For Protein Separation

Proteonavi

SHISEIDO

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1 Protein separation in reversed-phase mode

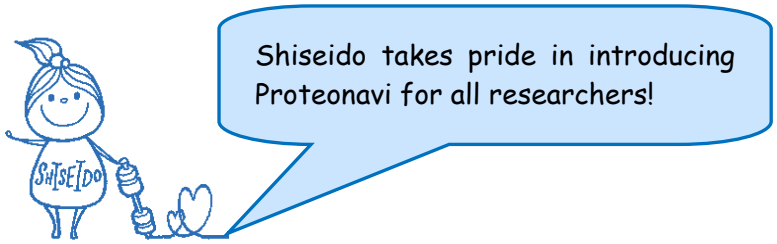
Chromatographic separations of proteins are based on their difference in either, size (size exclusion chromatography), electric charges (ion exchange chromatography), or hydrophobicity (reversed-phase chromatography).

"Proteonavi" was developed to separate proteins and peptides in reversed phase mode. In reversed-phase mode, it is known that proteins and peptides with higher-order structure are denatured to some extent in course of being retained on the stationary phase after introduction to the column. Once the retention with denaturation is occurred, molecules will not migrate until the organic content of the mobile phase is raised to an appropriate level under a gradient program.

In reversed-phase separation, peak shapes are often deteriorated when secondary interactions, such as electrostatic interactions with partial bare silica or metal imprities, exist between the stationary phase and proteins. Listed below are common facts in analytical and preparative separations of proteins and peptides.

1. Stationary phase with long alkyl chains (C_{18}) shows irreversible adsorption of protein.
2. Stationary phase with shorter alkyl chains (C_1, C_4, or C_8) have a lower durability under acidic mobile phases.
3. Stationary phase with shorter alkyl chains are influenced by silica, as a starting material, showing a lot variation.
4. Stationary phase with shorter alkyl chains often shows insufficient separation.

All of the above issues are related to chemistry to introduce alkyl chains to silica. The type of alkyl chain and the way to anchor it on silica seem to be the keys of preparing an appropriate stationary phase for protein and peptide analysis. Proteonavi has a C_4-modified surface designed after comprehensive basic research.



2 Properties of Proteonavi

2-1 Property values

Proteonavi utilizes high-purity silica with few metal impurities, and shows minimal irreversible adsorption for proteins and peptides. Its pore size is as wide as 30 nm, so that large proteins are able to have enough interactions with the stationary phase.

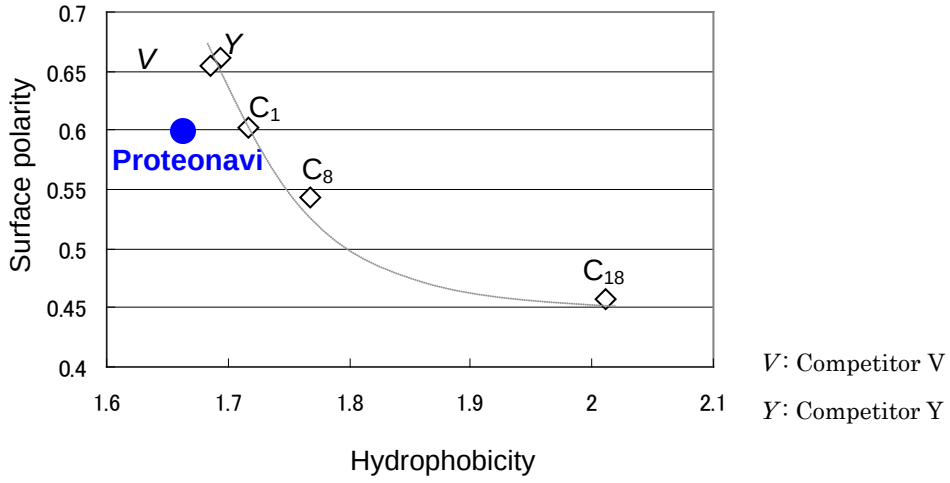
Property values

Functional group	Pore size (nm)	Particle size (µm)	Specific surface area (m^2/g)	Carbon content (%)	Alkyl group density (µmol/m^2)	pH	USP
C_4	30	5	105	3.3	3.2	2 - 10	L26

2-2 Evaluation of hydrophobicity and surface polarity

Nature of reversed-phase columns can be studied by measuring separation factors of different pairs of standard compounds. The strength of hydrophobicity can be expressed as a separation factor of toluene and benzene, showing a retention increase generated by one methyl group. Similarly, surface polarity is calculated between methyl benzoate and benzene, observing an influence of ester moiety on retention.

Results obtained with CAPCELL PAK SG 300 C_1, C_8, and C_{18}, Proteonavi and two other commercially available columns for protein separation were plotted on the hydrophobicity/surface polarity graph below.



Hydrophobicity is the most fundamental parameter in reversed-phase columns. Generally, a stationary phase with a large carbon content (larger alkyl density and/or longer alkyl chain) shows a large value. A large hydrophobicity is preferred when a

large retention or a high loadablility is required. A large value, however, may not be always good for macromolecules with a structure of higher degrees, such as proteins, since a long alkyl chain (C18) is more likely to cause an irreversible adsorption accompanied by their denaturation.

Surface polarity contributes to the retention and separation of polar compounds, and therefore, generates selectivity. Residual silanol groups on silica surface are thought to be one of the sources of surface polarity. While peak shapes of neutral compounds are hardly influenced by them, acidic silanol groups, if any, are known to cause a peak tailing for those having a basic moiety, such as proteins.

CAPCELL PAK SG300 columns form a characteristic curve in the graph, showing the influences of alkyl chain length on both values. However, even C_1, the one having the shortest chain, shows smaller polarity values than those of other products (*V* and *Y*), which can be attributed to the polymer-coated surface of CAPCELL PAK series.

