

HPLC Column

Technical Guide

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(1) Introduction

Thank you for choosing GL Sciences' HPLC columns. GL Sciences' HPLC columns are subjected to a rigorous array of QC test in the ISO 9001 compliant facility, with special emphasis on reagent purity, raw material traceability and consistency in raw materials, and final products. To maintain and maximize peak performance of GL Sciences' HPLC columns, and ensure long life and stability, please read the following before use.

(2) Specifications

(2-1) Recommended Operating Pressure (Maximum Operating Pressure)

GL Sciences' HPLC columns can tolerate up to pressures shown below. Although the columns are packed by high pressure slurry method, it is recommended to keep the operating pressure under the pressure shown below to maintain peak performance and ensure long column life and stability.

● Recommended Operating Pressure for Analytical Columns

| Analytical Columns | Particle Size | Recommended Operating Pressure (MPa) |
|---|-------------------------------------|--------------------------------------|
| Inertsil series, InertSustain series, InertSustainSwift series | 1.9 μm , 2 μm | 80 |
| Inertsil series, InertSustain series, InertSustainSwift series | 3 μm HP | 50 |
| Inertsil series, InertSustain series, InertSustainSwift series, Titansphere | 3~10 μm | 20 |
| InertCore C18 | 2.4 μm | 100 |
| InertSphere Sugar-1 | 5 μm | 15 |
| Capillary EX columns | 3 μm , 5 μm | 20 |
| Capillary EX Nano columns | 3 μm , 5 μm | 15 |

● Recommended Operating Pressure for Guard Columns

| Guard Columns | Recommended Operating Pressure (MPa) |
|--|--------------------------------------|
| Guard Column for UHPLC | 80 |
| Cartridge Guard Column E Cartridge Guard Column Ei GL-Cart, Pre-clean ORG PREP Guard Cartridge | 20 |
| Conventional Guard Column Conventional Mini Guard Column Capillary Micro Guard Preparative Guard Column | 20 |

(2-2) Temperature and pH Range

The recommended operating pH range and temperatures are shown below. To maximize column lifetime, set the pH of the mobile phase within the range shown below. Additionally, use mixed mobile phase such as organic solvent and buffer rather than 100 % buffer. When operating at high pH, lower operating temperatures are recommended for longer column lifetime. Working at high temperatures may also result in shorter column lifetimes. Please note that the column lifetime will vary depending upon the operating temperature, the type, and concentration of buffer used.

| Columns | Maximum Operating Temperature | pH Range | | |
|---|-------------------------------|--------------------|------------|------------|
| | | 20 ~ 40 °C | 40 ~ 50 °C | 50 ~ 60 °C |
| Inertsil series, Titansphere | 60 °C | 2 - 7.5 | | |
| InertSustain C18, InertSustain AQ-C18 InertSustainBio C18, InertSustainSwift C18 | | 1 - 10 (*) | | 1 - 7 |
| InertSustain C8, InertSustain Phenylhexyl, InertSustainSwift C8 | | 1 - 10 (*) | 1 - 9 (*) | 2 - 7 |
| InertSustain Amide | | 2 - 8.5 (*) | | 2 - 7 |
| InertSustain Phenyl, InertSustain NH2 | | 2 - 7.5 | | |
| InertCore C18 | | 2 - 7.5 | | |
| InertSphere Sugar-1 | 80 °C | 2 - 14 (5 ~ 80 °C) | | |

* For method development at low pH (pH 1 ~ 2), the usage of either trifluoroacetic acid, formic acid, acetic acid or phosphate buffer is recommended. At high pH (pH 10), the buffer concentration should be adjusted at 5 mM using buffers such as trimethylamine. In case an organic solvent is not present in the buffer mobile phase, set the pH in the range within 2 ~ 8.

Lower operating temperatures are recommended for longer column lifetime at pH 1 ~ 2 or pH 9 ~ 10. Additionally, it is recommended to have an organic solvent (e.g., methanol) present in your mobile phase.

(2-3) Column End-fittings

The specification of GL Sciences' end-fitting styles are summarized below. The chromatographic separation and result can be negatively impacted if the style of the column end-fittings does not match the existing tubing ferrule settings.

● End-fitting Styles for Analytical Columns

| Analytical Columns | Particle Size | End-fitting Style |
|--|--|-------------------|
| InertSustain AQ-C18 | 1.9 μm | UP Type |
| InertSustainSwift C18, InertSustainSwift C8 | 1.9 μm , 3 μm HP | UP Type |
| InertSustain C18, InertSustain C8, InertSustain Phenyl Inertsil series | 2 μm | W Type |
| InertSustain AQ-C18, InertSustainC18, InertSustain C8, InertSustain Phenylhexyl, InertSustain Phenyl, InertSustain Amide, Inertsil series, Unisil Q series, Titansphere | 3 μm HP, 3 ~ 10 μm | W Type |
| UHPLC PEEK, PEEK columns | All Particle Sizes | UP Type |
| InertCore C18 | 2.4 μm | UP Type |
| InertSphere Sugar-1 | 5 μm | W Type |
| Capillary EX columns | 3 μm , 5 μm | UP Type |
| Capillary EX Nano columns | 3 μm , 5 μm | UP Type |

* Each end-fitting style differs in the required length of the tubing protruding from the ferrule.
The UP type requires a 2.4 mm ferrule depth.
The W type requires a 3.3 mm ferrule depth.
For more details, please see page 5.

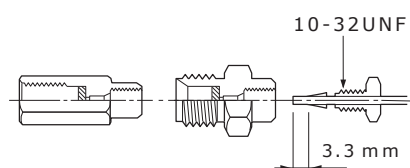
● End-fitting Styles for Guard Columns

| Guard Columns | End-fitting Styles |
|--|--------------------|
| Cartridge Guard Column E Cartridge Guard Column Ei GL-Cart, Pre-clean ORG PREP Guard Cartridge | W Type |
| Guard Column for UHPLC | UP Type |
| Conventional Guard Column Conventional Mini Guard Column Capillary Micro Guard Preparative Guard Column | W Type |

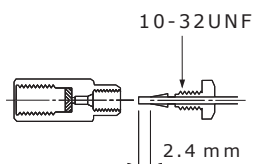
● Various End-fitting Styles

Various column manufacturers have employed different types of chromatographic column connectors. The following explains the differences in each type.

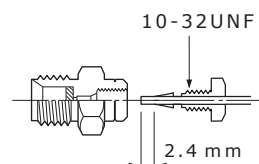
● W Type



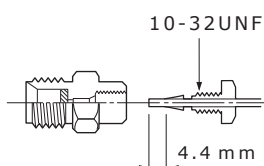
● UP Type (Parker style)



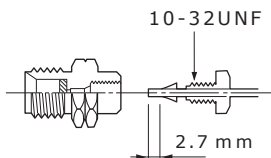
● OS Type



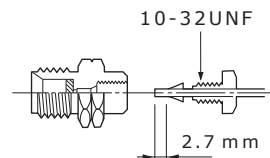
● T Type



● ON Type



● HA Type



(3) Shipping Solvent

(3-1) Various Shipping Solvents

GL Sciences' HPLC columns are shipped in the solvents shown below. Please be reminded that normal-phase columns are shipped in non-aqueous solvents.

| | Columns | Particle Size, Dimensions | Shipping Solvent |
|------------------------|---|-------------------------------------|--|
| Reversed-Phase Columns | <ul style="list-style-type: none"> • InertSustain C18, C8, AQ-C18, Phenylhexyl, Phenyl, Amide • InertSustainSwift C18, C8 • Inertsil ODS-3V, ODS-4V, Amide, HILIC, ODS-HL, ODS-P, ODS-EP, ODS-80A, C8-4, C8-3, C8, C4, Ph-3, Ph, WP300 C18, WP300 C8, WP300 C4, ODS, C30, Peptides, Acrolein, Sulfa • InertCore C18 • MonoTower C18, MonoClad C18-HS | All Particle Sizes & All Dimensions | Acetonitrile/Water |
| Reversed-Phase Columns | • Inertsil ODS-4, ODS-3, ODS-SP, ODS-2 | 5 µm 250 x 4.6 mm I.D. | Methanol/Water |
| | | 5 µm 150 x 4.6 mm I.D. | |
| | | Other Particle Sizes, Dimensions | Acetonitrile/Water |
| Normal-Phase Columns | <ul style="list-style-type: none"> • InertSustain NH2 • Inertsil NH2, Diol, CN-3, SIL-100A, SIL-150A, WP300 SIL, WP300 Diol, • Titansphere TiO | All Particle Sizes & All Dimensions | n-Hexane/Ethanol |
| Ion-Exchange Columns | • Inertsil AX, CX | All Particle Sizes & All Dimensions | 100 % Methanol |
| Polymer Columns | • InertSphere Sugar-1 | All Particle Sizes & All Dimensions | 100 mM Sodium Hydroxide Aqueous Solution |

(3-2) Column Equilibration Prior to Use

It is important to first understand the mobile phase compatibility before changing to a different mobile phase system. This understanding will help to avoid precipitating mobile phase buffers on your column as well as in your system. Before injecting the sample, thoroughly equilibrate the column with the mobile phase to be used to ensure stable chromatographic separation. For more details, please refer to the following.

● Column Equilibration Procedure for Reversed-Phase Columns (C18)

If the mobile phase does not contain buffers or additives, equilibrate the column and system with the mobile phase to be used for at least 30 minutes. The column may be considered equilibrated once a steady column back pressure and baseline are observed.

If the mobile phase contain buffers or additives, equilibrate the column and system with a water/organic solvent mixture, using the same content as in the buffered mobile phase. For example, equilibrate the column and system with 20 % acetonitrile in water for at least 30 minutes. Then, introduce and equilibrate with 20 % acetonitrile / 80 % buffered mobile phase for at least 30 minutes. The column may be considered equilibrated once a steady column back pressure and baseline are observed.

