# The HPLC Preparative Scale-Up of Soybean Phospholipids

Application

**Food and Flavors**


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## Abstract

**Classes of major soybean phospholipids can be separated from a crude mixture, under both analytical and preparative conditions, using a matched set of columns, normal phase conditions, and a small amount of volatile salt added to the mobile phase. At higher concentrations of phosphatidylcholine, however, two distinct peaks, whose areas changed with the mass injected, were observed. These two peaks were determined by mass spectrometry to be the same or, at least, very similar.**


## Introduction

Phospholipids have an important role in animal and plant systems since they are commonly found in cellular and intracellular membranes where they fulfill the role of primary structural components. Phospholipids are also widely used in the food, cosmetic and industrial manufacturing industries and account for 80% of worldwide plant oil production. The structure of a typical phospholipid is depicted in Figure 1. Phospholipids are the major components of soybean lipids and are often desired in a pure form as standards for chromatography and other studies. Preparative amounts are therefore needed and HPLC is often the first choice for this purpose. This application note describes both the development of an analytical normal phase HPLC separation of the most common phospholipids found in crude soy lecithin extracts and the possibility of performing a linear scale-up to preparative columns filled with the same packing.

## Properties of Phospholipids

From a chromatographic viewpoint, phospholipids are in a class of polar lipids and are amphipathic. They contain an ionic portion due to the phosphate moiety and they may contain additional hydrophilic or ionic portions due to the presence of choline, serine, ethanolamine, myo-inositol and/or glycerol functionality. All phospholipids contain a hydrophobic portion due to the presence of the fatty acid moieties and their complexity results from the two fatty acids on the 1,2-diacylglycerol portion. These fatty acid moieties may be the same or different. The main phospholipids present in soybeans along with their abbreviations are shown in Table 1. In a typical soybean extract, about half of the extractables are phospholipids with neutral lipids, mainly triglycerides, and carbohydrates making up the remainder. Since phospholipids are generally very complex and difficult to prepare in a pure form, most commercially prepared mixtures contain varying amounts of these extractables.

Phospholipids generally have low water solubility and can be dissolved in nonpolar solvents. Since their ultraviolet (UV) absorbance arises only from the double bond in the fatty acid portion, they provide low response with UV detectors. Refractive index-, evaporative light scattering- or mass spectrometric-detectors are, therefore, more frequently used.

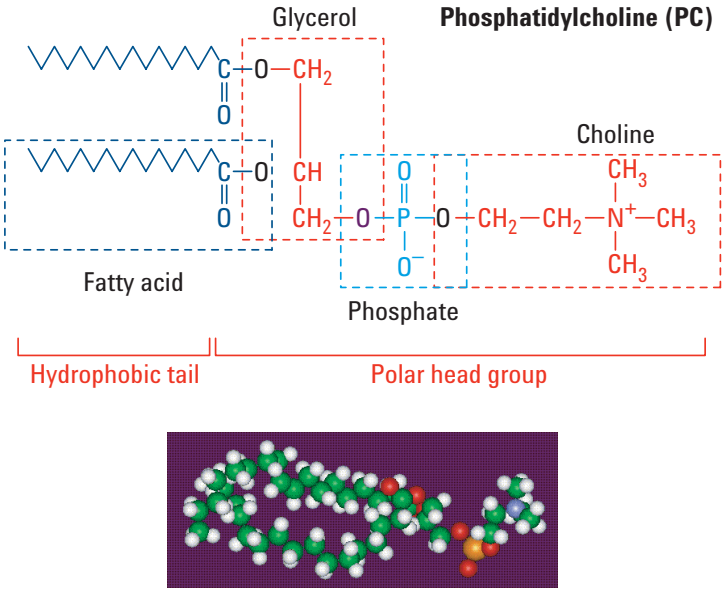


**Figure 1. Structure of typical phospholipid.**

**Table 1. Important Phospholipids in Commercial Plant Oil Extracts**

| Phospholipid | Abbreviation |
|---|---|
| Phosphatidylethanolamine | PE |
| Phosphatidylserine | PS |
| Phosphatidylinositol | PI |
| Phosphatidylcholine | PC |

