Efficient Peptide Purification by HPLC - Effect of Pore Size, Particle Size and Chemistry

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Introduction

Reversed-phase HPLC is an invaluable tool also for the analytical and preparative separation of peptides and proteins. Owing to the availability of different pore sizes and particle sizes, the alkyl-bonded silica gel products are economically the first choice for both analytical and preparative separations.

Although the surface area decreases with increasing pore size, large-pore silica gel products are popular for various separation purposes. A wrong pore size gives, however, poor chromatographic performance. It is important to select the right pore size in separation where a high resolution and high yield are required. This study shows how a wrong pore size affects the resolution and performance. We also refer to the efficient choice of media and the advantages of PROTEIN-RP column designed for separation of peptides and proteins.

Retention Mechanism for Peptides and Proteins (1)
## -Concentration of organic solvent vs. Retention time-
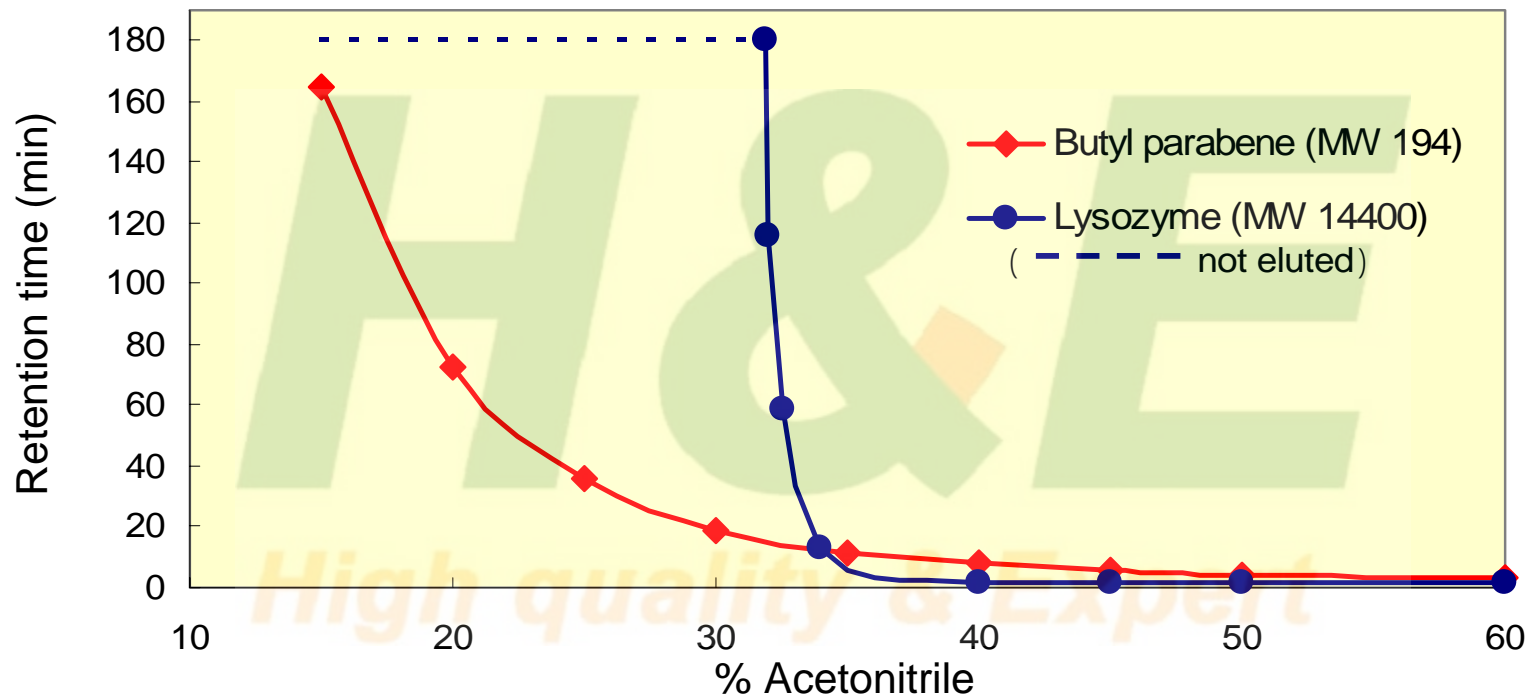
Small organic molecules(━) are retained/eluted by a distribution mechanism as shown in the linear relationship. On the other hand, peptides and proteins (━) are retained/eluted by an adsorption-desorption (on-off) mechanism. Due to this mechanism, the pore size plays a key role in determination of resolution and loading amount in separation of peptides and proteins.

Retention Mechanism for Peptides and Proteins (2)

Small organic compounds

Mobile phase

Stationary phase

Peptides and proteins
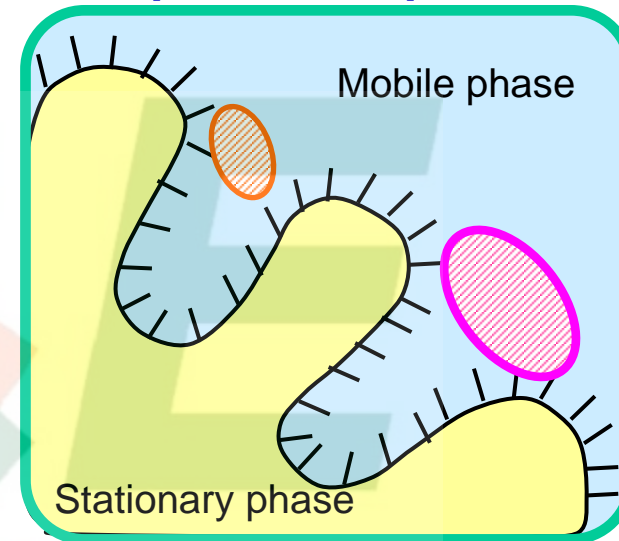
Mobile phase
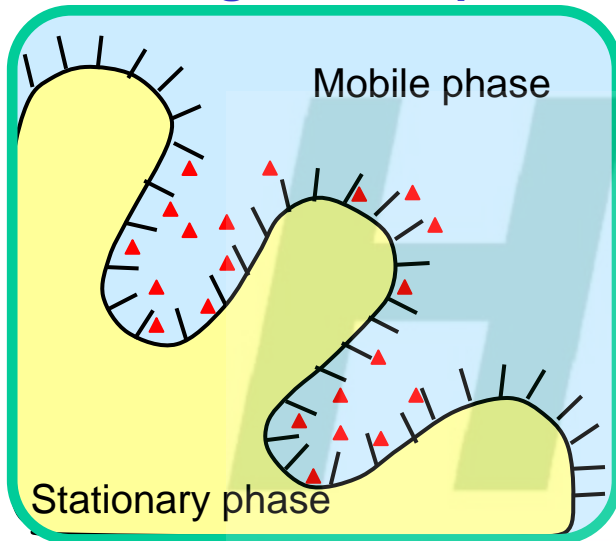
Stationary phase

The *marks* represent small organic compounds.

The small organic compounds are easily entered into the pores and interact with the ligands on the stationary phase.

They mobilize with distributing between the stationary phase and mobile phase.

The *ovals* represent peptides and proteins.

The large molecules cannot enter the pores and merely interact with the ligands on the surface of stationary phase.

HPLC Conditions and Analytes

HPLC conditions

Stationary phase: **C4**

Pore size : **12, 20, 30 nm**

Particle size : 5μm

Column size : 150× 4.6 mmI.D.

