Application News

High Performance Liquid Chromatograph Nexera™-e

Analysis of Kakkonto using Comprehensive Two-Dimensional LC Nexera-e

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User Benefits

◆ Nexera-e system can be used for comprehensive two-dimensional separation.
◆ Accurate quantitation of individual components can be obtained (glycyrrhizic acid).

■ Introduction

Kakkonto (traditional Chinese medicine) is believed to be effective against cold, headache, stiff shoulders, etc., because it has the properties to promote sweating and provide anti-inflammatory and analgesic action. It consists of various natural products such as puerariae radix, ephedra, licorice, paeoniae radix, etc. It contains many compounds such as ephedrine, glycyrrhizic acid, and cinnamic acid. The Nexera-e comprehensive two-dimensional LC system is useful to separate such a complex sample.

Fig. 1 shows the appearance (a), and flow path diagram (b) of the Nexera-e. In a comprehensive 2D-LC system configuration generally two pumps and two columns are used and the fractions from first dimension are continuously transferred to a second column (second dimension). This transfer is performed by filling two identical loops alternately through a synchronized valve switching.

Generally, different separation modes are selected for first dimension (1D) and second dimension (2D) in comprehensive two-dimensional chromatography. This combination can usually provide higher resolution compared to the one-dimensional separation. Traditional Chinese medicine such as Kakkonto contains many relatively-polar compounds, so pH is also an important parameter to be considered.

This article introduces a comprehensive two-dimensional separation using a semi-micro reversed-phase column and neutral pH mobile phase for 1D and an ultra-high speed reversed-phase column and acidic pH mobile phase for 2D. The standard solution of glycyrrhizic acid was analyzed and calibration curve was created. Furthermore, three commercial Kakkonto extract granule from different manufacturers were analyzed and the concentration of glycyrrhizic acid was determined.

■ Analytical Conditions

Table 1 shows the analytical conditions. Neutral and acidic phosphate buffer were used for 1D and 2D, respectively.

In general, when both of the 1D and 2D are analyzed in the reversed-phase HPLC mode, as the concentration of the organic solvent in the fractionation solution divided into loops by the 1D LC increases, the separation and peak shape may deteriorate due to the solvent effect in the 2D LC analysis. LabSolutions™ is equipped with an "auto-gradient function" to solve the above problems, and makes it easy to create complex gradient conditions, such as changing the gradient elution conditions of the second dimension in multiple steps according to the gradient elution of the first dimension. The auto-gradient function was used this time. Fig. 2 shows the time program of 1D and 2D.
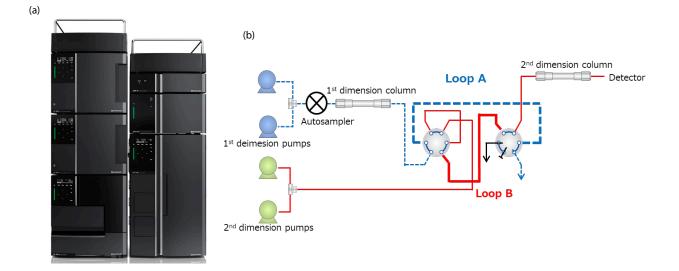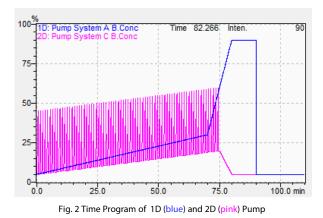

Fig. 1 Nexera™-e system
(a) appearance (b) flow path diagram

Table 1 Analytical Conditions

System	: Nexera-e
<1D>	
Column	: Shim-pack™ XR-ODSII
	(100 mm × 1.5 mm I.D., 2.2 μm)*1
Mobile Phase A	: 10 mmol/L (Sodium) phosphate buffer (pH: 6.9)
Mobile Phase B	: Acetonitrile
Flow Rate	: 0.05 mL/min
Time Program	: B Conc. 5% (0 min) - 30% (70 min) - 90% (80-90 min)
	- 5% (90.01-110 min)
<2D>	
Column	: Shim-pack Velox C18 (50 mm × 2.1 mm I.D., 2.7 μm) *2
Mobile Phase A	:10 mmol/L (Sodium) phosphate buffer (pH: 2.7)
Mobile Phase B	: Acetonitrile
Flow Rate	: 1.5 mL/min
Time Program	: Initial B Conc. 5% (0.01 min) - 45% (0.5 min)
	-5% (0.51-1.00 min)
	Final B Conc. 20% (0.01 min) - 65% (0.5 min)
	-20% (0.51-1.00 min)
Loop Vol.	: 50 μL
Modulation Time	: 1 min
Column Temp.	: 40 ˚C
Injection Vol.	: 2 μL
Vial	: SHIMADZU LabTotal™ Vial for LC 1.5 mL, Glass *3
Detection	: 254 nm (SPD-M40)

*1 P/N：228-59907-94, *2 P/N： 227-32009-02, *3 P/N：227-34001-01

Fig. 3 Comprehensive 2D-Plots of Glycyrrhizic Acid Standard Solution

Table 2 Repeatability of Retention Time and Blob Area (%RSD, n=6)

