High-Performance Liquid Chromatographic Separations of Soy Phospholipids

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ABSTRACT: High-performance liquid chromatography methods to quantitate soy phospholipids vary as to which phospholipids are analyzed, degree of method ruggedness, precision, time, and standards. Fluid and deoiled soy lecithins were analyzed by three different high-performance liquid chromatography methods, including the American Oil Chemists' Society (AOCS) Method Ja 7b-91. The other methods include an isocratic mixed phase (normal-phase column and reverse-phase solvent) method with ultraviolet detection, and a binary gradient normal-phase (proposed International Lecithin and Phospholipid Society) method with light-scattering detection. A set of 20 analyses were repeated on three different days for fluid and deoiled product by the three methods. The statistical comparison involved the selected methods and the phosphatidylcholine and phosphatidylethanolamine measured data, which were the common analytes among the methods. The precision for the mixed phase method and the Proposed International Lecithin and Phospholipid Society method was better than that for the AOCS method. Selection of reference standards was an important issue in defining the results. Column conditioning varied by 2–3 h for the Proposed International Lecithin and Phospholipid Society method, 16 h for the mixed phase method, and 2–3 d for the AOCS method. The ruggedness for the methods showed the following descending order: proposed International Lecithin and Phospholipid Society, mixed phase, AOCS.

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KEY WORDS: Gradient, HPLC, lecithin, methods, phospholipids, separations, standards.

Phospholipids include a wide range of compounds that are often found in a matrix with other lipid classes (1–3). Phospholipids are widespread across plant and animal species and tissue types, and individual analytes may vary among themselves as to their structure and function (4–6). The components and matrix must be defined and characterized to separate a multicomponent lecithin.

Not only is there variation of structure and characteristics within the group, but the matrix may also contain other components that can interfere when trying to analyze the sample. Also, differences between analytes or portions of the analytes are important because of their effect on detection or response. The degree of saturation of the fatty acids on a phospholipid may be different when samples are from different vegetable lecithins. Ultimately, this may affect detector response, depending on which detector type is used (7,8).

High-performance liquid chromatography (HPLC) columns (9) generally contain silica or bonded phase. Silica columns are primarily used because compounds are separated by their polarity. The mobile phase can be isocratic or gradient. An isocratic system is the simpler or more stable system but can limit the number of analytes that can be determined. Gradients open the possibility of separating additional analytes.

The detection system used initially in HPLC analyses was largely ultraviolet (UV) and is still used extensively. Some major problems result from the UV cut-off because most wavelengths used are in the 200 nm range (10–13). Also, what is measured is largely absorption of the double bond. The response varies with the species being analyzed, and the standard must be that species when using UV.

UV and refractive index (RI) detection are sensitive to gradient conditions, and result in baseline drift and high background noise. RI detection is limited to isocratic runs and is generally less responsive than UV. UV and RI detection have shortcomings that cause less-than-favorable conditions for analysis. UV methods have been used successfully for the major phospholipids with the proper selection of analytical conditions.

Another type of detector that is growing in popularity is the light-scattering detector (14). It displays good linearity within a narrow range. An advantage to this detector is that it is not affected as much by gradient conditions.

There is still a need for an increase in the number of analytes run routinely, as well as a need to be able to analyze lecithin from different sources. The goal of this study was to evaluate various methods with which multiple components could be analyzed.

Past experiences with various methods for analyzing phospholipids lead to the evaluation of the following three methods: (i) mixed phase method: the "in-house method," originally developed for a limited number of analytes. Phosphatidylethanolamine (PE), phosphatidylcholine (PC), and lysoPC (LPC) are analyzed in a Quality Control setting where reproducibility and ruggedness are important (15); (ii) AOCS method: allows for an increased number of analytes that can be separated while still using a similar system (16); (iii) Pro-

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posed International Lecithin and Phospholipid Society (ILPS) method: Involves the use of a gradient mobile phase and an evaporative light-scattering detector, which allows for an increased number of analytes (17). The major difference in the mechanics of this method is the use of a gradient, a diol-bonded type of silica column, and a light-scattering detector.

