

## CHAPTER VI

### METHOD DEVELOPMENT FOR THE SEPARATION OF PHOSPHOLIPIDS BY SUBCRITICAL FLUID CHROMATOGRAPHY

#### 6.1 INTRODUCTION

Super/subcritical Fluid Chromatography (SFC) has been used successfully for the analysis of a variety of compounds including foods, polymers, and pharmaceuticals.<sup>1-7</sup> Supercritical carbon dioxide exhibits solvating power comparable to hexane and has been primarily utilized as a fluid for the separation of relatively non-polar compounds.<sup>8</sup> For this reason, many SFC methods have been developed as substitutes for normal-phase HPLC methods. Commonly used normal-phase HPLC mobile phases consist generally of chlorinated solvents such as chloroform and methylene chloride. Traces of water in these mobile phases cause retention times to fluctuate in the normal-phase mode. These solvents also present an environmental hazard, which can be eliminated in part by using a mobile phase of carbon dioxide which may or may not have been modified with a small percentage of organic solvent. Additionally, the quantity of solvent required and the high disposal costs commonly encountered with many normal-phase HPLC solvents may be dramatically reduced by employing carbon dioxide based mobile phases.

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<sup>1</sup> *J.W. King, J. Chromatogr. Sci.*, **28** (1990) 9.

<sup>2</sup> *K.S. Nam, J.W. King, J. High Resolut. Chromatogr.*, **17** (1994) 577.

<sup>3</sup> *K. Fudor-Csorba, J. Chromatogr.*, **624** (1992) 353.

<sup>4</sup> *F.P. Schmitz, E. Klesper, J. Supercrit. Fluid*, **3** (1990) 29.

<sup>5</sup> *M.W. Raynor, K.D. Bartle, K. Davies, A. Williams, A.A. Clifford, J.M. Chalmers, B.W. Cook, Anal. Chem.*, **60** (1988) 427.

<sup>6</sup> *P. Macaudiere, M. Claude, R. Rosset, A. Tambute, J. Chromatogr. Sci.*, **27** (1989) 383.

<sup>7</sup> *J.T.B. Strode III, L.T. Taylor, A.L. Howard, M.A. Brooks, D. Ip, J. Pharm. Biomed. Anal.*, **12** (1994) 1003.

<sup>8</sup> *C.R. Yonker, R.D. Smith, "Supercritical Fluid Extraction and Chromatography", B.A. Charpentier, M.R. Sevenants (Ed.), ACS Symposium Series, Vol. 406, American Chemical Society, Washington, DC (1989) 52.*

More polar analyte separations can be achieved by adding polar solvents such as methanol directly to the CO<sub>2</sub>.<sup>9</sup> In many cases, high modifier percentages are needed to elute highly polar and/or high molecular weight compounds from a packed column in a timely manner. When using modified fluids, the critical pressure (P<sub>c</sub>) and temperature (T<sub>c</sub>) of the mixture lies between the P<sub>c</sub> and the T<sub>c</sub> of the modifier and CO<sub>2</sub>.<sup>10</sup> Consequently, the combined mobile phase mixture may no longer be supercritical at a stated condition after incorporation of the modifier. Supercritical and subcritical fluids as well as enhanced-fluidity liquid mixtures (i.e. common HPLC eluents that have been pressurized with high pressures of CO<sub>2</sub>) exhibit several advantageous properties compared with normal liquids including higher diffusivities and lower viscosities. The lowered viscosity, as compared to HPLC mobile phases, allows the use of higher flow rates, due to a decreased pressure drop along the length of the column. Numerous reports have illustrated that carbon dioxide based mobile phase separations can be achieved in significantly less time without a subsequent compromise in efficiency than via HPLC.<sup>11-17</sup>

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<sup>9</sup> M.L. Lee, K.E. Markides (Ed.), "Analytical Supercritical Fluid Chromatography and Extraction", Chromatography Conferences, Inc., Provo, UT (1990) 100.

<sup>10</sup> R.C. Reed, T.K. Sherwood, Properties of Gases and Liquids, 2nd ed., McGraw-Hill, NY (1966).

<sup>11</sup> E. Stahl, K.W. Quirin, D. Gerard, "Dense Gases for Extraction and Refining", translated by M.R.F. Ashworth, Springer-Verlag, New York (1988) 176.

<sup>12</sup> L.G. Randall (Ed.), "Ultrahigh Resolution Chromatography", ACS Symp. Ser. 250, American Chemical Society, Washington, DC (1984) 135.

<sup>13</sup> D.R. Gere, R. Board, D. McManigill, Anal. Chem., **54** (1985) 736.

<sup>14</sup> Y. Cui, S.V. Olesik, Anal. Chem., **63** (1991) 1812.

<sup>15</sup> S.T. Lee, S.V. Olesik, Anal. Chem., **66** (1994) 4498.

<sup>16</sup> S.T. Lee, S.V. Olesik, J. Chromatogr. A, **707** (1995) 217.

<sup>17</sup> Y. Cui, S.V. Olesik, J. Chromatogr. A, **691** (1995) 151.

Phospholipids are commonly found in plant and animal tissue and serve as structural components in membranes in addition to playing a role in enzyme activation.<sup>18</sup> For this reason, both their biochemical and functional activities as related to their molecular structure are explored widely. Most commonly, phospholipid mixtures are used as emulsifying additives, thus, they have found many uses in the foods, cosmetics, and pharmaceutical industries.<sup>19</sup>

Phospholipid separations are most commonly performed by thin-layer chromatography (TLC) and normal-phase high-performance liquid chromatography (HPLC). TLC is traditionally used to qualitatively separate classes of phospholipids, but the quantitative separation of individual species is a common problem.<sup>20</sup> Recently, however, several reports have been published which describe more successful separations of individual phospholipid species by HPLC.<sup>21-24</sup> Most separations although are limited by the mode of detection. For example, when UV is utilized, low wavelengths (~200 nm) must be chosen or analyte derivatization is necessary. In a few instances, evaporative light scattering detection (ELSD) has been utilized. Specifically, Olsson et al. separated via HPLC phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, and phosphatidylcholine from extracted brain tissues on a Nucleosil Diol packed column with a linear mobile phase gradient consisting of a mixture of hexane:2-propanol:*n*-butanol:tetrahydrofuran:isooctane:water and a second mixture of 2-propanol:*n*-butanol:tetrahydrofuran:isooctane:water.<sup>21</sup>

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<sup>18</sup> *T.M. Devin*, "Textbook of Biochemistry With Clinical Correlations", 3rd ed., Wiley-Liss, New York (1992) 427.

