2006

Soybean sphingolipid content as affected by palmitate content and by seed development stage

Liping Wang
Iowa State University

Soybean sphingolipid content as affected by palmitate content and by seed development stage

by

Liping Wang

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Food Science and Technology

Program of Study Committee:
Tong Wang, Major Professor
Earl G. Hammond
Walter R. Fehr

Iowa State University

Ames, Iowa

2006

UMI Number: 1439834

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

In Memory of My Mother

TABLE OF CONTENTS

LIST OF ABBREVIATIONS iv

CHAPTER 1. GENERAL INTRODUCTION 1

LITERATURE REVIEW 1
THESIS ORGANIZATION 10
LITERATURE CITED 10

CHAPTER 2. HPLC QUANTIFICATION OF SPHINGOLIPIDS IN SOYBEANS WITH MODIFIED PALMITATE CONTENT 14

ABSTRACT 14
INTRODUCTION 15
MATERIALS AND METHODS 17
RESULTS AND DISCUSSION 21
ACKNOWLEDGEMENT 27
LITERATURE CITED 27

CHAPTER 3. EFFECT OF SEED DEVELOPMENT STAGE ON SPHINGOLIPID AND PHOSPHOLIPID CONTENTS IN SOYBEAN SEEDS 36

ABSTRACT 36
INTRODUCTION 37
MATERIALS AND METHODS 38
RESULTS AND DISCUSSION 42
ACKNOWLEDGEMENT 48
LITERATURE CITED 48

CHAPTER 4. GENERAL CONCLUSIONS 57

APPENDIX A. ANALYSIS OF VARIANCE TABLES FOR CHAPTER 2 58

APPENDIX B. ANALYSIS OF VARIANCE TABLES AND ADDITIONAL DATA FOR CHAPTER 3 59

ACKNOWLEDGEMENTS 63

LIST OF ABBREVIATIONS

μ	micro
ANOVA	analysis of variance
APCI-MS	atmospheric pressure chemical ionization mass spectrometry
c	normal fatty acids
$^{\circ}$C	degrees Celsius
C16:0	palmitate
C18:0	stearate
C18:1	oleat
C18:2	linoleate
C18:3	linolenate
Cer	ceramide
DA	dihydroxysphingoid base with α-hydroxyl fatty acids
DAF	days after flowering
DGDG	digalactosyldiacylglycerol
DN	dihydroxysphingoid base with normal fatty acids
LOD	limit of detection
dwb	dry weight basis
ELSD	evaporative light—scattering dectector
EMS	ethyl methanesulfonate
ER	endoplasmic reticulum
ESG	esterified sterylglucoside
g	gram
GalCer	galactosylceramide
GC	gas chromatography
GIPC	glycosyl inositol phosphoryl ceramide
GlcCer	glucosylceramide
GLM	general linear model
GSL	glycosphingolipid
h	α -hydroxyl fatty acids
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography

hr	hour
L	liter
$LSD_{0.05}$	least significant difference at the 0.05 probability level
M	mole per liter
MAG	monoacylglycerol
mg	milligram
MGDG	monogalactosyldiacylglycerol
min	minute
mL	milliliter
mol	mole
MS	mass spectrometry
nmol	nanomole
NMU	*N*-nitroso *N*-methylurea
NPE	*N*-acylphosphatidyl- ethanolamine
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PL	phospholipid
PS	phosphatidylserine
PSL	phosphosphingolipid
SB	sphingoid base
r	correlation coefficient
SG	sterylglucoside
SL	sphingolipid
SM	sphingomyeline
SPT	serine palmitoyl-transferase
TA	trihydroxysphingoid base with α-hydroxyl fatty acids
TLC	thin layer chromatography
TN	trihydroxysphingoid base with normal fatty acids

CHAPTER 1. GENERAL INTRODUCTION

LITERATURE REVIEW

Sphingolipids (SL) are a structurally diverse class of lipids found in the cell membranes of all eukaryotes and some prokaryotes. They were noted by J. L. Wilhelm in 1884 during the study of the chemical constituents of ox brain, and were named after the riddle of the ancient Greek Sphinx (*1*). E. Klenk identified the chemical structure of sphingosine in 1929 (*1*). Carter et al. proposed the term "sphingolipid" as a designation for lipids containing sphingosine in 1947 (*1*). Since then, numerous researchers have explored their structures, biosynthesis, biological functions, and health benefits. SL have been recognized as second messengers involved in cell regulation, signal transduction, and cell apoptosis (*2*). However, most studies focus on the SL of mammals or fungi. SL from plants have only been investigated in recent years. This review will emphasize plant SL and will cover five parts: 1) a brief introduction of the diversity of SL; 2) biosynthesis of SL in plants; 3) biological functions of SL in plants; 4) potential health benefits of plant SL; and 5) SL in soybeans and strategies to maximize SL content in soybean seeds.

Diversity of Sphingolipids

SL are a collection of different derivatives with a common backbone known as the long chain sphingoid base (LCSB). More than 300 molecular structures have been identified and described so far. Based on their functional groups, SL have been classified into four groups: sphingoid bases, ceramides, glycosphingolipids, and phosphosphingolipids. Representative substructures of these four groups are shown in Figure.1.

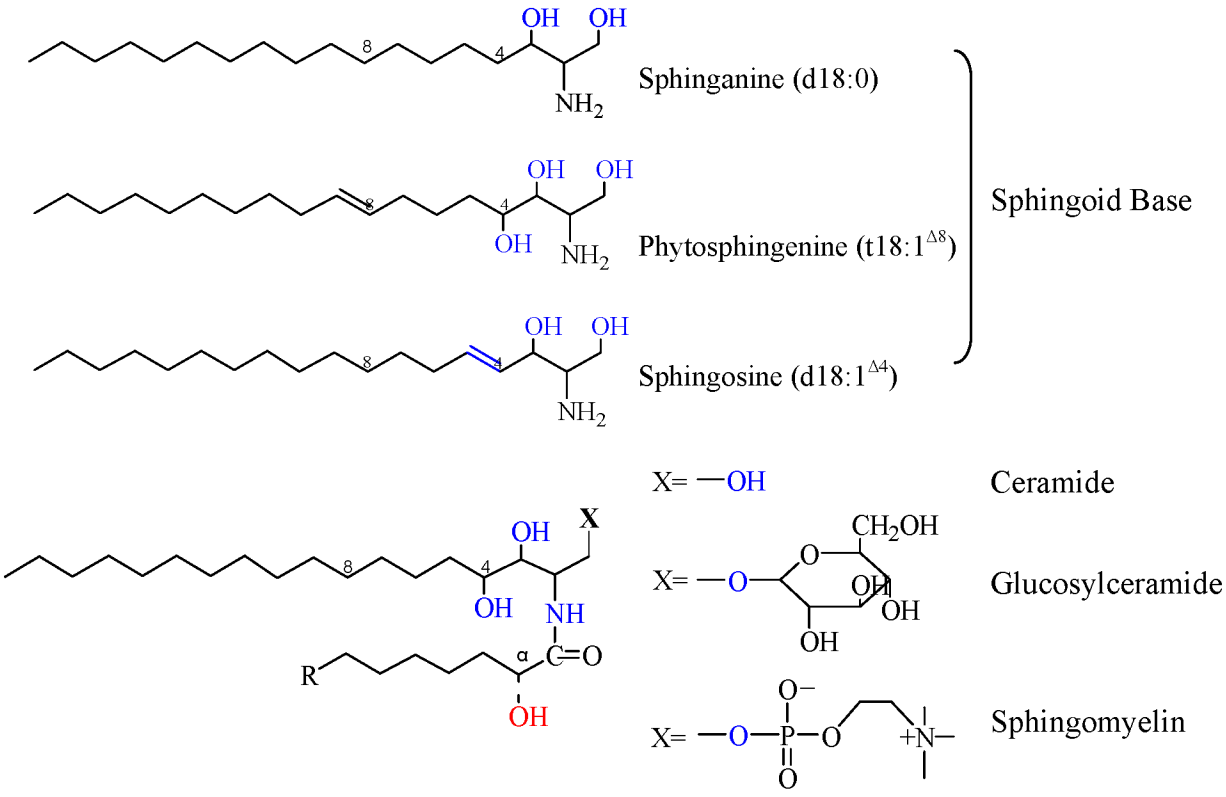

Figure 1. General structures of sphingolipids.

