

Comprehensive two-dimensional HPLC analysis coupled with mass spectrometric detection and informative data processing for lipid analysis

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Introduction

In lipidomics, phospholipids are the attractive targets of analysis since lipids are important and essential components of biological membranes. However, conventional HPLC system by a single separation mode performs poorly on biological lipid sample, because it contains various kinds of lipids with common moieties that govern their behavior on column. In such a case, comprehensive two-dimensional (2D) LC will be a

powerful tool. This system was capable of characterizing phospholipids both quantitatively and qualitatively when coupled with triple quadrupole and ion trap-TOF type of mass spectrometer respectively. Reliable identification of lipid species was performed by acquiring m/z values of related parent and fragment ions at high accuracy with the ion trap-TOF mass spectrometer and matching the data to commercially available data-base.

Methods and Materials

Comprehensive 2D LC System

A mixture of glycerophospholipids (GPLs) consisting of phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylcholine (PC) was used as a model sample. Comprehensive 2D LC system (Nexera-e, Shimadzu Corporation, Japan) consisted of two flow lines: one was for the first dimension separation and the other for the second dimension. A mixture of lipids was roughly classified by the micro-scale normal phase chromatography in the first dimension. All eluents were collected in two loops alternately. Changing the flow line connection, the entire eluents were introduced into

the second dimension serially that employed reversed phase high speed (UHPLC) analyses. The lipids of interest were monitored with triple quadrupole (LCMS-8050) or ion trap-TOF (LCMS-IT-TOF) mass spectrometer (Shimadzu Corporation, Japan) followed by a contour analysis with ChromSquare software (Chromaleont, Italy). Using the LCMS-IT-TOF data, lipid identification analysis was performed by the data-base matching software named Lipid Search (MKI, Japan) that compares the experimental and theoretical m/z value of parent and fragment ions.

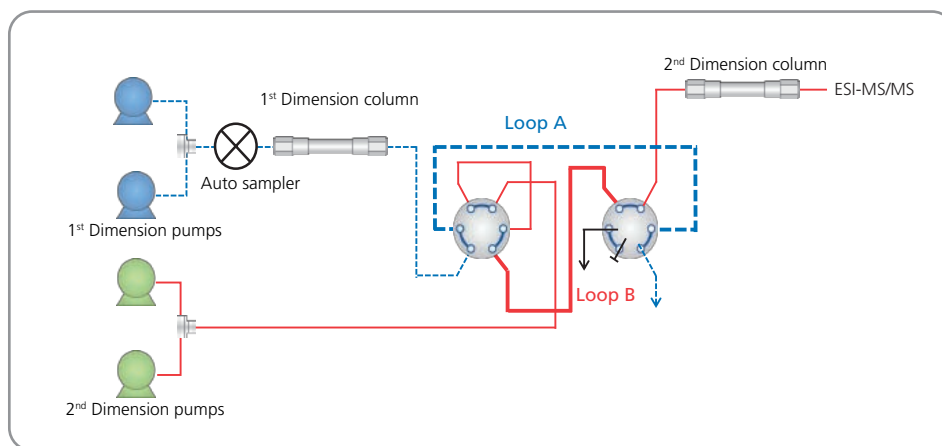
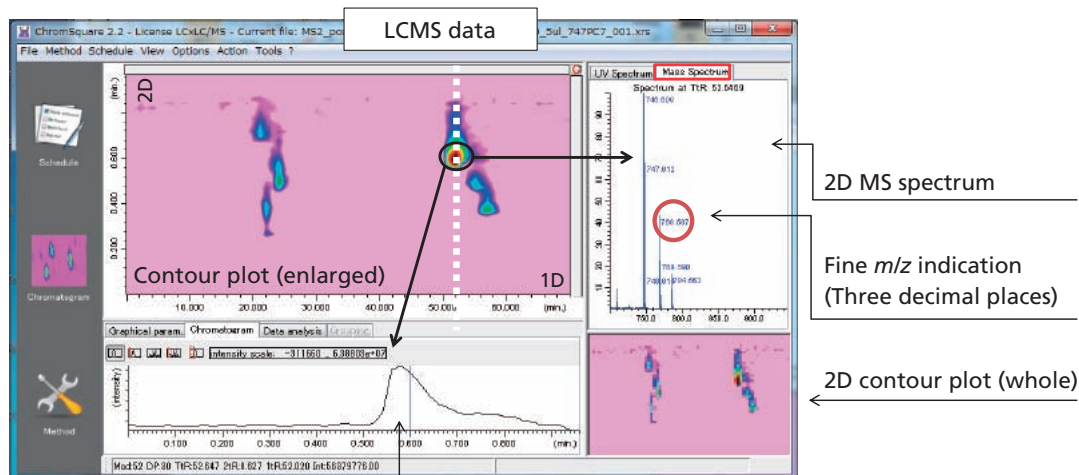


