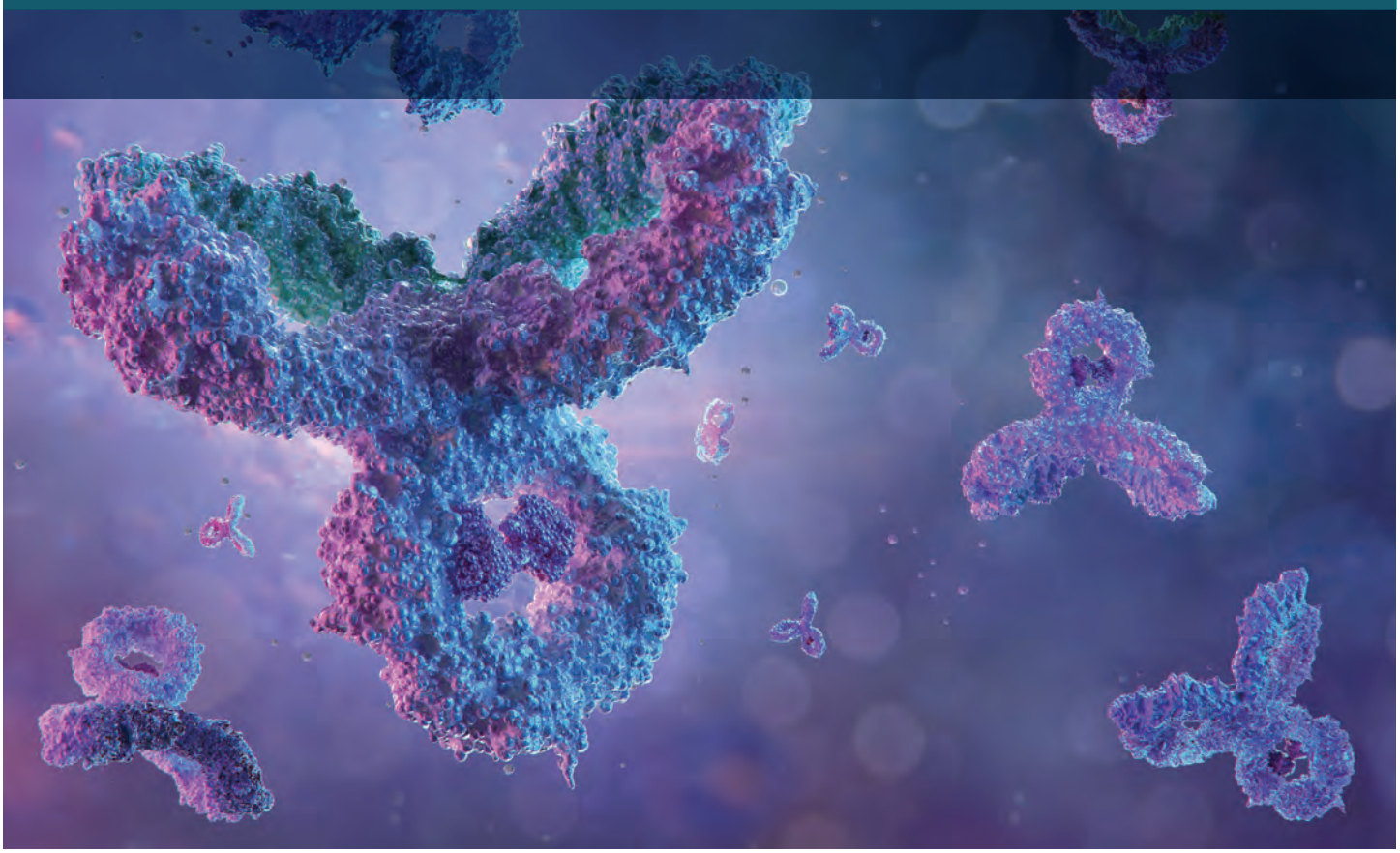
Agilent

Trusted Answers

Agilent Biocolumns

Peptide Mapping

Application Compendium



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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.

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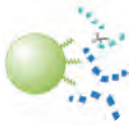

Peptide Mapping

Reversed-phase chromatography (<150 Å)

Reliably characterizes primary sequence and detects PTMs

AdvanceBio Peptide Mapping

Protein identification and PTM analysis

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

AdvanceBio Peptide Plus

Ideal formic acid performance for MS detection

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

AdvanceBio EC-C18

Ideal for samples susceptible to stainless steel on-column artifacts

Attribute	Advantage
PEEK hardware	Eliminates on-column metal interactions

Getting Started

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

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

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

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

Step 1:

Sample preparation

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

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

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

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

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

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

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

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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

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Step 2:

Selection of cleavage agents

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

Table 2. Cleavage Type.

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

Step 3:

Denaturation, reduction, and alkylation

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

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

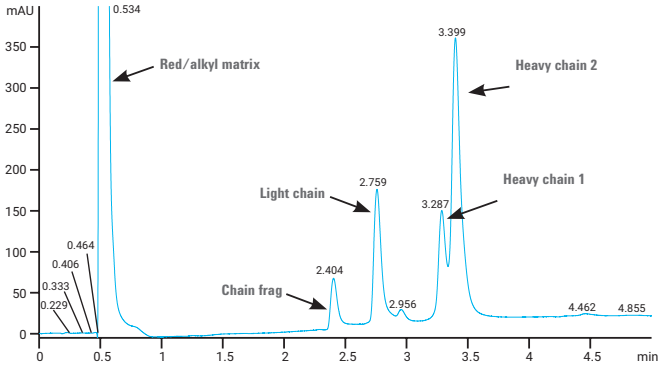

Figure 1. Reversed-phase separation of a reduced and alkylated monoclonal antibody prior to digestion protocol using an Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C8, 2.1 x 50 mm column (Agilent p/n 857750-906). The separation was performed at 0.5 mL/min, 75 °C using water (0.1% TFA)/ACN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

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Step 4:

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

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

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

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

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

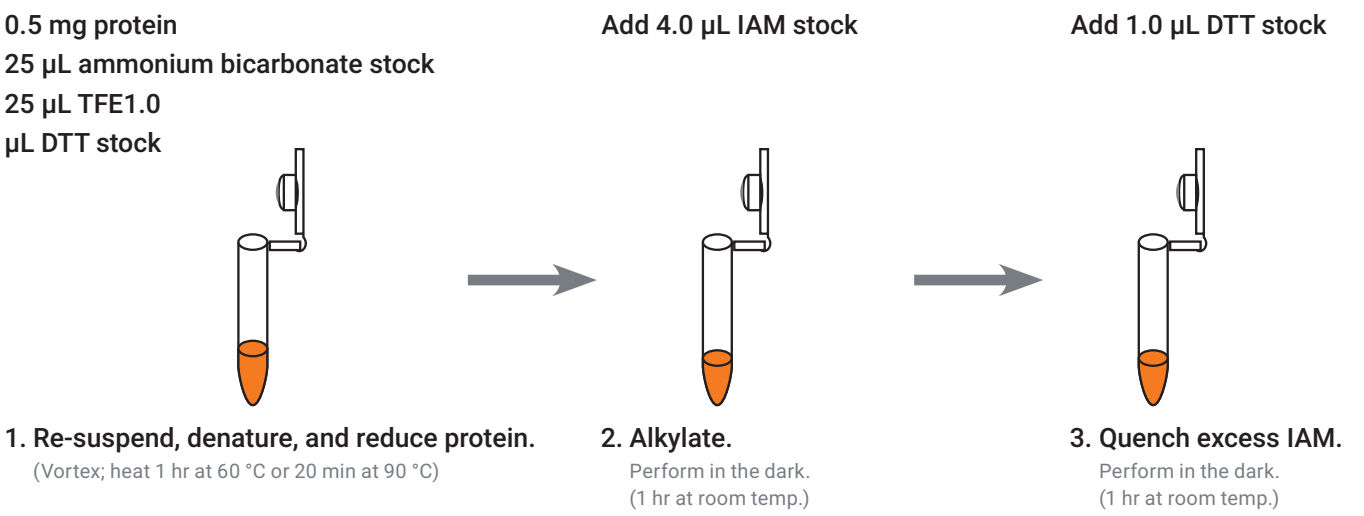
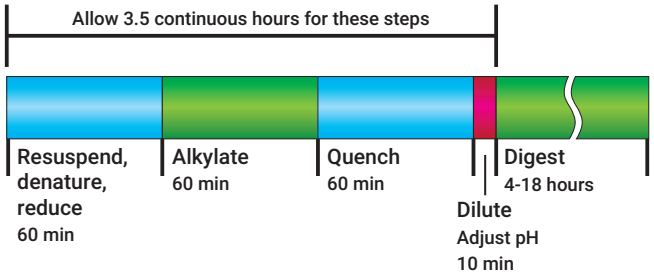


Figure 3 – Expected timeline for digestion procedure



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

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Reduction, alkylation, digestion solution preparation: Summary

100 mm ammonium bicarbonate:

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

Trypsin stock:

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

200 mM DTT:

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

200 mm IAM

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

Trypsin digestion protocol

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

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

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Step 5:

Cleanup and enrichment of digests

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

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

Trypsin digestion protocol

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

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

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

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

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

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

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

Jacob D. Jaffe, Ph.D.

Assistant Director - Proteomics Platform

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

Reversed-Phase Chromatography: The Superior Choice for Peptide Mapping

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

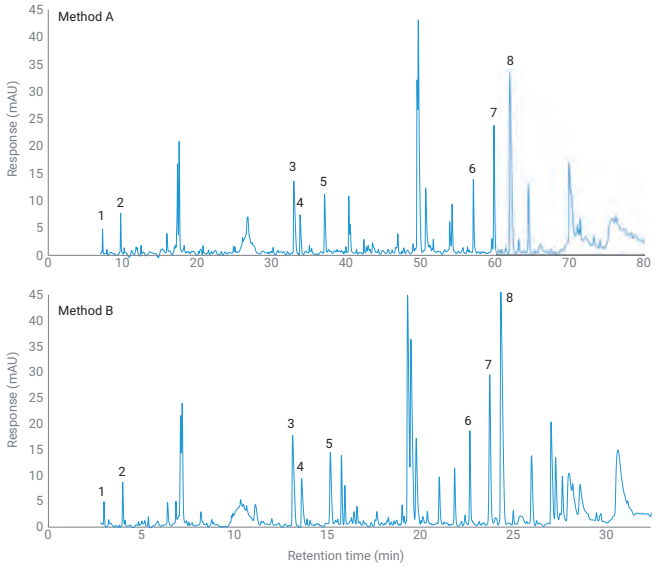
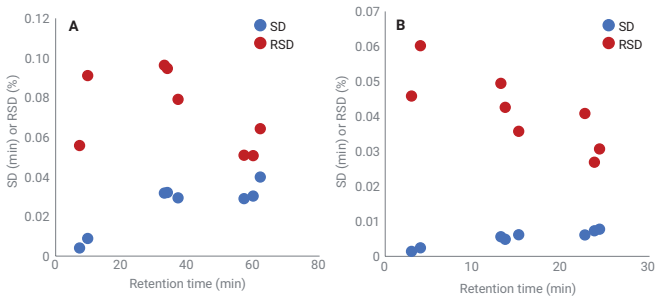


Figure 4. Chromatograms of a tryptic digest of the NISTmAb separated by the Agilent 1290 Infinity II Bio LC with Methods A and B. Eight peaks were picked for subsequent retention time precision and peak capacity calculations.



