YMC GENERAL CATALOG

# 13

# Information

# Column handling

## 1 Introduction

YMC Co., Ltd. produces HPLC columns under strict quality control and delivers to customers only those products that pass the column tests prior to shipment. In order to ensure peak functionality and long life of the column, please follow these instructions.

## 2 Shipping solvent

The solvent enclosed at shipment is described in the COLUMN INSPECTION REPORT or instruction manual included with each column. Please pay attention to the miscibility of the solvent with the mobile phase used in your analysis. If you intend to store the column for a long time, replace the mobile phase in the column with the shipping solvent or solvent specified in the Column Inspection Report.

## 3 Mobile phase

- Reversed-phase columns can be used with both aqueous and non-aqueous solvents. However, repeated alternate use of solvents with extremely different polarity may diminish column performance. Usable general organic solvents include acetonitrile, methanol and THF.
- CN columns can be used in both normal-phase and reversed-phase modes. However, a column should be dedicated for each separation mode. When using the column in a normal-phase mode, replace the solvent in the column with 2-propanol . (Be sure to set the flow rate so that the pressure is at 15 MPa or less.)
- SIL columns are usually used with non-aqueous solvents. Use *n*-hexane, chloroform or other weak solvents and add isopropanol and ethyl acetate or similar as appropriate to speed up elution of high-polarity components.
- Amino columns (Polyamine II and $NH_2$) can be used with both aqueous and non-aqueous solvents. However, repeated alternate use of solvents with extremely different polarity may diminish column performance.
- Solvent should flow in the direction of the arrow as indicated on the column label.
- The usable pH ranges of columns vary by product. See the instruction manual included with each column. If the instruction manual is not available, please contact us.

## 4 Mobile phase replacement and column cleaning (general methods)

**(A) Reversed-phase columns**

- When a mobile phase containing neither buffer nor salts is used, wash the column with a solution having the same solvents as that of the mobile phase, but with a higher organic solvent concentration.
- When a mobile phase containing buffer or salts is used, replace it with a mixture of water and organic solvent (at the same ratio as the mobile phase) containing neither buffer nor salts.
  When a mobile phase containing buffer or salts is used at about 50 mM, it can be replaced directly with about 60% acetonitrile aqueous solution.
- After using a column near the usable pH limit, washing the column with water alone may cause column deterioration. Use the mixture of water and organic solvent described above or about 60% acetonitrile aqueous solution to replace the mobile phase.
- When column pressure increases, flow the solution through the column in the reverse direction (the opposite direction of the arrow shown on the column label). A solution having the same composition as that of the mobile phase, but with a higher organic solvent concentration is usually used as the cleaning solution, but consideration should be given with respect to the sample characteristics to select a solvent which easily dissolves the sample.
- When macromolecules, including proteins and sugars, adsorb to the column, it is usually difficult to wash out the macromolecules with organic solvents. When a sample containing macromolecules is analyzed, it is preferable to conduct pretreatment and/or use a guard column.

**(B) Normal-phase columns**

- Wash the column with a solution having the same solvents as that of the mobile phase, but with a high polar component concentration that is higher than that of the mobile phase. If polar compounds absorb to the column, flush with isopropanol or similar solvent.
- When a mobile phase containing acid or alkali is used, replace it with a non-acid, non-alkali solvent before storage.
  (eg., *n*-hexane/2-propanol/acetic acid (90/10/0.1)
  → *n*-hexane/2-propanol (90/10))

## 5 Guard columns

- Use a guard column with the same packing material and the same or smaller inner diameter as that of the main column.
- A YMC guard cartridge is composed of a cartridge holder and a guard cartridge column. The cartridge holder can be used repeatedly. A guard cartridge with an inner diameter of 4.0 mm can be used with analytical columns with inner diameters of 4.0 to 6.0 mm.
- For an analytical column for which both a guard cartridge and a packed column-type guard column are available, select the guard cartridge or the packed column-type guard column according to the expected replacement frequency of the guard column. We recommend a guard cartridge if samples containing a lot of impurities require the guard column to be replaced frequently.

## 6 Other environments

- The upper limit of column pressure is about 20 MPa for a column with a length of 150 mm or less and 25 MPa for a column with a length of 250 mm. Note that the upper limit of column pressure for a column with an inner diameter of 10 mm or more is about 10 MPa.
- Repeated injection of samples may cause the column pressure to increase. Filter samples through a YMC Duo-Filter (0.2 µm) before injection. Use a precolumn filter for a sample that may clog the frit of the column.
- The upper limit of column temperature for reversed-phase columns and SIL columns is 50℃. However, we recommend using the columns between 20℃ and 40℃ because some conditions, such as pH of the mobile phase, may affect the column life. For recommended column temperatures for column types other than reversed-phase and SIL columns, see the instruction manual included with each column.

