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LC-MS/MS Analysis of Lysophospholipids Associated with Soy Protein Isolate

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Approximately 20−25% of babies consuming formula are fed formulas containing soy protein as the sole protein source. Isoflavone phytoestrogens are considered as major bioactive components present in soy protein isolate (SPI) and the most widely studied phytochemicals in human diets and soy infant formulas. In the present study, LC-MS/MS analysis of SPI phytochemical extract resulted in identifications of fifty-six lysophospholipids (lyso-PL), including eighteen lysophosphatidylcholines (lyso-PC), twelve lysophosphatidylethanolamines (lyso-PE), eleven lysophosphatidylinositols (lyso-PI), eleven lysophosphatidic acids (lyso-PA) and four lysophosphatidylglycerols (lyso-PG). The LC-MS/MS conditions were first developed using commercially available standard mixtures. Under these conditions, lyso-PL compounds could be separated by LC and yielded unique fragments and neutral losses in the electrospray ionization mass spectrometry (ESI-MS) with both positive- and negative-ion modes, quite indicative of the polar headgroups for different subclasses. This is the first characterization of lyso-PL in SPI. Lyso-PLs are important cell signaling and growth factor molecules experimentally linked to cardiovascular function and disease, cancer, and neurological disorders. These findings suggest that biological effects of PLs associated with SPI in infants clearly needs to be considered as part of an overall evaluation of potential health benefits.

KEYWORDS: Soy protein isolate (SPI); SPI phytochemical; lysophospholipids; LC-MS/MS

INTRODUCTION

The soybean is considered by some to be one of the most important dietary factors responsible for disparities in breast cancer rates between Western and Asian countries (*1*). Many experimental studies suggest significant health benefits of soybeans, including the prevention of heart disease through reductions in serum cholesterol and reducing the risks of some cancers (*1*−*5*). In addition, the American Academy of Pediatrics recommends soy-based formula as safe and effective as an alternative to breast-feeding or milk-based formulas. Soy formulas are made with soy protein isolate (SPI), which is the sole protein source. In the United States, approximately 25% of infants who are formula-fed received soy formula (*6*). The long-term health consequences of early SPI intake have not been fully explored. Much of the putative health benefit of SPI has been attributed to the isoflavones bound to SPI. The two most studied isoflavones are genistein and daidzein, both of which have been demonstrated to have significant in vivo and in vitro effects. There are, however, many other phytochemicals bound to SPI, including phytosterols and saponins. Although these latter phytochemicals have not been studied for their health effects as rigorously as those of the isoflavones, they have been reported to have bioactivity in several in vitro systems (*7*).

We have been interested in determining the profile of lyso-PLs in SPI used to prepare soy-based infant formula. In the present study, lyso-PL components associated with SPI were extracted and directly analyzed by LC-MS/MS without the multistep purification and separation process.

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MATERIALS AND METHODS

Standards and Chemicals. Four lipid standards were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). They were L-α-lysophosphatidylcholine mixture (prepared by the action of phospholipase A on soybean L-α-phosphatidylcholine), containing primarily C-18 unsaturated fatty acids; L-α-lysophosphatidylethanolamine mixture (prepared from egg yolk), containing primarily stearic and palmitic acids; L-α-lysophosphatidylinositol mixture (prepared by the action of phospholipase A on soybean L-α-phosphatidylinositol), containing primarily palmitic and stearic acids; and L-α-lysophosphatidic acid (oleoyl-*sn*-glycero-3-phosphate, prepared by the action of phospholipase A on L-α-phosphatidic acid), dioleoyl. L-α-lysophosphatidylglycerol mixture was purchased from Larodan Lipids (Surrey, England). Soy protein isolate (SPI 670) was a kind gift from Protein Technologies International (St. Louis, MO).

Preparation of a Phytochemical Concentrate of SPI. SPI (4 g) was extracted with 50% (100 mL) and 80% (100 mL) aqueous methanol and 100% methanol (100 mL) at 5 °C. The extracts were combined and concentrated on a rotary evaporator under reduced pressure at room temperature until the methanol was removed. The concentrated extract was passed through a C18 (60 cm^3/10 g) Mega Bond Elute cartridge from Supelco (Bellefonte, PA), after activation with 2 volumes of methanol then 2 volumes of water. The cartridge was then rinsed with 2 volumes of water. The chemicals not retained by the C18 cartridge and eluted with water were sugars and some simple organic acids. The