## Chromatographic Separation of Phospholipids

Because of their chemical properties, phospholipids may be separated by both normal phase and reversed-phase chromatography. For preparative scale-up from analytical dimensions, the use of silica normal phase chromatography has advantages when compared to reverse phase chromatography (RPC): 1) The mobile phases are more volatile; thus, collected fractions can easily be recovered. 2) The viscosity of the common normal phase solvents is low, allowing higher flow rates to be employed, thereby improving throughput. 3) Using the adsorption mechanism, class separations of major phospholipids are easier to perform compared to RPC. 4) In normal phase work, since phospholipids absorb poorly in the UV, detection is better accomplished using the evaporative light scattering detector (ELSD) that allows gradient systems to be employed and provides excellent sensitivity in organic mobile phases.

Row and Kang demonstrated successful separation of phospholipids using normal phase chromatography but they operated at 208- or 210-nm in the UV [1]. In their work, they used a ternary gradient system. However, since our preparative chromatograph employed a binary pumping system, we had to adapt our gradient conditions for both the analytical and preparative chromatography accordingly.

## Evaporative Light Scattering as a Detection Principle

The ELSD detects compounds based somewhat on a compound's mass, and not on its spectroscopic characteristics. It is nearly universal and it responds to all compounds as long as they are less volatile than the mobile phase. As seen in Figure 2, the chromatographic flow stream enters the heated nebulizer and is nebulized using a stream of dry nitrogen; the resultant mist containing the analyte is sequentially directed through the heated evaporator where is loses the solvent and becomes a particle, which is then directed through the polychromatic light beam. The particles cause photon scattering as they traverse the path of the light beam. The signal generated is proportional to the analyte particle size present. The solvent is evaporated and does not interfere with the analyte measurement. The presence or absence of chromophores, fluorophores, or electroactive groups has no impact on the signal. The ELSD can be optimized for analyte sensitivity and baseline stability by adjustment and optimization of evaporation and nebulization temperatures. For preparative applications, the upper flow rate capability of the ELSD is 4 mL/min, and thus requires post-column splitting of the chromatographic effluent.

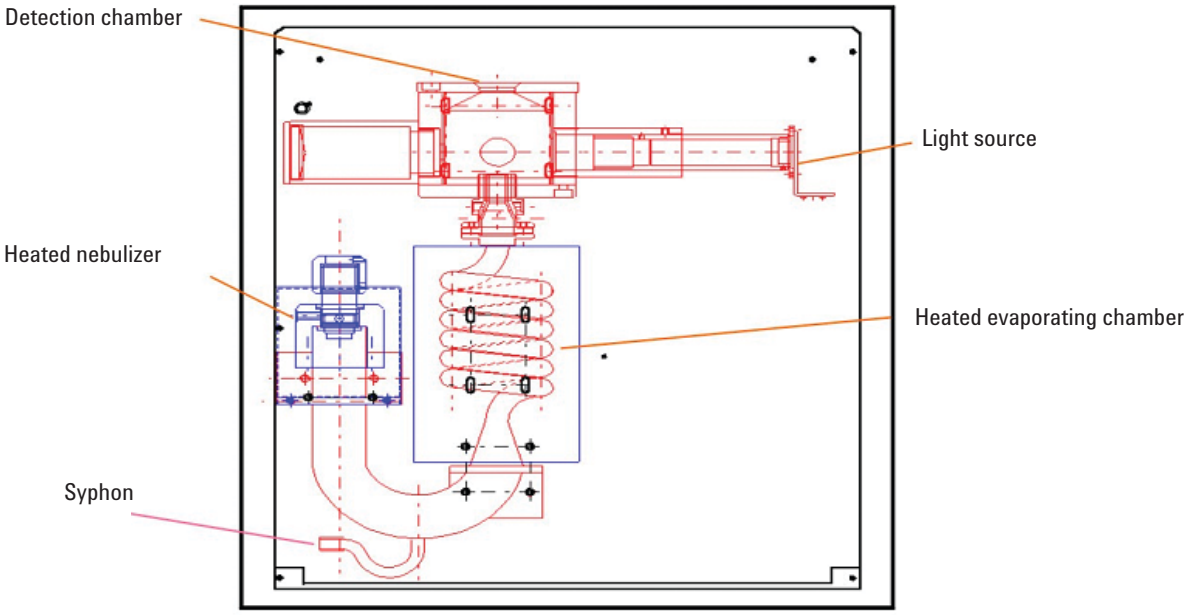


**Figure 2. Schematic of ESA Chromachem ELSD.**

## Scaling Up from Analytical to Preparative Chromatography

In scaling up to a preparative separation, it is recommended that initial method development use an analytical-sized column. Since flow rates and sample injection sizes are lower than for prep, solvent costs are lowered and less sample is used, which is important when sample mass is limited or for samples that are relatively expensive. Once the separation is optimized using an analytical (or scalar) column, linear scale-up to a preparative column of the same materials is easily achieved.

