BIOCHEMICAL FACTORS EFFECTING THE PRODUCTION OF SOY-FREE TOFU PREPARED USING YELLOW SPLIT PEAS (*PISUM SATIVUM*)

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AUTHORIZATION TO SUBMIT THESIS

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ABSTRACT

Tofu has been a staple source of protein for over a millennium. Tofu is traditionally made from soybeans (*Glycine max*) because of its high protein and fat content, but with increased pressures away from soy, other crops, like peas are being explored. Some research has been done on the production of soy-free tofu using freeze dried isolates, but there is a paucity of work that investigates manufacturer friendly techniques from non-soy sources. The purpose of this thesis is to review the effects of unconventional treatments and different formulations of a pea-based tofu on texture (Texture Profile Analysis), secondary structure (FTIR), surface hydrophobicity (Bromophenol blue binding), and molecular weight (microfluidics).

Yellow split peas (*Pisum sativum*) have a protein makeup that is functionally similar to soy, but previous work on peas produced pea tofu with low hardness values. Two studies were performed to assess the feasibility of producing pea tofu with suitable texture. One study examined physically disrupting the curd and pasteurization; the other examined cook time and changes to the formulation; and both examined fat addition to mimic soy.

The first study tested 8 tofus; a control, a pasteurized, a disrupted, a disrupted+pasteurized, and the 4 previous but with added fat. This increased the hardness of tofu from 175.9 g (control) to 323.0 g (disrupted+pasteurized) in no fat added tofu and from 48.8 g (control) to 127.8 g (disrupted+pasteurized) in fat added tofu. This treatment prevented the fat added tofu from falling apart during pasteurization.

Chemical changes in the structure of the could be correlated with textural properties. Changes in the amide I region of the FTIR spectra and β-Sheet structures correlated related to surface hydrophobicity and tofu hardness. The surface hydrophobicity decreased with the degree of pasteurization and increased with disruption.

In the second study, cook time, $MgCl_2$, and fat addition were varied. By changing these parameters tofus with hardness ranging from 39.4 g to 110.9 g, in the range of commercially available tofu, could be produced. By varying the $MgCl_2$ and fat content changes in the overall trend could be observed in α-helices and β-Sheets respectively in the FTIR spectra. SEM images were taken of all varieties of tofu and exhibited similar structures to those seen in historical data reported for soy tofu. Overall the research determined that peas can be used to make a tofu with similar parameters to that of commercially produced soy tofu.

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Furthermore, I would like to thank my committee, Dr. Rasco for her insights on protein structure and perspectives on my research and its applications into the broader food industry and Dr. Joyner for her providing her rheological expertise. I would also like to sincerely thank Dr. Armando McDonald who was kind enough to provide access to FTIR instrumentation and provide guidance on its used and interpretation of spectra.

DEDICATION

This work is dedicated to my parents who have always supported and tolerated my curiosity.

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CHAPTER 1: LITERATURE REVIEW

1.1 Abstract

For nutritional and environmental reasons consumers are shifting their diets to include more plant-based proteins. While soy has long been the mainstay of vegetable protein foods, allergies and aversion to genetically modified organisms (GMOs) are pushing manufacturers to offer more options produced from other types of legumes. A potential market for replacing soy is tofu. Tofu is a staple throughout Eastern Asia and is rapidly growing in popularity in other regions of the world. While the first historical evidence of soy tofu showed up about millennia ago, there has been no real success preparing tofu without soy until recently.

Field peas (*Pisum sativum*) may have the potential to accommodate this market need. Field peas are a relatively underutilized crop in the United States, and there is current push to increase application and value by producers and processors. The legumin protein in peas is similar in gel forming ability to glycinin in soy. This similarity makes peas applicable to some gel applications such as tofu than other legumes. However, there has been no real success in the production of tofu from dry peas due to a lack of research in this area and a concern about flavor and color.

1.2 Introduction

Originally, meat substitutes were targeted to vegetarians and vegans. With emerging dietary lifestyles like flexitarians, a person that loosely follows a vegetarian diet, but occasionally eats meats, vegetable proteins are being consumed by and increasing number of people. Currently, about 33 % of consumers that regularly eat meat replacers are trying to reduce the meat in their diet rather than eliminate it (Behn, 2011). The shift is likely caused by health concerns, such as the role of saturated fatty acids in cardio vascular disease (Pan et al., 2012),

and red meats association with colon cancer. Conversely, vegetable proteins, when consumed as part of the whole plant or seed, often have the benefit of increased fiber, the influence on intestinal health is well documented. Legumes often contain soluble oligosaccharides such as the trisaccharide raffinose and the tetrasaccharide stachyose (Fig 1.1). These oligosaccharides are synthesized sequentially by binding a galactinol to a sucrose molecule to form raffinose and then an additional galactinol is bound to form stachyose (Peterbauer, Mucha, Mach, & Richter, 2002). Plants biosynthesize raffinose and stachyose for storage and to transport energy. These sugars also act as a cryoprotectant, since legumes, like peas, are a cool-season crop and often endure frost and snow (Sprenger & Keller, 2000). While legumes have the necessary enzymes to process these oligosaccharides humans do not; they pass into the intestine where they promote intestinal health by acting as prebiotics stimulating growth of beneficial bacteria like *Bifidobacterium spp* (Hayakawa et al., 1990).

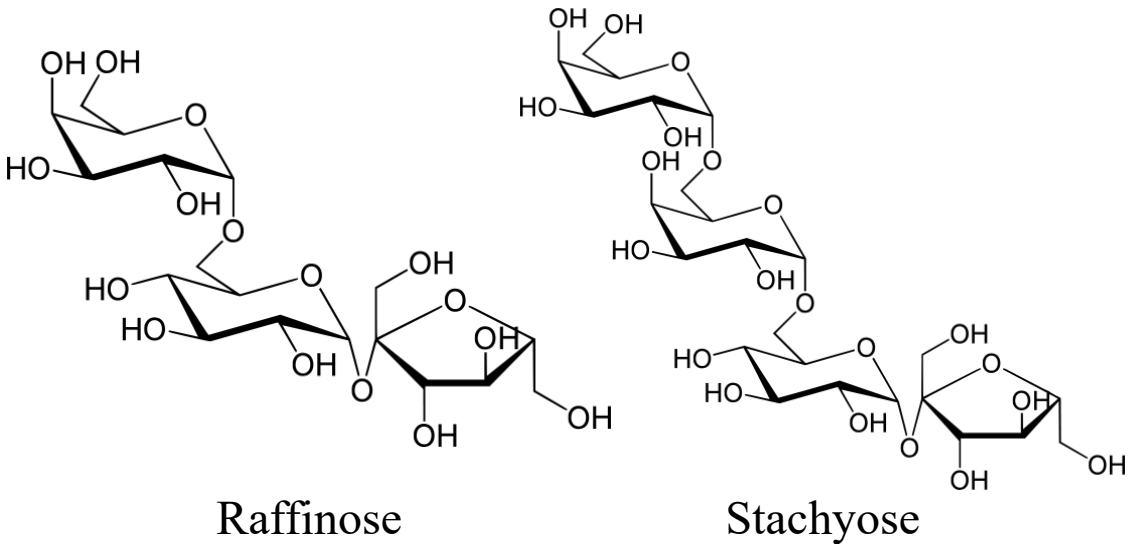

Figure 1.1 A representation of the structures of Raffinose and Stachyose.

Consuming more vegetable protein has the ecological benefits of potentially reducing the demand for meat. Some crops are grown almost exclusively to feed livestock, requiring

manhours, fertilizer, fuel, water, and land for crop production intended for feed. The animals, cattle in particular, generate greenhouse gasses in the form of methane and nitric oxide; contributing an estimated 35.5 Tg CO_2-equivalents in 2010 (Boadi, Benchaar, Chiquette, & Massé, 2004). By reducing the amount of livestock by half due to decreased demand and replacing these dietary requirements with plant-based protein agricultural emissions could be reduced by 40 % (Westhoek et al., 2014). As consumers become more attuned to these issues, greener more health-conscious products are introduced to the market to facilitate consumers' needs. Foods made from soy beans (Glycine max) have long been a panacea for filling these niches, but as consumers grow wary of allergens and GMOs, new alternatives need to be found.

1.3 Allergens

While it is difficult to track trends of the number of individuals with allergies, allergy related hospital visits can serve as a metric. In the U.K. food anaphylaxis hospitalizations doubled between 1998 and 2012 for children 4 and under (Turner et al., 2015). In the U.S. between 2000 and 2009 food anaphylaxis hospitalizations doubled for people 18 and under (Rudders, Arias, & Camargo, 2014). This increase has been observed in Western countries, but instances of increased allergies among Asian individuals raised in the West are increasing disproportionally, indicating that there is likely an interaction with Eastern Asian genetics and Western lifestyle (Tang & Mullins, 2017). However, since the data is number of hospital visits it is possible that the instances of allergic reactions are not increasing, but that people are seeking medical attention more readily or the severity of the allergic reactions is increasing. There are 18 allergenic proteins in soy and while the individual impact of each is unclear (Cordle, 2004), soy has been deemed one of the 8 most common allergenic foods by the FDA (FALCPA, 2004). It is estimated that 0.3 % of the general population exhibit allergic reactions to soy (Young,

Stoneham, Petruckevitch, Barton, & Rona, 1994), and 0.4 % for children under 3 (Bock, 1987). However, allergies to beans and peas are rare making them potential alternatives to soy in processed food. However, beans from the lupin plant (*Lupinus mutabilis*) are considered to be a common allergen by the European Food Safety Authority (EFSA Panel, 2005) and also in Canada.

1.4 GMOs

Many consumers are looking to buy organic non-GMO foods. While there is little conclusive research to support the health benefits of foods deemed organic there is ample research on willingness to buy organic. Burton, Rigby, Young, & James (2001) found that typical male and female shoppers were willing to pay 26 % and 49 % more for organic foods, respectively, and that committed organic shoppers would pay up to 4 times as much to avoid GMOs. When breakfast cereal was studied, consumers were willing to pay 18 cents more per box if it had a GMO-free claim on the label (Batte, Hooker, Haab, & Beaverson, 2007). A study on beef found that information about an organic product is more effective at increasing willingness to buy than the sensory attributes of the product (Napolitano, Braghieri, Piasentier, Favotto, Naspetti, and Zanoli). In Taiwan where tofu is a staple, more affluent individuals were willing to pay more for non-GMO tofu, but lower earners opted for the cheaper GMO variety, indicating price is a concern (Jan, Fu, & Huang, 2007). If a tofu could be made with a cheaper legume that is not genetically modified, then both cost conscious and organic shopper segments could be satiated. With a changing climate and increasing population a new protein source is needed, and the answer to the world's food demands might not be soy after all (Fleischer, 1973).

1.5 Reading the Pulse of the Market

With a growing consumer mistrust of soy, pulse crops can provide alternatives soy products. The designation “pulse” is largely semantic, referring to members of the legume family that are harvested and sold dry rather than fresh or for its oil (“What are Pulses?,” n.d.) Soy does not qualify because it is harvested largely for its oil and lecithin, altering how it is taxed. Plant based proteins are becoming more prominent because of its availability, nutritional composition, and its perceived sustainability over animal proteins. The storage stability, nitrogen fixing nodules, and high protein content have made pulse crops a staple. Many nations manage a strategic food reserve to buffer bad crop years caused by draught, natural disaster, in order to prevent price spikes and potential famine. Because pulses are stored dry, they do not have a fixed storage stability and can last indefinably if moisture and pests are kept out, with storage stability studies lasting 10 years (Ockenden, Falk, & Lott, 1997). Crops with long storage-life that contain copious amounts of carbohydrates and protein are ideal for meeting global nutrient demands. In 2010 worldwide production of pulses like chickpeas (*Cicer arietinum*), lentils (*Lens culinaris*) and dried peas (*Pisum sativum*) reached 68.8 billion kg (FAO, 2013).

1.6 Increasing Demand

The United Nations (UN) predicts the global population to be 10 billion by 2080 (Alexandratos & Bruinsma, 2012). To keep up with the resulting food demands, food production will need to increase by 60 % (Alexandratos & Bruinsma, 2012), putting increased strain on the soil and environment. Much of this population growth will take place during the development of sub-Saharan Africa (FAO, 2013) where a limiting factor to small scale agriculture in Africa is access to fertile soil (B. Vanlauwe & Giller, 2006). Chemical fertilizers are too expensive for smaller farms (Bernard Vanlauwe et al., 2011). Considering 25 % of Africans operate small

farms, there is a need to decrease dependence on chemical fertilizers (Gueye & Ndeso-Atanga, 1992). Nitrogen is one of the most limiting elements in soil (Bernard Vanlauwe et al., 2011), and in 2014 pulses fixed 3-6 million tons of Nitrogen into the worlds soils (FAO, 2016), so much like the development of Australia, pulses' nitrogen fixing nodules can help prepare and maintain the soil (FAO, 2013).

1.7 Increasing Supply

The US views pulses as a secondary crop, and in 2010 was ranked seventh in global pulse production (FAO, 2013). However, with the potential a looming boon to health and agriculture as well as the Western increase of allergens, the secondary status may change in the near future. Change does appear to be happening in the US, as the tonnage of pulses harvested grew from 2 million kg in 2002, to 3.5 million kg in 2016 (Thornsbury, Wells, & Bond, 2013). Most of this increase came from the increased production of dry peas which increased nearly 6-fold. Though some of the increase represents exported product; export revenue increased from $338.6 million in 2013 to $475.8 million in 2016 (Thornsbury et al., 2013). Peas, like other legumes are a nitrogen fixing crop that is high in protein and widely consumed by humans. Unlike soy, peanuts and lupin dry peas are rarely allergenic (Wensing et al., 2003). Peas are gaining popularity with large meat producers like Tyson branching into pea protein derived burgers (Doris, 2017) to address demands of vegetarian markets. Traditionally peas have been sold in a dried form, needing to be soaked before use, peas are now working their way into more casual ready to eat products like extruded puffs and workout protein powder.

1.8 Composition of dry field peas

Peas are a cool season crop with dicotyledonous seeds that grow in pods. Peas are self-pollenating, not needing bees to fertilize its flower to produce seeds. After harvest, peas are

dehulled, split mechanically, and dried. The composition varies significantly based on crop year, location and cultivar (Nikolopoulou, Grigorakis, Stasini, Alexis, & Iliadis, 2007). Carbohydrates typically range from 33.4 % - 49.6 % (Nikolopoulou et al., 2007) with some researchers reporting as high as 70.9 %, but these researchers measured carbohydrates by difference and did not differentiate between starch and fiber (Karaca, Low, & Nickerson, 2011) (Table 1). Fiber can range from 4.3 % – 18.4 % (Wang et al., 2010; Nikolopoulou et al., 2007), but typically is around 14 % (Ma, Boye, & Hu, 2017). Peas' fat content ranges from 0.7 % - 3.95 % (Nikolopoulou et al., 2007); (Wang et al., 2010); (Karaca et al., 2011), which is why peas are valued as a protein source with a low-fat content. The protein content of peas ranges from 18.8 – 32.5 %, but averages in the low to mid 20s (Nikolopoulou et al., 2007) (Ma et al., 2017); (Wang et al., 2010); (Karaca et al., 2011). Pea protein has an inverse relationship with its carbohydrate content, but a positive correlation with phytic acid content (Nikolopoulou et al., 2007), which is regarded as one of peas 4 antinutrients along with tannins, trypsin inhibitors, and amylase inhibitors.

Table 1.1: Mean values of physicochemical and cooking characteristics of six field pea cultivars grown in 2006 and 2007. Taken from: Wang et al (2010)

	Cultivar mean[C]							
	CDC Striker	**Cooper**	**Cutlass**	**Eclipse**	**SW Marquee**	**SW Sergeant**	**Mean[D]**	**Range[D]**
	***n* = 20**	***n* = 20**	***n* = 20**	***n* = 20**	***n* = 20**	***n* = 20**	***n* = 120**	***n* = 120**
Physical characteristics								
100 seed weight (g)	22.6c[B]	26.8a	21.2d	23.2b	19.6e	19.8e	22.2	16.6–31.7
Seed size (mm)	6.7c	7.1a	6.5d	6.8b	6.4e	6.4e	6.7	6.1–7.5
WHC[A] (g H_2O/kg seed)	1040.5e	1139.2b	1063.7d	1066.4d	1183.6a	1088.3c	1096.9	936.0–1316.6
Cooking time (min)	11.0d	13.5c	15.9b	21.7a	9.6d	14.4c	14.3	6.2–33.5
Firmness (N/g seed)	18.4c	16.6e	19.1b	20.4a	17.4d	20.0a	18.6	14.5–31.3
Composition (g/kg dry matter)								
Protein (N × 6.25)	263a	242c	239d	249b	248b	245c	248	213–284
Starch	456c	476a	474a	472ab	472ab	470b	470	433– 496
Crude fiber	55.9a	48.5d	52.9b	49.7c	50.7c	56.7a	52.4	43.4–62.2
Fat	9.6b	8.5d	9.2c	8.1e	8.0e	10.1a	8.9	6.7–11.7
Ash	25.7d	27.4abc	27.9a	27.3bc	27.6ab	27.0c	27.2	23.0–31.3
Phytic acid	7.8 cd	8.8a	7.5d	8.2bc	8.4ab	7.8cd	8.1	4.2–11.9

[A]WHC = water hydration capacity (g H_2O/kg seed).
[B]Means within a row with the same letter are not significantly different ($p > 0.05$) as determined using Duncan's multiple range test.
[C]*n* = number of samples for each cultivar (5 sites × 2 years × 2 duplicates = 20).
[D]*n* = number of samples (6 cultivars × 5 sites × 2 years × 2 duplicates = 120).

1.8.1 Antinutrients

Peas store phosphorus in the form of phytic acid (myo-inositol hexaphosphate) and depending on the cultivar peas' phytic acid content can ranged from 1.19 % to 1.33 % (Alonso, Orúe, & Marzo, 1998)). Phytic acids bind to proteins preventing proteolysis (Reddy, Sathe, & Pierson, 1988), and bind to Ca^{+2} ions that promote amylase activity indirectly reducing carbohydrate bioavailability (Deshpande & Cheryan, 1984). Tannins are large polyphenolic antioxidants that are considered an antinutrient which reduce the bioavailability of protein and

carbohydrates by forming complexes. However, legumes with low to moderate amounts of tannins, have greater digestibility of protein (Reed, 1995). Whole peas contain 0.237 % tannins, but there is a reduction in tannin content when the hull is removed, if the peas are soaked (water soluble tannins leached into soak water), and if the peas are cooked (heat degradation) (Ma et al., 2017). Other pea antinutrient are the enzyme inhibitors. The trypsin inhibitors found in peas are the Bowman–Birk type (Wiseman, Al-Mazooqi, Welham, & Domoney, 2003), a class of antifungal proteins (Ye, Ng, & Rao, 2001). These proteins have a molecular weight of 8-20 kDa, and inhibit proteolytic enzymes that lyse proteins at serine, e.g. trypsin, chymotrypsin, and elastase (Mello, Tanaka, & Silva-Filho, 2003). Trypsin inhibitors can be completely inactivated with heat (microwaved in water for 25 min) (Ma et al., 2017). Peas also contain amylase inhibitors in the form of lectins, a class of proteins that binds to carbohydrates (Schroeder et al., 1995), and cause erythrocytes to aggregate (Habiba, 2002). Cooking the peas for 10 min (120 °C) or 20 minutes (100 °C) is enough to completely inactivate the lectins (Habiba, 2002), so the reason that carbohydrates are not fully digested has to do with the carbohydrates themselves.

1.8.2 Carbohydrates

In raw peas, the resistant starch represents about 20-30 % of the total native starch and is unavailable to digestive enzymes (Ma et al., 2017; Chung, Liu, & Hoover, 2009). In cooked legumes, between 8-10 % resistant starch has been reported and is attributed to the recrystallization of amylose (Ma et al., 2017; Chung, Liu, & Hoover, 2009). The undigested starch is fermented into butyrate in the large intestines by bacteria both serving as a prebiotic and a butyrate source for intestinal epithelial cells. This has been associated with preventing malignance transformation of colon cells *in-vitro* (Whitehead, Young, & Bhathal, 1986). Starch resistance is aided by phytic acid which inhibits digestive enzymes by either binding to amylase

itself or calcium which catalysis amylase (Thompson & Yoon, 1984). Approximately, 40 % of peas are starch, of which about 40 % is high molecular weight amylopectin, 24 % is low molecular weight amylopectin, and 36 % is amylose (Simsek, Tulbek, Yao, & Schatz, 2009). The starch is packed into large granules that have a higher instance of being damaged than smaller granules. This damage increased the granules swelling ability (Gujska, D.-Reinhard, & Khan, 1994), which could improve yields of pea products or act as a bulking agent.

1.9 Protein

Peas contain about 20 % protein (Wang et al., 2010), which is typical for most pulse crops, but soy contains about 40 % protein (Piper & Boote, 1999). This is an issue in terms of the yields when replacing soy with peas, but peas can compensate for this with its starch related increase in water holding capacity and its reduced amount of trypsin inhibitors. Peas contain 10 % less trypsin inhibitors than soy, resulting in a greater amount of protein being absorbed by humans (Schatz & Endres, 2009). This will not improve the yield, but ensures that more protein gets to the consumer, which is a benefit in terms of preventing malnourishment.