The sphingoid bases (SB) are one group of SL. The predominant SB is a linear alkyl amino alcohol of 18 carbons with different degrees of unsaturation at carbon 4 and/or carbon 8, and/or with a hydroxyl group at carbon 4. The configuration of the double bond in the carbon 8 position may be *cis* or *trans*, while the double bond in the carbon 4 typically has the *trans* configuration. The abbreviations of the SB are designed by (in order of appearance in the abbreviations) the number of hydroxyl groups (d for dihydroxy and t for trihydroxy), the chain length, and the number of double-bonds. For example, t18:1$^{\Delta 8}$ represents phytosphingenine, which has an 18 carbon chain with one double bond at carbon 8 and three hydroxyl groups. The SB in plants are sphingenine (d18:1$^{\Delta 8}$), phytosphinganine (t18:0), and phytosphingenine (t18:1$^{\Delta 8}$), whereas sphingosine (d18:1$^{\Delta 4}$) is predominant in mammals, and sphinganine (d18:0) or phytosphinganine is in yeast (*3*).

A second group of SL, ceramides (Cer), are formed by the addition of long chain fatty acids to SB in an amide linkage. Besides the diversity of SB, the fatty acyl group of Cer varies in the chain length, the degree of unsaturation, and/or the occurrence of a hydroxyl group in the α or ω carbon. Similar to the abbreviation of free fatty acids, Cer are designated by an h for hydroxyl fatty acids or c for normal fatty acid with the carbon number and the degree of unsaturation. Generally, h14:0 − h26:0 are the predominant fatty acyl moieties of plant Cer, while c8:0 − c18:0 are in animal Cer (*4*). The fatty acid composition of Cer may be different with tissues and maturity stages (*4, 5*).

Glycosphingolipids (GSL) are a third group of SL, typically found in the membranes of eukaryotic cells (*6*). Based on their glycosyl head group at the primary alcohol of Cer, GSL have been classified into two groups. One is glycosyl inositol phosphoryl ceramide (GIPC), in which inositol-1-phosphate is linked to the primary hydroxyl of Cer to form a phosphodiester and the inositol end is further extended by oligosaccharide chains. GIPC is a free membrane lipid and membrane anchors of covalently bound proteins (*3*). The other group of GSL is cerebroside, which is formed by the linkage of a monosaccharide to the terminal hydroxyl of Cer. Cerebroside serves as the precursor of more complex SL such as sulfatides and gangliosides. The simplest cerebrosides are glucosylceramides (GlcCer) commonly found in animals, plants, and fungi, and galacosylceramide (GalCer) which are unique in mammals (*4*).

Phosphosphingolipids (PSL) are a fourth group of SL. Compared with the glycerophospholipids, PSL are formed by connecting phosphatyl groups to the terminal hydroxyl of Cer. Sphingomyelin (SM) is a major PSL unique to animal cells, in which

phosphoryl choline attaches to the terminal hydroxyl of Cer (*7*). SM usually is quantified with glycerophospholipids as part of total phospholipids (PL).

The animal or plant cells also contain a number of other SL, such as sphingosine-1-phosphate (S1P), ceramide phosphate, and lyso-sphingolipid, which may be presented in small amount but highly bioactive (*3*). They are not described in detail in this review. But the understanding the bio-functions of these trace SL may be important in disease controls (*3*).

Some of the SL mentioned above have been identified with the development of advanced spectrometric techniques. Most of them have not been quantified accurately. Based on a report by Heinz (*8*) and the recalculated data published by Sperling et al. (*3*), the approximate SL contents in 1 kg fresh weight of plant tissues, including about 10% lipid on a dry weight basis (dwb), were: S1P, 30 nmol; SB, 1−2 μmol; free Cer, 10−30 μmol; GlcCer, 100−400 μmol; and glycerophospholipids, 2−5 mmol. The absolute quantities of SL and their specific molecular distribution vary with the organelles, cells, and tissues studied, and with the analytical methods (*3*).

Biosynthesis of Sphingolipids in Plants

SL have been found in relatively high concentrations in the plasma membrane and other organelle membranes, such as the endoplasmic reticulum (ER) and the Golgi apparatus. Spassleva et al. (*9*) reviewed plant SL and pointed out that GlcCer were not only present in the plasma membrane, but in the tonoplast, chloroplast, and mitochondria membranes. The GlcCer in the outer monolayer of plasma membrane account for about 7−30% of membrane lipids (*3*). The distributions of GlcCer in other membranes remain to be investigated and quantified.

The pathway of SL biosynthesis has been well studied in mammals and fungi (*4*). Because it is difficult to identify enzymes and genes in plants, the pathway of plant SL biosynthesis has only been proposed recently as shown in Figure 2. The *de novo* SL biosynthesis starts from the condensation of palmitoyl-CoA and serine, generating the 3-ketosphinganine by serine palmitoyl−transferase on the cytosolic side of ER (*10*). The 3-ketosphinganine is reduced to sphinganine at the same location. Once formed in plants, sphinganine has several possible fates: (a) it may be acylated to form dihydroceramides directly by Cer synthase using acyl-CoA as acyl donor, (b) be phosphorylated to S1P, (c) be hydroxylated to 4-hydroxysphinganine. Cer synthesis takes place at the cytoplasmic leaflet of the ER, and Cer can be further converted to GlcCer by GlcCer synthase or to other complex SL at the cytosolic surface of the Golgi apparatus using uridine diphosphoglucose (UDP-glucose) as the sugar donor or sterylglucoside (SG) as the glucose donor (*2*). In addition, the GlcCer can be converted to Cer by GlcCerase to change the membrane structures and to regulate the cell bioactivity (*2*). For the SL biosynthesis pathway, the condensation of palmitoyl-CoA with serine is thought to the rate control step because this reaction is irreversible, and free SL and Cer do not accumulate as biosynthetic intermediates in health cells (*2, 4, 11*).

Some important steps in plant Cer biosynthesis are the modifications of SB and the fatty acyl chain (*2, 4*). These modifications provide specific substrates to synthesize unique molecules in plant tissues. Three major SB modifications in plants are Δ4- desaturation, Δ8-desaturation, and C-4 hydroxylation. Wright et al. (*12*) examined the hydroxylation of free sphinganine and suggested that the SB hydroxylation and desaturation may occur principally after the Cer formation. The modification of fatty acyl chain includes the Cer hydroxylation to form the majority of the α-OH fatty acyl Cer. Lych et al. (*2*) have pointed out that

sphinganine or sphingosine serve as substrates of Cer instead of 4-hydroxysphinganine, which is a product of the Cer turnover rather than a precursor of the Cer formation.

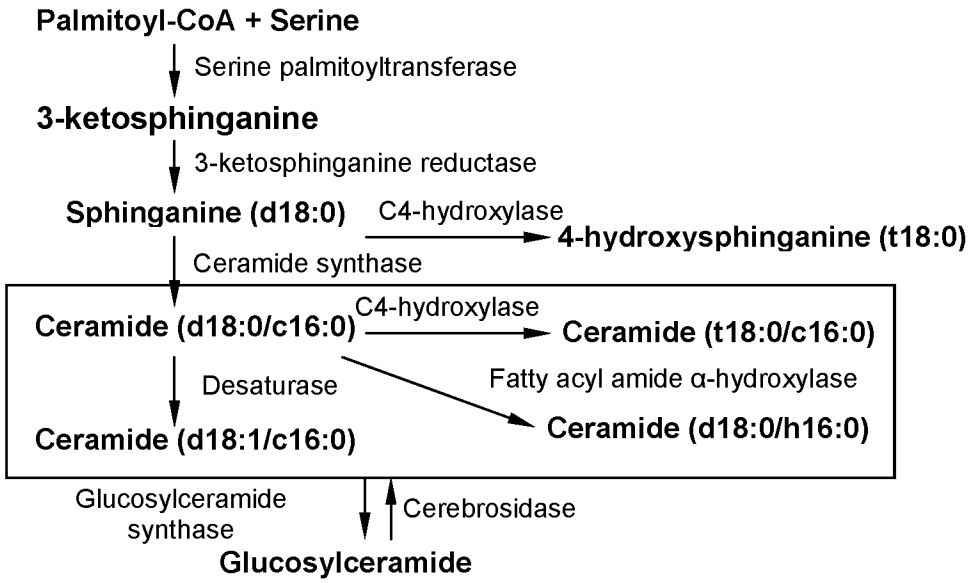

Figure 2. Pathway of ceramide and glucosylceramide biosynthesis in plant *(4)*

Biological Functions of Sphingolipids in Plants

SL are both structural and functional lipids. Little is known about the biological functions of SL in plants. Based on a review by Sperling et al. (*3*), the roles of plant SL are considered to be involved in membrane stability, environmental stress, and cell signaling.

