Optimization of the Effective Separations for Peptides and Proteins Using High Durable Packing Materials for HPLC

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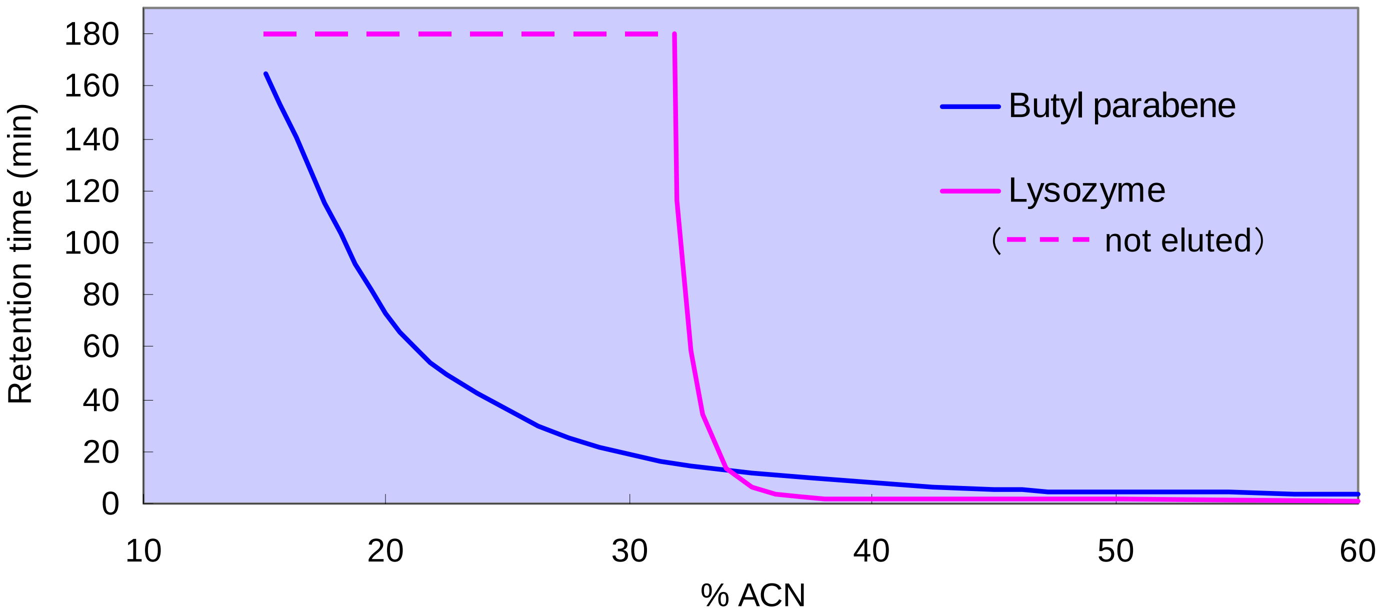
Introduction

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)

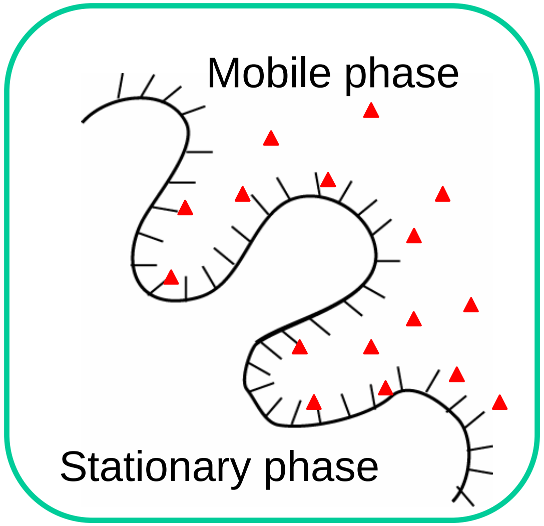
Concentration of organic solvent vs. Retention time



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

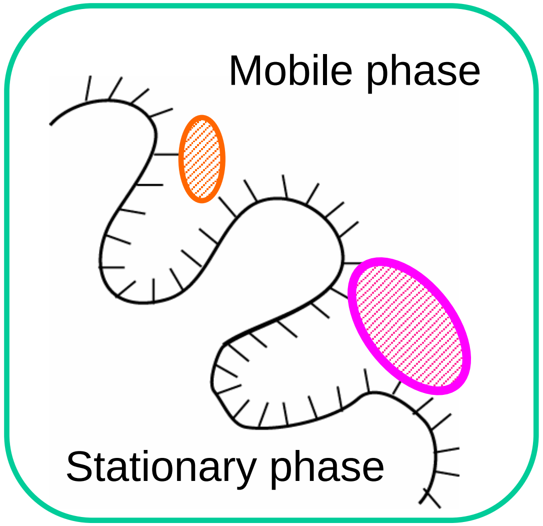


The ▲ *marks* represent small organic compounds.

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.

Peptides and proteins



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.

Experimental Conditions and Analytes

All separations were performed on YMC–Pack C4 (Butyl) 5 micron 150✕4.6 mmi.d. columns with pore sizes of 12, 20 and 30 nm.

Flow rate: 1.0 mL/min, Detection: UV at 220 nm,

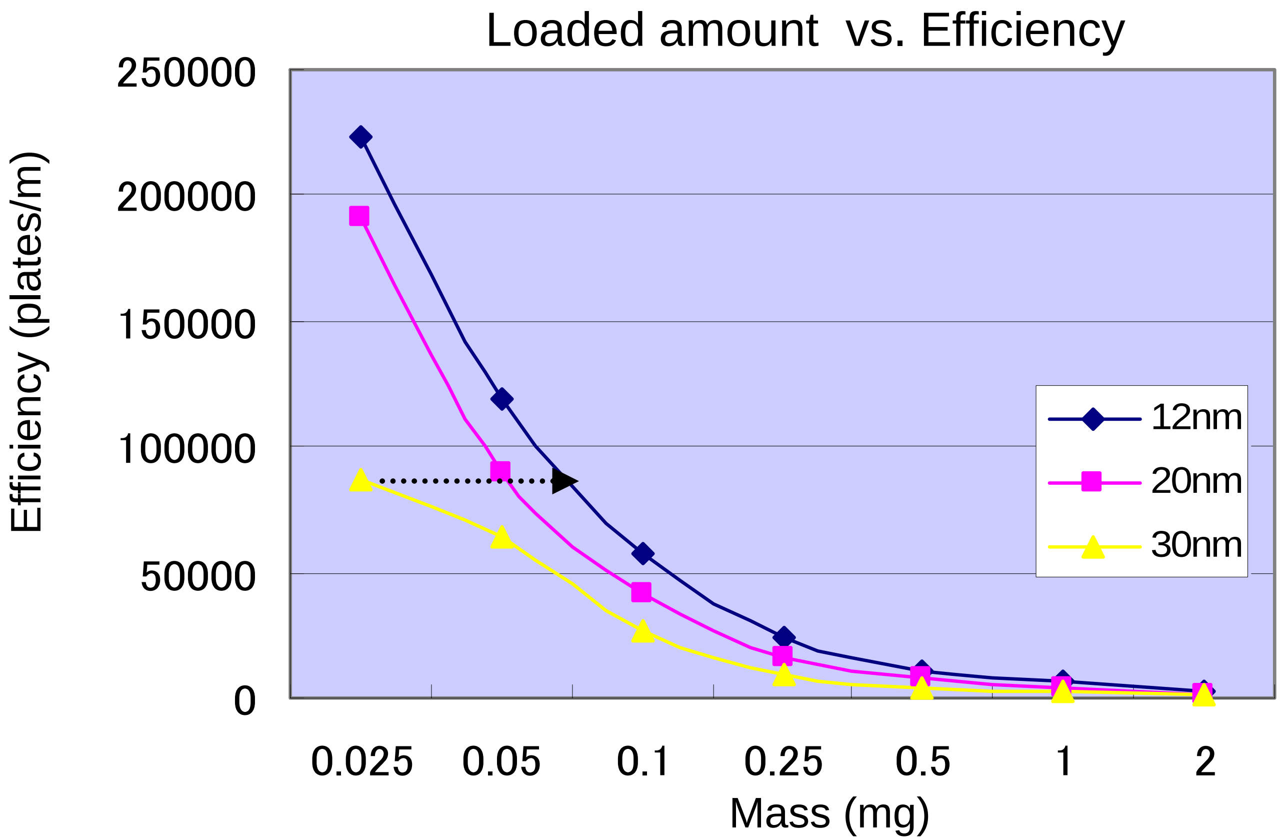
Eluent: A solvent 0.1% TFA, B solvent ACN with 0.1% TFA,
10-90%B(0-20min), 90%B(20-25min)

