility to PVDF filters for common LC solvents and is superior in terms of protein binding and cleanliness.
Learn more at **agilent.com/chem/filtration**

Captiva PES Filters				
Diameter	**Pore Size (um)**	**Certification**	**Housing**	**Part Number**
15	0.2	LC/MS	Polypropylene	5190-5096
4	0.45	LC	Polypropylene	5190-5095
4	0.2	LC/MS	Polypropylene	5190-5094
15	0.45	LC	Polypropylene	5190-5097
25	0.2	LC/MS	Polypropylene	5190-5098
25	0.45	LC	Polypropylene	5190-5099

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Step 2:

Selection of cleavage agents

There are two methods employed for the cleavage of peptide bonds, chemical and enzymatic. Chemical cleavage involves the use of nucleophilic non-enzymatic reagents such as cyanogen bromide (CNBr) to chemically cleave the peptide bond at a specific region while proteolytic enzymes, such as trypsin, have been proven highly useful for a variety of site specific cleavage locations. The cleavage method and agent will depend on the protein under test and the specific outcome expectations of the analysis. Additionally, the selection process involves careful examination of the entire peptide mapping process and considerations for related characterizations. The most common cleavage agent used for peptide mapping is trypsin due to its well defined specificity. Trypsin hydrolyzes only the peptide bonds in which the carbonyl group is followed either by an arginine (Arg) or lysine (Lys). Several common cleavage agents and their specificity are shown in table 2.

Table 2. Cleavage Type.

Cleavage	Cleavage Agent	Specificity
Enzymatic	Trypsin	C-terminal side of Arg & Lys
	Pepsin	Non-specific
	Chymotrypsin	C-terminal side of hydrophobic residues
	Glutamyl endopeptidase	C-terminal side of Glu and Asp
Chemical	Cyanogen bromide	C-terminal side of Met
	Dilute acid	Asp and Pro
	BNPS-skatole	Trp

Step 3:

Denaturation, reduction, and alkylation

For the proteolytic enzyme to efficiently cleave the peptide chains, most samples need to be denatured, reduced, and alkylated, using various reagents. Denaturation and reduction can often be carried out simultaneously by a combination of heat and a reagent, like 1,4-dithiothreitol (DTT), mercaptoethanol, or tris(2-carboxyethyl)phosphine. Most used is DTT, which is a strong reducing agent that reduces the disulfide bonds and prevents inter- and intra-molecular disulfide formation between cysteines in the protein. By combining denaturation and reduction, renaturation – a problem when using heat solely as the denaturation agent – due to reduction of the disulfide bonds can be avoided. Following protein denaturation and reduction, alkylation of cysteine is necessary to further reduce the potential renaturation. The most commonly used agents for alkylation of protein samples prior to digestion are iodoacetamide (IAM) and iodoacetic acid (IAA).

Figure 1 provides a good example of a reversed-phase chromatographic separation method used to evaluate the reduction and alkylation completeness of a monoclonal antibody prior to digestion.

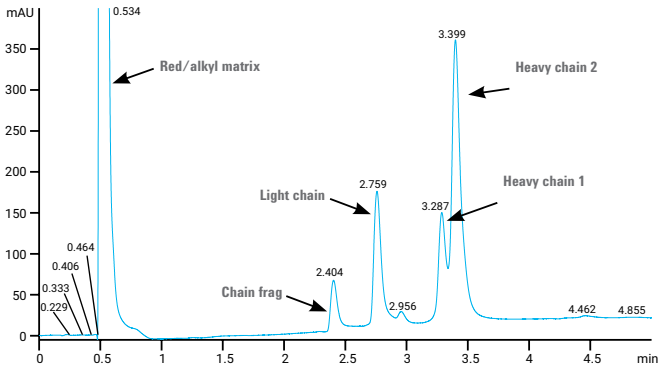

Figure 1. Reversed-phase separation of a reduced and alkylated monoclonal antibody prior to digestion protocol using an Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C8, 2.1 x 50 mm column (Agilent p/n 857750-906). The separation was performed at 0.5 mL/min, 75 °C using water (0.1% TFA)/ACN (0.08%) multi-segmented conditions on an Agilent 1290 Infinity LC.

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Step 4:

As already mentioned, trypsin is the most commonly used protease for digestion due to its well defined specificity. Since trypsin is a protein, it may digest itself in a process called autolysis. However, calcium, naturally present in most samples, binds at the Ca^2 binding loop in trypsin and prevents autolysis. With the modified trypsin presently used in most laboratories, autolysis is additionally reduced and not typically a large concern.

Tryptic digestion is performed at an optimal pH in the range 7.5-8.5, and commonly at 37 °C. To provide an optimal pH for the enzymatic cleavage, a buffer is added (usually 50 mM triethyl ammonium bicarbonate (tABC) or 12.5 mM ammonium bicarbonate (ABC) prior to the addition of trypsin. A 2-amino-2-hydroxymethyl propane-1,3-diol (Tris) buffer may also be used for this purpose, but it should be taken into consideration that the Tris buffer is incompatible with MS analysis, such as MALDI and ESI-MS, and needs to be depleted through solid phase extraction (SPE) or ZipTips. To ensure a sufficient – but not too high – amount of enzyme to perform the digestion, it is crucial to have the right enzyme-to-protein ratio.

Proteins may act differently in different environments and when model proteins were digested in a mixture vs. separately, less effective digestions have been observed. One reason could be increased competition for the trypsin cleavage sites, when more proteins are digested together. Additionally, there can be many factors and conditional parameters that could affect the completeness and effectiveness of digestion of proteins, causing a variety of anticipated outcomes. If these factors are more carefully understood or controlled, the digestion results can be greatly improved. The pH of the reaction, digestion time and temperature and the amount of cleavage agent used are all critical to the effectiveness of the digestion.

- **Digestion pH.** In general, the pH of the digestion mixture is empirically determined to ensure the optimization of the performance of a given cleavage agent. For example, when using cyanogen bromide as a cleavage agent, a highly acidic environment (e.g. pH 2, formic acid) is necessary; however, when using trypsin as a cleavage agent, a slightly alkaline environment (pH 8) is optimal. As a general rule, the pH of the reaction milieu must not alter the chemical integrity of the protein during the digestion or the course of the fragmentation reaction.
- **Digestion time & temperature.** Time and temperature play an important role for optimum digestion. To minimize chemical reactions, a temperature between 25 °C and 37 °C is adequate – and recommended – for most protein digestions (e.g., trypsin digestions are commonly run at 37 °C). However, the type and size of protein will ultimately determine the temperature of the reaction due to protein denaturation as the temperature of the reaction increases. Reaction time is also a factor for consideration in optimizing the digestion protocol. If sufficient sample is available, an experimental study should be considered in order to determine the optimum time to obtain a reproducible map while avoiding incomplete digestion. Time of digestion varies from 2 h to 30 h depending on sample size and type, while the reaction is stopped by the addition of an acid, which does not interfere in the map or by freezing.
- **Concentration of cleaving enzyme.** The concentration of the cleaving agent should be minimized to avoid its contribution to the map patterns. An excessive amount of cleavage agent is commonly used to accomplish a reasonably rapid digestion time (i.e. 6 to 20 hours); however, careful consideration should be given to these increased amounts. A protein-to-protease ratio between 10:1 and 200:1 is generally used and it is recommended that the cleavage agent be added in two or more stages to optimize cleavage. In many standard trypsin digestion procedures, the trypsin is added in this manner. Nonetheless, the final reaction volume remains small enough to facilitate separation – the next step in peptide mapping. To sort out digestion artifacts that might interfere with the subsequent analysis, a blank determination is performed using a digestion control with all the reagents, except the test protein.

Figure 2 – Trypsin digestion procedure (Parts I-V)

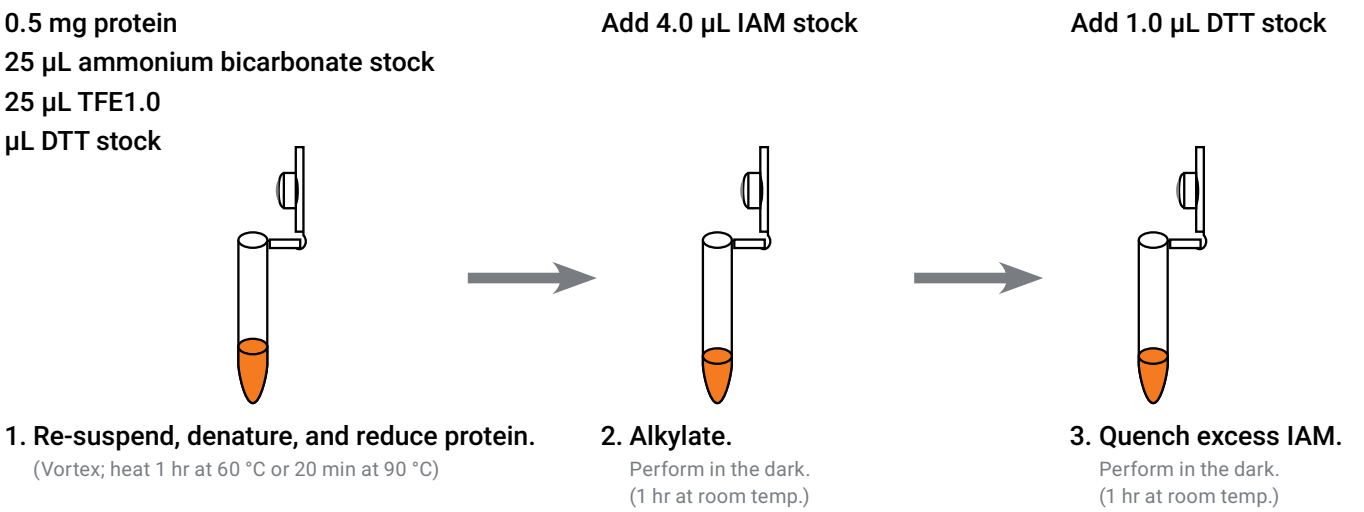
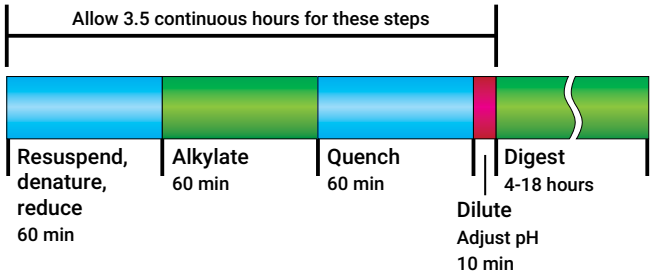


Figure 3 – Expected timeline for digestion procedure



The Trypsin digestion method described below and summarized in figure 2 and 3 is a common procedure routinely used for the reduction, alkylation, in-solution digestion, and cleanup of protein (0.5 mg). This procedure is scalable for smaller amounts of proteins and additionally provides a useful list of Agilent reagents and part numbers.

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Reduction, alkylation, digestion solution preparation: Summary

100 mm ammonium bicarbonate:

Add 100 mL water to 0.7906 g ammonium bicarbonate. Store in refrigerator at 4 °C for up to 2 months.

Trypsin stock:

Modified trypsin can be purchased: Agilent Proteomics Grade Trypsin (P/N 204310), see next page "Reagents and equipment"). It is lyophilized and may be stored in this form at -20 °C for more than one year without significant loss in activity. When required, prepare trypsin stock solution by hydrating the lyophilized trypsin in 100 µL of 50 mM acetic acid, to a final concentration of 1 µg/mL. To minimize freeze-thaw cycles and to increase storage stability, divide the hydrated trypsin into ten separate tubes of ~10 µL each. Store each aliquot at -20 °C in a non-frost-free freezer. This 1 µg/µL solution is used to prepare the trypsin intermediate solution as needed (see below). Note that the Agilent Proteomics Grade Trypsin comes with technical literature that provides an alternate protocol for tryptic digestion. We have used the method below and find it to be straightforard and reliable.

200 mM DTT:

Add 1 mL water to 0.031 g DTT in a 1.5 mL Eppendorf tube. Vortex. Divide the DTT solution into convenient (e.g., 100 µL) aliquots in microcentrifuge tubes. Store each aliquot at -20 °C for up to one month in a non-frost-free freezer. Do not thaw and re-freeze.

200 mm IAM

(prepare just before use): Add 1 mL water to 0.037 g IAM in a 1.5 mL Eppendorf tube. Vortex.

Trypsin digestion protocol

Resuspension, denaturing, and reduction of protein	
1	Add 0.5 mg total protein to 0.5 mL Eppendorf tube.
2	Add 25 µL ammonium bicarbonate stock solution.
3	Add 25 µL TFE denaturation agent.
4	Add 1.0 µL DTT stock solution.
5	Vortex to mix.
6	Heat under one of the following sets of conditions to denature: ✓ 60 °C for 45 minutes to 1 hour ✓ 90 °C for 20 minutes (hydrophilic proteins) to 1 hour (hydrophobic proteins)
7	Cool to room temperature.
Akylation	
1	Add 4.0 µL IAM stock solution.
2	Vortex briefly.
3	Incubate sample in the dark (foil-covered rack) at room temperature for 1 hour.
Quenching of excess IAM	
1	Add 1.0 µL DTT stock solution to destroy excess IAM.
2	Allow to stand for 1 hour in the dark (foil-covered rack) at room temperature.
Dilution and pH adjustment	
1	Add 300 µL water to dilute denaturant.
2	Add 100 µL ammonium bicarbonate stock solution to raise pH.
3	Optionally check pH by placing 0.5 to 1 µL on a strip of pH indicator paper. Typical value is 7.5 to 8.0. It is more important to check pH when the pH of the starting sample is unknown.
4	Add more base (ammonium bicarbonate) if pH is not in the 7 to 9 range.

Digestion	
1	Make fresh stock solution of trypsin in trypsin storage solution. Allow 15 min for complete re-suspension.
2	If you plan to digest less than 20 µg total protein, prepare trypsin intermediate solution by diluting stock 10-fold by adding 45 µL ultrapure water. This 100 ng/µL solution may be stored at -20 °C for 2 months without significant loss of activity. CAUTION: If IAM is not destroyed, it will slowly alkylate lysines.
3	Add trypsin stock solution at 1:20 to 1:50 by mass of enzyme:substrate. For example, for 500 µg protein, add between 10 and 25 µg trypsin (10 to 25 µL trypsin stock).
4	Vortex briefly.
5	Place tube in heater and incubate at 37 °C for 4 to 18 hours.
6	Cool solution.
Lowering of pH to halt trypsin activity	
1	Add 1 µL neat formic acid or TFA to lower the pH and stop trypsin activity. If you are planning to desalt, use TFA because it aids in the peptide binding to the resin during cleaning.
2	Vortex briefly.
3	If you are concerned about the pH of the original sample, check pH (3.0 to 3.3 typically). Add more acid if pH is greater than 4.
Digestion Cleanup	
1	Depending on sample origin, it may be necessary to desalt prior to MS analysis.
2	If desalting is not necessary, but the sample appears opaque, filter the sample prior to MS. Use Agilent spin filters, P/N 5185-5990. The opacity may be caused by cellular debris in the sample.
3	**Dilute an aliquot of sample as necessary for analysis.** If protein has a molecular weight of 50 kDa, and if digestion went to completion, solution is about 20 pmol/µL. If you have a less complex sample, dilute to achieve a 50 fmol/µL solution.

Step 5:

Cleanup and enrichment of digests

Prior to peptide mapping, cleanup and/or enrichment is usually required for the successful analysis of peptide maps. There are many types of methods to accomplish cleanup and enrichment dependent on sample type and targeted objective. For example, enrichment for specific PTMs (e.g., phosphorylation, ubiquitination and glycosylation) is performed by affinity purification using PTM-specific antibodies or ligands, while phosphopeptides can be enriched by IP using anti-phospho-specific antibodies or by pull-down using TiO2, which selectively binds phosphorylated serine, tyrosine or threonine.

After peptide enrichment, salts and buffers can be removed using either graphite or C-18 tips or columns, and detergents can be removed using affinity columns or detergent-precipitating reagents. Dilute samples can also be concentrated using concentrators of varying molecular weight cutoff (MWCO) ranges. Once purified, peptide samples are then ready for the final preparation for MS analysis, which varies based on the type of analysis. For LC/MS or LC-MS/MS analysis, the proper choice of mobile phases and ion-pairing reagents is required to achieve good LC resolution and analytical results. MALDI-MS requires combining the peptide sample with specific matrices (crystalline energy absorbing dye molecules), which are then dried on MALDI plates prior to analysis.

Trypsin digestion protocol

Item needed	Example
Ammonium bicarbonate, reagent grade	Sigma catalog #A-6141
Dithiothreitol (DTT), >99+%	Sigma catalog #D-5545
Iodoacetamide (IAM), 97%	Sigma-Aldrich catalog #I-670-9
Trifluoroethanol (TFE), 99+%	Sigma-Aldrich catalog #T63002-100G
Trypsin, modified	Agilent Proteomics Grade Trypsin (p/n 204310)
Water, 18 megohm or equivalent	Agilent p/n 8500-2236
Formic acid, analytical grade or trifluoroacetic acid, sequencing grade	Agilent p/n G2453-85060
Eppendorf Safe-Lock microcentrifuge tubes, natural, not siliconized	Eppendorf p/n 022363611 (0.5 mL, box of 500), or p/n 022363204 (1.5 mL, box of 500)
Micropipettors and tips: 1-1000 µL range	
Tube heater/shaler	Eppendorf Thermomixer
pH indicator strips, pH ranges 2.5-4.5 and 7.0-9.0	EM Science ColorpHast strips, catalog #700181-2
Analytical balance	
Bond Elut OMIX Tips, 10 µL (elution volume 2-10 µL)	1x96 tips (Agilent p/n A5700310); 6x96 tips (Agilent p/n A5700310K)
Bond Elut OMIX Tips, 100 µL (elution volume 10-100 µL)	1x96 tips (Agilent p/n A57003100); 6x96 tips (Agilent p/n A57003100K)

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For small volumes of peptides for cleanup: Bond Elut OMIX tips

Bond Elut OMIX (10 µL volume) method for peptide digest cleanup

Item needed	Example
Sample	Adjust sample to a 0.5 %-1.0 % trifluoroacetic acid (TFA) concentration using a 2.5 % TFA solution
Pretreatment	Aspirate 10 µL of 50 % acetonitrile (ACN):water and discard solvent. Repeat.
Conditioning and Equilibratio	Aspirate 10 µL of 1.0 % TFA solution and discard solvent. Repeat.
Sample Application	Aspirate up to 10 µL of pre-treated sample into OMIX Tip. Dispense and aspirate sample 3-5 cycles for maximum efficiency. Up to 10 cycles may be used for improved binding.
Rinsing	Aspirate 10 µL of 0.1% TFA buffer and discard solvent. Repeat.
Elution	LC/MS or LC/MS/MS Analysis: Aspirate 2-10 µL of 0.1% Formic Acid or 0.1% Acetic Acid in either a 50-75 % acetonitrile or 50-75 % methanol solution and dispense into an autosampler vial or well plate.

For best results, set the pipettor to match the tip volume – 10 µL – for equilibration, sample application, and rinsing steps. For elution, aliquot the exact volume of elution solution into a separate container and maintain your pipettor at the maximum volume setting to match the tip volume, 10 µL.

For high-throughput peptide applications: Automated sample prep solutions for peptide mapping

"Using the combination of extremely consistent, parallelized digestion with automated reversed-phase cleanup via AssayMAP... has enabled us to contemplate collaborative studies of previously unheard of scales and throughputs."

Jacob D. Jaffe, Ph.D.

Assistant Director - Proteomics Platform

See more information about automated sample prep for peptide mapping on **page 73.**

Reversed-Phase Chromatography: The Superior Choice for Peptide Mapping

The selection of a column and method to generate peptide maps ultimately depends on the protein being mapped and the goals of the workflow. The most widely used peptide mapping column method, especially among the biopharmaceutical industry, is reversed-phase chromatography (RPC). Excellent resolving power and the use of volatile mobile phases (compatible with mass spectrometry) has resulted in this technique becoming the predominant HPLC method for most peptide separations. It is superior to other modes of HPLC separations with respect to both speed and efficiency. Figure 4 shows chromatograms of a tryptic digest of the NISTmAb separated by the Agilent 1290 Infinity II Bio LC with Methods A and B. Eight peaks were picked for subsequent retention time precision and peak capacity calculations. For full method parameters, see app note 5994-2718EN.

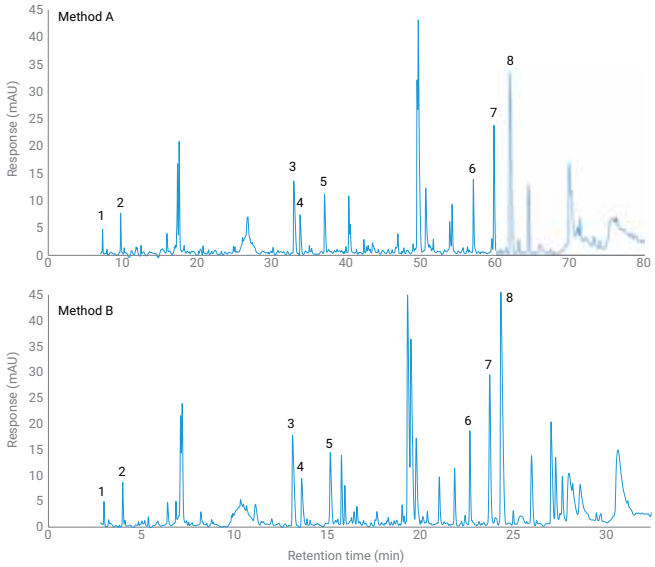
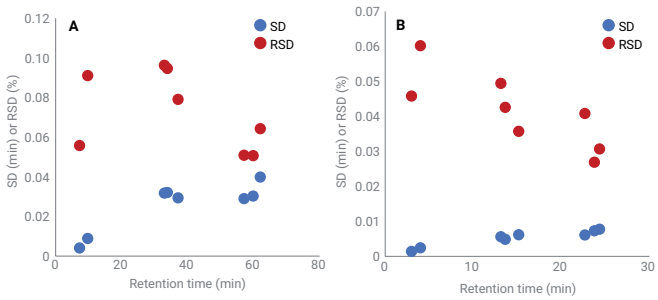


Figure 4. Chromatograms of a tryptic digest of the NISTmAb separated by the Agilent 1290 Infinity II Bio LC with Methods A and B. Eight peaks were picked for subsequent retention time precision and peak capacity calculations.



Method	Gradient Slope (%B/min)	Ø SD (min)	Ø RSD (%)	PC,4σ (–)
Method A	0.39	0.026	0.073	428
Method B	0.98	0.005	0.039	348

Figure 5. Absolute (SD) and relative (RSD) retention time precision values of Methods A and B acquired with the Agilent 1290 Infinity II Bio LC. The gradient slope and peak capacity are depicted in the table.

Requirements for a Successful Peptide Mapping Separation

The general approach in developing a practical RPC method for peptide mapping requires a good understanding of peptide specific column requirements and chromatographic method development. Although many of the same chromatographic principles apply to the separation of peptides compared to small molecule separations, there are a number of condition specific variables for optimizing the peptide method and achieving a reproducible and robust separation. Column selection, column quality, mobile phase selection and detection requirements are all important components to peptide mapping separations that can vastly improve the quality of your peptide maps.

Column selection

The most important aspect for achieving a reliable, well-resolved peptide mapping separation is the selection of a suitable column. The column pore size, particle type and size, bonded phase chemistry and stability (chemical and packed bed) all play a significant role in facilitating the peptide mapping separation, optimization strategy and spectrometric analysis. For peptide separations, the preferred column pore sizes range from 100 Å to 120 Å, while the optimum phase selection is typically C18. Although some commercial columns offer pore sizes for peptides down to 60 Å, these are typically related to smaller peptide fragment separations or standards analysis. Likewise, there are smaller bonded phase carbon chain lengths used, but these have relationships to specific methods and have limited practicality for achieving retention across a broad spectrum of peptide hydrophobicity.

Separations of peptides deliver smaller plate numbers due to their higher diffusion coefficients, and have favored the use of smaller diameter totally porous column materials at slower flows. This has spawned an increase in sub-2 µm packings for achieving more efficient peptide maps. However, more recently, superficially porous columns have become increasingly popular for biological separations – especially among the biopharmaceutical industry – because they address the limitations of protein and peptide mass diffusion. These columns offer a shorter diffusion path allowing the separations of larger molecules at high linear velocities without the system backpressure increases associated with the smaller particles. Figure 6 provides an example of a rapid high resolution peptide map achieved using a superficially porous column.

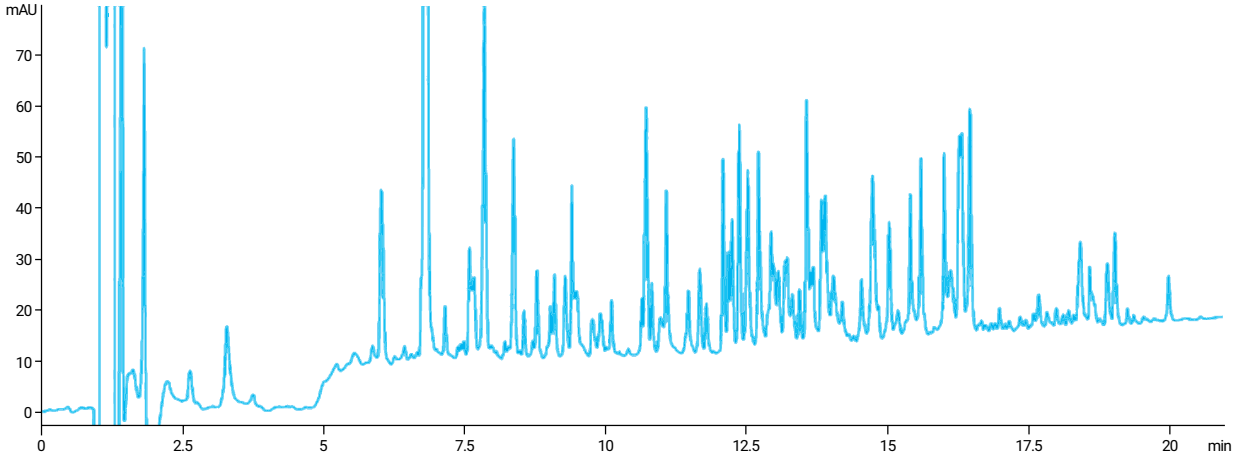


Figure 6. Reversed-phase separation of BSA using an Agilent AdvanceBio Peptide Mapping 2.1 x 150 mm column (Agilent P/N 653750-902). The peptide mapping separation was performed at 0.3 mL/min, 40 °C using water (0.1% TFA)/ACN (0.08%) linear gradient.

Column quality – run-to-run reproducibilty and stability – is a critical, and sometimes overlooked, requirement for maintaining reproducible and robust peptide mapping separations. Reversed-phase separations of peptides are commonly carried out at low pH (pH<3) and elevated temperatures (>40 °C).

Peptide maps rely on repeatable operation of the column for delivering precise mapping fingerprints and repeated validation protocols. When choosing a column for peptide mapping, column quality should be at the forefront of the decision making process. Figure 7 provides an excellent example of a reproducible peptide map of a monoclonal antibody tryptic digest separated under low pH and elevated temperature conditions during an LC/MS analysis.

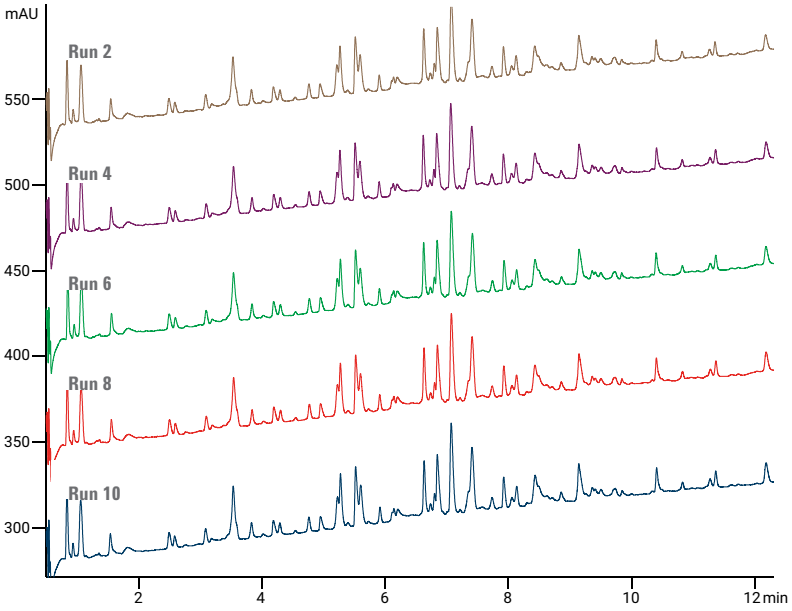


Figure 7. Five replicate injections of a monoclonal antibody tryptic digest using a 3.0 x 150 mm Agilent AdvanceBio Peptide Mapping column (Agilent P/N 653950-302) on an Agilent 1200 LC system coupled to a 6520 Q-TOF. Separation was performed at 0.3 mL/min, 40 °C using water (0.1% FA)/ACN (0.1% FA) gradient.

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Mobile phase selection

The most commonly used solvent in peptide mapping is water with acetonitrile as the organic modifier to which not more than 0.1% of ion pairing agent is recommended. Under certain circumstances, propyl alcohol or isopropyl alcohol can be added to solubilize the digest components, provided that the addition does not unduly increase the viscosity of the components. Buffered mobile phases containing phosphate are used to provide some flexibility in the selection of pH conditions, since shifts of pH in the 3.0-5.0 range enhance the separation of peptides containing acidic residues (e.g. glutamic and aspartic acids). Sodium or potassium phosphates, ammonium acetate, phosphoric acid at a pH between 2 and 7 (or higher for polymer-based supports) have also been used with acetonitrile gradients. Acetonitrile containing trifluoroacetic acid is used quite often.

Mobile phases used in RPC for the analysis of proteins and peptides contain an additive which works as an ion-pairing agent. This component increases the hydrophobicity of peptides by forming ionic pairs with their charged groups. As a consequence, interaction of the peptides with the hydrophobic stationary phase is possible and, therefore, so is their improved separation through increased retention. More common additives such as trifluoroacetic acid (TFA), formic acid (FA), and acetic acid (AcOH) can yield very low pHs and promote protein unfolding and denaturation. Thus, molecules such as peptides, elute in sharper and more symmetrical bands. The ion-pairing agent most widely used for the separation of proteins and peptides is TFA for both its compatibility (high volatility) with mass spectrometry and affinity for the charged peptide.

Detection

Detection for peptides is usually 210 nm to 220 nm and/or 280 nm (figure 7). Detection at 280 nm is often performed in parallel with detection at 210 nm in peptide mapping. Tryptophan, tyrosine, and phenylalanine are sensitive at 280 nm while 210 nm detection is relatively unselective for a host of other biologicals in the sample matrix. However, sensitivity at 210 nm and 220 nm is two to four fold higher than 280 nm. Additionally, of some importance to the detection profile for peptide maps is the blending of 0.1% TFA in water (A-solvent) and 0.08% TFA (B-solvent) in ACN which is used to minimize baseline drift caused by changes in absorbance over the course of the elution gradient. Figure 8 provides an example comparison of a peptide mapping separation as wavelength is varied between 220 nm and 280 nm and details the differences in absorbance sensitivity and UV peak profiles.

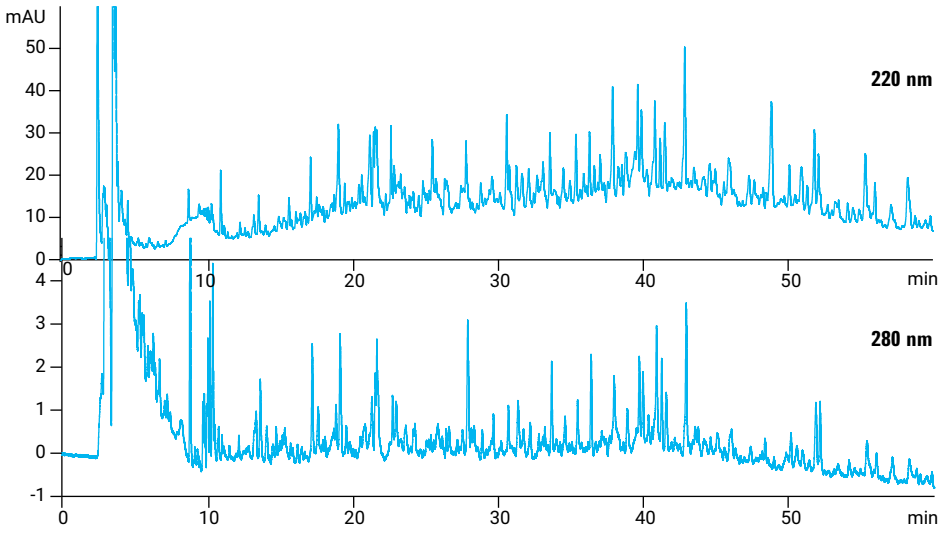

Figure 8. AdvanceBio Peptide Mapping column (Agilent P/N 651750-902), 2.1 x 250 mm, profiling e.coli digest at 220 nm (top) and 280 nm (bottom) on an Agilent 1290 Infinity LC.

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Developing an Efficient Peptide Mapping Method

The general approach to developing an RPC method for a peptide mapping separation is the same employed by typical RP method development practices, however, there are special requirements unique to peptide mapping development. This section will provide a recommended basic approach for preparing a well resolved peptide map via (1) optimization of gradient conditions for retention, (2) variables for changing selectivity and (3) further optimizing column conditions to improve the compromise between run time and resolution. At each step of this method development process careful attention should always be given to sample type and the intended purpose of your peptide mapping experiment.

(1) Optimizing the gradient conditions

A low pH ACN buffer gradient is always highly recommended for the separation of peptides, because it:

- Facilitates the separation of a wide range of peptide types and structures.
- Suppresses ionization of silanols, which can have undesirable interactions with basic amino side chains in the molecule, resulting in poor peak shapes.
- Helps to denature the peptide fragment improving retention and resolution.
- Allows for low UV detection (<210 nm) for maximizing detection sensitivity.
- Provides narrower bands due to the lower viscosity of the mobile phase.
- Increases retention of small poorly retained peptides by ion-pairing with the free amino terminus and basic amino acids (when TFA is used in buffer).

Propanol or iso-propanol (IPA) can be substituted for ACN as the organic modifier to provide better recovery of hydrophobic peptides. However, they are more viscous, resulting in higher column backpressure and somewhat broader bands in some cases. These solvents also require a higher wavelength for detection (>220 nm) and have a loss in detection sensitivity.

Most peptides are eluted with less than 60 % ACN, but occasionally a higher ACN concentration is required. A good starting point for an initial peptide mapping development run is 0 to 60 % in 45 minutes (2%/min). However a flatter gradient often is necessary in the final method to obtain the desired resolution. Gradient steepness, or the %B/min, determines the average retention (k') of a sample band during its migration through a column. The value of k' depends on the column dimensions, flow, sample weight and gradient steepness.

(2) Variables for changing selectivity of the peptide map

The Chromatographers working with biological samples generally postpone a change of column conditions until band spacing has been improved. Changes in temperature and gradient steepness are convenient to perform (no change in mobile phase or column) and should be explored first to improve band spacing for optimizing a peptide mapping separation.

A change in temperature is a powerful means of changing selectivity and could result in retention switching for particular peptide residues. Elevating the temperature of a peptide mapping separation produces narrower bands, lowers system backpressure and changes selectivity. An initial temperature of 30-50 °C is recommended; however, the optimum temperature for a particular mapping separation will depend on many factors based on digestion type and composition. Some very hydrophobic peptides require a temperature of 60-80 °C for maximum recovery, while selectivity for a given sample will often be best for a particular temperature in the range of 30-60 °C.

Figure 9 details a comparison between two identical gradient regions when temperature was increased from 30 °C (top chromatogram) to 60 °C (bottom chromatogram) for a myoglobin tryptic digest. At an elevated temperature of 60 °C, the separation profile details changes in band shapeand peak position highlighted by the peaks 1-7. Clearly some of the notable changes in this region of the chromatogram are the improved separation between peaks 1, 2 and 3 and the band positioning differences (selectivity) between peaks 4 and 5.

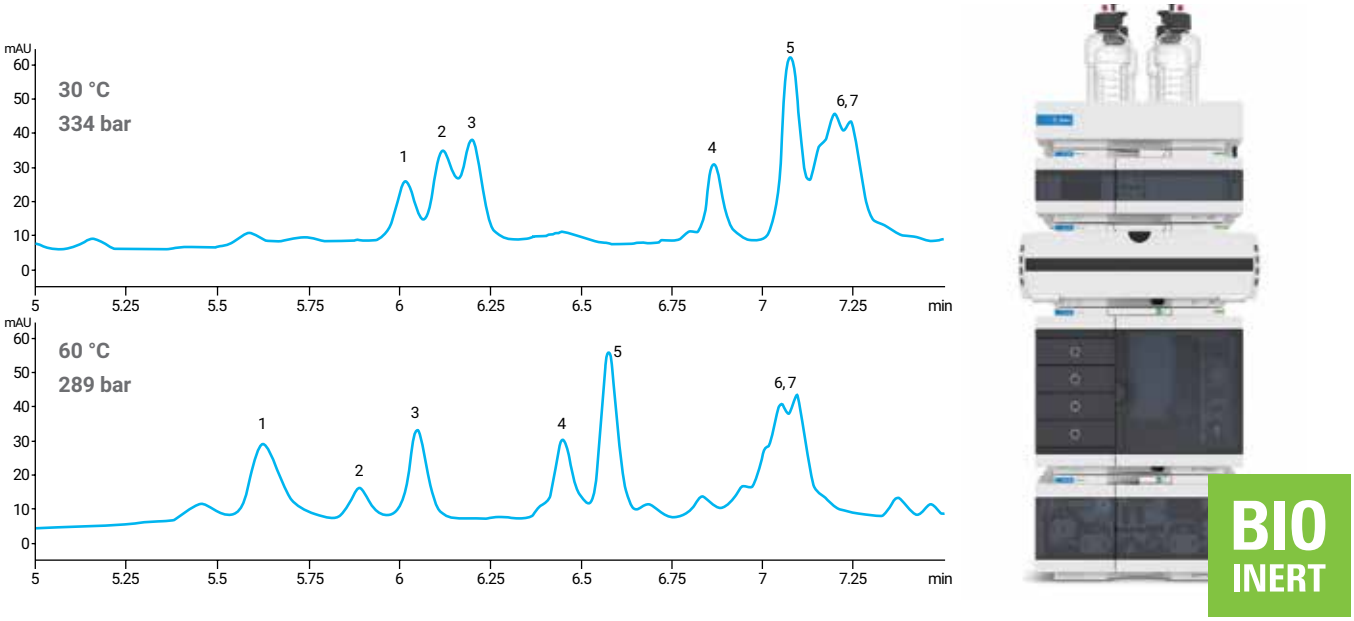

Figure 9. Myoglobin tryptic digest gradient separation at 5.0-8.0 min of a 20 min gradient with a 2.1 x 150 mm AdvanceBio Peptide Mapping column (Agilent P/N 653950-302). Both separations were completed with a water (1.0 % TFA)/ACN (0.08% TFA) linear gradient, 0.3 mL/min at 215 nm on an Agilent 1260 Infinity Bio-inert Quaternary LC system. The top chromatogram was separated at a temperature of 30 °C and the bottom chromatogram was completed at a temperature of 60 °C.

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Changes in gradient steepness can also dramatically improve band spacing and change selectivity of the peptide mapping separation. Gradient steepness can be varied in two ways by either keeping the flow rate constant and changing the elution time to shorter (Increasing steepness) or longer (decreasing steepness) run times or by keeping run time constant and changing the flow rate.

Figure 10 demonstrates selectivity changes resulting from varying gradient steepness. Using a myoglobin tryptic peptide digest, a steep gradient run time of 15 minutes (top chromatogram) was compared to longer gradient run time of 40 minutes (bottom chromatogram), while both separations were maintained at a flow of 0.6 mL/min at 50 °C. A comparison on the chromatograms – and identifying the same peaks (asterisks) in each separation – shows numerous changes to band spacing, peak counts and peak shape.

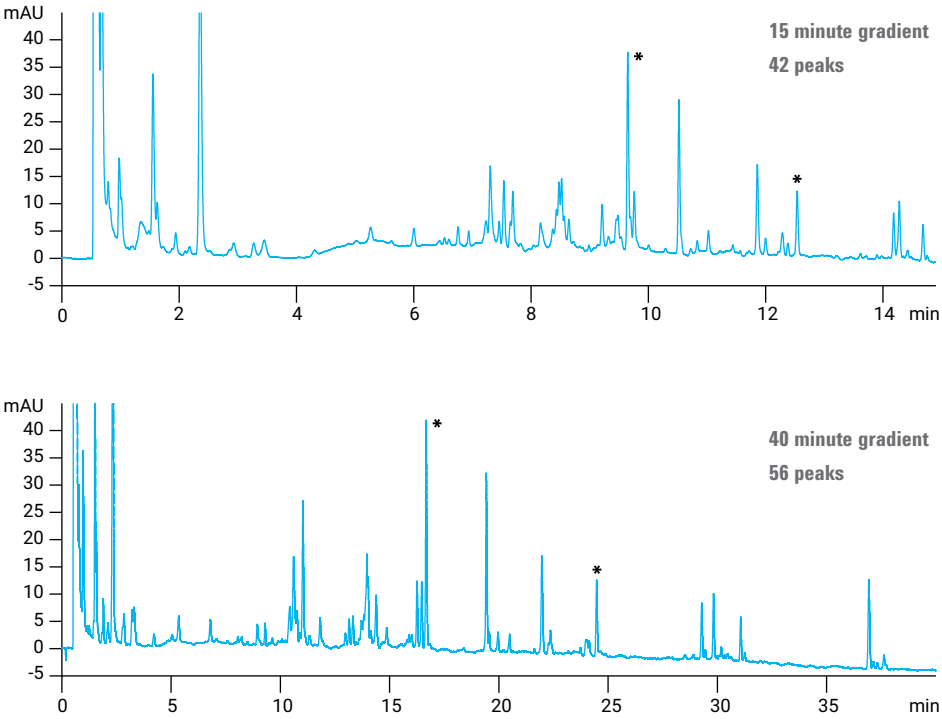

Figure 10. Myoglobin tryptic digest gradient separations with a 2.1 x 150 mm AdvanceBio Peptide Mapping column (Agilent P/N 653950-302) on an Agilent 1260 Infinity Bio-inert Quaternary LC system using water (1.0 %TFA)/ACN (0.08% TFA) linear gradient, 0.6 mL/min at 50 °C. The top chromatogram was completed in 15 minutes while the bottom chromatogram was completed in 40 minutes. Asterisks in each chromatogram represent same peaks.

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(3) Adjusting column conditions for further optimization

Once the gradient has been optimized in terms of retention (k') and selectivity (a), further improvements in separation are possible by varying column length and flow rate. The choice of which column condition to vary in gradient elution is essentially the same as for an isocratic separation. In both cases, larger values of efficiency (N) can be obtained at the expense of longer run times. For minor improvements in resolution, where an increase in the run time is less important, it is convenient to reduce flow rate. However, when a larger increase in resolution is needed, an increase in column length is usually preferred. If resolution is greater than required after optimizing selectivity, this excess resolution can be traded for a shorter run time by increasing flow rate and/or reducing column length. figure 11 provides an example of improved peptide mapping resolution for a myoglobin tryptic digest when column length was increased from 150 mm to 250 mm. In this comparison, conditions and gradient time were held constant while column length was increased from 150 mm to 250 mm. A red box was added to the same areas of the separations to highlight the increased resolution enabled by the 250 mm length and to emphasize the gains in peak capacity per unit time.

The gradient elution, subsequent variables associated in optimizing selectivity and the column condition optimizations discussed in (1), (2) & (3) above are proven basic strategies for improving any separation strategy including peptide mapping. The methods described above can be best outlined in the steps below:

Peptide mapping method development steps	
1	Select the initial gradient conditions: column length, mobile-phase composition, flow rate, temperature, and detection. The initial separation should be optimized for retention (k'). This requires a gradient that is not too steep.
2	Adjust the gradient range. This is used to minimize run time by eliminating wasted space at the beginning and end of the chromatogram.
3	Vary selectivity. If overlapping bands are observed or run time is too long, options discussed for selectivity adjustments can be tried.
4	Consider gradient shape. Additional band spacing may be achieved with the use of a non-linear gradient shape as an option to further improve the separation.
5	Adjust column conditions. When band spacing and selectivity are optimized, consider varying run time and/or column length to improve resolution and/or analysis speed.

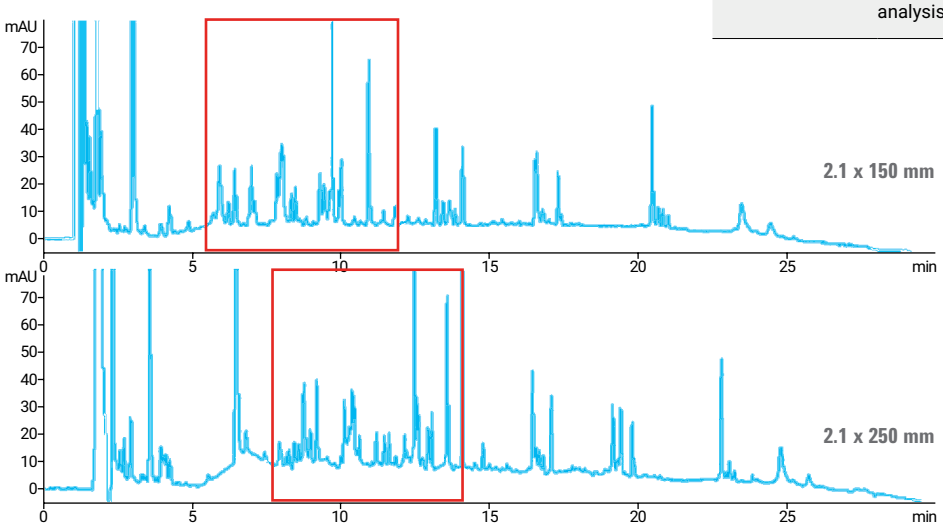

Figure 11. Effect of column length on resolution, a peptide mapping comparison using a myoglobin tryptic digest (Agilent P/N 651750-902). Areas highlighted in red indicate equivalent areas of separation to emphasize resolution and peak shape. The separations were performed using an Agilent AdvanceBio Peptide Mapping Column, 2.1 x 150 mm (Agilent P/N 651750-902), on an Agilent 1260 Infinity Bio-inert Quaternary LC system using a water (1.0 % TFA)/ACN (0.08% TFA) linear gradient, 10-60 % B in 30 minutes, 0.3 mL/min, 45 °C.

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Peptide Mapping Characterizations by Mass Spectrometry

The use of RPC with mass spectrometry has made this combined technique the method of choice for characterizing peptides and peptide maps. For example, in the biopharmaceutical industry, establishing and monitoring the sequence identity of a therapeutic target is critical, and the stability of a protein biologic is an important aspect of therapeutic development for monitoring modifications such as oxidation, reduction, glycosylation, and truncation. MS can be used as a non-regulatory purity test for establishing the genetic stability of a product throughout its lifecycle.

Peptides are analyzed by mass spectrometry by direct infusion of the isolated peptides – or by the use of on-line LC/MS for structure analysis – and then correlated to the protein amino acid sequence. The identified peptides thus confirm the specific amino acid sequences covered by the peptide map, as well as the identity of the protein. Mass spectrometric peptide mapping is applied to:

- Confirm the identity of a specific protein.
- Get detailed characterization of the protein, such as confirmation of N-terminal and C-terminal peptides, high sequence coverage peptide maps, amino acid substitutions, etc.
- Screen and identify post translational modifications. (e.g. glycosylations, disulfide bonds, N-terminal pyroglutamic acid, methionine and tryptophan oxidation, etc.)

In general, types of MS analysis include electrospray and MALDI-TOF-MS, as well as fast-atom bombardment (FAB). Tandem MS has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. Using electrospray ionization (ESI) or MALDI-MS, proteolytic peptides can be ionized intact into the gas phase and their masses accurately measured. Most peptide separations are performed on electrospray ionization (ESI) LC/MS instruments due to the convenience of LC coupling and better quality of tandem mass spectra for confident protein identification. For example, a quadrupole time-of-flight (QTOF) MS instrument often gives more structural information, especially for larger peptides, due to its high resolving power and mass accuracy.

Based on MS information, proteins can readily be identified in which measured masses are compared to the predicted values derived from the intact protein or protein database to elucidate mass and sequence coverage information. The goal of a characterization of a protein through peptide mapping is to reconcile and account for at least 95 % sequence coverage of the theoretical composition of the protein structure. figure 12 is an example of a highly optimized peptide map of erythropoietin protein (EPO) digest using ESI-MS. The optimized chromatographic conditions and MS parameters have enabled 100 % sequence coverage and highlight a well characterized peptide mapping separation.

Use Agilent Peptide Mapping standards to ensure your system is operating at peak performance for the application.

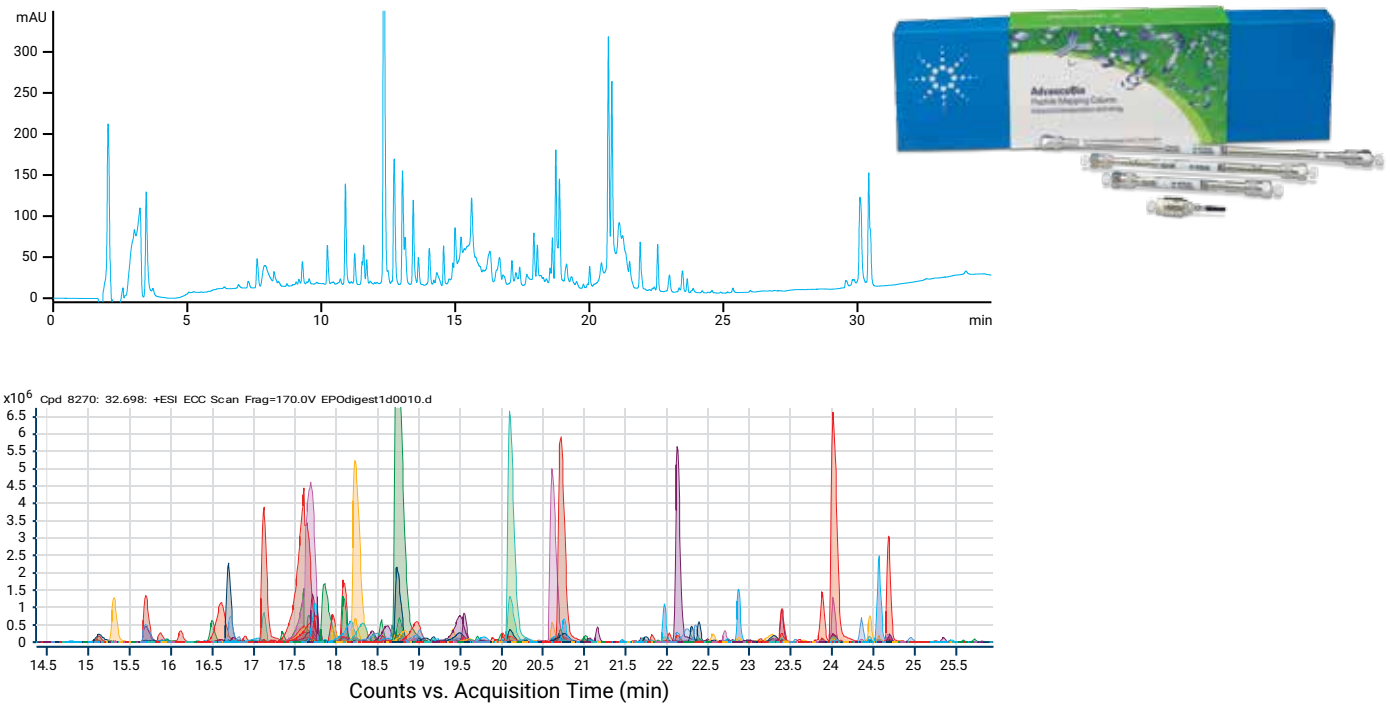


Figure 12. The top chromatogram displays a fully optimized EPO digest peptide mapping separation performed on a 2.1 x 150 mm AdvanceBio Peptide Mapping column. The bottom chromatogram shows the qualitative analysis (using a molecular feature extractor) for sequence coverage generated by on an Agilent Q-TOF.

Ordering Information

For peptide mapping, Agilent recommends:

AdvanceBio Peptide Mapping – the first choice for most applications

Description	Part Number	Fast Guard Part Number
54.6 x 150 mm, 2.7 µm	653950-902	850750-911
53.0 x 150 mm, 2.7 µm	653950-302	853750-911
52.1 x 250 mm, 2.7 µm	651750-902	851725-911
52.1 x 150 mm, 2.7 µm	653750-902	
52.1 x 100 mm, 2.7 µm	655750-902	

*Fast Guards extend column lifetime without slowing down the separation or affecting resolution.

Peptide Quality Control Standard

Use Agilent's 10-Peptide Quality Control Standard, the same standard Agilent uses to QC its columns, to evaluate your column performance over its lifetime. It can be used for HPLC or LC/MS. Approximately 20 injections per vial.

Description	Part Number
Peptide quality control standard, 71 µg in 2 mL vial	85190-0583

AdvanceBio Peptide Plus – advance your confidence for protein/peptide analysis

Description	Part Number
4.6 x 150 mm, 2.7 µm	693975-949
3.0 x 150 mm, 2.7 µm	693975-349
2.1 x 250 mm, 2.7 µm	693775-949
2.1 x 150 mm, 2.7 µm	695775-949
2.1 x 50 mm, 2.7 µm	699775-949
4.6 mm guard (3/pk)	820750-940
3.0 mm guard (3/pk)	823750-952
2.1 mm guard (3/pk)	821725-954
HSA Peptide Standard Mix	G2455-85001

AdvanceBio EC-C18 – for samples susceptible to stainless steel on-column artifacts

Description	Part Number
AdvanceBio EC-C18 2.7 µm, 2.1 x 150 mm PEEK lined	673775-902
AdvanceBio EC-C18 2.7 µm, 2.1 x 100 mm PEEK lined	675775-902
AdvanceBio EC-C18 2.7 µm, 2.1 x 50 mm PEEK linedm	679775-902

Peptide sample preparation for mass spec analysis, intelligently automated

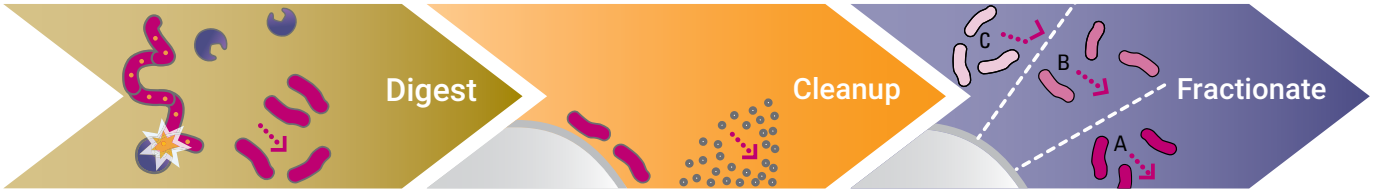
Manual sample preparation of peptides is a time-consuming process. If you are doing peptide mapping applications on MS, you are likely looking for increased throughput. And you are going to be reliant on a highly reproducible end-to-end workflow to ensure your results are consistent. AssayMAP transforms digestion, cleanup, and fractionation workflows to enable previously unachievable precision and throughput:

- Improved reproducibility, due to decreased human error – <5 % CVs
- Increased throughput – up to 384 samples each day
- Significantly reduces hands-on time – freeing up scientists to do analytical work
- Faster method development – the automated platform enables you to quickly optimize methods

AssayMAP Peptide Sample Prep Solution is based on the powerful combination of miniaturized, packed bed chromatography, the state-of-the-art Bravo Liquid Handling Platform and a simple, applications-based user interface that creates an open access environment for both novices and experienced users and simplifies the most challenging sample preparation workflows.

AssayMAP peptide sample prep solution

For Mass Spec Analysis



Digestion:

- In-solution digestion with user-supplied reagents
- Parallel process up to 4x96-well plates
- 1 manual pipetting step

Benefits:

- Reduce user variability
- Improve throughput and reproducibility

Cleanup:

- Quantitative separation method using reversed-phase cartridges
- Parallel process 1x96-well plate

Benefits:

- 10 µL elution equals short dry down times or "dilute and shoot" method
- Process control – every sample is treated identically

Fractionation:

- Strong cation exchange (SCX) cartridges generate up to 6 fractions to simplify the sample using step-wise elution with pH or salt
- Parallel process 1x96-well plate

Benefits:

- Increases LC/MS throughput by taking fractionation offline, reducing long LC gradient times
- Powerful enrichment tool for simplifying samples and isolating target peptides prior to analysistreated identically

Total workflow benefit:

- User interfaces for workflows are standardized for ease-of-use and linked for workflow integration.
- AssayMAP reduces the need for sample replicates and requires fewer repeated samples.

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Achieve total workflow reproducibility with Agilent AssayMAP solution for sample prep before mass spec analysis

The AssayMAP Peptide Sample Prep Solution was used to digest 64 replicates each of two sample types: BSA in urea and guanidine HCL. The samples were cleaned using AssayMAP reversed-phase cartridges and analyzed using an Agilent AdvanceBio Peptide Mapping column, Agilent 1290 Infinity LC, and an Agilent 6550 iFunnel Q-TOF mass spectrometer. The experiment was repeated on day two to examine reproducibility. %CV was determined for 25 peptides within each sample as shown in Table 1. The different %CV bins are shown. Illustrating the contributions of the total average %CV. To further showcase the reproducibility, peak area for representative peptides are shown in figure 13.

The AssayMAP sample prep took about four hours per day, with only two hand-on hours per day. Manual sample prep for the same workflow would take about eight hours per day, with four hands-on hours each day.

Total workflow CVs were <4%. The full workflow included AssayMAP Peptide Sample Prep system, an Agilent AdvanceBio Peptide Mapping Column, the 1290 Infinity LC System, and an Agilent 6550 iFunnel Q-TOF mass spectrometer.

For more details about this application, see Agilent publication 4991-2474EN.

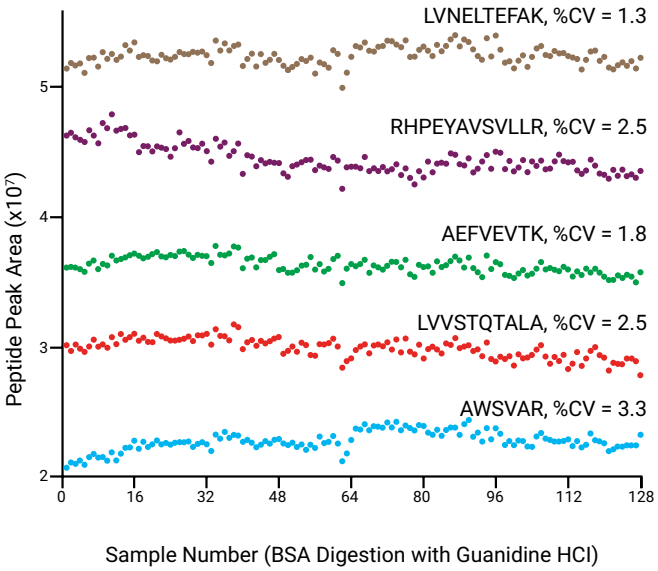

Figure 13. Scatter plots showing peak area of 4 peptides over 2 days.

Table 1. – %CV by day with different %CV bins.

	Urea (n=64, 62)		Guanidine HCl (n=64, 62)	
25 Peptides	**Day 1**	**Day 2**	**Day 1**	**Day 2**
Average Peak Area %CV	3.3	3.7	2.3	2.6
Peptides with %CV<5	23	21	25	23
Peptides with 5>%CV<10	2	3		1
Peptides with %CV>10		1		1

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Application Note

Peptide Mapping

Agilent

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Robust and Reliable Peptide Mapping

The Agilent 1290 Infinity II Bio LC System as the new platform for UV and MS-based primary structure and PTMs analysis of mAbsm

Author

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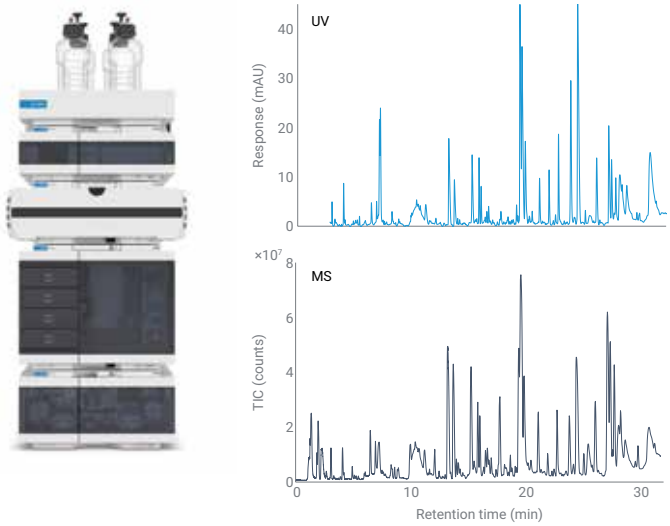
Abstract

The Peptide mapping is the gold standard for elucidating the primary structure of monoclonal antibodies (mAbs). However, the key to successful peptide mapping is a robust and reliable LC system for high-quality peptide separation. In this application note, we present the Agilent 1290 Infinity II Bio LC as the system of choice for peptide mapping. Recreation of a published comprehensive peptide-mapping method for the NISTmAb showed exceptionally good relative retention time deviations below 0.1% even for very shallow gradients. Further method development decreased the total run time by 60%, keeping the excellent relative standard deviations and peak capacity values. Additionally, the 1290 Infinity II Bio LC was connected directly to the Agilent 6545XT AdvanceBio LC/Q-TOF as an example of a method development setup, facilitating easy method transfer throughout the biopharmaceutical production chain.



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Introduction

Peptide mapping is a widely used technique for analyzing the primary structure and post-translational modifications (PTMs) of biopharmaceuticals in today's industrial biotechnology. Typically, bottom-up approaches are employed by denaturation, alkylation, and digestion of a mAb. Subsequently, resulting peptides are separated by HPLC or UHPLC using reversed-phase or even hydrophilic interaction liquid chromatography, in some cases. Detection is either carried out with mass spectrometry (MS), to identify a drug substance, or ultraviolet (UV) absorbance in quality control (QC) environments, by comparison of the chromatographic profile to a reference map. Peptide mapping can be used as part of the acceptance criteria for the evaluation of biological products, which is described in ICH Guideline Q6B.[1] By using LC/MS or UV, changes in the peptide map—for example, increased oxidation or deamidation,[2] the appearance of new sequence variants,[3] or changes in the glycan composition[4]—can be evaluated. Therefore, precision and robustness, especially when using a UV detector, are of utmost importance to release and develop safe and potent biopharmaceuticals.

This application note showcases the new 1290 Infinity II Bio LC as a novel platform for peptide mapping. Exploiting the high-precision, binary Agilent 1290 Infinity II Bio High-Speed Pump and a biocompatible, iron-free flow path, the system is especially suited to biomolecules like peptides, proteins, and metabolites.

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System coupled to the Agilent 6545XT AdvanceBio LC/Q-TOF comprised the following modules:

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116A) equipped with a Standard Flow Quick Connect Bio Heat Exchanger (G7116-60071) and two Agilent Thermal Equilibration Devices (G7116-60013)
- Agilent 1290 Infinity II Variable Wavelength Detector (VWD) (G7114B), equipped with a Bio Micro Flow Cell VWD, 3 mm, 2 µL, RFID.
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6545XT)

Software

- Agilent MassHunter workstation data acquisition (B.09.00)
- Agilent MassHunter Qualitative Analysis (B.10.00)
- Agilent MassHunter BioConfirm (B.10.00)

Columns

- Agilent AdvanceBio Peptide Mapping, 2.1 × 250 mm, 2.7 µm (part number 651750-902)
- Agilent AdvanceBio Peptide Mapping Fast Guards, 2.1 × 5 mm, 2.7 µm (part number 851725-911)
- Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm (part number 959759-902)
- Agilent ZORBAX RRHD Eclipse Plus C18 Fast Guards, 2.1 × 5 mm, 1.8 µm (part number 821725-901).

Chemicals

LC-grade acetonitrile, ammonium bicarbonate, tris (2-carboxyethyl) phosphine, and 2-iodoacetamide were purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Formic acid was purchased from VWR (Darmstadt, Germany). Trypsin (porcine, mass spectrometry-grade) was obtained from G-Biosciences (St. Louis, USA).

Sample preparation

0.8 mg of the Agilent-NISTmAb (part number 5191-5744) in 100 µL ammonium bicarbonate (100 mM) was denatured and reduced by the addition of 2 µL of tris(2-carboxyethyl)phosphine (TCEP, 200 mM) and incubated at 60 °C for 1 hour. After the alkylation with 4 µL of 2-iodoacetamide (IAM, 200 mM, 1 hour at RT), quenching of excess IAM with 2 µL of TCEP (1 hour at RT), and subsequent dilution with 0.8 mL of 25 mM ammonium bicarbonate, the enzyme trypsin was added (20:1, NISTmAb to trypsin w/w). After the overnight digestion at 37 °C, the pH of the resulting suspension was decreased below pH 4 by the addition of 2 µL of formic acid.

Results and Discussion

Tryptic digests of protein biopharmaceuticals such as mAbs present a highly complex mixture of numerous peptides. To determine and analyze the primary structure of these biopharmaceuticals, very long and shallow gradients are deployed, which can range up to several hours' run time, putting high demands on the instrumentation. Showcasing the suitability of the 1290 Infinity II Bio LC for this challenging analysis, we chose to recreate an LC/UV and MS method previously published by the National Institute of Standards and Technology (NIST) for the tryptic digest of the NISTmAb.[5] For this, the AdvanceBio Peptide Mapping column with a length of 250 mm was used with a total method run time of 2.5 hours (Method A, Table 1). Additionally, a second LC method was developed to decrease run time by exploiting the sub-2 µm particles of the ZORBAX RRHD Eclipse Plus column (Method B, Table 2). Figure 1 shows the chromatograms of both methods detected with the Agilent 1290 Infinity II Variable Wavelength Detector (VWD).

Similar peptide patterns can be observed in both chromatograms. However, most analytes could be eluted after 30 minutes with Method B compared to 80 minutes with the originally published NIST Method A. As a consequence, the total run time could be decreased by 60%. To systematically evaluate the precision and robustness of the 1290 Infinity II Bio LC, eight peaks were chosen in both methods. Subsequently, retention time standard deviations were calculated based on 10 consecutive injections (Figure 2).

Methods A and B both show relative retention time deviations below 0.1%, displaying the exceptional performance of the 1290 Infinity II Bio High-Speed Pump even at very shallow gradient slopes of 0.39 and 0.98% B/min, respectively. To evaluate the comprehensive separation character of the LC methods, 4σ peak capacities were calculated as a measure of the quality of the separation. Due to the extended run time of Method A, the corresponding peak capacity value was the highest with 428. However, combining the outstanding average RSD of 0.039% with a high peak capacity of 348, Method B stands as a serious alternative, with a greatly decreased run time compared to the published peptide-mapping method provided by the NIST.

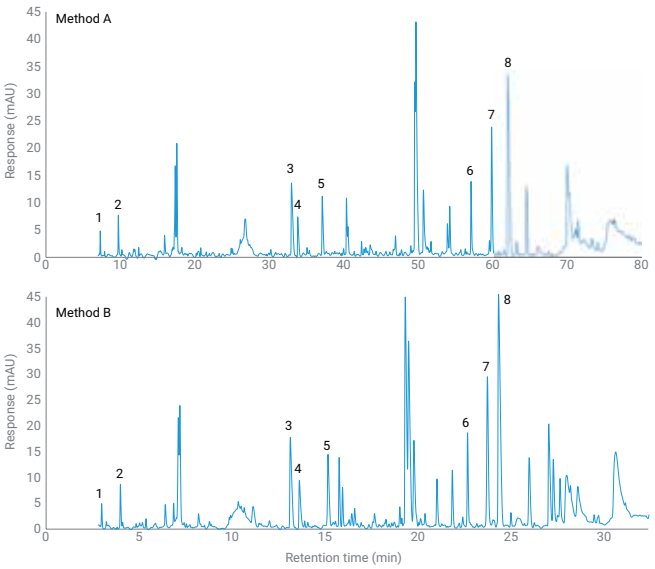
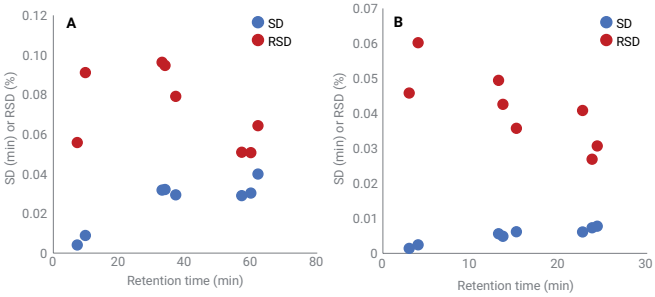


Figure 1. Chromatograms of a tryptic digest of the NISTmAb separated by the Agilent 1290 Infinity II Bio LC with Methods A and B. Eight peaks were picked for subsequent retention time precision and peak capacity calculations.



Method	Gradient Slope (%B/min)	Ø SD (min)	Ø RSD (%)	PC,4σ (–)
Method A	0.39	0.026	0.073	428
Method B	0.98	0.005	0.039	348

Figure 2. Absolute (SD) and relative (RSD) retention time precision values of Methods A and B acquired with the Agilent 1290 Infinity II Bio LC. The gradient slope and peak capacity are depicted in the table.

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A typical workflow for peptide mapping in a biotechnological environment uses a UV and MS detector in sequence. With this setup, method development can be done with both detectors, using the MS for the identification of peptides. After establishing the method, the analysis can be easily transferred to the UV detector for high-throughput analysis in a QC environment. To demonstrate this case, the 1290 Infinity II Bio LC was directly connected to the 6545XT AdvanceBio LC/Q-TOF, and the tryptic digest of the NISTmAb was reanalyzed with Method B. MS detection was carried out in iterative MS/MS mode as shown in Table 3. Resulting chromatograms are depicted in Figure 3.

Even though no special measures were taken against peak broadening the resolution remained more than sufficient for reliable MS detection. Identification and confirmation of the primary structure of mAbs can conveniently be carried out by using the AgilentMassHunter BioConfirm software. Comparing the identified peptides on the MS and/or MS/MS level with a reference sequence of the biopharmaceutical of choice, PTMs can be analyzed and quantified relatively. With this approach, the so-called PENNY peptide (GFYPSDIAVEWESNGQPENNYK)6 and the corresponding deamidated isoform could be identified. The PENNY peptide is part of the conserved region (Fc) shared by nearly all human or humanized mAbs, which can be used as decent indicator for induced deamidation. A zoomed-in view of these peptides is depicted in Figure 4.

After identifying the peptides, relative quantification can also be carried out by UV detection in this case, owing to the great separation capability of the optimized peptide-mapping Method B in combination with the excellent retention time precision of the 1290 Infinity II Bio LC.

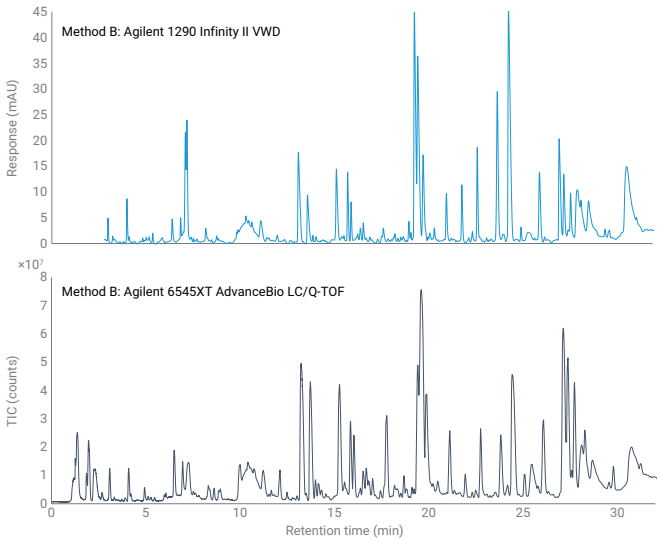

Figure 3. Chromatograms of a tryptic digest of the NISTmAb detected in sequence with the Agilent 1290 Infinity II VWD equipped with the biocompatible micro flow cell (upper) and the Agilent 6545XT AdvanceBio LC/Q-TOF (lower).

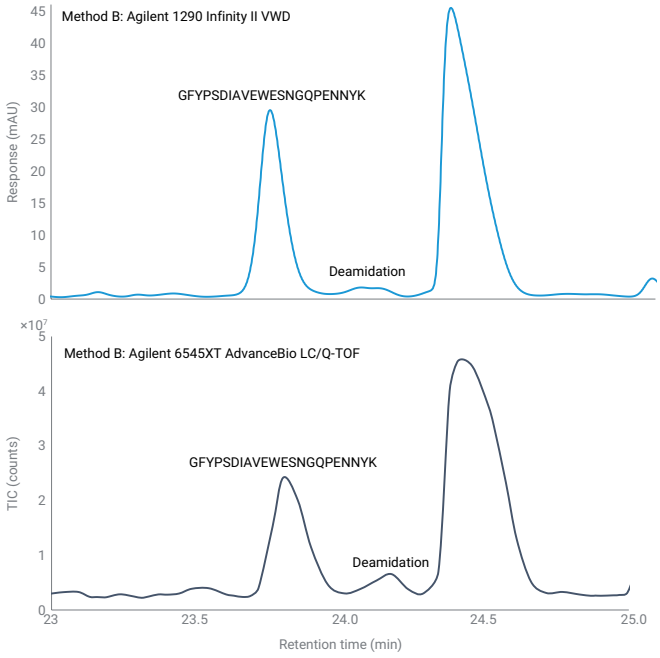

Figure 4. Magnified view of the previous chromatogram to highlight the separation of the PENNY peptide (GFYPSDIAVEWESNGQPENNYK) and corresponding deamidated isoform.

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Conclusion

Critical quality attributes (CQA) such as sequence or glycosylation variants, oxidation, and deamidation can be analyzed by peptide mapping. However, it is mandatory that the used method and instrumentation are robust and reliable to deliver the best results possible. In this application note, we showed that the new 1290 Infinity II Bio LC can live up to these high expectations. Retention time precision deviations below 0.1% could be routinely achieved by recreating a comprehensive published peptide-mapping method for the NISTmAb. By optimizing this method, the total run time could be decreased by 60% without compromising the excellent precision and separation quality thanks to the 1290 Infinity II Bio High-Speed Pump. To present the usability in a method development environment, it was shown that the capability to connect the 1290 Infinity II Bio LC directly to a 6545XT AdvanceBio LC/Q-TOF enables the straightforward method transfer to a high-throughput QC environment. To sum up, the 1290 Infinity II Bio LC can be the new platform for UV and MS-based primary structure and PTMs analysis of mAbs.

References

1. ICH HARMONISED TRIPARTITE GUIDELINE PHARMACEUTICAL DEVELOPMENT Q6B. (1999). Available at: https://database.ich.org/sites/default/files/Q6B Guideline.pdf.
2. Li, X. *et al*. High Throughput Peptide Mapping Method for Analysis of Site Specific Monoclonal Antibody Oxidation. *J. Chromatogr. A* **2016**, *1460*, 51–60.
3. Li, Y. *et al*. Characterization of Alanine to Valine Sequence Variants in the Fc Region of Nivolumab Biosimilar Produced in Chinese Hamster Ovary Cells. *MAbs* **2016**, *8*, 951–960.
4. Wang, T. *et al*. Application of a Quantitative LC-MS Multiattribute Method for Monitoring Site-Specific Glycan Heterogeneity on a Monoclonal Antibody Containing Two N-Linked Glycosylation Sites. *Anal. Chem*. **2017**, *89*, 3562–3567.
5. Mouchahoir, T.; Schiel, J. E. Development of an LC-MS/MS Peptide Mapping Protocol for the NISTmAb. *Anal. Bioanal*. Chem. **2018**, *410*, 2111–2126.High Stakes.
6. Chelius, D.; Rehder, D. S.; Bondarenko, P. V. Identification and Characterization of Deamidation Sites in the Conserved Regions of Human Immunoglobulin Gamma Antibodies. *Anal. Chem*. **2005**, *77*, 6004–6011.

Application Note

Peptide Mapping

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Separation of Deamidated Peptides with an Agilent AdvanceBio Peptide Plus Column

Authors

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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

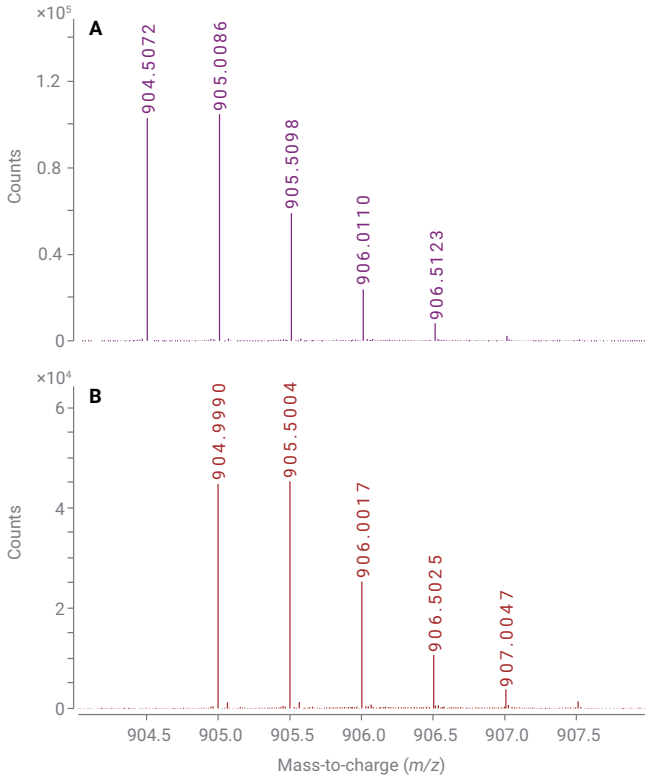

Figure 1. Mass spectrum of VVSVLTVLHQDWLNGK (A) and a deamidated variant of that peptide (B), showing the overlap between mass spectra.

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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)

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

LC 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

MS conditions

Parameter	Agilent 6546 Q-TOF
Column	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 voltage	1255 V
Skimmer voltage	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
Data analysis	Agilent BioConfirm software B.08

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.

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.

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.

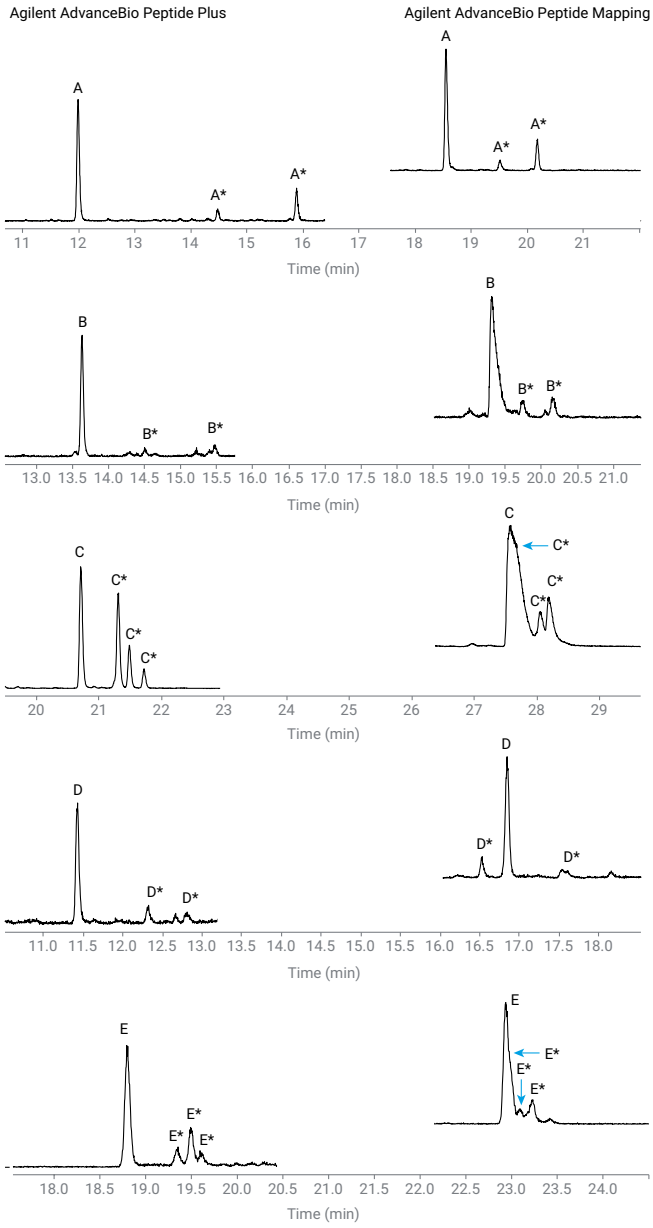

Figure 2. Separation of peptides and their deamidated variants (indicated by *) on the Agilent AdvanceBio Peptide Mapping column (elevated and eluting later) and the Agilent AdvanceBio Peptide Plus column (eluting earlier) under the same conditions with 0.1% formic acid mobile phase modifier.

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 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.

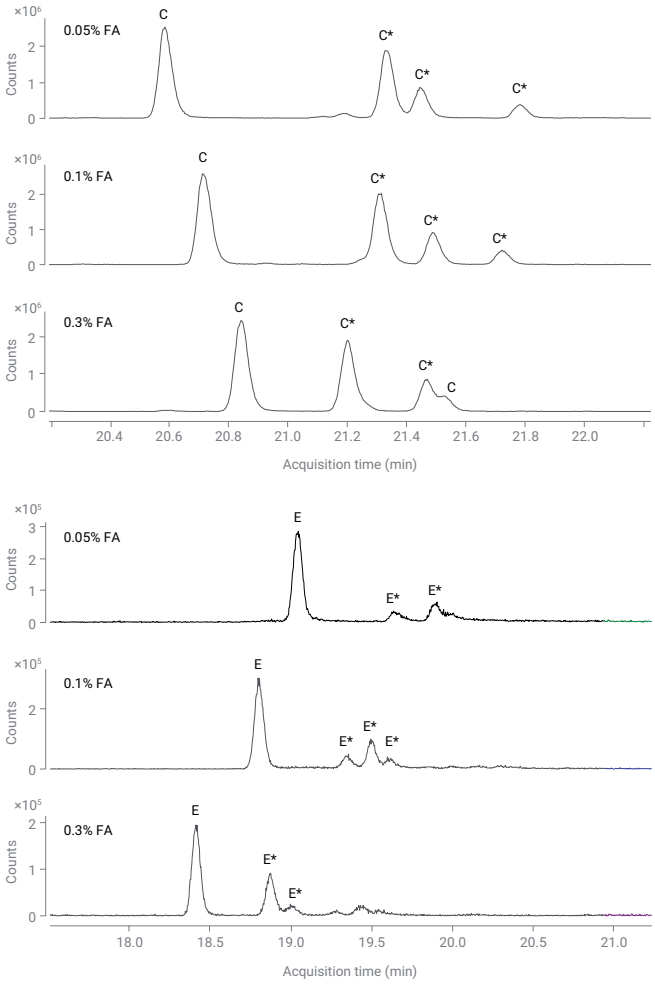

Figure 3. Separation of peptides on Agilent AdvanceBio Peptide Plus with increasing concentrations of formic acid mobile phase modifier. Selectivity for deamidated peptide variants over their native forms is maximized at lower concentrations.

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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]

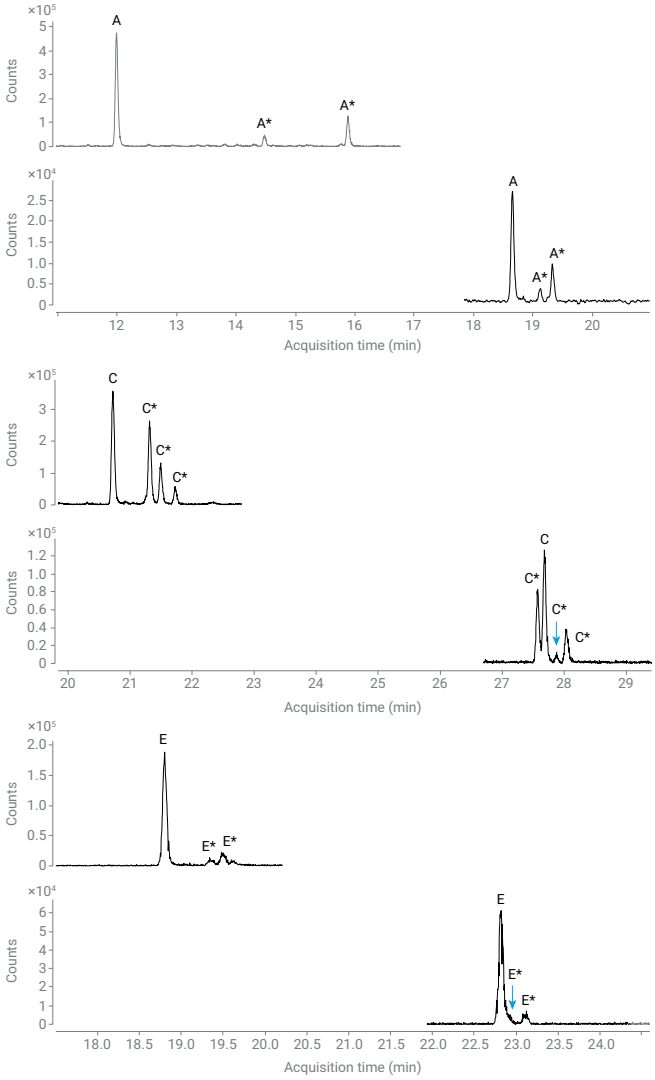

Figure 4. Separation of peptides and their deamidated variants on Agilent AdvanceBio Peptide Plus with 0.1% formic acid mobile phase modifier compared to 0.1% TFA.

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.

Application Note

Peptide Mapping

Agilent

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Analysis of a Synthetic Peptide and Its Impurities

Using mass spectrometry compatible mobile phases with an Agilent AdvanceBio Peptide Plus column

Authors

Andrew Coffey and

Veronica Qin

Agilent Technologies Inc.

Abstract

Conventionally, chromatographic peptide separation with UV detection is performed using C18 reversed-phase HPLC columns and mobile phases containing trifluoroacetic acid (TFA) as ion pair reagent. However, although TFA provides improved resolution, it can suppress the mass spectrometry (MS) signal. Formic acid (FA) is a preferred ion pair reagent for MS detection but can result in suboptimal separation with many traditional C18 columns. This application note presents the use of the Agilent AdvanceBio Peptide Plus column to separate synthetic peptide impurities with MS-compatible FA as a mobile phase modifier.

Introduction

Most peptide drugs are manufactured using solid-phase peptide synthesis. Synthetic peptide-related impurities can come from raw materials, manufacturing processes, or be generated by degradation during manufacturing or storage.1 Traditionally, peptide separation is achieved using reversed phase columns with trifluoroacetic acid (TFA) as a mobile phase modifier and UV as a detector. However, TFA is not ideal for mass spectrometry (MS) since it can suppress the MS signal.

To identify the impurity peaks in an LC/MS method, formic acid (FA) is a preferred mobile phase modifier but results in suboptimal separation with traditional C18 columns.

TFA (pKa ~0.23) can lower the pH to protonate residual (incompletely alkylated or endcapped) silanol sites on the stationary phase surface, leaving no negative charge to interact with positively charged peptides facilitating good peak shape. In addition, TFA anions form an ion pair with positively charged peptides, increasing their hydrophobicity and increasing their retention time. By contrast, FA (pKa ~3.77) is a weaker acid than TFA and cannot lower the pH enough to protonate all the silanol sites so the interaction between the silanols and peptides is not masked completely. This often leads to broader peaks, increased tailing, and overall lower resolution and peak capacity compared with using TFA as a modifier.

The Agilent AdvanceBio Peptide Plus stationary phase possesses a hybrid, positively charged surface, and can provide better peak shape and separation with FA as a modifier than traditional C18 columns. This note describes an LC method to separate synthetic peptide impurities using FA as a mobile phase modifier that can be run with either UV or MS detection, therefore making method transfer between LC/UV and LC/MS easier. Both LC/MS and LC/MS/MS are used to positively identify some of the impurities found in the sample, synthetic bivalirudin, Figure 1.

Bivalirudin is a 20 amino acid synthetic peptide that reversibly inhibits thrombin.

Quality control of the synthetic peptide requires the identification and determination of impurities. The amino acid sequence of bivalirudin (FPRPGGGGNGDFEEIPEEYL) has a monoisotopic mass of 2178.9858 Da.

Therefore, using LC/MS can accurately determine the mass of the peptide, but by also using MS/MS analysis it is possible to confirm the sequence through the predicted fragmentation pattern, as shown in Table 1.

Table 1.

Seq	No.	b	y	No. (+1)
F	1	148.076	2179.993	20
P	2	245.129	2032.925	19
R	3	401.230	1935.872	18
P	4	498.282	1779.771	17
G	5	555.304	1682.718	16
G	6	612.325	1625.697	15
G	7	669.347	1568.675	14
G	8	726.368	1511.654	13
N	9	840.411	1454.632	12
G	10	897.433	1340.589	11
D	11	1012.460	1283.568	10
F	12	1159.528	1168.541	9
E	13	1288.571	1021.472	8
E	14	1417.613	892.430	7
I	15	1530.697	763.387	6
P	16	1627.750	650.303	5
E	17	1756.793	553.250	4
E	18	1885.835	424.208	3
Y	19	2048.899	295.165	2
L	20	2161.983	132.102	1

H-D-Phe-Pro-Arg-Pro-Gly-Gly-Gly-Gly-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH

Figure 1. The amino acid sequence of synthetic bivalirudin.

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher.

Sample preparation

Aged synthetic peptide bivalirudin trifluoroacetate hydrate was purchased from Selleckchem and reconstituted with 0.1 % FA in water to 1 mg/mL.

Instrumentation

For HPLC experiments, an Agilent 1290 Infinity LC was used comprising:

- Agilent 1290 Infinity binary pump (G4220A)
- Agilent 1290 Infinity autosampler (G4226A)
- Agilent 1290 Infinity thermostatted column compartment (G1316C)
- Agilent 1260 Infinity II diode array detector (DAD) (G7115A)

For LC/MS experiments, the same 1290 Infinity LC configuration was used with an Agilent 6545XT AdvanceBio LC/Q-TOF detector.

Data processing

LC/UV data was processed using Agilent OpenLab 2.2 CDS. LC/MS data was processed using Agilent MassHunter BioConfirm B.08.00 software. MS/MS spectra were used to confirm the identities of the synthetic peptides and their impurities.t

Method conditions

Parameter	HPLC Conditions
Column	Agilent AdvanceBio Peptide Plus, 2.1 × 150 mm (p/n 695775-949)
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 17 2 17 22 37 24 95 26 95 26.1 17
Post time	5 minutes
Injection volume	5 µL (UV); 1 µL (MS)

Conditions

Parameter	Value
Instrument	Agilent 6545XT AdvanceBio LC/Q-TOF
Source	Dual Agilent Jet Stream
Gas Temperature	350 °C
Drying Gas Flow	10 L/min
Nebulizer Gas	30 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 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* 100 to 1,700 (MS); *m/z* 50 to 1,700 (MS/MS)
MS Scan Rate	8 spectra/s
MS/MS Scan Rate	3 spectra/s
Acquisition Mode	Positive, extended dynamic range (2 GHz)
Collision Energy	3.6 × (*m/z*)/100 − 4.8

Results and discussion

Figure 2 shows the separation profile of an aged bivalirudin peptide sample using FA as a mobile phase modifier with UV detection. LC/MS/MS is used to identify several major impurity peaks in the profile, as shown in Table 2, with very low mass error.

Common impurities include deletion sequences (where an individual amino acid is missing), the presence of incompletely removed protecting groups or modifications of the peptide during removal of the protecting groups, loss of water and, in this particular peptide sequence, Asn is prone to deamidation, which could occur during manufacture or upon storage.

A total of five peaks were selected to illustrate the techniques used for identification using a combination of LC/MS and LC/MS/MS.

Table 2. Peak identification of aged bivalirudin peptide and major impurities.

Peak	Mass (Da)	Peak ID	Target mass (Da)	Mass error (ppm)
1	2,049.9467	Deletion of Glu	2,049.9432	1.71
2	2,178.9894	Product	2,178.9858	1.65
3	2,121.9663	Deletion of Gly	2,121.9644	0.90
4	2,160.9764	Loss of H_2O	2,160.9705	2.73
5	2,179.9742	Deamidation	2,179.9698	2.02

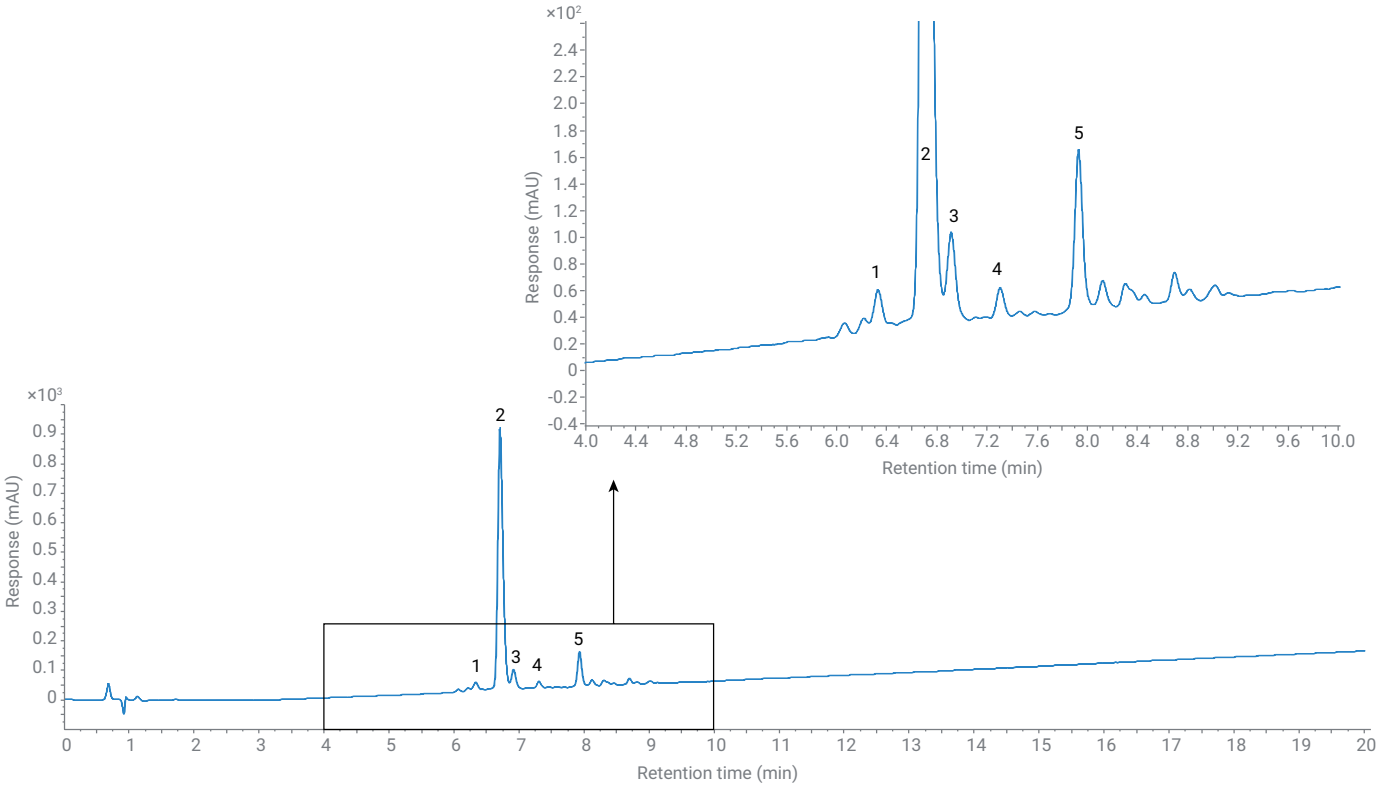

Figure 2. LC/UV chromatogram of synthetic bivalirudin. A zoomed baseline region of synthetic bivalirudin is shown.

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The major component of the LC/UV chromatogram, Peak 2, gave an MS spectrum shown in Figure 3. This corresponds to [M + 2H]2+ and [M + 3H]3+ m/z which, when deconvoluted, gives the mass of 2178.9894 corresponding to the full-length peptide sequence of bivalirudin, FPRPGGGGNGDFEEIPEEYL.

A similar approach is used to identify the earlier eluting impurity, Peak 1. In this case, a similar MS spectrum is obtained (Figure 4A), however, the mass of the impurity is 2049.9467 following deconvolution. The mass difference is –129 Da indicative of the loss of glutamic acid. By closer inspection of the LC/MS/MS spectrum, it is possible to identify the position of the missing Glu residue (Figure 4B).

The BioConfirm software has identified the b15 and y4 fragments for FPRPGGGGNGDFEEIPEYL, a 19 amino acid sequence, indicating that the sequence is missing a glutamic acid at position 17 or 18.

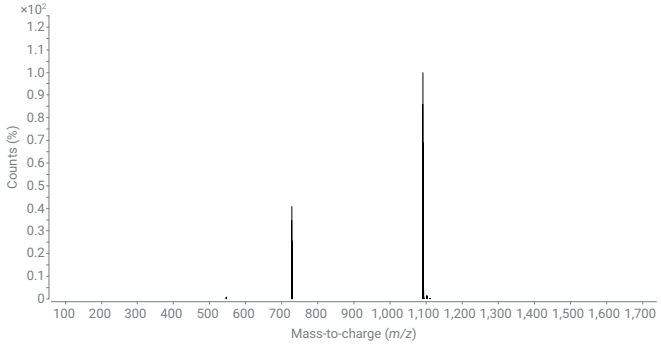

Figure 3. MS spectrum of the main product (peak 2)

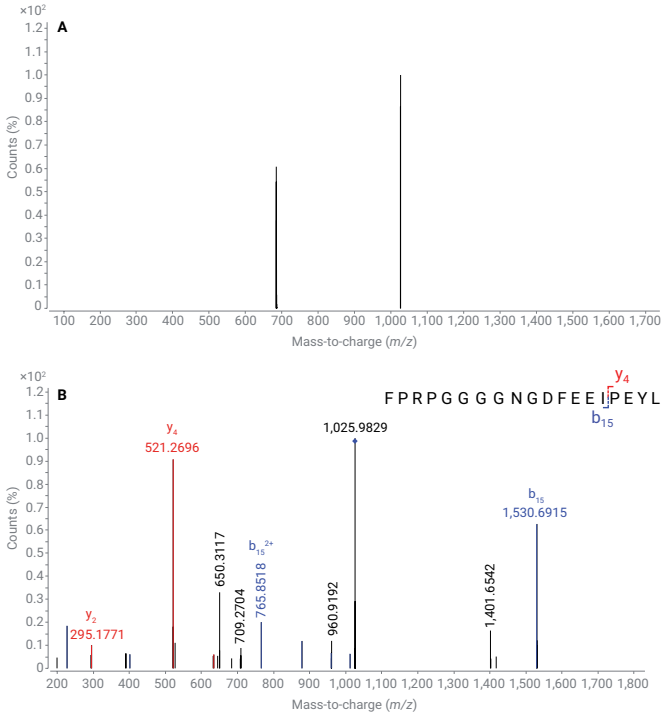

Figure 4. (A) MS spectrum of impurity, peak 1.
(B) MS/MS spectrum of impurity, peak 1.

Analysis of impurity peak 3 gives a mass difference of −57 Da, indicative of a missing glycine (Figure 5). Impurity peak 4, meanwhile has a mass difference of 18 indicative of dehydration through loss of H2O (MS spectrum not shown).

Finally, analysis of impurity peak 5 gives a mass difference of +1 Da, indicative of deamidation (Figure 6A). A closer look at the MS/MS data for this impurity reveals the software has identified that Asn at position 9 (N) has been converted to Asp (D) through deamidation.

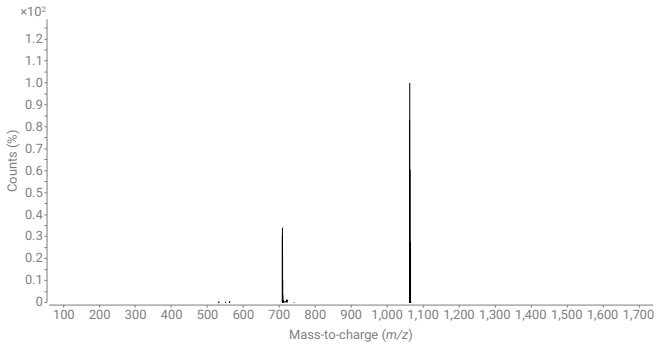

Figure 5. MS spectrum of impurity, peak 3

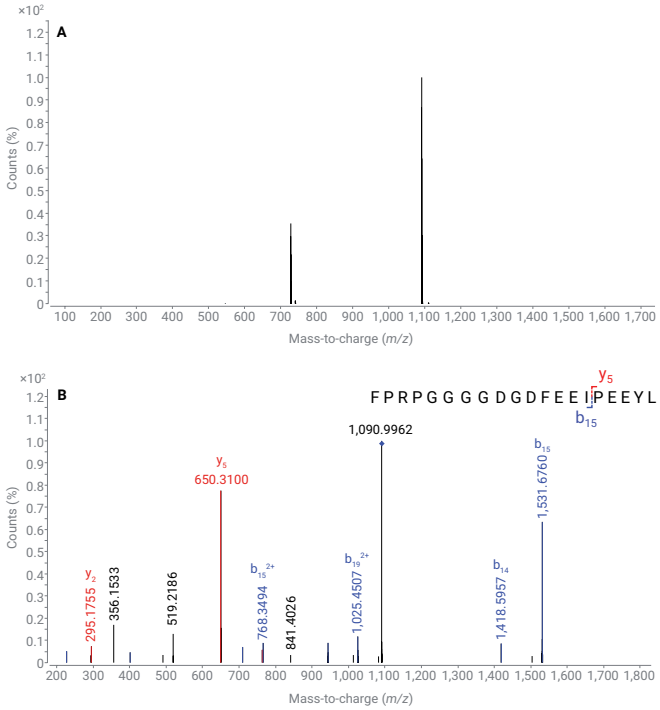

Figure 6. (A) MS spectrum of impurity, peak 5.
(B) MS/MS spectrum of impurity, peak 5.

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Conclusion

In this study, an Agilent AdvanceBio Peptide Plus column was used with formic acid as a mobile phase modifier to analyze a synthetic peptide and its impurities. The method employed can be easily transferred between LC/UV and LC/MS.

References

1. Eggen, I. *et al*. Control Strategies for Synthetic Therapeutic Peptide APIs Part III: Manufacturing Process Considerations. *Pharm. Technol*. **2014**, *38(5)*.

Application Note

Peptide Mapping

Agilent

Trusted Answers

High-Resolution Mapping of Drug Conjugated Peptides in an ADC Digest

Peptide map comparison of mAb and drug conjugated mAb

Authors

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Agilent Technologies Inc.

Introduction

Currently, antibody drug conjugates (ADCs) are prime protein drugs for biotherapeutic use. When a cytotoxic drug is conjugated to a biotherapeutic monoclonal antibody (mAb), there are several options for the conjugation site. As part of the characterization of ADCs, it is important to be able to identify these conjugation sites. This can be done using peptide mapping. The specificity of the enzyme to cleave the mAb into peptide fragments results in different cleavage patterns, and, hence, peptide fragments, around the conjugation site. High-resolution peptide mapping can be used to identify peptides that are produced as a result of conjugation of the cytotoxic drug. This Application Note demonstrates the use of the Agilent AdvanceBio Peptide Mapping Column and an Agilent 1290 Infinity LC system for ADC peptide mapping analysis. For analysis using UV, it is important to have high resolution to identify the individual peptides, therefore, the method was developed with an optimized flow rate and gradient time for increased peak capacity. Comparison of a peptide map of Trastuzumab biotherapeutic mAb and its cytotoxic drug conjugate, ADC, revealed the peptide map differences corresponded to drug-conjugated peptides. These hydrophobic peptides were resolved on the AdvanceBio Peptide Mapping Column.

Materials and Methods

Therapeutic proteins, ADC, and Trastuzumab were purchased from a local pharmacy. All chemicals and solvents were HPLC grade. Tryptic digestion of mAbs was carried out as described elsewhere1. Before the digestion of the mAbs with trypsin, the disulfides were reduced and alkylated under denaturing conditions.

An Agilent 1290 Infinity LC system with the following confi guration was used for the study:

- Agilent 1290 Infinity Binary Pump with integrated vacuum degasser (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector (DAD) (G4212A)with 10 mm Max-Light flow cell (G4212-60008)
- AdvanceBio Peptide Mapping Column (p/n 651750-902)

Conditions

Parameter	Value
Column	Agilent AdvanceBio Peptide Mapping, 2.1 × 250 mm, 2.7 µm (p/n 651750-902)
Mobile phase	A) 0.1 % TFA in water B) 0.08 % TFA in 90 % ACN
Gradient	Time (min) %B 0 3 60 50 65 90 66 90 70 3
Injection volume	5 µL (10 µg/µL)
Flow rate	0.5 mL/min
Data acquisition	210 nm/4 nm, 252 nm/4 nm
Thermostatted column compartment	60 °C
Sample thermostat	5 °C
Postrun time	10 minutes

Results and Discussion

Peak capacity is often used as an evaluation criterion to measure the performance of a column under given chromatographic conditions. mAb digestion produces many peptides for analysis. Therefore, it is necessary to develop a method that can increase peak capacity. Also, peak capacity is essential in a peptide mapping study so small impurity peaks or sample heterogeneity can be addressed. Due to the heterogeneous nature of ADC with glycosylation and cytotoxic drug conjugates, tryptic-digested ADC will generate more complex peptides than unconjugated mAb. To monitor the tryptic-digested ADC peptide mixture, optimization of gradient time and flow rates is critical to achieve high peak capacity.

The peak capacity values were calculated by dividing the gradient time by the average peak width of fi ve peptide peaks at baseline (5σ). Figure 1 depicts the effect of gradient time and flow rate on peak capacity. The results suggested that a 0.5 mL/min flow rate and 60 minutes gradient time gave the highest peak capacity values for the 2.1 × 250 mm, 2.7 µm column. These would, therefore, be the optimum conditions for identifying the peptides that have cytotoxic drug conjugation with high resolution.

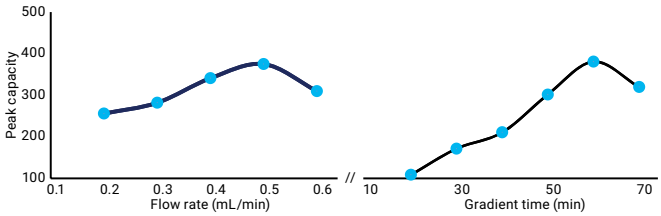

Figure 1. Effect of flow rate and gradient time on peak capacity.

Figure 2 shows the peptide map of the tryptic digested ADC. The peptide map shows excellent performance with baseline separation and resolution across the entire gradient profile. There was a significant improvement in separation time with the 250 mm column (60 minutes) compared to a 150 mm column (220 minutes) as previously reported in the literature for peptide mapping of ADC2. A peak capacity value of 354 was obtained and the RSD values demonstrate the excellent reproducibility of retention time and peak area and, thus, the precision of the system (Table1).

To identify peptides that have the cytotoxic drug attached, the peptide digests of the mAb and its conjugate, ADC, were analyzed by monitoring the UV trace at 252 nm (Figure 3). Peptide maps of ADC are different from those of Trastuzumab. It is clearly evident that the more hydrophobic drug-bonded peptides in ADC are eluted later (~ 40 to 60 minutes). Comparing the two peptide maps shows a group of later-eluting peptides identified in the ADC digest that are not present in the digest of the mAb. These hydrophobic peptides are the ones with the cytotoxic drug conjugation.

Table 1. RSD of retention time and area (n = 5) of peaks shown in Figure 2.

	Mean RT (min)	RSD RT (%)	Mean area (mAU/min)	RSD Area (%)
Peak 1	5.37	0.13	369.2	0.76
Peak 2	14.27	0.06	106.1	1.66
Peak 3	28.84	0.02	202.61	0.09
Peak 4	35.86	0.02	193.83	0.58

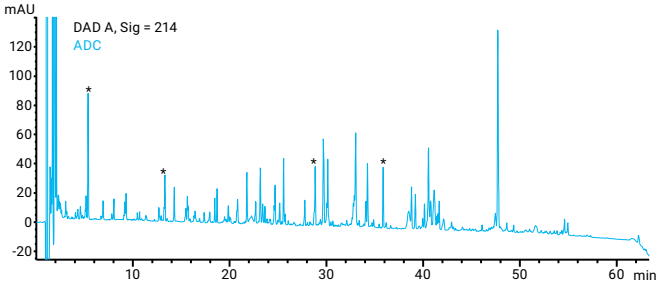

Figure 2. Peptide map of tryptic-digested ADC separated on an Agilent AdvancedBio Peptide Mapping column (*peaks selected for RSD calculations).

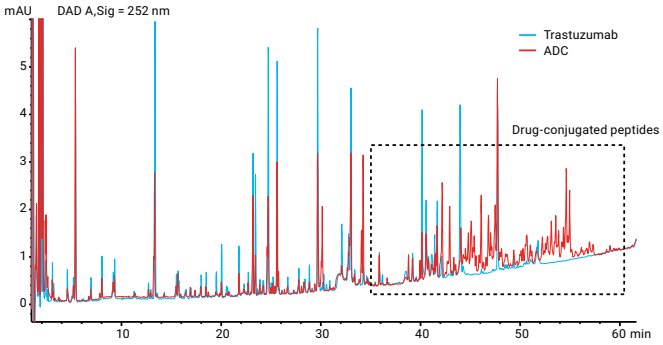

Figure 3. Overlay of peptide map of tryptic-digested ADC and Trastuzumab separated on an Agilent AdvancedBio Peptide Mapping Column.

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Conclusions

High-resolution peptide maps are obtained when the 250 mm Agilent AdvanceBio Peptide Mapping Column is used with an Agilent 1290 Infinity LC System. By comparing the peptide maps of the mAb and its conjugate, ADC, it is possible to identify the peptides conjugated with a cytotoxic drug in the ADC digest. Additionally, we demonstrated that the AdvanceBio Peptide Mapping Column provided resolution across the range of peptide types. Good peak shapes and reproducibility were obtained for the analysis of the more hydrophobic conjugated peptides, enabling identifi cation and quantitation.

References

1. Gudihal, R.; Waddell, K. Identification of Oxidation Sites on a Monoclonal Antibody Using an Agilent 1260 Infinity HPLC-Chip/MS System Coupled to an Accurate-Mass 6520 Q-TOF LC/MS; ***Agilent Technologies Application Note***, publication number 5990-8768EN, **2011**.
2. Kim, M. T.; *et al.* Statistical Modeling of the Drug Load Distribution on Trastuzumab Emtansine (Kadcyla), a Lysine-Linked Antibody Drug Conjugate. ***Bioconjugate Chem***. **2014**, ***25***, pp 1223-1232.

Application Note

Peptide Mapping

Analysis of Tryptic Digests of a Monoclonal Antibody and an Antibody-Drug Conjugate with the Agilent 1290 Infinity II LC

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Abstract

An Agilent 1290 Infinity II LC in combination with an Agilent AdvanceBio Peptide Maping column was used to analyze tryptic digests of the monoclonal antibody (mAb) trastuzumab, or trade name Herceptin, and the antibody-drug conjugate (ADC) ado-trastuzumab emtansine, or trade name Kadcyla. The use of the highly efficient Agilent V380 Jet Weaver mobile phase mixer reduced the baseline noise caused by the trifluoroacetic acid (TFA) modifier significantly, enabling detection of low abundant peptides by UV at 214 nm. By modifying the gradient steepness, peak capacities of approximately 300, 450, and 900 could be obtained for total analysis times of 25, 45, and 205 minutes, respectively.

Introduction

Monoclonal antibodies (mAbs) have emerged as important therapeutics for the treatment of cancer and autoimmune diseases, among others1,2. The successes of mAbs have triggered the development of various next-generation formats including antibody-drug conjugates (ADCs), which combine a specific mAb and a cytotoxic drug by a stable linker1,2. The promise of ADCs is that highly toxic drugs can selectively be delivered to tumor cells, thereby substantially lowering side effects typically experienced with classical chemotherapy. Peptide mapping is an important methodology in the analysis and characterization of these molecules. Hundreds of peptides with varying physicochemical properties present in a wide dynamic concentration range exist in mAb and ADC tryptic digests, demanding the best in terms of separating power. This Application Note describes how an Agilent 1290 Infinity II LC in combination with an Agilent AdvanceBio Peptide Map column is successfully applied to tackle these challenging separations.

Experimental

Instumentation

An Agilent 1290 Infinity II LC was used, comprising:

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity II Diode Array Detector (G7117B) equipped with a 10 mm flow cell

An Agilent 1290 Infinity II LC was used, comprising:

- Agilent Jet Weaver mixer, both 35 and 100 µL configurations were tested (G4220-60006)
- Agilent High-Performance Jet Weaver mixer, 380 µL (G4220-60012)

Samples and sample preparation

Trastuzumab (Herceptin) and ado-trastuzumab emtansine (Kadcyla) were obtained from Roche (Basel, Switzerland).

A 100 µg amount of protein, diluted in 0.05 % Rapigest/100 mM Tris-HCl, pH 8, was reduced at 60 °C for 30 minutes by the addition of 5 mM dithiothreitol, and alkylated at 37 °C for 1 hour by adding 10 mM iodoacetamide. Trypsin was subsequently added at an enzyme-to-substrate ratio of 1:25 (w:w). Digestion proceeded for 16 hours at 37 °C. The final concentration was 0.5 µg/µL.

Method parameters

Parameter	Value
Column	Agilent AdvanceBio Peptide Mapping column, 2.1 × 250 mm, 2.7 µm (p/n 651750-902)
Mobile phase	A) 0.05 % TFA in water/acetonitrile 99:1 (v:v) B) 0.045 % TFA in acetonitrile
Flow rate	0.35 mL/min
Gradient	0 to 60 %B in various gradient slopes (see peak capacity) 60 to 90 %B in 0.5 minutes and hold for 4.5 minutes 7 minutes post time at 0 %B Example for 40 minutes gradient: 0 to 40 minutes – 0 to 60 %B 40 to 40.5 minutes – 60 to 90 %B 40.5 to 45 minutes – 90 %B
Temperature	60 °C
Injection	5 µL Needle wash flush port, 5 seconds, 0.05 % TFA in water/acetonitrile 20:80 (v:v)
Detection	Signal 214/4 nm, Reference 360/60 nm Signal 252/4 nm, Reference 360/60 nm (for ADC) >0.025 minutes (0.5 seconds response time) (10 Hz)

Results and Discussion

Agilent AdvanceBio Peptide Mapping column

The Agilent AdvanceBio Peptide Mapping column is packed with 2.7 µm superficially porous C18 particles with 120 Å pore size. It is a state-of-the-art column for peptide mapping, enabling high-resolution separations in short analysis times. Figure 1 shows the results for a fast analysis (25 minutes total analysis time) of a tryptic digest of Herceptin.

Agilent Jet Weaver mixers

For comprehensive peptide mapping, the system should be able to detect high as well as low abundant peptides. Peptide mapping is generally carried out with UV detection at 214 nm and a water/acetonitrile mobile phase containing trifluoroacetic acid (TFA) because of its beneficial effects on peptide retention and peak shape. It is known that these conditions contribute to increased baseline noise due to the UV absorption of the TFA modifier3. The noise will depend on column dimensions and flow, gradient slope, and system gradient formation.

When mobile phase mixing is inadequate, small variations present in the mobile phase composition may persist after passage through the column, and reach the detector. In the case of TFA, which has significant UV absorbance at low wavelengths (for example, 214 nm), small fluctuations in TFA concentration and water/acetonitrile ratio will be visible with UV or DAD. Excessive noise can be tackled by increasing the mobile phase mixing performance to stabilize solvent composition as much as possible.

The impact of mixing has been tested with the selected column and samples by comparison of three state-of-the-art Jet Weaver mobile phase mixers.

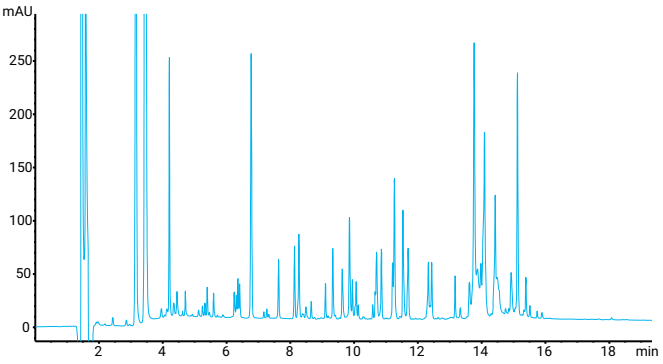

Figure 1. Analysis of a Herceptin tryptic digest, 20-minute gradient, UV detection at 214 nm.

- Agilent V35 Jet Weaver: internal volume of 35 µL, most commonly used because of the small impact on delay volume
- Agilent V100 Jet Weaver: internal volume of 100 µL, used for applications that require higher mixing performance and low delay volume
- Agilent V380 High-Performance Jet Weaver: internal volume of 380 µL, used for applications that need best mixing

Figure 2 shows the results of a blank (mobile phase A) injection with a 40-minute gradient carried out with the various Jet Weaver mixers. It is clear that despite the excellent behavior of the V35 Jet Weaver for most analyses, its performance for the detection of low-level compounds under these particular conditions is poor. Increasing the mixing volume to 100 µL significantly improves baseline stability, and by installing the 380 µL mixer, the noise caused by TFA is nearly eliminated. It is striking how the small system peaks present in the blank analysis (retention time 23 to 27 minutes) are not detectable with the V35 Jet Weaver whereas they are easily visible with the V380 mixer. Therefore, the V380 High-Performance Jet Weaver was selected for further analyses.

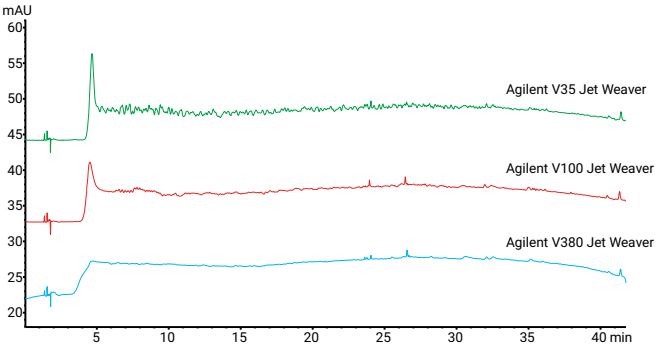

Figure 2. Analysis of a blank solution with an Agilent V35 (green), Agilent V100 (red), and Agilent V380 (blue) Jet Weaver mixer, 40-minute gradient, UV detection at 214 nm.

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Switching between a low-volume and high-volume mixer will change the delay volume of the setup, and will affect retention times and potentially selectivity. To maintain the separation, some delay time should be added to the gradient to correct for this. This time shift for the gradient enabled nearly identical retention times and selectivity to be obtained with all three mixers (Figure 3).

Considerations on peak capacity

As shown in Figure 3, the tryptic digests are complex samples, and high peak capacity is mandatory for adequately characterizing mAbs and ADCs. With the current system and column configuration, the peak capacity can be adjusted according to the desired performance by changing only the gradient steepness and gradient time4.

As illustrated, the Kadcyla tryptic digest was analyzed with 12 different gradient times between 5 and 200 minutes. Four peptides were selected to calculate the peak capacity at 4 sigma (= 13.4 %) peak height. Figure 4 and Table 1 show the results. Short gradients with peak capacity below 250 can be used for fast (high productivity) analyses, whereas long gradients will result in peak capacities close to 900. The Herceptin tryptic digest was injected with some selected gradient conditions as a control, and peak capacities were in accordance to expectations. Figure 5 shows the results for these analyses. Note that the slope of the curve flattens strongly from 120 minutes onward, and that, in fact, working at slower slopes is pointless.

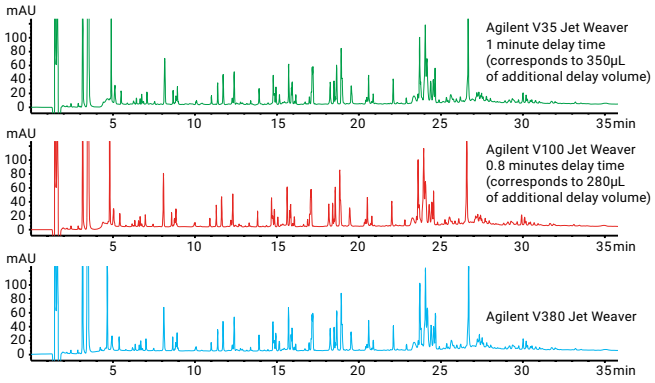

Figure 3. Analysis of Kadcyla tryptic digest with an Agilent V35 (green), Agilent V100 (red), and Agilent V380 (blue) Jet Weaver mixer, 40-minute gradient, UV detection at 214 nm.