<sup>19</sup> *B.F. Szuhaj (Ed.)*, "Lecithins: Sources of Manufacture and Uses", Am. Oil Chem. Soc., Champaign, 1989.

<sup>20</sup> *W.W. Christie*, "Lipid Analysis", 2nd ed., Pergamon Press, New York (1992) 107.

<sup>21</sup> *N.U. Olsson, A.J. Harding, C. Harper, N. Salem Jr.*, *J. Chromatogr. B*, **681** (1996) 213.

<sup>22</sup> *A. Sakamoto, M. Novotny*, *J. Microcol. Sep.*, **8** (1996) 397.

<sup>23</sup> *R. Szücs, K. Verleysen, G. Duchateau, P. Sandra, B. Vandeginste*, *J. Chromatogr. A*, **738** (1996) 25.

<sup>24</sup> *J. Becart, C. Chevalier, J.P. Biesse*, *J. High Resolut. Chromatogr.*, **13** (1990) 126.

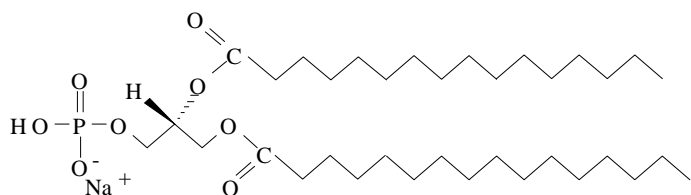
In 1992, Lafosse et al. reported on the use of the evaporative light scattering detection for the analysis of pharmaceuticals by both liquid and supercritical fluid chromatography.<sup>25</sup> In particular, the first analysis of phospholipids by SFC with ELSD was described briefly. The separation of phosphatidylcholine, phosphatidic acid, phosphatidylinositol, and phosphatidylethanolamine from soya lecithin was isocratically achieved in 22 minutes on a Zorbax Silica column (4.6 mm x 25 cm,  $d_p = 5 \mu\text{m}$ ) with a mobile phase consisting of carbon dioxide modified with a mixture of methanol:water:triethylamine (95:4.95:0.05) in a 78.4:21.6 (w/w) ratio at a column outlet pressure of 278 bar, 45 °C, and a mobile phase flow rate of 4.3 mL/min. The only SFC parameter specifically discussed was column temperature. Increased detector sensitivity and reduced analysis time were achieved by working at a low temperature, 30 °C. No SFC vs HPLC comparisons were made.

The main goal of this Chapter was to demonstrate further the applicability of SFC to phospholipid analysis. Specifically, the objective was to develop a qualitative analytical SFC method for the separation of five phospholipids varying in polarity and ionic nature. Structures for these compounds can be found in **Figure 6.1**. This work differs from the work described by Lafosse in that a systematic method development approach was undertaken to examine the effect of several SFC parameters on peak resolution and peak shape of five phospholipids. The parameters investigated were: stationary phase composition, acidic modifier additive concentration, modifier ramp rate, and column outlet pressure. In contrast to the Lafosse work, the fatty acid substituents of each phospholipid were well characterized in this study. For example, phosphatidyl choline, as assayed by Lafosse, contains mostly palmitic acid (16:0) or stearic acid (18:0) in the *sn*-1 position, and unsaturated C<sub>18</sub> fatty acids such as oleic, linoleic, or linolenic

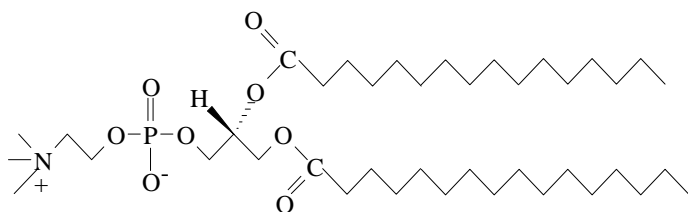
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<sup>25</sup> M. Lafosse, C. Elfakir, L. Morin-Allory, M. Dreux, J. High Resolut. Chromatogr. **15** (1992) 312.

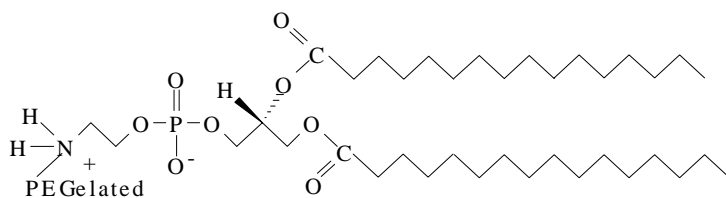
1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate (DPPA)



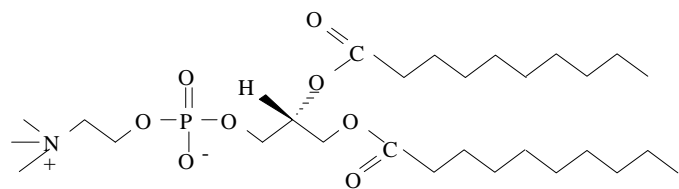
1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine (DPPC)



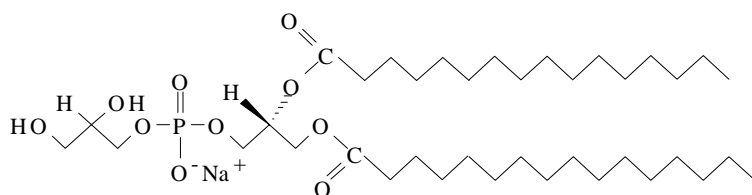
1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)] (DPPE-PEG)



1,2-Dicaproyl-*sn*-Glycero-3-Phosphocholine (DCPC)



1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (DPPG)



**Figure 6.1.** Chemical Structures of Phospholipids Investigated

acid in the *sn*-2 position.<sup>18</sup> In our separation, the fatty acid substituents of the choline substituted phospholipid were known to be palmitic acid in both the *sn*-1 and *sn*-2 positions.

