Phospholipid Fatty Acid Composition and Stereospecific Distribution of Soybeans with a Wide Range of Fatty Acid Composition

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ABSTRACT: Phospholipid (PL) fatty acid composition and stereospecific distribution of 25 genetically modified soybean lines with a wide range of compositions were determined by gas chromatography and phospholipase A_2 hydrolysis. PL contained an average of 55.3% phosphatidylcholine, 26.3% phosphatidylethanolamine, and 18.4% phosphatidylinositol. PL class proportions were affected by changes in overall fatty acid composition. PL fatty acid composition changed with oil fatty acid modification, especially for palmitate, stearate, and linolenate. Stereospecific analysis showed that saturated fatty acids were primarily located at the *sn*-1 position of all PL, and changes of the saturates in PL were largely reflected on this position. Oleate was distributed relatively equally between the *sn*-1 and *sn*-2 positions. Linoleate was much more concentrated on *sn*-2 than on *sn*-1 position for all PL. Linolenate was distributed relatively equally at low concentration but preferred *sn*-2 position at high concentration.

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KEY WORDS: Fatty acid stereospecific distribution, gas chromatography, genetic modification of soybeans, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phospholipase A_2, phospholipid, thin-layer chromatography.

Modification of the fatty acid composition of soybean oil to make it more competitive in various segments of the food and industrial oil markets (1) has been an important objective of plant breeding and molecular genetics in recent years. Altered fatty acid compositions have been developed through traditional plant breeding (2) and application of chemical mutagens (3–6) that have extended the range of the five major fatty acids found normally in soybean oil (palmitate, stearate, oleate, linoleate, and linolenate). Aside from the triglycerides (TG), which typically make up more than 99% of refined soybean oil, soybeans also contain 0.3 to 0.6% phospholipids (PL), with phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) being the major classes (7). These PL are the major components of the cell membranes that form the outer boundary of the cell and divide it into various compartments. It is important that these membrane PL be in the proper physical state for cells to perform their metabolic tasks, and this proper physical state requires that PL have the correct balance of saturated and unsaturated fatty acids, as well as a balanced PL class composition (8). The arrangement of fatty acyl groups within individual PL classes can be an important factor in determining their physical characteristics and is carefully controlled (9).

PL compositional changes in the membranes of organisms in response to environmental temperature have been studied extensively (10,11). Soybeans produced under drought and high-temperature conditions contain PL with altered fatty acid compositions that affect the ability of the seed to maintain optimum rates of metabolism and germination (12). Information about the effect of genetic modification of soybean on PL fatty acid composition is limited. Mounts *et al*. (13) reported a study on PL, tocopherols, and sterols in several soybeans with low linolenate and increased saturate percentages. They concluded that there was little impact of fatty acid composition on the proportions of the PL classes, but considerable change occurred in the molecular species present in each class.

Do soybeans with fatty acid composition modified far from its typical range have PL that are altered in ways that may be detrimental to the plant? In this study, soybean lines with the wide ranges of the five major fatty acids were analyzed for PL composition and stereospecific distribution to determine the effect of alteration in the overall fatty acid composition on the composition and distribution of PL.

EXPERIMENTAL PROCEDURES

Materials. Commercial soybean cultivars and experimental soybean lines were provided by the Agronomy Department at Iowa State University (Ames, IA). PL and lyso PL standards

TABLE 1
Typical Soybean Fatty Acid Percentage and the Composition Range Examined in This Study

	Palmitate	Stearate	Oleate	Linoleate	Linolenate
Typical	11	4	24	54	7
Range	3.1–33.3	2.4–24.2	7.8–35.3	35.3–68.3	2.7–16.3

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TABLE 2
Average Soybean Crude Lipid and PL Compositions and Their Standard Deviations (SD)

	Lipid (%)[a]	PC[b]	PC (%)[c]	PE[b]	PE (%)[c]	PI[b]	PI (%)[c]	Tot. PL[b]	Tot. PL (%)[d]
Average	23.7	4.7	55.3	2.3	26.3	1.6	18.4	8.5	3.7
SD	3.8	1.4	3.5	0.8	2.8	0.5	2.5	2.4	1.2

[a]Crude lipid in the bean.
[b]mg/g bean.
[c]Individual phospholipid (PL) relative to total PL.
[d]Total PL in crude lipid. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

(from soybean) and snake venom from *Crotalus adamentus,* containing phospholipase A_2, were obtained from Sigma Chemical Co. (St. Louis, MO). All organic solvents were reagent grade.

