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Application News

High Performance Liquid Chromatograph Nexera™ X3

Automated Preparation of Calibration Curves Using the Autosampler's Dilution Function

-Application to Organic Acid Analysis-

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User Benefits

- The auto-dilution function equipped in the autosampler eliminates the need for manual dilution during calibration curve creation and sample preparation.
- Samples can be automatically diluted to create a calibration curve by specifying the desired dilution factor in the batch table.
- Because the same method file can be used across different dilution factors, only minor adjustments are needed when changing dilution conditions.

■ Introduction

In LC quantification, the dilution of standard and sample solutions is typically performed manually using pipettes, which is a labor-intensive and time-consuming process. Recent advancements have called for automation to reduce labor and improve efficiency. By automating the dilution process, workflows can be streamlined, leading to increased productivity. The Nexera series autosamplers are equipped with an automatic pretreatment function that enables automated dilution. Using the auto-dilution function, samples can be automatically prepared at any desired dilution factor and directly introduced into the analytical column. This article presents a method to automate the creation of calibration curves and subsequent quantification workflows using the autosampler's auto-dilution function.

■ Pretreatment Program and Operational Overview

The method file consolidates information such as instrument parameters, analytical parameters, and the pretreatment program. The pretreatment program within the method file allows for the setting of any desired dilution factor, however, multiple method files are required for each dilution factor. In contrast, the batch add-in (Fig. 2 on the next page) allows the use of a single method file to set multiple dilution factors, thereby preventing human errors such as incorrect settings. The pretreatment program and its settings window are presented in Table 1 and Fig. 1, respectively. In this study, a rinse solution was used as the diluent. A volume of the undiluted solution corresponding to the desired dilution factor is aspirated from the vial and dispensed with the rinse solution into a pre-set empty vial (mixing vial) in the autosampler (total 100 µL). The mixture in the mixing vial is then stirred, after which the specified volume of the mixture is aspirated and introduced 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

