

# Application News

High Performance Liquid Chromatograph Nexera™ XR

## Simple Labor-Saving Calibration Curve Creation Using Autosampler Automatic Dilution Function Part 2

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### User Benefits

- ◆ The autosampler's automatic dilution function reduces manual dilution preparation and organic solvent consumption.
- ◆ Simply specify the desired dilution ratio in the batch table, and use the same method file to automatically dilute solutions and create a calibration curve.
- ◆ Setting and management are easy when changing HPLC conditions since a single method file is used regardless of the dilution ratio.

### Introduction

The dilution of standard and sample solutions for HPLC analysis is generally performed manually, using pipettes. However, such work is labor-intensive and time-consuming. In recent years, automation for the purpose of labor-saving is desired to improve work efficiency and productivity. When the organic solvent is a diluent, a large amount of solvent is consumed to prepare standard solutions for calibration curves in volumetric flasks, but the volume of sample solution required for HPLC analysis is only a few tens of  $\mu\text{L}$  or less.

Using the automatic dilution function equipped with Nexera Autosamplers, it is possible to prepare a sample diluted at a user-defined factor and introduce it directly into the analytical column. The Application News 01-00717 describes a simple method for creating calibration curves using ultrapure water as a diluent. This article introduces the analysis using organic solvent as a diluent.

### Pretreatment Program and Operation Overview

A method file contains information such as LC parameters, analytical parameters, and the pretreatment program. The pretreatment program can set various dilution ratios, such as a 100-fold dilution. In addition, when the program is used with the batch add-in (Fig. 2 on the next page), a single method file can be used regardless of the dilution ratio, thereby preventing human errors such as setup mistakes.

The dilution factor and conditions related to the mixing process are configured using the LabSolutions™ workstation. The setup window for the autosampler pretreatment is shown in Fig. 1. Pretreatment program commands are shown in Table 1. In this article, the rinse solution was used as a diluent.

A volume corresponding to the dilution ratio is aspirated from the stock solution vial and dispensed with the diluent into an empty vial (mixing vial) previously set in the autosampler (final volume is 100  $\mu\text{L}$  in this example). The solution in the vial is mixed using the aspiration/dispensing function (pipetting). Finally, a specific amount of the solution is aspirated and injected into the column.

Table 1 Pretreatment Program

Line	Command
1	a3=100/a2
2	n.drain
3	disp 600.0,rs
4	d.rinse
5	vial.n a0,a1
6	n.strk ns
7	aspir a3,ss
8	air.a 0.1,ss
9	d.rinse
10	vial.n rn,sn
11	n.strk ns
12	disp 100.1,rs
13	mix 1,5,40,ss,35
14	n.drain
15	disp 100.0,rs
16	d.rinse
17	inj.p
18	v.inj
19	wait 2.0
20	goto f0
21	end

