Effect of Processing on Sphingolipid Content in Soybean Products

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ABSTRACT: Soybeans are believed to be a rich source of sphingolipids, a class of polar lipids that has received 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 soybeans, was measured in several processed soybean products to illustrate which product(s) GlcCer is partitioned into during processing and 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 isolates (SPI) by laboratory-scale methods that simulated industrial practices. GlcCer was isolated from the samples by solvent extraction, solvent partition, and TLC and was quantified by HPLC. GlcCer remained mostly within the defatted soy flakes (91%) rather than in the oil (9%) after oil extraction. Only 52, 42, and 26% of GlcCer from defatted soy flakes was recovered in the acid-washed SPC, alcohol-washed SPC, and SPI products, respectively. 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: Cerobroside, HPLC, processing, quantification, soybean, sphingolipids.

Sphingolipids are found primarily in the plasma membrane of all eukaryotes and some prokaryotes (1). They are a constituent in most foods, and soybeans are considered a rich source (1). Several sphingolipid classes exist, but all sphingolipid species contain a sphingoid base backbone (usually an 18-carbon amino alcohol). The backbone is usually N-acylated to a long-chain FA and attached to a polar head group, such as a sugar or phosphorylcholine residue, to form the various sphingolipid classes. In soybeans, glucosylceramide (GlcCer) is the major sphingolipid type. The backbone is N-acylated and contains glucose as the only polar head group (2). GlcCer belongs to the sphingolipid class cerobrosides (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 to reduce plasma cholesterol by 30% in experimental animals (4). The amounts of sphingolipids fed to experimental animals in some *in vivo* studies are comparable to the amounts estimated to be 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 the sphingolipid content in foods, and certain available information may not be accurate. Far fewer studies have attempted to investigate the effects of processing on the sphingolipid contents in foodstuffs, including soy products. In the present 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), which did not cause structural alteration of the GlcCer molecules in their quantification. In many earlier studies, lipid samples or sphingolipid molecules have been hydrolyzed or derivatized for quantification. However, these treatments may underestimate the actual sphingolipid contents and may produce artifacts. The analytical procedures developed in our previous study were relatively accurate and reproducible.

EXPERIMENTAL PROCEDURES

Soybean preparation for oil extraction. A conventional soybean genotype, IA1008, was purchased from The Committee for Agricultural Development (Ames, IA). All procedures used to obtain seed compositional data for the IA1008 soybean genotype are described in Reference 6. The IA1008 seed contained 36.0% protein (13% moisture basis), 17.6% oil (13% moisture basis), and 8.2% moisture. FA were analyzed and their percentages were as follows: 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 yield between 6 and 8 cotyledons fragments, or "meats," per seed and then dehulled. Industrial cracking typically yields 4–6 meats/seed (7). The soy meats were then conditioned by heating them to 60°C (they were not simultaneously treated with moisture or steam as in the industry; see Ref. 7). Conditioned meats were flaked to a typical flake thickness of 0.02–0.05 cm. The flakes were stored at −10°C temporarily. All other processes were conducted on a laboratory scale using techniques simulating typical industrial practices.

Oil extraction. Oil was extracted from 350 g of full-fat soy flakes (as-is basis) using a laboratory-scale apparatus that