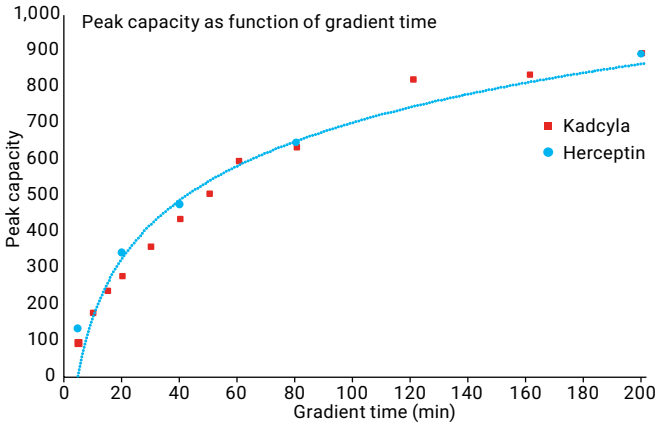

Figure 4. Peak capacity as a function of gradient time. UV detection at 214 nm (detector set at 40 Hz to ensure sufficient data points in fast analyses).

Table 1. Peak capacity according to gradient for Kadcyla and Herceptin.

	Peak capacity	
Gradient time (min)	**Kadcyla**	**Herceptin**
5	95	134
10	177	
15	238	
20	280	342
30	359	
40	438	475
50	506	
60	596	
80	635	646
120	822	
160	836	
200	893	891

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Further comments on the analysis of the Herceptin and Kadcyla digests

A 40-minute gradient resulted in a peak capacity of approximately 450, and these conditions, a good compromise between analysis time and separation performance, were used to highlight the difference between the mAb and the ADC. The protein sequence of Kadcyla is identical to Herceptin; the difference is in the conjugation of the cytotoxic agent emtansine to lysine residues. Figure 6 shows an overlay of both samples, with detection at 214 nm and 252 nm. Overall, the chromatograms are similar except for the cluster of peaks eluting late in the gradient (between 25 and 35 minutes). These are peptide-drug conjugates all containing emtansine. The complexity stems from the fact that a high number of lysine residues are available for conjugation. Figure 7 shows a detail of the chromatograms recorded at 252 nm. The addition of the drug to the peptide increases hydrophobicity and, therefore, retention. Since this cytotoxic agent has a UV absorbance at 252 nm, the conjugates are better observed with the detector set at this wavelength. The repeatability of the developed method was evaluated by five replicate injections of the Kadcyla tryptic digest. The overlay in Figure 8 shows that the injection and retention time precision are excellent.

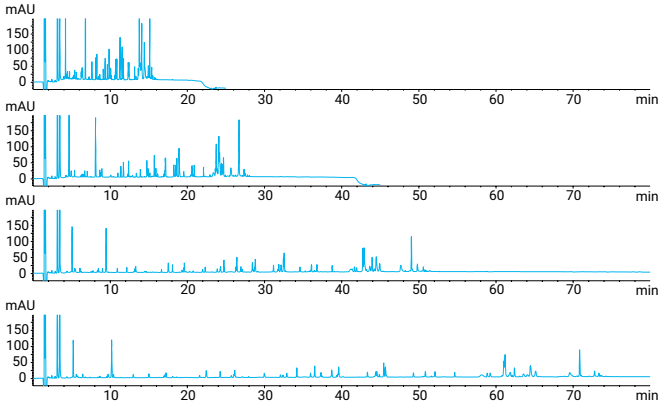

Figure 5. Analysis of a Herceptin tryptic digest with a 20, 40, 80, and 120-minute gradient, UV detection at 214 nm. Note, for comparison, that the y-scale was increased with the Δ-gradient factor.

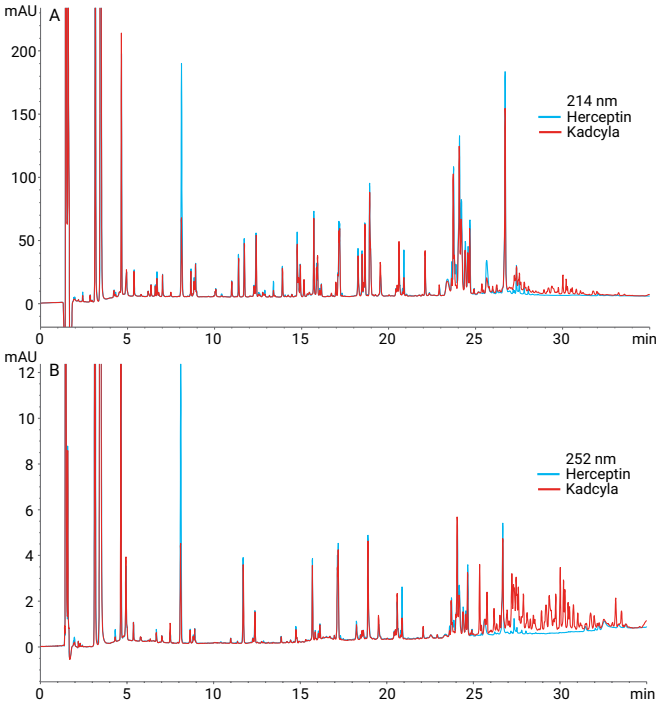

Figure 6. Comparison of analysis of a Herceptin (blue) and Kadcyla (red) tryptic digest with a 40-minute gradient, UV detection 214 nm and 252 nm.

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Conclusions

High peak capacities can be achieved for the analysis of tryptic digests of mAbs and ADCs with an Agilent 1290 Infinity II LC in combination with an Agilent AdvanceBio Peptide Map column. The Agilent V380 Jet Weaver mobile phase mixer is effective in reducing the noise caused by the TFA modifier. This opens opportunities to quantify low abundant species in complex mixtures. The excellent precision obtained with the current setup suggests that this is a valuable tool for detailed analysis of protein biopharmaceuticals such as mAbs and ADCs. A next level of detail can be obtained when analyzing these digests on an LC×LC setup as described recently5,6.

References

1. Sandra, K.; Vandenheede, I.; Sandra, P. Modern chromatographic and mass spectrometric techniques for protein biopharmaceutical characterization. ***J. Chromatogr. A*** **2014**, *1335*, 81–103.
2. Fekete, S.; *et al.* Chromatographic, electrophoretic, and mass spectrometric methods for the analytical characterization of protein biopharmaceuticals. ***Anal. Chem.*** **2016**, *88*, 480–507.
3. Huesgen, A. G. Performance of the Agilent 1290 Infinity Quaternary Pump using Trifluoroacetic Acid (TFA) as the mobile phase modifier, ***Agilent Technologies Technical Overview,*** publication number 5990-4031EN, **2009**.
4. Vanhoenacker, G.; *et al*. Tryptic digest analysis using the Agilent 1290 Infinity LC System, ***Agilent Technologies Application Note,*** publication number 5990-4031EN**, 2009**.
5. Vanhoenacker, G.; *et al.* Comprehensive two-dimensional liquid chromatography of therapeutic monoclonal antibody digests, ***Anal. Bioanal. Chem.*** **2015**, *407*, 355-366.
6. Sandra, K.; *et al.* Multiple heart-cutting and comprehensive two-dimensional liquid chromatography hyphenated to mass spectrometry for the characterization of the antibody-drug conjugate ado-trastuzumab emtansine, ***J. Chromatogr. B***, doi: 10.1016/j.jchromb.2016.04.040.

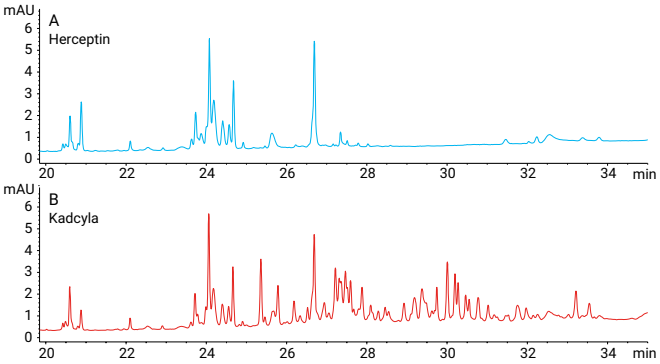

Figure 7. Detail of the comparison of an analysis of a Herceptin and Kadcyla tryptic digest with a 40-minute gradient, UV detection at 252 nm.

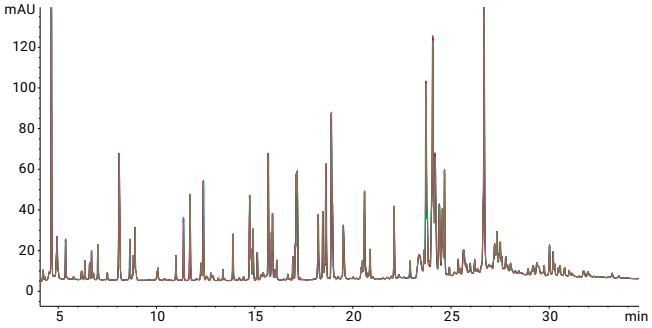

Figure 8. Overlay of five replicate injections of a Kadcyla tryptic digest with a 40-minute gradient, UV detection at 214 nm.

Application Note

Peptide Mapping

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Characterization of Viral Vector Particles Using the Agilent 6545XT AdvanceBio LC/Q-TOF

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Abstract

This application note describes a workflow for the characterization and determination of critical quality attributes (CQAs) of intact adeno-associated viruses (AAVs), together with post-translational modification (PTM) identification of the capsid proteins. The workflow comprised an Agilent 1290 Infinity II LC coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF, with Agilent MassHunter BioConfirm 10.0 software used for data analysis.

Introduction

Peptide AAVs are the main viral vectors for gene therapy and have been successful in treating inherited retinal diseases and spinal muscular atrophy. An AAV is composed of an icosahedral protein shell with a single-stranded genome of approximately 4.7 kb. The intact AAVs act as a vehicle to protect and deliver oligonucleotide therapeutics. There are 13 known serotypes that transduce different cell types, allowing increased selectivity for therapeutics. As AAVs continue to be explored as therapeutic delivery platforms, it is vital to ensure that all the CQAs of the therapeutic product are maintained. Characterizing viral capsid proteins yields several challenges. The protein shell is composed of three capsid proteins, VP1, VP2, and VP3, that assemble into a 3.9 megadalton structure in a ratio of 1:1:10 with 60 capsids per virion. In addition to the low molar ratios of VP1 and VP2, all three proteins have overlapping sequences at the C-terminus. Traditionally, SDS-PAGE is used to establish the molecular weight of the capsid proteins, however, this technique provides an approximate molecular weight and may not be able to distinguish between different serotypes. Mass spectrometry (MS) is a promising method to overcome these challenges and determine CQAs of the capsid proteins. This application note describes a workflow for intact analysis and peptide mapping, including PTM identification of the viral capsid proteins. The tools used for this workflow include a 1290 Infinity II LC coupled to the 6545XT AdvanceBio LC/Q-TOF, using MassHunter BioConfirm 10.0 software for data analysis.

Experimental

Instrumentation

Agilent 1290 Infinity II LC including:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B) fitted with 20 µL loop for intact analysis, 40 µL loop for peptide-mapping analysis
- Agilent 1290 Infinity II thermostatted column compartment (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF

Materials

AAV8 was produced by Lake Pharma (Worcester, MA, USA). Molecular weight cutoff filters and (*tris*(2-carboxyethyl) phosphine) (TCEP) were purchased from Millipore Sigma. Trypsin and rAsp-N were purchased from Promega).

Sample preparation

For intact analysis, AAVs underwent a buffer exchange three times at 10,000 g with a 10 kDa molecular weight filter. The buffer contained 5 mM TCEP, 80% H_2O, and 20% acetonitrile with 0.1% formic acid (v/v). After the sample was collected, it was incubated at room temperature before injection. For peptide mapping, the AAVs underwent denaturation, reduction, alkylation, and digestion. The enzymes used in this experiment were trypsin and rAsp-N.

LC/MS analysis

LC/MS analysis was performed on a 1290 Infinity II LC coupled to a 6545XT AdvanceBio LC/Q-TOF system with a dual Agilent Jet Stream source. Agilent MassHunter Acquisition (B.09.00) workstation software with the large molecule SWARM autotune feature was used for intact analysis. The instrument was further calibrated and operated in standard mass mode. The iterative MS/MS feature was used for the peptide-mapping workflow.

Data processing

All MS data were processed with MassHunter BioConfirm 10.0 software.

Table 1. Liquid chromatography parameters for intact analysis.

Parameter	Value
Column	Agilent ZORBAX RRHD 300-Diphenyl, 2.1 × 150 mm, 1.8 µm
Mobile Phase A	Water, 0.1% formic acid
Mobile Phase B	Acetonitrile, 0.1% formic acid
Flow Rate	0.4 mL/min
Injection Volume	20 µL
Gradient	0–30 min: 30–40% B; 30–38 min: 40–90% B; 38 –39 min: 90% B; 39–40 min: 90–30% B; 40–45 min: 30% B
Post Time	0 minutes
Column Temperature	60 °C

Table 2. Agilent 6545XT AdvanceBio LC/Q-TOF parameters for intact analysis.

Parameter	Value
Source	Dual Agilent Jet Stream
Gas Temperature	325 °C
Gas Flow	13 L/min
Nebulizer	35 psig
Sheath Gas Temperature	375 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	5,000 V
Nozzle Voltage	500 V
Fragmentor	180 V
Acquisition Rate	1 spectra/sec
Reference Mass	922.0098

Table 3. Liquid chromatography parameters for peptide mapping analysis.

Parameter	Value
Column	Agilent AdvanceBio Peptide Mapping, 2.1 × 150 mm
Mobile Phase A	Water, 0.1% formic acid
Mobile Phase B	Acetonitrile, 0.1% formic acid
Flow Rate	0.4 mL/min
Injection Volume	40 µL
Gradient	0–3 min: 3% B; 3–50 min: 3–35% B; 50–60 min: 35–97% B; 60–62 min: 97% B; 62–62.5 min: 97–3% B; 62.5–65 min: 3% B
Post Time	5 minutes
Column Temperature	60 °C

Table 4. Agilent 6545XT AdvanceBio LC/Q-TOF parameters for peptide mapping analysis.

Parameter	Value
Source	Dual Agilent Jet Stream
Gas Temperature	325 °C
Gas Flow	13 L/min
Nebulizer	35 psig
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Fragmentor	170 V
Acquisition Rate	5/3 spectra/sec for MS and MS/MS
Reference Mass	121.0509, 922.0098

Results and Discussion

Intact analysis on the 6545XT AdvanceBio LC/Q-TOF

While SDS-PAGE is a rapid and simple way to verify the molecular weight of AAV capsid proteins, it is not specific enough to resolve different proteoforms, such as acetylation or phosphorylated versus unmodified forms. High-resolution Q-TOF MS provides ample sensitivity, resolves PTMs, and determines accurate intact molecular mass of the proteins. Further aiding this is the large molecule SWARM autotune feature on the 6545XT AdvanceBio LC/Q-TOF, which provides excellent sensitivity for the capsid proteins by improving their transmission throughout the mass spectrometer. In addition, the ultralow TOF vacuum (e^{-8} torr) allows increased spectral clarity due to the increased mean free path of the protein molecules.

Sample preparation before LC/MS is critical for obtaining high-quality mass spectra, and can further be highlighted with our work on AAVs. Figure 1 displays the total ion chromatograms (TICs) and raw mass spectra of the capsid proteins with and without sample preparation. The buffers of the original solution are introducing contaminants into the mass spectrometer. In addition, the separation between the capsid proteins is improved after buffer exchange. The raw data show that the protein has also increased 1.5 times in abundance, and the spectrum is much cleaner. The deconvoluted data are not shown here, but the spectra are clearer due to the lack of sodium and potassium adducts, leaving the interpretation of the data much simpler. Additionally, this will increase the robustness of the workflow, allowing longer times between instrument maintenance.

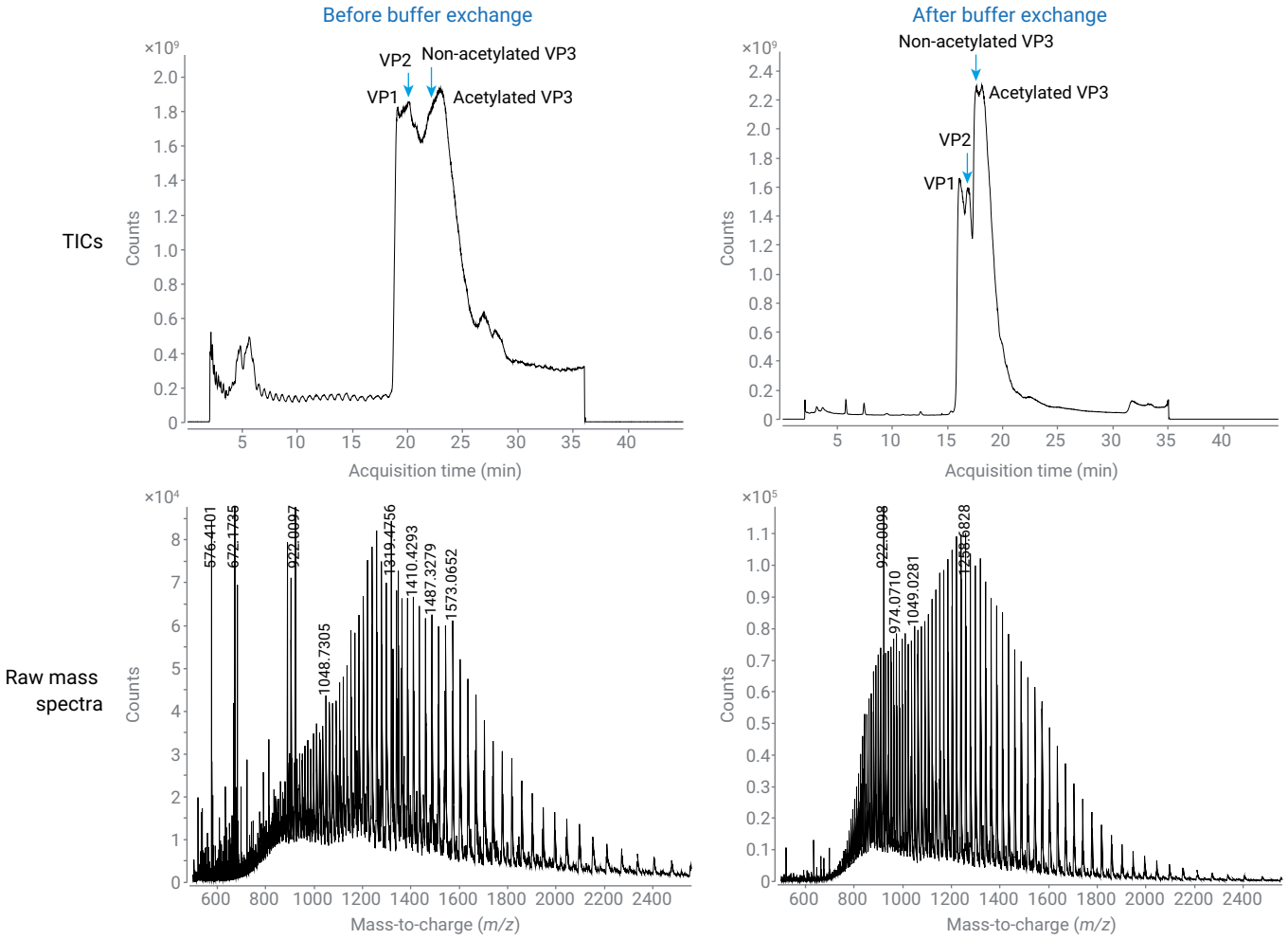

Figure 1. TICs of AAV capsid proteins and raw spectra of VP1 with and without sample preparation.

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Figure 2 presents the raw and deconvoluted spectra of VP1. With this workflow, we were able to detect three phosphorylation sites on VP1 with less than 10 ppm error. The accurate mass data confirmed that VP1 is missing its N-terminal amino acid residue and that the new N-terminus is acetylated. There are currently very few reports of PTM analysis on AAV capsid proteins, including phosphorylation on VP1. VP2 is chromatographically separated from VP1. While mass spectrometry can separate these proteins by mass, having chromatographic separation allows less ion suppression of these two low-abundant proteins. The accurate mass data confirm at least two phosphorylation sites on VP2, and likely a third in Figure 3.

Figure 4 shows that the unmodified form of VP3 is mostly chromatographically separated from acetylated VP3. Again, the deconvoluted spectra determine that both acetylated and unmodified VP3 are present with high mass accuracy. N-terminal acetylation of proteins is a common PTM and is involved in protein stability, folding, and interactions with other proteins. While VP1 was fully acetylated, approximately 70% of VP3 was acetylated. While it is not clear at this time why VP3 was not fully acetylated, it may affect the overall structure of the capsid shell of the virus. The spectral clarity provided by the improved vacuum on the 6545XT AdvanceBio LC/Q-TOF in combination with the large molecule SWARM autotune feature show all three viral capsid proteins with their PTMs with high mass accuracy, under 10 ppm for all proteoforms.

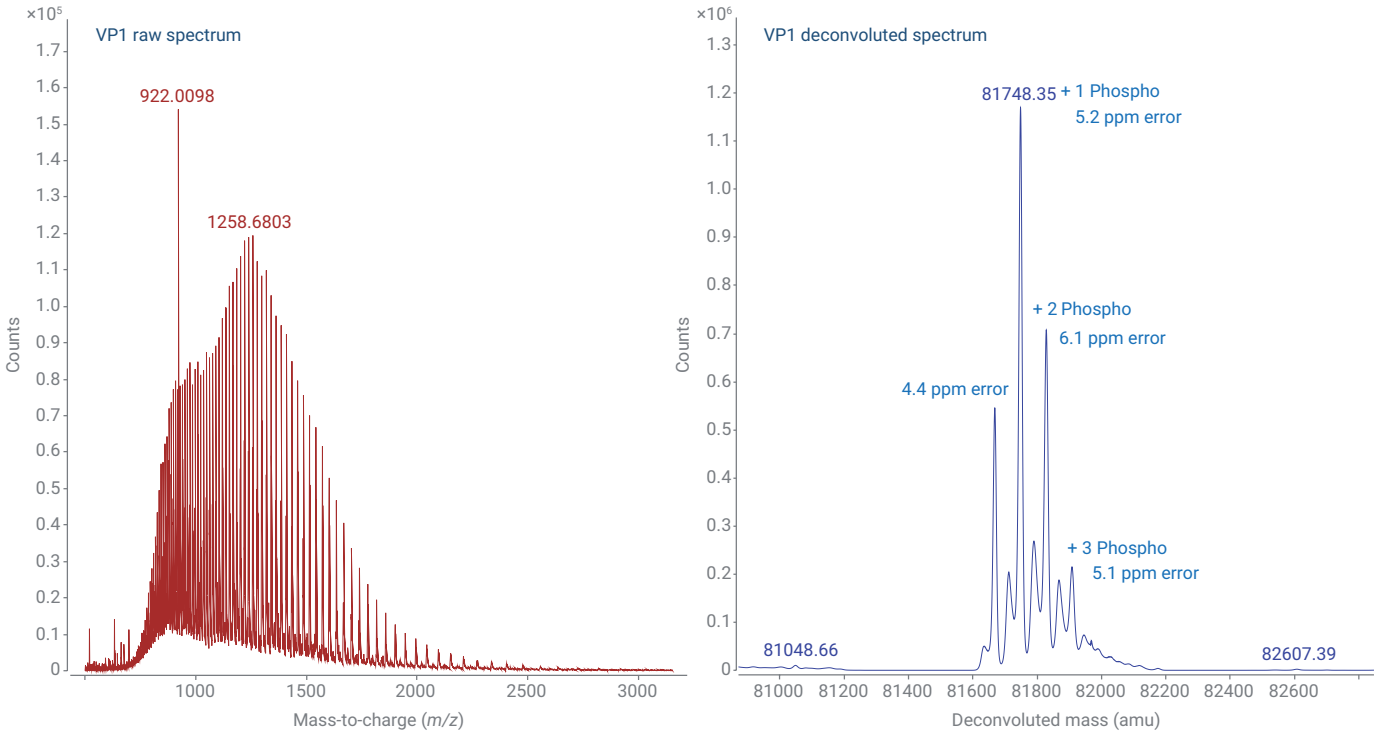

Figure 2. Raw and deconvoluted spectra of VP1 capsid protein. The native and phosphorylated forms of the protein have excellent mass accuracy.

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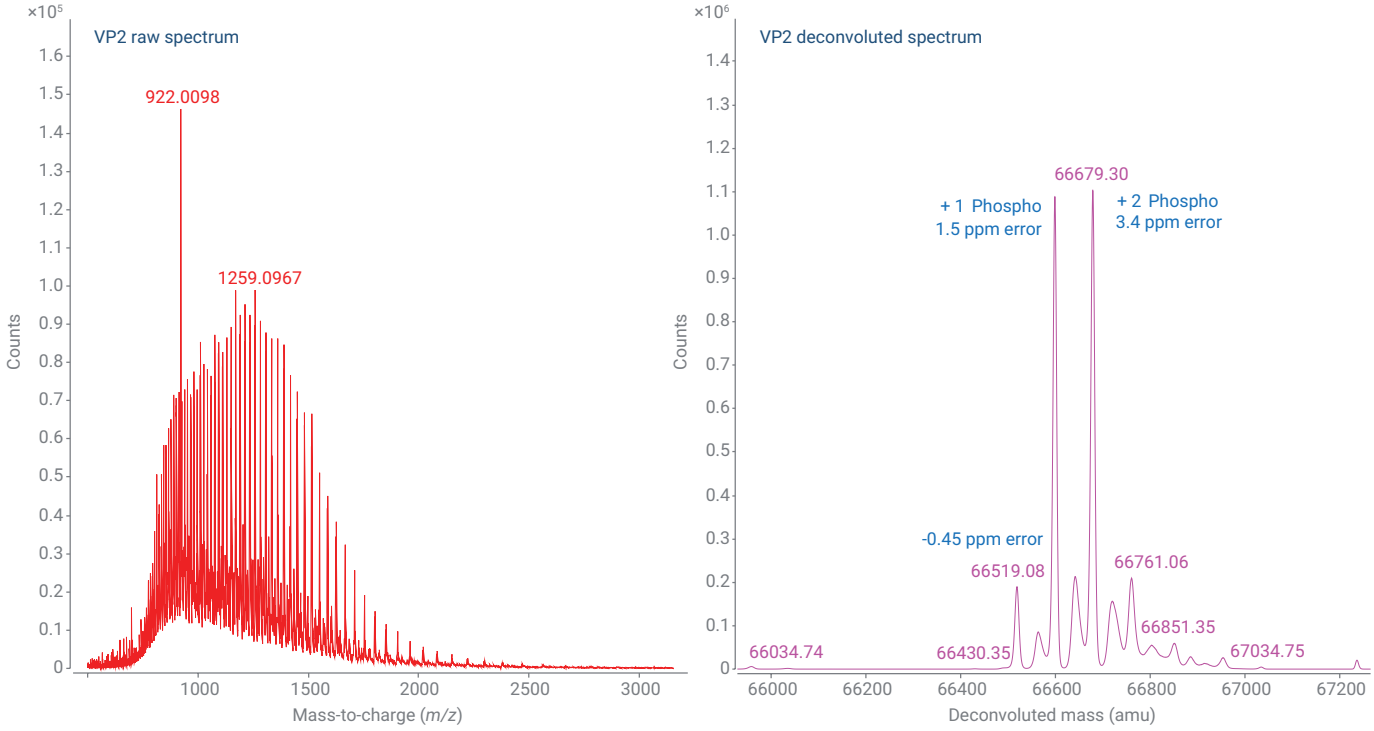

Figure 3. Raw and deconvoluted spectra of VP2 capsid protein. The native and phosphorylated forms of the protein have excellent mass accuracy, all under 5 ppm error.

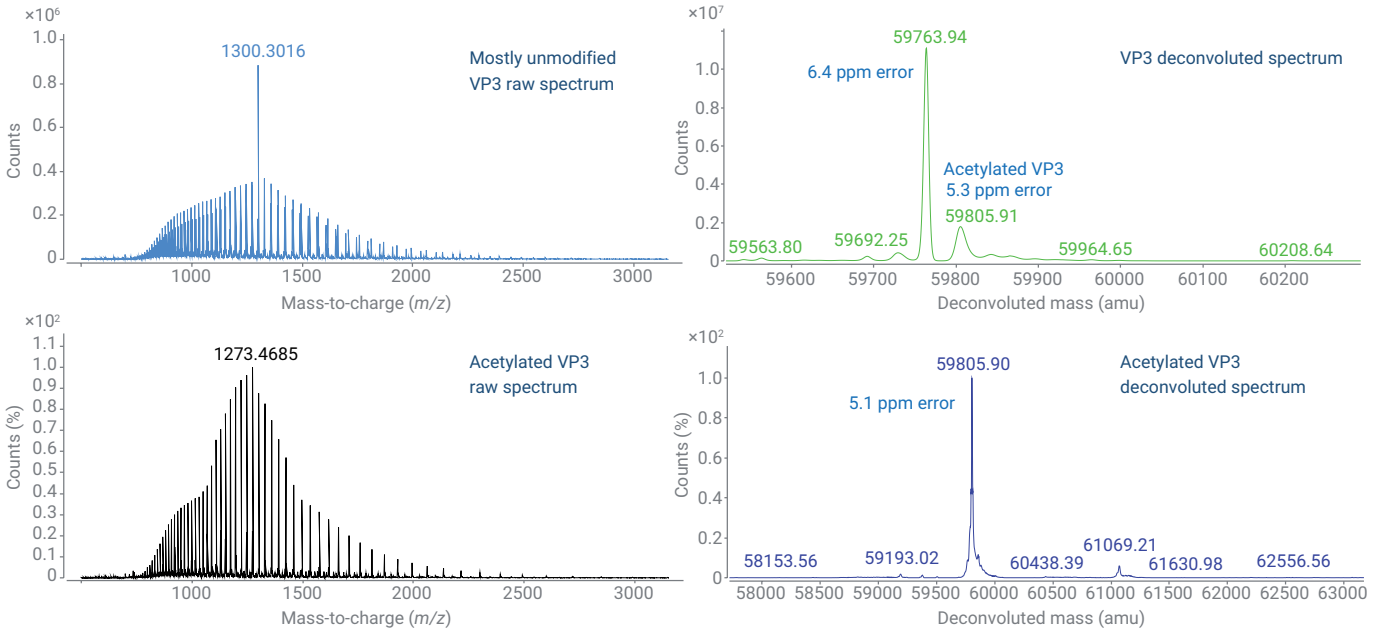

Figure 4. Raw and deconvoluted spectra of VP3 capsid protein. The unmodified and acetylated forms are mostly chromatographically separated, with excellent mass accuracy for each proteoform.

Peptide mapping on the 6545XT AdvanceBio LC/Q-TOF

Peptide mapping of biotherapeutics is an essential method to determine protein sequence and PTMs, required by the ICH, FDA, and other regulatory agencies. Although gene therapy via AAV is an emerging field, it is conceivable to imagine a future requirement for peptide mapping of the capsid proteins. As of January 2020, the FDA recommends providing information regarding primary and secondary structure including PTMs for human gene therapy drug substances. The 6545XT AdvanceBio LC/Q-TOF's iterative MS/MS feature excludes peptides from all previous runs for isolation and fragmentation, allowing selection and detection of low-abundant peptides. In addition, MassHunter BioConfirm 10.0 allows multiple runs to be selected to give a total sequence coverage. This feature is useful for combining results from iterative MS/MS runs as well as using multiple enzymes.

Determining identity of PTMs such as oxidation and deamidation is vital in determining protein stability. To have confidence with peptide mapping, all identified peptides had less than 10 ppm error and at least one MS/MS spectrum to confirm peptide sequence and to localize PTMs. Furthermore, the false discovery rate was set to 1%. AAV8's sequence has several regions where there are frequent lysine and arginine residues, rendering it difficult to obtain full sequence with trypsin alone. Therefore, rAsp-N was used to complete sequence coverage.

The largest protein, VP1, had a total sequence coverage of 97.7%, as shown in Figure 5. The solid lines represent identification of the peptide by MS/MS. The blue and green lines come from two iterative runs of the tryptic digestion, while the black and red lines come from two iterative runs of the rAsp-N digestion. MS/MS data confirm site-specific phosphorylation as shown in Figure 6. The red annotations display peptide fragments that contain the phosphorylated serine. Other common PTMs such as asparagine deamidation and methionine oxidation are present, but in low abundance, as expected. Figure 7 shows examples of these low-level modifications with the relative quantitation feature in BioConfirm 10.0. VP2 and VP3 had 98.5 and 100.0% sequence coverage, respectively. While there have been reports of N-glycosylation in AAV8, no N-glycosylation was found. This discrepancy may be due to the differences in vector expression systems.

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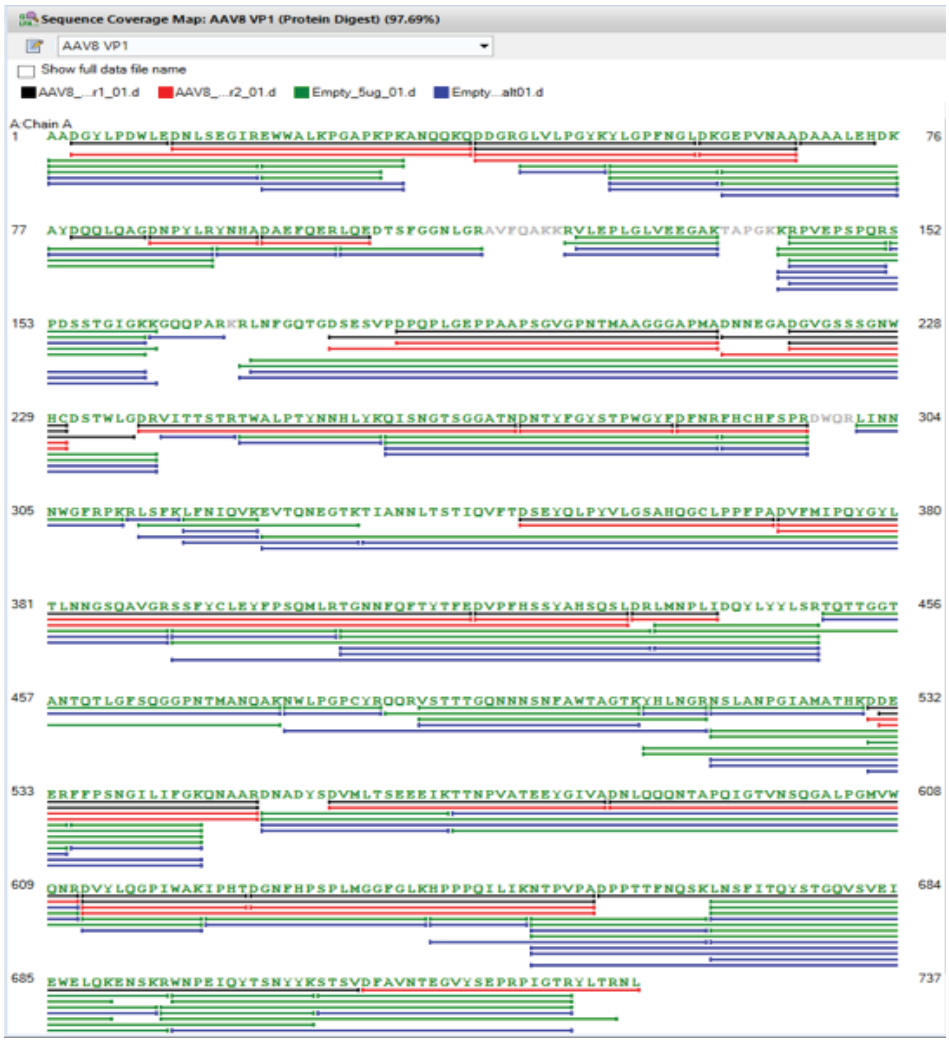

Figure 5. Agilent MassHunter BioConfirm 10.0 screenshot showing sequence coverage of VP1 with iterative MS/MS and using trypsin and rAsp-N as complementary enzymes. This protein has 97.7% sequence coverage.

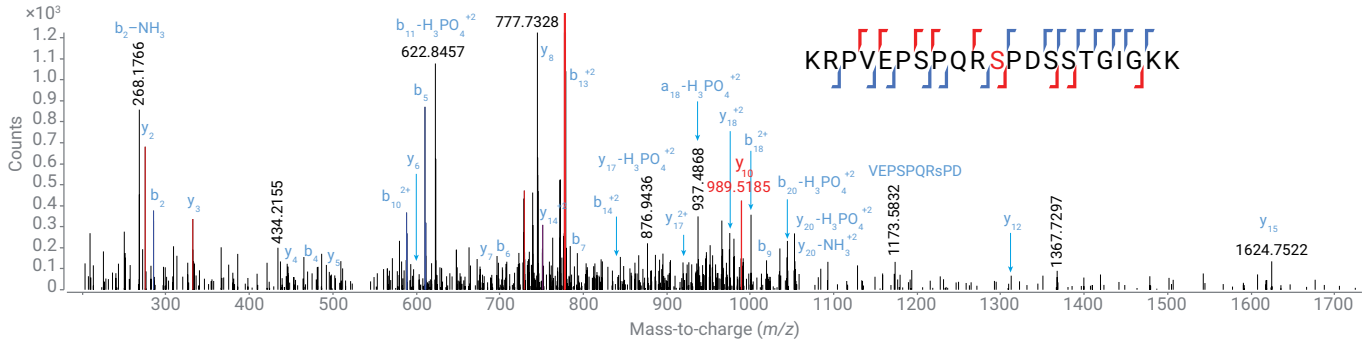

Figure 6. One example of site-specific phosphorylation with MS/MS confirmation. The annotated peptide has red markings when it contains the phosphorylated serine residue.

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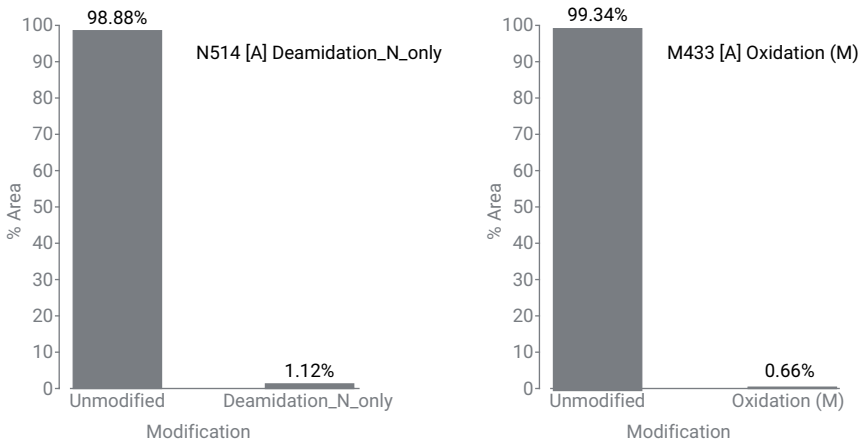

Figure 7. Examples of common PTMs in proteins: methionine oxidation and asparagine deamidation. Both peptides are minimally modified, as expected.

Conclusion

The use of AAV particles as vehicles for gene therapy has shown great promise, making characterization of the capsid proteins CQAs vital to the drug approval process. Here, we show a workflow from sample preparation through data analysis that determines the accurate mass of the capsid proteins and identifies PTMs. A 1290 Infinity II LC coupled to an 6545XT AdvanceBio LC/Q-TOF with MassHunter BioConfirm 10.0 provides a reliable and accurate solution for analysis of AAV capsid proteins.

Acknowledgements

Agilent would like to thank Dominique Garceau, Tristan Cano, Caitlin Jaeger, and William Hermans from Lake Pharma for being involved in sample production. An additional thanks to Brian Liau and Ravindra Gudihal of Agilent Singapore for sharing ideas.

References

1. Dalkara, D. *et al*. *In vivo*–Directed Evolution of a New Adeno-Associated Virus for Therapeutic Outer Retinal Gene Delivery from the Vitreous. *Sci. Transl. Med*. **2013**, *5(189)*, 189ra76-189ra76.
2. Xie, Q. *et al*. The Atomic Structure of Adeno-Associated Virus (AAV-2), a Vector for Human Gene Therapy. **2002**, *99(16)*, 10405–10410.
3. Wu, Z.; Asokan, A.; Samulski, R. J. Adeno-Associated Virus Serotypes: Vector Toolkit for Human Gene Therapy. *Mol. Ther*. **2006**, *14(3)*, 316–327.
4. Bui, H. *et al*. (**2014**) ASMS Poster WP-681.
5. Jin. X. *et al*. Direct Liquid Chromatography/Mass Spectrometry Analysis for Complete Characterization of Recombinant Adeno-Associated Virus Capsid Proteins. *Hum. Gene Ther. Methods* **2017**, *28(5)*, 255–267.
6. Giles, A. R. *et al*. Deamidation of Amino Acids on the Surface of Adeno-Associated Virus Capsids Leads to Charge Heterogeneity and Altered Vector Function. *Mol. Ther.* **2018**, *26(12)*, 2848–2862.
7. Van Vliet, K. *et al*. Adeno-associated virus capsid serotype identification: Analytical methods development and application. J. Virol. Methods **2009**, *159(2)*, 167–177.
8. Arruda, V. R. *et al*. It's All About the Clothing: Capsid Domination in the Adeno-Associated Viral Vector World. *J. Thromb. Haemost*. **2007**, *5(1)*, 12–15.
9. Office of Medical Products and Tobacco, Center for Biologics Evaluation and Research. *Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs). Silver Spring*, MD. **2020**, 28–29.

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Application Note

Peptide Mapping

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Trusted Answers

Seamless Method Transfer to the Agilent 1290 Infinity II Bio LC System

Peptide-mapping analysis shows excellent performance and high method compatibility compared to the Agilent 1290 Infinity II LC System

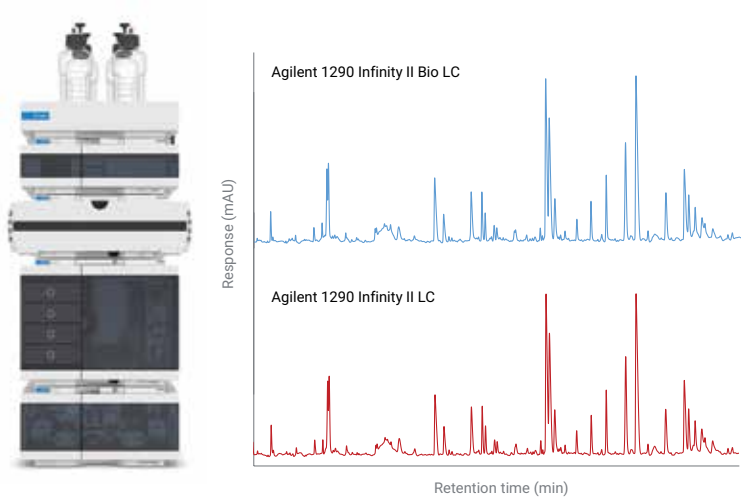
Author

André Feith

Agilent Technologies, Inc.

Abstract

Peptide mapping requires reliable and robust methods with high precision for analyzing the primary structure and post translational modifications (PTMs) of biopharmaceuticals. However, method transferability and compatibility can be an issue for validated methods. This application note shows that method transfer can be easy and convenient with the new Agilent 1290 Infinity II Bio LC System. Building on the excellent average relative retention time deviation of 0.039% for 12 selected peptides, it was discovered that the retention times only deviated by 0.17% between the 1290 Infinity II Bio LC System and the Agilent 1290 Infinity II LC System. By combining the LC systems with the Agilent 6545XT AdvanceBio LC/Q-TOF, additional comparative statistical analysis of peak abundances revealed no significant differences between both systems, rendering the new 1290 Infinity II Bio LC the ideal choice for UV- or MS-based peptide-mapping workflows.



Introduction

Method transfer and compatibility from one instrument to another are important for laboratories across different industries.1 Especially in the biopharmaceutical industry, instrument-to-instrument method transfer is highly important for validated methods. To demonstrate the seamless method transfer from the 1290 Infinity II LC to the 1290 Infinity II Bio LC, the peptide-mapping workflow was chosen because of its considerable relevance in the evaluation of biological products as described in ICH Guideline Q6B.2 Employing a tryptic digest of the NISTmAb, this application note will show that method transfer can be straightforward thanks to the 1290 Infinity II Bio LC.

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System and the Agilent 1290 Infinity II LC System coupled to the Agilent 6545XT AdvanceBio LC/Q-TOF comprised the following modules:

Agilent 1290 Infinity II Bio LC:

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116A) equipped with a Standard Flow Quick Connect Bio Heat Exchanger (G7116-60071) and two Agilent Thermal Equilibration Devices (G7116-60013)
- Agilent 1290 Infinity II Variable Wavelength Detector (VWD) (G7114B), equipped with a Bio Micro Flow Cell VWD, 3 mm, 2 µL, RFID.
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6545XT)

Agilent 1290 Infinity II Bio LC:

- Agilent 1290 Infinity II High-Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116A) equipped with a Standard Flow Quick Connect Heat Exchanger (G7116-60015) and two Agilent Thermal Equilibration Devices (G7116-60013)
- Agilent 1290 Infinity II Variable Wavelength Detector (VWD) (G7114B), equipped with a Micro Flow Cell VWD, 3 mm, 2 µL, RFID.
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6545XT)

Software

- Agilent MassHunter workstation data acquisition (B.09.00)
- Agilent MassHunter Qualitative Analysis (B.10.00)
- Agilent MassHunter Mass Profiler (B.10.00)

Columns

- Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm (part number 959759-902)
- Agilent ZORBAX RRHD Eclipse Plus C18 Fast Guards, 2.1 × 5 mm, 1.8 µm (part number 821725-901)

Chemicals

LC-grade acetonitrile, ammonium bicarbonate, tris (2-carboxyethyl)phosphine, and 2-iodoacetamide were purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Formic acid was purchased from VWR (Darmstadt, Germany). Trypsin (porcine, mass spectrometry-grade) was obtained from G-Biosciences (St. Louis, USA).

Sample preparation

0.8 mg of the Agilent-NISTmAb (part number 5191-5744) in 100 µL of ammonium bicarbonate (100 mM) was denatured and reduced by the addition of 2 µL of tris(2-carboxyethyl)phosphine (TCEP, 200 mM) and incubated at 60 °C for 1 hour. After the alkylation with 4 µL of 2-iodoacetamide (IAM, 200 mM, 1 hour at RT), quenching of excess IAM with 2 µL of TCEP (1 hour at RT), and subsequent dilution with 0.8 mL of 25 mM ammonium bicarbonate, the enzyme trypsin was added (20:1, NISTmAb to trypsin w/w). After the overnight digestion at 37 °C, the pH of the resulting suspension was decreased below pH 4 by the addition of 2 µL of formic acid.

Results and Discussion

To show the excellent performance and method transfer between the 1290 Infinity II Bio LC and the 1290 Infinity II LC, a tryptic digest of the NISTmAb was analyzed with UV and MS detection. Both systems were equipped with capillaries of the same length and diameters to have similar extra column volumes. However, the 1290 Infinity II Bio LC featured a completely iron-free flow path especially suited for sticky biomolecules. For both analyses, the same ZORBAX RRHD Eclipse Plus column and method parameters were used (Table 1). Figure 1 shows the chromatograms of the peptide maps acquired by both systems. Excellent similarities between the peptide patterns are visible, with

Table 1. Peptide-mapping method for the Agilent 1290 Infinity II LC and Bio LC..

Parameter	Value
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm + Fast Guard 2.1 × 5 mmm
Solvent	A) Water + 0.1% formic acid B) Acetonitrile + 0.1% formic acid
Gradient	0.00 min – 2% B 44.00 min – 45% B 44.01 min – 97% B 50.00 min – 97% B 50.01 min – 2% B 60.00 min – 2% B
Flow rate	0.300 mL/min
Temperature	40 °C with thermal equilibration devices installed
Detection	VWD: 214 nm, 10 Hz/MS: see Table 2
Injection	Injection Volume: 15 µL Sample temperature: 4 °C Wash: 3 s in water (Flush Port)

Table 2. Source and MS parameters for the All Ions MS/MS analysis of peptides.

Parameter	Value
Instrument	Agilent 6545XT AdvanceBio LC/Q-TOF
Gas Temperature	300 °C
Drying Gas Flow	13 L/min
Nebulizer	40 psig
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	500 V
Fragmentor	175 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Acquisition Rate	Positive, extended dynamic range (2 GHz)
Mass Range	m/z 100 to 1,700
Acquisition Rate	6 spectra/sec
Collision Energy	All ions MS/MS—0 V, 10 V, 25 V

For better evaluation, three generic resolution (R_s) values were calculated for both separations (Figure 1) and also showed exceptionally good comparability. To analyze the performance of the 1290 Infinity II Bio LC and 1290 Infinity II LC regarding retention time precision, 12 peptides were chosen, and the corresponding relative standard deviations (RSD) of the retention times were calculated based on 10 consecutive injections. Figure 2 depicts that all RSD values, irrespective of the system, are below 0.1%, showcasing the excellent performance of the Agilent 1290 Infinity II Bio High-Speed Pump and 1290 Infinity II High-Speed Pump. The average RSD value of the 12 peptides even gets as low as 0.039% for the 1290 Infinity II Bio LC, rendering this system an excellent choice for robust and reliable peptide mapping. However, besides high performance, method compatibility between different LC systems is also very important for numerous labs.

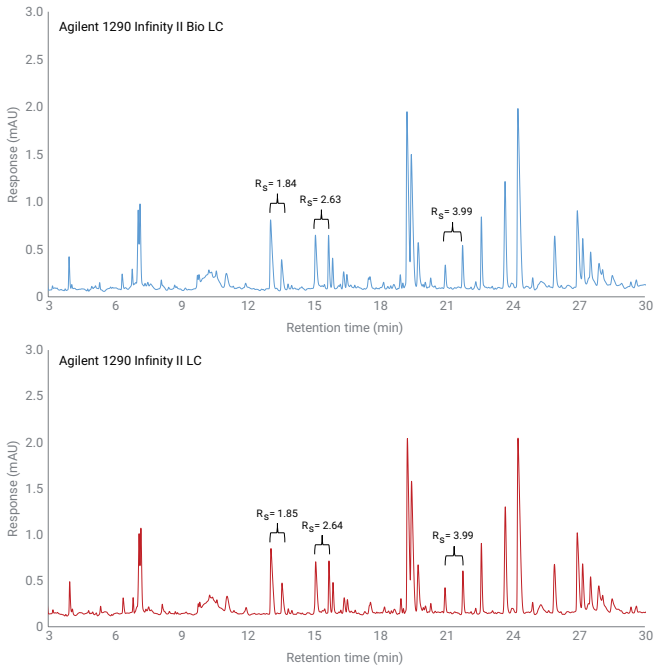


Figure 1. Chromatograms of a tryptic digest of the NISTmAb separated by the Agilent 1290 Infinity II Bio LC and the Agilent 1290 Infinity II Bio LC with the same method (Table 1).

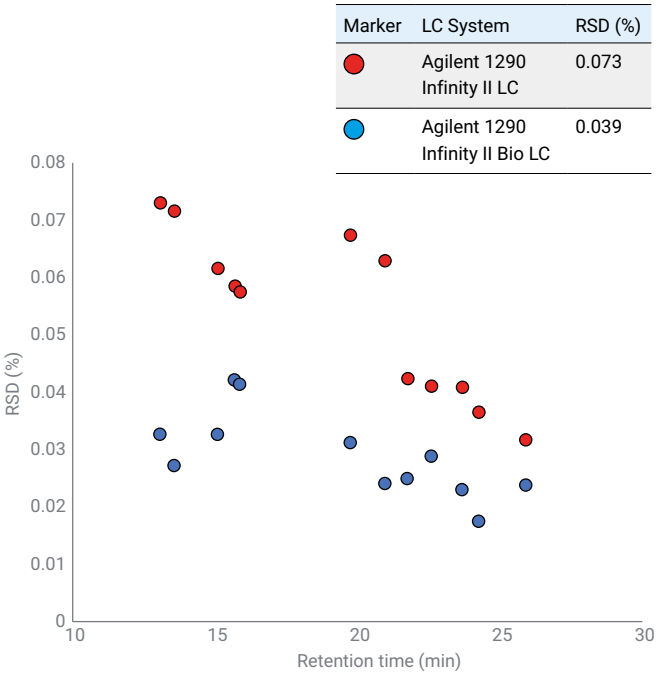


Figure 2. Relative retention time precision (RSD) values for the Agilent 1290 Infinity II Bio LC and the Agilent 1290 Infinity II LC

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LC System	Average Peptide Retention Times (min)											
	1	2	3	4	5	6	7	8	9	10	11	12
Agilent 1290 Infinity II LC	13.082	13.577	15.104	15.704	15.887	19.751	20.968	21.769	22.599	23.684	24.261	25.907
Agilent 1290 Infinity II Bio LC	13.062	13.559	15.084	15.677	15.860	19.743	20.961	21.742	22.585	23.663	24.249	25.907

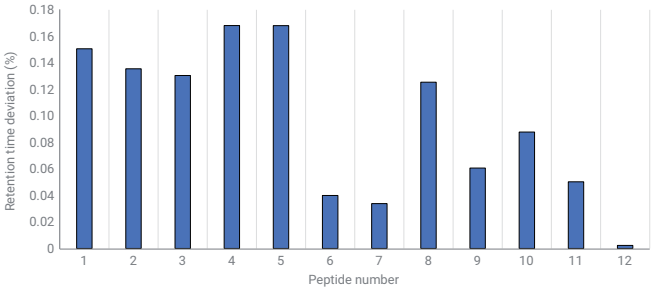

Figure 3. Average retention times for the 12 chosen peptides and their deviations between the two LC systems.

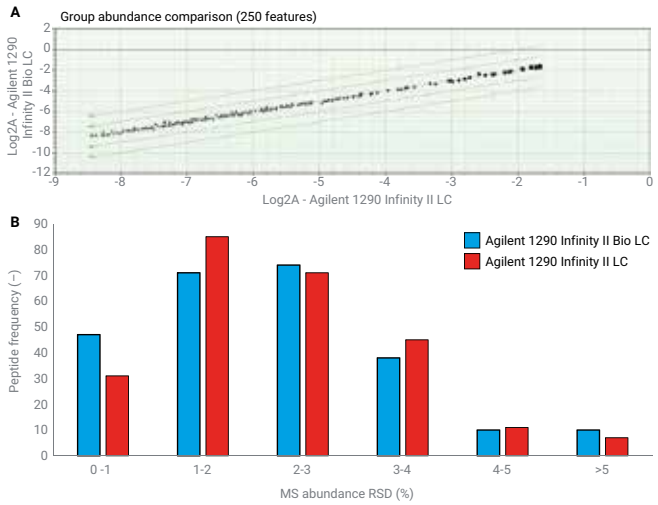

Figure 4. (A) Statistical correlation analysis based on 10 consecutive injections. Log fold values are depicted as black dots for the 250 highest abundance peaks identified by Agilent MassHunter Mass Profiler (B.10.00). (B) Histogram for the RSD values of the peptide abundances for the Agilent 1290 Infinity II Bio LC and 1290 Infinity II LC.

Absolute retention times need to be in certain windows to identify analytes in a validated or compliance environment. By determining the average retention times of the 12 peptides for both LC systems and calculating the deviation of the retention times between the 1290 Infinity II Bio LC and 1290 Infinity II LC, the performances were evaluated. Average peptide retention times are depicted in the table of Figure 3 and corresponding deviations are shown as bar plots. Minimal deviations of up to 0.17% between the LC systems were calculated, showing seamless method transfer between the 1290 Infinity II Bio LC and 1290 Infinity II LC.

To further investigate the method compatibility, both systems were coupled to the Agilent 6545XT AdvanceBio LC/Q-TOF. In an untargeted approach, the MS detector was used in All Ions mode (Table 2), periodically fragmenting all precursor ions in the collision cell. These information-rich data sets were then evaluated with the Agilent MassHunter Mass Profiler (B.10.00) software for both LC systems to get a holistic view of the differences in the abundance of identified peaks. Ten consecutive injections of a tryptic digest of the NISTmAb on both LC systems were the basis for subsequent statistical analysis. The 250 most abundant peaks were evaluated by correlation analysis, and the corresponding log-fold changes are depicted in Figure 4A. If a peak does not differ in both systems, it will cluster around the 1x line in Figure 4A, signaling no significant difference in the peak area. However, if there is a peak with a two-times higher abundance in one system, it would be located around the 2x line. Looking at the graphical data results, it becomes clear that there are no significant differences for most peaks. Up to 75% of the peaks varied with 10% or less in abundance. Even more strikingly, the RSD for the abundances over 10 injections were nearly the same for the 1290 Infinity II Bio LC and 1290 Infinity II LC (Figure 4B). Over 90% of the peptide peaks had an area RSD value of 4% or less.

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Conclusion

Method transfer can sometimes be a laborious and difficult process for many labs when configuring and installing a new LC system. This application note showed that this is not the case for the Agilent 1290 Infinity II Bio LC. By running the same NISTmAb peptide-mapping method on the 1290 Infinity II Bio LC and 1290 Infinity II LC, it was shown that the method could be seamlessly transferred with retention time deviations of only up to 0.17% between the systems. Thanks to the 1290 Infinity II Bio High-Speed Pump, the average relative retention time deviations after 10 injections also showed an excellent value with 0.039%. By coupling both systems with the 6545XT AdvanceBio LC/Q-TOF, a comprehensive statistical analysis of peak abundances showed no significant differences and excellent average RSD of 2.8%. Combining these findings, it is clear that efficient and convenient method transfer between the 1290 Infinity II Bio LC and 1290 Infinity II LC can easily be achieved. The 1290 Infinity II Bio LC is therefore the ideal choice for peptide-mapping workflows regardless of the detection method, with the benefit of an iron-free flow path.

References

1. Agilent 1290 Infinity with ISET. *Agilent Technologies user manual*, publication number G4220-90314, **2015**.
2. ICH HARMONISED TRIPARTITE GUIDELINE PHARMACEUTICAL DEVELOPMENT Q6B, **1999**. Available at: https://database.ich.org/sites/default/files/Q6B_Guideline.pdf.

Additional Application Notes

Publication Number	Title
5991-1813EN	High Resolution Glycopeptide Mapping of EPO Using an Agilent AdvanceBio Peptide Mapping Column
5991-2085EN	Peptide Mapping of Glycoprotein Erythroprotein by HILIC LC/MS and RP-LC/MS
5991-3585EN	Fast and Efficient Peptide Mapping of a Monoclonal Antibody (mAb): UHPLC Performance with Superficially Porous Particles
5991-4920EN	Comparison of Biosimilar and Innovator Monoclonal Antibody Rituximab Using the Agilent 1260 Infinity Bio-inert LC System and Agilent OpenLAB Match Compare Software
5991-6338EN	Peptide Mapping: A Quality by Design (QbD) Approach

Additional Information

For high throughput protein digestion for peptide mapping, the AssayMAP Bravo Platform allows for automated sample preparation. More information can be found at www.agilent.com and in the following documents.

Part Number	Title
5991-6273EN	Agilent AssayMAP Bravo Platform: Automated Protein and Peptide Sample Preparation for Mass Spec Analysis
5991-6478EN	Rapid Antibody Digestion Enabled by Automated Reversed-Phase Desalting on the Agilent AssayMAP Bravo Platform

Peptide standards are available to assist with method development and system checks.

Part Number	Title
5190-0583	10 peptide standard
G2455-85001	HSA peptides standard
G1990-85000	Trypsin digest methylated BSA standard

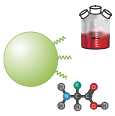
Amino Acid Analysis

Background

Determining the amino acid composition of a protein is a well-established technique that is used with other techniques to confirm the correct structure. Acid hydrolysis is typically used to hydrolyze the protein into its constituent amino acids before analysis. Amino acids are also key ingredients in the cell culture medium used to prepare recombinant proteins. It is often desirable to monitor the consumption of individual amino acids during the fermentation reaction and so the same chromatographic approach may be used.

Amino acids are inherently zwitterionic and possess a variety of side chains, including neutral, hydrophobic, hydrophilic, acid, and basic groups. They also lack a suitable UV chromophore, making the separation and detection of the twenty or so naturally occurring amino acids challenging. Agilent introduced a unique method of amino acid analysis combining precolumn derivatization, employing the liquid handling capabilities of the multisampler, together with reversed-phase separation to baseline resolution of all the common amino acids.

To perform precolumn derivatization the sample is first neutralized in borate buffer at high pH to ensure that the amino terminus of each amino acid is neutralized. Primary amines are then reacted with ortho-phthaldehyde (OPA) and secondary amines (proline, hydroxyproline etc.) are reacted with 9-fluorenylmethylchloroformate (FMOC-Cl). This enables the subsequent separation by reversed-phase to be performed, but the high pH conditions required to obtain baseline resolution means that the latest pH stabilized columns provide the longest column lifetime.



Amino Acid and Cell Culture Analysis

Small molecule chromatography (<150 Å)

Delivers robust, high-resolution separations

AdvanceBio Amino Acid Analysis (AAA)

LC/UV or LC/FLD with sample derivatization

Attribute	Advantage
Exceptional resolution	More reliable results
High pH–resistant C18 stationary phase	Longer column lifetimes
HPLC and UHPLC compatible	Increased flexibility

AdvanceBio MS Spent Media

LC/MS without sample derivatization

Attribute	Advantage
HILIC LC separation/ MS detection	One method for multiple metabolite classes
No sample derivatization needed	Use any LC/MS system
PEEK-lined stainless steel column hardware	Excellent peak shape and recovery

Getting Started

The AdvanceBio Amino Acid Analysis kit combines all the reagents and calibration standards necessary for the analysis into a single part number, 5190-9426. Each component can also be ordered separately if needed. The 'How-To" Guide on the following pages contains detailed instructions for mobile phase preparation, setting up the automated, online amino acid derivatization in the autosampler, and the LC method.

The AdvanceBio Amino Acid Analysis column is packed with C18 silica particles that have been chemically modified for high pH stability. Amino acid separations are most efficient at high pH, and this improvement over previous amino acid analysis solutions significantly extends the lifetime of the column under these mobile phase conditions. To maximize the benefit of this advancement, take care to never store the column in mobile phase A. Short term storage should be in mobile phase B, while long-term storage should be in 50 % acetonitrile. The column is meant to withstand short-term exposure to basic pH during a gradient, but prolonged exposure to high pH will still lead to shortened column lifetimes.

Amino Acid Analysis: “How-To” Guide

Accurate results with AdvanceBio end-to-end solution

The Agilent AdvanceBio Amino Acid Analysis (AAA) end-to-end solution optimizes workflow efficiency by combining the advantages of the Agilent InfinityLab LC Series instrumentation and column technology with proven precolumn derivatization chemistry. It is part of the AdvanceBio family that delivers consistent, exceptional performance for the complete characterization of proteins, antibodies, conjugates, new biological entities, and biopharmaceuticals.

This complete, single vendor solution (including chemicals/standards, columns, and application support) provides fast, sensitive, and automated amino acid analysis. It is based on the latest InfinityLab LC Series instrument and column technology. The automated online derivatization in the Agilent 1290/1260 Infinity II vialsampler eliminates tedious manual procedures and delivers reproducible reaction results. AdvanceBio AAA columns provide the speed and resolution of sub-2 µm columns, but with 50 % less backpressure and reduced risk of column clogging.

The AdvanceBio AAA solution has evolved from proven Agilent ortho-phthalaldehyde/ 9-fluorenyl-methyl chloroformate (OPA/FMOC) reagents for amino acid derivatization. Together with AdvanceBio AAA columns and standards, these reagents provide an ideal, quantitative and qualitative amino acid analysis that combines speed and sensitivity. When used according to the protocol described in this document, the AdvanceBio AAA solution enables the user to separate the amino acids commonly found in protein/peptide hydrolysates.

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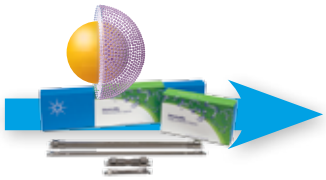
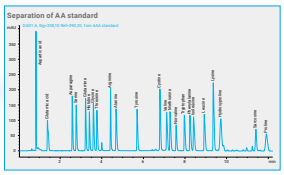
Expert applications and technical support

AdvanceBio AAA reagents and standards
Ready to use

Agilent analytical LC systems
Efficient LC solutions



AdvanceBio AAA columns
Fast, rugged amino acid separation



Fast, reliable data

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AdvanceBio AAA columns: Superficially porous particle (SPP) technology

AdvanceBio AAA columns are based on Agilent's innovative 2.7 µm superficially porous particle (SPP) Poroshell technology—particles consist of a 1.7 µm solid core with a 0.5 µm porous shell.

The 2.7 µm SPPs provide 40-50 % lower backpressure with 80-90 % of the efficiency of sub-2 µm totally porous particles. The SPPs have a narrower particle size distribution than totally porous particles, which results in a more homogeneous column bed, and reduces dispersion in the column. At the same time, the thin porous shell provides lower resistance to mass transfer. What's more, since the columns incorporate a 2 µm frit, they are as resistant to clogging as 3.5 and 5 µm columns.

Until recently, all silica-based SPP materials possessed limited lifetime in higher pH buffers, including phosphate buffers. To achieve longer lifetimes, it is necessary to protect the base particle by either surface modification or special bonding modification. The surface is chemically modified using a proprietary process to form an organic layer that is resistant to silica dissolution at high pH conditions.

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AdvanceBio AAA columns: Superficially porous particle (SPP) technology

The AdvanceBio AAA columns ensure excellent selectivity for amino acid analysis.

Fast and rugged amino acid separation

- The speed and resolution of a sub-2 µm column with up to 50 % less backpressure
- More forgiving for dirty samples, due to 2 µm frits
- Unique chemical modification for high pH stability and column lifetime
- Guard column options reduce your operating costs by extending the life of the columns

Everyday efficiency with confidence

- Higher speed and higher resolution thanks to the operating power up to 600 bar and 5 mL/min
- Injector programming for automated online derivatization
- High-sensitivity UV detection based on diode array technology for uncompromised sensitivity for simultaneous multiwavelength detection
- Optional full spectral detection for identification and peak purity analysis
- Wide flexibility for other LC or UHPLC applications with 100 % HPLC compatibility

Agilent detectors – flexibility in detection

Multiple Wavelength Detector:

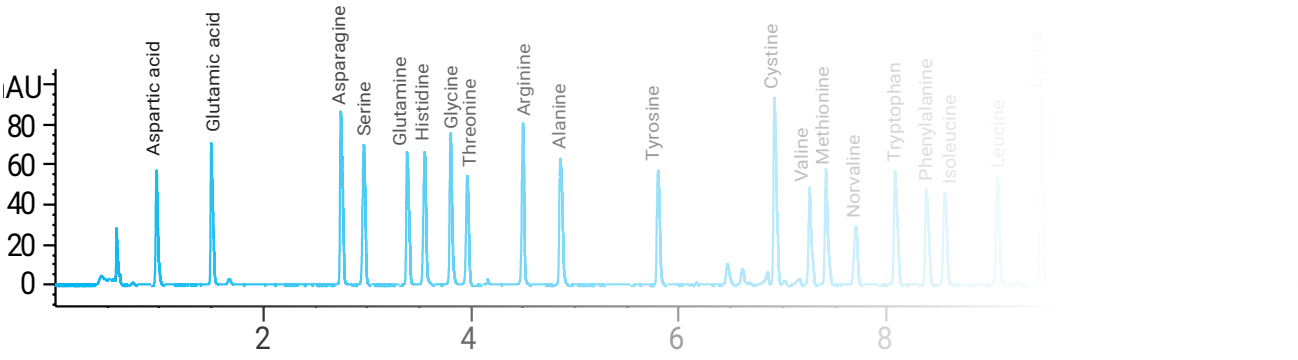
Uncompromised sensitivity for simultaneous multiwavelength detection.

Diode Array Detector with spectral data:

Identification and peak purity analysis with more selectivity and fewer matrix effects.

Fluorescence Detector:

Superior sensitivity in the multi-signal mode in the femto-mole range.



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Advance your confidence: Agilent AdvanceBio Amino Acid Analysis (AAA)

Achieve fast, sensitive, and reproducible separation of amino acids in biological samples

Steps for AAA analysis

1. Prepare HPLC mobile phases
2. Prepare amino acid standards
3. Prepare Internal Standard (ISTD) stock solution
4. Perform online derivatization
5. Set parameters for detection
6. Run high throughput routine analysis
7. Ensure system suitability per European Pharmacacopoeia (Ph. Eur.)
8. Optimize cell culture media and protein hydrolysate standard

Learn more about analyzing amino acids with utmost confidence, visit

www.agilent.com/chem/advancebioaaa

Step 1:

Prepare HPLC mobile phases

Mobile phase A:

10 mM Na_2HPO_4, and 10 mM $Na_2B_4O_7$ pH 8.2

To prepare 1 L, weigh out 1.4 g anhydrous Na_2HPO_4 and 3.8 g $Na_2B_4O_7 \bullet_{10} H_2O$ in 1 L water. Adjust to approximately pH 8.4 with 1.2 mL concentrated HCl, then add small drops of acid and adjust to a final pH of 8.2. Allow stirring time for complete dissolution of borate crystals before adjusting pH. Filter through 0.45 µm regenerated cellulose membranes (p/n3150-0576).

Mobile phase B:

Acetonitrile:methanol:water (45:45:10, v:v:v)

All mobile-phase solvents are HPLC grade.

Mobile phase A is consumed at a faster rate than mobile phase B. Therefore, we recommend preparing 2 L of mobile phase A for every 1 L of mobile phase B.

Injection diluent

The injection diluent is 100 mL of mobile phase A and 0.4 mL concentrated H_3PO_4. This solution is prepared in a 100 mL bottle that should be stored at 4 °C.

0.1 N HCl

Extended amino acid and internal standard stock solutions are prepared in 0.1 N HCl solution. To prepare 0.1 N HCl, add 4.2 mL concentrated HCl (36%) to a 500 mL volumetric flask that is partially filled with water. Mix, and fill to the mark with water. Store at 4 °C.

Derivatization reagents

Derivatization reagents (borate buffers, OPA, and FMOC) are ready-made solutions supplied by Agilent. Simply transfer these reagents from their container into an autosampler vial. Recommended precautions include:

- OPA is shipped in ampoules under inert gas to prevent oxidation. Once opened, the OPA is potent for about 7 to 10 days. We recommend transferring 100 µL aliquots of OPA to microvial inserts and storing in a refrigerator. Replace the OPA autosampler microvial daily.
- FMOC is stable in dry air but deteriorates in moisture. It should also be transferred to microvial inserts in 100 µL aliquots, and stored in a refrigerator. Like the OPA, an open FMOC ampoule transferred to 10 microvial inserts is potent for about 7 to 10 days.
- Borate buffer can be transferred to a 1.5 mL autosampler vial without a vial insert. Replace every three days.

Step 2:

Prepare amino acid standards

Solutions of 17 amino acids (AA) in five concentrations are available from Agilent (10 pmol/µL to 1 nmol/µL) for calibration curves. Store solutions at 4 °C.

To make the extended amino acid (EAA) stock solution, weigh:

- 59.45 mg asparagine
- 59.00 mg hydroxyproline
- 65.77 mg glutamine
- 91.95 mg tryptophan

Add the weighed out amino acids to a 25 mL volumetric flask, fill halfway with 0.1 N HCL and shake or sonicate until dissolved. Then fill to mark with water for a total concentration of 18 nmol/µL of each amino acid.

For the high-sensitivity EAA stock solution, take 5 mL of this standard-sensitivity solution and dilute with 45 mL water (1.8 nmol/µL). Solutions containing extended standards are unstable at room temperature. Keep them frozen and discard at first signs of reduced intensity.

Step 3:

Prepare Internal Standard (ISTD) stock solution

For primary amino acid ISTD stock solutions, weigh 58.58 mg norvaline into a 50 mL volumetric flask. For secondary amino acids, weigh 44.54 mg sarcosine into the same 50 mL flask. Fill halfway with 0.1 N HCl and shake or sonicate until dissolved. Finally fill to mark with water for a final concentration of 10 nmol for each amino acid/µL (standard sensitivity). For high-sensitivity ISTD stock solution, take 5 mL of standard-sensitivity solution and dilute with 45 mL of water. The final concentration of the high-sensitivity ISTD is 1 nmol for each amino acid/µL. Store at 4 °C.

Calibration curves may be made using two to five standards depending on experimental need. Typically 100 pmol/µL, 250 pmol/µL, and 1 nmol/µL are used in a three-point calibration curve for "standard sensitivity" analysis.

The following tables should be followed if an internal standard or other amino acid (for example, the extended amino acids) is added. Table 1 describes "standard sensitivity" concentrations typically used in UV analysis. Table 2 is typically used for "high sensitivity" fluorescence analysis.

Table 1. Standard sensitivity calibration standards

	Concentration of Final AA Solution (pmol/µL)		
	900	**225**	**90**
Take 5 mL of 18 nmol EAA Dilute with water	5 mL -	5 mL 15 mL	5 mL 45 mL
Diluted EAA mix	5 mL	20 mL	50 mL
Take 5 mL of diluted EAA mix	5 mL	5 mL	5 mL
Add 10 nmol ISTD solution	5 mL	5 mL	5 mL
EAA-ISTD mix	10 mL	10 mL	10 mL
Take 100 µL of EAA-ISTD mix	100 µL	100 µL	100 µL
For 1 nmol AA, add	900 µL	-	-
For 250 pmol AA, add		900 µL	-
For 100 pmol AA, add	-	-	900 µL
Final AA solution with EAA and 500 pmol/µL ISTD	1 mL	1 mL	1 mL

Table 2. High sensitivity calibration standards

	Concentration of Final AA Solution (pmol/µL)		
	90	**22.5**	**9**
Take 5 mL of 1.8 nmol EAA Dilute with water	5 mL -	5 mL 15 mL	5 mL 45 mL
Diluted EAA mix	5 mL	20 mL	50 mL
Take 5 mL of diluted EAA mix	5 mL	5 mL	5 mL
Add 1 nmol ISTD solution	5 mL	5 mL	5 mL
High-sensitivity EAA-ISTD mix	10 mL	10 mL	10 mL
Take 100 µL of EAA-ISTD mix	100 µL	100 µL	100 µL
For 100 nmol AA, add	900 µL	-	-
For 25 pmol AA, add		900 µL	-
For 10 pmol AA, add	-	-	900 µL
Final AA solution with EAA and 50 pmol/µL ISTD	1 mL	1 mL	1 mL

Step 4:

Perform online derivatization

Depending on the autosampler model, the automated online derivatization program differs slightly. For the Agilent G7129A well plate automatic liquid sampler with 100 µL capillary* (WPALS), with injection program is as follows:

1. Draw 2.5 µL from borate vial (p/n5061-3339)
2. Draw 1.0 µL from sample vial
3. Mix 3.5 µL in wash port five times
4. Wait 0.2 minutes
5. Draw 0.5 µL from OPA vial (p/n 5061-3335)
6. Mix 4.0 µL in wash port 10 times default speed
7. Draw 0.4 µL from FMOC vial (p/n 5061-3337)
8. Mix 4.4 µL in wash port 10 times default speed
9. Draw 32 µL from injection diluent vial
10. Mix 20 µL in wash port eight times
11. Inject
12. Wait 0.1 minutes
13. Valve bypass

* Note: other autosampler models may have a different volume capillary installed, which will require adjustment of volumes

The location of the derivatization reagents and samples is up to the analyst and the ALS tray configuration. Using the G7129A with a 2 × 56 well plate tray (p/n G2258-44502), the locations are:

- Vial 1: Borate buffer
- Vial 2: OPA
- Vial 3: FMOC
- Vial 4: Injection diluent
- P1-A-1: Sample

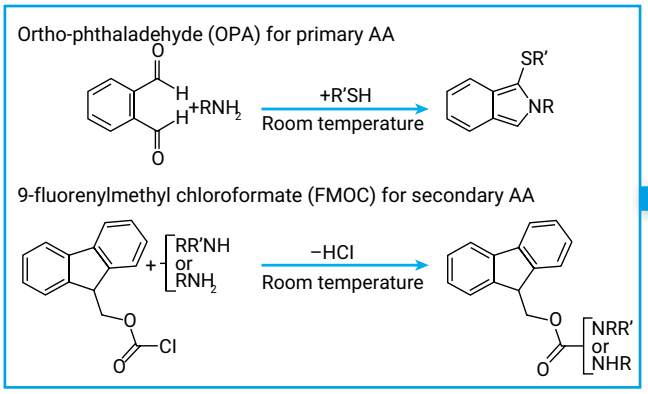
Note: Use the correct vials, closures, and pumps parameters

Conical vial inserts with polymer feet (Figure 1A) are required to hold the OPA and FMOC reagents because of the limited volumes involved. The inserts are compatible with wideopening screw-top (Figures 1B and 1C) or crimp-top vials. For this procedure, an airtight seal is needed for both FMOC, which is highly volatile, and OPA, as it slowly degrades in the presence of oxygen. Snap-cap vials should therefore not be used in this procedure. Be careful not to use vials or caps designed for other instruments, to prevent damage to the auto injector.

Pump parameters for all methods include compressibility (×10-6 bar) A: 40, B: 80, with minimal stroke A, B of 20 µL.

Figure 1. Insert, vial, and cap for amino acid analysis using the Agilent 4226A autosampler: A) Conical insert (Agilent p/n 5181-1270), B) amber wide opening vial (Agilent p/n 5182-0716), and C) screw cap (Agilent p/n 5182-0721).

Increase precision with Autosampler automation



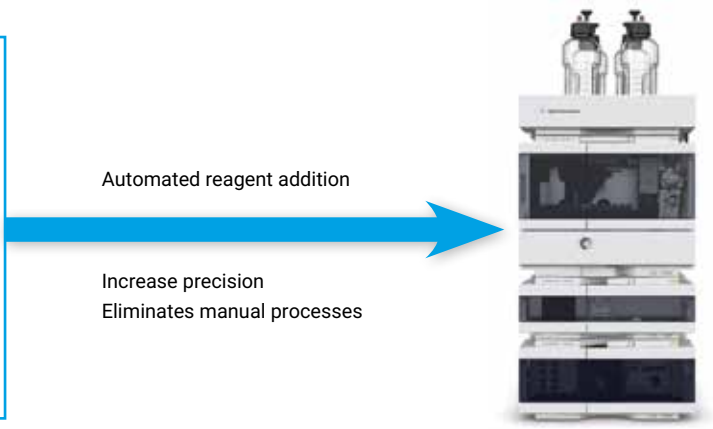


Figure 2. Online derivatization of OPA and FMOC: Separation of polar amino acids on RP-phase and detection by UV and Fluorescence

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Step 5:

Set parameters for detection

Thermostatted column compartment (TCC)

Left and right temperatures should be set to 40 °C. Enable analysis when the temperature is within ± 0.8 °C.

Diode array detector (DAD)

Signal A: 338 nm	10 nm bandwidth	Reference wavelength 390 nm	20 nm bandwidth
Signal B: 262 nm	16 nm band-width	Reference wavelength 324 nm	8 nm bandwidth
Signal C*: 338 nmix	10 nm band-width	Reference wavelength 390 nm	20 nm bandwidth

*Signal C is not required if the instructions below are followed.

To detect both OPA and FMOC derivitized amino acids in a single chromatogram it is necessary to switch detector wavelength between the last eluting OPA derivitized amino acid, lysine (peak 20 in the standard), and before the first eluting FMOC derivitized amino acid, hydroxyproline (peak 21 in the standard).

Determining the appropriate transition point using the DAD is possible by initially collecting two channels (Signal A 338 nm, to detect OPA derivitized amino acids and Signal B 262 nm, to detect FMOC derivitized amino acids). This will determine the ideal point at which to switch the wavelength during the run. Subsequent runs can be made using a single channel with the detector timetable function used to program a wavelength switch from 338 to 262 nm at the appropriate time. Between the elution of OPA-lysine and FMOC-hydroxyproline, allow time for both OPA and FMOC derivitized amino acids to be detected in a single chromatogram.

Peak width settings of > 0.01 minutes are used for all columns.

Fluorescence detection

FLD should always be the last detector module in the flow stream to avoid damage to the pressure sensitive flow cell (max 20 bar).

Peak width 0.01 min, stop time 18 min (adjust as needed)

Excitation 340 nm; Emission 450 nm; Filter 390 nm (Default filter)

Timetable Signal:

0.00 min Excitation 340 nm, Emission 450 nm; Gain (as needed)

5.53 min Excitation 260 nm, Emission 325 nm;

PMT Gain 10 (as needed; transition between lysine and hydroxyproline)

To determine the transition point needed with fluorescence detection (FLD), it is necessary to perform two separate runs: the first using Excitation 340 nm, Emission 450 nm to detect the OPA derivitized amino acids and the second using Excitation 260 nm, Emission 325 nm to detect the FMOC derivitized amino acids. Both OPA and FMOC derivitized amino acids can be detected in a single chromatogram, using the detector timetable function. This function programs a wavelength switch at the appropriate point after the last eluting OPA derivitized amino acid, lysine (peak 20 in the standard) and before the first eluting FMOC derivitized amino acid, hydroxyproline (peak 21 in the standard).

Note: If the concentration of amino acids is below 100 pmol, we recommend the use of fluorescence detection.

Gradient program

Time (min)	% B
0	2
0.35	2
13.4	57
13.5	100
15.7	100
15.8	2
18	end

Flow rate: 1.5 mL/min for 4.6 mm id columns and 0.62 mL/min for 3 mm id columns.

Injection volume: 1 µL with needle wash at the port for 7 s.

Typical Separations

A separation of 20 amino acids using an AdvanceBio AAA column is shown in Figure 3.
The following parameters are noted:

- No change in elution profile of amino acids with and without NaN_3 in mobile phase.
- NaN_3 is used only as a preservative to contain bacterial/fungal growth.
- Filtering the mobile phase using 0.45 µm filter is highly recommended. Note: If the concentration of amino acids is below 100 pmol, we recommend the use of fluorescence detection.

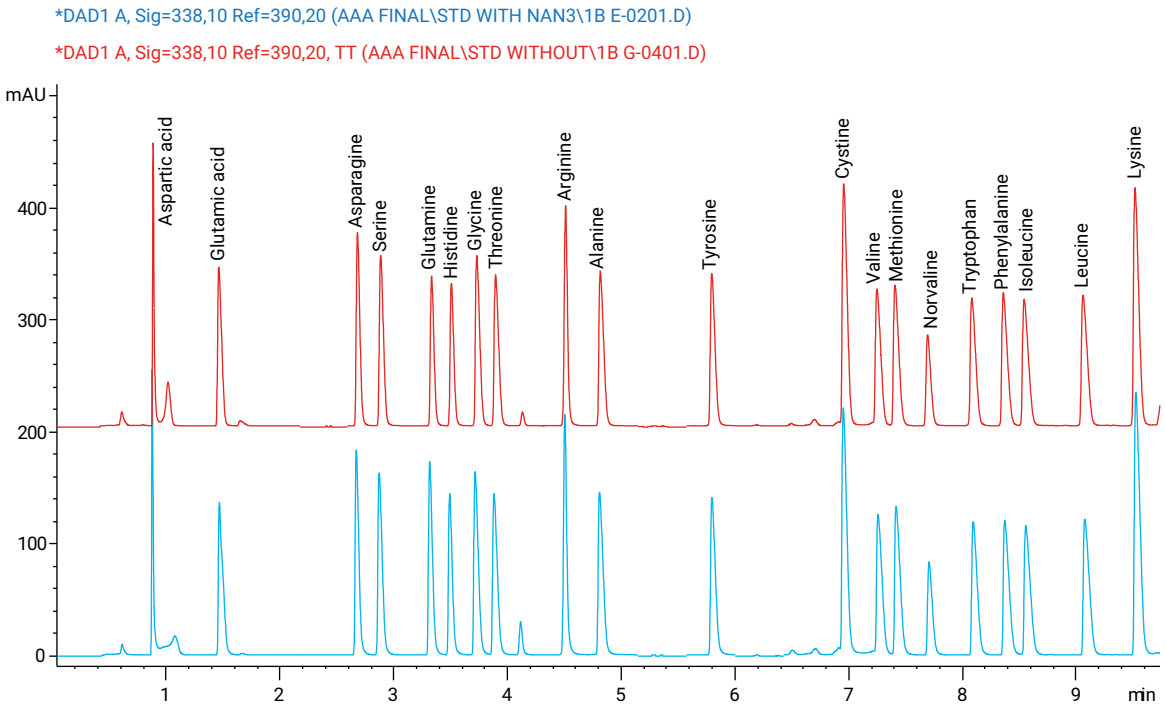

Figure 3. Separation of 20 amino acid standards using an Agilent AdvanceBio AAA 4.6 x 100 mm column with and without 5 mM sodium azide in mobile phase.

Note: Addition of 5 mM sodium azide (NaN_3) to mobile phase A is optional to prevent microbiological growth and extend shelf life of buffers.

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Step 6:

Run high-throughput amino acid analysis

The chromatogram in Figure 4 illustrates typical routine standard sensitivity in high-throughput applications that can be obtained using Agilent AdvanceBio AAA columns. These separations were produced using the Agilent 1260 Infinity II HPLC binary system with AdvanceBio AAA, 100 mm, 2.7 µm columns of different internal diameters, and DAD detection. A single run can be completed in under 20 minutes (including re-equilibration) with adequate resolution. The primary amino acids (1-20, OPA-derivatized), were monitored at 338 nm, while the secondary amino acids (21-23, FMOC-derivatized), were monitored at 262 nm.

The first 20 amino acids in Figure 4, the primary amino acids, are derivatized with OPA. The last three, hydroxyproline, sarcosine, and proline, are derivatized with FMOC. A programmable wavelength switch from 338 to 265 nm takes place after lysine (peak 20) elutes and before hydroxyproline (peak 21) elutes.

- The method can easily be scaled to different column dimensions.
- In this case, the only changes to the method were made by altering the flow rate, changed geometrically with the diameter of the column.
- The low-volume heat exchanger was used with short red tubing to minimize extra column volume.

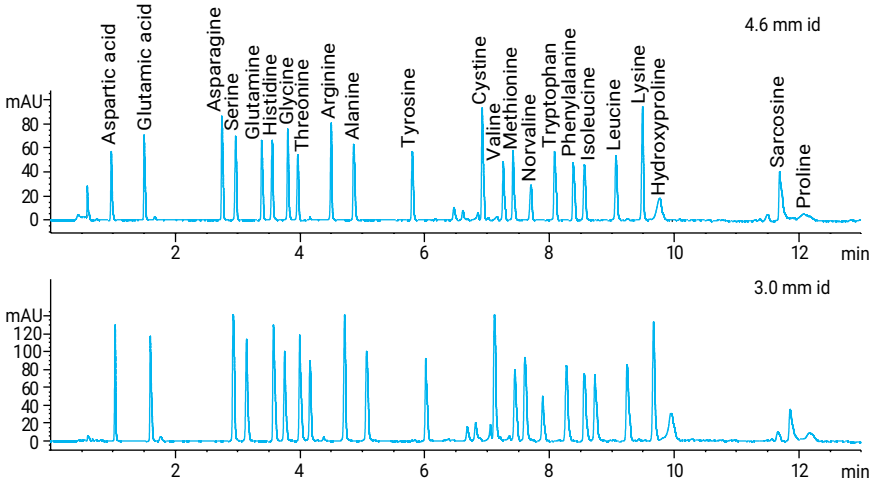

Figure 4. Separation of AA standards using Agilent AdvanceBio AAA columns of different internal diameters using the amino acid method.

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Retention time and area precision for 100 pmol and 1000 pmol analysis (n=6)

Table 3. Retention time and area RSD precision for amino acids (100 pmol) separated using an AdvanceBio AAA, 4.6 x 100 mm column (six replicates.)

Amino Acids	Average RT	RT RSD (%)	Area RSD (%)
1. Aspartic acid	0.851	1.270	1.066
2. Glutamic acid	1.428	0.973	1.850
3. Asparagine	2.639	0.605	1.790
4. Serine	2.835	0.629	1.820
5. Glutamine	3.285	0.470	1.560
6. Histidine	3.465	0.430	1.220
7. Glycine	3.681	0.477	1.920
8. Threonine	3.837	0.440	1.950
9. Arginine	4.458	0.251	2.150
10. Alanine	4.764	0.280	3.060
11. Tyrosine	5.762	0.128	1.650
12. Cystine	6.870	0.067	1.900
13. Valine	7.201	0.084	2.47
14. Methionine	7.363	0.073	1.82
15. Norvaline	7.602	0.073	1.72
16. Tryptophan	8.055	0.054	1.57
17. Phenylalanine	8.341	0.051	1.66
18. Isoleucine	8.503	0.047	1.72
19. Leucine	9.000	0.030	1.70
20. Lysine	9.428	0.028	1.66
21. Hydroxyproline	9.747	0.021	4.13
22. Sarcosine	10.980	0.026	1.15
23. Proline	11.620	0.021	4.36

Table 4. Retention time and area RSD precision for amino acids (1000 pmol) separated using an AdvanceBio AAA, 4.6 x 100 mm column (six replicates).

Amino Acids	Average RT	RT RSD (%)	Area RSD (%)
1. Aspartic acid	0.837	0.151	2.60
2. Glutamic acid	1.400	0.512	2.19
3. Asparagine	2.583	0.124	2.13
4. Serine	2.772	0.114	1.74
5. Glutamine	3.220	0.092	1.80
6. Histidine	3.405	0.077	1.39
7. Glycine	3.598	0.068	1.48
8. Threonine	3.766	0.059	2.26
9. Arginine	4.422	0.027	1.66
10. Alanine	4.685	0.031	1.87
11. Tyrosine	5.695	0.034	2.04
12. Cystine	6.794	0.030	2.22
13. Valine	7.118	0.025	2.40
14. Methionine	7.281	0.025	1.78
15. Norvaline	7.573	0.019	1.77
16. Tryptophan	7.970	0.024	2.03
17. Phenylalanine	8.238	0.027	1.98
18. Isoleucine	8.413	0.025	2.17
19. Leucine	8.925	0.020	1.81
20. Lysine	9.357	0.022	2.00
21. Hydroxyproline	9.718	0.014	3.14
22. Sarcosine	10.961	0.015	5.91
23. Proline	11.911	0.011	2.58

Step 7:

Ensure system suitability as per European Pharmacopoeia

The The European Pharmacacopoeia (Ph. Eur.) defines requirements for the qualitative and quantitative composition of amino acids and mixtures of amino acids. The requirements for allowed impurities are also defined. Manufacturers of amino acids are legally bound to prove that their amino acids meet these specifications before they can distribute their products in Europe.

Leucine (Leu) is a branched-chain α-amino acid, produced by the fermentation process. During this process, isoleucine can be produced as a by-product. The European Pharmacopoeia states that leucine and isoleucine should have a resolution of not less than 1.5 [1]

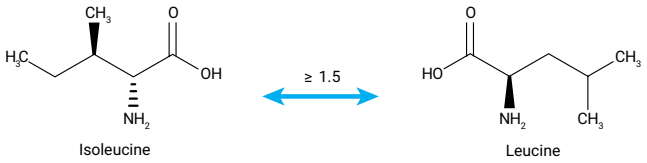


Table 5. System suitability testing using Agilent AdvanceBio AAA columns and AA standards

System Suitability	AdvanceBio AAA, C18, 4.6 × 100 mm, 2.7 µm	AdvanceBio AAA, C18, 3.0 × 100 mm, 2.7 µm
Resolution between Leucine and Isoleucine (≥1.5)	4.5	4.6

Reference:

1. European pharmacopoeia 9.0 (2.2.56)
Amino Acid Analysis

Step 8:

Optimize cell culture media and protein hydrolysate standard

Cell cultures are widely used to produce biopharmaceuticals and other biologically active compounds. The composition of the cell culture media affects the yield and structure of the desired products and must be carefully optimized. Cell culture media is typically composed of mixtures of amino acids, vitamins, carbohydrates, inorganic salts, as well as different peptides, proteins, and other compounds. As the cells grow, they consume nutrients and release target biopharmaceuticals as well as waste products. Amino acids serve as the building blocks of proteins, as well as intermediates in many metabolic pathways. Therefore, amino acids are typically added to cell culture media to provide nutritional requirements for the cells.

Determination of amino acid flux in cultured cells is an important indicator of the metabolic rate and health of those cells. It can also be used as an indicator of the remaining carbon and nitrogenous fuel available. This is especially true in hepatocyte and hepatoma cell lines, where extensive gluconeogenesis, urea production, and protein synthesis may consume larger quantities of amino acids than other cell types.

HPLC with precolumn derivatization is a standard technique in the analysis of amino acids. Precolumn derivatization of free amino acids in solution for HPLC separations with UV or fluorescence detection is at times done manually offline. Some immediate drawbacks to offline derivatization are sources of error due to operator skill, competence, and laboratory technique; extra sample manipulation; extra time required; and increased risk of contamination. Automated online derivatization minimizes these error sources, immediately improves precision, and saves time. A rugged high-resolution HPLC method including online derivatization, therefore, can increase productivity compared to offline methods.

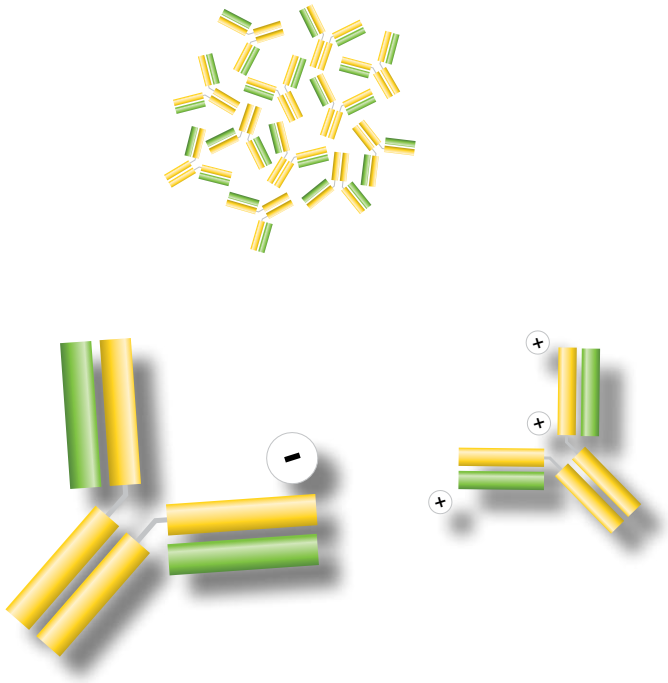
Amino acid compositional analyses of commonly used cell culture media and protein hydrolysate are shown in Figures 5-8. This analysis confirms that the amino acid composition of cell culture media accurately matches with their theoretical composition. Such applications are useful for monitoring and adjusting amino acid composition. This analysis is an essential part of optimizing the manufacturing process to ensure high quality and optimum yield of the final biopharmaceutical product.

The following cell culture media are used for compositional analysis using amino acid method with an AdvanceBio AAA 4.6 x 100 mm column (Figures 5-8).

1. Minimum Essential Medium Eagle (MEM) M4655: L-arginine, L-Cystine, L-Glutamine, L-Histidine, L-Isoleucine, L- Leucine, L-Lysine, L-Methionine, L- Phenylalanine, L-Threonine, L-Tryptophan, L- Tyrosine, and L-Valine.

2. Non-Essential Amino Acid (NEAA) Cell Culture Supplement M7145: L-Alanine, L-Asparagine, L-Aspartic acid, L-Glutamic acid, Glycine, L-Proline, and L-Serine.

3. RPMI 1640 R0083: L-arginine, L-Asparagine, L-Cystine, Glycine, L-Histidine, Hydroxy-L-Proline, L-Isoleucine, L-Leucine, L-Lysine, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine, and L-Valine.



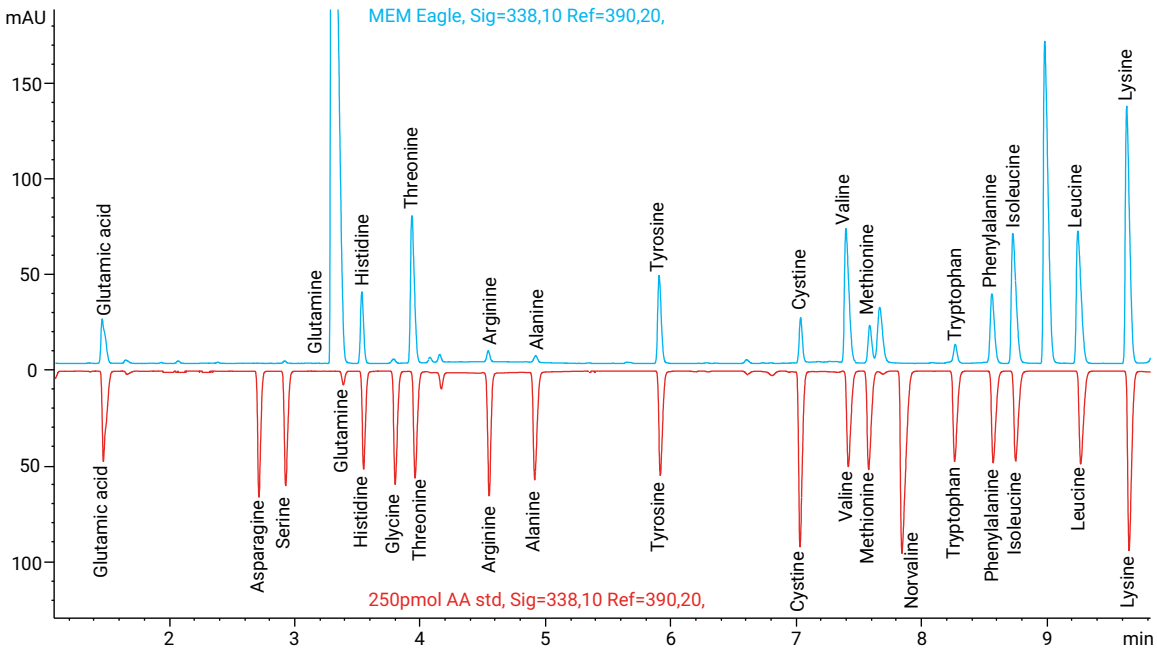


Figure 5. Amino acid analysis of Eagles MEM media (blue trace) and comparison with amino acid standards using the Agilent AdvanceBio AAA solution.

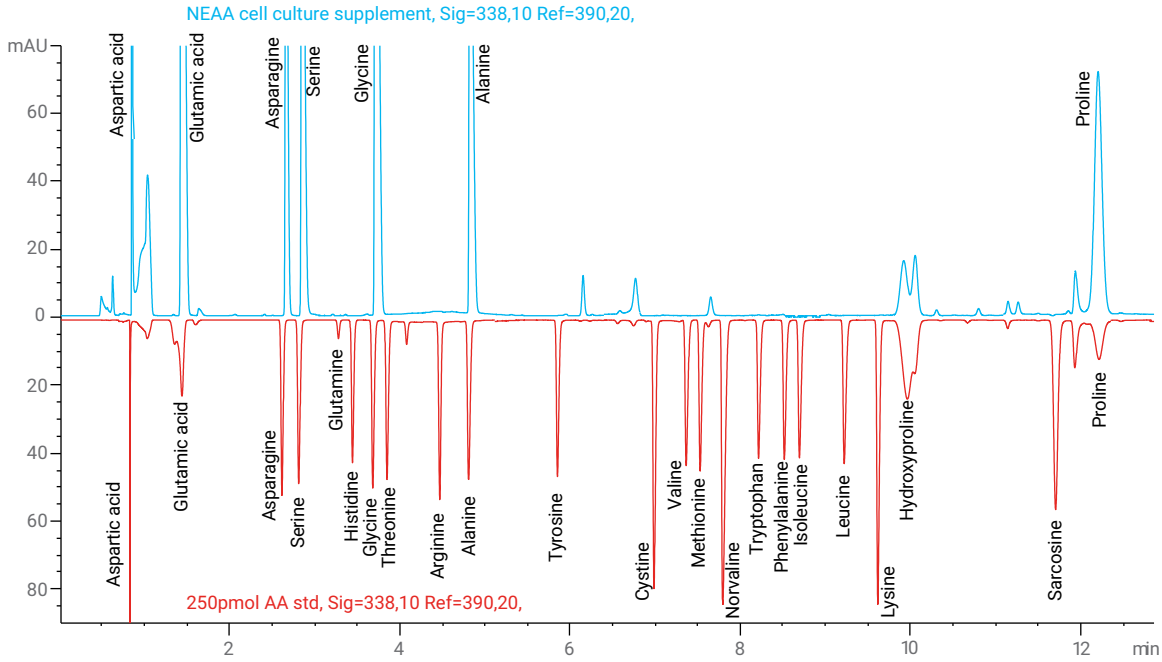


Figure 6. Amino acid analysis of Non-Essential Amino Acid (NEAA) media (blue trace) and comparison with amino acid standard using the Agilent AdvanceBio AAA solution.

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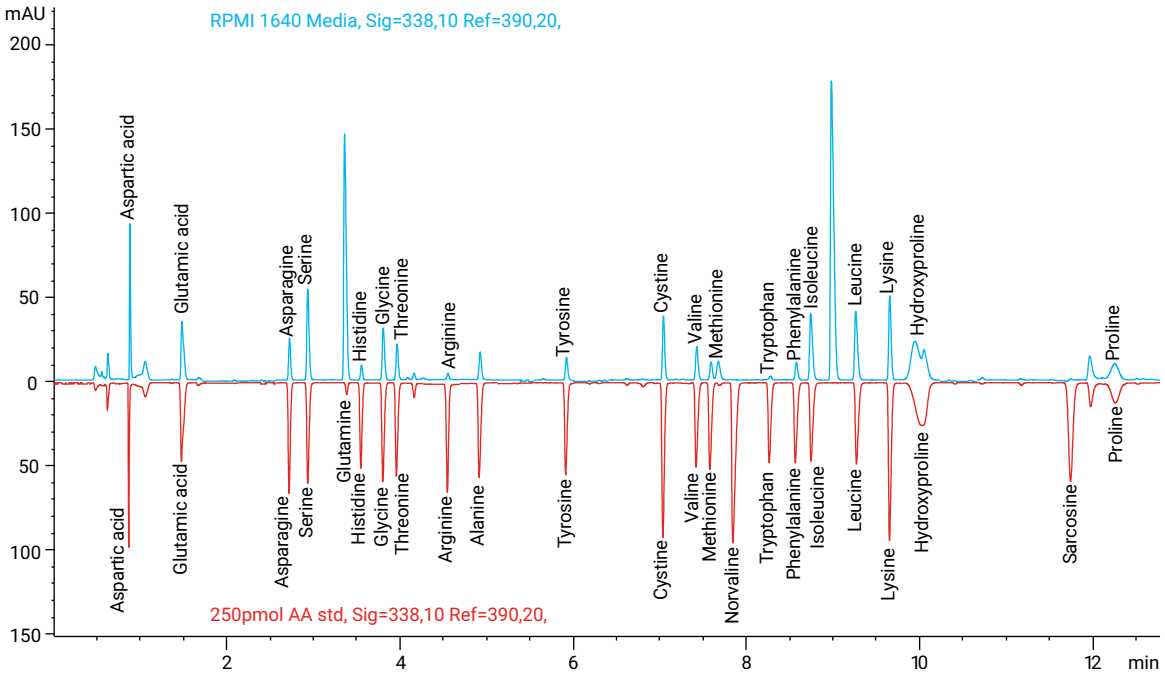

Figure 7. Amino acid analysis of RPMI 1650 media (blue trace) and comparison with amino acid standard using the Agilent AdvanceBio AAA solution.

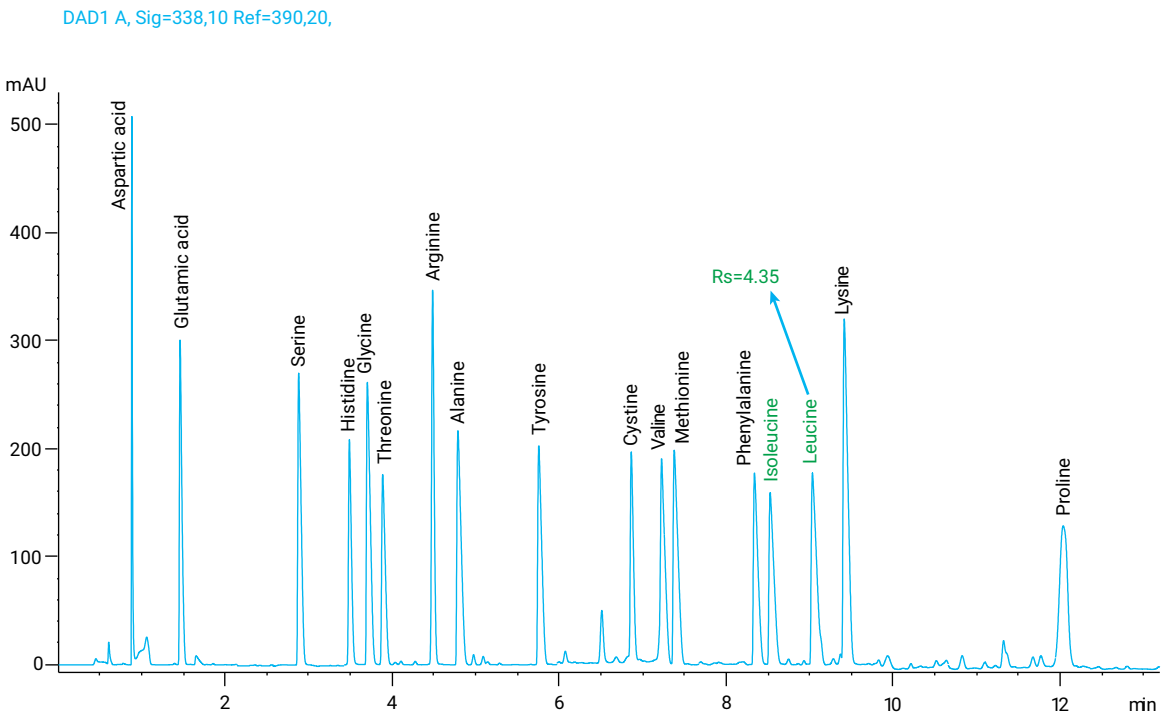

Figure 8. Amino acid analysis of protein hydrolysate. The resolution between leucine and Isoleucine with the AdvanceBio AAA, 4.6 x 100 mm, 2.7 µm column is much higher than the reported value for system suitability requirement.

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Maintenance and troubleshooting

The Agilent AdvanceBio AAA solution includes technical and application support. The following maintenance and troubleshooting tips are recommended to keep your InfinityLab LC Series instrument systems running smoothly.

Daily Maintenance:

- Replace derivatization reagent, borate buffer, amino acid standards, and wash water, which are placed in autosampler tray.
- Recalibration of retention times and response factors.
- Check column and guard column performance using system suitability report.
- Every two days replace mobile phase A and B with freshly made solvents

Troubleshooting:

Poor chromatographic resolution

- Exhausted guard column
- Damaged analytical column
- Post column band broadening due to too long connections.
- Always use short red tubing with the low-volume heat exchanger be to minimize extracolumn volume

Low Intensity Chromatogram

- OPA reagent has deteriorated
- FMOC reagent has deteriorated
- Glycine contamination

Ordering Information

Columns, supplies and chemicals	Size	Part No.
AdvanceBio AAA LC column	4.6 x 100 mm, 2.7 µm	655950-802
AdvanceBio AAA guard columns	4.6 x 5 mm, 2.7 µm, 3/pk	820750-931
AdvanceBio AAA LC column	3.0 x 100 mm, 2.7 µm	695975-322
AdvanceBio AAA guard columns	3.0 x 5 mm, 2.7 µm, 3/pk	823750-946
Borate Buffer	0.4 M in water, pH 10.2, 100 mL	5061-3339
FMOC Reagent	2.5 mg/mL in ACN, 10 x 1 mL ampoules	5061-3337
OPA Reagent	10 mg/mL in 0.4 M borate buffer and 3-mercaptoproprionic acid, 6 x 1 mL ampoules	5061-3335
Dithiodipropionic Acid (DTDPA) reagent	5 g	5062-2479
Inserts, with polymer feet	250 µL, 100/pk	5181-1270
Vial, screw top, amber with write-on spot	2 mL, certified, 100/pk	5182-0716
Cap, screw, green, PTFE/white silicone septum	100/pk	5182-0721
Vial, screw top, clear, flat bottom	for LC, 6 mL, certified, 100/pk	9301-1377
Cap, screw	for 6 mL vials, 100/pk	9301-1379
Septum	for 6 mL vials, 100/pk	9301-1378
AA standard	1 nmol/µL, 10 x 1 mL	5061-3330
AA standard	250 pmol, 10/pk	5061-3331
AA standard	100 pmol/µL, 10 x 1 mL	5061-3332
AA standard	25 pmol/µL, 10 x 1 mL	5061-3333
AA standard	10 pmol/µL, 10 x 1 mL	5061-3334
Amino acids supplement kit		5062-2478

Learn more about the Agilent AdvanceBio family of innovations, designed specifically for biomolecule characterization, visit
www.agilent.com/chem/advancebio

Application Note

Amino Acid Analysis

Agilent

Trusted Answers

Determination of Amino Acid Composition of Cell Culture Media and Protein Hydrosylate Standard

The Agilent AdvanceBio Amino Acid Solution

Authors

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Agilent Technologies, Ltd

Abstract

This study presents a method for analyzing primary amino acids in cell culture media using the Agilent AdvanceBio Amino Acid Analysis (AAA) solution with absorbance detection. Derivitization using an online injector program with OPA and FMOC decreases sample preparation time, and increases reproducibility over traditional offline methods. The effectiveness of this solution for routine analysis was confirmed using a system suitability test and retention time and area precision studies. The AdvanceBio AAA solution provides sensitive and high-resolution separation of all amino acids in cell culture media. The limit of detection (LOD), limit of quantification (LOQ), and linearity for selected amino acids for qualitative assays are also reported.

Introduction

Amino acids are the basic building blocks of proteins. They constitute all proteinaceous material of the cell including the cytoskeleton and the protein component of enzymes, receptors, and signaling molecules. In addition, amino acids are used for the growth and maintenance of cells. Cell culture media plays a key role in the biopharma industry. A large proportion of the amino acids supplied from cell culture media are diverted to pathways that could influence the fate of the cells in a culture. The identification of the optimal concentration of amino acids is important in fed batch and perfusion culture. Therefore, the design of an amino acid supplementation strategy might be streamlined by identifying the amino acid demands of a cell culture due to host cell growth and product production.

HPLC with precolumn derivatization is commonly used for the analysis of amino acids. Precolumn derivatization of free amino acids in solution for HPLC separations with UV or fluorescence detection is sometimes done manually, offline. Some immediate drawbacks to offline derivatization are sources of error due to operator skill, competence, and laboratory technique. Other drawbacks include extra sample manipulation, extra time required, and increased risk of contamination. Automated online derivatization minimizes these error sources, immediately improves precision, and saves time. Thus, a rugged high-resolution HPLC method including online derivatization, can increase productivity compared to offline methods. Consistent automated OPA derivatization, using the injector programming of the HPLC's autosampler and highly efficient Agilent AdvanceBio AAA columns, generate a rapid-reproducible amino acid method ideal for cell culture media. This method is convenient because the cell media samples are simply transferred to autosampler vials and analyzed. The selectivity of the AdvanceBio AAA column and the mobile phase gradient provides high resolution of 23 amino acids.

Materials and Methods

Instrumentation

Analyses were performed using an Agilent 1290 Infinity LC, which was equipped with an Agilent 1290 Infinity binary pump delivery system (G4220A), Agilent 1290 Infinity autosampler (G4226A), Agilent 1290 Infinity thermostatted column compartment (G1316C), and Agilent 1290 Infinity DAD (G4212A).

Reagents, samples, and materials

Cell culture media for compositional analysis, Minimum Essential Medium Eagle (M4655), Non-Essential Amino Acid (M7145), RPMI 1640 (R0083), Na2HPO4, and Na2B4O7•10H2O, were bought from Sigma-Aldrich. Protein hydrolysate was obtained from Fisher Scientific. Acetonitrile and methanol used were bought from Lab-Scan (Bangkok, Thailand). HPLC grade and highly purified water from a Milli-Q water purification system (Millipore Elix 10 model, USA) was used.

Column

Agilent AdvanceBio AAA, C18, 4.6 × 100 mm, 2.7 µm (p/n 655950-802)

Preparation of HPLC mobile phase

Mobile phase A contained 10 mM Na_2HPO_4, and 10 mM $Na_2B_4O_7$, pH 8.2. Mobile phase B contained acetonitrile, methanol, and water (45:45:10, v:v:v). Since mobile phase A is consumed at a faster rate than B, it is convenient to make 2 L of A for every 1 L of B produced.

Injection diluent

The injection diluent was 100 mL mobile phase A, plus 0.4 mL concentrated H_3PO_4 in a 100 mL bottle, stored at 4 °C. To prepare 0.1 N HCl, 4.2 mL concentrated HCl (36%) was added to a 500 mL volumetric flask that was partially filled with water, mixed, then filled to the mark with water. This solution was then used for making extended amino acid and internal standard stock solutions. It was stored at 4 °C.

Agilent AdvanceBio AAA standards and reagents kit, p/n 5190-9426, includes:

Part number	Component
5061–3339	Borate buffer: 0.4 M in water, pH 10.2, 100 mL
5061–3337	FMOC reagent, 2.5 mg/mL in ACN, 10 × 1 mL ampules
5061–3335	OPA reagent, 10 mg/mL in 0.4 M borate buffer and 3-mercaptoproprionic acid, 6 × 1 mL ampules
5062–2479	Dithiodipropionic acid (DTDPA) reagent, 5 g
5061–3330	AA standard, 1 nmol/µL, 10 × 1 mL
5061–3331	AA standard 250 pmol, 10/pk
5061–3332	AA standard, 100 pmol/µL, 10 × 1 mL
5061–3333	AA standard, 25 pmol/µL, 10 × 1 mL
5061–3334	AA standard, 10 pmol/µL, 10 × 1 mL
5062–2478	Amino acids supplement kit, 1 g each

Derivatization reagents

Derivatization reagents (borate buffers, OPA, and FMOC) are ready-made solutions supplied by Agilent, and were transferred from their containers to autosampler vials. Precautions included:

- OPA is shipped in ampules under inert gas to prevent oxidation. Once opened, the OPA is potent for approximately 7 to 10 days. Therefore, 100 µL aliquots of OPA were transferred in microvial inserts and refrigerated. The OPA autosampler microvial was then replaced daily. Each ampule lasted 10 days (one vial/day).
- FMOC is stable in dry air, but deteriorates in moisture. Therefore, FMOC was transferred in 100 µL aliquots to microvial inserts and refrigerated. An open FMOC ampule transferred to 10 microvial inserts should last 10 days.
- Borate buffer was transferred to a 1.5 mL autosampler vial without a vial insert, and replaced every 3 days.

Preparation of amino acid standards

- Solutions of 17 amino acids in five concentrations are available from Agilent (10 pmol/µL to 1 nmol/µL) for calibration curves. Each 1 mL ampule of standards was divided into 100 µL portions in conical vial inserts, and stored at 4 °C.
- The extended amino acid (EAA) stock solution was produced by weighing 59.45 mg asparagine, 59.00 mg hydroxyproline, 65.77 mg glutamine, and 91.95 mg tryptophan into a 25 mL volumetric flask. This flask was filled halfway with 0.1 N HCL, and shaken or sonicated until the amino acids were dissolved. It was then filled to mark with water to produce a total concentration of 18 nmol/µL of each amino acid.
- For the high-sensitivity EAA stock solution, 5 mL of this standard-sensitivity solution was diluted with 45 mL water (1.8 nmol/µL). Solutions containing extended standards were unstable at room temperature, and were kept frozen, and discarded at the first signs of reduced intensity.

Internal Standard (ISTD) stock solution

For primary amino acid ISTD stock solutions, 58.58 mg norvaline was weighed into a 50 mL volumetric flask. For secondary amino acids, 44.54 mg sarcosine was weighed into the same 50 mL flask. This flask was filled halfway with 0.1 N HCl, and shaken or sonicated until dissolved, then filled to mark with water for a final concentration of 10 nmol each amino acid/µL (standard sensitivity). For high-sensitivity ISTD stock solution, 5 mL of standard-sensitivity solution was diluted with 45 mL of water, and stored at 4 °C.

Calibration curves may be made using two to five standards, depending on experimental need. Typically, 100 pmol/µL, 250 pmol/µL, and 1 nmol/µL are used in a three-point calibration curve for standard analytical sensitivity analysis. The following tables should be followed if an internal standard or other amino acids (for example, the extended amino acids) are added. Table 1 describes standard analytical sensitivity concentrations typically used in UV analysis.

Table 1. Chromatographic parameters used for intact and reduced analysis.

	Concentration of Final AA solution (pmol/µL)		
	900	**225**	**90**
Take 5 mL of 18 nmol EAA	5 mL	5 mL	5 mL
Dilute with water	–	15 mL	45 mL
Diluted EAA mix	5 mL	20 mL	50 mL
Take 5 mL of diluted EAA mix	5 mL	5 mL	5 mL
Add 10 nmol ISTD solution	5 mL	5 mL	5 mL
EAA–ISTD mix	10 mL	10 mL	10 mL
Take 100 µL of EAA–ISTD mix	100 µL	100 µL	100 µL
For 1 nmol AA, add:	900 µL	–	–
For 250 pmol AA, add:	900 µL	–	
For 100 pmol AA, add:	–	–	900 µL
Final AA solution with EAA and 500 pmol/µL ISTD	1 mL	1 mL	1 mL

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Online derivatization

Depending on the autosampler model, the automated online derivatization program differs slightly. For the Agilent G4226A well plate automatic liquid sampler (WPALS), the injection program was:

1. Draw 2.5 µL from borate vial (p/n 5061-3339).
2. Draw 1.0 µL from sample vial.
3. Mix 3.5 µL in wash port five times.
4. Wait 0.2 minutes.
5. Draw 0.5 µL from OPA vial (p/n 5061-3335).
6. Mix 4.0 µL in wash port 10 times default speed.
7. Draw 0.4 µL from FMOC vial (p/n 5061-3337).
8. Mix 4.4 µL in wash port 10 times default speed.
9. Draw 32 µL from injection diluent vial.
10. Mix 20 µL in wash port eight times.
11. Inject.
12. Wait 0.1 minutes.
13. Valve bypass.

The location of the derivatization reagents and samples is up to the analyst and the ALS tray configuration. Using the G1367C with a 2 × 56-well plate tray (p/n G2258-44502), the locations were:

- Vial 1: Borate buffer
- Vial 2: OPA
- Vial 3: FMOC
- Vial 4: Injection diluent
- P1-A-1: Sample

Thermostatted column compartment (TCC)

Left and right temperatures were set to 40 °C. Analysis was enabled when the temperature was within ±0.8 °C.

Diode array detector (DAD)

Signal A: 338 nm, 10 nm bandwidth, reference wavelength 390 nm, 20 nm bandwidth.

Signal B: 262 nm, 16 nm bandwidth, reference wavelength 324 nm, 8 nm bandwidth.

Signal C: 338 nm, 10 nm bandwidth, reference wavelength 390 nm, 20 nm bandwidth.

To detect both OPA and FMOC derivatized amino acids in a single chromatogram, it was necessary to switch detector wavelengths. This switch took place between the last eluting OPA derivatized amino acid, lysine (peak 20 in the standard), and the first eluting FMOC derivatized amino acid, hydroxyproline (peak 21 in the standard).

With the DAD, determining the appropriate transition point was possible by initially collecting two channels. Signal A, 338 nm, detected OPA derivatized amino acids, and signal B, 262 nm, detected FMOC derivatized amino acids. From this analysis, the ideal point at which to switch wavelength during the run was determined. Subsequent runs were then made using a single channel, with the detector timetable function used to program a wavelength switch from 338 to 262 nm at the appropriate time between the elution of OPA-lysine and FMOC-hydroxyproline. This switch allowed both OPA and FMOC-derivatized amino acids to be detected in a single chromatogram. Peak width settings of >0.01 minutes were used for all columns.:

Linearity, limit of detection (LOD), and limit of quantification (LOQ) determination

As an example extended amino acid (EAA) stock solution, asparagine (59.45 mg), glutamine (65.77 mg), and tryptophan (91.95 mg) were used for linearity, LOD, and LOQ determination. These standards were weighed into a 25 mL volumetric flask, which was filled halfway with 0.1 N HCl, and mixed or sonicated until they dissolved. The flask was then filled to mark with water for a total concentration of 18 nmol/µL of each amino acid.

Linearity was studied in the range of 0.9–1,000 pmol/µL of these standard amino acids. Appropriate AA standard solutions were prepared in triplicate and injected into the chromatograph. The LOD and LOQ were estimated from the calibration function. LOD and LOQ were calculated as 3 (SD(a)/b) and 10 (SD(a)/b), respectively, where SD(a) is the standard deviation of the intercept, and b is the slope of the calibration function.

Gradient program	
Time (min)	**%B**
0	2
0.35	2
13.4	57
13.5	100
15.7	100
15.8	2
18	end
Flow rate: 1.5 mL/min for 4.6 mm id	

Table 3. Retention time and area RSD precision for amino acids (1,000 pmol) separated on an Agilent AdvanceBio AAA, 4.6 × 100 mm, column (n = 6).

