HPLC Quantification of Sphingolipids in Soybeans with Modified Palmitate Content

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Efficient separation and accurate quantification of sphingolipids (SL) are important for studying SL concentrations and biological functions. The objectives of this study were to develop effective methods for the separation and quantification of SL and to determine the relationship between palmitate and SL contents of mature soybean seeds. Methods using column chromatography and high-performance liquid chromatography−evaporative light scattering detector (HPLC-ELSD) were developed to separate and quantify glucosylceramide (GlcCer) and ceramide (Cer) in 15 soybeans lines in which palmitate content ranged from 3.7 to 40.7%. There were significant differences among the lines for GlcCer (83.4−397.6 nmol/g) and major Cer contents (8.4−20.7 nmol/g) on a dry weight basis. The correlations of palmitate content with GlcCer and Cer concentrations were not significant. The results indicated that the palmitate content of soybean seed did not affect their GlcCer and Cer contents. Genetic factors other than those that control palmitate content seemed to be responsible for the variation among soybean lines for GlcCer and Cer contents.

KEYWORDS: Sphingolipids; glucosylceramide; ceramide; palmitate content; HPLC quantification; soybean

INTRODUCTION

Sphingolipids (SL) are a class of complex membrane lipids found in all eukaryotic and some prokaryotic cells, where they provide membrane structures and are involved in the regulation of cell metabolism (*1*, *2*). The de novo biosynthesis of SL starts from the condensation of palmitoyl-CoA and serine to generate 3-ketosphinganine by serine palmitoyl-transferase (*3*). After a series of enzymatic reactions, ceramide (Cer), glucosylceramide (GlcCer), and other complex SL are formed. The formation of 3-ketosphinganine has been reported as a regulatory step in SL biosynthesis (*4*−*6*).

Palmitoyl-CoA is one of the saturated fatty acid moieties involved in triacylglycerol biosynthesis. In conventional soybean seeds, palmitate accounts for about 10−12% of the total fatty esters. Alleles for elevated or reduced palmitate level have been developed by treating soybean seeds with ethyl methanesulfonate (EMS) or *N*-nitroso-*N*-methylurea (NMU) (*7*, *8*). By combining independent mutant alleles, soybean lines have been developed with palmitate content ranging from <4% to >40%. Merrill et al. (*6*) studied the influence of extracellular precursors on the formation of sphingoid bases (SB) and reported that a high concentration of free palmitic acid in cell culture enhanced the long-chain SB biosynthesis. On the basis of the results reported in this previous paper, it is possible that SL content may be positively correlated with the palmitate content of soybean seeds. The relationship between the two traits in soybean seeds that differ in palmitate content has not been evaluated.

Separation and quantification of SL are important for studying SL concentrations and biological functions. However, it has been difficult to isolate SL completely and effectively, particularly the free Cer, because SL contents are typically <1% of the total extractable lipid (*9*, *10*). Researchers have used different approaches to identify and quantify these minor lipids. Ohnishi et al. (*11*) identified SL in soybean seeds using gas chromatography−mass spectrometry (GC-MS) and found that GlcCer and Cer were the predominant SL. They also showed that in soybeans, the major Cer species were trihydroxysphingenines with normal fatty acids (TN) or with α-hydroxyl fatty acids (TA), which were structurally different from mammalian Cer with dihydroxysphingosine and normal fatty acids (DN) or α-hydroxyl fatty acids (DA). The structures of four Cer are shown in **Figure 1**. The quantification of Ohnishi et al. (*11*) was based on the amounts in the extracts recovered from TLC bands. This method is not considered to be accurate because the extracts might contain other lipid components with polarity similar to SL.