● Column Equilibration Procedure for Normal-Phase Columns

If the mobile phase does not contain buffers or additives, equilibrate the column and system with the mobile phase to be used for at least 30 minutes. The column may be considered equilibrated once a constant column back pressure and baseline are observed.

If the mobile phase contain buffers or additives, equilibrate the column and system with a mixture of solvents, using the same content as in the buffered mobile phase. For example, if the mobile phase is n-hexane / ethanol / acetic acid (900 /100 / 1), equilibrate the column and system with n-hexane / ethanol (900 / 100) for at least 30 minutes. Then, introduce and equilibrate with the mobile phase to be used for at least 30 minutes. The column may be considered equilibrated once a steady column back pressure and baseline are observed. When using normal-phase column for reversed-phase methods, the column must be properly equilibrated before use. For more details, please refer to section (3-3).

● Column Equilibration Procedure for HILIC Columns

(InertSustain Amide, Inertsil Amide, Inertsil HILIC)

If the mobile phase does not contain buffers or additives, equilibrate the column and system with the mobile phase to be used for at least 120 minutes. The column may be considered equilibrated once a constant column back pressure and baseline are observed.

If the mobile phase contain buffers or additives, equilibrate the column and system with a water/organic solvent mixture, using the same content as in the buffered mobile phase. For example, if the mobile phase is 90 % acetonitrile / 10 % ammonium acetate aqueous solution, equilibrate the column and system with 90 % acetonitrile in water for at least 30 minutes. Then, introduce and equilibrate with the mobile phase for at least 120 minutes. The column may be considered equilibrated once a steady column back pressure and baseline are observed.

● PEEK and UHPLC PEEK Columns

Avoid the use of tetrahydrofuran and chloroform as it can weaken the PEEK hardware and cause it to become brittle. The column equilibration procedure shall be the same as a stainless steel hardware column prior to the analysis.

(3-3) Column Equilibration Procedure for Normal-Phase Columns to be used for Reversed-Phase Methods

GL Sciences' normal-phase columns can be used for both normal-phase and reversed-phase separations. As shown at section (3-1), these columns are originally shipped in non-aqueous solvents and is ready to use for normal-phase conditions. If you intend to use the column for reversed-phase separations, you will require to condition the column with the following procedure:

【 For Inertsil NH2 (5 μ m, 150 x 4.6 mm I.D.) 】

- a) Flush the column and system with isopropyl alcohol at 0.5 mL/min for at least 60 minutes.
- b) If the mobile phase contain buffers or additives, equilibrate the column and system with a water/organic solvent mixture, using the same content as in the buffered mobile phase. For example, equilibrate the column and system with 90 % acetonitrile in water at 0.5 mL/min for at least 60 minutes.
- c) Then, introduce and equilibrate with 90 % acetonitrile / 10 % buffered mobile phase for at 0.5 mL/min for at least 60 minutes.
- d) The column may be considered equilibrated once a steady column back pressure and baseline are observed.
- e) Finally, inject the sample several times and make sure the retention times are not shifting.

● Difference in Column Stability depending on the Column Equilibration Procedure

As illustrated in the following page, the shift in retention times are not occurring in Figure B, which the column was properly equilibrated. Columns that were improperly equilibrated will most likely show results illustrated in Figure A.

HPLC Conditions

Column : Inertsil NH2
(5 μ m, 150 x 4.6 mm I.D.)
Eluent : A) CH₃CN
B) H₂O
A/B = 75/25, v/v
Flow Rate : 1.0 mL/min
Col. Temp. : 40 °C
Detection : RI

Sample :
1. Fructose
2. Glucose
3. Sucrose
4. Maltose
5. Lactose

Figure A.

The column was equilibrated only with a water/organic solvent mixture, using the same content as in the mobile phase. In other words, the column was not initially flushed with isopropyl alcohol.

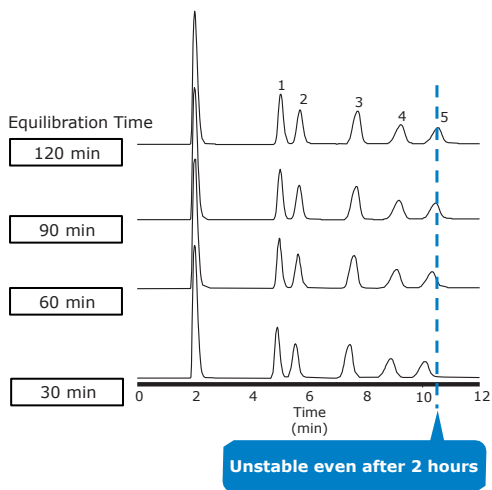
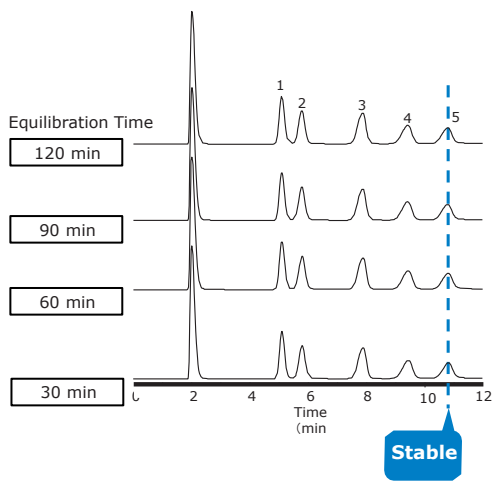


Figure B.

The column was properly equilibrated as per the procedure described in page 8, section (3-3).



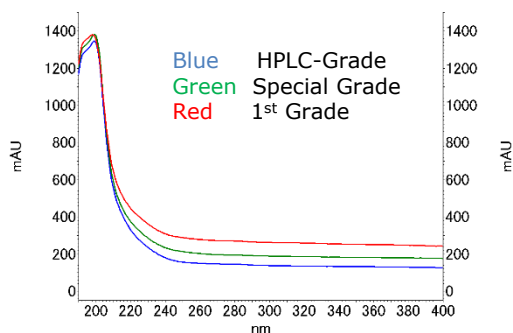
(4) Mobile Phase

(4-1) Various Grades of Solvents

To maintain maximum column performance, use high quality HPLC or MS-grade solvents. Solvents containing suspended particulate materials will clog the outside surface of the inlet frit of the column. This will result in higher operating back pressure. Additionally, the amount of contaminants/impurities or stabilizing agents (additives) contained in the solvents depends on the grades of solvents. These contaminants and stabilizing agents do have UV (ultraviolet) absorbance resulting in interfering chromatographic results. Select the appropriate grade of solvent depending on your method and application.

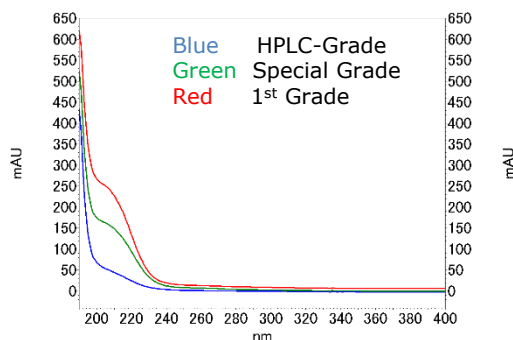
The UV absorbance curve is shown below between various grades of solvents.

●Methanol



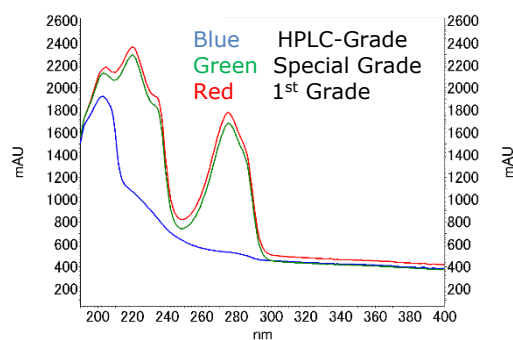
A significant difference in UV absorbance was not observed.

●Acetonitrile



Both special and 1st grade solvents showed absorbance at lower wavelengths.

●THF (Tetrahydrofuran)



When using THF, make sure using an HPLC grade. Other grades of THF contain antioxidants that can be detected by UV detectors and therefore affect the UV background. Additionally, the use of old THF may also result in a high UV background due to the formation of peroxides.

(4-2) Degassing Mobile Phase Solvents

When solvents are in contact with the atmosphere, air gradually dissolves into the solvent. The amount of dissolved air in solvents depends on the composition of mobile phase, temperature, and pressure. When the aqueous and organic solvents are mixed, they each contribute to the total dissolved air content of the mixture. Whether we are making isocratic mixtures or gradients, the amount of gas in solution is in proportion to the respective solvent volumes. The problem with this situation is that the solubility of air in the mixture is less than that of individual components. When such a situation exists, the solution is supersaturated with air, generating an unstable condition in which air will bubble out from the solution resulting in causing the following problems.