Flow rate : 1.0 mL/min

Temperature : 37

Detection : UV at 220 nm

Eluent : A) water / TFA (100/0.1), B) acetonitrile / TFA (100/0.1)
10-90%B(0-20min), 90%B(20-25min)

Peptides and proteins in this study

- Angiotensin II, Human   MW   1046
- Insulin Chain B, Oxidized, from Bovine Pancreas   MW   3495
- Insulin, from Bovine Pancreas   MW   5700
- Lysozyme, from Egg White   MW 14400
- Ovalbumin (Albumin, from Chicken Egg)   MW 45000
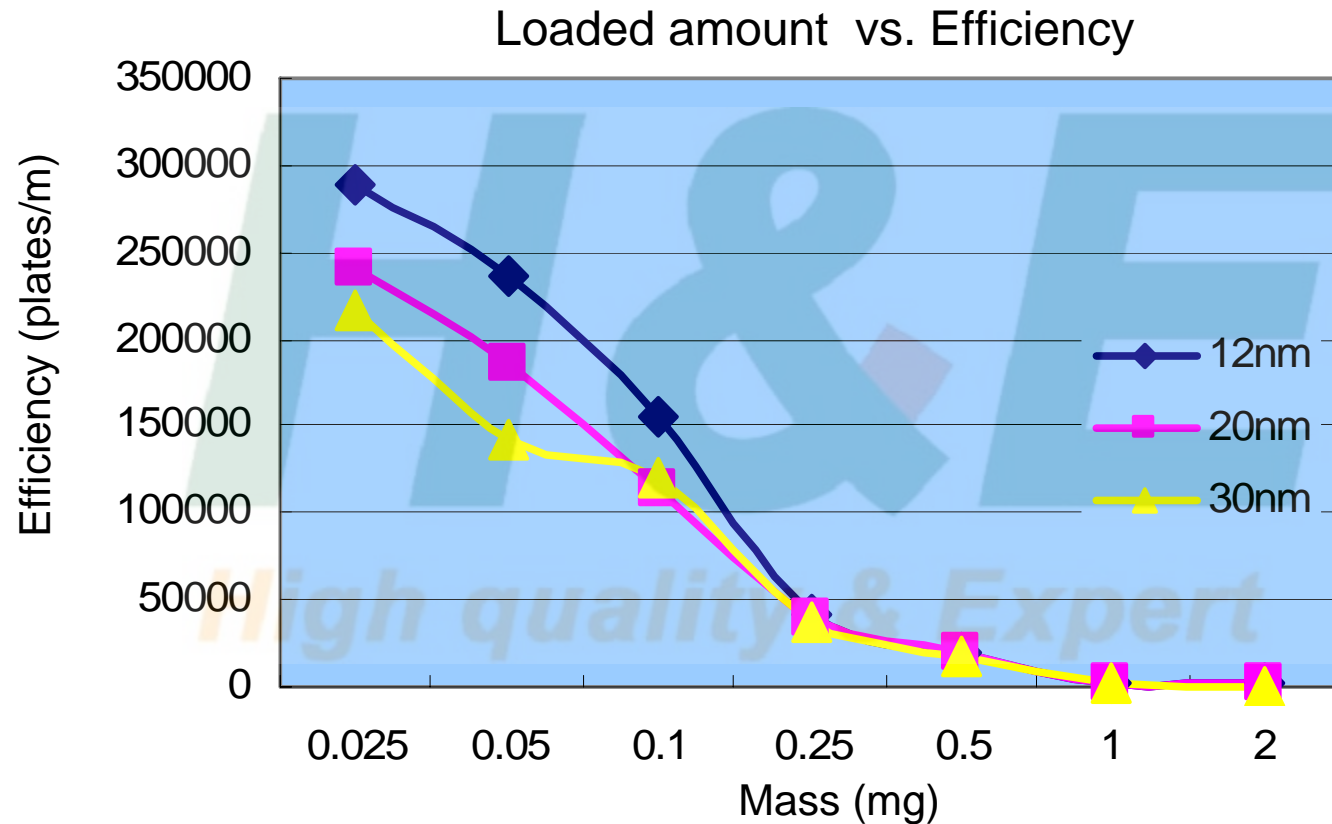- BSA (Albumin, from Bovine Serum)   MW 67000

Impact of Pore Size on Efficiency (1)
-Angiotensin II (MW 1046)-

Loaded amount vs. Efficiency
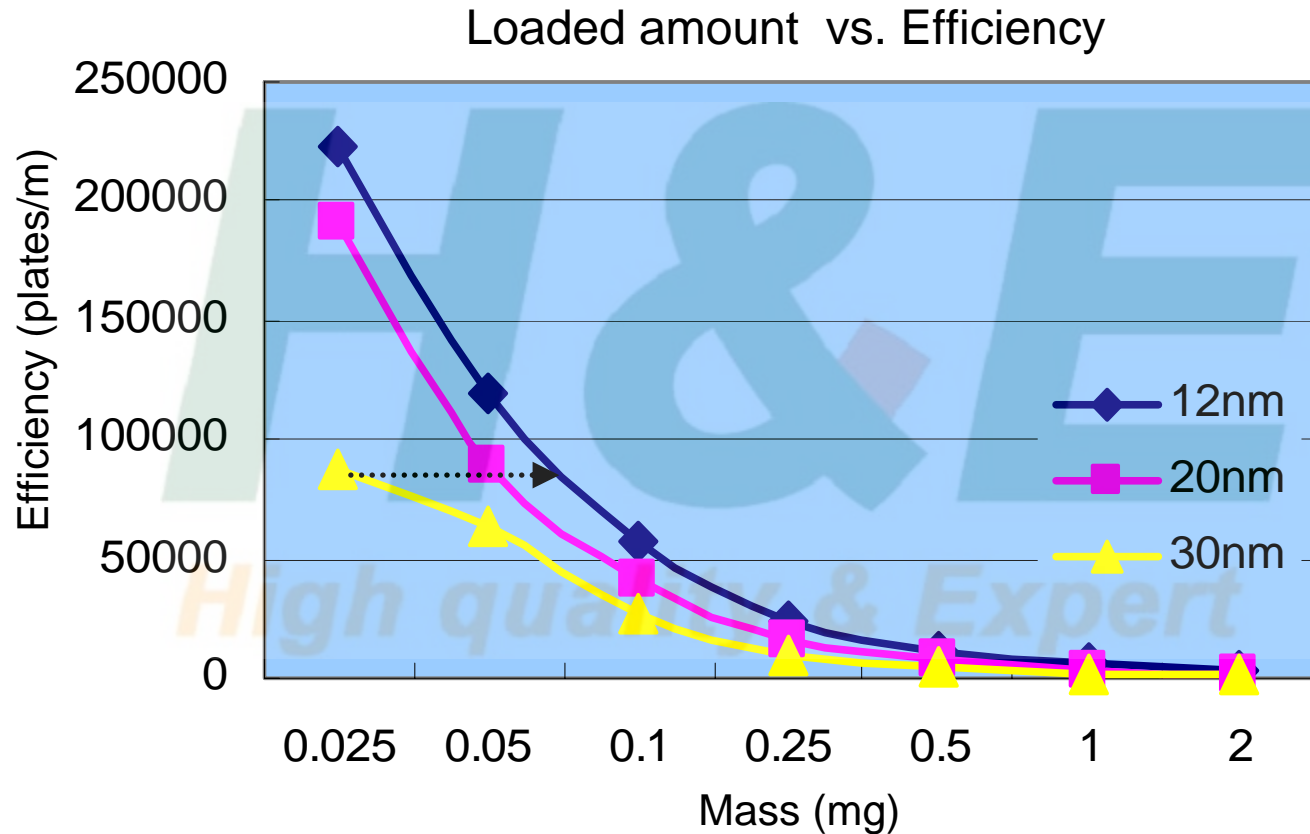
- **12 nm pore size is most efficient at all the loading levels.**