Iwamori et al. (*12*) determined that the free Cer concentration in rat brain was 1.5 μmol/g on a dry weight basis by quantifying benzoylated Cer with high-performance liquid chromatography (HPLC)−ultraviolet (UV) detector. Other studies presented

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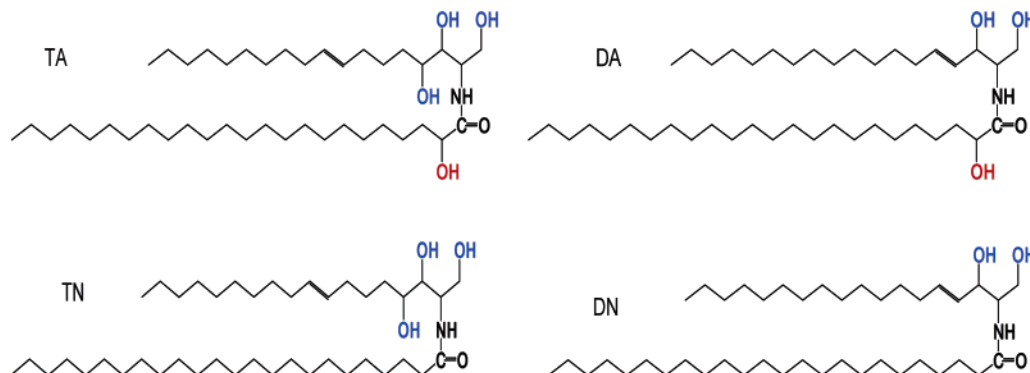† Department of Food Science and Human Nutrition.
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Figure 1. Ceramide structures. Abbreviations: TA, trihydroxysphingenine with α-hydroxyl fatty acids; TN, trihydroxysphingenine with normal fatty acids; DA, dihydroxysphingosine with α-hydroxyl fatty acids; DN, dihydroxysphingosine with normal fatty acids.

methods to quantify SL using high-performance thin layer chromatography (HPTLC) and fluorescence spectrodensitometry (*13*) or reverse phase chromatography and GC-MS (*14*). Even though some of these methods can be used to determine the molecular species of free Cer or Cer moieties, they all require derivatizations, which not only cause the partial degradation of SL but also are time-consuming. In addition, derivatization reactions may not be complete if the conditions are not fully tested or optimized. In recent years, tandem mass spectrometry has become one of the most powerful and accurate techniques in analyzing complex lipid mixtures. Sullards et al. (*15*) developed a method to determine SL molecular species and their compositions directly by using HPLC-MS-MS. However, the high cost of the tandem mass detector limits its wide use as a routine analytical method. HPLC coupled with an evaporative light scattering detector (ELSD) has been widely used in the lipid analysis. In general, HPLC-ELSD is a convenient tool that is better for quantification, whereas HPLC-MS is better for molecular characterization.

Several methods have been reported on the quantification of SL using HPLC-ELSD (*16*−*18*). None of them were satisfactory in the separation and quantification of free Cer. McNabb et al. (*19*) analyzed yeast Cer and SB without any purification and derivatization. The resolution between Cer and SB was acceptable, but the peaks of DN and DA overlapped. Zhou et al. (*20*) effectively separated DN from DA by using a cyanopropyl bonded column without derivatization. However, they could not separate DA from TA and DN from TN. Farwanah et al. (*21*) developed methods using both HPLC-ELSD and HPLC−atmospheric pressure chemical ionization mass spectrometry (APCI-MS) and found that HPLC-ELSD was suitable for separating stratum sorneum Cer with a detection limit of 500 ng. Although the four ceramides were well resolved by their method, one of the two peaks of TA still overlapped with the peak of TN.

In our study, HPLC-ELSD was used to separate and quantify GlcCer and Cer in soybean seeds. SL concentrations of 15 soybean lines that differed in the palmitate content were determined to examine the effect of palmitate content on SL concentration.

MATERIALS AND METHODS

Chemicals and Standards. All solvents used were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium methylate (5.4 M in methanol) was purchased from Sigma Chemical Co. (St. Louis, MO). Soybean GlcCer and phospholipid (PL) standards were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Bovine brain Cer from Matreya, Inc. (Pleasant Gap, PA, was used as DA and DN standards. Commercial Cer products ceramide III (*N*-stearoylphytosphingosine)

and ceramide VI [*N*-(2-hydroxyoctadecanoyl)phytosphingosine] were used as TN and TA standards. They were generously provided by Goldschmidt (Essen, Germany). Standards of monoacylglycerol (MAG), monogalactosyldiacylglycerol (MGDG), plant sterols, ergosterol, steryl-glucoside (SG), esterified sterylglycoside (ESG), and digalactosyl diacylglycerol (DGDG) were purchased from Matreya. All standards were stored in a freezer, and standard solutions were freshly prepared with chloroform/methanol (4:1, v/v).

