Ion-Exchangers Product Line(1)

NEW

- **GigaCap Q-650M**
  - Highest capacity (BSA; >162 mg/ml)
  - High flow rate
  - Small & mid MW proteins
- **SuperQ-650**S,M,C
  - Higher capacity (-155mg/ml)
  - Oligonucleotide
  - small & mid MW proteins
- **QAE-550**C
  - High capacity (-78 mg/ml)
  - Stronger retention
- **DEAE-650**S,M,C
  - Capacity; -35mg/ml
  - High resolution
  - Large MW proteins (Factor VIII)
  - Oligonucleotide

NEW

- **GigaCap S-650M**
  - Highest capacity(IgG; 136-176 mg/ml)
  - High flow rate
  - Peptide
  - small MW proteins
- **MegaCap II SP-550EC**
  - Higher capacity(Insulin; 95-120mg/ml)
  - High flow rate
  - Peptide
  - small MW proteins
- **SP-550C**
  - High capacity(-120mg/ml)
  - Peptide, Small proteins
  - Stronger retention
- **SP-650**, **CM-650**S, M, C
  - Capacity; -60mg/ml
  - High resolution
  - Large MW proteins

Ion-Exchangers Product Line(2)

- **TSKgel PW bulk resin series**
  - 20 micron, 30 micron
  - Same selectivity to TSKgel PW series analytical column
  - Higher resolution
  - Higher flow rate due to higher mechanical stability of resin than Toyopearl
  - Applicable to 300 L columns
  - Prepacked columns available at 55 mm I.D. x 20 cm and 108 mm I.D. x 20 cm (20 micron)*
  - Prepacked column available at 210 mmI.D. x 30 cm (30 micron)*
  - * ; Special inquiry products

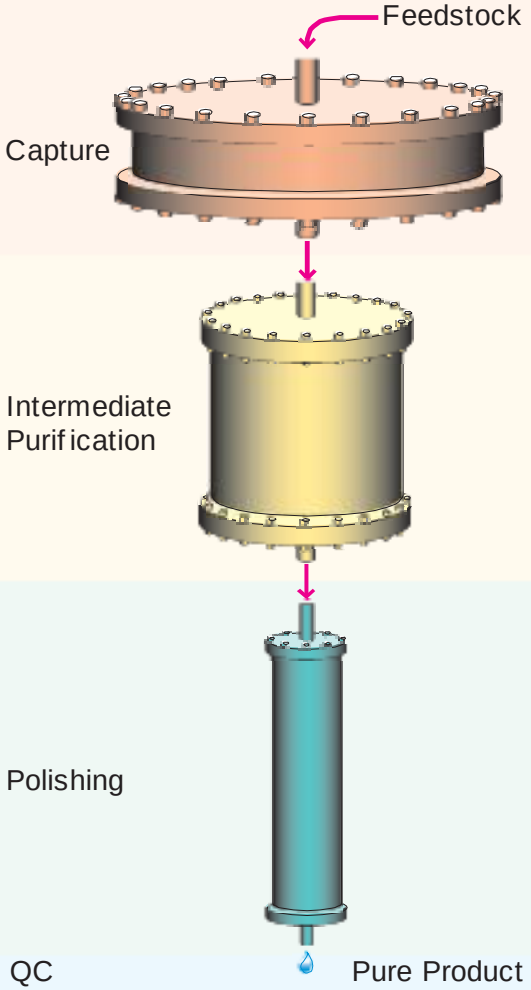
- **TSKgel DEAE-5PW (20), (30)**
  - Sharper peak
  - Better resolution for mid- and larger size proteins
- **TSKgel SuperQ-5PW (20), (30)**
  - Higher capacity than TSKgel DEAE-5PW
  - Applicable to synthetic oligonucleotide, siRNA, etc.
- **SP-5PW**
  - For proteins separation
- **SP-3PW**
  - High capacity and selectivity for insulin
  - For smaller proteins and peptides

- Separation of Peptide

Separation of Peptide on Ion-Exchangers

- Purification strategy
- Key factors on separation of peptide
- Cation-exchangers
- Anion-exchangers

Selection of IEC Resins with Different Particle Size



Process step	Bead size	Process media	
		Anion	*Cation*
Capture	200µm		Toyopearl MegaCap SP-550EC
	100µm	GigaCap Q-650M Toyopearl SuperQ-650C Toyopearl DEAE-650C Toyopearl QAE-550C	GigaCap S-650M, CM-650M Toyopearl SP-650C Toyopearl SP-550C Toyopearl CM-650C
Intermediate Purification	65µm	Toyopearl SuperQ-650M Toyopearl DEAE-650M	Toyopearl SP-650M Toyopearl CM-650M
	35µm	Toyopearl SuperQ-650S Toyopearl DEAE-650S	Toyopearl SP-650S Toyopearl CM-650S
Polishing	30µm	TSKgel SuperQ-5PW (30) TSKgel DEAE-5PW (30)	TSKgel SP-5PW (30)
	20µm	TSKgel SuperQ-5PW (20) TSKgel DEAE-5PW (20)	TSKgel SP-5PW (20)
QC	10µm	TSKgel SuperQ-5PW 7.5mmIDx7.5cm TSKgel DEAE-5PW 7.5mmIDx7.5cm	TSKgel SP-5PW 7.5mmIDx7.5cm TSKgel CM-5PW 7.5mmIDx7.5cm

Same selectivity HPLC columns are available for most process media

Key Factors on Peptides

- Molecular mass (weight)
  - Applicable to size-exclusion chromatography
- Ionic properties; isoelectric point (pI)
  - pI is determined by total amounts of acidic and basic amino acids
  - Applicable to Ion-exchange chromatography
- Hydrophobicity (hydrophilicity)
  - All amino acids contribute to hydrophobicity.
  - Applicable to reversed-phase chromatography
  - Applicable to normal phase chromatography/HILIC
- Metal chelating property (IMAC)
  - His, Met, Tyr, Cys are contribute to interaction.
  - Applicable to metal ion chelating affinity chromatography (IMAC)

Key Factors on Chromatographic Matrix

- Selectivity
  - Functional groups; ionic, hydrophobic, affinity
- Capacity and adsorption strength
  - Amounts and species of functional group
  - e.g. DEAE/QAE, C18/C8
- Particle size
  - 20, 30, 60 100, 200 micron
  - Dependent on purification step, purity (impurity), column volume
- Pore size
  - Larger pore for larger molecule, but causes lower surface.
  - Smaller pore for smaller molecule but causes exclusion from inner surface
- Lot to Lot variation of matrix/resin
  - Selectivity, capacity, recovery, etc.
- Supply of large amounts of resin
  - Scale-up ability and productivity

