Determination of Phospholipids in Vegetable Oil by Fourier Transform Infrared Spectroscopy

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ABSTRACT: A method was developed to determine the total phospholipid content in vegetable oil by Fourier transform infrared spectroscopy (FTIR). Calibration curves of l-α-phosphatidylcholine (PC), l-α-phosphatidylethanolamine (PE), and l-α-phosphatidylinositol (PI) in hexane were generated at different concentrations. The optimal phospholipid absorption bands between 1200–970 cm^{-1} were identified and used for quantitative determination. High $R^2 \geq 0.968$ were observed between band areas and phospholipid standard concentrations. Phospholipids from crude soybean oil were obtained by water degumming, and purification was performed on a silicic acid column. The phospholipid contents of purified phospholipid extract, degummed and crude soybean oil determined from calibration equations were >90, 0.0113, and 1.77%, respectively. High correlations of determination ($R^2 \geq 0.933$) were observed between the FTIR method and thin-layer chromatography–imaging densitometry method for the determination of phospholipid content. FTIR was found to be a useful analytical tool for simple and rapid quantitative determination of phospholipids in vegetable oil.

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KEY WORDS: Crude soybean oil (CSBO), Fourier transform infrared (FTIR), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI).

The phospholipid content of crude soybean oil (CSBO) is approximately 2% (1) of which 90% is removed during the degumming process to improve color and enhance oxidative stability (2). The major phospholipids in CSBO are phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) and comprise approximately 39, 23, and 20% of the total phospholipids, respectively (3).

Phospholipid separation and quantitation is possible using thin-layer chromatography (TLC) (3,4), gas chromatography (GC) and high-performance liquid chromatography (5–7). These chromatographic methods are largely used for routine analytical requirements in the fats and oils industry. The residual phosphorus in processed oil is an important quality measure and is routinely determined by ashing of the oil followed by colorimetric determination of phosphorus (8). This is a lengthy, tedious, and often inaccurate method. There is interest in replacing these methods with automated instrumental methods that improve analytical speed, accuracy, and efficiency and that also address increased environmental concerns about the use of large volumes of solvents and reagents in quality control laboratories (9).

Fourier transform infrared spectroscopy (FTIR) has potential as a powerful alternative analytical technique in the fats and oils industry. FTIR analysis is rapid and provides comprehensive chemical data with peak integration as well as digital background subtraction. The middle infrared (IR: 4000–400 cm^{-1}) is an extremely useful area of study, providing qualitative information on functional groups as well as offering the opportunity for quantitative analysis. Quantitative FTIR analysis of a compound in a mixture is relatively simple if an isolated absorption peak can be identified for each component. Difficulties arise if the characteristic peaks of the individual components overlap. This problem can be solved by applying methods such as classical, partial, and inverse least-squares methods to the spectral data (10).

The most widespread use of infrared spectroscopy in fats and oils has been to determine the percentage of *trans* in fatty acids and their esters (11); this method employs the *trans* alkene band at 965 cm^{-1}. FTIR has also been extensively used to study orientation in functionalized phospholipid monolayers (12), to determine conformational disorder in phospholipid bilayers quantitatively (13), and to determine water penetration in lipid bilayer membranes (14). Determination of intramuscular fat phospholipid in different commercial meats (15) has been performed by FTIR, and a high correlation is found between results from FTIR and the Iatroscan method from different muscle samples. Qualitative studies of oxidation products formed in heated frying oils have been carried out using GC–FTIR (16,17). Lanser *et al.* (18) have reported a method to estimate free fatty acid in oils involving the deconvolution of absorption bands over the region 2000–1600 cm^{-1} in order to distinguish the otherwise overlapping bands at 1710 cm^{-1} for free acid and 1746 cm^{-1} for triglyceride ester. Correlation with titration methods gave good results for the estimation of free fatty acid in damaged soybean. The potential benefits of quantitative FTIR in lipid

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analysis include rapid and reproducible analyses, amenability to automation, and a reduction in chemical waste and analytical costs (19).

The objective of this research was to develop a rapid method to identify and quantify phospholipids from vegetable oil as an alternative to oil ashing and colorimetric quantitation currently in use for routine analysis.

MATERIALS AND METHODS

Chemicals and reagents. All solvents used were glass-distilled analytical grade (Fisher Scientific, Fairlawn, NJ). CSBO and refined bleached deodorized soybean oil (RBDO) were obtained from Riceland Foods Inc. (Stuttgart, AR). Silicic acid and soybean PC, PE, and PI standards (purity > 90%) were purchased (Sigma Chemical Co., St. Louis, MO). High-performance TLC plates (20 × 20 cm, Silica Gel G plates (Analtech, Newark, DE) were used.