●Problems Possibly Caused by Insufficient Degassing of Solvents

| Places where air bubbles may have formed | Problematic Symptoms | Estimated Chromatographic Results |
|--|--|---|
| Pump | Unstable flow of solvent. Fluctuation of flow of solvent. | Shift in retention times. Change in peak areas. |
| Column | Decrease in column efficiency. | Distorted peak shapes. Less column efficiency. |
| Detector | Interference on detection of analyte. | Baseline drift or baseline noise. Decrease in sensitivity of detector. |

●Degassing Methods

The following describes the degassing methods and their features.

| Methods | Operation Procedures | Features |
|---------------------|---|---|
| Vacuum | Attach an aspirator to the mobile phase bottle and degas by vacuuming for at least 15 minutes while agitating the mobile phase to remove air bubbles. | Can be done at a low cost, but the solvent content can be changed. |
| Sonication | Place the mobile phase bottle into an ultrasonic cleaner and sonicate for at least 10 minutes. | Safe and easy, but air bubbles cannot be removed completely. |
| Vacuum + Sonication | Place the mobile phase bottle into an ultrasonic cleaner and attach an aspirator. Vacuum for at least 2 minutes while sonicating the solvent. | The solvent can be degassed in a short time, however, the solvent content can change very easily. |
| Helium Sparge | A stream of helium bubbles will sweep dissolved air out of solvents. | Very effective and can reduce the dissolved air in common solvents to levels below the saturation level of mixtures. However, the cost of helium gas is high. |
| Degasser | A membrane degasser uses a tube made of semi-permeable membrane passing through a vacuum chamber. Gasses diffuse through the membrane and are removed. Solvents are retained in the tube. | Easy-to-use and the solvent content does not change so much. |

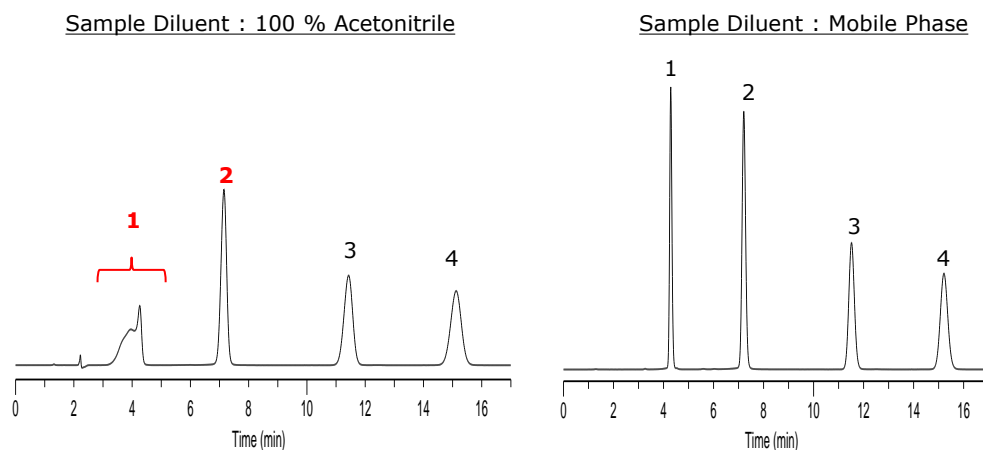
- Cautions on Degassing

Low temperature promotes gas solubility. Therefore, solvents stored at a cold place may contain a large volume of air. Additionally, air may be present in a mixture of acetonitrile/water as the temperature decreases after mixing these two solvents. Solvents that cool overnight should be stabilized to room temperature and fully degassed prior to introducing work into the system.

(5) Sample Diluent

(5-1) The Effect of Difference in Elution Strength between Sample Diluent and Mobile Phase

Ideally, the sample diluent should have a composition as close as possible to that of the mobile phase used for the separation *i.e.* the diluent should match the initial mobile phase conditions. As illustrated below, injecting a solvent stronger than the mobile phase can cause peak shape problems, such as peak splitting.



HPLC Conditions

Column : InertSustain AQ-C18 (5 μ m, 150 x 4.6 mm I.D.)
Eluent : A) CH₃CN
 B) H₂O
 A/B = 10/90 , v/v
Col. Temp. : 40 °C
Detection : UV 280 nm
Injection Volume : 10 μ L

Sample

1. 5-Hydroxymethyl-2-furaldehyde
2. 2-Furfural
3. 2-Acetylfuran.
4. 5-Methyl-2-furfural

(5-2) The Effect of Difference in pH between Sample Diluent and Mobile Phase

Peak splitting or fronting of peaks may be observed when the pH of the sample diluent and that of mobile phase are too different. Such problem can be overcome by diluting the sample solution with the mobile phase or decreasing the injection volume.

(6) Column Cleaning and Storage

Residual ion-pairing reagents, acids, or buffer salts in the column will promote and encourage hydrolysis of the bonded phase resulting in shorter column lifetimes. Additionally, storage in highly aqueous mobile phases for a long period can lead to splitting peaks or less column efficiency as the sample matrices such as lipids can accumulate in the column. To prolong column lifetimes, clean the column every single time after the analysis or whenever possible.

(6-1) Cleaning Reversed-Phase Columns

- When the mobile phase does not contain any buffered mobile phases or ion-pairing reagents
Use high concentration organic solvent to remove the highly lipophilic contaminants. Increase the content of organic solvent up to 100 %. Then, flush the column with 5 column volumes. When observing excessive back pressure, reduce and adjust the flow rate.

| | |
|--------------------------|----------------------------|
| Example Column Dimension | 4.6 mm I.D. x 250 mm |
| Method Flow Rate | 1 mL/min |
| Method Mobile Phase | acetonitrile/water = 65/35 |

Step 1 : Clean the column with 100 % acetonitrile at 1 mL/min for at least 30 minutes.

- When the mobile phase contain buffered mobile phases
Clean the column with a water/organic solvent mixture, using the same content as in the buffered mobile phase. For example, clean the column with 20 % acetonitrile in water for at least 30 minutes. Then, clean it with 100 % acetonitrile.

| | |
|--------------------------|---|
| Example Column Dimension | 4.6 mm I.D. x 250 mm |
| Method Flow Rate | 1 mL/min |
| Method Mobile Phase | 10 mM KH ₂ PO ₄ /acetonitrile = 80/20 |

Step 1 : Clean the column with 20 % acetonitrile in water at 1 mL/min for at least 30 minutes.

Step 2 : Clean the column with 100 % acetonitrile at 1 mL/min for at least 30 minutes.

- * When using the column again for the analysis, follow the procedures below to avoid precipitating mobile phase buffers on the column.

Step 1 : Equilibrate the column with 20 % acetonitrile in water at 1 mL/min for at least 30 minutes.

Step 2 : Equilibrate the column with the buffered mobile phase to be used at 1 mL/min for at least 30 minutes.

Step 3 : The column may be considered fully equilibrated once a constant back pressure and stable baseline are observed.

●When the mobile phase contain ion-pairing reagents

Depending on the ion-pairing reagent type, precipitation may occur when cleaning the column with 100 % water and extreme care is required. Clean the column with a water/organic solvent mixture, using the same content as in the mobile phase containing an ion-pairing reagent. For example, clean the column with 10 % acetonitrile in water for at least 30 minutes. Then, clean it with water/acetonitrile = 50/50 for at least 30 minutes.

The content of organic solvent should be increased further when using ion-pairing reagents containing long alkyl chains to effectively remove out from the column.

| | |
|--------------------------|---|
| Example Column Dimension | 4.6 mm I.D. x 250 mm |
| Method Flow Rate | 1 mL/min |
| Method Mobile Phase | 10 mM KH ₂ PO ₄ + 2 mM *IPCC-09 (pH:2.5)/acetonitrile = 90/10 |

Step 1 : Clean the column with 10 % acetonitrile in water at 1 mL/min for at least 30 minutes.

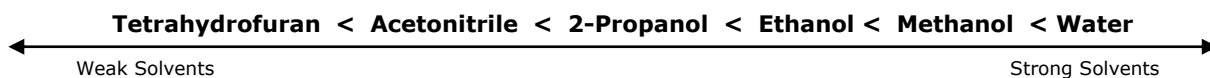
Step 2 : Clean the column with acetonitrile/water = 50/50 for at least 30 minutes.

* IPCC-09: Sodium 1-nonanesulfonate

* Please be aware that removal of 100 % of the ion-pairing reagent may not be possible. Due to the fact that ion-pairing reagents can alter column selectivity, it is strongly recommended to dedicate columns to ion-pairing methods to avoid problems with reproducibility.

(6-2) Cleaning HILIC Columns

Under HILIC mode, polar analytes are retained with high organic mobile phases. The following describes the elution strength of solvents used in HILIC mode.



●Cleaning InertSustain Amide Columns

To avoid precipitating mobile phases buffers within the column, clean the column with a water/organic solvent mixture, using the same content as in the buffered mobile phase. Clean the column with acetonitrile/water = 50/50 to remove highly polar contaminants.

If the column still shows shift in retention time or distorted peak shapes, clean the column with 100 % water for at least 30 minutes. After cleaning the column, make sure to thoroughly equilibrate the column with the mobile phase to be used in the analysis prior to use. Store the InertSustain Amide column in 100 % acetonitrile.

| | |
|--------------------------|---|
| Example Column Dimension | 4.6 mm I.D. x 250 mm |
| Method Flow Rate | 1 mL/min |
| Method Mobile Phase | 5 mM CH ₃ COONH ₄ /acetonitrile = 10/90 |

Step 1 : Clean the column with acetonitrile/water = 90/10 at 1 mL/min for at least 30 minutes.

Step 2 : Clean the column with acetonitrile/water = 50/50 at 1 mL/min for at least 30 minutes.

●Cleaning Inertsil HILIC Columns

To avoid precipitating mobile phases buffers within the column, clean the column with a water/organic solvent mixture, using the same content as in the buffered mobile phase. Clean the column with 100 % water to remove highly polar contaminants.

| | |
|--------------------------|---|
| Example Column Dimension | 4.6 mm I.D. x 250 mm |
| Method Flow Rate | 1 mL/min |
| Method Mobile Phase | 5 mM CH ₃ COONH ₄ /acetonitrile = 10/90 |

Step 1 : Clean the column with acetonitrile/water = 90/10 at 1 mL/min for at least 30 minutes.

Step 2 : Clean the column with 100 % water in water at 1 mL/min for at least 30 minutes.

●Cleaning InertSustain NH₂ Columns

To avoid precipitating mobile phases buffers within the column, clean the column with a water/organic solvent mixture, using the same content as in the buffered mobile phase. Clean the column with acetonitrile/water = 50/50 to remove highly polar contaminants.

If the column still shows shift in retention time or distorted peak shapes, clean the column with 50 mM ammonium formate (or ammonium acetate) aqueous solution/acetonitrile = 50/50 for at least 30 minutes. After cleaning the column, make sure to thoroughly equilibrate the column with the mobile phase to be used in the analysis prior to use. Store the InertSustain NH₂ column in 100 % acetonitrile.

| | |
|--------------------------|---|
| Example Column Dimension | 4.6 mm I.D. x 250 mm |
| Method Flow Rate | 1 mL/min |
| Method Mobile Phase | 5 mM CH ₃ COONH ₄ /acetonitrile = 10/90 |

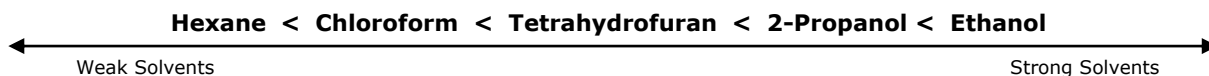
Step 1 : Clean the column with acetonitrile/water = 90/10 at 1 mL/min for at least 30 minutes.