Key Factors on Chromatography

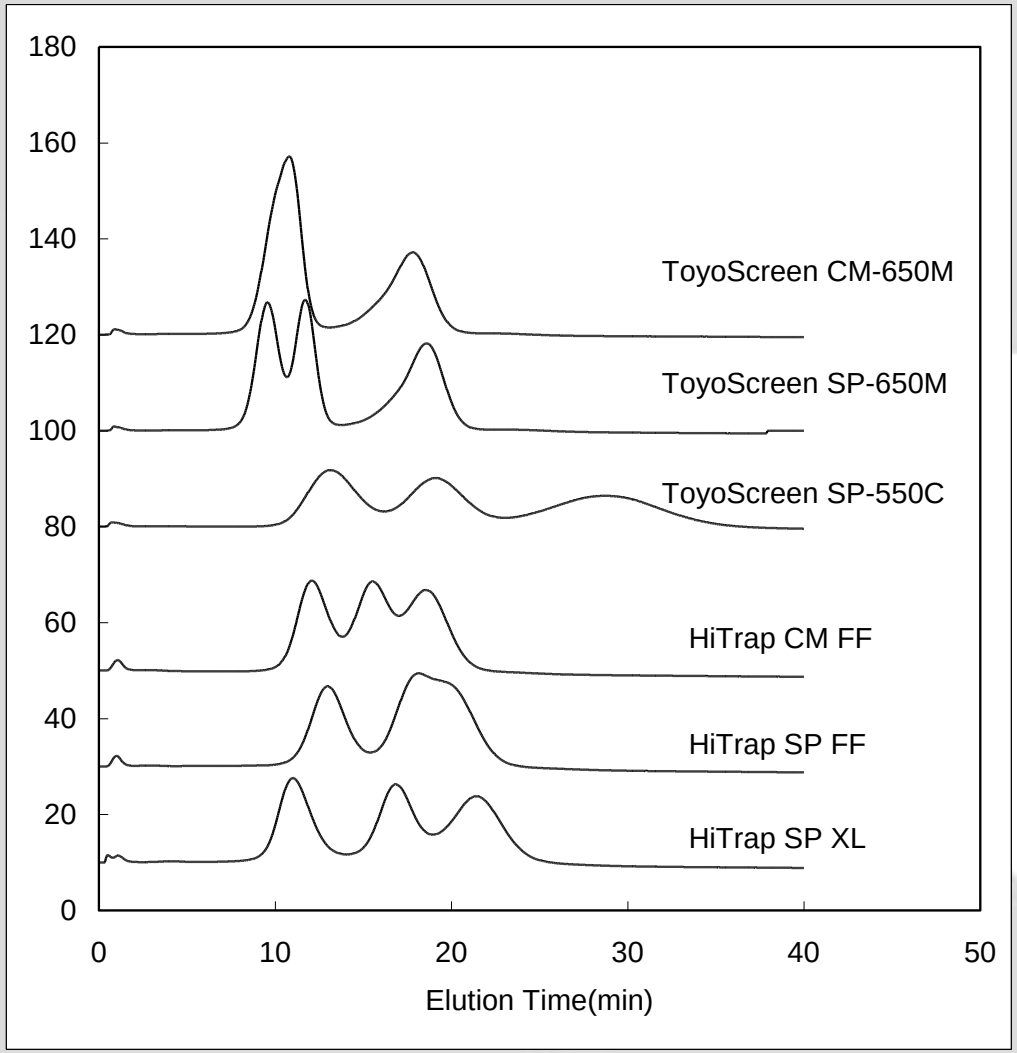
- Mobile phase/Eluent
  - Buffer species; phophate, acetate, citrate, Tris,
  - Buffer pH; acidic, basic and neutral
  - Salt as counter ion; NaCl, citrate, acetate, etc.
- Linear or step gradient
  - Higher resolution or easy operation
- Flow rate
  - Productivity
  - Dependent on rigidity of resin/matrix
- Temperature
  - Seasonal temperature change affects to separation.
- Combination of all factors above decide and contributes to the separation performance.
  - Loading capacity, resolution, purity, recovery, reproducibility, etc.

Property of Recombinant Insulin

- MW; ca. 5,800 - 6,100
- Ip; originally ca. 5.5
  - varies around 5.0 – 7.0 by substitution of amino acids
- Impurity for recombinant products
  - Amino acids substitute analogues
  - Dimer
  - Host cell protein

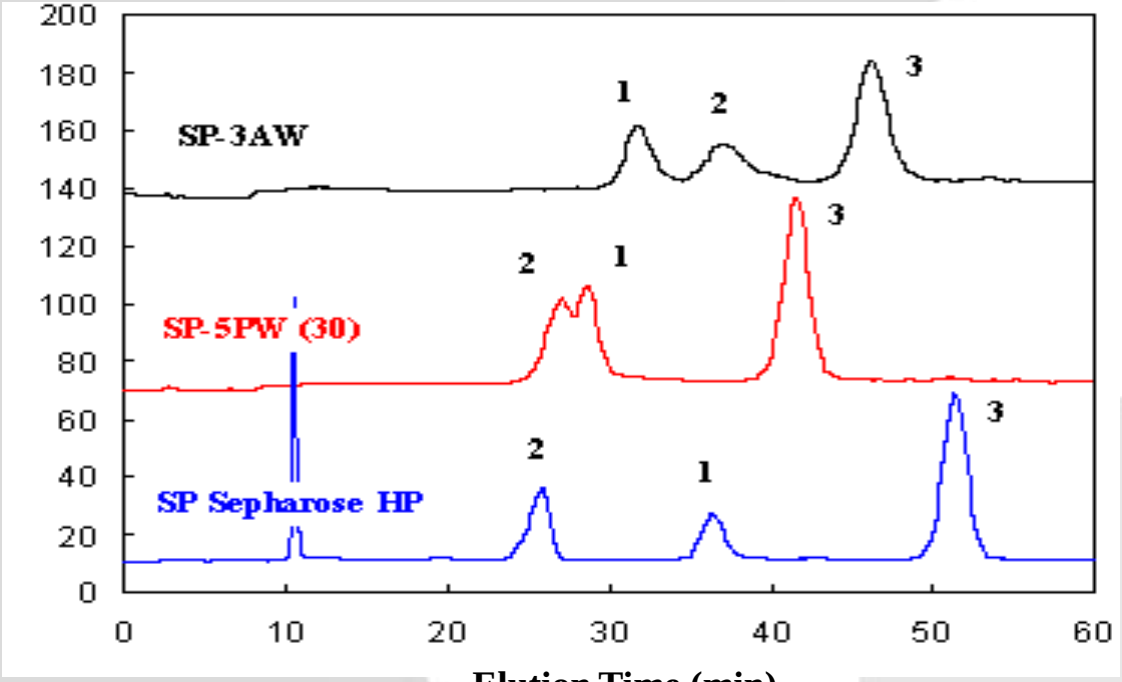
- Cation-Exchanger

Retention and Selectivity on CIEC for protein Separation



Condition
Column Size: ToyoScreen 6.4 mm I.D.*3cm, 1mL
HiTrap 7mm I.D.*2.5cm, 1mL
Eluent :a) 20mM Phosphate Buffer (pH6.0)
b) 20mM Phosphate Buffer
+ 0.5M NaCl (pH6.0)
Gradient: a) to b) 30min Linear
Flow rate: 1mL/min
Detection: UV (280nm)
Injection: 50μL
Sample: alpha-Chmotrypsinogen A, Cytochrom C, Lysozyme 1g/L

Comparison of Elution Behavior of Insulin: SP-3PW(30), SP-5PW(30) and SP Sepharose HP