Membrane Stability. SL are important membrane components. GlcCer make up about 5−30 mol % of the total lipid in the plasma membrane and 9−23 mol % of the total lipid in the tonoplast (*2*). SL tend to combine with sterols to form raft−like domains, which are thought to increase the membrane stability and to decrease the permeability of membranes because of the long chain hydroxyl acyl group and the intra- or inter-molecular hydrogen bonds between amide and hydroxyl groups (*13*). The highly hydroxylated SL in plants may

function as a barrier to stabilize membranes. In addition, a small amount of free Cer can significantly stabilize SL−sterol rafts (*3*).

Environmental Stress. Plant GlcCer also demonstrate unusual physical behaviors and cause gel-phase metastability, both of which are very important in tolerating environmental stresses (*3*). Studies have shown that tri-OH SB with a *cis* double bond at the C8 position are more prevalent in chilling resistant and frost tolerant plants, monounsaturated hydroxy fatty acid chains (particularly h24:1) are common in frost tolerant plants, and saturated hydroxy fatty acids are present exclusively in sensitive plants (*14, 15*).

Cell Signaling. There is limited information indicating that SL are important cellular mediators in plants. It has been reported that free SB have negative effects on the cell growth and viability when they were presented at elevated concentrations (*11, 16*). Liang et al. (*17*) provided evidences that Cer and its derivatives Cer-1-P play a role in mediating cell death in *Arabidopsis thaliana*.

Potential Health Benefits of Consuming Plant Sphingolipids

SL can be obtained from the diet as well as from de novo biosynthesis. The amount of SL and their molecular species vary considerably with different dietary sources. In a comprehensive review, Vesper et al. (*18*) listed the approximate content of SL in 29 food products and suggested that cream, cheese, and egg are good sources of SM (1.3−2.2 μmol/g). Sugawara and Miyazawa (*19*) determined the glycolipids from 48 edible plant sources and found that cerebroside was one of the major groups, and accounted for about 12% of the total glycolipid content. Among these edible plants, soybean has been suggested as a potential source of GlcCer.

Currently, there are no nutritional requirements for dietary SL. They still are important dietary components. Exogenous SL can be hydrolyzed in the small intestine and converted to bioactive molecules such as SB, Cer, and S1P, which regulate the cell growth, differentiation, and apoptosis (*2*). The potential health benefits of SL have been studied in recent years. Some animal studies showed that milk SL (SM and GalCer) inhibited colon carcinogenesis when fed to mice with induced colon tumors (*20*−*22*). SL from bovine brain reduced plasma cholesterol level by about 30% when fed to mice (*23*). However, the Cer backbones of these dairy SL are somewhat different from those of plant SL. In plants, sphinganine (d18:0) and sphingosine (d18:$1^{\Delta 4}$) are found in trace amount, whereas sphingadienine (d18:$2^{\Delta 4, 8}$) and phytosphingenine (t18:$1^{\Delta 8}$) are the predominant SB (*4*). The α-hydroxyl fatty acids are almost exclusively fatty acids with 16, 20, 22, and 24 chain lengths in GlcCer (*4*). The different structures of plant SL may lead to unique heath benefits.

Few studies have been conducted on the physiological and biological activities of plant SL. Aida et al. (*24*) investigated the properties and the physiological effects of cerebroside from soybean, maize, wheat grain, rye grain, and rice bran. They suggested that dietary plant cerebroside had a potential physiological function similar to that of animal SL from bovine brain. Gossioux (*25*) reviewed cosmetic uses of plant Cer and indicated that plant Cer were preferable to animal Cer for moisturizing skin because of their structural similarity to the Cer of the stratum granulosum. Sugawara et al. (*26*) investigated the digestion of maize cerebroside in rats and found that the digestibility of maize cerebroside was similar to that of the cerebroside of mammalian origins. However, the isomers of sphingadienine (d18:$2^{\Delta 4, 8}$) obtained from maize cerebroside were poorly absorbed in digestive tract. A preliminary study by Sullard et al. (*27*) showed that wheat and soybean Cer had a toxicity in a human

cancer cell compared to the Cer derived from SM. Sugawara et al. (*28*) reported that apoptosis induction by wheat–flour SB (d18:1$^{\Delta 8}$) reduced the viability of human colon cancer cells in a dose-dependent manner, which was similar to that caused by animal SB (d18:1$^{\Delta 4}$). By feeding soybean GlcCer to mice with induced colon tumors, Symolon et al. (*29*) found that soybean GlcCer reduced colonic cell proliferation and the number of aberrant colonic crypt foci. Their results suggested that the soybean GlcCer should be further examined as a possible inhibitor of colon cancer.

Sphingolipids in Soybeans

Soybean is one of the most important oil seed crops and is widely used in food products. The main polar lipids of soybeans are PL or soy lecithin, and they have been widely used as an emulsifier in food products and as health-promoting ingredients in certain functional products and nutritional supplements. The minor polar lipids, such as SL, have not been given much attention in food products, even though they have functionalities similar to or better than SL from animals or fungi and may confer health benefits (23, 25, *27, 29*). The reason might be their relatively low content and the limited amount of biological information.

SL in soybeans have been investigated for several decades. Ohnishi and Fujino (*30*) first identified SL in soybean seeds and reported that Cer and GlcCer were the major SL components. They found that the composition of these two SL classes were similar in both immature and mature soybean seeds. The principle molecular species were t18:1$^{\Delta 8}$/h24:0 for Cer and d18:2$^{\Delta 4, 8}$/h16:0–glucose for GlcCer. Ohnishi et al. (*31*) investigated the SL in soybean leaves and found that t18:1$^{\Delta 8}$ (t18:0) / c16:0 (c18:0) were the predominant Cer and t18:0 (d18:2$^{\Delta 4, 8}$)/c16:0 (c18:0)–glucose were the predominant GlcCer. These two studies did

not report the absolute content of SL in soybean samples. Sugawara et al. (*19*) quantified GlcCer content using high-performance liquid chromatography (HPLC) with an evaporative light scattering detector (ELSD). They reported that the GlcCer concentration in soybean was 110 nmol/g (dwb). Recently, Gutierrez et al. (*32*) determined the effect of processing on soybean GlcCer content and found that about 91% of the GlcCer as retained in defatted soy flakes and 9% of the GlcCer remained in the oil after hexane extraction. Gutierrez et al. (*33*) also quantified GlcCer content in various soybean genotypes and showed that the GlcCer concentration ranged from 142 to 492 nmol/g (dwb).

Thus, SL content in soybeans might be maximized by controlling soybean seed development and by selecting genotypes. In this study, we hypothesized that soybean seeds with elevated palmitate content may have higher SL concentrations because the increase in the concentration of initial substrate (palmitoyl-CoA) would enhance the production of SL. We also hypothesized that soybean seeds would have higher concentrations of SL and PL at immature stages than at mature stages because SL and PL are mostly found in the membrane and the relative proportion of membrane components in the seeds would decrease during development.

THESIS ORGANIZATION

This thesis consists of a general introduction, followed by two research papers and a general conclusion section. The papers are in the journal format required by the American Chemical Society.

LITERATURE CITED

(1) http://www.cyberlipid.org/history/history1.htm, Cyberlipid Center, 2005.

(2) Lynch, D.V.; Dunn, T. M. An introduction to plant sphingolipids and a review of recent advances in understanding their metabolism and function. *New Phytol.* **2004**, *161*, 677−702.

(3) Sperling, P.; Warnecke, D.; Heinz, E. Plant sphingolipids. In *Lipid Metabolism and Membrane Biogenesis*; Daum G., Ed.; Springer-Verlag: Berlin, Germany, **2004**; pp 337−380.

(4) Warnecke, D.; Heinz, E. Recently discovered functions of glucosylceramides in plants and fungi. *Cell Mol. Life Sci.* **2003**, *60*, 919−941.

(5) Kondo, Y.; Nakano, M. Kinetic changes in free ceramide and cerebroside during germination of black gram. *Agri. Biol. Chem.* **1986**, *50*, 2553−2559.

(6) Robson, K. J.; Stewart, M. E. 6−hydroxy-4-sphingenine in human epidermal ceramides. *J. Lipid Res.* **1994**, *35*, 2060−2068.

(7) Limonnier, L. A.; Dillehay, D. L.; Vespremi, M. J. Sphingomyelin in the suppression of colon tumors: prevention versus intervention. *Arch. Biochem. Biophys.* **2003**, *419,* 129−138.

(8) Heinz, E. Plant glycolipids: Structure, isolation and analysis. In *Advances in Lipid Methodology*; Christie, W.W., Ed.; The Oily Press: Dundee, Scotland, **1996**; pp 211−332.

