Phospholipid Analysis

^{31}P NMR

Analytical Services

Avanti®
Polar Lipids, Inc.

Quantitative Phospholipid Analysis of Soy Lecithin and Krill Lecithin by ^{31}P NMR

Katherine L Lanier; Jeff D Moore; Dale Smith; Shengrong Li; Ben Davis; and Walter A. Shaw.

September, 2008

Avanti Polar Lipids, Inc.
Alabaster, AL. 35007

Introduction:

At Avanti® Polar Lipids, Inc. we strive for accurate and reliable analytical methods to define the quality of our products. The same analytical methods are the basis for the analysis of samples submitted to Avanti's® Lipid Analytical Service Division as well. As analytical technology grows more precise and sensitive, we strive to develop methods that are more exact and rigorous. The quantitative ^{31}P NMR method recently developed at Avanti® provides more accurate results with easier sample preparation, a better limit of detection, single point calibration, and faster analysis time over that of our existing HPLC based methods.

Principle:

Since its invention, Nuclear Magnetic Resonance Spectroscopy (NMR) has changed the face of structural analysis for chemists world wide. But it has not been until recently, with the advent of higher field strength magnets and precision probes, that we are able to use NMR for purity and quantitative analysis in addition to structural analysis. The basis of quantitative NMR relies on the concept that all isotopes have a direct relationship between peak area and the number of atoms excited. If one peak in the spectra, suitably resolved from all other peaks, can be identified and has a known molar concentration, then the remaining peaks can be calibrated against this standardized peak. The sharp peaks and broad range of chemical shifts of Phosphorous NMR (^{31}P) make this isotope an ideal candidate for quantitative NMR. The gyromagnetic ratio (½ spin) of the ^{31}P nucleus and high natural abundance allows for more sensitive detection and simple NMR pulse sequences. (Skoog, et al. 1998) (Schiller, et al. 2002) . ^{31}P NMR lends itself particularly well to phospholipid (PL) analysis since most PL classes contain a single phosphorous atom resulting in one sharp resolved peak per PL class. By using external standards of known and validated concentrations, the different PL classes can be quantified, resulting in a weight/weight % analysis for a broad range of matrices.

Historically, the use of ^{31}P NMR analysis for the quantification of PL's in samples has proven difficult because of poor resolution and chemical shift instability resulting from PL interactions within a matrix. Previous studies used organic solvents with highly controlled composition, temperature and pH to overcome these obstacles. Cation chelators such as EDTA were used by many studies to further decrease linewidth of peaks by reducing ion activity in different matrices. (Estrada, et al. 2008). However, since many samples contain differing amounts of retained water, the organic solvent composition would be altered, changing the chemical shifts of PLs. (Schiller, et al. 2002). To overcome the innate water content of samples, researchers either took extra sample preparation steps, such as lyophilization, or utilized detergent/water solutions. Detergent solutions, such as Triton X or

Deoxycholate have been previously documented to show PL resolution by class, and sometimes by fatty acid chain length as well. (Schiller, et al. 2002). (Pearce, et al. 2000). These detergent solutions work by creating fewer molecule micelles in which phospholipids aggregate, giving clear solutions at relatively high concentrations. The micelle capability of a detergent solution is dependent on its aggregation number or behavior. The smaller aggregates allow for a more homogenous solution, thus producing more resolved spectra. The principle behind this is as the aggregation size decreases, the opportunity for mixed micelle formation also reduces, further limiting PL/PL interactions (Schiller, et al. 2002). Figure 1 below displays the resolution of common phospholipids in a synthetic mixture, while Table 1 lists common phospholipids and their chemical shifts in a detergent based solvent system.

