Development of effective purification method for peptides and proteins by silica gel based reversed phase packing material

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Introduction

Reversed-phase HPLC is an invaluable tool for the analytical and preparative separtion 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.

We reported that optimum pore size of gel gave good peak shape and good separation in analytical and preparative/process scale. Based on these results, we attempted to purify insulin with cost effectively. Actually, in the production by chromatography, cost performance would be most important thing. This study, we show cost-effective purification of insulin by optimizing necessary factors.

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Comparison of *preparative* and *analytical* chromatography

Analytical

Peak shape
Peak resolution
Column choice
Analytical time

Preparative

Recovery
Purity
Cost performance
Safety in production

In preparative scale, factors to be considered are

easily recovery, cycle time, recovery, purity, loading amount, solvent, etc.

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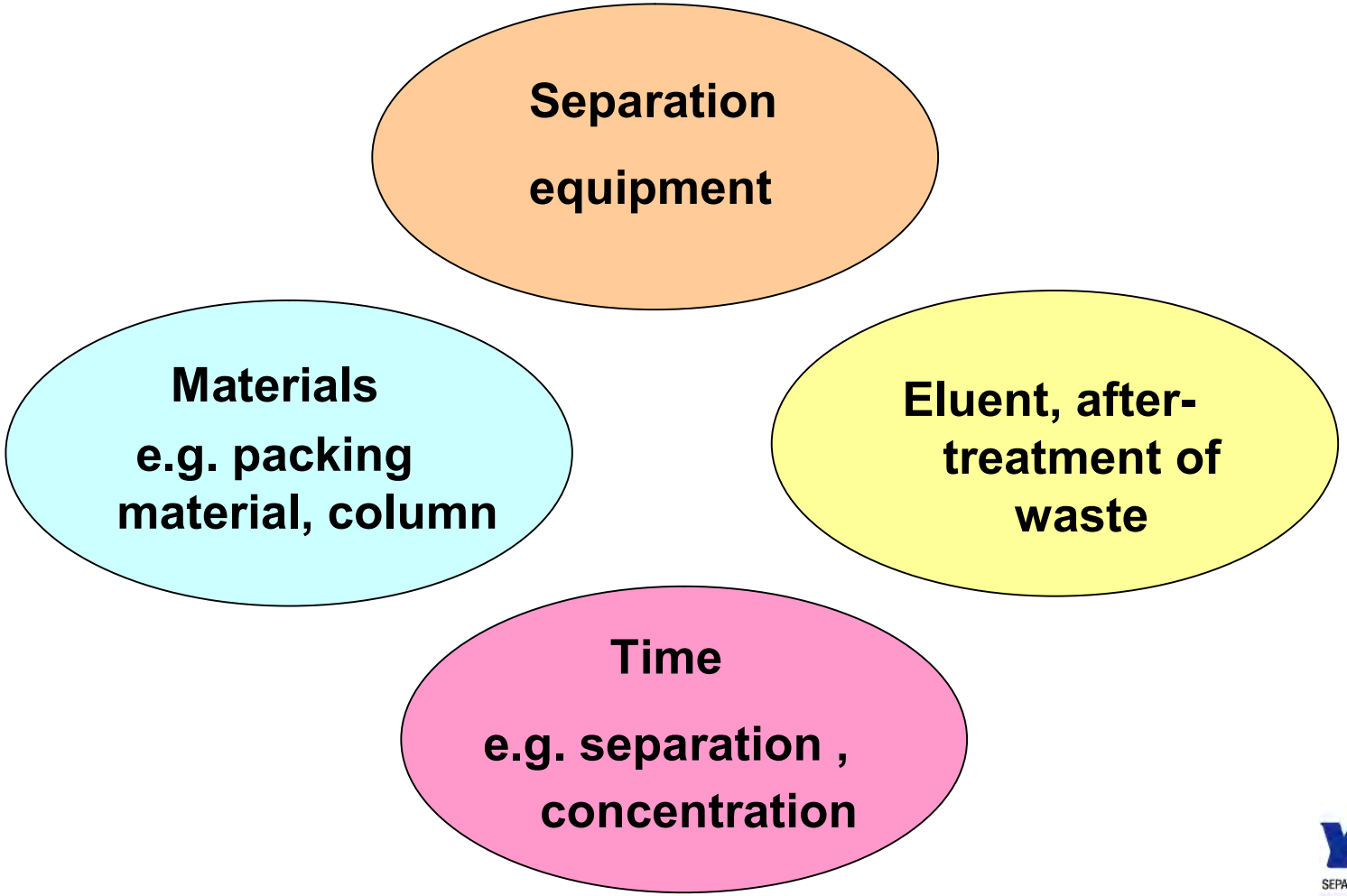
Preparative production cost includes



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Flow chart of preparative chromatography

Set up separation conditions
(eluent, time of interval, flow rate, etc)