The primary focus of the study was testing the three methods, each having its advantages and disadvantages. The methods are similar in type but have basic differences. The objective was to determine how well the results from the three different methods correlated with each other. As part of this study, any differences that may exist between the matrix types of standard fluid lecithin and deoiled lecithin were investigated.

A small study was carried out to investigate what role standards play in determining concentration with the light-scattering detector. The same samples were analyzed with a commercially-available fractionated standard, which was purified from soy lecithin, and a fluid lecithin reference standard, which was recommended by the ILPS. The phospholipid concentrations of the fluid lecithin standard were determined by nuclear magnetic resonance. The standards are the only variable, whereas all other factors are constant in this study.

EXPERIMENTAL PROCEDURES

The design was to analyze two lecithin types, standard fluid grade and deoiled. Twenty analyses were done with each of the three methods for each type. The standard used for the three methods was a commercially-available fractionated standard.

The mixed phase method is an isocratic method with a Porasil (Waters, Milford, MA) 10-micron silica column and a UV detector (205 nm). The mobile phase consists of acetonitrile/methanol/phosphoric acid (130:5:1.5, vol/vol/vol). The American Oil Chemists' Society (AOCS) method is also isocratic, with a LiChrosorb Si-60 5-micron silica column (EM Science, Gibbstown, NJ), a UV detector (206 nm), and a mobile phase of *n*-hexane/2-propanol/acetate buffer of pH 4.2 (8:8:1, vol/vol/vol). The ILPS gradient method incorporates a binary solvent system of *n*-hexane/2-propanol/acetic acid/triethylamine (81.5:17:1.5:0.08, by vol) and 2-propanol/water/acetic acid/triethylamine (84.5:14:1.5:0.08, by vol) and a LiChroCart 100 diol 5-micron column (EM Science). The gradient starts with the ratio of 93% A/7% B and changes over 20 min to 100% B. The UV detector is replaced with a light-scattering detector (ACS, Model 750/14; Polymer Laboratories, Inc., Amherst, MA).

Sample preparation was basically the same for the three methods investigated. Approximately 350 mg of the lecithin reference standard was dissolved in 25 mL of the appropriate solvent. Sample weights were adjusted to ensure a linear response, and 0.05% butylated hydroxytoluene was added to each sample. An aliquot was filtered through a 0.45-micron filter and 20 μL was analyzed under the stated HPLC conditions within 24 h after preparation to avoid loss.

RESULTS

A standard curve (Fig. 1) is plotted (peak area vs. concentration of single fractionated standards) for an indication of the linear working range. Responses of the light-scattering detector for phosphatidic acid (PA), PE, PC, phosphatidylinositol (PI), and LPC are linear in the range tested, except for a small deflection with PA, and they are consistent with reports from other laboratories (7,11,14).

The chromatogram (Fig. 2) for the mixed phase method shows three well-resolved components. The chromatogram displays a stable baseline and symmetrical peaks. Figure 3 is the chromatogram for the AOCS method. The baseline is not as stable, and peak symmetry is not as good when compared with the other methods. The best chromatographic performance results from the ILPS method (Fig. 4). The components are well resolved and show peak symmetry. These factors can ultimately play a role in integration and results. Additional analytes are separated by the ILPS method.

Table 1 compares the PC, PE, and PI data for the three HPLC methods. The average is the grand average, and the coefficient of variation represents the total of the three-day block of data for each method. The deoiled PC results from the ILPS gradient method are significantly different from the mixed phase or AOCS results. The average values for the three methods are similar, with a slight decrease in PC from the ILPS gradient method. The AOCS fluid PC results are significantly different from the mixed phase or ILPS method results, and one observes the variance with the ILPS method.

The PE results for the three methods are significantly different. All three method averages are statistically different, and the ILPS gradient method value is considerably higher than the other two methods. The precision of the ILPS gradient method is the best. PE fluid results from the ILPS gradient method are significantly different from the mixed phase or the AOCS method. The ILPS gradient PE results are approximately 2% higher for both the fluid and deoiled lecithin.

The PI results from the AOCS and ILPS gradient methods are significantly different. The ILPS gradient data have better precision, but the method yields lower results.

