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Method development for sphingolipid quantification in soybeans and soy products

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Method development for sphingolipid quantification in soybeans and soy products

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CHAPTER 1. GENERAL INTRODUCTION

Literature Review

Sphingolipid structure

Sphingolipids were discovered by J.L.W. Thudichum, a German practitioner, in 1884 (1). Thudichum first isolated and characterized sphingolipid molecules from bovine brain extracts (2) and named their backbones "sphingosin" (1). Today, the generic term for a non-specific sphingolipid base is sphingoid. Sphingolipids are considered a very complex lipid group and include the following classes: free sphingoid bases, ceramides, sphingophospholipids, and glycosphingolipids (2). The sphingophospholipid and glycosphingolipid classes also contain subclasses based on headgroup compositions. All sphingolipids contain a sphingoid base which share a common core structure, 2-amino, 1, 3-dihydroxy-octadecane or named sphinganine (abbreviated d18:0, d=dihydroxy base) (fig. 1) (3).

Bases may deviate from the core structure through the following variations: 1) alkyl chain length, although C18 is most common, 2) double bonds at C4 and/or C8 (for example, *trans*-4-sphingenine or commonly named sphingosine), 3) branching methyl groups, and 4) the presence of an additional hydroxyl group, usually at C4 (for example, 4-hydroxy-sphinganine or t18:0, t= trihydroxy base) (3). Over 70 sphingoid species have been identified, but the most common mammalian sphingolipid backbone is sphingosine (d18:1$^{\Delta 4}$). Other less common backbones are sphinganine (d18:0) and 4-hydroxysphinganine (t18:0) (4). In plants, the major backbone is 4,8-sphingadienine (d18:2$^{trans\ 4,\ cis\ or\ trans\ 8}$), and other less common backbones include sphinganine (d18:0), 8-sphingenine (d18:1$^{trans\ or\ cis\ 8}$), 4-

hydroxysphinganine (t18:0), and 4-hydroxy-8-sphingenine (t18:0 $^{trans\ or\ cis\ 8}$) (5). Sphingoid bases are normally present in low concentrations in nature compared to complex sphingolipids.

The next more complex sphingolipid class is ceramides. Ceramides are formed as the 2-amino group on the sphingoid base is acylated with a long-chain fatty acid (fig. 1). A variety of ceramide species exist not only due to the variations in the backbone, but also in the fatty acid (i.e. chain length, typically C16-C30, presence of α-hydroxy group, etc.) (6). A variety of polar head groups may attach to the 1-hydroxy position on the ceramide backbone to form even more complex sphingolipids, such as the glycosphingolipids and sphingomyelin (2).

A ceramide molecule is converted into a sphingomyelin molecule as phosphorylcholine is attached to the 1-ol position of its backbone (fig. 1). Other less complex sphingophospholipids, whose backbones are not acylated to a fatty acid but have a phosphate-containing group attached to it include, but are not limited to, sphingosine-1-phosphate and lysosphingomyelin (7). These compounds are nearly absent in cells, but they may play important roles as signaling molecules (2). Sphingosine-1-phosphate has been found to be mitogenic, as discussed in a later section.

Glycosphingolipids are formed when a carbohydrate group(s) becomes attached to the 1-ol position of a ceramide backbone (fig. 1). Glycosphingolipids include the following sub-classes: cerobrosides, globosides, gangliosides and sulfatides (2). Cerobrosides are the simplest of glycosphingolipids because they contain a single sugar residue. Common cerobrosides are glucosylceramide (GlcCer), containing a glucose residue, and galactosylceramide, containing a galactose residue. Globosides contain more than one sugar

residue. Gangliosides are like globosides, although they also contain varying amounts of sialic acid residues (fig. 1). Sulfatides are 3-sulfate esters of galactosylceramide (2).

Animal products contain a very complex mixture of sphingolipids, typically containing glycosphingolipids, sphingomyelin, ceramides, and sulfatides (6). Sphingomyelin is usually the dominating sphingolipid (8). Plant tissues mainly contain GlcCer and ceramides. GlcCer is usually the predominant sphingolipid in most plants (8). Dairy, meat, and soybean products are considered excellent sources of dietary sphingolipids (8).

Sphingolipids in disease prevention

Sphingolipids are primarily located in the outer leaflet of the plasma membrane in all eukaryote and some prokaryotic cells (9). Lesser quantities of sphingolipids are found in the lumen of intracellular vesicles, all organelles associated with membrane trafficking (9), lipoproteins, lamellar permeability barrier of the skin, and membrane rich tissue, such as the liver, pancreas, and neuronal tissue (10). They provide structural integrity to the membrane, engage in cell-to-cell and cell-to-extracellular matrix interactions by acting as ligands and receptors, and serve as binding sites for bacteria, toxins and viruses (11). Until recently, this was all that was known on the biological functions of sphingolipids. In the mid-1980s, it was discovered that sphingosine, a sphingolipid and a sphingolipid metabolite, could competitively inhibit activation of protein kinase C (PK-C) in cell cultures (10). PK-C is an initiator of several cellular responses, such as growth and differentiation, which are associated with the development of carcinogenesis. This discovery resulted in a significant increased effort to further investigate the role of sphingolipid metabolites (i.e. ceramide, sphingosine, and sphingosine-1-phosphate) as intracellular signal transduction molecules

(11). Since then, sphingolipid metabolites have been found to act as mediators of cell growth, differentiation, and programmed cell death (apoptosis) (11).

Sphingolipid metabolites act as lipid "second messengers" for a variety of agonists, such as cytokines, growth factors, hormones, and chemotherapeutics, to conduct a variety of functions (11). For example, certain agonists, such as platelet-derived growth factor (PDGF), stimulate the production of sphingosine-1-phosphate, which is mitogenic and inhibits apoptosis (10). Other agonists, such as tumor necrosis factor α (a cytokine), stimulate the production of both ceramide and sphingosine, which are able to induce apoptosis and act as growth inhibitors. Since sphingolipid metabolites affect several aspects of cell regulation, their affect on cancer was investigated in *in vitro* and *in vivo* studies.

In vitro studies. Ceramide and sphingosine were found to be toxic for a variety of transformed cell lines (HL60, Jurkett, HT29, and CHO cells) and even inhibited cell transformation during the early events of carcinogenesis (10). These results have and continue to be extended to *in vivo* feeding studies to determine the effect of dietary sphingolipids and their metabolites on skin and colon cancer. Models for these cancers have been designed since topical application of sphingolipids is most feasible.

Colon cancer model studies. The first of these studies was conducted by Dillehay et al. (12). In this study, CF1 mice were treated with a colon carcinogen (1,2-dimethylhydrazine, DMH) and supplemented with 0 (control), 0.025, 0.05, and 0.1% sphingomyelin in their diets (w/w). The control group was fed a standard AIN76A diet, which contained very low sphingolipid contents. The development of aberrant colonic crypt foci (ACF-an early biomarker of tumor development) was studied among mice fed sphingomyelin at 0.05% of their diets. In this group, the occurrence of aberrant colonic crypt foci was significantly reduced by 50% in

comparison to the control group (12). Mice fed sphingomyelin at all levels had a colon tumor incidence of 20% compared with 47% for the control group (12). The tumors were not classified as benign adenomas or adenocarcinomas in that study (12), but they were distinguished in a follow-up investigation.

In the follow-up investigation by Schmelz et al. (13), CF1 treated mice were supplemented with the same amounts of sphingomyelin in their diets and were fed these diets 12 weeks longer than in the first study. Similar to the first study, ACF was significantly reduced in the mice fed sphingomyelin (0.1% of diet) by up to 70% compared to the control group. Aberrant crypts per focus also were studied by Schmelz et al. (13) because they were thought to better correlate with the development of tumors than ACF. Aberrant crypts per focus were significantly reduced by 30% in mice fed sphingomyelin (0.1% of diet) compared to the control group. The incidence of colonic tumors were not reduced in the sphingomyelin fed mice, but the proportion of benign adenomas vs. adenocarcinomas was higher in the sphingomyelin fed mice than in the control group. It has been suggested that sphingomyelin may prevent adenomas from progressing into adenocarcinomas or promote reversion of adenocarcinomas into adenomas (13).

The amount of sphingomyelin fed in the above studies was comparable to the estimated sphingolipid amounts consumed in the American diet (0.01% to 0.02% of the diet) (10). Sphingolipids other than sphingomyelin, including glucosylceramide, lactosylceramide, and ganglioside, also were shown to reduce ACF by 50-80%, further indicating that sphingolipids may suppress colon carcinogenesis through the release of their metabolites by hydrolysis (11).

Sphingolipid hydrolyzing enzymes, including sphingomyelinase, glucoceramidase, and ceramidase, are primarily found in the small intestine (6). Intact sphingolipids that are not digested or absorbed in the small intestine are able to reach the colon. Hydrolysis also occurs in the colon, but it is likely done by colonic microflora because germ-free mice exhibit significant reduced hydrolysis (11). The colonic cells are directly exposed to the metabolites produced in the colon, as well as those not absorbed in the small intestine. These finding have stimulated a hypothesis for the mechanism in which sphingolipids may suppress colon carcinogenesis in rodents and possibly how they may in humans.

Hertervig et al. (14) has found that the activity of sphingomyelinase is reduced in both human and rodent colorectal carcinoma compared to healthy tissue, which decreases the production of both ceramide and sphingosine. Dudeja et al. (15) reported that the membrane fluidity of colonic cells was rapidly altered after a single DMH treatment, which was believed to be a result of decreased sphingomyelinase activity and loss of sphingomyelin turnover. If reduction of sphingomyelinase acitivity is discovered to be a defect that contributes to the development of colon cancer, dietary sphingolipids may by-pass this defect and supply the bioactive metabolites, sphingosine and ceramide. Sphingolipids may have important implications to the prevention or treatment of human colon cancer, but to date, neither human clinical (10) or epidemiological studies have been performed to evaluate their influence in this regard (10). Currently, there is also no nutritional requirement for sphingolipids because humans are able to synthesize them (10). Synthesis of sphingolipids is initiated by condensation of L-serine and palmitoyl-CoA and takes place in the endoplasmic reticulum (ER) and the Golgi apparatus of cells (2). However, several studies suggest that dietary sphingolipids may be beneficial to humans as well.

A study that best represents a human model has utilized $APC^{Min-/+}$ (Min) mice (11). Min mice develop a condition related to the human genetic disease familial adenomatous polyposis (FAP) (11). FAP leads to the spontaneous development of intestinal tumors and colon cancer due to one defective gene product (11). The incidence of intestinal tumors in Min mice fed a mixture of sphingolipids (0.1% of the diet) was reduced by up to 53% (11). It also was found that both ceramide and sphingosine induced apoptosis in human adenocarcinoma lines (HT29 cells) and several human colon cancer lines (SW480, HCT 116, and T84) (10). These results provide evidence that dietary sphingolipids may inhibit or prevent the progression of human colon cancer.

Effects of sphingolipids on skin carcinoma. In vitro studies have shown that sphingosine is able to inhibit activation of PK-C by tumor promoter phorbol esters, such as 12-O-tetradecanoylphorbor-13-acetate (TPA) (10). PK-C seems to play a major role in epidermal growth and differentiation (16), and its activation results in increased ornithine decarboxylase activity (ODC) (17). Increased activity of ODC, the rate-limiting enzyme in polyamine synthesis, has been well correlated with tumor promotion in skin and other organs by TPA (17). In early studies ODC activity was measured in the skin of mice treated with TPA alone or TPA and sphingosine to measure the efficacy of sphingosine in inhibiting TPA-induced ODC activity.

In vivo studies by Gupta et al. (16) and Enkvetchakul et al. (17) found that ODC activity was significantly less in mice treated with both TPA and sphingosine than in mice treated with TPA alone (control). Both investigators found that inhibition of TPA by sphingosine was dose dependent. In comparison to the control group, TPA induction of ODC was decreased by more than 50% with doses between 15-20 μmole sphingosine/mouse (16)

and 10-40 μmole sphingosine/mouse (17). These results suggested that sphingosine also may inhibit papilloma (benign tumors) and skin carcinoma development. Birt et al. (18) investigated this hypothesis using sphingosine as well as several of its analogs.

Sphingosine and its analogs N-acetylsphingosine (NAS), N-methylsphingosine (NMS), octylamine (OCT), sterylamine (STR) were evaluated for their ability to prevent papilloma and skin carcinoma development in female Sencar mice (18). Skin tumor development was induced with an initiator, 7,12-dimethylbenz[a]anthracene (DMBA), and a promoter, TPA. Two application protocols of the initiator, promoter and sphingoid were evaluated in this study to form two carcinogenesis studies. The efficacy of sphingosine and its analogs and their ability to inhibit TPA-induced ODC activity and hyperplasia (abnormal cell proliferation) at low (0.05 μmol) and high concentrations (20 μmol) also were assessed.