⇩

Choosing packing material (functional group)

⇩

Choosing particle size

⇩

Optimization of preparative conditions

⇩

Preparative chromatography

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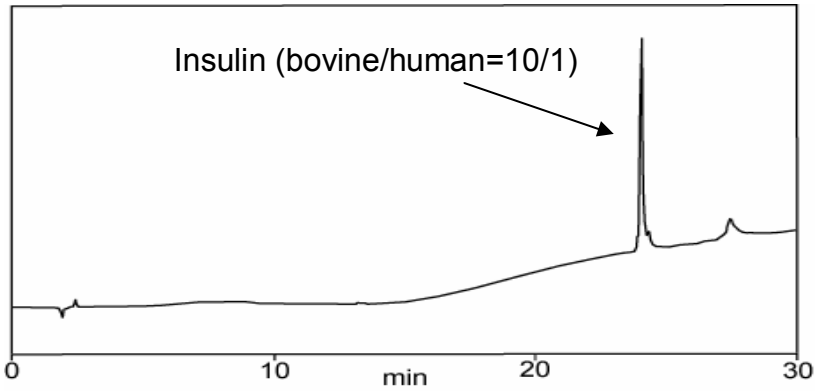
Recommend combination of MW and pore size for peptides and proteins

MW		C18	C8	C4
500 ↓ 5000 ↓ 20000 ↓ 100000	**Pore size** **120 Å**	◎	○	△
	200 Å	○	◎	○
	300 Å	△	○	◎

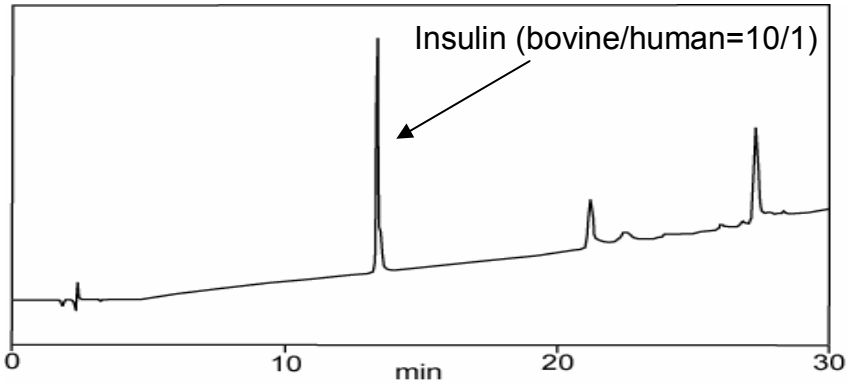
Initial conditions for separation of insulin

comparison of solvent, MeOH vs ACN

Methanol (10-90%B)



Acetonitrile (10-90%B)



Initial analytical conditions

Column	: YMC-Pack C8, S-5μm,20nm (150mmX4.6mmI.D.)
Flow rate	: 1.0mL/min
Eluent	: A) water / TFA (100/0.1)
	B) _____ / TFA (100/0.1)
Detection	: UV at 220 nm
Temperature: ambiennt	
Sample	: **Insulin (bovine/human=10/1)**

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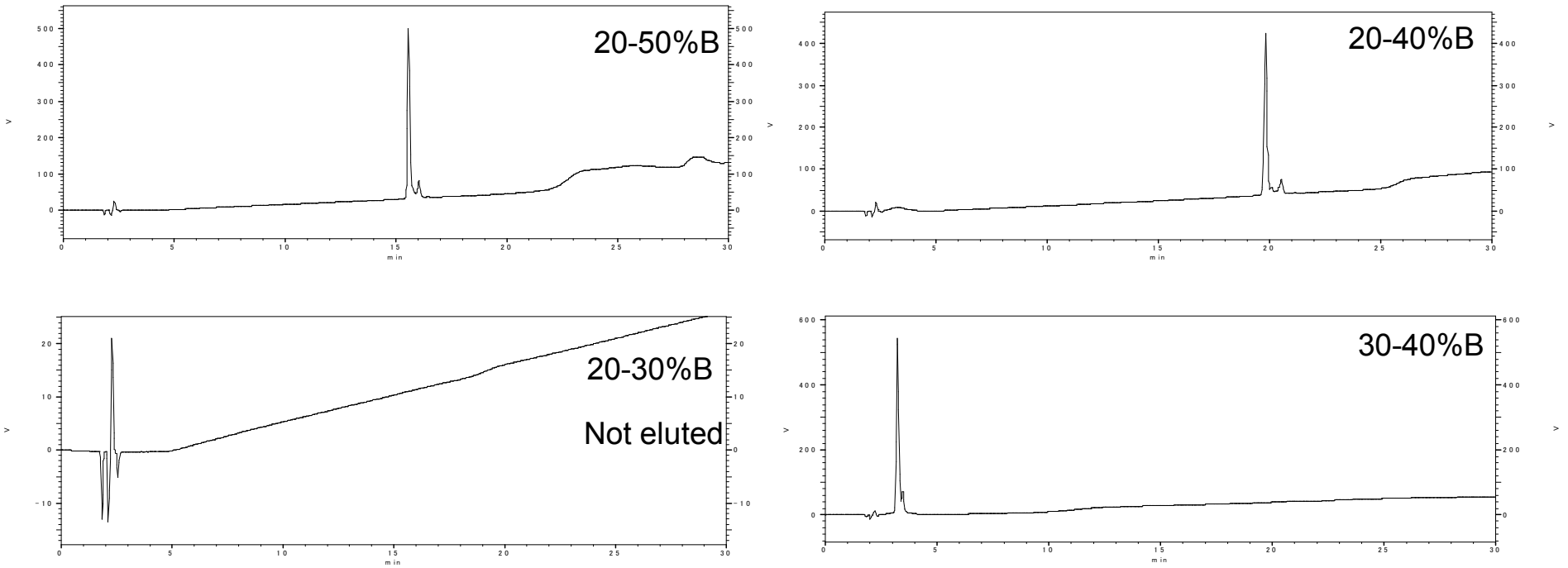
Optimization of gradient condition with ACN



