8. Methods in Developing Mobile Phase Condition for C_{18} Column

Introduction

In reversed phase HPLC, octadecyl group bonded silica columns (C_{18}, ODS) are the most widely employed. A proper mobile phase condition for C_{18} columns can be achieved by referring to publications, application notes from manufactures and your own experiences. This section shows traditional methods of developing mobile phase condition. The following columns are used as examples because of their popularity.

Packing material : COSMOSIL 5C_{18}-MS-II, COSMOSIL 5C_{18}-AR-II
Column size (I.D. x length) : 4.6 mm I.D x 150 mm

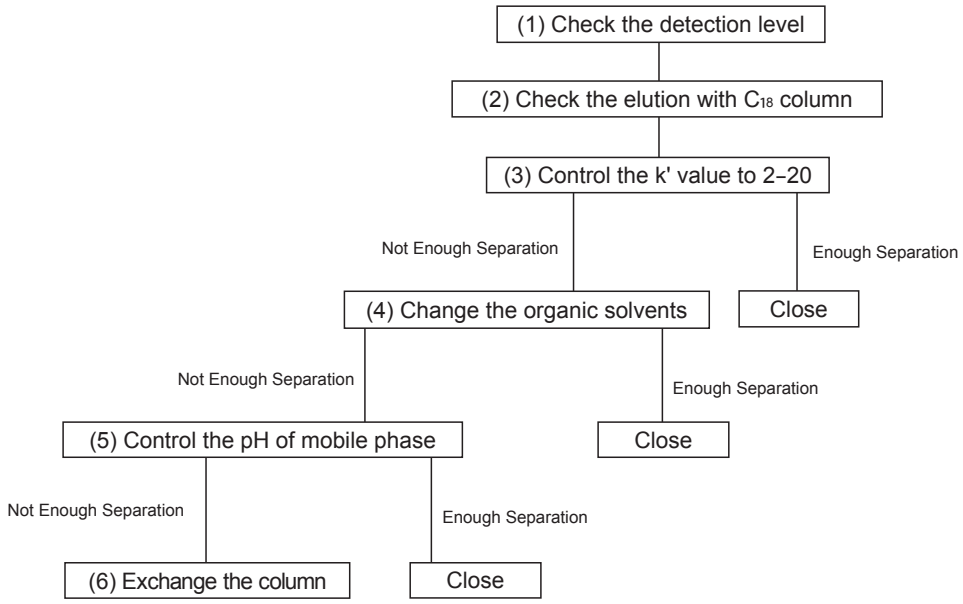
Methods for Developing Mobile Phase Conditions

In the Isocratic method, the mobile phase composition remains constant throughout the run. In the gradient method, the mobile phase composition changes. Each method needs strict preparation of the mobile phase and control of the column temperature to achieve good separation.

● Isocratic Method

Methods for developing mobile phase condition generally proceed as follows. First, elute the samples with strong solvents to check whether the samples can be detected. Then, separate the samples by controlling the retention time through changing the mobile phase condition. Increasing the concentration of strong elution efficiency solvents results in shorter retention time and decreasing in concentration of them results in longer retention time. If your samples are ionizable, such as acid and amine group, pH control with buffers is highly advisable.. The ionization control method or ion-pair chromatography is used to increase the retention of ionized samples. The method uses ion-pair reagents (e.g., alkyl benzene sulfonate for basic compounds, quaternaty ammonium for acidic compounds) into in the mobile phase to form ion pairs with samples.

(e.g.,) Procedures for Basic Condition Setting



1. Check the detection of samples with strong elution solvents. In this step, check the detection by connecting the injector directly to the detector without a column.
2. Consult references and carbon numbers and check the elution with C_{18} column using aqueous mobile phase with methanol.
3. Control the k' value to 2–20 by changing the amount of the methanol in the mobile phase.
4. If the separation is not enough, change the methanol to acetonitrile or add tetrahydrofuran to change the selectivity.
5. If tailing peaks occur for basic compounds, control the pH by adding buffers to the mobile phase.
6. If separation is not satisfactory after step 5, change the column to other C_{18} columns or columns with different stationary phases such as alkyl–based, aromatic–based and others.

● Gradient Method

Gradient method changes organic solvent composition continuously in the mobile phase. It is useful for shortening the separation time of samples with wide range of hydrophobicity and molecular weight, with long elution time, and with great changes in retention time by slight changes in organic solvent composition. It is also useful for large molecule weight compounds like peptides. Gradient method is not compatible with RI detector. Gradient method development is beyond the scope that can be discussed here.

Easy Method to Set the Reversed Phase Condition

Reversed phase chromatography does not have an easy method to set mobile phase conditions, unlike normal phase chromatography mobile phase conditions that can simply be determined by thin layer chromatography. Therefore, the composition of the mobile phase (concentration of organic solvent) is often determined by repeated trial and error. If you know the structure of the analytes, here is a general instruction on configuring an appropriate mobile phase organic solvent concentration.

