Lipid Components That Reduce Protein Solubility of Soy Protein Isolates

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ABSTRACT: A lipid fraction from a commercial soy protein isolate (SPI), previously found to be detrimental to SPI solubility, was analyzed by size-exclusion liquid chromatography, by high-performance liquid chromatography (HPLC), and for chemical composition. The molecular weight of most of this material was greater than 1,100 daltons. This lipid fraction was water-soluble yet required a strong nonpolar solvent mixture to elute it from a C_{18} HPLC column. The lipid material was alkaline (pH 8.7) and composed of 3.0% nitrogen, 1.6% phosphorus, 17.5% nonvolatile crude fatty acids (primarily hydroxylated), 10.4% long-chain bases, 9.9% hexuronic acid, 3.2% hexosamine, and 6.6% total sugar. The molecular weight, chemical composition, and physical characteristics (solubility characteristics, surfactant characteristics, and appearance) of this material were all similar to those reported for phytoglycolipid.
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KEY WORDS: High-performance liquid chromatography, lipids, phytoglycolipid, size-exclusion chromatography, solubility, soy protein isolate.

In a previous investigation (1), the lipids associated with a commercial soy protein isolate (SPI) were found to contribute to decreased protein solubility, modification of protein thiols, and protein oxidation as measured by protein carbonyl content. A fraction of the commercial SPI lipid extract (designated Fraction B), which was eluted from a carboxymethyl (CM)–cellulose column with ≥50% methanol in chloroform and represented approximately 5% of the total lipid extract, was highly detrimental to protein solubility. The adverse effect of Fraction B lipids on protein solubility was not reversible by treatment with a reducing agent, and little effect on protein thiols or protein carbonyl content was contributed by this lipid fraction.

This investigation was designed to further characterize Fraction B lipids to elucidate the mechanism of their detrimental effect on SPI solubility. Because the addition of antioxidants during SPI processing inhibits the formation of insoluble proteins (2), the effect of added *tert*-butylhydroquinone (TBHQ) on Fraction B lipids was also examined.

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MATERIALS AND METHODS

Protein isolation and lipid extraction. Laboratory isolates were prepared by dispersing hexane-defatted Forrest var. soybean flour in water (1 part flour to 10 parts water), followed by additions of 1 N NaOH as needed until a pH of 9 was achieved and maintained for 15 min. After centrifuging at 1,500 × *g* for 10 min, the supernatant was adjusted to pH 4.5 with 1 N HCl to precipitate proteins. After centrifugation at 1,500 × *g* for 10 min, the precipitate was washed once with water, and the protein isolate was adjusted to pH 7 with 1 N NaOH. Samples were freeze-dried after being frozen overnight at −15°C. Added TBHQ was first dispersed in a premix by adding 0.5 g Tenox 20 (Eastman Chemical Co., Kingsport, TN) into a 50-mL volumetric flask with 40 mL water. The flask with the premix was suspended for 30 min in a Bandelin Sonorex Super RK106 sonicator (Bandelin Electronics, Berlin, Germany). The water temperature was maintained at 50°C. After sonication, the premix was made to 50 mL with water. A portion of the premix was then added to the processing water, prior to dispersing the hexane-defatted flour, to provide 200 ppm TBHQ to the lipids associated with the defatted flour.

Lipid extracts were obtained from hexane-defatted Forrest var. soybean flour, freeze-dried SPIs, and commercial SPI (Pro Fam 970, sample A; Archer Daniels Midland, Decatur, IL). Composition and solubility data for these materials were described in previous investigations (1,2).

Lipids were extracted by the modified method of Bligh and Dyer (3,4), and without the use of acid and subsequent neutralization with NH_4OH or by the modified Folch procedure (4,5). Flour/solvent ratios (wt/vol) were 1:5 for the commercial samples, 1:10 for the laboratory-prepared hexane-defatted flours, and 1:18 for the laboratory-prepared SPI. Materials were extracted twice, and the solvents were combined prior to phase separation.

Fractionation and chemical characterization of lipid extracts. Lipid extracts were fractionated by CM–cellulose column chromatography by the method of Comfurius and Zwaal (6). This was performed with preswollen CM-cellulose, CM-52 (Whatman, Clifton, NJ). Lipids eluted with 50% methanol in chloroform (vol/vol) were designated Fraction A. All lipids subsequently eluted with 100% methanol were designated as Fraction B.

