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Phospholipids determination in vegetable oil by thin-layer chromatography and imaging densitometry

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A rapid reliable method was developed to measure vegetable oil phospholipid content by thin-layer chromatography–imaging densitometry. Phospholipid samples were obtained from crude soybean oil by water degumming and then analyzed and quantified by thin-layer chromatography and imaging densitometry. Phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) and phosphatidyl inositol (PI) standard curves were generated. The total phospholipid content of the oil was 1.12–1.26%, of which 27.1 ± 1.86% was PC, 22.7 ± 0.45% was PE, while PI accounted for 17.2 ± 0.85% of the total. A high correlation coefficient of 0.985 was found for reproducibility of pixel area (OD*mm) at a constant concentration of standard phospholipid. Regression analysis of the pixel area vs the phospholipid (PC, PE, and PI) weights (μg) generated R^2 greater than 0.983. Thin-layer chromatography–imaging densitometry proved a useful tool for rapid determination and quantification of the major phospholipids in soybean oil. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

The measurement of phospholipids is important in determining the stability and quality of vegetable oils. Phospholipids are undesirable in the oil since they are responsible for oil discoloration during deodorization and steam distillation. They affect the stability of the oil by chelating metal ions, therefore increasing oxidative processes (Arruda and Dimick, 1991). Crude soybean oil contains *ca.* 2% phospholipid, 90% of which is removed by hydration during oil degumming (Nash *et al.*, 1984). Spectrophotometric methods have been employed to determine the concentration of phospholipids and phosphorus in vegetable and animal tissues (Skidmore and Entenman, 1962; Keenan *et al.*, 1968; Zhukov and Vereshchagin, 1976; AOCS, 1994). However, many of these methods are tedious and inaccurate. Thin-layer chromatography (TLC) provides quick separation and identification of a variety of compounds with differing polarities. There are a number of high-performance liquid chromatographic (HPLC) methods available for the analysis of phospholipids in vegetable oils (Chapman, 1980; Mounts and Nash, 1990; Singleton, 1993; Singleton and Stikeleather, 1995). HPLC separations using gradient elution can also be done using a single mixed solvent system (Ackman, 1993; Padley *et al.*, 1994). However, TLC offers a much higher sample throughput, because of its ability to perform numerous separations simultaneously and it can analyze much cruder samples than either HPLC or gas chromatography (Padley *et al.*, 1994). A limitation has been accurate quantification of separated species, which could be done by imaging densitometry. An advantage of this technique is its ability to analyze the whole TLC plate at once, hence making it a fast method for routine work. Video scanners can analyze > 20 samples on a TLC layer in the same time it takes for one HPLC assay (Ackman, 1993). Densitometry *in situ* on chromatographic plates directly determines the concentration of analytes by absorptiometry.

Recent analytical progress has made direct quantitative TLC attractive and applicable to many areas of research and routine laboratory work. The trend towards widespread use of TLC was accelerated by work of Stahl, who demonstrated its potential use in lipid research (Skidmore and Entenman, 1962). Villé *et al.* (1995) were able to determine phospholipid content of meat extracts using a fourier transform infra-red method. The data were found to give good correlation with data obtained using an Iatroscan. Chapman (1980) analyzed the phospholipids in crude soybean oil using

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two-dimensional TLC, and found that the major phospholipids were phosphatidyl choline (PC) 39%, phosphatidyl ethanolamine (PE) 23% and approximately 20% phosphatidyl inositol (PI). Touchstone and Sherma (1979) found that the R_f for the individual phospholipids was generally the same on any conventional TLC relative to HP-TLC when the same mobile phase was used. A combination of column chromatography and quantitative TLC showed that soy lecithins consisted of *ca.* 82% mixture of the major phospholipids of soybeans, PC, PE and PI (Barierjee and Ackman, 1990). TLC–imaging densitometry is a cost-effective, simple and easy chromatographic technique that can be adapted for qualitative and quantitative analyses of a complex lipid mixture. TLC is extremely versatile and considered one of the most important analytical techniques in current lipid research (Touchstone and Dobbins, 1978).

The objectives of this research were to develop a rapid, reliable TLC method for the separation of phospholipids from vegetable oils, and to quantitate the phospholipids by imaging densitometry.

MATERIALS AND METHODS

Chemicals and reagents

All solvents and chemicals used were analytical-reagent grade (Fisher Scientific, Fair Lawn, NJ). Crude (CSBO) and refined bleached deodorized (RBD) soybean oil were obtained from Riceland Foods Inc. (Stuttgart, AR). Silicic acid and soybean PC, PE and PI standards (purities greater than 90%), were purchased (Sigma Chemical Co., St. Louis, MO).