Soybean Planting, Seed Harvest, and Fatty Ester Determination. Seeds of 15 soybean lines developed by the soybean breeding program at Iowa State University were planted in adjacent plots at the Agricultural Engineering and Agronomy Research Center near Ames, IA, in May 2004. The selected lines had different combinations of the major genes that control palmitate content (*22*, *23*, *27*). At maturity, 10 individual plants of each line were harvested and threshed separately. The fatty ester composition of a five-seed bulk of each plant was determined with the procedure described by Hammond (*24*). The seeds from plants of each line with similar palmitate percentages were pooled, and about 50 g of each pooled sample was stored in a well-ventilated room for further analysis.

Total Lipid Extraction of Soybean Seeds. The total lipid of soybean seeds was obtained by the solvent extraction of two replicates of mature seeds. For each replicate, about 15 g of seeds was ground in a Wiley mill equipped with a 20-mesh delivering tube (Thomas Scientific, Swedesboro, NJ). Ten grams of the ground seeds was sequentially 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 two extracts were filtered and pooled prior to the removal of solvents with a rotary evaporator. To remove the water-soluble sugars and proteinaceous components, the crude lipid extract was redissolved in chloroform/methanol (2:1, v/v) and purified according to the method reported by Folch et al. (*25*). The lower layer of the solvent phase was collected and desiccated with anhydrous Na_2SO_4 before solvent evaporation. The purified total lipid extract was redissolved in 0.5 mL of chloroform after the filtration and evaporation of residual solvents and stored in a freezer.

Separation and Fractionation of Total Lipid. The two replicates of the total lipid extract were each fractionated into different lipid classes by column chromatography. To separate 1 g of the total lipid extract, 10 g of silica gel (60A, Sorbent Technology, Atlanta, GA) was mixed with hexane to form a mobile slurry. The silica slurry was added to a glass column with standard fritted glass bed support (19 × 300 mm) (ACE Glass Inc., Vineland, NJ). Compressed air was gently applied to the glass column to compress the silica and to force solvent and air through the column. The top of the silica column should be below the solvent level. About 100 mL of chloroform was added to rewash the column and to equilibrate the silica. The total lipid extract in chloroform was loaded slowly onto the top of silica along the column wall. The packed column was sequentially eluted with 100 mL of chloroform, 100 mL of chloroform/acetone (95:5, v/v), 100 mL of chloroform/methanol (4:1, v/v), 50 mL of methanol, and 50 mL of methanol/water (9:1, v/v). The neutral lipid fraction (the first two eluates), intermediate polar lipid fraction (the third eluate, containing SL), and polar PL

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Table 1. Binary Gradient Program for Ceramide Separation

time, min	composition of mobile phase[a]	
	A, %	B, %
0	94	6
2	94	6
6	91	9
21	89	11
22	88	12
30	88	12
38	0	100
48	0	100
49	94	6
59	94	6

[a] A was hexane, and B was 2-propanol/ethyl acetate/88% formic acid (50:50: 0.5, v/v). Mobile phase flow rate was 0.65 mL/min.

fraction (the last two eluates) were obtained after column separation. During collections, each eluate was monitored on a TLC plate (Sigma, St. Louis, MO), which was developed with chloroform/methanol/ether/ hexane/acetic acid (100:20:20:10:1.5, v/v) and visualized with permanganate stain (KMnO$_4$/K$_2$CO$_3$/0.5% NaOH/H$_2$O, 3:20:5:300) to confirm that no SL were lost in either the neutral oil fraction or the polar PL fraction. After the solvents were evaporated, the intermediate polar lipid fraction was used for the HPLC quantification of SL.