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Fatty acid methyl esters (FAME) and trimethylsilyl (TMS) derivatives of FAME were prepared according to the method of Weiss (7) without the addition of diazomethane. Standard FAME mixture D-102 was purchased from Serdary Research Laboratories (London, Ontario, Canada). DL-α-hydroxybehenic acid methyl ester, DL-α-hydroxypalmitic acid methyl ester, and DL-α-hydroxyhexacosanoic acid were purchased from Sigma Chemical Co. (St. Louis, MO). DL-α-hydroxyhexacosanic acid was converted to the methyl ester with boron trifluoride by the method of Morrison and Smith (8). All standard hydroxy fatty acids were converted to TMS derivatives by the method of Carter and Graver (9). Gas–liquid chromatography (GLC) was done in a Hewlett-Packard Model 5890A chromatograph (Hewlett-Packard, San Fernando, CA) equipped with a hydrogen flame-ionization detector, a Hewlett-Packard Model 3392A integrator, and a 2 m × 2 mm i.d. glass column packed with 10% DEGS on Chromosorb W AW (Alltech Associates, Inc., Deerfield, IL). The column temperature was 190°C (isothermal), injector and detector temperatures were 225°C, and high-purity. He was used as carrier gas at 25 mL/min flow rate. Fatty acid derivatives were tentatively identified by cochromatography with standards or from plots of log retention times (relative to internal standards) against carbon number. Response factors of all components were taken as 1.0, and relative composition was calculated on the basis of peak area. All determinations were performed in triplicate. Total nonvolatile crude fatty acids were determined gravimetrically (10,11) after the petroleum ether was completely evaporated.

Long-chain bases were extracted into a chloroform phase after lipid hydrolysis according to the method of Weiss (7). After the chloroform was evaporated at 50°C under a stream of dry nitrogen, the long-chain bases were resuspended in ethyl acetate and determined colorimetrically while using the molar absorption coefficient 2.73×10^4 for phytosphingosine (12,13).

Hexuronic acid was assayed by the method of Bitter and Ewins (14), with glucuronic acid as the standard. Hexosamine was quantitatied by hydrolyzing approximately 2 mg lipids in 1.5 mL 0.5 N HCl for 16 h at 100°C. After cooling to room temperature, the hydrolysate was neutralized with an equal volume of 0.5 N NaOH. One-mL aliquots were assayed for hexosamine against a glucosamine–HCl standard by the method of Elson and Morgan (15), as modified by Boas (16) and Rondle and Morgan (17).

Total sugars were determined by the anthrone method of Yomamoto and Rouser (18), with glucose as the standard. Sterols were quantitatied by the method of Sperry and Webb (19). Peroxide value (PV) was determined by the colorimetric ferric thiocyanate method (20). Total carbonyls were determined according to the method of Yukawa *et al.* (21).

Size-exclusion chromatography (SEC). SEC was performed on a Sephadex LH-20 (Pharmacia, Piscataway, NJ) with 100% methanol as the mobile phase. This was accomplished with a Beckman Model 110A pump (Beckman Instruments, Inc., Palo Alto, CA) with either a 250-, 500-, or 1000-μL injection loop attached to a 2.0 × 50-cm column and a 1.0 × 30-cm column connected in tandem. Peaks were monitored with a Hitachi model 100-10 Spectrophotometer (Beckman Instruments, Inc.) set at 210 nm. The flow rate was 2.0 mL/min. Internal standards used were methyl oleate (MW 297) (Sigma Chemical Co.), phosphatidylcholine dioleoyl (MW 804), and *bis*-phosphatidic acid (approximate MW 1170) (Serdary Research Laboratories).

TABLE 1
Solvent Gradient for High-Performance Liquid Chromatography Separation of Lipids

Time (min)	Buffer[a]	2-Propanol	Acetonitrile	Flow rate (mL/min)
0.0	70	0	30	2.0
0.5	70	0	30	2.0
15.0	23	62	15	1.5
25.0	20	65	15	1.5
28.0	20	65	15	1.5
32.0	70	0	30	1.5
34.0	70	0	30	2.0

[a]50 mM phosphate buffer (pH 6.0).

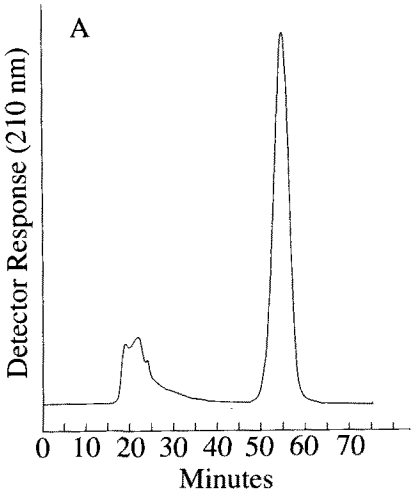
High-performance liquid chromatography (HPLC). HPLC separations were accomplished on a Hewlett-Packard HP 1090L System (Hewlett-Packard, Palo Alto, CA), equipped with a diode array ultraviolet (UV) detector and HP Analytical DOS Chemstation controller.

