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Abstract
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Keywords
31 P NMR, soybean phospholipids, aqueous extraction, enzyme-assisted aqueous extraction, emulsion stability

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^{31}P NMR Phospholipid Profiling of Soybean Emulsion Recovered from Aqueous Extraction

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The quantity and composition of phospholipids in full-fat soybean flour, flakes, and extruded flakes and in the cream fraction recovered after aqueous extraction (AEP) and enzyme-assisted aqueous extraction (EAEP) of these substrates were studied with ^{31}P NMR. Extruded flakes had significantly more phosphatidic acid (PA) than flakes and flour prior to aqueous extraction. The PA content of the cream recovered after AEP and EAEP of extruded flakes was similar to that of the starting material, whereas the PA content of the creams from flour and flakes significantly increased. Changes in the PA content could be explained by the action of phospholipase D during the processing step and aqueous extraction. Total phospholipids in the oil recovered from the creams varied from 0.09 to 0.75%, and free oil yield, which is an indicator of cream stability, varied from 6 to 78%. Total phospholipid did not correlate with emulsion stability when it was lower than 0.20%. Inactivation of phospholipase D prior to aqueous extraction of flour resulted in a cream emulsion less stable toward enzymatic demulsification and containing less PA and total phospholipids than untreated flour. The phospholipid distributions in the cream, skim, and insolubles obtained from AEP flour were 7, 51, and 42%, respectively.

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INTRODUCTION

Aqueous extraction processing (AEP) uses water as extraction medium, and the process has been applied to many oilseeds including soybeans (*1*−*6*). AEP is a more environmentally friendly oil extraction method compared to the conventional hexane extraction and has the advantage of extracting proteins simultaneously with the oil (*7*). The oil extraction yield of AEP is greatly affected by the starting materials, especially the extent of cotyledon cell wall disruption (*7*). Grinding and extrusion have been used as mechanical pretreatments to improve oil extraction yield (*7*, *8*). A reduction of soy flour particle size from 1200 to 100 μm increased AEP oil extraction recovery from 22 to 65% (*6*). Extrusion treatment formed insoluble aggregates that trapped the released oil (*9*) and led to a yield of 68%, although it disrupted cotyledon cells (*8*). However, an extraction yield comparable to hexane extraction can be obtained with protease treatment of extruded soy flakes (*10*). This technology has been named enzyme-assisted aqueous extraction processing (EAEP). Seemingly, the protease releases the oil trapped in insoluble aggregates formed during extrusion and possibly also hydrolyzes the lipid body membranes, thus facilitating oil recovery (*7*, *9*).

Most of the oil obtained during aqueous extraction is recovered in the form of a stable emulsion. Only a small fraction of the oil was released as free oil. Destabilization of this emulsion is critical to obtaining free oil, which will thus improve the commercial viability of the process. Recent developments that use enzyme(s) to assist demulsification showed that enzyme concentration, temperature, and incubation time greatly affected oil extraction yield (*11*). Up to 98% of free oil could be recovered after demulsification by treating the EAEP cream of extruded flakes with a mixture of alkaline serine endopeptidase and phospholipase A2 (*11*). The cream emulsion stability is affected by the starting material (i.e., flour, flakes, or extruded flakes) and the presence of proteases during the extraction (i.e., AEP vs EAEP) (*10*, *12*−*14*). AEP and EAEP creams from soybean flour are composed mainly of triacylglycerides and water. Phospholipids, proteins, and carbohydrates are also present in these creams but in minor quantities (*12*). As phospholipids and proteins both are important natural surfactants that reduce the interfacial tension of the oil−water interface, they might have played significant roles in determining the cream stability. Phospholipids found in crude soybean oil include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), *N*-acyl phosphatidylethanolamine (APE), phosphatidylserine (PS), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), and the corresponding lyso-phospholipids (*15*). Because of their structural differences, especially in the head groups, the effects of these phospholipids on the properties of modeled emulsion systems are not the same (*16*−*19*). We hypothesize that the phospholipid profile and content in the cream emulsions vary depending on the starting materials and aqueous extraction methods used, therefore resulting in creams with various stability levels.

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Some of the methods used for analysis of the phospholipids include thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), and ^{31}P nuclear magnetic resonance spectroscopy (^{31}P NMR). ^{31}P NMR is easy to perform, allowing quick access to results, and has a higher selectivity than HPLC and TLC because only phosphorus-containing molecules are detected in a single signal. Unlike HPLC, ^{31}P NMR does not need various standards to calibrate the different phospholipids (*20*).

The aim of this study was to examine the effect of phospholipids on the emulsion stability of creams obtained from the AEP and EAEP of soy flour, flakes, and extruded flakes by profiling and quantifying the phospholipids with ^{31}P NMR.

MATERIALS AND METHODS

Materials. Full-fat soy flour was obtained from Natural Products, Inc. (Grinnell, IA). Full-fat soy flakes were prepared from variety IA 92M91-N201 soybeans. Protex 6 L (P6L, alkaline serine endopeptidase, optimum pH and temperature of 8.0 and 50 °C, respectively) were provided by Genencor International, a Division of Danisco (Rochester, NY). Soy phosphatidylcholine (soy PC) containing about 40, 16, 11, and 33 wt % of PC, PE, PI, and others, respectively, was from Avanti Polar Lipids, Inc. (Alabaster, AL). The potassium EDTA solution (K-EDTA) (0.1 N, pH 7.5) was prepared by titrating 0.1 N EDTA with solid K_2CO_3 until the pH was 7.5. The cesium EDTA solution (Cs-EDTA) (0.2 N, pH 8.5) was prepared by titrating 0.2 N EDTA with solid CsOH until all of the substances were dissolved (pH ~6.0) and then raising the pH to 8.5 by the addition of solid K_2CO_3. Triphenol phosphate (TPP) used as an internal standard in ^{31}P NMR analysis was from Sigma-Aldrich (St. Louis, MO). Laboratory grade soy lecithin and other chemicals were from Fisher Scientific (Fair Lawn, NJ).