HPLC Method for Cer Quantification. A Beckman Coulter HPLC system (Beckman Instruments, Inc., Fullerton, CA) consisting of an autosampler 508, a solvent delivery system module 126, and an ELSD 2000 (Alltech Associates, Inc., State College, PA) was used. The intermediate polar lipid fraction was separated with a 5-μm Chromegasphere Si60 column (150 × 3.2 mm) (ES Industries, West Berlin, NJ). Two mobile phases and a gradient program were applied (**Table 1**). Solvent A was hexane, and solvent B was 2-propanol/ethyl acetate/ 88% formic acid (50:50:0.5, v/v). The detection conditions were a drift tube temperature of 60 °C, gain of 4, nitrogen flow rate of 0.8 L/min, and impactor in the off mode.

HPLC Method for GlcCer Quantification. The HPLC method for the separation and quantification of GlcCer was adopted from that of Gutierrez et al. (*26*) with modifications. Mobile phase B was changed to methanol/methyl *tert*-butyl ether (75:25, v/v), and mobile phase A was hexane/tetrahydrofuran (99:1, v/v). The detection conditions were a drift tube temperature of 60 °C, gain of 1, nitrogen flow rate of 1.4 L/min, and impactor in the off mode. The HPLC column, gradient program, and mobile phase flow rate were the same as reported by Gutierrez et al. (*26*).

Statistical Analysis. The fatty ester composition of each soybean line was evaluated with three replications of five-seed bulks. GlcCer and Cer were evaluated with two replications of the lipid extract of each soybean line. The data were analyzed as a completely randomized design with SAS v. 9.1 (SAS Institute, Inc., Cary, NC). Soybean line was considered to be a fixed effect. An *F* test based on the analysis of variance was used to determine the significance of differences for SL and fatty ester contents among the 15 soybean lines. The correlation (*Corr*) procedure of SAS was used to compute the correlation coefficient (*r*) between GlcCer and Cer contents and the correlation coefficients between palmitate content and GlcCer and Cer contents.

RESULTS AND DISCUSSION

Chromatographic Separation and Quantification of GlcCer. The intermediate polar lipid fraction was used for the quantification of GlcCer content. The GlcCer and other glycolipids could be separated and quantified in a single HPLC run even in the presence of certain nonpolar lipids and PL (**Figure 2**). Because plant sterols had a retention time (11.388 min) similar to that of ergosterol (10.855 min), ergosterol was used in the standard mixture. The elution order was ergosterol, ESG, MGDG, SG, GlcCer, DGDG, and PL. The peaks of GlcCer and SG were sharp without splitting compared with the

chromatogram obtained with the method reported by Gutierrez et al. (*26*). The system pressure was high and became elevated after only several injections when methanol/2-propanol (50:50, v/v) was used as solvent B in their method. To improve the separation efficiency and stability, substitutes for solvent B were tested that had suitable solvent polarity and strength parameters. Methanol/methylene chloride (70:30, v/v) and methanol/methyl *tert*-butyl ether (75:25, v/v) were found to have better resolutions than the solvents used by Gutierrez et al. (*26*). However, the use of methylene chloride occasionally produced bubbles in our pump system and had environmental and safety concerns. Thus, methanol/methyl *tert*-butyl ether was used as solvent B in our final HPLC method. The detection conditions were optimized and the most sensitive detection conditions obtained by changing the drift tube temperature from 69 to 60 °C and decreasing the nitrogen flow rate from 2.5 to 1.4 L/min.

The power relationship ($Y = 143010X^{1.2556}$, $r^2 > 0.99$) between peak area (*Y*) as integrator counts and amount of GlcCer (*X*) was found in the 2−12 μg injection range. The limit of detection (LOD) of GlcCer was determined as about 100 ng at a signal-to-noise ratio of 2.

When this HPLC method was used to separate and quantify Cer, it gave acceptable separation of DN and DA when 1% of formic acid was added to solvent B, but the resolutions among DA, TA, and TN were poor. The peaks of these three Cer overlapped and combined with the peak of ESG. A new HPLC method was needed to separate and quantify these Cer subclasses.