Amino acid	RT RSD (%)	Area RSD (%)	Amino acid	RT RSD (%)	Area RSD (%)
1. Aspartic acid	0.151	2.60	13. Valine	0.025	2.4
2. Glutamic acid	0.512	2.19	14. Methionine	0.025	1.78
3. Asparagine	0.124	2.13	15. Norvaline	0.019	1.77
4. Serine	0.114	1.74	16. Tryptophan	0.024	2.03
5. Glutamine	0.092	1.8	17. Phenylalanine	0.027	1.98
6. Histidine	0.077	1.39	18. Isoleucine	0.025	2.17
7. Glycine	0.068	1.48	19. Leucine	0.020	1.81
8. Threonine	0.059	2.26	20. Lysine	0.022	2
9. Arginine	0.027	1.66	21. Hydroxyproline	0.014	3.14
10. Alanine	0.031	1.87	22. Sarcosine	0.015	5.01
11. Tyrosine	0.034	2.04	23. Proline	0.011	2.58
12. Cysteine	0.030	2.22			

System suitability as per the European Pharmacopoeia (Ph. Eur.)

The European Pharmacacopoeia (Ph. Eur.) defines requirements for the qualitative and quantitative composition of amino acids and mixtures of amino acids. The requirements for allowed impurities are also defined. Manufacturers of amino acids are legally bound to prove that their amino acids meet these specifications before they can distribute their products in Europe. Leucine (Leu) is a branched-chain α-amino acid that is produced by a fermentation process (Figure 2). During this process, isoleucine can be produced as a by-product. The Ph. Eur. states that leucine and isoleucine should have a resolution of not less than 1.5 [1].

Ten concentration points for three amino acids were assayed in triplicate. The three standard amino acids showed good linearity in the tested range. The area response obeyed the equation y = mx + C, where the intercept C was zero within 95 % confidence limits, and the square correlation coefficient (R2) was always greater than 0.99. Figure 3 shows the linearity curve for asparagine, glutamine, and tryptophan in the concentration range evaluated.

The LOD and LOQ were approximately 0.9 pmol and 3.8 pmol, respectively, using UV detection, indicating that the method was sensitive. Table 5 shows the observed LOD and LOQ values of asparagine, glutamine, and tryptophan.

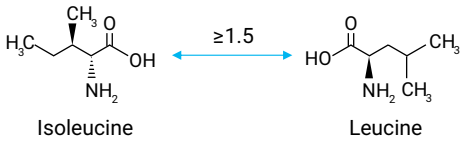

Figure 2. Isoleucine and leucine chemical relationship.

Table 4. System suitability testing using AdvanceBio AAA columns and AA standards.

System suitability	Agilent AdvanceBio AAA, C18, 4.6 × 100 mm, 2.7 µm	Agilent AdvanceBio AAA, C18, 3 × 100 mm, 2.7 µm
Resolution between leucine and isoleucine (≥1.5)	4.5	4.6

Table 5. LODs and LOQs for three amino acids.

Asparagine		Glutamine		Tryptophan	
Concentration (pmol)	S/N ratio	Concentration (pmol)	S/N ratio	Concentration (pmol)	S/N ratio
0.9 (LOD)	5.3	0.9 (LOD)	3.0	0.9 (LOD)	4.5
1.9 (LOQ)	10.8	3.8 (LOQ)	13.8	3.8 (LOQ)	20.5

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Results and Discussion

High-throughput routine analysis

The chromatogram in Figure 1 illustrates the standard analytical sensitivity achieved in high-throughput separations of amino acids. This chromatogram was obtained using an Agilent 1290 Infinity LC with an Agilent AdvanceBio AAA, 4.6 × 100 mm, 2.7 µm column using the amino acid method with DAD detection. A single run was completed in 18 minutes (including re-equilibration) with adequate resolution. The primary amino acids (1-20, OPA-derivatized), shown in Figure 1, were monitored at 338 nm, while the secondary amino acids (21-23, FMOC-derivatized) were monitored at 262 nm.

Precision of retention time and area (n = 6)

Tables 2 and 3 summarize the average retention times and area RSDs for all the amino acids for the 100 and 1,000 pmol from six replicates of an amino acid method. The retention time RSDs for all amino acid peaks, including the early eluting peak 1 were less than 1.2%, demonstrating excellent gradient reproducibility. Peak area RSDs were less than 5 %, indicating precise sample injection. The RSD values demonstrate the robustness and precision of the amino acid method.

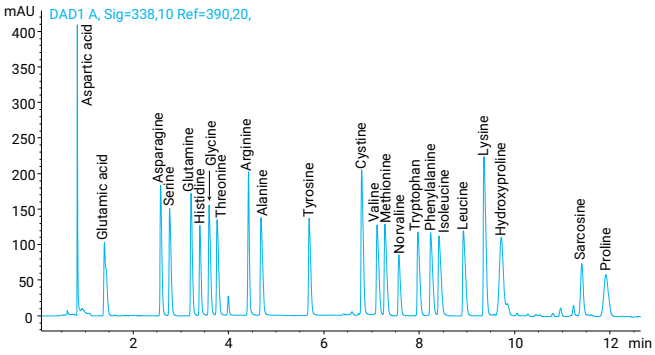

Figure 1. Separation of amino acid standard (1 nmol) on an Agilent AdvanceBio AAA 4.6 × 100 mm, 2.7 µm column, using the amino acid method.

Table 2. Retention time and area RSD precision for amino acids (100 pmol) separated on an Agilent AdvanceBio AAA, 4.6 × 100 mm, column (n = 6).

Amino acid	RT RSD (%)	Area RSD (%)	Amino acid	RT RSD (%)	Area RSD (%)
1. Aspartic acid	1.270	1.066	13. Valine	0.084	2.47
2. Glutamic acid	0.973	1.85	14. Methionine	0.073	1.82
3. Asparagine	0.605	1.79	15. Norvaline	0.073	1.72
4. Serine	0.629	1.82	16. Tryptophan	0.054	1.57
5. Glutamine	0.470	1.56	17. Phenylalanine	0.051	1.66
6. Histidine	0.430	1.22	18. Isoleucine	0.047	1.72
7. Glycine	0.477	1.92	19. Leucine	0.03	1.7
8. Threonine	0.440	1.95	20. Lysine	0.028	1.66
9. Arginine	0.251	2.15	21. Hydroxyproline	0.021	4.13
10. Alanine	0.280	3.06	22. Sarcosine	0.026	1.15
11. Tyrosine	0.128	1.65	23. Proline	0.021	4.36
12. Cysteine	0.067	1.9			

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Amino acid analysis of cell culture media and protein hydrolysate standard

We analyzed the amino acid composition of commonly used cell culture supplements. These standards included: Minimum Essential Medium Eagle (MEM), Non-Essential Amino Acid (NEAA), RPMI 1640 R0083, and protein hydrolysate standard. The results were then compared with the amino acid standards. Figures 4 to 7 show the overlays of amino acid composition of the media and the amino acid standards.

It is evident that the amino acid composition of cell culture supplements, as determined by this method, matches accurately with their theoretical composition. In addition, baseline resolution of isoleucine and leucine was observed with a resolution factor of 4.35 for the protein hydrolysate standard, meeting the regulatory requirements for these components significantly better than competitive columns. Such applications are useful in monitoring and adjusting amino acid composition, which is an essential part of optimizing the manufacturing process to ensure high quality and optimum yield of the final biopharmaceutical product.

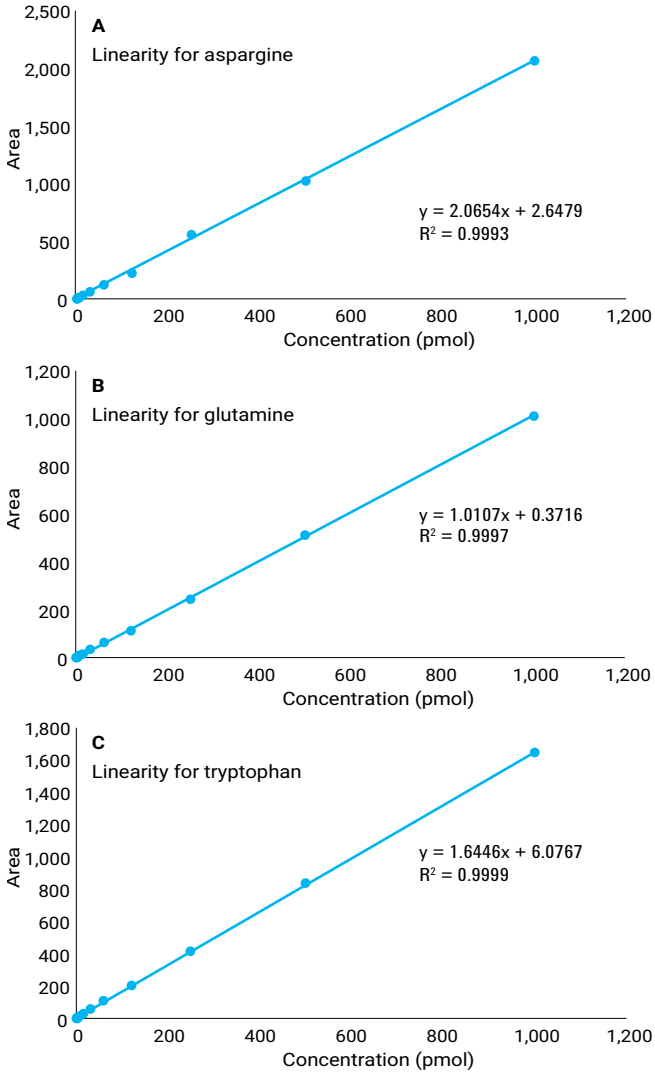

Figure 3. Linearity curve with 10 standard concentrations of asparagine, glutamine, and tryptophan ranging from 0.9 to 1,000 pmol, showing excellent coefficient values.

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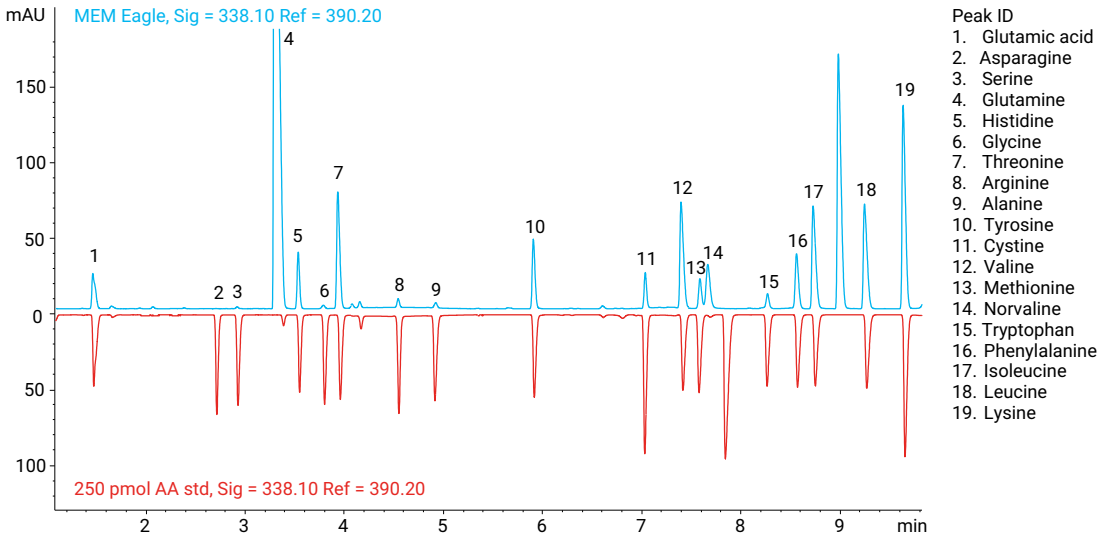

Figure 4. Amino acid analysis of MEM media (blue trace) mirrored with AA standards (red trace).

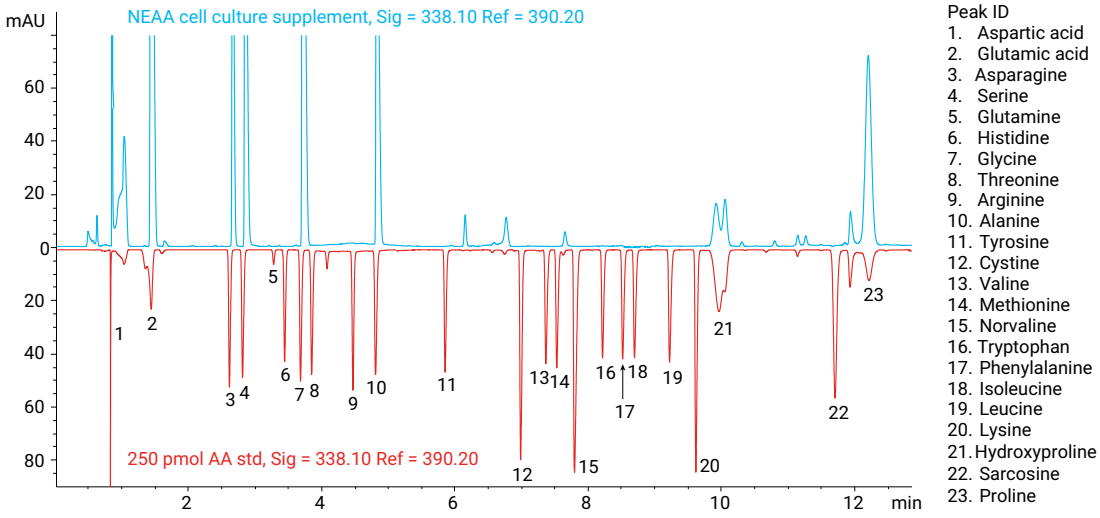

Figure 5. Amino acid analysis of NEAA media (blue trace) and comparison with AA standards (red trace).

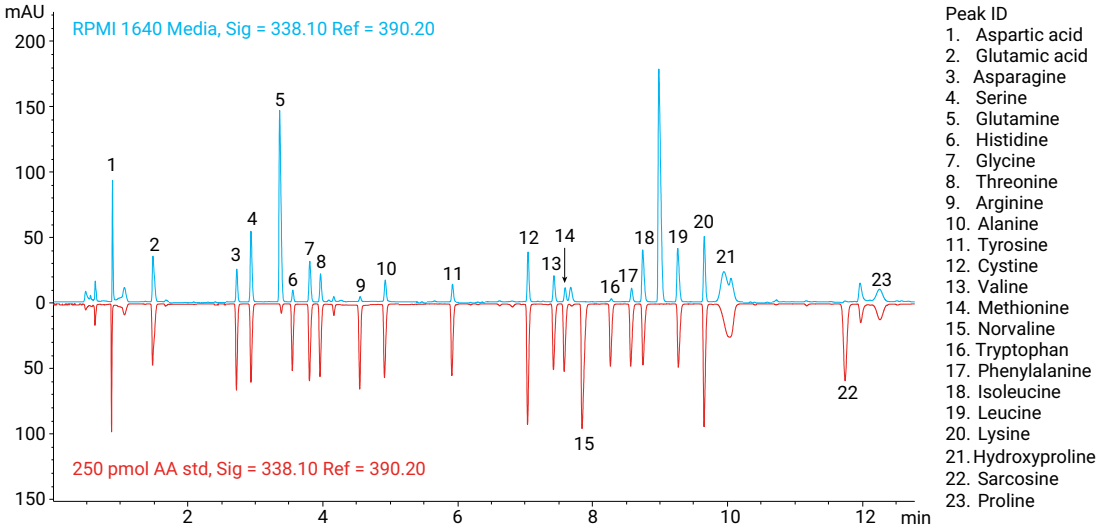

Figure 6. Amino acid analysis of RPMI 1650 media (blue trace) and comparison with AA standards (red trace).

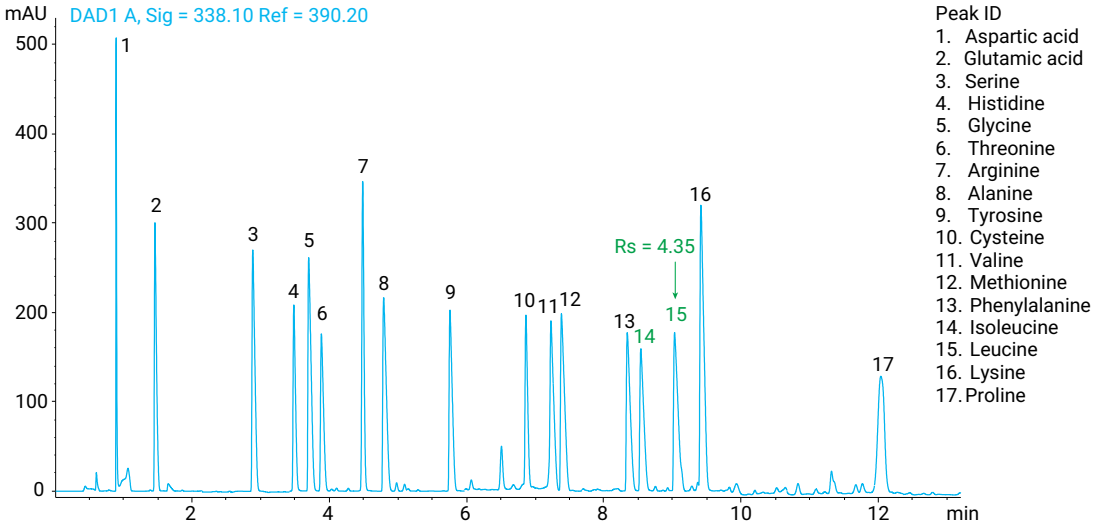

Figure 7. Amino acid analysis of protein hydrolysate standard. The resolution between leucine and isoleucine with the Agilent AdvanceBio AAA, 4.6 × 100 mm, 2.7 µm column is much higher than the reported value for system suitability requirement for this pair.

Conclusion

Amino acid analysis is an important approach for the characterization of protein and peptide-based products. Studying the roles of amino acids during bioprocesses leads to better understanding the feeding strategy, and to improving the yield and quality of the product. In addition, the determined amino acid composition can confirm sample identity, and give a measure of sample purity. This Application Note demonstrates several Agilent tools for the analysis of amino acids. We first used the Agilent 1290 Infinity LC and the Agilent AdvanceBio AAA kit for the automated online derivatization of amino acids using OPA/FMOC chemistries. The derivitized amino acids were then separated on an AdvanceBio AAA LC column to achieve a fast, sensitive, and reproducible separation of amino acids. Area and RT precision of the method were excellent, and met the system suitability requirement. Linearity curves with 10 standard concentrations of three amino acids, ranging from 0.9 pmol to 1 nmol, had excellent coefficient of linearity values, indicating that the method was quantitative and accurate. The LOD and LOQ for the amino acids were 0.9 pmol and 3.8 pmol, respectively, indicating that the method was sensitive. In addition, this method was able to separate and detect amino acids from cell culture media and protein hydrolysate standard. The amino acid composition determined using this method correlated well with their theoretical compositions..

Reference

1. European Pharmacopoeia 9.0 (2.2.56) *Amino Acid Analysis*

For More Information

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Application Note

Amino Acid Analysis

Agilent

Trusted Answers

Automated Amino Acid Analysis Using an Agilent Poroshell HPH-C18 Column

Authors

William Long
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Abstract

In this application note, an automated precolumn OPA/FMOC amino acid method, previously developed on 3.5 and 1.8 µm Agilent ZORBAX Eclipse Plus C18 columns, is expanded to include 2.7 µm Agilent Poroshell HPH-C18 superficially porous columns. This column exhibits good lifetime and transferability to different column dimensions, both of which are shown in this work. Applications of the column to fermentation products are also shown.

Introduction

Superficially porous particle (SPP) technology is based on particles with a solid core and a superficially porous shell. These particles consist of a 1.7 µm solid core with a 0.5 µm porous shell. In total, the particle size is about 2.7 µm. The 2.7 µm superficially porous particles provide 40 to 50 % lower backpressure and 80 to 90 % of the efficiency of sub-2 µm totally porous particles. The superficially porous particles have a narrower particle size distribution than totally porous particles. This results in a more homogeneous column bed, and reduces dispersion in the column. At the same time, the thin porous shell provides lower resistance to mass transfer. The result is minimal loss of efficiency at higher flow rates [1]. Additionally, since the columns incorporate a 2 µm frit, they are as resistant to clogging as 3.5 and 5 µm columns. Until recently, all silica-based SPP materials possessed limited lifetime in higher pH buffers, including phosphate buffers. To achieve these longer lifetimes, it is necessary to protect the base particle by either surface modification or special bonding modification. The surface of Agilent Poroshell HPH-C18 particles are chemically modified to form an organic layer, resistant to silica dissolution at high pH conditions, using a proprietary process. The continuous improvement in HPLC columns and instrumentation presents an opportunity to improve HPLC methods. A proven ortho-phthalaldehyde/9-fluorenylmethyl chloroformate (OPA/FMOC)-derivatized amino acid method developed on HP 1090 Series HPLC systems, and later updated for the Agilent 1100 Series, has now evolved further taking advantage of the Agilent 1260 Infinity Binary LC and superficially porous Agilent Poroshell HPH-C18 columns [2-8].

Experimental

Preparation of HPLC mobile phase

Mobile phase A contained 10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, pH 8.2, and 5 mM NaN_3. For 1 L, weigh 1.4 g anhydrous Na_2HPO_4 plus 3.8 g $Na_2B_4O_{7\cdot10}H_2O$ in 1 L water plus 32 mg NaN_3. Adjust to about pH 8.4 with 1.2 mL concentrated HCl, then add small drops of acid to pH 8.2. Allow stirring time for complete dissolution of borate crystals before adjusting pH. Filter through 0.45 µm regenerated cellulose membranes (p/n 3150-0576). Mobile phase B contains acetonitrile:methanol:water (45:45:10, v:v:v). All mobile-phase solvents were HPLC grade. Since mobile phase A is consumed at a faster rate than B, it is convenient to make 2 L of A for every 1 L of B.

The injection diluent was 100 mL mobile phase A, plus 0.4 mL concentrated H_3PO_4 in a 100 mL bottle, stored at 4 °C.

To prepare 0.1 N HCl, add 4.2 mL concentrated HCl (36%) to a 500 mL volumetric flask that is partially filled with water. Mix, and fill to the mark with water. This solution is for making extended amino acid and internal standard stock solutions. Store at 4 °C

Derivatization reagents (borate buffers, OPA, and FMOC) are ready-made solutions supplied by Agilent. They simply need to be transferred from their container into an autosampler vial. Some precautions include:

- OPA is shipped in ampoules under inert gas to prevent oxidation. Once opened, the OPA is potent for about 7 to 10 days. We recommend transferring 100 µL aliquots of OPA to microvial inserts. Label with name and date, cap, and refrigerate. Replace the OPA autosampler microvial daily. Each ampoule lasts 10 days.
- FMOC is stable in dry air but deteriorates in moisture. It should also be transferred in 100 µL aliquots to microvial inserts. Label with name and date, cap tightly, and refrigerate. Like the OPA, an open FMOC ampoule transferred to 10 microvial inserts should last 10 days (one vial/day).
- Borate buffer can be transferred to a 1.5 mL autosampler vial without a vial insert. Replace every three days.

Preparation of amino acid standards

Solutions of 17 amino acids in five concentrations are available from Agilent (10 pmol/µL to 1 nmol/µL) for calibration curves. Divide each 1 mL ampoule of standards (p/n 5061-3330 through 5061-3334) into 100 µL portions in conical vial inserts. Cap and refrigerate aliquots at 4 °C. To make the extended amino acid (EAA) stock solution, weigh 59.45 mg asparagine, 59.00 mg hydroxyproline, 65.77 mg glutamine, and 91.95 mg tryptophan into a 25 mL volumetric flask. Fill halfway with 0.1 N HCL and shake or sonicate until dissolved. Fill to mark with water for a total concentration of 18 nmol/µL of each amino acid. For the high-sensitivity EAA stock solution, take 5 mL of this standard-sensitivity solution and dilute with 45 mL water (1.8 nmol/µL). Solutions containing extended standards are unstable at room temperature. Keep them frozen and discard at first signs of reduced intensity.

For primary amino acid ISTD stock solutions, weigh 58.58 mg norvaline into a 50 mL volumetric flask. For secondary amino acids, weigh 44.54 mg sarcosine into the same 50 mL flask. Fill half way with 0.1 N HCl and shake or sonicate until dissolved, then fill to mark with water for a final concentration of 10 nmol each amino acid/µL (standard sensitivity). For high-sensitivity ISTD stock solution, take 5 mL of standard-sensitivity solution and dilute with 45 mL of water. Store at 4 °C. Calibration curves are made using two to five standards depending on experimental need. Typically, 100 pmol/µL, 250 pmol/µL, and 1 nmol/µL are used in a three-point calibration curve for standard-sensitivity analysis.

Pump parameters

Pump parameters for all methods include compressibility (×10–6 bar) A: 35, B: 80, with minimal stroke A, B of 20 µL.

Online derivatization

Depending on the autosampler model, the automated online derivatization program differs slightly. For the Agilent G1376C well plate automatic liquid sampler (WPALS), with injection program:

1. Draw 2.5 µL from borate vial (p/n 5061-3339).
2. Draw 1.0 µL from sample vial.
3. Mix 3.5 µL in wash port five times.
4. Wait 0.2 minutes.
5. Draw 0.5 µL from OPA vial (p/n 5061-3335).
6. Mix 4.0 µL in wash port 10 times default speed.
7. Draw 0.4 µL from FMOC vial (p/n 5061-3337).
8. Mix 4.4 µL in wash port 10 times default speed.
9. Draw 32 µL from injection diluent vial.
10. Mix 20 µL in wash port eight times.
11. Inject.
12. Wait 0.1 minutes.
13. Valve bypass

The location of the derivatization reagents and samples is up to the analyst and the ALS tray configuration. Using the G1367C with a 2 × 56 well plate tray (p/n G2258-44502), the locations were:

• Vial 1: Borate buffer

• Vial 2: OPA

• Vial 3: FMOC

• Vial 4: Injection diluent

• P1-A-1: Sample

Thermostatted column compartment (TCC)

Left and right temperatures were set to 40 °C. Enable analysis when the temperature is within ± 0.8 °C. See Table 5 for which heat sink to use.

Diode array detector (DAD)

Signal A: 338 nm, 10 nm bandwidth, and reference wavelength 390 nm, 20 nm bandwidth.

Signal B: 262 nm, 16 nm bandwidth, and reference wavelength 324 nm, 8 nm bandwidth.

Signal C: 338 nm, 10 nm bandwidth, and reference wavelength 390 nm, 20 nm bandwidth.

The DAD was programmed to switch to 262 nm, 16 nm bandwidth, reference wavelength 324 nm, 8 nm bandwidth, after lysine elutes, and before hydroxyproline elutes. Signal C was determined by examining signal A and B timeframes between peaks 20 and 21, then choosing a suitable point to switch wavelengths. Once the switch time was established and programmed into the method, signals A and B were optional.

Peak width settings of > 0.01 minutes were used for all columns.

Results and Discussion

As can be seen in Figure 1, using the same chromatographic conditions, the separation was very similar. The elution order of the mixture on both columns was the same, and as shown in Figure 2, the relationship of retention times of the amino acid samples was highly correlated between an Eclipse Plus C18 and a Poroshell HPH-C18, with a correlation co-efficient of 0.997. As can be seen in the chromatograms, the retention times were slightly less on the Poroshell HPH-C18 column. Some chromatographic differences are notable. Thus, separation of leucine and lysine looks better on Poroshell HPH-C18, while the separation between lysine and hydroxyproline and the sarcosine/proline pair looks worse. As suggested in previous application notes, the chromatography can be altered to enhance resolution of desired peak pairs.

Conditions for Figure 1.

Parameter	Value	
Column:	Agilent Poroshell HPH C18, 4.6 × 100 mm, 2.7 µm (p/n695975-702) or Agilent Eclipse Plus C18, 4.6 × 100 mm, 3.5 µm (p/n959961-902)	
Flow rate:	1.5 mL/min	
Gradient:	Time (min)	% B
	0	2
	0.35	2
	13.4	57
	13.5	100
	15.7	100
	15.	8 2
	18	end

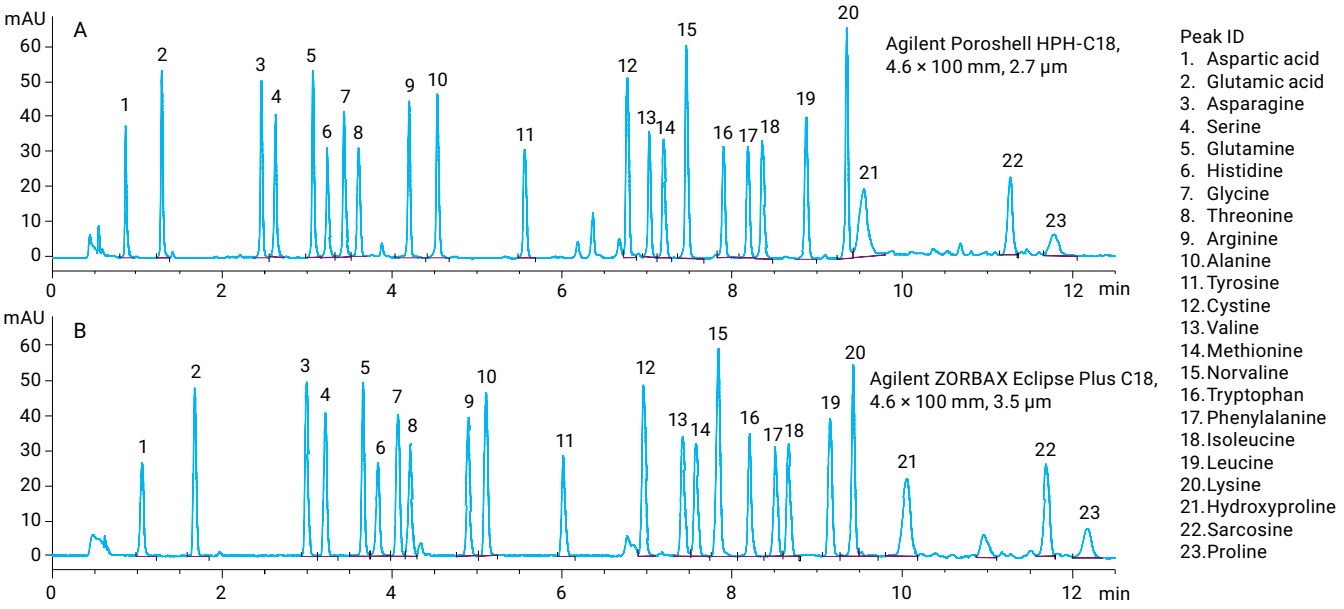

Figure 1. Comparison of an Agilent Poroshell HPH C18 to an Agilent ZORBAX Eclipse Plus C18 column using the Amino Acid Method.

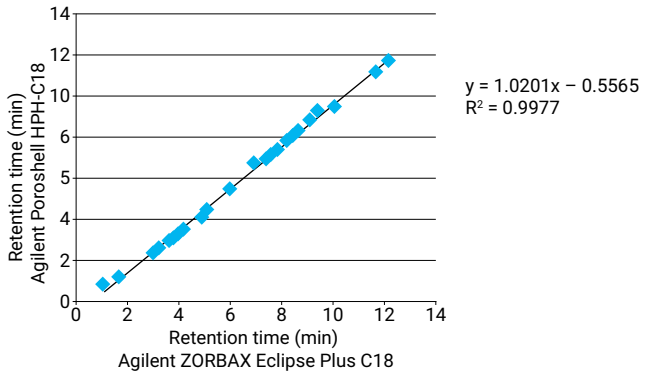

Figure 2. Correlation of retention times using Agilent Poroshell HPH-C18 and Agilent ZORBAX Eclipse Plus C18 columns.

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Column dimensions

The method can easily be scaled to different column dimensions. In this work, three column dimensions were studied. All columns were 100 mm in length with 4.6, 3.0, or 2.1 mm internal diameter, as shown in Figure 3. In this case, the only changes to the method were made by altering the flow rate. Table 1 lists the gradient program used throughout. Flow rates are changed geometrically with the diameter of the column. The flow rate used with the 4.6 × 100 mm column was 1.5 mL/minute. The flow rates for the 3 and 2.1 mm columns were 0.62 and 0.21 mL/min, respectively. In all cases, the low-volume heat exchanger was used with short red tubing to minimize extra column volume. Using the Agilent 1260 Infinity Binary LC with low dispersion heating and tubing, the column pressure was approximately 175 bar. We observed that retention time of all analytes increased slightly (without changing selectivity) as columns were changed from larger to smaller internal diameter. This is due to the increase in gradient delay time. As the flow rates are scaled and consequently reduced from larger to smaller column ids, the gradient delay volume remains constant, thereby increasing the time it takes for the gradient to reach the column. The difference in retention between various column ids could potentially be reduced or eliminated by scaling the gradient delay volume on the LC system (adding or removing capillary length/diameter/volume between the pump and column inlet).

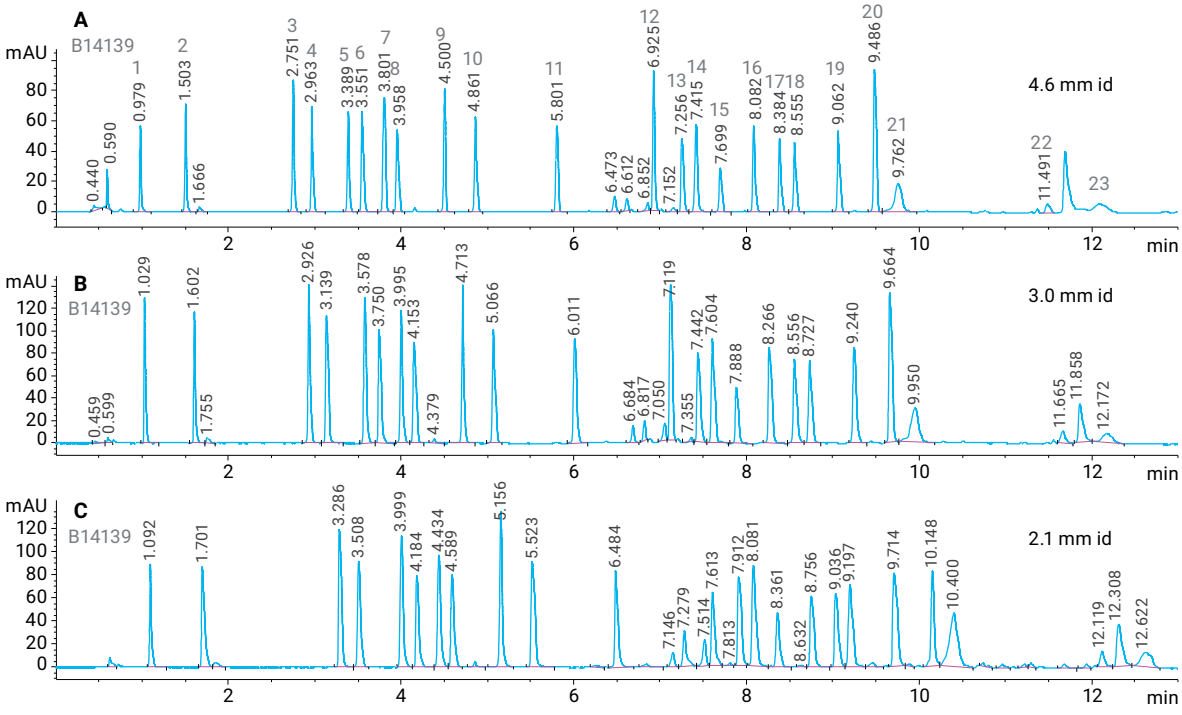

Figure 3. Agilent Poroshell HPH-C18 100 mm columns of different inside dimension using the amino acid method.

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Lot-to-lot variability

Batch-to-batch or lot-to lot reproducibility is also an import factor in method development. It is recommended that, before a method is adopted, one of the earliest validation steps is to examine the method performance on at least three columns made from different lots.

Following good validation practice, three columns loaded with particles from different production batches were examined for 4.6, 3.0, and 2.1 × 100 mm columns. The overlays of these three sets are shown in Figures 4A-C. As can be seen in Figure 4A, the amino acid separation on the 4.6 × 100 mm column achieved good peak as well as baseline separation shape for all compounds. No change in elution order was noted, and lot-to-lot reproducibility looked good. A slight change in retention time can be seen in Figure 4A though the k' remained constant. However, a slight change in the wavelength switch time is required as it is tied to the elution times of lucine and hydroxyproline. Similar reproducibility is evident in Figures 4B and 4C for the smaller id columns.

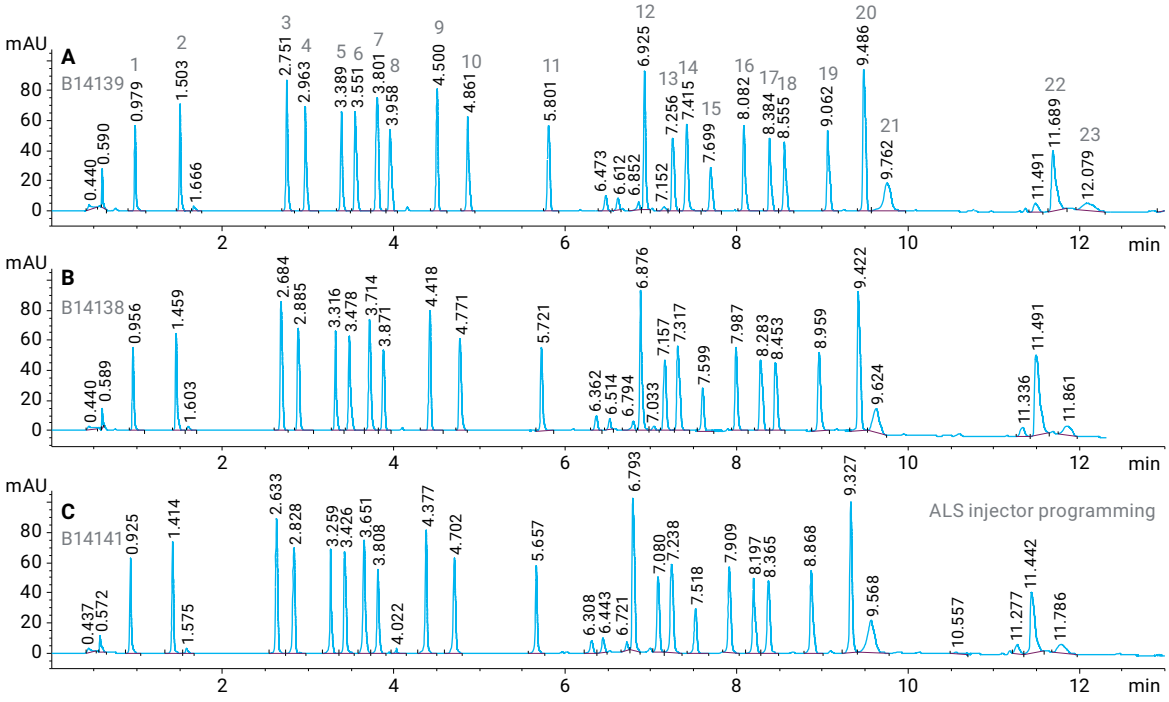

Figure 4A. Separation of amino acid and internal standards on three lots of Agilent Poroshell HPH-C18, 4.6 × 100 mm, 2.7 µm (p/n 695975-702).

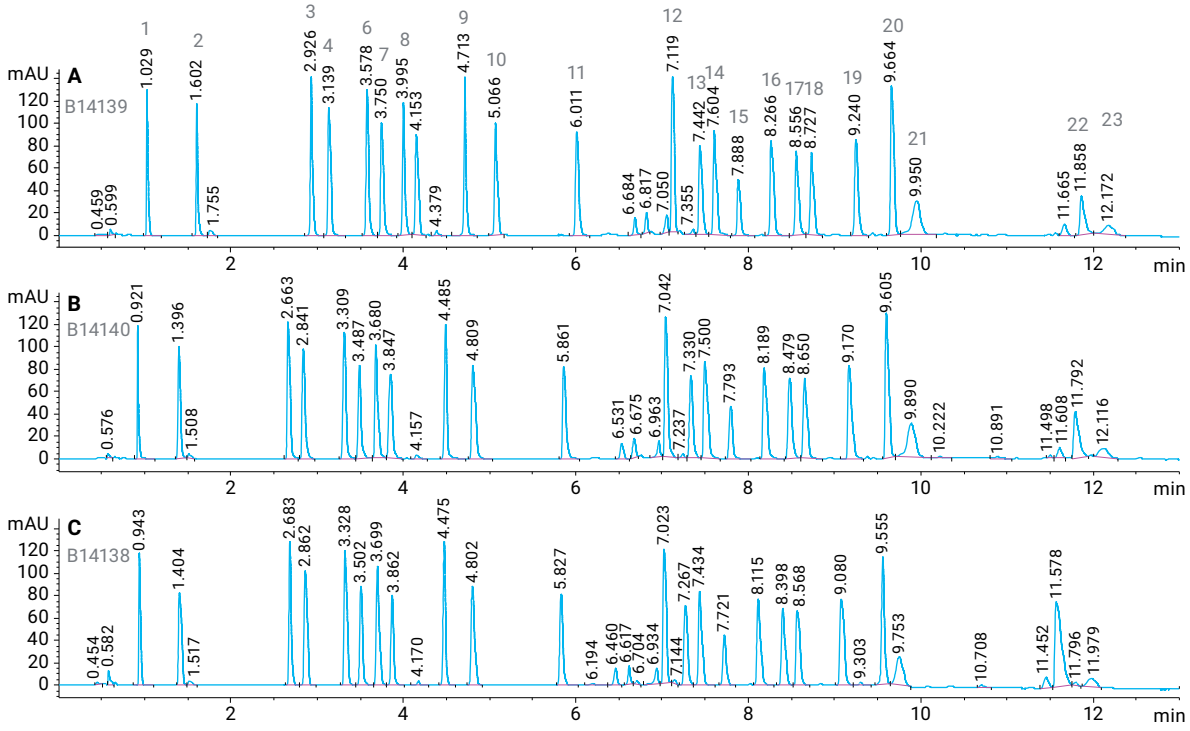

Figure 4B. Separation of amino acid and internal standards on three lots of Agilent Poroshell HPH-C18, 3 × 100 mm, 2.7 µm (p/n 695975-502).

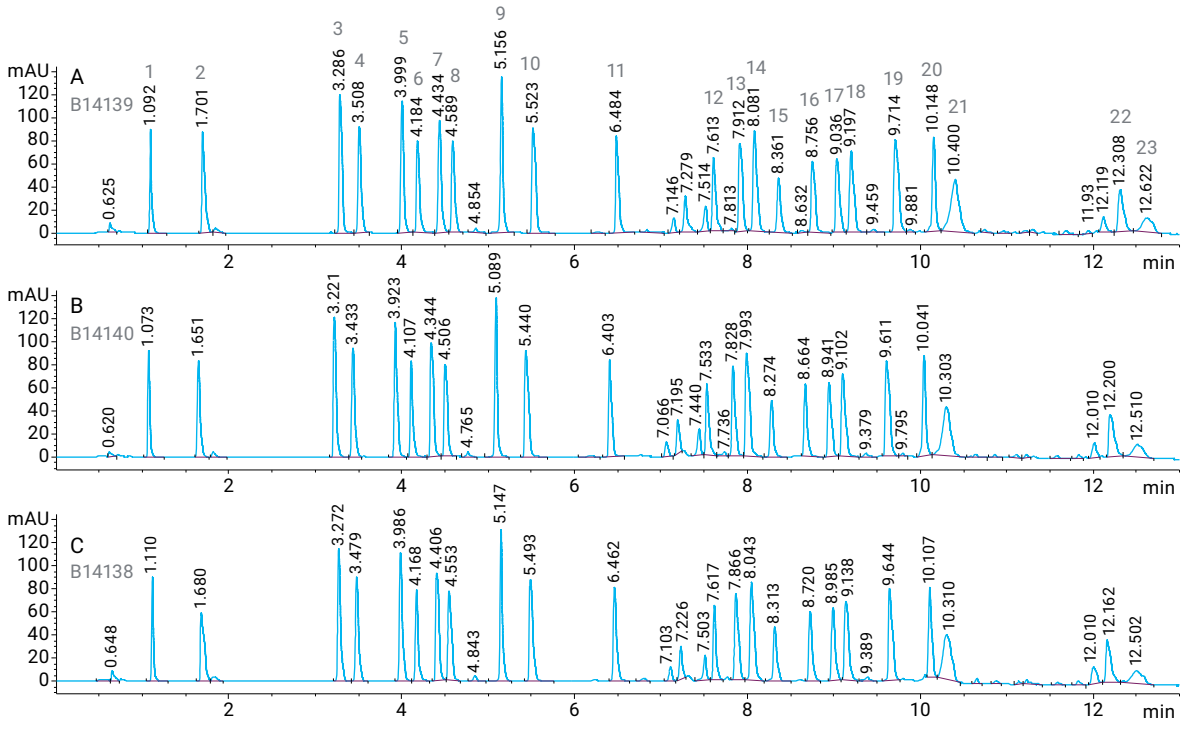

Figure 4C. Separation of amino acid and internal standards on three lots of Agilent Poroshell HPH-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-702).

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Lifetime

Column lifetime is an important consideration for chromatographers analyzing amino acid samples. Most silica columns lose efficiency after prolonged exposure to these conditions. Kirkland et al. [9] and Tindall and Perry [10] discussed possible reasons for the reduced lifetime of silica columns in phosphate buffer, but both agree that columns do not last as long.

There are two approaches to achieving high pH stability in silica HPLC columns. One way is to employ special bonding chemistry, as in the Agilent ZORBAX Extend C18 column. This column uses bidentate bonding to protect the silica from dissolution at high pH. Another way to achieve high pH stability is to modify the silica itself, making it less soluble. The surface of Poroshell HPH particles are chemically modified to form an organic layer, resistant to silica dissolution at high pH conditions, using a proprietary process [11].

Figure 5 is an overlay of four chromatograms. Single 4 L bottles of mobile phase A and B were prepared. A single 2.1 × 100 mm column was used for lifetime testing from a series of 500 analyses over a period of four weeks. In this series, approximately 102 injections were made each week using freshly opened amino acid standard mix and reagents. At the end of the sequence, the column was flushed with 100 % B mobile phase for 40 minutes and the instrument was shut down. In this manner, the method was run for 3.5 days and the column was stored with no analysis for 3.5 days. This simulated typical practice in a lab where samples are run for an extended time, and then a column is washed and stored. Storing a column in 100 % mobile phase B was recommended in the original amino quant methods, and is common practice in many successful laboratories that frequently run amino acids. A realistic lifetime study was carried out, showing excellent lifetime of the column over one month of use, with over 500 standard injections, shutting down and storing after each sequence. As can be seen in Figure 5, the 17 amino acid sample lost no resolution and only a slight retention time shift was seen.

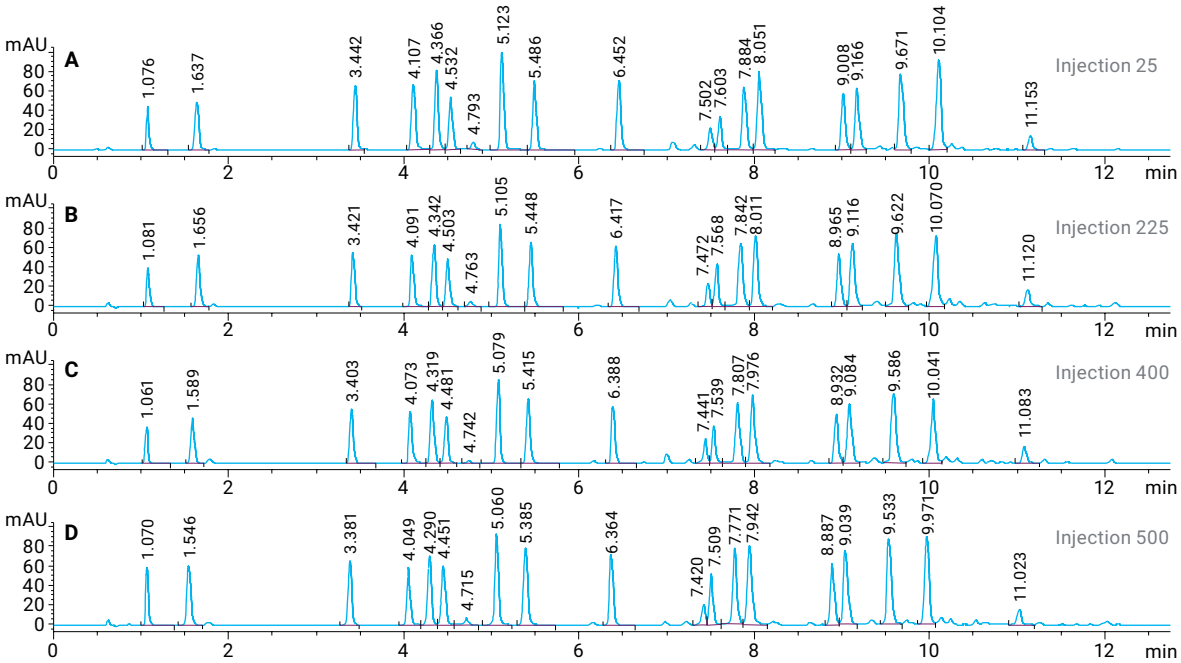

Figure 5. Column lifetime test using an Agilent Poroshell HPH-C18, 2.1 × 100 mm column running an amino acid method.

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Conclusions

Agilent Poroshell HPH-C18 has selectivity similar to totally porous Agilent ZORBAX Eclipse Plus C18. This allows easy transfer of existing methods such as the amino acid method. In this work, no changes to the chromatographic conditions were made although changes in the gradient could be done to improve resolution on selected amino acids. In most cases, Poroshell HPH-C18 was slightly less retentive than totally porous Eclipse Plus C18. The method was investigated with 4.6, 3.0, and 2.1 mm × 100 mm columns. Use of the low volume column heater is recommended. In total, four particle lots were investigated, requiring only slight changes to the wavelength switch time. A realistic lifetime study was carried out, showing excellent lifetime of the column over one month of use, with over 500 standard injections, shutting down and storing after each sequence.

References

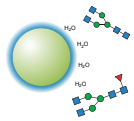
1. Wang, X.; Barber, W. E.; Long, W. J. Applications of superficially porous particles: High Speed, high efficiency or both? *J. Chromatogr.* A. **2012**, 1228, 72-88.
2. Schuster, R.; Apfel, A. A new technique for the analysis of primary and secondary amino acids; Application note, Hewlett-Packard Publication number 5954-6257, **1986**.
3. Schuster, R. Determination of amino acids in biological, pharmaceutical, plant and food samples by automated precolumn derivatization and high-performance liquid chromatography. *J. Chromatogr.* B. **1988**, 431, 271-284.
4. Henderson, Jr., J. W.; Ricker, R. D.; Bidlingmeyer, B. A.; Woodward, C. Rapid, Accurate, Sensitive and Reproducible HPLC Analysis of Amino Acids; Application note, Agilent Technologies, Inc. Publication number 5980-1193E, **2000**.
5. Woodward, C.; Henderson, Jr., J. W.; Todd Wielgos, T. High-Speed Amino Acid Analysis (AAA) on Sub-Two Micron Reversed-phase (RP) Columns; Application note, Agilent Technologies, Inc. Publication number 5989-6297EN, **2007**.
6. Gratzfeld-Huesgen, A. Sensitive and Reliable Amino Acid Analysis in Protein Hydrolysates using the Agilent 1100 Series HPLC; Application note, Agilent Technologies, Inc. Publication number 5968-5658EN, **1999**.
7. Greene, J.; Henderson, Jr., J. W.; Wikswo, J. P. Rapid and Precise Determination of Cellular Amino Acid Flux Rates Using HPLC with Automated Derivatization with Absorbance Detection; Application note, Agilent Technologies, Inc. Publication number 5990-3283EN, **2009**.
8. Henderson, Jr., J. W.; Brooks, A. Improved Amino Acid Methods using Agilent ZORBAX Eclipse Plus C18 Columns for a Variety of Agilent LC Instrumentation and Separation Goals; Application note, Agilent Technologies, Inc. Publication number 5990-4547EN, **2010**.
9. Kirkland, J. J.; van Straten, M. A.; Claessens, H. A. Reversed-phase high-performance liquid chromatography of basic compounds at pH 11 with silica-based column packings. *J. Chromatogr.* A. **1998**, 797, 111-120.
10. Tindall, G. W.; Perry, R. L. Explanation for the enhanced dissolution of silica column packing in high pH phosphate and carbonate buffers. *J. Chromatogr.* A. **2003**, 988, 309-312.
11. Anon. Extending Column Lifetime in Pharmaceutical Methods with High pH-Stable Poroshell HPH Chemistries; Technical overview, Agilent Technologies, Inc. Publication number 5991-5022EN, **2014**.

Glycan Analysis

Background

Glycosylation is an important post-translational modification as glycans play a key role in protein recognition and biotherapeutic efficacy. Glycosylation patterns in human cells generally follow a typical antenna-like pattern based on a common core structure, and it is the goal of recombinant protein manufacturers to try to replicate that profile using cell culture. Mammalian cell lines are required but the glycosylation pathway is complex, and not all clones will generate the desired glycan profile. Regulatory authorities recognize this as a major challenge and provide instructions in how to determine the glycan fingerprint. This involves the use of a specific enzyme, PNGase F, to cleave N-linked glycans, labeling them with a fluorophore to increase detection sensitivity, and then separating them using hydrophilic interaction chromatography (HILIC) columns (often in combination with a fluorescence detector, although mass spectrometry may also be used).

Agilent provides several kits containing all the components needed to manually perform the deglycosylation and labeling reaction with a variety of labels including 2-aminobenazamide (2-AB), InstantPC, and APTS. Alternatively, much higher throughput can be obtained using a fully automated AssayMAP platform capable of handling a greater number of samples in a fraction of the time. This approach is illustrated in the application note featured on page 66.



Glycan Analysis

Hydrophilic interaction chromatography

Fast, high-resolution, reproducible glycan separation

AdvanceBio Glycan Mapping

An amide HILIC column

Attribute	Advantage
2.7 µm superficially porous particle	High resolution at low back pressure
1.8 µm totally porous particles	Maximum resolution
Fluorescence and MS compatible	Easy method transfer

Getting Started

HILIC, or hydrophilic interaction chromatography, is a normal phase chromatography. In some ways the opposite of the more familiar reversed phase chromatography, with gradients starting at high organic solvent content rather than high aqueous content. The mechanism of interaction of analytes with the stationary phase is a partitioning from the high organic eluent into the aqueous layer present on the surface of the stationary phase. Water is also the strong eluting solvent. Ample re-equilibration time at the end of the method and minimized sample injection volumes are critical for reproducible chromatography and good peak shape. AdvanceBio Glycan Mapping columns are available in superficially porous 2.7 µm columns suitable for use on all HPLC instruments, or in fully porous 1.8 µm columns designed for use on UHPLC instruments. Appropriate method conditions to achieve the optimum resolution for closely related glycan structures can be found in the Workflow "How-To" guides detailed in this section.

Glycan Mapping: A "How-To" Guide

Introduction

Post-translational modifications to the primary amino acid sequence, including glycosylation, have functional consequences and can impact efficacy and immunogenicity of a biopharmaceutical. The structure of the glycan also contributes to the clearance rates of the protein in plasma and the ability of the monoclonal antibody to trigger the immune response required for efficacy. Many regulatory authorities consider glycosylation to be one of the critical quality attributes of biomolecules. Therefore, it must be characterized and quantified, with acceptable ranges determined, as part of the development process for a glycoprotein innovator, biosimilar, or biobetter pharmaceutical.

Agilent's AdvanceBio Glycan Mapping solutions provide optimized workflows designed to deliver reproducibility in the analysis of fluorescently-labeled glycans for accurate identification and quantification.

Sample Preparation - AdvanceBio N-Glycan Sample Preparation Kits

The mapping of the N-linked glycan component of a glycoprotein, including monoclonal antibodies, requires the N-glycans to be enzymatically cleaved from the protein amino acid backbone using PNGase F. The cleaved N-glycans can be analyzed by hydrophilic interaction chromatography (HILIC) with MS detection. More commonly, N-glycans can labeled with one of a variety of fluorophores and analyzed using HILIC chromatography followed by either fluorescence or MS detection.

AdvanceBio glycan sample preparation kits provide all the reagents needed to prepare samples. Agilent's sample preparation kits are modular, generally separated into sub-kits for deglycosylation, labeling and cleanup. In addition, modules for each of the separate steps of the workflow have separate part numbers for flexibility. The workflow yields samples that are suitable for analysis by liquid chromatography, typically by HILIC.

Column Selection

Agilent AdvanceBio Glycan Mapping columns are designed and manufactured to deliver fast, high resolution, reproducible glycan identification using HILIC chromatography. AdvanceBio Glycan Mapping columns apply technology that optimizes results for MS and fluorescence detection. Choose from two UHPLC configurations: 2.7 µm superficially porous, for high resolution and lower backpressure, or 1.8 µm for highest resolution.

AdvanceBio Glycan Mapping	**Amide bonded phase for rapid equilibration and enhanced selectivity for glycans.**
1.8 µm	Based on a fully porous particle for high speed separations and high throughput applications. Stability to 1200 bar for use with the Agilent 1290 Infinity II LC.
2.7 µm	Based on Poroshell technology to give a superficially porous particle with reduced diffusion distances to give high resolution separations at lower pressures and enable the use of longer column lengths for increased separation efficiency.

Speed of Analysis

The AdvanceBio Glycan Mapping 1.8 µm columns provide high throughput N-glycan analysis where speed is the primary concern either due to the number of samples or to the immediate requirement for data. These columns deliver superior results in 40 % less time than the competition.

Conditions

Parameter	Value
Column A	AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm
Column B	Competitor sub-2 µm glycan column
Instrument	Agilent 1290 Infinity LC with 1260 Infinity Flourescence Detection
Column Temperature	55 °C
Sample thermostat	105 °C
Mobile Phase	A: 100 mM NH_4Formate. pH 4.5 B: ACN
FLD	Excitation = 260 Emission = 430
Injection Volume	2 µl in 70:30 ACN: 100mM NH_4Formate
Sample	Agilent 2-AB labeled N-linked Human IgG glycan library (p/n5190-6996)

Resolution

High resolution separations are achieved using the AdvanceBio glycan mapping 2.7 µm media, in longer 250 mm columns. This increased resolution enables accurate quantitation of target glycans and changes to the protein glycosylation profile, which may have occurred during expression.

Time	%A	%B	Flow rate (mL/min)
0	20	80	0.5
25	40	60	0.5
26	100	0	0.5
27	40	80	0.5

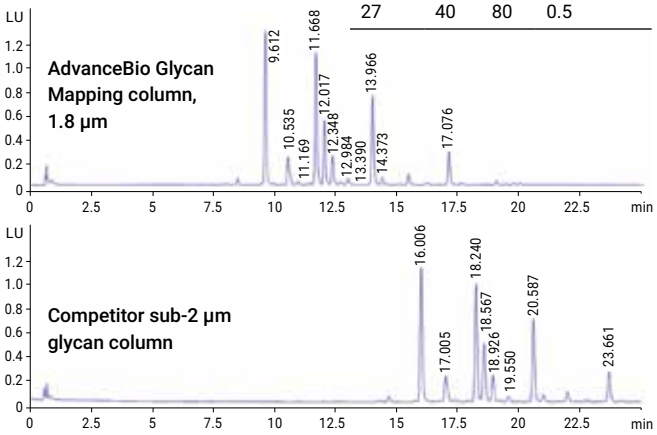


Figure 1. The Agilent AdvanceBio Glycan Mapping column delivers better resolution, narrower bands, and higher peak capacity than the non-Agilent sub-2 µm column in a 2.1 x 150 mm configuration.

Ensure consistent results with glycan standards for performance testing and retention mapping

Missed information in the early stages of development can cause major setbacks downstream. What's more, production process inconsistency may lead to changes in glycosylation, which can negatively impact immunogenicity and efficacy. Agilent reference standards help you make sure that critical data have been captured, and that every workflow component is working optimally. Agilent offers a full line of pre-labeled glycan standards and libraries as well as unlabeled glycan standards. A full list including structure diagrams and part numbers is available on pages 12-15.

Separation of a 2-AB labeled dextran ladder

Conditions

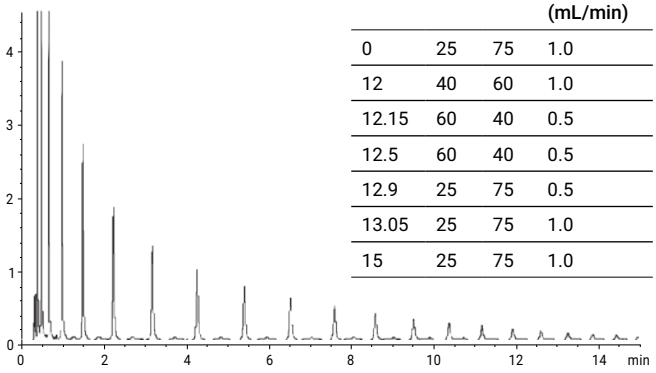
Parameter	Value
Column	AdvanceBio Glycan Mapping, 859700-913 2.1 × 150 mm, 1.8 µm
Mobile Phase	A: 100 mM NH_4Formate. pH 4.5 B: ACN
FLD	Excitation = 260 Emission = 430
Injection Volume	2 µl (10pmol total glycan/1 µl 75:25 ACN:water)
Sample	Agilent 2-AB labeled dextran ladder (p/n GKSB-503)

Flexible, high-performance LC instruments

Robust and easy to use:
The 100 % bio-inert Agilent 1260 Infinity II Bio-inert LC delivers outstanding results with its low surface activity, corrosion resistance, active seal wash, and quaternary buffer mixing.

New Benchmarks in Efficiency:
The Agilent 1290 Infinity II LC is the next generation in UHPLC, providing maximum analytical, instrument, and laboratory efficiency, with pressures up to 1300 bar and flows up to 5 mL/min.

Better efficiency and interaction-free results: Agilent bio-inert LC supplies improve chromatographic reliability with sharper peaks and high reproducibility.



Time	%A	%B	Flow rate (mL/min)
0	25	75	1.0
12	40	60	1.0
12.15	60	40	0.5
12.5	60	40	0.5
12.9	25	75	0.5
13.05	25	75	1.0
15	25	75	1.0

Figure 2. This analysis uses the agilent dextran ladder standard, together with an AdvanceBio Glycan Mapping column to correlate retention times of unkown glycans. From 5990-9384EN.

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Conditions

Mobile phase

AdvanceBio Glycan Mapping columns are shipped with storage buffer acetonitrile: water and are ready to use for HILIC separations. HILIC columns are compatible with aqueous buffers and acetonitrile, which are most commonly used for glycan analysis. A typical mobile phase for glycan analysis is:

Buffer A: 100 mM ammonium formate in water, pH 4.5

Buffer B: Acetonitrile (mass spectrometry compatible)

The operating pH range of AdvanceBio Glycan Mapping columns is pH 2 to 7. AdvanceBio Glycan Mapping columns are silica-based columns with an amide HILIC phase. All silica has some solubility in pH>6 aqueous mobile phases, therefore using the column above pH 7 reduces the column lifetime.

Sample injection

For maximum resolution, inject 1 to 2 µL of your samples. Samples should first be dissolved in H_2O then made up to 70:30 acetonitrile: water. The autosampler should be cooled to preserve sample integrity. In addition, samples should be filtered before injection into the column. For UHPLC analysis, we recommend injecting 1 µL aqueous. The column inlet frit is nominally 0.5 µm for the AdvanceBio Glycan Mapping 1.8 µm columns, and 2 µm for the AdvanceBio Glycan Mapping 2.7 µm columns. Samples should therefore be filtered through a 0.2 µm sample filter.

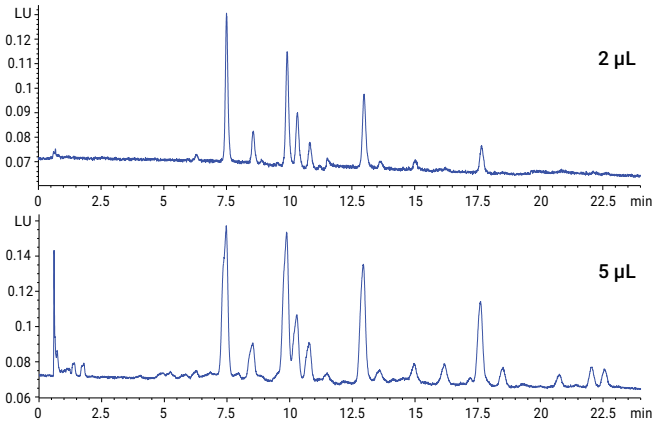


Figure 3. Demonstrates the outcome from injecting 5 µL (bottom chromatogram) on a AdvanceBio Glycan Mapping 1.8 µm, 2.1 x 150 mm column, peaks become broader and resolution is lost compared to 2 µL injection (top chromatogram). From 5991-6183EN.

Flow rates

For high resolution separations, a flow rate of 0.5 mL/min should be used. Whereas for high speed separations up to 1.0 mL/min can be used. Always run high aqueous cleanup at reduced flow rates.

Maximum operating pressure is 1200 bar for the 1.8 µm column, and 600 bar for the 2.7 µm column. Optimal column lifetime is achieved when operating up to 80 % of the maximum pressure.

AdvanceBio Glycan Mapping 2.7 µm column suggested gradients

Suggested gradient for resolution

Time	Eluent A	Eluent B	Flow
0	20 %	80 %	0.5 mL/min
32	40 %	60 %	0.5 mL/min
33	80 %	20 %	0.5 mL/min
35	80 %	20 %	0.5 mL/min
36	20 %	80 %	0.5 mL/min
45	20 %	80 %	0.5 mL/min

Suggested gradient for speed

Time	Eluent A	Eluent B	Flow
0	20 %	80 %	0.7 mL/min
12	40 %	60 %	0.7 mL/min
12.5	80 %	20 %	0.5 mL/min
13.5	80 %	20 %	0.5 mL/min
16	20 %	80 %	0.5 mL/min
17	20 %	80 %	0.7 mL/min
18	20 %	80 %	0.7 mL/min

AdvanceBio Glycan Mapping 1.8 µm column suggested gradients

Suggested gradient for resolution

Time	Eluent A	Eluent B	Flow
0	20 %	80 %	0.5 mL/min
32	40 %	60 %	0.5 mL/min
33	80 %	20 %	0.5 mL/min
35	80 %	20 %	0.5 mL/min
36	20 %	80 %	0.5 mL/min
45	20 %	80 %	0.5 mL/min

Suggested gradient for speed

Time	Eluent A	Eluent B	Flow
0	25 %	75 %	1.0 mL/min
12	40 %	60 %	1.0 mL/min
12.5	80 %	20 %	0.5 mL/min
13.5	80 %	20 %	0.5 mL/min
14	25 %	75 %	0.5 mL/min
15	25 %	75 %	1.0 mL/min
20	25 %	75 %	1.0 mL/min

It may be necessary to adjust the start and end point to obtain highest resolution for samples containing different types of glycans. Larger glycan structures may require 75 to 55 % acetonitrile gradient for optimum results for example.

Temperature

The typical operating temperature is 60 °C to achieve sharp peaks. Higher temperatures can be used but may shorten column lifetime. For longer column lifetimes, 40 °C is recommended. Selectivity and resolution may change with temperature.

Detection

Mass spectrometry can be used to detect N-glycans. However, fluorescence detection is the most commonly used analytical method employed for labeled N-glycans. Excitation and emission wavelengths are dye-specific, and values for the Agilent 1260 Infinity fluorescence detector can be found in the instruction manual pertaining to the sample preparation kit.

Maximizing column lifetime and performance

Column Conditioning

The AdvanceBio Glycan Mapping columns are designed for the separation of N-linked glycans cleaved from glycoproteins and glycopeptides. For the HILIC mechanism to work effectively, the column must be fully equilibrated before use.

- Flush the column with 100 % acetonitrile for a minimum of 10 column volumes
- Flush the column aqueous phase containing 15 % acetonitrile for a further 10 column volumes
- Finally, flush the column with the mobile phase to be used at the start of the analysis for 20 column volumes.

To check that the column is fully equilibrated, two to three analysis runs may be made done to check for reproducibility.

Troubleshooting high backpressure

If the solvent flow appears to be restricted (unusually high column backpressure), first check to see that solvent flow is unobstructed up to the column inlet. If the restriction is before the column, replace the appropriate piece of tubing or filter that is plugged. If the column is plugged, do not backflush the column, instead replace the column.

Storing your column

Acetonitrile: water (95:5) is recommended as the long-term storage solvent. It may be necessary to flush the column with 60 % acetonitrile: 40 % water to remove buffer before switching to the storage solvent. Before storing the column, tightly cap the end fittings with the end plugs to prevent the packing from drying out.

Columns can be safely stored for short periods in the mobile phases. However, to protect equipment, it is best to remove salts from the instrument and column by purging the column with the same mobile phase without the buffer. For example, using 90:10 ACN:H_2O to remove a 90:10 acetonitrile:0.01 M formate buffered mobile phase.

For short term storage, re-equilibration is faster when the column is stored in 80 % acetonitrile:20 % 5 mM ammonium formate. Several (3 to 6) injections should be made to verify column equilibration.

Ordering information

AdvanceBio Glycan Mapping Columns

1.8 µm, stable to 1200 bar

Description	Part number
2.1 x 100 mm	858700-913
2.1 x 150 mm*	859700-913
Fast Guards, 2.1 mm, 1.8 µm	651750-913

* Recommended initial column size

2.7 µm superficially porous, stable to 600 bar

Description	Part number
2.1 x 100 mm	685775-913
2.1 x 150 mm*	683775-913
2.1 x 250 mm	651750-913
Fast Guards, 2.1 mm, 2.7 µm	821725-906
4.6 x 100 mm	685975-913
4.6 x 150 mm	683975-913
4.6 x 250 mm	680975-913

* Recommended initial column size

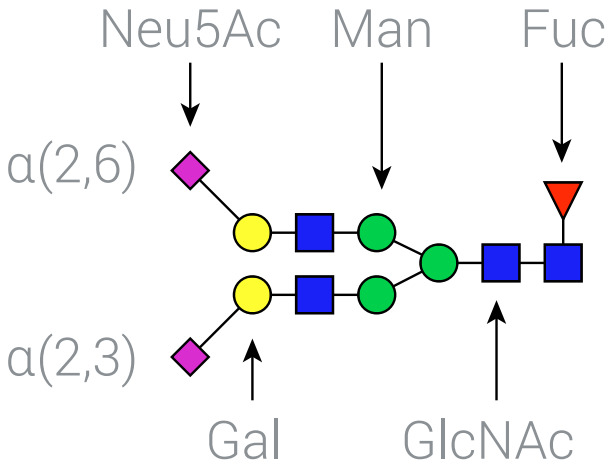
For glycan standards, see 5994-2202EN on pages 12-15

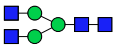
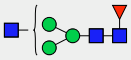
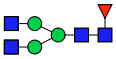
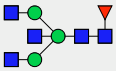
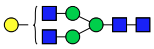
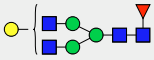
AdvanceBio Glycan Standards InstantPC, 2-AB, 2-AA, APTS, InstantAB, Unlabeled

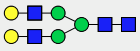
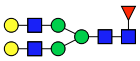
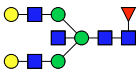
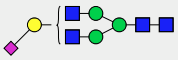
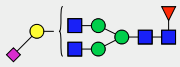
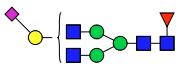
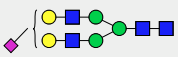
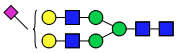
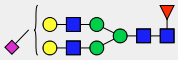
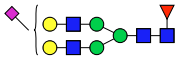
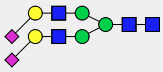
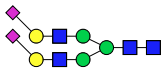
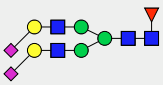
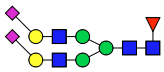
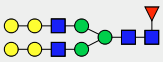
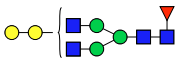
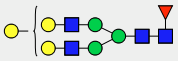
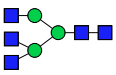
Glycan standard structures

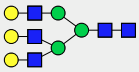
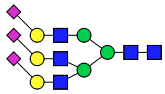
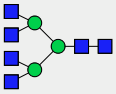
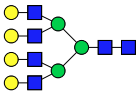
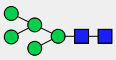
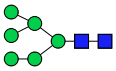
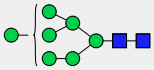
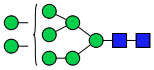
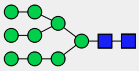
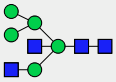
Glycan graphical representations follow the recommendations of the Consortium for Functional Glycomics[3] (CFG) and were drawn using GlycoWorkbench 2.1[4]. Neu5Ac = N-acetylneuraminic acid;
Gal = galactose;
Man = mannose;
GlcNAc = N-acetylglucosamine;
Fuc = fucose.

The α(2,3) sialic acid linkage is found on glycoproteins produced in Chinese hamster ovary (CHO) cells[5]. In contrast, human intravenous immunoglobulin (IVIG) IgG Fc N-glycans are predominantly α(2,6)-sialylated[6].



Glycan	ProZyme name	Oxford name[1]	CFG structure	Unlabeled[2]	InstantPC	InstantAB	2-AB	2-AA	APTS
Complex-type Native N-Glycans									
G0-N	NGA2-N	A1			GKPC-401		GKSB-401		GKSP-401
G0	NGA2	A2		GKC-004300	GKPC-301	GKIB-301	GKSB-301	GKSA-301	GKSP-301
G0F-N	NGA2F-N	F(6)AI			GKPC-402		GKSB-402		GKSP-402
G0F	NGA2F	F(6)A2		GKC-004301	GKPC-302	GKIB-302	GKSB-302	GKSA-302	GKSP-302
G0FB	NGA2FB	F(6)A2B		GKC-004311			GKSB-303		
G1	NA2G1	A2G1		GKC-014300	GKPC-317	GKIB-317	GKSB-317		GKSP-317
G1F	NA2G1F	F(6)A2G1		GKC-014301	GKPC-316	GKIB-316	GKSB-316	GKSA-316	GKSP-316

Glycan	ProZyme name	Oxford name[1]	CFG structure	Unlabeled[2]	InstantPC	InstantAB	2-AB	2-AA	APTS
G2	NA2	A2G(4)2		GKC-024300	GKPC-304	GKIB-304	GKSB-304	GKSA--304	GKSP-304
G2F	NA2F	F(6)A2G(4)2		GKC-024301	GKPC-305	GKIB-305	GKSB-305	GKSA-305	GKSP-305
G2FB	NA2FB	F(6)A2BG(4)2		GKC-024311			GKSB-306		
G1S1 α(2,3)		A2G(4)1S(3)1			GKPC-329				
G1S1 α(2,6)		A2G(4)1S(6)1			GKPC-319				
G1FS1 α(2,3)		FA2G(4)1S(3)1			GKPC-330				
G1FS1 α(2,6)		FA2G(4)1S(6)1			GKPC-320				
G2S1 α(2,3)	A1(α2,3)	A2G(4)2S(3)1			GKPC-321				
G2S1 α(2,6)	A1(α2,6)	A2G(4)2S(6)1		GKC-124300	GKPC-311	GKIB-311	GKSB-311	GKSA-311	GKSP-311
G2FS1 α(2,3)	A1F(α2,3)	F(6)A2G(4)2S(3)1			GKPC-325				
G2FS1 α(2,6)	A1F(α2,6)	F(6)A2G(4)2S(6)1		GKC-124301	GKPC-315	GKIB-315	GKSB-315	GKSA-315	GKSP-315
G2S2 α(2,3)	A2(α2,3)	A2G(4)2S(3)2			GKPC-322				
G2S2 α(2,6)	A2(α2,6)	A2G(4)2S(6)2		GKC-224300	GKPC-312	GKIB-312	GKSB-312	GKSA-312	GKSP-312
G2FS2 α(2,3)	A2F(α2,3)	F(6)A2G(4)2S(3)2			GKPC-323				
G2FS2 α(2,6)	A2F(α2,6)	F(6)A2G(4)2S(6)2		GKC-224301	GKPC-313	GKIB-313	GKSB-313	GKSA-313	GKSP-313
G2F w/2 α-gal	NA2Ga2F	F(6)A2G(4)2Ga(3)2			GKPC-318		GKSB-318		GKSP-318
G1F w/1 α-gal	NA2G 1FGa1	F(6) A2G(4)1Ga(3)1			GKPC-403				
G2F w/1 α-gal	NA2FGa1	F(6)A2G(4)2Ga(3)1			GKPC-404				
A3	NGA3	A3		GKC-005300		GKIB-307	GKSB-307	GKSA-307	

Glycan	ProZyme name	Oxford name[1]	CFG structure	Unlabeled[2]	InstantPC	InstantAB	2-AB	2-AA	APTS
G3	NA3	A3G(4)3		GKC-035300			GKSB-308	GKSA-308	
G3S3 α(2,6)	A3(a2,6)	A3G(4)3S(6)3		GKC-335300			GKSB-314		
A4	NGA4	A4		GKC-006300			GKSB-309	GKSA-309	
G4	NA4	A4G(4)4		GKC-046300			GKSB-310		
High Mannose-type Native N-Glycans									
Man5	MAN-5	M5		GKM-002500	GKPC-103	GKIB-103	GKSB-103	GKSA-103	GKSP-103
Man6	MAN-6	M6		GKM-002600	GKPC-104	GKIB-104	GKSB-104	GKSA-104	GKSP-104
Man7	MAN-7	M7		GKM-002700	GKPC-105	GKIB-105	GKSB-105	GKSA-105	GKSP-105
Man8	MAN-8	M8		GKM-002800	GKPC-106	GKIB-106	GKSB-106	GKSA-106	GKSP-106
Man9	MAN-9	M9		GKM-002900	GKPC-107	GKIB-107	GKSB-107	GKSA-107	GKSP-107
Hybrid-type Native N-Glycan									
Hybrid	HYBR	M5A1B					GKSB-111		
Native N-Glycan Cores									
NF	NF			GKR-001001					
NN	NN			GKR-002000			GKSB-100		
NNF	NNF			GKR-002001					
Man1	MNN	M1		GKR-002100					
Man1F	MNNF	F(6)M1		GKR-002101					
Man3				GKR-002300			GKSB-101		
Man3F				GKR-002301			GKSB-102		

Return to Contents Section

Glycans	Unlabeled	InstantPC	InstantAB	2-AB	2-AA	APTS
N-Glycan Libraries						
Human IgG N-Glycan Library	GKLB-005	GKPC-005	GKIB-005	GKSB-005	GKSA-005	GKSP-005
CHO mAb N-Glycan Library		GKPC-020				
CHO mAb N-Glycan Library plus CHO mAb Glycoprotein		GKPC-020-P				
Human α1-acid glycop rotein N-Glycan Library	GKLB-001		GKIB-001	GKSB-001	GKSA-001	
Bovine Fetuin N-Glycan Library	GKLB-002		GKIB-002	GKSB-002	GKSA-002	
RNase B N-Glycan Library (High Mannose)			GKIB-009			
Biantennary and High Mannose Partitioned Library			GKIB-520	GKSB-520		GKSP-520
Sialylated Biantennary N-Glycan Library			GKIB-232	GKSB-232		GKSP-232
α(2,6) Sialylated Biantennary N-Glycan Library				GKSB-262		GKSP-262
α(2,3) Sialylated Triantennary N-Glycan Library		GKPC-233	GKIB-233	GKSB-233		GKSP-233
α(2,6) Sialylated Triantennary N-Glycan Library		GKPC-263		GKSB-263		GKSP-263
α(2,3) Sialylated Tetraantennary N-Glycan Library		GKPC-234	GKIB-234	GKSB-234		GKSP-234
α(2,6) Sialylated Tetraantennary N-Glycan Library		GKPC-264		GKSB-264		GKSP-264
Alignment Standards						
Glucose Unit (GU) Ladder		GKPC-503	GKIB-503	GKSB-503	GKSA-503	GKSP-503
Internal Migration Standards for Capillary Electrophoresis (CE)						GKSP-500

References

1. Harvey DJ, *et al*. Proposal for a standard system for drawing structural diagrams of N- and O-linked carbohydrates and related compounds. *Proteomics*. **2009**, *9*(15):3796–801.
2. Not all unlabeled glycans are shown.
3. Varki A, et al. Symbol Nomenclature for Graphical Representations of Glycans. *Glycobiology*. **2015** *Dec; 25*(12): 1323–1324.
4. Ceroni A, *et al*. GlycoWorkbench: a tool for the computer-assisted annotation of mass spectra of glycans. *J Proteome Res*. **2008** *Apr;7*(4):1650-9.
5. Lee EU, *et al*. Alteration of terminal glycosylation sequences on N-linked oligosaccharides of Chinese hamster ovary cells by expression of beta-galactoside alpha 2,6-sialyltransferase. *J Biol Chem*. **1989**, *264*(23), 13848-55.
6. Anthony RM, *et al*. Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc. *Science*. **2008**, *320*(5874), 373-6.

Application Note

Pharma & Biopharma

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Trusted Answers

Streamlined Workflows for N-Glycan Analysis of Biotherapeutics Using Agilent AdvanceBio Gly-X InstantPC and 2-AB Express Sample Preparation with LC/FLD/MS

Authors

John Yan and Aled Jones
Agilent Technologies, Inc

Introduction

This Application Note describes the preparation and analysis of released N-glycans from biotherapeutic glycoproteins using two labels, InstantPC and 2-aminobenzamide (2-AB). N-Glycan analysis is vital to the development and production of biotherapeutics, as glycosylation can influence the therapeutic function of the final drug product. The workflows described here use the Agilent AdvanceBio Gly-X with InstantPC and Gly-X 2-AB Express kits (formerly ProZyme) for the release of N-glycans using PNGase F followed by instant glycosylamine labeling with InstantPC or reductive amination labeling with 2-AB Express, respectively. Labeled N-glycans were separated by hydrophilic interaction liquid chromatography (HILIC), with detection using both fluorescence and mass spectrometry (MS). Gly-X sample preparation offers a high level of reproducibility and throughput, with a one hour preparation time for InstantPC and two hours for 2-AB Express. In addition, the InstantPC label offers improved fluorescence response and MS ionization efficiency.

Introduction

The characterization of N-glycans is an essential part of the biotherapeutic development process, as the structure of N-linked glycans can influence the function of glycosylated biotherapeutics, frequently making glycosylation a critical quality attribute (CQA).[1] N-Glycan analysis often involves the labeling of released glycans with a tag to allow for detection by fluorescence (FLD), and to enhance ionization for mass spectrometry (MS), followed by N-glycan separation, detection, and relative quantitation. Many of the frequently used fluorescent tags such as 2-AB[2] are limited concerning MS sensitivity compared with recently introduced dyes such as InstantPC, and pre-existing N-glycan sample preparation workflows can be time-consuming.[3] However, 2-AB has been used for over 20 years and so is well-established in the literature and in many laboratories.

This Application Note presents streamlined workflows for preparation of InstantPC and 2-AB labeled N-glycans coupled with analysis using Agilent LC/FLD/MS instrumentation. Gly-X N-glycan sample preparation kits for InstantPC or 2-AB Express labeling (formerly ProZyme) include all reagents for N-glycan sample preparation: denaturation, deglycosylation, labeling, and sample cleanup, as illustrated in Figure 1.

Experimental

N-Glycan sample preparation

Agilent AdvanceBio Gly-X N-glycan prep with InstantPC (p/n GX96-IPC) and Gly-X 2-AB Express (p/n GX96-2AB) Kits were used to prepare labeled N-glycans from monoclonal antibody rituximab (Rituxan, lot number M190170) and Fc fusion protein etanercept (Enbrel, lot number 1092537), 40 µg protein per preparation. Four replicates of each sample were analyzed with fluorescence/MS detection and relative percent glycan peak areas calculated.

InstantPC and 2-AB labeled samples were prepared by standard manual protocols. The Gly-X in-solution deglycosylation protocol uses a three-minute denaturation at 90 °C, opening up the glycoprotein target to enable a five-minute deglycosylation reaction at 50 °C with PNGase F. Following in-solution deglycosylation, InstantPC labeled samples are prepared by one-minute glycosylamine labeling of released N-glycans (Figure 2), followed by vacuum-driven cleanup of free dye using HILIC solid-phase extraction (SPE).

N-Glycan samples labeled with 2-AB were prepared using the standard Gly-X 2-AB Express protocol with reductive amination chemistry. Following the Gly-X five-minute deglycosylation with PNGase F, released N-glycans are converted from the glycosylamine form ($-NH_2$) to free reducing end form (–OH) to allow for 2-AB labeling with reductive amination. N-Glycans are then desolvated by vacuum filtration onto a solid-state matrix followed by an on-matrix 2-AB labeling step. This process eliminates the need for glycan drying prior to the 2-AB labeling step, thereby reducing total sample preparation time.

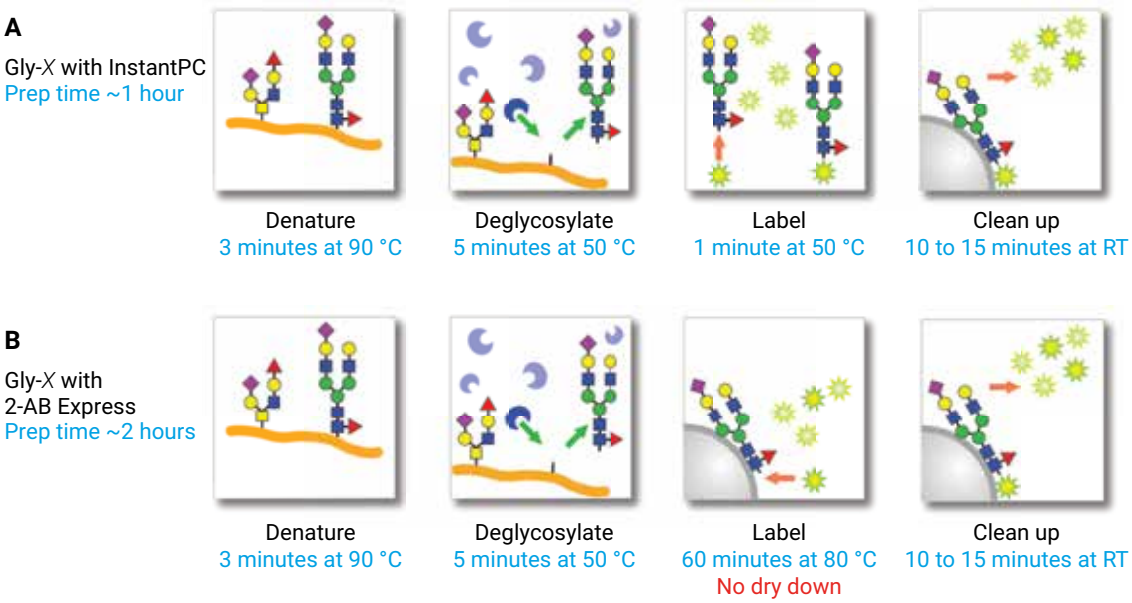

Figure 1. Gly-X N-glycan sample prep.
A) InstantPC workflow with in-solution deglycosylation and labeling followed by on-matrix cleanup;
B) 2-AB workflow with deglycosylation in-solution, followed by on-matrix labeling and cleanup.

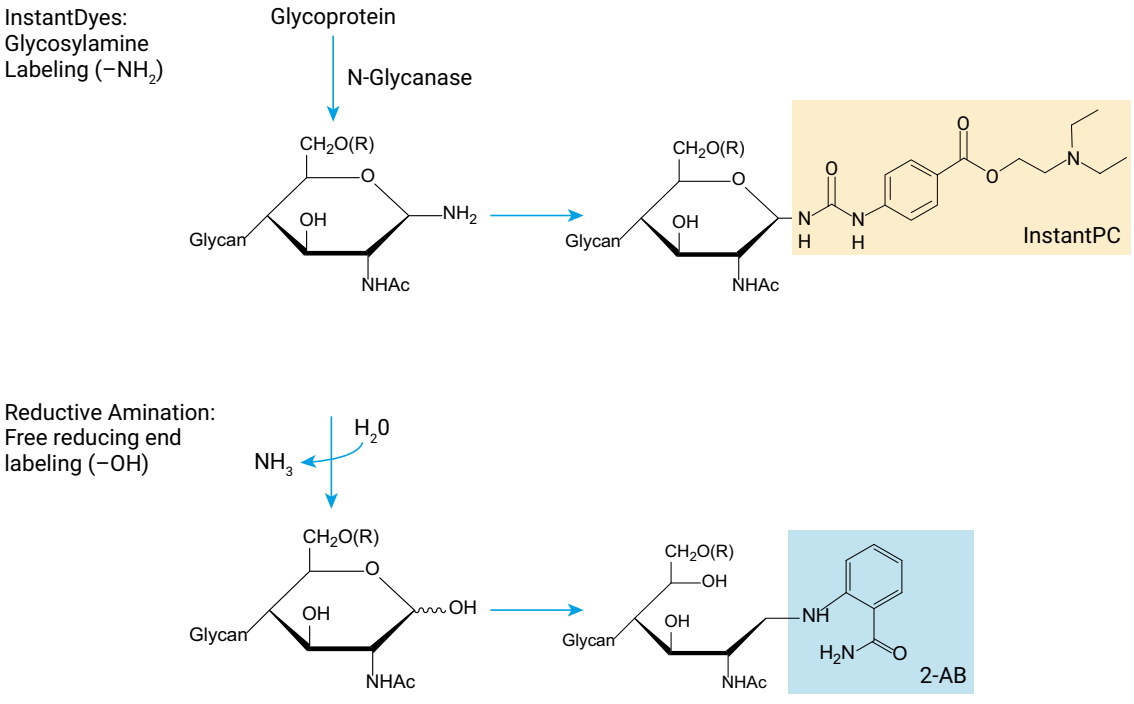

Figure 2. Comparison of InstantPC glycosylamine labeling and traditional reductive amination with 2-AB.

N-Glycan analysis

InstantPC and 2-AB labeled N-glycans were separated by hydrophilic interaction liquid chromatography (HILIC) using an Agilent AdvanceBio Glycan Mapping column, 2.1 × 150 mm, 1.8 µm (p/n 859700-913) with an Agilent 1290 Infinity II LC system with in-line fluorescence detection (Table 1) coupled to an Agilent AdvanceBio 6545XT LC/Q-TOF (Table 2).

All HILIC separations were conducted under the conditions described in Table 1. A fixed flow splitter (IDEX Health & Science p/n UH-427) was used post-FLD, diverting approximately 50% of the flow to waste and 50% to the MS. Agilent MassHunter BioConfirm software was used for data processing, with a personal compound database (PCD).

Materials

LC/MS grade acetonitrile and water were purchased from Honeywell Research Chemicals.

Instrumentation

Labeled N-glycan samples were separated using an Agilent AdvanceBio Glycan Mapping column (Table 1 shows the method details) on an Agilent LC/MS setup composed of:

- Agilent 1290 Infinity II high speed pump (G7120A)
- Agilent 1290 Infinity II 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 MassHunter Acquisition
- Agilent MassHunter Qualitative Analysis software

Table 1. Agilent 1290 Infinity II UHPLC HILIC/FLD conditions.

Conditions, Agilent 1290 Infinity II UHPLC HILIC/FLD

Parameter	Value	
Column:	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n 859700-913)	
Column Temp:	40 °C	
Mobile phase:	A) 50 mM ammonium formate, pH 4.5 B) Acetonitrile	
Gradient:	Time (min) 0 2 48 49 51.5 52 60	% B 80 75 62 40 80 80 80
Flow rate:	0.5 mL/min	
Injection volume:	1 µL (equivalent to glycans from 0.4 µg protein)	
Detection:	Agilent 1260 Infinity II FLD InstantPC: λEx 285 nm, λEm 345 nm 2-AB: λEx 260 nm, λEm 430 nm	

Table 2. Agilent 6545XT Q-TOF parameters.

Agilent 6545XT Q-TOF parameters	
Source	Dual AJS ESI
Gas temperature	150 °C
Drying Gas Flow	9 L/min
Nebulizer	35 psi
Sheath gas temperature	300 °C
Sheath gas flow	10 L/min
Vcap	3,000 V
Nozzle Voltage	500 V
Fragmentor	120 V
Skimmer	65 V
Mass Range	m/z 600 to 3,000
Scan Rate	1 spectra/sec
Acquisition Mode	High resolution (4 GHz)

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Results and Discussion

HILIC Separation of InstantPC and 2-AB N-Glycans

HILIC separation of labeled N-glycans from Rituxan and Enbrel labeled with InstantPC or 2-AB results in well resolved peaks for major glycan species with the 60-minute method used (Figures 3 and 4). Rituxan (Figure 3A, InstantPC; Figure 4A, 2-AB), an IgG, has an N-glycan profile typical of monoclonal antibodies with one N-glycosylation site in the Fc region produced in Chinese hamster ovary (CHO) cells: predominantly neutral biantennary complex N-glycans with core fucose, some Man5, and a relatively low proportion of sialylated glycans. The N-glycan profile of Enbrel (Figure 3B, InstantPC; Figure 4B, 2-AB), an Fc fusion protein, contains a higher level of sialylated glycans owing to two additional N-glycosylation sites in the fusion partner, TNF-α receptor (TNFR) extracellular domain, in addition to the single N-glycan site in the Fc portion.5

The HILIC retention time of 2-AB N-glycans is shorter than for InstantPC N-glycans, although the elution order of N-glycan species is comparable. Critical pairs such as G0F/Man5 and Man5/G1, which are often monitored during the development process of biotherapeutics, are well separated with both InstantPC and 2-AB labels, leading to confident determination of relative percentage composition. G1F isomers G1F[6] and G1F[3] are also separated. Relative percent areas, standard deviation, and relative standard deviation are reported in Tables 3 through 6, and show a low degree of variability between the four sample preparation replicates. This variability rises for lower abundance glycans.

An added benefit of InstantPC is the separation of isoforms G2S1[6]/[3] and G2FS1[6] from Enbrel (Figure 3B) compared to 2-AB (Figure 4B) using the previously described chromatography conditions. Analysis with fluorescence detection of InstantPC and 2-AB labeled N-glycans from biotherapeutics Rituxan and Enbrel results in comparable relative percent areas for major glycoforms G0F, G1F[6]/[3], G2F, G2S2, and G2FS2.

FLD and MS detection of InstantPC and 2-AB N-Glycans

InstantPC displays higher fluorescence and MS signal compared to 2-AB (Figure 5), when using the same amount of glycoprotein starting material (40 µg), and injecting the same relative volume for HILIC separations (1 µL of 100 µL kit eluent). Individual spectra for InstantPC and 2-AB labeled Man5 illustrates the higher MS signal of InstantPC (Figure 6).

Table 3. Absolute trastuzumab biosimilar concentrations determined in the different CHO clones grown on two different media.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.75	0.01	1.55
G0	1.47	0.02	1.18
G0F	46.82	0.07	0.15
Man5	1.21	0.01	0.83
G1[6]	0.75	0.02	2.67
G1F[6]	31.21	0.11	0.35
G1F[3]	9.27	0.05	0.54
G2F	7.04	0.04	0.51
G2FS1[6]	0.67	0.02	2.29
G2FS1[3]	0.37	0.06	15.98
G2FS2	0.45	0.03	6.67

Table 4. Figure 3B relative % area, SD, and %CV values for Enbrel N-glycans labeled with InstantPC, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0	1.10	0.02	2.09
G0F	19.36	0.16	0.84
Man5	5.08	0.03	0.52
G1[6]	0.48	0.00	0.00
G1F[6]	10.48	0.04	0.39
G1F[3]	3.97	0.01	0.25
G2	2.08	0.01	0.55
G1FS1	1.84	0.05	2.49
G2F	4.26	0.09	1.99
G2S1[6]	1.18	0.01	0.49
G2S1[3]	13.91	0.04	0.31
G2FS1[6]	0.89	0.00	0.00
G2FS1[3]	20.54	0.08	0.37
G2S2	4.26	0.01	0.14
G2FS2	10.54	0.08	0.78

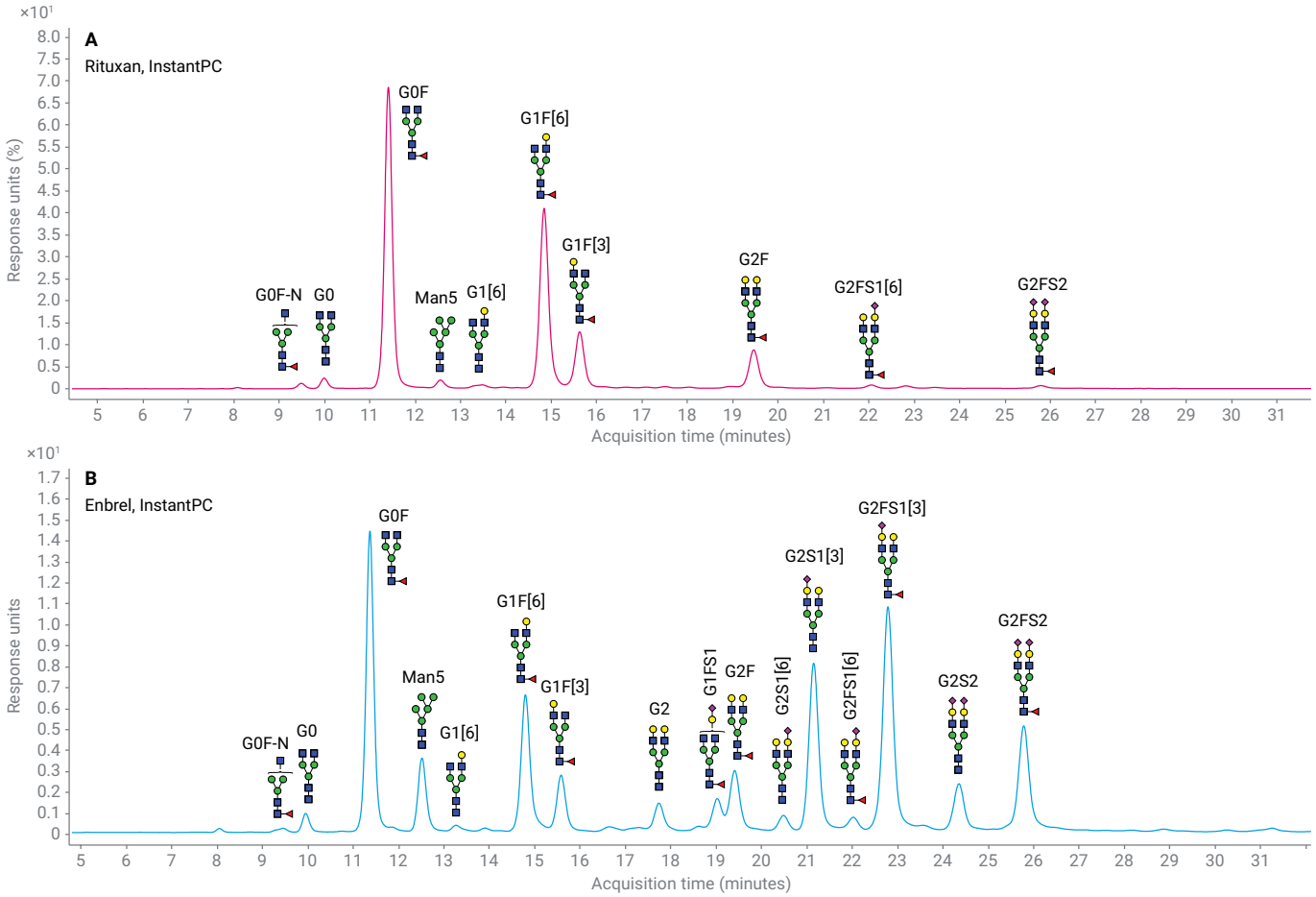

Figure 3. HILIC-UHPLC fluorescence profile of A) Rituxan and B) Enbrel N-glycans labeled with InstantPC. N-Glycan relative percent areas are shown in Table 3 and Table 4, n = 4.

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Table 3. Absolute trastuzumab biosimilar concentrations determined in the different CHO clones grown on two different media.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.75	0.01	1.55
G0	1.47	0.02	1.18
G0F	46.82	0.07	0.15
Man5	1.21	0.01	0.83
G1[6]	0.75	0.02	2.67
G1F[6]	31.21	0.11	0.35
G1F[3]	9.27	0.05	0.54
G2F	7.04	0.04	0.51
G2FS1[6]	0.67	0.02	2.29
G2FS1[3]	0.37	0.06	15.98
G2FS2	0.45	0.03	6.67

Table 4. Figure 3B relative % area, SD, and %CV values for Enbrel N-glycans labeled with InstantPC, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0	1.10	0.02	2.09
G0F	19.36	0.16	0.84
Man5	5.08	0.03	0.52
G1[6]	0.48	0.00	0.00
G1F[6]	10.48	0.04	0.39
G1F[3]	3.97	0.01	0.25
G2	2.08	0.01	0.55
G1FS1	1.84	0.05	2.49
G2F	4.26	0.09	1.99
G2S1[6]	1.18	0.01	0.49
G2S1[3]	13.91	0.04	0.31
G2FS1[6]	0.89	0.00	0.00
G2FS1[3]	20.54	0.08	0.37
G2S2	4.26	0.01	0.14
G2FS2	10.54	0.08	0.78

Table 5. Figure 4A relative % area, SD, and %CV values for Rituxan N-glycans labeled with 2-AB, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.78	0.09	11.94
G0	1.64	0.05	3.12
G0F	44.89	0.39	0.87
Man5	1.54	0.14	8.83
G1F[6]	31.39	0.09	0.27
G1F[3]	10.40	0.14	1.34
G2F	7.52	0.16	2.10
G2FS1	1.17	0.03	2.13
G2FS2	0.67	0.02	3.58

Table 6. Figure 4B relative % area, SD, and %CV values for Rituxan N-glycans labeled with 2-AB, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.32	0.02	7.44
G0	1.27	0.07	5.34
G0F	20.18	0.45	2.22
Man5	5.50	0.34	6.17
G1[6]	0.45	0.02	3.89
G1F[6]	10.35	0.33	3.18
G1F[3]	3.92	0.17	4.39
G2	2.21	0.15	6.78
G2F/G1FS1	7.00	0.25	3.63
G2S1	15.19	0.17	1.09
G2FS1	20.10	0.32	1.59
G2S2	4.19	0.25	5.95
G2SF2	9.35	0.74	7.93

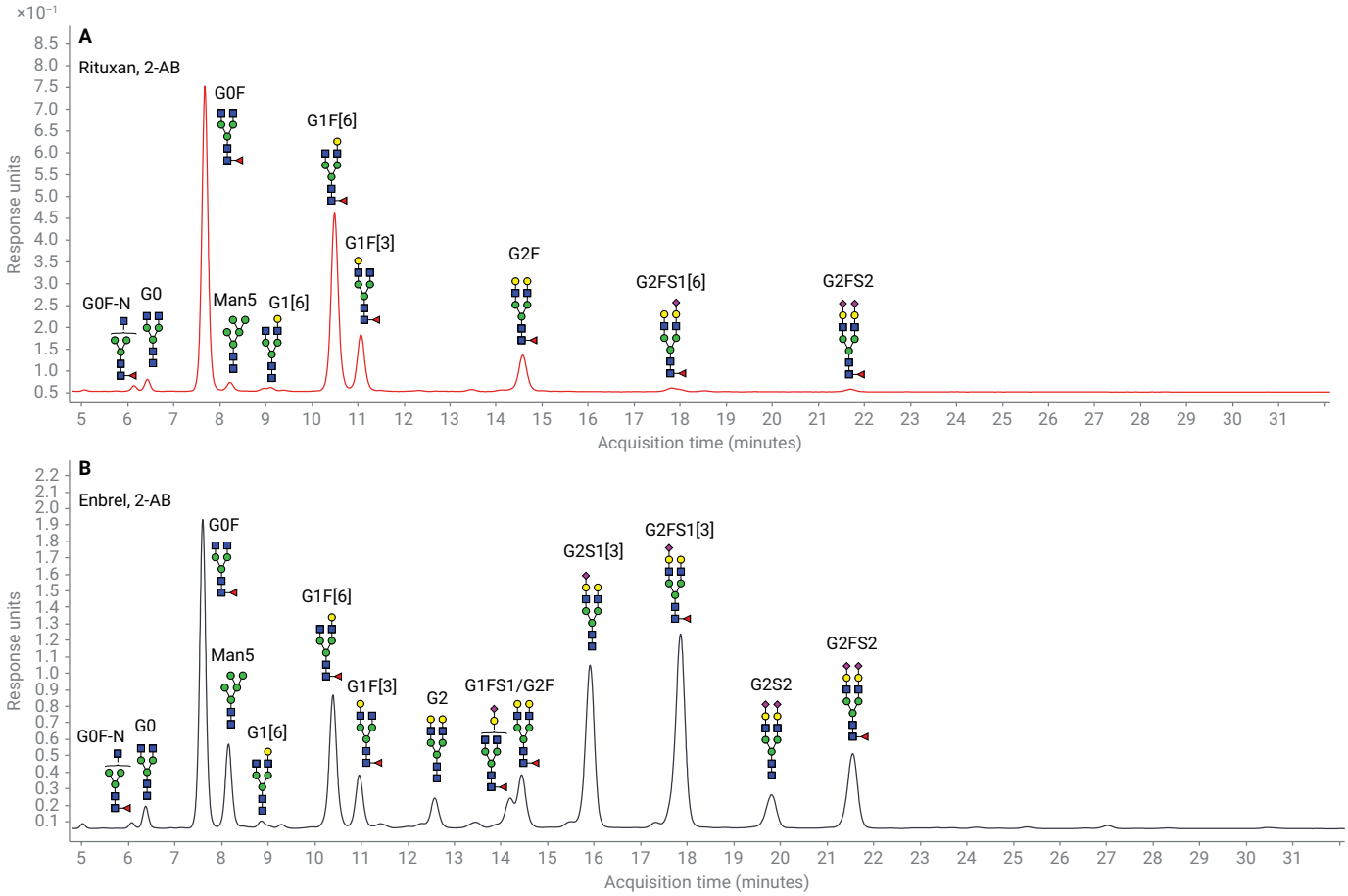

Figure 4. HILIC-UHPLC fluorescence profile of A) Rituxan and B) Enbrel N-glycans labeled with 2-AB. N-Glycan relative percent areas are shown in Table 5 and Table 6, n = 4.

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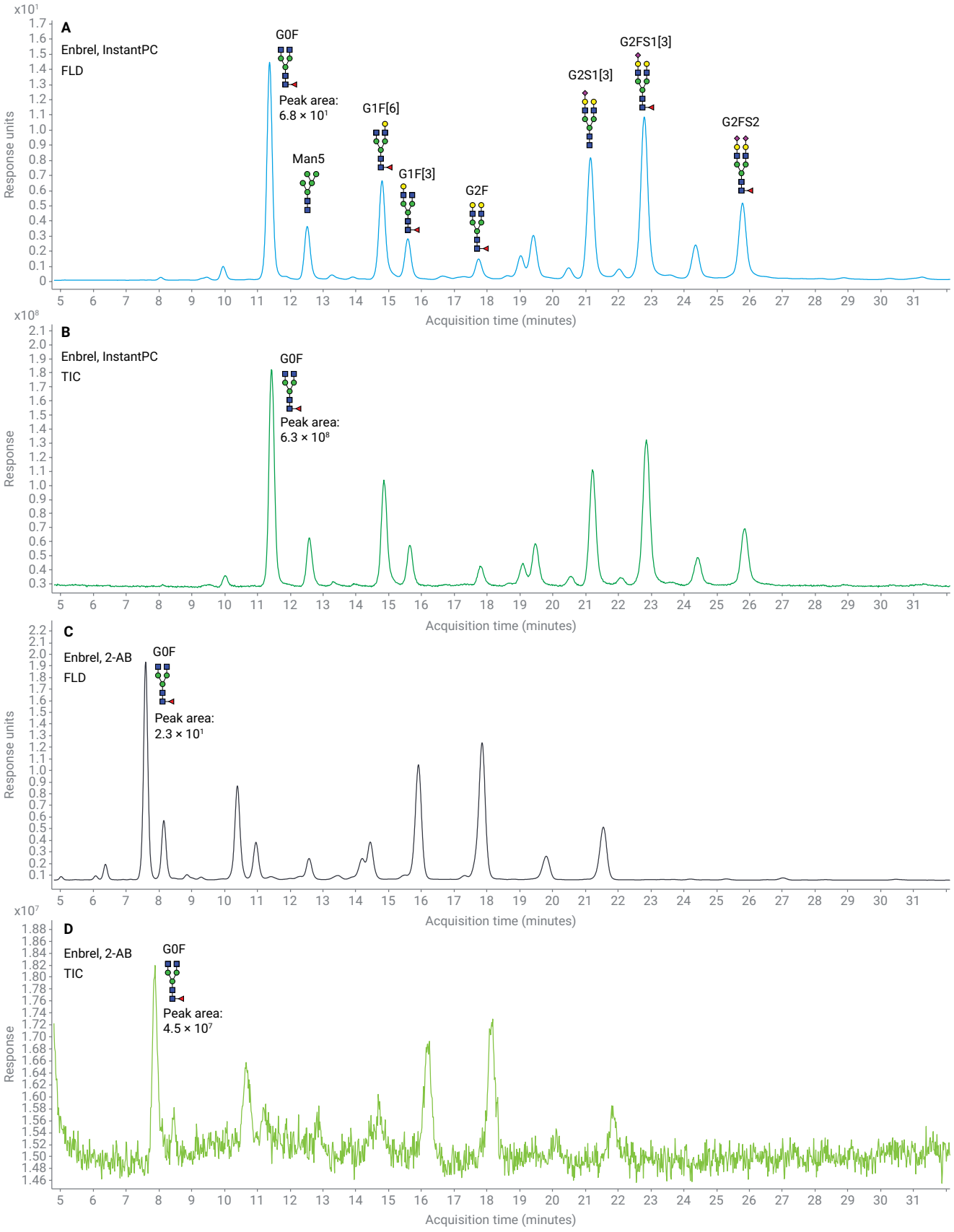

Figure 5. FLD and MS of InstantPC and 2-AB labeled N-glycans from Enbrel. A) InstantPC FLD; B) InstantPC TIC (total ion chromatogram); C) 2-AB FLD; D) 2-AB TIC.

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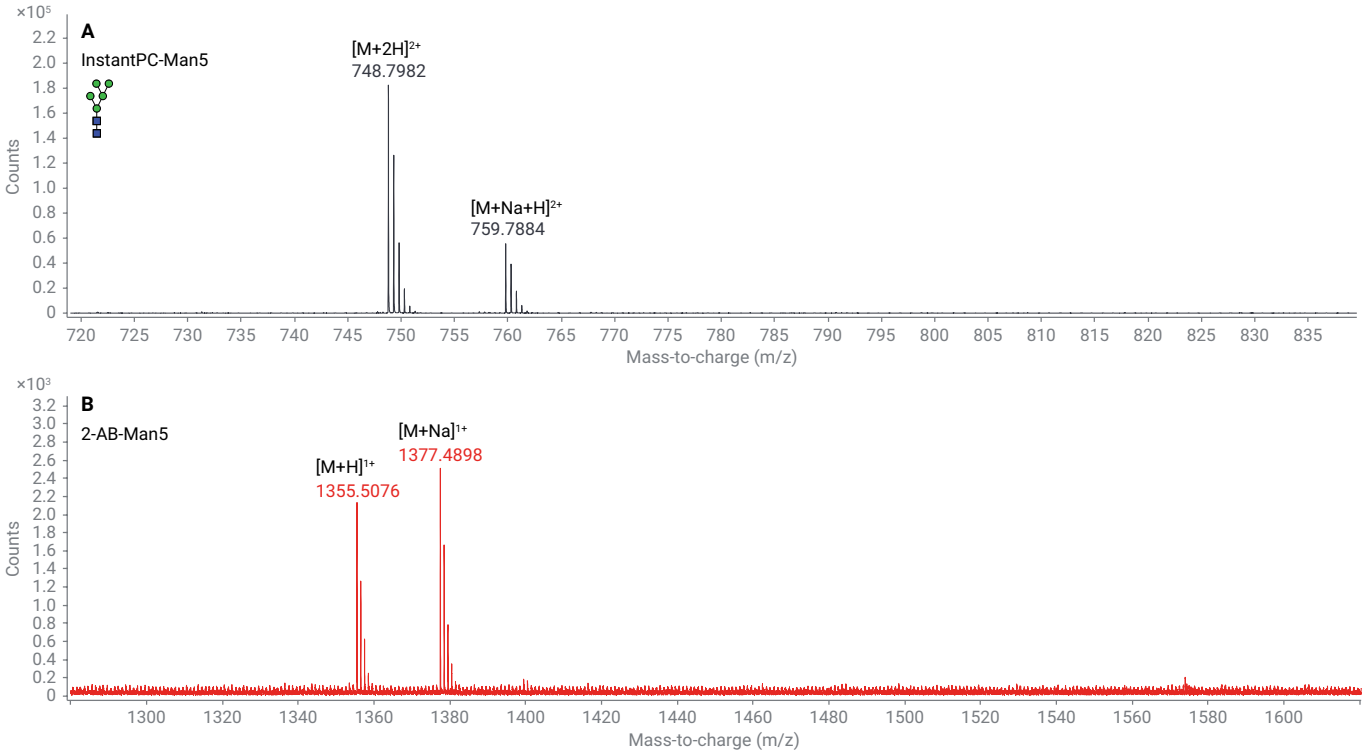

Figure 6. Mass spectrum comparison of Man5 from Enbrel, labeled with A) InstantPC and B) 2-AB.

Conclusions

Glycosylation is a feature of many biotherapeutic proteins and is often a CQA that must be monitored. N-Glycan analysis is important in the development and production of therapeutic proteins. Gly-X N-glycan sample preparation workflows enable a five minute release of N-linked glycans suitable for labeling both by glycosylamine labeling with InstantPC and reductive amination chemistry with 2-AB. These workflows allow for instant glycosylamine labeling with InstantPC or no dry down on-matrix reductive amination labeling with 2-AB. Glycan species were profiled by relative fluorescence peak area % and peak assignments confirmed by high resolution mass spectrometry. Compared to 2-AB, InstantPC labeled glycans display higher FLD signal and greater MS ionization efficiency in positive mode, allowing for confident detection of low abundance glycan species. Although the performance benefits of InstantPC are clear, 2-AB is an N-glycan label that has been used for many years. Therefore, a rapid 2-AB workflow enables continuity with historical 2-AB N-glycan data sets.

References

1. 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.
2. Anumula, K. R. Advances in Fluorescence Derivatization Methods for High-Performance Liquid Chromatographic Analysis of Glycoprotein Carbohydrates. *Anal. Biochem.* **2006**, *350*, 1–23.
3. Kimzey, M. et al. Development of a 5-Minute Deglycosylation Method for High Throughput N-Glycan Analysis by Mass Spectrometry. ProZyme Technical Note, Bulletin 4001, Rev E.
4. Yan, J. *et al.* Preparation of Released N-Glycan Samples from Monoclonal Antibodies Using Agilent AdvanceBio Gly-X 2-AB Express for LC-Fluorescence Analysis. Agilent Technologies Application Note, publication number 5994-0682EN, **2019**.
5. Houel, S.; *et al*. N- and O-Glycosylation Analysis of Etanercept Using Liquid Chromatography and Quadrupole Time-of-Flight Mass Spectrometry Equipped with Electron-Transfer Dissociation Functionality. *Anal. Chem.* **2014**, *86*, 576–584.

Application Note

Pharma & Biopharma

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Trusted Answers

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

Authors

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Agilent Technologies, Inc.

Introduction

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 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.

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

Table 1. Reversed-phase UHPLC conditions.

Conditions, Agilent 1290 Infinity II LC System

Parameter	Value			
Column:	Agilent InfinityLab PoroShell 120EC-C18, 2.1 × 75 mm, 2.7 µm (p/n 697775-902)			
Column Temp:	30 °C			
Mobile phase:	A) Methanol:acetonitrile:water (4:8:88) B) Acetonitrile			
Gradient:	Time (min) 0 6 6.25 7.3 7.5 10	% A 100 100 20 20 100 100	% B 0 0 80 80 0 0	 Isocratic elution Wash Re-equilibration
Flow rate:	0.4 mL/min			
Injection volume:	10 µL			
Detection:	Agilent 1260 Infinity II FLD λEx 373 nm, λEm 448 nm			

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

6545XT AdvanceBio LC/Q-TOF parameters	
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)

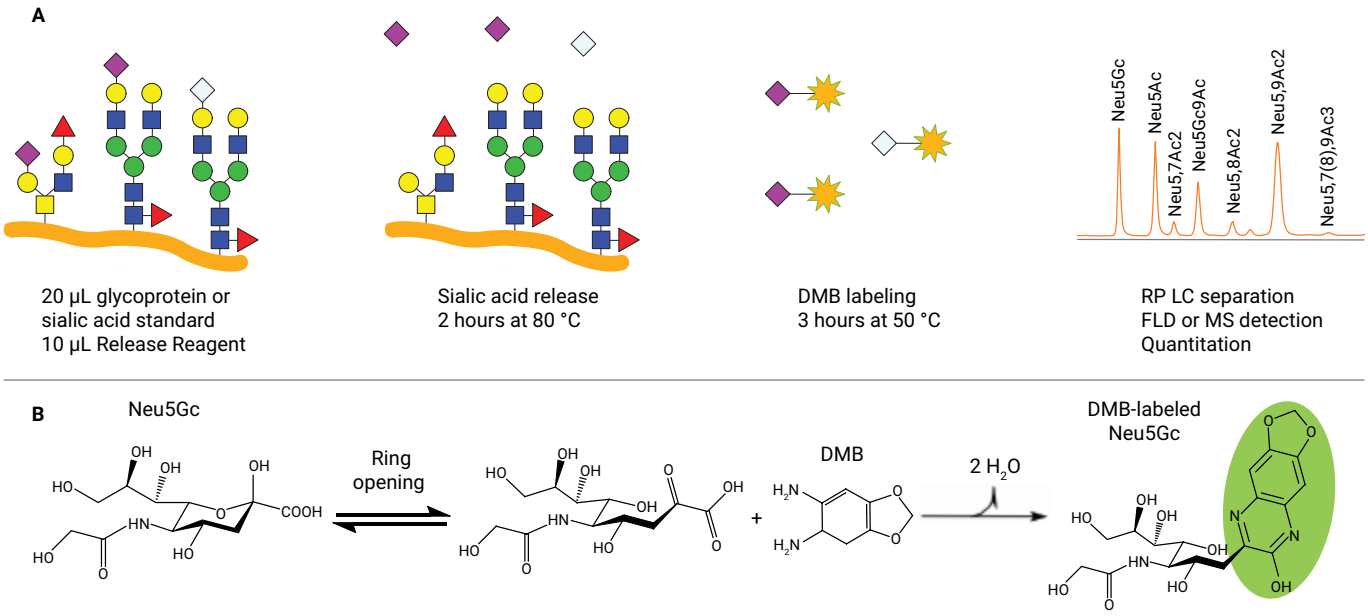

Figure 1. Sialic acid release and DMB labeling workflow (A) overview (B) DMB labeling mechanism of sialic acid Neu5Gc.

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 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.

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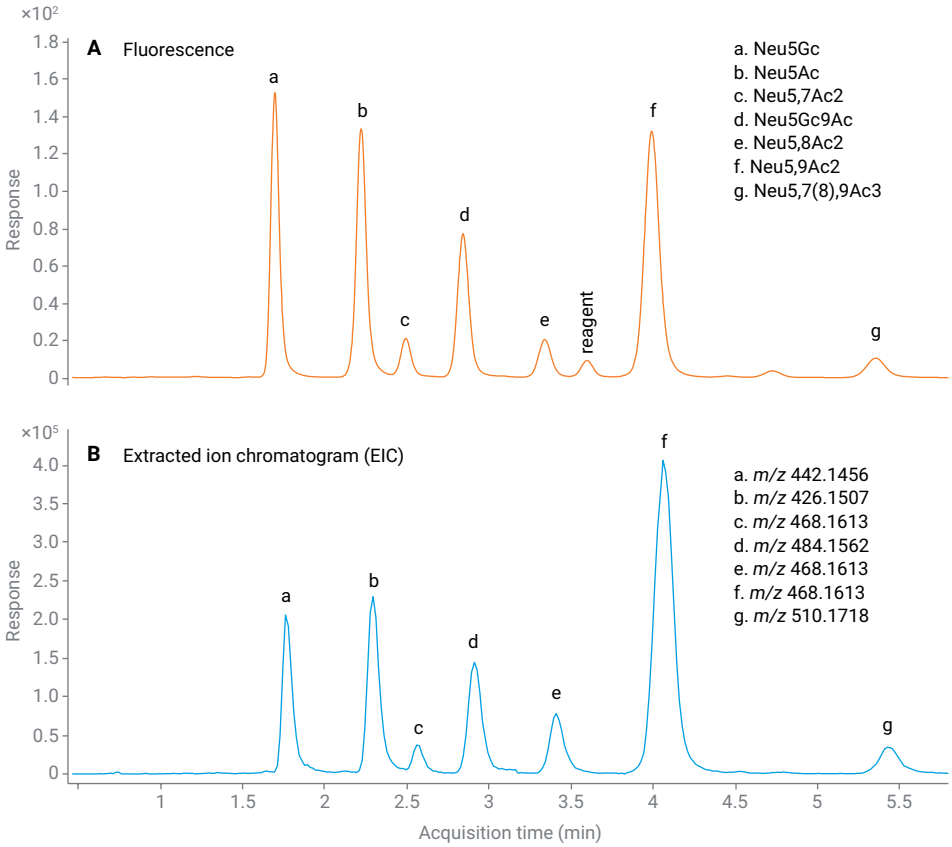

Figure 2. UHPLC chromatogram of DMB-labeled SARP. (A) fluorescence; (B) extracted ion chromatogram of DMB-labeled sialic acid species, [M+H]1+.