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

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

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

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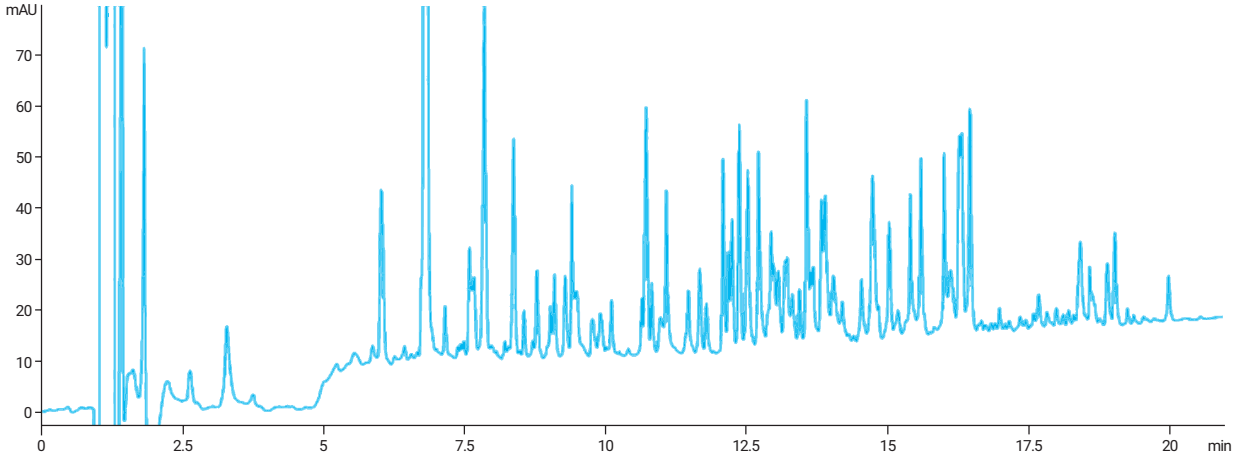

Figure 6. Reversed-phase separation of BSA using an Agilent AdvanceBio Peptide Mapping 2.1 x 150 mm column (Agilent P/N 653750-902). The peptide mapping separation was performed at 0.3 mL/min, 40 °C using water (0.1% TFA)/ACN (0.08%) linear gradient.

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

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

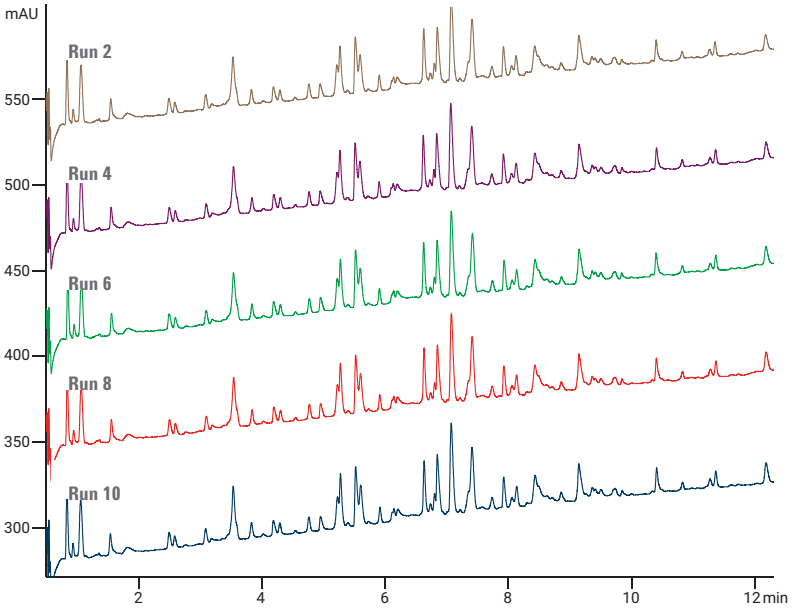

Figure 7. Five replicate injections of a monoclonal antibody tryptic digest using a 3.0 x 150 mm Agilent AdvanceBio Peptide Mapping column (Agilent P/N 653950-302) on an Agilent 1200 LC system coupled to a 6520 Q-TOF. Separation was performed at 0.3 mL/min, 40 °C using water (0.1% FA)/ACN (0.1% FA) gradient.

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Mobile phase selection

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

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

Detection

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

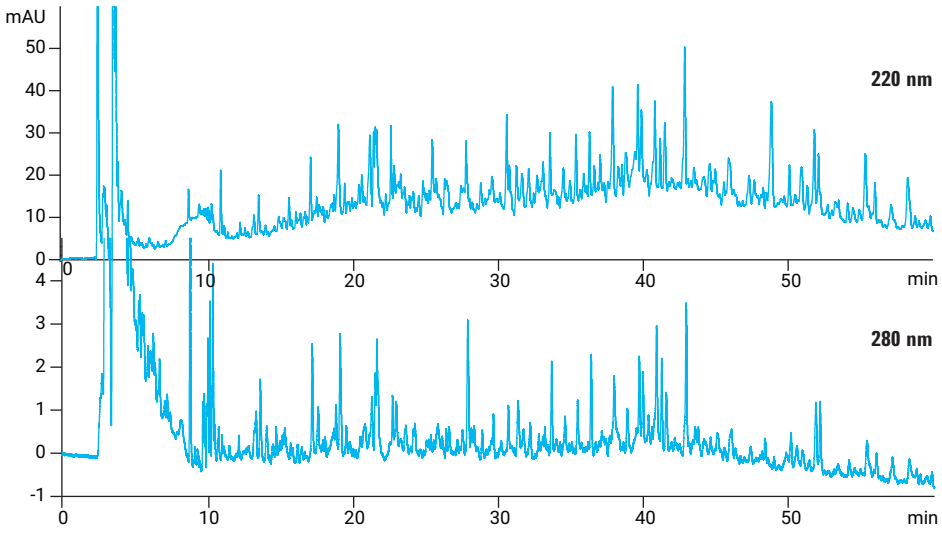

Figure 8. AdvanceBio Peptide Mapping column (Agilent P/N 651750-902), 2.1 x 250 mm, profiling e.coli digest at 220 nm (top) and 280 nm (bottom) on an Agilent 1290 Infinity LC.

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Developing an Efficient Peptide Mapping Method

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

(1) Optimizing the gradient conditions

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

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

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

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

(2) Variables for changing selectivity of the peptide map

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

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

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

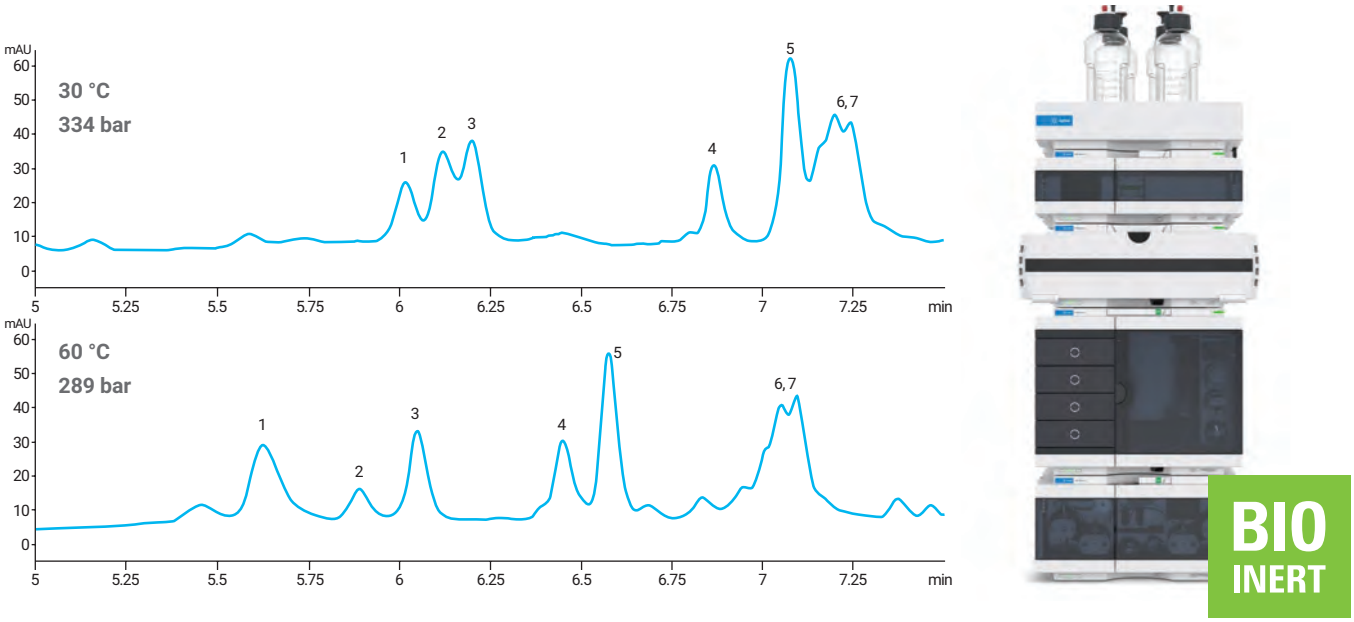

Figure 9. Myoglobin tryptic digest gradient separation at 5.0-8.0 min of a 20 min gradient with a 2.1 x 150 mm AdvanceBio Peptide Mapping column (Agilent P/N 653950-302). Both separations were completed with a water (1.0 % TFA)/ACN (0.08% TFA) linear gradient, 0.3 mL/min at 215 nm on an Agilent 1260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

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

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

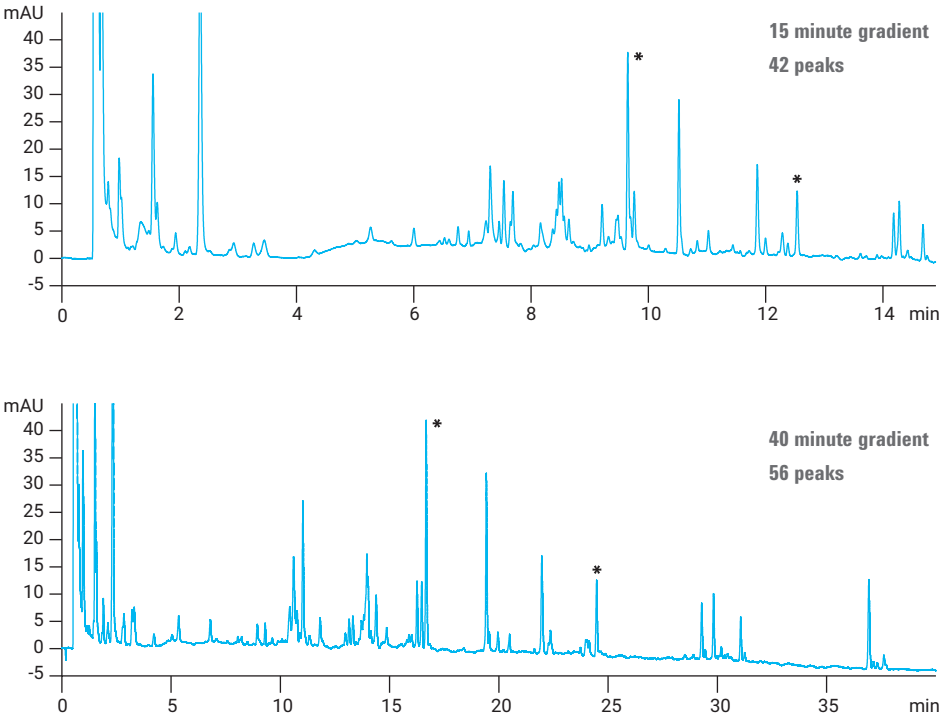

Figure 10. Myoglobin tryptic digest gradient separations with a 2.1 x 150 mm AdvanceBio Peptide Mapping column (Agilent P/N 653950-302) on an Agilent 1260 Infinity Bio-inert Quaternary LC system using water (1.0 %TFA)/ACN (0.08% TFA) linear gradient, 0.6 mL/min at 50 °C. The top chromatogram was completed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

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(3) Adjusting column conditions for further optimization

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

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

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

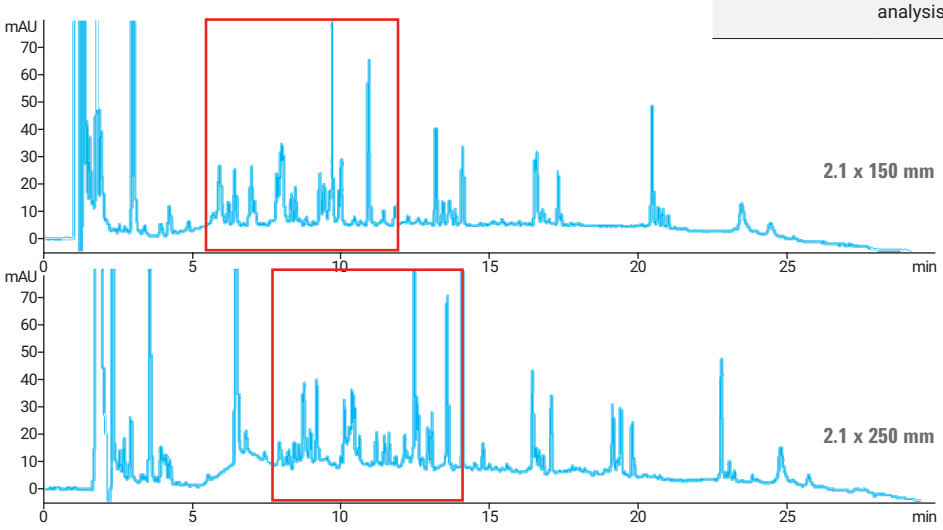

Figure 11. Effect of column length on resolution, a peptide mapping comparison using a myoglobin tryptic digest (Agilent P/N 651750-902). Areas highlighted in red indicate equivalent areas of separation to emphasize resolution and peak shape. The separations were performed using an Agilent AdvanceBio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