Initially, we adjusted our gradient to approximate the one used by Row and Kang [1]. Using 2.5% methanol (MeOH), 2.5% isopropanol (IPA), and 95% hexane as solvent A, and using 40% IPA and 60% MeOH as solvent B, we achieved a successful separation of the four standard phospholipids but found that our retention times often varied from run to run. In earlier normal phase work [2], Christie found that the addition of a small amount of ionic material to the most polar solvent, in his case, isopropanol-water, gave better resolution and extended the column life. However, several of his suggested ionic additives were nonvolatile. Since the ELSD requires the use of a volatile mobile phase, we chose a low concentration of ammonium acetate (10-mM) but added it to both mobile phase constituents. Higher concentrations could be used to give improved retention behavior, but at >10-mM levels there is danger of salt precipitation, and should therefore be avoided.

Using this hexane-methanol-isopropanol-ammonium acetate ($NH_4Ac$) gradient system allowed us to fine tune the separation, especially relative to isocratic methods of the past. The final set of chromatographic conditions are depicted in Table 2. Figure 3 shows the optimized separation on a silica-gel scalar column as a function of the injected amount of phospholipid standards: PE, PS, PI, and PC. The small amount of $NH_4Ac$, a volatile salt, gave much better retention behavior for PC, a zwitterion with a quaternary amine functionality (see Figure 1). The salt addition also assured minimal retention variation with load at low injection mass. Surprisingly, at higher injection masses, we observed two peaks for PC.

**Table 2. Final Set of Experimental Conditions**

| | | |
|---|---|---|
| **Chromatography** | | |
| **Analytical runs:** | | |
| Column: | Agilent Prep SIL Scalar Column, 4.6 × 150 mm, 10 µm | |
| Mobile phase: | A = 95:2.5:2.5% hexane-isopropanol-methanol<br>B = 40:60% isopropanol-methanol<br>Both solvents contain 10-mM ammonium acetate | |
| Flow rate: | 1.5 mL/min | |
| Gradient: | Time, min | %B |
| | 0 | 0 |
| | 20 | 18.7 |
| | 20.2 | 100 |
| | 25 | 100 |
| | 25.1 | 0 |
| Temperature: | Ambient | |
| **Preparative runs:** | | |
| Column: | Agilent Prep SIL Column, 21.2- × 150 mm, 10 µm | |
| Mobile phase: | Same as above | |
| Flow rate: | 31.9 mL/min | |
| Gradient: | Same as above | |
| Temperature: | Ambient | |
| **Detection** | | |
| Detector: | Chromachem ELSD, (ESA, Cambridge, MA) | |
| Temp. nebulizer: | 45 °C | |
| Temp. evaporator: | 65 °C | |
| Filter: | High | |
| Gas pressure: | 23 psi | |
| Attenuation: | 1 | |
| **Sample** | | |
| Dissolved in: | $CHCl_3$:Hexane 2:1 | |
| Analytical: | 5 mg/mL | |
| Prep: | 25 mg/mL | |