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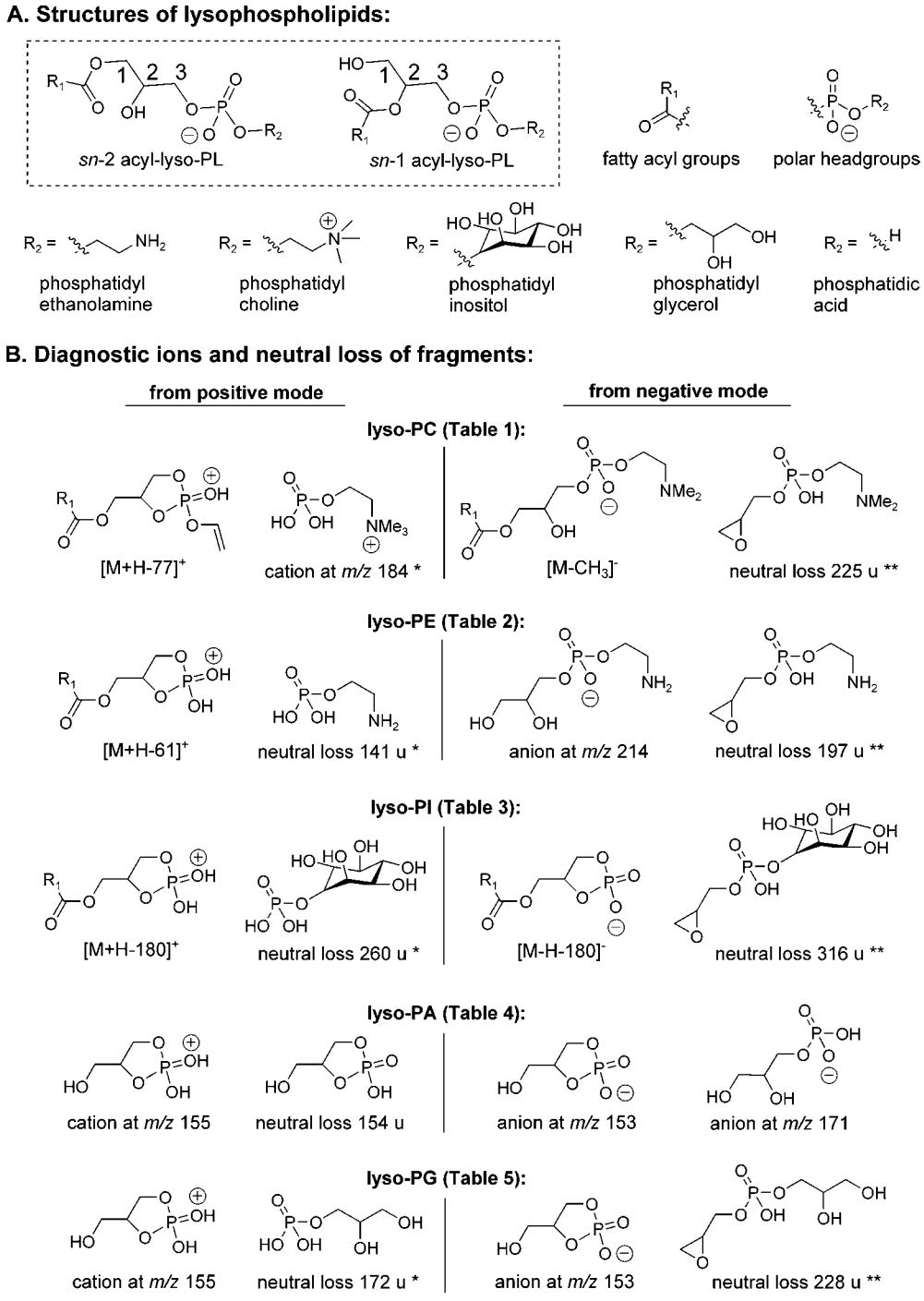

Figure 1. Structures of lyso-PLs, MS diagnostic ions and neutral loss of fragments used in this study. Structures of diagnostic ions and neutral loss of fragments were identified tentatively.

phytochemicals retained by the cartridge were eluted with 50% aqueous methanol (50 mL), methanol (50 mL), and acetone (50 mL). All three effluents were combined to give a fraction of interest, which was rotary evaporated under reduced pressure at room temperature followed by drying in a freeze-dryer.

LC-MS/MS Analysis. Lyso-PLs in complex mixture were directly analyzed by LC-MS/MS. LC-MS/MS was performed using a Bruker Esquire-LC multiple ion trap mass spectrometer equipped with an Agilent 1100 series liquid chromatograph. An HP ChemStation was used for data collection and manipulation. A 150 × 4.6-mm i.d. Eclipse XDB−C8 column (Agilent Technologies, Wilmington, DE) was used with LC solvent at a flow rate of 0.8 mL/min. The LC gradient was 0.1% formic acid/acetonitrile (solvent B) in 0.1% formic acid/H_2O (solvent A) as follows: 10−15% in 15 min; 15−18% from 15 to 16 min; 18−22% from 16 to 33 min; 22−40% from 33 to 40 min; held at 40% from 40 to 45 min; 40−42% from 45 to 49 min; 42−45% from 49 to 50 min; 45−50% from 50 to 65 min; 50−65% from 65 to 75 min; 65−85% from 75 to 76 min; 85−100% from 76 to 80 min; held at 100% from 80 to 85 min and finally back to 10% in 90 min. Lyso-PLs in the eluant were monitored by a diode-array detector and analyzed by automatic MS/MS with both negative and positive ion modes. For optimum MS analysis, 10 mM ammonium acetate (for negative-ion mode) or 2% formic acid (for positive-ion mode) in methanol were used as ionization reagents and added at a flow rate of 0.2 mL/min via a tee in the eluant stream of the HPLC just prior to the mass spectrometer. Conditions for ESI-MS analysis of HPLC peak in both