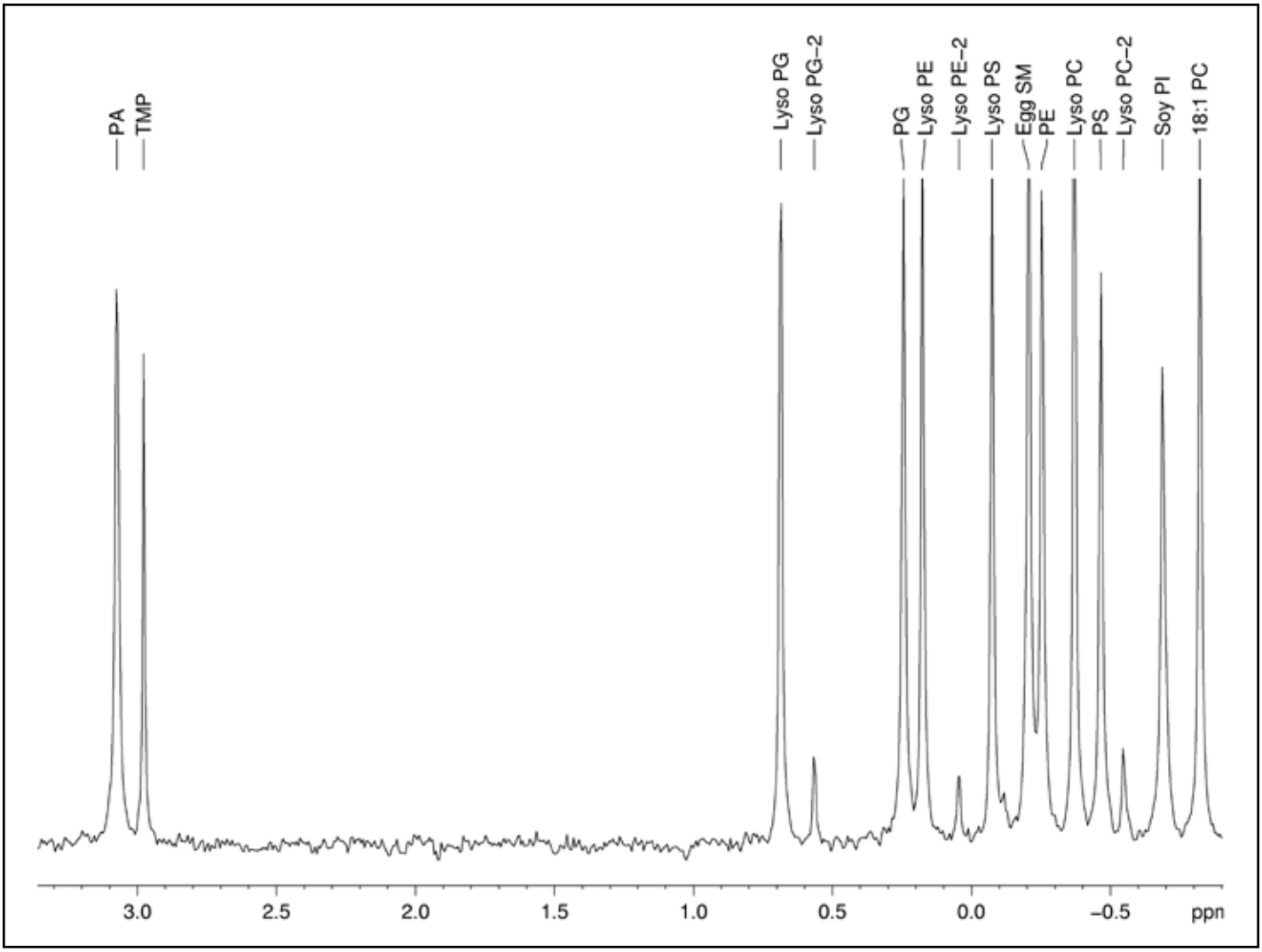

Figure 1. ^{31}P NMR analysis of a synthetic mixture of common phospholipids. Please note, all NMR analysis was completed on the Bruker Avance III 400mHz system with 5mm BBO probe and 24 place autosampler

Common Phospholipids and their chemical shifts in detergent solvent at pH 7.7	
Phospholipid	ppm
Lysophosphatidic Acid (LPA)	3.5
LPA-2	3.4
Phosphatidic Acid (PA)	3.1
Lysophosphatidylglycerol (LPG)	0.69
LPG-2	0.57
Phosphatidylglycerol (PG)	0.24
Lysophosphatidylethanolamine (LPE)	0.18
Cardiolipin (CA)	0.18
N Acyl PE	0.16
LPE-2	0.04
Lysophosphatidylserine (LPS)	-0.07
Glycerophosphocholine (GPC)	-0.18
Sphingomyelin	-0.21
Phosphatidylethanolamine (PE)	-0.25
Lysophosphatidylinositol (LPI)	-0.25
Lyso Ether PC	-0.30
Lyso Plasmalogen PC	-0.33
Lysophosphatidylcholine (LPC)	-0.35
LPI-2	-0.37
Phosphocholine	-0.41
Phosphatidylserine (PS)	-0.47
Diether PC	-0.52
LPC-2	-0.55
Phosphatidylinositol (PI)	-0.69
Ether/Ester PC	-0.75
Phosphatidylcholine (PC)	-0.82
Plasmalogen PC	-0.82

Table 1

Even though the use of detergent solvents can greatly increase resolution, some compounds will still not completely resolve, as can be seen in in the table above. In these instances, LC/Mass Spec analysis can be used to determine the chemical species. Temperature and pH conditions still should be maintained as these also effect the chemical shift of certain PL's. The chemical shift of PA is particularly susceptible to pH changes, with its strongest response around a neutral pH. (Puppato, et al. 2007). It has also been shown through testing at Avanti® that the chemical shift of PA can change from 2.32ppm to 3.08ppm with changes in concentration alone. Shown below in Figure 2 is an overlay of a serial dilution of 18:1 PA compared against an internal standard of TMP (seen at 2.98ppm). As the concentration of PA decreases, the peak shifts downfield and even co-resolves with TMP at a concentration of 1.56mM.