Lipid extraction and class separation. Approximately 10 g of seed (fresh weight, about 9% moisture) was ground with a Wiley mill equipped with a 20-mesh delivering tube. Duplicate samples of 2.00 g ground beans were extracted with 30 mL chloroform/methanol (2:1, vol/vol) for 1 h with stirring. The sample was filtered and washed with additional solvent, and 0.75% KCl, equal to 20% of the final total volume, was added to the filtrate. The lower chloroform phase was collected, and the solvent was removed with a rotary evaporator at 40˚C. Total crude lipid was measured gravimetrically. Neutral and polar lipid class separation was achieved by solid-phase extraction by a modification of the method of Mounts *et al.* (13). A 900-mg silica cartridge (Alltech Associates, Inc., Deerfield, IL) was loaded with about 0.4 g of crude lipid dissolved in chloroform. Neutral lipid, TG, was eluted with 30 mL chloroform and sampled for fatty acid composition analysis by gas chromatography (GC); polar lipids were sequentially eluted with 10 mL chloroform/methanol (1:1, vol/vol), 20 mL methanol, and 15 mL chloroform/methanol/water (1:2:0.8, vol/vol/vol). Phase separation of the last elution was obtained by adding 7.6 mL chloroform and 3.0 mL water, and the lower chloroform layer was combined with the other two elutions. Solvent was removed by a rotary evaporator, and the polar lipids were redissolved in 0.5 mL chloroform/methanol (8:2, vol/vol).

PL class separation. To separate the major PL classes, 0.15 mL of the total polar lipid solution was streaked on a 20 × 20 cm, 500-µ Adsorbsil-plus1 preparative plate (Alltech), and the plate was developed with chloroform/methanol/acetic acid/water (100:45:5:2, by vol). Bands were visualized by spraying with 0.1% 2′,7′-dichlorofluorescein (Sigma) in methanol and viewing under ultraviolet radiation. PC, PE, and PI bands (R_f were 0.28, 0.70, and 0.56, respectively) were identified by comparison with standards. The bands were scraped from the plate and extracted five times with 15 mL chloroform/methanol/water (1:2:0.8, vol/vol/vol) (14). Phase separation was performed as already described, and the chloroform layer was evaporated under nitrogen. Next, 1 mL of freshly distilled ethyl ether was added to redissolve the PL.

PL quantitation and stereospecific analysis. From the 1-mL ether solution of PL, 0.2 mL was taken for PL fatty acid analysis and quantitation. The remaining 0.8 mL was used for the stereospecific distribution analysis. A modified Robertson and Lands procedure (15) was used for PL *sn*-2 position fatty acid hydrolysis. The PL samples were shaken gently overnight at 37˚C with 0.2 mL of 5 mg/mL snake venom in a 50:50 mixture of 0.1 M borate buffer at pH 7.0 and 0.01 M calcium chloride. The hydrolyzed mixture was dried under nitrogen and suspended in 0.2 mL chloroform/methanol (8:2, vol/vol). The *sn*-2 position fatty acid and lyso PL were separated by thin-layer chromatography (TLC) by using the same system used for PL class separation to make sure there was no unhydrolyzed PL, which might bias the stereospecific analysis. R_f values for free fatty acid, lyso PC, lyso PE, and

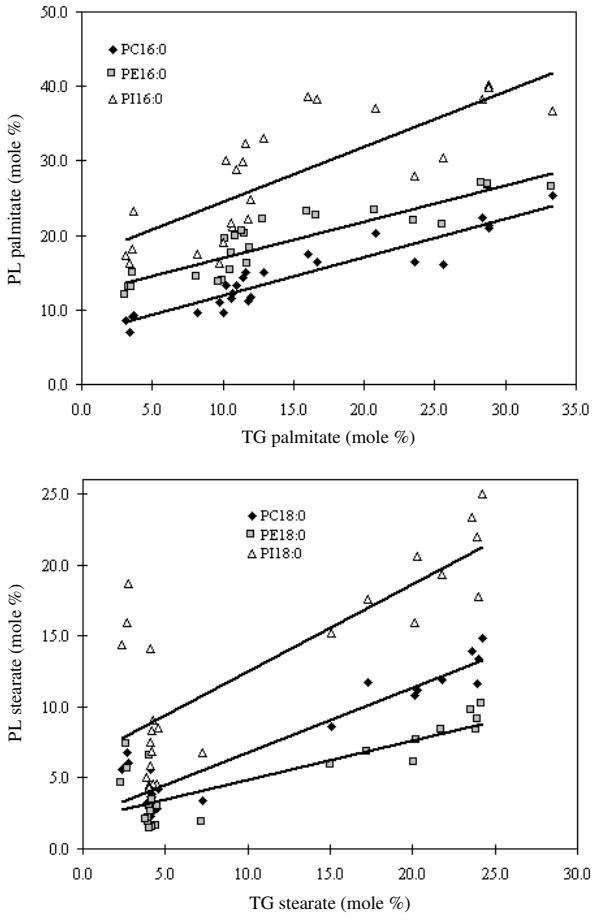

FIG. 1. Percentages of saturated fatty esters vs. triglyceride (TG) percentages for each phospholipid (PL) class. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

