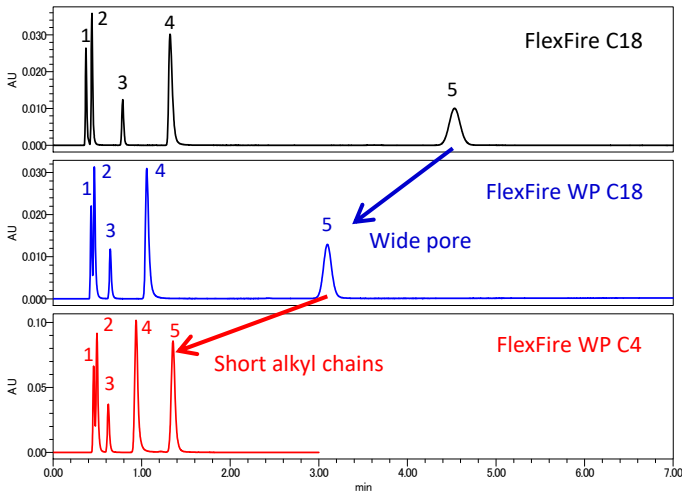
Technical report No.30

Analysis of peptides/proteins and monoclonal antibodies by UHPLC

1. Introduction

We released “FlexFire WP C4” in February2020. The FlexFire WP C4 uses a 300Å silica gel substrate for biopharmaceutical analysis. The wide pores target low molecular compounds to hundreds of thousands of high molecular compounds, leading to the characterization of the primary structure. This report contains the latest data that could not be included in the catalog and other WP series data. Please use it.



Conditions;

Column:	FlexFire C18, 2.6μm (2.0x50mm) FlexFire WP C18, 2.6μm (2.0x50mm) FlexFire WP C4, 2.6μm (2.0x50mm)
Mobile phase	Acetonitrile/10mM $HCOONH_4$=40/60
Flow rate:	0.3mL/min
Temperature:	40℃
Sample:	1.Uracil 2.Caffeine 3.Phenol 4.Amitriptyline 5.Naphthalene
injection volume:	0.1μL
System:	Waters ACQUITY UPLC H-Class PLUS

2. Retention and separation due to different pore sizes

The physical properties differ greatly between the pore size of 120Å and 300Å. Since the pores are large = the surface area is small, the shorter the wide pores and the shorter the alkyl chains, the shorter the retention.

■ Specification of comparison target column

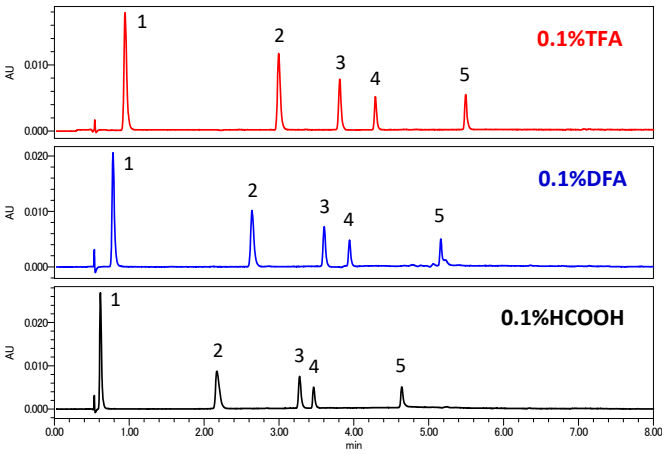
	FlexFire C18	FlexFire WP C18	FlexFire WP C4
Particle size	2.6μm	2.6μm	2.6μm
Chemistry	Octadecyl	Octadecyl	Butyl
Surface area	340m^2/g	170m^2/g	170m^2/g
Pore volume	1.0mL/g	1.4mL/g	1.4mL/g
Pore diametter	11nm	30nm	30nm
Carbon	22%	15%	5%

■ **Retention comparison with Develosil QC samples**

Obtained retention varies with surface area and carbon content. If the retention is extremely strong, it may take less time to move to a column with wide pores or a column with short chain alkylation rather than the difference in alky l chains in the same series.

