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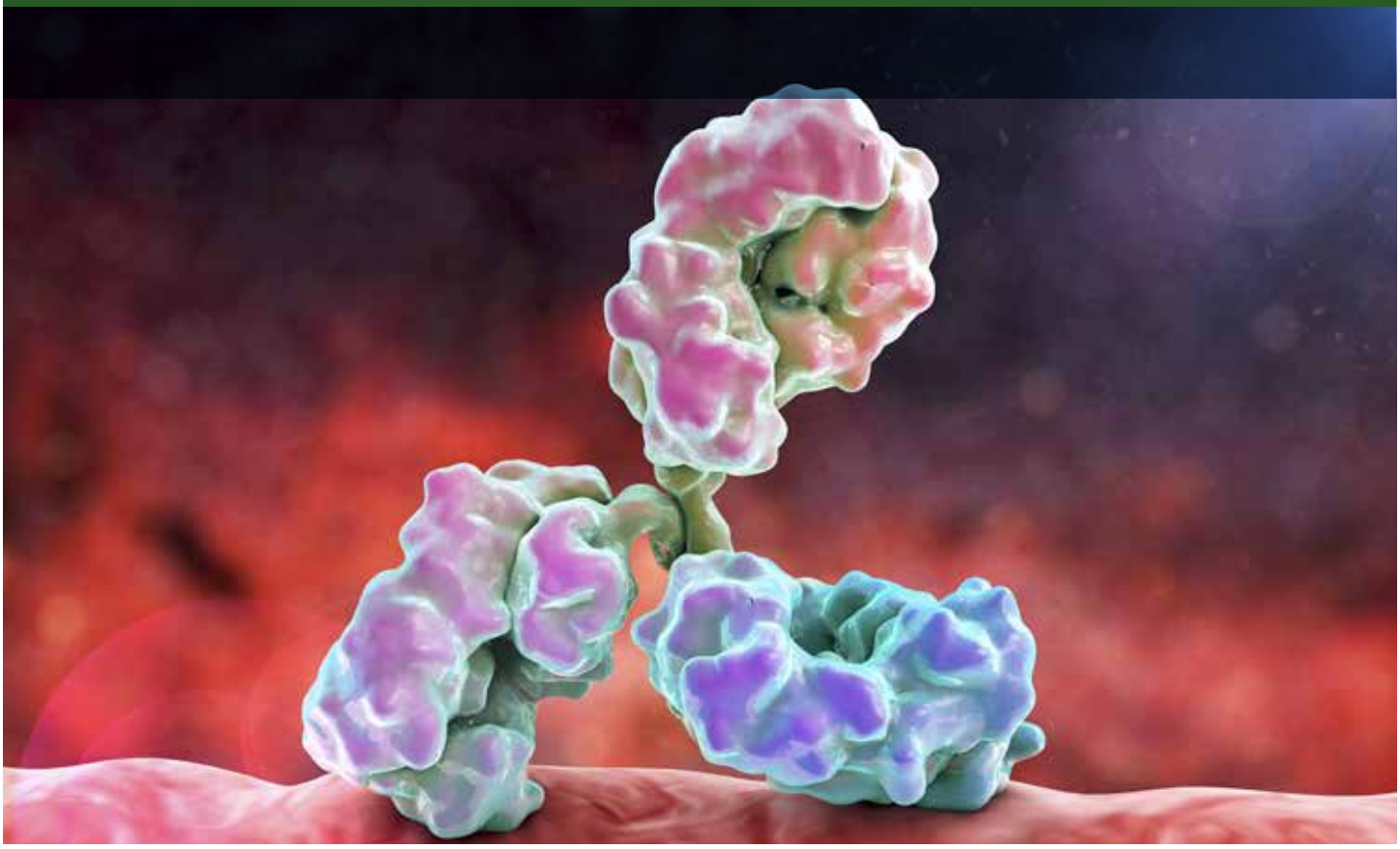
Agilent Biocolumns

Critical Quality Attributes

Application Compendium



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Critical Quality Attributes Application Compendium- Part I

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 CrossLab, 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.

Best regards,

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Agilent CrossLab Group

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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.

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

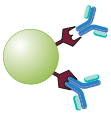
Background

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 4 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

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 4 minute) results.

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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. HCl has the advantage of having a lower refractive index than other eluents, reducing baseline noise, however it can lead to shorter column lifetimes than other eluents such as citric acid.

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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 and IgG2 and no affinity for IgG3, whereas Agilent Bio-Monolith Protein G columns have high affinity for human subclasses IgG1, IgG2, and IgG3. 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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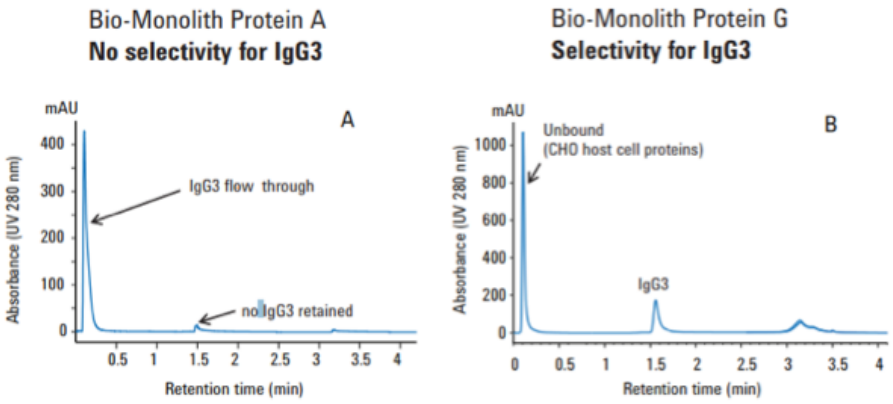


The Bio-Monolith Protein A has no affinity for IgG3 but the Bio-Monolith Protein G does.

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

HPLC system considerations - Protect your proteins during analysis

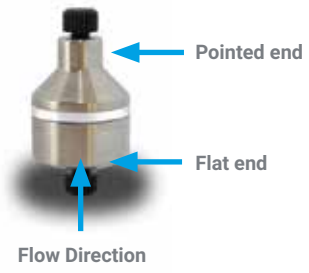
These monolithic columns are compatible with HPLC and UHPLC systems, however an ideal choice for this type of analysis is the Agilent 1260 Infinity II bio-inert LC. It handles challenging solvent conditions with ease, such as extreme pH values of pH 1 to pH 13, and buffers with high salt concentrations. 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. However, 1.0 mL/min gives shaper and taller peaks and better signal-to-noise ratio.

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.
(Do you want to/have instructions to include here to make up 1 or 2 L of this)
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. However, HCl has a lower refractive index compared to other eluents. Therefore if a low concentration sample is used and baseline noise and artifact peaks are of concern, HCl can be used as an eluent.

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	Citric acid	0.1 M	2.5 to 3.0
	Glycine	0.1 M	2.5 to 3.0
	Acetic acid	5–20 %	
	HCl	12 mM to 0.1 M	
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 %	
	HCl	12 mM to 0.1 M	

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Fast separation protocols

The Bio-Monolith Protein A and Protein G columns are designed for rapid separations. The columns can be operated up to 3 mL/min. The capability for fast mAb capture and elution at various flow rates is demonstrated with IgG_3, using a Bio-Monolith Protein G column in Figure 2, with 1.0, 1.5, 2.0, and 2.5 mL/min flow rates. Table 3 shows the flow rates and operating gradients, these conditions can be used for Bio-Monolith Protein A as well, just adjust the eluting buffer (mobile phase B) to pH 2.5 to 3.0.

Conditions

Parameter	Value
Column:	Bio-Monolith Protein G
Injection:	IgG_3 (2 mg/mL)
Mobile phase A:	5 µL
Mobile phase B:	50 mM sodium phosphate buffer, pH 7.4
Temp.:	0.1 M citric acid, pH 2.0
HPLC:	25 °C
Detection:	Agilent 1260 Bio-inert Quaternary LC

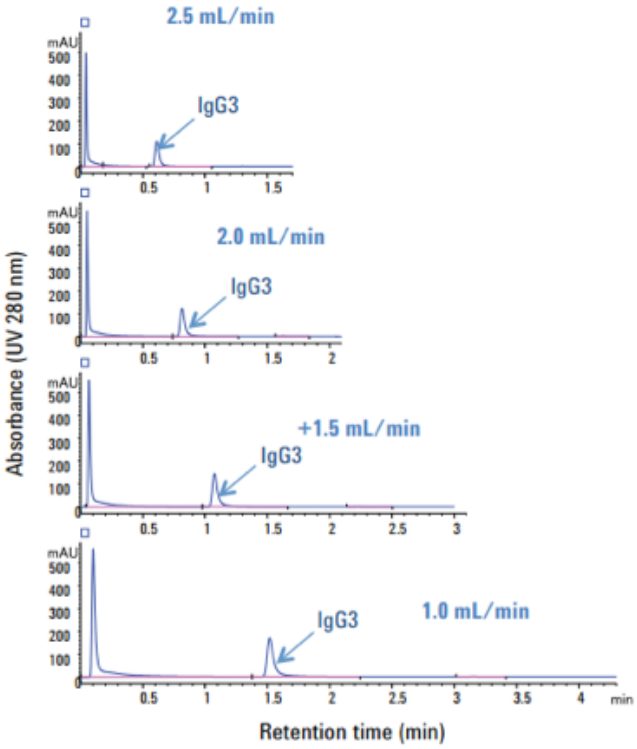

Figure 2. Column: Bio-Monolith Protein G

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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.

Table 3. Flow rates and operating gradients for fast separation of IgG3

1.0 mL/min		
Time (min)	**% A**	**% B**
0	100	0
0.4	100	0
0.5	0	100
2.0	0	100
2.1	100	0
4.2	100	0

1.5 mL/min		
Time (min)	**% A**	**% B**
0	100	0
0.3	100	0
0.4	0	100
1.7	0	100
1.8	100	0
3.2	100	0

2.0 mL/min		
Time (min)	**% A**	**% B**
0	100	0
0.2	100	0
0.3	0	100
1.2	0	100
1.3	100	0
2.2	100	0

2.5 mL/min		
Time (min)	**% A**	**% B**
0	100	0
0.1	100	0
0.2	0	100
0.8	0	100
0.9	100	0
1.7	100	0

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
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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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.

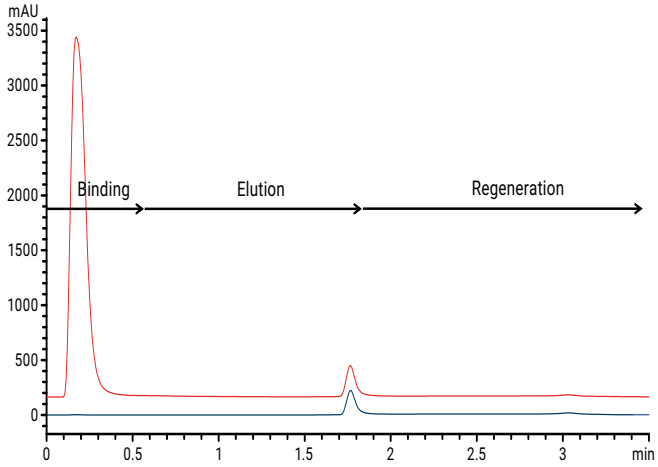

Figure 1. UV 280 nm Agilent Bio-Monolith Protein A chromatogram of a trastuzumab-producing CHO clone, clone 9 (red), and of a Herceptin originator diluted in 50 mM Na-phosphate pH 7.4 to 0.2 mg/mL (blue). Note that the supernatant was diluted 1:1 in phosphate buffer.

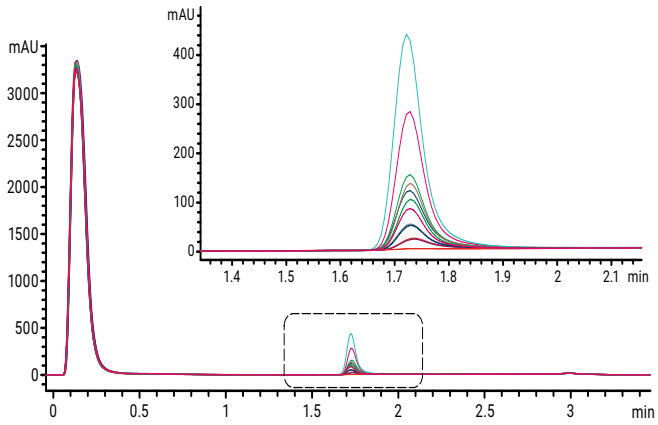

Figure 2. Overlaid UV 280 nm Agilent Bio-Monolith Protein A chromatograms of 12 trastuzumab-producing CHO clones.

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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.

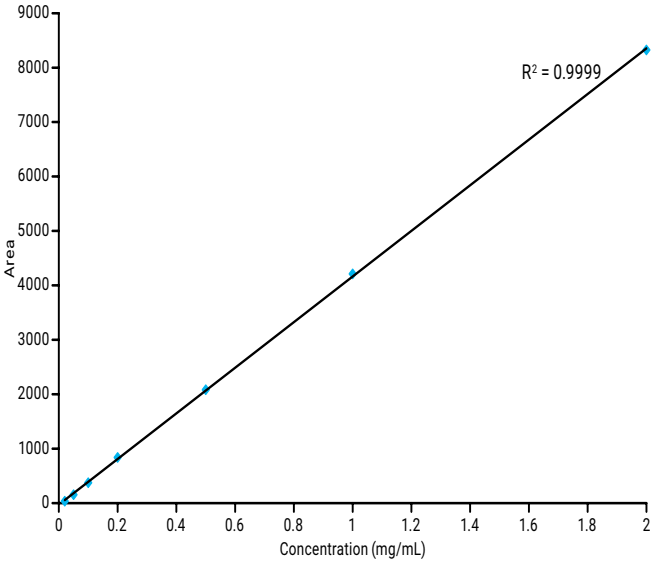

Figure 3. Herceptin Agilent Bio-Monolith Protein A calibration curve, 0.02 to 2 mg/mL.

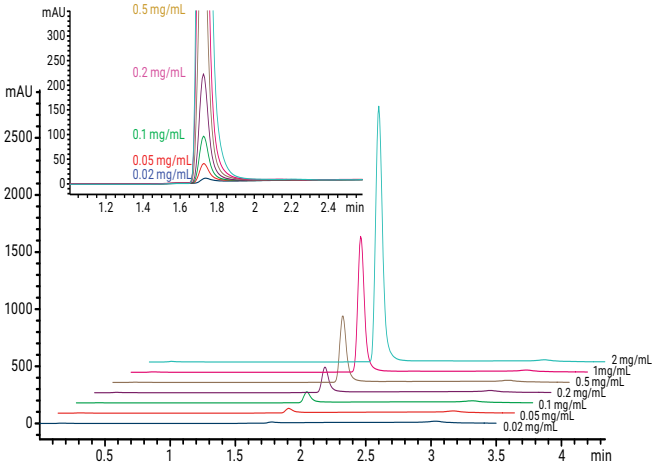

Figure 4. Overlaid UV 280 nm Agilent Bio-Monolith Protein A chromatograms of Herceptin calibration points.

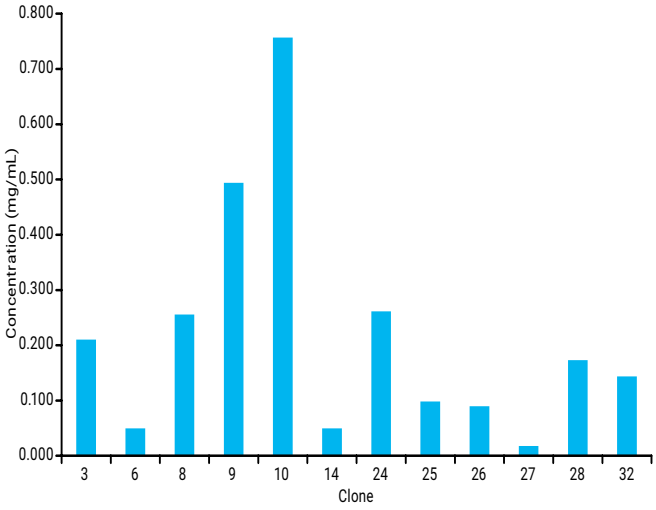

Figure 5. Graphical representation of the biosimilar mAb titer, expressed in mg/mL, in the different trastuzumab CHO clones.

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.

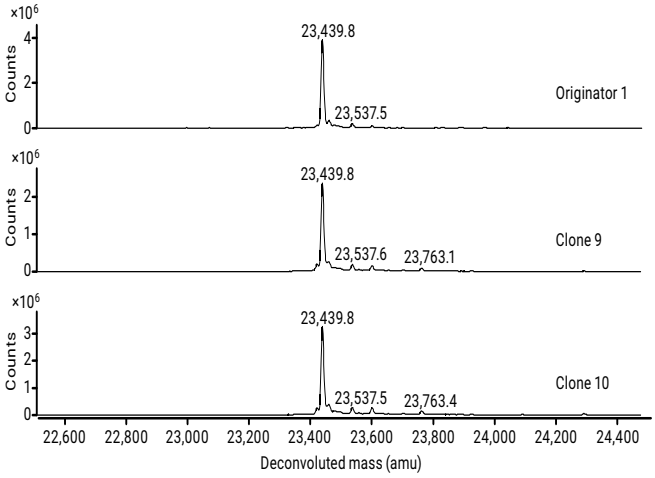

Figure 6. Deconvoluted light chain spectra of a Herceptin originator and two trastuzumab-producing clones.

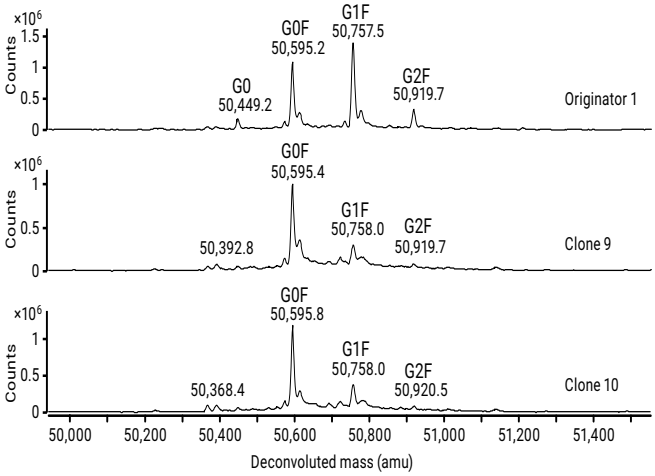

Figure 7. Deconvoluted heavy chain spectra of a Herceptin originator and two trastuzumab producing clones. The abbreviations G0, G0F, G1, and G2F refer to the N-glycans attached to the mAb backbone..

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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	

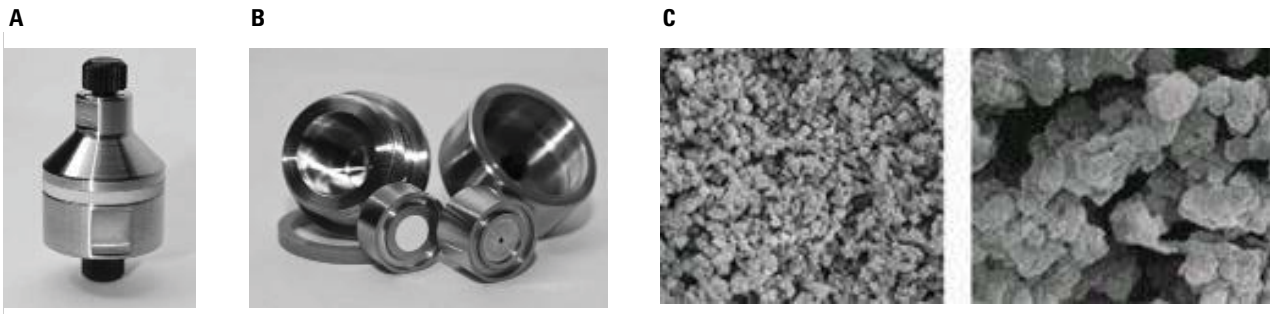

Figure 1. UV 280 nm Agilent Bio-Monolith Protein A chromatogram of a trastuzumab-producing CHO clone, clone 9 (red), and of a Herceptin originator diluted in 50 mM Na-phosphate pH 7.4 to 0.2 mg/mL (blue). Note that the supernatant was diluted 1:1 in phosphate buffer.

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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.

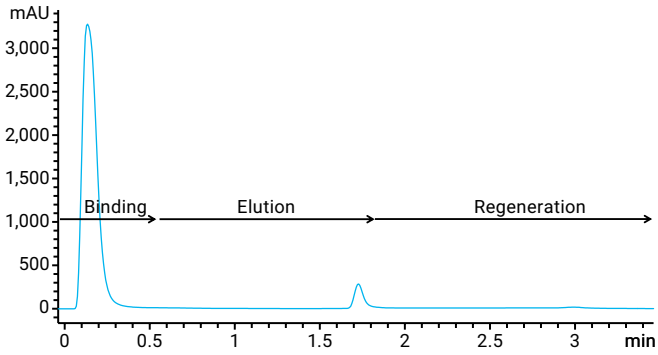

Figure 2. UV 280 nm Protein A chromatogram showing the supernatant of a trastuzumab-producing CHO clone. Injection volume was 50 µL. Peak width at half height was 0.10 minutes for the unbound material and 0.06 minutes for the retained mAb.

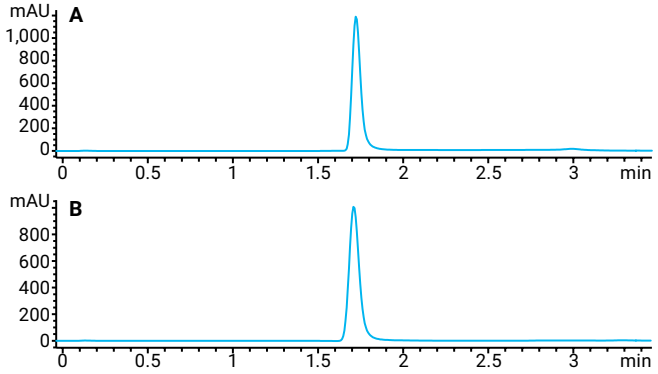

Figure 3. UV 280 nm Protein A chromatogram of Herceptin originator diluted in 50 mM Na-phosphate, pH 7.4, to 0.5 mg/mL (50-µL injection, 25-µg column load). Elution was achieved using citric acid (A) and acetic acid (B). Peak width at half height is 0.057 and 0.067 minutes for citric acid and acetic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

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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.

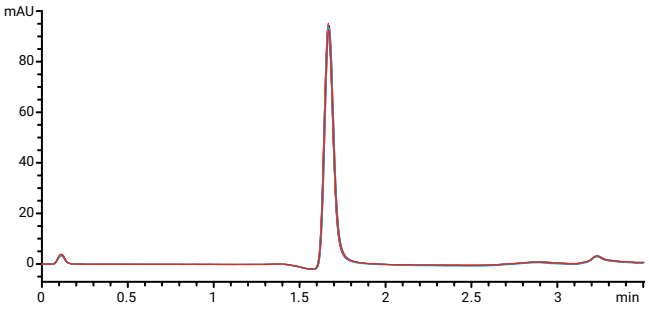

Figure 4. Replicate (n = 10) UV 280 nm Protein A chromatograms of a Herceptin originator diluted in 50 mM Na phosphate, pH 7.4, to 0.5 mg/mL (injection volume 5 µL). Elution was achieved using acetic acid.

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.

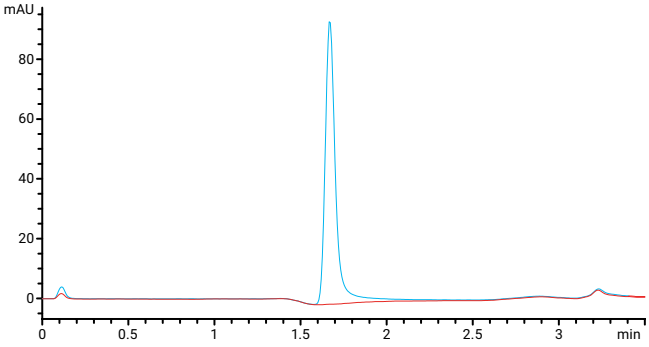

Figure 5. UV 280 nm Protein A chromatograms of a Herceptin originator diluted in 50 mM Na phosphate, pH 7.4, to 0.5 mg/mL, and a blank buffer analyzed after a sequence of 10 Herceptin injections. Elution was achieved using acetic acid, and injection volumes were 5 µL.

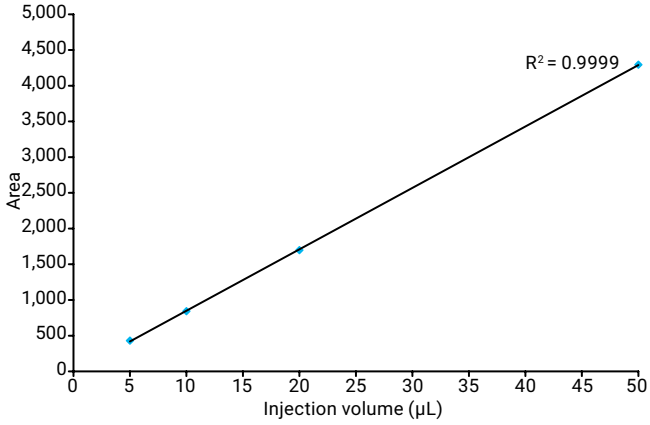

Figure 6. Linearity obtained by increasing the injection volume of a Herceptin originator (0.5 mg/mL) from 5 to 50 µL.

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.

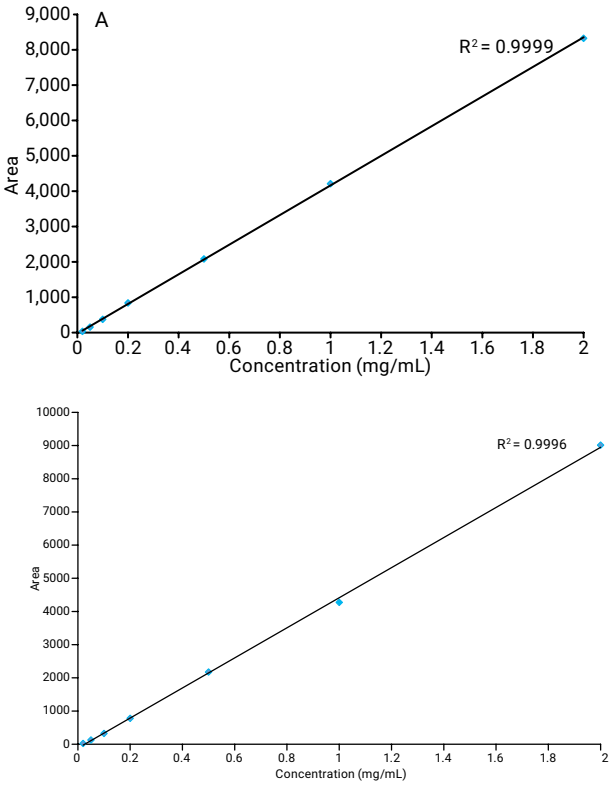

Figure 7. Herceptin Protein A calibration curve (0.02 to 2 mg/mL) using citric acid (A) and acetic acid (B) as elution buffer.

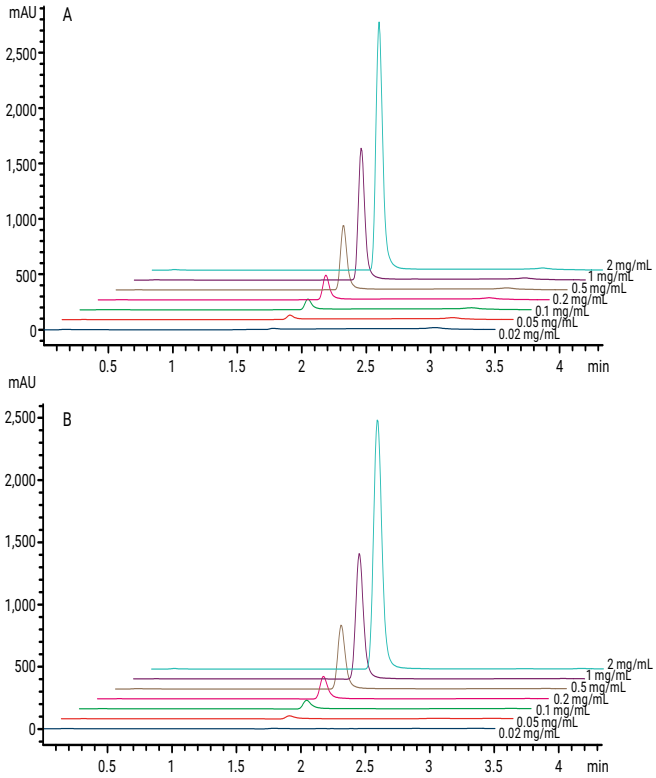

Figure 8. Overlaid UV 280 nm Protein A chromatograms of Herceptin calibration points using citric acid (A) and acetic acid (B) as elution buffer.

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.

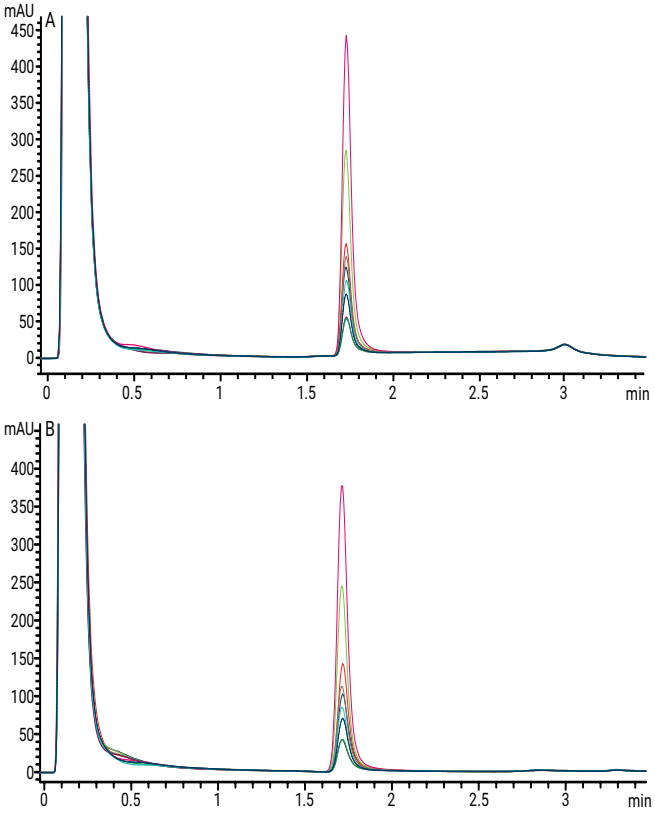

Figure 9. Overlaid UV 280 nm Protein A chromatograms of nine trastuzumab-producing CHO clones using citric acid (A) and acetic acid (B) as elution buffer.

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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

Agilent Bio-Monolith Protein A and G

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.

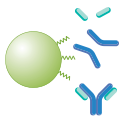
Intact & Subunit Purity

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)

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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

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.

Application Note

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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

Authors

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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.

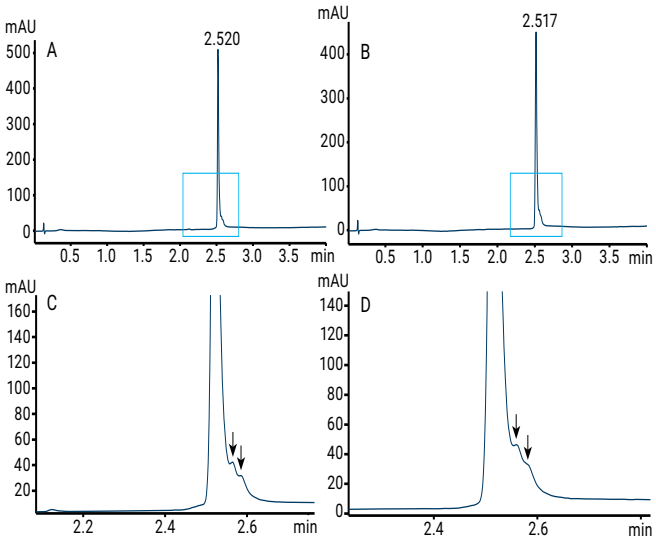

Figure 1. RP-HPLC analysis of innovator rituximab (A) and biosimilar rituximab (B) separated on an Agilent AdvanceBio RP-mAb Diphenyl 2.1 × 50 mm, 3.5 µm column. C and D show zooms.

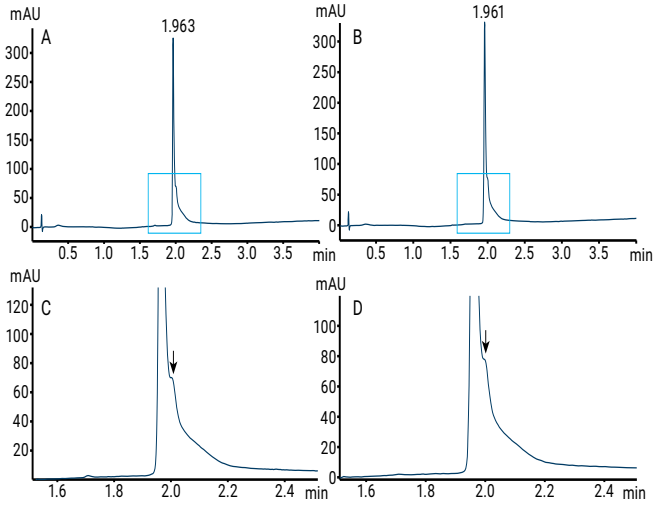

Figure 2. RP-HPLC profile of intact innovator rituximab (A) and biosimilar rituximab (B) on an Agilent AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm column. C and D show zooms.

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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.

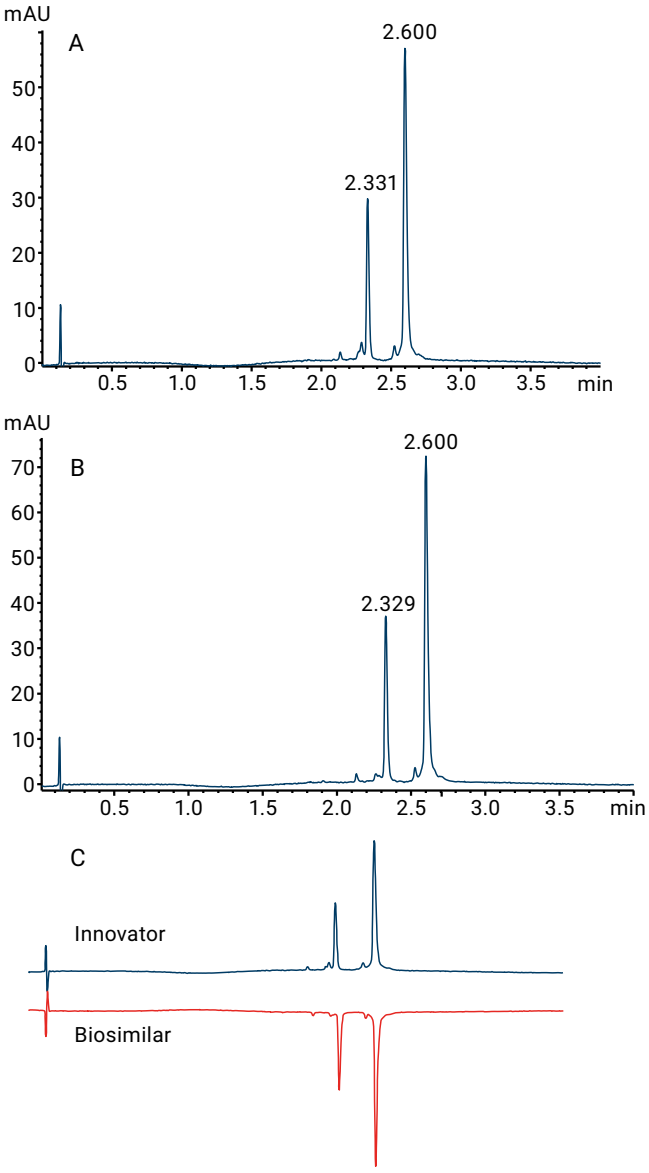

Figure 3. RP-HPLC profiles of innovator rituximab (A) and biosimilar rituximab (B) separated on an Agilent AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm column. Mirror plot image overlays (C).

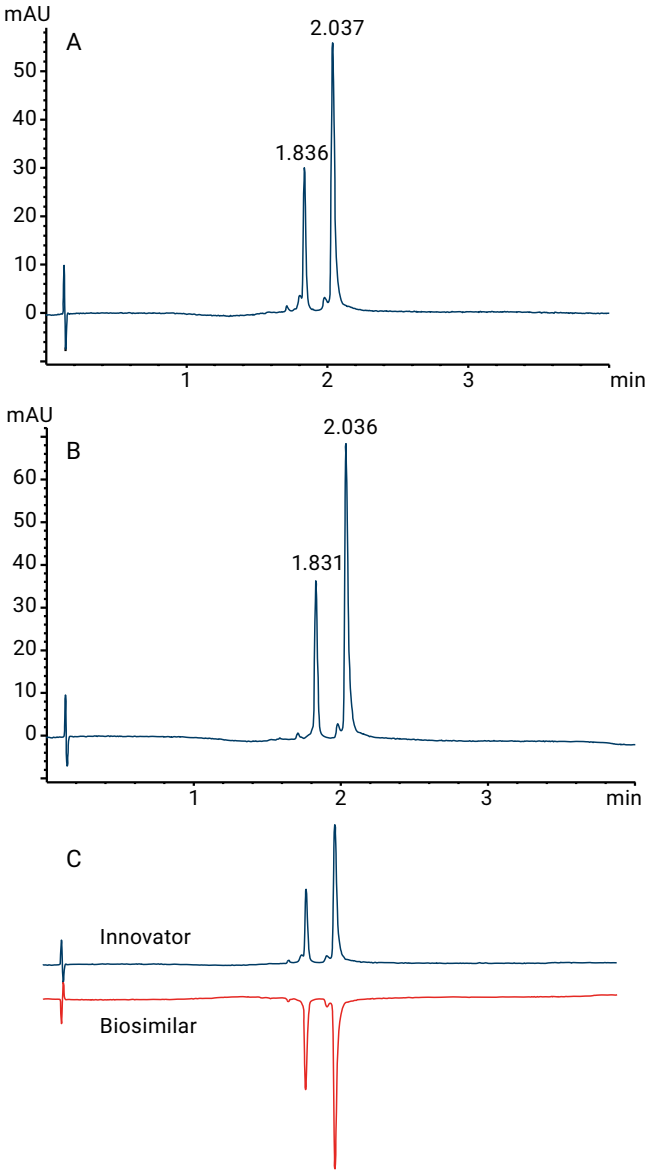

Figure 4. RP-HPLC profiles of innovator rituximab (A) and biosimilar rituximab (B) separated on an Agilent AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm column. Mirror plot image overlays (C).