(9) Spassieva, S.; Hille, J. Plant sphingolipids today-are they still enigmatic? *Plant Biol.* **2003**, *5*, 125−136.

(10) Lynch, D. V. Enzymes of sphingolipid metabolism in plants. *Method Enzymol.* **2000**, *311*, 130−149.

(11) Merrill, A. H.; Wang, E. Biosynthesis of long-chain (sphingoid) bases from serine by LM cells. Evidence for introduction of the 4-trans-double bond after de novo biosynthesis of N-acylsphinganine(s). *J. Biol. Chem.* **1986**, *261*, 3764 – 3769.

(12) Wright, B. S.; Snow, J. W.; Lynch, D. V. Synthesis of 4-hydroxysphinganine and characterization of sphinganine hydroxylase activity in corn. *Arch. Biochem. Biophys.* **2003**, *415*, 184−192.

(13) Boggs, J. Lipid intermolecular hydrogen bonding: influence on structural organization and membrane function. *Biochim. Biophys. Acta.* **1987**, *906*, 353−340.

(14) Imai, H.; Ohnishi, M.; Hotsubo, K.; Kojima, M. Ito S. Structure and distribution of cerebroside containing unsaturated hydroxyl fatty acids in plant leaves. *Biosci. Biotechnol. Biochem*. **1995**, *59*, 1309−1313.

(15) Imai, H.; Ohnishi, M.; Hotsubo, K.; Kojima, M. Ito S. Sphingoid base composition of cerebrosides from plant leaves. *Biosci. Biotechnol. Biochem.* **1997**, *61*, 351−353.

(16) Wang, H.; Li, J.; Bostock, R. M.; Gilchrist, D. G. Apoptosis: a functional paradigm for programmed plant cell death inducted by a host selective phytotoxin and invoked during development. *Plant Cell* **1996**, *8*, 375−391.

(17) Liang, H.; Yao, N.; Song, J. T.; Luo, S.; Lu, H.; Greenberg, J.T. Ceramide phosphoylation modulates programmed cell death in plants. *Gene Dev.* **2003**, *17*, 2636−2641.

(18) Vesper, H.; Schmelz, E.; Merrill, A. H. Sphingolipids in food and the emerging importance of sphingolipids to nutrition. *J. Nutr.* **1999**, *129*, 1239−1250.

(19) Sugawara, T.; Miyazawa, T. Separation and determination of glycolipids from edible plant sources by high-performance liquid chromatography and evaporative light-scattering detection. *Lipids* **1999**, *34*, 1231−1237.

(20) Schmelz, E. M.; Sullards, M. C.; Dillehay, D. L.; Merrill, A. H. Colonic cell proliferation and aberrant crypt foci formation are inhibited by dairy glycosphingolipids in 1, 2-dimethylhydrazine-treated CF1 mice. *J. Nutr.* **2000**, *130*, 522−527.

(21) Dillehay, D. L.; Web, S. K.; Schmelz, E. M.; Merrill, A. H. Dietary sphingomyelin inhibits 1, 2-dimethylhydrazine-induced colon cancer in CF1 mice. *J. Nutr.* **1994**, *124*, 615−620.

(22) Limonnier, L. A.; Dillehay, D. L.; Vespremi, M. J. Sphingomyelin in the suppression of colon tumors: prevention versus intervention. *Arch. Biochem. Biophys.* **2003**, *419*, 129−138.

(23) Kobayashi, T.; Shimizugawa, T.; Osakabe, T.; Watanabe, S.; Okuyama, H. A long-term feeding of sphingolipids affected the levels of plasma cholesterol and hepatic triacylglycerol but not tissue phospholipids and sphingolipids. *J. Nutr. Res.* **1997**, *17*, 111−114.

(24) Aida, K.; Takakuwa, N.; Kinoshita, M.; Sugawara, T.; Imai, H.; Ohnishi, M. Properties and physiological effects of plant cerebroside species as functional lipids. In *Advanced research on plant lipids*; Murata et al., Ed.; Kluwer Academic Publishers: The Netherlands, **2003**; pp 233−236.

(25) Gossioux, P. Plant ceramides. *Parfums Cosmetiques Aromes* **1992**, *103*, 55−61.

(26) Sugawara, T.; Kinoshita, M.; Ohnishi, M.; Nagata, J.; Saito, M. Digestion of maize sphingolipids in rats and uptake of sphingadienine by Caco-2 cells. *J. Nutr.* **2003**, *133*, 2777−2782.

(27) Sullard, M. C.; Lynch, D. V.; Merrill, A. H. Structure determination of soybean and wheat glucosylceramides by tandem mass spectrometry. *J. Mass Spectrom.* **2000**, *35*, 347−353.

(28) Sugawara, T.; Kinoshita, M.; Ohnishi, M.; Miyazawa, T. Apoptosis induction by wheat−flour sphingoid bases in DLD-1 human colon cancer cells. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 2228−2231.

(29) Symolon, H.; Schmelz, E. M.; Dillehay, D. L.; Merrill, A. H. Dietary soy sphingolipids suppress tumorigenesis and gene expression in 1, 2-dimethylhydrazaine-treated CF1 mice and Apc Min/+ mice. *J. Nutr.* **2004**, *134*, 1157−1161.

(30) Ohnishi, M.; Fujino, Y. Sphingolipids in immature and mature soybeans. *Lipids* **1982**, *17,* 803−813.

(31) Ohnishi, M.; Seisuke, I.; Fujino,Y. Chemical characterization of ceramide and cerebroside in soybean leaves. *Agri. Biol. Chem.* **1982**, *46*, 2855−2861.

(32) Gutierrez, E.; Wang, T. Effect of processing on sphingolipid content in soybean products. *J. Am. Oil Chem. Soc.* **2004**, *81*, 971−977.

(33) Gutierrez, E.; Wang, T.; Fehr, W. R. Quantification of sphingolipids in soybeans. *J. Am. Oil Chem. Soc.* **2004**, *81*, 737−742.

CHAPTER 2. HPLC QUANTIFICATION OF SPHINGOLIPID IN SOYBEANS WITH MODIFIED PALMITATE CONTENT

A paper has been accepted by the *Journal of Agricultural and Food Chemistry*

Liping Wang,[†] Tong Wang,[*,†] and Walter R. Fehr[§]

ABSTRACT

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

[†] Department of Food Science and Human Nutrition, Iowa State University, Ames, Iowa 50011
[§] Department of Agronomy, Iowa State University.
*Address correspondence to this author at the Department of Food Science and Human Nutrition, 2312 Food Sciences Building, Iowa State University, Ames, IA 50011-1061 [telephone (515) 294-5448; fax (515) 294-8181; e-mail tongwang@iastate.edu].

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 dihydroxysphingosines 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 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-hydroxy-octadecanoyl)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, sterylglucoside (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 hr and with 50 mL of water-saturated 1-butanol for 8 hr. 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 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 ($KMnO_4/K_2CO_3$/0.5% NaOH/H_2O, 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 contains 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 $Y=2\times10^7X^{1.7635}$ for TA-2 and $Y=5\times10^6X^{1.7701}$ 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 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 hr. The second aliquot was transesterified with 0.5 mL of 0.5 M $NaOCH_3$ in methanol at room temperature for 1 hr. 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.

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 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 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 trihydroxysphingenines for Cer and dihydroxysphingedienines 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.

ACKNOWLEDGEMENT

The authors thank Center for Designing Foods to Improve Nutrition (CDFIN) at Iowa Stated University for financial support.

LITERATURE CITED

(1) Lynch, D. V.; Dunn, T. M. An introduction to plant sphingolipids and a review of recent advances in understanding their metabolism and function. *New Phytol.* **2004**, *161*, 677−702.

(2) Warnecke, D.; Heinz, E. Recently discovered functions of glucosylceramides in plants and fungi. *Cell. Mol. Life Sci.* **2003**, *60*, 919−941.

(3) Cantatore, J. L.; Murphy, S. M.; Lynch, D. V. Compartmentation and topology of glucosylceramide synthesis. *Biochem. Soc. Trans.* **2000**, *28*, 748−750.

(4) Merrill, A. H.; Williams, R. D. Utilization of different fatty acyl-CoA thioesters by serine palmitoyltransferase from rat brain. *J. Lipid Res.* **1984**, *25*, 185−188.

(5) Merrill, A. H.; Nixon, D. W.; Williams, R. D. Activities of serine palmitoyltransferase (3-ketosphinganine synthase) in microsomes from different rat tissues. *J. Lipid Res.* **1985**, *26*, 617−622.