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Peptide Mapping Characterizations by Mass Spectrometry

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

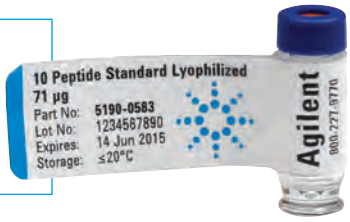
Peptides are analyzed by mass spectrometry by direct infusion of the isolated peptides – or by the use of on-line LC/MS for structure analysis – and then correlated to the protein amino acid sequence. The identified peptides thus confirm the specific amino acid sequences covered by the peptide map, as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

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

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

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

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



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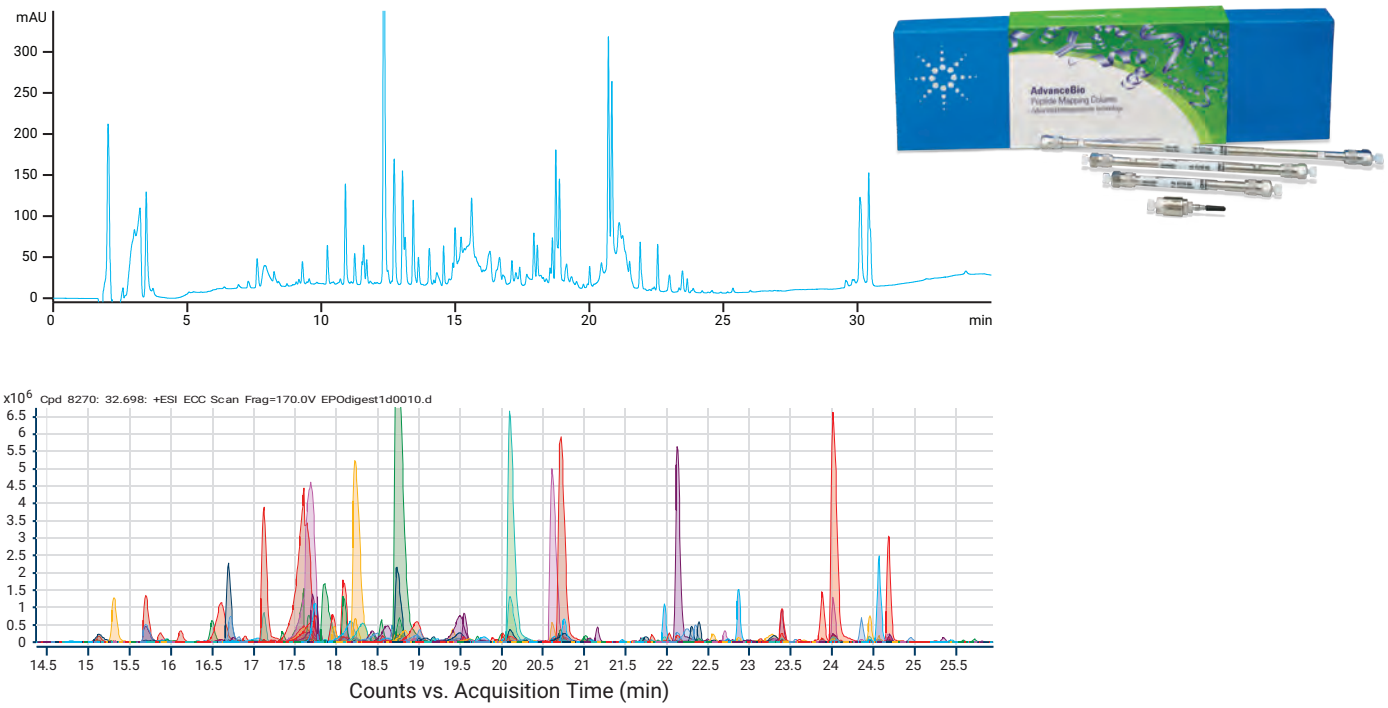

Figure 12. The top chromatogram displays a fully optimized EPO digest peptide mapping separation performed on a 2.1 x 150 mm AdvanceBio Peptide Mapping column. The bottom chromatogram shows the qualitative analysis (using a molecular feature extractor) for sequence coverage generated by on an Agilent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

Ordering Information

For peptide mapping, Agilent recommends:

AdvanceBio Peptide Mapping – the first choice for most applications

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

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

Peptide Quality Control Standard

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

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

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

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

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

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

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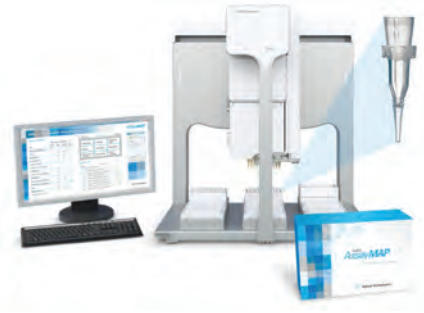
Peptide sample preparation for mass spec analysis, intelligently automated

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:

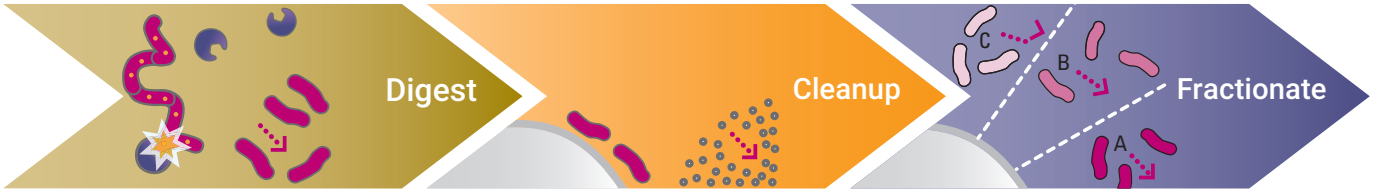
- Improved reproducibility, due to decreased human error – <5 % CVs
- Increased throughput – up to 384 samples each day
- Significantly reduces hands-on time – freeing up scientists to do analytical work
- Faster method development – the automated platform enables you to quickly optimize methods



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 13.

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

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

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

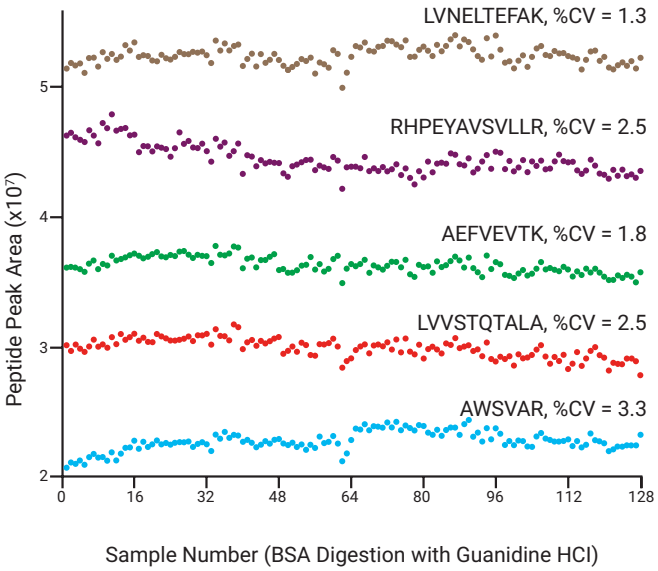


Figure 13. Scatter plots showing peak area of 4 peptides over 2 days.

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

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

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Application Note

Peptide Mapping

Agilent

Trusted Answers

Robust and Reliable Peptide Mapping

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

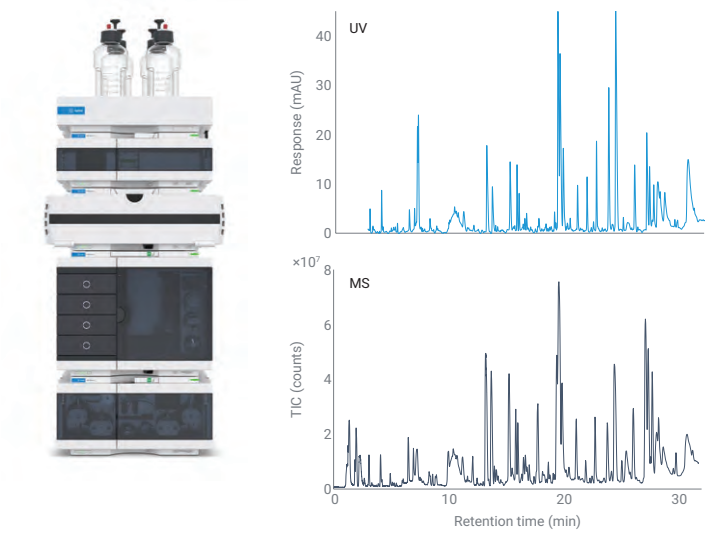
Author

André Feith

Agilent Technologies, Inc.

Abstract

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



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Introduction

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

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

Experimental

Equipment

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

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

Software

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

Columns

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

Chemicals

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

Sample preparation

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

Results and Discussion

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

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

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

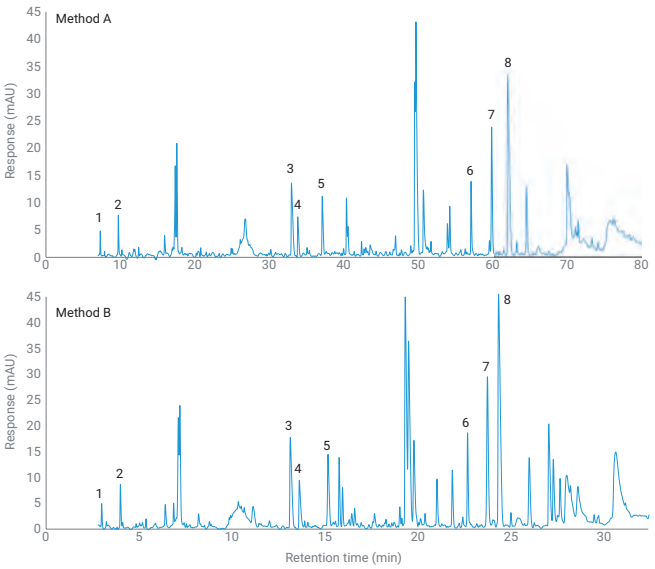
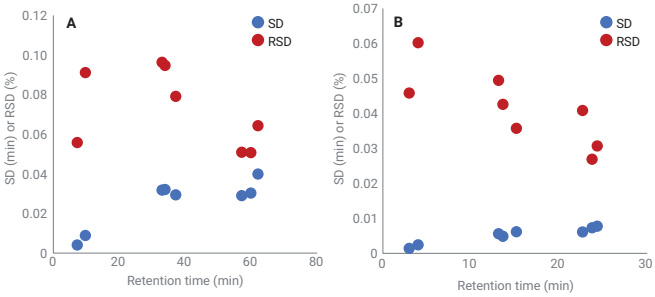


Figure 1. Chromatograms of a tryptic digest of the NISTmAb separated by the Agilent 1290 Infinity II Bio LC with Methods A and B. Eight peaks were picked for subsequent retention time precision and peak capacity calculations.



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

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

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

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

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

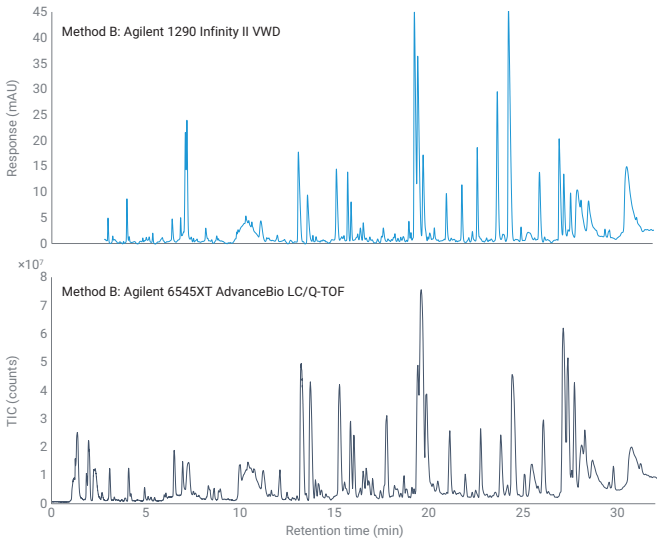

Figure 3. Chromatograms of a tryptic digest of the NISTmAb detected in sequence with the Agilent 1290 Infinity II VWD equipped with the biocompatible micro flow cell (upper) and the Agilent 6545XT AdvanceBio LC/Q-TOF (lower).