Figure 1 Flow diagram of the comprehensive 2D LC - MS system

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MS chromatogram of 2D at selected position

Figure 2 Contour plot analysis by the dedicated software

LC/MS/MS Analysis

HPLC conditions (Nexera-e system)	
1D Column	: Nucleosil SIL (150 mm L. x 1 mm I.D., 3 µm)
Mobile phase	: A : Isooctane / Acetone / Ethyl Acetate / Acetic acid = 40/20/20/0.03 (v/v/v/v) B : Isooctane / 2-propanol / Water / Acetic acid / 28 % Ammonium hydroxide = 40/51/9/0.03/0.03 (v/v/v/v/v)
Flow rate	: 0.02 mL / min.
Time program	: B Conc. 30 % B (0 min) → 40 % (25 min.) → 100 % (40 min) → 100 % (55 min) → 30 % (55.1 min) → 30 % (55.1 min) → STOP (70 min)
Column Temp.	: 40 °C
Injection Vol.	: 5 µL
Loop Vol.	: 20 µL
2D Column	: Phenomenex Kinetex C18 (50 mm L. x 4.6 mm I.D., 2,6 µm)
Mobile phase	: A : Methanol / Water / Acetic acid / 28 % Ammonium hydroxide = 100/10/0.05/0.05 (v/v/v/v) B : 2-propanol / Acetic acid / 28 % Ammonium hydroxide = 100/0.05/0.05 (v/v/v)
Flow rate	: 3.5 mL/min (50 % split to MS)
Time program	: B Conc. 10 % B (0 min) → 50 % (0.75 min.) → 10 % (0.76 min) → STOP (1 min) The initial B Concentration was changed by a stepwise method
MS conditions	
LCMS-8050	
Ionization	: ESI (Positive)
MRM	: 31 MRM transitions Dwell time 5 msec. / Pause time 1 msec.
LCMS-IT-TOF	
Ionization	: ESI (Positive/Negative)
Scan	: m/z 700-800

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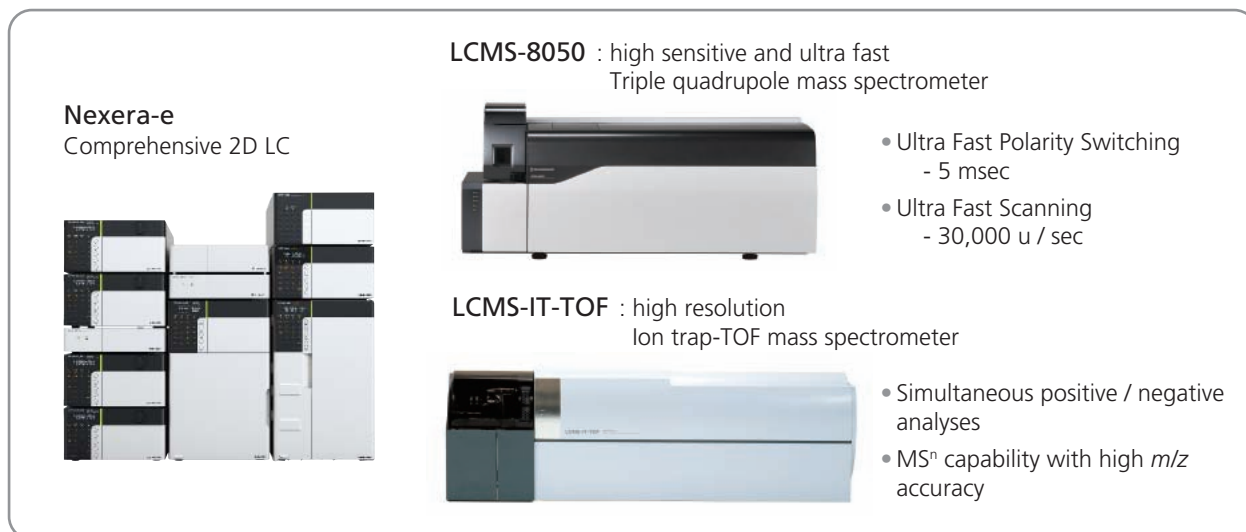