2. Analysis of Peptides

Columns with a pore size of 120Å class are often used for the analysis of low molecular weight peptides, but nowadays, biopharmaceutical analysis is drawing attention, and there is a demand for columns that can be used for a wide range of analysis from low molecular weight to high molecular weight.



Conditions:

Column: Develosil FlexFire WP C4, 2.6μm (2.0x50mm)

Mobile phase:
A) Water + 0.1%TFA
B) Acetonitrile + 0.1%TFA
A) Water + 0.1%DFA
B) Acetonitrile + 0.1%DFA
A) Water + 0.1%HCOOH
B) Acetonitrile + 0.1%HCOOH

Gradient:

min	mL/min	%A	%B	Curve
0.00	0.3	100	0	
4.76	0.3	40	60	6
8.42	0.3	80	20	6
8.50	0.3	100	0	6

Temperature: 40℃
Detection: UV260nm
Sample:
1.Gly-Tyr (0.20mg/mL)
2.Val-Tyr-Val (0.21mg/mL)
3.Methionine_Enkephalin (0.18mg/mL)
4.Angiotensin II (0.17mg/mL)
5.Insulin (0.20mg/mL)
Injection volume: 0.2μL
Sysytem: Waters ACQUITY UPLC H-Class PLUS
Mixer: 100μL

■ **Peptide analysis in different mobile phases**

In the analysis of small peptides, even if it is not TFA mobile phase, Analysis is possible.

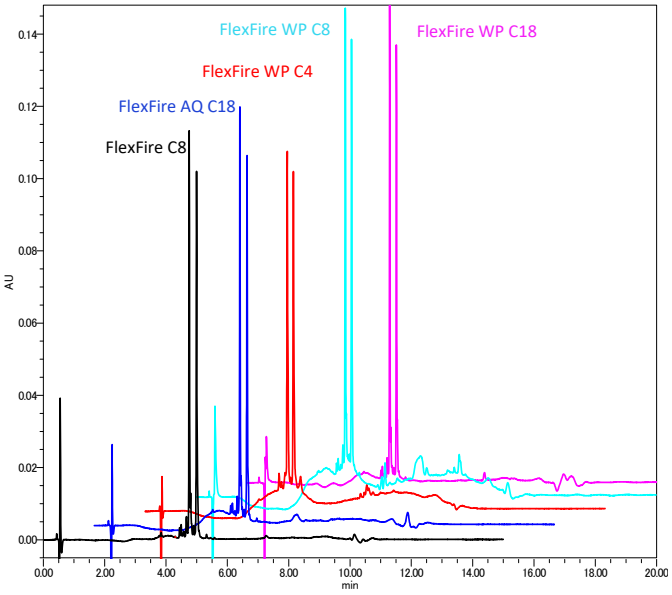
Measurement in negative mode of LC/MS If contamination of the ion source is a concern, use 0.1% formic acid mobile Phase analyzing can help you minimize risk.

3. Analysis of cyclic peptides

There are multiple types of peptides, including dipeptides, tripeptides, oligopeptides and polypeptides.

Among them, cyclic peptides are known to have excellent physical properties such as metabolic stability and membrane permeability.

It can be handled in the same way as chain peptides in analysis.



Conditions:

Column:
Develosil FlexFire WP C18, 2.6μm (2.0x50mm)
Develosil FlexFire WP C8, 2.6μm (2.0x50mm)
Develosil FlexFire WP C4, 2.6μm (2.0x50mm)
Develosil FlexFire AQ C18, 2.6μm (2.0x50mm)
Develosil FlexFire C8, 2.6μm (2.0x50mm)

