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Flaking as a Pretreatment for Enzyme-Assisted Aqueous Extraction Processing of Soybeans

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Abstract Soybean moisture content (7.2–12.8%) and conditioning temperature (51–79 °C) during flaking were evaluated to determine their effects on oil and protein extraction and oil distribution among fractions produced in enzyme-assisted aqueous extraction processing (EAEP). Extractions were performed by using two-stage counter-current EAEP at a 1:6 solids-to-liquid ratio with 0.5% protease (wt/g extruded flakes) at pH 9.0 and 50 °C for 1 h. Oil extraction improved when using soybeans with moisture contents ranging from 8.0 to 12.0% for flaking but was not affected by conditioning temperature. Oil extraction was reduced when moving away from 10% moisture with the lowest values at 7.2 and 12.8% moisture. Free oil extraction increased as soybean moisture content increased from 7.2 to 12.8% although total oil extraction was reduced at 12.8% moisture. Higher (79 °C) and lower conditioning temperatures (51 °C) improved free oil extraction and reduced cream emulsion formation. Skim oil content was not significantly affected by soybean moisture content and the conditioning temperature, although an undesirable high oil content in the skim was observed at 8% moisture and at 55 °C. The cream with a high oil yield was easily demulsified compared with cream containing a low oil yield (95 vs. 76.5% de-emulsification). Due to differences in cream stability, similar oil recoveries (78–80%) were obtained for treatments yielding creams with either low or high oil yields. Mean protein extraction of 95% was achieved for all treatments and was not significantly affected by soybean moisture content at flaking or conditioning temperature.

Keywords Enzyme-assisted aqueous extraction · Oil extraction · Protein extraction · Flaking · Soybeans · Emulsion stability

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Introduction

The soybean is the most widely produced oilseed on a worldwide basis. Production of soybeans is expected to continue to meet growing demand for soybean oil for human consumption and biodiesel production, and for soybean meal for animal feeding [1]. Although the primary goal of soybean processing is to produce animal feed protein, 17–20% of the soybean is edible oil [2]. Most soybeans (>97%) are extracted by using direct solvent extraction with the petroleum distillate hexane, achieving approximately 95% oil extraction [3]. The growing importance of vegetable proteins for use as functional food ingredients and biobased products (adhesives and plastics) along with increasingly restrictive environmental regulations and health concerns regarding use of hexane have revived interest in developing simultaneous aqueous extraction of oil and protein from many oil-bearing seeds [4–12]. Aqueous extraction processing (AEP) is an environmentally clean technology, enabling simultaneous recovery of oil and protein but the process has challenges that must be solved before industry will adopt these process technologies. The main challenges to AEP are low oil

extraction, formation of difficult-to-break emulsions (cream) that impede free oil recovery, risk of microbial growth due to many wet processing steps, and absence of high-value uses for the resulting protein- and sugar-rich effluent (skim) [13, 14].

Complete rupture of cell walls is critical to improving oil extraction in AEP so that oil can be washed from cells and recovered either as an emulsified cream, or even more preferable, as free oil [3]. Low oil extraction efficiency ($\sim$65%) [15] when using soy flour increased to 97% [16] by adding flaking, extrusion and enzyme-catalyzed proteolysis treatments (known as enzyme-assisted aqueous extraction processing, EAEP). Since higher extraction yields have been associated with low solids-to-liquid ratios (usually 1:10 using single-stage extraction) [16–18], reducing water usage without loss in extraction efficiency is another important challenge in aqueous extraction. The use of two-stage countercurrent EAEP reduced the amount of water used for extraction by 40–50%, while achieving slightly greater oil and protein extraction yields than standard single-stage EAEP [19]. In addition to reducing costs of concentrating the components present in the oil-lean skim fraction by reducing water use, the option of heat inactivation or absence of heat inactivating the enzyme in the skim to be recycled to the first extraction stage allows recovery of proteins with very different degrees of hydrolysis and functionalities. Oil and protein extraction yields of 95 and 89% and 98 and 92%, respectively, were obtained when using an enzyme in both extractions and when the skim containing inactivated enzyme was recycled to first-stage extraction.

Two-stage countercurrent EAEP was scaled up by Moura et al. [20] in order to identify potential problems that might occur on an industrial commercial scale. Previous laboratory-scale experiments were performed using two-stage countercurrent EAEP with 0.08 kg of extruded soybean flakes [19] while 2 kg of extruded soybean flakes were used in scale-up trials [20]. Oil and protein extraction yields of 99 and 96%, respectively, were achieved in the scale-up trials of two-stage countercurrent EAEP. These values were similar to those obtained at laboratory scale, but higher residual oil content in the skim fraction resulted in larger scale reducing free oil recovery. Modifications in extraction parameters (pH 8.0 for 15 min and pH 9.0 for 1 h) improved oil distribution among the fractions, increasing oil yield in the cream fraction from 76 to 86% while reducing oil yield in the skim fraction from 23 to 12%.