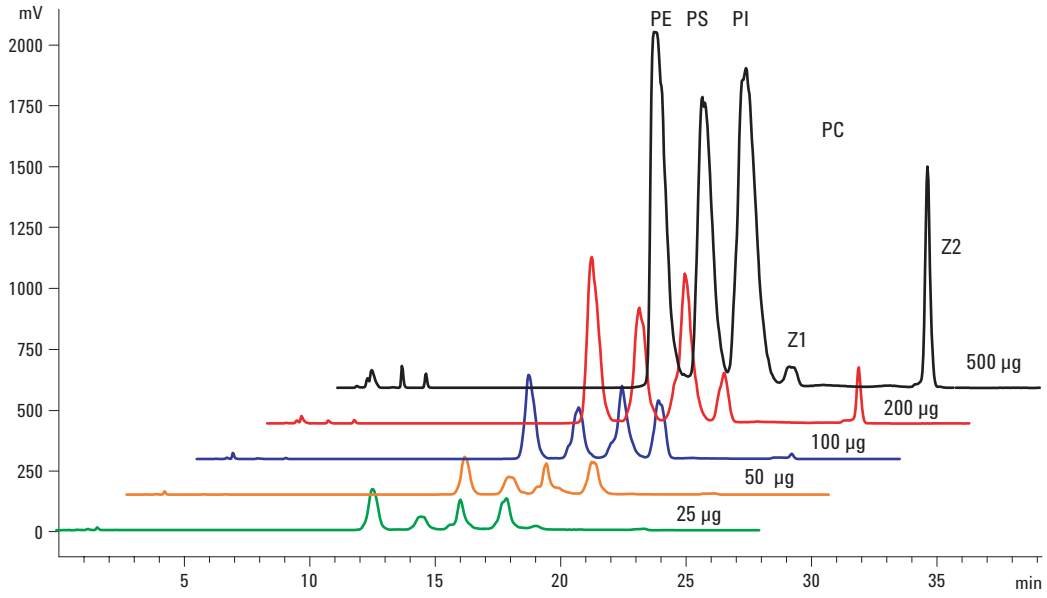


**Figure 3. Agilent prep 4.6 × 150 mm, 10 µm – Mixture of soy phospholipid standards. - Loadability.**

## Study of PC Elution Behavior

As stated above, at higher injection masses, in Figure 3, we observed two peaks for PC, one eluting at a 16.1 minute (PC-Z1) and the second 21.8 minutes (PC-Z2). As sample mass increased (above 75 µg on the 4.6 × 150-mm column), the latter peak increased while the former decreased but the total area increased as expected. As can be seen in Figure 4, with increasing sample mass, the peak areas for all of the phospholipids, including PC, increased. Typical of the ELSD, response was nonlinear, especially at the lowest concentrations (less than 50 µg); however, since the double peak phenomenon occurred with PC only, we suspected that it had something to do with the zwitterion functionality. We, therefore, investigated this aspect more thoroughly.

