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Glycerophospholipids Analysis by Two-Dimensional Liquid Chromatography Coupled with a Triple Quadrupole Mass Spectrometer

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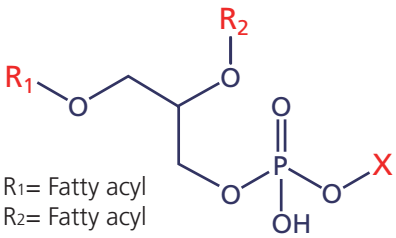
1. Introduction

Glycerophospholipids (GPLs) are the major component of biological membranes. They can not only act as a barrier from the external environment, but can also play a key role in a variety of biological processes including membrane trafficking and signal transduction. Thus, analysis of GPLs is one of the most important studies in the metabolomics field. Although reversed phase (RP) HPLC coupled with electrospray ionization (ESI) MS is an effective strategy for lipidomics, there is still room for further improvement of the analytical methods. One drawback to performing comprehensive identification and precise quantification of minor species of GPLs is ion suppression by major components. To solve this problem, we established a new strategy using two-dimensional liquid chromatography (2D-LC) coupled with ESI-MS/MS (2D-LC-ESIMS/MS).



X	PA	H
	PC	Choline
	PE	Ethanolamine
	PG	Glycerol
	PS	Serine
	PI	Inositol

Fig. 1 General structures of glycerophospholipids

2. Methods

Fig. 2 shows the flow diagram of the complete 2D-LC-ESI-MS/MS system. The system comprises 3 flow lines: one for the first dimension separation with a normal phase column, the second for the concentration of the target fraction, and the third for the second dimension separation with a reversed phase column.

A mixture of GPLs was separated by normal phase chromatography in the first dimension. An online dilution for the target fraction was performed in order to concentrate the target GPLs on a trapping column. The concentrated GPLs were separated by reversed phase chromatography in the second dimension, followed by multiple reaction monitoring in the mass spectrometer.

The detailed analytical conditions are shown in Table 1 and Table 2.

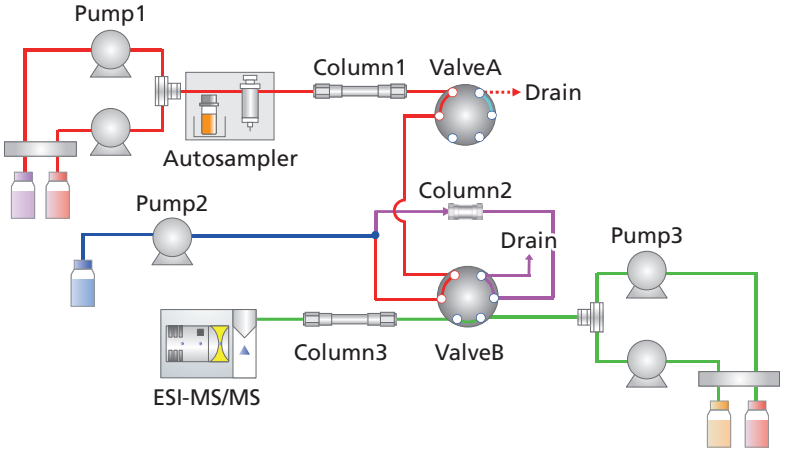


Fig. 2 Flow diagram of 2D-LC-ESI-MS/MS system

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Table 1 Analytical conditions

[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%(8.5 min)→100% (16-25 min) →40% (25-39 min)
Flow Rate	: 0.3 mL/min
Column Temp.	: 40℃
Fraction Time	: PG(5.5-9 min), PI(8.5-12.5 min), PS(16-20 min)
[Column2]	: COSMOSIL Guard Column HILIC (10 mm L. × 4.6 mm I.D., 5 µm)
Mobile Phase	: 0.05% acetic acid in acetonitrile
Flow Rate	: 2.1 mL/min
[Column3]	: L-Column ODS2 (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% (0-5 min)→55% (20 min)→90% (22-25 min)→0% (25-29 min)
Flow Rate	: 0.15 mL/min
Detector	: Shimadzu LCMS-8040 (ESI positive, MRM mode)

Table 2 MRM transactions

#	Compound	Polarity	Adductlon	MRM transition	Range (pgon column)	Correlation Coefficient	% RSD (at 200 pg)
1	16:0-16:0 PG	+	NH^4	740.45 > 551.50	50-1000	0.9999	3.96
2	16:0-18:1 PG	+	NH^4	766.50 > 577.45	50-1000	0.9994	1.31
3	16:0-20:4 PG	+	NH^4	788.50 > 599.45	50-1000	0.9998	5.43
4	16:0-18:2 PG	+	NH^4	764.50 > 575.45	50-1000	0.9999	4.16
5	18:0-18:0 PG	+	NH^4	796.50 > 607.50	50-1000	0.9999	4.41
6	18:0-20:4 PG	+	NH^4	816.55 > 627.50	50-1000	0.9991	3.62
7	18:0-22:6 PG	+	NH^4	840.45 > 651.50	50-1000	0.9999	3.96
8	22:6-22:6 PG	+	NH^4	884.45 > 695.50	50-1000	0.9998	8.1
9	16:0-16:0 PS	+	H	736.70 > 551.45	50-1000	0.9999	2.54
10	18:0-18:0 PS	+	H	792.80 > 607.55	50-1000	0.9991	4.45
11	16:0-20:4 PS	+	H	784.70 > 599.40	50-1000	0.9998	3.11
12	18:0-20:4 PS	+	H	812.70 > 627.55	50-1000	0.9977	8.11
13	18:0-22:6 PS	+	H	836.70 > 651.60	50-1000	0.9986	6.6
14	18:1-18:1 PS	+	H	788.80 > 604.40	50-1000	0.9996	4.17
15	16:0-16:0 PI	+	NH^4	828.45 > 551.50	50-1000	0.9998	6.24
16	18:0-18:0 PI	+	NH^4	884.60 > 607.55	50-1000	0.9998	2.81
17	16:0-18:1 PI	+	NH^4	854.45 > 577.45	50-1000	0.9993	3.7
18	18:0-20:4 PI	+	NH^4	904.50 > 627.50	50-1000	0.9994	5.57