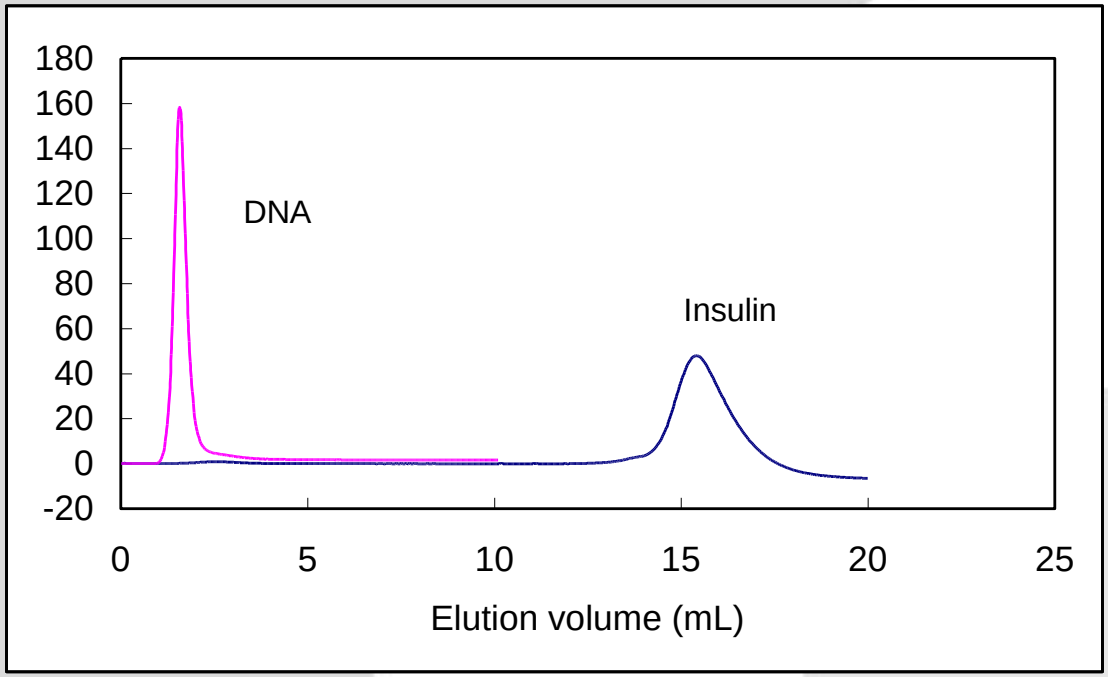
Column Size; 7.5mmI.D.x 7.5cm, Flow Rate; 1.0 ml/min

Sample; 1) Trypsinogen, 2) Insulin, 3) Lysozyme (0.5mg/ml, 100ul injection)

Eluent; A) 20mM Citrate buffer(pH 3.0) / Ethanol = 80:20 , B) A + 1.0 M NaCl (pH 3.0) / Ethanol = 80: 20Elution; 60 min. Linear Gradient from A) to B)

TSKgel SP-3PW(30) has stronger retention on insulin.

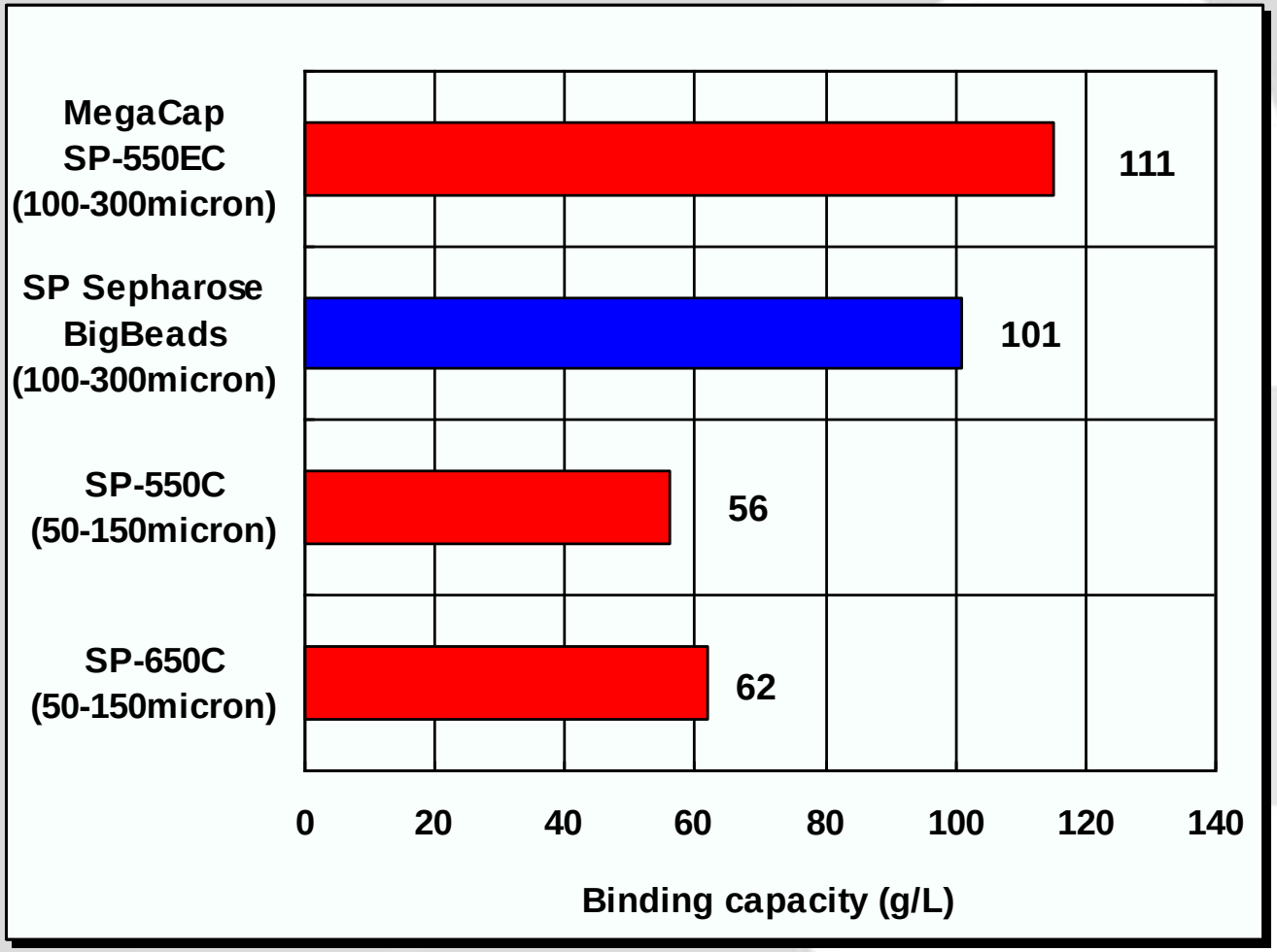
Separation of Insulin on Toyopearl MegaCap II SP-550EC



Condition

Column: TOYOPEARL MegaCap II SP-550EC(7.5mmID*7.5cm)
Eluent: (A) 0.1M Citrate Buffer (pH3.0)
(B) 0.1M Citrate Buffer (pH6.2)
Gradient: (A) --> (B) step at 10min
Flow rate: 1mL/min Sample: DNA, Insulin (STD, human) 1g/L
Injection: 100uL Detection: UV(280nm)

Comparison of Capacity for Insulin on Cation-Exchangers



∗ GigaCap S-650M has also higher capacity for insulin.