Step 2 : Clean the column with acetonitrile/water = 50/50 at 1 mL/min for at least 30 minutes.

(6-3) Cleaning Normal-Phase Columns

Normal-phase separations depend upon polar adsorptive interactions, which the bonded phase is polar and the mobile phase is non-polar. Polar analytes will be more strongly retained than non-polar analytes in normal-phase chromatography. Clean the column with polar solvents to remove highly polar contaminants.

The following describes the elution strength of solvents used in normal-phase mode.



- Cleaning Inertsil SIL-100A, Inertsil SIL-150A, Inertsil WP300 SIL, Inertsil NH2, Inertsil CN-3, Inertsil Diol, InertSustain NH2 Columns

Clean the column with ethanol or 2-propanol. Because alcohol solvents are quite viscous, adjust the flow rate to avoid excessive column back pressure.

Example Column Dimension 4.6 mm I.D. x 250 mm
Method Flow Rate 1 mL/min
Method Mobile Phase n-hexane/2-propanol/acetic acid = 90/10/0.1

Step 1 : Clean the column with 100 % 2-propanol at 0.2 mL/min for at least 60 minutes.

(6-4) Column Storage

Residual ion-pairing reagents, acids or salts in the column will promote and encourage hydrolysis of the bonded phase resulting in shorter column lifetimes. Additionally, storage in highly aqueous mobile phases for a long period can lead to splitting peaks or less column efficiency as the sample matrices such as lipids can accumulate in the column. To prolong column lifetimes, clean the column every single time after the analysis or whenever possible. After cleaning the column properly (refer to section 6-1 to 6-3), store the column by referring to the following table.

| Bonded Phase | Storage from 1~10 Days | Storage longer than a few weeks |
|---------------------------------|--|---------------------------------|
| ODS, C8, C4, Ph HILIC, Amide | Mobile Phase (without salts, additives) | 100 % Acetonitrile |
| CN, NH2, Diol, SIL | Mobile Phase (without salts, additives) | 100 % n-Hexane |
| AX, CX | Mobile Phase (without salts, additives) | 100 % Acetonitrile |

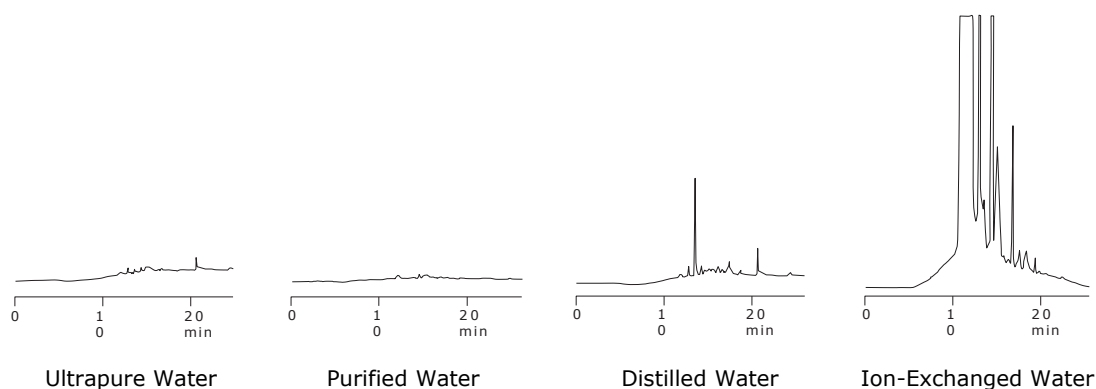
- * Columns should be stored in a cool and dark place.
- * Columns stored for a long period of time should be cleaned prior to use.

(7) Tips to Increase HPLC Column Lifetime

To increase column lifetime, follow these important guidelines.

(7-1) Column Handling

- Do not drop or bump columns, to avoid a deterioration of the column performance. Make sure to disconnect the column from the system after confirming the display of the pressure gauge showing zero "0" value. Avoid rapid pressure fluctuation to extend column lifetime.
- Use a pump equipped with an inline filter to prevent particulates from worn pump seals or contaminants from mobile phases entering the column.
- To maximize column lifetime, use the column within the operating conditions described at section (2). Please note that working in combinations of extreme pH, temperature and pressure will result in shorter column lifetime.
- Residual ion-pairing reagents, acids or salts in the column will promote and encourage hydrolysis of the bonded phase resulting in shorter column lifetimes. To prolong column lifetimes, clean the column every single time after the analysis or whenever possible. For more details, please read section (6).
- Only use ultrapure water in the mobile phase. Routinely maintain the water purification system to ensure it is functioning properly. The usage of contaminated water can cause noisy or drifting baselines and detecting ghost peaks, which are frequently observed in gradient methods. Additionally, use freshly prepared purified water and avoid storing ultrapure water (or aqueous solvents). Ultrapure water will absorb contaminants from the laboratory atmosphere and from containers.
- Comparison of Residual Contaminants in Water between various Water Purification Process



(7-2) Guard Columns

Guard columns are connected in between the sample injector and analytical column to protect analytical column against contamination by sample particulates and strongly retained compounds. GL Sciences offer two different types of guard columns, a cartridge format requiring an exclusive holder and packed with the same material as in the analytical column. The packed guard column type is packed with the same material as in the analytical column too, but to a stainless steel hardware format.

● Selecting the Appropriate Column Protection System

It is best to choose a guard column that contains the same packing material as your analytical column. The monolithic silica type guard cartridge, SILFILTER STD C18 is compatible and can be used with other C18 analytical columns.

● Selecting the Appropriate Guard Column Size

Particle Size : Select a guard column that contains the same packing material and particle size as your analytical column.

Internal Diameter : Select a guard column with the same I.D. size or similar as your analytical column.

| Format | Product | Analytical Column I.D. | Guard Column I.D. | Guard Column Length |
|---|---|------------------------|-------------------|---|
| Cartridge Type An Exclusive Holder is Required | Cartridge Guard Column E (First Choice Guard Column) | 1.0 ~ 2.1 mm | 1.0 ~ 1.5 mm | 10 mm |
| | Cartridge Guard Column Ei (Metal-Free Guard Column) | 2.1 ~ 4.6 mm | 2.1 ~ 4.0 mm | 10, 20 mm |
| | Guard Column for UHPLC (Can Tolerate Pressures up to 80 MPa) | 1.0 ~ 3.0 mm | 1.0 ~ 3.0 mm | 10 mm |
| | SILFILTER [®] STD C18 (Compatible with Other C18 Columns) | 3.0 ~ 4.6 mm | 3.0 mm | 10 mm |
| | GL-Cart (Available only in one size, but Economical) | 4.0, 4.6 mm | 4.6 mm | 5 mm |
| Packed Guard Column Type Holder Not Required | Packed Guard Column (Packed to a Stainless Steel Hardware) | 1.0 ~ 4.6 mm | 1.0 ~ 4.6 mm | 33, 50 mm |
| | Packed Mini Guard Column (Short Size) | 4.0, 4.6 mm | 4.0 mm | 10 mm |
| | Preparative Guard Column | 6.0 ~ 100 mm | 6.0 ~ 100 mm | 50 mm 75 mm (For 50 mm I.D.) 100 mm (For 100 mm I.D.) |

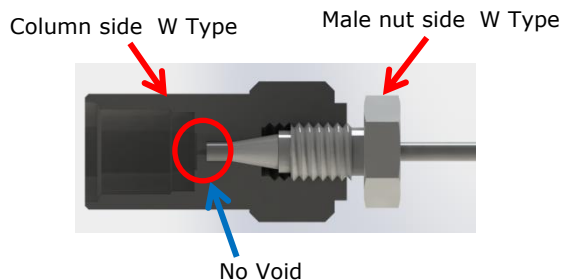
● Guard Column Installation

Column connectors and tubings are required separately to connect guard columns to analytical columns. Make sure to select the appropriate connectors depending on the type of end-fittings used on the analytical column, otherwise, chromatographic performance will be negatively impacted due to extra dead volume.

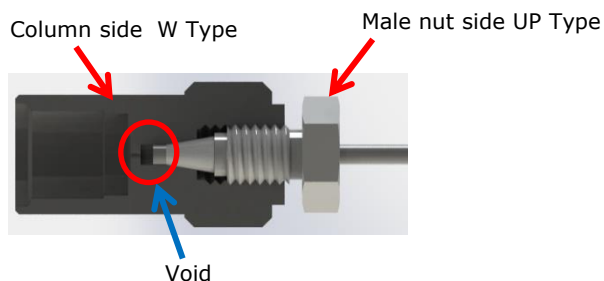
●Type of End-fittings

End-fitting styles differ among various manufacturers and have different seating depths. Always make sure to select the appropriate end-fittings, otherwise, chromatographic performance will be negatively impacted due to extra dead volume. For more details on the end-fitting styles, please see section (2-3).

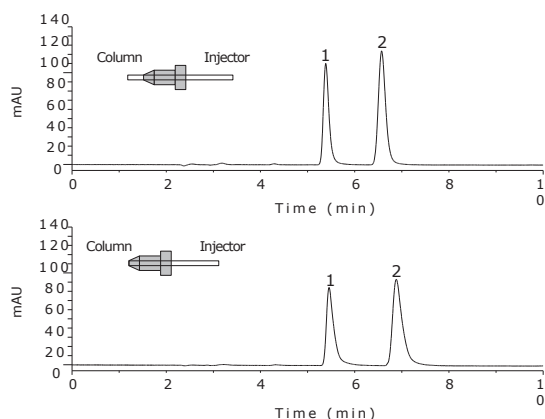
●Proper Column Connection



●Improper Column Connection



●Influence of Extra Dead Volume on Chromatographic Performance



(7-3) Sample Preparation

●Sample Filtration

Particulates often contribute to column contamination. To maximize column lifetime, filtrate sample through a disposable syringe filter to remove particulates.



