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Application News

No.L472

High Performance Liquid Chromatography

Improved Analytical Workflow for Phospholipids by Nexera-e and Co-Sense for Impurities

Together, the comprehensive two-dimensional Nexera-e, offering exhaustive analysis of diverse compounds, and the Co-sense series, permitting high resolution and enrichment of specific fractions, make it possible to compare the overall compound patterns in the target sample. Combination of the Nexera-e and Co-Sense series also allows precise separation of the elution fraction of interest.
With this method, the two workflows consisting of the differential analysis of a sample with a complex matrix, and a detailed analysis of the compounds of those results to reveal differences, can be conducted simultaneously, quickly and conveniently. Here, we introduce an actual example using phospholipids.

Comprehensive Separation of Glycerophospholipids by Nexera-e

Glycerophospholipids (GPLs), phospholipids with a glycerol backbone that play an important role in the field of metabolomics, are known to serve as a structural component of biological cell membranes. Depending on the type of hydrophilic compound that is attached to the phospholipid via an ester bond, the glycerophospholipids are classified into five groups, as follows:
Phosphatidylglycerol (PG), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Phosphatidylserine (PS), Phosphatidylcholine (PC)
One characteristic in common is an alkyl side chain which is coupled to each of the glycerin moieties. In analysis using the Nexera-e, it is possible to distinguish between the polar groups which can be identified using the normal phase mode in the first dimension. Further, in the second dimension, it is possible to determine differences in chain length using the reversed phase mode. Fig. 1 shows an example of separation of these phospholipids using analysis by typical LC-MS/MS. The analytical conditions are shown in Table 1. Using the MRM mode, a chromatogram is obtained for each individual compound, but the overall picture of the sample is difficult to grasp. Depending on the compound, overlapping of peaks other than the compound of interest may result in poor peak shape and insufficient peak intensity. Also, the reduced quantitative accuracy due to ion suppression is a concern. Next, Fig. 2 shows a contour plot obtained using comprehensive two-dimensional separation of these glycerophospholipids (each 500 µg/L) by MS/MS detection. The overall separation is clear, and the existing state of each phospholipid is easy to visualize. Separation of PE, PC, etc. according to chain length is also clear and with a relatively high sensitivity, but separation based on chain length for some of other phospholipids lacks separation and sensitivity. These include PG and PS. Then, coexisting substances were eliminated by using the heart-cut feature of the Nexera-e, and to compensate for insufficient separation and sensitivity, analysis by Co-Sense for Impurities was performed. As for the analytical conditions using the Nexera-e, please refer to the Application News L462.

Table 1 Analytical Conditions for 1D Separation of Phospholipids

Column	: L-Column2 ODS (100 mm L. × 1.5 mm I.D., 3 µm)
Mobile Phase	: A: MeOH / Water / Acetic Acid / 28 % Ammonia = 80 / 20 / 0.05 / 0.05 (v/v/v/v) B : 2-Propanol / Acetic Acid / 28 % Ammonia = 100 / 0.05 / 0.05 (v/v/v)
Time Program	: B Conc. 0 % (0 - 5 min) → 55 % (20 min) → 90 % (22 - 25 min) → 100 % (25.1 - 30 min) → 0 % (30.1 - 35 min)
Flowrate	: 0.15 mL/min
Column Temp.	: 40 °C
Detector	: Shimadzu LCMS-8050 (ESI positive, MRM)

