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Effect of Seed Development Stage on Sphingolipid and Phospholipid Contents in Soybean Seeds

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Glucosylceramide (GlcCer) and ceramide (Cer) are the predominant sphingolipids (SL) in soybeans. They have been recognized as functional components in plants and may have health benefits for humans. The objective of this study was to evaluate the changes in SL and phospholipid (PL) contents that occurred during seed development. Soybean seeds of three cultivars (IA1008, IA1010, and IA1014) were harvested at 5-day intervals from 28 days after flowering (DAF) to 68 DAF (mature seed). SL and PL contents of seeds were quantified using high-performance liquid chromatography (HPLC) with an evaporative light scattering detector (ELSD). SL and PL contents decreased significantly during seed development. Averaged across cultivars, Cer content on a dry weight basis decreased from 51.4 nmol/g at 28 DAF to 22.2 nmol/g at 68 DAF, whereas GlcCer content decreased from 522.8 nmol/g at 28 DAF to 135.8 nmol/g at 68 DAF. PL percentage of the total lipid decreased from 9.1% at 28 DAF to 3.5% at 68 DAF.

KEYWORDS: Sphingolipid; glucosylceramide; ceramide; phospholipid; developing soybean seeds; HPLC-ELSD

INTRODUCTION

Sphingolipids (SL) and phospholipids (PL) are structural and functional lipids found in cell membranes. Soybean SL and PL have attracted considerable interest because of their health benefits (*1*−*4*). Soybean PL, known as soy lecithin, has been utilized as a health-promoting ingredient in functional food products. SL has potential applications in the inhibition of colon cancer, reduction of low-density lipoprotein (LDL) cholesterol, and protection against bacteria toxins and infections (*1*). Soybean glucosylceramide (GlcCer), the predominant SL class in soybeans, has been identified as an inhibitor of colon cancer (*4*).

Few studies have been conducted on the content of the total SL in soybean seed. Ohnishi et al. (*5*) reported that ceramide (Cer) and GlcCer were the predominant SL in both immature and mature soybean seeds and that these SL were present in higher concentrations in immature seeds than in mature seeds. Sugawara et al. (*6*) and Gutierrez et al. (*7*) found that the GlcCer content of mature soybean seeds was about 110−472 nmol/g on a dry weight basis. No research has been reported on the content of SL and PL during soybean seed development. Consumption of immature soybeans as a green vegetable, commonly known as edamame, is becoming more popular in the United States. Therefore, it is important to know the contents of these minor, but potentially health-promoting components during different stages of seed development.

The development of soybean seed is commonly divided into three stages (*8*). The cell division of seed is completed at an early stage of development by 20−25 days after flowering (DAF). Seed growth occurs from about 25 to about 60 DAF when the seed reaches physiological maturity. During this stage, the majority of the lipids, proteins, and carbohydrates are synthesized and accumulated in the pre-existing cells. The last stage is when the seed loses moisture and its color changes from green to its mature color.

In this study, it was hypothesized that the contents of SL and PL would decrease during seed development due to a reduction in the relative proportion of membrane components in the seed. To test the hypothesis, the contents of SL and PL were measured during soybean seed development. The contents of protein, total lipid, and other lipid components also were measured to determine the nutritional value of immature seeds at different stages of development when they are consumed as a vegetable.

MATERIALS AND METHODS

Chemicals and Standards. All solvents and reagents were obtained from Fisher Scientific (Fair Lawn, NJ). The ceramide VI (trihydroxysphinganine with α-hydroxystearic acid, TA) used as the soybean Cer standard was donated by Goldschmidt (Essen, Germany). The soy GlcCer and PL standards including phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylcholine (PC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The conjugated sterol standards, including sterylglucoside (SG)

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and esterified sterylglucoside (ESG), were purchased from Matreya (Pleasant Gap, PA).

