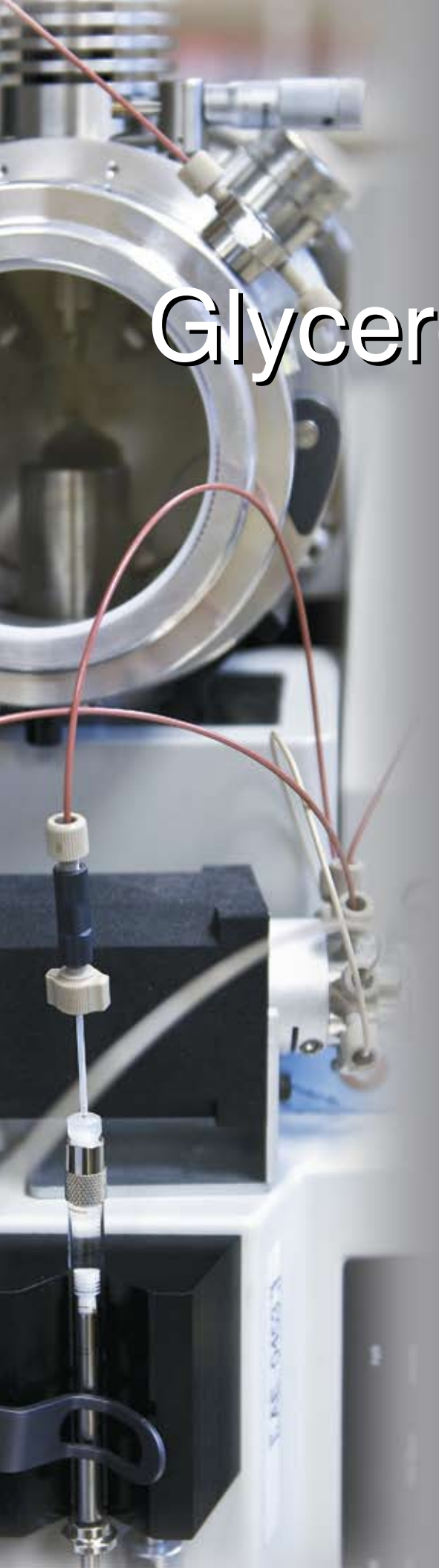

Glycerolipid Analysis

LC/MS/MS

Analytical Services

Avanti®
Polar Lipids, Inc.

Molecular Characterization and Quantitation of Glycerophospholipids in Commercial Lecithins by High Performance Liquid Chromatography with Mass Spectrometric Detection

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Introduction:

Lecithin is a loosely defined material that contains predominately neutral lipids and glycerophospholipids (GPLs) extracted from natural products such as soybean, algae and marine sources. The glycerophospholipids contained therein have beneficial pharmacologic and nutritional properties. Lecithin or modified lecithin products administered alone or in combination with drugs are used as emulsifiers, drug carriers, nutritional supplements and active nutraceuticals. The number and molecular diversity of GPL structures present within lecithins are quite large and dependant on their source. Molecular characterization and content of the GPLs is becoming a prerequisite for their use according the current good manufacturing practices (cGMPs) employed in the pharmaceutical and nutraceutical industries.

Avanti Polar Lipids® Analytical Services utilizes high performance liquid chromatography (HPLC) with a hybrid triple quadrapole - linear ion trap mass spectrometry (MS/MS-LIT) to characterize the molecular structures and quantify their respective content of the major GPLs in lecithins. This methodology circumvents classical approaches of thin-layer chromatography or HPLC fractionation of GPL groups followed by gas chromatography for fatty acid composition and or reversed phase separation for molecular species against (non)-available standards.

Principle:

A mass spectrometric detector provides an additional dimension to separation and quantitation of complex samples when utilized in conjunction with high performance liquid chromatography. Rather than identity and measurement of a compound based on retention time and peak response of a molecularly identical standard, mass spectrometry allows for identification based on the molecular weight of the compound(s) and quantitation using internal standards of similar structure. Full resolution of all compounds in a sample is not required due to the added third dimension of unique molecular weights for individual analytes. Further collisional dissociation of these specific molecular ions provides additional structural specificity as well as confirmation of analyte structure.

