Analyze MAb and BSA digests by UHPLC with UV detection and Agilent ZORBAX RRHD 300SB-C18

Application Note

BioPharma

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Abstract

A new reversed-phase media, Agilent ZORBAX RRHD 300SB-C18 1.8 µm, is used for the analysis of trypsin-digested monoclonal antibody and bovine serum albumen. The robustness of the media is demonstrated using different acidic eluents, temperatures and flow rates. Good reproducibility is also evident.

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Introduction

Agilent ZORBAX RRHD 300SB-C18 1.8 µm is a new reversed-phase media designed for UHPLC of proteins and peptides. The use of 1.8 µm particles in a column designed for UHPLC systems significantly reduces analysis time in HPLC. Protein digest, enzymatic cleavage of the protein, into peptide fragments is used to confirm the identity of a protein through database matching of the fragments and in the qualitation or identification of amino acid modifications. To analyze trypsin digests, denaturing conditions are required, and so reversed-phase HPLC is normally employed. In this example, we use ZORBAX Rapid Resolution High Definition (RRHD) columns, which benefit from improved packing processes to achieve stability up to 1200 bar for use with the Agilent 1290 Infinity LC.

Analysis of trypsin-digested MAb at different digestion times

Monoclonal antibody was obtained from Reactive Biolabs, and BSA, Trypsin Gold (digestion grade), TFA and formic acid from Sigma Aldrich. An Agilent 1290 Infinity LC system was used for all analyses.

Protocol for trypsin digestion

Add Trypsin Gold to a final protease:protein ratio of 1:20 (w/w) in tris-HCl buffer pH 8.0 - it is desirable that the protein concentration is 1 mg/mL. Incubate at 50 °C, for at least 4 h to overnight (16 h) (method adapted from *Trypsin Digestion of Proteins in Solution*, Promega, Madison WI, USA).

Depending on the nature of the investigation, protein digestion times and analysis temperatures may vary. Notwithstanding, monoclonal antibody and its digested fragments are well resolved with ZORBAX RRHD 300SB-C18 1.8 µm, as shown in Figure 1.

Conditions

Column:	Agilent ZORBAX RRHD 300SB-C18 1.8 µm
Sample:	Humanized monoclonal antibody (MAb) (1 mg/mL) (Reactive Biolabs)
Flow rate:	0.6 mL/min (~ 680 bar)
Mobile phase A:	0.1% TFA
Mobile phase B:	0.01% TFA in ACN
Gradient:	1 to 100% B in 20 min
Injection:	5 µL
Temp:	25 °C or 50 °C
Digest time:	As indicated
Detection:	280 nm

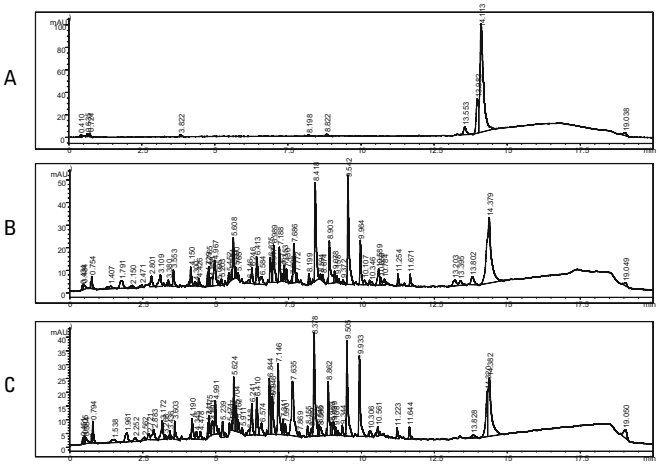

Figure 1. *Resolution of monoclonal antibody and its digested fragments at different temperatures and digestion times using Agilent ZORBAX RRHD 300SB-C18 1.8 µm. A, humanized monoclonal antibody at 25 °C; B, 10 h trypsin-digested MAb at 50 °C; C, 16 h trypsin-digested MAb at 50 °C*