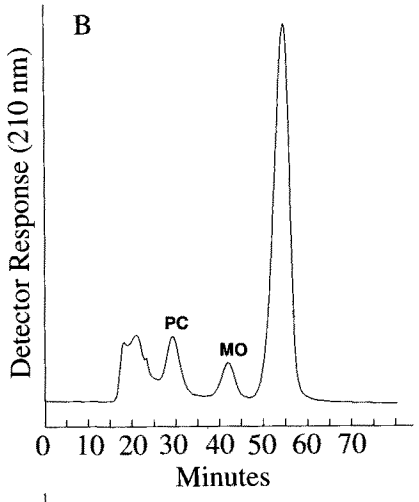
Lipids were separated on a Bondesil C18-5-μm column, 4.6 × 250 mm (Varian, Sugarland, TX), and a 4.6 × 20–mm Suplecosil C_{18} guard column (Supleco, Bellefonte, PA). The solvent gradient and flow are shown in Table 1. Each chromatogram was prepared by subtracting a blank run from the sample run. Peaks were monitored at 210 nm. The column temperature was 50°C. Concentrating samples or bringing them to near-dryness was accomplished by rotary evaporating at 50°C and by 508 mm Hg vacuum.

RESULTS AND DISCUSSION

Characterization of fraction B from commercial SPI (Pro Fam 970 A). SEC of the commercial SPI lipid Fraction B on Sephadex LH-20 is represented in Figure 1A. This material consisted of two distinct fractions. The larger peak, eluted at 55 min, appeared to be at or below the molecular weight cutoff of 100 for Sephadex LH-20. The set of peaks eluting from 17 to 25 min corresponded to molecular weights ranging from approximately 1,100 to beyond the upper molecular weight exclusion limit for Sephadex LH-20 in methanol (approximately 4,000.)

Two chromatograms were obtained from the reverse-phase HPLC separations of the commercial SPI (Pro Fam 970 A) lipid Fraction B. Figure 2A demonstrates the result of injecting the 100%-methanol fraction as is (approximately 0.0125 mg nonvolatile material/25 μL). Figure 2B illustrates the results of concentrating the lipid fraction to near-dryness on a rotary evaporator and resuspending it in water/acetonitrile, 70:30 (vol/vol) (0.25 mg/25 μL). Similar chromatograms





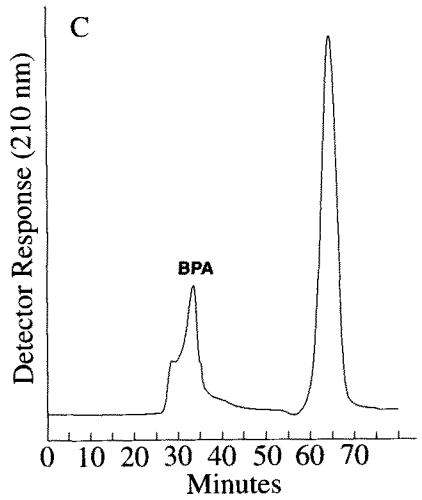
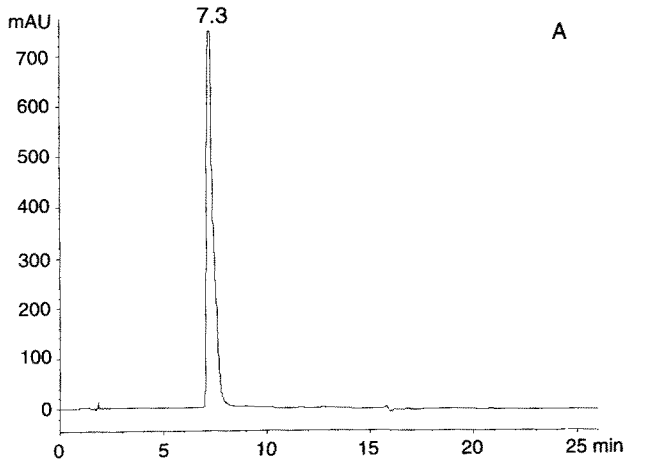
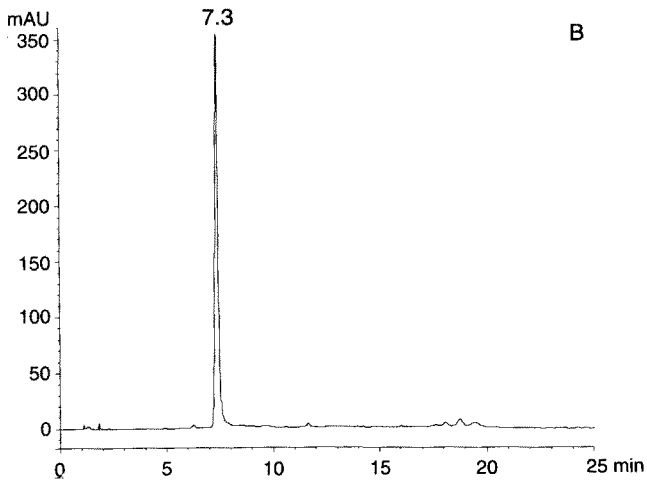