● Sample Preparation for Biological Samples

Proteins are present in biological samples which can interfere the quantitation of the target analytes. Additionally, the protein matrices can reduce the column lifetime, especially under reversed-phase HPLC. In some cases, some target analytes can bond with protein matrices leading to a negative impact on the accuracy, precision, and robustness of the method.

● Major Deproteinization Methods

《Protein Precipitation》

Add organic solvent to the biological sample to denature it. Centrifuge the precipitated sample solution. Take the supernatant portion of the sample and then filtrate.

《Ultrafiltration》

The primary basis for separation is molecular size. Proteins, which are larger than the membrane pores will be retained at the surface of the membrane.

《Protein Hydrolysis》

Protein hydrolysis is the breakdown of protein into smaller peptides and free amino acids using enzymes.

(8) Benefits of Scaling Down Column Internal Diameter Size

By scaling down to a narrower internal diameter column, we can significantly reduce the flow rate and reduce costs by reducing solvent consumption. Depending on the detector type, improvement in sensitivity can be obtained.

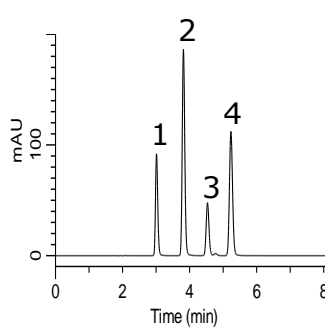
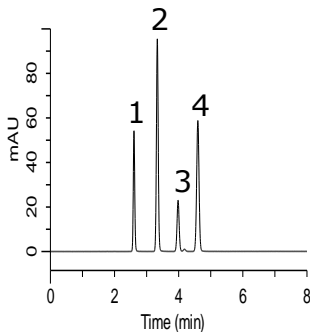
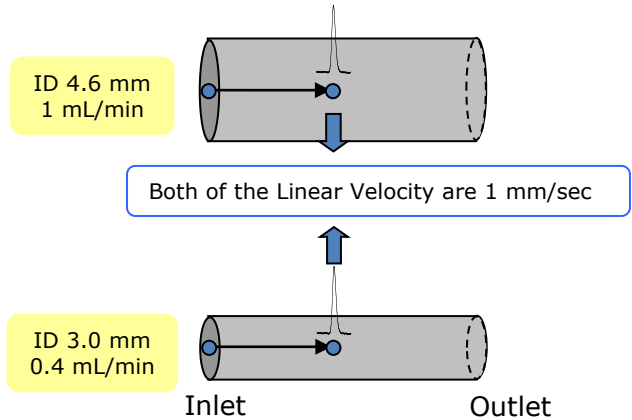
However, caution needs to be taken when scaling down the column. The following adjustments are required to maximize the column performance.

(8-1) Adjust the Flow Rate (Linear Velocity)

To maintain equivalent separation and retention times when transferring a method it is important to keep the linear velocity constant between the original and new method.

The linear velocity is related to the flow rate and internal diameter of the column.

$$\text{Linear Velocity (mm/s)} = \frac{\text{Flow Rate (mm}^3\text{/s)}}{\text{Column Cross-Sectional Area (mm}^2\text{)}}$$



HPLC Conditions

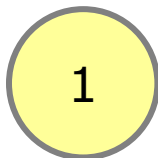
System : GL7700 HPLC
 Column : InertSustainSwift C18
 (3 μ m, 150 mm)
 Eluent : A) CH₃CN
 B) H₂O
 A/B = 65/35, v/v
 Column Temp. : 40 °C
 Detection : UV 254 nm
 Injection Vol. : 2.0 μ L

1. Acetophenone
2. Benzene
3. Toluene
4. Naphthalene

ID 4.6 mm
Flow Rate 1.0 mL/min

ID 3.0 mm
Flow Rate 0.4 mL/min

60 % Less Solvent Consumption

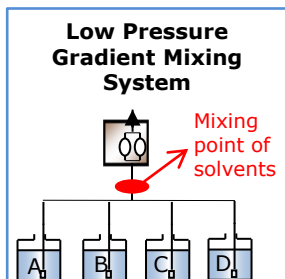


Ratio of Column
Cross-Sectional
Area



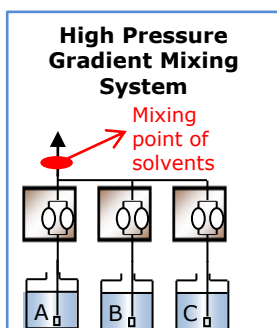
(8-2) Gradient Delay

A low pressure gradient mixing system (quaternary pump) has one pump, which is used to deliver the mobile phase to the system. Caution needs to be taken when scaling down the column internal diameter size.



The point where mixing of the solvents begins is right before the pump unit. Once the solvents are delivered to the pump, the plunger draws the solvent into the pump head(s) and creates a turbulent environment where the mobile phase mixes together.

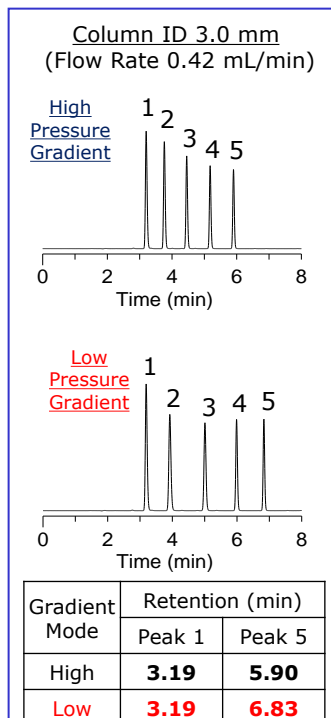
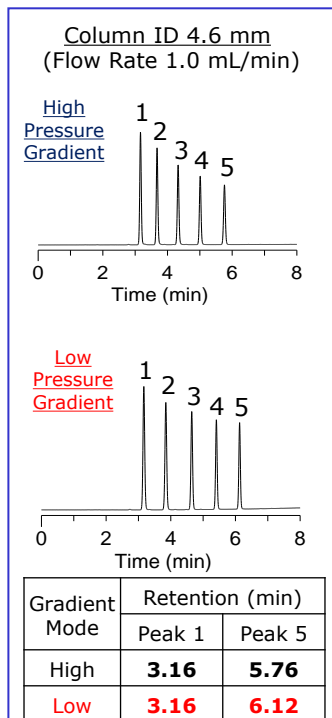
Because the mobile phase is not under pressure at the mixing point of solvents, quaternary pumps are typically considered low pressure gradient mixing system.



In a high pressure gradient mixing system, we can prepare one solvent per pump, with each pump providing flow for a specific solvent. The solvents are then combined in a mixing chamber that is located after the pumps. This creates a high pressure proportioning environment because the solvents are already under pressure before they reach the mixing point where the mixing occurs.

● Variation in Gradient Delay Volume between Low/High Pressure Gradient Systems

The biggest impact these two mixing modes have on chromatography is on the gradient delay volume, specifically on the mixer and tubing volume. Gradient delay volume is measured from where the mobile phase begins mixing to where it reaches the column. Generally, low pressure gradient system shows larger gradient delay volume as the mixing of the mobile phase begins is right before the pump unit. As shown below, the gradient delay can be observed more on the low pressure gradient system when using narrower ID size at low flow rates.



HPLC Conditions

Column : InertSustainSwift C18
 Standard : 3 μ m, 150 x 4.6 mm I.D.
 Semi-micro : 3 μ m, 150 x 3.0 mm I.D.
 Eluent : A) CH₃CN
 B) H₂O
 A/B = 50/50 - 6min - 100/0, v/v

Column Temp. : 40 °C
 Detection : PDA 270 nm
 Injection Volume : 0.5 μ L
 Mixer Volume : The volume of solvent delivered in 1 minute
 Standard approx. 1.0 mL
 Semi-micro approx. 0.4 mL

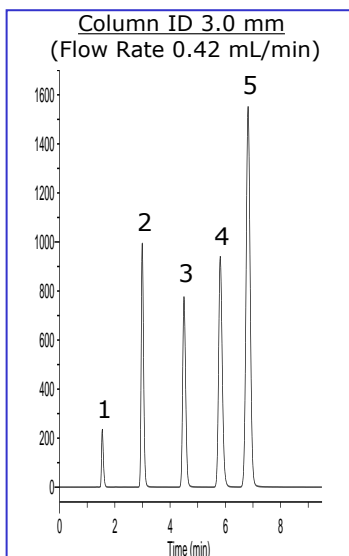
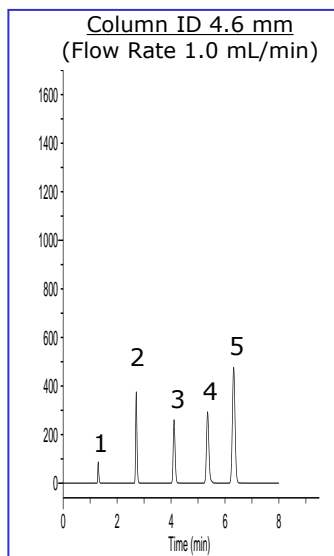
Sample:

1. 4-Methylphenol
2. 4-Ethylphenol
3. 4-Propylphenol
4. 4-Butylphenol
5. 4-Pentylphenol

(8-3) Adjusting the Sample Injection Volume

It is recommended to adjust the sample injection volume when scaling down the column to a narrower internal diameter size.

- When the injection volume is the same, the sensitivity will increase when using a concentration-dependent UV detector.



