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Lipids analysis by 2 dimensional LC coupled with triple quadrupole mass spectrometer

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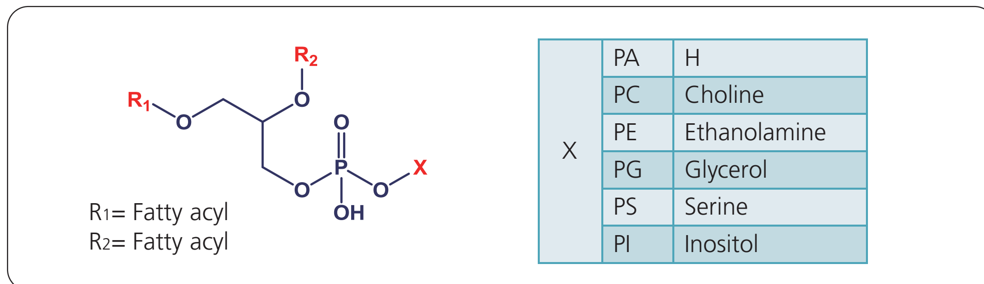
1. Introduction

Glycerophospholipids (GPLs) are the major components of biological membranes. Not only can GPLs act as a barrier from outer environment, but also they play a key role in a variety of biological processes including membrane trafficking, signal transduction, and so on. Thus, analysis of GPLs is one of the most important studies in metabolomics field. Although ESI-MS coupled with RP-HPLC is an effective strategy for lipidomics, it is necessary to further improve analytical methods. One drawback to perform comprehensive identification and precise quantification of minor species of GPLs using ESI-MS is interference from matrices or other components. In order to solve this problem, we managed to establish a new strategy to analyze GPLs by using 2DLC-MS/MS, i.e. two-dimensional liquid chromatography coupled with triple quadrupole mass spectrometer.
General structures of glycerophospholipids are depicted in Fig. 1.



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

Fig. 1 General structures of Glycerophospholipids

2. Methods

A two-dimensional LC coupled with triple quadrupole mass spectrometer system comprises three flow lines: one is for primary class separation in normal phase mode (1st dimension), another is for trapping and concentration of target lipid class, and the third is for secondary separation in reversed phase mode (2nd dimension). The system with electrospray ionization was operated in multiple-reaction-monitoring (MRM) mode. The schematic of two-dimensional LC system is displayed in Fig. 2, followed by detailed analytical conditions.