Peptides and proteins used in the separations.

- 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)
- Albumin, Chicken Egg (MW 45000)
- Albumin, from Bovine Serum (MW 67000)

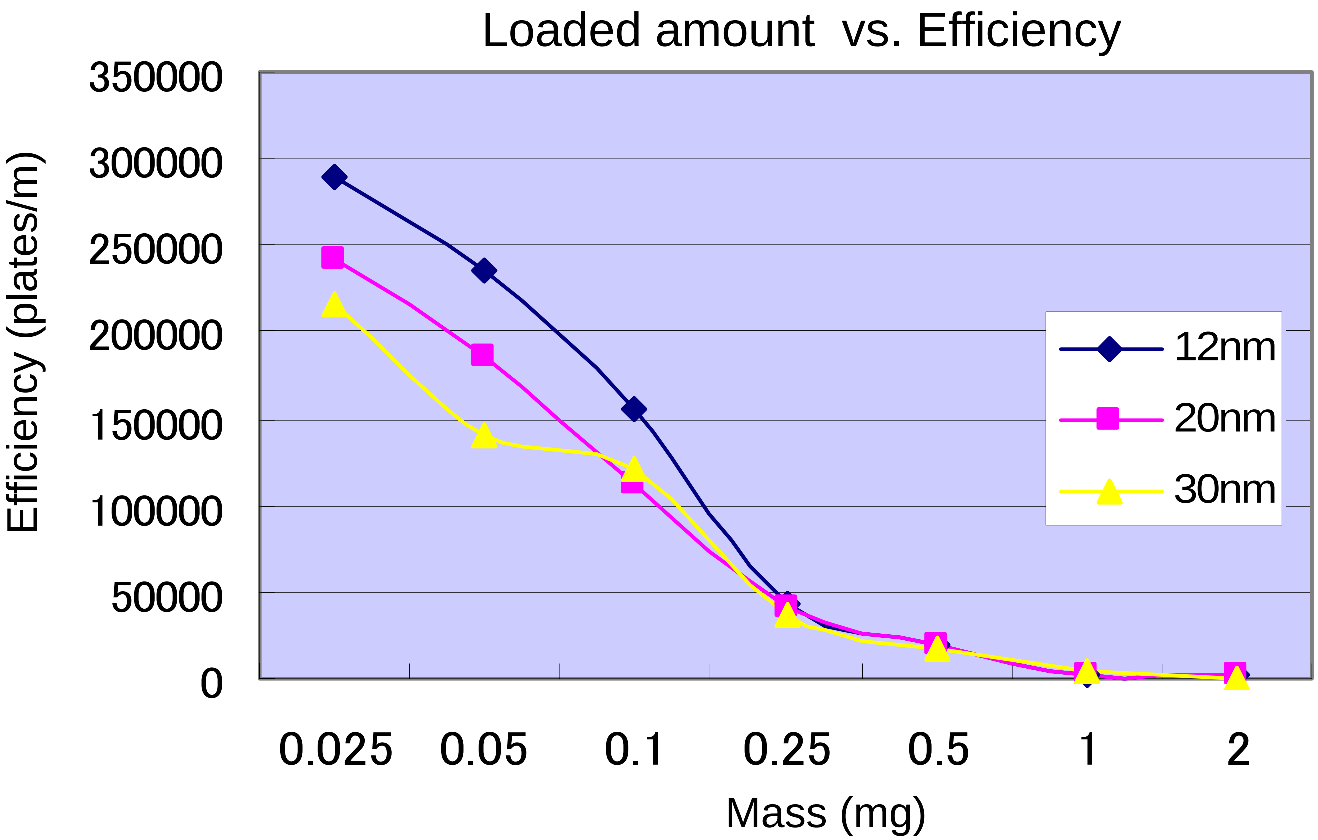
Impact of Pore Size on Efficiency (1)

Angiotensin II (MW 1046)



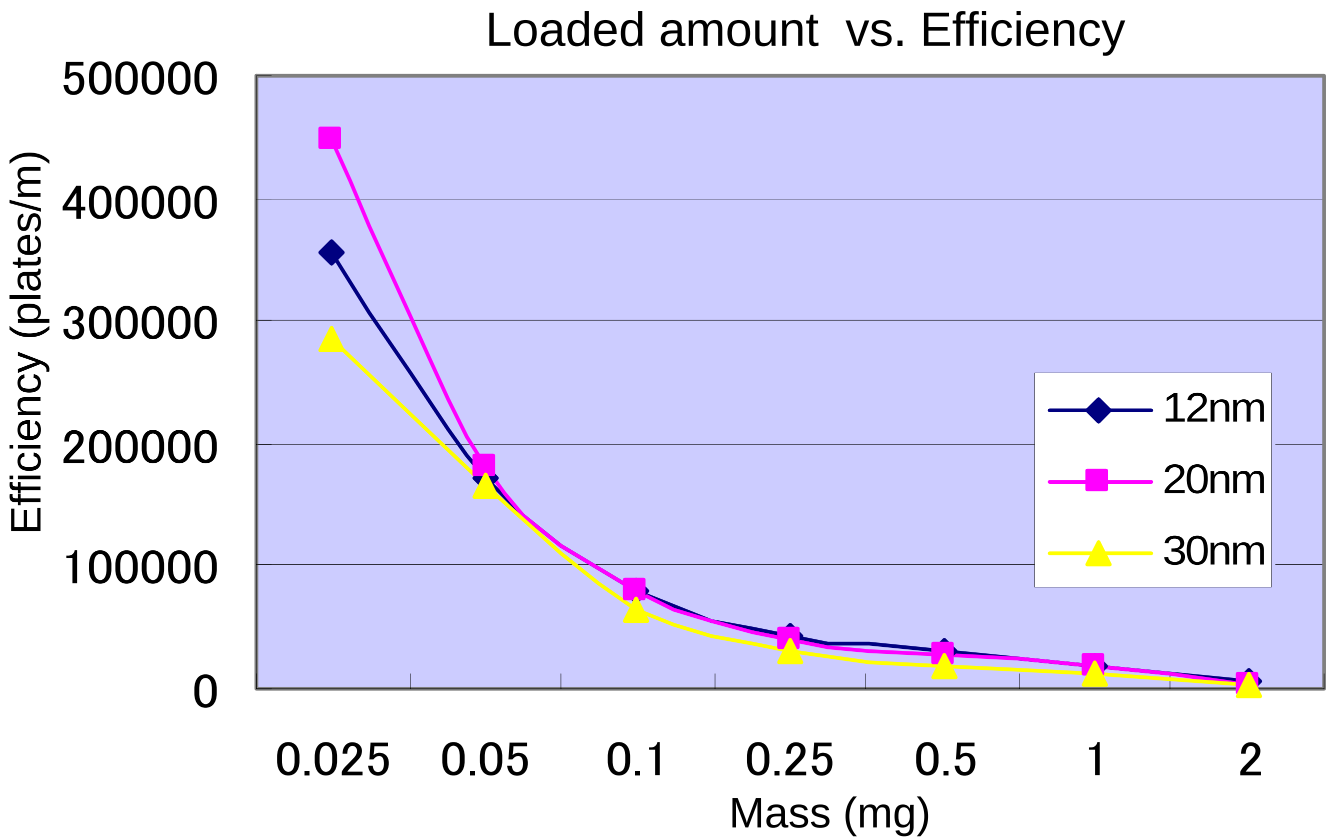
- 12 nm pore size is most efficient at all the loading levels.
- 12 nm pore size enables a threefold loading level compared with 30 nm pore size. (········▶)

