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Application News

No. L548

High-Performance Liquid Chromatography

Fermentation Processes Monitoring Using a Nexera™ Dual Injection System

When microorganisms decompose substances to produce useful materials, that process is called fermentation. Fermentation is used not only for producing foods, but in recent years is also being widely used in industrial fields. In such fields, organic acids, sugars, amino acids, and other groups of compounds are multilaterally measured to understand the fermentation process or optimize conditions. In the case of HPLC analysis, the appropriate separate mode and detection method can differ depending on the component class. This requires multiple independent pieces of equipment for each analysis.

This article introduces an example of monitoring a fermentation process using a dual injection system to evaluate two types of analysis simultaneously with only one system.

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Overview of Dual Injection System

Using the optional dual injection function available on Nexera SIL-40 series autosamplers, samples can be injected into two flow to run two independent analysis in one platform simultaneously. The two sets of results obtained by the system are integrated in one data file to ensure data traceability to specific samples. The corresponding method and batch files are also integrated in one file to simplify analysis operations.

Fig. 1 shows a flow channel diagram of a dual injection system used for two analyses. In one channel, organic acids are analyzed using an ion-exclusion column, post-column pH-buffering, and an electrical conductivity detector, whereas in the other channel, sugars are analyzed using a ligand exchange column and a refractive index detector. Because organic acid analysis and sugar analysis involve different column temperatures, columns are temperature-controlled separately in two CTO-40S column ovens.

Analysis of Organic Acids

Flow channel (1) was used to analyze organic acids. Fig. 2 shows a chromatogram for a standard mixture solution of organic acids (100 mg/L each of citric, malic, lactic, formic, and acetic acids) obtained using the analytical conditions shown in Table 1.

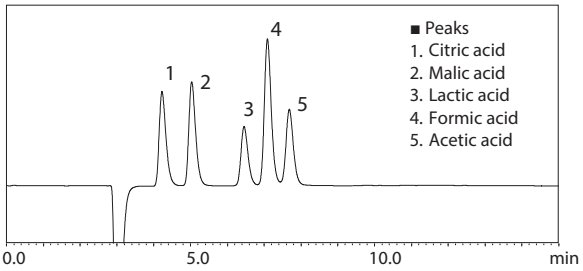

Fig. 2 **Chromatogram of Standard Organic Acid Mixture Solution**

Table 1 **Analytical Conditions for Organic Acid Analysis**

Column:	Shim-pack™ Fast-OA (100 mm L × 7.8 mm I.D., 5 µm) (two used)
Guard column:	Shim-pack™ Fast-OA (G) (10 mm L × 4.0 mm I.D., 12 µm)
Mobile phase flowrate:	0.8 mL/min
pH buffer solution flowrate:	0.8 mL/min
Mobile phase:	Aqueous 5.0 mmol/L *p*-toluenesulfonic acid solution
pH buffer solution:	Aqueous mixture of 5.0 mmol/L *p*-toluenesulfonic acid, 20 mmol/L Bis-Tris, 0.1 mmol/L EDTA 4H
Column temperature:	40 °C
Injection volume:	10 µL
Detector:	Electrical conductivity detector

For details regarding analysis using the Shim-pack Fast-OA column and post-column buffering, refer to the Technical Report, "High-Speed Analysis of Organic Acids Using Shim-pack Fast-OA and pH-Buffered Electrical Conductivity Detection" (C190-E237).

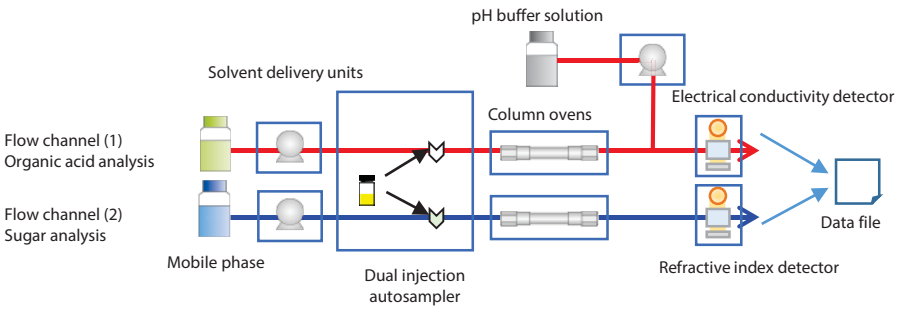

Fig. 1 **Dual Injection Flow Channel Diagram**

Analysis of Sugars

Flow channel (2) was used to analyze sugars. Fig. 3 shows a chromatogram for a standard mixture solution of sugars (1000 mg/L each of glucose, fructose, mannose, and lactose) obtained using the analytical conditions shown in Table 2.