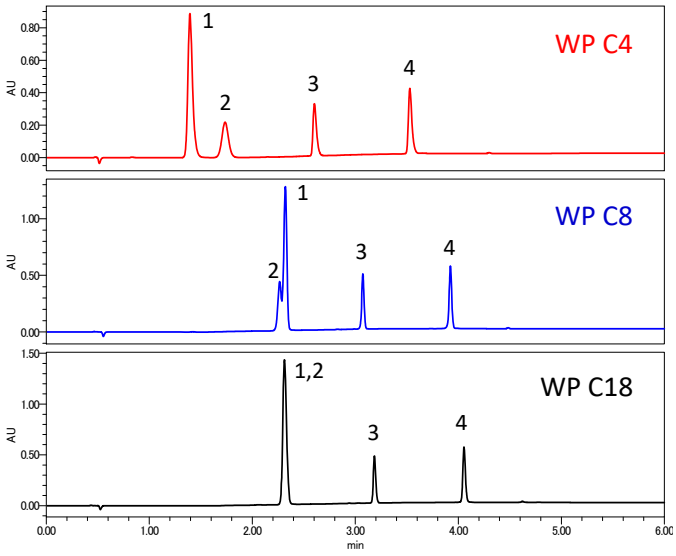
Mobile phase: A) Water + 0.1%TFA B) Acetonitrile + 0.1%TFA

Gradient:

min	mL/min	%A	%B	Curve
0.00	0.3	100	0	
4.76	0.3	60	40	6
8.42	0.3	20	80	6
8.50	0.3	100	0	6

Temperature: 40℃
Sample: Colistin (0.51mg/mL)
Injection volume: 0.5μL
System: Waters ACQUITY UPLC H-Class PLUS
Mixer: 100μL

FlexFire



Conditions:

Column: Develosil FlexFire WP C4, 2.6μm (2.0x50mm)
Develosil FlexFire WP C8, 2.6μm (2.0x50mm)
Develosil FlexFire WP C18, 2.6μm (2.0x50mm)

Mobile phase: A) Water + 0.1%TFA B) Acetonitrile + 0.1%TFA

Gradient:

min	mL/min	%A	%B	Curve
0.00	0.3	100	0	
4.76	0.3	60	40	6
8.42	0.3	20	80	6
8.50	0.3	100	0	6

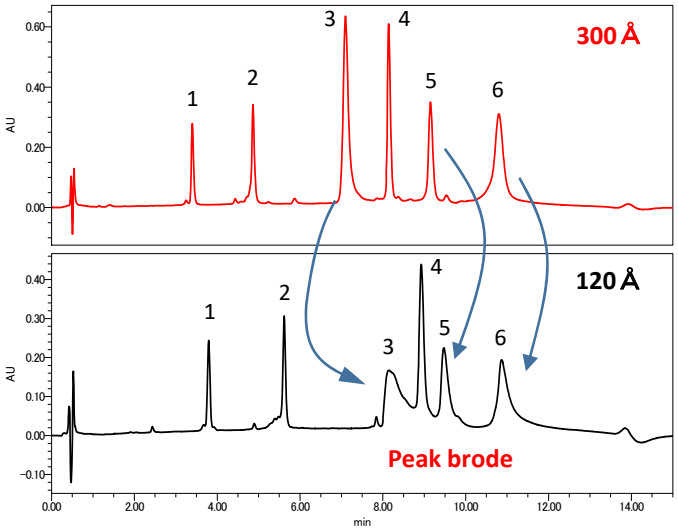
Temperature: 40℃
Sample: Cyclo (-RGDSP)
Cyclo (-GRGESP)
Cyclo (-RGDfK)
Cyclo (-RGDfC)
Injection volume: 0.5μL
System: Waters ACQUITY UPLC H-Class PLUS
Mixer: 100μL

3. Analysis of Proteins

Unlike the peptides described in the previous section, the molecular weight of proteins becomes large at once.

Molecules that do not fit in the pores cause peak broadening and peak collapse.

The sharp peaks that cannot be obtained with a 120Å pore size column can be obtained with a 300Å column.