Compound	Retention Time (1D)(%RSD)	Retention Time (2D)(%RSD)	Blob Area (%RSD)
Glycyrrhizic acid	0.01	<0.01	3.59

Fig. 4 Calibration Curve of Glycyrrhizic Acid

Fig. 2 Time Program of 1D (blue) and 2D (pink) Pump

■ Repeatability and Calibration Curve of Glycyrrhizic Acid

Fig. 3 shows the 2D contour plot of standard solution of 50 mg/L of glycyrrhizic acid standard solution. The 2D contour plot was generated by the dedicated software ChromSquare. The horizontal axis shows the 1D retention time and the vertical axis shows the 2D retention time. The blob of glycyrrhizic acid is identified by the black rectangle. Table 2 shows the retention time repeatability for 1D and 2D separation, repeatability for blob area of a 50 mg/L standard solution in 6 repeated analyses. Repeatability for blob area was 3.59% and repeatability for retention time of 1D was 0.01% , and 2D was less than 0.01%. Fig. 4 shows the calibration curve (50 to 1000 mg/L) of glycyrrhizic acid. Good linearity was obtained with coefficient of determination $r^2 = 0.9996$.
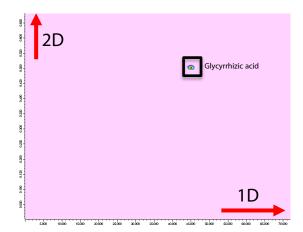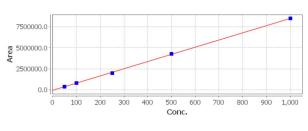
■ Quantitative Analysis of Glycyrrzihic Acid in the Kakkonto Extract Granule

Quantitative analysis was performed using three different kinds of commercial Kakkonto extract granule.

The sample preparation protocol is shown in Fig. 5. 1.5 g of Kakkonto extract granule was extracted with 15 mL of 50% methanol aqueous solution, and the extracts were centrifugated (3,500 rpm, 10 min). The supernatant was then filtered through a 0.2 μm membrane filter. Finally, 2 μL of Kakkonto after sample preparation was injected into LC.
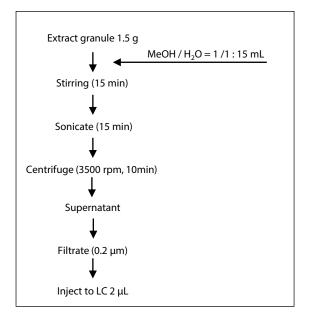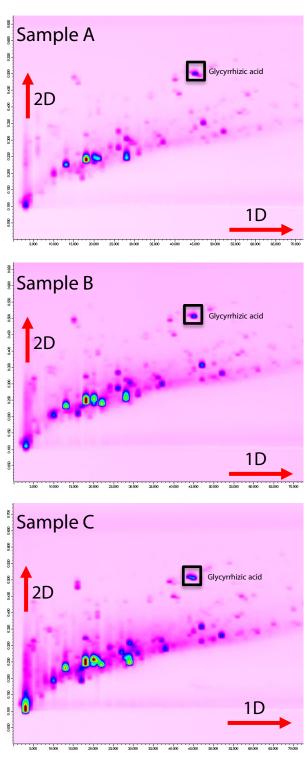

Fig. 5 Sample Preparation Protocol

Sample A

2D

1D

Glycyrrhizic acid

Sample B

2D

1D

Glycyrrhizic acid

Sample C

2D

1D

Glycyrrhizic acid

Fig. 6 Comprehensive 2D-Plots of Sample A, Sample B and Sample C

Table 3 Repeatability of Glycyrrzihic Acid in Sample A (n=6)

Compound	Retention Time (1D)(%RSD)	Retention Time (2D)(%RSD)	Blob Area (%RSD)
Glycyrrhizic acid	0.83	0.94	0.59

Fig. 6 shows the 2D contour plots of three Kakkonto extracts granule. Multiple peaks were observed on each chromatogram. The chromatographic patterns were different among different products which can be confirmed easily from the 2D contour plot.

As for Sample A, 6 repeated analyses were performed. Glycyrrhizic acid was identified using the information of retention time. Table 3 shows the repeatability of retention time and blob area of glycyrrhizic acid. Good repeatability is also confirmed in the real sample.

Then glycyrrhizic acid contained in the Kakkonto extract granule was quantitated. Table 4 shows the quantitative values of glycyrrhizic acid contained in three Kakkonto products. Please note that these values are the concentration after sample preparation.

Table 4 Quantitative Values of Glycyrrhizic Acid in Kakkonto Extract Granule

Sample	Quantitative Value (mg/L)
Sample A[1]	528.7
Sample B[2]	557.6
Sample C[3]	982.8

[1] n=6, [2] n=3, [3] n=3

■ Conclusion

Comprehensive two-dimentional LC analysis of glycyrrhizic acid was performed using Nexera-e system. Repeatability of 50 mg/L glycyrrhizic acid standard solution was confirmed. Repeatability for blob area was 3.59% and repeatability for retention time of 1D was 0.01% , and 2D was less than 0.01%. Three different kinds of Kakkonto extract granule were analyzed and the concentration of glycyrrhizic acid was successfully determined.