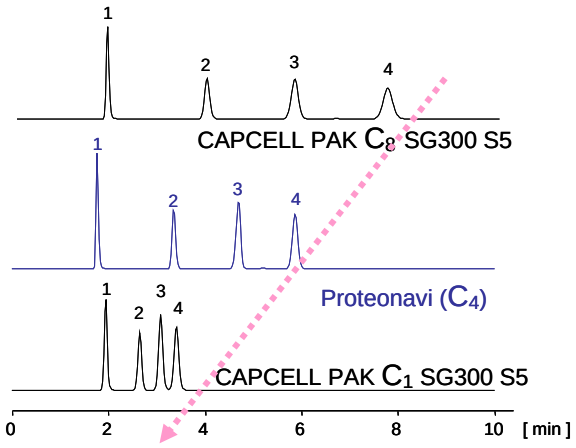
Proteonavi shows the surface polarity close to that of CAPCELL PAK C_1, in spite of its C_4 structure. Its entirely different surface coating process provides a new balance of hydrophobicity and surface polarity for protein and peptide analysis.

3. Features of Proteonavi

3-1 Specifically large retention of proteins

Proteonavi, having C_4 chains, showed overall retention of standard neutral small compounds, which is somewhat between C_1 and C_8 columns, as expected. However, it retains six standard proteins more than the C_8 column, apparently because of its structure designed specifically for protein separation. The large retention does not contradict with the unique property values discussed at the section before.

Neutral low molecular organic compounds

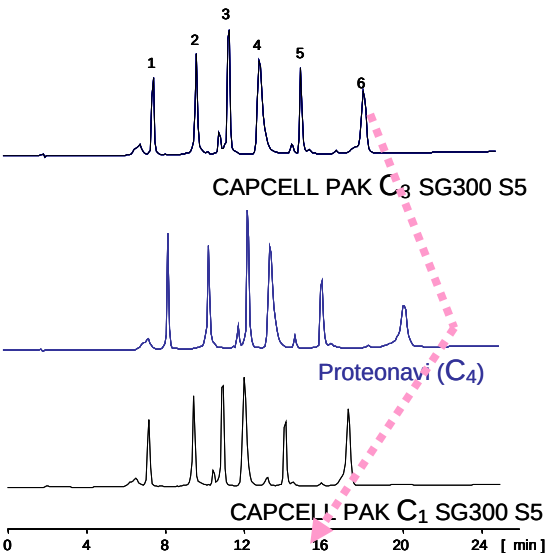


1. Uracil（M.W. 112.1）
2. Methyl benzoate（M.W. 136.2）
3. Toluene（M.W. 92.1）
4. Naphthalene（M.W. 128.2）

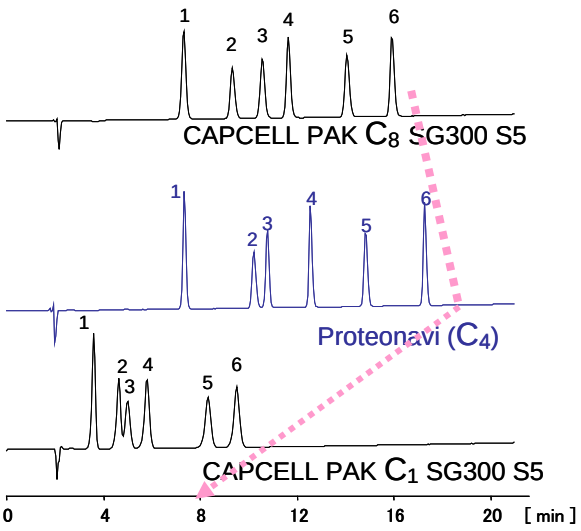
【HPLC Conditions】

Mobile phase	: CH_3CN / H_2O = 40 / 60
Column size	: 2.0 mm i.d. x 150 mm
Flow rate	: 200 µL/min
Temp.	: 40 ºC
Detection	: UV 254 nm
Inj. vol.	: 2 µL

Standard proteins



Standard peptides



Standard proteins

1. Ribonuclease A (M.W. : 13.7 kDa)
2. Cytochrome C (M.W. : 12.4 kDa)
3. Lysozyme (M.W. : 14.3 kDa)
4. B.S. albumin (M.W. : 69.0 kDa)
5. Myoglobin (M.W. : 17.0 kDa)
6. Ovalbumin (M.W. : 45.0 kDa)

【HPLC Conditions】

Column size	: 2.0 mm i.d. x 150 mm
Mobile phase	: A) 0.1vol% TFA / H_2O
	B) 0.1vol% TFA / CH_3CN
	B 20% (0 min) → 70% (25 min) Gra.
Flow rate	: 200 µL/min
Temp.	: 40 ºC
Detection	: UV 220 nm
Inj. vol.	: 2 µL
Sample	: 6 Proteins (STD)
	Equal amount of 1000µg/mL for 1 to 3 and 5, 2000µg/mL for 4, and 3000µg/mL for 6 were mixed.