Impact of Pore Size on Efficiency (2)
Insulin Chain B, Oxidized (MW 3495)



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

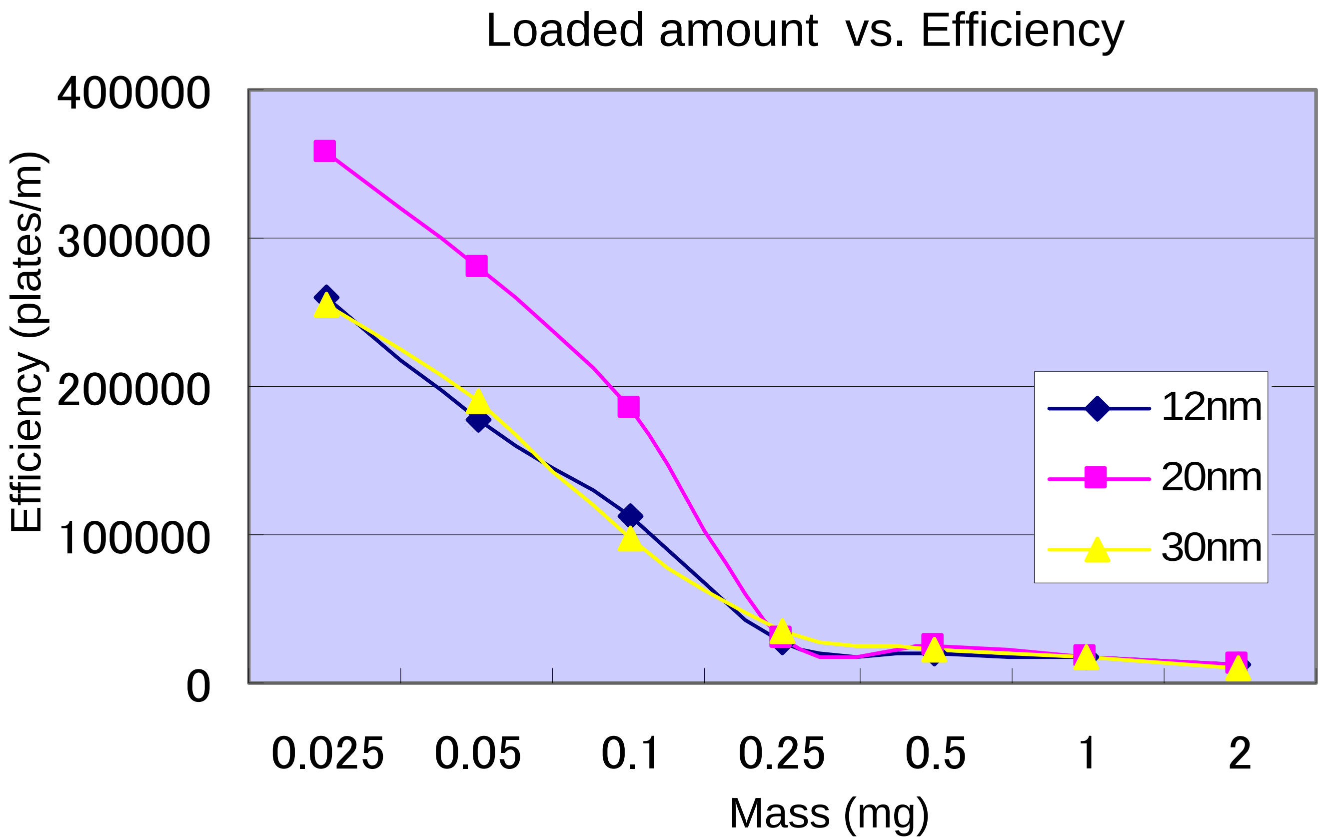
Impact of Pore Size on Efficiency (3)
Insulin from Bovine Pancreas (MW 5700)