Band determination for analysis. The phospholipid bands were determined using 4.1 mg/mL PC, 3.6 mg/mL PE, and 2.7 mg/mL PI dissolved in hexane. A standard phospholipid mixture containing 39% PC, 22% PI, and 27% PE (4 mg/mL hexane) was prepared and used to determine band assignments for calibration of the FTIR method. This standard phospholipid mixture of PE, PC, and PI approximated that in soybean oil.

Calibration standards. Calibration curves were generated using the standard phospholipid mixture at concentrations of 4, 2.0, 1.0, 0.50, 0.25, and 0.13 mg/mL hexane. These standard solutions were used to generate calibration equations, determine reproducibility, and validate the FTIR method.

FTIR. An Impact 410 FTIR instrument (Nicolet, Madison, WI) was used for analysis. A sodium chloride stainless steel sealed precision pathlength cell (Nicolet, Madison, WI) with a total volume of 0.11 mL and a pathlength of 0.1 mm was used. A nominal resolution of 4 cm^{-1} and scan number of 100 were used to generate the spectra using automatic gain. Calibration standards and phospholipid extract samples were loaded into salt plate cells with a microsyringe. The salt plates were cleaned three times with ethanol and then twice with hexane after each sample. The FTIR bands between 1765 and 740 cm^{-1} were compared and examined statistically by correlating with concentration. Hexane was subtracted from each spectrum to eliminate the functional groups common to the sample and solvent (20).

Sample preparation. Phospholipid samples from CSBO were prepared for analysis by adding distilled water (2% vol/vol) to 500 g CSBO, followed by heating at 90°C for 30 min with stirring. The oil was centrifuged at 5,000 rpm for 20 min and decanted before the phospholipid-rich layer was washed three times with 100 mL acetone. The crude phospholipid extract was then dried under vacuum to remove the residual acetone remaining after washing. Four replicate preparations were obtained and designated PL_1, PL_2, PL_3, and PL_4.

The phospholipid extracts were further purified by passing through a column of silicic acid (70–230 mesh) 30 cm long and 2.5 cm in diameter. Approximately 4.5 wt% phospholipid extract was introduced to the top of the column. Nonpolar components were eluted with 50 mL of chloroform and the phospholipids subsequently eluted with 100 mL of methanol. The solvent was then evaporated under vacuum.

The phospholipid content in the crude and the degummed soybean oil was determined by dissolving 20 mg sample/mL hexane. Each spectrum was subtracted from a spectrum containing 20 mg RBDO/mL to remove the C-H and C-O-C stretching, bending, and wagging vibrations due to triglycerides. Standard phospholipid calibration equations were used to estimate the phospholipid content in the subtracted spectra.

TLC–imaging densitometry (TLC–ID). This was performed as an additional method of comparison for FTIR (6). Phospholipid standards used for FTIR calibration were spotted on TLC plates and developed using a solvent system consisting of 75:25:3 of chloroform/methanol/ water (vol/vol/vol). The TLC plates were developed in iodine and then scanned using the Biorad® model GS-670 ID system (Richmond, CA). Regression models were generated using pixel area vs. phospholipid concentration.

Statistical analysis. The SAS Inc. (Cary, NC) 1988 computer program was used to generate linear regression equations and Pearson correlation coefficients for phospholipid band area vs. concentration. Since the concentration of more than one phospholipid would vary from one phospholipid extract to another, a multivariate calibration approach was used in order to account for contributions of additional components to the absorption spectrum. Regression coefficients were determined between the FTIR method and the TLC–ID method.

RESULTS AND DISCUSSION

Determination of FTIR bands for phospholipid analysis. A preliminary study was performed to identify the optimal bands for phospholipid analysis. Typical FTIR spectra for individual standards of PC, PI, and PE subtracted from a hexane spectrum are shown in Figure 1. Differences and similarities in functional group vibration were observed. PC, PI, and PE spectra all had a vibration band at 1200–1145 cm^{-1} due to the PO_2 group vibration. PC and PI had a medium PO_2 vibration at approximately 980 cm^{-1}, while none was observed in the PE spectrum. These differences made it difficult to measure individual phospholipids in an oil. Figure 2 shows a spectrum of 2 mg/mL standard phospholipid mixture (39% PC, 22% PI, and 27% PE) with and without hexane subtraction. The spectra of phospholipid in hexane and that of the subtracted sample are shown. The phospholipid standard mixture gave a better representation of phospholipid present in soybean oil compared to the individual phospholipid spectra (Fig. 1). Differences were observed between the phospholipid standard mixture spectrum in hexane and the subtracted spectrum in the region between 1200 and 970 cm^{-1}. Hexane was chosen as the solvent since phospholipids dissolved well and there are no interfering bands between 1200 and 970 cm^{-1} in the hexane spectrum. Subtraction was also important to increase calibration accuracy. One of the fundamental strengths of