Standard peptides

1. Methionine-Enkephalin (M.W. 574)
2. Bradykinin (M.W. 1060)
3. Leucine-Enkephalin (M.W. 556)
4. Angiotensin II (M.W. 1046)
5. Neurotensin (M.W. 1673)
6. Angiotensin I (M.W. 1296)

【HPLC Conditions】

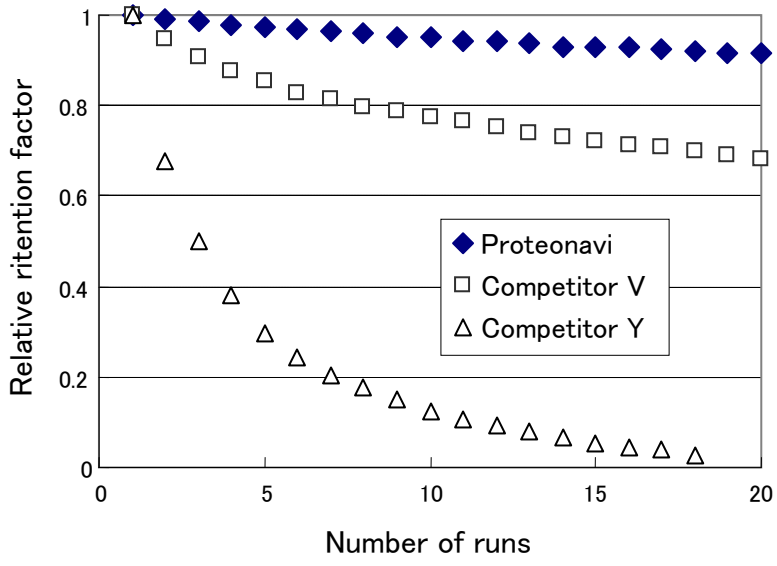
Column size	: 2.0 mm i.d. x 150 mm
Mobile phase	: A) 0.1vol% TFA / H_2O
	B) 0.1vol% TFA / CH_3CN
	B 15% (0 min) → 30% (20 min) Gra.
Flow rate	: 200 µL/min
Temp.	: 40 ℃
Detection	: UV 220 nm
Inj. vol.	: 2 µL
Sample	: 6 Peptides (STD) 100µg/mL each

3-2 Outstanding acidic durability

Acidic mobile phases containing trifluoroacetic acid (TFA) are often used in protein separation in reversed-phase chromatography. Acidic hydrolysis of alkyl chain is known to occur more or less on any silica-based columns, which is more pronouncing for those with shorter alkyl chains. Partial loss of alkyl groups generally causes a decrease of overall retention of analytes and a change of selectivity toward them.

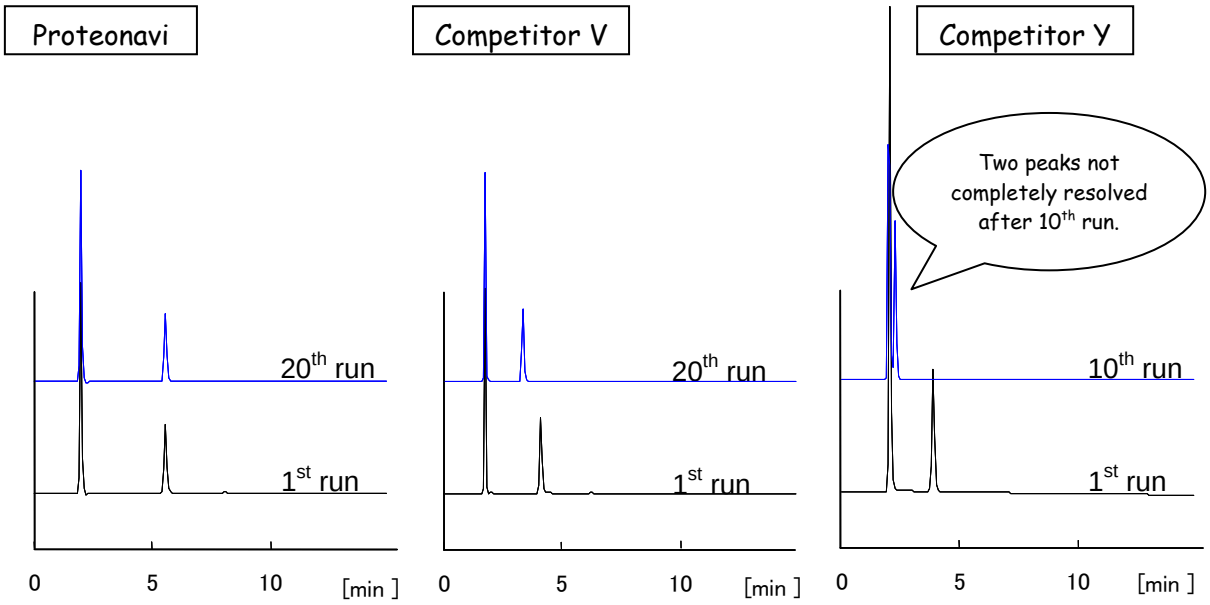
In order to evaluate durability of different columns under acidic conditions, an accelerated test was performed in the conditions shown below. Retention time change of benzylalcohol was examined under the strongly acidic mobile phase at the elevated temperature.

As shown in the graph, all of the columns tested showed loss of retention as time passed. While the column Y showed a rapid drop, and V lost approximately 30% of its retention during the process, Proteonavi kept more than 90% of the initial retention time until the end.



【HPLC Conditions】
Column size : 4.6 mm i.d. x 150 mm
Mobile phase : 1 vol%TFA, CH_3OH / H_2O = 10 / 90
Flow rate : 1 mL/min
Temp. : 60 ºC
Detection : UV 254 nm
Inj. vol. : 7µL
Sample : Uracil (50 µg/mL), Benzylalcohol (1000 µg/mL)
Runtime : 60 min per run

Actual chromatograms of the first and 20th runs (10th run for the column Y) are shown below. The absence of change in uracil peak (shape and position) at t_0 indicates that the test hardly damaged silica support in all of the columns. Retention time of benzyl alcohol, on the contrary, showed a significant difference from one column to another.