Conditions;

Column: Develosil FlexFire WP C4, 2.6μm (2.0x50mm)
Develosil FlexFire AQ C18, 2.6μm (2.0x50mm)

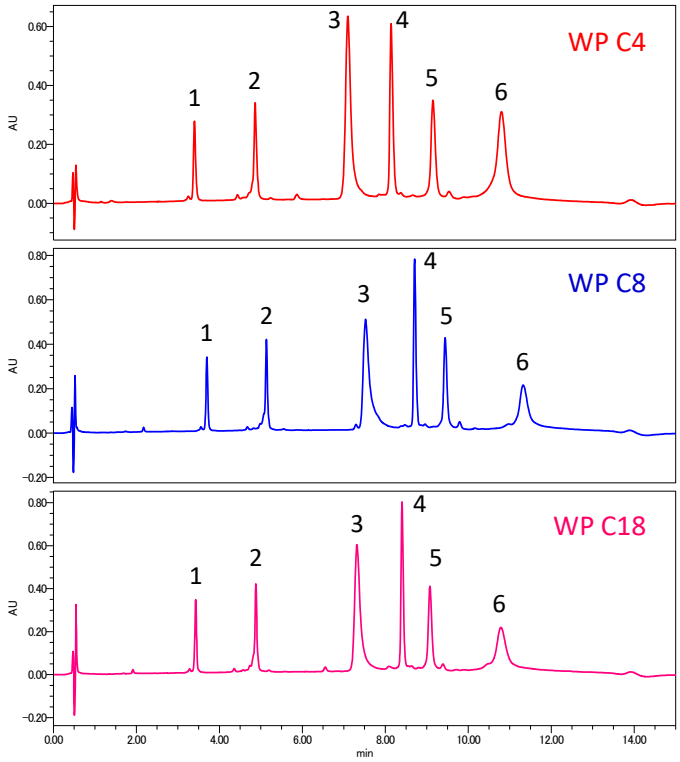
Mobile phase: A) Water + 0.1%TFA B) Acetonitrile + 0.1%TFA

Gradient:

min	mL/min	%A	%B	Curve
0.00	0.3	80	20	
12.60	0.3	40	60	6
12.63	0.3	80	20	6

Temperature: 40℃
Detection: UV210nm
Sample: 1. Ribonuclease A (13KDa)
2. Cytochrome C (11KDA)
3. BSA (67KDa)
4. Myoglobin (14KDa)
5. Enolase(46KDa)
6. Phosphorylase B (97KDa)
Injection volume: 10μL
System: Waters ACQUITY UPLC H-Class PLUS
Mixer: 100μL

FlexFire



Conditions;

Column:	Develosil FlexFire WP C4, 2.6μm (2.0x50mm) Develosil FlexFire WP C8, 2.6μm (2.0x50mm) Develosil FlexFire WP C18, 2.6μm (2.0x50mm)
Mobile phase:	A) Water + 0.1%TFA B) Acetonitrile + 0.1%TFA

Gradient:

min	mL/min	%A	%B	Curve
0.00	0.3	80	20	
12.60	0.3	40	60	6
12.63	0.3	80	20	6

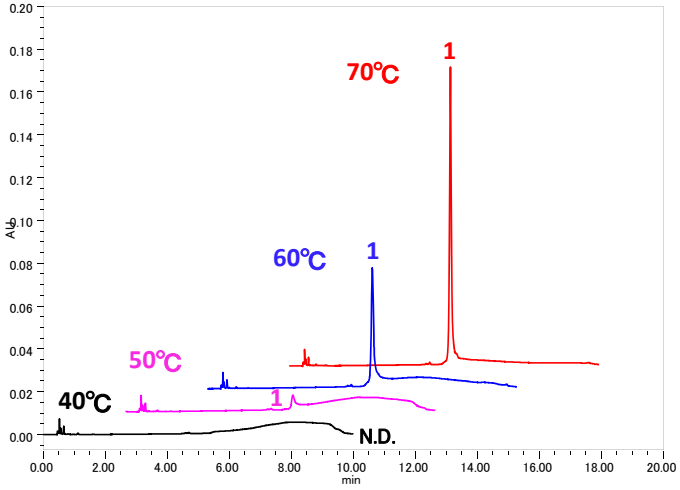
Temperature:	40℃
Detection:	UV210nm
Sample:	Ribonuclease A (13KDa) Cytochrome C (11KDa) BSA (67KDa) Myoglobin (14KDa) Enolase (46KDa) Phosphorylase (97KDa)
Injection volume:	10μL
System:	Waters ACQUITY UPLC H-Class PLUS
Mixer:	100μL

4. Analysis of mAb

Monoclonal antibodies have received a great deal of attention as antibody drugs. In addition to identifying proteins of biological origin, it is expected to treat diseases for which there is no effective treatment such as cancer and infectious diseases.