The two remaining challenges to adopting two-stage countercurrent EAEP are: (1) producing a skim fraction with a still lower oil content, because methods to recover oil from skim are inadequate; and (2) efficiently and completely de-emulsifying the highly stable cream fraction. In terms of oil extractability (oil present in the cream, skim, and free oil), two-stage countercurrent EAEP [19, 20] and standard EAEP processing [16] (96–99%) are as efficient as commercial hexane oil extraction (95.0–97.5%) [3]; however, oil recovery is reduced by the oil contained in the skim fraction and the difficulty in de-emulsifying the cream fraction to obtain free oil. Our past work has shown that the cream fraction generated by using single-stage EAEP could be completely de-emulsified by employing enzyme and pH treatments, thereby achieving 82% total oil recovery (14% of the remained in the skim fraction) [19]. In order to assess oil recovery in two-stage countercurrent EAEP (free oil obtained from extraction + free oil from cream de-emulsification), experiments to reduce cream stability to enable efficient de-emulsification are necessary.

Although moisture and conditioning temperature for flaking soybeans are important in traditional crushing plants, these parameters have not been evaluated for oil extraction and oil distribution in EAEP. We determined how moving away from the normal moisture and temperature values used in traditional soybean crushing and past EAEP research affected EAEP performance. In the present work, we evaluated the effects of different soybean moisture contents and conditioning temperatures before flaking soybeans on: (1) oil and protein extraction yields; (2) oil distribution among the fractions produced in two-stage countercurrent EAEP; and (3) resistance to chemical and/or enzymatic de-emulsification of cream. In order to identify the best combination of soybean moisture and conditioning temperature, a central composite rotatable design (2^2), with three center points and four axial points, was used.

Materials and Methods

Full-Fat Soybean Flakes

Full-fat soybean flakes were prepared from variety 92M91-N201 soybeans (Pioneer a DuPont Company, Johnston, IA, USA) harvested in 2007. The soybeans were cracked into 4–6 pieces by using a corrugated roller mill (model 10X12SGL, Ferrell-Ross, Oklahoma City, OK, USA) and hulls were removed from the meats by aspirating with a multi-aspirator (Kice Industries, Wichita, KS, USA). Soybean meats (6.6–9.3% moisture) were adjusted to achieve the range of 7.2–12.8% moisture contents (Table 1) by drying the meats in an oven at 50 °C or spraying water onto the meats while mixing in a Gilson mixer (model 59016A, St. Joseph, MO, USA) and tempering for 24 h in a cold room. A Grain Analysis Computer GAC2100 (Dickey-john Corporation, Auburn, IL, USA) was used to estimate moisture content.

After adjusting the moisture content, the meats were conditioned at temperatures ranging from 51 to 79 °C (Table 1) by using a triple-deck seed conditioner (French

Table 1 Variables and levels evaluated in the experimental design to optimize oil and protein extraction from soybeans

Treatment	Coded levels		Uncoded levels	
	Moisture (%) (X_1)	Temperature (°C) (X_2)	Moisture (%)	Temperature (°C)
1	+1	+1	12.0	75.0
2	+1	–1	12.0	55.0
3	–1	+1	8.0	75.0
4	–1	–1	8.0	55.0
5	0	0	10.0	65.0
6	0	0	10.0	65.0
7	0	0	10.0	65.0
8	+1.41	0	12.8	65.0
9	–1.41	0	7.2	65.0
10	0	+1.41	10.0	79.0
11	0	–1.41	10.0	51.0

Complete 2^2 factorial design parameters, with two independent variables in two levels, three repetitions in the central point and four in the axial points

Oil Mill Machinery Co., Piqua, OH, USA). The conditioned meats were flaked to approximately 0.25 mm thickness by using a smooth-surface roller mill (Roskamp Mfg, Inc., Waterloo, IA, USA). About 20 kg of soybeans were processed for each treatment.

Extrusion of Soybean Flakes

The moisture content of the flakes was increased to 15% just prior to extruding by spraying water onto the flakes while mixing in the Gilson mixer. A twin-screw extruder (ZSE 27-mm diameter, American Leistritz Extruders, Somerville, NJ, USA) was used to extrude the flakes. High-shear geometry screws were used in co-rotational orientation. The extruder barrel (1,080 mm length) was composed of 10 heating blocks that were set to achieve the temperature profile 30–70–100–100–100–100–100–100–100–100 °C. The extruder was manually fed to achieve 10.5 kg/h of extruded flakes output rate. Screw speed of 90 rpm was used based on our previous work [20] and the extruded material was not collected in water because doing so is unnecessary. The collets were cooled to room temperature, placed in polyethylene bags and stored in a cold room at 4 °C until extracted. The extruded flakes contained 21.5 ± 1.2% oil (as is), 36.6 ± 0.3% protein (as is) and 11.9 ± 0.7% moisture.