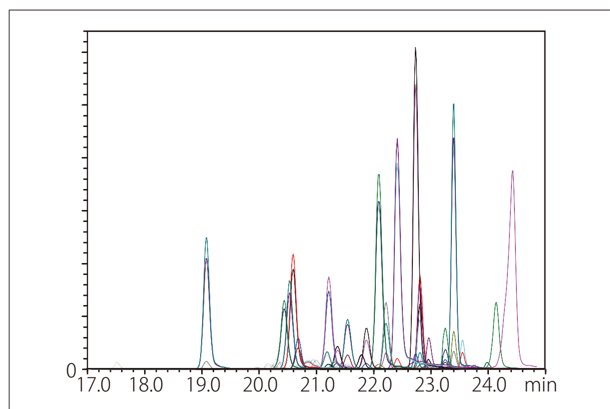

Fig. 1 1D Reversed Phase Separation of Phospholipids

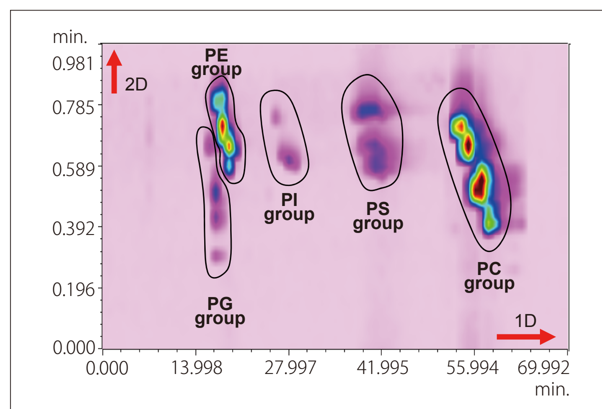

Fig. 2 2D Plot of All Positive MRM Transitions of Phospholipids

Fine Separation of PG and PS Fractions in Glycerophospholipids by Co-Sense for Impurities

In general, when performing the analysis of trace compounds in complex matrices by mass spectrometry, comprehensive separation by Nexera-e affords characteristic results, but when focusing on individual compounds, the sample must be diluted due to the serial combination use of different separation modes, possibly causing decreased sensitivity or deteriorated peak shapes. After enriching the compound group fractioned in the vicinity of the peak of interest separated in the first dimension, Co-Sense for Impurities, which supports separation in a different mode, can be used to remove unnecessary coexisting substances or maintain a constant concentration using a trap column, thus making this a valid analytical method when used in conjunction with the Nexera-e.

Table 2 shows the analytical conditions using the Co-Sense for Impurities. Mobile phase compositions similar to those used for the Nexera-e, including the column type, were applied to both the first and second dimensions. Aside from the targets, consisting of the eight PG and six PS types (concentration 10 µg/L), a matrix consisting of eight PG, eight PC, eight PE, four PI, five PA (phosphatidyl acid), four TG (triglyceride), and five DG (diglyceride) types were prepared, respectively. With respect to PG and PS, two types of samples at approximately 100-fold concentrations were prepared. Fig.3 shows a comparison of the MRM transition chromatograms for just the first dimension separation for PG (16:0-18:1) and PS (18:1-18:1), and separation using Co-Sense for Impurities, respectively. Compared to the first dimension separation results, there is clearly an increase in sensitivity due to enrichment in the case of PG, and for PS, a decrease in coexisting substances due to the heart-cut technique. Thus, based on the results of this comprehensive separation study using the Nexera-e, it is possible to conduct complementary analysis relatively easily and quickly using Co-Sense for Impurities.