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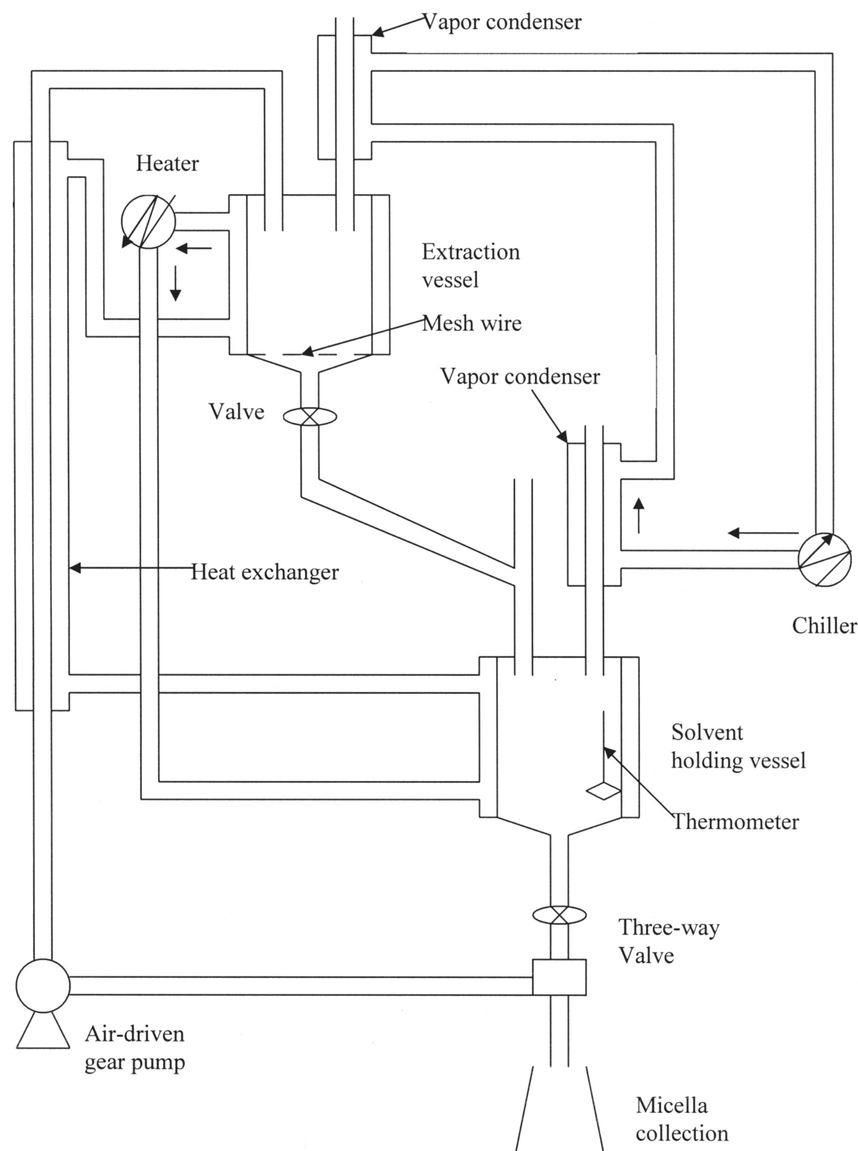

FIG. 1. Diagram of the oil extraction system.

allowed a percolation extraction (Fig. 1). Briefly, a solvent vessel held hexanes at about 60°C; solvent was then pumped by an air-driven gear pump to an extraction vessel (containing soy flakes) placed above it. 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. The solvent cycled the system continuously for 6 min (from the solvent vessel to the extraction vessel) while the flow was maintained to keep the soy flakes submerged in solvent at all times (ratio of solvent to flakes = 1.6). 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 the total oil 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 separate crude oil fractions collected after two separate oil extractions from the 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 h. After phospholipid precipitation, the degummed oil was separated from the gum by centrifugation at $1,000 \times g$ for 20 min.

For alkali refining, the degummed oil was neutralized according to AOCS Official Method Ca 9d-52 (8), assuming an FFA percentage of 0.5 in the soybean oil (the typical percentage of FFA in crude soybean oil is 0.4; see Ref. 7). After insoluble soap was formed, the neutralized oil was separated from the soap using centrifugation at $1,000 \times g$ for 20 min.

Soy protein concentrate (SPC) and soy protein isolate (SPI) preparation. Most commercial SPC are produced by