This monoclonal antibody can also be analyzed by HPLC and UHPLC and is widely used for product stabilization and quality control. Also, the analytical conditions vary depending on the type of column.

Here, we will introduce what the FlexFire WP series can do in monoclonal antibody analysis.



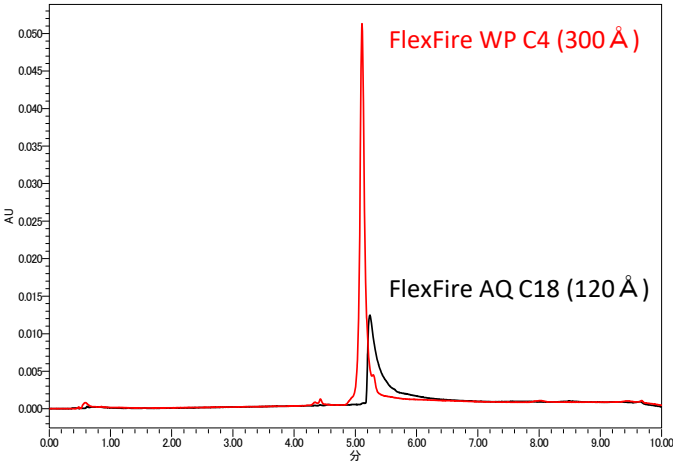
Conditions:

Column:	Develosil FlexFire WP C4, 2.6μm (2.0x50mm)
Mobile phase:	A) Water + 0.1%TFA B) Acetonitrile + 0.1%TFA

Gradient:

Time	mL/min	%A	%B	Curve
0.00	0.3	80	20	
8.40	0.3	40	60	6
8.42	0.3	80	20	6

Temperature:	40℃, 50℃, 60℃, 70℃
Detection:	UV280nm
Sample:	1. Intact Mouse IgG1 (5.0mg/mL)
Injection volume:	1.0μL
System:	Waters ACQUITY UPLC H-Class PLUS
Mixer:	100μL



Conditions:

Column: Develosil FlexFire WP C4, 2.6μm (2.0x50mm)
Develosil FlexFire AQ C18, 2.6μm (2.0x50mm)

Mobile phase: A) Water + 0.1%TFA
B) Acetonitrile + 0.1%TFA

Gradient:

Time	mL/min	%A	%B	Curve
0.00	0.3	80	20	
8.40	0.3	40	60	6
8.42	0.3	80	20	6

Temperature: 70℃
Detection: UV280nm
Sample: 1. Intact Mouse IgG1 (5.0mg/mL)
Injection volume: 1.0μL
System: Waters ACQUITY UPLC H-Class PLUS
Mixer: 100μL

