PARAMETERS AFFECTING ENZYME-ASSISTED AQUEOUS EXTRACTION OF EXTRUDED SUNFLOWER MEAL

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ABSTRACT

Microscopic observation of sunflower meal before and after extraction indicated that extensive cellular disruption was achieved by extrusion, but that unextracted oil remained sequestered as coalesced oil within the void spaces of disrupted cotyledon cells. A full factorial design experiment was defined to develop aqueous extraction processing (AEP) with and without enzymes to improve vegetable oil extraction yields of extruded sunflower meal. This experimental design studied the influence of four parameters, agitation, Liquid/solid (L/S) ratio, and cellulase and protease addition, on extraction yield of lipid and protein. Agitation and addition of cellulases increased oil extraction yield, indicating that emulsification of oil and alteration of the geometry of the confining cellular matrix were important mechanisms for improving yields. Protease and liquid- solid ratio of the extraction mixture did not have significant effects, indicating key differences with previously established soy oil extraction mechanisms. Maximum yields attained for oil and protein extraction were 39% and 90%, respectively, with the aid of a surfactant.

Key words: aqueous extraction, protease, cellulase, oil, sunflower, enzymes

47 **1. Introduction**

48 Pressing, with single screw extruders, is usually the first step of oil production. For seeds
49 with high oil content such as sunflower, extraction yields of 70% to 85% can be achieved
50 (Kartika, Pontalier & Rigal, 2006; Evon, 2008). However, to maximize yields, residual oil in the
51 extruded meal is extracted with an organic solvent, most commonly hexane. An important part of
52 the Green Chemistry (Anastas & Warner, 1998) movement is to develop technologies that are
53 environmentally friendly and reduce the use of petroleum-derived materials. Aqueous extraction
54 processing (AEP) and enzyme-assisted aqueous extraction processing (EAEP) are safe water-
55 based extraction processes that, with the use of enzymes, have succeeded in achieving free oil
56 yields as high as 88% in soybean oil extraction (Moura, Campbell, Mahfuz, Jung, Glatz &
57 Johnson, 2008, Moura & Johnson, 2009).

58 In an immiscible oil-water system, the ability to extract oil is dependent on its mobility
59 within the solid matrix confining the unextracted portion (Campbell & Glatz, 2009). Therefore,
60 one important factor in AEP/EAEP is the geometry of the confining matrix as determined by the
61 nature of the oilseed itself, as well as the mode of comminution used to disrupt cells. In soy,
62 grinding and extruding produced substrates with very different physical geometries from which
63 the oil must escape (Campbell & Glatz, 2009). In the case of extrusion, oil was released from a
64 matrix of insoluble denatured protein, while in flour from flakes; oil was released from partially
65 disrupted cells.

66 Cellulases increase the extraction yield of oil from ground sunflower in EAEP by cellular
67 disruption (Dominguez, Nunez & Lema, 1995; Sineiro, Dominguez, Nunez & Lema, 1998) but
68 could also act by modifying the geometry of cells previously disrupted, thus facilitating oil

transfer out of the remaining matrix. Furthermore, Campbell and Glatz have established that emulsification is a key parameter in the extraction mechanism for EAEP of soybean flour (Campbell & Glatz, 2009). In an aqueous environment, where the extract (oil) is immiscible with the solvent (water), extraction is increased when coalesced oil entrapped within ruptured cells can be emulsified into smaller, more mobile droplets by turbulent forces in the extraction medium.

Another important factor for soy oil extraction is the nature of the oil-water interface. Campbell and Glatz proposed that the mechanism, by which protease increases oil yields in soy flour extraction, is by disruption of a viscoelastic interfacial protein film at the oil-water interface, facilitating emulsification. Badr and Sitohy demonstrated that at pH 5 proteases can also increase the yields of sunflower oil from dehulled chopped seeds, which they attributed to a disruption of lipid-protein complexes (Bair & Snyder, 1980).

The objectives of this work were to identify the conditions to increase the oil recovery yield from extruded meal, using aqueous extraction or enzyme-assisted aqueous extraction instead of the classical hexane extraction procedure.

2. Materials and methods

2.1. Preparation of Extruded Sunflower Meal

Common variety sunflower kernels (with hulls) obtained from Toulgrain, Inc. (Toulouse, France) were extruded in an Omega 20 single screw bench top press-extruder (Eurl Laplace Co., Pau, France), equipped with a heated collar around the die housing. Steady-state exit temperature of the extruded cake was measured to be around 100 °C (+/- 5 °C) with an infrared thermometer.