Preparing Full-Fat Soy Flakes. The soybeans were cracked using a corrugated roller mill (model 10X12SGL, Ferrell-Ross, Oklahoma City, OK) and aspirated in a multiaspirator (Kice, Wichita, KS) to separate into meat and hull fractions. The meats were conditioned at 60 °C using a triple-deck seed conditioner (French Oil Mill Machinery Co., Piqua, OH). The conditioned meats were flaked using a smooth-surfaced roller mill (Rosskamp Manufacturing, Inc., Waterloo, IA) to approximately 0.25 mm thickness and 3−5 mm in width. For satisfactory extrusion, the moisture content of the flakes was adjusted from 10 to 14% by spraying water onto the flakes while they tumbled in a Gilson mixer (model 59016A, St. Joseph, MO). The moistened and unmoistened flakes were placed separately into double polyethylene bags and kept at 4 °C.

Extrusion. The extrusion was carried out with a Micro ZSE-27 twin-screw extruder (American Leistritz Extruders, Somerville, NJ). The unit was equipped with a 4 mm diameter die. The length and diameter of each screw were 1080 and 27 mm, respectively. The screw configuration used in the experiments consisted of conveying elements (length/diameter (L/D) = 8), a kneading element (L/D = 5.4), a conveying element (L/D = 4.6), a kneading element (L/D = 3.4), a conveying element (L/D = 4.6), a kneading element (L/D = 3.4), a conveying element (L/D = 2.2), a kneading element (L/D = 2.2), and a conveying element (L/D = 8). The barrel consisted of 10 independently controlled heating sections. The barrels also had jackets in which air was circulated at controlled flow rates via solenoid valves to achieve consistent temperatures during processing. The barrel temperatures, measured via Fe-CuNi thermoelements inserted in the bottom of each barrel during the process, were as follows: feed section, 30 °C; section 1, 70 °C; sections 2−9, 100 °C. The output under these conditions was 11 kg/h of extruded flakes, and the passage time of the material was 1 min. Processed material was fed through until equilibrium conditions were reached before material was collected for experimental use. After extrusion, the extruded flakes were stored in a refrigerator at 4 °C in double polyethylene bags.

Aqueous Extraction. Starting material (300 g) was mixed with 3 L of water (to achieve a 1:10 solid-to-water ratio) in a 3 L jacketed glass reactor with a bottom drain valve (Chemglass, Vineland, NJ). The temperature was kept at 50 °C via a water circulator (HAAKE Phoenix P1, Thermo HAAKE, Portsmouth, NH). The pH was adjusted to 9.0 with 2 N NaOH. EAEP was performed with the addition of 0.5% P6L w/w (g of enzyme/g of flour, as is). The extraction without enzyme addition was termed AEP. The slurry was stirred via an external stirrer at 600 rpm for 1 h. The pH of the slurry was kept nearly constant by adding 2 N NaOH periodically. After 1 h of extraction, the AEP or EAEP slurry was then cooled in a 4 °C refrigerator for about 1 h. The slurry was then centrifuged at a speed of 3000*g* for 15 min at 25 °C using an HS-4 swinging bucket rotor (Sorvall RC-5B, Newtown, CT). A solid phase, called "insolubles", and a liquid phase were obtained after centrifugation by decanting. The liquid phase was further separated to the cream (an oil-rich and protein-lean fraction) and skim (a protein-rich and oil-lean fraction) fraction in a 4 L separatory funnel overnight at 4 °C. After the phase separation, the cream was collected from the top and immediately subjected to crude oil extraction. When free oil was observed on top of the cream after the phase separation, it was collected along with the cream for quantification purpose.

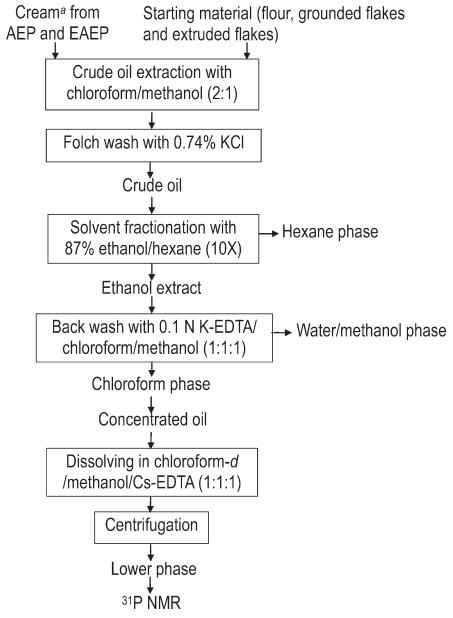

Figure 1. Simplified scheme for sample preparation for ^{31}P NMR. [a] Cream might contain some free oil.