- **12 nm pore size enables a threefold loading level compared with 30 nm pore size.** ·······▶
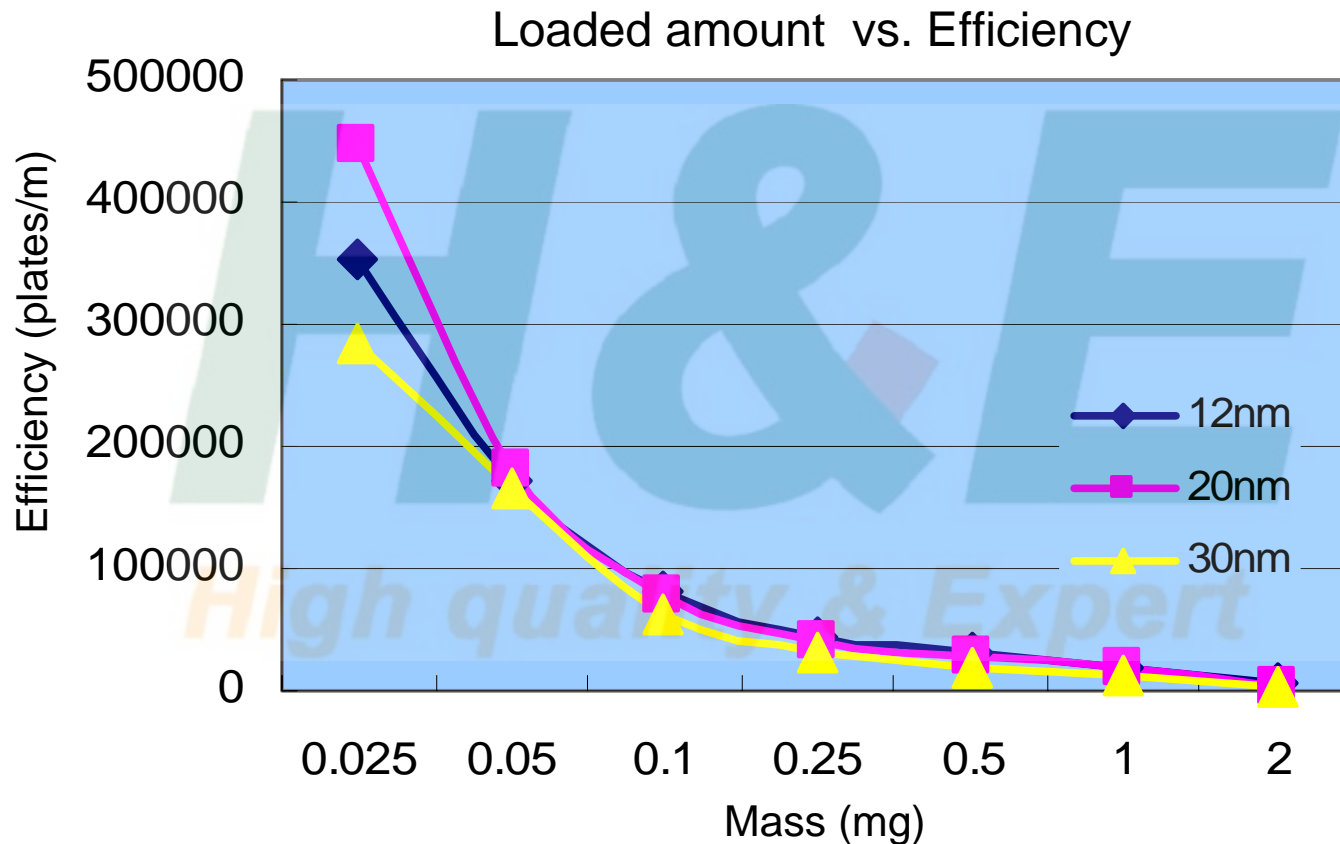
Impact of Pore Size on Efficiency (2)
-Insulin Chain B, Oxidized (MW 3495)-

Loaded amount vs. Efficiency

- **12 nm pore size is most efficient at all the loading levels.**

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Impact of Pore Size on Efficiency (3)
-Insulin (MW 5700)-

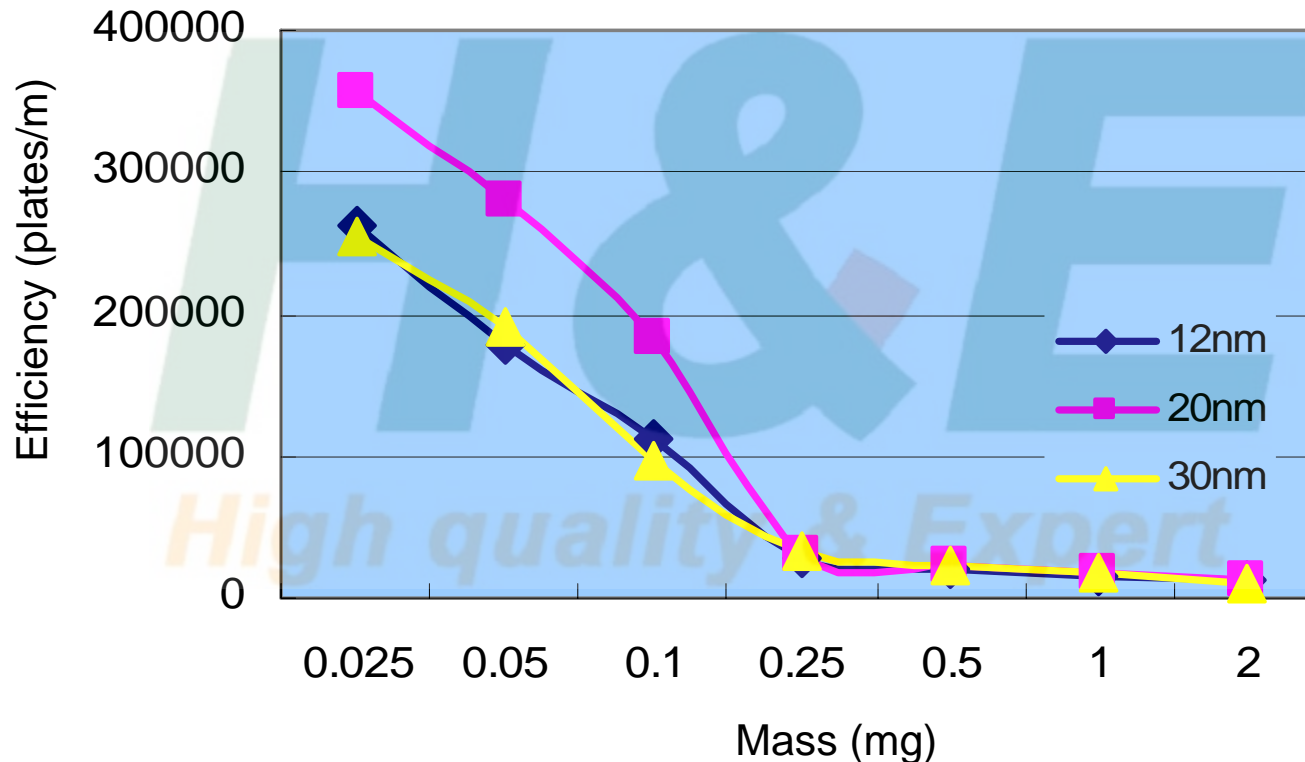Loaded amount vs. Efficiency

- **20 nm pore size is the best choice for preparative samples up to the 0.1 mg loading level.**

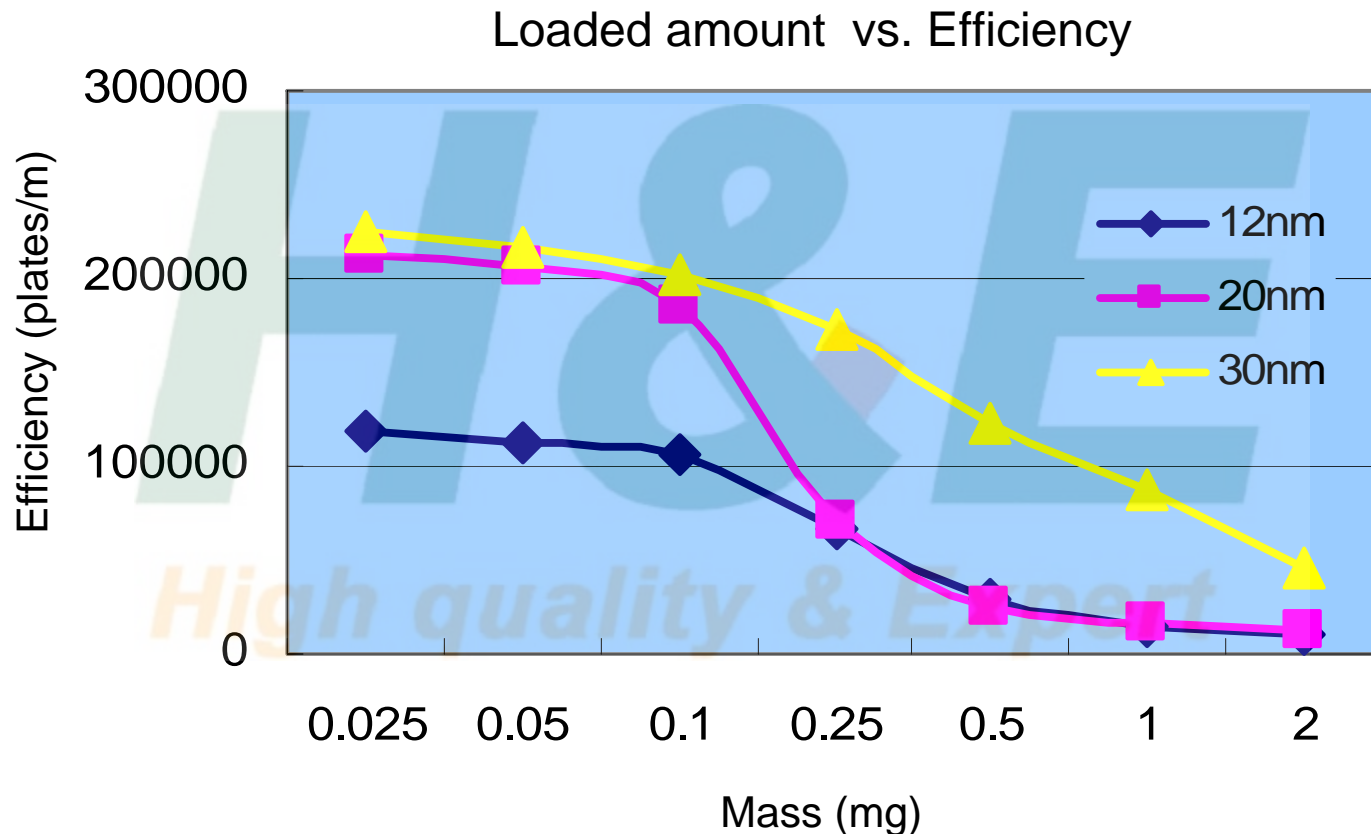# Impact of Pore Size on Efficiency (4)
-Lysozyme (MW 14400)-