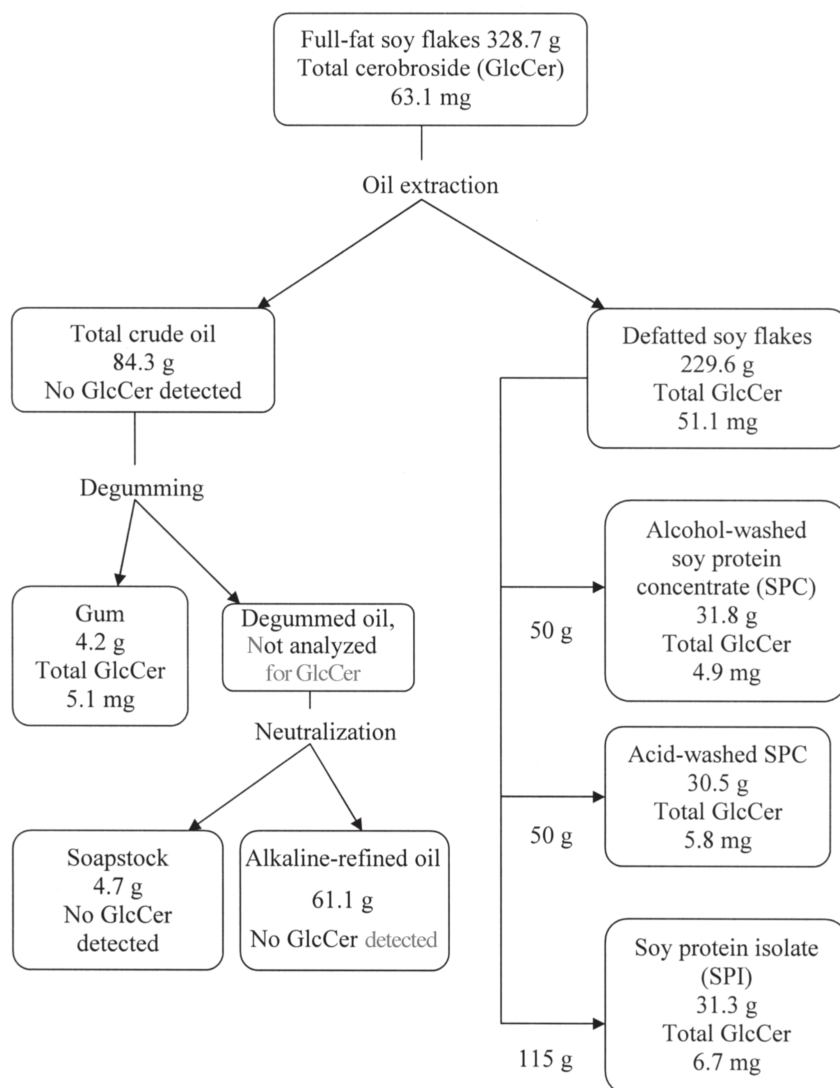

FIG. 2. Overall soybean processing scheme and mass balance of GlcCer. Product masses (dry wt basis) are based on the average of duplicate processing.

either an aqueous ethanol wash or an acid wash process; therefore, both preparation procedures were used in this study to produce SPC. Conventional procedures were used with slight modifications (9).

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

(i) Acid-wash procedure for SPC (Fig. 3). DSF were mixed with water in a typical 10:1 (water/DSF) ratio (9). The pH of the mixture was brought 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 by 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 and 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 by centrifugation.

(iii) SPI procedure (Fig. 5). Soy protein and soluble sugars were extracted from the 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 adjusted to 4.5 to allow precipitation of the soy protein. The mixture was refrigerated at 4°C for 1 h before the precipitate was removed to allow larger curd formation. The precipitate was recovered by centrifugation. This procedure is most commonly practiced for the production of SPI (9).

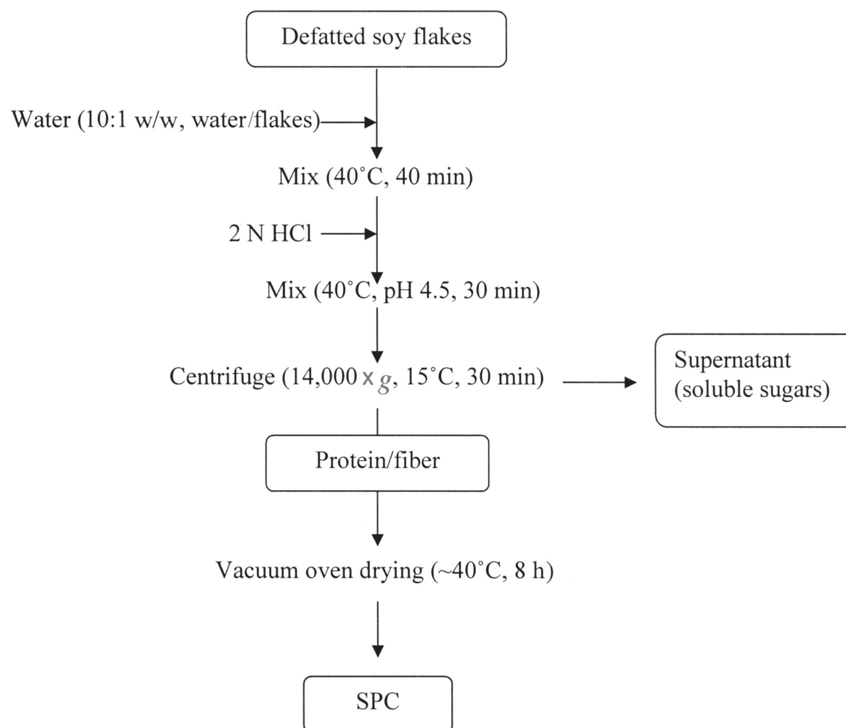

FIG. 3. Procedure for producing SPC using the acid-washed method. For abbreviation see Figure 2.