The data being compared in Table 2 differ only in which standard was used to calculate the final phospholipid concentration. The run consists of a single fractionated standard and a reference standard. There is a significant difference between the single standard and the lecithin standard for the PC results. The lecithin standard is significantly lower than the single standard, indicating a difference in the standards where the same method and detection are used. This decrease in the PC value is in addition to the decrease observed in the previously mentioned study, when the same standard but a different method was used.

There is a significant difference between the single PE standard and the lecithin standard. The PE is significantly decreased when the lecithin standard is used. However, the reference lecithin standard values are similar to the values ob-

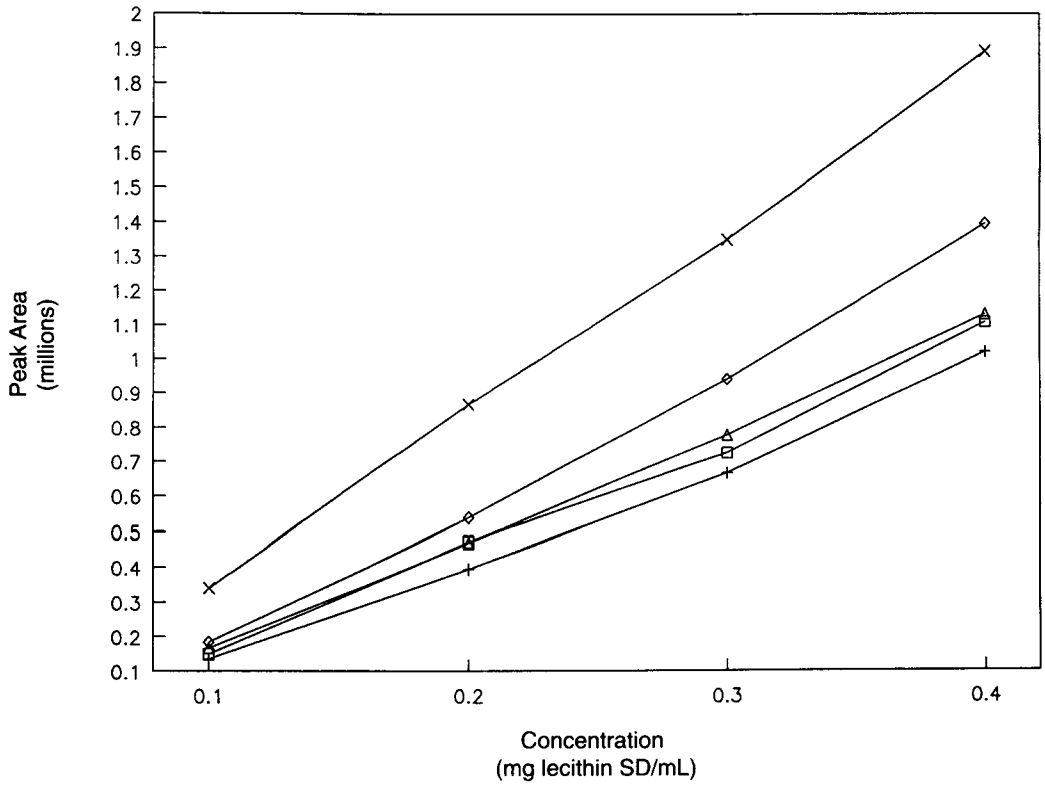

FIG. 1. Concentration dependence of detector response for a high-scattering detector. □, phosphatidic acid; +; phosphatidylethanolamine; ◇, phosphatidylcholine; △, phosphatidylinositol; x, lysophosphatidylcholine.