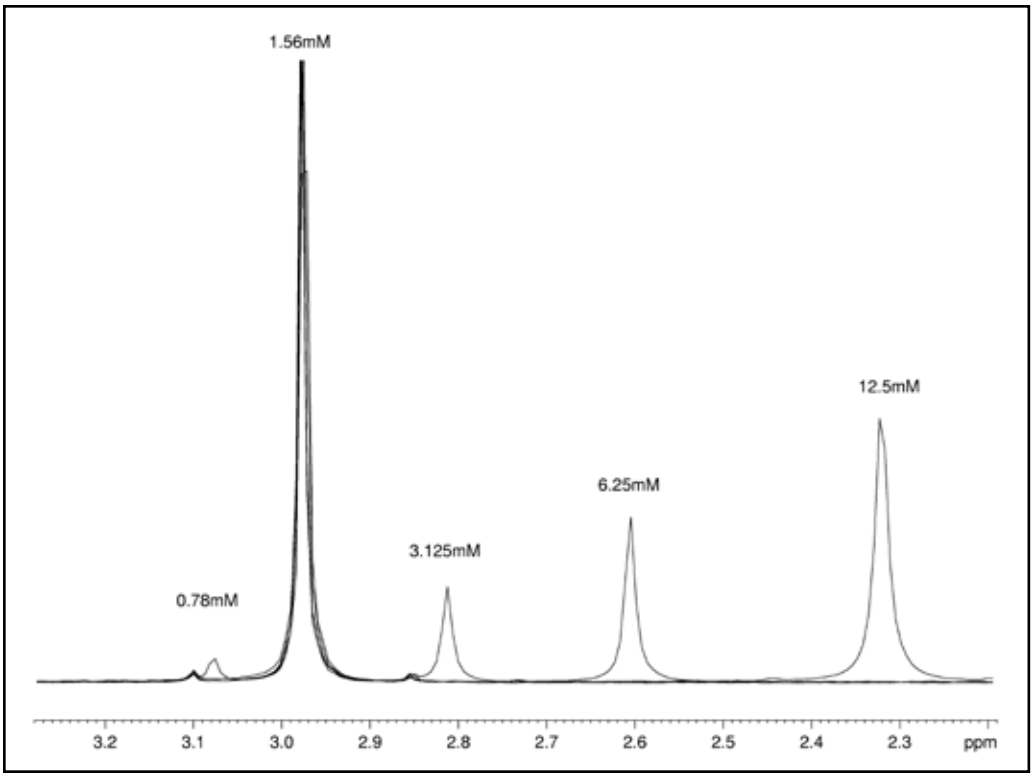

Figure 2. Overlay of 18:1 PA spectra at varied concentrations.

As earlier mentioned, PL classes in detergent systems can be further resolved by fatty acid chain length. Figure 3 is an overlay of differing chain length synthetic PCs. As can be seen, 18:1 and 18:0 chains can be almost completely resolved. Also notice the trend that shorter or more unsaturated chains lengths are shifted upfield. Since all lipids derived from natural sources contain differing chain lengths of PL's, it is important to ensure that all peaks are integrated properly.

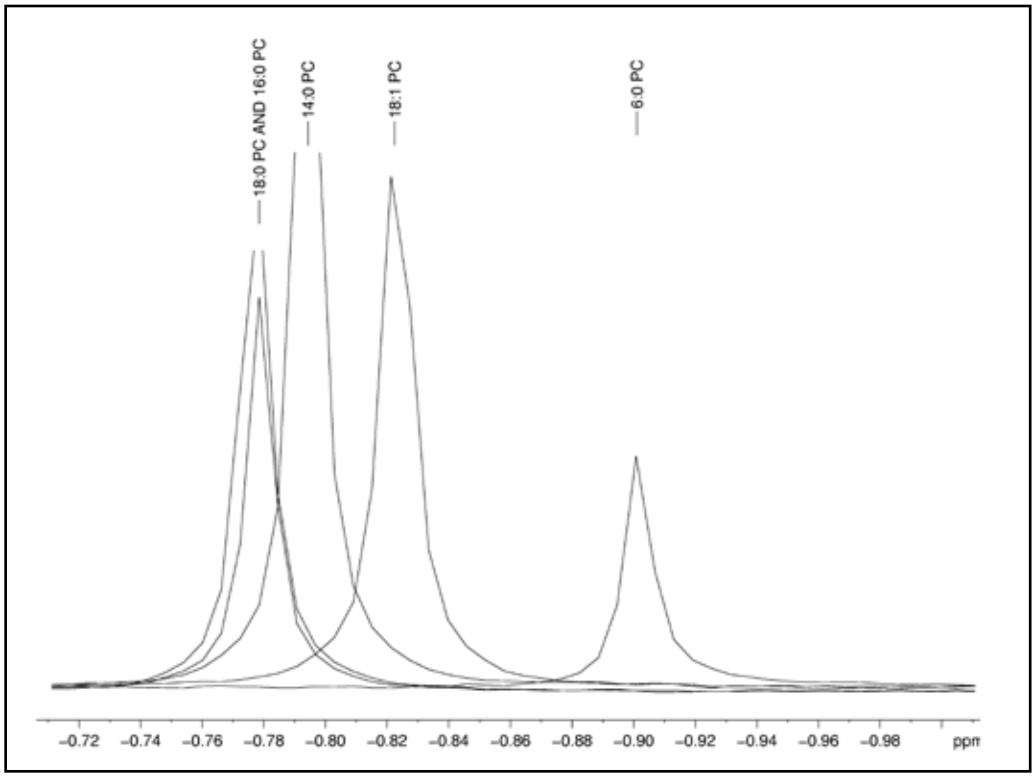

Figure 3. Overlay of PC's varying in fatty acid chain length

However in most instances, PL classes from natural sources are resolved as one sharp peak even with the presence of a mixture of chain lengths. A factor for the poor resolution of different chain length PLs from lipids derived from natural sources is the interactions of mixed micelles. In Figure 4, a mixture of 21:0, 14:0 and 06:0 PC was analyzed. The spectra resulted in the coresolution of 14:0PC and 21:0PC, with almost full resolution of the 06:0PC. Furthermore, the 06:0PC is shifted slightly downfield in the mixture compared to analysis by itself as seen in Figure 3.