## 6.2 EXPERIMENTAL

A Gilson SFC system (Middleton, WI) consisting of a model 308 liquid CO<sub>2</sub> pump, a 306 modifier pump, a 233 XL injector, a 811 C dynamic mixer, a 831 column oven, and a 821 pressure regulator were used for all separations. An Alltech Varex Mark II ELSD (Deerfield, IL) modified earlier for SFC was used as the detector unless stated otherwise.<sup>26</sup> All data were collected and analyzed using Gilson Unipoint software. All phospholipid solutions were prepared at 1.0 mg/mL in methanol. The injection volume in all cases was 25 µL. The column effluent was split between a variable pressure controlling restrictor and the ELSD using a three-way union. The effluent was introduced to the ELSD using a silica capillary (2-3 m, 50 µm i.d.). The ELSD conditions were as follows: CO<sub>2</sub> gas flow rate entering detector = 0.3 L/min., nitrogen flow rate = 0.7 mL/min., and drift tube temperature = 70 °C. The split ratio (ELSD:Variable restrictor) was 1:3. The columns investigated in this study were: a) Valuepak Amino, 4.6 mm X 15 cm, 5 µm (Keystone Scientific, Bellefonte, PA), b) Deltabond Cyano, 4.6 mm X 25 cm, 5 µm (Keystone Scientific), c) Hypersil Silica, 4.6 mm X 25 cm, 5 µm (Keystone Scientific), and d) Luna Octyl, 4.6 mm X 25 cm, 5 µm (Phenomenex, Torrance, CA). All chromatographic conditions can be found in the Figure captions.

SFE/SFC grade carbon dioxide without helium headspace was obtained from Air Products and Chemicals Inc. (Allentown, PA). HPLC grade methanol, ethanol, and trifluoroacetic acid were purchased from EM Science (Gibbstown, NJ), Aaper Alcohol (Shelbyville, KY), and Sigma Aldrich (St. Louis, MO) respectively. All phospholipids

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<sup>26</sup> *J.T.B Strode, L.T. Taylor, J. Chromatogr. Sci.* **34** (1996) 261.

were donated by the DuPont Merck Pharmaceutical Company but they were originally obtained from Avanti Polar Lipids (Alabaster, AL). No further purification was performed.

## **6.3 RESULTS AND DISCUSSION**

The objective of this study was to develop a separation of five phospholipids that vary in polarity and ionic nature. Several chromatographic parameters including stationary phase composition, modifier additive, modifier additive concentration, modifier gradients, and column outlet pressure were investigated in an attempt to produce a baseline resolved separation in under 20 minutes. Due to the poorly UV absorbing nature of these analytes, Evaporative Light Scattering Detection (ELSD) was employed.

### **Effect of Stationary Phase**

Since SFC best emulates normal-phase chromatography, several normal-phase packed columns were investigated. They were: a) Valuepak Amino, b) Deltabond Cyano, and c) Hypersil Silica. The SFC conditions were: 230 bar CO<sub>2</sub>, 40 °C, 2.0 mL/min. liquid CO<sub>2</sub>, 40% (v/v) methanol-modified CO<sub>2</sub>. No peaks were observed for each singly injected phospholipid after a run time of 60 min., thus indicating that 40% methanol-modified CO<sub>2</sub> did not have sufficient solvent strength to solubilize the phospholipids or to break the hydrogen bonding interactions that may exist between the hydrophilic portions of the phospholipids and the highly active stationary phases. To remove the possibility that light scattering detection may have been inadequate, the column was removed, and each phospholipid was injected separately under the same conditions. A peak was observed for each of the phospholipids by ELSD, therefore, the detector was assumed to be operating properly concluding that each phospholipid did have sufficient solubility in 40% methanol-modified CO<sub>2</sub>.

Due to the lipophilicity of the phospholipids, the separation was next attempted on a reversed-phase column (5  $\mu\text{m}$  Luna Octyl). A modifier gradient was utilized due to the varying chemical characteristics of the phospholipids investigated as well as to ensure elution of all 5 analytes. The mobile phase consisted of a mixture of  $\text{CO}_2$  and 50/50 (v/v) (0.01% trifluoroacetic acid (TFA)) ethanol/methanol. Use of these conditions resulted in a fairly successful separation wherein 4 chromatographic peaks were observed (**Figure 6.2**). Sufficient resolution was observed between DCPC and DPPA [ $R_s = 1.4$ ], however, DCPC tailed greatly. Under these conditions, DPPC and DPPG were unresolvable. A sharp peak for DPPE-PEG was observed, however, an unidentified impurity peak eluted just prior to DPPE-PEG. Although a totally successful separation was not achieved at this point, we were satisfied that all five phospholipids were eluted from the column.

### Effect of Modifier Additive

Secondary interactions between basic and acidic compounds with acidic silanols on the stationary phase surface may result in strong analyte retention and peak tailing in HPLC.<sup>27</sup> The addition of acidic and basic additives directly to the  $\text{CO}_2$  modifier has been investigated in SFC in order to increase apparent solvent strength as well as to reduce unfavorable secondary interactions.<sup>28-32</sup> For example, Berger et al. reported on the separation of benzene polycarboxylic acids by packed column SFC using methanol-modified  $\text{CO}_2$  mixtures that contained a small amount of a very polar additive. Using 5.7% (v/v) (0.5% trifluoroacetic acid) methanol-modified  $\text{CO}_2$ , 1,2-, 1-3-, and 1,4-

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<sup>27</sup> L.R. Snyder, J.L. Glajch, J.J. Kirkland, Practical HPLC Method Development, John Wiley and Sons, New York (1988).