Chromatographic Separation and Quantification of Cer. A new method was developed for the separation of four Cer enabling the baseline separation of Cer standards according to the number and the position of hydroxyl groups in the Cer. Because the standard TA was produced with a biofermentation process following N-acylation, the TA contained racemic isomers of the α-OH fatty acid moiety. Two separated peaks labeled TA-1 and TA-2 were observed. The elution order was DN, TA-1, TN, DA, ESG, and TA-2 (**Figure 3**). In the chromatogram of the intermediate polar lipid fraction of one soybean sample, TA and TN peaks were observed, as well as a small peak at the retention time of DA (**Figure 3B**), but no DN was detected in the soybean sample. Because TA and TN were reported to be the predominant Cer in soybeans (*11*), they were considered further in this study. When the chromatogram of standard mixtures of Cer (**Figure 3A**) was compared with that of the soybean sample (**Figure 3B**), no peak was observed around the retention time of TA-1. This suggested that only isomer TA-2 may be present in soybean. Because the product description of TA did not mention the relative percentage of TA-2, the proportion of TA-2 was estimated by comparing the peak area of TA-2 with the total peak area of TA. This proportion of two areas was relatively constant (55.0 ± 0.4%, $n = 5$) at various levels of injections. Therefore, a factor of 55% was used to quantify TA in soybean seeds.

The power relationships ($r^2 > 0.99$) between peak area (*Y*) and amount of Cer (*X*) were found in the 0.5−5 μg injection range. Calibration curve equations were $Y = 2 \times 10^7 X^{1.7635}$ for TA-2 and $Y = 5 \times 10^6 X^{1.7701}$ for TN. The LODs were about 100 ng for TA-2 and 50 ng for TN at a signal-to-noise ratio of 2.

Effect of Chemical Treatments on the Quantification of Cer Subclasses. Cer standard mixtures could be separated from ESG when they were injected in similar amounts. However, the ESG content in soybeans was reported to exceed the Cer content by 13-fold (*11*, *18*). The similar polarity and the
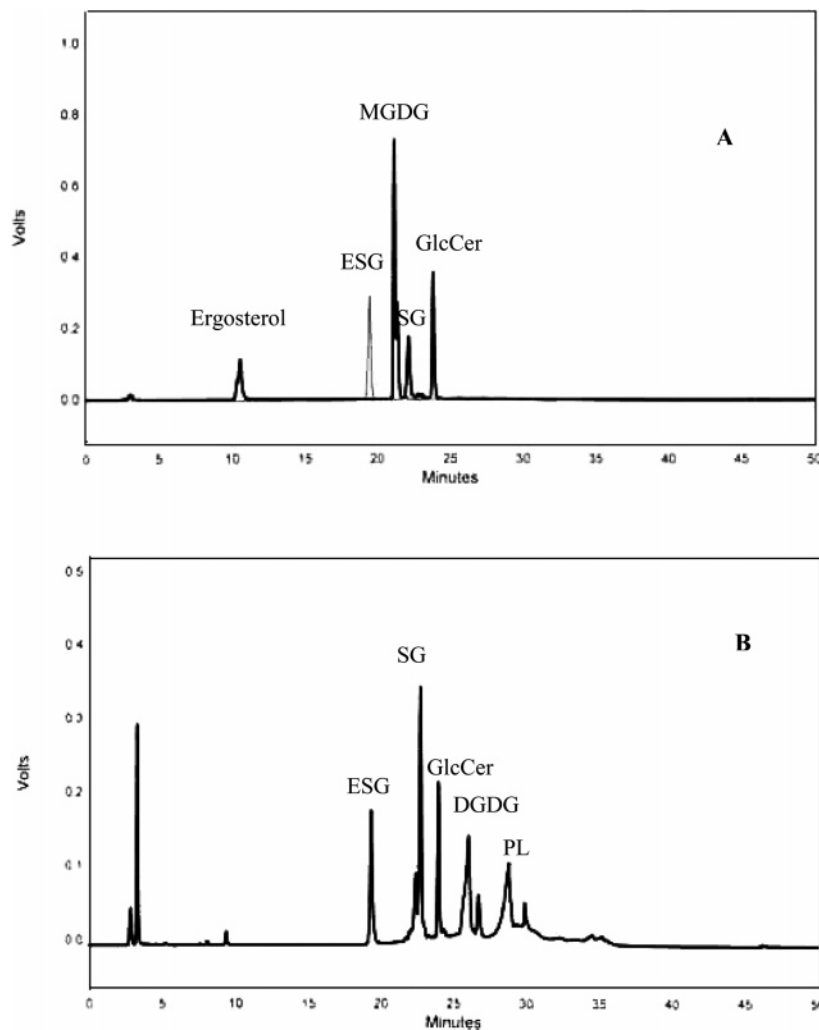
Figure 2. HPLC-ELSD chromatograms of the glucosylceramide separation: (**A**) standard mixtures; (**B**) one intermediate polar lipid fraction of soybean prepared by silica column separation. Abbreviations: ESG, esterified sterylglucoside; SG, sterylglucoside; GlcCer, glucosylceramide; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol. Ergosterol, SG, MGDG, and GlcCer were injected together at 10 μg each. ESG was injected separately at 10 μg.