# Mobile phases for ODS columns

**The composition of mobile phase greatly affects the separation in HPLC. To optimize a separation, it is necessary to consider the interaction of the solutes, stationary (or solid) phase, as well as the mobile phase.**
**For ODS columns, commonly used in HPLC, various mobile phases are available. Attention needs to be paid to many points when deciding on the mobile phase composition. The variables include the following factors: miscibility of solvents, effects on detection methods (eg., UV or MS), effects on the column (eg., column deterioration due to pressure or pH), separation reproducibility and the stability of solutes. Useful solvents for ODS columns and some helpful tips for establishing optimum separation conditions are described below.**

## General solvents

Water, acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF) are important solvents for use with ODS columns.
It is important to use high purity water purified by ion-exchange, distillation, reverse osmosis, or the like. The presence of organic substances or ionic impurities may cause problems, including ghost peaks during short wavelength UV detection.
ACN is frequently used as an HPLC solvent, due to its low UV absorption and low viscosity. MeOH has a higher viscosity and often shows different separation selectivity from that of ACN. THF is sometimes used to influence selectivity along with ACN and MeOH. The cyclic ether structure of THF may result in different separation characteristics from those of ACN and MeOH. Be aware that THF has significant UV absorption and take notice of its viscosity and peroxides. Usually, THF containing no antioxidants is used for HPLC.
Find the appropriate separating conditions by using these three types of organic solvents individually or in combination.

## Buffers and reagents

Acetic acid, phosphoric acid and trifluoroacetic acid (TFA) are generally used as acidic modifiers. The buffers normally used include phosphate buffer (sodium, potassium, ammonium) and acetate buffer. Monobasic phosphates yield about pH4.6 and are used as convenient pH adjusters by themselves.
In order to separate an ionic compound, such as amine and carboxylic acid, with good repeatability, the pH of mobile phase must be controlled. It is desirable to adjust the pH of the mobile phase so that it is one or more units away from the pKa of the solute. Near the pKa, peak broadening or splitting may be observed.
These buffers are usually used at a concentration of about 10 mM. However, depending on dissociation of solutes and interaction with the stationary phase, these buffers may be used at 50 to 100 mM. When acids or alkalis that are likely to deteriorate ODS are used, caution must be taken regarding their concentrations and pH. TFA and phosphoric acid are usually used at concentrations of 0.1% or less.
Approximately 60% acetonitrile aqueous solution is a convenient solvent for storage after the use of acids or buffers (salts).
Tetrabutylammonium salts and sodium perchloric acid may be used as ion pair reagents for retention of highly polar compounds on ODS or for improvement of separation and peak shape. When these additives are used, it is necessary to use a reagent with the shortest alkyl chains available. If sodium dodecylsulfate, (SDS;which contains long alkylchains) is used, the SDS is likely to be retained on ODS and sometimes cause problems with repeatability.

## Others

Ethanol, 2-propanol, ethyl acetate, or chloroform may be added to the mobile phase in order to improve retention or separation of solutes. In some cases, hexane is used as a mobile phase. When a hydrophobic solvent is used as a mobile phase, caution is required regarding the miscibility of the solvent with the mobile phase in the column.

# HPLC Column Performance

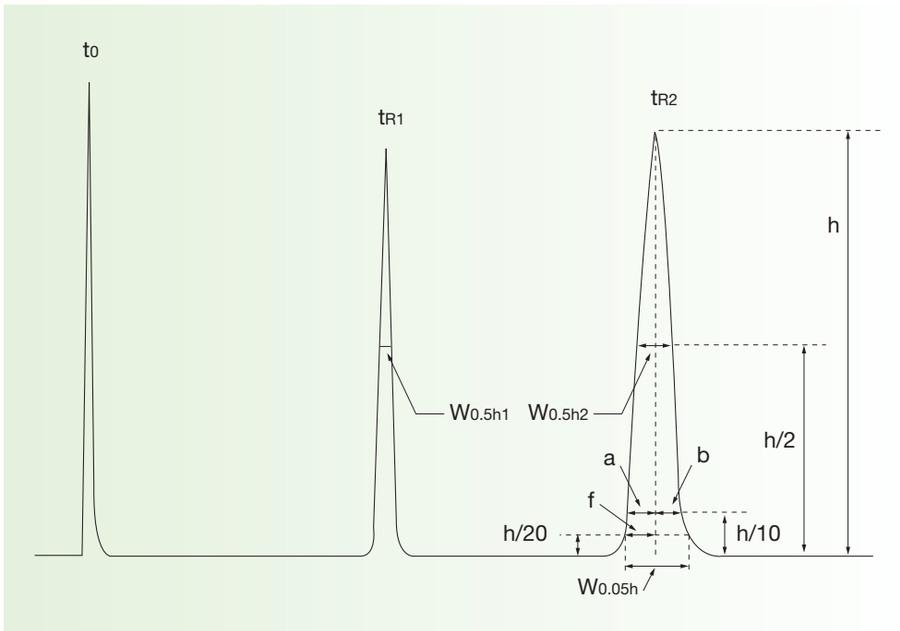
**Important factors used in the evaluation of column performance include column efficiency, capacity, separation characteristics of solutes, peak shape, and column pressure. Elution curve management in partition chromatography, including ODS, at YMC Co., Ltd. is described below.**

Column efficiency, an important characteristic for evaluation of column performance, is generally measured in terms of theoretical plate number. Usually, the theoretical plate number is calculated using bandwidth at half-height. Narrower peak widths result in higher theoretical plate numbers. Longer columns and smaller packing material particle size tend to result in higher theoretical plate numbers. Due to a variety of factors, one column does not always show the same theoretical plate number. This may be caused by differences between linear velocity and solute diffusion in the column or because of interaction between solutes and the mobile phase or the stationary phase.