Crude Oil Extraction. The procedure for preparing the phospholipid NMR samples from the starting material (flour, flakes, and extruded flakes) and from the cream fraction recovered after aqueous extraction is summarized in **Figure 1**. The flakes and extruded flakes (~80 g) were milled in a coffee grinder (Smart Grind, Black & Decker, Towson, MD) to a fine powder. The flour (~80 g) was used as is. Methanol (200 mL) was added to the powder, and the mixture was homogenized for 5 min with an Ultra-Turrax T25 homogenizer (Ika Works, Wilmington, NC) at 9500 rpm, followed by the addition of chloroform (400 mL) and another 5 min of homogenization. The mixture was then stirred for 4 h at room temperature. The meal recovered from Buchner funnel filtration was mixed with fresh methanol (100 mL) and chloroform (200 mL) for a second extraction. The filtrate from the two extractions was combined, and the solvent was removed with a rotary evaporator (Buchi Corp., New Castle, DE) at 40 °C. The crude oil was then subject to a Folch wash with chloroform/methanol/0.74% aqueous solution of KCl (8:4:3, by vol) (*21*). The final crude oil was weighed gravimetrically after removal of the residual solvent with a vacuum oven at 22 °C and then stored at −26 °C until analysis.

The crude oil in the AEP and EAEP cream was obtained according to the same procedure described in the previous section with the exception that only one solvent extraction was performed instead of two.

Concentrating Phospholipids by Solvent Fractionation with Hexane and Ethanol. The phospholipids were concentrated by solvent partitioning between hexane and 87% ethanol with a procedure adapted from Galanos and Kapoulas (*22*). Briefly, hexane (100 mL) and 87% of ethanol (100 mL) were mixed and equilibrated in a separatory funnel. The upper hexane phase and lower ethanol phase were collected as solvents A and B, respectively. Crude oil (~10 g) was dissolved in 45 mL of solvent A and 15 mL of solvent B in a 200 mL separatory funnel. After a 5 min phase

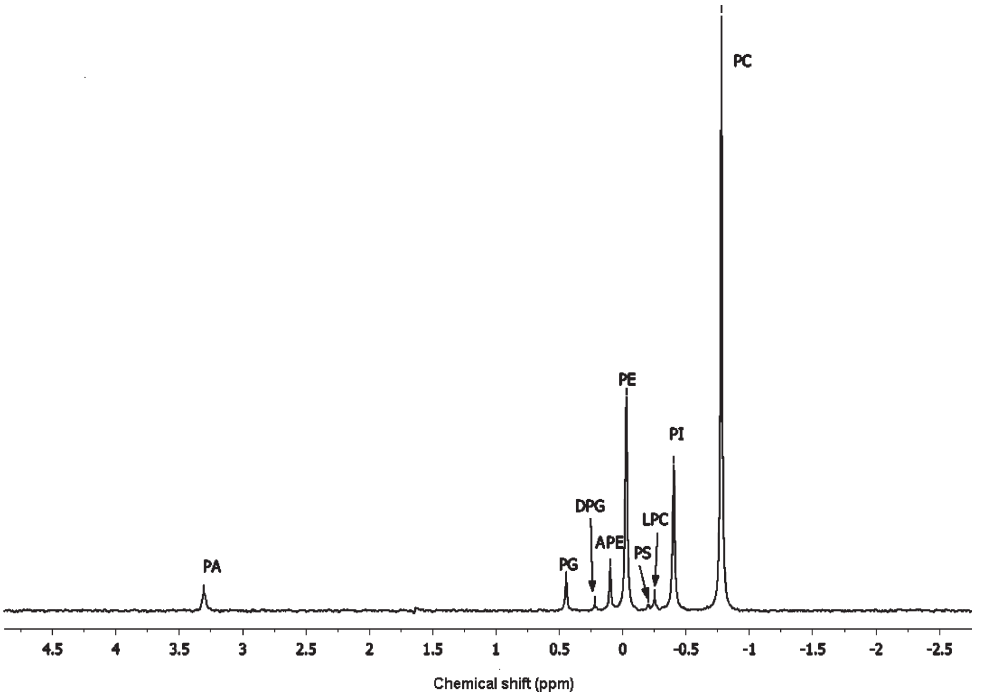

Figure 2. ^{31}P NMR spectrum of the phospholipids from soy flakes. For abbreviations, refer to Abbreviations Used.

equilibration, the lower ethanol phase was collected. Fresh solvent B (15 mL) was added to the upper phase. Then a second ethanol phase was collected after phase equilibration and combined with the first one. The extraction was repeated 10 times, and the pooled ethanol extracts were mixed with chloroform (130 mL) and 0.1 N K-EDTA (111 mL, pH 7). The chloroform phase was then collected and dried with sodium sulfate. The concentrated oil freed of solvent was stored in a desiccator at −26 °C.