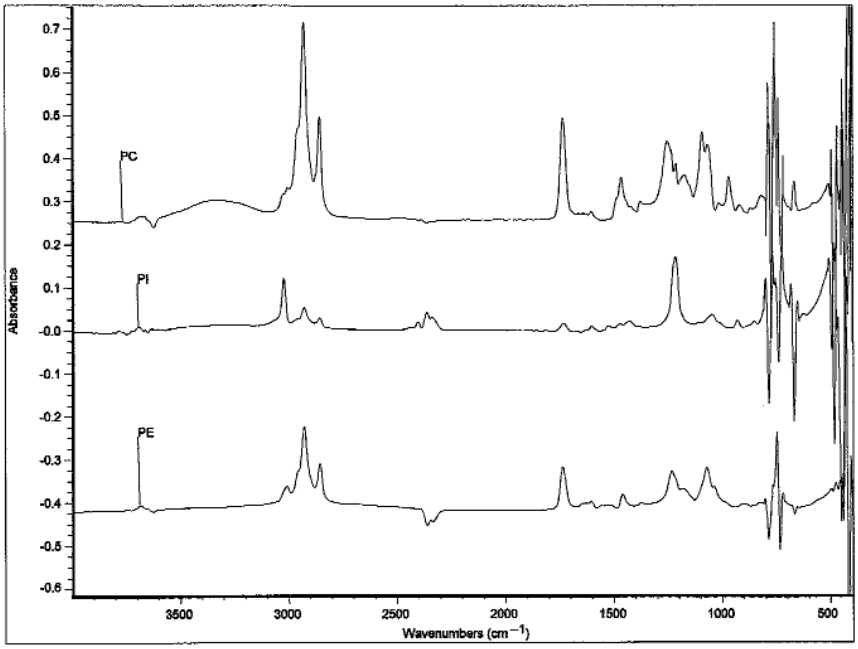

FIG. 1. The Fourier transform infrared spectroscopy (FTIR) spectra of 4.1 mg/mL phosphatidylcholine (PC), 2.9 mg/mL phosphatidylinositol (PI), and 3.64 mg/mL phosphatidylethanolamine (PE) solution in hexane with hexane subtracted. One hundred scans were co-added with a resolution of 4 cm^{-1} (*y* axis not on common scale).

FTIR is its ability to ratio spectra in order to see small differences that normally might not be apparent in the original spectra (17). These differences are not apparent in the unratioed standard (Fig. 2). Subtraction of the hexane spectrum yielded the O-H vibration band at about 3400 cm^{-1} that was not evident in the original spectrum. Table 1 shows the FTIR band assignments for phospholipids. Common vibrational regions in the phospholipid spectra were essentially the PO_2 and P-O-C vibrations. The region 1200–970 cm^{-1} contained very weak solvent absorption.

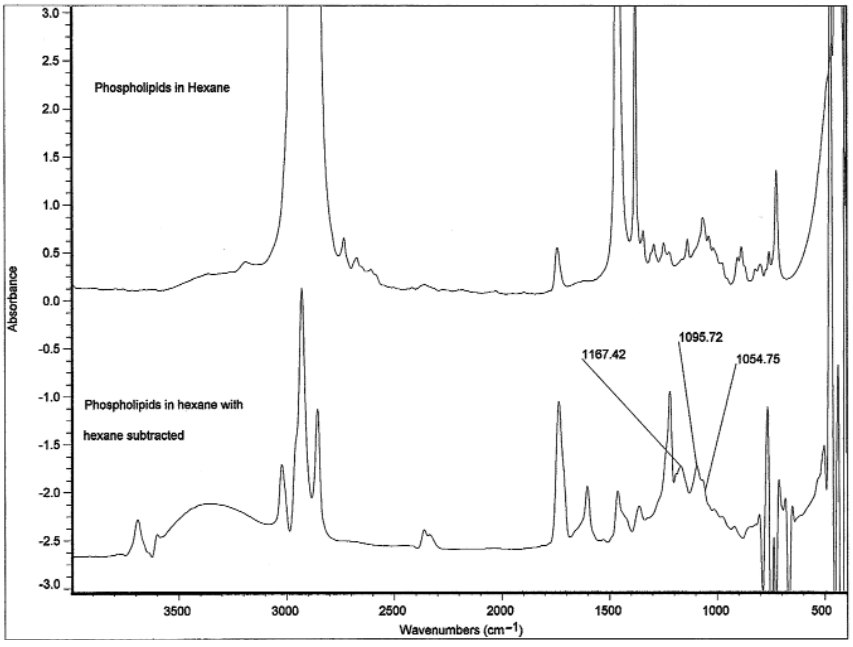

FIG. 2. The FTIR spectra of 2 mg/mL phospholipid standard mixture (39% PC, 22% PI, and 27% PE) with and without hexane subtraction. One hundred scans were co-added with a resolution of 4 cm^{-1} (*y* axis not on common scale). See Figure 1 for abbreviations.