For these and other reasons, column efficiency is solute specific and the measurment of efficiency must be conducted under nearly identical HPLC conditions for results to be directly comparable.

Retention and separation characteristics for solutes on the column are evaluated by the capacity factor and separation factor values. These values are indexes of the packing material characteristics and, in contrast to the retention time, do not depend on column inner diameter and length.

Elution peak shape is also an important factor for evaluation of column performance. The asymmetry factor is a relatively simple evaluation measure, generally calculated at 10% of peak height.




| | | |
|---|---|---|
| $t_0$ | Void volume, Column dead-time | |
| $t_R$ | Retention time | |
| h | Peak height | |
| $W_{0.5h}$ | Bandwidth at half-height | |
| N | Theoretical plate number | $N=5.54\times(t_R/W_{0.5h})^2$ |
| k' | Capacity factor | $k'=(t_R-t_0)/t_0$ |
| $\alpha$ | Separation factor | $\alpha=k'_2/k'_1$ |
| $R_S$ | Resolution | $Rs=1.18\times(t_{R2}-t_{R1})/(W_{0.5h1}+W_{0.5h2})$ |
| $A_S$ | Asymmetry factor | $As=b/a$ |
| Tf | Tailing factor | $Tf=W_{0.05h}/2f$ |

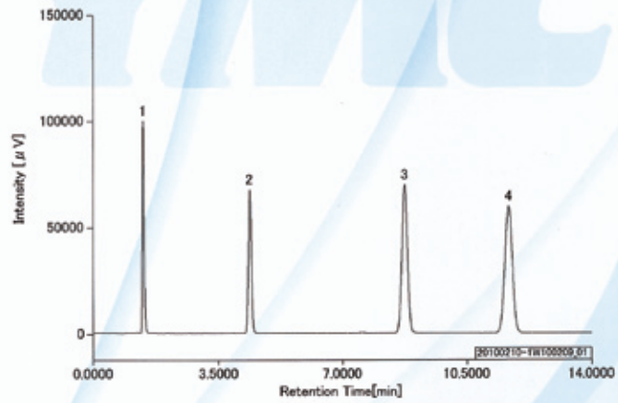
# Inspection reports

YMC Co., Ltd. conducts quality control of packing materials to ensure lot-to-lot and column-to-column reproducibility. YMC Co., Ltd. tests in principle the performance of all columns and delivers only those products that meet strict specifications.
A test report of the kind that is shipped with each column is shown below.
The analysis conducted at the delivery inspection is not only a method for column performance evaluation but also a useful test method for determination of column life. YMC Co., Ltd. describes all analytical conditions in the test reports of main products, including sample concentration, for convenience in evaluation of column deterioration by the end user.

## YMC HPLC COLUMN INSPECTION REPORT

**Serial No.:**

| | |
|---|---|
| Product code: | TA12S05-1546WT |
| Name, Particle: | YMC-Triart C18/ S-5 um/ 12 nm |
| Gel lot: | 9402 |
| Column size: | 150 × 4.6 mm I.D. |
| Eluent: | acetonitrile/water (60/40) |
| Flow rate: | 1 mL/min |
| Temperature: | ambient |
| Detection: | UV at 270 nm |
| Injection volume: | 5 uL |
| Pressure: | 4.4 MPa |
| Shipping solvent: | acetonitrile |




| Sample Components | Retention time [min] | Capacity factor [k'=(tRn-tR1)/tR1] | Theoretical plates [5.54×(tR/W0.5)^2] | USP Tailing factor [TF=W0.05/2f] |
|---|---|---|---|---|
| 1. Uracil (0.01 mg/ml) | 1.39 | 0.00 | 4025 | 1.37 |
| 2. Methyl benzoate (0.1 ul/ml) | 4.37 | 2.16 | 12948 | 1.07 |
| 3. Naphthalene (0.04 mg/ml) | 8.75 | 5.32 | 14412 | 1.02 |
| 4. Butyl benzoate (0.3 ul/ml) | 11.66 | 7.42 | 14298 | 1.01 |

[System No.104] [Inspected by T.OKADA]

YMC Co., Ltd.

# FAQ

## Q What are the features of YMC ODS Columns?

**A** We have 12 different kinds of ODS columns. They are divided into 3 groups; Silica-based columns, Polymer-based columns, such as YMC-Pack Polymer C18 and hybrid silica based, Triart series.
To break down further within the Silica-based columns, they are divided in 3 groups, *Pro* series, YMC-Pack series (excl. Polymer C18) and J'sphere series. *Pro* series features in a superior performance and excellent reproducibility. YMC-Pack ODS series and J'sphere series are conventional type columns. *Pro* series with an efficient endcapping technology is superior to YMC-Pack ODS series and J'sphere series. You can find more information on our column introduction page.

## Q How different are ODS-A, AM and AQ?

**A** ODS-A and AM are conventional ODS. ODS-AQ provides a lower rate of carbon content and is suitable for separation of hydrophilic compounds. ODS-A and -AM have the same basic physical properties such as the base material, the rate of carbon content, and the separation characteristics; however -AM is produced under more strict Standards of Quality Control.