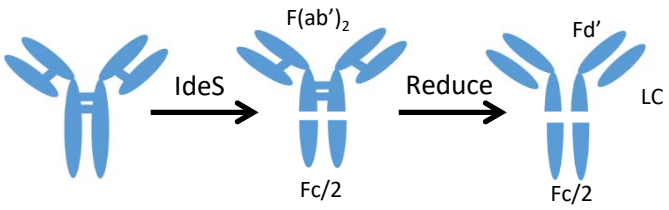
■ **Comparison with 120Å column**

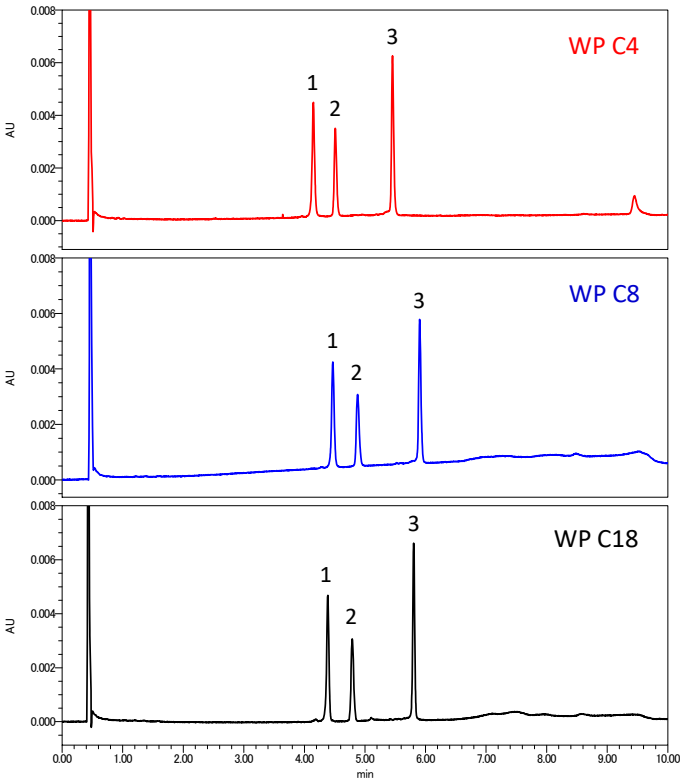
The molecular weight of Intact Mous IgG 1 used in this data is approximately 150 kDa.

For compounds with large molecular weight and structure, matching pore size is important.

4-2. Fragmentation of monoclonal antibodies

Monoclonal antibodies can be fragmented (lower molecular weight) by enzymes. Three fragments have been obtained by reducing F(ab')2 and Fc/2 obtained by IdeS digestion.





Conditions:

Column: Develosil FlexFire WP C4, 2.6μm (2.0x50mm)
Develosil FlexFire WP C8, 2.6μm (2.0x50mm)
Develosil FlexFire WP C18, 2.6μm (2.0x50mm)

Mobile phase: A) Water + 0.1%TFA
B) Acetonitrile + 0.1%TFA

Gradient:

min	mL/min	%A	%B	Curve
0.00	0.3	100	0	
4.76	0.3	40	60	6
8.42	0.3	80	20	6
8.50	0.3	100	0	6

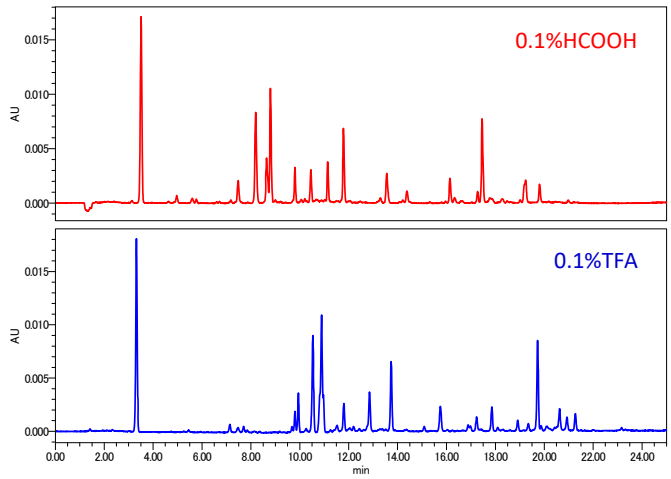
Temperature: 70℃
Detection: UV260nm
Sample: mAb subunit
1. Fc/2
2. LC
3. Fd'
Injection volume: 1.0μL (0.25ug)
Sysytem: Waters ACQUITY UPLC H-Class PLUS
Mixer: 100μL