The protein content of peas can be further improved by pin milling coupled with air fractionation. The starch in peas is heavier than the protein, so this method is effective for producing high protein pea flours of up to 63 % (Vose, Basterrechae, Gorin, Finlayson, & Youngs, 1976). Purer protein is typically isolated by isoelectric focusing or salting out with sodium chloride. Both methods require drying, and both isolation and drying have an effect on the functionality of the protein. Isoelectric focusing produced isolates with a higher percent protein, similar fat content, and lower fiber and ash than salting out (Sumner, Nielsen, & Youngs, 1981) which retains more impurities and incorporates salt (Arakawa & Timasheff, 1984). Isoelectric focusing has better retention of solubility overall but salting out improves the

other functional properties (Sumner et al., 1981). Common types of drying used on isolates is spray drying, freeze drying, and drum drying.

1.9.1 Protein Solubility

Drying effects protein functionality in a variety of ways, which may vary between pulses and methods of preparation, but the specifics of how spray driers are run can affect the protein functionality (Table 2). There are instances when the inconsistencies in spray drying operation will reduce the functionality of a protein and erroneously make it seem like freeze drying is the more effective method, so it is best to look at several different pulse crops. Freeze drying has the highest solubility retention, reaching 100 % solubility at pH of 10, 87 % soluble at pH of 7, 2 % at pH 4.5, which is the isoelectric point, and regain 56 % of its solubility at pH of 3 (Sumner et al., 1981). Spray drying is almost as effective at retaining solubility, but is lower than freeze drying at some pHs, and drum drying loses most of the solubility even at a favorable pH of 10 (Sumner et al., 1981). However, on fava beans (*Viciu faba*) and lentils (*Lens culinaris*) spray drying was the most effective method for retaining solubility (Cepeda, Villarán, & Aranguiz, 1998; Joshi, Adhikari, Aldred, Panozzo, & Kasapis, 2011). The researchers working on the lentils attributed the high solubility to spray dryings regular particle size and minimal denaturation because of low heat (Joshi et al., 2011) and disaccharides have been shown to undergo hydrogen binding with proteins and prevent folding during moisture loss (Allison, Chang, Randolph, & Carpenter, 1999). While disaccharides could prevent folding during freeze drying because of the loss of moisture, disaccharides do not act as cryoprotectants, so the low temperature required for freeze drying can still denature the proteins (Allison et al., 1999), but pulses contain oligosaccharides in the raffinose family (raffinose and stachyose) that act as cryoprotectants (Sprenger & Keller, 2000). Peas contain 0.93 % raffinose / 6.47 % stachyose

(Wang, Hatcher, & Gawalko, 2008) as opposed to 0.0 % raffinose / 1.81 % stachyose in fava beans (Khalil & Mansour, 2016), and 0.40 % raffinose / 1.81 % stachyose (Hefnawy, 2011). This difference could also be from the concentration of protein, spray pressure, or droplet size.

Table 1.2: Properties of pea and soy protein isolates, dry basis isolates, dry basis. Taken from: Sumner et al (1981).

Product	PNal			SNal	Pll			Sll
	Spray	Freeze	Drum	Spray	Spray	Freeze	Drum	Spray
pH	7.0	7.0	7.0	7.0	4.5	4.5	4.5	5.0
Moisture%	5.0	2.4	7.8	3.8	8.5	4.2	11.0	5.9
Crude protein %	85.8	83.0	83.2	93.4	88.5	90.0	85.9	89.0
Crude fat%	5.3	4.5	2.8	0.1	5.3	4.4	1.8	0.2
Crude fiber %	0.5	0.7	0.6	0.2	0.2	0.2	0.3	0.2
Ash%	5.2	5.0	5.0	4.0	2.7	2.8	3.1	3.7
NFE%	3.2	6.8	8.4	2.3	3.3	2.6	8.9	6.9
Nitrogen solubility %								
pH 3.0	53	53	14	43	56	56	7	34
pH 4.5	1	3	0	3	3	2	2	0
pH 7.0	63	64	16	61	72	87	3	30
pH 10.0	81	94	34	91	100	100	23	71
Emulsification %	28	42	52	29	38	38	38	33
Fat absorption %	104	230	204	145	90	122	127	100
Water absorption %	250	205	283	698	132	112	191	155
Foaming%	433	75	335	203	412	143	198	250
Color L	87.7	74.6	74.4	84.0	82.2	62.8	62.6	84
a	-0.3	1.5	1.6	2.1	1.3	5.0	5.8	1.3
b	14.7	20.4	21.8	13.2	15.3	22.1	20.6	13.8
Flavor	8.9	8.1	6.5	8.6	8.6	7.4	7.8	5.9

[a] L. (100 white, 0 black); a (+red, -green); b (+yellow, -blue).
[b] 1 "' raw pea flavor;10"' bland.

1.9.2 Emulsification Capacity

Emulsification capacity is highest for drum drying at 52 % with freeze drying and spray drying having capacities of 42 % and 28 % respectively (Sumner et al., 1981). Again, the trend reverses in fava beans, with spray drying having higher emulsion capacity than freeze drying (Cepeda et al., 1998). Since soy lecithin is a common emulsifier, emulsion capacity is important function for non-soy legumes. Since the amount of phospholipids, like lecithin, retained in protein extracts is seldom reported in articles, making practical comparisons between protein extracts cannot be made.

1.9.3 Absorbed Fat

The percent of absorbed fat is highest for freeze dried at 230 %, with drum dried and spray dried at 204 % and 104 % respectively (Sumner et al., 1981). In this instance the trend (freeze drying higher functionality than spray drying) seen in peas are consistent with fava (Cepeda et al., 1998).

1.9.4 Percent Water Absorbed

The percent water absorbed is highest from drum dried proteins at 283 %, with spray drying and freeze drying at 250 % and 205 % respectively (Sumner et al., 1981). In this instance the trends (spray drying higher function than freeze drying) seen in peas are consistent with fava and lentils (Cepeda et al., 1998; (Joshi et al., 2011).

1.9.5 Foaming Percent

The foaming percent is highest with spray drying at 433 %, with drum drying and freeze drying at 335 % and 75 % respectively (Sumner et al., 1981). It is noteworthy that the foaming percent of freeze dried pea protein is the only non-solubility related functional property that is higher with isoelectric focusing, 143 %, than when salted out (Sumner et al., 1981).

1.9.6 Flavor

The flavor is the best in spray and freeze-dried isolates with drum dried isolates having an unpleasant pea flavor (Sumner et al., 1981). This flavor is a combination of the bitter astringency of the tannins (Reed, 1995) and from the volatile compound hexanol, which accounts for 55 % of the volatiles in peas (Jakobsen, Hansen, Christensen, Brockhoff, & Olsen, 1998). Both of these are decreased during cooking, improving the palatability.

1.9.7 Glycinin and Legumin

The Svedberg unit (S) displays the sedimentation rate of a particle under ultra-centrifugation, which is a function of protein size. While this is not a precise measurement of the size, it is commonly used to group proteins of similar size. Pea's two most abundant proteins are analogous in structure to those of soy. Glycinin (11S) and β-conglycinin (7S) (Fig. 1.2) proteins account for 70 % of the total protein in soy (Poysa, Woodrow, & Yu, 2006), with glycinin making up 40-60 % and β-conglycinin 30-40 % (Krishnan & Nelson, 2011). Glycinin is formed heterogeneity from six of five different subunits and can range from ~300 kDa to ~380 kDa (Williams, 2011). The five sub-units that can potentially comprise a glycinin, each itself made of an acidic polypeptide of about ~35 kDa, comprised of three fragments of ~22 kDa, ~13 kDa, and ~9 kDa and a basic polypeptide of about ~20 kDa, comprised of two fragments of ~20 kDa and 1 kDa (Staswick, Hermodson, & Nielsen, 1984). The variability in fragments causes variability in the sub-units; resulting in molecular weights of ~54 kDa, ~54.4 kDa, ~55.7 kDa, ~58 kDa, and ~63.7 kDa (Nielsen, 1989). The sub-units are held together by hydrophobic interaction as well as a single disulfide bond, (Rawel & Rohn, 2002). The formation of disulfide bridges gives the protein its ability to form a strong gel (Meng, 2015). Glycinin's pea counter-part is legumin, also a globular storage protein of ~360 kDa (Schwenke et al., 2001), which is also an 11S hexamer

comprised of acidic (~40 kDa) and basic subunits (~21 kDa – ~24 kDa) (Bacon, Lambert, Phalp, Plumb, & Wright, 1987) bound by disulfide bonds, but differs by having 63 % the amount of cysteine residues as glycinin (O'Kane, Happe, Vereijken, Gruppen, & Van Boekel, 2004b). The reduced cysteine content could lower legumin gel's potential hardness.

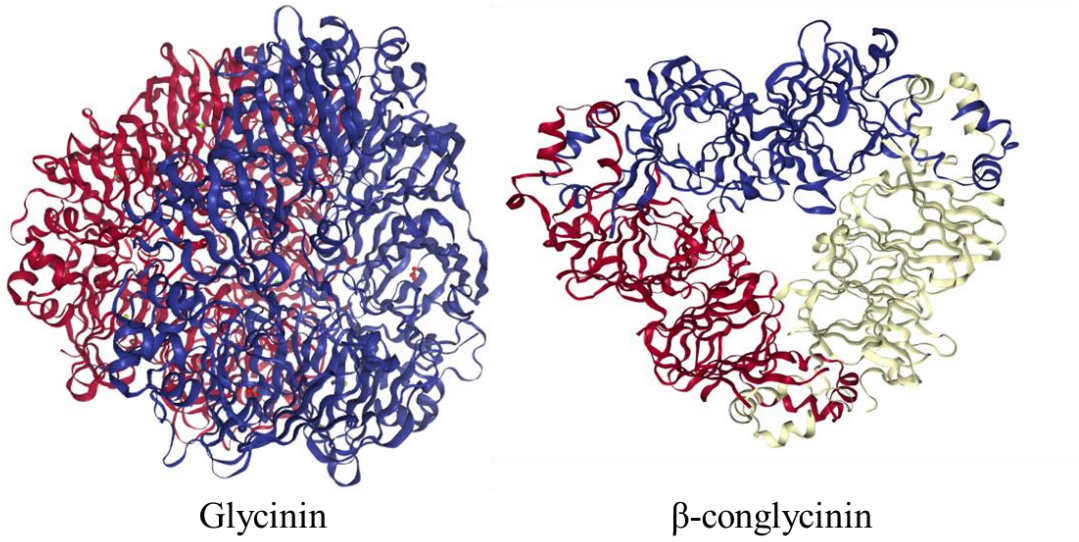

Figure 1.2: Representations of crystal structures of native soybean glycinin homohexamer β-conglycinin β-homotrimers, where red, white and blue each represent an individual protein making up the trimer. Taken from: Rose et al (2016).

1.9.8 β-Conglycinin and Vicilin

β-Conglycinin is a 7S globular soy protein with 3 subunits, α (~67 kDa), α' (~71 kDa), and β (~50 kDa) (Maruyama et al., 1999). β-Conglycinin can exist as one of six species; $\alpha'\beta_2$, $\alpha\beta_2$, $\alpha\alpha'\beta$, $\alpha_2\beta$, $\alpha_2\alpha'$, α_3 (Thanh & Shibasaki, 1978), so the molecular weight of β-Conglycinin ranges between ~150 kDa – ~200 kDa (Williams, 2011). β-Conglycinin has a lower cysteine content so its gels are less hard and more elastic (likely means springy or flexible) than glycinin gels, possible because one of the sub-units, β, cannot form disulfide bonds (Williams, 2011). Vicilin is a 7S trimeric protein with a molecular weight of about ~170 kDa (O'Kane, Happe,

Vereijken, Gruppen, & Van Boekel, 2004a). The subunits' molecular weights are around ~33 kDa, ~50 kDa, and ~71 kDa (Gatehouse, Croy, Morton, Tyler, & Boulter, 1981). There is some variation in sub-unit molecular weight because the ~50 kDa subunit can be cleaved in two places (Shewry, 1995). If the proteolysis occurs at site 1 then polypeptides of ~19 kDa and ~31 kDa are formed (fig 1.3). If the proteolysis occurs at site 2 then polypeptides of ~12.5 kDa and ~33 kDa are formed (Shewry, 1995). If the proteolysis occurs at both sites 1 and 2 then polypeptides of ~19 kDa, ~13.5 kDa, and ~12.5 kDa are formed (Shewry, 1995). Furthermore, the region that dissociates into the ~12.5 kDa polypeptide can become glycosylated (Gatehouse et al., 1981), increasing its molecular weight to 16 kDa (Shewry, 1995).

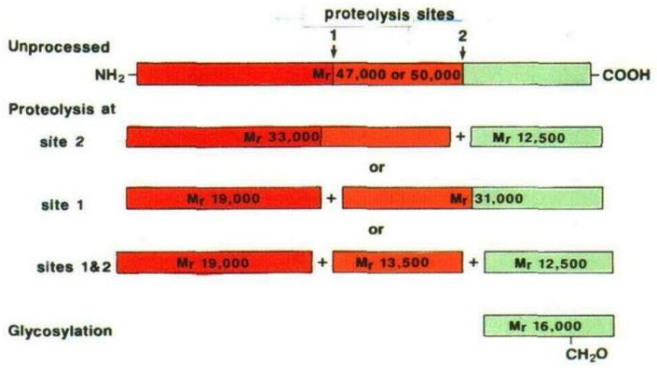

Figure 1.3: Break down of vicilin's 50 kDa sub-unit's polypeptide. Taken from: Sumner et al (1981).

1.10 Tofu

Tofu is a high protein food originating in China during the Han Dynasty (Han, Rombouts, & Nout, 2001). Today, tofu is marketed by its texture ranging from silken, soft, firm, extra firm, and super firm. There is no standard of identity for tofu texture, so the terminology and texture can vary between producers; e.g. a firm tofu from one manufacturer could have the mechanical properties of an extra firm from another. Tofu is prepared by grinding the soy beans, filtering the insoluble material, heating the milk, and mixing with a positively charged ion. The three most common coagulants used are glucono-delta-lactone (GDL), calcium sulfate, and magnesium

chloride. GDL is a fermented by *Acetobacter suboxydans* (Myers, 2001) from glucose and is different from the others in its mechanism. GDL works by hydrolyzing into gluconic acid when heated denaturing proteins slowly, so a single block of soft gel similar to Jell-O® forms instead of individual curds. GDL is added to soy milk which is filled into its packaging and during pasteurization the gluconic acid drops the pH during GDL hydrolysis and this coagulates the soy milk into silken tofu. Since silken tofu is not pressed it has a high yield, but a much lower protein content than firmer tofu.

Salts are added during tofu production to induce coagulation. Calcium sulfate (gypsum) is a calcium salt that is commonly used in dry wall and Plaster of Paris. Magnesium chloride (nigari or bittern) is the original coagulant used, as it was a by-product of sea salt production, and when used in the correct proportion leaves a sweet oceany flavor akin to fresh fish. Both calcium sulfate and magnesium chloride work by forming cross-linked proteins with divalent cations (Prabhakaran, Perera, & Valiyaveettil, 2006). In order for the cations to have access to the cysteine amino acids the soy milk is heated before coagulant addition, this allows the 11S subunits to dissociate (Wu, Hua, Chen, Kong, & Zhang, 2017) exposing the sulfated and hydrophobic regions. This dissociation occurs at 65 °C at low ionic strength solutions and 75 °C at higher ionic strength (Matsudomi, Mori, Kato, & Kobayahi, 1985), this temperature difference could potentially assist re-aggregation after the salts are added. Curds are given time to form during which, the hydrophobic regions of different proteins aggregate, while positive ions (H^+, Ca^{2+}, or Mg^{2+} pending use of GDL, Calcium sulfate, or magnesium chloride respectively) (Kohyama, Sano, & Doi, 1995) will react with the negative portions of the protein to neutralize the net charge and disulfide bonds form between the adjacent proteins to form linear or branched strands of glycinin (Nakamura, Utsumi, & Mori, 1984). The formed curds are then pressed in a

cloth to remove excess water and increase firmness. Considering, the similarities of glycinin and legumin in structure it is likely that the process of making pea-based tofu is analogous. Pea legumin also denatures exposing its subgroups' sulfated regions when treated with heat (75 °C - 85 °C) (Mession, Chihi, Sok, & Saurel, 2015). Furthermore, β-conglycinin in soy tofu has been found to improve the mechanical properties by providing elasticity to the gel (Williams, 2011), similarly vicilin has been shown to prevent legumin from self-associating and improved the network between aggregates, which would improve the elasticity. By using the same process of heating to expose cysteine regions, adding positively charged ions to neutralize the negatively charged regions and promote disulfide binding, and pressing out excess moisture, pea proteins can be used as a stand in for soy. However, soy's high protein, high fat, and low carbohydrate content makes it ideal for tofu, and research that has looked at using different beans has encountered issues.

1.10.1 Alternative Tofu

Tofu production is artisanal in nature. Many bench top experiments are varied because the particular coagulant, the rate the coagulant is mixed in, the concentration of the coagulant, the press time, the press force (Cai, Chang, Shih, Hou, & Ji, 1997), and the cultivar (Kim & Wicker, 2005). Increasing the mixing and/or coagulant amount, increases the reaction rate and forms firmer curds with lower moisture which produces a less homogenous tofu (Zhu, Wu, Saito, Tatsumi, & Yin, 2016). While the higher protein and lower water content of the individual curds of the fast-coagulated tofu implies it will be firmer, the block of tofu does not hold together as well.

The goal of tofu is to produce a firm, cohesive, and smooth curd (Kim & Wicker, 2005), regardless if the tofu is made of soy or other legumes. There have been attempts to make

alternative tofus in the past, but for most non-soy tofus, the issue is a lack of firmness (Mohamed, Johan, & Bakar, 1989). One potential issue is that these studies use calcium sulfate as the coagulant. Calcium sulfate is insoluble in water, this slows the reaction with proteins and increases water retention, reducing firmness. However, magnesium chloride is water soluble, reacts quickly to form lower moisture, firmer curds (Johnson, 1984). Calcium sulfate's insolubility can result in settling while it is being added. This is not an issue on a manufacturing scale, but on the bench top scale the percent of coagulant left behind is much higher. Furthermore, phytic acid binds to calcium sulfate making the coagulant inert (Liu & Chang, 2004), and has been shown to disproportionally inhibit glycinin's ability to form a gel (Saio & Watanabe, 1977), which is the protein that contributes most to tofu hardness. While both calcium and magnesium ions bind to phytic acid it has been found that Ca^{+2}'s ability to inhibit enzyme activity was lowered more than Mg^{+2}'s ability to reduce enzyme activity when each was incubated with phytic acid beforehand (Deshpande & Cheryan, 1984). This implies that there was more Mg^{+2} available in the system than Ca^{+2}, indicating Ca^{+2}'s greater affinity for phytic acid. This issue is exacerbated by Mg^{+2} being a more effective ion for salting out (Zhang & Cremer, 2010). If the researchers are using the same amount of coagulant for their alternative legumes without factoring in the amount of phytic acid specific to each sample they may not be fully coagulating the protein. Unfortunately, researchers cannot simply adjust coagulant amounts because the rate of coagulant addition affects the curding (Cai & Chang, 1998). Ergo, the rate the coagulant added needs to be determined based on the total coagulant needed, the concentration of the coagulant solution, and the concentration of the protein in the legume milk.

Palatable tofus have been made by replacing up to 40 % of the soy bean with lupin beans (Jayasena, Khu, & Nasar-Abbas, 2010), but the inclusion of soy undercuts many of the purposes

of making non-conventional tofu. Cai, McCurdy, & Baik, (2002) attempted making protein curds with soy, fava beans, peas, chickpea, and mung beans, but used a method more fitting for analysis of legume components than food production. The legumes were milled to flour and extracted twice with water. The soluble portions were combined and freeze dried. To make the tofu, the dried extract was reconstituted to 3 % protein, without factoring in the starch content, and boiled for 10 min. This is the most aggressive heat treatment in tofu literature. The mechanical properties of curd made with each bean deviated greatly within its own sample set. Furthermore, tofu manufacturers start with whole beans and soak them prior to grinding, skipping the soaking step could prevent enzymatic activity that would be relevant to manufacturers and future research.

1.10.2 Cultivars Requirements for Tofu

Soy gels made using only glycinin or β-conglycinin showed that glycine formed a hard, brittle gel, and β-conglycinin formed a soft flexible gel (Williams, 2011), so the ratio of these proteins will need to be in balance depending on consumer acceptance. Soy has been used to produce tofu for millennia, so there has been time to develop cultivars with proper characteristics. Cultivars with a higher 11S/7S ratio are firmer and more cohesive (Wu et al., 2017), but the smoothness decreased at ratios greater than 2.0 11S to 7S (Ji, Cai, & Chang, 1999). As previously mentioned, pea legumin and vicilin can mimic soy glycine and β-conglycinin respectively. Peas ratio of legumin to vicilin is between 0.2-1.5 depending on cultivar (Casey, Sharman, Wright, James, & Guldager, 1982). This is below the 2.0 ratio where any of the quality attributes start to decline, and well below the optimum for hardness which is considered the most important for tofu, but with time pea cultivars could be developed with appropriate ratios. While traditional selective breading is an option for improving the ratio, there

are extrinsic factors that can affect the ratio. Drier years and silty soils have been shown to improve the ratio (Mertens, Dehon, Bourgeois, Verhaeghe-Cartrysse, & Blecker, 2012).

