

## CHAPTER I

### Introduction

Lipids play a variety of roles in biological systems. They are molecules that are insoluble in water and soluble in organic solvents. The diversity of lipids can be very high due to (a) the complexity of the hydrophobic acyl chains (i.e. carbon number and degree of unsaturation) and (b) the nature of the hydrophilic polar head group. Thus, over a thousand different lipid molecular species can be theoretically devised which exhibit a wide polarity range. Lipids can be divided into non-polar lipids (e.g. triglycerides and cholesterol) and polar lipids (e.g. phospholipids, glycolipids, and sphingolipids) based on the number of primary products formed after hydrolysis, **Figure 1**. The structure of selected lipids and their primary hydrolysis products are shown in **Figure 2**. In general, non-polar lipids yield no more than two types of primary products after hydrolysis. For example, triglycerides only yield fatty acids and one mole of glycerol when hydrolyzed; while polar lipids could yield three or more types of primary products under the same reaction. For example, phospholipids which are discussed in this thesis yield glycerol, fatty acids, inorganic phosphate, and an organic base. Although the role of lipids in the cellular area is still not fully understood, they are generally believed to be (a) used for energy storage, (b) serve as structural components of cell membranes, and (c) constitute important signaling molecules. Many publications<sup>1-4</sup> have reported that phospholipids are important constituents for the above functions in all living cell membranes due to their amphiphilic structure.

Phospholipids are composed of a glycerol backbone with fatty acids esterified at adjacent carbon positions; while a polar head group is attached to a phosphate ester group at the remaining carbon. The fatty acids can vary in length from 14 to 22 carbons and can have from 0 to 6 double bonds, **Figure 3**.<sup>1</sup> The amphiphilic nature of membrane phospholipids has been well known to form the bi-layer that is necessary for proteins to function and interact. A second function of phospholipids is to serve as the source of arachidonic acid, which is required for the formation of lipid mediators such as leukotrienes and prostaglandins.<sup>2</sup> They are also the precursors for platelet-activating factor and signaling molecules such as inositol triphosphate and diacylglycerol.<sup>3</sup> Moreover, synthetic phospholipids have been used as drug carriers in the pharmaceutical industry.<sup>4</sup>

Phospholipids nomenclature is based on the polar head group. Choline(PC) -, ethanolamine(PE)-, inositol(PI)-, and serine(PS)-glycerophospholipid are the most common phospholipids. Each one has a different biochemical function. PC not only plays a critical role in the manufacture of primary cell membrane components, but it is also essential in the synthesis of acetylcholine.<sup>5</sup> Clinical studies have shown that PC can increase serum acetylcholine levels, which play a crucial role in many brain processes. PE is a key component of membrane bilayers. It has a smaller head group, and it can hydrogen bond through its ionizable amine group.<sup>6</sup> PS has been suggested to be a nutritional supplement for the elderly, as there is evidence from clinical trials that it improves brain function, and in particular cases it reduces the risk or intensity of cognitive dysfunction.<sup>7</sup> PI is essential for the regulation of proteins at the cell interface. It is the main source of diacylglycerols that serve as signaling molecules in animal and

plant cells.<sup>8</sup> Since each phospholipid has a different function, analysis and purification of individual components seems to be important.

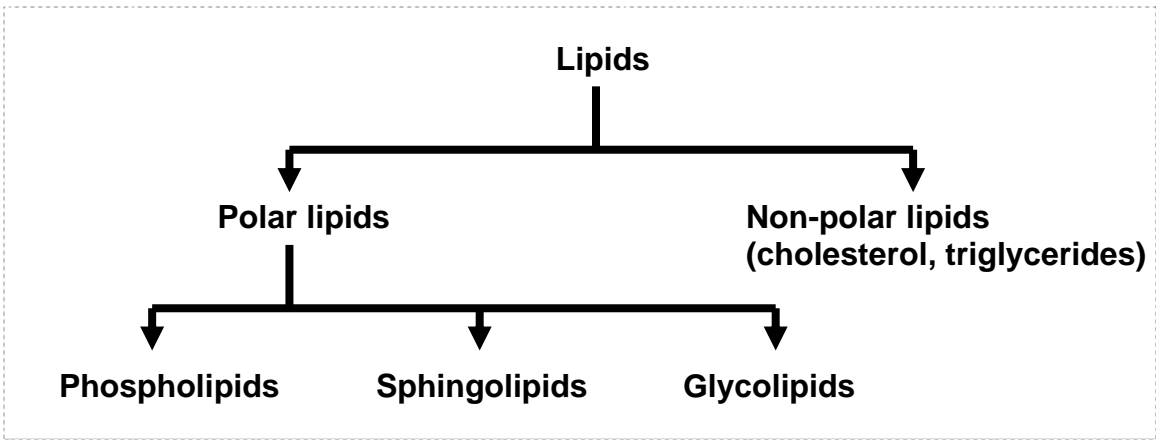
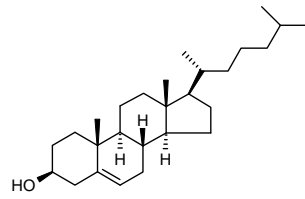
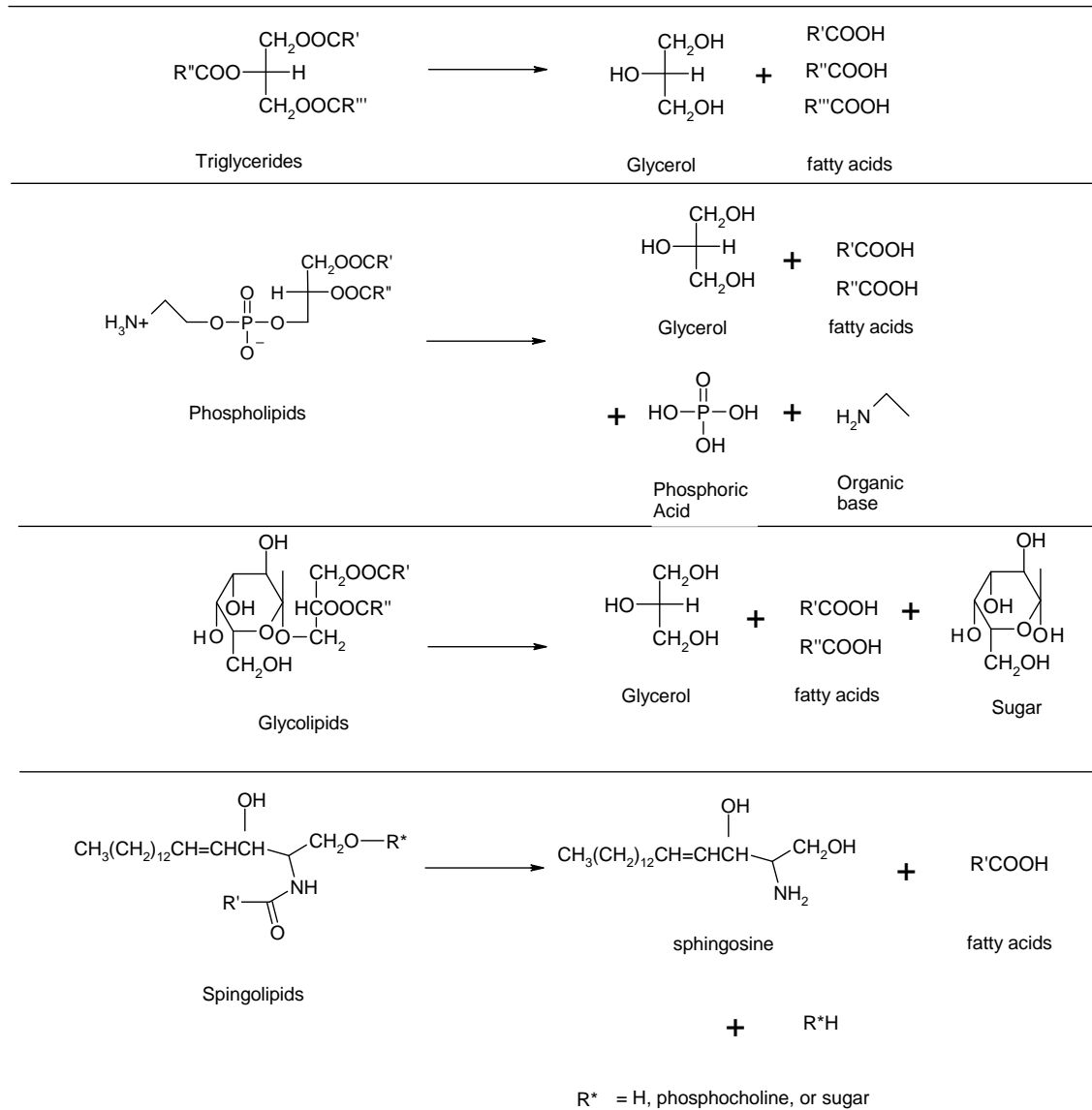


Figure 1. Classification of Lipids

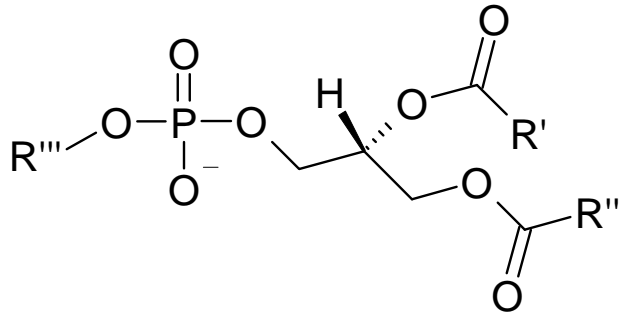


Cholesterol



R<sup>\*</sup> = H, phosphocholine, or sugar

Figure 2. Structure of selected lipids and their primary hydrolysis products



- R', R'' = fatty acid chains
- R''' = head group

Figure 3. Basic Phospholipids Structure

## CHAPTER II

### Previous Chromatographic Methods for Analysis of Phospholipids

Lipidomics is a rapidly growing field that deals with lipids and their structures, but it also provides insight as to how specific phospholipids play roles in normal physiological and disease states. One of the major priorities of lipidomics today is the development of a practical lipids analytical system which exhibits high resolution, high throughput, and user-friendliness in order to measure constituents in the diverse field of low abundance lipids. Identification of each lipid in a mixed condition is the goal which further necessitates analytical methods that have the ability to discriminate between endogenous and exogenous phospholipids in the biological system.

As stated previously, phospholipids are composed of a glycerol backbone with fatty acids esterified at adjacent carbon positions.<sup>1</sup> The third carbon position has a phosphate group attached to a polar head group. In order to advance the field of lipidomics, analytical techniques capable of discriminating between polar head groups, double bonds, and fatty acid chain lengths are required. Coupled, highly efficient, rapid chromatographic and spectrometric techniques may satisfy this need.

There are many analytical methods available for qualitative and quantitative determination of phospholipids. A recent review has summarized the methods that have been historically used such as thin layer chromatography, gas chromatography, and high performance liquid chromatography.<sup>9</sup> For example, Iacono and Ishikawa<sup>10</sup> used preparative thin-layer chromatography for phospholipids separation. The liquid extracts from platelets and erythrocytes were separated using a ternary solvent mixture, CHCl<sub>3</sub>-

MeOH-H<sub>2</sub>O (65:30:5, v/v). The phospholipid bands were located by spraying the plate with dichlorofluorescein for viewing under ultraviolet light. Both phosphatidylcholine-phosphatidylserine and phosphatidylinositol-sphingomyelin pairs were resolved in this case, but this mode of separation was time-consuming and lacked quantitative accuracy.

The use of gas chromatography (GC) for the direct analysis of intact phospholipids was not possible, owing to their non-volatile nature. Thus, most GC methods rely on hydrolysis of phospholipids to diacylglycerols followed by methyl transesterification which produces fatty acid methyl esters that are subsequently analyzed by GC.<sup>11</sup>

High performance liquid chromatography (HPLC) was first used for separation of PL over 30 years ago. Ultraviolet (UV) detection at low wavelengths (200-210 nm) was initially employed. For example, elution of phospholipids from both a diol- and a cyanopropylsilica bonded stationary phase (25cm x 4.6mm, d<sub>p</sub> = 5 μm) was reported by Andrews.<sup>12</sup> Six phospholipids were separated in a gradient mode with solvent A (100% acetonitrile) and solvent B (acetonitrile-water, 3.5:1, v/v) as the mobile phase components. In 1998, Mawatari and Murakami<sup>13</sup> applied HPLC-UV for the study of changes in human erythrocyte membrane phospholipids as a function of peroxidation by lipoxygenase. An amino-silica bonded column with acetonitrile/methanol/0.2% triethylamine (67:22:11, v/v) as the mobile phase mixture was used. The phospholipids were detected at 210 nm. Commonly used solvents, such as methanol and chloroform, which have a low UV absorption, however, were observed to cause baseline drift during gradient elution.

Evaporative light scattering detection (ELSD), on the other hand, does not have the limitations experienced with UV detection under gradient elution conditions<sup>14</sup>. For



example, phospholipids from brain tissue extracts were separated on a diol-silica bonded packed column with a very complex linear mobile phase gradient consisting of a solvent mixture A of hexane-2-propanol-n-butanol-tetrahydrofuran-isooctane-water and a second solvent mixture B of 2-propanol-n-butanol-tetrahydrofuran-isooctane-water.<sup>15</sup>

Mass spectrometry has been widely used for phospholipid identification. Soft ionization methods, such as matrix-assisted laser desorption ionization (MALDI)<sup>16,17</sup> and electrospray ionization (ESI)<sup>18,19</sup> have been used during the last decade. Unlike “harder” ionization methods, soft ionization methods provided minimal fragmentation. Protonated molecules and other adducts that are easily related to the molecular structure are usually present in the mass spectrum. Ham, et al.<sup>16</sup>, for example, studied the phospholipid composition of normal human eye tear extracts by using MALDI-TOF/MS analysis. p-Nitroaniline and butyric acid were used for formation of the ionic crystal MALDI matrix. Later, Jones, et al.<sup>17</sup> were able to profile phospholipids directly from mouse tissues via the use of MALDI-FTMS. High reproducibility and good accuracy were shown. Hayakawa, et al.<sup>20</sup> were able to quantify eight phospholipid classes in human high-density lipoprotein by using normal phase HPLC/ESI/MS. A silica column with a gradient mobile phase consisting of methanol-propanol-water-28% ammonium hydroxide (83:10:6:1, v/v) was used.