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

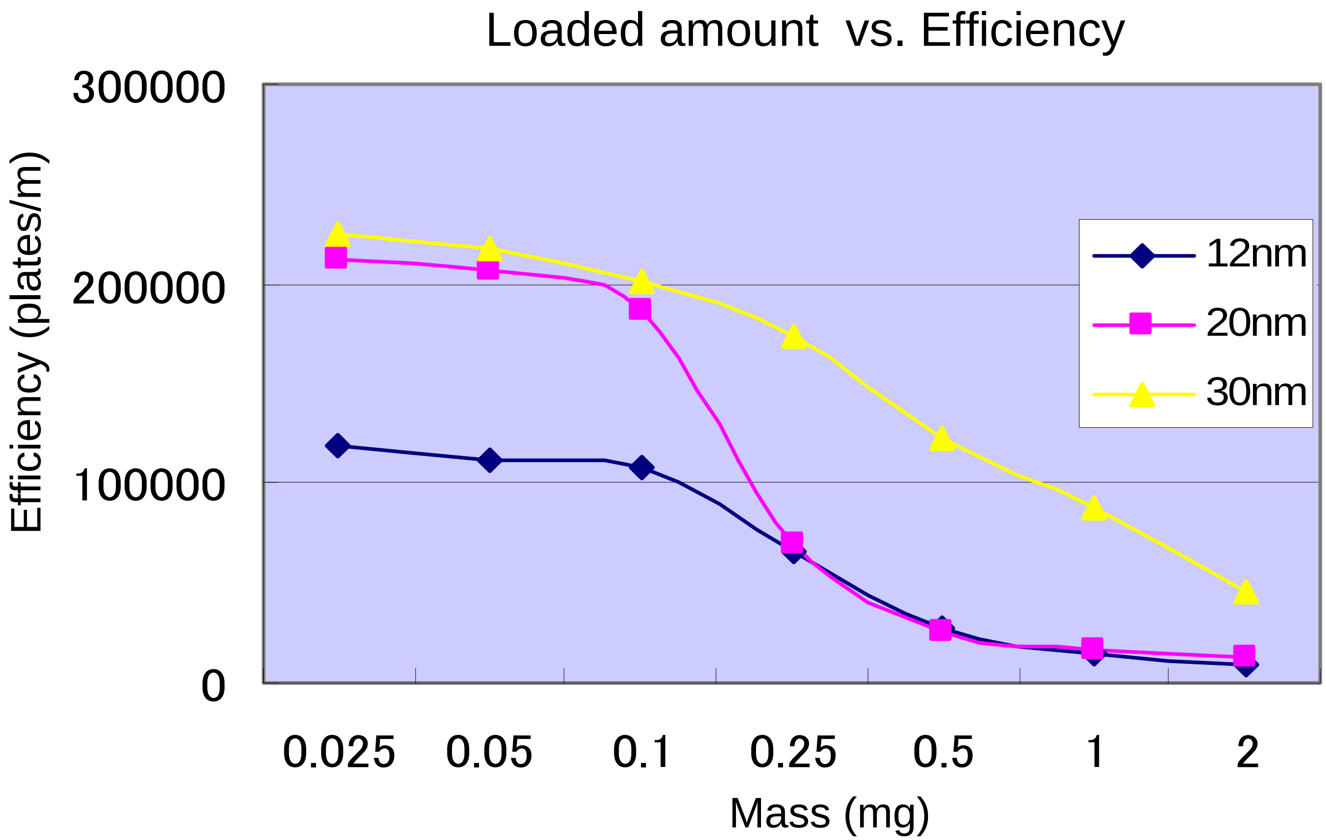
Impact of Pore Size on Efficiency (4)

Lysozyme from Egg White (MW 14400)



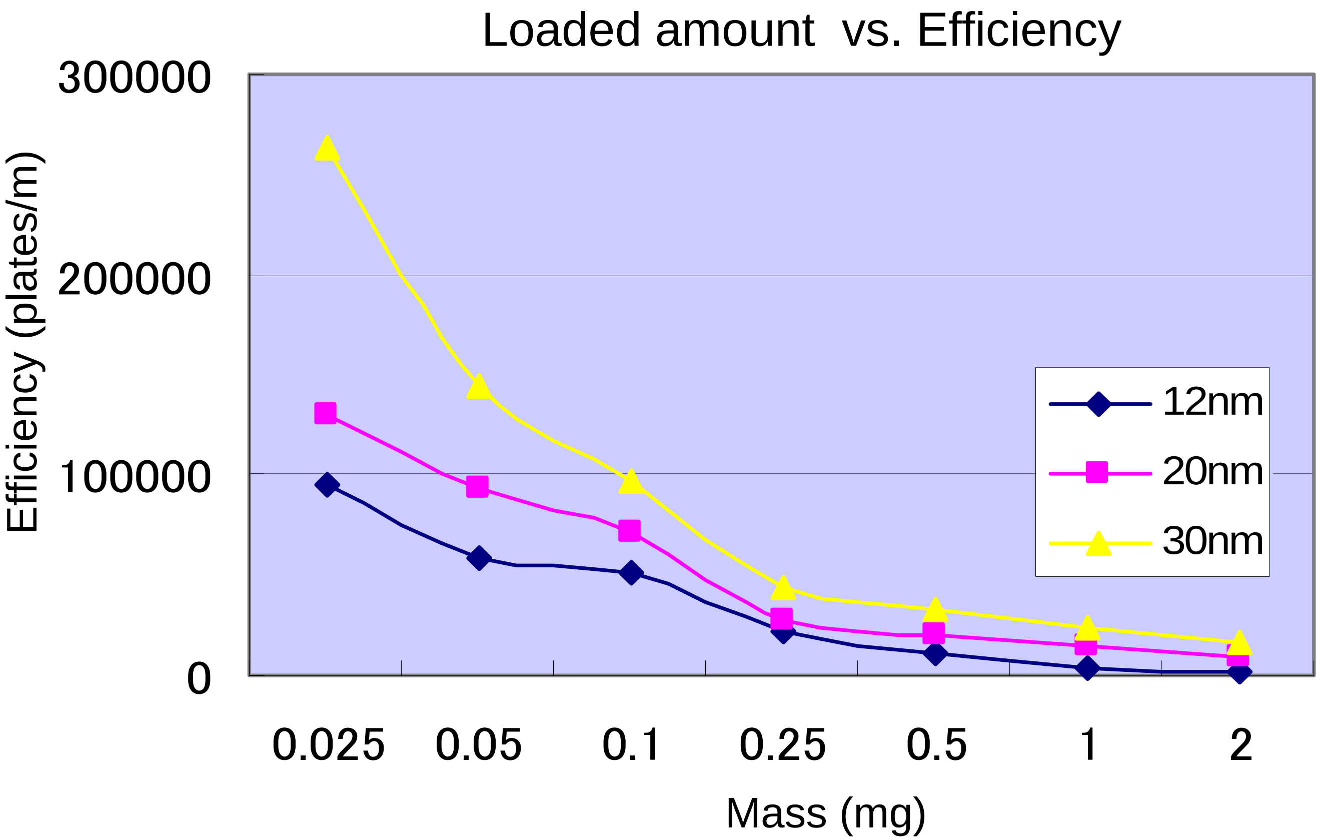
- **20 nm pore size is efficient below the 0.2 mg loading level.**
- **20 nm pore size would be suitable to large peptides and small proteins.**

Impact of Pore Size on Efficiency (5)
Albumin, Chicken Egg (MW 45000)



- **30 nm pore size is most efficient at all the loading levels.**
- **At low loading levels, 20 nm pore size also shows good efficiency.**

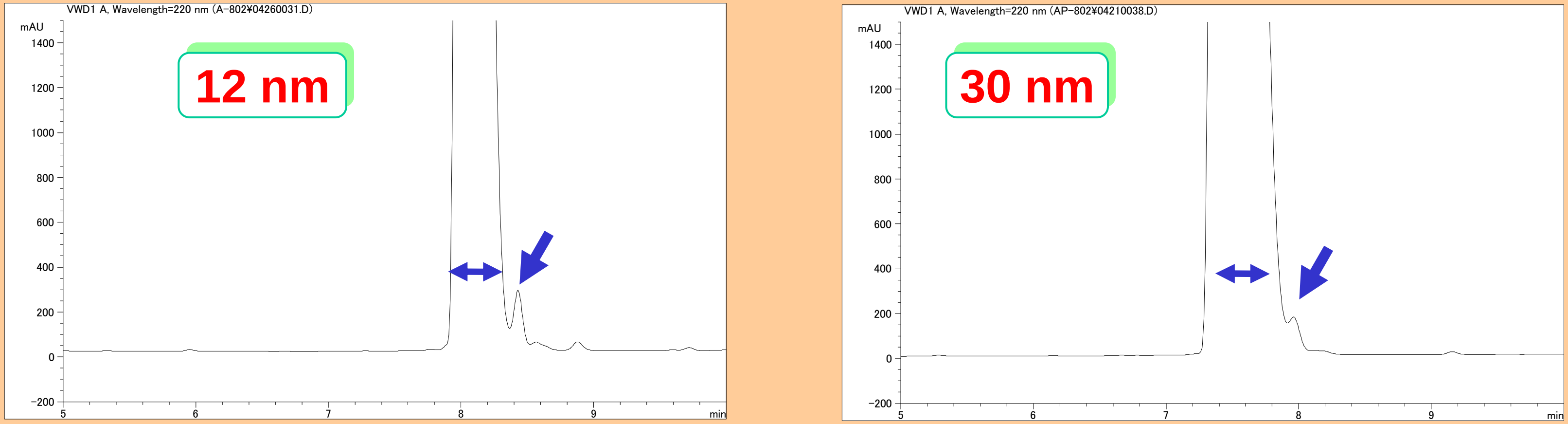
Impact of Pore Size on Efficiency (6)
Albumin from Bovine Serum (MW 67000)