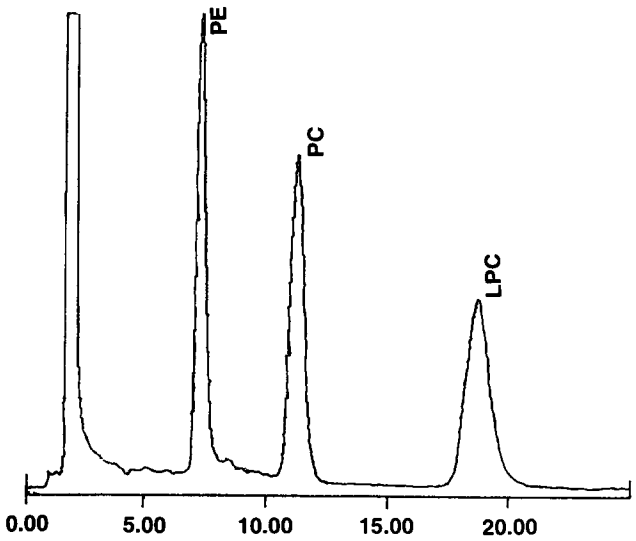

FIG. 2. High-performance liquid chromatographic separation of lecithin by the mixed phase method. PE, phosphatidylethanolamine; PC, phosphatidylcholine; LPC, lysoPC.

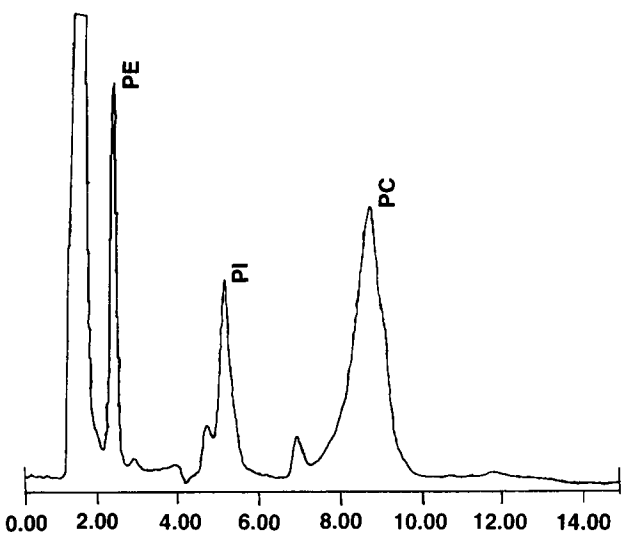

FIG. 3. High-performance liquid chromatographic separation of lecithin by AOCS Method Ja 7b-91. PI, phosphatidylinositol; other abbreviations as in Figure 2.

tained from the mixed phase and AOCS methods with the single fractionated standard, as shown in Table 1.

There is no significant difference between the single PI standard and the lecithin standard. The PI for the fluid product indicates the same trend as for the deoiled lecithin. For PI, there is only a method difference and no significant differences due to selection of standards.

In reviewing the different standards with the same method, the precision is similar for the purified single standard compared with the reference lecithin fluid standard. However, the

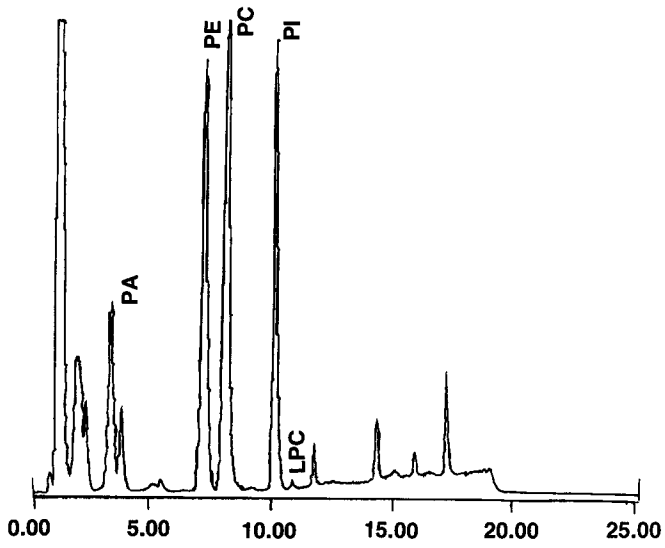

FIG. 4. High-performance liquid chromatographic separation of lecithin by the International Lecithin and Phospholipid Society method. PA, phosphatidic acid; other abbreviations as in Figures 2 and 3.