TABLE 3
Correlation Among PL and Crude Lipid Contents and Triglyceride (TG) Fatty Acid Composition

		Lipid (%)[a]	PC[b]	PC (%)[c]	PE[b]	PE (%)[c]	PI[b]	PI (%)[c]	Tot. PL[b]	Tot. PL (%)[d]
Total saturated	*r*	–0.51	0.40	–0.18	0.48	0.48	0.26	–0.30	0.42	0.61
	prob[e]	0.01	0.06	0.43	0.02	0.02	0.23	0.17	0.05	0.00
Palmitate	*r*	–0.60	0.32	–0.13	0.50	0.61	0.10	–0.52	0.35	0.62
	prob	0.00	0.14	0.56	0.02	0.00	0.66	0.01	0.10	0.00
Stearate	*r*	–0.06	0.20	–0.10	0.13	0.01	0.25	0.13	0.20	0.18
	prob	0.78	0.35	0.65	0.56	0.96	0.25	0.55	0.36	0.41
Oleate	*r*	0.57	–0.30	–0.18	–0.26	–0.09	–0.10	0.35	–0.27	–0.49
	prob	0.00	0.16	0.42	0.23	0.67	0.64	0.10	0.22	0.02
Linoleate	*r*	0.46	–0.37	0.29	–0.49	–0.55	–0.28	0.22	–0.41	–0.59
	prob	0.02	0.08	0.18	0.02	0.01	0.20	0.31	0.05	0.00
Linolenate	*r*	–0.56	0.19	0.21	0.13	–0.04	0.02	–0.24	0.15	0.39
	prob	0.01	0.39	0.35	0.55	0.86	0.93	0.27	0.50	0.07

[a]Crude lipid in bean.
[b]mg/g bean.
[c]Individual PL relative to total PL.
[d]Total PL in crude lipid.
[e]Probability of $r = 0$. See Table 2 for abbreviations.

lyso PI were 0.90, 0.08, 0.34, and 0.19, respectively. Free fatty acid and lyso PL bands were scraped from the plate, and the methyl ester conversion reaction was performed directly on silica.

*Fatty acid methyl ester (*FAME*) preparation and GC*. For TG fatty acid composition analysis, a 0.5-mL aliquot of the neutral lipid eluted from the cartridge was evaporated under nitrogen and reacted with 0.3 mL of 1.0 M sodium methoxide in methanol at ambient temperature for 40–60 min with occasional shaking (16). For PL fatty acid determination and quantitation, a 0.2-mL aliquot of PL was evaporated under nitrogen and reacted with 0.3 mL of the sodium methoxide reagent. Methyl heptadecanate (Sigma) was used as internal standard and added to the reaction mixture before termination of the reaction. Water was added to stop the reaction, and 0.3 mL hexane was used to extract the FAME. For transesterification of lyso PL on silica, a similar procedure was applied, but without adding internal standard, and 0.5 mL sodium methoxide was used. To esterify free fatty acid on silica (17), 0.5 mL of 2% sulfuric acid in methanol was added, and the mixture was reacted at 80°C for 1 h. The FAME (1 μL) were analyzed with a Hewlett-Packard (HP) (Avondale, PA) 5890A gas chromatograph, equipped with a flame-ionization detector and capillary DB-23 (15-m length, 0.25-mm i.d., and 0.25-μm film thickness) fused column from J&W Scientific (Deerfield, IL). Oven temperature was 220°C; inlet and detector temperatures were 250˚C; split ratio was 10:1. Theoretical correction factors were calculated (18) and applied to correct the FAME weight percentages. Mole percentages were calculated and reported.

RESULTS AND DISCUSSION

PL content. Table 1 shows the typical soybean fatty acid composition (19) and the composition range selected for this study. Table 2 presents the average percentage of the amounts and relative proportions of the crude lipid and PL. The extraction recoveries for PC, PE, and PI from the scraped silica were 94.2, 99.6, and 98.8%, respectively, when known amounts of these standards were applied to a TLC plate and developed with the solvent. In total, 25 soybean samples were analyzed. On average, the soybeans contained 23.7% crude lipid, and the crude lipid contained 3.7% total PL. Of the total PL, PC accounted for 55.3%, PE 26.3%, and PI 18.4%. PL reportedly makes up 1.5 to 5.0% of the crude hexane extractables from soybean seed and contains 35 to 46% PC, 25 to 27% PE, and 13 to 18% PI (12). Soybean PL also contains a minor amount of phosphatidylserine (PS), phosphatidic acid (PA), and lyso PC (7). The presence of PA and lyso PC usually has been associated with active phospholipase during seed storage (7,13) or laboratory sample manipulation.