In the first carcinogenesis study, mice were treated with DMBA. One week after DMBA treatment, the mice received bi-weekly applications of TPA and sphingolipid treatment for 15 weeks (18). The TPA treatment was followed 30 min after the sphingolipid application. NAS, NMS, OCT, and STR were evaluated at 0.05 μmol and 0.50 μmol. Papilloma incidence and multiplicity was not affected by most of the sphingoids investigated, with the exception of OCT. OCT elevated papilloma multiplicity. Skin carcinoma development was inhibited by 0.05 μmol NAS and at both NMS concentrations (0.05 and 0.50).

In the second carcinogenic study, sphingolipids and TPA were applied as in the first study, but after the final treatment, they were treated with the sphingoid bases in a DMBA solution for another 10 weeks (18). SPH, NAS, and NMS at 0.05 μmol and 0.5 μmol were assessed. Papilloma development also was not reduced by any of the sphingolipids studied;

however, NAS inhibited skin carcinoma development at 0.50 μmol and SPH at both doses. At the levels that inhibited carcinoma development for both carcinogenic studies, the sphingoid bases did not decrease TPA-induced ODC activity or hyperplasia and papilloma development. High levels of SPH (5-10 μmole/application) were found to be as effective as TPA (3.2 nmol/application) in inducing hyperplasia. In another study by Enkvetchakul et. al (19), high doses of SPH (10 μmol) also increased tumor development when it was applied with both DMBA and TPA.

The mechanisms in which sphingoid bases may enhance hyperplasia or tumor promotion were not determined. One suggestion was that perhaps the large sphingosine concentration initiated the production of sphingosine-1-phosphate, which inhibits apoptosis and is mitogenic (19). Based on *in vitro* studies, such a result is possible, and the results of *in vivo* studies should be thoroughly and carefully analyzed.

The mechanism by which sphingosine 1-phosphate initiates action is not entirely clear. It has been found that the enzyme that catalyzes the conversion of sphingosine to sphingosine 1-phosphate, sphingosine kinase, has a much lower affinity for 1-deoxy analogues by as much as ten times (10). Naturally occurring or synthetic bases that are not normal substrates of this enzyme could be used to better investigate the role of sphingolipid metabolites on carcinoma development (10). What is known, through both *in vitro* and *in vivo* studies, is that increased intake or exposure to sphingolipids, ceramide, or sphingosine has several health-promoting effects and may act as important chemopreventive agents regarding skin cancer and colon cancer. No *in vivo* studies were found that support the mutagenic properties of sphingosine 1-phosphate. Information on the mechanism leading to the results of the above studies is needed.

Effects of sphingolipids on cholesterol. In a short-term feeding study of 2 weeks, rats were fed sphingomyelin at 0.5% and 2% of their diets (20). Plasma cholesterol was unaffected in the rats fed sphingomyelin, but hepatic cholesterol content decreased and triacylglycerol levels remained unchanged. The investigators of this study proposed that dietary sphingolipids may decrease absorption of dietary cholesterol and/or increase fecal excretion of steroids (20). In a long-term feeding study of two generations, rats were fed sphingolipids at 1% of their diets (21). Total plasma cholesterol was reduced by 30% in the rats fed sphingolipids, but hepatic cholesterol and triacylglycerol contents increased in comparison to the control group (21). Dietary sphingolipids were suspected to accelerate uptake of low density lipoproteins or suppress secretion of very low density lipoproteins from the liver. These studies also suggests that dietary sphingolipids may be non-toxic because no deleterious effects were found in those experimental animals fed sphingolipids in their diet at a relatively high level.

These studies also demonstrate that dietary sphingolipids may influence plasma and liver lipid levels in humans, and their affects may be dependent upon feeding period. Further research is necessary to determine if sphingolipids are able to reduce cholesterol. The future research should evaluate changes in lipoprotein fractions as a result of sphingolipid intake. Sphingomyelin and cholesterol are known to strongly interact, which may interfere with cholesterol absorption if this interaction is not broken in the intestine (8).

Recent evidence suggests that increased ceramide levels in intestinal cells, a major site of cholesterol synthesis, decreases cholesterol production (8). Dietary sphingolipids deserve more attention as a possible measure in preventing cardiovascular diseases, especially since sphingolipids form close associations with cholesterol in the body.

Not only are sphingolipids found in plasma membranes, but also in serum lipoproteins, particularly in low-density lipoproteins (LDL) (6). Sphingomyelin is the predominant sphingolipid in LDL and is in close association with cholesterol together forming microdomains or caveolae (6). Sphingomyelin influences several areas of cholesterol metabolism and transport, such as conversion of cholesterol to bile acids and cholesterol esters, control of β-hydroxyl-β-methyl glutarate (HMG)-CoA reductase activity, and cholesterol transport out of the cell (6). Cholesterol also influences sphingomyelin metabolism and transport.

In vivo studies have shown that diets supplemented with cholesterol affected sphingomyelin metabolism (6). In another experiment, it was shown that 25-hydroxycholesterol, an inhibitor of cholesterol synthesis, stimulated sphingomyelin production in Chinese hamster ovary cells (6). Due to close interactions between cholesterol and sphingomyelin, the affect of sphingomyelin and sphingomyelinase on the development of atherosclerosis was investigated.

A few studies suggest that sphingomyelin and sphingomyelinase may contribute to the development of atherosclerosis. Growth of smooth muscle cells and human blood monocytes is stimulated by oxidized lipoproteins via the sphingomyelin signaling pathway (6). Unusual large amounts of sphingomyelin have been found in aortic lesions due to decreased turnover and increased synthesis of sphingomyelin in arterial tissue (6). Foam cell formation by macrophages is promoted by hydrolysis of LDL sphingomyelin by extracellular sphingomyelinase enriched in atherosclerotic tissues (6). The association between endogenous sphingomyelin and atherosclerosis requires further investigation, but dietary sphingomyelins are not likely to be a risk. Studies have shown through radiolabeled

compounds that most sphingolipid metabolites are retained in the intestinal mucosa, and only a small amount actually is transferred to the body via the blood or lymph. (11)

Effects of sphingolipids on pathogenic protection. Many bacteria, viruses, and toxins utilize sphingolipids, particularly glycosphingolipids, to adhere to cells (6). Some of these pathogenic agents include cholera toxin, *Clostridium botulinum* type B neurotoxin, *Escherichia coli (E. coli)*, *Pseudomonas aeruginosa*, HIV-1 gp120, and influenza viruses (6). Synthetic sphingolipids have been produced to successfully prevent bacterial and viral infections (6). It is reasonable to assume that sphingolipids in foods bind to pathogens and remove them from the intestine (6). The primarily compound in human milk that protects against pathogens is assumed to be glycosphingolipids (6). Rats fed buttermilk powder were better protected against *Listeria monocytogenes* than those fed skimmed milk powder (8). Membrane lipids are concentrated in buttermilk more so than in skim milk (8). In another study, preterm newborn infants fed a milk formula supplemented with gangliosides had significantly fewer *E. coli* in their feces than infants fed a control formula (6).

Sphingolipid content in food and quantification methods

Foods from most food groups have been analyzed for their sphingolipid contents, but the list is short and may not be accurate. Table 1 represents most of the sphingolipid contents reported in the literature for a variety of foods. Most of the values in the table are derived from quantification studies. A few values originate from qualitative investigations but commonly are accepted as a measure of total sphingolipid content in the literature because data are scarce (6, 8). Therefore, the accuracy of some of the values in the table is questionable.

Single studies were conducted for most of the foods listed, and in many cases, quantification was incomplete. For example, sphingomyelin was the only sphingolipid type quantified for most of the fruit and vegetables listed, even though cerobroside is the predominating sphingolipid class in plants (6). Although animal-derived foods contain a much wider variety of sphingolipid types, sphingomyelin was the primary sphingolipid type measured for most of the animal products listed in Table 1. Table l also lists values from a unique study where both sphingomyelin and glycosphingolipids were measured in a variety of meats and fish. In another study by different investigators, only sphingomyelin was measured for the same type of meat and fish products (Table 1). Although the values reported by these two investigations seem similar, they were obtained by using indirect means of sphingolipid measurement, which may produce artifacts or underestimate actual sphingolipid contents.

The other sphingolipid contents reported in Table 1 also were not obtained by direct quantification of the intact sphingolipid molecules, but through their derivatives or their products of hydrolysis. Sphingomyelin was digested and quantified either through its phosphorus (22, 23), choline (24), or fatty acid (25) group. Glycosphingolipids were hydrolyzed and quantified either through their carbohydrate headgroup (26-28) or sphingoid backbone (29). The released glycosphingolipid backbone also may have been derivitized with fluorescamine, a fluorescent marker, in order to be quantified (25). All these methods involve many steps to obtain the sample to be quantified. For example, most quantification schemes involved the following procedures: 1) separation of polar lipids and neutral lipids by use of silica acid columns, 2) alkaline treatment to remove contaminating glycerides or liberation of fatty acids, 3) thin-layer chromatography and extraction from plates, 4) chemical

hydrolysis or derivitization involving harsh conditions (for example a derivitization treatment may involve subjecting the sphingolipid molecule to methanolic HCl for 18hr at 70°C), and 5) GC, HPLC, or spectrophotmetric analysis for quantification. The many steps involved increase the possibility of interference due to artifacts and/or underestimation of actual sphingolipid concentration due to degradation.

Because most values in Table 1 are mainly derived from a single study, they do not take into account seasonal variation, stage of plant maturity, processing/preparation, or other aspects that may influence sphingolipid concentration in a particular food. Only a few investigators have reported how certain factors affect sphingolipid contents in food. Kashani et al. (30) reported that roasting does not affect sphingolipid contents in pistachio nuts (1.8mg/g as-is basis). Whitaker (26) reported the effect of plant maturity on cerobroside contents in tomato and bell peppers (Table 1). Zeisel et al. (22) investigated human milk obtained at different times during a feeding (foremilk, middle milk, hind milk), stage of lactation, and time of day. Foremilk was milk collected before a feeding. Middle milk was that fed to the infant. Hind milk was that collected after a feeding. The study found that sphingomyelin content in human milk varied only between fractions collected at different times during a feeding. Hind milk contained significantly larger sphingomyelin contents than foremilk and middle milk. In another study, it was found that stage of lactation affected ganglioside concentrations in human milk (31). This investigation did not measure sphingomyelin, which is the major sphingolipid in milk (6). Bovine milk has not been thoroughly investigated in the same manner as human milk. It may be more important to investigate the effects of processing on sphingolipid contents of bovine milk. Table 1 includes a few values from dairy foods, although their sphingolipid contents cannot be

correlated to processing effects because they did not originate from the same starting milk material. This is true for all the related foods in Table 1.

Due to the convincing evidence that sphingolipids may provide positive health benefits, knowledge of sphingolipid contents of more foods and the variables that may influence their content is needed. It would be ideal to have an analytical method that is complete, less laborious, and involves quantification of the intact molecule, not a part of it. Because very limited information exists on the sphingolipid content of soybeans and soy products, and consumption of soy is currently encouraged due to its other proven health benefits, it became the focus of this research. The objectives of the research were: 1) to develop an ideal method for sphingolipid isolation and quantification of all sphingolipid classes and sphingolipid molecular species in soybean, 2) to determine the effect of soybean genotype, stage of seed development, and growth conditions on sphingolipid contents, and 3) to determine how sphingolipids are partitioned during the production of soy products (refined oil, oil refining by-products, soy concentrate, and soy isolate).

Thesis Organization

This thesis contains a general introduction, followed by two research papers and a general conclusion. The papers are in the required journal formats.