(6) Merrill, A. H.; Wang, E.; Mullins, R. E. Kinetics of long-chain (sphingoid) base biosynthesis in intact LM cells: effects of varying the extracellular concentrations of serine and fatty acid precursors of this pathway. *Biochemistry* **1988**, *27*, 340−345.

(7) Fehr, W. R.; Hammond, E. G. Soybeans and soybean products having low palmitic acid content. U.S. Patent 5 585 535, 1996.

(8) Fehr, W. R.; Hammond, E. G. Elevated palmitic acid production in soybeans. U.S. Patent 5 750 846, 1998.

(9) Heinz, E. Plant glycolipids: structure, isolation and analysis. In *Advances in Lipid Methodology*; Christie, W. W., Ed.; The Oily Press: Dundee, Scotland, **1996**; pp 211−332.

(10) Sperling, P.; Warnecke, D.; Heinz, E. Plant sphingolipids. In *Lipid Metabolism and Membrane Biogenesis*; Daum G., Ed.; Springer-Verlag: Berlin, Germany, **2004**; pp 337−380.

(11) Ohnishi, M.; Fujino, Y. Sphingolipids in immature and mature soybeans. *Lipids* **1982**, *17*, 803−813.

(12) Iwamori, M.; Costello, C.; Moser, H. W. Analysis and quantitation of free ceramide containing nonhydroxy and 2-hydroxy fatty acids, and phytosphingosine by high-performance liquid chromatography. *J. Lipid Res.* **1979**, 20, 86−96.

(13) Alvarez, J. G.; Touchstone, J.; Storey, B. T.; Grob, R. L. Determination of sphingolipid sphingoid bases by HPTLC−fluorescence spectrodensitometry. *J. Liq. Chromatpgr.* **1989**, *12*, 3115−3120.

(14) Guaudi, K.; Chaminade, P.; Baillet, A.; Ferrier, D.; Bleton, J.; Gousaud, S.; Tchapla, A. Contribution to liquid chromatographic analysis of cutaneous ceramides. *J. Liq. Chromatogr. Relat. Technol.* **1999**, *22*, 379−400.

(15) Sullard, M. C.; Lynch, D. V.; Merrill, A. H. Structure determination of soybean and wheat glucosylceramides by tandem mass spectrometry. *J. Mass Spectrom.* **2000**, *35*, 347−353.

(16) Vaghela, M. N., Kilara, A. Quantitative analysis of phospholipids from whey protein concentrates by high-performance liquid chromatography with a narrow−bore column and an evaporative light-scattering detector. *J. Am. Oil Chem. Soc.* **1995**, 72, 729−733.

(17) Conforti, F. D., Harris, C. H.; Rinehard, J. T. High-performance liquid chromatographic analysis of wheat flour lipids using an evaporative light scattering detector. *J. Chromatogr.* **1993**, *645*, 83−88.

(18) Sugawara, T.; Miyazawa, T. Separation and determination of glycolipids from edible plant sources by high-performance liquid chromatography and evaporative light-scattering detection. *Lipids* **1999**, *34*, 1231−1237.

(19) McNabb, T. J.; Cremesti, A. E.; Brown, P. R.; Fischl, A. S. The separation and direct detection of ceramides and sphingoid bases by normal-phase high-performance liquid chromatography and evaporative light-scattering detection. *Anal. Biochem.* **1999**, *15*, 242−250.

(20) Zhou, Q.; Zhang, L.; Fu, X.; Chen, G. Quantitation of yeast ceramides using high-performance liquid chromatography−evaporative light-scattering detection. *J. Chromatogr. B* **2002**, *15*, 161−169.

(21) Farwanah, H.; Nuhn, P.; Neubert, R.; Rauth, K. Normal−phase liquid chromatographic separation of stratum corneum ceramides with detection by evaporative light scattering and atmospheric pressure chemical ionization mass spectrometry. *Anal. Chim. Acta* **2003**, *492*, 233−239.

(22) Fehr, W. R.; Welke, G. A.; Hammond, E. G.; Duvick, D. N.; Cianzio, S. R. Inheritance of reduced palmitate acid content in seed oil of soybean. *Crop Sci.* **1991**, *31*, 88−89.

(23) Narvel, J. M.; Fehr, W. R.; Ininda , J.; Welke, G. A.; Hammond, E. G.; Duvick, D. N.; Cianzio, S. R. Inheritance of elevated palmitate in soybean seed oil. *Crop Sci.* **2000**, *40*, 635−639.

(24) Hammond, E. G. Organization of rapid analysis of lipids in many individual plants. In *Essential oils and waxes*; Lindkins et al., Eds.; Modern Methods of Plant Analysis, Vol. 12; Springer-Verlag: Berlin, Germany, **1991**; pp 321−330.

(25) Folch, J.; Lees, M.; Stanley, G. H. S. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1957**, *226*, 495−509.

(26) Gutierrez, E.; Wang, T.; Fehr, W. R. Quantification of sphingolipids in soybeans. *J. Am. Oil Chem. Soc.* **2004**, *81*, 737−742.

(27) Stoltzfus, D. L.; Fehr, W. R.; Welke, G. A. Relationship of elevated palmitate to soybean seed traits. *Crop Sci.* **2000**, *40*, 52−54.

(28) Takakuwa, N.; Saito, K.; Ohnishi, M.; Oda, Y.; Determination of glucosylceramides contents in crop tissues and by products from their processing. *Bioresour. Technol.* **2005**, *95*, 1089−1092.

(29) Lynch, D. V.; Fairfield, S. C. Sphingolipid long-chain base synthesis in plants. *Plant Physiol.* **1993**, *103*, 1421−1429.

(30) Warnecke, D.; Heinz, E. Recently discovered functions of glucosylceramides in plants and fungi. *Cell. Mol. Life Sci.* **2003**, *60*, 919−941.

(31) Nakayama, M.; Kojima, M; Ito, S. Enzymatic formation of plant cerebroside: properties of UDP-glucose: ceramide glucosyltranferase in radish seedlings. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 1882−1886.

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.

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

Lines	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.

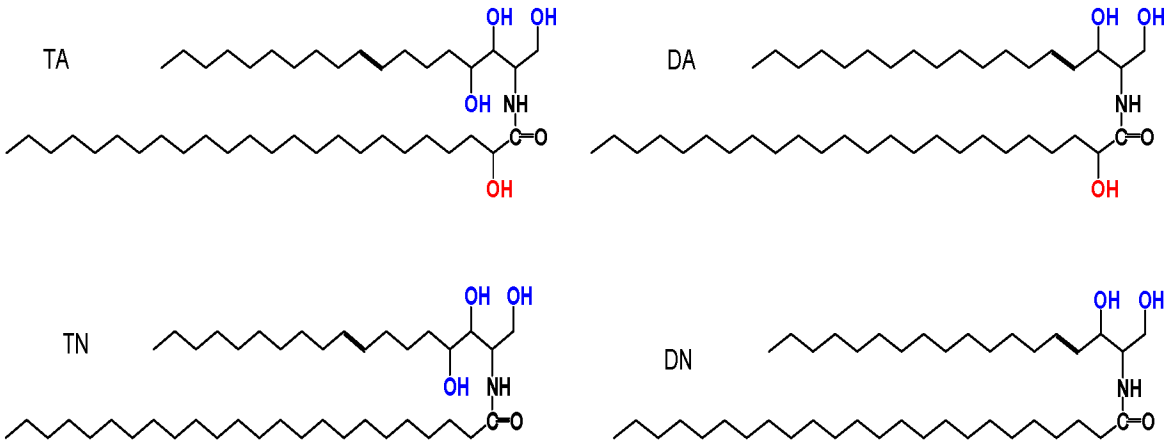

Figure 1. Ceramide structures.

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.