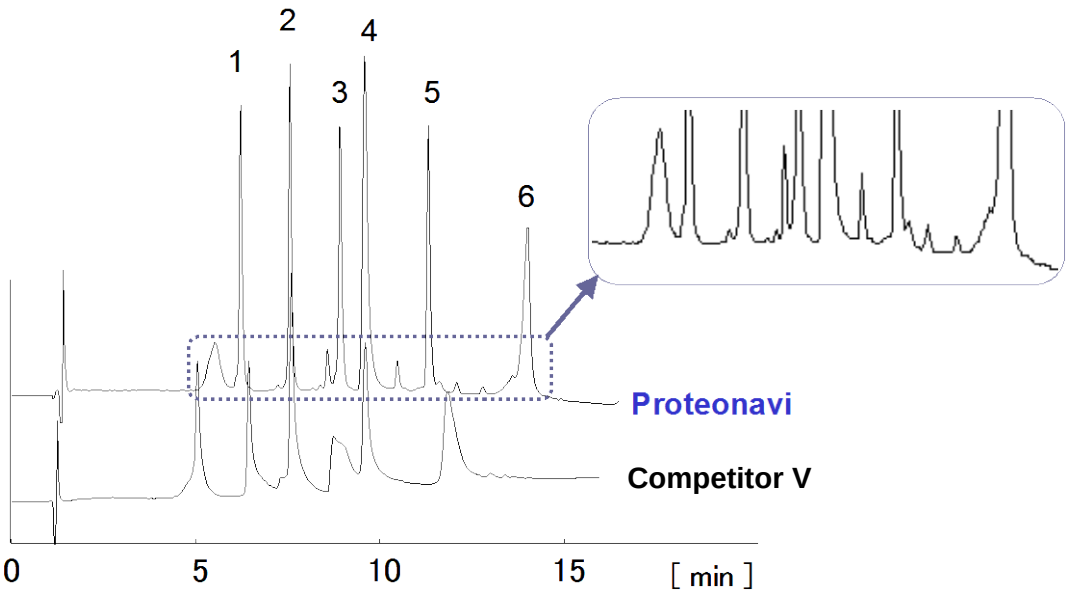
3-3 Minimal protein adsorption and high column efficiency

The primary source of protein retention on reversed phase is hydrophobic interaction between hydrophobic parts of amino acids residues and alkyl chains in the stationary phase. When secondary interactions, such as coulombic interactions by acidic silanols or metal impurities in the stationary phase, exist to a large extent, peak shapes of proteins will be deteriorated due to their slow kinetics. Proteonavi shows better peak profiles and higher resolution among standard proteins, in comparison with conventional wide-pore columns.

【HPLC Conditions】

Column size	: 2.0 mm i.d. x 150 mm
Mobile phase	: A) 0.1 vol% TFA / H_2O
	B) 0.1 vol% TFA / CH_3CN
	B 20% (0 min) → 70% (15 min) Gra.
Flow rate	: 200 µL/min
Temp.	: 40 ºC
Detection	: UV 220 nm
Inj. vol.	: 2 µL
Sample	: 6 Proteins (STD)

1. Ribonuclease A (M.W. : 13.7 kDa)
2. Cytochrome C (M.W. : 12.4 kDa)
3. Lysozyme (M.W. : 14.3 kDa)
4. B.S. albumin (M.W. : 69.0 kDa)
5. Myoglobin (M.W. : 17.0 kDa)
6. Ovalbumin (M.W. : 45.0 kDa)



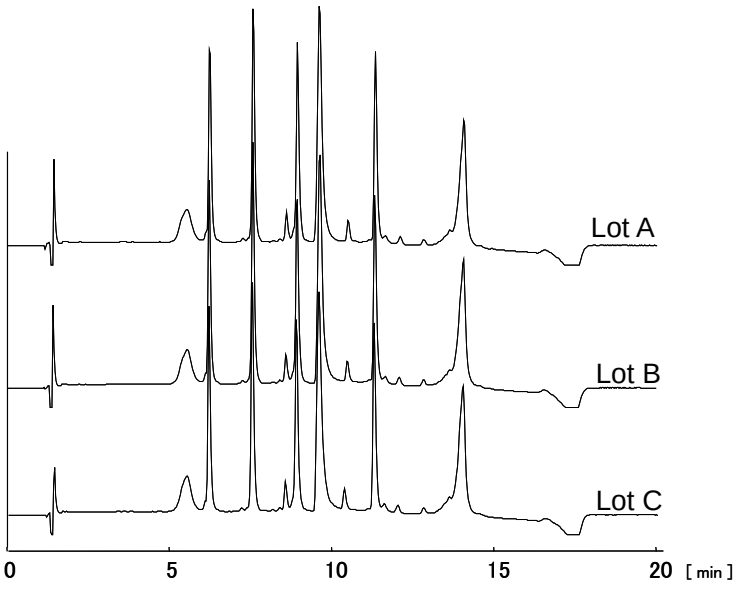
3-4 Excellent lot reproducibility

Lot-to-lot variation in separation is often discussed in protein analysis. Proteonavi has excellent reproducibility of retention time and separation.

【HPLC Conditions】

Column size	: 2.0 mm i.d. x 150 mm
Mobile phase	: A) 0.1 vol% TFA / H_2O
	B) 0.1 vol% TFA / CH_3CN
	B 20% (0 min) → 70% (15 min) Gradient
Flow rate	: 200 µL /min
Temp.	: 40 ºC
Detection	: UV 220 nm
Inj. vol.	: 2 µL
Sample	: 6 Proteins (STD)

1. Ribonuclease A (M.W. : 13.7 kDa)
2. Cytochrome C (M.W. : 12.4 kDa)
3. Lysozyme (M.W. : 14.3 kDa)
4. B.S. albumin (M.W. : 69.0 kDa)
5. Myoglobin (M.W. : 17.0 kDa)
6. Ovalbumin (M.W. : 45.0 kDa)