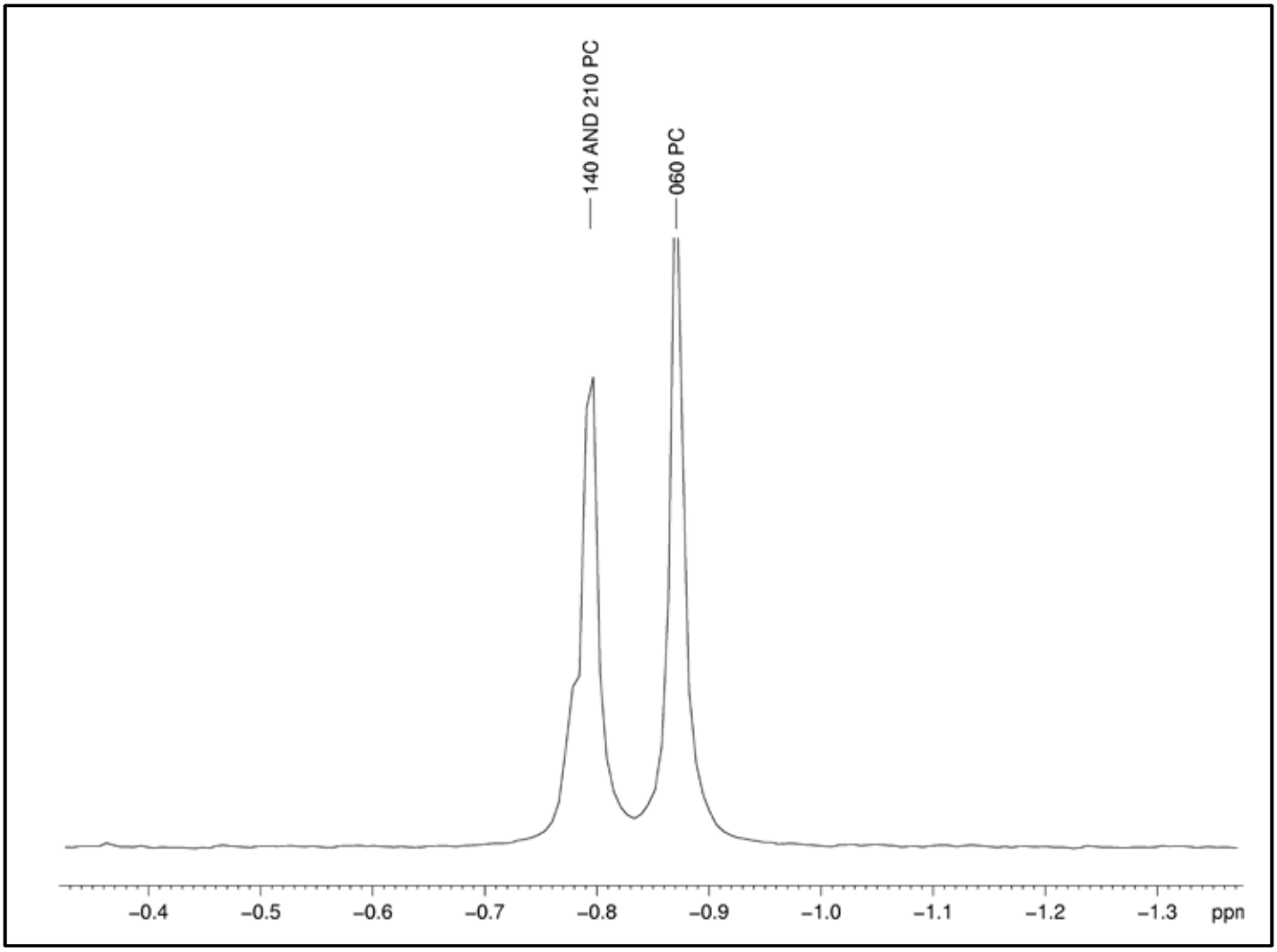

Figure 4. Analysis of a mixture of different fatty acid chain length PCs.

Another important observation to mention when dealing with detergent based solvents is that acyl migration of lyso PL's is induced in mixed micelle systems, although the mechanism is still unknown (Schiller, et al. 2002; and Puppato, et al. 2007).

Determine Relaxation Times:

In order to ensure optimum accuracy, the delay time between the excitation of nuclei must be considered. Many studies used a variety of delay times from 10 seconds (Pearce, et al. 2007) to as short as 1.86 seconds (Meneses, et al. 1988). To reduce excess analysis time, we performed thorough delay time studies where a synthetic mixture of PC, PG, PA, PE, LPE, and PS was assayed at 1, 3, 5, and 10 seconds in triplicate. The average integrated peak area of each PL at each delay time was compared to the average integrated peak area of the 10 second delay in terms of %RSD.

Comparison of D1 times on peak response. D1=10 sec, run time @ NS 512 is 1 hour and 34.57min			
	% RSD of 1 sec vs 10 sec	% RSD of 3 sec vs 10 sec	% RSD of 5 sec vs 10 sec
PA	0.07	0.12	3.27
PG	0.07	0.12	0.60
LPE	5.18	0.29	0.39
PE	0.43	0.82	1.04
PS	0.29	0.55	0.81
PC	0.52	0.22	0.18
Run Time NS=512	51.57min	34.45min	17.33min

Table 2

As can be seen in in the table above, a delay time of even one second does not give a significant difference in the peak response over a 10 second delay. However, notice LPE, a minor component in the mixture, does have a much higher %RSD when comparing the 1 second delay peak response to that of the 10 second delay. This is due to increased noise of the 1 second delay spectra. The LPE variance is due to a much smaller integrated area at a delay of 1 second compared to that with a 3 second delay. A 3 second delay provides greater signal to noise with acceptable run time.