Expressed oil was collected, weighed, and centrifuged. The resulting precipitate was rinsed three times with cyclohexane, dried, and weighed to determine the fraction of foots in the expressed oil. The resulting cake was cooled and then ground in a Pulverisette 19 (Fritsch Ltd. Idar-Oberstein, Germany) knife mill with a 2 mm outlet screen. Extruded meal was stored at -20 °C until use.

2.2. Extraction

The appropriate quantity of extruded meal was added to 1 L of DI water in a 2 L jacketed reactor with an agitator, maintained at 50 °C with a water bath and at constant pH 6.5 using a 716 DMS Titrino autotitrator (Metrohm Ltd., Herisau, Switzerland) with 1 N NaOH. Samples were collected by siphon into a 500 mL bottle, weighed, and centrifuged (Sigma 6-16k) at 3000g for 15 min at 20 °C. The supernatant was discarded and the remaining residual solid was weighed, freeze dried, and weighed again for moisture determination. Freeze-dried precipitate was ground in a coffee grinder for approximately 30 s and then stored in a dessicator until oil and protein content determination. Yield was calculated as one minus the fraction of total material remaining in the residual fraction. Protein dissolution was defined as the protein extraction yield plus the fraction of dissolved protein entrained in the solid-fraction, estimated by multiplying the liquid fraction protein concentration by the mass of water in the solid fraction. The liquid fraction protein content was determined by mass balance based on the protein content of the residual fraction.

For microscopy experiments, extraction was carried out by placing extruded meal in 500 mL centrifuge bottles with DI water for a solid-liquid ratio of 1:10. Bottles were placed on a stir plate in a water bath maintained at 50 °C, and agitated with a magnetic stir bar at 1000 rpm. Centrifugation (3000g 15 min at 20 °C) resulted in two distinct layers in the centrifuge bottles.

Therefore, samples for microscopy were from the bulk mixture before centrifugation and from each of the two layers after centrifugation.

2.3. Full Factorial Design Experiment

To elucidate the effects of enzyme, solid-liquid ratio, and agitation, a randomized full factorial design experiment was conducted using two continuous two-level parameters: solid-liquid ratio (0.05 and 0.10) and agitation rate (160 and 350 rpm), plus two discrete parameters: with and without protease Protex 7L and with and without cellulase Multifect CX 13L, kindly provided by Genencor (Rochester, NY), both 2% w/w solid, giving a total of 16 possible experimental conditions. Cellulase Multifect CX 13L, with a specific activity of 3,900 CMC/g, exhibits significant activity towards cellulose, hemicelluloses, β -glucans and arabinoxylans. The Protex 7L (also named Multifect Neutral) has an activity of 1600 AU (Azo Unit)/g define by hydrolysis of Azo-casein substrate at pH 7.5 for 5 minutes at 30°C. The active pH ranges of these enzymes overlap in the pH 6 to 7 region, and so pH 6.5 was selected for all of these experiments. Measured responses were oil extraction yield, protein dissolution, and non-lipid material dissolution. Trials for the full factorial design experiment were not replicated, while all other trials reported were made in triplicate. Error estimation for analysis of variance (carried out using JMP 7 software from SAS Institute, Inc. Cary, NC) was based on the assumption of interactions of an order higher than two, being nonsignificant.

2.4. Analytical Methods

Oil was extracted from residual samples four times for 10 min, at 105 °C and 95 bar with cyclohexane using an ASE 200 Accelerated Solvent Extractor (Dionex Corp, Sunnyvale, CA). Extract was transferred from vials to preweighed glass beakers (dried 1 h at 103 °C, cooled to room temperature on the bench top), rinsing twice with cyclohexane. Cyclohexane was

evaporated by placing beakers in a boiling water bath and then drying them for 1 h in a 103 °C oven. Beakers were cooled to room temperature on the bench top, and weighed again to determine mass of oil. Protein content was determined by the Kjeldahl total nitrogen method using a nitrogen to protein conversion factor of 6.25 g protein per g nitrogen. Residual moisture content was determined by loss of mass upon freeze-drying. Moisture gained during sample storage was analyzed simultaneously with oil content determination, by measuring the loss of mass upon drying samples at 103 °C for 24 h. This was used to correct the oil content determination.