Appropriate optimization was not achieved in these analyses because of poor peak resolution between bovine and human.

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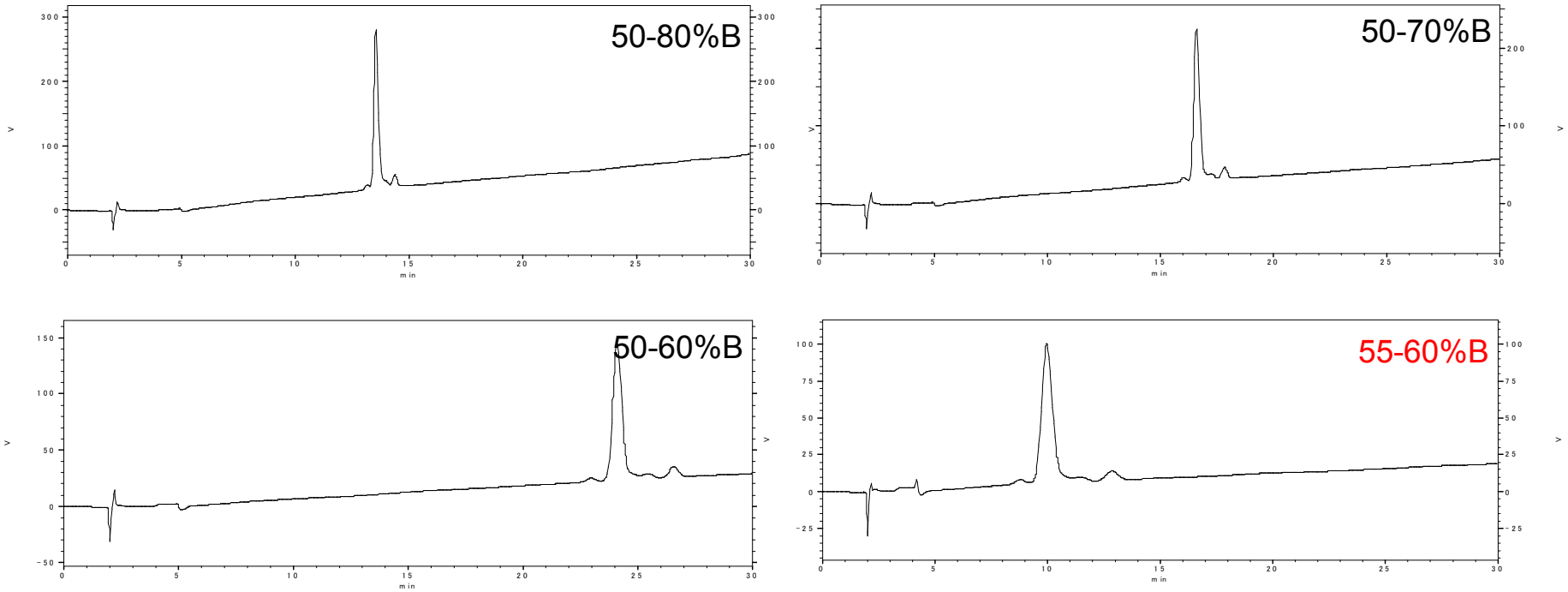
Optimization of gradient condition with MeOH



By using methanol as eluent, better separation was obtained in condition of 55-60%B solution.

Methanol was better than ACN because it gave good separation, cost effective and better safety.