Although HPLC affords reasonable resolution of phospholipids, long elution and column re-equilibration times remain a problem. Supercritical fluid chromatography (SFC), on the other hand, uses a supercritical (or near critical) mobile phase, **Figure 4**. The SF possesses intermediate properties between gas and liquid. It has lower viscosity and higher diffusivity than conventional liquids which translates into higher optimum

SFC flow rates and lower packed column pressure drops, **Table 1**. More efficient mass transfer means longer columns (e.g. higher plate counts and mixed stationary phases) can be used than is possible in traditional HPLC. SFC also affords a normal phase separation mechanism without many of the disadvantages (such as traces of water) of traditional normal phase chromatography. Although organic modifiers are sometimes mixed with carbon dioxide in order to separate highly polar analytes, SFC, nevertheless, requires a smaller amount of organic solvent than HPLC. Furthermore, preparative scale SFC separations are rather trivial in that carbon dioxide-based mobile phase are more economical and their removal from the analyte of interest is essentially a spontaneous process. Thus, SFC should have greater potential for phospholipid separations compared to traditional HPLC methods.<sup>21</sup>

Several reports have been published regarding the SFC of phospholipids.<sup>22-25</sup> The first paper used SFC-ELSD and was reported by Lafosse, et al. in 1992.<sup>25</sup> The separation of PC, PA (phosphatidic acid), PE, and PI was achieved in 22 minutes by using a Zorbax bare silica column (25cm x 4.6mm,  $d_p = 5 \mu\text{m}$ ) and an isocratic mobile phase consisting of carbon dioxide modified with a mixture of methanol-water-triethylamine (95:4.95:0.05, v/v/v). Later, Eckard, et al.<sup>26</sup> were able to separate five phospholipids using SFC-ELSD. A Luna octyl-bonded silica packed column employing a mobile phase gradient of CO<sub>2</sub> with a mixture of ethanol-methanol (50:50, v/v) containing 0.1% TFA was used. Taylor and King<sup>27</sup> in another study used supercritical fluid extraction (SFE) on-line coupled with SFC-ELSD at 50°C to obtain enriched fractions of several major phospholipids. A silica

# Phase Diagram

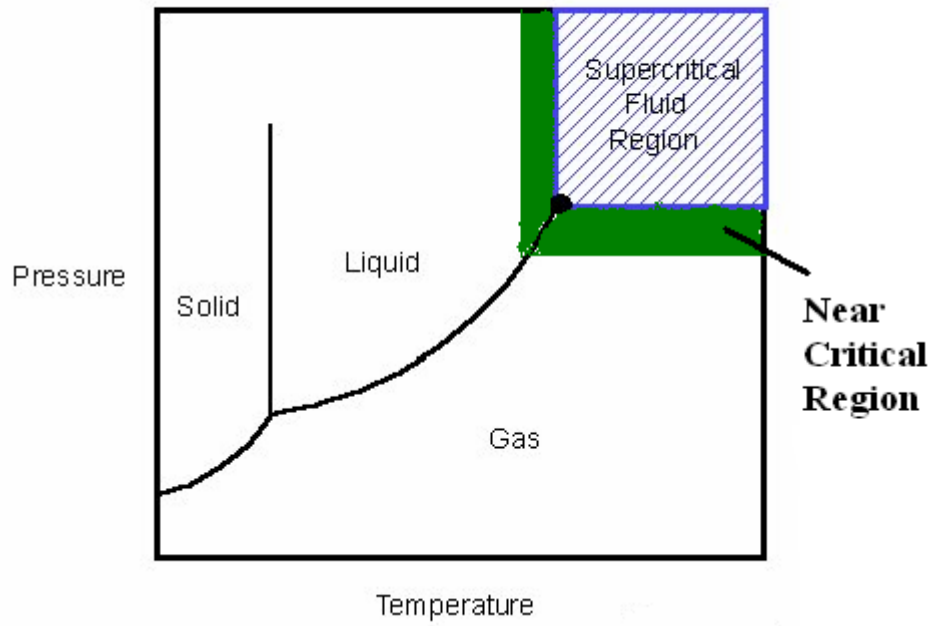


Figure 4. Phase diagram of carbon dioxide

	Density (g/mL)	Dynamic Viscosity (g/cm-sec)	Diffusion Coefficient (cm <sup>2</sup> /sec)
Gas (ambient)	0.0006-0.002	0.0001-0.003	0.1-0.4
Supercritical fluid (T <sub>c</sub> = 31.3 °C, P <sub>c</sub> = 72.9 atm in the case of CO <sub>2</sub> )	0.2-0.5	0.0001-0.0003	0.0007
Liquid (ambient)	0.6-1.6	0.002-0.03	0.000002-0.00002

Table 1. Physical properties of gas, supercritical fluid, and liquid

column (25cm x 4.6mm,  $d_p = 5 \mu\text{m}$ ) was used with a mobile phase consisting of carbon dioxide modified with a mixture of ethanol-water (9:1, v/v). In 2001, Wang, et al.<sup>28</sup> used SFC-UV to separate six phospholipids in 15 minutes with carbon dioxide modified with ethanol containing 0.05% (v/v) triethylamine as an additive. A  $C_{18}$  column and UV detection at 214 nm were used.

The purpose of the research reported here was to develop alternative analytical methods for the analysis of high molecular weight phospholipids. Chapter III deals with the elution of four phospholipids (PC, PE, PI, and PS) from a variety of stationary phases. Two commonly used and two newly developed silica-based stationary phases were studied: diol, end-capped cyanopropyl, 2-ethylpyridine, and 4-ethylpyridine. The influence of basic, acidic, and ionic mobile phase additives (incorporated into the primary methanol modifier) on the elution of the phospholipids was studied. The additives that were studied include isopropylamine, trifluoroacetic acid, and ammonium acetate. In Chapter IV, supercritical fluid fractionation of selected components from lecithin is discussed. The optimized chromatographic conditions previously developed were used to identify the corresponding phospholipids in the extracts.

## CHAPTER III

### Feasibility of Phospholipids Separation via Packed Column Supercritical Fluid Chromatography with Mass Spectrometric and Light Scattering Detection

#### 3.1 Introduction

Carbon dioxide (CO<sub>2</sub>) is most commonly used as the SF mobile phase due to its low physical parameters ( $T_c = 31.3^\circ\text{C}$ ;  $P_c = 72.9 \text{ atm}$ ),<sup>29</sup> inertness to most analytes, non-toxicity, and non-flammability. However, CO<sub>2</sub> has weak solvating power which limits the application of SFC to intermediate polar and non-polar analytes. Polar organic solvents such as methanol can be added to CO<sub>2</sub> to increase the solvating power of the mobile phase; nevertheless, these binary mobile phases are still not sufficient to elute certain highly polar and ionic analytes. Thus, a small amount of very polar chemicals called “additive” was added to expand the range of applicability to more polar compounds. Recently, Pinkston et al.<sup>30</sup> reported that low levels of various ammonium salts (e.g. ammonium acetate, ammonium formate, and ammonium carbonate) were effective carbon dioxide mobile phase additives for the successful elution of polar and even ionic organic materials such as sulfonate and carboxylate salts, polyamines, and quaternary ammonium salts from packed columns.

Zheng et al.<sup>31,32</sup> have studied the SFC of ionic compounds in more detail. Sodium arylsulfonate salts were able to be eluted in the presence of various ammonium salt additives in the methanol modified CO<sub>2</sub>. Two elution mechanisms were proposed depending on the stationary phase<sup>32</sup>: Ion pairing between additive and analytes was suggested to be the major mechanism on a Deltabond cyanopropyl silica-based stationary

phase; while modification of the silanol sites by the additives was suggested on the bare silica stationary phase, **Figure 5**. Later, these workers reported that one secondary amine (propranolol hydrochloride) and two quaternary amine salts (benzyltrimethylammonium chloride and cetylpyridinium chloride) were successfully eluted from similar columns with the addition of sodium alkylsulfonate additive to the methanol-modified, CO<sub>2</sub> based mobile phase. An ion-pairing interaction between the positively charged analytes and anionic sulfonate additives was believed to be the major retention mechanism here.

Beyond the Deltabond cyano column, two basic stationary phases, 2-ethylpyridine and 3-aminopropyl bonded silica, were also used for the elution of the three amine salts. The amine analytes were readily eluted from the pyridine columns without the need of additive. A similar separation of the amine analytes without mobile phase additives was observed by using a strong silica-based anion exchange (SAX) column which suggested that the two basic pyridine stationary phases were positively charged in the presence of a mixture of methanol and CO<sub>2</sub>.<sup>33</sup> The results above were then expanded to the elution of polypeptides. Four groups of polypeptides that contained a large variety of amino acid groups, with up to 40 mers and with a molecular mass greater than 4500 Dalton, have been found to be eluted from the protonated 2-ethylpyridine silica-based column using 0.1% trifluoroacetic acid as additive in the methanol modified CO<sub>2</sub> mobile phase.<sup>34</sup>

We now wish to report the extension of this study of ionic analytes to four, high molecular weight, commercially available phospholipids, **Figure 6**. Zwitterionic phosphatidylcholine (PC) and phosphatidylethanolamine (PE) constituted one analyte class. Anionic phosphatidylinositol (PI) and phosphatidylserine (PS) formed a second class. A variety of packed column stationary phases have been investigated with

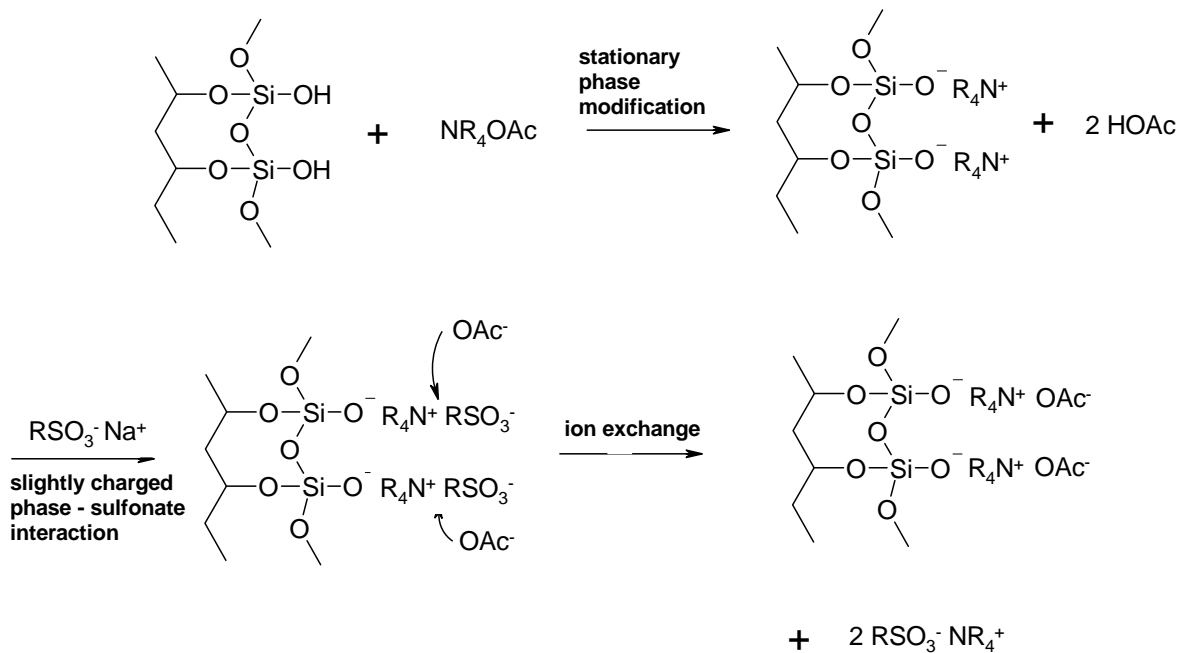


Figure 5. Proposed mechanism for modification of bare silica phase by ionic additive.<sup>33</sup>



methanol-modified CO<sub>2</sub>-based mobile phases. Detection has been via evaporative light scattering (ELSD) and electrospray mass spectrometry (MS). For screening purposes, ELSD proved to be highly useful; while, MS was invaluable for identification of the specific peak components. Traditional diol- and cyanopropyl-silica bonded stationary phases have been studied. Both 2-ethylpyridine- and 4-ethylpyridine-silica bonded phases, which exhibit greater basic character, have been studied. In the latter case, we were interested to determine if placement of the alkyl group on the pyridine ring is critical in the mechanism of separation. The success of acidic and ionic additive incorporation into the methanol mobile phase in our previous studies has also prompted experiments in this area.

While identification of these molecular phospholipids has been previously and successfully achieved with HPLC/ESI/MS, this is the first report, to our knowledge, of using SFC coupled on-line with MS for phospholipid identification.

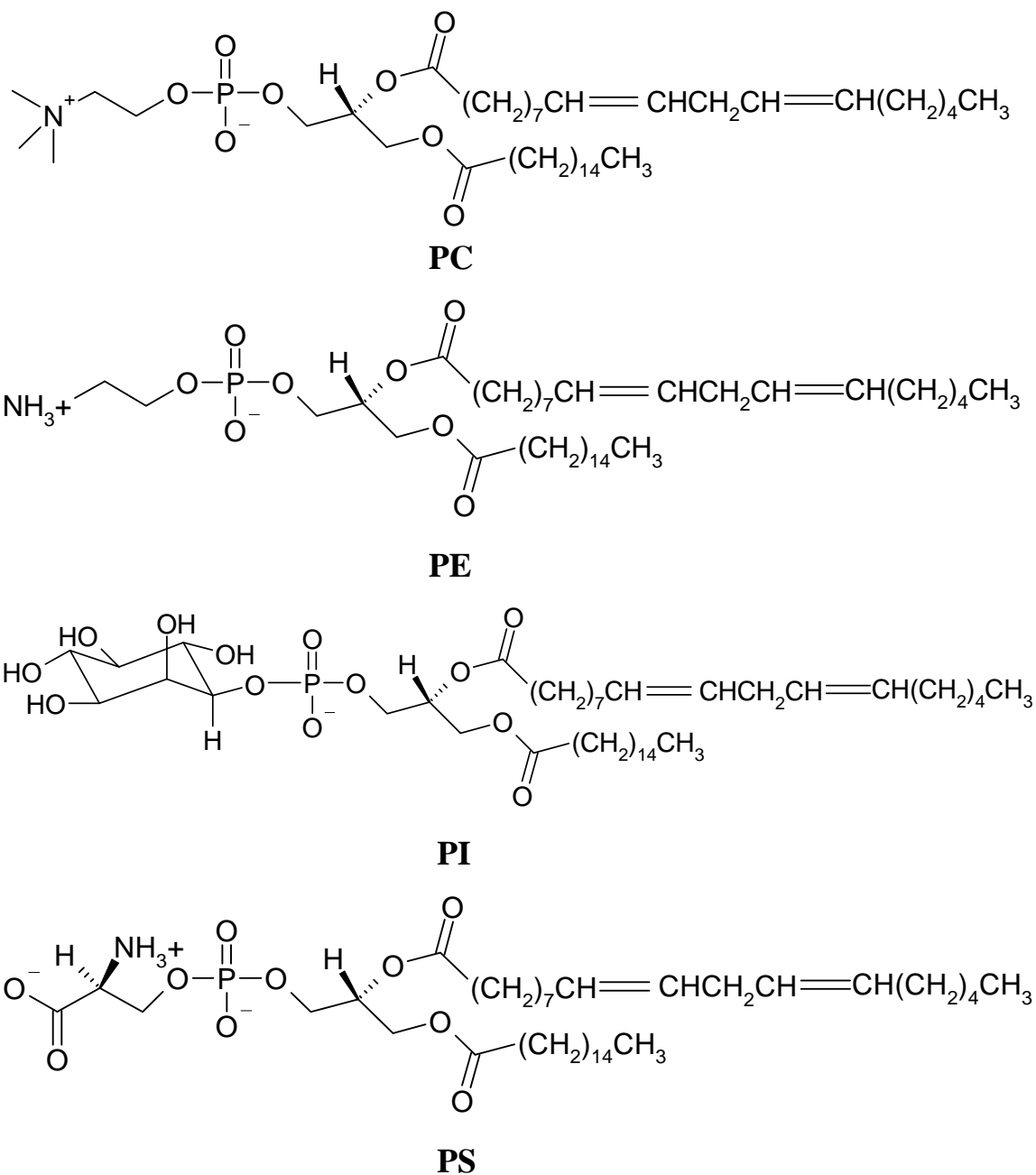


Figure 6. Molecular structures of selected phospholipids: phosphatidylcholine (PC) (M.M.=757 Da), phosphatidylethanolamine (PE) (M.M.=715 Da), phosphatidylinositol (M.M.=833 Da), phosphatidylserine (PS) (M.M.=758 Da)

## **3.2 Experimental**

### **3.2.1 Chemicals**

Methanol was HPLC grade, (EMD, Durham, NC, USA). The carbon dioxide was SFE/SFC grade (Air Products and Chemicals, Inc., Allentown, PA, USA) without helium-head pressure. Ammonium acetate (AA) (99%, ACS grade), trifluoroacetic acid (TFA) (99%), and isopropylamine (IPA) (99%) were obtained from Sigma Aldrich (Milwaukee, WI, USA). The following standards (Sigma Aldrich) were used for the study: a) L- $\alpha$ -phosphatidylethanolamine (PE), b) L- $\alpha$ -phosphatidylcholine (PC), c) L- $\alpha$ -phosphatidylinositol ammonium salt (PI), d) L- $\alpha$ -phosphatidyl-L-serine (PS). Each standard solution was prepared by dissolving 2.5 mg of phospholipid in 10 mL of methanol. A mixture of all four components was then prepared by combining 1 mL of each individual standard solution.

### **3.2.2 SFC-ELSD**

The SFC was a Berger Instruments Analytical SFC system (Newark, DE, USA) with a Hewlett Packard (Little Creek, DE, USA) Model 1050 Diode Array Detector, which employed a 13  $\mu$ L high pressure flow cell (10 mm path length) and Berger Instruments 3D SFC ChemStation software, version 3.4. Mobile phase flow was 2 mL/min (measured in the liquid state). The post-column effluent was split such that 0.8 mL of the total flow was directed into a Varex MKIII evaporative light-scattering detector (ELSD) (Alltech Associates Inc., State College, PA, USA) with drift tube temperature at 85°C.

The chromatographic columns were PrincetonSFC 2-ethylpyridine, PrincetonSFC 4-ethylpyridine (Princeton Chromatography Inc., Cranbury, NJ, USA), Supelcosil LCDiol and Supelcosil Discovery Cyano (Supelco Park, Bellefonte, PA, USA). The column dimensions were 25 cm in length and 4.6 mm I.D., with a silica particle size of 5  $\mu\text{m}$  for each stationary phase. Each stationary phase had a pore size of 60  $\text{\AA}$ .

Unless otherwise specified, chromatographic conditions were as follows: injection 10  $\mu\text{L}$ , mobile phase flow 2  $\text{mL min}^{-1}$ , column outlet 125 bar, and column oven 40°C. The mobile phase gradient composition was: 15% modifier in  $\text{CO}_2$  for 1 minute after injection, modifier concentration increased to 55% at 4% per minute, and was held for 5 minutes at 55%. The modifier consisted of either pure methanol or methanol with 5 mM additive.