Initially, a lecithin matrix must be characterized for the major GPL groups and respective molecular species of which it is comprised. This is performed by using the MS/MS to scan specifically for molecules within a GPL group to the exclusion of all others. For example, phosphatidylserines (PS)

undergo a neutral loss of 87 daltons (u) from their de-protonated exact mass ([M-H]-) when assayed in negative ionization mode under instrument conditions which induce collisional dissociation (CID), i.e. fragmentation. This molecular – MS/MS reaction can be utilized to detect all the PS molecular species by performing a neutral loss scan for -87 u. The mass spectrometer returns a spectrum of masses equivalent to the [M-H]- for each PS species detected. These masses can be interpreted to a presumptive structure using a molecular weight library for GPLs. The presumptive identity is assigned by total number of acyl carbons and double bonds within the molecule (CC:DB), such as 36:4 PS. This 36:4 PS designation however could be equivalent to 5 different molecules to include 14:0-22:4 PS, 16:0-20:4 PS, 16:1-20:3 PS, 18:1-18:3 PS and 18:2-18:2 PS, positional ions withstanding.

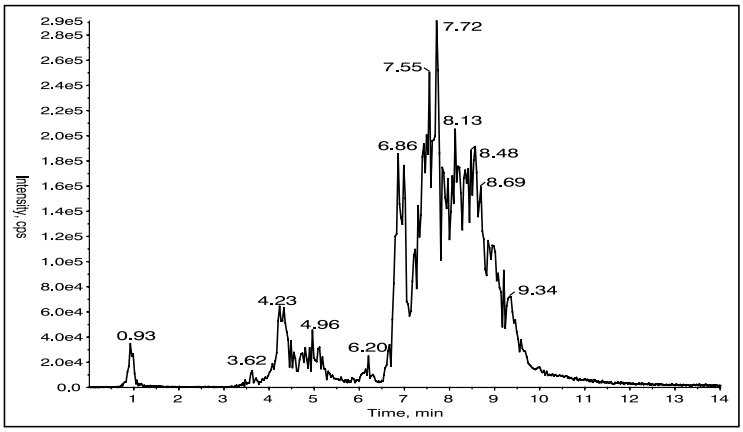
Secondly, the identified PS, [M-H]- masses and their corresponding [M-87]- u fragmentation reactions are optimized within the MS/MS quadrapoles. This provides for very sensitive and selective analysis of each PS species. This technique is known as multiple reaction monitoring (MRM). This MRM profile for PS species coupled with in-time separation afforded by reversed phase HPLC allows for possible quantitation without isotope correction between M+2 isotopes and CC:DB+1 molecular species. The measurement of each PS species is calculated from the linear area / concentration response of internal standards spiked into the lecithin sample prior to injection. These internal standards are synthetic GPLs which are exogenous in nature and have a defined concentration and stability.

Thirdly, a collision mass spectrum is generated by coupling the HPLC/MS/MS with the LIT of the hybrid instrument. As the PS peaks elute from the HPLC column their corresponding MRM peak is detected by the MS/MS. Resultant peaks of a minimum preset intensity triggers the LIT to collect a spectrum designed to deliver the molecular fragmentation of the peak. Interpretation of this spectrum allows for assignment of the final molecular species.

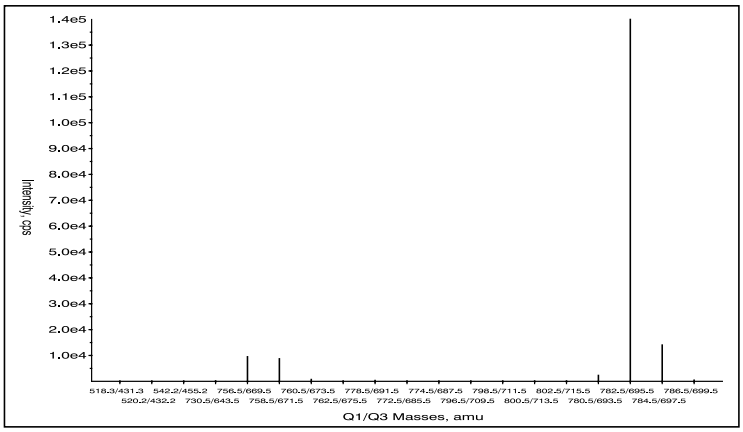
The technique described above can be performed for phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) species as well as PS based on their respective and specific mass spectral fragmentation properties. Unfortunately, phosphatidic acid and phosphatidylglycerol do not have group specific fragmentation mechanisms which are conducive to the present method conditions. These two GPL groups were not measured for this reason.