## Q How different are YMC-Pack Polyamine II, PA-G and $NH_2$?

**A** Polyamine II and PA-G are chemically bonded with polyamine where $NH_2$ is with aminopropyl group. Polyamine II and PA-G are superior to $NH_2$ in durability. They also have difference in selectivity. Polyamine II and PA-G have different ligand structure of polyamine. Polyamine II is superior to PA-G in durability.

## Q What is "Endcapping"?

**A** General ODS(C18) packing material is a silica gel bonded with octadecyl groups. This is the result of a reaction between silanol groups and octadecyl groups on the silica surface. However, some silanol groups remain after the reaction. It is impossible for all the silanol groups to react because of steric hindrance of octadecyl groups. Such residual silanol groups create a secondary interaction in chromatography, which, in many cases, affects chromatograms by, in general, causing a peak tailing of basic compounds or irreversible absorption to the column. Therefore, a secondary silanization on residual silanol groups with unbulky silanization reagents should be performed. This process is called "endcapping". Trimethylsilane(TMS) is commonly used in "endcapping" process.

## Q Are there ODS columns used with 100% aqueous mobile phase?

**A** "Hydrosphere C18" and "ODS-AQ" columns can be used with 100% aqueous mobile phase. On conventional ODS columns, retention time is shortened due to the incompatibility between water and material surface with high hydrophobicity. Water tends to be expelled from the pores on material. The retention time hardly shortened on "Hydrosphere C18" and "ODS-AQ" because they are capable of solvation between mobile phase and hydroplilic surface by reducing the density of C18 functional groups.

## Q What is the upper limit of column pressure?

**A** Column length of 150mm or less: Approx. 20 MPa,
Column length of 250mm or less: Approx. 25 MPa
*Note: If the inner diameter is 10mm or more: Approx. 10 MPa

## Q What are the applicable pH range and temperature?

**A** **Applicable range of pH:**

| Column Type | pH range |
|---|---|
| Triart C18 : | 1 - 12 |
| *Pro* series ODS : | 2 - 8 |
| *Pro* C18 RS : | 1 - 10 |
| J'sphere ODS-H80 : | 1 - 9 |
| PolymerC18 : | 2 - 13 |
| Reversed-phase(Other than mentioned above) | |
| Normal phase(SIL,Polyamine II) : | 2.0 - 7.5 |

**Applicable temperature:**
upper limit:50℃
Recommendable range:20 - 40℃
*The given data is subject to change depends on product types. Those data should be confirmed with instruction manual when the column is used.

## Q How should we store the columns?

**A** When columns are not used for a long time, keep them in a cool place after replacing the shipping solvent as indicated in the attached inspection report. Do not keep the column in the mobile phase with salt or acid regardless of whether or not it is for a short period of time. Close the airtight stopper tightly to prevent the solvent from volatilizing.

## Q How can we evaluate the performance of columns?

**A** Perform an inspection test under the same conditions as the inspection report attached to the column at the time of purchase. Columns are evaluated to be effective and have no change in performance if the result indicates no irregularity in retention time, theoretical plate number, peak asymmetry, etc. Columns which indicate no irregularity in the said criteria after using several years from purchase, however, may have changes in separation characteristics for compounds such as ionic compounds.
It is advisable to avoid using them for method development. Reproducibility may not be obtained with new columns.

## Q What is the shipping solvent?

**A** Triart, J'sphere series: Acetonitrile (100)
*Pro* series: Acetonitrile/Water (60/40)
ODS-A, AM, AQ, etc.: Methanol/water (70/30)
* Indicated in the instruction manual.

## Q How do we clean the columns?

**A** 1. Remove highly hydrophobic absorbate.
Use solvent with a stronger elution ability than mobile phase. For example, cleaning reversed-phase columns, use solvent with increased ratio of organic modifier and flush the volume of 10 times as much as the column volume.
2. Renaturation of gel surface condition
Irregularities are observed in Peak asymmetry and retention time. In silica-based packing material, separation behavior may be affected by the conditions of residual silanol groups whether in dissociation or in non-dissociation. Washing with acidic solvents may be effective in such case. Washing with a mixed solvent of 0.1% aqueous phosphoric acid solution and organic solvent* can perform the renaturation of silanol groups to the dissociation state.
*Ratio of organic solvent: 10 to 60% .

## Q Do we need Guard columns?

**A** To analyze samples containing a lot of contaminants, guard columns are effective and can improve the durability of main columns. We recommend guard columns with the same packing materials as main columns. Guard columns with different material may cause defects in peak asymmetries and reproducibility. We have them in 2 types; conventional type and cartridge type. We recommend cartridge type if guard columns require a frequent replacement. Inner diameter should be the same as the main column or smaller.

## Q What is the "Connector type (W)"?

**A** "Connector type (W)" indicates Waters connectors. Most of the columns in the market are this type. Connectors, other than Waters, are Shimazu, JASCO, Hitachi, etc. Connectors differ in the length of tubing section coming out from the tip of ferrule. The connector types of column and tubing system should be the same, or the tubing and the column may fail to fit well and cause leakage and defects in peak asymmetry. If your system has something other than Waters, a connection adapter or a ferrule replacement may be required. * PEEK inch screw thread built-in ferrule will not have this problem.