The silicic acid column was prepared by introducing approximately 40 g of silicic acid in 100 ml of chloroform into a Kontes column (Vineland, NJ), 22×350 column (ID $\times$ length (mm)). High performance-TLC 20×20 cm and 5×10 cm silica gel G plates (Analtech, Newark, DE) were used.

Phospholipid sample preparation

The overall experimental design for the preparation of soybean phospholipid from crude soybean oil is shown in Fig. 1. Distilled water (2% v/v) was added to 500 g of crude soybean oil and heated at 90°C for 30 min with stirring. The oil was then centrifuged at 30*g* for 10 min, decanted and the phospholipid rich layer washed three times with 100 ml of acetone. The crude phospholipid extract was dried under vacuum to remove residual acetone left after washing.

A recovery study was performed by adding a 0.5% (w/w) phospholipid standard mixture (approximately 27% PC, 23% PE and 17% PI) in RBD soybean oil with an initial phospholipid content of 50 ppm. This sample was then subjected to degumming and analyzed similarly to the CSBO (Fig. 1).

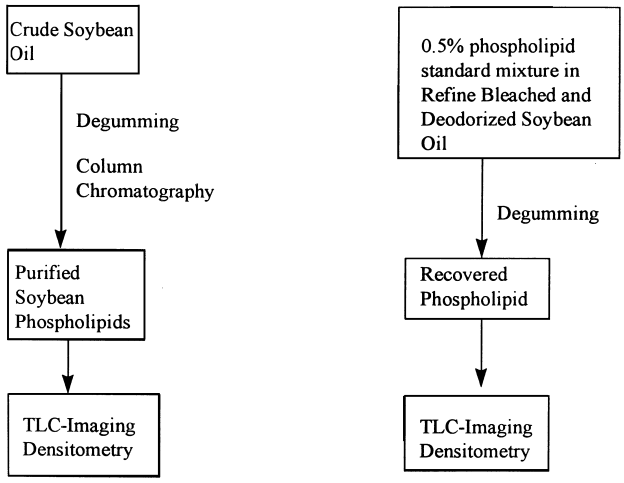

Fig. 1. A schematic flow diagram of phospholipid preparation and determination by thin-layer chromatography–imaging densitometry.

Column chromatography

The crude phospholipid extracts from CSBO and RSBO were dissolved in 90:10 chloroform/methanol and applied to a silicic acid column (4.5% by weight of the silicic acid). Non-polar lipids were first eluted from the crude phospholipid extract using chloroform. The phospholipids were then eluted using 150 ml methanol, collected into a flask and the solvent evaporated off under vacuum.

TLC

A modification of the AOCS method Ja-86 (AOCS, 1994) was used. Instead of a two-dimensional method, the one-dimensional method was used. The developing solvent was composed of 75:25:3 (v/v) of chloroform:methanol:water. For calibration, standard phospholipid concentrations (PC, PE and PI) were dissolved in a 95:5 chloroform:methanol solvent mixture to prepare standard concentrations of 0.228, 0.114, 0.050, 0.026, 0.013, and 0.006 g ml^{-1}. Silica plates were developed with solvent prior to spotting in order to remove impurities from the adsorbent layer. The plates were air dried for 10 min and oven activated for 20 min at 100°C before use. The same volume of phospholipid extract and phospholipid standards were spotted on the plates. A filter paper was placed in the developing chamber and the developing solvent added. The chamber was allowed to saturate for 10 min before chromatography. The spotted plates were placed in the developing chamber for 40 min, and then air dried for 10 min and placed in a 90°C oven for 10 min.

Detection of phospholipids

Ammonium molybdate–perchloric acid spray and iodine were the two methods compared for the detection

of phospholipids (Singleton, 1993; Padley *et al.*, 1994). Ammonium molybdate–perchloric acid spray was prepared by dissolving 3 g ammonium molybdate in 50 ml of distilled water. To this solution was added 5 ml of a 6 N HCl solution and 13 ml of a 70% perchloric acid solution. Phospholipids turned blue gray on a white background after spraying with ammonium molybdate and heating the plate at 80°C for 10 min. In a separate experiment, approximately 20 mg of iodine crystals were placed in a chamber and left to stand for 5 min. In iodine vapor, the phospholipids were yellow on a white background.