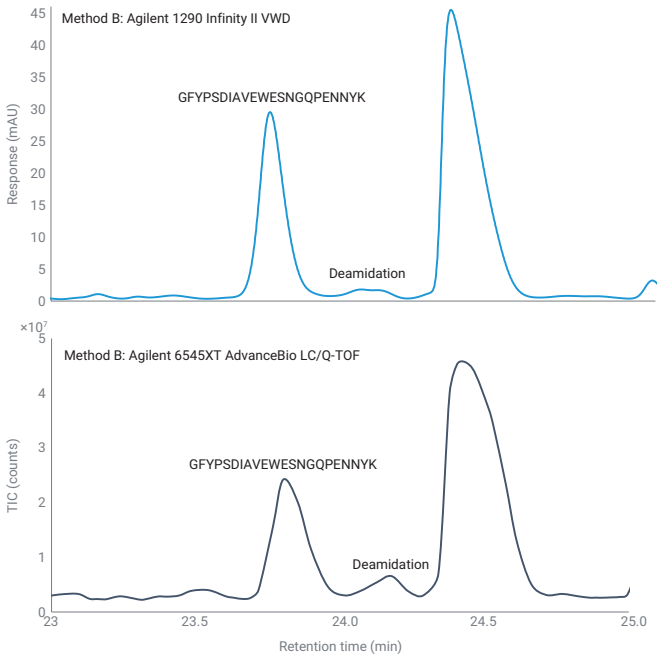

Figure 4. Magnified view of the previous chromatogram to highlight the separation of the PENNY peptide (GFYPSDIAVEWESNGQPENNYK) and corresponding deamidated isoform.

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Conclusion

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

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Application Note

Peptide Mapping

Agilent

Trusted Answers

Separation of Deamidated Peptides with an Agilent AdvanceBio Peptide Plus Column

Authors

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Agilent Technologies Inc.

Abstract

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

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Introduction

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

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

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

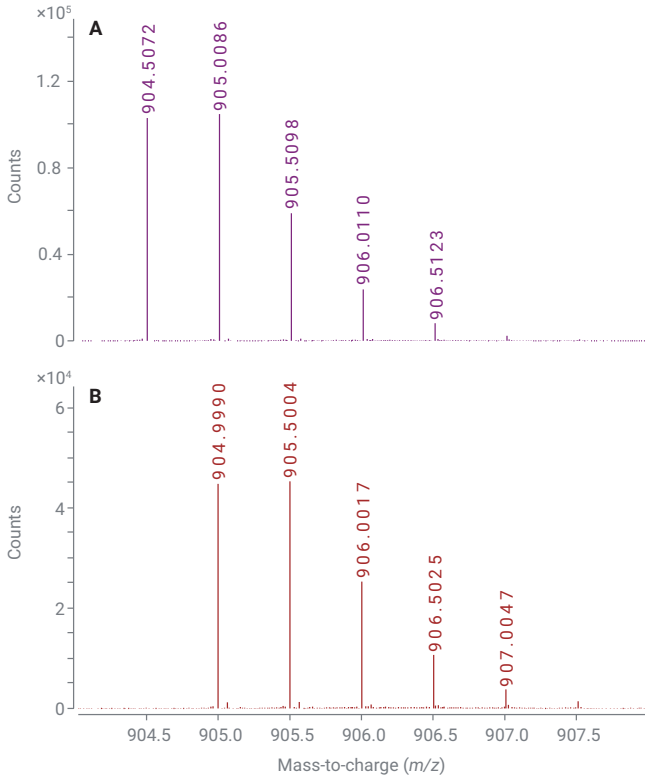

Figure 1. Mass spectrum of VVSVLTVLHQDWLNGK (A) and a deamidated variant of that peptide (B), showing the overlap between mass spectra.

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Experimental

Materials

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

Instrumentation

LC system

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

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

MS system

Agilent 6546 LC/Q-TOF.

Data processing

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

Results and discussion

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

Table 1.

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

LC conditions

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

MS conditions

Parameter	Agilent 6546 Q-TOF
Column	Agilent Jet Stream
Gas temperature	323 °C
Drying gas flow	13 L/min
Nebulizer gas	35 psi
Sheath gas temperature	275 °C
Sheath gas flow	11 L/min
Capillary voltage	4,000 V
Nozzle voltage	0 V
Fragmentor voltage	1255 V
Skimmer voltage	65 V
Oct 1 RF Vpp	750 V
Mass range	*m/z* 300 to 1,700
MS scan rate (spectra/s)	5
Acquisition Mode	Positive mode, extended dynamic range (2 GHz) Centroid data format
Data analysis	Agilent BioConfirm software B.08

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Column type

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

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

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

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

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

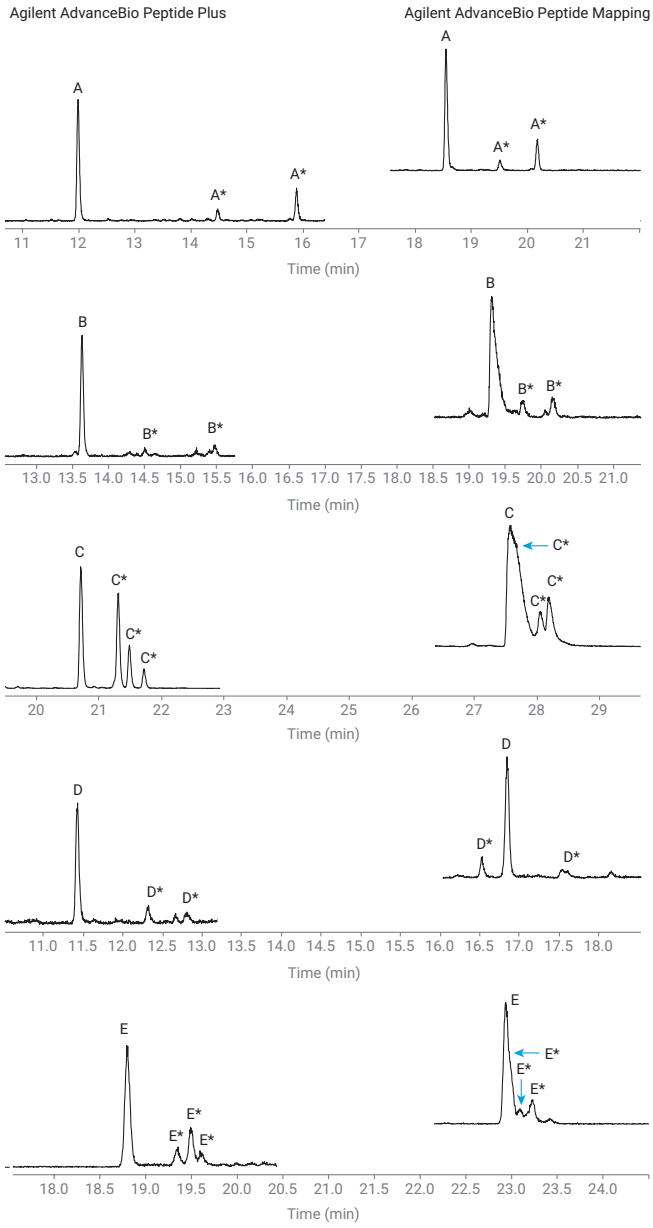

Figure 2. Separation of peptides and their deamidated variants (indicated by *) on the Agilent AdvanceBio Peptide Mapping column (elevated and eluting later) and the Agilent AdvanceBio Peptide Plus column (eluting earlier) under the same conditions with 0.1% formic acid mobile phase modifier.

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Mobile phase

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

Figure 3 shows the separation of peptide C from its variants when the aqueous and organic mobile phases are modified with 0.05%, 0.1 or 0.3% formic acid. Dropping the concentration to 0.05% increased selectivity and resolution, while at 0.3% formic acid, resolution was somewhat reduced. A similar pattern is observed for peptide E.

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

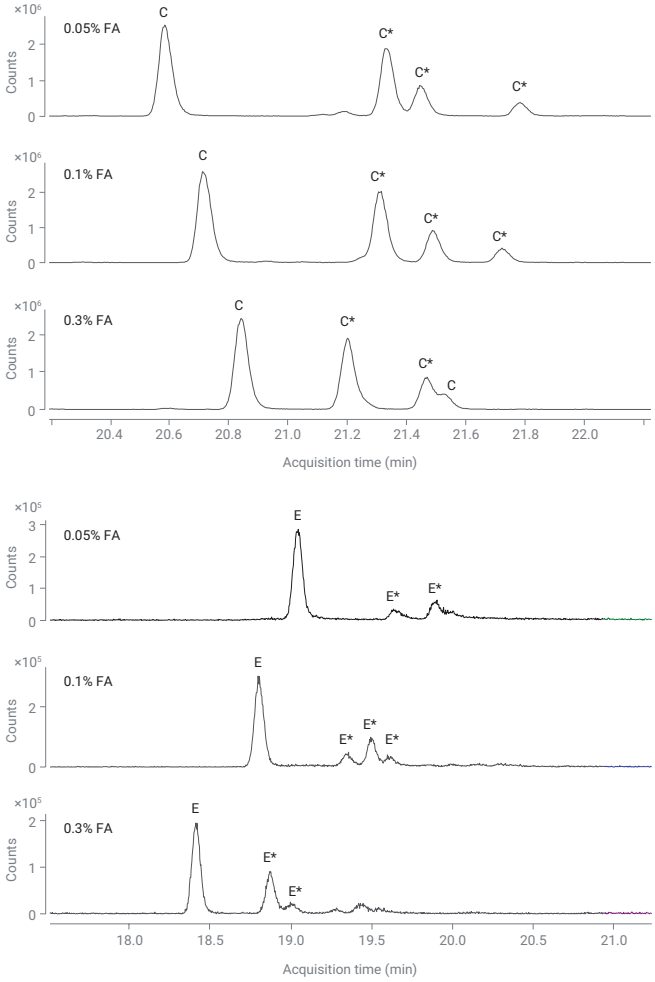

Figure 3. Separation of peptides on Agilent AdvanceBio Peptide Plus with increasing concentrations of formic acid mobile phase modifier. Selectivity for deamidated peptide variants over their native forms is maximized at lower concentrations.

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

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

Conclusion

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

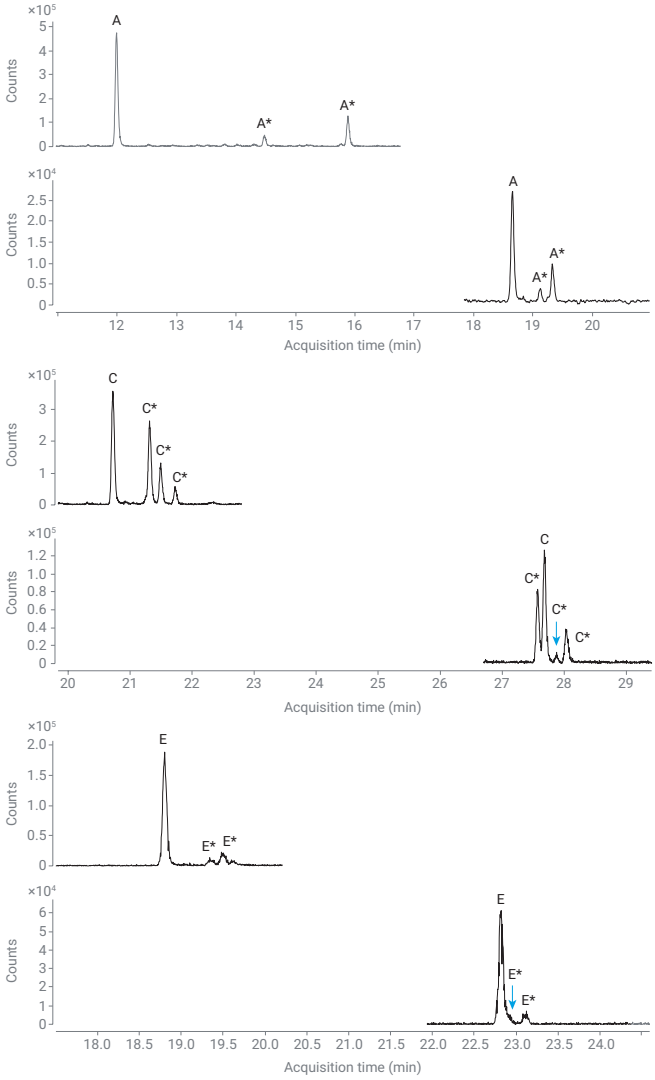

Figure 4. Separation of peptides and their deamidated variants on Agilent AdvanceBio Peptide Plus with 0.1% formic acid mobile phase modifier compared to 0.1% TFA.