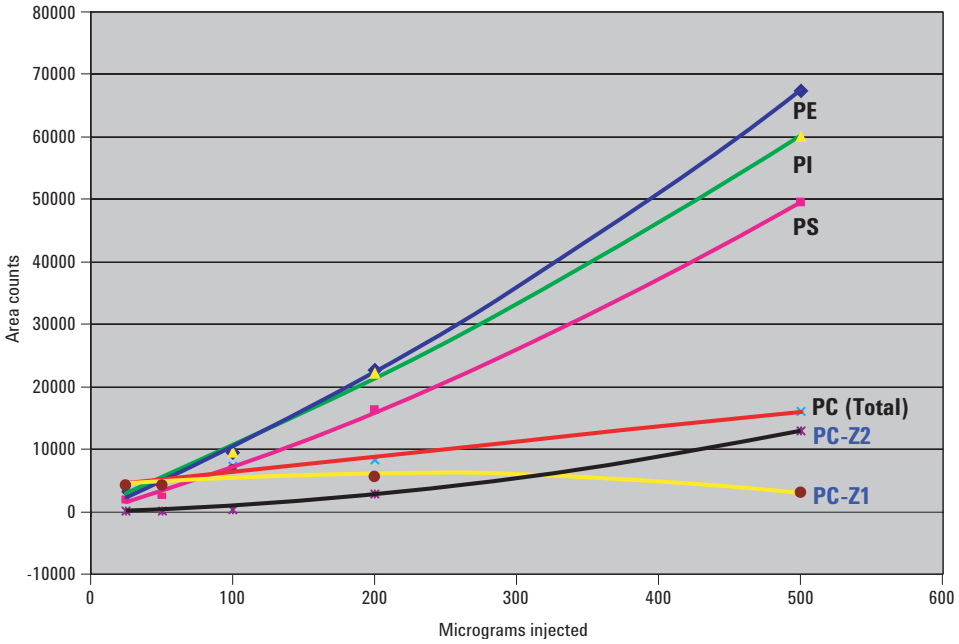


**Figure 4. Area counts for ELSD versus micrograms injected (analytical column, 4.6 × 150 mm).**

Using liquid chromatography/mass spectrometry (LC/MS), we attempted to determine the structure of the two peaks observed with PC. Since in normal phase chromatography ionization could be a problem, we first investigated MS ionization approaches. MS sensitivity is related to the formation of stable ions in the source. Using electrospray ionization, a minimal signal was observed; so, we resorted to atmospheric pressure photometric ionization (APPI), a more useful ionization technique for neutral organic compounds. Since PC is zwitterionic, its natural ionic character allowed it to be detected in either positive or negative APPI. To enhance sensitivity, acetone and toluene dopants were added post-column as a 50:50 mixture. From other studies [3] it is known that soybean PC consists of multiple phospholipid entities with varying fatty acid content. We were able to assign certain *m/z* values to individual PCs on the basis of the fatty acid portion of the 1,2-diacylglycerol functionality. Figure 5 shows the raw, unsmoothed mass spectral data of the two PC peaks (PC-Z1 and PC-Z2).

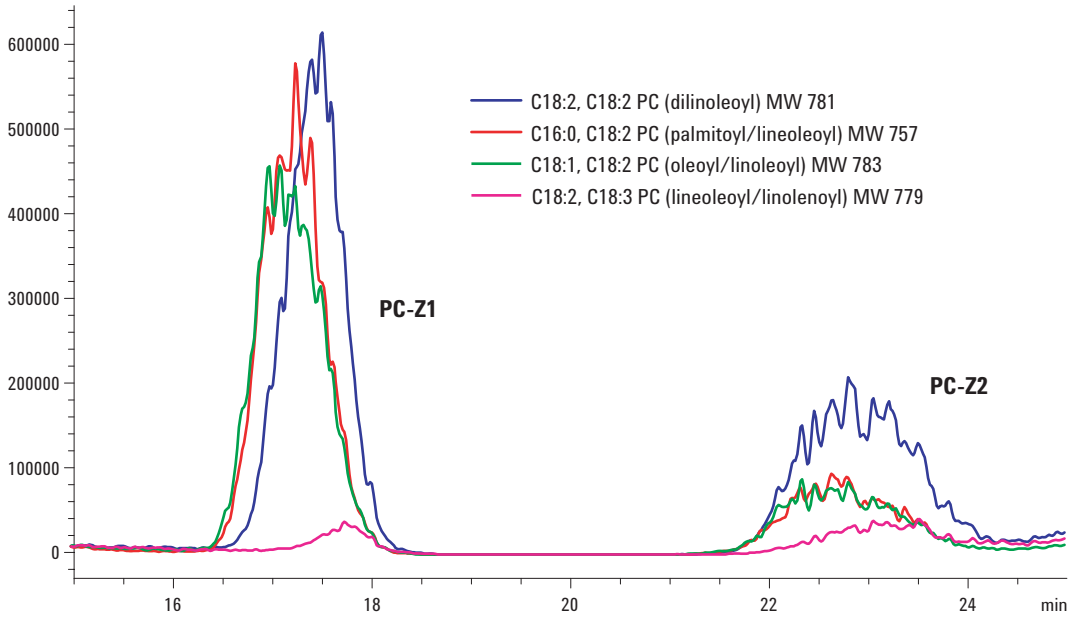


**Figure 5. Overlay of the most common phosphatidylcholine (lecithin) (PC) species present in crude soy lecithin – Extracted ions from LC/MS (unsmoothed data).**