Instrument Precision and Accuracy:

The level of precision and accuracy for any sample analysis is equivalent to the innate precision and accuracy of the instrument and method. In order to test the precision of the instrument and current method, we ran one sample of a mixture of synthetic PG, PC, PA, PS, and PE for a 24 hour period, with a 10 second delay, giving us 19 replicate spectra. Each peak was analyzed by the average, standard deviation and % RSD of the integrated peak areas for the 19 replicates, as seen in the table below.

Average, standard deviation and % RSD for integrated peak areas of 19 replicate spectrum			
	Peak Area	Standard Deviation	% RSD
PA	153887006	2236064	1.45
PG	154661061	1390090	0.90
PE	156539243	2440379	1.56
PS	133714077	2030841	1.52
PC	137003256	1638062	1.20

Table 3

This test proves the precision of the instrument and method, as well as allow for a 24 hour stability period for sample preparations. Note that all peak areas have an excellent relative standard deviation of less than 2%.

Instrument accuracy was determined by preparing various single component samples of reference standard materials. Samples were prepared at known concentrations confirmed by a second method.

Accuracy analysis of single component reference standards			
Sample	Actual [mM]	Measured [mM]	% Accuracy
14:0 PG	7.30	7.43	101.8
16:0 PC	6.91	6.82	98.7
14:0 CA	3.95	3.86	97.7
18:1 PS	6.14	6.31	102.8
16:0 PG	7.07	7.22	102.1
18:1 PC	6.27	6.31	100.7
Soy PI	5.83	6.21	106.5
16:0 LPC	5.19	5.24	101.1

Table 4

Linearity:

The linearity of a method describes the upper and lower limits of detection and quantitation, and the response behavior of the analytes. A linear response is ideal, allowing for one point calibrations. Linearity of the ^{31}P NMR method was determined by analyzing serial dilutions of a mixture of synthetic PC, PE, PG, PA, PS and Soy PI in triplicate. All components exhibited a linear response, and had the same response curve, allowing for a single point calibration of one standard for all components of a mixture.

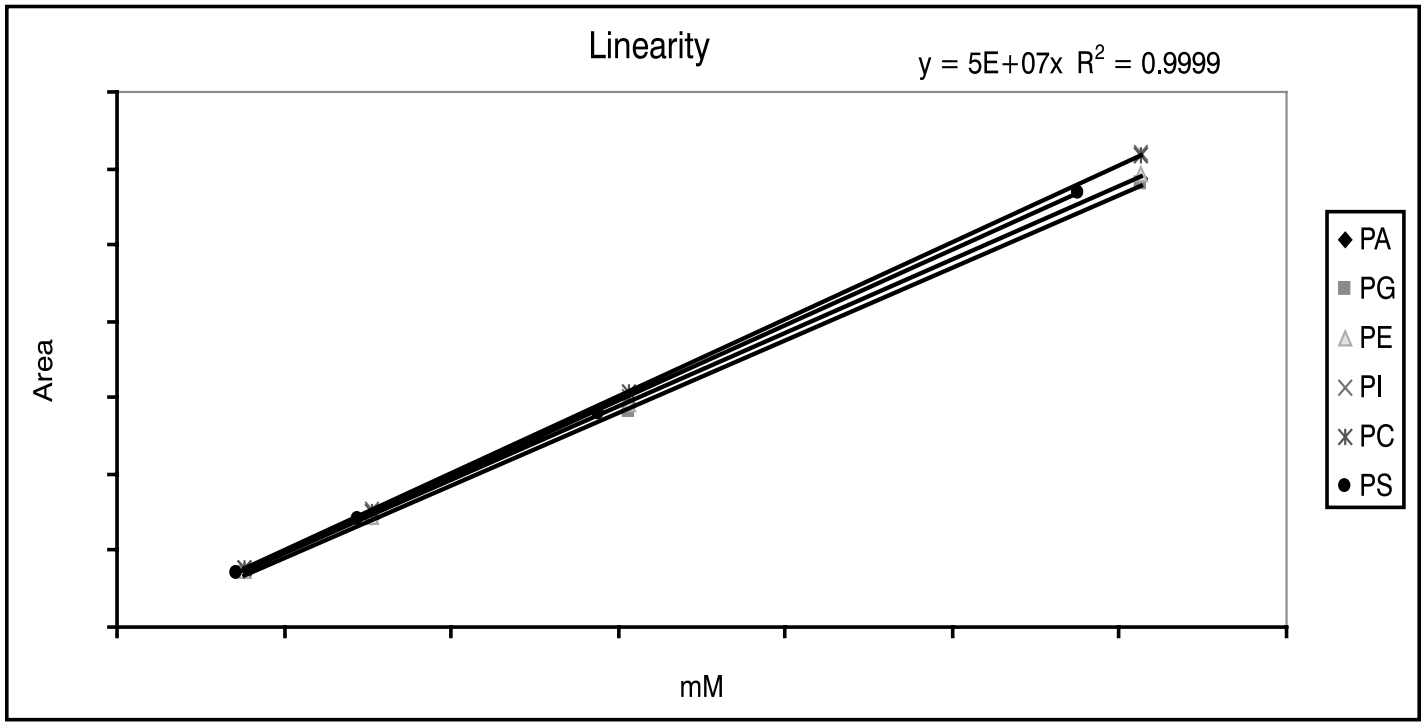

Figure 5. Concentration of the serial dilutions of PA, PE, PI, PC, PS, and PG plotted against the resulting integrated peak area.