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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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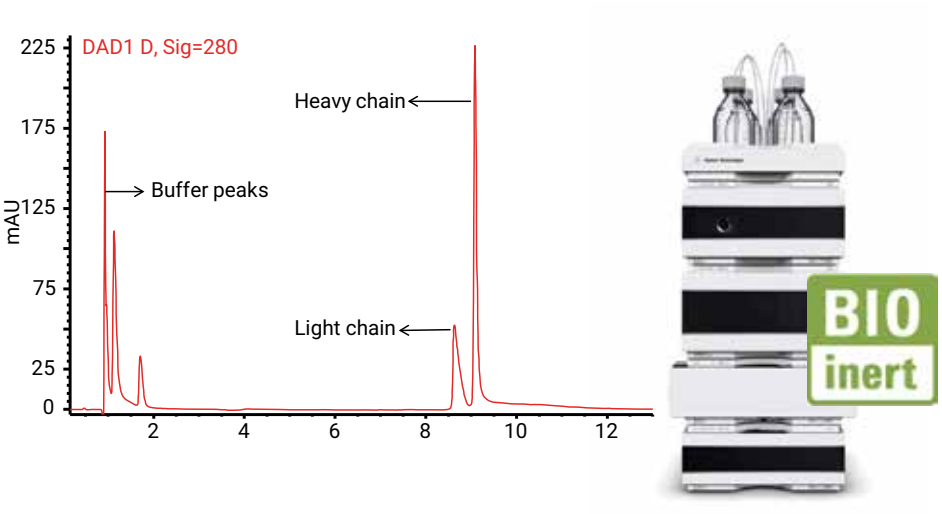
Abstract

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.

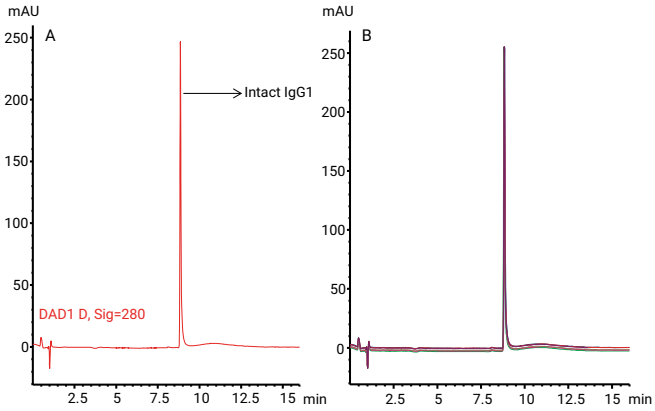

Figure 1. RP HPLC profile of intact IgG1 on an Agilent ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm column (A), and an overlay of six replicates (B).

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.

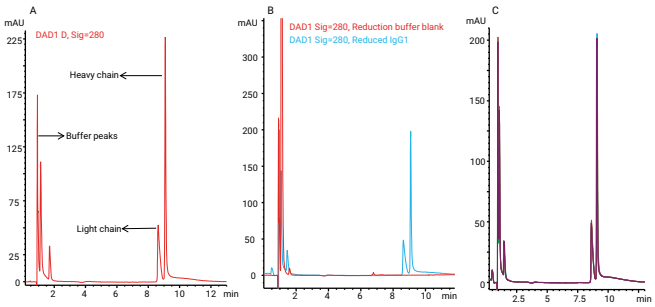

Figure 2. RP HPLC profile of (A) Reduced and alkylated IgG1, (B) overlaid with buffer blank, (C) overlay of six replicates.

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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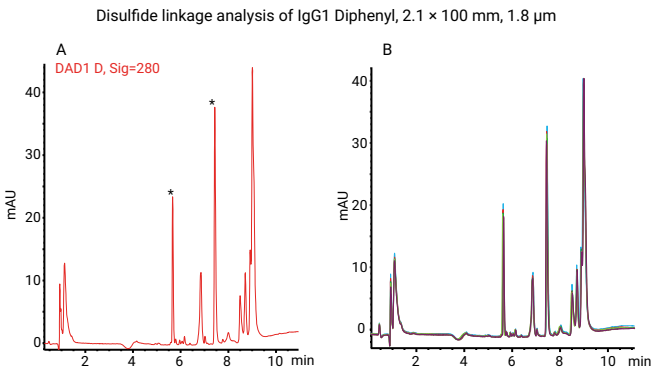

Figure 3. RP HPLC profile of (A) Lys C digested IgG1 and (B) overlay of six replicates. Peaks selected for RT and area RSDs are indicated by an asterix.

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

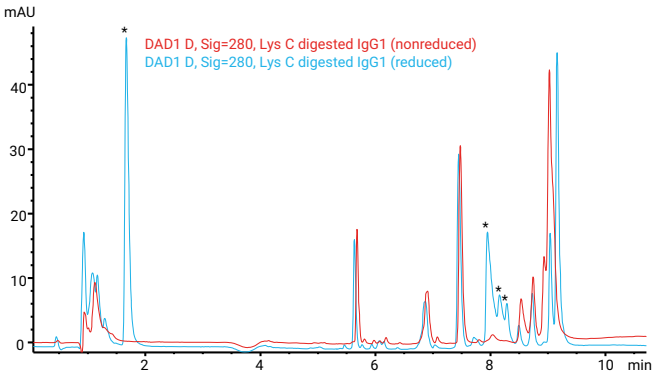

Figure 4. Comparison of peptide maps of Lys C digested IgG1 under nonreducing condition (red trace) followed by reduction (blue trace). Peptides bound through disulfide linkages are indicated by an asterix.

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.

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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.
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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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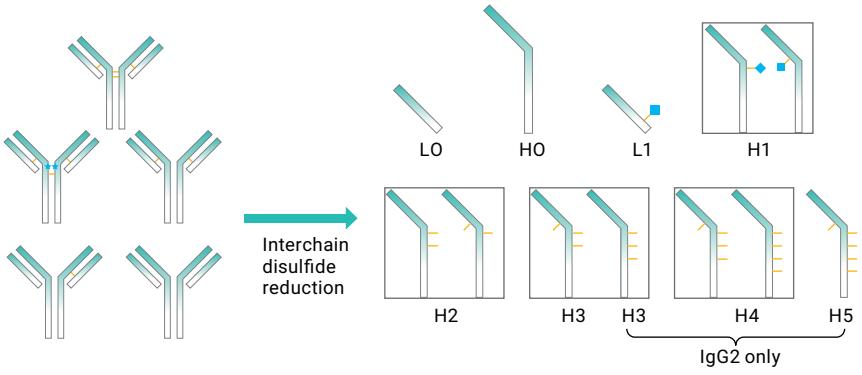
Abstract

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.

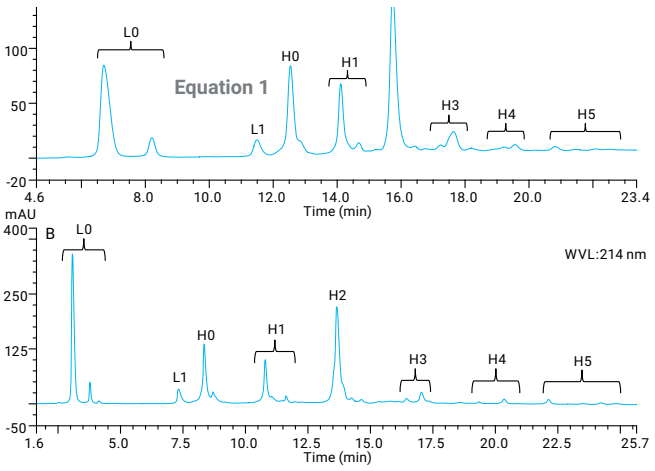

Figure 1. Comparison of reduced RP profiles obtained using a C4 HPLC column (A) and an Agilent ZORBAX RRHD SB300-C8 (B) for a cysteine-conjugated IgG2 ADC.

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.

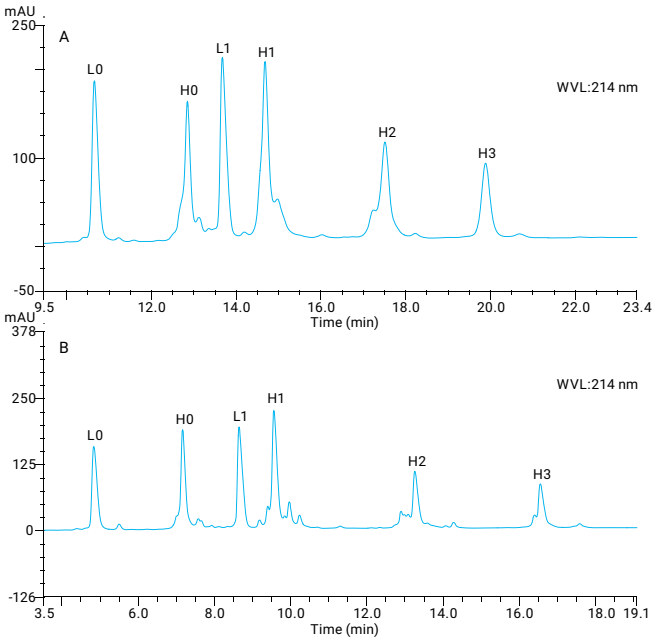

Figure 2. Comparison of PLRP-S HPLC (A) and Agilent ZORBAX RRHD SB300-C8. 1.8 µm (bottom) profiles for a cysteine-conjugated IgG2 ADC.

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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.

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

ZORBAX RRHD 300SB

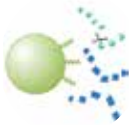
Publication Number	Title
5990-9668EN	Ultra High Speed and High Resolution Separations of Reduced and Intact Monoclonal Antibodies with Agilent ZORBAX RRHD Sub-2 µm 300 Diphenyl UHPLC Column
5990-7988EN	Analysis of Oxidized Insulin Chains using Reversed-Phase Agilent ZORBAX RRHD 300SB-C18
5990-7989EN	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

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.

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.

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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

Pprocedure	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.

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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

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	
	Dilute acid	C-terminal side of Met
	BNPS-skatole	
		Asp and Pro
		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.

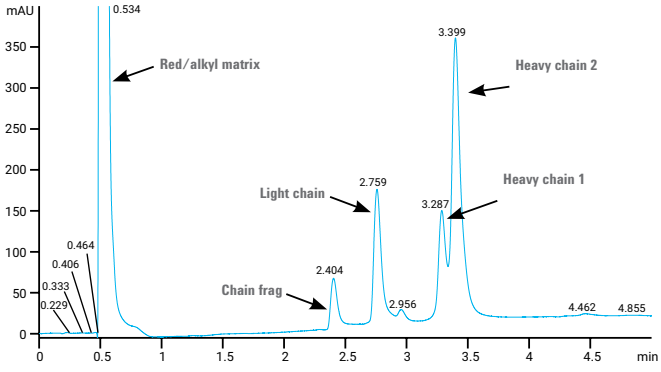

Figure 1. Reversed-phase separation of a reduced and alkylated monoclonal antibody prior to digestion protocol using an Agilent Zorbax Rapid Resolution High Definition (RRHD) 300SB-C8, 2.1 x 50 mm column (Agilent p/n 857750-906). The separation was performed at 0.5 mL/min, 75 °C using water (0.1% TFA)/ACN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

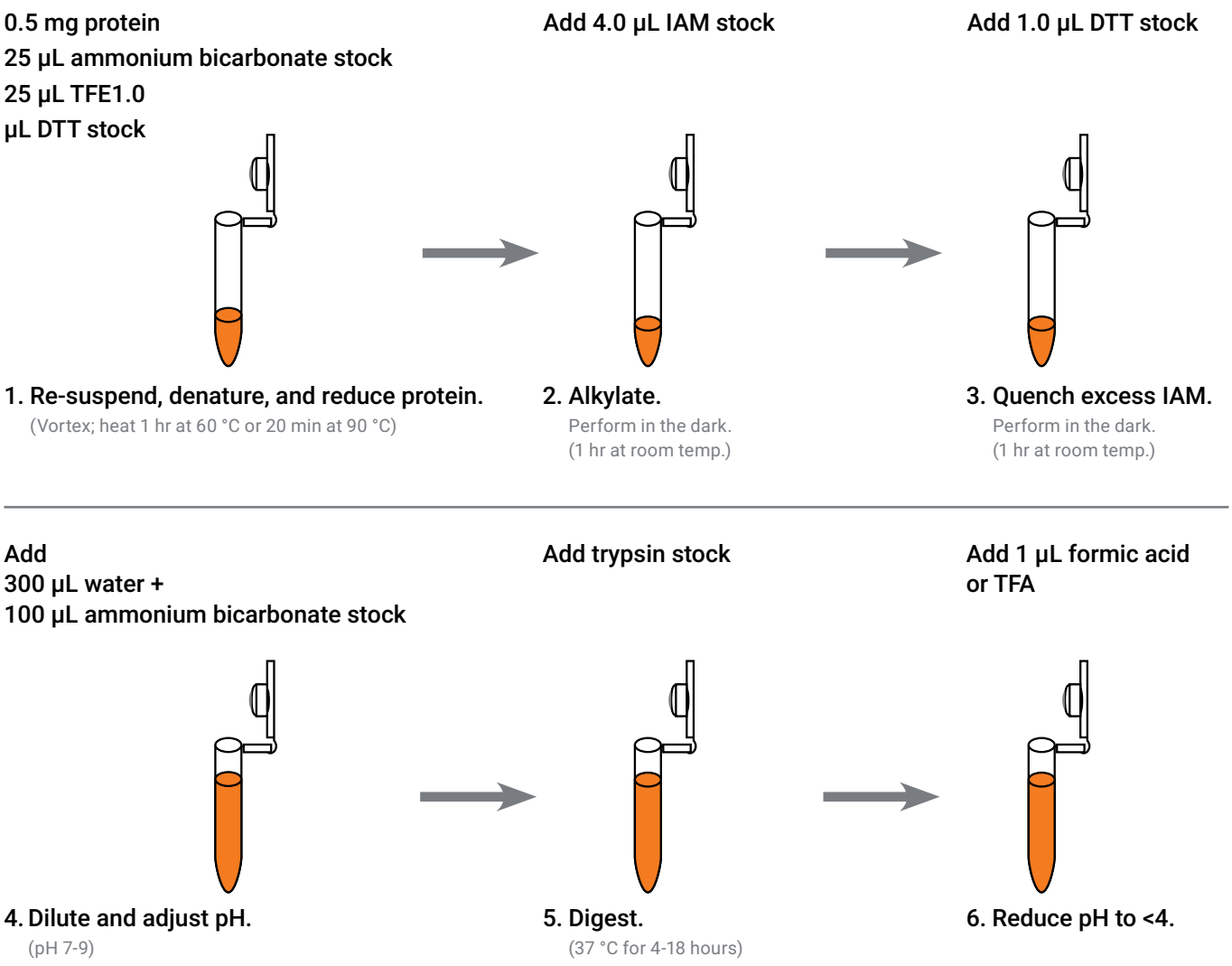
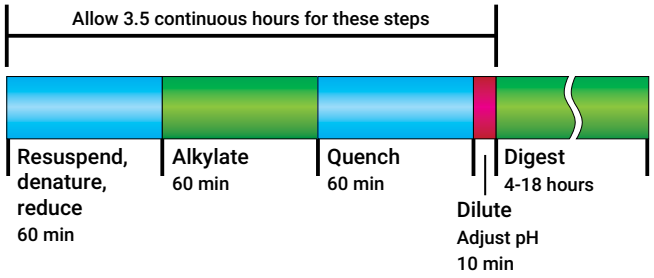


Figure 3 – Expected timeline for digestion procedure



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 displays a well resolved peptide separation using bovine serum albumin and demonstrates the multitude of peptide peak fragments that can be resolved by employing RPC for peptide mapping.

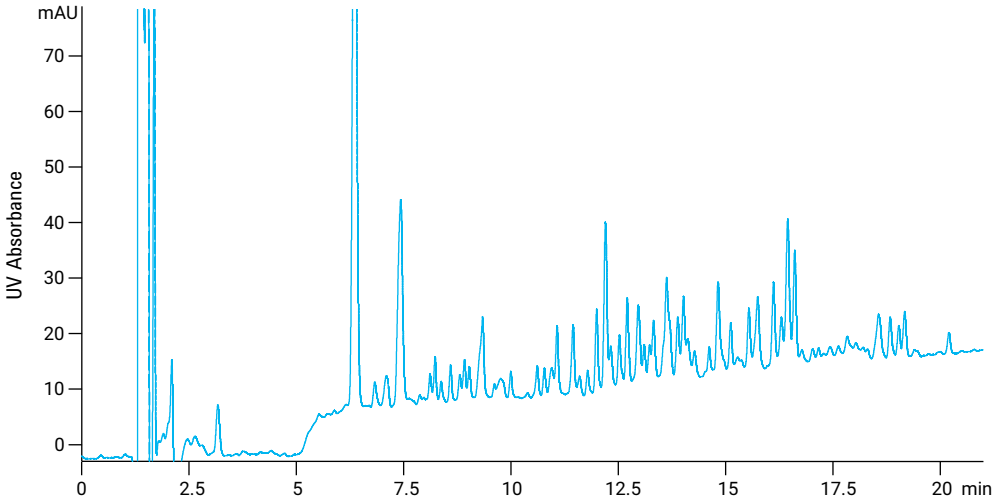

Figure 4. Reversed-phase separation of BSA using an Agilent Polaris C18-A, 2.0 x 150 mm column (Agilent P/N A2001150X020).

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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 5 provides an example of a rapid high resolution peptide map achieved using a superficially porous column.

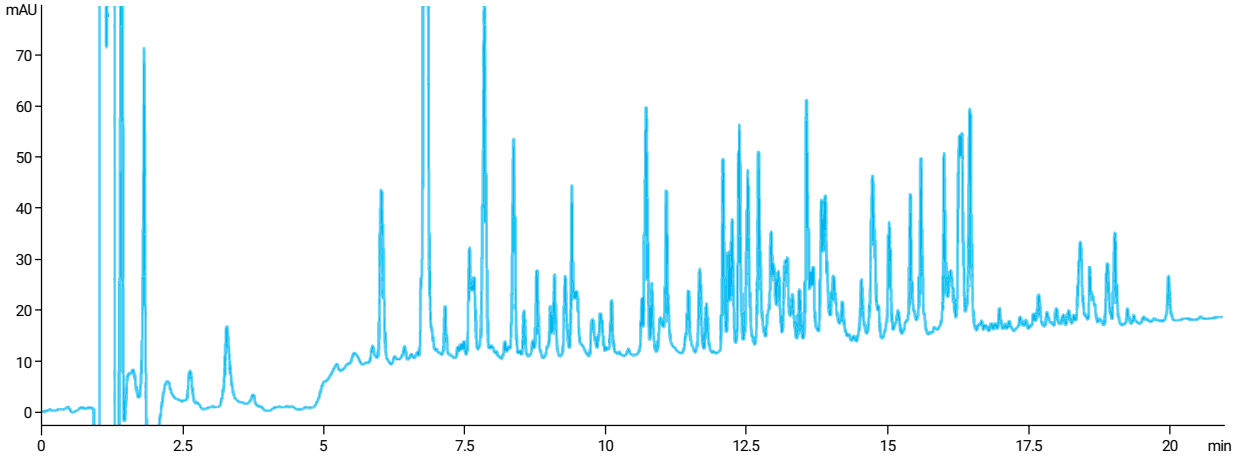

Figure 5. Reversed-phase separation of BSA using an Agilent AdvanceBio Peptide Mapping 2.1 x 150 mm column (Agilent P/N 653750-902). The peptide mapping separation was performed at 0.3 mL/min, 40 °C using water (0.1% TFA)/ACN (0.08%) linear gradient.

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 6 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.

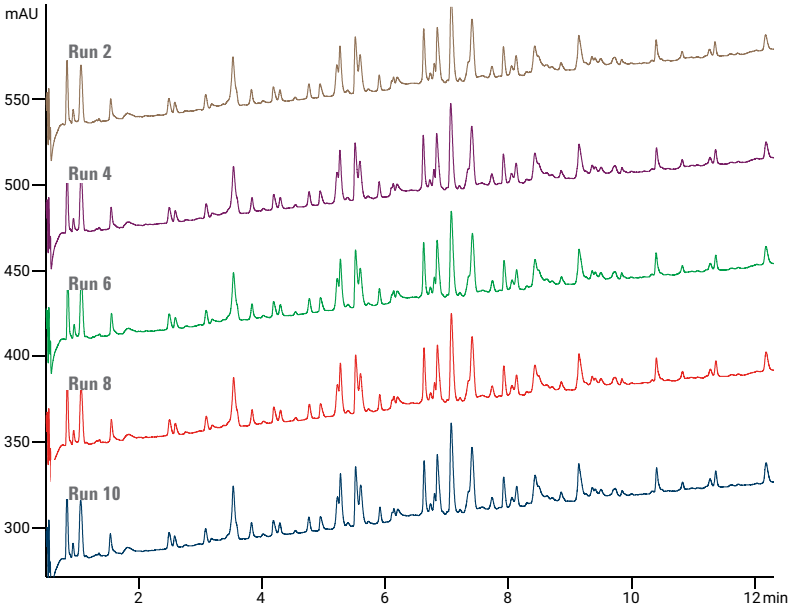

Figure 6. Five replicate injections of a monoclonal antibody tryptic digest using a 3.0 x 150 mm Agilent AdvanceBio Peptide Mapping column (Agilent P/N 653950-302) on an Agilent 1200 LC system coupled to a 6520 Q-TOF. Separation was performed at 0.3 mL/min, 40 °C using water (0.1% FA)/ACN (0.1% FA) gradient.

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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 7 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.

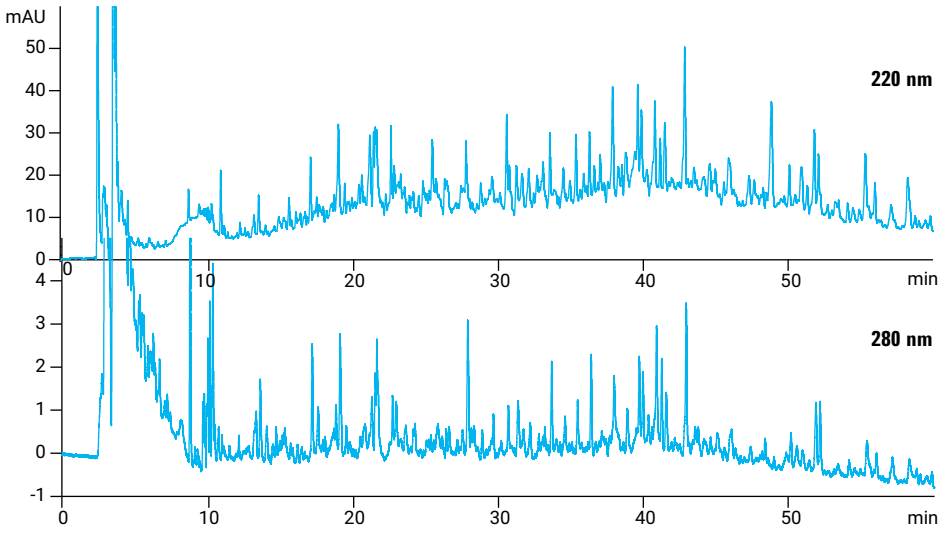

Figure 7. AdvanceBio Peptide Mapping column (Agilent P/N 651750-902), 2.1 x 250 mm, profiling e.coli digest at 220 nm (top) and 280 nm (bottom) on an Agilent 1290 Infinity LC.

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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 8 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.

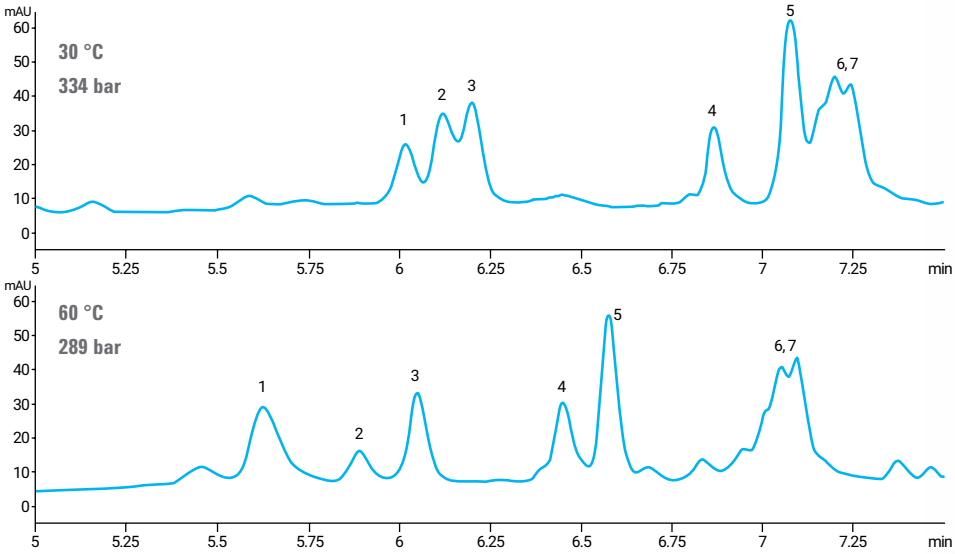

Figure 8. Myoglobin tryptic digest gradient separation at 5.0-8.0 min of a 20 min gradient with a 2.1 x 150 mm AdvanceBio Peptide Mapping column (Agilent P/N 653950-302). Both separations were completed with a water (1.0 % TFA)/ACN (0.08% TFA) linear gradient, 0.3 mL/min at 215 nm on an Agilent 1260 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 9 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.

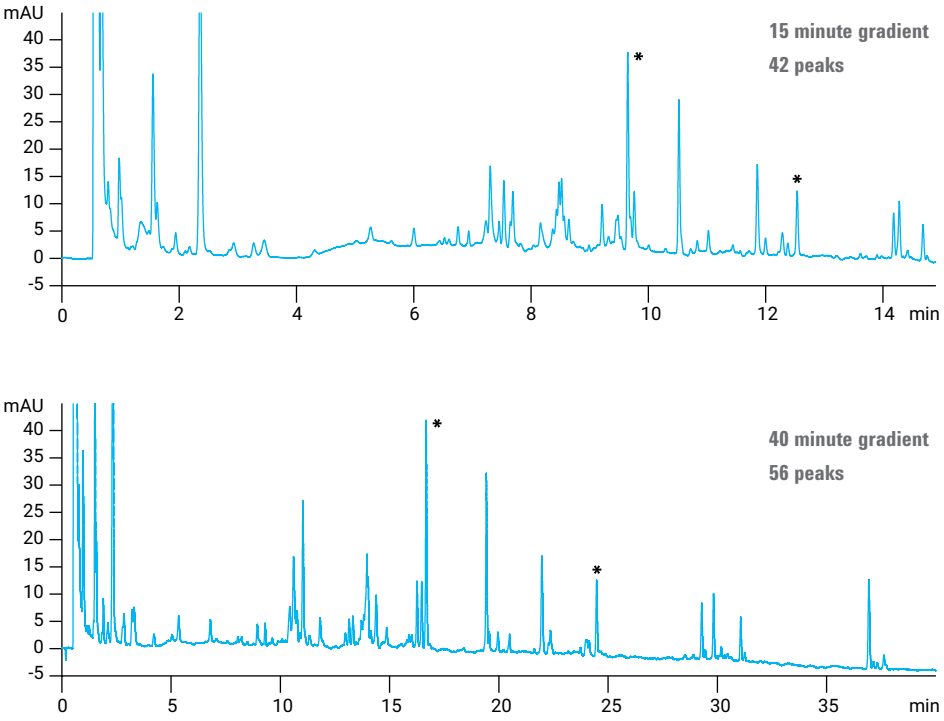

Figure 9. Myoglobin tryptic digest gradient separations with a 2.1 x 150 mm AdvanceBio Peptide Mapping column (Agilent P/N 653950-302) on an Agilent 1260 Infinity Bio-inert Quaternary LC system using water (1.0 %TFA)/ACN (0.08% TFA) linear gradient, 0.6 mL/min at 50 °C. The top chromatogram was completed 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 10 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.

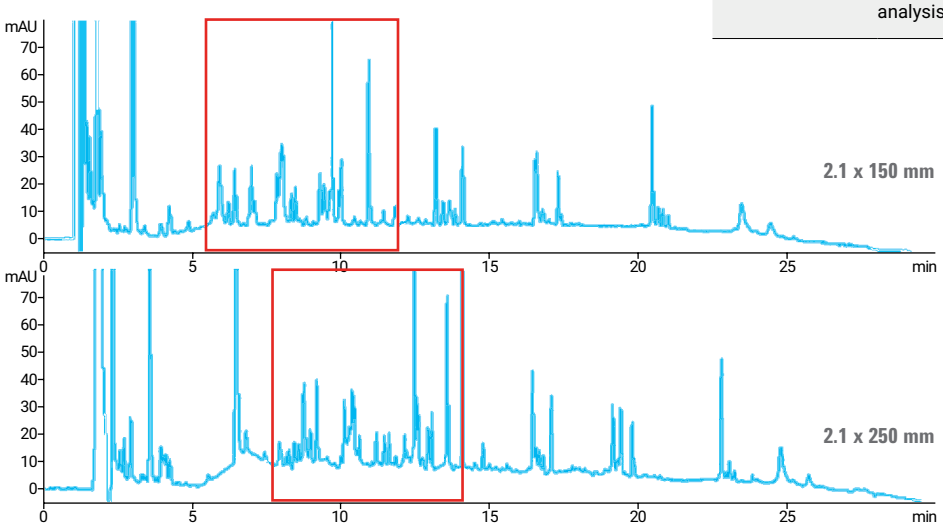

Figure 10. Effect of column length on resolution, a peptide mapping comparison using a myoglobin tryptic digest (Agilent P/N 651750-902). Areas highlighted in red indicate equivalent areas of separation to emphasize resolution and peak shape. The separations were performed using an Agilent AdvanceBio 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 11 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.

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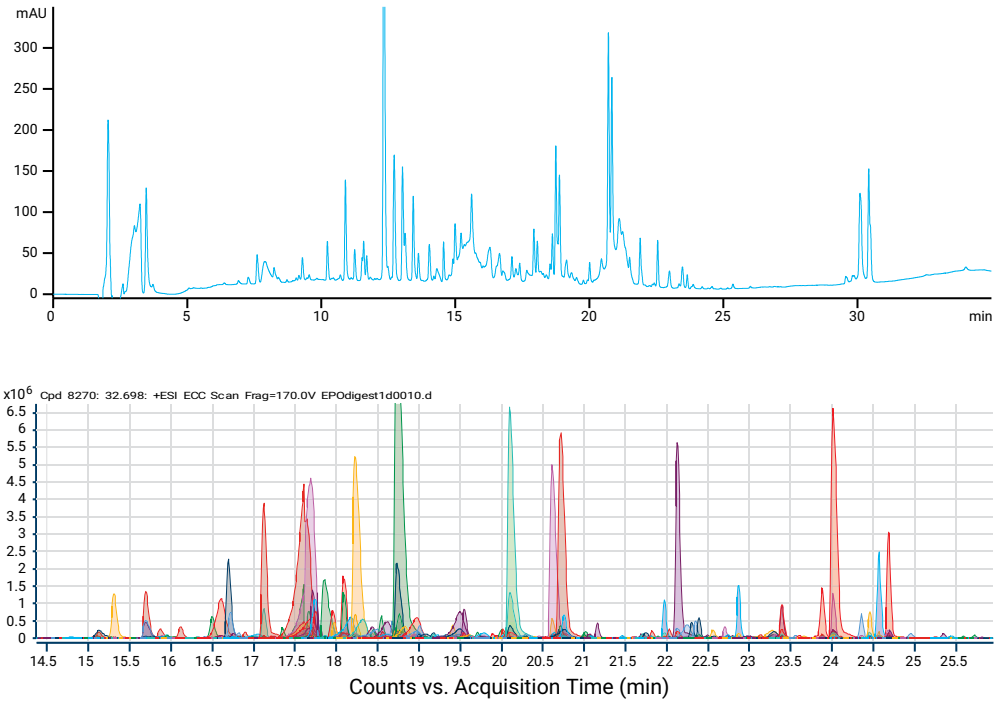

Figure 11. The top chromatogram displays a fully optimized EPO digest peptide mapping separation performed on a 2.1 x 150 mm AdvanceBio Peptide Mapping column. The bottom chromatogram shows the qualitative analysis (using a molecular feature extractor) for sequence coverage generated by on an Agilent 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
4.6 x 150 mm, 2.7 µm	653950-902	850750-911
3.0 x 150 mm, 2.7 µm	653950-302	853750-911
2.1 x 250 mm, 2.7 µm	651750-902	851725-911
2.1 x 150 mm, 2.7 µm	653750-902	
2.1 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	671950-902
3.0 x 150 mm, 2.7 µm	671950-302
2.1 x 250 mm, 2.7 µm	677950-902
2.1 x 150 mm, 2.7 µm	675950-902
2.1 x 50 mm, 2.7 µm	673750-902
4.6 mm guard (3/pk)	820750-932
3.0 mm guard (3/pk)	823750-947
2.1 mm guard (3/pk)	821725-946
HSA Peptide Standard Mix	G2455-85001

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Peptide sample preparation for mass spec analysis, intelligently automated

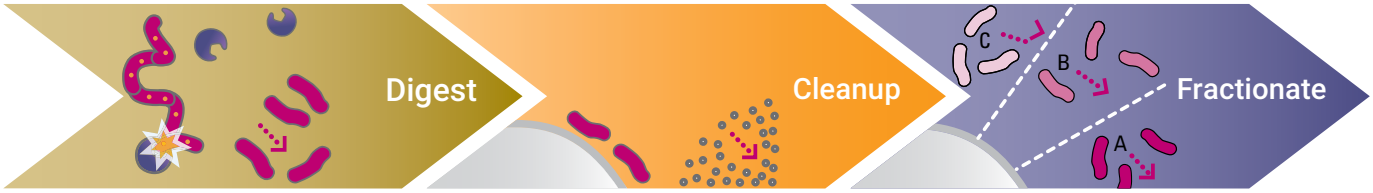
Manual sample preparation of peptides is a time-consuming process. If you are doing peptide mapping applications on MS, you are likely looking for increased throughput. And you are going to be reliant on a highly reproducible end-to-end workflow to ensure your results are consistent. AssayMAP transforms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

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 12.

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.

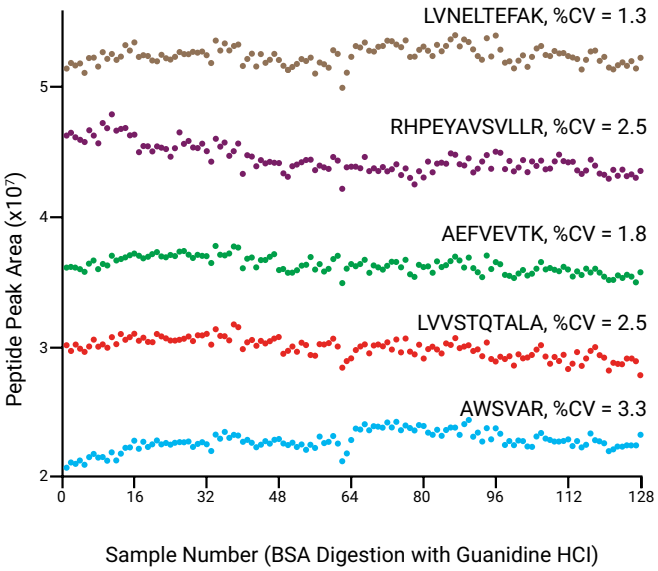


Figure 12. Scatter plots showing peak area of 4 peptides over 2 days.

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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With a wealth of biocolumns and small columns available, Agilent has introduced the LC columns and Sample Prep NAVIGATOR to help you choose the right column for your application.

The NAVIGATOR presents four easy search options:

- By part number – cross-reference for LC columns and sample prep products to find the best Agilent replacement
- By column – recommendations based on method
- By compound – drop down list
- By USP method

In addition, the tool offers column support to optimize chromatography, sample prep product recommendations, and quick access to technical support resources and other tools.

Application Note

Peptide Mapping

Agilent

Trusted Answers

Reproducible LC/MS Peptide Separation Using Agilent AdvanceBio Peptide Plus Columns

Authors

Suresh Babu C.V.

Agilent Technologies Inc.

Introduction

Peptide separation is of paramount importance during the characterization of biopharmaceuticals. Peptide mapping serves as a primary quality control (QC) step in pharmaceutical development. It involves enzymatic digestion to produce peptide fragments, followed by reversed-phase separation and identification by mass spectrometry. Due to the inherent complexity of proteolytic digests of protein mixtures, high-efficiency and high-resolution peptide separation is very important. The quality attributes of peptide mapping such as retention time and peak area/height are critical for reliable peptide maps. Hence, rigorous characterization of peptide fragments requires a robust and reproducible LC/MS method for confident evaluation of the results.