concentration difference make the complete separation of these components extremely difficult in the soybean lipid extract. To eliminate the interference of ESG, saponification has been used in most SL quantification studies (*11*, *18*), and transesterification was assumed to achieve a similar purpose. However, Gutierrez et al. (*22*) investigated the recovery of a Cer standard after the two treatments and found that only 76.5% of Cer was recovered after saponification and 75% of Cer was recovered after transesterification. About 25% of Cer was degraded during these chemical treatments.

Experiments were conducted to examine the effect of chemical treatments on the degradation of soybean Cer. The intermediate polar lipid fraction of one soybean sample was equally divided into three parts. The first aliquot was saponified with 1 mL of 0.5 M NaOH in methanol/water (19:1, v/v) at 50 °C for 1 h. The second aliquot was transesterified with 0.5 mL of 0.5 M NaOCH$_3$ in methanol at room temperature for 1 h. The third aliquot was used as the control. After the reactions were carried out, 0.1 M acetic acid was added to neutralize the solution, and calculated volumes of chloroform and water were added as described in the method of Folch et al. (*25*). Solvents in the lower layer were removed at room temperature under nitrogen. The residues of the two treated samples were dissolved in the same volume of chloroform as that of the third control aliquot.

The three samples were analyzed using the HPLC method developed for the separation of Cer.

The chromatograms of the control sample and the two samples after transesterification and saponification are shown in **Figure 3B**. The area between 14 and 34 min was enlarged and is presented as an inset in **Figure 3B**, and the chromatogram of TA and TN standard mixtures was added into the inset. Transesterification and saponification removed most of the ESG, but the peak areas of TA-2 of the two chemically treated samples decreased compared with the peak area of the control sample. Only 45% of peak areas of TA-2 was retained after the transesterification treatment, and 28% was retained after the saponification treatment. The peak area of TN was 26% greater in the transesterified sample than in the control sample. Some artifacts such as MAG may have been produced during transesterification and contributed to the increase in the peak area of TN. To evaluate this possibility, drops of dilute MAG standard solution were added to the transesterified sample to spike the TN peak. The peak area of TN increased dramatically. In addition, an unknown peak with a retention time of about 16 min, which is close to the retention time of DN, was observed in two chemically treated samples. It probably was a degradation product from the complex lipids. A further MS determination is needed to identify this unknown.
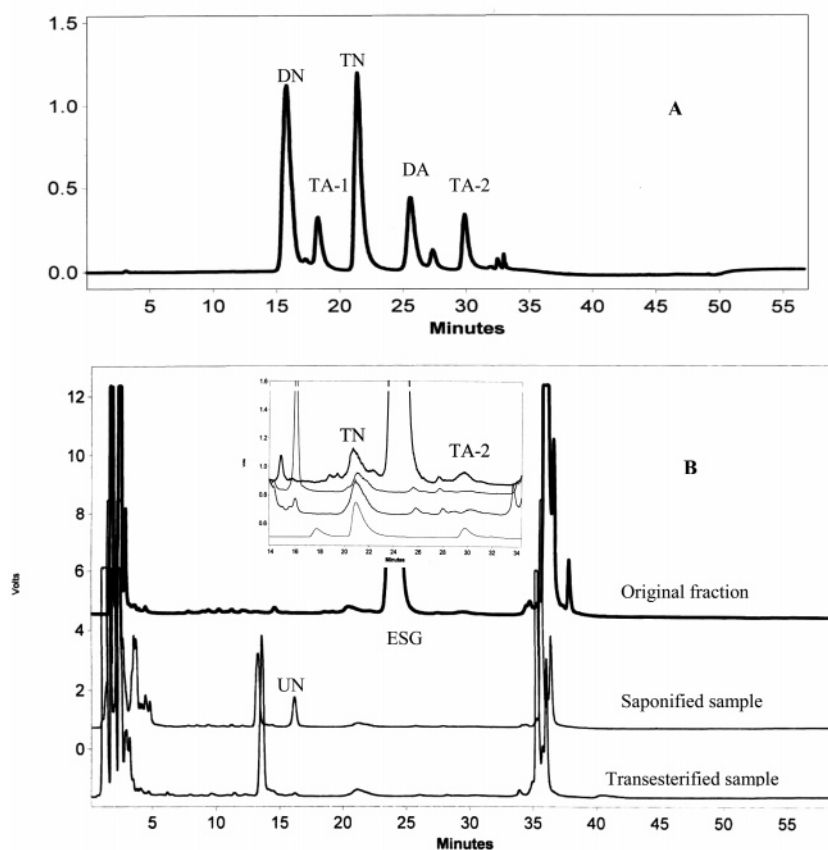
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Figure 3. HPLC-ELSD chromatograms of the ceramide separation: (**A**) ceramide standard mixtures; (**B**) intermediate polar lipid fraction of a soybean sample and its chemically treated samples. The insert in B is the enlarged chromatogram between 14 and 34 min. The bottom chromatogram in the insert is the standard mixture of TA and TN. TA, TN, and bovine brain Cer (DA and DN) were injected at 10 μg each. Abbreviations: TA, trihydroxysphingenine with α-hydroxyl fatty acids; TN, trihydroxysphingenine with normal fatty acids; DA, dihydroxysphingosine with α-hydroxyl fatty acids; DN, dihydroxysphingosine with normal fatty acids; ESG, esterified sterylglucoside; UN, unknown.