^{31}P NMR Analysis. Concentrated oil (80−90 mg) and TPP (~10 mg, solid) were dissolved in chloroform-*d* (1 mL), methanol (1 mL), and Cs-EDTA (1 mL, 0.2 N, pH 8.5). After vigorous shaking, the sample was centrifuged and the lower phase was transferred to a 5 mm NMR tube (Kimble/Kontes, Vineland, NJ). The ^{31}P NMR spectra were obtained from a Varian VXR-400 spectrometer (Varian, Inc., Palo Alto, CA) with a Bruker Magnet (Bruker BioSpin, Billerica, MA) operating at 162 MHz. Samples were analyzed with an inverse gated decoupling pulse sequence to suppress any nuclear overhauser effect (*23*). The NMR spectroscopic scan conditions were as follows: probe temperature, 29 °C; pulse width, 22 μs; sweep width, 9718 Hz; acquisition time, 1.2 s; relaxation delay, 10 s; and number of scans, 256. The chemical shifts were reported relative to TPP (δ −17.8). The relative distribution of phospholipids was expressed in mole percent related to the sum of all phospholipids that was detected by ^{31}P NMR. The data processing was completed with MestReNova software (Mestrelab Research SL, Spain). The chemical shifts of various phospholipid classes were determined by comparing our spectra of soy PC with the spectra analyzed by a commercial laboratory, Spectral Service GmbH (Cologne, Germany). The phospholipid content (percent) in the crude oil extracted from cream was calculated as follows: phospholipid content (%) = 100[(phospholipids (g) in the concentrated oil)/(starting oil (g) in the solvent fractionation)].

Phospholipid Recovery from Solvent Fractionation with Hexane and Ethanol. Artificial crude soybean oil was prepared by dissolving 2.6 g of soy lecithin and 60 g of soybean oil (Wesson Vegetable Oil, ConAgra Foods, Inc., Omaha, NE) in 100 mL of hexane. Hexane was removed after the lecithin was well mixed with the oil. The phospholipids in this artificial oil were quantified with the same procedure used for quantification of the phospholipids in the AEP and EAEP cream and then compared to the theoretical phospholipid content in the bulk lecithin. The recovery yield was calculated as follows: recovery (%) = [phospholipid content in the oil (%) × soybean oil (g) × 100]/[phospholipid content in soy lecithin (%) × soy lecithin added (g)].

Enzyme Inactivation. To inactivate phospholipase D, soy flour was autoclaved with live steam for 15 min at 121 °C and 16.5 psi prior to aqueous extraction.

Oil Extraction Yield and Free Oil Yield Calculation. The oil content in each fraction was determined using the acid hydrolysis Mojonnier method (AOAC method 922.06). The oil extraction yield was expressed as the difference between the residual oil present in the insoluble fraction and the initial amount of oil present in the starting material: oil extraction yield (%) = 100[1 − [(oil (g) in insoluble fraction, db)/(oil (g) in starting material, db)]].

The demulsification of the cream fraction (cream + free oil (if any)) was performed with 2.5% P6L at 25 °C for 20 min as previously described by Jung et al. (*11*). The free oil yield (%) after demulsification was calculated as follows: free oil yield = [free oil (g) + hexane-washed free oil (g)]/[cream (g) × oil content (%) in (cream + free oil fraction)].

Statistical Analysis. Oil extraction yield, quantity of cream and crude oil, phospholipid content in crude oil and in concentrated oil, phosphatidic acid in crude oil, and free oil yield after cream demulsification were analyzed by using analysis of variance (ANOVA) and Tukey's test to detect significant differences among different treatments using a general linear modeling procedure from SAS (version 9.1, SAS Institute, Inc., Cary, NC). The level of significance was set at α = 0.05. Each of the six treatments, which were from various combinations of starting materials and aqueous extraction methods, was repeated twice. Each crude oil was split into half so that two concentrated oil replicates were obtained. Duplicate NMR samples were prepared from each concentrated oil, and each NMR sample was analyzed once.

RESULTS AND DISCUSSION

^{31}P NMR Phospholipid Quantification. The use of the chloroform−methanol−aqueous−Cs-EDTA NMR reagent led to good resolution of the NMR spectra with adequate separation of most of the phospholipid classes as illustrated in **Figure 2**. This spectrum was obtained from soy flakes and represents typical results obtained from various substrates. None of the samples we prepared contained LPA, but some of the commercial samples did. The chemical shift of LPE, located upfield of PG, was observed in a small quantity (lower than 2% in moles) in some commercial samples, but was not detected in soy flakes because the amount of LPE might have been below the ^{31}P NMR detection limit. When LPE appeared in a sample spectrum, it was integrated into the signal area of PG. As shown previously, the effect of pH on the chemical shifts of phospholipids varied

Table 1. Phospholipid Composition of Soy Lecithin and Artificial Oil after Concentration,[a] ^{31}P NMR Chemical Shifts of Each Phospholipid, and Their Molecular Weight Used in Quantification

	phospholipid composition (mol %)									
	PC	PI	PE	PA	LPC	PS	APE	DPG	PG	LPA[b]
lecithin, bulk	35.83a	17.47a	32.77a	5.50a	1.64a	0.80a	2.24a	0.81a	2.71a	0.21a
artificial oil	37.59b	16.67a	32.58a	5.11a	1.74a	0.62a	2.52a	0.43b	2.74a	0.00b
chemical shift δ[c]	−0.78	−0.40	−0.03	3.31	−0.25	−0.21	0.10	0.21	0.45	4.10
mol wt (g/mol)	770	835	725	685	515	797	990	683	758	430

[a] Means in the same column with different letters are significantly different ($p < 0.05$). [b] LPA was detected only in commercial soy lecithin and soy PC. [c] The chemical shifts were relative to the internal standard TPP (δ −17.8).

with the particular NMR reagents used (*24*). Because the prolonged exposure to alkaline pH (at pH 10.6) might cause basic hydrolysis and thus produce lyso-phospholipids (*25*), and acidic conditions led to broader NMR signals and increased variability (*20*), Cs-EDTA with pH 8.5 was used in our quantifications. The chemical shift of phospholipids relative to the internal standard TPP (δ −17.8) and the molecular weights used to calculate the phospholipid content are summarized in **Table 1**.