Separation of trypsin-digested BSA fragments

Conditions

Column:	ZORBAX RRHD 300SB-C18 1.8 µm
Sample:	Trypsin-digested BSA (1 mg/mL) (Sigma Aldrich)
Flow rate:	0.6 mL/min (~ 680 bar)
Mobile phase A:	0.1% TFA
Mobile phase B:	0.01% TFA in ACN
Gradient:	1 to 100% B in 20 min
Injection:	5 µL
Temp:	25 °C
Digestion time:	16 h
Detection:	280 nm

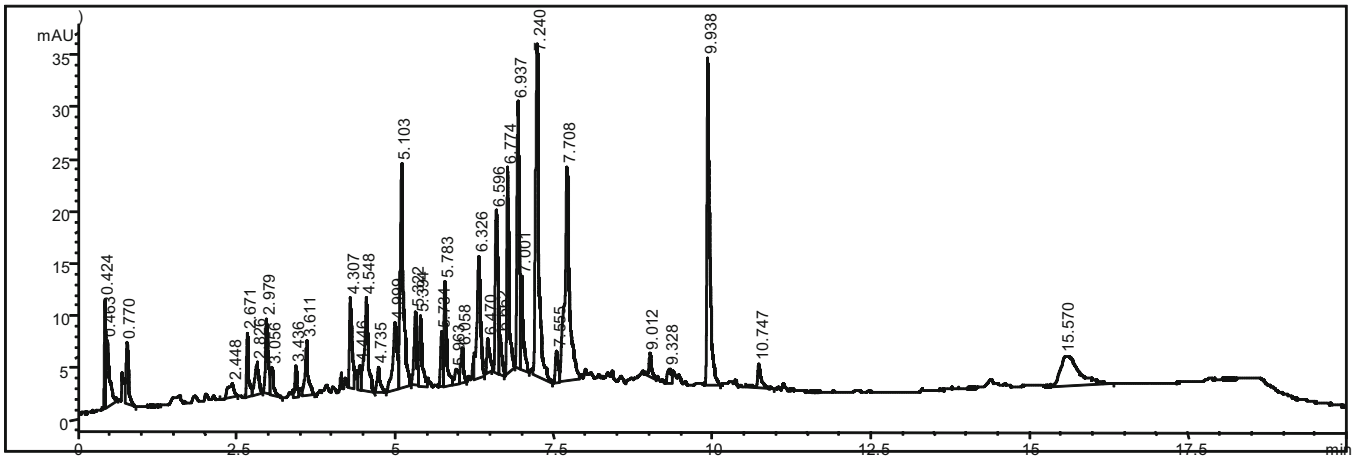

Figure 2. *Fragments from digested BSA are well separated using Agilent ZORBAX RRHD 300SB-C18 1.8 µm*

Analysis of trypsin-digested MAb with formic acid

TFA reduces the signal and trace sensitivity of the method when using HPLC-MS. This can limit the utility of the method, making it more difficult to identify minor peptides in the digest when complete coverage for database matching is needed. Formic acid can be used as an alternative to TFA for protein digest analysis using UHPLC-MS. The analysis of monoclonal antibody digest is shown in Figure 3.

Conditions

Column:	ZORBAX RRHD 300SB-C18 1.8 µm
Sample:	Trypsin-digested MAb (1 mg/mL)
Flow rate:	0.6 mL/min (~ 720 bar)
Mobile phase A:	0.1% formic acid
Mobile phase B:	0.01% formic acid in ACN
Gradient:	0.5 to 100% B in 20 min
Injection:	5 µL
Temp:	25 °C
Digestion time:	16 h
Detection:	280 nm