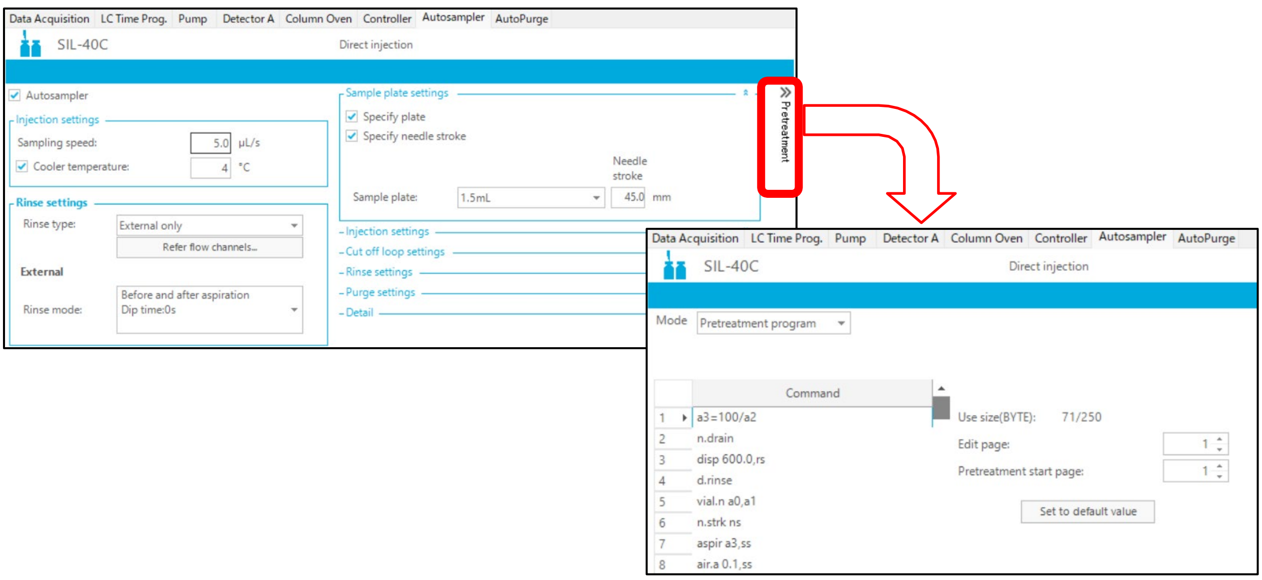


Fig. 1 Setup Window for Autosampler Pretreatment Program

■ How to Set up the Batch Table

A batch add-in*, "SIL pretreatment variables", is applied in advance to LabSolutions™ to set the vial locations of the undiluted solution and the desired dilution factors in the batch table. Fig. 2 shows the SIL pretreatment variable settings window of the batch table, where the plate number (A0) and vial number (A1) for the undiluted solution, as well as the dilution factor (A2), are specified. Empty vials for automatic dilution (mixing vials) are placed at the locations specified by the tray number and vial number in the batch table.

* For applying the batch add-in, please contact us.

■ Automated Dilution Analysis of Organic Acid Standard Solution

Automated dilution analysis was conducted using a 1000 mg/L mixed standard solution of ten organic acids. The analytical conditions are shown in Table 2 (pretreatment program : Table 1). 1.0 mL polypropylene vials were used as mixing vials, and septum-sealed vials were used for the undiluted standard solution. Rinse solution (ultrapure water) was used as the diluent. Fig. 3 shows the chromatogram of the 100-fold diluted organic acid mixed standard solution (concentration after auto-dilution : 10 mg/L for each organic acid). The Nexera Organic Acid Analysis System (pH-buffered electric conductivity detection) was employed for this analysis.

Table 2 Analytical Conditions and Target Compounds

System	: Nexera Organic Acid Analysis System
Sample	: (1) Phosphoric acid、(2) Citric acid、(3) Pyruvic acid、(4) Malic acid、(5) Succinic acid、(6) Lactic acid、(7) Formic acid、(8) Fumaric acid、(9) Acetic acid、(10) Pyroglutamic acid
Mobile phase	: 5 mmol/L p-toluensulfonic acid (Reagents kit for Organic Acid Analysis System*1)
pH buffering solution	: 5 mmol/L p-toluensulfonic acid, 20 mmol/L Bis-Tris*2, 0.1 mmol/L EDTA*3 (Reagents kit for Organic Acid Analysis System *1)
Column	: Shim-pack™ SCR-102H (300 mm×8 mm I.D., 7 µm)*4×2
Guard column	: SCR-102H (50 mm ×6 mmI.D. , 10 µm)*5
Analytical conditions	
Flow rate	: 0.8 mL/min (mobile phase and pH buffering)
Mixer	: Organic Acid Analysis Plumbing Kit (MR)*6
Injection Vol.	: 20 µL
Needle stroke	: 45 mm
Sample aspiration speed	: 5 µL/sec
Vial for mixing	: Shimadzu Vial, LC, 1 mL, Polypropylene*7
Vial for standard	: Shimadzu Vial, LC, 1.5 mL, Polypropylene*8
Vial for sample	: Shimadzu Vial, LC, 1.5 mL, Polypropylene*8
Diluent	: Ultrapure water
Column Temp.	: 45 °C
Detection	: Conductivity

*1 P/N : 228-61465-91
*2 Bis-(2-hydroxyethyl)iminotris(hydroxymethyl)methane
*3 Ethylenediaminetetraacetic acid、*4 P/N : 228-17893-91
*5 P/N : 228-17924-91、*6 P/N : 228-77532-41
*7 P/N : 228-31600-91、*8 P/N : GLC-IVS-100

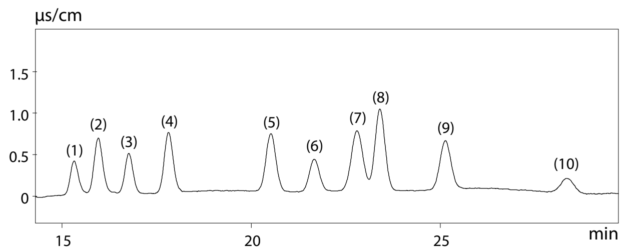


Fig. 3 Chromatogram of Organic Acid Solution after Automated Dilution
(Dilution factor : 100-fold, Concentration after auto-dilution : 10 mg/L)
(Peak numbers correspond to those listed in Table 2)

■ Repeatability and Linearity of the Calibration Curve

Table 3 shows the repeatability (%RSD) of retention times and peak areas obtained from six repeated analyses of each organic acid at 10 mg/L, prepared by automated 100-fold dilution of a 1000 mg/L mixed standard solution using ultrapure water. Table 4 presents the coefficients of determination (r^2) for each organic acid based on calibration curves constructed at five concentration levels : 10, 50, 100, 500, and 1000 mg/L. All organic acids demonstrated excellent linearity, with r^2 values greater than 0.999.