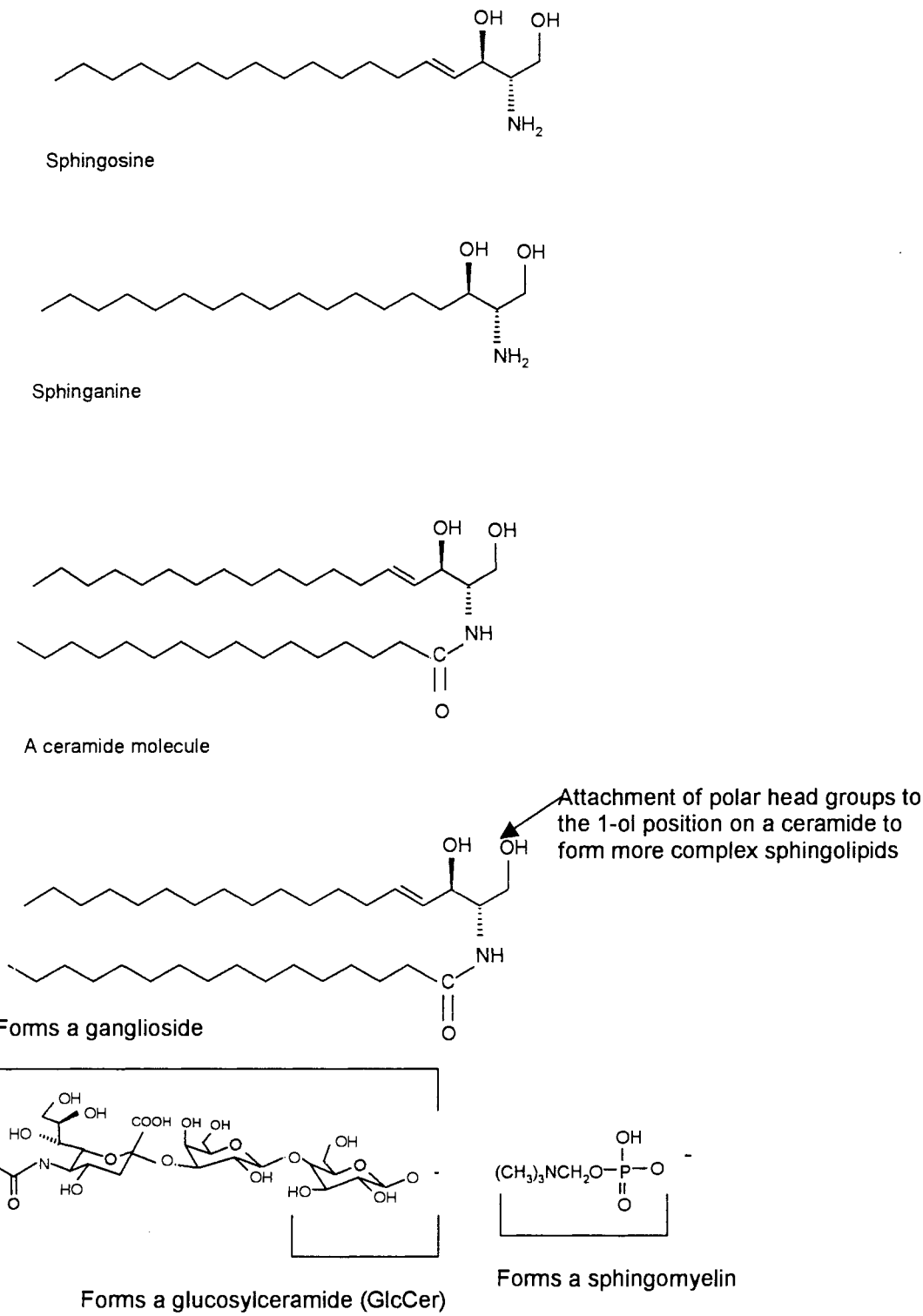

Figure 1. Sphingolipid structures

Table 1: Compliation list of sphingolipid contents in several foods[a-b]

Food Item	*Sphingolipid content μmol/kg*	*Sphingolipid measured*	*Ref.*	*Sphingolipid content μmol/kg*	*Sphingolipid measured*	*Ref.*
Dairy						
Bovine milk (whole)	119	SM	22	82	SM	24
Human milk	158-200	SM	22	107	SM	32
Nonfat Dry Milk (dw)	203	All	29			
Swiss Cheese (dw)	167	All	29			
Yogurt (dw)	138	All	29			
Butter	460	SM	24			
Margarine	15	SM	24			
Meat						
Beef	448	SM and GS	25	390	SM	23
Pork	335	SM and GS	25	350	SM	23
Chicken	589	SM and GS	25	530	SM	23
Turkey	497	SM and GS	25	390	SM	23
Ham	309	SM and GS	25			
Lamb	498	SM and GS	25			
Beef steak	506	SM	24			
Beef liver	1850	SM	24			
Fish						
Salmon	301	SM and GS	25	160	SM	23
Catfish				100	SM	23
Cod	118	SM and GS	25			
Herring	184	SM and GS	25			
Egg	2250	SM	24			
Legumes						
Soybean (dw)	128	Cer and CB	33			
Fullfat soy flakes (dw)	609	All	29			
Soyflour (dw)	610	All	29			

a: In many studies, sphingolipid content based on dry weight (dw) or fresh weight(fw) and/or stage of maturity was not specified.

b: SM-sphingomyelin, CB-cerobrosides, GS-glycosphingolipids, Cer-ceramides

Table 1. (continued)

Food Item	*Sphingolipid content μmol/kg*	*Sphingolipid measured*	*Ref.*	*Sphingolipid content μmol/kg*	*Sphingolipid measured*	*Ref.*
Legumes						
Isolated soy protein (dw)	211	All	29			
Peanuts	78	SM	24			
Peanut butter	9	SM	24			
Bread						
Whole wheat	11	SM	24			
Vegetables						
Potato	26	SM	24	43 (fw)	CB	27
Cauliflower	183	SM	24			
Lettuce	50	SM	24			
Bell pepper (immature) (fw)	45	CB	26			
Bell pepper (mature) (fw)	36	CB	26			
Fruits						
Apple	15	SM	24	69 (fw)	CB	28
Tomato	32	SM	24			
Tomato (immature) (fw)	10.1	CB	26			
Tomato (mature) (fw)	9.8	CB	26			
Orange	24	SM	24			
Banana	20	SM	24			

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Chapter 2. Method Development for Quantification of Sphingolipids in Soybeans

A modification of this paper is to be submitted to the *Journal of American Oil Chemists' Society*

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Abstract

Soybeans are believed to be a relatively rich source of sphingolipids, which are a class of polar lipids with desirable biological activities. Analytical methods for sphingolipids vary, and quantitative data for sphingolipids in food are scarce, including soybeans. The objectives of this study were to develop a method for quantification of sphingolipids in soybeans without alteration of their chemical structure and to determine whether genotype, stage of maturity, and growing location affect sphingolipid content in soybean. Separation of neutral lipids and interfering polar lipids from sphingolipids by saponification, transesterification, and solvent partition was studied. Solvent partition and TLC purification was the most accurate sample preparation method for HPLC quantification. There were significant differences for cerobroside concentrations among genotypes with a range of 142 to 492 nmol/g (dry wt basis). The differences in cerobroside concentration between immature and mature seeds of one genotype or between two seed productions locations of one genotype were not significant. The relative composition of cerobroside molecular species in two genotypes was analyzed, which was determined by tandem mass spectrometry.

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Key words: sphingolipids, cerobroside, soybean, quantification, mass spectrometry, mass spectrometry, HPLC

Introduction

Sphingolipids are found primarily in the plasma membrane of all eukaryotes, some prokaryotes, and in all foods, with soybean considered a rich source (1). Sphingolipids include free sphingoid bases, ceramides, sphingophospholipids, and glycosphingolipids. Sphingoid bases, usually 18-carbon amino alcohol, are N-acetylated to a long-chain fatty acid to form ceramides. Polar head groups, such as sugar residues and phosphorylcholine, attach to the 1-ol position of ceramide to form more complex sphingolipids.

Soybean contains two classes of sphingolipids, ceramide (Cer) and cerobroside. Cerobroside is the predominating class in soybeans (2). Cerobrosides are simple glycosphingolipids because they are associated with only one sugar residue. The only types of cerobroside found in soybean are glucosylceramides (GlcCer), which contain a glucose molecule (2).

Until recently, sphingolipids were recognized only as structural lipids. It has been discovered that their metabolites (i.e. ceramides, sphingosine) are involved in intracellular signaling, cell growth, differentiation, and apoptosis (3). Dietary sphingolipids have been shown to protect mice from skin and colon cancer (3) and decrease plasma cholesterol by 30% in rats (4).

Dietary sphingolipids have important positive health implications, but information on their total content in foodstuffs, including soybean, is sparse and may not be accurate. Most data on foodstuffs have been obtained from incomplete, single studies in which total

sphingolipid in the food may not have been measured and/or the effects of processing/preparation or other aspects influencing sphingolipid content were not considered. Almost all quantification studies have employed means of chemical hydrolysis or derivitization, which require many steps and may produce artifacts and/or underestimate sphingolipid concentrations. Because data for food are scarce, values from characterization or qualitative studies usually are cited, which were not designed for accurate sphingolipid quantification. The objectives of this study were to develop a method for total quantification of sphingolipids in soybeans without alteration of their chemical structure and to develop a database reflecting the effect of soybean genotype, stage of maturity, and growing location on sphingolipid concentration in the seed, and to determine relative composition of GlcCer molecular species in soybean by using tandem mass spectrometry.

Experimental Procedures

Solvents and standards. All chemicals, except sodium methylate and petroleum ether, were obtained from Fisher Scientific (Fairlawn, NJ, USA). Sodium methylate (5.4M) was obtained from Sigma Chemical Co. (St. Louis, MO, USA), and petroleum ether (b.p. 20°-40°C) from J.T. Baker (Phillipsburg, NJ, USA). Soybean GlcCer standard was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). All other standards, including Cer were obtained from Matreya, Inc. (State College, PA, USA).

Seed selection. To determine the effect of genotype on sphingolipid content, mature seeds from 10 soybean genotypes with different fatty acid compositions and protein contents were obtained from the Iowa State University soybean breeding program (Table 1). The genotypes were planted in adjacent plots at the Agricultural Engineering and Agronomy Research

Center near Ames, IA on June 6, 2002. Individual plants were harvested from the plots when the plants were mature. A 5-seed bulk sample from each plant was analyzed for fatty acid composition by gas chromatography. The seeds from plants of each genotype with similar fatty acid composition were bulked together to obtain the sample used for analysis. IA1008, one of the genotypes grown at Ames, was grown by S.R. Cianzio at the Iowa State University-University of Puerto Rico nursery near Isabela, Puerto Rico in 2002. Mature seeds from Ames and Isabela were used to determine effect of production location on sphingolipid content. To evaluate effect of seed maturity on sphingolipid content, seeds of Pioneer 3981 were collected while they were immature and when they were fully mature. This genotype was grown by X.B Yang of Iowa State University near Ames, IA.

Moisture, protein, oil, and fatty acid compositional analyses. Mature seeds were dried according to AOCS official method Ca2c-25 (5) to determine moisture percentage. Immature beans were dried in a vacuum oven at 55°C until a constant weight was reached, usually 4.5 hr. Protein and oil contents were determined using a near-infrared analyzer, Grainspec (Foss Electric North America, Eden Prairie, MN, USA). The near-infrared analyzer was calibrated according to the methods described by Hardy et al. (6). For fatty acid analysis, five seeds of each genotype were crushed together at 40,000 psi using a hydraulic press (Pasadena Hydraulics, Inc., El Monte, CA, USA). Hexane (1 mL) was added to the crushed beans for oil extraction. The hexane:oil mixture (200 μL) was transferred into gas chromatography (GC) vials along with 500μL of a 1N sodium methoxide solution to produce fatty acid methyl esters (FAME). After 2 hr of reaction, FAMEs were analyzed by a 5890 Series II (Hewlett-Packard, Avondale, PA) GC equipped with a flame ionization detector and capillary column (15 m length, 0.25 mm id, 0.2 μm film thickness) (Supelco, Bellefonte, PA,

USA). The oven temperature was 220°C, inlet and detector temperatures were both 250°C, and the split ratio was 1:100. Protein and oil analyses were performed in duplicate. Moisture and fatty acid analyses were replicated four times.

Lipid extraction. Soybeans were ground using a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) equipped with a 20-mesh delivering tube. Ten grams of ground sample (as-is basis) was sequentially extracted with 50 mL of each of the following solvents for 4 hr each with stirring: one with hexane, one with chloroform:methanol (v:v, 2:1), and twice with water-saturated butanol. For the second water-saturated butanol extraction, the soybean cake was sonicated while in solvent for 45 sec (Ultrasonic Liquid Processor, Model XL2020, Farmingdale, NY, USA; sonicator setting: continuous mode at setting 3). The four extracts were pooled, and the solvent was removed by a rotary evaporator at 60°-70°C. The crude lipid was purified using the Folch method (7) and measured gravimetrically.

Analytical techniques investigated for sphingolipid isolation in soybeans.

i. Saponification to remove phospholipids and neutral lipids from sphingolipids.

Saponification is the most commonly used method to remove glycerol lipids. To test if it has any degradative effect on sphingolipids, saponification was performed on sphingolipid standards. A 2 mg sample of Cer standard was treated with 1.6 mL of 1M KOH for 6 hr at 40°C with stirring, and a 3 mg sample was treated with 2.4 mL in the same manner. The treatment condition was considered mild because an estimated 3.5 mL 1M methanolic KOH is required to saponify 1 g of soybean oil (based on moles of esterified fatty acids in 1 g soybean oil), and 1 g of crude soybean oil is estimated to contain 0.12 mg of Cer (8). The amounts of Cer saponified (2 mg and 3 mg) for this experiment would be in 16 and 25 g of crude soybean oil, which would have required about 55.2 and 86.3 mL of the KOH solution

for saponification, respectively. After 6 hr, the samples were neutralized with 1M acetic acid, washed using the Folch method, and quantified with HPLC and an evaporative light scattering detector (ELSD) using the conditions outlined under in the HPLC quantification section of this paper.

ii. Transesterification to remove phospholipids and neutral lipids from sphingolipids. Oil samples (2 g), and Cer and GlcCer standards (1 mg) were separately treated with 0.2 ml of 5.4 M sodium methylate and 3.2 g of methanol for either 20 or 45 min at ambient temperature to convert glycerol fatty esters to methyl esters. After the reaction, samples were washed using the Folch method or purified by solvent partition extraction and quantified with HPLC/ELSD.

iii. Solid phase extraction (SPE) of sphingolipids from other lipids. To determine the efficacy of sphingolipid separation from saponified and transesterified samples, model systems were created and silica cartridges (5000 mg) (Alltech Associates, Inc., Deerfield, IL, USA) were used to remove 6 mg Cer from 2 g oleic acid and 6 mg Cer from 2 g soybean methyl esters. The cartridge was eluted with hexane:diethyl ether (v:v, 95:5) to remove the fatty acids and other unsaponifiable neutral lipids followed by acetone, methanol, and methanol:water (v:v, 95:5) to collect the alkali-stable sphingolipids.

iv. Direct separation of sphingolipids from neutral lipids by solvent partition. A modification of the petroleum ether/87% ethanol partition extraction procedure (9) was made to separate polar and neutral lipids. Both solvents were saturated with each other before extraction. Lipid was dissolved into 25 mL of petroleum ether in which 8.2 mL of 87% ethanol was added, and the funnel was shaken thoroughly. The equilibrated lower ethanol phase was transferred to a second funnel containing 25 mL petroleum ether, and the funnel

was shaken thoroughly so that the neutral lipids extracted by ethanol would be redistributed into the petroleum ether phase. The equilibrated lower ethanol phase was transferred to a flask to complete one cycle of extraction. To begin another cycle, 8.2 mL of 87% ethanol was added to the first funnel to extract the polar lipids and the ethanol layer was transferred to the second funnel containing petroleum ether. Eight cycles were performed to complete one extraction. Solvent partition was the only investigated technique adopted into this study's methodology for sphingolipid isolation and quantification (fig. 1).