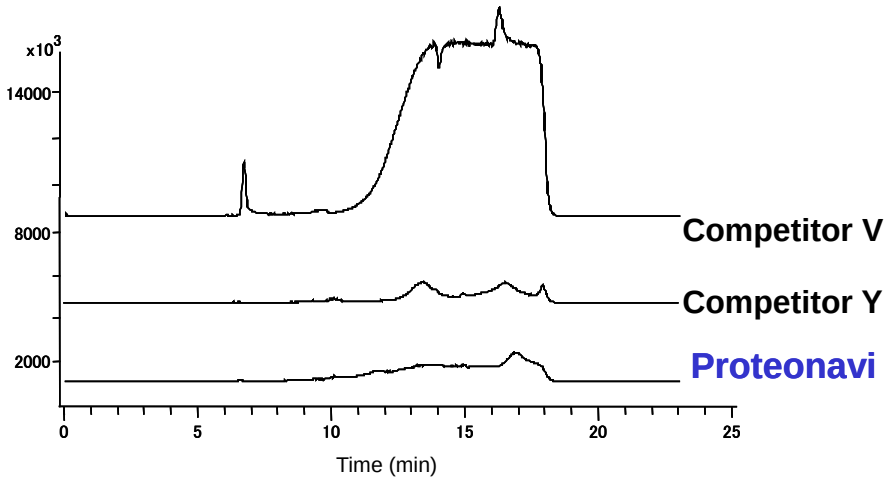


3-5 Minimal column bleed

"Column bleed" used to be a term used in gas chromatography with universal detectors, such as flame ionization detector. The recent development of interfacing technologies in mass spectrometry brought the term into the field of LC. Column bleed of an LC column is a synonym of a signal level of total ion chromatogram (TIC) obtained with LC-MS under gradient conditions at full scan mode.

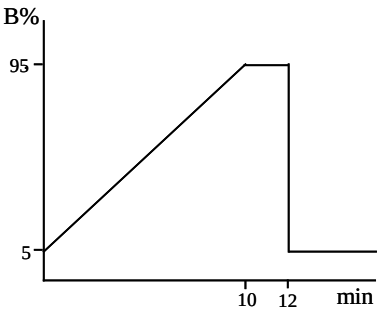
The source of column bleed is a bonded phase on silica. It may affect ionization efficiency of analytes of interest, or will be potential impurities in preparative separation. Proteonavi was designed to minimize the level of column bleed. A comparison among conventional wide-pore columns and Proteonavi is shown below. These background chromatograms were obtained under gradient conditions without sample introduction.

Total Ion Chromatogram（TIC）



【HPLC conditions】

Column size	: 2.0 mm i.d. x 150 mm
Mobile phase	: A) 0.1 vol% TFA / H_2O,
	B) 0.1 vol% TFA / CH_3CN
Flow rate	: 200 µL/min
Detection	: LC-MS（AccuTOF）



4 Scale up from analytical to preparative size

4-1 Scale up process

Proteonavi is suitable to large-scale preparative separation, taking advantage of its low-column bleed nature and high separation efficiency.

The followings are a common process to set up conditions for preparation separation.

Step ***1***

Pre-optimizing conditions in analytical scale

1) Mobile phase optimization
2) Know temperature dependence of separation
3) Study loadability by increasing sample amount.

Step ***2***

Determination of size

Select the inner diameter and particle size of a preparative column.

Step ***3***

Optimizing conditions in preparative scale

1) Study sample load and peak resolution
2) Column pressure and flow rate

Step ***4***

Running real samples

The table below gives a conversion factor to a calculate flow rate or sample size for each column size under the same linear velocity. In practice, a flow rate tends to be lowered in a large scale separation because of a pump capacity or safety reasons.

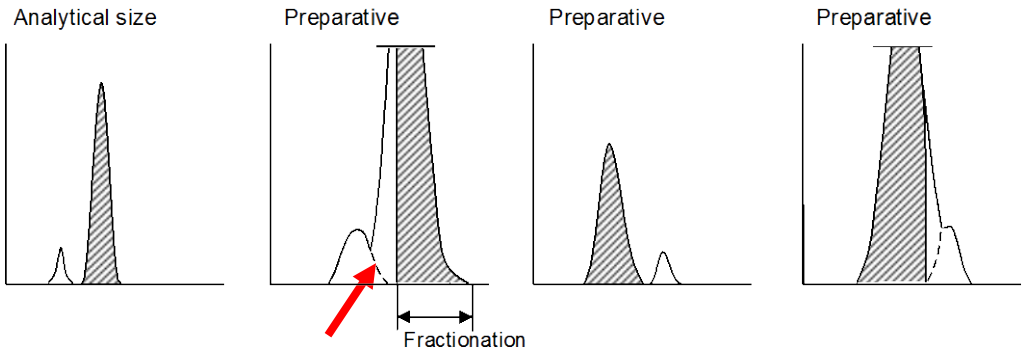
Conversion factors for flow rate and sample size

Inner diameter of column（mm i.d.）	Relative Flow rate or sample size
4.6	1
10	5
20	19
30	43

Here are the tips in preparative separation.

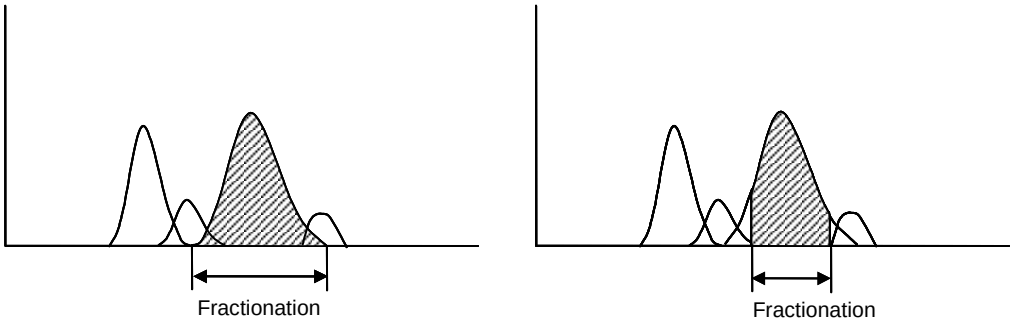
i ） Impurities on the edges

When impurities exist on the either edge of target peak, sample load and timing of fractionation will be determined by compromise between throughput and purity.