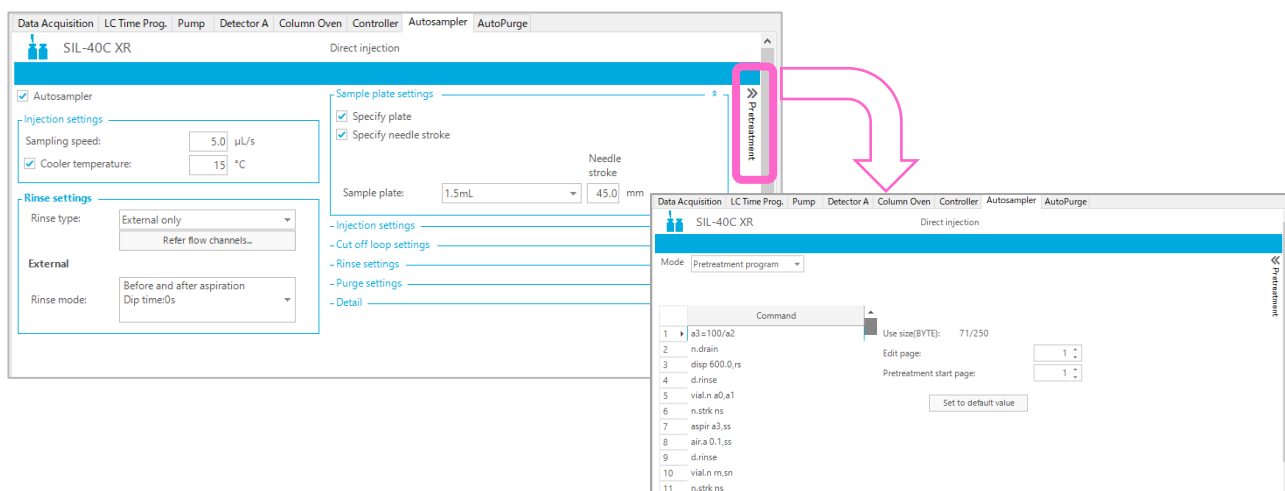


Fig. 1 Setup Window for Autosampler Pretreatment Program

### ■ Setting up a Batch Table

A batch add-in called the SIL pretreatment variable is pre-applied to LabSolutions to populate the batch table with the location of the stock solution vial and any dilution ratio<sup>\*1</sup>. Fig. 2 shows the SIL pretreatment variable setup window for the batch table. Set the plate number (A0), vial number (A1), and dilution ratio of the stock solution vial (A2) in columns A0-A2 of the SIL pretreatment variables. Place empty vials for automatic dilution (mixing) at the locations specified by the tray and vial numbers in the batch table. If the sample type (standard) and level number are set as shown in Fig. 2, a calibration curve is automatically generated after the analysis. Note that separate analytical parameter settings are required.

\*1 Contact Shimadzu for information about applying the batch add-in.

### ■ Automatic Dilution Analysis for Mixed Standard Solution

An automatic dilution analysis was performed using a mixed standard solution of coumarin and cinnamaldehyde at a concentration of 100 mg/L each (prepared with acetonitrile). Table 2 shows the analytical conditions. The pretreatment program is the same as in Table 1. 1.0 mL polypropylene vials were used for mixing, and septum vials were used for the stock solutions (standard and sample). Acetonitrile was used as the diluent for the rinse solution<sup>\*2</sup>. Fig. 3 shows the chromatograms of mixed standard solution (concentration after automatic dilution: 1.0 mg/L each) diluted 100-fold with acetonitrile.

\*2 For HPLC equipped with a multi-rinse function, use the rinse solution specified in the parameter settings of an autosampler.

Table 2 Analytical Conditions

System:	Nexera XR
Column:	Shim-pack™ GIST-HP C18 <sup>*3</sup> (150 mm × 3.0 mm I.D., 3 μm)
Flowrate:	0.8 mL/min
Mobile Phase:	A) Water B) Acetonitrile
Time Program:	50 % B (0-2.0 min) → 60 % B (4.0 min) → 100 % B (4.1 - 5.0 min) → 50 % B (5.1 - 5.5 min)
Column Temp.:	40 °C
Sample:	100 mg/L Coumarin and Cinnamaldehyde in Acetonitrile
Injection Volume:	5 μL
Needle Stroke:	45 mm
Vial for Mixing:	Shimadzu Vial, LC, 1 mL, Polypropylene <sup>*4</sup>
Vial for Stock Solution and Sample:	SHIMADZU LabTotal™ for LC 1.5 mL, Glass <sup>*5</sup>
Diluent:	Rinse solution (Acetonitrile)
Detection:	Ch1 (Coumarin): 276 nm, Ch2 (Cinnamaldehyde): 288 nm (SPD-M40)

\*3 P/N: 227-30040-05, \*4 P/N: 228-31600-91, \*5 P/N: 227-34001-01

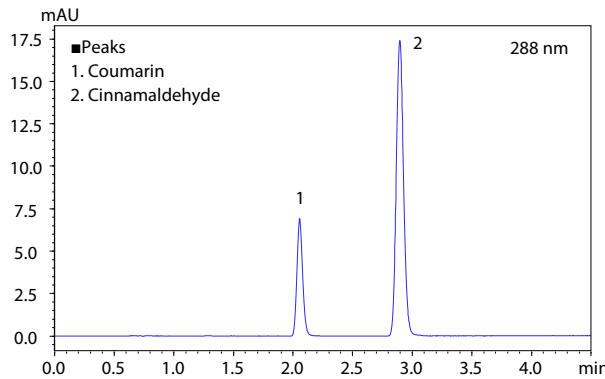
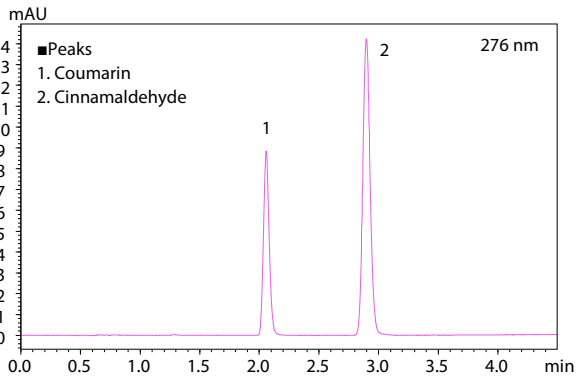


