(8) Methods for Developing Mobile Phase Conditions for C_{18} Columns

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 manufacturers, and your own experience. This section shows traditional methods for developing mobile phase conditions. 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

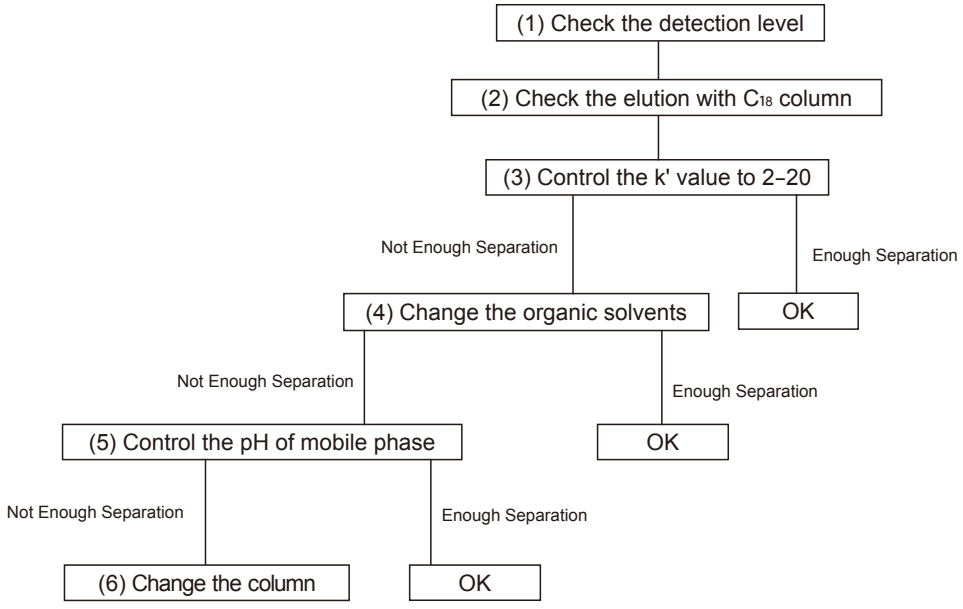
Methods for Developing Mobile Phase Conditions

In isocratic methods, the mobile phase composition remains constant throughout the run. In gradient methods, the mobile phase composition changes. Each method requires precise preparation of the mobile phase and control of the column temperature to achieve good separation.

● Isocratic

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 with the mobile phase condition. Increasing the concentration of strongly eluting solvents results in shorter retention time, and decreasing their concentration results in longer retention time. If your samples are ionizable, such as acids and amines, pH control with buffers is highly advisable. Buffers and/or ion-pair reagents are used to increase the retention of ionizable samples. Ion-pair reagents (e.g., alkyl benzene sulfonate for basic compounds, quaternary ammonium for acidic compounds) in the mobile phase form ion pairs with samples, increasing their hydrophobicity and retention.

(Example) Procedure for Basic Condition Setting



1. Check the detection of samples with strongly eluting solvents. In this step, check the detection by connecting the injector directly to the detector without a column.
2. Consult references and consider carbon numbers, then check elution time with a C_{18} column using an aqueous mobile phase with methanol.
3. Control the k' value to 2–20 by changing the amount of methanol in the mobile phase.
4. If the separation is not sufficient, change the methanol to acetonitrile or add tetrahydrofuran to change the selectivity.
5. If peak shape is poor with basic or acidic compounds, control the pH by adding buffers to the mobile phase.
6. If separation is still not satisfactory, change the column to another C_{18} column or a column with different chemistry, such as other alkyl-based, aromatic, or ionic interaction-based stationary phases.

● Gradient

Gradient elution changes the organic solvent ratio in the mobile phase continuously. It is useful for shortening the separation time of samples with a wide range of hydrophobicity and molecular weight, which would otherwise have a long elution time and high sensitivity to slight changes in solvent composition. It is also essential for high-molecular weight compounds like peptides. Gradient elution is not compatible with RI detectors, as it produces baseline interference. Gradient method development relies on the user's experience and is beyond the scope of this note.

Easy Method to Determine Reversed Phase Conditions

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

Suitable concentration of organic solvent for basic chemical skeleton + Effects from substituents
= Estimated optimal 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.