Enzyme-Assisted Aqueous Extraction Processing

A bacterial alkaline endoprotease (Protex 6L) obtained from Genencor International (Rochester, NY, USA), a division of Danisco, was used to assist extraction. Protex 6L has its highest activity at pH 7.0–10.0 and a 30–70 °C temperature. The 0.5% enzyme dosage in the extraction was based on the weight of extruded flakes (as is) and was selected based on our previous work [16].

Two-stage countercurrent EAEP was performed over 4 days for each treatment of the experimental design. In actual practice two-stage countercurrent EAEP would be a continuous process; 4 days were required in laboratory simulation only due to materials handing issues and complexity. For each treatment, extruded flakes were subjected to two-stage extraction and the liquid fraction (skim + cream + free oil) obtained in the second extraction stage of one trial was recycled to the first extraction stage of the next trial (incoming fresh flakes) on the following day (Fig. 1). On the first day of extraction, the first extraction stage of EAEP was performed with 2 kg of extruded flakes at 1:6 solids-to-liquid ratio. The slurry was adjusted to pH 9.0 before adding 0.5% Protex 6L (wt/extruded flakes) and stirred for 1 h at 120 rpm and 50 °C. The reaction was carried out in a 20-L jacketed glass reactor. The slurry obtained in the first extraction stage was centrifuged at 3,000×g to remove the insoluble fraction. After removing the insoluble fraction, the liquid phase (skim, cream and free oil) was placed in a separatory funnel (5-L jacketed reactor) and allowed to settle overnight at 4 °C. After settling, the liquid phase was separated into three fractions (skim, cream and free oil).

The insoluble fraction obtained in the first extraction stage (1st insolubles) was then subjected to a second extraction stage. Prior to the second stage of extraction, the 1st insoluble fraction was dispersed in water to obtain 1:6 solids-to-liquid ratio and reaction conditions were the same as used in the first extraction stage. The slurry obtained in the second extraction stage was centrifuged to separate the insoluble and liquid fractions. The liquid phase was recycled to the first extraction stage on the next day. The extractions on the second, third and fourth days were performed in the same manner. Since steady-state extraction is achieved from the second extraction trial [20], samples from the third and fourth extraction trials were collected

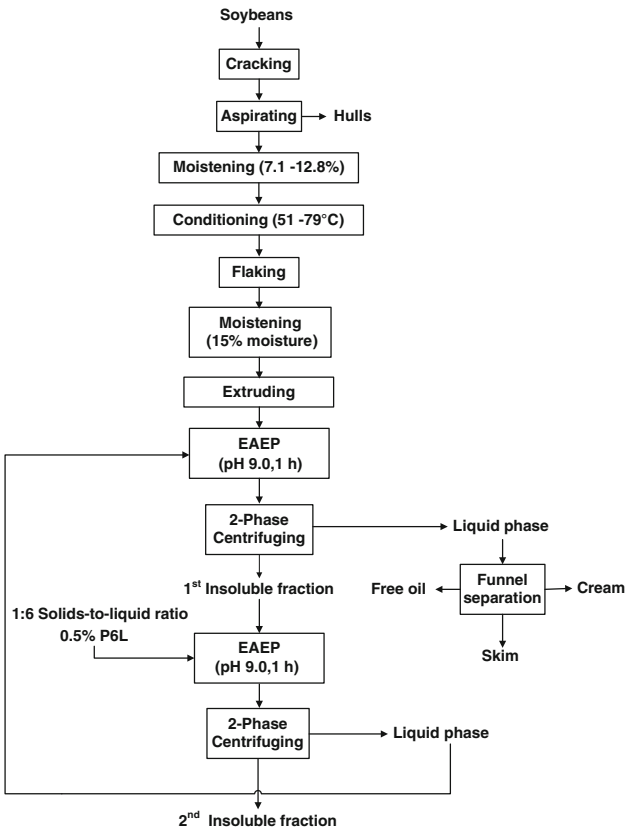

Fig. 1 Process flow diagram for two-stage countercurrent EAEP of flaked and extruded soybeans

and analyzed to determine proximate compositions of the fractions and mass balances for oil, protein and solids.