This study demonstrates high-efficiency peptide separation using the MS-compatible Agilent AdvanceBio Peptide Plus column with the mobile phase containing formic acid modifier. Different sample types were screened to evaluate the column performance for peptide separation. The results showed excellent retention time, peak area, peak height, and full width half maximum (FWHM) reproducibility.

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Materials and Methods

Samples

HSA Peptides Standard (G2455-85001)

10 Peptide Standard (5190-0583)

mAb tryptic digest

LC system

Agilent 1290 Infinity II LC

MS system

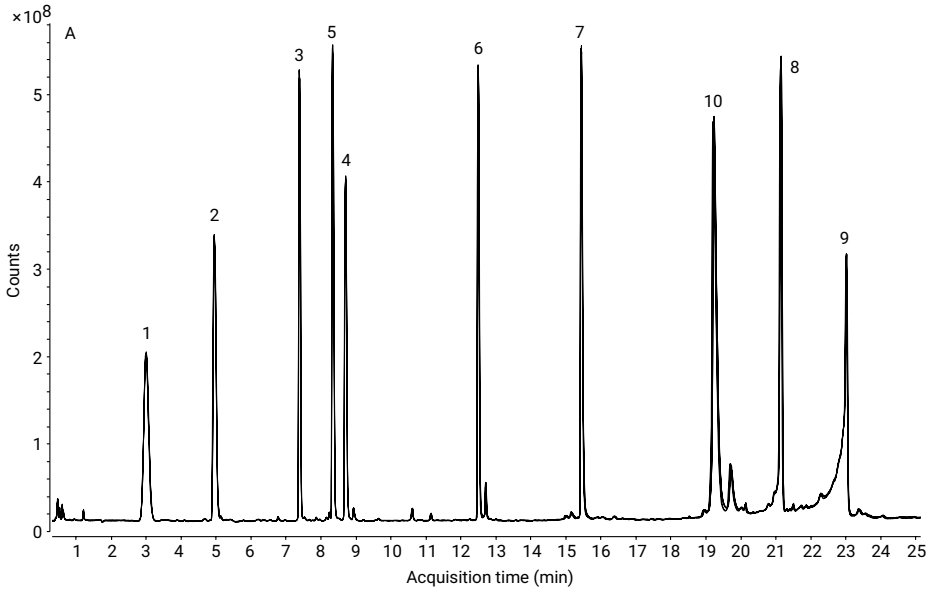
Agilent 6545XT AdvanceBio LC/Q-TOF

Conditions

Parameter	Value
Column	Agilent AdvanceBio Peptide Plus 2.1 × 150 mm, 2.7 µm, 120 Å (675950-902)
Mobile phase	A) 0.1 % FA in water B) 0.1 % FA in ACN
Column temperature	55 °C
Gradient	3–40 %B in 30 minutes
Flow rate	0.5 mL/min
Ion mode	Positive ion mode, dual AJS ESI
Drying gas temperature	325 °C
Drying gas flow	13 L/min
Sheath gas temperature	275 °C
Sheath gas flow	12 L/min
Nebulizer	35 psi
Capillary voltage	4,000 V
Fragmentor voltage	175 V
Skimmer voltage	65 V
Oct RF Vpp	750 V
Data analysis	Agilent BioConfirm software B.08

Results and Discussion

Figures 1A and 1B show LC/MS separations for two different peptide standard mixtures. The selected peptide sequences are composed of varying amino acid residues, length, and mass, and, thus, are useful to evaluate column performance. The results show the highly efficient, baseline separation of the peptide standards. The column provided excellent TIC peak shapes with <0.16 minutes FWHM. The narrow TIC peak width was obtained using formic acid as a mobile phase modifier. The RSD values demonstrate the robustness of the LC/MS method.

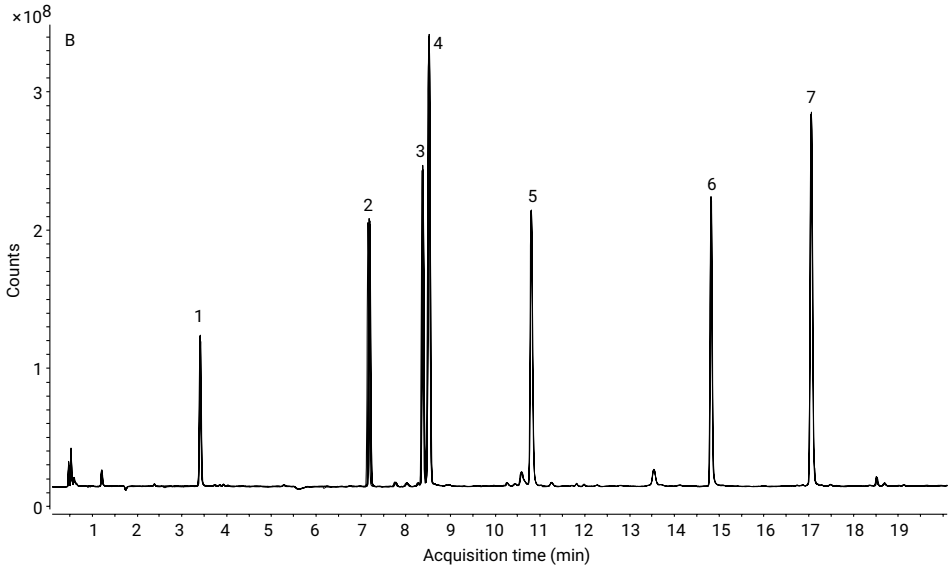


Peak ID

1. Bradykinin frag 1–7
2. Bradykinin
3. Angiotensin II (human)
4. Neurotensin
5. Angiotensin I (human)
6. Renin substrate porcine
7. [Ace-F-3,-2H-1] Angiotensinogen (1–14)
8. Ser/Thr Protein Phosphatase (15–31)
9. [F14] Ser/Thr Protein Phosphatase (15–31)
10. Melittin (honey bee venom)

% RSD

	RT	Area	Height	FWHM
Peak 1	0.17	2.03	1.65	0.44
Peak 2	0.10	1.08	0.96	0.48
Peak 3	0.07	0.85	0.66	0.00
Peak 4	0.05	0.77	0.47	0.65
Peak 5	0.06	0.82	0.77	0.00
Peak 6	0.04	0.42	0.49	0.55
Peak 7	0.05	0.50	0.80	0.50
Peak 8	0.03	1.49	0.73	0.63
Peak 9	0.02	2.54	1.02	1.25
Peak 10	0.08	0.52	1.06	1.26



Peak ID

1. AAFTE[CAM][CAM]QAADK
2. YLYEIAR
3. LVNEVTEFAK
4. KVPQVSTPTLVEVSR
5. RP[CAM]FSALEVDETYVPK
6. AVMDDFAAFVEK
7. HPYFYAPELLFFAK

% RSD

	RT	Area	Height	FWHM
Peak 1	0.001	0.014	0.021	0.011
Peak 2	0.002	0.011	0.011	0.010
Peak 3	0.001	0.009	0.008	0.012
Peak 4	0.001	0.013	0.018	0.014
Peak 5	0.001	0.013	0.005	0.013
Peak 6	0.000	0.008	0.006	0.009
Peak 7	0.001	0.005	0.005	0.009

Figure 1. Overlaid total ion chromatograms of peptide standard mixture (five replicate injections) using an Agilent AdvanceBio Peptide Plus column. A) Agilent 10 Peptide Standard (p/n 5190-0583). B) Agilent HSA Peptides Standard (p/n G2455-85001).

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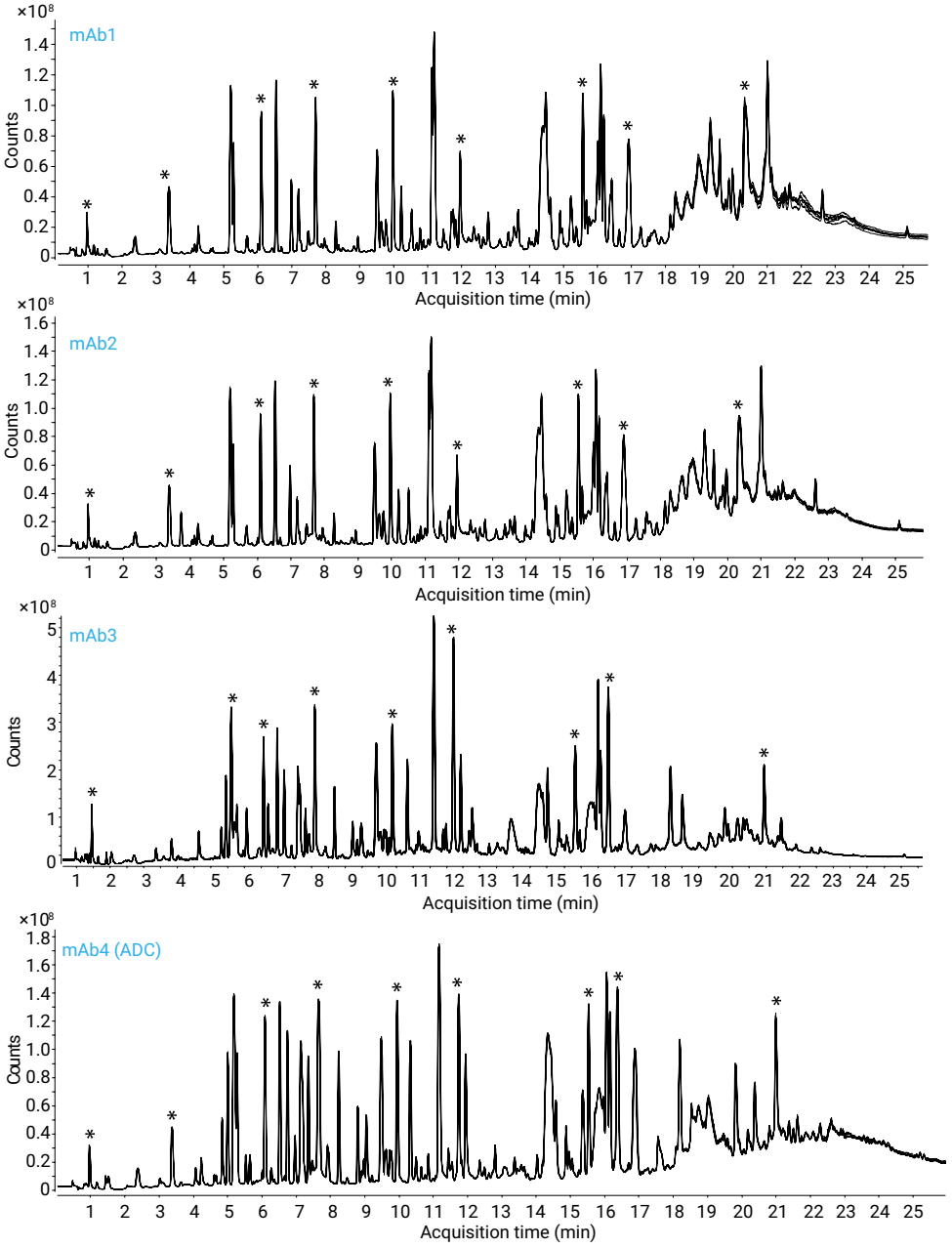

% RSD

	RT	Area	Height	FWHM
Peak 1	0.72	4.55	3.23	2.52
Peak 2	0.08	2.13	0.61	0.60
Peak 3	0.06	5.64	1.51	0.92
Peak 4	0.06	6.35	1.96	0.88
Peak 5	0.04	1.37	0.57	1.07
Peak 6	0.03	8.80	2.18	2.40
Peak 7	0.02	4.15	1.67	0.97
Peak 8	0.04	1.14	0.73	0.50
Peak 9	0.09	8.77	7.90	6.10

% RSD

	RT	Area	Height	FWHM
Peak 1	0.36	0.44	2.40	3.30
Peak 2	0.14	2.61	0.37	0.75
Peak 3	0.07	3.00	1.97	0.92
Peak 4	0.11	1.20	0.87	0.86
Peak 5	0.07	2.76	0.52	0.86
Peak 6	0.04	3.31	0.82	1.70
Peak 7	0.05	3.52	1.09	0.79
Peak 8	0.03	1.05	0.97	0.93
Peak 9	0.04	9.92	2.37	3.21

% RSD

	RT	Area	Height	FWHM
Peak 1	0.11	0.39	0.51	1.24
Peak 2	0.02	5.87	2.04	1.71
Peak 3	0.07	4.06	1.61	1.39
Peak 4	0.06	4.13	1.19	0.78
Peak 5	0.06	3.57	1.84	0.85
Peak 6	0.04	2.85	0.87	0.66
Peak 7	0.04	6.92	3.77	4.33
Peak 8	0.02	2.62	1.03	0.53
Peak 9	0.01	3.11	1.30	0.95

% RSD

	RT	Area	Height	FWHM
Peak 1	0.22	4.48	4.42	3.82
Peak 2	0.09	1.86	1.17	0.79
Peak 3	0.04	7.63	2.42	2.02
Peak 4	0.13	4.78	2.03	2.04
Peak 5	0.07	0.81	0.29	0.00
Peak 6	0.01	4.68	1.91	0.87
Peak 7	0.03	7.06	3.65	1.53
Peak 8	0.04	3.71	3.48	1.79
Peak 9	0.03	6.31	7.21	4.43

Figure 2. Overlaid total ion chromatograms of mAb tryptic peptide map (five replicate injections) using an Agilent AdvanceBio Peptide Plus column.

Figure 2 shows the LC/MS peptide map separation of four different mAbs. Various mAb preparations were chosen to study the column performance as well as LC/MS method robustness. The result shows excellent peptide separation for different sets of peptide maps with improved peak shapes. There is no observable shift in the chromatographic profile between the replicate injections. The RSD values illustrate that the column and LC/MS method are robust.

Conclusions

This study demonstrates:

- High efficiency LC/MS peptide separation using formic acid as a mobile phase modifier with an Agilent AdvanceBio Peptide Plus column
- Excellent reproducibility of retention time, peak area, peak height, and peak width

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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

Suresh Babu C.V.

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.

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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.

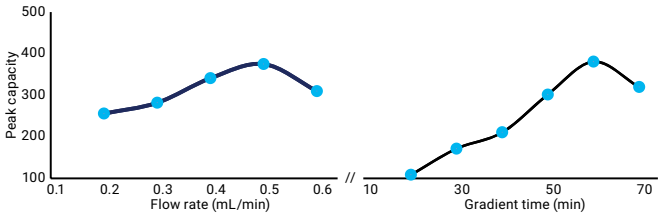

Figure 1. Effect of flow rate and gradient time on peak capacity.

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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

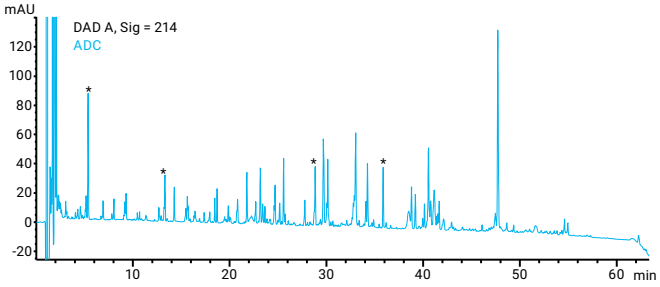

Figure 2. Peptide map of tryptic-digested ADC separated on an Agilent AdvancedBio Peptide Mapping column (*peaks selected for RSD calculations).

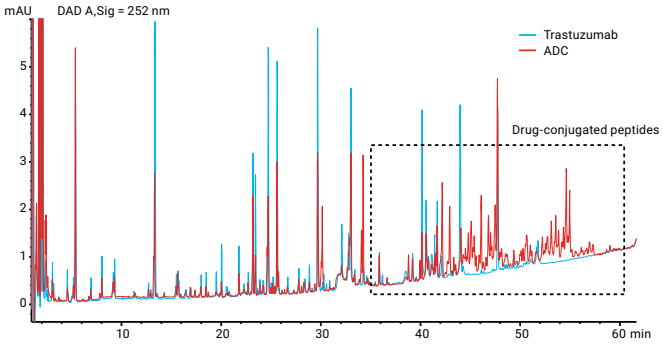

Figure 3. Overlay of peptide map of tryptic-digested ADC and Trastuzumab separated on an Agilent AdvancedBio Peptide Mapping Column.

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

Agilent

Trusted Answers

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.

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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.

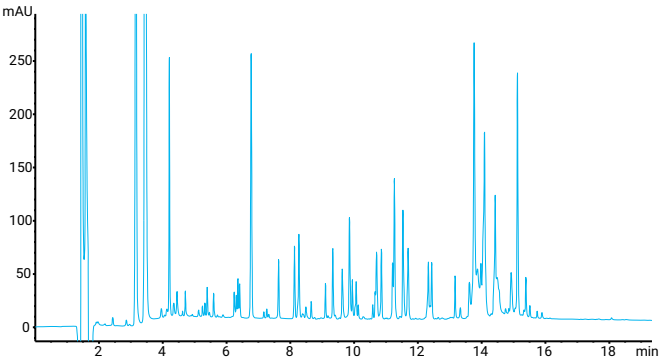

Figure 1. Analysis of a Herceptin tryptic digest, 20-minute gradient, UV detection at 214 nm.

- 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.

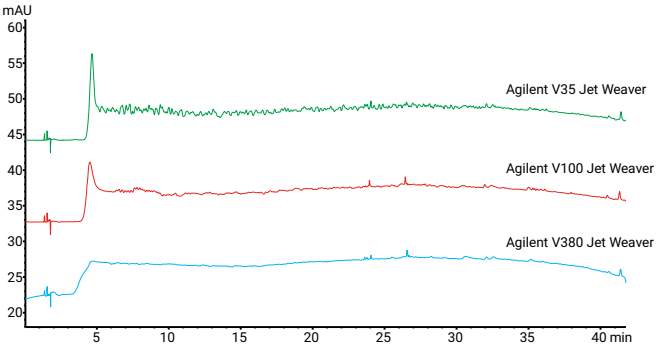

Figure 2. Analysis of a blank solution with an Agilent V35 (green), Agilent V100 (red), and Agilent V380 (blue) Jet Weaver mixer, 40-minute gradient, UV detection at 214 nm.

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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.

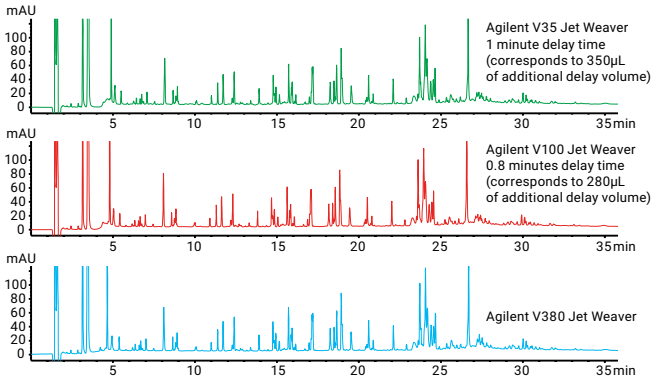

Figure 3. Analysis of Kadcyla tryptic digest with an Agilent V35 (green), Agilent V100 (red), and Agilent V380 (blue) Jet Weaver mixer, 40-minute gradient, UV detection at 214 nm.

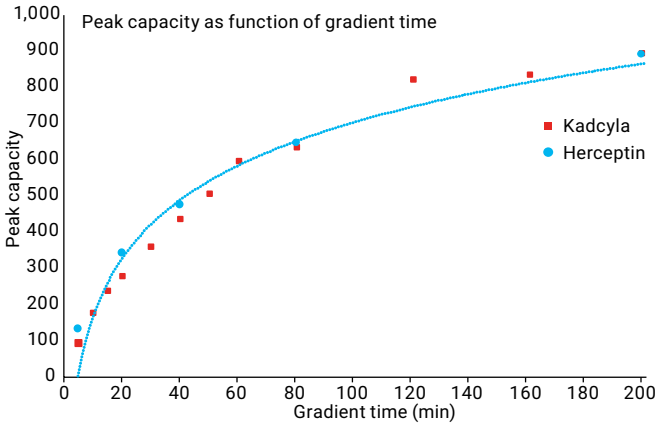

Figure 4. Peak capacity as a function of gradient time. UV detection at 214 nm (detector set at 40 Hz to ensure sufficient data points in fast analyses).

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

Gradient time (min)	Peak capacity	
	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.

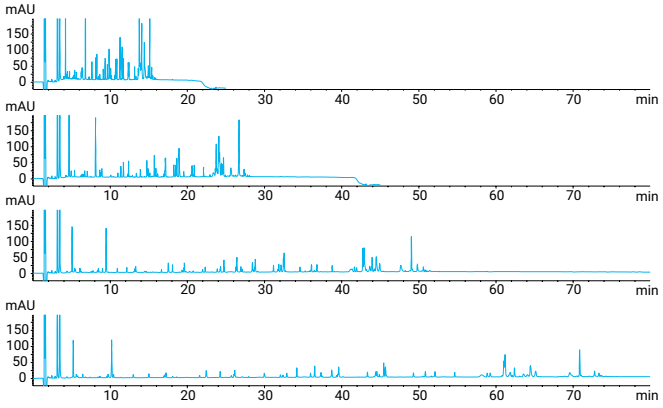

Figure 5. Analysis of a Herceptin tryptic digest with a 20, 40, 80, and 120-minute gradient, UV detection at 214 nm. Note, for comparison, that the y-scale was increased with the Δ-gradient factor.

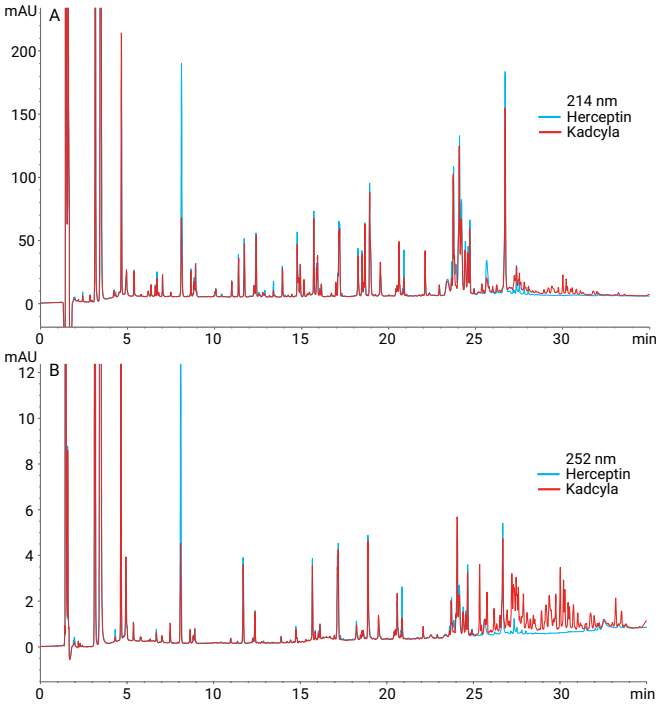

Figure 6. Comparison of analysis of a Herceptin (blue) and Kadcyla (red) tryptic digest with a 40-minute gradient, UV detection 214 nm and 252 nm.

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.

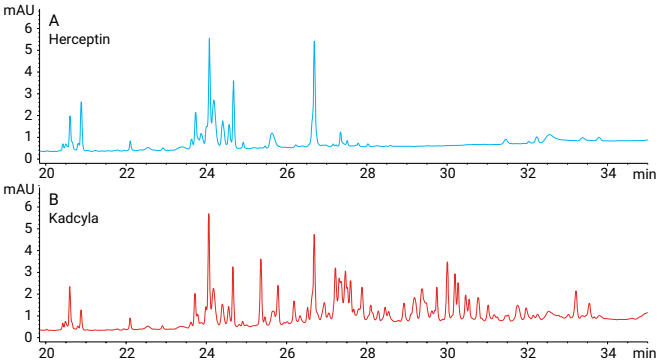

Figure 7. Detail of the comparison of an analysis of a Herceptin and Kadcyla tryptic digest with a 40-minute gradient, UV detection at 252 nm.

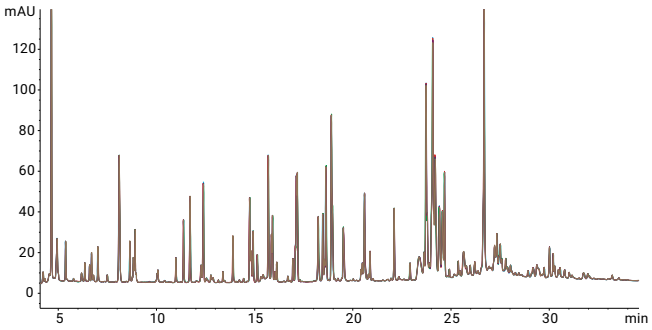

Figure 8. Overlay of five replicate injections of a Kadcyla tryptic digest with a 40-minute gradient, UV detection at 214 nm.

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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

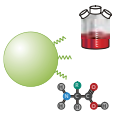
Amino Acid Analysis

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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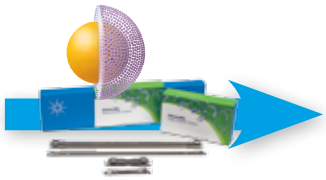
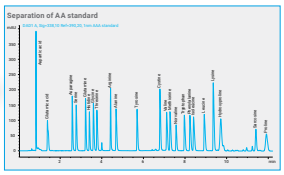
Expert applications and technical support

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:

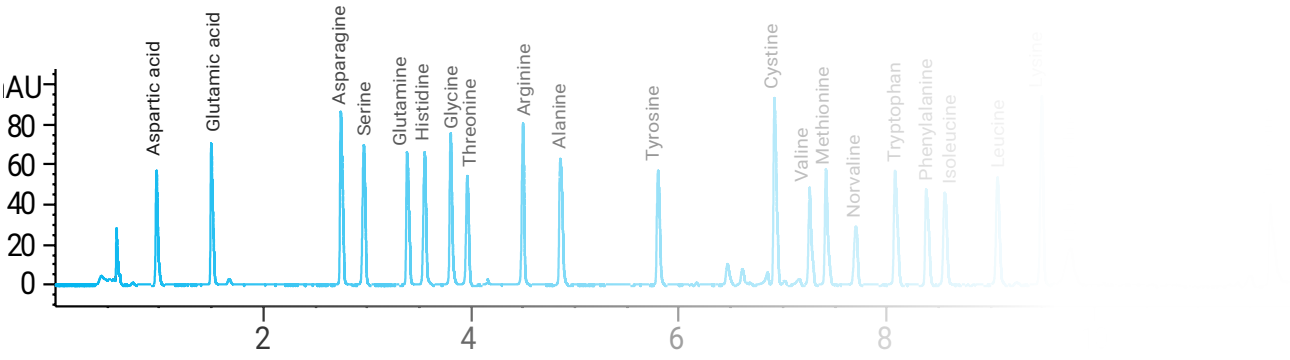
Uncompromised sensitivity for simultaneous multiwavelength detection.

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

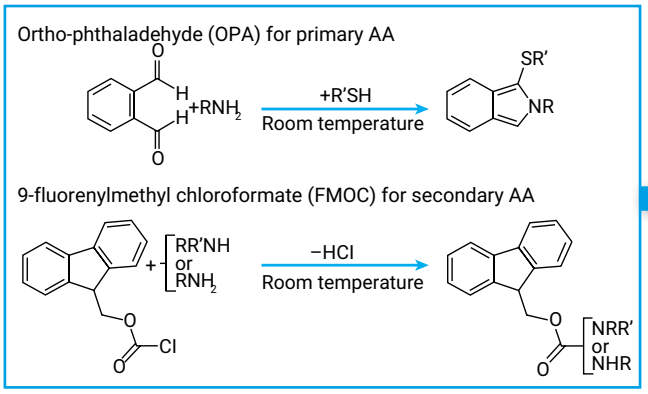
Note: Use the correct vials, closures, and pumps parameters

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



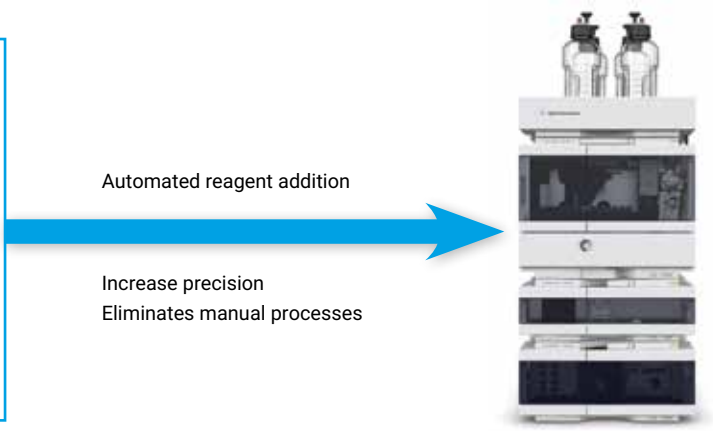


Figure 2. Online derivatization of OPA and FMOC: Separation of polar amino acids on RP-phase and detection by UV and Fluorescence

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.

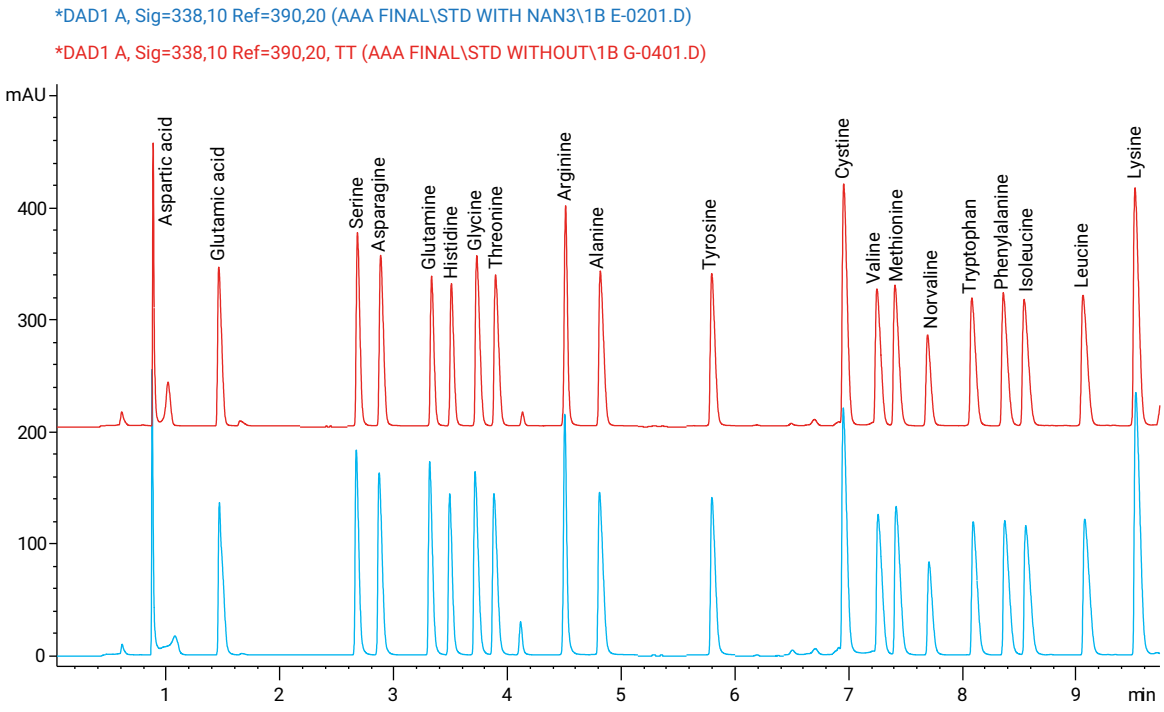


Figure 3. Separation of 20 amino acid standards using an Agilent AdvanceBio AAA 4.6 x 100 mm column with and without 5 mM sodium azide in mobile phase.

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.

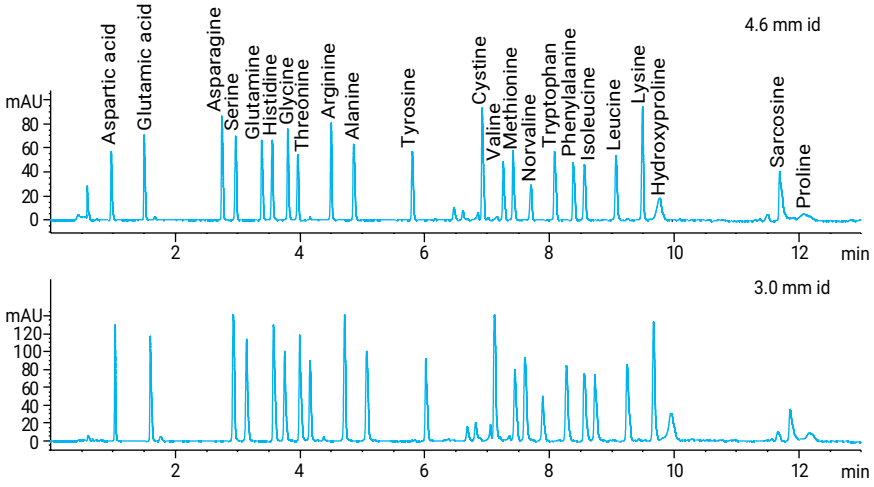


Figure 4. Separation of AA standards using Agilent AdvanceBio AAA columns of different internal diameters using the amino acid method.

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. Cysteine	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).

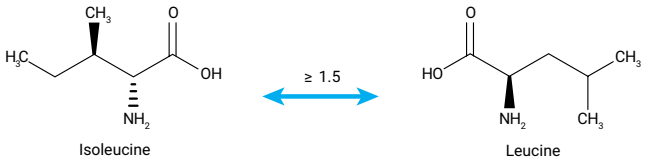
Amino Acids	Average RT	RT RSD (%)	Area RSD (%)
1. Aspartic acid	0.837	0.151	2.60
2. Glutamic acid	1.400	0.512	2.19
3. Asparagine	2.583	0.124	2.13
4. Serine	2.772	0.114	1.74
5. Glutamine	3.220	0.092	1.80
6. Histidine	3.405	0.077	1.39
7. Glycine	3.598	0.068	1.48
8. Threonine	3.766	0.059	2.26
9. Arginine	4.422	0.027	1.66
10. Alanine	4.685	0.031	1.87
11. Tyrosine	5.695	0.034	2.04
12. Cysteine	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]



Isoleucine Leucine

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*

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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.

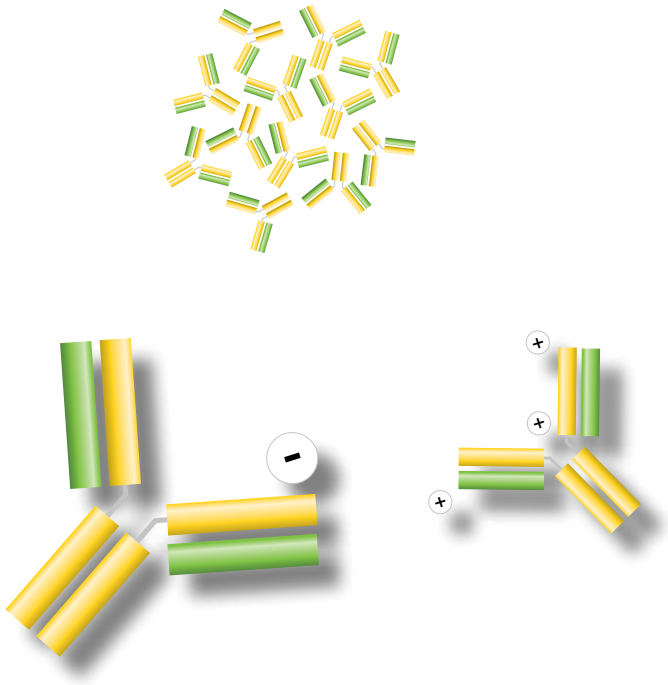
Amino acid compositional analyses of commonly used cell culture media and protein hydrolysate are shown in Figures 5-8. This analysis confirms that the amino acid composition of cell culture media accurately matches with their theoretical composition. Such applications are useful for monitoring and adjusting 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.



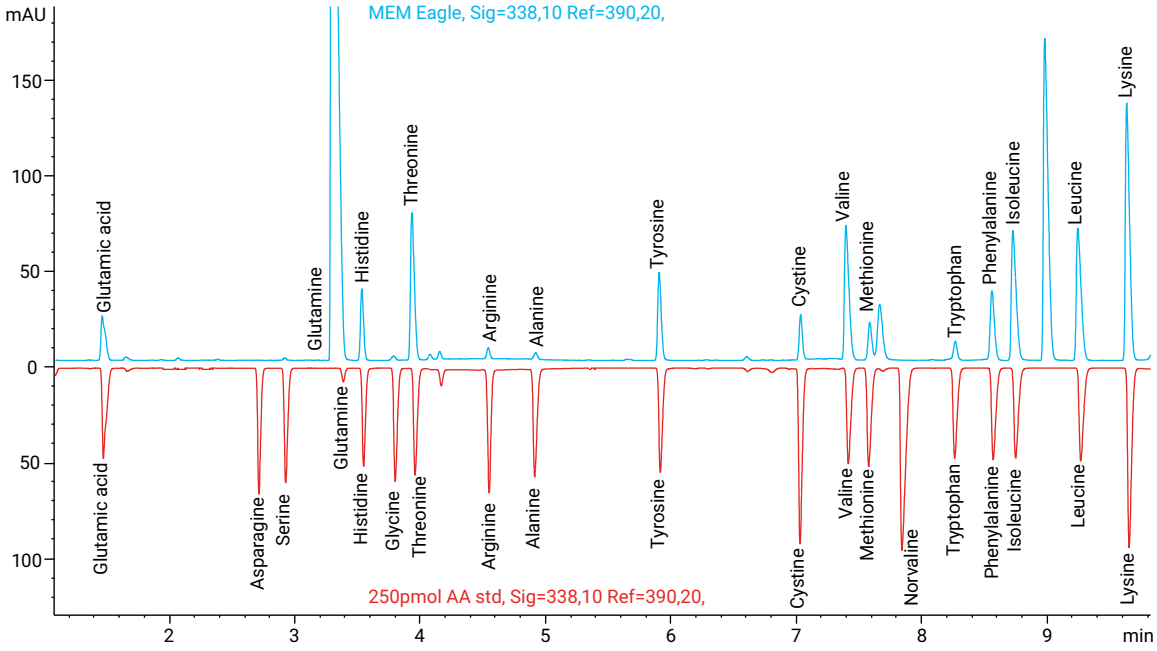


Figure 5. Amino acid analysis of Eagles MEM media (blue trace) and comparison with amino acid standards using the Agilent AdvanceBio AAA solution.

