

# High-Throughput Optimization of Fermentation Condition Using Dual Injection System

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## 1. Introduction

Fermentation is the process in which microorganisms decompose the substrates through metabolism to produce organic compounds such as sugars and organic acids. This process has been employed in not only the food industry but also various industries such as the pharmaceutical and chemical industries. To measure sugars and organic acids, and multilaterally investigate their level, contributes to optimizing the fermentation conditions. However, each compound class often requires a dedicated separation and detection condition. For this reason, several independent HPLC platforms specialized for each purpose are necessary to obtain the complete data set in a certain period.

In this study, we examined the simultaneous analysis of sugars and organic acids with the Nexera™ Dual Injection System, which enables us to run two independent analyses in one platform with a single method file. The system enabled separation and detection at the same time in the different analytical conditions for each of the sugars and organic acids. We give an example of optimizing the fermentation condition in the yogurt, in which the organic acid is generated while the sugar is consumed. We also varied the fermentation conditions such as the ingredient type and investigated if the Dual Injection System is useful to monitor how the fermentation is going quickly.

## 2. Materials and Method

### 2-1. System

The Nexera dual injection system (Shimadzu Corporation) with two UHPLC flow paths in one platform was used (Figure 1) The organic acid analysis and the sugar analysis were carried out in the first and second flow channels, respectively. The autosampler equipped with two injection ports (both were in the loop injection mode) injected the sample from one vial into two flow paths and then both analyses started at the same time.