1.11 A Unique Approach is Needed

While soy has many similarities to pulse crops, there are still many differences that need to be addressed. To this end, if viable soy-free tofus are to be produced, the production process needs to be changed to accommodate the increase in starch, decrease in protein, and near elimination of fat when compared to soy. All previous attempts ran beans “as is” and did not attempt to adjust the fat content. Soy beans are used as an oil crop because it has a relatively high fat content, about 20 %. Protein confirmation and hydrophobic interactions are shown to play an important part of tofu gel formation, but all studies previously cited on the matter of gel formation were performed on soy protein isolate or glycinin faction prepared from defatted soy protein isolate (Kohyama et al., 1995; Matsudomi et al., 1985; Nakamura et al., 1984). There has been little to no research on the effects lipids have on soy protein gelation in terms of curding. Since hydrophobic interactions lead to aggregation after the hydrophobic portions of the protein are exposed a fat globule could serve as a nucleation site that could attract proteins to form micelles around it, regulating the number or rates of curding. It is likely that by removing the excess starch and adding fat peas could provide a suitable alternative to soy in tofu manufacturing.

1.12 Conclusions

Tofu is a protein rich food, traditionally made from soy, which has been consumed for millennia. With current consumer trends leading to avoidance of food products containing soy and looming food demands, resulting from increasing global populations, alternatives to soy-based tofu are needed. While pulses are defined differently from soy due to how they are traded

on the market, botanically there are many similarities. Pulses, like the yellow field pea, contain similar protein subclasses as soy, and theoretically have the potential to replace soy in tofu. While there are many similarities, the fundamental differences between the two crops are too great to directly apply traditional tofu manufacturing practices to achieve a desired product. No publications exist exploring the interrelationship between biochemical composition, processing methods, and product quality for application of non-soy crops in tofu production. Future research should focus on this area to improve the feasibility of non-soy tofus.

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CHAPTER 2: EFFECT OF PROCESSING ON THE CHEMISTRY, TEXTURE, AND MICROSTRUCTURE OF TOFU MADE FROM YELLOW FIELD PEA (*PISUM SATIVUM*)

2.1 Abstract

Tofu, made by coagulating soy milk, is a nutritious food originating in China and is widely consumed in current global markets. However, due to allergenicity and consumer perceptions of risks from consuming genetically modified organisms consumer demand for soy alternatives is increasing. In this study, tofu was made from yellow split peas (*Pisum sativum*). The effects of pasteurization, fat addition and disrupting then re-pressing the curds were studied. Pasteurization because the tofu to take on water in non-disrupted samples causing the fat added tofu to fall apart, but disrupted samples became firmer with pasteurization. Texture profile analysis indicated that the disrupted+pasteurized tofu had improved mechanical properties by increasing tofu hardness from ~175 g force from the control to ~325 g force for the disrupted+pasteurized no fat added sample. A similar trend was observed for the fat added samples where hardness increased from ~50 g force to ~75 g force. FTIR spectroscopy of the amide I region showed a reduction of ordered structures correlated to an increase in hardness. The shifts in β-sheet structures followed the same trends as surface hydrophobicity. Surface hydrophobicity increased with both pasteurization and disruption. Similarly, the molecular weights showed that shear and heat separately degraded the proteins, indicating a breakdown of proteins into smaller polypeptides and exposure of hydrophobic interiors. These changes in biochemical parameters allowed for a tofu to be made from the yellow field pea.

2.2 Introduction

As populations grow in developing nations and developed nations become more health conscious there has been an increase in demand for plant-based protein sources. High protein soy foods, like tofu, have long been a staple for vegetarians or cultures that lack graze land.

However, allergies and mistrust of genetically modified organisms (GMO) are pushing other consumers and consequently farmers to look into different crops, particularly pulses.

While there is little research to back up negative health claims of GMOs, consumer perception tends to be negative. Typical male and female shoppers were willing to pay 26% and 49 % more for organic foods, respectively (Burton, Rigby, Young, & James, 2001). However, since cost is a major factor in a consumer's willingness to buy, only more affluent individuals are willing to pay more for GMO-free tofu (Jan, Fu, & Huang, 2007). In addition to this, there has been an increase in allergy related hospitalizations in western cultures (Turner et al., 2015) (Rudders, Arias, & Camargo, 2014). It is estimated that 0.3% of the general population (Young, Stoneham, Petruckevitch, Barton, & Rona, 1994) and 0.4% of children under 3 (Bock, 1987), exhibit allergic reactions to soy. For these reasons, lower valued pulse crops like the yellow field pea (*Pisum sativum*) have the potential to fulfill consumer needs in products like tofu.

For tofu production, soy is traditionally used because it is a readily available protein with a good amino acid profile. Soy based tofu is formed from two main proteins; glycinin a hexamer which provides firmness, and β-conglycinin a trimer which provides elasticity (Williams, 2011). The proteins dissociate into their subunits when heated (Mession, Chihi, Sok, & Saurel, 2015)], and re-associate based on hydrophobic interactions (Kohyama, Sano, & Doi, 1995). For tofu production, soy milk, containing these proteins, is heated and a cationic coagulant is added. Heating the milk dissociates the glycinin and β-conglycinin, so when the cationic coagulant is added it can bind to the newly exposed negatively charged groups, precipitating the proteins into curds. The curds are then pressed to remove moisture and form a cohesive block. The heating of the milk is a sufficient microbiological kill step but occurs before packaging. Therefore, a pasteurization step is added to kill any contaminants from packaging and to extend shelf-life.

This extension increases the shelf-life from days to weeks, allowing tofu to be shipped and stored. Furthermore, this pasteurization increases hardness of the tofu (Huang, Fu, and Chi-Tang Ho, 2003).

Peas have the potential to be used as an alternative in tofu manufacturing if some of the processes are adjusted to account for lower lipid content and fewer disulfide linkages between proteins (O'Kane, Happe, Vereijken, Gruppen, & Van Boekel, 2004b). Peas contain proteins that are structurally analogous to soy proteins. Both pea legumin and soy glycinin are hexameric proteins of approximately 360 kDa (Schwenke et al., 2001) (Williams, 2011), where each subunit is made of an acidic and basic polypeptide (Bacon, Lambert, Phalp, Plumb, & Wright, 1987) and connected by a disulfide bond (O'Kane et al., 2004b) (Rawel & Rohn, 2002), and both pea vicilin and soy β-conglycinin are trimetric proteins of approximately 170 kDa (O'Kane, Happe, Vereijken, Gruppen, & Van Boekel, 2004a) (Maruyama et al., 1999). While the proteins of soy and peas are similar few studies exist describing protein curd formation from peas and to the authors' knowledge, no work has been published on tofu production from peas which effectively mimics commercial conditions.

Aside from proteins, a major difference in composition between soy and the yellow field pea is lipid content. Up to 30 % (w/w) of the neutral lipids in soy milk can be homogenized by the proteins and the rest by endogenous phospholipids (Tomotada, Motoyoshi, & Guo, 1996). Furthermore, the phospholipids increase the amount of protein that form soluble particles; proteins in particle form, increase the uniformity of the curds in tofu (Tomotada et al., 1996). While the yellow field pea contains 1.36% phospholipids (Hefnawy, 2011) compared to soy's 0.89% (L. Wang, Wang, & Fehr, 2006), peas have a range of total lipids between 0.7% - 3.95 %

(w/w) (N. Wang, Hatcher, Warkentin, & Toews, 2010)(Nikolopoulou, Grigorakis, Stasini, Alexis, & Iliadis, 2007), as opposed to soybeans' 15.4-25.9% (Piper & Boote, 1999).

For this study, the amount of fat in the pea milk was increased to that of soy milk, in order to examine the effects of lipids on the gel network. In addition to this, our preliminary findings demonstrated that heated peas proteins formed curds with a soft texture which could not stay together in a uniform mass. This might be attributed to fewer disulfide bonds present in peas proteins; legumin has 63% the amount of cysteine as glycinin (O'Kane et al., 2004b), resulting in a weaker gel. To account for this, we hypothesized that physically disrupting the pea protein gel would allow for more moisture expulsion and void removal during the second pressing which would lead to improve the texture. We also hypothesized that the large discrepancy in lipid content between soy and yellow field peas would have an effect on protein structure and tofu quality. Therefore, the objectives of this study were to examine the effects of lipid addition and protein gel disruption on the texture and microstructure of tofu made from the yellow field pea.

2.3 Materials and Methods

Yellow split peas packaged by Columbia Bean & Produce (Moses Lake, WA, U.S.A.) were purchased at Winco (Moscow, ID, U.S.A.).

Dried Refined Japanese Nigari (Magnesium chloride) from Handy Pantry (Salt Lake City, UT, U.S.A) purchased on Amazon (Seattle, WA, U.S.A.)

2.3.1 Tofu Production

2.3.1.1 Pea Milk Preparation

Pea milk and pea tofu preparation was based on the method used by Kim & Wicker (2005) with modifications. Several aliquots of 400 g of dry peas (Columbia Bean & Produce,

Moses Lake, WA, USA) were soaked for 16 hours in 1000 mL beakers. The amount of water needed for grinding was calculated with the following equation:

(Start pea weight / 200 g) × 550 mL – (end pea weight – start pea weight) = water to add

After water addition, peas were ground using a 120 v, 60 Hz, 900 v Ninja blender model#: NJ600WM (SharkNinja Operating LLC, Needham, MA, USA) on setting 3 for 2 min. The slurry was filtered through a cotton cloth with a thread count of 160 per inch by placing the cloth over a strainer, pouring the slurry into the cavity, bringing the corners together to form a bundle, and squeezing by hand to remove the insoluble material. The cloth was rinsed, and the pea milk was filtered a second time. The pea milk was allowed to rest in a refrigerator at approximately 5° C for 1 hour to allow remaining suspended solids to settle. The pea milk was then decanted to remove settled solids.

2.3.1.2 Curd Formation

Pea milk (474.75 g) was brought from approximately 5 °C to 80 °C over a period of approximately 9 min. Milk was removed from heat and 25.24 g of 0.82 M magnesium chloride solution was added over the course of 10 sec, stirring constantly. The solution was then allowed to rest for a period of 10 min to allow for curd formation. The curds were poured into a 12.8 × 9.1 × 7.9 cm tofu mold lined with a cotton cloth with a thread count of 160 per inch. The cloth was folded once over the curds and a piece of plastic wrapped cardboard was placed on the cloth before the other sides of the cloth were folded. The addition of the plastic wrapped cardboard was to mitigate excessive wrinkling in the tofu cloth and prevent molding defects. A 5 kg weight was placed on top of the mold cover for 10 min. The tofu was removed from the mold and held at 32 °C in a proofing oven until secondary processing. For samples containing fat, methods were the same as described above, but prior to bringing the milk to 80 °C, 456.83 g pea milk and

17.93 g corn oil were homogenized with a Cuisinart Smart Stick 200 W Variable Speed Handheld Immersion Blender (Cuisinart, Stamford, CT, USA) for 2 min on the highest setting. The amount of oil addition, 3.8 % (w/w), is slightly lower than that of soy milk, but the highest addition level that would reliably produce a mass that resembled soy tofu. All samples were prepared in triplicate.

2.3.1.3 Secondary Processes

Each block of tofu was quartered. One quarter was used as a control and the three other quarters underwent one of three secondary processes. The three secondary processes were pasteurization, disruption, and disruption+pasteurization. For pasteurized samples, tofu was placed in a water bath at 98 °C for 5 min. The samples were removed from the water bath and excess moisture was removed by gently dabbing with a cloth. The pasteurization temperature of 98 °C for 5 min was selected because preliminary tests showed that was ample time for the center of a block to reach 72 °C and be above for 15 sec, per pasteurization FDA guidelines (U. S. Food and Drug Administration, 2017). For the disrupted samples, tofu was broken by hand into ~1 mm pieces. The disrupted tofu was then wrapped in a cloth, and pressed with a 2 kg weight in a 55 mm × 55 mm × 60 mm mold for 5 min. The disrupted and pasteurized tofu was disrupted as previously described and after being unmolded, was pasteurized at 98 °C for 5 min as previously described. After unmolding samples from the secondary process, all sample were wrapped in plastic wrap, and refrigerated 4 °C overnight.

2.3.1.3 Freeze Drying

For microscopy, surface hydrophobicity, and Fourier-transform infrared spectroscopy (FTIR) analysis samples were frozen in liquid nitrogen, stored at -80 °C, and once all samples

were collected they were freeze dried in a Freezone 6 (Labconco, Kansas City, Missouri, USA) at -56 °C and 0.02 mbar. Dried samples were stored frozen.

2.3.2 Texture Profile Analysis

Texture profile analysis (TPA) was performed with a TA,XT.plus (Stable Micro Systems Ltd., Godalming, Surrey, United Kingdom). A 25 mm diameter cylindrical plastic probe attached to a 5 kg load cell was used for all TPA measurements. A pre-test speed of 1.00 mm/s, test speed of 0.83 mm/s, and post-test speed of 2.00 mm/sec was used with a trigger force of 5.0 g to compress the tofu a distance of 30% of the block thickness (1 cm). Rest time between cycles was 2.0 sec. Hardness, cohesiveness, and springiness data were collected. Samples were removed from refrigeration 30 min before testing. To ensure uniformity, samples were cut into 1 cm cubes using 2 blades fastened together 1 cm apart.

For a comparison, 8 brands of commercially available tofu were purchased from local grocery outlets. Tofus corresponding to silken, soft, firm, and extra firm was obtained. Tofu was prepared and analyzed for texture, as described for experimental treatments.

2.3.3 Moisture Content

The AOAC Official Method 950.46 was used to determine the moisture. The mass of a 4 oz Gilson SC-402 tin and its lid was recorded. The tin was tared and a ~2 g sample of tofu was placed in the tin. The sample was macerated and spread into a thin layer. The final weight of the sample was recorded. The tin containing tofu was held at 135 °C for 2 hours. After cooling for 15 min final sample weights were recorded. The moisture was calculated by the following equation:

$$1 - [(\text{End weight} - \text{tin and lid}) / \text{sample weight}] = \text{moisture content}$$

2.3.4 FTIR Analysis

IR spectra were recorded with a NICOLET iS10 Fourier transform IR spectrometer (Thermo Fisher Scientific, Waltham, MA). The instrument was purged continuously with dry air. For transmission measurements, samples were allowed 45 min to come up to room temperature. For each spectrum, 64 scans were collected from 400-4,000 cm^{-1} at a resolution of 4.00 cm^{-1}. Spectra were obtained by the

method of attenuated total reflection (ATR), using a diamond, 45° angle of incidence, and 1 bounce. For spectral analysis, the spectra for lipids were first subtracted using Omnic 9 software (Thermo Fisher Scientific, Waltham, MA). After subtraction of the lipid spectra, spectra were analyzed on OriginPro 2018 (OriginLab, Northampton, MA). After baseline correction, spectra were normalized and the amide I region of the spectra was deconvoluted. To accomplish this, the second derivative was used to find hidden peaks. Using 50 iterations, the amide I region was fitted with Lorentzian peaks with the full width at half maximum set to 20 cm^{-1}. Areas under the model corresponding to α-helices (1650-1658) and β-sheets (1630-1638) were quantified and divided by the sum of areas of all peaks discovered in the amide I region.

2.3.5 Molecular Weight Analysis

A portion of each freeze-dried sample was ground and vortexed with an excess of hexane for 30 min at 450 RPM in a shaker incubator at 27 °C. The samples were centrifuged at 340 *g* for 1 min. The excess hexane was decanted, and the remaining hexane evaporated off samples. To solubilize proteins, microcentrifuge tubes containing 10.0 mg of sample were vortexed with a 50 mM pH 7 sodium phosphate buffer containing 150 mM NaCl and 8 M urea for 15 min, then centrifuged at 9875 *g* for 5 min. The resulting supernatant was then diluted 1:1 with a 50 mM pH 7 sodium phosphate buffer containing 150 mM NaCl and vortexed. The supernatant dilution was

completed in order to lower urea concentration to be within specifications of protocols for subsequent microfluidic analysis on an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). Extracted samples were analyzed reduced and unreduced. Analysis was completed in accordance to the instrument manufacturer's specifications. 2-mercaptoethanol was used as the reducing agent.

2.3.6 Surface Hydrophobicity

The method used to determine surface hydrophobicity was modified from Chelh, Gatellier, and Santé-Lhoutellier (2006). A Bromophenol blue (BPB) solution of 1 mg/mL was prepared with DI water and shielded from light until use. Tubes containing freeze dried samples corresponding to 100 mg of protein and 10 mL of 20 mM Na-Phosphate buffer with a pH of 7.0 were preheated to 40 °C. Protein content determination was completed via nitrogen combustion on a LECO Nitrogen Analyzer (LECO Corp. At. Joseph, MI). Samples were vortexed at 450 rpm for 30 min in a shaker incubator set to 40 °C. Samples were cooled for 5 min and 2000 µL of BPB solution was added. Samples were vortexed for 10 min at with a 27 °C shaker incubator at 450 rpm and centrifuged for 15 min at 2000 *g*. The samples were diluted by transferring 100 µL of each sample into a microcentrifuge with 900 µL of buffer and vortexed briefly. Samples were transferred to cuvettes and ran on an Eppendorf biophotometer at 595 nm.

2.3.7 Microscopy

A piece of freeze dried sample, approximately 2 mm x 2 mm, was mounded to a slide. A Quanta 200F SEM (FEI Company, Hillsboro, OR, USA) was used to complete scanning electron microscopy (SEM) backscattering electron (BSE) imaging at 130 Pa and an accelerating voltage of 15.0 kV.

2.3.8 Statistical Analysis

All samples were analyzed in triplicate at a significance level of P=0.05. Analysis of variance with a Tukey's post hoc analysis was completed with Statistical Analysis Software (SAS Institute Inc., Cary, North Carolina).

2.4 Results and Discussion

2.4.1 Texture Profile Analysis

Hardness (fig 2.1) was significantly ($P<0.05$) higher in the no fat added treatments. Within a fat added or no fat added treatments, neither pasteurization nor disruption alone significantly ($P<0.05$) effected hardness. However, disrupted+pasteurized samples were significantly ($P<0.05$) harder; in tofu hardness is commonly considered the most important quality attribute (Johnson, 1984). For the pasteurized fat added treatments, the resulting tofu appeared watery and was difficult to handle (fig 2.2). In fact, the samples were so easily friable, that they were omitted from TPA analysis. It should also be mentioned that in preliminary studies increasing pressing weight had little effect on texture (data not shown). To put these values in perspective, the commercial tofus hardness ranged from 51-54 g force for silken and soft varieties, 54-86 g force for firm varieties, and 64-242 g force for extra firm varies across different brands. As stated in the introduction there is no standard for tofu firmness and it varies widely between manufacturers. It is interesting that values similar to the silken and extra firm can be obtained through the variability of the experimental treatments. Because of this inconsistency in soy based tofus, they could not be used as a gold standard for comparison. To this end, laboratory made soy tofu was not able to be reformed after disruption and therefore could not be used a standard to measure against within this study.

The springiness (fig 2.1) of the tofu behaved similarly between the no fat added and the fat added samples with a noteworthy difference. In the no fat added samples the springiness of the disrupted and the disrupted+pasteurized samples were significantly lower than that of the control. Since the springiness of the other samples were not significantly different from the control, the difference likely came from the disruption. Furthermore, in the no fat added treatments, pasteurization significantly increased the springiness of the disrupted+pasteurized samples. The fat added samples followed the same trend, but the added fat cushioned the reduction in springiness to the point where the high temperature pasteurization brought the tofu subjected to the disrupted+pasteurized treatment springiness values up to the point where there was no significant difference from the control ($P>0.05$). Pasteurization increased springiness by approximately 0.10 in both no fat added, and fat added samples.

The cohesiveness (fig 2.1) of the samples followed similar trends to springiness, in that disruption decreased the cohesiveness and fat addition reduced the overall loss of cohesiveness. The exception to this was the fat added pasteurized treatments that fell apart so easily they were not testable by TPA. The key difference was that pasteurization after disruption did not significantly increase the cohesiveness compared to the non-pasteurized disrupted sample, where it did increase springiness (fig 2.1).

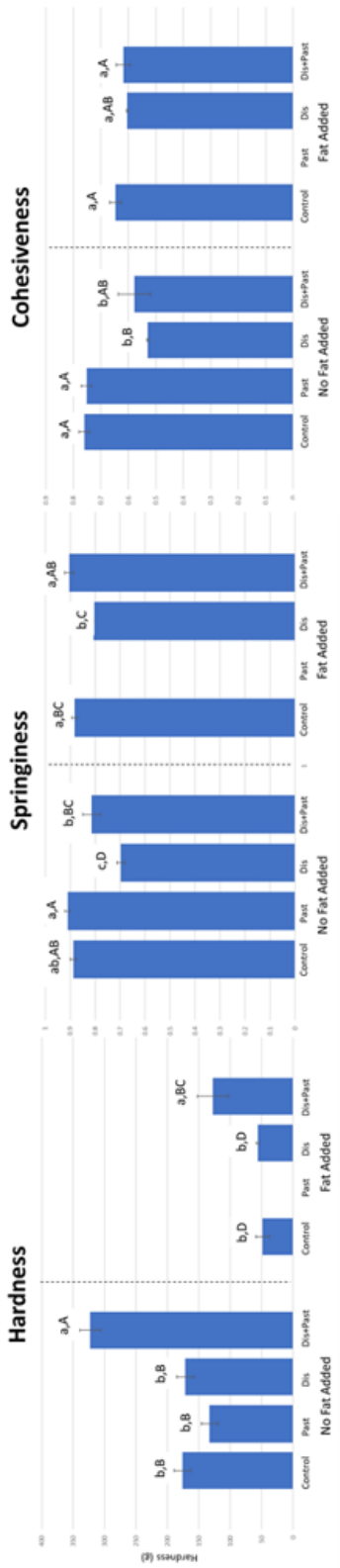

Figure 2.1: Graphical representation of Hardness (left), Springiness (center), and Cohesiveness (right) data for control, pasteurized (Pas), disrupted (Dis), and disrupted+pasteurized (Dis + Past) pea tofu processed with and without fat addition. Values with a common lower-case letter within a fat treatment represent tofus that are significantly different ($P<0.05$). Values with a common upper-case letter are significantly different ($P<0.05$) across fat treatments.