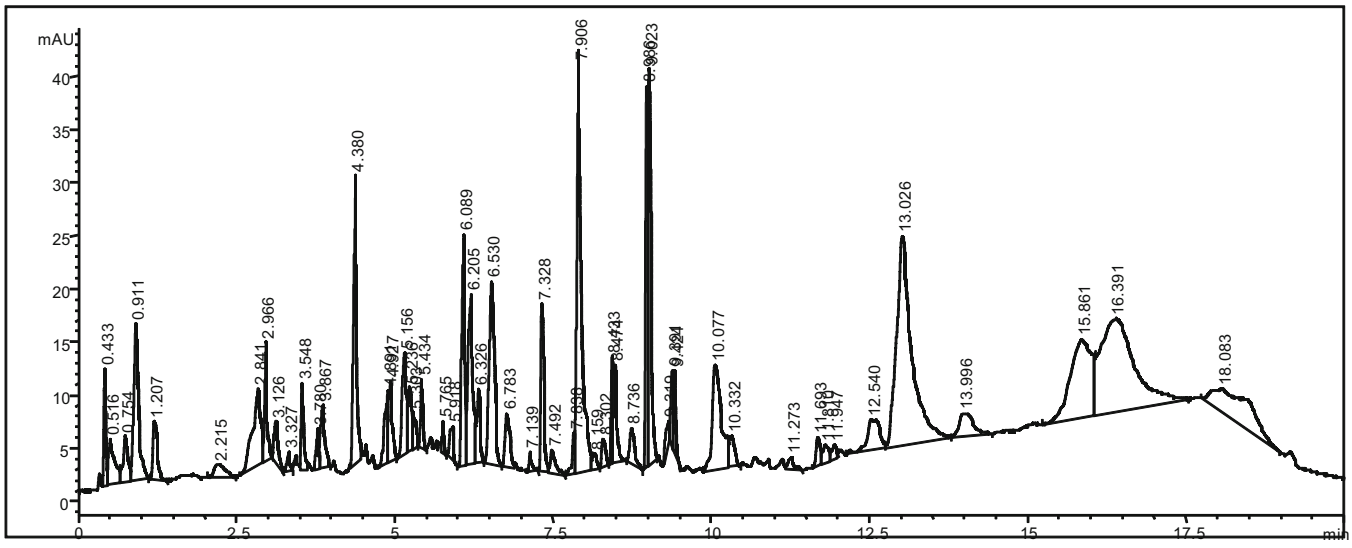

Figure 3. *Agilent ZORBAX RRHD 300SB-C18 1.8 µm is used with formic acid to analyze trypsin-digested MAb*

High pressure separation of trypsin-digested MAb and BSA

If fast analysis times are required, the ZORBAX RRHD 300SB-C18 1.8 µm handles the increased pressure required to separate digested MAb and BSA in 17 minutes (Figure 4).

Conditions

Column:	ZORBAX RRHD 300SB-C18 1.8 µm
Sample:	Trypsin-digested BSA and MAb (1 mg/mL)
Flow rate:	1 mL/min (~ 1010 bar)
Mobile phase A:	0.1% TFA
Mobile phase B:	0.01% TFA in ACN
Gradient:	1 to 100% B in 20 min
Injection:	5 µL
Temp:	25 °C
Digestion time:	16 h
Detection:	280 nm