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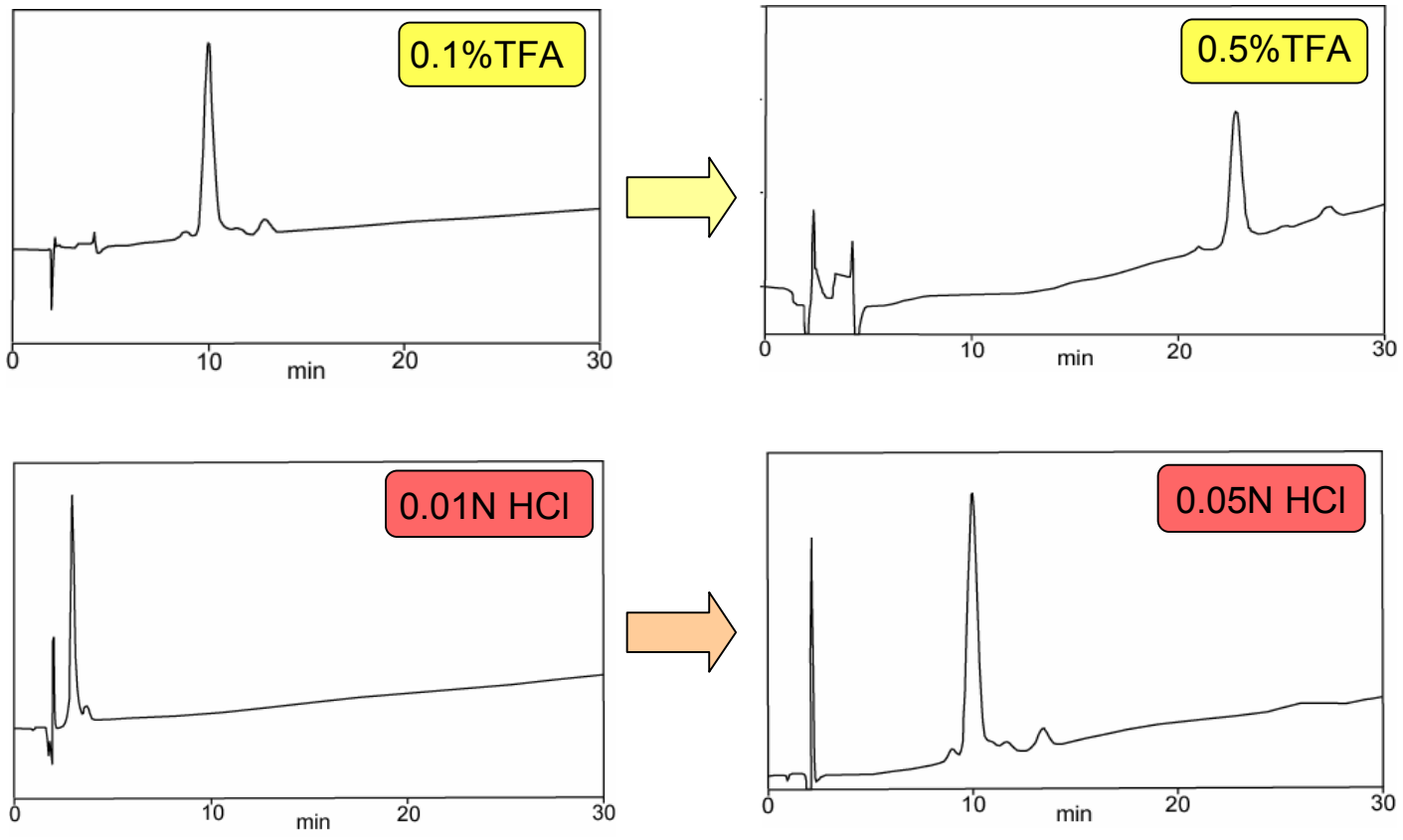
Influences of *additive type and concentration*



Separations with 0.1%TFA and 0.05N HCl show appropriate retention time and good resolution.