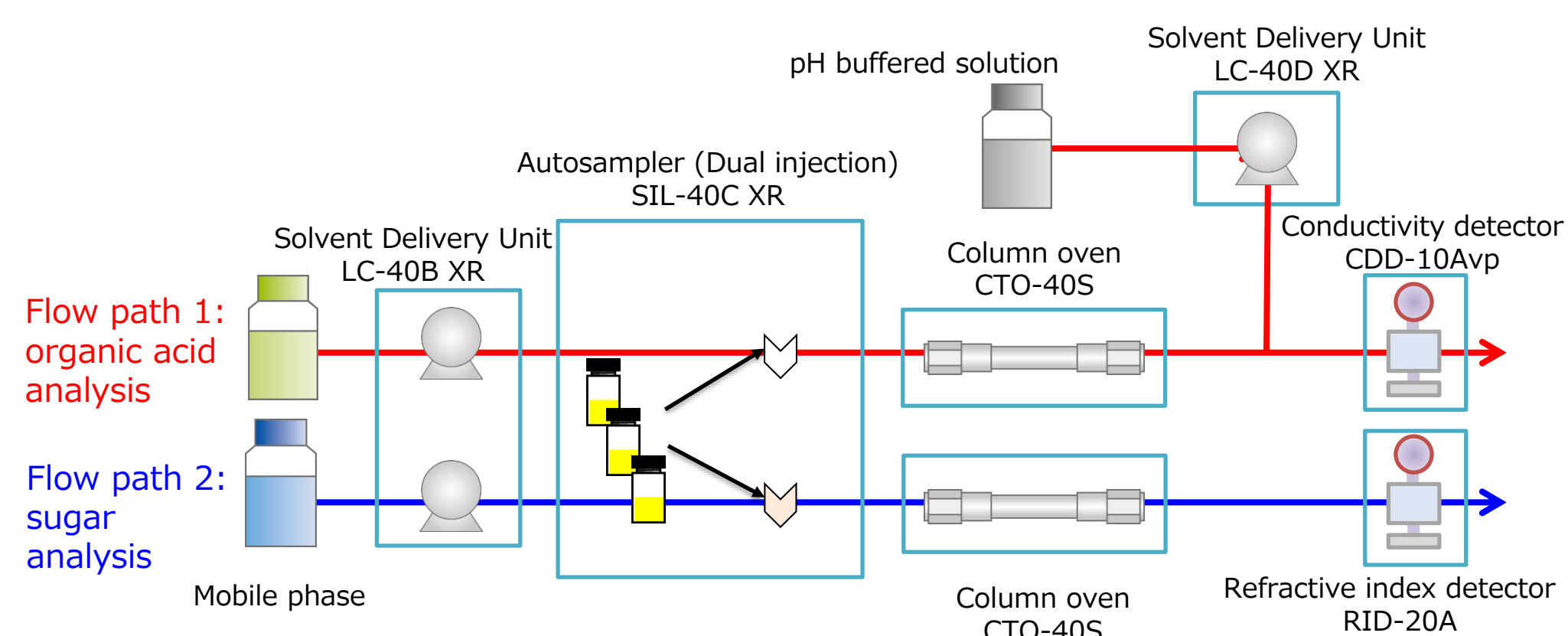


Figure 1 Flow diagram of the Nexera with Dual Injection function

### 2-2. Samples

A spoon of yogurt was added to the milk and fermented at 40 ° C using a commercial yogurt cooker. A part of the sample was collected at a regular time interval. The collected sample was prepared in the following procedure:

- 1) 250 mg of yogurt was taken into an Eppendorf tube, and 1 mL of 5.0 mmol/L p-toluenesulfonic acid solution and 250 μL of chloroform were added.
- 2) After shaking for 1 min, the mixture was centrifuged at 10,000 rpm for 2 min.
- 3) The supernatant was collected and filtered through a 0.2μm pore membrane filter.

### 2-3. Analytical conditions

#### <Organic acid analysis>

A combination of the ion exclusion chromatography and the post-column pH buffering conductivity detection provide high selectivity and sensitivity of the organic acid in a complex sample such as food. The ion exclusion chromatography separates the organic acid substances based on the magnitude of the exclusion between H<sup>+</sup> type ion exchange group of the stationary phase and the acidic mobile phase.

The retention is therefore affected by the pKa value. On the other hand, this separation conditions gives a negative impact on sensitivity. As the majority of the molecules eluted from the column are undissociated due to the acidic mobile phase, the signal on the conductivity detector tends to be low. The post-column addition of the neutral buffer solution enhances the ionization of the organic acid molecule leading to an improvement in the conductivity signal.

And, we used the high-speed organic acid analysis column, Shim-pack™ Fast-OA, which elutes organic acids faster than conventional columns. The analytical conditions are shown in Table 1.

Table 1 Analytical conditions for organic acids

Column	: Shim-pack Fast-OA 2 columns in series (100 mm L. x 7.8 mm I.D., 5 μm)
Guard column	: Shim-pack Fast-OA (G) (10 mm L. x 4.0 mm I.D.)
Mobile phase	: 5.0 mmol/L p-toluenesulfonic acid aq.
pH buffer	: 5.0 mmol/L p-toluenesulfonic acid aq. with 20 mmol/L Bis-Tris, and 0.1 mmol/L EDTA
Temperature	: 40 °C
Detector	: Conductivity detector
Injection volume	: 10 μL

#### <Sugar analysis>

The separation was performed with a Shim-pack SCR-101C column using a combination of the gel permeation mode and the ligand exchange mode. The refractive index detector was used for the detection. The analytical conditions is shown in table 2.

Table 2 Analytical condition for sugars

Column	: Shim-pack SCR-101C (300 mm L. x 7.9 mm I.D., 10 μm)
Guard column	: Shim-pack SCR (C) (50 mm L. x 4.0 mm I.D.)
Mobile phase	: Water
Temperature	: 80 °C
Detector	: Refractive index detector
Injection volume	: 10 μL

## 3. Results

### 3-1. Method development using dual injection system

Simultaneous analysis method of sugars and organic acids was developed with the dual injection system.

#### (1) The linearity of target compounds

Aiming to monitor the fermentation process in yogurt, lactic acid and lactose were selected as the target substances. As a control of the organic acid level, citric acid was also monitored. The calibration curves of each compound showed a wide dynamic range (Table 3)

Table 3 Linearity of the target compounds

organic acid	Range (mg/L)	Coefficient (r2)	sugar	Range (mg/L)	Coefficient (r2)
lactic acid	10 -1,000	0.9999	lactose	10 -2,000	0.9999
citric acid	10 -1,000	0.9999			

#### (2) Fermentation monitoring of yogurt sample

The results of the sample taken 0 and 8 hours after fermentation are shown in Figure 2. The sample in the same vial presented two chromatograms of lactose, citric acid, and lactic acid. Each component was able to be measured without interferences.