## Q What is required in system and flow rate for using semi-micro columns?

**A** Flow rate on Semi-micro column (hereafter columns in 1.0 to 2.0 mm inner diameters will be mentioned as semi-micro columns) is 50 to 200 µL/min in general. It can be increased if the length of column is short and back pressure is low. Commonly used HPLC System is applicable, however, with pumps, flow cell of detectors and tubing system designed for semi-micro column is more suitable.

## Q How do I carry out a scale-up?

**A** ::Step 1::
Determine separation conditions by using analytical columns.

::Step 2::
Study the preparative scale. Set the particle size of the packing material and the inner diameter of the column in consideration of the sample volume.

::Step 3::
Optimize the separation conditions by using analytical columns with inner diameter of 4.6 mm or 6.0 mm packed with the selected packing material. If the particle size of the packing material is the same as in Step 1, this process can be omitted. If the preparative scale is more than 100 mm in inner diameter, another process using a column with a diameter of 20 mm will follow to determine the loadability and calculate the running cost.

::Step 4::
Proceed with the preparative separation.

## Q What should I do when the column pressure rises up?

**A** Following solutions are introduced depends on causes.

1. Frit clogging
   Flush the column in reverse flow according to "How do we clean the columns? 1. Remove high hydrophobic absorbate". Reduce the flow rate accordingly in order to keep the column pressure adequate when you flush the column.
2. Absorbate to packing material
   Wash the column in reverse flow under the method in "How do we clean the columns? 1. Remove high hydrophobic absorbate" when flashing the column.

* If pressure increase is observed often after both 1 and 2, pretreat a sample or use guard columns to prevent the problem.

## Q What are the solutions for poor peak shapes?

**A** Following solutions are introduced depends on causes.

1. Inappropriate Mobile phase
   In the case of ionic analyte if pKa of the analyte and pH of mobile phase are close, it causes defects in the peak shape.
   Set the pH of mobile phase distant from pKa.
2. Influence by dissolving solvent of sample
   If dissolving solvent of sample and mobile phase are not the same, it causes defects in the peak shape.
   Dilute the sample solution with mobile phase or reduce the injection volume.
3. Overloaded sample injection
   Overload will cause defects in the peak shape.
   Reduce injection volume.
4. Insufficient equilibration time
   When variance in pH is wide between the current and previous mobile phase or the buffer concentration of mobile phase is low, column equilibration may take a while.
5. Column contamination and degradation
   In the case of contamination, wash the column according to "How do we clean the columns? 1. Remove high hydrophobic absorbate". If column is in degradation, it is impossible to regenerate. The column should be replaced.
6. System problem
   Dispersion may occur within tubing between injector and column or the flow cell of detector which results in peak tailing and/or broading. System should comply with semi-micro use.

## Q What are the solutions for ghost peaks?

**A** Following solutions are introduced depends on causes.

1. Injector fouling
   If the ghost peak appears when injecting mobile phase only, wash the injector.
2. Gradient Analysis
   When hydrophobic impurities are eluted by a stronger solvent, it appears as a ghost peak. Clean the column according to "How do we clean the columns? 1. Remove highly hydrophobic absorbate". If you still can not eliminate them, the cause should be impurities of solvent.
   Use a higher grade solvent. Trap the impurities by attaching guard column between the solvent delivery pump and the mixing chamber.

## Q What should I do if columns are dried?

**A** Flush the column with solvent such as MeOH for other than silica, hexane for silica and remove air under pressure lower than half of what used in usual analysis. After all of the air is removed, check the performance by tracing the conditions on the inspection report which is sent with the product at the time of purchase.

## Q What should I do if the column fails to provide reproducibility?

**A** 1. Inappropriate mobile phase conditions
   - It may become difficult to obtain reproducibility in ionic compounds analysis if pH of mobile phase is not controlled or buffer concentration is low. Increase the buffer concentration.
   - Retention time fluctuates widely due to a slight variance of pH in cases where the pH of mobile phase is set close to the pKa of analyte. Set the pH of mobile phase distant from pKa.

2. System variance
   It may become difficult to obtain reproducibility in chromatogram by using different systems. The manufacturer of pumps, detectors and injectors should be the same, or extra column volume such as mixing chamber, detector cell, and plumbing will be different and fail to obtain reproducibility between systems. Also, using a column heater from a different manufacturer may affect the retention time due to the required temperature difference between the systems. Using a same system throughout a sequence of analysis is recommended.
3. Column histories
   Reproducibility of chromatogram may not be obtained between the same type of columns. This is due to the column histories. For example, in some cases, change in surface condition of packing material that are caused by using columns with mobile phase containing ion pair reagent or absorption of highly hydrophobic sample fails to obtain reproducibility. Dedicating a column per separation purpose is recommendable.
4. Using 100% aqueous mobile phase
   Reproducibility of chromatogram on ordinary ODS columns will not be obtained by using 100% aqueous mobile phase due to a short retention time. Columns, can be used in 100% aqueous mobile phase, and are recommended and available from every manufacturer. For YMC columns, "Hydrosphere C18" and "ODS-AQ" can be used in 100% aqueous mobile phase.
5. Grade difference in mobile phase
   Reproducibility of chromatogram may not be obtained by using a different grade of solvent in mobile phase. Impurities contained in solvent can act like salts in mobile phase and affect the separation. Solvent in HPLC grade is recommended.