HPLC Conditions

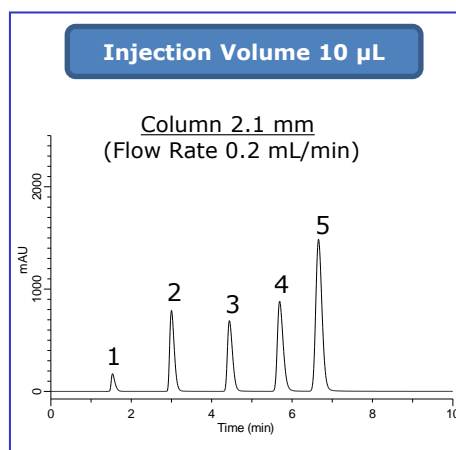
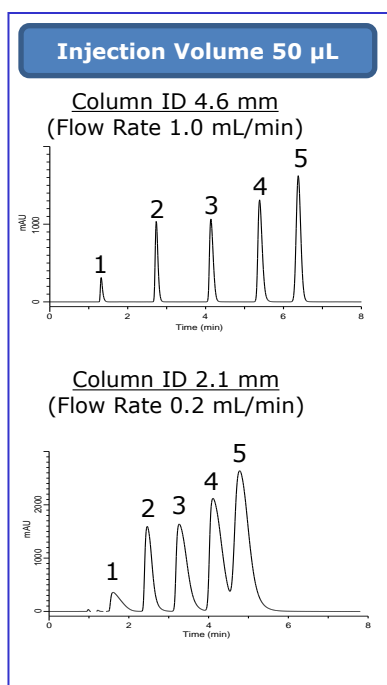
Column : InertSustain C18
 Standard : 3 μ m, 150 x 4.6 mm I.D.
 Semi-micro : 3 μ m, 150 x 3.0 mm I.D.
 Eluent : A) CH₃CN
 B) H₂O
 A/B = 65/35, v/v

Column Temp. : 40 °C
 Detection : UV 254 nm
 Injection Volume : 10 μ L

Sample:

1. Uracil
2. Acetophenone
3. Benzene
4. Toluene
5. Naphthalene

- When scaling down the column to a narrower internal diameter size, but with the same column length, the sample loading volume is generally proportional to the column's cross-sectional area. Therefore, deteriorated peak shapes may be observed when the polarity of mobile phase and that of injection solvent are significantly different. In such case, the injection volume should be reduced proportionally to the flow rate or the sample solution should be diluted with the mobile phase.



(8-4) HPLC Tubing

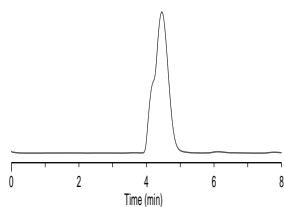
It is necessary to select the appropriate tubing in your HPLC system depending on the internal diameter size of an analytical column and flow rate of the mobile phase.

Extra-column volume and internal diameter of the tubing connecting the column outlet and detector inlet can greatly impact instrument band spreading, which will effect the peak shape and efficiency. It is recommended to decrease the internal diameter of tubing as it can decrease band spreading. Make sure not to select the internal diameter of the tubing too narrow as it can generate high back pressure.

● Effect of Extra-Column Volume on Chromatographic Separation

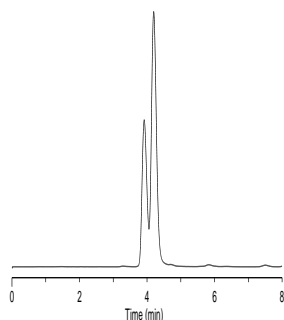
As shown below, larger extra-column volume will negatively impact the chromatographic separation. Caution needs to be taken especially when using semi-micro HPLC systems.

Tubing ID [0.50 mm](#)
Tubing Length 0.4 m



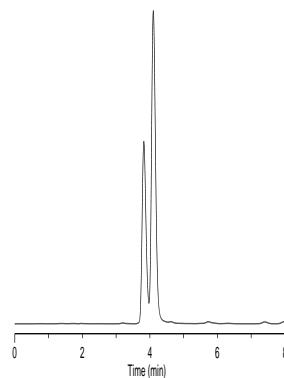
Rs NA
Efficiency NA

Tubing ID [0.25 mm](#)
Tubing Length 0.4 m



Rs 1.055
Efficiency 3,908

Tubing ID [0.13 mm](#)
Tubing Length 0.4 m



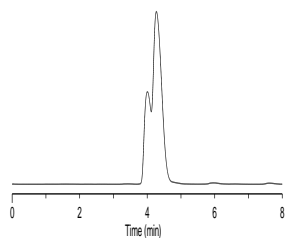
Rs 1.302
Efficiency 5,673

HPLC Conditions

Column : InertSustain C18 HP (3 μ m, 150 \times 2.1 mm I.D.)
Eluent : CH₃CN / H₂O = 50/50, v/v
Flow Rate : 0.2 mL/min
Column Temperature : 40 $^{\circ}$ C
Detection : UV 254 nm
Sample : o, p-Cresol

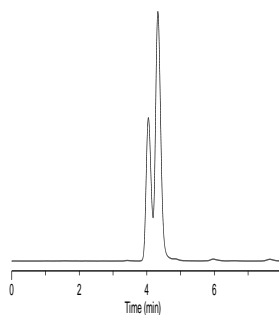
As shown below, the usage of narrower internal diameter tubing delivers better peaks even under the same tubing volume.

Tubing ID [0.50 mm](#)
Tubing Length 0.2 m
Tubing Volume **39.25 μ L**



Rs NA
Efficiency NA

Tubing ID [0.25 mm](#)
Tubing Length 0.8 m
Tubing Volume **39.25 μ L**

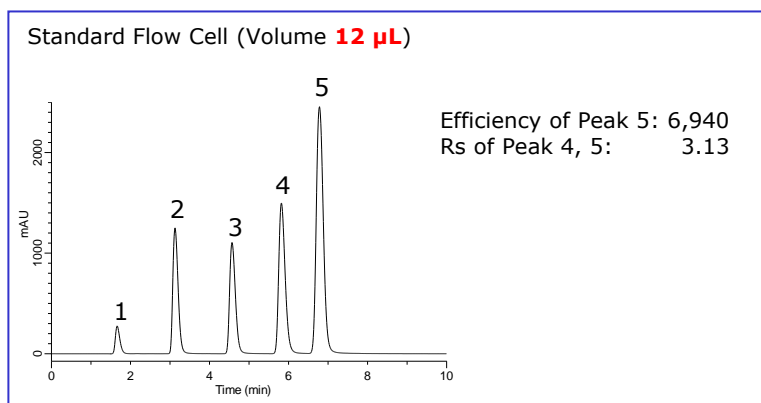


Rs 1.009
Efficiency 3,765

(8-5) Detector Flow Cell Volume

When scaling down the column to a narrower internal diameter size, the sample diffusion occurring not only at the tubing and the column, but at the detector flow cell can also influence chromatographic results. When using narrower internal diameter columns, it is important to use smaller volume flow cells.

● Comparison of Chromatographic Results using different Volumes of Detector Flow Cell (Column ID 2.1 mm, Flow Rate at 0.2 mL/min)

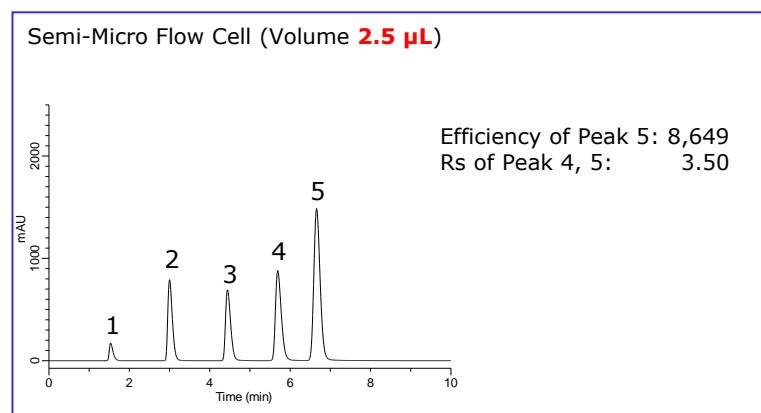


HPLC Conditions

Column : InertSustain C18
(2 μ m, 150 x 2.1 mm I.D.)
Eluent : A) CH₃CN
B) H₂O
A/B = 65/35, v/v
Column Temp. : 40 °C
Detection : PDA, 254 nm
Injection Volume : 10 μ L

Sample:

1. Uracil
2. Acetophenone
3. Benzene
4. Toluene
5. Naphthalene



If the structure (e.g., optical path length) of the flow cell is the same, lower volume flow cell will deliver better resolution, however, the sensitivity will decrease.

● Comparison of Efficiency between Column Internal Diameter Size and Flow Cell Volume

| Column ID (mm) | Standard Flow Cell | Semi-Micro Flow Cell |
|----------------|--------------------|----------------------|
| 2.1 | 6,940 | 8,649 |
| 3.0 | 10,137 | 10,713 |
| 4.6 | 19,231 | 19,195 |

* The HPLC system was optimized to be used for a 4.6 mm ID analytical column.

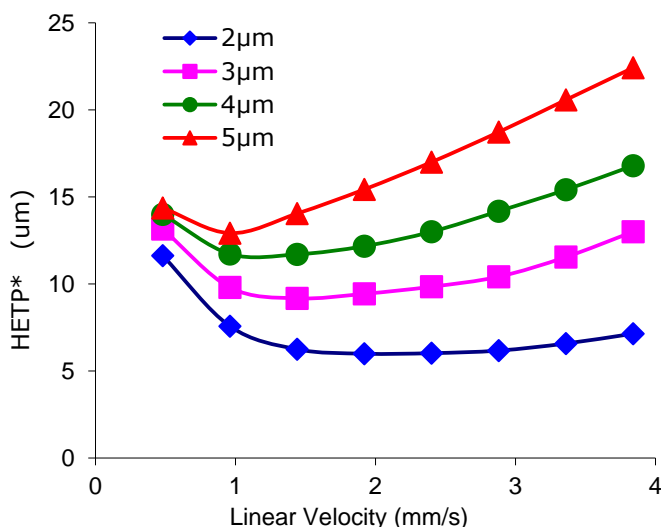
* The column length was 150 mm.