Seed Planting and Harvest. Three soybean cultivars developed by the soybean breeding project at Iowa State University that differed in seed size and protein content were selected for this study. IA1008 is a cultivar with the seed size and protein content of conventional soybeans. IA1010 is a cultivar with the large seeds preferred for consumption as a green vegetable. IA1014 is a cultivar with large seeds and high protein content for use in making tofu and other soyfoods. The three cultivars were planted in adjacent plots at the Agricultural Engineering and Agronomy Research Center near Ames, IA, in May 2004. On July 10 when the plants were flowering, one flower at one node on each of 600 plants of each cultivar was tagged. The petals of the tagged flowers were emerging from the sepals but were not completely open. Tagged pods equivalent to about 30 g of fresh seed weight were collected every 5 days from 28 to 68 DAF, when the seeds were mature and yellow in color. The number of pods harvested at each date was estimated on the basis of the data reported by Schnebly et al. (*9*). The fresh seed weight was measured by shelling enough pods to obtain 30 seeds. Three replicates of 10 fresh seeds were weighed, and the weight was divided by 10. The remaining pods were blanched in boiling water for about 5 min to deactivate enzymes and stored in a freezer until analysis.

Total Lipid Extraction. To improve the efficiency of the solvent extraction, all immature soybean seed samples were ground in a mortar and a pestle, whereas all mature seeds were ground in a Wiley mill (Thomas Scientific, Swedesboro, NJ) equipped with a 20-mesh delivering tube. After grinding, two replicates of the ground seeds were used for total lipid extraction. For each replicate, 10 g of the ground seeds was extracted by stirring with 50 mL of chloroform/methanol (2:1, v/v) for 4 h and with 50 mL of water-saturated 1-butanol for 8 h. The solvent extracts were filtered. The residual solid matter was air-dried in a fume hood and saved for the protein determination. Solvents in the extracts were removed with a rotary evaporator to obtain the crude lipid. To remove the water-soluble sugars and proteinaceous components, the crude lipid was purified using the method described by Folch et al. (*10*). The lower layer of the solvent−lipid mixture was dried with anhydrous Na_2SO_4. After filtration and evaporation of solvents, the purified total lipid was weighed, redissolved in 0.5 mL of chloroform, and stored in a freezer.

Lipid Class Separation by Silica Column Chromatography. Because the amount of total lipid extract from each replicate of immature seeds was too low to be used in the separation and quantification of the SL contents, the two replicates of the total lipid extract of each immature seed sample were combined. The total lipid was separated into the neutral lipid fraction, the intermediate polar lipid fraction, and the polar lipid fraction using the procedures described by Wang et al. (*11*). The intermediate polar lipid fraction mainly contained SL, glycolipid, and some PL. To accurately quantify the PL content, half of the intermediate polar lipid fraction and half of the polar lipid fraction were combined and marked as the PL fraction for the HPLC quantification of PL.

Moisture Content, Protein Content, and Fatty Ester Composition of Soybean Seeds. Moisture content was measured from two replicates of each ground soybean seed sample. For each replicate, 5 g of the ground seeds was dried in a vacuum oven at 55 °C until a constant weight was obtained. Protein content was determined on the two replicates of residual solid matter obtained after total lipid extraction by measuring the N content with an automated N analyzer (Rapid N III, Elementar Americas, Inc., Mt. Laurel, NJ) and multiplying the N content by 6.25. Fatty ester composition of the neutral oil fraction was analyzed with a 5890 series II gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector and a capillary column (15m × 0.25 mm, 0.25 μm film thickness) (Supelco, Bellefonte, PA). Transesterification and GC quantification were done according to the methods reported by Palacios et al. (*12*).

HPLC Method for Sphingolipid and Conjugated Sterol Quantification. Soybean GlcCer content was quantified using the HPLC method reported by Wang et al. (*11*). Because ESG and SG also are membrane lipid components, they were quantified in the same run for the GlcCer quantification with HPLC-ELSD. Only the major Cer, TA, was determined and quantified in this study using the HPLC method reported by Wang et al. (*11*). Different concentrations of the GlcCer, ESG, and SG standard mixtures and the TA standard were used for establishing standard calibration equations (**Table 1**).