Comparison of DBC for r-Insulin

	TSKgel SP-3PW (30)	TSKgel SP-5PW (30)	SOURCE 30S
Matrix	**polymethacrylate**	**polymethacrylate**	**polystyrene divinylbenzen**
Particle size	**30 micron**	**30 micron**	**30 micron**
Insulin capacity	**49 g/L**	**24 g/L**	**45 g/L**

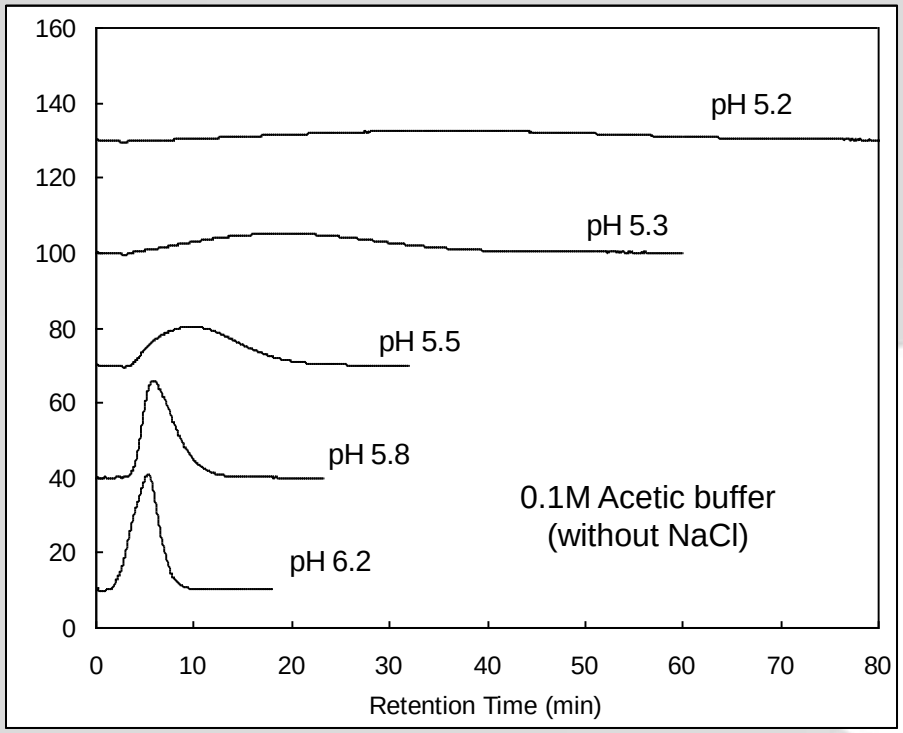
Column; 4.6 mm I.D. x 7.5 cm

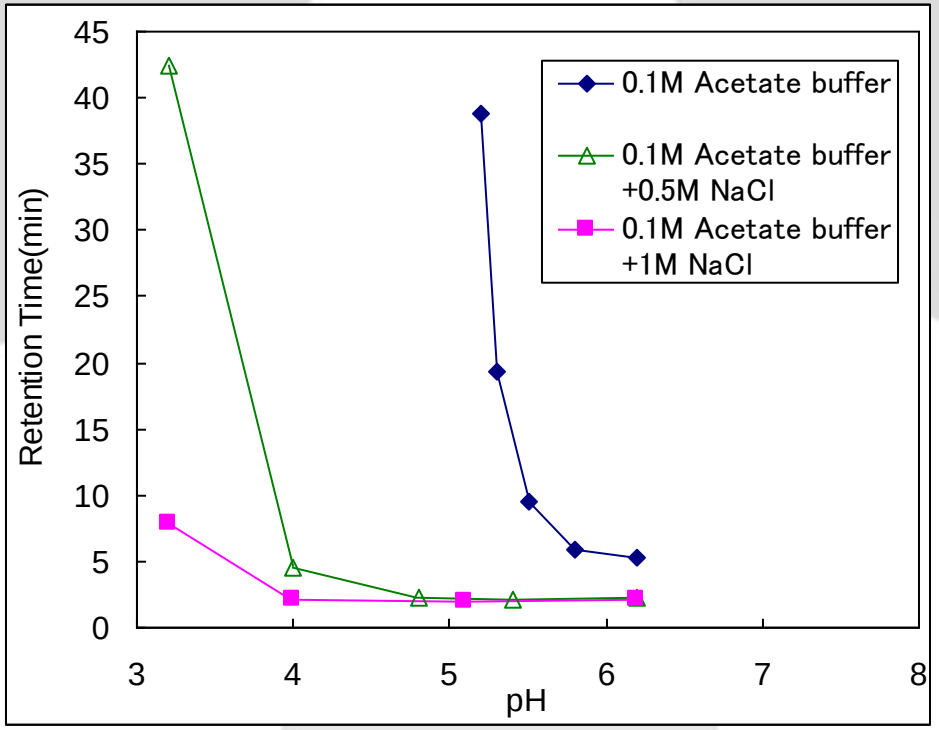
Eluent; Gradient elution with 1-propanol by acidic buffer (pH 3.0) containing neutral salt

Flow rate; 0.75 ml/min (270 cm/hr)

Sample; recombinant insulin (7.2 g/L), DBC; calculated by 10 % breakthrough

Effect of pH in Eluent for Insulin Separation by CIEC





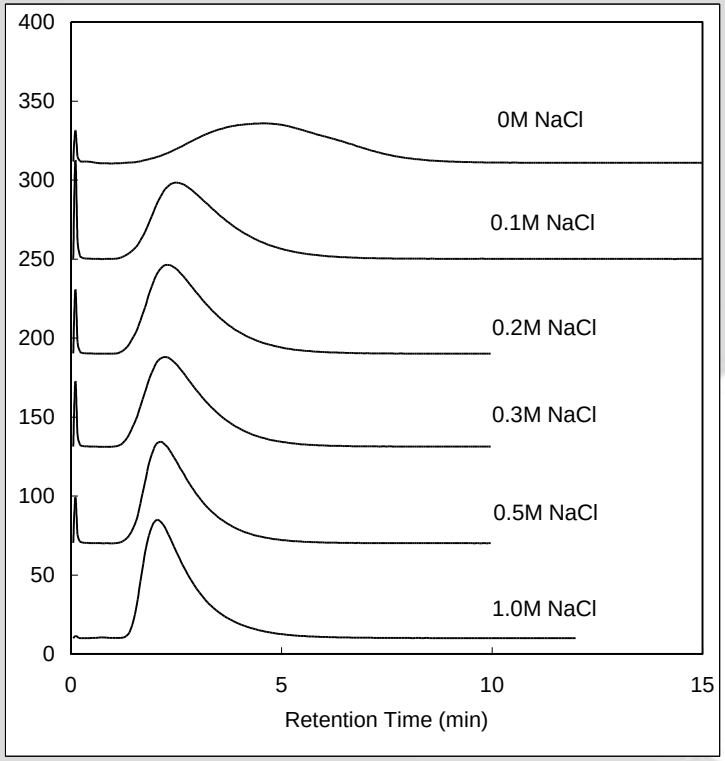
Condition

Column: TOYOPEARL MegaCap II SP-550EC (7.5mmID*7.5cm)