Table 1. ESI-MS Data for Lysophosphatidylcholines Associated with SPI

		Positive Ionization mode, *m/z* (rel intens. %)					Negative Ionization mode, *m/z* (rel intens. %)					
no. in	rt	Major ions in MS		Product ions in CID spectra			Major ions in MS		Product ions in CID spectra			
fig 2	(min)	$[M+H]^+$	$[head]^+$	parent	$[M+H-H_2O]^+$	$[head]^+$	$[M+HCOO]^-$	$[M-CH_3]^-$	parent	$[M-CH_3]^-$	$[acyloxyl]^-$	structure of lyso-PC
1	38.4	570 (100)		570	552 (100)	184 (44)	614 (100)	554 (63)	614	554 (100)		triOH-18:1a/lyso-PC
									554		329 (100)	
2	46.4	536 (43)[a]	184 (47)	536	518 (100)	184 (18)	580 (100)	520 (61)	580	520 (100)		lyso/OH-18:2a-PC
				518	500 (78)	184 (100)						
3	47.1	536 (42)[a]	184 (10)	536	518 (100)	184 (6)	580 (100)	520 (45)	580	520 (100)		OH-18:2a/lyso-PC
				518	500 (100)	184 (46)			520		295 (100)[b]	
4	48.1	536 (34)[a]	184 (40)	536	518 (100)	184 (67)	580 (100)	520 (31)	580	520 (100)		OH-18:2a/lyso-PC
				518	500 (100)	184 (19)			520		295 (100)	
5	49.0	536 (41)[a]	184 (18)	518	500 (100)	184 (89)	580 (100)	520 (62)	580	520 (100)		OH-18:2a/lyso-PC
									520		295 (63)[b]	
6	51.0	536 (100)		536	518 (100)	184 (26)	580 (100)	520 (59)	580	520 (100)		OH-18:2a/lyso-PC
7 (Std-A)	54.9	468 (100)	184 (32)	468	450 (75)	184 (100)	512 (100)	452 (74)	512	452 (100)	227 (21)	lyso/14:0a-PC
									452		227 (100)	
8 (Std-B)	56.4	468 (100)	184 (18)	468	450 (100)	184 (18)	512 (100)	452 (63)	512	452 (100)		14:0a/lyso-PC
									452		227 (100)	
9 (Std-C)	56.8	518 (66)	184 (100)	518	500 (100)	184 (86)	562 (100)	502 (82)	562	502 (100)		lyso/18:3a-PC
10 (Std-D)	58.2	518 (100)		518	500 (100)	184 (21)	562 (100)	502 (85)	562	502 (100)		18:3a/lyso-PC
									502		227 (100)	
11 (Std-E)	61.9	520 (100)		520	502 (83)	184 (100)	564 (100)	504 (59)	564	502 (100)	279 (7)	lyso/18:2a-PC
									504		279 (100)	
12 (Std-F)	63.4	520 (100)		520	502 (100)	184 (24)	564 (100)	504 (64)	564	502 (100)	279 (1)	18:2a/lyso-PC
									504		279 (100)	
13 (Std-G)	64.8	496 (100)	184 (26)	496	478 (100)	184 (99)	540 (100)	480 (84)	540	480 (100)		lyso/16:0a-PC
14 (Std-H)	66.7	496 (100)	184 (4)	496	478 (100)	184 (22)	540 (100)	480 (75)	540	480 (100)		16:0a/lyso-PC
15 (Std-I)	68.7	522 (100)	184 (46)	522	504 (100)	184 (96)	566 (100)	506 (58)	566	506 (100)	281 (6)	lyso/18:1a-PC
16 (Std-J)	70.1	522 (100)		522	504 (100)	184 (27)	566 (100)	506 (44)	566	506 (100)		18:1a/lyso-PC
									506		281 (100)	
17 (Std-K)	74.4	524 (100)	184 (34)	524	506 (75)	184 (100)	568 (100)	508 (50)	568	508 (100)	283 (6)	lyso/18:0a-PC
18 (Std-L)	75.7	524 (100)		524	506 (100)	184 (25)	568 (100)	508 (52)	568	508 (100)		18:0a/lyso-PC

[a] Ions at *m/z* 518 $[M+H-H_2O]^+$ are base peaks in spectra of these compounds **2**–**5**. [b] Ions at *m/z* 277 are $[acyloxyl-H_2O]^-$ and present in CID spectra of ions at *m/z* 520 from compounds **3** and **5** at relative intensity of 26 and 100%, respectively.

negative- and positive-ion modes included a capillary voltage of 3200 V, a nebulizing pressure of 33.4 psi, a drying gas flow of 8 mL/min, and a temperature of 250 °C. Parameters that control the API interface and the mass spectrometer were set via the Smart Tune with compound stability of 50% and trap drive level of 50%. Ion Charge Control (ICC) was on, including target, 5000; maximum accumulation time, 50.00 ms; scan, *m/z* 80.00−850.00; averages, 10; and rolling averaging, off. Conditions for automatic MS/MS were width of the isolation, 4.0; fragmentation amplitude, 1.00 V; and number of parents, 1.

RESULTS AND DISCUSSION

Analysis of Lyso-PLs Associated with SPI. Lyso-PL standards were used to develop the LC-MS/MS method for analysis of lyso-PLs in the SPI extract. **Figure 1** shows the basic chemical structures and unique fragments and neutral losses discussed in this study.

Lysophosphatidylcholine (*Lyso-PC*). LC-MS/MS analysis of lyso-PCs standard mixture resulted in a total ion chromatogram (TIC) of multiple peaks (**Std-A** to **L** in **Figure 2**). **Std-A** to **L** exhibited very similar positive-ion mass spectra with the base peaks for $[M+H]^+$ ions and minor peaks for ions at *m/z* 184. Collision-induced dissociation (CID) of $[M+H]^+$ ions yielded the simple and characteristic spectra with two major peaks for the ion $[M+H-H_2O]^+$ and an ion at *m/z* 184 (**Table 1**). The ion at *m/z* 184 is a phosphocholine cation and an important indicator for the presence of glycerophospholipid (**Figure 1**) (*8*, *9*). Compounds **7**−**18** were identified as lyso-PCs by direct comparison with LC-MS/MS data of their corresponding standard lyso-PCs **A**−**L**, respectively (**Figure 2**). Using negative-ion mode, lyso-PC produced very abundant adduct ions $[M+HCOO]^-$ due to neutralization of the positive charge of the choline nitrogen atom charge with a formic anion. CID of $[M+HCOO]^-$ were dominated by the loss of formic ether to yield the base peaks of $[M-CH_3]^-$ (**Figure 2** and **Table 1**). When the ions $[M-CH_3]^-$ were used as precursor ions, the most abundant ions generated from CID were carboxylate anions (R_1COO^-), due to loss of the neutral cyclic glycerophosphate ester (**Figure 1**). For the structures of compounds **1**−**6**, their CID pathways indicate that they are lyso-PCs (**Figure 1** and **Table 1**). Calculation of $[M+H]^+$ and $[M+HCOO]^-$ suggests that **1** is triOH-18:1a/lyso-PC and **2**−**6** are five isomers of OH-18:2a/lyso-PC, respectively. Their retention behaviors on RP-LC having shorter retention times on the C8 sorbent than other PCs and product carboxylate anion (R_1COO^-) from precursor