Figure 3 Overview of comprehensive 2D LC- MS system

Result

Quantitative Analysis of GPLs by the Detection of Triple Quadrupole Mass Spectrometer

The GPLs mixture was comprised of 500 µg/L of each of PG, PE, PI, PS and PC. The whole 2D contour plot of ESI-positive MRM (31 transitions) by LCMS-8050 showed the separation of these compounds. The repeatability (n=5) of retention times and blob areas that correspond to peak

areas in ordinary quantitation and linearity for 50-5000 µg/L of 3 PC compounds are shown in Table 1. Necessary information for compound-identification is shown in the upper contour plot in Figure 4.

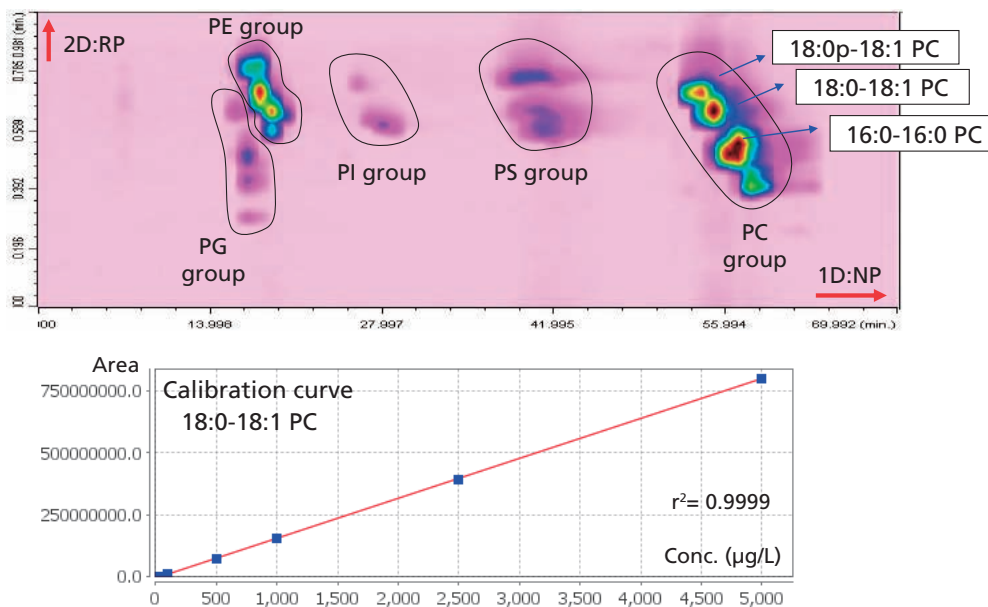


Figure 4 Comprehensive separation and calibration curve for 18:0-18:1 PC

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Table 1 Repeatability of 5 analyses in %RSD for 3 PC compounds

Compound	MRM transition	Total retention time	Retention time(2D)	Blob Area
16:0-16:0 PC	m/z 734.6 > 184.1	0.007	0.9	6.8
18:0-18:1 PC	m/z 788.6 > 184.1	0.013	1.1	8.9
18:0p-18:1 PC	m/z 772.6 > 184.1	0.013	1.2	6.4

Qualitative Analysis of GPLs by the Detection of Ion trap-TOF Mass Spectrometer

The GPLs mixture was comprised of 50 mg/L of each of PG, PE and PC. The contour plot of ESI- positive/negative scan by LCMS-IT-TOF shows the separation of these compounds. PC compounds were detected more sensitively in ESI positive, whereas PE and PG compounds in negative mode. Then MS/MS analysis to identify the peak (RT 22.3 min, *m/z* 769.5) was performed. Obtained data from both MS and MS/MS in negative mode was

contributed to automatic lipid identification using the data-base matching software "Lipid Search" that compares the experimental and theoretical *m/z* values of both parent and fragment ions and it successfully indicated 16:0-20:4 PG as the most expecting candidate. Additionally, MS/MS fragment peak assignments support the reliability of 16:0-20:4 PG identification.