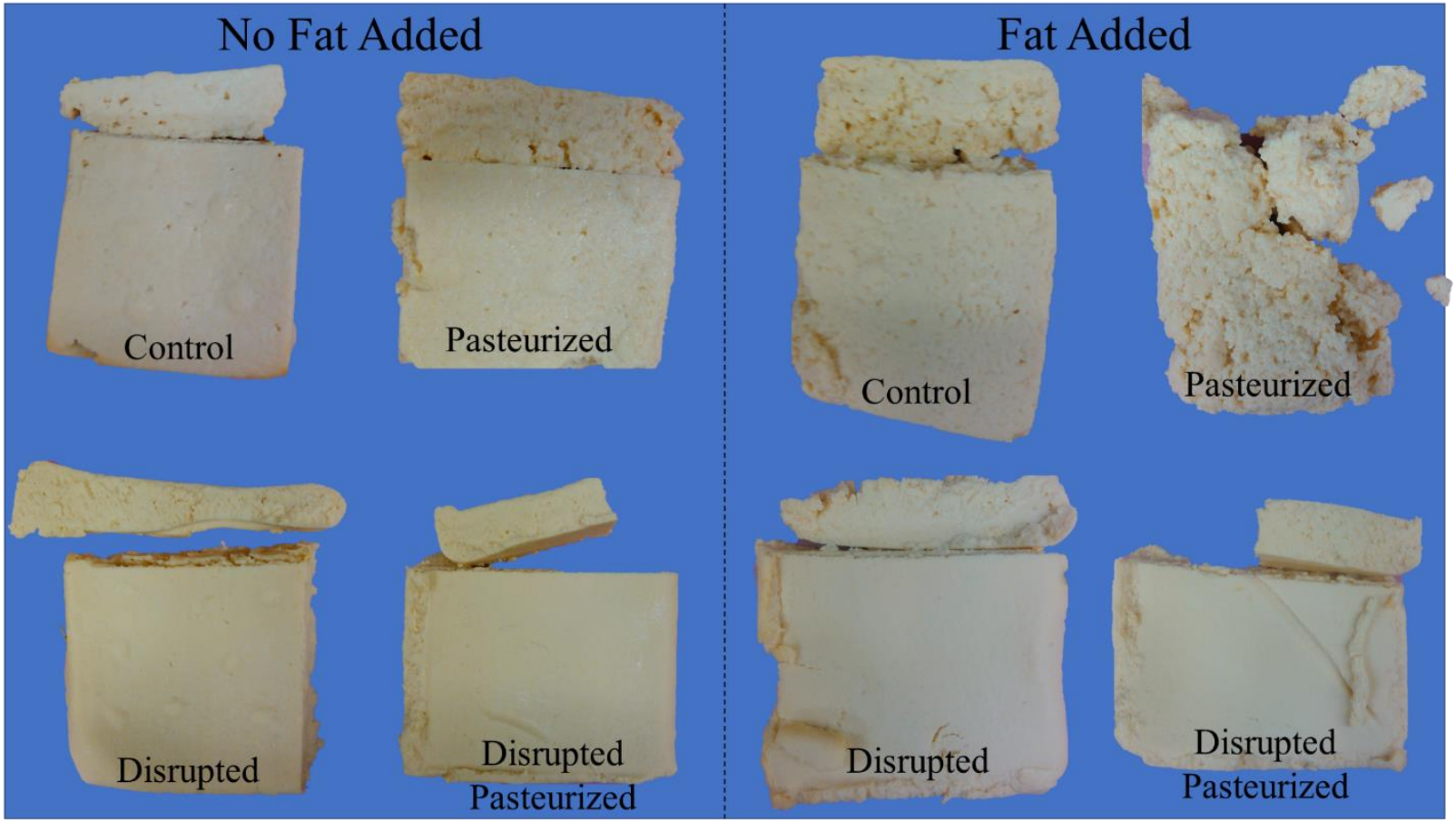

Figure 2.2: Visual representation of control, pasteurized, disrupted, and disrupted + pasteurized treatments. The four tofu samples on the left represent no fat added treatments and samples on the right represent fat added treatments.

2.4.2 Percent Solids

The percent solids (fig 2.3) were significantly lower in the non-disrupted pasteurized samples than the control. However, if the sample was disrupted prior to pasteurization, the samples absorbed less water and were not significantly different from the control ($P>0.05$). Other studies investigating soy-free tofu (Cai, McCurdy, & Baik, 2002) (Cai, Klamczynska, & Baik, 2001) (Mohamed, Johan, & Bakar, 1989) which were already suffering from low firmness, do not include a pasteurization step and would likely have incorporated more water, further reducing firmness if they were pasteurized.

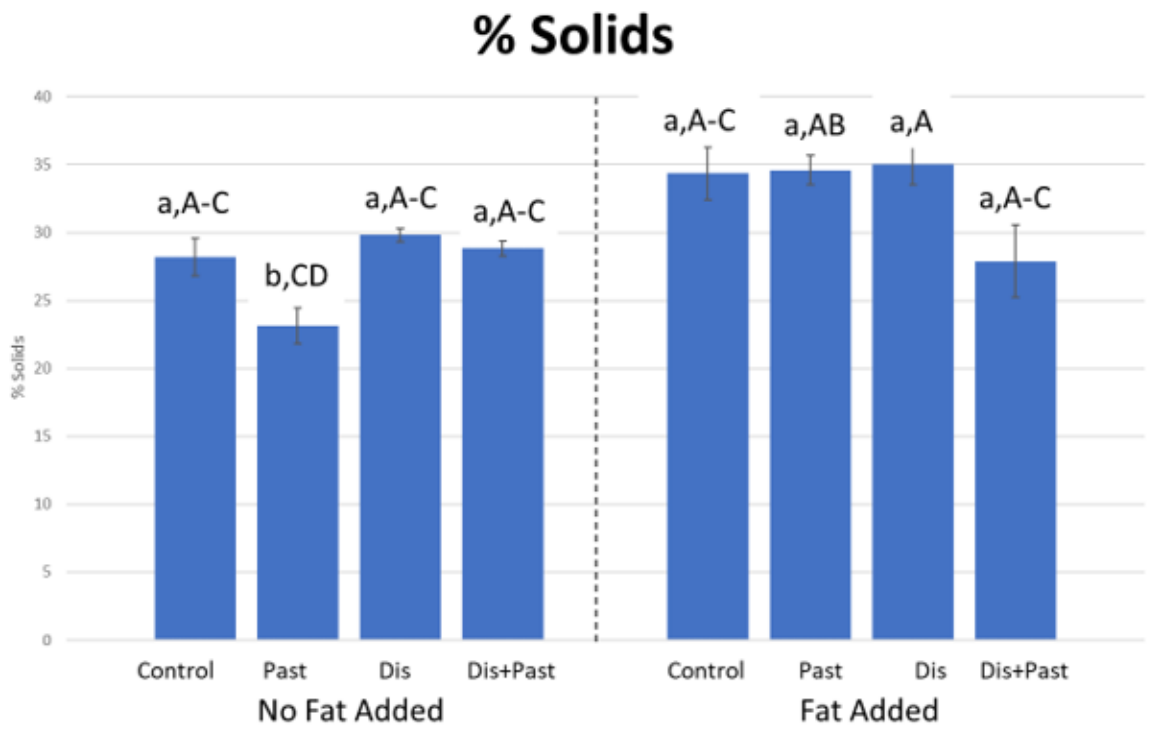

Figure 2.3: A graphical representation of % Solids data for control, pasteurized (Past), disrupted (Dis), and disrupted+pasteurized (Dis + Past) pea tofu processed with (right) without (left) fat addition (3.8 %). Values with a common lower case letter within a fat treatment represent tofus that are significantly different ($P<0.05$). Values with a common upper case letter are significantly different ($P<0.05$) across lipid treatments.

2.4.3 FTIR Analysis

The amide I region (1700-1600) was deconvoluted, examining the percent of the peak comprised of α-helices (~1653-1658) and β-sheets (~1630-1638) (fig 2.4). Other areas in the amide I region were assessed but found to be more variable with no significant differences between treatments (data not shown). The α-helices were not significantly different within a disruption treatment between the no fat and fat added samples. Within the no fat added treatments, the presence of α-helices in pasteurized sample was significantly higher than the control, the disrupted sample was not significantly different ($P>0.05$) from the control, and the disrupted-pasteurized sample was significantly lower than the control ($P<0.05$).

The percent of α-helices in the tofu with fat added was significantly higher ($P<0.05$) in the pasteurized sample than in the control (fig 2.4). Both the tofus made with the disrupted method and disrupted+pasteurized method were significantly lower ($P<0.05$) in α-helix content than the control and pasteurized samples. Disruption reduces the percent of α-helices in the fat added tofu more than in the no fat added samples. Despite the increase of α-helices occurring in the pasteurized sample, the disrupted+pasteurized sample is lower in α-helical content than all other samples. While no conclusions can be made on why there was an additive effect on α-helices with the combination of disruption and pasteurization treatments, the trends relate to with the tofu hardness (fig 2.1). Given the fact that α-helices are a more ordered structure with lesser molecular mobility than other secondary protein structures, it is likely that the loss of α-helices led to greater interprotein interactions. This in turn, may have allowed for an increased ability to form a gel.

The percent of β-sheets in the no fat added treatments was significantly different ($P<0.05$) in the pasteurized sample than in the control (fig 2.4). The disrupted treatment yielded

tofu that was more variable and was not significantly different than the control or pasteurized treatments ($P>0.05$) in β-sheet content. The disrupted+pasteurized treated samples were significantly lower ($P<0.05$) in β-sheets than the control, but the opposite was observed for tofu not pasteurized or disrupted samples (fig 2.4). The β-sheets in both the no fat and fat added samples followed similar trends and appear to be inversely proportionate to the α-helices except for the disrupted+pasteurized sample, where both are decreased. In the control, pasteurized, and disrupted samples approximately 29 % of the amide I region is α-helices and β-sheets, except for the disrupted+pasteurized sample, which is 22.7 % and 25.5 % α-helices and β-sheets in the no fat and fat added samples, respectively. The variability of the unordered structures in the amide I prevents determination of which specific structures are leading to changes in tofu texture observed in (fig 2.1). However, the shift towards an increase in unordered structure fits well with an increase in tofu firmness.

Przybycien and Bailey (1991) found that the surface area of the β-sheets improves intermolecular interactions and improves the gel structure. The β-sheets in the experimental tofus appear to correlate with hardness except for the disrupted+pasteurized sample, which has the lowest percent of β-sheets but the highest hardness (fig 2.1 & 2. 4. While the decrease of ordered structures could be increasing the mobility of the proteins allowing for greater interprotein interactions and gel formation, another explanation is that the increase in hardness is a result of decreased moisture and voids from this particular treatment. This would simply increase the density of the tofu influencing texture. A combined effect of shifts in protein secondary structure and increased tofu density are likely both responsible for the differences observed in tofu texture (fig 2.1-2.4).

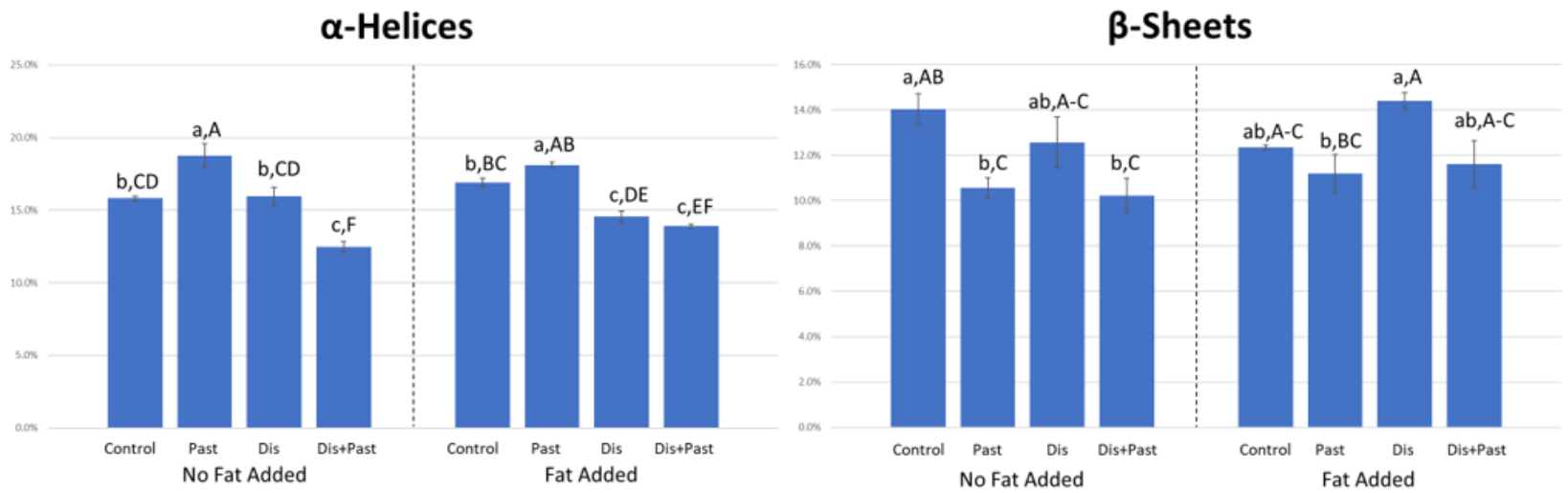

Figure 2.4: A graphical representation of α-helical (left) and β-Sheets (right) content for control, pasteurized, disrupted, and disrupted+pasteurized pea tofu processed with and without lipid addition. Values with a common lowercase letter within a lipid treatment represent tofus that are not significantly different ($P<0.05$). Values with a common uppercase letter are not significantly different ($P<0.05$) across lipid treatments.

2.4.4 Molecular Weight Analysis

Due to molecular similarities to glycinin and β-conglycinin, vicilin and legumin are key to tofu production. In the no fat samples, vicilin dissociated into subunits and polypeptides. This was evident by the increase in the 16, 19, and 31 kDa polypeptides as well as the 33 kDa peak (fig 2.5) (Shewry, 1995). The 33 kDa peak represents both a subunit of vicilin and a polypeptide of the 50 kDa vicilin subunit, depending where the subunit is cleaved (fig 2.5) (Shewry, 1995). The increase in the 33 peak is cumulative between the pasteurization and disruption, suggesting that shear from disruption and heat denature separately. The fat added samples exhibited an increase in the 33 kDa peak as well, but the 16, 19, and 31 kDa polypeptides were stable throughout treatments (fig 2.5). The ability of pea proteins to associate with and emulsify lipids is well documented (Gharsallaoui et al., 2010). One of the key characteristics of a protein emulsifier, is its ability to denature and rapidly disperse along the oil water interface (Gharsallaoui et al., 2010). This denaturation and association with lipids will lead to conformational changes (Gharsallaoui et al., 2010). Therefore, it is possible these polypeptides fully dissociated when the lipids were homogenized, so they would not dissociate with additional shear. These results are further confirmed with reduction of disulfide bonds by 2-ME (fig 2.5). Furthermore, the reduced sample is showing increases in the basic (21 and 24 kDa) and acidic (40 kDa) polypeptides, indicating these subunits had re-associated using disulfide bonds, similar to soy tofu. It should be noted that the electropherograms for all reduced experimental treatments are too similar to differentiate from one another. For this reason, only one plot representing all reduced samples is shown in figure 2.5. Both the no fat and fat added tofus had an increase in peak area at 54 kDa. The 54 kDa peak was disulfide linked and completely disappeared with reduction (fig 2.5). This molecular weight is not typically associated with tofu, rather it is a

protein associated with chloroplast (Franklin & Hoffman, 1993). However, some researchers have reported that with succinylation, legumin's acidic polypeptide could be approximately 54 kDa, indicating either the breakdown of the 60 kDa subunit or loss of the succinyl group (Schwenke et al., 1990). In either case, changes to this peak appear to be associated with experimental treatments. It is possible these proteins are dissociating and interacting with vicilin and/or legume and influencing physical properties. For this reason, the proteins in the 54 kDa range may warrant future investigation.

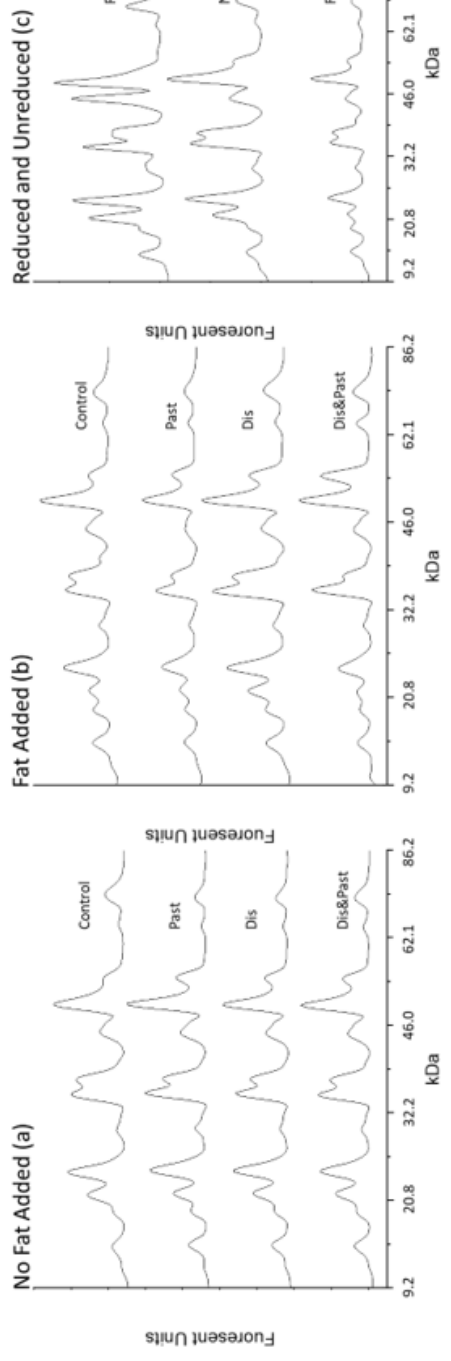

Figure 2.5: Electropherograms representing protein extract molecular weight distributions of no fat added (a) and fat added (b) treatments for control, pasteurized, disrupted, and disrupted+pasteurized pea tofu. Electropherograms representing no-fat added, fat added, and proteins extracted from a tofu in the presence of 2-ME, a reducing agent (c).

2.4.5 Surface Hydrophobicity

Both pasteurization and disruption had significant ($P<0.05$) effects on the surface hydrophobicity of the samples, for both no fat added samples and fat added samples (fig 2.6). For each treatment the fat added samples were significantly ($P<0.05$) lower than the no fat added counterpart. These findings are indicative of conformational changes to the proteins of the pea milk, where fewer hydrophobic regions of the protein are exposed to the aqueous phase of the solution. In soy the β-conglycinin protein, which is analogous to vicilin, associates with phospholipids to promote the formation of soluble particles, the phospholipids then provide binding sites for neutral lipids (Tomotada et al., 1996). Peas contain comparable amounts of phospholipids as soy, but much less neutral lipid. With the addition of neutral lipid, we hypothesize that hydrophobic binding sites of pea proteins associate with the lipids promoting the formation of soluble protein particles. Therefore, the decrease in dye binding by the fat added samples can be attributed to hydrophobic regions of the pea proteins binding to the added lipids and becoming unavailable to the dye. Wang et al. (2011) suggested that surface hydrophobicity is directly proportionate to β-sheets. This holds true within this study, as the trends in treatment values between β-sheets (fig 2.4) are nearly identical to those observed for surface hydrophobicity (fig 2.6). Is should also be mentioned that only one range of the amide I region of the FTIR spectra was assessed. There may have also been variability in the 1600-1620 and 1660-1700 bands of the amide I region, but these are typically variable due to overlapping of fitted peaks.