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Application Note

Peptide Mapping

Agilent

Trusted Answers

Analysis of a Synthetic Peptide and Its Impurities

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

Authors

Andrew Coffey and

Veronica Qin

Agilent Technologies Inc.

Abstract

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

Introduction

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

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

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

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

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

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

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

Table 1.

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

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

Figure 1. The amino acid sequence of synthetic bivalirudin.

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Experimental

Reagents and chemicals

All reagents were HPLC grade or higher.

Sample preparation

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

Instrumentation

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

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

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

Data processing

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

Method conditions

Parameter	HPLC Conditions
Column	Agilent AdvanceBio Peptide Plus, 2.1 × 150 mm (p/n 695775-949)
Column temperature	60 °C
Mobile phase	A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile
Flow rate	0.4 mL/min
Gradient	Time (min) %B 0 17 2 17 22 37 24 95 26 95 26.1 17
Post time	5 minutes
Injection volume	5 µL (UV); 1 µL (MS)

Conditions

Parameter	Value
Instrument	Agilent 6545XT AdvanceBio LC/Q-TOF
Source	Dual Agilent Jet Stream
Gas Temperature	350 °C
Drying Gas Flow	10 L/min
Nebulizer Gas	30 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Fragmentor	125 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	*m/z* 100 to 1,700 (MS); *m/z* 50 to 1,700 (MS/MS)
MS Scan Rate	8 spectra/s
MS/MS Scan Rate	3 spectra/s
Acquisition Mode	Positive, extended dynamic range (2 GHz)
Collision Energy	$3.6 \times (m/z)/100 - 4.8$

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Results and discussion

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

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

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

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

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

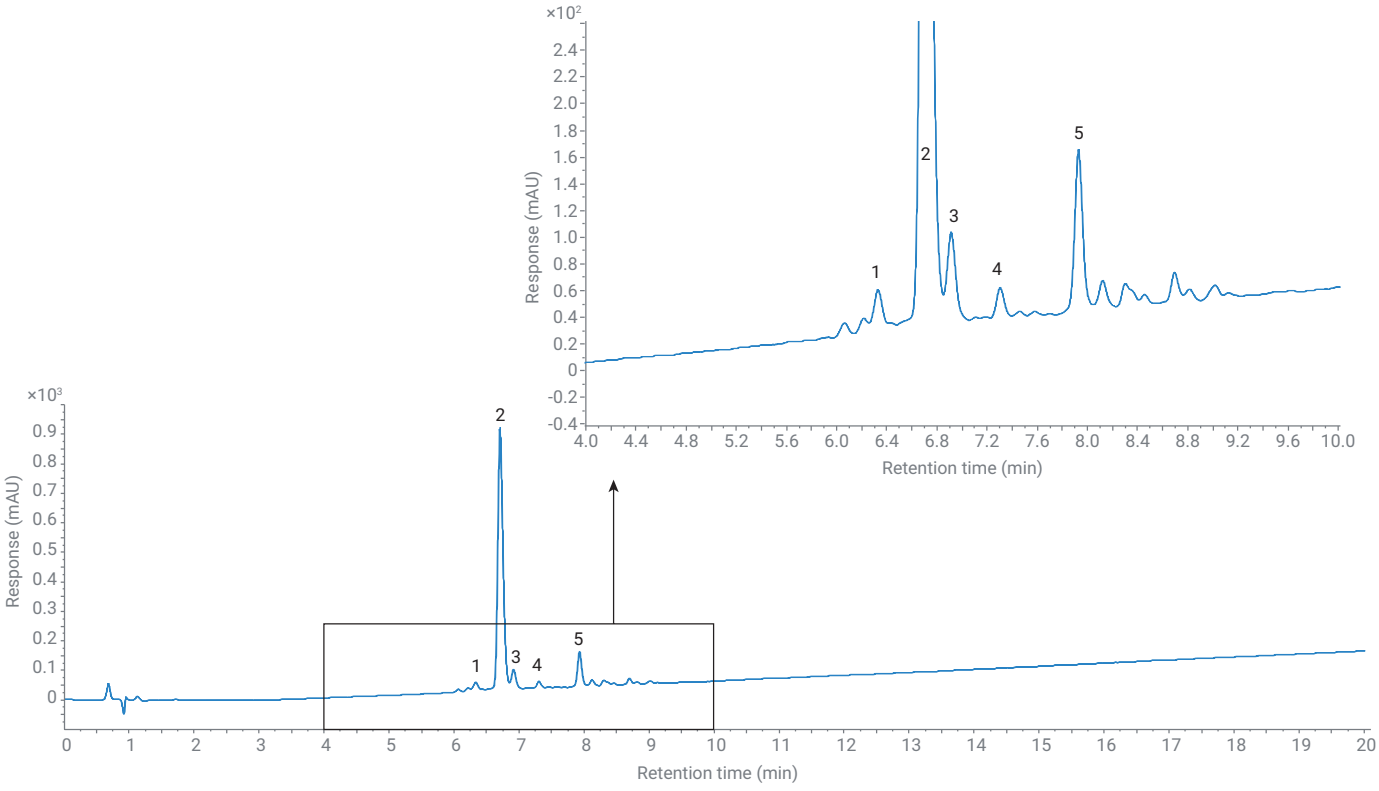

Figure 2. LC/UV chromatogram of synthetic bivalirudin. A zoomed baseline region of synthetic bivalirudin is shown.

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

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

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

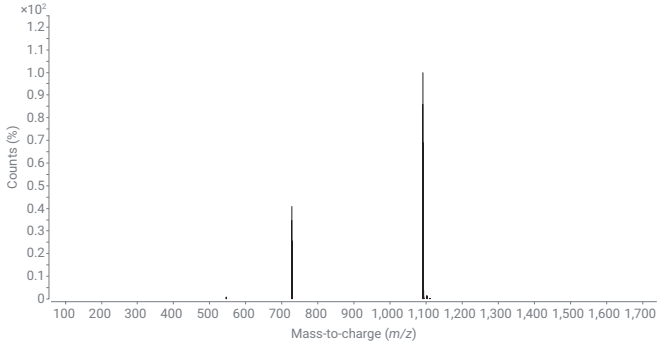

Figure 3. MS spectrum of the main product (peak 2)

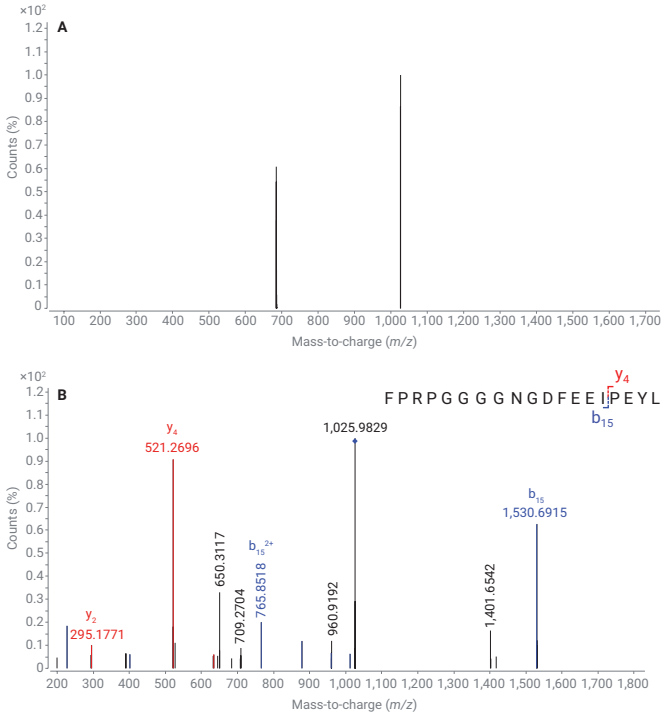

Figure 4. (A) MS spectrum of impurity, peak 1. (B) MS/MS spectrum of impurity, peak 1.

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

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

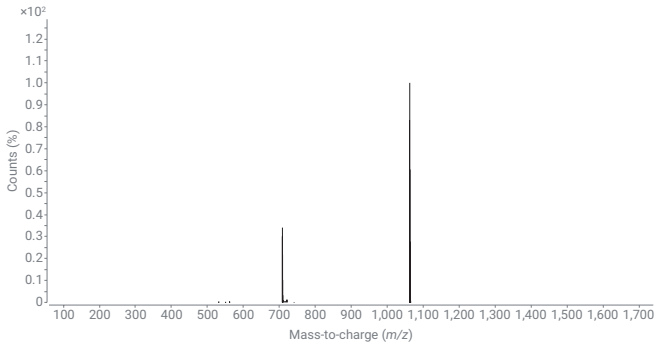

Figure 5. MS spectrum of impurity, peak 3

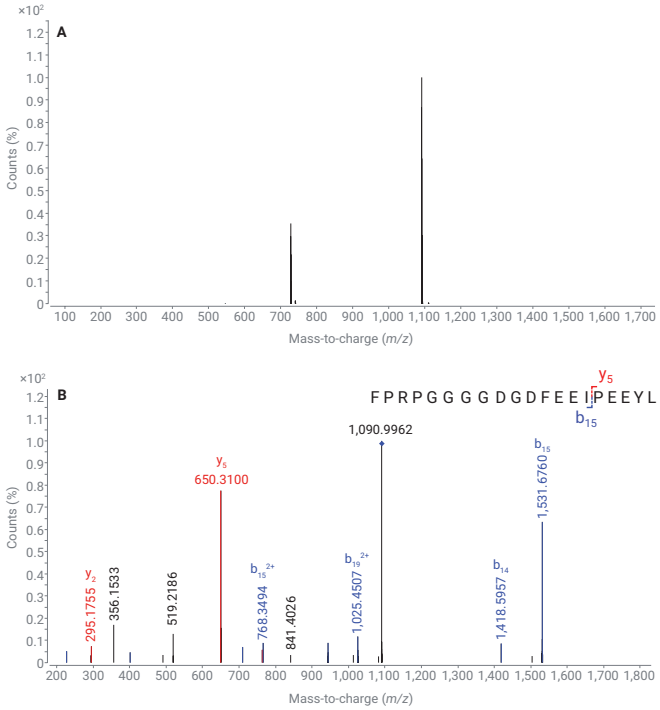

Figure 6. (A) MS spectrum of impurity, peak 5.
(B) MS/MS spectrum of impurity, peak 5.

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Conclusion

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

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Application Note

Peptide Mapping

Agilent

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

Peptide map comparison of mAb and drug conjugated mAb

Authors

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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.

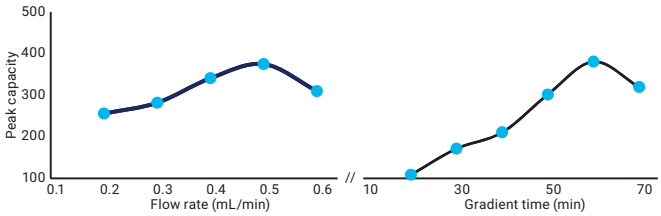

Figure 1. Effect of flow rate and gradient time on peak capacity.

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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

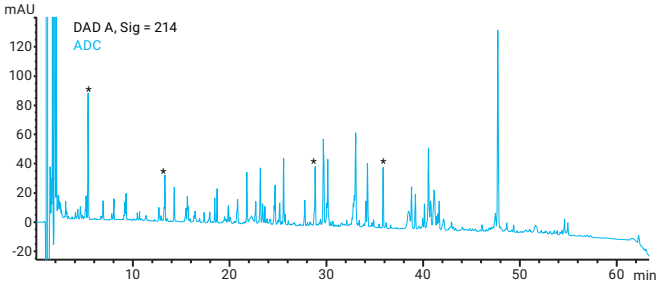

Figure 2. Peptide map of tryptic-digested ADC separated on an Agilent AdvancedBio Peptide Mapping column (*peaks selected for RSD calculations).