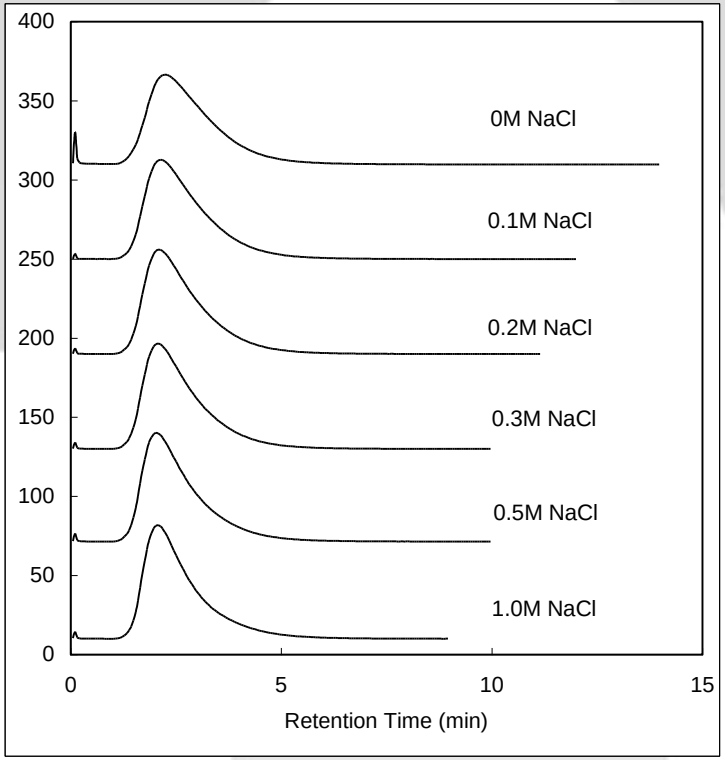
Eluent: 0.1M Aceticate buffer Flow rate: 1mL/min Detection: UV(280nm)

Sample: Insulin (Human) 1g/L Injection: 100uL

Effect of Salt and Buffer for Insulin Separation by CIEC



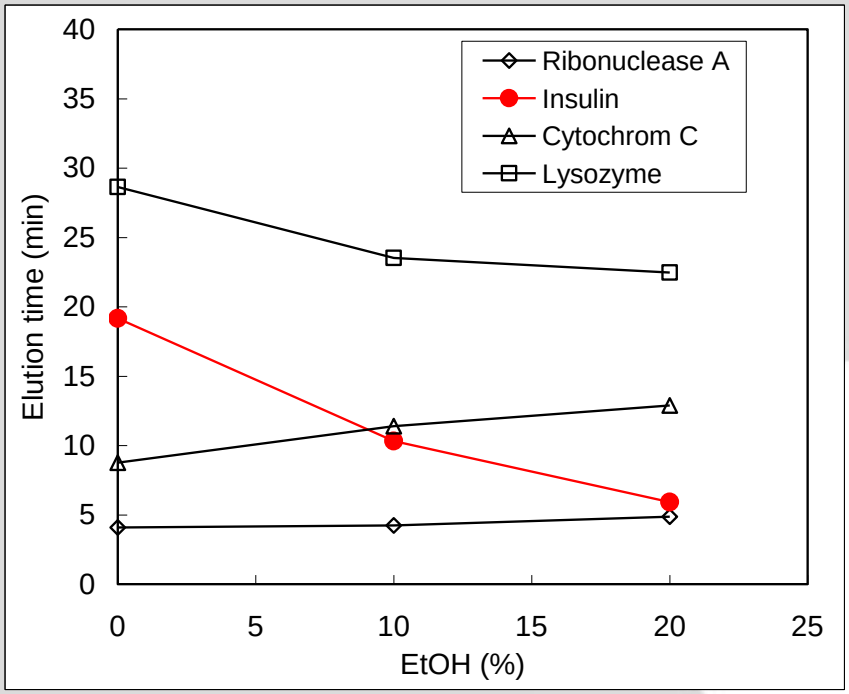
Eluent; Acetate buffer (pH 6.2)

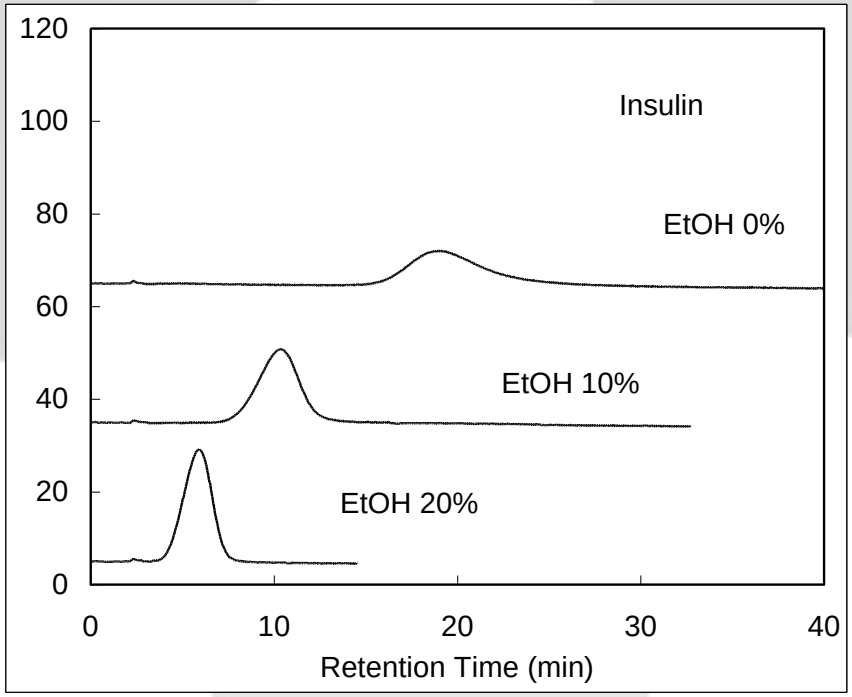


Eluent; Citrate buffer (pH 6.2)