De-emulsification of Cream

Cream fractions obtained from Treatment 1 (12.0% moisture, 75 °C) and Treatment 5 (10.0% moisture, 65 °C) were subjected to de-emulsification. Twenty-five-g aliquots of the well-mixed cream obtained after funnel separation of free oil, cream and skim fractions were transferred into 100-mL reactors equipped with shaft stirrers in a Tornado IS6 Overhead Stirring System (Radleys Discovery Technologies, Shire Hill, Saffron Walden, UK). For enzymatic de-emulsification, 2.5% (w/w) Protex 6L was added to the cream, at an initial pH of 9.0 ± 0.3, before incubating at 50 °C for 1 and 3 h with constant stirring at 80 rpm. The controls without enzyme were incubated at the same conditions.

For chemical de-emulsification, the pH of the cream was adjusted to 4.5 with 2 N HCl, the temperature was raised to 50 °C and maintained for 2 min. After the de-emulsification treatment, the samples were transferred into 50-mL centrifuge tubes and cooled to 15 °C in an ice bath and then centrifuged at 3,000×*g* for 15 min at 20 °C. Free oil was collected with a Pasteur pipette and any remaining free oil was collected with two hexane washes. Hexane was used only for accurately quantifying free oil; we do not envision using hexane in commercial practice. After the hexane was evaporated, the total free oil yield (%) was determined as shown in Eq. 1.

$$\text{Free oil} = \frac{[\text{free oil(g)} + \text{hexane-washed free oil(g)}]}{[\text{cream(g)} \times \text{oil content}\,(\%)]} \quad (1)$$

De-emulsification was performed in duplicate from creams coming from two independent extractions.

Oil, Protein and Solids Recoveries

Oil, protein and dry matter (solids) contents of the skim, insoluble, and cream fractions as well as the extruded flakes were determined. Oil contents were determined by using the acid hydrolysis Mojonnier method (AOCS method 922.06), protein contents by using the Dumas method and a conversion factor of 6.25 (vario MAXCN Elementar Analysensysteme Gmbh, Hanau, Germany), and total solids by weight after drying samples in a vacuum-oven at 110 °C for 3 h (AACC Method 44–40). Extraction yields were expressed as percentages of each component in each fraction relative to the initial amounts in the extruded flakes. Total oil and protein extraction values consider all oil and protein extracted from the extruded flakes that were present in the liquid fractions (i.e., skim, cream, and free oil). Chemical analyses were performed in duplicate with samples obtained from two different extraction batches.

Experimental Design and Statistical Analysis

In order to identify the best combination of soybean moisture content and conditioning temperature to favor oil and protein extraction, a complete 2^2 factorial design of the central rotational type was established, with three central points and four axial points, based on Response Surface Methodology [21]. Soybean moisture content and conditioning temperature, the independent variables, were evaluated according to the following coded levels: $-\alpha$, -1, 0, $+1$, $+\alpha$. Coded and uncoded levels and their corresponding independent variables are shown in Table 1. Dependent variables (i.e., evaluated responses) were total oil and protein extraction yields, free oil yield, oil yield in the cream, and oil yield in the skim fraction. Data were analyzed by using Statistica version 8.0. The significance of each model was tested by Analysis of Variance (ANOVA).

Table 2 Experimental design for optimizing total oil extraction and oil distribution among EAEP fractions

Treatment	Moisture (%)	Temperature (°C)	Total oil extraction (%)	Free oil yield (%)	Cream oil yield (%)	Skim oil yield (%)	Insolubles oil yield (%)
1	12.0	75.0	98.2	56.6	27.6	14.0	1.8
2	12.0	55.0	97.7	45.6	36.4	15.8	2.3
3	8.0	75.0	96.8	47.0	36.1	13.6	3.2
4	8.0	55.0	97.2	33.9	38.8	24.5	2.8
5	10.0	65.0	96.9	33.2	48.1	15.6	3.1
6	10.0	65.0	97.5	18.0	66.4	13.1	2.5
7	10.0	65.0	98.0	20.9	63.2	13.9	2.0
8	12.8	65.0	94.7	55.0	24.9	14.8	5.3
9	7.2	65.0	94.0	17.4	59.8	16.9	6.0
10	10.0	79.0	97.9	47.9	36.3	13.7	2.1
11	10.0	51.0	96.7	56.7	27.6	12.4	3.3

Results and Discussion

Oil Extraction and Oil Distribution Among Fractions

In industrial soybean crushing plants, cracked and dehulled soybeans are conditioned before flaking by using a vertical-stack cooker to heat the meats to 60–70 °C while maintaining 10.5–11.5% moisture (slight injection of steam is required). This treatment makes the meats plastic in texture which is necessary to produce thin, non-fragile flakes with minimum fines and maximum cell wall rupture that facilitates efficient oil extraction [22]. In Table 2, we present the effects of soybean moisture content and conditioning temperature before flaking soybeans on total oil extraction and oil distribution among the fractions (cream, skim and free oil) for 11 treatments.