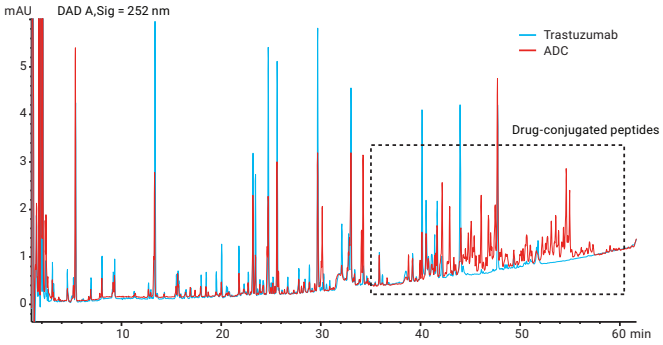

Figure 3. Overlay of peptide map of tryptic-digested ADC and Trastuzumab separated on an Agilent AdvancedBio Peptide Mapping Column.

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.

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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)

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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.

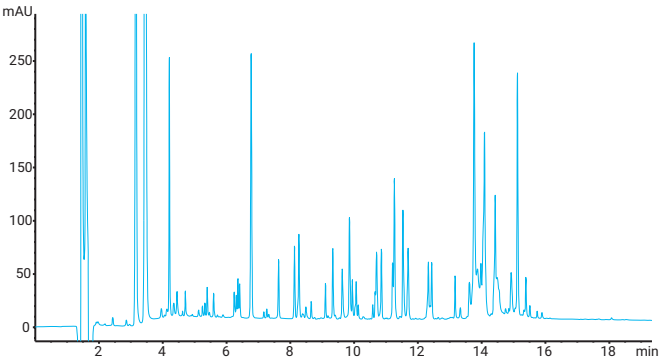

Figure 1. Analysis of a Herceptin tryptic digest, 20-minute gradient, UV detection at 214 nm.

- 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.

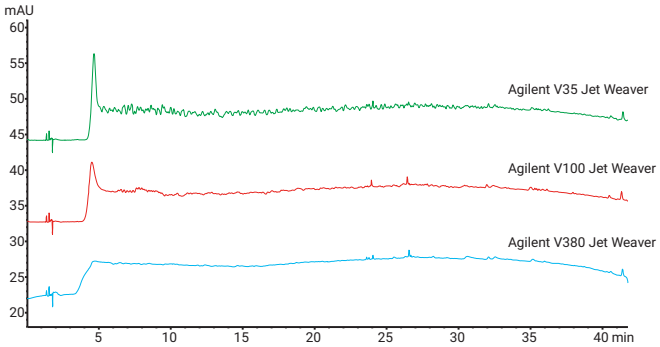

Figure 2. Analysis of a blank solution with an Agilent V35 (green), Agilent V100 (red), and Agilent V380 (blue) Jet Weaver mixer, 40-minute gradient, UV detection at 214 nm.

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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.

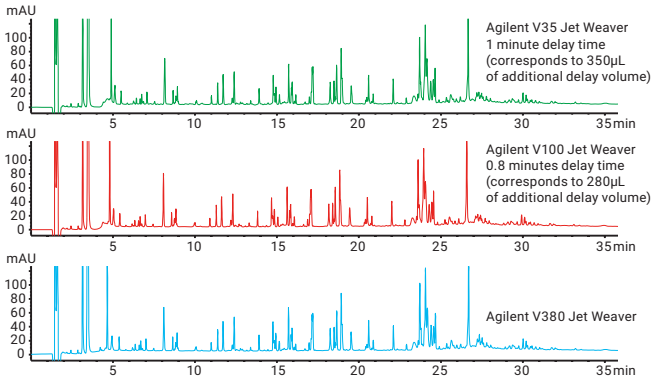

Figure 3. Analysis of Kadcyla tryptic digest with an Agilent V35 (green), Agilent V100 (red), and Agilent V380 (blue) Jet Weaver mixer, 40-minute gradient, UV detection at 214 nm.

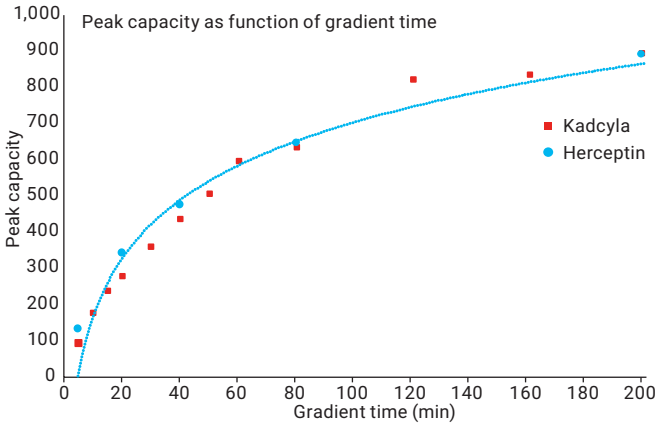

Figure 4. Peak capacity as a function of gradient time. UV detection at 214 nm (detector set at 40 Hz to ensure sufficient data points in fast analyses).

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.

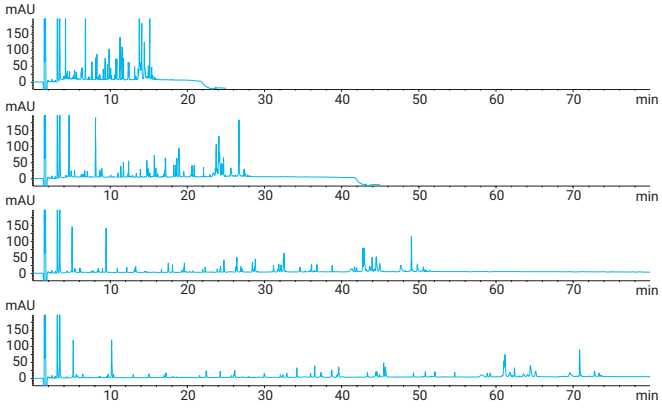

Figure 5. Analysis of a Herceptin tryptic digest with a 20, 40, 80, and 120-minute gradient, UV detection at 214 nm. Note, for comparison, that the y-scale was increased with the Δ-gradient factor.

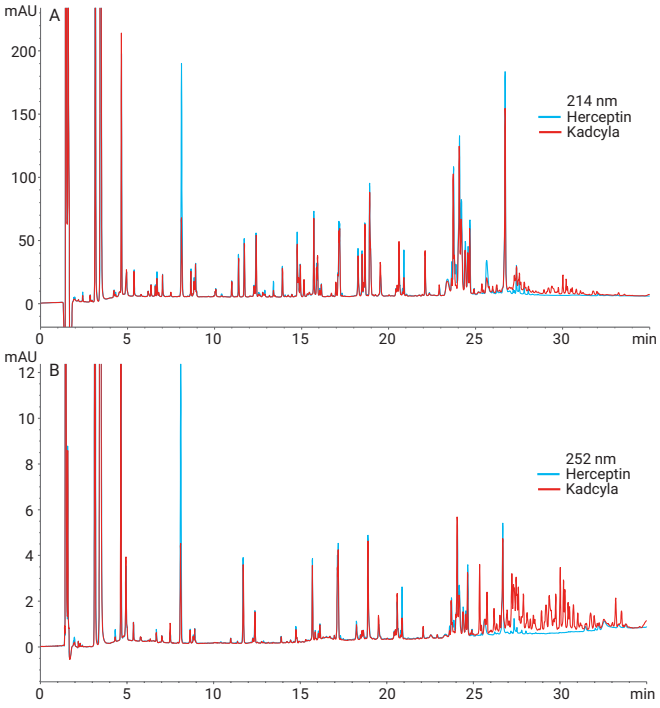

Figure 6. Comparison of analysis of a Herceptin (blue) and Kadcyla (red) tryptic digest with a 40-minute gradient, UV detection 214 nm and 252 nm.

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Conclusions

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

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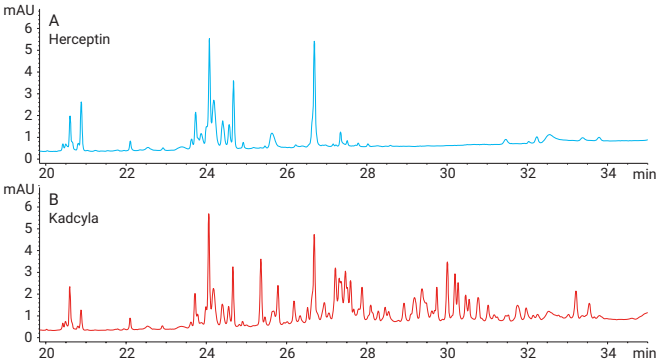

Figure 7. Detail of the comparison of an analysis of a Herceptin and Kadcyla tryptic digest with a 40-minute gradient, UV detection at 252 nm.

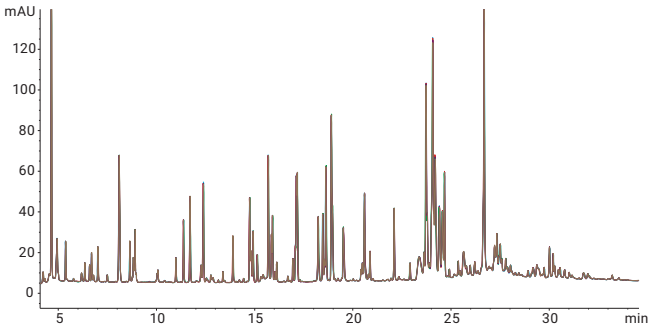

Figure 8. Overlay of five replicate injections of a Kadcyla tryptic digest with a 40-minute gradient, UV detection at 214 nm.

Application Note

Peptide Mapping

Agilent

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

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Abstract

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

Introduction

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

Experimental

Instrumentation

Agilent 1290 Infinity II LC including:

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

Materials

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

Sample preparation

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

LC/MS analysis

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

Data processing

All MS data were processed with MassHunter BioConfirm 10.0 software.

Table 1. Liquid chromatography parameters for intact analysis.

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

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

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

Table 3. Liquid chromatography parameters for peptide mapping analysis.

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

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

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

Results and Discussion

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

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

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

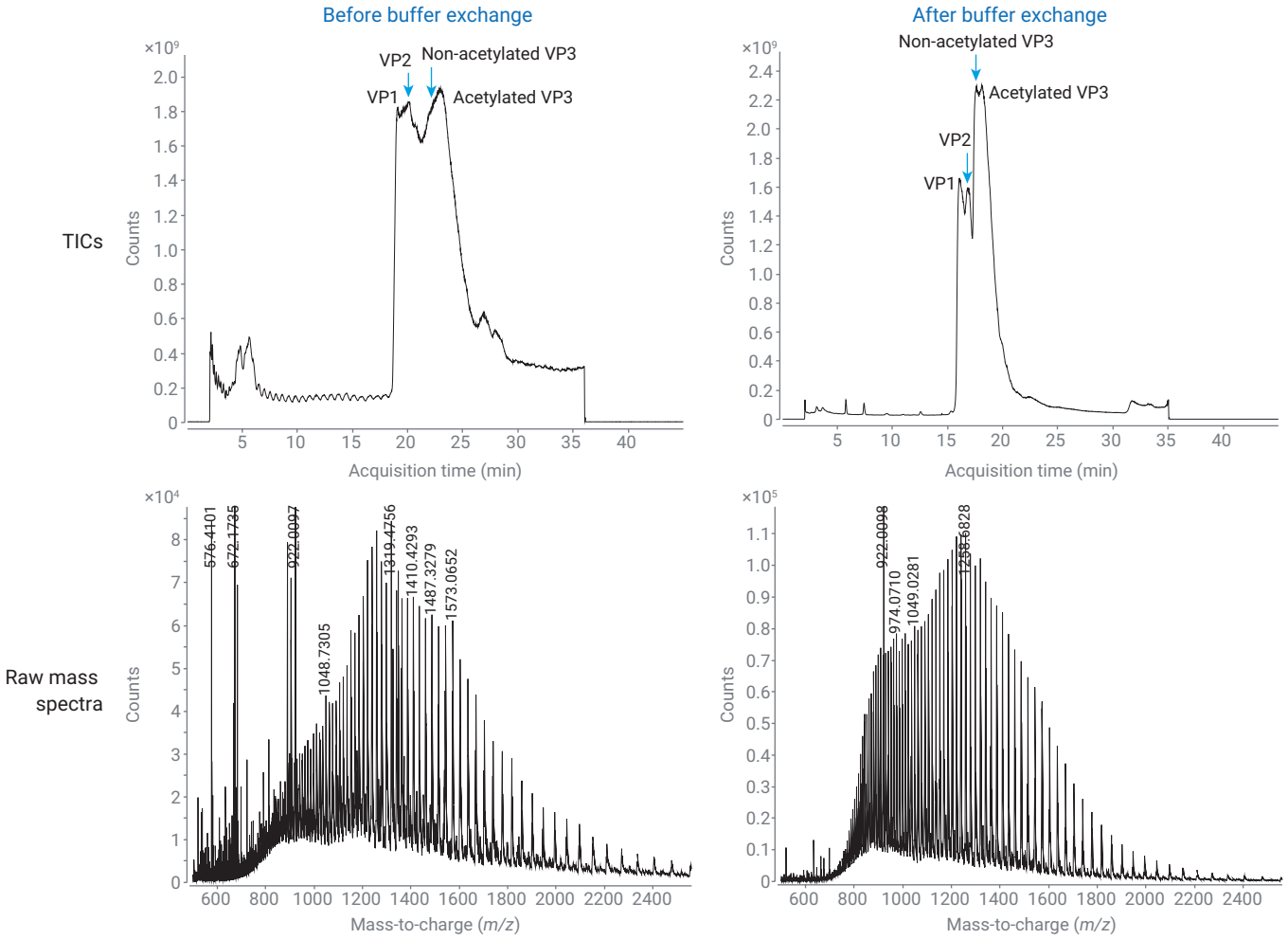

Figure 1. TICs of AAV capsid proteins and raw spectra of VP1 with and without sample preparation.