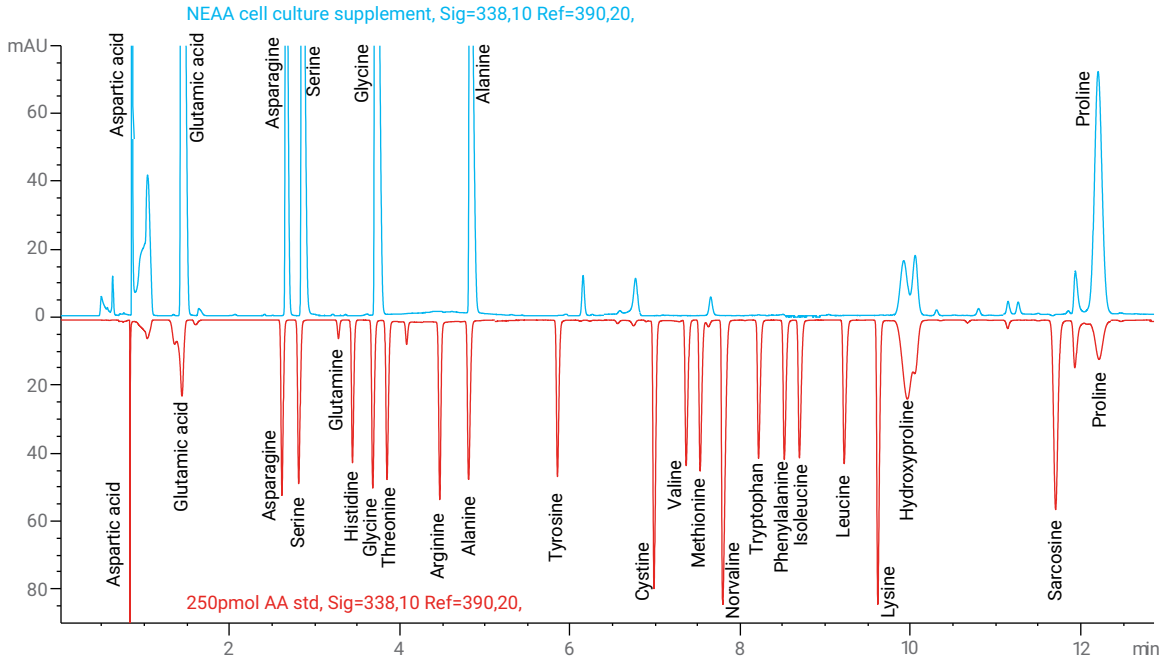


Figure 6. Amino acid analysis of Non-Essential Amino Acid (NEAA) media (blue trace) and comparison with amino acid standard using the Agilent AdvanceBio AAA solution.

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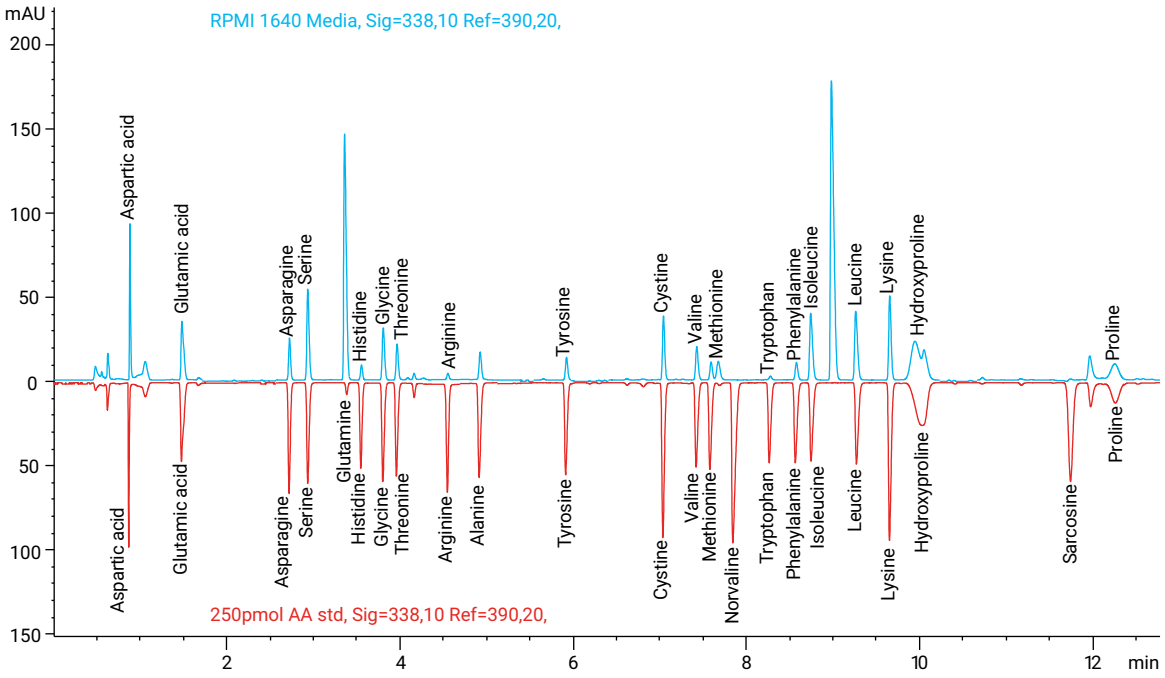

Figure 7. Amino acid analysis of RPMI 1650 media (blue trace) and comparison with amino acid standard using the Agilent AdvanceBio AAA solution.

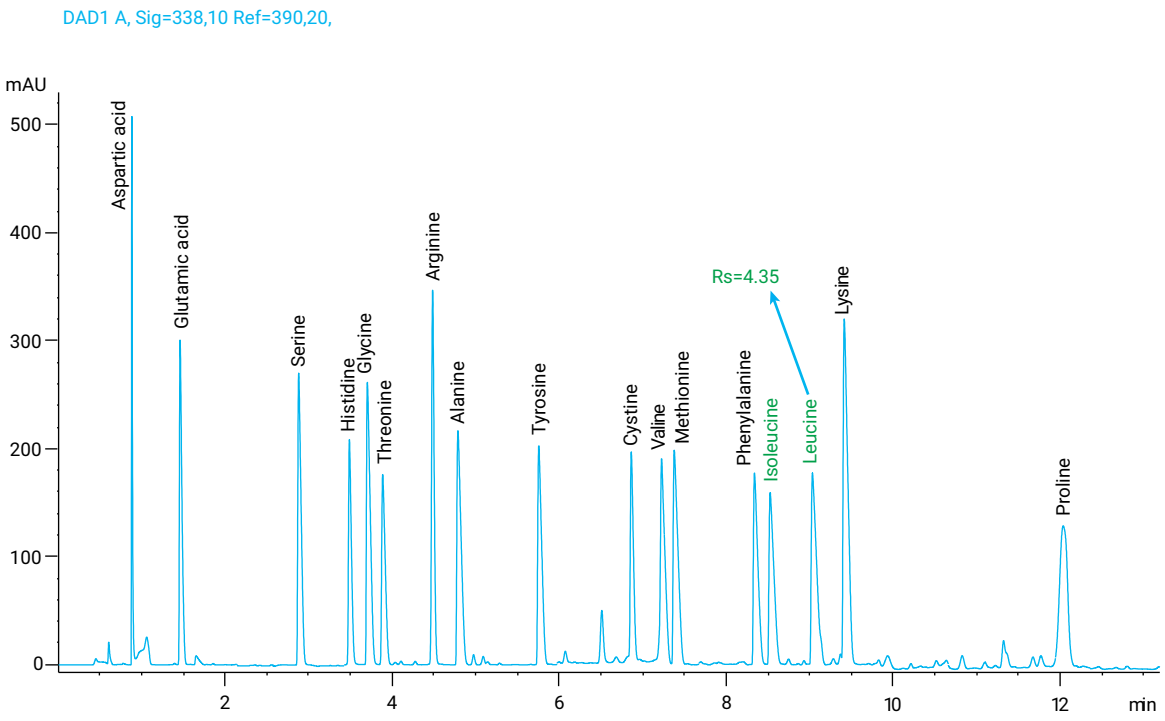

Figure 8. Amino acid analysis of protein hydrolysate. The resolution between leucine and Isoleucine with the AdvanceBio AAA, 4.6 x 100 mm, 2.7 µm column is much higher than the reported value for system suitability requirement.

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

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.

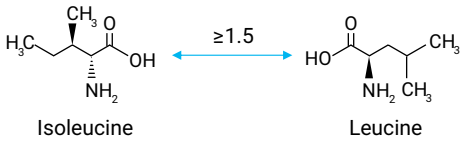

Figure 2. Isoleucine and leucine chemical relationship.

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.

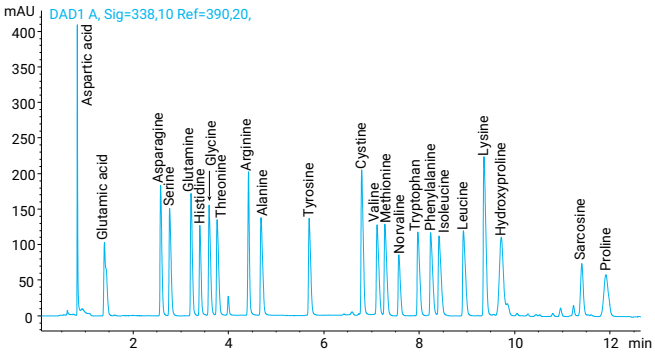

Figure 1. Separation of amino acid standard (1 nmol) on an Agilent AdvanceBio AAA 4.6 × 100 mm, 2.7 µm column, using the amino acid method.

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.

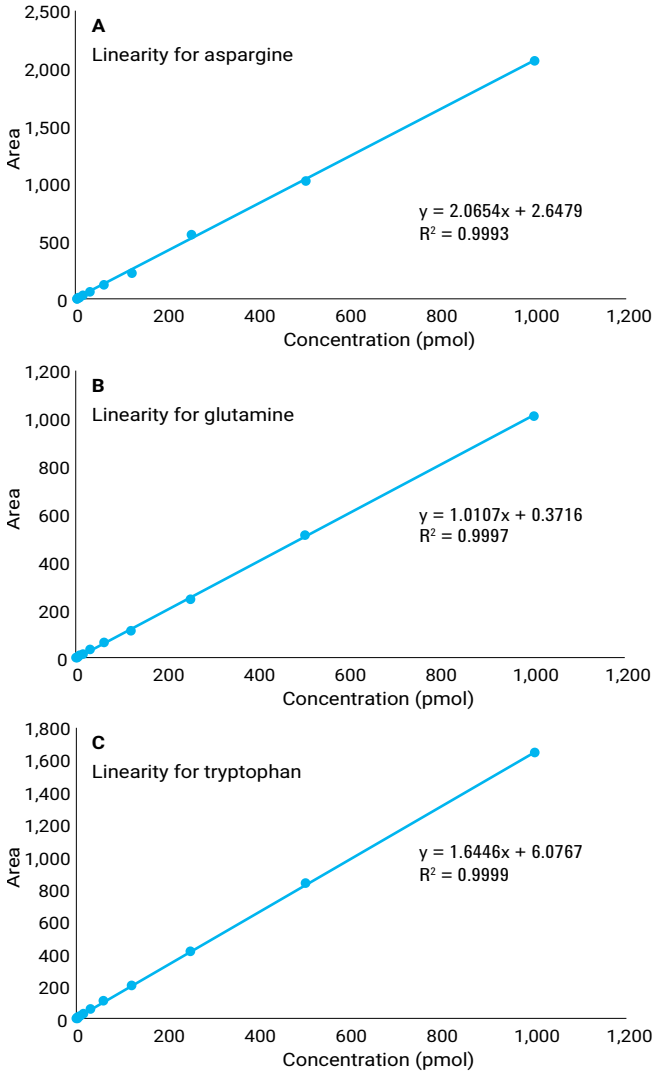

Figure 3. Linearity curve with 10 standard concentrations of asparagine, glutamine, and tryptophan ranging from 0.9 to 1,000 pmol, showing excellent coefficient values.

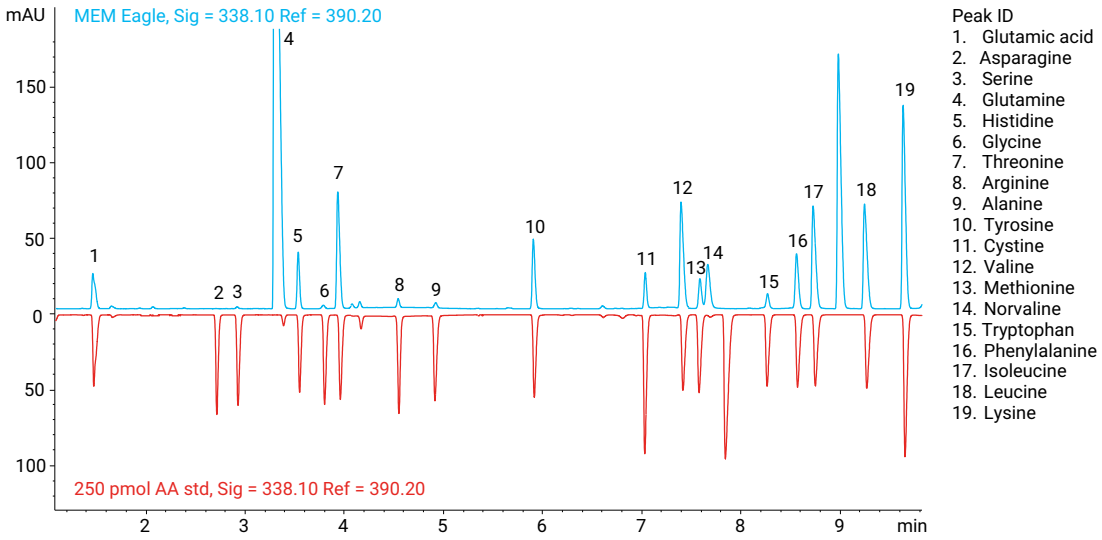

Figure 4. Amino acid analysis of MEM media (blue trace) mirrored with AA standards (red trace).

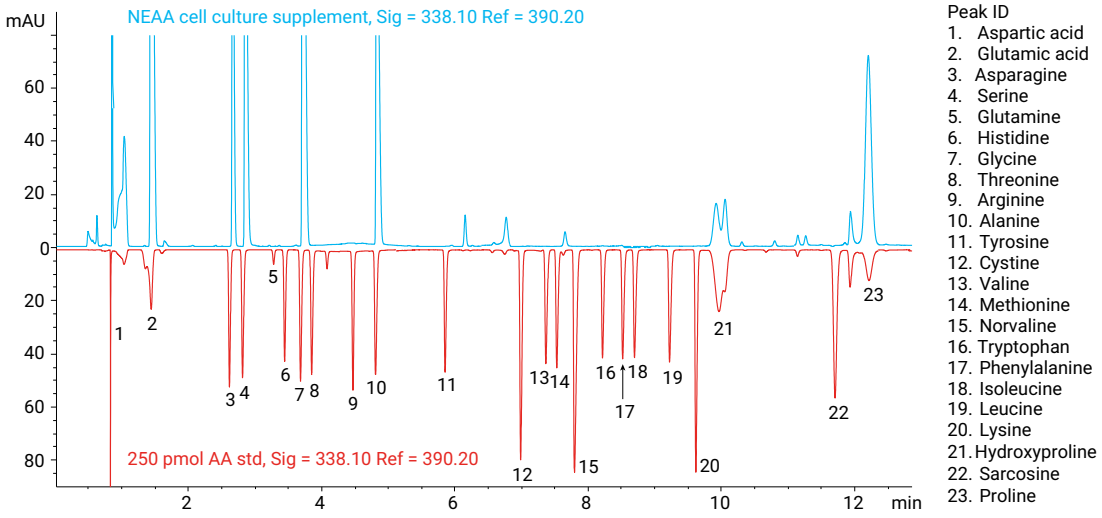

Figure 5. Amino acid analysis of NEAA media (blue trace) and comparison with AA standards (red trace).

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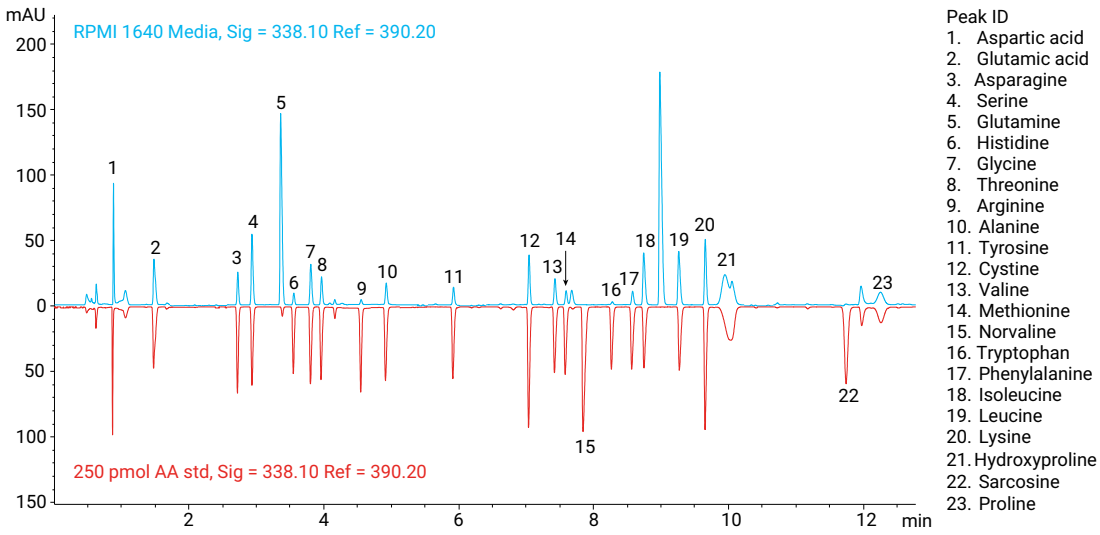

Figure 6. Amino acid analysis of RPMI 1650 media (blue trace) and comparison with AA standards (red trace).

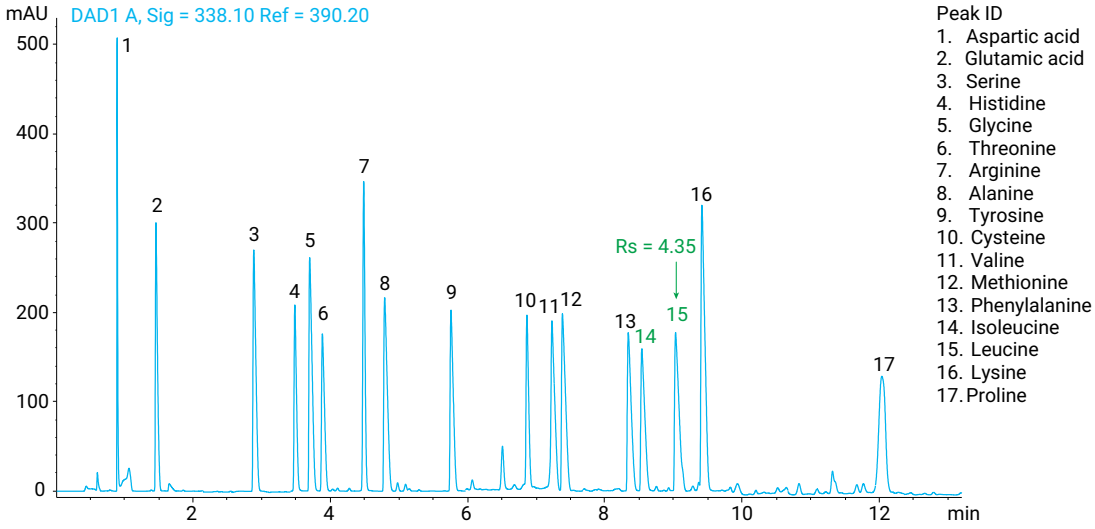

Figure 7. Amino acid analysis of protein hydrolysate standard. The resolution between leucine and isoleucine with the Agilent AdvanceBio AAA, 4.6 × 100 mm, 2.7 µm column is much higher than the reported value for system suitability requirement for this pair.

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

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

Application Note

Amino Acid Analysis

Agilent

Trusted Answers

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

Authors

William Long
Agilent Technologies, Inc

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.

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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

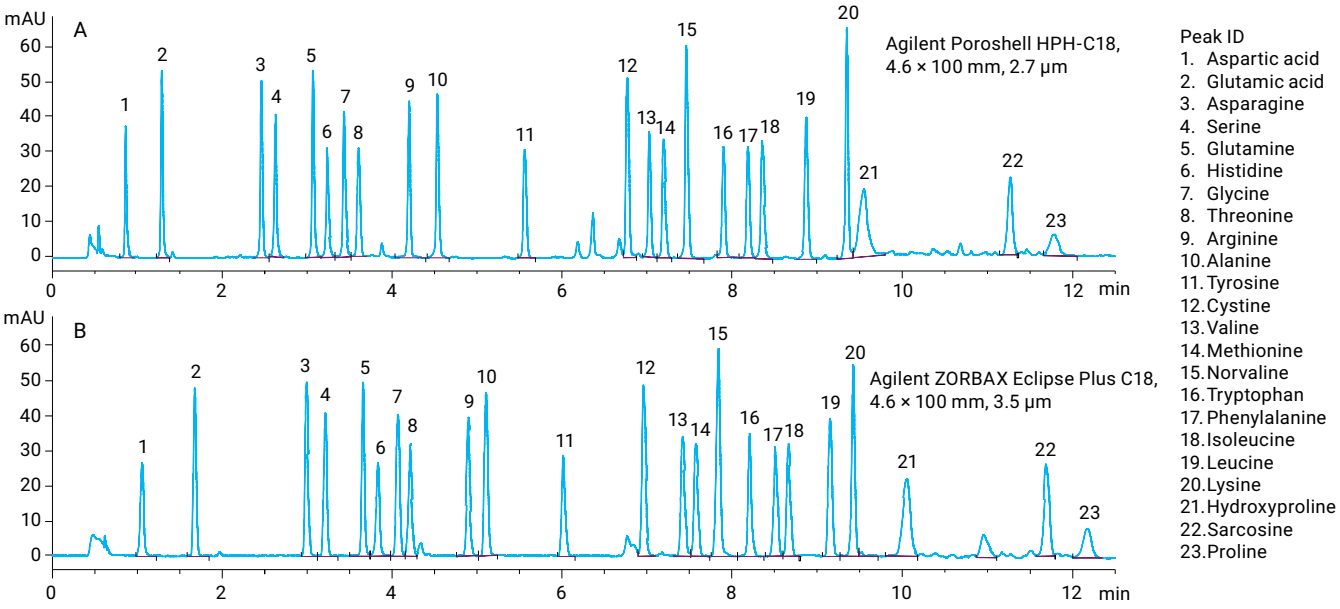

Figure 1. Comparison of an Agilent Poroshell HPH C18 to an Agilent ZORBAX Eclipse Plus C18 column using the Amino Acid Method.

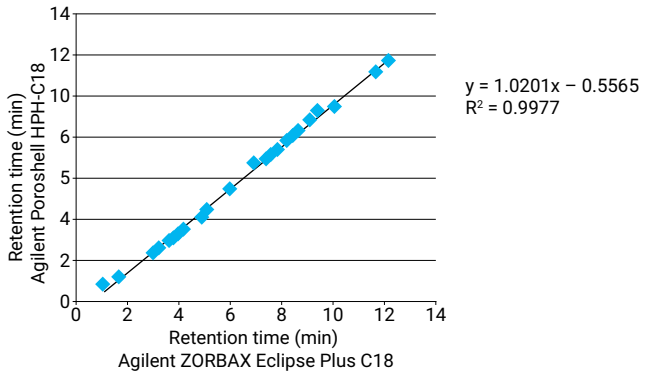

Figure 2. Correlation of retention times using Agilent Poroshell HPH-C18 and Agilent ZORBAX Eclipse Plus C18 columns.

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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).

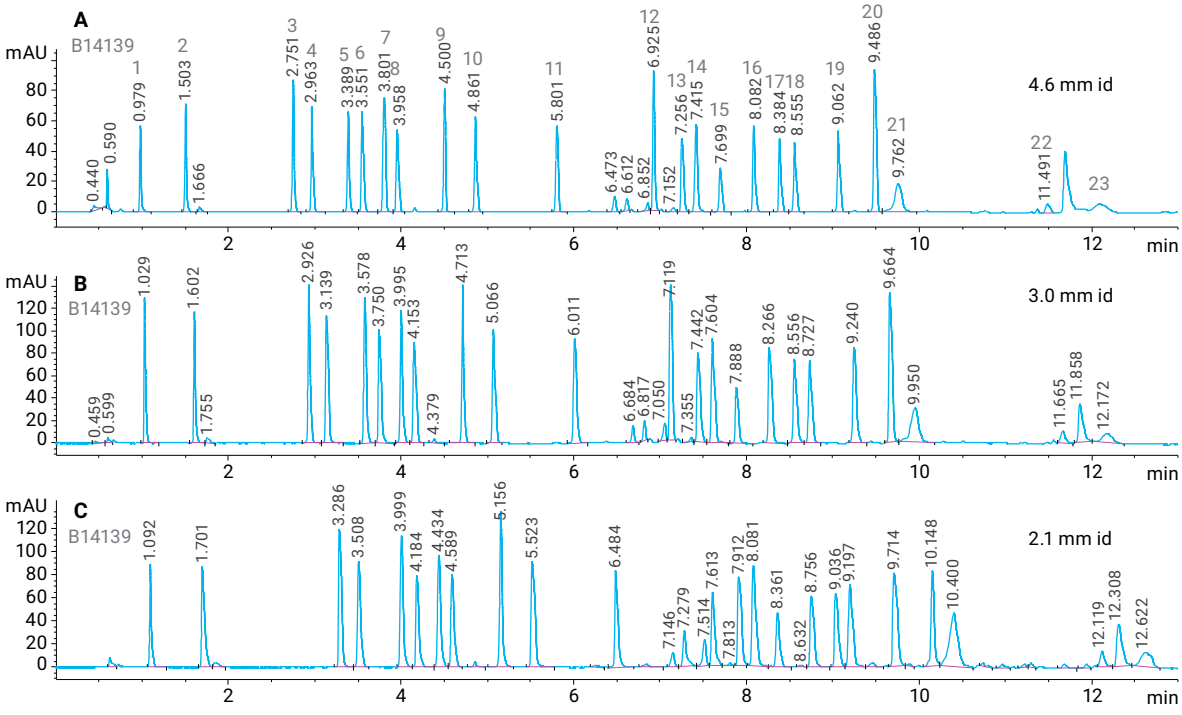

Figure 3. Agilent Poroshell HPH-C18 100 mm columns of different inside dimension using the amino acid method.

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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.

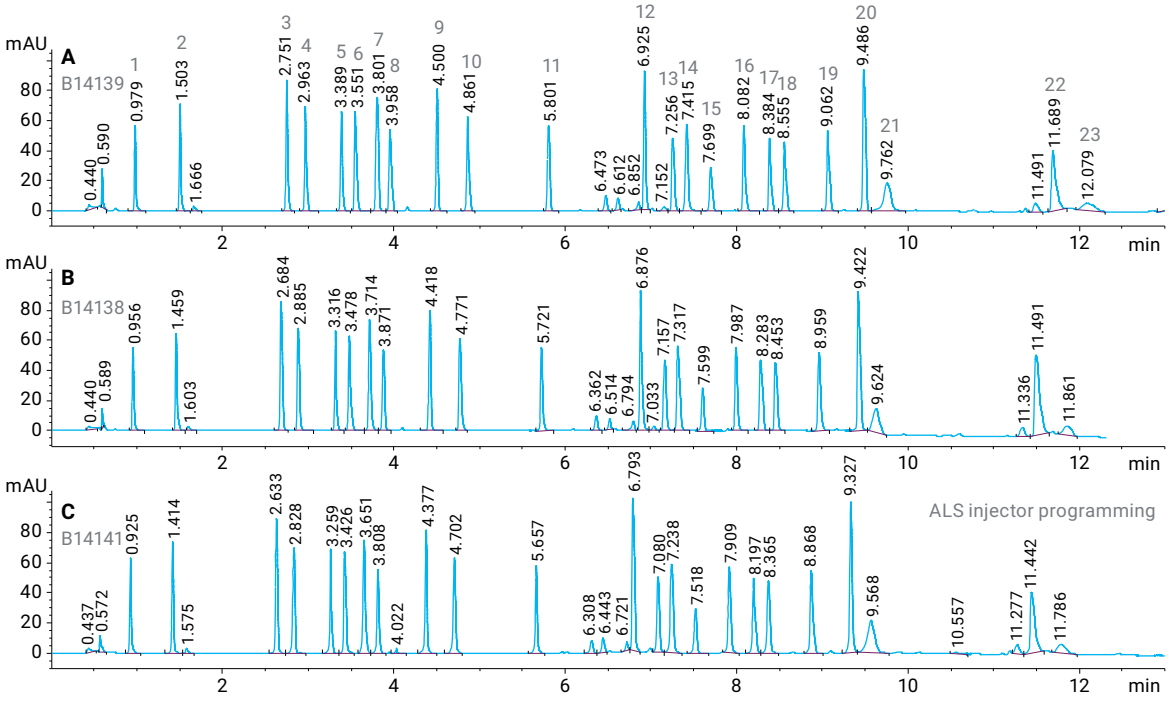

Figure 4A. Separation of amino acid and internal standards on three lots of Agilent Poroshell HPH-C18, 4.6 × 100 mm, 2.7 µm (p/n 695975-702).

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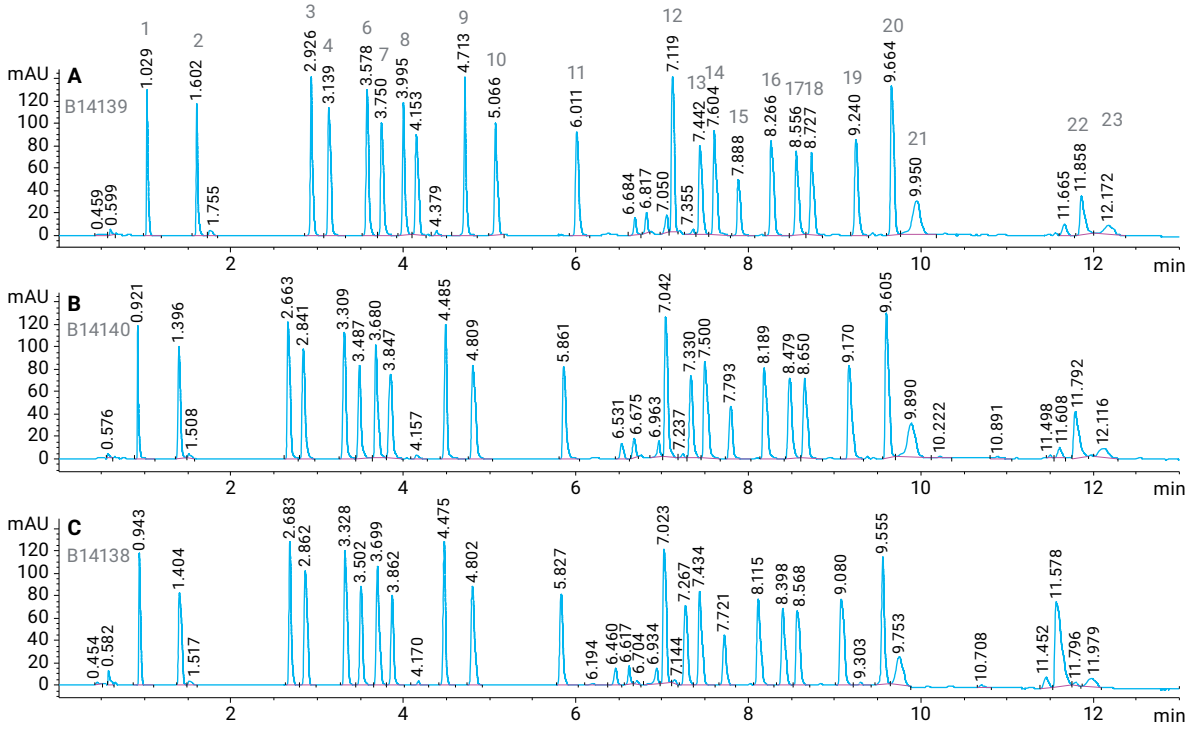

Figure 4B. Separation of amino acid and internal standards on three lots of Agilent Poroshell HPH-C18, 3 × 100 mm, 2.7 µm (p/n 695975-502).

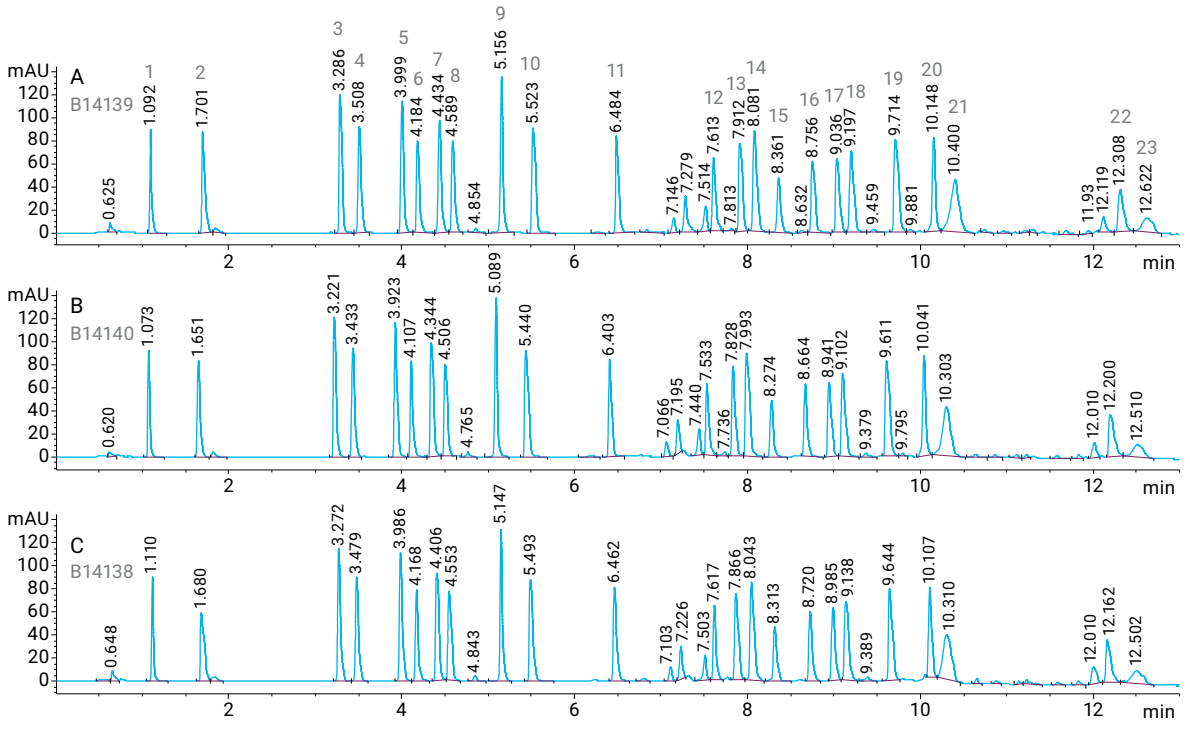

Figure 4C. Separation of amino acid and internal standards on three lots of Agilent Poroshell HPH-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-702).

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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.

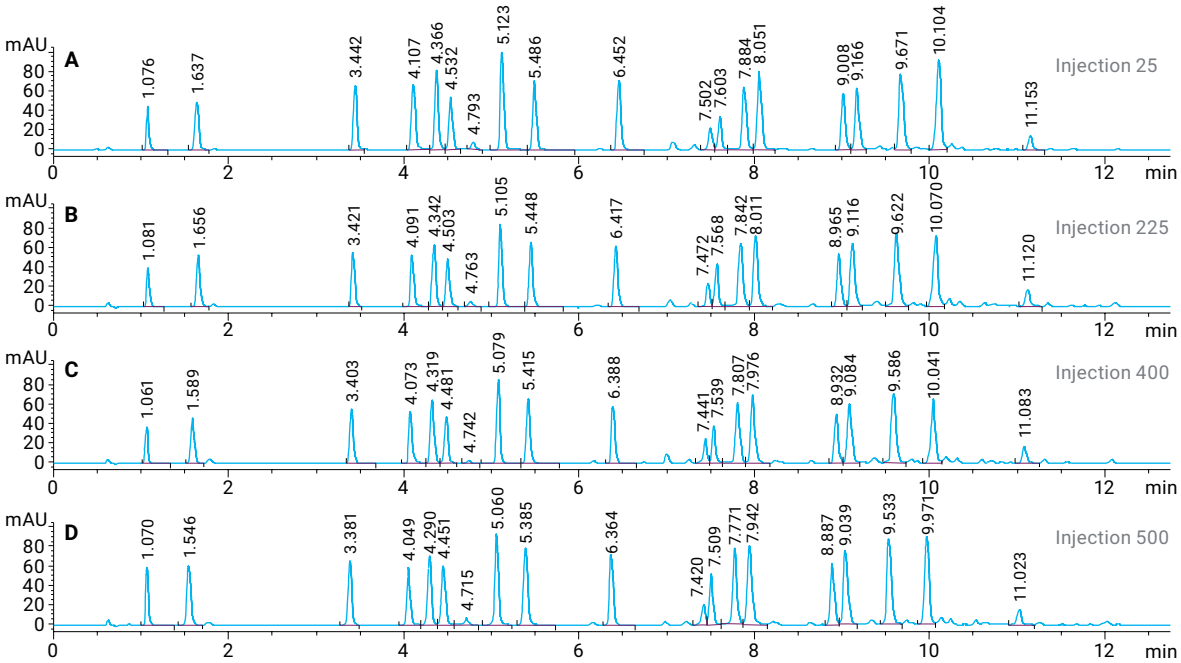

Figure 5. Column lifetime test using an Agilent Poroshell HPH-C18, 2.1 × 100 mm column running an amino acid method.