FIG. 1. Size exclusion chromatography of: A) commercial SPI (Pro Fam 970 A; Archer Daniels Midland Co., Decatur, IL) lipid Fraction B (0.125 mg nonvolatile material/250 µL); B) same as A, with internal standards of phosphatidylcholine (PC), and methyl oleate; and C) same as A, with internal standard of *bis*-phosphatidic acid (BPA).





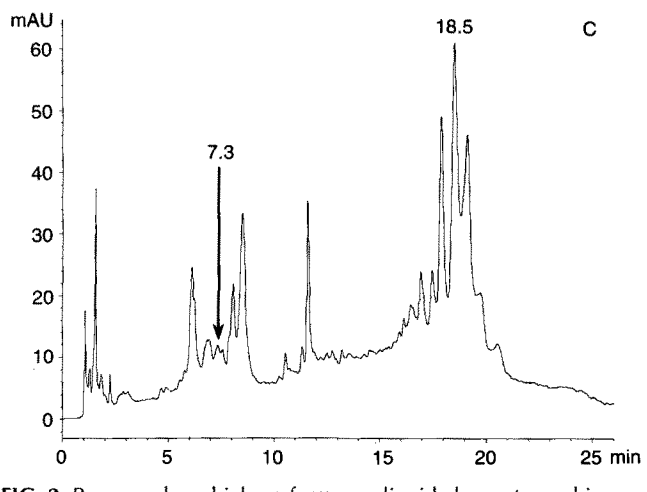


FIG. 2. Reverse-phase high-performance liquid chromatographic separation of commercial SPI (Pro Fam 970 A) lipid extract Fraction B: A) 100%-methanol fraction as is (approximately 0.0125 mg nonvolatile material); B) concentrated 2 to 1 (approximately 0.025 mg nonvolatile material); and C) concentrated to near-dryness and suspended in water/acetonitrile 70:30 (vol/vol) (approximately 0.25 mg nonvolatile material). Company source as in Figure 1.

were obtained from commercial SPI Fraction B, with or without the use of acid and subsequent neutralization with NH_4OH during the lipid extraction. Bringing the material to near-dryness on the rotary evaporator (see Fig. 2B) was the method previously used for adding Fraction B lipids back to the reduced-lipid SPI during processing (1). The dominant

peak at 7.3 min in Figure 2A, if nonvolatile, would be expected to be approximately twenty times as large on Figure 2B; instead it is virtually absent.

Collecting and injecting the volatile material, represented by the small molecular weight peak from the Sephadex LH-20 columns (eluted at 55 min), on the reverse-phase HPLC column demonstrated that this peak corresponded almost entirely to the HPLC peak at 7.3 min (Fig. 3A). Separation of the set of large-molecular weight peaks (eluted at 17 to 25 min) from the Sephadex LH-20 columns by reverse-phase HPLC (Fig. 3B) demonstrates that this material corresponds to the nonvolatile material shown in Figure 2C. Because the dominant peaks produced by this lipid material required a strong nonpolar solvent mixture [approximately 22 parts buffer (pH 6.0), 63 parts 2-propanol, and 15 parts acetonitrile] for elution, it is interesting that this material was soluble only in pyridine, water, and solvent mixtures containing a substantial portion of water. Making the eluting buffer more acidic (pH 2.0) had little effect on the elution profile of this material.