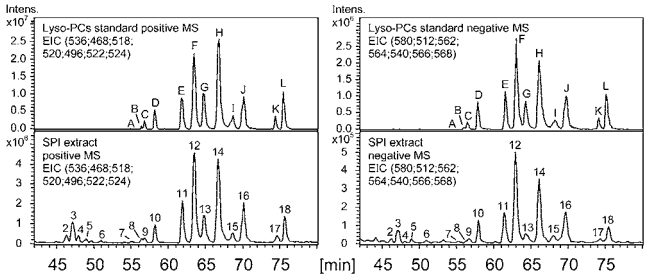

Figure 2. LC-MS/MS chromatograms of standard lyso-PCs and lyso-PCs in SPI, and mass spectra of *sn*-1 acyl-lyso-PC and *sn*-2 acyl-lyso-PC. **1**, triOH-18:1a/lyso-PC; **2**, lyso/OH-18:1a-PC; **3**, OH-18:2a/lyso-PC; **4**, OH-18:2a/lyso-PC; **5**, OH-18:2a/lyso-PC; **6**, OH-18:2a/lyso-PC; **7** (**A**), lyso/14:0a-PC; **8** (**B**), 14:0a/lyso-PC; **9** (**C**), lyso/18:3a-PC; **10** (**D**), 18:3a/lyso-PC; **11** (**E**), lyso/18:2a-PC; **12** (**F**), 18:2a/lyso-PC; **13** (**G**), lyso/16:0a-PC; **14** (**H**), 16:0a/lyso-PC; **15** (**I**), lyso/18:1a-PC; **16** (**J**), 18:1a/lyso-PC; **17** (**K**), lyso/18:0a-PC; and **18** (**L**), 18:0a/lyso-PC. Compound **1** is a trace component in SPI, and LC/MS/MS data are shown in Table 1.

Table 2. ESI-MS Data for Lysophosphatidylethanolamines Associated with SPI

		positive ionization mode, *m/z* (rel intens %)						negative ionization mode, *m/z* (rel intens. %)				
		major ions in MS		product ions in CID spectra				product ions in CID spectra				
no. in fig 3	rt (min)	$[M+H]^+$	$[M+H-141]^+$	parent	$[parent-H_2O]^+$	$[parent-61]^+$	$[parent-141]^+$	parent[a]	$[acyloxyl]^-$			structure of lyso-PE
1	46.8	494 (3)	476[b] (100)	476[b]	458[b] (100)	415[b] (40)	335[b] (34)	492	295 (100)		196 (10)	OH-18:2a/lyso-PE
2	55.5	426 (100)	285 (26)					424	227 (100)		196 (48)	14:0a/lyso-PE
3	55.9	476 (29)	335 (100)	476	458 (7)		335 (100)	474	277 (100)			lyso/18:3a-PE
4	57.3	476 (100)	335 (27)	476	458 (100)	415 (19)	335 (18)	474	277 (100)	214 (5)		18:3a/lyso-PE
5	60.6	478 (91)	337 (100)	478	460 (11)		337 (100)	476	279 (100)		196 (2)	lyso/18:2a-PE
6	62.3	478 (100)	337 (51)	478	460 (100)	417 (2)	337 (21)	476	279 (100)		196 (5)	18:2a/lyso-PE
7 (Std-A)	63.3	454 (49)	313 (100)	454	436 (16)		313 (100)	452	255 (100)	214 (2)		lyso/16:0a-PE
8 (Std-B)	65.2	454 (100)	313 (30)	454	436 (100)	393 (4)	313 (20)	452	255 (100)	214 (2)	196 (2)	16:0a/lyso-PE
9	67.1	480 (100)	339 (33)	480	462 (62)		339 (100)	478	281 (100)			lyso/18:1a-PE
10	68.9	480 (100)	339 (29)	480	462 (100)	419 (9)	339 (16)	478	281 (100)		196 (7)	18:1a/lyso-PE
11 (Std-C)	73.1	482 (58)	341 (100)	482	464 (24)		341 (100)	480	283 (100)			lyso/18:0a-PE
12 (Std-D)	74.3	482 (100)	341 (30)	482	464 (100)	421 (5)	341 (19)	480	283 (100)	214 (4)	196 (4)	18:0a/lyso-PE

[a] $[M-1]^-$ are base peaks in MS. [b] Ions at 476, 458, 415, and 335 are $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, $[M+H-H_2O-61]^+$ and $[M+H-H_2O-141]^+$, respectively.

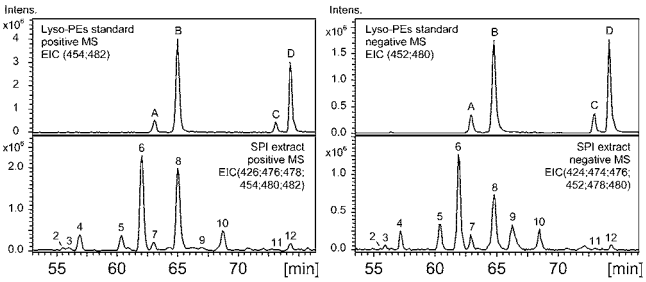

Figure 3. LC-MS/MS chromatograms of standard lyso-PEs and lyso-PEs in SPI, and mass spectra of *sn*-1 acyl-lyso-PE and *sn*-2 acyl-lyso-PE. **1**, OH-18:2a/lyso-PE; **2**, 14:0a/lyso-PE; **3**, lyso/18:3a-PE; **4**, 18:3a/lyso-PE; **5**, lyso/18:2a-PE; **6**, 18:2a/lyso-PE; **7** (**A**), lyso/16:0a-PE; **8** (**B**), 16:0a/lyso-PE; **9**, lyso/18:1a-PE; **10**, 18:1a/lyso-PE; **11** (**C**), lyso/18:0a-PE; and **12** (**D**), 18:0a/lyso-PE. Compound **1** is a trace component in SPI, and LC/MS/MS data are shown in Table 2.