Thin layer chromatography (TLC) purification of lipid. After solvent partition of sphingolipids from neutral lipids, preparative silica chromatography plates (500-μ Absorbosil Plus 1, Alltech) were used to separate GlcCer from other polar lipids. The lipid extract from solvent partition was streaked onto the 20 × 20 cm plate that also was cochromatographed with GlcCer standard. The plate was developed with chloroform:methanol:ether:hexane:acetic acid (v:v, 100:20:20:10:1.5). Only the GlcCer standard on the plate was sprayed with 2′,7′-dichlorofluorescein (Sigma) in methanol (0.1%) and visualized under UV light. The GlcCer silica band was identified, scraped, and extracted five times with approximately 30 mL of methanol:water (v:v, 95:5). The extracts were pooled, and the solvent was evaporated using a rotary evaporater. The extracted lipid was redissolved into chloroform:methanol (v:v, 2:1) for HPLC analysis.

HPLC quantification of sphingolipids. A Beckman Coulter (Fullerton, CA, USA) HPLC system equipped with auto sampler 508, solvent delivery system module 126, silica column (250 mm length, 2.1mm i.d., from Alltech), and an evaporative light scattering detector (ELSD 2000, Alltech) was used. Two mobile phases and a gradient program were created: "A" was hexane:tetrahydrofuran (99:1, v:v), and "B" was isopropanol:methanol (50:50, v:v).

The gradient elution program is presented in Table 2. A second gradient program, with slight modification of the first, was created for the second replicate of samples and quantification because a new column was used, and separation of lipids using the gradient program for the first replicate could not be achieved. The mobile phase flow rate was 0.3 mL/min. Nitrogen at a 2.5 L/min flow rate was used to evaporate the solvent in the heated (68°C) chamber of the ELSD. A standard calibration curve for each replicate of analysis was made with soybean GlcCer standard (purity greater than 98%) using the above HPLC/ELSD conditions. The two curves are presented as follows (X represents mg/mL of standard and Y represents peak area):

For 1st replicate analysis: $Y= 13900000X^{1.4200}$ $R^2= 0.9980$

For 2nd replicate analysis: $Y= 20000000X^{1.5438}$ $R^2= 0.9975$

A standard solution used for the calibration curves was run several times on the same day the samples were analyzed to detect any changes in the detector's original response during HPLC analysis. Reproducibility was good for each replicate analysis, and the coefficient of variation, on average, was 2.9%.

Mass spectrometry (MS). MS analyses were performed on a PE Sciex API 3000 triple quadrupole tandem mass spectrometer equipped with a turboionspray source. The ionspray needle was held at 5500 V while the inlet voltage (orifice) was kept low (<50 V) to minimize collisional decomposition of molecular ions prior to entry into the first quadrupole.

Individual soybean sphingolipid species were identified using precursor ion scans of unique molecular dissociations, which are structurally specific for sphingolipids. Dried GlcCer extracts of the mature Pioneer 3981 (~4 mg) and of the B01472B013 (~3 mg) seed were solubilized in 1 mL 50:50 $CHCl_3/CH_3OH$. Aliquots of each GlcCer solution (52.5 μL of

the Pioneer 3981 solution and 70 μL of the B01472B013 solution) were diluted to a final volume of 1 mL with 5 mM ammonium formate in 99:1 CH_3OH/HCOOH. This solution was infused at a flow rate of 10 μL min^{-1} by a syringe pump. Precursor ion spectra were acquired by scanning Q1 over a 200 u mass range (670 u – 870 u) in 0.1 u steps with a dwell time of 1.0 ms. Nitrogen was used to collisionally activate precursor ion dissociation in Q2, which was offset from Q1 by 50 eV to maximize formation of molecularly distinctive "N" product ions. Q3 was set to pass these product ions (*m/z* 262.4) to the detector. Data were acquired for 15 min, and the resulting spectra (peak intensities of individual GlcCer species) were from an average of ~450 scans.

Statistical data analysis. GlcCer was extracted from each genotype in two replications conducted two months apart. For each replication, all genotypes were prepared together for GlcCer isolation (fig. 1). After GlcCer extracts from all genotypes were prepared, they were analyzed by HPLC/ELSD. Data were analyzed by analysis of variance (ANOVA) using SAS software ($P \leq 0.05$) (10). Tukey Kramer's mean comparison ($P \leq 0.05$) was used to determine differences between genotypes.

Results and Discussion

Effects of sample preparation methods on sphingolipid quantification

i. Saponification. An alkaline saponification treatment was initially considered essential if Cer was to be quantified because separation of Cer and esterified steryl glucoside (ESG) was difficult under several HPLC conditions. ESG is a glycolipid found in soybean, and its concentration in soybean is typically 38mg/100g soybean (as-is basis) (11). Such alkaline treatment would remove ESG in the sample by hydrolysis of the ester linkage between the

fatty acid and sugar molecule. Cer standards treated alone with methanolic KOH caused some molecular degradation and the recovery of Cer was only 76.5 ± 0.7%. This result was likely since nearly complete sphingolipid saponification occurs after 10 hr in 1M methanolic KOH under reflux (12). Samples were not under reflux to avoid saponification of sphingolipid; however, certain hydrolysis of the Cer standards still occurred.

SPE was performed to examine the efficacy of isolating sphingolipids from the free fatty acids after acidification of the saponified sample. A model system was created to represent a saponified and acidified sample, which consisted of 6 mg Cer standard added into 2 g of pure oleic acid. Lipid extraction of 10 g of seed (as-is basis) typically yielded 2 g crude lipid in this study. The amount of Cer recovered with the polar solvents, only about 20%, was quantified by HPLC. A significant amount of oleic acid was recovered in the polar fractions. Hexane:diethyl ether (95:5, v:v) was chosen as the initial eluting solvents because this solvent combination was recommended by Christie (12) to elute free fatty acids from silica columns. Christie also suggested that the amount of diethyl ether in hexane could be increased if elution of FFA was incomplete with 5% diethyl ether in hexane. Increasing diethyl ether in hexane would risk more Cer loss during elution of the fatty acids in this study. The sphingolipid degradation and difficulty in separating free fatty acid from sphingolipid by SPE make saponification an inappropriate method of sample preparation.

In most studies involving isolation of both Cer and GlcCer from plant material, including one using soybean leaves, saponification was conducted after silica column chromatography (13-17). These studies utilized larger columns because they extracted total lipids from much greater amounts of starting material (11.8 kg to 110 g), and they were all performing characterization or qualitative studies. We chose to use 5000 mg silica columns

and expected that other polar lipids, such as esterified steryl glucoside and phospholipids, would interfere with the separation of sphingolipids from neutral lipids because only 50 mg of polar lipids can be retained by the cartridge (Alltech representative). Our samples likely contained much more than 50 mg of polar lipids. For this reason, the samples were saponified before solid phase extraction.

ii. Transesterification. The effects of transesterification on sphingolipid quantification were investigated because this technique is considered a milder and a more rapid derivitization treatment than saponification (12). There was 75 ± 3.0% of the Cer standards recovered when it was treated for 20 min. Only 43 ± 20.5% of the GlcCer standards were recovered after 45 min of treatment. The treatment thought not to cause sphingolipid degradation was: 0.00108 mole sodium methylate per 50 mg lipid treated for 10 min at 50°C (12). Our conditions included 0.001 mole sodium methylate per 2 g lipid at ambient temperature for up to 45 min. The conditions used in this study were not more severe than those suggested (12). GlcCer was treated for 45 min because it was later found, after treatment of Cer standards, that this time was necessary for complete transesterification of a lipid sample containing a significant amount of phospholipids.

SPE also was used to isolate Cer from a model system representing a transesterified sample. Cer (6 mg) was added to 2 g of soybean methyl esters to form the model. Recovery of Cer was greater than 100%, which suggested possible production of an artifact during the transesterification of neutral oil. To validate this assumption, 2 g of purified soybean oil obtained by passing through a silica cartridge column was transesterified alone under the same conditions as the Cer standards. HPLC analysis of the soy oil sample verified that an artifact was produced in a significant amount that had a similar retention time as the Cer

standard, accounting for the greater than 100% recovery in the model system (fig. 2). Therefore, Cer quantification by transesterification was not considered.

GlcCer degradation by transesterification might be less if other neutral lipids were present in the sample, as in an actual extracted soybean lipid sample, as opposed to GlcCer standard receiving the treatment alone. Two extracted soybean lipid samples from the same genotype were compared with and without transesterification treatment. One sample was not treated with sodium methylate, and the other was treated in the same manner the GlcCer standards were treated. The samples were purified by solvent partition separation and TLC purification using multiple plates. The experiment was repeated twice, and recovery of GlcCer from the tranesterified samples was 65% and 133% relative to the untreated samples. When GlcCer was added to 2 g of purified soybean oil and transesterified, the recovery was 127%. One possible explanation for these inconsistent results was that an artifact also may be generated during transesterification, and its formation may be sensitive to slight treatment differences. The above experiments were conducted two weeks apart.

iii. Solvent partition separation. This extraction procedure was preformed with both Cer and GlcCer standards alone. It was expected that recovery of Cer might be low because it is a relatively neutral lipid; its recovery was proved to be only 43 ± 0%. Recovery of GlcCer, a more polar lipid, was high (91.5 ± 2.1%) and consistent using this technique. Recovery of GlcCer in a model sample (GlcCer standard in 2 g purified oil) was 93.0 ± 0.04%. This extraction procedure was chosen as a final procedure for GlcCer quantification.

Overall, saponification caused some sphingolipid degradation. This treatment together with SPE did not allow separation of polar lipids from neutral lipids. Transesterification also may cause some degradation and/or produce artifacts that would

interfere with both Cer and GlcCer quantification. Solvent partition extraction resulted in good recovery and quantification of GlcCer, but severe loss of Cer. Only GlcCer was quantified in this study. GlcCer is the major sphingolipid class in soybean (2, 3, 8). Ohnishi et al. (8) reported the GlcCer content in soybean to be almost three times more than Cer.

Final procedure for GlcCer isolation and quantification. After recovery of total lipids and the Folch wash, two petroleum ether/ethanol extractions were performed to further remove contaminating neutral lipids. Silica plates were used to isolate GlcCer from other polar lipids (fig. 1), and GlcCer was quantified using HPLC.

Effect of soybean genotype on GlcCer content. The 10 soybean genotypes grown at Ames, IA, were significantly different for GlcCer concentration (Table 1). The genotypes utilized for this study were not of a single genetic background, so differences in GlcCer content among them may not be due only to differences in their seed composition.

The GlcCer contents of the two conventional genotypes, IA1008 and IA2021, were found to be significantly different. GlcCer content may vary among conventional soybean genotypes grown in the same environment with typical fatty acid and/or protein compositions. It also was found that the two genotypes with the highest palmitic contents, A97-877006 and A00-815004, had the highest GlcCer contents. A possible explanation for the greater GlcCer concentration in the genotypes with elevated palmitic is that the biosynthesis of sphingolipids begins with the condensation of serine and palmitoyl-CoA by serine palmitoyltransferase (18). The *de novo* studies by Merrill et al. (19) and Paumen et al. (20) showed that sphingoid or ceramide biosynthesis increased with palmitic acid in the medium compared with cells receiving no exogenous fatty acids or exposed to other types of fatty acids (C15-C18). Their studies were not conducted with plant tissues and cerobroside

concentration was not measured; however, sphingolipid synthesis in plants in believed to closely simulate that of other cell types (18).