Avanti Polar Lipids Analytical Services' LC/MS/MS method for GPLs uses these advantageous fragmentation mechanisms to measure the individual compounds for PC, PE, PI and PS and there lyso counterparts (LPC, LPE, LPI and LPS) within a lecithin sample on a weight percentage basis. This document will report the molecular characterization and quantitation of major GPLs in crude soy lecithin and PS modified soy lecithin samples. Figure 1 demonstrates the HPLC/MS/MS-LIT technique described above for an aged PS modified soy lecithin. Tables 1 and 2 report the detected GPLs in crude soy lecithin and a PS modified soy lecithin sample different than that demonstrated in figure 1. Table 3 reports comparison of assay of total GPL groups between LC/MS/MS and 31P-NMR performed by Avanti Polar Lipids.

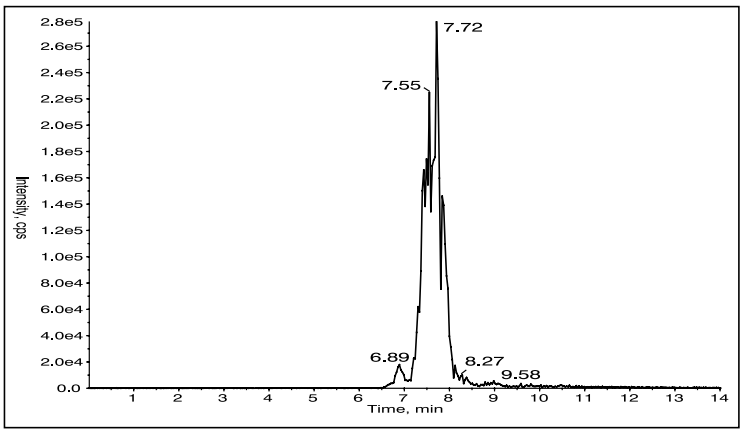
Figure 1: Detection of PS molecular species in aged PS modified soy lecithin.



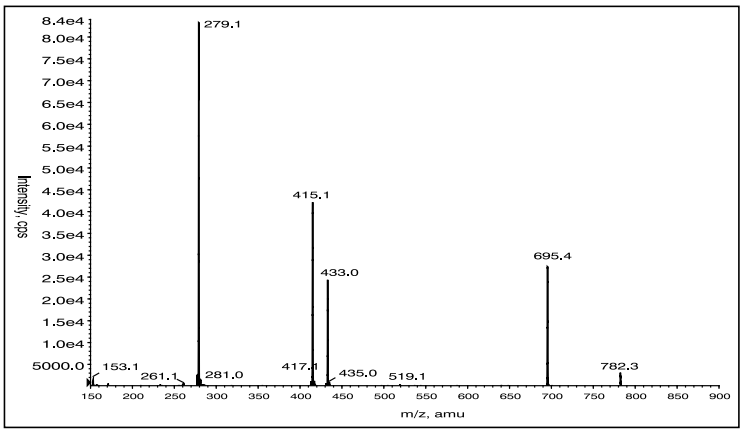
HPLC/MRM chromatogram demonstrates resolution of molecular species. Peaks at 0.9, 3-6.5 and 6.5-10 minutes are LPS, oxidized PS and PS species respectively.



Major MRM of 7.7 min peak is 782.5 / 695.5 (M-H-87) u for 36:4 PS.



Extracted ion chromatogram of 782.5 / 695.5 u. Note the separation of the [M+2] isotope peak of 36:3 PS (784.5/697.5 u) at 6.89 min.