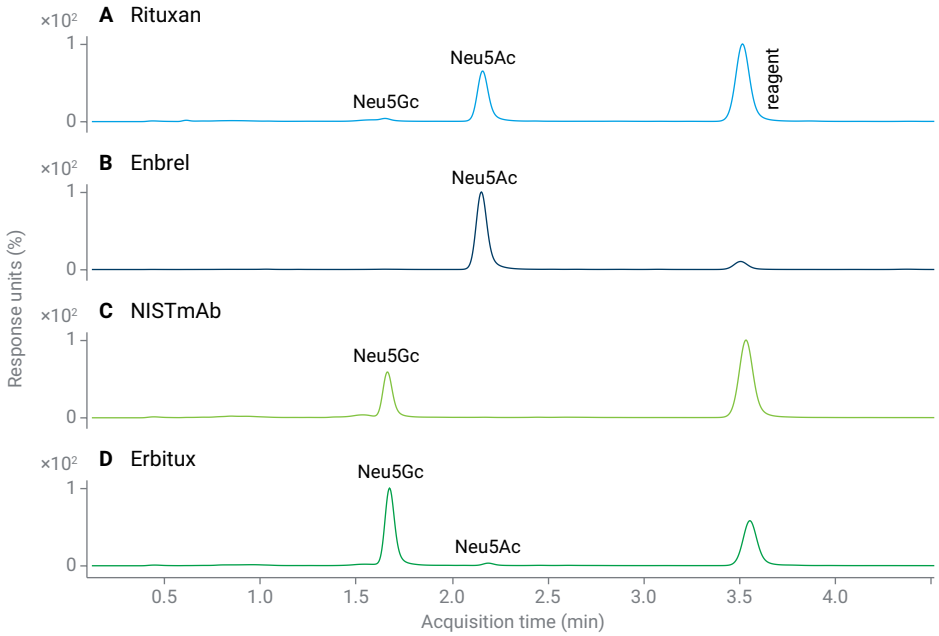

Figure 3. UHPLC fluorescence profiles of DMB-labeled sialic acids from different glycoproteins (A) Rituxan; (B) Enbrel; (C) NISTmAb; and (D) Erbitux.

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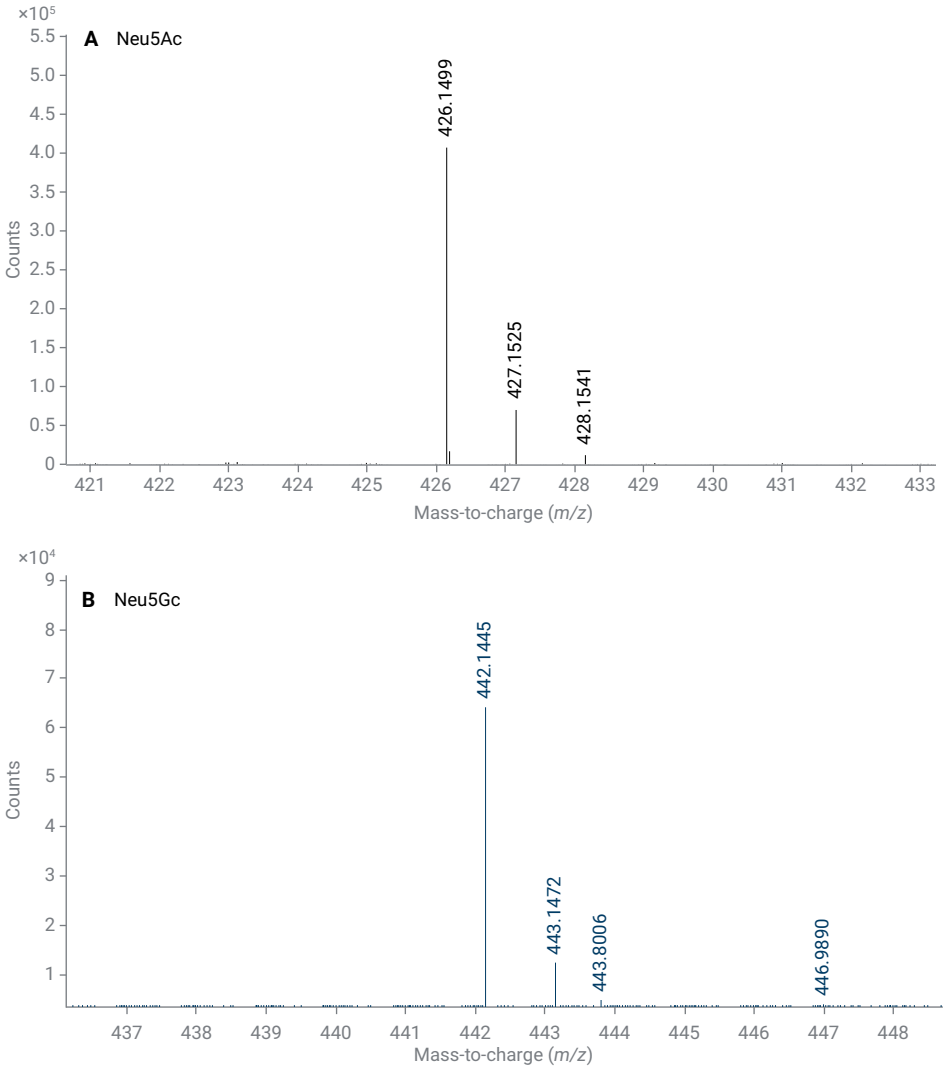
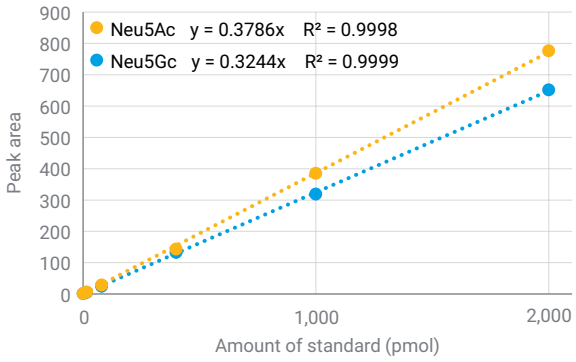


Figure 4. Mass spectrum of DMB-labeled sialic acid (A) Neu5Ac from Enbrel; and (B) Neu5Gc from Erbitux.



Sialic Acid	LOD (pmol)	LOQ (pmol)
Neu5Ac	0.016	0.053
Neu5Gc	0.0162	0.040

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

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

Column	Concentration (mg/ml)	RSample 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.

		GKK-407		Agilent AdvanceBio Sialic Acid Profiling and Quantitation Kit	
Glycoprotein	Sialic acid	pmol/µg	%CV	pmol/µg	%CV
Rituxan	Neu5Gc	ND	-	40.02	1.75%
	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	-	10.9%	3.72
	Neu5Ac	201	1.47%	222	4.44%

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

	Agilent AdvanceBio Sialic Acid Profiling and Quantitation Kit		Agilent AdvanceBio Total Sialic Acid Quantitation Kit	
Peak	(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%

Conclusions

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 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

Application Note

Pharma & Biopharma

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Trusted Answers

Comparison of Relative Quantification of Monoclonal Antibody N-glycans Using Fluorescence and MS Detection

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Introduction

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).

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Introduction

Monoclonal antibodies (mAbs) are modified by N-glycans during biosynthesis in cell culture. Typical mAbs contain two N-glycosylation sites, one in each of the Fc regions of the molecule. Some mAbs contain additional glycosylation sites, including N- or O-glycosylation in the Fab region. Glycans can affect the function of the mAb, so it is important to monitor the glycosylation profile using appropriate analytical methods.

Popular methods for glycan analysis involve NMR, CE-LIF, HPLC with fluorescence detection (FLD), and more recently, LC/MS. Both CE-LIF and HPLC-FLD require that the glycans are labeled with a dye to permit optical detection. Conventionally, the dyes that have been used also increase the ionization efficiency of glycans in comparison to the unlabeled species, but only to the point where the most abundant compositions can be detected using MS. More recently, a novel dye (InstantPC from Prozyme Inc., depicted in Figure 1) has been developed, which moderately improves fluorescence activity and greatly improves ionization efficiency for MS analysis. Using such a tag, researchers can now use MS (in the form of accurate mass or tandem MS) for identification of glycans from LC separations. Furthermore, they have the option of relative quantification using MS rather than fluorescence detection.

This application note investigates the performance of InstantPC in the context of relative quantification of N-glycans released from two mAb preparations. Quadruplicate samples of the mAb samples were processed using the InstantPC kit from ProZyme, Inc. The samples were then separated by HILIC on a UHPLC system using FLD and MS detection. The LC separation conditions were optimized for maximum chromatographic separation. In doing so, the goal was to decrease the number of overlapping peaks that would otherwise not be discernable using FLD detection alone. As a result, we were able to compare the relative quantification results from the two detection methods for nearly all significant glycan structures. Accurate mass and tandem MS spectra were acquired for all glycan compositions, and were used for identification of the glycans present in the mAb preparations. Figure 2 shows the entire workflow.

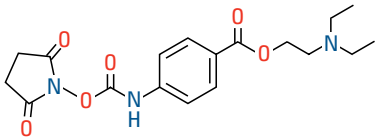

Figure 1. Diagram of InstantPC (ProZyme, Inc), an amine reactive instant label for fluorescence and MS detection of glycans.

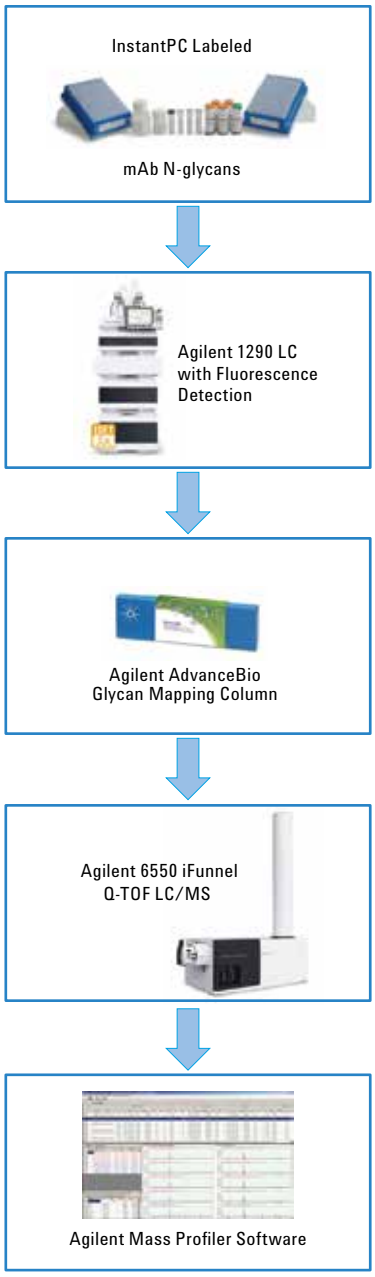

Figure 2. Workflow used for identification and quantification of InstantPC-labeled N-glycans from mAbs.

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Experimental

The Agilent LC/MS System used in this work comprised the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity High Performance Autosampler (G4226A) with an Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Fluorescence Detector (G1321B)

MS system

Agilent 6550 iFunnel Q-TOF LC/MS system with dual-nebulizer AJS source.

Columns

Agilent AdvanceBio Glycan Mapping column, 2.1 × 150 mm, 1.8 µm connected to a second AdvanceBio Glycan Mapping column, 2.1 × 100 mm.

Software

- Agilent PCDL Manager (Version B.07.00 Build 7024.0) and Agilent Mass Profiler (Version B.07.01 Build 99.0)
- Agilent MassHunter Workstation Software, Version B.05.01, Build 5.01.5125.1

Solvents and samples

All reagents and solvents used were of the highest purity available.

Chromatographic conditions

Parameter	Value	
Column Temp:	40 °C	
Mobile phase:	A) 50 mM ammonium formate pH 4.4 B) Acetonitrile	
Gradient:	Time (min)	% B
	0	75
	32	69
	48	60
	48.5	25
	50.5	25
	52	75
Flow rate:	0.4 mL/min	
Injection volume:	2 µL in 20 % DMSO (1 µg of IgG equivalent)	
Autosampler Temp:	4 °C	
FLD:	Ex. 285 Em. 345	
Column Temp:	40 °C	

Results and Discussion

FLD chromatograms from both mAb 1 and mAb 2 revealed that each molecule was modified by a very similar set of glycoforms, as shown in Figure 3. Some structures have been annotated in the figures, and represented by symbols according to the guidelines of the Consortium for Functional Glycomics (CFG) [1].

Using accurate mass and tandem mass spectrometry information, FLD peaks were assigned to glycan compositions in the form:

HxNxFxSgx + Core

H = galactose or mannose,
N = N-acetylglucosamine,
F = fucose,
Sg = N-glycolylneuraminic acid,
and Core = trimannosyl core common to all N-glycans.

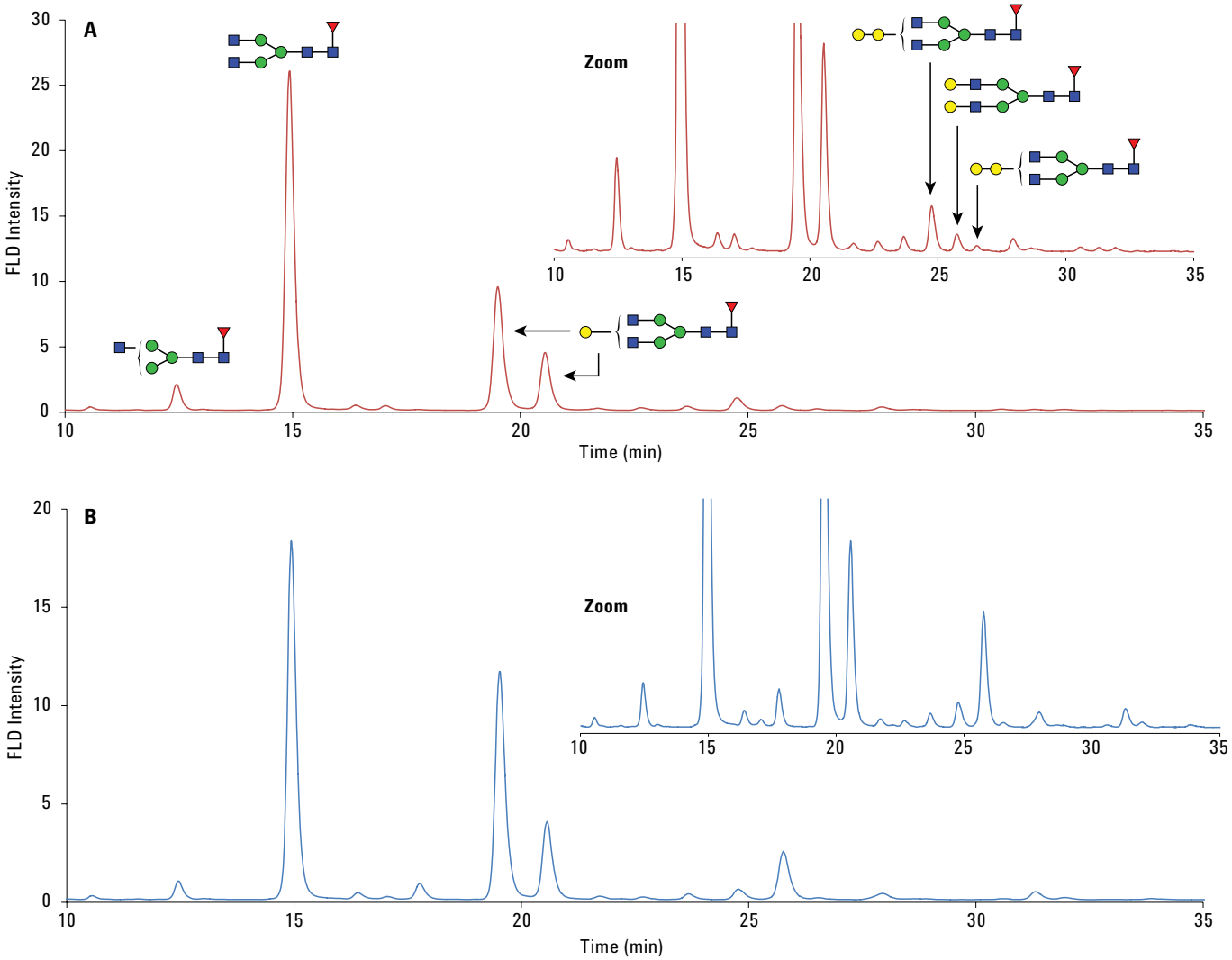

Figure 3. FLD chromatograms of InstantPC-labeled N-glycans released from mAb 1 and mAb 2. A) FLD chromatogram for mAb 1. B) FLD chromatogram for mAb 2.

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Based on peak area from the FLD chromatograms, each composition was quantified as a relative sum percentage based on the total FLD area for all compositions. The results are shown as a histogram in Figure 4.

Overall, 21 glycan compositions were quantified based on the FLD data. The criteria used for inclusion in the FLD quantification were a relative abundance of 0.1 % or greater, and reasonable resolution from neighboring peaks. One exception was the pair H2N1F1Sg1 + Core/H2N3F1 + Core. These coeluting compositions were abundant enough to merit inclusion in the FLD quantification. Therefore, the FLD signal area from this peak was divided into two portions according to the relative abundance of each as determined by MS.

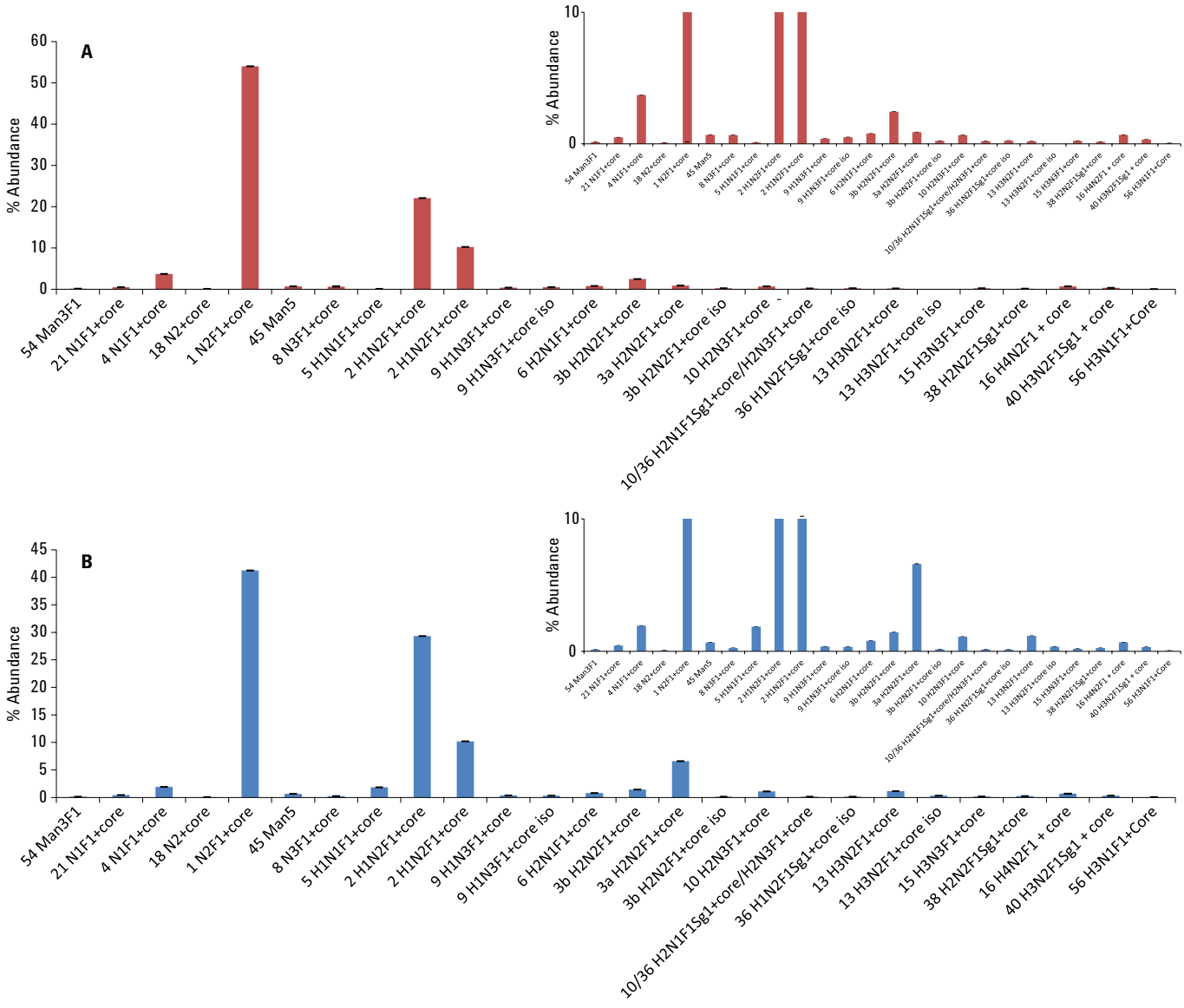

Figure 4. A) Relative FLD quantification of mAb 1 glycans. B) Relative FLD quantification of mAb 2 glycans. Error bars represent ± standard deviation of quadruplicates having gone through the entire workflow. Integration of FLD signals was performed using Agilent MassHunter Qualitative Analysis Software. Insets show the same data zoomed to better display components with <10 % relative abundance.

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InstantPC imparts high ionization efficiency to N-glycans. Thus, it is possible to perform relative quantification using the peak area from extracted ion chromatograms from MS detection. To assess this possibility, we compared FLD chromatograms with ion chromatograms. Figure 5 shows that the FLD and MS chromatograms were highly similar. There is a corresponding MS peak for every FLD peak that was detected.

Encouraged by the high similarity seen in Figure 5, we performed relative quantification of glycans from mAb 1 and mAb 2 based on the MS data. Ion chromatograms for each feature (defined as a mass-retention time pair, which includes signals from all charge states and adducts) were created using Agilent Mass Profiler software. In this case, no lower threshold for detection was imposed. The features determined using Mass Profiler were identified using a Personal Compound Database (PCD) constructed for these experiments.

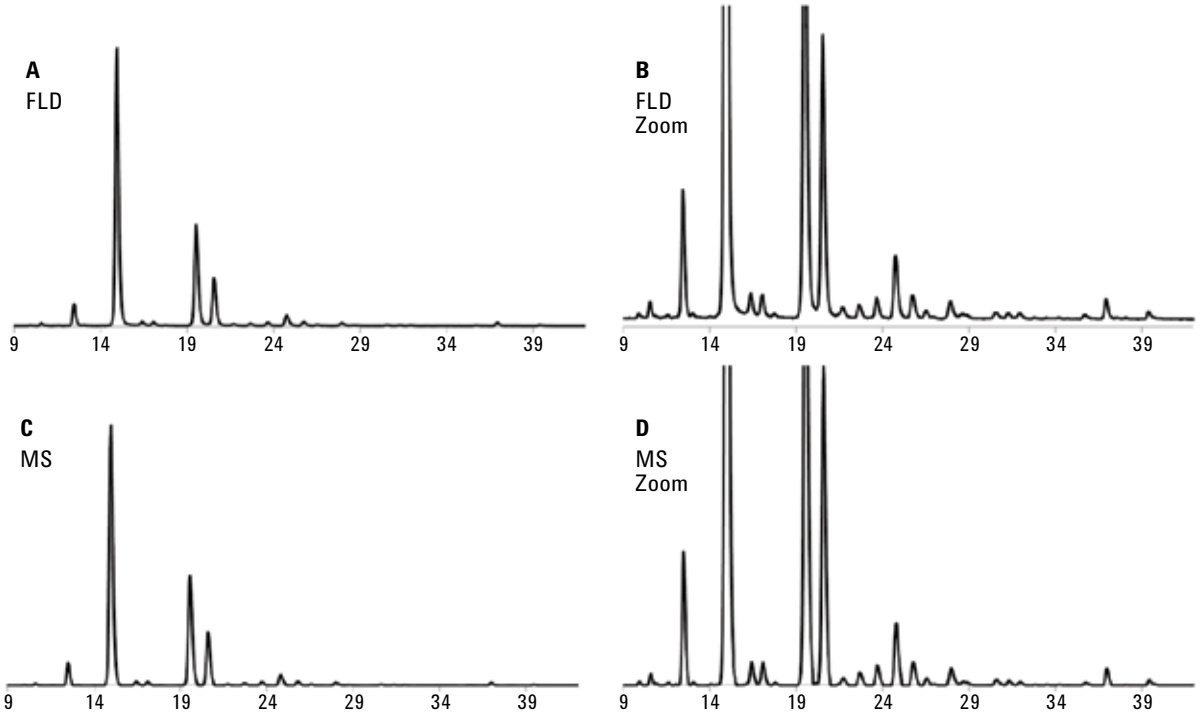

Figure 5. Comparison of FLD and MS chromatograms for mAb 1. A) FLD chromatogram of mAb 1 glycans. B) Zoom of FLD chromatogram of mAb 1 glycans. C) MS chromatogram of mAb 1 glycans. D) Zoom of MS chromatogram of mAb 1

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The PCD contains accurate mass and retention time information for mAb glycans. The database was constructed based on a combination of tandem MS information from the current work in addition to knowledge of glycan biosynthetic rules. Figure 6 shows an example of the utility of tandem MS for assigning glycan compositions. In particular, the example shown in Figure 6B illustrates a common case where mass alone may be insufficient for assignment of composition, due to the fact that the mass of NeuGc + fucose is isobaric with that of NeuAc + galactose. Tandem MS resolves the ambiguity, because the presence of the fragment ion at m/z 673 provides strong evidence that the structure contains an antenna with NeuGc.

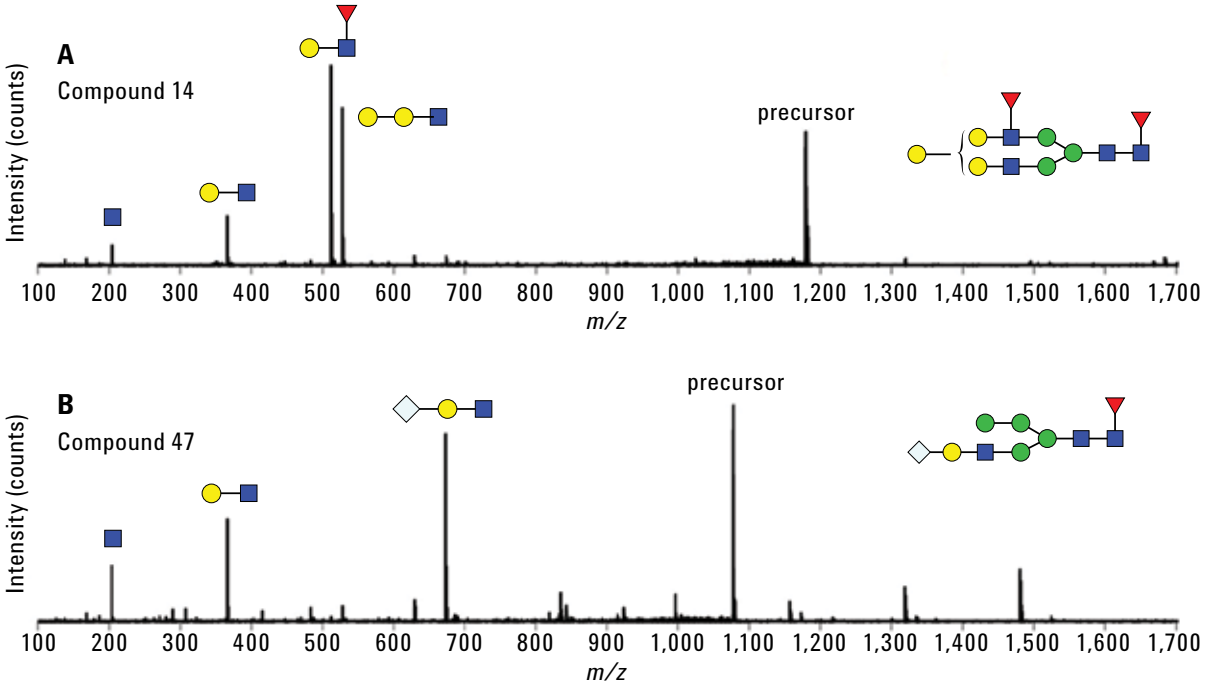

Figure 6. Tandem MS data were acquired for all glycans. MS/MS aided in compound identification when accurate mass was insufficient. The two examples above are consistent with gal-gal and outer arm fucose (A) and NeuGC (B) modifications.

Figure 7 shows the results of the MS-based quantification of mAb 1 and mAb 2 glycans.

As a result of the mass selectivity provided by Q-TOF detection, it was possible to quantify more compositions than from the FLD detection. In this case, a total of 35 compositions were quantified. The average RSD was 3.2 % for mAb 1 and 3.9 % for mAb 2 for all features independent of abundance. For those features equal to or greater than 0.1 % relative abundance, RSDs were 2.7 % and 3.4 % respectively.

Finally, we directly compared the relative quantification of glycans from mAb 1 and mAb 2 using FLD and MS. Figure 8 shows the results from each method plotted on a single histogram.

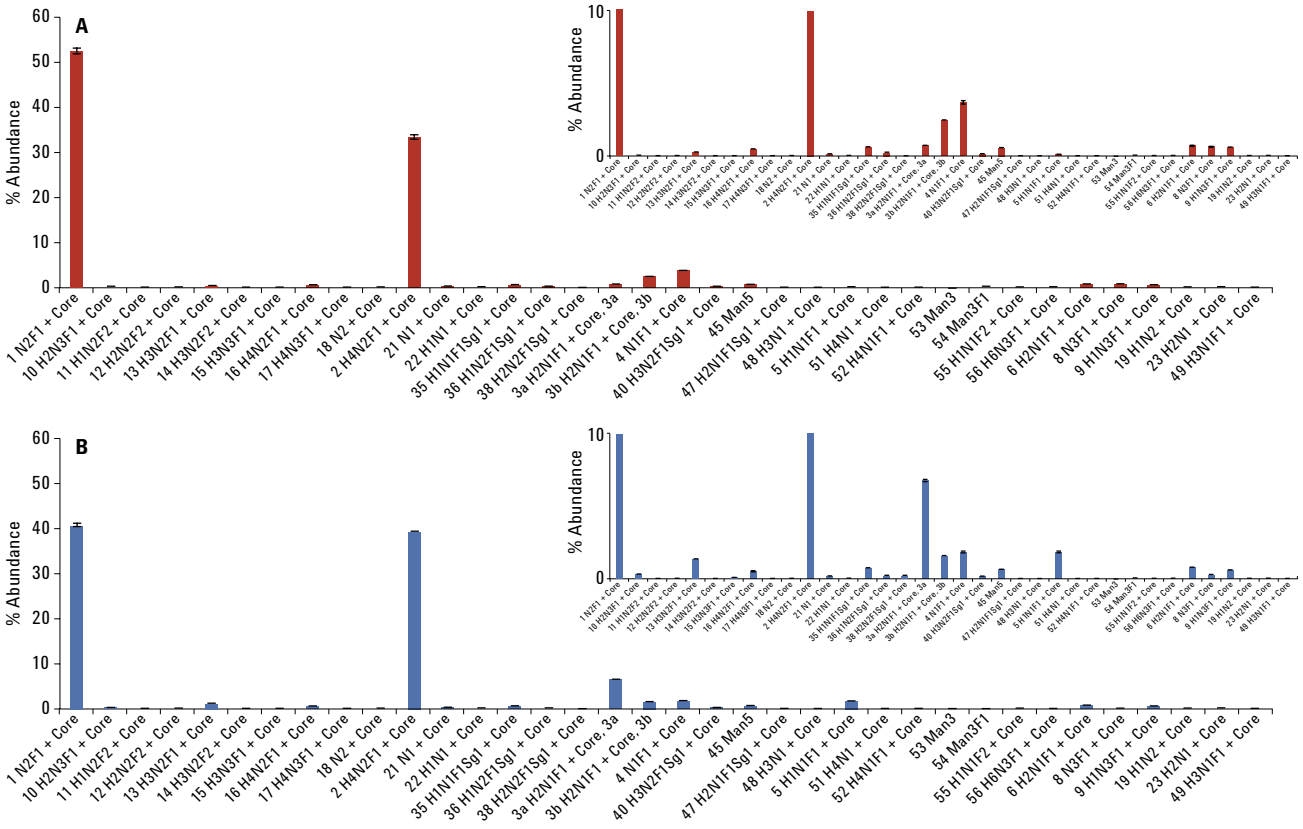

Figure 7. Relative MS quantification of InstantPC labeled N-glycans released from mAb 1 and mAb 2. A) Relative MS quantification of mAb 1 glycans. B) Relative MS quantification of mAb 2 glycans. Error bars represent ± standard deviation of quadruplicates having gone through the entire workflow. Insets show the same data zoomed to better display components with <10 % relative abundance.

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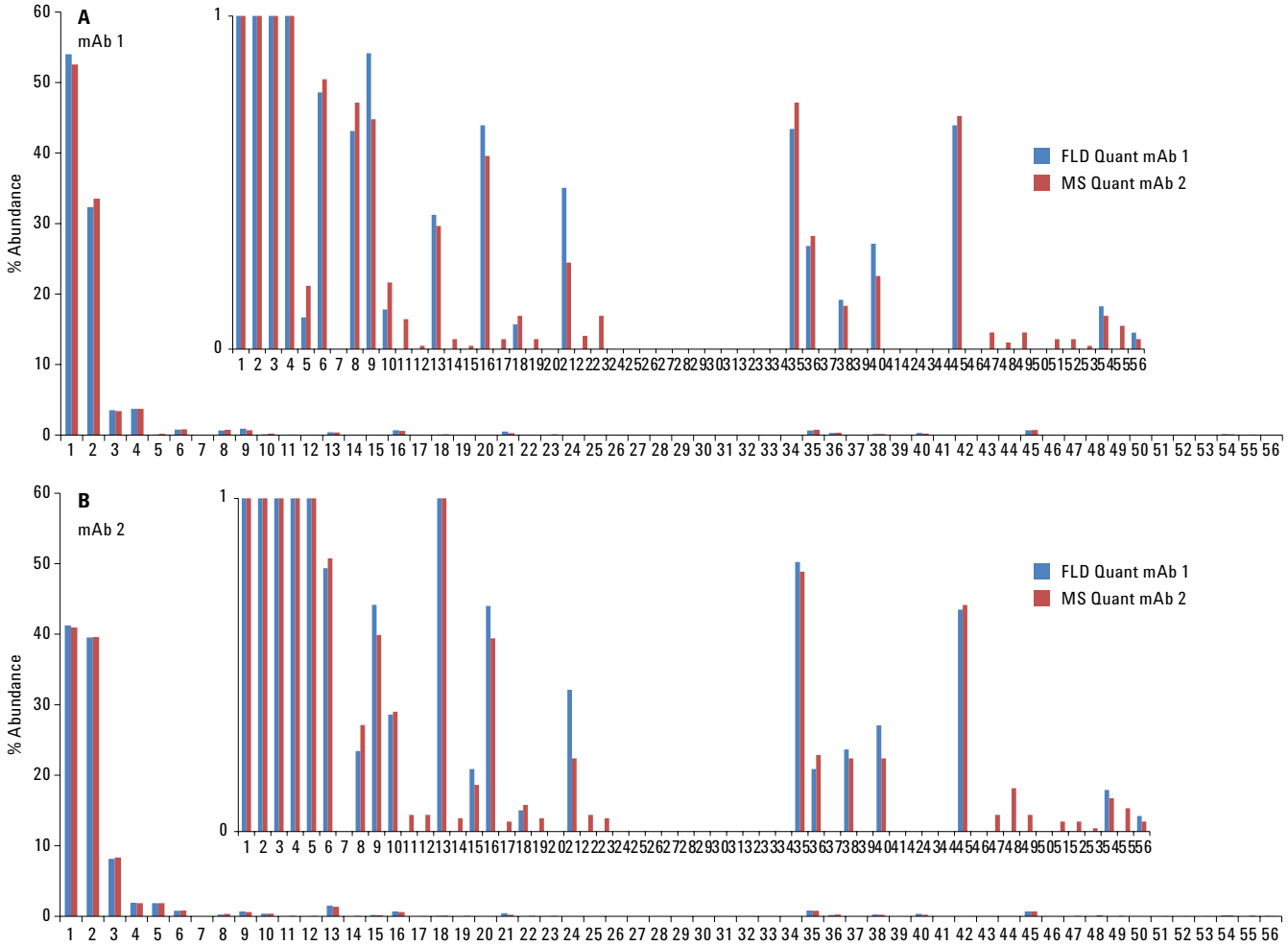

Figure 8. Comparison of fluorescence and relative MS abundance (area sum percentage) of InstantPC-labeled N-glycans from mAb 1 and mAb 2. The X-axis represents individual glycan compositions quantified in the study.

Conclusions

As shown in Figure 8, the relative quantification results from FLD and MS were highly similar. Some small differences in the results from the two methods can be explained by the different numbers of compositions quantified in the two methods (21 from FLD, 35 from MS). Based on the results of this study, the combination of Prozyme's InstantPC label and an Agilent LC/MS system provides the researcher with the capability to perform MS-based quantification of glycans from mAbs. Still, FLD will likely remain a gold standard detection method for this compound class. In that case, high quality Q-TOF MS data greatly facilitate peak assignment by offering accurate mass and tandem mass information for each of the InstantPC-labeled glycans detected using FLD.

Acknowledgements

We would like to thank NIST for providing the two mAb samples used in this work.

References

1. http://glycomics.scripps.edu/CFGnomenclature.pdf

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Application Note

Glycan Analysis

Agilent

Trusted Answers

A Comprehensive Approach for Monoclonal Antibody N-linked Glycan Analysis from Sample Preparation to Data Analysis

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Introduction

Monoclonal antibodies (mAbs) and their derivatives comprise a very important class of biopharmaceutical molecules with a wide range of applications. Due to the heterogeneous nature of these mAbs, comprehensive analytical characterization is required. These analyses include determining the complete amino acid sequences of the mAbs and their variants, as well as characterization of post-translational modifications (PTMs) including glycosylation, oxidation, and deamidation.

Glycosylation plays an important role in many biological processes. It also affects the therapeutics' efficacy, stability, pharmacokinetics, and immunogenicity[1]. Glycan characterization usually involves techniques such as NMR, HPLC, or mass spectrometry (MS). Since glycans are very diverse in composition/structures and are poorly ionized by electrospray, the MS-based approach for glycan characterization has been challenging. InstantPC is a novel fluorescence tag from ProZyme Inc. (Figure 1) that has been developed to improve MS ionization efficiency, and sensitivity for N-glycan molecules.

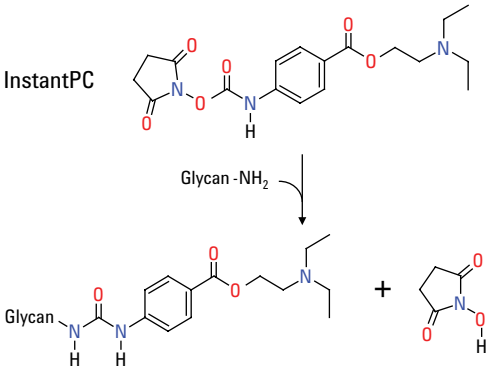

Figure 1. Diagram of InstantPC-labeled N-glycans released from an mAb.

The traditional method of glycan analysis is laborious, and involves many steps, starting with enzymatic glycan release by PNGaseF (overnight), followed by sample cleanup, labeling with a fluorescence tag by reductive amination (2-AB or InstantPC), and finally cleanup of the released labeled N-glycans prior to LC-FLD or LC/MS analysis[2,3]. Despite the significant improvement of MS sensitivity using fluorescent tags, the labor intensiveness of manual sample preparation, low reproducibility, and limitation to scale-up on sample processing have been major issues for the biopharmaceutical industry.

This study demonstrates how to increase sample throughput for glycan characterization workflows using the Agilent AssayMAP Bravo liquid handling platform. The solution incorporates the Agilent 1290 Infinity II LC system, Agilent AdvanceBio Glycan Mapping column, Agilent highly sensitive fluorescence detection (FLD), and the Agilent 6545XT AdvanceBio LC/Q-TOF. The Q-TOF data are analyzed automatically with Agilent MassHunter BioConfirm B.09.00 software (Figure 2). This solution dramatically improves productivity by allowing convenient sample preparation, streamlined data acquisition, and data analysis. This solution provides the flexibility to perform quantitation based on FLD or MS signals with accurate mass peak assignment from an N-glycan mass database.

Experimental

Sample preparation

Four monoclonal antibody (mAb) samples were used in this study:

- The monoclonal antibody standard, RM 8671, was from National Institute of Standards & Technology (NIST) A.K.A. NISTmAb.
- Formulated Herceptin (Trastuzumab) was from Genentech (So. San Francisco, California, USA).
- Sigma SiLu mAb was purchased from Sigma-Aldrich (SiLu Lite, P/N: MSQC4).
- CHO mAb1 was expressed and purified from the Agilent R&D lab.Agilent 6550 iFunnel Q-TOF LC/MS system with dual-nebulizer AJS source.

All mAb samples were diluted with DI water to 1.0 µg/µL prior to sample preparation using the AssayMAP Bravo liquid handling system (G5542A) with the GlykoPrep-plus Rapid N-Glycan Sample Preparation with InstantPC (96-ct) from ProZyme Inc. A detailed procedure for the sample preparation is described in ProZyme's application note (product code: GPPNG-PC). After the final cleanup step, the eluted, released, labeled N-glycans had a final volume of 50 µL, so that each 1 µL of the prepared sample contained N-glycans from 1 µg of mAb.

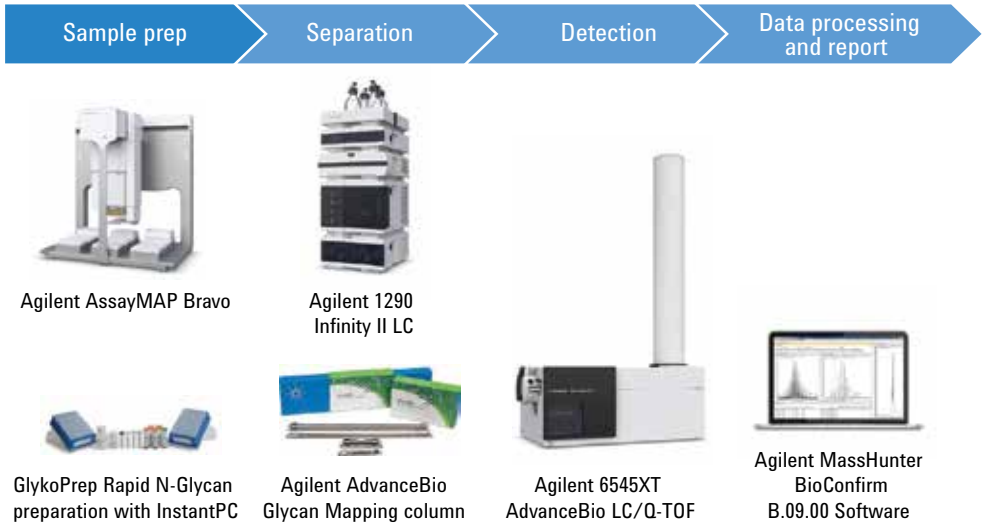

Figure 2. mAb Glycan characterization workflow.

LC/MS analysis

LC/MS analyses were conducted on a 1290 Infinity II LC system equipped with an Agilent 1260 Infinity Fluorescence Detector (G1321B), coupled with a 6545XT AdvanceBio LC/Q-TOF system with a Dual Agilent Jet Stream source. The detector was set to λEx = 285 nm, λEm = 345 nm, with PMT gain = 10. Glycans were chromatographically separated with an AdvanceBio Glycan Mapping column (2.1 × 100 mm, 1.8 µm). Tables 1 and 2 list the LC/MS parameters used. Approximately 1–2 µL of each N-glycan sample were injected for LC/MS analysis..

Data processing

The InstantPC-labeled released N-glycans were analyzed using the Released Glycans Workflow of MassHunter BioConfirm B.09.00 software. This analytical workflow uses the Agilent Personal Compound Database (PCD) glycan database. The PCD glycan database provides accurate glycan identification and confirmation. Finally, a summarized report of the analyses was generated in PDF format using the Report Builder program in BioConfirm B.09.00.

Table 1. Liquid chromatography parameters

Conditions, Agilent 1290 Infinity II LC System

Parameter	Value	
Column:	Agilent AdvanceBio Glycan Mapping, 2.1 × 100 mm, 1.8 µm	
Column Temp:	40 °C	
Thermostat:	4 °C	
Solvent:	A) 50 mM formic acid adjusted to pH 4.5 with ammonium hydroxide B) Acetonitrile	
Gradient:	Time (min)	% B
	0-0.5	75-71
	0.5-1.6	71-67.5
	1-22	67.5-60
	22-22.5	60-40
	22.5-23.5	40 (0.7 mL/min)
	23.5-24	40-75 (0.7 mL/min)
	24-30	75 (0.9 mL/min)
Flow rate:	0.4 mL/min	
Injection volume:	2.0 µL	

Agilent 1260 Infinity Fluorescence Detector (G1321B) was used.
The detector was set to λEx = 285 nm, λEm = 345 nm, with PMT gain = 10.

Table 2. MS Acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF system	
Gas temperature	150 °C
Drying Gas Flow	9 L/min
Nebulizer	35 psi
Sheath gas temperature	300 °C
Sheath gas flow	10 L/min
Vcap	3,000 V
Nozzle Voltage	500 V
Fragmentor	120 V
Skimmer	65 V
Acquisition mode	Low mass range, HiRes (4 GHz)
Mass Range (MS)	m/z 300–1,700
Acquisition rate	2 spectra/sec

Results and Discussion

LC-FLD analysis of released labeled glycans is one of the most widely used approaches to determining therapeutic protein glycosylation. We have previously published application notes showing optimized separation of several mAb glycan profiles using various column dimensions and run conditions[4,5]. The separation method in this report represents the best overall performance with maximum peak resolution and excellent robustness for the different mAb N-glycan samples in this study.

Figure 3 shows the representative chromatograms of N-glycans (FLD and MS EIC) from the NISTmAb. The FLD chromatogram (Figure 3 top, zoom in) reveals that more than 15 glycan peaks were detected. The glycosylation pattern of the major abundant glycans, such as the G0F, G1F isoforms, and G2F was comparable between the fluorescent and MS data (Figure 7).

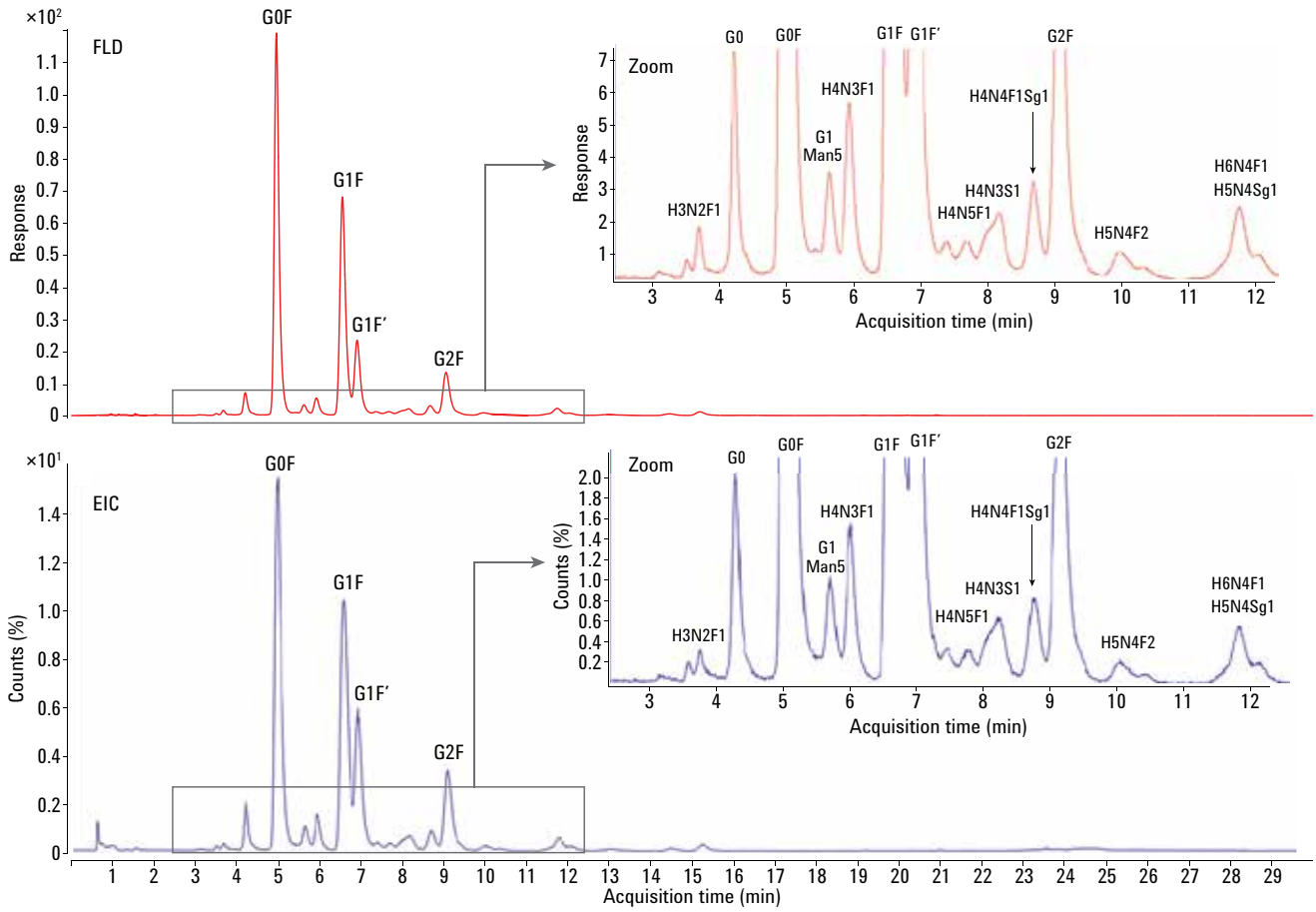

Figure 3. FLD chromatogram and mass spectra (EIC) of InstantPC-labeled N-glycans from NISTmAb

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While fluorescence detection does not allow for direct structure elucidation, MS analysis of mAb glycans can be used to determine glycan monosaccharide composition. In the case of many mAb N-glycans, this composition is sufficient to achieve a high-confidence structural assignment. The combination of the positively charged InstantPC tag and sensitive Agilent Jet Stream (AJS) electrospray ionization (ESI) source technology dramatically increases MS detection sensitivity for N-glycans. In addition, we have optimized the MS parameters to maximize the sensitivity of the InstantPC-labeled N-glycans while minimizing in-source fragmentation of these fragile molecules.

The optimized conditions have significantly improved the MS spectrum quality, leading to accurate N-glycans identification and relative quantification results. Figure 4 shows the MS spectrum of an InstantPC-labeled N-glycan (G2F) where only the doubly charged ions of its protonated form, $[M+2H]^{2+}$, as well as its adducts $[M+H+Na]^{2+}$ and $[M+H+K]^{2+}$ were observed (Note: InstantPC tag causes a mass increment of 261.1477 Da compared to the free reducing end form of the glycan).

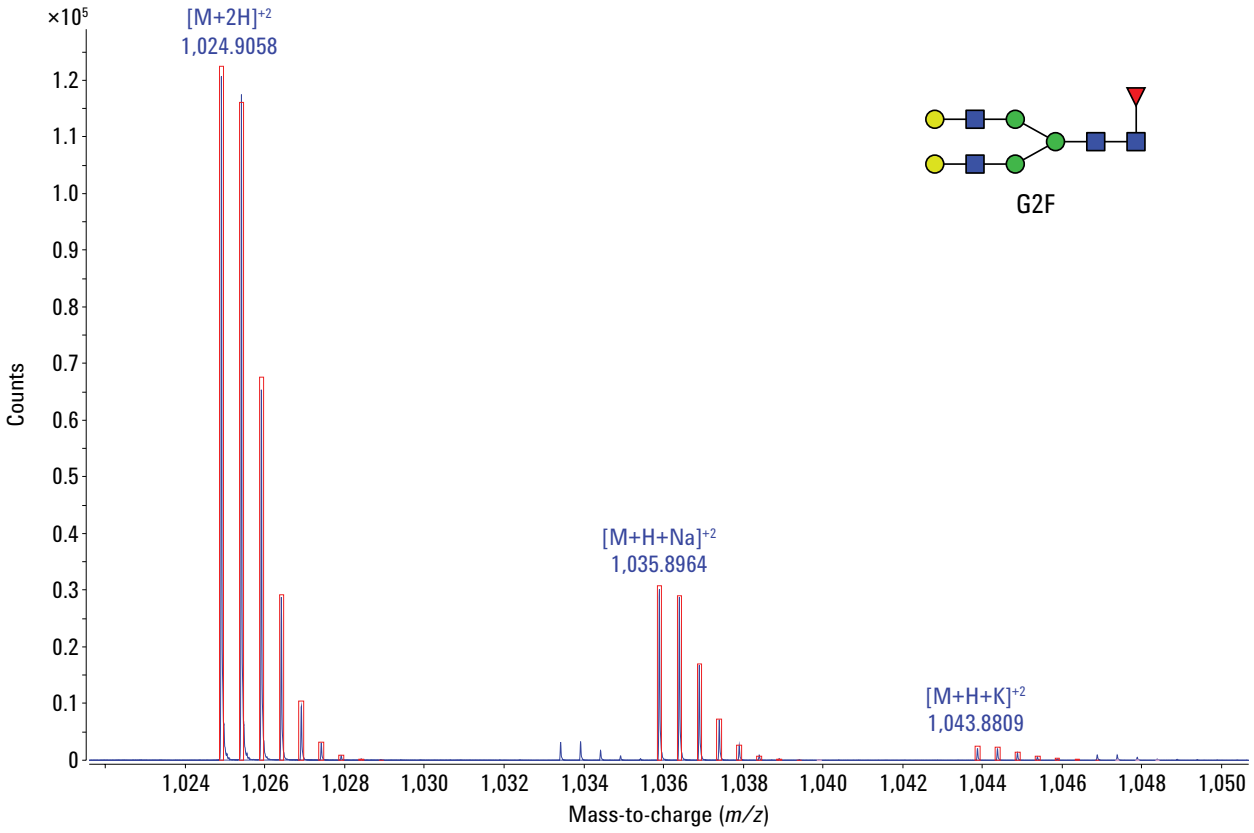

Figure 4. Representative spectrum of an InstantPC-labeled N-glycan (G2F). Excellent isotopic fidelity of the charge states of the InstantPC-labeled G2F glycan and its adducts. The red boxes represent the theoretical isotopic pattern, and the blue lines represent the actual raw MS spectrum.

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We have introduced a workflow in MassHunter BioConfirm software for released glycan profiling. This workflow enables the easy setup of sample batch analysis. The software can accommodate many commercial or customized fluorescent tags. A Personal Compound Database (PCD) containing accurate mass and structural information of glycans is used for identification using the Agilent proprietary Find by Formula algorithm. Subsequently, a summary analytical report can be created in a customer-defined report format. Figure 5 shows the extracted ion chromatograms (EICs) of the identified glycans.

The Biomolecules results table (Figure 6) in BioConfirm allows quick review of detailed glycans information including names, mass, retention time, peak area, composition, and database matching score. Multiple IDs are displayed for glycans with possible isoform structures. It also allows users to review the TIC of the sample as well as the individual glycan MS spectra. In addition, multiple data files can be processed and analyzed in batch mode. The user may use peak areas of the selected glycans in the results table for relative quantitative analysis.

InstantPC-labeled glycans were previously shown to give similar relative quantitation results for MS and FLD analysis4. The FLD chromatogram for the CHO mAb1 sample was integrated using the Agilent MassHunter Qualitative Analysis software. The relative sum abundance of the top seven most abundant N-glycans was calculated and compared against the same data from the MS analysis (Figure 7). To get equivalent results, do not saturate the MS detector. An ideal quantity for this workflow would be to inject N-glycans released from approximately 0.5 µg of mAb.

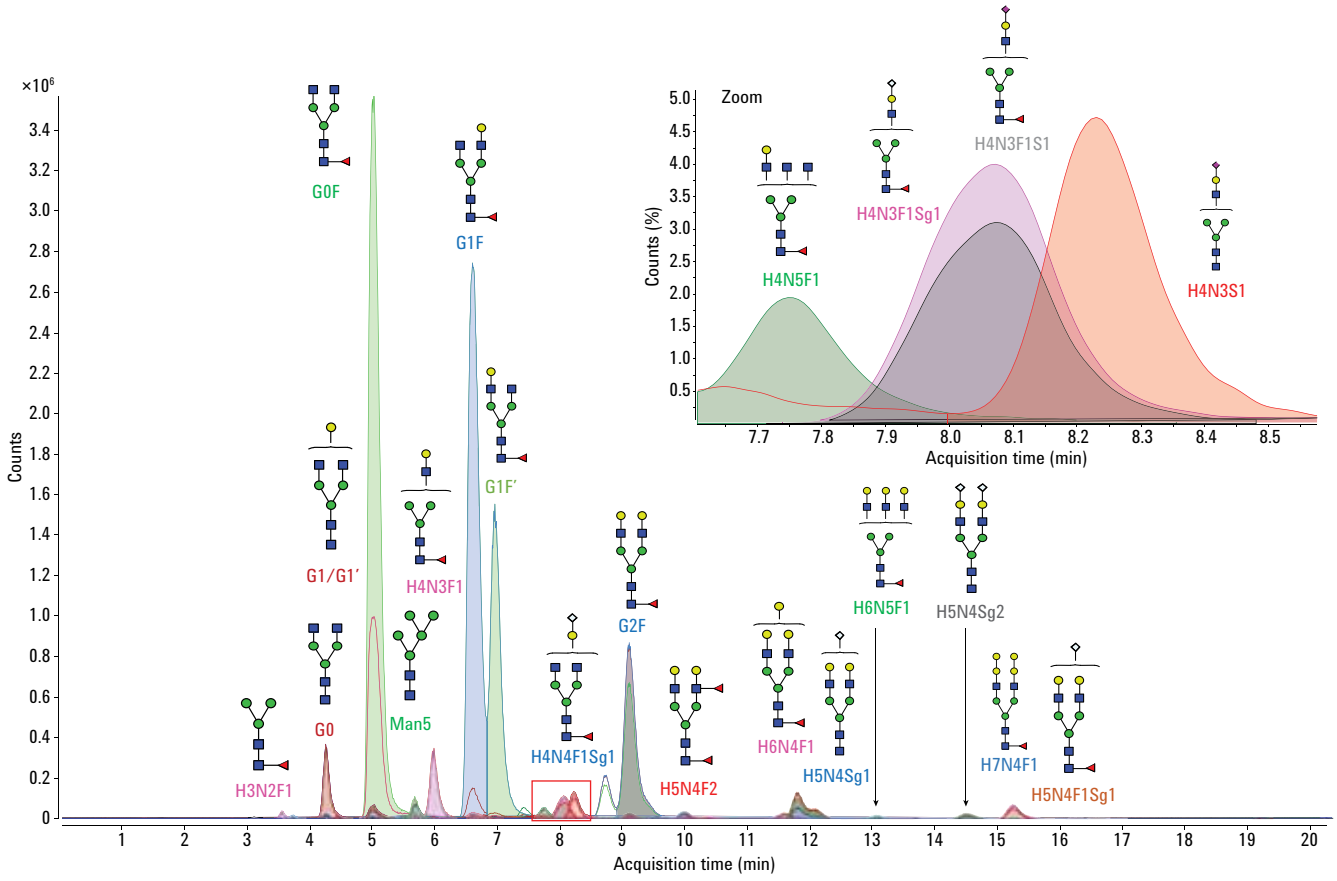

Figure 5. Extracted ion chromatograms of the identified glycans from NISTmAb. Inset: zoom of EICs of identified glycans eluted in the retention time range of 7.6–8.6 minutes.

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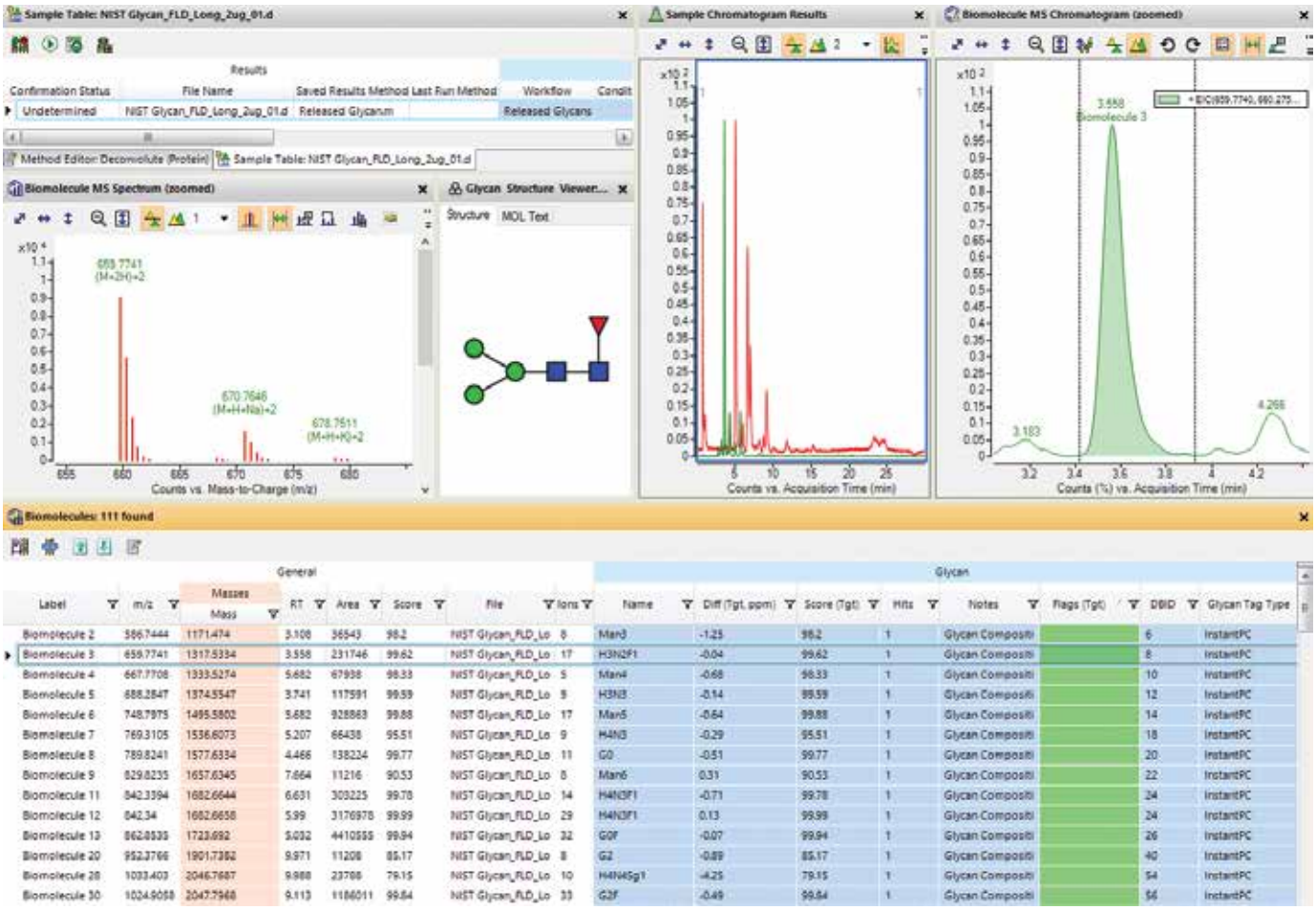

Figure 6. Screenshot of Agilent MassHunter BioConfirm B.09.00 software with representative glycan profiling results.

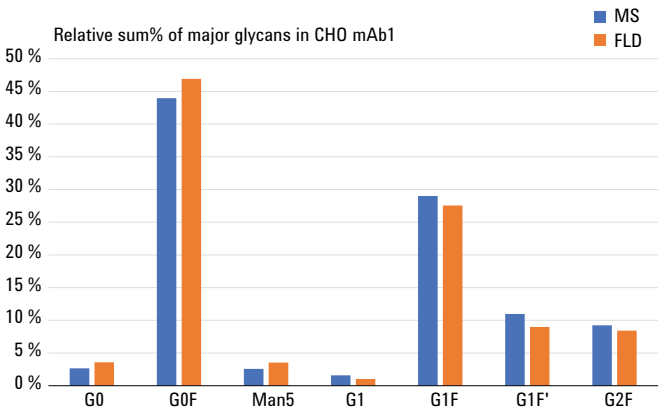

Figure 7. Relative sum % of the major N-glycans in the CHO mAb1 (0.5 µg), comparing results from MS-based quantitation (blue) with FLD-based quantitation (orange).

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To summarize and compare the MS results, the top five most abundant N-glycans for each mAb sample were used to calculate relative sum %. Figure 8 presents the data.

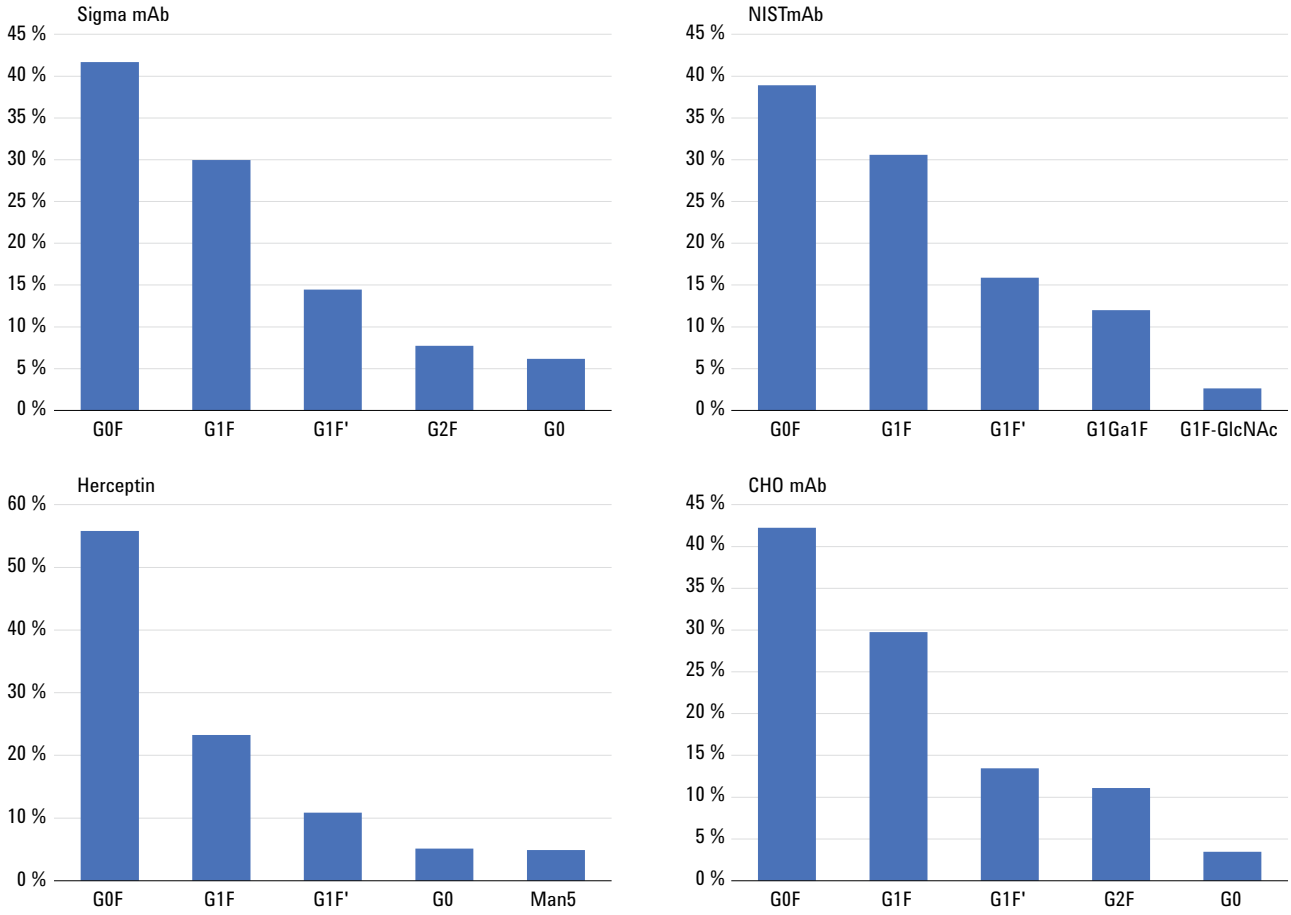

Figure 8. Relative sum % of the top five N-glycans in each of the four mAb samples.
Note: The NISTmAb contained a structure suspected to be G1F with an additional alpha-1,3-galactose, and this was labeled as G1Ga1F.

The BioConfirm B.09.00 software allows users to generate their own glycan profile reports using the Report Builder program. Figure 9 shows an example of a released glycan report. In the Report Builder, users can customize the report sections with information such as Sample Information, Sample Chromatogram, Biomolecule Summary, and Biomolecule Details. The corresponding glycan structures are displayed along with the identified glycans.

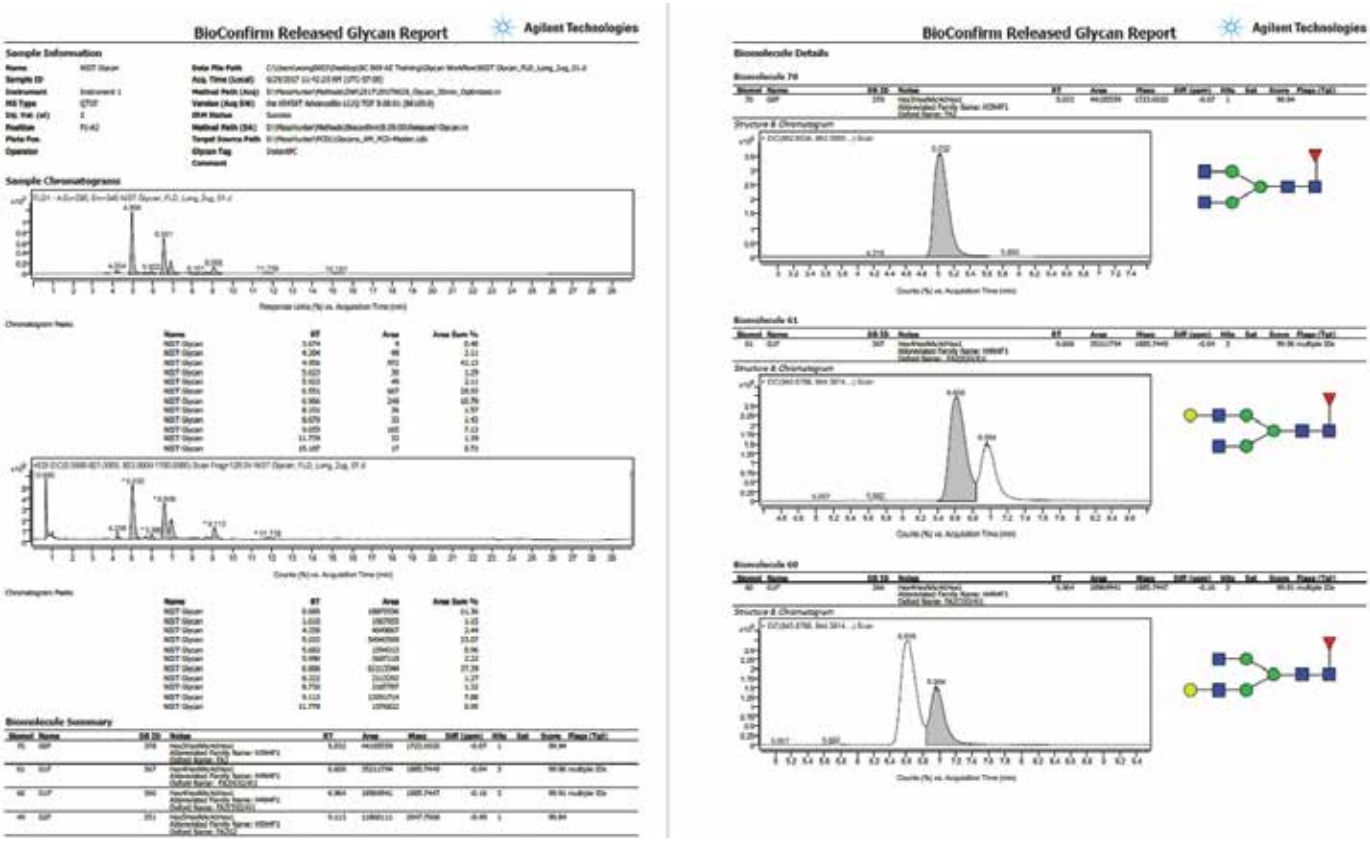

Figure 9. Agilent MassHunter BioConfirm B.09.00 Software – Released Glycan Report.

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Conclusions

This study demonstrated the performance of the Agilent AssayMap Bravo, 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software, when used as an integrated solution for released glycan analysis.

- This workflow combines high-throughput sample preparation with excellent chromatographic separation using the Agilent AdvanceBio Glycan Mapping column.
- The easy setup and use of the glycan database included with BioConfirm B.09.00 provided the ability to accurately profile, identify, and perform relative quantification.
- The 6545XT-based glycan analysis generated similar quantitative results to that of fluorescence analysis, making it possible to compare different N-glycans across different mAb samples.
- The Report Builder function in BioConfirm B.09.00 provides the ability to create custom reports.

In conclusion, the Agilent solution automated the entire process of N-linked glycan analysis from sample preparation to data analysis with high precision. This approach provided high sensitivity and best quantitation for glycan analysis using fluorescence and additional identification by mass spectrometric detection.

References

1. Rademacher, T. W; Williams, P; DwekMark, R. A. "Agalactosyl glycoforms of IgG autoantibodies are pathogenic" *P. Natl. Acad. Sci*. **1994**, *91*, 6123-6127.
2. Anumula, K. R. "Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates" *Anal. Biochem*. **2006**, *350*, 1-23.
3. N-Glycan Analysis of mAbs and Other Glycoproteins with UHPLC and Fluorescence Detection, *Agilent Technologies*, publication number 5991-5253EN.
4. Comparison of Relative Quantification of Monoclonal Antibody N-glycans Using Fluorescence and MS Detection, *Agilent Technologies*, publication number 5991-6958EN.
5. Analysis of Monoclonal Antibody N-glycans by Fluorescence Detection and Robust Mass Selective Detection Using the Agilent LC/MSD XT, *Agilent Technologies*, publication number 5991-8071EN.

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Application Note

Glycan Analysis

Agilent

Trusted Answers

Fast and Efficient HILIC Methods for Improved Analysis of Complex Glycan Structures

Authors

James Martosella,
Oscar Potter,
Danny Mancheno,
and Jia Liu
Agilent Technologies, Inc

Introduction

N-linked glycosylation is a critically important and very complex post-translational modification. It therefore needs to be controlled and monitored throughout development, processing, and manufacture of drug glycoproteins. Therapeutic protein characteristics, including safety, efficacy, and serum half-life, can be affected by differences in their glycosylation pattern, and so the analysis of these patterns is an important part of the characterization of therapeutic glycoproteins, particularly mAbs. Separation using HILIC with fluorescence detection is a robust method for glycan analysis, whereas HILIC/LC can also be coupled to mass spectrometry to obtain important mass and structure information.

One of the growing challenges in HILIC/LC, however, is achieving high-resolution separations with fast analysis times. With ever-increasing demands placed on biocharacterization for higher throughput, researchers are looking for improved separation (HILIC) methods, but not at the cost of lost separation performance. Since glycans include many closely related structures, it is critical to achieve the highest resolution possible, and preferably during a fast analysis time.

In this work, we used a sub-2 µm UHPLC HILIC column with amide chemistry for high-throughput glycosylation profiling. Specifically, we profiled 2-AB labeled human IgG and bovine fetuin N-linked glycans using a 1.8 µm, 2.1 × 150 mm column with fluorescence detection. Rapid, sensitive and selective separations were achieved to provide ultra-high resolution of these complex glycans in run times as short as 9 minutes. In a run time comparison to a currently available UHPLC glycan column, we observed a 40 % reduction in analysis speed for human IgG N-linked glycans under identical conditions.

Materials and Methods

Conditions, recombinant human IgG1

Parameter	Value
Sample:	Agilent 2-AB labeled IgG N-linked glycan library, 200 pmol (p/n5190-6996)
Column:	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n859700-913)
Mobile phase:	A, 100 mM NH_4 formate, pH 4.5; B, ACN
Inj vol:	2 µL in 70/30 ACN:water
Column temp:	55 °C
Sample thermostat:	10 °C
Detection:	Fluorescence, excitation 260 nm, emission 430 nm
Instrument:	Agilent 1290 Infinity LC with 1260 Fluorescence Detector

Conditions, bovine fetuin

Parameter	Value
Sample:	AdvanceBio 2-AB bovine fetuin N-linked library (p/n GKSB-002)
Column:	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n859700-913)
Mobile phase:	A, 100 mM NH_4 formate, pH 4.5; B, ACN
Inj vol:	5 µL (20 pmol) in 70/30 ACN:water
Column temp:	55 °C
	Other conditions as above

Conditions, dextran ladder

Parameter	Value
Sample:	Agilent 2-AB labeled dextran ladder standard GKSB-503
Column:	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n859700-913)
Mobile phase:	A, 100 mM NH_4 formate, pH 4.5; B, ACN
Inj vol:	2 L 75:25 ACN:water (10 pmol total glycan)
	Other conditions as above

Conditions, mass spectrometry, recombinant human IgG1

Parameter	Value
Instrument:	Agilent 6550 iFunnel Q-TOF LC/MS
Source:	Agilent Dual JetStream
Drying gas temp:	200 °C
Drying gas flow:	12 L/min
Sheath gas temp:	250 °C
Sheath gas flow:	12 L/min
Nebulizer pressure:	25 psi
Capillary voltage:	3,500 V
Nozzle voltage:	500 V
Fragmentor voltage:	250 V
Skimmer voltage:	45 V
Octupole RF Vpp:	550 V
MS range:	100 to 1,700 m/z
MS scan rate:	2 spectra/s

The workflow is shown in Figure 1.

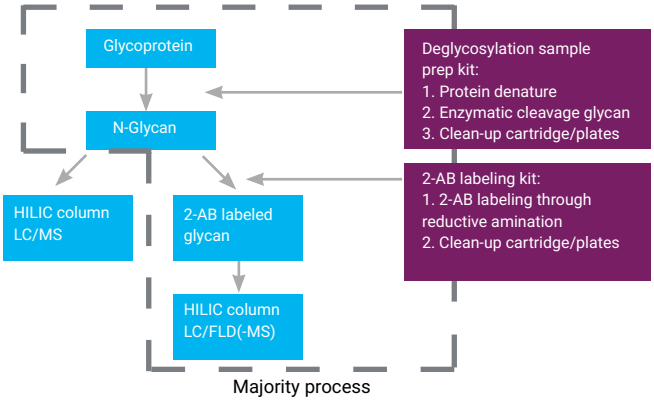
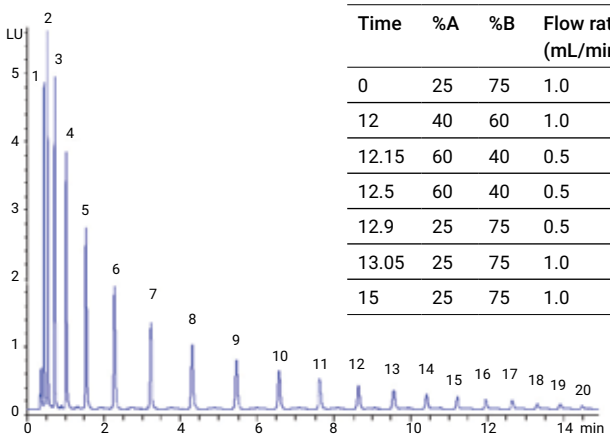


Figure 1. Total workflow solution used in an investigation of 2-AB-labeled antennary glycans using an AdvanceBio Glycan Mapping HILIC column with fluorescence detection.

Results and Discussion

Dextran ladder

Figure 2 shows the separation of a 2-AB labeled dextran ladder. In this separation, a homopolymeric series of 20 glucose oligomers were efficiently baseline resolved in less than 15 minutes.



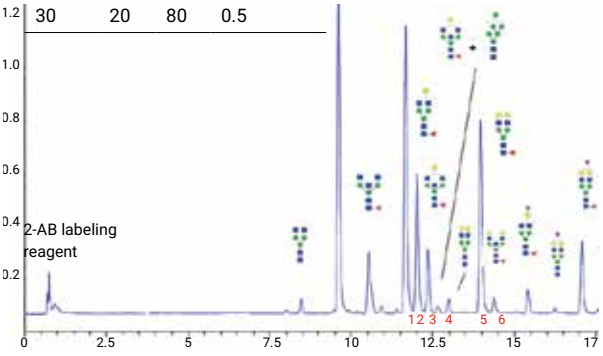
Time	%A	%B	Flow rate (mL/min)
0	25	75	1.0
12	40	60	1.0
12.15	60	40	0.5
12.5	60	40	0.5
12.9	25	75	0.5
13.05	25	75	1.0
15	25	75	1.0

Figure 2. The Agilent AdvanceBio Glycan Mapping column separates a 2-AB labeled dextran ladder to baseline in less than 15 minutes

Human IgG glycans

Figure 3 demonstrates an ultra-high resolution separation of 2-AB labeled N-linked human IgG glycans.

Time	%A	%B	Flow rate (mL/min)
0	20	80	0.5
25	40	60	0.5
26	100	0	0.5
27	20	80	0.5
30	20	80	0.5

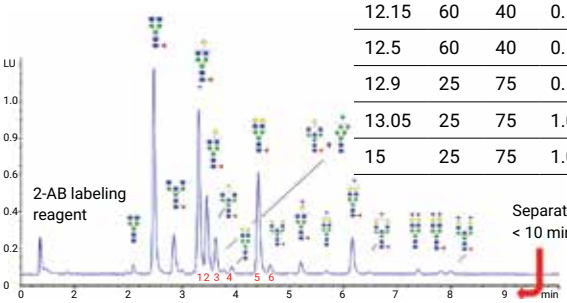


Avg RT (min)	RS 2,1	RS 3,2	RS 4,3	RS 6,5	Avg PW (min)	Bp (bar) at 30 % B	Peak capacity
12.7	2.60	2.90	5.43	2.81	0.0741	298	221

Figure 3. Very high resolution of 2-AB labeled N-linked human IgG glycans on the Agilent AdvanceBio Glycan Mapping column.

Fast separation in less than 10 minutes was also achieved, as shown in Figure 4.

Time	%A	%B	Flow rate (mL/min)
0	25	75	1.0
12	40	60	1.0
12.15	60	40	0.5
12.5	60	40	0.5
12.9	25	75	0.5
13.05	25	75	1.0
15	25	75	1.0



Avg RT (min)	RS 2,1	RS 3,2	RS 4,3	RS 6,5	Avg PW (min)	Bp (bar) at 30 % B	Peak capacity
3.93	1.63	1.70	3.05	2.00	0.059	298	135

Figure 4. The Agilent AdvanceBio Glycan Mapping column separates 2-AB labeled N-linked human IgG glycans in less than 10 minutes.

MS N-linked glycans

Figure 5 shows eight representative mass spectra from the ultra-high resolution separation of human IgG glycans. The spectra were generated by Q-TOF analysis (experimental). All spectra matched theoretical masses to within 6 ppm.

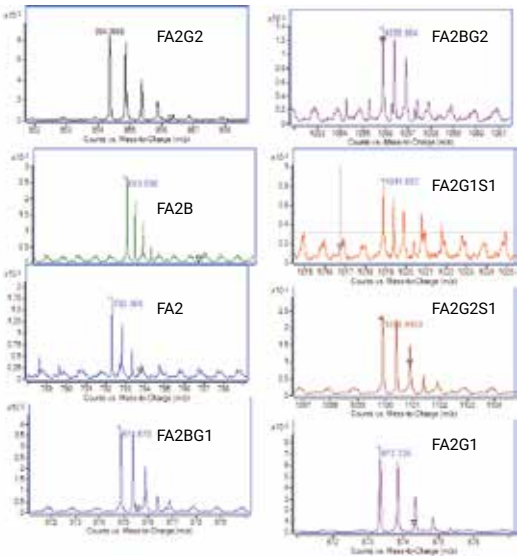


Figure 5. Eight representative mass spectra from the ultra-high resolution separation of human IgG glycans (fluorescence detector separations are displayed on the panel to the left).

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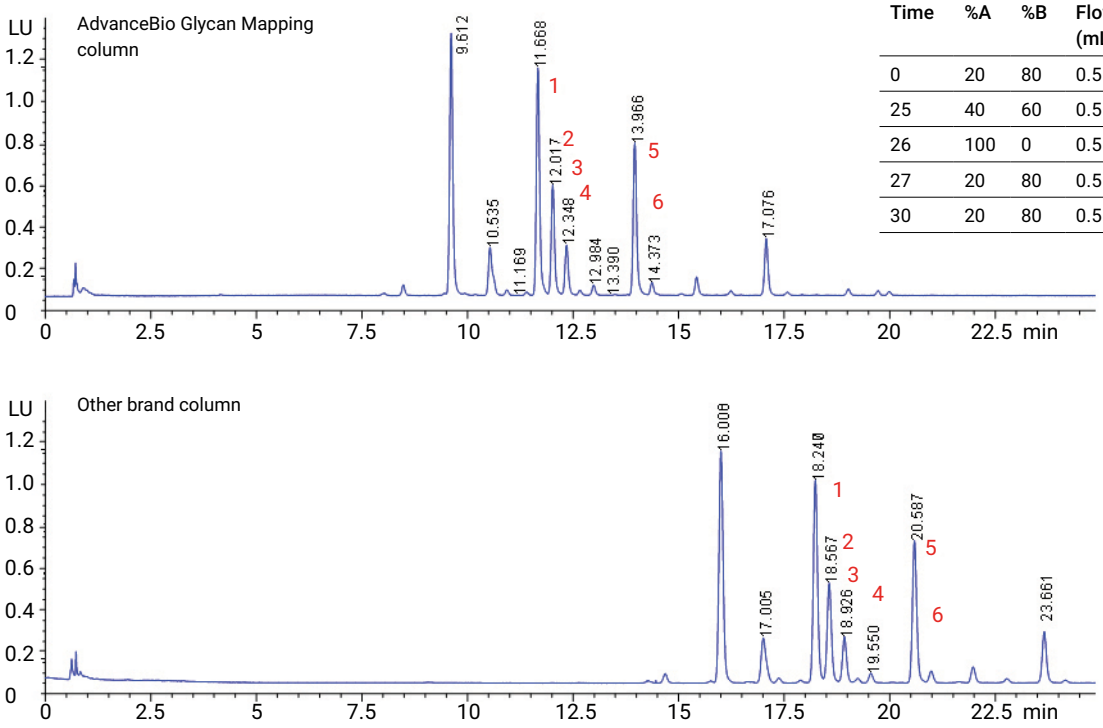
Sub-2 µm HILIC comparison

Figure 6 shows the results of a comparison of glycan amide columns. Using the same chromatographic conditions, the AdvanceBio Glycan Mapping column delivered better resolution and narrower bands, with higher peak capacity, at a 40 % faster separation time than another brand of sub-2 µm HILIC column in a 2.1 × 150 mm configuration.

Time	%A	%B	Flow rate (mL/min)
0	20	80	0.5
25	40	60	0.5
26	100	0	0.5
27	20	80	0.5
30	20	80	0.5



Column	Avg RT (min)	RS 2,1	RS 3,2	RS 4,3	RS 6,5	Avg PW (min)	Bp (bar) at 30 % B	Peak capacity
Agilent AdvanceBio Glycan Mapping	12.7	2.60	2.90	5.43	2.81	0.0741	298	221
Other brand	20.2	1.77	1.94	3.39	2.10	0.1085	349	214

Figure 6. The AdvanceBio Glycan Mapping column delivers better resolution and narrower bands, with higher peak capacity at a 40 % faster separation time.

Fetuin glycans

Finally, we revealed the fast and highly efficient performance of the AdvanceBio Glycan Mapping column in a separation of 2-AB labeled bovine fetuin N-linked glycans (Figure 7).

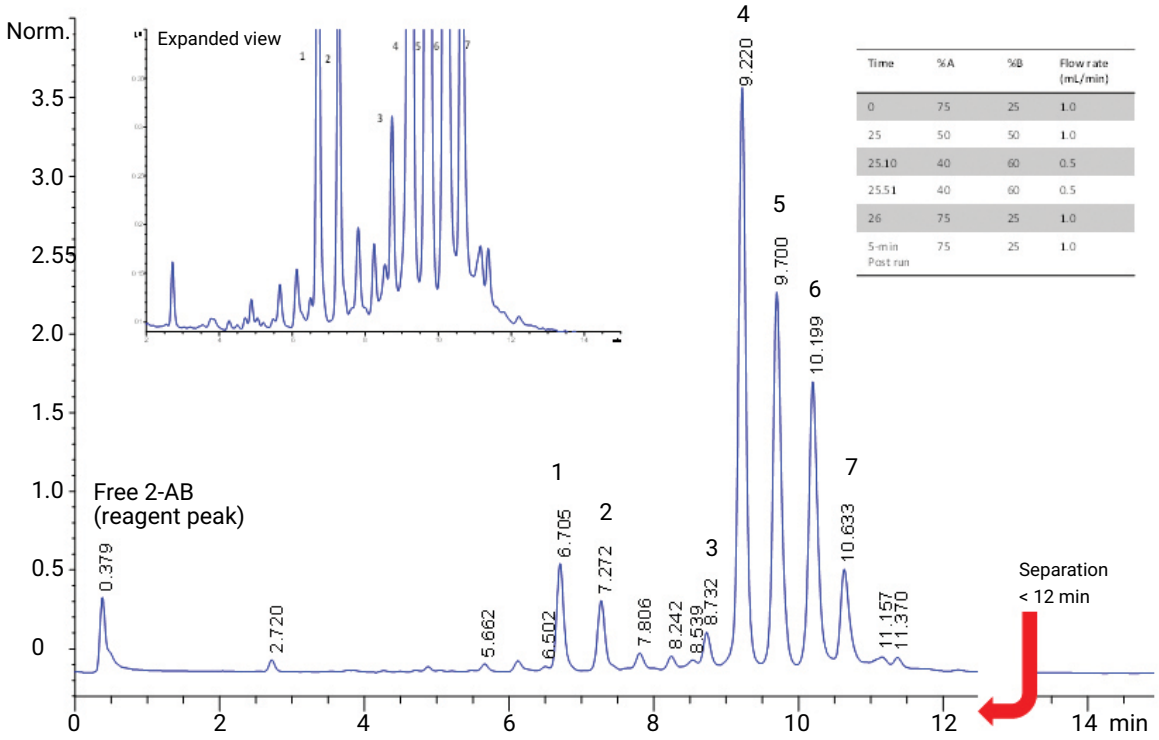


Figure 7. Fast and highly efficient separation of 2-AB labeled bovine fetuin N-linked glycans, 2.1 × 150 mm 1.8 µm AdvanceBio Glycan Mapping column.

Table 1. Glycan nomenclature and structure assignments for the optimized rapid separation of bovine fetuin 2-AB labeled N-linked glycans displayed in top chromatogram of Figure 7.

Peak	Retention	GU value	Glycan structure	Structures
1	6.70	9.4	A2G2S2	
2	7.27	9.8	A2G2S2	
3	8.73	10.8	A3G3S3, A3G3S2 (trace)	
4	9.22	11.2	A3G3S3, A3G3S2 (trace)	
5	9.70	11.6	A3G3S3, A3G3S4 (trace)	
6	10.20	12	A3G3S4, A3G3S3	
7	10.63	12.4	A3G3S4	

Galactose N-acetylglucosamine
Fucose Mannose N-acetylneuramic acid

Conclusions

The Agilent 1.8 µm HILIC amide AdvanceBio Glycan Mapping column provided separation of N-linked glycans with high speed, excellent resolution and increased efficiency. In a 2.1 × 150 mm configuration and under identical chromatographic conditions, the column enabled a well-resolved separation of 2-AB labeled IgG N-linked glycans, with a 40 % reduction in elution time compared to another brand of sub-2 µm HILIC column. A separation of 2-AB labeled bovine fetuin N-linked glycans demonstrated the column's excellent analytical selectivity and resolving power for separating these complex biantennary and triantennary glycans.

Acknowledgement

This work was presented in a poster session at the 62nd ASMS Conference on Mass Spectrometry and Allied Topics, 15–19 June, 2014, Baltimore.

Application Note

Glycan Analysis

N-Glycan Analysis of mAbs and Other Glycoproteins with UHPLC and Fluorescence Detection

The Agilent 1290 Infinity Binary LC System with the Agilent AdvanceBio Glycan Mapping Column

Authors

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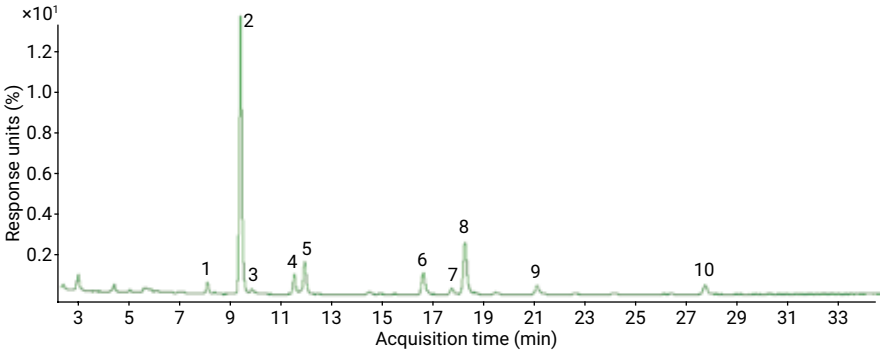
Oscar Potter
Agilent Technologies, Inc.

Waldbronn, Germany

Abstract

This Application Note describes the analysis of N-linked glycans with hydrophilic interaction chromatography (HILIC) using the Agilent 1290 Infinity Binary LC together with the Agilent 1260 Infinity Fluorescence Detector and the Agilent 6530 Accurate-Mass Q-TOF LC/MS. Enzymatic glycan release with PNGase F followed by derivatization with 2-aminobenzamide (2-AB) was conducted on monoclonal antibodies (mAbs) and two other glycoproteins, fetuin and ovalbumin. The excellent resolution provided by the Agilent AdvanceBio Glycan Mapping column allowed detection and identification of all major N-glycans in the mAb sample. Furthermore, the highly complex N-glycans released from fetuin and ovalbumin were well resolved.



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Introduction

Glycosylation is one of the most frequently observed post translational modifications. Mammalian glycoproteins contain three major types of glycans: N-linked, O-linked, and glycosylphosphatidylinositol (GPI) lipid anchors, which consist of one or more monosaccharide units. A single glycosylation site can generate considerable heterogeneity of the mass and charge of glycoproteins. These oligosaccharides are involved in many biological regulation and recognition processes, for example, protein sorting, immune and receptor recognition, inflammation, pathogenicity, metastasis, and other cellular processes[1,2]. In addition, properties such as safety, efficacy, and the serum half-life of therapeutic proteins can be affected by their glycosylation pattern.

Recombinant monoclonal antibody therapeutics (mAbs) represent the largest group of therapeutic proteins. The efficacy of these therapeutics is highly dependent on the correct glycosylation pattern of the mAbs and, so far, all licensed therapeutic mAbs are immunoglobulins G (IgGs)[3]. Human IgG has a single conserved N-linked glycosylation site located on the Fc region of each heavy chain at Asn2974 (Figure 1), resulting in the presence of two N-glycans per IgG. This typically consists of a handful of major structures and numerous minor structures[5]. The combination of glycans at each of the two glycosylation sites on the Fc region leads to large numbers of different glycoforms in each batch of mAb production.

The glycan structure plays a critical role in complement activation and receptor affinity[6], which affect the efficacy of therapeutic mAbs. Moreover, non-human glycans are a safety issue due to induced immune responses. Therefore, analysis of the glycan pattern is an important part of the characterization of therapeutic glycoproteins, especially mAbs.

This Application Note uses symbolic glycan structures according to the Consortium for Functional Glycomics (CFG), as shown in Figure 2. Assigned glycans are also described by the Oxford glycan nomenclature and by another style of nomenclature, which is popular for mAb glycans, shown here in italics.

Figure 2A shows the general nomenclature used to describe sugar residues of different glycan structures on proteins. Figure 2B shows some predominant glycan structures present on the Asn-297 site in human IgG. In general, N-glycans have a core structure, containing two b-D-N-acetylglucosamine (GlcNac) and three mannose (Man) units. IgG Fc N-glycans are predominantly biantennary complex-type structures, partially core-fucosylated (for example, FA2 or G0F).

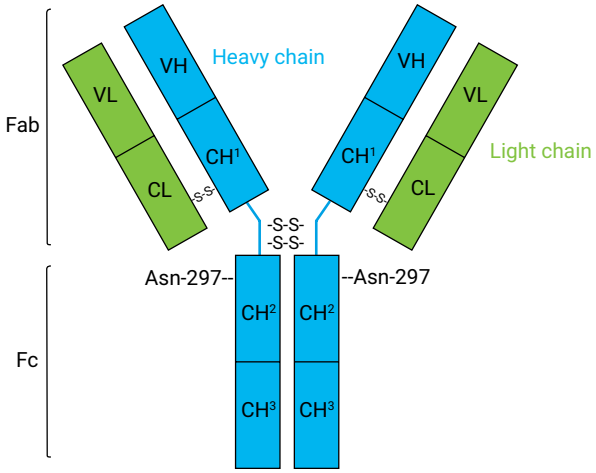

Figure 1. IgG antibody structure.

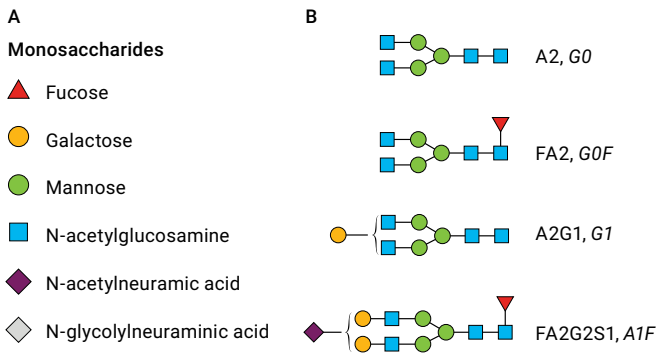

Figure 2. Glycan structure and isoforms. A) Monosaccharide description after the Consortium for Functional Glycomics, B) predominant glycan structures of human IgGs.

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Different strategies for the analysis of N-glycans have been described. Many methods are based on enzymatic release of N-glycans from the protein by PNGase F. Due to the lack of intrinsic chromophores, it is also common to derivatize the glycans with a fluorescent label prior to analysis[7]. Each N-glycan contains a single reducing end site that can be reacted with an excess of fluorescent label, such that each N-glycan will be attached to one fluorophore. The processed sample is, therefore, appropriate for relative quantification by separation with fluorescence detection without the need for any quantitation standards or calibration. 2-AB is a stable, neutral label that is popular for N-glycan analysis[7,8,9]. Figure 3 illustrates 2-AB labeling by reductive amination (Schiff's base intermediate not shown).

Subsequent purification using hydrophilic interaction chromatography/solid phase extraction (HILIC/SPE) is performed to remove the large excess of 2-AB so that it does not interfere with the HILIC/FLD analysis.

Here, we show enzymatic release of N-glycans using PNGase F with subsequent derivatization with 2-AB prior to separation by HILIC UHPLC, with fluorescence detection and identification by on-line quadrupole time-of-flight mass spectrometry (Q-TOF/MS).

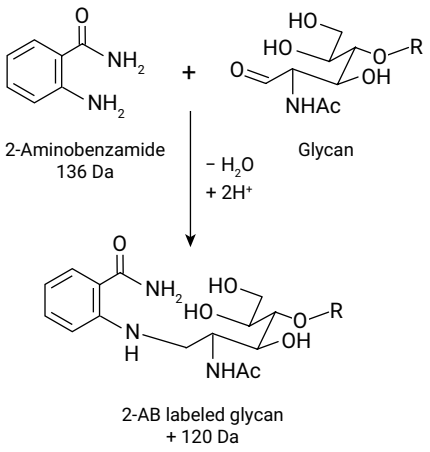

Figure 3. Labeling of a glycan with 2-aminobenzamide (2-AB).

Experimental

Reagents, samples, and materials

The Agilent 1290 Infinity Binary LC System consisted of the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A) with 35 µL Jet Weaver
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Fluorescence Detector (G1321B) with standard flow cell

Reagents, samples, and materials

Agilent 6530 Accurate-Mass Q-TOF LC/MS

Column

Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n 859700-913)

Software

Agilent OpenLAB CDS ChemStation Edition for LC & LC/MS Systems Rev. C.01.05 [38] and Agilent MassHunter Workstation Software, Version B.05.01, Build 4.0.479.0. Glycan structures were created with GlycoWorkbench, Version 2.1, stable (146).

Sample preparations

Deglycosylation procedure:

N-glycans were released from a monoclonal antibody, fetuin, and ovalbumin using PNGase F. This enzyme cleaves asparagine-linked high mannose as well as hybrid and complex oligosaccharides from the glycoproteins and leaves the glycans intact. Fetuin has three N-glycosylation sites (Asn-81, Asn-138, and Asn-158) and four O-linked sites (Ser-253, Thr-262, Ser-264, and Ser-323)11. Ovalbumin has only one glycosylation site, whereas the mAb contains two glycosylation sites. The amount of PNGase F was adjusted to the amount of N-glycosylation sites. The proteins were deglycosylated according to instructions for 3 hours at 37 °C. The reaction was then stopped, and the sample was vacuum-dried for further processing.

2-AB-labeling for fluorescence detection and sample cleanup

The dried glycan samples were labeled with 2-aminobenzamide according to the protocol for 3 hours at 65 °C. After the labeling procedure, the samples were purified using the HILIC cleanup cartridges according to the instruction manual. After the cleanup procedure, the samples were vacuum-dried and reconstituted in ultrapure water:acetonitrile 30:70 (v/v) for analysis.

Solvents and samples

Buffer A was 100 mM ammonium formate in water, pH 4.5 and buffer B was acetonitrile. All solvents were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). The monoclonal antibody was RAT Anti-DYKDDDDK Tag Antibody. Ammonium formate, fetuin and ovalbumin, PNGase F from Elizabethkingia miricola, GlycoProfil 2-AB Labeling Kit, and GlycoProfil Glycan Cleanup Cartridges were purchased from Sigma-Aldrich Corp., St. Louis, USA.

Instrumental conditions

	Antibody standard gradient	Fetuin gradient	Ovalbumin gradient
Starting flow rate	0.5 mL/min	0.5 mL/min	0.5 mL/min
Gradient	0 minutes 85 % B	0 minutes 75 % B	0 to 6 minutes 85 % B
	5 minutes 75 % B	45 minutes 50 % B	10 minutes 80 % B
	35 minutes 64 % B	47 minutes 40 % B, flow 0.5 mL/min	60 minutes 70 % B
	40 minutes 50 % B	47.01 minutes, flow 0.25 mL/min	65 minutes 50 % B, flow 0.5 mL/min
	42 minutes, flow 0.5 mL/min 42.01 minutes, flow 0.25 mL/min	49 minutes 0 % B	65.01 minutes, flow 0.25 mL/min
	43 minutes 0 % B	51 minutes 0 % B	68 minutes 0 % B
	48 minutes 0 % B	51.01 minutes 75 % B, flow 0.25 mL/min	73 minutes 0 % B
	50 minutes 85 % B 50.01 minutes, flow 0.25 mL/min	52.00 minutes, flow 0.5 mL/min	74 minutes 85 % B, flow 0.25 mL/min
	51 minutes, flow 0.5 mL/min		75.00 minutes, flow 0.5 mL/min
Stop time	51 minutes	52 minutes	75 minutes
Post time	20 minutes	20 minutes	20 minutes
Injection volume	5 µL	1 µL	1 µL
Thermostat autosampler	5 °C		
Column temperature	60 °C		
FLD	Ex. 260 nm, em. 430 nm		
Peak width	> 0.013 minutes (0.25 seconds resp. time) (37.04 Hz)		

MS parameters	
Gas temperature	250 °C
Sheath gas temperature	250 °C
Gas flow	8 L/min
Sheath gas flow	8 L/min
Nebulizer	25 psi
Vcap	3,500 V
Nozzle	1,000 V
Fragmentor	200 V
Skimmer	45 V
Oct 1 RF Vpp	550
Collision energies	15 and 30 V
Mode	MS and targeted MS/MS

Results and Discussion

Analysis of N-glycans from monoclonal antibodies

Figure 4 shows the separation of the mAb N-glycans. The mAb glycan pattern was optimally resolved, allowing separation and integration of all major N-glycans. Relative quantification was made based on the calculation of the peak area percentage. High intensity of the labeled glycans was achieved by setting the optimal wavelengths for glycan detection on the Agilent 1260 Infinity Fluorescence Detector, using 260 nm as excitation wavelength and 430 nm as emission wavelength[10].

The resulting HILIC glycan profile was assigned to the corresponding glycan structures based on the parent ion mass observed and the related MS/MS spectra. The parent masses were entered into the GlycoMod tool from Expasy to find related glycan structures.

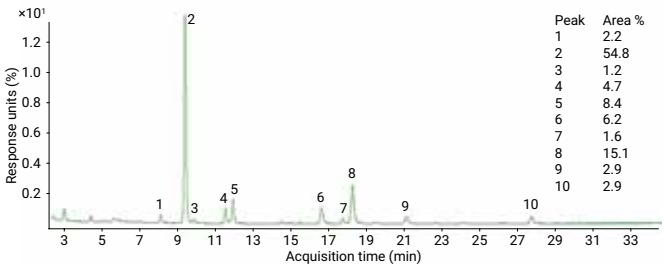

Figure 4. Separation of mAb N-glycans with fluorescence detection with 260 nm as the excitation wavelength.

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GlycoMod predicts possible glycan structures (labeled or unlabeled) from the experimentally determined masses (http://web.expasy.org/glycomod/). Another helpful tool for glycan assignment and glycan structure design is GlycoWorkbench15, which was used in this work to prepare glycan structure cartoons.

As an example of the workflow, the N-glycan FA2G1Sg1 with a parent mass of 1026.88 [z = 2] (peak 7 and 8) was chosen. The glycan databases revealed the two most likely glycan structures for this mass (Figure 5).

MS/MS data was then used to distinguish between these two potential structures. Figure 6 shows the collision-induced dissociation (CID) MS/MS spectrum of the N-glycan FA2G1Sg1. The MS/MS data confirm the presence of a type of sialic acid, N-glycolylneuraminic acid (NeuGc), which results in strong signals for fragment ions at m/z 308 (NeuGc) and m/z 673 (NeuGc attached to galactose and N-acetylglucosamine). Meanwhile, there are no signals at m/z 292 or m/z 657, which would have indicated the presence of N-acetylneuraminic acid (NeuAc). Therefore, these results provided strong evidence that the structure was FA2G1Sg1 (containing NeuGc) rather than A2G2S1 (which contains NeuAc). The decision to assign a structure with a core fucose was also supported by the MS/MS data, based on the lack of strong fragment ion signals at m/z 512 or m/z 350, which would have been present if the fucose had instead been attached in the outer arm region.

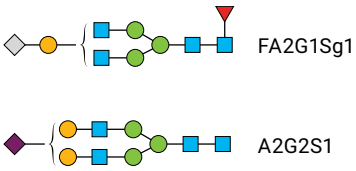

Figure 5. Two most likely glycan structures for the parent mass of 1,026.88 [z = 2], FA2G1Sg1 and A2G2S1.

All other peaks were similarly assigned using their MS and MS/MS spectra. The assigned structures are given in Table 1. The results show that the mAb mainly contains core fucosylated complex glycans, including several structures with NeuGc. These findings are typical for an IgG antibody produced by rat cells. NeuGc does not normally occur in human glycoproteins and is undesirable in therapeutic proteins[12]. The sialic acids occurring in human glycoproteins are typically N-acetylneuramic acids.

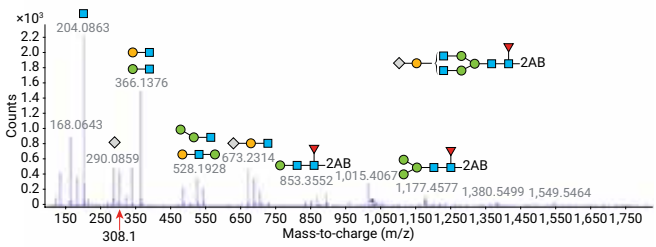

Figure 6. MS/MS spectra of FA2G1Sg1 - 1026.88 [z = 2] - 1931.6876 Da.

Table 1. Overview of masses and assigned 2AB- glycan structures of rat monoclonal antibody.

Peak	Oxford	Biopharma mAb style	Mass detector (Da)	Structure
1	FA1	G0-GlcNAc	A2G2S2	
2	FA2	G0F	792.3130 [z = 2]	
3	M5	Man5	1,355.5 [z = 1]	
4,5	FA2G1	G1F	873.34 [z = 2]	
6	FA1G1Sg1	G1FSg1-GlcNAc	925.34 [z = 2]	
7,8	FA2G1Sg1	G1FSg1	1,026.88 [z = 2]	
9	FA2G2Sg1	Ag1F	1,107.9135 [z = 2]	
10	FA2G2Sg2	Ag2F	1,261.446 [z = 2]	

Analysis of antibodies from fetuin and ovalbumin

N-glycans from two more proteins, fetuin and ovalbumin, were released by PNGase F, derivatized with 2-AB and analyzed using HILIC/UHPLC with online MS. Figure 7 shows the separation of bovine fetuin N-glycans. This glycosylation profile was dominated by complex non-fucosylated biantennary and triantennary glycans containing NeuAc. Nine major peaks could be assigned using Q-TOF/MS detection. Table 2 shows the assigned glycan structures.

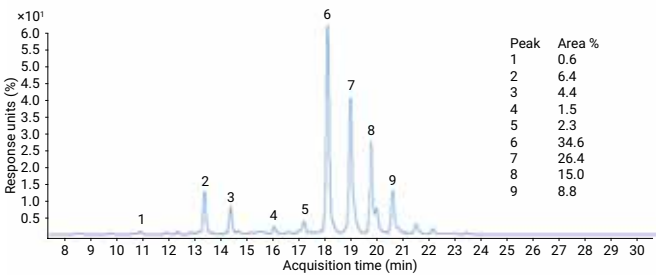

Figure 7. Separation of 2-AB-labeled fetuin.

Table 2. Detailed information of N-glycan ovalbumins.

Peak	Oxford	Structure
1	A2G2S1	
2,3	A2G2S2	
4	A3G3S2	
5	A3G3S3, A3G3S2 (trace)	
6	A3G3S3, A3G3S2 (trace)	
7	A3G3S3, A3G3S4 (trace)	
8	FA3G3S4, A3G3S3	
9	A3G3S4	

GlycoMod Figure 8 shows the separation of ovalbumin glycans. Ovalbumin is N-glycosylated only at one site (Asn292), but a complex glycosylation pattern can be associated to this site[13]. Due to the complexity of the glycan profile, the gradient had to be adjusted to a longer separation time to achieve higher resolution. The high performance of the AdvanceBio Glycan Mapping column allowed resolution of over 50 peaks with a good signal-to-noise (S/N) ratio. Twenty major peaks were assigned based on the parent ion-mass data (Table 3). Detailed structural conclusions were not achievable due to the high chance of isobaric structures occurring, several of which cannot necessarily be distinguished from the MS/MS data. Instead, the N-glycans are described in terms of their monosaccharide composition.

Compared to the relatively simple glycan pattern of the mAb, the two other glycoproteins had a greater variety of glycan structures. No fucosylated glycans were detected in ovalbumin in contrast to the mAb glycans, which matches previously reported findings that avian egg glycoproteins are non-fucosylated[14].

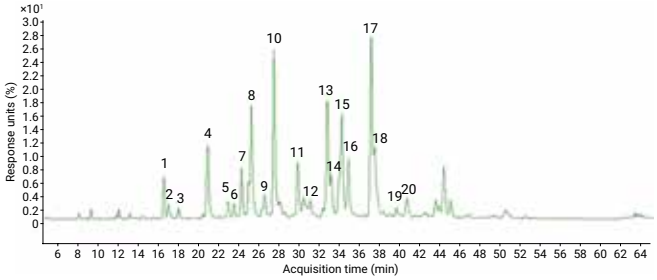

Figure 8. Separation of N-glycans released from ovalbumin.

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Table 3. Assigned masses and monosaccharide composition of ovalbumin N-glycans; H = hexoses, i.e. galactose or mannose; N = N-acetylglucosamine.

Peak	Mass + 2AB (Da)	Calculated mass (Da)	PComposition (short form)	PComposition (long form)
1,2	1,234.48 [M]	1,114.48	H3N3	$(HexNAc)_1+(Man)_3(GlcNAc)_2$
3	1,193.45 [M]	1,073.45	H4N2	$(Hex)_4(HexNAc)_2$
4	1,437.56 [M]	1,317.56	H3N4	$(HexNAc)_2+(Man)_3(GlcNAc)_2$
5	1,396.53 [M]	1,276.53	H4N3	$(Hex)_1(HexNAc)_1+(Man)_3(GlcNAc)_2$
6, 7	1,640.64 [M]	1,520.64	H3N5	$(HexNAc)_3+(Man)_3(GlcNAc)_2$
8	1,355.506 [M]	1,235.51	H3N5	$(Hex)_2+(Man)_3(GlcNAc)_2$
9	1,599.61 [M]	1,479.61	H4N4	$(Hex)_1(HexNAc)_2+(Man)_3(GlcNAc)_2$
10	1,843.73 [M]	1,723.73	H3N6	$(HexNAc)_4+(Man)_3(GlcNAc)_2$
11	1,802.74 [M]	1,682.74	H4N5	$(Hex)_1(HexNAc)_3+(Man)_3(GlcNAc)_2$
12, 14	2,046.884 [M]	1,926.88	H3N7	$(HexNAc)_5+(Man)_3(GlcNAc)_2$
13	1,517.56 [M]	1,397.56	H6N2	$(Hex)_3+(Man)_3(GlcNAc)_2$
15	1,761.6574 [M]	1,641.66	H5N4	$(Hex)_2(HexNAc)_2+(Man)_3(GlcNAc)_2$
16	2,005.8098 [M]	1,885.81	H4N6	$(Hex)_1(HexNAc)_4+(Man)_3(GlcNAc)_2$
17	2,249.9728 [M]	2,129.97	H3N8	$(HexNAc)_6+(Man)_3(GlcNAc)_2$
18	1,964.82 [M]	1,844.82	H5N5	$(Hex)_2(HexNAc)_3+(Man)_3(GlcNAc)_2$
19, 20	2,208.87 [M]	2,088.87	H4N7	$(Hex)_1(HexNAc)_5+(Man)_3(GlcNAc)_2$

Conclusions

The Agilent 1290 Infinity Binary LC System, together with the Agilent 1260 Infinity Fluorescence Detector and Agilent 6530 Accurate-Mass Q-TOFLC/MS, was an ideal combination for the analysis of released N-glycans that were derivatized with 2-aminobenzamide. Sample preparation using PNGase F for the release of N-linked glycans followed by 2-AB derivatization with subsequent HILIC sample cleanup was demonstrated for one monoclonal antibody and two other glycoproteins.

The Agilent AdvanceBio Glycan Mapping column demonstrated excellent resolving power, allowing separation and identification of all major N-glycans in a rat mAb sample. Complex biantennary and triantennary N-glycans from fetuin and ovalbumin were also analyzed with very high resolution. Optimized fluorescence excitation and emission wavelengths of 260 and 430 nm provided better S/N ratios. Electrospray ionization Q-TOF MS analysis allowed assignment of different glycan structures or monosaccharide compositions, depending on the complexity of the sample.

References

1. Rademacher, T. W; Williams, P; DwekMark, R. A. Agalactosyl glycoforms of IgG autoantibodies are pathogenic. *P. Natl. Acad. Sci.* **1994**, 91, pp 6123-6127.
2. Peracaula. R; *et al.* Glycosylation of human pancreatic ribonuclease: differences between normal and tumor states. *Glycobiology* **2003**, 13, pp 227-244.
3. Jefferis, R. Glycosylation of recombinant antibody therapeutics. Biotechnol. Progr. 2005, 21, pp 11-16. 4. Arnold, J. N; *et al.* Human immunoglobulin glycosylation and the lectin pathway of complement activation. *Adv. Exp. Med. Biol.* **2005**, 564, pp 27-43.
4. Fernandes, D. Demonstrating comparability of antibody glycosylation during biomanufacturing. *Euro. Biopharm. Re.* **2005**, Summer, pp 106-110.
5. Abès, R; Teillaud, J. L. Impact of Glycosylation on Effector Functions of Therapeutic IgG. *Pharmaceuticals* **2010**, 3, pp 146-157.
6. Ruhaak, L. R; *et al.* Glycan labeling strategies and their use in identifi cation and quantifi cation. *Anal. Bioanal. Chem.* **2009**, 397, pp 3457-3481.
7. Royle, L; *et al.* HPLC-based analysis of serum N-glycans on a 96-well plate platform with dedicated database software. *Anal. Biochem.* **2008**, 376, pp 1-12.
8. Huhn, C; *et al.* IgG glycosylation analysis. *Proteomics* **2009**, 9, pp 882-913.
9. Melmer, M; *et al.* HILIC analysis of fl uorescence-labeled N-glycans from recombinant biopharmaceuticals. *Anal. Bioanal. Chem.* **2010**, 398, pp 905-914.
10. Ding, W; *et al.* Identifi cation and Quantifi cation of Glycoproteins Using Ion-Pairing Normal-phase Liquid Chromatography and Mass Spectrometry. *Mol. Cell. Proteom.* **2009**, 8, pp 2170-2185.
11. Leibiger, H; *et al.* Variable domain-linked oligosaccharides of a human monoclonal IgG: structure and infl uence on antigen binding. *Biochem. J.* **1999**, 338, pp 529-538.
12. Anumula, K. R. Advances in fl uorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates. *Anal. Biochem.* **2006**, 350, pp 1-23.
13. Montreuil, J; Vliegenthart, J. F. G; Schachter, H., Eds.; Glycoproteins II. Elsevier B. V., Amsterdam, **1997**.
14. Ceroni, A; *et al.* GlycoWorkbench: a tool for the computer-assisted annotation of mass spectra of glycans. *J. Proteome Res.* **2008**, 7, pp 1650-1659.

Application Note

Glycan Analysis

Agilent

Trusted Answers

Agilent Solutions for High-throughput N-linked Glycan Profiling from Biotherapeutics

Authors

Arunkumar Padmanaban, Sreelakshmy Menon, and Suresh Babu CV

Agilent Technologies India Pvt Ltd Bangalore India

Abstract

This Application Note presents an automated high-throughput sample preparation for 2-AB labeled N-linked glycans from innovator and biosimilar monoclonal antibody (mAb) followed by liquid chromatography (LC) analysis.
Agilent AssayMAP Bravo liquid handling platform was used for the automated sample preparation involving glycan cleavage and release from mAb, 2-AB labeling, and purification. The downstream ready samples were then analyzed using an Agilent 1290 Infinity LC system with Agilent AdvanceBio Glycan Mapping columns. The glycan distribution and heterogeneity between the samples were deduced by comparing the chromatogram from both innovator and biosimilar mAb.

The study highlights the high-throughput application of the AssayMAP Bravo platform for automated and reproducible sample preparation for glycan profiling, followed by fast chromatographic separation using a 1290 Infinity LC system.

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Introduction

Monoclonal antibodies (mAbs) are leading the race in biotherapeutics, and have revolutionized the way diseases are treated and intervened. Patents for most of the 20 clinically approved[1] first-generation mAbs have either expired, or are about to expire. This has increased the opportunity for generating generic versions, referred to as biosimilars. Regulatory bodies such as the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) have published guidelines for the characterization of biosimilar protein therapeutics including post-translation modifications[2]. Glycosylation modification results in structural heterogeneity that changes an mAb's target binding capacity, stability, charge, and mass[3]. During the course of developing the antibodies from the cell line, the glycans are monitored for cell line alteration and culture optimization. This requires a high-throughput sample preparation to screen several cell culture conditions in parallel. The Agilent Bravo Automated Liquid Handling Platform is a precise liquid-handling system designed for high-throughput applications like glycan profiling. Agilent Bravo with Agilent AssayMAP technology combines the automation with miniature 5 µL pack bed cartridges for sample preparations, enabling high-throughput chromatography and sample preparations in a shorter time[4].