TABLE 1
FTIR Band Assignment and Regression Equations for Standard Phospholipid

Bands	Vibrational mode	Wavelength (cm^{-1})	Equations[a]	R^2
1	C=O	1765–1720	y = –0.0245 + 0.329(PL)	0.995
2	PO_2	1200–1145	y = –0.1056 + 0.483(PL)	0.987
3	P-O-C ν_{asym}	1145–970	y = 0.1560 + 0.153(PL)	0.990
4	P-O-C + PO_2	1200–970	y = 0.0453 + 0.312(PL)	0.968
5	PO_2	830–740	y = 0.7010 + 0.512(PL)	0.618

[a]$y = a + bx$ where x is the concentration of the standard phospholipid mixture (39% PC, 22% PI, and 27% PE), a is the intercept, b is the slope, and y is the FTIR-corrected area. Abbreviations: FTIR, Fourier transform infrared spectroscopy; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

Figure 3 shows the PL_4 spectrum with hexane subtracted. This was very similar to the spectrum of standard phospholipid mixture (Fig. 2). Figure 4 shows the differences between PL_1 and PL_4 at the PO_2 stretching around 783 cm^{-1}. The difference seen may be attributed to the presence of water in the phospholipid miscella making the PO_2 vibration weaker and producing a weak band. This phosphate-stretching region shows intensity differences probably due to increased water–phosphate interaction in PL_1. This phenomenon has the effect of reducing the peak at 783 cm^{-1} in the PL_1 while the same band is very evident in PL_4. The band at 783 cm^{-1} is sharper and more defined when the phospholipid is less hydrated, an effect that signifies the reorientation effects due to increased lattice order and increased vibrational energy at the characteristic phospholipid group (21). Reduction in band width and increase in peak height indicates a reduction in the mobility of various functional groups. Two other phosphate bands found in the spectra of hydrated phospholipids are the P-O-C asymmetric and symmetric stretching bands of the phosphate group near 1167 and 1085 cm^{-1}, and the $(CH_3)_3N$ stretching band near 972 cm^{-1}.

The P=O vibration lies between 1300 and 1250 cm^{-1} (18). However, this region is too extensive and overlaps with C-O-C vibrations. Ville *et al.* (15) found the absorption pattern between 1282 and 1020 cm^{-1} useful for calculating a phospholipid calibration curve. However, at about 1210 cm^{-1}, the vibration for C-O-C is fairly strong and therefore interferes with the calibration. Figure 5 shows the spectra of a phospholipid standard mixture and that of a phospholipid extract, PL_2, containing some triglyceride. This sample shows that some triglyceride was still present after the degumming process. There is an increase in the peak area at 1210 cm^{-1} due to the C-O-C vibration that makes this peak unreliable for quantitative phospholipid determination.

Broad phosphate stretches are evident at 1169, 1084, and 1063 cm^{-1} (Fig. 6). The quaternary amine headgroup (PC and PE) is characterized by 1063 and 1084 cm^{-1} due to asymmetrical and symmetrical stretching at this headgroup.

Band 1 (1765–1720 cm^{-1}) corresponds to a strong C=O due to the ester groups present in the phospholipid molecule (Table 1). This band is not a good indication of the presence of phospholipids because it can also be attributed to other lipid components and derivatives, such as triglycerides, but it can be used in conjunction with other bands to determine the presence of phospholipids in a given sample. The bands selected for quantitative phospholipid determination were those between 1200 and 950 cm^{-1} and the C=O band between 1765 and 1720 cm^{-1}. The band at 830–740 cm^{-1} was not used for

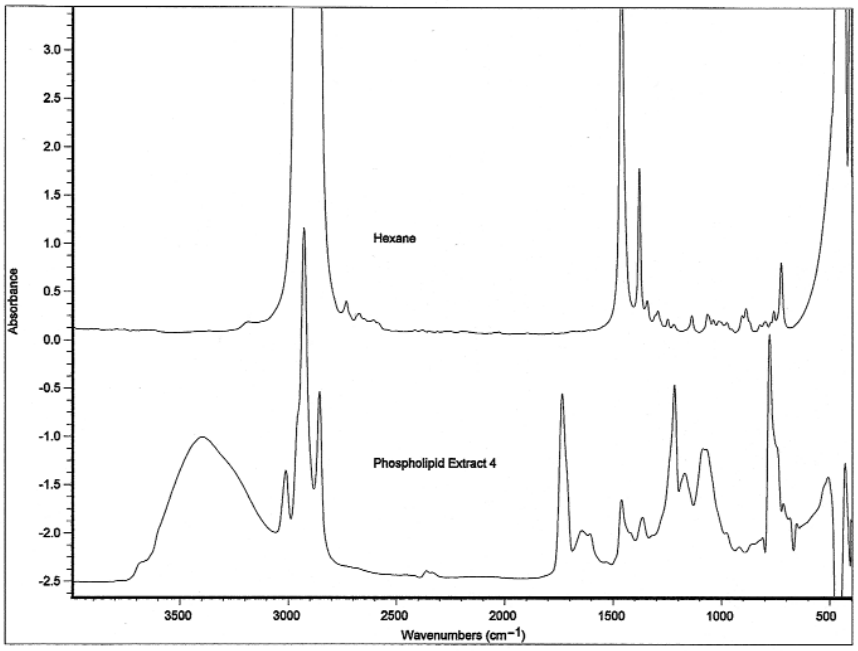

FIG. 3. FTIR spectrum of hexane and PL_4 (20 mg/mL in hexane) solution with hexane subtraction. One hundred scans were co-added with resolution of 4 cm^{-1} (y axis not on common scale). See Figure 1 for abbreviation.