$[M-CH_3]^-$ of **1**, **3** − **5** support these assignments of hydroxyacyl-lyso-PC structures (**Table 1**). The numbers of double bonds and hydroxyl groups could be determined by their MS data, but their positions could not be elucidated here.

The discrimination between regioisomers of lyso-PC (*sn*-1 or *sn*-2 lyso subspecies) is difficult because of their closely related structures. With a similar CID pathway for lyso-PC regioisomers from ESI-MS, the peak intensity ratio of product ions at *m/z* 104 and 147 generated from $[M+Na]^+$ ion were used previously for distinguishing between *sn*-1 and *sn*-2 lyso-PC regioisomers (*10*). However, these two ions are not present in the product ion spectra of lyso-PC in the present study. However, using this MS/MS condition, cleavage of the monoacyl glycerol part and polar headgroup part (**Figure 1**) was favored for *sn*-1 lyso-PC regioisomer (**Std-A**, **C**, **E**, **G**, **I,** and **K**) resulting in prominent phosphocholine cations with more than 80% relative intensity. In contrast, phosphocholine cations from *sn*-2 lyso-PC regioisomer (**Std-B**, **D**, **F**, **H**, **J,** and **L)** were never prominent.

Lysophosphatidylethanolamine (*Lyso-PE*). SPI components **7**, **8**, **11,** and **12** were identified as standard lyso-PEs **A**, **B**, **C,** and **D**, respectively, on the basis of their almost identical LC-MS/MS data, including retention time in LC and MS data in both positive and negative ions (**Figure 3** and **Table 2**). The two most abundant ions in positive-ion mass spectra of all compounds correspond to the protonated molecular ions $[M+H]^+$ and $[M+H-141]^+$. The neutral loss of 141 Da resulted from the loss of phosphoethanolamine (**Figure 1**) and is quite indicative of lyso-PE (*8*). In negative ion MS, the CID pathway of $[M-H]^-$ was characterized by an abundant loss of 197 Da (neutral cyclic glycerophosphate ester) and formation of carboxylate anions (R_1COO^-) from fatty acyl groups (**Figure 1**). Compounds **1**−**6**, **9**, and **10** were identified as lyso-PE on the basis of their same MS behavior as that of standard lyso-PEs in both positive and negative ions (**Table 2**). The regioisomers (*sn*-1 and *sn*-2 lyso-PE) yielded the same fragments, but relative intensities of the same fragments in positive-ion spectra of the isomers were strikingly different. While relative intensities of the ion $[M+H-141]^+$ in the product ion spectra of *sn*-2 lyso-PE were lower than 22%, the MS data of *sn*-1 lyso-PE was consistent with a favored loss of phosphoethanolamine neutral species (141 Da) and formation of $[M+H-141]^+$ as base peaks in MS and product ion spectra. This difference in ion intensity, along with the different retention behaviors in HPLC, can readily distinguish the regioisomers of lyso-PE. In a previous study, the minor product ions $[M+Na-61]^+$, $[M+Na-163]^+$and ion at *m/z* 164 were used for discrimination between sodiated lyso-PE regioisomers (*10*).

Lysophosphatidylinositol (*Lyso-PI*). Direct comparison of LC-MS/MS analysis of lyso-PI standards (**Std A** − **J**) and SPI extract led to identification of 10 lyso-PIs (compounds **2**−**11**) in the SPI extract (**Figure 4** and **Table 3**). Compound **1** was identified as HO-18:2a/lyso-PI by a mass fragmentation pattern very similar to that of standard lyso-PIs **A**−**J**. The fragmentation pattern for lyso-PI with a positive ion mode was dominated by the cleavage of monoacyl glycerol and polar headgroup (260 Da) to generate abundant monoacyl glycerol cations. The neutral loss of inositol (180 Da) resulted in the ions $[M+H-\text{inositol}]^+$, and CID of ions $[M+H-\text{inositol}]^+$ produced an abundant ion at *m/z* 155, corresponding to $[\text{glycerolphosphate}-H_2O]^+$. Formation of carboxylate anion (R_1COO^-) as a dominant ion by elimination of a neutral cyclic glycerophosphate ester (316 Da) indicates that the major dissociation pathway involved in CID of the $[M-H]^-$ from lyso-PI is identical to that for lyso-PC and lyso-PE (**Figure 1**). The fragment at *m/z* 241 represents the inositolphosphate minus water and characterizes the polar headgroup of PI (*8*). Also, the fragment obtained from the neutral loss of inositol (180 Da) can be used as indicator for lyso-PI. The product-ion patterns from $[M+H]^+$ of lyso-PC and lyso-PE exhibit the critical features for distinction between regioisomers, but analysis of lyso-PI in this LC/MS/MS condition did not generate product-ion data of $[M+$