FlexFire

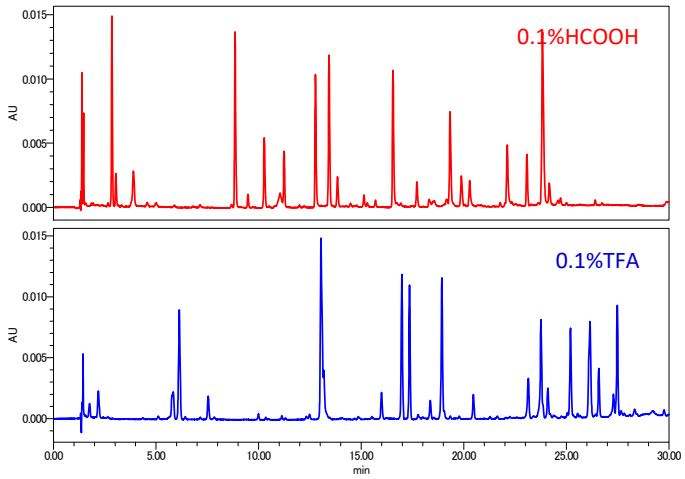
5. Intact protein/mAb peptide mapping

The fragment obtained by IdeS digestion/reduction of the monoclonal antibody is reduced to around 25KDa. It is possible to further reduce the molecular weight by digesting the intact protein or mAb with an enzyme such as trypsin.

■BSA



■mAb



Conditions;

Column:	Develosil FlexFire WP C4, 2.6μm (2.0x150mm)
Mobile phase:	A) Water + 0.1%HCOOH B) Acetonitrile + 0.1%HCOOH
	A) Water + 0.1%TFA B) Acetonitrile + 0.1%TFA

Gradient:

min	mL/min	%A	%B	Curve
0.00	0.3	100	0	
30.0	0.3	50	50	6
30.1	0.3	100	0	6

Temperature:	40℃
Detection:	UV280nm
Sample:	Tryptic digest of BSA
Injection volume:	10μL
system:	Waters ACQUITY UPLC H-Class PLUS
Mixer:	100μL

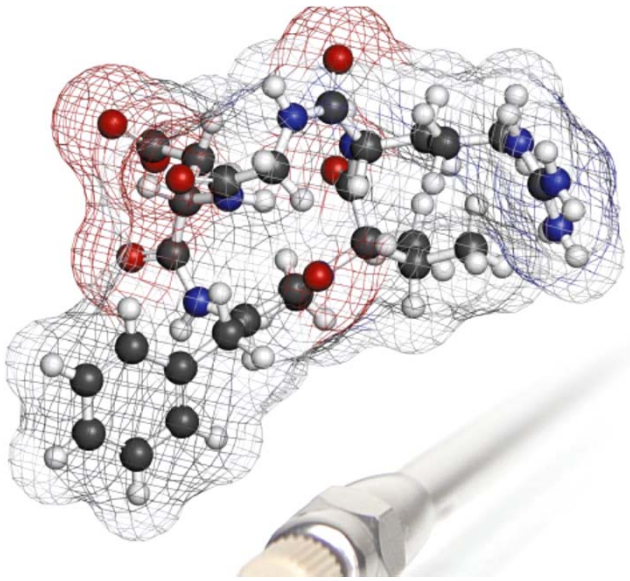
Conditions;

Column:	Develosil FlexFire WP C4, 2.6μm (2.0x150mm)
Mobile phase:	A) Water + 0.1%HCOOH B) Acetonitrile + 0.1%HCOOH
	A) Water + 0.1%TFA B) Acetonitrile + 0.1%TFA

Gradient:

min	mL/min	%A	%B	Curve
0.00	0.3	100	0	
30.0	0.3	50	50	6
30.1	0.3	100	0	6

Temperature:	70℃
Detection:	UV280nm
Sample:	Tryptic digest of mAb
Injection volume:	10μL
system:	Waters ACQUITY UPLC H-Class PLUS
Mixer:	100μL



6. Finally

The FlexFire wide pore series can perform from detection of intact proteins and mAbs to fragmented peptides only on this column.

The process does not require cumbersome column and mobile phase changes.

In addition, this series can be used with UHPLC, and a metal-free column can be selected.

■Contact us

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