Table 1. Standard Calibration Equations for Seven Classes of Lipids

lipid class[a]	calibration equation[b]	r^2
GlcCer	$Y = 759199X - 10^6$	0.9977
ESG	$Y = 502954X - 10^6$	0.9946
SG	$Y = 992497X - 4 \times 10^6$	0.9977
Cer	$Y = 2 \times 10^7 X^{1.7635}$	0.9987
PC	$Y = 13273X^{1.9964}$	0.9999
PE	$Y = 23698X^{1.9336}$	0.9992
PI	$Y = 45383X^{1.7452}$	0.9973

[a] GlcCer, glucosylceramide; ESG, esterified sterylglucoside; SG, sterylglucoside; Cer, ceramide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol. Lipid subclasses were analyzed using HPLC columns and analytical conditions specific for GlcCer, Cer (TA-2), and PL. [b] *X* is the amount of lipid in μg and *Y* is the peak area.

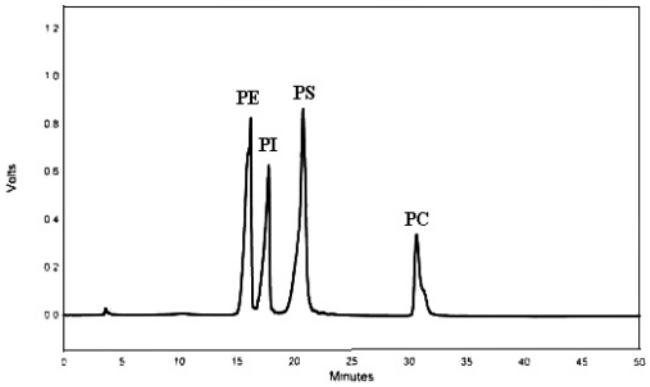

Figure 1. HPLC chromatogram of the soy PL standard mixture: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. The injection amount for each standard was 10 μg.

HPLC Method for Phospholipid Quantification. A Pholipidec certified silica column (250 × 4.6 mm, 5 μm particle size) with a guard column (20 × 4 mm) from Advanced Separation Technology Inc. (Astec, Whippany, NJ) was used to separate and quantify PL. The HPLC method was modified from the method provided by Astec. Mobile phase A was chloroform/methanol/30% ammonia (80:19:1, v/v), and mobile phase B was chloroform/methanol/30% ammonia/water (50:48:1:1, v/v). In the binary program, B was increased from 0 to 100% in 25 min, held at 100% for 15 min, and returned to 0 in 2 min. The mobile phase flow rate was at 1 mL/min. For each run, the HPLC column was re-equilibrated for 6 min with 100% A. The detection conditions of ELSD were a drift tube temperature of 55 °C, gain of 4, nitrogen flow rate of 1.7 L /min, and impactor in the off mode. Peaks of PE, PI, PS, and PC were sharp and baseline separated (**Figure 1**). Different concentrations of standard mixtures of PE, PS, PI, and PC were used for establishing standard equations. Because PS was present in <1% of the total PL, only PE, PI, and PC were quantified and calculated with the standard calibration equations as presented in **Table 1**.

Statistical Analysis. The data were analyzed as a randomized complete-block design with SAS v. 9.1 (SAS Institute, Inc., Cary, NC). Cultivars and seed development stages were considered to be fixed effects. Seed weight had three replications; moisture, protein, and total lipid contents had two replications; and SL, PL, conjugated sterol, neutral oil, and fatty ester contents had one replication for each seed development stage of each cultivar. For the contents that were measured with only one replication, the interaction between cultivar and seed development stage was used to evaluate the significance of the main effects of cultivar and seed development stage with an *F* test.