Few studies have reported the sphingolipid content in soybean with which to compare our 10 soybean genotypes. The most frequently cited value in the literature for sphingolipid content in soybean is based on a qualitative study by Ohnishi et al. (8). Ohnishi et al. analyzed one genotype and reported Cer (38 nmol/g, as-is basis) and GlcCer (91 nmol/g, as-is basis) amounts in the mature seeds. Other authors have interpreted the results found by Ohnishi et al. (8) and reported soybean to have a sphingolipid content of 2,400 nmol/g (as-is basis) (2, 3, 21), which represents the total glycolipid content found in soybean. This may not accurately reflect the sphingolipid content in soybean because it is based on glycolipid content and one soybean genotype that was studied by Ohnishi et al. (8).

Effect of maturity stage on sphingolipid content. The immature seed of Pioneer 3981 was harvested on August 29, 2002 and the mature seeds on September 24, 2002. The moisture content was 68% for the immature seed and 14% for the mature seed. The GlcCer content for the immature Pioneer 3981 seeds (378 nmol/g, dry wt basis) was greater than the GlcCer content for the mature seeds (209 nmol/g, dry wt basis), but the difference between these two seed types was not significant ($P \leq 0.05$). The effect of seed maturity was investigated because sphingolipids are primarily membrane lipids, so it was expected that sphingolipid concentration would be higher in immature seeds because other seed components, such as triacylglycerides and protein, would have not been fully synthesized and deposited in the seed as in the mature seeds. Ohnishi et al. (8) reported immature beans to have a sphingolipid concentration of 612 nmole/g (as-is basis) while the GlcCer content in the mature seeds of the same genotype was 128 nmol/g (as-is basis). The magnitude of difference between the

GlcCer contents of the immature and mature seeds in our study may have varied from that by Ohnishi et al. (8) due to differences in the genotype, growing location, and stage of maturity that were evaluated.

Effect of environment on sphingolipid content. The GlcCer content of the genotype IA1008 (142 nmol/g, dry wt basis) when grown in Ames, IA was not significantly different than its content when grown in Isabela, Puerto Rico (208 nmol/g, dry wt basis) ($P \leq 0.05$). The protein and oil contents were significantly different between the IA1008 seeds grown at different locations, but their fatty acid compositions were not ($P \leq 0.05$). The IA1008 seeds from Ames were comprised of 36.0% protein and 18.5% oil, while the seeds collected from Puerto Rico contained 34.4% protein and 20.0% oil (13% moisture basis).

GlcCer molecular species composition by MS. Mature seed of Pioneer 3981 and B0147B013 were analyzed. Data from the two genotypes cannot be compared because they were not grown at the same production location. Table 3 presents the MS peak intensities for the GlcCer species analyzed.

Peak intensities also were not compared among different GlcCer molecular species within the same genotype to calculate relative percentages of GlcCer species. We did not compare different GlcCer species because the mass detector response likely varies for each type of molecular species based on two factors, gas-phase basicity and kinetics associated with fragmentation. Gas-phase basicity refers to a molecules' ability to accept a proton and become ionized. Kinetics associated with fragmentation refers to the extent a molecule is fragmented, and some molecules are more easily fragmented than others. Regarding sphingolipid analysis, the kinetics associated with fragmentation has a much larger influence on response differences that occurs between species.

The characteristics and proportions of the GlcCer species in the two soybean genotypes analyzed seem to agree with known information regarding GlcCer composition in soybean (Table 3). Ohnishi et al. (8) reported that soybean GlcCer molecules were composed primarily of two sphingoid backbones. The primary backbone was reported to be 4, 8-sphingadiene (69%), while 4-hydroxy-8-sphingenine (17%) was the second most abundant sphingoid found in soybean GlcCer molecules. Sullards et al. (22) reported that >95% of soybean GlcCer molecules were comprised of 4, 8-sphingadiene. In both studies by Ohnishi et al. (8) and Sullards et al. (22), the major GlcCer species was found to be 4, 8-sphingadiene, and the primary fatty acid component was α-hydroxypalmitic. Ohnishi et al. (8) also reported that α-hydroxylignoceric acid is the major fatty acid component found in the GlcCer species containing a 4-hydroxy-8-sphingenine backbone.

Acknowledgement

This research was supported by the U.S. Department of Agriculture (USDA)-Center for Designing Foods to Improve Nutrition (CDFIN) grants

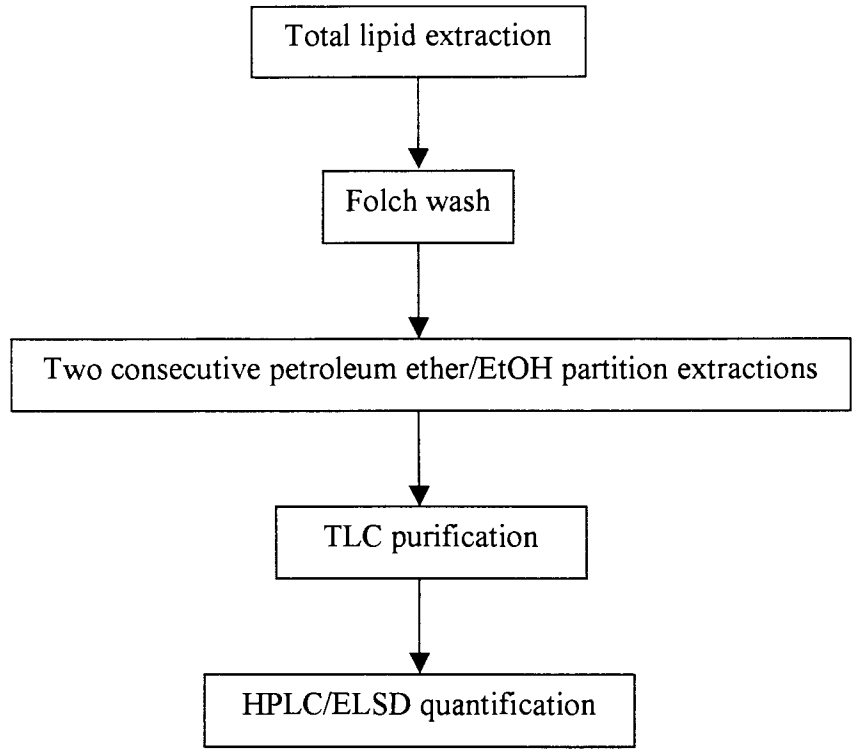

Figure 1. Procedures for GlcCer quantification in soybeans.

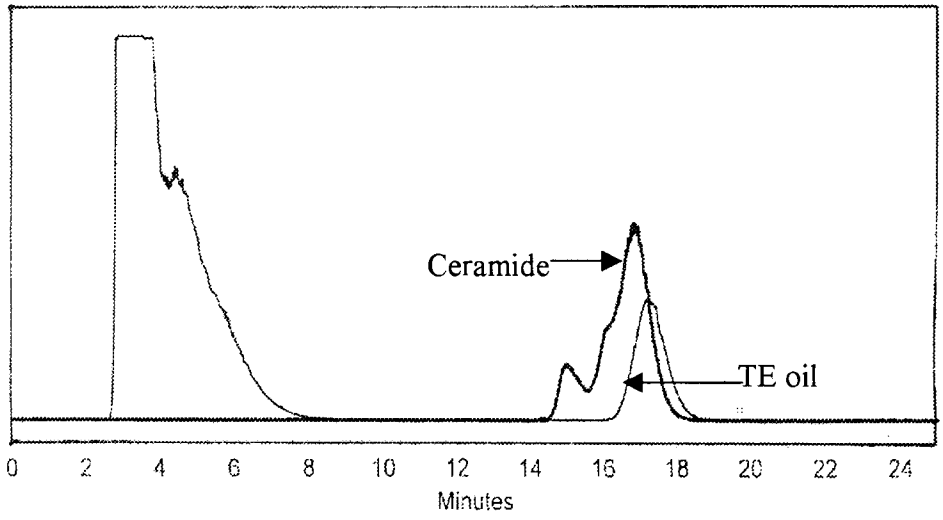

Figure 2. Chromatographs for ceramide standard vs. transesterified (TE) pure soybean oil.

Table 1. Mean composition and cerobroside (GlcCer) content of 10 soybean genotypes grown near Ames, IA.

Genotype and selectively modified trait(s)	*Protein*[a] *(%)*	*Oil*[a] *(%)*	*Palmitic (%)*	*Stearic (%)*	*Oleic (%)*	*Linoleic (%)*	*Linolenic (%)*	*GlcCer (nmol/g dry wt basis)*
IA1008 Conventional	36.0	18.5	10.8	4.4	26.3	51.0	7.4	142
IA2021 Conventional	36.0	19.3	10.6	4.7	25.4	52.4	6.8	283
IA2041 High protein	41.0	16.3	9.9	4.4	25.8	52.5	7.3	201
A00-815004 High palmitic	34.0	14.6	41.3	4.5	10.0	34.6	9.6	389
A97-877006 Mid palmitic	34.0	15.0	27.0	4.6	14.1	45.3	9.3	493
FA22 High oleic	38.8	16.7	8.1.	3.4	51.8	31.8	4.9	306
B0147B013 Low palmitic	36.4	16.7	3.4	3.0	24.6	60.1	8.9	168
AX7019-12 Mid palmitic/stearic	33.6	15.0	20.6	24.0	8.7	39.2	7.5	246
A97-552013 Low linolenic	37.0	17.4	10.1	5.0	27.9	55.6	1.3	229
A99-144085 High stearic	35.1	16.9	8.0	28.1	20.3	40.8	2.9	197
MSD[b]	1.3	0.9	2.1	2.8	4.7	4.5	0.9	122

a: Protein and oil based on 13% seed moisture content
b: MSD=minimum significant differences between means in each column determined by Tukey Kramer's mean comparison($P \leq 0.05$).

Table 2.Gradient program of mobile phase for HPLC of cerobroside[a]

Time (minutes)	*Solvent A (%)*	*Solvent B (%)*
0	95	5
5	90	10
10	90	10
22	0	100
24	0	100
34	100	0
36	100	0
51	100	0

a: Solvent A: hexane:tetrahydrofuran = 99: 1 v:v, Solvent B: methanol:isopropanol = 50:50 v:v

Table 3. MS relative peak mean intensities for GlcCer species in two genotypes.

Genotype	*Backbone 4,8-sphingadiene*								*Backbone 4-hydroxy-8-sphingenine*		
	h[a]14:0	h15:0	h16:0	h17:0	h18:0	h20:0	h22:0	h24:0	h18:0	h22:0	h24:0
Pioneer 3981	0.6	0.4	100	0.2	0.3	0.1	2.0	1.6	0.1	1.8	1.9
B0147B013	0.5	0.5	100	0.2	0.5	0.2	2.1	2.0	0.1	1.6	2.0

a: h= 2-hydroxy fatty acid

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Chapter 3. Effect of Processing on Sphingolipid Content in Soybean Products

A paper to be submitted to the *Journal of American Oil Chemists' Society*

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Abstract

Soybean is believed to be a rich source for sphingolipids, a class of polar lipids receiving attention for their possible cancer-inhibiting activities. The effect of processing on the sphingolipid content of various soybean products has not been determined. Glucosylceramide (GlcCer), the major sphingolipid type in soybean, was measured in several processed soybean products to describe which product(s) GlcCer is partitioned into during processing and/or where it is lost. Whole soybeans were processed into full-fat flakes from which crude oil was extracted. Crude oil was refined by conventional methods and defatted soy flakes were further processed into alcohol-washed and acid-washed soy protein concentrates (SPC) and soy protein isolate (SPI). Most processes were conducted in a laboratory-scale that simulated industrial practices. GlcCer was isolated from the samples by solvent extraction, solvent partition, and TLC, and was quantified by HPLC. GlcCer mostly remained with the defatted soy flakes (91%) rather than with the oil (9%) after oil extraction. Recovery of GlcCer from the defatted soy flakes through the acid-washed SPC (52%), alcohol-washed SPC (42%), and SPI (26%) products was poor. All protein products had a similar GlcCer concentration of about 281 nmol/g (dry wt basis). The minor quantity of GlcCer in the crude oil was almost completely removed by water degumming.

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Key words: sphingolipids, cerobroside, soybean, processing, quantification, HPLC

Introduction

Sphingolipids are found primarily in the plasma membrane of all eukaryotes and some prokaryotes (1). They are constituents in most foods, and soybean is considered a rich source (1). Several sphingolipid classes exist, but all sphingolipid species contain a sphingoid backbone, usually an 18 carbon amino alcohol. The backbone is usually N-acetylated to a long-chain fatty acid and/or attached to a polar head group, such as a sugar or phosphorylcholine residue, to form the various sphingolipid classes. In soybean, glucosylceramide (GlcCer) is the major sphingolipid type whose backbone is N-acetylated and contains glucose as the only polar head group (2). GlcCer belong to the sphingolipid class cerobroside (2).

