

TECHNICAL NOTE

2. Preparation of mobile phase for HPLC

1) Organic solvent/ aqueous mixed mobile phase

1)-1. Preparation of methanol : water = 70 : 30 (v/v) 1L

- ① Measure 700 ml of methanol in a measuring cylinder.
- ② Measure 300 ml of distilled water in a measuring cylinder.
- ③ Mix ① and ② thoroughly and degas.

The better approach is to prepare the mobile phase gravimetrically rather than volumetrically. Following is example of preparation.

Composition table for mobile phase 1L using methanol and water

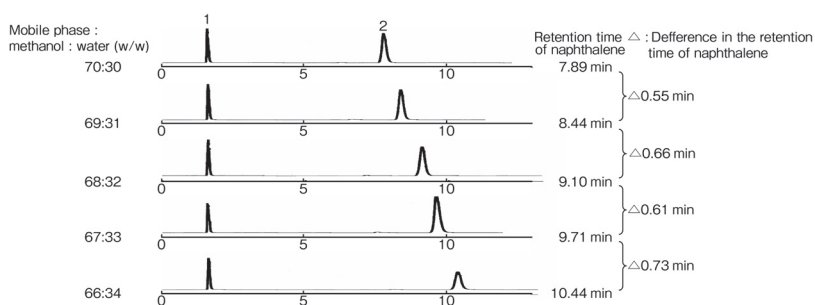
Methanol / Water	Methanol (g)	Distilled water (g)
90/10 (v/v)	711.9	99.8
80/20 (v/v)	632.8	199.6
70/30 (v/v)	553.7	299.5
60/40 (v/v)	474.6	399.3
50/50 (v/v)	395.5	499.1
40/60 (v/v)	316.4	598.9
30/70 (v/v)	237.3	698.7
20/80 (v/v)	158.2	798.6
10/90 (v/v)	79.1	898.4

Composition table for mobile phase 1L using acetonitrile and water

Acetonitrile / Water	Acetonitrile (g)	Distilled water (g)
90/10 (v/v)	707.4	99.8
80/20 (v/v)	628.8	199.6
70/30 (v/v)	550.2	299.5
60/40 (v/v)	471.6	399.3
50/50 (v/v)	393.0	499.1
40/60 (v/v)	314.4	598.9
30/70 (v/v)	235.8	698.7
20/80 (v/v)	157.2	798.6
10/90 (v/v)	78.6	898.4

Caution : Methanol and acetonitrile are hazardous substances, do not use for medical purpose. Always process in a laboratory hood and wear an eye protection and a mask.

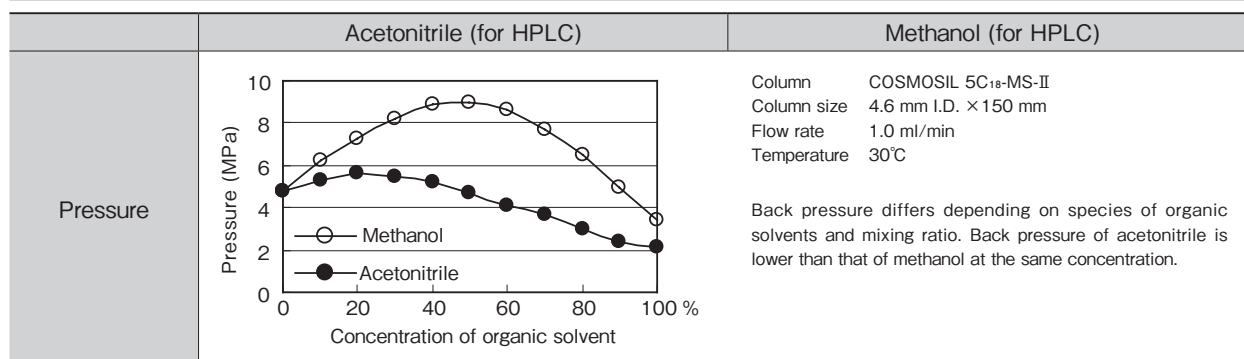
Influence of organic solvent composition in mobile phase on the retention time. 1% difference in the composition significantly changes the retention.



Column COSMOSIL 5C₁₈-MS-II
 Column size 4.6 mm I.D. × 150 mm
 Flow rate 1.0 ml/min
 Detection 254 nm 0.16 AUFS
 Temperature 30°C
 Sample 1 uracil
 2. naphthalene

Special attention should be paid to measure correct amount of organic solvent as the retention time is significantly changed by 1% different composition.

Differences between acetonitrile and methanol in reversed phase liquid chromatography



TECHNICAL NOTE

	Acetonitrile (for HPLC)	Methanol (for HPLC)
Elution strength	<p>Column COSMOSIL 5C₁₈-MS-II Column size 4.6 mm I.D. × 150 mm Flow rate 1.0 ml/min Temperature 30°C</p> <p>Elution strength of acetonitrile/water is stronger than methanol/ water at the same concentration. Within the range of about 30%-80% in the concentration of the organic solvent, elution strength of acetonitrile/water is almost equivalent to that of methanol/water which was raised methanol concentration about 10% (ex : acetonitrile/water = 60/40 → methanol/water = 70/30)</p>	
Absorbance	Acetonitrile has a lower UV absorbance in far UV region (less than 250 nm) .	Methanol has a higher UV absorbance than acetonitrile in far UV region (less than 250 nm).
Degas of mobile phase	When acetonitrile is mixed with water, it is endothermic. It is difficult to degas.	When methanol is mixed with water, it is exothermic. It is easy to degas.