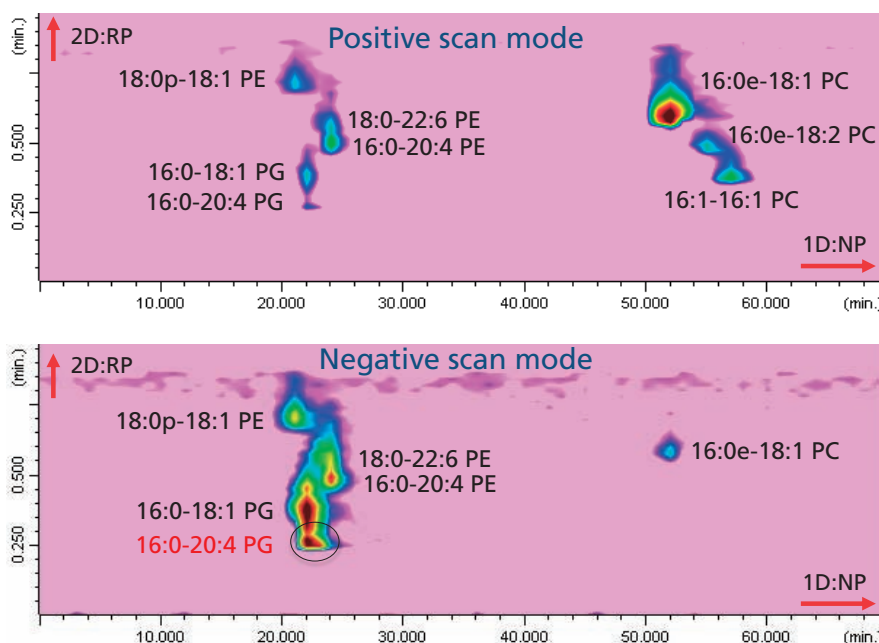


Figure 5 Contour plot of positive/negative scan mode

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Candidate for lipid identification analysis and comparison of parent m/z value between theoretical and observed

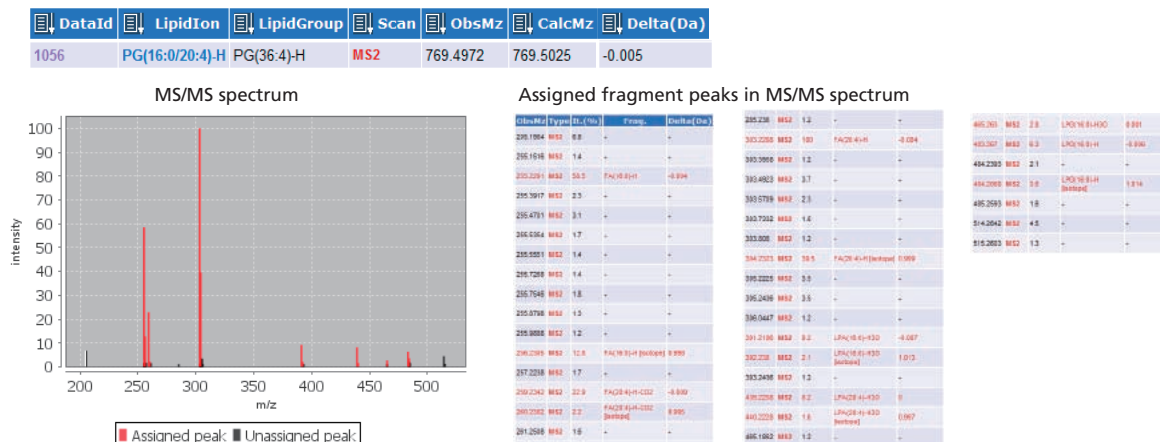


Figure 6 Lipid identification analysis using the data-base matching software

Conclusions

- Comprehensive 2D LC analysis by combination use of normal phase and reversed phase afforded sufficient orthogonal separation of phospholipids.
- Excellent repeatability and linearity of blob area and retention time by comprehensive 2D LC - triple quadrupole mass spectrometer setup was confirmed.
- Reliable identification of target lipid in 2D contour plot was achieved by data-base matching analysis based on high resolution ion trap-TOF mass spectrometer data.