This Application Note presents a workflow solution for profiling N-glycans from mAb using an Agilent AssayMAP Bravo platform. The system automates the N-glycan purification and derivatization using 2-aminobenzamide (2-AB) dye, which is then followed by Agilent HPLC analysis using Agilent Glycan Mapping columns. We showcase the ease-of-use of an AssayMAP Bravo for the automated sample preparation required for high-throughput profiling studies.

Experimental

Materials

Agilent's AdvanceBio GlykoPrep-plus Rapid N-Glycan Sample Preparation with 2-AB kit, and 2-AB labeled Oligomannose 5 and 6 were used to prepare and analyze the samples. An Agilent AdvanceBio Glycan Mapping column, 2.1 × 150 mm, 1.8 µm (p/n 859700-913), and Agilent 2-AB labeled human IgG N-linked glycans standards (p/n GKSB-005) were purchased from Agilent Technologies. Innovator rituximab and biosimilar product were purchased commercially from a local pharmacy. All other chemicals were procured as HPLC grade from Sigma.

Sample preparation

Reagents from an Agilent AdvanceBio GlykoPrep-plus kit with an AssayMAP protocol were used for the sample preparation. An innovator and biosimilar sample was diluted to 1 mg/mL, then loaded onto three columns of the 96-well plate (24 replicates each). After placing the sample plates and reagents plates as specified in the guideline[5], the samples were processed by launching the N-Glycan Sample Prep: RX digestion & 2-AB labeling module from Agilent VWorks software. The protocol consists of five modules that are performed in sequential order to immobilize the samples, digest the glycans, elute, label with 2-AB, and complete a final cleanup to remove the excess dyes. The final purified labeled glycan from each well were then transferred to HPLC vials and analyzed immediately, or stored at –80 °C.

Figure 1 presents a schematic diagram of the complete workflow.

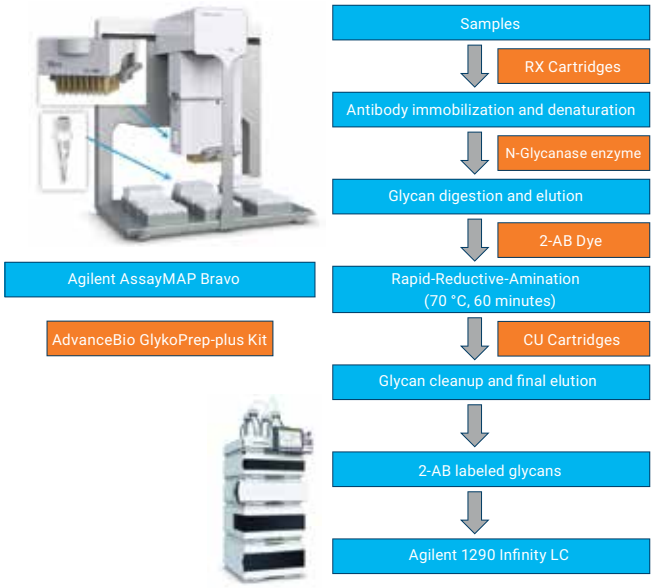

Figure 1. Schematic workflow for the glycan isolation, labelling, purification followed by LC analysis using Agilent solutions. Components of the Agilent AssayMAP steps are shown in blue; the AdvanceBio kits are shown in orange.

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Instrumentation

Agilent 1290 Infinity LC System including:

- Agilent 1290 Infinity Binary Pump G4220A
- Agilent 1290 Infinity Autosampler G4226A
- Agilent 1290 Infinity TCC G1316 C
- Agilent 1260 Fluorescence Detector G1321 B

The LC method described earlier was adopted for this study[6]. Every sample was analyzed in quadruplet injections, followed by a blank injection.

Software

- Agilent VWorks Automation Control 11.4.0.1233
- Agilent AssayMAP Launch Pad 3.0
- Agilent N-Glycan Sample Prep: RX digestion & 2-AB labeling protocols 1.0
- Agilent ChemStation C.01.06

Results and Discussion

Agilent AssayMAP sample preparation

The samples were processed following a protocol consisting of five modules, as presented in Figure 2. The software suite consists of a deck layout, labware table, and application settings tab for each module to be performed (Figure 3). The user was prompted to place the appropriate consumables and reagents listed in the labware table in the specified deck positions. After setting up all labware, the protocol was executed, and AssayMAP Bravo completed the protocol, and a confirmation message was displayed to proceed to the next module.

The final Cleanup Protocol module eluted the labeled and purified glycans in an aqueous buffer into a clean 96-well plate. The samples were then analyzed in quadruplets, along with blanks, using the Agilent AdvanceBio Glycan Mapping column for the downstream LC analysis.

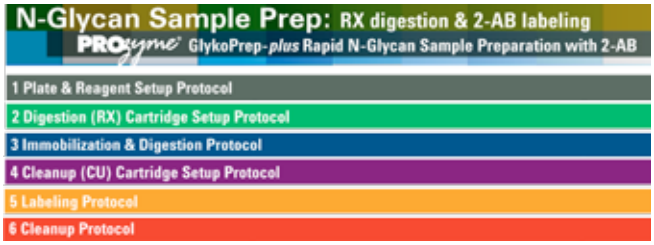

Figure 2. Agilent VWorks modules of an Agilent AssayMAP N-Glycan Sample Preparation.

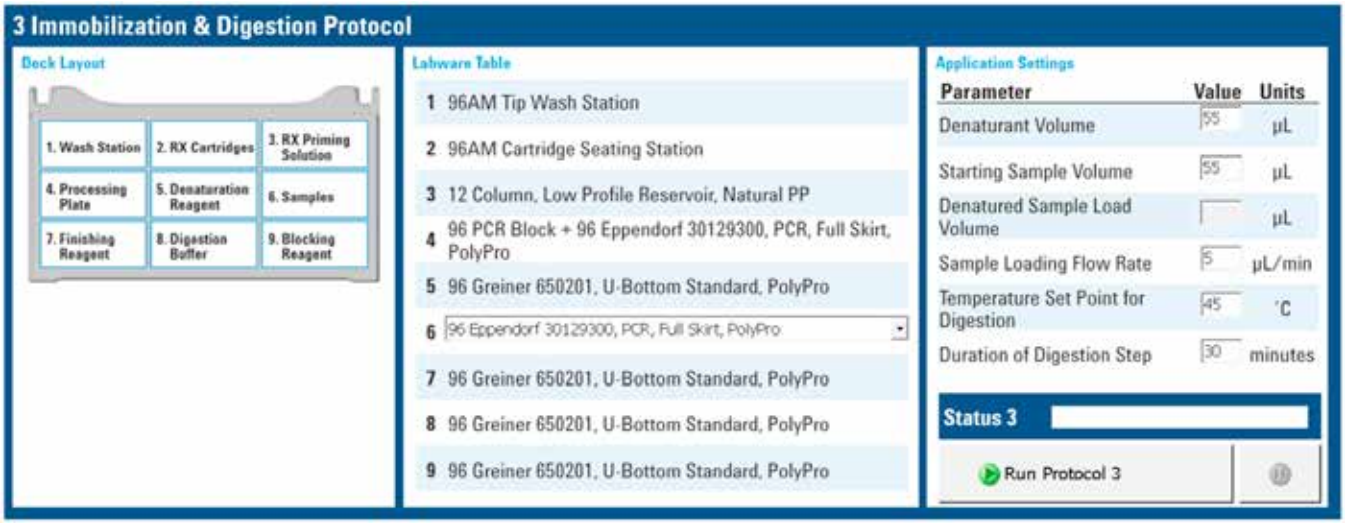

Figure 3. The deck layout, labware table, and application settings of the Immobilization and Digestion Protocol, one of the five sample preparation modules in Agilent VWorks software.

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HPLC Analysis

The N-glycan profiles were compared between the innovator and biosimilar mAbs using a fast analysis method with the Agilent 1290 Infinity system. The Agilent 2-AB labeled IgG N-linked glycan library was used as the standard to assess the column performance, and to annotate the peaks in mAb samples. The 1290 Infinity system with high backpressure enabled the analysis of the samples in less than 6 minutes, saving analysis time per sample, and increasing sample throughput.

Each sample was HPLC analyzed in replicates of four. Figure 4 presents the peak area of four major glycans species from 96 replicates, demonstrating the excellent column-to-column reproducibility of the AssayMAP micro chromatography pipette tips. The Reproducibility Standard Deviation (RSD) calculated for peak area and peak height showed a coefficient of variation (CV) of less than 6 % for all glycan species. This demonstrates the very robust and reproducible sample preparation capability of the AssayMAP Bravo system.

Glycan profiling and comparison of innovator and biosimilar rituximab

The chromatograms of the innovator and biosimilar rituximab were compared with the standard N-linked IgG glycan library, and the peaks corresponding to glycans were annotated. Separate standards comprising 2-AB labeled oligomannose 5 and 6 were also used to annotate additional peaks. The glycosylation pattern of the major abundant glycans, such as the G0F, G1F isoforms, and G2F was comparable between the innovator and biosimilar product (Figure 5). Small differences in the low abundant glycans were observed; the biosimilar sample contained lower amounts of mannose (Man5), as shown in the zoomed view of Figure 6. Despite minor differences in some low abundant glycans, the glycan profile of the innovator and biosimilar rituximab can be concluded to be comparable.

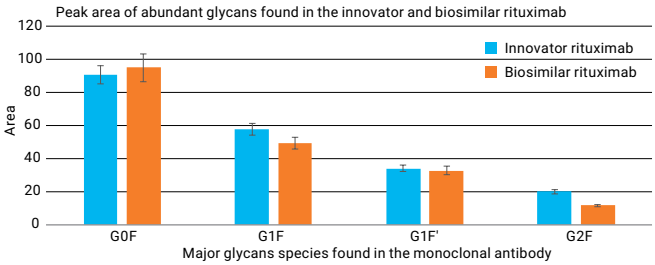

Figure 4. Peak area of major glycan species from 96 replicates for each innovator and biosimilar sample.

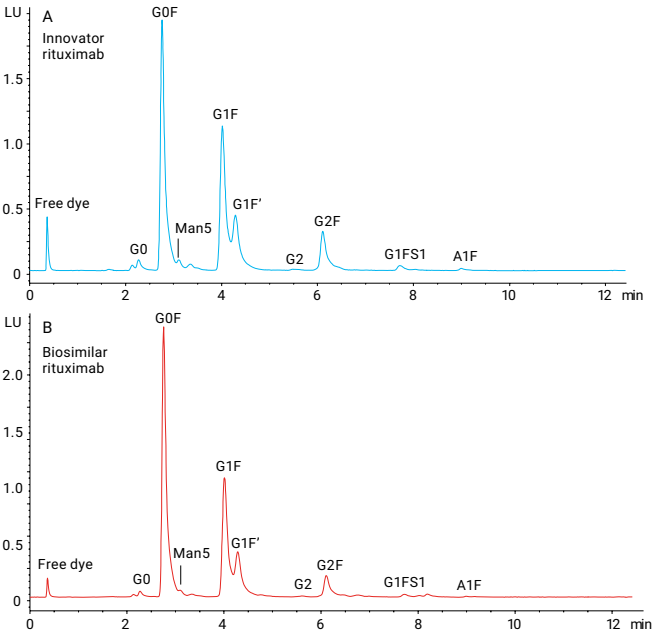

Figure 5. Glycosylation pattern of innovator and biosimilar rituximab.

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The area percentage for each N-glycan core was calculated and compared between both samples. Table 1 shows the ratio of N-glycans between the innovator and biosimilar rituximab. The table shows a similar N-glycan fingerprint profile for both innovator and biosimilar.

Both samples showed a similar trend in the distribution of N-glycans, with G0F as the major glycoform followed by G1F, G1F', and G2F. Apart from the annotated glycans, there were a few unknown glycan forms that may be assigned with an orthogonal detection system. A correlation graph (Figure 7) plotted for the area percentage of both samples shows high similarity, with an R2 of 0.973.

Table 1. Glycan area percentage between innovator and biosimilar mAbs.

Percentage of N-glycans		
N-glycans	**Innovator**	**Biosimilar**
G0	1.5	0.8
G0F	42.2	50.5
Man5	1.7	1.2
G1F	28.6	26.7
G1F'	13.0	11.6
G2	0.3	0.2
G2F	8.7	5.5
G1FS1	0.9	0.6
A1F	0.2	0.5

Conclusions

This study highlights the versatility of the Agilent AssayMAP Bravo system for a high-throughput sample preparation.

- The Agilent VWorks Automation Control software suite simplifies the sample preparation with ready-to-go protocols, resulting in downstream-compatible samples with minimal hands-on operation.
- Monoclonal antibody samples from a 96-well plate were processed, in parallel, for enzymatic glycan cleavage, separation, derivatization with 2-AB, and purification.
- The AssayMAP demonstrated excellent reproducibility in the glycan purification, and performed robustly.
- The purified samples were then analyzed using an Agilent 1290 Infinity LC system with the Glycan Mapping column.
- The glycan species were well-resolved in a shorter time, and were annotated using standards.
- The distribution of the glycan species between the innovator and biosimilar were assessed, and the data suggest comparable glycan profiles for the innovator and biosimilar rituximab used in this study.

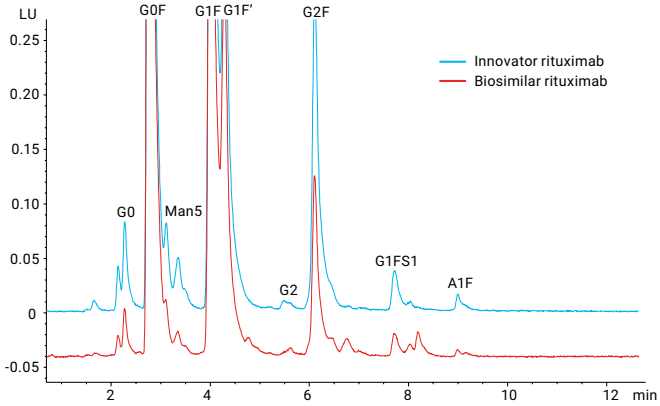

Figure 6. Zoomed view of Figure 5: the glycosylation pattern of innovator and biosimilar rituximab, showing minor differences in the low abundant glycans.

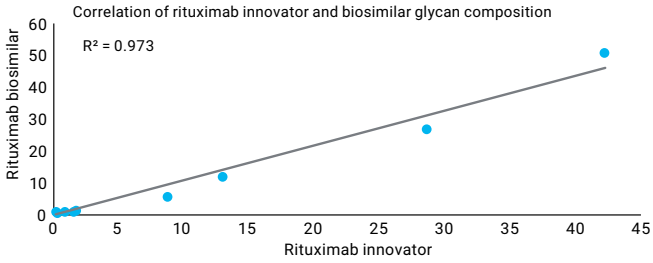

Figure 7. Linear graph showing the comparison of the area % of glycans released from innovator and biosimilar rituximab.

References

1. Chames, P.; Baty, D. Bispecific antibodies for cancer therapy. mAbs **2009**, 1:6, 539-547.
2. Scientific Considerations in Demonstrating Biosimilarity to a Reference Product: Guidance for Industry. Food and Drug Administration. Center for Biologics Evaluation and Research (CBER). April 2015. http://www. fda.gov/downloads/Drugs/GuidanceComplianceRegulatory Information/Guidances/UCM291128. pdf
3. Zhang, Q.; et al. A Perspective on the Maillard Reaction and the Analysis of Protein Glycation by Mass Spectrometry: Probing the Pathogenesis of Chronic Disease. Journal of Proteome Research **2009**, 8(2), 754–769.
4. Agilent AssayMAP Cartridges Automated Sample Preparation, Agilent Technologies Data Sheet, publication number 5991 -1774EN, **2013**.
5. AssayMAP with ProZyme GlykoPrep-plus Rapid N-Glycan Sample Preparation with 2-AB Protocol Guide, Agilent Technologies Manual, publication number G5409-90012EN, Rev May **2013**.
6. Martosella, J.; et al. A Novel HILIC Column for High Speed N-linked Glycan Analysis, Agilent Technologies Application Note, publication number 5991-4886EN, **2014**.

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Additional Application Notes

Part Number	Title
5991-4801EN	Sensitive and Reproducible Glycan Analysis of Human Immunoglobulin G
5991-4886EN	A Novel HILIC Column for High Speed N-linked Glycan Analysis
5991-7024EN	Analysis of N-Linked Glycans from Antibody-Drug Conjugate (ADC) Using the Agilent AssayMAP Automated Sample Preparation and Agilent 1290 Infinity LC System
5991-8071EN	Analysis of Monoclonal Antibody N-Glycans by Fluorescence Detection and Robust Mass Selective Detection using the Agilent LC/MSD XT
5991-8796EN	Profiling Glycosylation of Monoclonal Antibodies at Three Levels Using the Agilent 6545XT AdvanceBio LC/Q-TOF

Additional Information

Glycan sample preparation kits are available, containing supplies and reagents for deglycosylation, glycan labeling, and sample clean-up. Detailed protocols may be found in the user guides. 5991-9561EN and 5991-9560EN.

Glycan standards are available to assist with method development, data analysis, and system checks.
See pages 12-15, or download 5994-2202EN.

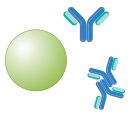
For more information on the glycan mapping workflow, visit www.agilent.com/chem/glycoscience

Aggregate/Fragment Analysis

Background

Proteins are often susceptible to aggregation, forming dimers and larger oligomers or high order structures because of exposure to stress conditions. This is particularly problematical in biotherapeutic protein manufacture since the target protein will be subjected to a wide variety of conditions that may induce aggregation. These conditions include changes in temperature and concentration during fermentation and changes in pH and concentration during downstream processing. Even shear forces (from impeller blades, stirrers, and other engineering plant equipment) can result in stress-related aggregation. The presence of aggregates, particularly very large multimers up to subvisible particles, are potentially harmful to health. It is therefore a prerequisite that the level of aggregation is quantified and determined and that limits are put into place.

Size exclusion chromatography is one of the techniques that is particularly suited to the separation of monomer peaks from higher-order aggregates. With a suitable concentration detector such as UV or DAD quantification is relatively straightforward. More complex molecules such as antibody drug conjugates, or ADCs, may be more challenging due to the presence of hydrophobic cytotoxic drugs that can result in nonideal behavior with many size exclusion chromatography columns. To address this issue, Agilent has developed a new stationary phase that demonstrates greatly reduced risk of secondary interactions. The new AdvanceBio SEC columns are therefore ideally suited to rapid separation and quantification of aggregates.



Aggregate/ Fragment Analysis

Size exclusion chromatography

Accurate, precise quantitation for a broad range of biomolecule separations

AdvanceBio SEC 1.9 and 2.7 µm

Versatile performance for routine and challenging applications

Attribute	Advantage
Hydrophilic polymer coating	Avoid secondary interactions
Increased analytical speed	Meet vital deadlines
Higher reproducibility	Reduce rework
Greater sensitivity	Quantitate aggregates, even at low levels
1.9 µm particle	Highest resolution
PEEK-lined stainless steel hardware option	Metal-free pathway for metal-sensitive samples and detection

Bio SEC-3 and Bio SEC-5

Extra wide pore and scale-up options

Attribute	Advantage
Compatibility with most aqueous buffers	Method flexibility
Wide range of pore size options, including 1000 Å and 2000 Å	Options for everything from peptides to VLPs
Analytical and semi-prep dimensions	Easy scale up or down

Getting Started

Since protein aggregation can occur as a result of external factors, one of the most important steps in aggregate analysis is sample preparation. It is necessary to ensure that the protein is fully dissolved in the mobile phase but is not subjected to factors that may alter the level of aggregation, such as sonication, temperature, pH, and excessive concentration. Size exclusion chromatography is a relatively straightforward technique relying on isocratic elution. The mobile phase conditions should ensure that there is no secondary interactions between the protein and the column stationary phase. Typically aqueous buffers such as 150 mM sodium phosphate or PBS (phosphate buffered saline) at neutral pH are used. Such mobile phase solutions are also ideal conditions for bacterial growth. It is therefore important to prepare fresh buffer regularly and to filter it through 0.2 µm filter before use. Under no circumstance should unused buffer be left on the instrument for a prolonged period.

Avoiding the temptation to add preservatives such as sodium azide to the mobile phase can allow low wavelengths (210–220 nm) to be used, greatly improving the sensitivity of the technique. Preservatives, such as 20 % ethanol, are recommended for column storage. However, care must be taken since the viscosity of the mobile phase containing organic modifiers is often significantly higher and column damage could ensue.

Size Exclusion Chromatography For Biomolecule Analysis: A "How-To" Guide

A Guide To Successful SEC

The chromatographic separation of biomolecules based on their size in solution is known as size exclusion chromatography (SEC). Unlike other modes of chromatography, it relies on the absence of any interaction between the analyte and the stationary phase packed in the column. This provides an ideal solution for separating and analyzing intact proteins from contaminants that can include aggregates, excipients, cell debris, and other impurities arising from degradation. SEC is therefore widely used in both development and manufacture for biotherapeutic molecule characterization.

In this guide, we discuss SEC separations, the effect of solute size and molecular weight, column selection choices, important mobile phase considerations, general rules for using SEC, and more.

Separation is straightforward and uncomplicated

With SEC, molecules are separated from largest to smallest in proportion to their molecular size in solution. Very large molecules are excluded from the packed bed and elute first, in the void volume. Smaller molecules will be able to penetrate the pores to various degrees depending on their size (Figure 1), with the smallest molecules diffusing furthest into the pore structure and eluting last.

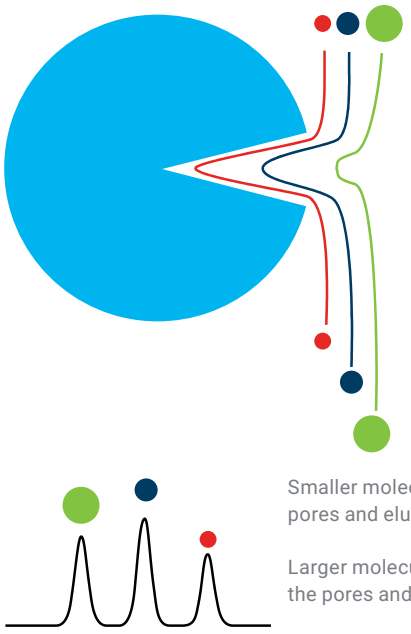


Figure 1. Molecules permeate the pores of the stationary phase to different extents depending on their size.

Size exclusion chromatography is suitable for separating and quantifying protein mixtures, and is therefore a valuable technique for quality control in recombinant protein manufacture. This includes measuring aggregates (dimers, trimers, tetramers, etc.) or separating low molecular weight excipients and impurities from larger molecular weight proteins (Figure 2).

Understanding and controlling aggregation in therapeutic proteins is essential as it will affect efficacy and lifetime, and could even result in a potentially serious immunogenic response. Regulations such as ICH(Q6B) clearly state that aggregates must be resolved from the desired product and quantitated.

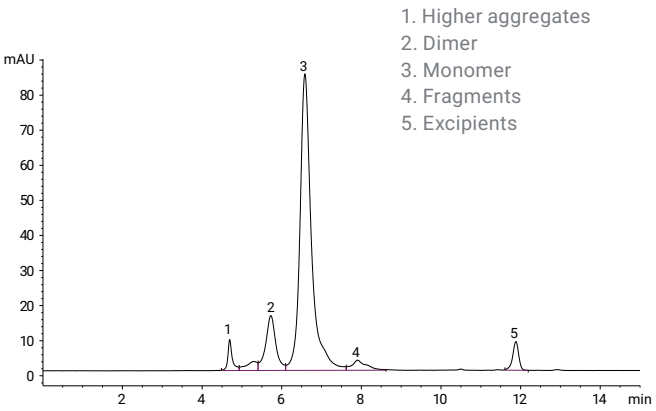


Figure 2. Separation of IgG aggregates and excipients.

Conditions, intact IgG monomer and dimer separation

Parameter	Value
Sample:	Polyclonal IgG
Sample conc:	150 mM sodium phosphate buffer
Column:	Agilent AdvanceBio SEC, 300 Å 7.8 x 300 mm,
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Flow rate:	1.0 mL/min
Temp.:	Ambient
Detector:	UV, 220 nm
Injection:	5 µL

Elution order typically follows molecular weight. Molecules with the highest molecular weight elute first. However, the true mechanism of SEC is based on size in solution. Most proteins are compact, but some protein molecules are cylindrical, so may elute earlier than expected due to their larger hydrodynamic radius in solution (Figure 3). Furthermore, different mobile phases can affect the elution order because of changes in size in solution (hydrodynamic radius or radius of gyration).

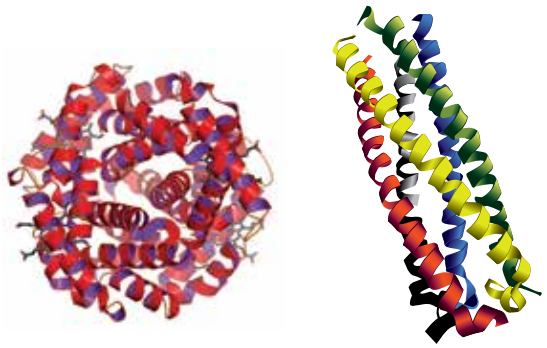


Figure 3. Comparison of compact globular protein versus cylindrical protein.

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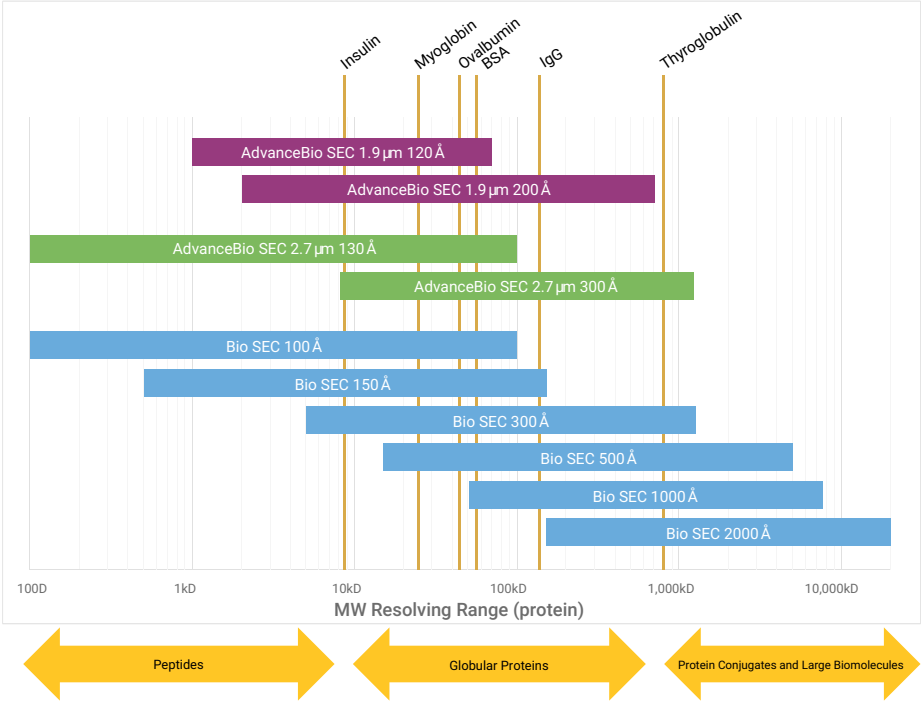
SEC-UV/DAD Method Development Guide

Choose initial columns and conditions for size-based separation of biomolecules, aggregation analysis, peptides, polypeptides, and proteins.



Recommended Initial Separation Conditions

Column:	AdvanceBio SEC or Agilent Bio SEC-5
Mobile phase:	150 mM phosphate buffer, pH 7.0*
Gradient:	Isocratic in 10-30 min range
Temperature:	Recommended: 10-30 °C, Maximum: 80 °C
Flow rate:	0.1-1.25 mL/min for 7.8 mm id columns 0.1-0.4 mL/min for 4.6 mm id columns 0.05-0.1 mL/min for 2.1 mm id columns
Sample size:	≤ 5 % of total column volume

*Other aqueous buffers with high and low salt can be used

For additional information, see application note: *Defining the Optimum Parameters for Efficient Size Separations of Proteins (publication no. 5990-8895EN)*
www.agilent.com/chem/library

After the initial chromatogram, additional changes may be needed to improve the separation, maintain protein solubility, or to decrease sample interaction with the chromatographic media. The ionic strength of the mobile phase can be adjusted up or down in strength to attain an optimized separation. pH can also be adjusted, usually ± 0.2 units. If further optimization is necessary, the upward or downward range should be expanded. A change of temperature or addition of an organic solvent can also be used.

For protocols requiring additional salt, these buffers are typical:
100-150 mM sodium chloride in 50 mM sodium phosphate, pH 7.0
100-150 mM sodium sulfate in 50 mM sodium phosphate, pH 7.0
50-100 mM urea in 50 mM sodium phosphate, pH 7.0. Other similar salts (e.g. KCl) and guanidine hydrochloride can also be used.

pH range: 2.0-8.5

Potential organic solvent additions include:
5-10 % ethanol (or other similar solvents such as methanol or acetonitrile) in 50 mM sodium phosphate, pH 7.0, 5 % DMSO in
50 mM sodium phosphate, pH 7.0. Note that it may be necessary to reduce the flow rate to keep below the maximum operating pressure when using higher viscosity mobile phases.

Temperature:
Typically, SEC separations are run at 10-30 °C. Separation of proteins and peptides may require higher temperature to improve resolution and recovery of proteins and hydrophobic peptides. SEC may be run in a cold room to maintain maximum biological activity of temperature sensitive proteins. Maximum operating temperature of Agilent Bio SEC columns is 80 °C. Note that higher temperatures can denature proteins.

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Instrumentation consideration for SEC

The SEC separation mechanism means that the elution volume, or retention time, is absolutely critical to the analysis. This requires high performance instrumentation to ensure precision and reproducibility. Isocratic pumps or gradient pumps operated in isocratic mode are suitable, and so refractive index (RI) detectors–as well as the more conventional UV or DAD detector–can be employed. To ensure baseline stability, especially when using an RI detector, online degassing of the mobile phase and thermostatted compartments are highly recommended. Operating at elevated temperatures increases the diffusion coefficient, leading to better resolution, better reproducibility, and reduced stress on the column. Therefore, thermostatted compartments are essential for a high performance system.

Agilent 1260 Infinity Bio-inert Quaternary LC System

Robust and reliable operation even under challenging solvent conditions

Buffers with high salt concentrations such as 2 M NaCl or 8 M urea and extreme pH values between 1 and 13 are commonly used in the analysis of biomolecules, posing a significant challenge for LC instruments. The dedicated design of the 1260 Infinity Bio-inert Quaternary LC handles these harsh solvent conditions with ease. Corrosion resistant titanium in the solvent delivery system and metal-free materials in the sample flow path create an extremely robust instrument, protecting not only your sample but your investment. The detector is also designed for biomolecule separations and does not affect the analysis of proteins, peak shape, and recovery.

Bio-inert flow cell with RFID tag, 10 mm, 13 µL (p/nG5615-60022)

Protect your proteins during analysis

Heat can denature proteins, and so it is important that your sample is kept at constant temperature in the whole LC flow path. The Agilent bio-inert autosampler with inert sample loop and ceramic needle can be cooled with an add-on thermostat. Bio-inert heat exchangers for the thermostatted column compartment keep the temperature constant.

Agilent offers a number of bio-inert and protein-friendly flow cells to enable reliable analysis of your protein under various conditions. For DAD use, the new Max Light Cartridge Cell LSS will mitigate light intensity, ensuring sample integrity. Learn more about flow cell options at **www.agilent.com/chem/bioflowcells**

Software solutions provide new insights

When working with size exclusion chromatography, there are several software options to support you:

- HPLC software: Agilent OpenLAB CDS ChemStation software helps you to acquire, review, and organize chromatographic data and perform quantitative analysis.
- GPC/SEC software: Available as part of the Agilent GPC/SEC system, providing more information based on molecular weight.
- Buffer Advisor software: Eliminates the tedious and error-prone method-development steps of buffer preparation, buffer blending, and pH scouting by creating salt and pH gradients quickly and easily.

Components of Size Exclusion Characterization

Sample Preparation

Sample preparation for size exclusion chromatography is similar to that for any protein analysis for HPLC methods. The most important aspect is that the sample must be soluble in the eluent and should ideally be dissolved in the mobile phase itself. Because of the larger column dimensions and low linear velocity as a result of relatively slow flow rates compared with other forms of HPLC (see "Column size", below), sample concentrations and injection volumes may need to be larger than normal. To protect the column from possible damage, we recommend that samples be filtered or centrifuged before use to remove particulates. However, filtration should not be used to address poor sample solubility–an alternative eluent may need to be found.

For effective sample preparation, it is also important to ensure that the methods used to dissolve the sample do not change the properties of the sample itself. Some proteins may aggregate (forming dimers and higher molecular weight multimers) or dissociate (forming lower molecular weight sub-units) under stress conditions. These may include freeze-thaw cycles, extremes of temperature, sonication, or even concentration. See the method development guide on page 5 for more information.

Captiva low protein binding filters

Regardless of what sample prep you are performing, it is a good idea to filter your sample with a low protein binding filter.

Agilent PES filters provide superior and consistent low protein binding for protein-related filtration. PES filter membranes are a better option than PVDF membranes for most LC analyses. Agilent PES has similar compatibility to PVDF filters for common LC solvents and is superior in terms of protein binding and cleanliness. Learn more at **www.agilent.com/chem/filtration**

Captiva PES Filters

Diameter (mm)	Pore size (µm)	Certification	Housing	Part Number
4	0.45	LC	Polypropylene	5190-5095
4	0.2	LC	Polypropylene	5190-5094
15	0.2	LC	Polypropylene	5190-5096
15	0.45	LC	Polypropylene	5190-5097
25	0.2	LC	Polypropylene	5190-5098
25	0.45	LC	Polypropylene	5190-5099

Column Selection

Column size

SEC columns are usually much larger than those used for other types of chromatography and are operated at comparatively low flow rates or slow linear velocities. The standard column dimensions for SEC are 7.8 x 300 mm, operated at 1.0 mL/min, compared to a reversed-phase column that is likely to be 2.1 or 4.6 x 150 mm and operated at 2-3 times greater linear velocities. This is not a column size effect but due to the SEC mechanism.

With SEC, there is no increase in concentration of samples typically seen with other chromatography techniques due to absorption or interaction with the stationary phase. Therefore, samples analyzed by SEC are injected in much larger volumes (5-20 µL), often at high concentrations (1-4 mg/mL). Run times are typically 10-12 minutes per column (assuming a conventional 7.8 x 300 mm column operated at 1.0 mL/min) and peaks are usually broad, so high data collection rates are not needed. For comparison or quantitation for protein aggregation, HPLC software is used. To obtain molecular weight distribution information for polydispersed polymers, specific SEC software is used.

Understanding the properties of your chosen column through the use of regular calibration is of paramount importance. By including a sufficiently large molecule–one that is too big to permeate any of the pores–it should be possible to determine the exclusion limit for the column. Similarly, using a very small molecule–one that is small enough to permeate all of the pore structure–it is possible to determine the total permeation limit of the column. You should then ensure that the separation you are trying to achieve occurs between these two limits. If the chromatogram of your sample includes excluded material or material that elutes at the total permeation point, it is an indication that you may need to use a different pore size column for your analysis.

Increasing analytical speed with shorter columns

It is usually necessary to use columns of 300 mm in length to obtain the degree of resolution you need for your analysis. However, to improve the speed of separation, you can consider using shorter column lengths. The separation can be accomplished in half the time by using a column 150 mm in length. However, the resolution will be impaired. Where high throughput is necessary, shorter columns can often be run at higher flow rates without risk of backpressure limits being reached, and so a further reduction in analysis time can be achieved. See Figure 5.

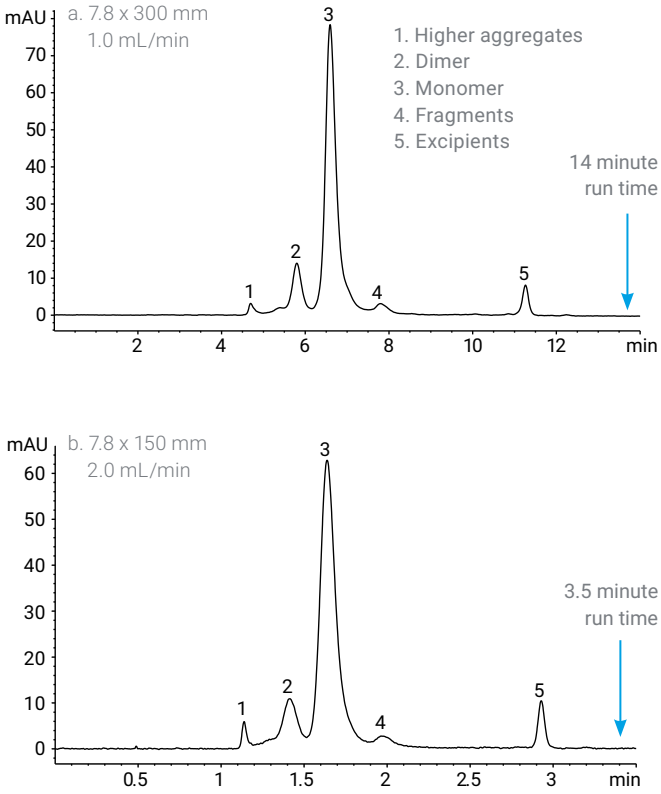

Figure 5. Comparison of Polyclonal IgG analysis using 300 mm vs. 150 mm AdvanceBio SEC columns to demonstrate time savings.

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Column media choice

Choose a size exclusion column suitable for your molecule type and size after determining the solubility of the sample and the mobile phase–water, buffer, or organic solvent–of your separation. Columns packed with polymer-based sorbents are frequently used for polymeric molecules with broad molecular weight distributions, such as heparin, starch, or cellulose. Proteins and molecules that have a discrete molecular weight are best suited to silica-based stationary phases (Table 1).

Agilent Bio SEC columns for biomolecule separations, including protein aggregation, and Agilent GPC columns for natural polymer analysis, including polysaccharide molecular weight determination.It is important to remember that proteins contain numerous amino acids with differing side chain functionalities: acidic, basic, hydrophobic, and neutral/hydrophilic. To prevent interactions occurring with silica columns, buffers are needed in the mobile phase.

Agilent suggests the appropriate molecular weight range for its columns and ideally your column choice should fall in the middle of the operating range.

Agilent Bio SEC columns for biomolecule separations, including protein aggregation, and Agilent GPC columns for natural polymer analysis, including polysaccharide molecular weight determination.

Size Exclusion Chromatography (SEC)

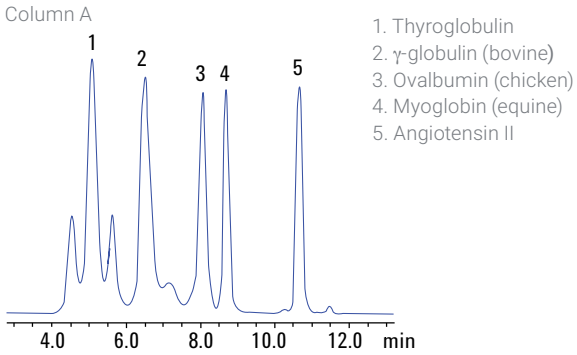
Table 1. Agilent offers a diverse variety of SEC columns to cover all of your size exclusion needs.

Application	Agilent Columns	Notes
Proteins		
SEC-UV/DAD, native SEC-MS, or LS analysis of mAbs, proteins, and peptides.	Agilent AdvanceBio SEC	The latest innovative technology that provides resolution to eliminate sample re-analyses and speed to reduce analysis time, so improving lab productivity. PEEK lined option for native SEC-MS.
Large biomolecules and samples with multiple weight components.	Agilent Bio SEC-5	More pore size options (100 Å, 150 Å, 300 Å, 500 Å, 1000 Å, and 2000 Å) to cover a wider range of analytes.
Globular proteins, antibodies.	ProSEC 300S	Single column option for protein analysis in **high salt** conditions.
Proteins, globular proteins.	ZORBAX GF-250/450	Legacy products that should be employed where protocols still require use of USP designation L35.

Pore size

Proteins are relatively small and compact compared to other biopolymers, and so 300 Å pore size is a good choice for an initial column selection. Figure 6 compares the resolution of a five-protein mix reference standard and a polyclonal IgG sample on columns with different pore sizes and clearly shows the effect of pore size on the resolution.

Agilent AdvanceBio SEC Protein Standards



Conditions

Parameter	Value
Column A:	AdvanceBio SEC 300 Å 4.6 x 300 mm, 2.7 µm (p/n PL1580-5301)
Column B:	AdvanceBio SEC 130 Å 4.6 x 300 mm, 2.7 µm (p/n PL1580-5350)
Sample:	Agilent AdvanceBio SEC Protein Standards (300 Å p/n 5190-9417 and 130 Å p/n 5190-9416)
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM phosphate buffer, pH 7.0
Flow rate:	0.35 mL/min
Detector:	UV, 220 nm

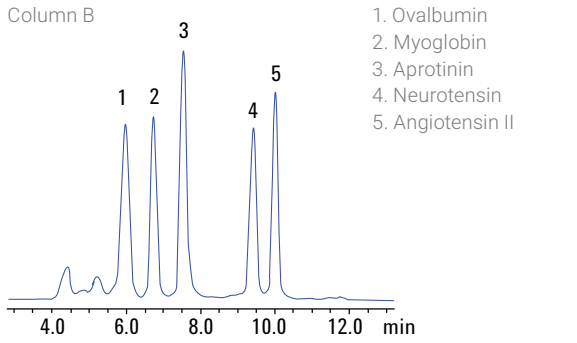
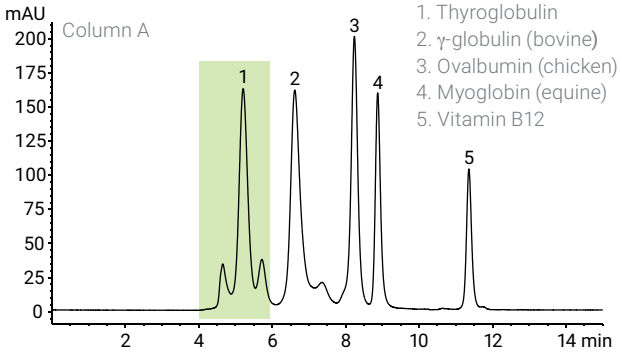


Figure 6: Comparison of pore sizes on the resolution of the Agilent AdvanceBio SEC 300 Å standard. The area highlighted in green shows the difference in resolution between the two pore sizes. The larger pore size is needed for analysis of larger proteins.

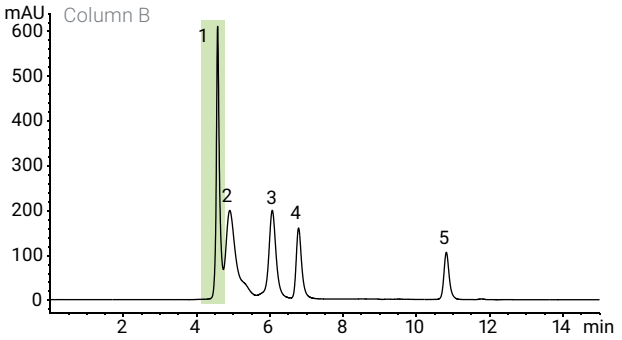
With 300 Å pores, the largest protein thyroglobulin and the IgG dimer are resolved, but as the pore size decreases, the largest proteins are excluded and there is no separation.

BioRad gel filtration standards mix



Conditions

Parameter	Value
Column A:	AdvanceBio SEC 300 Å 4.6 x 300 mm, 2.7 µm (p/n PL1580-5301)
Column B:	AdvanceBio SEC 130 Å 4.6 x 300 mm, 2.7 µm (p/n PL1580-5350)
Sample:	BioRad gel filtration standards mix
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM phosphate buffer, pH 7.0
Flow rate:	0.35 mL/min
Detector:	UV, 220 nm



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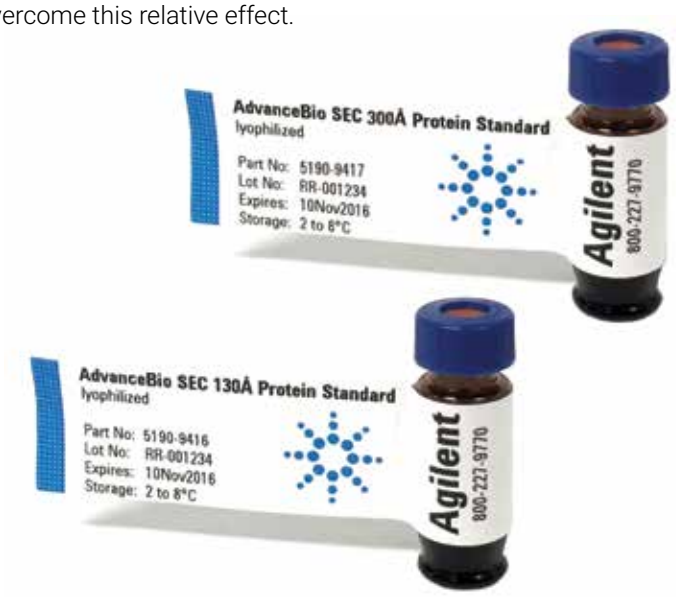
Evaluating SEC permeation ranges

With proteins, it is important to recognize that the SEC mechanism works by separating solutes depending on their size in solution and not their molecular weight. This is evident when comparing the calibration plot of the proteins/peptides with the pullulan/ polysaccharide and PEG/PEO curves, as shown in Figure 7. The pullulan/ polysaccharide and PEG/PEO calibrants provided quite similar calibration curves but the protein/peptide curve is shifted and a different shape. Proteins are composed of complex polypeptide chains that form three-dimensional structures. These structures are affected by the environment to which they are exposed, such as pH or ionic strength. The chains will form the shape that is most suited to them and so their structure and size may vary.

To demonstrate that elution time is due to size rather than molecular weight, consider the retention times of calibrants with a molecular weight of approximately 50,000, in which there is significant difference (Figure 8). The PEG elutes just after 7 minutes, the polysaccharide elutes at just over 7.5 minutes but the protein elutes at approximately 9.5 minutes.

This clearly demonstrates that the SEC separation mechanism is based on the actual size and not molecular weight. Therefore, when using calibration curves it is important to specify what calibrants have been used. For example, it can be stated that the sample of interest has a pullulan/polysaccharide equivalent molecular weight of 50,000. See page 16 for advanced detectors that overcome this relative effect.



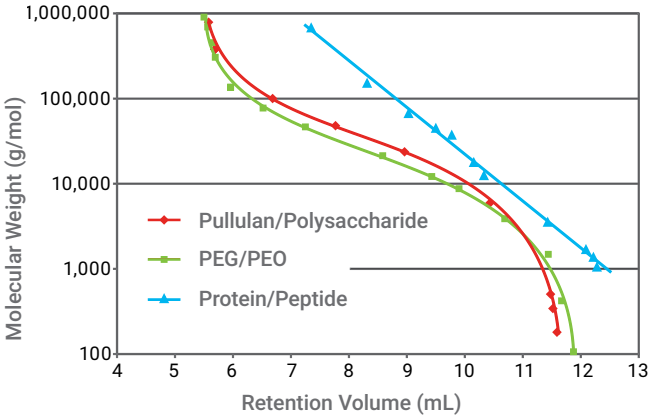


Figure 7: Comparison of calibration plots generated for three types of calibrant.

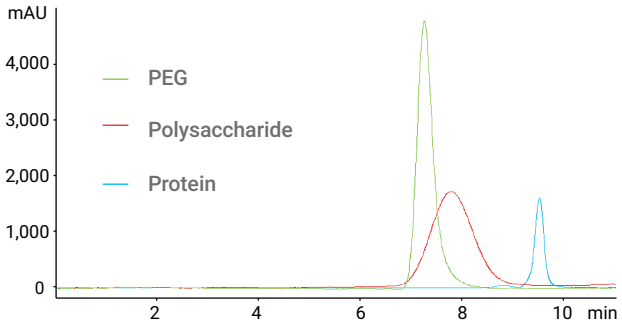


Figure 8: Overlay of chromatograms obtained for calibrants of similar molecular weight.

130 Å AdvanceBio SEC calibration standard (p/n5190-9416 130 Å AdvanceBio SEC calibration standard, 2 mL vial)

A protein mix consisting of 5 carefully selected proteins (Ovalbumin, Myoglobin, Aprotinin, Neurotensin, Angiotensin II – molecular weights ranging from 43 to 1.1 kDa) designed to calibrate Agilent's 130 Å AdvanceBio SEC columns. This standard can be used regularly to calibrate the column and ensure ideal system performance in various applications involving protein purification and analysis.

300 Å AdvanceBio SEC calibration standard (p/n5190-9417 300 Å AdvanceBio SEC calibration standard, 2 mL vial)

A protein mix consisting of 5 carefully selected proteins (Thyroglobulin, γ-Globulin, Ovalbumin, Myoglobin, Angiotensin II) designed to calibrate Agilent's 300 Å AdvanceBio SEC columns. This standard can be used regularly to calibrate the 200 Å and 300 Å AdvanceBio SEC columns and ensure ideal system performance in various applications involving protein purification and analysis.

Particle size

Particle size is also an important consideration in column selection. Smaller particle sizes provide more efficient separation, and therefore higher resolution, but at the risk of degrading (shearing/deforming) larger proteins. Figure 9 shows a comparison between 3 µm and 5 µm particles in Agilent Bio SEC columns. There is a greater risk of higher backpressure and columns becoming blocked if samples and eluents are not prepared carefully. Filtration is recommended to remove insoluble matter and debris. The use of a guard column or in line filter can also extend column lifetime.

Column diameter

Column diameter can also be important depending on the amount of sample. If only limited amounts of material are available, 4.6 mm id columns (operated at 0.35 mL/min) are useful. But it is important to minimize system volumes when using the smaller id columns to prevent excessive dispersion and loss of resolution. SEC is considered to be a non denaturing technique when aqueous eluents are used, and so it is extremely useful for fractionation of complex samples or isolation of a sample component for further analysis. Larger diameter columns, such as 21.2 mm as found in the Agilent SEC-3 and SEC-5 product range, mean that lab prep separations can be performed using analytical HPLC systems.

Comparison between 3 µm and 5 µm SEC particles

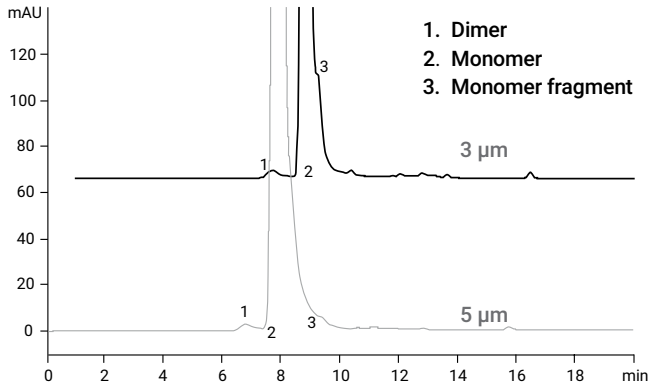


Figure 9: Comparison of Agilent Bio SEC-3 versus Agilent Bio SEC-5 particles. The 3 µm column gives better separation.

Conditions, analysis of monoclonal antibody

Parameter	Value
Column A:	Bio SEC-3, 300 Å 7.8 x 300 mm, 3 µm (p/n5190-2511)
Column B:	Bio SEC-5, 300 Å 7.8 x 300 mm, 5 µm (p/n5190-2526)
Sample:	Humanized monoclonal antibody
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM sodium phosphate, pH 7
Flow rate:	1 mL/min
Detector:	UV, 220 nm

Agilent AdvanceBio SEC columns 7.8 x 300 mm and 4.6 x 300 mm

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Method Parameters

Flow rate

For some applications the speed of analysis is crucial. A shorter column can be used to reduce the analysis time–150 mm versus the conventional 300 mm–or flow rates can be increased or both. However, this could have a detrimental effect on resolution, because SEC relies on diffusion into and out of a pore to create differential path lengths through the column. Nonetheless, as shown in Figure 10, it is possible to obtain sufficient resolution to quantify an IgG dimer and monomer in under 4 minutes when using a 150 mm column at a flow rate of 2 mL/min.

Conditions

Parameter	Value
Column:	AdvanceBio SEC 300 Å, 7.8 x 150 mm, 2.7 µm (p/nPL1180-3301)
Sample:	IgG (2 mg/mL)
Eluent:	150 mM phosphate buffer, pH 7.0
Flow rate::	0.5, 1.0, 1.5 mL/min (52, 102, 152 bar)
Detector:	UV, 220 nm
Injection:	5 µL

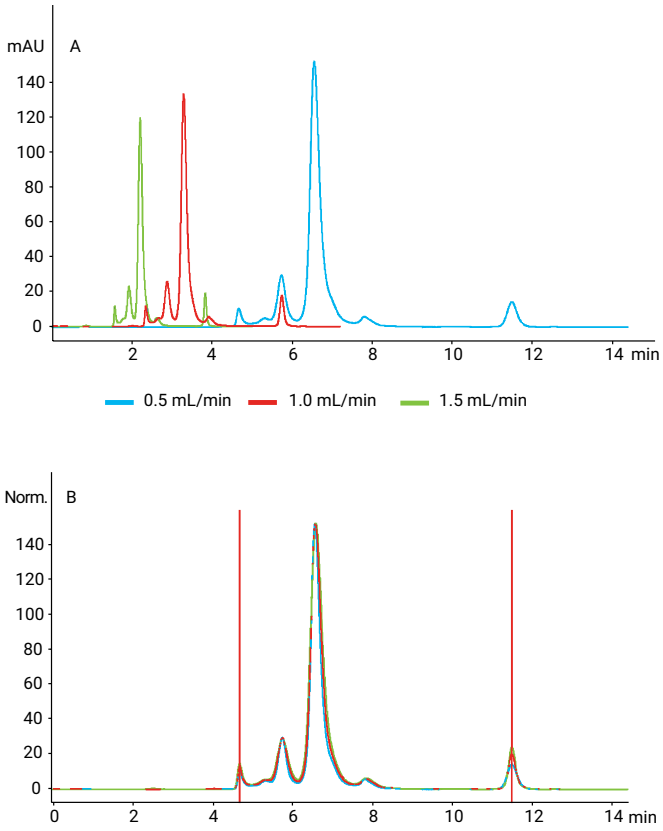

Figure 10: Increasing the flow rate reduces the analysis time from 12 to 4 minutes (A). When the retention times are normalized and overlayed (B) it is evident that the retention times are consistent and that there is minimal reduction in resolution.

Troubleshooting your SEC method

Problem	Source	Solution
Lower than expected recovery, or a broadening of the peaks	Hydrophobic analytes	Add a small amount (10-20 %) of organic modifier (acetonitrile or methanol) to mobile phase
Peaks that appear when they should not, based on molecular weight, or peak tailing	Ionic interactions or basic proteins	Increase the ionic strength–salt concentration at 50-100 mM intervals; add to phosphate buffer
Poor peak shapes	Non specific adsorption	Increase salt concentration or try an Agilent 1260 Infinity Bio-inert Quaternary LC system
Poor retention/resolution of analytes	Insufficient pore size for molecule size	Check your pore size; see pages 6 and 12 for more information

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Mobile phase selection

Secondary interactions can cause difficulties

To overcome undesirable secondary interactions, it may be necessary to perform method optimization. Such interactions may lead to an analyte eluting later than expected and could give the appearance of a lower molecular weight. Slight adjustments in the mobile phase composition–pH, ionic strength, or organic modifiers–can help to overcome such difficulties (Figure 11). It may also be necessary to refine the choice of pore size, combine columns in series, reduce analysis flow rate, or change temperature to achieve the desired separation.

Conditions

Parameter	Value
Column:	Agilent Bio SEC-3 300 Å 4.6 mm x 300 mm, 3 µm (p/n5190-2513)
Sample:	Protein (1 mg/mL 20 mM phosphate buffer, pH 7)
Instrument:	150 mM phosphate buffer, pH 7.0
Flow rate:	0.35 mL/min
Detector:	UV, 220 nm A: Eluent 20 mM phosphate buffer, pH 7 + 50 mM NaCl B: Eluent 20 mM phosphate buffer, pH 7 + 100 mM NaCl C: Eluent 20 mM phosphate buffer, pH 7 + 400 mM NaCl
Injection:	5 µL

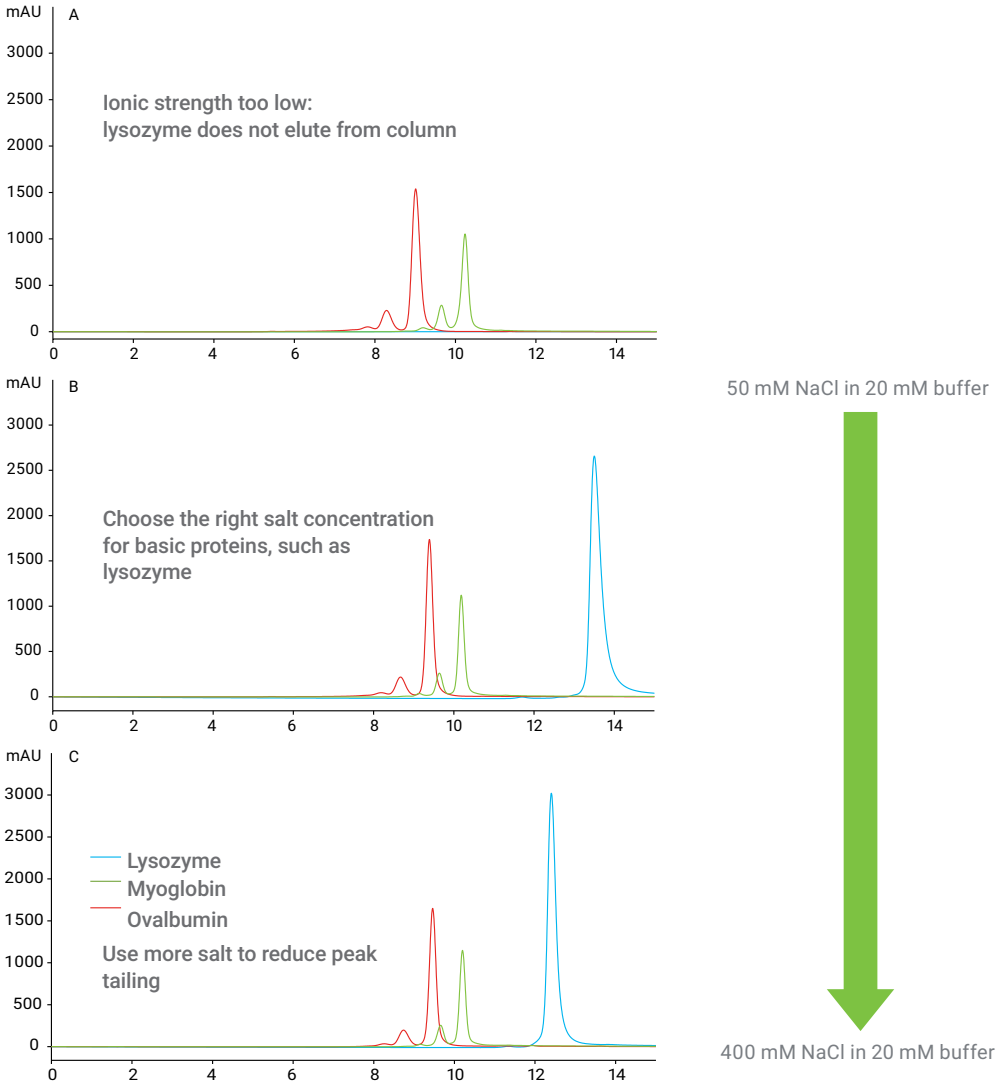

Figure 11: Effect of too much or too little ionic strength on achieving your desired separation.

Calibration

Once you have chosen a column, it will be necessary to construct a calibration with standards of a known molecular weight. Each time you change your column or make changes to the mobile phase, you will need to repeat the calibration. The calibration curve is obtained by plotting retention time against molecular weight (Figure 12). It is particularly important to choose standards appropriate to the molecule of interest. For a protein separation, use protein molecular weight standards. Pullulan molecular weight standards should be used for a polysaccharide separation.

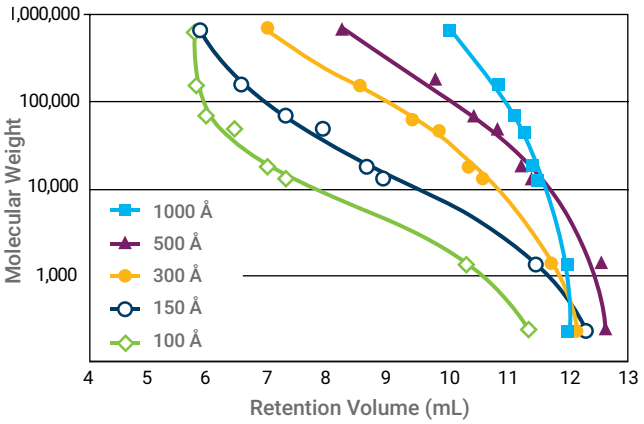

Figure 12: Calibration curves obtained by plotting retention time against molecular weight.

		Retention Volume				
Proteins	**MW**	**1000 Å**	**500 Å**	**300 Å**	**150 Å**	**100 Å**
Thyroglobulin	670,000	10.07	8.23	7.03	5.82	5.77
γ-globulin	158,000	10.88	9.80	8.57	6.55	5.79
BSA	67,000	11.13	10.44	9.44	7.29	6.00
Ovalbumin	45,000	11.28	10.83	9.89	7.90	6.40
Myoglobin	17,000	11.44	11.28	10.42	8.66	7.05
Ribonuclease A	12,700	11.52	11.41	10.58	8.93	7.32
Vitamin B12	1,350	12.00	12.59	11.78	11.49	10.30
Uracil	112	12.08	12.68	12.21	12.13	11.41

Conditions

Parameter	Value
Column:	Agilent Bio SEC-5 7.8 x 300 mm, 5 µm (p/n5190-2521)
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM Na phosphate, pH 7.0
Flow rate:	1.0 mL/min
Detector:	UV

Ideally, the standards should be dissolved in the mobile phase and care should be taken to ensure that the sample has dissolved fully. If the solution appears cloudy, it will be necessary to take further action. Centrifugation or filtration should be used to remove insoluble matter before injection. However, it may be necessary to look at alternative mobile phase conditions that will improve sample solubility since physical processes could be altering the molecular weight composition.

Advanced detection techniques

Further SEC considerations include choice of detector. UV or diode array (DAD) is commonly used for protein separations. Best results, i.e. highest sensitivity, for peptides and proteins will normally be obtained at 220 nm. Although some buffer solutions or organic modifiers may have too much background absorbance at low wavelengths, in which case 254 nm or 280 nm may be necessary. A drawback of UV detection is that some molecules do not possess a chromophore, but since analytes are eluted isocratically, it is possible to use an RI detector instead. The addition of advanced light scattering detection significantly increases the performance of SEC. Static light scattering determines accurate molar masses, independent of column calibrations and unwanted interactions and is complemented with dynamic light scattering to study the molecular size. Light scattering has increased sensitivity to large moieties allowing for discovery of aggregation at much lower quantities (Figure 13). It is important to select a detector with low dead volume to ensure this extra information is obtained without sacrificing chromatographic performance.

In special cases, mass spectrometry (MS) may be used with SEC, either in native mode (standard aqueous buffer mobile phase) or denaturing (organic solvent included in the mobile phase). In the case of MS, smaller i.d. columns are often preferable due to the low flow rates used for native mode MS. In some cases there is benefit to PEEK-lined columns to eliminate undesirable sample-metal interactions. AdvanceBio SEC 1.9 µm is available in 2.1 mm i.d. and PEEK-lined hardware for these circumstances.

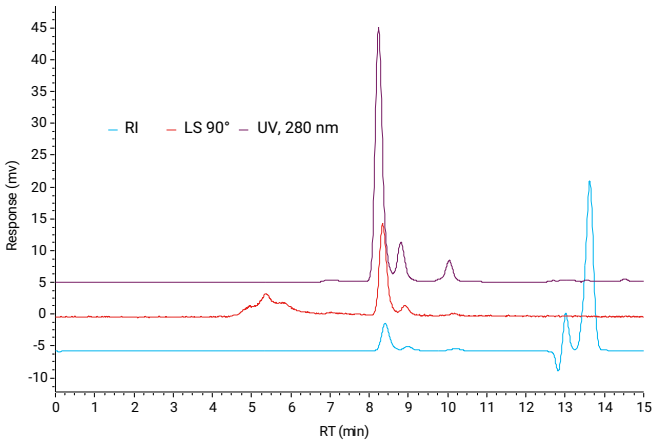

Figure 13: Results of using different detectors on a protein separation.

Conditions

Parameter	Value
Column:	Agilent AdvanceBio 300 Å, 7.8 x 300 mm, 2.7 µm
Sample:	Degraded monoclonal antibody
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System with Agilent 1260 Infinity Multi-Detector GPC/SEC
Mobile phase:	150 mM sodium phosphate, pH 7.0
Flow rate:	0.8 mL/min
Temp.:	30 °C
Detector:	UV, 280 nm + RI + LS 90°
Injection:	5 µL

Conjugated proteins

Therapeutic proteins are subjected to aggregation and degradation during all stages of development, such as expression, refolding, downstream processing, formulation, sterilization, and storage. Although aggregates/degradents are present in extremely low concentrations, they may have a big impact on the quality of biologics, leading to activity loss, decreased solubility, and increased immunogenicity. Size exclusion chromatography is the standard method used to characterize protein aggregation and is also required for regulatory submission and approval.

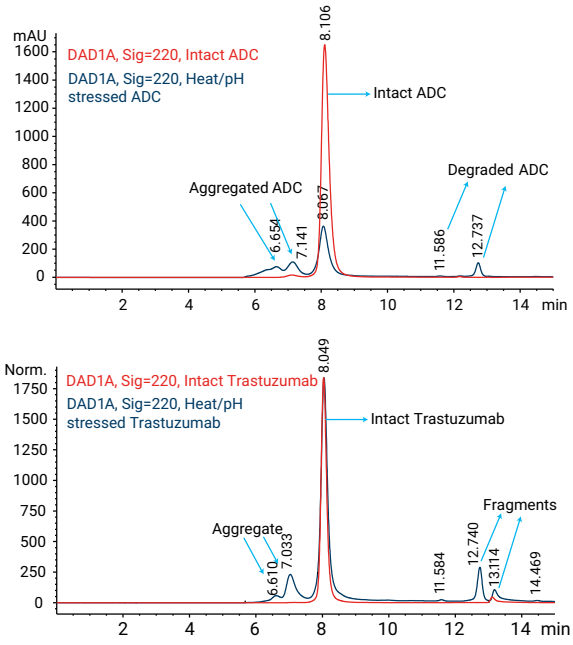

Figure 14: The same aqueous mobile phase was used for the mAb and the more hydrophobic ADC.

To improve the delivery, increase half life, and increase potency, proteins, including monoclonal antibodies, can be conjugated. Water-soluble polymers, such as polyethylene glycol, are conjugated with the protein to enhance pharmacological activities, increase their half life in the blood stream, and reduce immunogenicity. More recently, there has been interest in antibody drug conjugates, ADCs, where monoclonal antibodies are conjugated to a cytotoxic agent for targeted drug delivery and increased efficacy of treatment. After conjugation, the same aggregation studies are required because the change in sample characteristics can present a greater challenge to achieving an SEC separation. Columns with very low non specific binding, such as AdvanceBio SEC, are required for the analysis of both the antibody and the ADC using aqueous mobile phases. See Figure 14.

Conditions

Parameter	Value
Column:	AdvanceBio SEC 300 Å 7.8 x 300 mm, 2.7 µm
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	PBS, 50 mM sodium phosphate containing 150 mM sodium chloride, pH 7.4
TCC Temp.:	Ambient
Injection:	10 µL
Flow rate::	0.8 mL/min
Detector:	UV, 220 nm

Sample preparation

- Ideally, samples should be dissolved in the mobile phase
- If the sample is cloudy, it may be necessary to change the mobile phase conditions
- Filtration or centrifugation can be used to clarify samples, but these processes could alter the molecular weight composition of the sample
- To dissolve a sample, gentle heating, vortexing, or sonication is sometimes used, but should be applied with caution because this can alter the molecular weight composition
- Care should also be taken to ensure the sample does not change during storage
- Samples should be made up fresh and analyzed as soon as possible
- Bacterial growth can develop quickly in buffer solutions
- Samples made up at high concentration can also change over time, leading to aggregation or even precipitation

Column selection

- To ensure sample integrity, SEC is carried out slowly on long columns
- Column lengths are typically 150 or 300 mm
- Normal flow rate is 1.0 mL/min on a 7.5 or 7.8 mm i.d. column, 0.35 mL/min on a 4.6 mm i.d. column, and 0.1 mL/min on a 2.1 mm i.d. column.
- Columns are often run in series to increase resolution in biopolymer applications
- Smaller particle sizes are used to increase resolution in protein applications
- Separations done on 150 mm columns with smaller particle sizes can reduce analytical time

Column media choice

- There should be no non specific interactions between analytes and column media
- Silica-based sorbents are used for analyzing peptides and proteins
- Polymer-based sorbents are for analyzing biopolymers

Column parameters

- **Pore size**–depends on the molecular weight range of the sample to avoid exclusion of sample components and maximize volume in the required separation region
- **Particle size**–use smaller particles for higher resolution (but higher backpressure)
- **Column length**–compromise between resolution and analysis time
- **Column i.d.**–use smaller columns for reduced solvent consumption and smaller injection volume

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Mobile phase

- Mobile phase should contain buffer/salt to overcome ionic interactions, but too much may cause hydrophobic interactions
- Do not alter the analyte to avoid degradation/ aggregation, etc
- Make up fresh mobile phase and use promptly, as bacterial growth is rapid in dilute buffer stored at room temperature
- Buffer shelf life is less than 7 days unless refrigerated
- Filter before use to remove particulates in water (less likely) or in buffer salts (more likely)
- High pH phosphate buffers (particularly at elevated temperature) can significantly reduce column lifetime when using silica columns

Learn more about Agilent biocolumns for SEC at

www.agilent.com/chem/advancebio

Application Note

Aggregate/Fragment Analysis

Agilent

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Elevate Your mAb Aggregate Analysis

High-resolution SEC with the Agilent 1290 Infinity II Bio LC SystemBio LC System

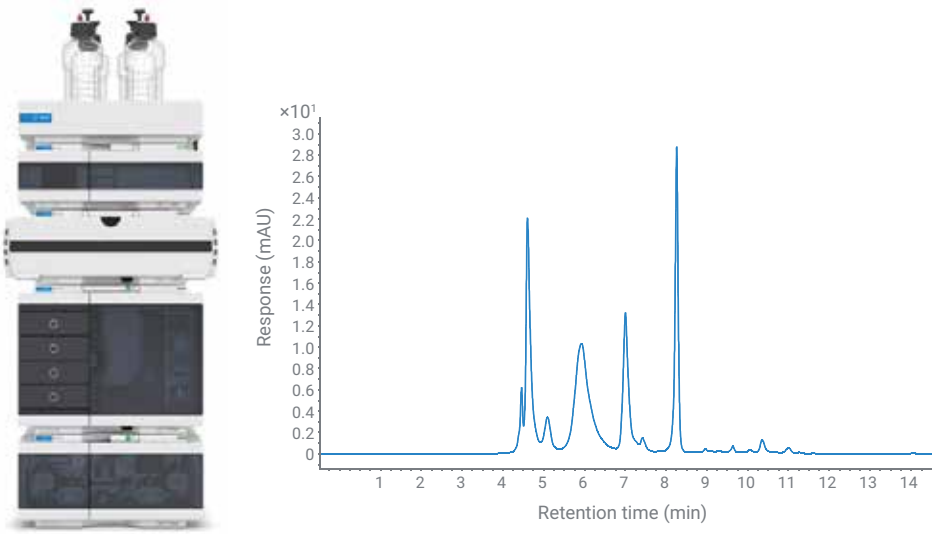
Author

Edgar Nägele

Agilent Technologies, Inc.

Abstract

This application note demonstrates the superior resolution in size exclusion chromatography (SEC) protein separation made possible by the Agilent 1290 Infinity II Bio LC System equipped with the Agilent AdvanceBio SEC column and ultralow dispersion capillaries. The biocompatible UHPLC system enables analysis using corrosive salty buffers and therefore saves maintenance expense. The resolution in SEC analysis was compared for capillaries of different inner diameter (0.17, 0.12, and 0.07 mm). A protein standard mixture and monoclonal antibodies (mAbs), including aggregates, were separated and compared for resolution. In addition, molecular weight was determined by the Agilent OpenLab GPC/SEC add-on software in one software solution, enabling a one-step workflow.



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Introduction

Modern biopharmaceuticals, such as mAbs, are highly heterogeneous compounds. Aggregation monitoring, one of the most important critical quality attributes (CQAs), is typically executed by SEC. With this technique, the identity of the compound can be determined by the calculation of the molecular weight after a standard column calibration. In addition, it confirms the purity by showing the presence of unwanted higher molecular weight compounds such as dimeric and higher aggregates. To achieve the necessary resolution, modern SEC columns with sub-2 µm particle material are recommended. To enable optimal performance, a combination of sub-2 µm columns and a UHPLC instrument with dead volumes as low as possible is preferred. Large dead volumes destroy the resolution obtained by these columns due to dispersion effects. In addition, the completely biocompatible 1290 Infinity II Bio LC perfectly copes with the high salt concentrations often found in SEC buffers, providing confident results at the lowest maintenance cost.

This application note demonstrates the use of modern sub-2 µm SEC columns on the 1290 Infinity II Bio LC and illustrates the benefit of using instruments with the lowest possible dead volumes. To demonstrate the effect of dead volume on the separation of proteins and aggregates, capillaries with different inner diameters were used. The well-characterized NISTmAb will be used to generate more aggregates by pH and thermal stress with subsequent separation of dimers, trimers, and higher aggregates.

Experimental

Instrument

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) including integrated Sample Thermostat (#101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with biocompatible Heat Exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2 µL

Additional parts

Agilent 1290 Infinity II Bio Ultra Low Dispersion Kit (G7132A#006)

Software

Agilent OpenLab Version 2.5 and GPC/SEC add-on software V. 1.2

LC Method

Parameter	Valve
Solvent	Phosphate-buffered saline (PBS), pH 7.4
Flow rate	0.35 mL/min
Isocratic separation	
Column temperature	30 °C
Sample temperature	4 °C
Needle wash	3 s water
Injection volume	5 µL
Detection (VWD)	280 nm, data rate 20 Hz

Column

Agilent AdvanceBio SEC, 200 Å, 4.6 × 300 mm, 1.9 µm (part number PL1580-5201)

Samples

- Protein mix for calibration (part number 5190-9417): thyroglobulin (670,000 Da), γ-globulin (150,000 Da), ovalbumin (45,000 Da), myoglobin (17,000 Da), angiotensin II (1,000 Da)
- Humanized monoclonal antibody (mAb) trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland). The trastuzumab was dissolved in 30 mM phosphate buffer, pH 6.8.
- Agilent NISTmAb, humanized IgG1κ mAb (part number 5191-5745)(Steinheim, Germany).

Protocol for pH/temperature-stressed NISTmAb

The mAb was diluted in the mobile phase to a final concentration of 2 mg/mL. pH stress was carried out as described elsewhere with slight modification:1 1 M HCl was slowly added dropwise to the sample solutions to change the pH from 6.0 to 1.0. Then, 1 M NaOH was added to adjust the pH to 10.0. Finally, 1 M HCl was added again to adjust the pH back to 6.0. There was approximately 1 minute waiting time between the pH shifts, with constant, slight stirring. The resulting solution was incubated at 60 °C for 60 minutes.

Solvents and chemicals

- **PBS:** One tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.4, at 25 °C.
- Chemicals were purchased from VWR, Germany.
- Fresh ultrapure water was obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 µm membrane point-of-use cartridge (Millipak).

Results and Discussion

Modern columns for SEC separation of proteins comprise material with sub-2 µm particles for optimum resolution. However, this requires instruments that have optimized low dead volume, because especially capillaries of larger inner diameters can destroy the achieved resolution. The separation of a mixture of five proteins, including three dimers, with the 1290 Infinity II Bio LC is shown in Figure 1A. To minimize dead volume and dispersion effects, capillaries with an inner diameter of 0.07 mm were used for the separation. Even the early-eluting dimer of thyroglobulin (4.947 min) was partially separated. To set up a calibration for molecular weight determination, all the proteins in this mixture were used to generate the calibration curve (Figure 1B). The best curve fit was obtained for a fourth order.

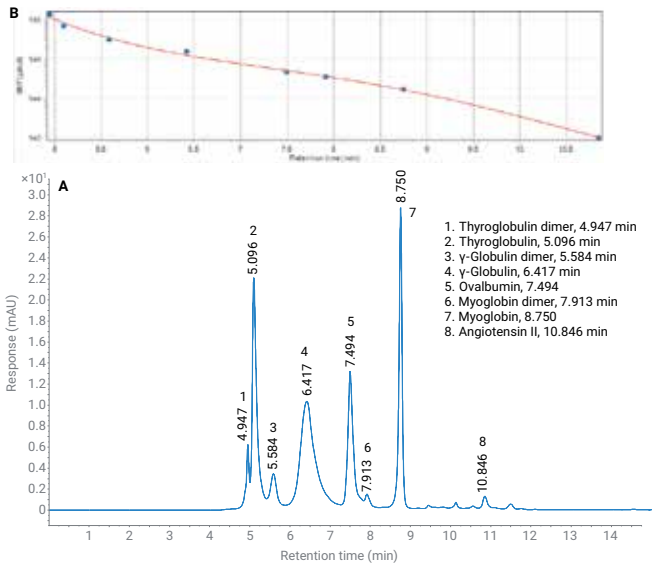
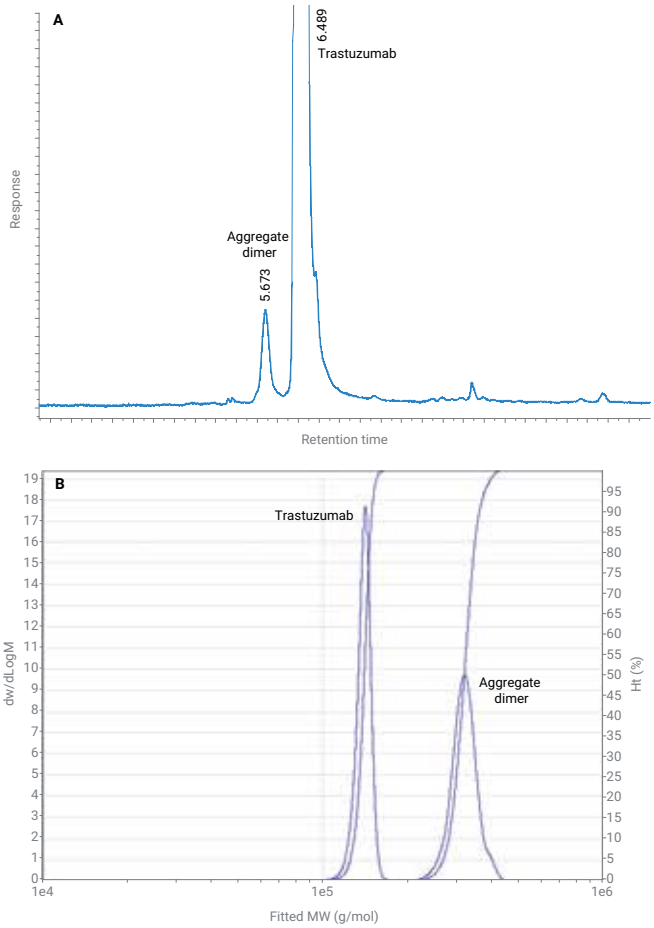


Figure 1. (A) Separation of a five-protein mixture including three dimers on the Agilent AdvanceBio SEC, 200 Å, 4.6 × 300 mm, 1.9 µm in combination with the Agilent 1290 Infinity II Bio Ultra Low Dispersion Kit comprising 0.07 mm capillaries. (B) Calibration curve of SEC for molecular weight determination with a protein mixture featuring some dimers.

The calibration was used to determine the molecular weight of the mAb trastuzumab and a comprised dimeric aggregate (Figure 2). The antibody elutes at 6.489 minutes and the corresponding dimeric aggregate elutes at 5.673 minutes (Figure 2A). The determined molecular weight at the peak maximum of trastuzumab and the dimer were Mp 141,566 Da and Mp 321,609 Da, respectively. The molecular weight distribution is shown in Figure 2B and the calculated molecular weights are outlined in the included table (2C).



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Compound	RT (min)	Mp (g/mol)
Trastuzumab Aggregate	5.673	321609
Trastuzumab	6.489	141566
D4	0.072	0.071
D6	0.065	0.262
D8	0.066	0.281

Figure 2. Trastuzumab and dimeric aggregate, determination of molecular weights. (A) SEC separation of the monomer of trastuzumab from a dimeric aggregate. (B) The molecular weight of trastuzumab and the dimeric aggregate. (C) Table with molecular weights of trastuzumab and its aggregate. Mp: molecular weight at peak maximum.

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The influence of the inner diameter of the used capillaries could be shown in a comparison of capillaries with an inner diameter of 0.07 (ULD), 0.12 and 0.17 mm. To demonstrate the effect, the resolution of the second and third peak of the protein mixture (shown in Figure 1) was determined (Figure 3). The best values of resolution for peak 2 and peak 3 could be obtained by means of capillaries with 0.07 mm inner diameter (table in Figure 3). For the determination of the peak width at half-height, peak 2 of the protein mixture was used. From the measured values, it could be seen that the peak width increases when using capillaries of larger inner diameters.

The influence of the capillaries on the separation of trastuzumab and its aggregate is shown in Figure 4. Here, it can be seen that an additional lower molecular weight compound was hidden under the main peak, which is only separated as a slight shoulder with the 0.17 mm capillaries and is more clearly visible using the 0.07 mm capillaries.

	Resolution		Width (min)
Capillaries (µm)	Peak 2	Peak 3	Peak 2
0.07	0.94	1.98	0.110
0.12	0.86	1.88	0.120
0.17	0.71	1.73	0.142

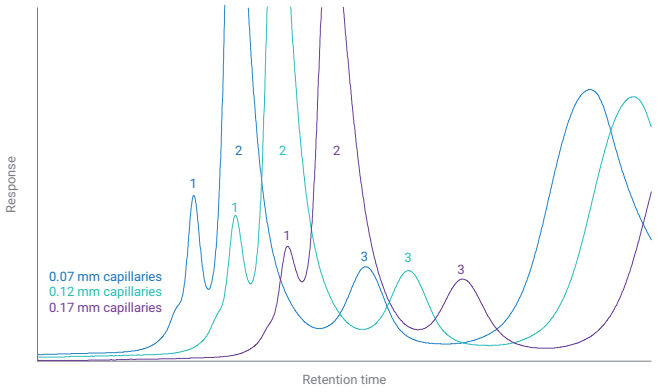

Figure 3. Comparison of capillaries with increasing inner diameters and their influence on resolution and peak width.

Capillaries (µm)	Resolution	Width (min) Herceptin	Width (min) Aggregate
0.07	3.34	0.096	0.189
0.12	3.05	0.107	0.205
0.17	2.83	0.121	0.216

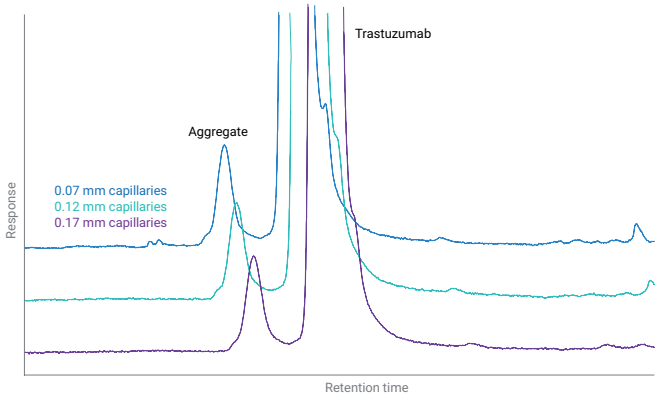

Figure 4. Resolution of trastuzumab from its dimeric aggregate and peak width depending on the inner diameter of the used capillaries.

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The RSD values of retention time and peak area are excellent for all capillaries (Table 1).

As another example, the well-characterized NISTmAb (humanized IgG1κ mAb) was used for separation from aggregates and determination of molecular weights (Figure 5).

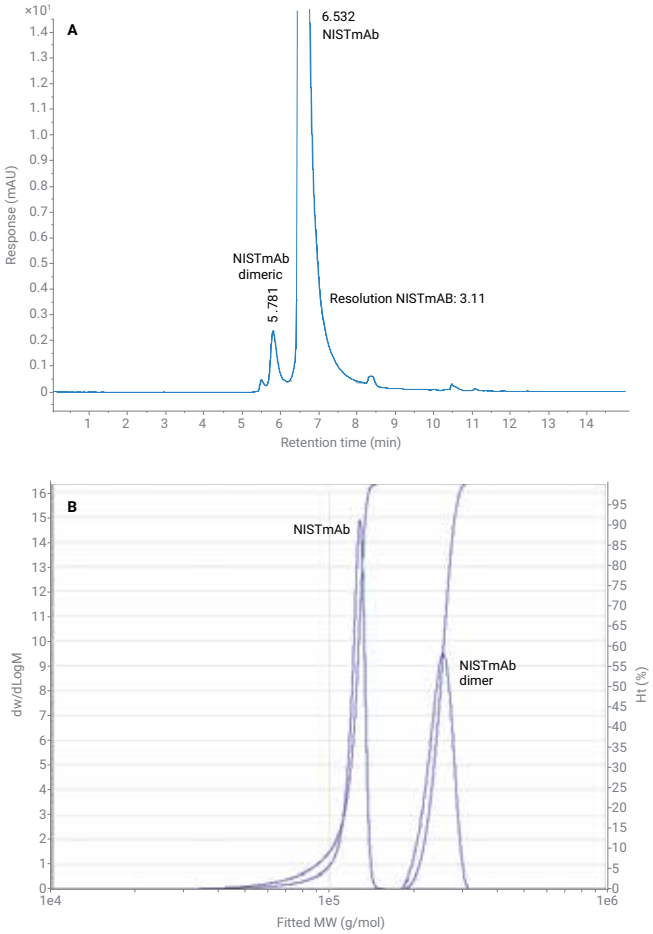

Figure 5. (A) Separation of NISTmAb from its main dimeric aggregate with resolution 3.11. (B) Molecular weights in the NISTmAb and its aggregate. (C) Table showing the values of major peak characterization from NISTmAb and its aggregate. (D) Tables showing the molecular mass of NISTmAb and its aggregate.

Table 1. Retention time and peak area RSDs of trastuzumab for all used capillaries. The increase in retention times is due to the increasing volumes of the different sets of capillaries.

	0.07 mm Capillaries		0.12 mm Capillaries		0.17 mm Capillaries	
	RT	Area	RT	Area	RT	Area
Average	6.464	1736.13	6.500	1727.05	6.554	1717.29
RSD (%)	0.02	0.10	0.01	0.28	0.01	0.25

C

RT (min)	Area	Height	Resol. USP	Tailing	Width 50%
5.781	23.80	2.08	7.59	1.940	1.940
6.532	3006.00	409.40	3.11	1.569	0.100

D

RT (min)	Mp (g/mol)
5.781	305626
6.532	144767

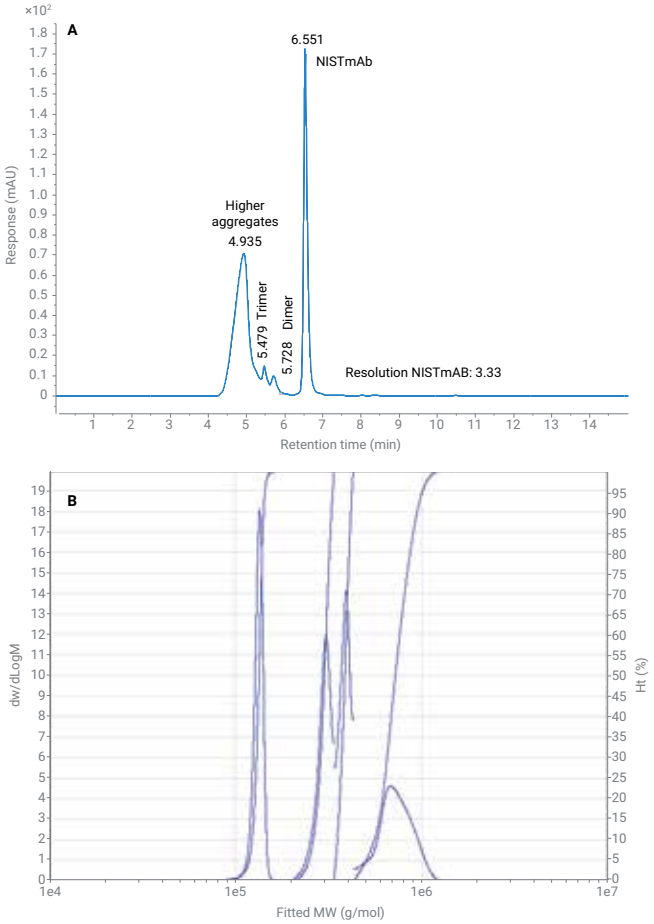

Figure 6. (A) Separation of aggregates from a pH-stressed NISTmAb. (B) Distribution of molecular weight of aggregates occurring under stress conditions from NISTmAb. (C) Table of values of major peak characterization of NISTmAb and its aggregates. (D) Tables showing the molecular mass of NISTmAb and its aggregates.

Under pH- and temperature-stress conditions (see Experimental section), this mAb can form higher aggregates (Figure 6). With the ultralow dispersion capillaries, the higher aggregates could be separated (Figure 6A). Their molecular weight distribution and values for peak characterization are outlined in Figure 6B and the associated tables.

C

RT (min)	Area	Height	Resol. USP	Tailing	Width 50%
4.913	1926.46	70.80	7.59	0.781	0.398
5.479	129.63	14.58	0.85	1.277	0.168
5.728	106.16	9.5	0.91	1.985	0.185
6.551	1208.07	172.21	3.33	1.116	0.110

D

RT (min)	Mp (g/mol)
4.913	706827
5.479	411615
5.728	321609
6.551	141918

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Conclusion

This application note demonstrates the capability of the 1290 Infinity II Bio LC together with the AdvanceBio SEC column to separate proteins and their aggregates with the highest resolution due to minimized system dead volume and ultralow dispersion capillaries. The 1290 Infinity II Bio LC is a completely biocompatible system capable of operating with highly salted buffers. This offers the lowest maintenance costs at the highest resolution performance.

References

1. Quantitation of mAb and ADC Aggregation Using SEC and an Aqueous Mobile Phase. *Agilent Technologies application note*, publication number 5991-6303EN, **2016**.

Published in the USA, October 22, 2020
5994-2709EN

Application Note

Aggregate/Fragment Analysis

Agilent

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High-Resolution, High-Throughput Size Exclusion Chromatography Analysis of Monoclonal Antibodies

Using an Agilent AdvanceBio SEC 200 Å 1.9 µm column

Author

Veronica Qin

Agilent Technologies, Inc.

Abstract

This Application Note demonstrates the use of the Agilent AdvanceBio SEC 200 Å 1.9 µm column for high-resolution and high-throughput size exclusion chromatography (SEC) analysis of a monoclonal antibody (mAb). The optimized sub-2 µm particle enables faster separations and uncompromised high resolution for accurate quantitation.

Introduction

Aggregates and fragments are critical quality attributes of biotherapeutic proteins that need to be well characterized. Size exclusion chromatography (SEC) is commonly used to analyze these size variants. There are cases where high-throughput SEC analysis is in high demand. Examples are in the early stage of drug development during clone selection, or process development, where large numbers of samples need to be analyzed daily. The AdvanceBio SEC 200 Å 1.9 µm column, with its unique, durable sub-2 µm particles, offers fast analysis with high resolution. These features significantly improve sample throughput, while delivering robust and accurate results.

Experimental

Materials

SILu Lite SigmaMAb universal antibody standard was purchased from MilliporeSigma and reconstituted with water to 1 mg/mL. Monobasic and dibasic sodium hydrogen phosphate and sodium chloride were purchased from MilliporeSigma. All chemicals used were ≥99.5 % pure. Water was purified from a Milli-Q A10 water purification system (Millipore). Solutions were prepared fresh daily, and filtered through a 0.22 µm membrane filter prior to use.

Instrumentation

An Agilent 1260 Infinity LC with the following configuration was used:

- Agilent 1260 Infinity II bio-inert quaternary pump (G5654A)
- Agilent 1260 Infinity II bio-inert multisampler (G5668A) with sample cooler (option #100)
- Agilent 1260 Infinity II multicolumn thermostat (G7116A) with bio-inert heat exchanger (option #019)
- Agilent 1260 Infinity II variable wavelength detector (G7114A)

Columns

- Agilent AdvanceBio SEC 200 Å 1.9 µm, 4.6 × 300 mm (p/n PL1580-5201)
- Agilent AdvanceBio SEC 200 Å 1.9 µm, 4.6 × 150 mm (p/n PL1580-3201)bio-inert flow cell (option #028)

Software

Agilent OpenLab 2.2 CDS.

Results and discussion

Materials

Figure 1 shows SEC chromatograms of mAb with aggregates and fragments using 300 mm columns with flow rates at 0.35, 0.4, and 0.5 mL/min. Excellent resolution of both dimer/monomer and monomer/fragment1 was achieved even at 0.5 mL/min (Table 1) by saving 28 % of run time versus 0.35 mL/min flow rate without compromising resolution.

Instrument conditions

Parameter	HPLC conditions
Column temperature	25 °C
Mobile phase	50 mM sodium phosphate, 200 mM NaCl, pH 7.0
Flow rate	0.3 to 0.7 mL/min
Injection volume	1 µL
Direction	UV at 220 nm

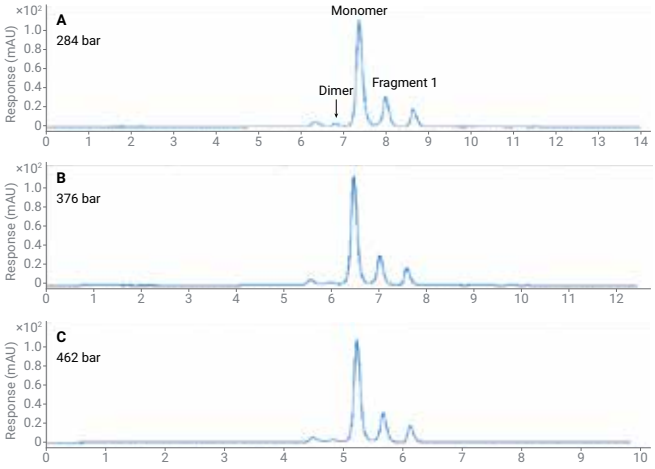

Figure 1. Size exclusion chromatograms of SigmaMAb (mixed with its F(ab')2 and Fc fragments) using 4.6 × 300 mm SEC columns running with 50 mM sodium phosphate, 200 mM NaCl, pH 7.0 at A) 0.35 mL/min; B) 0.4 mL/min; C) 0.5 mL/min.

Aggregates and fragments are critical quality attributes of biotherapeutic proteins that need to be well characterized. Size exclusion chromatography (SEC) is commonly used to analyze these size variants. There are cases where high-throughput SEC analysis is in high demand. Examples are in the early stage of drug development during clone selection, or process development, where large numbers of samples need to be analyzed daily. The AdvanceBio SEC 200 Å 1.9 µm column, with its unique, durable sub-2 µm particles, offers fast analysis with high resolution. These features significantly improve sample throughput, while delivering robust and accurate results.

Table 1. Peak tailing factor and resolution under different flow rates.

Flow Rate (mL/min)	Tailing Factor (Monomer)	Resolution (Dimer/Monomer)	Resolution (Monomer/Fragment 1)
0.35	1.18	1.98	2.37
0.4	1.16	1.96	2.36
0.5	1.14	1.91	2.29

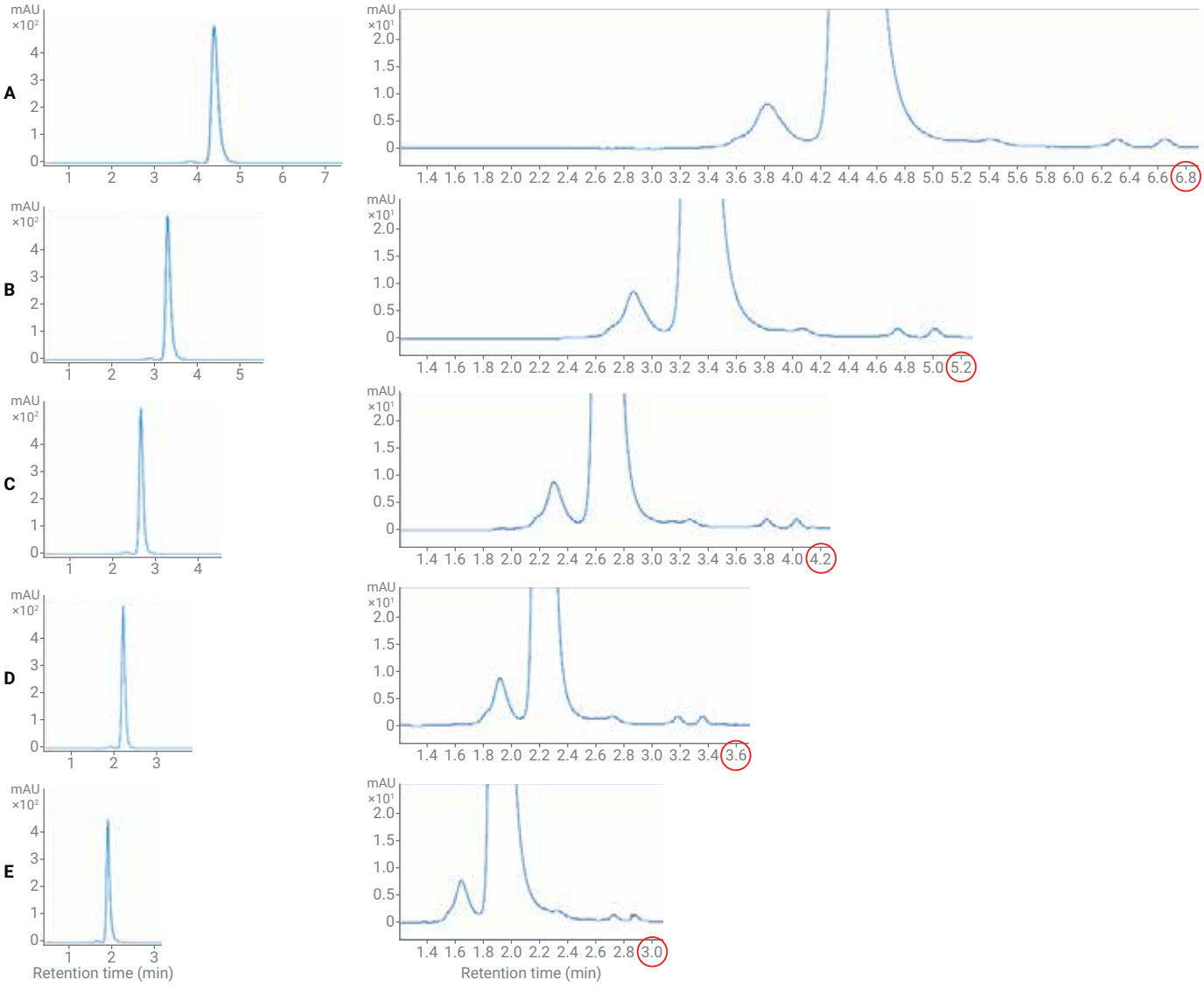

Figure 1. Size exclusion chromatograms of SigmaMAb using 4.6 × 150 mm SEC columns running with 50 mM sodium phosphate, 200 mM NaCl, pH 7.0 at A) 0.3 mL/min; B) 0.4 mL/min; C) 0.5 mL/min; D) 0.6 mL/min; E) 0.7 mL/min.

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The unique particles in this column enable excellent stability at much higher flow rates with high resolution of dimer/monomer for accurate quantitation of the dimer peak area (Table 2).

Table 2 calculates the effect of flow rate on sample throughput. When increasing the flow rate from 0.3 to 0.7 mL/min, 480 samples can be analyzed per day, which is a 2.3-fold improvement in throughput. Compared to running the 300 mm column at 0.3 mL/min, which can only analyze 105 samples per day, the throughput increases 4.6-fold..

Conclusion

This study demonstrates the ability of the AdvanceBio SEC 200 Å 1.9 µm column to be used for fast analysis of mAb aggregates. The durable particles enable running at a higher flow rate without loss of high resolution. By reducing column length from 300 to 150 mm, and by increasing flow rate from 0.3 to 0.7 mL/min, we can enhance sample throughput 4.6-fold.

Table 2. Effect of flow rate on resolution, monomer area percentage, and sample throughput.

RT	Run time (min)	Backpressure (bar)	Resolution	Dimer Area %	Samples per hour	Samples per day 24-hours
0.3	6.8	164	1.81	2.33	8-9	211
0.4	5.2	218	1.79	2.35	11-12	276
0.5	4.2	272	1.78	2.35	14	342
0.6	3.6	324	1.77	2.39	16-17	400
0.73	3.0	380	1.58	2.30	20	480

Published in the USA, March 27, 2020
5994-0828EN

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Application Note

Aggregate/Fragment Analysis

Agilent

Trusted Answers

Sensitive Native Mass Spectrometry of Macromolecules Using Standard Flow LC/MS

Authors

David L. Wong

Agilent Technologies, Inc.

Abstract

Native mass spectrometry can be used for a variety of protein-based applications, such as protein-protein interaction, protein-ligand binding, protein complex structures, protein folding and antibody-drug conjugates. Most of the native MS analyses are using a nano-electrospray approach which faces significant challenges.

This application note describes a robust and sensitive LC/MS method using standard LC flow for the analysis of native protein analysis. The workflow comprised the Agilent 1290 Infinity II LC, the AdvanceBio SEC column, the 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software.

Introduction

Native mass spectrometry (MS) has emerged as a widely used technique for the characterization of intact proteins and noncovalent protein complexes. Various sizes of protein complex structures (protein-ligand binding or protein-protein interaction) ranging from a few kDa to more than 1 MDa have successfully been analyzed and studied by this technique despite its tremendous analytical challenges.1-4 Without organic solvent and acid to enhance sample desolvation and ionization, native MS analysis of protein samples at neutral pH conditions tends to have fewer charges per molecule and much lower abundance MS signals at higher m/z ranges. In the past decade, the nano-electrospray ionization (nESI) approach has become a crucial method used in native protein analysis. The nESI forms fine charged droplets, which can dramatically increase the sample desolvation and ionization efficiency while preserving the noncovalent protein-protein complexes. However, it has commonly been observed that the neutral aqueous protein samples tend to aggregate easily under the unstable nanoflow rate condition and cause the nanospray emitter to clog. Also, well trained or experienced researchers are needed to produce good-quality MS data using the nESI technique.

In this study, we demonstrate a highly sensitive analytical flow LC/MS methodology for the analysis of native proteins and protein complexes. This workflow uses the AdvanceBio size exclusion chromatography (SEC) column for online sample separation. The 6545XT AdvanceBio LC/Q-TOF, featuring large molecule SWARM autotune and 30,000 m/z extended mass range, was used for rapid and reproducible native protein analysis (Figure 1).

Experimental

Materials and methods

Monoclonal antibody standard, RM 8671, was purchased from the National Institute of Standards and Technology (NIST), often referred to as NIST-mAb. The formulated Herceptin (trastuzumab) was obtained from Genentech (South San Francisco, California, USA). The formulated trastuzumab emtansine (TDM1, ADC) was also from Genentech. All other protein samples and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

To perform native MS analysis, it is crucial to preserve the protein samples at neutral pH and volatile aqueous solutions, such as ammonium acetate or ammonium formate. Therefore, sample desalting and buffer exchange are usually needed prior to the MS analysis. Briefly, protein stock solutions (1 to 10 mg/mL) were desalted and solvent exchanged into 100 mM ammonium acetate using Bio-Rad Bio-Spin P-6 (6,000 MW limit) or P-30 (40,000 MW limit) cartridges. The cartridge was first fully equilibrated with 100 mM ammonium acetate buffer. Protein sample was then pipetted to the top of the column and centrifuged for 5 min at 1000 × g. The protein was then buffer exchanged into the 100 mM ammonium acetate and was ready for MS analysis. This desalting protocol caused minimal sample loss and much less structural alteration of the protein molecule.

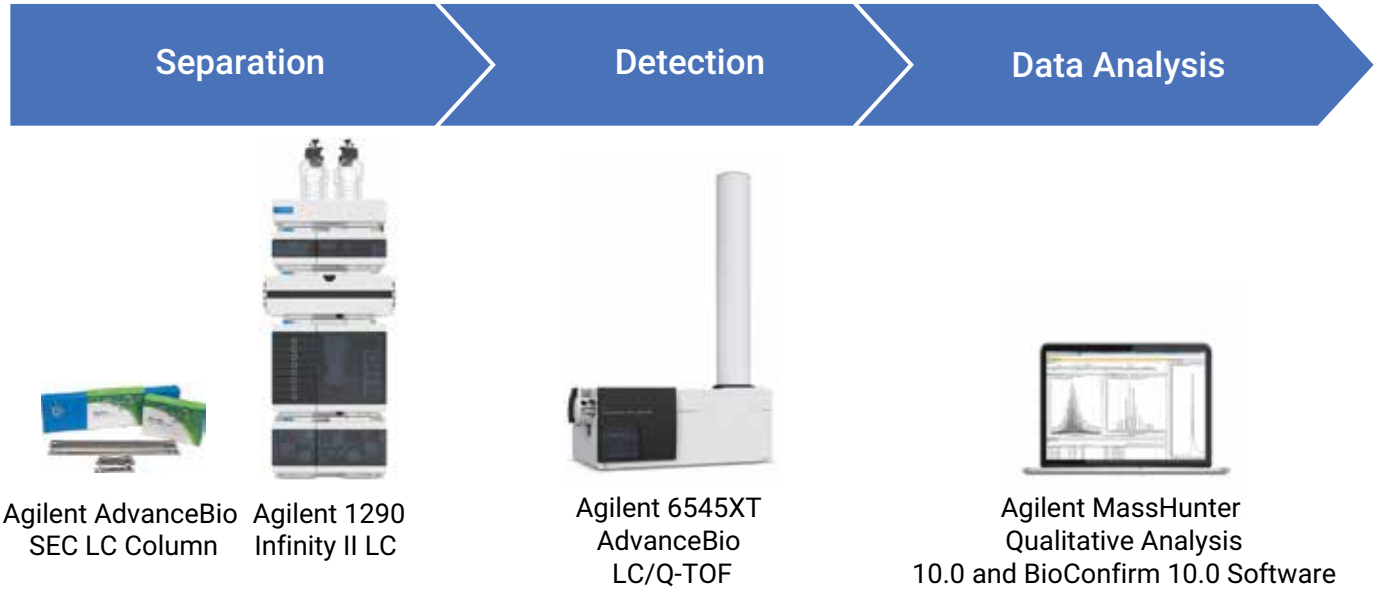

Figure 1. Analytical components of the native protein analysis workflow.

Instrumentation

Agilent 1290 Infinity II LC including:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF)

LC/MS analysis

LC/MS analyses were conducted on a 1290 Infinity II LC coupled with a 6545XT AdvanceBio LC/Q-TOF system equipped with an Agilent Jet Stream source. Agilent MassHunter Acquisition (B.09.00) workstation software with the large molecule SWARM autotune feature was used.

LC separation was obtained with an Agilent AdvanceBio SEC guard column (4.6 × 30 mm, 200 Å, 1.9 µm).

Tables 1 and 2 list the LC/MS parameters used.

Data processing

All MS data of the native intact mAbs or protein complexes were processed using Agilent MassHunter Qualitative Analysis 10.0 and BioConfirm 10.0 software.

Results and discussion

Method optimization for native protein and protein complex analysis

To overcome the challenges of native protein analysis, some key method developments and optimizations were made:

- The use of offline desalting cartridges (Bio-Rad Bio-Spin P-30) for sample preparation (desalting and buffer exchange) prior to the MS analysis and online SEC column further separated the target protein from background salts, which led to higher MS sensitivity and improved MS data quality.
- The use of a conventional flow rate (0.2 mL/min) of 100 mM ammonium acetate buffer not only eliminated the sample aggregation but also improved LC/MS analytical reproducibility for well-preserved native protein samples.
- The 6545XT AdvanceBio LC/Q-TOF system was equipped with large molecule SWARM autotune for optimizing macromolecular ions transmission, and the extended mass range of up to m/z 30,000 for the native protein complex analysis with high sensitivity.

Table 1. Liquid chromatography parameters.

	Agilent 1290 Infinity II LC
Column	AdvanceBio SEC (200 Å, 4.6 × 30 mm, 1.9 µm) (p/n: PL1580-1201)
Thermostat	4 °C
Solvent (A)	100 mM NH4OAc (pH 7)
Isocratic Elution	0–5 min, 100% A
Column temperature	Room temperature
Flow rate	0.2 mL/min
Injection volume	1–5 µL

Table 2. Native MS data acquisition parameters.

	Agilent 1290 Infinity II LC		
Sample Type	Myoglobin	Intact mAbs	Intact Macroprotein Complexes
Source	Agilent Jet Stream	Agilent Jet Stream	Agilent Jet Stream
Dry Gas Temperature	150 °C	365 °C	150 °C
Dry Gas Flow	10 L/min	12 L/min	10 L/min
Nebulizer	30 psig	35 psig	30 psig
Sheath Gas Temperature	150 °C	300 °C	150 °C
Sheath Gas Flow	10 L/min	12 L/min	10 L/min
VCap	5000 V	5500 V	5000 V
Nozzle Voltage	2000 V	2000 V	2000 V
Fragmentor	250 V	300 V	250 V
Skimmer	100 V	220 V	100 V
Quad AMU	m/z 500	m/z 1000	m/z 3000
Mass Range	m/z 300–7000n	m/z 3000–10000n	m/z 5000–25000n
Acquisition Rate	1.0 spectrum/s	1.0 spectrum/s	1.0 spectrum/s
Acquisition Mode	Positive, extended (m/z 10,000 mass range	Positive, extended (m/z 10,000 mass range	Positive, extended (m/z 25,000 mass range

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Native MS analysis of intact myoglobin (with heme)

Native MS analysis of noncovalent interactions of myoglobin has been well-studied.4 In myoglobin, heme is noncovalently attached to the globin through hydrogen bonds and hydrophobic interactions. When the heme is attached to the globin, the protein is referred to as holomyoglobin (the native conformation). Monitoring of the charge state distributions of myoglobin ions in mass spectra of ESI-MS has been used in protein folding/unfolding studies.4 The apomyoglobin (with no heme) with high charge states indicated the disruption of the native heme-protein interaction, which led to a considerable degree of protein unfolding. As shown in Figure 2A, myoglobin was denatured in the organic and acid solvent, and under harsh MS source conditions.

The charge envelope of the denatured myoglobin ranged from m/z 1,000 (17+) to 3,500 (5+) while the most intense charged ion was 12+.Most of the native holomyoglobin was denatured into apomyoglobin and heme (inset in Figure 2A). Our optimized native MS analysis of myoglobin clearly demonstrated that the native conformation of myoglobin was retained (Figure 2B). Only trace amounts of apomyoglobin and heme could be detected. The charge envelope of holomyoglobin was from 9+ to 5+ and the charge state of 8+ was the most abundant ion. The overall MS signal intensities of the native MS ions were about 1/10 of those in the denatured MS spectrum.

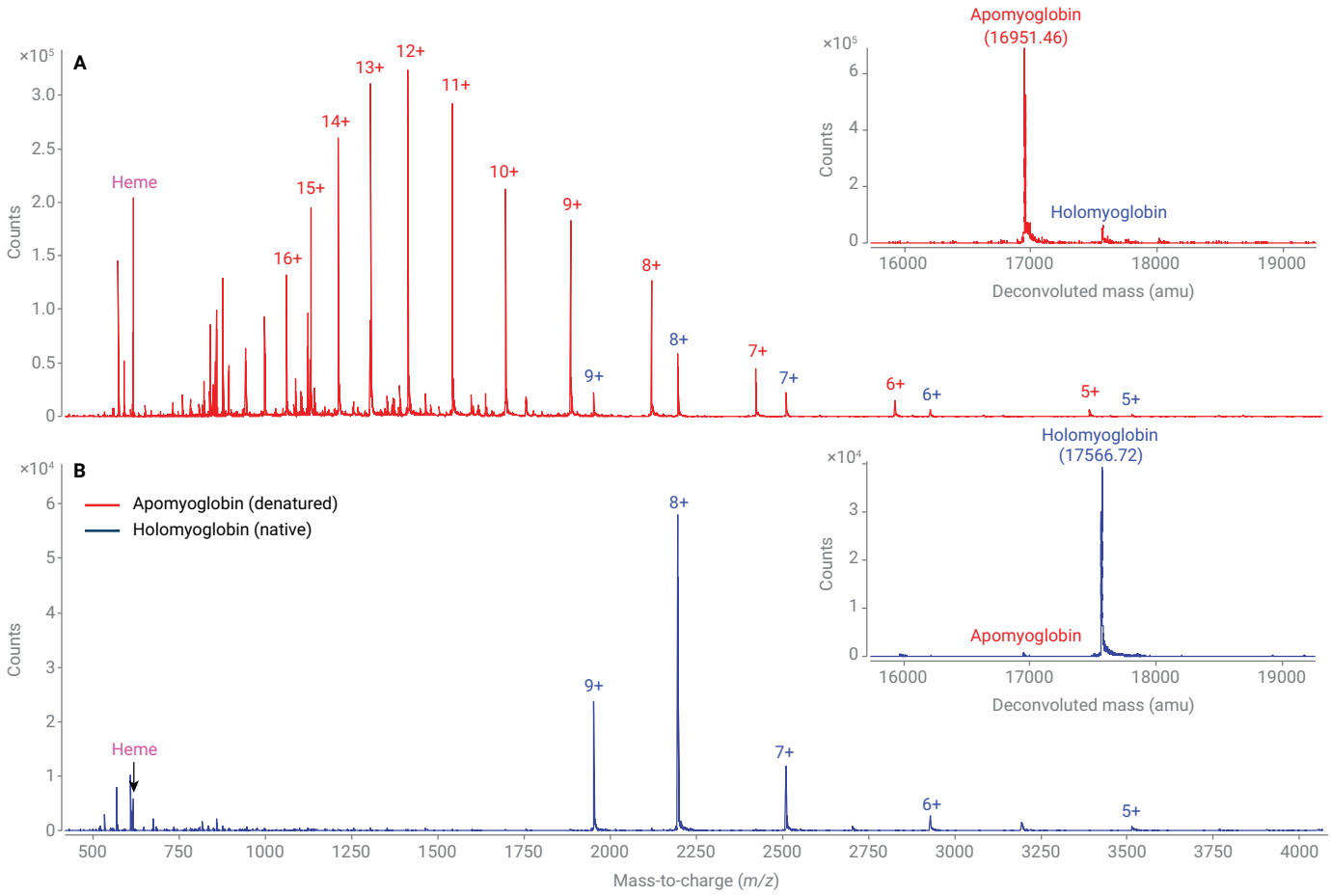

Figure 2. LC/MS analysis of intact myoglobin sample.
A) Myoglobin sample was analyzed under denatured LC/MS conditions (previous studies).
The heme group was dissociated from the protein complex and the majority of the protein was apomyoglobin (inset figure).
B) Native MS analysis of myoglobin. The holomyoglobin (with heme) structure was preserved and only trace amount of heme was detected.

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The native MS analysis results confirmed that ionic strength of the SEC column mobile phase also played a key role in maintaining the protein native conformation.2 Figure 3 demonstrates the native MS analysis of alcohol dehydrogenase (ADH, tetramer) under two mobile phase conditions. Even though both mobile phases were at neutral pH, protein dissociation products (dimer) were observed when ammonium formate was substituted for ammonium acetate in the mobile phase (Figure 3A and 3B). Also, the charge state envelope of the intact native ADH in the 50 mM ammonium formate was shifted to a lower m/z range compared to that in the 100 mM ammonium acetate (Figure 3C).

The results indicate that use of ammonium formate in the mobile phase increases the number and extent of multiply charged ions (max at 26+ compared to 23+ using ammonium acetate, Figure 3), although the ADH species may still be considered an intact protein tetrameric complex. Therefore, we believe that the 100 mM ammonium acetate solution offered better structural protection to protein complexes during the native MS analysis.

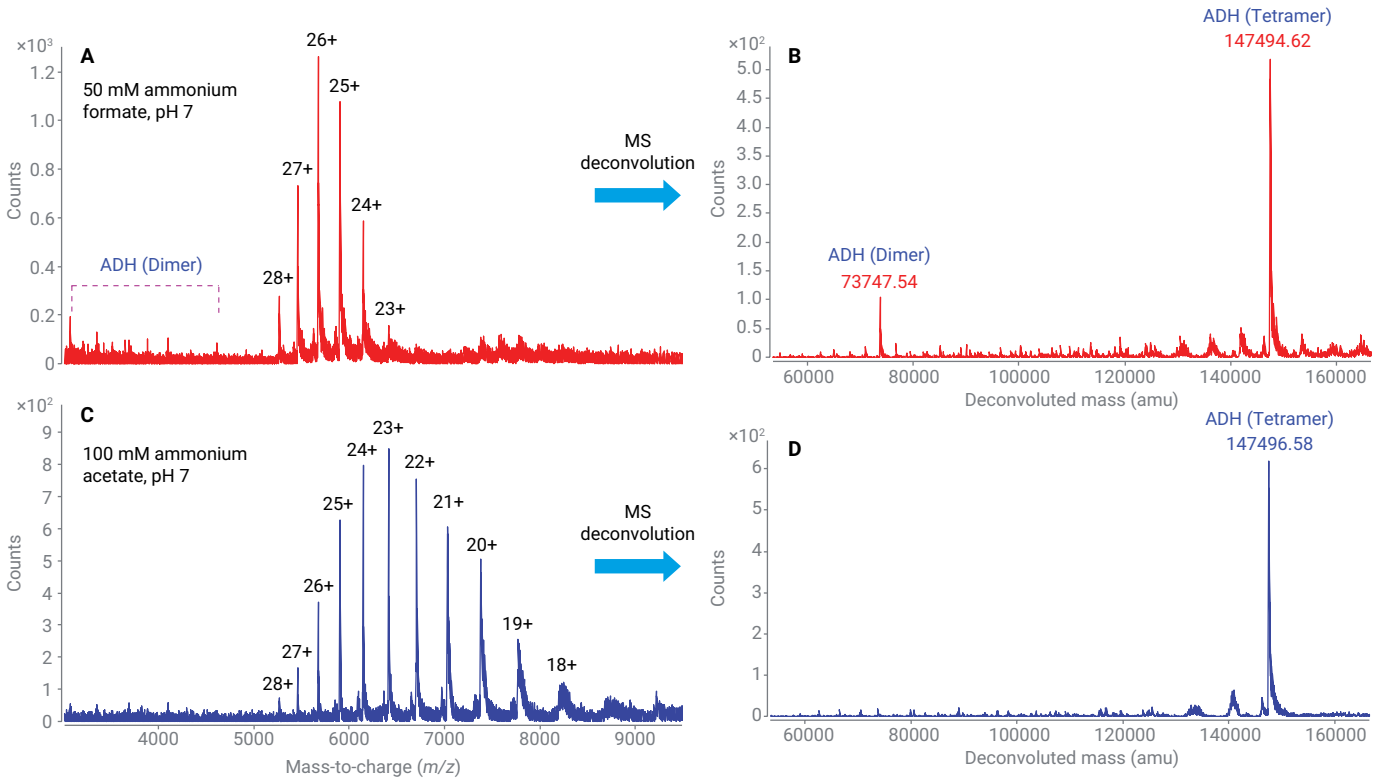

Figure 3. Native alcohol dehydrogenase (ADH, tetramer) analysis under various solvent conditions.
A) Native ADH in 50 mM ammonium formate, pH 7. C) Native ADH in 100 mM ammonium acetate, pH 7. B) and D) deconvoluted spectrum of both samples.

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Native MS aanalysis of intact mAbs

Monoclonal antibodies (mAbs) and their derivative products have quickly become an important class of biopharmaceutical molecules with a wide range of therapeutic applications. Native MS analysis of mAbs can provide valuable information, such as: protein folding, mAb aggregation (mAb dimer or trimer), antibody drug conjugates (ADCs), bispecific mAbs, etc.

In this study, we applied the online SEC method for rapid and robust native mAbs MS analysis. Approximately 0.5 to 1.0 µg of mAb was injected onto an AdvanceBio SEC guard column using a 5 min isocratic flow at 0.2 mL/min of 100 mM ammonium acetate solvent. The Q-TOF source conditions were optimized for excellent quality of native MS spectra over the mass range from m/z 5,000 to 10,000.