* The efficiency was determined by using Naphthalene under a specific analytical condition.

(9) Tips on Maximizing Efficiency on UHPLC Columns

In HPLC, if the particle size of the packing material is decreased, separation efficiency increases. As shown in the following van Deemter plot, smaller particles provide lower HETP values and these are achieved at a higher linear velocity, which a high throughput analysis can be operated by using a higher flow rate.

However, some optimizations (e.g., tubing volume, data sampling rate) are required to fully maximize the performance of UHPLC columns. Poor peak shapes or resolution may be observed when using and connecting to unoptimized LC systems.



* HETP : HETP is an acronym for the Height Equivalent to the Theoretical Plate. It arises from the Plate Theory and is numerically equal to the column length divided by efficiency (the number of theoretical plates) of the column.

(9-1) Reduce Extra-Column Volume

● Tubing Volume

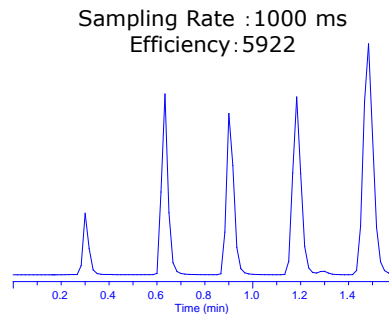
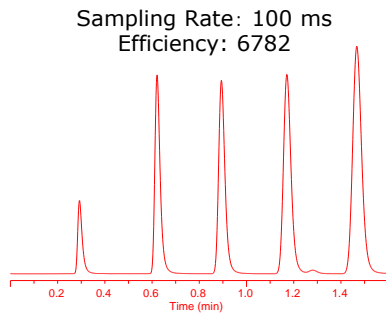
Select the appropriate tubing in your HPLC system depending on the internal diameter size of an analytical column and flow rate of the mobile phase. For more details, please see section (8-4).

● Detector Flow Cell Volume

The sample diffusion occurring at the detector flow cell can influence chromatographic results. For more details, please see section (8-5).

(9-2) Data Sampling/Acquisition Rate

The data sampling rate determines how often a detector-signal data point is recorded to construct the chromatogram. Generally, each peak should ideally be defined by at least 20 data points. Insufficient data points may cause less separation efficiency or irreproducible quantitation results. When using UHPLC columns, the sampling rate should be set less than 100 ms.



Enlarged view
from 0 to 60
seconds

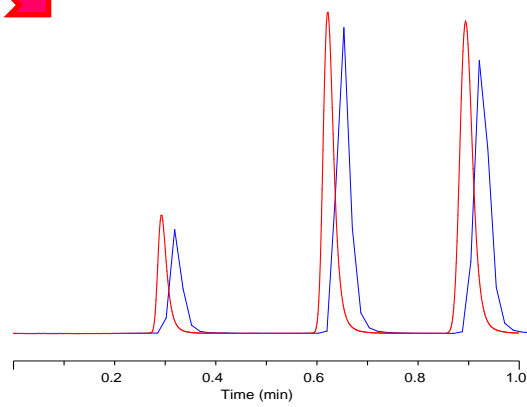


HPLC Conditions

Column : InertSustain AQ-C18
(1.9 μ m, 50 x 2.1 mm I.D.)
Eluent : A) CH₃CN
B) H₂O
A/B = 65/35, v/v
Column Temp. : 40 °C
Detection : UV, 254 nm (Response: 0.1 sec)
Injection Volume : 0.2 μ L

Sample:

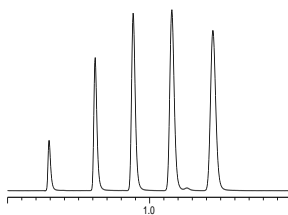
1. Uracil
2. Acetophenone
3. Benzene
4. Toluene
5. Naphthalene



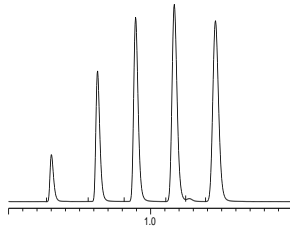
(9-3) Response Time/Time-Constant

Response time describes how fast the detector signal follows a sudden change of absorbance in the flow cell. To achieve an optimal signal-to-noise ratio, the response time should be set three times higher than the period of baseline noise. However, higher response time can produce shorten and broaden peaks. As the peak widths are very narrow when using UHPLC columns, the response time should be adjusted lower.

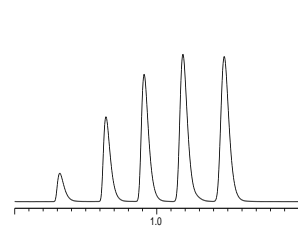
Response Time: 0.05 sec
Efficiency: 7137



Response Time: 0.5 sec
Efficiency: 5879



Response Time: 1.5 sec
Efficiency: 2993



HPLC Conditions

Column : InertSustain AQ-C18
(1.9 μ m, 50 x 2.1 mm I.D.)
Eluent : A) CH₃CN
B) H₂O
A/B = 65/35, v/v
Column Temp. : 40 °C
Detection : UV, 254 nm (Sampling Rate: 100 ms)
Injection Volume : 0.2 μ L

Sample:

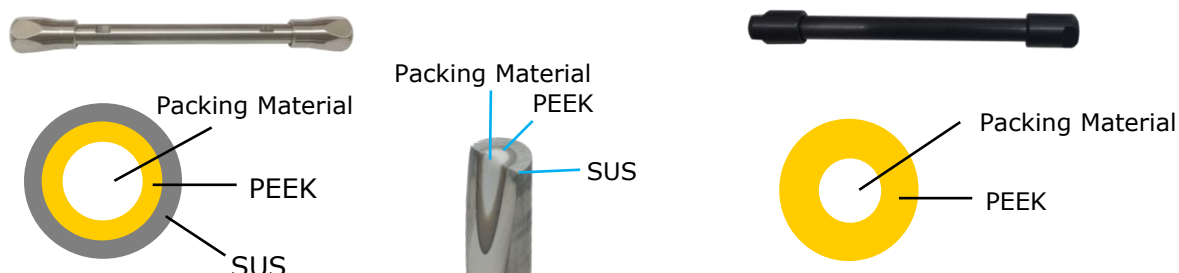
1. Uracil
2. Acetophenone
3. Benzene
4. Toluene
5. Naphthalene

(10) Benefits of Metal-Free PEEK Columns

(10-1) Benefits of Metal-Free PEEK Columns

Analytes containing phosphate groups creates the formation of phosphate-iron complexes, which often lead to deteriorated peak shape or poor precision of quantitative result. In the past, the residual metallic impurities in the packing material were assumed to be the cause of such problem. However, the influence of the material of the column hardware also provides a big impact to such trace analytes in high sensitivity analysis. Some improvement may be observed when using mobile phases containing phosphate buffer or EDTA, however, these are not compatible with LC/MS(/MS) applications.

The metal-free PEEK columns, including all wetted parts are made of polyetherether ketone (PEEK), are available to improve chromatographic results with better peak shape and S/N for bio-chromatographic applications.

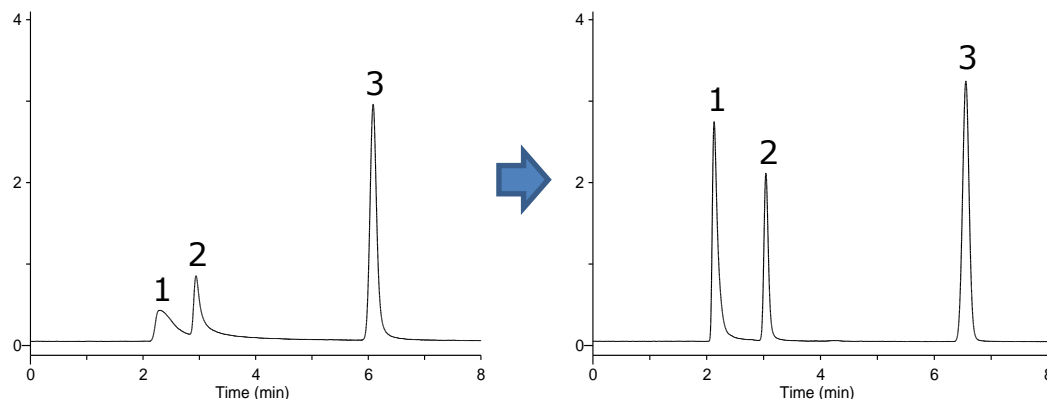


UHPLC-PEEK Columns
(Available for 3 and Sub-2 μm particles)

PEEK Columns
(Available for 4 to 10 μm particles)

● Comparison of Chromatographic Results between SUS and Metal-Free PEEK Columns

Analytes such as ATP contains phosphate groups, are highly chelating compound. As shown below, Metal-Free PEEK column delivers improved peak shape and S/N compared to a SUS hardware column.



SUS Hardware Column

Metal-Free PEEK Column

1. ATP
 2. ADP
 3. AMP
- 500 $\mu\text{g/L}$ each

HPLC Conditions

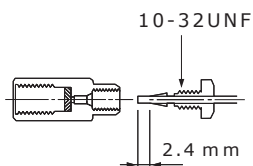
Column : InertSustain AQ-C18
(3 μm , 150 x 3.0 mm I.D.)
Eluent : 5 mM HCOONH_4 in H_2O
Column Temp. : 40 $^\circ\text{C}$
Detection : UV, 260 nm
Injection Volume : 10 μL

(10-2) Handling of Metal-Free PEEK Columns

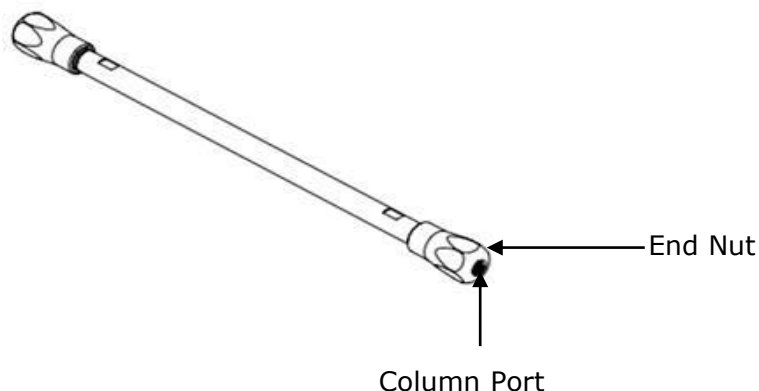
Please note the handling of these PEEK columns are different from those traditional metal stainless steel hardware columns. Please read the following before use.