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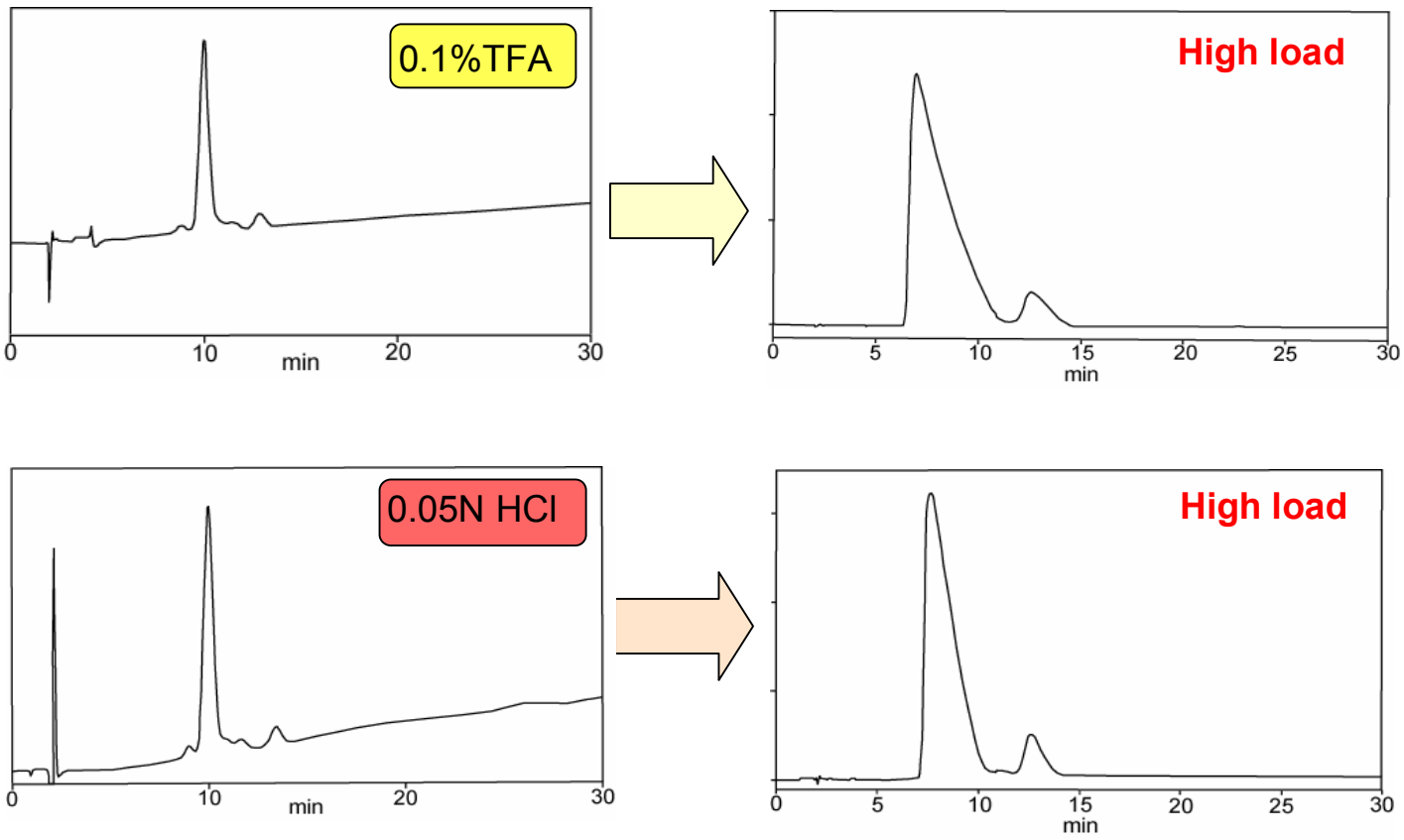
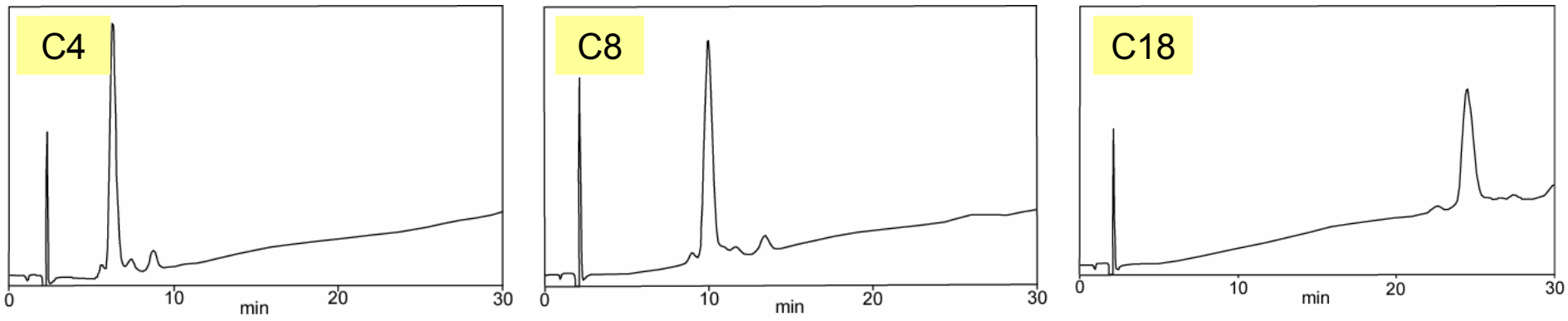
Comparison of peak shape under high load



Using TFA additive shows broader than the case of HCl.

In terms of cost effective and peak shape, HCl was chosen as an additive.

Comparison of packing material/functional group



	C4	C8	C18
Durability for acids	○	◎	◎
Cost performance	△	◎	○
Peak resolution	○	◎	○
Peak shape	◎	◎	○
Retention time	◎	◎	△

◎ Excellent,
○ Good,
△ Moderate

C8 medium is best choice for the separation of insulin.