The phospholipids detection limit of ^{31}P NMR is relatively high, 1−5 mg/mL, compared to other analytical methods such as TLC and HPLC (*15*). The phospholipid content in some AEP and EAEP creams was particularly low, and therefore concentration of phospholipids was required before ^{31}P NMR analysis. Concentrating phospholipids was achieved on the basis of the differences in solubility of phospholipids and neutral lipids in organic solvents (*22*). To determine phospholipid recovery with our procedure, artificial oil to which some lecithin was added was analyzed after concentration (**Table 1**). The phospholipid recovery was found to be 78.87 ± 0.24%, which might be explained by the loss of some phospholipids in the hexane phase during the purification (**Figure 1**). Overall, all phospholipids were well recovered except DPG and LPA, for which a significant amount was lost in the concentrating process. On the basis of their chemical structures, it is more likely that DPG, being a little more hydrophobic than other phospholipids, might have stayed in the hexane phases, whereas LPA, with a free hydroxy group as well as a phosphate group, was likely lost in the water phase of the subsequent chloroform−methanol−K-EDTA washes due to its hydrophilic nature. DPG and LPA are minor components (< 2%) in soybean phospholipids, and optimizing the recovery of all phospholipid classes was not our focus; therefore, no further attempt was given to recover them. It was assumed that similar recovery yield would be obtained for each individual phospholipid. With our method, the phospholipid contents in concentrated oil from various creams were amplified by 50−103 times that in crude oil (**Table 2**).

Effect of Starting Material and Aqueous Extraction Method on Oil Extraction Yield and Cream Emulsion Stability. Oil extraction yields obtained after AEP and EAEP of flour, flakes, extruded flakes, and autoclaved flour ranged from 51 to 95%, a higher yield indicating a better oil extractability (**Table 2**). The highest oil extraction yield was achieved by EAEP of extruded flakes, followed by EAEP flour, and then AEP flour. Thermal treatment (autoclaving) of the flour before AEP significantly reduced the oil extraction yield, from 78 to 51%. This change in the oil extraction yield could be due to the formation of protein aggregates that trapped the released oil, as observed for extruded flakes (*9*). The free oil yield after enzymatic demulsification of the cream and free oil fraction (if any) ranged from 6 to 78%, illustrating wide variability in the stability of the creams. The cream of EAEP extruded flakes was less stable toward demulsification, with a free oil yield of 78%, whereas the creams from AEP and EAEP of flour, AEP of autoclaved flour, and EAEP of flakes were more stable, with free oil yields of <20%. Thermal pretreatment of the flour significantly decreased cream stability, with free oil yield increasing from 7 to 15%, and so did the extrusion pretreatment of flakes before EAEP (78 vs 16% of EAEP flakes). Such an impact of the extrusion pretreatment on cream stability was, however, not observed for AEP (54 vs 49% for flakes and extruded flakes, respectively). These results confirmed that the pretreatment of starting materials and the presence of a protease during aqueous extractions affected the cream stability.

The difference between the amount of cream (g) and crude oil (g) is a rough estimate of water incorporated in the cream emulsion network because oil and water are the major constituents of the cream. The water contents in the cream recovered from EAEP flour and AEP flour were approximately 47 and 70%, respectively, 37% from AEP autoclaved flour, 39% from both AEP and EAEP flakes, and 27 and 22% from AEP and EAEP extruded flakes, respectively. The greater capacity of the cream to hold water was found to generally correspond to a low free oil yield, except for the cream from AEP flakes. When the cream (AEP and EAEP flour) held >40% water, the free oil yield was below 10%; when the cream (AEP autoclaved flour and EAEP flakes) contained 30−40% water, its free oil yield was around 15%; when the cream (AEP and EAEP extruded flakes) had <20% of water, the free oil yield could be as high as 78%. However, AEP flakes gave free oil yield (54%) 6 times as high as EAEP flakes did, even though they held the same amount of water in the cream. As suggested by Wu et al. and Kuehler et al. (*26*, *27*), peptide sizes affect emulsion stability. Seemingly, the proteolytic enzymatic breakdown of soy proteins during EAEP of flakes strengthened the interactions between hydrolyzed proteins and phospholipids, which further stabilized the emulsions. Our results also suggest some correlations between the phospholipid content of the cream and free oil yield with higher phospholipid content resulting in a more stable emulsion. Indeed, the phospholipid contents in the creams recovered after AEP of flour and EAEP of extruded flakes were among the highest (0.75%) and lowest (0.11%) values, respectively, whereas their free oil yields were 78 and 7%, respectively (**Table 2**).