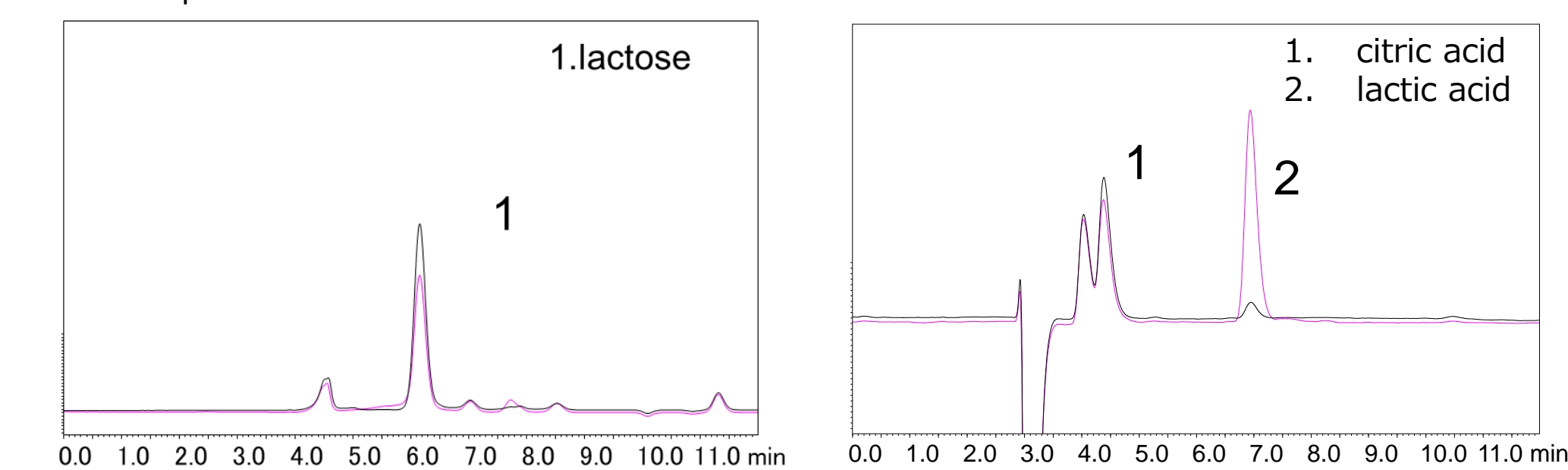


Figure 2 Chromatogram of a yogurt sample (black: 0 hour fermentation, red: 8 hour fermentation).

A portion of the yogurt was collected at several time points (0.0, 1.0, 2.0, 3.5, 5.5, 7.0, and 8.5 hours), and measured lactose, citric acid and lactic acid with the dual-injection system. Since it could inject the sample every 15 minutes, three data were able to be obtained for each sampling point in a small standard deviation (< 1.0%, n=6).

The results of the sugar and organic acid levels in yogurt are illustrated in Figure 3. The simultaneous analysis of these two classes of components enable presenting the activity of microorganisms in yogurt consuming lactose, producing lactic acid, and being independent of citric acid level. Finally, the fermentation process terminated in almost eight hours.