Fig. 3 Chromatograms for Mixed Standard Solution of  
Coumarin and Cinnamaldehyde  
(Dilution Ratio: 100, Concentration after Automatic Dilution: 1.0 mg/L each)

### ■ Repeatability

The mixed standard solution (Dilution Ratio: 500, the concentration after automatic dilution was 0.2 mg/L each) was analyzed six times consecutively using the autosampler automatic dilution function. The repeatability (%RSD) of the retention time and the peak area are shown in Table 3.

Table 3 Repeatability (%RSD) in Six Replicate Analyses

Compound	Retention time	Peak area
Coumarin	0.09	1.46
Cinnamaldehyde	0.06	1.82

Analysis	Tray Name	Vial#	Sample Name	Sample ID	SIL Pretreatment Variables	Sample Type	Level#	Inj. Volume	Method File
1	2	3	2mix_100	x1000	A0=2,A1=51,A2=1000	1:Standard	1	5	45_5.5m.lcm
2	2	4	2mix_100	x500	A0=2,A1=51,A2=500	1:Standard	2	5	45_5.5m.lcm
3	2	5	2mix_100	x200	A0=2,A1=51,A2=200	1:Standard	3	5	45_5.5m.lcm
4	2	6	2mix_100	x100	A0=2,A1=51,A2=100	1:Standard	4	5	45_5.5m.lcm
5	2	7	2mix_100	x50	A0=2,A1=51,A2=50	1:Standard	5	5	45_5.5m.lcm
6	2	8	2mix_100	x20	A0=2,A1=51,A2=20	1:Standard	6	5	45_5.5m.lcm
7	2	9	2mix_100	x10	A0=2,A1=51,A2=10	1:Standard	7	5	45_5.5m.lcm

Plate No. →  
Vial No. →  
Dilution ratio →

SIL Pretreatment Variables

A0: 2

A1: 51

A2: 10

A3:

A4:

A5:

A6:

A7:

OK

Cancel

Fig. 2 Setup Window for Batch Table SIL Pretreatment Variables

## ■ Calibration Curve

Three calibration curves created with automatic dilution function for coumarin and cinnamaldehyde (concentration range of 0.1-10 mg/L each) were analyzed.

Excellent linearities with coefficients of determination ( $r^2$ ) of 0.999 or greater were obtained. Calibration curves are displayed in Fig. 4.

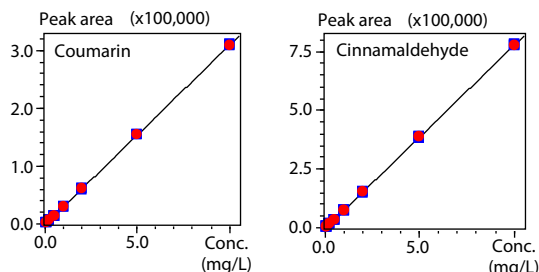


Fig. 4 Calibration Curves (n = 3)

## ■ Analysis of Cinnamon

A Sample of commercial *Cinnamomum cassia* was used. The pretreatment protocol is the same as the process up to filtration in Fig.3 from Application News No. 01-00233. Note that samples were manually diluted with acetonitrile at the final step of pretreatment in No. 01-00233-EN, but the sample was automatically diluted with an autosampler in this article. The pretreatment protocol is shown in Fig. 5. Acetonitrile was used as the extraction solvent. Lipids were removed using a dispersive solid phase extraction (dSPE) cartridge (Merck Supel™ QuE Z-Sep+). The cartridge eliminates the need to carry out conditioning before loading samples, which simplifies operations.