Loaded amount vs. Efficiency

- **20 nm pore size is efficient below the 0.2 mg loading level.**

- **20 nm pore size would be suitable to large peptides and small proteins.**

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Impact of Pore Size on Efficiency (5)
-Ovalbumin (MW 45000)-

Loaded amount vs. Efficiency

- **30 nm pore size is most efficient at all the loading levels.**

- **At low loading levels, 20 nm pore size also shows good efficiency.**

Impact of Pore Size on Efficiency (6)
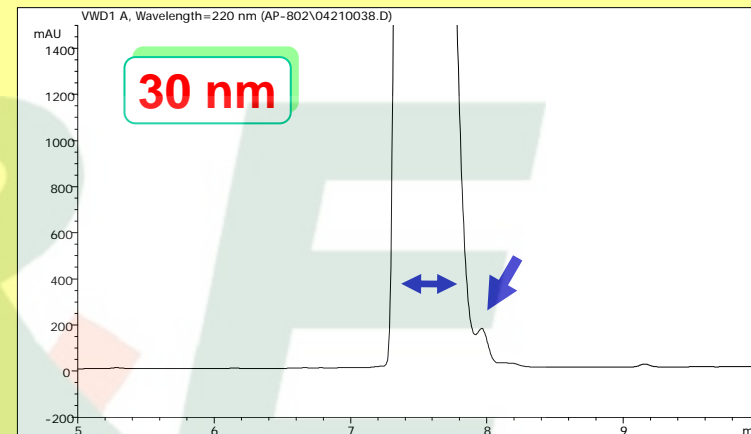## -BSA (MW 67000)-
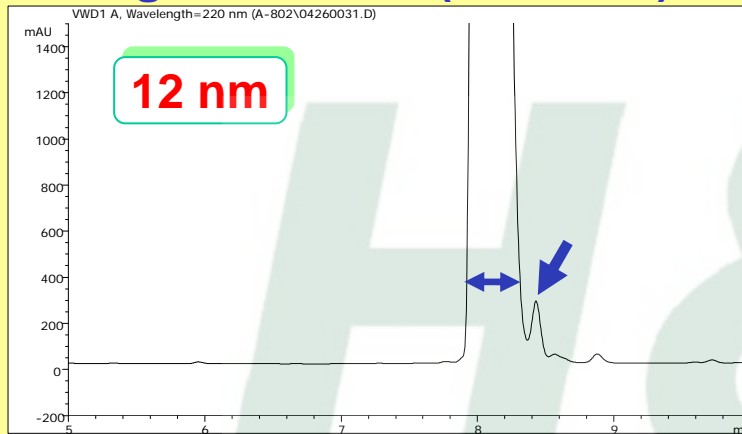

Loaded amount vs. Efficiency
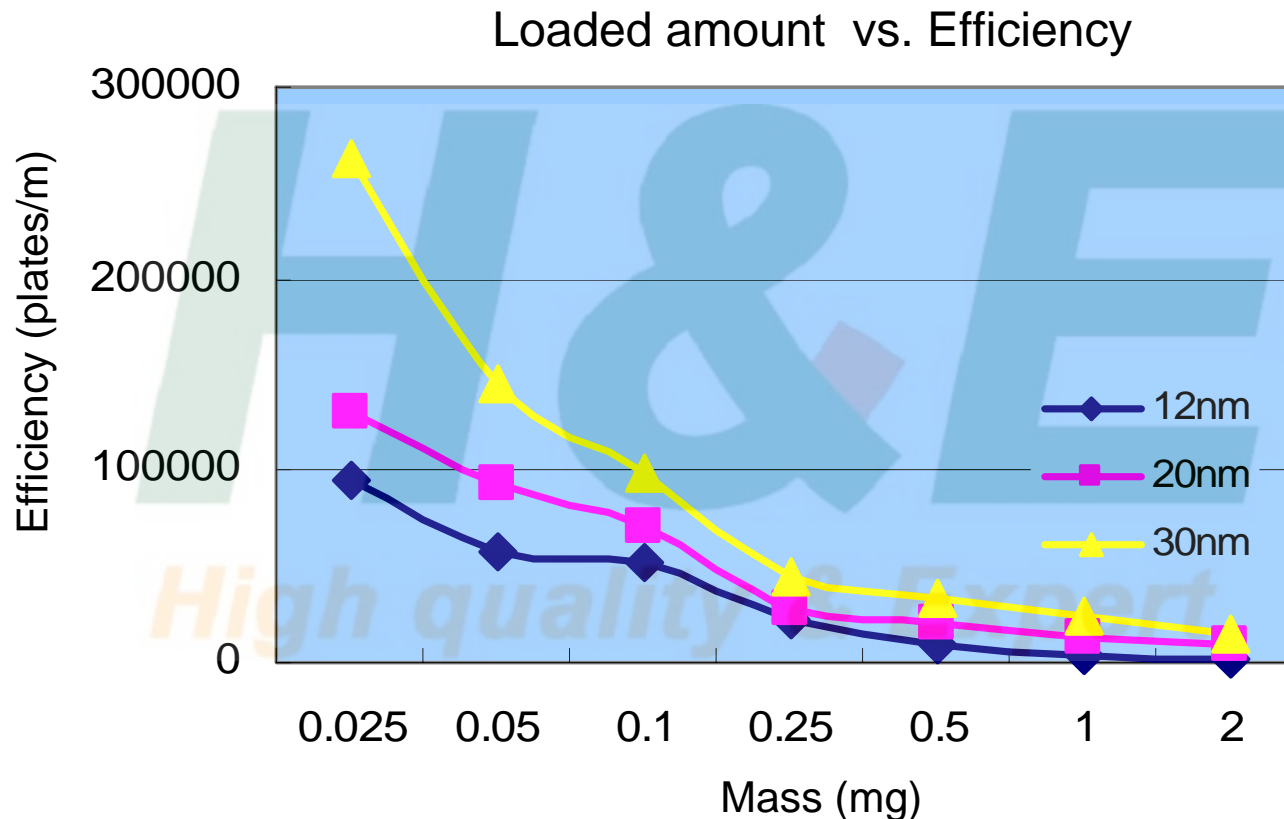
- **30 nm pore size gives the highest efficiency at all the loading levels.**

- **12 nm and 20 nm pore sizes are too small to give a good peak shape and resolution.**
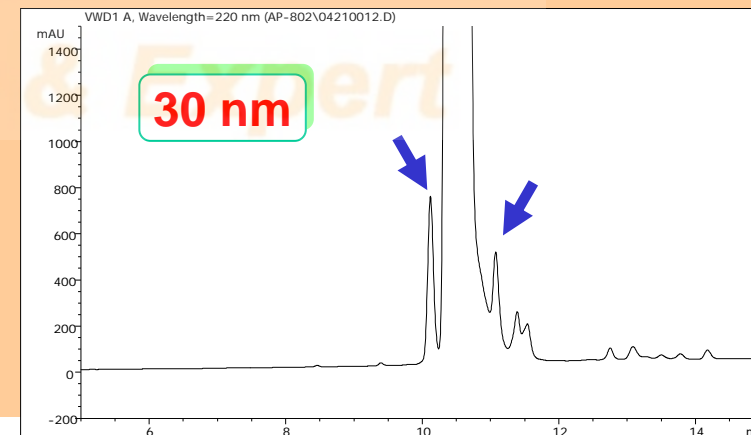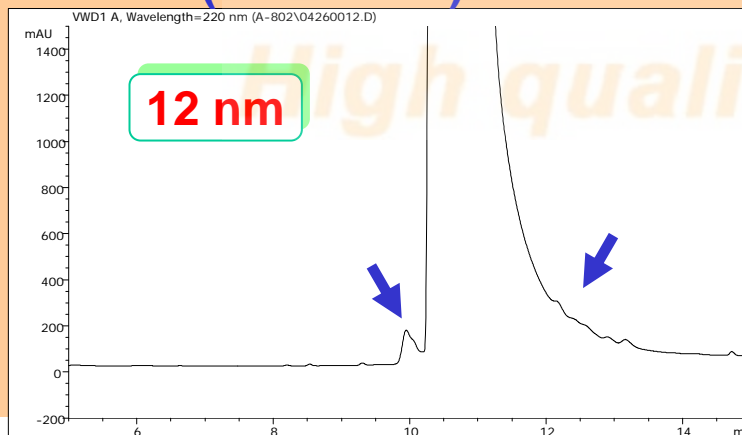
Comparison of peaks on C4 with 12 nm and 30 nm pore sizes