Table 2. Significance of Differences among the Means of Three Cultivars and of Nine Seed Development Stages As Measured in Days after Flowering (DAF)

seed component	DAF[a]	cultivar[a]	seed component	DAF[a]	cultivar[a]
seed weight	**	**	PL % in total lipid	**	ns
moisture	**	**	PE mol % in PL	**	**
protein	**	**	PI mol % in PL	**	**
total lipid	**	**	PC mol % in PL	**	**
GlcCer	**	ns	neutral oil	**	**
Cer	**	**	C16:0	**	*
SL mol % in the polar lipid	**	*	C18:0	**	ns
SG	**	ns	C18:1	*	**
ESG	**	ns	C18:2	ns	**
PL	**	ns	C18:3	ns	**

[a] **,* mean squares significant at the 0.01 and 0.05 probability level, respectively. ns, mean squares not significant at the 0.05 probability level.

RESULTS AND DISCUSSION

Seed Weight, Moisture, Protein, and Total Lipid Contents. Significant differences among the three cultivars were observed for mean fresh seed weight (**Tables 2** and **3**). The fresh seed weight and moisture content of the cultivars changed significantly during seed development (**Tables 2** and **3**). The mean fresh seed weight of the three cultivars increased continuously from 28 to 53 DAF. From 53 DAF to seed maturity at 68 DAF, seed weight declined due to the reduction in seed moisture.

Protein and total lipid contents were significantly different among the three cultivars and among the nine development stages (**Tables 2** and **3**). During seed development, the percentage of protein in IA1008 did not change significantly, whereas the protein content in IA1010 seed increased 10.4% from 28 to 68 DAF and that in IA1014 increased 14.8% during the same period. The percentage of the total lipid of the three cultivars increased significantly from 28 to 68 DAF by 24.2% for IA1008, by 20.7% for IA1010, and by 27.5% for IA1014.

Sphingolipid Contents. The intermediate polar lipid fraction was used for the SL quantification. Significant differences were observed for Cer content among the three cultivars, but not for GlcCer (**Tables 2** and **4**). GlcCer and Cer contents decreased significantly during seed development (**Tables 2** and **4**). Averaged across cultivars, Cer content on a dry weight basis decreased 56.8% from 51.4 nmol/g at 28 DAF to 22.2 nmol/g at 68 DAF, and GlcCer content decreased 74.0% from 522.8 nmol/g at 28 DAF to 135.8 nmol/g at 68 DAF. The changes of GlcCer and Cer during seed development were similar to those observed in previous studies. Ohnishi et al. (*5*) reported a decrease in Cer content from 150 nmol/g in immature seeds to 37.5 nmol/g in mature seeds and in GlcCer content from 462 nmol/g in immature soybean seeds to 91 nmol/g in mature seeds, on an as-is moisture basis. Because they did not report the moisture contents and the days after flowering of the immature seeds, it is not possible to make a direct comparison of their SL contents with those in our study. Their Cer content expressed as a percentage of GlcCer was 32 mol % for immature seeds and 41 mol % for mature seeds, which were much higher than the range from 9.8 mol % at 28 DAF to 16.3 mol % for mature seeds in our study (**Table 4**).

The difference in the analytical methods of Ohnishi et al. (*5*) and those we used may partially explain the differences in the levels of Cer and GlcCer observed in the two studies. In a previous paper, we discussed that certain Cer subclasses could not be well resolved from MAG, the polarity of which is similar to that of Cer (*11*). In our study reported herein, only the major Cer subclass, TA, was quantified. As a result, our Cer to GlcCer percentages were slightly underestimated. Ohnishi et al. (*5*) obtained GlcCer and Cer contents by weighing the amount of extracts recovered from the thin layer chromatography silica band, which may contain MAG and result in an overestimation of the Cer to GlcCer percentage. No other studies have been conducted in the quantification of both GlcCer and Cer contents in soybean seeds. Lynch et al. (*13*) reviewed recent studies in plant SL and pointed out that Cer content was about 10−20% of GlcCer in plant tissues. Our results seemed to be in that range even though only the major Cer was quantified.