Figure 4 demonstrates the LC/MS analysis of intact NIST mAb standard under the denaturing MS conditions (Figure 4A and 4B) as well as the native MS conditions (Figure 4C and 4D). In both conditions, all major glycoforms of the NIST mAb were well resolved (Figure 4A and 4C, inset). The charge state distribution of denatured NIST mAb spanned the mass range of m/z 2,000 to 5,000 (30+ to 75+), while the native NIST mAb had a charge envelope in the range of m/z 5,000 to 10,000 (15+ to 30+). As shown in the MS deconvoluted spectra (Figure 4B and 4D), low ppm in mass errors were obtained for all major glycoforms. We also achieved very good agreement with the data for the intact NIST mAb analysis under both MS conditions.

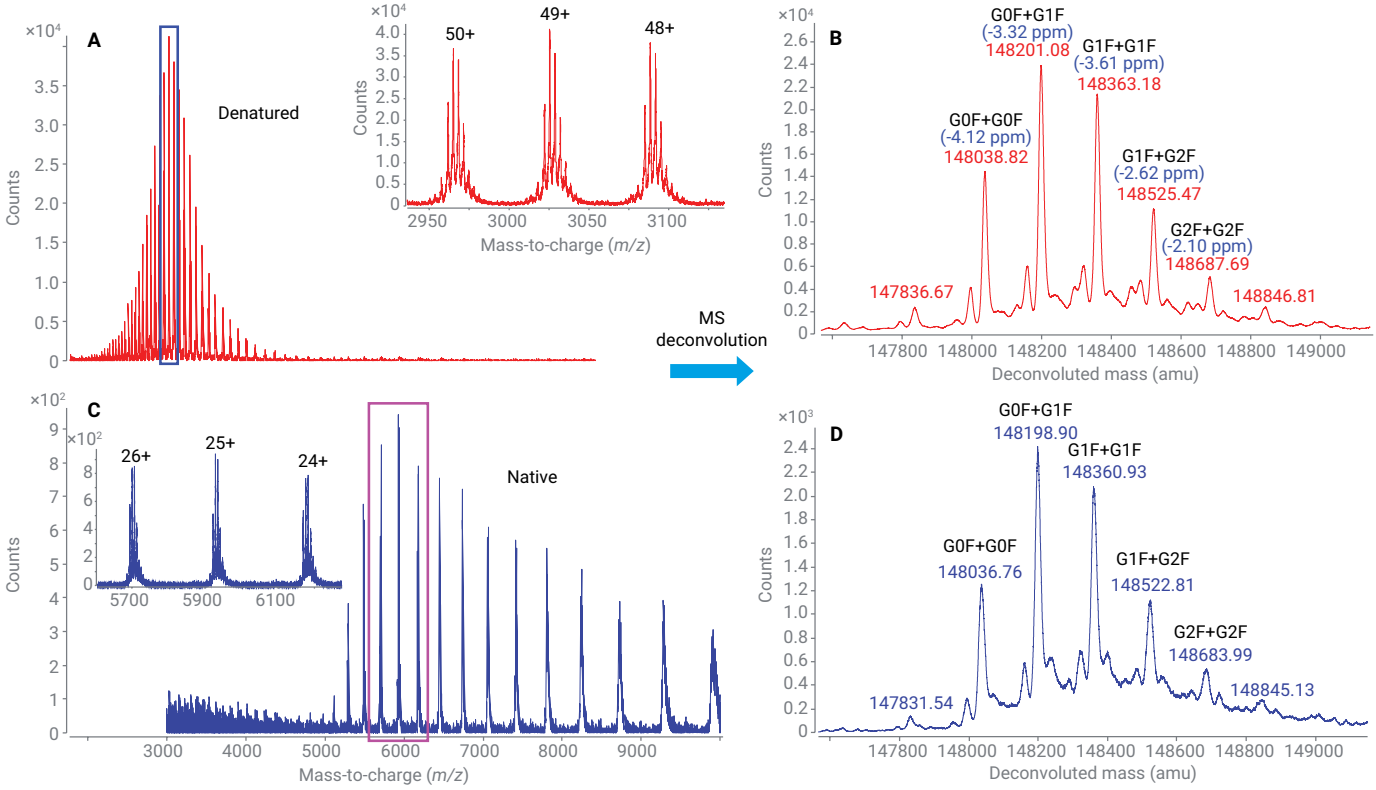

Figure 4. LC/MS analysis of NISTmAb under: A) denaturing MS conditions (acetonitrile and 0.1% formic acid) (previous work, Ref. 5) and C) native MS conditions (in 100 mM ammonium acetate, pH 7). The deconvoluted MS spectra of both samples are shown in B) and D), respectively.

Similarly, native MS analysis of a biotherapeutic drug (trastuzumab, brand name: Herceptin) and its ADC (trastuzumab emtansine, T-DM1) was performed and compared. Figure 5A illustrates the native mass spectrum of intact Herceptin, showing a nicely distributed charge envelope from m/z 5,000 to 10,000 with charge states between 15+ and 28+. The most prominent charge state was at 24+ which indicated the intact Herceptin was in its native/folded conformation. High mass accuracies for the major glycoforms were achieved as shown in the inset deconvoluted spectrum.

Native MS analysis enables probing of protein molecules while preserving their native structural conformation. As this method minimizes the interferences from organic solvent and acid in the mobile phase, it is an ideal analytical tool for noncovalent protein complexes or acid labile protein conjugates, such as some ADCs. Figure 5 shows the native raw and deconvoluted (inset) MS spectrum of T-DM1. The average DAR value calculated using the BioConfirm DAR Calculator was 3.5 (Figure 5B, inset), which is consistent with the DAR values of the intact ADC reported by Genentech (the manufacturer).

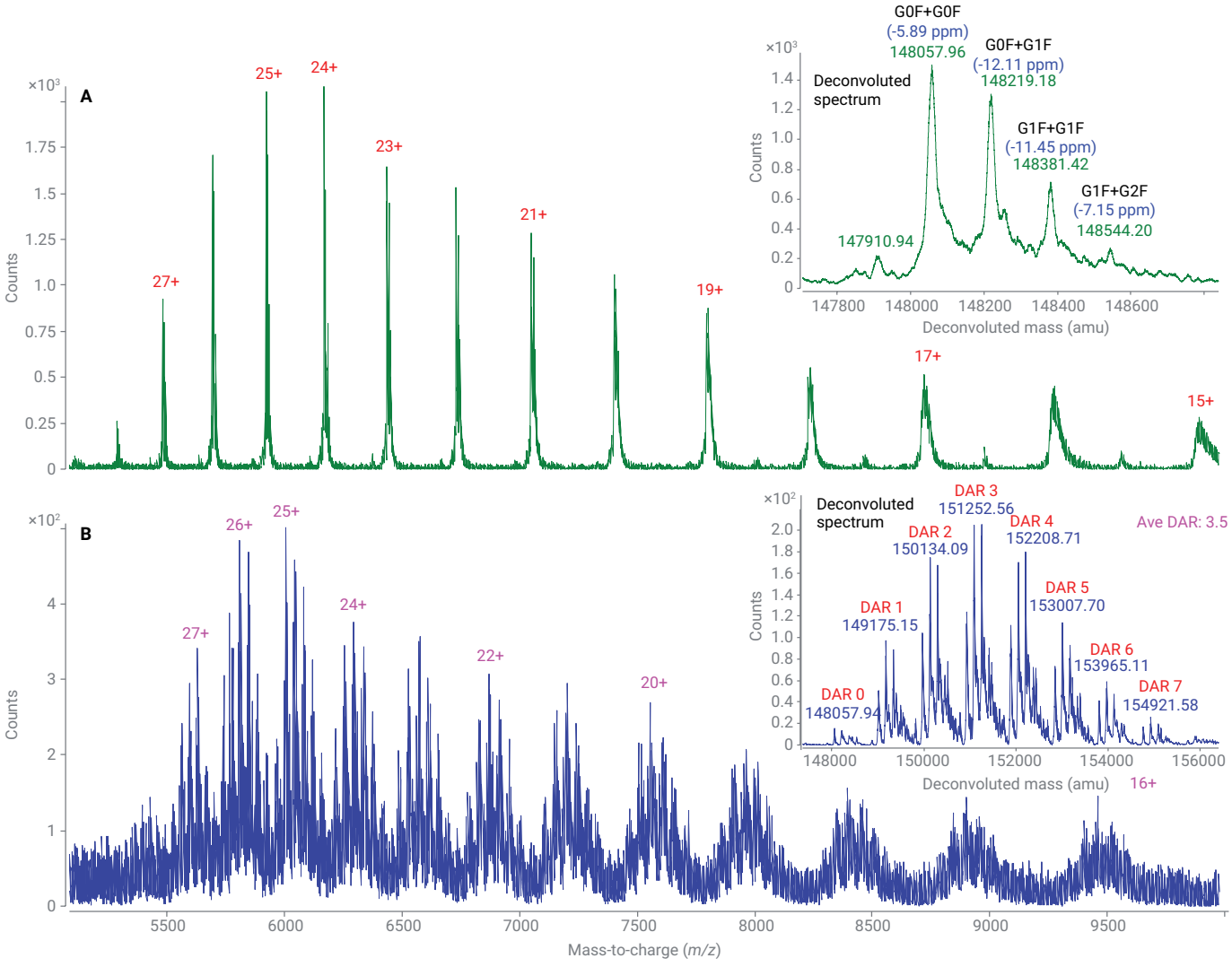

Figure 5. Native LC/MS analysis of mAb and its antibody drug conjugate (ADC): A) Herceptin and B) T-DM1. The deconvoluted MS spectra of both samples are shown in the inset figures.

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Native MS analysis of intact protein complexes

The optimized native MS analysis method was also evaluated by three protein complex samples. They were: tetrameric pyruvate kinase (PK, 232 kDa), hexameric glutamate dehydrogenase (GDH, 335 kDa), and tetrameric β-galactosidase (466 kDa). The 6545XT AdvanceBio LC/Q-TOF system offers large molecule SWARM autotune for optimizing macromolecular ions transmission in the extended mass range up to m/z 30,000. It is an ideal LC/MS system for native protein complex analysis. Figure 6A shows the native mass spectrum of the tetrameric pyruvate kinase. Two major charge envelopes ranging from m/z 6,000 to 10,000 with charge state of 24+ to 37+ were detected.

The deconvoluted spectrum revealed that there were two multi-proteoform complexes of PK tetramers in the sample: full-length pyruvate kinase and truncated PK tetramer (three intact subunits plus one PK proteoform with N-terminal cleavage).[3]

The 6545XT system also demonstrated excellent detection sensitivity for protein complexes at higher m/z (>m/z 10,000). Figure 6B and 6C show the native MS spectrum of GDH and β-galactosidase. Both of their protein charge envelopes were greater than m/z 8,000, whereas the most abundant ions were at m/z 9,566 (35+) for GDH and m/z 10,832 (43+) for β-galactosidase, respectively. The molecular mass of the intact hexameric GDH was determined to be 334,754 and 465,788 Da for the tetrameric β-galactosidase with 1 to 10 µg sample injections.

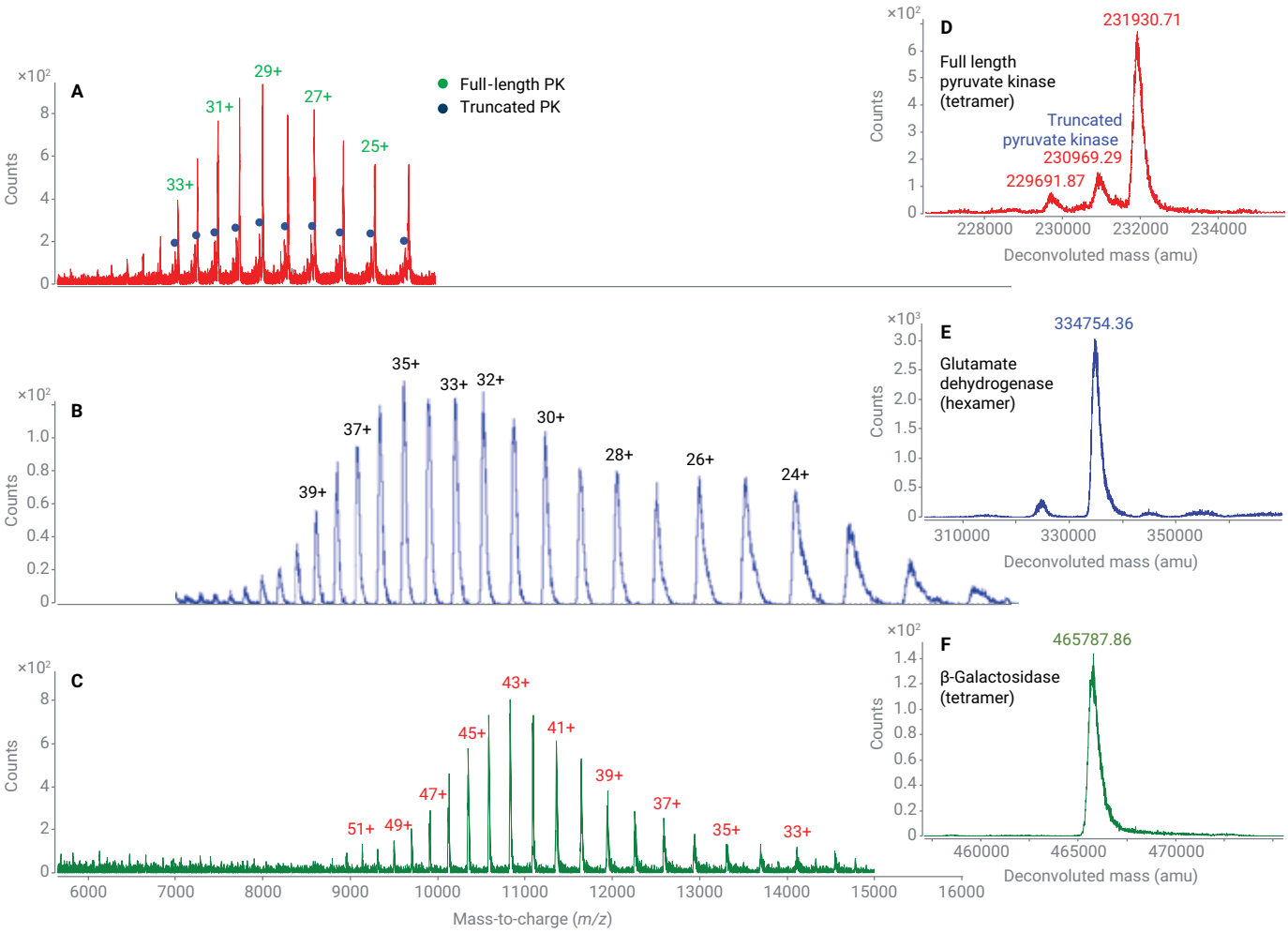

Figure 6. Native LC/MS analysis of various intact protein complexes. A) Pyruvate kinase (PK, tetramer, 232 kDa), B) glutamate dehydrogenase (GDH, hexamer, 335 kDa) and C) β-galactosidase (tetramer, 466 kDa). The deconvoluted spectra are shown in D) to F), respectively. The raw MS spectrum in Figure 6B was smoothed using the mMass open-source MS software tool

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Conclusion

We have developed a highly sensitive and robust LC/MS workflow methodology for native protein analysis. This optimized workflow utilizes the 1290 Infinity II LC with the AdvanceBio SEC column, the 6545XT AdvanceBio LC/Q-TOF with extended mass range up to m/z 30,000, and MassHunter BioConfirm software. The following benefits have been demonstrated by this native MS analysis method:

- Use of an online SEC column at typical analytical LC flow rates eliminates the challenging issues (protein aggregation and instable spraying flow) associated with nanoESI analysis.
- The optimized native MS conditions provide high confidence in ADC characterization, with accurate determined DAR values.
- The large molecule SWARM autotune feature, along with the extended mass range of the 6545XT AdvanceBio LC/Q-TOF, enables the sensitive detection and characterization of native intact macroprotein complexes.

References

1. Heck, A. J. Native Mass Spectrometry: A Bridge Between Interactomics and Structural Biology. *Nat. Methods* **2008**, *5*, 927–933.
2. Freeke, J.; Robinson, C. V.; Ruotolo, B. T. Residual Counter Ions can Stabilise a Large Protein Complex in the Gas Phase. *Intl. J. Mass Spec*. **2010**, *298*, 91–98.
3. Schachner, L. F.; Kelleher, N. L. Standard Proteoforms and Their Complexes for Native Mass Spectrometry. *JASMS* **2019**, *30*, 1190–1198.
4. Konermann, L., Rosell, F. L., Mauk, A. G.; Douglas, D. J. Acid-Induced Denaturation of Myoglobin Studied by Time-Resolved Electrospray Ionization Mass Spectrometry. *Biochemistry* **1997**, *36(21)*, 6448–6454.
5. Wong, D. L. Precise Characterization of Intact Monoclonal Antibodies by the Agilent 6545XT AdvanceBio LC/Q-TOF. *Agilent Technologies application note*, publication number 5991-7813EN, **2017**.

Published in the USA, March 12, 2020
5994-1739EN

Application Note

Aggregate/Fragment Analysis

Agilent

Trusted Answers

Analysis of Camelid Single-Domain Antibodies Using Agilent AdvanceBio SEC 120 Å 1.9 µm and AdvanceBio HIC Columns

Authors

Te-Wei Chu and Greg Staples

Agilent Technologies, Inc.

Abstract

This application note describes the use of size exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC) for the analysis of camelid single-domain antibodies (nanobodies). Nanobodies are a growing class of single-domain antibody fragments used for therapeutic purposes. The Agilent AdvanceBio SEC 120 Å 1.9 µm column provides a unique advantage over other SEC columns for high-resolution separation of nanobody aggregates and fragments. The Agilent AdvanceBio HIC column enables analysis of nanobody post-translational modifications (PTMs) such as glutamine/pyroglutamate conversion. An SEC-based native LC/MS method is also demonstrated for greater understanding of PTM and impurity characterization.

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Introduction

Despite the success of biotherapeutics such as monoclonal antibodies (mAbs), many significant drawbacks still exist for this class of drugs. For example, IgG mAbs, the most widely used biologic drugs, have a complex structure and rather large size (150 kDa). The large size hampers their efficient in vivo delivery to diseased cells such as those found in tumors. Alternatively, single-domain antibodies, also known as nanobodies, provide tremendous opportunity in terms of reaching their intended targets.[1]

Nanobodies are small (~15 kDa), natural single-domain proteins derived from the camelid heavy chain antibody (Figure 1). They are recombinantly produced antigen-binding V_{HH} fragments with binding affinity equivalent to that of conventional IgG mAbs. Due to their small size, nanobodies can bind to antigen motifs that are frequently inaccessible to conventional mAbs, providing access to presently "undruggable" targets. In addition, the relatively simple protein conformation offers many advantages to drug developers such as ease of manufacturing and different administrative routes.[1] These promising features make nanobodies and V_{HH} fragment-derived biologics the rising stars in the biopharma research and development pipeline.[2]

This application note presents thorough characterization of two V_{HH} fragments (anti-PD1 and anti-PDL1 single-domain antibodies) using SEC and HIC, both with UV detection. Furthermore, we demonstrate SEC-based native LC/MS analysis of the two samples. The Agilent AdvanceBio SEC 120 Å 1.9 µm column is designed for aqueous SEC separation of small biomolecules such as proteins in the molecular weight range of 1 to 80 kDa. The column is perfectly suitable for nanobody analysis. The proprietary sub-2 µm hydrophilic polymer-coated silica packing technology enables higher resolving separation of aggregates and fragments compared to SEC columns from other vendors. HIC analysis allows reserved-phase-like separation of protein variants in a native condition. The Agilent AdvanceBio HIC column exhibits optimal hydrophobicity and selectivity for nanobody PTM characterization. Using a generic HIC method (without organic solvent modifier), a common PTM, glutamine/pyroglutamate conversion is revealed. The combination of these approaches offers a complete solution for in-depth analysis of nanobody purity and critical quality attribute assessment.

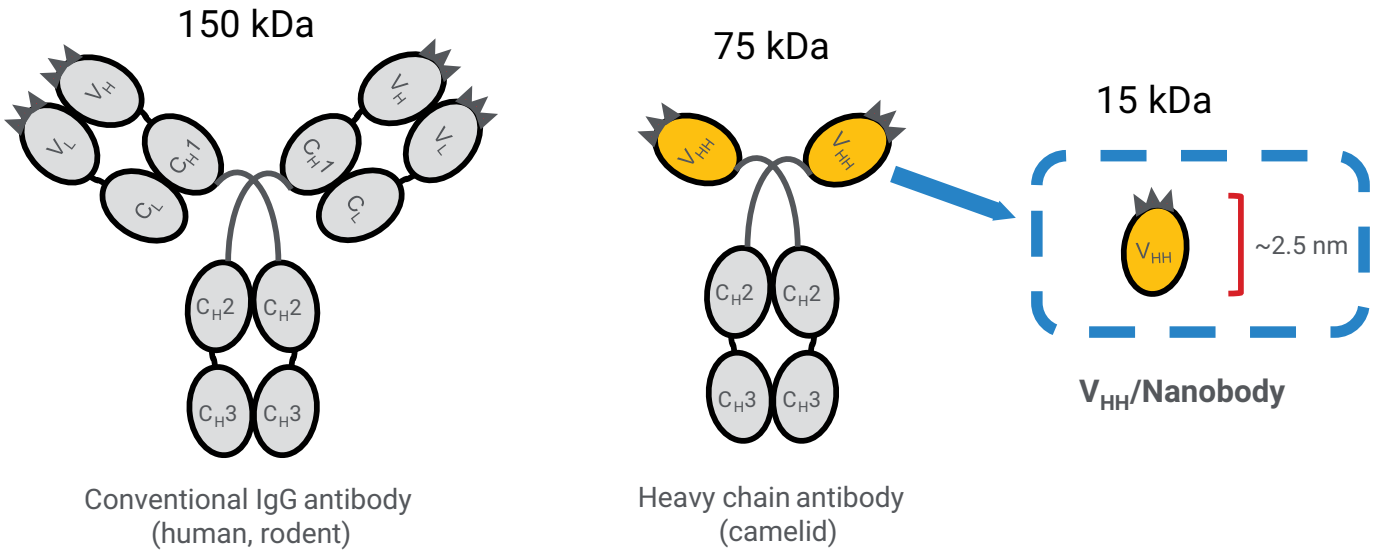

Figure 6. Schematic of a V_{HH} single-domain antibody (nanobody) compared to a conventional mAb.

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Experimental

Samples and chemicals

- Llama anti-PD1 single-domain antibody [F12A8]; purchased from ProSci Inc (Poway, CA)
- Llama anti-PDL1 single-domain antibody [F2G2]; purchased from ProSci Inc (Poway, CA)
- Glutaminyl-peptide cyclotransferase; purchased from R&D Systems (Minneapolis, MN)
- Human anti-IL8 IgG monoclonal antibody; produced in house from CHO cells

All chemicals and solvents used were HPLC grade or higher. Sodium phosphate monobasic and dibasic, sodium chloride, and ammonium sulfate were from Sigma-Aldrich. Water was purified using a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipore).

Columns

- Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
- Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 150 mm (p/n PL1580-3250)
- Agilent AdvanceBio HIC, 4.6 × 100 mm (p/n 685975-908)

Instrumentation

For HPLC experiments, an Agilent 1260 Infinity II Bio-inert LC system was used comprising:

- Agilent 1260 Infinity II Bio-inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-inert Multisampler (G5668A) with sample cooler (option 100)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with bio-inert heat exchanger (option 019)
- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A)

For LC/MS experiments, an Agilent 6224 accurate-mass time-of-flight (TOF) LC/MS and 1290 Infinity II LC were used comprising::

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B)
- Agilent 1290 Infinity II Thermostatted Column Compartment (G7116B)
- Dual ESI Agilent 6224 accurate-mass time-of-flight (TOF) LC/MS

Results and discussion

SEC is a gold standard technique for characterizing aggregation of biologics. The chromatographic separation mechanism is unique in that analytes are not retained by the stationary phase; instead, they are separated based on accessibility to available particle pore volume. Therefore, careful selection of column pore size based on the protein analyte's size in solution (hydrodynamic radius) is important. Nanobodies are small proteins with molecular weight of approximately 15 kDa, translating to a hydrodynamic radius of approximately 25 Å. SEC columns packed with narrow pore (120 to 130 Å) particles offer linear separation in the range suitable for such an application.[3,4] Figure 2 shows an SEC separation of anti-PD1 single-domain antibody (sdAb) using a standard method with sodium phosphate pH 7 as the mobile phase (to maintain the native state of the protein). Excellent separation of sdAb monomer from impurities, i.e., high-molecular weight (HMW) and low molecular weight (LMW) species, can be seen with the AdvanceBio SEC 1.9 µm 120 Å column.

The resolution of HMW and LMW species as well as the peak width and tailing factor of the monomer peak are significantly better compared to columns packed with larger (2.7 µm) particles. Compared to other vendors' offering of sub-2 µm SEC technology, the AdvanceBio SEC 1.9 µm 120 Å column exhibits best-in-class performance. This is due to careful design of particle surface bonding coverage to eliminate undesirable secondary interactions.[3,4]

Method conditions

Parameter	HPLC Conditions (SEC Analysis)
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile phase	150 mM sodium phosphate, pH 7.0
Flow rate	0.35 mL/min
Column temperature	25 °C
Injection volume	5 µL, 1 mg/mL
Total run time	15 min
Detection	UV at 214 nm

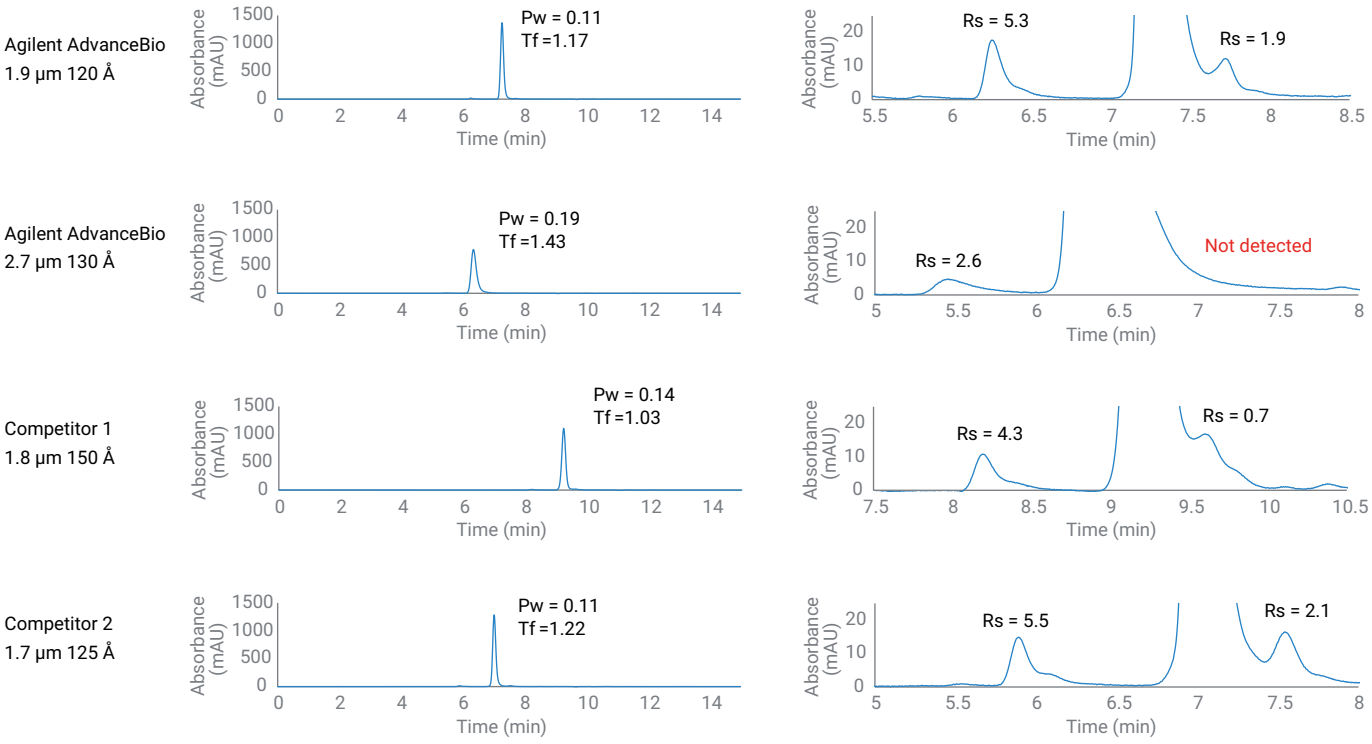

Figure 2. SEC of anti-PD1 single-domain antibody. Right panels show the magnified baseline of the same run on the left. Resolution of the HMW aggregate peak (eluted earlier) or the LMW fragment peak (eluted later) compared to the monomer peak are shown.

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SEC is a relatively straightforward chromatographic method where the column is run in isocratic mode. Method development and optimization involve the selection of mobile phase parameters (pH, salt concentration, etc.) to minimize potential secondary interactions such as ionic or hydrophobic interaction. Figure 3 shows SEC salt plot studies for method optimization of anti-PD1 and anti-PDL1 sdAb analysis. A shorter column of 15 cm length was selected for this experiment to achieve higher throughput and speed up the method optimization process. Results showed that anti-PD1 sdAb had minimal or no secondary interaction with the column at the range of mobile phase NaCl concentration used. This is evident from the similar chromatographic peak shapes and retention times at each mobile phase condition. In contrast, anti-PDL1 sdAb had slightly deteriorated peak shape with delayed retention time at higher salt concentration mobile phase, indicative of potential hydrophobic interaction between analytes and the column. Interestingly, at 250 mM NaCl and above, a secondary (shoulder) peak became evident.

Method conditions

Parameter	HPLC Conditions (SEC Salt Plot Study)
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 150 mm (p/n PL1580-3250)
Mobile phase	20 mM sodium phosphate, pH 7.0 with concentration of sodium chloride indicated in Figure 3
Flow rate	0.35 mL/min
Column temperature	25 °C
Injection volume	2 µL, 1 mg/mL
Total run time	7 min
Detection	UV at 214 nm

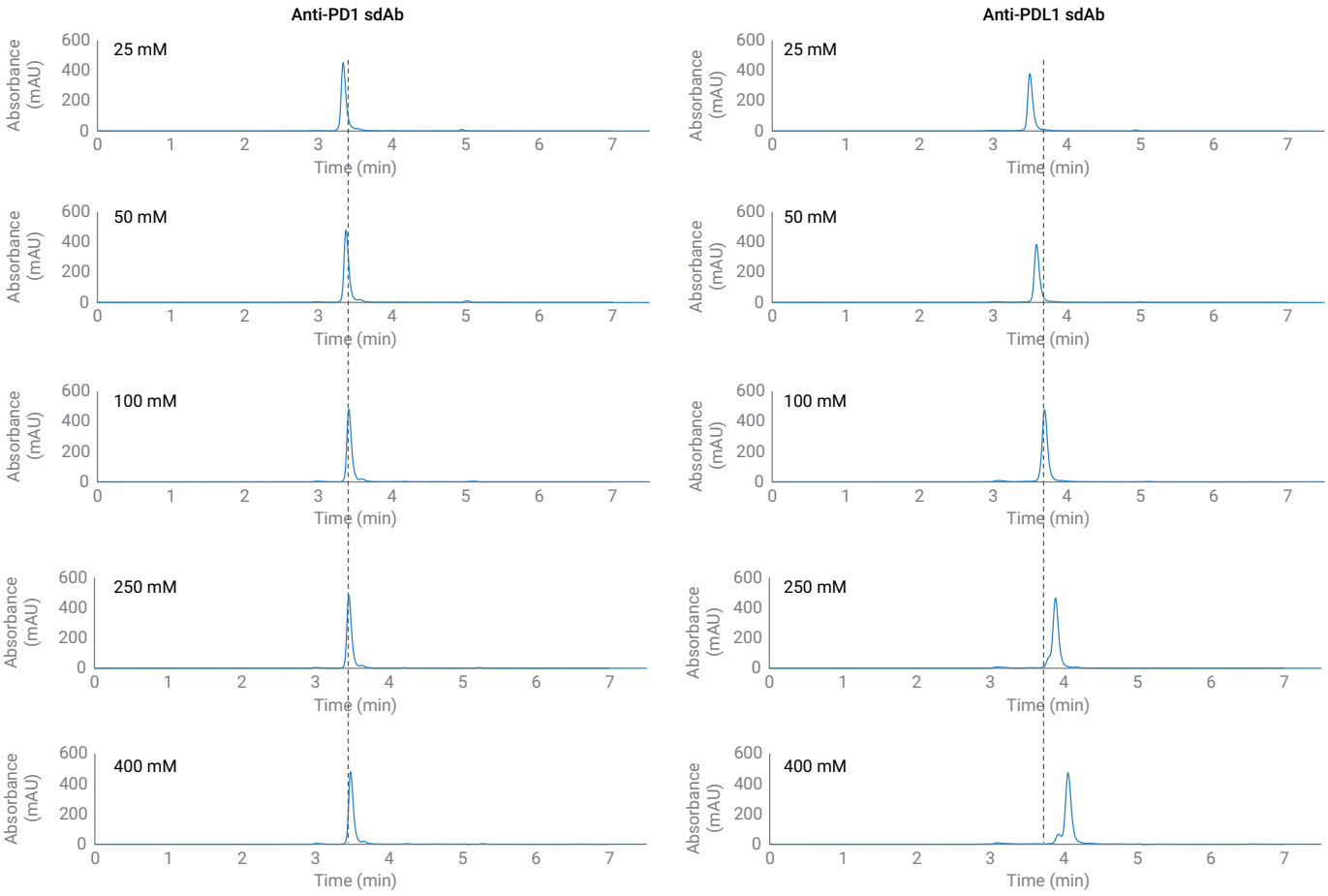

Figure 3. Salt plot studies of anti-PD1 (left) and anti-PDL1 (right) single-domain antibodies using the Agilent AdvanceBio SEC 1.9 µm 120 Å column.

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This phenomenon is commonly encountered with challenging proteins that contain highly hydrophobic motifs or which have extreme isoelectric points. As nanobodies contain only the variable domain of an antibody, the amino acid sequence varies significantly when comparing one nanobody to another, potentially causing large differences in protein physicochemical characteristics. The method demonstrated here is useful for fast screening of SEC mobile phase conditions to determine the optimum conditions for analyzing different samples. The data inform the use of lower salt concentration (50 mM or below) in the mobile phase for anti-PDL1 sdAb.

To further characterize the two nanobodies, native SEC-LC/MS experiments were conducted (Figure 4). Results showed that the AdvanceBio SEC 1.9 µm 120 Å column was suitable for SEC-MS, where low concentrations of volatile aqueous buffer (i.e., 50 mM ammonium acetate) are used. Excellent ion chromatograms can be seen for both sdAb samples, together with high-resolution mass spectra. The deconvoluted MS results showed accurate molecular weight measurement for both samples: anti-PD1 sdAb (16,528 Da) and anti-PDL1 sdAb (16,895 Da).

Method conditions

Parameter	Native SEC-LC/MS Conditions
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile phase	50 mM ammonium acetate, pH 7.0
Flow rate	0.35 mL/min
Column temperature	25 °C
Injection volume	20 µL, 1 mg/mL
Total run time	15 min
MS Detection	Min range: 300 m/z Max range: 7,000 m/z Ion polarity: Positive
MS Source Parameters	5Gas temperature: 325 °C Gas flow: 5 L/min Nebulizer: 30 psi Vcap: 5,500 V Fragmentor: 250 V Skimmer: 65 V Octopole RF peak: 750 V

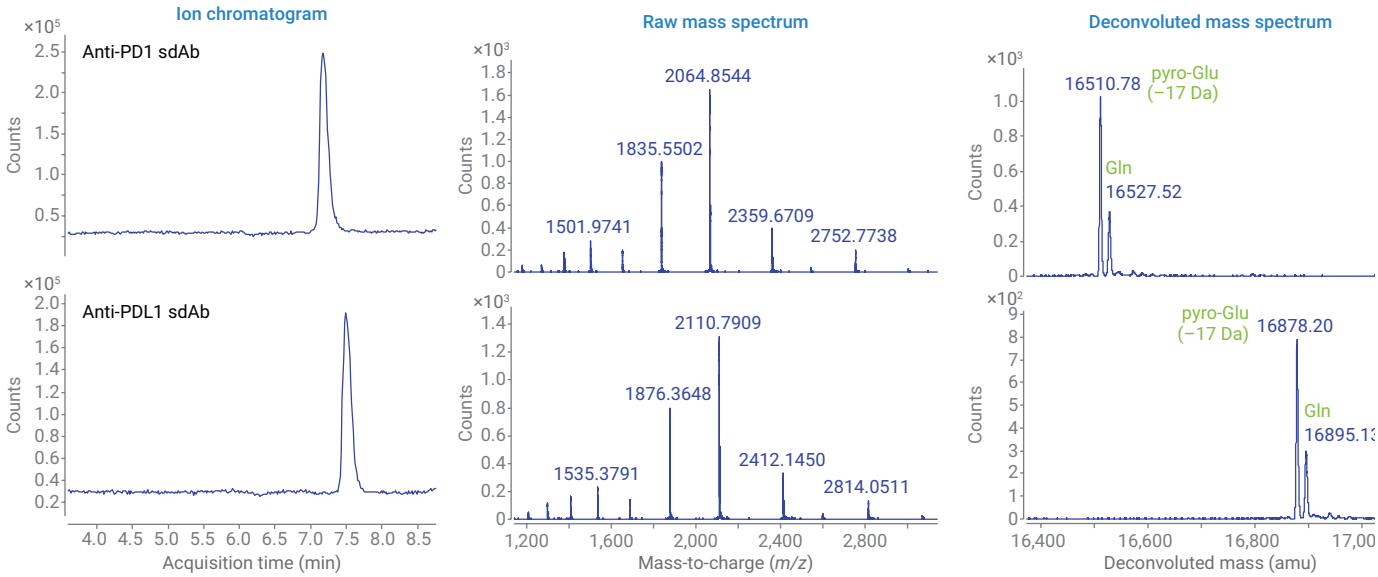

Figure 4. Native SEC-LC/MS analysis of anti-PD1 (top) and anti-PDL1 (bottom) single-domain antibodies.

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Interestingly, MS results revealed a large amount of a –17 Da modification on both samples. This is a typical mass shift associated with cyclization of N-terminal glutamine (Gln) to form pyro-glutamic acid (pyro-Glu).[5] The Gln/pyro-Glu conversion commonly occurs at the heavy chain variable domain of the antibody; thus, it is not surprising to observe this PTM in nanobodies (V_{HH} fragment). Both sdAb samples analyzed here were produced in bacteria (E. coli). It has been well documented that production in prokaryotic systems may result in proteins being recovered as inclusion bodies, thus leading to unusual PTMs.[5] Because the N-terminal Gln residues of V_{HH} are near the complementarity-determining region (CDR), pyro-Glu formation can potentially have significant impact on target binding.[1] Therefore, careful characterization and documentation of this PTM is typically required.[5]

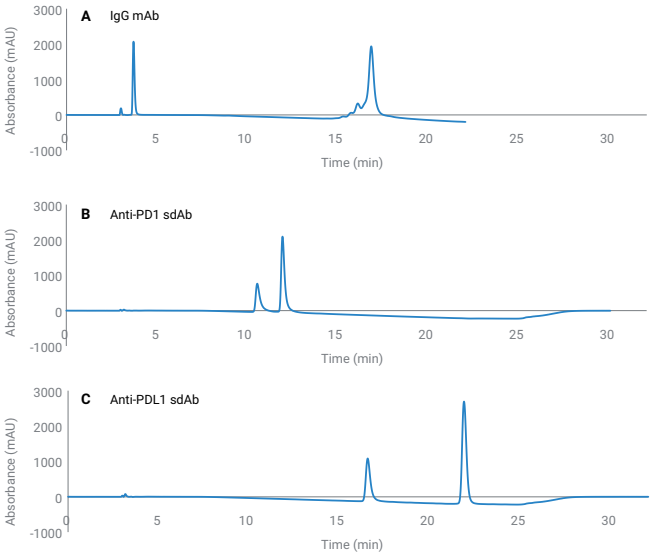

Figure 5. Hydrophobic interaction chromatograms of (A) an IgG mAb, (B) anti-PD1 single-domain antibody, and (C) anti-PDL1 single-domain antibody.

Method conditions

Parameter	HPLC Conditions (HIC analysis)
Column	Agilent AdvanceBio HIC, 4.6 × 100 mm (p/n 685975-908)
Mobile phase	A) 2 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0 B) 50 mM sodium phosphate, pH 7
Gradient	0 min: 50% B 2 min: 50% B 17 min: 100% B 20 min: 100% B 22 min: 50% B 32 min: 50% B
Flow rate	0.4 mL/min
Column temperature	30 °C
Injection volume	5 µL, 0.8 mg/mL (mAb) 5 µL, 1.0 mg/mL (sdAb)
Detection	UV at 214 nm

To characterize protein Gln/pyro-Glu conversion, chromatographic methods such as ion-exchange chromatography and HIC can be used. Here, HIC was chosen because, in addition to PTM analysis, it also provided an assessment of hydrophobicity for the two sdAb samples. HIC uses a salting-out mechanism to separate intact, native proteins based on hydrophobicity under near physiological conditions. Figure 5 shows the HIC separation of anti-PD1 and anti-PDL1 sdAbs and an IgG mAb using a generic ammonium sulfate gradient method. For both Nbs, two chromatographic peaks were well resolved, indicating that both samples contained two species of different hydrophobicity. This result was consistent with the LC/MS data showing the Gln/pyro-Glu conversion. In addition, HIC data suggested that the anti-PDL1 sdAb was very hydrophobic. The anti-PDL1 sdAb retention time was much longer than anti-PD1 sdAb and the IgG mAb (with a molecular weight that is 10 times larger). These results help explain the observations from the SEC salt plot study (Figure 3), suggesting major physicochemical property differences between the two single-domain antibodies.

To further confirm whether the two peaks separated using HIC were indeed sdAb species that contained Gln or pyro-Glu, a biochemical approach was developed using glutaminyl-peptide cyclotransferase (QPCT) (Figure 6). The enzyme is known to catalyze the conversion of N-terminal glutaminyl residues of proteins to pyroglutamyl groups.[6] Indeed, treatment of both sdAb samples with QPCT resulted in chromatograms containing only the pyro-Glu peak. The experiments presented here using the AdvanceBio HIC column demonstrated excellent selectivity for nanobody PTM analysis.

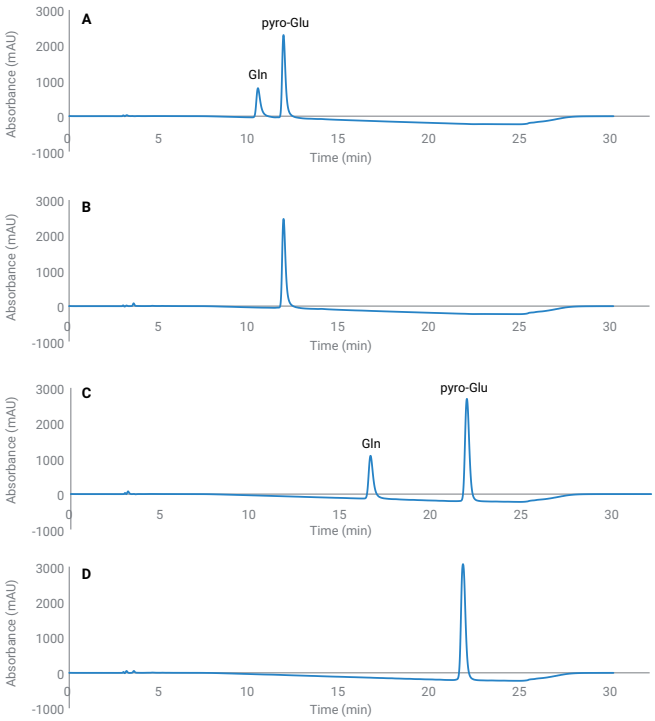

Figure 6. HIC analysis of (A) anti-PD1 sdAb, (B) anti-PD1 sdAb + QPCT, (C) anti-PDL1 sdAb, and (D) anti-PDL1 sdAb + QPCT. For enzyme treatment, 20 µL of sdAb (1 mg/mL) was incubated with 4 µL QPCT at 37 °C for 16 to 18 hours.

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Conclusion

This Nanobodies are revolutionary, new biotherapeutic modalities that offer many advantages over conventional mAb therapy. For research and development of this novel class of biologic, it is of utmost importance to characterize and document quality attributes that can be formed or changed during the process of production and storage. These attributes have been shown to impact drug potency, pharmacokinetics, immunogenicity, and safety. Reliable and robust analytical tools and methods are needed. This application note presents SEC, HIC, and SEC-LC/MS techniques that can successfully be applied to nanobody characterization. Important critical quality attributes (CQAs) such as HMW aggregates and LMW fragments GluGlncan be characterized using SEC in a high-resolution and high-throughput manner. Subtle changes on the molecules such as post-translational Gln/pyro-Glu conversion can be detected using HIC and SEC-MS approaches. The methods described here offer guidance for careful analysis of nanobodies in native, non-denaturing modes.

References

1. Bannas, P.; Hambach, J.; Koch-Nolte, F. Nanobodies and Nanobody-Based Human Heavy Chain Antibodies as Antitumor Therapeutics. *Front Immuno*l. **2017**, *8*, 1603.
2. Morrison, C. Nanobody Approval Gives Domain Antibodies A Boost. *Nat. Rev. Drug Discov*. **2019**, *18*, 485–487.
3. Kondaveeti, S. Analysis of Covalent High Molecular Weight Insulin. *Agilent Technologies Application Note*, publication number 5994-1566EN, **2020**.
4. Kondaveeti, S.; Chu, T-W.; Coffey, A. High Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins. *Agilent Technologies Application Note*, publication number 5994-1829EN, **202**0.
5. Jefferis, R. Posttranslational Modifications and the Immunogenicity of Biotherapeutics. *J. Immunol. Res*. **2016**, *2016*, 5358272.
6. Busby, W. Jr. *et al*. An Enzyme(s) That Converts Glutaminyl-Peptides into Pyroglutamyl-Peptides. Presence in Pituitary, Brain, Adrenal Medulla, and Lymphocytes. *J. Biol. Chem*. **1987**, *262*, 8532–6.

Published in the USA, April 15, 2020
5994-1869EN

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Application Note

Aggregate/Fragment Analysis

Agilent

Trusted Answers

High Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins

Authors

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Agilent Technologies, Inc.

Abstract

Protein denaturation processes involving aggregation are among the factors impeding the development of stable protein drug formulations. The use of size exclusion chromatography (SEC) HPLC for determining purity and aggregates of these proteins is a relatively straightforward technique. Regular calibration of SEC methods ensures better reproducibility, leading to improved accuracy, and enables earlier detection of potential problems with samples and batches. Agilent AdvanceBio SEC 120 Å 1.9 µm columns are compared to columns with sub-2 µm particle technology from other vendors. Analysis of recombinant human growth hormone (hGH), granulocyte colony-stimulating factor (hG-CSF), and interferon α-2b (INF α-2b) proteins demonstrate the superior performance of the AdvanceBio column for small protein therapeutic applications

Introduction

In recent years, there has been a large increase in the development of biologically derived therapeutics, known as biologics, to treat a myriad of diseases. Some of the biologic drugs include small protein therapeutic agents such as growth factors and cytokines because of their key roles in regulating the production, maturation and activity of blood, muscle and bone cells. For example, human growth hormone (hGH) is used to stimulate growth in children and adults exhibiting slow or subnormal growth due to hormonal deficiencies.[1] Granulocyte colony-stimulating factor (hG-CSF) is employed to treat cancer patients undergoing chemotherapy, to help raise white blood cell levels that have been reduced by cytotoxic therapeutic agents.[2] Interferons are a class of glycoproteins that have multiple therapeutic uses but are known to form partially unfolded species as well as aggregates particularly when exposed to pH or thermal degradation.[3].

Protein denaturation processes involving aggregation are among the prime factors impeding the development of stable protein drug formulations. The United States Pharmacopeia monograph method recommends size exclusion chromatography (SEC) HPLC for determining purity and aggregates of these proteins. SEC is a relatively straightforward technique. SEC relies on simple diffusion into the pore structure of the stationary phase; larger molecules cannot permeate the particles, and elute first, while smaller molecules diffuse readily into the pores, and elute later. Agilent AdvanceBio SEC 120 Å 1.9 µm columns are designed for aqueous size exclusion chromatography (SEC) of biomolecules. The particles have been manufactured using proprietary technology to combine optimum pore size and pore volume for separating molecules such as smaller proteins and peptides.

Experimental

Equipment and materials

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).

Instrumentation

Agilent 1260 Infinity II Bio-inert LC instrument comprising:

- Agilent 1260 Infinity II Bio-inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-inert Multisampler (G5668A) with sample cooler (option #100)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with bio-inert heat exchanger (option #019)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with bio-inert flow cell (option #028)

Software

OpenLab 2.2 CDS

Method conditions

Parameter	HPLC conditions
Column	AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile phase	150 mM Sodium phosphate, pH 7.0
Flow rate	0.30 or 0.35 mL/min (as shown in text)
Column temperature	25 °C
Injection volume	2 µL, 1 mg/mL
Total run time	Low molecular weight protein standard mix Human growth hormone, rhGH Human granulocyte colony stimulating factor, rG-CSH
Total run time	15 or 20 minutes (depending on flow rate)

Results and discussion

Proteins are complex molecules containing numerous side chain functionalities: acidic, basic, neutral, and hydrophobic. Finding the optimum conditions to avoid secondary interactions can be challenging, however the AdvanceBio SEC product range has a polymeric surface coating applied to the silica particle that overcomes many of these issues. The mechanism of separation relies on differences in size of molecules in solution (hydrodynamic radius). Protein structures are often compact and globular in nature, and proteins often aggregate under stress conditions such as extremes of temperature, pH, or salt composition and for dimers and larger units. This is a particular issue for protein molecules, where the presence of aggregated proteins can lead to adverse effects if administered as a therapeutic molecule. SEC provides the ideal tool for quantifying and monitoring protein aggregation. Figure 1 represents the SEC separation of low molecular weight protein and peptide standards. The calibration curve of these standards based on their retention time is shown in Figure 2. One can estimate the optimal molecular range for this column to be 1 to 80 kDa.

Peak	Protein/Peptide	Molecular Weight (Da)
1	Ovalbumin	44,000
2	Myoglobin	17,000
3	Aprotinin	6,700
4	Neurotensin	1,700
5	Uridine	244

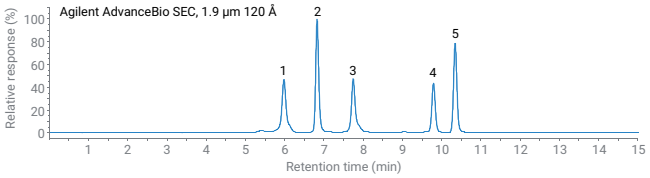

Figure 1. Size-exclusion chromatogram of low molecular weight protein and peptide mix at 0.35 mL/min.

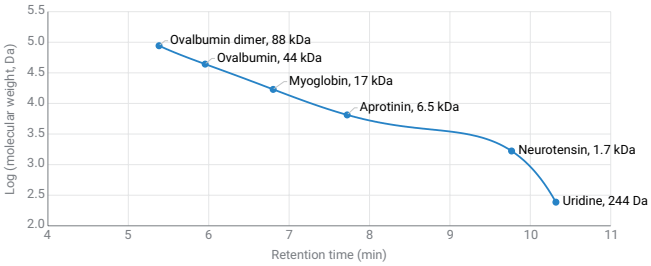

Figure 2. AdvanceBio SEC 1.9 µm 120 Å calibration curve of low molecular weight protein and peptide standards.

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Even if the intention is to use the AdvanceBio SEC column for quantification of monomer and dimer content, it is still good practice to regularly perform a calibration using appropriate molecular weight standards. Regular calibration ensures better reproducibility, leading to improved accuracy, and enables earlier detection of potential problems, reducing system downtime and troubleshooting. For protein separations, the standards should be a range of well-characterized proteins covering the entire operating range of the column. The proper choice of standards provide two key aspects for the successful use of SEC: There should be minimal, secondary interactions between the analyte and the stationary phase. The pore size should be chosen to match the size of molecules being analyzed.

This application note demonstrates high resolution separation with an Agilent AdvanceBio SEC 120 Å 1.9 µm column for size-exclusion chromatography (SEC) analysis of the recombinant hGH and hG-CSF therapeutic proteins compared to current competition with sub-2 µm particle technology. By further optimizing the mobile phase conditions, the SEC separation of nondegraded and thermally degraded interferon alpha-2b (IFN α-2b) is also compared.

By comparing the retention time of the analyte of interest with the calibration curve, it is possible to determine if there are any signs of secondary interactions. Peaks that elute earlier or later than expected or have poor shape are signs that the mobile phase conditions may not be sufficiently optimized. Figure 3 shows the size-exclusion chromatogram of hG-CSF on the AdvanceBio SEC 1.9 µm 120 Å column where the retention time corresponds well to that of a protein of around 20 kDa.

Figure 4 shows the close up of the baseline of hG-CSF run on the AdvanceBio SEC 1.9 µm 120 Å column as well as other sub-2 µm columns from other vendors. The chromatogram at the bottom of the diagram is indicative of problems associated with secondary interactions (later than expected elution time and tailing peak)

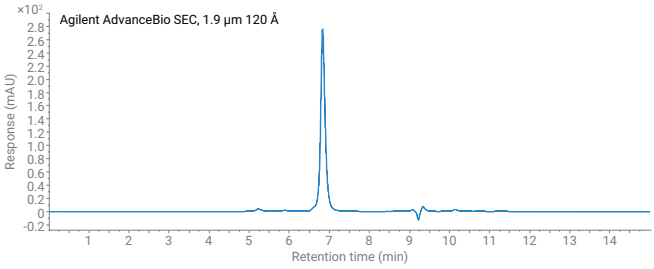

Figure 3. Size-exclusion chromatogram of hG-CSF on an Agilent AdvanceBio SEC 1.9 µm 120 Å 4.6 × 300 mm column at 0.35 mL/min.

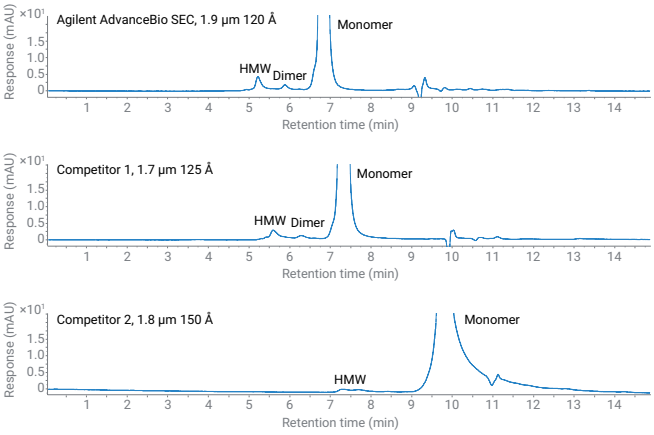

Figure 4. Close up of size-exclusion chromatograms of hG-CSF at 0.35 mL/min.

Many other biotherapeutic proteins have similar molecular weights and are therefore also suitable for analysis on the same AdvanceBio SEC 1.9 µm 120 Å column. The recombinant form of hGH, somatropin, may contain some impurities due to post-translational modification or as a result of downstream processing. Figure 5 shows the size-exclusion chromatogram of somatropin carried out under the same conditions as described previously. The inset shows the zoomed baseline region where dimer and higher molecular weight aggregates are evident.

Other proteins may require further method development to obtain the optimum peak shape and resolution. A series of experiments with different mobile phase conditions was used to determine the optimum composition for peak shape and protein recovery of IFN α-2b as shown in Table 2.

Table 1. Peak area data for high molecular weight (HMW), dimer, and monomer peaks for hG-CSF.

	AdvanceBio SEC 1.9 µm 120 Å				Competitor 1, 1.7 µm 125 Å				Competitor 2, 1.8 µm 150 Å			
	RT (min)	%Area	Rs USP	Peak Tailing	RT (min)	%Area	Rs USP	Peak Tailing	RT (min)	%Area	Rs USP	Peak Tailing
HMW	5.22	2.61		1.16	5.59	2.49		1.28	7.40	2.01		1.37
Dimer	5.88	1.02	2.41	1.11	6.27	0.83	1.68	1.26	N.D.			
Monomer	6.82	96.37	3.77	1.13	7.31	96.68	3.04	1.11	9.74	97.99		2.13

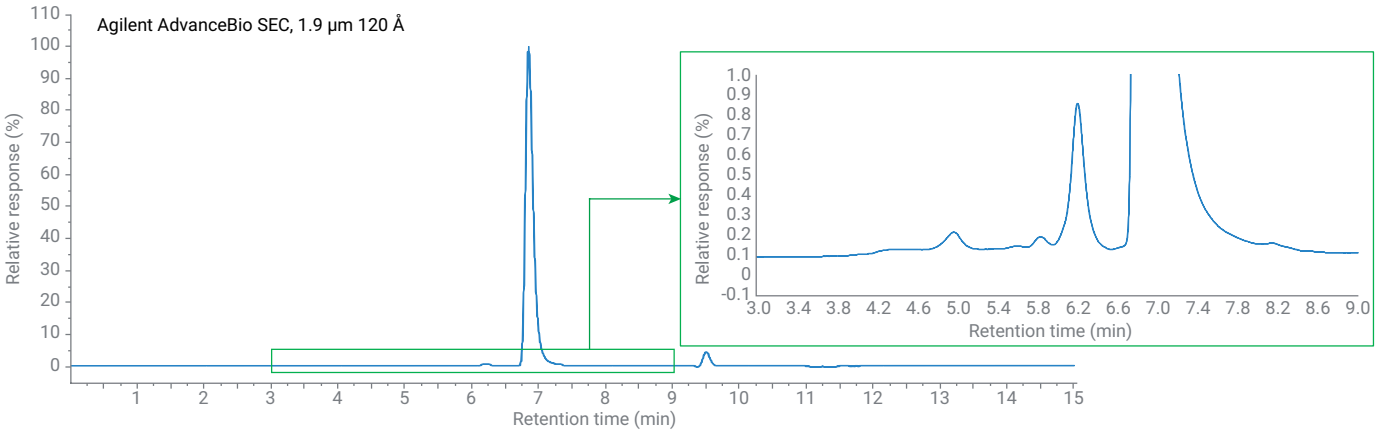

Figure 5. Size-exclusion chromatogram of somatropin (rhGH).

Table 2. Peak shape data during method optimization for IFN α-2b.

NaCl (mM)	Peak Width (min)	Tailing	Resolution HMW-Monomer	Resolution Monomer-LMW
100	0.20	2.88	1.94	1.98
150	0.18	2.65	2.25	2.31
200	0.16	2.52	2.26	2.66
250	0.15	2.39	2.84	2.86
400	0.14	2.08	3.32	3.59

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The size-exclusion chromatograms of interferon alpha-2b reference material run on three different sub-2 µm SEC columns is shown in Figure 6, along with the retention time and peak tailing data. The difference in column performance may lead to a difference in resolution when separating IFN α-2b impurities by SEC therefore the experiment was repeated using a degraded sample.

In the case of interferon alpha-2b, it has been suggested that the partial unfolding of the molecule is involved in the formation of aggregates, but that the partially unfolded species are somewhat stable.3 Furthermore, the presence of O-glycosylation can also reduce the thermal stability of these molecules4. The choice of cell line for recombinant protein manufacture is a critical parameter since E. coli cell lines do not introduce glycosylated variants.

Method conditions

Parameter	Optimized HPLC Conditions for INF α-2b
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile phase	200 mM Sodium phosphate + 250 mM NaCl, pH 6.5
Flow rate	0.35 mL/min
Column temperature	25 °C
Injection volume	2 µL, 1 mg/mL
Samples	Interferon alpha-2b (INF α-2b) Heat stressed interferon alpha-2b (INF α-2b): 60 °C for 30 min
Total run time	15 min

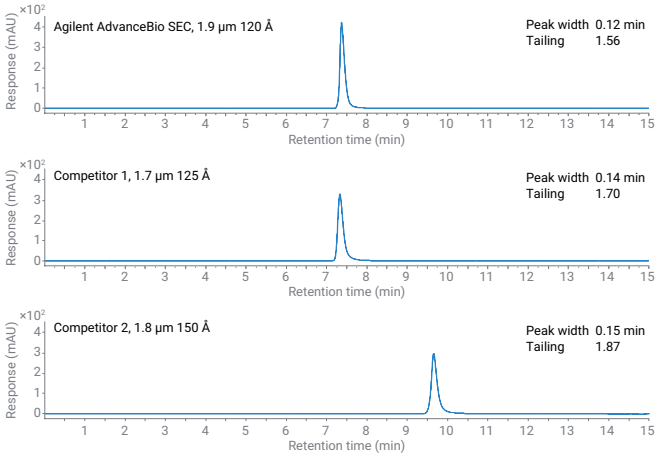

Figure 6. Size-exclusion chromatograms of interferon α-2b.

The size-exclusion chromatograms of interferon alpha-2b reference material run on three different sub-2 µm SEC columns is shown in Figure 6, along with the retention time and peak tailing data. The difference in column performance may lead to a difference in resolution when separating IFN α-2b impurities by SEC therefore the experiment was repeated using a degraded sample.

In the case of interferon alpha-2b, it has been suggested that the partial unfolding of the molecule is involved in the formation of aggregates, but that the partially unfolded species are somewhat stable.3 Furthermore, the presence of O-glycosylation can also reduce the thermal stability of these molecules4. The choice of cell line for recombinant protein manufacture is a critical parameter since E. coli cell lines do not introduce glycosylated variants.

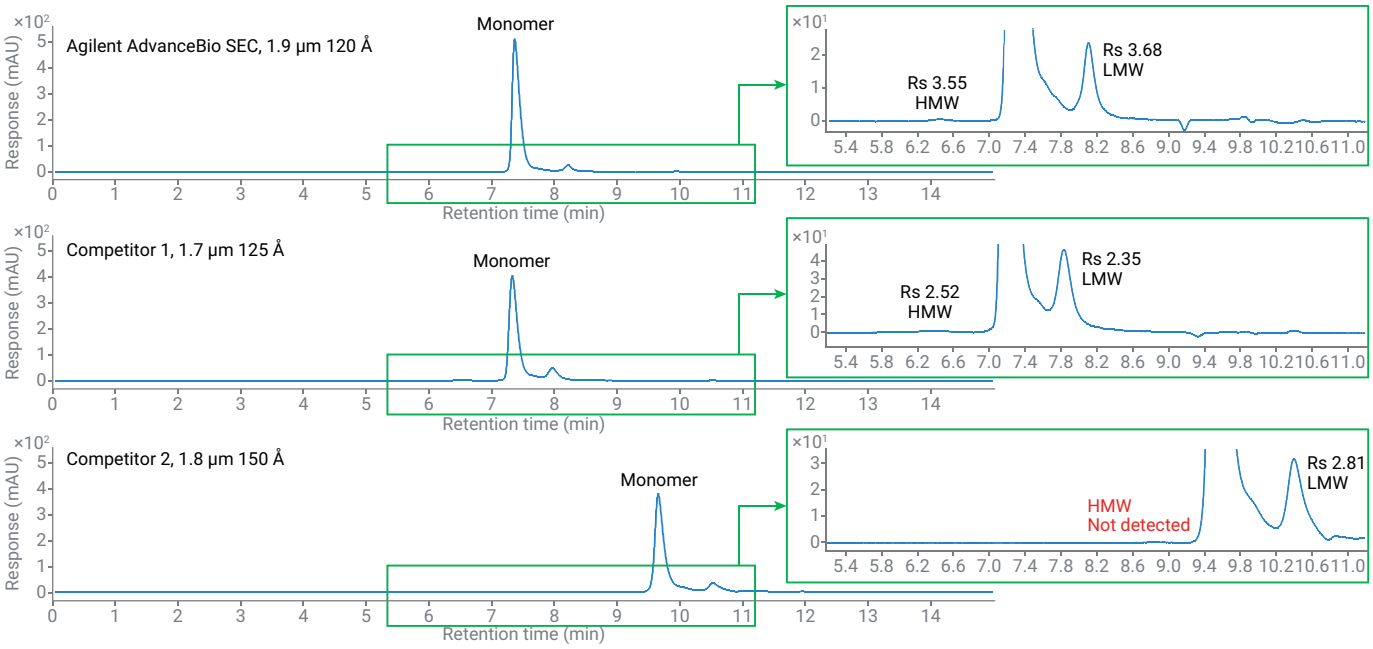

Figure 7. Size-exclusion chromatograms of interferon α-2b.

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Conclusion

Agilent AdvanceBio SEC offers a range of column dimensions and different pore sizes suitable for differently sized molecules. The featured AdvanceBio SEC 120 Å 1.9 µm column demonstrates superior performance with high resolution SEC analysis of small protein therapeutic applications when compared to columns of similar particle size and pore size characteristics from other vendors.

Calibrating your AdvanceBio SEC size exclusion column with appropriate standards ensures you understand the correct working range. These standards allow you to use calibration curves to estimate the molecular size of unknown molecules. However, regular calibration with a selection of standards is beneficial, and can be used to monitor column performance over time, allowing early detection of potential problems. In turn, corrective action can be taken, ultimately reducing system downtime and improving productivity.

References

1. Bayol, A. *et al. Pharmeuropa Bio*. 2004, **2004***(1)*, 35–45.
2. Advani, S. H. *et al. Indian J. Med. Paediatr. Oncol*. **2010** (Jul–Sep), *31(3)*, 79–82.
3. Sharma, V. K.; Kalonia, D. S. *Pharmaceutical Research* **2003**, *20*, 1721–1729
4. Johnston, M. J. W. *et al. Pharmaceutical Research* **2011**, *28*, 1661–1667

Published in the USA, March 30, 2020
5994-1829EN

Application Note

Aggregate/Fragment Analysis

Agilent

Trusted Answers

Analysis of Covalent High Molecular Weight Insulin

Improvements in speed and resolution with high performance size exclusion chromatography

Author

Sandeep Kondaveeti

Agilent Technologies, Inc.

Abstract

The analysis of insulin was done with an HPLC size exclusion chromatography (SEC method using an Agilent AdvanceBio SEC 120 Å pore size, sub-2 µm hydrophilic polymer coated silica packing material. The results of the analysis were compared to traditional methods and competitor columns for performance and efficiency. Resolution of insulin and high molecular weight (HMW) proteins were significantly improved over results from traditional methods with the AdvanceBio column. Chromatographic run times were shorter, and high-throughput insulin sample analysis became a reality.

Introduction

Insulin is a small polypeptide hormone that controls blood glucose homeostasis and is widely used in diabetes treatment. Genetic engineering techniques have enabled biopharma companies to develop diverse, long-acting insulin analogs. It has long been known that, when subjected to acidic conditions and high temperature, insulin monomers form amyloid-like fibrils.[1] For the insulin analogue manufacturer, this is especially problematic, since insulin analogs can have a higher propensity for aggregation than native insulin.[2] One of the critical quality control attributes for injectable insulin is the control of insulin fibrillation, commonly known as high molecular weight (HMW) proteins. The current US (USP) and European (EP) pharmacopoeia monograph methods for HMW aggregates determination are based on HPLC size exclusion chromatography (SEC).[3,4] According to the EP method, the use of a "hydrophilic silica gel for chromatography R (5 to 10 µm) with a pore size of 12 to 12.5 nm, of a grade suitable for the separation of insulin monomer from covalent dimer and polymers" with a length of 30 cm and a minimum internal diameter of 7.5 mm are prescribed. However, the method requires a lengthy 35 minute run time that is cost prohibitive for any laboratory performing high throughput sample analysis. The application presented here describes a SEC method developed using an Agilent AdvanceBio SEC 120 Å pore size, sub-2 µm hydrophilic polymer coated silica packing material. Some of the advantages of this method include faster run times and higher resolving separations of insulin and covalent insulin HMW compared to traditional pharmacopoeia methods.

Method conditions

Parameter	HPLC Conditions
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile phase	Arginine (1.0 g/L)/acetic acid/acetonitrile (65/15/20 v/v/v)
Flow rate	0.30 mL/min
Column temperature	25 °C
Injection volume	2 µL
Samples	Human insulin control, Heat-stressed insulin (60 °C for six hours)
Total run time	15 min

Experimental

Equipment and materials

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 preparation

To perform native MS analysis, it is crucial to preserve the protein samples at neutral pH and volatile aqueous solutions, such as ammonium acetate or ammonium formate. Therefore, sample desalting and buffer exchange are usually needed prior to the MS analysis. Briefly, protein stock solutions (1 to 10 mg/mL) were desalted and solvent exchanged into 100 mM ammonium acetate using Bio-Rad Bio-Spin P-6 (6,000 MW limit) or P-30 (40,000 MW limit) cartridges. The cartridge was first fully equilibrated with 100 mM ammonium acetate buffer. Protein sample was then pipetted to the top of the column and centrifuged for 5 min at 1000 × g. The protein was then buffer exchanged into the 100 mM ammonium acetate and was ready for MS analysis. This desalting protocol caused minimal sample loss and much less structural alteration of the protein molecule.

Instrumentation

Agilent 1260 Infinity II Bio-inert LC instrument comprising:

- Agilent 1260 Infinity II Bio-inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-inert Multisampler (G5668A) with sample cooler (option #100)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with bio-inert heat exchanger (option #019)
- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A)

Software

Agilent OpenLab 2.2 CDS.

Sample preparation

The control human insulin (Sigma, I2643) and heat-stressed insulin samples were prepared as per the Ph. Eur.

Sample was reconstituted and diluted to 4.0 mg/mL in 0.01 N hydrochloric acid solution, then further diluted to 2 mg/mL as the final concentration.

Results and discussion

The focus of this application is a performance evaluation of the AdvanceBio SEC 120 Å, 1.9 µm, 4.6 × 300 mm column under the conditions provided by the USP and EP monographs for the analysis of HMW species in insulin samples. The acidic mobile phase prescribed by both of these pharmacopeial methods is composed of 0.65 g/L L-arginine, 15% acetic acid, and 20% (v/v) acetonitrile. This mobile phase provides an assessment of the levels of covalent HMW present in these preparations while disrupting noncovalent insulin self-association and column interactions. The featured AdvanceBio SEC 1.9 µm columns are designed with hydrophilic polymer coating to minimize undesired secondary effects between analyte and surface particle chemistry. The columns provide superior resolution and accurate HMW protein quantification.

Figure 1 shows the chromatogram for system suitability with Ph. Eur. insulin control standard. Monomer and HWM species for the 1.9 µm column have a resolution of 4.03, which far exceeds the resolution of the monograph system suitability requirement of ≥2.0. Peak areas (Table1) for HMW proteins in the insulin control sample are within the <1% suitability requirement. Note the total analysis time required for this method is approximately 15 minutes using AdvanceBio SEC 120 Å, 1.9 µm, 4.6 × 300 mm column compared to reported 35 minutes in Ph. Eur. monograph with traditional hydrophilic silica column using higher particle size.

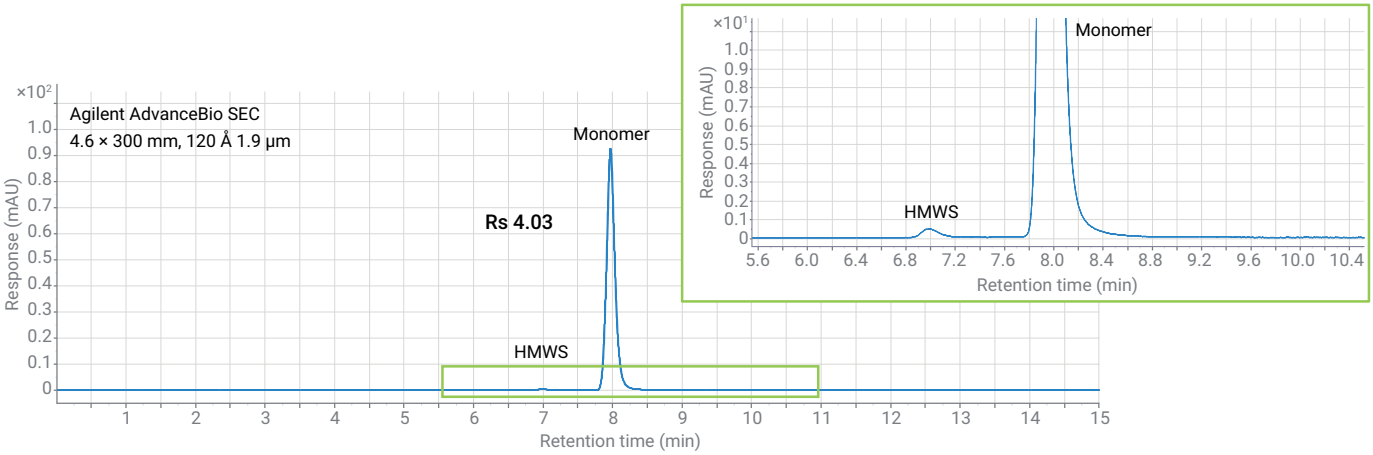

Figure 1. Size-exclusion chromatograms of human insulin control with monomer and HMW species.

Table 1. Results summary for SEC analysis of human insulin control.

Peak ID	RT (min)	% Area	Resol. USP	Tailing	Width 50%
Insulin HWM	6.99	0.67		1.48	0.15
Insulin Monomer	7.96	99.33	4.03	1.04	0.15

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The insulin SEC HMW determination method in the EP monograph prescribes an SEC particle size of 5 to 10 µm while the USP monograph does not specify a particle size limit. As part of this study, a comparison was performed among AdvanceBio SEC 120 Å 1.9 µm column and other SEC column vendors with sub-2-µm particles and equivalent 300 mm length with 4.6 mm id (Figure 2). The 1.9 µm Agilent column demonstrates a significant increase (>50%) in resolution as compared to the competitor columns. The improved resolution is also apparent in the insulin monomer peak tail, in which lower molecular weight fragment peaks are better resolved with Agilent SEC compared to the vendor 2 SEC column. Significant peak tailing observed in the vendor 2 SEC column might be due to undesired secondary interactions. It is important to note that different elution times of insulin peaks are due to pore size differences between these columns.

The percent peak areas of HMW species in the heat stressed insulin sample exceeds 1% for all the columns, indicating that sample would not pass the suitability test limits. However, higher % aggregates were resolved using Agilent SEC 120 Å, 1.9 µm column compared to other vendor columns.

Data are summarized in Table 2. Improvements are seen in efficiency for monomer and covalent dimer peaks in the AdvanceBio SEC 120 Å, 1.9 µm, 4.6 × 300 mm column method, again lending to the increased resolution and reduced run times with the updated method. According to USP (USP37-NF32S1) and EP guidelines, a 50% reduction in particle size and a 25% change in the column inner diameter for isocratic methods are the maximum allowable adjustments. Based on these requirements, featured SEC methods for insulin analysis with sub-2 µm particle size and 4.6 mm id would require further method validation and optimization to incorporate modern particle technology into established methods.

Table 2. Results summary of competitor SEC analysis for stressed insulin sample.

	AdvanceBio SEC 1.9 µm 120 Å			Vendor 1 SEC 125 Å 1.7 µm			Vendor 2 SEC 150 Å 1.8 µm		
Peak ID	%Area	Peak Tailing	Peak Width 50%	%Area	Peak Tailing	Peak Width 50%	%Area	Peak Tailing	Peak Width 50%
HMWS	1.93			1.57			1.10	2.01	
Insulin Monomer	97.66	1.00	0.13	97.85	1.10	0.13	98.80	1.37	0.14
LMWS	0.41			0.58			0.20	97.99	

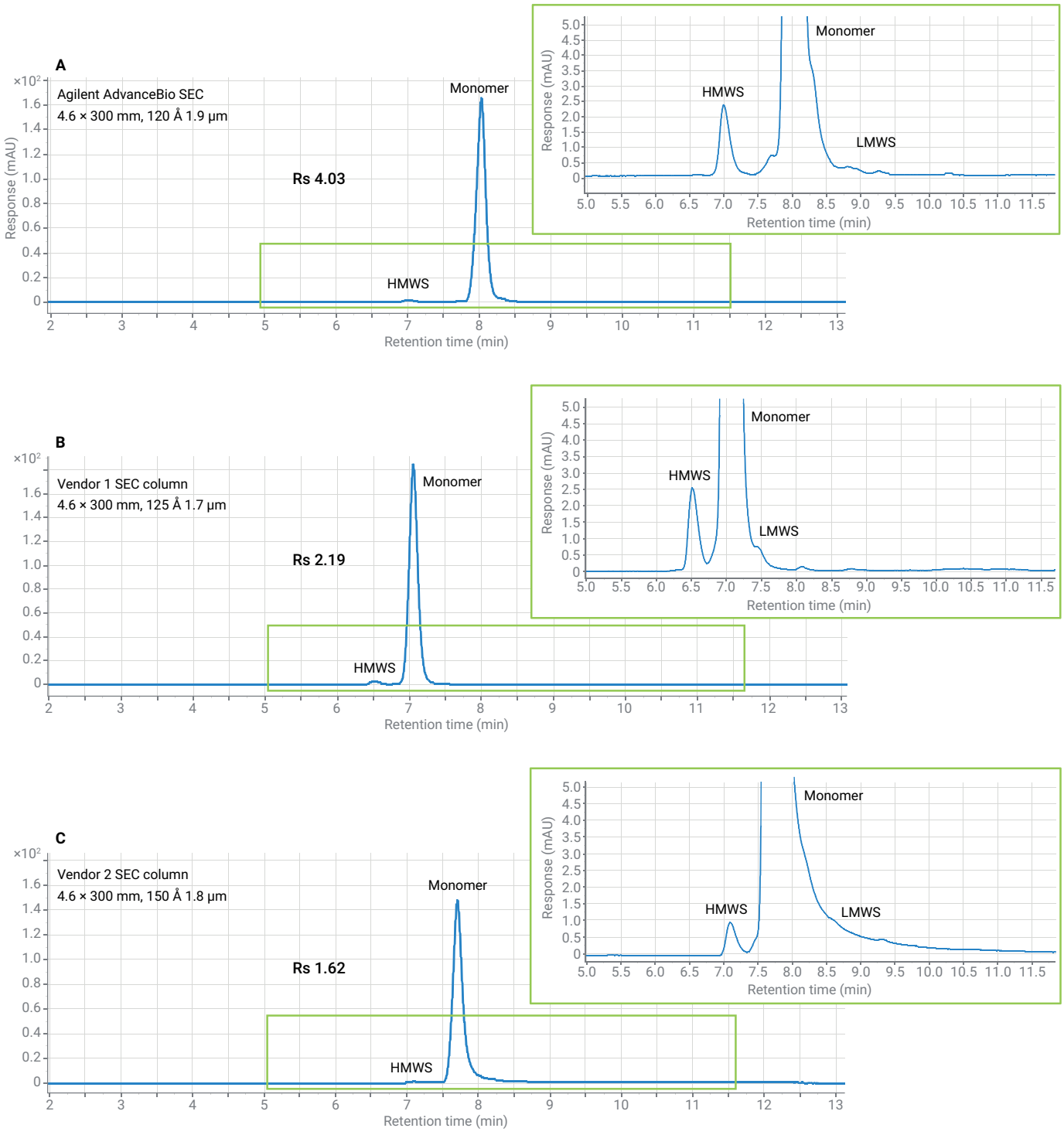

Figure 2. Competitor SEC analysis of stressed insulin with aggregates and low molecular weight fragments.

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Conclusion

Size exclusion chromatography is the USP and EP standard method for the analysis of covalent HMW insulin in therapeutic preparations. The chromatographic profiles demonstrating the performance of this method using SEC columns of different competitors have been presented. Based on these results the use of Agilent BioAdvance SEC 120 Å pore size, AdvanceBio SEC 1.9 µm column, and Agilent Infinity II Bio-inert liquid chromatography instrumentation for this SEC-based analysis provides significant improvements in resolution compared to traditional SEC-HPLC methods while reducing analysis time and mobile phase use.

References

1. Yang, Y., *et al*. An Achilles' Heel in an Amyloidogenic Protein and Its Repair: Insulin Fibrillation and Therapeutic Design." *J. Biol. Chem*. **2010**, 10806–10821.
2. Librizzi; Fabio; Rischel. The Kinetic Behavior of Insulin Fibrillation is Determined by Heterogeneous Nucleation Pathways. *Protein Science* **2005**, *12*, 3129–3134.
3. Insulin Monograph, *USP Pharmacopeial Forum*, *31(5)*, 1375.
4. European Pharmacopeia 5.0, *Human Insulin Monograph* **2005**, *838*, 1800–1802.

Published in the USA, February 6, 2020
5994-1566EN

Application Note

Aggregate/Fragment Analysis

Agilent

Trusted Answers

Calibrating your Agilent AdvanceBio SEC Columns

Technical Overview

Authors

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Introduction

Agilent AdvanceBio SEC columns are specifically designed for aqueous size exclusion chromatography (SEC) of biomolecules. The particles have been manufactured using proprietary technology to combine optimum pore size and pore volume for separating molecules such as proteins, polysaccharides, or aqueous soluble polymers.

Even if the intention is to use the AdvanceBio SEC column for quantification of monomer and dimer content, it is still good practice to regularly perform a calibration using appropriate molecular weight standards. Regular calibration ensures better reproducibility, leading to improved accuracy, and enables earlier detection of potential problems, reducing system downtime and troubleshooting.

For protein separations, the standards should be a range of well characterized proteins covering the entire operating range of the column. For polysaccharide analysis, pullulan polysaccharide molecular weight standards should be used, and for synthetic polyethylene glycol or polyethylene oxide, PEG/PEO standards are suitable.

SEC is a relatively straightforward technique. It relies on simple diffusion into the pore structure of the stationary phase; larger molecules cannot permeate the particles, and elute first, while smaller molecules diffuse readily into the pores, and elute later. This provides two key aspects for the successful use of SEC:

- There should be no secondary interactions between the analyte and the stationary phase
- The pore size should be chosen to match the size of molecules being analyzed

Proteins are complex molecules containing numerous side chain functionalities: acid, basic, neutral, and hydrophobic. Finding the optimum conditions to avoid secondary interactions can be challenging, however the AdvanceBio SEC product range has a polymeric surface coating applied to the silica particle that overcomes many of these issues.

The mechanism of separation relies on differences in size of molecules in solution. Protein structures are often compact and globular in nature, and proteins often aggregate under stress conditions such as extremes of temperature, pH, or salt composition and for dimers and larger units [1]. This is a particular issue for molecules such as monoclonal antibodies, where the presence of aggregated proteins can lead to adverse effects if administered. SEC provides the ideal tool for quantifying and monitoring protein aggregation. In comparison, polysaccharides and synthetic polymers such as polyethylene oxide do not possess a wide range of functional groups, but are often made up of a series of closely related oligomers. In this case, SEC can provide the analysis of molecular weight, molecular weight distribution, and branching information [2].

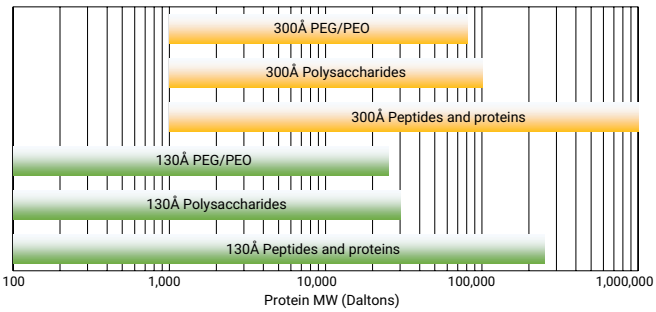

Figure 1. Resolving ranges for Agilent AdvanceBio SEC columns.

Calibrating your Agilent AdvanceBio SEC column

Choose the eluent for analysis. The samples need to be soluble in the mobile phase, and there should be no secondary interactions that lead to molecules eluting earlier or later than expected. For proteins, we recommend 150 mM sodium phosphate, pH 7.0 as this eliminates sodium chloride, which may lead to corrosion, from your HPLC system. However, other mobile phases including phosphate buffered saline (PBS), or high ionic strength mobile phases such as 0.1 M sodium phosphate/0.1 M sodium sulphate may still be used (see Pages 10–11).

Dissolve the standards in the mobile phase. Ensure that the solution is clear and tha there is no insoluble material present. If necessary, filter the sample. Consider changing the mobile phase to ensure that the samples fully dissolve.

Record the chromatogram for each of the standards, and plot the retention time (x-axis) versus the molecular weight (y-axis) to create the calibration curve. Notice that the y-axis is plotted on a logarithmic scale.

Conditions

Parameter	Value
Column:	Agilent AdvanceBio SEC 130 Å, 2.7 µm, 7.8 × 300 mm (p/nPL1180-5350) Agilent AdvanceBio SEC 300 Å, 2.7 µm, 7.8 × 300 mm (p/nPL1180-5301)
Samples:	Agilent polyethylene glycol calibration kit, PEG-10, 10 × 0.2 g (p/nPL2070-0100) Agilent polyethylene oxide calibration kit, PEO-10, 10 × 0.2 g (p/nPL2080-0101) AdvanceBio SEC 130 Å Protein Standard, lyophilized, 1.5 mL (p/n5190-9416) AdvanceBio SEC 300 Å Protein Standard, lyophilized, 1.5 mL (p/n5190-9417) Protein standards (Sigma-Aldrich) Samples prepare at 0.5 – 1.0 mg/mL in mobile phase
Eluent A:	150 mM Sodium phosphate buffer, pH 7.0, PBS, pH 7.4 (10 mM phosphate, 140 mM NaCl) or 100 mM Sodium phosphate + 100 mM sodium sulfate, pH 7.0
Flow rate:	1.0 mL/min
Detector:	RI for polysaccharides and PEG / PEO standards UV, 220 nm for peptides and proteins
System:	Agilent 1260 Infinity Bio-inert LC (with additional refractive index detector)

Peptide and Protein Calibration

Proteins and peptides are generally very compact molecules containing multiple hydrogen bonds, electrostatic interactions, or covalent bonds such as disulphide bridges. Choosing a range of standards covering the operating range of the column for calibration is preferred. It is also possible to use prepared standard mixtures, as peaks are likely to be clearly defined and well resolved (Figures 3A and 3B).

The presence of a diverse range of side chain functionality from different amino acids may mean undesirable secondary interactions can occur, and so different mobile phase compositions may need to be tested.

The peaks shown are generally very sharp and well defined since they represent a single molecular species. It is quite common for proteins to contain aggregates, and these too are typically well resolved – evident as an earlier eluting peak or series of peaks.

		Retention time (min)	
Protein/Peptide	**MW**	**130 Å**	**300 Å**
Thyroglobulin	670,000	4.60	5.14
γ-Globulin	150,000	4.90	6.53
BSA	66,000	5.53	7.57
Ovalbumin	44,300	6.04	8.13
Myoglobin	17,600	6.77	8.79
Cytochrome C	12,327	6.95	8.92
Aprotinin	6,511	7.56	9.38
Neurotensin	1,672	9.42	10.54
Angiotensin-II	1,040	9.94	10.82

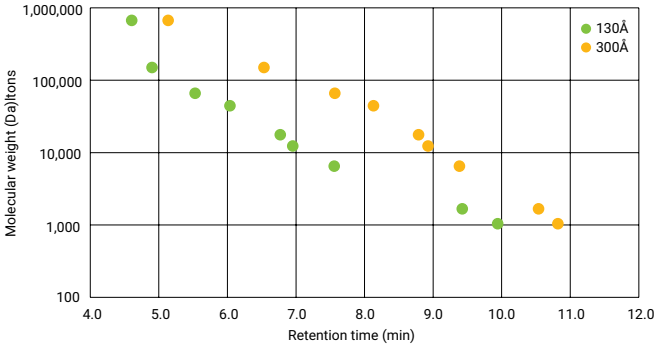

Figure 2. Peptide and protein calibration on Agilent AdvanceBio SEC columns.

Representative chromatograms for protein and peptide standards

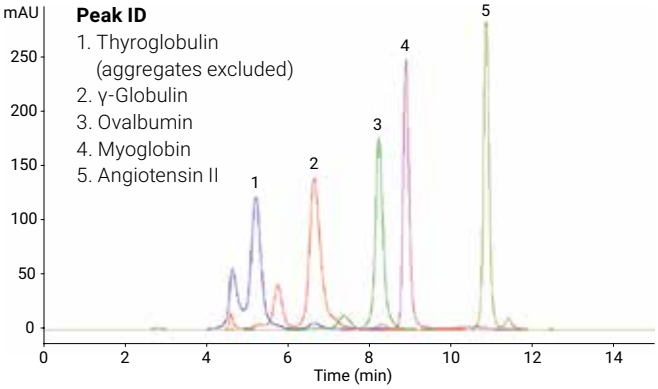

Figure 3A. Individual peptide and proteins contained in Agilent AdvanceBio SEC 300 Å Protein Standard (p/n5190–9417) on an Agilent AdvanceBio SEC 300 Å column.

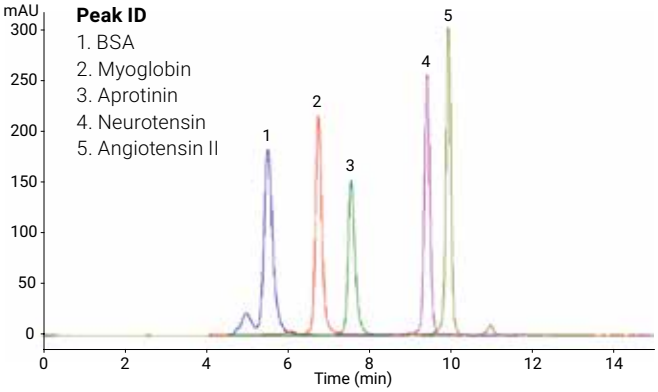

Figure 3B. Individual peptides and proteins contained in Agilent AdvanceBio SEC 130 Å Protein Standard (p/n5190–9416) on an Agilent AdvanceBio SEC 130 Å column.

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Polysaccharide Calibration

Calibration with polysaccharide standards should be used when your analyte is also a polysaccharide, for example starch or cellulose. There are many types of polysaccharide, including linear, branched, or sulfated molecules. Unlike peptides or proteins, they comprise of a range of chain lengths and can extend to very large sizes. Therefore, it is important to ensure that the column you are using is capable of resolving the molecular weight range of polysaccharide you are investigating.

The mass difference of 162 for each additional hexose means that separating individual oligomers is very difficult unless the sample is sufficiently small (Figure 5C), achieved using two columns in series for additional resolution.

The combination of two columns run in series can increase resolution to the extent that individual oligomers are becoming visible in the 5,800 Da polysaccharide sample.

This sample contains oligomers of approximately 30–40 units, with a mass difference of 162 Da each.

		Retention time (min)	
Polysaccharide	**MW**	**130 Å**	**300 Å**
788K	788,000	4.48	4.74
380K	380,000	4.58	4.84
100K	100,000	4.73	5.33
48K	48,000	4.89	6.19
23.7K	23,700	5.39	7.42
5.8K	5,800	7.16	9.22
Maltotriose	504	9.46	10.72
Maltose	342	9.73	10.82
Glucose	180	10.01	11.09

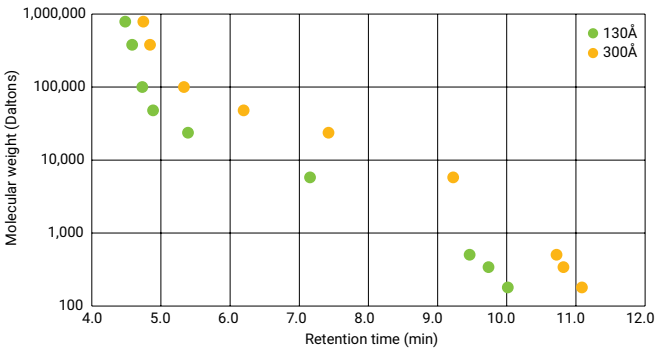

Figure 4. Polysaccharide calibration on Agilent AdvanceBio SEC columns.

Representative chromatograms for polysaccharide standards

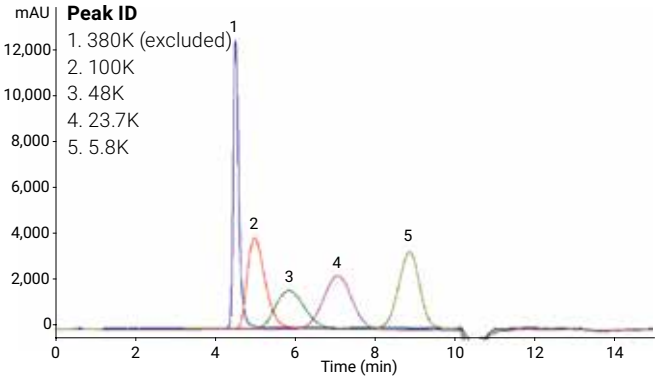

Figure 5A. Polysaccharide standards on an Agilent AdvanceBio SEC 300 Å column.

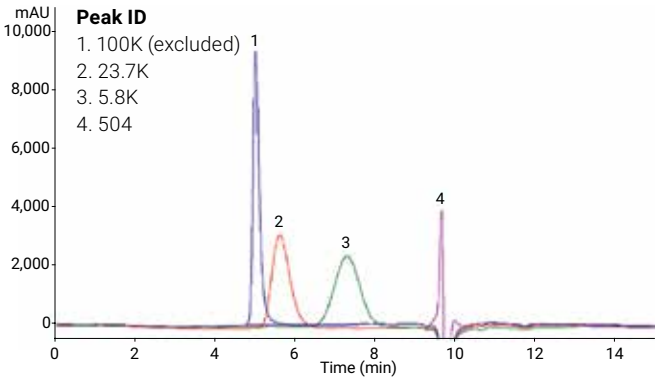

Figure 5B. Polysaccharide standards on an Agilent AdvanceBio SEC 130 Å column.

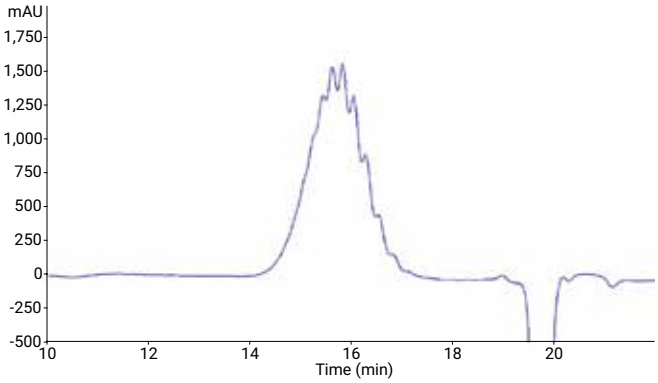

Figure 5C. Polysaccharide 5.8K run on Agilent AdvanceBio SEC columns 300 Å + 130 Å in series.

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PEG/PEO Calibration

Polyethylene glycol (PEG) and polyethylene oxide (PEO) are synthetic polyethers, available in a range of sizes covering a diverse molecular weight range. Similar to polysaccharides, they comprise of a range of chain lengths, however the mass difference is just 44 Da.

		Retention time (min)	
PEG/PEO	MW	130 Å	300 Å
905K	905,000	4.41	4.73
692K	692,000	4.41	4.73
498.6K	498,600	4.58	4.83
305.5K	305,500	4.55	4.85
135.8K	135,800	4.65	4.93
77.35K	77,350	4.71	5.20
46.47K	46,470	4.77	5.67
21.3K	21,300	5.11	6.81
12.14K	12,140	5.65	7.74
8.73K	8,730	6.10	8.28
3.87K	3,870	7.26	9.32
1.48K	1,480	8.59	10.26
420	420	9.79	11.03
106	106	10.64	11.44

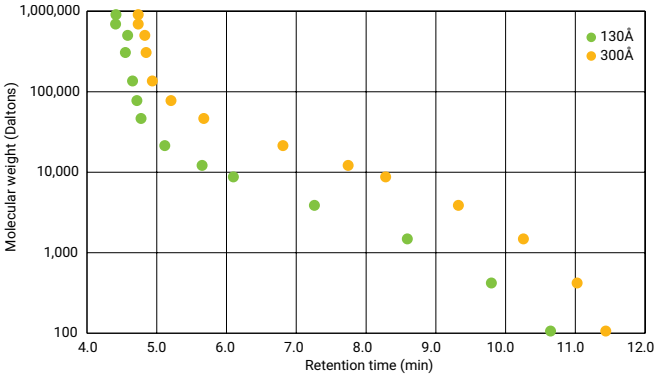

Figure 6. PEG/PEO Calibration on Agilent AdvanceBio SEC columns.

Representative chromatograms for PEG/PEO standards

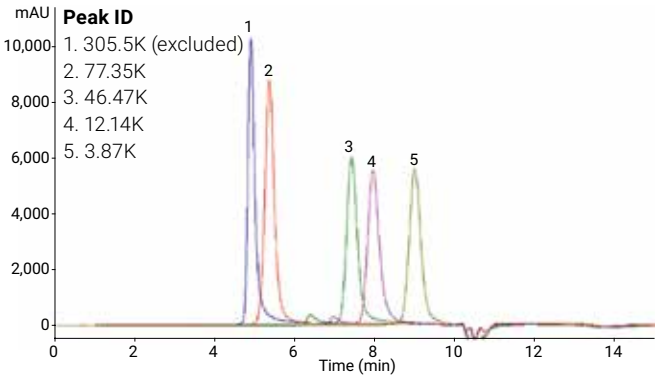

Figure 7A. PEG/PEO standards on an Agilent AdvanceBio SEC 300 Å column.

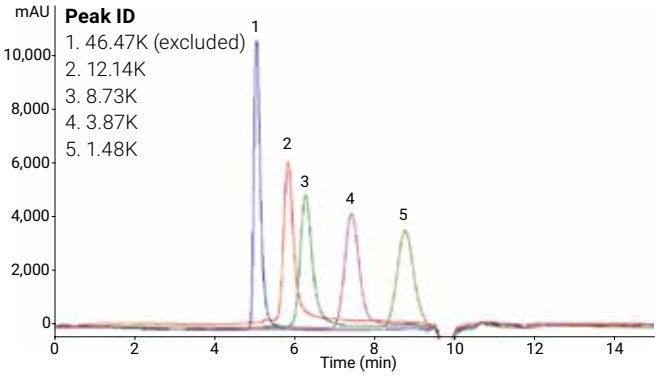

Figure 7B. PEG/PEO standards on an Agilent AdvanceBio SEC 130 Å column.

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Effect of Mobile Phase Composition

The impact of mobile phase on the size of the molecule in solution, or in overcoming secondary interactions needs to be taken into consideration, as this will influence the retention time and alter the shape of the calibration curve. This is clearly visible in the comparison of three different mobile phases: 150 mM sodium phosphate, pH 7.0; PBS, pH 7.4 (approximately 10 mM sodium phosphate, 140 mM NaCl); 0.1 M sodium phosphate + 0.1 M sodium sulfate, pH 7.0 (Figure 8).

For molecules that do not present secondary interactions, such as polysaccharides, the effect of mobile phase composition is minimal (Figure 9).

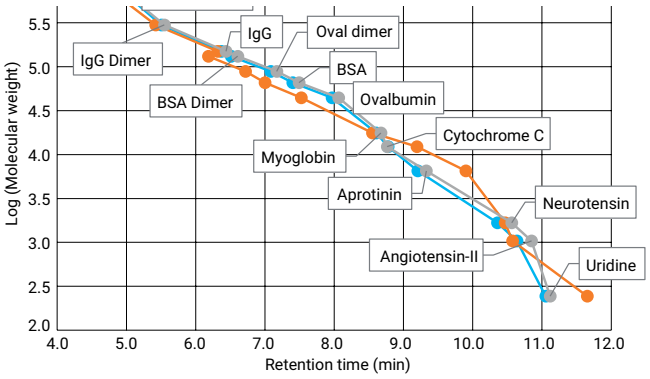

Figure 8. Effect of mobile phase composition on column calibration with protein standards.

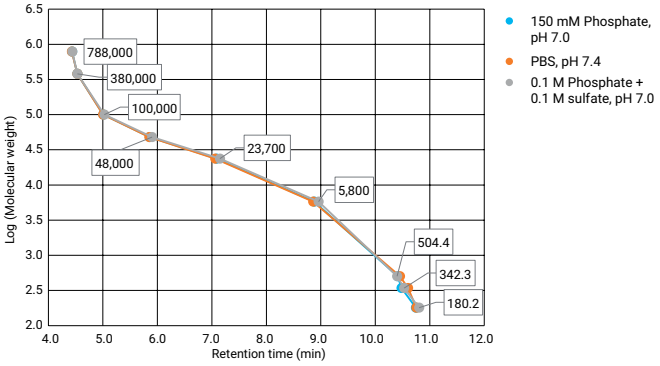

Figure 9. Effect of mobile phase composition on column calibration with polysaccharide standards.

When choosing a mobile phase for peptide or protein analysis by SEC, it is important to determine the effect that differences in pH or ionic strength may have on the sample of interest. Figures 10A, 10B, and 10C show a noticeable shift in the retention time of BSA under different mobile phase conditions. Resolution factors between dimer and monomer peaks may also be affected, so method optimization and method robustness should be fully explored.

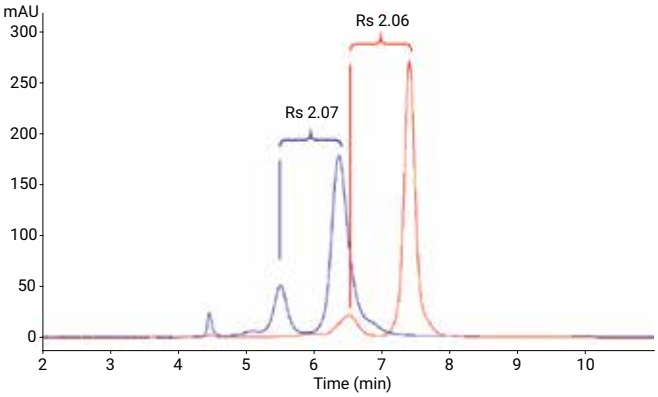

Figure 10A. γ-Globulin and BSA on an Agilent AdvanceBio SEC 300 Å column using 150 mM sodium phosphate, pH 7.0.

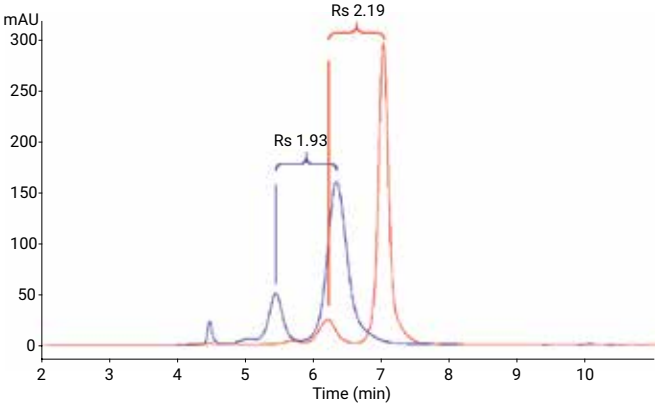

Figure 10B. γ-Globulin and BSA on an Agilent AdvanceBio SEC 300 Å column using PBS, pH 7.4.

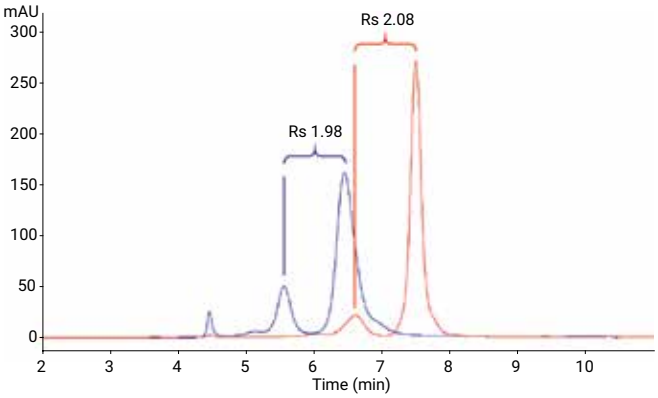

Figure 10C. γ-Globulin and BSA on an Agilent AdvanceBio SEC 300 Å column using 100 mM sodium phosphate + 100 mM sodium sulfate, pH 7.0.

Conclusion

Calibrating your Agilent AdvanceBio SEC size exclusion column with appropriate standards ensures you understand the correct working range, and can allow you to use calibration curves to estimate the molecular size of unknown molecules. However, regular calibration with a selection of standards is beneficial, and can be used to monitor column performance over a period of time, allowing early detection of potential problems. In turn, corrective action can be taken, ultimately reducing system downtime and improving productivity.

Agilent offers a range of column dimensions and different pore sizes suitable for differently sized molecules. Together with the extensive range of Agilent LC instrumentation, consumables, and technical support, we can provide the ultimate workflow solution for your needs.

Ordering Information

Part Number	Description
PL1180-5301	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 7.8 × 300 mm
PL1180-3301	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 7.8 × 150 mm
PL1180-1301	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 7.8 × 50 mm guard
PL1580-5301	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 4.6 × 300 mm
PL1580-3301	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 4.6 × 150 mm
PL1580-1301	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 4.6 × 50 mm guard
5190-9417	Agilent AdvanceBio SEC 300 Å Protein Standard, lyophilized, 1.5 mL
PL1180-5350	Agilent AdvanceBio SEC 130 Å, 2.7 µm, 7.8 × 300 mm
PL1180-3350	Agilent AdvanceBio SEC 130 Å, 2.7 µm, 7.8 × 150 mm
PL1180-1350	Agilent AdvanceBio SEC 130 Å, 2.7 µm, 7.8 × 50 mm guard
PL1580-5350	Agilent AdvanceBio SEC 130 Å, 2.7 µm, 4.6 × 300 mm
PL1580-3350	Agilent AdvanceBio SEC 130 Å, 2.7 µm, 4.6 × 150 mm
PL1580-1350	Agilent AdvanceBio SEC 130 Å, 2.7 µm, 4.6 × 50 mm guard
5190-9416	Agilent AdvanceBio SEC 130 Å Protein Standard, lyophilized, 1.5 mL

See Also

1. Size exclusion chromatography for Biomolecule analysis: A "How-To" Guide; 5991-3651EN.

2. GPC/SEC standards: Product guide; 5990-7996EN.

3. Calibrating GPC columns: A Guide to Best Practice; 5991-2720EN.

References

1. Critical Reviews in Therapeutic Drug Carrier Systems **1993**, ***10(4)***, 307-377.

www.agilent.com/cs/library/slidepresentation/Public/1-Conventional_GPC_-_Polymers_ans_Molecular_Weight.pdf

Printed in the Printed in the USA, January 9, 2017
5991-7799EN

Application Note

Aggregate/Fragment Analysis

Agilent

Trusted Answers

Size Exclusion Chromatography of Biosimilar and Innovator Insulin

Using the Agilent AdvanceBio SEC column

Authors

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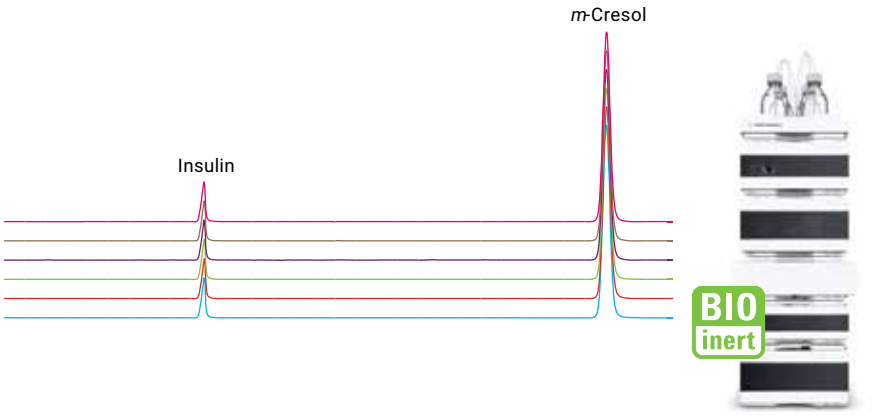
Agilent Technologies, Inc.

Abstract

Insulin is a small polypeptide hormone that controls blood glucose homeostasis. Genetic engineering techniques have enabled biopharma companies to develop diverse, long-acting insulin analogs. There is no pharmacopeia method available for the analysis of insulin analogs. An SEC method identifying innovator and biosimilar insulin analog, following a draft EP method, was developed using an Agilent AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm column. The effectiveness of this method, for routine analysis, was confirmed using a system suitability test, and retention time (RT) and area precision studies using innovator insulin as a reference material. This Application Note also presents the application of this column for detecting impurities with molecular masses greater than that of insulin for quantitation studies.



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Introduction

Novel insulin analogs are alternatives to human insulin products. Clinical trials have demonstrated equal or superior efficacy outcomes when these analogs are compared with human insulin. Insulin analogs are currently the long-acting basal human insulin on the market. Insulin analog was approved for use by the US Food and Drug Administration (USFDA) in April 2000. Unlike small molecules, biotherapeutics are created using biological processes. Each manufacturer uses an in-house developed process for the production of drug substance and drug product. These production methods can result in impurities derived from the drug substance, such as aggregates and degradation products. Due to the increased demand for antidiabetic drugs, it is a crucial yet challenging task to produce drugs free from impurities, and provide safe medicine free from side effects. In the biopharma industry, LC with UV detection is a versatile tool for lot release and characterization studies[1]. Size exclusion chromatography (SEC) is the method of choice for purity analysis, and for detecting aggregates of drug product. This Application Note describes a SEC-UV approach to determine the molecular similarity between insulin biosimilar and its innovator reference, following system suitability and method precision analysis[2]. These tests ensure that the method can generate results of acceptable accuracy and precision. The criteria selected is based on critical chromatographic parameters and their variation within acceptable limits, which are defined during the method evaluation experiments. An excellent correlation coefficient was observed for the linearity curve of insulin in the range of 10.6 to 3,400 µg/mL, indicating that the method is quantitative. Use of the Agilent AdvanceBio SEC column to monitor and separate impurities with molecular masses greater than the drug product, as determined by forced-stress studies, is also shown.

Table 1. Chromatographic parameters used for SEC HPLC.

Parameters	Conditions
Mobile phase	200 mL of anhydrous acetic acid, 300 mL of acetonitrile, and 400 mL of water, adjusted to pH 3.0 with concentrated ammonia, and diluted to 1,000.0 mL with water.
TCC temperature	Ambient
Isocratic run	Mobile phase A
Injection volume	10 µL
Flow rate	0.5 mL/min
UV detection	276 nm

Materials and Methods

Instruments

- A completely biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC System with a maximum pressure of 600 bar was used, consisting of:
- 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 DAD VL (G1315D with Bio-inert standard 10-mm flow cell)
- Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm (p/nPL1180-5350)
- Software
- Agilent ChemStation B.04.03 (or higher)
- Size exclusion chromatography parameters
- Table 1 shows the chromatographic parameters for size exclusion chromatography using an Agilent 1260 Infinity Bio-inert LC System.

Reagents, samples, and materials

Commercial innovator and biosimilar insulin were purchased from a local pharmacy, and stored according to manufacturer's instruction. Acetic acid and ammonia 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.

Procedure

A 10 µL volume of mobile phase was injected as a blank, followed by individual linearity levels in triplicate. Area and retention time (RT) of each level were used to calculate standard deviation (SD) and relative standard deviation (RSD %) values. Limits of detection (LODs) and limits of quantitation (LOQs) were established from the lower linearity level injections. The average area of each linearity level was plotted against the concentration of insulin to determine the calibration curve for the monomers.

Linearity and range

The calibration curve was constructed with nine standard concentrations of innovator insulin in the range 10.6 to 3,400 µg/mL.

LOQ and LOD

The insulin concentration that provides a signal-to-noise ratio (S/N) of > 3 was considered as the LOD, and S/N > 10 was considered as the LOQ.

Preparation of insulin aggregates

Aggregates of insulin were prepared following temperature stress. Briefly, about 3.4 mg/mL of the drug product was incubated at 60 °C for 6 hours in a polypropylene tube. Samples were cooled to room temperature, and immediately analyzed.

System suitability

As per the draft monograph, the following are the system suitability requirements:

- Symmetry factor: Maximum 2.0 for the peak due to insulin analog
- Peak-to-valley ratio: Minimum 2
- Total of all impurities with a retention time less than that of insulin analog: Not more than 0.3 % of the total area of the peaks, disregarding any peak with a RT greater than that of the insulin peak

Results and Discussion.

Separation and detection

The biosimilar insulin was compared using the innovator as the reference standard. The optimized SEC HPLC separation of intact biosimilar and innovator insulin on the AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm column achieved excellent separation. Homogenous profiles without any indication of aggregation were demonstrated within a total run time of 55 minutes. A peak due to the preservative m-cresol was also observed, eluting at approximately 49 minutes (Figure 1).

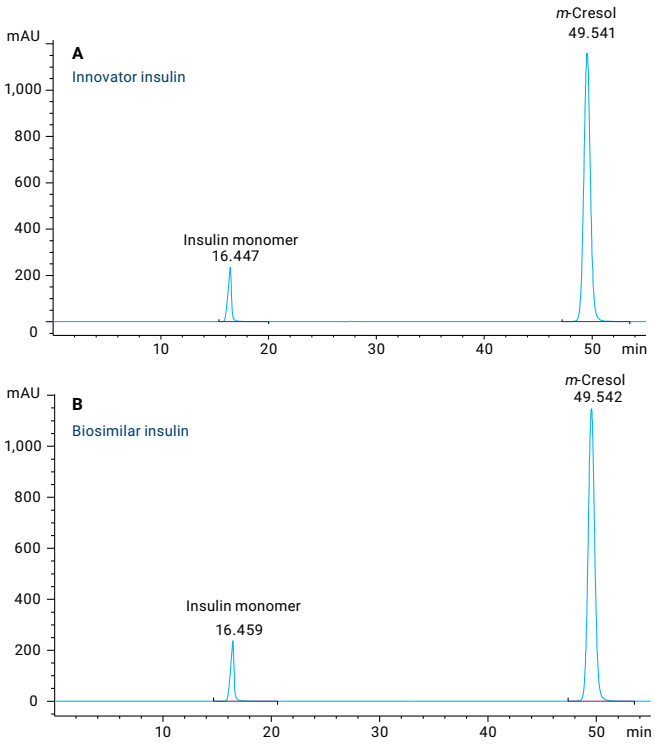

Figure1. SEC HPLC profile of insulin innovator and biosimilar on an Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm column.

Precision of retention time and area

Figure 2 shows the overlays of six replicates of innovator and biosimilar insulin, demonstrating excellent separation reproducibility. Table 2 lists the average RTs and peak area RSDs for the insulin monomer from six replicates. The RT and peak area RSDs for the insulin monomer were within the acceptable limit of ±3 % and ±5 %, respectively, demonstrating the excellent reproducibility and precision of this method.

System suitability

Table 3 tabulates the acceptance criteria for this system suitability study for insulin analog, and Table 4 presents the summary of the system suitability results.

These results of the system suitability test for insulin innovator and biosimilar demonstrate that the method performed using an Agilent Bio-inert LC and an AdvanceBio SEC column meets the stringent performance requirements for insulin QA/QC analysis.

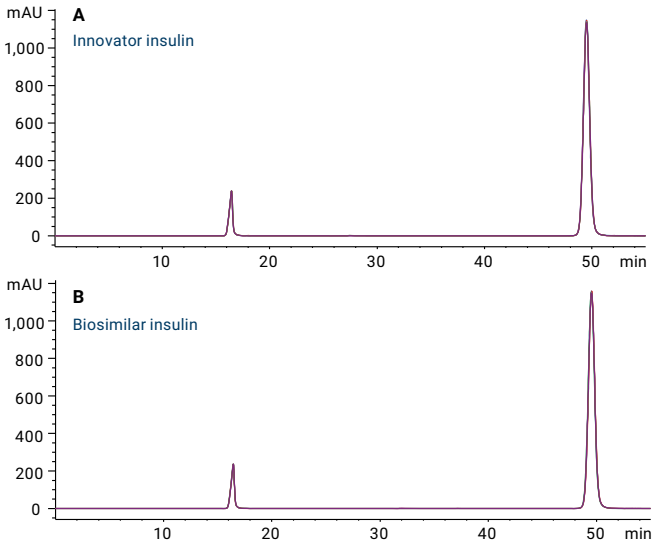

Figure2. Overlay of six replicates of innovator and biosimilar insulin separated on an Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm column.

Table 2. RT and peak area precision (n = 6).

	RT		Peak area	
Sample	Mean (min)	RSD	Mean (mAU/min)	RSD
Innovator insulin	16.450	0.057	5,544.91	0.285
Biosimilar insulin	16.460	0.044	5,459.55	0.662

Table 3. Acceptance criteria.

Parameter	Limit
Symmetry factor	Maximum 2.0 for the peak due to insulin analog
Peak-to-valley ratio	Minimum 2
Total of all impurities with an RT less than that of insulin analog	Not more than 0.3 % of the total area of the peaks

Table 4. Summary of system suitability test results.

	Results on an Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm column			
Sample	Symmetry factor	Peak-to-valley ratio	Total of all impurities with an RT less than that of insulin analog	Passed (Yes/No)
Innovator insulin	1.71	–	0.167	Yes
Biosimilar insulin	1.72	–	0	Yes

Table 5. LOD, LOQ, and S/N results (n = 3) for insulin innovator.

Concentration (µg/mL)	S/N	Average area
10.6 (LOD)	11.9	12.8
31.8 (LOQ)	34.7	37.4

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LOD and LOQ

The LOD and LOQ were tested for insulin innovator, and were found to be 11.3 µg/mL and 28 µg/mL, respectively, indicating that the method is sensitive. Table 5 shows the observed LOD and LOQ values of insulin innovator.

Linearity

Linearity curves for insulin innovator were constructed from the LOD level to the label claim (3.4 mg/mL) in the study, using the area response and concentration of insulin. Figure 3 shows the linearity curve for insulin in the concentration range 10.6 to 3,400 µg. The R2 value observed was more than 0.99, suggesting excellent dose-dependent correlation between the peak area and the concentration of insulin.

Aggregation/degradation analysis and quantification

The impurity profile of biotherapeutics is of increasing importance in drug safety. Although aggregates are present in extremely low concentrations, they may have a big impact on the quality of the product. The AdvanceBio SEC column is designed to have minimum interaction with biomolecules, enabling distinct baseline separation of insulin aggregates. These insulin aggregates elute from the AdvanceBio SEC column at 11.181 and 13.884 minutes, respectively, as shown in Figure 4.

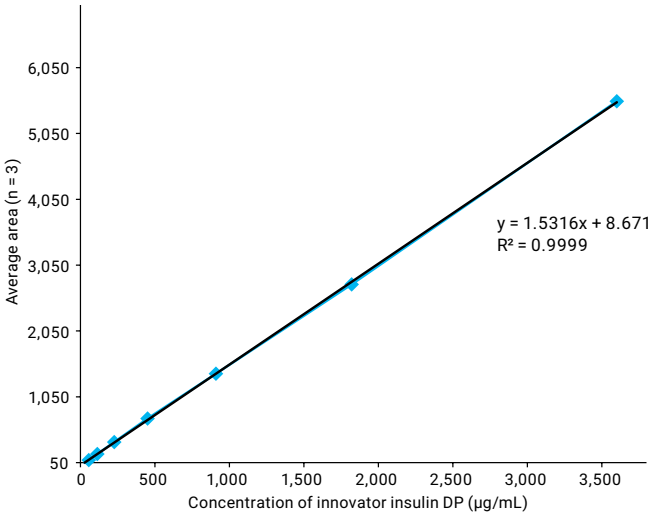

Figure 3. Linearity curve with standard concentrations of insulin ranging from 10.6 to 3,400 µg/mL, showing excellent coefficient value.

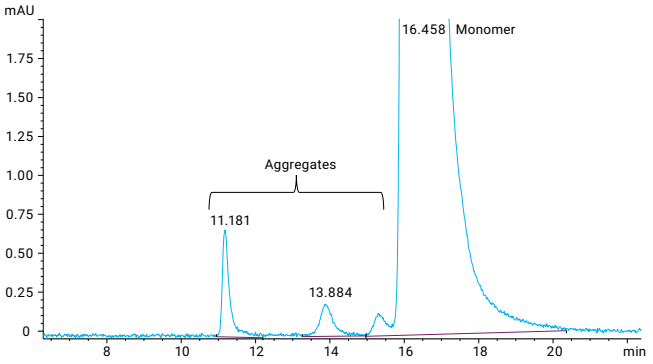

Figure 4. An Agilent AdvanceBio SEC profile of heat stressed insulin showing baseline separation of insulin aggregates.

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Economic value and lifetime studies

A laboratory head or group leader may primarily consider cost, particularly when comparing the cost of the AdvanceBio SEC column and other column types. In SEC separation, apart from the cost of the operator and instrument, the most expensive component is the cost of the column itself. If the columns do not last long enough, or there are column-to-column reproducibility issues, multiple columns may need to be screened. Ensuring batch-to-batch reproducibility through control of the entire production process is essential. Figure 5 shows the separation of AdvanceBio 130 Å protein markers on four separate batches of the AdvanceBio SEC 130 Å media, ensuring thorough control of the entire production process.