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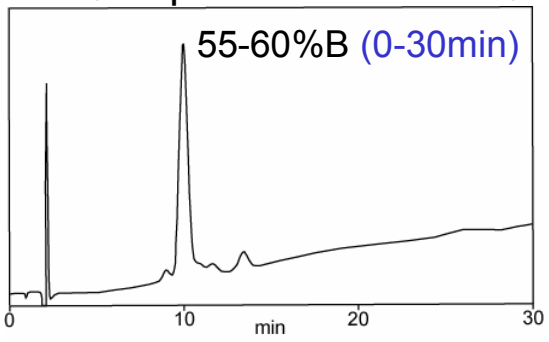
Optimization of analysis time and flow rate

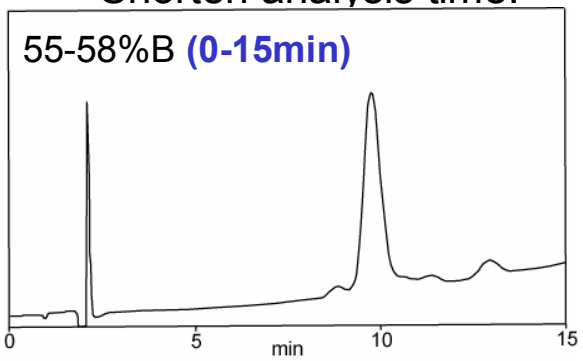
1) Optimized initial conditions

（No peak in 15-30 min）



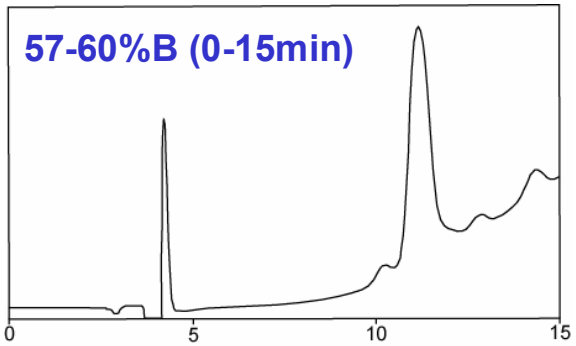
2) Half time of 1cycle time

Shorten analysis time.



3) Fine optimization

Flow rate : 0.5 mL/min, gradient curve



Column	: YMC-Pack C8, S-5μm, 20 nm (150mmX4.6mmI.D.)
Flow rate	: **1.0mL/min** ⇒ **0.5 mL/min**
Eluent	: A) water / HCl (100/0.05N)
	B) methanol / HCl (100/0.1)
Detection	: UV at 220 nm
Temp.	: ambiennt
Sample	: Insulin (bovine/human=10/1)

Fine optimization of gradient curve and flow rate, shorten analysis time and reduce eluent amount were achieved.