In order to established stable, reversible chromatographic conditions, the mobile phase was allowed to flow through the packed column for 30 min prior to injection of the sample. Between each change of mobile phase additive, the stationary phase was washed with pure methanol at 1  $\text{mL min}^{-1}$  for an hour (i.e. about 20 column volumes) in order to either purge previous additive solution from the system or to remove analytes that had irreversibly interacted with the stationary phase. After this time period, the next additive solution was introduced to the SFC system, and the column was equilibrated again for 30 min prior to injection.

### **3.2.3 SFC-MS Instrumentation**

The SFC system (i.e. different from SFC/ELSD) was a Berger Instruments Analytical SFC Instrument (Newark, DE, USA) with an Agilent Technologies Model

1100 Diode Array Detector, which employed a 13  $\mu\text{L}$  high pressure flow cell (10 mm path length) and Berger Instruments 3D SFC ProNT software. A zero-dead volume chromatographic tee was installed just before the SFC outlet pressure regulator in order to connect the SFC system to the mass spectrometer. The SFC column effluent was pumped directly without any split into the Thermo Instrument TSQ triple quadrupole mass spectrometer (Thermo Finigan, San Jose, CA, USA), equipped with an APCI source in MS mode configured for analyzing positively charged ions. The settings were as follows: APCI vaporization temperature, 400°C; discharge current, 4 mA; heated capillary temperature, 350°C. The  $m/z$  range for measurement was 300-900 for PC and PE with 0.7 s scan cycle time. The same scan time with  $m/z$  300-1000 was employed for PI and PS.

### **3.3 Results and Discussion**

#### **3.3.1 Elution with Primary Mobile Phase Modifier**

**Figure 7** shows SFC-ELSD traces for the separation of the four component synthetic phospholipid mixture with pressurized  $\text{CO}_2$  modified with pure methanol on diol, cyanopropyl, 2-ethylpyridine, and 4-ethylpyridine silica-bonded stationary phases. The modifier composition ranged from 15% to 55%. Each phospholipid was preliminarily identified by retention time comparison with the respective phospholipid standard. Only two (PC and PE) of the four analytes eluted with retention times less than 15 minutes under the stated gradient conditions from the four properly conditioned stationary phases. The elution order was PC followed by PE. While cyano and diol phases yielded reasonably good peak shapes for these two analytes, a shoulder or split

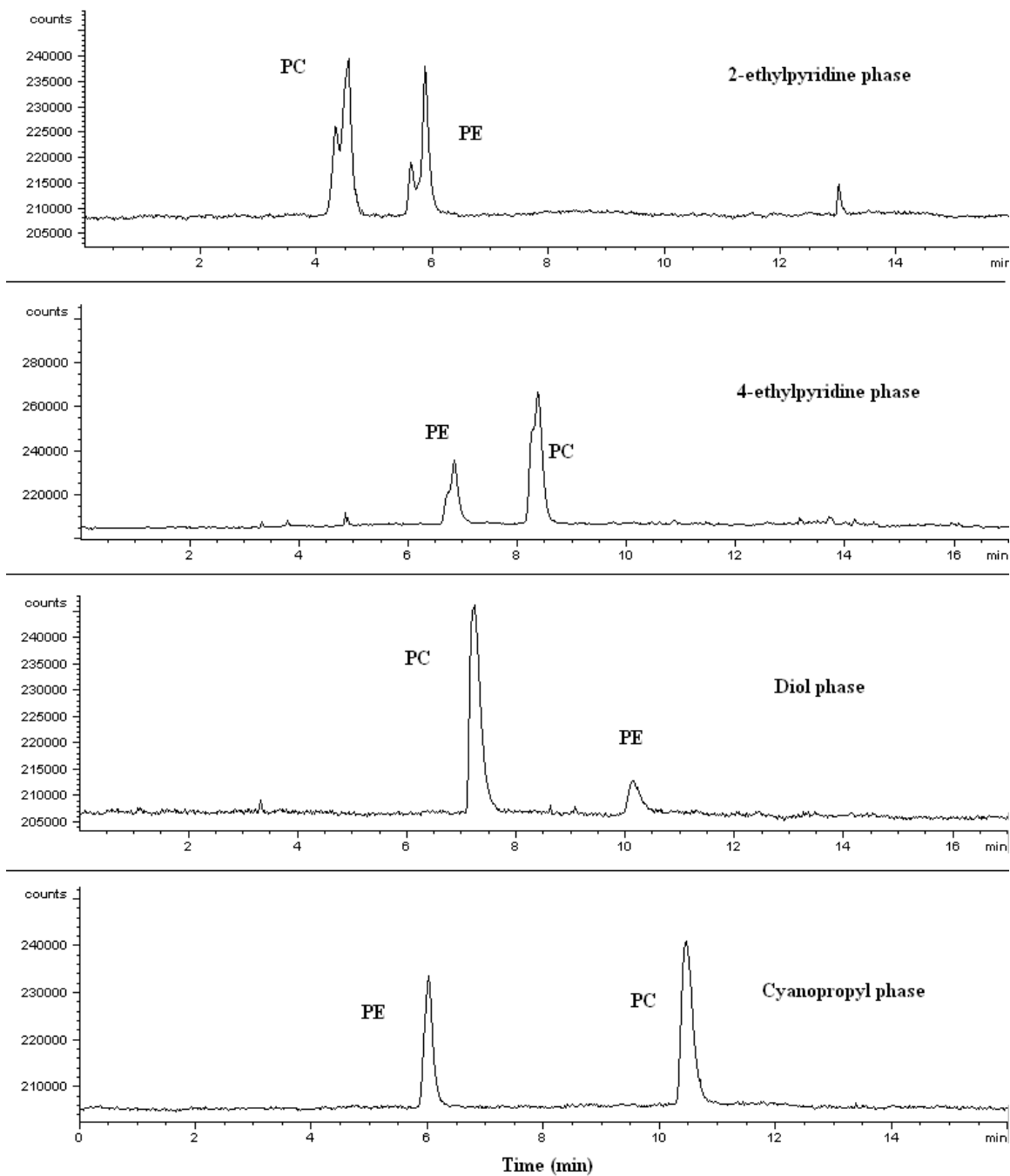


Figure 7. SFC-ELSD traces for the separation of phospholipid mixture with pressurized CO<sub>2</sub> modified with pure methanol on various stationary phases. See Experimental Section for chromatographic conditions.

peak was apparent for PE and PC on the two pyridine stationary phases which indicated either a mixed mode of separation or the presence of a mixture of related components in our “working” mixture. The failure of PI and PS to elute from any of the phases under these conditions is probably due to their existence in an anionic form (as opposed to a zwitterionic form) which would strongly and irreversibly interact with the stationary phase and/or silica support. In support of this hypothesis, it has been previously shown that the two pyridine phases are most probably positively charged in the presence of CO<sub>2</sub>.<sup>32</sup>

To gain a better perspective on the nature of the split peaks, mass spectrometric detection was directly coupled with SFC. Mass spectra obtained via SFC/APCI-MS of the components eluting from the 4-ethylpyridine phase are shown in **Figures 8 and 9**. For PC, which was the earliest eluting component, the major chromatographic peak gave a prominent ion at  $m/z$  758, which corresponds to the protonated PC molecule, **Figure 8**. On the other hand, the visible chromatographic shoulder gave  $m/z$  782. Thus, the mass difference between the two chromatographic components was 24 mass units. Sodium ion adducts are fairly common in the HPLC/MS of phospholipids, but the mass difference here is 24 not 22 (i.e.  $[M+Na]^+ - [M+H]^+$ ).<sup>16</sup> Alternatively, a different polyunsaturated fatty acid chain with the same polar head group could account for the chromatographic shoulder. The commercially available PC that was used here is stated to have a molecular mass of 757 Da, and it can be represented as PC (16:0/18:2) where 16 and 18 correspond to the total number of carbon atoms in the two acyl chains; while, 0 and 2 refer to the total number of double bonds in the respective chain. A difference of 24 mass units could be explained by the addition of two methylene carbons to one of the chains and the

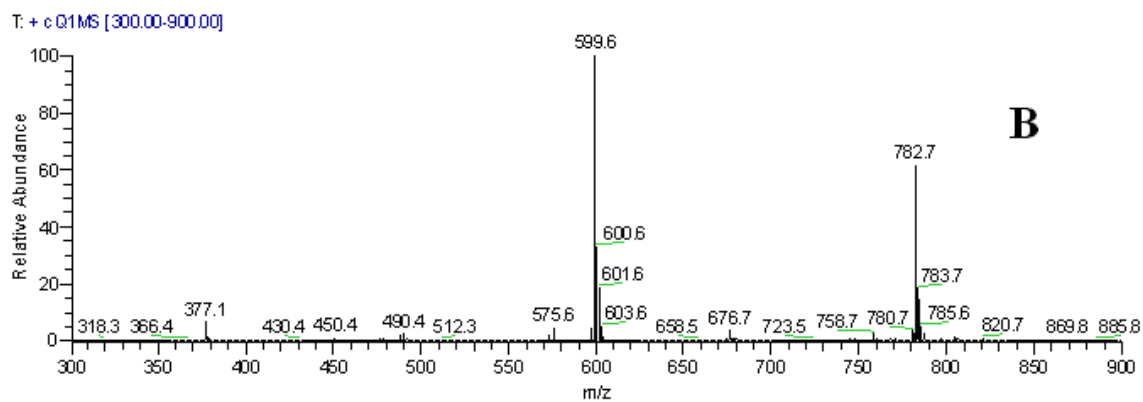
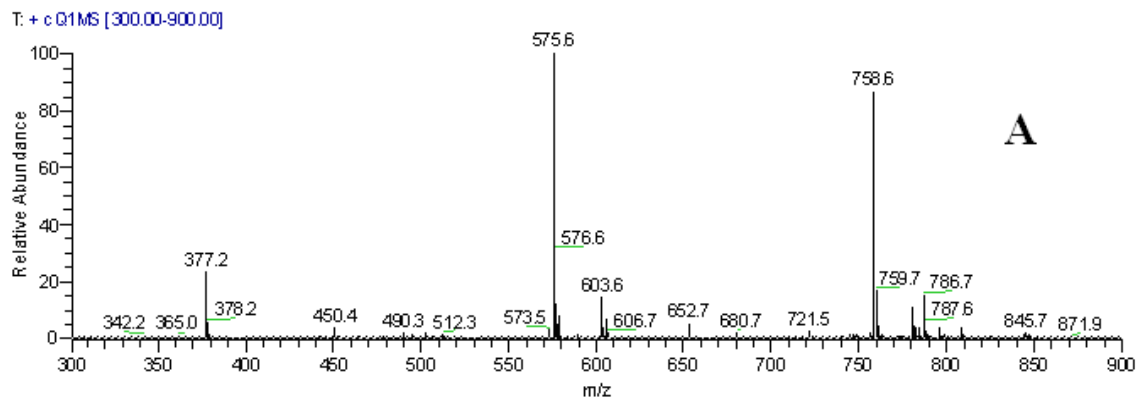


Figure 8. Mass spectra of the two PC peak components eluting on the 4-ethylpyridine column with only methanol as the modifier. A = major peak; B = shoulder eluting in front of the main peak.



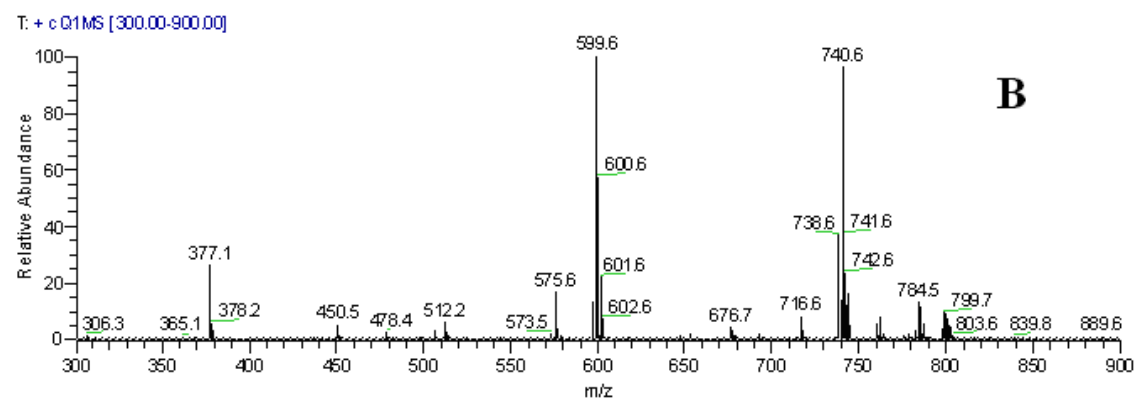
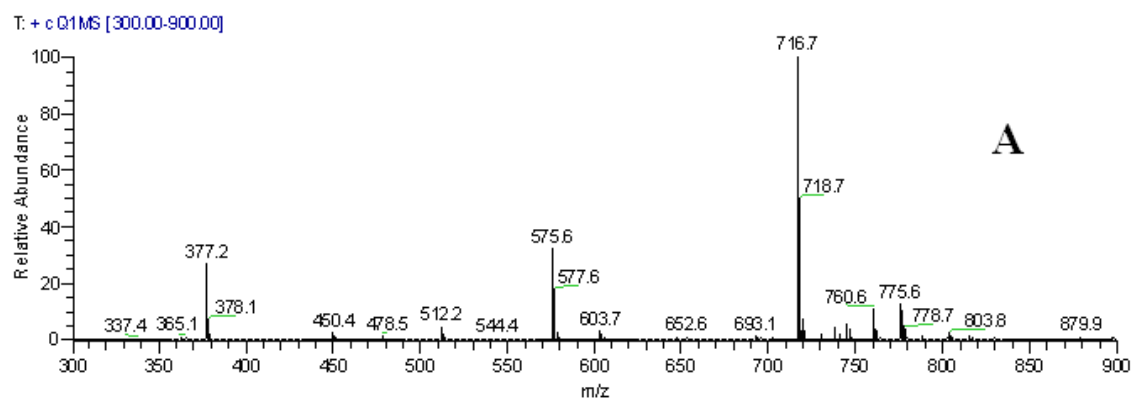


Figure 9. Mass spectra of the two PE peak components that elute from the 4-ethylpyridine column with only methanol as the modifier. A = main peak; B = shoulder

incorporation of two additional double bonds (e.g., in other words: PC (16:0/20:4)). Fragment ions with  $m/z$  575 and  $m/z$  599 were also observed for both the main peak and shoulder, respectively, which corresponds to the loss of the phosphatidylcholine head group (183 Da) in each case. These fragment ions lend support to our postulated structures.

A similar interpretation can be applied to our PE data with the 2-ethylpyridine column, **Figure 9**. The protonated molecule associated with the major chromatographic peak at  $m/z$  716 corresponds to PE (16:0/18:2). The chromatographic shoulder again yielded  $m/z$  740 assignable to PE (16:0/20:4) with an anticipated additional two carbons and two double bonds. Two fragment ions were observed with  $m/z$  599 and 575 which are suggested to arise from the loss of the phosphatidylethanolamine head group (141 Da). In contrast, no hint of multiple chromatographic peaks was apparent for either PC or PE with the diol and cyano phases. However, the lone mass spectrum of the component(s) eluting as the single PC peak on the diol and cyano phases gave  $m/z$  values of 758 and 782 which suggested that both components had co-eluted on these two columns. Identical results were observed in the mass spectrum of the PE peak with ions  $m/z$  716 and  $m/z$  740 corresponding to PE (16:0/18:2) and PE (16:0/20:4) respectively. One therefore concludes that the two pyridine phases with only methanol as the modifier separate both zwitterionic analytes by polarity completely and in part by hydrophobicity. The diol and cyano phases on the other hand appear to be only effective in separating PC and PE by polarity.

### 3.3.2 Elution with Basic Additive and Primary Modifier

The failure of both anionic PS and PI to elute from all of the four stationary phases with only methanol as the fluid modifier prompted us to search for an additive to the methanol which might be effective. Isopropylamine (IPA) proved to be satisfactory for this assignment. The best chromatographic resolution of all four analytes was achieved when pressurized CO<sub>2</sub> with 5 mM IPA in methanol was used with a diol column, **Figure 10**. The four analytes were baseline separated in 15 minutes with reasonable peak intensity. **Figures 11 and 12** show the mass spectra of the PI and PS chromatographic peak components obtained via SFC/APCI-MS on the diol stationary phase. The parent PI ion peak is the protonated isopropylamine adduct ion  $m/z$  894 [PI (16:0,18:2) neutral + IPA neutral + proton]. A fragment cation peak with  $m/z$  575 was observed which corresponds to loss of the phosphatidylinositol head group (260 Da) and the IPA additive (59 Da). Surprisingly, no additional components with varying numbers of double bonds (i.e. fatty acid constituents) were found with PI as was observed with PC and PE. This observation strongly supported our acquisition of a single, relatively sharp peak in all chromatographic runs of PI. Much less intense peaks observed at  $m/z$  892 and 922 could however suggest another conclusion, **Figure 11**. For example,  $m/z$  892 could be due to PI (16:0, 18:3).