One of our objectives is to ensure extended column lifetime throughout our customers' development processes. This extended column lifetime provides extra benefits, as the downtime is greatly reduced. Figure 6 shows six overlaid chromatograms of the 250 injections of 3 mg/mL insulin drug substance taken at an interval of 50 runs. Table 6 shows the RT, area, tailing factors, and theoretical plates from the selected runs.

The results clearly demonstrate that there is virtually no change in RT, area, as well as tailing factor over the course of 250 injections. The theoretical plates, a measure of the efficiency of the column, also do not vary significantly.

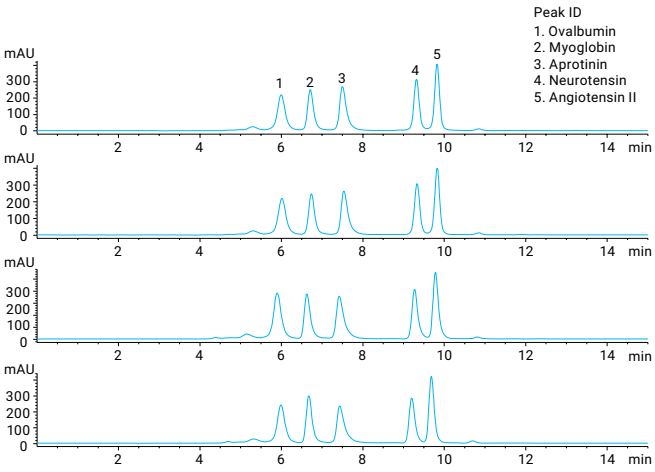

Figure 5. Separation of Agilent AdvanceBio 130 Å protein standards on four separate batches of an Agilent AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm media.

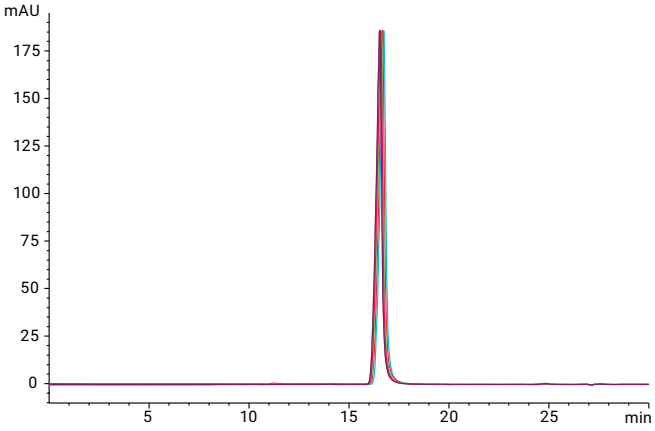

Figure 6. Overlay of six chromatograms for the 250 injection at an interval of 50 runs.

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Conclusion

Size exclusion chromatography is the workhorse for detecting and monitoring aggregates and monomers for biopharmaceuticals. This Application Note demonstrates the suitability of an Agilent AdvanceBio SEC 130 Å column as an excellent choice to study insulin analogs. We used the draft pharmacopeia method to develop a simple UV-based approach to define the molecular similarity between biosimilar and innovator insulin drug product using an AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm column. RT and area precision of the method were excellent, and met the system suitability requirements. A linear relationship between the peak area and eight standard concentrations of the insulin drug product was observed, with an outstanding coefficient of linearity value. The observed LOD and LOQ was found to be 10.6 and 31.8 µg/mL, respectively, indicating the sensitivity of the method. The AdvanceBio SEC column was able to separate and monitor aggregates analyzed by forced stress study. We have also shown the greater economic benefits of using an AdvanceBio SEC column, some of which are reducing lot-to-lot manufacturing variations, and prolonged column lifetime with reproducible and robust outcomes. This simple and reproducible method, coupled with a bio-inert and corrosion-resistant instrument is considered to be reliable and suitable for routine quality checks of insulin throughout the development process.

Table 6. Observed RT, area, tailing factor, and theoretical plates for 250 injections of insulin drug substance.

Injection no.	RT (min)	Area	Tailing factor	Theoretical plates
1	16.657	3944	0.899	16,001
50	16.671	3966	0.890	15,849
100	16. 681	3968	0.898	15,982
150	16.622	3942	0.893	15,942
200	16.634	3953	0.895	15,919
250	16.634	3963	0.890	15,944

References

1. Kannan V; Narayanaswamy P; Gadamsetty D; Hazra P; Khedkar A; Iyer, H. A tandem mass spectrometric approach to the identification of O-glycosylated glargine glycoforms in active pharmaceutical ingredient expressed in Pichia pastoris. *Rapid Communications in Mass Spectrometry* **2009**, 23(7), 1035-42.
2. Pharmeuropa, Vol. 23, No. 2, April **2011**.

Published in the USA, May 1, 2016
5991-6872EN

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Application Note

Aggregate/Fragment Analysis

Agilent

Trusted Answers

A Comprehensive Workflow to Optimize and Execute Protein Aggregate Studies

Combining Size Exclusion Chromatography with Method Development and Light Scattering

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Abstract

This Application Note illustrates a comprehensive aggregate analysis workflow to:

- Optimize mobile phase conditions for high-performance size exclusion chromatography (SEC) of monoclonal antibodies
- Characterize aggregation profiles that include monomers, dimers, and higher-order aggregates

We used Agilent Buffer Advisor software to automate complex SEC optimization experiments that use the full capabilities of the bio-inert quaternary pump of the Agilent 1260 Infinity II Bio-inert LC system to mix a wide range of buffer compositions, automatically, in real time during a series of fast LC runs. The Agilent 1260 Infinity Bio-MDS multidetector suite provided dynamic light scattering detection capability to reveal higher-order protein aggregates, determine absolute molecular weights, and augment quantitative measurements made by a UV detection system.

Introduction

Some monoclonal antibodies (mAbs) and proteins are prone to aggregating spontaneously in solution[1,2]. For many biopharmaceutical applications, the extent of such aggregation must be characterized and quantified precisely under a variety of conditions. Size exclusion chromatography (SEC) is a powerful technique to characterize and quantify protein aggregation, but accurate measurements require excellent chromatography under conditions that accommodate natural protein conformation. To improve chromatographic peak shape for a particular protein, and thus improve resolution, it is often necessary to evaluate a variety of different mobile phase conditions.

The utility of optimizing buffer conditions is sometimes overlooked with SEC techniques. Historically, buffer conditions were optimized to overcome undesirable nonspecific interactions with stationary phase materials, but optimizing for column deficiencies can introduce a risk of disrupting the very aggregation that the technique seeks to measure. However, the inert surface coating of Agilent AdvanceBio SEC columns helps to reduce secondary interactions across a wide range of buffer conditions, and provides greater flexibility to optimize buffer chemistry for protein conformation and chromatographic resolution. The sheer complexity of parameter-scouting experiments has been another impediment to routinely optimizing SEC buffer conditions for aggregate analysis. Optimization experiments required the design of complex tables of mobile phase possibilities, and the tedious manual creation of many different solutions to evaluate the matrix of salts, buffers, and pH variations experimentally. However, modern tools have greatly streamlined workflows for optimizing SEC conditions, characterizing and quantifying aggregates, and deploying optimized techniques in daily use.

This Application Note demonstrates the utility of a full workflow solution for aggregation studies to:

- Automatically mix a specified list of LC buffers from four simple stock solutions and to adjust pH and buffer concentrations in real time using Agilent Buffer Advisor software and a high-resolution bio-inert quaternary LC pump
- Measure higher-order protein aggregates with dynamic light-scattering detection, complementing UV detection to extend high-sensitivity across a broad mass range
- Characterize absolute molecular weight and hydrodynamic radius of aggregated and monomeric proteins by light-scattering detection

For the present analyses, we used an Agilent AdvanceBio SEC 150 mm column to provide fast separations for rapid screening. It is important to note that AdvanceBio SEC columns come in longer (and shorter) variations, so the methods can easily be adapted to the 300 mm format, or even multiplexed columns, where additional resolution may be required. Monoclonal antibody samples in this study include commercially available rituximab and a commercially available rituximab biosimilar.

Materials and Methods

Reagents, samples, and materials

Monobasic and dibasic sodium hydrogen phosphate and sodium chloride were purchased from VWR. All the chemicals and solvents used were ≥ 99.7 % pure. >18 MΩ Water was used from a Milli-Q A10 water purification system (Millipore, USA). Solutions were prepared fresh daily and filtered through 0.22 µm membrane filter prior to use.

Instrument

Agilent 1260 Infinity II Bio-inert LC System for aggregate analysis, comprising:

- Agilent 1260 Infinity II Bio-inert Quaternary Pump (G5654A)
- Agilent 1260 Infinity II Bio-inert Multisampler with sample cooler and multi-wash (G5668A)
- Agilent 1260 Infinity II Multicolumn Thermostat with bio-inert heat exchangers (G7116A)
- Agilent 1260 Infinity II Diode Array Detector WR with bio-inert standard flow cell (G7115A)
- Agilent 1260 Infinity Bio-SEC Multi Detector System (G7805AA)
- Agilent AdvanceBio SEC 300 Å, 7.8 × 150 mm, 2.7 µm column (p/n PL1180-3301) or
- Agilent AdvanceBio SEC, 2.7 µm 300 Å 7.8 × 300 mm column (p/n PL1180-5301)

Instrument conditions

Parameter	Value
Mobile phase	See Table 1
Flow rate	0.8 mL/min
Temperature	25 °C
Injection	1–25 µL (dependent on sample concentration)
Detection	220 nm, 280 nm, LS 90°, and DLS
Samples	Rituximab innovator, rituximab biosimilar, and BSA

Results and Discussion

To identify optimal mobile-phase compositions for each analyte mixture, we evaluated three different mobile phases across four different pH levels, representing a matrix of 12 experimental conditions. Buffer compositions were:

- 150 mM Sodium phosphate
- 10 mM Sodium phosphate + 140 mM NaCl (emulating PBS at different pH values)
- 100 mM Sodium phosphate + 150 mM NaCl

Each mobile phase was tested at pH 6.2, 6.6, 7.0, and 7.4.

To execute each experiment, we specified buffer composition and pH for each treatment in Agilent Buffer Advisor. The software automatically calculated the appropriate mixtures of stock solutions A–D to achieve the desired mobile phase specifications in real time during the LC parameter-scouting run. Table 1 summarizes the 12 experimental conditions.

These conditions were used to analyze a commercial sample of rituximab, a sample of rituximab biosimilar, and a commercial BSA protein standard mixture (10 mg/mL solution for instrument calibration purposes).

By using an Agilent AdvanceBio SEC 300 Å 150 × 7.8 mm column, we were able to perform screening experiments in less than 10 minutes per sample.

Table 1. Experimental conditions and corresponding mobile phase compositions.

	User-specified parameters				Software-calculated parameters			
Experiment	pH	Buffer (mM)	NaCl (mM)	Total conc. (mM)	% A	% B	% C	% D
1	6.2	150	0	150	25.0	0.0	57.0	18.0
2	6.6	150	0	150	25.0	0.0	42.3	32.7
3	7.0	150	0	150	25.0	0.0	26.3	48.7
4	7.4	150	0	150	25.0	0.0	13.8	61.2
5	7.4	10	140	150	67.0	28.0	0.9	4.1
6	7.0	10	140	150	67.0	28.0	1.8	3.2
7	6.6	10	140	150	67.0	28.0	2.9	2.1
8	6.2	10	140	150	67.0	28.0	3.9	1.1
9	6.2	100	150	250	20.0	30.0	36.3	13.7
10	6.6	100	150	250	20.0	30.0	26.1	23.9
11	7.0	100	150	250	20.0	30.0	15.6	34.4
12	7.4	100	150	250	20.0	30.0	7.8	42.2

A = Water
B = 500 mM NaCl
C = 200 mM NaH_2PO_4
D = 200 mM Na_2HPO_4

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An initial review of the chromatographic data revealed that the mobile phase conditions impacted the peak shapes of the rituximab innovator and rituximab biosimilar during the experiment, as shown in Figure 1 and Figure 2. The two versions of the molecule appear similar in profile, and demonstrate the same unusual behavior with the mobile phase composition of 10 mM sodium phosphate with 140 mM NaCl. This mobile phase composition produced a noticeable increase in peak tailing with a reduction in peak height. This type of behavior may also be seen with other proteins, illustrating the need to carefully evaluate the effect of mobile phase composition for both method development and method robustness.

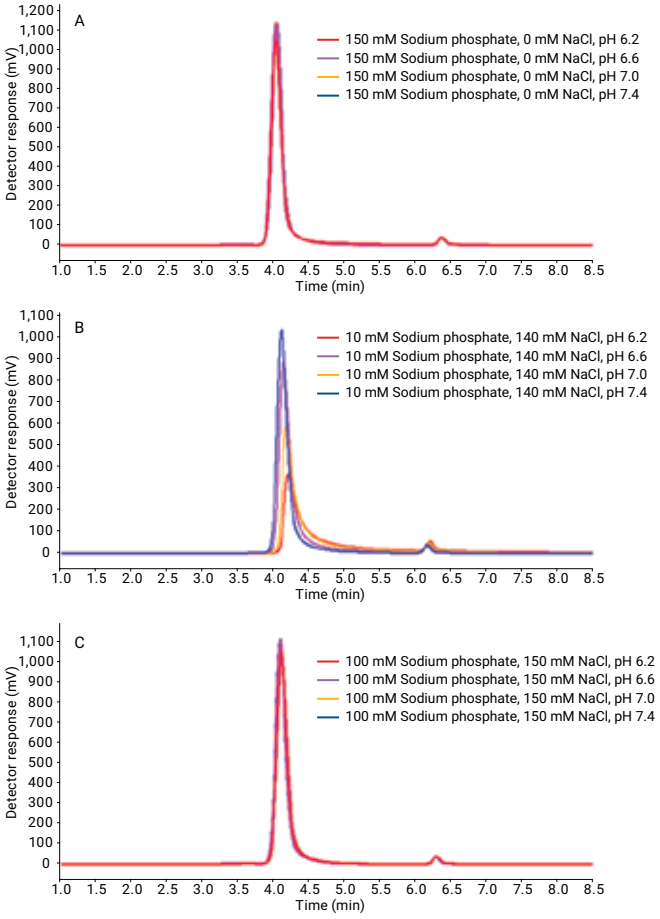

Figure 1. Rituximab innovator (1 µL injection) run consecutively at 12 different mobile phase conditions (Experiments 1–12: see Table 1).

For quantification of aggregate content, it is necessary to use the UV detector. Integration of monomer peak and aggregate peak (where detected) enables the peak area aggregate percentage to be determined. Results are reported in Figure 3A for rituximab innovator and Figure 3B for rituximab biosimilar. The most consistent results were obtained at pH 7.0 using 150 mM sodium phosphate or 100 mM sodium phosphate with 150 mM NaCl (Experiments 3 and 11).

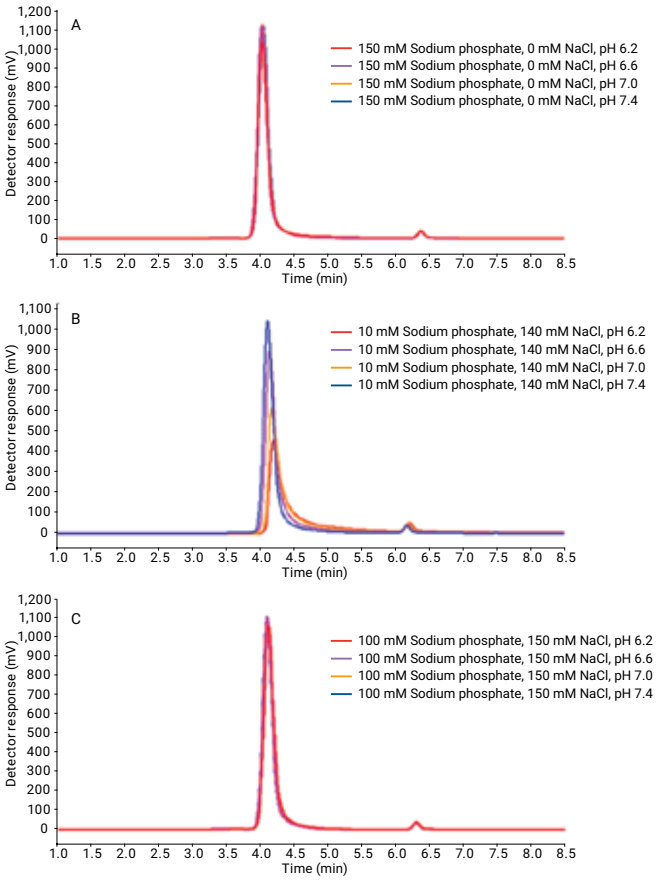

Figure 2. Rituximab biosimilar (1 µL injection) run consecutively at 12 different mobile phase conditions (Experiments 1–12: see Table 1).

Figure 4, showing chromatograms obtained at pH 7.0 in the three different buffer compositions, clearly illustrates that aggregates (highlighted with an arrow) were not detected when 10 mM sodium phosphate with 140 mM NaCl was used as mobile phase for the analysis of this molecule.

Adding a light scattering detector to the aggregate analysis workflow is optional, but it reveals more useful information about protein aggregates. Following a simple instrument calibration with a single injection of a well-defined molecule, in this case BSA, the inter-detector delay and instrument constants can be determined rapidly. Agilent Bio-SEC software can be used to report molecular weight information for individual peaks from any other chromatogram obtained under the same column and flow rate conditions.

Figure 5 shows the light scattering analysis results of the rituximab innovator versus rituximab biosimilar run under the high salt conditions of Experiment 11. Focusing analysis on the monomer peak only, the light scattering analysis reported molecular weights close to the accurate mass values seen in a previous Application Note[1]. The biosimilar molecule is expected to have a slightly higher mass due to the presence of C-terminal lysine variants not evident in the originator molecule.

The formation of larger aggregates and subvisible particles is a particular concern in biopharmaceutical applications. The sensitivity of light-scattering detection toward highly aggregated samples complements the concentration data obtained from UV detection.

Despite the very similar results for monomers and dimers analyzed by UV detection, the light scattering detector proved more responsive to higher order aggregates, and revealed some differences in the more extensive aggregation of the rituximab innovator and biosimilar under certain mobile phase conditions as shown in Figure 6.

The addition of DLS capability further enhances the level of information that can be gained, providing hydrodynamic radius measurements to be made (Figure 7).

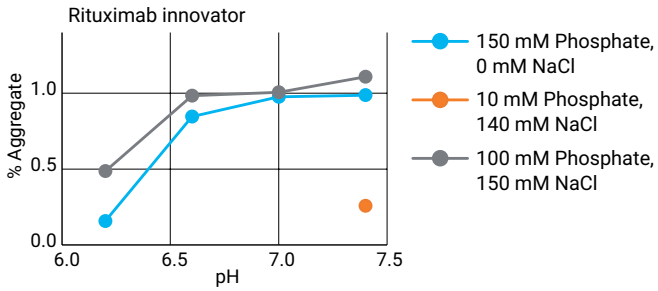

Figure 3A. Peak area percentage of aggregate content of rituximab innovator (Experiments 1–12: see Table 1). Points are shown for experiments where aggregates were detected.

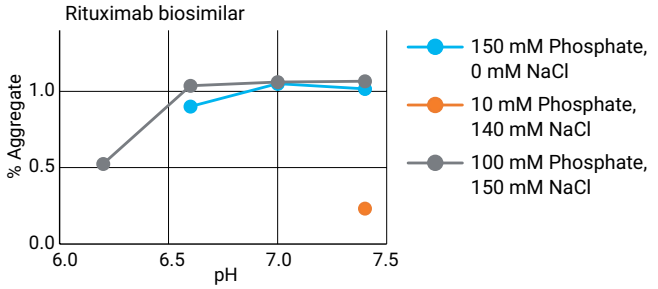

Figure 3B. Peak area percentage of aggregate content of rituximab biosimilar (Experiments 1–12: see Table 1). Points are shown for experiments where aggregates were detected.

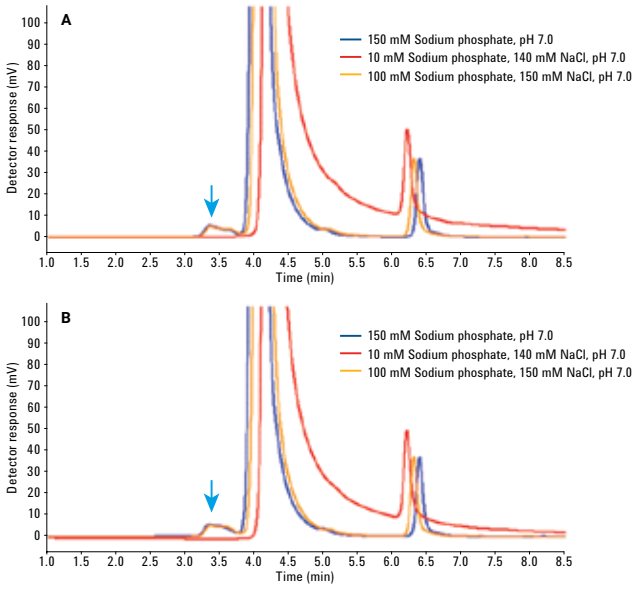

Figure 4. Baseline zoom of UV 220 nm signals of rituximab innovator (A) and rituximab biosimilar (B) run with different buffer salt concentrations at the optimized pH 7.0 (Experiments 3, 6, and 11 in Table 1).

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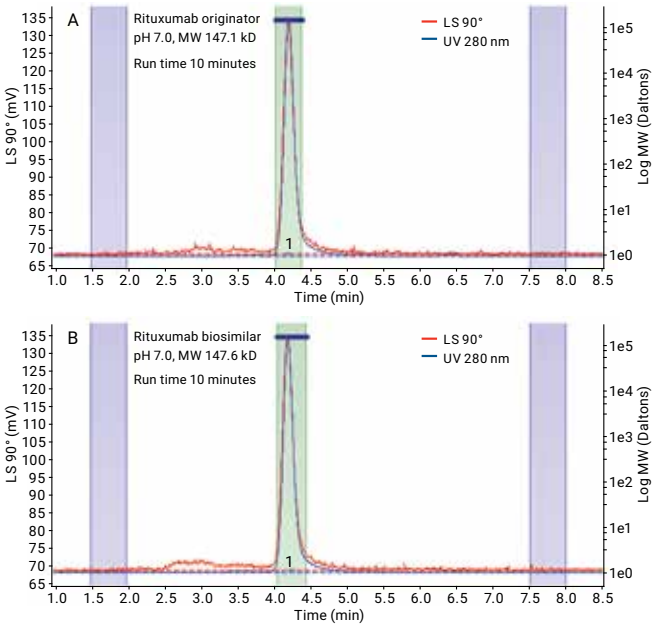

Figure 5. Comparison of LS analysis rituximab innovator (A) and rituximab biosimilar (B) run at 100 mM sodium phosphate with 150 mM NaCl, pH 7.0 (Experiment 11 in Table 1).

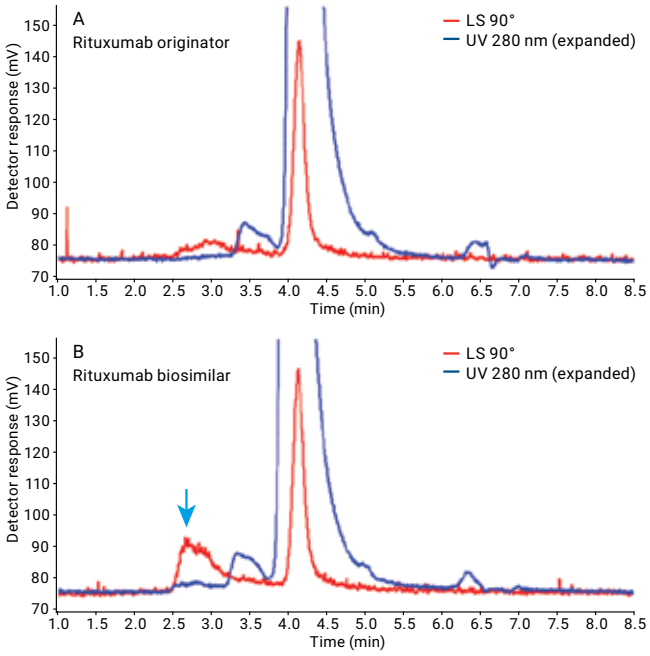

Figure 6. Comparison of LS analysis rituximab innovator (A) and rituximab biosimilar (B) run at 150 mM sodium phosphate, pH 7.0 (Experiment 3 in Table 1).

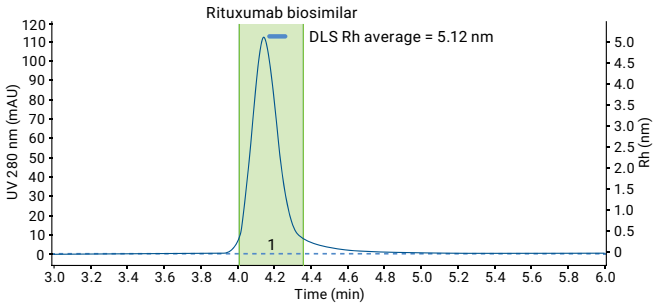

Figure 7. DLS analysis of rituximab biosimilar showing hydrodynamic radius results from LS detector.

Conclusion

The Agilent 1260 Infinity II Bio-inert LC System containing a fully bio-inert flow path, with Agilent Buffer Advisor software, provides a simple way of performing method optimization for size exclusion chromatography for protein aggregate quantitation. Faster separations are possible using a shorter Agilent AdvanceBio SEC 300 Å 150 mm column, which greatly increases throughput and reduces the time required for screening a wide range of analysis conditions. To gain more resolution and higher accuracy, use a longer 300 mm column.

The AdvanceBio SEC column shows additional benefits such as low nonspecific binding. The Bio-MDS Multidetector Suite with Bio-SEC software can be used to reveal low levels of high molecular weight aggregates that are difficult to detect by any other means. Ultimately, the Bio-MDS can be used to determine protein molecular weight, or determine hydrodynamic radius information in conjunction with DLS detection. This suite of technologies comprises a comprehensive workflow solution to optimize SEC conditions rapidly, quantify aggregates accurately across their entire molecular weight range, and characterize the aggregation dynamics of monoclonal antibodies in relevant buffer conditions.

References

1. Guidance for Industry Immunogenicity Assessment for Therapeutic Protein Products, U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Biologics Evaluation and Research (CBER), **2014**.
2. Mahler, H-C.; *et al.* Protein Aggregation: Pathways, Induction Factors and Analysis, *J. Pharm. Sci.* **2008**, *98(9)*.
3. Schneider, S. 2D-LC/MS Characterization of Charge Variants Using Ion Exchange and Reversed-Phase Chromatography, *Agilent Technologies Application Note* publication number 5991-6673EN **2016**

Published in the USA, November 1, 2016
5991-7476EN

Application Note

Aggregate/Fragment Analysis

Agilent

Trusted Answers

Size Exclusion Chromatography in the Presence of an Anionic Surfactant

Intact Protein Profiling

Authors

Andy Coffey
Agilent Technologies, Inc

Abstract

Sodium dodecyl sulfate (SDS, or SLS) is a well known anionic detergent, frequently used to denature proteins. It is commonly used in polyacrylamide gel electrophoresis (SDS-PAGE), where a remarkably consistent level of binding across a wide range of proteins imparts a reliable charge-to-mass ratio. This allows separation of denatured proteins based on relative size due to their relative ion mobility. Conversely, size exclusion chromatography (SEC) for size-based separation of proteins is normally performed under nondenaturing conditions using predominantly aqueous buffers as mobile phase.

This Application Note used SEC with light scattering detection to investigate the impact of varying SDS concentration on the protein molecule. This was achieved by studying the changes in RT, apparent molecular weight, and hydrodynamic radius.

Introduction

To denature proteins, sodium dodecyl sulfate (SDS) is used above its critical micelle concentration (CMC). The CMC of SDS in pure water is 8.2 mM (approximately 0.2 % w/v). However, the presence of buffer salts or changes in pH and temperature may reduce the CMC significantly. CMC values of around 1.0 mM (approximately 0.04 % w/v) are observed in phosphate buffered saline (PBS), for example. A micelle forms when approximately 62 SDS molecules coalesce into a spherical shape with a hydrophobic core surrounded by an anionic surface, as depicted in Figure 1. Typically, SDS is used at significantly higher concentrations of 2 % w/v for sample preparation for SDS-PAGE, but also with a reducing agent to cleave disulfide bonds within the protein molecule. The resulting saturated SDS-protein complex has, on average, 1.4 g SDS per gram of protein [1]. Such denatured proteins are considered to adopt a rigid cylindrical shape resulting in the observation that ion mobility in gel electrophoresis is proportional to molecular weight. By omitting the reduction of disulfide bonds, it has been observed that the ratio of SDS to protein decreases significantly and the time to reach equilibrium may be doubled [2].

In contrast, adding SDS (approximately 10:1 mol ratio) has been found to arrest heat denaturation of BSA solution, which otherwise leads to creation of high molecular weight oligomers [3], or reduces the level of noncovalent aggregation. Historically, it was recommended to use 0.1 % w/v SDS in the mobile phase to reduce retention time (RT), and improve peak shape in protein size exclusion chromatography (SEC) [4].

The mechanism by which SDS denatures a protein is not fully understood. Since both the detergent molecule and the protein possess complementary ionic and hydrophobic regions, it is clear that a combination of mechanisms may be involved. Thermodynamic studies have suggested that interactions are not ionic alone. It is conceivable that SDS first begins to bind through an ionic interaction with positively charged amino acids side chains (Lys and Arg) on the surface of the protein. Increasing SDS concentration leads to higher levels of incorporation, and it is postulated that ultimately, the flexible protein chain is decorated with SDS micelles rather than the rigid cylinder model originally proposed [5].

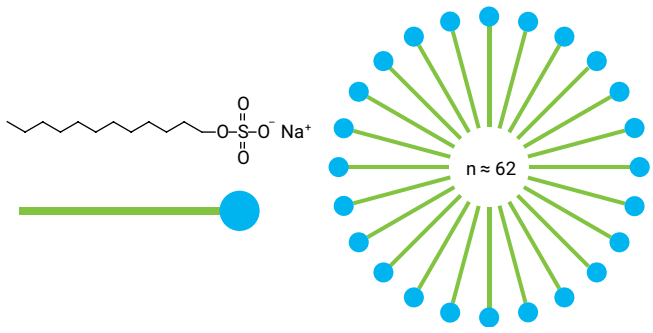

Figure 1. Cartoon depicting SDS micelle formed at concentrations above CMC (aggregation number ~ 62).

Bovine serum albumin (BSA) is a well studied protein due to its capability of acting as a carrier of smaller molecules between tissues and cells. The structure and size (hydrodynamic radius) of the BSA monomer has been determined using multiple analytical techniques. Its primary structure is 583 amino acids with a molecular weight of 66,463 Da, Figure 2. Commercial samples frequently contain varying amounts of dimer and higher-order aggregates, and different isolation techniques may result in varying amounts of these oligomers. Since BSA contains 35 cysteine residues, there are 17 disulfide bridges and one free thiol group. It is believed that BSA oligomers are predominantly covalently linked through disulfide bonds involving the extra thiol group. Therefore, in the absence of reducing agents, denaturation with SDS will not result in such covalently linked dimers and higher aggregates reverting to a monomeric form.

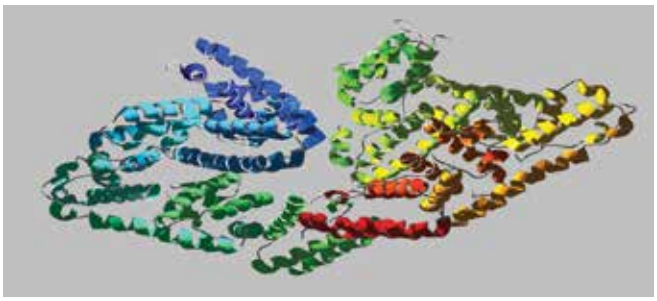

Figure 2. Native heart-shaped conformation of bovine serum albumin.

To explore the effect of SDS denaturation on the characteristics of BSA, a series of experiments were performed. Using SEC of the nonreduced BSA protein, the amount of dimer and higher-order aggregates could be determined. The addition of a light scattering detector with dynamic light scattering (DLS) capability allowed the measurement of both molecular weight and hydrodynamic radius (Rh). The eluent chosen for this series of experiments was phosphate buffered saline (PBS), containing 10 mM phosphate buffer, and 140 mM NaCl, pH 7.4 (Eluent A). A second mobile phase was prepared in an identical manner, but with the addition of 2 % w/v SDS (Eluent B). The HPLC instrument was then run with increasing levels of SDS incorporated in the mobile phase by varying the proportions of Eluent A and Eluent B.

Throughout the experiment, the same BSA sample was used, prepared at a concentration of 10 mg/mL in PBS alone.

The experiment was designed to determine:

- If SDS can denature BSA oligomers
- What effect is observed on the molecular weight measured using inline light scattering detection
- What change in hydrodynamic radius is observed

Conditions

Parameter	Value
Column:	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 7.8 × 300 mm (p/n PL1180-5301)
Samples:	BSA (Sigma-Aldrich)
	Sample prepared 10 mg/mL in Eluent A
Eluent A:	PBS, pH 7.4 (10 mM phosphate, 140 mM NaCl)
Eluent B:	PBS, pH 7.4 (10 mM phosphate, 140 mM NaCl) + 2.0 % w/v SDS
Gradient:	Isocratic elution at 0 % B; 10 %B; 20 % B; 30 % B; 40 % B; 50 % B; 60 % B; 70 % B
Flow rate:	0.8 mL/min
Detector:	UV, 280 nm; LS 15°, LS 90°
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC with Agilent 1260 Infinity Bio-SEC multidetector system.

Results and Discussion

Following two blank injections to allow for the column to become conditioned in the mobile phase, duplicate injections of BSA 10 mg/mL were made.

Figure 3 shows overlaid chromatograms (UV 280 nm signal) of four individual experiments at different mobile phase compositions. Table 1 contains RT information relating to the entire experimental series.

From Table 1, it is apparent that there is little change in total peak area for different runs. This indicates that increasing SDS concentration does not result in the absorption of the protein, or loss of protein through induced aggregation. RTs become shorter but stabilize from 0.6 to 1.4 % SDS (w/v), as seen in Figure 4. This shortening of RT also results in lower resolution between the peaks, so it proved difficult to accurately determine how much dimer and higher-order aggregates became denatured. It is clear that these multimer peaks are still abundant and must, therefore, be predominantly covalently linked.

Table 1. RT and total peak area.

	RT (min)			
% SDS In mobile phase	Higher-order aggregates	Dimer	Monomer	Total peak area
0.0 %	6.00	6.46	7.29	3,632
0.2 %	5.22	5.59	6.45	3,633
0.4 %	4.96	5.23	5.96	3,668
0.6 %	4.89	5.15	5.83	3,588
0.8 %	4.89	5.13	5.80	3,571
1.0 %	4.89	5.11	5.77	3,572
1.2 %	4.88	5.11	5.76	3,536
1.4 %	4.90	5.10	5.75	3,566

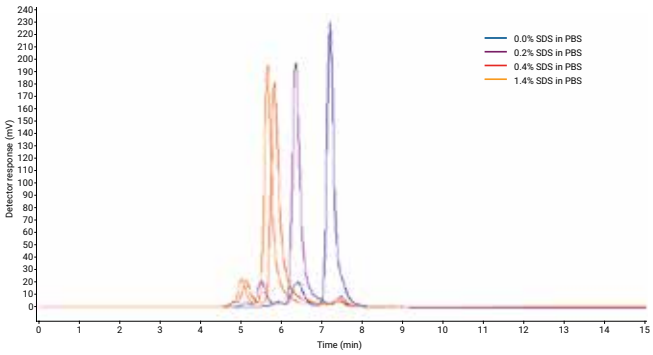

Figure 3. SEC chromatograms of BSA with increasing SDS concentration in mobile phase.

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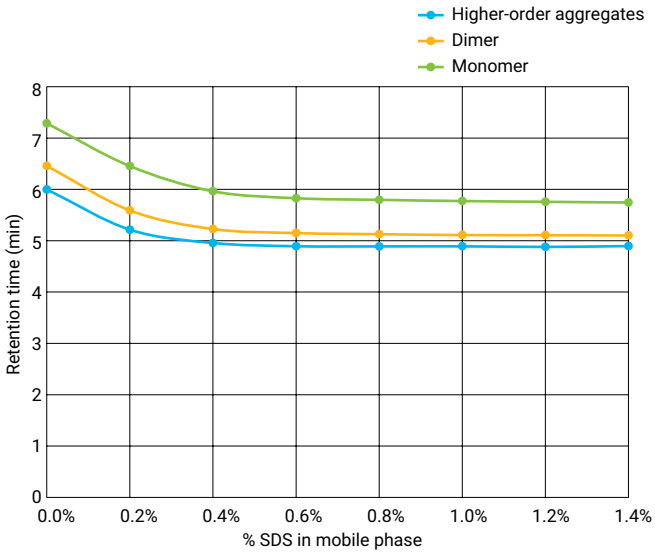

Figure 4. Effect of % SDS in mobile phase on RT.

In SEC, shorter RT is indicative of an increase in the size of a molecule in solution, but does not necessarily infer an increase in molecular weight. To ascertain the molecular weight of the three major peaks of the chromatogram, the BioSEC software was used to calculate the molecular weight using the relationship described in the following formulae [6]:

Where:

(*LS*) = Light scattering detector signal

K_{LS} = LS detector constant

dn/dc = Specific refractive index increment*

(*UV*) = UV detector signal (280 nm)

ε = Extinction coefficient

* Using the approximation of *dn/dc* ≈ 0.186 mL/g for nonglycosylated proteins

Figure 5 shows the regions of each peak chosen to perform the light scattering molecular weight determination to try to minimize interference due to anticipated peak overlap. Table 2 shows the results for the molecular weight measurements. This table includes columns where the relative number of monomer units has been calculated. It is important to recognize that the molecular weight of the higher-order aggregates peak is nearly always three times larger than the molecular weight of the monomer peak, indicating it is a trimer. The molecular weight of the dimer peak is two times larger than the molecular weight of the monomer peak, as expected. In addition, the molecular weight of each of the peaks increases with increasing SDS concentration until a plateau is reached at 0.6% w/v SDS, in agreement with the stabilization of RTs at this point.

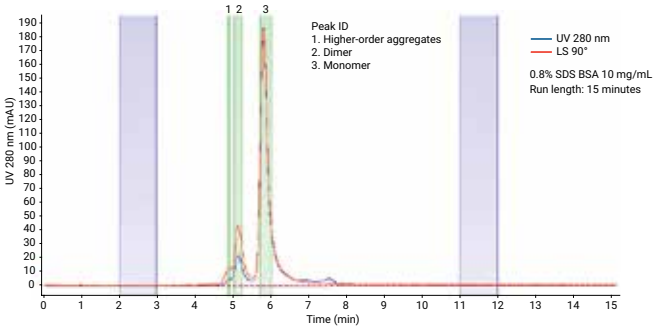

Figure 5. Molecular weight calculation regions for (1) higher-order aggregates, (2) dimer, and (3) monomer peaks of BSA.

Table 2. Molecular weight analysis results from light scattering detection.

	Mass gain			SDS:BSA (g/g)		
SDS w/v%	Higher-order aggregates	Dimer	Monomer	Higher-order aggregates	Dimer	Monomer
0.0	202,197	136,383	67,838	3.0	2.0	1.0
0.2	242,541	194,876	107,905	2.2	1.8	1.0
0.4	560,154	359,239	203,737	2.7	1.8	1.0
0.6	667,318	423,418	212,596	3.1	2.0	1.0
0.8	615,726	404,898	207,028	3.0	2.0	1.0
1.0	624,648	404,634	205,868	3.0	2.0	1.0
1.2	617,385	405,618	206,376	3.0	2.0	1.0
1.4	612,031	406,699	206,614	3.0	2.0	1.0

Table 3. Mass gain for BSA with increasing surfactant concentration, showing steady state at concentrations >0.6%.

	Mass gain			SDS:BSA (g/g)		
SDS w/v%	Higher-order aggregates	Dimer	Monomer	Higher-order aggregates	Dimer	Monomer
0.0	0	0	0	0.0	0.0	0.0
0.2	40,344	58,493	40,067	0.2	0.4	0.6
0.4	357,957	222,856	135,899	1.8	1.6	2.0
0.6	465,121	287,035	144,758	2.3	2.1	2.1
0.8	413,529	268,515	139,190	2.0	2.0	2.1
1.0	422,451	268,251	138,030	2.1	2.0	2.0
1.2	415,188	269,235	138,538	2.1	2.0	2.0
1.4	409,834	270,316	138,776	2.0	2.0	2.0

Since the observed molecular weight increases in line with the concentration of SDS present in the mobile phase, it may be inferred that each molecular species is actually gaining mass through accumulation of associated SDS. However, the observed mass gain is considerable and higher than expected, particularly since this BSA sample has not been reduced (Table 3). The steady state mass gain under the analysis conditions is 2 g SDS per 1 g protein, suggesting that SDS micelles may be accumulating along the protein molecule regardless of the number of oligomers it contains.

Closer inspection of the concentration-dependant UV trace of the chromatograms obtained at 0.0 % SDS and 1.4 % SDS shows further differences. There is clearly a reduction in resolution for the higher molecular weight species, however some smaller peaks eluting after the monomer peak have become evident in the 1.4 % SDS mobile phase composition (Figure 6). It was not possible to identify these lower molecular weight species.

Figure 7A shows the signals from the LS 90° detector for BSA at 0.0 % SDS and 1.4 % SDS concentrations. Since the light scattering detector is mass sensitive rather than concentration-dependant, it is not surprising that the signal obtained under the higher surfactant concentration conditions is much larger than the signal under native conditions.

In addition to the increased response seen in Figure 7A at the highest SDS concentration, there is also a vertical offset. The reason for this becomes apparent when the DLS data are analyzed to determine the Rh. Not only do we see an increase in the Rh of the monomer peak (from around 3.8 to 5.7 nm, close to literature values) [7,8,9], there is a continual background of particles detected of approximately 2.4–2.8 nm in size, almost certainly due to SDS micelles in solution.

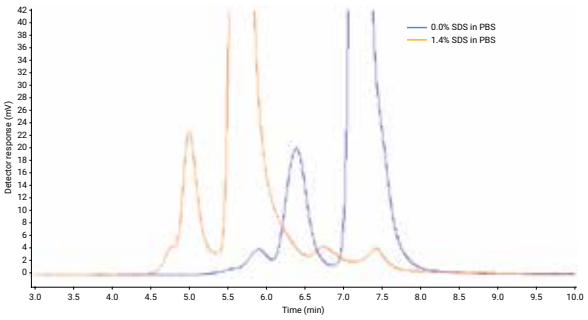

Figure 6. Close up of the UV 280 nm signal of BSA in PBS mobile phase containing 0.0 % SDS and 1.4 % SDS.

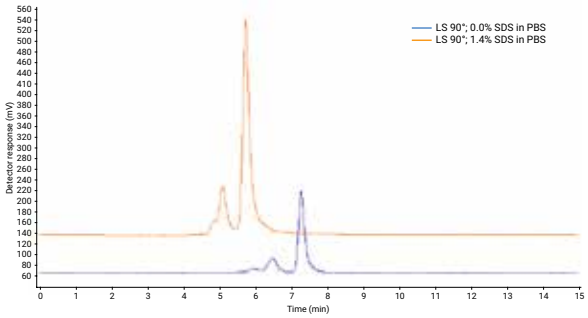

Figure 7A. Overview of the LS 90° signal of BSA in PBS mobile phase containing 0.0 % SDS and 1.4 % SDS.

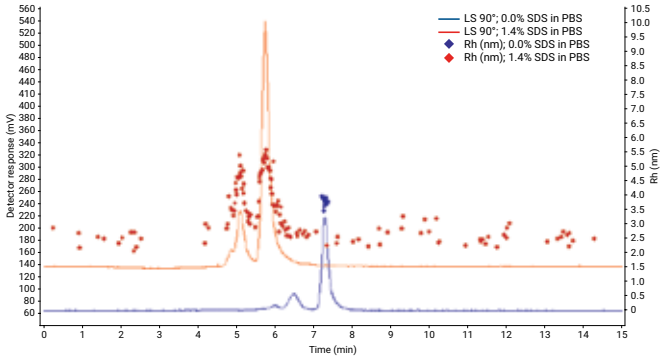

Figure 7B. Overview of the LS 90° signal of BSA in PBS mobile phase containing 0.0 % SDS and 1.4 % SDS with hydrodynamic radius (Rh) data added.

Conclusions

At the outset, this experiment was designed to determine if SDS can denature BSA oligomers using SEC with light scattering detection. It was found that accurate quantification of oligomer content was not possible through loss in resolution as the individual peaks corresponding to monomer, dimer, and higher-order aggregates eluted closer together. However, it is apparent the three individual species remain intact and become saturated with SDS to the same extent.

Simply denaturing the protein, making the molecule unfold, would be expected to give a larger structure in solution, leading to a shorter RT in SEC. However, as the SDS concentration was increased from 0.0 to 0.4 % w/v, the molecular weight of the monomer, dimer, and higher-order aggregates increased proportionally. At higher SDS concentrations (0.6–1.4 % w/v) the RTs (and, therefore, size in solution) and the molecular weight determined by light scattering stabilized as the molecular species were saturated by SDS.

Using DLS capability to determine hydrodynamic radius shows an increase in size comparable to literature values for a reduced BSA sample. The size increase does not reflect a change in conformation of the BSA sample run under nonreducing conditions, but replicates the observed increase in molecular weight as the molecule complexes with and becomes saturated by SDS micelles.

It is clear that, although the Agilent AdvanceBio SEC 300 Å column is able to tolerate SDS in the mobile phase, as seen by the good peak shape and reproducible recovery, the SEC separation is badly affected by the dramatic increases in size and molecular weight of the protein species being analyzed. The use of surfactants in the mobile phase for SEC should be avoided wherever possible.

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References

1. Reynolds, J. A.; Tanford, C. The gross conformation of protein-sodium dodecyl sulfate complexes. *J. Biol. Chem*. **1970**, *245(19)*, 5161-5165.
2. Pitt-Rivers, R.; Impiombato, F. S. A. The binding of sodium dodecyl sulphate to various proteins. *The Biochemical Journal* **1968**, *109*, 825-830.
3. Aoki, K.; Hiramatsu, K.; Kimura, K.; Kaneshina, S.; Nakamura, Y.; Sato, K. Heat Denaturation of Bovine Serum Albumin. I : Analysis by Acrylamide-gel Electrophoresis. *Bulletin of the Institute for Chemical Research, Kyoto University* **1969**, *47(4)*, 274-282
4. Hagarová, D.; Horváthová, M.; Žúbor, V.; Breier, A. Optimization of conditions for size-exclusion chromatography of proteins. *Chemical Papers* **1991**, *45(3)*, 341-348.
5. Shirahama, K.; Tsujii, K.; Takagi, T. Free-boundary electrophoresis of sodium dodecyl sulfate-protein polypeptide complexes with special reference to SDS-polyacrylamide gel electrophoresis. *J. Biochem.* **1974**, *75*, 309-319.
6. Wen, J.; Arakawa, T.; Philo, J. S. Size-Exclusion Chromatography with On-Line Light-Scattering, Absorbance, and Refractive Index Detectors for Studying Proteins and Their Interactions. *Anal. Biochem*. **1996**, *240*, 155-166.
7. Lorber, B.; Fischer, F.; Bailly, M.; Roy, H.; Kern, D. Protein Analysis by Dynamic Light Scattering: Methods and Techniques for Students. *Biochem. and Mol. Biol. Ed.* **2012**, *40(6)*, 372-382.
8. Valstar, A.; Almgren, M.; Brown, W. The Interaction of Bovine Serum Albumin with Surfactants Studied by Light Scattering. *Langmuir* **2000**, *16(3)*, 922-927.
9. Tanner, R. E.; Herpigny, B.; Chen, S.-H.; Rha, C. K. Conformational change of protein sodium dodecylsulfate complexes in solution: A study of dynamic light scattering. *J. Chem. Phys*. **1982**, *76(8)*, 3866-3872.

Published in the USA, November 2, 2017
5991-7989EN

Additional Application Notes

AdvanceBio SEC

Part Number	Title
5994-0876EN	Size Exclusion Chromatography Method Development of NIST mAb Using an Agilent AdvanceBio SEC 200 A 1.9 µm column
5991-6791EN	Analysis of PEGylated Proteins with Agilent AdvanceBio SEC Columns
5991-7165EN	High-throughput and Sensitive Size Exclusion Chromatography (SEC) of Biologics Using Agilent AdvanceBio SEC Columns
5991-6458EN	Fast, High-Resolution Size Exclusion Chromatography of Aggregates in Biotherapeutics
5991-6304EN	Separate and Quantify Rituximab Aggregates and Fragments with High-Resolution SEC
5991-6303EN	Quantitation of mAb and ADC Aggregation Using SEC and an Aqueous Mobile Phase
5991-6302EN	Agilent AdvanceBio SEC Columns for Aggregate Analysis: Instrument Compatibility
5991-6474EN	Advantages ofAgilent AdvanceBio SEC Columns for Biopharmaceutical Analysis

Bio SEC-3

Part Number	Title
5991-2463EN	Choosing the Right Calibration for the Agilent Bio SEC-3
5991-3955EN	Determination of Protein Molecular Weight and Size Using the Agilent1260 Infinity Multi-Detector Bio-SEC Solution with Advanced Light Scattering Detection
5990-9894EN	Optimum Pore Size for Characterizing Biomolecules with Agilent Bio SEC Columns

Additional Information

A number of standards are available to assist with method development, molecular weight estimates, and system QC testing.

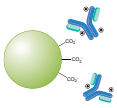
Part Number	Title
5190-9416	AdvanceBio SEC 130 Å Protein Standard, lyophilized, 1.5 mL
5190-9417	AdvanceBio SEC 300 Å Protein Standard, lyophilized, 1.5 mL
PL2070-0100	Polyethylene glycol calibration kit, PEG-10, 10 x 0.5 g
PL2080-0101	Polyethylene oxide calibration kit, PEO-10, 10 x 0.2 g
PL2090-0101	Pullulan Polysaccharide calibration kit
5191-5744	Agilent-NISTmAb standard 1/pack
5191-5745	Agilent-NISTmAb standard 4/pack

Charge Variant Analysis

Background

The presence of positively charged and negatively charged amino acids and negatively charged glycans (sialic acids) means that large proteins exist as multiple charged species and there are several side reactions that can result in a change in the net charge. Understanding which amino acids or glycans are involved and their specific location within a large biotherapeutic protein is of paramount importance. Variants within the antigen binding region of an antibody are likely to have a more profound effect on function.

Ion exchange chromatography can enable the separation of some charge variants, particularly those positioned on the surface of the protein (rather than hidden within the structure). Nonetheless, separating a molecule may have a net charge of +50 from a variant that is +49 or +51 is still a considerable challenge. Elimination of pore structure and therefore pore diffusion by using nonporous particles goes some way to improving peak shape and gaining resolution. It is often necessary to revert to weak cation exchange columns and to perform extensive method optimization to determine the most appropriate conditions for a particular molecule.



Charge Variant Analysis

Ion exchange chromatography

Enhances the accuracy and speed of biomolecule characterization

Bio MAb

Ideal for monoclonal antibodies

Attribute	Advantage
Rigid, non-porous particles	High-efficiency separations
Hydrophilic, polymeric layer	Eliminates non-specific binding
High density WCX chemistry	High ion exchange capacity ideal for MAbs

Bio IEX

Ideal for proteins and peptides

Attribute	Advantage
Rigid particles with hydrophilic coating	Eliminates non-specific binding
Strong/weak anion, cation chemistries	A column for every separation

Getting Started

Since most proteins contain more basic amino acids than acidic amino acids, most charge variant separations will require cation exchange. However, every protein is different and finding the conditions to deliver the best resolution you require will likely require considerable optimization. Strong cation exchange columns are often easier to work with, however for monoclonal antibodies a weak cation exchange column may be the only way to achieve the desired resolution.

Before beginning method development, it is crucial to determine the isoelectric point, or pI, of the target protein. If the pH of initial mobile phase conditions is too close to the pI of the protein, the protein will not be retained on the column. Depending on how widely the pI of the charge variants differs, the pH may need to be a minimum of 0.5 to 2 pH units away from the isoelectric point of the main species. Proteins may be eluted by either a salt gradient (using high ionic strength to disrupt protein adsorption to the column) or a pH gradient (proteins elute when the pH equals the pI).

It is worthwhile considering an instrument that allows screening of several different columns during method development. It is difficult to predict the outcome of even small changes to method conditions such as ionic strength and pH; both of these factors will influence the net charge on the protein and, in the case of weak ion exchange columns, the net charge on the column too. A rigorous "Quality by Design" approach is recommended. Software to develop a matrix or systematic design of experiments is advisable. Buffer advisor software that can utilize the quaternary HPLC pump capabilities of an Agilent 1260 Infinity II Bio-inert LC can save considerable method development time. Several of the application notes listed in this section, including the “How-To” Guide and the featured application note illustrate how to use buffer advisor to test a range of mobile phase conditions. When the optimum conditions for separation require very low ionic strength buffers at pH levels at the extreme limits of the buffering range then PEEK columns may also be advisable.

Like size exclusion chromatography, ion exchange conditions are typically nondenaturing; the separation is conducted on the intact, native protein. This means that the method is not MS compatible unless combined as the first dimension in a 2D-LC setup. However, quantification can be achieved by UV detection.

Ion-Exchange Chromatography for Biomolecule Analysis: A "How-To" Guide

Introduction

Proteins are made up of chains containing numerous amino acids, several of which possess acidic or basic side chain functionalities. This results in an overall charge on the surface of the protein that can be controlled by adjusting the pH of the surrounding solution. The isoelectric point, pI, is the pH at which the net charge of the protein is neutral (the number of positive charges is equal to the number of negative charges). If the pH is below this value, the protein will possess an overall positive charge and can be retained on a negatively charged cation-exchange sorbent; if the pH is above the pI, the protein will be negatively charged overall and can be retained on an anion-exchange sorbent.

In this "How-To" Guide we discuss ion-exchange (IEX) chromatography, column selection choices, important mobile phase considerations, general rules of thumb for using IEX, instrument considerations, and more.

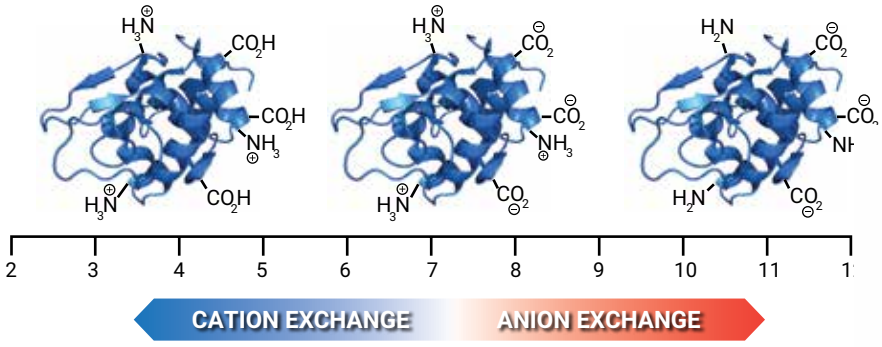
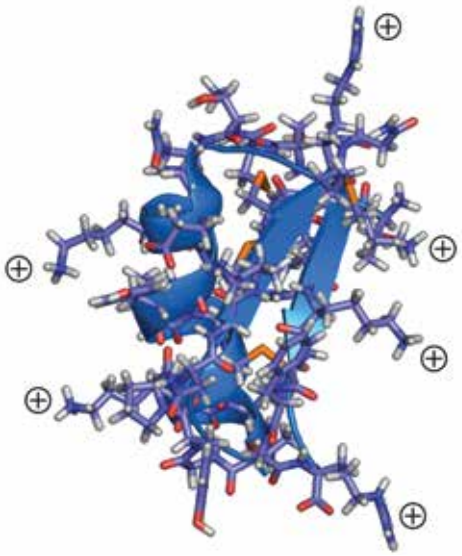


Figure 1. Effect of pH on net protein charge



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Separation, based on ionic charge, is typically performed under non-denaturing conditions

Ion-exchange is a widely used method for separating biomolecules based on differences in ionic charge. It is a mild, non-denaturing technique that does not require organic solvents and is therefore frequently used for characterization of proteins in their native or active form, and for purification.

Proteins contain a variety of functionalities that can give rise to differences in charge. Acidic groups include C-terminal carboxylic acids, acidic side chains of aspartic and glutamic acid, and acidic groups arising from sialic acid in glycosylated proteins; basic groups include N-terminal amines and basic side chains of arginine, lysine, and histidine. The overall charge of the molecule is therefore dependent on the pH of the surrounding solution and this in turn will affect the ion-exchange method that can be used. The mobile phase must maintain a controlled pH throughout the course of the separation, and so aqueous buffers are used as eluents.

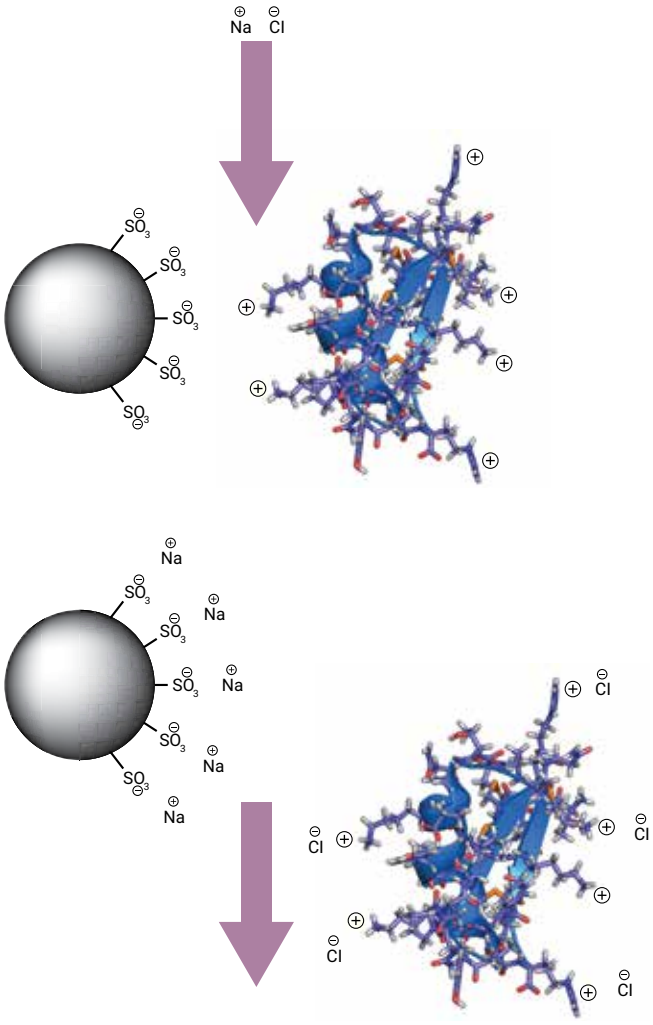

Figure 2. Separation mechanism of ion-exchange

The technique of ion-exchange is therefore suitable for separating proteins with differing isoelectric points, but it is equally valuable in separating charged isoforms of a single protein. In the increasingly important field of biopharmaceuticals, where proteins are manufactured through bioengineering and isolated from fermentation reactions, it is important to identify charged isoforms as these indicate a difference in primary structure of the protein. A difference in primary structure could indicate a change in glycosylation, or degradation pathways such as loss of C-terminal residues or amidation/deamidation. They can also result in a change in stability or activity and could potentially lead to immunologically adverse reactions. Ion-exchange is used to separate and quantify charge variants during the development process and also for quality control and quality assurance during manufacture of biotherapeutics. With large molecules such as monoclonal antibodies (mAbs) it is also important to consider the size and structure of the molecule (mAbs are typically 150 kD), particularly as the chromatographic interactions will only occur with surface charges.

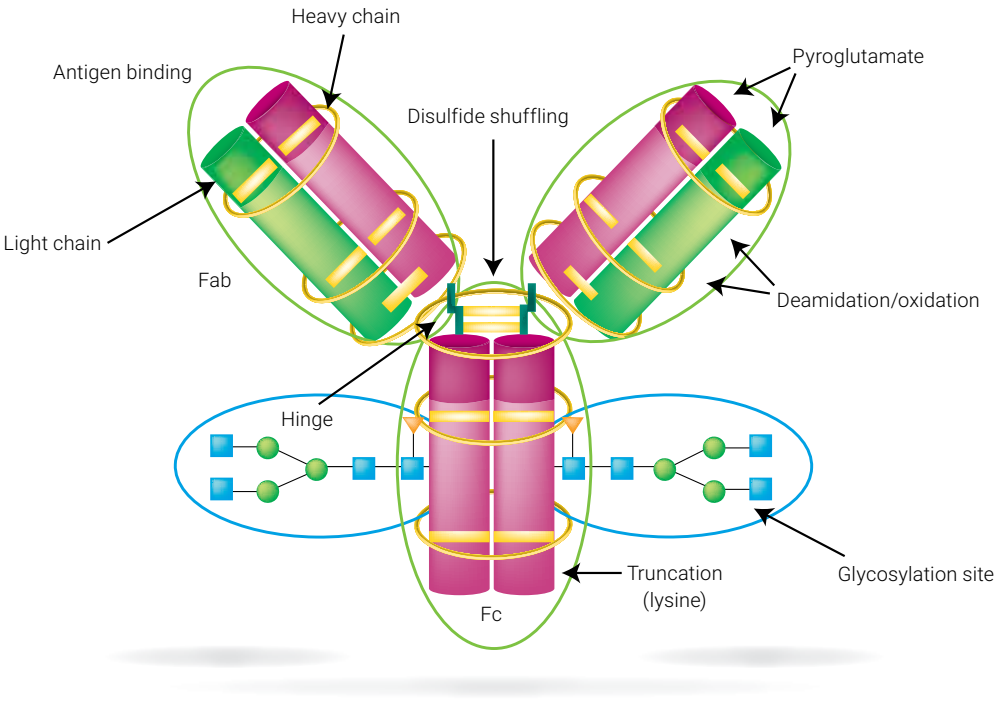

Figure 3. Charged variants of monoclonal antibodies arise through different levels of glycosylation, deamidation, and oxidation of amino acids, and through lysine truncation of heavy chains

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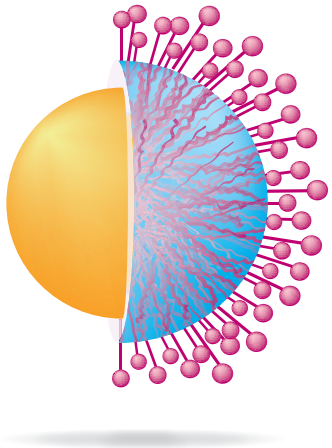
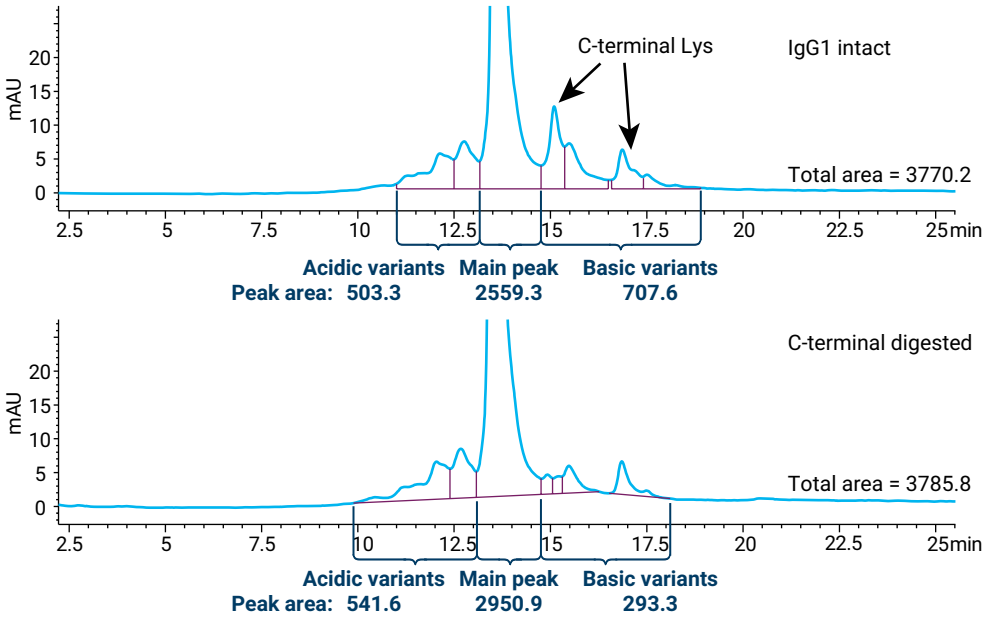
Agilent Bio MAb HPLC columns: superior performance from the inside out

- Particles, coating, and bonding are resistant to high pressures, promoting higher resolution and faster separations
- Hydrophilic coating eliminates most nonspecific interactions
- A highly uniform, densely packed, weak cation-exchange (WCX) layer chemically bonded to the hydrophilic, polymeric coating



Use Bio MAb to identify C-terminal truncation on heavy chains



Conditions

Parameter	Value
Column:	Bio MAb, PEEK 5190-2407 4.6 x 250 mm, 5 µm
Sample:	5 µL of 1 mg/mL of intact or C-terminal digested IgG1
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC
Mobile Phase:	A: 10 mM Na phosphate buffer, pH 5.5 B: A + 0.5 M NaCl
Flow Rate:	0.85 mL/min
Gradient:	10 to 35 % B from 0-25 min
Detector:	UV, 225 nm
Injection:	5 µL

Figure 4. Calculation of C-terminal digested IgG1 using an Agilent Bio MAb 5 µm column on the Agilent 1260 Infinity Bio-inert Quaternary LC. The column delivers high resolution, enabling better peak identification and accurate quantification

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Understanding the requirements for a successful ion-exchange separation

Step 1:

Sample preparation

Sample preparation for ion-exchange chromatography is not unlike that for any protein analysis. The most important aspect is that the sample must be soluble in the eluent and should ideally be dissolved in the mobile phase itself. To protect the column from possible damage we recommend that samples are filtered before use to remove particulates, but filtration should not be used to compensate for poor sample solubility – an alternative eluent may need to be found.

Captiva Low Protein Binding Filters

Agilent Captiva Premium PES Syringe Filters provide superior and consistent low protein binding for protein-related filtration. The polyethersulfone (PES) filter membranes are a better option than polyvinylidene difluoride (PVDF) membranes for most LC analyses. Agilent PES has similar compatibility to PVDF filters for common LC solvents and is superior in terms of protein binding and cleanliness.
Learn more at **www.agilent.com/chem/filtration**

Captiva Premium PES Syringe Filters

Diameter (mm)	Pore size (µm)	Certification	Housing	Part Number
4	0.2	LC/MS	Polypropylene	5190-5094
4	0.45	LC	Polypropylene	5190-5095
15	0.2	LC/MS	Polypropylene	5190-5096
15	0.45	LC	Polypropylene	5190-5097
25	0.2	LC/MS	Polypropylene	5190-5098
25	0.45	LC	Polypropylene	5190-5099

AssayMAP Automated Protein and Peptide Sample Preparation

AssayMAP sample preparation, an automated solution for protein purification, digestion, peptide cleanup, and peptide fractionation, minimizes hands-on time and maximizes workflow reproducibility and efficiency. Standardized user interfaces simplify the workflow while enabling flexible control over key assay parameters. The level of data quality and increased capabilities achievable with AssayMAP technology provide unmatched ability to scale from discovery to validation and production.

- Reproducible results
- Reduced hands-on time
- Simple, user-customizable protocols
- Increased throughput, 8 to 384 samples per day
- Easy method transfer

Learn more about AssayMAP technology:
www.agilent.com/lifesciences/assaymap

For an intact protein analysis workflow such as the one presented in this guide, target proteins can be quantitatively purified on the AssayMAP platform using Protein A or Protein G microchromatography cartridges, then fed to HPLC columns to separate and detect intact protein charge variants.

For effective sample preparation it is also important to ensure that methods used to dissolve the sample do not change the properties of the sample itself.

Step 2:

Column selection - Ion-Exchange

Application	Agilent Columns	Notes
Monoclonal antibodies	Bio MAb	Thorough characterization of monoclonal antibodies includes the identification and monitoring of acidic and basic isoforms. Agilent Bio MAb HPLC columns feature a unique resin specifically designed for high-resolution charge-based separations of monoclonal antibodies.
Peptides and proteins	Bio IEX	Agilent Bio IEX columns are packed with polymeric, nonporous, ion-exchange particles. The Bio IEX columns are designed for high resolution, high recovery and highly efficient separations.
Proteins, peptides and deprotected synthetic oligonucleotides Globular proteins and peptides Very large biomolecules/ high speed	PL-SAX 1000 Å PL-SAX 4000 Å PL-SAX 1000 Å PL-SAX 4000 Å	The strong anion exchange functionality, covalently linked to a fully porous chemically stable polymer, extends the operating pH range. In addition, the anion-exchange capacity is independent of pH. For synthetic oligonucleotides, separations using denaturing conditions of temperature, organic solvent, and high pH are all possible. The 5 µm media delivers separations at high resolution with the 30 µm media used for medium pressure liquid chromatography.
Small peptides to large proteins Globular proteins Very large biomolecules/ high speed	PL-SCX 1000 Å PL-SCX 4000 Å PL-SCX 1000 Å PL-SCX 4000 Å	PL-SCX is a macroporous PS/DVB matrix with a very hydrophilic coating and strong cation-exchange functionality. This process is controlled to provide the optimum density of strong cation-exchange moieties for the analysis, separation and purification of a wide range of biomolecules. The 5 µm media delivers separations at higher resolution with the 30 µm media used for medium pressure liquid chromatography.
Antibodies (IgG, IgM), plasmid DNA, viruses, phages and other macro biomolecules Viruses, DNA, large proteins Plasmid DNS, bacteriophages Proteins, antibodies	Bio-Monolith QA Bio-Monolith DEAE Bio-Monolith SO_3 Bio-Monolith QA Bio-Monolith DEAE Bio-Monolith SO_3	Strong cation-exchange, strong and weak anion-exchange phases. Bio-Monolith HPLC columns are compatible with preparative LC systems, including Agilent 1100 and 1200 Infinity Series.

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Column Media Choice

As with most chromatographic techniques, there is a range of columns to choose from. With ion-exchange the first consideration should be “anion or cation-exchange?” There is also the choice of strong or weak ion-exchange. In most circumstances it is best to start with a strong ion-exchange column. Weak ion-exchangers can then be used to provide a difference in selectivity if it is required.

The functional group in a strong cation-exchange column is sulfonic acid, resulting in the stationary phase being negatively charged in all but the strongest acidic mobile phases. Conversely, the functional group in a strong anion-exchange column is a quaternary amine group, which is positively charged in all but the most basic mobile phases. Strong ion-exchange columns, therefore, have the widest operating range. Weak ion-exchange sorbents (carboxylic acids in weak cation-exchangers and amines in weak anion-exchangers) are more strongly affected by the mobile phase conditions. The functionalities are not dissimilar to the charged groups on proteins themselves and the degree of charge can be influenced by ionic strength as well as mobile phase pH.

This can result in a change in resolution that may be subtly controlled and optimized through careful choice of operating conditions. Weak ion-exchangers are therefore an additional tool and can sometimes provide selectivity that is not met by a strong ion-exchange column.

Pore Size

Where resolution is more important than capacity, rigid, spherical non-porous particles (with an appropriate surface functionality), as provided by the Agilent Bio IEX product range can be beneficial. For the analysis of exceptionally large biomolecules, or where maximum speed is sought, the Agilent Bio-Monolith column can provide optimum results. Some stationary phases, such as PL-SCX or PL-SAX sorbents, are fully porous with 1000 or 4000 Å pores. It is important to ensure the pores are sufficiently large to allow proteins to fully permeate the structure unhindered. This then provides greater surface area and hence greater loading capacity, which is more suited to preparative separations.

Exceptional separating power

The hydrophilic, polymeric layer and densely packed ion-exchange functional groups provide extremely sharp peak shapes and high resolution of a mixture of proteins with a broad range of isoelectric points (pI).

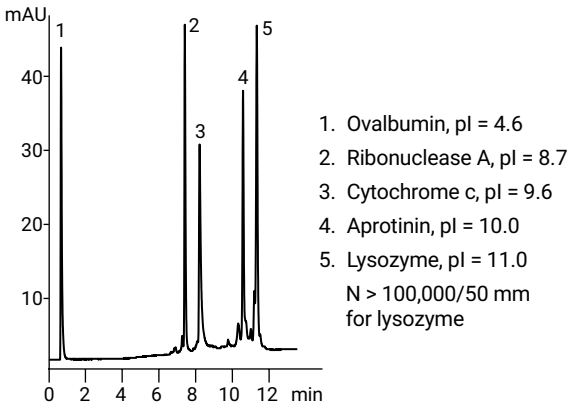

Figure 5. Exceptional separating power of Agilent Bio SCX

Conditions

Parameter	Value
Column:	Bio SCX, stainless steel 5190-2423 4.6 x 50 mm, 3 µm
Buffer: A:	10 mM phosphate, pH 6.0
Flow Rate:	0.5 mL/min
Gradient:	0-1.0 M NaCl, 15 min
Detector:	280 nm

Particle Size

Particle size is an important consideration in column selection. Smaller particle sizes provide more efficient separation, but at the cost of higher operating pressure. Because biomolecules are relatively large and have slower rates of diffusion, smaller particle sizes do not necessarily provide the same level of improvement in resolution that might be seen with small molecules. Furthermore, eluents comprising aqueous buffers are relatively viscous and care must be taken to ensure back pressures are not excessive.

Column Hardware

Particle size is an important consideration in column selection. Smaller particle sizes provide more efficient separation, but at the cost of higher operating pressure. Because biomolecules are relatively large and have slower rates of diffusion, smaller particle sizes do not necessarily provide the same level of improvement in resolution that might be seen with small molecules. Furthermore, eluents comprising aqueous buffers are relatively viscous and care must be taken to ensure back pressures are not excessive.

Separation of protein standards on Agilent 3 µm ion-exchange columns by cation-exchange chromatography

Conditions

Parameter	Value
Column A:	Bio SCX, stainless steel 5190-2423, 4.6 x 50 mm, 3 µm
Column B:	Bio WCX, stainless steel, 5190-2443 4.6 x 50 mm, 3 µm
Column C:	Bio MAb, stainless steel, 5190-2403 4.6 x 50 mm, 3 µm
Sample:	Ribonuclease A, cytochrome c, lysozyme and protein mix
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC
Mobile Phase:	A: 10 mM Sodium phosphate, pH 5.7 B: A + 1 M NaCl
Flow Rate:	0.5 mL/min
Gradient:	0 min - 100 % A : 0 % B 25 min - 0 % A : 100 % B
Temp.:	Ambient
Detector:	Agilent 1260 Infinity Bio-inert Quaternary LC with diode array detector at 220 nm

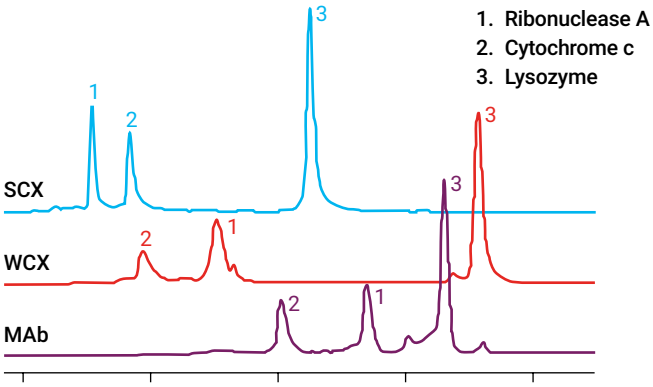

Figure 6. Separation of protein standards on Agilent 3 µm ion-exchange columns by cation-exchange chromatography

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Achieve faster analysis time with smaller particles and shorter column lengths – speed up your separation by 30 %

Stainless steel columns are used, but salt gradients can prove aggressive and cause corrosion if left in contact with the column. PEEK columns do not suffer from this problem and can be beneficial for molecules that are metal-sensitive, though they operate at lower back pressures. For a metal-free sample flow path, a PEEK column run with a bio-inert instrument such as the Agilent 1260 Infinity Bio-inert Quaternary LC should be used.

Conditions

Parameter	Value
Column A:	Bio WCX, stainless steel 5190-2445 4.6 x 250 mm, 5 µm
Column B:	Bio WCX, stainless steel 5190-2443 4.6 x 50 mm, 3 µm
Sample:	0.5 mg/mL
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC
Mobile Phase:	A: 20 mM Sodium phosphate, pH 6.5 B: A + 1.6 M NaClGradient: 0 min - 100 % A : 0 % B
Gradient:	0 to 50 % B
Temp.:	Ambient
Injection:	10 µL
Detector:	UV, 220 nm

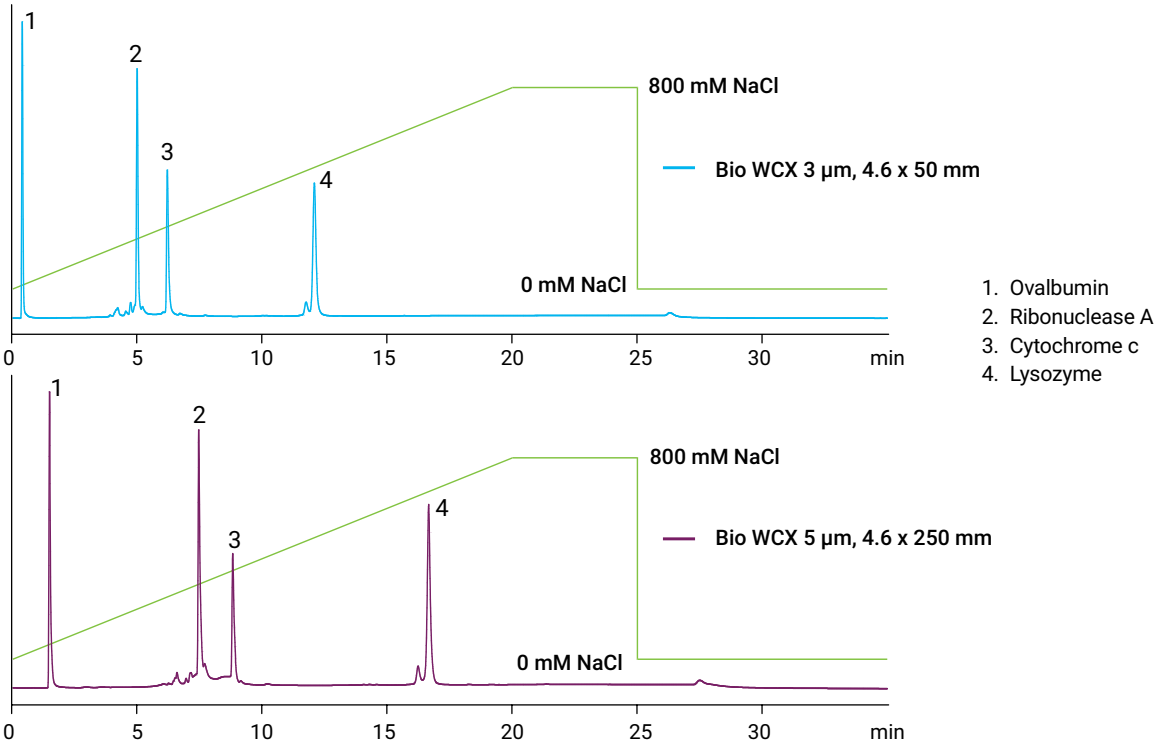

Figure 7. Protein separation on Agilent Bio WCX columns (4.6 x 50 mm, 3 µm and 4.6 x 250 mm, 5 µm) at a flow rate of 1 mL/min. Faster analysis times were achieved with smaller particle size and shorter column length – samples eluted from the longer column in 17 minutes but in only 12 minutes from the shorter column

Column Diameter

Column diameter can also be important, depending on the amount of sample being analyzed. If only limited amounts of material are available, 2.1 mm id columns (operated at 0.35 mL/min) are useful. But it is important to minimize system volumes between the column and detector when using smaller id columns to prevent excessive dispersion and loss of resolution.

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Step 3:

HPLC system considerations

An ideal choice for this type of analysis is the Agilent 1260 Infinity Bio-inert Quaternary LC. It handles challenging solvent conditions with ease, such as extreme pH values of pH 1 to pH 13, and buffers with high salt concentrations.

Corrosion-resistant titanium in the solvent delivery system and metal-free materials in the sample flow path create an extremely robust instrument.

Detection

For biomolecules such as proteins that consist of multiple amino acids linked via amide bonds, UV detection at 210 nm or 220 nm will give the best signal strength and sensitivity. However, some of the eluents commonly employed in ion-exchange have a strong background absorbance at low wavelengths, and so it may be necessary to use 254 nm or 280 nm instead. These wavelengths are only sensitive to amino acids with aromatic or more conjugated side chains, which will result in much lower sensitivity.

Optimize interaction-free chromatography

Agilent Bio-inert LC supplies provide robust, interaction-free results to ensure increased system efficiency – while improving chromatographic reliability with sharper peaks and more reproducible analysis.

Learn more: **www.agilent.com/chem/biosupplies**

The Agilent 1260 Infinity Bio-inert Quaternary LC is an ideal HPLC instrument for ion-exchange chromatography

Step 4:

Flow rate

Typical flow rate for use with 4.6 mm id columns is 0.5 to 1.0 mL/min. For some applications the speed of analysis is crucial. Shorter columns can be used to reduce the analysis time – 50 mm instead of the conventional 150 mm or 250 mm – or flow rates can be increased, or both (taking care not to exceed column pressure limitations).

Smaller particle sizes provide increased resolution

Conditions

Parameter	Value
Column A:	Bio WCX, stainless steel 5190-24414.6 x 50 mm, 1.7 µm
Column B:	
Sample:	0.5 mg/mL
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC
Mobile Phase:	A: 20 mM sodium phosphate, pH 6.5 B: A + 1.6 M NaCl
Gradient:	0 to 50 % B
Temp.:	Ambient
Injection:	10 µL
Detector:	UV, 220 nm

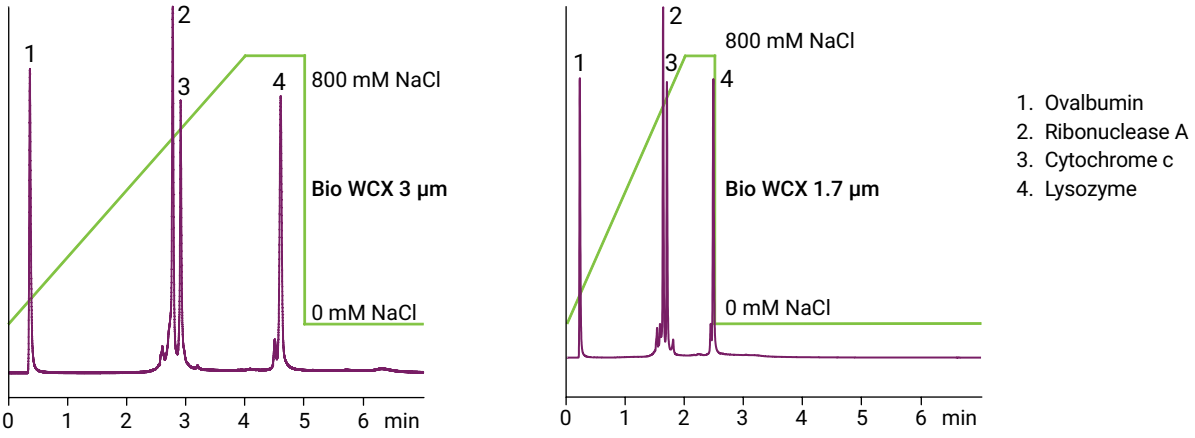

Figure 8. Reduce analysis time – without sacrificing peak shape and resolution – by increasing flow rate

Mobile Phase Selection

Step 5:

Initial mobile phase selection will be dictated by the pI of the protein and the method of analysis, i. e. cation- or anion-exchange. Figure 10 shows the range of buffers commonly available.

The role of the buffer is to control the change in pH during the separation and therefore maintain a consistent charge on the compounds being analyzed. It is important to remember that a buffer will only satisfactorily perform this role if it is within one pH unit of its dissociation constant, pKa. Phosphoric acid or phosphates possess three dissociation constants:

$$H_3PO_{4\,(aq)} \xrightleftharpoons{pK_{a1} = 2.12} H^+_{(aq)} + H_2PO_4^-{}_{(aq)}$$

$$H_2PO_4^-{}_{(aq)} \xrightleftharpoons{pK_{a2} = 7.21} H^+_{(aq)} + HPO_4^{2-}{}_{(aq)}$$

$$HPO_4^{2-}{}_{(aq)} \xrightleftharpoons{pK_{a3} = 12.67} H^+_{(aq)} + PO_4^{3-}{}_{(aq)}$$

Phosphate buffers in the range pH 6 to 7 are therefore suitable for cation-exchange chromatography, typically in concentrations of 20 to 30 mM, and have the advantage of low background absorbance at 210 nm. It is important to make up buffers systematically and accurately, as even minor differences in ionic strength or pH can affect the retention time of proteins to different extents, and could result in poor resolution and variability in the chromatographic profile.

Unlike strong ion-exchange columns that are fully ionized under normal operating conditions, it is important to realize that the buffer pH and ionic strength can affect the degree to which a weak ion-exchange column is ionized. This is one of the tools available to alter selectivity, to achieve a desired separation.

However, to elute biomolecules from the column, a competing ion must be introduced. Typically, this will be accomplished by a linear sodium chloride gradient. Eluent A will comprise the buffer adjusted to the appropriate pH. Eluent B will contain the same concentration of buffer with a higher concentration of sodium chloride, perhaps 0.5 M, with the pH then adjusted to the same value.

Conditions

Parameter	Value
Column A:	Bio MAb, PEEK 5190-2407 4.6 x 250 mm, 5 µm
Sample:	Mix of three proteins, dissolved in PBS (phosphate buffered saline) pH 7.4 Ribonuclease A: 13,700 Da, pI 9.6 Cytochrome c: 12,384 Da, pI 10-10.5 Lysozyme: 14,307 Da, pI 11.35
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile Phase:	A: Water B: 1.5 M NaCl C: 40 mM NaH_2P0_4 D: 40 mM Na_2HP0_4 By combining predetermined proportions of C and D as determined by the Buffer Advisor Software, buffer solutions at the desired pH range and strength were created.
Flow Rate:	1 mL/min

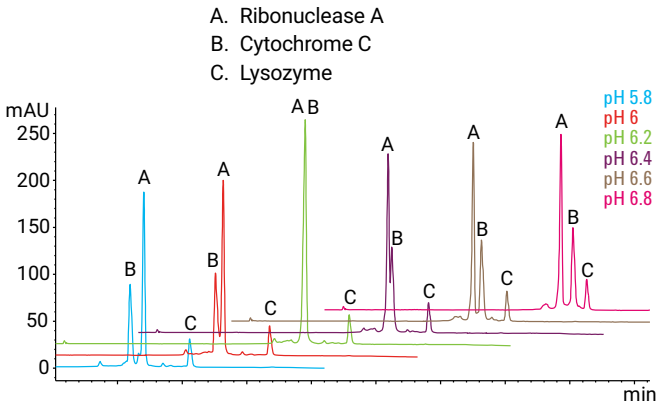

Figure 9. pH scouting for the separation of a three-protein mix using dynamically mixed quaternary gradients

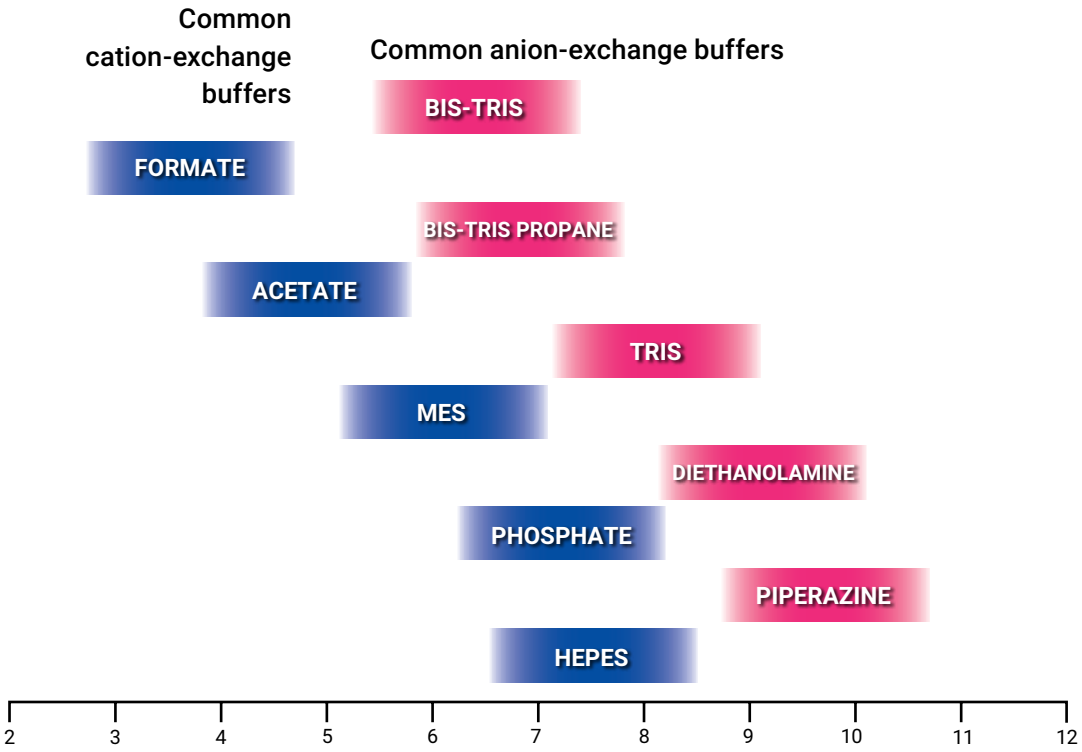

Figure 10. Commonly available buffers for ion-exchange

Developing an Effective Ion-Exchange Method

It must be remembered that biomolecules such as monoclonal antibodies are incredibly complex. A typical mAb comprises over 1,300 individual amino acids. Of these, perhaps 130 have acidic side chains and 180 have basic residues. The likelihood is that a monoclonal antibody will have a net positive charge at neutral pH and therefore should be separated using a cation-exchange column. However, it is difficult to predict the actual isoelectric point, pI, of such a molecule, and so some method development or optimization should be anticipated.

Sample Preparation

- Samples should ideally be dissolved in the mobile phase (eluent A).
- If the sample is cloudy, it may be necessary to change the mobile phase conditions.
- Filtration or centrifugation can be used to clarify samples, but these processes could alter the composition of the sample.
- Samples should be made up fresh and analyzed as soon as possible.
  Refrigeration can increase the “shelf life” of samples.
- Bacterial growth can develop quickly in buffer solutions.

Column Media Choice

- The choice between anion- and cation-exchange depends on the isoelectric point of the protein(s) of interest.
- Strong ion-exchangers are a good first choice, with weak ion-exchange offering a difference in selectivity if it is required.

Column Selection

- Pore size: proteins of interest must be able to freely permeate the particles. Non-porous spherical particles provide highest resolution for analytical separations, where column loading capacity is not a major concern.
- Particle size: use smaller particles for higher resolution (which results in higher back pressure).
- Column length: shorter 50 mm columns can be used for more rapid separations, particularly with smaller particles, and longer 250 mm columns where additional resolution may be required.
- Column id: use smaller columns for reduced solvent consumption and smaller injection volumes (beneficial if sample is limited).

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Mobile Phase

- The mobile phase should contain buffer to maintain the desired operating pH, typically 20 mM. The pH and ionic strength of the buffer can affect resolution on weak ion-exchange products and so the optimum conditions should be found experimentally.
- Addition of sodium chloride to the mobile phase will alter the pH. Re-adjust as necessary.
- Make up fresh mobile phase and use promptly because bacterial growth is rapid in dilute buffer stored at room temperature.
- Buffer shelf life is less than seven days unless refrigerated.
- Filter before use. Particulates can be present in water (less likely) or in buffer salts (more likely).

Column Conditioning and Equilibration

For reproducible ion-exchange separation, the column equilibration and cleanup phases of the gradient are critical. Protein elution is achieved by increasing the ionic strength or changing the eluent pH, or both, and so at the end of each analysis the column must be equilibrated back to the starting conditions, ionic strength, and pH. If this is not done, the next column run will have a different profile as the protein will interact differently with the column.

Software

One additional tool that can be used to simplify your workflow is the Agilent Buffer Advisor Software.

Agilent Buffer Advisor Software eliminates the tedious and error-prone method development steps of buffer preparation, buffer blending and pH scouting, by providing a fast and simple way to create salt gradients (Figure 11) and pH gradients (Figure 12). Using the mixing principle of the 1260 Infinity Bio-inert Quaternary pump, the Buffer Advisor Software facilitates dynamic mixing of solvents from only four stock solutions, simplifying the bioanalysis workflow and significantly reducing the time required for buffer preparation. In addition, buffers are prepared more accurately, which makes for more robust method transfer to other laboratories.

To create a salt gradient, an increasing amount of salt solution from channel D is mixed with the acidic and basic buffer components from channels A and B, and with water for dilution from channel C.

Fast buffer scouting with Agilent Buffer Advisor software.
Watch video: **agilent.com/chem/bufferadvisor-video**

Initial screening of twenty experiments was achieved from just four mobile phase eluents instead of needing forty different solutions. The software automatically blends the buffers to create the desired pH and buffer strengths. The gradient timetable can then be programmed in the quaternary, as shown in Figure 13.

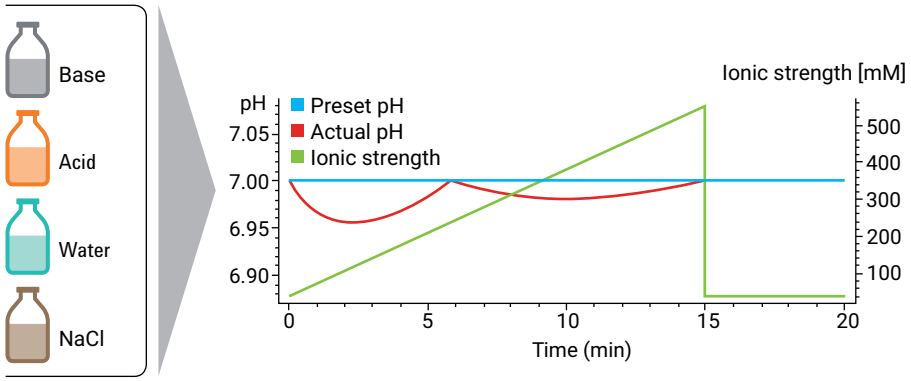

Figure 11. Salt gradients are easily created from stock solutions with Agilent Buffer Advisor Software

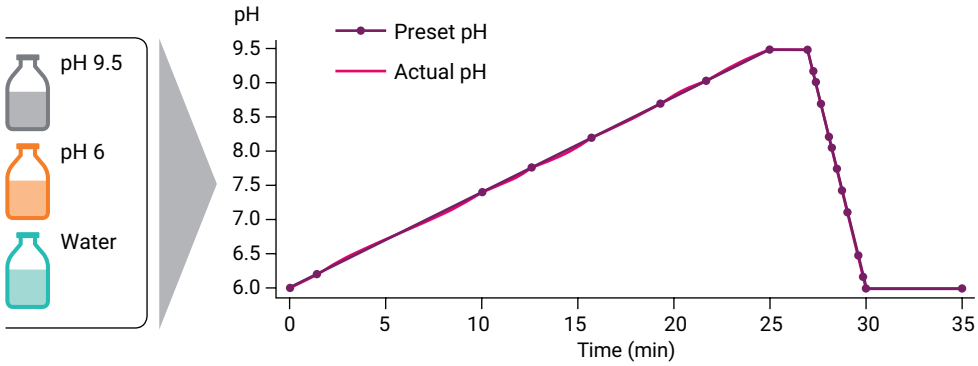

Figure 12. Optimizing buffer strength for a monoclonal antibody separation – pH gradients are easily created from stock solutions

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Conditions

Parameter	Value
Column A:	Bio WCX, stainless steel 5190-2443 4.6 x 50 mm, 3 µm
Column B:	Bio SCX, stainless steel 5190-2423 4.6 x 50 mm, 3 µm
Sample:	IgG monoclonal antibody
Sample Conc.:	2 mg/mL (in 20 mM sodium phosphate buffer, pH 6.0)
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC
Mobile Phase:	A: Water B: 1.5 M NaCl C: 40 mM NaH_2P0_4 D: 40 mM Na_2HP0_4 By combining predetermined proportions of C and D as determined by the Buffer Advisor Software, buffer solutions at the desired pH range and strength were created.
Flow Rate:	1.0 mL/min
Gradient:	Conditions for chromatograms shown: pH 5.0 to 7.0, 10 to 25 mM buffer strength 0 to 500 mM NaCl, 0 to 15 min 500 mM NaCl, 15 to 20 min DOE experiments pH 5.0 to 7.0 0 to 200 mM, 0 to 250 mM, and 0 to 300 mM
Temp.:	Ambient
Injection:	5 µL
Detector:	UV, 220 nm

Automated method development for optimized charged-variant separations

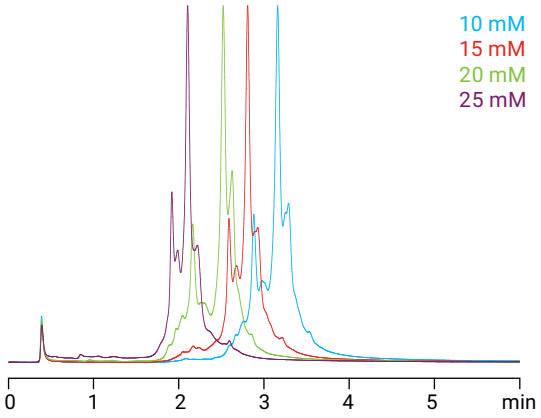

Figure 13. Optimizing buffer strength at pH 6.5 from the screening chromatograms of a monoclonal IgG separation

Conditions

Parameter	Value
Column A:	Bio MAb, PEEK 5190-2407 4.6 x 250 mm, 5 µm
Sample: thermostat	IgG monoclonal antibody
Mobile Phase:	A: 10 mM sodium phosphate buffer, pH 6.0 B: 10 mM sodium bicarbonate buffer, pH 9.5
Flow Rate:	1.0 mL/min
Gradient:	Time (min) Mobile phase (% B) 0 0 25 100 27 100 30 0
Post time:	5 min
Temp.:	30 °C
Data acquisition:	214 and 280 nm
Acquisition rate:	20 Hz
Flow cell:	60 mm path
Injection:	10 µL (needle with wash, flush port active for 7 s)
Detector:	

Bio MAb columns enable precise quantitation, robust methods

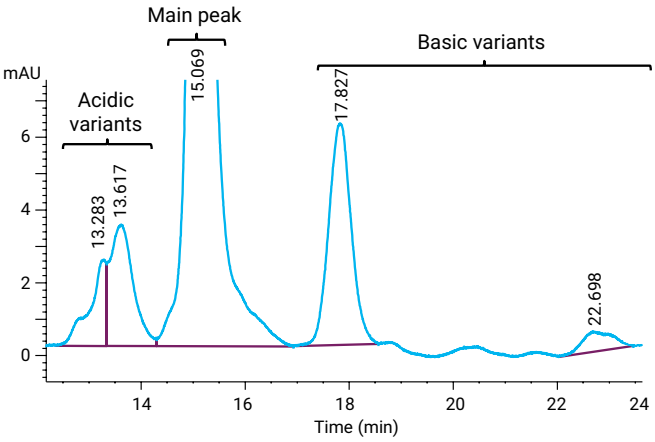

Figure 14. pH gradient-based cation-exchange chromatogram of an IgG1 separation using an Agilent Bio MAb PEEK, 4.6 x 250mm, 5 µm column

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Application Note

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Convenient Customization of Your Cation Exchange Analysis

Combining the Agilent 1260 Infinity II Bio Prime LC System, Agilent Buffer Advisor Software, and pH gradients for high-resolving charge variant analysis

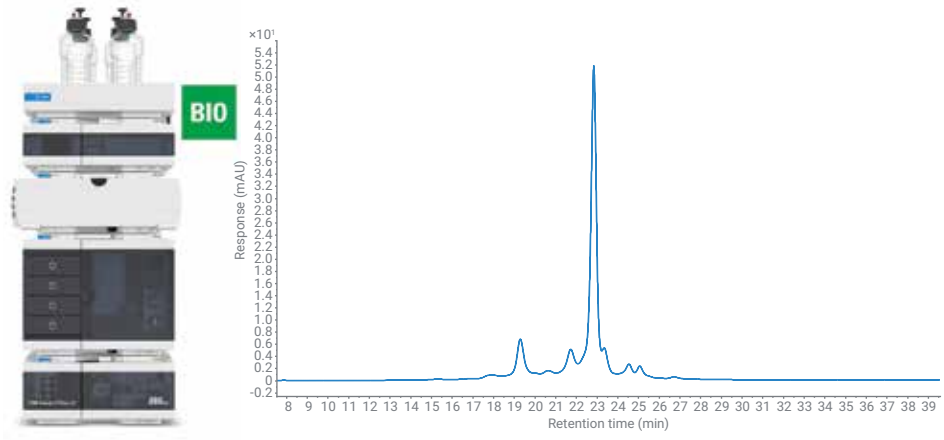
Author

Sonja Schneider
Agilent Technologies, Inc

Abstract

Charge variants separation of monoclonal antibodies can be a challenging task for the chromatographer. Due to the microheterogeneity of the analyzed monoclonal antibody, extensive method development can be necessary to find the optimal desired resolution. Outperforming many traditional salt gradients, the resolving power of pH gradients enables the separation of charge variants in a very efficient way. This application note demonstrates high-resolving and reproducible charge variant analysis of two monoclonal antibodies, trastuzumab and NIST mAb, with different types of pH gradients.

The Agilent 1260 Infinity II Bio Prime LC System, with a completely iron-free flow path and featuring an Agilent 1260 Infinity II Bio Flexible Pump, enables the use of Agilent Buffer Advisor Software to facilitate dynamic mixing of solvents from only four stock solutions.



Introduction

Therapeutic monoclonal antibodies (mAbs) are highly heterogeneous molecules and are composed of a large number of variants. These are naturally occurring in this kind of biopharmaceutical and are not necessarily considered impurities. Proteins in solution have mostly polar and charged amino acids at the protein interface to aqueous media, while the hydrophobic residues tend to self-associate due to hydrophobic interactions. These amino acids on the "outside" of the proteins that are in contact with surrounding liquid are more predisposed to modifications.[1]

Variants, also called protein microheterogeneity, might originate from post-translational modifications during antibody production. In addition, modifications after purification processes, formulation and/or storage can be formed.[2] However, if the variants are present in the pharmaceutical protein, their biological activity might differ and immunogenicity might be enhanced.[2] Hence, the microheterogeneity of the mAbs is subject to extensive analytical characterization to ensure safety and efficacy of the biopharmaceutical.

Cation exchange chromatography (CEX) is considered the gold standard for charge variant analysis of monoclonal antibodies.[3] Classic salt gradients have high resolving power once the method is fully optimized. However, the amount of effort required to develop a high-resolving ion exchange method for protein separation can be very high. Salt concentration, mobile phase pH values, and additives are only a few of the parameters to be optimized. In addition, every molecule and especially biological molecules might show different behavior and the developed methods are not tolerant to large changes in experimental parameters, especially with respect to pH values.[1,4]

pH gradient-based CEX, also known as chromatofocusing, enables high-resolving as well as robust methods for the separation of mAb charge variants.[1,4,5] In typical ion-exchange chromatography (IEX), the molecules are eluted from the column by increasing the ionic strength (mostly with salts like NaCl) of the buffer. In contrast, with pH gradients, the bound molecules are eluted with the changing pH of the buffer. This alters their net surface charge to enable the elution of the bound molecules at their isoelectric point (pI), where the molecule is electrically neutral.

Wide pH gradient methods are more generic and can separate variants from different antibodies within a single buffer system.[1] Also, the method development of pH gradient-based methods is more straightforward and significantly shorter compared to conventional ionic strength-based IEX.

Wide pH gradient methods are more generic and can separate variants from different antibodies within a single buffer system.[1] Also, the method development of pH gradient-based methods is more straightforward and significantly shorter compared to conventional ionic strength-based IEX.

The 1260 Infinity II Bio Prime LC System is the next generation of Agilent high-end liquid chromatography systems, specially designed for conditions used in bio chromatography: The sample flow path is completely free of stainless steel (SST) or iron; all capillaries and fittings throughout the multisampler, multicolumn thermostat, and detectors are built of MP35N, a nickel-cobalt alloy. With this material, potential corrosion from high salt-containing buffers is reduced and protein modifications caused by the presence of ferric ions (e.g. oxidation and protein complex formation) can be avoided.

The 1260 Infinity II Bio Flexible Pump, as a quaternary pump, enables the use of Buffer Advisor Software to facilitate dynamic mixing of solvents from only four stock solutions, simplifying the bioanalysis workflow and significantly reducing the time required for buffer preparation. With Buffer Advisor Software, quaternary salt gradients as well as pH gradients can be generated quickly and simply by the calculation of pump timetables for IEX.

This application note presents the analysis of charge variants for trastuzumab and the NIST mAb reference standard with two different pH gradients.

Experimental

Equipment

The Agilent 1260 Infinity II Bio Prime LC System comprised the following modules:

- Agilent 1260 Infinity II Bio Flexible Pump (G7131C)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with standard flow biocompatible heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2 µL

Software

Agilent OpenLab CDS version 2.5 or later versions

Columns

Bio MAb, NP5, 2.1 × 250 mm, PEEK (part number 5190-2411)

Chemicals

All solvents were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, sodium chloride, tris (tris(hydroxymethyl)aminomethane), imidazole, hydrochloric acid, and piperazine hexahydrate were obtained from Sigma-Aldrich (Steinheim, Germany).

Samples

The Agilent 1260 Infinity II Bio Prime LC System comprised the following modules:

- Agilent-NISTmAb (part number 5191-5744)
- Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland). Trastuzumab was dissolved in 30 mM phosphate buffer, pH 6.8

Buffer preparation

The buffers were prepared according to the Stock Solution Recipes from Buffer Advisor Software (see Figure 1).

Quaternary phosphate-based buffer system—calculated by Buffer Advisor

A: Water
B: 1,700 mM sodium chloride
C: 44.5 mM sodium phosphate monobasic
D: 55 mM sodium phosphate dibasic

Note: This setup can be used for salt as well as pH gradient elution—enabling direct comparison and increasing the possibilities for method development.

Binary wide pH gradient buffer system—calculated by Buffer Advisor

With Buffer Advisor, it is also possible to create wide range pH gradients, also termed Composite Buffer. In these cases, only the C and D channels are employed to create the gradient. This experiment used a pH gradient described by Farnan and Moreno[1] and inserted the buffer composition as a User Mixture in the stock solution composition of Buffer Advisor (2.4 mM tris, 1.5 mM imidazole, 11.6 mM piperazine, HCl for pH adjustment to pH 6 & C and 10.5 & D). With this option, the user can construct self-made buffer compositions to enable the desired pH range. Buffer Advisor calculates the ionic strength (IS) as well as the buffering capacity (BC) for both buffer mixtures.
A: Water
B: n/a
C: pH = 6; IS = 22.5 mM; BC = 6.19 mM
D: pH = 10.5; IS = 0.717 mM; BC = 2.31 mM

Note: When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method. So, for example, for solvent B in the phosphate-buffered gradient with 1,700 mM NaCl, use Sodium Chloride 1.5 M rather than Generic Aqueous or Water in the solvent selection field in the pump method. High amounts of salts change the compressibility of the solvent, and hence using the preconfigured solvent tables enables best pump performance.

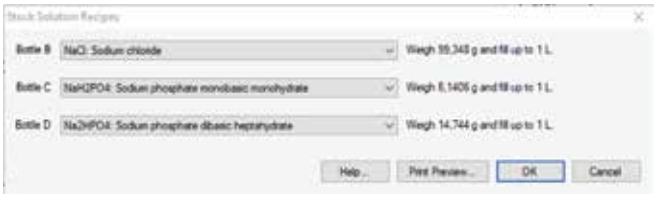


Figure 1. Agilent Buffer Advisor Stock Solution Recipe—quaternary phosphate buffer system.

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Method

Table 1. Quaternary phosphate-based buffer system—salt gradient chromatographic conditions.

Parameter	Value
Solvent	A) Water B) 1,700 mM sodium chloride C) 44.5 mM sodium phosphate monobasic D) 55 mM sodium phosphate dibasic
Gradient	Gradient from 0 to 30 minutes from 10 to 110 mM NaCl in 30 mM phosphate buffer, pH 6.8 with 500 mM NaCl washing step from 30 to 31 minutes Stop time: 31 minutes Post time: 15 minutes
Flow rate	0.200 mL/min
Temperature	30 °C
UV Detection	280 nm 10 Hz
Injection	Injection volume: 4 µL Sample temperature: 8 °C Needle wash: 3 s in water

Table 2. Quaternary phosphate-based buffer system—pH gradient chromatographic conditions.

Parameter	Value
Solvent	A) Water B) 1,700 mM sodium chloride C) 44.5 mM sodium phosphate monobasic D) 55 mM sodium phosphate dibasic
Gradient	Gradient from 0 to 30 minutes from pH 7 to 8.4 in 30 mM phosphate buffer, pH 6.8 with 500 mM NaCl washing step from 30 to 31 minutes Stop time: 31 minutes Post time: 15 minutes
Flow rate	0.200 mL/min
Temperature	30 °C
UV Detection	280 nm 10 Hz
Injection	Injection volume: 4 µL Sample temperature: 8 °C Needle wash: 3 s in water

Table 3. Binary wide pH gradient buffer system/Farnan pH gradients.

Parameter	Value
Solvent	A) n/a B) n/a C) pH = 6; IS = 22,4 mM; BC = 6,14 mM (Farnan Buffer[1]) D) pH = 10,5; IS = 0,717 mM; BC = 2,31 mM (Farnan Buffer[1])
Gradient	Trastuzumab gradient: Gradient from 0 to 50 minutes from pH 8.3 to 10 with a subsequent "wash" step from 51 to 55 minutes at pH 10.5 Stop time: 55 minutes Post time: 20 minutes NISTmAb gradient: Gradient from 0 to 45 minutes from pH 8.9 to 10.5 Stop time: 50 minutes Post time: 20 minutes
Flow rate	0.200 mL/min
Temperature	30 °C
UV Detection	280 nm 10 Hz
Injection	Injection volume: 4 µL Sample temperature: 8 °C Needle wash: 3 s in water

Results and discussion

With Buffer Advisor, it is possible to calculate both salt as well as pH gradients. With the quaternary phosphate buffer described in the Experimental section of this application note, it is possible to calculate both versions for the separation of trastuzumab charge variants.

Figure 2 displays the overlay of two chromatograms, separating trastuzumab charge variants with a flat salt gradient (blue) and a phosphate-buffered pH gradient from pH 7 to 8.4 (green). The separations by salt and pH gradient are comparable, with slight improvements in resolution when using the pH gradient.

One of the features of Buffer Advisor Software is the improved calculation of linear gradients (salt as well as pH) by adding additional gradient steps within the given gradient to enable perfect linearity without major deviations from the desired/preset pH. To enable this functionality, the Optimize Gradient box in the *4. Create % Timetable* section in the Buffer Advisor user interface (UI) needs to be checked (see red circle in Figures 3A and 3B).

Figures 3A and B showcase the difference between the preset and the actual pH if the box is unchecked (A) and checked (B). With no further optimization from Buffer Advisor, the actual pH can deviate up to 0.4 units from the preset pH, which makes it difficult for the user to rely on the running gradient linearity. By checking the Optimize Gradient box (Figure 3B), additional steps are inserted into the original gradient to ensure linearity of the pH gradient. The Result Pump Gradient Timetable on the bottom left displays the additional inserted steps resulting in the actual gradient being as close as possible to the preset gradient. This gradient timetable can then be exported into the method in OpenLab for an easy transfer without additional time needed for typing.

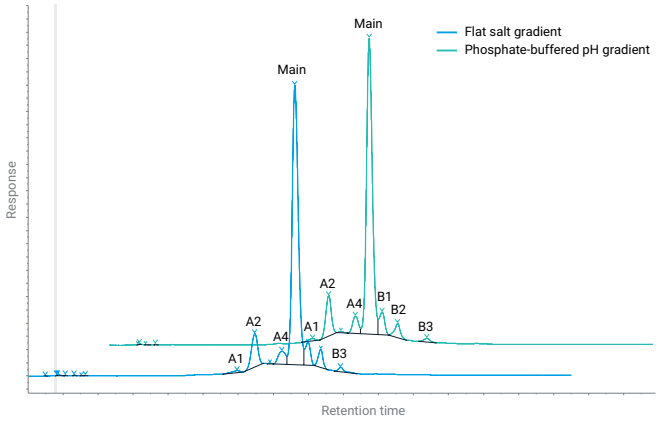

Figure 2. Overlay of two chromatograms for the separation of charge variants with a flat salt gradient (blue) as well as a phosphate-buffered pH gradient from pH 7 to 8.4 (green).

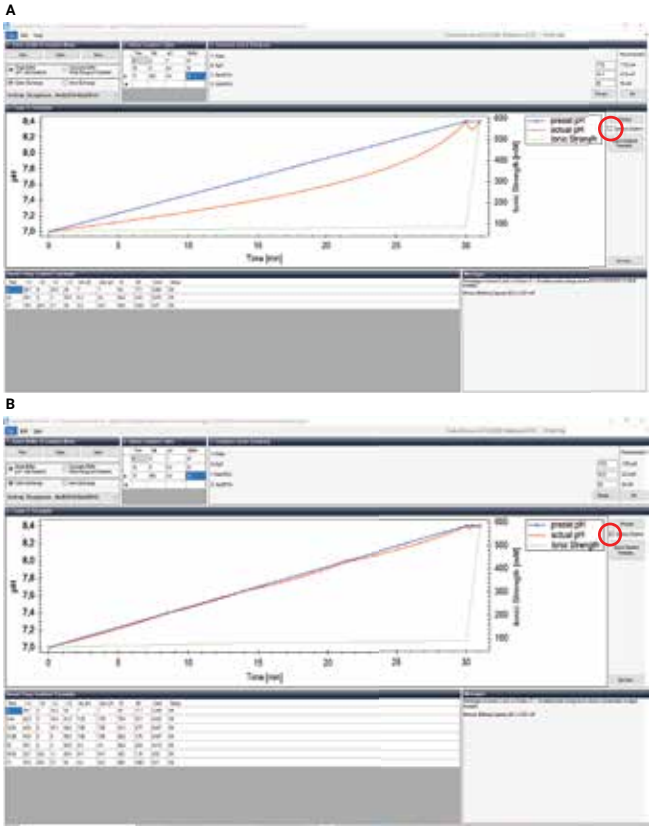

Figure 3. Optimize Gradient function in Agilent Buffer Advisor software to enable highly linear pH gradient. A shows no optimization, B displays the optimized gradient.

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Figure 4 shows an overlay of seven subsequent runs of the charge variant analysis of trastuzumab with the phosphate-buffered pH gradient from pH 7 to 8.4. Excellent reproducibility was found for retention time (RT) and area with relative standard deviation (RSD) of less than 0.085% apart from the first two peaks. Due to the minimal area as well as height of the variants A3 and B3, the area reproducibility is higher than 1%.

Within the phosphate-buffered system, method development is limited, especially if the pI of the molecules of interest is not in the pH buffering range between 6 and 8. If the pI of the molecule is too high, elution is not possible using this buffer system. For example, the NIST mAb with a pI of 9.186 needs a different buffer system to enable elution from the CEX column.

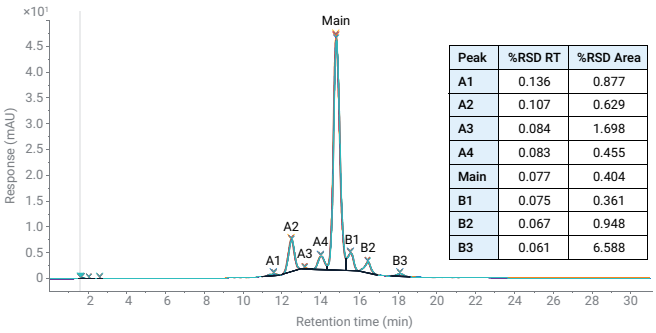
A more generic approach is to use a wide-range pH gradient composed of more than one buffer system. This setup is also incorporated in Buffer Advisor Software under the name Composite Buffer (Wide Range pH Gradient). The pH gradient with pH range 6.0 to 10.5 from Farnan and Moreno (2009)[1] is a suitable buffer system to analyse the charge variants of monoclonal antibodies.

This system was further used and the method optimized for trastuzumab and NIST mAb.

Figure 5 shows the separation of trastuzumab charge variants using the wide-range pH gradient, narrowed from pH 8.3 to 10 to achieve optimal resolution. Compared to the phosphate-buffered pH gradient (see Figure 2), it was possible to resolve two more acidic variants—A1 to A6—eluting before, and one more basic variant eluting after the main peak. Especially the zoomed view in Figure 5B shows the excellent resolution of different charge variants around the main peak, with sharper peaks and enhanced resolving power compared to the shallow salt and pH gradient shown in Figure 2.

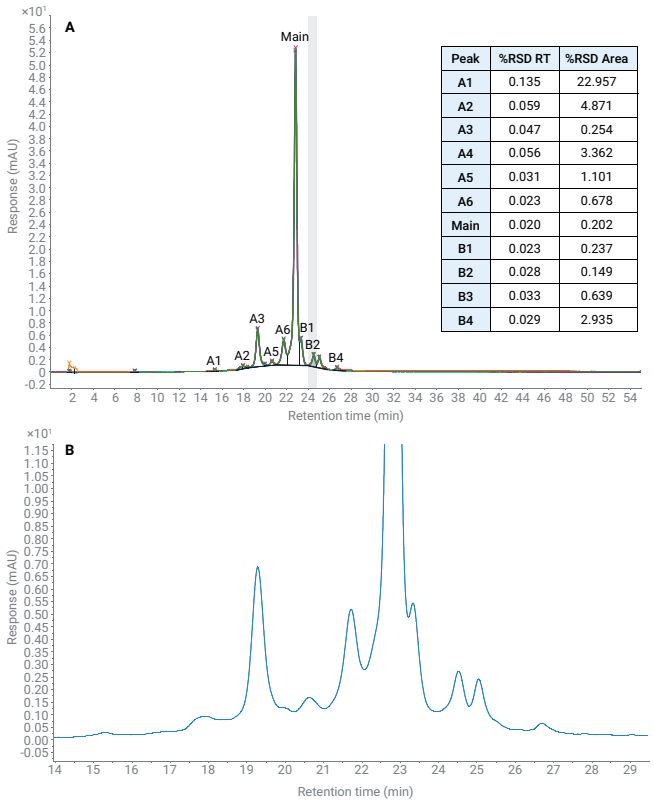
The precision of RT and area was evaluated for all resolved variants (see peak table in Figure 5). Even for extremely small peaks, the precision of RT was excellent, with values below 0.06% RSD except for the first variant A1. The area precision showed excellent values for most of the peaks except for the extremely small ones.

The pH gradient used by Farnan and Moreno[1] has also proven to be ideal for the analysis of the NISTmAb (see Figure 6). For the NISTmAb, the pH gradient was modified to a different pH range due to the different pI of the NISTmAb. With this developed shallow gradient from pH 8.9 to 10.5, it was possible to separate three acidic and two basic variants.



Peak	%RSD RT	%RSD Area
A1	0.136	0.877
A2	0.107	0.629
A3	0.084	1.698
A4	0.083	0.455
Main	0.077	0.404
B1	0.075	0.361
B2	0.067	0.948
B3	0.061	6.588

Figure 4. Overlay of seven consecutive runs of trastuzumab analyzed with phosphate-buffered pH gradient from pH 7 to 8.4 including the precision table for retention time (RT) and area.



Peak	%RSD RT	%RSD Area
A1	0.135	22.957
A2	0.059	4.871
A3	0.047	0.254
A4	0.056	3.362
A5	0.031	1.101
A6	0.023	0.678
Main	0.020	0.202
B1	0.023	0.237
B2	0.028	0.149
B3	0.033	0.639
B4	0.029	2.935

Figure 5. Overlay of seven consecutive runs of trastuzumab using a wide pH gradient from pH 8.3 to 10 including the precision table for retention time (RT) and area A. Zoomed view of a single injection B.

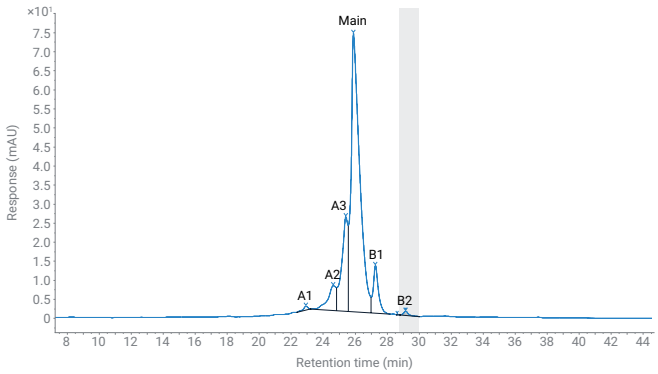


Figure 6. Separation of the NISTmAb with a pH gradient modified according to Farnan and Moreno1 from pH 8.9 to 10.5.