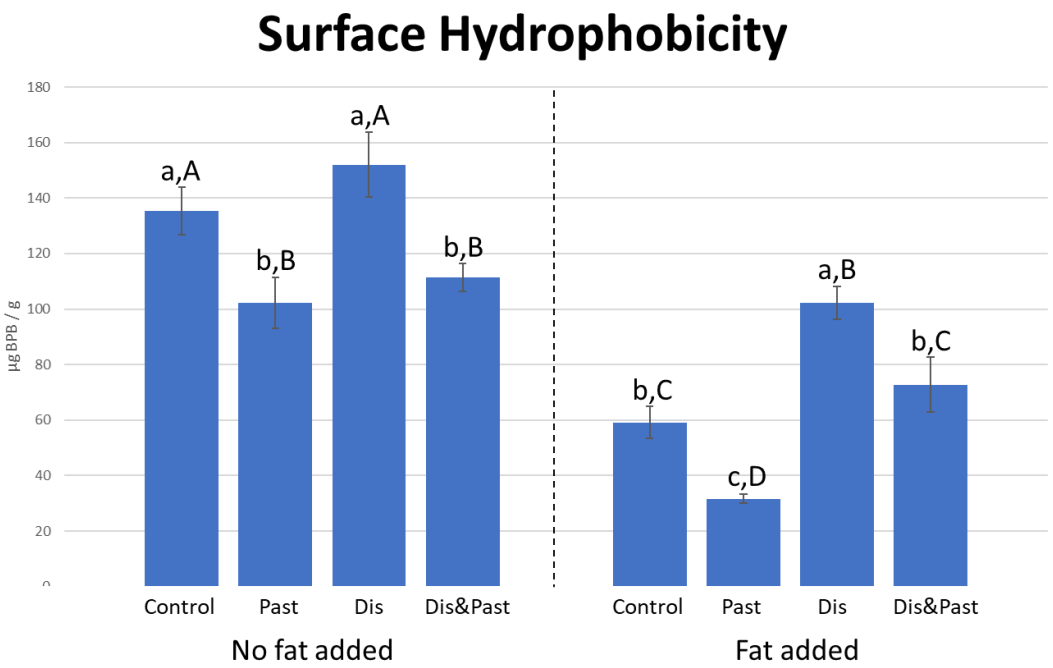

Figure 2.6: A graphical representation of surface hydrophobicity data for control, pasteurized, disrupted, and disrupted+pasteurized pea tofu processed with and without lipid addition. Values with a common lower-case letter within a lipid treatment represent tofus that are not significantly different ($P<0.05$). Values with a common uppercase letter are not significantly different ($P<0.05$) across lipid treatments.

2.4.6 Structure

There was a visual difference between the no fat added and the fat added sample (fig 2.2). The addition of fat increased curd size in all samples. It is possible that proteins associated with the fat increasing the volume of the curds by incorporating the lipids into the network. Fat addition increased the number and apparent volume of voids in the non-disrupted samples. Control samples have medium size curds with some voids. The pasteurized samples have larger, more discreet curds and many large voids. It is likely that the pasteurization exacerbates existing voids that are not visible in the control, to increase the number and severity of the voids. The disrupted samples had smaller curds and fewer voids. The fat added disrupted and disrupted+pasteurized samples had more voids than their no fat added counterparts. It is likely

that void space provides an open space for the tofu to collapse into when compressed, preventing permanent physical deformation.

2.4.7 Microscopy

Historically, the microstructure of tofu is described as having sheets and branched structures with rounded voids that form a honey comb shape (Noh, Park, Pak, Hong, & Yun, 2005) (Prabhakaran, Perera, & Valiyaveettil, 2006)(Kao, Su, & Lee, 2003) (Metussin, 1992). The no fat added treatment microstructure did not form sheets with the pasteurized sample, rather it formed a honey comb with much larger voids than the control (fig 2.7a-b). It is hypothesized that the size of the voids likely increased from the absorption of water caused by in increased in heat induced organized protein segregation. This is evident by the increase in branch structure width (fig 2.7b). The disrupted no fat added sample formed thicker aggregates with minor branching in between (fig 2.7c), while the disrupted+pasteurized sample formed small, densely packed aggregates connected by some honeycomb structures (fig 2.7d). Considering the structure of figure 2.7d, the increase in hardness observed in figure 2.1 was likely a direct result of the increased density of the no fat added disrupted+pasteurized sample or the additive effect of modifications to protein secondary structure and changes to tofu microstructure.

The branching structures and aggregates in the fat added samples are much thicker than the no fat added samples (fig 2.7e-h). Since the surface hydrophobicity of the fat added samples was lower in the fat added samples, the oil is likely being surrounded by the hydrophobic portions of the proteins, bulking the structures. The fat added tofu control (fig 2.7e) had thick honey comb structures. Interestingly, the solid phase of the control fat added sample was continuous with little to no breaks. The voids that were present were large and macroscopic (fig

2.2) in nature. This is interesting, because one would expect the increase in the continuous solid phase to lead to increased tofu hardness, which was not the case in this study. It is likely that this was caused by the increase in protein lipid interactions and decrease in interprotein hydrophobic interactions (fig 2.6). In studies with other it is known that lipids can coat the surface of proteins, having a softening and plasticizing effect (Schober et al 2010; Schober et al 2011). The pasteurized sample (fig 2.7f) was similar to the control but had larger voids. This increase in aggregation is similar to what was observed with the no fat added pasteurized treatment (fig 2.7b). The disrupted sample (fig 2.7g) was comprised mostly of aggregates and the few honey comb structures that were present were not ordered and appear to be associated with individual aggregates. The disrupted+pasteurized sample (fig 2.7h) was also comprised mostly of aggregates but were larger than the disrupted sample (fig 2.7g). Overall, these data demonstrate that smaller more densely packed aggregates with little void space related well with increased hardness values (fig 2.1 & 2.7). There does appear to be an association between the voids and springiness, but it is not as evident in the microstructure as it is macroscopically (fig 2.1, 2.2, & 2.7).

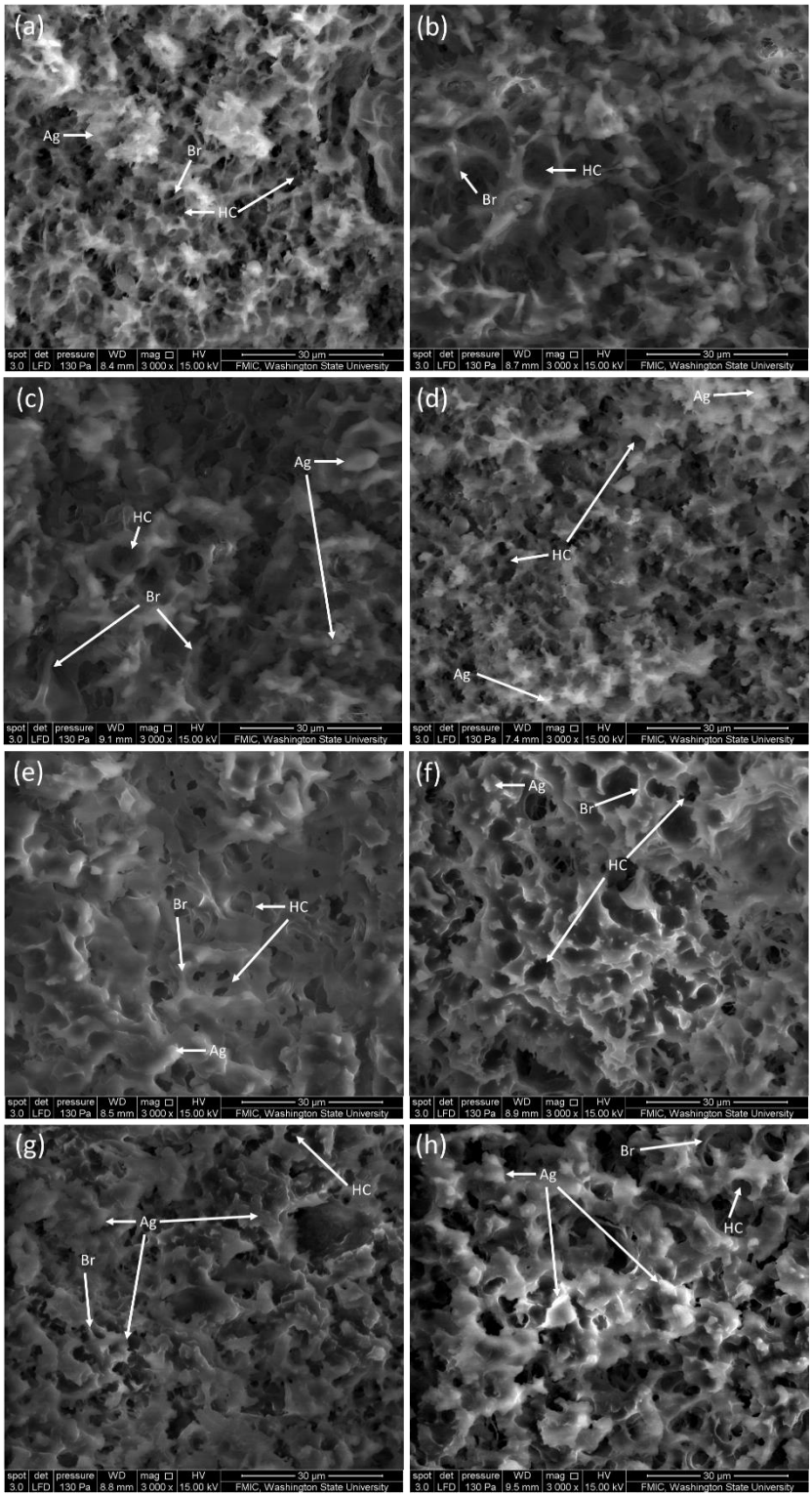

Figure 2.7: Scanning electron micrographs of no fat added control (a), no fat added pasteurized (b), no fat added disrupted (c), no fat added disrupted+pasteurized (d), fat added control (e), fat added pasteurized (f), fat added disrupted (g), and fat added disrupted+pasteurized (h). All micrographs were taken at 3,000 x magnification Ag designates aggregates, Br designates branches, and HC designates honey comb structures.

2.5 Conclusion

Pea based tofu forms the same way soy tofu does; heat dissociates proteins to their subunits which reassociation based on hydrophobic interactions and form disulfide bonds. Fat addition alters how pea proteins aggregate, reducing the surface hydrophobicity and producing a smoother curd. Most tofu processes provide an adequate kill step for safety but manufactures add a pasteurization step to increase shelf-life. Heating the tofu during pasteurization can also improve the texture, but the absorption of water negates the improvement. If the tofu is disrupted prior to pasteurization and repressed, voids are removed, the tofu does not become water logged, and the texture is improved. In addition to this, experimental treatments all have an effect on protein structure and biochemistry. Through the removal of voids and modification of protein structure, disruption made pasteurization a possibility for fat added pea tofu and increased the feasibility of the no fat added variety. While the addition of fat reduced tofu hardness, it produced a more aesthetically appealing tofu and prevented losses in springiness and cohesiveness.

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CHAPTER 3: THE EFFECT OF PROCESSING AND FORMULATION ON TEXTURAL AND BIOCHEMICAL PROPERTIES OF PEA-BASED TOFU

3.1 Abstract

Tofu is a healthy source of protein with cultural significance to much of the world. Soy allergies and consumer mistrust of genetically modified organisms has resulted in an increasing demand for soy free foods. Peas are an underutilized crop that do not require allergen labeling and are rarely genetically modified. Peas contain less protein than soy and vary in protein composition. This, coupled with the fact that peas contain more starch than soy and small amounts of lipids, requires that an alternative procedure for pea tofu production be developed to prevent excessive starch gelatinization while promoting curd development. Pea milk was extracted in a similar method to soy milk with an additional decanting step. In doing so, a pea-based tofu with acceptable texture can be developed. To accomplish this a response surface design (RSM) was utilized to determine optimal oil addition, cook time, and salt concentration. The salt used was 0.82 M $MgCl_2$ solution. Testing ranges were from 0.0 - 4.2 % for oil addition, 60-134 min for cook time, and 5.0-9.2 % for $MgCl_2$ addition. Treatments had varying effects on tofu texture, and most formulations fell within the parameters of commercially produced tofu. Cook time was directly proportional to the hardness, and could be used to match the soft, firm, and extra firm texture targets of conventional soy tofu. This will help satisfy the growing demand for alternatives to soy-based foods.

3.2 Introduction

Chapter 2 looked at the trends seen in tofu fat was added, but the study focused on individual variables and not the synergistic effects of multiple variables on tofu texture. Response Surface Methodology systematically alters multiple variables and creates a surface that span the X×Y×Z axes. Variables that effect the mechanical properties of tofu are heat treatments, coagulant concentrations, and oil addition.

In traditional tofu, soy proteins need to dissociate to expose the hydrophobic regions. This occurs between 65 °C and 75 °C depending on ionic strength (Matsudomi, Mori, Kato, & Kobayahi, 1985), with the higher the ionic strength increasing the temperature required for dissociation. Other research found that some of the proteins remained undissociated after 30 minutes (Wu, Hua, Chen, Kong, & Zhang, 2017), and pea proteins are more thermally stable dissociating between 75 °C and 85° C (Mession, Chihi, Sok, & Saurel, 2015).

The change in dissociation temperator is based on ionic strength (Matsudomi, et al., 1985) which will change when the coagulent is added. The degree of dissociation prior to coagulent addition will effect the amount of protein avilable to react with the coagulent. Unless the proteins are fully reacted at that point, excess coagulent is likely to have an effect on the system.

The dissociaction exposes hydrophobic regions on the protein that determine how the proteins aggregate. In a protein isolate, proteins exclusivly incteract with other proteins, but the addition of oil allows for protein lipid interaction.

Considering the ionic strength affects the temperature required for dissociation, time affects how much is dissociated, and oil will affect how any dissociated proteins aggregate, there is an ample amount of synergistic interaction in the system. The objective of this study is to

determine the synergistic effects of different processing factors (cook time, $MgCl_2$, and oil addition).

3.3 Materials and Methods

3.3.1 Materials

Yellow split peas packaged by Columbia Bean & Produce (Moses Lake, WA, U.S.A.) were purchased at Winco (Moscow, ID, U.S.A.). The Dried Refined Japanese Nigari ($MgCl_2$) was purchased from Handy Pantry (Salt Lake City, UT, U.S.A).

3.3.3 Response Surface Methodology

A variation of Response Surface Methodology (RSM) used by (Smith, Bean, Herald, & Aramouni, 2012; McCarthy, Gallagher, Gormley, Schober, & Arendt, 2005) was used to determine the effects of cook time, $MgCl_2$ addition, and oil addition. Preliminary tests were run to approximate the range of values to be used. A central composite design was prepared in Stat Ease 10 (Stat-Ease Corporation, Minneapolis, MN). Five levels of cook times (45-150 min), $MgCl_2$ (4.2-10 % (w/w)), and oil (0.0-4.2 % (w/w)) were chosen (Table 3.1), and 11 combinations of the variables at the selected levels was completed. Error was assessed using 5 replicates of one treatment combination. The sequential model sum of squares (SMSS), lack-of-fit tests, and the multiple correlation coefficient (R^2) were used to select a model (mean = no model, linear or quadratic) for each response. Treatment variables were used as factors for model selection using the method of Schober et al (2005).

Table 3.1: Coded variable levels for Time, $MgCl_2$, and Oil for the experimental RSM design.

Table 3.1
Experimental Design

	Coded Levels[a]		
Treatment	**Time**	**$MgCl_2$**	**Oil**
1	-1	+1	+1
2	0	0	0
3	-1.414	0	0
4	0	0	+1.414
5	+1.414	0	0
6	0	0	0
7	0	0	0
8	0	-1.414	0
9	0	0	0
10	0	0	0
11	-1	-1	+1
12	+1	-1	+1
13	+1	+1	-1
14	0	+1.414	0
15	0	0	-1.414

[a]Coded levels: Time (min): -1.414 = 45 min, -1 = 60.38 min, 0 = 97.5 min, +1 = 134.62 min, +1.414 = 150 min; $MgCl_2$ (w/w): -1.414 = 4.2%, -1 = 5.05%, 0 = 7.1%, +1 = 9.15%, +1.414 = 10%; Oil (w/w): -1.414 = 0.0%, -1 = 0.62%, 0 = 2.1%, +1 =3.58%, +1.414 = 4.2%.

3.3.4 Pea Milk Preparation

Dry peas (800 g) were soaked for 16 hours at room temperature. The amount of water needed for grinding is calculated with the following equation:

(Start pea weight / 200 g) x 550 mL – (end pea weight – start pea weight) = water to add

Peas were blended with the water using a 120v, 60 Hz, 900 v Ninja blender model#: NJ600WM (SharkNinja Operating LLC, Needham, MA, USA) on setting 3 for 2 min. A cotton cloth (160 thread count) was used to filter the slurry, by pouring the slurry into the cloth, cinching the cloth into a satchel, and squeezing out the soluble fraction. The cloth was rinsed, and the pea milk was filtered a second time. The pea milk was refrigerated at approximately 5°C for 1 hour so suspended solids could settle and decanted.

3.3.5 Tofu Preparation

Pea milk (481 g) plus corn oil treatments (0.0-4.2 %) were added to a 1000 mL beaker. The mixture was blended with a Cuisine Art immersion blender for 2 min to homogenize. The beaker was placed in a 98 °C water bath for the duration of a given cook time (45-150 min). After heating, $MgCl_2$ (4.2-10 %) was added over the course of 10 sec, stirring constantly. After 10 min the curds were poured into a 12.8 x 9.1 x 7.9 cm tofu mold lined with a cotton cloth. The cloth was folder once over the curds and a perforated piece of plastic was placed on the cloth before the other sides of the cloth was folder over to prevent molding defects. A 5 kg weight was placed on top of the mold for 10 min. The tofu was removed from the mold and cooled for 10 min before being wrapped in plastic wrap and refrigerated.

An additional sample set of tofu was prepared in duplicate using the method previous described. The tofu was cut into strips and slid into a 50 mL centrifuge. The tube was submerged in liquid nitrogen until tofu was frozen. The samples were held in a -80 °C freezer until being freeze dried in a Freezone 6 (Labconco, Kansas City, Missouri, USA) at -56 °C and 0.02 mbar. The freeze-dried samples were stored in a -80 °C freezer until biochemical analysis was performed.

3.3.2 Pea Milk Timed Heat Treatments

To assess changes occurring to proteins while in the pea milk, pea milk was prepared using the method described above. A sample set of pea milk was modified to contain 3.8 % corn oil by homogenizing 481 g pea milk and 19 g corn oil with a Cuisinart Smart Stick 200 W Variable Speed Handheld Immersion Blender (Cuisinart, Stamford, CT, USA) for 2 min on the highest setting. The oil addition level was selected based on preliminary findings. Non-fat and fat added milks were transferred into 12 each tared 50 mL centrifuge tubes and the masses was

recorded. Centrifuge tube stands were modified to sink in a water bath, and the samples were placed in the stands, and the stands were placed in a water bath at 98 °C and weighted to ensure remained under the water bath water. Two of each sample was removed after every 30 min and transferred to an ice bath for 30 minutes. The samples were freeze dried and held in a -80 °C freezer.

3.3.6 Texture Profile Analysis

Samples were equilibrated to room temperature for 30 min prior to testing. Two blades fastened together 1 cm apart were used to cut the samples into 1 cm cubes. A 25 mm diameter cylindrical plastic probe was attached to a TA,XT.plus (Stable Micro Systems Ltd., Godalming, Surrey, United Kingdom) equipped with a 5 kg load cell. Texture profile analysis (TPA) was run with a pre-test speed of 1.00 mm/s, test speed of 0.83 mm/s, post-test speed of 2.00 mm/sec, a trigger force of 5.0 g, a compression distance of 30 % of the block thickness (1 cm), and a 2 s rest between the compressions.

3.3.7 Percent Yield

The mass of the block of tofu was measured on a Mettler Toledo New Classic SG digital balance. The following equation was used to determine the percent yield.

$$(\text{Mass of tofu}/\text{mass of pea milk + corn oil}) \times 100 = \%\ \text{yield}$$

3.3.8 Percent Retained Solids

Retained solids are the percent of solids that went into the system and were not pressed out. The value was calculated by the following equation:

$$(\text{pea milk solids (g)} + MgCl_2\ \text{solids (g)})/(\text{tofu solids (g)} - \text{oil added (g)}) \times 100 = \%\ \text{retained solids}$$

3.3.9 Moisture Content

To determine the moisture content the AOAC Official method 950.46 was used. A 4 oz Gilson SC-402 tin and its lid of known weight was tared, and a 2 g sample of tofu was added. The sample was spread into a thin layer along the interior of the tin, and the weight of the sample was recorded. The sample was dried uncovered in a 135 °C oven for 120 min. The samples were taken out of the oven, capped with their respective lids, and cooled for 15 min before being weighed again. The following equation was used to calculate the moisture content:

$$1 - [(\text{End weight} - \text{tin and lid}) / \text{sample weight}] = \text{moisture content}$$

3.3.10 FTIR Analysis

Freeze dried samples were equilibrated to room temperature for 45 min prior to testing A NICOLET iS10 Fourier transform IR spectrometer (Thermo Fisher Scientific, Waltham, MA) that was purged continuously with dry air and equipped with a diamond ATR, was used to measure the IR spectra. Each spectrum was measured with 64 scans at a resolution of 4.00 cm^{-1}. The angle of incidence was 45°, and 1 bounce was used for measurements between 4000 cm^{-1} - 500 cm^{-1}. The spectra for added oils were subtracted using Omnic 9 software (Thermo Fisher Scientific, Waltham, MA) prior to deconvolution. The resulting spectra were analyzed on OriginPro 2018 (OriginLab, Northampton, MA). Baselines were corrected, and spectra were normalized before the amide I region of the spectra was deconvoluted using the second derivative to find hidden peaks. The amide I region was fitted with Lorentzian peaks using 50 iterations and a full width at half maximum set to 20 cm^{-1}. Areas under the model corresponding to α-helices (1650-1658) and β-sheets (1630-1638) were quantified and divided by the sum of areas of all peaks discovered in the amide I region.