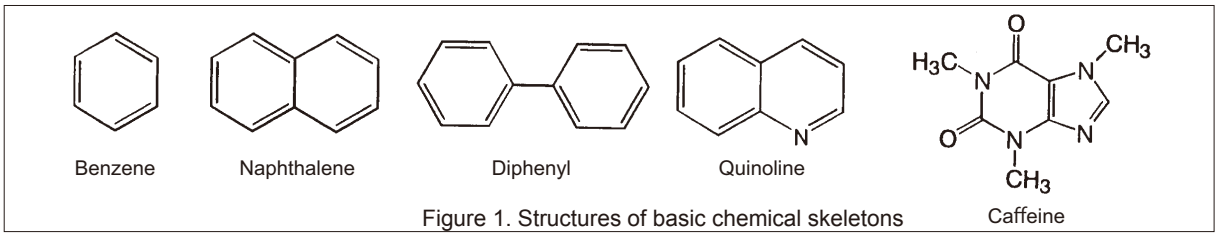
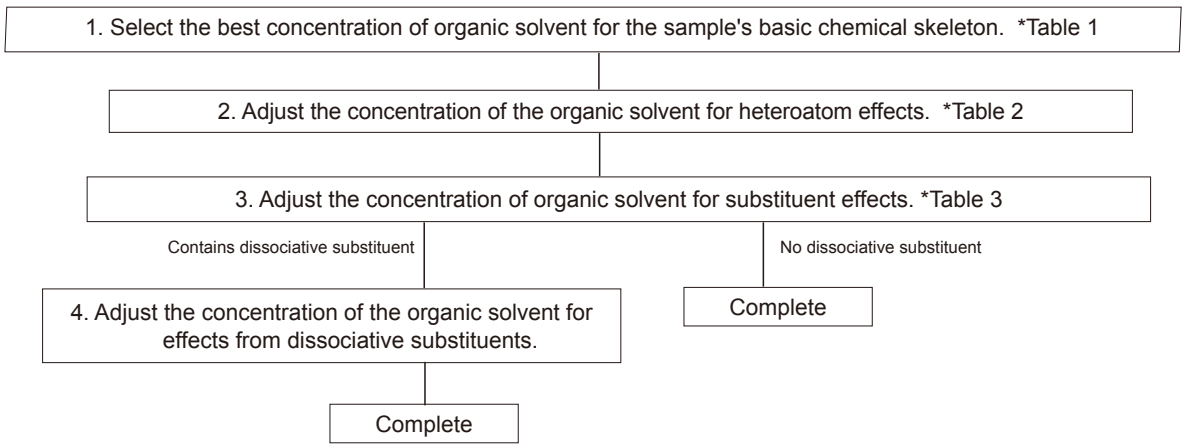


Figure 1. Structures of basic chemical skeletons



1. Select a compound from Figure 1 with a similar basic skeleton to the target sample. 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 effects of heteroatoms as shown in Table 2.

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

Hetero rings, polycyclic aromatics		Sample	5C18-MS-II	5C18-AR-II
Conjugate ring (1 ring)		Benzene	+10%	+10%
Heterocyclic heteroatom	S (1 atom)	Thiophene	±0%	±0%
	O (1 atom)	Furane	−5%	−5%
	N (1 atom)	Pyridine	−20%	−10%
Carbonyl group (1 group)		Quinone	−5%	−5%
Double Bond (1 bond)		−	−5%	−5%

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

Table 3. Organic solvent concentration adjustment due to substituents

Substituent	Methanol Concentration	
	5C18-MS-II	5C18-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
$-CH_2-$ (Alkyl chain) MeOH concentration of basic skeleton:	
100–90%	+10% for every 4 carbons
90–80%	+10% for every 3 carbons
80–60%	+10% for every 2 carbons
< 60%	+10% for each carbon
Phenyl group MeOH concentration of basic skeleton:	
100–90%	+5% for each phenyl group
90–60%	+10% for each phenyl group
< 60%	+20% for each phenyl group

Column: COSMOSIL 4.6 mm I.D. × 150 mm

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

* Effect may differ somewhat due to the position of the substituent.

4. Compounds with dissociative substituents are extremely sensitive to slight pH changes. Maintain consistent mobile phase pH to obtain reproducible data. Table 4 shows the influence of acidic (pH 2) and neutral (pH 7) conditions on methanol concentration (for approximately the same retention time).

Table 4. Effect of dissociative substituent on organic solvent concentration

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 5C18-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

● Examples

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

(1) 5-Benzyloxyindole

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

Substituent: (Phenyl) + (-OCH_2- [equal to -OCH_3])
=(+10%) + (+0%)

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

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

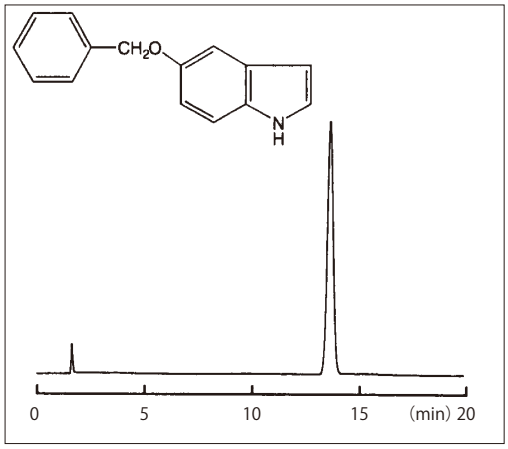


Figure 2. Analysis of *5*-Benzyloxyindole

(2) Homovanillic Acid

<Calculation> Basic skeleton: Benzene = 60%

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

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

Basic skeleton + Substituent = Acidic pH (2): 30-20%
Neutral pH (7): 10-0%

<Result> (pH 2) 30% Methanol: Retention time=5.7 min
20% Methanol: Retention time=11.7 min

(pH 7) 10% Methanol: Retention time=4.0 min
0% Methanol: Retention time=12.1 min

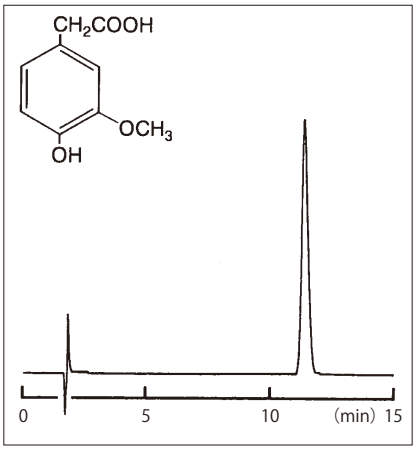


Figure3. Analysis of 20% Methanol (pH 2)

* Actual results may have about ±10% error in organic solvent concentration due to unique properties of the analyte.