Because SL is one of the important membrane lipids that affect membrane properties, the molar percentage of SL (sum of GlcCer and Cer) in the polar lipids (sum of GlcCer, Cer, SG, ESG, and PL) was calculated. SL molar percentage in the polar lipids decreased significantly from 2.8 mol % at 28 DAF to 1.4 mol % in mature seeds (**Tables 2** and **4**). It is possible that this change may be related to changes in the membrane physicochemical properties, such as the ion permeability, fluidity, and bilayer stability during seed development. Berglund et al. (*14*) studied the bilayer permeability and monolayer behavior of GlcCer in PL mixtures and found that the membrane permeability for glucose significantly increased when GlcCer concentration was >7.5 mol % of PL. Steponkus et al. (*15*) reviewed the plasma membrane changes resulting from freeze-induced cell dehydration and reported that a low proportion of GlcCer in mixtures containing PL and SL resulted in an increase in gel mixability. Similarly, cell dehydration during soybean seed development may be accompanied by changes in membrane physicochemical properties. However, no studies have been reported regarding the influence of GlcCer concentration on cell dehydration during seed development.

Phospholipids. Significant differences were observed for the PL percentage in the total lipid among the seed development stages, but not among cultivars (**Tables 2** and **4**). Averaged across cultivars, the PL percentage in the total extractable lipid decreased from 9.1% at 28 DAF to 3.5% at 68 DAF as the neutral oil content increased from 81.5% at 28 DAF to 92.9% at 68 DAF. The PL content on a dry weight basis decreased by 44.4% from 18.7 μmol/g at 28 DAF to 10.4 μmol/g at 68 DAF. The decrease in PL content during seed development supported our hypothesis that there were higher relative proportions of membrane components in immature seeds than in mature seeds. Compared with the changes in other polar lipids, PL content seemed to decrease more slowly than GlcCer, Cer, and SG contents. It is possible that accumulation of oil bodies slowed the reduction rate of the total PL content because PL occurs as the exclusive components of the half-unit membrane in the oil bodies, in addition to being the predominant components of plasma membranes where SL and SG are located.

Significant differences for PL subclasses were observed among cultivars and seed development stages (**Tables 2** and **5**). Averaged across cultivars, PC content in the total PL decreased from 79 mol % at 28 DAF to 62 mol % at 68 DAF, which was accompanied by increases in PE contents from 11 mol % at 28 DAF to 23 mol % at 68 DAF and in PI contents from 10 mol % at 28 DAF to 15 mol % at 68 DAF. Among the three PL subclasses, PC was the predominant PL component followed by PE and PI in both immature and mature seeds. Our data for PL contents of soybean seeds differed from those reported in previous studies (*16*−*18*). Privett et al. (*16*) analyzed the PL in developing soybean seeds and found that phosphatidic acid (PA) and PI accounted for >50% of the total PL in immature seeds, whereas PC was the major one in mature seeds

Table 3. Composition of Developing Soybean Seeds[a]

cultivar and component	days after flowering									
	28	33	38	43	48	53	58	63	68[b]	$LSD_{0.05}$
IA1008										
seed wt, mg	223.1	301.7	367.5	471.7	489.4	503.6	251.3	237.8	219.6	11.4
moisture, %	74.5	72.8	70.3	66.4	61.6	55.1	17.7	10.4	7.8	0.6
protein, %	41.3	41.4	40.8	42.1	40.5	42.4	43.2	42.2	41.2	0.7
total lipid, %	18.2	19.7	21.1	21.1	22.3	20.1	22.4	22.9	22.6	1.1
IA1010										
seed wt, mg	126.9	239.0	449.2	537.1	721.9	785.4	423.2	373.7	337.9	2.5
moisture, %	80.1	76.9	73.1	70.2	66.0	64.0	42.6	18.4	10.5	0.6
protein, %	40.2	40.2	38.3	40.2	41.3	42.2	40.0	42.7	44.4	2.4
total lipid, %	18.4	18.7	18.7	19.2	19.1	18.9	18.4	18.9	22.2	1.9
IA1014										
seed wt, mg	126.4	243.7	339.4	427.5	504.0	527.7	523.5	297.7	286.4	2.2
moisture, %	79.0	75.9	71.6	68.9	65.6	62.0	52.2	19.4	11.3	1.2
protein, %	41.9	44.6	43.8	44.9	45.8	45.4	49.2	46.5	48.1	2.0
total lipid, %	16.7	19.0	22.0	20.9	22.3	21.2	20.4	21.4	21.6	1.3

[a] Protein and total lipid are reported on a dry weight basis. [b] Mature seed.