## Q I still have poor retention after adding an ion pair reagent to mobile phase. Why?

**A** This is caused by excess of ion pair reagent. In general, when the concentration of ion pair reagent is higher, a stronger retention is observed. However in cases where the concentration of ion pair reagent is above a certain level, the retention may become poor because of micell formation. Good separation is achieved with the concentration of ion pair reagent, 5 mM to 20 mM. Set the concentration as low as possible to avoid short column life due to high ion pair reagent concentration.

# Troubleshooting

## 1 On organic solvents for reversed-phase liquid chromatography

Reversed-phase liquid chromatography frequently employs such organic solvents as methanol, acetonitrile or tetrahydrofuran. Although HPLC grade products of these types of solvents are available, it seems some users have trouble when using a reagent grade solvent instead of HPLC grade, causing them to waste considerable amounts of time. How do the two solvent grades differ?

～～ Methanol/acetonitrile ～～

Reagent grade solvents contain larger quantity of impurities absorbing UV than HPLC grade solvents do, which makes it difficult to use them in gradient elution or trace analysis. Especially when detection is conducted in a short wavelength, significant differences appear in baseline noise or detection sensitivity. In some cases (or in some wavelengths) it could be feasible to use a reagent grade solvent however we recommend using HPLC grade solvents to obtain a stable chromatogram.

～～ Tetrahydrofuran ～～

Tetrahydrofuran easily generates peroxides. To compensate for this tendency, it is commonly mixed with antioxidants. The antioxidants cause a ghost peak so a solvent that does not contain antioxidants should be used in HPLC. The peroxides in tetrahydrofuran also have great impact on the baseline stability (with differences between grades greater than those of other organic solvents), which prompts a strong recommendation to use HPLC grade solvents with very small quantities of impurities.

## 2 Eluent conditions

Although a column is apt to be thought of as a cause of HPLC analysis not showing proper data trace, many cases are attributed to other causes than a column which include improper maintenance operations. This article discusses the case in which the grade of a solvent has impact on peak shapes. Here is a chromatogram of the basic compound analysis with eluent of acetonitrile/ water. Peak 2 represents the basic compound. Figures below show chromatogram of two operations conducted under identical conditions except that the acetonitrile used was of different grades. One was HPLC grade (Figure 1); the other was reagent grade (Figure 2). While the peak shape was broadened with HPLC grade acetoniteile, it improved greatly when a reagent grade was used. The peak shape differences were observed depending on acetonitrile products of different makers even though they were of the same special grade. This may be because traces of impurities contained in acetonitrile behave in the same way as modifier added to an eluent.

Replacing eluent with acetonitrile/ 5 mM ammonium acetate produced a chromatogram like that in Figure 2 either with reagent or HPLC grade acetonitrile.

To avoid the influence of different grades, solvent specialized for HPLC must be used. Even compounds which have dissociation groups can be analyzed with eluent containing no acid or salt, though eluents with additives such as salt must be used when reproducibility is important.

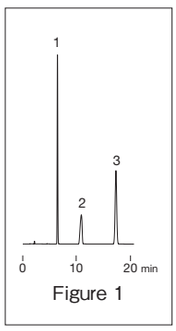


Figure 1

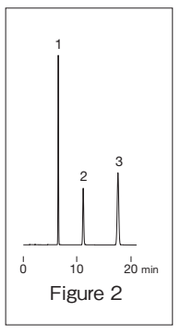


Figure 2

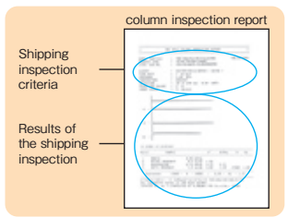
## 3 Peak shape anomaly

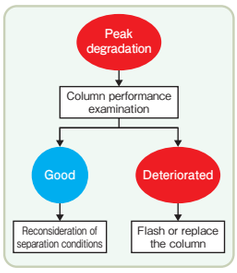
Common problems during HPLC operations include peak shape anomalies such as peak tailing and double peaks. In order to remove these problems, the cause must be precisely determined. The majority of cases are the result of inappropriate conditions of separation, including inappropriate selection of column or solvent, or use of an old column which has a void to the packing at the top of the packing. Here we discuss the method of determining the cause of the problem with peak shapes.

The simplest way is to test the column performance according to "shipping inspection criteria" in the column inspection report, which is included with every column. If the examination reveals no peak shape anomalies, the cause will be the result of inappropriate selection of separation condition. The separation condition such as eluent selection must be reconsidered.

If on the contrary, the same examination reveals any anomaly, the column can be deficient. Flushing (when the impurity could have accumulated) or replacement of the column is necessary. We recommend examining column performances on a regular basis and under identical conditions.

YMC provides analytical criteria including sample concentrations described in column inspection reports for principal products to help customers examine the performance of the column purchased.