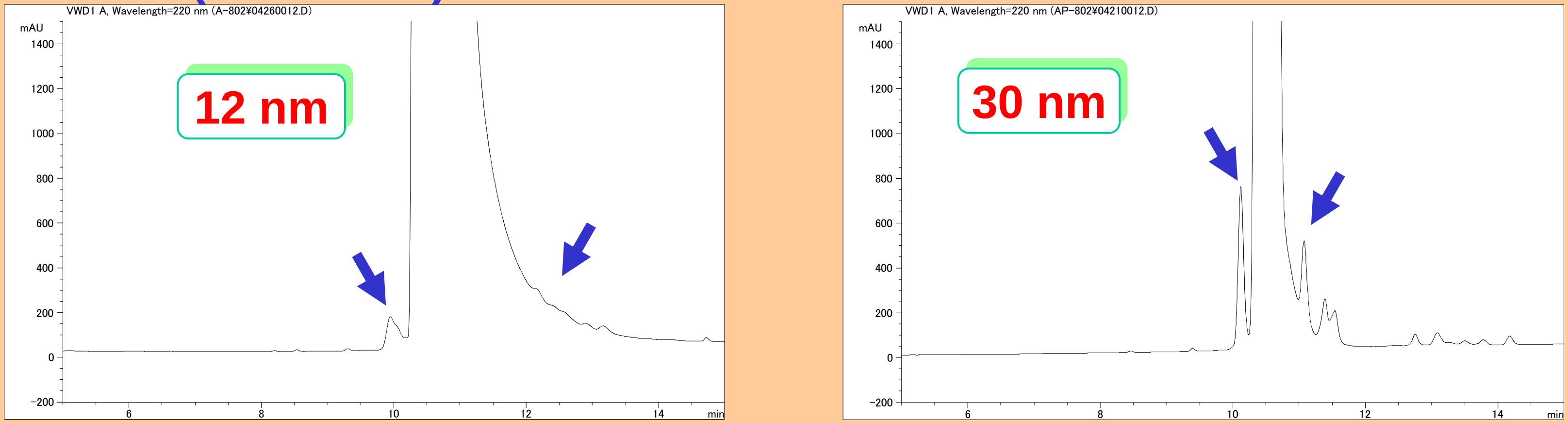
- **30 nm pore size gives the highest efficiency at all the loading levels.**
- **12 nm and 20 nm pore sizes are too small to give a good peak shape and resolution.**

Comparison of peaks on C4 with 12 nm and 30 nm pore sizes

Angiotensin II (MW 1046)

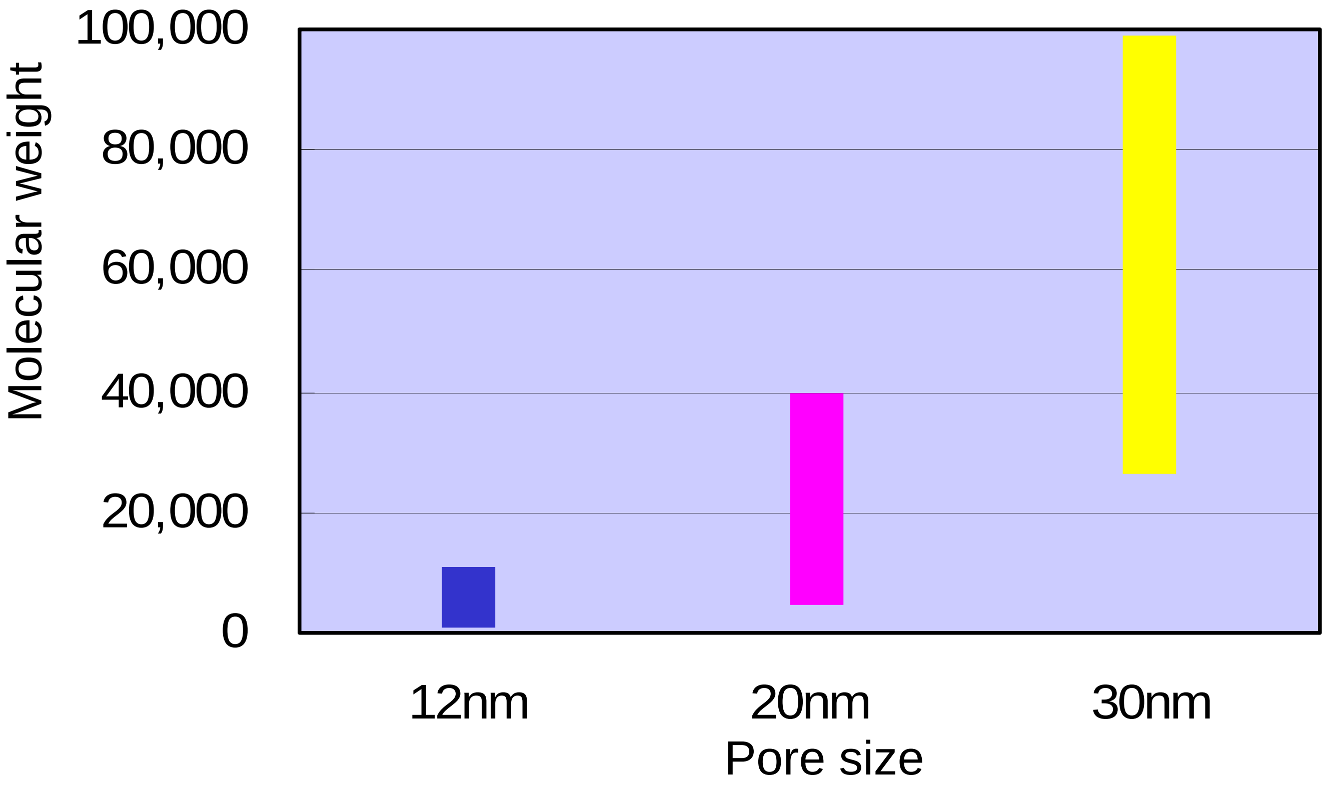


BSA (MW 67000)