Table 3. ESI MS/MS Data for Standard Lysophosphatidylinositols Associated with SPI

no. in fig 4	rt (min)	positive-ion mode *m/z* (rel intens. %)[a] major ions in MS				negative-ion mode *m/z* (rel intens. %) product ions in CID spectra						structure of lyso-PI
		$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H-180]^+$	$[M+H-260]^+$	parent[b] $[M-H]^-$	$[parent-180]^-$	ion at 315	$[acyloxyl]^-$			
1	47.8	613 (0)	577 (44)[c]	415 (9)[c]	335 (100)[c]	611	431 (93)	315 (11)	295 (100)	277 (13)	241 (61)	HO-18:2a/lyso-PI
2 (Std-A)	57.2	595 (10)	577 (7)	415 (0)	335 (100)[d]	593	413 (74)	315 (63)	277 (91)	241 (100)	223 (94)	lyso/18:3a-PI
3 (Std-B)	59.0	595 (16)	577 (69)	415 (19)	335 (100)	593	413 (50)	315 (32)	277 (100)	241 (14)	223 (5)	18:3a/lyso-PI
4 (Std-C)	62.3	597 (8)	579 (7)	417 (5)	337 (100)	595	415 (35)	315 (19)	279 (100)	241 (69)	223 (59)	lyso/18:2a-PI
5 (Std-D)	64.1	597 (2)	579 (48)	417 (20)	337 (100)	595	415 (57)	315 (25)	279 (100)	241 (26)	223 (7)	18:2a/lyso-PI
6 (Std-E)	65.3	573 (0)	555 (7)	393 (4)	313 (100)	571	391 (18)	315 (82)	255 (100)	241 (86)	223 (60)	lyso/16:0a-PI
7 (Std-F)	67.3	573 (3)	555 (79)	393 (40)	313 (100)	571	391 (69)	315 (27)	255 (100)	241 (42)	223 (4)	16:0a/lyso-PI
8 (Std-G)	68.4	599 (2)	581 (7)	419 (8)	339 (100)	597	417 (16)	315 (65)	281 (88)	241 (100)	223 (56)	lyso/18:1a-PI
9 (Std-H)	71.1	599 (3)	581 (59)	419 (15)	339 (100)	597	417 (87)	315 (9)	281 (100)	241 (65)	223 (9)	18:1a/lyso-PI
10 (Std-I)	76.0	601 (0)	583 (6)	421 (1)	341 (100)	599	419 (18)	315 (75)	283 (100)	241 (28)	223 (14)	lyso/18:0a-PI
11 (Std-J)	77.9	601 (15)	583 (66)	421 (36)	341 (100)	599	419 (44)	315 (26)	283 (100)	241 (31)	223 (3)	18:0a/lyso-PI

[a] MS with positive-ion mode of the lyso-PI was less sensitive than that with negative-ion mode, and no lyso-PI yielded CID spectra of $[M+H]^+$ or $[M+H-H_2O]^+$. [b] Lyso-PIs yielded predominant base peaks for $[M-H]^-$ without other significant peaks in their mass spectra. [c] Ions at 577, 415, and 335 are $[M+H-2H_2O]^+$, $[M+H-H_2O-180]^+$ and $[M+H-H_2O-260]^+$, respectively. [d] Data from CID of ion $[M+H-260]^+$ (monoacyl glycerol) are not shown in this table.

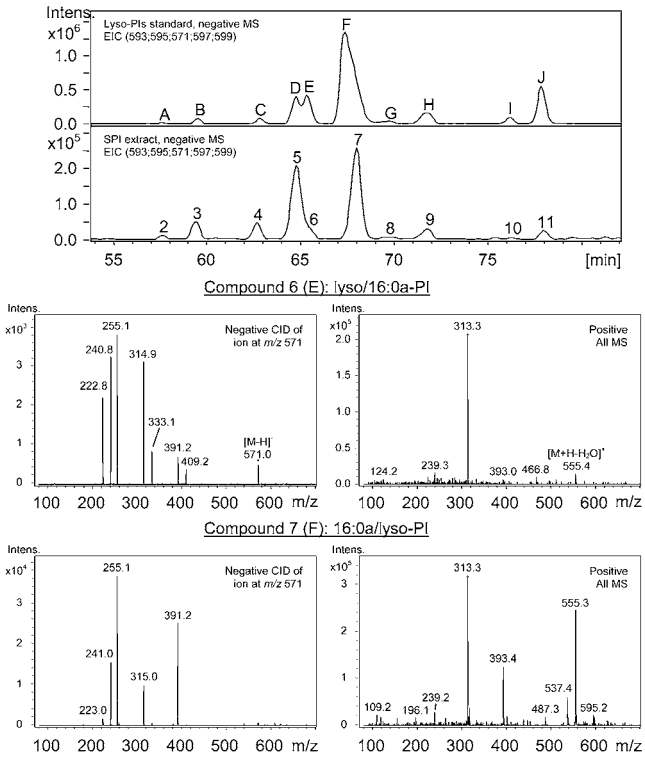

Figure 4. LC-MS/MS chromatograms of standard lyso-PIs and lyso-PIs in SPI, and mass spectra of *sn*-1 acyl-lyso-PI and *sn*-2 acyl-lyso-PI. **1**, OH-18:2a/lyso-PI; **2** (**A**), lyso/18:3a-PI; **3** (**B**), 18:3a/lyso-PI; **4** (**C**), lyso/18:2a-PI; **5** (**D**), 18:2a/lyso-PI; **6** (**E**), lyso/16:0a-PI; **7** (**F**), 16:0a/lyso-PI; **8** (**G**), lyso/18:1a-PI; **9** (**H**), 18:1a/lyso-PI; **10** (**I**), lyso/18:0a-PI; and **11** (**J**), 18:0a/lyso-PI. *Compound **1** is a trace component in SPI and LC/MS/MS data is shown in Table 3.