Angiotensin II   (MW 1046)
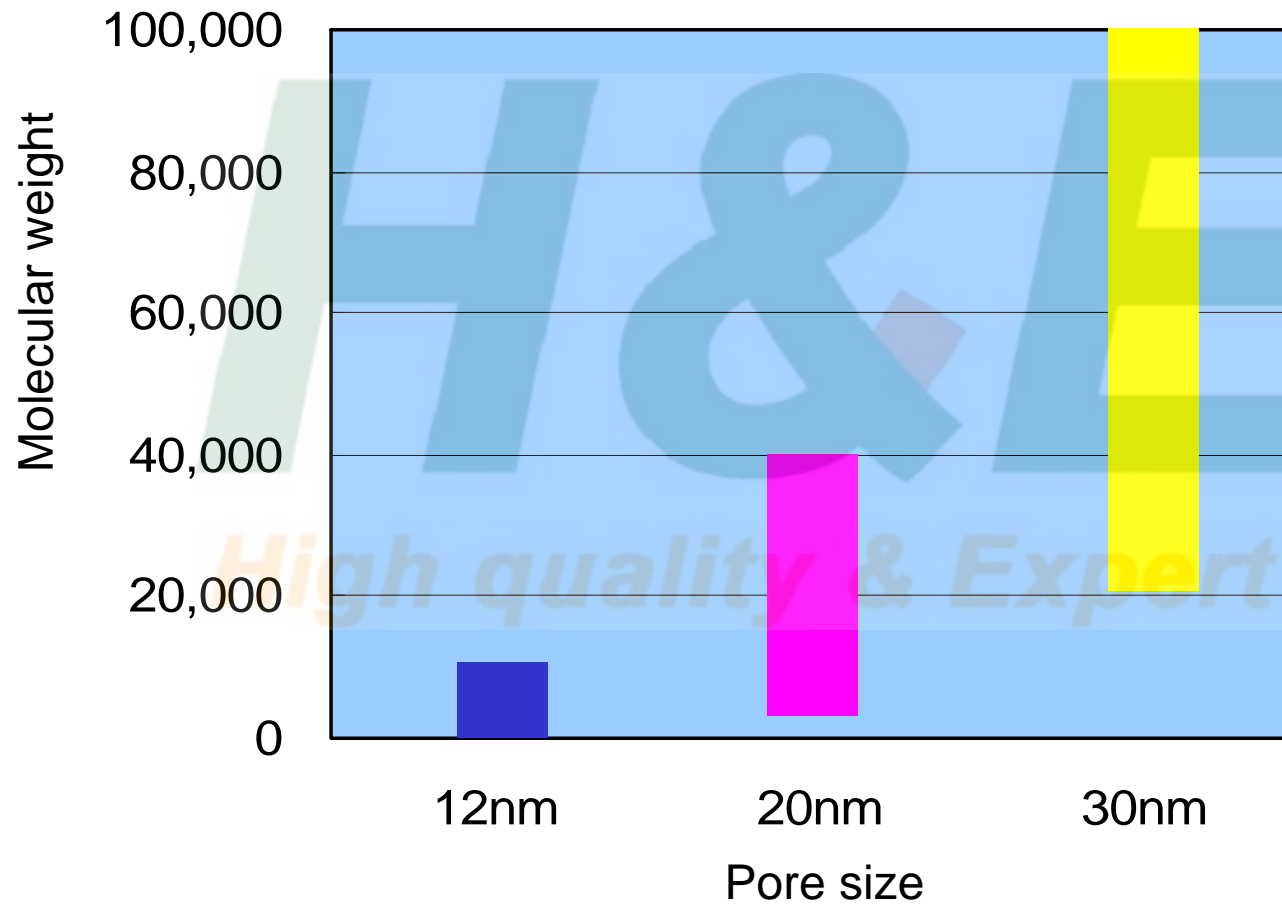

BSA   (MW 67000)

● It is important to choose an appropriate pore size for achieving a good peak shape.

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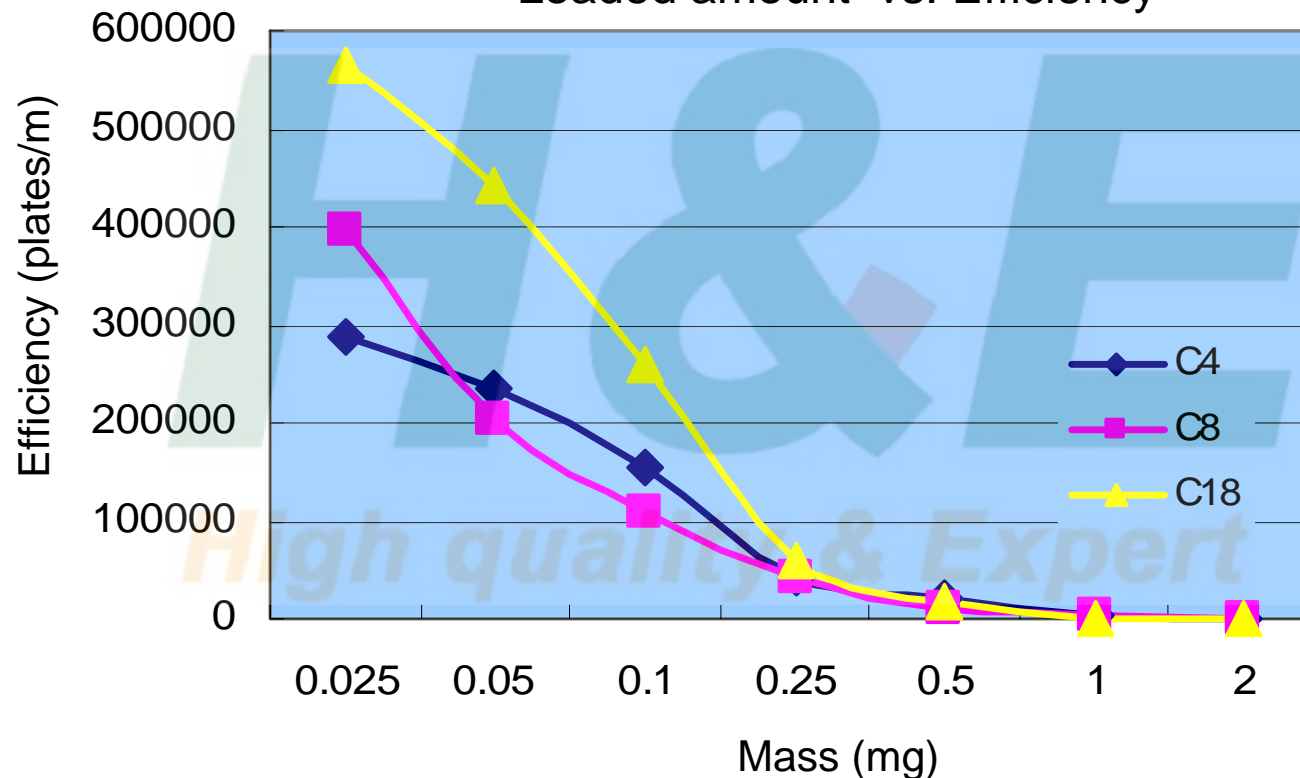# Optimum pore size gel for peptides and proteins

Comparison of C18, C8, C4 ligands on gel with 12 nm pores

Insulin Chain B, Oxidized (MW 3495)