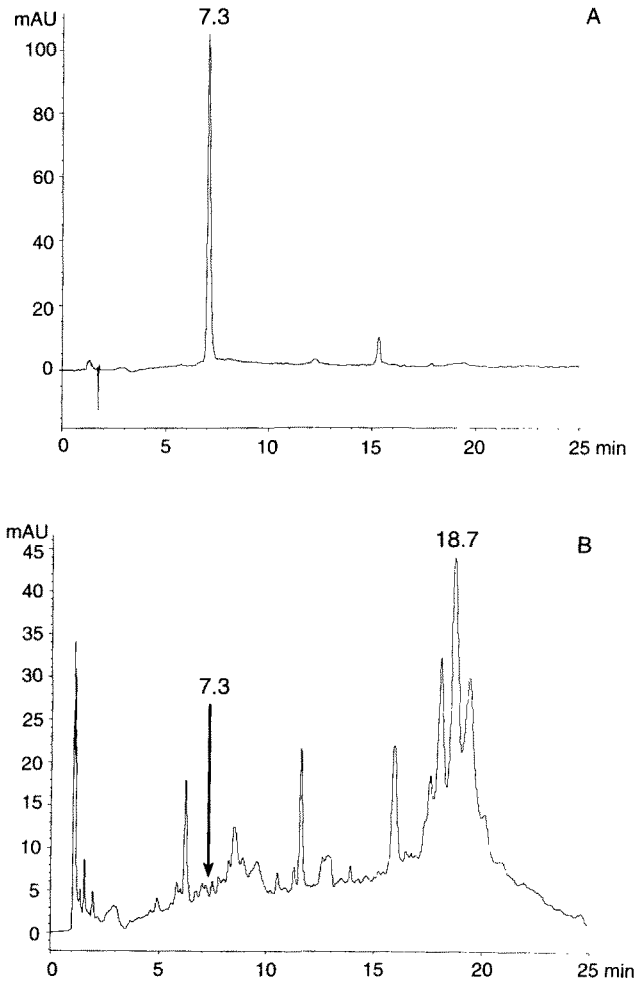


FIG. 3. Reverse-phase high-performance liquid chromatography separation of commercial SPI (Pro Fam 970 A) lipid extract Fraction B peaks resulting from separation on Sephadex LH-20 (Pharmacia, Piscataway, NJ): A) Sephadex LH-20 peak at 55 min; and B) Sephadex LH-20 peaks at 17 to 25 min. Company source as in Figure 1.

Examination of the ultraviolet spectrum (190–400 nm) of the peaks in Figure 2B revealed that every major peak that eluted beyond 2.5 min possessed similar spectra: a single sharp peak with an apex at 195 nm (Fig. 4A–C). The HPLC peak at 1.5 min (Fig. 4D) and at 2.2 min (Fig. 4E) exhibited a second peak at approximately 260 nm, possibly the result of fatty acids with conjugated triene unsaturation (22). The UV spectrum for the large volatile peak in Figure 2A had a single broad peak with an apex at approximately 200 nm (Fig. 4F).

At low concentrations (0.4 mg/mL), aqueous solutions of the nonvolatile material behaved as a strong surfactant. It produced an abundance of foam when agitated, and the foam persisted for more than 2 h. Because surfactants are often strong protein denaturants (23–26) and the rate of protein oxidation by lipid free radicals can be stimulated in the presence of a surfactant (27), the surface-active properties of lipid Fraction B may contribute to its strong detrimental effect on soy-protein solubility observed earlier (1).

The chemical composition (Tables 2 and 3) and physical characteristics of the material designated as Fraction B in this investigation indicated that a substantial portion of this material is a phytoglycolipid (PGL). Work by Carter *et al.* (11,28) led to the structural determination of PGL, which contains, in addition to inositol, both nitrogen and phosphorus at a 2:1 ratio. Half of the nitrogen was found as an unsaturated long-chain basic constituent, phytosphingosine or dehydrophytosphingosine. In addition, PGL contained a substantial amount of carbohydrate, including inositol, hexuronic acid, hexosamine, mannose, galactose, and arabinose. Total sugar content of flaxseed PGL, purified by DEAE-cellulose chromatography, was 8.1% (29). The anthrone-negative carbohydrates of DEAE-cellulose–purified flaxseed PGL were reported to include hexosamine (9.6%) and hexuronic acid (15.4%). Qualitative analysis for inositol was positive. This same lipid from flaxseed contained approximately 17% by weight of nonvolatile crude fatty acids (11). The fatty acids of both flaxseed and bean-leaf PGL consisted primarily of saturated long-chain ($C_{22:0}$ to $C_{26:0}$) hydroxylated fatty acids (29). Ultracentrifugal evaluation indicated a molecular weight for PGL of approximately 2,500.

PGL, which accounts for about 5% of the total phospholipids found in soybean, corn, flaxseed, peanut, sunflower, cottonseed, and wheat, is unique in that it possesses the structural features of both a glycolipid and a phospholipid (10). Purified PGL is a white, amorphous powder that is insoluble in all common solvents except basic ones, such as pyridine, morpholine, aqueous alkali (10), and dimethyl sulfoxide (29). The solution of PGL in an aqueous base gave a clear soapy solution (11). PGL is the only documented compound that possesses chemical and physical characteristics similar to those exhibited by the lipid Fraction B in this investigation (10,11,28,29).