Table 3 Repeatability (%RSD) of Each Organic Acid in Six Replicate Analyses

	Retention time	Peak area
(1) Phosphoric acid	0.01	1.48
(2) Citric acid	0.01	1.46
(3) Pyruvic acid	0.01	2.27
(4) Malic acid	0.01	2.83
(5) Succinic acid	0.02	2.83
(6) Lactic acid	0.01	2.33
(7) Formic acid	0.01	1.63
(8) Fumaric acid	0.01	0.92
(9) Acetic acid	0.02	2.62
(10) Pyroglutamic acid	0.04	2.70

Table 4 Linearity of Each Organic Acid in Six Replicate Analyses

	Calib. range (mg/L)	Coefficient of determination (r^2)
(1) Phosphoric acid	10~1000	0.9999
(2) Citric acid	10~1000	0.9999
(3) Pyruvic acid	10~1000	0.9999
(4) Malic acid	10~1000	0.9999
(5) Succinic acid	10~1000	0.9999
(6) Lactic acid	10~1000	0.9999
(7) Formic acid	10~1000	0.9999
(8) Fumaric acid	10~1000	0.9999
(9) Acetic acid	10~1000	0.9999
(10) Pyroglutamic acid	10~1000	0.9999

Tray Name	Vial#	Sample Name	Sample ID	SIL Pretreatment	Data Comment	Sample Type	Level	Inj. Volume	Method File
1	1	Organic Acid	100-fold	A0=1;A1=6;A2=100	A0:Plate No. A1:Vial No. A2:Dilution ratio	1:Standard:(I)	1	20	Auto dilution OA STD.lcm
1	2	Organic Acid	20-fold	A0=1;A1=6;A2=20	A0:Plate No. A1:Vial No. A2:Dilution ratio	1:Standard	2	20	Auto dilution OA STD.lcm
1	3	Organic Acid	10-fold	A0=1;A1=6;A2=10	A0:Plate No. A1:Vial No. A2:Dilution ratio	1:Standard	3	20	Auto dilution OA STD.lcm
1	4	Organic Acid	2-fold	A0=1;A1=6;A2=2	A0:Plate No. A1:Vial No. A2:Dilution ratio	1:Standard	4	20	Auto dilution OA STD.lcm
1	5	Organic Acid	no dilution			1:Standard	5	20	No dilution OA STD.lcm

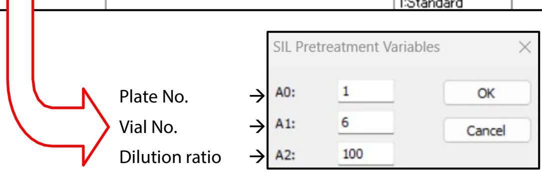


Fig. 2 Batch Table Screen for SIL Pretreatment Variable Settings (Batch Add-in)

■ Comparison of Peak Areas with Manual Dilution

Table 5 presents the accuracy of peak areas obtained from automated dilution (dilution factor: 20-fold, calibration level: 50 mg/L), using the peak areas from manually prepared samples as the 100% reference. The results confirm that the automated dilution achieved comparable performance to manual dilution.

Table 5 Peak Area Accuracy in Automated Dilution
(Mean of six replicate measurements)

	50 mg/L
(1) Phosphoric acid	-0.3%
(2) Citric acid	0.9%
(3) Pyruvic acid	0.6%
(4) Malic acid	-0.1%
(5) Succinic acid	-0.3%
(6) Lactic acid	-0.1%
(7) Formic acid	0.0%
(8) Fumaric acid	-1.4%
(9) Acetic acid	-0.6%
(10) Pyroglutamic acid	0.7%

■ Analysis of Organic Acids in Bath Salt

Quantitative analysis of organic acids in a commercially available bath salt was performed using the autosampler's auto-dilution function. For sample pretreatment, 1 g of the bath salt was diluted to a final volume of 10 mL with ultrapure water and then filtered through a 0.45 µm membrane filter. Fig. 4 shows the chromatogram of the bath salt sample after 50-fold dilution using the auto-dilution function. Table 6 summarizes the analytical results (concentrations after automatic dilution).