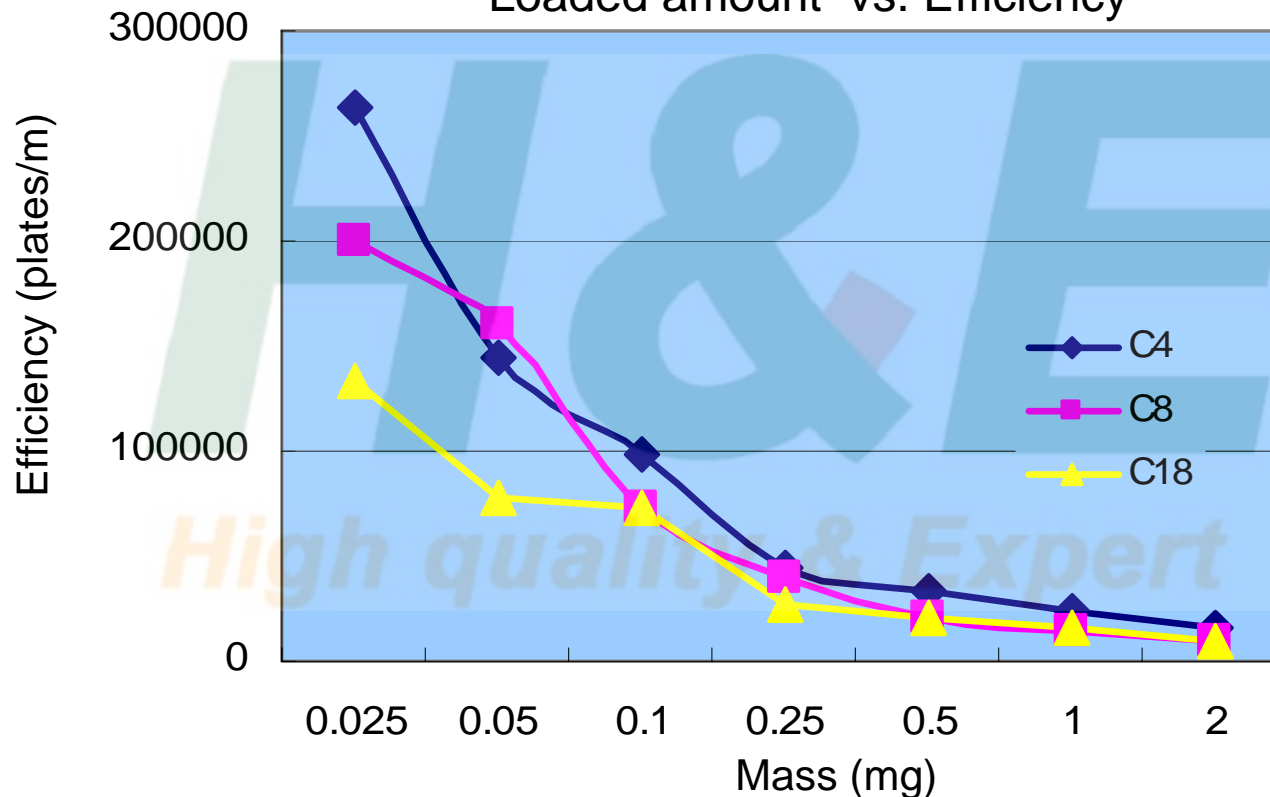Loaded amount vs. Efficiency

- **C18 alkyl chain is most efficient at all loading levels.**

- **In separation of low-MW peptides, the combination of small pore size and hydrophobic alkyl chain would be favorable.**

Comparison of C18, C8, C4 ligands on gel with 30 nm pores

BSA (MW 67000)
Loaded amount vs. Efficiency

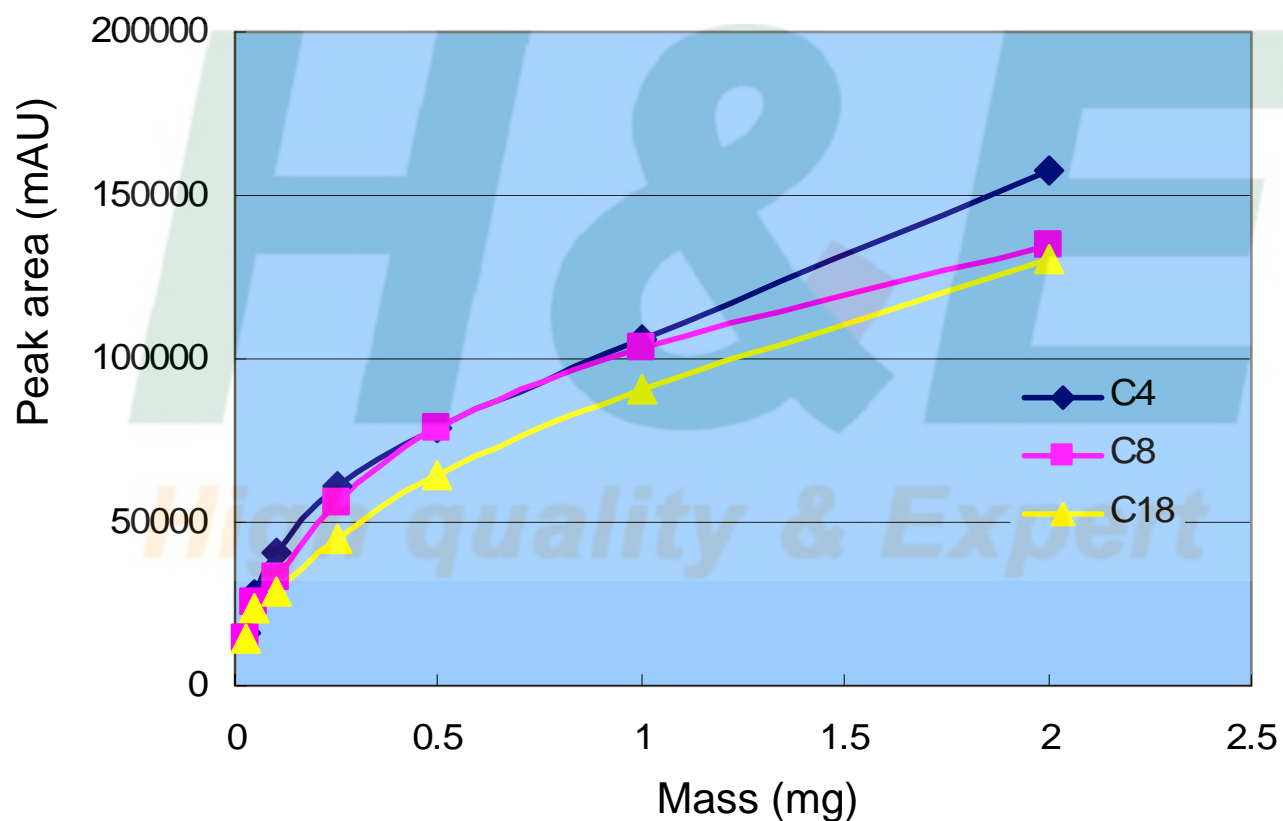- **At almost all the loading levels, C4 ligand shows good efficiency.**

- **For separation of proteins, the combination of 30 nm pore size and short alkyl chain would be the best choice.**

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Impact of ligands on recoveries
-C18, C8, C4 (20 nm)-

Insulin (MW 5700)

Loaded amount vs. Peak area

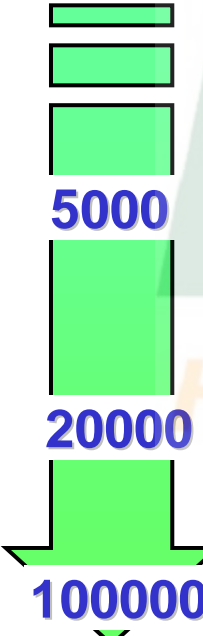- **The higher the hydrophobicity of stationary phase, the lower the recoveries of peptides.**

Effects of ligand on separation

Ligand	C18	C8	Ph	C4	CN	PROTEIN -RP
Hydrophobicity of gel	high				low	low
Suitable MW of sample	low				high	medium ~ high
Recovery of sample	low				high	high
Column durability	good				poor	good

- **It is also necessary to select an appropriate ligand for efficient preparative separation.**

- **The higher the molecular weight is, the less hydrophobic the favorable gel is. However, the less hydrophobic ligand results in shorter column-life, meanwhile, the hydrophobic ligand results in lower sample recovery.**

Optimized stationary phase for separation

MW		C18	C8	C4
5000 20000 100000	12 nm			
	20 nm			
	30 nm			

: excellent,   : good,   : moderate

Optimization of analytical conditions (1)
-Initial conditions-

Peptides and small proteins

- Amyloid β-protein (1-40)   MW   4300
- Insulin, from Bovine pancreas   MW   5700
- Cytchrome C, from Horse heart   MW 12400
- α-Lactalbumin, from Human milk   MW 14200
- Lysozyme, from Egg white   MW 14400
- Myoglobin, from Horse skeletal muscle   MW 17000

Initial conditions

Stationary phase	: **C8, 20 nm** pore size
Particle size	: **5 micron**
Column size	: **150 x 4.6 mm i. d.**
Elution	: **gradient elution**
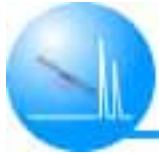
Optimization of analytical conditions (2)
-Gradient elution-