Effects of processing and TBHQ on lipid Fraction B. The occurrence of Fraction B lipids in those associated with Forrest var. hexane-defatted soybean flour and corresponding SPI (1,2) was also examined. Extraction of the lipids from the de-

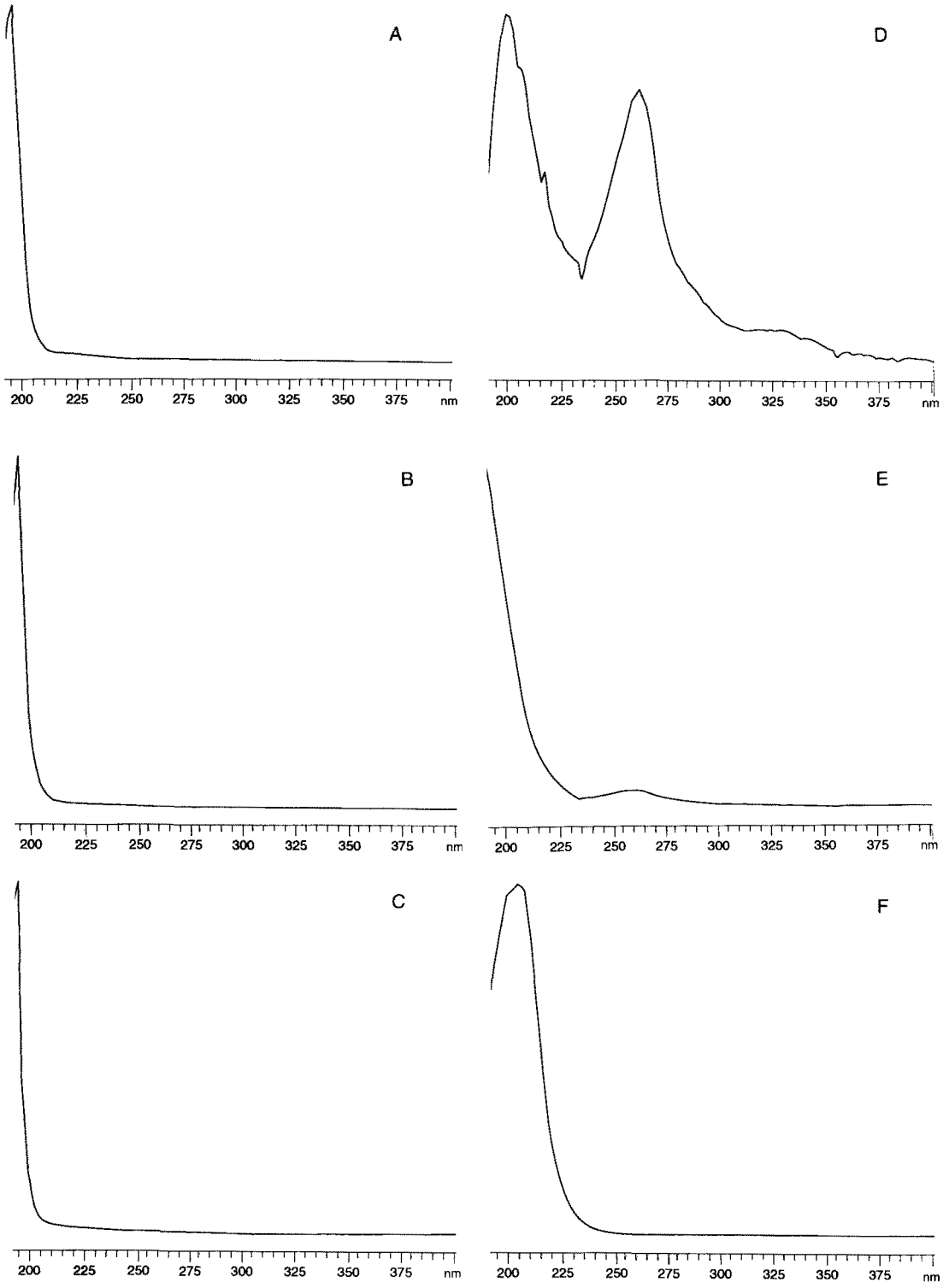


FIG. 4. Ultraviolet spectrums (190–400 nm) of selected high-performance liquid chromatography peaks from Figure 2: A) peak eluted at 18.5 min in Figure 2C; B) peak eluted at 11.6 min in Figure 2C; C) peak eluted at 8.5 min in Figure 2C; D) peak eluted at 2.2 min in Figure 2C; E) peak eluted at 1.5 min in Figure 2C; and F) peak eluted at 7.3 min in Figure 2A.

fatted Forrest var. soybean flour and subsequent separation of these lipids by CM-cellulose chromatography were accomplished by the same methods as those used for the commercial SPI lipid extracts. This resulted in 4.1% ± 0.06 Fraction B and 94.9% ± 0.58 Fraction A. Extraction of the hexane-defatted flour by the modified Folch procedure resulted in only