Both TA and TN were quantified during the initial HPLC analysis. The TN content was ~2 times greater than the TA content. This result disagreed with the Cer composition reported by Ohnishi et al. (*11*), who showed that TA accounted for >70% of the total free Cer in soybean seeds. The insufficient separation of TN and MAG may have caused the overestimation of the TN content. Therefore, TA was the only Cer subclass reported in our study.

Fatty Ester Composition of Soybean Lines with Modified Palmitate Content. The palmitate contents of the 15 soybean lines were significantly different (P < 0.01), ranging from 3.7 to 40.7% (**Table 2**). Palmitate content had significant negative correlations with oleate ($r = -0.93$) and linoleate ($r = -0.98$) contents and significant positive correlations with stearate ($r = 0.52$) and linolenate ($r = 0.67$) contents. Oleate and linoleate were the two fatty esters most influenced by an increase in palmitate content. As the palmitate content increased, the oleate content decreased from a high of 26.4% to a low of 8.9% and the linoleate content decreased from a high of 61.2% to a low of 34.0%. Stearate and linolenate contents of lines with 3.7% palmitate were lower than those for lines with the highest palmitate. Our results agreed with those of Stoltzfus et al. (*27*), who evaluated lines with elevated palmitate and reported significant positive correlations of palmitate with stearate and linolenate and significant negative correlations with oleate and linoleate.

GlcCer and Cer Contents of Soybean Seeds with Modified Palmitate Content. Significant differences (P < 0.01) were observed for GlcCer and Cer contents among the 15 soybean