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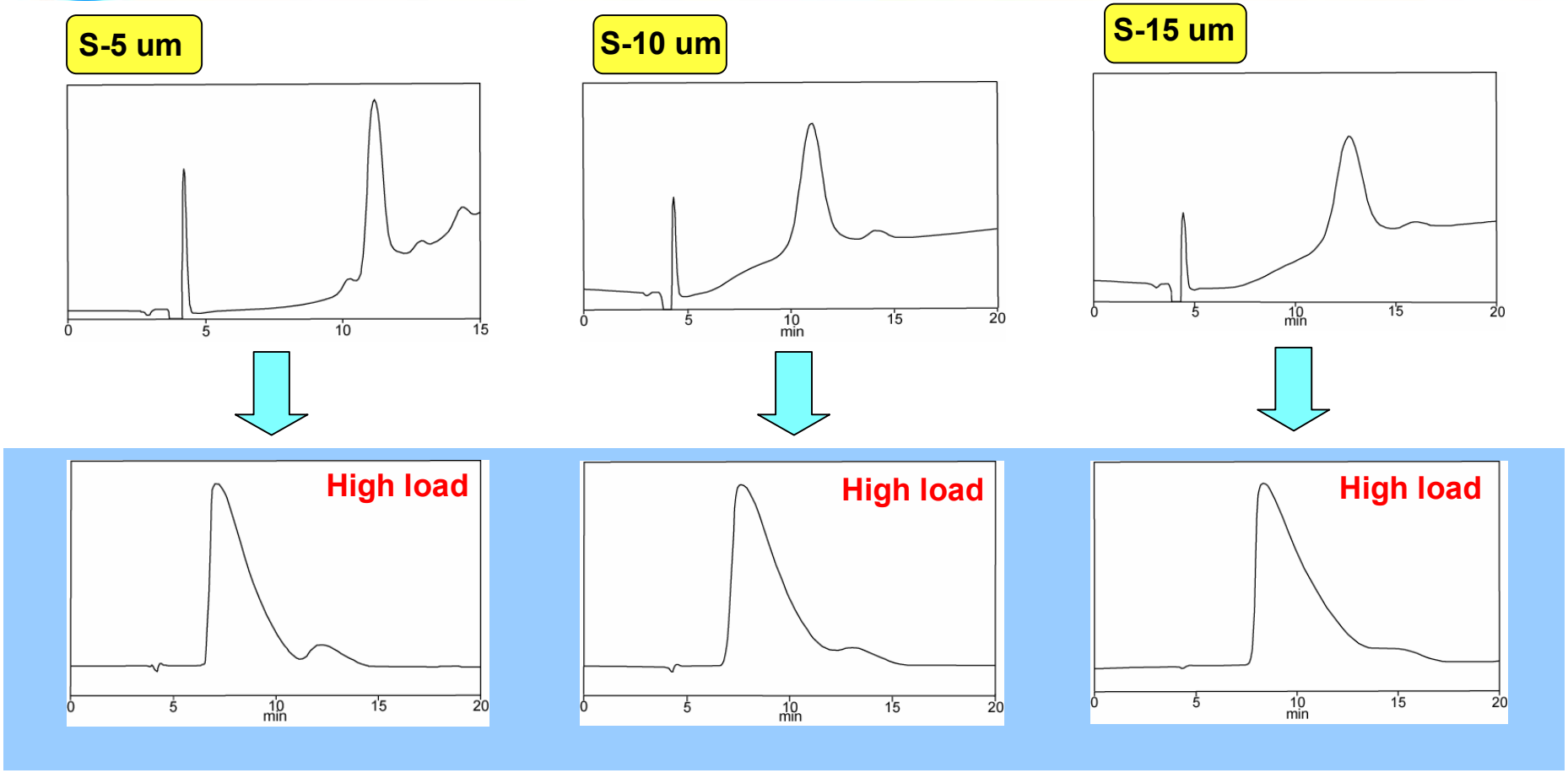
Influence of particle size (C8, 20 nm)



In preparative, peak shape of each particle size are similar.

It is better to use larger particle size for cost effective production.

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Preprative conditions and cost performances

Conditions	Analytical	Preparative
Particle size	5 μm	15 μm
Packing material	C8, 20 nm	C8, 20 nm
Eluent	ACN / H_2O/ TFA	MeOH / H_2O/ HCl
Flow rate	1.0 mL/min	0.5 mL/min

Cost performance of each factor

Solvent (MeOH)	**: 84% Decrease than ACN**
Additive (HCl)	**: 95% Decrease than TFA**
Eluent (By reducing flow rate)	**: 50% Decrease than initial condition**
Preparative time of 1 cycle	**: 50% Decrease than initial condition**
Particle size (S-15 um)	**: 95% Decrease than 5 μm**

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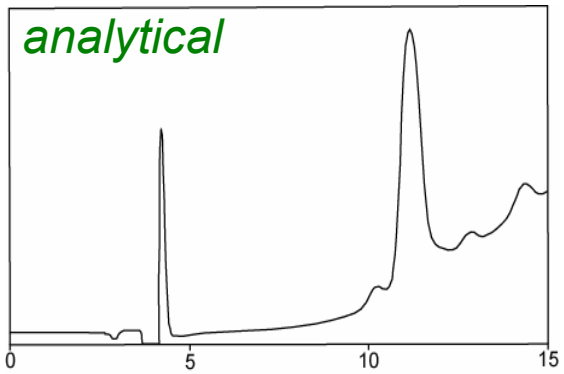
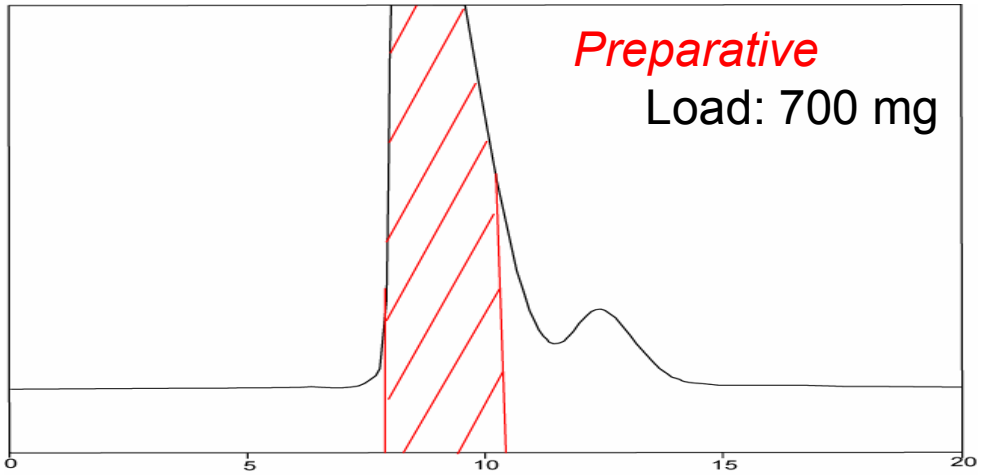
Preparative separation of insulin (1)