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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.

For More Information

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

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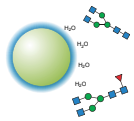
Glycan Analysis

Background

Glycosylation is an important post-translational modification as these sugar molecules play a key role in protein recognition and biotherapeutic efficacy. Human glycosylation profiles follow a typical antenna-like pattern 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 necessary 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 to increase detection sensitivity, and then separating them using HILIC chromatography columns (often in combination with a fluorescence detector, although mass spectrometry may also be used).

Agilent provides a kit containing all the components needed to manually perform the deglycosylation and labeling reaction with 2 aminobenzoic acid (2AB). 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 166.



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, uses reversed-phase type eluents with gradients starting at high organic solvent 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. It is therefore important to minimize the amount of water present in the sample matrix and to allow sufficient time for the column to re-equilibrate and stabilize at the end of each gradient. Following the protocol contained in the sample preparation kit should ensure the 2AB labeled N-glycans are ready for analysis. 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 highly optimized 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.

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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 half-life of the protein in plasma and the ability of the monoclonal antibody to trigger the immune response required for efficacy. 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 pharmaceuticals.

Agilent AdvanceBio Glycan Mapping solution provides an optimized workflow designed to deliver reproducibility in the analysis of 2-aminobenzamide (2AB) labeled glycans, for accurate identification and quantification.

Sample Preparation - AdvanceBio N-Glycan Sample Preparation Kit

The mapping of the N-linked glycan component of a glycoprotein, including monoclonal antibodies, requires the N-glycans to be enzymatically cleaved, using PNGase F, from the protein amino acid backbone. The cleaved N-glycans can be analyzed by hydrophilic interaction chromatography (HILIC) with MS detection. Alternatively, N-glycans can labeled with the fluorophore, 2-aminobenzamide (2AB), 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. The kit is composed of four components that perform the steps of N-glycan release, N-glycan purification, labeling with 2-aminobenzamide, and labeled glycan cleanup. In addition, kits 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 hydrophilic interaction chromatography (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.

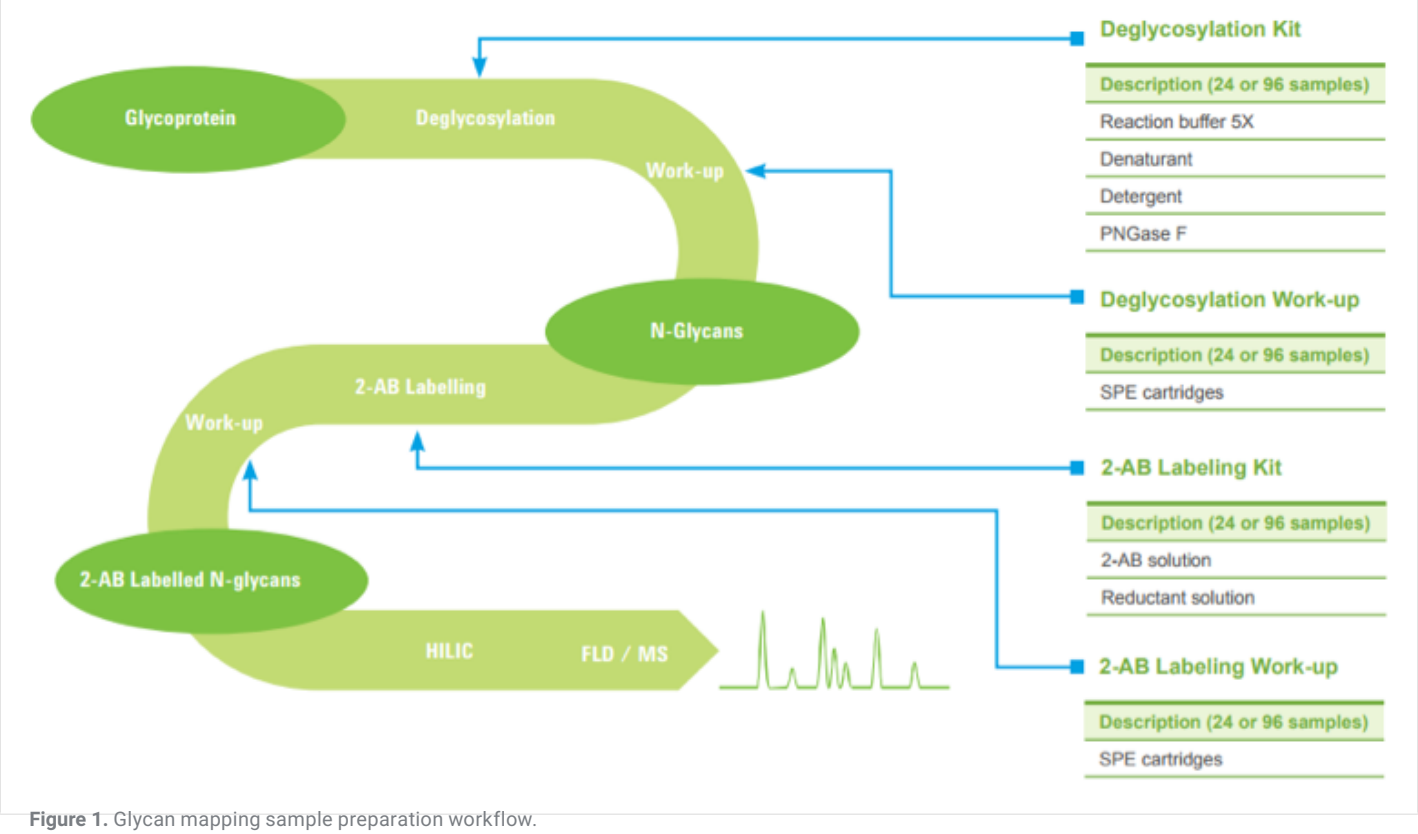

Figure 1. Glycan mapping sample preparation workflow.

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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.

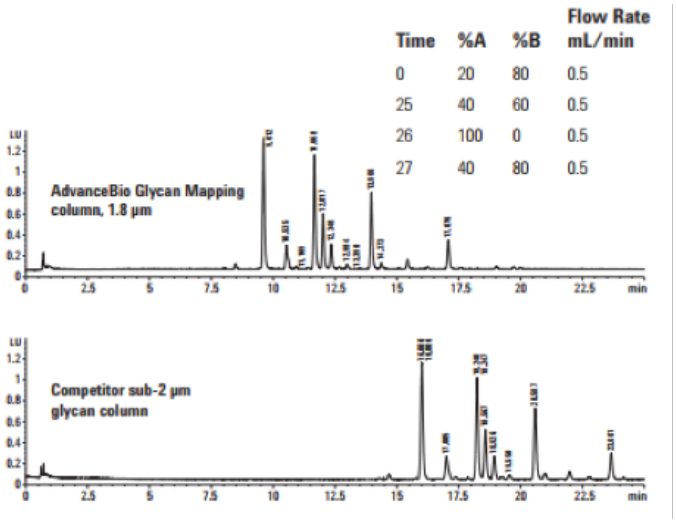

Figure 2. The Agilent AdvanceBio Glycan Mapping column delivers better resolution, narrower bands, and higher peak capacity than the non-Agilent sub-2 µm column in a 2.1 x 150 mm configuration.

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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. Choose from two options:

- Dextran ladder standard facilitates data analysis and reporting
- IgG N-linked glycan standard confirms workflow efficiency

The two standards are available with the 2AB label attached and also without the 2AB label for use as sample preparation reference materials.

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.

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/n5190-6998)

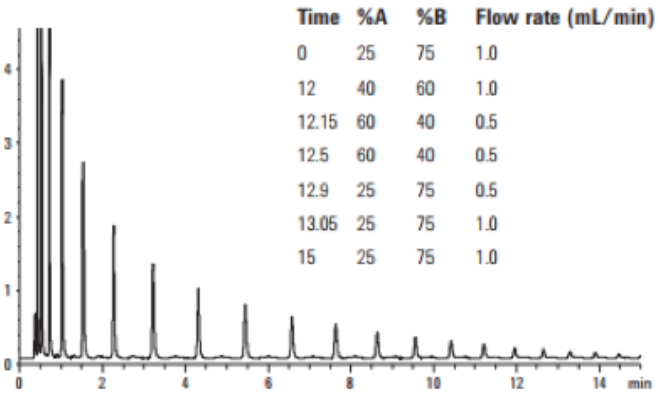

Figure 3. This analysis uses the agilent dextran ladder standard, together with an AdvanceBio Glycan Mapping column to correlate retention times of unkown glycans. From 5990-9384EN.

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Conditions

Mobile phase

AdvanceBio Glycan Mapping columns are shipped with acetonitrile: water and are ready to use for HILIC separations. HILIC columns are compatible with 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 a HILIC amide 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, a chiller should be used. In addition, samples should be filtered before injection into the column. 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.

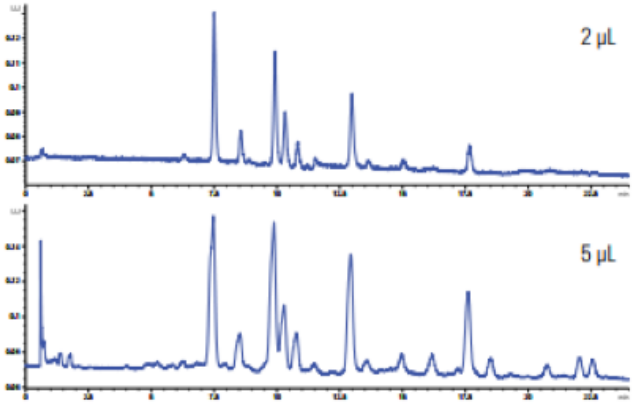

Figure 4. Demonstrates the outcome from injecting 5 µL (bottom chromatogram) on a AdvanceBio Glycan Mapping 1.8 µm, 2.1 x 150 mm column, peaks become broader and resolution is lost compared to 2 µL injection (top chromatogram). From 5991-6183EN.

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

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

Both gradients provide 1.25 %/mL slope. It may be necessary to adjust the start and end point to obtain highest resolution for samples containing different types of glycan. 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, it is recommended to detect 2AB labeled N-glycans with an Agilent 1260 Infinity fluorescence detector, using an excitation wavelength of 260 nm and an emission wavelength of 430 nm, with an 8 µL cell

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.

Cleaning the column

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

Glycan Standards

Description	Part number
Dextran ladder standard, 10 µg, 0.5 mL vial	5190-6997
2AB labeled dextran ladder standard, 200 pmol	5190-6998
IgG N-linked glycan library, 20 µg, 0.5 mL	5190-6995
2AB labeled IgG N-linked glycan library, 200 pmol	5190-6996

* Recommended initial column size

Glycan Standards

Description	Part number
AdvanceBio N-glycan sample preparation kit (24 samples)	5190-8000
AdvanceBio N-glycan deglycosylation kit (24 samples)	5190-8001
AdvanceBio N-glycan deglycosylation cleanup cartridges (24 samples)	5190-8002
AdvanceBio 2AB glycan labeling kit (24 samples)	5190-8003
AdvanceBio 2AB glycan labeling cleanup cartridges (24 samples)	5190-8004
AdvanceBio N-glycan sample preparation kit (96 samples)	5190-8005
AdvanceBio N-glycan deglycosylation kit (96 samples)	5190-8006
AdvanceBio N-glycan deglycosylation cleanup cartridges (96 samples)	5190-8007
AdvanceBio 2AB glycan labeling kit (96 samples)	5190-8008
AdvanceBio 2AB glycan labeling cleanup cartridges (96 samples)	5190-8009
96 well plate for deglycosylation and labeling	5190-8010

* Recommended initial column size

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 labelled 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:	ProZyme Glyko 2-AB bovine fetuinN-linked library
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 labelled dextran ladder standard (p/n5190-6998)
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.

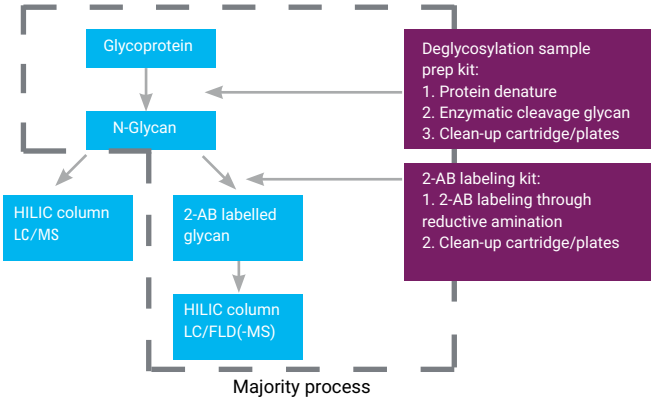

Figure 1. Amino acid analysis of protein hydrolysate. The resolution between leucine and Isoleucine with the AdvanceBio AAA, 4.6 x 100 mm, 2.7 µm column is much higher than the reported value for system suitability requirement.

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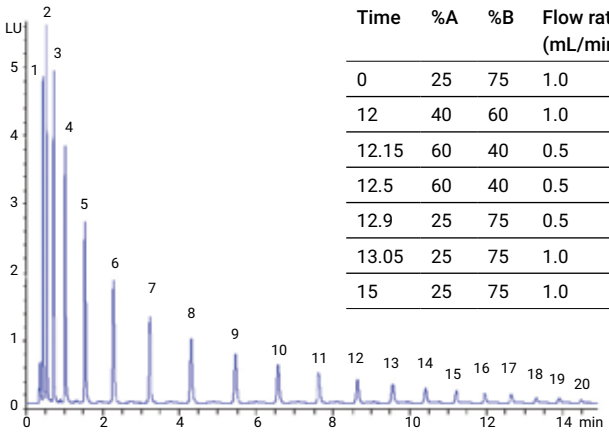
Results and Discussion

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.



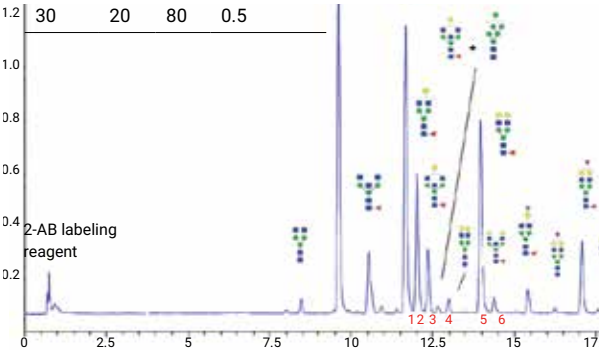
Time	%A	%B	Flow rate (mL/min)
0	25	75	1.0
12	40	60	1.0
12.15	60	40	0.5
12.5	60	40	0.5
12.9	25	75	0.5
13.05	25	75	1.0
15	25	75	1.0

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

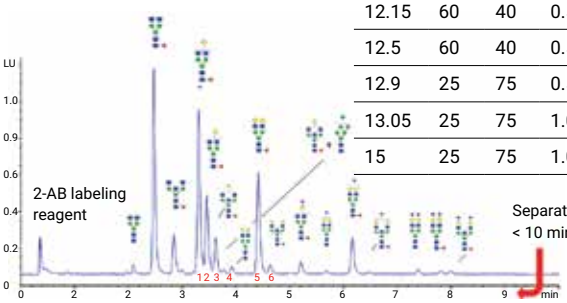


Avg RT (min)	RS 2,1	RS 3,2	RS 4,3	RS 6,5	Avg PW (min)	Bp (bar) at 30 % B	Peak capacity
12.7	2.60	2.90	5.43	2.81	0.0741	298	221

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.

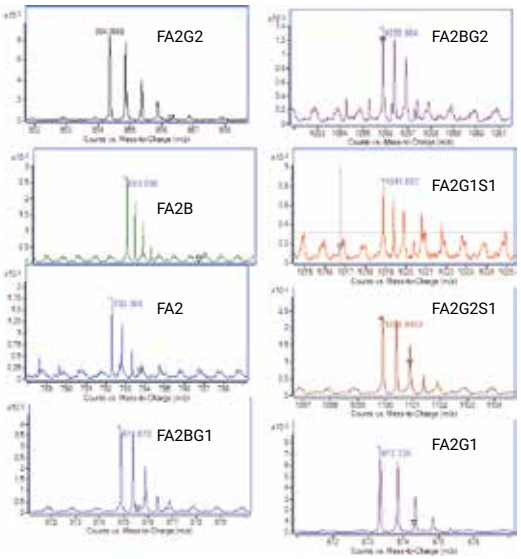


Figure 5. Eight representative mass spectra from the ultra-high resolution separation of human IgG glycans (fluorescence detector separations are displayed on the panel to the left).

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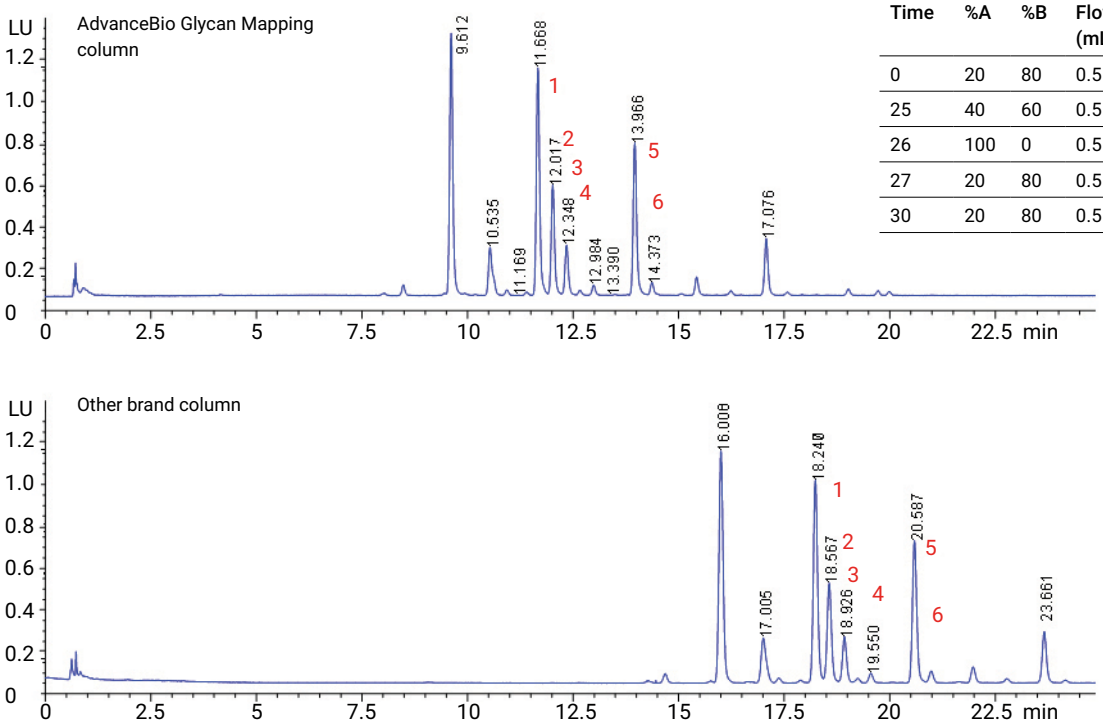
Sub-2 µm HILIC comparison

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.

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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).

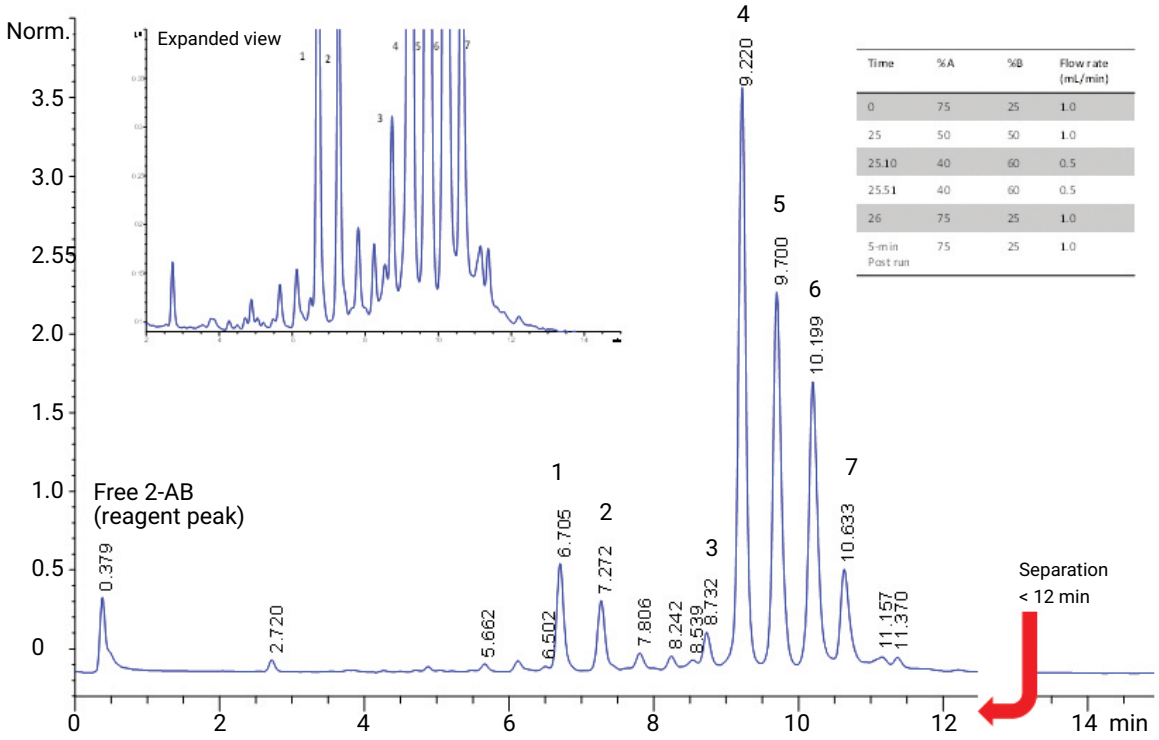

Figure 7. Fast and highly efficient separation of 2-AB labeled bovine fetuin N-linked glycans, 2.1 × 150 mm 1.8 µm AdvanceBio Glycan Mapping column.

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

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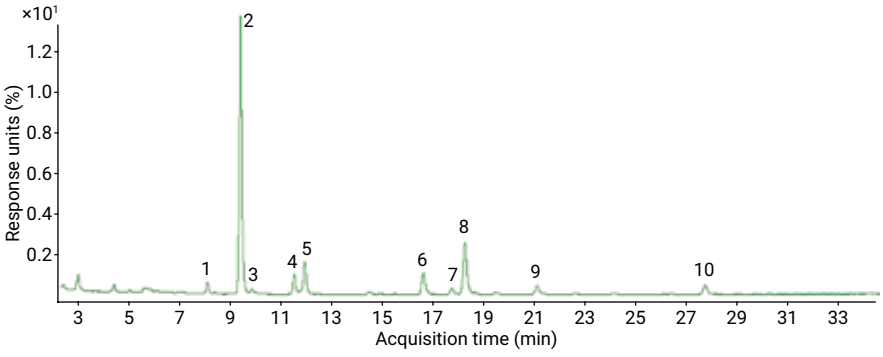
Abstract

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).

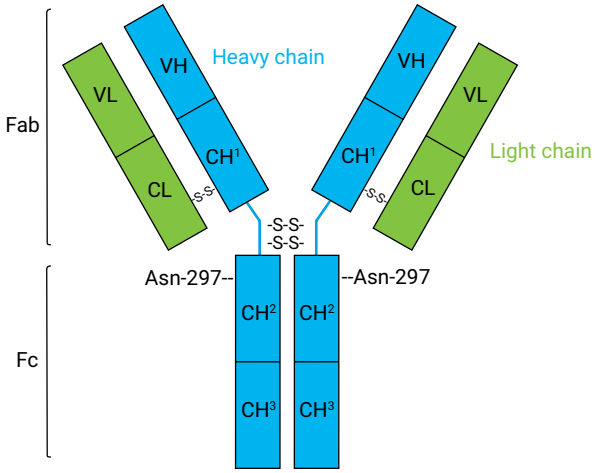

Figure 1. IgG antibody structure.

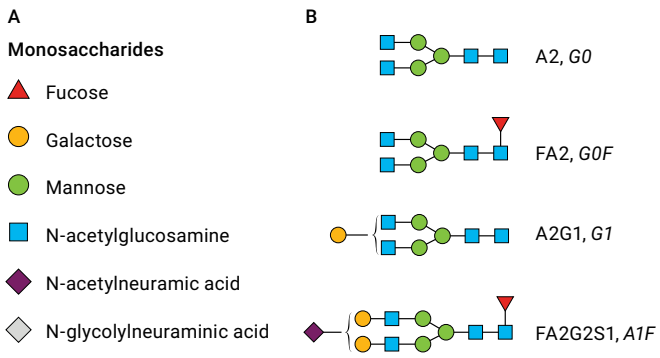

Figure 2. Glycan structure and isoforms. A) Monosaccharide description after the Consortium for Functional Glycomics, B) predominant glycan structures of human IgGs.

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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).

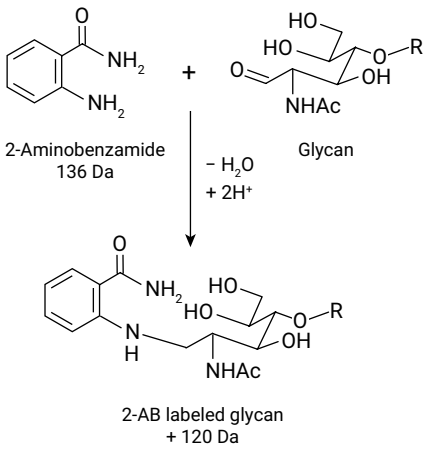

Figure 3. Labeling of a glycan with 2-aminobenzamide (2-AB).

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.

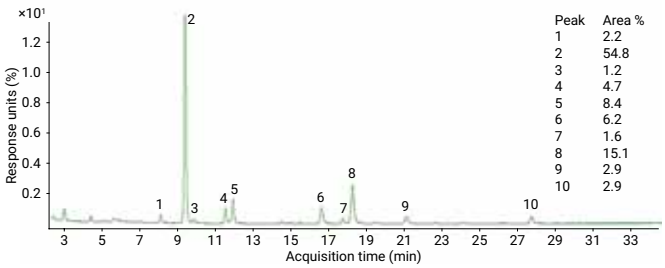

Figure 4. Separation of mAb N-glycans with fluorescence detection with 260 nm as the excitation wavelength.

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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.

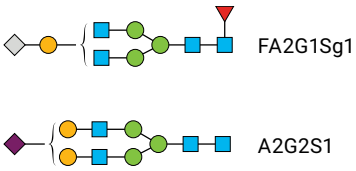

Figure 5. Two most likely glycan structures for the parent mass of 1,026.88 [z = 2], FA2G1Sg1 and A2G2S1.

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.

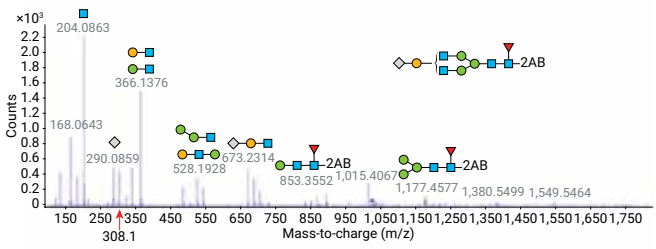

Figure 6. MS/MS spectra of FA2G1Sg1 - 1026.88 [z = 2] - 1931.6876 Da.

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.

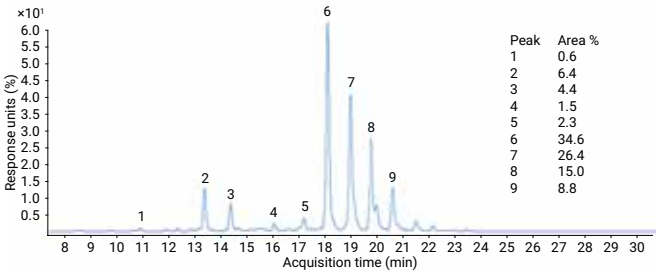

Figure 7. Separation of 2-AB-labeled fetuin.

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].

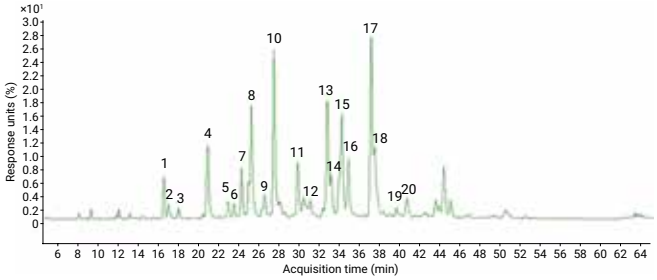

Figure 8. Separation of N-glycans released from ovalbumin.

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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.

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Application Note

Glycan Analysis

Agilent

Trusted Answers

Agilent Solutions for High-throughput N-linked Glycan Profiling from Biotherapeutics

Authors

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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

ProZyme's GlykoPrep-plus Rapid N-Glycan Sample Preparation with 2-AB kit, and 2-AB labeled Oligomannose 5 and 6 were purchased from ProZyme. 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 5190-6996) 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 a ProZyme 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 ProZyme 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.

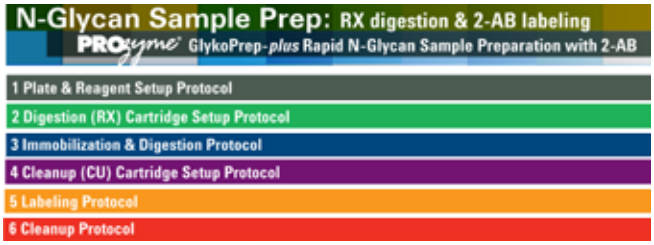

Figure 2. Agilent VWorks modules of an Agilent AssayMAP N-Glycan Sample Preparation.

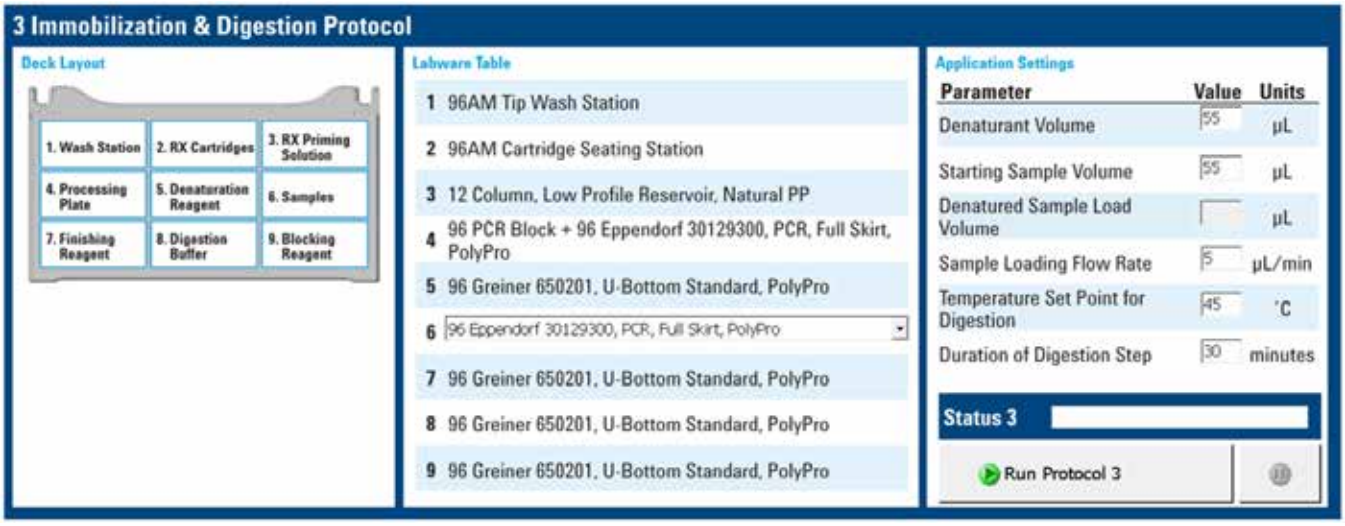

Figure 3. The deck layout, labware table, and application settings of the Immobilization and Digestion Protocol, one of the five sample preparation modules in Agilent VWorks software.

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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.

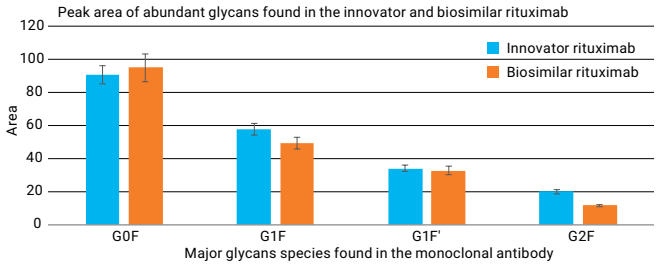

Figure 4. Peak area of major glycan species from 96 replicates for each innovator and biosimilar sample.

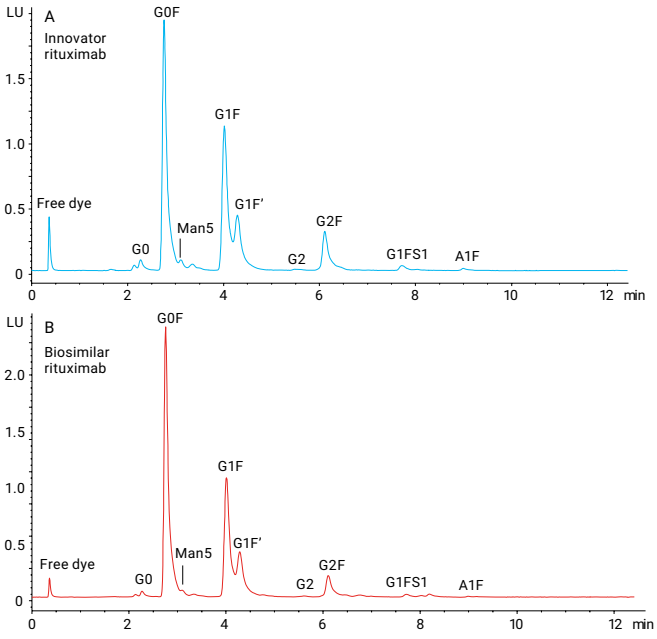

Figure 5. Glycosylation pattern of innovator and biosimilar rituximab.

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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.

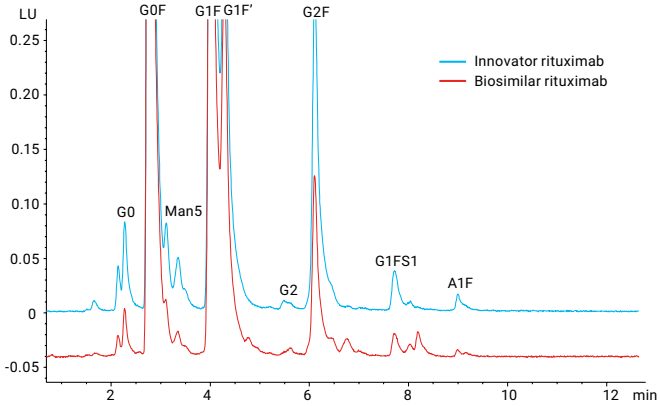

Figure 6. Zoomed view of Figure 5: the glycosylation pattern of innovator and biosimilar rituximab, showing minor differences in the low abundant glycans.

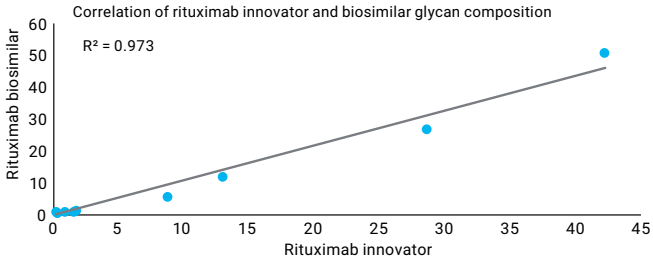

Figure 7. Linear graph showing the comparison of the area % of glycans released from innovator and biosimilar rituximab.

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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

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 following user guides.

Part Number	Title
5991-9561EN	AdvanceBio N-Glycan Sample Preparation Kit User Guide (96 Samples) p/n 5190-8005
5991-9560EN	AdvanceBio N-Glycan Sample Preparation Kit User Guide (24 Samples) p/n 5190-8000

Glycan standards are available to assist with method development, data analysis, and system checks.

Part Number	Title
5190-6997	Dextran ladder standard
5190-6998	Dextran ladder standard, 2-AB labeled
5190-6995	IgG N-linked glycan library
5190-6996	IgG N-linked glycan library, 2-AB labeled

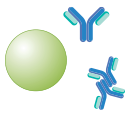
A video overview of the AdvanceBio Glycan Mapping Workflow, including sample preparation, can be found online.

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

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

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.