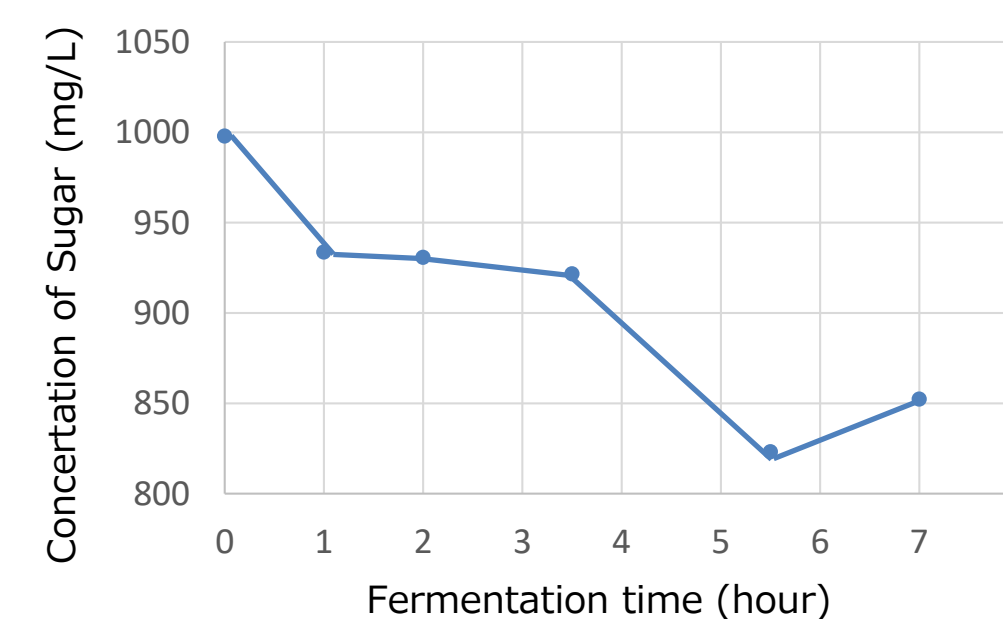


Figure 3 Content of sugar in yogurt

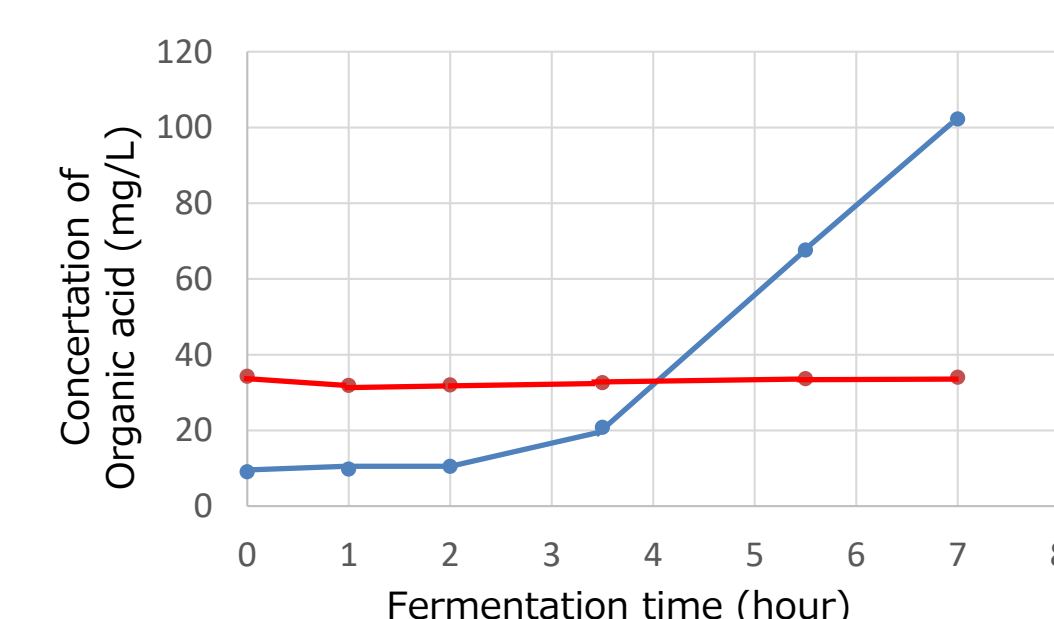


Figure 4 Content of organic acids in yogurt (blue: lactic acid; red: citric acid)

### 3-2. Optimizing the fermentation condition

Milk added with various concentrations of lactose was fermented in 8 hours. The levels of lactose consumption and lactate production were monitored to evaluate the effect of lactose content on fermentation (Table 4, 5). The higher the initial lactose content in milk, the less lactic acid was produced. It is essential to monitor both lactose (substrate) and lactic acid (products) for the optimization of fermentation conditions.