2.5. Particle Size Distribution of Extruded Meal

Particle size distribution of extruded meal was determined by sieving. 250 g of extruded meal was placed in a sieve-shaker equipped with four different sieve sizes: 1.25 mm, 0.80 mm, 0.50 mm, and 0.25 mm. Material was fractionated for 15 min at a frequency of 50 s⁻¹, and then weighed from each screen. **As the entire meal was used for the experiments, specific extrusions were done for particle size distribution determination.**

2.6. Differential Scanning Calorimetry (DSC)

The extent of protein denaturation was determined by measuring the heat absorbed by 12 mg samples of dry material, heated at a rate of 10°C per minute from room temperature to 190°C using a Pyris 1 differential scanning calorimeter (Perkin Elmer, Waltham, MA).

2.7. Microscopy

Samples were fixed and embedded following Bair and Snyder (Boy & Snyder, 1980) with minor modifications, at the Centre de Microscopie Electronique Appliquée in Toulouse, France. Sections were made at the Iowa State University NanoImaging Facility using a Reichert Ultracut S ultramicrotome (Leeds Precision Instruments, Minneapolis, MN). Thick sections

were contrast stained using 1% toluidine blue. Light microscopy images were made using a Zeiss Axioplan 2 light microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

3. Results and discussion

3.1. Extruded meal characterization

Sunflower kernels were extruded at bench scale with a single screw press-extruder. The composition of the extruded meal used in all experiments was determined as 9.0% (+/- 0.1) moisture, 20.6% (+/- 0.1) oil (dry basis), and 30.1% (+/- 0.4) protein (dry basis). Based on the mass of oil expelled during extrusion, the oil content of the entire seed (kernel plus hull) prior to extrusion was 44% and oil extraction yield was 68%.

The mass-weighted particle size distribution profile of the extruded meal has been determined (data not shown). The only fraction where hulls were not clearly visible was the smallest one, <0.25 mm. The largest fraction appeared to be mostly hulls, with the other fractions containing a mix of seed particles and hull. The high oil content of the extruded meal caused considerable clumping, making sieve separation ineffective for the smaller particle size ranges.

The destruction of the cells was defined by microscopic analysis before and after extrusion (figures 1). Before extrusion (figure 1a) cotyledon cells ranged from 50 to 100 μm in length, and 20 to 40 μm in diameter while protein bodies' ranged from 1 to 10 μm in diameter. The protein bodies filled a smaller proportion of the cytoplasmic volume compared to soy protein bodies (Bair & Snyder, 1980; Mantese, Medan & Hall, 2006). Oil bodies, that is oil storage organelles delimited by a protein-phospholipid membrane, occupied the space between protein bodies.

After extrusion, intact cotyledon cells were not observed (Figure 1b). Regions of disrupted cotyledon cells, with few recognizable structures, are seen between regions of intact sclerenchyma cells, the hollow structural and vascular tissue making up the bulk of the sunflower pericarp (Mantese et al., 2006). Some disrupted cell wall material can be seen on the outer regions of the disrupted cotyledon tissue (images not shown). Lipid was observed mostly as coalesced oil, in the outer regions of the extruded cellular matrix inside and outside disrupted cells. No intact oil bodies are visible. Some lipid is also observed in the interior of sclerenchyma cells of the pericarp. Sunflower pericarp is low in lipids (generally less than 5%) and it is unlikely that the lipid observed here occurs *in vivo*. A likely explanation for this observation is that the heat and pressure during extrusion causes some oil to fill the void spaces in the sclerenchyma cells.

After centrifugation, residual material settled into two distinct layers in the centrifuge bottles: a lower coarse layer making up about 80% of residual volume, and an upper layer of fine gray material making up the remainder. The lower layer consisted of a mixture of pericarp and disrupted cotyledon tissue, while the upper layer contained only cotyledon cells with some seed coat particles (Figure 2). As before, no intact cotyledon cells were observed; all cells have undergone at least some extraction of the cytoplasmic material but oil remains in some cells as coalesced oil droplets. Furthermore, coalesced oil is again prominent in sclerenchyma cells. These results are similar to those observed in soybean, where unextracted oil is sequestered inside disrupted cells as coalesced droplets too large to pass out of the matrix (Campbell & Glatz, 2009). A notable difference from soybean, however, is the entrapment of oil in the void spaces of the pericarp sclerenchyma cells.