Imaging densitometry

The system used for the quantitation of phospholipids was a Bio- Rad® model GS-670 imaging densitometry. The plates were scanned immediately after visualization. The pixel areas were then determined for each of the standards and the concentrations of the unknown soybean phospholipid samples determined from the standard curves.

Statistical analysis

The experimental design was a complete randomized design on 10 replicate samples each of phospholipid extract and standard phospholipid PC, PE and PI. For recovery studies, eight replicates of phospholipids recovered from the RSBO were used in a complete block design. Pixel area reproducibility was determined by a repeated-measures analysis of variance at $p < 0.05$ (SAS 88). The Pearson correlation coefficients were used in the statistical analysis.

RESULTS AND DISCUSSION

Percent recovery of phospholipids

The total phospholipid yield from crude soybean oil was found to be approximately 86%, while the individual percentages of the major phospholipids were 27.1, 22.7 and 17.2% of PC, PE and PI, respectively. Approximately 1.5–2.2% crude phospholipid by weight of oil was obtained from the crude soybean degumming process. The recoveries of the purified phospholipids by degumming and quantification by TLC–imaging densitometry are summarized in Table 1. In determining the effectiveness of degumming, Smiles *et al.* (1988) were able to remove approximately 86–90% of the phospholipids in soybean oil. They also found that the levels of PC, PE, and PI were not significantly altered by the type of degumming agent used. Carelli *et al.* (1997) found that recovery of phospholipids from triolein and sunflower oil spiked with known concentrations of PE, PC, PI and phosphatidic acid (PA) ranged from 94 to 107%. The study gave good precision with the coefficients of variation for the four independent tests ranging from 1 to 9%. Przybylski and Eskin (1991) used TLC/flame ionization detector (FID) to determine phospholipid composition of canola oils during early stages of processing. The yield of phospholipids from spiked oil samples was > 90%, showing that the two-dimensional TLC/FID had a high degree of accuracy. In the present study, approximately 98% of the standard phospholipids added to RBD soybean oil was recovered, of which 26.8, 22.1 and 16.4% were PC, PE and PI, respectively. The starting concentrations were 27% PC, 23% PE and 17% PI and at a total concentration 0.5% in the RSBO, it was possible to recover most of the major phospholipid components from the refined oil. The percentage recovery for the RSBO was much higher than that for the yield of phospholipids from CSBO. This difference may be due to losses encountered during the purification of the phospholipids by column chromatography.

Table 1. Percentage recovery of phospholipids ($n = 10$)

	% PC	% PE	% PI	Total % PL[a]
CSBO[b]	27.1 ± 1.86	22.7 ± 0.45	17.2 ± 0.85	86 ± 2.1
RBD SBO[c]	26.8 + 1.23	22.1 + 0.82	16.4 + 1.37	98 + 1.2

[a]Total amount of phospholipids from soybean oil, $n = 8$.
[b]Losses occurred during purification of phospholipids.
[c]Refined bleached deodorized soybean oil with less than 50 ppm initial phospholipid content.
PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, PI = phosphatidyl inositol, PL = phospholipids.

Reproducibility of phospholipid analysis

The reproducibility of image pixel peak area with concentration of phospholipid is shown by ANOVA in Table 2. The R^2 was 0.985 and at $\alpha = 0.05$ there were no significant differences in the pixel areas for the same concentrations of phospholipids by imaging densitometry. The coefficient of variation signified that the method gave good precision for area spotted at any given phospholipid concentration.

Phospholipid determination

It was extremely difficult to obtain a uniform spray on the plates using the ammonium molybdate method. Sensitivity at the same concentration of phospholipid was much higher using iodine vapor than the ammonium molybdate–perchloric acid method. Therefore, all plates were visualized using iodine. Iodine has been found to be an extremely sensitive method for the detection of lipids (Erdahl *et al.*, 1973; Ackman, 1993). The main disadvantage with this method is that it may be reversible, requiring readings to be taken immediately after development in order to reduce error. However, very consistent routine TLC–imaging densitometry had to be followed. The efficiency of the spotting

time of TLC development, as well as the visualization technique, required strict monitoring. It is important that the amount of phospholipid applied to the plate, the development time, as well as the scanning time, are monitored to obtain consistency in the results.