3. Results

Comparison between conventional reversed phase LC analysis and 2D analysis

Fig. 3 shows the typical 1stD chromatogram of GPLs mixture and 2ndD chromatogram of PS. GPLs were separated in terms of their classes by 1stD chromatography. The GPLs in a same class were trapped onto the second column. They were then quantitated by 2ndD chromatogram and MRM. Pure GPL samples were analyzed to create calibration curves. Linearity and reproducibility for each compound is summarized in table 2. Every sample showed excellent linearity. Some typical calibration curves are shown in Fig. 4.

In order to evaluate the effect of matrix with different concentration, a series of samples were prepared for PS and PG, respectively (Table 3). The mixture of PG, PE, PI and PC were used as the matrix for PS sample. The matrix for PG sample was prepared, similarly. These samples and standards which did not contain matrix were analyzed by 2D-LC-ESI-MS/MS system and conventional LC-ESI-MS/MS system. Same reversed phase column was used for both systems. The ratio of peak areas was calculated by following equation:

[Ratio] = [Area (Sample)] / [Area(STD)]

With the conventional system, due to insufficient separation, the intensity of PS fluctuated. It may be difficult to quantify real samples precisely using conventional LC method. Interestingly, not only suppression but also enhancement was observed. With 2D-LC-ESI-MS/MS system, the interference from other components was avoided and reliable quantification was achieved (Table 4).

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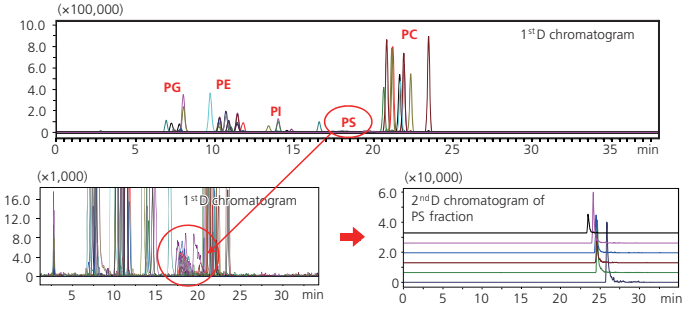

Fig. 3 Typical Chromatograms of GPLs mixture sample

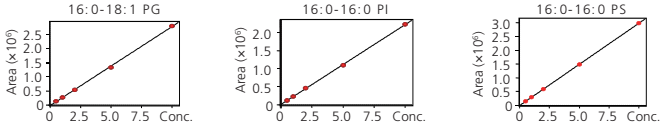

Fig. 4 Calibration curves of 16:0-18:1 PG, 16:0-16:0 PI and 16:0-16:0 PS

Table 3 Matrix sample preparation

	PS or PG [µg/L]	Matrix [µg/L]	Ratio
Sample1	25	25	1:1
Sample2	25	225	21:9
Sample3	25	2475	1:99
STD	25	0	-

Table 4 Comparison between Conventional RP LC and 2D LC results

#	Compounds	Ratio (%)					
		Conventional RP LC			2D-LC		
		Sample1	Sample2	Sample3	Sample4	Sample5	Sample6
1	16:0-16:0 PS	93.82	87.94	66.11	96.36	99.88	100.02
2	18:0-18:0 PS	82.99	139.4	113.7	104.89	97.69	91.26
3	16:0-20:4 PS	101.17	81.72	69.00	115.78	101.28	89.71
4	18:0-20:4 PS	95.07	110.4	83.55	112.45	104.91	91.67
5	18:0-22:6 PS	114.68	117.3	54.75	103.30	116.60	101.30
6	18:1-18:1 PS	100.77	139.4	105.4	96.83	84.69	96.30
7	16:0-16:0 PG	99.20	89.06	70.86	89.02	77.90	82.29
8	16:0-18:1 PG	106.30	90.63	88.89	103.40	89.22	86.91
9	16:0-20:4 PG	106.60	93.12	108.31	92.95	83.80	95.06
10	16:0-18:2 PG	111.36	93.16	100.80	110.94	93.70	100.13
11	18:0-18:0 PG	109.47	91.78	80.70	92.50	83.22	83.19
12	18:0-20:4 PG	107.14	91.22	79.98	112.72	98.66	83.67
13	18:0-22:6 PG	95.24	85.02	77.39	86.13	77.35	84.73
14	22:6-22:6 PG	112.99	94.39	102.40	94.40	80.37	84.88

4. Conclusions

This system offers automated concentration of the target GPLs and removal of undesired metabolites to help achieve excellent linearity and reproducibility. Furthermore, utilizing two analytical columns with different retention characteristics will permit reliable separation of various classes of GPLs. Comparison between conventional reversed phase LC method and the 2D-LC method for matrix effect demonstrated the great advantage of the 2D-LC method.

5. Acknowledgement

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