## 4 Column Pressure Increases

Pressure increase is a common problem in HPLC. The solution to pressure increase in reversed phase separation is discussed herein.

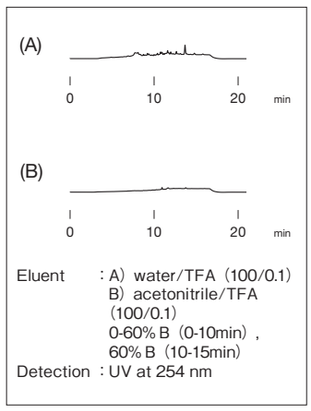
If the system pressure increases, you should disconnect the column, run the system without a column, and determine the line pressure. If the line pressure is high, piping or tubing may be clogged. If there is no excessive line pressure, then the column pressure may be high. The column needs washing. Washing by reversed direction flow will be very effective. Although the relative proportion of the organic solvent of mobile phase is generally increased from washing, the key consideration is to choose, in accordance with the characteristics of the sample, an appropriate solvent that easily dissolves the adsorbed material. Reversed phase separation often causes protein to be adsorbed by the packing material, resulting in high pressure. This problem can be fixed effectively by gradient washing with acetonitrile/ water containing 0.1% TFA, rather than washing with an organic solvent. If the cause is believed to be insoluble material in samples or precipitation of a sample in separation, washing or replacing a frit (filter) might be successful.

However, in reality, a column will become difficult to restore despite washing once it has undergone pressure increase.

It is desirable that column pressure should be prevented by conducting sample preparation, such as protein removal, using a guard column, and conducting sample filtration.

## 5 The Cause of the Ghost Peak

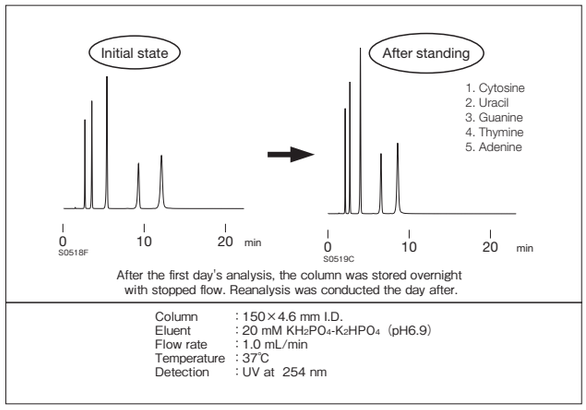
There was a case where during the blank run as a part of a preliminary study of gradient elution without a sample, an infinite number of peaks as in figure (A) appeared. When conducting a similar operation where the column was disconnected, the ghost peak disappeared as shown in figure (B). Consequently, the cause must have been the column.
However, despite flushing or replacing the column, the baseline would not improve. Therefore, many factors other than columns were examined; the cause was found to be water used to prepare eluents. Standard distilled water (inadequate for HPLC) had been mistakenly used, rather than HPLC grade distilled water which leads to an excellent baseline as in figure (B).
As discussed above, the water purity can have a great impact on gradient elution. Even HPLC grade distilled water will become contaminated as time passes after purchase, causing ghost peaks. A condition which has no significant influence on isocratic elution could cause a problem in gradient elution. In gradient elution, a column is equilibrated with an eluent with low content of organic solvent so that impurities in the eluent are adsorbed and concentrated in the column. After initiating analysis, content of organic solvent increases and impurities begin to be eluted resulting in the ghost peak. The heights of ghost peaks are dependent on the duration of equilibration. The ghost peak did not appear without a column because of the absence of adsorption and concentration during equilibration.
In gradient analysis, solvent requires great care especially of its grade distinction and storage.




Eluent : A) water/TFA (100/0.1)
B) acetonitrile/TFA (100/0.1)
0-60% B (0-10min),
60% B (10-15min)
Detection : UV at 254 nm

## 6 Regenerating Columns

In reversed phase HPLC, column deterioration causes poor peak shapes or shortened retention time. The column deterioration results from packing materials' chemical alteration such as loss of bonded phase like C18 or dissolution of silica-gel as the base material. Consequently columns in such condition are difficult to restore and reuse.
Meanwhile, 100% aqueous mobile phase in an ODS column sometimes entails steep reduction in retention of compounds as in the figure right. Many may think the reduction of this retention time is due to the column deterioration. However this is not the case. Rather, the cause is considered to be the decrease of apparent hydrophobicity of packing material due to polarity difference between the water of mobile phase and the surfaces of packing material bonded with C18 functional groups and become difficult to solvate. Coping with this and restoring the initial retention time is easily achieved by flushing the column with 10 times its volume of mobile phase containing 50% organic solvent. This situation is believed to result from the decrease of the repulsion between the eluent and the C18 functional groups. If the retention time reduction occurs when using 100% aqueous mobile phase, try to flush with organic solvent/water mixture to regenerate the column.



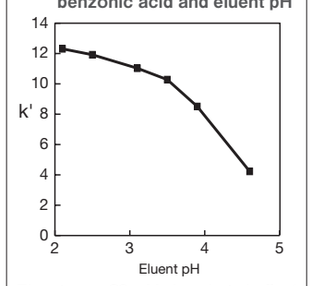

After the first day's analysis, the column was stored overnight with stopped flow. Reanalysis was conducted the day after.