Crude lipid percentage, amounts of PC, PE, PI, and total PL (mg/g bean), relative percentage of individual PL classes, and PL percentage in crude lipid were correlated with TG fatty acid percentages to explore the effect of oil composition on the quantity of PL. The results are summarized in Table 3. These data suggested that soybean oil percentage decreased with increased levels of total saturate ($r = –0.51$), palmitate ($r = –0.60$), and linolenate ($r = –0.56$), and increased with in-

TABLE 4
Slopes and R^2 of the Linear Regressions of the Percentage of Various Fatty Esters in Each PL Class vs. the Percentage of the Fatty Ester in TG

		$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
PC	Slope	0.52	0.46	0.14	0.33	0.53
	R^2	0.88	0.88	0.07 (0.20)[a]	0.34	0.57
PE	Slope	0.49	0.28	0.17	0.18	0.46
	R^2	0.80	0.67	0.11 (0.10)	0.20 (0.02)	0.47
PI	Slope	0.74	0.62	0.26	0.27	0.57
	R^2	0.61	0.62	0.35	0.57	0.70

[a]Numbers in parentheses indicate *P* value of the R^2 being equal to zero; R^2 without parentheses have $P < 0.001$. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; R^2, regression coefficient. See Tables 2 and 3 for other abbreviations.

TABLE 5
Slopes and R^2 of Linear Regressions for Stereospecific Plots of Fatty Esters in PC, PE, and PI

			$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
PC	*sn*-1	Slope	1.74	1.67	0.88	1.21	0.59
		R^2	0.92	0.97	0.87	0.76	0.74
	sn-2	Slope	0.15	0.33	1.11	0.59	1.34
		R^2	0.09 (0.14)[a]	0.52	0.96	0.36	0.89
		Slope sum	1.89	2.00	1.99	1.80	1.93
PE	*sn*-1	Slope	1.96	1.72	0.78	0.97	0.63
		R^2	0.94	0.89	0.86	0.42	0.68
	sn-2	Slope	0.19	0.15	1.14	0.90	1.19
		R^2	0.1 (0.12)	0.46	0.94	0.41	0.84
		Slope sum	2.15	1.87	1.92	1.87	1.82
PI	*sn*-1	Slope	1.86	1.77	0.86	1.05	0.11
		R^2	0.97	0.95	0.61	0.57	0.21 (0.02)
	sn-2	Slope	0.15	0.22	1.08	0.64	1.76
		R^2	0.17 (0.04)	0.73	0.85	0.15 (0.05)	0.92
		Slope sum	2.01	1.99	1.94	1.69	1.87

[a]Numbers in parentheses indicate P value of the R^2 being equal to zero; R^2 without parentheses have $P < 0.001$. See Tables 2–4 for abbreviations.

creased levels of oleate ($r = 0.57$) and linoleate ($r = 0.46$). In contrast, the percentage of total PL in total lipid increased with increased levels of total saturate ($r = 0.61$), palmitate ($r = 0.62$) and linolenate ($r = 0.39$), and decreased with increases in oleate ($r = –0.49$) and linoleate ($r = –0.59$). Total PL and oil contents were not correlated ($r = –0.06$, not presented in Table 3). When oil content increases, either the size or the number of the oil bodies should increase, so that the PL half membrane surrounding the oil body should also increase. But the other organelle membranes also can be coextracted with oil and may mask the effect of oil body PL.

In general, the quantity of total PL did not correlate with any of the fatty acids ($P \geq 0.05$), and neither did the amount of individual PL, except for PE, which was positively correlated with total saturate and palmitate, and negatively with linoleate. PL class compositions (percentage of each PL class relative to total) were also correlated with various TG fatty acids. There were significant positive correlations of PE percentage with total saturate and palmitate and a negative correlation with linoleate. There also was a negative correlation between PI percentage and palmitate. These results suggest that fatty acid composition of soybean affects PL class composition, in contrast to Mounts *et al.* (13), who reported that PL class composition was unaffected by soybean genetic modification.