Effect of Starting Material and Aqueous Extraction Method on Cream Phospholipid Compositions. Figures 3−6 display the phospholipid compositions of flour, flakes, extruded flakes, and various creams recovered after AEP and EAEP extraction. The major phospholipids in the starting materials were PC (43−50%), PE (21−24%), PI (16−17%), and PA (3−10%) (**Figure 3**). The values of PC, PE, and PI were slightly lower than the ones reported in the literature (PC, 55%; PI, 17%; and PE, 28%; the weight ratio used by Wang et al. (*28*) is converted to a molar ratio by using the MW in **Table 1**), which can be explained by the fact that minor phospholipids were not included in their TLC quantification. Extruded flakes contained a significantly higher amount of PA (10 vs 4 and 3% for flour and flakes, respectively) and lower amounts of PC (43 vs 50 and 48% for flour and flakes, respectively) and PE (21 vs 24 and 23% for flour and flakes, respectively) (**Figure 3**), which

Table 2. Oil Extraction Yield, Quantity of Cream and Crude Oil, Phospholipid Content in Crude Oil and in Concentrated Oil, Phosphatidic Acid in Crude oil, and Free Oil Yield[a] after Cream Demulsification as a Function of Starting Material and Aqueous Extraction Procedure[b]

starting material	aqueous extraction method	oil extraction yield (%)	cream quantity (g)	crude oil quantity (g)	phospholipid content in crude oil[e] (wt %)	phospholipid content in concentrated oil[f] (wt %)	phosphatidic acid in crude oil[g] (mol %)	free oil yield (%)
flour	AEP	77.56 ± 8.85bc	67.47 ± 4.40e	19.55 ± 2.76c	0.75 ± 0.05a	36.7 ± 30.30a	13.67 ± 0.59ab	7.38 ± 1.85d
	EAEP[d]	83.82 ± 0.28b	47.03 ± 0.54b	24.71 ± 1.09bc	0.32 ± 0.03b	19.18 ± 2.15b	21.79 ± 0.15a	6.07 ± 0.66d
autoclaved flour	AEP	50.92 ± 0.85a	40.68 ± 0.68ab	25.69 ± 0.72bc	0.23 ± 0.05bc	16.17 ± 2.28b	7.53 ± 0.87c	15.44 ± 1.41c
flakes	AEP[c]	56.52 ± 5.29a	25.98 ± 1.07ad	16.08 ± 3.59c	0.14 ± 0.09bc	14.48 ± 1.17b	15.76 ± 4.67ab	53.57 ± 15.70ab
	EAEP[d]	59.95 ± 4.81ac	22.87 ± 0.16d	14.21 ± 1.98c	0.20 ± 0.00bc	11.39 ± 0.42bc	19.87 ± 2.54a	16.38 ± 3.51c
extruded flakes	AEP[c]	64.83 ± 3.52ac	44.80 ± 0.05bc	32.53 ± 1.71ab	0.09 ± 0.01c	6.25 ± 0.49d	6.10 ± 0.27c	48.90 ± 0.76b
	EAEP[d]	95.47 ± 0.32b	49.33 ± 1.14bc	37.93 ± 5.65a	0.11 ± 0.01c	8.99 ± 1.44c	8.65 ± 1.52bc	77.91 ± 8.56a

[a] The free oil yield was determined after demulsification, which was performed with 2.5% P6L at 25 °C for 10 min. [b] Means in the same column with different letters are significantly different ($p < 0.05$). [c] The oil extraction yield and free oil yield of AEP flakes and AEP extruded flakes were from Jung (*23*). [d] The oil extraction yield and free oil yield of EAEP flour, EAEP flakes, and EAEP extruded flakes were from Jung et al. (*22*). [e] The phospholipid content in crude oil is the weight percentage of total phospholipids in the crude oil extracted from the cream. [f] The phospholipid content in concentrated oil is the weight percentage of total phospholipids in the concentrated oil obtained after solvent fractionation with 87% ethanol and hexane. [g] The phosphatidic acid in crude oil is the molar percentage of phosphatidic acid in the phospholipids of crude oil extracted from the cream.

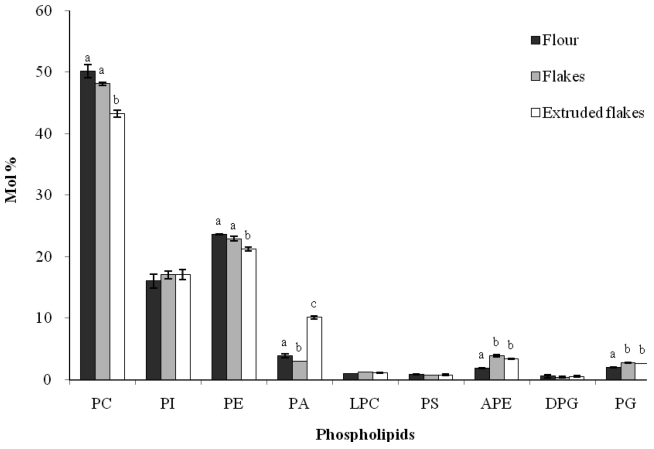

Figure 3. Phospholipid composition of the starting materials. The letters within each phospholipid category denote significant difference ($p < 0.05$). Phospholipids without statistical letters are not significantly different at the $p < 0.05$ level within that category.

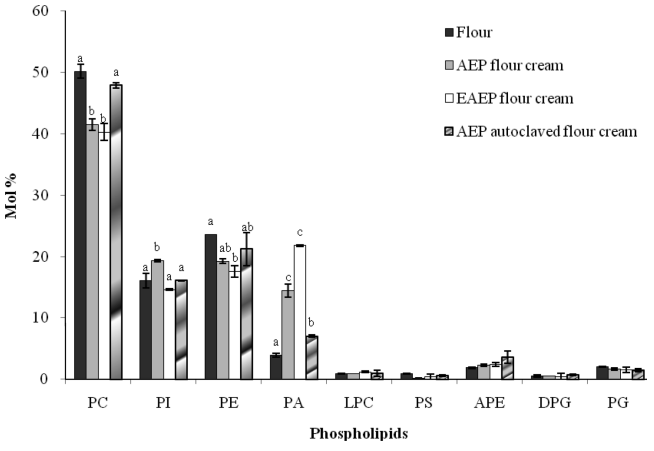

Figure 4. Phospholipid composition of flour, AEP and EAEP flour cream, and AEP autoclaved flour cream. The letters within each phospholipid category denote significant difference ($p < 0.05$). Phospholipids without statistical letters are not significantly different at the $p < 0.05$ level within that category.