Oil extraction was not significantly affected by conditioning temperature (51–79 °C). Higher oil-extraction values (96.7–98.2%) were obtained when using soybeans ranging from 8 to 12% moisture. Oil extraction was reduced when using lower (7.2%) and higher (12.8%) moisture levels (Treatment 8, 12.8% moisture, 65 °C and Treatment 9, 7.2% moisture, 65 °C). High (12.8%) and low (7.2%) moisture contents may alter plasticity of the meats, which adversely affects cell wall rupture during flaking and extrusion, thus reducing oil extraction.

In addition to high oil extraction yield, high free oil yield and low oil yield in the cream and skim fractions are desirable to maximize oil recovery. Higher free oil yields (>50%) and lower oil yields in cream (<30%) were observed in Treatment 1 (12.0% moisture, 75 °C), Treatment 8 (12.8% moisture, 65 °C), and Treatment 11 (10.0% moisture, 51 °C): however, only Treatment 1 (12% moisture, 75 °C) and Treatment 11 (10.0% moisture, 51 °C) had high total oil extraction yields. Oil partitioning in the cream fraction or in the free oil fraction could be related to the effect of soybean moisture content, storage time of whole beans and flakes prior to extrusion, on the activity of phospholipase D. Yao and Yung [23] reported that variations in phospholipid profiles of cream fractions were associated with phospholipase D activity, which produces phosphatidic acid, increasing emulsion stability.

Minimum phospholipase D activity has been observed when soybeans were stored at 12% moisture at 40 °C [24]. In previous work, cracking and flaking soybeans moistened to 12, 14, and 16% increased enzyme activity in flaked soybeans by 1.5–1.8 times compared with whole beans, whereas flaking at 10% moisture did not increase phospholipase activity over whole beans [24]. We found high oil extraction, high free oil yield and low oil yield in the cream fraction in Treatment 1 (12% moisture, 75 °C) and Treatment 11 (10.0% moisture, 51 °C). Treatment 1 corresponds to the moisture content with previously reported minimum phospholipase activity and Treatment 10 corresponds to the moisture content at which flaking does not increase enzyme activity. However, characterizing the phospholipid profile in all fractions is necessary to better understand the oil distribution among the fractions. Although oil content in the skim fraction was not significantly affected by soybean moisture content and temperatures used, an undesirable high oil content was observed at 8% moisture and 55 °C.

Statistical Analysis

Estimated regression models and coefficients of determination for total oil extraction, free oil yield and oil yield in the cream are shown in Table 3. Only parameters significant at $p < 0.05$ were used in the regression models. The coefficients of determination (R^2) for models of total oil extraction, free oil yield and oil yield in the cream were 0.52, 0.72, and 0.79, respectively, indicating the regression

Table 3 Regression models and coefficient of determination (R^2) for total oil extraction, free oil yield and oil yield in the cream

Estimated regression models	R^2
% Estimated total oil = $97.76 - 1.25X_1^2$	0.52
% Estimated free oil yield = $30.15 + 9.40X_1 + 12.71X_2^2$	0.72
% Estimated oil yield in cream = $59.21 - 7.53X_1 - 9.08X_1^2 - 14X_2^2$	0.79

X_1 coded level corresponding to moisture variable, X_2 coded level corresponding to temperature variable

models were able to explain 52, 72, and 79% of the variation between observed values for total oil extraction, free oil yield and oil yield in the cream, respectively. A possible source of variation, not accounted by the regression models, is the phospholipase D activity during storage of beans and flakes before extrusion and extraction. Our material was prepared over about 2 months, which may have impacted phospholipase D activity. Tables 4, shows the analysis of variance of the models. For all cases, the regression was significant ($F_{calculated} > F_{tables}$) and the F-test, for the lack of fit, was not statistically significant ($F_{calculated} < F_{tables}$), indicating that the models do not show lack of fit and thus can be used for predictive goals in the range of the parameters evaluated. Based on the estimated regression models, response surfaces were built to express total oil extraction, free oil yield extraction, and oil yield in the cream (Figs. 2, 3 and 4, respectively). According to the estimated regression model and Fig. 2, oil extraction decreases as soybean moisture content gets further away from the center point (10% moisture), with lower extraction values in the axial points (± 1.41). According to the estimated regression model and Fig. 3, free oil yield increases when the moisture value increases from -1.41 to $+1.41$, for any of the conditioning temperatures tested; however, this increase is sharper when high and low temperature values are used (levels $\pm$ 1.41).