3.3.11 Surface Hydrophobicity

A modified method of Chelh, Gatellier, and Santé-Lhoutellier (2006) was used to measure surface hydrophobicity. DI water was used to prepare a 1 mg/mL solution of bromophenol blue (BPB). BPB is a dye that binds to hydrophobic regions of proteins and fluoresces at 595 nm. The BPB solution was covered completely with aluminum foil and mixed with a magnetic stirrer for at least 180 min. Freeze dried samples corresponding to 100 mg of protein and 10 mL of 40 °C 20 mM Na-Phosphate buffer with a pH of 7.0 were transfer into 50 mL tubes. A shaker incubator heated to 40 °C was used to vortex samples at 450 rpm for 30 min. After cooling for 5 min, 2000 µL of BPB solution was added. Samples plus BPB solution were placed in a shaker incubator set to 450 rpm and 27 °C for 10 min. After this, samples were centrifuges for 15 min at 2000g. The supernatants were diluted by transferring 100 µL of each sample into a microcentrifuge containing 900 µL of buffer. The dilutions were vortexed briefly. Samples were transferred to cuvettes and ran on an Eppendorf biophotometer at 595 nm.

3.3.12 Molecular Weight Analysis

Fifty milliliter centrifuge tubes were filled with approximately 0.5 g of freeze-dried sample which was then ground and vortexed at 450 RPM for 30 min with an excess of hexane and centrifuged for 1 min at 340 g. The supernatant was decanted, and the tubes were left uncovered until the samples dried. This was completed to remove lipids, which caused loss of resolution to electropherograms in preliminary experiments.

A 50 mM sodium phosphate buffer of pH 7 containing 8 M urea was prepared. Ten mg ± 1.0 mg of the defatted sample was transferred to a microcentrifuge tube and 1 mL of the buffered urea solution was added. The samples were vortexed for 15 min before being centrifuged for 5 min at 9875 *g*. The resulting supernatant was diluted 1:1 with a pH 7 sodium phosphate buffer to

obtain a 4 M urea concentration, which is within the specifications for analysis with an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA) was used to analyze the samples. Analysis was completed in accordance to the instrument manufacturer's specifications.

3.3.13 Color Analysis

Tofu color was assessed by L*, a* and b* color parameters according to the CIELAB international system of color measurement. To accomplish this, a > 10 mm^2 piece of tofu was placed on Konica Minolta Spectrophotometer CM-5 (Konica Minolta Sensing Americas, Inc. New Jersey, USA) equipped with an 8mm aperture and instrument set to D65 with a 10 % light angle. Excess moisture was blotted from the surface of the sample prior to analysis.

3.3.14 Microscopy

A pieces of freeze dried sample, approximately 2 mm x 2 mm, was mounded to a slide. Scanning electron microscopy (SEM) backscattering electron (BSE) imaging was completed with a Quanta 200F SEM (FEI Company, Hillsboro, OR, USA) at 130 Pa and an accelerating voltage of 15.0 kV.

3.4 Results and Discussion

3.4.1 Texture Profile Analysis

The linear model for hardness (fig 3.1a) was significant (P=0.0165). The hardness of the tofu was significantly affected by cook time (P=0.0049), increasing the hardness by 50 g between 60 min and 134 min when all other variables are held constant. The hardness was not significantly affected by the $MgCl_2$ (P=0.3175), reducing the hardness by 10 g between 5.1 % and 9.1 % when all other variables are held constant. The hardness was not significantly affected by oil addition (P=0.1397), decreasing the hardness by approximately 20 g between 0.62 % and 3.55 %.

The linear model for cohesiveness (fig 3.1b) was significant (P=0.0199). The cohesiveness of the tofu was significantly affected by cook time (P=0.0224), increasing the cohesiveness by 0.02 between 60 min and 134 min when all other variables are held constant. The cohesiveness was not significantly affected by the $MgCl_2$ (P=0.1135), reducing the cohesiveness by 0.07 between 5.1 % and 9.1 % when all other variables are held constant. The cohesiveness was significantly affected by oil addition (P=0.0471), decreasing the cohesiveness by 0.04 between 0.62 % and 3.55 %.

The quadratic model for springiness (fig 3.1c) was not significant (P=0.2672). The springiness of the tofu was not significantly affected by cook time (P=0.1836), $MgCl_2$ (P=1.0000), or oil addition (P=0.2336). Interactions between the factors reduces predictability of individual factors.

The cook time is the only significant factor in determining the hardness, and one of two significant factors effecting cohesiveness. Soy proteins have been shown to not immediately dissociate, with some remaining undissociated after 30 min of heating (Wu et al., 2017). This could be exacerbated in pea proteins, which require higher temperatures to dissociate (Wu et al., 2017; Mession et al., 2015).

The $MgCl_2$ was not a significant factor for any mechanical property. The rate of coagulant addition does affect the mechanical properties (Cai & Chang, 1998). The concentration of $MgCl_2$ was adjusted by changing the amount of the stock $MgCl_2$ solution. While the duration of $MgCl_2$ addition was constant for all samples (10 seconds), the amount was not. This means the rate $MgCl_2$ varied between treatments. This could potentially have led to confounding variable.

The oil addition did not significantly affect the hardness. This does go against previous work which showed a significant decrease. The preliminary work looked at formulations that

contained either 0 % or 3.9 % oil and were not held in a water bath. Since the model does show a trend of decreasing hardness with oil addition, the significance of the oil addition is likely lost in the noise of the $MgCl_2$ addition and significance of the heat treatment. The oil addition also effected the cohesiveness, which is likely because as the percent of protein in the resulting tofu decreases with oil addition. Hypothetically, this would shift the balance away from the gel forming component and towards a liquid. Although, it is plausible the decrease is partially caused by redistribution of protein subunits, as shown by the surface hydrophobicity assay discussed later (3.4.5). Overall, while $MgCl_2$ concentration and oil level did have decrease hardness, the decrease in these values can be overcome by increasing cook time.

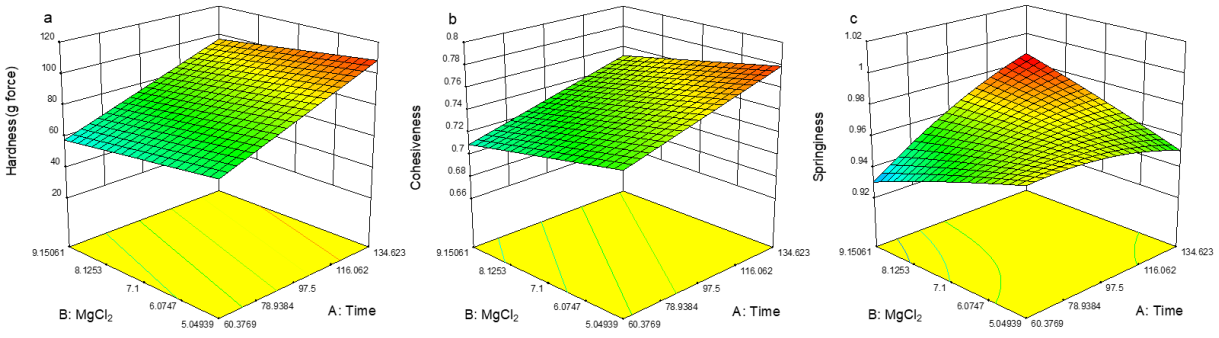

Figure 3.1: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) at 2.14 % oil addition with the vertical axis being Hardness (a), Cohesiveness (b), and Springiness (c).

3.4.2 Percent Yield

The linear model for percent yield (fig 3.2a) was significant (P=0.0316). The percent yield of the tofu was not significantly affected by cook time (P=0.2124), decreasing the percent yield by approximately 3 % between 60 min and 134 min when all other variables are held constant. The percent yield was not significantly affected by the $MgCl_2$ (P=0.5353), increasing the percent yield by approximately 1 % between 5.1 % and 9.1 % when all other variables are

held constant. The percent yield was significantly affected by oil addition (P=0.0076), increasing the percent yield by approximately 4 % between 0.62 % and 3.55 % addition levels.

The percent yield was most affected by the oil addition, which is to be expected since the oil is incorporated into the gel and the bulk of the water is removed during pressing. While the percent yield is important, greater attention must be given to the present solids. This value represents additional nutrition that gets to the consumer and less loss for the manufacturer.

3.4.3 Percent Retained Solids

The quadratic model for percent retained solids (fig 3.2b) was significant (P=0.0075). The retained solids of the tofu were not significantly affected by cook time (P=0.3133). The retained solids were not significantly affected by the $MgCl_2$ (P=0.1763). There was an interaction between cook time and $MgCl_2$ concentration; at low $MgCl_2$ concentrations retained solids were decreased by 3 %, and at high $MgCl_2$ concentrations retained solids were increased by 6 %. The retained solids were significantly affected by oil addition (P=0.0027), increasing the retained solids by approximately 6 % between 0.62 % and 3.55 %.

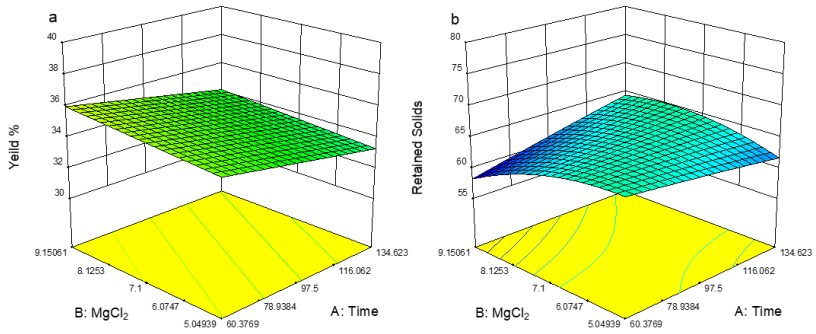

Figure 3.2: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) at 2.14 % oil addition with the vertical axis being % Yield (a) and retained solids (b).

It is important to note that retained solids accounted for only the solids from the milk and $MgCl_2$. Since the oil is completely incorporated by the curds the only solids that can be removed

during pressing come from the pea milk and $MgCl_2$. Therefore, it is important to focus on the how much of the pea solids are lost and $MgCl_2$ is left because it is difficult to determine how much of the salt is incorporated. The oil addition had little effect on retained solids at the lower levels. However, after a retained solids value of 2.4 % is reached, the values increase rapidly as it is a quadratic function. This may represent a critical value of when proteins with higher binding affinities to lipids becomes saturated and excess oil is free to bind to unassociated protein subunits.

3.4.4 FTIR Analysis

The range of 1700-1600 cm^{-1}, or amide I, was deconvoluted and the percent of the peak comprised of α-helices (~1654) and β-sheets (~1636) (Shevkani, Singh, Kaur, & Rana, 2015) was determined. The areas in the amide I region associated with unordered structures were found to be too variable to effectively assess (data not shown). This is a common issue due to the overlapping of hidden peaks in the upper and lower sections of the amide I region.

The α-helices in the milk samples were not significantly affected by the heat treatment while the β-sheets were (fig 3.3). In the no fat added milk, the β-sheets decreased initially, over the first 60 min and gradually went up before falling again after 120 min. This can be attributed to conformational changes to the proteins and protein dissociation. Both the fat added and no fat added samples showed similar trends. However, the fat added sample tended to have a slightly lower percent β-sheet for each time when compared to the no fat added samples. These results indicate that changes to the β-sheet structures was not associated with changes to the α-helical structures and was likely associated with shifts in other secondary structure in areas of the amide I band that are more difficult to assess.

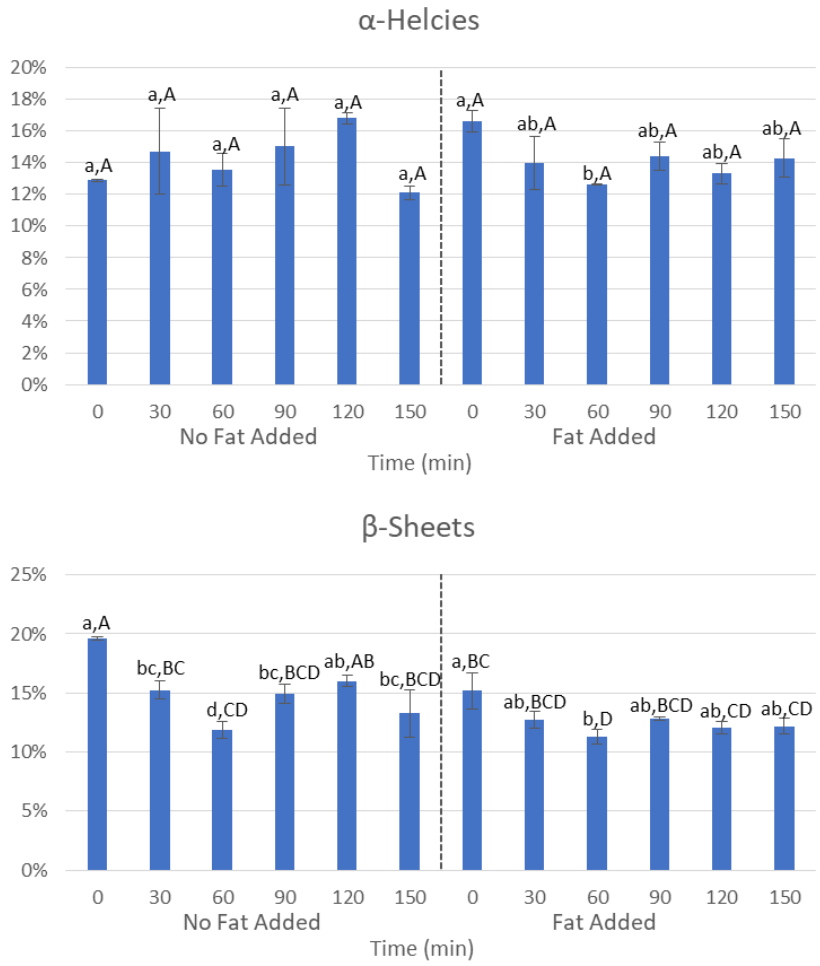

Figure 3.3: A graphical representation of the effect of heating pea milk at 98 ˚C for 0, 30, 60, 90, 120, and 150 min periods on α-helical (top) and β-sheet (bottom) structures. Values with a common lower-case letter within a fat treatment represent milks that are not significantly different ($P<0.05$). Values with a common upper-case letter are not significantly different ($P<0.05$) across fat treatments.

The quadratic model for α-helices (fig 3.4a) was significant ($P=0.0252$). The α-helices of the tofu was not significantly affected by cook time ($P=0.6790$, $MgCl_2$ ($P=0.4520$), or oil addition ($P=0.4520$) alone. However, the interaction of time and $MgCl_2$ ($p=0.0824$), and the quadratic value of oil addition ($P=0.0014$) did have significant affects.

The quadratic model for β-sheets (fig 3.4b) was significant ($P=0.0406$). The β-sheets of the tofu was not significantly affected by cook time ($P=0.7054$), $MgCl_2$ ($P=0.2189$), or oil addition ($P=0.1573$) alone. However, the interaction of $MgCl_2$ and oil addition ($P=0.0162$) did

have a significant affect; at low levels of oil addition the percent of β-sheets increased from 7 % to 14 % between the $MgCl_2$ concentration of 5.0 % and 9.2 %. At high levels of oil addition β-sheets decrease from 13 % to 10 % between the $MgCl_2$ concentration of 5.0 % and 9.2 %. This may be related to ionic mediated dissociation of vicilin and legumin (Matsudomi et al., 1985). For this reason, the dissociation of the proteins is likely related to the low percent of β-sheets in low ionic strengths. Since β-sheets, correlate with retained solids, it is possible that the solids being retained are high in β-sheets and would thus be protein. Much of the decrease associated with time seen in the milk (fig. 3.3) occurred before the majority of the tofu measurements, so it is not as evident when in the tofu samples, and any related change would be lost in the effect of the $MgCl_2$.

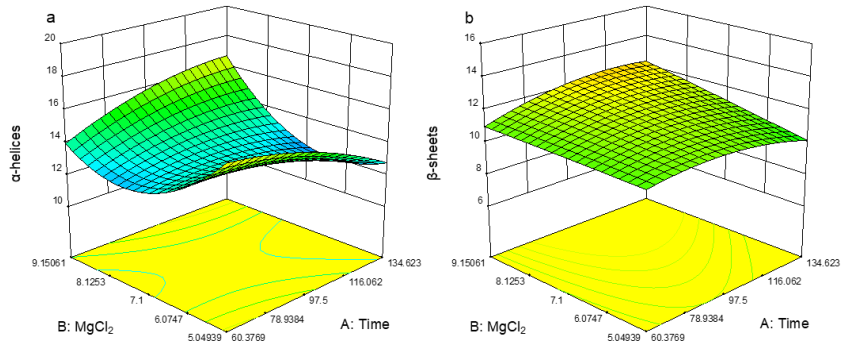

Figure 3.4: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) at 2.14 % oil addition with the vertical axis being α-helices (a) and β-sheets (b).

3.4.5 Surface Hydrophobicity

The quadratic model for surface hydrophobicity (fig 3.5) was significant (P=0.0074). The surface hydrophobicity of the tofu was not significantly affected by cook time (P=0.3269). The surface hydrophobicity was significantly affected by the $MgCl_2$ (P=0.0498). The cook time and $MgCl_2$ did have an interaction with oil levels; at low oil concentrations, surface hydrophobicity was directly proportionate to cook time and $MgCl_2$ concentrations, but at high oil levels the surface hydrophobicity was inversely proportionate to cook time and $MgCl_2$ concentrations (see

appendix fig. A.3.8). The surface hydrophobicity was significantly affected by oil addition (P=0.0038), decreasing the surface hydrophobicity by approximately 25-50 µg BPB between 0.62 % and 3.55 % if all other factors are held constant. The decrease in surface hydrophobicity is likely caused by the reorientation of exposed hydrophobic regions of the proteins to associate with the surface of oil globules.

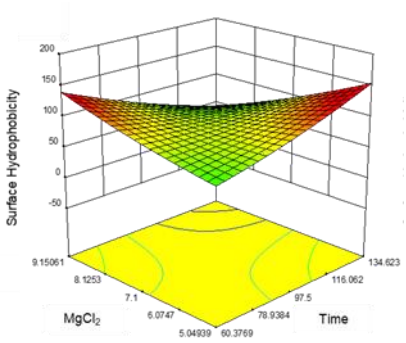

Figure 3.5: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) on surface hydrophobicity (vertical axis) with oil at 0.620 % (a), 2.14 % (b), and 3.55 % (c).

At low ionic strength, time increased the surface hydrophobicity, and at high ionic strength time decreased surface hydrophobicity. Lower ionic strength promotes aggregation of legumin (Gueguen, Chevalier, & Schaeffer, 1988), so there would be less dissociation and thus less exposure of the hydrophobic interior resulting in less binding of BPB. At low oil concentrations the cook time increased the surface hydrophobicity, but at higher oil concentrations, time decreased the surface hydrophobicity. As the proteins are heated they dissociate into subunits, decreasing molecular weight and exposing hydrophobic portions, as oil is added the newly exposed hydrophobic regions can associate with the lipids. This is seen in the decrease in surface hydrophobicity as the sample is cooked longer, with higher ionic strengths, and oil is added. The change in the trend from increasing to decreasing surface hydrophobicity

when oil levels are increased is likely from newly exposed hydrophobic surfaces on the dissociated protein subunits coating the surface of oil globules.

When the heat-treated milk was evaluated, the surface hydrophobicity rapidly increased between 0 and 30 min indicating that without the influence of the $MgCl_2$ the subunits dissociated more quickly and less erratically (fig 3.6). This also demonstrates that the changes occurring to the protein structure happen rapidly upon heat exposure.

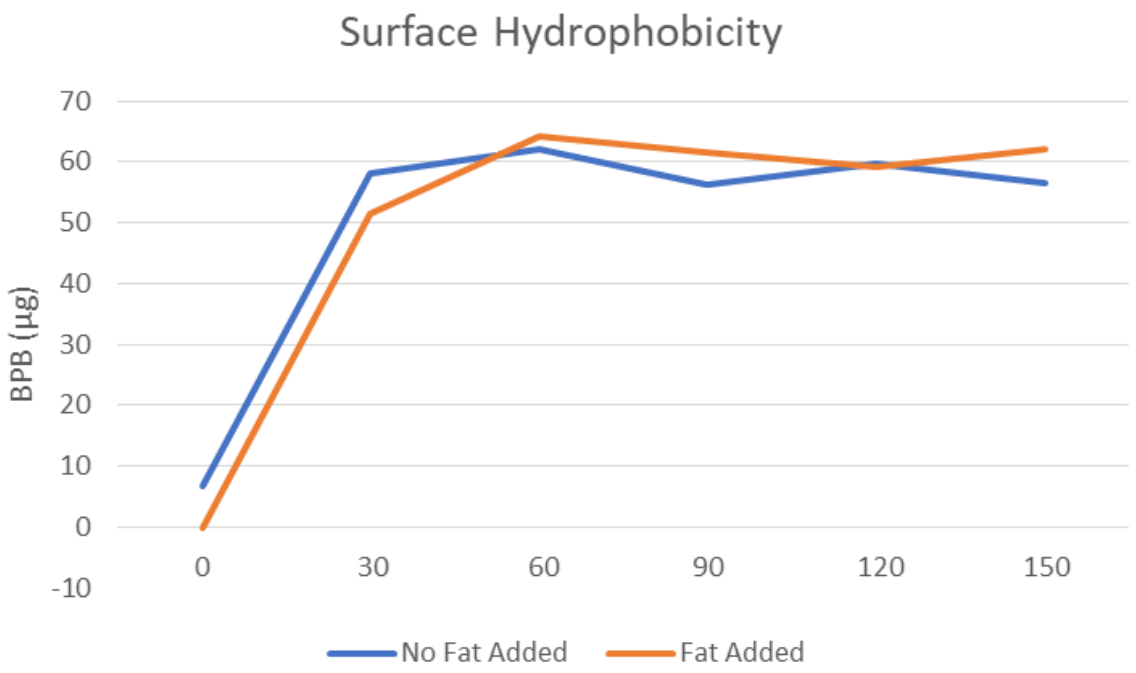

Figure 3.6: A graphical representation of the effect of heating and 98 °C on surface hydrophobicity of no fat added and fat added pea milk (3.8%).