To further confirm molar phosphorus response linearity, comparison between results obtained from a four point calibration and one point calibration yielded a +/-0.04% difference in wt/wt% results.

Sample Analysis:

The ultimate aim of this method is to analyze and quantify the PL content of any mixture, natural or synthetic. As seen above, synthetic mixtures are readily analyzed with precision and accuracy. In natural mixtures we must consider matrix contribution and interactions. For this study, two common natural matrices were analyzed, Soy Lecithin and Krill Oil.

Soy Lecithin Analysis:

Soy Lecithin is a common natural source of PC, PE, PI and PA derived from soybean and contains mostly 16:0 and 18:2 fatty acid. It is commonly used in the industry as an emulsifier, and as a nutritional supplement for phospholipids as well. Purification by column chromatography or precipitation techniques can be used to increase certain PL content, as well as decrease other matrix components, such as glycerides and free fatty acids. The following spectra, Figure 6, is an analysis of Soy Lecithin used in this study. As can be seen, the matrix contains PA, LPA, PG, PE, LPE, PI, PC and LPC. The average wt/wt% of each PL is reported in Table 5 following the spectra.

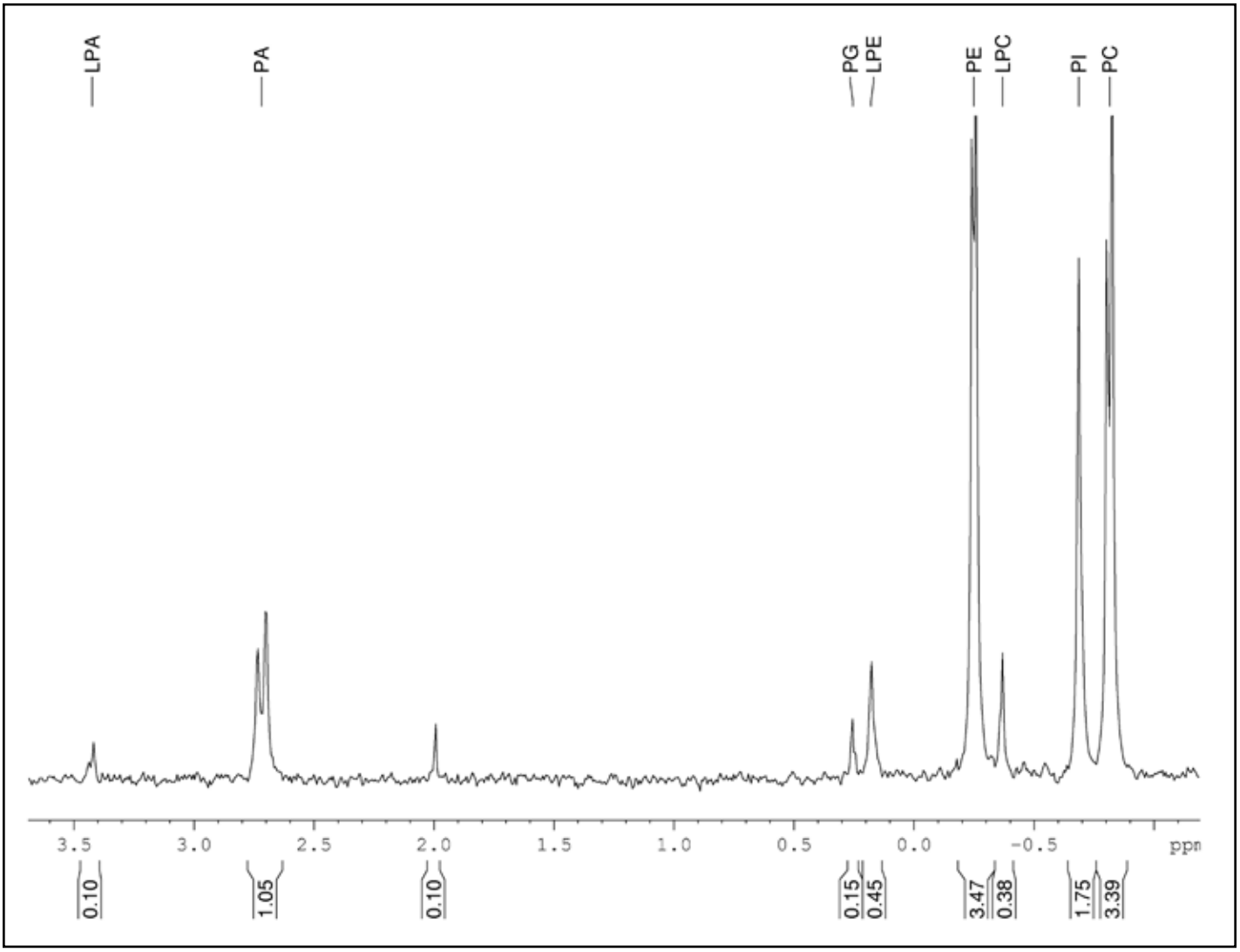

Figure 6. ^{31}P NMR analysis of Soy Lecithin

Average concentration and wt/wt% of PL's found in Soy Lecithin. The MW used in calculations is also provided. Samples prepared ten times at ~10mg/mL total weight.			
PL	[mM]	MW (g/mol)	Average wt/wt %
LPA	0.09	468.5	0.4 (+/-0.07)
PA	1.05	746.0	7.0 (+/-0.14)
PG	0.15	820.0	1.1 (+/-0.12)
LPE	0.45	492.5	2.0 (+/-0.02)
PE	3.38	770.0	23.1 (+/-0.74)
LPC	0.35	534.5	1.7 (+/-0.20)
PI	1.67	907.0	13.5 (+/-0.70)
PC	3.34	812.0	24.1 (+/-0.70)

Table 5

For accuracy testing, samples of Soy Lecithin were spiked with 1.23mM of 18:1 PC, PS, PE, PA and Soy PI, and 1.15mM 18:1 PS. These samples where then compared to non spiked preparations. All preparations were done in triplicate to ensure precision. These results were then compared in terms of percent recovery of the spiked samples as seen in the table below.