The extent of protein denaturation and solubility has a crucial influence on the oil yield with aqueous extraction in soybeans (Rosenthal, Pyle, Niranjana, Gilmore & Trinca, 2001; Campbell & Glatz, 2009). Therefore, the conformational state of sunflower protein in the extrudate was analyzed, and compared to a control sample of pressed, ground sunflower seeds which had not been exposed to the heat of extrusion. The heat absorption profile of the extrudate is shown in Figure 3. An obvious peak occurs at 150 °C, which is slightly lower than the 155 °C denaturation temperature determined by Rouilly et al. (2003) for untreated sunflower of similar moisture content (10%). The peak area divided by the protein content of the samples, both extruded and pressed, gives a specific heat of denaturation of 9.7 (+/- 0.4) J/g protein. By comparison, Rouilly et al. report denaturation enthalpies of 8.6 J/g protein. Therefore, the extrusion conditions used here did not affect the conformational state of the sunflower proteins (Rouilly, Orliac, Silvestre, Rigal, 2003).

3.2. Aqueous extraction and enzyme assisted aqueous extraction

3.2.1. Full Factorial Design Experiment results

The measured responses for oil extraction yield, protein dissolution, and non-lipid material dissolution are shown in Table 1. The results indicate that in aqueous extraction conditions (trial 6, 7, 12 and 13) this is about 30% for oil (with the exception of Trial 12), comparable to similar extraction conditions from Bayberry (Zhang, Li, Yin, et al. 2012), 56% for proteins and 27% for the others. The highest recovery yields are obtained when extraction is managed with both enzymes, with an oil recovery yield of 40% and a protein recovery yield of 85%.

Significant parameters were identified by ANOVA. Table 2 summarizes the parameter estimates determined after elimination of the insignificant terms, the resulting analysis of

variance, and statistical significance. For oil extraction yield, only agitation and cellulase had significant main effects, while solid to liquid ratio and protease had significant interaction effects. Dissolution of non-lipid material, on the other hand, was not affected by agitation at all, with protease having the most important effect. The effect of cellulase was also significant, but the increase in dissolution caused by cellulase was much smaller than that of protease. Only protease had a significant effect on protein dissolution with an average increase of 28%. The goodness of fit and significance for the three responses after elimination of the insignificant parameters, are shown in Figure 4. Each of the models fit the data well, with actual values plotted against predicted values randomly distributed around a line of a slope of one on the fit test plot. The p-values for all models were less than 0.02.

3.2.2. Influence of parameters

Sunflower protein from defatted meal generally has low nitrogen solubility, less than 30% at pH 6.5 and low ionic strength, but this increases with salt addition (Kabirullah & Mills, 1983; Canella, Castriotta, Bernardi & Boni, 1985) and hydrolysis (Kabirullah & Mills, 1981). A protein solubilization of 85% with protease indicates a very high degree of disruption. Assuming protein can only be extracted from disrupted cells, as has been previously established (Campbell & Glatz, 2009), at most 15% of the cells remained intact after extrusion, and it could be even less considering the low solubility of sunflower proteins under these conditions.

Cellulase addition was made to facilitate the oil's exit from the solid residue, by promoting disruption of cells that were still intact after extraction and by promoting the modification of the disrupted structures. Comparisons between the significant parameters, for the three different responses, indicate that cellulases affected both oil extraction yield (3%) and

dissolution of non-lipid material, but not dissolution of protein. Cellulose degradation occurs but was limited and did not change the dissolution and the transfer of the entrapped molecules. As no intact sunflower cotyledon cells were observed in microscopic images of extruded sunflower meal, one possible explanation could be that extrusion succeeded in achieving near complete cellular disruption prior to extraction. There are no noticeable differences in these images between material extracted with and without cellulase. Nonetheless, the entrapment of oil droplets inside the confines of the cell wall of disrupted cells, suggests that the effect of cellulase is to disrupt this confining matrix.

Agitation can increase oil yield through several physical mechanisms depending on its intensity, and at the highest levels it can increase oil release by rupturing intact cells. The shear and turbulence created by agitation can also break up the solid matrices that entrap oil within the confines of disrupted cells. A third possibility is that the turbulence of agitation increases the emulsification of oil within the cells, creating smaller oil droplets that exit more efficiently out of the cellular matrix into the bulk fluid. Since agitation did not affect dissolution of protein or other non-lipid material, it is unlikely that it caused significant additional cell rupture, since extrusion alone achieved a high degree of cellular disruption as indicated by microscopic observation. Consequently, as increasing agitation from 160 rpm to 350 rpm increased oil extraction yields by an average of 8%, it can be assumed that this effect comes from modification of the droplet sizes.