In previous studies, secondary structure, α-helices and β-sheets, is often proportional to surface hydrophobicity and textural properties of tofu. Within these studies, it is typical to have one or two variables, and measures the treatment effect on the resulting gel. It is typically found protein secondary structures are proportional to gel textural properties. In this study, exposure to heat, ion concentration, and oil addition were studied. It was found that treatment effects were not necessarily proportional to resulting data. The findings of this study suggest that the synergy

between treatments has a large influence on secondary structure, surface hydrophobicity, and gel strength. It is evident that quantification of α helices and β sheets alone may not be a good indicator of gel strength and do not necessarily have to correlate to gel textural properties. To this end, care must be taken in subsequent studies to ensure that protein structure is not falsely correlated to gel textural properties and casual relationships must be demonstrated.

3.4.6 Molecular Weight Analysis

The model for the ~21 kDa peak (fig 3.7a) was not significant (P=0.6912) The ~24 kDa peak of the tofu was not significantly affected by cook time (P=0.6951), $MgCl_2$ (P=0.8952), or oil addition (P=0.5566).

The quadratic model for the ~24 kDa peak (fig 3.7b) was not significant (P=0.3449). The ~24 kDa peak of the tofu was not significantly affected by cook time (P=0.8578), $MgCl_2$ (P=0.5219), or oil addition (P=0.2670). The most significant component of the model (P=0.0582) was the effect of the oil squared, which is what contributed to the drastic increase of the amount of ~24 kDa polypeptide. The ~24 kDa peak is a polypeptide of legumin.

The ~24 kDa peak increasing with time at higher oil levels could indicate that as legumin breaks down into more base components the polypeptide was retained by the oil, possibly contributing to the retained solids.

The linear model for the ~54 kDa peak (fig 3.7c) was not significant (P=0.0761), nor was it significantly affected by $MgCl_2$ (P=0.4190) or oil addition (P=0.5192). However, the ~54 kDa peak was significantly affected by time (P=0.0170) decreasing by approximately 1.5 % between 60 min and 134 min when all other variables are held constant.

One of the key proteins in peas is legumin. The ~21 and ~24 kDa polypeptides are two of the alkaline polypeptides that make up a legumin sub-unit. Legumin breaks down into sub-units

that are reported to be approximately between 54 and 60 kDa (Schwenke et al., 1990). The breakdown of the ~54 in to the ~21 and ~24 is consistent with tofu's glycinin, breaking into sub-units during heating, and indicated that pea-based tofu follows a similar mechanism of formation as conventional soy tofu. This hypothesis is further supported by the surface hydrophobicity data that indicated an increase in dissociation with increased cook time. The amount that cook time increases the ~21 and ~24 peaks increases with oil addition. However, oil addition does not affect the ~54 peak as much, indicating that legumin's break down is not augmented by oil addition but that the retention of ~21 and ~24 polypeptides is. The other key protein in peas is vicilin, which is comprised of sub units of ~33 kDa, ~50 kDa, and ~71 kDa.

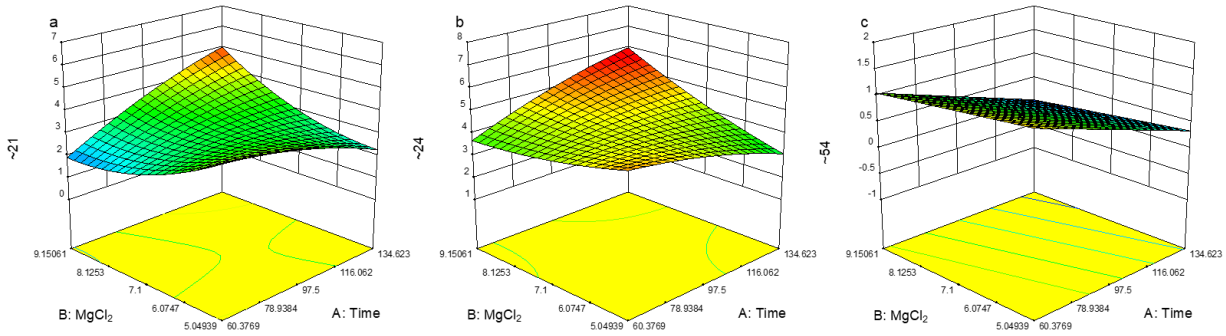

Figure 3.7: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) at 2.14 % oil addition with the vertical axis being ~21 (a), ~24 (b), and ~54 (c).

The quadratic model for the ~33 kDa (fig 3.8a) was not significant (P=0.3262). The ~33 kDa of the tofu was significantly affected by cook time (P=0.4447), $MgCl_2$ (P=0.6695), or oil addition (P=0.4023). The change in the ~33 kDa, is difficult to interpret because the ~50 kDa sub-unit is comprised in part by a polypeptide that is also ~33 kDa.

The quadratic model for the ~50 kDa (fig 3.8b) was significant (P=0.0334). The ~50 kDa peak of the tofu was not significantly affected by cook time (P=0.8635), $MgCl_2$ (P=0.4259), or oil addition (P=0.2310) alone. However, the combined effect of the $MgCl_2$ and oil addition was significant (P=0.0178) as well as the quadratic effect of the oil addition (P=0089). The ~33 and

~50 peaks follow the same trend as the ~21 and ~24 peaks, where cook time becomes augments the peak more at high oil addition.

The linear model for the ~71 kDa (fig 3.8c) was not significant (P=0.0603). The ~71 kDa of the tofu was significantly affected by cook time (P=0.0244), reducing the ~71 kDa by 2 % between 60 min and 134 min when all other variables are held constant. The ~71 kDa was not significantly affected by the $MgCl_2$ (P=0.1228), reducing the ~71 kDa by 2 % between 0.62 % and 3.55 % when all other variables are held constant. The ~71 kDa was not significantly affected by oil addition (P=0.5415). The decrease in the ~71 kDa, a subunit of vicilin, is inversely proportionate to time and thus hardness, likely indicating that the subunit is associating with other protein during gel formation.

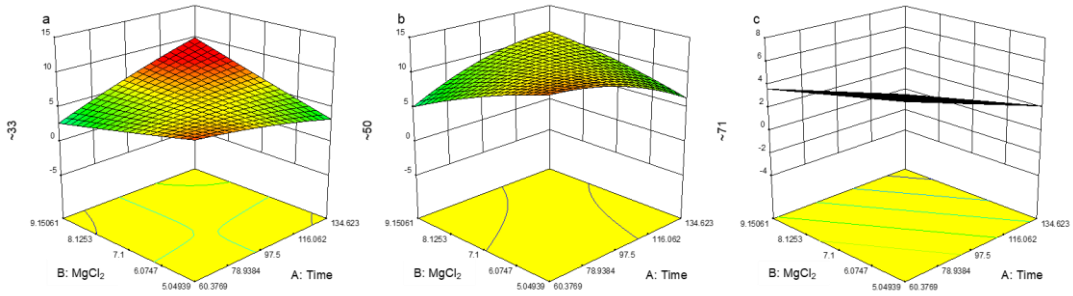

Figure 3.8: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) at 2.14 % oil addition with the vertical axis being ~33 (a), ~50 (b), and ~71 (c).

3.4.7 Color Analysis

The linear model for L* value (fig 3.9a) was significant (P=0.0190). The L* value of the tofu was significantly affected by cook time (P=0.0056), decreasing the L* value by 1.2 between 60 min and 134 min when all other variables are held constant. The *L value was not significantly affected by the $MgCl_2$ (P=0.4689), exhibiting no change in the *L value between 5.1% and 9.1% when all other variables are held constant. The L* value was not significantly affected by oil addition (P=0.1147), increasing the L* value by approximately 0.8 between 0.62 % and 3.55 %.

The linear model for a* value (fig 3.9b) was significant (P=0.0032). The a* value of the tofu was significantly affected by cook time (P=0.0004), decreasing the a* value by approximately 0.6 between 60 min and 134 min when all other variables are held constant. The a* value was not significantly affected by the $MgCl_2$ (P=0.3397), exhibiting no change in the a* value between 5.1 % and 9.1 % when all other variables are held constant. The a* value was not significantly affected by oil addition (P=0.9424), exhibiting no change in the a* value between 0.62 % and 3.55 % when all other variables are held constant.

The 2FI model for b* value (fig 3.9c) was significant (P=0.0049). The b* value of the tofu was not significantly affected by cook time (P=0.6500), decreasing the b* value by 1.2 between 60 min and 134 min when all other variables are held constant. The b* value was not significantly affected by the $MgCl_2$ (P=0.6127), exhibiting no change in the b* value between 5.1 % and 9.1 % when all other variables are held constant. The b* value was not significantly affected by oil addition (P=0.0222), increasing the b* value by approximately 0.8 between 0.62 % and 3.55 %. However, preliminary result showed that tofu did a look visible lighter when compared to non-fat.

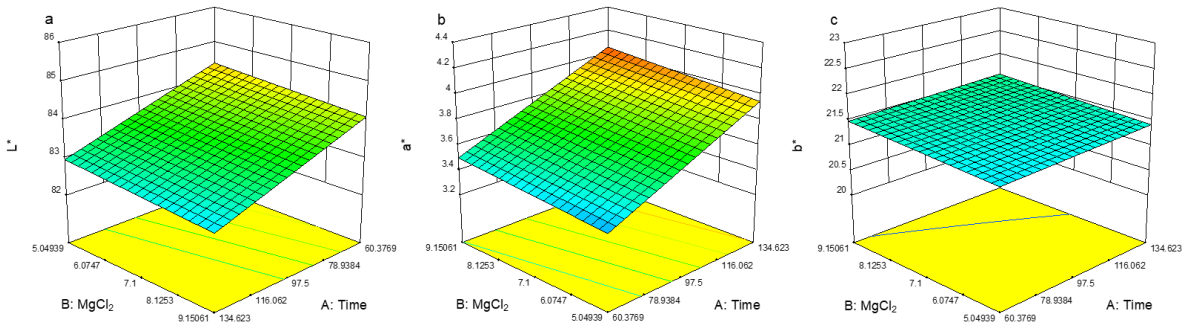

Figure 3.9: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) at 2.14 % oil addition with the vertical axis being L* (a), a* (b), and b* (c).

The decrease in lightness associated with cook time is likely due to decreased water content, resulting in an increased concentration of pigments and a darker color. However, it

should be mentioned that when no fat added samples are compared to fat added samples the fat added are visibly lighter color, but these results indicate that the amount of oil added does not increases the lightening after a certain point.

The positive a* indicates a very slight reddening, but the stronger color is the yellowing, as indicated by the b* values. Tofu prepared from yellow split peas, had a more yellow appearance than traditional tofu, which would be considered a product defect (Kim & Wicker, 2005). Fortunately, this effect can be mitigated through the addition of oil and modification of heat treatments. For reference Kim and Wicker (2005) reported L* values of 90 and 93 for soy tofu, with the pea tofu ranged from 82 to 85. To put these values in perspective, an image of pea tofu has been included (fig 3.10). This tofu is similar to soy-based tofu in appearance and is only slightly more yellow than a soy-based tofu.

Figure 3.10: An image representing a tofu made from (top) and an image representing a tofu made from yellow field peas at the zero points of the RSM design and the methods described within this study (bottom).

3.4.8 Microscopy

All but two SEM images (fig 3.11a and 3.11d) displayed honeycomb structures, a commonly seen structure in tofu. The voids in the honeycombs increase as cook time increases, with the smallest voids in the 45 min cook time (fig 3.11b), and progressively larger voids in the

60 min (fig 3.11g), 97.5 min (fig 3.11c, 3.11e, 3.11f, 3.11j, and 3.11k), and 134 min (fig 3.11h and 3.11i). The longest time, 150 min (fig 3.11d), did not form honeycombs. Since time correlates with hardness this is unexpected since previous work showed tofu with denser honeycombs and smaller voids had higher hardness values. Following the increase in time it can be seen that the shorter cook times have a denser structure in terms of smaller voids, but the branches that connect the aggregates become thicker, to the point where aggregates are no longer discrete.

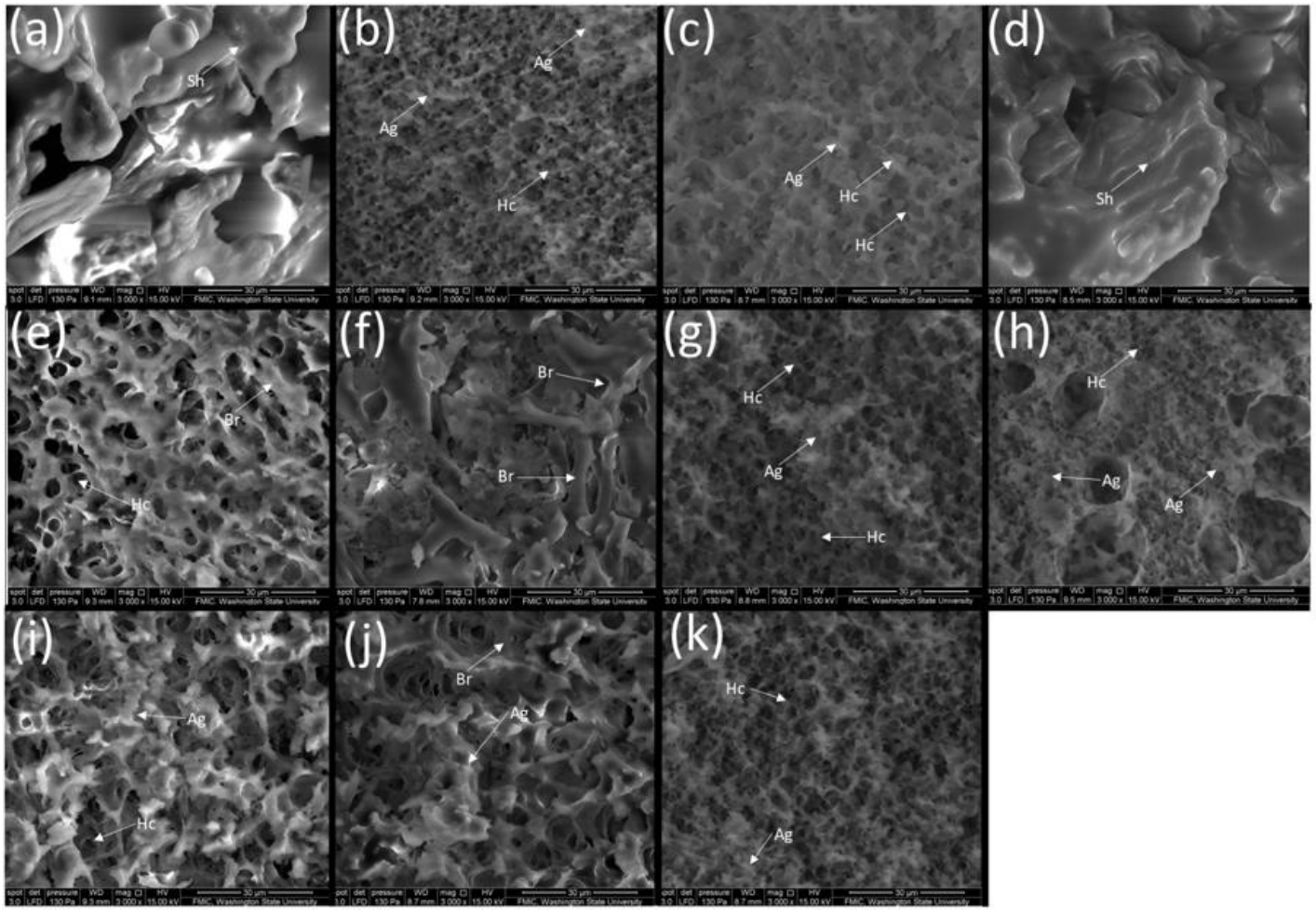

Figure 3.11: SEM images of RSM treatments **a)** 1 (-1, +1, 0), **b)** 3 (-1.414, 0, 0), **c)** 4 (0, 0, +1.414), **d)** 5 (+1.414, 0, 0), **e)** 7 (0, 0, 0), **f)** 8 (0, -1.414, 0), **g)** 11 (-1, -1, +1), **h)** 12 (+1, -1, +1), **i)** 13 (+1, +1, -1), **j)** 14 (0, +1.414, 0), **k)** 15 (0, 0, -1.414). All micrographs were taken at 3,000 x magnification Ag designates aggregates, Br designates branches, HC designates honey comb structures, and Sh designates sheets.

3.5 Conclusion

The sample set demonstrated a range of tofu textures could be obtained by varying cook time, $MgCl_2$, and oil content. Cook time was the most important factor for determining hardness, the most important quality characteristic of tofu. Furthermore, it was determined that the synergistic effects between treatments influence gel strength and protein secondary structure. Gel strength does not necessarily have to correlate to any one factor. The biochemical assays showed that there is thermal dissociation of legumin and vicilin, which form a matrix that is effected by the hydrophobic interactions. These interactions allow for the incorporation of lipid to make a visibly smoother, whiter tofu. This data can act as a template by manufacturers looking for a low-cost high protein food.

3.6 References

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Chapter 4: Conclusions and Future Work

Research covered in chapters 2 and 3 produced a variety of tofus. Chapter 2 showed that biochemical factors like surface hydrophobicity and secondary structure could be used as indicators of hardness. However, Chapter 3 demonstrated that those trends are conditional on certain variables. Chapter 2 used a more traditional method of heating and brought the temperature up quickly to 80 °C, while the study in Chapter 3 raised the temperature more gently in a water bath and held it for a variety of times. The slow rate of heating over a long time made a tofu that had better cohesiveness and springiness. This is potentially achieved by more fully dissociating the proteins. Also, Chapter 3's varying of the coagulant and oil showed that some of the biochemical trends were contingent on the $MgCl_2$ and oil content. The α-helices were affected by an interaction between the $MgCl_2$, the β-sheets were significantly affected by $MgCl_2$ but most notable at low levels of oil addition, and the yellowness of the tofu was inversely related to the $MgCl_2$ at low levels of oil addition, but the trend reversed to be directly related at high levels of oil. This demonstrates that work done on isolates will not relate well to real world applications.

For the future, similar work needs to be repeated with soy. This would give a better understanding of the role lipids play in traditional tofu. For pea-based tofu, the logical next step would be to design and conduct sensory study. To assess relevant attributes a descriptive analysis with a trained panel is advisable. Firmness, grittiness, springiness, bitterness, grassiness, and sweetness should be assessed within this study. Informal tastings of tofus made in chapter 3 of this thesis showed that extended heating reduced bitter flavors, which is often noted in the preparation of foods containing dried peas, such as split pea soup. A shelf life study should be run as part of the sensory to note changes and determine the best option for packaging; water

packed or vacuum packed. A potential peripheral to the study could be assessing the acceptability of a no fat added variety. Manufacturers have produced reduced fat soy tofu in the past, but they were plagued with off flavors.

One of the important next steps would be scale up. Determining the capabilities of the equipment to extract the right amount of the right components of the peas. Once this is completed, quickly and reliably altering the percent solids of the milk could provide interesting results. Some soy tofu researchers found that lowering the solids content of the milk to below 6 % resulted in a smaller curd with low water holding capability and higher firmness. Pending sensory results on grittiness, it could be profitable to increase the amount of solids in the milk to produce a larger curd.

$MgCl_2$ is the coagulant that is most practical for a benchtop study, but others are used in industry. Preliminary findings demonstrated that calcium sulfate is an effective coagulant. Calcium sulfate would also provide a source of calcium but can produce a chalky tofu. Other coagulants like GDL are used to prepare a silken tofu. Silken tofu was not evaluated in the study, but extended heat treatments, prior to $MgCl_2$ addition resembled silken tofu.

Appendix A Supplemental Tables:

Table A.2.1 TPA Comparisons of experimental pea tofu and commercially available soy tofu.

Hardness (g force)			Springiness			Cohesiveness	
No fat dis+past	323.0		Sunrise soft*	0.99		Sunrise extra firm*	0.85
Sunrise extra firm*	241.9		Sunrise extra firm*	0.98		Soyganic Extra Firm*	0.85
Soyganic Extra Firm*	205.0		House Extra Firm*	0.98		Nasoya extra Firm*	0.84
No fat control	175.9		Sunrise medium firm*	0.98		House Firm*	0.83
No fat disrupted	171.7		Azumya extra Firm*	0.98		House Extra Firm*	0.81
No fat pasteurized	132.5		House Firm*	0.98		Azumya Firm*	0.80
Fat dis+past	127.8		Azumya silken*	0.98		Azumya extra Firm*	0.79
House Firm*	85.9		Azumya Firm*	0.97		Sunrise medium firm*	0.79
Sunrise medium firm*	80.5		Nasoya extra Firm*	0.97		No fat control	0.76
Nasoya extra Firm*	79.9		Soyganic Extra Firm*	0.95		No fat pasteurized	0.75
Azumya extra Firm*	67.4		No fat pasteurized	0.91		Sunrise soft*	0.73
House Extra Firm*	64.4		Fat dis+past	0.90		Azumya silken*	0.72
Fat disrupted	56.3		No fat control	0.89		Fat control	0.65
Azumya Firm*	54.4		Fat control	0.88		Fat dis+past	0.62
Sunrise soft*	54.1		No fat dis+past	0.81		Fat disrupted	0.60
Azumya silken*	50.8		Fat disrupted	0.81		No fat dis+past	0.58
Fat control	48.8		No fat disrupted	0.70		No fat disrupted	0.53
Fat pasteurized	28.5		Fat pasteurized	0.31		Fat pasteurized	0.24

*Commercial soy tofu

Table A.3.1 TPA Comparisons of experimental pea tofu and commercially available soy tofu.