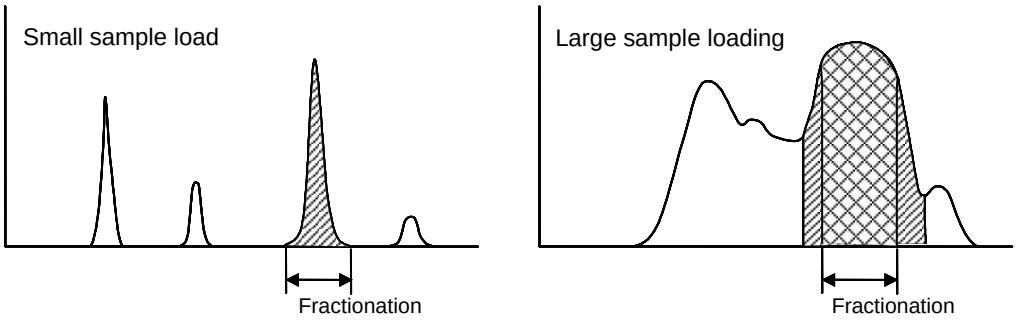


ii ） Purity

Purity of collected sample is always inversely related to recovery or throughput.



iii ） Throughput

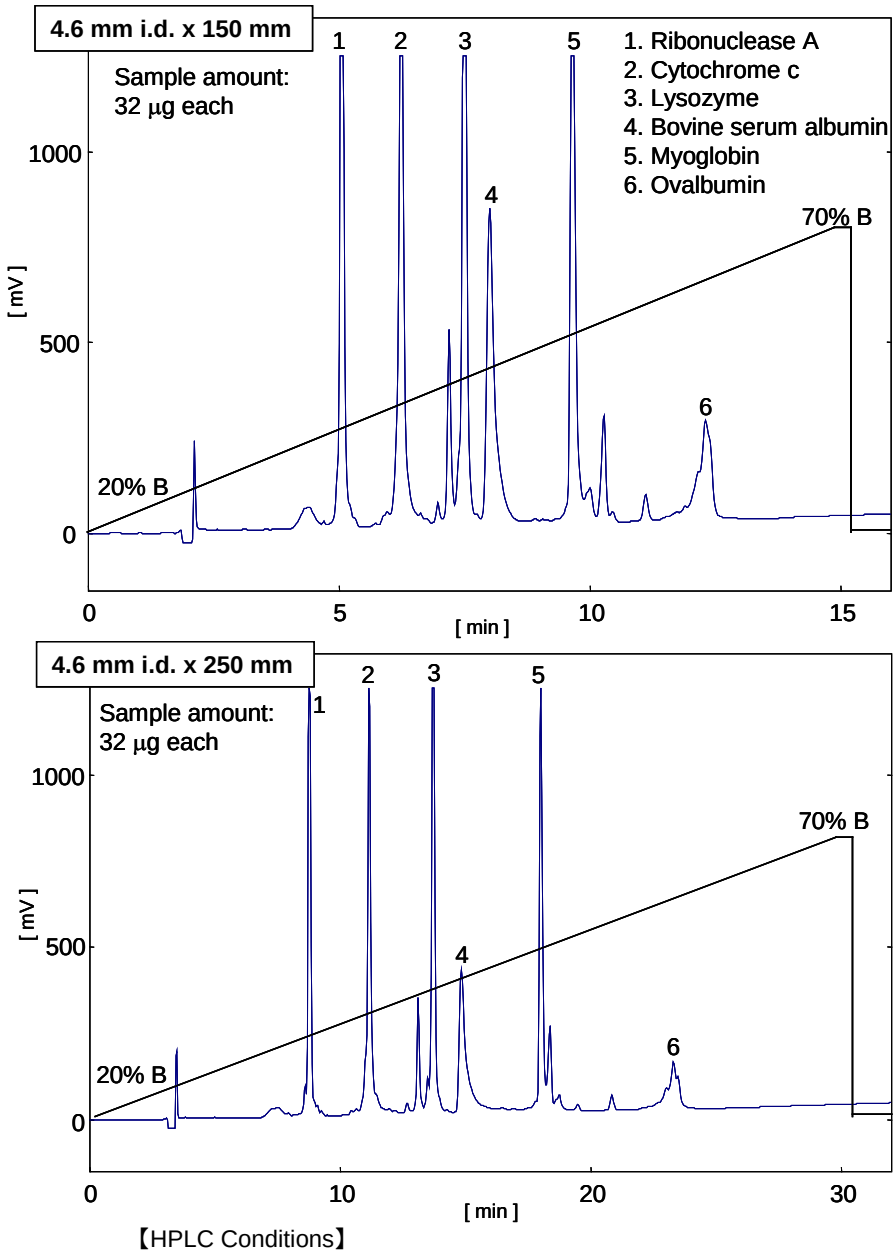


How to obtain the maximum production efficiency?

Although quite a few theoretical works have been published in preparative separation, optimization of fractionation with purity evaluation of each fraction is always necessary. A composition of mixture and target purity differs in every case. Theory does not help you much (-_-).