Dietary sphingolipids have gained a great deal of attention because their metabolites are bioactive and have been shown to inhibit colon and skin carcinogenesis (3) and reduce plasma cholesterol by 30% in experimental animals (4). The amounts of sphingolipid fed to experimental animals in some *in vivo* studies are comparable to the estimated amounts consumed in the American diet (0.01 to 0.02% of the diet) (5). Dietary sphingolipids may have important positive health implications; however, few studies have been carried out to determine sphingolipid content in many foods, and certain available information may not be accurate. Fewer studies have attempted to investigate the effects of processing on sphingolipid contents in foodstuffs, including soy products. In our study, the effect of soybean processing on the GlcCer content of various soybean products was determined using analytical procedures we developed in a previous study (6) that do not cause structural

alteration of GlcCer molecules for their quantification. In many earlier studies, lipid samples and/or sphingolipid molecules have been chemically hydrolyzed or derivitized for quantification. These treatments may underestimate actual sphingolipid contents and/or produce artifacts. The analytical procedures developed in our previous study were accurate and reproducible.

Experimental Procedures

Soybean preparation for oil extraction. A conventional soybean cultivar, IA1008, was purchased from the Committee for Agricultural Development, Ames, IA. All procedures used to obtain seed compositional data for the IA1008 were described in Gutierrez et al. (6). The IA1008 seed was comprised of 36.0% protein (13%-moisture basis), 17.6% oil (13%-moisture basis), and 8.2% moisture. The fatty acids analyzed and their percentages were: palmitic 10.9%, stearic 4.2%, oleic 22.1%, linoleic 54.1%, and linolenic 8.1%.

The soybean seeds were flaked at the Center for Crops Utilization Research at Iowa State University, Ames, IA. Two kilograms of the seed was cracked to produce 6-8 meats or cotyledon pieces per seed that were dehulled. Industrial cracking typically yields 4 or 6 meats/seed (7). The soy meats were conditioned by heating them to 60°C (they were not simultaneously treated with moisture or steam as in industry (7). Conditioned meats were flaked to a typical flake thickness of 0.02-0.05 cm (7). The flakes were stored at -10°C for 1.5 weeks. All other processes were conducted on a laboratory-scale using techniques simulating typical industrial practices.

Oil Extraction. Oil was extracted from 350 g full-fat soy flakes (as-is basis) using a lab-scale apparatus, which allowed a percolation extraction (fig. 1). A solvent vessel held hexanes at

60°C. Solvent was then pumped from this vessel to an extraction vessel, which contained soy flakes. Both the solvent vessel and extraction vessel were jacketed to maintain the solvent at 60°C. Solvent percolated down through the flakes and drained into the solvent vessel. Solvent continuously cycled the apparatus for 6 min from the solvent vessel to the extraction vessel, and the flow was maintained to keep the soy flakes submerged in solvent at all times (solvent:flake ratio= 1.6:1). After 6 min, the solvent was allowed to drain from the flakes for 3 min before repeating the above cycle with a fresh solvent. Six cycles or stages of extraction were used to complete total extraction. Duplicate oil extractions were performed. Hexanes were evaporated using a rotary evaporator to yield crude oil.

Oil refining. All refining steps were applied to the two crude oil fractions collected after two separate oil extractions from full-fat soy flakes (fig. 2).

For degumming, crude oil was hydrated with water at 3% of its weight and maintained at 60°C in a water bath with stirring for approximately 1.5 hr. After phospholipid precipitation, degummed oil was separated from the gum by centrifugation at 1,000 x g for 20 min.

For alkali refining, degummed oil was neutralized according to the AOCS Ca 9d-25 method (8) assuming a free fatty acid (FFA) percentage of 0.5 in the soybean oil. The typical free fatty acid percentage in soybean oil is 0.4 (7). After formation of insoluble soap, the neutralized oil was separated from soap using centrifugation at 1,000 x g for 20 min.

Soy protein concentrate and soy protein isolate preparation. Most commercial soy protein concentrates are produced by either an aqueous ethanol wash or acid wash process; therefore, both preparation procedures were used to produce SPC for this study. This study utilized conventional procedures (9).

Portions (50 g) of defatted soy flakes (DSF) from each extraction were used to produce acid-washed SPC and alcohol-washed SPC. For SPI production, 115 g of DSF from each extraction were used (fig. 2). Duplicate SPC and SPI preparations were performed. The protein contents in the above products were determined by the Dumas method AOAC 990.03 (10) using a Rapid NIII nitrogen analyzer (Elementar Americas, Inc., Mt. Laurel, NJ, USA)

i. Acid-wash procedure for SPC (fig. 3): DSF was mixed with water in a typical 10:1 (water:DSF) ratio (9). The pH of the mixture was bought to the isoelectric point for soy protein, pH 4.5, and was maintained at this pH for 30 min at 40°C with stirring (9). The protein precipitate was separated from soluble sugars in the supernatant using centrifugation.

ii. Alcohol-wash procedure for SPC (fig. 4): DSF was mixed with a 60% ethanol solution in a 10:1 (alcohol:DSF) ratio. Conventional alcohol wash procedures use between 60%-80% ethanol solutions (9). The mixture was stirred for 40 min at 40°C, and the protein precipitate was separated from soluble sugars in the supernatant using centrifugation.

iii. SPI procedure (fig. 5): Soy protein and soluble sugars were extracted from DSF by adjusting the pH of the 10:1 (water:DSF) mixture to 8.5. The supernatant containing protein and soluble sugars was separated from the precipitate, collected, and its pH was adjusted to 4.5 to allow precipitation of soy protein. The mixture was refrigerated at 4°C for 1 hr before the precipitate was removed to allow larger curd formation. The precipitate was recovered using centrifugation. This procedure is most commonly practiced for the production of soy protein isolates (9).

Sphingolipid extraction from full-fat soy flakes, defatted-soy flakes, SPC, and SPI samples. Total lipids were extracted from 10 g of ground sample using the methods previously described (6). However, hexanes were not used in the sequential extraction scheme for the

samples, except for full-fat soy flakes. Lipids in the other sample types were extracted through one extraction with chloroform:methanol (2:1, v:v) and two extractions with water-saturated butanol.

Sphingolipid extraction from oil and oil-refining by-products. Approximately 2 g of crude soybean oil was used for sphingolipid quantification, as described earlier (6). A 2 g of refined oil, gum and soapstock were analyzed in this study for their GlcCer content. GlcCer was isolated through solvent partition extraction and TLC.

HPLC quantification. A Beckman Coulter (Fullerton, CA) HPLC system equipped with an auto sampler 508, solvent delivery system module 126, silica column (250 mm length, 2.1 mm i.d., from Alltech), and an evaporative light scattering detector (ELSD 2000, Alltech) was used for GlcCer quantification. Two mobile phases and a gradient program (Table 1) were created: "A" was hexane:tetrahydrofuran (v:v, 99:1), and "B" was isopropanol:methanol (v:v, 50:50). The flow rate was 0.3 mL/min. Nitrogen at a flow rate of 2.5 L/min flow rate was used to evaporate the solvent in the heated (68°C) chamber within the ELSD. The GlcCer standard with purity greater than 98% was used to create standard calibration curves where X represents mg/mL of standard and Y represents peak area:

1^{st} Standard curve: $Y= 10000000X^{1.1.6708}$ $R^2= 0.9970$

2^{nd} Standard curve: $Y= 10000000X^{1.5758}$ $R^2= 0.9940$

A second curve was made during analyses because the silica column degraded. The new curve was created for a new column with the same specification as the first column. A standard solution from the calibration curve frequently was run with samples to detect any changes in the detector's original response during HPLC analysis. Reproducibility of duplicate injections was good, and the average coefficient of variation was 2.6%.

Statistical analysis. All treatments, including oil extraction, oil refining, and SPC and SPI preparations were conducted in duplicate. One GlcCer extract was produced from each duplicated product and analyzed. Analysis of variance (ANOVA) using the SAS program (11), was used to determine the reproducibility of duplicate treatments and how GlcCer concentration was affected by the treatments. ANOVA was used to evaluate the recovery of GlcCer in the protein products. Tukey-Kramer's mean comparison ($P \leq 0.05$) was used to determine minimum significant differences.

Results and Discussion

Processing. Although this processing experiment was conducted on a laboratory scale, we simulated industrial practices as much as possible to accurately determine how GlcCer is partitioned into common soy products during processing. Our seed conditioning steps prior to oil extraction closely resembled those used for industrial scale processing. We were unable to conduct a typical continuous, countercurrent extraction of oil from the soy flakes (7). Instead, percolation extraction was performed with six extraction cycles completing an extraction. The greatest amount of oil was extracted during the first cycle, and the amount of oil extracted progressively decreased with following cycles (fig. 6). More than six extraction cycles was not necessary as shown in fig. 6. The efficiency of the extraction technique used for this study was good. Full-fat soy flakes typically contain about 20% oil (7), and the extracted oil in this study resulted in 24.0% of the full-fat flake (as-is basis) used for extraction.

Crude oil was refined according to conventional practices. Crude oil was degummed and alkali refined but not bleached and deodorized as in industry (7). It was necessary to

examine the degumming and alkali refining steps because these processes are most likely to remove sphingolipids from oil because they both remove polar substances from oil including phospholipids and free fatty acids. Gum or lecithin from soybean oil has been used as a source of GlcCer for qualitative studies (2), indicating that sphingolipids may be enriched in these relatively polar by-products. However, information on the approximate amount of sphingolipids in soy lecithin or in soapstock is not available, which may be valuable sources of GlcCer. Bleaching and deodorization would more than likely remove only insignificant amounts of sphingolipids from the oil if there were any GlcCer left after alkali refining.

Soy protein meal is commonly processed into SPC or SPI. SPC must have a protein content between 65-72% (dry wt basis), while SPI must have a protein content between 90-92% (dry wt basis) (9). The SPC products prepared in this study had more than 65% protein (dry wt basis), and the SPI product contained 90.7% protein (dry wt basis) (Table 2). The SPC and SPI products were prepared from defatted-soy flakes using typical industrial extraction parameters with one exception. In industry, SPC produced through the acid-wash method and SPI are usually neutralized and spray-dried to recover protein (9). Due to the lab-scale quantities of SPC and SPI produced for this study, the samples were not neutralized or spray-dried. No negative effect on GlcCer content was anticipated for not neutralizing the protein products.

GlcCer content in various soybean products. GlcCer contents (ppm, dry wt basis) between the duplicate oil and protein samples were not significantly different ($P \leq 0.05$), indicating the processing procedures used to produce each product were reproducible. The GlcCer contents for the soybean products produced are shown in Table 2.

For the oil samples, GlcCer was only detected in the degumming by-product, i.e. gum or lecithin. GlcCer is a polar lipid and was removed with other polar lipids during degumming. If degumming removed all of the GlcCer that was present in the crude oil, as the data suggested. The two crude oil fractions collected after oil extraction from full-fat soy flakes would have contained at least 5.1 mg of GlcCer (fig. 2). The 2 g of crude oil used for analysis would have contained 0.12 mg GlcCer. In our previous investigation (6), we have shown the GlcCer isolation procedures could result in 7% loss of GlcCer, giving about 0.11 mg recoverable GlcCer in 2 g crude oil for HPLC quantification. The quantity of GlcCer that would be injected into HPLC if the crude oil contained 0.11 mg GlcCer would be 3.3 μg based on μL of the GlcCer extract from crude oil, which was near the HPLC/ELSD detection limit of 2.4 μg for GlcCer. This may explain why GlcCer was not detected in the crude oil. In our previous investigation (6), GlcCer was detected in 2 g of crude oil because total lipids were extracted from 10 g of ground soybean seed by sequential solvent extraction using more polar solvents than hexanes, such as chloroform:methanol (2:1, v:v) and water-saturated butanol. The 2 g crude oil extracted in this manner contained much more GlcCer than 2 g of crude oil extracted with hexanes only. Refined oil and soapstock also may have contained trace amounts of GlcCer, but HPLC/ELSD analysis may have not been sensitive enough to detect any GlcCer.

Because GlcCer is a relatively polar lipid class, it was expected that most of it would remain in the DSF and in the purified soy protein products. GlcCer was nearly equally concentrated in all the soy protein products prepared for this study (Table 2). No significant difference in GlcCer content was found among the DSF, acid-washed SPC, alcohol-washed SPC, and SPI products. The processing conditions adopted for this study are very similar to

typical industrial practices; therefore, commercially produced soybean products, like the types produced for this study, may not differ significantly in their GlcCer contents if they originated from the same soybean genotype.

Few studies have reported the sphingolipid content of soy products with which to compare our results. Ahn et al. (12) measured total sphingolipids in a commercially purchased SPI sample (211 nmol/g dry wt basis) and a full-fat soy flake sample (609 nmol/g dry wt basis) by molecular hydrolysis of the sphingolipids and quantification of their backbones. In our study, the GlcCer content for SPI was 297 nmol/g (dry wt basis) and 268 nmol/g (dry wt basis) for full-fat soy flakes. Even though the SPI sphingolipid contents for both studies are similar, it cannot be concluded that these values reflect the GlcCer contents in all SPI samples because sphingolipid contents vary with genotype (6). Ceramide, the only other sphingolipid class that is a minor contributor to total sphingolipid content in soybean (13), was not measured in our study.

Fate of GlcCer during processing. After oil extraction and production of defatted soy flakes, 89% of the GlcCer content in the starting full-fat soy flake material was recovered in the DSF and crude oil (fig. 2). Most of the recovered GlcCer remained with the DSF (91%) rather than with the crude oil (9%), but recovery of GlcCer from the defatted soy flakes through the alcohol-washed SPC (42%), acid-washed SPC (52%), and SPI (26%) products was poor. These recoveries were not found to be significantly different ($P \leq 0.05$).