A strikingly opposite situation, however, was observed for PS on the diol column. Three pairs of mass spectral peaks, **Figure 12**, were observed (e.g the components of each pair were separated by 24 mass units) even though there had been no chromatographic resolution to suggest that there were multiple eluting components associated with PS. These pairs were  $m/z$  819/843;  $m/z$  760/784; and  $m/z$  732/756. The

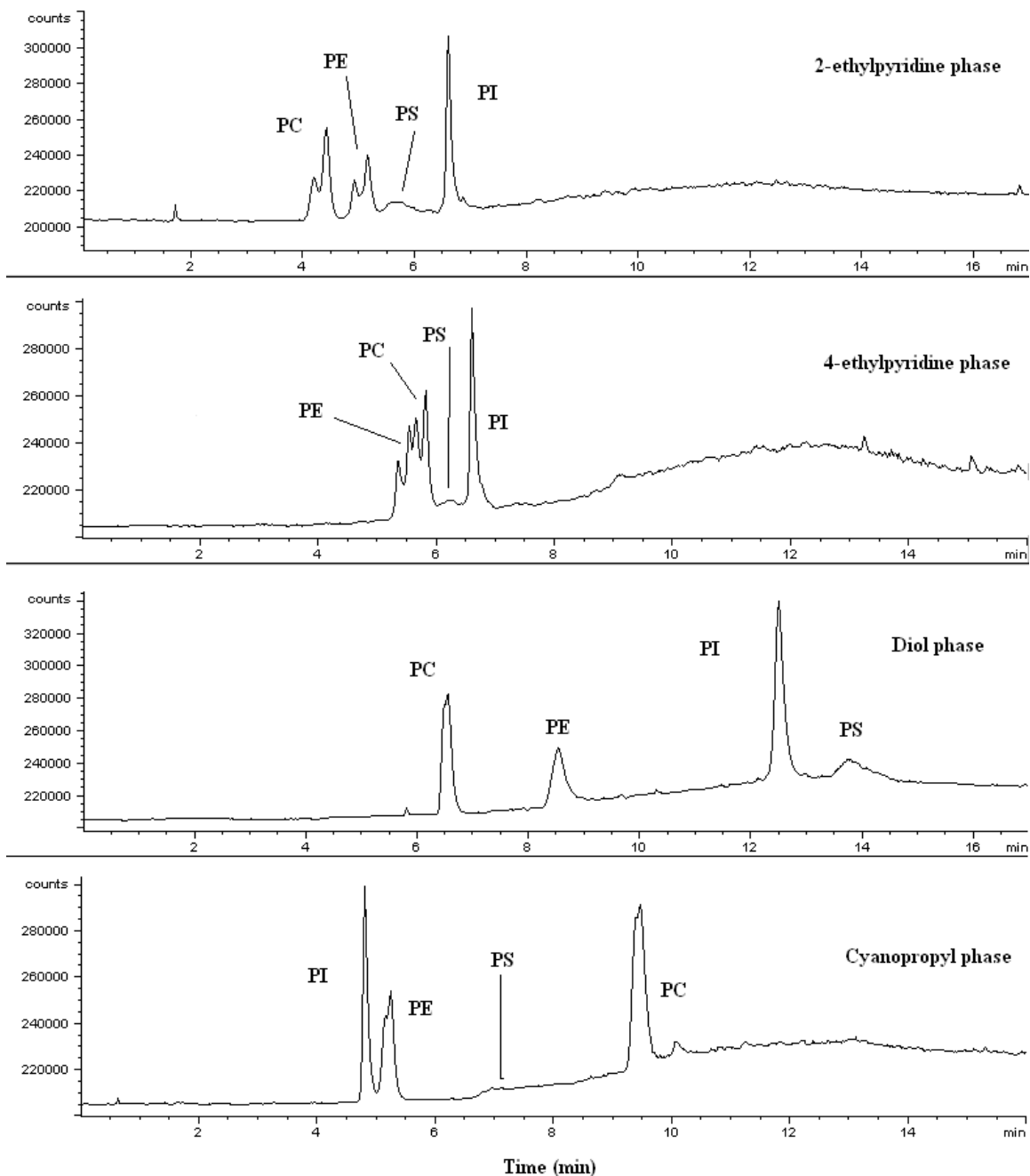


Figure 10. SFC-ELSD of phospholipid mixture with modified CO<sub>2</sub> on the four stationary phases. The modifier consisted of methanol with 5.0 mM isopropylamine. Chromatographic conditions: flow rate 2 mL/min, methanol/additive concentration raised from 15% to 55% at 4% per minute and held for 5 minutes at 55%.

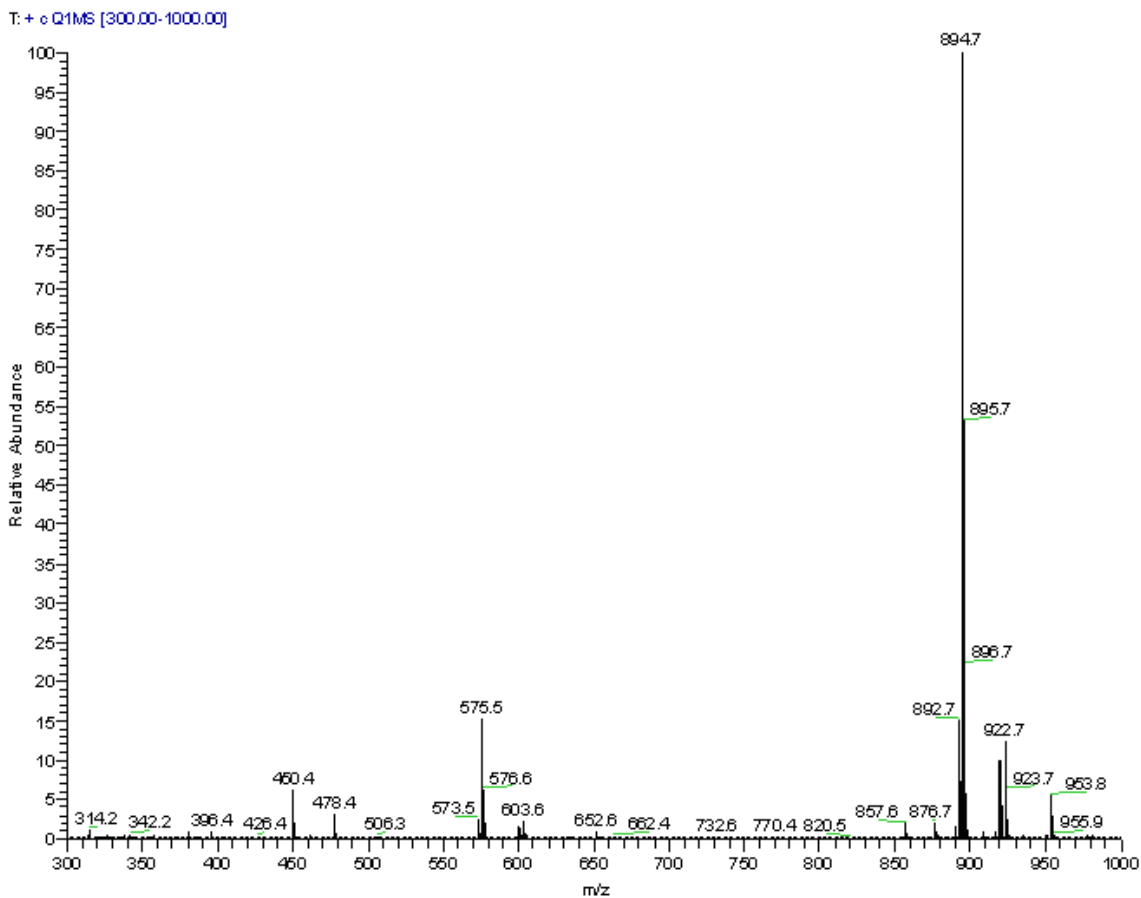


Figure 11. Mass spectrum of PI peak components eluting on the diol column. Methanol is the primary modifier. Isopropyl amine (5.0 mM in methanol) was used as the additive.

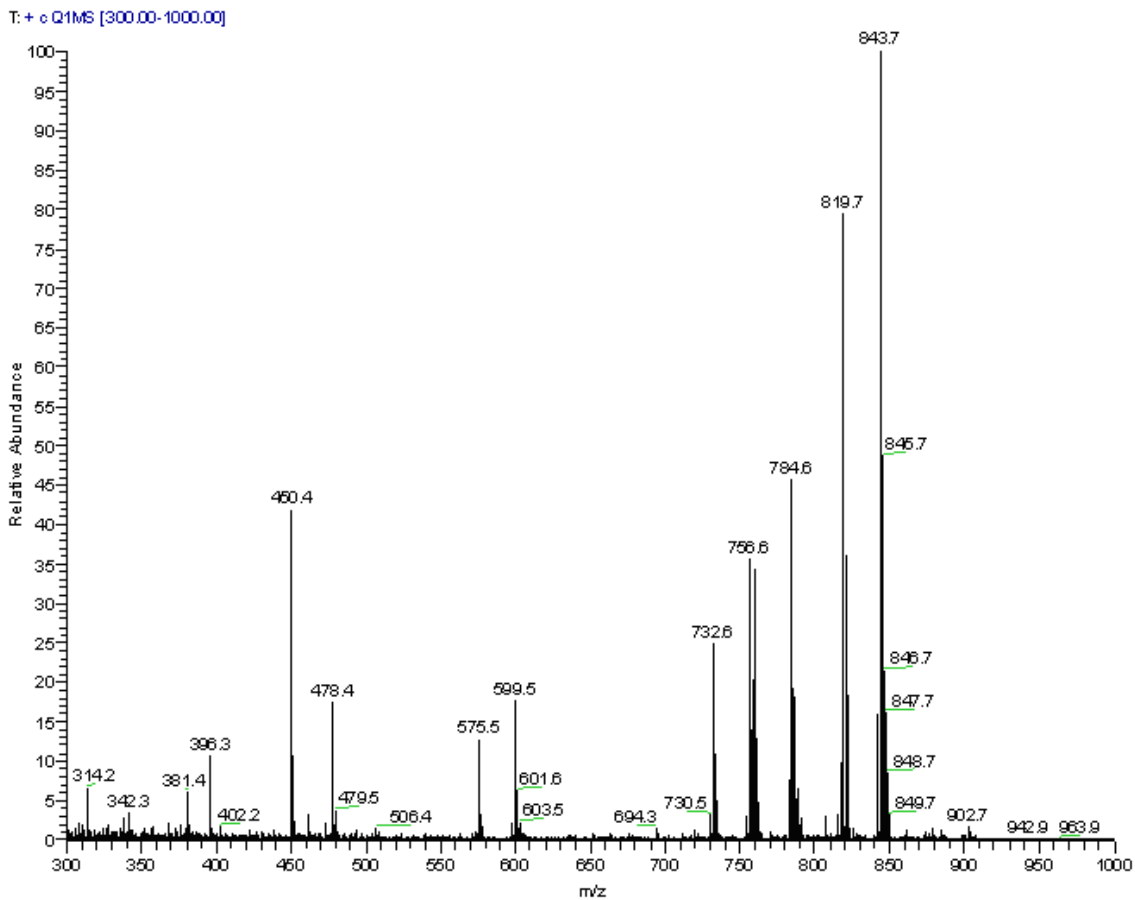


Figure 12. Mass spectrum of PS peak component eluting on the diol column. Methanol is the primary modifier. Isopropyl amine (5.0 mM in methanol) was used as the additive.

highest mass peak pair can be assigned to a protonated IPA adduct of PS (PS anion + proton + IPA + proton): (16:0/18:2;  $m/z$  819) and (16:0/20:4;  $m/z$  843). The next highest mass pair can be assigned to the protonated PS molecule (loss of IPA  $m/z$  59). The fragment ions at  $m/z$  599 and 575 are thought to arise from the highest mass pairs by loss of both the neutral phosphatidylserine head group (185 Da) and the IPA adduct (59 Da). The same fragment ion pair at  $m/z$  599 and 575 could also be formed from the protonated PS molecule (16:0/18:2;  $m/z$  760) after loss of only the phosphatidylserine head group (185 Da). A difference of 28 mass units between  $m/z$  756/784 and between  $m/z$  732/760 suggests the presence of structures in the mixture which contain two methylene groups fewer in their acyl chains. While we feel that it is unlikely, it should be noted that these latter structures could have been produced in the mass spectrometer

### 3.3.3 Elution with Acidic Additive and Primary Modifier

The success of basic IPA additive mobile phase incorporation led to a study of an acidic additive to the CO<sub>2</sub>-methanol mobile phase. No doubt the basic additive afforded major improvements in both polar analyte solubility in the mobile phase and the more favorable interaction of the polar analyte with the less active IPA-modified stationary phase. Trifluoroacetic acid (TFA) was thus selected for study with the four stationary phases and four phospholipids. **Figure 13** shows the SFC-ELSD trace for pressurized CO<sub>2</sub> modified with methanol containing TFA on the four stationary phases. Both zwitterionic phospholipids (PC and PE) were successfully eluted and baseline resolved with all four phases. The retention times of PC and PE varied widely on the four phases. The retention order was the same for the 2-ethylpyridine and cyano phases (PE before

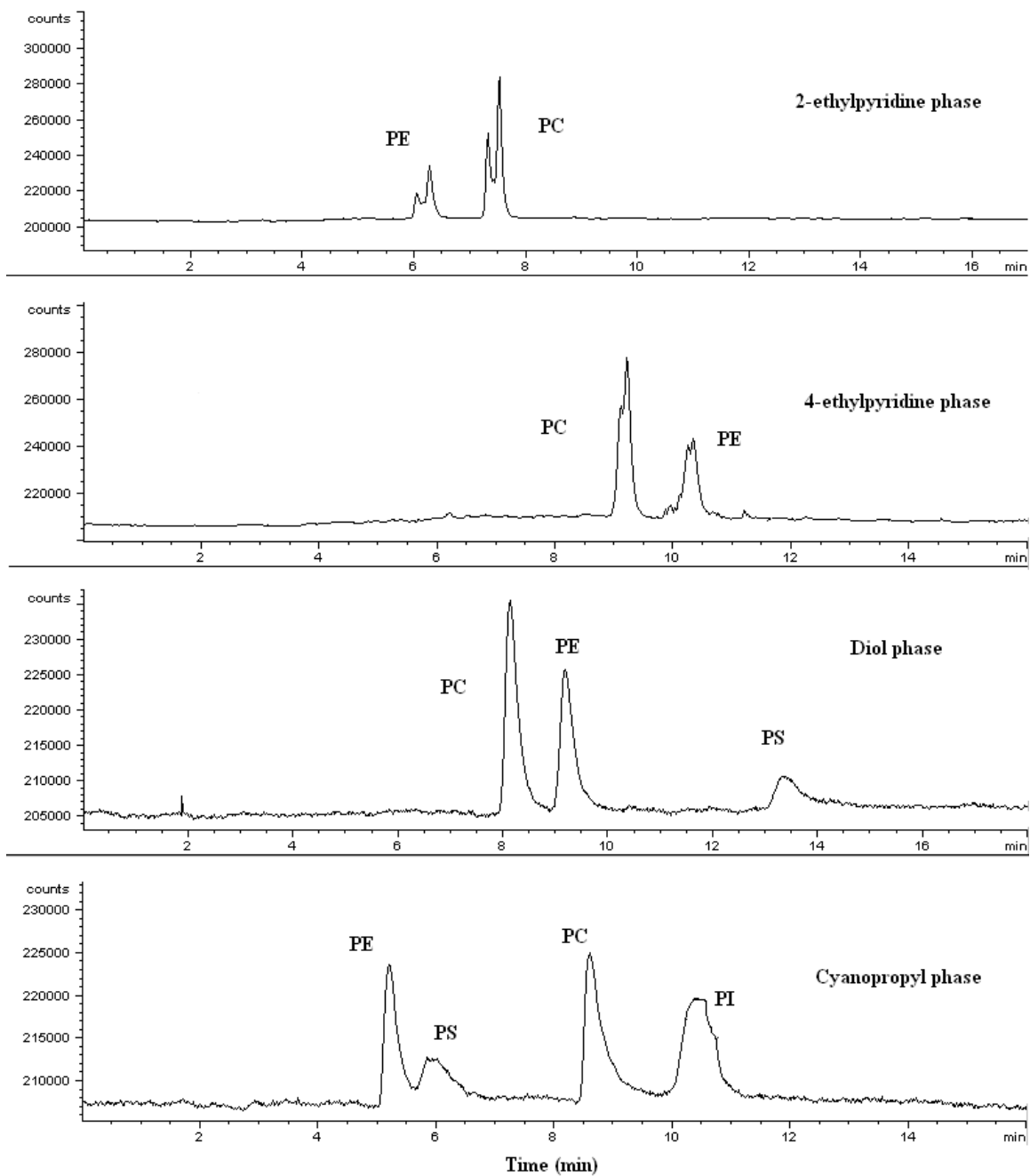


Figure 13. SFC-ELSD of phospholipid mixture with modified CO<sub>2</sub> on the four stationary phases. The modifier consisted of methanol with 5 mM trifluoroacetic acid. Chromatographic conditions: flow rate 2 mL/min, methanol/additive concentration raised from 15% to 55% at 4% per minute and held for 5 minutes at 55%.