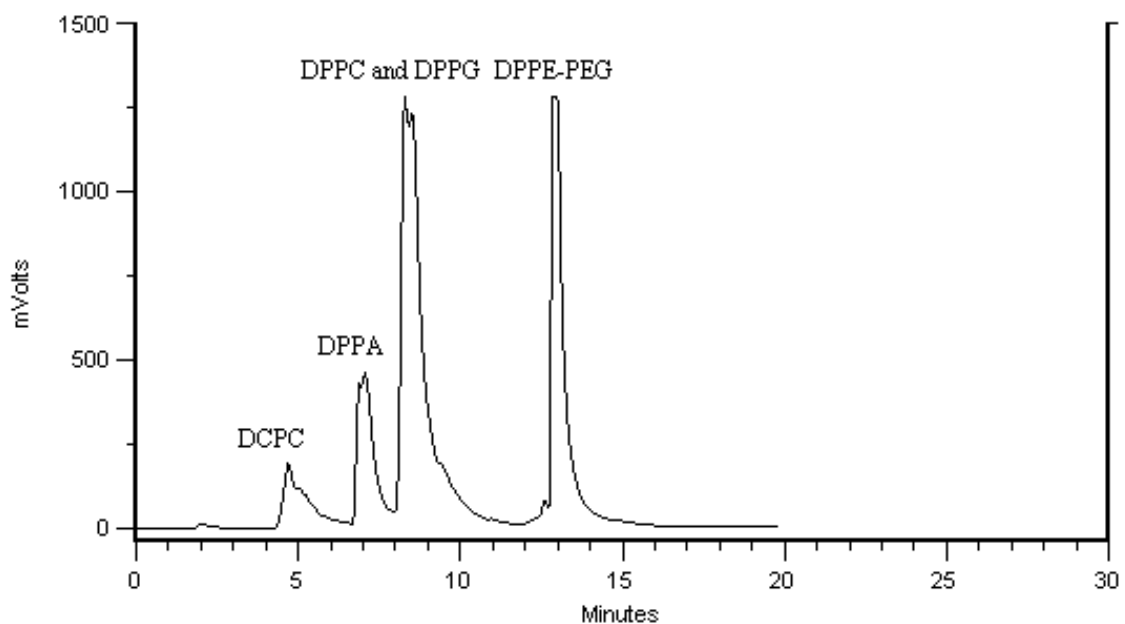
<sup>28</sup> T. Berger, J. Deye, J. Chromatogr. Sci., **29** (1991) 26

<sup>29</sup> M. Ashraf-Khorassani, M.G. Fessahaie, L.T. Taylor, T.A. Berger, J.F. Deye, J. High Resolut. Chromatogr., **11** (1988) 352.

<sup>30</sup> L.J. Mulcahey, L.T. Taylor, J. High Resolut. Chromatogr., **13** (1990) 393.

<sup>31</sup> T.A. Berger, W.H. Wilson, J. Chromatogr. Sci., **31** (1993) 127.

<sup>32</sup> T.A. Berger, J.F. Deye, J. Chromatogr. Sci., **29** (1991) 141.



**Figure 6.2.** Chromatogram A of 5 phospholipids. Order of elution: DCPC, DPPA, DPPC, DPPG, DPPE-PEG. Mobile Phase, 9% (50/50 (v/v) ethanol/methanol (0.01% (v/v) trifluoroacetic acid)) for 2 min., ramp to 25% in 10 min. (2.0%/min.), 40% at 10.1 min.; oven temperature, 70 °C; outlet pressure, 125 bar, flow; 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 µm Luna Octyl. 25 µg of each phospholipid in methanol injected.

DCPC - 1,2-Dicaproyl-*sn*-Glycero-3-Phosphocholine

DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate

DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine

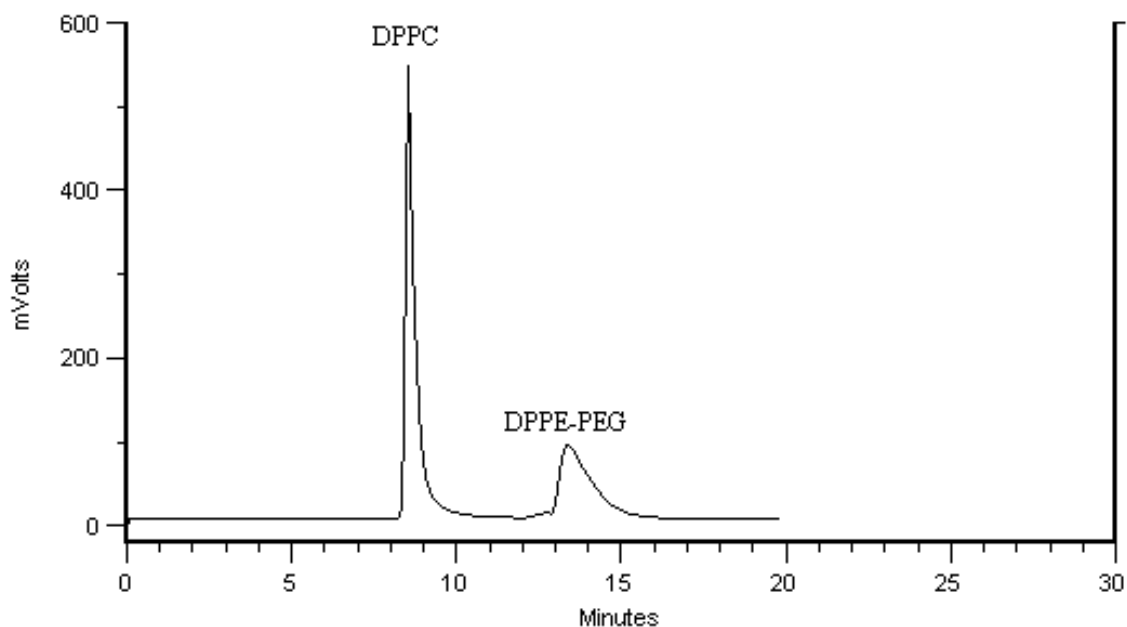
DPPG - 1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

benzenedicarboxylic acids and 2-, 3-, 4-hydroxybenzoic acids were eluted from a Diol packed column. It was reported that without the use of the very polar additive, elution of all analytes was not achievable even at higher methanol modifier concentrations (9 and 11%).

Trifluoroacetic acid (TFA) was, therefore, added to the ethanol/methanol modifier (0.01% (v/v)) in hopes of neutralizing the charged phosphate group on DPPA as well as to reduce secondary interactions between the polar functionality on the phospholipid and any acidic silanols on the stationary phase. Only a solution of DPPA, DPPC, and DPPE-PEG was injected since DCPC and DPPA at this point coeluted with DPPA and DPPC respectively. Three visual observations can be made. First, only DPPC and DPPE-PEG were initially thought to be observed (**Figure 6.3**). Second, the peak shape of DPPC appeared to be the same with and without the modifier additive. Third, in absence of the additive, detrimental effects on the peak shape of DPPE-PEG (i.e. decreased peak height, broadening) were observed. Clearly, when the acidic additive was present, the secondary interactions were lessened thereby resulting in a favorable peak shape for DPPE-PEG. A mechanism that may be used to explain the additive's advantageous effect on the peak shape of DPPE-PEG is stationary phase modification. It is well known that complete coverage of the silanol sites with the octyl groups is impossible. For instance, the polar ethanolamine functionality on the phospholipid may hydrogen bond to the residual silanol groups on the stationary phase surface. When introduced, the additive may preferentially absorb to these exposed silanol groups thus reducing any interactions with the phospholipids.<sup>28</sup>