TABLE 1
Comparison of High-Performance Liquid Chromatography Methods for Lecithin Analyses[a]

		PC		PE		PI	
Treatment	Method	%	CV	%	CV	%	CV
Deoiled	Mixed phase	22.4	1.1	18.0	1.1	ND	
	AOCS	22.3	3.5	18.6	1.8	16.9	3.4
	ILPS	21.4	0.6	20.9	0.6	13.9	0.6
Fluid	Mixed phase	15.4	2.1	11.0	1.8	ND	—
	AOCS	13.8	4.3	11.1	2.2	11.0	3.9
	ILPS	15.4	1.2	13.6	1.2	9.9	1.2

[a]PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; CV, coefficient of variation; ND, not determined; ILPS, International Lecithin and Phospholipid Society; AOCS, American Oil Chemists' Society.

TABLE 2
Comparative Analysis of Single Component Lecithin Standard and Both Being Analyzed by ILPS Method[a]

		PC		PE		PI	
Treatment	Standard	%	CV	%	CV	%	CV
Deoiled	Single	21.5	0.6	20.9	0.6	13.9	0.6
	Lecithin	19.7	0.6	18.0	0.6	13.3	0.6
Fluid	Single	15.4	1.2	13.6	1.2	9.9	1.2
	Lecithin	14.3	1.4	11.4	1.2	9.6	1.2

[a]For abbreviations, see Table 1.

values for PC and PE are lower with the fluid lecithin standard. The PI, although showing no significant difference, does indicate a possible low trend.

DISCUSSION

The study indicates that there is not only a method variation affecting the data but also a day component of the variation. It appears that the daily method set-up may be an important factor for the analysis. The ILPS method shows increased precision for both within- and between-day as compared to that of the mixed phase and AOCS methods. This is probably due to column stability. The chromatogram shown for the gradient method indicates baseline stability and peak symmetry.

Column conditioning appears to correlate with precision of the data from the methods. The major problem that can occur with silica columns, when water is present, is the difficulty of getting a column or system to equilibrate. This may result in nonreproducible data. This problem will carry over to a greater extent after each gradient sample run. Conditioning is an important factor in controlling retention time and reproducibility. The diol column is not as sensitive to water, so that stabilization time is minimized. Even though the method requires a gradient, the column appears to stay stable, and precision of the analytes is improved.

The order of the methods for the number of analytes that can be quantitated is: the mixed phase has the fewest, then the AOCS, followed by the ILPS gradient method. This shows an improvement in the number of analytes that can be observed, along with increased precision.

Investigating matrix effects with the methods indicates that the deoiled lecithin results are more precise than those for the standard fluid. It may be because the deoiling process concentrates the analytes or removes components that may interfere with the analyses.

The ILPS method with the light-scattering detector shows that PC decreased, PE increased, and PI decreased as compared with the AOCS and mixed phase methods. This is attributed to the type of detector and column used. However, the mixed phase results compare better with those of the AOCS method than either of them do with the gradient method, which is probably because both the AOCS and the mixed phase methods are based on UV detection. The gradient method values are significantly different when compared with the AOCS and mixed phase methods. This study was not designed to establish which results are accurate. Problems with the commercially-available fractionated standards have been observed over the years. It is understood in the industry that the type of standard, e.g., egg vs. soy, plays an important role in the analysis. Previously, variation of the standard of approximately 10% from lot to lot or run was observed. The solvent in which the sample is stored may be important. Basically, the type of standard used and whether the standard is affected by the type of detection or variation of the HPLC method used are important issues.

The standards studied indicate that the single purified soy standard yields higher results than a fluid soy lecithin reference standard. This requires further investigation.

Overall, the gradient method allows for an increase in the number of phospholipid analytes that can be analyzed. The

precision appears to be better for the ILPS gradient system. This may be partially due to increased column stability. The ILPS gradient method requires the least amount of stabilizing time. Also, the study indicates that there is a difference between the light-scattering detector and the UV detector data. Differences in quantitation are observed between the ILPS gradient and the other two methods. The degree of accuracy for the methods and standards may have to be determined.

This study identified major issues that must be addressed for phospholipid assays, such as the differences that are due to the reference standard used and the accuracy of the results based on the choice of detection.

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