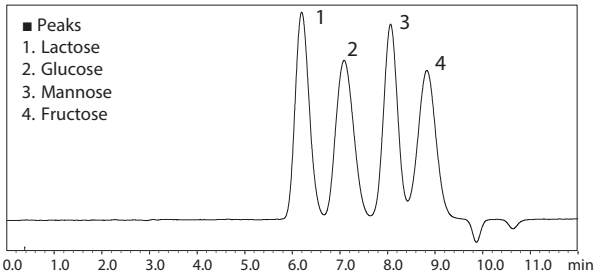

Fig. 3 **Chromatogram of Standard Sugar Mixture Solution**

Table 2 **Analytical Conditions for Sugar Analysis**

Column:	Shim-pack SCR-101C (300 mm L × 7.9 mm I.D., 10 µm)
Guard column:	Shim-pack guard column SCR (C) (50 mm L × 4 mm I.D., 10 µm)
Flowrate:	1.0 mL/min
Mobile phase:	Water
Column temperature:	80 °C
Injection volume:	10 µL
Detector:	Refractive index detector

Reproducibility

The average retention time and relative standard deviation of area (%RSD) values from six analysis repetitions are shown in Table 3 for the standard organic acid mixture solution (200 mg/L each) and in Table 4 for the standard sugar mixture solution (1000 mg/L each). For all components, the results indicated a %RSD of less than 1 % for both retention time and area.

Table 3 **Retention Time and Area Reproducibility of Organic Acids (n=6)**

Component	Average Retention Time (min)	Retention Time %RSD	Area Value %RSD
Citric acid	4.21	0.023	0.25
Malic acid	5.02	0.020	0.07
Lactic acid	6.44	0.018	0.39
Formic acid	7.08	0.015	0.34
Acetic acid	7.67	0.013	0.53

Table 4 **Retention Time and Area Reproducibility of Sugars (n=6)**

Component	Average Retention Time (min)	Retention Time %RSD	Area Value %RSD
Lactose	6.20	0.013	0.08
Glucose	7.10	0.055	0.09
Mannose	8.07	0.010	0.13
Fructose	8.83	0.008	0.11

Calibration Curves

Fig. 4 shows the calibration curves and Table 5 the calibration range and contribution rate for the organic acids. Fig. 5 shows the calibration curves and Table 6 the calibration range and contribution rate for the sugars.

The contribution rates over $R^2 = 0.9998$ obtained for the five organic acid components and four sugar components indicate good linearity.