Suitable concentration of organic solvent for basic chemical skeleton + effects from substituents
= The best organic solvent concentration

● Condition Setting

Select the condition based on retention time of the basic chemical skeleton shown in Figure 1, then adjust for the effect of hetero atom and substituents.

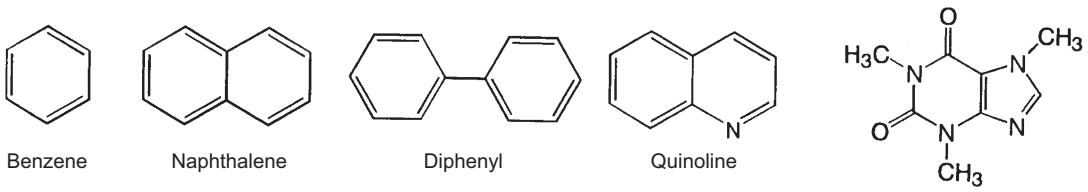
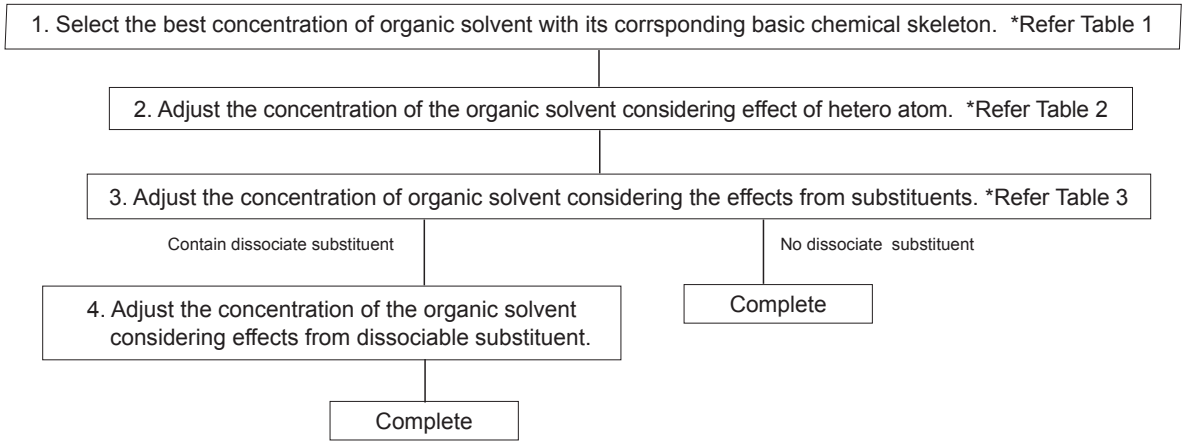


Figure 1. Structures of basic chemical skeletons



1. Choose a compound of similar basic chemical structure as the target sample in Figure 1. Select the best organic solvent concentration.

Table 1. Retention time of the basic chemical skeleton

Basic skeleton	Column	Retention Time Under Different Methanol Concentrations (min)						
		80%	70%	60%	50%	40%	30%	20%
Benzene	$5C_{18}$-MS-II	–	4	7	11	20	–	–
	$5C_{18}$-AR-II	–	4	7	13	23	–	–
Naphthalene	$5C_{18}$-MS-II	5	8	18	–	–	–	–
	$5C_{18}$-AR-II	5	10	22	–	–	–	–
Diphenyl	$5C_{18}$-MS-II	8	13	–	–	–	–	–
	$5C_{18}$-AR-II	7	15	–	–	–	–	–
Quinoline	$5C_{18}$-MS-II	–	–	–	–	6	11	–
	$5C_{18}$-AR-II	–	–	–	–	8	17	–
Caffeine	$5C_{18}$-MS-II	–	–	–	–	–	4	9
	$5C_{18}$-AR-II	–	–	–	–	–	4	9

Column: COSMOSIL 4.6 mm I.D. × 150 mm Flow Rate: 1.0 ml/min Detection: UV 254 nm

2. Adjust the organic solvent concentration considering effect of hetero atom as shown in Table 2.

Table 2. Organic solvent concentration adjustment from hetero rings or polycyclic aromatics

Hetero Rings, polycyclic Aromatics		Sample	$5C_{18}$–MS–II	$5C_{18}$–AR–II
1 of Conjugate Ring		Benzene	+10%	+10%
Heterocyclic Hetero Atom	1 of S	Thiophene	±0%	±0%
	1 of O	Furane	–5%	–5%
	1 of N	Pyridine	–20%	–10%
1 of Carbonyl Group		Quinone	–5%	–5%
1 of Double Bond		–	–5%	–5%

3. Adjust the concentration of organic solvent considering effect from substituents as shown in Table 3.

Table 3. Organic solvent concentration adjustment from substituents

Substituent	Methanol Concentration	
	$5C_{18}$–MS–II	$5C_{18}$–AR–II
–F	0	0
–Cl	+10%	+10%
–Br	+10%	+10%
–I	+20%	+15%
$-CONH_2$	–40%	–40%
$-COCH_3$	–10%	–10%
$-COOCH_3$	0	0
$-OCH_3$	0	0
$-CHCH_2O$	–10%	–10%
–CH2OH	–30%	–30%
–OH	–30%	–30%
$-NO_2$	–10%	–5%
–CN	–20%	–15%
$-NH_2$	–40%	–30%
$-SCH_3$	+10%	+10%