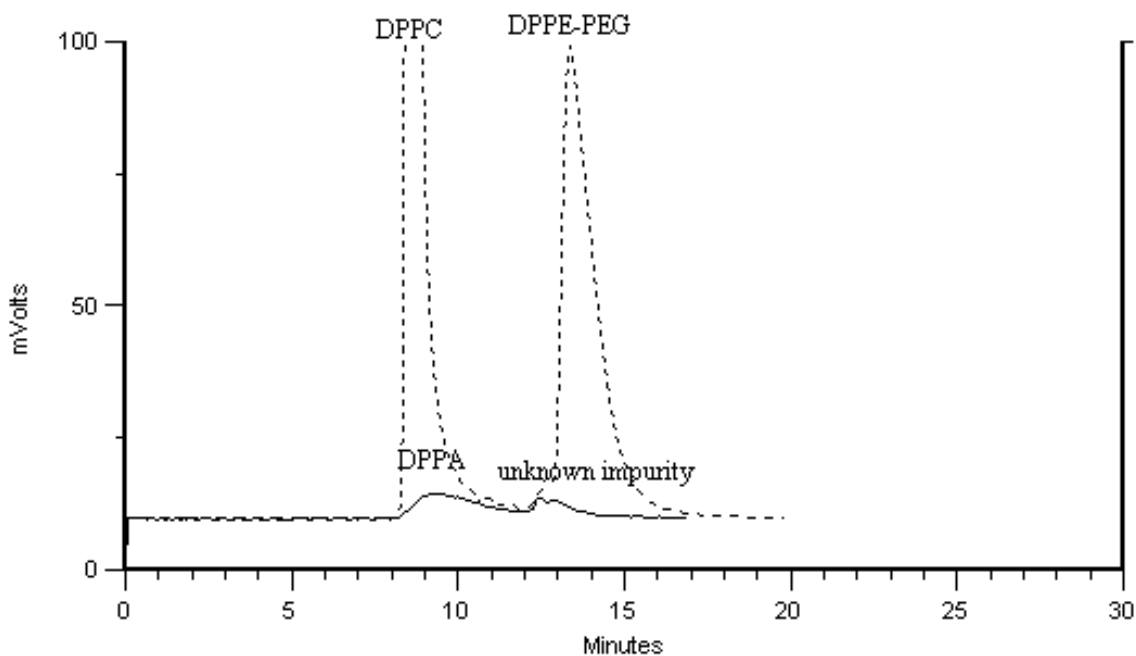
In order to decipher whether coelution of DPPA and DPPC was occurring or whether DPPA was not entirely eluting from the column, DPPA was singly injected. By overlaying the chromatograms of the phospholipid mixture and DPPA alone (**Figure 6.4**) without the additive, the coelution of DPPA and DPPC was obvious. The peak shape of DPPA in the absence of TFA was also not desirable due to its excessive peak width. The addition of trifluoroacetic acid to the modifier allowed the efficient elution of DPPA from



**Figure 6.3.** Chromatogram B of 3 phospholipids. Order of elution: DPPC, DPPE-PEG. Mobile Phase, 9% (50/50 (v/v) ethanol/methanol for 2 min., ramp to 25% in 10 min. (2.0%/min.), 40% at 10.1 min.; oven temperature, 70 °C; outlet pressure, 125 bar, flow, 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5  $\mu$ m Luna Octyl. 25  $\mu$ g of each phospholipid in methanol injected.

DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine

DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]



**Figure 6.4.** Chromatogram C of 3 phospholipids. Order of elution: DPPA = DPPC, DPPE-PEG.

— DPPA alone      ..... Phospholipid mixture

Mobile Phase, 9% (50/50 (v/v) ethanol/methanol for 2 min., ramp to 25% in 10 min. (2.0%/min.); 40% at 10.1 min.; oven temperature, 70 °C; outlet pressure, 125 bar; flow, 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 µm Luna Octyl. 25 µg of each phospholipid in methanol injected.

DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate

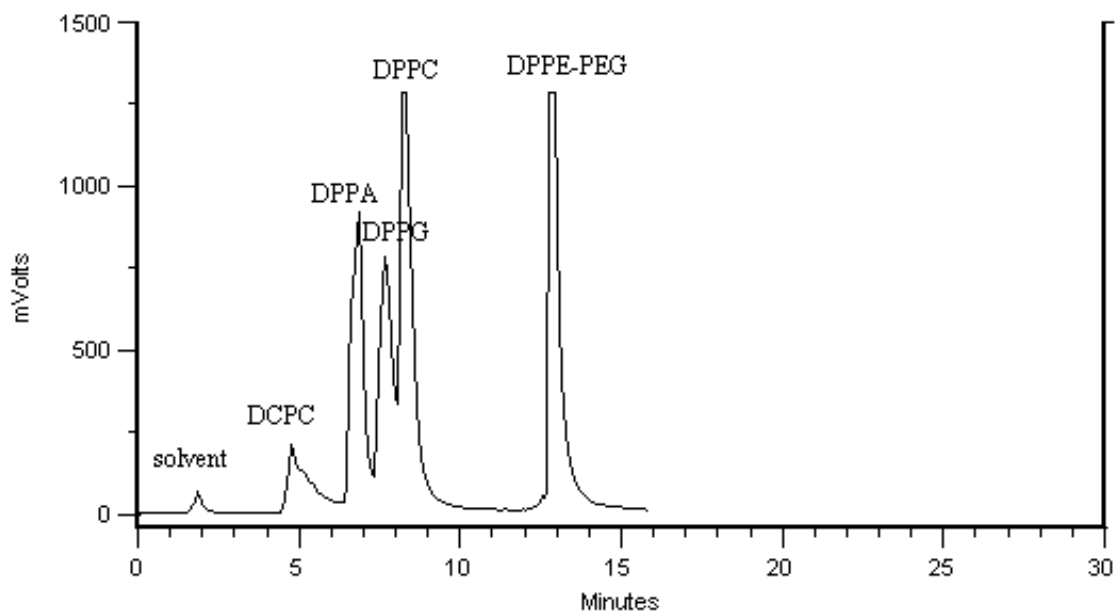
DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine

DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

the column with improved peak shape and its complete separation from DPPC. The additive's usefulness can be attributed to neutralization of the DPPA as well as to stationary phase modification. Since charged species are believed to not exhibit significant solubility in pure CO<sub>2</sub>, some of the charged DPPA may have precipitated out on the column; therefore, giving rise to a low detector response. Alternatively, it is possible that a mixture of both charged and neutral DPPA existed in solution, thereby giving rise to a large peak width and subsequent tailing.