% Recovery results for Soy Lecithin spiked sample analysis.			
PL	Theoretical Spiked [mM]	Measured Spiked [mM]	% Recovery
PA	2.28	2.17 (+/-0.07)	95.2
PG	1.38	1.28 (+/-0.03)	92.8
PE	4.61	4.47 (+/-0.10)	97.0
PS	1.15	1.12 (+/-0.06)	97.4
PI	2.90	2.91 (+/-0.04)	100.3
PC	4.57	4.55 (+/-0.12)	99.6

Table 6

Sample precision was performed by preparing 10 separate samples and calculating the standard deviation and relative standard deviation of each PL in the matrix. As can be seen in the table below, the lower limit of quantitation for Soy Lecithin is 2% wt/wt with the precision below a preferable 8%.

Precision analysis results for 10 sample preparations of Soy Lecithin.			
PL	Average wt/wt%	Standard Deviation	%RSD
PA	6.83	0.20	2.96
PG	1.32	0.18	13.89
LPE	2.13	0.13	6.29
PE	22.92	0.65	2.84
LPC	1.82	0.19	10.64
PI	13.61	0.26	1.91
PC	23.89	0.60	2.52

Table 7

Krill Lecithin Analysis:

Krill oil, derived from shrimp-like crustaceans from the Antarctic Ocean, is a newly discovered source of omega-3 fatty acids known for its aid in lowering cholesterol and its ability to help reduce the symptoms of premenstrual syndrome. Krill oil contains mostly PC and triglycerides carrying mainly eicosapentanoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6). (Sampalis, et al. 2003). The following spectrum, Figure 7, is an analysis of Krill Lecithin used in this study. Krill lecithin contains mostly PC and LPC with smaller amounts of PE, LPE and PA. Table 8 below lists the average wt/wt% of each PL.