Equations were generated to determine the content of phospholipids in a crude soybean phospholipid sample, and in purified samples. Standard curves for the individual phospholipids, generated by plotting the pixel area (OD*mm) (corrected for any attenuation changes) of six spots vs the amount (μg), gave high correlation coefficients. Great linearity was achieved over the concentration range with regression coefficients in the range of 0.990–0.998. The regression equations generated for the pixel area and weights (μg) for individual phospholipid standards as well as a mixture of the three phospholipid standards, are shown in Table 3. From the equations generated, approximately 1.12% phospholipid was present in the purified fraction. This lower amount of phospholipid was due to losses encountered during column chromatography. These losses can be reduced by increasing the solvent polarity, therefore reducing the phospholipid content left in the silicic acid column. The R^2 obtained from the calibration curves were much greater than 0.983, signifying the high accuracy of the TLC–imaging densitometric method.

The TLC plate of samples of soybean phospholipid and individual standards consisting of PC, PI and PE is

Table 2. Pixel area reproducibility of phospholipid standard mixture (n = 10)

Source	DF	SS	Mean square	Mean response	Root MSE	F_{value}	Pr > F	R^2	C.V.
Model	10	0.1465	0.0366	0.700	0.012	240.3	0.0001	0.985	1.762
Error	15	0.0023	0.0001						

Table 3. Correlation equations for individual phospholipids—pixel peak areas vs weight (μg)

Components	Equations[a]	R^2	R^b
PC	0.541 + 0.0111 (Phospholipid μg)	0.995	0.998
PE	0.315 + 0.013 (Phospholipid μg)	0.983	0.991
PI	0.473 + 0.015 (Phospholipid μg)	0.988	0.994
Standard mixture[c]	0.779 + 0.010 (Total Phospholipids μg)	0.989	0.995

[a] $y = b + ax$ where y = pixel area and x = phospholipid weight μg.
[b] Correlation coefficient, $p < 0.05$, d.f. = 10.
[c] Phospholipid mixture consisting of approximately equal amounts of PC, PE and PI.
PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, PI = phosphatidyl inositol.

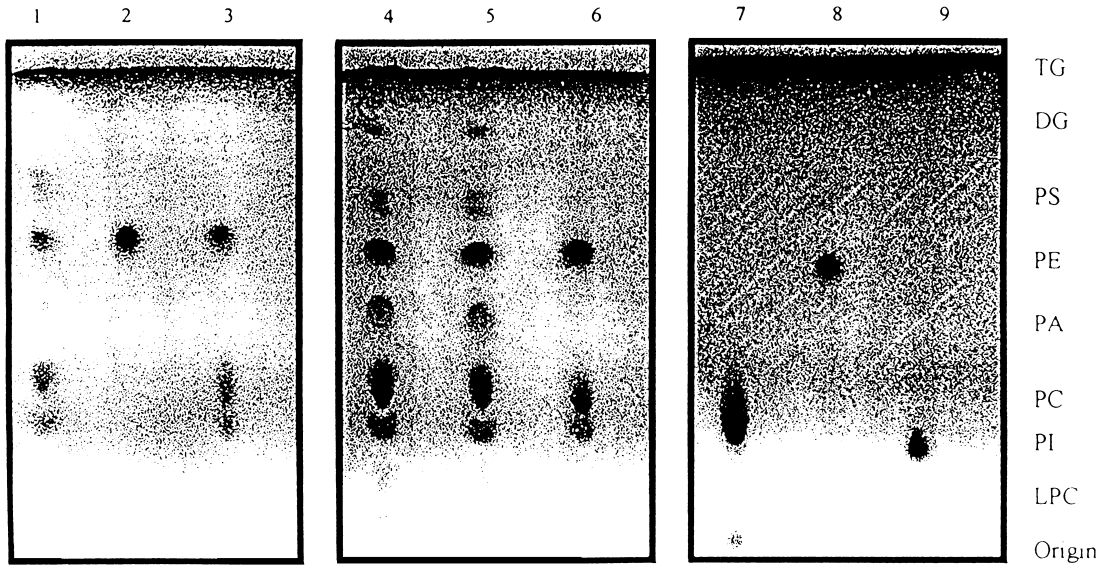

Fig. 2. Thin-layer chromatography of phospholipids from soybean oil and individual phospholipid standards. Samples: 1 and 3, standard phospholipid mixture containing PC, PE and PI; 4, 5, and 6, phospholipid extract samples; 2 and 8, PE; 7, PC; and 9, PI. TG = triglyceride, DG = diglyceride, PS = phosphatidyl serine, PE = phosphatidyl ethanolamine, PA = phosphatidic acid, PC = phosphatidyl choline, PI = phosphatidyl inositol, LPC = lyso-phosphatidyl choline.