Column : 150×4.6 mm I.D.
Eluent : 20 mM $KH_2PO_4$-$K_2HPO_4$ (pH6.9)
Flow rate : 1.0 mL/min
Temperature : 37°C
Detection : UV at 254 nm

## 7 The pH Adjustment of Eluents.

Analysis of ionic compounds by reversed-phase HPLC is conducted with the pH of eluent controlled using acid or buffering agent. However, a separation with a pH range which is not optimum for the compound of interest could cause problems such as double peak or peak broadening. Even if the peak shape is satisfactory, retention time reproducibility could in some cases not be obtained.
The relation between retention of benzonic acid and pH value is shown in the figure below. Although the k' falls within relatively narrow limits in the region where the pH ranges from 2 to 3.5, it varies widely in the region where the pH ranges from 3.5 to 4.5. The pKa of benzonic acid is 4.2 and it is noticeable that the region where the k' most widely varies is near the pKa. If the eluent pH is adjusted to the region near the pKa with the wide variation of the k', the result might not be reproducible since the slight error of the pH adjustment could be of great impact on separation. In fact, the eluent pH variation of just 0.1 significantly affects separation.
Consequently, it is desirable that the eluent pH should be more than 1 off the pKa. If the pKa is unknown, the eluent pH should be adjusted to within the region where the impact on separation seems minimal, after having deliberately considered the relation between the eluent pH and the retention time.
When considering the pH value, it is also important to confirm the influence on separation using several eluents with their pHs adjusted to be slightly different from each other.

**Relation beween retention (k') of benzonic acid and eluent pH**




Eluent : 20 mM phosphate buffer/acetonitrile (85/15)

# Basic Data

## Unit Conversion

### Pressure

| MPa | bar | psi | kgf/cm$^2$ | atm |
|---|---|---|---|---|
| 1 | 10 | 145.04 | 10.20 | 9.87 |
| 0.1 | 1 | 14.504 | 1.020 | 0.987 |
| $6.90\times10^{-3}$ | 0.069 | 1 | 0.070 | 0.068 |
| 0.0981 | 0.981 | 14.223 | 1 | 0.968 |
| 0.101 | 1.013 | 14.696 | 1.033 | 1 |

### Length

| m | in | ft | yd | mile |
|---|---|---|---|---|
| 1 | 39.37 | 3.28 | 1.094 | $6.21\times10^{-4}$ |
| 0.025 | 1 | 0.083 | 0.028 | $0.15\times10^{-4}$ |
| 0.305 | 12 | 1 | 0.33 | $1.89\times10^{-4}$ |
| 0.91 | 36 | 3 | 1 | $5.68\times10^{-4}$ |
| 1609.3 | 63360 | 5280 | 1760 | 1 |

### Weight

| kg | oz | lb |
|---|---|---|
| 1 | 35.274 | 2.204 |
| 0.0283 | 1 | 0.0625 |
| 0.454 | 16 | 1 |

### Volume

| L | gal(UK) | gal(US) |
|---|---|---|
| 1 | 0.22 | 0.26 |
| 4.55 | 1 | 1.201 |
| 3.79 | 0.83 | 1 |

### Temperature

| K | ℉ | ℃ |
|---|---|---|
| 0 | -459.67 | -273.15 |
| 255.37 | 0 | -17.8 |
| 273.15 | 32 | 0 |
| 298.15 | 77 | 25 |
| 310.93 | 100 | 37.8 |
| 373.15 | 212 | 100 |

formula: ℃=(℉-32)×5/9　℉=℃×9/5+32

### Ratio Scale

| ppb | ppm | % |
|---|---|---|
| 1 | $10^{-3}$ | $10^{-7}$ |
| $10^3$ | 1 | $10^{-4}$ |
| $10^7$ | $10^4$ | 1 |

## SI Prefixes

| da (deca) | h (hecto) | k (kilo) | M (mega) | G (giga) | T (tera) | P (peta) | E (exa) | Z (zetta) | Y (yotta) |
|---|---|---|---|---|---|---|---|---|---|
| $10^1$ | $10^2$ | $10^3$ | $10^6$ | $10^9$ | $10^{12}$ | $10^{15}$ | $10^{18}$ | $10^{21}$ | $10^{24}$ |

| d (deci) | c (centi) | m (milli) | μ (micro) | n (nano) | p (pico) | f (femto) | a (atto) | z (zepto) | y (yocto) |
|---|---|---|---|---|---|---|---|---|---|
| $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-6}$ | $10^{-9}$ | $10^{-12}$ | $10^{-15}$ | $10^{-18}$ | $10^{-21}$ | $10^{-24}$ |

* 1 Å (ångström)= 0.1 nm = $10^{-10}$ m

## Column Area Ratio

| Inner Diameter | 1.0 | 2.0 | 3.0 | 4.6 | 10.0 | 20.0 | 30.0 | 50.0 |
|---|---|---|---|---|---|---|---|---|
| Ratio | 0.0473 | 0.189 | 0.425 | 1 | 4.73 | 18.90 | 42.53 | 118.15 |