Initial conditions

Column	: **YMC-Pack C8 (5μm, 20nm)**
	150 X4.6 mmI.D.
Eluent	: A) water / TFA (100/0.1)
	B) acetonitrile / TFA (100/0.1)
Flow rate	: 1.0 mL/min
Temperature	: 37
Detection	: UV at 220 nm

1.	Cytchrome C	MW	12400
2.	Insulin	MW	5700
3.	Amyloid β-protein	MW	4300
4.	Lysozyme	MW	14400
5.	α-Lactalbumin	MW	14200
6.	Myoglobin	MW	17000

10 – 90%B (0-20min)

(04082003.D)
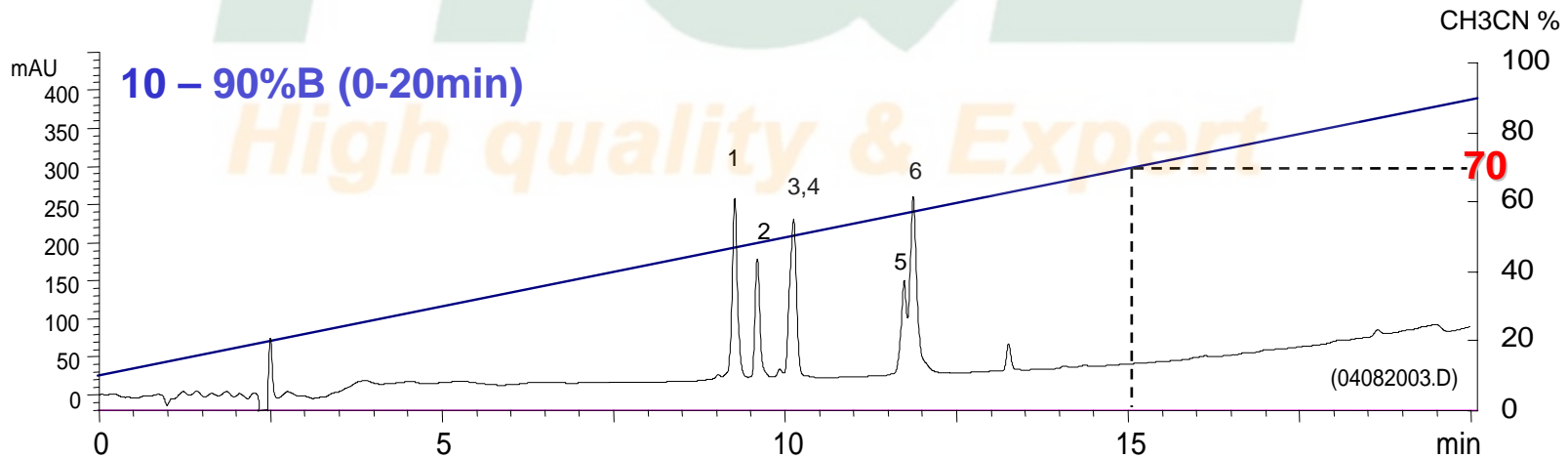
Optimization of analytical conditions (3)
-Initial ACN concentration-

20 – 70%B (0-20min)

04082005.D

25 – 70%B (0-20min)

04082006.D

30 – 70%B (0-20min)

04082007.D

35 – 70%B (0-20min)

04082008.D
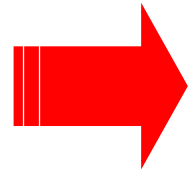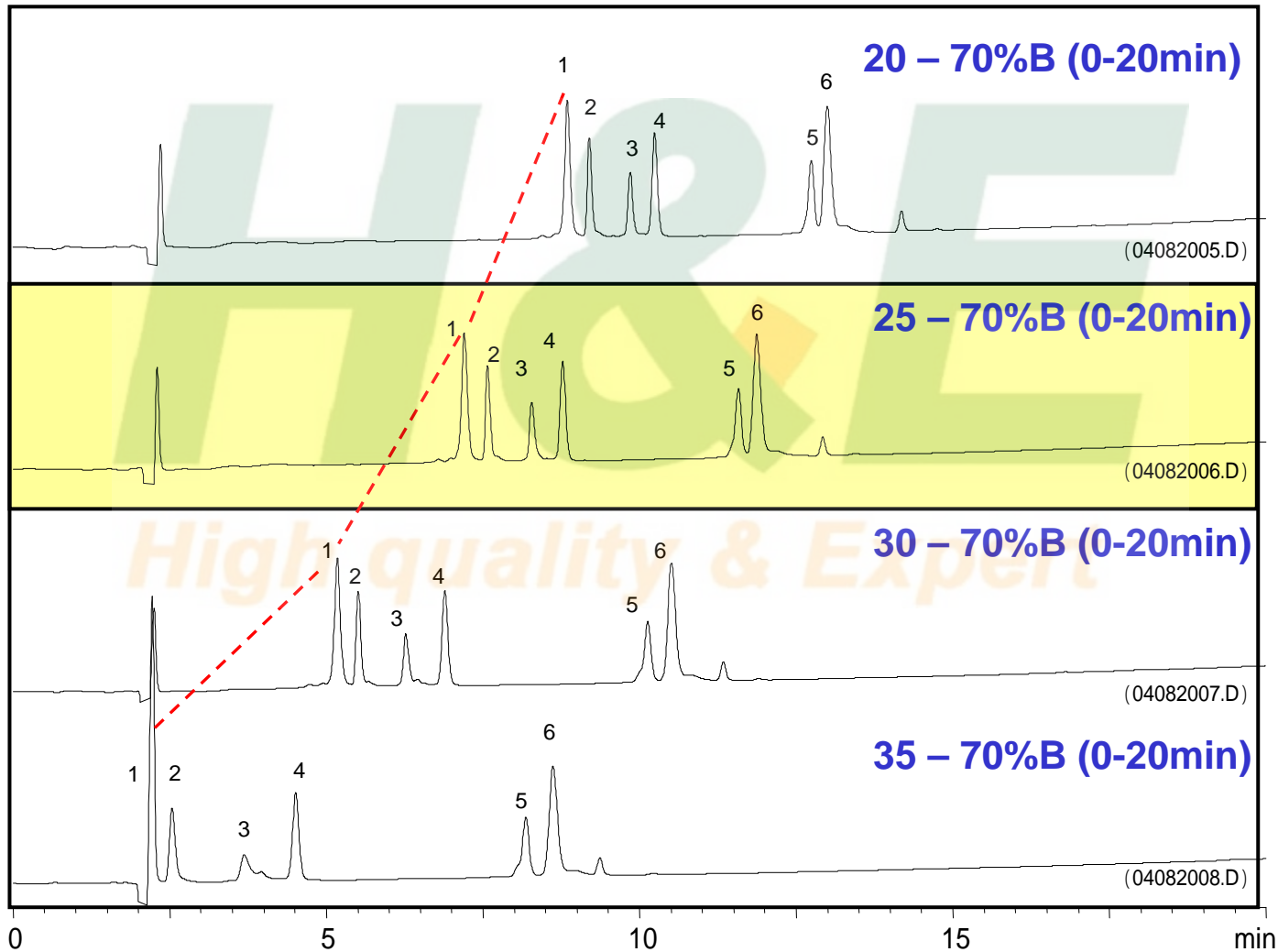
Optimization of analytical conditions (4)
-Final ACN concentration-

25 – 55%B (0-20min)

(04082010.D)

25– 60%B (0-20min)

(04082002.D)

25 – 65%B (0-20min)

(04082009.D)

25 – 70%B (0-20min)