2) Organic solvent/ buffer mixed mobile phase

2)-1. Preparation of methanol : 20 mmol/l phosphate buffer (pH2.5) = 80 : 20 (v/v) 1L

- Preparation of 20 mmol/l phosphate buffer (pH2.5)
 - ① Prepare 20 mmol/l sodium dihydrogenphosphate aqueous solution.
 - ② Prepare 20 mmol/l phosphoric acid aqueous solution.
 - ③ Adjust the pH to 2.5 by mixing ① with ② .
 - ④ Filter ③ under reduced pressure to remove insoluble substance that may deteriorate pump-seal and clog columns (0.45 μm or smaller pore size is recommended).

(Easy method)

- ① Dissolve 1.31 g of sodium dihydrogenphosphate and 1.05 g of phosphoric acid in distilled water to make 1 L solution.
- ② Filter the solution under reduced pressure to remove insoluble substance (0.45 μm or smaller pore size is recommended).
- ③ Confirm that the solution is pH2.5.

- Preparation of methanol : 20 mmol/l phosphate buffer (pH2.5) = 80 : 20 1L
 - ① Measure 800 ml of methanol in a measuring cylinder.
 - ② Measure 200 ml of 20 mmol/l phosphate buffer (pH2.5) in a measuring cylinder.
 - ③ Mix ① and ② thoroughly and degas.

2)-2. Preparation of methanol : 20 mmol/l phosphate buffer (pH7.0) = 80 : 20 (v/v) 1L

- Preparation of 20 mmol/l phosphate buffer (pH7.0)
 - ① Prepare 20 mmol/l sodium dihydrogenphosphate aqueous solution.
 - ② Prepare 20 mmol/l di-sodium hydrogenphosphate aqueous solution.
 - ③ Adjust the pH7.0 by mixing ① with ② .
 - ④ Filter ③ under reduced pressure to remove insoluble substance that may deteriorate pump-seal and clog columns (0.45 μm or smaller pore size is recommended).

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(Easy method)

- ① Dissolve 1.14 g of sodium dihydrogenphosphate and 1.49 g of di-sodium hydrogenphosphate in distilled water to make 1L solution.
- ② Filter the solution under reduced pressure to remove insoluble substance (0.45 μm or smaller pore size is recommended).
- ③ Confirm that the solution is pH7.0.

• Preparation of Methanol : 20 mmol/l phosphate buffer (pH7.0) = 80 : 20 1 L

- ① Measure 800 ml of methanol in a measuring cylinder.
- ② Measure 200 ml of 20 mmol/l phosphate buffer (pH7.0) in a measuring cylinder.
- ③ Mix ① and ② thoroughly and degas.

The better approach is to prepare the mobile phase gravimetrically rather than volumetrically. Following is example of preparation.

methanol : 20 mmol/l phosphate buffer	methanol (g)	20 mmol/l phosphate buffer (pH2.5) (g)	20 mmol/l phosphate buffer (pH7.0) (g)
90/10 (v/v)	711.9	99.8	99.9
80/20 (v/v)	632.8	199.6	199.8
70/30 (v/v)	553.7	299.4	299.7
60/40 (v/v)	474.6	399.2	399.6
50/50 (v/v)	395.5	499.0	499.5
40/60 (v/v)	316.4	598.8	599.4
30/70 (v/v)	237.3	698.6	699.3
20/80 (v/v)	158.2	798.4	799.2
10/90 (v/v)	79.1	898.2	899.1

Caution : Methanol and acetonitrile are hazardous substances, do not use for medical purpose. Always process in a laboratory hood and wear an eye protection and a mask.

3) Preparation of ion pair reagent containing mobile phase

3)-1.Preparation of 5 mmol/l sodium 1-butanefulfonate containing 20 mmol/l phosphate buffer (pH2.5)

- ① Prepare 5 mmol/l sodium 1-butanefulfonate containing 20 mmol/l sodium dihydrogenphosphate aqueous solution.
- ② Prepare 5 mmol/l sodium 1-butanefulfonate containing 20 mmol/l phosphoric acid aqueous solution
- ③ Adjust the pH to 2.5 by mixing ① with ② .
- ④ Filter ③ under reduced pressure to remove insoluble substance that may deteriorate pump-seal and clog columns (0.45 μm or smaller pore size is recommended).

(Easy method)

- ① Dissolve 1.31 g of sodium dihydrogenphosphate, 1.05 g of phosphoric acid and 0.80 g of sodium 1-butanefulfonate in distilled water to make 1L solution.
- ② Filter the solution under reduced pressure to remove insoluble substance (0.45 μm or smaller pore size is recommended).
- ③ Confirm that the solution is pH2.5.

※ 0.5M sodium 1-butanefulfonate aqueous solution is also available from Nacalai Tesque.