### **Effect of Modifier Additive Concentration**

Previously, the addition of trifluoroacetic acid to the modifier was shown to significantly improve the peak shape of DPPA and DPPE-PEG and to alter the selectivity of DPPA and DPPC. The modifier additive concentration was increased from 0.01% (v/v) to 0.1% (v/v) with a modifier ramp rate of 2.0%/min (same conditions as in **Figure 6.2**). Upon comparing the chromatograms with 0.01% (**Figure 6.2**) and 0.1% (v/v) TFA (**Figure 6.5**), the higher additive concentration was a definite improvement. Six peaks were observed in this chromatogram including the solvent peak, DCPC, DPPA, DPPG, DPPC, and DPPE-PEG (**Figure 6.5**). Additionally, the peak shape/height of DPPA was further improved in the presence of a higher concentration of TFA. The selectivity of the separation was also altered where the elution order of DPPC and DPPG reversed. To explain these phenomena, the structures of DPPC and DPPG (**Figures 6.1**) were compared in terms of polarity. DPPG is the more polar of the two due to its glycerol-functionality versus the choline-functionality. In a reversed-phase process, the more polar compounds are expected to elute from the non-polar stationary phase first which indeed was observed in the separation with the higher additive concentration. The reversal in the elution order between DPPC and DPPG can be explained by the following reasoning. By increasing the additive concentration from 0.01% (v/v) to 0.1% (v/v), the stationary phase surface became less active. Consequently, partitioning of the nonpolar



**Figure 6.5.** Chromatogram D of 5 phospholipids. Order of elution: DCPC, DPPA, DPPG, DPPC, DPPE-PEG. Mobile Phase, 9% (50/50 (v/v) ethanol/methanol (0.10% (v/v) trifluoroacetic acid)) for 2 min., ramp to 25% in 10 min. (2.0%/min.), 40% at 10.1 min.; oven temperature, 70 °C; outlet pressure, 125 bar, flow; 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5  $\mu$ m Luna Octyl. 25  $\mu$ g of each phospholipid in methanol injected.

DCPC - 1,2-Dicaproyl-*sn*-Glycero-3-Phosphocholine

DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate

DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine

DPPG - 1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

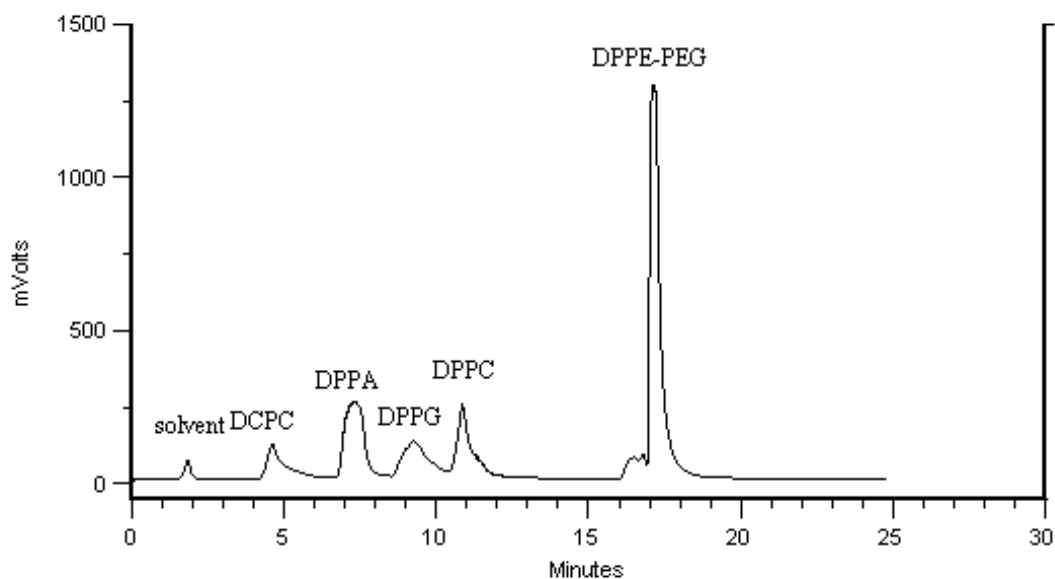
functionality of the DPPG with the octyl stationary phase was believed to have increased at the higher additive concentration due to less interaction of analyte with the silanol groups. For these reasons, reversal in the elution order and better peak shape as well as improved separation of DPPG and DPPC was observed.

### **Effect of Modifier Ramp Rate**

Similar to reversed-phase HPLC, mobile phase gradients are commonly employed in packed-column super/subcritical fluid chromatography. During the gradient, the stronger solvent, methanol, is increased in concentration at a certain rate. Initially, the separation of DPPC and DPPG was not achieved. Although an improved separation was observed at the higher modifier additive concentration, the resolution between DPPG and DPPC remained unsatisfactory. In hopes of improving the separation, the modifier ramp rate was reduced from 2.0%/min. to 0.5%/min. (**Figure 6.6**). Simply by lowering the modifier ramp rate, or allowing more time for the analytes to partition with the lipophilic stationary phase, baseline resolution between DPPG and DPPC became apparent. However, the peak shape of DPPA and DPPC became worse.

### **Effect of Pressure**

At this point while the resolution between all of five phospholipids was satisfactory, the peak shapes were less than desirable. The pressure was increased from 125 bar to 135 bar, while the modifier ramp rate was held at 0.5%/min. (**Figure 6.7**). By simply increasing the pressure by 10 bar, the peak shape and response of all analytes was improved without a significant loss in resolution, thereby resulting in the optimized separation of all five phospholipids. The resolution values ( $R_s$ ) between the following adjacent peaks were: 1.6 (DCPC and DPPA); 1.6 (DPPA and DPPG); 1.1 (DPPG and DPPC); and 10.1 (DPPC and DPPE-PEG). Other peak parameters including peak width



**Figure 6.6.** Chromatogram E of 5 phospholipids. Order of elution: DCPC, DPPA, DPPG, DPPC, DPPE-PEG. Mobile Phase, 9% (50/50 (v/v) ethanol/methanol (0.10% (v/v) trifluoroacetic acid)) for 2 min., ramp to 15% in 14 min. (0.5%/min.), 40% at 14.1 min.; oven temperature, 70 °C; outlet pressure, 125 bar, flow; 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 µm Luna Octyl. 25 µg of each phospholipid in methanol injected.