The percentage of GlcCer recovered in each soy protein product was based on the GlcCer content in the amount of DSF used to prepare the SPC and SPI products. The estimated total GlcCer content in the total amount of DSF produced was 51.1 mg (fig. 2). The 50 g of defatted soy flake used for the SPC preparations would contain 11.1 mg GlcCer,

and 115 g of defatted soy flake used for SPI preparation would contain 25.6 mg GlcCer. The two SPC preparation procedures performed in this study did not differ in their ability to retain GlcCer in the protein product. Although the recoveries did not significantly differ, more GlcCer tended to be lost during the production of SPI. GlcCer, being a polar lipid, may have been lost to the aqueous supernatant formed during the production of all these products. The supernatants were not analyzed in this study.

Acknowledgements

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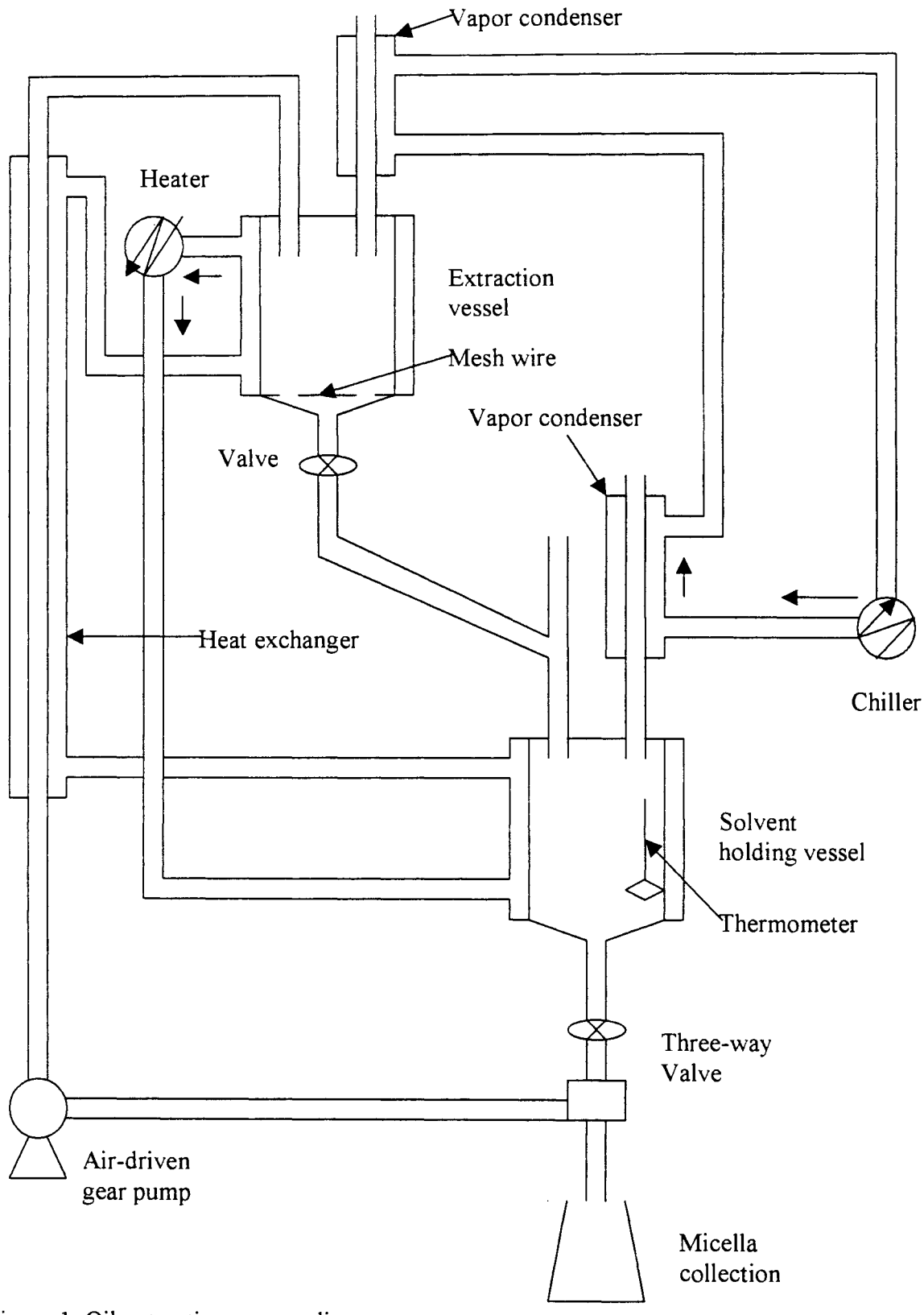

Figure 1. Oil extraction system diagram

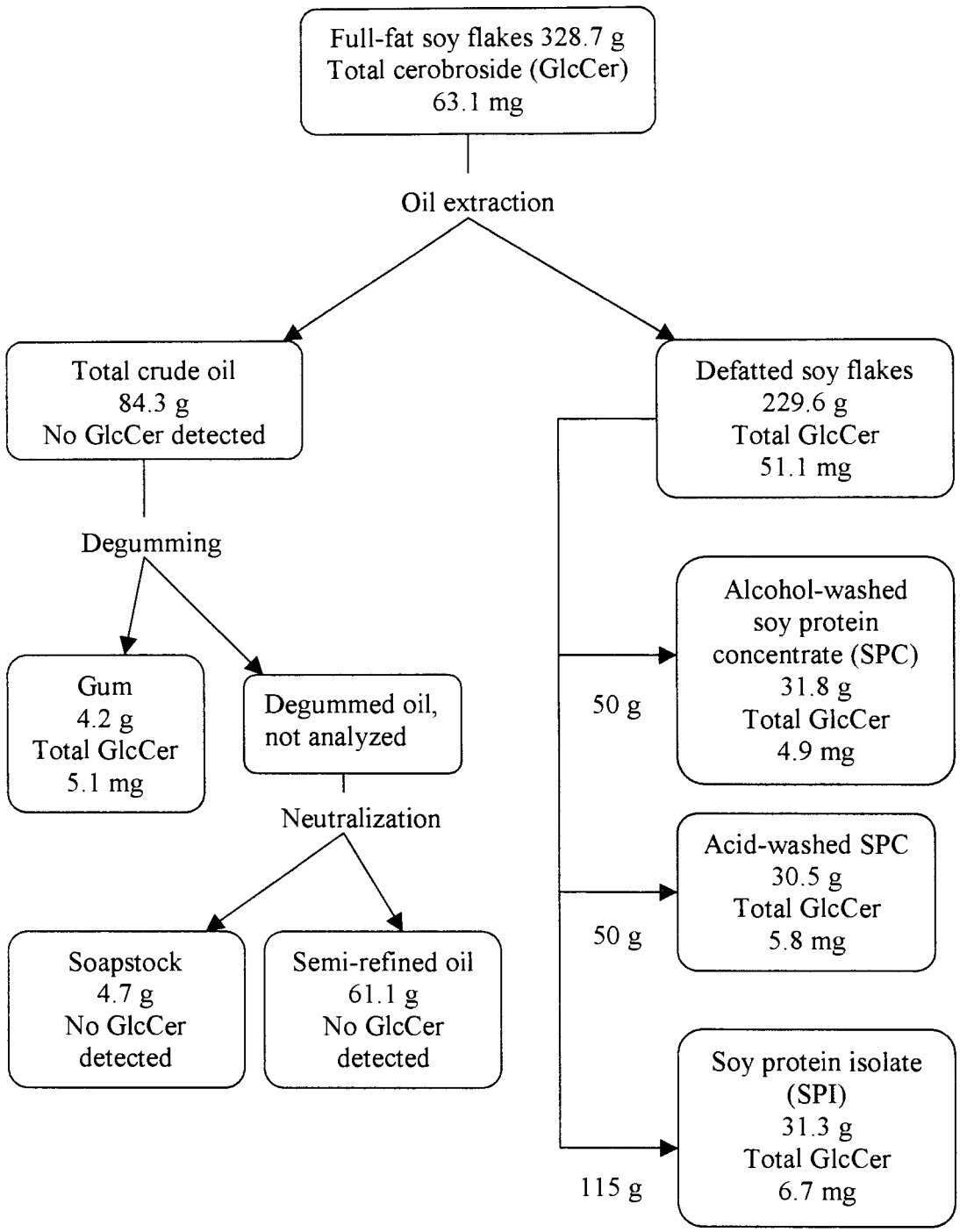

Figure 2. Overall soybean processing scheme and mass balance of GlcCer. Product masses (dry wt basis) are based on average of duplicate processing steps.

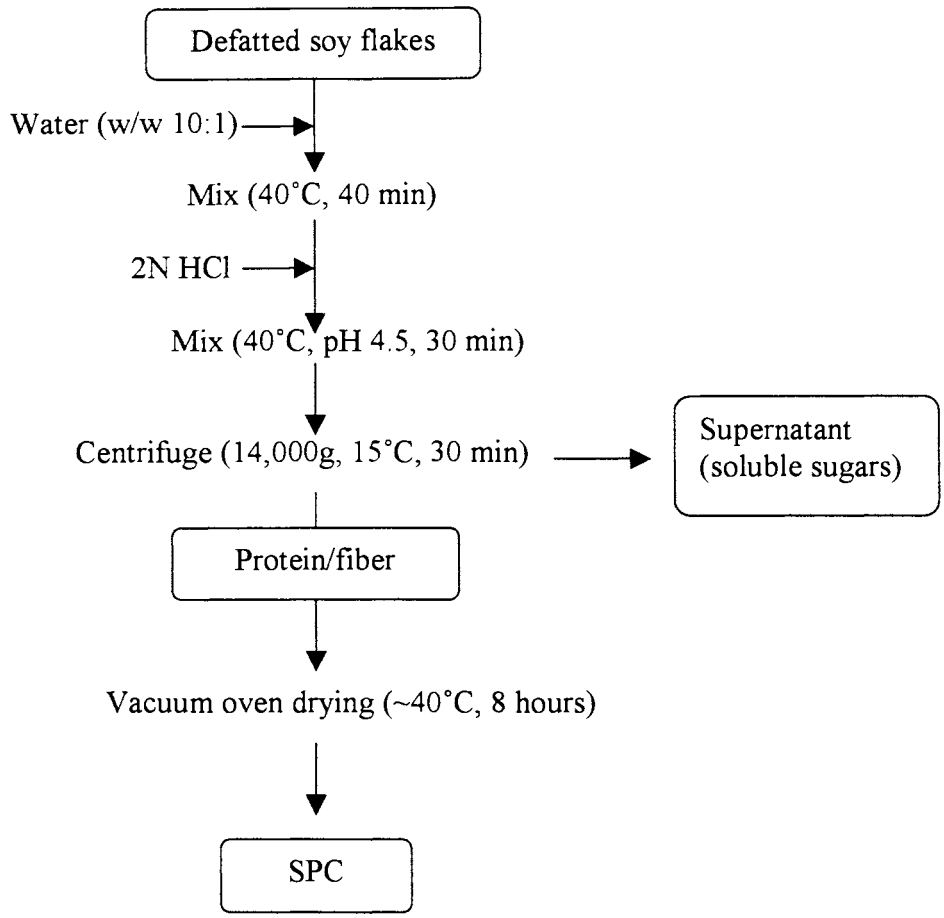

Figure 3. Procedure for producing soy protein concentrate (SPC) using acid-washed method