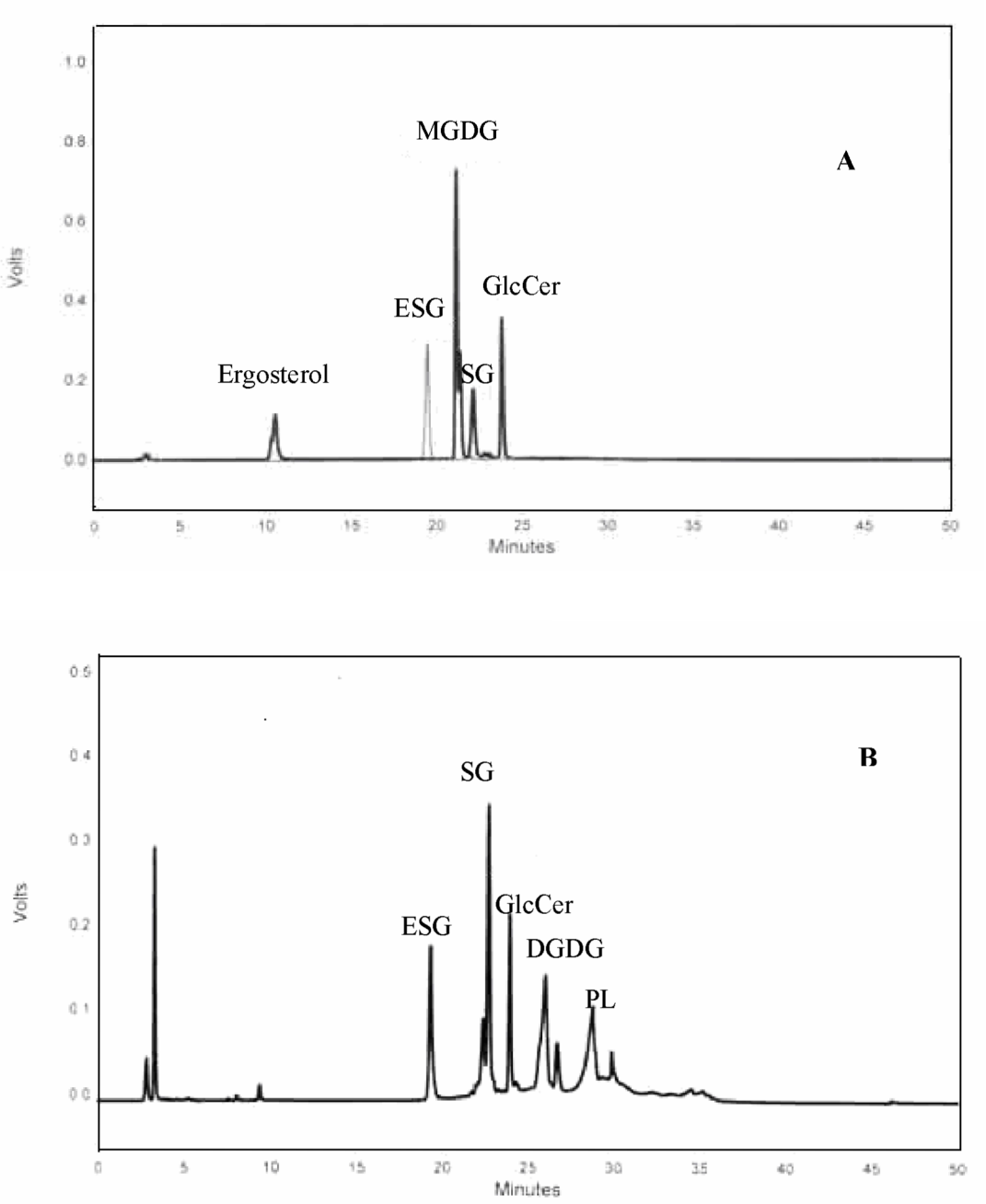

Figure 2. HPLC−ELSD chromatograms of the glucosylceramide separation

(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.

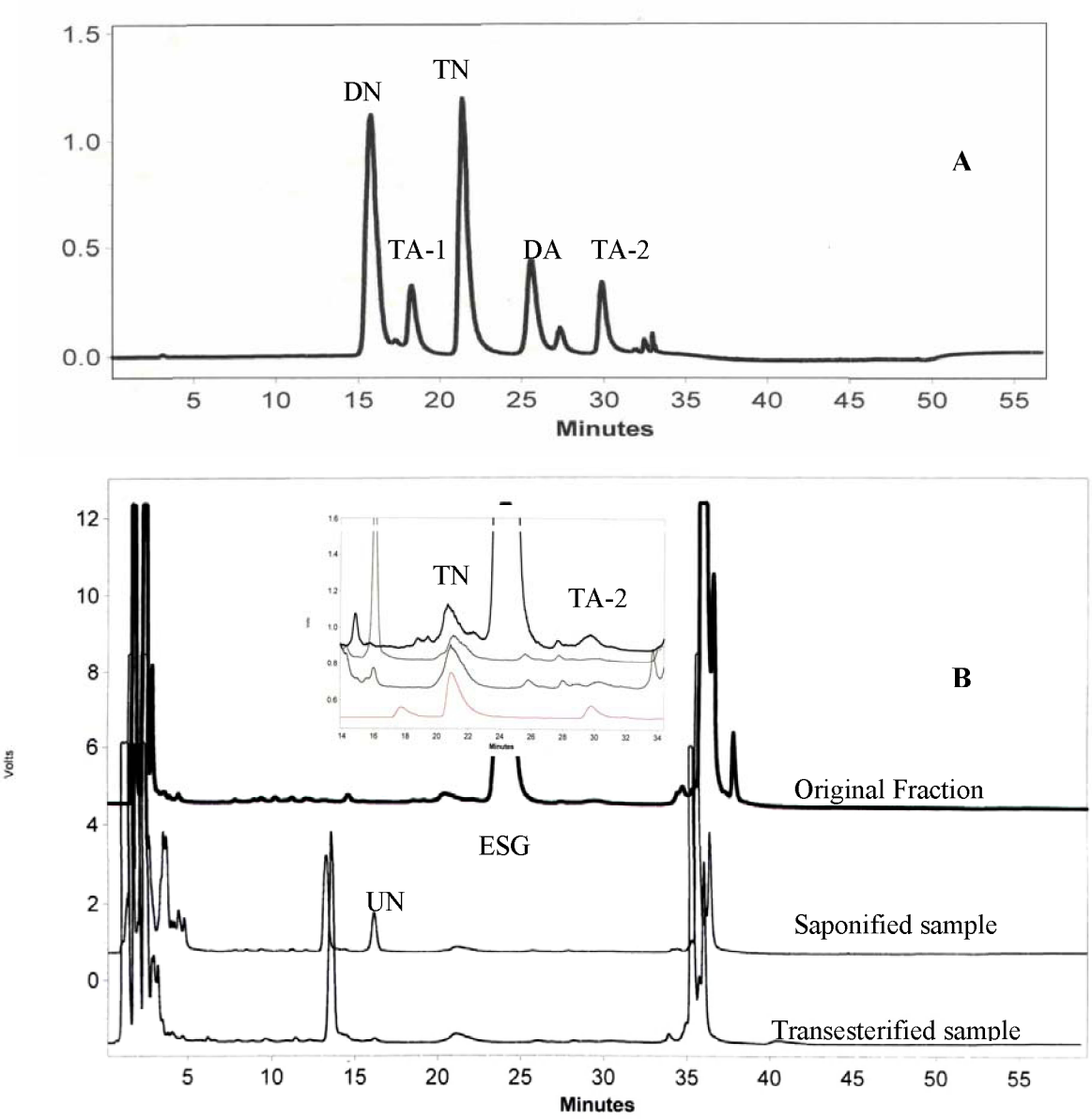

Figure 3. HPLC−ELSD chromatograms of the ceramide separation.

(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.

CHAPTER 3. EFFECT OF SEED DEVELOPMENT STAGE ON SPHINGOLIPID AND PHOSPHOLIPID CONTENTS IN SOYBEAN SEEDS

A paper has been accepted by the *Journal of Agricultural and Food Chemistry*

Liping Wang,[†] Tong Wang,[*, †] and Walter R. Fehr[§]

ABSTRACT

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

[†] Department of Food Science and Human Nutrition, Iowa State University.
[§] Department of Agronomy, Iowa State University.
*Address correspondence to this author at the Department of Food Science and Human Nutrition, 2312 Food Sciences Building, Iowa State University, Ames, IA 50011-1061 [telephone (515) 294-5448; fax (515) 294-8181; e-mail tongwang@iastate.edu].

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, have been utilized as a health-promoting ingredient in functional food products. SL have 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 seeds. 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) 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 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 hr and with 50 mL of water-saturated 1-butanol for 8 hr. 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 × 0.25mm, 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).

HPLC Method for Phospholipid Quantification. A Pholipidec certified silica column (250 × 4.6 mm, 5μm 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, PI, PS, 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 two factorial 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.

RESULTS AND DISCUSSION

Seed Weight, Moisture, Protein, and Total Lipid Contents. Significant differences among the three cultivars were observed for mean fresh seed weight (Table 2 and 3). The fresh seed weight and moisture content of the cultivars changed significantly during seed development (Table 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 (Table 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 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 (Table 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 the cultivars (Table 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 (Table 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 from 11 mol % at 28 DAF to 23 mol % at 68 DAF and 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 (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 (Table 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 (Table 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.