Table 2 Analytical Conditions for Co-Sense for Impurities

[Column1]	: Shim-pack XR-SIL (100 mm L. × 3.0 mm I.D., 2.2 µm)
Mobile Phase	: A : Isooctane / Acetone / Ethyl Acetate / Acetic Acid = 40 / 40 / 20 / 0.03 (v/v/v/v)
	B : Isooctane / 2-Propanol / Water / Acetic Acid / Ethanolamine = 40 / 51 / 9 / 0.03 / 0.03 (v/v/v/v/v)
Time Program	: B Conc. 40 % (0 min) → 50 % (2.5 min) → 100 % (5 - 7.5 min) → 40 % (7.6 - 16.5 min)
Flowrate	0.6 mL/min
Column Temp.	: 40 °C
Fraction Time	: PG (1.1 - 1.8 min) PS (16 - 20 min)
[Column2]	: COSMOSIL Guard Column HILIC (10 mm L. × 2 mm I.D., 5 µm)
Mobile Phase	: Acetonitrile
Flowrate	: 5.4 mL/min
[Column3]	: L-Column2 ODS (100 mm L. × 1.5 mm I.D., 3 µm)
Mobile Phase	: A : Methanol / Water / Acetic Acid / 28 % Ammonium Hydroxide = 80 / 20 / 0.1 / 0.1 (v/v/v/v)
	B : 2-Propanol / Acetic Acid / 28 % Ammonium Hydroxide = 100 / 0.1 / 0.1 (v/v/v)
Time Program	: B Conc. 0 % → 60 % (2 min) → 90 % (4 - 8 min) → 0 % (8.1 min)
Flowrate	: 0.15 mL/min
Detector	: Shimadzu LCMS-8050 (ESI negative, MRM)

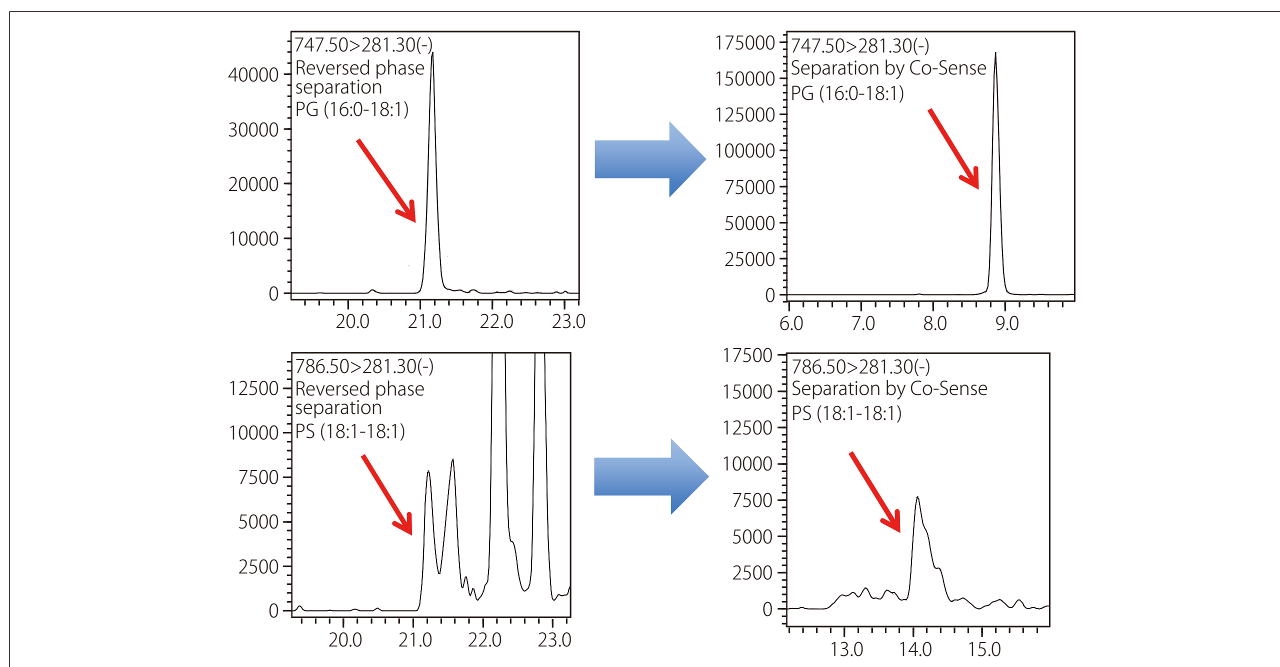

Fig. 3 Comparison of 1D Reversed Phase and 2D Co-Sense MRM Separations of PG (16:0-18:1) and PS (18:1-18:1)

First Edition: Jan. 2015

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