Clearly, one can determine that there was partial resolution by the type of fatty acid moiety present. Note that the same species are present in both peaks PC-Z1 and PC-Z2. Furthermore, as can be seen in Table 3, if one explores the peak height ratios of the individual PCs within the PC-Z1 and PC-Z2 umbrellas, they are similar enough to deduce that the peaks are the same. In addition, based on fatty acid ratio studies of the individual PCs in purified PC as determined by gas chromatography [3], for a first approximation, the fatty acid composition of the PCs depicted in Figure 5 are similar. This observation also led us to believe that these two PC peaks were the same compound (Table 3). Our speculation is that the two peaks are a result of the competitive silanol binding between the positively charged ammonium ion in the mobile phase modifier and the positively charged portion of PC. At low PC concentrations, the ammonium binds more to the silica silanol groups and the concentration of unbound PC-Z1 is insufficient to displace it. As the concentration of PC increases, however, some of the PC adsorbs to the silanol sites in competition with the ammonium ion. This strongly held PC (identified as PC-Z2) elutes only at very high concentrations of IPA-MeOH. As larger amounts of crude soy lecithin are injected, the available PC increases so that more binds to the surface, resulting in an increase in its elution peak, PC-Z2. If one estimates the total amount of available silanol groups (assuming 8-µmoles/$m^2$ for a totally hydroxylated surface) on the surface of the packing inside of the column, the number of µmoles of PC potentially bound to the surface at saturation (around 600 µmoles) is consistent with the calculated level of binding sites available.

## Preparative Studies of Crude Soy Lecithin

Figure 6 depicts the preparative scale-up of the same phospholipid standard to a 21.2-mm × 150-mm column packed with the same material as the scalar column. The flow rate was increased to 31.88 mL/min to achieve the same linear velocity and separation time as under the analytical conditions. The same relative amounts injected onto the preparative column allowed as much as 20 mg of total phospholipid before the ELSD detector showed signs of overload as noted in the upper chromatogram. Note that the same PC-Z1 and PC-Z2 peaks can be observed in the chromatogram of Figure 6.

**Table 3. Table of Relative Extracted Ion Currents**

| Fatty acid composition | Weight,% typical soybean* | Relative weight.% | Relative height PC-Z1 | Relative height PC-Z2 |
|---|---|---|---|---|
| C18:2/ C18:2 PC (dilinoleoyl) | 47.5 | 50 | 39 | 45 |
| C16:0/ C18:2 PC (palmitoyl/ lineoleoyl) | 30.0 | 31 | 31 | 24 |
| C18:1/ C18:2 PC (oleoyl/ linoleoyl) | 10.5 | 13 | 28 | 21 |
| C18:2/ C18:3 PC (lineoleoyl/ linolenoyl) | 6.1 | 6 | 3 | 9 |

*B. Herslof, U. Olsson, and P. Tingvall in I. Hanin and G. Pepeu,Eds. (1990). Phospholipids: Biochemical, Pharmaceutical, and Analytical Considerations, Plenum Press, New York, pp. 295–298.