Table 4. Lipid Composition of Developing Soybean Seeds[a]

days after flowering	SL				PL[e]			conjugated sterols	
	Cer,[b] nmol/g	GlcCer, nmol/g	Cer mol % of GlcCer	SL mol % in the polar lipid[c]	%[d]	μmol/g	neutral oil,[d,f] %	SG, μmol/g	ESG, μmol/g
28	51.4	522.8	9.8	2.8	9.1	18.7	81.5	1.8	0.6
33	43.4	511.5	8.5	2.7	7.8	18.1	86.0	1.9	0.6
38	44.5	428.0	10.4	2.5	6.3	16.1	87.2	2.1	0.5
43	33.3	330.0	10.1	1.9	6.4	16.7	88.3	1.8	0.5
48	32.6	286.9	11.4	1.9	5.8	14.6	89.8	1.2	0.5
53	27.0	199.5	13.5	1.7	5.1	12.1	90.2	1.0	0.4
58	20.7	163.1	12.7	1.7	4.0	10.2	91.3	0.7	0.3
63	22.1	146.7	15.1	1.7	3.8	9.0	92.3	0.5	0.6
68	22.2	135.8	16.3	1.4	3.5	10.4	92.9	0.5	0.6
$LSD_{0.05}$	14.0	86.2	5.1	0.6	1.8	4.9	2.2	0.6	0.1

[a] The molecular weights used in calculation were Cer, 654; GlcCer, 714; SG, 576; ESG, 814; PC, 758; PE, 716; and PI, 857. [b] Cer referred only to the major component TA. [c] Polar lipids were the sum of GlcCer, Cer, SG, ESG, and PL. [d] Percentage of the neutral oil and PL were calculated on the basis of the total lipid extract. Other components were calculated on a dry weight basis. [e] PL was the sum of PC, PE, and PI. [f] The neutral oil was obtained by silica column fractionation and may include TAG, FFA, DAG, tocopherols, and pigments.

Table 5. Means of Phospholipid Subclass and Fatty Ester Content Averaged across Cultivars

days after flowering	PL composition, mol %			fatty ester composition, %				
	PE	PI	PC	C16:0	C18:0	C18:1	C18:2	C18:3
28	11.0	10.0	79.0	16.7	6.7	25.5	41.8	9.7
33	14.9	9.0	76.1	14.8	5.0	25.6	44.4	9.5
38	12.3	10.0	77.7	11.8	5.3	28.4	45.2	8.9
43	12.1	10.9	77.0	10.7	5.0	28.7	46.7	8.8
48	15.3	12.3	72.4	9.8	5.0	33.9	43.3	7.8
53	15.3	13.6	71.1	9.4	4.7	35.0	43.2	7.6
58	17.8	14.0	68.2	9.3	4.5	35.4	42.8	7.6
63	17.7	12.9	69.4	9.2	4.5	33.8	44.7	7.5
68	22.9	14.9	62.2	9.3	4.6	33.8	44.8	7.4
$LSD_{0.05}$	3.4	2.5	4.7	2.2	1.0	7.4	6.8	1.6

(45%) followed by PE and PI. Wilson et al. (*17*) determined phosphorus contents of separated PL and reported that *N*-acylphosphatidylethanolamine (NPE) (>50 mol %) and PA (~15 mol %) were the two major PL subclasses in immature soybean seeds, whereas PC (46 mol %), PE (25 mol %), and PI (17 mol %) were the major ones in mature seeds. Wilson et al. (*18*) also reported that NPE (>50%) and PC (>14%) were the major PL in immature seeds, whereas PI content (~30%) was higher than the others in mature seeds. In our study, NPE was not detected in the PL fraction. NPE has been considered to be a potential diacylglycerol donor for oil biosynthesis, and it would be degraded before seed maturation (*19*). The discrepancy among studies may be due to the differences in the analytical methods used.