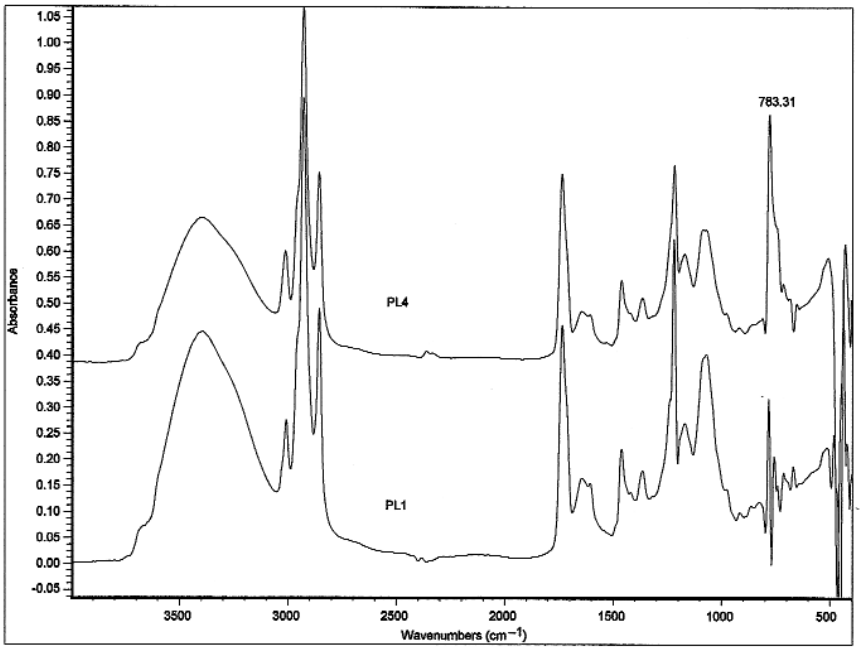

FIG. 4. FTIR spectra of 20 mg/mL PL_1 and PL_4 in hexane with hexane subtraction. One hundred scans were co-added with a resolution of 4 cm^{-1} (*y* axis not on common scale). See Figure 1 for abbreviation.

phospholipid analysis because it varied with the orientation effects at the water–phosphate group.

Quantitative determination of phospholipid. The quantitative phospholipid analysis was restricted to wavelengths that showed increasing intensity with increasing concentration. These were at 1765–1720 cm^{-1} (band 1), 1200–1145 cm^{-1} (band 2), 1145–970 cm^{-1} (band 3), and 1200–970 cm^{-1} (band 4). Figure 7 shows the calibration curves for FTIR area vs. the concentration of phospholipid standards while Table 1 shows regression equations for individual phospholipid bands. The R^2 ranged from 0.618 to 0.995. Band 5 (830–740 cm^{-1}) gave the lowest R^2 at 0.618 possibly owing to water binding at the PO_2 site, weakening the vibration at this group, and reducing the peak area (Fig. 4). The reproducibility of FTIR phospholipid band concentration is shown in Table 2. FTIR was found to be reproducible at low phospholipid concentrations, even as low as 0.25