Column; Toyopearl MegaCap II SP-550EC

Effect of Organic solvent for Insulin Separation by CIEC



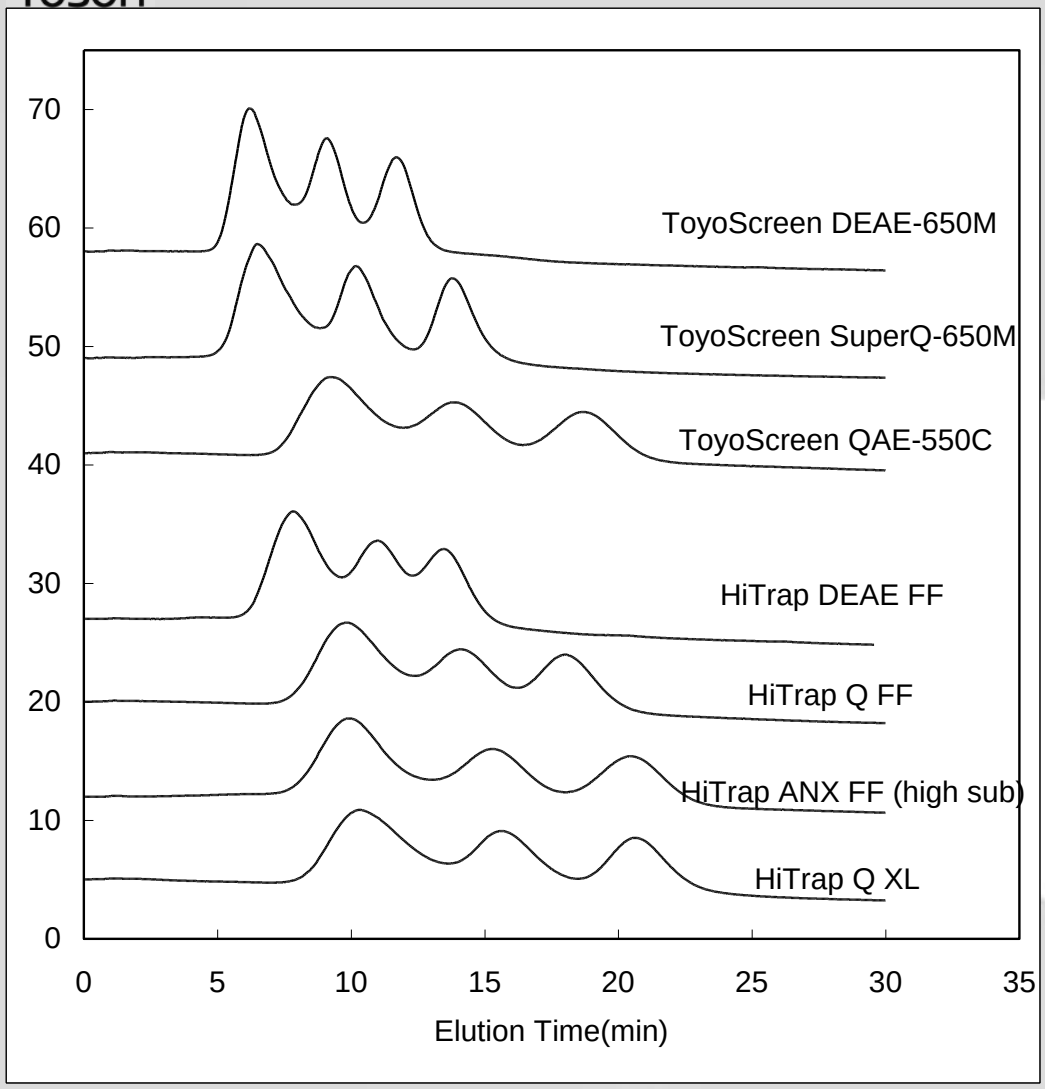


Condition
Column: IEC-011-LPC (SP resin for Insulin, 20-50um)
Eluent: (A) EtOH / (0.1M Citrate buffer (pH 5.0))
(B) EtOH / (0.1M Citrate buffer + 1.0M NaCl (pH5.0))
Gradient: (A) --> (B) 60min linear
Flow rate: 1ml/min
Detection: 280nm(UV)
Sample: Standard proteins 1g/L
Injection: 50uL

Condition
Column: IEC-011-LPC (SP resin for Insulin, 20-50um)
Eluent: (A) EtOH / (0.1M Citrate buffer (pH 5.0))
(B) EtOH / (0.1M Citrate buffer + 1.0M NaCl (pH5.0))
Gradient: (A) --> (B) 60min linear
Flow rate: 1ml/min
Detection: 280nm(UV)
Sample: Insulin 1g/L
Injection: 50uL

- Anion-Exchanger

Retention and Selectivity on AIEC for protein Separation



Condition
Column Size: ToyoScreen 6.4 mm I.D.*3cm, 1mL
HiTrap 7mm I.D.*2.5cm, 1mL
Eluent :a) 20mM Tris-HCl (pH8.0)
b) 20mM Tris-HCl + 0.5M NaCl (pH8.0)
Gradient: a) to b) 30min Linear
Flow rate: 1mL/min
Detection: UV (280nm)
Injection: 50μL
Sample: Transferrin, Ovalbumin,
Trypsin inhibitor 1g/L

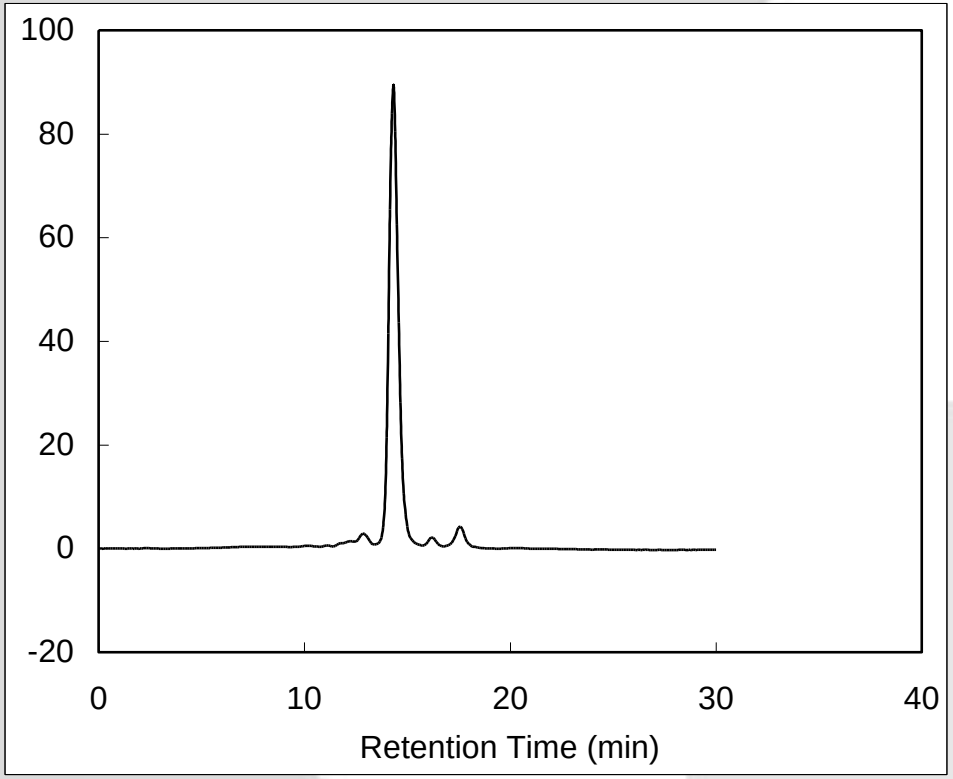
Dynamic Binding Capacity of Anion-Exchangers for Insulin

	Binding Capacity(g/L-gel) (10% Leakage)
DEAE-650M	29
QAE-550C	83
SuperQ-650M	162
DEAE Sepharose FF	66