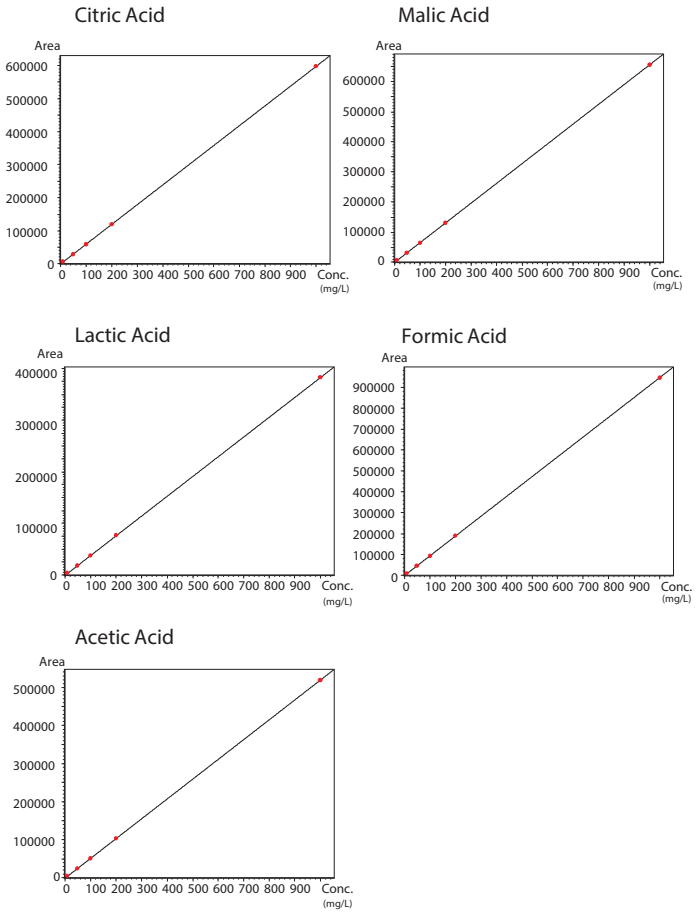

Fig. 4 **Calibration Curves for Organic Acids**

Table 5 **Linearity of the Organic Acids**

Compound	Range (mg/L)	Coefficient (R^2)
Citric acid	10-1000	0.9999
Malic acid	10-1000	0.9999
Lactic acid	10-1000	0.9999
Formic acid	10-1000	0.9999
Acetic acid	10-1000	0.9999

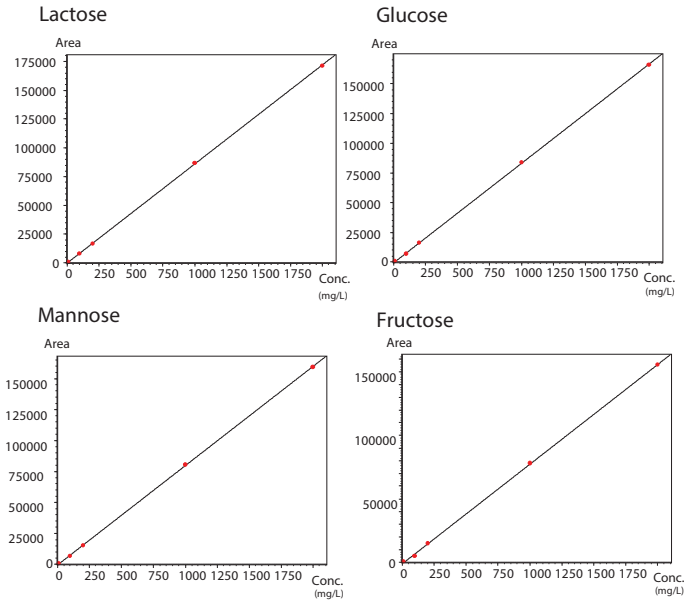

Fig. 5 **Calibration Curves of Sugars**

Table 6 **Linearity of the Sugars**

Compound	Range (mg/L)	Coefficient (R^2)
Lactose	10-2000	0.9999
Glucose	10-2000	0.9999
Mannose	10-2000	0.9998
Fructose	10-2000	0.9998

Evaluation of Carryover

Carryover was evaluated for organic acids (citric acid and lactic acid) and a sugar (lactose). To rinse the autosampler, the interior and exterior of the sample needle were rinsed with water.

Organic acid carryover evaluation results are shown in Fig. 6 and sugar carryover evaluation results in Fig. 7. Carryover was 0.0055 % for citric acid, 0.0069 % for lactic acid, and 0.0098 % for lactose, which are sufficiently low to confirm that carryover did not affect quantitation results.

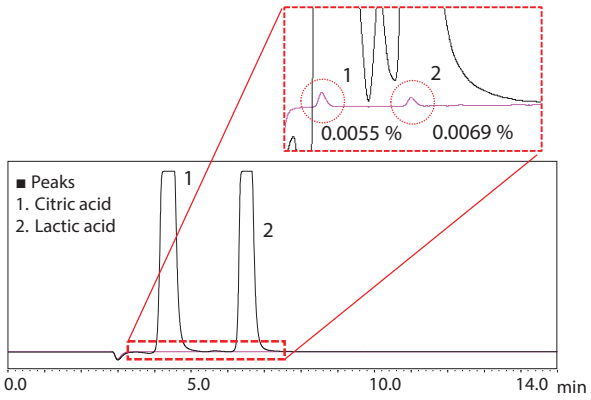

Fig. 6 **Evaluation of Citric and Lactic Acid Carryover**

Black line: Aqueous standard mixture solution of 100 g/L citric acid and lactic acid
Red line: Blank