Scale up study (150mmX50mmI.D., DAU-50-700)



Scale up



Analytical conditions

Column	:YMC-Pack C8, 5 μm, 20nm 150mmX4.6mmI.D.
Flow rate	:0.5mL/min
Eluent	: A) water / HCl (100/0.05N) B) methanol / HCl (100/0.05N) 57-60%B (0-15min, linear)
Detection	:UV at 220nm
Temperature	:ambient

Preparative conditions

Column	:YMC ＊ GEL C8, 15 μm, 20nm 150mmX50mmI.D., (DAU-50-700)
Flow rate	:60mL/min
Eluent	: A) water / HCl (100/0.05N) B) methanol / HCl (100/0.05N) 57-60%B (0-15min, linear)
Detection	:UV at 220nm
Temperature	:ambient

after fractionating

Purity	99%
Recovered amount	620 mg
Recovery	88%

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Preparative separation of insulin (2)

Loadability of insulin

Loaded amount	200 mg	700 mg
Purity	99%	99%
Recovered amount	190 mg	620 mg
Recovery	95%	88%

The separation in 200 mg loaded shows high recovery, but low recovered amount.

In production scale, higher loaded amount would give high recovery.

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Choice of column size

3000 kg insulin purification per year

Column size →	50 mmI.D.	200 mmI.D.	600 mmI.D.
Loaded amount / day*	68 g	1056 g	4896 g
Product amount / year	24 kg	360 kg	**3560 kg**
Cost of packing material (ratio)	1.0	16	144

* Cycle time: 15 min, Loading was performed 96 times per day.

To achieve expected production amount,

it is necessary to use 600 mmI.D. column.

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Improvement of cost performance (1)

Comparison of cost performance in different conditions

	Example conditions	Optimized conditions
1 cycle time	30 min (48 times/day)	15 min (96 times/day)
Eluent	Acetonitrile	Methanol
Flow rate*	17 L	8.6 L
Packing material	C18, 10μm	C8, 15μm
Eluent cost	Above conditions →	**95% Decrease**
Cost of gel		**90% Decrease**
Production efficiency		**100% Increase**

*By using 600 mmI.D. column.

High flow rate and high column puressure cause not only increasing eluent amount but also increasing equipment cost.

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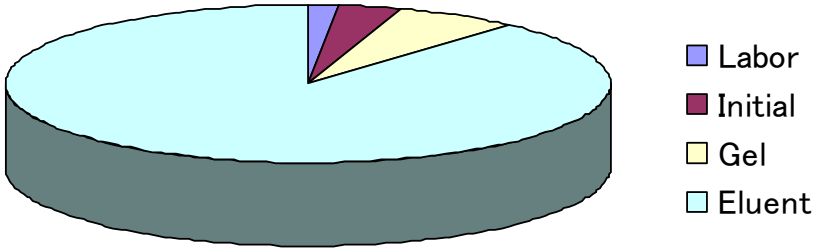
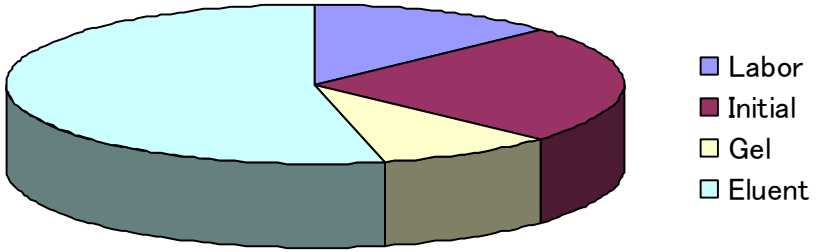
Improvement of cost performance (2)

Example conditions



Optimized conditions



Total cost	30,550,000 US$	3,810,000 US$
Product amount/year	1,780 kg	3,560 kg
Total cost / Product 1 kg	17,162 $ / kg	1,070 $ / kg
Initial cost / Product 1 kg	566 $ / kg	278 $ / kg
Labor cost / Product 1 kg	309 $ / kg	149 $ / kg
Gel cost / Product 1 kg	1132 $ / kg	107 $ / kg
Eluent cost / Product 1 kg	15,102 $ / kg	631 $ / kg

By optimizing conditions, total cost is extremely lower than the example case.

Summary

- In preparative production, by choosing optimum conditions as we shown this study, you can make purification effectively.
- It is important to choose best combination of pore size, functional group and particle size for the preparative purification of peptides and proteins.
- YMC can offer not only various packing materials but also preparative products (e.g. dynamic axial column, process equipments). We also offer the information of every stage of purification process.

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