Fig. 6 shows chromatograms obtained by diluting *Cinnamomum cassia* extracts 100-fold with acetonitrile using an automatic dilution function. The two target compounds were well separated from the contaminants. The analytical results (concentration after automatic dilution) are shown in Table 4. Table 4 also shows the analytical results obtained when standard solutions for calibration curves were prepared manually, and the 100-fold dilution with acetonitrile of the pretreated cinnamon extract was performed manually.

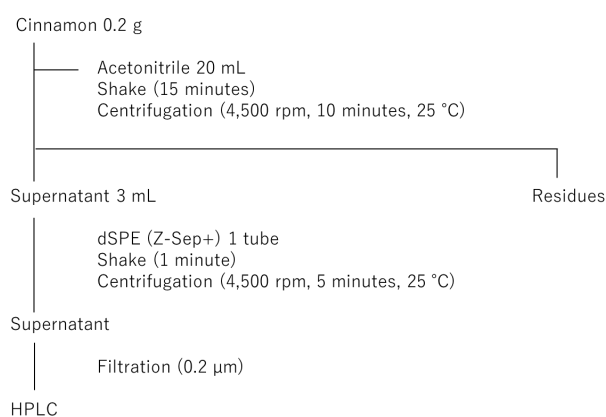


Fig. 5 Pretreatment Protocol

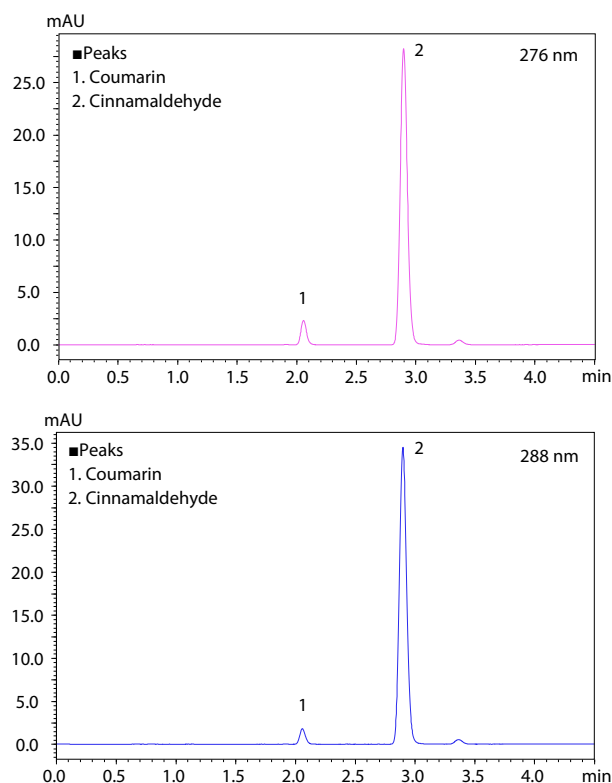


Fig. 6 Chromatograms of *Cinnamomum Cassia* Obtained by Automatic Dilution (Dilution Ratio: 100)

Table 4 Analytical Results (n = 3)

Compound	Concentration (mg/L)*6	
	Automatic	Manual
Coumarin	0.28 (0.72)	0.27 (0.38)
Cinnamaldehyde	1.90 (1.08)	2.02 (0.59)

\*6 Numbers in parentheses indicate %RSD, n = 3.

## ■ Conclusion

By automatically preparing standard solutions for calibration curves at any dilution ratio and analyzing them directly, it was possible to create a calibration curve easily and accurately. In addition, when performing the determination of an actual sample, dilution could be performed automatically. It was confirmed that automatic determination was possible with only simple pretreatment. The calibration curve creation method described in this article is expected to lead to labor-saving for analysts and solvent-saving from a sustainability viewpoint.

## Related Applications

1. Simultaneous Quantitative Analysis of Coumarin and Cinnamaldehyde in Cinnamon Produced in Different Regions  
[Application News No. 01-00233](#)
2. Simple Labor-Saving Calibration Curve Creation Using Autosampler Automatic Dilution Function  
[Application News No. 01-00717](#)

Contact Shimadzu for information about applying batch add-ins.

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Some products may be updated to newer models.



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Shim-pack

> Shim-pack GIST Series

HPLC Column Solutions

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