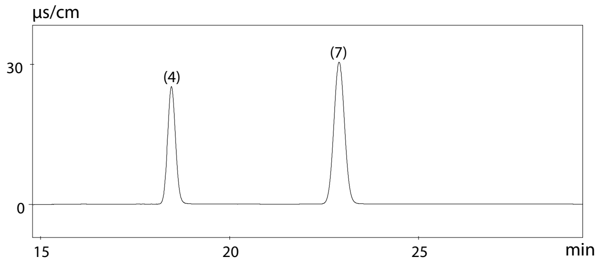


Fig. 4 Chromatogram of Bath Salt after Automated Dilution

Table 6 Analytical Results of Bath Salt

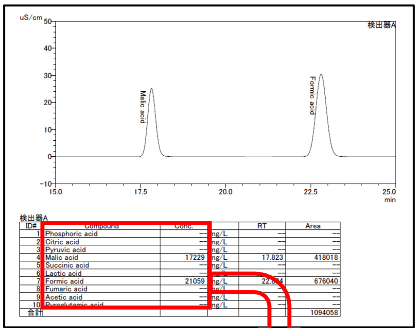
	Conc. (mg/L)	%RSD (n=3)
(1) Phosphoric acid	ND	-
(2) Citric acid	ND	-
(3) Pyruvic acid	ND	-
(4) Malic acid	344.58	1.53
(5) Succinic acid	ND	-
(6) Lactic acid	ND	-
(7) Formic acid	421.18	1.84
(8) Fumaric acid	ND	-
(9) Acetic acid	ND	-
(10) Pyroglutamic acid	ND	-

■ Automatic Calculation of Concentrations in Undiluted Sample Solution

When the dilution factor of the sample is entered in the dilution factor cell in the batch table, the concentration displayed in the analysis results will represent the concentration of the target compound in the undiluted sample solution (before dilution). Fig. 5 shows the dilution factor and report output settings screen, and Fig. 6 shows an example of a report generated from the bath salt analysis.

Sample Name	Dil. Factor	SIL Pretreatment	Report Output	Report Format File
blank	1		✓	Report_organic acid.lsr
Organic Acid	1	A0=1;A1=6;A2=100	✓	Report_organic acid.lsr
Organic Acid	1	A0=1;A1=6;A2=20	✓	Report_organic acid.lsr
Organic Acid	1	A0=1;A1=6;A2=10	✓	Report_organic acid.lsr
Organic Acid	1	A0=1;A1=6;A2=2	✓	Report_organic acid.lsr
Organic Acid	1		✓	Report_organic acid.lsr
blank	1		✓	Report_organic acid.lsr
Bath salt	50	A0=1;A1=54;A2=50	✓	Report_organic acid.lsr

Fig. 5 Dilution Factor and Report Output Settings in Batch Table



Compound	Conc.	
Phosphoric acid	-----	mg/L
Citric acid	-----	mg/L
Pyruvic acid	-----	mg/L
Malic acid	17229	mg/L
Succinic acid	-----	mg/L
Lactic acid	-----	mg/L
Formic acid	21059	mg/L
Fumaric acid	-----	mg/L
Acetic acid	-----	mg/L
Pyroglutamic acid	-----	mg/L

Fig. 6 Report Generated from the Bath Salt Analysis

■ Conclusion

By utilizing the auto-dilution function in the autosampler, the standard undiluted solution for calibration curve creation can be automatically diluted at any desired dilution ratio. This eliminates the need for manual sample dilution and streamlines the calibration curve creation workflow. Additionally, since the auto-dilution function can also be applied to sample solutions, only the preparation of undiluted standard and sample solutions is required, allowing for a fully automated process from calibration curve creation to quantitation. As a result, the reduction in pretreatment work contributes to improved work efficiency and productivity.

Please contact us for information on applying batch add-in.

＜Related Applications＞

1. Simple Labor-Saving Calibration Curve Creation Using Autosampler Automatic Dilution Function, Application News No.01-00717-EN

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