Black line: 20 g/L aqueous lactose solution
Red line: Blank

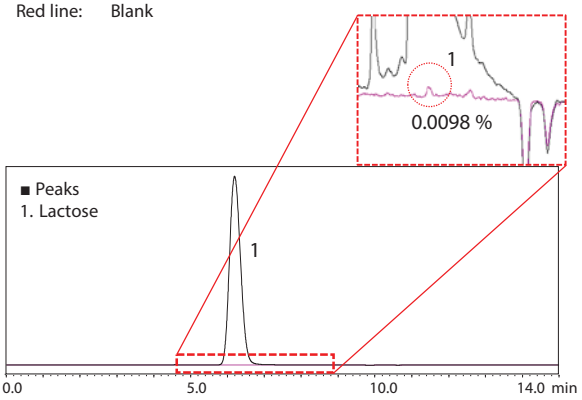

Fig. 7 **Evaluation of Lactose Carryover**

Sample Pretreatment

Yogurt was added to milk and fermented by heating it to 40 °C in a commercial yogurt maker. Samples were then obtained by sampling the mixture at fixed intervals after starting fermentation. Samples were pretreated according to the following procedure.

(1) Weigh 1 g of yogurt. Add 4 mL of 5 mmol/L aqueous *p*-toluenesulfonic acid solution and 1 mL of chloroform.
(2) Shake vigorously for one minute and then separate by centrifuge for one minute at 10,000 rpm.
(3) Collect the supernatant and filter it through a 0.45 µm pore filter.
(4) Use the filtrate diluted by ten times as the sample for analysis.

Analysis of Yogurt

For this article, samples were analyzed at fixed intervals (0.0, 1.0, 2.0, 3.5, 5.5, 7.0, and 8.5 hours) after fermentation started.

The chromatogram from analyzing organic acids in the sample acquired 3.5 hours after fermentation started is shown in Fig. 8 and the chromatogram from analyzing sugars in the same sample is shown in Fig. 9. Organic acids detected were citric and lactic acids and the sugar detected was lactose. These results were used for monitoring the fermentation process.

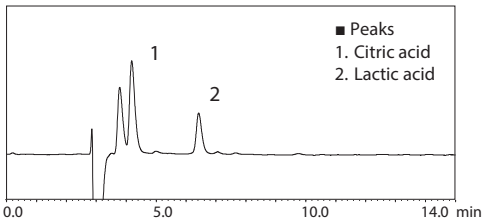

Fig. 8 **Chromatogram from Analyzing Organic Acids in Yogurt after 3.5 Hours of Fermentation**

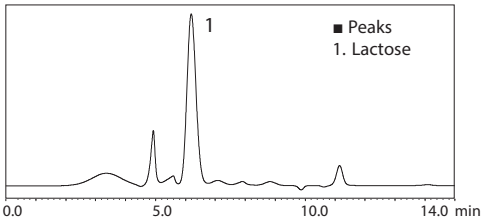

Fig. 9 **Chromatogram from Analyzing Sugars in Yogurt after 3.5 Hours of Fermentation**

Evaluation of Yogurt Recovery Rate

The sample taken 3.5 hours after fermentation started was used to evaluate the recovery rate of added organic acids and sugars. In step (1) of the pretreatment process, 1 g of yogurt was weighed. Then 2.6 mL of a 5 mmol/L aqueous *p*-toluenesulfonic acid solution, 0.7 mL of a 400 mg/L aqueous organic acid mixture solution, 0.7 mL of a 2000 mg/L aqueous sugar mixture solution, and 1 mL of chloroform were added to the yogurt. Chromatograms with and without the standard organic acids added are shown in Fig. 10, the recovery rates of organic acids are indicated in Table 7, chromatograms with and without the standard sugars added are shown in Fig. 11, and the recovery rates of sugars are indicated in Table 8. Good recovery rates between 94.6 and 101.8 % were obtained for both organic acids and sugars.