Table 2. Fatty Ester Composition and SL Content of Seeds from 15 Soybean Lines

line	fatty ester composition, %					SL content,[a] nmol/g		Cer mol % of GlcCer
	C16:0	C18:0	C18:1	C18:2	C18:3	GlcCer	Cer	
B01769B019	3.7	3.1	23.7	61.2	8.3	83.4	8.4	10.1
IA2066	3.7	3.1	26.4	57.9	8.9	161.0	14.2	8.8
A22	6.6	4.2	24.0	56.7	8.6	298.1	16.4	5.5
IA1008	10.6	4.2	25.3	51.9	7.9	284.2	17.9	6.3
IA2021	10.6	4.4	22.2	55.2	7.7	285.9	16.5	5.8
A30	13.5	4.4	24.1	50.6	7.5	229.9	12.4	5.4
A24	15.3	4.3	20.6	49.9	9.8	264.5	17.3	6.5
A27	17.5	5.0	25.6	45.3	6.5	397.6	19.0	4.8
A21	18.5	4.7	21.5	47.6	7.8	233.7	10.0	4.3
A97877006	25.8	4.2	16.4	43.9	9.6	280.7	15.1	5.4
A96496018	28.2	4.1	13.2	43.9	10.5	252.6	20.7	8.2
A28	33.0	6.0	12.2	37.3	11.5	297.8	19.2	6.4
97HP417	39.8	5.3	9.9	34.6	10.5	258.5	18.1	7.0
97HP12	39.8	4.9	9.7	34.4	11.2	269.9	19.1	7.1
97HP307	40.7	4.5	8.9	34.0	11.9	186.1	10.2	5.5
LSD$_{0.05}$	2.1	1.0	4.3	4.0	1.7	41.8	2.3	1.5

[a] The molecular weights used in calculation were Cer/654 and GlcCer/714. GlcCer and Cer contents were calculated on a dry weight basis.

lines. The content of GlcCer ranged from 83.4 to 397.6 nmol/g among the lines (**Table 2**). The average GlcCer content of the conventional soybean cultivars IA1008 and IA2021 was 285.0 nmol/g, which partially agreed with reported data using different methods (*18, 26, 28*). Sugawara et al. (*18*) determined that the GlcCer content of a commercial soybean was 110 nmol/g. Gutierrez et al. (*26*) reported that GlcCer content was 142

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nmol/g in IA1008 and 283 nmol/g in IA2021. Takakuwa et al. (*28*) quantified GlcCer content in different soybean tissues and reported that soybean cotyledon contained 280 nmol/g GlcCer. The correlation between palmitate and GlcCer contents was not significant ($r = 0.19$, $P = 0.30$). The two lines with a palmitate content of 3.7% were significantly different from each other in GlcCer content, and one of the lines was not significantly different in GlcCer content from the line with the highest palmitate content of 40.7%. The content of Cer ranged from 8.4 to 20.7 nmol/g among the 15 soybean lines (**Table 2**). The correlation between palmitate and Cer contents was not significant ($r = 0.29$, $P = 0.12$). The Cer content of one of the lines with the lowest palmitate content was not significantly different from that of the line with the highest palmitate. These results indicate palmitate content does not have a significant influence on GlcCer or Cer content in the soybean seed. Genetic factors other than those controlling palmitate contents seemed to be responsible for the significant differences among lines for the two SL components.

The molar percentage of Cer to GlcCer was significantly different among the soybean lines ($P < 0.01$). The range among lines was from 4.3 to 10.1 mol % of Cer to GlcCer (**Table 2**). Lynch et al. (*29*) reported that Cer concentration in plant tissues was 10−20% of the GlcCer content and that this concentration may be affected by environmental stress, diversity of tissues, and different analytical methods. A significant positive correlation ($r = 0.70$, $P < 0.01$) was observed between Cer and GlcCer contents. Few studies have been reported on the relationship between GlcCer and Cer in plants. Warnecke et al. (*30*) reported that plant Cer may be synthesized during GlcCer turnover instead of being the precursors of GlcCer biosynthesis because Cer and GlcCer in plants have different SB moieties. The major SB moieties in soybean are trihydroxysphingenine for Cer and dihydroxysphingedienine for GlcCer (*11*). Nakayama et al. (*31*) reported that the formation of GlcCer in radish seedlings was enhanced by the addition of Cer with trihydroxy SB rather than the addition of the Cer with dihydroxy SB.

Conclusions. The HPLC methods developed in this study can be effectively used to quantify GlcCer and Cer (TA), as well as certain glycolipids in soybeans, without any chemical treatment of the extracted lipid. SL contents were significantly different among soybean lines that differed in palmitate content. No significant correlations were observed between SL and palmitate contents among the 15 soybean lines. A significant and positive association ($r = 0.70$) was observed between GlcCer and Cer contents.

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