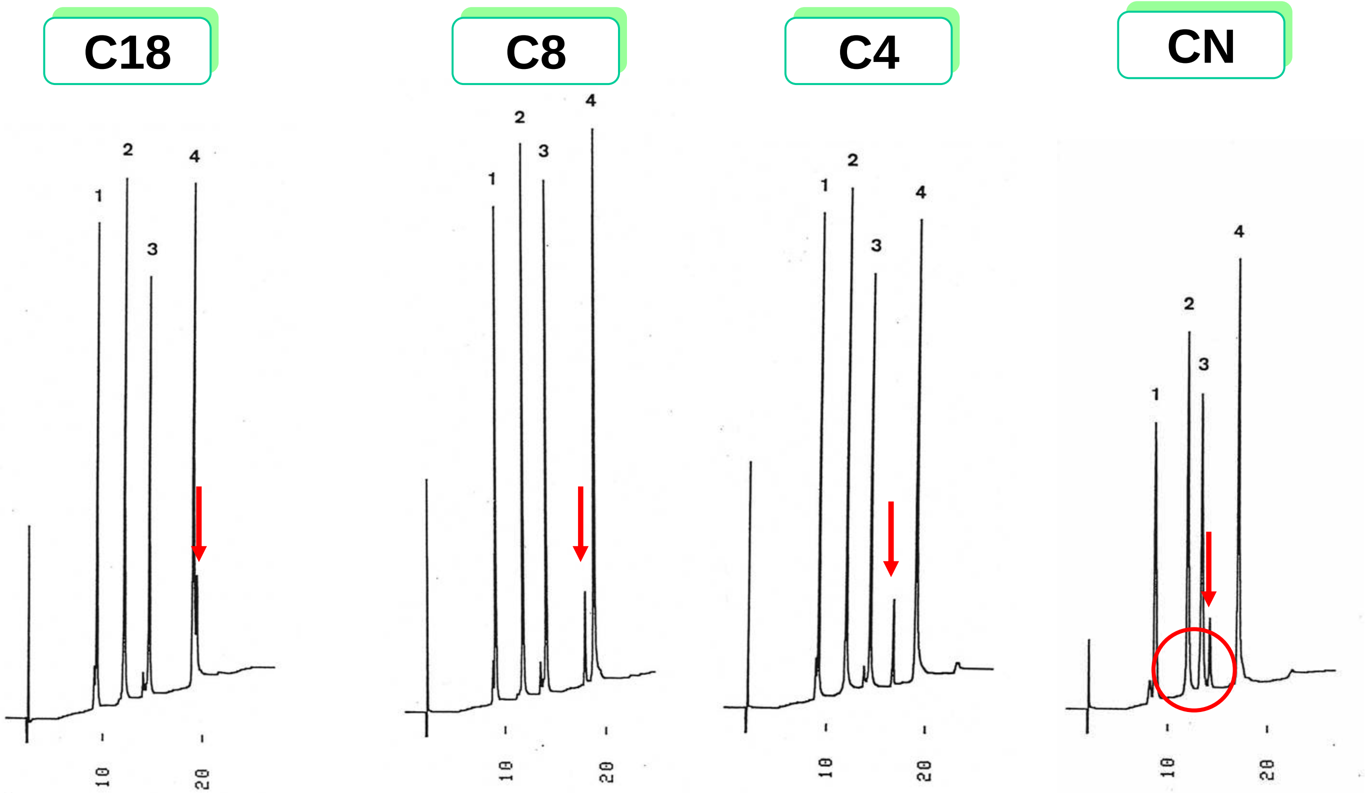
- It is important to choose an appropriate pore size for achieving a good peak shape.

Optimum pore size gel for peptides and proteins



- The 12 nm or 20 nm pore size is ideal for peptides and proteins with a MW ranging from 200 to 40000. The 30 nm pore size is suitable to proteins with a MW of ≧ 40000.

Comparison of C18,C8,C4,CN ligands
Analysis of low molecular proteins



C18　　C8　　C4　　CN

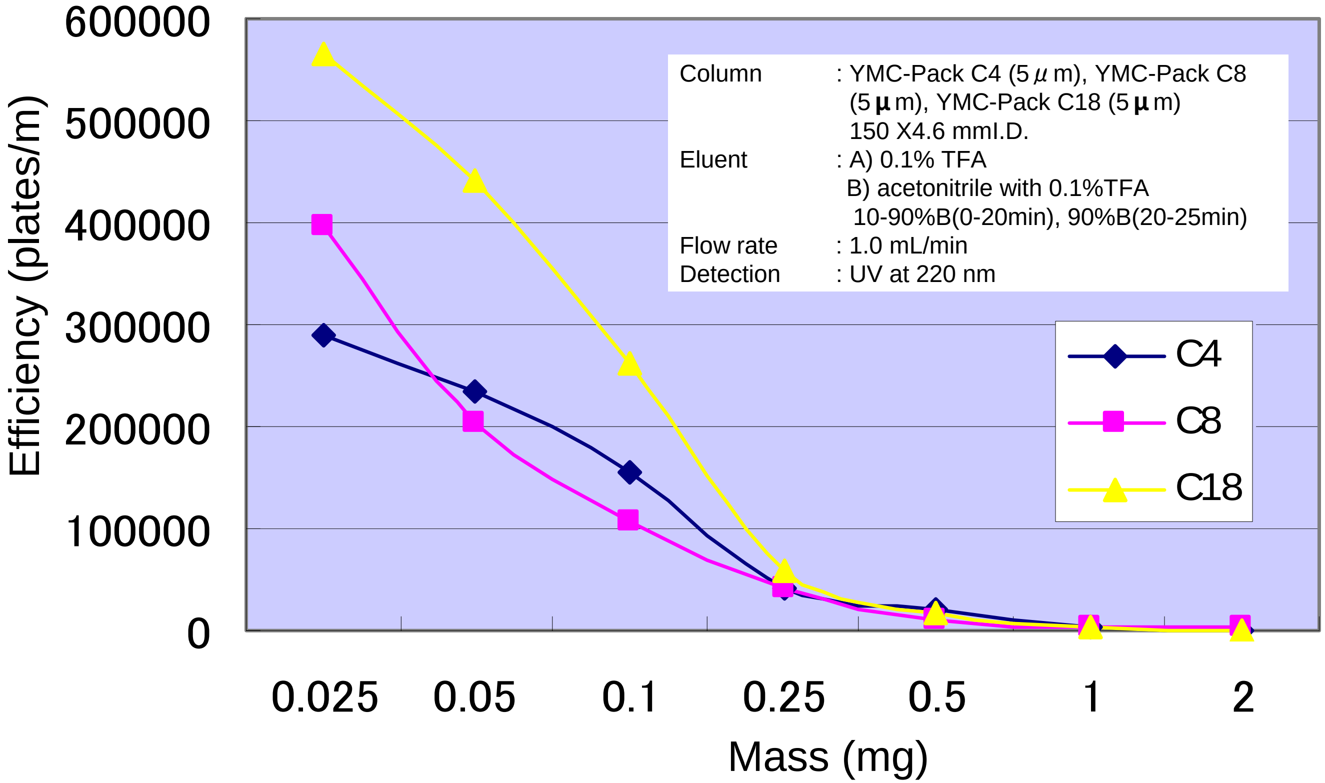
Column : YMC-Pack Wide Pore (5μm,30nm)
150 X4.6 mmI.D.
Eluent : A) acetonitrile/water/TFA (5/95/0.1))
B) acetonitrile/water/TFA (60/40/0.1)
30-90%B(0-20min), 90%B(20-25min)
Flow rate : 1.0mL/min
Temperature : 37 ℃
Detection : UV at 220 nm