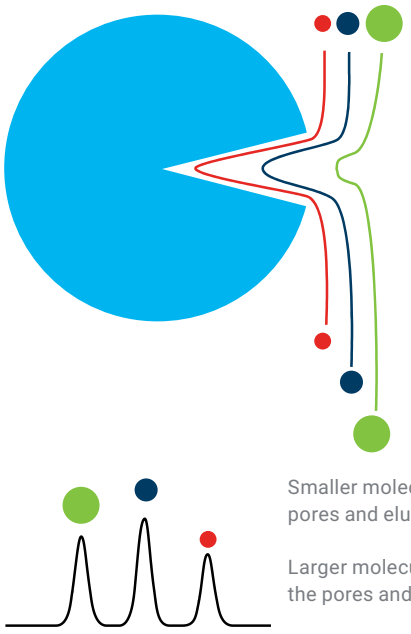


Figure 1. Molecules permeate the pores of the stationary phase to different extents depending on their size.

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.

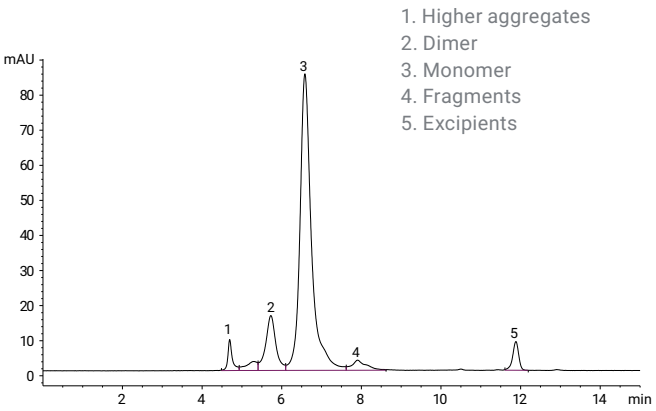


Figure 2. Separation of IgG aggregates and excipients.

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).

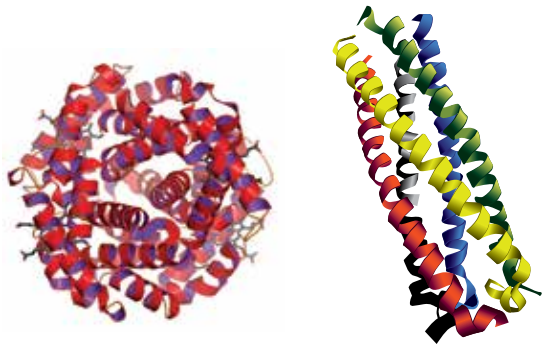


Figure 3. Comparison of compact globular protein versus cylindrical protein.

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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

Peptides, polypeptides, proteins, mAbs MW >0.1-1,250 kDa

Peptides, polypeptides, proteins, mAbs MW >0.1-10,000 kDa

Select column based on molecular weight range and pore size

AdvanceBio SEC (2.7 µm)

Pore Size	MW Range (kDa)
130 Å	0.1-100
300 Å	5-1,250

Agilent Bio SEC-5 (5 µm)

Pore Size	MW Range (kDa)
100 Å	0.1-100
150 Å	0.5-150
300 Å	5-1,250
500 Å	15-5,000
1000 Å	50-7,500
2000 Å	>10,000

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: 0.1-0.4 mL/min for 4.6 mm id columns
0.1-1.25 mL/min for 7.8 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 flow cells to enable reliable analysis of your protein under various conditions. 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

Comprehensive molecular characterization

SEC can be used to determine the average molecular weight of polymeric analytes, including naturally occurring molecules (polysaccharides, starches, etc) and synthetic polymers (polyethyleneglycol or polyethylene oxide) (Figure 4). For proteins or more complex samples, including vaccines, a more sophisticated form of data analysis with dedicated software is often required. In combination with the appropriate detectors, valuable information on conformation of the sample can be obtained. See page 17 for more information about detector choices.

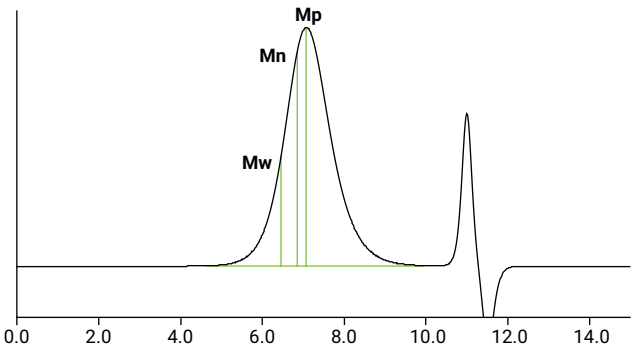


Figure 4. SEC separation of polysaccharide showing Mw, Mn, and Mp.

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

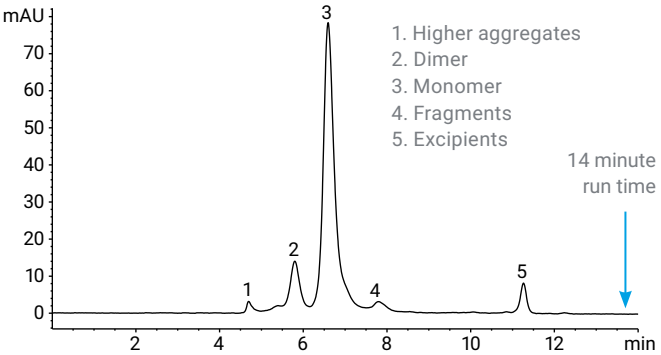
SEC columns are usually much larger than those used for other types of chromatography and are operated at comparatively low flow rates or slow linear velocities. The standard column dimensions for SEC are 7.8 x 300 mm, operated at 1.0 mL/min, compared to a reversed-phase column that is likely to be 2.1 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.



Conditions

Parameter	Value
Sample	Polyclonal IgG
Column:	AdvanceBio SEC, 7.8 x 300 mm
Flow rate:	A, 100 mM NH4 formate, pH 4.5; B, ACN
Sample	Polyclonal IgG
Column:	AdvansceBio SEC, 7.8 x 150 mm
Flow rate:	2.0 mL/min

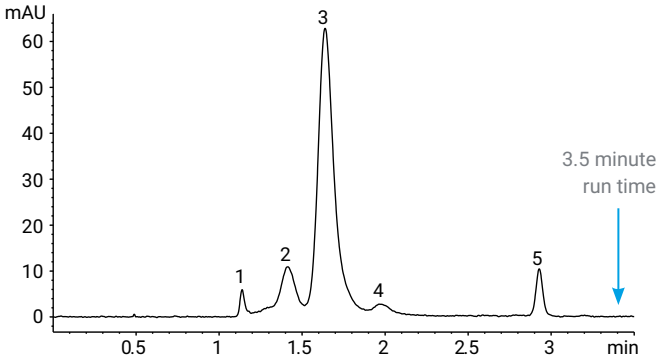


Figure 5. Comparison of analysis using 300 mm columns vs. 150 mm columns to demonstrate time savings.

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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)

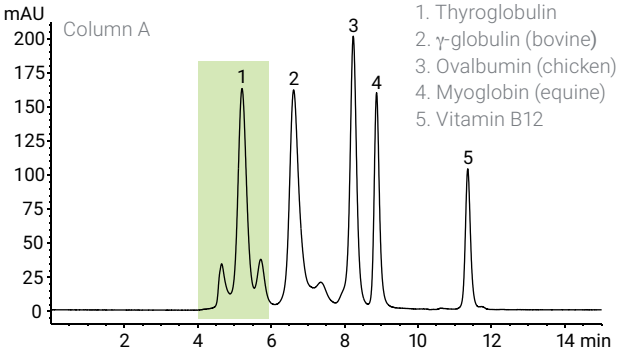
Table 1. Absolute trastuzumab biosimilar concentrations determined in the different CHO clones grown on two different media.

Application	Agilent Columns	Notes
Proteins		
SEC-UV/DAD, 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
SEC-MS analysis of mAbs, proteins, and peptides	Agilent Bio SEC-3	Provides stable baselines with MS detection
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
Water Soluble Analytes		
Low MW polymers and oligomers, oligosaccharides, PEGs, lignosulfonates	2 or 3 PL aquagel-OH ✓ PL aquagel-OH 8 µm ✓ PL aquagel-OH 20 5 µm ✓ PL aquagel-OH MIXED-M 8 µm	The PL aquagel-OH analytical series has a pH range of 2-10, compatibility with organic solvent (up to 50 % methanol), mechanical stability up to 140 bar (2030 psi), and low column operating pressures
Polydisperse biopolymers, polysaccharides, cellulose derivatives	2 or 3 PL aquagel-OH ✓ PL aquagel-OH MIXED-H 8 µm ✓ PL aquagel-OH 60/50/40 8 µm	
Very high MW polymers, hyaluronic acids, starches, gums	PL aquagel-OH 60/50/40 15 µm in series	

Pore size

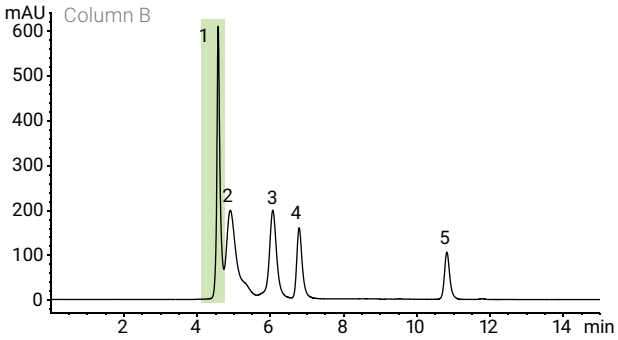
Proteins are relatively small and compact compared to other biopolymers, and so 300 Å pore size is a good choice for an initial column selection. Figure 6 compares the resolution of a five-protein mix reference standard and a polyclonal IgG sample on columns with different pore sizes and clearly shows the effect of pore size on the resolution.

BioRad gel filtration standards mix



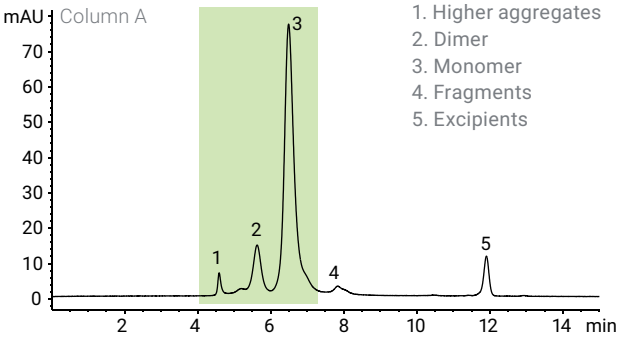
Conditions

Parameter	Value
Column A:	AdvanceBio SEC 300 Å 4.6 x 300 mm, 2.7 µm (p/nPL1580-5301)
Column B:	AdvanceBio SEC 130 Å 4.6 x 300 mm, 2.7 µm (p/nPL1580-5350)
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



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.

Polyclonal IgG separation



Conditions

Parameter	Value
Column A:	AdvanceBio SEC 300 Å 4.6 x 300 mm, 2.7 µm (p/nPL1580-5301)
Column B:	AdvanceBio SEC 130 Å 4.6 x 300 mm, 2.7 µm (p/nPL1580-5350)
Sample:	Polyclonal IgG
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

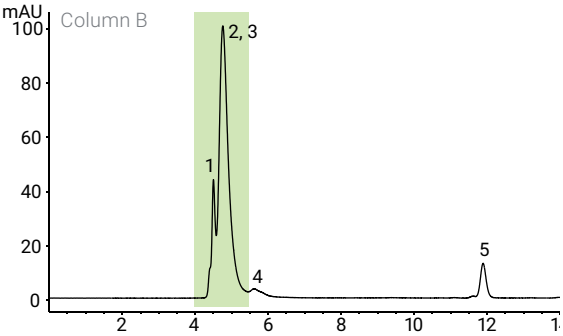


Figure 6: Comparison of pore sizes on the resolution of BioRad gel filtration standards and polyclonal IgG. The area highlighted in green shows the difference in resolution between the two pore sizes. The larger pore size is needed for analysis of the larger proteins.

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.

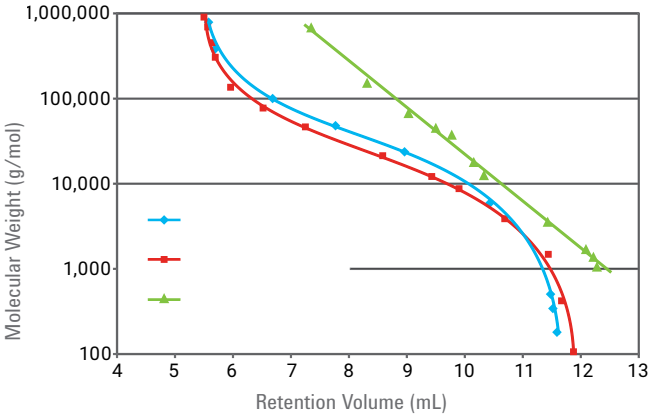


Figure 7: Comparison of calibration plots generated for three types of calibrant.

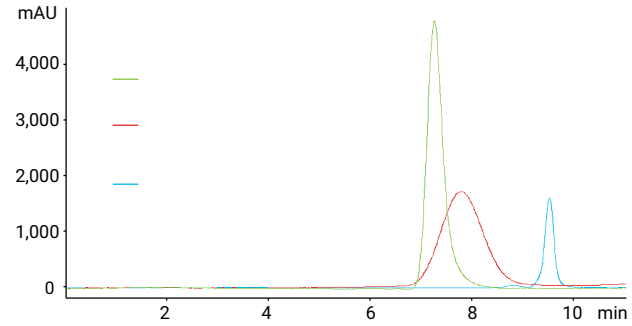
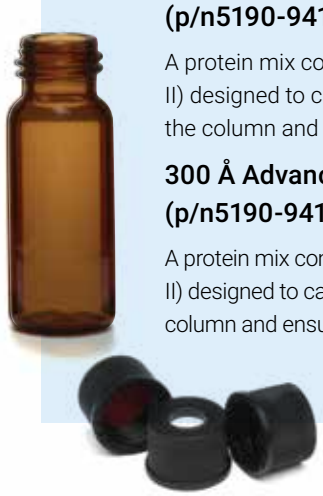


Figure 8: Overlay of chromatograms obtained for calibrants of similar molecular weight.



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) 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 column 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, but at the risk of degrading (shearing/deforming) the protein. Figure 9 shows a comparison between Agilent 3 µm Bio SEC-3 and 5 µm Bio SEC-5 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 Agilent Bio SEC-3 and Agilent Bio SEC-5

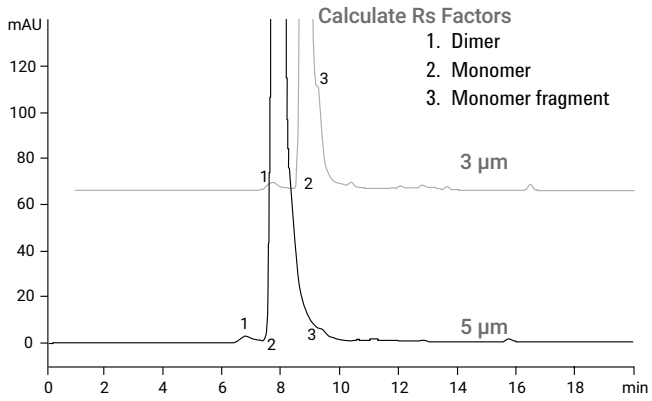


Figure 9: Comparison of Agilent Bio SEC-3 versus Agilent Bio SEC-5 columns. The 3 µm column gives better separation.

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

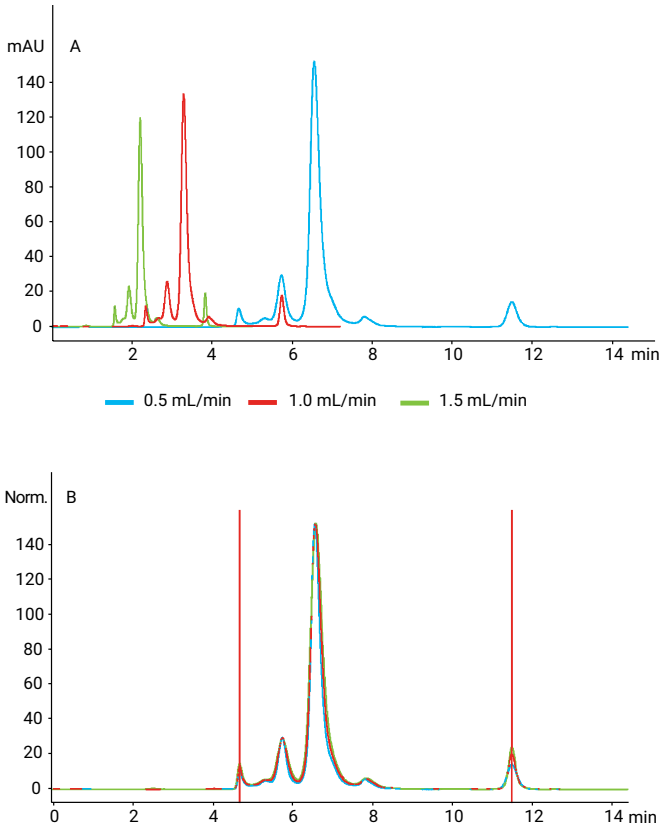

Figure 10: Increasing the flow rate reduces the analysis time from 12 to 4 minutes (A). When the retention times are normalized and overlayed (B) it is evident that the retention times are consistent and that there is minimal reduction in resolution.

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 page 11 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

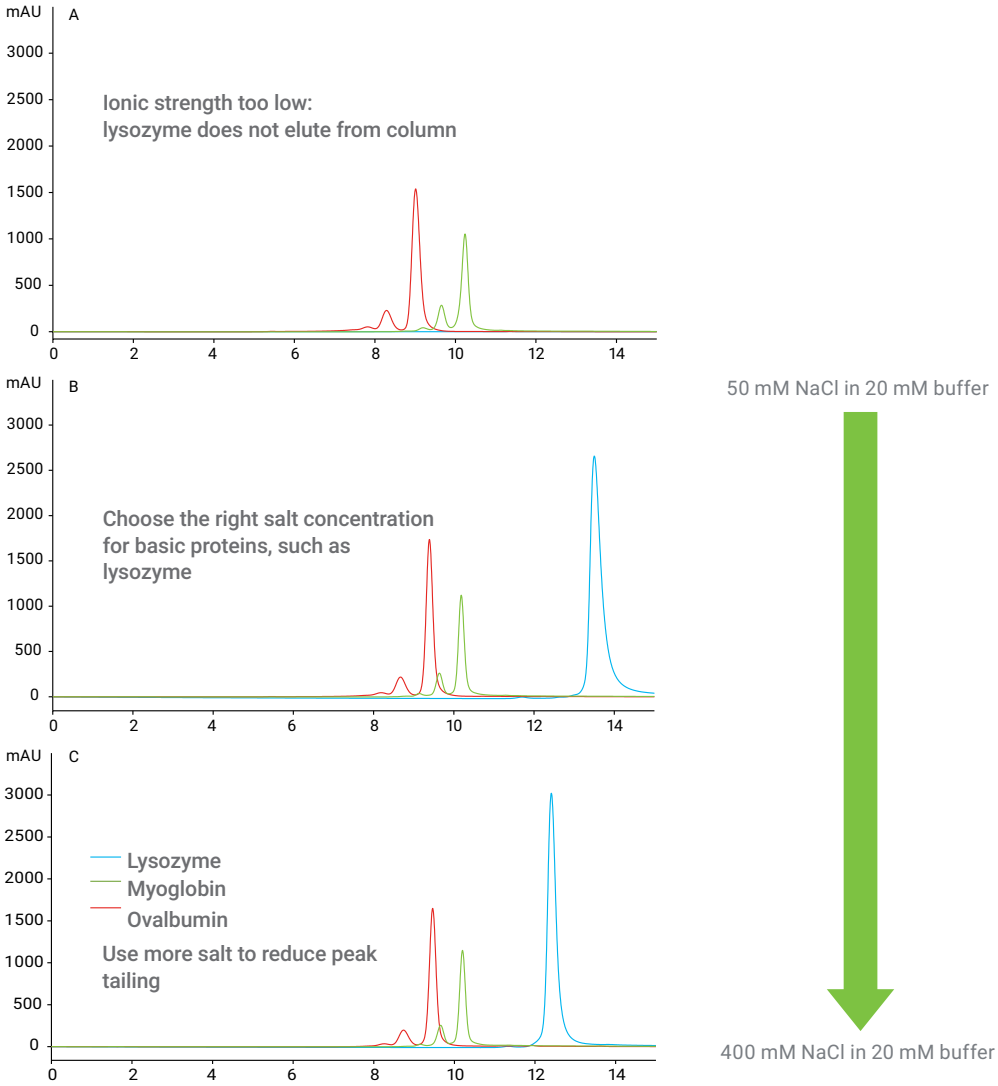

Figure 11: Effect of too much or too little ionic strength on achieving your desired separation.

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.

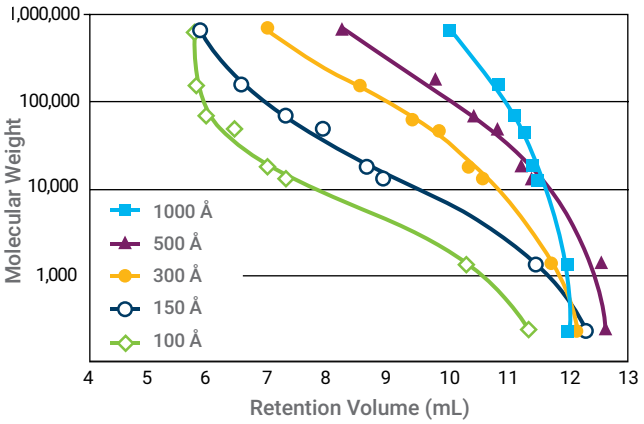

Figure 12: Calibration curves obtained by plotting retention time against molecular weight.

		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.

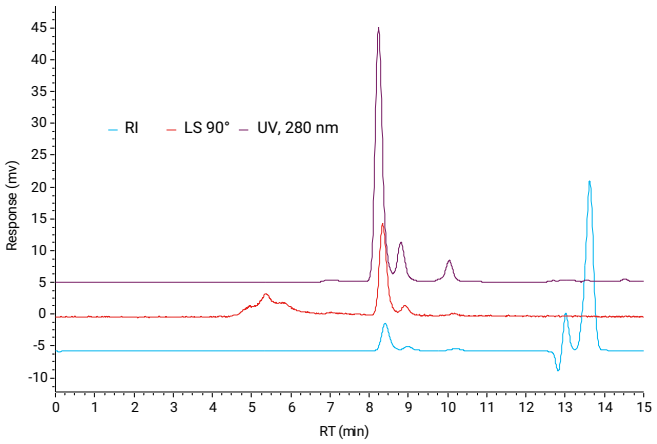

Figure 13: Results of using different detectors on a protein separation.

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.

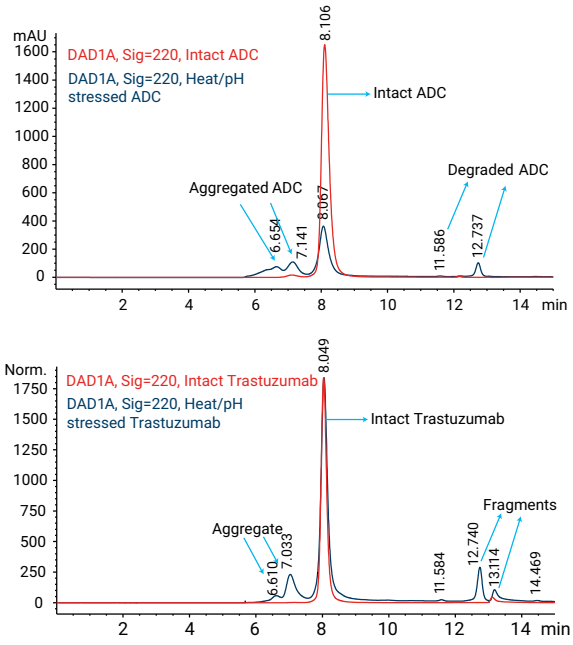

Figure 14: The same aqueous mobile phase was used for the mAb and the more hydrophobic ADC.

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 250 or 300 mm
- Normal flow rate is 1.0 mL/min on a 7.5 or 7.8 mm id column and 0.35 mL/min on a 4.6 mm id 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 id**–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

Trusted Answers

Size Exclusion Chromatography of Biosimilar and Innovator Insulin

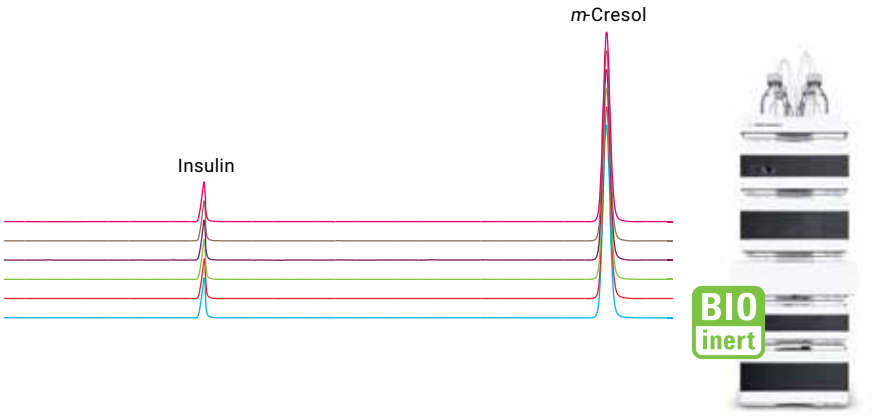
Using the Agilent AdvanceBio SEC column

Authors

M. Sundaram Palaniswamy and Andrew Coffey
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).

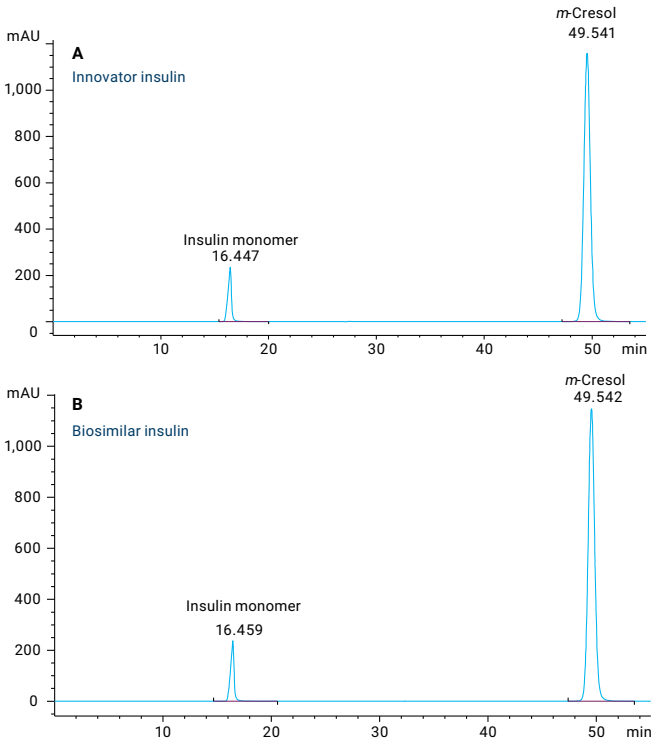

Figure1. SEC HPLC profile of insulin innovator and biosimilar on an Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm column.

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.

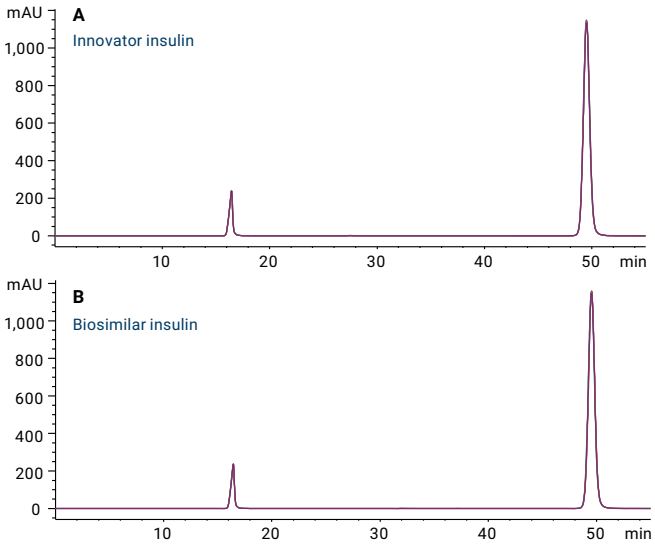

Figure2. Overlay of six replicates of innovator and biosimilar insulin separated on an Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm column.

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.

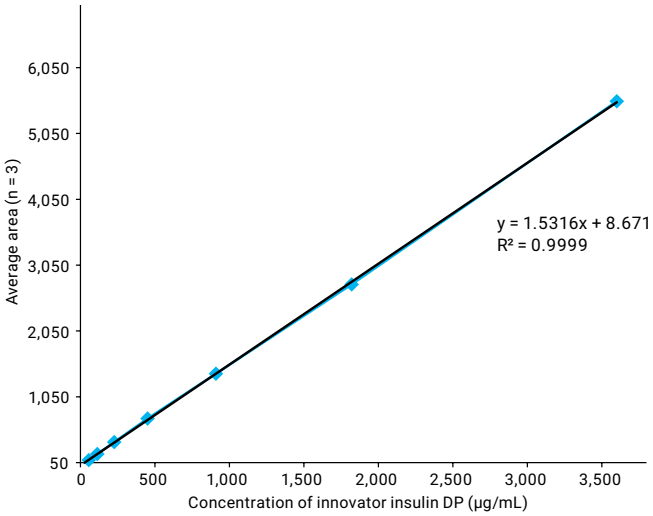

Figure 3. Linearity curve with standard concentrations of insulin ranging from 10.6 to 3,400 µg/mL, showing excellent coefficient value.

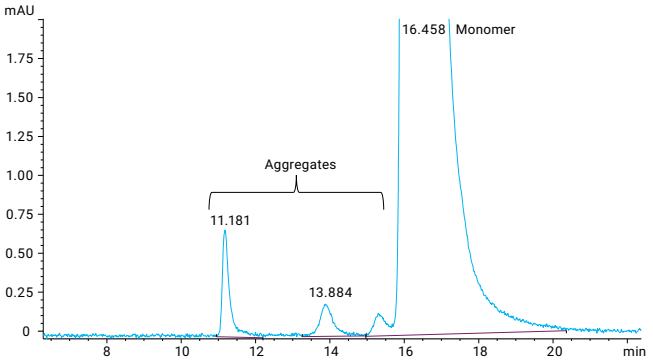

Figure 4. An Agilent AdvanceBio SEC profile of heat stressed insulin showing baseline separation of insulin aggregates.

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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.

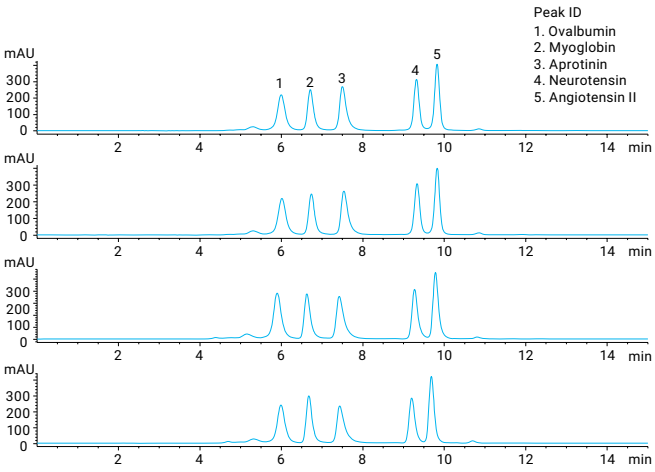

Figure 5. Separation of Agilent AdvanceBio 130 Å protein standards on four separate batches of an Agilent AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm media.

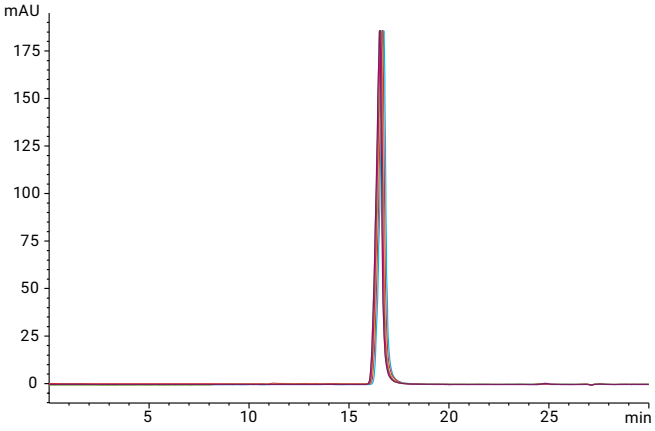

Figure 6. Overlay of six chromatograms for the 250 injection at an interval of 50 runs.

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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**.

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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

Authors

Andy Coffey and
Matthew Rain
Agilent Technologies, Inc

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.

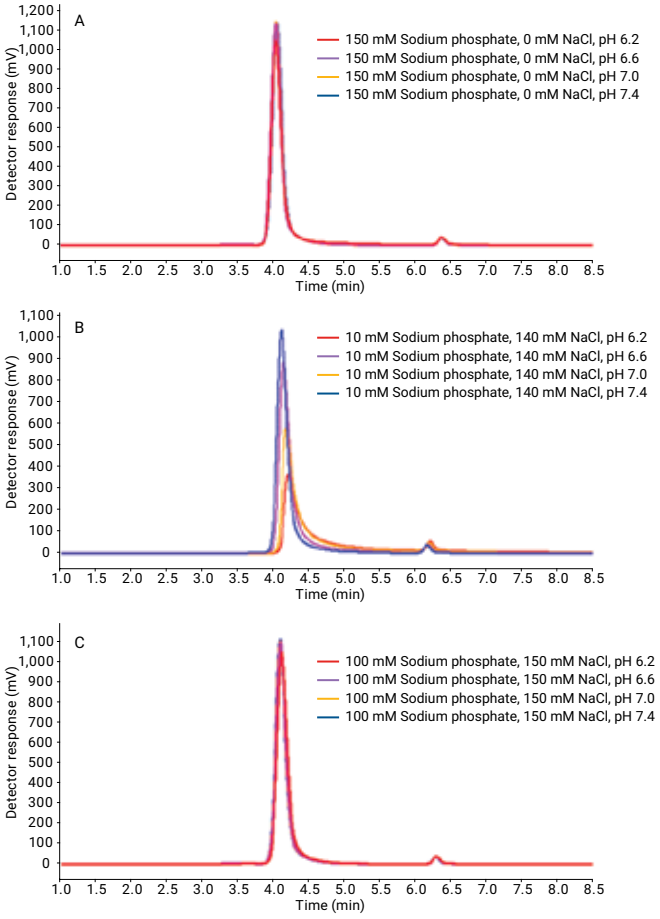

Figure 1. Rituximab innovator (1 µL injection) run consecutively at 12 different mobile phase conditions (Experiments 1–12: see Table 1).

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).

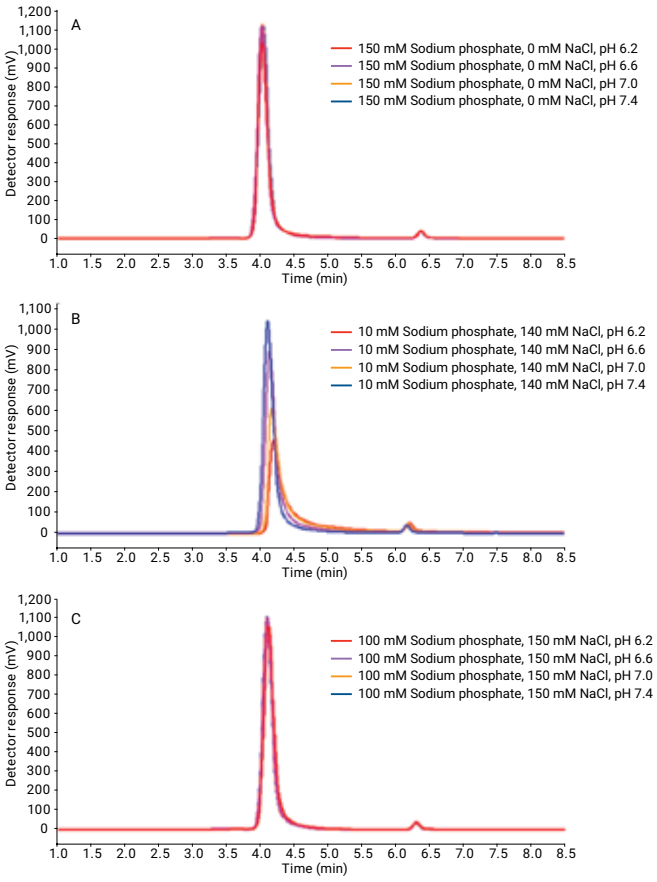

Figure 2. Rituximab biosimilar (1 µL injection) run consecutively at 12 different mobile phase conditions (Experiments 1–12: see Table 1).

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).

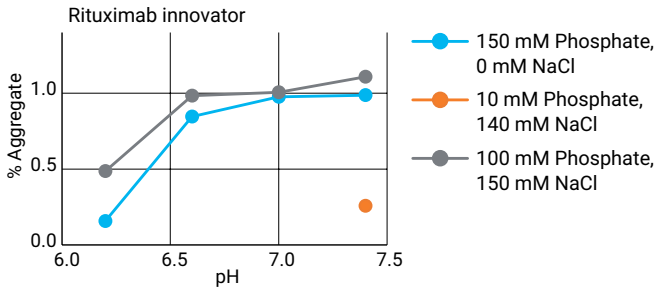

Figure 3A. Peak area percentage of aggregate content of rituximab innovator (Experiments 1–12: see Table 1). Points are shown for experiments where aggregates were detected.