Sphingolipid extraction from full-fat soy flake, DSF, SPC, and SPI samples. Total lipids were extracted from 10 g of ground protein sample using the methods described previously (6). However, hexanes were used in the sequential extraction scheme only for full-fat soy flakes. Lipids in the other sample types were extracted through one extraction with chloroform/methanol (2:1, vol:vol) and two extractions with water-saturated butanol.

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

HPLC quantification. A Beckman Coulter (Fullerton, CA) HPLC system equipped with a model 508 autosampler, model 126 solvent delivery system module, a silica column (250 mm length, 2.1 mm i.d.; Alltech, Deerfield, IL), and an ELSD (ELSD model 2000; Alltech) was used for GlcCer quantification. Two mobile phases and a gradient program (Table 1) were created: Solvent A was hexane/tetrahydrofuran (99:1, vol/vol), and solvent B was isopropanol/methanol (50:50, vol/vol). The flow rate was 0.3 mL/min, and nitrogen (2.5 L/min flow rate) was used to evaporate the solvent in the heated (68°C) chamber in the ELSD. A GlcCer standard (purity greater than 98%) was used to establish standard calibration curves (X represents mg/mL of the standard, and Y represents the peak area):

first standard curve: $Y = 10{,}000{,}000X^{1.1.6708}$ $\quad R^2 = 0.9970$ [1]

second standard curve: $Y = 10{,}000{,}000X^{1.5758}$ $\quad R^2 = 0.9940$ [2]

A second curve was made during analyses because the silica column degraded, and a new curve was created for a new column with the same specifications as the first column. A standard solution from the calibration curve was frequently run with samples to detect any changes in the detector's original response during HPLC analysis. The reproducibility of duplicate injections was good, and the average CV 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. ANOVA, using the SAS program (11), was used to determine the reproducibility of duplicate treatments and how the GlcCer concentration was affected by the treatments. ANOVA was also 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. This processing experiment was conducted on a laboratory scale. However, we simulated industrial practices as much as possible to determine how GlcCer is partitioned into soy products during processing. Our seed conditioning steps prior to oil extraction closely resembled those used for

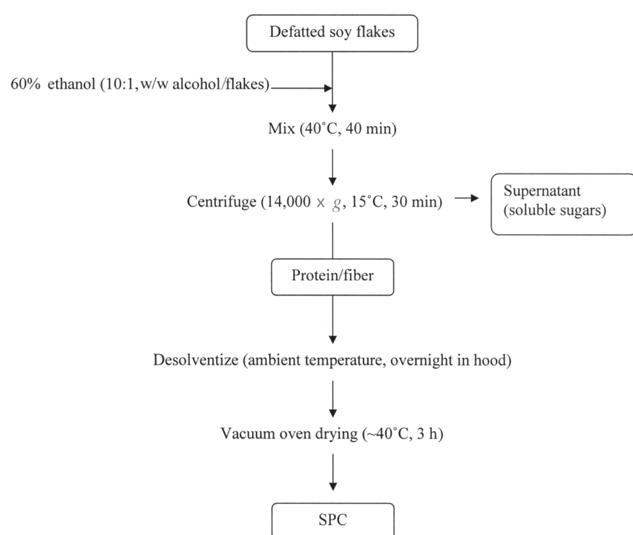

FIG. 4. Procedure for producing SPC using the alcohol-washed method. For abbreviation see Figure 2.

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 one extraction. The greatest amount of oil was extracted during the first cycle, and the amount of oil extracted progressively decreased with the following cycles (Fig. 6). More than six extraction cycles were not necessary, as shown in Figure 6, and four extraction cycles resulted in nearly complete oil extraction. The efficiency of the extraction used for this study was good. Full-fat soy flakes typically contain about 20% oil (7), and the extracted crude oil in this study represented 24.0% of the full-fat flakes (as-is basis) used for extraction.