LIT spectrum of 782.5 / 695.5 peak at 7.3 – 8.0 min.
782.3 u = [M-H]-,
695.4 u = [M-H-87]-,
433.0 u = [695.4 – C18H30O]-,
415.1 u = [695.4 – C18H32O2]-,
279.1 u = [M-H]- of 18:2 acid.
Minor peaks of 277.1 (18:3) and 281.0 (18:1) u are detectable (not shown).
Interpretation: 36:4 PS is predominately 18:2-18:2 PS with minor amounts of 18:1-18:3 PS.

Table 1: Glycerophospholipid Profile of Crude Soy Lecithin

GPL (CC:DB)	Analyte Retention Time (min)	Analyte Mass Ranges (amu)	Structure from LIT Spectrum	%/W	%Rsd of Triplicate Assay	% of GPL / Group
Phosphatidylcholines						
18:2 LPC	1.5	578.3/504.2 amu	18:2 LPC	1.58	3.84	9.20
17:1 LPC ISTD	1.5	566.4/492.4 amu	17:1 LPC			
16:0 LPC	1.7	554.3/480.2 amu	16:0 LPC	0.52	9.35	3.04
18:1 LPC	1.9	580.4/506.3 amu	18:1 LPC	0.31	14.72	1.80
25:0 PC ISTD	6.4	694.5/620.4 amu	12:0-13:0 PC			
31:1 PC ISTD	10.0	776.5/702.4 amu	17:0-14:1 PC			
36:4 PC	10.5	840.6/766.5 amu	18:2-18:2 PC	0.35	4.97	2.03
34:2 PC	10.9	816.6/742.5 amu	16:0-18:2 PC	5.63	3.32	32.82
36:3 PC	11.2	842.6/768.5 amu	18:1-18:2 PC	3.91	6.13	22.80
32:0 PC	11.3	792.6/718.5 amu	16:0-16:0 PC	0.62	8.82	3.62
34:0 PC	11.7	820.6/746.5 amu	16:0-18:0 PC	0.15	16.16	0.85
34:1 PC	11.7	818.6/744.5 amu	16:0-18:1 PC	1.77	3.65	10.28
37:4 PC ISTD	11.4	854.6/780.5 amu	17:0-20:4 PC			
38:4 PC	11.5	868.6/794.5 amu	18:0-20:4 PC	0.02	11.54	0.12
36:2 PC	12.0	844.6/770.5 amu	18:1-18:1 PC	1.97	9.70	11.47
36:1 PC	12.7	846.6/772.5 amu	18:0-18:1 PC	0.20	10.75	1.18
43:6 PC ISTD	13.2	934.7/860.6 amu	21:0-22:6 PC			
36:0 PC	13.4	848.6/774.5 amu	18:0-18:0 PC	0.13	45.99	0.77
Phosphatidylethanolamines						
16:0 LPE	1.4	454.3/313.2 amu	16:0 LPE	0.44	7.9	3.26
17:1 LPE ISTD	1.3	466.6/325.5 amu	17:1 LPE			
18:2 LPE	1.3	478.3/337.2 amu	18:2 LPE	1.20	4.0	8.83
25:0 PE ISTD	5.9	594.4/453.4 amu	12:0-13:0 PE			
36:4 LPE	9.3	738.5/597.5 amu	18:2-18:2 PE	0.40	6.1	2.93
36e_0 PE	9.3	760.6/619.6 amu	18:0e-18:0 PE	0.01	19.9	0.10
31:1 PE ISTD	9.5	676.5/535.5 amu	17:0-14:1 PE			
34:2 PE	9.7	714.5/573.5 amu	16:0-18:2 PE	0.39	3.5	2.89
36:3 PE	10.0	740.5/599.5 amu	18:1-18:2 PE	0.71	2.2	5.23
34:1 PE	10.4	716.5/575.6 amu	16:0-18:1 PE	6.57	5.5	48.29
36:2 PE	10.8	742.5/601.5 amu	18:1-18:1 PE	2.61	2.9	19.18
34e:2 PE	11.0	730.5/589.4 amu	16:0e-18:2 PE	0.02	5.6	0.13
37:4 PE ISTD	11.0	754.5/613.5 amu	17:0-20:4 PE			
34:0 PE	11.2	718.5/577.5 amu	16:0-18:0 PE	0.24	9.1	1.79
36:1 PE	11.5	744.5/603.5 amu	18:8-18:1 PE	0.91	4.0	6.66
36:0 PE	12.2	746.5/605.5 amu	18:0-18:0 PE	0.10	7.8	0.72
43:6 PE ISTD	12.8	834.6/693.6 amu	21:0-22:6 PE			