An opposite trend to free oil was observed for oil yield in the cream (Table 3 and Fig. 4). Oil yield in the cream decreased as soybean moisture content rose from -1.41 to $+1.41$ (7.2–12.8%). Lower oil yields in the cream fraction were obtained when the conditioning temperature got further away from the center point, with lowest values at ± 1.41 levels. The best flaking treatment for total oil extraction and favorable oil distribution among the fractions should take into consideration, not only high oil extraction and free oil yield, low oil yield in the cream, and in the skim fraction, but also stability of the cream against de-emulsification. Depending on cream stability, it may be preferable to have a low free oil yield and a high oil yield in the cream fraction, if the cream can be easily and completely de-emulsified to obtain free oil. For this reason we decided to de-emulsify the creams obtained with

Table 4 Analysis of variance of the estimated regression models

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-test
Total oil extraction ($R^2 = 0.52$; $F_{0.95-1,9} = 5.12$; $F_{0.95-7,2} = 19.35$)				
Regression	9.58	1	9.58	**10.08**[a]
Residual	8.56	9	0.95	
Lack of fit	7.93	7	1.13	3.65[b]
Pure error	0.62	2	0.31	
Total	18.14			
Free oil yield ($R^2 = 0.72$; $F_{0.95-2,8} = 4.46$; $F_{0.95-6,2} = 19.33$)				
Regression	1694.19	2	847.09	**10.07**[a]
Residual	672.97	8	84.12	
Lack of fit	542.121	6	90.35	1.38[b]
Pure error	130.85	2	65.42	
Total	2367.16			
Oil yield in cream fraction ($R^2 = 0.79$; $F_{0.95-3,7} = 4.35$; $F_{0.95-5,2} = 19.30$)				
Regression	1743.80	3	581.268	**8.70**[a]
Residual	467.676	7	66.81	
Lack of fit	276.699	5	55.34	0.57[b]
Pure error	190.88	2	95.44	
Total	2211.39			

Values in bold are statistically meaningful ($P < 0.05$)

[a] F-ratio (regression/residual)

[b] F-ratio (lack of fit/pure error)

Treatment 1 (high free oil yield and low oil yield in the cream) and Treatment 5 (center point − low free oil yield and high oil yield in cream).

Cream Composition and Stability Against De-emulsification

After funnel separation, three liquid streams are recovered, the protein- and sugar-rich, oil-lean skim fraction, the oil-rich cream fraction and some free oil floating on the surface. Two strategies can be applied to recover free oil from the cream fraction. One strategy is to keep the free oil with the cream fraction and apply the de-emulsification treatment to this slurry. When working with a small quantity of material (the amount of free oil being negligible compared to the cream fraction), separating the free oil from the cream can be challenging and this solution might be preferred. The presence of free oil with the cream increases the volume of material to be de-emulsified and consequently increases the amount of enzyme for de-emulsification, but the enzyme is recycled to up-stream extraction and, therefore, the cost of increased enzyme quantity is not an issue. Separating the free oil fraction from the cream before de-emulsification requires an additional processing step, but decreases enzyme quantity and also allows

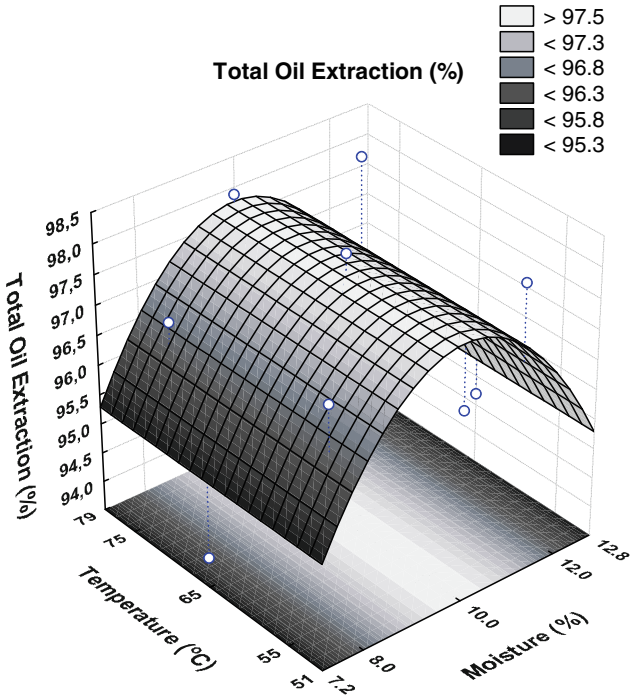

Fig. 2 Effects of soybean moisture content and conditioning temperature for flaking on total oil extraction