PC). On the other hand, the retention order was reversed for the 4-ethylpyridine and diol phases. Partial resolution of both phospholipids via fatty acid content was clearly seen on only the 2-ethylpyridine phase. The two anionic phospholipids (PI and PS) were strongly retained on the two pyridine columns. They failed to elute within 15 minutes. In contrast, PS eluted from both the diol and cyano phases as a broad peak, while PI did not elute from the diol column. An explanation for the failure of one anionic phospholipid to elute from the diol column; while the other one eluted may be explained by noting the structure of the two phospholipids. The amino group and the carboxylic acid group of PS are both thought to be protonated at  $\text{pH} < 7$ ; while the phosphate group would probably remain unprotonated. Thus, a more easily eluted zwitterionic PS would be formed under the chromatographic conditions. On the other hand, the phosphate group of PI would likely remain unprotonated, and would remain anionic, at  $\text{pH} < 7$ . Thus, TFA would not alter PI's strong interactions with the polar diol phase and/or silanol solid support active sites. The less active, end-capped cyano phase may account for the fact that PI elutes here but not from the diol phase. It should be further noted that the retention time of PS decreased over 50% in going from the diol to the cyano phase. Retention of PS was also found to be sensitive to temperature on the cyano column. A  $10^{\circ}\text{C}$  increase caused PS to elute approximately two minutes later.

#### **3.3.4 Elution with Ionic Additive and Primary Modifier**

The employment of ammonium acetate as an additive gave unique results, **Figure 14**. Under these conditions, PS only eluted from the 2-ethylpyridine phase and in this case was a very broad peak with a retention time of approximately 10 minutes. PI on the

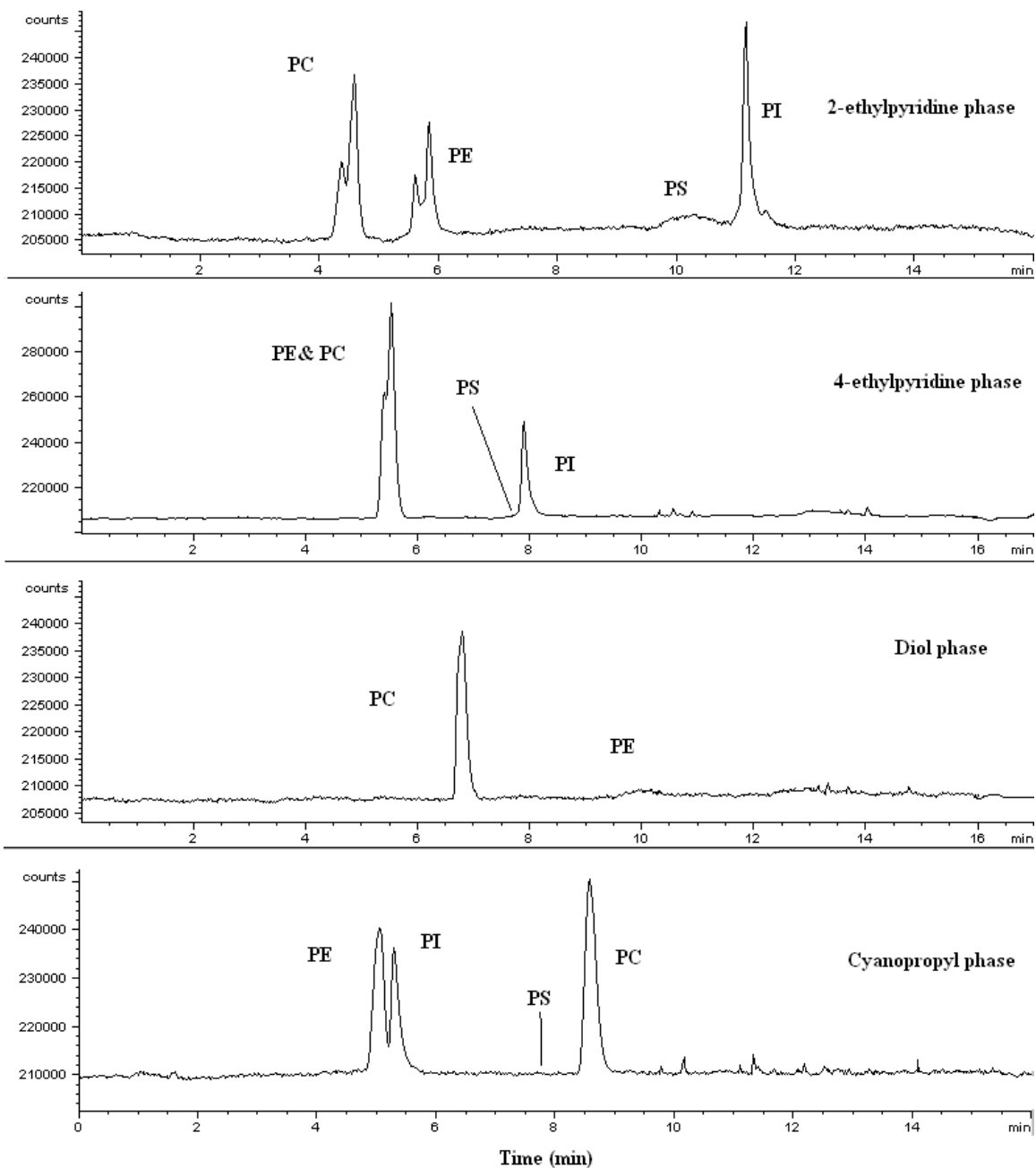


Figure 14. SFC-ELSD of phospholipid mixture with modified CO<sub>2</sub> on the four stationary phases. The modifier consisted of methanol with 5 mM ammonium acetate. Chromatographic conditions: flow rate 2 mL/min, methanol/additive concentration raised from 15% to 55% at 4% per minute and held for 5 minutes at 55%.

other hand, only failed to elute from the diol phase. Retention time of the relatively sharp peak varied from 5.3 min on the cyano phase to 11.2 min on the 2-ethylpyridine phase. In contrast to previous results, the 4-ethylpyridine phase was unable to resolve PC and PE; whereas good peak separation in terms of both polarity and fatty acid content was observed with the 2-ethylpyridine phase. Also, when AA was used as additive, the two anionic phospholipids eluted between PE and PC with some partial overlap. In both cases, PS eluted as a broad peak and showed low detector sensitivity compared to the other three analytes. With the cyano stationary phase, better resolution was observed with both acidic and basic additives relative to the ionic additives. Interestingly, the retention of PI changed with the nature of the additive. It had the longest retention time when TFA was used as the additive, but the shortest elution time in the case of IPA. The resolution of the four components was better when TFA was used although PE and PS were slightly overlapped. A temperature increase of 10°C gave a better resolution between these peaks, but that caused PS and PC to be slightly overlapped instead.

The effect of various additives on the elution of phospholipids from the two isomeric ethylpyridine columns is interesting. The four phospholipids were successfully eluted from both columns within 12 minutes with all additives except TFA. However, it took a longer time for the four phospholipids to elute from the 4-ethylpyridine stationary phase. That can be explained by noting the greater accessibility of active silanol sites on the 4-ethylpyridine stationary phase than on the 2-ethylpyridine phase. The nitrogen on the 4-ethylpyridine column is further removed from the silanol sites which should not allow for as much hydrogen bonding interaction and thus provide a more active chromatographic surface. The elution order of PC and PE was also found to differ on the

two ethylpyridine columns with various additives. Since the PC head group has a larger surface charge on the nitrogen group, a stronger interaction between the positively charged PC head group and active sites on the 4-ethylpyridine column may retain PC longer than PE. Only the zwitterionic analytes (PC and PE) were eluted when TFA was used. This observation no doubt is due to the strong interaction between the TFA-protonated pyridine groups and the negatively charged phosphate group associated with PI and PS. In general, there was no significant difference in peak shape on the two ethylpyridine columns. The 2-ethylpyridine phase, however, showed better resolution of the phospholipids.

### **3.3.5 Effect of Number of Theoretical Plates**

Supercritical fluid mobile phases have lower viscosity and higher diffusivity than conventional liquids which means longer columns (e.g. higher plate counts and mixed stationary phases) can be used than is possible in traditional HPLC. Therefore, coupled 2-ethylpyridine columns (e.g. increase the efficiency) were used to study the effect of increased plate count on PC resolution, **Figure 15**. The resolution ( $R_s$ ) of a single column and a double columns system were 0.63 and 0.91 respectively. Although an improved separation was observed, the resolution between the split peak and main peak remained unsatisfactory.

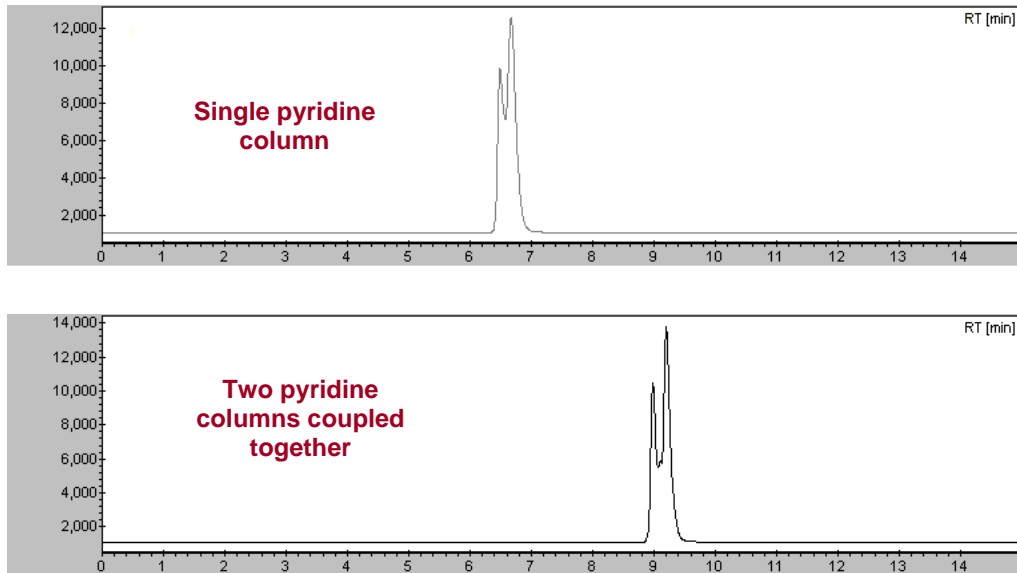


Figure 15. Effect of number of theoretical plates on separation of PC

### 3.4 Conclusion

The feasibility for chromatographic resolution of four phospholipids that differ mainly in polar head group has been demonstrated via packed column SFC. Zwitterionic lipids were easily separated on all four phases with only methanol as the modifier. Separation of the anionic lipids was achieved with additive along with methanol incorporation. Elution time and elution order were greatly influenced by both the nature of the mobile phase additive and the stationary phase functionality. The basic and ionic additives appeared to be more effective than the acidic one since all lipids did not elute with the acidic additive. The 2-ethylpyridine phase appears to have much potential in that not only is separation via head group achieved but simultaneously separation via fatty acid constituent appears highly possible. The use of stacked, 2-ethylpyridine-packed columns in future SFC-MS experiments whereby a greater number of theoretical plates is feasible should enhance this type of information. The best overall normal phase resolution of all four phospholipids was found on the diol column when IPA was used as an additive.

## CHAPTER IV

### The Isolation and Separation of Phospholipids from Soybean Lecithin by Supercritical Fluid Technologies

#### 4.1 Introduction

Crude lecithin is a by-product of the edible vegetable oil refining process. Demand for the phosphatidylcholine(PC)-enriched fraction of lecithin has increased due to its critical role in both the pharmaceutical and industrial field. For example, clinical studies have shown that PC can lower serum cholesterol levels and is used for the treatment of neurological disorders.<sup>35, 36</sup> Lecithin with higher PC content has also been shown to be a better oil-in-water emulsifier than the mixture of phospholipids currently employed for industrial use. Although PC and “lecithin” have been used interchangeably, commercial or crude lecithin is actually a complex mixture of polar and neutral lipids along with other impurities. Neutral lipids consist of triglycerides which are readily soluble in acetone. Nowadays, the major commercial source of PC is soybean lecithin. It contains 35% non-polar lipids and 64.3% polar lipids which comprise 86% phospholipids, 14% glycolipids, and 0.7% moisture.<sup>37</sup> The main components of polar lipids in soybean lecithin and their molecular structures are given in **Table 2**<sup>38, 39</sup> and **Figure 16, 17**. Since PC-content lecithin is in great demand in the pharmaceutical and industrial fields, enrichment or fractionation of phospholipids from crude lecithin is important.

De-oiling of non-polar lipids from lecithin with hexane or acetone is important prior to fractionation of polar lipids (i.e. phospholipids and glycolipids). Various methods have been used for fractionation of the de-oiled lecithin. For example, Sotirhos et al.<sup>40</sup>

Glycolipids	Cerebrosides (CE), Monogalactosyl diglyceride (MGDG), Digalactosyl diglyceride (DGDG), Acylated Steryl Glucoside (ASG)
Phospholipids	Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Phosphatic acid (PA), Lyso-phospholipids

Table 2. Main components of polar lipids from soybean lecithin.<sup>38, 39</sup>



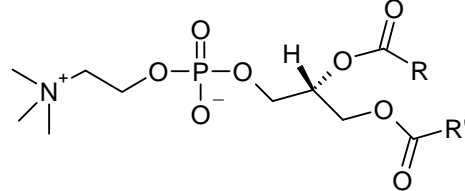
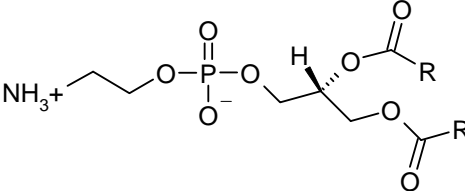
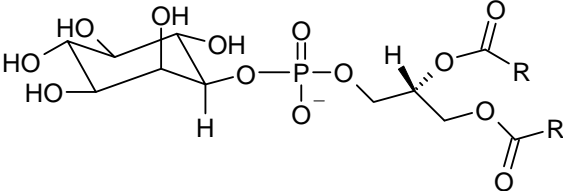
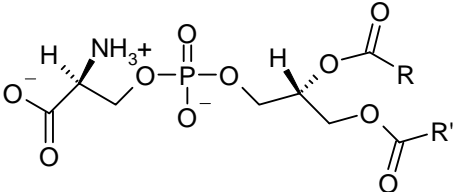
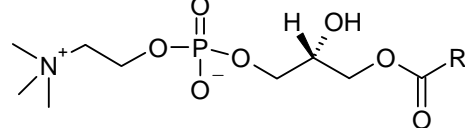
Phosphatidylcholine (PC)	 <p style="text-align: center;"><math>R, R' = (CH_2)_n</math></p>
Phosphatidylethanolamine (PE)	 <p style="text-align: center;"><math>R, R' = (CH_2)_n</math></p>
Phosphatidylinositol (PI)	 <p style="text-align: center;"><math>R, R' = (CH_2)_n</math></p>
Phosphatidylserine (PS)	 <p style="text-align: center;"><math>R, R' = (CH_2)_n</math></p>
Lyso-Phosphatidylcholine (lyso-PC)	 <p style="text-align: center;"><math>R' = (CH_2)_n</math></p>

Figure 16. Molecular structures of phospholipids isolated from soybean lecithin via supercritical fluid fractionation