Table 1 (cont): Glycerophospholipid Profile of Crude Soy Lecithin

GPL (CC:DB)	Analyte Retention Time (min)	Analyte Mass Ranges (amu)	Structure from LIT Spectrum	%/W	%Rsd of Triplicate Assay	% of GPL / Group
Phosphatidylinositols						
17:1 LPI ISTD	0.6	583.3/241.0 amu	17:1 LPI			
18:2 LPI	0.6	595.3/241.0 amu	18:2 LPI	0.66	7.27	8.71
16:0 LPI	0.6	571.3/241.0 amu	16:0 LPI	0.67	22.56	8.84
18:1 LPI	0.7	597.3/241.0 amu	18:1 LPI	0.11	16.04	1.45
25:0 PI ISTD	1.9	711.4/241.0 amu	12:0-13:0 PI			
36:5 LPI	4.8	855.5/241.0 amu	18:2-18:3 PI	0.19	11.91	2.51
31:1 PI ISTD	4.9	793.5/241.0 amu	17:0-14:1 PI			
34:3 LPI	5.2	831.5/241.0 amu	16:0-18:3 PI	0.41	2.09	5.41
36:4 PI	5.6	857.5/241.0 amu	18:2-18:2 PI	0.98	17.40	12.93
34:2 PI	6.0	833.5/241.0 amu	16:0-18:2 PI	3.29	6.73	43.40
36:3 PI	6.5	859.5/241.0 amu	18:1-18:2 PI	0.59	15.96	7.78
37:4 PI ISTD	6.7	871.5/241.0 amu	17:0-20:4 PI			
36:2 PI	7.3	861.5/241.0 amu	18:0-18:2 PI	0.63	5.59	8.31
36:1 PI	8.1	863.5/241.0 amu	18:0-18:1 PI	0.05	5.91	0.66
43:6 PI ISTD	9.1	951.6/241.0 amu	21:0-22:6 PI			