1. Ribonuclease A	MW 13700
2. Cytochrome C	MW 12400
3. Lysozyme	MW 14400
4. Myoglobin	MW 17000

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

Insulin Chain B, Oxidized (MW 3495)

Loaded amount vs. Efficiency



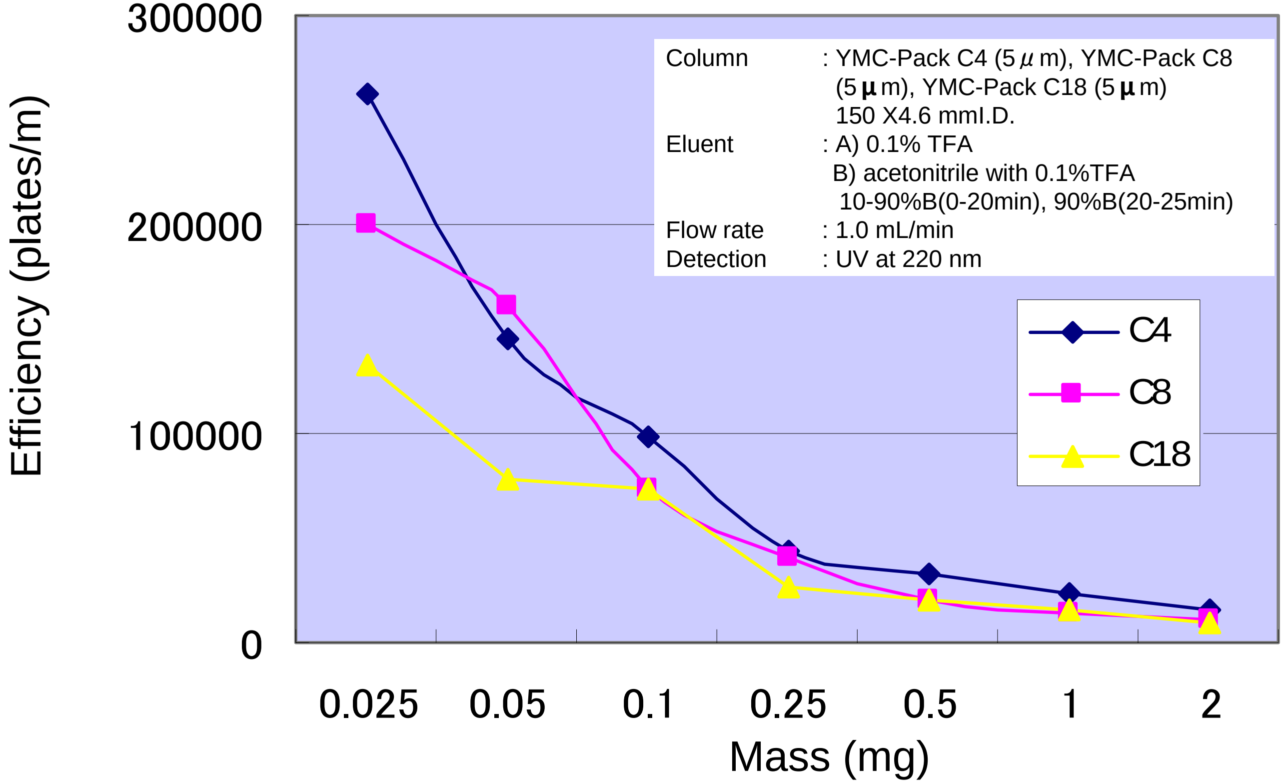
Column : YMC-Pack C4 (5 μm), YMC-Pack C8 (5 μm), YMC-Pack C18 (5 μm) 150 X4.6 mmI.D.
Eluent : A) 0.1% TFA
B) acetonitrile with 0.1%TFA
10-90%B(0-20min), 90%B(20-25min)
Flow rate : 1.0 mL/min
Detection : UV at 220 nm

- **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

Albumin from Bovine (MW 67000)

Loaded amount vs. Efficiency



Column : YMC-Pack C4 (5 μm), YMC-Pack C8 (5 μm), YMC-Pack C18 (5 μm)
150 X4.6 mmI.D.
Eluent : A) 0.1% TFA
B) acetonitrile with 0.1%TFA
10-90%B(0-20min), 90%B(20-25min)
Flow rate : 1.0 mL/min
Detection : UV at 220 nm

- **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.**

Effects of ligand on separation

Ligand	C18	C8	Ph	C4	CN
Hydrophobicity of gel	high				low
Suitable MW of sample	low				high
Column durability	good				poor
Recovery of sample	low				High

- 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.

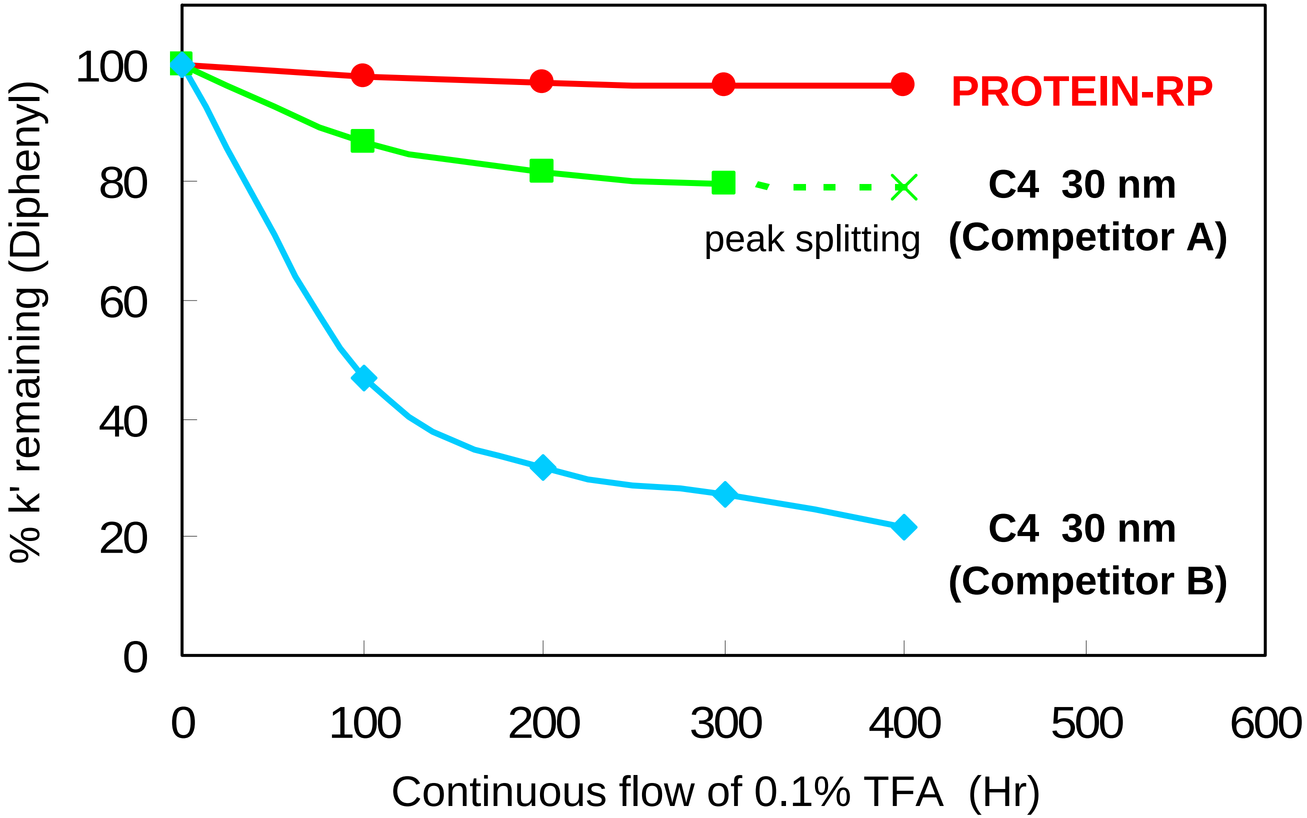
Column designed for separation of Peptides and Proteins