Crude oil was also refined according to conventional practices. Crude oil was degummed and alkali-refined but not subsequently bleached and deodorized as in industry (7). It was necessary to examine the degumming and alkali-refining steps because these processes, both of which remove polar substances from oil (e.g., phospholipids and FFA), are most likely to remove sphingolipids from the oil. Furthermore, 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, and they may be valuable sources of GlcCer. The subsequent oil-refining steps, bleaching and deodorization, were likely to remove only insignificant amounts of sphingolipids from the oil if any GlcCer were left after alkali refining.

Soy protein meal is commonly processed into SPC or SPI. SPC must have a protein content of between 65 and 72% (dry wt basis), whereas SPI must have a protein content of between 90 and 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)

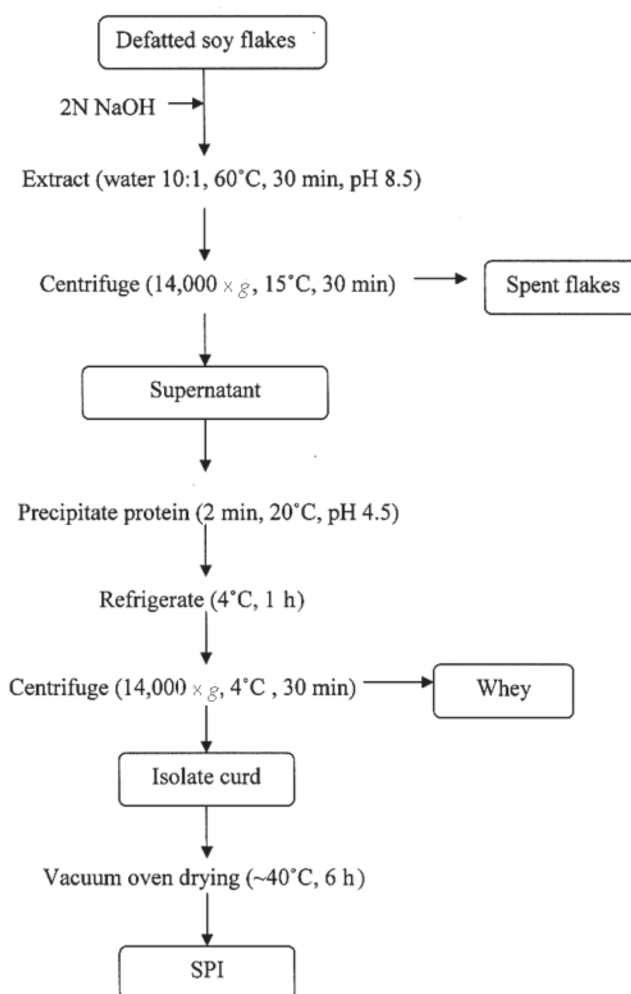

FIG. 5. Procedure for producing SPI. For abbreviation see Figure 2.

(Table 2). The SPC and SPI products were prepared from DSF using typical industrial extraction parameters, with one exception. In industry, SPC produced through the acid-wash method and SPI are usually neutralized and then spray-dried to recover protein (9). Because of the lab-scale quantities of SPC and SPI produced for this study, these samples were neither neutralized nor spray-dried. No harmful effect was anticipated from not neutralizing the protein products.

GlcCer content in various soybean products. GlcCer con-

TABLE 1
Gradient Program of the Mobile Phase in HPLC Analysis[a]

Time (minutes)	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 (vol/vol); solvent B, methanol/isopropanol = 50:50 (vol/vol).

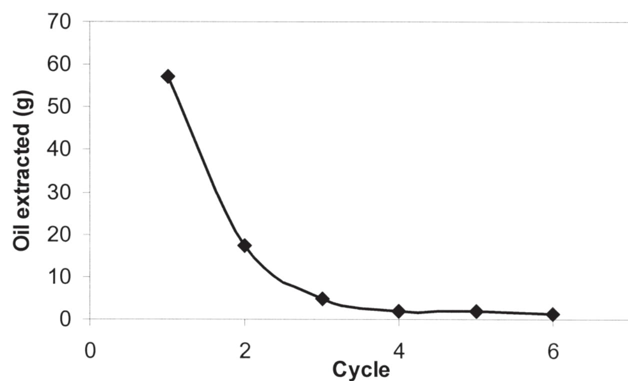

FIG. 6. Quantity of oil extracted from 350 g of full-fat soy flakes (as-is basis) at each percolation extraction cycle (amount at each cycle is the average from duplicate oil extractions).