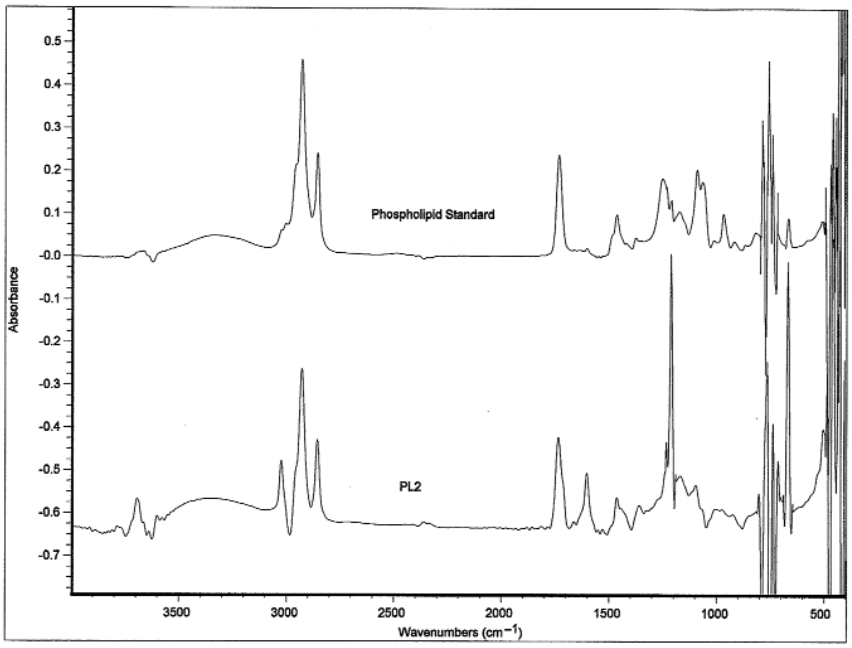

FIG. 5. FTIR spectra of standard phospholipid mixture (2 mg/mL) and PL_2 extract (10 mg/mL) with hexane subtraction. One hundred scans were co-added with a resolution of 4 cm^{-1} (*y* axis not on common scale). See Figure 1 for abbreviation.

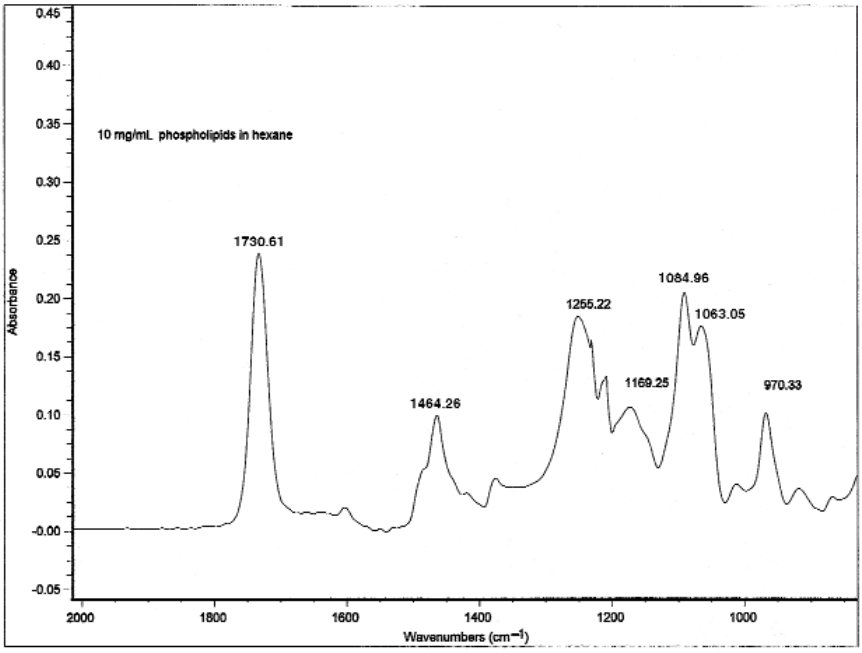

FIG. 6. The spectrum of 10 mg/mL PL_3 in hexane with hexane subtraction.

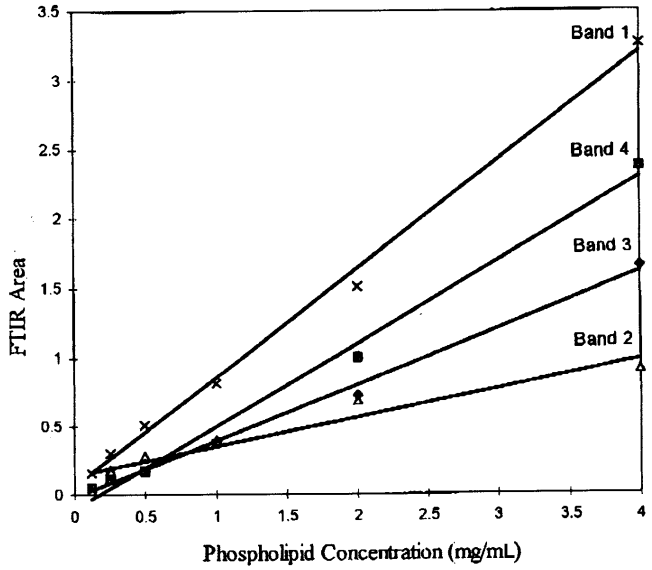

FIG. 7. The standard phospholipid mixture (39% PC, 22% PI and 27% PE) calibration curves for FTIR. Band 1 (X), 1765–1720 cm^{-1}; band 2 (△), 1200–1145 cm^{-1}; band 3 (◆), 1145–970 cm^{-1}; band 4 (■), 1200–970 cm^{-1}. See Figure 1 for abbreviations.