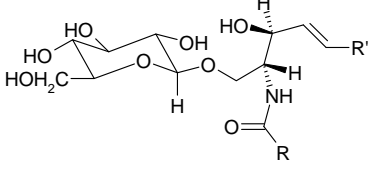
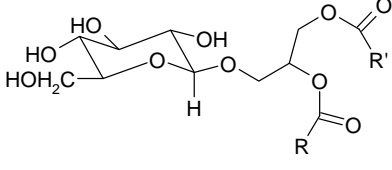
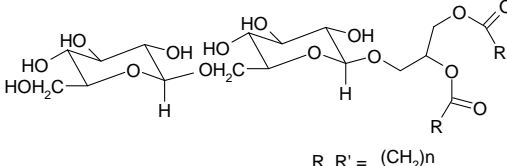
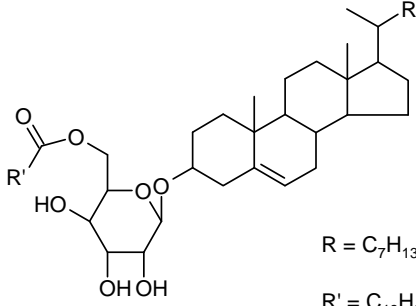
Cerebrosides (CE)	 <p style="text-align: center;"><math>R, R' = (CH_2)_n</math></p>
Monogalactosyl diglyceride (MGDG)	 <p style="text-align: center;"><math>R, R' = (CH_2)_n</math></p>
Digalactosyl diglyceride (DGDG)	 <p style="text-align: center;"><math>R, R' = (CH_2)_n</math></p>
Acylated Steryl Glucoside (ASG)	 <p style="text-align: center;"><math>R = C_7H_{13}</math> <math>R' = C_{18}H_{35} (18:1)</math></p>

Figure 17. Proposed molecular structures of glycolipids isolated from soybean lecithin via supercritical fluid fractionation

used ethanol to extract the polar lipids from soybean lecithin. The crude mixture of phospholipids was then separated into classes by HPLC on a normal phase silica column with hexane-*isopropanol*-water (6:8:0.55 to 6:8:1.5, v/v). Further separation of PC, PE, and PI was achieved by using RP-HPLC on a C<sub>18</sub> column with methanol-acetonitrile-water (8:1:1, v/v). In another study, an enzymatic method was used to increase the PC content in soybean lecithin. After the deoiling step, several phospholipases were tested for their ability to increase PC content by transphosphatidylation in the presence of choline chloride. Soybean lecithin containing about 80% PC could be converted to products having 90% PC. PI was found to be resistant to this enzymatic conversion.<sup>41</sup>

Chemical modification such as acetylation of the phospholipid (PL) head group is another way to indirectly enrich the PC level of the deoiled lecithin matrix. In this regard, soybean lecithin was first de-oiled by using acetone and then acetylated with acetic anhydride. It was found that PE is more reactive towards acetic anhydride than PC. The acetylated PE (N-Acetyl-PE) could then be removed by fractionation with acetone. The PC level was, thus increased without using chromatography.<sup>42,43</sup> Acetylation of the head group in phospholipids derived from soybean lecithin has also been found to increase the solubility of phospholipids in supercritical carbon dioxide, and N-acetylated phosphatidylethanolamine was found to be the most soluble component of the acetylated phospholipids. Since acetylation of the head group involved primarily PE, separation of PE from the nonacetylated PL's by dissolution in acetone or supercritical CO<sub>2</sub> was possible.<sup>44</sup>

Although the chromatographic methods afford reasonable recovery of PC from crude lecithin, long column re-equilibration times remained a problem. Hence,

conventional extraction processes with organic solvents have been used more frequently. For example, 200g of egg yolks were subjected to extraction with 1L of acetonitrile in a Soxhlet extractor at 50°C for approximately an hour. The extract was concentrated by rotary evaporation and crystallized from hexane and acetone giving about 9g PC.<sup>45</sup> Recently, Wang et al. have reported the fractionation of PLs in egg-yolk lecithin<sup>46, 47</sup> and soybean lecithin.<sup>48, 49</sup> In these reports, a comparison of two extraction sequences was studied. 1) The crude lecithin was first extracted with acetone for deoiling, and then the PLs were extracted from the deoiled material with ethanol or 2) crude lecithin was first extracted with ethanol and then acetone was used to remove the oil from the ethanol extract. Better enrichment of PC was found by using the latter sequence, although the cholesterol content of this PC-enriched fraction was higher.

The major disadvantage of these methods is the large volume of solvent and the cost and time for removal of that solvent. In addition, high temperature is usually required during solvent removal which may promote isomerization of extracted components. In this regards, traces of residual hexane in the extract may give rise to a bitter flavor, which would not be suitable for human consumption. Thus, supercritical CO<sub>2</sub> which requires less organic solvent usage affords a reasonable alternative for natural product extractions.

## **4.2 Supercritical Fluid Extraction**

A brief introduction of supercritical fluid (SF) properties was given in Chapter III. Compared to the fixed solvating strength of a liquid organic solvent, the solvating strength of SF can vary by adjusting temperature and pressure. Relatively high diffusivity

and low viscosity of SF can also reduce extraction time. Another advantage of SF is the easy removal of the extracting phase from the extracted product by isothermal pressure reduction, so the labor or time usage for solvent evaporation can be greatly reduced. Carbon dioxide (CO<sub>2</sub>) is the most useful SF because it is non-toxic, non-flammable, relatively inexpensive, and exhibits low critical parameters ( $P_c = 73 \text{ atm}$ ,  $T_c = 31^\circ\text{C}$ ).

Since 1980, there has been rapid development in supercritical fluid extraction (SFE) for sample preparation in the food and pharmaceuticals industry. For example, decaffeination of coffee and tea,<sup>50</sup> cholesterol extraction from butter,<sup>51</sup> removal of nicotine from tobacco,<sup>52</sup> and removal of unsaturated fatty acids from fish oils<sup>51</sup> are some applications of SFE. Since the extraction can be carried out in the absence of light and in an oxygen free environment, photochemical isomerization or oxidative degradation of the extract can be prevented. In addition, the mild extraction conditions using CO<sub>2</sub> as the extraction phase can reduce the possibility of thermal degradation of the extracted product.<sup>53</sup> Due to the non-polar nature of CO<sub>2</sub>, it has solvating power similar to liquid hexane at 40°C and 300-700 atm. It therefore can remove neutral lipids; while the polar lipids remain in the crude lecithin. After the de-oiling process, recovery of economically valuable PLs can be achieved by addition of modifier to the CO<sub>2</sub> extraction phase.

Various reports have described the supercritical fluid extraction of PLs from different sources. PC and PE, for example, have been extracted from inedible eggs, a waste product of the egg industry, using supercritical CO<sub>2</sub> with ethanol as a co-solvent.<sup>54</sup> Dunford and Temelli<sup>55</sup> have shown the extraction of canola meal by using a CO<sub>2</sub>/ 10% ethanol at 70°C and 55.2 MPa. PLs recovery increased from 20.8% to 30.4% when the canola meal was soaked with ethanol before the CO<sub>2</sub>/ ethanol extraction step. Later,

Montanari et al.<sup>56</sup> showed the extraction of PL from soybean flakes. The de-oiled flakes were extracted for 30 min with CO<sub>2</sub>/ 10% ethanol at pressure from 16.6 to 68.9 MPa and temperatures from 60°C to 80°C. A PC enriched fraction was obtained at 19.4 MPa and 80°C. In another paper<sup>27</sup>, these workers used an on-line SFE/SFC system to fractionate de-oiled PL extracts from soybean flakes. The PL enriched fractions were then transferred to a SFC with a neutral alumina column and a mobile phase consisting of 25% ethanol:water (9:1 v/v) in CO<sub>2</sub>. The pressure and temperature were 350 bar and 50°C respectively. Recently, Teberikler et al.<sup>57</sup> selectively extracted PC from de-oiled soybean lecithin using CO<sub>2</sub>/ 10% ethanol at moderate pressure and temperature. A 95% PC-enriched fraction was isolated at 60°C and 20.7 MPa.

The objective of this research was to further enhance the purity of individual phospholipid fractions obtained from crude soybean lecithin by using SFE. Enhanced fractional purity has been achieved by incorporating an adsorbent (i.e. silica gel) with the lecithin matrix. To achieve this goal, neutral lipids were first removed from the crude lecithin by using pure CO<sub>2</sub>. The effect of CO<sub>2</sub> pressure, oven temperature, and modifier percentage on phospholipid fractionation was studied.

## **4.3 Experimental**

### **4.3.1 SFE**

An Isco-Suprex (Lincoln, NE) AutoPrep (AP-44) system was used for all extractions. Prior to each extraction, a 100 mg lecithin sample was mixed with 2 g of Ottawa Sand and placed in a 3 mL stainless steel extraction vessel. The accompanying solid analyte trap was packed with C<sub>18</sub>-silica and after each extraction the analyte was

rinsed from the trap with 5 mL of a mixture of dichloromethane and methanol (50:50). The fixed restrictor was heated to 55°C during the extraction, while the solid phase trap was held at 30°C during both the analyte collection and rinsing steps.

Unless otherwise specified, all samples were initially extracted in the static mode for 2 minutes. The extraction conditions were as follows: CO<sub>2</sub> pressure 460 atm, and extraction temperature 40°C. The dynamic conditions were CO<sub>2</sub> flow rate 2 mL/min, and extraction time was either 30 min or 60 min. The trapped extracts were rinsed into glass vials which were then purged with nitrogen in order to dry the extract. The dried extracts were re-dissolved in 2 mL of methanol for SFC-ELSD and SFC-MS analysis. For the chromatographic conditions, please refer back to Chapter III.

## **4.4 Results and Discussion**

The goal of our study was to fractionate the individual phospholipids inherent to crude soybean lecithin and to assay the extracted fractions by a method that is described in a recent publication via supercritical fluid chromatography with evaporative light scattering and mass spectrometric detection.<sup>25</sup> In order to achieve this goal, neutral, less polar lipids had to be removed.

### **4.4.1 Deoiling of crude soybean lecithin**

Pure non-polar CO<sub>2</sub> with no modifier was used since its polarity is comparable to the polarity of the neutral (i.e. fatty acids) lipids in the crude sample. **Figure 18** shows the SFC/ELSD trace of the soybean extract obtained with CO<sub>2</sub> at 360 atm and 40°C. Surprisingly, most of the neutral lipids were extracted during the first 30 minutes. A

repeat extraction with pure CO<sub>2</sub> for 30 minutes removed any remaining oil in the sample. Extraction for an additional 30 minutes concluded that no more oil was present in the sample. Similar results were obtained at a higher CO<sub>2</sub> pressure (460 atm).

#### **4.4.2 Pressure effect on SFE of PC from de-oiled lecithin**

In order to ascertain the optimized extraction conditions for phospholipids fractionation, independent experiments varying CO<sub>2</sub> pressure, extraction temperature, and modifier percentage were conducted. **Figure 19** shows the SFC/ELSD trace of various extracts produced at different pressures using 5% methanol modified CO<sub>2</sub> at 40°C. No phospholipid was detected at 260 atm. When CO<sub>2</sub> pressure was increased to 360 atm, the resulting extract revealed the presence of phospholipids with PC as the dominant species. Even more PC was extracted at 460 atm. Berger<sup>58</sup> earlier reported that the density of a methanol-CO<sub>2</sub> mixture changes at different pressures. For example, the CO<sub>2</sub> density was stated to increase from 0.825 g/mL to 0.850 g/mL when the pressure increases from 171 atm to 199 atm at 40°C and constant modifier percent (5%). Thus, the additional PC extracted at 460 atm can be explained by the increased CO<sub>2</sub> density (or solvating power) at higher pressure.



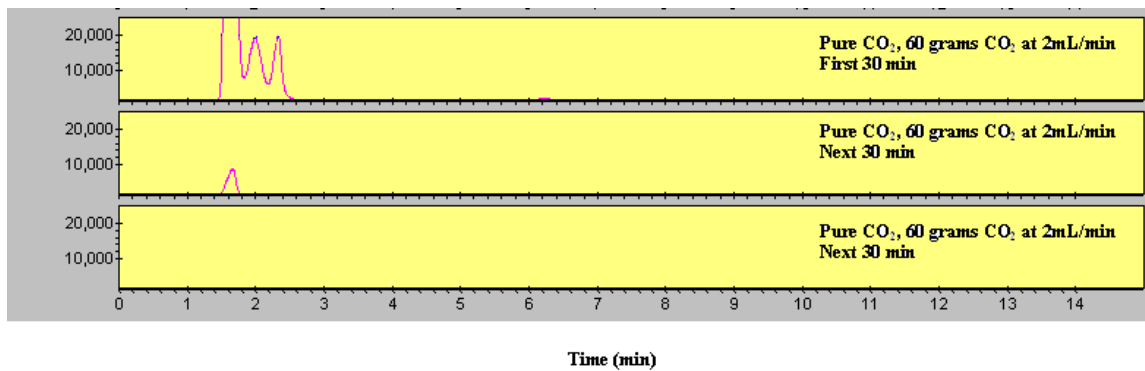


Figure 18. SFC/ELSD trace of soybean lecithin extract with pure CO<sub>2</sub>. Pressure: 360 atm, Temperature: 40°C, mass of soybean lecithin: 110 mg

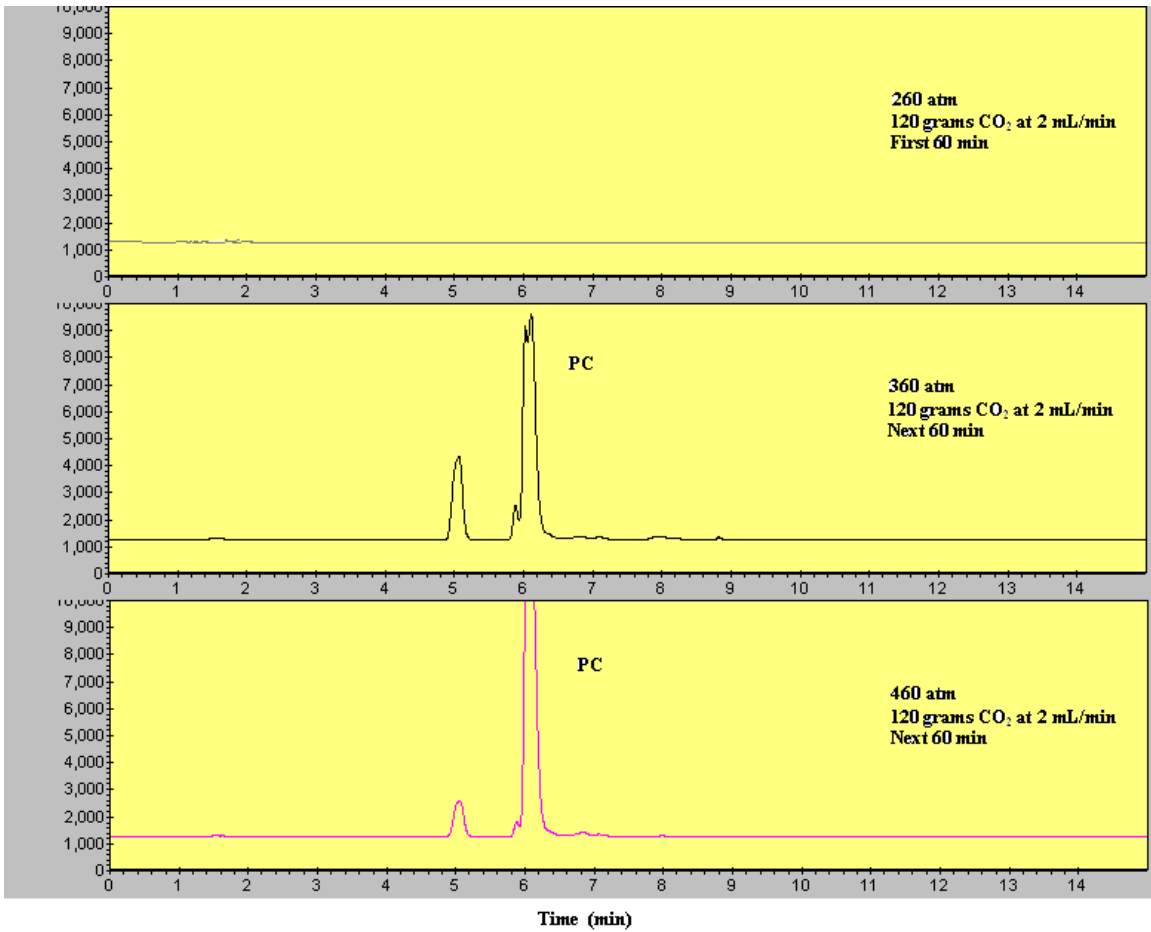


Figure 19. SFC/ELSD trace of soybean lecithin extracts at different pressure using 5% methanol/ CO<sub>2</sub> at 40°C.

#### **4.4.3 Temperature effect on SFE of PC from de-oiled lecithin**

The effect of temperature on the amount of polar lipids extracted was next studied. In general, temperature can give rise to two opposite effects in SFE when other parameters are kept constant. An increase in temperature can improve the extractability of the solute by increasing the vapor pressure of the solute. However, if the extraction is thermodynamically controlled, an increase in temperature will decrease the fluid density and thus decrease analyte extractability. We first tried to extract the deoiled lecithin using 5% modified CO<sub>2</sub> at 60°C and 260 atm. As was expected, no phospholipid was extracted. Surprisingly, similar results were observed with higher pressures (360 and 460 atm) at the same modifier percentage and same temperature. Based upon these results, CO<sub>2</sub> fluid density appears to be a more dominant factor in phospholipid extraction than analyte vapor pressure.