Relationship of PL and TG fatty acid composition. Figures 1 and 2 show the effect of changes in TG fatty ester percentage on PL fatty ester composition. Table 4 summarizes the slopes and regression coefficients (R^2) of these linear plots. In general, these plots show considerable scatter, but they illustrate a number of important relations. PI had greater palmitate and stearate percentages than did PC and PE. PC had the lowest palmitate percentage, whereas PE had the lowest stearate percentage. With the increase of TG palmitate and stearate, all three PL showed corresponding increases in these saturated esters. The slope of the PI plot was greater than those for PC and PE, suggesting that PI was the most sensitive PL to saturated fatty acid alteration. Note that for the PI palmitate plot, some points were well above and below the line. The group of points above the line had typical TG stearate level, whereas the group below the line had elevated TG stearate. Similarly, for the PI stearate plot, the group of points above the line had decreased TG palmitate, and the group of points below the line had elevated TG palmitate. The PC and PE plots also showed similar patterns of deviation, but less strongly than those of the PI plots. These deviations suggest that the presence of either of these saturates in PL suppresses the incorporation of the other.

The unsaturated fatty ester percentages of all PL also were positively correlated with their TG percentages (Fig. 2 and Table 4), but these plots again showed considerable scatter. In the oleate plot, the data points well above the regression lines had either low percentages of palmitate and stearate or low percentage linolenate. Generally, PC, PE, and PI contained similar percentage of oleate and linolenate. PC and PE contained much higher levels of linoleate than PI. The slopes of oleate and linoleate plots were smaller than those for the saturated esters and linolenate. All the slopes in Table 4 are less than one, indicating that PL acyl group percentages change less than those for TG as acyl percentages vary. These results are consistent with those of Mounts *et al.* (13).

PL fatty acid stereospecific distribution. Figures 3–7 show plots for PC, PE, and PI of saturated and unsaturated fatty ester percentages on the *sn*-1 and *sn*-2 positions as the total percentage of each fatty ester in a PL class varies. Table 5 presents a summary of the linear regressions fitted to these plots. Palmitate and stearate were predominantly located at the *sn*-1 position of all PL. The changes of palmitate in PC, PE, and PI were almost exclusively reflected on the *sn*-1 position. The changes of palmitate in *sn*-2 position were not statistically significant from zero for PC ($P = 0.14$) and PE ($P = 0.12$) at the 5% probability level, and barely significant ($P = 0.04$) in PI. The slopes of *sn*-1 and *sn*-2 regression lines theoretically should sum to 2.00 (20) and were 1.89 for PC, 2.15 for PE,

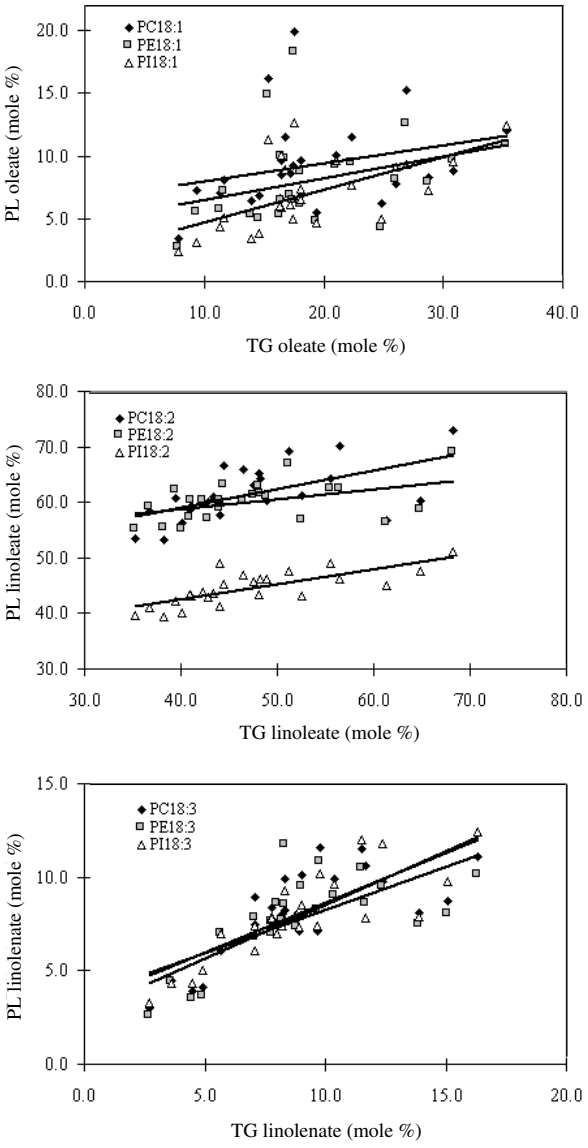

FIG. 2. Percentages of unsaturated fatty esters vs. TG percentages for each PL class. See Figure 1 for abbreviations.