TABLE 2
Reproducibility of FTIR Phospholipid Concentration[a]

Concentration	Band 1		Band 2		Band 3		Band 4		Band 5	
(mg/mL)	Mean[b]	SD[c]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0.16	—		—		—		0.13 ± 0.02		—	
0.26	0.21 ± 0.08		0.13 ± 0.14		0.19 ± 0.14		0.29 ± 0.07		—	
0.60	0.58 ± 0.01		0.41 ± 0.05		0.45 ± 0.02		0.57 ± 0.04		0.12 ± 0.14	
1.25	1.24 ± 0.04		0.93 ± 0.08		1.01 ± 0.11		1.23 ± 0.05		0.73 ± 0.15	
2.50	2.49 ± 0.06		1.34 ± 0.19		1.44 ± 0.16		2.54 ± 0.08		1.43 ± 0.09	
5.00	4.92 ± 0.03		2.57 ± 0.19		2.72 ± 0.08		4.89 ± 0.15		2.38 ± 0.25	

[a]The corrected area of four replicate samples of the standard phospholipid mixture (39% PC, 22% PI, and 27% PE) were used to generate the band concentrations.
[b]Mean concentration of four replicate samples.
[c]Standard deviation. For other abbreviations, see Table 1.

TABLE 3
Determination of Phospholipid Content by FTIR

Phospholipid	Extract[a]	Concentration[b] (mg/mL)	RSD	Phospholipid in extract[c] (%)	Phospholipid (%)	
					CSBO[d]	DCSBO[e]
PL_1	Band 1	34.84	2.97	174.2		
	Band 2	9.67	0.87	48.4		
	Band 3	8.48	1.58	42.4		
	Band 4	19.76	1.06	98.8	1.65	0.0170
PL_2	Band 1	30.44	1.23	152.2		
	Band 2	9.46	0.24	47.3		
	Band 3	8.41	2.08	42.1		
	Band 4	19.44	1.30	97.2	1.54	0.0074
PL_3	Band 1	35.42	2.03	177.1		
	Band 2	9.95	0.73	49.7		
	Band 3	9.24	2.53	46.2		
	Band 4	18.28	1.70	91.4	1.82	0.0118
PL_4	Band 1	34.80	1.57	174.0		
	Band 2	9.59	1.34	48.0		
	Band 3	6.60	1.63	33.0		
	Band 4	18.04	1.52	90.2	1.79	0.0093

[a]Phospholipid extract, 20 mg/mL in hexane, with hexane subtracted.
[b]Four replicate sample spectra used.
[c]Percent phospholipid = concentration from linear equation/20 mg/mL × 100.
[d]Crude soybean oil (CSBO) (20 mg/mL in hexane).
[e]Degummed crude soybean oil (DCSBO) (20 mg/mL in hexane). See Table 1 for abbreviation.

mg/mL in hexane. Bands 2 and 3 gave lower concentration values than did bands 1 and 4.

Band 4 gave a good estimate of phospholipid content and therefore was used to determine the phospholipid content of the CSBO and degummed soybean oil. Crude phospholipid extracted from CSBO by water degumming was found to be approximately 1.56% (w/w), and after purification it was approximately 1.12% phospholipid (w/w). The concentration as well as percentage of phospholipid in the extracts was determined (Table 3). Band 1 gave phospholipid percentages much higher than 100. The reason for this is that even though the C=O region produced a high linear correlation (Table 1) it may not give an accurate measurement of phospholipid alone because of the presence of nonphospholipid components that

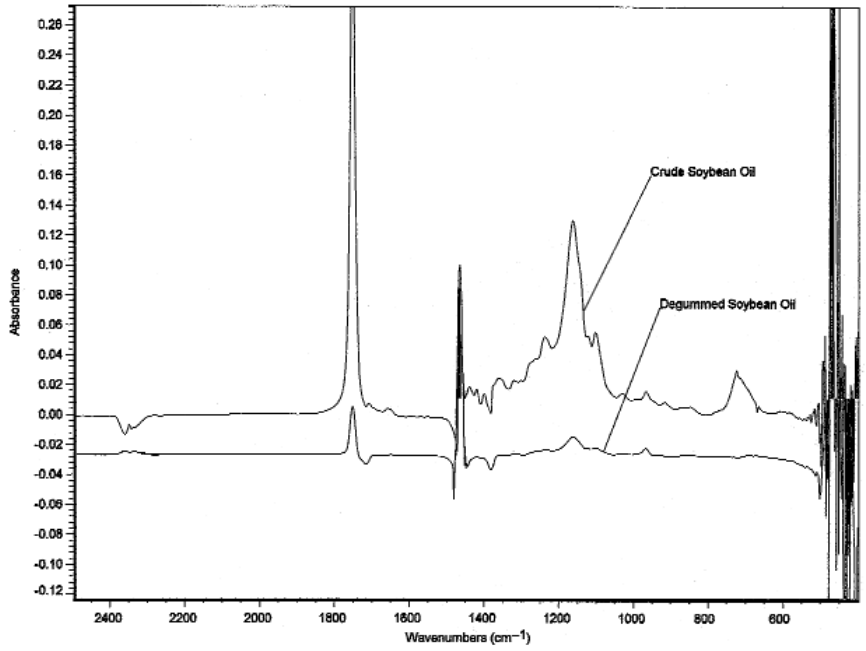

FIG. 8. The spectra of 20 mg/mL crude soybean oil and degummed soybean oil in hexane subtracted from 20 mg/mL refined bleached deodorized soybean oil in hexane. One hundred scans were co-added with a resolution of 4 cm^{-1} (*y* axis not on common scale).