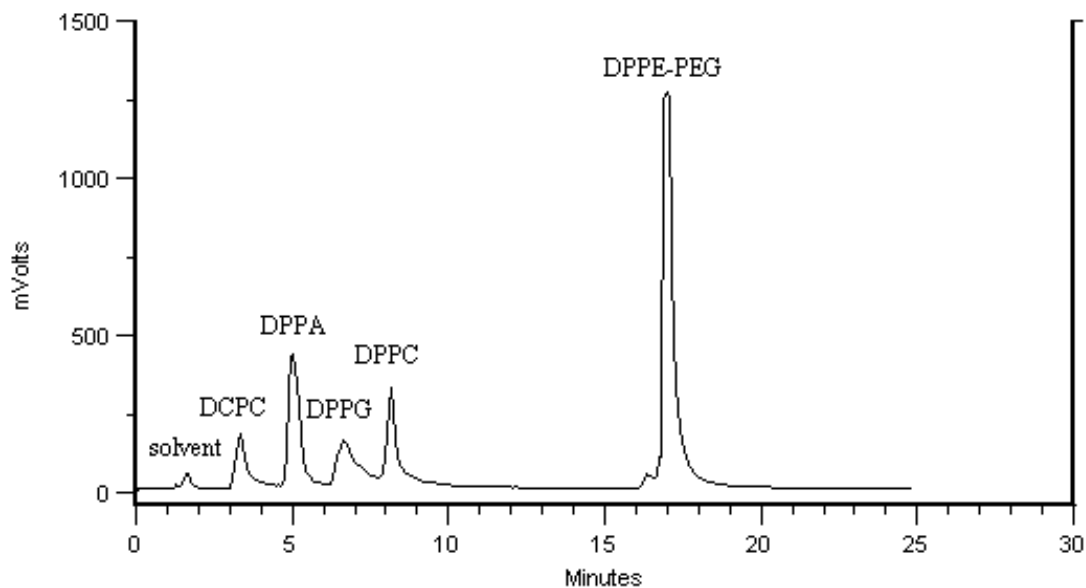
DCPC - 1,2-Dicaproyl-*sn*-Glycero-3-Phosphocholine

DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate

DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine

DPPG - 1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]



**Figure 6.7.** Chromatogram F of 5 phospholipids. Order of elution: DCPC, DPPA, DPPG, DPPC, DPPE-PEG. Mobile Phase, 9% (50/50 (v/v) ethanol/methanol (0.10% (v/v) trifluoroacetic acid)) for 2 min., ramp to 15% in 14 min. (0.5%/min.), 40% at 14.1 min.; oven temperature, 70 °C; outlet pressure, 135 bar, flow; 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 µm Luna Octyl. 25 µg of each phospholipid in methanol injected.

DCPC - 1,2-Dicaproyl-*sn*-Glycero-3-Phosphocholine

DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate

DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine

DPPG - 1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

at half height ( $w_{1/2}$ ), capacity factor ( $k'$ ), and selectivity factor ( $\alpha$ ), for the optimized separation can be found in **Table 6.1**. Specifically, the capacity factor ( $k'$ ) is a measurement that describes how long the component is retained on the stationary phase versus how long it resides in the mobile phase. Generally,  $k'$  values should be greater than 1 so that there is sufficient separation from the injection solvent and no greater than 10 where the analysis time would be too great.<sup>33</sup> As can be seen from the calculated capacity factors, all  $k'$  values for the phospholipid peaks ranged between 1-10. Another value that is used to evaluate the chromatographic separation is the selectivity factor ( $\alpha$ ). This value can be used to compare how close two adjacent peaks are to one another. Obviously, an  $\alpha$  of greater than 1 must be achieved to ensure separation. Once again, all  $\alpha$  values were at least 1.3. The third parameter is peak resolution ( $R_s$ ). Resolution expresses how well two adjacent peaks are separated from one another. Adequate peak separation must be ensured for quantitative purposes. Baseline separation is represented by a  $R_s$  value of 1.5 while a  $R_s$  of 1.0 represents that the separation between the two peaks is 90% complete.<sup>33</sup> The separation of 4 of the 5 phospholipids resulted in baseline resolution ( $R_s > 1.5$ ), while the resolution between DPPG and DPPC was 1.1. Although this method was developed to serve qualitative purposes, it may prove to be a successful quantitative method due to good retention and separation in a timely manner.

### **Comparison of the Reproducibility of Two ELSDs**

It was of great interest to examine whether this method could be transferred to another ELSD detector. Three phospholipids, DPPA, DPPC, and DPPE-PEG, each exhibiting a low, medium, and high ELSD response, were chosen for this evaluation. For this study, two ELSD detectors were compared in terms of area count reproducibility for

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<sup>33</sup> *R.W. Yost, L.S. Ettre, R.D. Conlon, Practical Liquid Chromatography, An Introduction, Perkin-Elmer, Norwalk, CT (1980).*

**Table 6.1.** Various Chromatographic Peak Parameters

Mobile Phase, 9% (50/50 (v/v) ethanol/methanol (0.1% (v/v) TFA)) for 2 min., ramp to 15% in 14 min. (0.5%/min.), 40% at 14.1 min.; oven temperature, 70 °C; outlet pressure, 135 bar, flow, 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 µm Luna Octyl. 25 µg of each phospholipid in methanol injected.

	DCPC	DPPA	DPPG	DPPC	DPPE-PEG
Retention Time (min.)	3.33	5.00	6.67	8.16	16.97
T <sub>r</sub> ' (min.)	1.68	3.35	5.02	6.51	15.32
w <sub>1/2</sub> (min.)	0.33	0.40	0.62	0.29	0.32
k'	1.0	2.0	3.0	3.9	9.3
α		2.0	1.5	1.3	2.4
R <sub>s</sub>		1.6	1.6	1.1	10.1

DCPC - 1,2-Dicaproyl-*sn*-Glycero-3-Phosphocholine

DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate

DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine

DPPG - 1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-rac-(1-glycerol)]

DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

a series of injections. The detectors compared were the Alltech Varex Mark II ELSD (Deerfield, IL) and the Sedere ELSD (Alfortville Cedex, France). ELSD and SFC conditions as well as area count comparisons (RSDs) are found in **Table 6.2**.