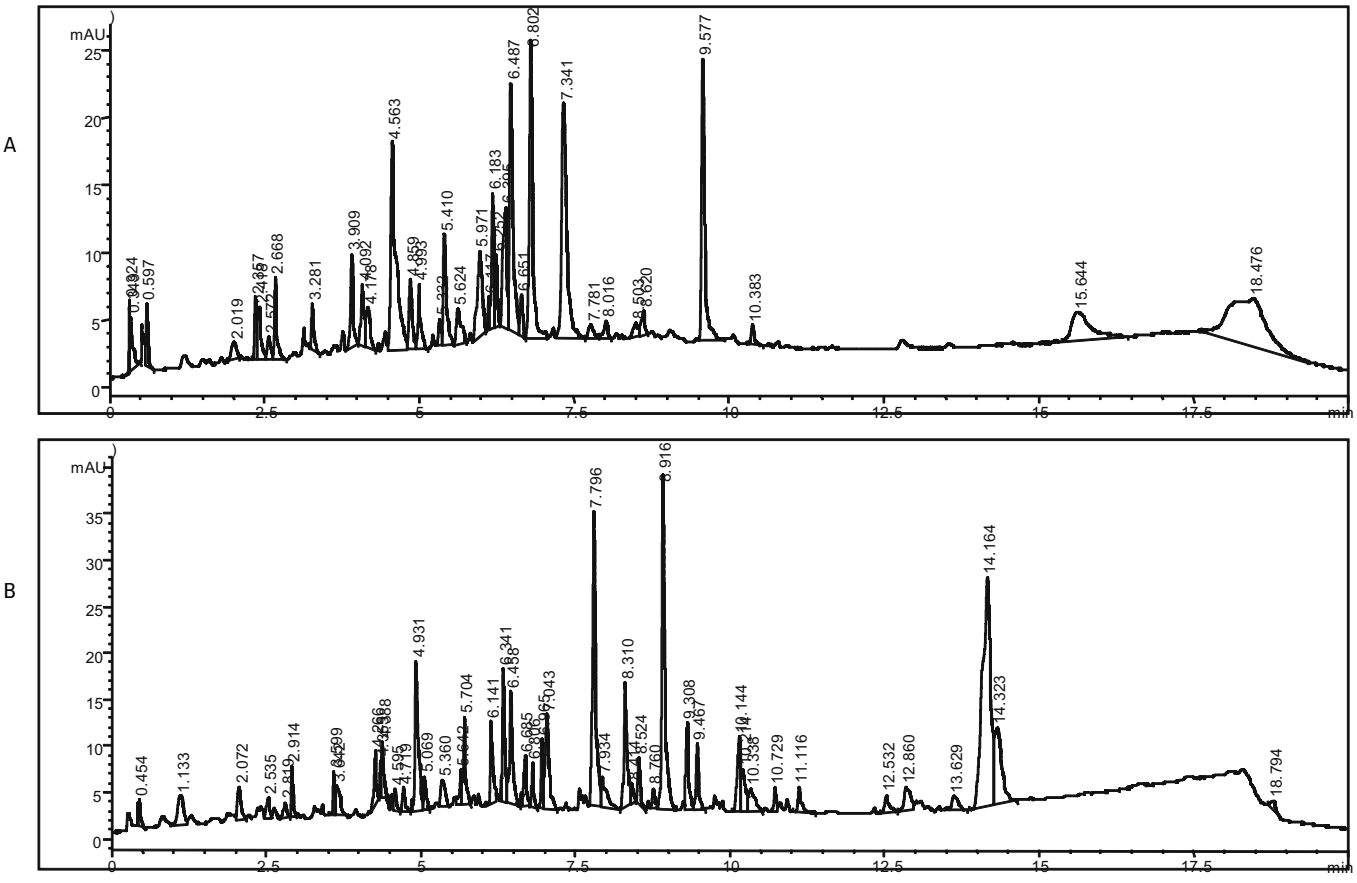

Figure 4. *High speed and high pressure deliver fast analysis of peptides using Agilent ZORBAX RRHD 300SB-C18 1.8 µm. A, trypsin-digested BSA; B, trypsin-digested MAb*

High temperature separations

Elevated temperatures may be needed to analyze highly bound peptides and proteins, to improve peak shape and recovery. However, this must be accomplished without damaging the integrity of the column. The ZORBAX RRHD 300SB-C18 column is stable to 90 oC. Figure 5 shows such an analysis using TFA. Similarly, Figure 6 indicates a high temperature separation achieved using formic acid.