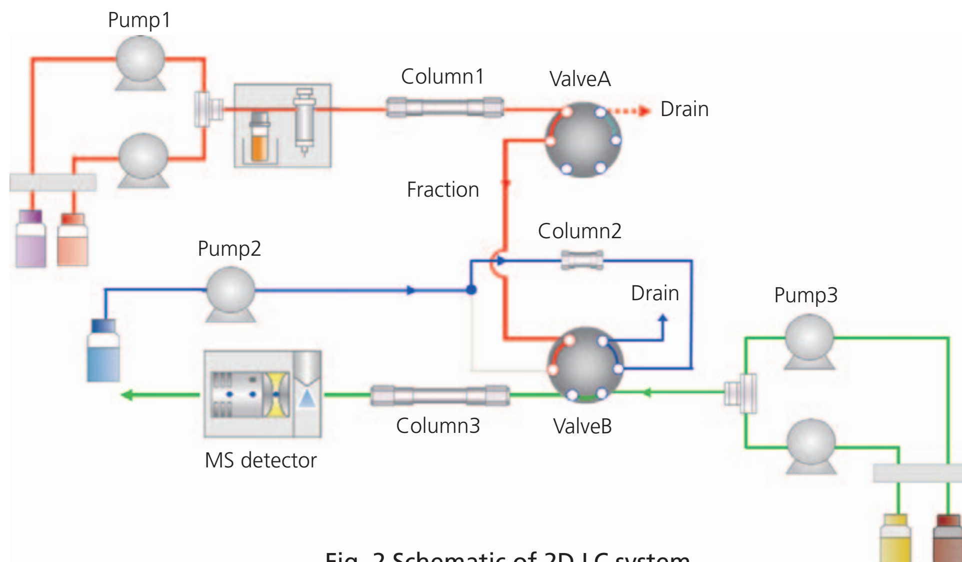


Fig. 2 Schematic of 2D LC system

[Column1]: Shim-pack XR-SIL (100 mm L. × 3.0 mm I.D., 2.2 µm)
Mobile Phase : A; isooctane / acetone / ethyl acetate (4 / 4 / 2, v/v/v) containing acetic acid (0.03%)
B; isooctane / 2-propanol / water (40 / 51 / 9, v/v/v) containing acetic acid and ethanolamine (each 0.03%)
Time Program : B CONC 40% (0 min) → 50% (8.5 min) → 100% (16-25 min) →40% (22.1-39 min)
Mixer : 40 µL
Flow Rate : 0.3 mL/min
Column Temp. : 40°C

[Column2]: COSMOSIL HILIC Guard Column(10 mm L. × 4.6 mm I.D., 5 µm)
Mobile Phase : acetonitrile containing acetic acid (0.05%)
Flow Rate : 2.1 mL/min
Fraction (Start-End) : PG(5.5-9 min), PI(8.5-12.5 min), PS(16-20 min)

[Column3]: L-column2 ODS(100 mm L. × 1.5 mm I.D., 3 µm)
Mobile Phase : A; methanol / water (8 / 2, v/v) containing acetic acid and ammonium hydroxide(28%) (each 0.1%)
B; 2-propanol containing acetic acid and ammonium hydroxide(28%) (each 0.1%)
Time Program : B CONC 0% (0-5 min) → 55 % (20 min) → 90% (22-25 min) → 0% (22-29 min)
Mixer : 20 µL
Flow Rate : 0.15 mL/min
Detection : LCMS-8040 (ESI positive & negative, MRM mode)

Table 1 MRM transitions of Lipids

#	Compound	Polarity	MRM transition	Range (pg on column)	Coefficient (r^2)	%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	8.10
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	6.60
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	3.70
18	18:0-20:4 PI	$+NH_4$	904.50 > 627.50	50-1000	0.9994	5.57

3. Results

3-1. Method development for glycerophospholipids

A sample solution of GPLs mixture was loaded into a 1stD separation column and the fraction of target GPLs was collected from the effluent by trapping. At the start point of trapping, two 2-position valves were serially switched and the effluent was automatically mixed with diluent and introduced onto a trapping column intended to retain sample within a narrow zone. At the end point of trapping, valves were returned to the original position and the concentrated target GPLs fraction was transferred to a 2ndD separation column connected in series to obtain resulting chromatogram. Fig. 4 shows the typical 1stD chromatogram of GPLs mixture and 2ndD chromatogram of PS.