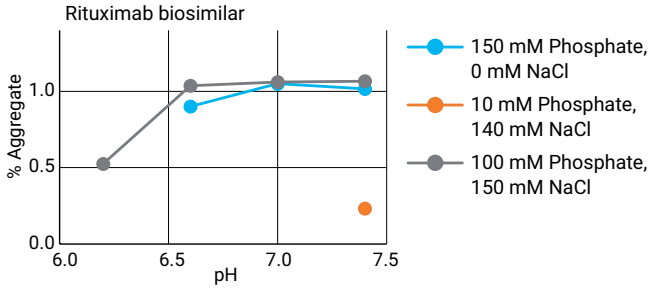

Figure 3B. Peak area percentage of aggregate content of rituximab biosimilar (Experiments 1–12: see Table 1). Points are shown for experiments where aggregates were detected.

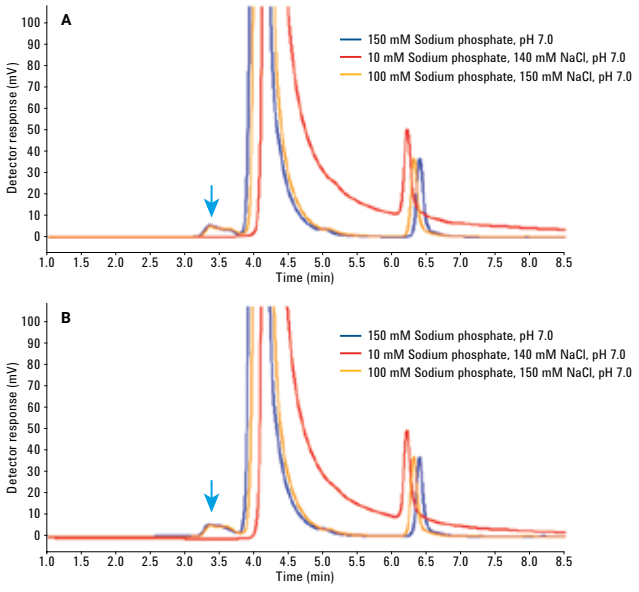

Figure 4. Baseline zoom of UV 220 nm signals of rituximab innovator (A) and rituximab biosimilar (B) run with different buffer salt concentrations at the optimized pH 7.0 (Experiments 3, 6, and 11 in Table 1).

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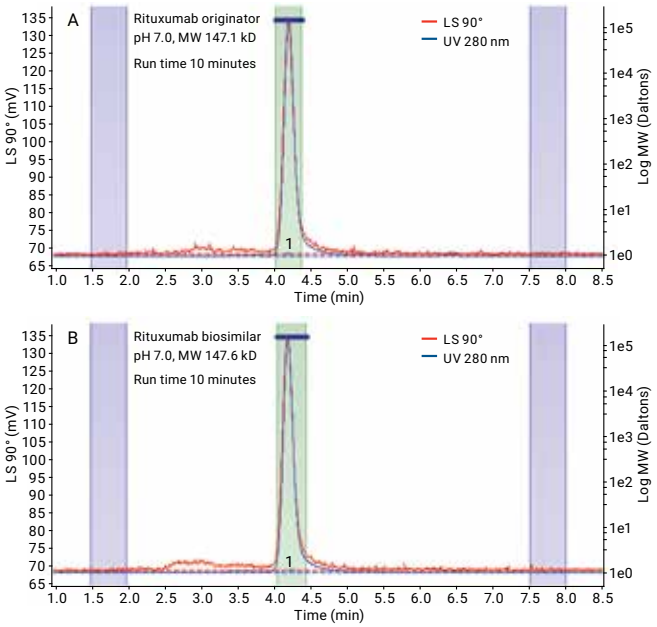

Figure 5. Comparison of LS analysis rituximab innovator (A) and rituximab biosimilar (B) run at 100 mM sodium phosphate with 150 mM NaCl, pH 7.0 (Experiment 11 in Table 1).

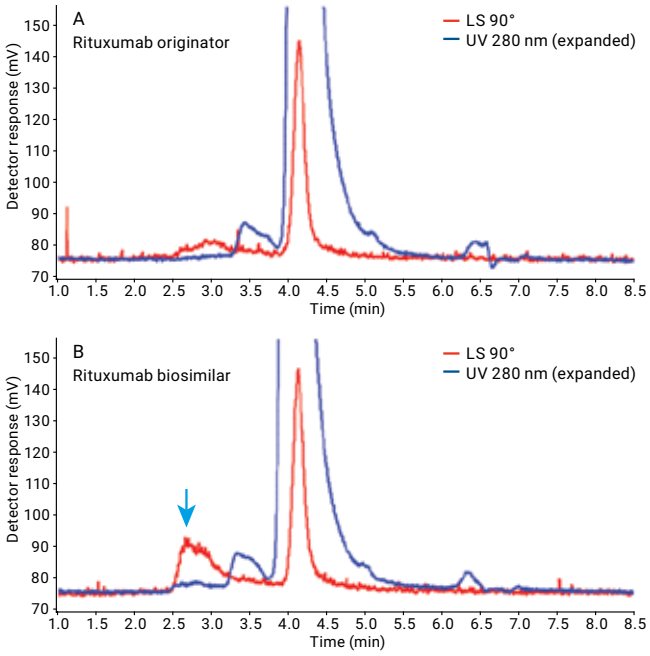

Figure 6. Comparison of LS analysis rituximab innovator (A) and rituximab biosimilar (B) run at 150 mM sodium phosphate, pH 7.0 (Experiment 3 in Table 1).

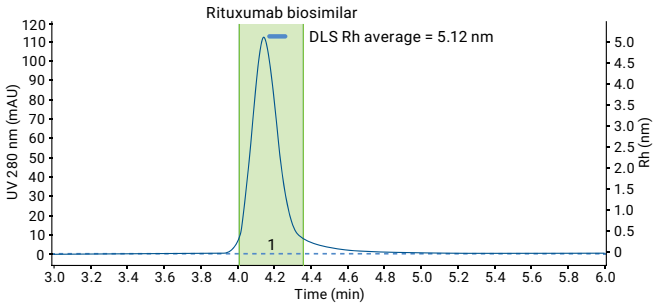

Figure 7. DLS analysis of rituximab biosimilar showing hydrodynamic radius results from LS detector.

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.

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Application Note

Aggregate/Fragment Analysis

Agilent

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Size Exclusion Chromatography in the Presence of an Anionic Surfactant

Intact Protein Profiling

Authors

Andy Coffey
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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].

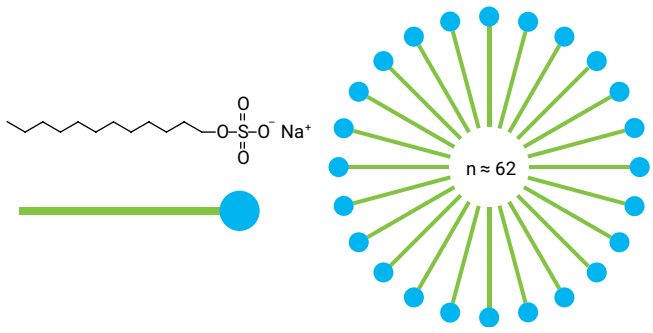

Figure 1. Cartoon depicting SDS micelle formed at concentrations above CMC (aggregation number ~ 62).

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.

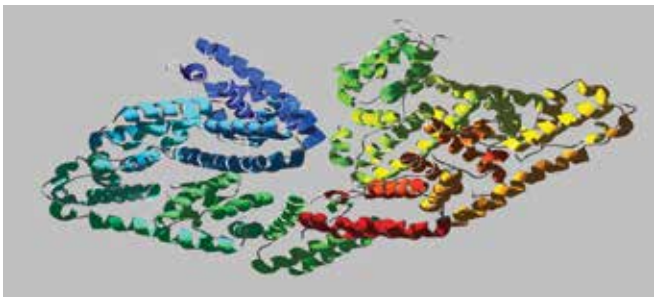

Figure 2. Native heart-shaped conformation of bovine serum albumin.

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

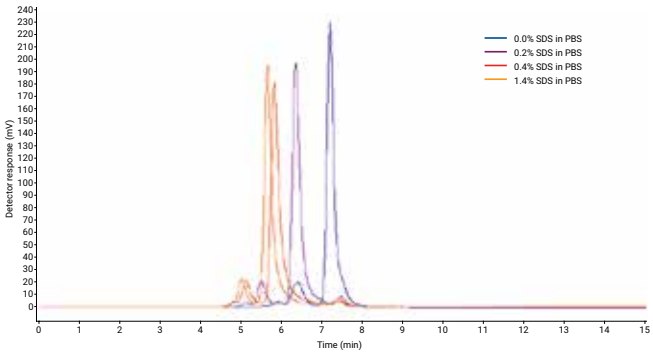

Figure 3. SEC chromatograms of BSA with increasing SDS concentration in mobile phase.

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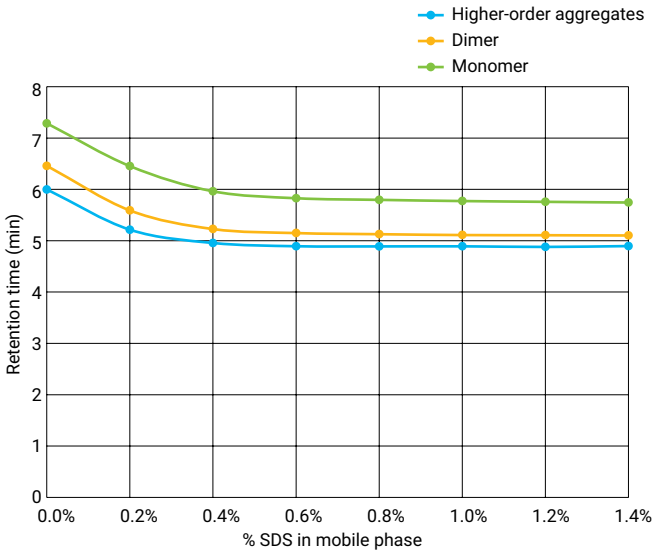

Figure 4. Effect of % SDS in mobile phase on RT.

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.

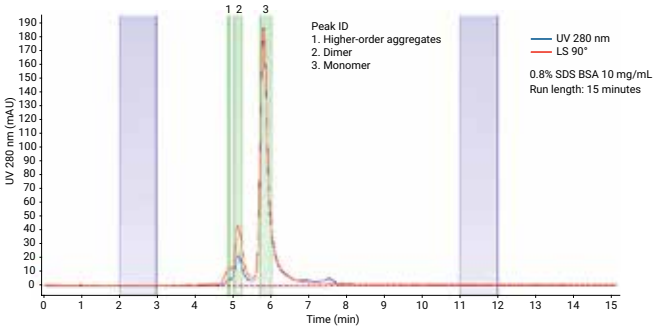

Figure 5. Molecular weight calculation regions for (1) higher-order aggregates, (2) dimer, and (3) monomer peaks of BSA.

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.

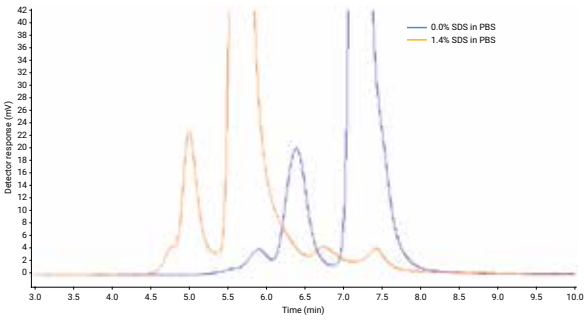

Figure 6. Close up of the UV 280 nm signal of BSA in PBS mobile phase containing 0.0 % SDS and 1.4 % SDS.

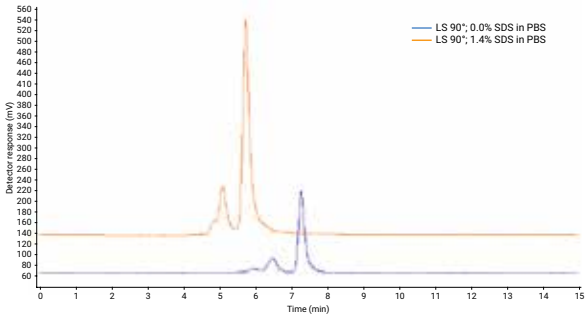

Figure 7A. Overview of the LS 90° signal of BSA in PBS mobile phase containing 0.0 % SDS and 1.4 % SDS.

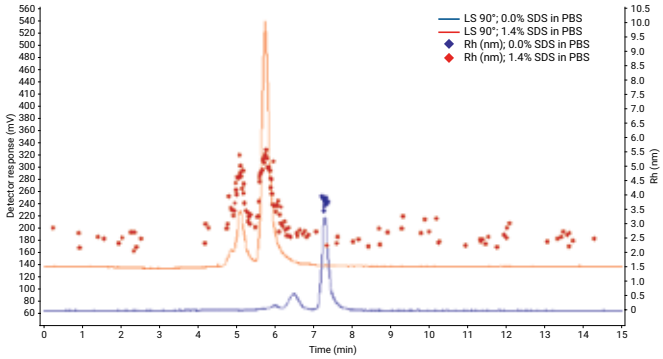

Figure 7B. Overview of the LS 90° signal of BSA in PBS mobile phase containing 0.0 % SDS and 1.4 % SDS with hydrodynamic radius (Rh) data added.

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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Agilent
Trusted Answers

Calibrating your Agilent AdvanceBio SEC Columns

Technical Overview

Authors

Andy Coffey
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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].

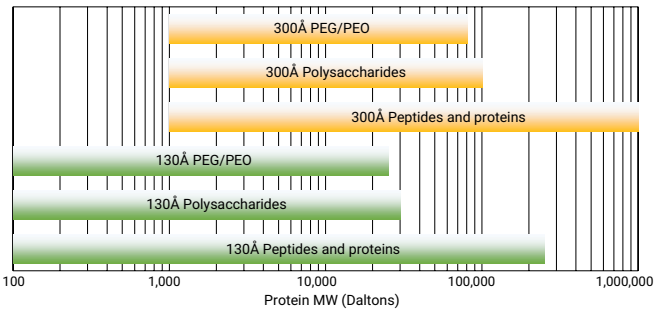

Figure 1. Resolving ranges for Agilent AdvanceBio SEC columns.

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)

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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

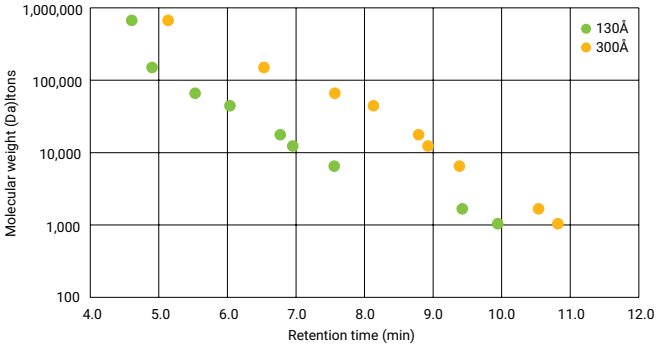

Figure 2. Peptide and protein calibration on Agilent AdvanceBio SEC columns.

Representative chromatograms for protein and peptide standards

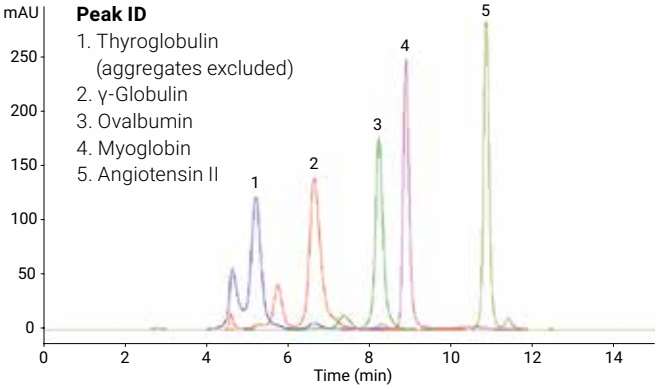

Figure 3A. Individual peptide and proteins contained in Agilent AdvanceBio SEC 300 Å Protein Standard (p/n5190–9417) on an Agilent AdvanceBio SEC 300 Å column.

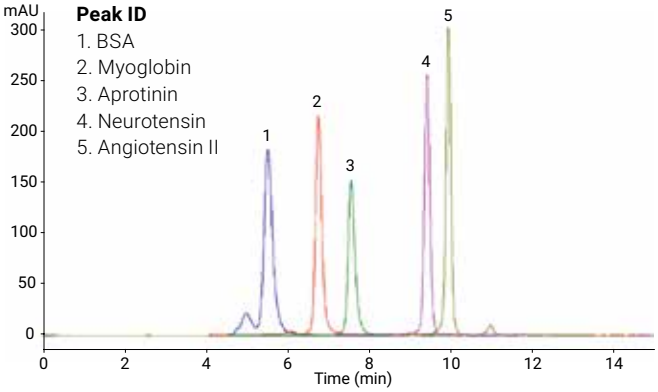

Figure 3B. Individual peptides and proteins contained in Agilent AdvanceBio SEC 130 Å Protein Standard (p/n5190–9416) on an Agilent AdvanceBio SEC 130 Å column.

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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

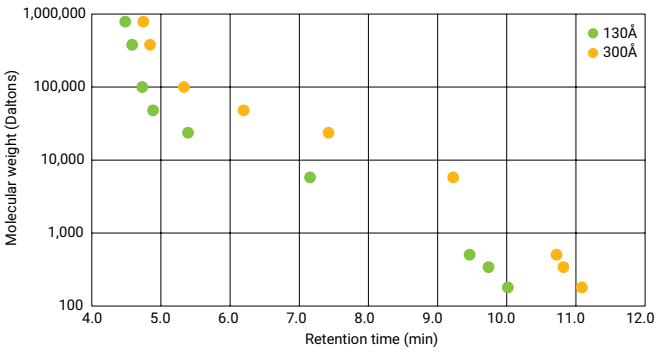

Figure 4. Polysaccharide calibration on Agilent AdvanceBio SEC columns.

Representative chromatograms for polysaccharide standards

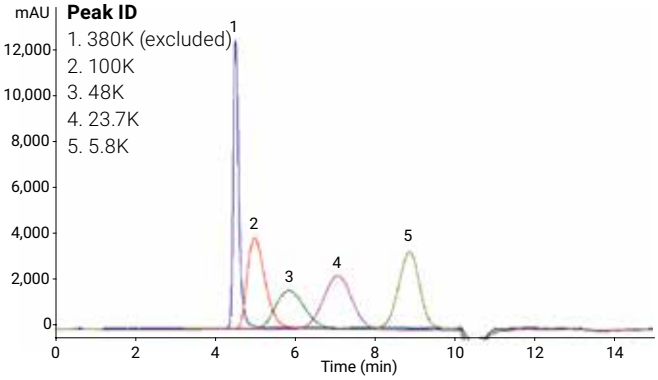

Figure 5A. Polysaccharide standards on an Agilent AdvanceBio SEC 300 Å column.

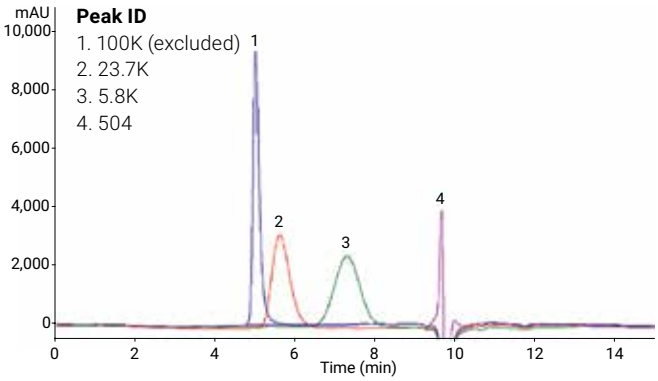

Figure 5B. Polysaccharide standards on an Agilent AdvanceBio SEC 130 Å column.

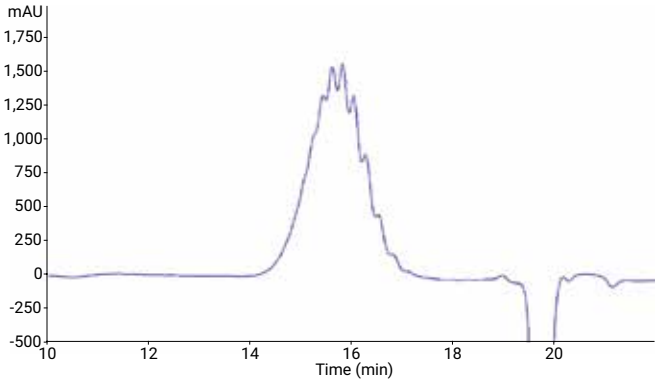

Figure 5C. Polysaccharide 5.8K run on Agilent AdvanceBio SEC columns 300 Å + 130 Å in series.

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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

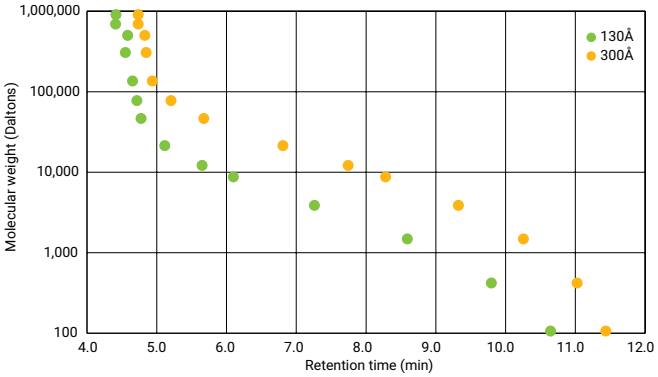

Figure 6. PEG/PEO Calibration on Agilent AdvanceBio SEC columns.

Representative chromatograms for PEG/PEO standards

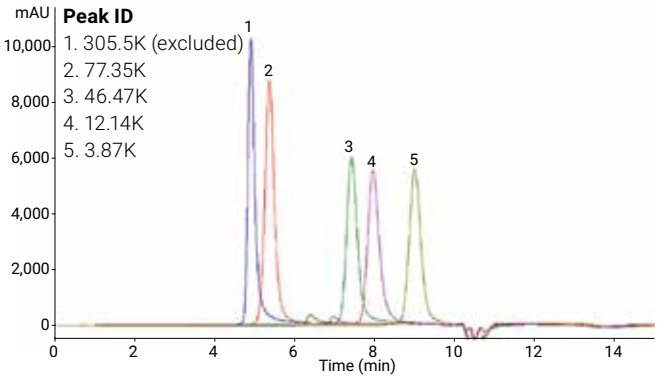

Figure 7A. PEG/PEO standards on an Agilent AdvanceBio SEC 300 Å column.

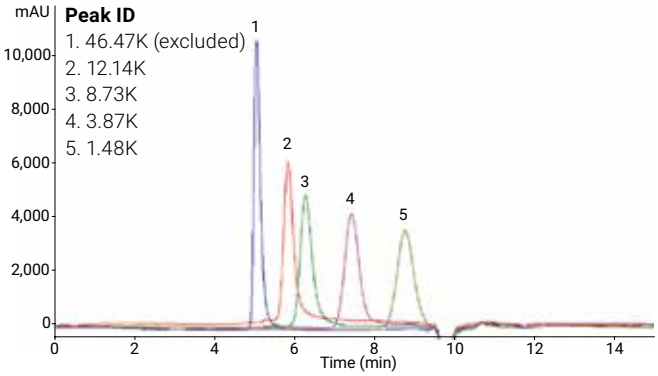

Figure 7B. PEG/PEO standards on an Agilent AdvanceBio SEC 130 Å column.

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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).

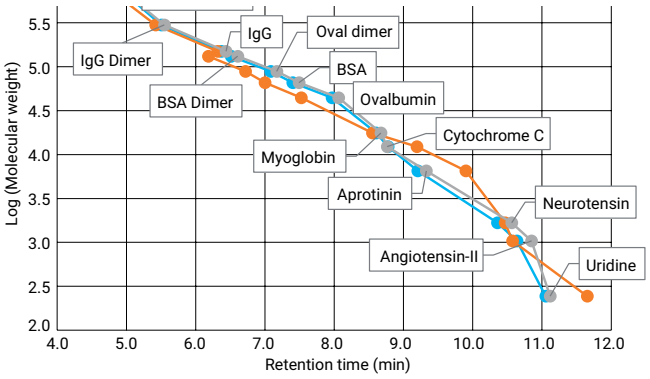

Figure 8. Effect of mobile phase composition on column calibration with protein standards.

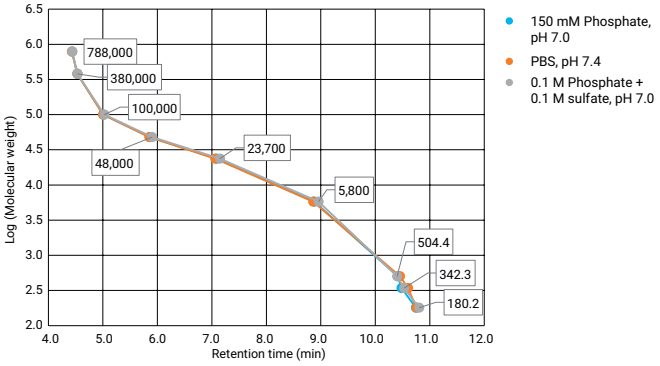

Figure 9. Effect of mobile phase composition on column calibration with polysaccharide standards.

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.

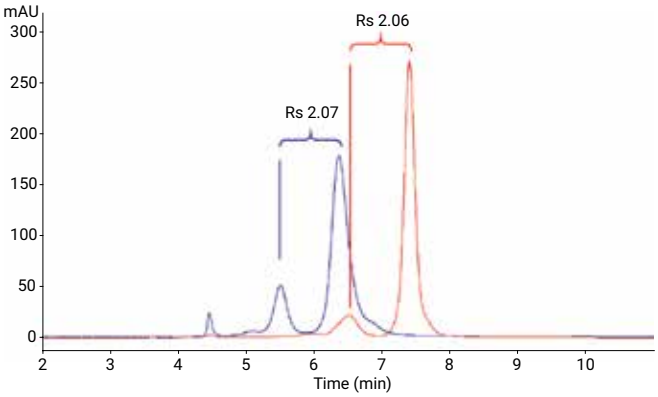

Figure 10A. γ-Globulin and BSA on an Agilent AdvanceBio SEC 300 Å column using 150 mM sodium phosphate, pH 7.0.

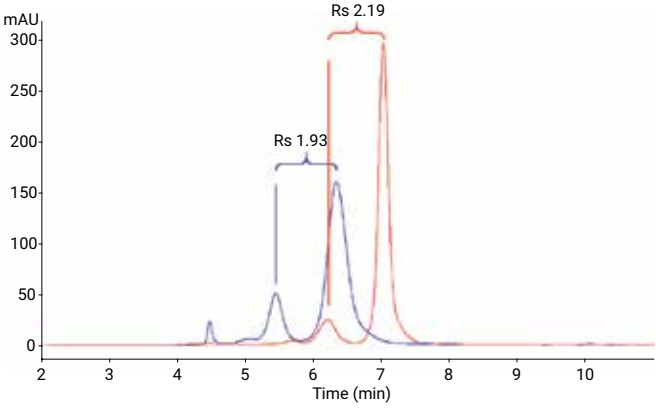

Figure 10B. γ-Globulin and BSA on an Agilent AdvanceBio SEC 300 Å column using PBS, pH 7.4.

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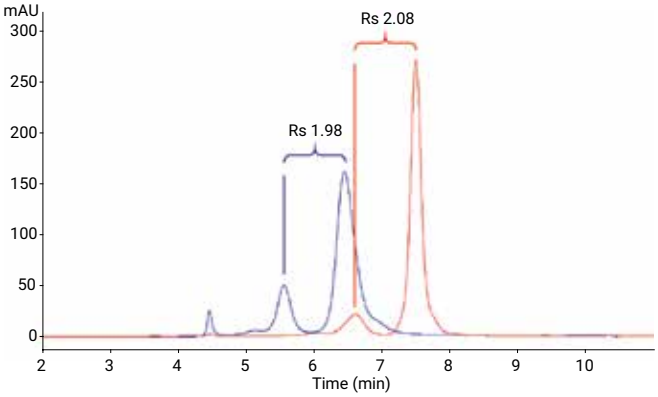

Figure 10C. γ-Globulin and BSA on an Agilent AdvanceBio SEC 300 Å column using 100 mM sodium phosphate + 100 mM sodium sulfate, pH 7.0.

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

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Additional Application Notes

AdvanceBio SEC

Part Number	Title
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
5991-3954EN	Detailed Aggregation Characterization of Monoclonal Antibodies Using the Agilent 1260 Infinity Multi-Detector Bio-SEC Solution with Advanced Light Scattering Detection
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
5991-0835EN	Development and partial validation of a SEC method for high-resolution separation and quantification of monoclonal antibodies

Additional Information

A number of standards are available to assist with method development, molecular weight estimates, and system QC testing.

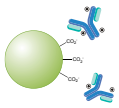
Part Number	Title
5190-9416	AdvanceBio SEC 130 Å Protein Standard, lyophilized, 1.5 mL
5190-9417	AdvanceBio SEC 300 Å Protein Standard, lyophilized, 1.5 mL
PL2070-0100	Polyethylene glycol calibration kit, PEG-10, 10 x 0.5 g
PL2080-0101	Polyethylene oxide calibration kit, PEO-10, 10 x 0.2 g
PL2090-0101	Pullulan Polysaccharide calibration kit

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 multiply 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.

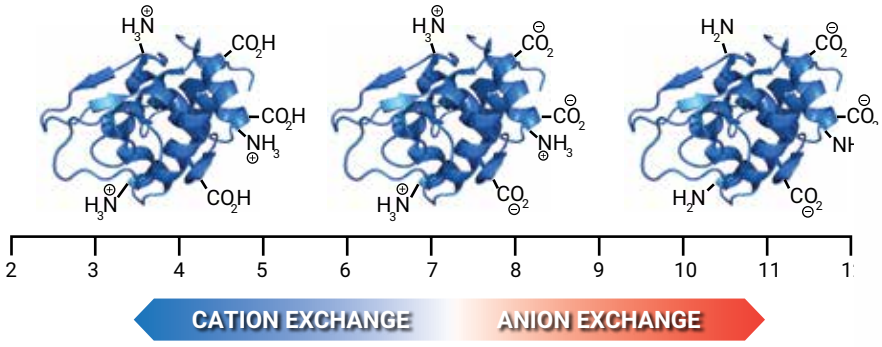
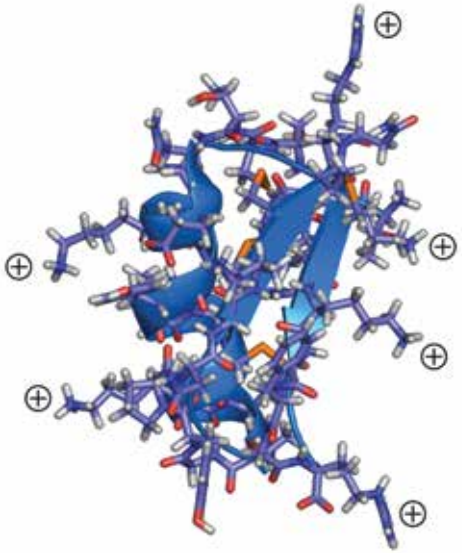


Figure 1. Effect of pH on net protein charge



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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.

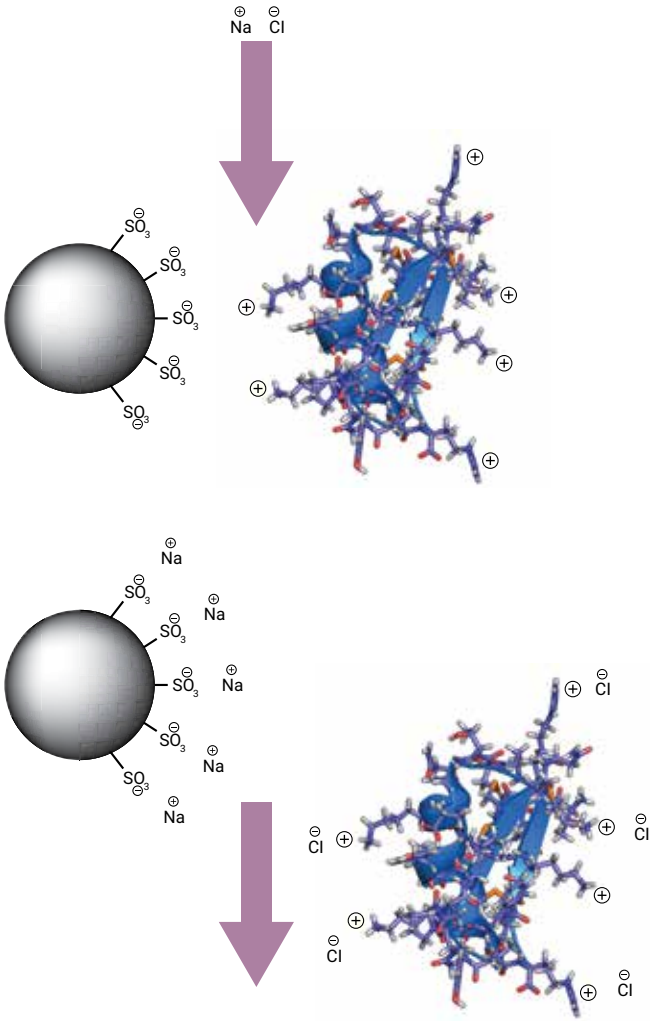

Figure 2. Separation mechanism of ion-exchange

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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.

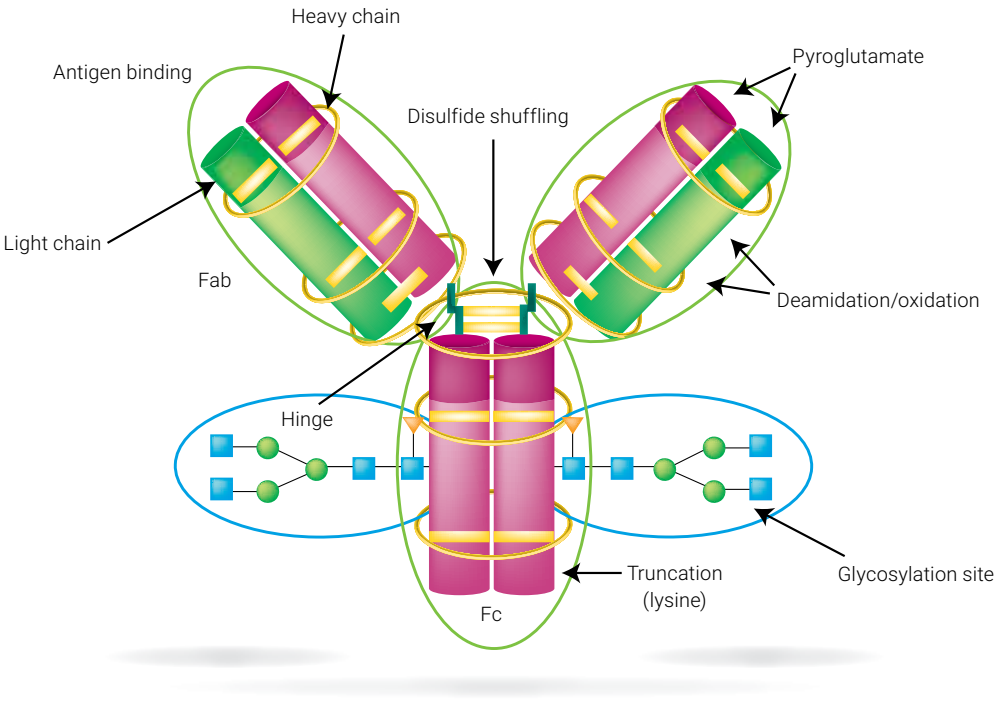
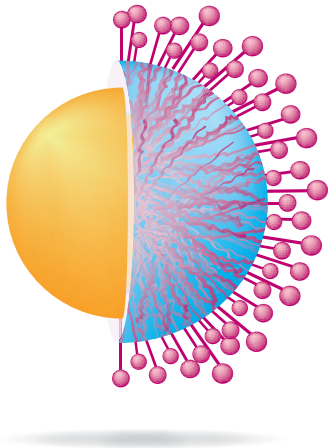


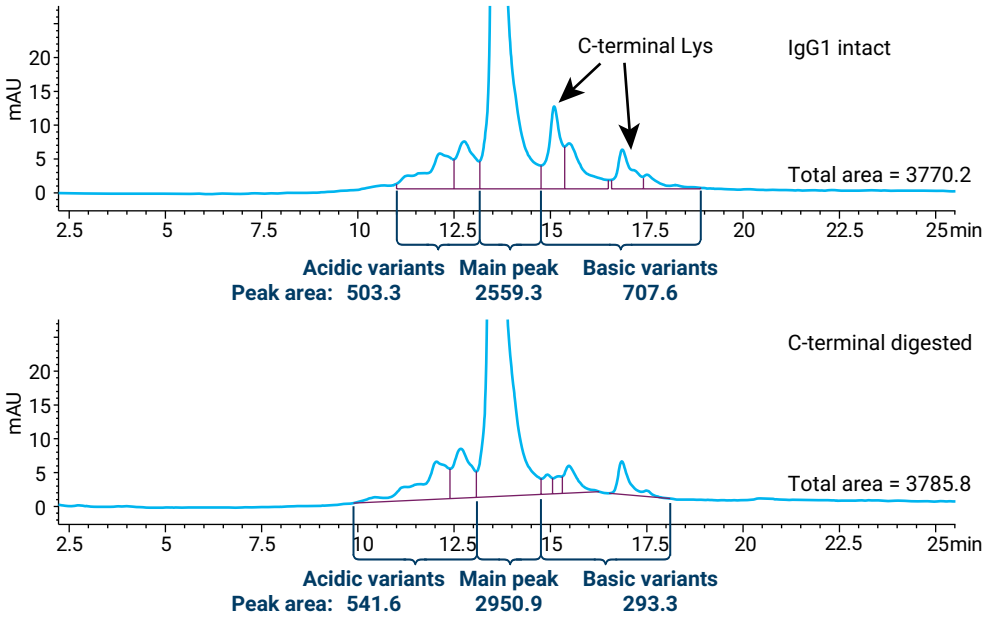
Figure 3. Charged variants of monoclonal antibodies arise through different levels of glycosylation, deamidation, and oxidation of amino acids, and through lysine truncation of heavy chains

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

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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.