Hardness (g force)			Springiness			Cohesiveness	
Sunrise extra firm*	241.9		Sunrise soft*	0.99		Sunrise extra firm*	0.85
Soyganic Extra Firm*	205.0		Sunrise extra firm*	0.98		Soyganic Extra Firm*	0.85
RSM 5	111.0		RSM 9	0.98		Nasoya extra Firm*	0.84
RSM 8	109.9		House Extra Firm*	0.98		House Firm*	0.83
RSM 7	104.8		Sunrise medium firm*	0.98		House Extra Firm*	0.81
RSM 13	100.5		Azumya extra Firm*	0.98		Azumya Firm*	0.80
RSM 6	97.9		House Firm*	0.98		Azumya extra Firm*	0.79
RSM 4	89.1		Azumya silken*	0.98		Sunrise medium firm*	0.79
House Firm*	85.9		RSM 6	0.98		RSM 9	0.78
RSM 9	85.9		Azumya Firm*	0.97		RSM 13	0.77
RSM 2	81.2		Nasoya extra Firm*	0.97		RSM 12	0.76
Sunrise medium firm*	80.5		RSM 5	0.97		RSM 10	0.76
Nasoya extra Firm*	79.9		RSM 11	0.96		RSM 7	0.76
RSM 14	79.2		RSM 2	0.96		RSM 8	0.76
RSM 11	74.8		RSM 8	0.96		RSM 11	0.75
RSM 12	73.1		RSM 14	0.96		RSM 6	0.75
Azumya extra Firm*	67.4		RSM 12	0.96		RSM 2	0.75
RSM 10	66.7		RSM 4	0.96		RSM 5	0.73
House Extra Firm*	64.4		RSM 10	0.96		Sunrise soft*	0.73
Azumya Firm*	54.4		RSM 13	0.95		RSM 4	0.72
Sunrise soft*	54.1		Soyganic Extra Firm*	0.95		RSM 14	0.72
Azumya silken*	50.8		RSM 7	0.95		Azumya silken*	0.72
RSM 1	44.7		RSM 3	0.94		RSM 1	0.70
RSM 3	39.4		RSM 1	0.92		RSM 3	0.68

*Commercial soy tofu

Appendix B Supplemental Figures:

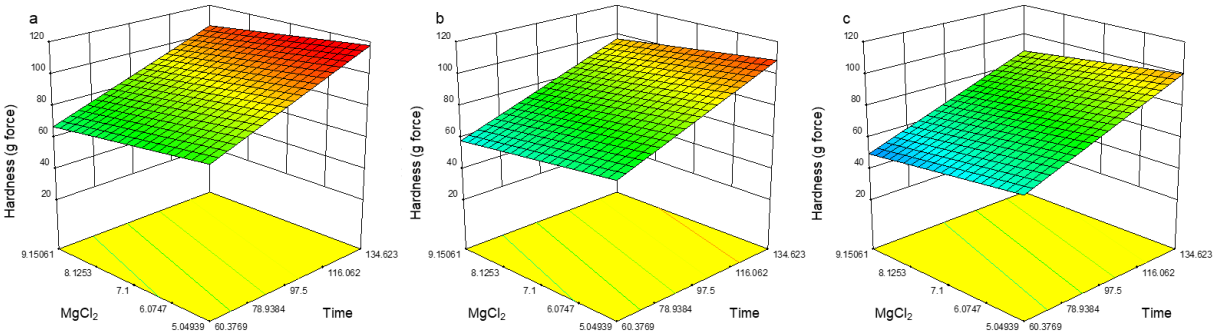

Figure B.3.1: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) on Hardness (vertical axis) with oil at 0.620 % (a), 2.14 % (b), and 3.55 % (c).

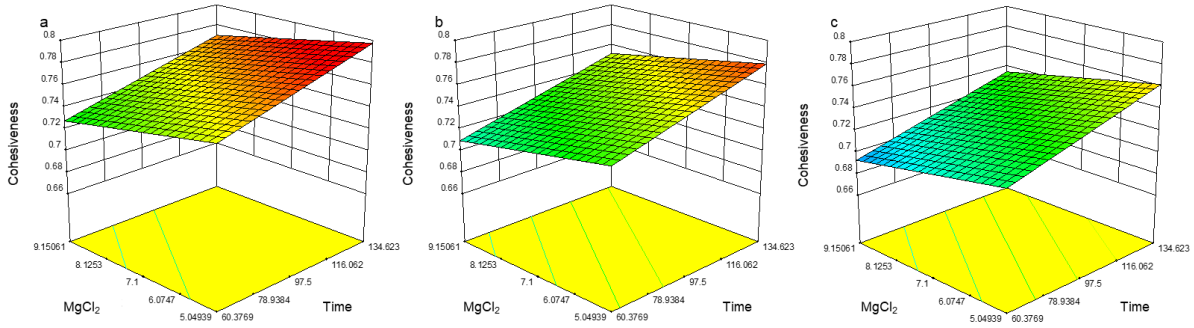

Figure B.3.2: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) on Cohesiveness (vertical axis) with oil at 0.620 % (a), 2.14 % (b), and 3.55 % (c).

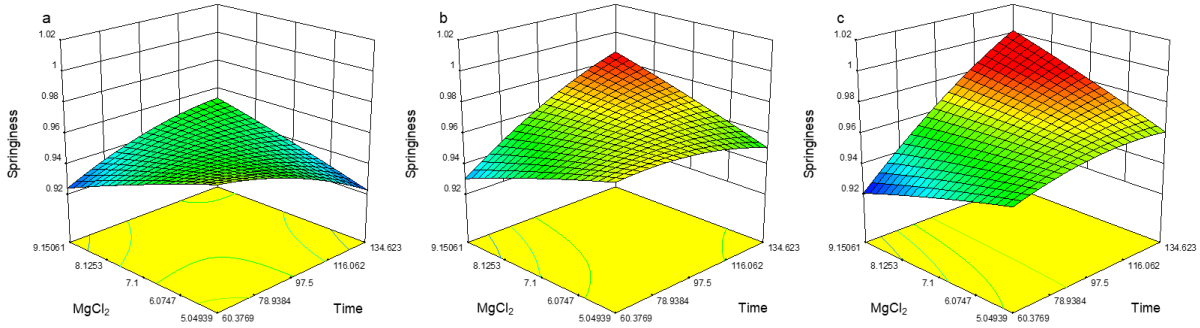

Figure B.3.3: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) on Springiness (vertical axis) with oil at 0.620 % (a), 2.14 % (b), and 3.55 % (c).

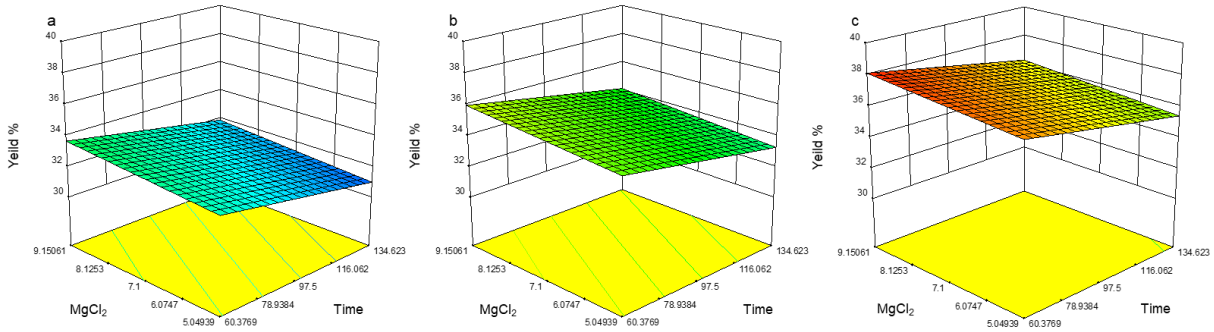

Figure B.3.4: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) on percent Yield (vertical axis) with oil at 0.620 % (a), 2.14 % (b), and 3.55 % (c).

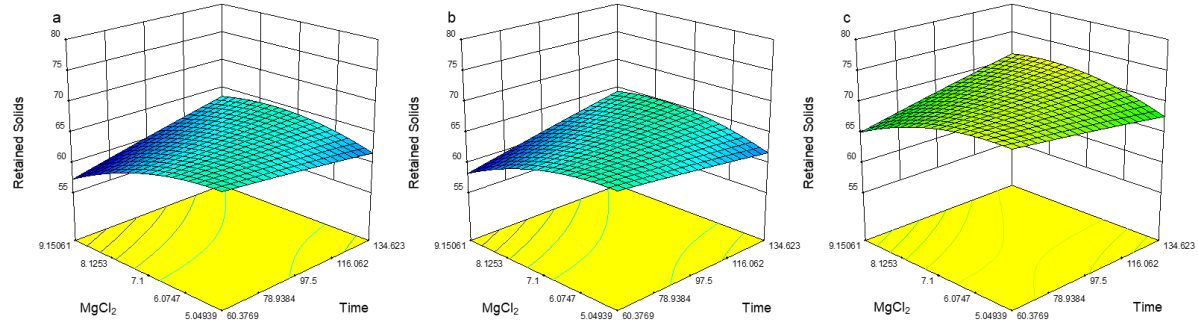

Figure B.3.5: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) on Retained Solids (vertical axis) with oil at 0.620 % (a), 2.14 % (b), and 3.55 % (c).

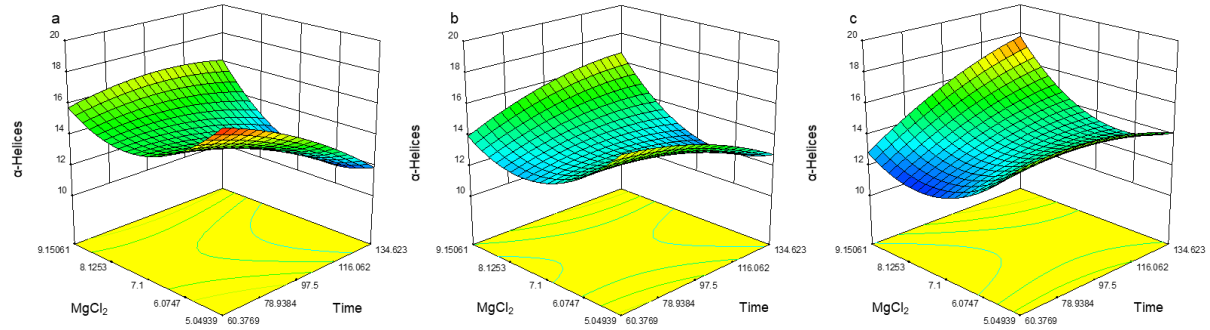

Figure B.3.6: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) on α-helices (vertical axis) with oil at 0.620 % (a), 2.14 % (b), and 3.55 % (c).

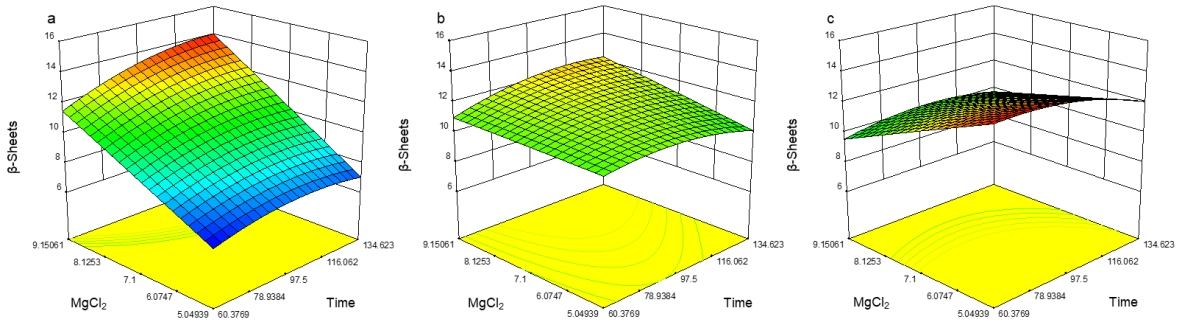

Figure B.3.7: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) on β-sheets (vertical axis) with oil at 0.620 % (a), 2.14 % (b), and 3.55 % (c).

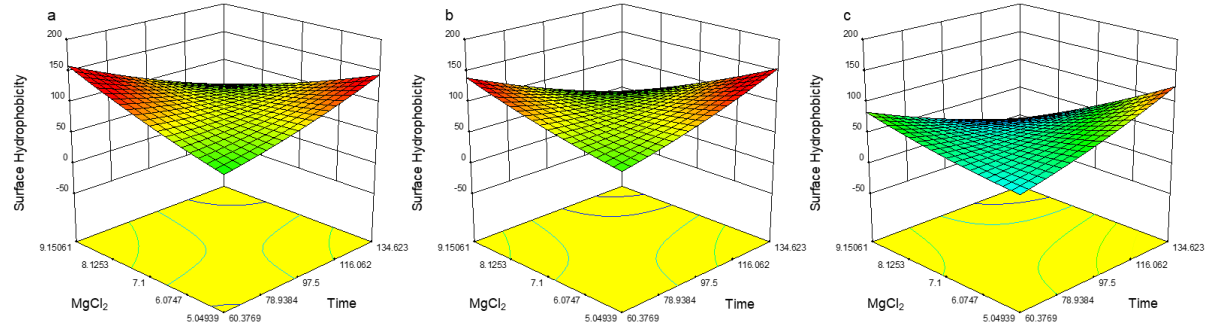

Figure B.3.8: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) on surface hydrophobicity (vertical axis) with oil at 0.620 % (a), 2.14 % (b), and 3.55 % (c).

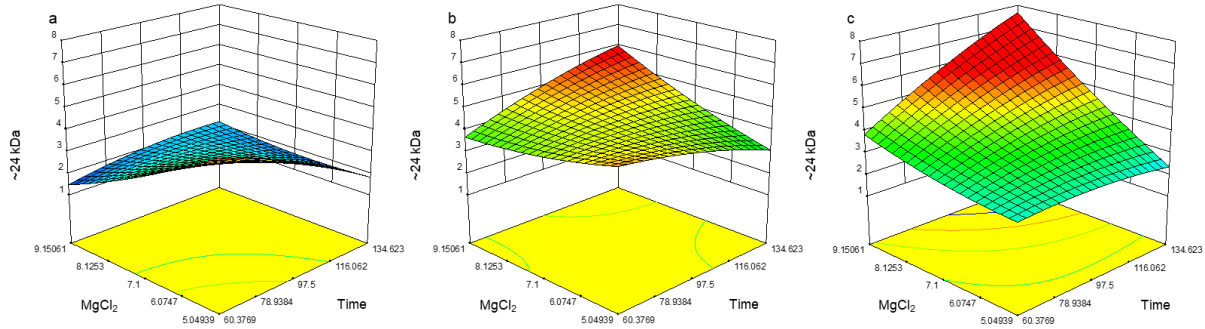

Figure B.3.9: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) on the ~24 kDa protein class (vertical axis) with oil at 0.620 % (a), 2.14 % (b), and 3.55% (c).

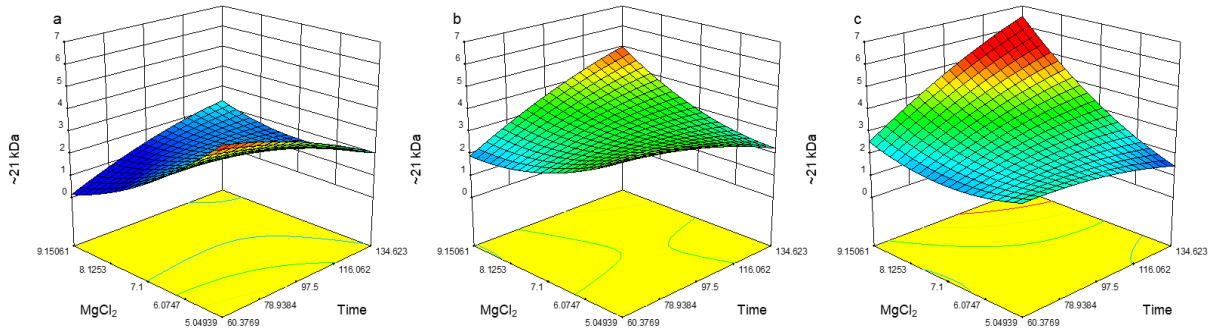

Figure B.3.10: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) on ~21 kDa protein class (vertical axis) with oil at 0.620 % (a), 2.14 % (b), and 3.55 % (c).

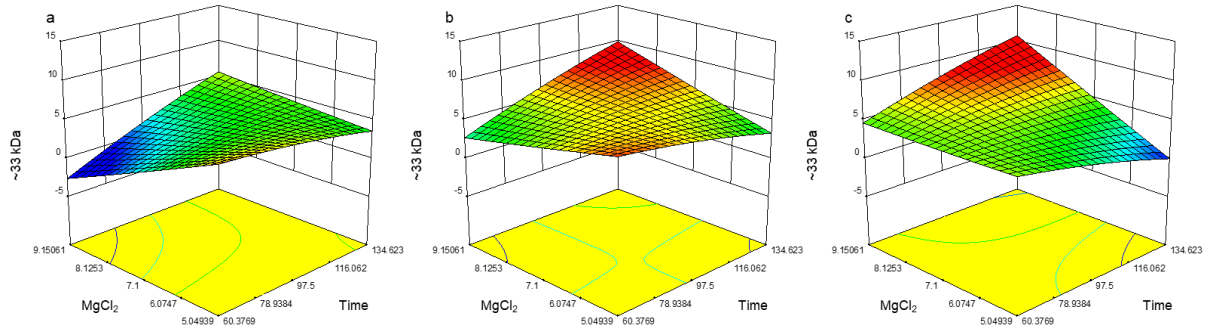

Figure B.3.11: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) on the ~33 kDa protein class (vertical axis) with oil at 0.620 % (a), 2.14 % (b), and 3.55 % (c).

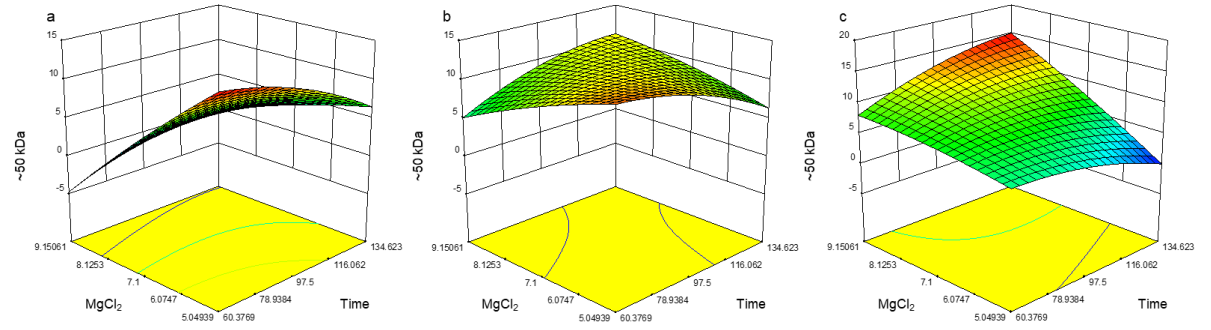

Figure B.3.12: A graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) on the ~50 kDa protein class (vertical axis) with oil at 0.620 % (a), 2.14 % (b), and 3.55 % (c).

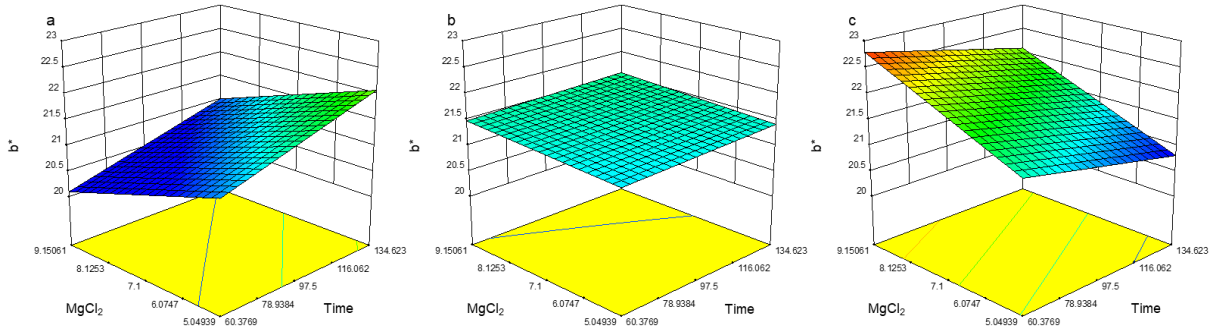

Figure B.3.13: Graphical representation of the effect of $MgCl_2$ (y axis) and Time (x axis) on b* (vertical axis) with oil at 0.620 % (a), 2.14 % (b), and 3.55 % (c).