ACKNOWLEDGEMENTS

The authors thank Center for Designing Foods to Improve Nutrition (CDFIN) at Iowa State University for financial support.

LITERATURE CITED

(1) Vesper, H.; Schmelz, E.; Merrill, A. H. Sphingolipids in food and the emerging importance of sphingolipids to nutrition. *J. Nutr.* **1999**, *129*, 1239−1250.

(2) Merrill, A. H.; Symolon, H.; Allegood, J. C.; Peng, Q.; Trotman-Pruett, S.; Sullards, M. C. Nutritional implications of sphingolipids: occurrence and roles in cell regulation. In *Nutrition and Biochemistry of Phospholipids*; Szuhaj, B. F., Nieuwenhuyzen, W., Eds.; AOCS Press: Champaigne, IL, **2003**; pp 61−69.

(3) Ghczy, M.; Boros, M. Phosphatidylcholine as drug substance and as excipient−the mechanism of biological activity. In *Nutrition and Biochemistry of Phospholipids*; Szuhaj, B. F., Nieuwenhuyzen, W., Eds.; AOCS Press: Champaigne, IL, **2003**; pp 234−241.

(4) Symolon, H.; Schmelz, E. M.; Dillehay, D. L.; Merrill, A. H. Dietary soy sphingolipids suppress tumorigenesis and gene expression in 1, 2-dimethylhydrazaine-treated CF1 mice and Apc Min/+ mice. *J. Nutr.* **2004**, *134*, 1157−1161.

(5) Ohnishi, M.; Fujino, Y. Sphingolipids in immature and mature soybeans. *Lipids* **1982**, *17*, 803−813.

(6) Sugawara, T.; Miyazawa, T. Separation and determination of glycolipids from edible plant sources by high-performance liquid chromatography and evaporative light-scattering detection. *Lipids* **1999**, *34*, 1231−1237.

(7) Gutierrez, E.; Wang, T.; Fehr, W. R. Quantification of sphingolipids in soybeans. *J. Am. Oil Chem. Soc.* **2004**, *81*, 737−742.

(8) Munnier-Jolain, N. G.; Ney, B. Sequential development of flowers and seeds on the mainstem of an indeterminate soybean. *Crop Sci.* **1993**, *33*, 768−771.

(9) Schnebly, S. R.; Fehr, W. R.; Hammond, E. G. Fatty ester development in reduced and elevated palmitate lines of soybeans. *Crop Sci.* **1996**, *36*, 1462−1466.

(10) Folch, J.; Lees, M.; Stanley, G. H. S. A simply method for the isolation and purification of total lipids from animal tissues, *J. Biol. Chem.* **1957**, *226,* 495−509.

(11) Wang, L.; Wang, T.; Fehr, W. R. HPLC quantification of sphingolipids in soybeans with modified palmitate content. *J. Agric. Food Chem.* **2006**, *54*, Accepted.

(12) Palacios, L.; Wang, T. Egg−yolk lipid fractionation and lecithin characterization. *J. Am. Oil Chem. Soc.* **2005,** *82*, 571−578.

(13) Lynch, D. V.; Dunn, T. M. An introduction to plant sphingolipids and a review of recent advances in understanding their metabolism and function. *New Phytol.* **2004**, *161*, 677−702.

(14) Herglund, A. H.; Norberg, P.; Quartacci, M. F.; Nilsson, R.; Liljenberg, C. Properties of plant plasma membrane lipid models−bilayer permeability and monolayer behaviour of glucosylceramides and phosphatidic acid in phospholipid mixtures. *Physiol. Plant.* **2000**, *109*, 117−122.

(15) Steponkus, P. L.; Lynch, D. V. Freeze/thaw−induced destabilization of the plasma membrane and the effects of cold acclimation. *J. Bioenerg. Biomembr.* **1989**, *21*, 21−41.

(16) Privett, O. S.; Dougherty, K. A.; Erdahl, W. L.; Stolyhowo, A. Studies on the lipid composition of developing soybeans. *J. Am. Oil Chem. Soc.* **1973**, *50*, 516−520.

(17) Wilson, R.; Rinne, R. W. Phospholipids in the developing soybean seed. *Plant Physiol.* **1974**, *54*, 744−747.

(18) Wilson, R.; Rinne, R. W. Lipid molecular species composition in developing soybean cotyledons. *Plant Physiol.* **1978**, *61*, 830−833.

(19) Wilson, R.; Rinne, R. W. Involvement of phospholipids in triglyceride biosynthesis by developing soybean cotyledons. *Plant Physiol.* **1976**, *57*, 556−559.

(20) Wojciechowski, Z. A. Biochemistry of phytosterol conjugates. In *Physiology and Biochemistry of Sterols*; Patterson et al., Eds.; American Oil Chemists' Society: Champaigne, IL, **1991**; pp 361−395.

(21) Moreau, R. A.; Whitaker, B. D.; Hicks, K. B. Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. *Prog. Lipid Res.* **2002**, *41*, 457−500.

(22) Rubel, A.; Rinne, R. W.; Canvin, D. T. Protein, oil, and fatty acid in developing soybean seeds. *Crop Sci.* **1972**, *44*, 739−741.

(23) Sangwan, N. K.; Gupta, K., Dhindsa, K. S. Fatty acid composition of developing soybeans. *J. Agric. Food Chem.* **1986**, *34*, 415−417.

Table 1. Standard Calibration Equations for Seven Classes of Lipids

Lipid class[a]	Calibration equations[b]	r^2
GlcCer	$Y = 759199X - 10^6$	0.9977
ESG	$Y = 502954X - 10^6$	0.9946
SG	$Y = 992497X - 4*10^6$	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.

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.

Table 3. Composition of Developing Soybean Seeds[a]

Cultivar and component	Days after flowering									$LSD_{0.05}$
	28	33	38	43	48	53	58	63	68[b]	
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 were reported on a dry weight basis.

[b] Mature seed.

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

DAF	SL				PL[e]		Neutral oil [d, f] %	Conjugated Sterols	
	Cer[b] nmol/g	GlcCer nmol/g	Cer mol% of GlcCer	SL,mol% in the polar lipid[c]	%[d]	μmol/g		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; 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

	PL composition, mol%			Fatty ester composition, %				
DAF	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

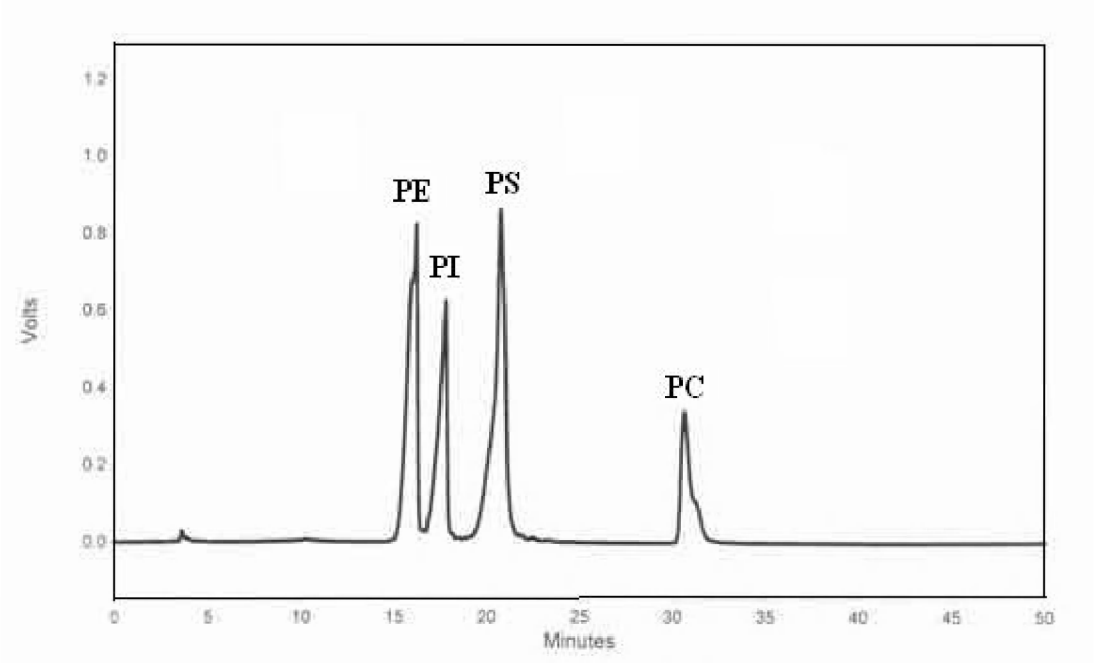

Figure 1. HPLC chromatogram of the soy PL standard mixture

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. The injection amount for each standard was 10 μg.