Conclusion

The advantage of pH gradients over salt gradients was demonstrated for the analysis of monoclonal antibodies. pH gradients have been shown to outperform even shallow salt gradients with simplifying method development on the one hand and the generation of high-resolution chromatographic results on the other hand. While the potential of salt gradient method development is rather limited—changes in gradient slope just increase peak width with no further changes in resolution[7]—pH gradients reveal possibilities to further increase resolution and maintain sharp peaks. This was showcased by the analysis of trastuzumab and NIST mAb, especially by the use of the wide range pH gradient, based on Farnan and Moreno.[1] Buffer Advisor Software facilitated dynamic mixing of four stock solutions for the phosphate-buffered systems, preventing time-consuming buffer preparation hands-on time in the lab. In addition, the wide-range pH gradient could be easily calculated by Buffer Advisor. Hence, all methods—developed with Buffer Advisor Software and run on the Agilent 1260 Infinity II Bio Prime LC System with Flexible pump, with its completely iron-free sample flow path—delivered highly reliable and reproducible results.

References

1. Farnan, D.; Moreno, G.T. Multiproduct High-Resolution Monoclonal Antibody Charge Variant Separations by pH Gradient Ion-Exchange Chromatography. *Anal. Chem*. **2009**, 81(*21*), 8846–8857.
2. Liu, H. *et al*. Heterogeneity of Monoclonal Antibodies. J. Pharm. Sci. **2008**, *97*, 2426–2447.
3. Vlasak, J.; Ionescu, R. Heterogeneity of Monoclonal Antibodies Revealed by Charge-Sensitive Methods. *Curr. Pharm. Biotechnol*. **2008**, *9*, 468–481.
4. Rea, J. *et al*. Validation of a pH Gradient-Based Ion-Exchange Chromatography Method for High-Resolution Monoclonal Antibody Charge Variant Separations. *J. Pharm. Biomed. Anal.* **2011**, *54*, 317–323.
5. Lingg, N. *et al*., Highly Linear pH Gradients for Analyzing Monoclonal Antibody Charge Heterogeneity in the Alkaline Range: Validation of the Method Parameters. *J. Chromatogr. A* **2014**, *1373*, 124–130.
6. Turner, A.; Schiel, J. E. Qualification of NISTmAb Charge Heterogeneity Control Assays. *Anal. Bioanal. Chem*. **2018**, *410(8)*, 2079–2093.
7. Schneider, S. How Shallow Can You Go? Refining Charge Variant Analysis of mAbs with the Agilent 1290 Infinity II Bio LC System. *Agilent Technologies application note*, publication number 5994-2692EN, **2020**.

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Application Note

Charge Variant Analysis

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How Shallow Can You Go?

Refining charge variant analysis of mAbs with the Agilent 1290 Infinity II Bio LC System

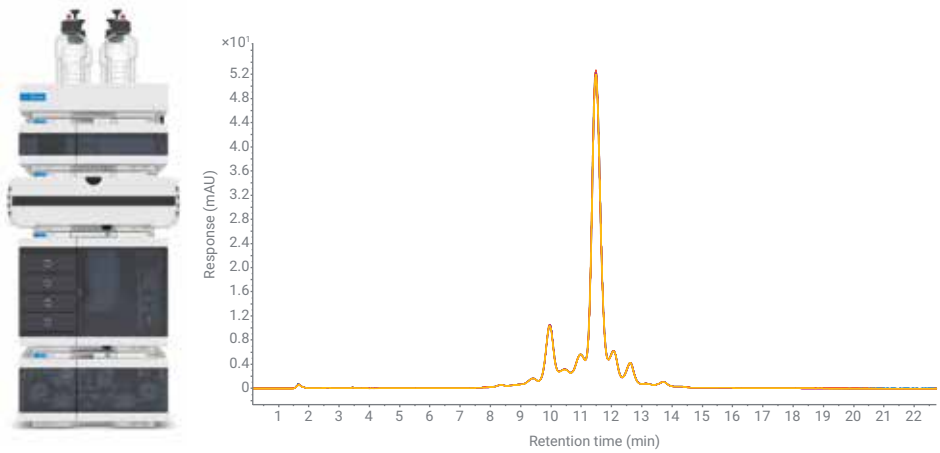
Author

Sonja Schneider
Agilent Technologies, Inc

Abstract

Charge variant analysis is a demanding application for applied liquid chromatography systems due to the use of highly corrosive buffer salts in combination with very shallow gradients for optimal separation. The evaluation of different salt gradients was performed on the Agilent 1290 Infinity II Bio LC System and analyzed for resolution as well as reproducibility.

The 1290 Infinity II Bio LC including High-Speed Pump, with its completely iron-free flow path, is optimally suited for the conditions used in biochromatography—avoiding potentially corrosive damage to the system. Excellent reproducibility even for highly challenging shallow gradients was determined, confirming the 1290 Infinity II Bio LC as the next generation of Agilent high-end liquid chromatography systems for high confidence in generated data.



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Introduction

Monoclonal antibodies (mAbs) are large and highly heterogeneous macromolecules, with a size of around 150 kDa, that are typically generated by recombinant production methods. They are generated in a complex biosynthetic process in which plenty of modifications can occur, leading to hundreds of different variants. Deamidation, oxidation, disulfide bridges, N-glycosylation, N- and C-terminal processing are some of the most common post-translational modifications (PTMs). All these modifications can occur during generation, but also manufacturing and storage contribute to the complexity of these macromolecules. PTMs form a complex isoform profile that needs to be extensively analyzed and monitored, as modifications in the final pharmaceutical might be associated with a loss of biological activity, affected half-life, or immunogenicity.[1] Some of the PTMs result in charge variants of the molecule, which are typically analyzed using ion-exchange chromatography (IEX).[2] Charge variants are considered one of the most important critical quality attributes (CQAs) and therefore strict acceptance criteria and quality controls are to be considered. It is of utmost importance to confirm that the product is correctly manufactured, and to identify and quantify any impurities.

Shallow gradient elution is very common in IEX of proteins. A typical salt gradient in ionic strength mode for the elution of proteins would be approximately 1 to 3 mM/min with a pH value set to a tolerance of ±0.02 pH units.[3]

The 1290 Infinity II Bio LC is equipped with a high-performance High-Speed Pump. The major advantage of binary pumps is that solvent mixing is much more accurate and precise when mixing small proportions of one of the solvent components compared to low-pressure mixing pumps (e.g., quaternary pumps). This type of mixing gives highly precise solvent compositions at the start and the end of a solvent gradient.[4] This is a basis for the generation of reproducible and accurate shallow gradients (below 1%/min from each channel).

The 1290 Infinity II Bio LC is the next generation of Agilent high-end liquid chromatography systems, specially designed for conditions used in biochromatography: high salt concentrations such as 2 M NaCl, up to 8 M urea, and high- and low-pH solvents such as 0.5 M NaOH or 0.5 M HCl. The complete flow path is completely free of stainless steel (SST) or iron; all capillaries and fittings throughout the multisampler, multicolumn thermostat, and detectors are built of MP35N, a nickel-cobalt alloy. With this material, potential corrosion from high salt-containing buffers is reduced and protein modifications caused by the presence of iron ions (e.g. oxidation, protein complex formation) can be avoided.

This application note presents the analysis of charge variants for trastuzumab and the NISTmAb reference standard. Different salt gradient slopes were tested to find the best resolution possible. The best performing gradient slopes were then evaluated for reproducibility.

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System comprised the following modules:

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with standard flow biocompatible heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2 µL

Software

Agilent OpenLab CDS Version 2.5

Columns

Agilent Bio MAb, NP5, 2.1 × 250 mm, PEEK (part number 5190-2411)

Chemicals

All solvents were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, sodium chloride, hydrochloric acid, sodium hydroxide, tris(hydroxymethyl)-aminomethane, imidazole, and piperazine hexahydrate were obtained from Sigma-Aldrich (Steinheim, Germany).

Samples

- Agilent-NISTmAb (p/n 5191-5744)
- Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland)
- The trastuzumab was dissolved in 30 mM phosphate buffer, pH 6.8

Note: As Adcetris contains many adjuvants, the concentration mentioned here is not the protein concentration only, but the total concentration of all components of the drug.

Buffer preparation

For 2 L of 30 mM phosphate buffer, pH 6.8, 4.45 g of sodium phosphate monobasic monohydrate and 7.44 g of sodium phosphate dibasic heptahydrate was weighed and added to an amber-colored 2 L bottle and filled up to 2 L using ultrapure water (› buffer A). 29.22 g of sodium chloride, for a total concentration of 500 mM, was added to an empty, amber-colored 1 L bottle and filled up to 1 L using the prepared phosphate buffer A (› buffer B). The pH values of both prepared buffers were checked and adjusted, if necessary, to pH 6.8 (the addition of high amounts of salt can change the pH). Both prepared buffers were filtered using a 0.2 µm membrane filter.

Method

Table 1. Salt gradient chromatographic conditions.

Parameter	Value
Solvent	A) 30 mM phosphate buffer, pH 6.8, B: 30 mM phosphate buffer, pH 6.8, 500 mM sodium chloride
Gradient	0 or 25 mM—150 mM NaCl in 30 minutes—different shallow gradients for method development 0 mM (trastuzumab) and 25 mM (NIST) to 100 mM NaCl in 30 minutes for reproducibility 25 to 50 mM NaCl in 30 minutes for reproducibility (very shallow gradient) 31 minutes—500 mM NaCl wash Stoptime: 35 minutes Post-time: 15 minutes
Flow rate	0.200 mL/min
Temperature	30 °C
Detection	280 nm, 10 Hz
Injection	Injection volume: 3 µL for trastuzumab and 2 µL for NIST Sample temperature: 10 °C Needle wash: 3 s in water

Note: When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method. For example, for Solvent B, including 500 mM sodium chloride, use “Sodium Chloride 0.5 M” rather than Generic Aqueous or Water in the solvent selection field in the pump method. High amounts of salts change the compressibility of the solvent, and so using the preconfigured solvent tables enables best pump performance.

Results and discussion

Method development

To achieve the desired resolution and enable optimal separation, extensive method development is necessary for charge variant analysis. Two parameters are essential to be successful: finding the optimal pH as well as the optimal gradient slope. Both factors can have a major impact on the separation. First, pH scouting is recommended to find the optimal pH for the separation. In earlier experiments, the pH of the used buffers was analyzed from pH 6.4 to 7.4 and was found to be optimal at pH 6.8 for both samples used: trastuzumab and the NISTmAb reference standard (data not shown). The next step is the determination of the ideal gradient slope to enable the efficient separation.

Figure 1 shows an overlay of charge variant analysis of trastuzumab at different gradient slopes ranging from 1% B/min (5 mM/min) down to 0.33% B/min (1.66 mM/min). The shallower the gradient, the higher the requirements to the pump performance. To deliver highly precise solvent compositions during the gradient, the pump needs to work accurately and precisely when mixing small proportions of the solvent components. It has to be considered, though, that for salt gradients, very shallow gradients do not always result in higher resolution, but simply increase peak width (e.g. 0.33% B/min in Figure 1). Therefore, the chosen gradient slope for reproducibility studies was found in the middle of the tested gradients with 0.66% B/min and 3.3 mM/min, which can still be considered shallow.

A similar method development procedure was carried out for the separation of charge variants of the NISTmAb reference standard (see Figure 2). The starting conditions for NIST contained a slighty higher amount of salt due to the higher isoelectric point (pI) of the NIST antibody (pI of ~9.2), compared to trastuzumab with a pI of ~9. For more effective separation of the charge variants of the NISTmAb reference standard, the gradients were slightly shallower compared to trastuzumab, so the most shallow gradient was at 0.17% B/min (0.83 mM/min), which is a challenging task for the pump. For further reproducibility studies, the 0.5% B/min (2.5 mM/min) gradient slope was chosen.

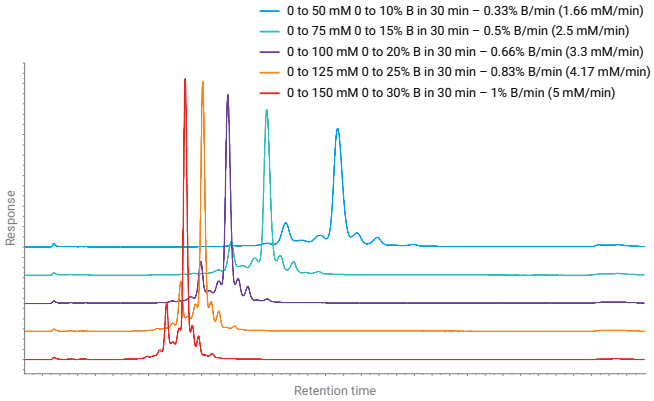

Figure 1. Method development for the separation of trastuzumab with different salt gradient slopes.

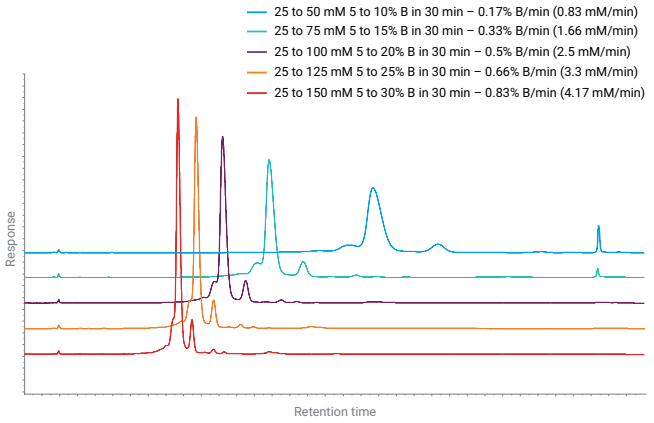

Figure 2. Method development for the separation of the NISTmAb reference standard with different salt gradient slopes.

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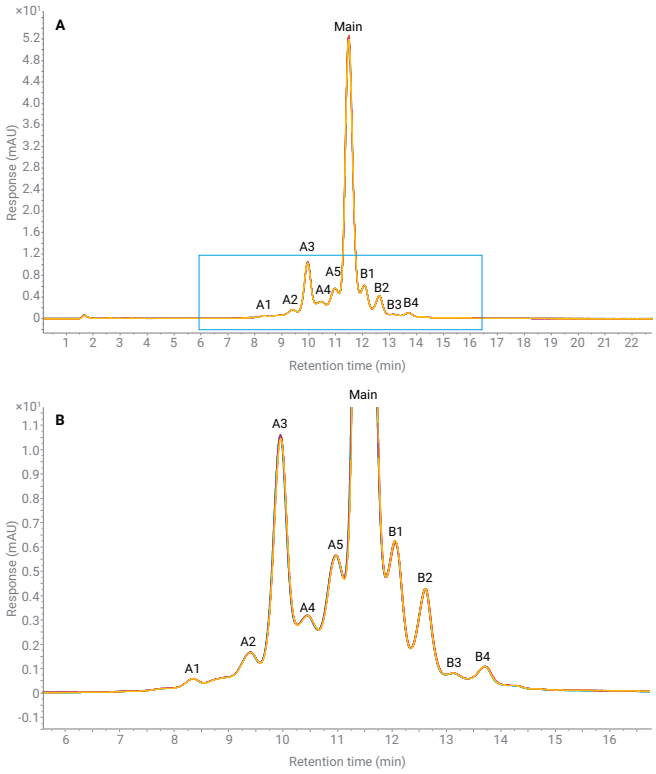
Reproducibility for trastuzumab charge variant separation

Figure 3 displays reproducibility studies for charge variant separation of trastuzumab (A) with a 0.66% B/min (3.3 mM/min) gradient slope. Figure 3B shows a zoomed view for better visualization of the separated variants. Variants marked with A represent the acidic variants eluting before the main peaks, whereas the basic variants B elute after the main peak. Five acidic variants were resolved before the main peaks and four basic variants eluted after the main peak. All variants and the main peak were evaluated for the precision of retention time (RT) and area. Both RT as well as area precision are excellent, with values below 0.052% relative standard deviation (RSD) for RT and below 0.82% RSD for area except for the two very small variant peaks A1 and B3.



Precision in RSD	RT (%)	Area (%)
A1	0.033	1.793
A2	0.016	0.701
A3	0.026	0.403
A4	0.023	0.813
A5	0.032	0.327
Main	0.033	0.313
B1	0.038	0.329
B2	0.048	0.254
B3	0.046	3.549
B4	0.051	0.812

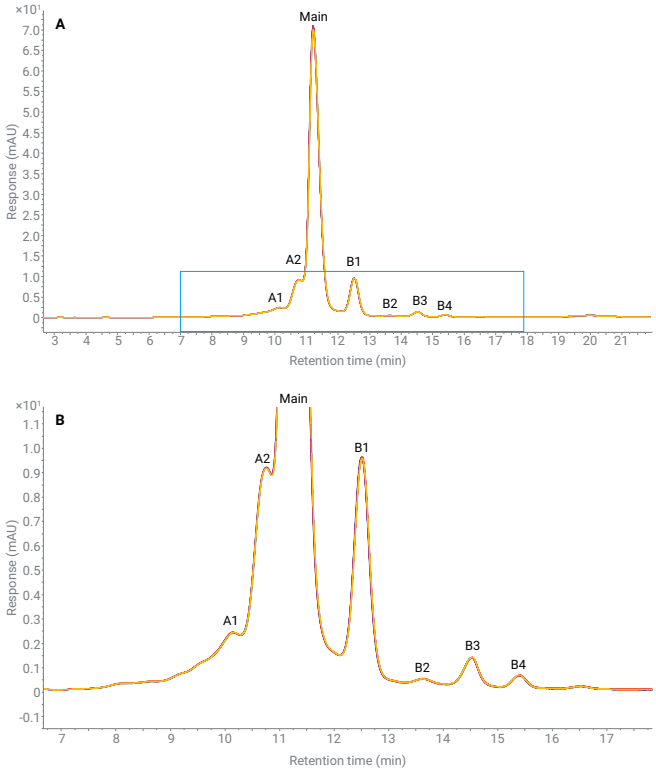
Figure 3. Reproducibility studies with seven subsequent runs for charge variant separation of trastuzumab (A) with 0.66% B/min (3.3 mM/min) gradient slope. (B) Zoomed view.

Reproducibility for NISTmAb charge variant separation

Figure 4 displays reproducibility studies for charge variant separation of the NISTmAb reference standard (A) with 0.5% B/min (2.5 mM/min) gradient slope.

Figure 4B displays the zoomed view with two acidic variants and four basic variants. Again, all variants and the main peak were evaluated for precision of retention time (RT) and area. Both RT as well as area precision are excellent, with values below 0.06% RSD for RT and below 0.55% RSD for area except for one very small variant peak, B2.

As shown in Figure 2, the shallowest gradient with 0.17% B/min (0.83 mM/min) does not deliver a better resolution compared to gradients such as 0.5% B/min (2.5 mM/min) (still shallow—used in the reproducibility studies).



Precision in RSD	RT (%)	Area (%)
A1	0.058	0.540
A2	0.039	0.374
Main	0.045	0.126
B1	0.042	0.141
B2	0.049	1.469
B3	0.033	0.354
B4	0.033	0.485

Figure 4. Reproducibility studies with seven subsequent runs for charge variant separation of the NISTmAb reference standard (A) with 0.5% B/min (2.5 mM/min) gradient slope. (B) Zoomed view.

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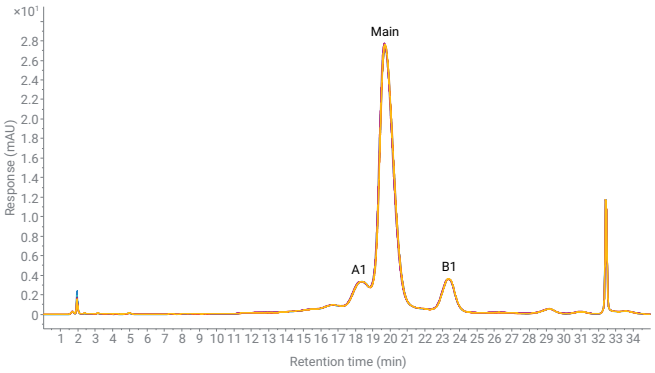
However, the 1290 Infinity II Bio High-Speed Pump managed the challenging gradient slope, which is displayed in Figure 5. RT precision over seven subsequent runs was very good (below 0.25% RSD), although the peaks became quite broad with the applied gradient. With increasing peak width, the peak height decreases, which negatively affects the area precision.



Precision in RSD	RT (%)	Area (%)
A1	0.238	6.608
Main	0.102	1.95
B1	0.08	8.157

Figure 5. Reproducibility studies with seven subsequent runs for charge variant separation of the NISTmAb reference standard with 0.17% B/min (0.83 mM/min) gradient slope.

Conclusion

Different salt gradient slopes were evaluated for resolution and reproducibility of the separation of charge variants for trastuzumab and NISTmAb on the 1290 Infinity II Bio LC. At first glance, shallower gradients seemed to improve resolution. However, for both mAbs, the evaluated most shallow gradients did not give the best resolution, as with decreasing slope, the peaks only began to broaden, which led to no further improvement of resolution. The methods with the best combination of high resolution and sharp peak shapes were further evaluated for reproducibility. For a gradient slope of 3.3 mM/min (trastuzumab) and 2.5 mM/min (NISTmAb), excellent reproducibility for RT but also area was documented. The RT precision was below 0.06% RSD for all evaluated peaks. The most shallow gradient tested for NISTmAb was also evaluated for reproducibility, and even for a super shallow gradient with 0.83 mM/min gradient slope, very good RT precision was found (<0.25% RSD). These data show that the 1290 Infinity II Bio LC with its completely iron-free flow path is optimally suited for the conditions used in biochromatography, leading to highly reproducible results.

References

1. Dick Jr., L. W. *et al*. Identification and Measurement of Isoaspartic Acid Formation in the Complementarity Determining Region of a Fully Human Monoclonal Antibody. J. Chromatogr. B Analyt. Technol. Biomed. *Life Sci*. **2009**, *877(30)*, 3841–3849.
2. Zhang, L. *et al*. Improving pH Gradient Cation-Exchange Chromatography of Monoclonal Antibodies by Controlling Ionic Strength. *J. Chromatogr*. *A* **2013**, *1272*, 56–64.
3. Farnan, D.; Moreno, G. T. Multiproduct High-Resolution Monoclonal Antibody Charge Variant Separations by pH Gradient Ion-Exchange Chromatography. *Anal. Chem*. **2009**, *81(21)*, 8846–8857.
4. The LC Handbook Guide to LC Columns and Method Development. *Agilent Technologies*, publication number 5990-7595EN, **2016**.
5. Goyon, A. et al. Determination of Isoelectric Points and Relative Charge Variants of 23 Therapeutic Monoclonal Antibodies. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci*. **2017**, *1065–1066*, 119–128.
6. Xie, L. *et al*. Demonstrating Analytical Similarity of Trastuzumab Biosimilar HLX02 to Herceptin with a Panel of Sensitive and Orthogonal Methods Including a Novel FcγRIIIa Affinity Chromatography Technology. *BioDrugs* **2020**, *34(3)*, 363–379.

Application Note

Charge Variant Analysis

Agilent

Trusted Answers

Charge Heterogeneity Analysis of Rituximab Innovator and Biosimilar mAbs

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Abstract

This Application Note describes the high-resolution separation of charge variants of innovator and biosimilar rituximab using an Agilent 1260 Infinity Bio-inert Quaternary LC, biocolumns, and an Agilent OpenLAB ChemStation Software tool. An Agilent Bio MAb, 4.6 × 250 mm, 5 µm PEEK ion exchange column features a unique resin designed for the charge-based separation of monoclonal antibodies (mAbs). The optimized salt-gradient showed the differences in acidic and basic charge variant profiles between innovator and biosimilar rituximab. Precision of retention time, height, and area of charge isoforms were well within the acceptable range. C-terminal digestion by Carboxypeptidase B (CPB) revealed the major lysine variant peaks in biosimilar rituximab.

Introduction

Recently, biosimilar products are increasing in popularity in biopharmaceuticals. mAbs can undergo various post-translational modifications (PTMs) including lysine truncation, deamidation, oxidation, glycosylation, and so forth, becoming heterogeneous in their biochemical and biophysical properties. Due to these modifications, charge variants can affect the efficacy, activity, and stability of mAbs as biotherapeutics. Hence, it is very important to characterize the charge heterogeneity in drug development that will serve as a quality control (QC) step in the biopharmaceutical industry. In addition, precise bioanalytical methods are necessary to demonstrate the similarity between a biosimilar and the innovator product.

Cation exchange chromatography (CEX) is the gold standard for charge-sensitive antibody analysis. In CEX, method parameters often need to be optimized for each protein, as ion exchange depends upon the reversible adsorption of charged protein molecules to immobilized ion exchange groups. This Application Note describes the salt-gradient method for separating the charge variants of innovator and biosimilar rituximab using an Agilent 1260 Infinity Bio-inert Quaternary LC and an Agilent Bio MAb NP5, 4.6 × 250 mm, PEEK ion exchange column. The method compares the CEX profiles of innovator and a rituximab biosimilar. Precision of retention time, height, area, and quantification of acidic, basic, and main forms was determined. Carboxypeptidase B (CPB) digestion was performed to study the contribution of C-terminal lysine variants.

Experimental

Instrumentation

An Agilent 1260 Infinity Bio-inert Quaternary LC, operating to a maximum pressure of 600 bar, was used for the experiments. The entire sample flow path was free of any metal components so that the sample did not come in contact with metal surfaces. Solvent delivery was free of any stainless steel or iron components.

Systems

- 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 (TCC) containing bio-inert click-in heating elements (G1316C option 19)
- Agilent 1260 Infinity Diode Array Detector with with 10 mm bio-inert standard flow cell (G1315D)
- Agilent Bio MAb NP5, 4.6 × 250 mm, PEEK (p/n5190-2407)

Software

- Agilent OpenLAB CDS ChemStation Edition, revision C.01.062
  Agilent Buffer Advisor, Rev. A.01.01

Table 1. Chromatographic parameters used for IEX chromatography.

Parameter	Conditions				
Mobile phase A	Water				
Mobile phase B	NaCl (850.0 mM)				
Mobile phase C	NaH_2PO_4 (41.0 mM)				
Mobile phase D	Na_2HPO_4 (55.0 mM)				
Gradient	**Time (min)**	**Mobile phase A (%)**	**Mobile phase B (%)**	**Mobile phase C (%)**	**Mobile phase D (%)**
	0	30.3	0	59.6	10.1
	2	26.0	5.0	56.9	12.1
	8	21.5	10.0	54.9	13.6
	20	13.3	19.0	51.9	15.8
	21	30.3	0	59.6	10.1
Injection volume	5 µL				
Flow rate	0.75 mL/min				
Data acquisition	280 nm/4 nm, Ref.: 360 nm/100 nm				
Acquisition rate	5 Hz				
TCC	Room temperature				
Sample thermostat	5 °C				
Post run time	10 minutes				

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Reagents, samples, and procedure

Innovator and biosimilar rituximab were purchased from a local pharmacy and stored according to the manufacturer's instructions. Sodium phosphate dibasic, sodium phosphate monobasic, sodium chloride, hydrochloric acid (HCl), and sodium hydroxide were purchased from Sigma-Aldrich. All the chemicals and solvents were HPLC grade, and highly purified water was from a Milli Q water purification system (Millipore Elix 10 model, USA). Carboxypeptidase B (C9584) was purchased from Sigma-Aldrich.

Ion exchange chromatography parameters

Table 1 shows the chromatographic parameters for ion exchange chromatography using a 1260 Infinity Bio-inert Quaternary LC. Rituximab (innovator and biosimilar) were diluted to 1 mg/mL in water, and the elution was monitored at 280 nm. Retention time (RT), area, and percent area were used to calculate standard deviation (SD) and relative standard deviation (RSD %) values. Relative percent area was used to quantify the charge variants of mAbs.

Carboxypeptidase B digestion

Biosimilar and innovator rituximab were diluted to 1 mg/mL using 10 mM sodium phosphate buffer, pH 7.5. To these, 0.25 units of CPB was added and incubated at 37 °C. At various time points, the reaction mixture was aliquoted and quenched with acetic acid before analysis.

Results and Discussion

The Agilent Buffer Advisor Software is an ideal tool to generate pH or ionic strength gradients for protein charge variant separation. It reduces the time required for method development. In this study, a series of method development scouting runs were carried out using the Buffer Advisor Software for optimal mAb charge variant separation. Figure 1 shows the charge variant profiles of innovator and biosimilar rituximab on a Bio MAb PEEK column, demonstrating high-resolution separation of charge variants in 20 minutes with three distinct peaks in biosimilar (Buffer: 30 mM, pH: 6.3, and NaCl: 0–161.5 mM). The Agilent Bio MAb columns contained a highly uniform, densely packed, weak cation exchange resin. Early and late-eluting peaks were called acidic and basic variants, respectively. The peak at 11.4 minutes was designated as the main peak. The overlay of five replicates of innovator and biosimilar rituximab shows excellent separation reproducibility (Figure 2). The average RTs and area RSDs for main peak are shown in the figure. The RSDs are within the acceptable range, which demonstrates the precision of the system.

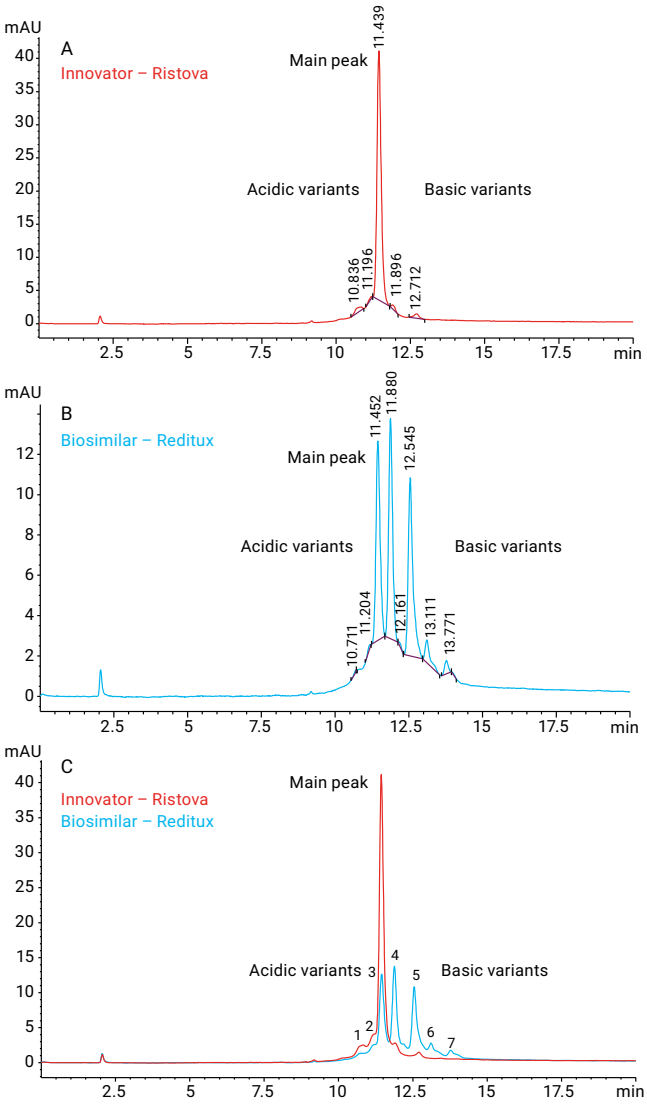

Figure 1. Charge variant profiles of innovator (A) and biosimilar (B) rituximab using an Agilent Bio MAb 5 µm column. C) Overlay of innovator and biosimilar rituximab. Peaks 1 and 2: acidic variants; 3: main form; 4, 5, 6 and 7: basic variants.

The high-resolution separation of mAbs facilitated the quantification of charge variants using peak areas. Table 2 summarizes the area percent of charge variants of five consecutive analyses. There was a significant difference in the area percent of the charge variants between two mAbs. The main form in the innovator rituximab was found to be 93.21 % and 29.78 % in biosimilar rituximab. The major charge variant in biosimilar rituximab was 69.46 % basic variants as compared to the innovator product (3.22 %).

Table 2. Charge variants quantification by area %, n = 5.

Innovator – Ristova	**RT (min)**	**Area %**
Acidic variant	10.84, 11.21	3.56
Main peak	11.44	93.21
Basic variant	11.9, 12.7	3.22
Biosimilar – Ristova	**RT (min)**	**Area %**
Acidic variant	10.73, 11.22	0.76
Main peak	11.45	29.78
Basic variant	11.87, 12.15, 12.59, 13.1, and 13.77	69.46

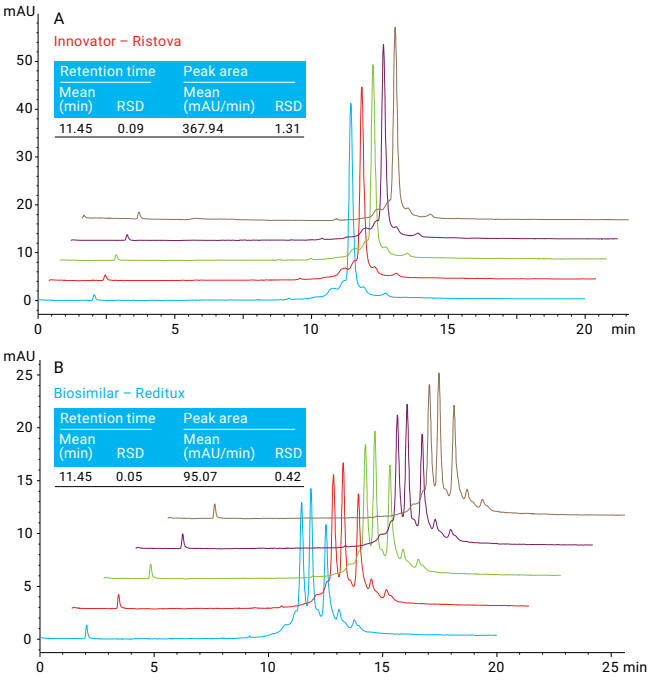

Figure 2. Overlay of five replicates of innovator (A) and biosimilar (B) rituximab on an Agilent 1260 Infinity Bio-inert Quaternary LC using an Agilent Bio Mab, 4.6 × 250 mm, 5 µm, PEEK column. Insert table shows the precision of retention time and area for main peak, n = 5.

To further characterize the basic variant mAU peaks, both mAbs were subjected to carboxypeptidase B digestion. Figures 3A and 3B show the overlay 50 of the IEX profiles before and after C-terminal cleavage of innovator and biosimilar rituximab, respectively. The disappearance of basic variant peaks after carboxypeptidase B treatment confirmed that the peaks correspond to lysine 20 variants. Figure 4 shows the overlay of the IEX profiles of biosimilar rituximab after CPB treatment and innovator rituximab without CPB treatment, 0 revealing the charge variant similarity between the mAbs.

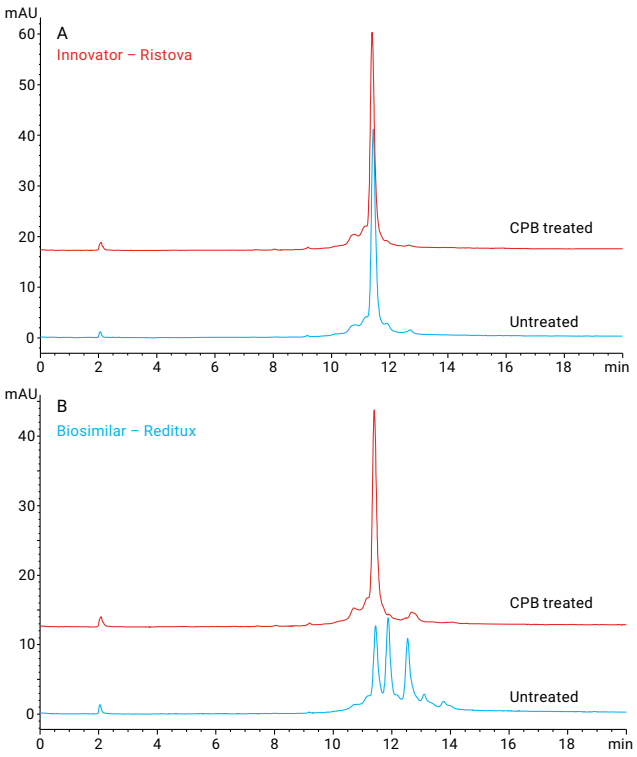

Figure 3. Characterization of basic charge variants. Separation of CPB treated (overnight) and untreated of innovator (A) and biosimilar (B) rituximab on an Agilent 1260 Infinity Bio-inert Quaternary LC using an Agilent Bio Mab, 4.6 × 250 mm, 5 µm, PEEK column.

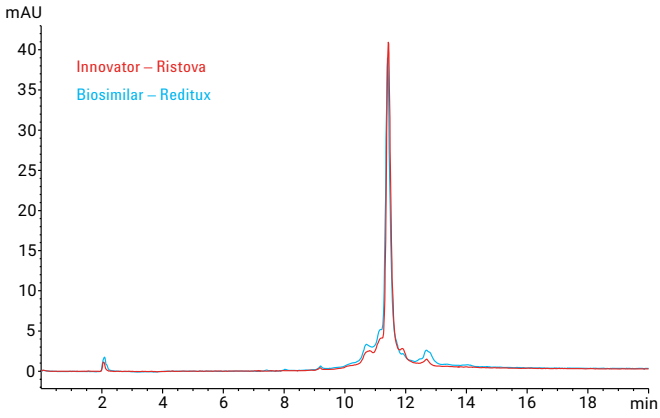

Figure 4. Overlay of innovator rituximab without CPB treatment (red) and biosimilar rituximab after CPB treatment (blue).

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Conclusion

The salt-gradient method described in this Application Note demonstrates the high-resolution separation of charge variant profiles of mAbs on an Agilent Bio MAb, 4.6 × 250 mm, 5 µm PEEK column. The innovator and biosimilar rituximab had different separation profiles with different degrees of acidic and basic variants. Carboxypeptidase B digestion confirmed that the major basic variant peaks in biosimilar correspond to lysine variants. The Agilent 1260 Infinity Bio-inert Quaternary LC with Bio MAb PEEK columns and reproducible method make this solution particularly suitable for the QA/QC analysis of mAbs for the biopharmaceutical industry.

References

Yan, He; et al. J. Sep. Sci. **2011**, 34, 548–555

Agilent publication number 5991-0895EN

Agilent publication number 5990-6844EN

Agilent publication number 5991-0565EN

Application Note

Charge Variant Analysis

Agilent

Trusted Answers

Faster Separations Using Agilent Weak Cation Exchange Columns

Authors

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Abstract

Ion exchange is a commonly used technique for the separation of complex protein mixtures. Traditionally, such separations are performed using shallow gradients of increasing salt concentration with long column lengths providing the necessary resolution. The columns have often been packed using large diameter particles to minimize backpressure. This Application Note demonstrates how analysis times can be significantly reduced, increasing throughput without compromising analytical performance, by exploiting the benefits of small particle size, non-porous ion exchange sorbents.

Introduction

Proteins, polypeptides and oligonucleotides are often analyzed by ion exchange chromatography because they are complex molecules with multiple charges on their surfaces. The technique is ideally suited to the separation of charged biomolecules as it is nondenaturing and can provide good performance and resolution.

Traditionally, this has meant using highly porous particles to enable such large molecules to permeate the particles. In turn, columns of 15 cm or 25 cm in length, packed with 5 µm or 10 µm particles are commonly used.

The advent of non-porous sorbents such as Agilent's Bio IEX range, comprising a rigid polymeric core particle with a grafted hydrophilic layer containing the ion-exchange functionality, can improve resolution. This is because the diffusion-limited band broadening associated with a molecule penetrating the core of a large particle is eliminated. In turn, this means smaller particles and shorter column lengths can be used to significantly improve throughput, greatly reducing analysis times. The benefits for improved productivity for tasks such as fraction analysis are immediately evident.

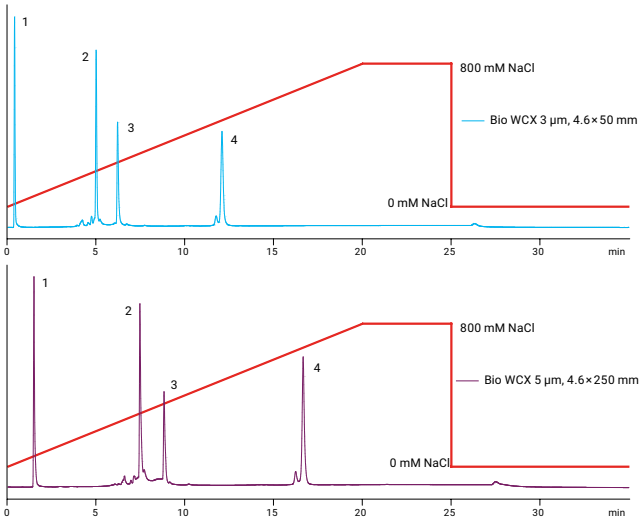

Figure 1. Protein separation on Agilent Bio WCX 5 µm 4.6 × 250 mm versus Agilent Bio WCX 3 µm, 4.6 × 50 mm (flow rate 1.0 mL/min).

Materials and methods

Agilent Bio IEX columns are packed with rigid polymeric, nonporous particles grafted with a functionalized hydrophilic polymer layer. The resultant 1.7, 3, and 5 µm rigid particles provide high resolution and high separation efficiency by reducing the band broadening effects resulting from diffusion limitations with totally porous particles. The chemically bonded hydrophilic coating significantly reduces the effects of nonspecific binding and results in greater levels of recovery.

Conditions, Bio-Monolith column

Parameter	Value
Columns:	Agilent Bio WCX 5 µm, 4.6 × 250 mm SS (p/n5190-2445) Agilent Bio WCX 3 µm, 4.6 × 50 mm SS (p/n5190-2443) Agilent Bio WCX 1.7 µm, 4.6 × 50 mm SS (p/n5190-2441)
Sample:	Ovalbumin (1), Ribonuclease A (2), Cytochrome c (3), Lysozyme (4)
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC
Mobile Phase:	A: 20 mM sodium phosphate, pH 6.5 B: A + 1.6 M NaCl
Gradient:	0 to 50 % B
Temp.:	Ambient
Injection volume:	10 µL
Conc.:	0.5 mg/mL
Detection:	UV, 220 nm

Results and Discussion

The performance of a column, as measured by plate count, is dependent on particle size and column length. From this it may be inferred that a shorter column packed with smaller particles can be used to achieve the same level of performance when compared to a longer column packed with larger particles (Figure 1). This is commonly found in practice. However, for gradient elution, further modifications to the method need to be employed to provide the additional benefits of shorter run times and greater productivity.

Converting gradient times into column volumes is a useful way of calculating the shorter gradient program and can provide the desired outcome in terms of higher speed separations (Table 1). However, smaller particle sizes may require higher flow rates to attain maximum performance. This is illustrated by the van Deemter curves shown in Figure 2.

To maximize the separation efficiency using the Agilent Bio WCX 3 µm, 4.6 × 50 mm column, the 4 minute gradient separation was carried out at 1.0, 1.5, 2.0, and 2.5 mL/min (Figure 3). As expected, the higher linear velocity created from higher flow rates improved the peak shape.

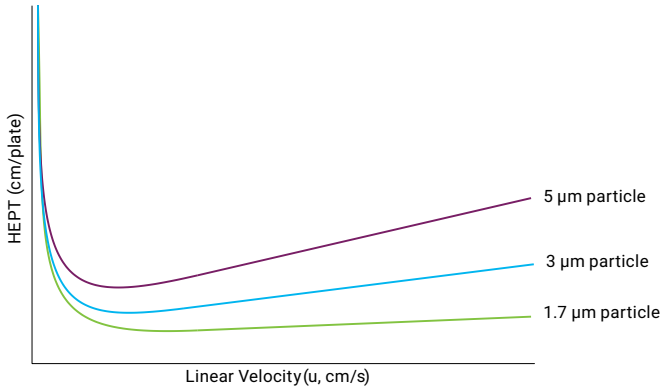

Figure 2. Typical van Deemter curves.

Table 1. Gradient Time to Column Volume Conversion

Time (minutes)	mM NaC	#CV
0	0	0.0
20	800	4.8
25	800	6.0
25.01	0	6.0
35	0	8.4

#CV = number of column volumes at 1.0 mL/min (4.6 Å~ 250 mm column)

Time (minutes)	mM NaCl	#CV
0	0	0.0
4	800	4.8
5	800	6.0
5.01	0	6.0
7	0	8.4

#CV = number of column volumes at 1.0 mL/min (4.6 Å~ 50 mm column)

In comparison, the Agilent Bio WCX 1.7 µm, 4.6 × 50 mm column provided sharper peaks under identical conditions (Figure 4).

Increasing the flow rate should mean that it is possible to further reduce the gradient time. This was investigated using the Bio WCX 1.7 µm, 4.6 × 50 mm column. The 0 to 800 mM NaCl gradient was reduced from 4 to 2 minutes.

It was found that at a flow rate of 1.7 mL/min the backpressure remained below 400 bar and still provided exceptional peak shape and resolution (Figure 5).

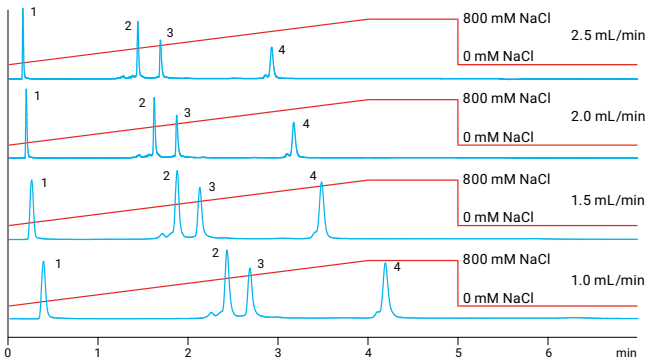

Figure 3. Effect of flow rate on chromatographic performance (Agilent Bio WCX 3 µm, 4.6 × 50 mm).

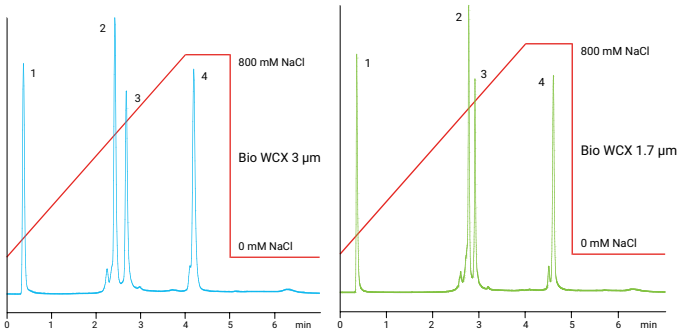

Figure 4. Comparison of Agilent Bio WCX 3 µm, 4.6 × 50 mm versus Agilent Bio WCX 1.7 µm

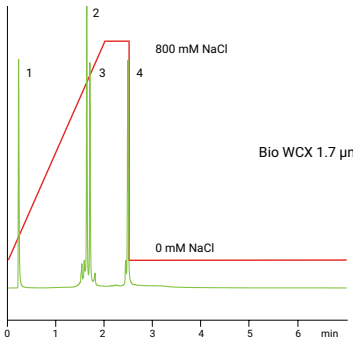

Figure 5. Agilent Bio WCX 1.7 µm, 4.6 × 50 mm for protein separations under 3 minutes (flow rate 1.7 mL/min)

Conclusions

We have shown that by using shorter 5 cm columns packed with smaller particle size (3 µm and 1.7 µm), Agilent Bio WCX products can lead to significant reductions in run times from 20 or 30 minutes down to less than 3 minutes, and still retain excellent peak resolution. This enables much higher throughput in time-critical applications.

The backpressure of 400 bar shows that, by reducing the analysis time dramatically from over 30 minutes to less than four minutes for the entire gradient, a 600 bar system such as the Agilent 1260 Infinity Bio-inert LC is still sufficient.

For More Information

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Application Note

Charge Variant Analysis

Optimizing Protein Separations with Cation Exchange Chromatography Using Agilent Buffer Advisor

Protein separation with the Agilent 1260 Infinity Bio-inert Quaternary LC System

Authors

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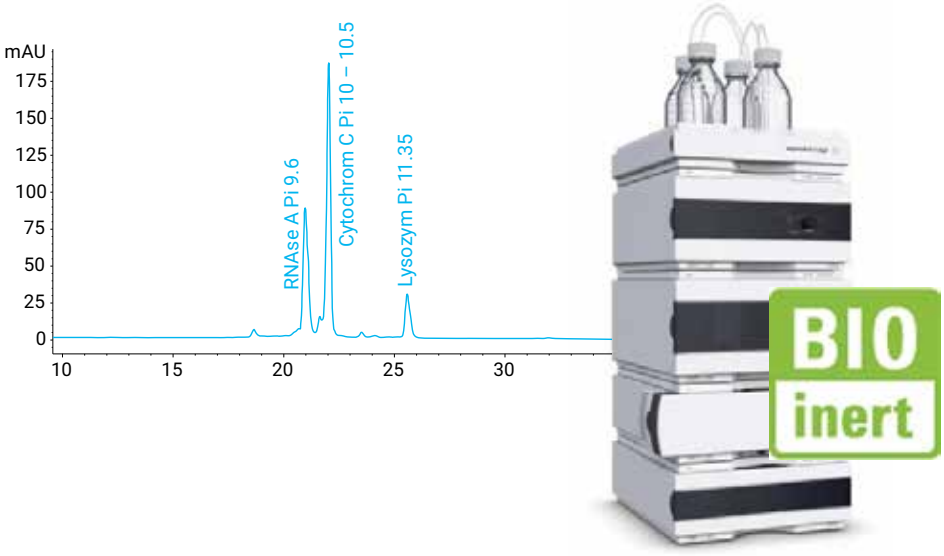
Abstract

This Application Note shows that the Agilent Buffer Advisor software in combination with the Agilent 1260 Infinity Bio-inert Quaternary LC System is an ideal solution for automated protein separation by ionic strength gradients. Usually, pH scouting using premixed two-component gradients is time-consuming and work-intensive. Dynamically mixed four-component gradients calculated by the Buffer Advisor software shorten and simplify the workflow for pH scouting. In addition, excellent retention time precision and pH consistency were gained using the gradients calculated by the Buffer Advisor software.



Introduction

Proteins consist of many different amino acids comprising weak acidic (carboxylic) and basic (amine) groups. Therefore, proteins are amphoteric molecules that exist mostly as zwitterions in a certain pH range. The pH where the protein has no net charge and does not interact with a charged medium is the isoelectric point (pI). In ion exchange chromatography (IEX), the unique relationship between net surface and pH can be used for optimal protein separation. The pH defines the number of charges on the protein and also helps to stabilize the native structure of the protein in the buffer used during analysis.

To ensure optimal binding and elution characteristics of proteins of interest to the IEX column, pH and ionic strength of the deployed buffer are important factors. Even small changes in these two parameters can affect the separation. As a consequence, pH scouting is an important method to find the optimal separating conditions when working with ionic strength gradients. In contrast to pH-gradients, the pH is kept constant in ionic strength gradients. By increasing the ionic strength (salt concentration) of the mobile phase, the less strongly bound proteins are eluted earlier than the stronger bound proteins.

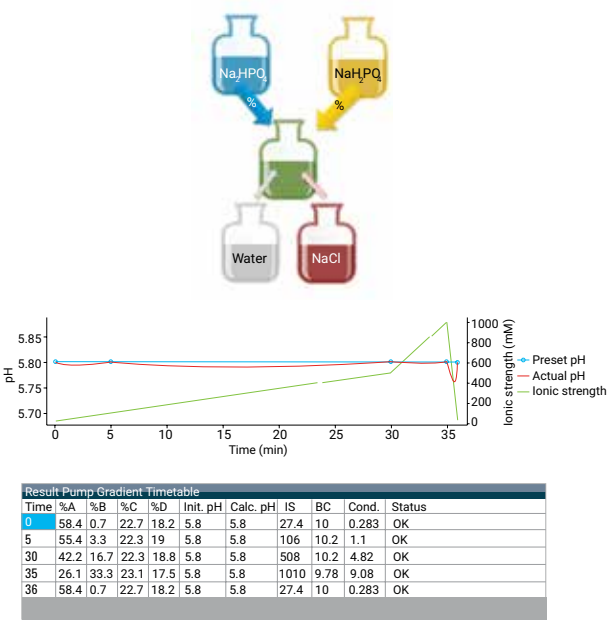
In general, a premixed two-component gradient is prepared for analysis with a starting buffer of low ionic strength and an elution buffer containing high ionic strength. This includes the following preparation steps:

- Dissolving the appropriate buffering compounds at defined concentration
- Titrating the pH with acid/base to the desired pH of the mobile phase
- Splitting the buffer and adding salt to one portion (elution buffer)
- Titrating the pH of the elution buffer with acid/base to the desired pH, if necessary

To perform pH scouting using premixed two-component gradients, prepare different bottles of buffer. To test, for example, six different pH values, it is necessary to prepare 12 bottles of premixed buffer. In contrast, with dynamically mixed four-component gradients, it is necessary to prepare only four bottles to generate various pH values. Further, dynamical mixing of a buffer eliminates the necessity to titrate the buffer solutions manually, which is typically time-consuming and errors prone. The application of dynamically mixed four-component gradients simplifies method development and reduces the time needed for buffer preparation to a large extent by just providing four bottles with stock solutions:

- Line A: Water
- Line B: Salt solution
- Line C: Acidic buffer component
- Line D: Basic buffer component

Using the four stock solutions, different buffers at different pH and salt concentration can be prepared. The Buffer Advisor software is a helpful tool to calculate the percentages of the stock solutions in order to achieve the desired pH, buffer concentration, and ionic strength (Figure 1).



Result Pump Gradient Timetable

Time	%A	%B	%C	%D	Init. pH	Calc. pH	IS	BC	Cond.	Status
0	58.4	0.7	22.7	18.2	5.8	5.8	27.4	10	0.283	OK
5	55.4	3.3	22.3	19	5.8	5.8	106	10.2	1.1	OK
30	42.2	16.7	22.3	18.8	5.8	5.8	508	10.2	4.82	OK
35	26.1	33.3	23.1	17.5	5.8	5.8	1010	9.78	9.08	OK
36	58.4	0.7	22.7	18.2	5.8	5.8	27.4	10	0.283	OK

Figure 1. Quaternary mixing to create a salt gradient with constant pH.

The Buffer Advisor software generates a timetable, which can be imported into the method of the 1260 Infinity Bio-inert Quaternary LC Pump using the Import Solvent Blending File function of the Agilent OpenLAB CDS ChemStation Edition software (Figure 2).

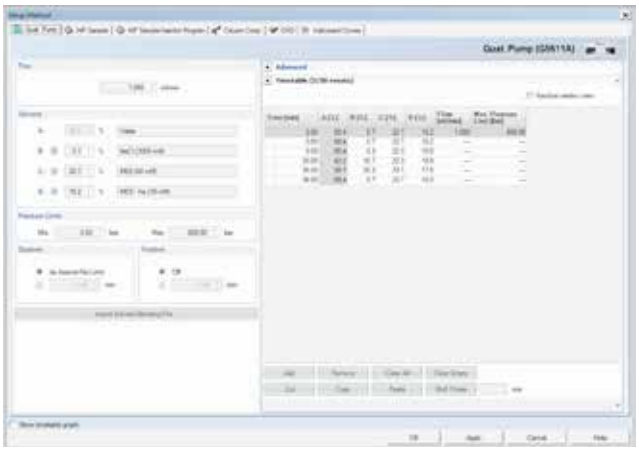


Figure 2. The generated Timetable can be imported into the method of the Agilent 1260 Infinity Bio-inert LC System through the Agilent OpenLAB CDS ChemStation Edition software.

The Buffer Advisor software can be applied for ionic strength or pH gradients in anion or cation exchange chromatography. The software provides a wide choice of different selectable buffers for single buffer (ionic strength gradients) or for composite buffer (pH gradients) applications. Depending on the proteins of interest and the used columns, the user can select buffers either for anion or cation exchange (Figure 3). To ensure optimal buffering capacity, recommended pH ranges and concentrations in which the buffers should be used are displayed.

A common issue in ion exchange chromatography with ionic strength gradients is the decrease in pH as an effect of added neutral salt like NaCl1,2. The Buffer Advisor software counteracts this issue by recalculation of the overall mobile phase composition considering the concentration of acidic and basic buffer (Line C and D) to maintain the desired constant pH. In addition, if the pH deviation gets too large, the Buffer Advisor software automatically inserts additional time points into the pump timetable.

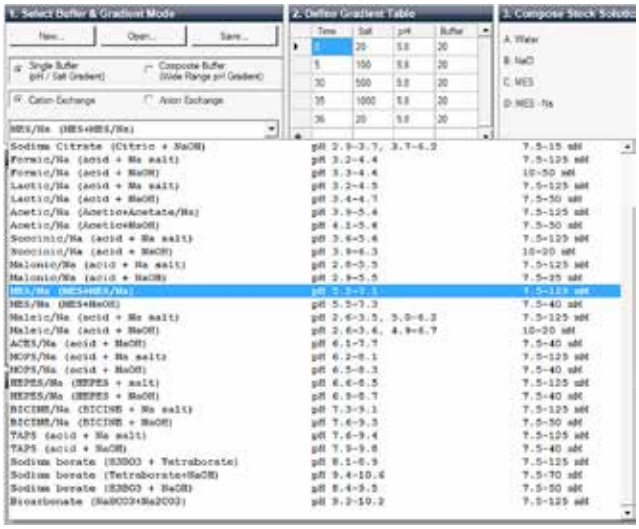


Figure 3. Buffer list for cation exchange chromatography, sorted by recommended pH range.

Experimental

Instrumentation

The Agilent 1260 Infinity Bio-inert Quaternary LC System consisted of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity High performance Bio-inert Autosampler (G5667A)
- Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C) with bio-inert solvent heat exchangers
- Agilent 1260 Infinity Diode Array Detector VL (G1315D with bio-inert standard flow cell, 10 mm)
- Agilent 1260 Infinity Bio-inert Analytical-scale Fraction Collector (G5664A)

Column

- Agilent Bio MAb Column, PEEK, 4.6 × 250 mm, 5 µm

Software

- Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, Rev. C.01.03 [32]
- Agilent Buffer Advisor, Rev. A.01.01

Solvents

Buffer A: H_2O

Buffer B: NaCl 3 M

Buffer C: MES (2-(N-morpholino) ethanesulfonic acid monohydrate) 60 mM

Buffer D: MES-Na (2-(N-morpholino) ethanesulfonic acid sodium salt) 35 mM

Sample

Mix of three proteins, solved in PBS (phosphate buffered saline), pH 7.4

Ribonuclease A: 13,700 Da pI 9.6

Cytochrom C: 1 2,384 Da pI 10–10.5

Lysozyme: 14,307 Da pI 11.35

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). MES (2-(N-morpholino)ethanesulfonic acid monohydrate) and MES-Na (2(N-morpholino)ethanesulfonic acid sodium salt) were purchased from Merck, Darmstadt, Germany. NaCl was purchased from VWR, Radnor, PA, USA.

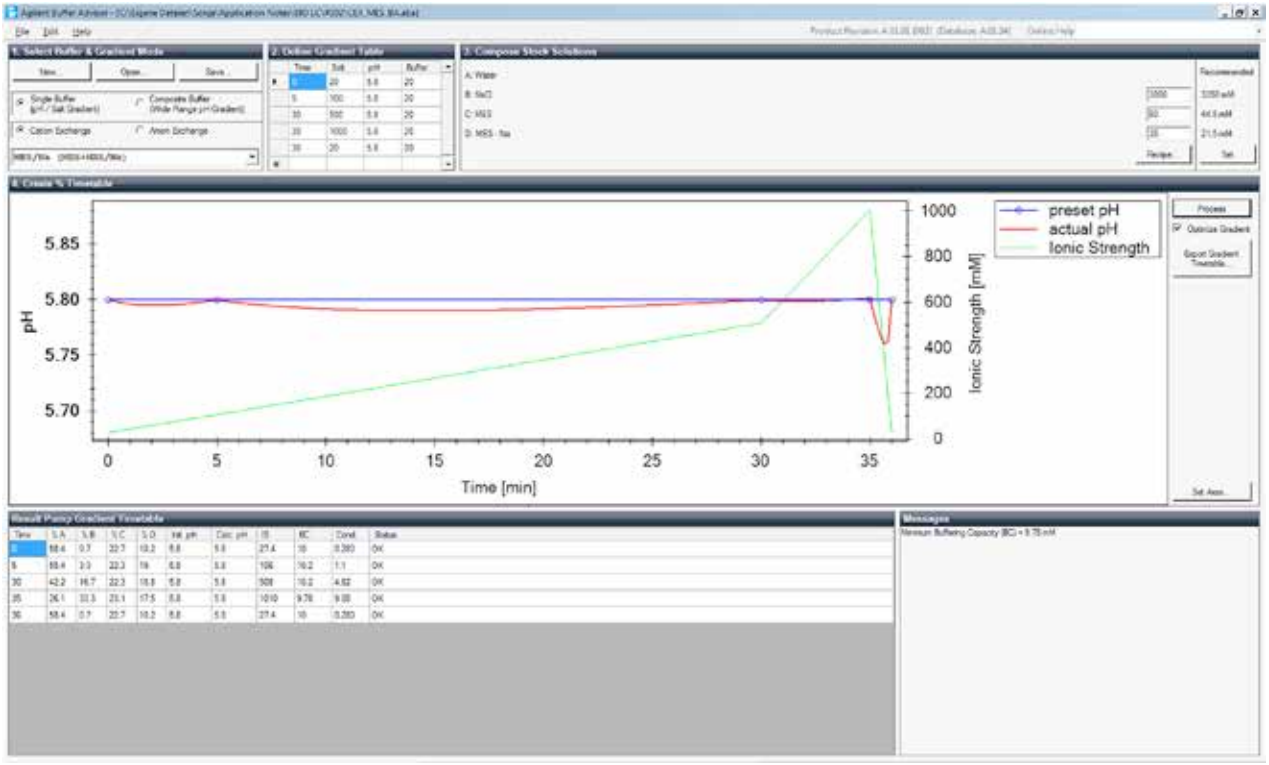


Figure 4. Agilent Buffer Advisor software, showing the steps described in "Results and discussion".

Conditions

Parameter	Value
Flow rate:	1 mL/min
Gradient:	0 min – 20 mM NaCl 5 min – 20 mM NaCl 30 min – 500 mM NaCl 35 min – 1000 mM NaCl 36 min – 20 mM NaCl
Injection volume:	10 µL
Thermostat:	4 °C
Temperature TCC:	25 °C
DAD:	280 nm/4 nm
Ref.:	Off
Peak width:	> 0.05 min (1.0 s response time)(5 Hz)

Figure 5. Stock solution recipes

Results and discussion

pH scouting was performed using calculations from the Agilent Buffer Advisor software for pH values from 5.8 to 6.8. A mix of three proteins (ribonuclease A, cytochrome C and lysozyme) was separated using a four-component salt gradient at six different pH values. Dynamically mixed four-component gradients were generated using the calculations from the software. The Buffer Advisor software simplifies the generation of different four-component gradients by calculating the percentage of the individual stock solutions in the mobile phase at defined time points (Figure 4).

1. After definition of the gradient parameters, such as time, maximum salt concentration, pH, and buffer concentration
2. The Buffer Advisor software calculates the needed stock concentrations. The Recipe button displays the absolute amount of needed chemicals for the preparation of the stock solutions (Figure 5). These proposed stock concentrations can be adjusted by the user.
3. Select the Process tab.
4. The Buffer Advisor software calculates the needed amount of each channel to maintain the correct pH during the complete chromatographic run. Furthermore, it calculates whether the pH, salt concentration and buffer concentration entered is suitable for the buffer system that was selected. The timetable displays also additional data, such as buffering capacity of the mobile phase.

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The pH scouting for the three-protein mix of ribonuclease A (A), cytochrome C (B) and lysozyme (C) demonstrates the benefits of the Agilent Buffer Advisor software (Figure 6). Even small pH changes of 0.2 have a strong influence on the retention of the proteins on the weak cation exchange (WCX) column. Changes in the elution order become obvious when the pH is changed from 5.8 to 6.8.

Manual preparation of corresponding buffers for premixed two-component gradients includes several steps. For each pH and for each prepared bottle (one with low and one with high ionic strength), a manual titration of the buffers is necessary. pH scouting for six different pH values in order to achieve the optimal resolution results in preparation of 12 solvent bottles (including weighing chemicals, pH adjustment). This is a very time-consuming procedure and highly prone to error and variation.

In contrast, the Buffer Advisor software is capable of automatically and reproducibly mixing all six separation conditions out of four stock solutions without any manual interference. The optimal resolution was achieved at pH 5.8 (Figure 6).

Based on the results, the user has various options on how to proceed:

1. Fine-tuning of the resulting pH values and gradients
2. Transfer of the dynamically mixed four-component gradient to other instruments through the OpenLAB CDS ChemStation timetable
3. Implementation of pH scouting results into two-component gradients using premixed buffers

In the last case, however, deviations from correct pH are expected, due to the pH optimization procedure of the Buffer Advisor software.

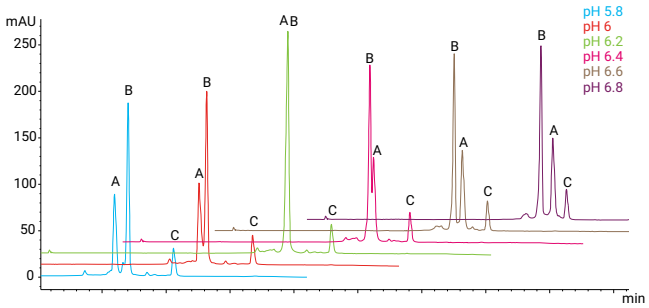

Figure 6. pH scouting for the separation of a three-protein mix using dynamically mixed quaternary gradients.

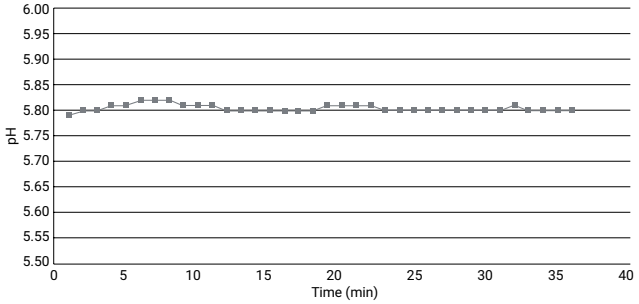

Figure 7. Off line pH measurement

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Conclusions

Using dynamically mixed four-component gradients, calculated by the Buffer Advisor software, shortens and simplifies the workflow for pH scouting. The employment of dynamically mixed gradients calculated with the Buffer Advisor software results in a significant decrease in buffer preparation time, particularly when compared to manual preparation of buffers for premixed two-component gradients. The Buffer Advisor software provides a wide range of prevalidated, user-selectable buffer systems for anion and cation exchange chromatography and delivers recipes for preparation of the most suitable stock solutions. Due to pH optimization of the software, resulting pH values are more accurate and precise than those resulting from premixed gradients formed out of manually prepared buffer solutions. The Buffer Advisor software counteracts this issue by the recalculation of the four-component gradient regarding the concentration of acidic and basic buffer to maintain the desired constant pH.

The Buffer Advisor software in combination with the Agilent 1260 Infinity Bio-inert Quaternary LC System is excellent for generating four-component gradients. The calculations of Buffer Advisor software lead to exact and reproducible protein analysis while providing an excellent tool for automated pH scouting and accurate ion exchange chromatography. The Buffer Advisor software is, therefore, an ideal tool for automatic development of analytical methods in ion-exchange chromatography, which can be seamlessly transferred to the corresponding QA/QC departments.

References

1. R. J. C. Brown & M. J. T. Milton. Observation of a combined dilution and salting effect in buffers under conditions of high dilution and high ionic strength, *Accred Qual Assur* 8(11): 505-510, **2003**.
2. A. E. Voinescu et al. Similarity of Salt Infl uences on the pH of Buffers, Polyelectrolytes, and Proteins, *J. Phys. Chem.* B 110: 8870-8876, **2006**.

Application Note

Charge Variant Analysis

Agilent

Trusted Answers

Fully Automated Characterization of Monoclonal Antibody Charge Variants Using 4D-LC/MS

Authors

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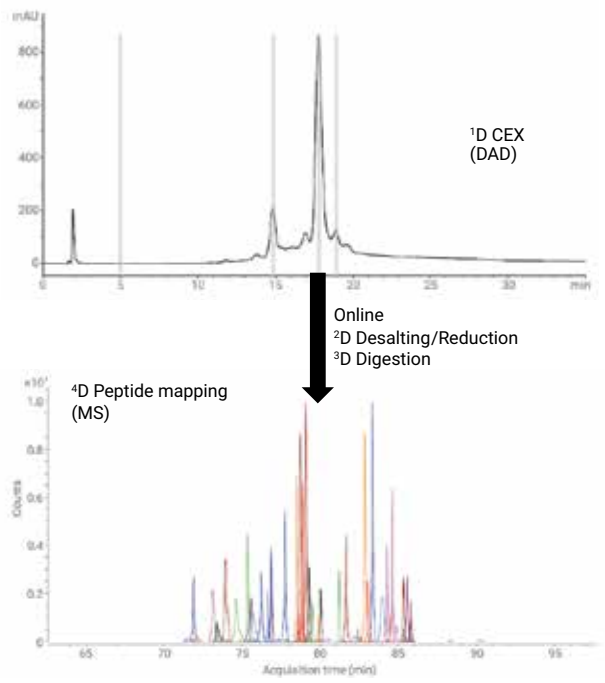
Abstract

This application note describes the fully automated and in-depth characterization of monoclonal antibody (mAb) charge variants by four-dimensional liquid chromatography/mass spectrometry (4D-LC/MS) using the Agilent InfinityLab 2D-LC Solution and the Agilent 6545 LC/Q-TOF system. Charge variants resolved by cation-exchange chromatography (CEX) are collected in loops installed on a multiple heart-cutting valve and consecutively subjected to online desalting, denaturation, reduction, and tryptic digestion prior to LC/MS-based peptide mapping.



Introduction

Protein biopharmaceuticals have emerged as important therapeutics for the treatment of various diseases including cancer, cardiovascular diseases, diabetes, infection, inflammatory, and autoimmune disorders.[1-3] Protein biopharmaceuticals come in many flavors and include monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), fusion proteins, hormones, growth factors, cytokines, therapeutic enzymes, blood factors, vaccines, and anticoagulants. Given their obvious benefits in terms of safety and efficacy, these molecules have substantially reshaped the pharmaceutical market, and today, over 350 products have been approved for human use in the United States and the European Union.[1-3] This represents approximately one quarter of the total pharmaceutical market, with mAbs being the fastest growing class of pharmaceuticals.

Together with a huge therapeutic potential, these molecules come with an enormous, analytically demanding structural complexity.[1,2] In contrast to small molecule drugs, biopharmaceuticals are large (mAbs have an MW of approximately 150 kDa) and heterogeneous. They are the product of one or a couple of genes. However, hundreds of possible variants that differ in post-translational modifications (PTMs), amino acid sequence, higher-order structure, etc. may coexist, all making up the profile, safety, and efficacy of the product.[1-3] Consequently, their in-depth structural characterization involves a significant number of analytical tools, with chromatography (LC) and mass spectrometry (MS) at the forefront.

A key technology to study charge variants that might arise from PTMs such as asparagine deamidation, C-terminal lysine truncation, N-terminal cyclization (pyroglutamate formation), sialylation, etc. is CEX. In CEX, electrostatic interaction between the anionic groups of the stationary phase and cationic groups on the protein surface form the basis of the separation. The protein is loaded on the column at a mobile phase pH below its isoelectric point (pI), and elution is achieved using a salt or pH gradient. CEX buffers are typically composed of nonvolatile constituents, making these methods incompatible with MS. Peak identification is a laborious task involving peak collection and desalting prior to MS analysis.[4] With the recent introduction of commercial and robust ^{2}D-LC instrumentation, this series of events is now commonly performed in an online automated manner.[5-9] Peaks eluting from the CEX column are stored in loops and subjected to online desalting using reversed-phase (RPLC) or size exclusion chromatography (SEC) prior to MS measurement. Both comprehensive (LC×LC) and (multiple) heart-cutting ^{2}D-LC (LC-LC) have been used.[5-9]

To unambiguously identify CEX peaks, however, peptide mapping is required. While protein measurement is indicative of identity and highlights dominant modifications with mass differences beyond the mass accuracy of the MS instrument, it typically does not provide the actual amino acid sequence, nor does it allow us to localize modifications. Addressing the latter, and inspired by previous work,[10-12] the current application note describes a fully automated online ^{4}D-LC/MS setup incorporating first dimension (^{1}D) CEX, peak collection, ^{2}D desalting, denaturation, reduction, ^{3}D trypsin digestion, and ^{4}D RPLC/MS-based peptide mapping for the in-depth characterization of mAb charge variants.

Experimental

Materials

Acetonitrile (HPLC-S), water (ULC/MS), and formic acid (ULC/MS) were obtained from Biosolve (Valkenswaard, The Netherlands). NaH_2PO_4, $Na_2HPO_4{\cdot}2H_2O$, NaCl, NH_4HCO_3, Tris base, and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO, USA). UltraPure Tris-HCl pH 7.5 was purchased from ThermoFisher Scientific (Waltham, MA, USA). Type I water was produced from tap water by an arium pro Ultrapure Lab Water System from Sartorius (Göttingen, Germany). Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland).

Sample preparation

Trastuzumab was diluted to 7 mg/mL in ^{1}D CEX mobile phase A (MPA: 10 mM sodium phosphate pH 7.65). Deamidation was induced by incubating trastuzumab at 37 °C for 3 days in high pH conditions (100 mM Tris pH 9.0) and subsequently buffer-exchanged to 7 mg/mL in ^{1}D CEX mobile phase A.

Instrumentation

An Agilent 1290 Infinity II 2D-LC system equipped with the multiple heart-cutting option, an additional Agilent 1260 Infinity II quaternary pump and Agilent 1260 Infinity II isocratic pump, two additional 2-position/6-port valves, and a zero dead volume T-piece were used. Stainless steel tubing with an internal diameter of 0.12 mm was applied. The configuration is schematically represented in Figure 1 and summarized in this application note. Diode array detection (DAD) was used in the first (CEX) and fourth dimension (RPLC). Additionally, an Agilent 6545 LC/Q-TOF with a Jet Stream ESI source was used for detection after the fourth and final dimension.

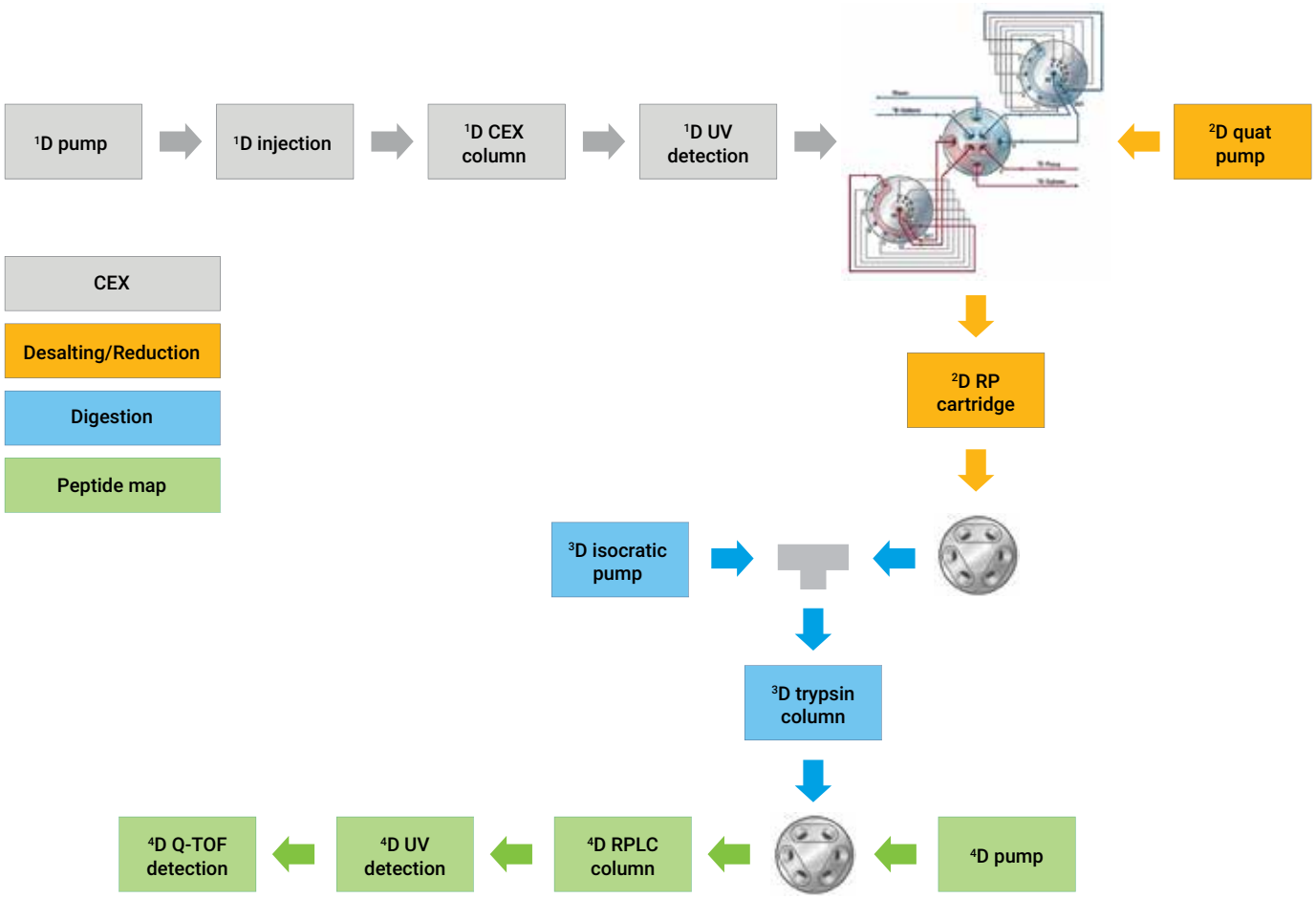


Figure 1. 4D-LC/MS configuration incorporating 1D CEX separation and charge variant peak collection using multiple heart-cutting, 2D RPLC-based desalting, denaturation, reduction, 3D trypsin digestion, and 4D RPLC-MS based peptide mapping.

Configuration

[1]D: Cation-exchange chromatography:

- G7120A Agilent 1290 Infinity II high-speed pump
- G7167B Agilent 1290 Infinity II multisampler with sample thermostat (option 101)
- G7116B Agilent 1290 Infinity II multicolumn thermostat (MCT) with valve drive installed (option 058) equipped with an Agilent InfinityLab quick change 2-position/6-port valve, 1300 bar (G4231C)
- G7117B Agilent 1290 Infinity II diode array detector with a 3.7 mm HDR InfinityLab Max-Light cartridge cell (G4212-60032)

Note: A short 3.7 mm detector flow cell was installed to reduce the signal intensity and prevent saturation of the UV signal

2D-LC with multiple heart-cutting

- Agilent 1290 Infinity valve drive (G1170A) with 2D-LC valve (G4236A)
- Two Agilent 1290 Infinity valve drives (G1170A) with multiple heart-cutting valves (G4242-64000) equipped with 40 µL loops

[2]D: Reversed-phase chromatography for desalting, denaturation, and reduction:

G7111B Agilent 1260 Infinity II quaternary pump with active inlet valve (AIV) (option 032)

[3]D: Trypsin digestion

- G7111B Agilent 1260 Infinity II quaternary pump with active inlet valve (AIV) (option 032)

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[4]D: Reversed-phase chromatography for peptide mapping:

- G7120A Agilent 1290 Infinity II high-speed pump
- G7116B Agilent 1290 Infinity II MCT with valve drive installed (option 058) equipped with an Agilent InfinityLab quick change 2-position/6-port valve, 1300 bar (G4231C)
- G7117B Agilent 1290 Infinity II DAD with a 10 mm InfinityLab Max-Light cartridge cell (G4212-60008)
- G6545A Agilent 6545 LC/Q-TOF with Jet Stream ESI source

Note: Orachrom StyrosZyme TPCK-Trypsin and Agilent AdvanceBio peptide mapping columns are both contained in different zones of one single column compartment and maintained at 40 and 60 °C, respectively.

Software

- Agilent OpenLab CDS ChemStation revision C.01.07 SR4 [505]
- 2D-LC add-on software revision A.01.04 [017]
- Agilent MassHunter for instrument control (B.09.00)
- Agilent MassHunter with BioConfirm add-on for data analysis (B.07.00)

Method

[1]D and [4]D were configured in the 2D-LC software, while [2]D and [3]D were controlled in a regular method setup and were programmed as repetitive events. The cycle time of these events was 110 minutes, identical to the [4]D cycle time programmed in the 2D-LC software. MassHunter acquisition was triggered by a remote start from the 2D-LC system.

Four heart-cuts were taken across the CEX analysis. The first heart-cut at 4.8 minutes is a blank cut, which enables preconditioning of all dimensions before the analysis of the actual CEX heart-cuts of interest.

[1]D: Cation-Exchange Chromatography			
Column	Agilent Bio MAb, nonporous (2.1 mm × 250 mm, 5 µm) (p/n 5190-2411)		
Temperature	30 °C		
Mobile phase A	10 mM sodium phosphate, pH 7.65		
Mobile phase B	10 mM sodium phosphate, pH 7.65 + 100 mM NaCl		
Flow rate	0.2 mL/min		
Gradient	**Time (min)**	**B (%)**	**CEX analysis**
	0	5	
	36	70	
	36.5	100	
	46	100	
	46.5	5	
	60	5	
Injection	100 µg		
Detection	220 and 280 nm		
Peak Width	> 0.025 min (10 Hz)		

Multiple Heart-Cutting [1]D > [2]D	
Sampling Timetable Trastuzumab	
Cut	**Time (min)**
1 – Blank	4.80
2 – Pre-peak	14.93
3 – Main Peak	17.55
4 – Post-peak	18.99

[2]D: Reversed-Phase Chromatography for Desalting, Denaturation, and Reduction (Manually Entered Repetitive Event)						
Column	Polymer-based desalting cartridge, 2.1 × 10 mm					
Temperature	23 °C					
Mobile Phase A	0.1% (v/v) formic acid in water					
Mobile Phase B	0.1% (v/v) formic acid in acetonitrile					
Mobile Phase C	20 mM DTT in 100 mM Tris-HCl, pH 7.5					
Gradient	**Time (min)**	**A (%)**	**B (%)**	**C (%)**	**Flow (mL/min)**	
	10	99	1	0	0.5	**Desalting and focusing**
	10.01	0	0	100	0.2	**Reduction**
	20	0	0	100	0.2	
	20.01	99	1	0	0.5	
	25	99	1	0	---	**Desalting and elution**
	25.01	40	60	0	---	
	27	40	60	0	0.5	
	27.01	40	60	0	0.015	**Elution and digestion**
	68	40	60	0	0.015	
	68.01	0	100	0	0.5	
	85	0	100	0	---	
	95	99	1	0	---	
	120	99	1	0	---	
Valve	27 min: Pos 1 & Pos 2 (start trypsin digestion) 67 min: Pos 2 & Pos 1 (start peptide mapping)					

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^{3}D: Trypsin Digestion (Manually Entered Repetitive Event)			
Column	Orachrom StyrosZyme TPCK-Trypsin PEEK (2.1 × 150 mm)		
Temperature	40 °C		
Mobile Phase	50 mM NH_4HCO_3, pH 8		
Gradient	**Time (min)**	**Flow (mL/min)**	**Digestion**
	25	0.06	
	25.01	0.135	
	67	0.135	
	67.01	0.06	
	135	0.06	
Valve	27 min: Pos 1 -> Pos 2 (start trypsin digestion) 67 min: Pos 2 -> Pos 1 (start peptide mapping)		

^{4}D: Reversed-Phase Chromatography for Peptide Mapping (Repetitive Event Controlled by 2D-LC Software)			
Column	Agilent AdvanceBio peptide mapping (2.1 × 150 mm × 2.7 µm) (p/n 651750-902)		
Temperature	60 °C		
Mobile Phase A	0.1% (v/v) formic acid in water		
Mobile Phase B	0.1% (v/v) formic acid in acetonitrile		
Flow Rate	0.4 mL/min		
Gradient	**Time (min)**	**B (%)**	
	0	1	
	8.5	1	
	9	100	
	15	100	
	16	1	
	20	1	**Load digest on peptide mapping column**
	64	1	
	64	1	**Peptide mapping**
	97	45	
	98	100	
	103	100	
	104	1	
	110	1	
DAD Detection	214 and 280 nm		
Peak Width	> 0.025 min (10 Hz)		

MS Detection	
Parameter	**Source**
Positive Ionization	
Drying Gas Temperature	300 °C
Drying Gas Flow	8 L/min
Nebulizer Pressure	35 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	8 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	1,000 V
Fragmentor	175 V

Parameter	**Acquisition**
Mode	Extended dynamic range (2 GHz)
Data Acquisition Range	*m/z* 100 to 3,200
	1 spectrum/s
	Centroid acquisition
Switch diverter valve to MS after 67 minutes	

Data processing

Measured signals were matched onto the trastuzumab light- and heavy-chain sequences using the BioConfirm algorithm incorporated in the MassHunter software. Mass tolerance for matching experimental data onto the sequence was set at 8 ppm. Extracted ion chromatograms (EICs) obtained at 20 ppm mass accuracy were used to monitor PTMs such as deamidation.

Results and Discussion

A scheme of the fully automated online 4D-LC/MS protein analyzer, incorporating CEX, peak collection, desalting, denaturation, reduction, trypsin digestion, and peptide mapping, is shown in Figure 1. CEX peaks are collected in 40 µL loops installed on a multiple heart-cutting valve and transferred one by one to a polymeric RP cartridge where desalting, denaturation, and reduction take place. The reduced mAb, trapped on the cartridge, is subsequently eluted into the trypsin column by raising the acetonitrile concentration. Using a T-piece, trypsin digestion buffer is mixed with the reversed-phase mobile phase to have optimal digestion conditions and to reduce the acetonitrile concentration.

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During the digestion, the trypsin column is in-line with the peptide mapping column, and generated peptides are focused at the head of the RPLC column. After 20 minutes, a valve switch initiates the elution of the digest into the MS.

The CEX chromatogram of the mAb trastuzumab is shown in Figure 2. Trastuzumab, commercialized as Herceptin, is a humanized IgG1 binding the HER2 receptor, thereby finding use in the treatment of HER2 positive metastatic breast cancer. With a pI of 8.45, the mAb is positively charged at the CEX mobile phase pH, thereby governing interaction with the negatively charged chromatographic resin. Upon eluting the mAb using a NaCl salt gradient, various charge variants were revealed, which were subsequently subjected to online peptide mapping. Figure 3A schematically presents the 4D-LC/MS experiment involving the analysis of three CEX peaks (pre-, main-, and post-peaks) and a CEX blank as shown in Figure 2. Figure 3B zooms in on the pressure and DAD profiles of one cycle and shows the desalting, denaturation, reduction, digestion, and peptide mapping of the main CEX peak. Over 90% sequence coverage could be obtained. Peptides identified are shown in Table 1, and an overlay of the LC/MS compound chromatograms is provided in Figure 4. Peptides not covered are typically small and/or hydrophilic and are not focused at the head of the peptide mapping column during the digestion. For this reason, they are diverted to waste.

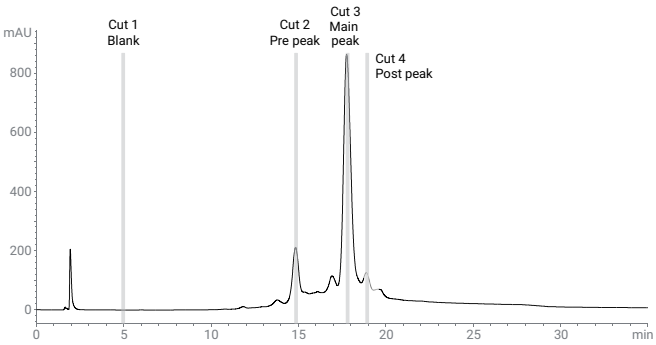

Figure 2. CEX chromatogram of the monoclonal antibody trastuzumab. Conditions according to reference 13. Heart-cuts taken are indicated in gray.

Next to sequence information, peptide mapping also reveals modifications and modification sites. Figure 5 shows the online peptide mapping of the CEX pre-, main-, and post-peak, and in particular the EICs of two peptides (i.e., light-chain peptide ASQDVNTAVAWYQQKPGK (LC 25–42) containing a potential deamidation site at position 30, and heavy-chain peptide WGGDGFYAMDYWGQGTLVTVSSASTK (HC 99–124) containing a potential isomerization site at position 102). From the data, it could be deduced that the pre-peak corresponds to a deamidated variant, with asparagine converted to aspartate at position 30 on one of the light chains. It could also be demonstrated that the post-peak carries an isoaspartate at position 102 on one of the heavy chains. This is clearly visualized by the peak doublets corresponding to the modified and nonmodified variants. These results are in accordance with those reported by Harris et al., who performed offline fraction collection and peptide mapping on Herceptin acidic and basic variants.4

The same experiment was performed on a high-pH stressed Herceptin sample (Figure 6). Such conditions are known to induce deamidation, thereby rendering the mAb more acidic. The CEX profile presented in Figure 6 shows an acidic shift, and the peptide map data of CEX peaks 1 and 2 show a double deamidated variant, with both light chains deamidated at position 30. CEX peaks 3 and 4 correspond to a single deamidation event, with one light chain deamidated at position 30. The difference between peaks 1 and 2, and 3 and 4 originate from another deamidation, this time at position 387 in the heavy chain. This deamidation site appears in two peptides (one fully cleaved and one miscleaved) that are apparently digested differently when a deamidation exists.

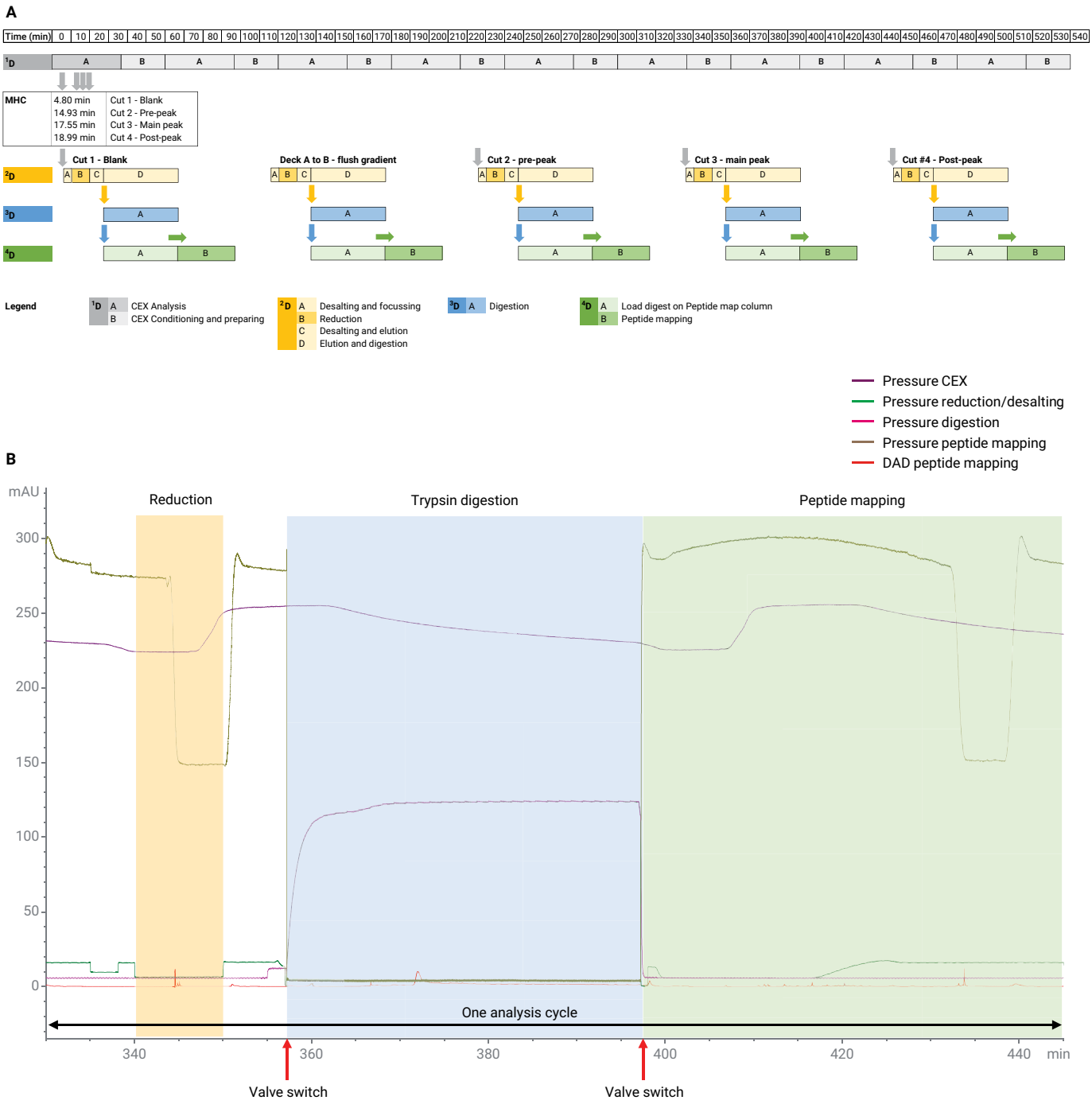

Figure 3. (A) Schematic representation of the different stages of the 4D-LC/MS experiment focusing on the pre-, main-, and post-peak as well as a CEX blank region as shown in Figure 2. (B) Focus on desalting, denaturation, reduction, digestion, and peptide mapping of the main CEX peak.

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Table 1. Peptides identified in the CEX main peak following online RPLC/MS-based peptide mapping.

RT	Mass	Vol	Vol %	Sequence	Seq Loc.	Tgt. Seq. Mass	Diff. (ppm)	Missed Cleavage
79.1	1880.9972	66635668	6.61	EVQLVESGGGLVQPGGSLR	HC(001-019)	1880.9956	0.8	0
76.8	1109.5539	25727274	2.55	LSCAASGFNIK	HC(020-030)	1109.5539	0.0	0
81.2	2180.0864	16055279	1.59	LSCAASGFNIKDTYIHWVR	HC(020-038)	2180.0837	1.2	1
76.6	1088.5410	13618352	1.35	DTYIHWVR	HC(031-038)	1088.5403	0.6	0
76.2	829.4442	24354704	2.42	GLEWVAR	HC(044-050)	829.4446	-0.5	0
71.9	1083.5360	19879112	1.97	IYPTNGYTR	HC(051-059)	1083.5349	1.0	0
72.1	1181.6059	3708836	0.37	GRFTISADTSK	HC(066-076)	1181.6041	1.6	1
73.1	968.4819	26423682	2.62	FTISADTSK	HC(068-076)	968.4815	0.4	0
79.3	2260.1184	317550	0.03	FTISADTSKNTAYLQMNSLR	HC(068-087)	2260.1158	1.1	1
81.3	3518.6474	320893	0.03	FTISADTSKNTAYLQMNSLRAEDTAVYYCSR	HC(068-098)	3518.6446	0.8	2
76.8	1309.6451	24858112	2.47	NTAYLQMNSLR	HC(077-087)	1309.6449	0.1	0
79.9	2568.1769	5903991	0.59	NTAYLQMNSLRAEDTAVYYCSR	HC(077-098)	2568.1737	1.2	1
71.6	1276.5392	2994056	0.30	AEDTAVYYCSR	HC(088-098)	1276.5394	-0.1	0
85.3	2783.2545	16863744	1.67	WGGDGFYAMDYWGQGTLVTVSSASTK	HC(099-124)	2783.2537	0.3	0
78.7	1185.6398	68405792	6.79	GPSVFPLAPSSK	HC(125-136)	1185.6394	0.4	0
77.7	1263.6494	36588096	3.63	STSGGTAALGCLVK	HC(137-150)	1263.6493	0.1	0
88.3	6655.2898	221285	0.02	DYFPEPVTVSWNSGALTSGVHTFPAVLQSS GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK	HC(151-213)	6655.2857	0.6	0
79.8	1374.7171	214690	0.02	VDKKVEPKSCDK	HC(214-225)	1374.7177	-0.4	3
84.9	2729.4093	7103687	0.71	THTCPPCPAPELLGGPSVFLFPPKPK	HC(226-251)	2729.4073	0.7	0
73.4	834.4277	13166738	1.31	DTLMISR	HC(252-258)	834.4269	1.0	0
82.0	2897.4175	220680	0.02	DTLMISRTPEVTCVVVDVSHEDPEVK	HC(252-277)	2897.4151	0.9	1
84.3	4556.2041	547132	0.05	DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	HC(252-291)	4556.1992	1.1	2
79.3	2081.0013	17858876	1.77	TPEVTCVVVDVSHEDPEVK	HC(259-277)	2080.9987	1.2	0
83.0	3739.7881	18901388	1.88	TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	HC(259-291)	3739.7828	1.4	1
78.6	1676.7966	5991668	0.59	FNWYVDGVEVHNAK	HC(278-291)	1676.7947	1.1	0
84.6	1807.0008	38113724	3.78	VVSVLTVLHQDWLNGK	HC(305-320)	1806.9992	0.9	0
83.3	2227.2022	63210428	6.27	VVSVLTVLHQDWLNGKEYK	HC(305-323)	2227.2001	0.9	1
82.4	2458.3080	2959244	0.29	VVSVLTVLHQDWLNGKEYKCK	HC(305-325)	2458.3043	1.5	2
81.6	2886.5495	183447	0.02	VVSVLTVLHQDWLNGKEYKCKVSNK	HC(305-329)	2886.5426	2.4	3
73.9	837.4964	38694668	3.84	ALPAPIEK	HC(330-337)	837.4960	0.5	0
75.4	1285.6662	875645	0.09	EPQVYTLPPSR	HC(348-358)	1285.6667	-0.4	0
75.6	1903.9366	36378636	3.61	EPQVYTLPPSREEMTK	HC(348-363)	1903.9350	0.8	1
80.5	2989.5263	1849832	0.18	EPQVYTLPPSREEMTKNQVSLTCLVK	HC(348-373)	2989.5253	0.3	2
78.4	1721.8701	179925	0.02	EEMTKNQVSLTCLVK	HC(359-373)	1721.8692	0.6	1
78.9	1103.6013	45037560	4.47	NQVSLTCLVK	HC(364-373)	1103.6009	0.4	0
81.6	2543.1245	29221288	2.90	GFYPSDIAVEWESNGQPENNYK	HC(374-395)	2543.1241	0.2	0
85.7	4398.0307	5032842	0.50	GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK	HC(374-412)	4398.0281	0.6	1
85.9	4954.3531	225838	0.02	GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK	HC(374-417)	4954.3502	0.6	2
82.8	1872.9144	57538216	5.71	TTPPVLDSDGSFFLYSK	HC(396-412)	1872.9146	-0.1	0
82.6	2429.2370	970108	0.10	TTPPVLDSDGSFFLYSKLTVDK	HC(396-417)	2429.2366	0.2	1
77.8	2986.3744	1042270	0.10	SRWQQGNVFSCSVMHEALHNHYTQK	HC(418-442)	2986.3715	1.0	1
78.5	2743.2427	46302832	4.60	WQQGNVFSCSVMHEALHNHYTQK	HC(420-442)	2743.2384	1.6	0
74.6	659.3487	26985572	2.68	SLSLSPG	HC(443-449)	659.3490	-0.5	0
76.7	1877.8787	2655889	0.26	DIQMTQSPSSLSASVGDR	LC(001-018)	1877.8789	-0.1	0
79.5	2551.2398	9011163	0.89	DIQMTQSPSSLSASVGDRVTITCR	LC(001-024)	2551.2371	1.1	1

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Table 1. Continued.

RT	Mass	Vol	Vol %	Sequence	Seq Loc.	Tgt. Seq. Mass	Diff. (ppm)	Missed Cleavage
75.6	1989.9932	10501849	1.04	ASQDVNTAVAWYQQKPGK	LC(025-042)	1989.9908	1.2	0
74.8	2286.1771	370620	0.04	ASQDVNTAVAWYQQKPGKAPK	LC(025-045)	2286.1757	0.6	1
84.3	1771.9519	27274394	2.71	LLIYSASFLYSGVPSR	LC(046-061)	1771.9509	0.6	0
85.8	4129.8936	10160642	1.01	SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK	LC(067-103)	4129.8892	1.1	0
85.3	4599.1803	2980494	0.30	SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIK	LC(067-107)	4599.1792	0.2	1
84.5	4755.2888	6157193	0.61	SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKR	LC(067-108)	4755.2803	1.8	2
82.2	2101.1217	3894068	0.39	RTVAAPSVFIFPPSDEQLK	LC(108-126)	2101.1208	0.4	1
84.0	1945.0220	32666390	3.24	TVAAPSVFIFPPSDEQLK	LC(109-126)	1945.0197	1.2	0
90.2	3666.8789	1457746	0.14	TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR	LC(109-142)	3666.8756	0.9	1
85.5	1739.8676	23403588	2.32	SGTASVVCLLNNFYPR	LC(127-142)	1739.8665	0.6	0
75.9	2676.2628	852142	0.08	VQWKVDNALQSGNSQESVTEQDSK	LC(146-169)	2676.2627	0.0	1
80.0	4160.0087	11663837	1.16	VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	LC(146-183)	4160.0033	1.3	2
79.7	4766.2746	403170	0.04	VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEK	LC(146-188)	4766.2683	1.3	3
78.8	3618.7073	16391463	1.63	VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	LC(150-183)	3618.7021	1.5	1
78.6	4224.9705	725845	0.07	VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEK	LC(150-188)	4224.9670	0.8	2
77.3	4490.1265	478190	0.05	VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHK	LC(150-190)	4490.1209	1.3	3
78.9	6290.0188	328711	0.03	VDNALQSGNSQESVTEQDSKDSTYSLSSTLT LSKADYEKHKVYACEVTHQGLSSPVTK	LC(150-207)	6290.0085	1.6	4
79.0	1501.7515	2071466	0.21	DSTYSLSSTLTLSK	LC(170-183)	1501.7512	0.2	0
74.0	2689.3218	547667	0.05	ADYEKHKVYACEVTHQGLSSPVTK	LC(184-207)	2689.3170	1.8	2
73.4	2083.0562	6116953	0.61	HKVYACEVTHQGLSSPVTK	LC(189-207)	2083.0521	2.0	1
75.3	1817.8988	25691042	2.55	VYACEVTHQGLSSPVTK	LC(191-207)	1817.8982	0.3	0

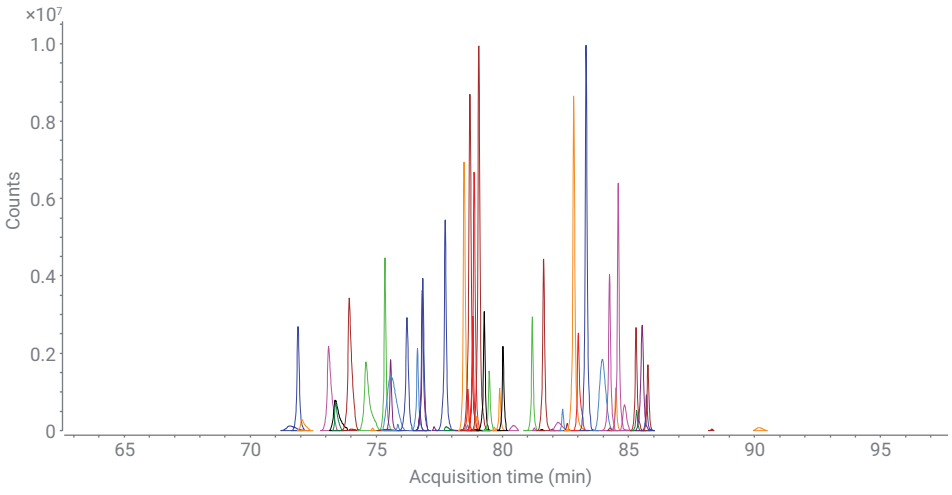

Figure 4. Overlaid RPLC/MS compound chromatograms of MS-identified peptides in the CEX main peak following online peptide mapping.

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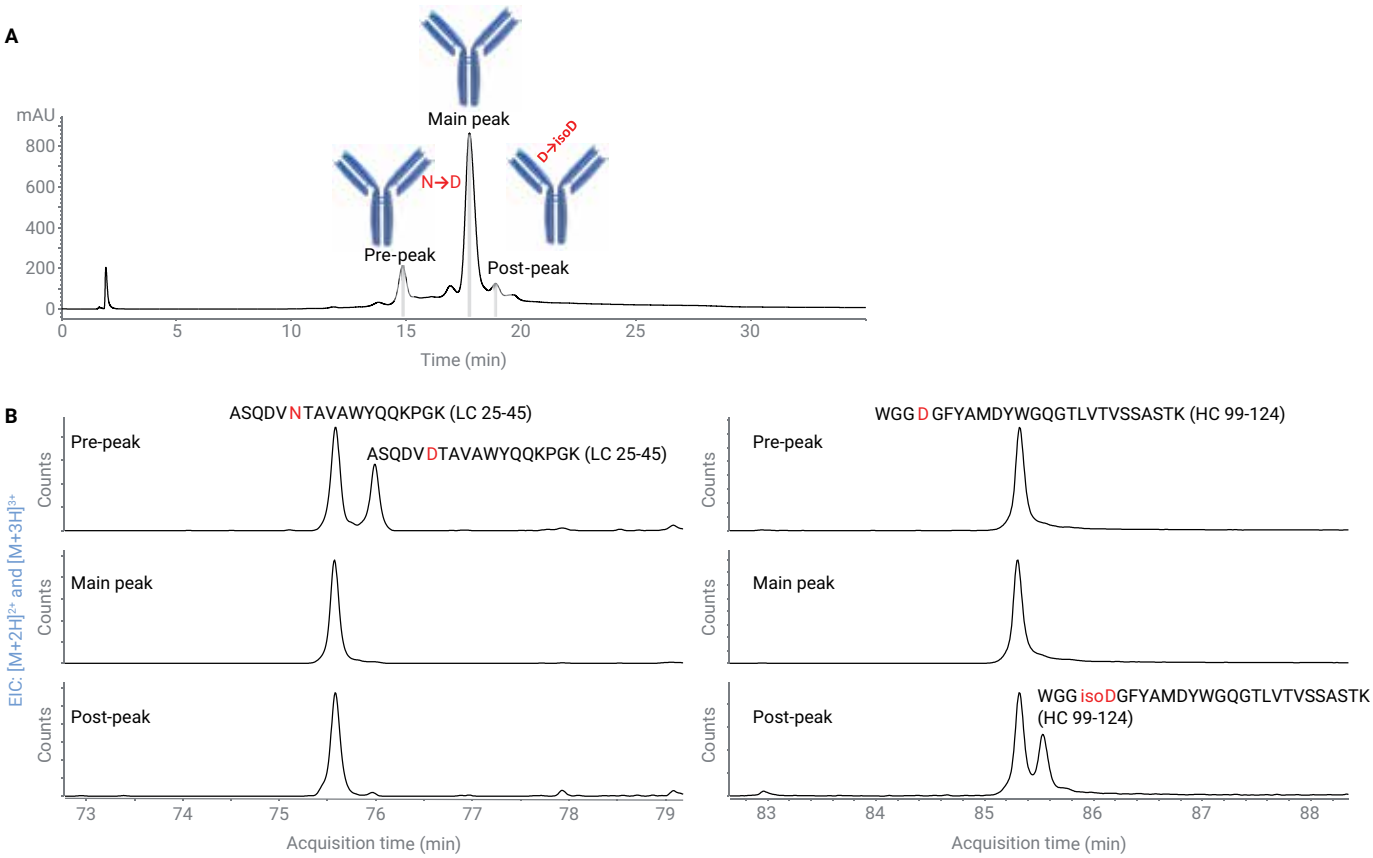

Figure 5. Online peptide mapping of trastuzumab CEX pre-, main-, and post-peaks. (A) the CEX chromatogram and (B) the extracted ion chromatograms of light-chain peptide ASQDVNTAVAWYQQKPGK (LC 25–42), deamidated light-chain peptide ASQDVDTAVAWYQQKPGK (LC 25–42), heavy-chain peptide WGGDGFYAMDYWGQGTLVTVSSASTK (HC 99–124) and isomerized heavy-chain peptide WGGisoDGFYAMDYWGQGTLVTVSSASTK (HC 99–124).

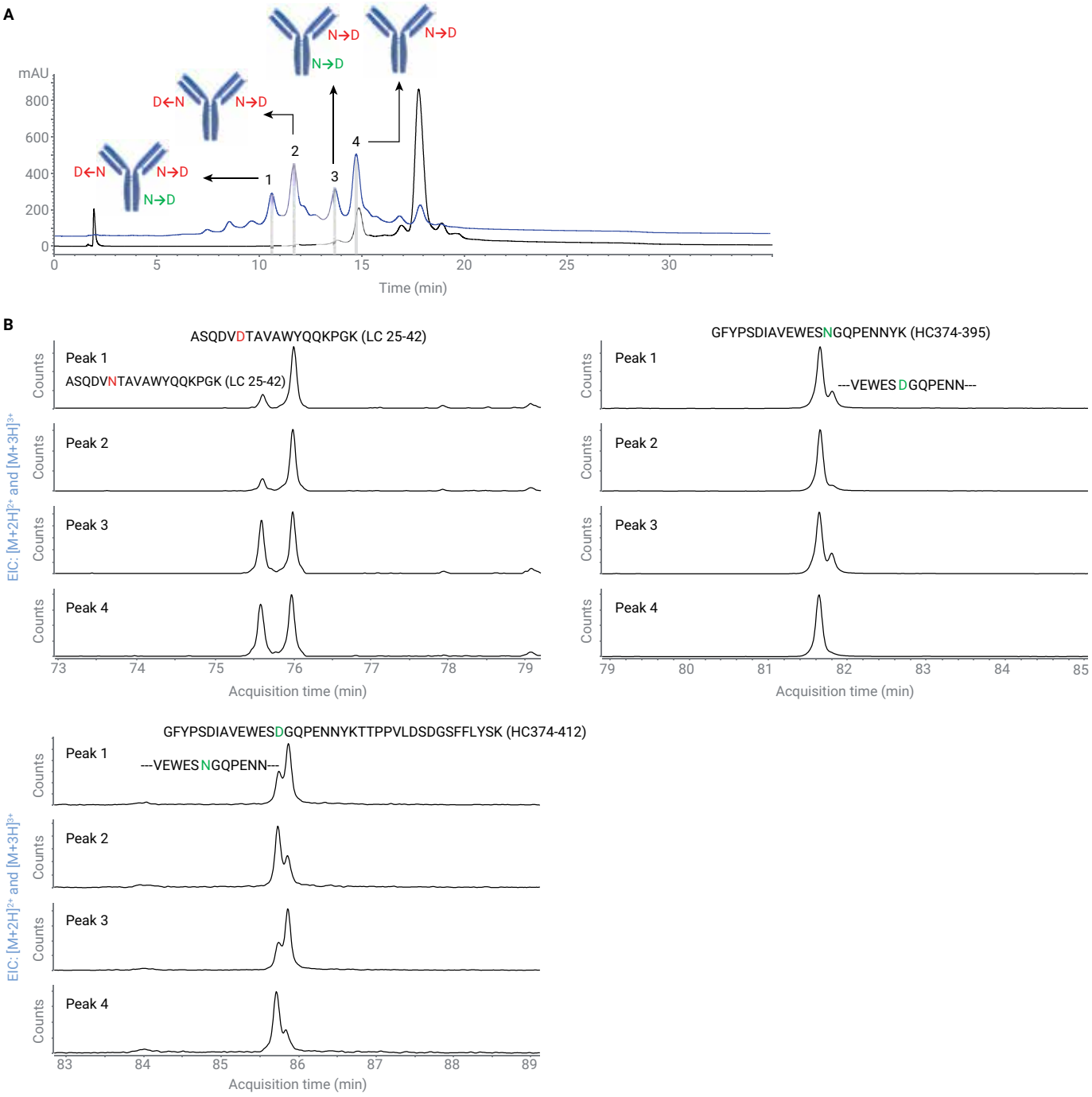

Figure 6. Online peptide mapping of high-pH stressed trastuzumab CEX peaks 1, 2, 3, and 4. (A) the overlaid CEX chromatograms of nonstressed and high-pH stressed trastuzumab and (B) the extracted ion chromatograms of light-chain peptide ASQDVNTAVAWYQQKPGK (LC 25–42), deamidated light-chain peptide ASQDVDTAVAWYQQKPGK (LC 25–42), heavy-chain peptide GFYPSDIAVEWESNGQPENNYK (HC 374–395), deamidated heavy-chain peptide GFYPSDIAVEWESDGQPENNYK (HC 374–395), heavy-chain peptide GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK (HC374–412), and deamidated heavy-chain peptide GFYPSDIAVEWESDGQPENNYKTTPPVLDSDGSFFLYSK (HC 374–412).

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Conclusion

A fully automated 4D-LC/MS protein analyzer incorporating 1D CEX separation and charge-variant peak collection using multiple heart-cutting, 2D RPLC-based desalting, denaturation, reduction, 3D trypsin digestion, and 4D RPLC/MS-based peptide mapping was described and successfully applied to characterize acidic and basic variants observed in the CEX profile of nonstressed and high-pH stressed trastuzumab. This multidimensional system is based on the InfinityLab 2D-LC Solution and the 6545 LC/Q-TOF system. A variant of this 4D-LC/MS design can readily be configured by replacing CEX in the first dimension by Protein A affinity chromatography, size exclusion chromatography, hydrophobic interaction chromatography, etc.

References

1. Sandra, K. *et al.* Modern Chromatographic and Mass Spectrometric Techniques for Protein Biopharmaceutical Characterization. *J. Chromatogr. A* **2014**, *1335*, 81–103.
2. Fekete, S. *et al.* Chromatographic, Electrophoretic and Mass Spectrometric Methods for the Analytical Characterization of Protein Biopharmaceuticals. *Anal. Chem.* **2016**, *88*, 480–507.
3. Walsh, G. Biopharmaceutical Benchmarks 2018. *Nat. Biotechnol*. **2018**, *32*, 992–1000.
4. Harris, R. J. *et al.* Identification of Multiple Sources of Charge Heterogeneity in a Recombinant Antibody. *J. Chromatogr. B* **2001**, *752*, 233–245.
5. Stoll, D. *et al.* Characterization of Therapeutic Antibodies and Related Products by Two-Dimensional Liquid Chromatography Coupled with UV Absorbance and Mass Spectrometric Detection. *J. Chromatogr. B* **2016**, *1032*, 51–60.
6. Sandra, K. *et al.* Characterizing Monoclonal Antibodies and Antibody-Drug Conjugates using 2D-LC-MS. *LCGC Europe* **2017**, *30*, 149–157.
7. Stoll, D. R. *et al.* Direct Identification of Rituximab Main Isoforms and Subunit Analysis by Online Selective Comprehensive Two-Dimensional Liquid Chromatography–Mass Spectrometry. *Anal. Chem.* **2015**, *87*, 8307-8315.
8. Sandra, K. *et al.* Multiple Heart-Cutting and Comprehensive Two-Dimensional Liquid Chromatography Hyphenated to Mass Spectrometry for the Characterization of the Antibody-Drug Conjugate Ado-Trastuzumab Emtansine. *J. Chromatogr. B* **2016**, *1032*, 119–130.
9. Schneider, S. 2D-LC/MS Characterization of Charge Variants Using Ion Exchange and Reversed-Phase Chromatography. *Agilent Technologies application note*, publication number 5991-6673EN, **2016**.
10. Gstöttner, C. *et al.* Fast and Automated Characterization of Antibody Variants with 4D HPLC/MS. *Anal. Chem.* **2018**, *90*, 2119–2125.
11. Goyon, A. *et al.* Streamlined Characterization of an Antibody-Drug Conjugate by Two-Dimensional and Four-Dimensional Liquid Chromatography/Mass Spectrometry. *Anal. Chem*. **2019**, *91*, 14896–14903.
12. Goyon, A. *et al.* From Proof of Concept to the Routine Use of an Automated and Robust Multi-Dimensional Liquid Chromatography Mass Spectrometry Workflow Applied for the Charge Variant Characterization of Therapeutic Antibodies. *J. Chromatogr. A* **2020**, doi: 10.1016/j.chroma.2019.460740.
13. Vandenheede, I. *et al.* Characterize mAb Charge Variants by Cation-Exchange Chromatography. *Agilent Technologies application note*, publication number 5991-5273EN, **2014**.

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Additional Application Notes

Publication Number	Title
5991-7442EN	Seamless Method Transfer from an Agilent 1260 Infinity Bio-inert LC to an Agilent 1260 Infinity II Bio-inert LC
5991-5273EN	Characterize mAb Charged Variants by Cation-exchange Chromatography
5991-5274EN	Characterize Fab and Fc Fragments by Cation-exchange Chromatography
5991-0895EN	Analysis of Intact and C-terminal Digested IgG1 on an Agilent Bio MAb 5 µm Column
5990-9629EN	pH Gradient Elution for Improved Separation of Monoclonal Antibody Charge Variants
5991-1407EN	High-resolution Analysis of Charge Heterogeneity in Monoclonal Antibodies Using pH-gradient Cation Exchange Chromatography
5991-1408EN	Protein Separation with pH Gradients Using Composite Buffer Systems Calculated by the Agilent Buffer Advisor Software
5991-4722EN	Reducing Cycle Time for Charge Variant Analysis of Monoclonal Antibodies
5991-3365EN	Simple Method Optimization in mAb Charge Variant Analysis using pH Gradients Generated from Buffer Advisor with Online pH and Conductivity Monitoring
5990-9270EN	Separation of Protein Standards on Agilent 3 µm Ion-Exchange Columns by Cation Exchange Chromatography
5990-9614EN	Analysis of proteins by anion exchange chromatography
5991-5221EN	Charge Profiling of 2AB-labelled N-linked Glycans

Best Practices and Troubleshooting

A systematic approach to troubleshooting is required to quickly determine the cause of any problems that may occur. Sample preparation, mobile phase, method conditions, instrument components, and column all play a part in the chromatographic separation and identifying the root cause can be difficult.

There are several small steps that one can take to avoid common problems, or to help recognize and diagnose problems when they do arise. Some examples are included in the guidelines indicated in the different sections of this document. Examples of other measures one can take include:

- Wherever possible a suitable reference standard should be regularly used and a performance record kept. This practice can help with spotting trouble before much time has been wasted. What's more the nature of the change observed can help identify the precise problem.
- Know the typical backpressure contributed by the LC system, the column, and the guard column if one is being used. When a pressure deviation is observed, it will be easier to pinpoint the source of the change and therefore which element needs either replacing or cleaning.

Some best practices directly related to the column are:

- When starting flow, start at a low flow rate (0.1 mL/min for example) and increase the flow rate gradually over a period of several minutes. While the backpressure of the method flow rate may not be near the maximum pressure, a sudden spike in pressure when starting flow from zero can damage the column.
- For gradient methods, make a cleaning step part of the method before re-equilibrating before the next injection. For reversed-phase methods this would be a high organic hold for several minutes. For an aqueous method like ion exchange for charge variant analysis, this could be to flow high salt mobile phase for several minutes. Often 100 % mobile phase B would be appropriate for this step.
- Be aware of the recommended minimum and maximum conditions for temperature, pressure, and pH during method development. Operating at the extremes of the recommended ranges will lead to shorter column lifetimes than more moderate conditions.

High-quality mobile phase solvents and additives are important for optimum performance, but pausing to consider a few other parameters will also go a long way to avoiding common problems:

- Consider solvent miscibility and solubility of any additives. Mobile phases A and B need to be fully miscible, but the sample solution also must be miscible with initial mobile phase conditions. Also, when changing mobile phase systems for cleaning or storing the column, think about the compatibility first, and decrease the flow rate as mixing can increase backpressure.
- Consult the column use guide for appropriate storage solvents.
- For aqueous chromatography, mobile phase "hygiene" is crucial. Aqueous mobile phases, especially those at neutral pH containing salts, are ripe for microbial growth. Filtering aqueous mobile phases through a 0.2 µm filter, storing in the refrigerator, and making fresh mobile phase often will minimize microbial growth that can lead to clogging.

Several resources exist with information to best care for LC columns, and to further troubleshoot problems that arise:

- The LC Handbook Guide to LC Columns and Method Development contains a wealth of information surrounding method development, and advice for troubleshooting based on the symptoms observed. **www.agilent.com/cs/library/primers/Public/LC-Handbook-Complete-2.pdf**
- Column user guides ship with each column, and detail appropriate temperature, pressure, and pH ranges, as well as compatible mobile phases, and instructions for cleaning and storing the column. If they have been misplaced, all column user guides may be found at **www.agilent.com/chem/biolc-columns-user-guides.**
- Customer support may be reached in the US and Canada at 800-227-9770. Please see **www.agilent.com** for support resources in your region

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Published in the USA, January 25, 2022
5991-9072EN

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