PC+PE+PI 38.35 %/W

Table 2: Glycerophospholipids in Phosphatidylserine Modified Soy Lecithin

GPL (CC:DB)	Analyte Retention Time (min)	Analyte Mass Ranges (amu)	Structure from LIT Spectrum	%/W	%Rsd of Triplicate assay	% of GPL / Group
Phosphatidylcholines						
18:2 LPC	1.4	578.3/504.2 amu	18:2 LPC	1.95	2.14	15.62
17:1 LPC ISTD	1.6	566.4/492.4 amu	17:1 LPC			
16:0 LPC	1.7	554.3/480.2 amu	16:0 LPC	0.56	4.87	4.47
18:1 LPC	2.0	580.4/506.3 amu	18:1 LPC	0.31	7.97	2.52
25:0 PC ISTD	6.5	694.5/620.4 amu	12:0-13:0 PC			
31:1 PC ISTD	10.0	776.5/702.4 amu	17:0-14:1 PC			
36:4 PC	10.5	840.6/766.5 amu	18:2-18:2 PC	0.32	7.07	2.53
34:2 PC	10.9	816.6/742.5 amu	16:0-18:2 PC	3.96	14.76	31.78
36:3 PC	11.3	842.6/768.5 amu	18:1-18:2 PC	2.37	5.92	19.00
32:0 PC	11.4	792.6/718.5 amu	16:0-16:0 PC	0.42	15.47	3.35
34:0 PC	11.7	820.6/746.5 amu	16:0-18:0 PC	0.06	2.28	0.46
34:1 PC	11.7	818.6/744.5 amu	16:0-18:1 PC	0.64	35.21	5.11
37:4 PC ISTD	11.4	854.6/780.5 amu	21:0-22:6 PC			
38:4 PC	11.6	868.6/794.5 amu	18:0-20:4 PC	0.02	27.61	0.18
36:2 PC	12.0	844.6/770.5 amu	18:0-18:2 PC	1.54	8.24	12.33
36:1 PC	12.7	846.6/772.5 amu	18:0-18:1 PC	0.12	8.25	0.98
43:6 PC ISTD	13.3	934.7/860.6 amu				
36:0 PC	13.4	848.6/774.5 amu	18:0-18:0 PC	0.21	17.75	1.67
Phosphatidylethanolamines						
16:0 LPE	1.6	454.3/313.2 amu	16:0 LPE	0.40	5.08	3.52
17:1 LPE ISTD	1.4	466.6/325.5 amu	17:1 LPE			
18:2 LPE	1.4	478.3/337.2 amu	18:2 LPE	1.27	3.90	11.10
25:0 PE ISTD	6.0	594.4/453.4 amu	12:0-13:0 PE			
36:4 LPE	9.3	738.5/597.5 amu	18:2-18:2 PE	0.59	3.38	5.14
36e:0 PE	9.3	760.6/619.6 amu	18:0e-18:0 PE	0.02	15.45	0.21
31:1 PE ISTD	9.5	676.5/535.5 amu	17:0-14:1 PE			
34:2 PE	9.8	714.5/573.5 amu	16:0-18:2 PE	0.47	1.44	4.12
36:3 PE	10.1	740.5/599.5 amu	18:1-18:2 PE	0.75	3.35	6.60
34:1 PE	10.5	716.5/575.6 amu	16:0-18:1 PE	5.10	0.38	44.65
36:2 PE	10.8	742.5/601.5 amu	18:1-18:1 PE	1.77	4.97	15.52
34e:2 PE	11.0	730.5/589.4 amu	16:0e-18:2 PE	0.01	16.99	0.10
37:4 PE ISTD	11.0	754.5/613.5 amu	17:0-20:4 PE			
34:0 PE	11.2	718.5/577.5 amu	16:0-18:0 PE	0.10	2.69	0.89
36:1 PE	11.6	744.5/603.5 amu	18:0-18:1 PE	0.87	6.49	7.63
36:0 PE	12.2	746.5/605.5 amu	18:0-18:0 PE	0.06	5.95	0.52
43:6 PE ISTD	12.8	834.6/693.6 amu	21:0-22:6 PI			

Table 2 (cont): Glycerophospholipids in Phosphatidylserine Modified Soy Lecithin

GPL (CC:DB)	Analyte Retention Time (min)	Analyte Mass Ranges (amu)	Structure from LIT Spectrum	%/W	%Rsd of Triplicate assay	% of GPL / Group
Phosphatidylinositols						
16:0 LPI	0.7	571.3/241.0 amu	16:0 LPI	0.52	26.36	8.12
18:2 LPI	0.6	595.3/241.0 amu	18:2 LPI	0.52	15.09	8.20
17:1 LPI ISTD	0.7	583.3/241.0 amu	17:1 LPI			
18:1 LPI	0.7	597.3/241.0 amu	18:1 LPI	0.07	3.98	1.04
25:0 PI ISTD	2.0	711.4/241.0 amu	12:0-13:0 PI			
36:5 PI	4.8	855.5/241.0 amu	18:2-18:3 PI	0.22	11.12	3.40
31:1 PI ISTD	5.0	793.5/241.0 amu	17:0-14:1 PI			
34:3 PI	5.3	831.5/241.0 amu	16:0-18:3 PI	0.51	10.54	7.97
36:4 PI	5.7	857.5/241.0 amu	18:2-18:2 PI	0.88	11.72	13.85
34:2 PI	6.1	833.5/241.0 amu	16:0-18:2 PI	2.58	4.56	40.49
36:3 PI	6.5	859.5/241.0 amu	18:1-18:2 PI	0.46	15.45	7.23
37:4 PI ISTD	6.8	871.5/241.0 amu	17:0-20:4 PI			
36:2 PI	7.4	861.5/241.0 amu	18:0-18:2 PI	0.56	16.30	8.83
36:1 PI	8.2	863.5/241.0 amu	18:0-18:1 PI	0.06	25.12	0.88
43:6 PI ISTD	9.1	951.6/241.0 amu	21:0-22:6 PI			
Phosphatidylserines						
18:3 LPS	0.6	518.3/431.3 amu	18:3 LPS	0.03	8.85	0.09
18:2 LPS	0.7	520.2/432.2 amu	18:2 LPS	0.28	10.86	0.80
17:1 LPS ISTD	0.7	508.3/421.3 amu	17:1 LPS			
25:0 PS ISTD	1.4	636.4/549.4 amu	12:0-13:0 PS			
36:6 PS	2.2	778.5/691.5 amu	18:3-18:3 PS	0.18	28.30	0.51
32:2 PS	2.6	730.5/643.5 amu	14:0-18:2 PS	0.06	8.60	0.18
36:5 PS	2.6	780.5/693.5 amu	18:2-18:3 PS	2.36	13.11	6.71
31:1 PS ISTD	2.8	718.6/631.6 amu	17:0-14:1 PS			
34:3 PS	2.9	756.5/669.5 amu	16:0-18:3 PS	0.80	13.00	2.28
36:4 PS	3.1	782.5/695.5 amu	18:2-18:2 PS	13.03	6.08	36.99
34:2 PS	3.5	758.5/671.5 amu	16:0-18:2 PS	8.75	8.31	24.84
36:3 PS	3.8	784.5/697.5 amu	18:1-18:2 PS	5.73	3.34	16.25
37:4 PS ISTD	4.0	796.5/709.5 amu	17:0-20:4 PS			
34:1 PS	4.2	760.5/673.5 amu	16:0-18:1 PS	1.61	23.66	4.56
34:0 PS	4.5	762.5/675.5 amu	16:0-18:1 PS	0.13	12.11	0.36
36:2 PS	4.7	786.5/699.5 amu	18:1-18:1 PS	2.27	17.55	6.44
43:7 PS ISTD	6.8	876.6/789.6 amu				