YMC-Pack PROTEIN-RP

■ Ligand	diphenyl derivative
■ Pore size	wide pore
■ Hydrophobicity of gel	low (similar to wide-pore C4)
■ Column durability	very stable under acidic conditions
■ Recovery of sample	excellent

- PROTEIN-RP is designed to overcome the major limitations of short alkyl chain reversed-phase column for separation of peptides and proteins, such as short column-life, low sample recoveries, poor peak shape.

Comparison of Stability under acidic conditions with TFA addition

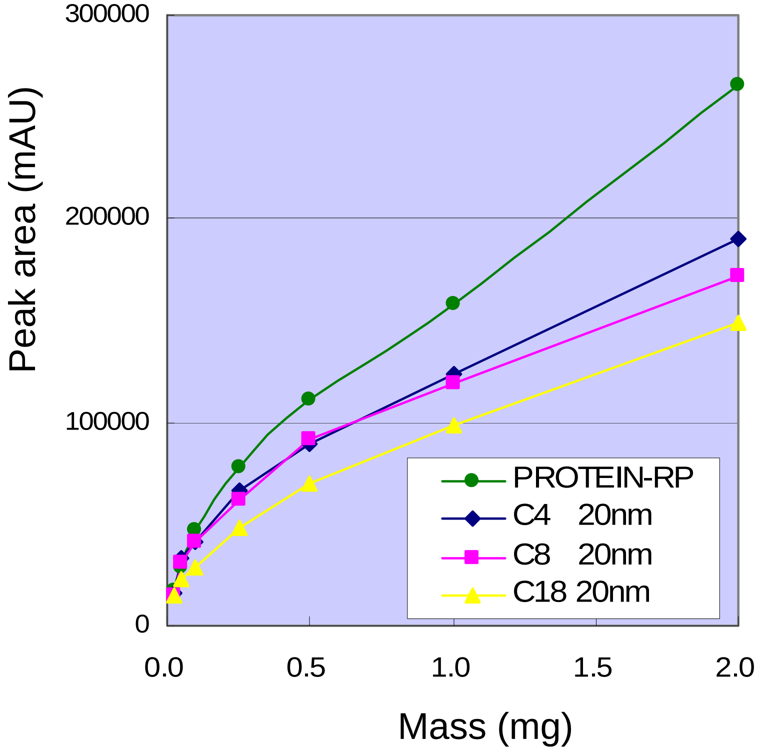


- PROTEIN-RP shows higher stability under typical aqueous acidic conditions used for separation of peptides and proteins.

Comparison of Sample recoveries of PROTEIN-RP with alkyl chain ligands

Insulin from Bovine (MW 5700)

Loaded amount vs. Peak area



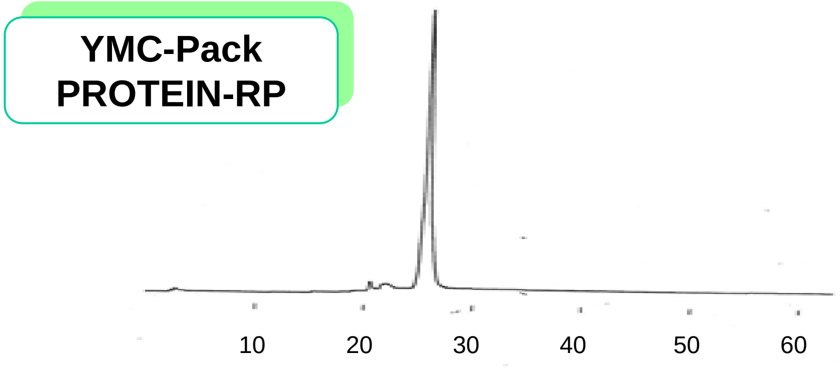
Recoveries of Peptides and Proteins

Samples	% Recoveries	
	PROTEIN-RP	Competitor C4 30nm
Ribonuclease A	93	95
Cytochrome C	94	89
Lysozyme	98	93
Myoglobin	97	85
BSA	94	92
Ovalbumin	90	73
Transferrin	94	98
Insulin(bovine)	97	73
Insulin chain B	82	76
α-Mating factor	93	82
Leu-Enkephalin	92	84
Gly-Gly-Gly-Gly	95	86

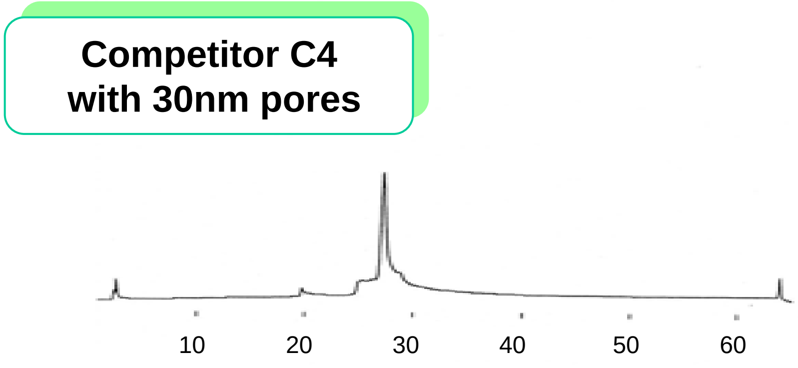
- Compared with typical alkyl chain ligands, PROTEIN-RP provides higher sample recoveries.

Analysis of high MW protein comparison of PROTEIN-RP with wide-pore C4

Lipoxidase (MW 96000)



YMC-Pack
PROTEIN-RP



Competitor C4
with 30nm pores

Column : 150 X4.6 mmI.D.
Eluent : A) water/TFA (100/0.1))
B) acetonitrile/TFA (100/0.1)
30-90%B(0-60min)
Flow rate : 1.0 mL/min
Temperature : 30 ℃
Detection : UV at 280 nm

- PROTEIN-RP provides excellent peak shape even in the case of high molecular weight protein such as Lipoxidase.

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.

- Due to proprietary bulky ligand, PROTEIN-RP has high durability under acidic conditions. Furthermore, the combination of proprietary ligands, appropriate bonding coverage and pore size provides high sample recoveries and excellent selectivity with good peak shape.

YMC