#### **4.4.4 SFE fractionation of PC from de-oiled lecithin**

Based on the previous results, an extraction pressure of 460 atm and a temperature 40°C were employed to study the effect of modifier percentage on extraction of phospholipids from crude soybean lecithin. The extraction time was 60 minutes and the liquid flow rate was 2mL/min. Trap conditions were unchanged from previously stated values, **Table 3. Figures 20 and 21** show the SFC/ ELSD traces of de-oiled soybean lecithin extracts at various modifier percentage. When 5% methanol modified CO<sub>2</sub> was used, a small amount of PC and an even lesser amount of two other unidentified polar lipids (I and II) were extracted. Re-extraction of the same sample with 5% methanol modified CO<sub>2</sub> for an additional 60 minutes revealed (in addition to those previously

Pressure of CO <sub>2</sub>	460 atm
Extraction temperature	40°C
Extraction time	60 minutes
Modified CO <sub>2</sub> flow rate	2 mL/ min
Trap temperature	30°C
Trap	C <sub>18</sub> Silica
Trap rinsing solvent	5 mL (dichloromethane : methanol, 1:1 v/v)

Table 3. Optimized dynamic SFE conditions for removal of PC from soybean lecithin

mentioned) a new known phospholipids (PE) and a new unidentified polar lipid (i.e. III). If the modifier were increased to 10% and 15%, no new chromatographic peaks were seen. Less PC and PE were detected which indicated that most of these phospholipids had been completely removed from the crude lecithin. When the extraction was accomplished with 25% and 35% methanol modified CO<sub>2</sub>, PI was extracted. More PI was detected by extracting for another 60 minutes under the same extraction conditions, but the concentration was very low. The usage of a large percent of modifier (35% methanol) for PI extraction is probably due to its lower solubility in methanol which has been reported previously.<sup>59</sup>

Although the two main phospholipids (PC and PE) were exhaustively extracted from the crude soybean, both appeared to be co-extracted which ran counter to our initial experimental goal of complete fractionation. Silica as a stationary phase has long been used for the separation of phospholipid mixtures.<sup>59</sup> For example, the phospholipids in de-oiled soybean lecithin can be extracted with ethanol and then separated by passing the extract through a column filled with silica gel using methanol/ acetone mixture as the mobile phase. A fraction high in PC content was collected. Based upon these findings, we were interested to see if the addition of silica adsorbent to the crude de-oiled soybean lecithin would afford a complete supercritical fluid fractionation of each phospholipid. To achieve this, silica gel was placed on top of the crude lecithin matrix inside the extraction vessel before extraction, **Figure 22**. The same extraction pressure (460 atm) and temperature (40°C) were used for comparison purpose.

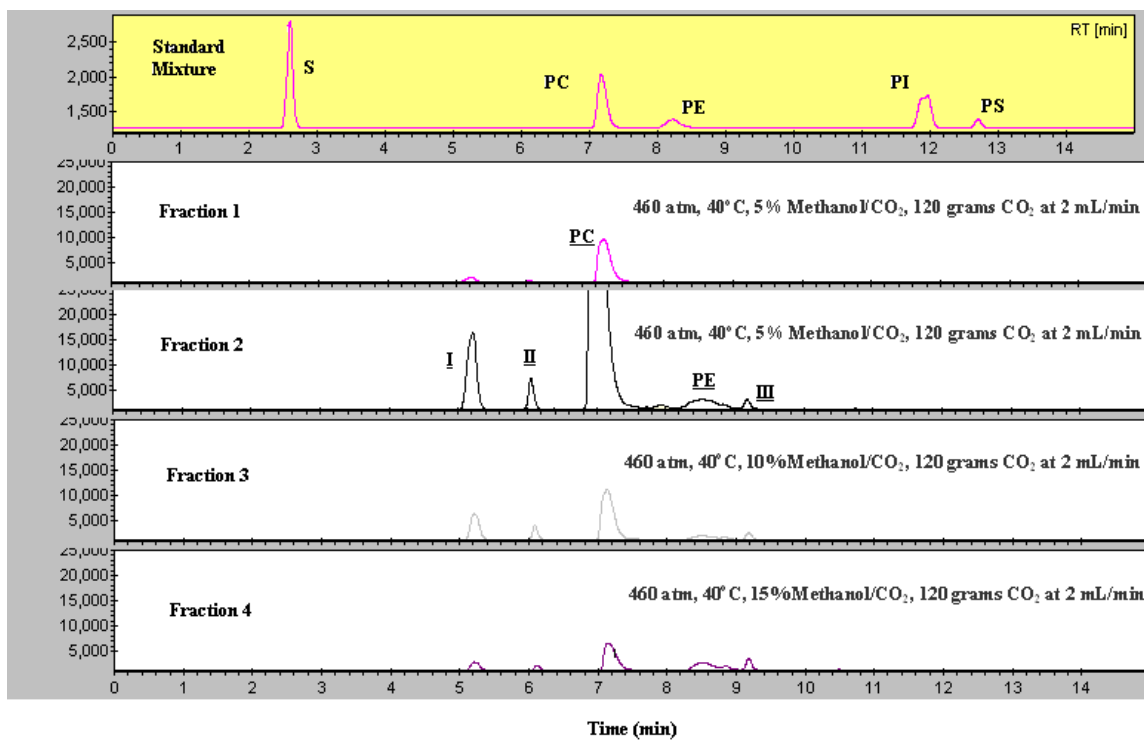


Figure 20. SFC/ ELSD trace of de-oiled soybean lecithin extracts at different modifier percentage without silica adsorbent

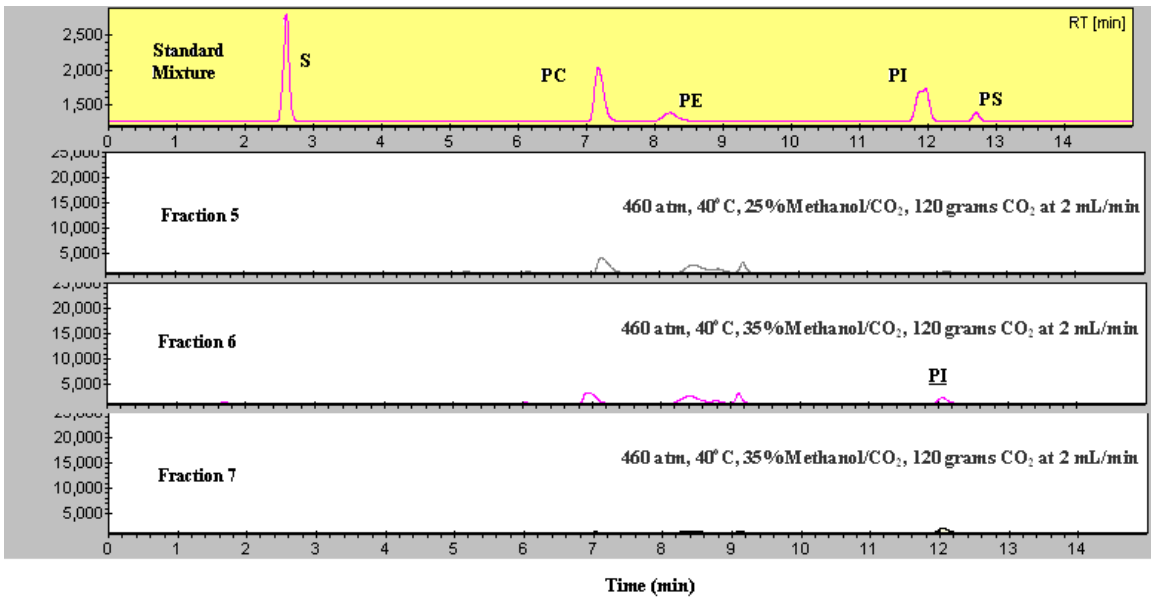


Figure 21. SFC/ ELSD trace of de-oiled soybean lecithin extracts at different modifier percentage without silica adsorbent (cont')

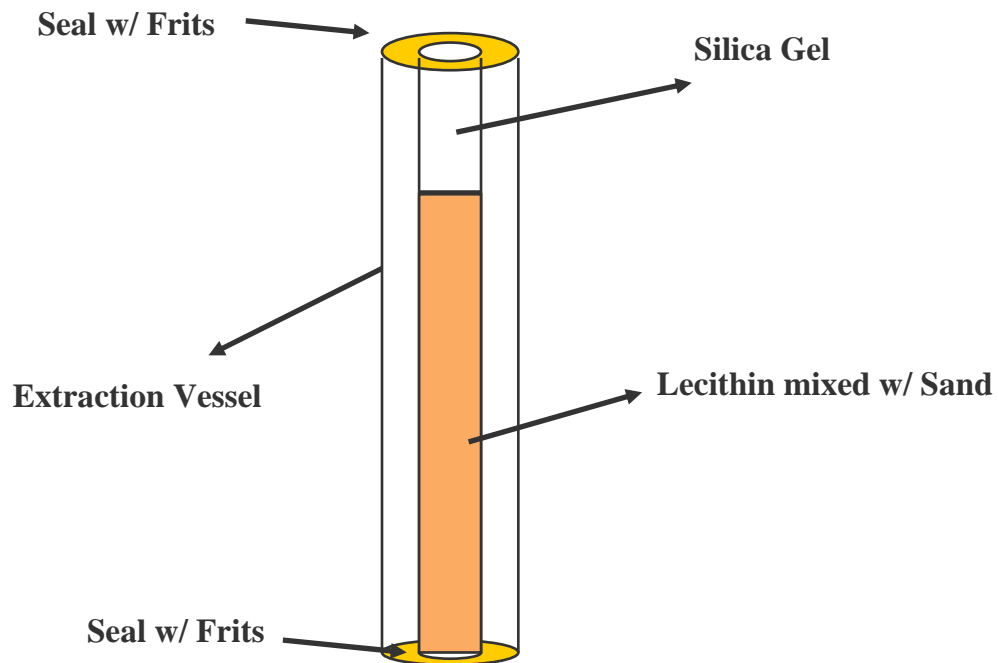


Figure 22. Packed extraction vessel with soybean lecithin and silica gel



#### 4.4.5 Internal adsorbent effect on SFE extraction of de-oiled lecithin

**Figure 23** shows the SFC/ ELSD trace of deoiled soybean lecithin extracts at different modifier percentages with silica as an internal absorbent. Under these conditions, the first four fractions contained none of our phospholipid standards. Fractions #1-3 (which were obtained with 5, 8, and 10% methanol modifier) suggested that components I and II were co-extracted. The third unidentified polar lipid appeared in fraction #4 after extraction with 15% modifier. Extending the extraction with 20% methanol modified CO<sub>2</sub>, the resulting SFC/ELSD trace showed the presence of a relatively large amount of PE eluting over a period of approximately one minute and residual polar lipid III. PC, the suspected phospholipid with the largest concentration, started to be extracted with residual PE when extraction was accomplished with 25% methanol modified CO<sub>2</sub>. The intensity of the PC peak increased markedly when a higher modifier percentage (30%) was used. Large amounts of pure PC were collected in a second and third extraction with 30% methanol modified CO<sub>2</sub> (i.e. fractions #8 and #9). Extending the extraction with the same modifier percentage depleted most of the PC but at the same time revealed the presence of a small amount of PI based upon retention time comparison with a standard. It is important to note that incorporation of silica with the crude lecithin matrix afforded almost pure PE and PC fractions; whereas without silica the PE and PC fractions were less pure.

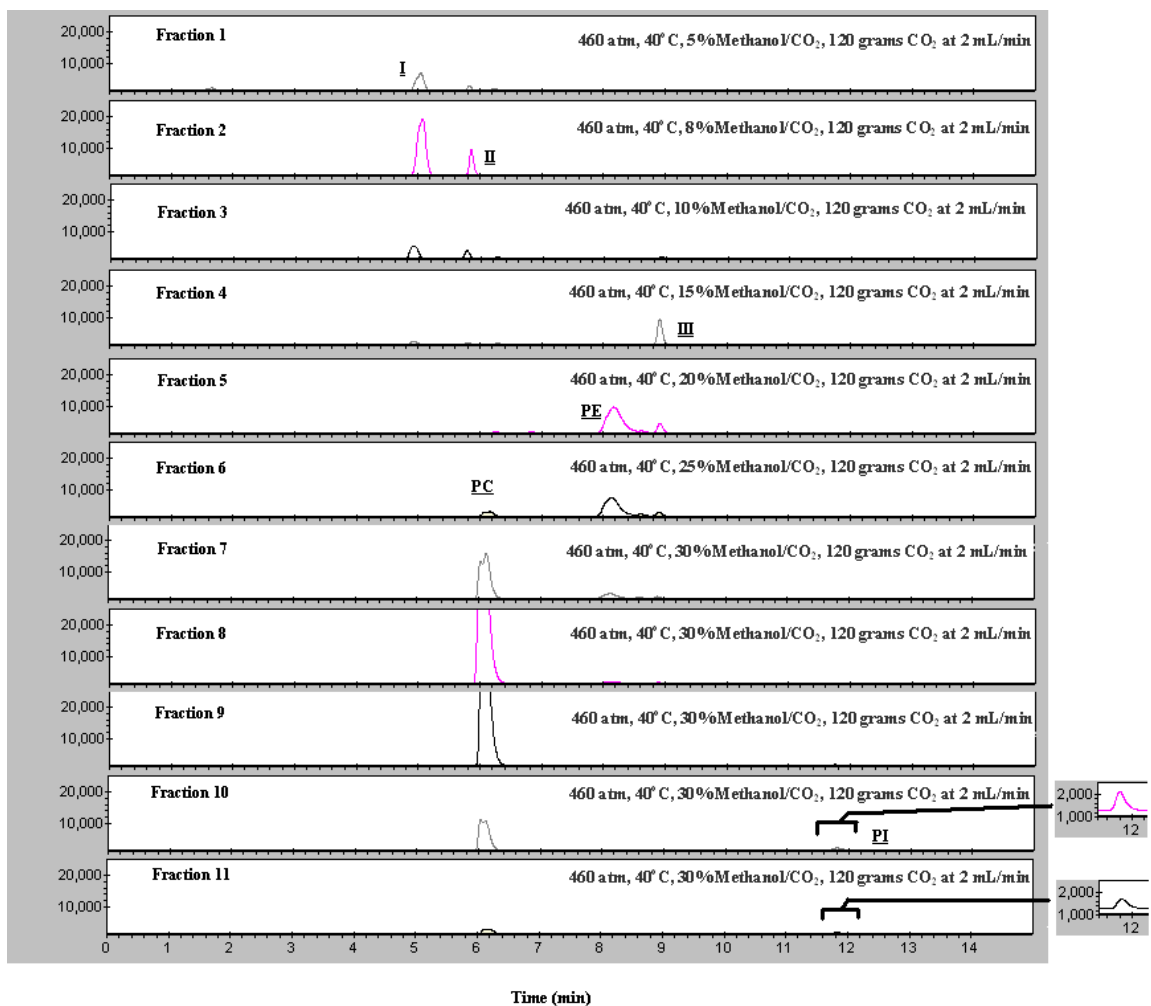


Figure 23. SFC/ELSD trace of soybean lecithin extracts at different modifier percentage with silica as the internal adsorbent

#### 4.4.6 Identification of the other polar lipids

The components in fractions #2 and #4 from **Figure 23** could not be identified based upon retention time comparison with our five standards. To gain a better perspective on the components giving rise to the chromatographic peaks, mass spectrometric detection was directly coupled with packed column SFC. Mass spectra obtained via SFC/APCI-MS of the components (I, II, and III) eluting from the diol phase in fractions #2 and #4 are shown in **Figures 24 26, and 27**. The unidentified polar lipid I in fraction #2 (retention time = 5.1 min) gave a prominent ion at  $m/z$  898.9, **Figure 24**. The mass difference between the prominent ion and the very weak fragment ion ( $m/z$  456.5) is 442 mass units. The large difference in mass units suggests that (if the component is a phospholipid) a head group with high molecular mass was lost in the fragmentation. It seems unlikely for any phospholipid found in soybean lecithin to carry such a large head group, **Figures 16 and 17**. Thus, we speculate that the prominent ion is due to the IPA adduct of acylated sterol glucoside where  $R = C_7H_{15}$  and  $R' = C_{18}H_{33}$  (18:2). The fragment ion may be due to  $[C_{25}H_{44}O_7]^+$ .