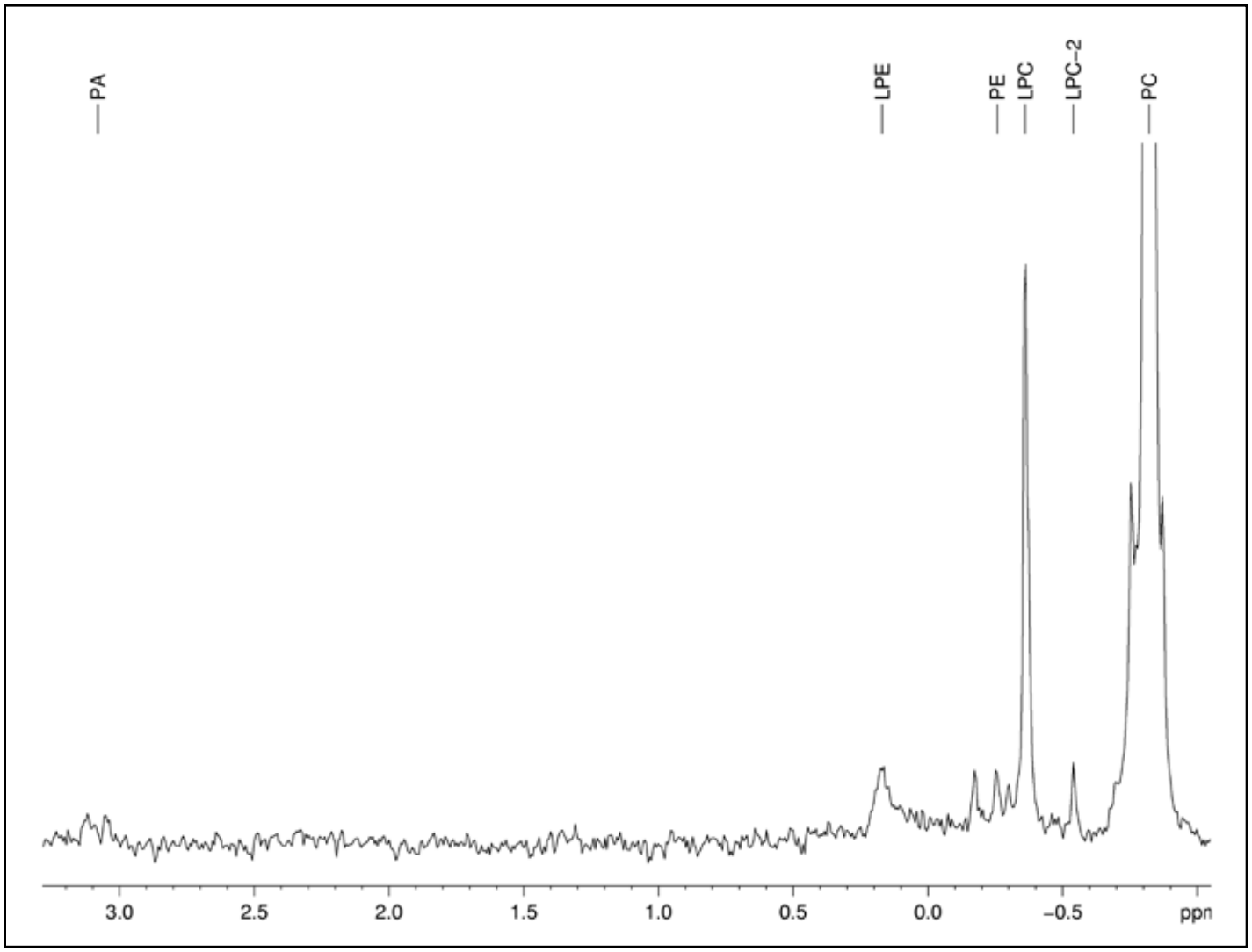

Figure 7. ^{31}P NMR analysis of Krill Lecithin

Average concentration and wt/wt% of PL's found in Krill Lecithin . The MW used in calculations is also provided. Samples prepared at ~10mg/mL total weight in triplicate.			
PL	[mM]	MW	Average wt/wt%
PA	0.19	746.0	1.28 (+/-0.20)
LPE	0.45	492.5	1.97 (+/-0.21)
PE	0.18	770.0	1.24 (+/-0.05)
LPC	1.36	534.1	6.49 (+/-0.14)
PC	9.05	812.0	65.76 (+/-0.16)

Table 8

For accuracy testing, samples of Krill Lecithin were spiked with 1.23mM 18:1 PG, PE, PA, 1.15mM PS, and 0.618mM 18:1 PC. An upfield shift in the polyunsaturated PC was observed preventing direct analysis of Soy PI. PI was therefore excluded from the spike recovery experiment. These samples were then compared to non-spiked preparations in terms of percent recovery of the spiked amounts as seen in the table below.

% Recovery results for Krill Lecithin spiked sample analysis.			
PL	Theoretical Spiked [mM]	Measured Spiked [mM]	% Recovery
PA	1.42	1.29 (+/-0.04)	90.9
PG	1.23	1.22 (+/-0.04)	99.2
PE	1.41	1.43 (+/-0.02)	101.4
PS	1.15	1.19 (+/-0.02)	103.5
PC	10.39	10.44 (+/-0.07)	100.5

Table 9

Sample precision was performed by preparing 6 separate samples and calculating the standard deviation and %RSD of each PL in the matrix. The table below reports the lower limit of quantitation for Krill Lecithins as 2% wt/wt with the %RSD of precision below a preferable 8%.

Precision analysis results for 6 sample preparations of Krill Lecithin.			
PL	Average wt/wt%	Standard Deviation	%RSD
PA	1.4	0.09	6.48
LPE	1.5	0.21	14.66
PE	1.3	0.21	17.05
LPC	5.8	0.11	1.95
LPC-2	0.7	0.15	22.95
PC	66.15	0.59	1.46

Table 10

In Conclusion:

This study reports the precision and accuracy of the quantitative ^{31}P NMR method developed at Avanti® for PL samples from both natural and synthetic sources. Through the use of a detergent solvent system, controlled at an optimum pH and temperature, we are able to increase resolution as well as solubilize diverse lipid matrices directly. Sample preparation requires no extraction, improving precision and accuracy of the assay. By utilizing a suitable delay time of 3 seconds and an auto sampler, we are able to analyze numerous samples within a 24 hour period, providing faster data production and turn around time.

Works Cited:

Estrada, Rosenda; Stolowich, Neal; and Yappert, M. Celcilia. (2008). Influence of temperature on ^{31}P NMR Chemical shifts of phospholipids and their metabolites in chloroform-methanol-water.
Analytical Biochemistry 380, 41-50.

London, Erwin; and Feigenson, Gerald W. (1979). Phosphorous NMR analysis of phospholipids in detergents.
Journal of Lipid Research, 20, 408-412

Meneses, Patricio; and Glonek, Thomas. (1988). High resolution ^{31}P NMR of extracted phospholipids.
Journal of Lipid Research 29, 679-689.

Pearce, John M.; and Komoroski, Richard A. (2000). Analysis of Phospholipid Molecular Species in Brain by ^{31}P NMR Spectroscopy.
Magnetic Resonance in Medicine 44,215-223.

Puppato, Andres; DuPre, Donald B.; Stolowich, Neal; and Yappert, M. Cecilia. (2007). Effect of temperature and pH on ^{31}P nuclear magnetic resonances of phospholipids in cholate micelles.
Chemistry and Physics of Lipids 150, 179-185.

Sampalis, Fotini; Bunea, Roxandra; Pelland, Marie France; Kowalski, Odile; Duguet, Natalie; and Dupuis, Sebastien. (2003). Evaluation of the effects of Neptune Krill Oil™ on the management of premenstrual syndrome and dysmenorrheal.
Alternative Medicine Review, 8 (2), 171-179.

Schiller, Jurgen; and Arnold, Klaus. (2002). Applications of high resolution ^{31}P NMR spectroscopy to the characterization of the phospholipid composition of tissues and body fluids- a methodological review.
Med Sci Monit, 8 (11), 205-222.

Skoog, Douglas A.; Holler, F. James; and Nieman, Timothy A. (1998). Principles of Instrumental Analysis. Fifth Edition. USA: Brooks/Cole Thomson Learning.

Avanti® Polar Lipids, Inc.