- All the wetted parts are made of PEEK in UHPLC PEEK / PEEK columns. The structure of the Column Port is especially delicate. Make sure not to over tighten the end-fittings. Over-tightening the end-fittings will cause damage to the connection parts.

- End-fittings are 1/16" Parker style, UP type.



- The connection of PEEK columns can be done with less strength/force when comparing from those traditional metal stainless steel hardware columns. Gently connect the PEEK columns to the tubing and check if there are no leakage. The tightening strength/force of the torque is approximately 0.8 N·m.
- Never use worn fittings. The usage of worn fittings will cause damage to the Column Port. Make sure to always use new fittings.
- As shown below, when installing or removing the column, hold the end-fitting and end nut to tighten or loosen the connection.



- When storing the column, use the provided column plugs and seal the column. Make sure not to over tighten the column plugs when sealing the column. Over-tightening the column plugs will cause damage to the connection parts.
- PEEK generally shows excellent solvent resistance to a wide range of organic solvents. However, the use of THF or chloroform damage the surface of PEEK and cause irreparable damage. Avoid the use of THF and chloroforms.

(11) Tips on Maximizing Purification Efficiency on Preparative Columns

The chemical properties of packing materials used for preparative separations must be chosen not only to achieve the selectivity necessary for optimal separations but also the loadability that enables maximum throughput. In this section, tips on considering the sample loadability and selection of appropriate column dimensions are described.

(11-1) Sample Loadability

In preparative HPLC, the goal is to isolate and purify the target analyte. The peak shape of target analyte may not look sharp, however, the resolution needs only to be maintained between the peak of interest and the nearest peaks.

●Step ① : Evaluate the Sample Loadability

- Prepare a sample solution as concentrated as possible.



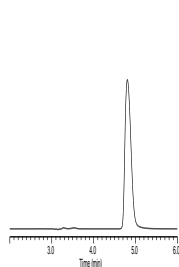
- Inject the sample solution with small increments of injection volumes to evaluate the sample loadability.

(To efficiently evaluate the sample loadability, use various injection volumes of 2 to 10 folds)

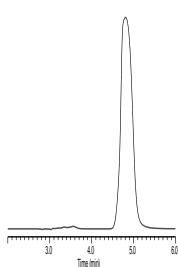


- Once the injection volume increases, the peak shape will start deteriorating. The injection volume where the peak shape deteriorated should be considered as the maximum sample loadability.

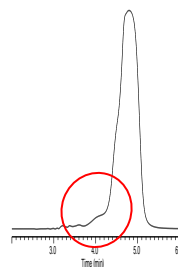
Injection Vol.: 10 μ L
(Loadability 1.0 mg)



Injection Vol.: 50 μ L
(Loadability 5.0 mg)



Injection Vol.: 100 μ L
(Loadability 10 mg)



Injection Vol.: 250 μ L
(Loadability 25 mg)



Column : Inertsil WP300 C18 (5 μ m, 250 x 4.6 mm I.D.)
Eluent : CH₃OH / H₂O = 90/10, v/v
Flow Rat : 1.0 mL/min
Column Temp. : 40 °C
Detection : UV 270 nm
Sample : *tert*-Butylbenzene (Approx. 10 %, Diluent: Eluent)

As shown above, the peak shape started to be deteriorated when injecting 100 μ L. The peak shape was completely deteriorated and split into two peaks when injecting 250 μ L. For this particular method and under this condition, we can determine the maximum sample loadability is approximately **100 μ L (10 mg)**.

● Step ② : Optimize Separation between the Target Analyte and nearest peaks

- Prepare a sample solution as concentrated as possible.

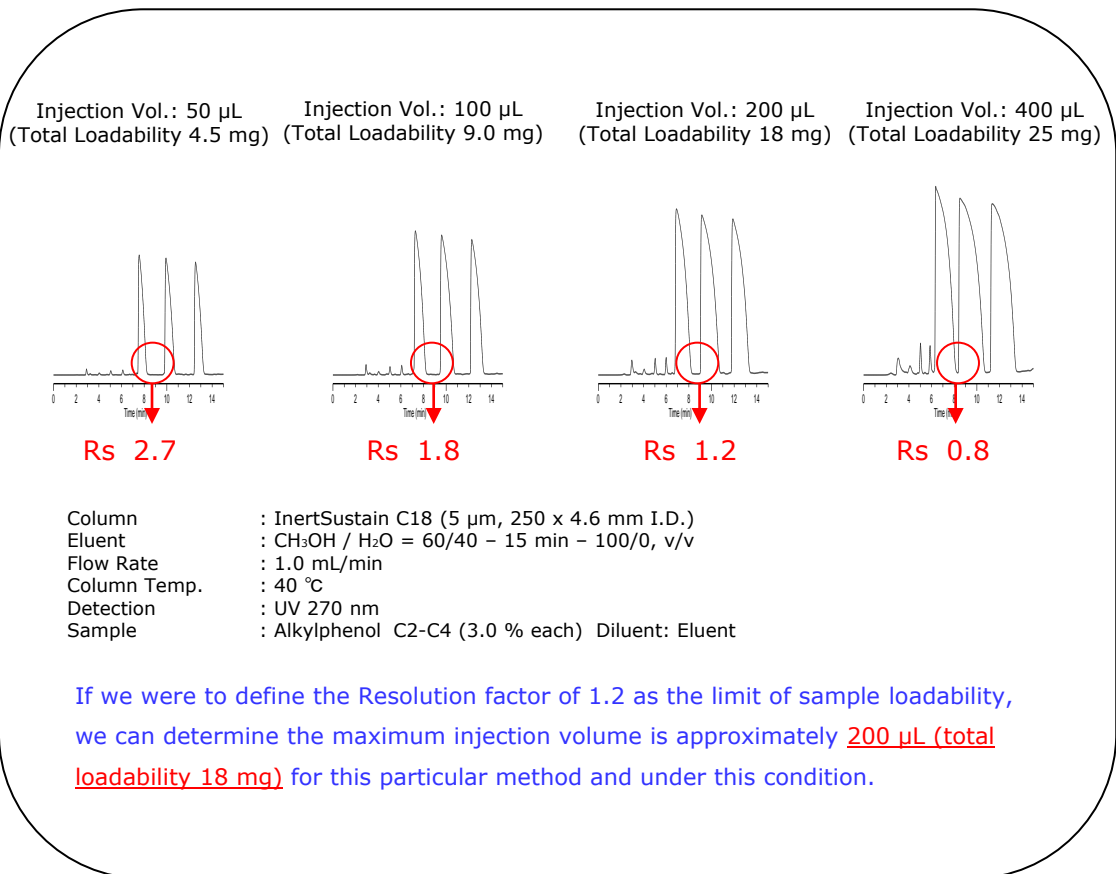


- Inject the sample solution with small increments of injection volumes to evaluate the resolution.

(To efficiently evaluate the resolution, use various injection volumes of 2 to 10 folds)



- Once the injection volume increases, the resolution is sacrificed as the column is in an overload situation. Resolution factor larger than 1.5 is referred as complete baseline separation between two neighboring peaks. Depending on the purification criteria, Resolution factor around 1.2 may be acceptable.



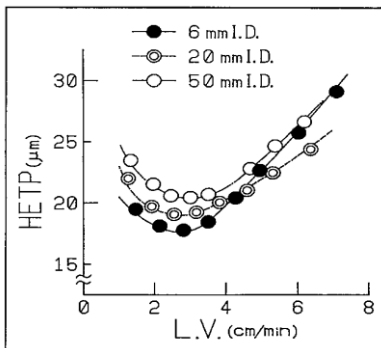
(11-2) Scale-Up

In the scale-up approach, a method developed for analytical purposes is directly applied to larger internal diameter columns. The most important part is to understand the ratio of cross-sectional area between the analytical and preparative column.

●Step ① : The flow Rate is Proportional to the Cross Sectional Area of the Column

The following HETP was plotted using three columns packed with 10 μm packing material having different internal diameters. Lower the HETP, higher the efficiency.

Regardless of the column internal diameter size, the optimal linear velocity is at 3.0 cm/min (0.5 mm/sec). As a conclusion, regardless of the column internal diameter size, when using a column packed with a 10 μm particle size with the same bonded phase, the flow rate should be adjusted to meet the linear velocity at 3.0 cm/min to maximize the efficiency of the column. The linear velocity of the mobile phase is determined by dividing the mobile phase flow rate by the cross-sectional area of the column. When changing the column to a different size, the linear velocity should be kept constant. To keep linear velocity constant, the flow rate should be adjusted in proportion to the column cross-sectional area, which is directly proportional to the square of the ratio of column diameters.



* The optimal linear velocity is dependent on the particle size and separation mode. Before scaling-up to a larger internal diameter column, make sure to use the same bonded phase and particle size in both analytical and preparative scale.

●Step ②: The Sample Loadability is Proportional to the Cross Sectional Area of the Column

The following was plotted using three columns having different internal diameters, which demonstrates the relationship between sample loadability and efficiency. For example, the sample loadability of each column are as follows when the efficiency is 2000.

ID 6 mm : Approx. 1 mg
 ID 20 mm : Approx. 10 mg
 ID 50 mm : Approx. 70 mg

As a conclusion, the injection volume should also be adjusted in proportion to the column cross-sectional area to achieve equivalent separation efficiency.