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

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

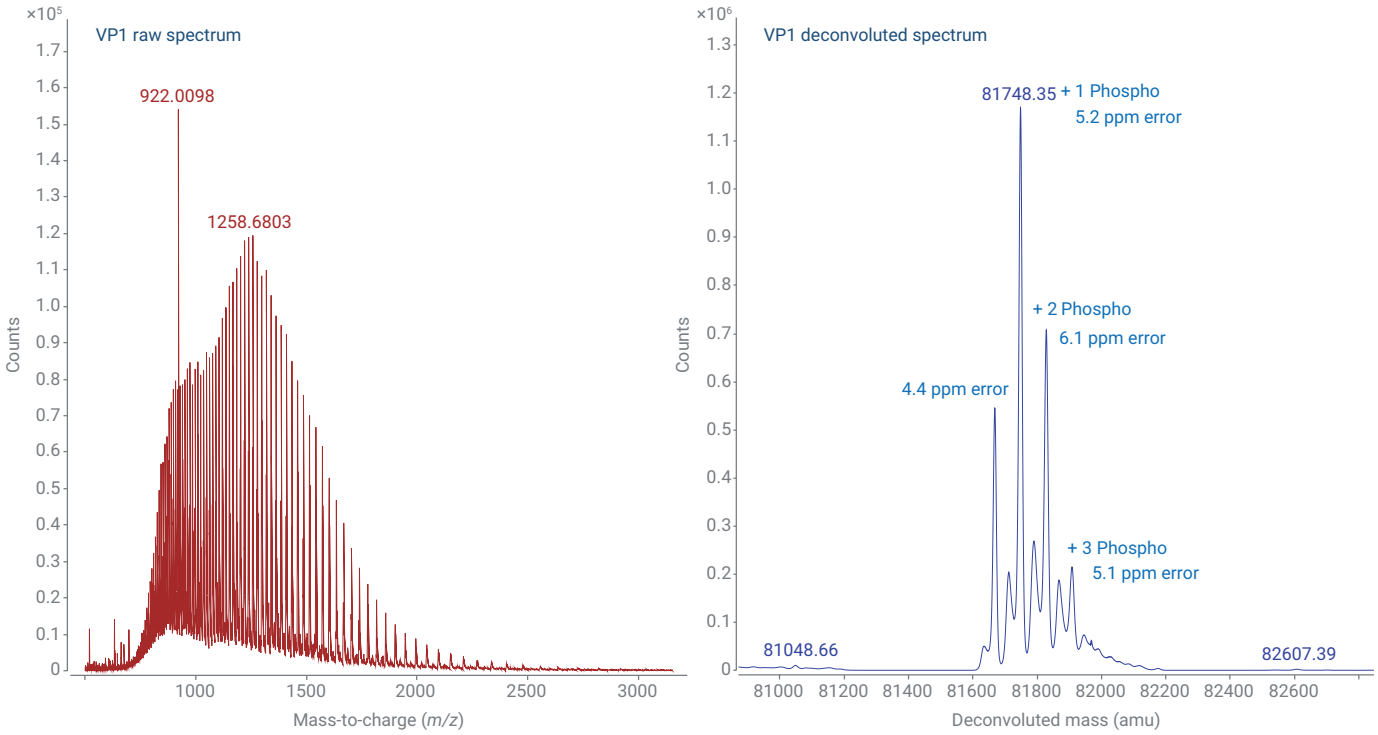

Figure 2. Raw and deconvoluted spectra of VP1 capsid protein. The native and phosphorylated forms of the protein have excellent mass accuracy.

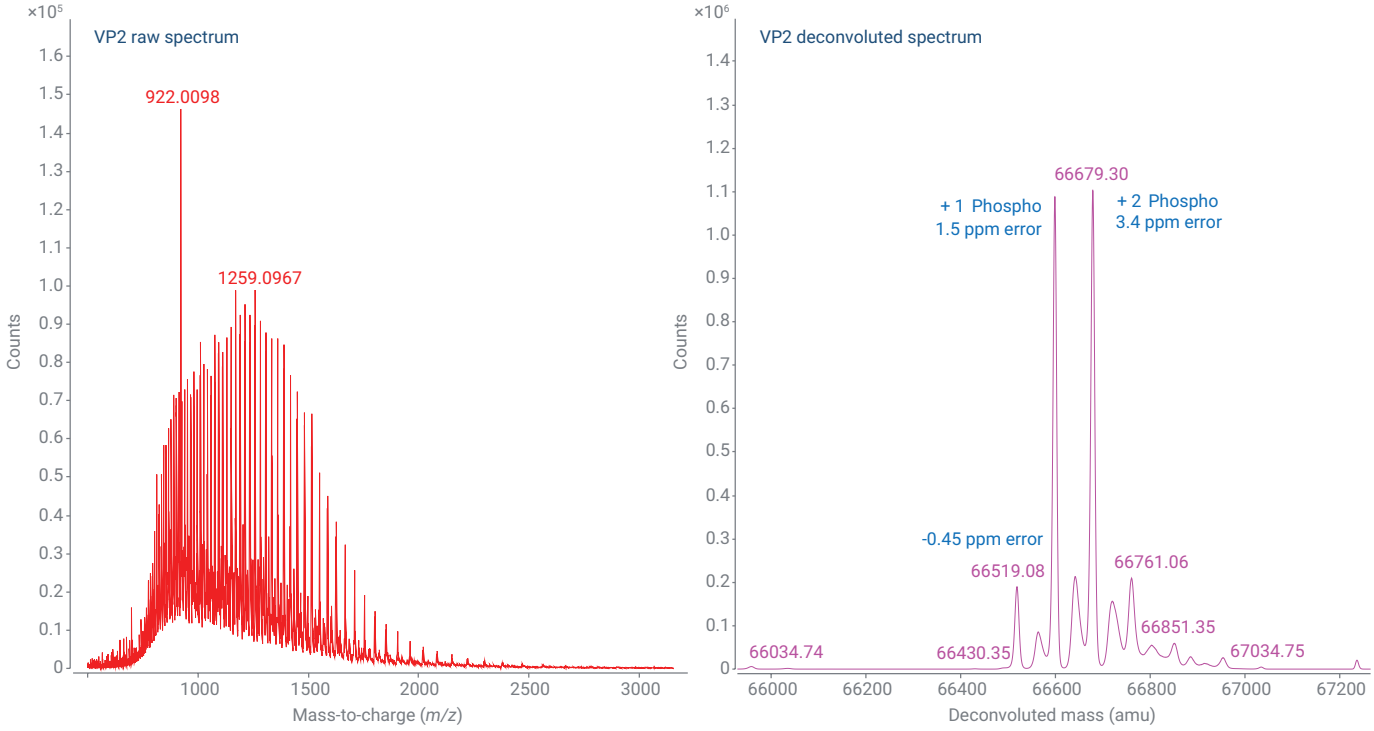

Figure 3. Raw and deconvoluted spectra of VP2 capsid protein. The native and phosphorylated forms of the protein have excellent mass accuracy, all under 5 ppm error.

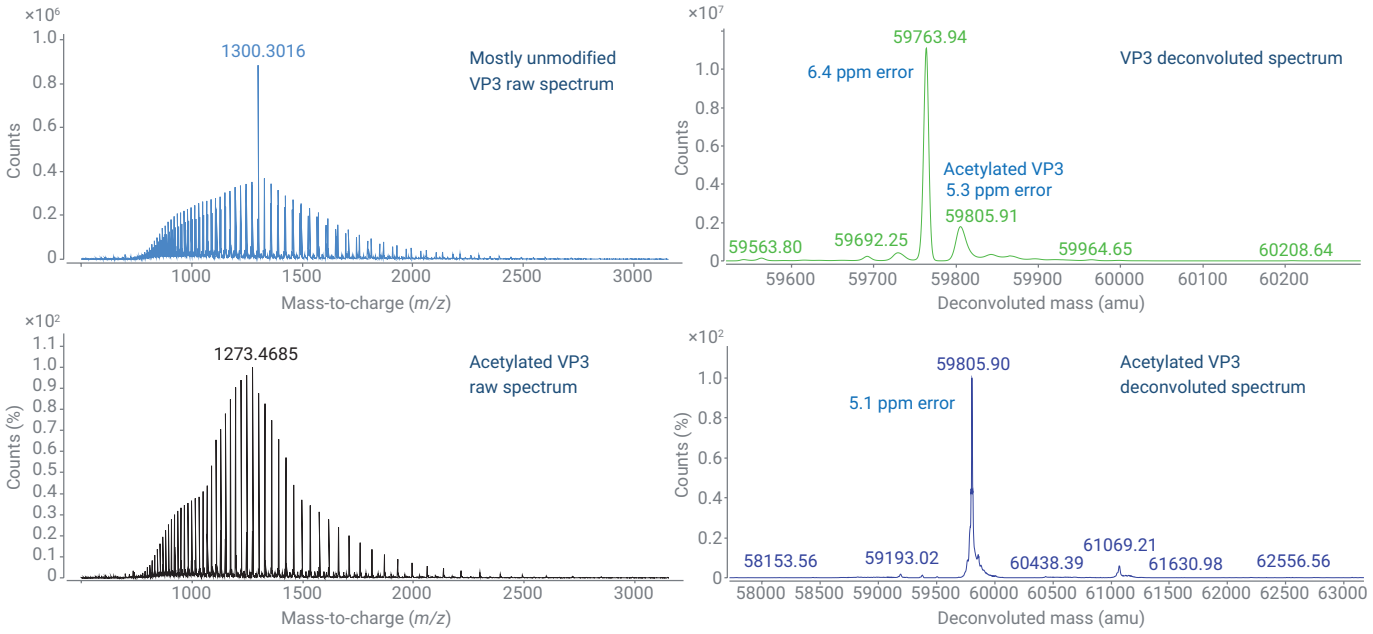

Figure 4. Raw and deconvoluted spectra of VP3 capsid protein. The unmodified and acetylated forms are mostly chromatographically separated, with excellent mass accuracy for each proteoform.