Chromatographic comparisons can be visualized in **Figure 6.8**. Area count precisions for both detectors were found to be excellent. Regardless of detector, for two peaks, the RSDs were under 3% while, one peak resulted in a RSD of under 8%. However, when comparing response (areas and peak height) as well as the RSDs, variances were observed amongst the two detectors. First, when comparing the response resulting for DPPA, the Varex detector response was 1.9 times greater than the Sedere detector, however, the precision of the area counts for the successive injections was comparable at approximately 3%. Visually, the peak width and peak shape produced with the two detectors was comparable. Second, the reverse trend was seen for DPPC. Although the peak height was greater with the Varex detector, the peak tailing of DPPC was more severe with the Sedere detector. Consequently, the reproducibility was greater, thus resulting in a 7% RSD vs 2% RSD obtained with the Varex. Third, once again the peak height and response was greater for DPPE-PEG with the Varex detector, however, greater peak tailing was observed. Therefore, the higher RSD of 7.4% resulted versus 1.9% for the Sedere detector. Overall, the reproducibilities were comparable and satisfactory regardless of detector manufacturer.

**Table 6.2.** Reproducibility Comparisons of Two ELSDs

Alltech Varex Mark II ELSD

CO<sub>2</sub> gas flow entering detector: 0.3 L/min.N<sub>2</sub> flow rate = 0.7 mL/min.

Drift tube temperature = 60 °C

Sedere ELSD

N<sub>2</sub> pressure = 0.3 bar

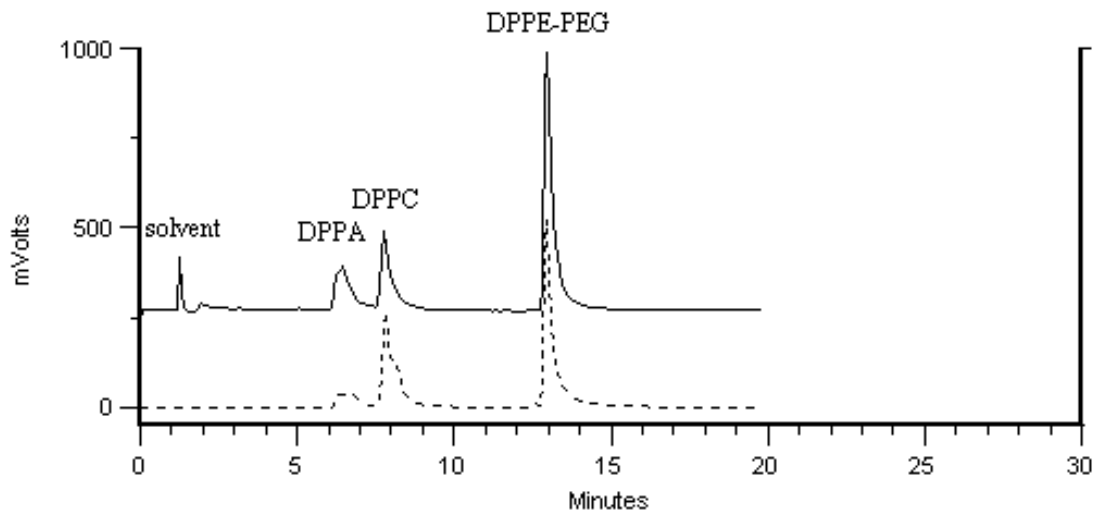
Drift tube temperature = 70 °C

Gain = 7

Mobile Phase, 9% (50/50 (v/v) ethanol/methanol (0.1% (v/v) TFA)) for 2 min., ramp to 15% in 14 min. (0.5%/min.), 40% at 14.1 min.; oven temperature, 70 °C; outlet pressure, 135 bar, flow, 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 µm Luna Octyl. 25 µg of each phospholipid in methanol injected.

Injection #	Varex Area Counts (DPPA)	Sedere Area Counts (DPPA)	Varex Area Counts (DPPC)	Sedere Area Counts (DPPC)	Varex Area Counts (DPPE- PEG)	Sedere Area Counts (DPPE- PEG)
1	5912372		7437424		18527042	
2	5441115		7361325		18606146	
3	5735241	2982946	7432577	10940631	19400954	15549362
4	5659742	3057047	7203082	10921601	21010416	15542923
5	5720216	2836616	7496150	10131608	19438068	14988550
6	5626522	3041959	7206636	10013061	22657764	14952279
7	5811993	2932888	7436769	9111561	20689258	15418418
RSD	2.6	3.0	1.6	7.4	7.4	1.9

DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-PhosphateDPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-PhosphocholineDPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]



**Figure 6.8.** Reproducibility comparison of 2 detectors. Order of elution: DCPC, DPPA, DPPG, DPPC, DPPE-PEG.

— Vorex ELSD    - - - Sedere ELSD

Mobile Phase, 9% (50/50 (v/v) ethanol/methanol (0.10% (v/v) trifluoroacetic acid)) for 2 min., ramp to 15% in 14 min. (0.5%/min.), 40% at 14.1 min.; oven temperature, 70 °C; outlet pressure, 135 bar, flow; 2.0 mL/min.; Column, 4.6 mm x 25 cm, 5 µm Luna Octyl. 25 µg of each phospholipid in methanol injected.

DPPA - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate

DPPC - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine

DPPE-PEG - 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]

## 6.4 SUMMARY

Subcritical fluid chromatography is a viable technique for the separation of phospholipids. A normal stationary phase was not effective due to high adsorption of the polar functionality of the phospholipids onto the polar stationary phase, however, a reversed stationary phase proved useful. Retention was attributed to partitioning of the lipophilic portions of the phospholipids with the non-polar octyl phase. Elution of each analyte and its peak shape was improved by the addition of an acidic modifier additive, trifluoroacetic acid. Secondary interactions between the phospholipids and the exposed silanol sites on the stationary phase were further reduced by an increase in additive concentration thus altering the column selectivity and improving resolution. The separation of all five phospholipids was achieved by optimizing the modifier gradient and pressure. Several chromatographic parameters used to describe the separation were compared, and good retention and resolution was achieved in a timely matter. Finally, two ELSD detectors were compared in terms of peak response (peak height, area) and peak shape. No significant differences were observed regardless of detector manufacturer.