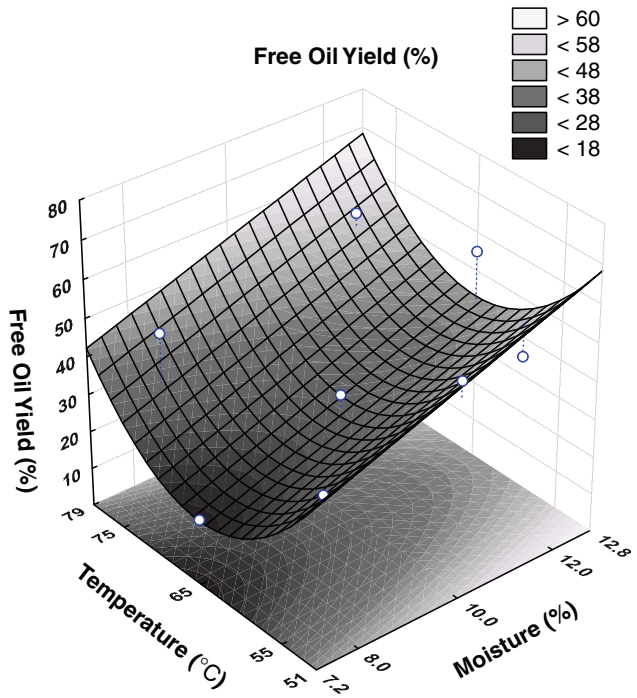

Fig. 3 Effects of soybean moisture content and conditioning temperature for flaking on free oil extraction

determining cream stability towards de-emulsification treatments (no interference with presence of free oil). In order to evaluate the stability of each cream fraction

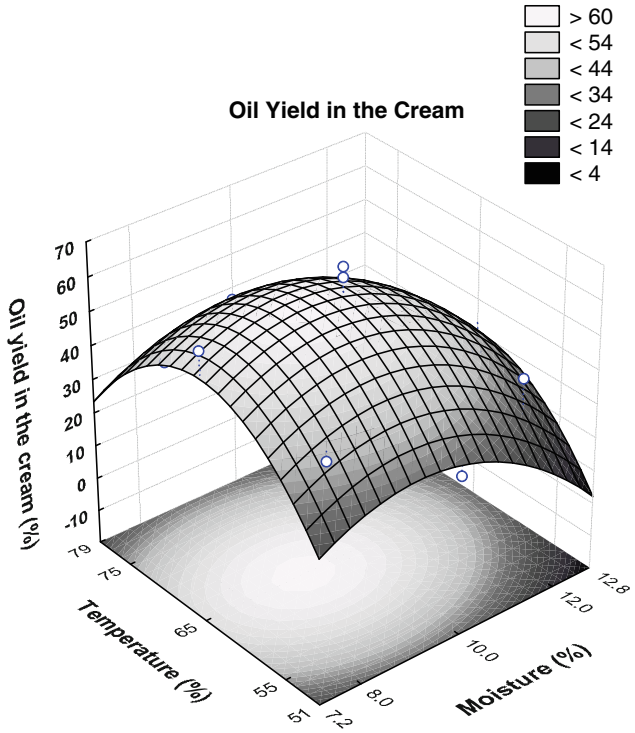

Fig. 4 Effects of soybean moisture content and conditioning temperature for flaking on oil yield in the cream fraction

regardless of the quantity of free oil present, the cream fraction was separated from the free oil obtained directly after extraction and only the cream was submitted to de-emulsification (enzyme treatment and pH adjustment).

The composition of the cream (oil, protein and moisture) was affected by both soybean moisture content at flaking and conditioning temperature (Table 5). The cream with higher oil yield was obtained when conditioning the beans at 10% moisture and 65 °C. Without enzyme addition, 65–69% of the oil present in the cream was recovered after 1 h of low-speed stirring, regardless of treatment conditions. The destabilization of the cream emulsion was attributed to residual protease that was added during the extraction step [25] and/or favored oil droplet coalescence during gentle stirring. Increasing the incubation time to 3 h did not further increase free oil yield recovery. When Protex 6L was added to the cream before incubation, flaking treatment conditions significantly affected oil yield, which increased by 18.5% and 32% after 1-h incubation, for flakes in Treatment 1 (12% moisture, 75 °C temperature) and Treatment 5 (10% moisture and 65 °C), respectively.