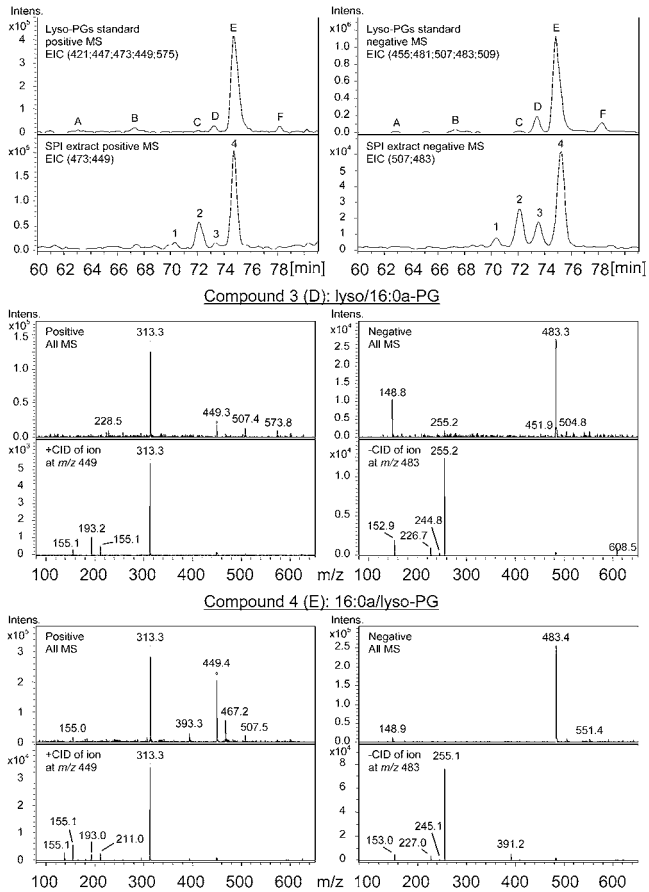

Figure 5. LC-MS/MS chromatograms of standard lyso-PGs and lyso-PGs in SPI, and mass spectra of *sn*-1 acyl-lyso-PG and *sn*-2 acyl-lyso-PG. (**A**), 14:0a/lyso-PG; (**B**), 16:1a/lyso-PG; **1**, lyso/18:2a-PG; **2** (**C**), 18:2a/lyso-PG; **3** (**D**), lyso/16:0a-PG; **4** (**E**), 16:0a/lyso-PG; and **F**, 18:1a/lyso-PG.

$H]^+$. However, the difference between the intensity ratio of ions at *m/z* $[M+H-H_2O]^+$ and $[M+H-260]^+$ in the mass spectra is still significant for discrimination between the regioisomers of lyso-PI (**Table 3**).

Lysophosphatidic acid (*Lyso-PA*). Using LC-MS/MS in negative ion mode, standard 18:1a/lyso-PA yielded a deprotonated molecular ion $[M-H]^-$ as base peak. CID of $[M-H]^-$ was characterized by an ion at *m/z* 171 resulting from elimination of the acyl part and an ion at *m/z* 153 formed by loss of water from the ion at *m/z* 171 (**Figure 1**). With the same CID pattern shown by their MS data, compounds **1**−**11** were identified as lyso-PA (**Table 4**). MS analysis of lyso-PA in positive ion mode had a much lower sensitivity in comparison to that in negative ion mode and only three lyso-PAs yielded positive MS data. Three major peaks were given in their mass spectra with $[M+H-H_2O]^+$ as base peak. Two other ions at *m/z* $[M+H-H_2O-154]^+$ and 155 were derived from the cleavage of carboxylate anion and dehydrated glycerophosphatidic acid (**Table 4**).

Lysophosphatidylglycerol (*Lyso-PG*). Four lyso-PGs were found as minor components in the SPI extract. Three of them, **2**−**4**, were identified by direct comparison with their corresponding lyso-PG standards **C**, **D,** and **E** (**Figure 5** and **Table**

Table 4. ESI-MS Data for Lysophosphatidic Acids Associated with SPI

		negative-ion mode, *m*/*z* (rel intens %)				
			product ions from CID spectra			
no. for lyso-PA	rt (min.)[a]	$[M–H]^-$ in MS	parent	ion at 171	ion at 153	structure of lyso-PA
std	(80.8)	435 (100)	435	171 (15)	153 (100)	18:1a/lyso-PA
1	39.8	483 (100)	483	171 (11)	153 (100)	triOH-18:1a/lyso-PA
2	52.4	449 (100)	449	171 (23)	153 (100)	OH-lyso/18:2a-PA
3	53.1	449 (100)	449	171 (13)	153 (100)	OH-18:2a/lyso-PA
4	54.2	449 (100)	449	171 (16)	153 (100)	OH-lyso/18:2a-PA
5	55.2	449 (100)	449	171 (5)	153 (100)	OH-18:2a/lyso-PA
6	67.5	431 (100)	431	171 (23)	153 (100)	lyso/18:3a-PA
7	69.1	431 (100)	431	171 (24)	153 (100)	18:3a/lyso-PA
8	73.7	433 (100)	433	171 (9)	153 (100)	lyso/18:2a-PA
9	74.7	433 (100)	433	171 (13)	153 (100)	18:2a/lyso-PA
10	77.2	409 (100)	409	171 (2)	153 (100)	lyso/16:0a-PA
11	78.1	409 (100)	409	171 (7)	153 (100)	16:0a/lyso-PA

	positive-ionization mode, *m*/*z* (rel intens. %)						
	major peaks in MS			product ions from CID spectra			
no. for lyso-PA	$[M + H - H_2O]^+$	$[M + H - H_2O - 154]^+$	ion at 155	parent	$[M + H - H_2O - 154]^+$	ion at 155	structure of lyso-PA
std	419 (100)	265 (77)	155 (59)	419	265 (100)	155 (15)	18:1a/lyso-PA
8	417 (100)	263 (51)	155 (15)	417	263 (100)	155 (18)	18:2a/lyso-PA
10	393 (100)	239 (27)	155 (28)	393	239 (40)	155 (100)	16:0a/lyso-PA

[a] Retention time in parentheses is from LC/MS/MS profile of standard in same condition.