ESG and SG Contents. The major conjugated sterols, SG and ESG, also are important components in the cell membranes. There were significant differences among the seed development stages, but not among the three cultivars for SG and ESG contents (**Tables 2** and **4**). Averaged across cultivars, SG content decreased 72% from 1.8 μmol/g at 28 DAF to 0.5 μmol/g at 68 DAF on a dry weight basis, whereas ESG content was similar for seeds at 28 DAF and 68 DAF (**Table 4**). SG was 3-fold higher than ESG in seeds at 28 DAF, but the contents of the two conjugated sterols were similar at 68 DAF. Few studies have been conducted on the content of conjugated sterols in soybean seed. Sugawara et al. (*6*) reported that in mature soybean seeds, SG content was 0.2 μmol/g and ESG content was 0.5 μmol/g on a dry weight basis, which were similar to our results for mature seeds. The changes in conjugated sterols were investigated by Wojciechowski (*20*), who reported that phytohormone levels and environmental factors controlled the interconversions between the free sterols and the conjugated sterols and indicated that ESG and SG contents may be altered substantially in response to changes in growth conditions, such as the dehydration and a reduction in photosynthesis. In a review, Moreau et al. (*21*) suggested that the levels of SG and ESG can be rapidly modulated by other

environmental and chemical factors such as light, temperature, water stress, ozone, copper ions, and exposure to various enzymes.

Fatty Ester Composition of Neutral Oil. Significant differences were observed among cultivars and seed development stages for neutral oil content (**Tables 2**, **4**, and **5**). Averaged across cultivars, neutral oil percentage in the total lipid increased significantly during seed development from 81.5% at 28 DAF to 92.9% at 68 DAF. Fatty ester composition also changed during seed development. Palmitate and stearate content decreased significantly and oleate content increased significantly from 28 to 68 DAF. Our finding was in general in agreement with the data reported by Rubel et al. (*22*). They found that palmitate, stearate, and linolenate contents decreased significantly while the oleate and linoleate contents increased from 24 to 40 DAF, but there was no significant change in the fatty ester composition from 54 to 72 DAF. Sangwan et al. (*23*) reported that oleate content decreased and linoleate increased from 45 DAF to maturity. The variation in results among studies may be due in part to the different soybean cultivars, environments, and analytical methods that were used.

The harvest of soybean for use as a green vegetable generally occurs when the seed has reached it greatest fresh weight. In our study, that occurred at 53 DAF. When the lipid contents were calculated on a fresh weight basis for a single seed, the greatest SL and PL contents were observed in immature seed at 48−53 DAF for the three cultivars studied. A single immature seed harvested at 48−53 DAF contained 34 μg of total SL in IA1008, 55 μg in IA1010, and 37 μg in IA1014, whereas a mature seed had 21 μg of SL in IA1008, 29 μg in IA1010, and 33 μg in IA1014. Similarly, PL contents in an immature seed at 48−53 DAF were 20 mg in IA1008, 28 mg in IA1010, and 20 mg in IA1014, whereas PL contents in a mature seed were 19 mg in IA1008, 18 mg in IA1010, and 24 mg in IA1014. These results indicated that green immature seeds harvested at their greatest fresh weight would have high nutritional value for SL and PL contents.

Conclusions. SL and PL are two major membrane components of soybean seeds. Our hypothesis was that the SL and PL contents would decrease during seed development as the relative proportion of membranes in the seeds decreased during development. There was a significant decrease in the two lipid components during seed development, which supported our hypothesis. The results also indicated that immature soybeans harvested at their greatest fresh weight will have high SL and PL contents.

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