

# Application News

## No. L443

### High Performance Liquid Chromatography

## Effective Method Development Using Online Blending of Organic Solvents for Mobile Phase Preparation

When investigating the analytical conditions during HPLC method development, selection of the mobile phase greatly influences the separation. In reversed-phase analysis, for example, when the retention and peak shape of an analyte vary greatly depending on the organic solvent used, the separation can often be improved by blending together various organic solvents. However, from the analyst's perspective, every organic solvent mixture prepared must be tested in the actual separation a process that requires a great deal of time and effort.

Here, we introduce a technique in which multiple organic solvents are blended online to prepare a mobile phase that is suitable for analysis.

### ■ Online Mobile Phase Blending System

In this system, a pump with a low-pressure gradient valve delivers an organic solvent mobile phase blend that has been prepared online by mixing multiple organic solvents via switching of a valve. An example of this flow path is shown in Fig. 1. This is a high-pressure gradient elution system in which each pump contains a low-pressure gradient valve that permits mixing of up to four different solutions in any desired ratios. This example illustrates the process in which aqueous mobile phase<sup>\*1</sup> delivered by pump A and organic mobile phase delivered by pump B are mixed entirely online.

\*1 For online preparation of aqueous mobile phases, refer to Application News No. L444 and L445.

### ■ Effects on Retention Due to Organic Solvents

Retention behavior may vary greatly depending on the type of organic solvent used in reversed-phase analysis. Acetonitrile and methanol are commonly used in HPLC analysis, and use of acetonitrile results in stronger elution from the column due to its lower polarity. In addition, depending on the analyte structure, the proton-donating ability of the solvent used for separation can influence retention. In this case, quite different retention behavior is displayed due to the differences between aprotic solvents such as acetonitrile and protic solvents such as methanol. Furthermore, this difference in proton-donating ability can also influence peak shape. In the case of silica-based reversed-phase column packing, since a hydrogen bond is formed between the silanol group on the surface of the packing material and methanol, the peak shape may be affected depending on the analyte species. Here, use of methanol rather than acetonitrile would provide a sharper peak. Therefore, since the effect on retention of an analyte can be significant depending on the type of organic solvent used for separation, in cases where separation is difficult using a single solvent or where a better peak shape is sought, both separation and peak shape can be improved by using a mixture of organic solvents.

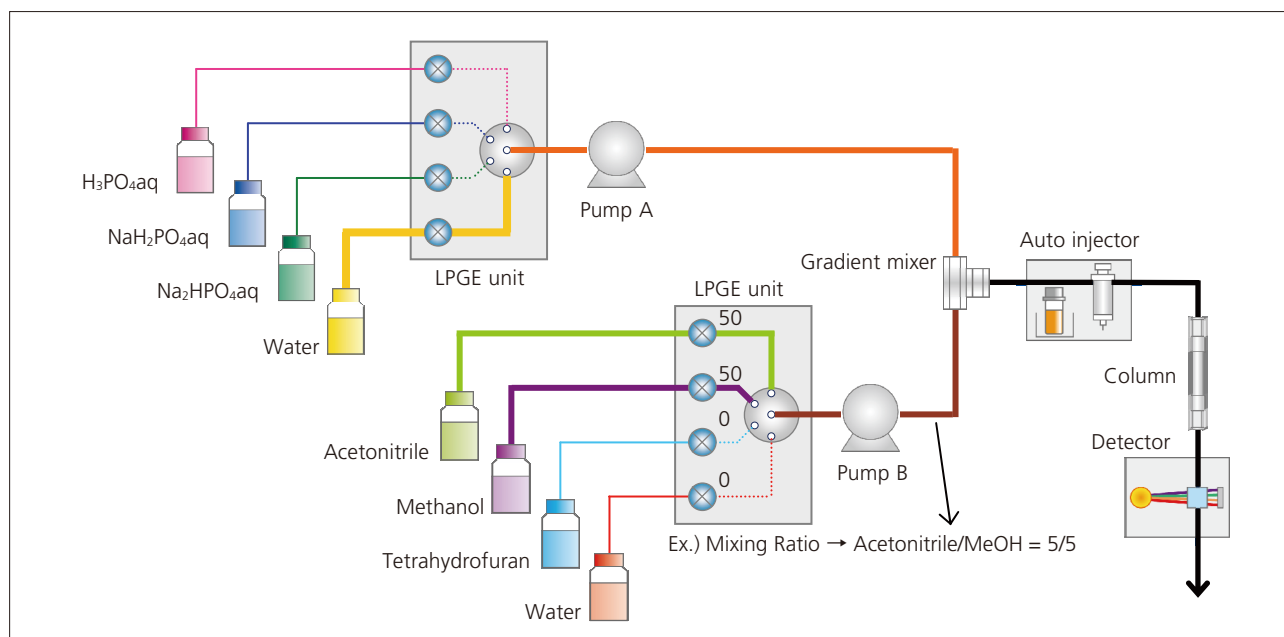


Fig. 1 Flow Diagram of Online Mobile Phase Blending System

### Online Blending of Organic Solvents

Fig. 2 shows the chromatograms obtained from analysis of a standard solution of caffeine using a different mixing ratio of (I) acetonitrile and (II) methanol as the mobile phase for each analysis. Tables 2 and 3 show the analytical conditions. It is clear that the retention time of caffeine varies greatly depending on the proportion of acetonitrile and methanol in the organic solvent mobile phase.