Table 5. ESI-MS Data for Lysophosphatidylglycerols Associated with SPI

		positive ionization mode, *m*/*z* (rel intens %)								negative ionization mode, *m*/*z* (rel intens %)					
		major ions in MS			product ions in CID spectra					$[M - H]^-$	product ions in CID spectra				
no. in fig 5	rt (min.)[a]	$[M + H]^+$	$[M + H - 36]^+$	$[M + H - 172]^+$	parent	$[parent - 136]^+$				in MS	parent	$[acyloxy]^-$			structure of lyso-PG
Std-A	(63.3)	457 (34)	421 (100)	285 (75)	421	285 (100)	211 (43)	193 (0)	155 (50)	455 (100)	455	227 (100)			14:0a/lyso-PG
Std-B	(67.9)	483 (6)	447 (57)	311 (100)	447	311 (100)	211 (0)	193 (54)	155 (21)	481 (100)	481	253 (100)	227 (12)	153 (6)	16:1a/lyso-PG
1	70.0	509 (8)	473 (15)	337 (100)						507 (100)	507	279 (100)	227 (0)	153 (9)	lyso/18:2a-PG
2 (Std-C)	72.0	509 (28)	473 (86)	337 (100)	473	337 (100)	211 (38)	193 (0)	155 (10)	507 (100)	507	279 (100)	227 (6)	153 (6)	18:2a/lyso-PG
3 (Std-D)	74.0	485 (0)	449 (14)	313 (100)	449	313 (100)	211 (10)	193 (19)	155 (6)	483 (100)	483	255 (100)	227 (8)	153 (16)	lyso/16:0a-PG
4 (Std-E)	75.4	485 (1)	449 (72)	313 (100)	449	313 (100)	211 (8)	193 (6)	155 (16)	483 (100)	483	255 (100)	227 (5)	153 (7)	16:0a/lyso-PG
Std-F	(79.0)	511 (0)	475 (64)	339 (100)	475	339 (100)	211 (3)	193 (20)	155 (28)	509 (100)	509	281 (100)	227 (7)	153 (6)	18:1a/lyso-PG

[a] Retention time in parentheses is from LC/MS/MS profile of standard in Figure 5.

5). Like other subtypes of lyso-PL discussed above, MS data of lyso-PG is characterized by the diagnostic fragments (cation at *m*/*z* 155 and anion at *m*/*z* 153) and neutral losses of 172 Da in positive-ion mode and 228 Da in negative-ion mode (**Figure 1**). Compound **1** also yielded these diagnostic MS data and was identified as lyso-PG (**Table 5**).

Application of LC-MS/MS to Analyze Lyso-PL. Lyso-PL compounds with closely related structures including *sn*-1 and *sn*-2 lyso-PL regioisomers could be separated using this LC condition. The *sn*-2 lyso-PLs are retained longer on the C8 sorbent than their corresponding *sn*-1 isomers and eluted at a higher organic percentage. Abundant molecular ion species including protonated and deprotonated molecular ions, their adduct ions, and ions for $[M - CH_3]^-$ and $[M + H - H_2O]^+$ are extremely valuable for determination of the molecular ion. Under this MS/MS condition, the product-ion patterns of lyso-PL subclasses, including lyso-PC, lyso-PE, lyso-PI, and lyso-PG, were dominated by the same CID pathway, which greatly simplifies the interpretation of ESI-MS data. The major pathway in positive-ion ESI-MS is cleavage of the monoacyl glycerol part and polar headgroup (**Figure 1**). For the negative ion mode, the most important feature is the generation of the acyloxy anion resulting from neutral losses of cyclic glycerophosphate ester, and this structural information readily distinguishes variants in polar headgroups and the fatty acyl groups of lyso-PL (**Figure 1**). Notably, the MS data in positive ion mode are consistent with a favored cleavage of monoacyl glycerol part and polar headgroup part from the *sn*-1 lyso-PL regioisomers leading to the more abundant production of monoacyl glycerol cations for lyso-PE or phosphocholine cations for lyso-PC, which therefore allows for the distinction between *sn*-1 and *sn*-2 lyso-PL regioisomers. Furthermore, lyso-PLs associated with SPI are analyzed by the LC-MS/MS method without the multistep purification and separation process to avoid the possibilities of isomerization and other structural change. This approach has allowed us to identify the individual lyso-PLs via the LC retention behaviors and unique diagnostic fragments and neutral losses of fragments. The methods were demonstrated to be well-suited for the rapid on-line characterization of lyso-PL from complex matrixes. Also, this method could be used for quantification of individual lyso-PL in complex mixtures by the integration of the areas of its well-separated peak using either TLC or extracted ion chromatogram (EIC) of MS if a corresponding standard is available.

In conclusion, the LC-MS/MS conditions were developed to analyze the potential bioactive components in SPI, and 56 lyso-PL components were identified. Lyso-PLs are important biosynthetic precursors of bioactive phospholipids and have biological activity in their own right. Whereas the phytochemical isoflavones were identified as the major bioactive ingredients for the health functionality of SPI, the PLs associated with SPI may also have health effects. It is yet to be determined if these

concentrations of lyso-PL components in SPI are sufficient to elicit health effects.

ABBREVIATIONS USED

SPI, soy protein isolate; ESI-MS, electrospray ionization mass spectrometry; API, atmospheric pressure interface; API-ES, atmospheric pressure interface-electrospray; ICC, ion charge control; TIC, total ion chromatogram; EIC, extracted ion chromatogram; CID, collision-induced dissociation; lyso-PL, lysophospholipids; lyso-PC, lysophosphatidylcholines; lyso-PE, lysophosphatidylethanolamines; lyso-PI, lysophosphatidylinositols; lyso-PA, lysophosphatidic acids; lyso-PG, lysophosphatidylglycerols.

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