Conditions

Column:	ZORBAX RRHD 300SB-C18 1.8 µm
Sample:	Trypsin-digested BSA and MAb (1 mg/mL)
Flow rate:	0.6 mL/min (~ 1010 bar)
Mobile phase A:	0.1% TFA
Mobile phase B:	0.01% TFA in ACN
Gradient:	1 to 100% B in 20 min
Injection:	5 µL
Temp:	50 °C
Digestion time:	16 h
Detection:	280 nm

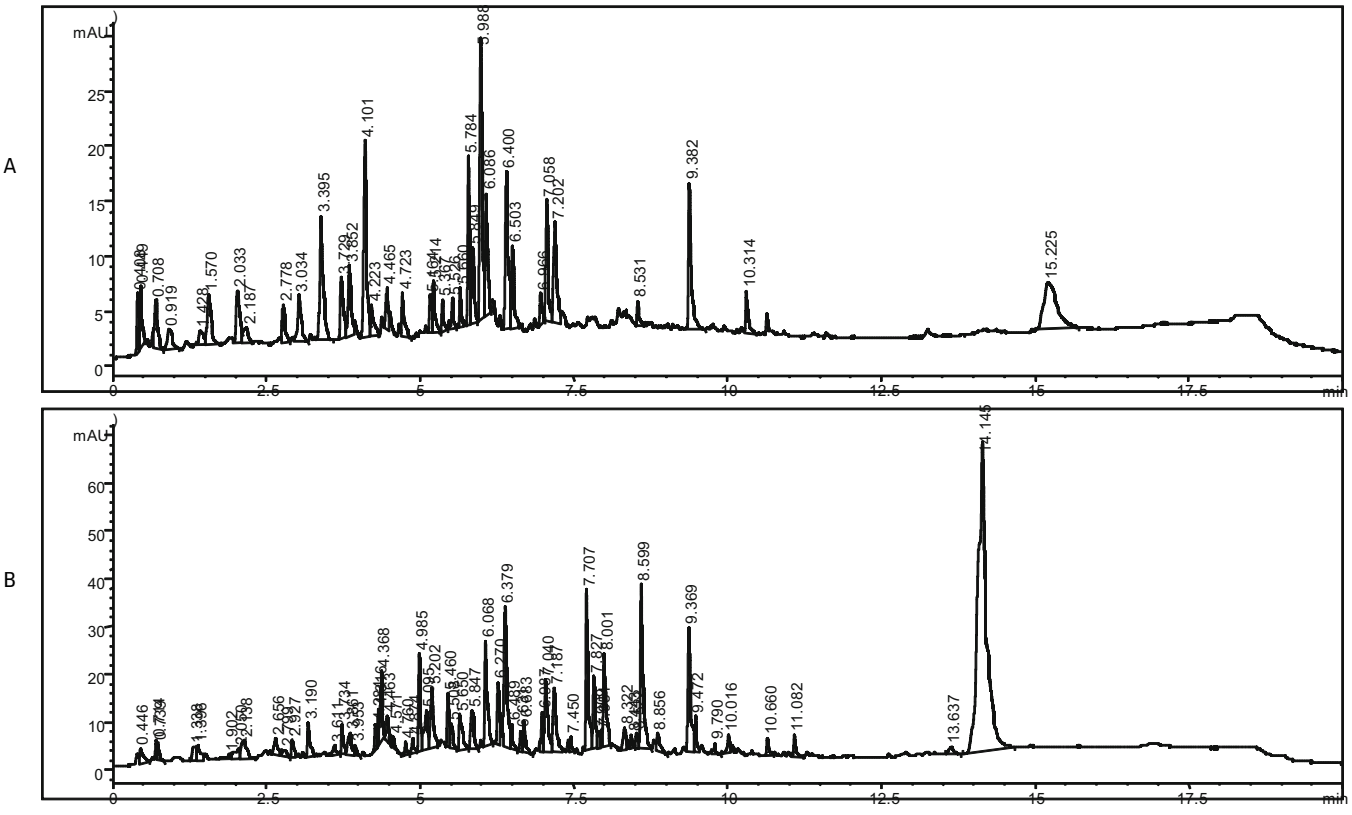

Figure 5. *Agilent ZORBAX RRHD 300SB-C18 with TFA eluent can be used at high temperature to efficiently analyze highly bound peptides and proteins without damaging the column's integrity. A, trypsin-digested BSA; B, trypsin-digested MAb*