The unidentified component II in fraction #2, surprisingly, exhibited a shoulder upon expansion of the chromatogram. Thus, the SFC/MS trace of component II (retention time = 5.8 min) in fraction #2 (from **Figure 23**) reveals two partially separated peaks in the  $m/z$  range 480 to 800, **Figure 25**. The front peak gives a major ion with  $m/z$  636.6, **Figure 26A**. A difference ( $m/z$  636 - 622) of 14 mass units between the two most intense peaks can be explained by the loss of one methylene carbon on one of the fatty acid chains. The mass difference between the parent ion peak and the fragment ion peak ( $m/z$  456) suggested that the mass of the head group was 180 Da. On the other hand, a

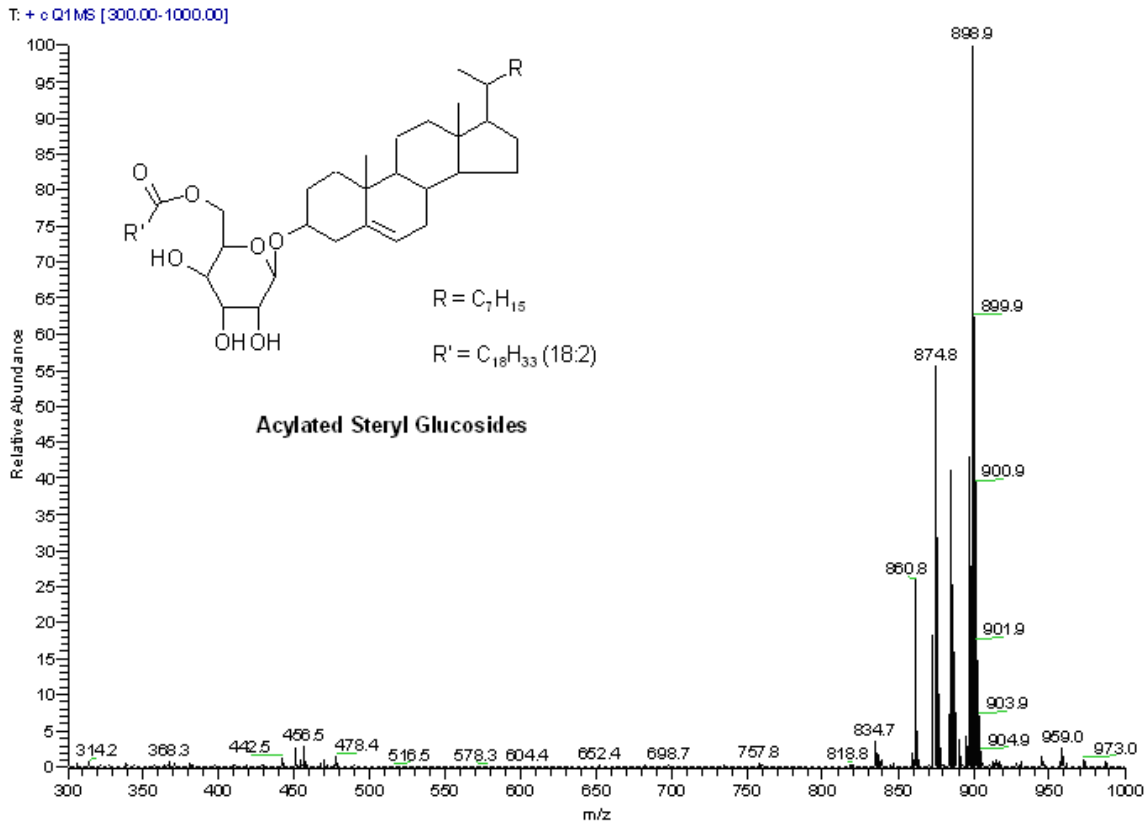


Figure 24. Mass spectrum of polar lipid I (retention time = 5.1min) eluting on the diol column with 8% methanol as the modifier (Fraction 2 from Figure 23)

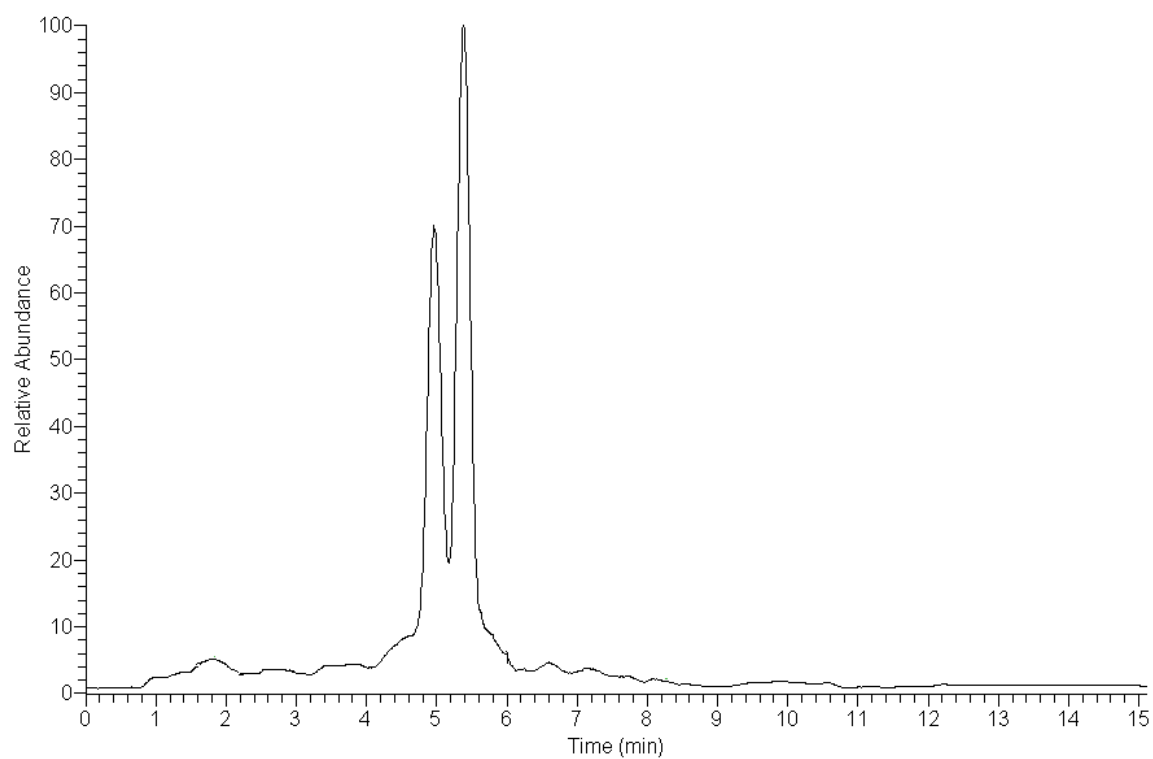


Figure 25. SFC/UV trace of polar lipid II (retention time = 5.8 min) in fraction #2 from Figure 23 ( $m/z$  range: 480-800)

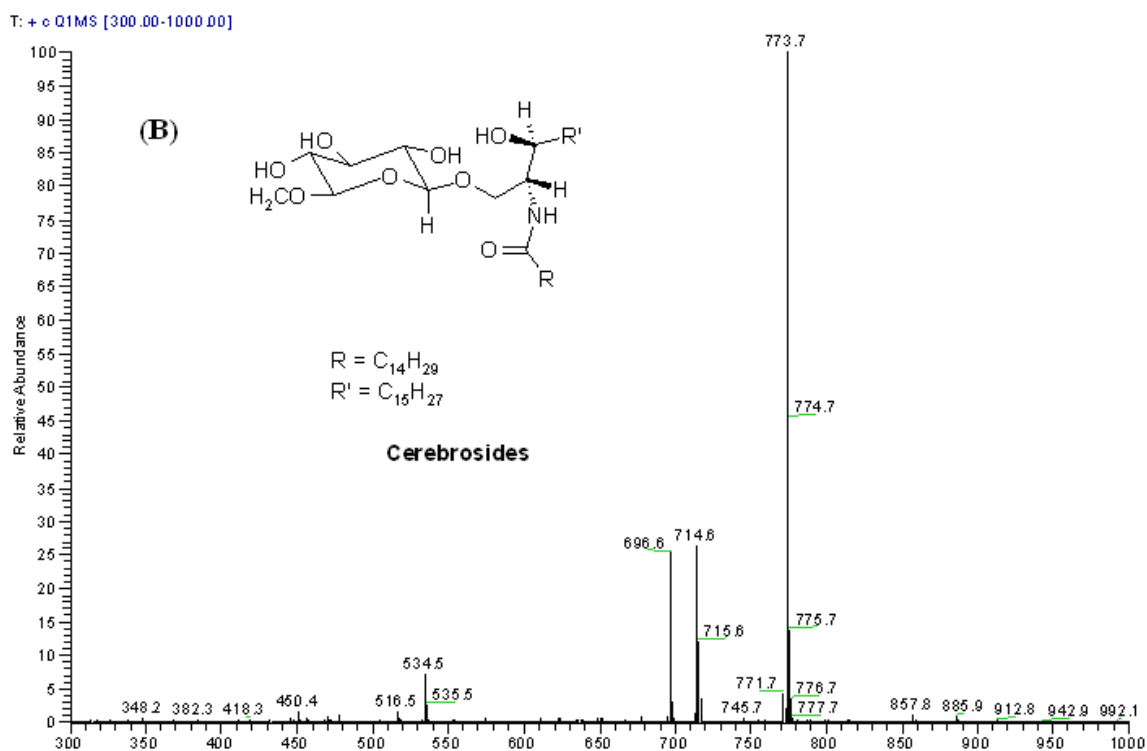
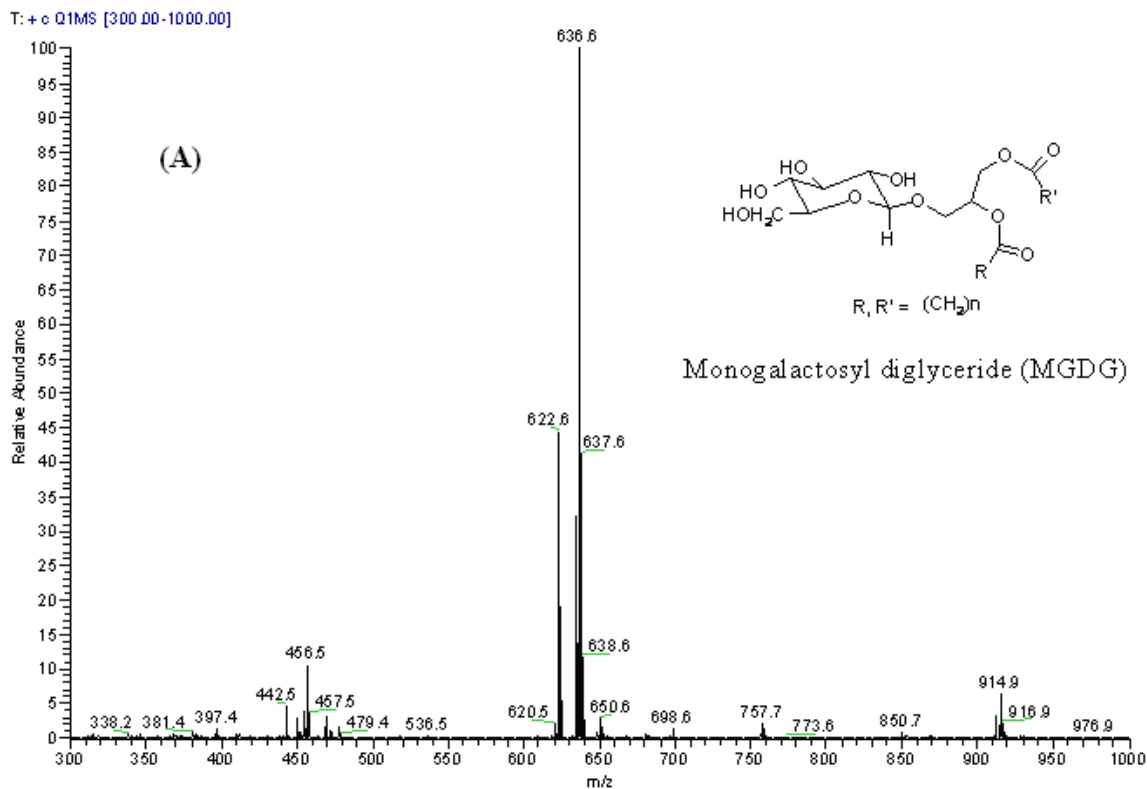


Figure 26. Mass spectra of polar lipid II eluting on the diol column with Fraction #2 from Figure 23. A = front peak; B = shoulder peak.

different mass spectrum was found for the shoulder peak, Figure 10B. The parent ion peak is believed to be the protonated IPA adduct parent ion  $m/z$  773. A fragment ion peak with  $m/z$  714 appeared which we believe is due to the loss of the IPA adduct (59 Da). A fragment ion with  $m/z$  534 gives a mass difference of 180 Da compared to the non-adduct peak. The same mass difference (180 Da) for both the main peak and shoulder suggested that they have the same head group. None of the phospholipids from **Figure 16** has a head group with 180 Da, so we conclude that these two peaks are either MGDG or cerebrosides.

The mass spectrum of the component III in fraction #4 (from **Figure 23**) is shown in **Figure 27**. Surprisingly, no additional component other than the prominent ion at  $m/z$  402 was found. Since the head groups from either glycolipids or phospholipids are all greater than 100 mass units. The fragment ions after loss of a head group were not expected to appear in the scan range. On the other hand, the number of carbons in a fatty acid for both glycolipids and phospholipids is usually 14 -22. With such a low  $m/z$  ratio (i.e. 402) for the parent ion, it is suggested that this molecule only contains one fatty acid chain. Thus, we concluded this fraction may be a lyso-phospholipids such as Lyso-PC.

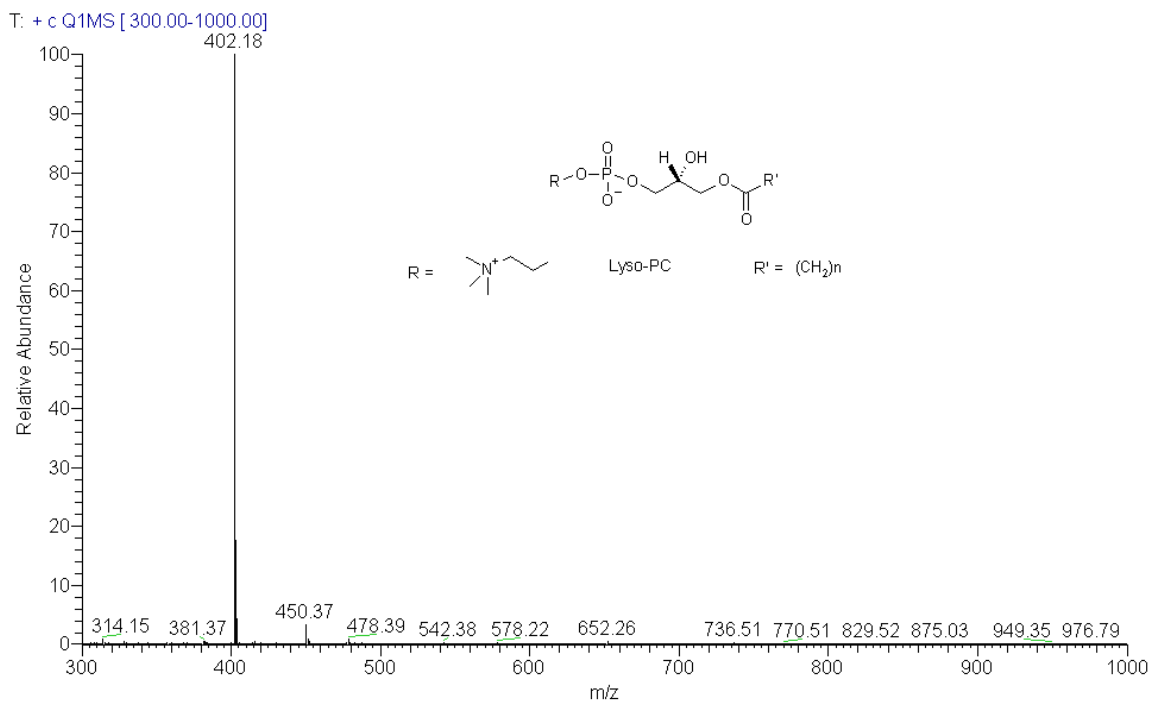


Figure 27. Mass spectrum of the polar lipid III (retention time = 8.9 min) eluting on the diol column with fraction #4 (from Figure 23) as the modifier.



#### **4.5 Conclusion**

The fractionation of crude soybean lecithin has been demonstrated via SFE. Neutral lipids (oil) can be removed from the crude sample using pure CO<sub>2</sub>. Partial fractionation of PE and nearly pure fractionated PC were obtained by varying the modifier concentration in the extraction fluid at constant pressure and temperature. Also, the presence of silica gel inside the extraction vessel was the key for getting these pure fractions. On the other hand, we were able to show that increased pressure and modifier percent had a positive effect on the selective extraction of PC, while increased temperature had a negative effect. A total of six components were isolated from crude soybean lecithin.

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