4-2 Separation efficiency, column size, and sample load

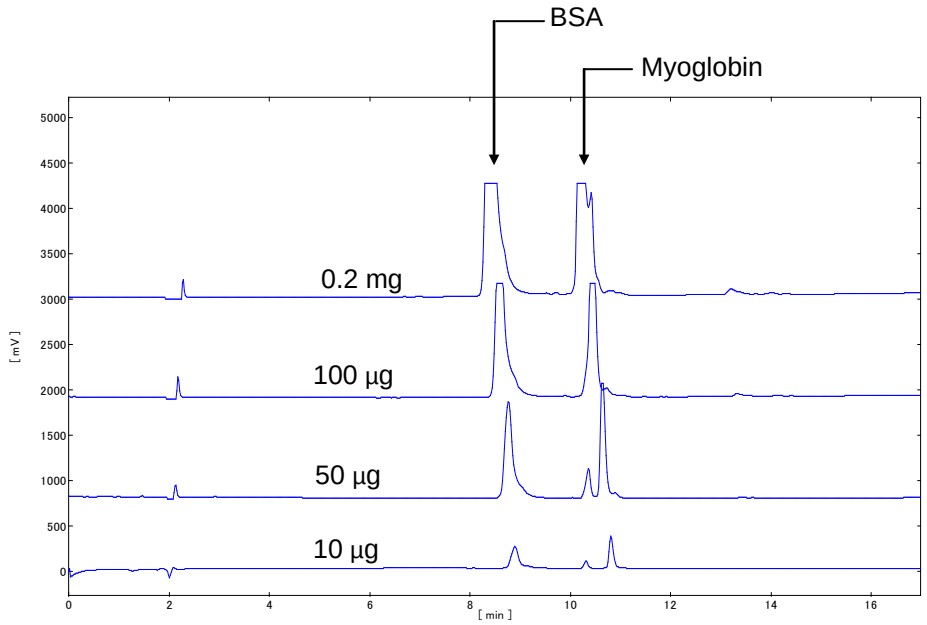
Separation efficiency at the same sample load was compared using Proteonavi columns of 150-mm and 250-mm long. The 250-mm column shows a higher resolution when an appropriate gradient time program is used. A resolution between peaks tends to be lowered as a sample size increases. However, the loss of separation efficiency by increased sample load will be moderate in a longer column.



【HPLC Conditions】

Mobile phase	: A) 0.1 vol% TFA / H_2O, B) 0.1 vol% TFA / CH_3CN
Flow rate	: 1 mL/min
Temperature	: 40 ºC
Detection	: 220 nm (Diodearray)
Sample dissolved in	: H_2O

Separation of two proteins of various sample loads were compared. (Detection wavelength was set at 225nm in order to avoid optical saturation in detection) The two proteins were baseline-separated even at 20-fold sample load in this particular case.



【HPLC Conditions】

Column	: Proteonavi , 4.6 mm i.d. x 150 mm
Mobile phase	: A) 0.1 vol% TFA / H_2O, B) 1vol% TFA / CH_3CN
	B% 20% (0 min) → 70% (15 min) → 70% (16 min)
	→ 20% (16.1 min) Gra.
Flow rate	: 200 µL/min
Temp.	: 40 ºC
Detection	: 225 nm (Diodearray)
Inj. vol	: 10 µL, 50µL, 100µL, 200µL (1000 µg/mL each)
Sample	: Bovine serum albumin (BSA), Myoglobin
Sample dissolved in	: H_2O

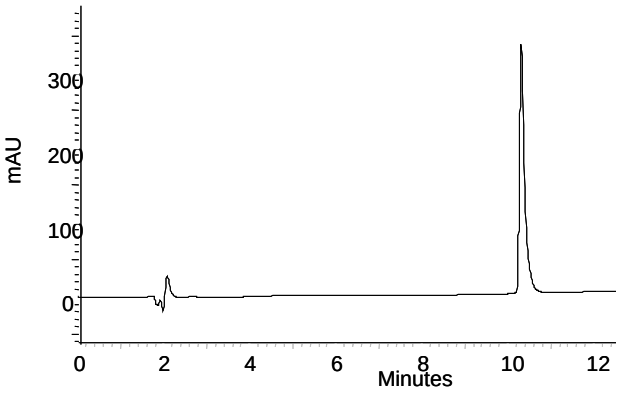
5 Application Data (FAQ)

5-1 Insulin（5.7 kDa）

Q. Can we analyze insulin with Proteonavi?

A. Shown below are the analytical results using Proteonavi. Insulin was detected by photodiodearray (PDA) and mass spectroscopy (MS).

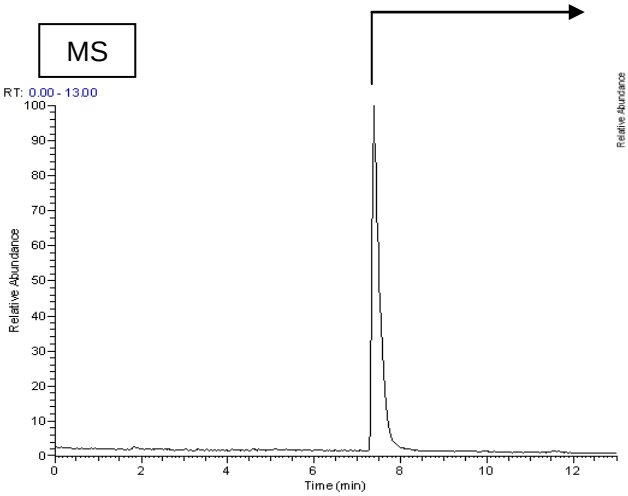
PDA



【HPLC Conditions】

Column	: Proteonavi ; 2.0 mm i.d. x 150 mm
Mobile phase	: A) 0.1 vol% TFA / H_2O
	B) 0.1 vol% TFA / CH_3CN
	B% 20% (0min) → 50% (15min) → 20%(15.1min) → 20% (25min) Gra.
Flow rate	: 200 µL/min
Temp.	: 40 ºC
Detection	: 224 nm (Diodearray)
Inj. vol.	: 2 µL (100 µg/mL)
Sample dissolved in	: M. phase