Campbell and Glatz (2009) showed that emulsification is an important extraction mechanism for AEP of soybean oil. To illustrate the level of droplet disruption that can be achieved in the given mixing system a turbulent inertial droplet breakup model from Vankova et al. has been used to estimate the maximum stable droplet diameter of oil in AEP of soybeans

(Vankova, Tcholakova, Denkov, Ivanov & Vulchev, 2007; Campbell & Glatz, 2009). According to this model and the agitator power number, the maximum stable droplet diameter would be in the range of 15 to 20 μm for the 160 rpm condition and 3 to 5 μm for the 360 rpm condition, assuming no viscoelastic protein film at the interface, and an interfacial surface tension of 5 mN/m, as measured for soy protein-oil systems (Campbell & Glatz, 2009).

Experiments designed to alter the oil-water interfacial conditions during extraction, confirm the influence of the droplet structure on oil recovery yield. The addition of 3% (w/w solid) sodium dodecyl sulfate (SDS) increased the extraction yield of oil from 28.4% (+/-1.4) to 39.0% (+/-0.6) at 160 rpm, S/L = 0.10 (+/- 95 % confidence interval, n = 3). At the same time, protein extraction increased from 57% (+/-1) to 90% (+/-2), which is even more than the increase with protease, which was 86.2% (+/-0.1). The addition of protease increased protein extraction almost as much as SDS addition, but protease had no effect on oil extraction yield.

3.3. Extraction model

The mechanism of protease action for oil yield enhancement in AEP of soybean is due to alteration of the oil-water interface by two possible mechanisms: 1) disruption of a viscoelastic protein film, or 2) creation of protein hydrolysates that are better emulsifiers than native proteins (Campbell & Glatz, 2009; Latif & Anwar, 2013). The same authors also hypothesized that higher solid-liquid ratios (S/L) reduced soybean oil extraction by increasing interfacial protein coverage, and therefore viscoelastic effects.

Unlike soybean extraction, neither proteases nor S/L had a measurable effect on oil extraction yield from sunflower, suggesting different release mechanisms for the two materials. Sunflower has lower protein content than soy, 30% compared to 40% for sunflower extrudate and soy flour, respectively. Nonetheless, the resulting protein extract concentrations are similar

to concentrations seen in soybean extractions, ranging from 8 mg/ml for S/L of 0.05 and no protease, to 25 mg/ml for S/L of 0.10 with protease, for sunflower extrudate. For soybean under the same conditions, protein concentrations were 19 mg/ml and 38 mg/ml, respectively (Campbell & Glatz, 2009). If the formation of a viscoelastic film impedes oil release in soy, this does not appear to be the case in sunflower, as neither increasing the protein concentration (and, hence, interfacial coverage) nor disrupting a film by hydrolysis, affects yield. This sunflower extrudate result also differs from that found for extraction of dehulled ground sunflower seeds, where S/L did affect oil extraction [1]. However, the ground seeds had much higher oil content (>40%) and were subjected to a larger range of S/L (0.05 to 0.2) and pH, although the latter had no effect.

If disruption and diffusion of oil droplets were important mechanisms for extraction, a greater oil concentration in the bulk would cause S/L to have a measureable effect. Droplets much smaller than the dimensions of a rupture in a cell wall would be able move into as well as out of disrupted cells. Therefore, the volume of disrupted cells with which the droplets can exchange, relative to the total volume, would affect yield. As relative cell volume increased (i.e. at higher S/L), so would the entrained fraction of oil in those cells, and the amount would be proportional to the concentration of freely exchanging bulk oil droplets. Evon hypothesized that increasing the relative amount of water (i.e. decreasing S/L) increased the amount of oil that could be stabilized in an emulsion, a phenomenon that would also be more apparent in material with higher oil content (Evon, Vandenbossche, Pontalier & Rigal, 2007; Evon, 2008). Since S/L effects were not observed here, it is possible that the oil concentration in this case was too low to have a measureable effect.

If hydrolyzed soy proteins increase extraction yield, because of improved emulsification properties over native soy proteins, this does not appear to be the case for the present sunflower material. In other studies of sunflower protein-stabilized emulsions, hydrolysis of up to 10% of the peptide bonds did not have an effect on the droplet diameter under conditions similar to those used in these experiments (Karayannidou et al., 2007). Sunflower protein hydrolyzates may not therefore be able to increase yield through enhanced emulsification.