Table 4 The change of the amount of lactose

	Initial concentration (mg/L)	Concentration after fermentation (mg/L)	Diminution (mg/L)
Milk A	839.5	733.7	105.8
Milk B	967.5	925	42.5
Milk C	1100.9	1068.6	32.2

Table 5 The change of the amount of lactic acid

	Initial concentration (mg/L)	Concentration after fermentation (mg/L)	Increment (mg/L)
Milk A	8.4	57.2	48.8
Milk B	8.8	50.0	41.3
Milk C	8.8	38.9	30.0

Figure 5 shows the relationship between the initial concentration of lactose and the variation of concentration of the target compounds. The variations of lactic acid aren't proportional with the initial lactose. Therefore, it is difficult to estimate the fermentation efficiency in advance. For the optimization of fermentation conditions, it is essential to monitor both lactose (substrate) and lactic acid (Products).

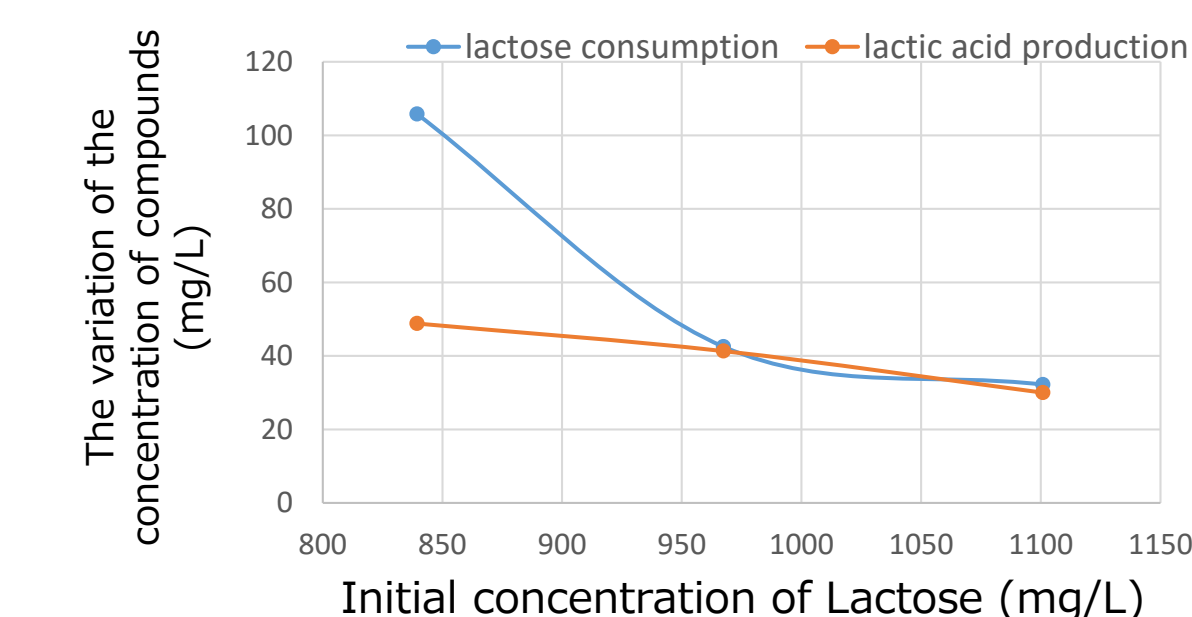


Figure 5 Content of lactose and lactic acid after fermentation (blue: lactose; orange: lactic acid)

## 4. Conclusion

A method for simultaneous analysis of organic acids and sugars as fermentation index was established. By using a dual injection system, the analysis of the two systems was integrated into one system.

The results indicated that the combination of two chromatograms acquired from the same sample at the same time reflected the real-time fermentation process well. Furthermore, this enables high-throughput scouting of fermentation conditions.

The application that determines two classes of components simultaneously with the Nexera Dual Injection System is beneficial across various fields. Additionally, it fulfills the demand for improving throughput and saving the installation space.