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

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

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

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

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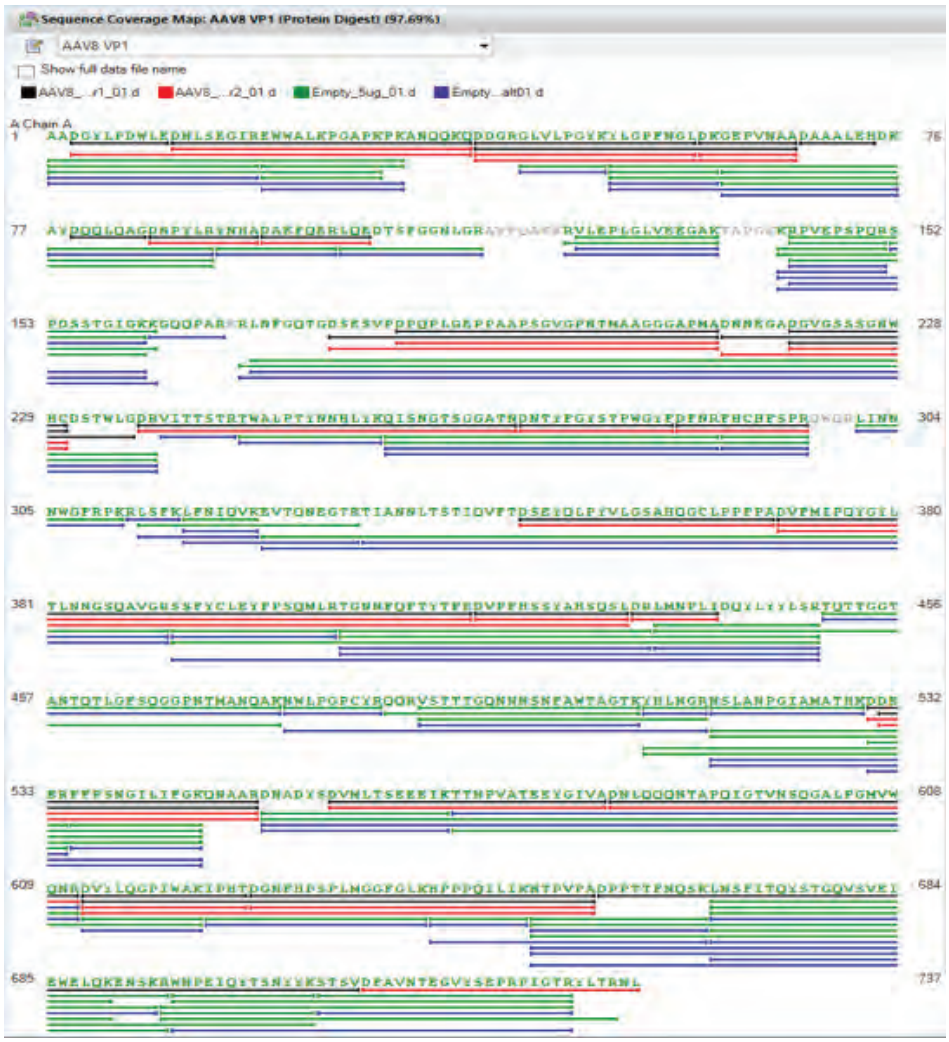

Figure 5. Agilent MassHunter BioConfirm 10.0 screenshot showing sequence coverage of VP1 with iterative MS/MS and using trypsin and rAsp-N as complementary enzymes. This protein has 97.7% sequence coverage.

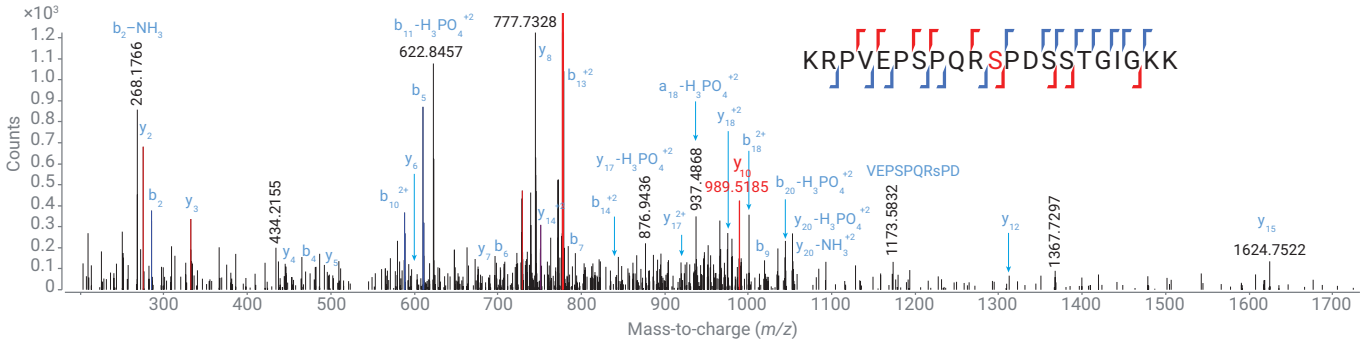

Figure 6. One example of site-specific phosphorylation with MS/MS confirmation. The annotated peptide has red markings when it contains the phosphorylated serine residue.

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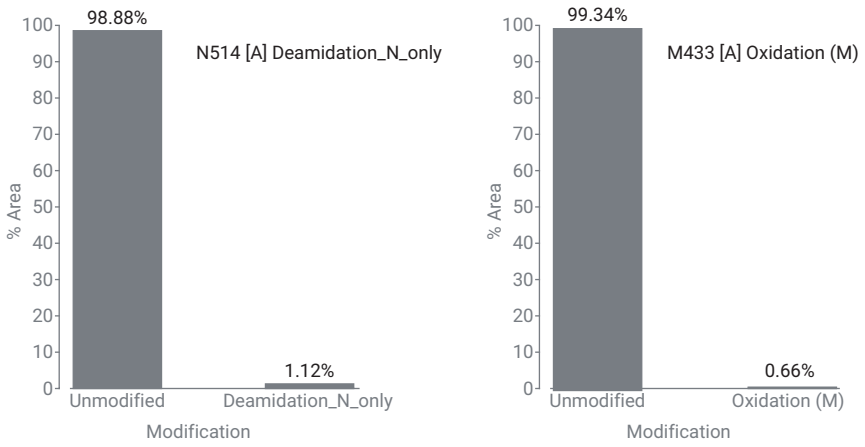

Figure 7. Examples of common PTMs in proteins: methionine oxidation and asparagine deamidation. Both peptides are minimally modified, as expected.

Conclusion

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

Acknowledgements

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Application Note

Peptide Mapping

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

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

Author

André Feith

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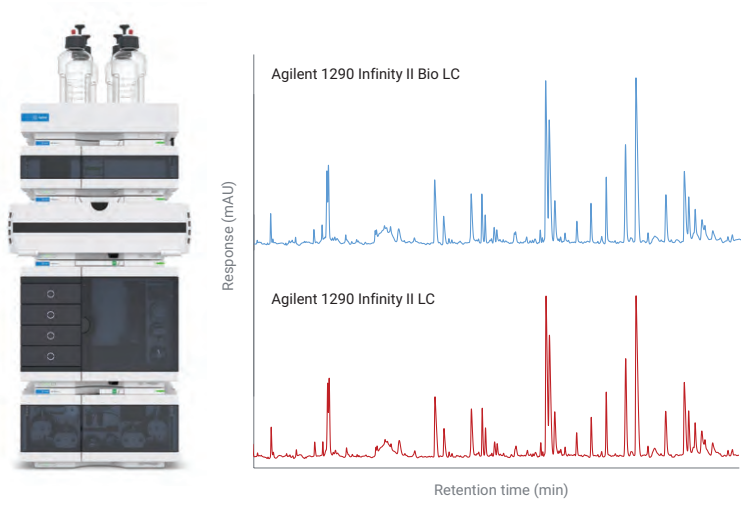
Abstract

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



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Introduction

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

Experimental

Equipment

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

Agilent 1290 Infinity II Bio LC:

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

Agilent 1290 Infinity II Bio LC:

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

Software

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

Columns

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

Chemicals

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

Sample preparation

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

Results and Discussion

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

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

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

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

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

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

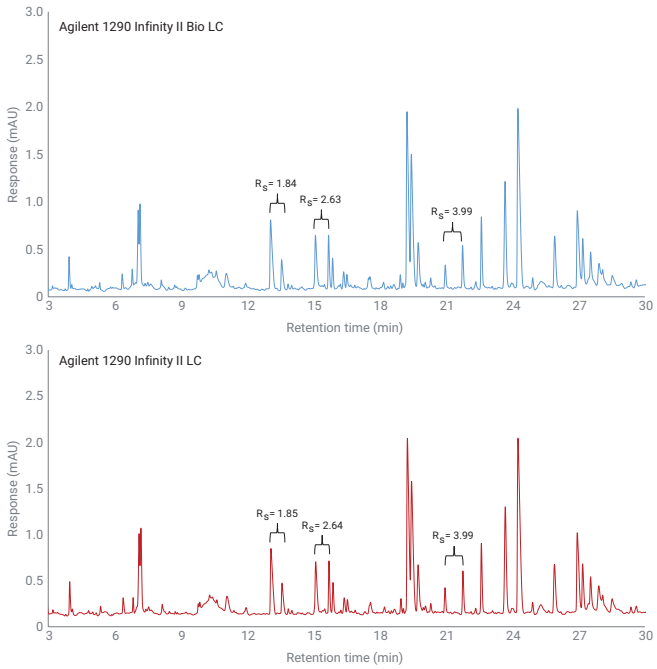

Figure 1. Chromatograms of a tryptic digest of the NISTmAb separated by the Agilent 1290 Infinity II Bio LC and the Agilent 1290 Infinity II Bio LC with the same method (Table 1).

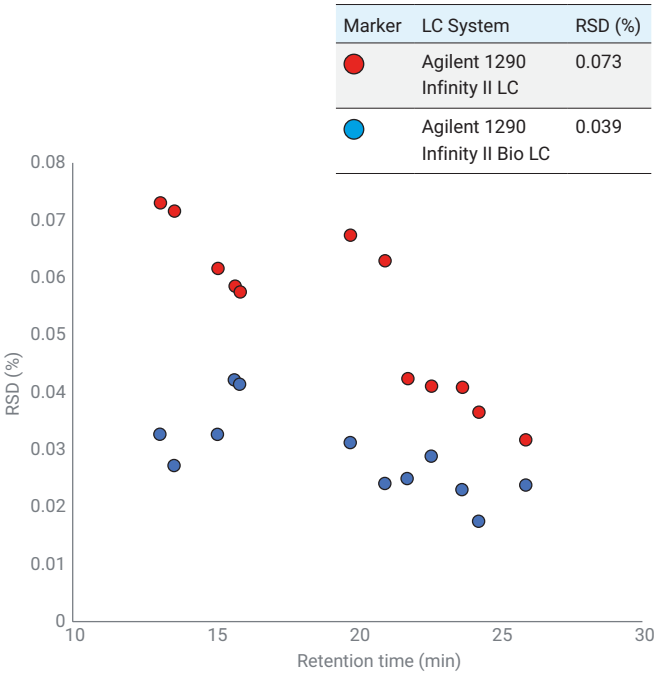

Figure 2. Relative retention time precision (RSD) values for the Agilent 1290 Infinity II Bio LC and the Agilent 1290 Infinity II LC

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

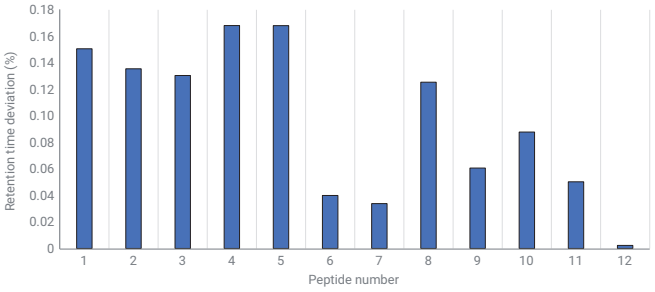

Figure 3. Average retention times for the 12 chosen peptides and their deviations between the two LC systems.

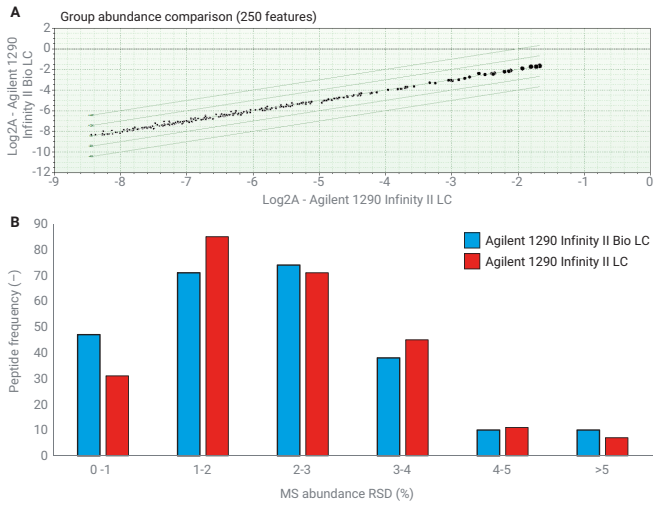

Figure 4. (A) Statistical correlation analysis based on 10 consecutive injections. Log fold values are depicted as black dots for the 250 highest abundance peaks identified by Agilent MassHunter Mass Profiler (B.10.00). (B) Histogram for the RSD values of the peptide abundances for the Agilent 1290 Infinity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

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

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

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Conclusion

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

References

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

Additional Application Notes

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

Additional Information

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

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

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

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

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