Comparison of Capacity for Insulin on Anion-Exchangers

	TOYOPEARL SuperQ-650M	DEAE Sepharose FF
Base material	Hydrophilic polymer	Cross linked agarose
Particle size	40-90 um	45-165 um
Ion exchange capacity	0.20-0.30 eq/L-gel	0.11-0.16 eq/L-gel
Binding capacity for Insulin	162 g/L-gel	66 g/L-gel
Rigidity	+++	+
Productivity	+++	+

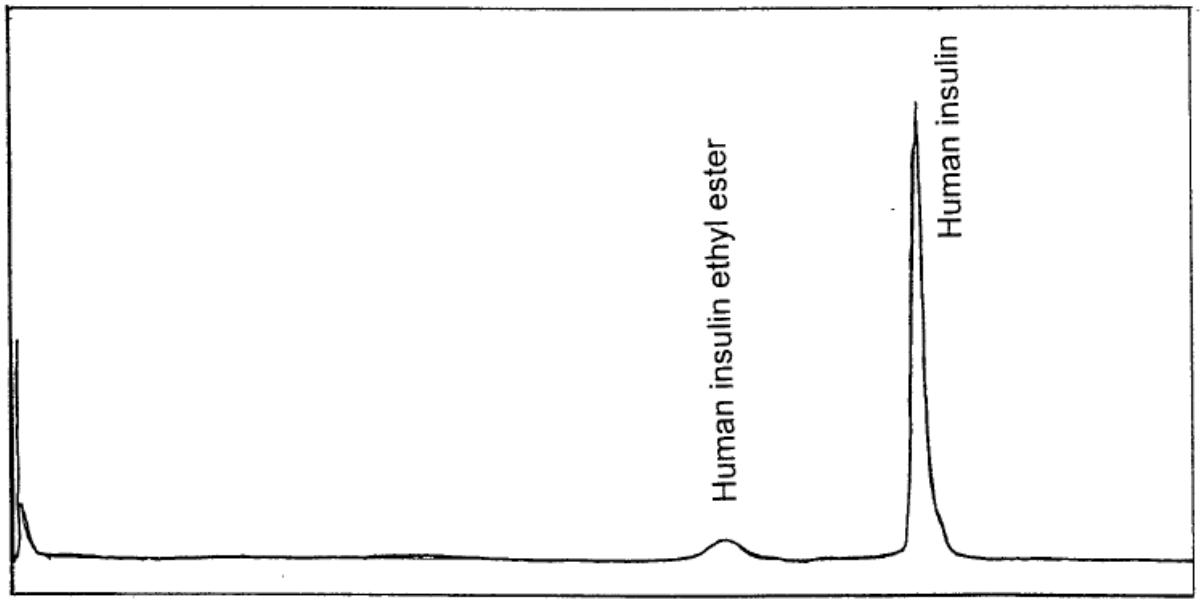
Separation of recombinant Insulin by IEC on TSKgel SuperQ-5PW Analytical Column



Condition

Column:	TSKgel SuperQ-5PW (7.5mmID x 7.5cm)
Eluent:	(A) EtOH/10mM Tris-HCl (pH 8.7)=1/1
	(B) 0.2M NaCl in (A)
Gradient:	(A) --> (B) 30min linear
Flow rate:	1ml/min
Detection:	280nm(UV)
Injection:	50uL

Separation of Insulin and DES-B30-Insulin by IEC on TSKgel SuperQ-5PW



Chromatogram of Example 13

Column; TSK-Gel Q-5PW (Tosoh) column (20 mL)

Elution; Buffer (A); 0.15% w/w triethanolamine, 42.5% w/w ethanol, pH 7.5.

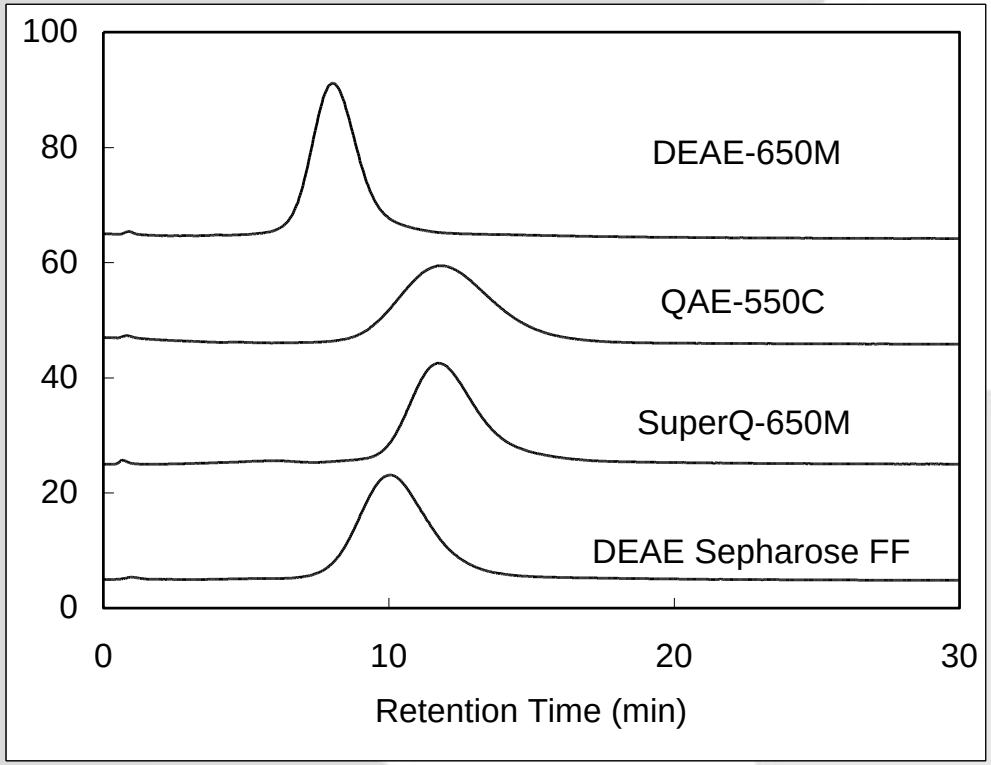
a linear gradient from 0 to 1.14% w/w sodium citrate tri-hydrate in Buffer (A)

Sample; A mixture of 7.7 mg/ml human insulin and 0.8 mg/ml human insulin ethyl ester (B30)

The mixture (2 mL) contained 4 mmol/l EDTA, 16% w/w ethanol, pH 7.5.