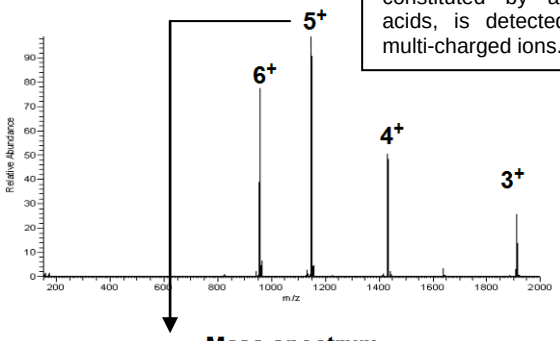
MS



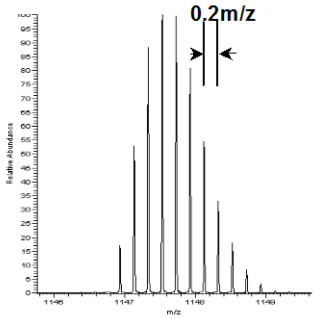
【LC-MS Conditions】

Column	: Proteonavi ; 2.0 mm i.d. x 150 mm
M.phase	: A) 0.1 vol% HCOOH / CH_3CN = 95 / 5
	B) 0.1 vol% HCOOH / CH_3CN =20 / 80
	B% 10%(0min)→70%(10min) → 10%(10.1min) → 10% (20min) Gra.
Flow rate	: 200 µL/min
Temperature	: 40℃
Detection	: ESI, Positive (LTQ-Orbitrap)
Inj. amount	: 1 nmol
Sample dissolved in	: Eluent

Protein, which is constituted by amino acids, is detected as multi-charged ions.



Mass spectrum



Isotope pattern

Particular pattern of large molecule by ^{13}C is obtained.
(Resolution: approximately 100,000)

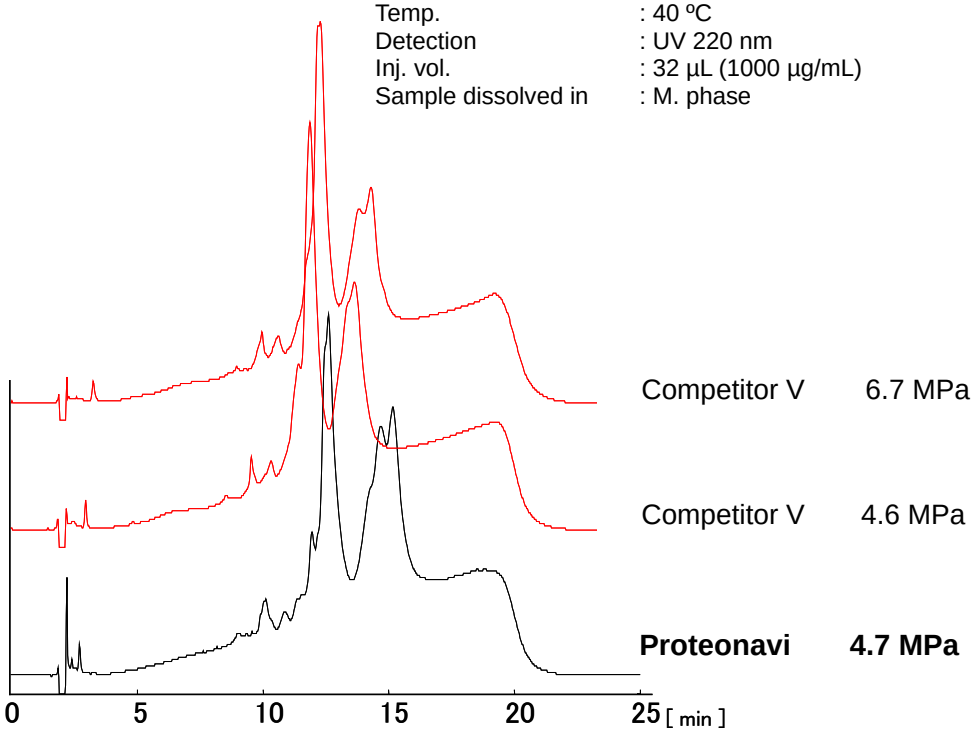
5-2 Thiogloblin (660kDa)

Q. How large molecules can Proteonavi separate?

A. There is no clear maximum molecular weight to be generally applied to all the proteins. However, very large molecules, such as thiogloblin, may not show a sharp peak profile any more. In addition, heterogeneity of the protein leads to the characteristic multi-peak profile in a chromatogram. Nevertheless, Proteonavi shows a slightly larger retention time and better separation between the last two major peak groups among the tested.

【HPLC Conditions】

Column	: Proteonavi ; 2.0 mm i.d. x 150 mm
Mobile phase	: A) 0.1 vol% TFA / H_2O B) 0.1 vol% TFA / CH_3CN B% 20% (0min) → 70% (15min → 70%(16min)→20% (16.1min) Gra.
Flow rate	: 200 µL/min
Temp.	: 40 ºC
Detection	: UV 220 nm
Inj. vol.	: 32 µL (1000 µg/mL)
Sample dissolved in	: M. phase



6 Product List

■ Analytical size

Product number	Particle size (μm)	Inner diameter (mm)	Length (mm)
80200	5	2.0	50
80201			150
80202			250
80203		4.6	50
80204			150
80205			250
11530		2.0	10 (cartridge, 2pcs)
11531		4.0	10 (cartridge, 2pcs)
12415	—	—	10L (cartridge holder)

※ Cartridge holder is needed when using cartridge.

■ Preparative size

Product number	Particle size (μm)	Inner diameter (mm)	Length (mm)
80214	5	10	150
80215			250
80224		20	150
80225			250
80234		30	150
80235			250

7 References

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