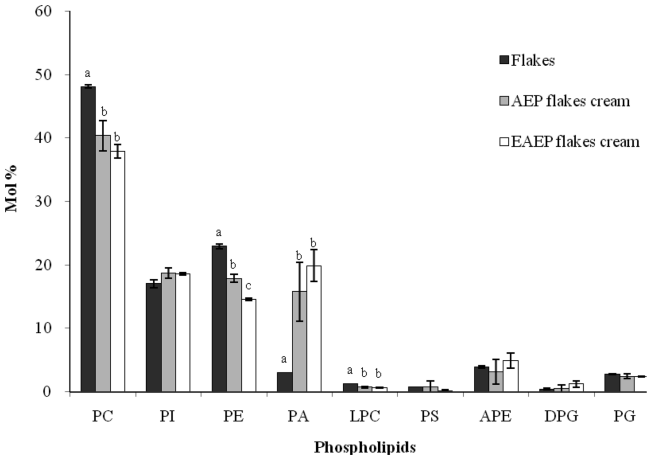

Figure 5. Phospholipid composition of flakes and AEP and EAEP flakes and cream. The letters within each phospholipid category denote significant difference ($p < 0.05$). Phospholipids without statistical letters are not significantly different at the $p < 0.05$ level within that category.

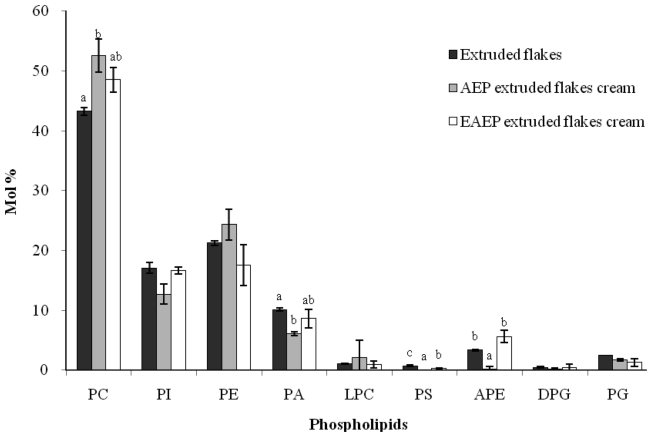

Figure 6. Phospholipid composition of extruded flakes, AEP and EAEP extruded flakes, cream. The letters within each phospholipid category denote significant difference ($p < 0.05$). Phospholipids without statistical letters are not significantly different at the $p < 0.05$ level within that category.

suggested that the phospholipid profile of extruded flakes has been modified. To obtain satisfactory extrusion, the moisture content of the flakes was increased to 14% prior to processing. It has been previously reported that phospholipase D activity is high at 14%

Table 3. Oil Mass Balances among Cream, Skim, and Insolubles Fractions of AEP Flour and AEP Autoclaved Flour and Phospholipid Distribution in Each Fraction of AEP Flour

	AEP flour		AEP autoclaved flour
fraction	phospholipid content[a] (wt %)	oil content[b] (wt %)	oil content[b] (wt %)
cream	0.30 ± 0.01	36.51 ± 0.86a	35.98 ± 2.59a
skim	1.97 ± 0.15	27.68 ± 0.39a	13.27 ± 4.42b
insolubles	1.80 ± 0.37	28.71 ± 2.40a	49.08 ± 0.85b
total	4.07 ± 0.23	92.89 ± 1.93a	98.33 ± 0.98b

[a] Phospholipid content is the weight percentage of phospholipids in the total crude oil, i.e., the sum of crude oil in cream, skim, and insolubles. [b] Means in the same row of oil content category with different letters are significantly different ($p < 0.05$).

moisture (*29*), and therefore the enzymatic hydrolysis of the PC and PE to form PA likely occurred during this step. Such moistening treatment was not applied to the flour and flakes, which had moisture contents of 7 and 10%, respectively.

After aqueous extraction, a significant increase of PA and decreases of PC and PE were observed in the flour and flake creams after both AEP and EAEP extractions (**Figures 4** and **5**). There was no statistical difference in PA content between AEP and EAEP creams. On the contrary, the amount of PA in AEP and EAEP creams of extruded flakes was statistically equal to or less than that in the starting material (**Figure 6**). The difference between the phospholipid profiles of the starting material and cream from flour and flakes, on one side, and extruded flakes, on the other, was likely due to the phospholipase D activity. Phospholipase D remained active in flour and flakes and thus hydrolyzed PC and PE during aqueous extraction. The extrusion at 100 °C might have inactivated the enzyme and, therefore, the PC and PE in the creams of extruded flakes did not decrease.