However, other studies have found significant increases in oil yield using proteases with chopped, rather than extruded, sunflower seeds (Badr & Sitohy, 1992). This contrast may be a result of differences in geometry of the matrices entrapping unextracted oil. In order for the turbulent forces to cause droplet breakup, eddies in the medium must be free to impinge on oil droplets, creating local pressure gradients around them. In sunflower extrudate, much oil was observed completely filling the sclerenchyma tissue void spaces, reducing the surface area available for energy transfer between turbulent eddies and oil droplets. The fraction of oil contained within the sclerenchyma tissue, would therefore be a theoretical limit to the amount of extraction that could take place in an aqueous environment without cellulolytic treatment, because of the geometrical barriers against emulsification.

It appears that the mechanism for oil transfer out of the matrix is different for sunflower extrudate. The results showed that introducing SDS leads to both increased oil and protein yield, while protease only increases protein yield. It could be that oil remained entrapped, after extrusion of sunflower kernel, in large structures involving proteins, and that these were too large to diffuse out of the solid residue. Protease addition could modify these structures, but as the hydrolyzed proteins have low emulsifying properties, only they are recovered while the lipids remain fixed on the residue. SDS addition seems to induce geometry changes in these structures,

allowing solubilisation of protein but also creation of smaller oil droplets that can then diffuse out. Changing the size of these structures can also be achieved with stronger agitation, but this action remains minimal since the oil recovery yield increase is only 3%.

The presence of insoluble protein inside the sunflower seed cotyledon cells could pose a major barrier to oil release and would explain this observation, and this insolubility may be caused by the extrusion (Jung, 2009). Hulls can also hinder the extraction because they contain mucilage that reduces the release of oil into the aqueous phase (Tabatabaei & Diosady, 2013).

4. Conclusions

Oil remaining in extruded sunflower meal after AEP/EAEP, was contained as coalesced oil droplets inside disrupted cotyledon cells and in void spaces of pericarp sclerenchyma cells. Agitation and cellulase treatment increased oil extraction yields, but protease and solid liquid ratio did not affect yields, contrary to observations for soybean. While emulsification may be an important extraction mechanism produced by agitation, the geometry of the cellular matrix entrapping coalesced oil may also be an important factor determining extraction yield, and is a possible explanation for the differences between sunflower and soybean oil extraction determination. Based on these observations, the extraction yields from both extrusion as well as AEP/EAEP could be improved if the kernels could be extruded in the absence of hulls.

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Figure captions

Figure 1- (a) Image of native sunflower cotyledon cells. Protein bodies are dark blue globules < 20 μm in length. Oil bodies fill the cytoplasmic space between protein bodies, 40X magnification. (b) Image of tissue after extrusion with features indicated: DC, region of disrupted cotyledon cells; S, region of intact sclerenchyma cells; SC, seed coat, 10X magnification.

Figure 2- Images of sunflower meal residue after extraction: (a) AEP Coarse layer sample (extracted without enzyme) showing intact sclerenchyma cells, 40X magnification; (b) EAEP coarse layer sample (extracted with cellulase) showing intact sclerenchyma cells, 10X magnification; (c) AEP fine layer sample 40X magnification (no enzyme); (d) EAEP fine layer sample, 40X magnification. CO, coalesced oil; S, region of sclerenchyma cells; DC, region of disrupted cotyledon cells (extracted with cellulase).

Figure 3- Differential scanning calorimetry profiles of sunflower meal that was extruded at 100°C and then ground and meal that was pressed at room temperature and then ground. Denaturation temperatures were near 150°C with denaturation enthalpies of 9.7 J/g protein for both treatments.

Figure 4- Fit tests of the multiple linear regression model for oil extraction yield, protein solubilization, and solubilization of non-lipid material using estimates determined after elimination of the insignificant effects (Table 3). E.g. predicted oil extraction yield = $0.29 - 0.0057 * \text{S/L} + 0.0003 * \text{protease} + 0.038 * \text{agitation} + 0.014 * \text{cellulase} + 0.018 * \text{S/L} * \text{protease}$. Actual values are plotted against predicted values overlaid on a line of a slope of one. Response mean values are shown as horizontal dashed lines. Prediction intervals are indicated by dashed lines on either side of the line of the slope of one.

FIGURES

Figure 1

Figure 2

Figure 3

Figure 4