Ref. EU patent WO0055184

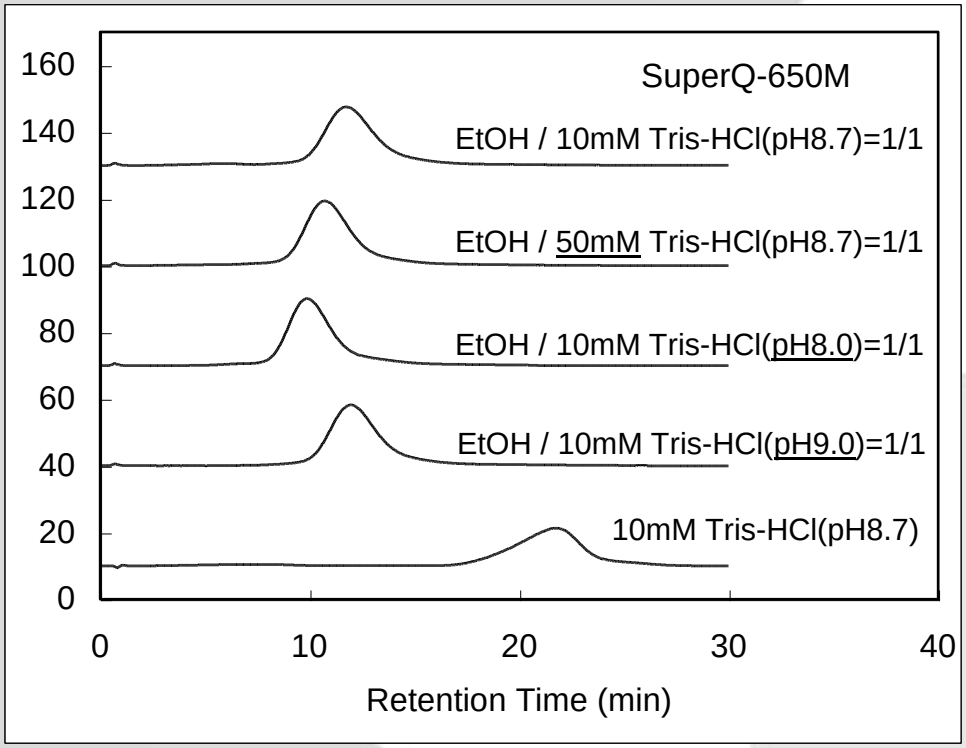
Retention and Selectivity of Insulin on AIEC resins



Condition

Column:	ToyoScreen (1mL)
Eluent:	(A) EtOH/10mM Tris-HCl (pH 8.7)=1/1
	(B) 0.2M NaCl in (A)
Gradient:	(A) --> (B) 30min linear
Flow rate:	1ml/min
Detection:	280nm(UV)
Injection:	50uL

Effect of pH in Eluent for Insulin Separation by AIEC

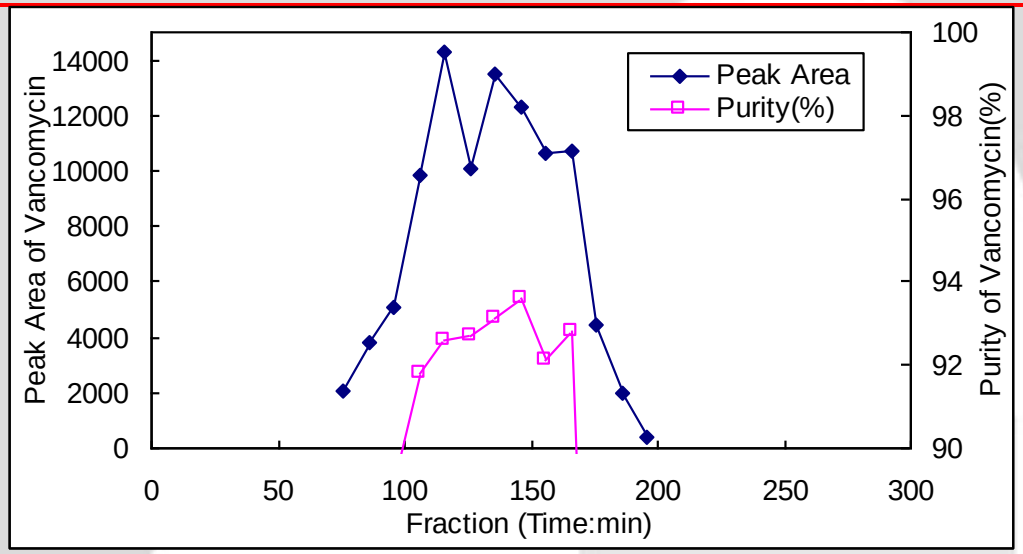


Condition

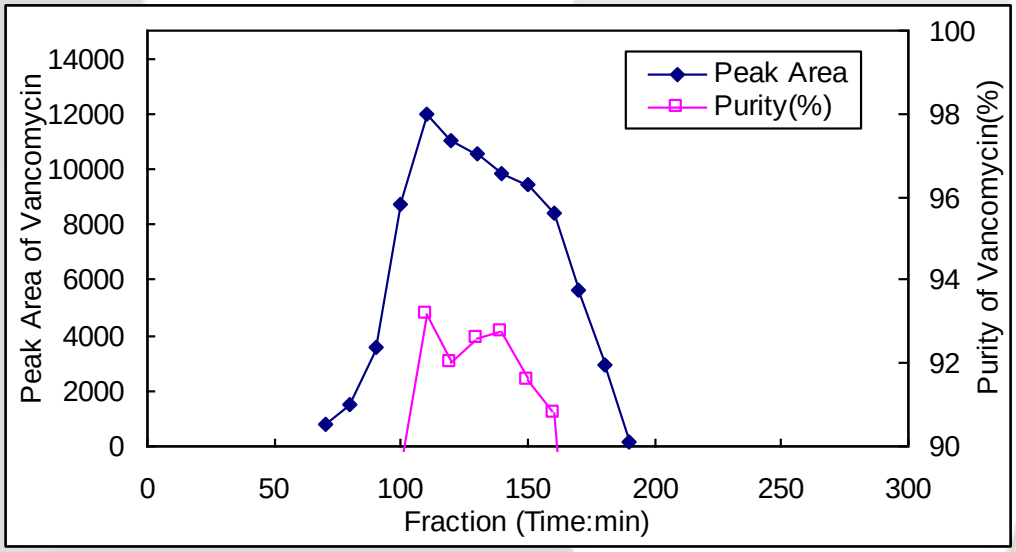
Column:	ToyoScreen SuperQ-650M (1mL)
Eluent:	(A) shown in figure
	(B) 0.2M NaCl in (A)
Gradient:	(A) --> (B) 30min linear
Flow rate:	1ml/min
Detection:	280nm(UV)
Injection:	50uL

Effect of Temperature on Separation and Purity of Peptide by AIEC

25 C



32 C



Reversed-phase Chromatography

- Selection of stationary phase
  - Monomeric vs. polymeric
  - ODS vs. other functional group C8, C4
  - Endocapping of silanol groups
  - Pore size; 8 – 12 nm
- Selection of Particle size
  - 20-100 micron
- Mobile phase
  - Organic modifier; methanol, acetonitrile
  - Acidic eluent; THF, HCl, acetic acid
  - Gradient method; linear or step
- Sample loading
  - Overloading conditions
- Column cleaning
  - CIP with NaOH for polymer resin
- Process econominc
  - Purity, recovery, production efficiency

Process R&D - TOYOSCREEN

- Easy to select the most effective resin for purification
- Selectivity, purity, recovery, capacity and elution conditions
- A-IEC, C-IEC, HIC; Kit and individual grade including AFC
- 1 ml and 5 ml column
- Installation to HPLC and/or Bio-LC system (e.g. AKTA Prime)