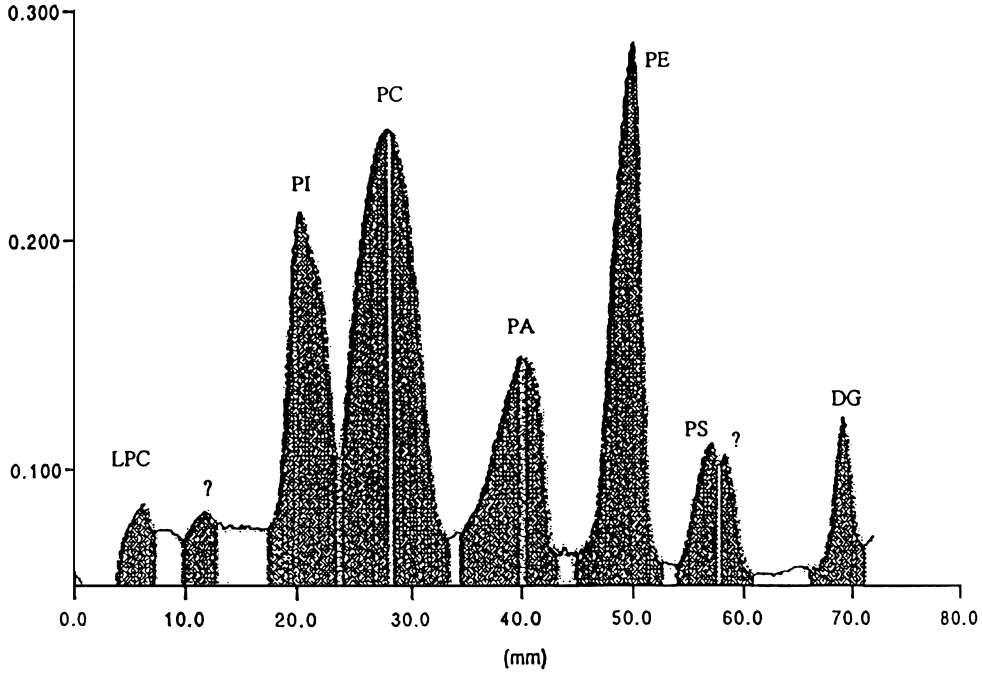

Fig. 3. Imaging densitometry of soybean phospholipids. LPC = lysophosphatidyl choline, PI = phosphatidyl inositol, PC = phosphatidyl choline, PA = phosphatidic acid, PE = phosphatidyl ethanolamine, PS = phosphatidylserine, DG = diglyceride.

shown in Fig. 2. The R_fs for PI, PE, and PC were 0.29, 0.32, and 0.67, respectively. The 75:25:3 chloroform:methanol:water solvent system gave good separation of individual phospholipids, making the one-dimensional TLC method useful for analysis of the purified phospholipid fraction. The crude phospholipid sample contained triglycerides with an R_f of 0.90. Keenan *et al.* (1968) quantitatively determined PE and other phosphatides in various rat tissues using a TLC method. However, using an 83% liquefied phenol/16% water/1% aqueous ammonia solvent system did not result in complete separation of PI from PA. Smiles *et al.* (1988) were able to achieve complete separation of PC, phosphatidyl serine (PS) and PA, but only partial resolution could be obtained between PE and PI by liquid chromatography. Imaging densitometry (Fig. 3) gave good resolution for the three major phospholipids. Hence, making it possible to quantitate peaks accurately. The resolution between the individual spots was > 1 for phospholipid samples containing $< 364\,\mu$g per spot area. At higher phospholipid concentrations, the resolution between peaks was reduced due to overlap of PC and PI.

CONCLUSION

A combination of calibration curves generated from three phospholipid standards, PE, PC and PI, were used to determine and quantify the major phospholipids present in crude soybean oil. TLC–imaging densitometry resulted in very high R^2. This shows that this method, when calibrated, can be used to generate quick results with standard errors $< 2\%$ (Ackman, 1993). One-dimensional TLC–imaging densitometry was used for the analysis of phospholipids in soybean oil. Tentative identification of phospholipid components was made possible using iodine, and quantitation of individual spots was achieved by imaging densitometry. The advantage of TLC–imaging densitometry is that rapid phospholipid determination of many samples is possible. TLC–imaging densitometry as a tool for quantitative research is rarely used and provides the opportunity for in-laboratory calibration and optimization of this technique as a means for simple and rapid determination of phospholipid content in the food industry today.

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