04082006.D
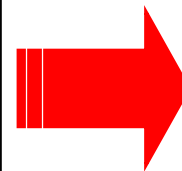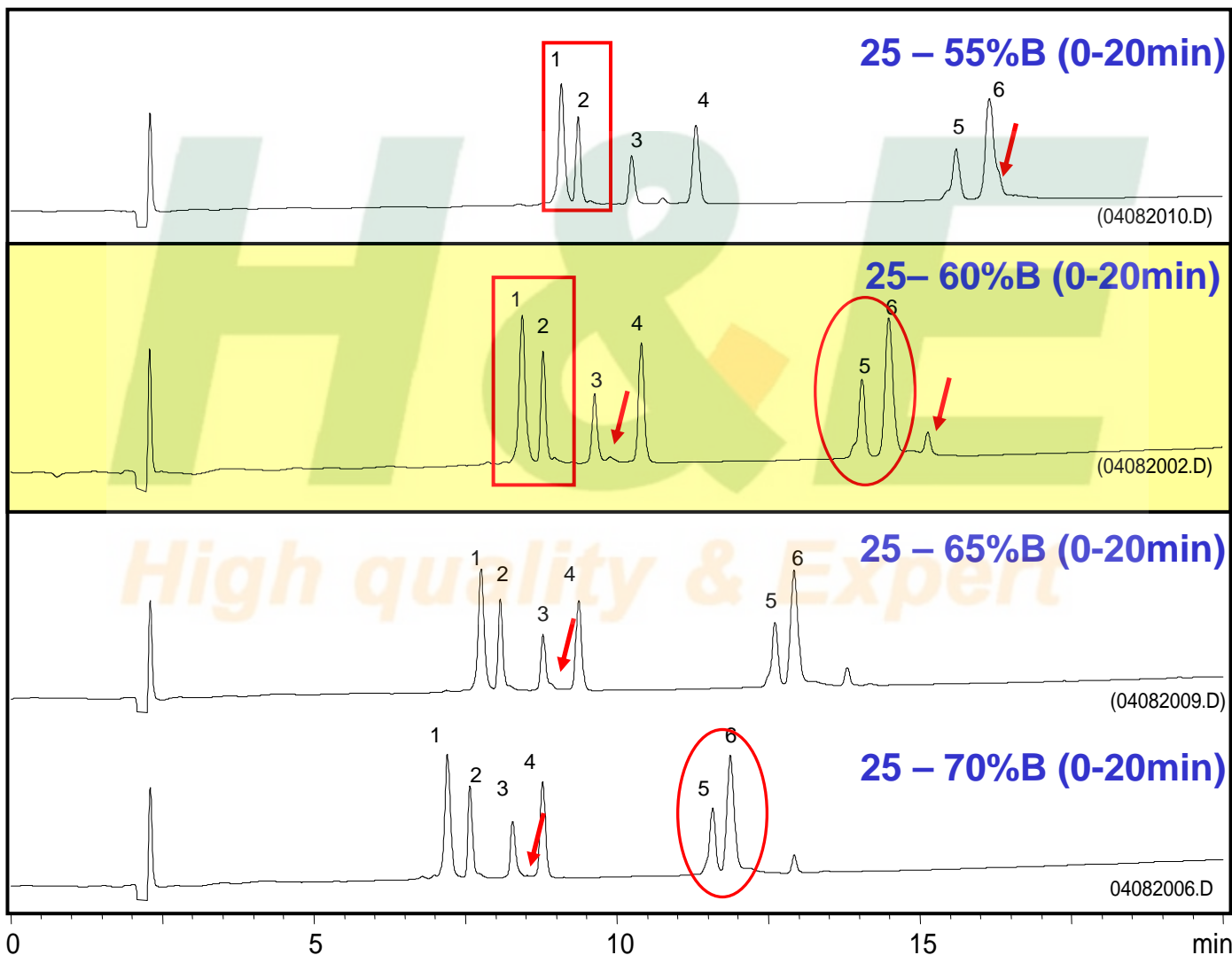
Optimization of analytical conditions (5)
-Optimized conditions-

Optimized conditions

C8, 20nm

Column	: 5μm, 150 X4.6 mmI.D.
Eluent	: A) water / TFA (100/0.1)
	B) acetonitrile / TFA (100/0.1)
	25-60%B(0-20min)
Flow rate	: 1.0 mL/min
Temperature	: 37
Detection	: UV at 220 nm

(04082002D)

C8, 12nm
(04073006D)

C8, 30nm
(04080204D)

C18, 20nm
(04072910D)

C4, 20nm
(04073002D)
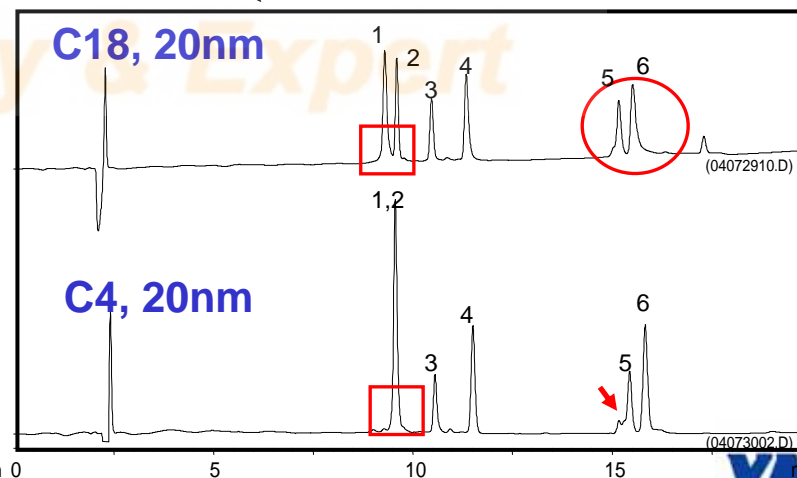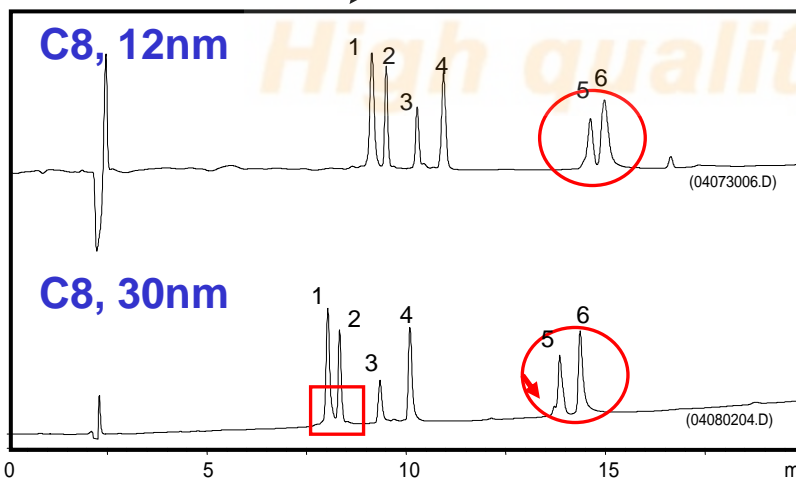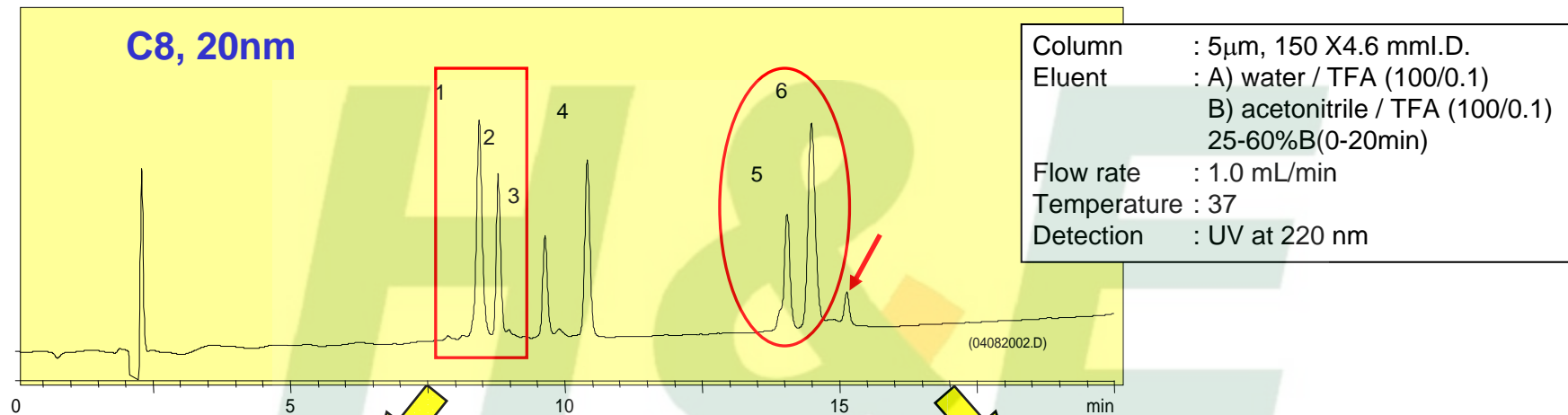
Conclusions

- It is important to choose the right pore size to achieve optimal separation of peptides or proteins. A too small or too large pore size results in poor resolution.

- The Ligand on the gel also plays an important role to achieve efficient separation. Appropriate hydrophobicity of the gel is essential for efficient separation.

- It is necessary to combine with appropriate pore size, hydrophobicity, particle size and column size to achieve higher recoveries or higher resolutions of peptides and proteins.

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