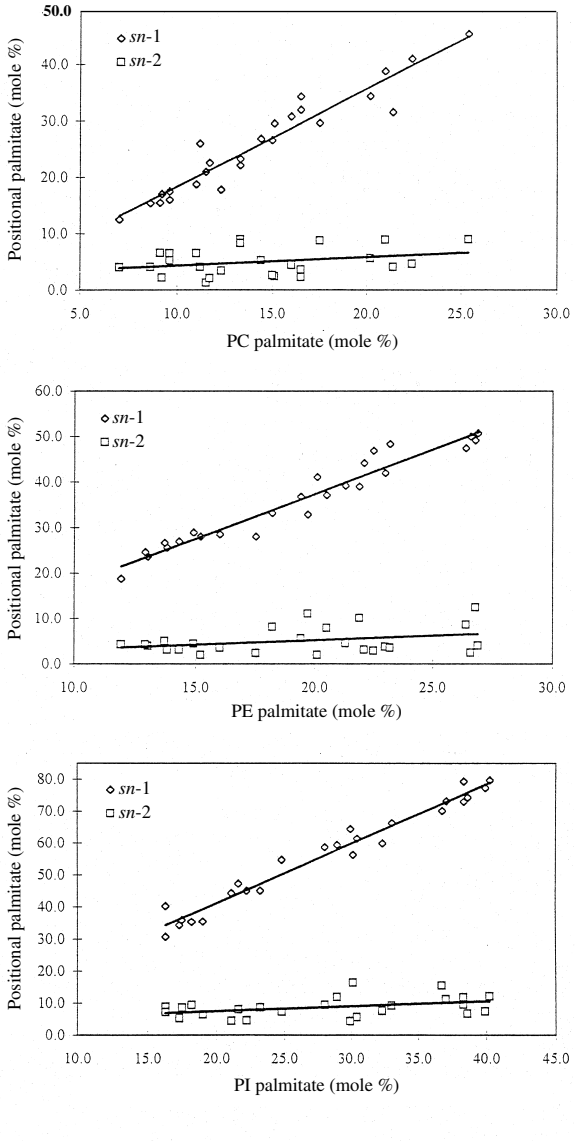

FIG. 3. Palmitate stereospecific distribution for PC, PE, and PI. See Figure 1 for abbreviations.

sn-1 position had a much greater change, as indicated by larger slopes. Although, statistically, the change at *sn*-2 position was significant ($P < 0.001$), the slopes were small and comparable to the palmitate *sn*-2 position slopes. The slope sums were 2.00, 1.87, and 1.99 for PC, PE, and PI, respectively. Therefore, saturated fatty acids primarily distributed at the *sn*-1 position of all PL, and this position accounted for the PL compositional change.

It is well documented that saturated fatty acids predominantly occupy the *sn*-1 and *sn*-3 positions of soybean TG, and these two positions reflected the saturated fatty acid change in oil (21–23). Harper (23) used soybean lines that had fatty acid composition ranges similar to those used in this study to analyze acyl group stereospecific distribution in TG. Similar graphs and linear regression were made. Harper (23) found that there was little saturate at the *sn*-2 position, and that the slopes of this position for palmitate and stearate were 0.04 and 0.08, as compared with the sums of *sn*-1 and *sn*-3 slopes of 2.94 and 2.91. Thus, the *sn*-2 position palmitate and stearate in PL had greater slopes than in TG.

Unsaturated fatty esters had a different distribution profile than those of the saturates. At low percentages of oleate in PL, oleate was equally distributed between the *sn*-1 and *sn*-2 positions. With an increase of oleate in the PL, more occupied the *sn*-2 position. Linoleate was much more concentrated on the *sn*-2 position than on the *sn*-1 position for all three PL, and for all three PL, the *sn*-1 slope was greater than the *sn*-2 slope. For all PL, linolenate was distributed relatively equally at both positions at low concentration, but as linolenate in PL increased, the *sn*-2 position accumulated significantly more linolenate than *sn*-1. This result again showed a great similarity to