CHAPTER 4. GENERAL CONCLUSION

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. The results indicated that 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.

Two major membrane components of soybean seeds, SL and PL, were also quantified during seed development. There was a significant decrease in the two lipid components during seed development, which proved our hypothesis that the SL and PL contents would decrease during seed development as the relative proportion of membranes in the seeds decreased during development. Cer content on a dry-weight basis decreased from 51.4 nmol/g at 28 DAF to 22.2 nmol/g at 68 DAF, while GlcCer deceased from 522.8 nmol/g at 28 DAF to 135.8 nmol/g at 68 DAF. The PL percentage in the total lipid decreased from 9.1% at 28 DAF to 3.5% at 68 DAF. The results also indicated that immature soybeans harvested at their greatest fresh weight would have high SL and PL contents.

APPENDIX A. ANALYSIS OF VARIANCE TABLES FOR CHAPTER 2

1 Cer

Source	d.f.	SS	MS	F	Pr>F
Line	14	407.09	29.08	25.07	<0.0001
Error	15	17.40	1.16		
Total	29	424.50			

2 GlcCer

Source	d.f.	SS	MS	F	Pr>F
Line	14	141766.03	10126.14	26.26	<0.0001
Error	15	5783.56	385.57		
Total	29	147549.59			

3 Cer mol% of GlcCer

Source	d.f.	SS	MS	F	Pr>F
Line	14	70.33	5.02	9.61	<0.0001
Error	15	7.56	0.76		
Total	29	77.89			

APPENDIX B. ANALYSIS OF VARIANCE TABLES AND ADDITIONAL DATA FOR CHAPTER 3

1 Seed weight

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	158019.4	79009.7	12931.2	<0.0001
DAF	8	1587423.2	198427.9	32475.9	<0.0001
DAF×Cultivar	16	316463.0	19778.9	3267.14	<0.0001
Error	54	329.9	6.11		
Total	80	2062235.5			

2 Moisture Content

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	710.27	4024.60	26089.9	<0.0001
DAF	8	32196.83	355.13	2302.8	<0.0001
DAF×Cultivar	16	1004.98	62.81	407.2	<0.0001
Error	27	4.16	0.15		
Total	53	33916.25			

3 Protein

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	216.41	108.20	127.22	<0.0001
DAF	8	77.2	9.65	11.35	<0.0001
DAF×Cultivar	16	63.39	3.96	4.66	0.0002
Error	27	22.96	0.85		
Total	53	379.98			

4 Total Lipid

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	37.12	18.56	38.61	<0.0001
DAF	8	76.90	9.61	19.99	<0.0001
DAF×Cultivar	16	35.06	2.19	4.56	0.0003
Error	27	12.98	0.48		
Total	53	162.06			

5 GlcCer

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	5925.57	2962.78	1.27	0.3081
DAF	8	552022.16	69002.8	29.69	<0.0001
Error	15	34867.25	2324.48		
Total	25	592814.98			

6 Cer

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	979.16	489.58	7.55	0.0044
DAF	8	2789.98	348.75	5.67	0.002
Error	15	923.38	61.56		
Total	25	4692.52			

7 SL mol% of Total Polar Lipid

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	1.13	0.57	5.05	0.0211
DAF	8	5.70	0.71	6.43	0.0011
Error	15	1.69	0.11		
Total	25	8.52			

8 SG

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	0.16	0.08	0.71	0.5066
DAF	8	9.24	1.15	1.52	<0.0001
Error	15	1.65	0.11		
Total	25	11.05			

9 ESG

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	0.015	0.007	1.06	0.3705
DAF	8	0.233	0.029	4.23	0.0079
Error	15	0.103	0.007		
Total	25	0.351			

10 PL

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	30.60	15.30	2.07	0.1611
DAF	8	321.25	40.16	5.43	0.0025
Error	15	111.03	7.40		
Total	25	462.88			

11 PL Percentage of the Total Lipid

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	0.89	0.44	0.43	0.6613
DAF	8	85.07	10.63	10.15	<0.0001
Error	15	15.72	1.05		
Total	25	101.69			

12 PE mol% of PL

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	54.58	27.29	7.53	0.0054
DAF	8	316.45	39.55	10.92	<0.0001
Error	15	54.32	3.62		
Total	25	425.35			

13 PI mol% of PL

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	30.27	15.14	7.41	0.0058
DAF	8	99.22	12.40	6.07	0.0014
Error	15	30.65	2.04		
Total	25	160.14			

14 PC mol% of PL

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	140.03	70.02	10.28	0.0015
DAF	8	697.41	87.17	12.80	<0.0001
Error	15	102.16	6.81		
Total	25	939.60			

15 Neutral Oil

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	37.68	18.84	12.58	0.0006
DAF	8	298.44	37.30	24.91	<0.0001
Error	15	22.46	1.50		
Total	25				

16 Palmitate

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	11.22	5.61	3.61	0.0524
DAF	8	173.88	21.73	13.99	<0.0001
Error	15	20.31	1.55		
Total	25	208.42			

17 Stearate

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	1.13	0.66	2.27	0.1380
DAF	8	12.28	1.53	5.53	0.0020
Error	15	4.40	0.29		
Total	25	18.01			

18 Oleat

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	220.51	110.26	6.41	0.0097
DAF	8	372.23	46.53	2.71	0.0459
Error	15	257.82	17.19		
Total	25	50.56			

19 Linoleate

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	272.88	136.44	9.40	0.0023
DAF	8	52.75	6.59	0.45	0.8692
Error	15	217.63	14.51		
Total	25	543.26			

20 Linolenate

Source	d.f.	SS	MS	F	Pr>F
Cultivar	2	12.65	6.33	8.14	0.0040
DAF	8	20.27	2.53	3.26	0.0232
Error	15	11.66	0.78		
Total	25				

21 SL and PL contents on a fresh weight basis for a single seed

DAF	Three soybean cultivars								
	IA1008			IA1010			IA1014		
	Cer	GlcCer	PL	Cer	GlcCer	PL	Cer	GlcCer	PL
	*u*g/seed		mg/seed	*u*g/seed		mg/seed	*u*g/seed		mg/seed
28	1.9	23.1	10.6	0.9	10.1	3.1	0.9	8.3	2.8
33	2.5	22.9	12.2	1.2	22.6	7.6	2	21.7	8.4
38	3.7	29.4	12.4	1.9	35	16.5	3.7	30	12.2
43	5.3	32.2	20.4	2.2	42	20.4	2.5	35.6	15.6
48	5.3	32.8	23.6	4.8	62.4	27.0	2.8	32.1	18.6
53	4.6	25.7	17.4	4.7	37.7	29.9	3.1	35.4	22.4
58	3.5	27.5	13.8	2	24.4	20.4	-	-	-
63	3.7	21.4	17.3	2.7	30	19.2	3.9	27.2	14.5
68	3.4	17.9	19.1	2.7	26.1	18.1	4.4	28.9	21.0

ACKNOWLEDGEMENTS

First of all, I want to thank my major professor, Dr. Tong Wang for everything she meant for me and everything she taught me in these years. She provided me with support and great advice throughout my research work and in the writing of my thesis. I admire her not only for her intelligence but also for her patience, optimism, and devotion to her family, work, and students. She always was there as an advisor and as a friend.

I also would like to express my sincere thanks to Drs. Earl Hammond and Walter Fehr, who served as my committee members. Dr. Earl Hammond was always available to kindly answer my questions and provide endless source of brilliant ideas whenever I had problems and went to him. Dr. Fehr generously selected and provided the soybean lines used in this study, in addition to providing excellent suggestions and guidance in writing my thesis. I also greatly appreciate Grace Welke, Dan Duvick, and other people in Dr. Fehr's group for their hard work in planting, harvesting soybean lines used in this study, and their help in analyzing palmitate contents of soybean lines.

I would like to thank my labmates, Luz Palacios, Elisabeth Gutierrez, Hui Wang, Guang Wang, and Margaret Jiang for their encouragement, help, and friendliness in and out of the lab. I also like to thank the faculties and staffs in Food Science and Human Nutrition department for their patience and supports.

Finally, I sincerely thank my husband Tao Wu for his support and love. I also need to thank the two most important persons in my life, my mother who gave me life and my daughter who is going to extend my life.