TABLE 2
Mean Protein and Cerebroside (GlcCer) Contents for the Soybean Products Produced

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
SPC (acid washed)	66.8	264.4	189.1
SPC (alcohol washed)	68.1	216.5	155.3
SPI	90.7	296.9	213.9
Crude oil	—	ND	ND
Gum	—	1678.9	1202.8
Soapstock	—	ND	ND
Alkaline refined oil	—	ND	ND
MSD[a]	—	113.4	78.4

[a]MSD, minimum significant differences between means in each column as determined by Tukey–Kramer's mean comparison ($P \leq 0.05$); SPC, soy protein concentrate; SPI, soy protein isolate; ND, not detected.

tents (ppm, dry wt basis) were not found to be significantly different between the duplicate oil and protein samples ($P \leq 0.05$), indicating that the processing procedures used to form each product were reproducible. The GlcCer contents of the soybean products are shown in Table 2.

For the oil samples, GlcCer was detected only 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 had removed all of the GlcCer present in the crude oil, as the data suggest, then the crude oil fraction obtained after oil extraction from the full-fat soy flakes would have contained at least 5.1 mg of GlcCer (Fig. 2). Two grams of crude oil, which was taken for analysis, would then have contained 0.12 mg of GlcCer. In our previous investigation (6), we showed that the GlcCer isolation procedures could potentially result in 7% loss of GlcCer, giving about 0.11 mg of recoverable GlcCer in 2 g of crude oil for HPLC quantification. The quantity of GlcCer that would be injected into the high-performance liquid chromatograph if the crude oil contained 0.11 mg of GlcCer would be 3.3 µg (based on microliters of the GlcCer extract from crude oil), which was near the HPLC/ELSD detection limit (2.4 µg) for GlcCer. This may explain why GlcCer was not detected in the crude oil. In our previous investigation, GlcCer was detected in 2 g of crude oil because those total lipids were extracted from 10 g of ground soybean seed by sequential solvent extraction using solvents more polar than hexanes, such as chloroform/methanol (2:1, vol:vol) and water-saturated butanol. Therefore, 2 g of 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 the HPLC/ELSD analysis may have not been sensitive enough to detect the trace amount of GlcCer.

Because GlcCer is a relatively polar lipid class, we expected most of it to remain in the DSF and in the purified soy protein products. GlcCer was nearly equally concentrated in all the soy protein products prepared in this study (Table 2). No significant differences in GlcCer content were found among the DSF, acid-washed SPC, alcohol-washed SPC, and SPI products. As mentioned, the processing conditions adopted for this study were very similar to typical industrial practices; therefore, commercially produced soybean products, such as the types produced in this study, may not differ significantly in their GlcCer contents if they have originated from the same soybean genotype.

Few studies have reported the sphingolipid content of soy products with which to compare our results. Ahn and Schroeder (12) have measured the 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 hydrolysis and quantification of its sphingoid base backbone. The GlcCer contents for both SPI and full-fat soy flakes in our study were 297 and 268 (nmol/g dry wt basis), respectively. Even though the SPI sphingolipid contents for both studies agreed well, we could not conclude that these values reflected the GlcCer contents in all SPI samples because the sphingolipid contents may vary with genotype, as suggested by Gutierrez *et al.* (6). Ceramide may be the only other sphingolipid present in soybeans, but it is a minor contributor to the total sphingolipid content (13). It was not measured in our study due to certain difficulties as discussed by Gutierrez *et al.* (6).

Fate of GlcCer during processing. After oil extraction and production of the DSF, 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 GlcCer remained with the DSF (91%) rather than with the crude oil (9%), but recovery of GlcCer from the DSF into the soy protein purification products was poor. Recovery of GlcCer in the alcohol-washed SPC, acid-washed SPC, and SPI products was 43, 52, and 26%, respectively.

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). Therefore, the 50 g of defatted soy flakes used for the SPC preparations would contain 11.1 mg of GlcCer, and the 115 g of defatted soy flakes used for the SPI preparation

would contain 25.6 mg of GlcCer. The two SPC preparation procedures used in this study did not differ in their ability to retain GlcCer in the protein product. 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 for GlcCer in this study.

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