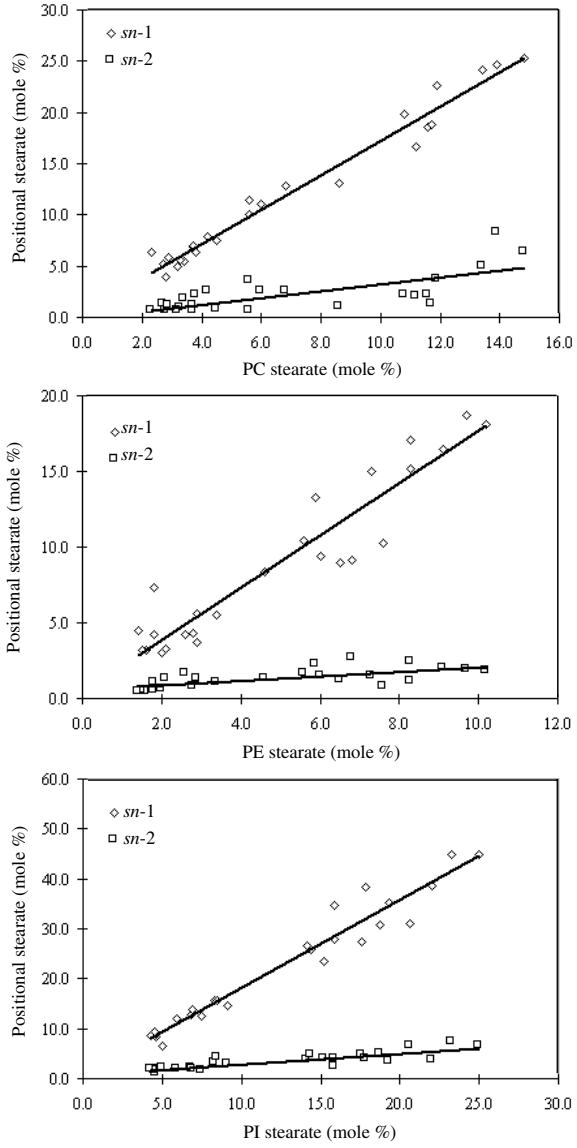

FIG. 4. Stearate stereospecific distribution for PC, PE, and PI. See Figure 1 for abbreviations.

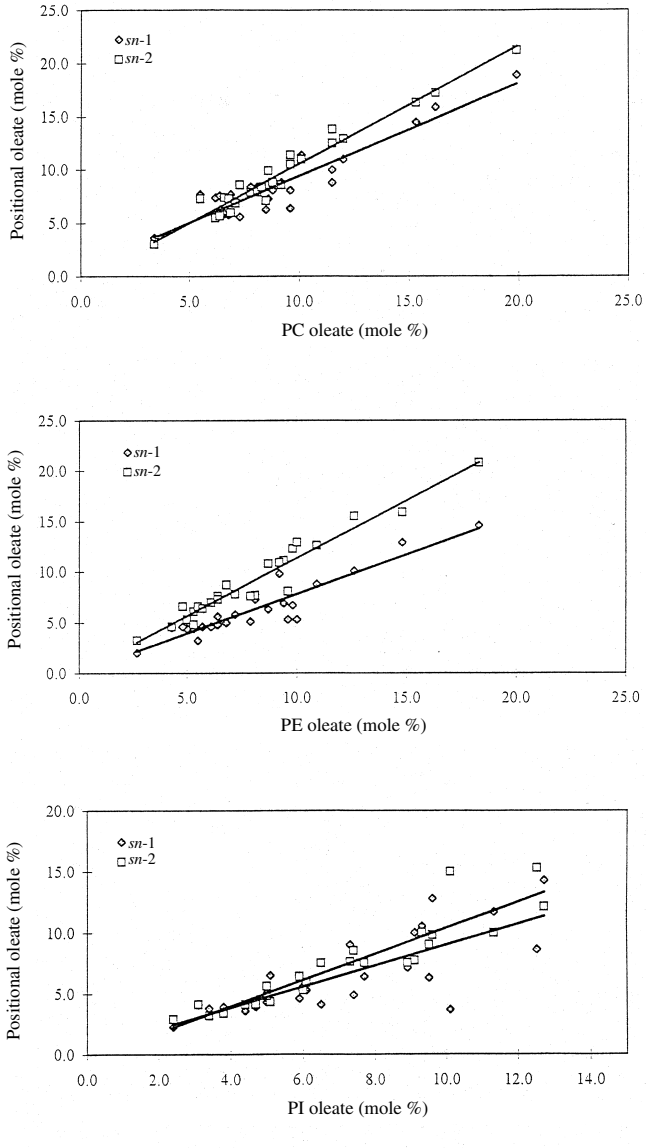

FIG. 5. Oleate stereospecific distribution for PC, PE, and PI. See Figure 1 for abbreviations.

their interrelated biosynthesis processes (24,25).