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).

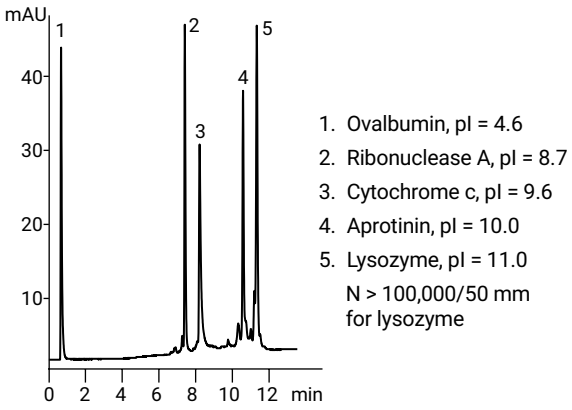

Figure 5. Exceptional separating power of Agilent Bio SCX

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

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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

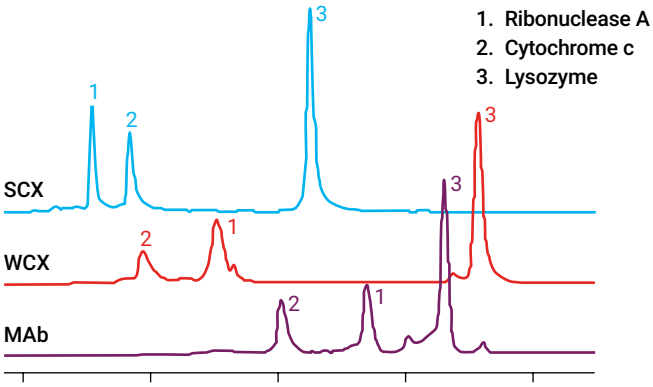

Figure 6. Separation of protein standards on Agilent 3 µm ion-exchange columns by cation-exchange chromatography

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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

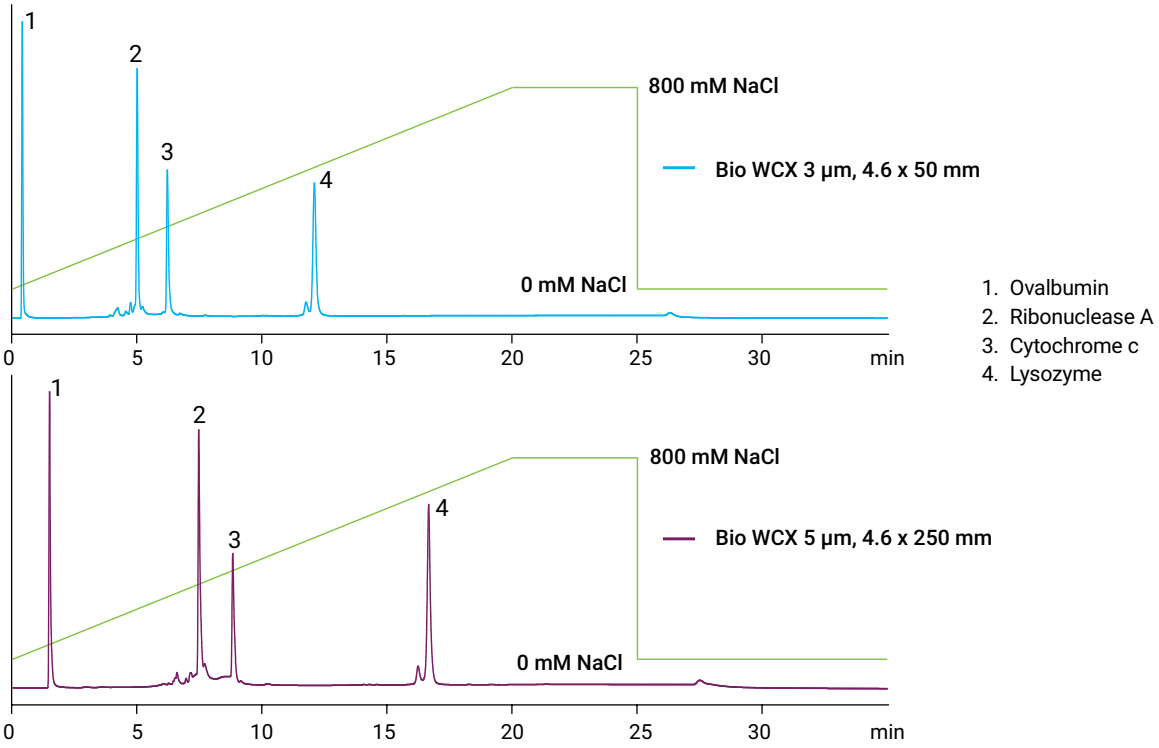

Figure 7. Protein separation on Agilent Bio WCX columns (4.6 x 50 mm, 3 µm and 4.6 x 250 mm, 5 µm) at a flow rate of 1 mL/min. Faster analysis times were achieved with smaller particle size and shorter column length – samples eluted from the longer column in 17 minutes but in only 12 minutes from the 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.

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

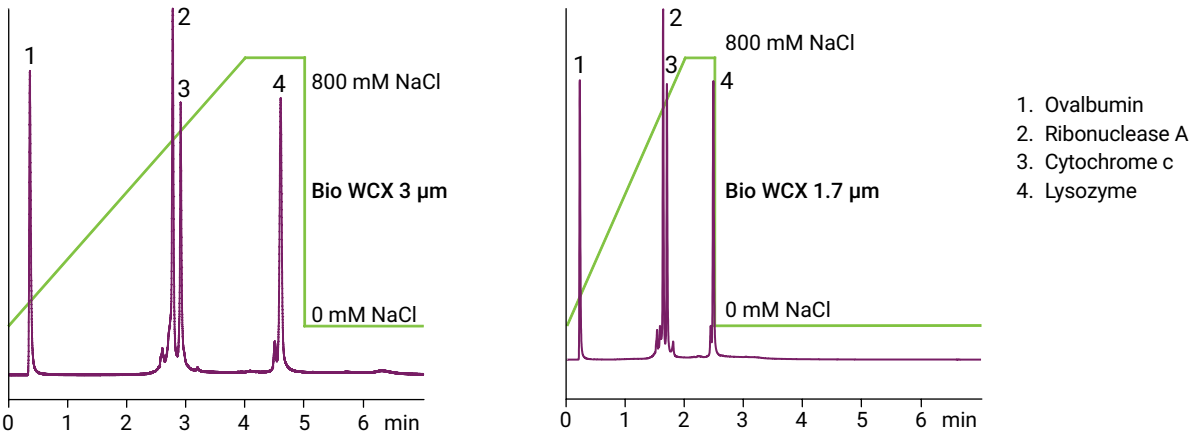

Figure 8. Reduce analysis time – without sacrificing peak shape and resolution – by increasing flow rate

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

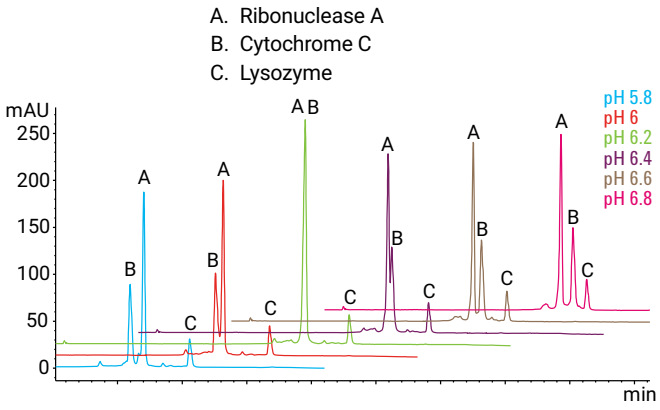

Figure 9. pH scouting for the separation of a three-protein mix using dynamically mixed quaternary gradients

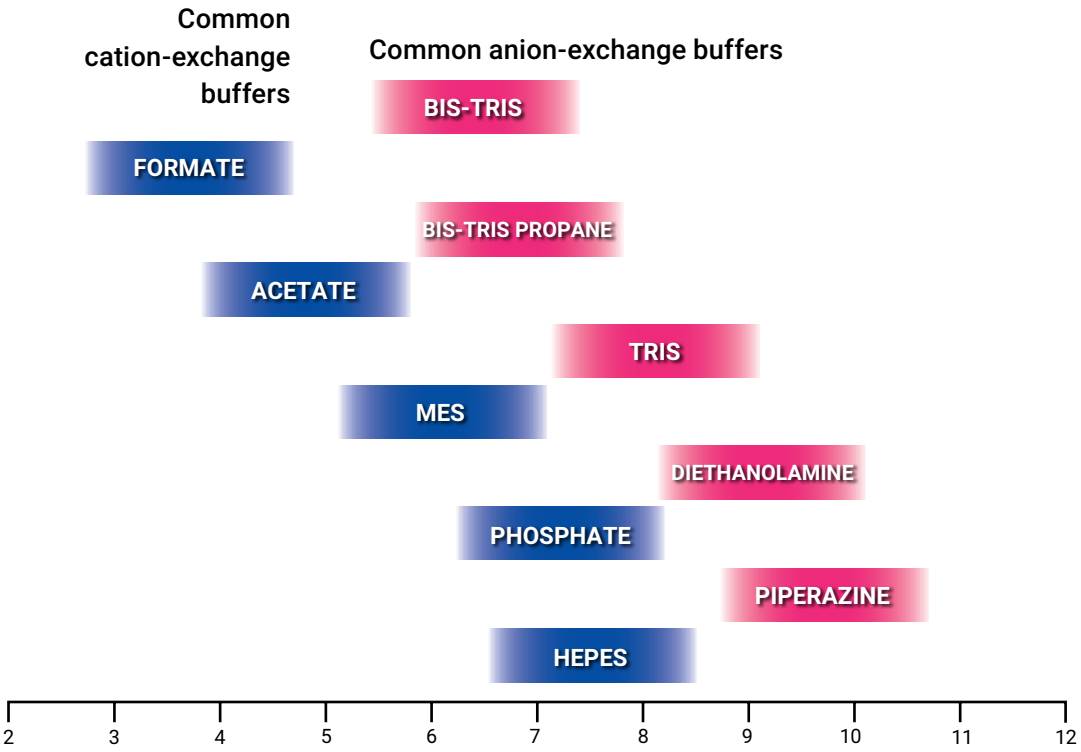

Figure 10. Commonly available buffers for ion-exchange

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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).

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.

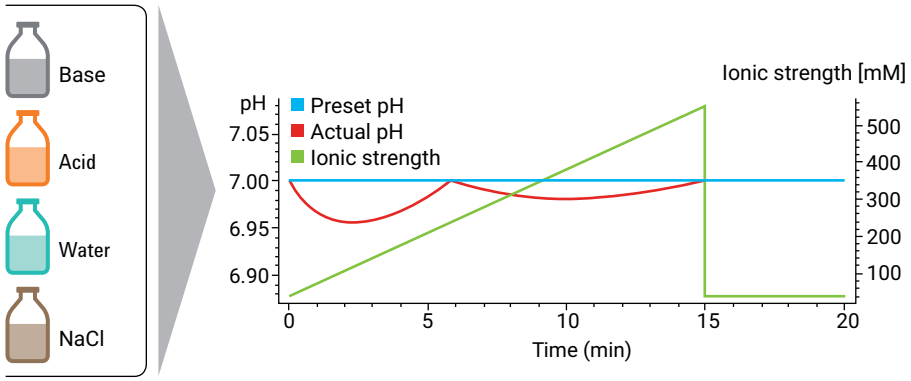

Figure 11. Salt gradients are easily created from stock solutions with Agilent Buffer Advisor Software

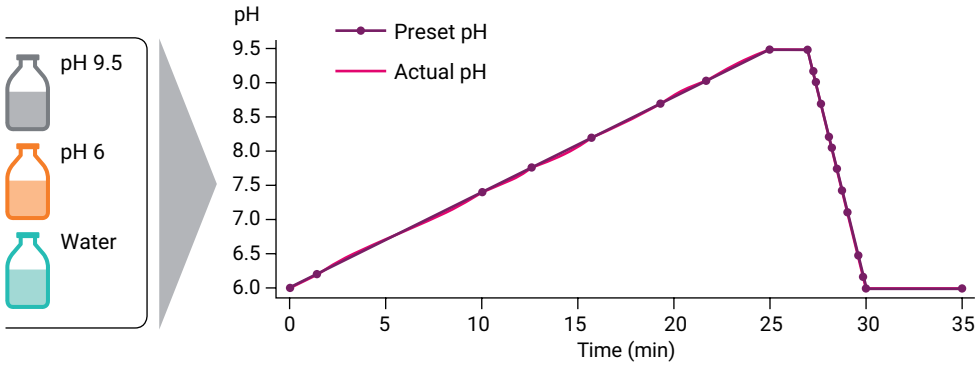

Figure 12. Optimizing buffer strength for a monoclonal antibody separation – pH gradients are easily created from stock solutions

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

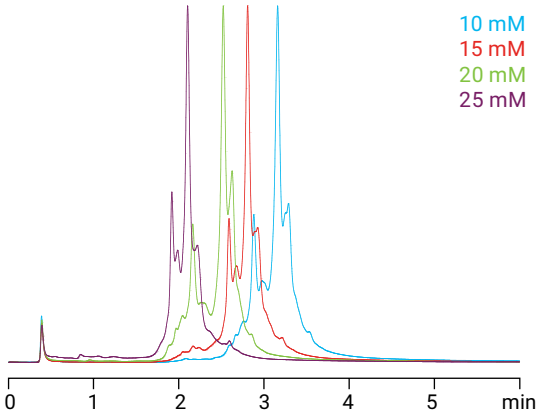

Figure 13. Optimizing buffer strength at pH 6.5 from the screening chromatograms of a monoclonal IgG separation

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

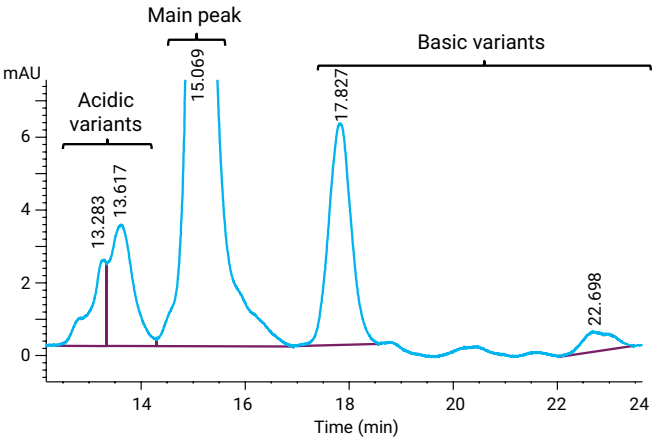

Figure 14. pH gradient-based cation-exchange chromatogram of an IgG1 separation using an Agilent Bio MAb PEEK, 4.6 x 250mm, 5 µm column

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Application Note

Charge Variant Analysis

Agilent

Trusted Answers

Charge Heterogeneity Analysis of Rituximab Innovator and Biosimilar mAbs

Authors

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Bangalore, India

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

Recombinant monoclonal antibodies (mAbs) are important biotherapeutics with a wide range of diagnostic and clinical applications. 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.

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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.

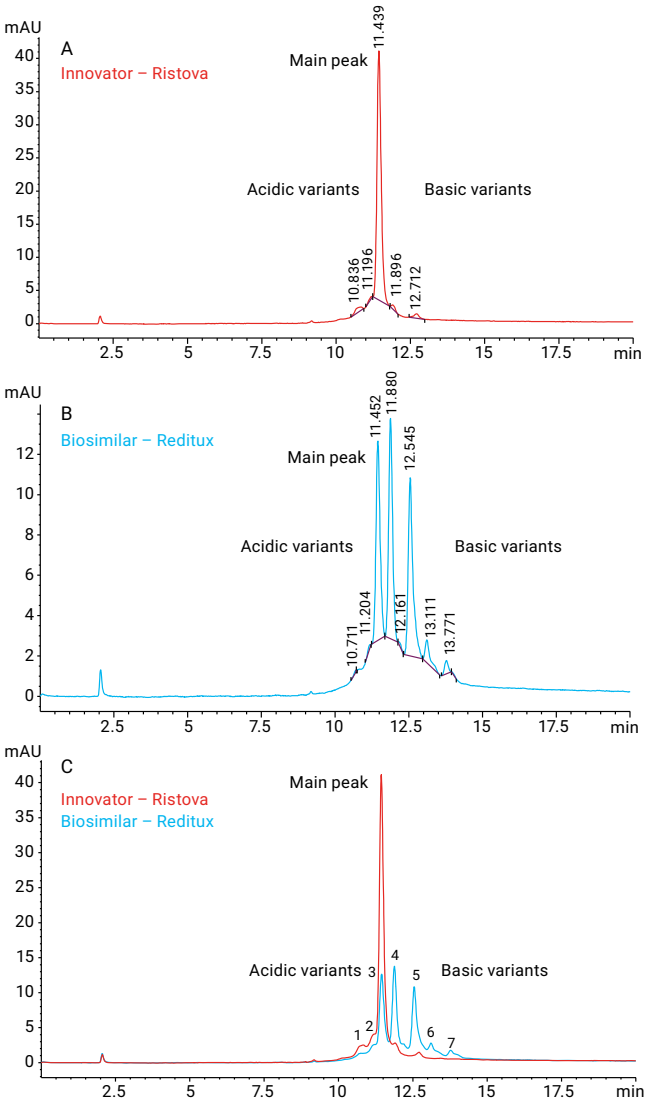

Figure 1. Charge variant profiles of innovator (A) and biosimilar (B) rituximab using an Agilent Bio MAb 5 µm column. C) Overlay of innovator and biosimilar rituximab. Peaks 1 and 2: acidic variants; 3: main form; 4, 5, 6 and 7: basic variants.

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

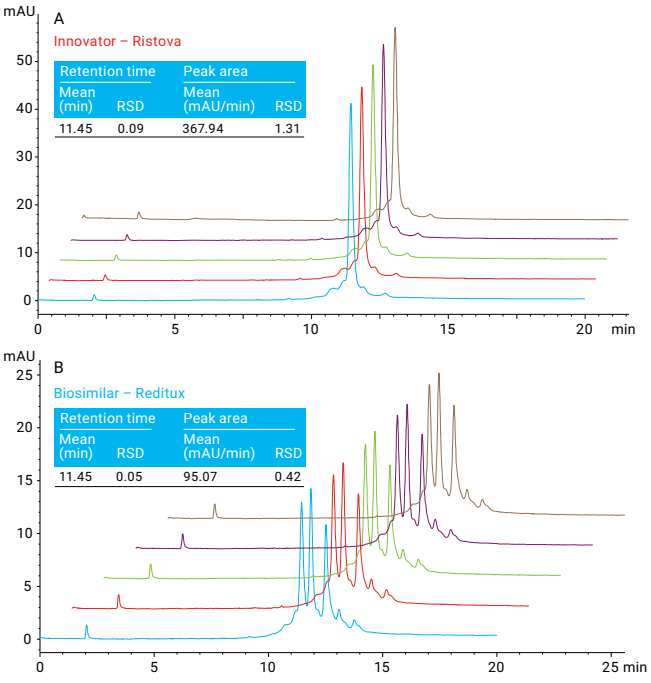

Figure 2. Overlay of five replicates of innovator (A) and biosimilar (B) rituximab on an Agilent 1260 Infinity Bio-inert Quaternary LC using an Agilent Bio Mab, 4.6 × 250 mm, 5 µm, PEEK column. Insert table shows the precision of retention time and area for main peak, n = 5.

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.

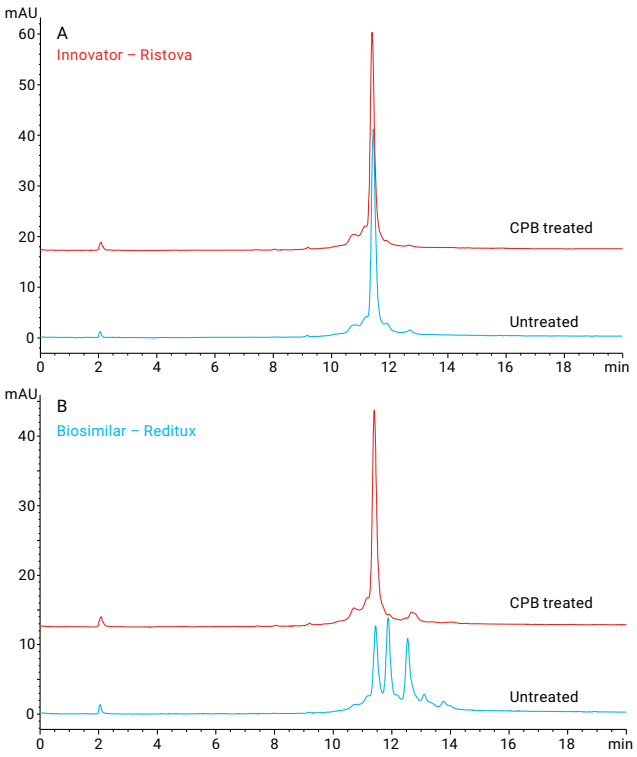

Figure 3. Characterization of basic charge variants. Separation of CPB treated (overnight) and untreated of innovator (A) and biosimilar (B) rituximab on an Agilent 1260 Infinity Bio-inert Quaternary LC using an Agilent Bio Mab, 4.6 × 250 mm, 5 µm, PEEK column.

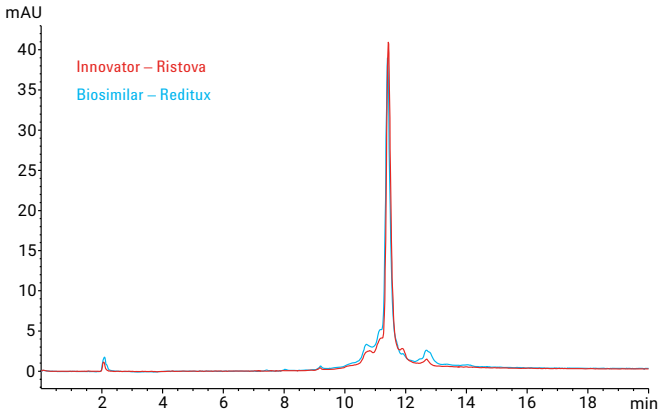

Figure 4. Overlay of innovator rituximab without CPB treatment (red) and biosimilar rituximab after CPB treatment (blue).

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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

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Optimizing Protein Separations with Cation Exchange Chromatography Using Agilent Buffer Advisor

Protein separation with the Agilent 1260 Infinity Bio-inert Quaternary LC System

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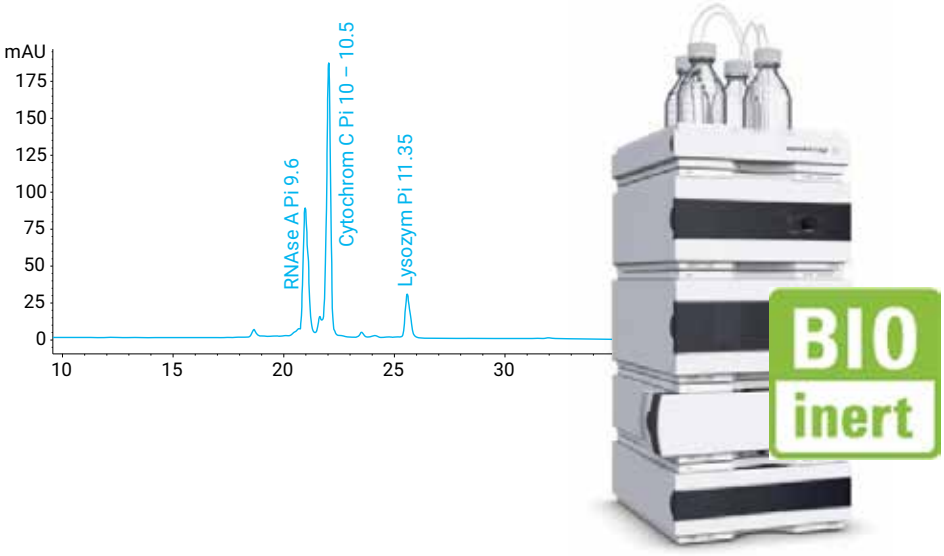
Abstract

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.



Verified for Agilent
1260 Infinity II LC
Bio-inert System

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.

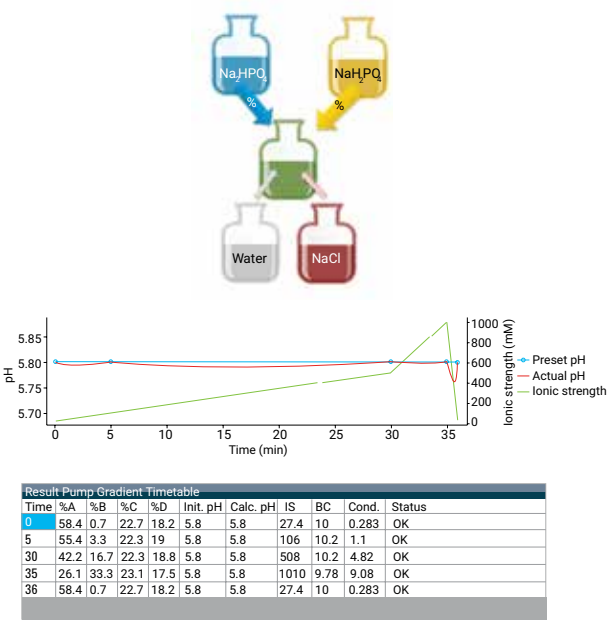
In general, a premixed two-component gradient is prepared for analysis with a starting buffer of low ionic strength and an elution buffer containing high ionic strength. This includes the following preparation steps:

- 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).

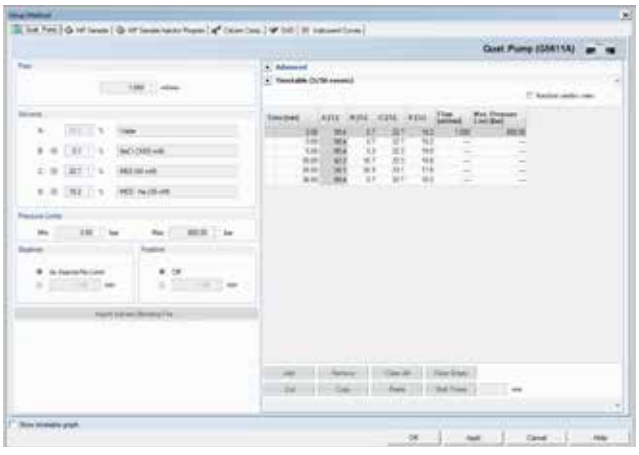


Figure 2. The generated Timetable can be imported into the method of the Agilent 1260 Infinity Bio-inert LC System through the Agilent OpenLAB CDS ChemStation Edition software.

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.

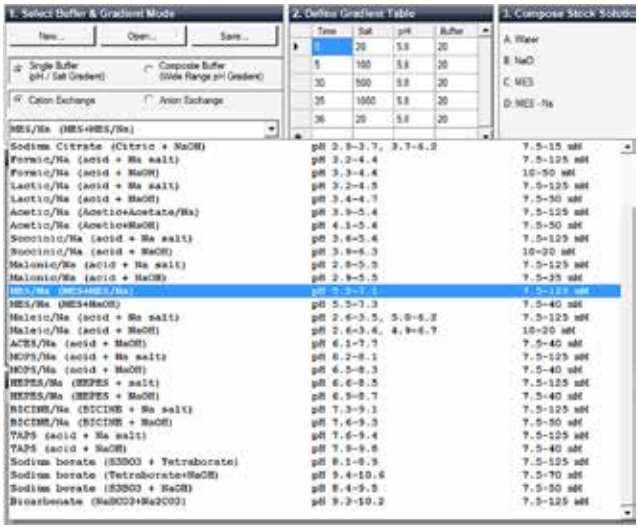


Figure 3. Buffer list for cation exchange chromatography, sorted by recommended pH range.

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.

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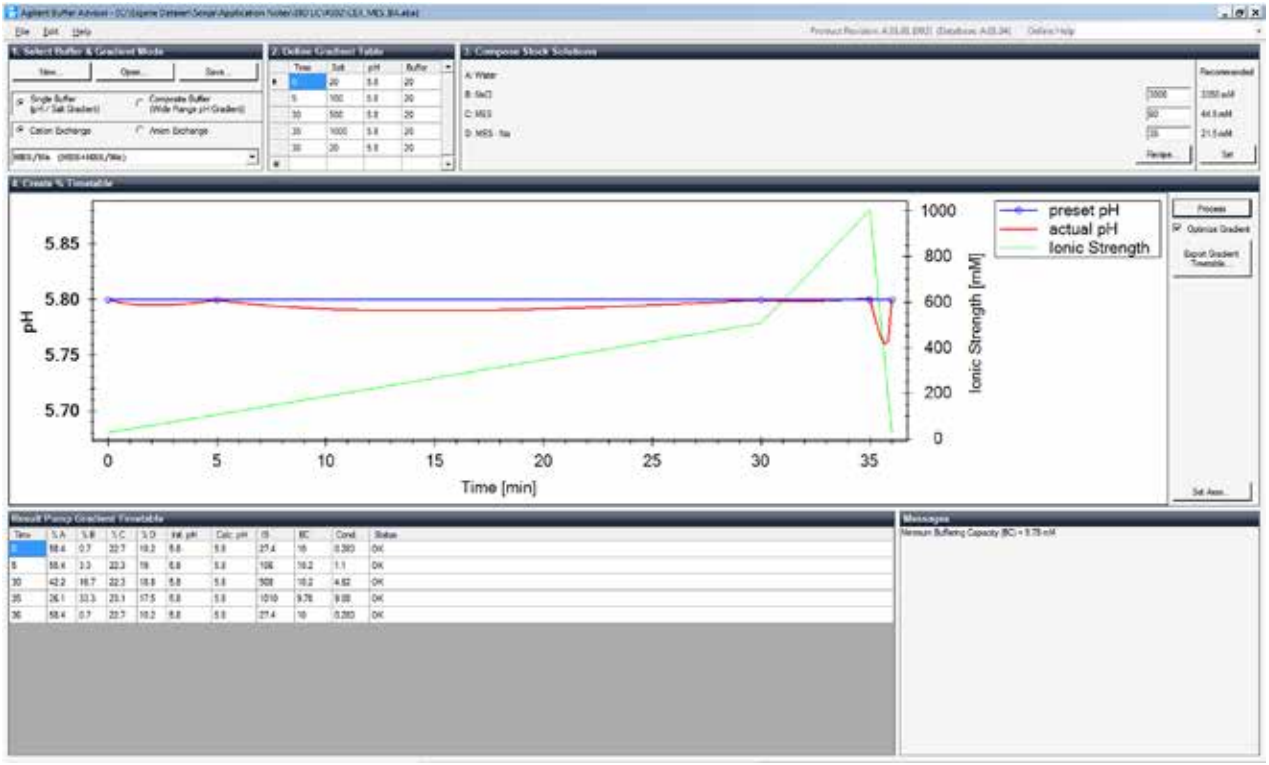

Figure 4. Agilent Buffer Advisor software, showing the steps described in "Results and discussion".

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.

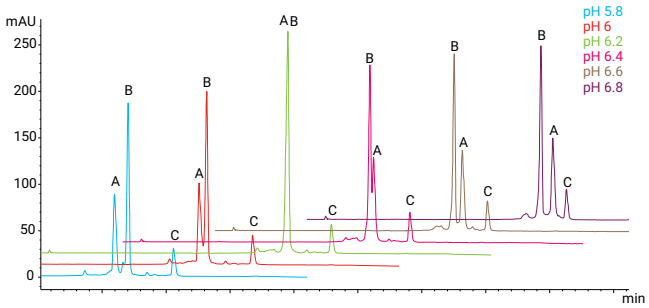

Figure 6. pH scouting for the separation of a three-protein mix using dynamically mixed quaternary gradients.

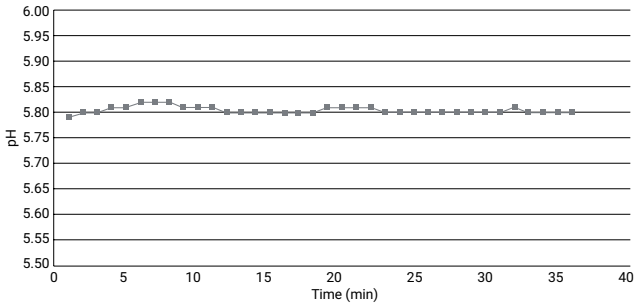

Figure 7. Off line pH measurement

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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

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Application Note

Charge Variant Analysis

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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.

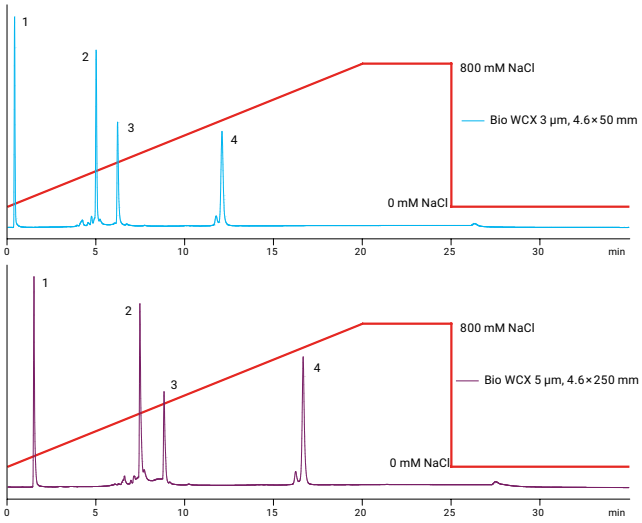

Figure 1. Protein separation on Agilent Bio WCX 5 µm 4.6 × 250 mm versus Agilent Bio WCX 3 µm, 4.6 × 50 mm (flow rate 1.0 mL/min).

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.

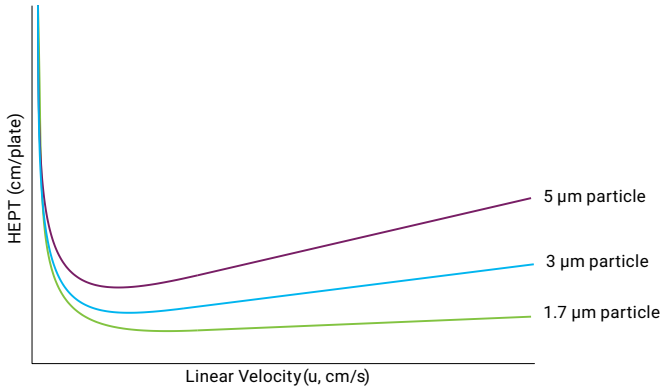

Figure 2. Typical van Deemter curves.

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)

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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).

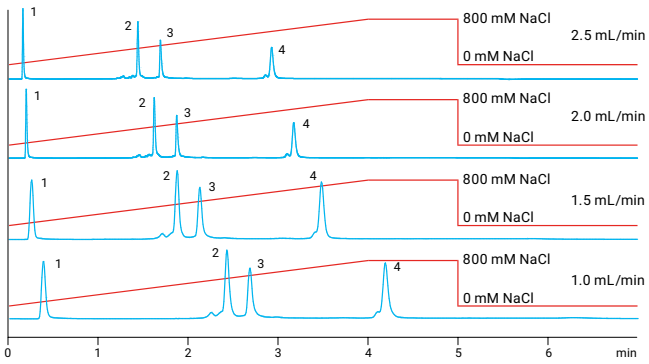

Figure 3. Effect of flow rate on chromatographic performance (Agilent Bio WCX 3 µm, 4.6 × 50 mm).

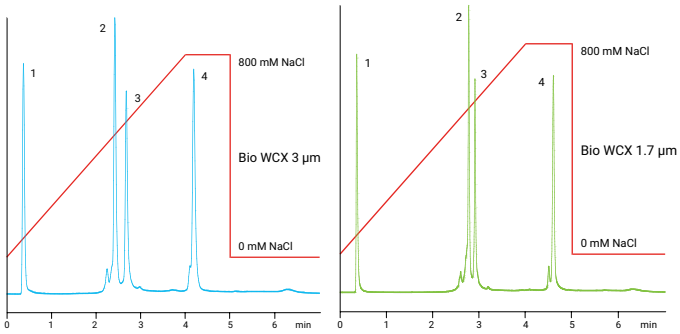

Figure 4. Comparison of Agilent Bio WCX 3 µm, 4.6 × 50 mm versus Agilent Bio WCX 1.7 µm

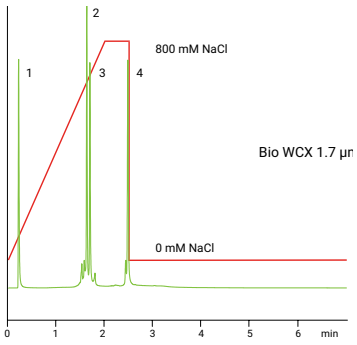

Figure 5. Agilent Bio WCX 1.7 µm, 4.6 × 50 mm for protein separations under 3 minutes (flow rate 1.7 mL/min)

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

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

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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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