Adjusting the pH to the isoelectric point of soy proteins (4–5) increased the free oil yield compared to the control (without pH adjustment). Better free oil yield was obtained with Treatment 5, similar to enzymatic de-emulsification. The chemical treatment, however, was not as effective as the enzymatic de-emulsification, with free oil yield of 70

Table 5 Cream composition and de-emulsification efficiency in function of soybean moisture content and conditioning temperature during flaking

	Treatment 1 (M:12%, *T*:75 °C)	Treatment 5 (M:10%, *T*:65 °C)
Cream composition		
Oil	51.4 ± 0.3 a	31.6 ± 1.5 b
Protein	3.9 ± 0.0 a	4.6 ± 0.0 b
Moisture	40.5 ± 0.3 a	59.7 ± 1.1 b
Enzymatic de-emulsification		
1 h incubation		
Control	64.6 ± 4.5 ab	68.7 ± 3.9 bc
2.5% P6L	76.6 ± 0.3 d	90.9 ± 1.6 f
3 h incubation		
Control	61.7 ± 0.8 a	66.5 ± 0.7 bc
2.5% P6L	76.5 ± 0.7 d	94.8 ± 0.8 f
Chemical de-emulsification		
pH 4.5	70.1 ± 2.0 c	84.2 ± 2.4 e

M moisture of the flakes, *T* conditioning temperature of flakes. Enzyme de-emulsification was performed at 50 °C and chemical de-emulsification for 2 min at 50 °C. Statistical analysis was performed separately on the data obtained for the cream composition and both de-emulsification treatments (pH and enzyme). Values within rows with different letters were significantly different ($P < 0.05$)

and 84% for the pH de-emulsification of the cream recovered from Treatment 1 (12% moisture, 75 °C temperature) and Treatment 5 (10% moisture and 65 °C), respectively, vs. 76 and 95%, for those subjected to 3 h of enzymatic de-emulsification.

We previously reported similar free oil yields after both pH and enzymatic treatments of the [cream + free oil] fraction recovered from a single-stage EAEP. Total de-emulsification was achieved when using 2.5% Protex 6L at 50 °C for 90 min or when adjusting the pH to 4.5 at 50 °C for 15 min [16]. This difference suggests that the creams recovered from the two-stage countercurrent EAEP may have different properties than those obtained with single-stage EAEP. The peptides present at the interface may be different as well as the quantity and type of phospholipids. The lower free oil yield recovery suggests that the proteins were more stabile to pH adjustment, which may be due to formation of peptides that have different solubility profiles. Two-stage EAEP reduced most peptides to <25 kDa molecular weight (data not shown), while single-stage EAEP yielded peptides with molecular weights <54.1 kDa [16]. Due to the higher degree of hydrolysis achieved in two-stage EAEP, protein solubility in the pH 4–5 range is greater.

Conditioning temperature and soybean moisture may have impacted phospholipase D activity and therefore the phospholipid profile in turn significantly impacted the stability of the cream emulsion [23]. Higher free oil yield after de-emulsification was not obtained from the cream having the higher oil content, suggesting that oil content could not be used to predict emulsion stability towards enzymatic and chemical de-emulsification. Although Treatment 1 (12% moisture and 75 °C) gave 24% less cream de-emulsification than Treatment 5 (10% moisture and 65 °C), integrating extraction and cream de-emulsification steps yielded similar oil recoveries (oil recovered as free oil) of 78 and 80%, respectively. Since both treatments yielded similar oil recoveries, the best treatment was selected based on the amount of enzyme used in the de-emulsification step. Treatment 5 produced twice the amount of cream as produced by Treatment 1, which used twice the amount of enzyme for de-emulsification. Since we envision recycling the enzyme from the de-emulsification to extraction, the amount of enzyme used during the de-emulsification should be slightly higher, due to expected reduced enzyme activity after de-emulsification, than the amount of enzyme necessary to perform the extraction. The amount of enzyme used during de-emulsification of creams from Treatment 1 and Treatment 5 are greater than the amount needed for extraction.

Effects of Soybean Moisture Content and Conditioning Temperature on Protein Extraction

Soybean moisture content at flaking and conditioning temperatures did not significantly affect total protein extraction. About 95% of protein in soybeans was extracted in all treatments.

Conclusions

Soybean moisture content and conditioning temperature at flaking affected the total oil extraction as well as the oil distribution among the EAEP fractions. High oil extraction yields were obtained when the soybean moisture content was around 10% and was not affected by the conditioning temperature. Free oil yield increased and cream formation decreased as soybean moisture content increased from 7.2 to 12.8%. This trend became more pronounced at low and high conditioning temperatures. Soybean moisture content and conditioning temperature significantly affected the resistance of the oil-rich cream to de-emulsification. Cream with a high oil yield was more easily de-emulsified than cream with a low oil yield, 95.0 vs 76.5%. Enzyme treatment was more efficient in de-emulsifying the cream than pH adjustment. Using soybeans with 12% moisture content and conditioning at 75 °C was best for oil extraction, oil distribution among the fractions, and enzyme use. Soybean moisture content and conditioning temperature did not

significantly affect protein extraction and all conditions achieved about 95% protein extraction.

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