Conditions

Column:	ZORBAX RRHD 300SB-C18 1.8 µm
Sample:	Trypsin-digested MAb (1 mg/mL)
Flow rate:	0.6 mL/min (~ 720 bar)
Mobile phase A:	0.1% formic acid
Mobile phase B:	0.01% formic acid in ACN
Gradient:	0.5 to 100% B in 20 min
Injection:	5 µL
Temp:	50 °C
Digestion time:	16 h
Detection:	280 nm

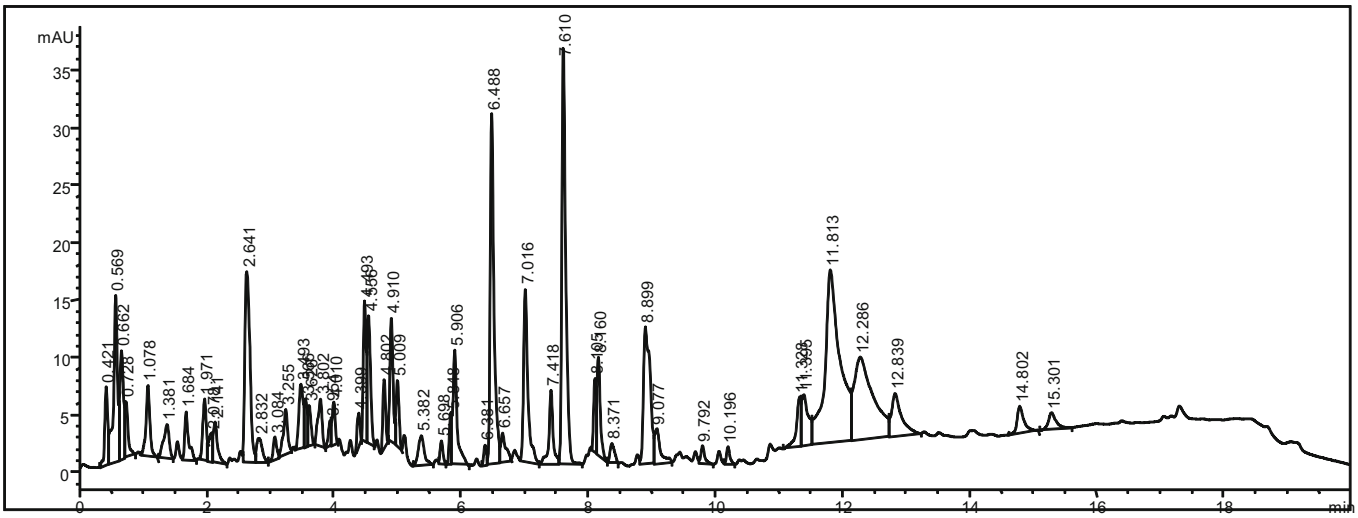

Figure 6. *High temperature separation of trypsin-digested MAb using an Agilent ZORBAX RRHD 300SB-C18 with formic acid eluent*

Conclusions

Rapid analysis of trypsin digests of monoclonal antibody and bovine serum albumen is accomplished using an ZORBAX RRHD 300SB-C18 1.8 µm column. The analysis time is reduced significantly from the 3 to 5 hours previously achieved with conventional HPLC. The efficiency of the column is evident over the range of temperature and flow rate elucidated in this study. In addition, the eluents routinely employed for reversed-phase analysis are acidic, containing trifluoroacetic acid or formic acid, which can limit the lifetime of many HPLC columns. With formic acid, preferred for UHPLC-MS, there is no deterioration in peak shape or efficiency. However, by using Agilent's StableBond technology it is possible to produce a 300Å pore-size media that is stable under acidic conditions, to provide the robust reproducible separations required for protein digest analysis.

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