TABLE 2
Chemical Characteristics of Commercial SPI Lipid Fraction B

	Solvent-free extract	As is extract
Phosphorus, %[a]	1.62 (0.01)[b]	1.3 (0.06)
Nitrogen, %	3.0 (0.33)	2.9 (0.07)
Nonvolatile crude fatty acids, %[d]	17.5 (0.39)	—
Long-chain bases, %[a]	10.4 (0.21)	—
Total sugar (as glucose), %[a]	6.6 (0.12)	—
Hexuronic acid, %[a]	9.9 (0.06)	—
Hexosamine, %[a]	3.2 (0.04)	—
Sterols, %[c]	n.d.[e]	—
pH of 0.1% aqueous solution	8.7	—
Peroxide value (meq/kg)[c]	—	9.5 (0.29)
Total carbonyls (as heptanal), %[a]	0.45 (0.004)	0.87 (0.055)

[a]Values are the means of triplicate determinations expressed by weight.
[b]Figures in parenthesis are standard errors.
[c]Values are the means of duplicate determinations expressed by weight.
[d]Value is the mean of triplicate determinations on duplicate preparations expressed by weight.
[e]n.d. = Not determined.

TABLE 3
Fatty Acid Composition of Lipid Fraction B

Fatty acid	Percentage by weight
C16:0	7
C18:0	2
C18:1	2
C18:2	7
C18:3	2
C22h:0[a]	19
C23h:0[a]	6
C24h:0[a]	34
C25h:0[a]	4
C26h:0[a]	8
Unidentified or minor	9

[a]Hydroxylated fatty acid.

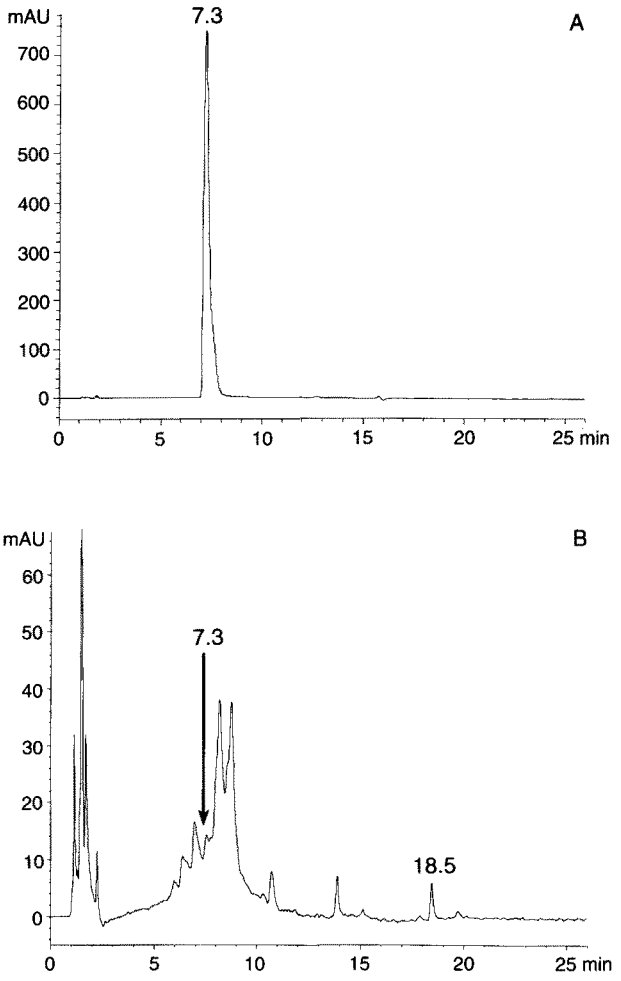


FIG. 5. Reverse-phase high-performance liquid chromatography separations of hexane-defatted flour (Forrest var.) lipid extract Fraction B: A) 100%-methanol fraction as is (approximately 0.004 mg nonvolatile material/25 µL); and B) concentrated to near-dryness and suspended in water/acetonitrile, 70:30 (vol/vol) (approximately 0.05 mg nonvolatile material/25 µL).

0.7% ± 0.09 Fraction B and 99.0% ± 1.2 Fraction A. Reverse-phase HPLC chromatograms of the hexane-defatted Forrest var. soybean flour lipid Fraction B (extracted by the modified Bligh and Dyer method) is shown as is (100%-methanol fraction) in Figure 5 after being brought to near-dryness and resuspended in water/acetonitrile (0.05 mg/25 µL). This demonstrated that the large, volatile peak at 7.3 min in Figure 5A already existed in the defatted flour or was an artifact formed during lipid extraction or subsequent fractionation. As before, all peaks beyond 2.5 min had the same basic UV spectrum as did the peaks in the commercial SPI lipid extract (see Fig. 4). The profile of peaks in the laboratory-prepared hexane-defatted flour was quite different from those of the commercial SPI Fraction B lipids, indicating that some type of lipid modification occurred during SPI processing.