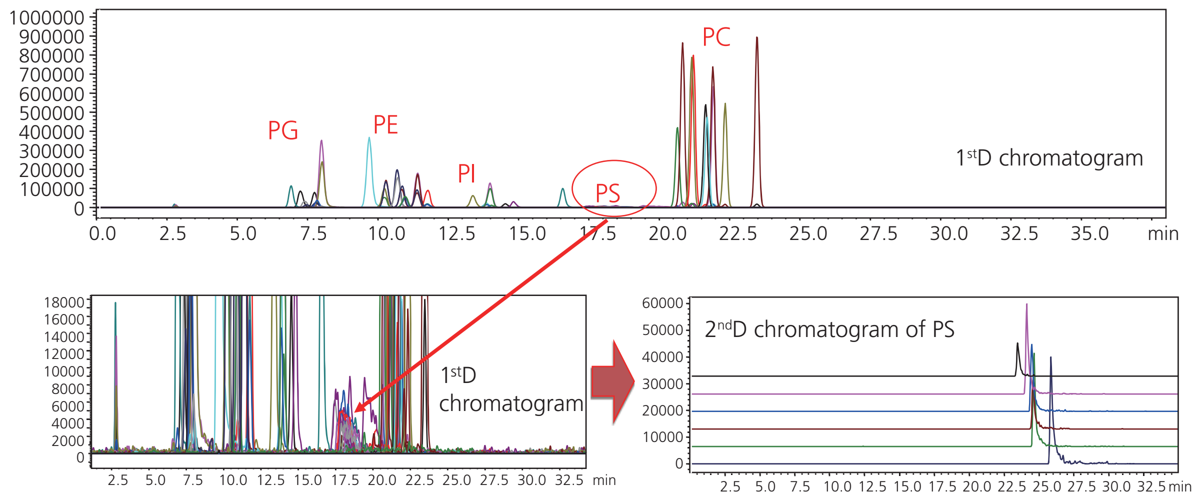


Fig. 3 Chromatograms of 1stD separation (mixtures including PG, PE, PI, PS and PC) and 2ndD separation (Fraction : PS)

Excellent linearity was acquired. Typical calibration curves are displayed in Fig. 4 and linearity and reproducibility results are summarized in Table 1.

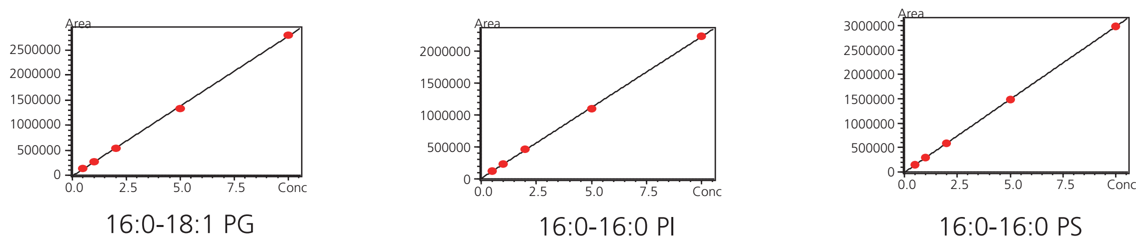


Fig. 4 Representative calibration curves (16:0-18:1 PG, 16:0-16:0 PI, 16:0-16:0 PS)

3-2. Comparison between conventional reversed phase LC and 2D analytical results

In the present study, PS and PG were selected to test the influence from other components. Results of conventional reversed phase analysis and 2D analysis were compared. In order to evaluate the effect of matrices with different concentrations, a series of samples were prepared for PS and PG, separately. For PS, the matrix including PG,PE, PI and PC was prepared firstly. PS standards were mixed with this matrix at three different ratios (1:1,1:9 and 1:99). (See Table 2.) As to PG, the matrix including PE, PI, PS and PC was added into PG standards with the same ratio as PS standards.

Due to insufficient separation of GPLs using conventional RPLC, the intensities of PS standards were affected at different levels, and also it is interesting to notice that not only suppression but also enhancement was observed (see Table 3). As a result, it was difficult to precisely quantify real samples using conventional reversed phase LC. With the help of 2D LC method, the interference from other components was avoided and reliable quantification was achieved.

Table 2 Final composition and concentration of matrix 1,2,3 and STD solution

	STD (µg/L)	Matrix (µg/L)	ratio
Matrix 1	25	25	1:1
Matrix 2	25	225	1:9
Matrix 3	25	2475	1:99
STD	25	0	-

Table 3 Comparison of Conventional RP LC and 2D LC results

#	Compounds	Conventional RP LC			2D LC		
		Matrix1 %	Matrix2 %	Matrix3 %	Matrix1 %	Matrix2 %	Matrix3 %
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

*The ratio was calculated by the following equation: Area (Matrix) /Area (STD)*100.
*Values in red mean significant ion interference from matrix was observed.

4. Conclusions

This 2D LC system offers automated target GPLs concentration and removal of undesired components. Furthermore, utilizing different column combinations or mobile phase compositions, this system permits reliable and sufficient separation of various classes of GPLs. It makes it easy to achieve reliable and accurate quantitation of minor species of GPLs.

5. Acknowledgement

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