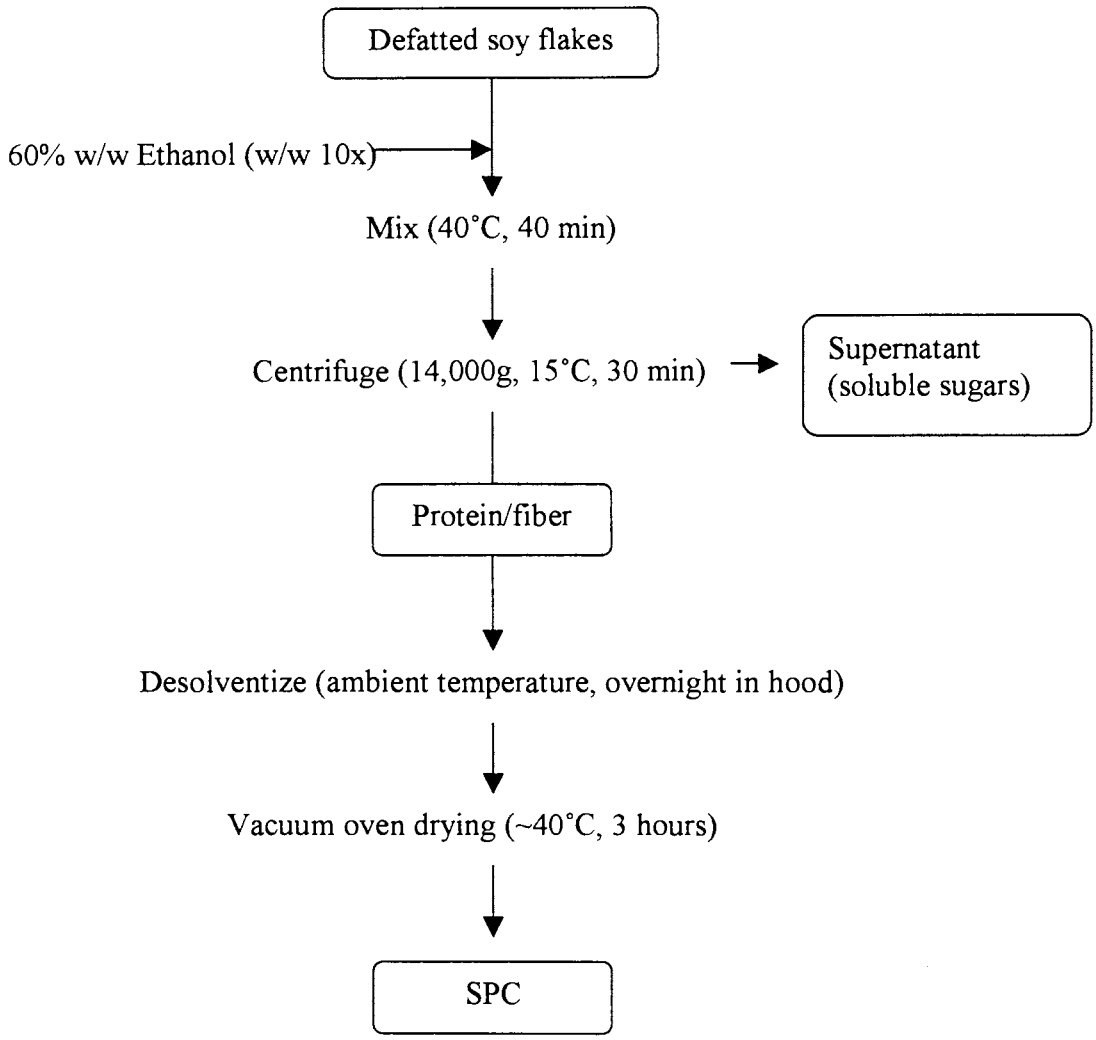

Figure 4. Procedure for producing soy protein concentrate (SPC) using alcohol-washed method

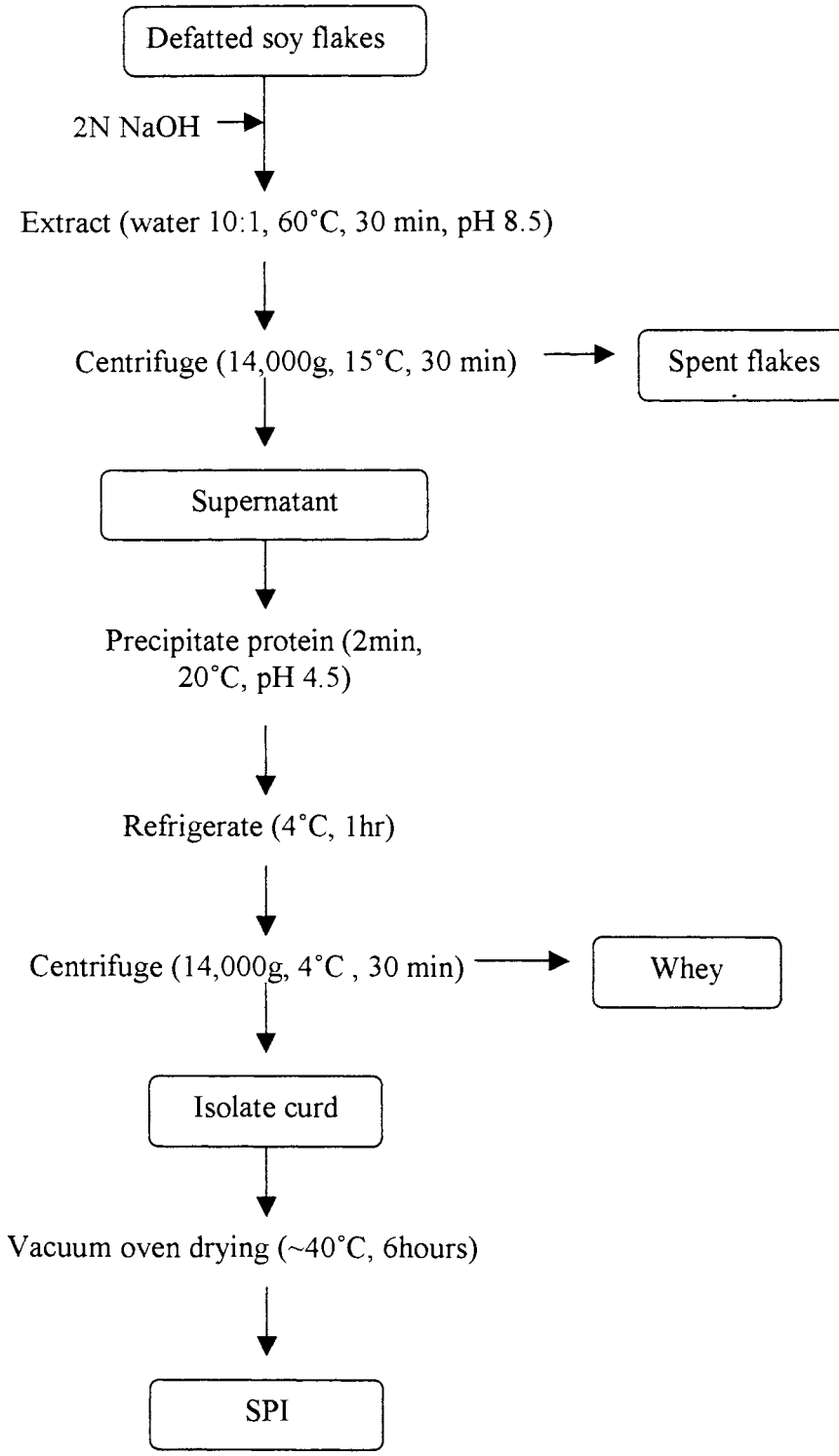

Figure 5. Procedure for producing soy protein isolate (SPI)

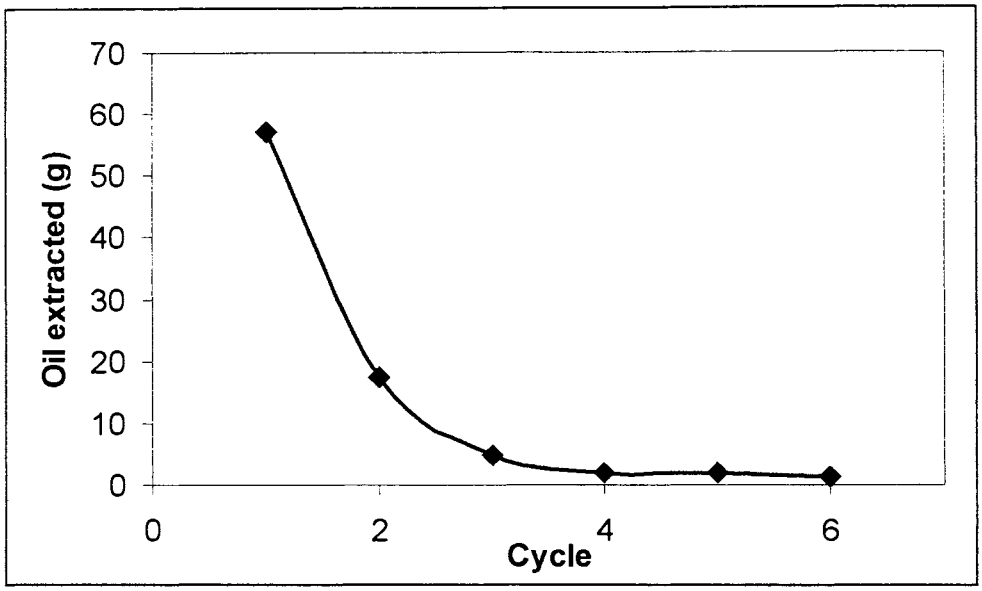

Figure 6. Oil amount extracted from 350 g full-fat soy flakes (as-is basis) at each percolation extraction cycle. The amount at each cycle is the average from duplicate oil extractions.

Table 1.Gradient program of mobile phase in HPLC analysis[a].

Time (min)	*Solvent A (%)*	*Solvent B (%)*
0	95	5
5	90	10
10	80	20
22	0	100
24	0	100
34	100	0
36	100	0
51	100	0

a: Solvent A: hexane:tetrahydrofuran = 99: 1 v:v, solvent B: methanol:isopropanol = 50:50 v:v

Table 2. Mean protein and cerobroside (GlcCer) contents for soybean products produced[a].

Soy product	*Protein content (%) (dry wt basis)*	*GlcCer nmol/g (dry wt basis)*	*GlcCer ppm (dry wt basis)*
Full-fat soy flakes	-	268.2	192.5
Defatted soy flakes	54.3	311.2	223.3
Soy protein concentrate, SPC (acid wash)	66.8	264.4	189.1
SPC (alcohol wash)	68.1	216.5	155.3
SPI	90.7	296.9	213.9
Crude oil	-	ND[b]	ND
Gum	-	1678.9	1202.8
Soapstock	-	ND	ND
Refined oil	-	ND	ND
MSD[a]	-	113.4	78.4

a: MSD=minimum significant differences between means in each column determined by Tukey Kramer's mean comparison ($P \leq 0.05$).
b: ND=not detected in sample

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Chapter 4. General Conclusions

Sphingolipids are a complex group of polar lipids that have been implicated to inhibit skin and colon carcinogenesis. Quantitative data on the sphingolipid content in foods and the factors that affect sphingolipid content in foods, including soybeans and soy products are scarce. In this research, effect of genotype, stage of seed development, and production location on sphingolipid content in soybean was investigated. The effect of processing on the sphingolipid content of various soybean products also was determined. Glucoslyceramide (GlcCer), the main sphingolipid type in soybean, was extracted and quantified using a novel method developed for this research. GlcCer was extracted from 10 g ground soybean seed or 10 g ground full-fat soy flakes using sequential extraction with hexane, chloroform:methanol (2:1, v:v), and water-saturated butanol. A modification of this sequential extraction scheme was conducted for all other samples in this research. Solvent partition and thin layer chromatography (TLC) methods were used to produce all GlcCer-enriched fractions, which were analyzed by high performance liquid chromatography (HPLC) and an evaporative light scattering detector (ELSD).

Ten soybean genotypes with different fatty acid and protein contents were analyzed. There were significant differences for GlcCer content among genotypes with a range of 142 to 492 nmol/g (dry wt basis). Immature and mature seeds from genotype Pioneer 3981 were harvested about a month apart. The GlcCer content for the immature Pioneer 3981 seeds (378 nmol/g, dry wt basis) was greater than the GlcCer content for the mature seeds (209 nmol/g, dry wt basis), but the difference between these two seed types was not significant. The GlcCer content of a conventional soybean genotype when grown in Ames, IA (142 nmol/g,

dry wt basis) was not significantly different than its content when grown in Isabela, Puerto Rico (208 nmol/g, dry wt basis).

After oil extraction and production of defatted soy flakes (DSF) from full-fat soy flakes, 89% of the GlcCer content contained in the starting full-fat soy flake material was recovered in the crude oil and the defatted soy flakes. Most of the GlcCer remained with the flakes (91%) rather than with the crude oil (9%). Recovery of GlcCer from the DSF through the soy protein purification products was poor. GlcCer was recovered from the defatted soy flakes in acid-washed soy protein concentrate (SPC) (52%), alcohol-washed SPC (42%), and soy protein isolate (SPI) (26%).

The GlcCer concentration of the soybean protein products was not significantly different from that of full-fat soy flakes. The GlcCer content range for full-fat soy flakes, DSF, acid-washed (SPC), alcohol-washed SPC, and SPI was 217 to 311 nmol/g (dry wt basis). The only soybean product whose GlcCer content significantly differed from that of the full-fat soy flakes was the lecithin fraction (1679 nmol/g, dry wt basis) collected after degumming of the crude oil. GlcCer was not detected in the crude oil, soapstock, or alkali-refined oil because it may have not been concentrated enough in these products to be detected by HPLC/ELSD.

Major GlcCer species in two soybean genotypes also was analyzed by using tandem mass spectrometry. The characteristics of GlcCer species analyzed in the soybean genotypes seem to agree with the current knowledge of GlcCer species in soybeans.

The results of this study contribute necessary information to the literature regarding sphingolipid content in soybean and soy products and the factors that may affect sphingolipid content in these foods, such as genotype and seed processing methods. Other factors that

were considered in this research should be more thoroughly studied including effect of soybean production location and stage of seed development. Other factors, such as seed storage conditions, and their affects on GlcCer content remain unknown and should be evaluated. Ceramide content in soybean was not quantified in this study but should be quantified in the future, even though it is a minor sphingolipid class in soybeans.

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