Substituent	Methanol Concentration
–CH2– (Alkyl–chain) MeOH concentration of basic skeleton	
100–90%	+10% (4 of $-CH_2-$)
90–80%	+10% (3 of $-CH_2-$)
80–60%	+10% (2 of $-CH_2-$)
< 60%	+10% (1 of $-CH_2-$)
–Phenyl MeOH concentration of basic skeleton	
100–90%	+5% (1 of – Phenyl)
90–60%	+10% (1 of – Phenyl)
< 60%	+20% (1 of – Phenyl)

Column: COSMOSIL 4.6 mm I.D. × 150 mm

Flow Rate: 1.0ml / min Detection: UV 254 nm

* Effect may shift somewhat by the position of the substituent.

4. Compounds with a dissociative substituent are extremely sensitive toward slight pH change. Maintain consistent mobile phase pH to obtain reproducible data. Table 4 shows the influence of acidic (pH 2) and neutral (pH 7) substituents to the retention.

Table 4. Effect of dissociate substituent to organic solvent

Dissociable Substituent	Change of Methanol Concentration (pH 2)	Change of Methanol Concentration (pH 7)
–COOH	–10~–20%	–30~–40%
$-SO_3H$	–20~–40%	–30~–40%
$-PO_4H_2$	–20%	–50%
$-BO_2H_2$	–20%	–20%
$-NH_2$ (molecular type)	–60%	–10%
$-NH_2$ (cyclic amine)	–50~–60%	–10~–20%
$-NH_2$ (ionic type)	–	–40~–50%

Column :COSMOSIL $5C_{18}$–MS–II, 4.6 mm I.D. x 150 mm
Buffer pH2 :20mmol/l H_3PO_4
pH7 :20mmol/l H_3PO_4/Na_2HP0_4=2/3
Flow Rate :1.0 ml/min
Detection :UV 254 nm

● Example of Condition Setting

Column: COSMOSIL 5C_{18}-MS-II 4.6 mm I.D. × 150 mm

(1) 5-Benzyloxyindole

<Calculation> Basic skeleton Naphthalene like + (hetero ring N)
=70%+ (-20%)
=50%

Substituent (Phenyl) + (-OCH_2- is equal to -OCH_3)
=(+10%) + (+0%)

Basic skeleton + Substituent = 50% + (+10%) = 60%

<Result> 60%Methanol (Methanol:Water=60:40)
Retention time=13.7 min

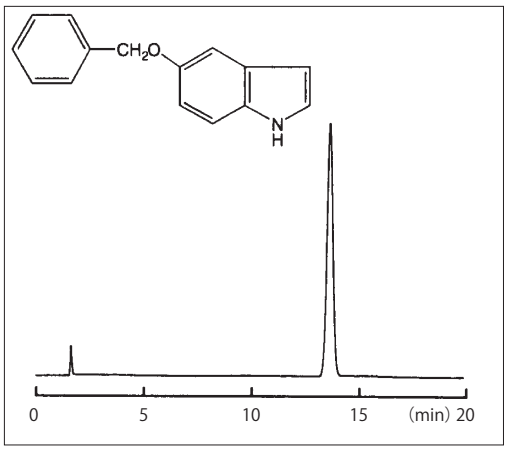


Figure 2. Analysis of 5-Benzyloxyindole

(2) Homovanillic Acid

<Calculation> Basic skeleton Benzene=60%

Nondissociative substituent (-OH) + (-OCH_3) + (-CH_2)
=(-30%) + (0%) + (+10%)
= -20%

Dissociable substituent -COOH = -10~-20% (pH 2)
-30~-40% (pH 7)

Basic skeleton + Substituent = Methanol concentration is
Acid range (pH 2) 30-20%
Neutral range (pH 7) 10-0%

<Result> (pH2) 30%Methanol : Retention time=5.7 min
20%Methanol : Retention time=11.7 min

(pH7) 10%Methanol : Retention time=4.0 min
0%Methanol : Retention time=12.1 min

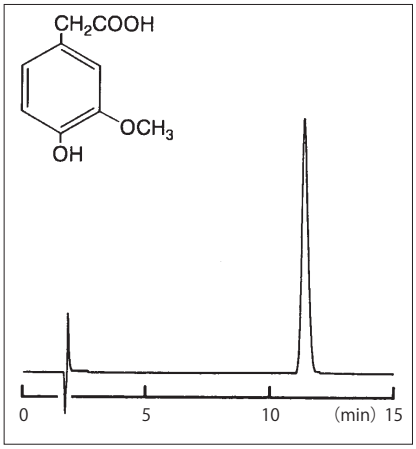


Figure3. Analysis of 20% Methanol (pH 2)

* Actual retention prediction results may have ±10% error in organic solvent concentration calculated.