Of the total Forrest var. SPI lipids (extracted by the modified Bligh and Dyer method) fractionated by CM-cellulose column chromatography, the lipid extract from the control SPI produced a Fraction A that accounted for 95.7% ± 0.44 and a Fraction B of 2.3% ± 0.08. Of the total material loaded on the column, the lipid extract from the SPI with TBHQ produced a Fraction A that accounted for 98.3% ± 0.64 and a Fraction B that accounted for 2.1% ± 0.07. The material unaccounted for in the control SPI lipid extract, while not as large as that from the commercial SPI, appears to have been eliminated by the addition of TBHQ during SPI processing.

The lipid Fraction B from the Forrest var. control SPI and the Forrest var. SPI processed with added TBHQ were also separated by reverse-phase HPLC (Fig. 6). Loading both materials without prior concentration (not shown) yielded a large volatile peak at 7.3 min, similar to the peaks resulting from the hexane-defatted flour and the commercial SPI. Both samples, when concentrated by rotary evaporating, exhibited no major peaks in the 6- to 9-min range, similar to the material obtained from the laboratory hexane-defatted flour. Each lipid fraction did exhibit a cluster of peaks in the 17- to 20-min range, similar to the commercial SPI Fraction B, further indicating lipid modification during SPI processing. The antioxi-

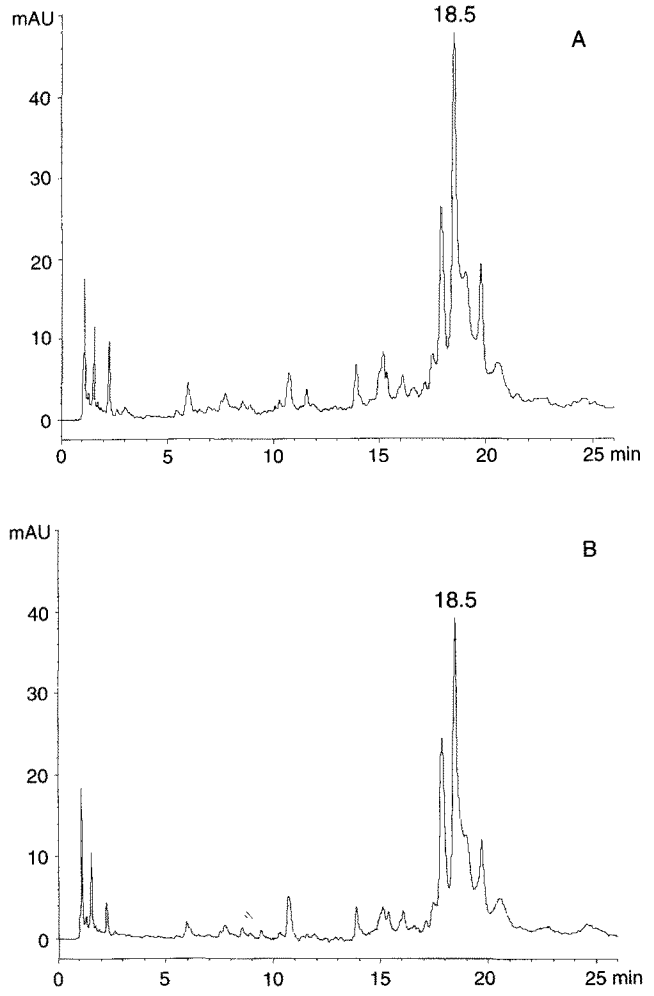


FIG. 6. Reverse-phase high-performance liquid chromatography separation of Forrest var. soy protein isolates (SPI) lipid extract Fraction B after concentrating to near-dryness and resuspending in water/acetonitrile, 70:30 (vol/vol): A) control SPI Fraction B (approximately 0.11 mg nonvolatile material/25 µL); and B) fraction B from SPI with added *tert*-butylhydroquinone (approximately 0.10 mg nonvolatile material/25 µL).

dant appeared to partially inhibit the formation of the material represented by the peaks at 18.5, 19.0, and 19.7 min, while having little effect on the material represented by the peak at 17.9 min.

All chemical and physical characteristics observed for the lipid fraction previously designated as Fraction B were similar to those previously described for PGL. The addition of TBHQ during processing of SPI resulted in both increased protein solubility (2) and a partial decrease in the presence of the lipids designated as Fraction B. Because this material exerts such a strong detrimental effect on protein solubility (1), this may, partially, account for the increased SPI solubilities obtained with added antioxidants. Because of its amphiphilic nature, Fraction B lipids may work in concert with lipid oxidation products to greatly reduce protein solubility.

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