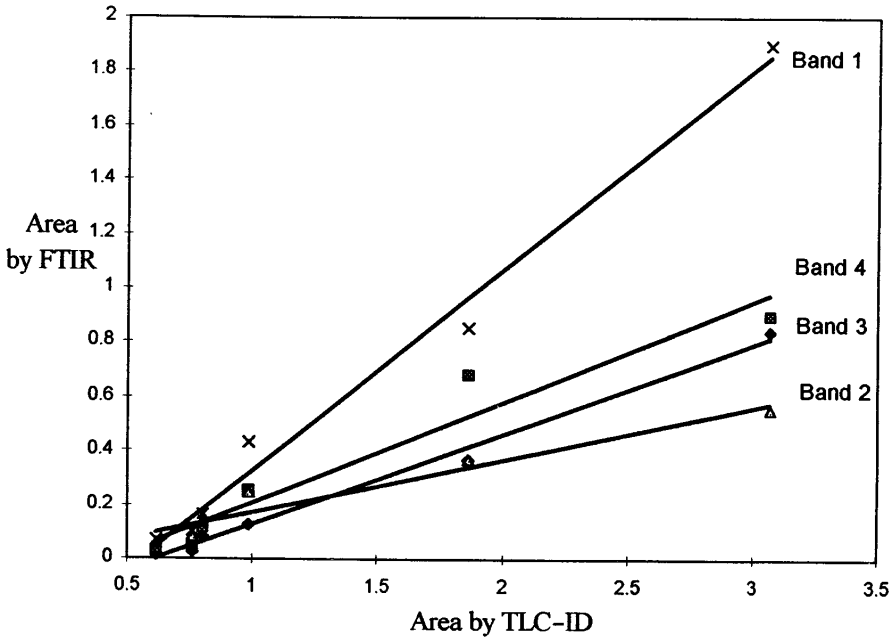

FIG. 9. Linear regression curves for corrected area of FTIR vs. the area by thin-layer chromatography–imaging densitometry (TLC–ID) for standard phospholipid mixture. See Figure 1 for abbreviation.

exhibit similar C=O stretching vibration. PL_1 had 98.8% phospholipid, whereas PL_4 had the lowest phospholipid content of the four extracts at 90.2% (Table 3). Figure 8 shows the spectra of the CSBO and that of the degummed oil subtracted from the RBDO spectrum in hexane. The CSBO phospholipid content using FTIR calibration equations was found to be approximately 1.77% (Table 3). This compares well with the expected amount, approximately 2–3%. The degummed oil was found to contain a phospholipid content of approximately 0.0113%.

Figure 9 shows the linear regression analysis between FTIR bands and TLC–ID used to generate a linear fit. High correlation coefficients were generated for the comparative regression analysis between FTIR and TLC–ID. When using TLC–ID, the measurement of the area depends on many factors that can vary from one TLC plate to another. The R^2 for band 1 was 0.993, band 2 was 0.933, band 3 was 0.937, while band 4 was 0.959. The FTIR area and accuracy as well as reproducibility of the phospholipid were greater than that of TLC–ID owing to the higher sensitivity that can be achieved by FTIR. TLC–ID had a higher area per concentration than FTIR. This may be due to impurities present on the TLC plates.

In this study, it was important to use a multivariate calibration of the FTIR method. The use of a multicomponent standard made the calibration more accurate than single standards that had slight differences in the vibrational bands. Differences were observed between phospholipid samples with and without interfering lipids, such as triglycerides. Reorientation effects at characteristic phospholipid groups were also observed.

The FTIR method proved useful at determining the phospholipid content in phospholipid extracts and residual phospholipids in degummed oil. The band from 1200–970 cm^{-1} due to P-O-C + PO_2 was found to be extremely useful in the quantitative determination of phospholipids. An FTIR run takes approximately 30 s for 100 scans and does not require highly trained personnel to run the instrument. It is therefore a rapid and reliable method that could provide substantial savings in the fats and oils industry in terms of time and labor. It also has the additional benefit of a reduction in industry solvent and reagent waste, since only about 0.1 mL solvent is required per FTIR run when compared to other instrumental methods, such as HPLC that require large solvent volumes (9,17). More research to obtain equations that account for the vibration due to other nonphospholipid components is still required to increase the accuracy as well as precision of this method.

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