To verify whether phospholipase D catalyzed the phospholipids hydrolysis, the flour was autoclaved prior to aqueous extraction. The cream obtained from AEP autoclaved flour was found to contain significantly less PA and more PC than the cream recovered from AEP of untreated flour (**Figure 4**), which supported that phospholipase D was involved in the differing levels of PA content in the creams. The cream from AEP autoclaved flour also contained less phospholipids than the one recovered from AEP flour, 0.23 vs 0.75%, respectively (**Table 2**).

For minor phospholipids, significant differences were found in APE and PG content of the starting materials (**Figure 3**), LPC content of the flakes and creams from AEP and EAEP flakes (**Figure 5**), and PS and APE content of the extruded flakes and creams from AEP and EAEP extruded flakes (**Figure 6**). The content of minor phospholipid for some treatments was very low and below NMR detected limits, thus influencing the accuracy of NMR area integration results. From our results, it appears that overall minor phospholipids were not greatly affected by either aqueous extraction or preprocessing. The result obtained from EAEP flour cream was different from those reported earlier by Chabrand and Glatz, who reported the presence of PC, LPC, PE, and LPA (*30*). Knowing that the presence of phospholipase D might affect phospholipid profiles, these differences could be attributed to the disparities in starting materials and changes in the extraction procedures applied.

Comparing the PA content of flour creams (14−22%) to extruded flakes creams (6−9%), we found that high PA content corresponded to low free oil yield, regardless of aqueous extraction method (**Table 2**). The same trend was observed in EAEP extruded flakes versus EAEP flakes and in AEP flour versus AEP autoclaved flour. This increase in emulsion stability with PA

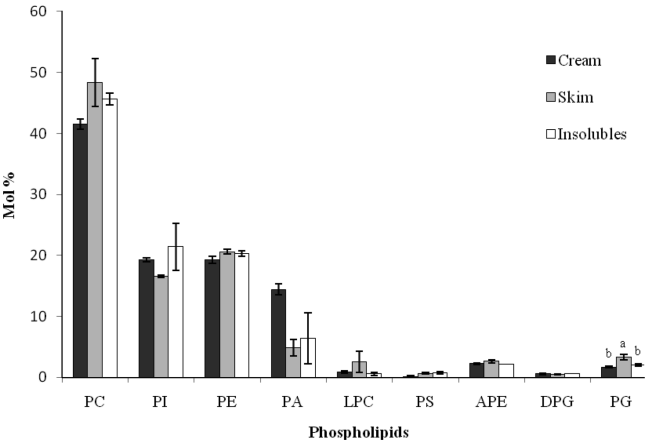

Figure 7. Phospholipid composition of the cream, skim, and insoluble fractions obtained from AEP flour. The letters within each phospholipid category denote significant difference ($p < 0.05$). Phospholipids without statistical letters are not significantly different at the $p < 0.05$ level within that category.

content could be attributed to PA being the most important fraction of the anionic phospholipids in stabilizing an emulsion (*19*). The headgroup identity of the phospholipids affected the surface adsorption of protein (*31*), the interaction of which with phospholipids was found to greatly influence the emulsion stability (*32*). Phospholipids containing unprotected charges, such as PA, were found to absorb more strongly to proteins compared to phospholipids with no net charge such as PE or shielded charges, such as PC (*16*). In addition, with the presence of negatively charged anionic molecules such as PA and PI, the lamellar liquid crystalline layers formed by structural phospholipids can incorporate large amounts of water. The ability of the lamellar layers to swell with water and thus to stabilize the emulsions increased with the amount of such negatively charged lipids in the emulsion mixture (*17*).

Distribution of Phospholipids in Cream, Skim, and Insolubles Fractions of AEP Flour. The total of phospholipid content in each fraction (i.e., cream, skim, and insolubles) of AEP flour was 4.1% (**Table 3**), which was comparable to the reported 3.7% of phospholipids in the soybean crude oil extracted with organic solvent (*28*). This confirmed the efficiency of our procedure for analyzing limited quantities of phospholipids extracted from emulsions. Recoveries of phospholipids in the skim and insoluble fractions were 51 and 42%, respectively, of the total phospholipids, whereas the cream fraction, having nearly 40% of the total oil, contained only 7% of the total phospholipids. A high proportion of phospholipids found in the insoluble fraction was expected because the processing of flour does not involve a massive rupture of the cotyledon cells (*9*) and thus the phospholipids reside with the intact cells. Because the skim is a protein-rich fraction and because of the strong interaction between proteins and phospholipids (*33*), the skim contained about half of the total phospholipids. The phospholipid profiles of cream, skim, and insolubles were not statistically different except for PG, which has a higher concentration in the skim than in the other fractions (**Figure 7**). The PA contents in the skim and insolubles were marginally lower than that in the cream at the $p = 0.1$ significance level. Therefore, there was no preferential partitioning of any individual phospholipid among the three fractions.

In conclusion, the cream emulsion stability was associated not only with the extent of cotyledon disruption by mechanical means and the enzymatic action but also with the PA content and the quantity of phospholipids present. Phospholipase D activity determines the production of PA and, therefore, is one of the controlling

factors of cream stability. Possibly, inhibition of the initial enzyme activity by blanching the beans before flaking and extrusion to minimize the production of PA may further decrease the stability of the EAEP cream emulsion recovered from extruded flakes.

ABBREVIATIONS USED

AEP, aqueous extraction processing; APE, *N*-acyl phosphatidylethanolamine; DPG, diphosphatidylglycerol; EAEP, enzyme-assisted aqueous extraction processing; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

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