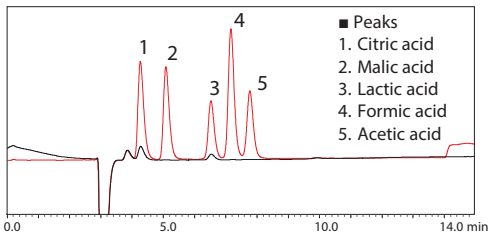

Fig. 10 **Chromatograms of Organic Acids (Red: With standard added; Black: Without standard added)**

Table 7 **The recovery rate of target component (Organic acid) (Additive concentration: 56 mg/L, calculated as concentration after pretreatment)**

Compound	Actual measurement of Spiked sample (mg/L)	Actual measurement of Unspiked sample (mg/L)	Recovery Rate (%)
Citric acid	65.9	10.0	99.8
Malic acid	57.0	Not detected	101.8
Lactic acid	61.4	8.4	94.6
Formic acid	56.4	Not detected	100.6
Acetic acid	56.1	Not detected	100.3

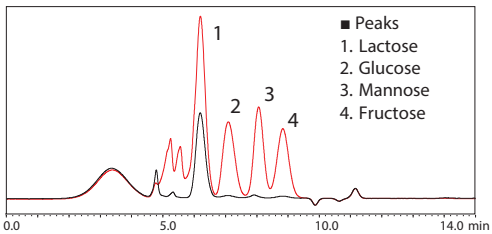

Fig. 11 **Chromatograms of Sugars (Red: With standard added; Black: Without standard added)**

Table 8 **The recovery rate of target component (Sugar) (Additive concentration: 280 mg/L, calculated as concentration after pretreatment)**

Compound	Actual measurement of Spiked sample (mg/L)	Actual measurement of Unspiked sample (mg/L)	Recovery Rate (%)
Lactose	544.0	257.1	102.4
Glucose	282.4	Not detected	100.9
Mannose	301.5	Not detected	107.7
Fructose	298.6	Not detected	106.6

Time-Course Evaluation of Changes during Yogurt Fermentation

Samples taken at fixed intervals (0.0, 1.0, 2.0, 3.5, 5.5, 7.0, and 8.5 hours) after fermentation started were analyzed to confirm the quantities of organic acids and sugars they contain.

The change in organic acid content after each fermentation interval is shown in Fig. 12 and the change in sugar content in Fig. 13. These results indicate that lactose is decomposed to form lactic acid as fermentation progresses.

Note that the indicated concentration values were converted based on the original yogurt solution.

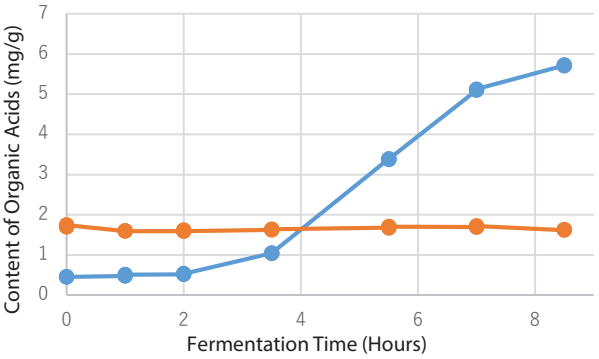

Fig. 12 **Content of Organic Acids in Yogurt (Blue: Lactic acid; Orange: Citric acid)**

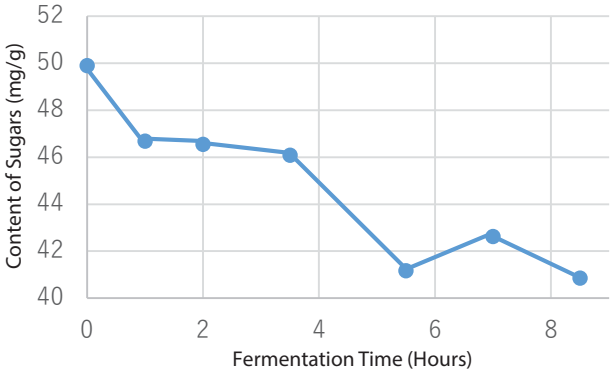

Fig. 13 **Content of Sugars in Yogurt**

Summary

Using the Nexera series dual injection system enabled two different types of analyses (organic acid analysis and sugar analysis) to be performed simultaneously in one system.

Using the dual injection system, we were able to monitor the progress of fermentation by evaluating time-course changes in organic acid and sugar content levels during fermentation. The dual injection system makes it easy to analyze multiple aspects of samples by analyzing samples and data from multiple groups of compounds simultaneously.

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