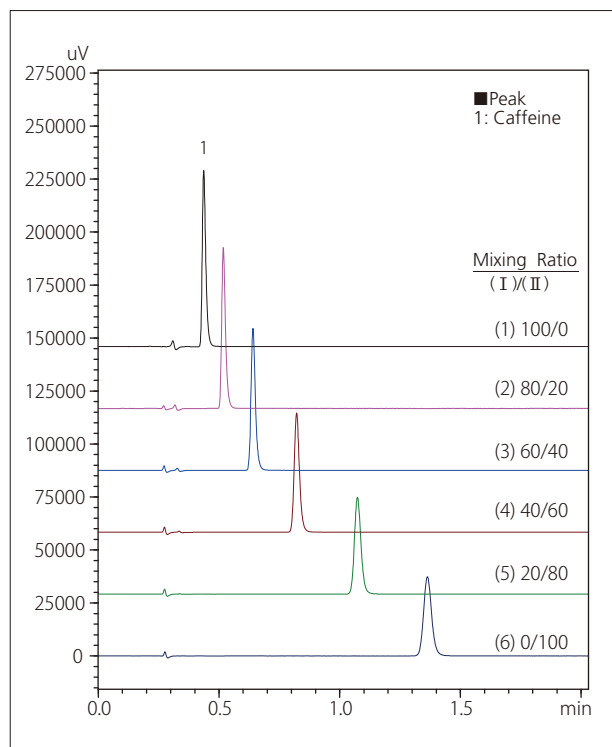


Fig. 2 Chromatograms of a Standard Solution of Caffeine (100 mg/L)

Table 2 Analytical Conditions

Column	: Shim-pack XR-ODS (50 mm L. x 3.0 mm I.D., 2.2 μm)
Mobile Phase	: A: Online blended 20 mmol/L Phosphate (Sodium) Buffer (I): 20 mmol/L Phosphoric acid (H <sub>3</sub> PO <sub>4</sub> ) (II): 20 mmol/L Disodium hydrogenphosphate (Na <sub>2</sub> HPO <sub>4</sub> ) Mixing Ratios of (I)/(II) are shown in Table 3
	: B: Methanol
	: A/B = 7/3 (v / v)
Flowrate	: 1.0 mL/min
Column Temp.	: 40 °C
Detection	: SPD-M20A at (260 nm)
Injection Vol.	: 1 μL

Table 3 Mixing Ratios of Organic Solvents (I) and (II)

No.	Mixing ratio (I)/(II)
(1)	100/0
(2)	80/20
(3)	60/40
(4)	40/60
(5)	20/80
(6)	0/100

### Comparison of Chromatograms and Retention Time Repeatability Obtained Using Online- and Offline-Blended Mobile Phases

Fig. 3 shows the chromatograms obtained from five repeat analyses of caffeine using a 60/40 mixing ratio of organic solvents I and II as the mobile phase prepared using online mixing, and the chromatogram of the same sample obtained using the same blend of mobile phase premixed offline.

The chromatograms obtained using online and offline mobile phase preparation show excellent correlation. In addition, the retention time repeatability obtained from five repeat analyses of the sample using online- and offline-blended mobile phases are shown in Table 4. The results obtained indicate excellent equivalence of retention time repeatability between the two methods.

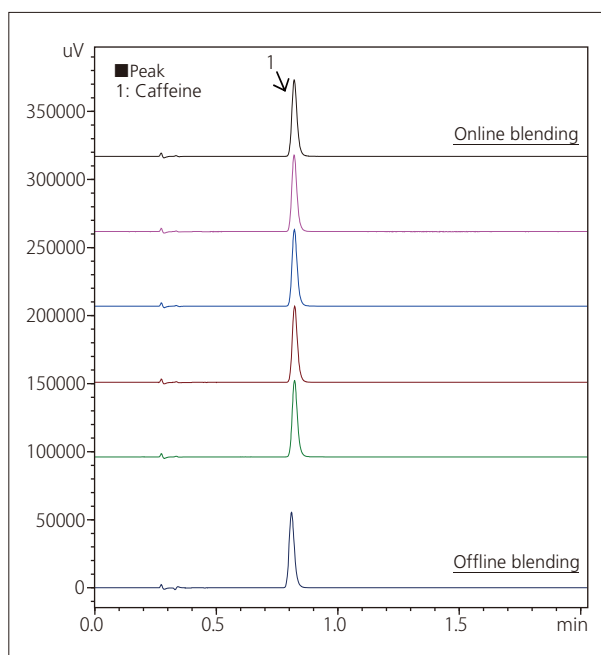


Fig. 3 Chromatograms Obtained Using Online Blending and Offline Blending (I)/(II) = 60/40

Table 4 Repeatability of Retention Time

Compound	Repeatability of retention time RSD (%) n5	
	Online blending	Offline blending
Caffeine	0.09	0.07