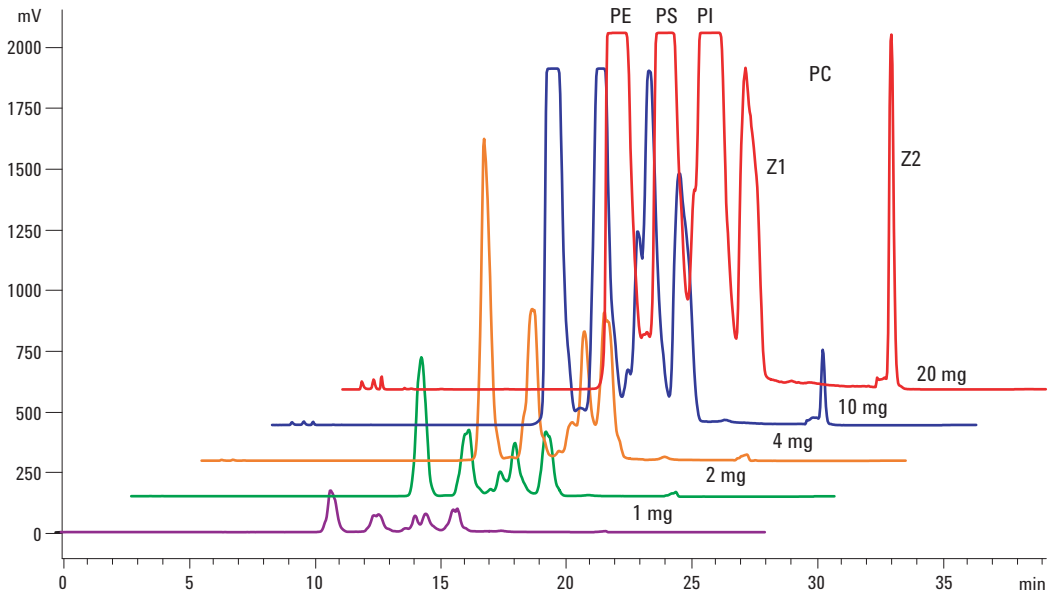


**Figure 6. Agilent prep 21.2 × 150 mm, 10-µm – Mixture of soy phospholipid standards. - Loadability.**

Finally, in Figure 7, we investigated the scale-up of a 20-mg injection of a crude soy lecithin sample which, by overlaying the standards run under the same conditions, revealed the presence of three phospholipids, including PE, PI, and PC. No PS is seen in this crude extract.

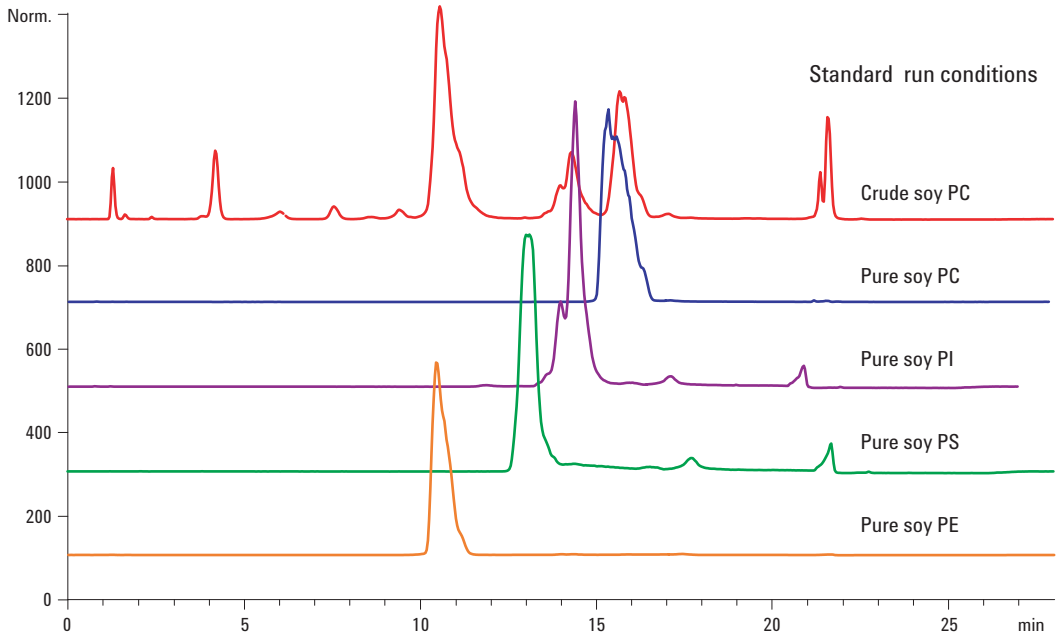


**Figure 7. Agilent prep – SIL, 21.2 × 150 mm,10 µm – Crude soy lecithin (PC) and pure individual standards.**

## Conclusion

These experiments show that the classes of major soybean phospholipids can be successfully separated from a crude mixture, under both analytical and preparative conditions, using a matching set of columns, normal phase conditions, and a small amount of volatile salt added to the mobile phase to cut down on possible electrostatic interactions. At higher concentrations of PC, however, two distinct peaks, whose areas change with mass injected, are observed. By mass spectrometric measurements of the eluted peaks, these two peaks were determined to be the same or, at least, very similar. It is suspected that PC is strongly binding to the silica silanol group in competition with the ammonium acetate, a mobile phase modifier, and is eluting at high gradient solvent polarity.

## Acknowledgement

We would like to thank ESA for the loan of the Chromachem ELSD detector for the duration of this study.

## References

1. Kyung Ho Row and Duk Hui Kang, (2003) *Amer. Biotech. Lab.*, 21(**9**) 40–43.
2. W. M., Christie, (1986) *J. Chromatogr.* **361**, 396–399.
3. B. Herslof, U. Olsson, and P. Tingvall in I. Hanin and G. Pepeu, Eds. (1990) Phospholipids: Biochemical, Pharmaceutical, and Analytical Considerations, Plenum Press, New York, pp. 295–298.

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