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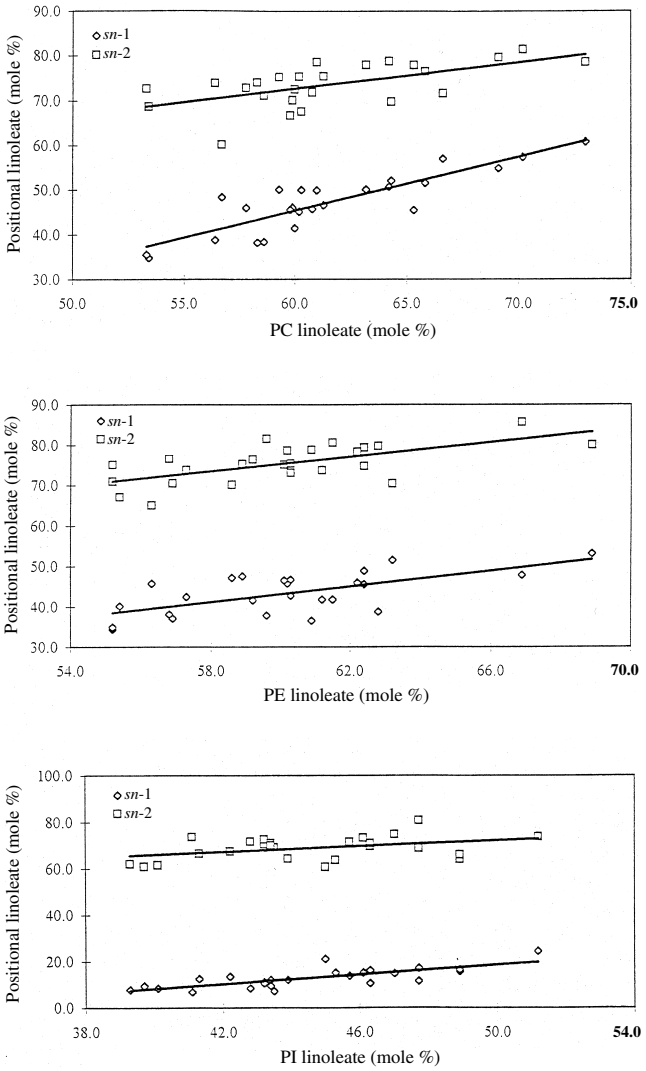

FIG. 6. Linoleate stereospecific distribution for PC, PE, and PI. See Figure 1 for abbreviations.

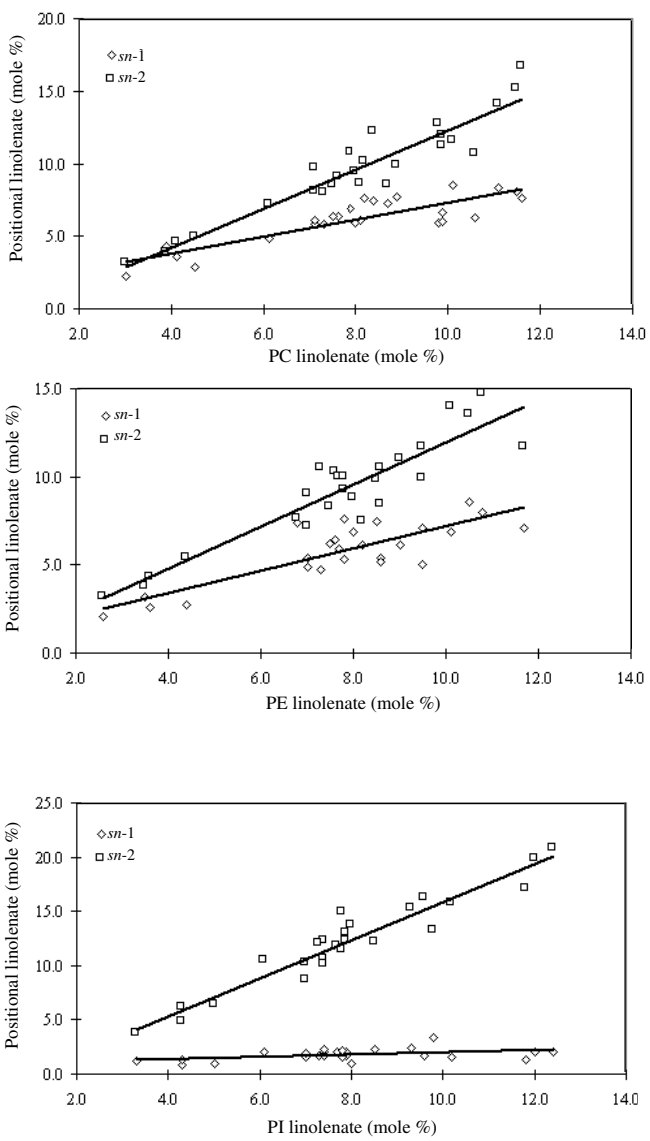

FIG. 7. Linolenate stereospecific distribution for PC, PE, and PI. See Figure 1 for abbreviations.

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