PC+PE+PI+PS 65.49 %/W

Table 3: Comparison of Total GPLs by LC/MS/MS versus ^{31}P-NMR

Crude Soy Lecithin						
^{31}P-NMR	%/W		LC/MS/MS	%/W		Diff
PC	12.1		PC	14.8		2.7
LPC	2		LPC	2.4		0.4
PE	10.5		PE	11.6		1.1
LPE	2.6		LPE	2		0.6
PI	7.1		PI	6.1		1
LPI	ND		LPI	1.4		NA
PS	ND		PS	ND		---
LPS	ND		LPS	ND		---
PS Modified Soy Lecithin						
^{31}P-NMR	%/W		LC/MS/MS	%/W		Diff
PC	11.9		PC	9.7		2.2
LPC	3.1		LPC	2.8		0.3
PE	10.6		PE	9.6		1
LPE	1.9		LPE	1.6		0.3
PI	7.3		PI	5.3		2
LPI	NA		LPI	1.1		NA
PS	31.6		PS	34.9		3.3
LPS	0.6		LPS	0.3		0.3

ND = none detected, NA = not available due to same ppm shift as PE.

Discussion:

The methodology and data reported represent the characterization and content assay of PC, PE, PI and PS in two commonly available, commercial lecithins processed from soybean. The data reported in tables 1 and 2 as % weight of total sample provides content of individual GPLs and normalized % of each relative to GPL group total. The variance of triplicate assays was not more than ~15% for GPLs of greater than 1%. The sensitivity is approximately 0.1% by weight for the samples assayed. Comparison of total % of GPL groups measured was not more than 3% of ^{31}P-NMR measurements. Additional information available through appropriate calculations could be mol% of each GPL species and mol% of fatty acids based on summation within GPL group or as a total. The reported technique overcomes the problem of isotope correction necessary with non-chromatographic approaches. Analysis time is faster than classical approaches with minimized sample preparation for a single analytical platform that requires 15 minutes per GPL group. The use of commercially available non-endogenous and non-isobaric internal standards for calibration and quantitation minimizes need for molecularly identical standards for all GPLs. This technique is potentially applicable to any source of these GPLs provided due diligence and care is taken in the initial characterization step for each new matrix. Necessity to assay phosphatidic acid and phosphatidylglycerol GPL groups would require some pre-fractionation step such as normal phase HPLC or solid phase extraction or molecular adduction with a volatile metal such as lithium to provide selective assay.